AN *IN VITRO* STUDY

OF

NEUTROPHIL CHEMOTAXIS

GAIL TODD

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To my father and my mother

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INDEX

Glossary of terms and abbreviations used

For the most part the abbreviations used in this thesis for biochemical compounds, prefixes or physical constants are those generally used in biological or biochemical literature. Their meaning is explicitly defined in the text or implicitly specified in the context in which they are used.

The following terms or abbreviations are less widely used and apply more specifically to chemotaxis research.

Chemotaxin; cytotaxin. These terms are used to describe chemical compounds that attract mobile cells. Generally speaking, the word "cytotaxin" is preferred. I have tended to use cytotaxin when discussing a specific compound or class of compounds.

CUF; SNF. These two abbreviations refer to partially purified chemotactic compounds isolated from casein. Details of the isolation procedure are given in Chapter II, but the abbreviations appear in Chapter I. Briefly, CUF refers to a chemotactic casein ultrafiltrate; SNF refers to the supernatant fraction of acid precipitated casein. The numerals following these abbraviations indicate the preparation number.

Gey's BSS; Gey's balanced salt solution. The formula for this physiological cell-suspending medium is given in the Appendix. It was usually prepared to contain 2% w/v of human serum albumin, in which case it is abbreviated Gey's BSS-2% HSA.

TBS: Triethanolamine-buffered-saline; 0,005M triethanolamine/HCl, pH 7,2 in 0,9% NaCl.

Boyden chamber; Sykes-Moore chamber: These two terms refer to the apparatus used to study chemotaxis *in vitro*. In both cases the principle is the same - i.e. a porous filter divides the chamber into a cell-containing compartment and a cytotaxin-containing compartment. Chemotaxis is shown by migration

of cells into or through the filter. The Boyden chamber is discussed in the Appendix. I did not use Sykes-Moore chambers. These are simpler in design and made of stainless steel.

Ultrafiltrate; dialysate concentrate and retentate: These terms are used to describe the fraction obtained when solutions are separated by procedures using semi-permeable membranes. The fraction passing through the membrane as a result of a hydrostatic pressure gradient across the membrane I have called the ultrafiltrate. The fraction passing through the membrane by diffusion down a concentration gradient I have referred to as the dialysate. The fraction retained by the membrane I have referred to as the concentrate or retentate.

Autoradiography; autofluorography: These terms both refer to techniques in which radioactivity is detected and localized by exposure of photographic emulsion in intimate contact with the radioactive source. Autofluorography differs from autoradiography in that, in the former case, the primary scintillator 2,5-diphenyloxazole (PPO) is included in the system to increase sensitivity for soft beta-emitting isotopes.

HPF: High-power field.

Introduction

When, at the beginning of 1972, my scientific attention was first drawn to the subject of cellular participation in the inflammatory response, I was struck by the need for an understanding of the cellular and molecular mechanisms whereby blood leucocytes are attracted to an area of injury. The literature at that time contained good technical accounts of methods available for studying chemotaxis *in vitro* and many reports of diverse compounds of biological origin with attractant, or chemotactic, properties for motile, phagocytic cells. In general, these reports tended to substantiate the belief that chemical substances generated at an inflammatory source attracted cells to that source in a teleologically appropriate way and they justified, by the consistent correlation observed, the relevance of *in vitro* procedures for studying the phenomenon. In other words, answers were available to the question "*What* substances attract?"; very few were available to the question, "*How* do they attract?".

My ambition, at the time, was to provide the answers to "*How*?" Five years, and a lot of work later, I must admit, in a sense, that this thesis is a documentary record of unfulfilled ambition! I have not produced the unequivocal answers that I had hoped for. I console myself, however, with the knowledge that I have enlarged my personal experience with a fascinating and striking biological phenomenon; I have defined, I hope, some of the problems in clearer experimental terms; and I have acquired a more confident scientific basis for pursuing my interests in this field.

For the sake of readability, I have, in compiling this thesis, relegated all general technical procedures to an Appendix at the end of the work. The remainder of the thesis is divided into five chapters, each of which deals with a more or less substantive aspect of the work.

Chapter I deals with my attempts to characterize the available *in vitro* techniques in terms of their ability to quantitate the chemotactic response, to quantitate cytotaxin activity and to discriminate between random and directional cell movement.

Chapter II records the results of my efforts to purify and characterize the cytotaxin present in casein. This work is incomplete inasmuch as I was able to take it only to the stage where I could identify the cytotaxins as oligopeptides with hydrophobic characteristics. I hope to resume and complete this work in the near future.

In Chapter III I describe the straightforward experiments I did to study some of the biochemical aspects of *in vitro* neutrophil chemotaxis.

In Chapter IV I give an account of experiments to indicate that cytotaxin treatment of neutrophils affects their surface charge and alters the avidity with which they adhere to foreign surfaces.

In Chapter V I take issue with the notion that esterase activation is an essential component of the chemotactic response. My own experimental results have left me unconvinced of the general validity of this hypothesis.

Chapter I

The study of leucocyte chemotaxis *in vitro*

Kinetics of cellular locomotion

The study of leucocyte chemotaxis *in vitro*.

The accumulation of leucocytes at the site of an inflammatory lesion is one of the most striking and fundamental phenomena in pathology. Not surprisingly, therefore, the events that cause phagocytic cells to leave the circulation and move, with directional purpose, towards an area of tissue injury, have occupied the interest of experimental pathologists since the end of the last century.

It has been assumed, with abundant subsequent experimental justification, that phagocytic cells leave the circulation in response to chemical stimuli emanating from the lesion. This assumption is implicit in the term "chemotaxis" which has been used to describe the phenomenon, and virtually all of the research that has been done on the subject has been predicated on the notion that it is chemical, rather than thermal, electrical or other physical factors, that mediate directional cellular movement.

In the early days of chemotaxis research, the phenomenon was studied by means of *in vivo* procedures in which, for example, emigration of leucocytes was observed directly in mesenteric spreads or transparent rabbit ear chambers. Others used techniques in which glass capillary tubes containing chemo-attractants were introduced into the living animal and cellular migration into the capillaries noted. Time-lapse cine-microphotography has been used to record the movement of viable cells *in vitro* and to obtain qualitative information regarding cellular locomotion. These historical aspects of chemotaxis research are well reviewed by Wilkinson (185), Harris (63) and McCutcheon (110).

A new era of chemotaxis research was initiated in 1962 when Boyden (24) described an apparatus in which a porous filter separated a

compartment containing viable cells suspended in a physiological medium from a second compartment containing a chemotaxin. Chemotaxis was measured by the extent to which cells traversed the filter.

The apparatus and procedures described originally by Boyden, were prototypes for many modifications and refinements, both in design and in experimental detail. In this chapter I present my own experience with some of the technical aspects of methods that employ the cell suspension-filter-chemotaxin principle and I consider the kinetics of cellular locomotion in these systems.

I did most of the work reported in this thesis with the standard Boyden chamber. A detailed description of this apparatus and of the techniques that I used are given in the Appendix and will not be repeated here. It is rather my present purpose to comment critically on factors that influence the choice of procedure and the validity of the conclusions that may be drawn.

Before discussing the four major modifications of the Boyden procedure, it is appropriate to comment upon two points of general relevance to all techniques that assay chemotaxis *in vitro* with filters.

Firstly, it has been my consistent experience that there is a considerable variation in response to the same chemotactic stimulus, not only by peritoneal cells harvested from different rabbits, but also by cells harvested from the same rabbit at different times. The reasons for this within- and between- rabbit biological variation are obscure, but the problem is a severe one and virtually precludes valid comparison between experimental variables that are not included in the same experiment with the same batch of cells. It also imposes a requirement for reproducibility from one experiment to the next that is more severe than that encountered with many other biological systems.

Secondly, the velocity with which cells traverse the thickness of the filter is directly related, *inter alia*, to the porosity of the filter used. This somewhat obvious fact has important technical implications. If the pore size is large for a particular cell type (for example 8µm for the polymorphonuclear leucocytes) and the time of incubation is excessive, cells may migrate through the filter, become detached and fall into the lower compartment. This cell loss from the under surface of the filter may result in cell counts that are *inversely* related to the intensity of the chemotactic response, and hence give entirely erroneous results.

If the pore size of the filter is too small, penetration of cells is retarded and, in a given time, only a lucky or robust few will penetrate the filter to any extent. Evaluation of the filters then requires "in-filter" counting procedures that are (as discussed later) technically tedious and discriminate less well between random movement and directional migration.

The four main methods for filter-assays of neutrophil chemotaxis are as follows:-

Method I: The standard Boyden technique in which cell traversal of a 3µm filter is quantitated by cell counts on the under surface of the filter.

Method II: The double-filter modification of the Boyden technique in which a second, 0,45µm, filter is placed underneath the 3µm filter to trap cells that detach from the lower surface of the latter. Cells are counted on the lower surface of the upper filter and the upper surface of the 0,45µm filter.

Method III: The method of cell-migration into the 3µm filter, in which cells are incubated for times short of those required for complete filter traversal. Chemotaxis is then assayed by

measuring the depth of penetration into the filter of the furthest two cells.

Method IV: The "in-filter cell count" method in which cells penetrate to a limited extent into a 0,65µm filter and are counted at an arbitrary plane of maximal density.

The following is a detailed discussion of these techniques.

Method I: The standard Boyden technique.

The technique originally described by Boyden (24) and adopted with minor modifications by Keller (75); Keller and Sorkin (84) and Wissler, Stecher and Sorkin (186) has a number of advantages to recommend it. It is widely used and its limitations are hence well recognized. The recommended porosity of the filter (3µm for neutrophils) is large enough to allow passage of cells yet small enough to maintain a chemotactic gradient and to prevent cells from falling through by gravity. Generally speaking the results are clearly defined and the nature of the migrating cell type can be easily identified histologically on the under surface of the filter.

A number of minor disadvantages associated with the technique have been reported. The dimensions of the chamber require large numbers of cells, making the apparatus unsuitable for clinical work; two operators are required to fill each chamber; and problems with cleaning may be encountered in chambers that are not machined from a single piece of Perspex or are not readily dismantled.

The major difficulty with the Boyden technique however, stems from a consideration of the kinetics of cell movement through a filter. Although I discuss this in more detail later in the chapter, it is appropriate to mention the technical implications at this point.

A population of cells resting upon the upper surface of a filter will, in the absence of chemotactic stimulation, move a certain distance into the filter by a process of random migration. If, after some time, the number of cells within the substance of the filter were plotted as a function of depth into the filter, a frequency distribution such as is depicted in Figure 1.1 would be expected.

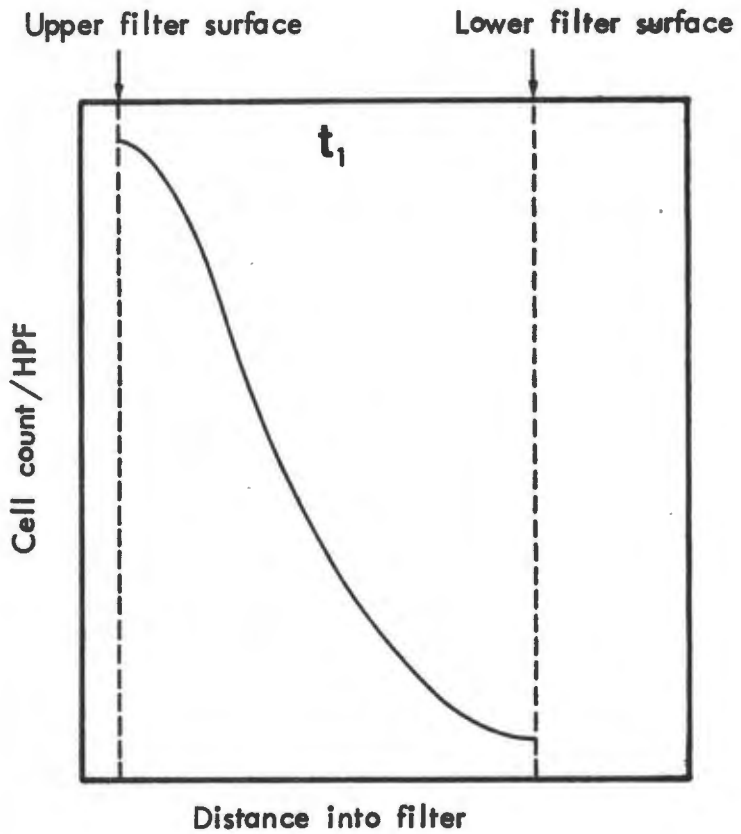

Figure 1.1

The frequency distribution of cells responding to a chemotactic stimulus would differ in that the population would migrate a greater depth into the filter to show after an equivalent period of time a roughly

normal distribution with the mode some distance from the surface but the entire population still confined within the substance of the filter. A cell count of the under surface of the filter at this time would, in the theoretical case, still be zero (Figure 1.2).

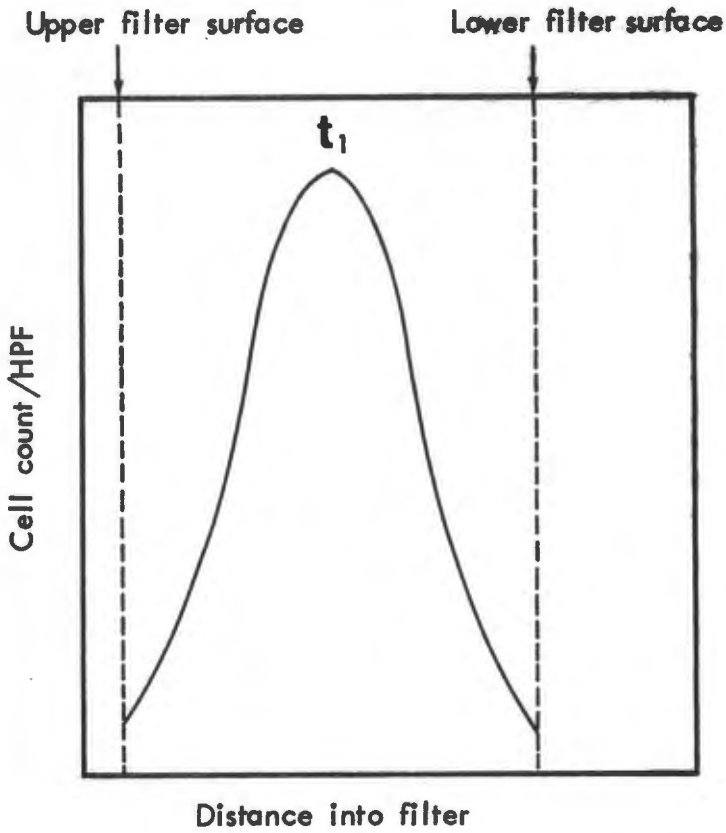

Figure 1.2

At the end of a further period of time the population would have migrated yet further into the filter. The mode would be a greater distance from the surface and the leading front of the cells would arrive and begin to accumulate on the under surface. This is represented in Figure 1.3 where the shaded area represents the "integral" of the frequency distribution curve that would be counted as cells on the

under surface of the filter.

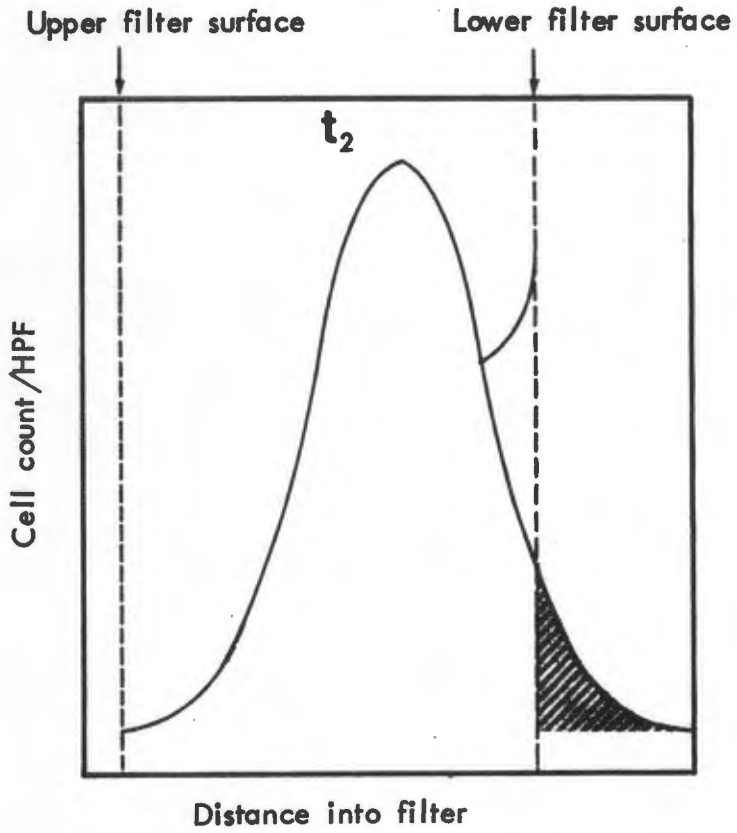

Figure 1.3

Theoretically, this integral value should increase progressively with time until the entire population of migrating cells had traversed the filter and come to adhere to the under surface. A graph of under-surface cell counts as a function of time should, therefore, reflect the sigmoid integral of a Gaussian curve. In practice, however, this does not occur since, after an undefined period of residence on the under surface, cells become detached and are lost to subsequent counting by falling into the lower chamber. As a result a relationship between

time and under-surface cell counts is observed which rises to a maximum and subsequently falls with progressive cell detachment. Since the intensity of a chemotactic response is related to the *velocity* with which cells move through the filter it is clear that a very active cell population may, after a given period of time, result in a *lower* under-surface cell count than that seen with a less active population of cells.

Furthermore, a uniformly migrating population of cells might give widely different under-surface cell counts as a result of variations in filter thickness. I have observed that the thickness of a single Millipore filter may vary, from one region to the next, by as much as 25μm (i.e. from 100 to 125μm).

To overcome these difficulties, various modifications have been introduced into the Boyden technique in an attempt to quantitate different degrees of chemotactic responsiveness or activity.

Method II: The double-filter technique.

The first attempts to circumvent the problems encountered with cell detachment were made by Keller and his co-workers who used a double-filter technique in which a second, cell-impermeable filter (0,45μm) was used to trap detached cells. The chemotactic response was then measured in terms of the sum of the cell counts on the under surface of the first filter and the upper surface of the second filter. In this way a true value for the "integral" was obtained.

This is undoubtedly a very valuable modification that overcomes most of the problems with the original technique. The method, as described by Keller, Borel, Wilkinson, Hess and Cottier (77), does however have the disadvantage that the cells are enumerated at only *one* point in time, with the result that the *velocity* of cell movement,

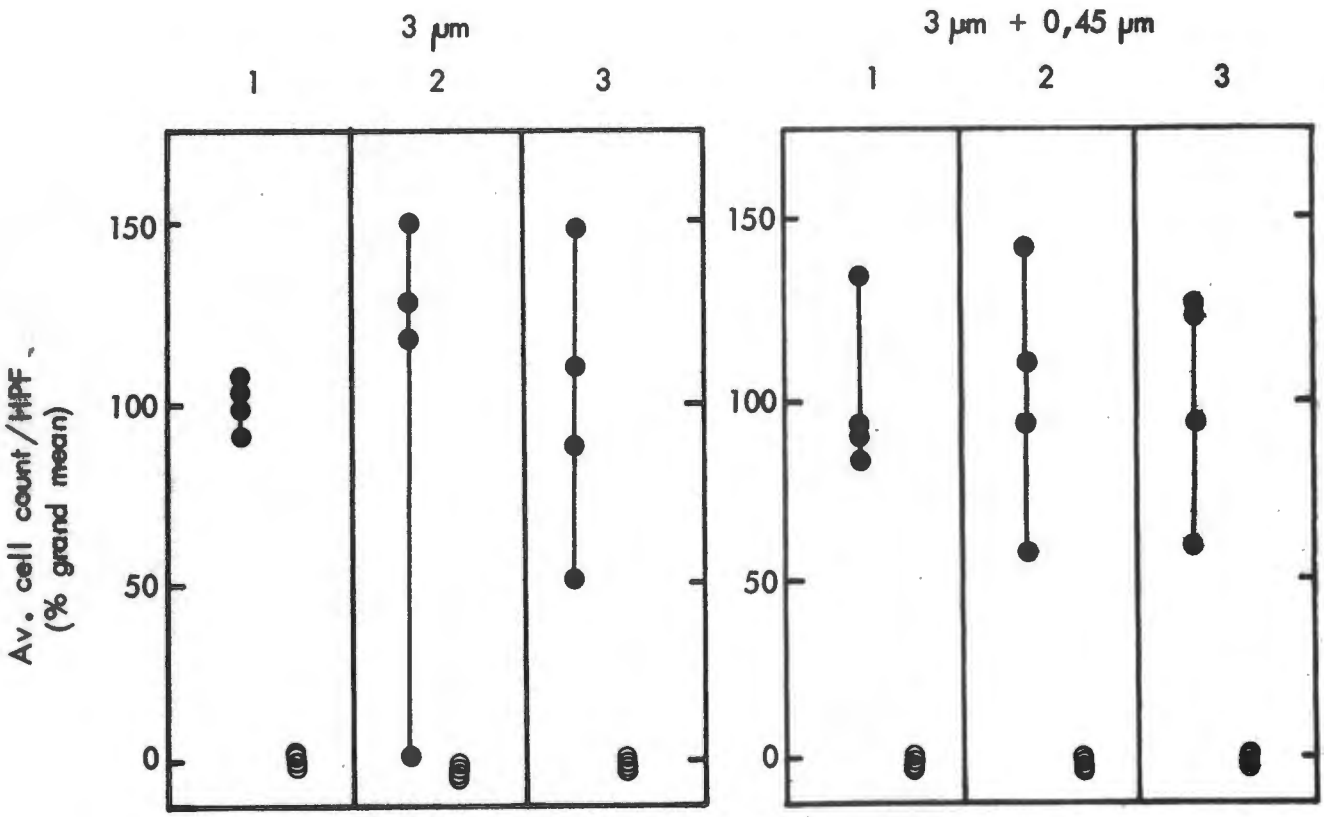

Figure 1.4 Chemotaxis assay by the method of Boyden (24) as modified by Keller (75), Keller and Sorkin (84), Wissler et al.(186) and Keller et al. (77).

Methods I and II

Results of three separate experiments (labelled 1, 2 and 3) in which $2x10^6$ cells/ml were incubated in the upper compartments of Boyden chambers and separated from 1% casein (solid symbols) or Gey's BSS/2% HSA (open symbols) by 3,0µm filters (*left*) or 3,0µm filters superposed upon 0,45µm filters (*right*).

In all cases, chambers were incubated for 180 min.

Each point represents the mean of four under-surface counts on single filters (*left*) or the sum of the means of four under-surface counts on the upper (3µm) filters and four upper-surface counts on the lower (0,45µm) filters (*right*).

Results are shown as the percentages of the grand mean obtained in the presence of cytotaxin for any particular set of experimental conditions.

or the length of time taken for the mode to traverse the filter, cannot be determined. Clearly, more than one incubation period is required to obtain an accurate estimate of the time-point at which the sigmoid curve inflects. With this further modification, i.e. different periods of incubation, the "time-to-inflection" of the sigmoid curve would be inversely related to the intensity of the response and would probably provide the most valid quantitative means for comparing the chemotactic activities of different solutions or the capacity for cell-migration of differing cell populations.

The disadvantages attendant upon the method are mainly technical in that care is required to ensure that air bubbles are not entrapped between or below the two filters and that cells are not dislodged when the filters are separated for final processing. The former is particularly difficult to avoid, since, to eliminate trapped air between the filters, they are wet in saline before assembling the chambers. When the chambers are subsequently filled the precise instant at which to load the cell compartment cannot be visually ascertained.

Method III: Cell-migration distance into the filter.

Weksler and Hill (180) were the first to quantitate chemotaxis in terms of the distance cells migrate into semi-permeable filters. Zigmond and Hirsch (194) developed this technique to surmount the problem of cell detachment from the under surface, and used it routinely after a careful study in which they compared it with generally accepted methods based upon cell counts.

In essence, the only modification introduced into the standard assay by their procedure involves a reduction of the incubation period such that migrating cells enter the body of the filter but do not move right through it. Chemotaxis is then assessed, in processed filters mounted with their upper surfaces uppermost, by measuring the distance

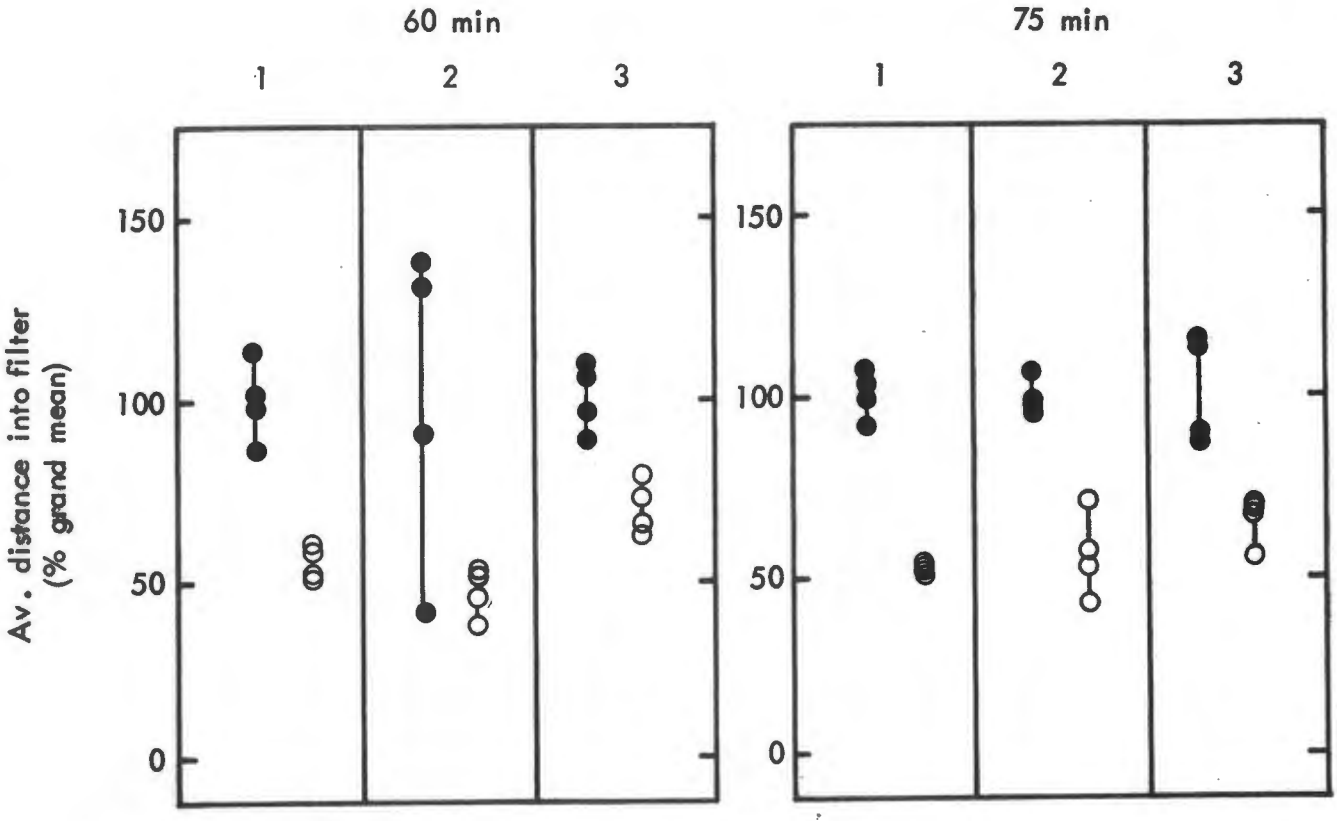

Figure 1.5 Chemotaxis assay by the methods of Weksler and Hill (180) as developed by Zigmond and Hirsch (194).

Method III

Results of 3 separate experiments (labelled 1, 2 and 3) in which cells ($2x10^6$/ml) were incubated in the upper compartment of Boyden chambers, separated from 1% casein (solid symbols) or Gey's BSS/2% HSA (open symbols) by 3μm filters.

After 60 min (*left*) or 75 min (*right*) of incubation, filters were scored for chemotaxis by measuring, with the fine-focus vernier, the furthest distance from the upper filter surface, that two cells could be brought into simultaneous focus.

Each point represents the mean of four readings taken at randomly selected, different areas on the same filter. Results are shown as percentages of the grand mean obtained in the presence of cytotaxin for any particular set of experimental conditions.

into the filter that the leading front of the cell population has moved. In practice this distance is determined by focusing upwards through the filter and taking readings with the optical micrometer on the fine-focus knob of the microscope (a) at the focal plane where two cells can first be brought into simultaneous focus, and (b) at the upper surface of the filter.

The technique is certainly less tedious than those involving cell counts and undoubtedly overcomes the problem of cell detachment and variations in filter thickness.

In considering this technique, two questions arise. Firstly, is it valid to describe the behaviour of an entire cell population by the performance of the two best performers? One's instinctive statistical sense is reluctant to accept this. Secondly, do these two cells that have migrated furthest, form part of a continuous population of cells, or are they members of a subset with a lower threshold for a quantal response to chemotactic stimulation? If the latter were the case, one would not be able to use this method to quantitate differences in chemotactic activity or responsiveness.

Zigmond and Hirsch (194) have reassured us as far as the second question is concerned by doing cell counts at different planes through the thickness of the filter and showing that the cells are, in fact, distributed continuously. My own experiments (reported later in this chapter) confirm this.

To justify the technique in the face of the first question, the authors produced experimental evidence to show that the distribution of cells through the filter in the *absence* of a chemotactic gradient conformed to that expected of a "random-walk" model. In fact, when the

logarithms of the cell counts were plotted as a function of the square of the distances into the filter, an extraordinarily good fit to a straight line was obtained (Zigmond and Hirsch (194); Figure 10) and the co-ordinates for the furthest two cells fell acceptably close to that line.

It is not clear from their publications whether or not a similar relationship would be obtained in the *presence* of a chemotactic gradient.

My own experiments (described below) indicate that this relationship does hold for cells responding chemotactically for as long as the mode of the responding cell population remains at the upper surface of the filter. Once the mode has entered the substance of the filter, (indicating that random movement alone can no longer be invoked to explain cell penetration into the filter) the linear relationship between $\log_e$ cell count and square of the distance moved is lost. It is, therefore, reasonable to conclude that the presence of a chemotactic gradient superimposes upon the random nature of cell movement an additional directional component that is itself variable in the extent to which individual cells in the population are affected by it. The complexity of the system in the presence of chemotactic material and the doubtful assumptions that are required regarding such factors as stability of the chemotactic gradient in the filter, make it difficult to justify the Zigmond and Hirsch (194) procedure on a rigorous kinetic or mathematical basis. The method can only be justified in an empirical way and I am not entirely satisfied that the proponents have done this adequately in the presence of a chemotactic gradient.

Method IV: In-filter cell counts

Ward and his co-workers have published extensively on several aspects of chemotaxis (16,17,165,166,167,168,169,170,171) and in most of these communications they refer to the original paper by Ward, Cochrane and Müller-Eberhard (170) for the technical details of the method used for studying cell movement.

This method involved a stainless steel circular chamber divided into two compartments by a 0,65µm filter. Initially the filled chambers were incubated for 3h at 37°C. (In a subsequent paper (167) where time studies were reported, the time was reduced to 60 min.) Chemotaxis was quantitated by microscopy according to the following method (taken verbatim from the original article (170); my italics),

"..... each filter contained two readily distinguishable planes of focus: an upper level was identified as the *upper surface* of the micropore filter containing PMN's and occasional mesothelial cells, monocytes and lymphocytes; the lower level was found to be the *lower surface* of the filter which contained PMN's which had migrated through the interstices of the filter. The chemotactic value of a test sample was determined by counting under high power magnification (utilizing a micrometer) five fields of the *lower surface* of the filter."

The text of the paper tells one that cell counts on the lower surface of the filter of 300-1000 per 5 high-power fields were observed and one is invited to accept, in the legend to plate 22, Figures 1(b) and 2(b) (170) that cells on the upper surface of the filter were still visible (though not in focus) when the cells on the under surface of the filter were in the focal plane.

I have two major reservations with regard to the data as presented by Ward et al. Firstly, neutrophils do not pass through a 0,65µm filter in 3h. Secondly, it is impossible, at 400x magnification,

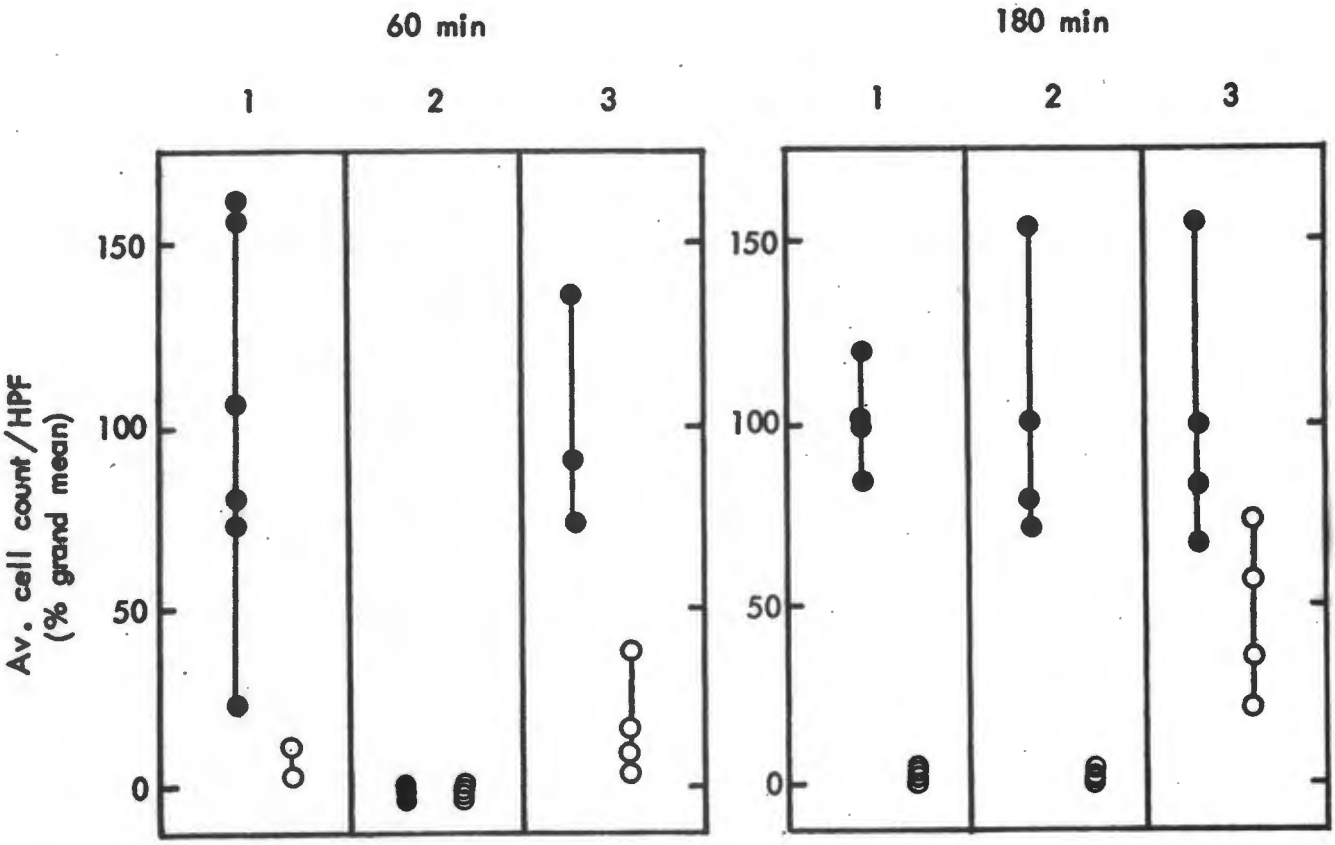

Figure 1.6 Chemotaxis assay by the methods of Ward et al.(170) and Ward and Becker (167).

Method IV

Results of three separate experiments (labelled 1, 2 and 3) in which cells ($2x10^6$/ml) were incubated in the upper compartments of Boyden chambers, separated from 1% casein (solid symbols) or Gey's BSS-2% HSA (open symbols) by 0,65μm filters.

After 60 min (*left*) or 180 min (*right*) of incubation, filters were scored for chemotaxis by counting cells at the planes of maximal density (usually 10-20μm) below the upper filter surfaces.

Each point represents the mean of the cell count in 4 high power fields on one filter. Vertical lines indicate the range observed. Results are expressed as percentages of the grand mean obtained in the presence of cytotaxin for any given set of experimental conditions.

Using this technique, under-surface cell counts were invariably zero.

to see discrete cells on the upper surface of a Millipore filter when the lower surface is in focus.

It is conceivable that Ward et al.'s technique requires cell counts at some plane within the filter other than the lower surface (it is difficult to see how it could be otherwise!), but if so they do not define that plane. Since cell distribution through the filter for different magnitudes of chemotactic stimulation vary, this is a serious omission. In my experience cell penetration of a 0,65µm filter is minimal and cells are only seen to a depth of 15 to 30µm in the case of cells responding to a chemotactic material. In the series of experiments I performed to evaluate Ward's technique I presumed that cells were counted in the filter at the focal plane where they appeared to be maximally concentrated (usually 15 to 30µm). Control filters were then counted at the same focal plane. (Had I done otherwise and followed the described method, all filters would have been negative with no cells visible on the lower filter surface even after 180 min in the presence of highly stimulating chemotactic material.)

The problems encountered in "in-filter" counting methods are tedious and numerous. Whether or not the cell observed at a particular focal plane is in fact in focus is a serious problem as the cells may become elongated in their passage through the Millipore filters and the same cell can be counted as "in focus" at several planes in the filter. Cells may also spread and contort, so becoming visible at different points in the same focal plane and thus appearing as several distinct cells.

All these factors considered I find little to recommend this technique.

If one takes into account the possible effects of chemotactic stimuli on the overall rate of random migration and the implications that this might have on chemotaxis, the use of a 0,65µm filter might be

considered in the same light as the morphological studies undertaken by Zigmond and Hirsch (194) in which an orientation of cells was used to assess the chemotactic activity of a variety of materials. As noted before, the degree of cell filter penetration is very small and it might well represent a directional orientation of the cells on the upper surface with pseudopod formation and penetration of the filter, analogous to the broad, thin veil (lamellipodium) at the front of an orientated neutrophil described by Zigmond and Hirsch (194).

Experimental study.

Despite these obvious theoretical merits and demerits of the various methods proposed, I felt it appropriate to examine these techniques with a formal comparative experimental study. Accordingly, three separate experiments were done. Each experiment was performed on a different day with peritoneal exudate cells from a different rabbit. In each experiment, four different techniques were used to measure the chemotactic response. Eight chambers were set up for each technique. In four of these, a chemotactic solution (1% w/v casein in saline) was placed in the lower compartment; the other four chambers were used to measure random migration into the filter by placing Gey's balanced salt solution supplemented with 2% w/v human serum albumin (Gey's BSS - 2% HSA) in the lower compartment. The filters used, the duration of incubation and the method employed to evaluate each filter differed for each technique according to the specifications of the author. In all other respects, conditions were the same. These experiments provided an estimate, on three separate occasions, of the following technical parameters:

(a) *Within-filter variation.* This was given by the distribution of counts for the four fields within each filter.

(b) *Between-filter variation.* This was given by the distribution of counts between each filter after subtraction of the contribution made by within-filter variation.

(c) *The ability of each technique to discriminate between directional and random migration.* This was given by comparing the range of mean filter counts obtained with and without cytotaxin.

Since the way in which the filters were evaluated differed from one technique to the other, the absolute values obtained could not be compared directly. They were therefore "normalized" for each technique by expressing each individual value as a percentage of the grand mean for the four filters incubated in the presence of a chemotactic gradient. Within- and between-filter variability (a & b above) were assessed by standard techniques for the analysis of variance. The discriminating capacity of each technique (c above) was assessed by expressing mean filter values in the presence and in the absence of cytotaxin as percentages of the grand mean obtained in the presence of cytotaxin. These results were not subjected to a statistical analysis. Examination of the results displayed graphically (Figures 1.4 to 1.6) sufficed to indicate the differences between the means and the extent to which values from stimulated and unstimulated cultures overlapped.

The manner in which the statistical calculations were performed is given in detail for one set of four filters in Table 1.1 and Table 1.2. The date for the remaining experimental results are summarized in tabular form in Tables 1.3(a) to (d). The analysis of variance of the four

methods of evaluating chemotactic responses are given in Table 1.4. The data used for Figures 1.4 to 1.6 are given in Table 1.5(a) to (d).

In principle, variability in the chemotaxis assay can be attributed to three major factors:

(i) *Variations in the results obtained from one high-power field to the next with any single filter.* Among the factors that contribute to the source of the variability, are heterogeneity of cell distribution on the filter (for example, cells tend to settle densely in depressions in the filter and sparsely on ridges); inhomogeneity in the filter thickness and internal structure; cell-to-cell variation; and, in the case of methods that require measurement of the distance cells have moved into the filter, optical and mechanical errors involved in focusing on a particular plane in the filter.

(ii) *Variations from one chamber to another.* This is reflected in significant differences between mean filter counts and may arise, *inter alia*, from the thermal heterogeneity within the incubators; trace contamination of chambers, individual filters, glassware or cell suspensions; difficulty with levelling of individual chambers; batch variability in quality of filters; and other factors that I have been at a loss to explain or identify.

(iii) *Biological variations between rabbits and cells harvested and prepared at different times.* Since I was particularly concerned with the technical aspects of the assay procedure, I have not included this source of variability in my analysis of the results obtained.

The analysis of variance to obtain estimates of within- and between-filter variability proceeded as in the following example (Table 1.1) showing the results obtained when cells that had traversed the thickness of a 3μm filter in 180 min were counted according to the method of Boyden (Method I). Four high-power fields were counted on the under surface of each of four filters giving sixteen counts in all.

Table 1.1

Technique: Method I, Experiment I.

Lower chamber: 1% w/v casein in saline

Filter	Cells per high-power field					
	1	2	3	4	Totals	Means
1	136	110	100	126	472	118
2	108	90	72	81	351	88
3	24	58	46	38	166	42
4	66	89	72	56	283	71

Expressing the results as percentages of the grand mean (79,5) the following "normalized" results were obtained.

Table 1.2

Technique: Method I, Experiment I.

Lower chamber: 1% w/v casein in saline

Filter	Cells per high-power field(% of grand mean)				Row Totals	Means
	1	2	3	4		
1	171,1	138,4	125,8	158,5	593,7	148,4
2	135,8	113,2	90,6	101,9	441,5	110,4
3	30,2	73,0	57,9	47,8	208,8	52,2
4	83,0	112,0	90,6	70,4	356,0	89,0

The following terms were then calculated:

(a) *The sum of the squares of the individual filters*

i.e. $(171,1)^2 + (138,4)^2 \ldots\ldots + (90,6)^2 + (70,4)^2 = 183\ 660,456.$

(b) *The sum of each row total squared, divided by the number in that row*

i.e. $\frac{(593,7)^2}{4} + \frac{(441,5)^2}{4} + \frac{(208,8)^2}{4} + \frac{(356,0)^2}{4} = 179\ 435,149$

(c) *The grand total squared and divided by the total number of observations*

i.e. $\frac{(593,7 + 441,5 + 208,8 + 356,0)^2}{16} = 160\ 000$

The analysis of variance is then as follows:

Source of variance	Sums of squares	Degrees of freedom	Mean squares
Between-filter	(b)-(c) = 19 435,2	3	6 478,4
Within-filter	(a)-(b) = 4 225,3	12	352,1
Total	(a)-(c) = 23 660,5	15	-

Testing the between-filter mean square in the above analysis, it gives a variance ratio of 6 478,4/352,1= 18,399 for $n_1 = 3$ and $n_2 = 12$. This is highly significant ($p < 0,001$) indicating that a real between-filter variability exists.

If (a) N, is the total number of observations and n_i the number in each row for i = 1 to k (in this case k = 4) and

(b)σ^2_B and σ^2_W are the components of the total variance that can be ascribed to between- and within-filter effects respectively, then the within-filter mean squares estimates σ^2_W and the between-filter mean squares estimates

$$\frac{N^2 - \Sigma n_i^2}{N(k-1)} \cdot \sigma^2_B + \sigma^2_W.$$

Substituting we have σ^2_W = 352,1 and

$$\left(\frac{256 - 64}{48}\right) . \sigma^2_B + 352,1 = 6\ 478,4$$

$$\therefore \sigma^2_B = 1\ 531,6$$

In summary, therefore, the data may be represented as follows:

Method	:	I
Experiment	:	1
Variance ratio	:	18,399
P	:	< 0,001
Component of variance		
-Within	:	352,1
-Between	:	1 531,6

These values are given for all experiments in tabular form in Table 1.4.

As can be seen from the tabulated results, within-filter variability was least with Method III, slightly greater with Methods I, II and IV incubated for 60 min, and greatest with Method IV incubated for 180 min. Significant between-filter variability was encountered in at least one experiment with all methods used. This tended to be lower with Method III, and much the same with all other techniques.

When the ability of different techniques to discriminate between stimulated and random migration was examined, all methods were able to discriminate although distinct differences between techniques was apparent (Figures 1.4 to 1.6). Those methods that relied on counting cells that had traversed the filter, Methods I and II (Figure 1.4) showed clear and obvious differences between cell movement in the presence and absence of cytotaxin.

Method III, in which filters were evaluated by measuring the distance into the filter that the furthest two cells had moved, showed overlap between results for stimulated and randomly migrating cells in only one experiment when a 60 min incubation time was used

(Figure 1.5). In terms of the relative magnitude of the values obtained for these two populations, the differences were by no means as striking as those seen with Methods I and II.

Method IV discriminated well in two experiments when the incubation time was 180 min. In the third experiment there was overlap between the ranges of mean filter counts with and without cytotaxin. When an incubation period of 60 min was allowed, as suggested by Ward and Becker (167), Method IV discriminated erratically and somewhat poorly between cells randomly migrating and cells responding to a chemotactic stimulus, compared to the other three methods (Figure 1.6).

While this work was in progress, Gallin, Clark and Kimball (52) described a technique in which ^{51}Cr-labelled neutrophils were allowed to migrate through two 5μm filters. After 3h incubation the lower filter (assumed to contain the bulk of the responding cell population) was removed and counted in a gamma-scintillation counter. Chemotaxis was then expressed in terms of counts per minute in the lower filter as a percentage of the counts per minute per $1x10^6$ granulocytes. This method has two advantages.

Firstly, cells do not traverse the entire thickness of the second, lower, filter, hence the detachment problem is overcome.

Secondly, use of radio-labelled cells avoids the tedious and variable procedure of visual counting.

Since I had already standardized my technique I was reluctant to change to another and hence did not explore the method as fully as I might have done. The one experiment which I did do was sufficient to indicate that there were certain difficulties associated with its use. In the first place, ^{51}Cr-labelled sodium chromate is not a stable marker for neutrophils. As much as 10 to 30% of the cell-bound label

Table 1.3(a)

Chemotactic response of rabbit polymorphonuclear leucocytes evaluated according to Method I; Boyden (24)

Expt no.	Filter (3µm)	Cell count per high-power field					Grand mean	Cell count as % grand mean				
		1	2	3	4	Mean		1	2	3	4	Mean
1	1	172	143	136	208	165	169	102,2	85,0	80,8	123,6	97,9
	2	148	150	164	156	155		88,0	89,2	97,5	92,7	91,8
	3	128	236	196	158	180		76,1	140,3	116,5	93,9	106,7
	4	216	149	160	172	174		128,4	88,6	95,1	102,3	103,6
2	1	192	184	172	160	177	118	162,7	155,9	145,8	135,6	150,0
	2	128	158	142	180	152		108,5	133,9	120,3	152,5	128,8
	3	140	161	136	124	140		118,6	136,4	115,3	105,1	118,9
	4	4	2	2	3	3		3,4	1,7	1,7	2,5	2,3
3	1	136	110	100	126	118	80	171,1	138,4	125,8	158,5	148,4
	2	108	90	72	81	88		135,8	113,2	90,6	101,9	110,4
	3	24	58	46	38	42		30,2	73,0	57,9	47,8	52,2
	4	66	89	72	56	71		83,0	112,0	90,6	70,4	89,0

The chemotactic response of rabbit peritoneal neutrophils to a 1% w/v solution of casein (Hammarsten) in the lower compartment of a Boyden chamber, scored in terms of an estimate of the number of cells that had traversed the entire thickness of a 3µm Millipore filter in 180 min and that thad remained attached to the lower filter surface i.e. cell counts of the adherent cell population at a magnification of 320x.

Table 1.3(b)

Table 1.3(b)

Chemotactic response of rabbit polymorphonuclear leucocytes evaluated according to Method II; Keller et al.(77)

Expt no.	Filter (μm)	Cell count per high-power field 1	2	3	4	Mean	Σ	Grand mean	Σ Cell count as % grand mean 1	2	3	4	Mean
1	1 3	104	120	161	176	140	140	151	68,7	79,3	106,4	116,3	92,7
	0,45	0	0	0	0	0							
	2 3	152	168	121	106	137	137		100,4	111,0	79,9	70,0	90,3
	0,45	0	0	0	0	0							
	3 3	188	201	208	212	202	202		124,2	132,8	137,4	140,1	133,6
	0,45	0	0	0	0	0							
	4 3	132	164	110	99	126	126		87,2	108,3	72,7	65,4	83,4
	0,45	0	0	0	0	0							
2	1 3	121	143	98	136	125	174	124	140,6	165,8	113,8	142,2	140,6
	0,45	52	61	42	39	49							
	2 3	33	28	64	71	49	72		67,4	39,0	66,6	59,3	58,1
	0,45	50	20	18	2	23							
	3 3	120	102	99	89	103	135		136,5	106,5	105,6	88,6	109,3
	0,45	48	29	31	20	32							
	4 3	88	108	80	76	88	113		92,6	111,3	76,4	87,8	92,0
	0,45	26	29	14	32	25							

3	1	3	30	52	36	41	40	62	105	51,5	83,9	51,5	48,6	58,8
		0,45	24	36	18	10	22							
	2	3	46	71	110	128	89	98		49,6	80,0	112,4	130,6	93,2
		0,45	6	13	8	9	9							
	3	3	120	116	100	99	109	131		133,4	132,5	118,2	115,3	124,8
		0,45	20	23	24	22	22							
	4	3	152	104	96	124	119	129		150,6	110,5	97,2	134,4	123,2
		0,45	6	12	6	17	10							

The chemotactic response of rabbit peritoneal neutrophils towards a 1% w/v casein (Hammarsten) solution assessed in terms of the number of cells which had traversed the entire thickness of a 3µm Millipore filter in 180 min. The results in the table give, for each pair of filters, the cells counted in 4 high-power (320x) fields on the lower surface of the 3µm filter and 4 high-power fields on the upper surface of the associated 0,45µm filter. The column headed "Σ" gives the sum of the means of these two sets of 4 counts. The 0,45µm filter served as a "trap" for cells that detached from the lower surface of the upper filter.

Table 1.3(c)

Table 1.3(c)

Chemotactic response of rabbit polymorphonuclear leucocytes evaluated according to Method III; Zigmond and Hirsch (194)

Expt no.	Incubation time (min)	Filter (3μm)	Distance from upper surface of filter (μm) 1	2	3	4	Mean	Grand mean	Distance as % of grand mean 1	2	3	4	Mean
1	60	1	38	52	39	59	47		79,4	108,8	81,6	123,4	98,3
		2	44	48	36	38	42	48	92,0	100,4	75,3	79,5	86,8
		3	51	51	44	48	49		106,7	106,4	92,0	100,4	101,4
		4	56	47	57	57	54		117,1	98,3	119,2	119,2	113,5
2	60	1	40	15	45	25	31		52,5	19,7	59,0	32,8	41,0
		2	105	95	100	100	100	76	137,7	124,6	131,1	131,1	131,1
		3	120	85	100	115	105		157,4	111,5	131,1	150,8	137,7
		4	80	55	60	80	69		104,9	72,1	78,7	104,9	90,2
3	60	1	70	60	70	100	75		101,8	87,3	101,8	145,5	109,1
		2	75	70	80	65	73		109,1	101,8	116,4	94,5	105,5
		3	70	50	60	65	61	69	101,8	72,7	87,3	94,5	89,1
		4	55	60	70	80	66		80,0	87,3	101,8	116,4	96,4

1	75	1	48	53	54	51	52	49	99,1	109,4	111,5	105,3	106,3
		2	45	30	53	49	44		92,9	61,9	109,4	101,2	91,4
		3	53	45	48	53	50		109,4	92,9	99,1	109,4	102,7
		4	46	48	50	49	48		95,0	99,1	103,2	101,2	99,6
2	75	1	80	85	90	100	89	94	85,1	90,4	95,7	106,3	94,4
		2	100	80	85	95	90		106,3	85,1	90,4	101,0	95,7
		3	100	110	95	90	99		106,3	116,9	101,0	95,7	105,0
		4	100	100	95	100	99		106,3	106,3	101,0	106,3	105,0
3	75	1	110	90	85	90	94	82	134,4	109,9	103,8	109,9	114,5
		2	90	100	85	95	93		109,9	122,1	103,8	116,0	113,0
		3	70	80	65	70	71		85,5	97,7	79,4	85,5	87,0
		4	70	65	70	75	70		85,5	79,4	85,5	91,7	85,5

The chemotactic response of rabbit peritoneal neutrophils towards a 1% w/v casein (Hammarsten) solution. Filters were scored by measuring, with the optical micrometer on the fine-focus knob of the microscope, the distance from the top of the filter to the furthest plane which contained at least two cells in focus at a 320x magnification.

Table 1.3(d)

Table 1.3(d)

Chemotactic response of rabbit polymorphonuclear leucocytes evaluated according to Method IV; Ward et al. (170) and Ward and Becker (167)

Expt no.	Incubation time (min)	Filter (0,65µm)	Cell count per high-power field 1	2	3	4	Mean	Grand mean	Cell count as % of grand mean 1	2	3	4	Mean
1	60	1	14	20	30	26	23		14,8	21,1	31,7	27,5	23,8
		2	51	69	81	74	69		53,9	72,9	85,5	78,1	72,6
		3	56	70	94	80	75		59,1	73,9	99,3	84,5	79,2
		4	82	100	99	120	100	95	86,6	105,6	104,5	126,7	105,9
		5	124	154	168	148	149		130,9	162,6	177,4	156,3	156,8
		6	131	149	160	173	153		138,3	157,3	168,9	182,7	161,8
2*	60	1	0	0	0	0	0		0	0	0	0	0
		2	0	0	0	0	0		0	0	0	0	0
		3	0	0	0	0	0	0	0	0	0	0	0
		4	0	0	0	0	0		0	0	0	0	0
3	60	1	71	68	49	61	62		83,4	79,8	57,5	71,6	73,1
		2	114	121	100	131	117		133,9	142,1	117,4	153,8	136,8
		3	63	76	98	70	77	85	74,0	89,2	115,1	82,2	90,1

1	180	1	140	96	154	189	145	174	80,8	55,4	88,9	109,1	83,6
		2	201	166	144	178	172		116,0	95,8	83,1	102,7	99,4
		3	220	196	124	138	170		127,0	113,1	71,6	79,7	97,8
		4	124	248	230	224	207		71,6	143,1	132,8	129,3	119,2
2	180	1	66	68	80	100	79	101	65,6	67,6	79,6	99,4	78,1
		2	167	181	142	121	153		166,1	180,0	141,2	120,3	152,0
		3	160	124	0	0	71		159,1	123,3	0	0	70,6
		4	120	0	148	132	100		119,3	0	147,2	131,3	99,4
3	180	1	52	61	74	46	58	72	72,9	85,5	103,7	64,4	81,6
		2	60	81	62	78	70		84,1	113,5	86,9	109,3	98,4
		3	48	58	84	0	48		67,3	81,3	117,7	0	66,6
		4	98	101	115	124	110		137,3	141,5	161,1	173,7	153,4

*Cells were concentrated on the upper surfaces of the filters and no penetration into the substance of the filters was evident.

The chemotactic response of rabbit peritoneal neutrophils to a 1% w/v casein (Hammarsten) solution.
Cells were counted, at a magnification of 320x, at varying distances into the filter, where the cell density appeared greatest. Individual cell counts for each of 4 high-power fields on each filter are given.

Table 1.4

Analysis of variance of four methods of evaluating the chemotactic response of rabbit polymorphonuclear leucocytes.

Technique	Incubation time (min)	Expt no.	Variance Within	Variance Between	Variance ratio	p
Method I Boyden(24)	180	1	369	0	0,46	N.S.
		2	168	4366	105,25	<0,001
		3	352	1532	18,40	<0,001
Method II Keller et al.(77)	180	1	314	439	6,59	<0,01
		2	309	1107	15,32	<0,001
		3	555	826	6,95	<0,01
Method III Zigmond and Hirsch(194)	60	1	185	74	2,49	N.S.
		2	271	1923	29,43	<0,001
		3	284	10	1,15	N.S.
	75	1	135	7	1,21	N.S.
		2	67	17	2,01	N.S.
		3	82	232	12,26	<0,001
Method IV Ward and Becker(167)	60	1+	254	2752	44,39	<0,001
		2*	-	-	-	-
		3x	227	1031	19,16	<0,001
		1	606	63	1,42	N.S.
		2	3082	576	1,75	N.S.
		3	808	1236	7,12	<0,01

+ Data obtained from 6 filter scores.

* No chemotactic response was evident

x Data obtained from 3 filter scores.

Three different rabbit peritoneal exudate cell populations (experiments 1 to 3) were tested for their response to 1% w/v casein (Hammarsten). Four filters were scored per method with four determinations being performed on each filter (i.e. 16 estimates of the chemotactic response per method, per experiment).

Table 1.5(a)

Directional and random movement through 3μm filters, scored according to Method I; Boyden (24)

Expt no.	Filter (3μm)	Directional movement* Cell count per high-power field					Grand mean	Random movement+ Cell count per high-power field				
		1	2	3	4	Mean		1	2	3	4	Mean
1	1	172	143	136	208	165	169	2	2	3	1	2
	2	148	150	164	156	155		0	0	0	0	0
	3	128	236	196	158	180		0	0	0	0	0
	4	216	149	160	172	174		3	4	3	2	3
2	1	192	184	172	160	177	118	0	0	0	0	0
	2	128	158	142	180	152		0	0	1	0	0
	3	140	161	136	124	140		0	0	0	0	0
	4	4	2	2	3	3		0	0	0	0	0
3	1	136	110	100	126	118	80	0	0	0	0	0
	2	108	90	72	81	88		0	0	0	0	0
	3	24	58	46	38	42		0	0	0	0	0
	4	66	89	72	56	71		0	0	0	0	0

*1% casein in lower compartment of Boyden chamber.

+ Gey's BSS - 2% HSA in lower compartment of Boyden chamber.

Cells adherent to the lower surface of each filter were counted in 4 high-power fields.

Table 1.5(b)

Table 1.5(b)

Directional and random movement through 3μm filter, scored according to Method II; Keller et al.(77)

Expt no.	Filter	(μm)	Directional movement* Cell count per high-power field 1	2	3	4	Mean	Σ	Grand mean	Random movement+ Cell count per high-power field 1	2	3	4	Mean	Σ
1	1	3	104	120	161	176	140	140		0	1	1	0	1	1
		0,45	0	0	0	0	0			0	0	0	1	0	
	2	3	152	168	121	106	137	137		0	0	0	0	0	0
		0,45	0	0	0	0	0		151	0	0	0	0	0	
	3	3	188	201	208	212	202	202		0	0	0	0	0	0
		0,45	0	0	0	0	0			0	0	0	0	0	
	4	3	132	164	110	99	126	126		2	4	2	1	2	2
		0,45	0	0	0	0	0			0	0	0	0	0	
2	1	3	121	143	98	136	125	174		0	0	0	0	0	0
		0,45	52	61	42	39	49			0	0	0	0	0	
	2	3	33	28	64	71	49	72		0	2	1	1	1	1
		0,45	50	20	18	2	23		124	0	0	0	1	0	
	3	3	120	102	99	89	103	135		2	1	2	1	2	2
		0,45	48	29	31	20	32			0	0	0	0	0	
	4	3	88	108	80	76	88	113		1	1	0	0	1	1
		0,45	26	29	14	32	25			0	0	0	0	0	

3	1	3	30	52	36	41	40	62		0	0	0	0	0	0
		0,45	24	36	18	10	22			0	0	0	0	0	
	2	3	46	71	110	128	89	98		1	0	2	0	1	1
		0,45	6	13	8	9	9		105	0	0	0	0	0	
	3	3	120	116	100	99	109	131		0	0	0	0	0	0
		0,45	20	23	24	22	22			0	0	0	0	0	
	4	3	152	104	96	124	119	129		0	0	0	0	0	0
		0,45	6	12	6	17	10			0	0	0	0	0	

*1% casein in lower compartment of Boyden chamber

+ Gey's BSS - 2% HSA in lower compartment of Boyden chamber.

The total number of cells traversing the entire filter thickness of a 3µm Millipore filter in 180 min was taken as a measure of cell movement for each filter, this is given as the result of each of 4 individual counts on the lower surface of the 3µm filter and 4 counts on the upper surface of the associated 0,45µm filter. The column headed "Σ" represents the sum of the means of these 2 sets of 4 counts for each filter.

Table 1.5(c)

Table 1.5(c)

Directional and random movement through 3μm filters, scored according to Method III; Zigmond and Hirsch (194)

Expt no.	Incubation time (min)	Filter (3μm)	Directional movement* Distance from upper surface of filter(μm) 1	2	3	4	Mean	Grand mean	Random movement+ Distance from upper surface of filter(μm) 1	2	3	4	Mean
1	60	1	38	52	39	59	47		25	34	26	26	28
		2	44	48	36	38	42	48	30	21	19	26	24
		3	51	51	44	48	49		26	24	27	18	24
		4	56	47	57	57	54		19	31	24	36	28
2	60	1	40	15	45	25	31		45	15	35	20	29
		2	105	95	100	100	100	76	30	45	50	30	39
		3	120	85	100	115	105		30	40	35	55	40
		4	80	55	60	80	69		45	40	30	25	35
3	60	1	70	60	70	100	75		40	65	60	50	54
		2	75	70	80	65	73	69	40	35	50	45	43
		3	70	50	60	65	61		50	40	35	55	45
		4	55	60	70	80	66		50	45	50	55	50

1	75	1	48	53	54	51	52		25	20	24	26	24
		2	45	30	53	49	44	49	27	24	26	22	25
		3	53	45	48	53	50		36	16	24	22	25
		4	46	48	50	49	48		20	26	25	24	24
2	75	1	80	85	90	100	89		65	50	45	50	53
		2	100	80	85	95	90	94	70	60	65	70	66
		3	100	110	95	90	99		40	35	45	40	40
		4	100	100	95	100	99		55	50	40	50	49
3	75	1	110	90	85	90	94		45	50	35	50	45
		2	90	100	85	95	93	82	65	50	50	60	56
		3	70	80	65	70	71		50	55	60	50	54
		4	70	65	70	75	70		50	60	50	55	54

* 1% casein in lower compartment of Boyden chamber.

\+ Gey's BSS-2% HSA in lower compartment of Boyden chamber.

The distance from the top of a 3μm Millipore filter to the furthest plane which contained at least two cells in focus at a magnification of 320x, was taken as the measure of cell movement. Measurements were made with the optical micrometer on the fine-focus knob of the microscope.

Table 1.5(d)

Table 1.5(d)

Directional and random movement through 3μm filters, scored according to Method IV; Ward et al.(170) and Ward and Becker (167)

Expt no.	Incubation time (min)	Filter (0,65μm)	Directional movement* Cell count per high-power field 1	2	3	4	Mean	Grand mean	Random movement+ Cell count per high-power field 1	2	3	4	Mean
1	60	1	14	20	30	26	23		10	6	7	21	11
		2	51	69	81	74	69		2	3	2	8	4
		3	56	70	94	80	75	95	–	–	–	–	–
		4	82	100	99	120	100		–	–	–	–	–
		5	124	154	168	148	149		–	–	–	–	–
		6	131	149	160	173	153		–	–	–	–	–
2x	60	1	0	0	0	0	0		0	0	0	0	0
		2	0	0	0	0	0		0	0	0	0	0
		3	0	0	0	0	0	0	0	0	0	0	0
		4	0	0	0	0	0		0	0	0	0	0
3	60	1	71	68	49	61	62		21	18	52	40	33
		2	114	121	100	131	117		4	2	6	8	5
		3	63	76	98	70	77	85	3	10	15	8	9
		4	–	–	–	–	–		10	15	17	18	15

1	180	1	140	96	154	189	145		3	4	7	10	6
		2	201	166	144	178	172	174	2	3	0	2	2
		3	220	196	124	138	170		0	2	0	0	1
		4	124	248	230	224	207		0	1	0	2	1
2	180	1	66	68	80	100	79		0	0	0	0	0
		2	167	181	142	121	153	101	0	0	0	0	0
		3	160	124	0	0	71		0	0	0	0	0
		4	120	0	148	132	100		0	0	0	0	0
3	180	1	52	61	74	46	58		29	92	42	44	52
		2	60	81	62	78	70	72	44	32	28	56	40
		3	48	58	84	0	48		21	20	29	30	25
		4	98	101	115	124	110		10	20	0	0	8

* 1% casein in lower compartment of Boyden chamber.

\+ Gey's BSS-2% HSA in lower compartment of Boyden chamber.

x No evident cellular response.

The cell count in each of 4 high-power fields (320x), at that focal plane within a 0,65µm Millipore filter where cell density appeared to be the greatest, was used to score each filter.

may be lost spontaneously during the course of a 3h incubation.

Secondly, minor variations in the diameter of the sealing bung in the Boyden chambers give rise (as a function of the square of the diameter) to variations in the area of the upper filter available for cells to settle and adhere. Without very accurately machined chambers, therefore, I obtained erratic results for between-filter radioactivity measurement that overshadowed differences due to variations in chemotactic responses.

Had circumstances permitted, I should have liked to have spent more time with this method. The use of a less labile radioactive cell marker; precautions to avoid chamber-to-chamber variations; and a careful study of the kinetics of two-filter traversal might make this a most useful technique.

Since completion of this aspect of my study, the use of specially treated nuclepore polycarbonate plastic filters[1], first employed by Horwitz and Garrett (65), has become more popular. Not having used these filters I cannot offer criticism of their use based upon experience. The following points, however, are relevant.

(a) The transparent, thin filters do offer certain advantages. They do not need dehydration and clearing, are easily marked for identification and reduce incubation times. It is claimed that problems of thickness, non-uniformity and inconsistent results with filter batches do not arise.

(b) With the filter being only 10μm in thickness (the approximate average diameter of a rabbit neutrophil), I can foresee problems arising regarding the exact position of a cell. The pores, running straight through the filter, offer easy

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(1) Nuclepore Corporation, Pleasanton, California, U.S.A.

access to the lower filter surface for pseudopodia formed by cells still resident on the upper surface. Thus the cell would be "in-focus" on both surfaces and a decision as to whether cell movement was directional or random would be difficult. The same problem is evident in the assay system of Ward et al.(170) discussed previously (Method IV).

(c) It has been shown by myself and others (81, 194) that chemotactic agents increase the intrinsic random cell motility as well as imposing an additional downward directional component to the movement. The use of these filters, therefore, would not allow one to relate the filter under-surface cell count to an increased random movement or to a chemotactic response in terms of the present methods for differentiating the two processes.

Thus, of the four methods that I examined in these experiments, the first method; that of Boyden (Method I) was able to discriminate between directional and random migration and gave obvious results when the lower chamber contained a cytotaxin. Although admittedly a useful device, the addition of a second filter to trap detached cells did not seem to me to offer any special advantages for most purposes for which I required the assay. I was able, with a single filter, to detect peaks of chemotactic activity in column chromatography effluents (Chapter II) and this method was able to distinguish, in a reasonably quantitative manner, different concentrations of chemotactic agent in the lower chamber (discussed later). I therefore adopted this method for most of the work reported in this thesis. In retrospect, I think that I was fortunate in that cell detachment, although it undoubtedly occurred in my experiments, was not of sufficient magnitude to vitiate

the conclusions I wished to draw.

The method of Zigmond and Hirsch (194) (Method III) was less tedious to perform. It gave more reproducible results and provided a definite measure of random migration that the other methods did not. My own experimental needs were for a technique that would distinguish clearly between directional and haphazard cell movement. This fact, coupled with my concern regarding the statistical admissibility of using the leading front of migrating cells to characterize the behaviour of the population, dissuaded me from using the technique routinely.

The technique described by Ward et al. (170) (Method IV) offered me no advantage and was altogether too ill-defined for me to adopt it.

In reflecting upon the *in vitro* techniques available for studying chemotaxis, I have found it of interest that, without exception, workers in the field have failed to give specific emphasis to the point that cell migration is, in essence, a displacement of a population of cells *as a function of time*. In other words, one should really attempt to measure chemotaxis in terms of *velocity*, a vector quantity with both magnitude and direction. Random movement, analogous to diffusion in simple physical systems, should be measured in terms of *scalar* quantity, such as the diffusion coefficient, that has magnitude only and included in its derivation a probabilistic error function that recognizes the essential features of random migration - i.e. *cellular redistribution*.

In the context of cellular migration through a filter, there is one entirely reliable feature that distinguishes directional from random migration; in the former the mode leaves the upper surface of the filter whereas in the latter it does not. I feel convinced, therefore, that the proper way to describe the magnitude of a chemotactic response is in terms of the velocity with which the mode moves through

the structure of the filter. This could readily be done with the improved chamber and the technical innovations described recently by Keller and co-workers (76,77,78). I suspect that this technical improvement will do much to further our understanding of chemotaxis.

The Kinetics of cellular locomotion *in vitro.*

Theoretical considerations.

The Boyden chamber technique for the *in vitro* study of neutrophil movement provides, at the start of an experiment (t_o), a population of cells that have settled to form a monolayer on the upper surface of a Millipore filter. The filter is of finite thickness (approximately 125μm) and is composed of a randomly oriented meshwork of cellulose ester fibres (122) compacted together to give an *average trans-filter porosity* of approximately 3μm. This pore size is sufficiently small to constitute an effective barrier to neutrophils, of average diameter 7-9μm falling through by gravity. The intricate structure of the filter is such that the interstices between the fibres have dimensions, in random directions, that allow relatively free passage of the neutrophils. Thus these cells, by following a tortuous course, may penetrate the substance of the filter and traverse its thickness to reach the lower surface. On reaching the under surface of the filter the cells adhere for a period of time after which they detach and fall into the lower chamber. This loss of cells may be prevented by the presence of a second filter, of considerably lower porosity, underneath the first (77). Cells are then able to leave the substance of the first filter and adhere to the upper surface of the second.

We may now consider two sets of experimental circumstances:

(a) Chemotaxin is absent from both upper and lower chambers *or* present on both sides of the filter as the same concentration. In either case there will be no chemotactic gradient across the filter.

(b) Chemotaxin is present at a concentration (C) in the lower chamber and absent from the upper. In this case there will be a concentration gradient across the thickness of the filter (l), maximal below and minimal above. This we refer to as a positive chemotactic gradient. As time proceeds, passive diffusion of chemotaxin across the filter will take place, so that the "steepness" of the gradient (dC/dl) will diminish. We assume, with sufficient validity for our present approximate purpose, that this gradient is uniform and linear, and remains so for the duration of the usual experiment.

Random migration.

Within a very short time of adhesion to the upper surface, cells begin to penetrate the substance of the filter, deriving the energy for this motility from metabolic processes. In the absence of a chemotactic gradient (and ignoring the insignificant effects of gravity) the movement of the cells should be random. This haphazard locomotion, with each cell undergoing many displacements in arbitrary directions, will result in the cell population becoming redistributed in a manner dictated by the random walk equation. Thus if we define $P(x,t)$ as the probability of a cell occupying a plane of the filter parallel to, and $x\mu m$ from the upper surface at any time $t > t_o$, we should find that the following relationship obtains

$$P(x,t) = \frac{N}{\sqrt{\pi\kappa \cdot t}} \cdot e^{-x^2/\kappa t} \qquad \ldots\ldots\ldots(1)$$

Here κ is a constant that incorporates the mean square velocity of the cells and the resistance to cell movement offered by the filter. These we assume remain constant during the course of the experiment.

The number of migrating cells (i.e. the value of the integral $\int_0^\infty P(x,t)\,dx$) is N.

Taking logarithms we have, from Equation (1)

$$\ln P(x,t) = \ln \frac{N}{\sqrt{\pi\kappa \cdot t}} - \frac{1}{\kappa t} \cdot x^2 \qquad \ldots\ldots\ldots(2)$$

Thus, at any particular time, a plot of the cell density as a function of distance from the upper surface of the filter should yield a curve determined by the parameters of Equation (1). A plot of the logarithm of the cell density as a function of the square of the vertical distance from the upper surface of the filter should yield a straight line with slope $\frac{-1}{\kappa t}$ and intercept $\ln \frac{N}{\sqrt{\pi\kappa \cdot t}}$

The effects (a) of increasing the mean square velocity (hence κ) and (b) of the passage of time, upon the theoretical frequency distributions of 10 000 cells have been calculated from equation (1) and equation (2) and are shown graphically in Figures 1.7 through 1.10. These diagrams illustrate the following essential characteristics of random cellular locomotion.

(a) The mode does not leave the upper surface of the filter.

(b) For a given mean square velocity, the distribution curve spreads, to become wider and flatter with the passage of time and the slope of the line drawn from equation (2) becomes less steep.

(c) At any particular time, the effect of increasing the value of κ is to spread the distribution curve and to diminish the slope of the line drawn from Equation (2).

Thus, if one wishes to study the effect of a chemotactic substance upon random migration (or the mean square velocity in random directions), one should prepare a series of Boyden chambers each containing a different concentration of chemotaxin, but having the same concentration of chemotaxin in the upper and lower compartments of any one chamber. The chambers should be incubated for the same length of time and in-filter cell counts done at different planes of the filter. The effect of chemotaxin on cell motility should then be evident as a progressive effect upon the *slope* of the line drawn from Equation (2) for each filter.

It is evident, from a consideration of Equation (2), that the value of the intercept will, in the presence of a constant κ and t, be influenced by the value of N. Thus the situation of the line will be determined both by the intrinsic motility of the cells *and* by the number of cells introduced into the chamber. In other words, the leading front of migrating cells, or the "toe" of the distribution curve as Zigmond and Hirsch (194) have called it, will penetrate further into the filter with increasing numbers of cells in the upper chamber, irrespective of the intrinsic motility of these cells. As discussed earlier, Zigmond and Hirsch have provided a convincing empirical justification for measuring random cell movement in terms of the distance from the top of the filter of the furthest focal plane at which two cells can be brought into simultaneous focus. If one considers the kinetics of random movement, it is, in my opinion, an

inescapable fact that the best measure of random migration is provided by the slope of the line drawn from Equation (2). A single point, i.e. the co-ordinates of the logarithm of 2 and the square of the distance moved, is insufficient to fix the line.

One further aspect of random migration requires consideration. This concerns the relationship between time and the density of cells on the lower surface of the filter. The problem has practical relevance, since many methods for evaluating cell migration in Boyden chambers involve lower-surface cell counts only. Without attempting a rigorous mathematical approach to this problem, it is nevertheless clear that there would be a delay in the appearance of any cells at the lower surface until (as exemplified by curve 3 in Figure 1.7; $\kappa = 50$, $t = 180$ min) the leading front of the distribution curve had reached the lower surface. Thereafter, cell numbers on the lower surface should increase as a continuous function of time until, in the limit, cells were evenly distributed throughout the filter. At *no* time should the cell count on the under surface be higher than that any any other plane in the filter. Practical considerations limit one's ability to examine, experimentally, these particular theoretical predictions. Generally speaking, the time required for cell density to equilibrate throught the filter would be in excess of that during which cells could be expected to remain viable or retain their initial motility. Cell loss by detachment from the under surface further invalidates the assumption that a complete theoretical treatment requires. It is, none the less, entirely to be expected that there would be an interval of time during which under-surface cell counts would increase progressively due to random movement alone. Consequently, if two cell-containing filters were compared during this interval, after incubation in the absence of

Figures 1.7 through 1.10

Figures 1.7 through 1.10.

The diagrams on the following 4 pages depict the theoretical, in-filter cell distribution that would be expected if random migration were to follow kinetics dictated by the random walk equation

$$P(x,t) = \frac{N}{\sqrt{\pi\kappa t}} \cdot e^{-x^2/\kappa t}$$

where $P(x,t)$ = av. cell count/HPF at any given value for x and t.

x = distance into the filter.

t = time

N = total migrating cell population

κ = mean square velocity

The graphs were drawn by a computer equipped with a plotter.

Figures 4.1 and 4.3 show the expected cell count $P(x,t)$ as a function of distance into the filter (x).

Figures 4.2 and 4.4 show $\ln P(x,t)$ as a function of x^2.

Arbitrary values assigned to N,κ and t are indicated on each figure.

Note that as κ is made to increase with constant t or as t is made to increase with constant κ; the expected cell distribution flattens into the filter and the slopes of the logarithmic plots increase. In no case does the distribution mode leave the upper surface of the filter.

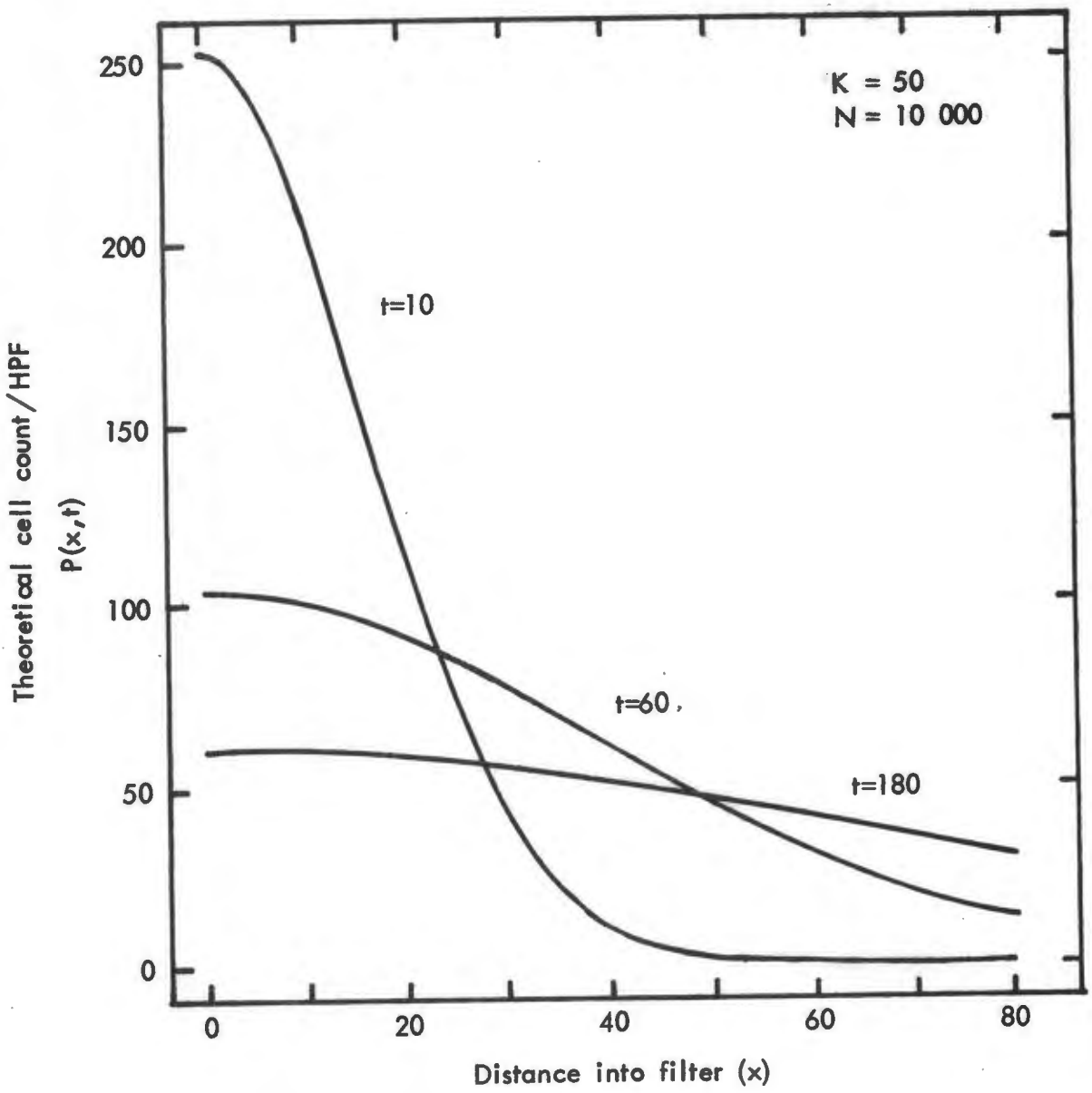

Figure 1.7

Theoretical in-filter cell distribution for constant κ and varying t. Both axes linear.

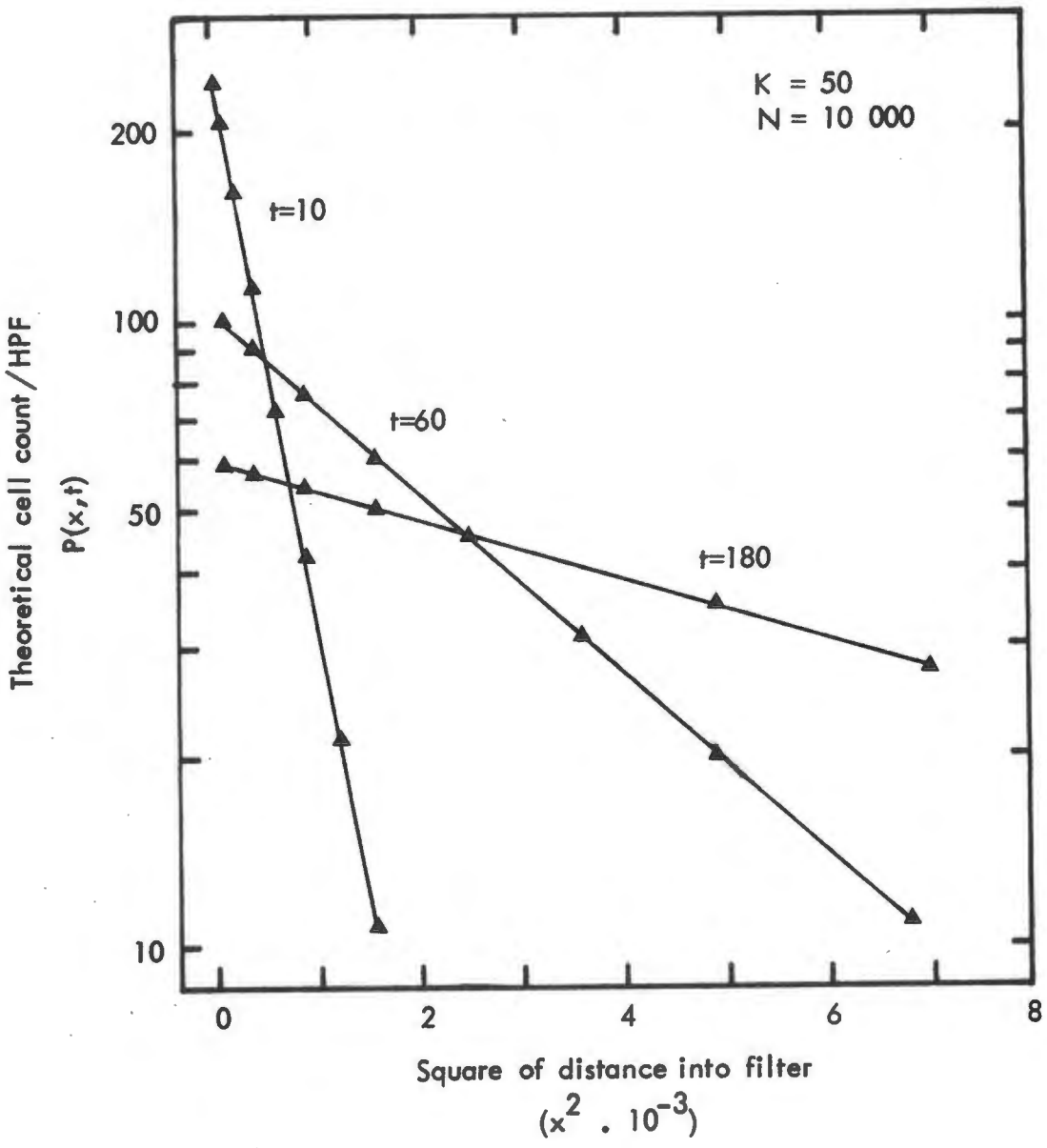

Figure 1.8

Theoretical in-filter cell distribution of constant κ and varying t.

$\ln P(x,t)$ vs x^2

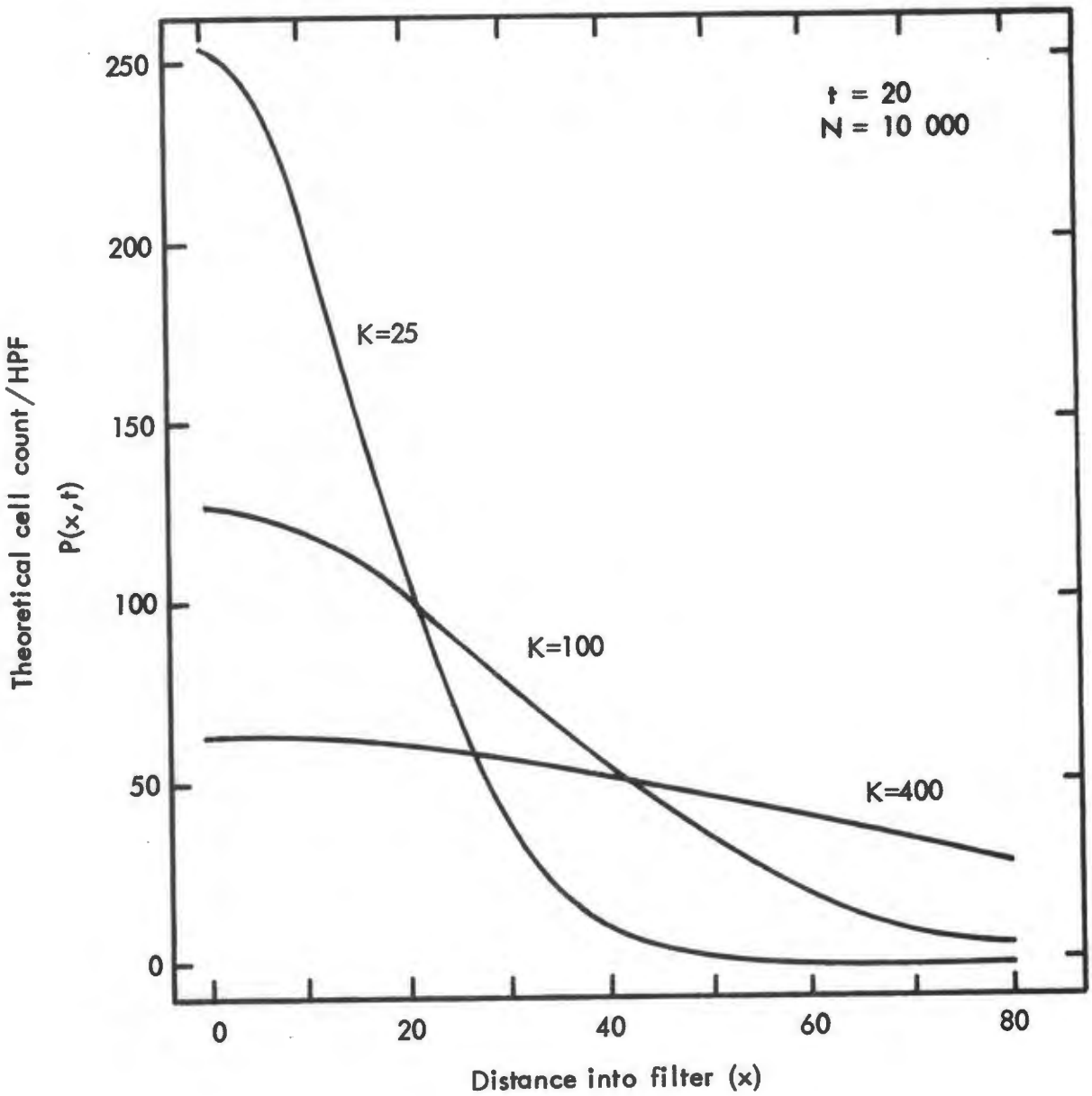

Figure 1.9

Theoretical in-filter cell distribution for constant t and varying κ. Both axes linear.

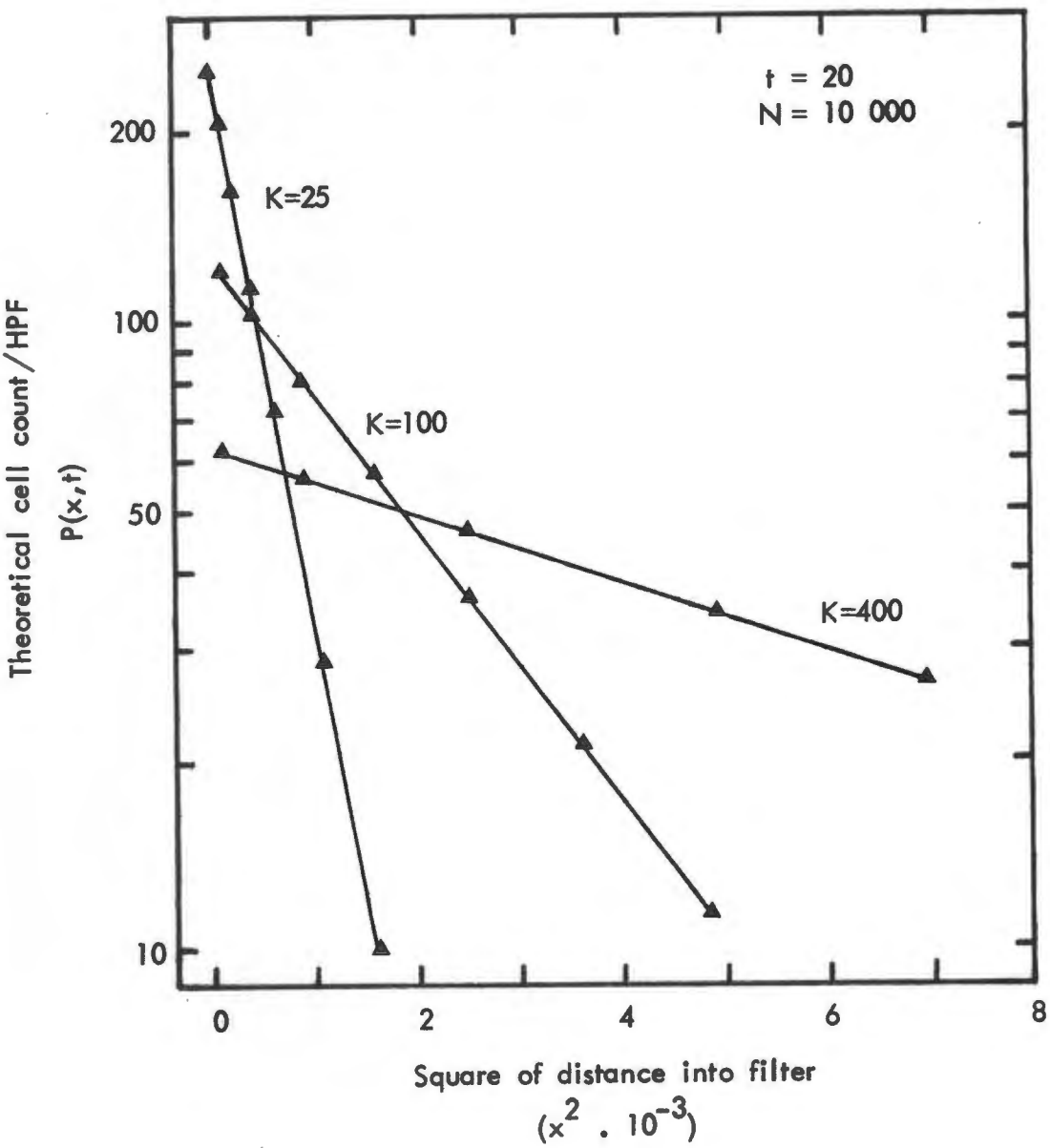

Figure 1.10

Theoretical in-filter cell distribution for constant t and varying κ.

$\ln P(x,t)$ vs x^2

a chemotactic gradient, and in the presence of different concentrations of chemotaxin, it might be found that the filter from the higher chemotaxin solution contained more cells on the lower surface. This would be so if, as Zigmond and Hirsch (194) and Keller and Sorkin (81) have shown, *one* of the effects of a chemotaxin is to increase random motility. One might, then, be tempted to conclude that directional cell movement, as a phenomenon distinct from random movement, does not exist. This confusion is evident in Wilkinson's (185) discussion of the equation of whether cells respond with directional movement to a chemotactic gradient or to an "absolute concentration" of chemotaxin.

Directional cell movement (chemotaxis)

Viable cells that settle upon and adhere to the upper surface of a filter will move randomly. In the presence of a positive chemotactic gradient, two things may happen.

(a) Firstly, the intrinsic random motility of the cells may increase. Stated more formally, each cell will, during a small interval of time, Δt, experience an acceleration in the direction of its random velocity vector, V_r. Since the cell has mass, one can infer from the elementary laws of motion, that the cell has been subject to a force, F_r. As a result of the acceleration the velocity of the cell will increase by an amount, ΔV_r, and hence it will suffer a displacement, Δx_r, during this interval of time. This requires that an amount of work, $F_r.\Delta x_r$, to be done. The subscript r is used to signify processes that are influenced by random movement.

(b) Secondly, a directional component may be impressed upon cellular movement so that the cells now move towards the lower surface of the filter.

In a more formal sense, the cells have experienced an additional acceleration, that need only involve a change in the direction of the velocity vector without a change in its magnitude. This implies the operation of a second force, F_d. (The subscript d denotes "directional".) The resulting change in velocity, ΔV_d, operating over the same interval of time, Δt, will effect a displacement, Δx_d, normal to the plane of the filter and in the direction of the lower surface. This requires that an amount of work, $F_d . \Delta x_d$, be done.

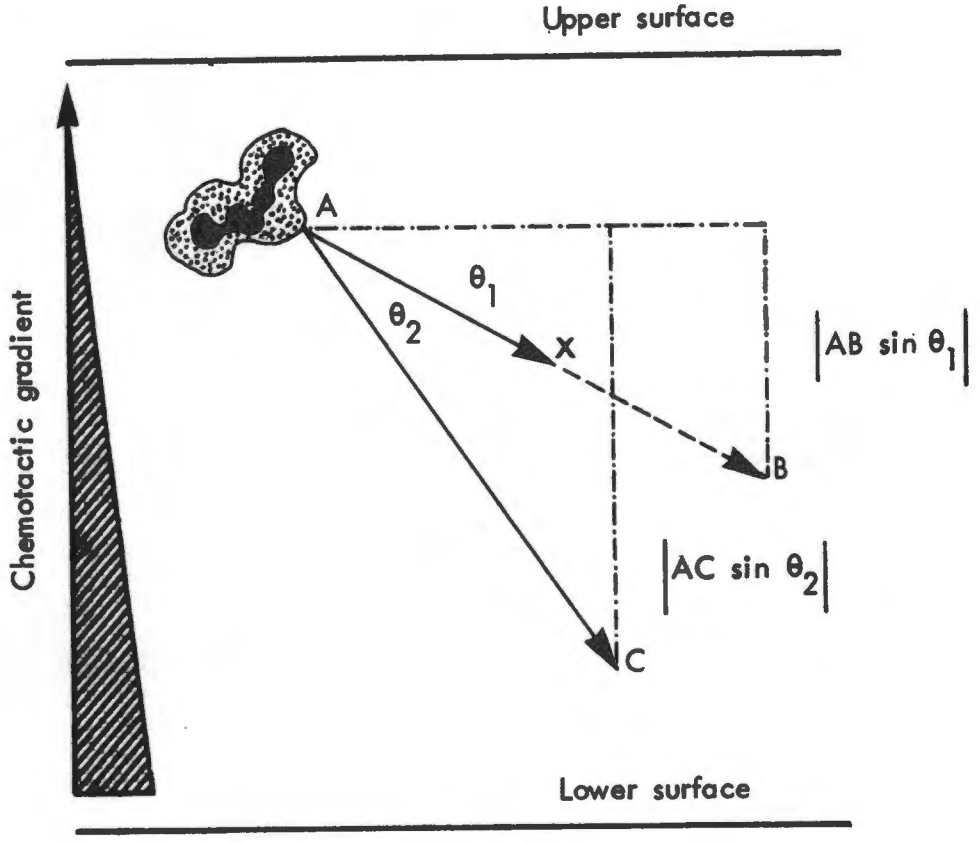

Figure 1.11

These concepts are summarized diagrammatically in Figure 1.11 for a cell at position A with an intrinsic random velocity indicated by the vector AX at angle θ_1 to the horizontal. Assume that the first effect of chemotaxin is to increase the magnitude of the velocity *in the same direction*, (V_r) from AX to AB. The second effect, i.e. that due to the chemotactic gradient, is to alter the direction of the velocity vector from AB to AC, so that it now lies at an angle θ_2 to the plane of the filter.

The acceleration due to the effect of chemotaxin upon the intrinsic motility is given by XB/Δt. The vertical acceleration towards the bottom of the filter is given by

$$1/\Delta t \quad (\; |AC \sin \theta_2| \; - \; |AB \sin \theta_1| \;).$$

We are thus able to distinguish, theoretically, two components of the chemotactic response, both of which require the expenditure of energy. The work of Zigmond and Hirsch (194) and Keller and Sorkin (81) provides evidence for the effect of chemotaxin on intrinsic motility.

Since, *all* cells capable of responding to the chemotactic gradient would be subject to the downward, directional acceleration, it is a necessary and sufficient condition for demonstrating the existence of this acceleration that the mode of the frequency distribution should leave the upper surface of the filter and traverse its thickness to pass through the lower surface.

On the basis of these theoretical concepts of the directional chemotactic response one can make the following useful experimental predictions.

(a) Movement of cell populations through the filter should follow the kinetics of bulk displacement of a normal distribution, truncated at the upper surface.

(b) If cell loss from the under surface is prevented, the curve representing under-surface cell counts as a function of time should be sigmoid in shape. The inflection of this curve should mark that time point at which the mode has traversed the filter.

(c) There should be a definite relationship between the steepness of the chemotactic gradient and the kinetics of cell movement. This could take one of two forms. Either (i) all cells respond in a continuous way to a gradient or (ii) cells have a different threshold for responsiveness, so that any cell, once its threshold has been exceeded, responds maximally to the directional component. In the former case, a unimodal, normal distribution of cells through the filter would be preserved with time. The time taken for the mode to pass through the filter should vary inversely with the steepness of the chemotactic gradient. In the latter case, a submaximal gradient should stimulate only a fraction of the cell population with the result that a bimodal distribution would be found.

Experimental

The following experiments were performed to examine the predictions of the theoretical considerations outlined above. In essence, they involved a study of the effects of varying the time of incubation and the concentration of chemotaxin on cell migration in the presence and absence of chemotactic gradients.

Materials and Methods

Rabbit peritoneal cells were obtained from sodium caseinate-induced exudates as described in the Appendix. The cells were adjusted to a concentration of $2x10^6$ cells per ml in Gey's BSS-2% HSA and 2,5 ml of this suspecsion ($5x10^6$ cells) were introduced into Boyden chambers with 3µm Millipore filters separating the upper and lower compartments.

Chemotactic stimuli were provided by solutions of casein in saline, pH 7,1, by SNF-cytotaxin or by CUF-cytotaxin. Dilutions of the concentrated preparations in saline, 0,005M triethanolamine pH 7,2 buffered saline (TBS), Gey's BSS or Gey's BSS-2% HSA were made as required.

At the end of the experimental period, filters were removed from the chambers and mounted on glass slides with the lower surface upwards. Dehydration, clearing and mounting of the filters rendered them transparent, so that in-filter cell counts could be performed on all cells in focus at different distances from the upper surface of the filter as measured on the micrometer scale of the fine-adjustment knob of the microscope.

Results

Neutrophil movement in the absence of a chemotactic gradient.

Two experiments were performed in which cellular movement was studied after incubation in the presence of different concentrations of partially purified casein cytotaxin (CUF-3). Triplicate chambers were used for each concentration; in each chamber the concentration of the cytotaxin above and below the filter was the same. Gey's BSS-2% HSA was used to dilute the cytotaxin concentrate and was also used

as the cell suspending medium. After incubation in-filter counts were performed at measured distances from the upper surface of the filters. Mean values for cell counts from each individual set of three filters were calculated; logarithms of these values were plotted as a function of the square of the distance into the filter and the regression lines fitted by the method of least squares. The results are summarized in Figures 1.12 and 1.13 and in Table 1.6.

As is evident from the data, a linear relationship was observed between the logarithm of the average cell count per high-power field and the square of the distance into the filter for all concentrations of cytotaxin. Furthermore, the slopes of the observed regression lines increased with increasing concentration of cytotaxin. In the first experiment the slope varied from $-6{,}14\times10^{-4}$ in the absence of cytotaxin to $-0{,}93\times10^{-4}$ in the presence of the highest concentration of cytotaxin. Corresponding values in the second experiment were $-3{,}63\times10^{-4}$ without cytotaxin and $-1{,}65\times10^{-4}$ in the presence of the highest concentration of chemotactic material.

In no case did the mode of the frequency distribution leave the upper surface of the filter.

These results indicate that neutrophil movement in the absence of a chemotactic gradient can be described by the random walk equation. Furthermore, they are entirely consistent with the results of Zigmond and Hirsch (194) and Keller and Sorkin (81) in showing that chemotactic materials have an effect upon the speed of random cellular movement.

Figure 1.12

Figure 1.12 Effect of varying cytotaxin concentration on random cell movement.

Results of an experiment in which in-filter cell counts were performed 180 min after incubating cells in Boyden chambers with the same concentration of cytotaxin (CUF-3) in the upper cell compartment and the lower test compartment of each chamber. The following concentrations of CUF-3 (38,9 mg protein/ml) were used:

CUF-3 diluted 1:50 (●—●); 1:100 (○—○); 1:200 (■—■); 1:500 (□—□); and no cytotaxin (▲—▲).

Each point represents the mean of 3 high-power field counts made on 3 separate filters. Counts were performed at measured distances from the upper surface of the filter. Results are plotted as the $\log_e$ of the cell count as a function of the square of the distance at which that count was recorded.

Lines fitted by least squares regression.

Note that good linear relationships were observed.

The slopes of the lines increased with increasing cytotaxin concentration (cf.Table 1.6).

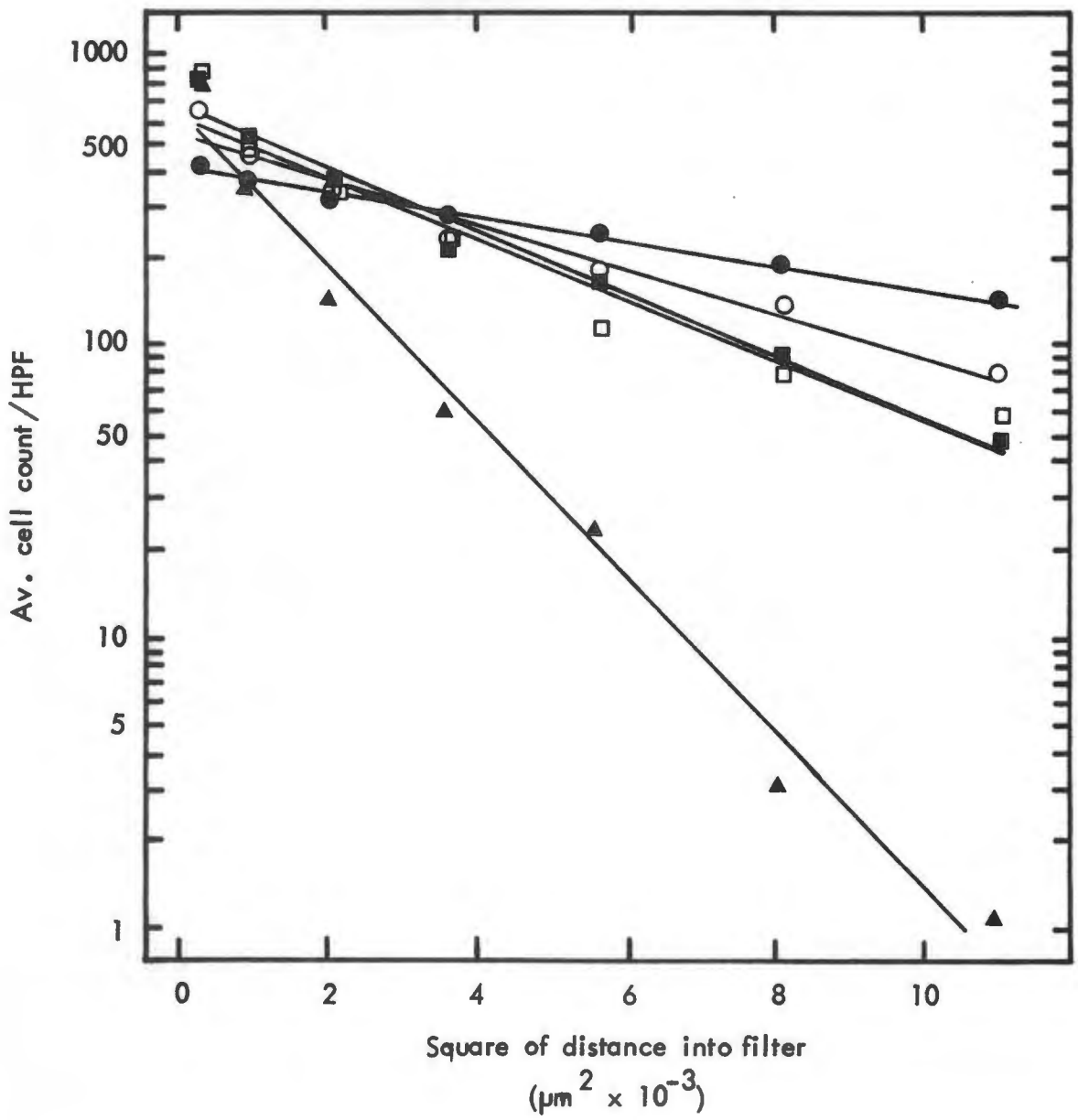
1000
500
100
50
10
5
1
Av. cell count/HPF
0
2
4
6
8
10
Square of distance into filter
(μm 2 x 10-3)

Figure 1.12

Figure 1.13

Figure 1.13 Effect of varying cytotaxin concentration on random cell movement.

Results of an experiment in which in-filter cell counts were performed 180 min after incubating cells in Boyden chambers with the same concentration of cytotaxin (CUF-3) in the upper cell compartment and the lower test compartment of each chamber. The following concentrations of CUF-3 (38,9mg protein/ml) were used:
CUF-3 diluted 1:50 (●—●); 1:100 (○—○); 1:200 (■—■); 1:400 (□—□); and no cytotaxin (▲—▲).

Each point represents the mean of 3 high-power field counts made on 3 separate filters. Counts were performed at measured distances from the upper surface of the filter. Results are plotted as the $\log_e$ of the cell count as a function of the square of the distance at which that count was recorded.

Lines fitted by least squares regression.

Note that good linear relationships were observed.

The slopes of the lines increased with increasing cytotaxin concentration (cf.Table 1.6)

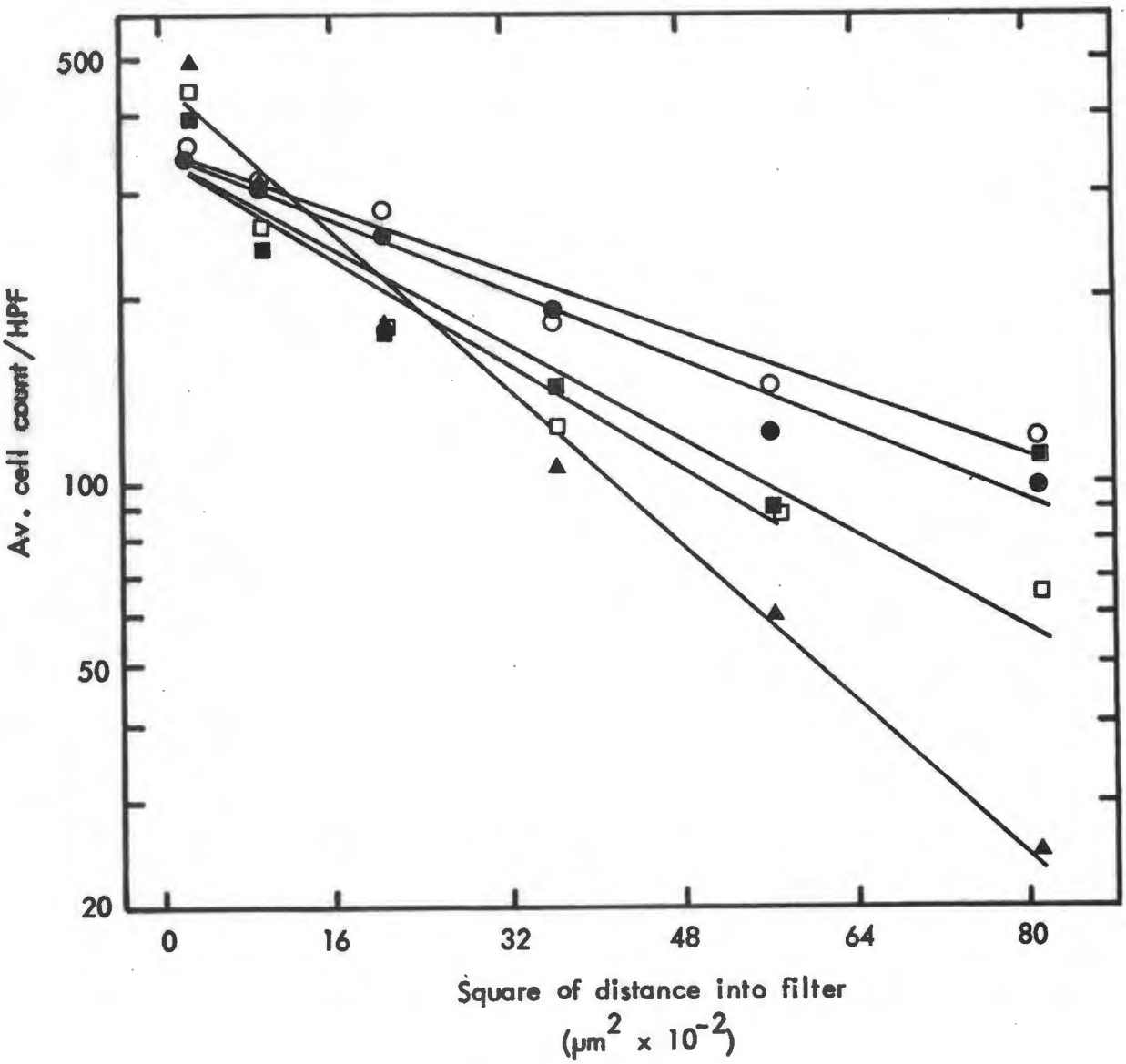
500
100
50
20
Av. cell count/HPF
0
16
32
48
64
80
Square of distance into filter
(μm² x 10⁻²)

Figure 1.13

Table 1.6

The effect of cytotaxin on random cell movement

Expt. no.	Slopes of least squares regression lines for Log_e cell count upon square of distance (μ^2) into filter					
	Dilution of chemotactic factor*					
	None	1/500	1/400	1/200	1/100	1/50
1	$-6,14x10^{-4}$	$-2,37x10^{-4}$	-	$-2,46x10^{-4}$	$-1,76x10^{-4}$	$-0,93x10^{-4}$
2	$-3,63x10^{-4}$	-	$-2,21x10^{-4}$	$-2,45x10^{-4}$	$-1,45x10^{-4}$	$-1,65x10^{-4}$

*CUF-3 was used as the cytotaxin. Protein concentration of the CUF-3 concentrate was 38,9 mg/ml.

Cell counts per high-power microscopic field (320x) were made at 15µm intervals into 3µm Millipore filters removed from Boyden chambers prepared to study the effects of cytotaxin on random cell movement starting at a depth of 15µm into the filter from its upper surface. Distances were measured on the optical micrometer of the fine-focus knob of the microscope. Cytotaxin was present at the same concentration on both sides of the filter so that no gradient of chemotactic material was set up across the filter but cells moved randomly in the presence of different concentrations of cytotaxin. Gey's BSS-2% HSA was used to dilute the cytotaxin.

Neutrophil migration in the presence of a chemotactic gradient;

(i) Cell movement as a function of time.

(a) In order to define the kinetics of cellular accumulation at the lower surface of the filter in the presence of a positive chemotactic gradient, Boyden chambers were incubated at 37°C with either 1% casein in 0,9% NaCl (test) or 0,9% NaCl (control) in the lower compartments and $5x10^6$ neutrophils in Gey's BSS-2% HSA in the upper compartments. Duplicate test chambers were removed at successive 30 min intervals for a total of 6h and the filters were prepared for under-surface cell counts. In the same experiment a suspension of the neutrophils was incubated at 37°C in Gey's BSS-2% HSA with occasional agitation; a sample of this suspension was taken for assay of cell viability by trypan blue exclusion each time a set of Boyden chambers was removed.

As can be seen from the results summarized diagrammatically in Figure 1.14, cellular accumulation at the lower surface was not linear with time. Cells appeared at the lower surface of the filters after 1h and accumulated with time, to reach a plateau after 2½h of incubation. This plateau was sustained for approximately 2h after which the number of cells adherent to the lower surface of the filters decreased, concomitant with a fall in the viable cell count.

(b) A second experiment was performed to define the progress of cellular movement through the filter with time in the presence of a chemotactic gradient.

Chambers containing $5x10^6$ cells each were incubated for varying

Figure 1.14

Figure 1.14 Kinetics of cellular movement in the presence of a chemotactic gradient.

Boyden chambers, charged with $6x10^6$ cells in their upper compartments and 1% casein in their lower compartments, were incubated at 37°C. At the indicated time points duplicate chambers were removed from the incubator and the filters prepared for under-surface cell counting (●—●). Each point represents the mean of 8 counts on the two filters.

A suspension of neutrophils in Gey's BSS-2% HSA was incubated under similar conditions and at each time point an aliquot was removed for trypan blue exclusion viability testing (O--O). Each point represents the mean of 2 determination.

Note that cellular accumulation at the lower filter surface was not linear with time. Cells started to appear at the lower surface after 1h and accumulated with time to reach a plateau after 2½h. Thereafter the number of adherent cells on the lower surface decreased with a concomitant fall in cell viability.

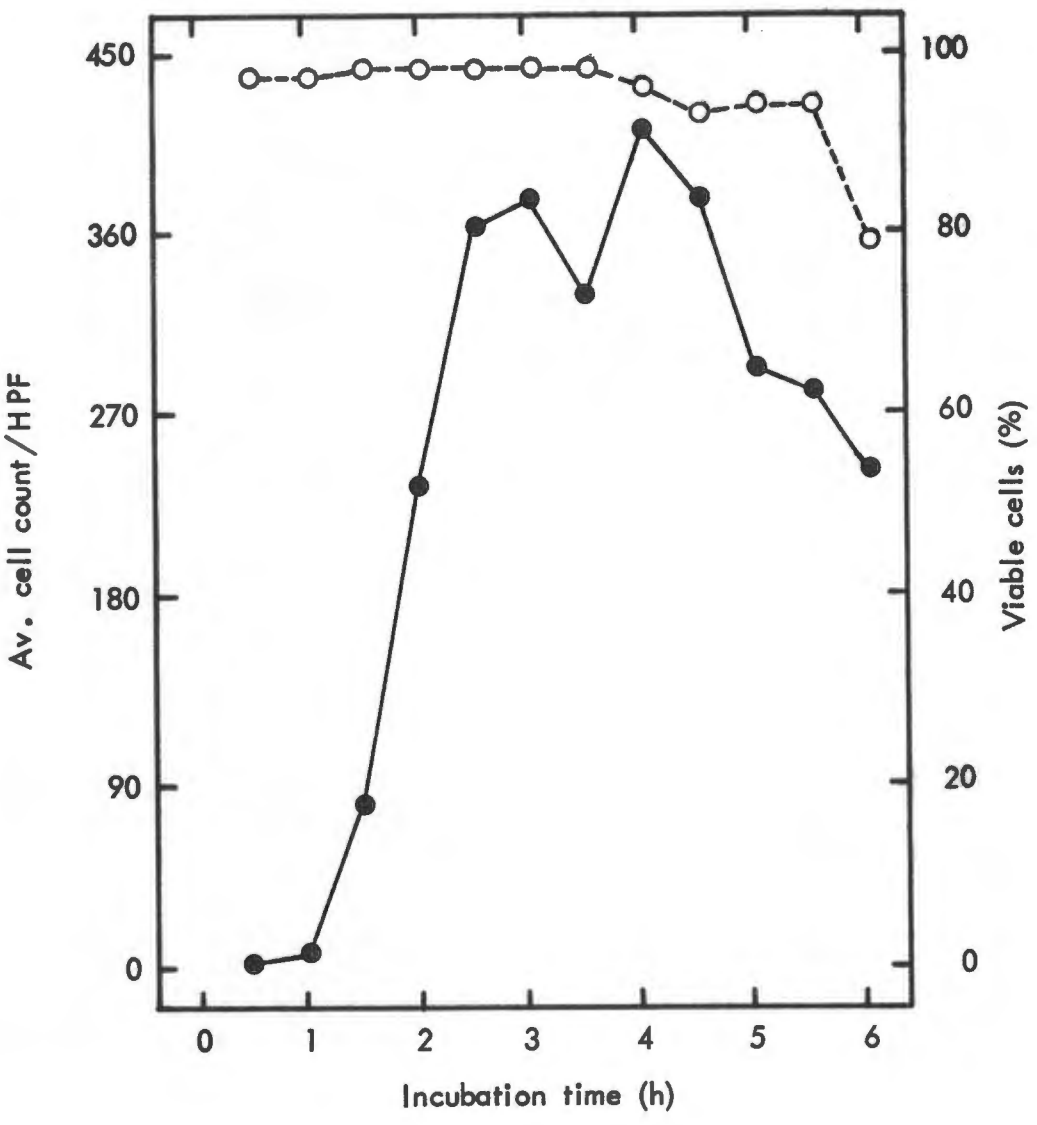
450
360
270
180
90
0
Av. cell count/HPF
100
80
60
40
20
0
Viable cells (%)
0
1
2
3
4
5
6
Incubation time (h)

Figure 1.14

lengths of time with 0,9% NaCl alone (control) or 0,9% NaCl containing 1% casein (test) in the lower compartments. At successive 15 min time points test and control filters were prepared for in-filter counting. The average cell counts of 4 high-power fields at focal planes progressively distant from the upper surface of the filter are shown, for each time point, in Figure 1.15. It is evident from these results that, by the time 15 min had elapsed, approximately 190 cells per high power field were seen in focus 15μm below the surface of the test filter; fewer than 20 cells per high-power field were encountered at the same depth in the control filter. With the passage of time, cells responding to a chemotactic gradient entered the filter in progressively greater numbers so that by 60 min a well-defined mode had left the upper surface and was evident 35μm into the filter. By 105 min the mode had penetrated further into the filter and appreciable accumulation of cells on the under surface was apparent. The filters examined at 150 min showed the great majority of cells (> 500 cells per high-power field) on the lower surface, with a minor population of cells still present within the filter.

Cells incubated without cytotaxin in the lower compartment did not show the bulk movement exhibited by cells responding to 1% casein, but penetrated the filter in typical "random-walk" fashion. This was evident in the fact that at no stage did the mode leave the upper surface of the filter and semi-log plots of cell counts as a function of the square of the distance into the filter yielded a family of straight lines.

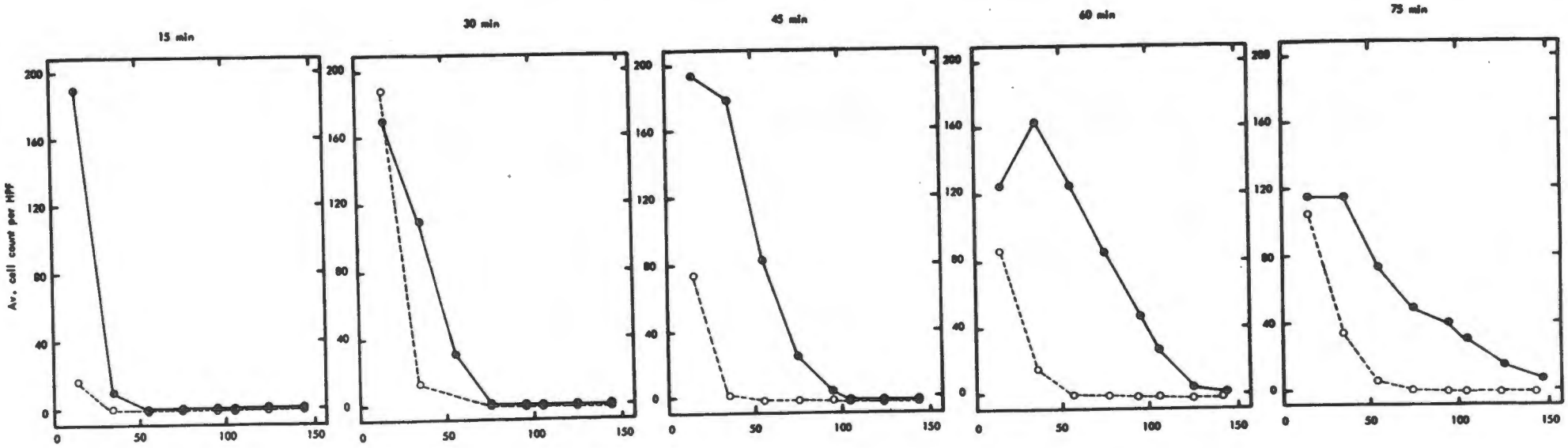

Figure 1.15: Kinetics of cell movement in presence or absence of a chemotactic gradient.

The sequence of diagrams above and on the next page summarizes the results obtained from an experiment in which Boyden chambers were each charged with $6x10^6$ cells in the upper compartments and either Gey's BSS-2% HSA (O--O--O) or 1% casein (●—●—●) in the lower compartments.

At the indicated time-points (15 min through 150 min) filters were removed and prepared for in-filter counting of migrated cells. Cell densities at various depths in the filters were determined by counting cells in the focal planes at the indicated distances (in μm) from the upper filter surfaces.

Each point represents the mean of 4 counts.

Numbers on the abscissae represent distances, from the upper filter surface, as measured with the vernier micrometer on the fine-focus knob of the microscope.

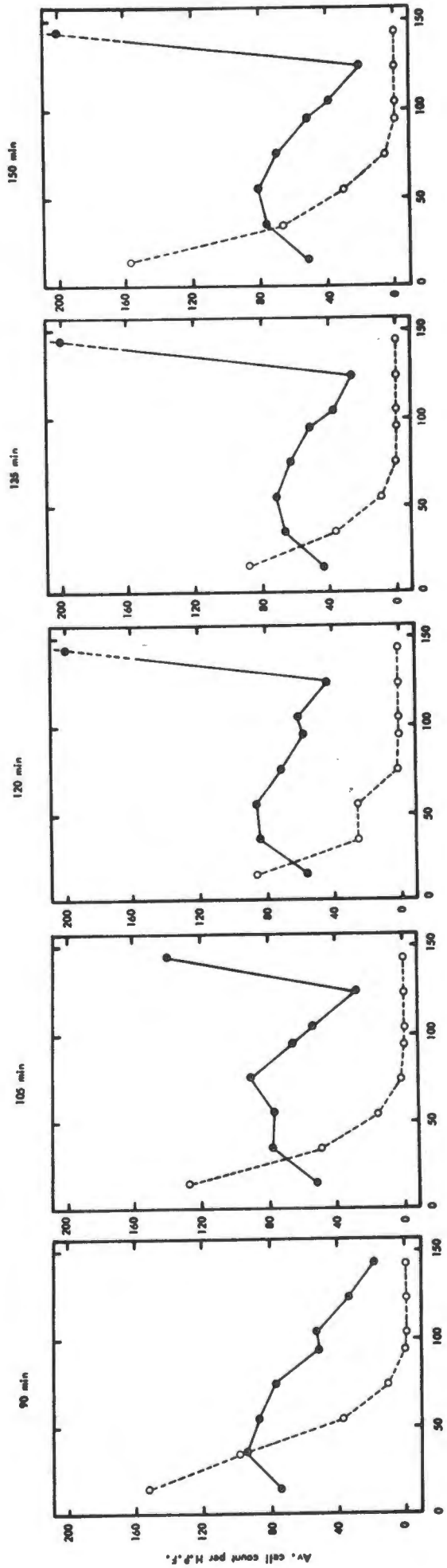

Figure 1.15 (continued)

Neutrophil migration in the presence of a positive chemotactic gradient:

(ii) Cell movement as a function of cytotaxin concentration.

(a) The effect of varying the cytotaxin concentration in the lower compartments of Boyden chambers upon the numbers of neutrophils accumulating on the under surfaces of filters incubated for 3h was studied in three series of experiments.

In the first series, comprising a total of 3 individual experiments, the chemotactic stimulus was provided by crude casein (Hammarsten) in concentrations varying from 0,001% w/v to 5% w/v (corresponding to protein concentration varying from 8µg/ml to 36mg/ml). Duplicate chambers were incubated for 3h for each casein concentration after which filters were removed and prepared for under-surface counts. The results, depicted diagrammatically in Figure 1.16 were consistent in showing a rapid increase in under-surface cell counts with increasing casein concentration to reach a maximum at approximately 1%. Further increase in the casein concentration resulted in a progressive fall in under-surface cell counts. A similar pattern of optimal response with supra-optimal inhibition has been described by Baum, Mowat and Kirk (12).

A second series of five experiments was performed, identical in design to the first, save for the fact that SNF-cytotaxin was added to the lower compartments instead of crude casein. The supernatant fluid concentrates, SNF XII, XIII and XIV, containing 5,75 mg protein/ml, 4,87 mg protein/ml and 4,26 mg protein/ml respectively were diluted with TBS to cover the range 0,01 to 1,00 mg protein/ml. The results of these

Figure 1.16

Figure 1.16 Cell movement as a function of casein (Hammarsten) concentration.

The indicated concentrations of casein (Hammarsten) dissolved in 0,9% NaCl were added to the lower compartments of Boyden chambers. The upper compartments received $6x10^6$ cells in Gey's BSS-2% HSA. Duplicate chambers were incubated for 3h at 37°C after which the filters were prepared for under-surface cell counting.

Each point represents the mean of 8 counts made on 2 filters.

There was a progressive increase in under-surface cell counts with increasing casein concentration reaching a maximum at approximately 1% with supra-optimal inhibition.

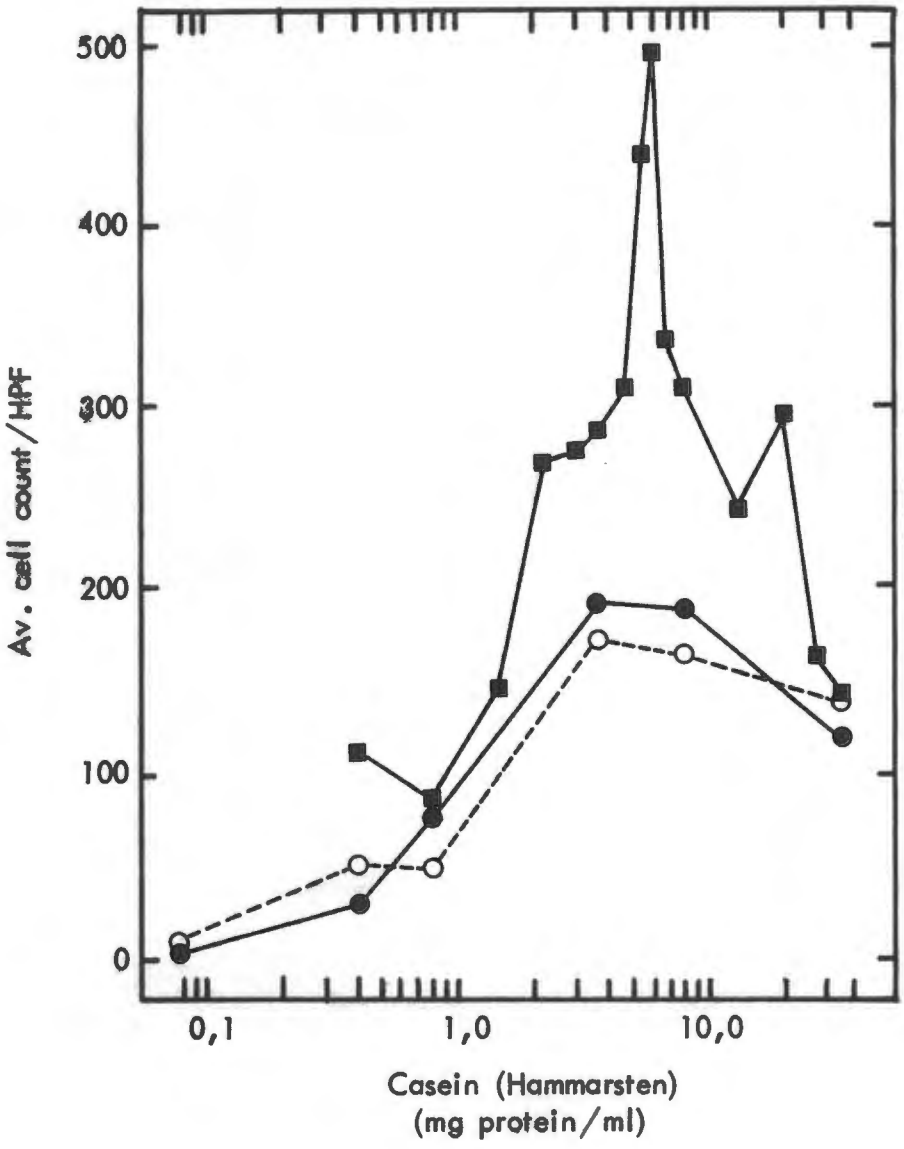
500
400
300
200
100
0
Av. cell count/HPF
0,1
1,0
10,0
Casein (Hammarsten)
(mg protein/ml)

Figure 1.16

experiments are presented in Figure 1.17 from which it can be seen that under-surface cell counts rose erratically with increasing cytotaxin concentration over the entire range of concentrations examined. There was no clear evidence of an "optimum" in the concentration-response relationship as was seen with the crude casein.

A third series of seven experiments was performed. These were identical in design to those of the previous two series save for the fact that CUF-cytotaxin was used to provide the chemotactic stimulus. Three different preparations of cytotaxin, 24,8 mg protein/ml, 38,9 mg protein/ml and 2,0 mg protein/ml, were studied over the protein concentration ranges 0,05 to 2,5 mg/ml, 0,008 to 4 mg/ml and 0,008 to 0,2 mg/ml respectively. The diluent was either 0,9% NaCl, Gey's BSS or Gey's BSS-2% HSA. The results of these experiments are depicted diagrammatically in Figure 1.18. Once again, the under-surface filter counts rose with increasing cytotaxin concentration. Only in one case (expt 4; Figure 1.18) was a peak in the concentration-response curve observed. In this case the cells responded very actively.

The results of these three series of experiments are noteworthy in the following respects. Firstly, under-surface cell counts at 3h were directly related to the concentration of cytotaxin in the lower compartment, but this relationship was not linear. In the case of crude casein, a distinct "optimum" cytotaxin concentration was observed above which cellular accumulation appeared to be inhibited. This pattern, though still apparent

Figure 1.17

Figure 1.17 Cell movement as a function of SNF-cytotaxin concentration.

SNF-cytotaxin concentrates were diluted with TBS to give the indicated concentrations of SNF-protein. The dilutions were then added to the lower compartments of duplicate Boyden chambers with the timed addition of $6x10^6$ cells in 3ml of Gey's BSS-2% HSA to the upper compartments. The chambers were incubated at 37^oC for 3h after which filters were prepared for under-surface counting.

Each point represents the mean of 8 counts made on the duplicate filters.

Three preparations of SNF-cytotaxin were tested:

SNF XII(5,75 mg protein/ml);Experiments 1 and 3

SNF XIII(4,87 mg protein/ml); Experiment 2

SNF XIV (4,26 mg protein/ml); Experiments 4 and 5

The under-surface cell counts rose erratically with increasing cytotaxin concentration over the entire range of concentrations tested and no clear evidence of an optimum in the concentration-response relationship was evident.

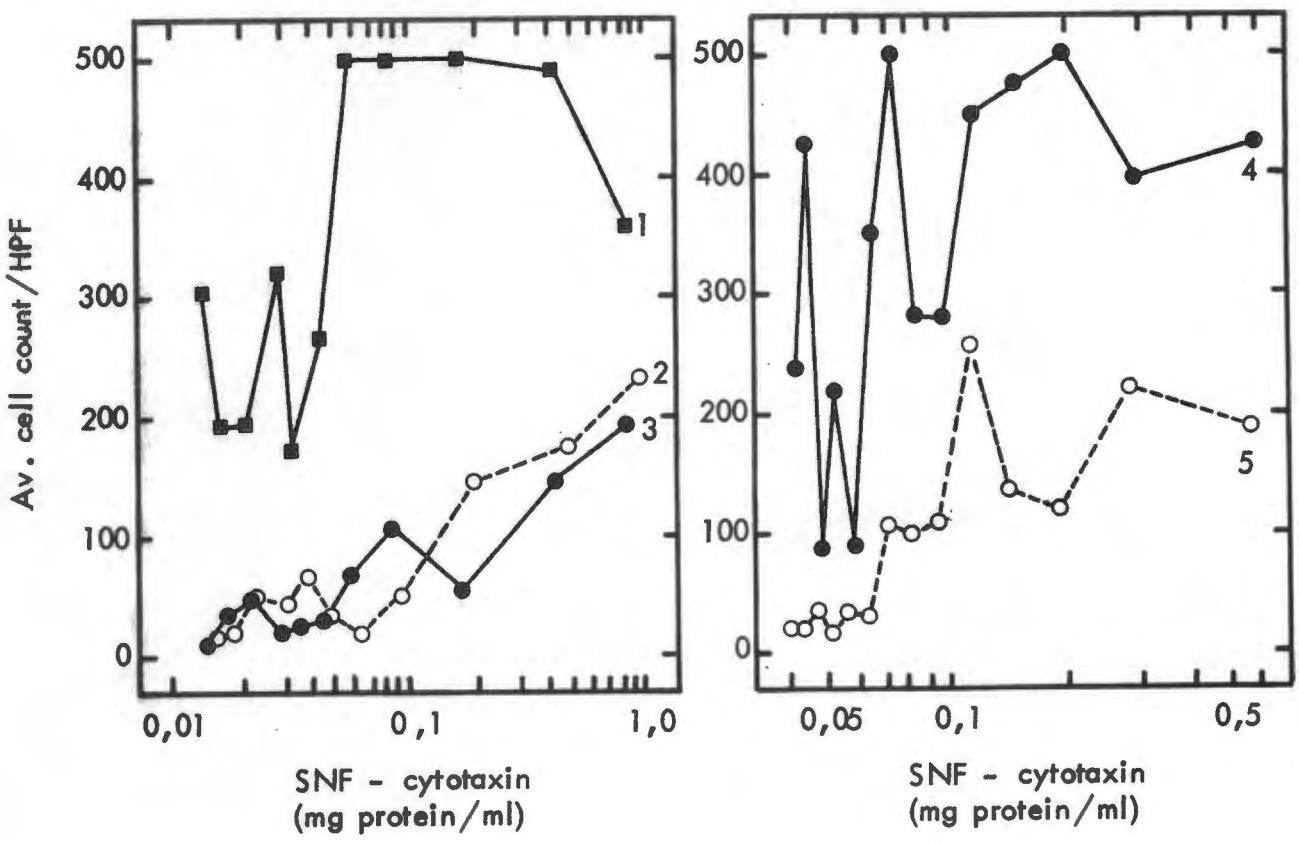
Av. cell count/HPF
500
400
300
200
100
0
0,01
0,1
1,0
SNF - cytotaxin
(mg protein/ml)
1
2
3
0,05
0,1
0,5
4
5

Figure 1.17

Figure 1.18

Figure 1.18 Cell movements as a function of CUF-cytotaxin concentration.

Three different preparations of CUF-cytotaxin were diluted to cover the indicated range of protein concentrations. Duplicate Boyden chambers were prepared for each concentration, the cytotaxin dilutions (2,5ml) being added to the lower compartments while the upper compartments received $6x10^6$ cells in Gey's BSS-2% HSA. Chambers were incubated at 37^oC for 3h and the filters processed for under-surface counts.

Each point represents the mean of 8 counts determined on 2 filters.

Diluents used were as follows:

0,9% NaCl : Experiments 4,5, 6 and 7.

Gey's BSS : Experiment 1

Gey's BSS-2% HSA: Experiments 2 and 3

CUF-cytotaxin preparations tested were as follows:

CUF-2: Experiment 5

CUF-3: Experiments 1,2,3,4 and 7.

CUF-4: Experiment 6.

The under-surface filter counts rose with increasing cytotaxin concentration and only in one case when the cells responded very actively was a peak in the concentration-response curve observed (Experiment 4)

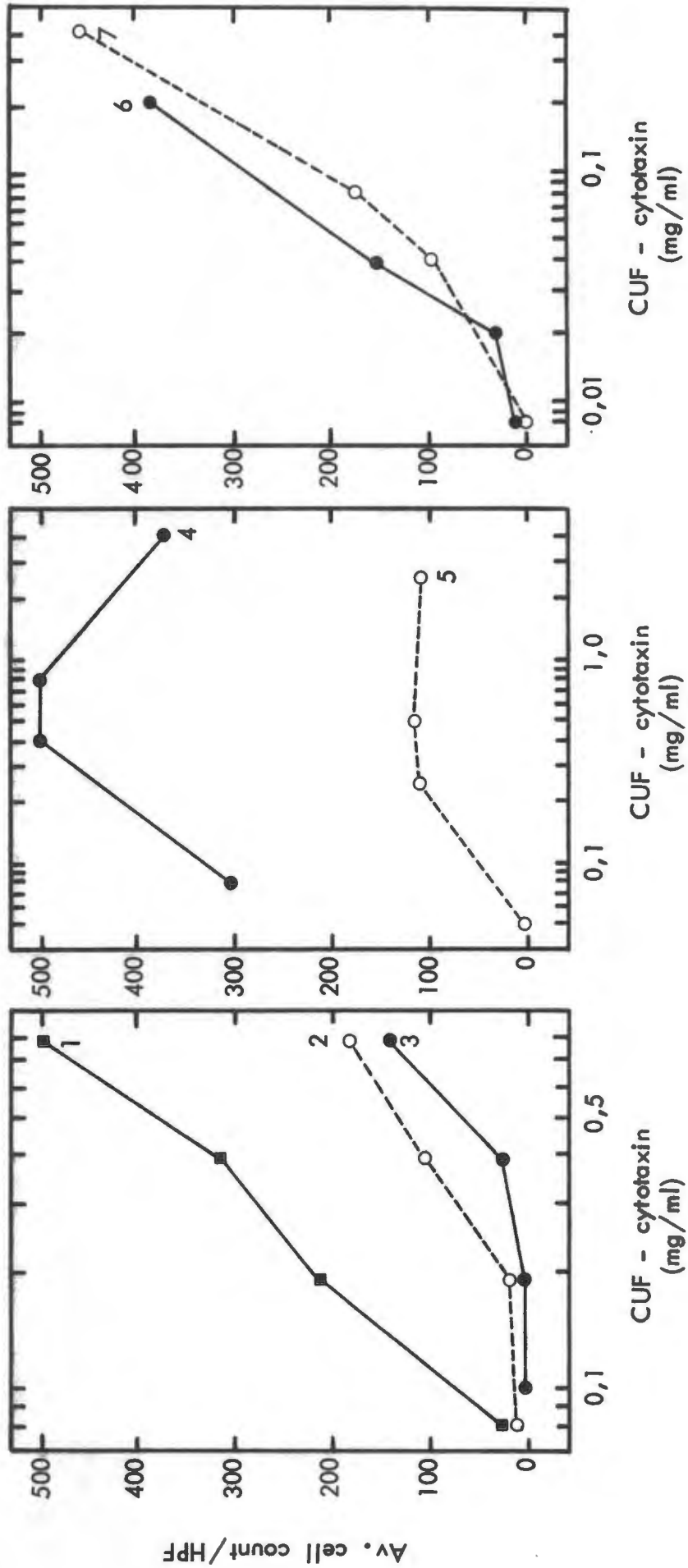
Av. cell count/HPF
500
400
300
200
100
0
0,1
0,5
1,0
0,01
CUF - cytotaxin (mg/ml)
1
2
3
4
5
6
7

Figure 1.18

in some experiments, was less evident with the semi-purified cytotaxins CUF and SNF. The reason for the inhibition at high cytotaxin concentrations is obscure and possibly reflects cellular detachment from the under surface with increasing intensity of chemotactic stimulation or inhibitory concentrations of contaminants in the cytotaxin preparation. Secondly, the magnitude of the cellular response to any given concentration of a particular cytotaxin varied considerably from one experiment to the next. This biological variation has already been alluded to earlier in this Chapter.

(b) Two experiments were performed to define the effects of different cytotaxin concentrations upon the kinetics of bulk cell movement through the filter.

In the first experiment varying concentrations of the partially purified (CUF-3)cytotaxin in 0,9% NaCl were used to provide the chemotactic stimulus. Cytotaxin was added to the lower compartments of Boyden chambers at protein concentrations varying from 0,00 to 0,78 mg/ml. Duplicate chambers were prepared for each concentration of cytotaxin and incubated at 37°C for 3h. At the end of the incubation period filters were removed for in-filter counting. The results of this experiment are summarized graphically in Figure 1.19 where the coordinates of each point represent the average of the high-power field cell counts on the two filters and the corresponding distance from the upper surface at which the count was taken.

In the second experiment two TBS dilutions of concentrated

SNF-cytotaxin were studied. The protein concentration of the concentrate was 4,26 mg/ml and that of the dilutions 0,02 mg/ml and 0,09 mg/ml respectively. Single chambers were prepared for each dilution, with the upper compartment containing $5x10^6$ peritoneal cells in 2,5ml of Gey's BSS-2% HSA. Following incubation at 37^oC for 3h, the filters were prepared for counting. Four fields of in-filter counts at different focal planes were made, from which the average cell count per focal plane into the filter was calculated. These average cell counts per high-power field were again plotted as a function of the distance of the focal plane from the upper filter surface. The two graphs showing the in-filter cell distribution observed for the two dilutions of cytotaxin are shown in Figure 1.20.

The data show that, as in other experiments, cells incubated in the absence of a chemotactic gradient (▲—▲—▲ ; Figure 1.19) migrate in a typically random fashion. When cytotaxin was added to the lower compartment, a directional component was added to the random cellular movement so that cells moved in bulk through the filter towards the lower surface. Cell counts performed on the filters from chambers incubated with a 1:500 or 1:200 dilution of CUF-3 (●—●—● or ○—○—○ ; Figure 1.19) showed the cells distributed with a single mode that penetrated further into the filter in the three hours of incubation with the 1:200 dilution than with the 1:500 dilution of cytotaxin. With higher concentrations of cytotaxin (1:100 or 1:50; ■—■ or □—□ ; Figure 1.19) in the lower compartment, cells had accumulated on the lower surface of the

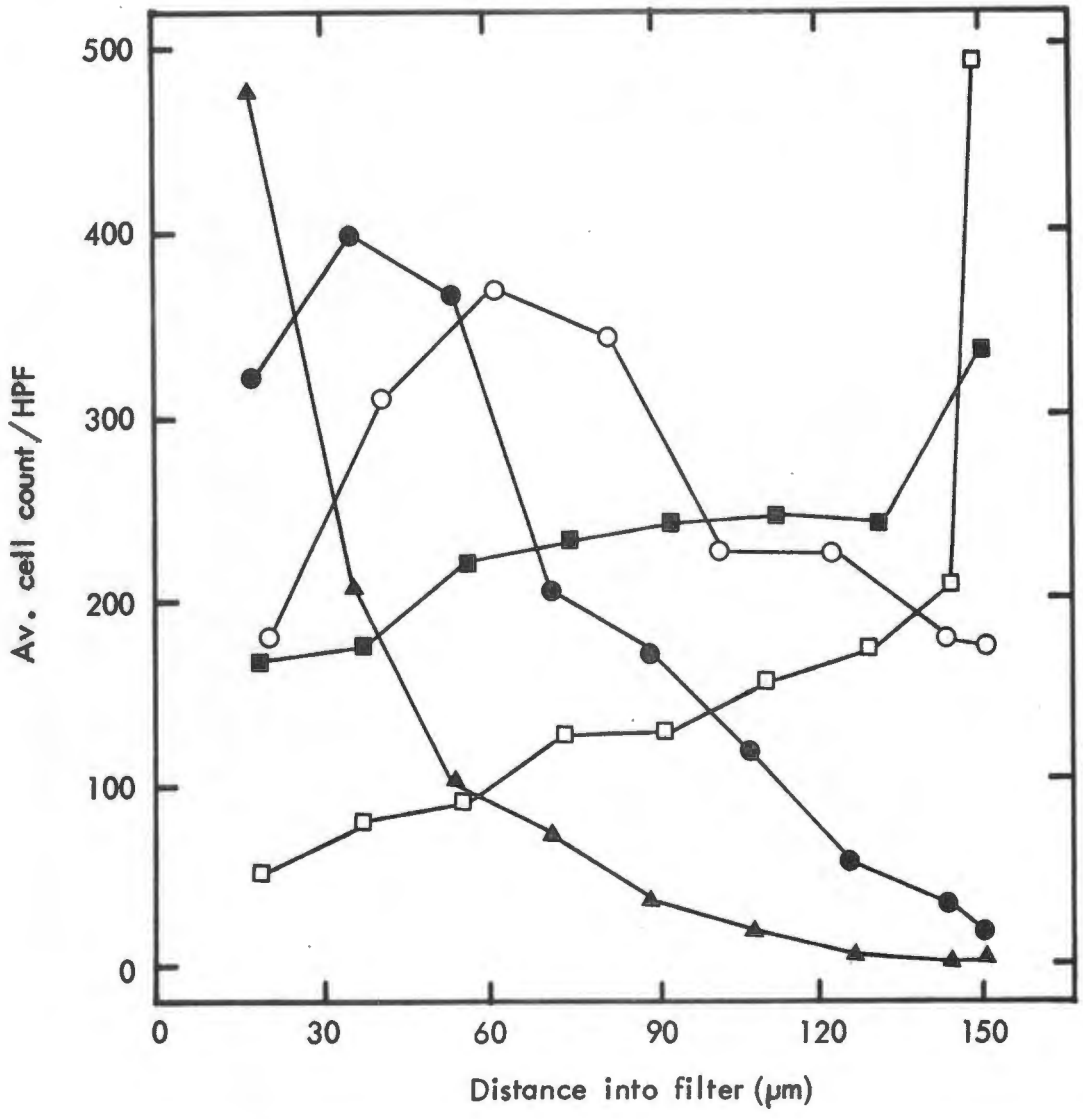

Figure 1.19 Effect of cytotaxin gradient on in-filter cell distribution.

Results of an experiment in which individual Boyden chambers were charged with $6x10^6$ cells in each upper compartment. The lower compartments contained partially purified cytotaxin (CUF-3; 39 mg protein/ml) at dilutions of 1:50 (□—□); 1:100 (■—■); 1:200 (○—○) or 1:500 (●—●). Control chambers (▲—▲) contained 0,9% NaCl in the lower compartments. In-filter counts were performed after 180 min of incubation. Each point represents the mean of 2 high power field counts made on separate filters at the indicated distances from the upper surface.

filters and the in-filters modes were no longer detectable.

In the second experiment in which the two concentrations of the SNF-cytotaxin (1:250 and 1:50) were compared, a similar in-filter cellular distribution was seen with a uni-modal population of cells distributed through the filter at the lower concentration of cytotaxin (●—●; Figure 1.20) the peak was situated approximately half way into the filter. With the higher concentration of cytotaxin (○—○; Figure 1.20) a suggestion of a peak was evident 80% of the distance into the filter with a large number of cells accumulated on the lower surface.

These results may be summarized by stating that cells responding to a chemotactic gradient traverse the filter as a uniformly distributed population with a single mode. The directional velocity of the migrating cells, as indicated by the penetration distance of the mode in a given time, is directly related to the steepness of the chemotactic gradient.

I interpret the uni-modality of the distribution curve as indicating the absence of a "threshold" effect in the dose-response relationship. Had such an effect been operative one would have expected a bi-modal distribution of cells at limiting concentrations of cytotaxin, composed of one population of cells migrating by random movement (i.e. cells whose "directional" threshold had not been exceeded) and a second population whose stimulatory threshold had been exceeded by the concentration of cytotaxin present.

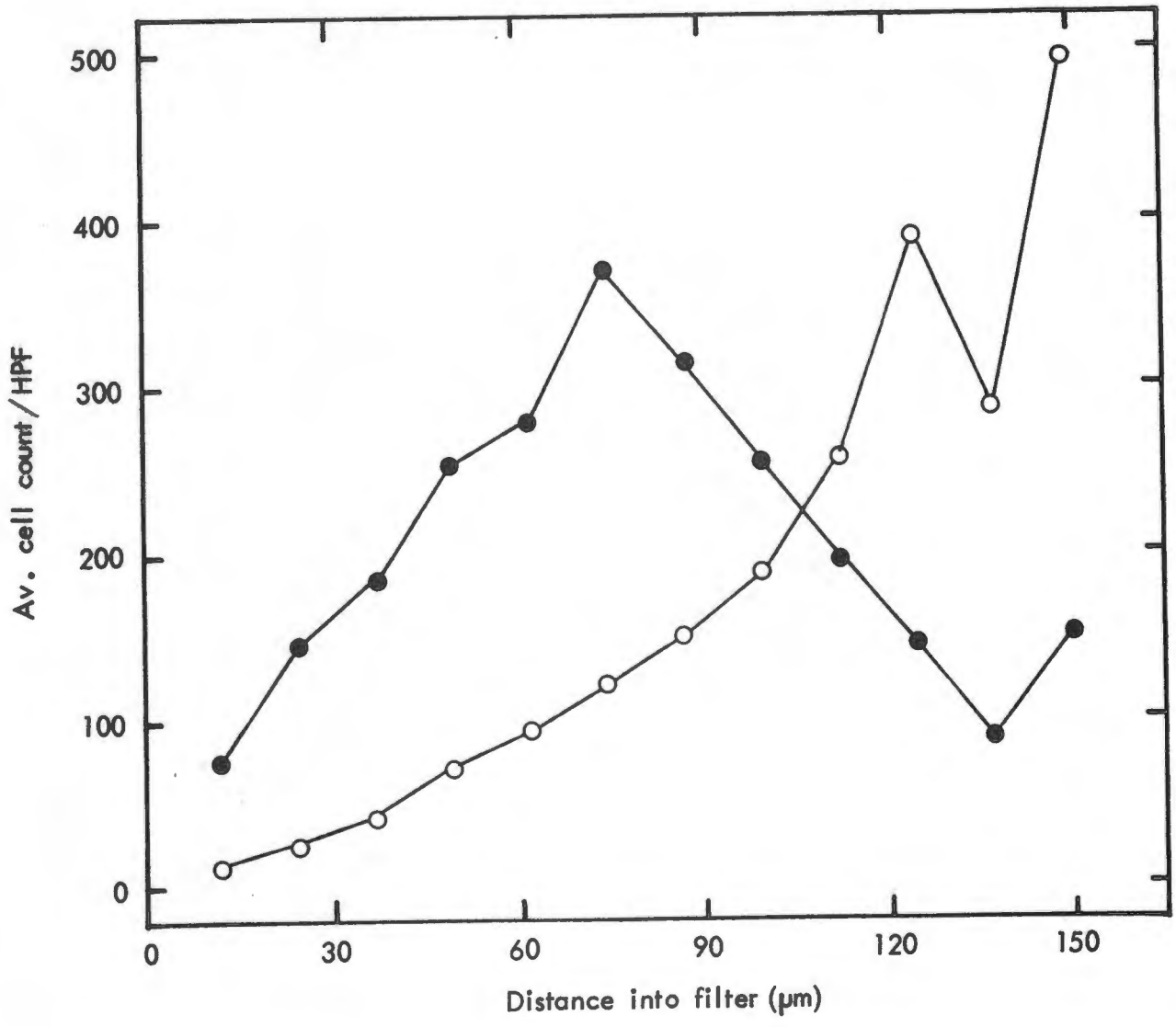

Figure 1.20 Effect of cytotaxin gradient on in-filter cell distribution.

Results of an experiment in which individual Boyden chambers were charged with $6x10^6$ cells in the upper compartment and a 1:50 (O—O) or 1:250 (●—●) dilution of cytotaxin (SNF) in the lower compartment. In-filter counts were performed after 180 min of incubation. Each point represents the mean of counts over 4 high-power fields on a single filter at the indicated distances from the upper surface.

In both cases, the mode has left the upper surface. In the case of cells responding to the lesser gradient (●—●) the mode is still within the filter after 180 min. In the other case (O—O) cells have accumulated on the lower surface.

Discussion

The experimental results presented in this section allow the conclusion that casein cytotaxin exerts two distinct effects upon the motility of rabbit peritoneal neutrophils.

The first effect is to increase the magnitude of the velocity vector, or the speed with which the neutrophils move. Zigmond and Hirsch (194) and Keller and Sorkin (81) have also noticed this effect. In the absence of the information provided by a chemotactic concentration gradient, this effect produces an increase in the mean velocity of the cells in random directions. The magnitude of this effect is directly related to the concentration of cytotaxin present. It would appear that the energy requirement for the increased speed of cellular movement is derived mainly from neutrophil glycolytic metabolism (191, this thesis Chapter III).

The second effect becomes apparent when a chemotactic concentration gradient is present, and consists of a change in the directional component of the cellular velocity vector which results in a displacement of the cells towards the source of the chemotactic gradient. Although the overall velocity of cellular movement towards the concentrated solution of cytotaxin was directly related to the steepness of the gradient, it was impossible to define, from my results, the way in which the purely directional acceleration was related to the steepness of the gradient. This difficulty stems from the fact that this experimental system does not allow one to dissociate, in any acceptable quantitative manner, the effect upon the magnitude of the velocity vector from the effect upon its direction.

In other words, an increasing rate of bulk cell traversal of the filter with increasing slope of the chemotactic gradient could result from either (a) increasing speed with *fixed* directional acceleration or (b) the summated effect of increasing speed *and* increasing directional acceleration.

It should perhaps be emphasized, in this regard, that the chemotactic compounds employed were impure. Although intuitively unlikely, it is not inconceivable that the two effects of the cytotaxin could have been produced by different compoenents of the chemotactic mixture. If this were the case it should be possible, with pure compounds, to demonstrate the pattern of increased random movement, without directional acceleration, even in the presence of a gradient of the appropriate stimulant of neutrophil speed. Similarly, the purely directional accelerant should cause coordinated bulk movement when present as a gradient, but have no effect upon random locomotion when present in the same concentration on both sides of the filter. Unfortunately the requirements for the appropriate reagents cannot be met at the present time.

Despite the fact that one cannot relate directional acceleration or chemotaxis in a quantitative manner to the intensity of the stimulation, it is nevertheless clear, that directional acceleration exists as a phenomenon distinct from the effect of chemotactic materials on speed of random movement.

Chapter II

The nature of casein cytotaxin

The nature of the molecular events that initiate directional cellular migration is obscure. Such mechanisms as have been proposed are either largely speculative or have been deduced from complex experimental systems in which it is difficult to define, in any satisfactory manner, the various components of the chemotactic response.

I felt it reasonable to assume, as a point of investigational departure, that chemotaxis must be initiated by interaction between some apparatus on the membrane of the responding cell and chemotactic molecules in the medium. This being the case, it seemed clear that one way in which this interaction could be studied would be by an experimental approach in which purified components were used to investigate, directly, the engagement of chemotaxin with putative cell membrane receptors for the biologically active compounds. I therefore decided to attempt to isolate a pure chemotactic substance.

When, in 1962, Boyden (24) introduced the technique for studying chemotaxis *in vitro*, he used, as chemotactic test substances, antibody-antigen mixtures that required, for chemotactic activity, incubation in the presence of heat labile factors present in fresh serum.

The first recorded *in vitro* studies in which casein was used as a cytotaxin were reported in 1965 by Keller and Sorkin (80). These authors found that casein (Hammarsten) was chemotactic for both polymorphonuclear leucocytes and macrophages without the necessity for prior incubation with fresh serum. They also noted that ultrafiltrates of casein solutions were chemotactic for polymorphonuclear leucocytes only, while the residual material ratained chemotactic activity for both polymorphonuclear leucocytes and macrophages and concluded that there were several cytotaxins present in casein (83). In describing this work the authors indicated that casein had previously been shown to exhibit

chemotactic activity *in vivo*. No reference to these experiments was given, nor have I been able to find specific references to the chemotactic property of casein in an extensive survey of the relevant early English literature.

Since this work was published, 1% w/v casein has been used as a positive control substance by numerous workers in laboratories throughout the world. No attempts to characterize the cytotaxin were made until Barth, Willerson, Asofsky, Sheagren and Wolff (11) established that their casein preparations, used for inducing experimental murine amyloidosis, contained small amounts of bacterial endotoxin. They ascribed a causal role to this contaminant in the genesis of the amyloidosis.

Baum, Mowat and Kirk (12) referred to these observations in their paper on human neutrophil chemotaxis and suggested that contaminating endotoxin was responsible for the chemotactic activity of the casein that they used.

Wilkinson (184), with doubtful experimental justification, concluded that the chemotactic activity of casein resided in the random coil configuration of the casein molecules themselves and was not attributable to a contaminant in the casein preparations that he used.

Casein appealed to me as a readily available, inexpensive source of starting material that was chemotactic as supplied without requiring activation by procedures that might introduce unnecessary experimental complexity.

At the initiation of this project the work of Baum et al.(12) and Wilkinson (184) had not been published so I had no precedent upon which to base my isolation procedure.

In the first instance I chose whole milk as a starting material for the fractionation.

The chemotactic properties of whole milk and crude milk fractions.

Whole, fresh, unpasteurized milk was obtained from a local herd of Jersey cattle. Skimmed milk was prepared from the whole milk by centrifugation at 7 000xg for 30 min at 4°C. This caused flotation of the cream to form a compact solidified surface layer that was easily removed. Any particulate cream remaining was removed by filtration through Whatmans 541 filter paper(1). The skimming process was found to be more efficient at low temperatures with the milk subjected to a high centrifugal force for prolonged periods of time.

The milk, apart from being extremely creamy, was found to contain a residue of insoluble material that pelleted under the conditions of centrifugation used. At the time no importance was attached to this material, hence further studies on its composition were not undertaken.

The casein was obtained from the skim milk by one of two procedures:

(a) Casein was precipitated according to the method first described by Hammarsten (60,61) as quoted by Oser (124). Acetic acid was used in preference to hydrochloric acid in conformity with Hammarsten's original work.

The method involved the slow acidification of a 1:2 aqueous dilution of skim milk by the addition of 10% acetic acid. The solution was continuously agitated and the pH gradually lowered to 4,5 at room temperature. After stirring at pH 4,5 for 30 min the precipitated casein was pelleted by centrifugation at 4 000xg for 10 min at 4°C and washed with distilled water.

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(1) W. & R. Balston Limited, Maidstone, England.

The yellow supernatant fluid was filtered through Whatman's 541 filter paper and the pH adjusted to 7,0 using 1N NaOH. It was then divided in two, half of which was sterilized by autoclaving at 10 psi for 10 min; the other half was sterilized by filtration through a 0,45µm Millipore filter. The precipitation process was repeated and the final precipitate was washed several times with distilled water before finally dissolving it in 0,9% NaCl and sufficient 1N NaOH to effect solution and a final pH of 7,1. The volume was adjusted with 0,9% NaCl to that of the starting milk volume. Any undissolved residue was removed by filtration and the filtrate was sterilized by autoclaving at 10 psi for 10 min.

(b) The skimmed, unpasteurized milk was treated with a calf rennet solution containing 4 mg/ml of protein. One millilitre of this rennet solution was sufficient to cause coagulation of 100 ml of the skimmed milk after incubation at pH 6,0 for 60 min at 37°C. The coagulated material was harvested by centrifugation at 4 000xg for 10 min at 4°C and washed several times with distilled water. The pelleted material had a rubbery texture and was difficult to dissociate into a suspension, in contrast to the granular, acid-precipitated material. The rennet casein was dissolved in 0,9% NaCl by the gradual addition of 1N NaOH to a final pH of 7,1 and a volume equal to that of the starting skim milk volume. The final solution was filtered using general purpose filter paper to remove insoluble material and sterilized by autoclaving at 10 psi for 10 min. The supernatant fluid from the precipitated rennet casein was filtered and brought to pH 7,1 with 1N NaOH.

Half of this supernatant fraction was sterilized by autoclaving at 10 psi for 10 min. The other half was sterilized by 0,45µm membrane filtration.

Chemotactic activity was assayed using the modified Boyden chamber technique as described in the Appendix.

Protein concentrations were measured according to a modified Folin Ciocalteau method detailed in the Appendix.

Results

As can be seen from the results summarized in Table 2.1 neutrophil chemotactic activity could be detected in whole milk, skim milk and neutral, autoclaved solutions of acid- or rennet-precipitated casein. No activity was demonstrable in the supernatant liquor of either of the casein preparations. The rennet casein preparation had the highest specific chemotactic activity in terms of response per mg of protein and was equivalent, in this respect, to a solution of commercially available casein prepared by acid precipitation.

Discussion

These results warrant comment in several general and specific respects. Perhaps the most important of these concerns the nature of casein itself.

In 1838 Mulder first reported the observation that a protein, "casein" could be precipitated from bovine milk by the addition of acid. This observation was later amplified by Hammarsten who described a technique for the purification of this protein after precipitation with dilute acetic acid. For many years the protein prepared in this way was regarded as a "pure" substance and it was not until the advent of more refined techniques for studying macromolecules that the composition

Table 2.1

The chemotactic properties of whole milk and milk fractions

No.	Sample	Treatment	Fraction	Sterilization	Volume (ml)	Protein (mg/ml)	Chemotaxis (Av. cell count/HPF)	Chemotaxis (Av.cell count/mg protein)	Recovery (%)	Purification (X)
1	Whole fresh unpasteurized milk	-	-	Sterile filtered	110	-	184	-	100	1
2	"	Centrifugation	Aqueous phase	Sterile filtered	100	16,41	369	23	182	-
3	Skimmed fresh unpasteurized milk	Acetic acid precipitation	Casein	10psi for 10min	100	7,53	66	9	3,3	0,4
4	"	"	Super-natant	10psi for 10min	100	1,14	1	0,9	0,5	0,04
5	"	"	"	Sterile filtration	100	2,52	4	1,6	2	0,07
6	"	Rennet precipitation	Casein	10psi for 10min	100	3,17	242	76	120	3
7	"	"	Super-natant	10psi for 10 min	50	4,26	1	0,2	0,2	0,009
8	"	"	"	Sterile filtration	50	-	47	-	12	-
9	Commercial BDH	-	1% w/v casein	10psi for 10min	-	5,40	374	-	-	-
10	-	-	0,9% NaCl	15psi for 20min	-	-	1	-	-	-

of casein (Hammarsten) became better defined. The biochemistry of milk proteins has recently been extensively reviewed (111,112,113) and little purpose would be served by my expanding on this subject. Suffice it to say that casein is now regarded as a heterogenous group of phosphoproteins which are precipitated from skim milk at pH 4,5 and 20°C. Considered in the context of contemporary knowledge of protein chemistry and the complexities of biological fluids, it would be very surprising if casein, prepared by so simple a procedure as that described by Hammarsten, were, in any sense of the word, "pure". Apart from the documented heterogeneity of the phosphoproteins present, it is highly probable that minor contaminants present in the skim milk would co-precipitate non-specifically at pH 4,5 with the major phosphoprotein fraction. It is, therefore, important to realize that casein (Hammarsten) is an operationally defined complex misture of compounds.

The coagulation of casein by rennin, the active gastric enzyme in a rennet solution, was first observed at the turn of the century by Hammarsten and has since been extensively studied by numerous workers. The subject has been well reviewed by Lindquist (99,100). Of relevance to the work in the thesis is the fact that the coagulation process is attended by the release of trichloracetic acid-soluble peptides of which a glycopeptide of M.W. 6 000 to 8 000 from the C-terminal end of the κ-chain of casein is the best defined (2,70,99,100). One may tentatively conclude, therefore, that casein coagulation involves proteolytic steps similar, in principle, to those found in the clotting of fibrinogen. It has been known for many years that the products of proteolysis may have biological activity particularly with respect to the inflammatory process (39,43,108,109,116,118,119,120,146).

Not only is casein itself a heterogenous product. It is now

clear that the casein content of milk may vary from one sample of milk to another, both in terms of concentration and, qualitatively, in terms of the chemical character of the casein components present. Some of these variations can be attributed to genetic factors, season and state of lactation (6,7,85,86,91,92,93,97,111,112,134,156,157,164).

It is also known that cows vary in the extent to which they are subject to inflammatory conditions of the udder. Mastitis of some degree is widespread amongst most herds and a bewildering array of causative micro-organisms has been identified (74). Inflammatory cells and other cellular debris are similarly present to a variable extent in virtually all samples of milk and were evident as a pellet in the centrifuged samples that I examined. It is clear, therefore, that growth of micro-organisms or inflammation in the udder could contribute varying amounts of chemotactic substances to the milk that have little to do with the casein itself.

In deciding upon the best source of starting material for attempts to isolate a cytotaxin, I abandoned fresh milk in view of the preparative difficulties that would be involved in handling large volumes and in view of the known wide variations in milk components referred to above. Furthermore, my own attempts to prepare chemotactic casein compared unfavourably with the commercial product. Rennet treatment was unsatisfactory for my purposes both for the fact that it gave a "rubbery" casein clot that was difficult to work with, and for the possible generation of complicating biologically active material that the rennet treatment might have caused.

For this reason I chose to base my further work on a large batch of casein (Hammarsten) obtained as lot numbers 0868390 and 300200 from a single commercial supplier(1).

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(1) British Drug House Limited, Poole, England.

THE CHEMOTACTIC FACTORS IN COMMERCIAL CASEIN

In 1967 Keller and Sorkin (83) reported chemotactic activity for polymorphonuclear leucocytes in ultrafiltrates of casein solutions, indicating that low molecular weight compounds might be the active components. With this information available, I developed the following procedure for the characterization of the chemotactic material, starting with acid precipitation of redissolved commercial casein as the initial step.

Detection of chemotactic activity in acid precipitated casein supernatant fluid.

Casein was dissolved in 0,9% NaCl by the slow addition of 1N NaOH (approximately 25 ml per 50g casein) to a final pH 7,1. The volume of the solution was then adjusted with 0,9% NaCl to give a 5% w/v casein solution. After stirring at room temperature for 60 min any undissolved material was sedimented by centrifugation (4 000xg; 60 min; $4^{o}C$) and the supernatant fluid was filtered through Whatman's 541 filter paper. Care was taken to ensure that local excesses of the alkali were avoided, since casein is reported to be susceptible to alkaline denaturation (35,36,45,46).

The casein was reprecipitated by the gradual addition of 1N HCl, with continuous stirring at room temperature, to pH 4,5. The solution was stirred for a further 30 min at this pH. By the careful and slow addition of acid, with adequate stirring, a fine flocculent precipitate could be readily obtained which was easy to handle and redissolve. The optimum pH for casein precipitation and for clarification of the supernatant fluid could be judged visually as that point in the titration when an abrupt change from turbidity to clarity occurred.

The precipitated material was removed by centrifugation (4 000xg; 10 min; 4°C) and washed twice with distilled water. The washed casein was redissolved in 0,9% NaCl and 1N NaOH was added to facilitate solution as before. The volume was adjusted with 0,9% NaCl to equal that of the initial 5% w/v casein solution. Finally, the reprecipitated and redissolved casein solution was sterilized by autoclaving at 10 psi for 10 min.

The supernatant fluid fraction was filtered and the pH was readjusted to pH 7,1 by the addition of 1N NaOH. It was then sterilized; half by autoclaving at 10 psi for 10 min and the other half by sterile filtration.

Chemotactic activities and protein concentrations were determined by the modified Boyden chamber and Lowry protein techniques respectively, as described in the Appendix.

As can be seen from the results presented in Table 2.2, acidification precipitated 98 to 99% of the protein from the initial solution, yet chemotactic activity was roughly equally divided between the precipitate and supernatant fluid. Expressed in terms of "specific chemotaxis", or cells migrated/mg of protein, the supernatant fluid was considerably more active (approximately 600 cells/HPF/mg protein) than either the redissolved casein precipitate or the initial material (both approximately 30 cells/HPF/mg protein). Although the kinetics of *in vitro* chemotaxis do not, strictly speaking, allow straightforward comparisons based upon unit weight of protein, these calculations nevertheless indicate that a substantial fraction of the chemotactic activity can be dissociated from casein by the simple expedient of acid precipitation and that a considerable degree of purification could be

Table 2.2

The effects of acid precipitation on solutions of casein (Hammarsten). A possible fractionation procedure for cytotaxin purification.

Expt no.	Sample	Sterilization	Volume (ml)	Protein (mg/ml)	Chemotaxis (Av.cell count/ HPF)	Chemotaxis (Av. cell count mg/protein)	Recovery (%)	Purification (X)
1	1/5 dilution 5% w/v casein	10psi for 10min	500	5,28	196	37	100	1
2	1/5 dilution 5% w/v casein (end)	10psi for 10min	500	4,04	107	26	55	0,7
3	Supernatant fluid	10psi for 10min	50	0,29	167	576	8,5	16
4		Sterile filtration	50	0,31	184	594	9,5	16
5	1% w/v casein control (+ve)	10psi for 10min	-	5,40	374	69	-	-
6	0,9% NaCl control (-ve)	15psi for 20min	-	-	1	-	-	-

achieved by this procedure. Despite the fact that only 18% of the chemotactic activity could be recovered in the supernate, I felt that the degree of purification achieved made the loss acceptable.

It is further apparent, from the data presented above, that the chemotactic activity was heat stable to the extent that it would withstand autoclaving at 120°C for 10 min. To ensure sterile preparations in subsequent procedures, acid supernatant fractions were sterile filtered and crude solutions of casein were autoclaved.

The stability of the chemotactic activity in the supernatant fluid to temperature extremes is evident from the data summarized in Table 2.3. Chemotactic activity could be preserved by storage at 4°C and -20°C for several months. For routine purposes, supernatant chemotactic solutions were stored at -20°C after sterile filtration.

Table 2.3

Temperature stability of the chemotactic components in acid-precipitated casein supernatant fluid.

Expt no.	Sample	Treatment	Chemotaxis (Av.cell count/HPF)
	Supernatant fluid	10psi; 10min; 120°C	167
1	Supernatant fluid	Sterile filtration; 20°;	184
	1% w/v casein (+ve) control	10psi; 10 min; 116°C	374
	0,9% NaCl (-ve) control	15psi; 20min; 121°C	1
	Supernatant fluid A	Stored 4°C	99
		Stored -20°C	137
2	Supernatant fluid B	Stored 4°C	265
		Stored -20°C	281
	1% w/v casein (+ve) control	Stored -20°C	192
	0,9% NaCl (-ve) control	Stored 20°C	1

Concentration and preliminary characterization of the chemotactic material in acid-precipitated casein supernatant fluid.

The clear, dilute solution of chemotactic material obtained by acid precipitation and rejection of the bulk protein I refer to as SNF-cytotaxin. For subsequent purification procedures I required:

(a) a means of concentrating the cytotaxin to a reasonable volume for column chromatography

(b) a means of detecting the cytotaxin chemically and

(c) a means of detecting the cytotaxin in column effluents by virtue of its biological activity.

In order to meet the requirement (c) it was necessary to choose buffers for subsequent chromatography that would not exert a toxic effect upon the neutrophils used in the biological assay. To devise a suitable method for the concomitant chemical detection of the cytotaxin, I needed to have some idea of whether it was a peptide, a carbohydrate or a lipid. The following preliminary experiments were, therefore, undertaken to serve the needs of subsequent purification steps.

The most obvious technique to attempt, in the first instance, for concentration of cytotaxin, was one that would use the semi-permeable properties of cellophane membranes[1]. To assess the applicability of this approach, 50ml of sterile SNF-cytotaxin was dialysed at 4°C for 12h against 2x1 000ml changes of sterile 0,9% NaCl. The contents of the dialysis bag were then tested for chemotactic activity using a modification of the Boyden chamber assay as described in the Appendix.

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(1) Union Carbide Corporation, Chicago, Illinois, U.S.A.

Table 2.4

The effect of dialysis on the chemotactic activity of acid-precipitated casein supernatant fluid.

No.	Sample	Treatment	Chemotaxis (Av.cell count/HPF)
1	SNF-cytotaxin	-	369
2	SNF-cytotaxin	Dialysis	145
3	1% w/v casein (+ve) control	-	333
4	0,9% NaCl (-ve) control	-	1

The results are summarized in Table 2.4 and show that dialysis resulted in the loss of 60% of the chemotactic activity of the SNF-cytotaxin preparation.

The molecular weight retention limit in membrane dialysis is approximately 12 000 Daltons indicating that the dialysable chemotactic material was of low molecular weight.

The ease with which chemotactic material passed through a dialysis membrane was verified in subsequent experiments (for example see Table 2.5).

Negative pressure filtration using cellophane membranes, had, therefore, to be abandoned as a means of concentrating the cytotaxin.

Samples of SNF-cytotaxin were therefore lyophilized for concentration.

The probable polypeptide nature of the cytotaxin was established in a preliminary experiment in which crude SNF-cytotaxin was digested with the proteolytic enzyme, pronase. Enzyme and products were separated after digestion by negative pressure filtration through a cellophane membrane. By this procedure, enzyme is retained and cytotaxin passes through into the filtrate.

Table 2.5

The effect of pronase digestion on the chemotactic activity of acid-precipitated casein supernatant fluid.

No.	Sample	Chemotaxis (Av.cell count/HPF)
1	Ultrafiltrate of SNF-cytotaxin	179
2	Ultrafiltrate of SNF-cytotaxin after pronase digestion	2
3	1% w/v casein (+ve) control	241
4	0,9% NaCl (-ve) control	2

Details of this experiment and the results obtained are given in Table 2.5 from which it is evident that chemotactic activity passed into the ultrafiltrate in the case of the control whereas no cytotaxin could be detected in that of the pronase digest. I, therefore, concluded that the cytotaxin was partly or wholely polypeptide in nature and that the techniques for the recognition of proteins would be most appropriate for its detection. A number of additional experiments confirming the polypeptide nature of the cytotaxins were performed. These are reported later in this chapter.

The fact that the cytotaxin passed through cellophane membranes meant that I would not be able to exchange the buffer in which it was dissolved by the simple experient of dialysis and I had hence to be prepared to perform all biological and chemical assays with the material dissolved in the buffer in which it found itself at the time.

I rejected phosphate buffers for the possibility that they might interfere with the detection and assay of phosphoproteins. Casein is known to be such a protein and I was concerned that the chemotactic material might be a phosphorus containing fragment of casein.

Triethanolamine was next considered and was found to have several advantages to recommend it. Firstly, it proved to be biologically inert and without detectable effect upon neutrophil chemotaxis in concentrations as high as 5mM. Secondly, it is known to be less nucleophilic than the alternative buffer, tris(hydroxy)methylaminomethane(tris) and less likely to have toxic effects upon migrating polymorphonuclear leucocytes. Thirdly, it was available in a highly purified form from a reputable supplier(1). Fourthly, its pK is relatively insensitive to temperature changes. Triethanolamine was therefore chosen as the buffering compound, at a concentration of 5mM and pH 7,1. All buffers were adjusted to isotonicity with NaCl for subsequent chemotactic assay. This 5mM triethanolamine/HCl pH 7,1; 0,15M NaCl I refer to as triethanolamine-buffered-saline (TBS).

Sephadex G-200 chromatography.

A 2,5x85cm column of Sephadex G-200(2) was prepared according to the instructions given by the suppliers and equilibrated with TBS in a Pharmacia K-25(2) chromatography column equipped with flow adaptors. Great care was taken to ensure sterility of the column, buffer and fraction collecting system to avoid spurious contributions of chemotactic material from bacterial contaminants. Bacterial cultures of the column effluent were taken routinely and all showed no growth after 24h.

The column was calibrated with the following protein markers

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(1) Boehringer Mannheim GmbH, Mannheim, West Germany.

(2) Pharmacia Fine Chemicals, Uppsala, Sweden

of known molecular weight: myoglobin[1] (17 800) and cytochrome C[2] (12 400). Blue dextran[3] (2 000 000) and L-tyrosine[4] (181,2) were used to estimate the void volume and the total column volume respectively.

Two hundred millilitres of sterile SNF-cytotaxin containing approximately 0,1mg protein/ml were lyophilized and the residue dissolved in 10 ml of TBS. After sterile filtration, 6 ml of the sample were introduced on to the column and eluted at 4°C, at 30 ml/h with TBS by upward flow. The effluent was collected as 5 ml fractions into sterile test tubes and alternate fractions were tested for their ability to elicit a chemotactic response from polymorphonuclear leucocytes. Owing to the large number of fractions to be tested, only one filter per fraction was used.

The optical density at 280 nm of alternate fractions was used to obtain approximate estimates of the protein concentration in the effluent.

The composite elution profile of chemotactic activity and protein concentration is shown diagrammatically in Figure 2.1.

As can be seen from the elution profile, only one peak of chemotactic activity was detected. This peak was clearly separated from the bulk of the protein present and eluted between cytochrome c (M.W. 12 400) and the total column volume. This elution profile corresponds to that of a compound with molecular weight less than 12 000 Daltons, in conformity with my previous results with dialysis fractions.

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(1) Horse skeletal muscle; Miles Seravac (Pty) Ltd., Epping Industria, Cape, South Africa.
(2) Horse heart; Miles Seravac (Pty) Ltd., Epping Industria, Cape,S. Africa
(3) Pharmacia Fine Chemicals, Uppsala, Sweden
(4) Merck GmbH, Dramstadt, West Germany.

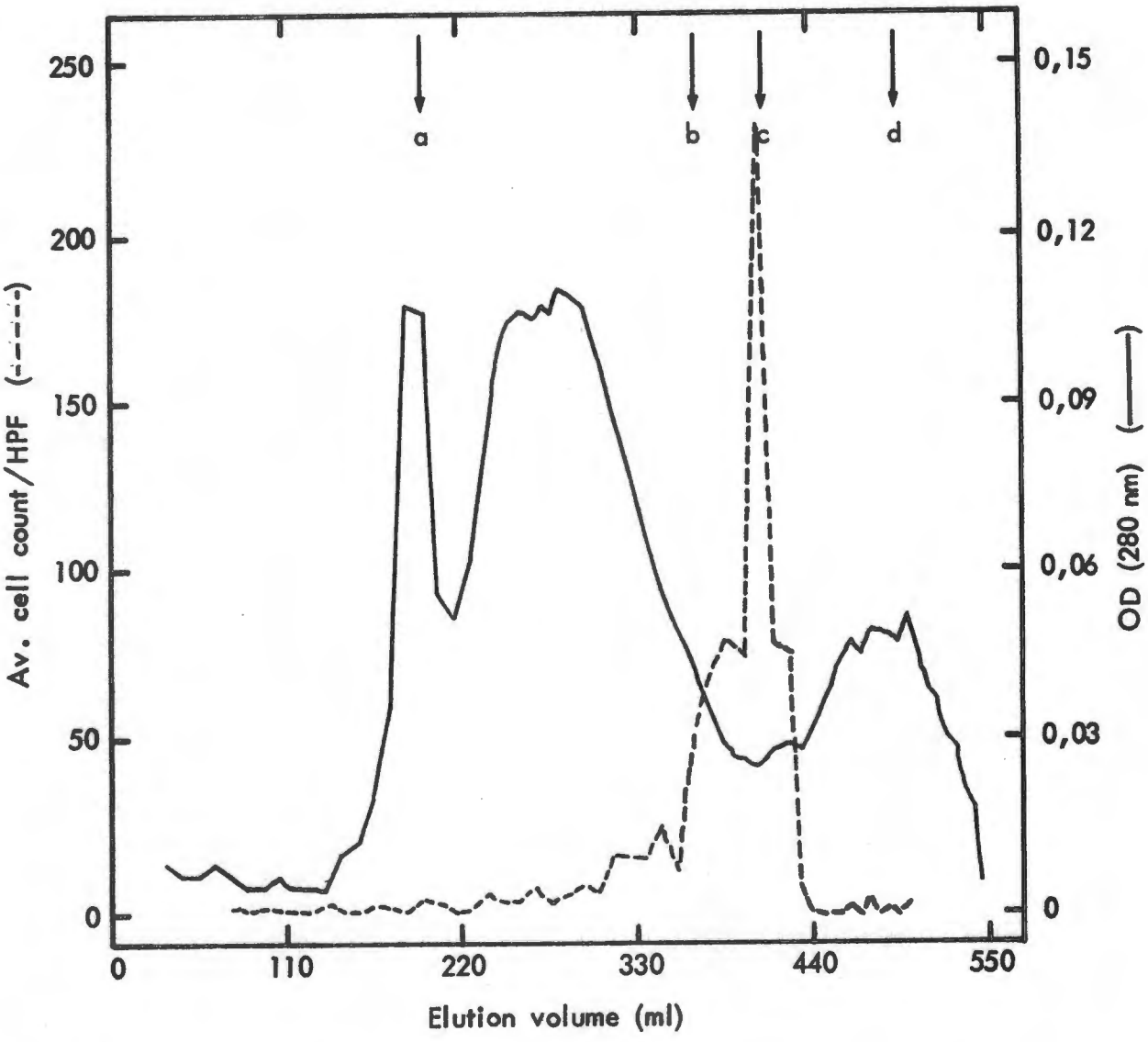

Figure 2.1 Gel chromatography of casein cytotaxin (SNF)

Elution profile of chemotactic activity present in 6,0ml of casein-SNF preparation chromatographed on a 2,5x85cm column of Sephadex-G200 equilibrated with TBS and eluted with the same buffer at a flow rate of 20ml/hr at 4°C.

Approximately 5ml fractions were collected and assayed directly for neutrophil chemotaxis (----). Absorbance at 280nm (——) was used for protein collection.

The lettered arrows indicate the volumes at which blue dextran (a); myoglobin (b); cytochrome C (c) and tyrosine (d) were eluted.

Amicon[1] *UM2 ultrafiltration.*

At this stage in the work I became acquainted with the Amicon range of molecular membrane filters with selective retention parameters in the useful low molecular weight range. The obvious benefits to be derived from a technique in which the cytotaxin could be concentrated without concomitant concentration of buffer salts, prompted me to evaluate the applicability of the UM2 membrane ultrafiltration procedure to the isolation of the cytotaxin. This particular membrane had a listed useful retentivity for compounds of molecular weight greater than approximately 1 000 Daltons. The following experiment illustrates the usefulness of this procedure.

Supernatant fluid from acid-precipitated casein was prepared as described. Two hundred millilitres of this solution were concentrated to 10 ml by positive nitrogen pressure in a sterile, model 404 Amicon stirred-cell ultrafiltration apparatus at 4°C. Samples of the starting SNF-cytotaxin, the concentrate and the ultrafiltrate were tested for chemotactic activity in the usual manner.

The results are given in Table 2.6 from which it can be seen that the UM2 ultrafiltration effectively retained and concentrated all chemotactic activity.

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(1) Amicon Corporation, Lexington, Massachusetts, U.S.A.

Table 2.6

The effect of Amicon UM2 ultrafiltration on the chemotactic activity of SNF-cytotaxin.

No.	Sample	Chemotaxis (Av. cell count/HPF)
1	SNF-cytotaxin	336
2	1/10 dil UM2 concentrate	500
3	1/20 dil UM2 concentrate	500
4	UM2 ultrafiltrate	1
5	1% w/v casein	500
6	Gey's BSS-2% HSA	1

Polyacrylamide gel bead molecular exclusion chromatography.

Sephadex G-200 chromatography with a useful discriminating molecular weight range from approximately 50 000 to 800 000 Daltons was inappropriate for the cytotaxin. Polyacrylamide beads[1] with a molecular weight fractionation range from approximately 1 500 to 20 000 Daltons was therefore chosen for the next chromatographic step.

Biogel P-10 (200-400 mesh) was swollen and sterilized by heat in distilled water for 6h in a boiling water bath. This was then used to pack a 2,5x92cm sterile column equipped with flow adaptors. This was equilibrated at 4°C with sterile TBS.

The column was calibrated by chromatographic separation of the marker substances listed in Table 2.7. Blue dextran was used to obtain the void volume (V_o) and sucrose the total column volume (V_t).

Acid-precipitated casein supernatant fluid was concentrated approximately 7-fold by Amicon UM2 ultrafiltration to a final protein concentration of approximately 6mg/ml. Two millilitres of this concentrate were added to the Biogel column and eluted by upward flow with TBS containing

(1) Biogel P-10 (200-400 mesh); Biorad Laboratories, Richmond, California, U.S.A.

Table 2.7

Calibration standards for Biogel P-10 chromatography

No.	Marker	Molecular Weight*	Method of Detection
1	Blue dextran(1)	$2x10^6$	OD_{280nm}
2	Myoglobin(2) (horse skeletal muscle)	17 800	OD_{280nm}
3	Cytochrome c(2) (horse heart)	12 400	OD_{280nm}
4	Insulin(3) (porcine)	5 780	γ-counter
5	Vitamin B_{12}(4) (Cytamen 1000)	1 460	OD_{280nm}
6	Sucrose(5) (ultra pure)	342,6	Anthrone carbohydrate reaction, OD_{570nm}.

*Molecular weights according to Andrews (5).

(1) Pharmacia Fine Chemicals, Uppsala, Sweden

(2) Miles Seravac (Pty) Ltd., Epping Industria, Cape, South Africa

(3) Nova Laboratories (Pharmaceuticals)(Pty) Ltd., Johannesburg, South Africa

(4) Glaxo-Allenbury South Africa (Pty) Ltd., Johannesburg, South Africa

(5) Schwarz Mann, Orangeburg, New York, U.S.A.

0,005% w/v chlorhexidine[1] at an approximate flow rate of 0,5 ml/min. Preliminary experiments indicated that chlorhexidine at this concentration did not inhibit chemotaxis. A total of 70 fractions were collected with the aid of an automatic drop counter in 150-drop fractions. Alternate fractions were assayed for chemotactic activity. The concentration of protein in the column effluent was monitored continuously as % transmission at 280 nm using the LKB 8300A Uvicord II[2] equipped with a LKB 6520 chopper bar recorder.

As can be seen from the elution profile of a typical experiment depicted in Figure 2.2, Biogel P-10 molecular exclusion chromatography resolved the chemotactic material into a major peak (Peak II) eluting with a V_e/V_t of 0,8 and two minor peaks, the first (Peak I) eluting with a V_e/V_t of 0,6 and the second (Peak III) with a V_e/V_t of 0,96.

The bulk of the protein in the SNF-cytotaxin was clearly separated from the chemotactic activity. Protein in the chemotactic fractions was present in too low a concentration to be detected by the ultraviolet monitoring system used.

Chromatography on Biogel P-10 polyacrylamide gel beads was repeated on four occasions for the marker substances and on six occasions with the chemotactic material. On each occasion the V_e/V_t was calculated for the molecular weight markers and for the three chemotactic peaks I, II and III. The results obtained are summarised in Table 2.8.

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(1) Hibitane R ; Imperial Chemical Industries Ltd., Cheshire, England.

(2) L.K.B. Produkter AB, Bromma 1, Sweden.

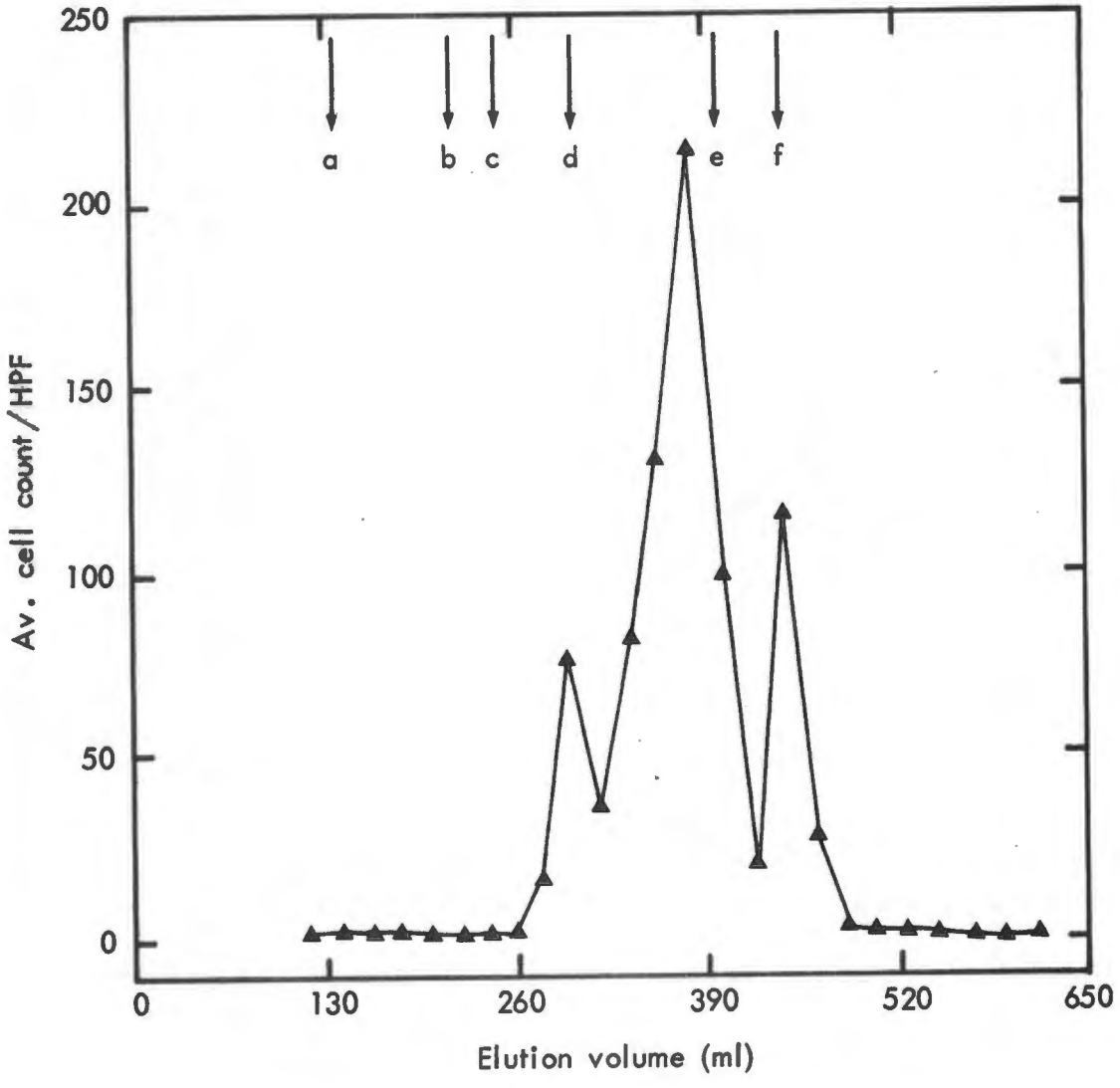

Figure 2.2 Gel chromatography of casein cytotaxin (SNF)

Elution profile of chemotactic activity present in 2,0ml of casein-SNF preparation chromatographed on a 2,5x92cm column of Biogel P-10 equilibrated with TBS and eluted with the same buffer at a flow rate of 20ml/hr at 4°C.

Ten millilitre fractions were collected and assayed directly for neutrophil chemotaxis.

The lettered arrows indicate the volumes at which blue dextran (a); myoglobin (b); cytochrome C (c); insulin (d); vitamin B_{12} (e) and sucrose (f) were eluted.

Table 2.8

Values for V_e/V_t obtained for marker substances (A) and chemotactively active peaks (B) on Biogel P-10 chromatography

	No.	Substance	V_e/V_t						
		Expt	1	2	3	4	5	6	Mean
A	1	Myoglobin	0,48	0,48	0,51	0,51	-	-	0,49
	2	Cytochrome c	0,55	0,55	0,57	0,57	-	-	0,56
	3	Insulin	-	-	0,69	0,68	-	-	0,68
	4	Vitamin B_{12}	0,90	0,89	0,91	0,92	-	-	0,90
	5	Sucrose	1,00	1,00	1,00	1,00	-	-	1,00
B	1	Peak I	0,68	0,66	0,69	0,67	0,66	0,68	0,67
	2	Peak II	0,85	0,81	0,82	0,86	0,82	0,86	0,84
	3	Peak III	0,97	1,00	0,99	1,01	1,00	0,96	0,99

When the logarithms of the molecular weight of the markers were plotted as a function of the mean V_e/V_t values observed, an excellent linear relationship was seen (Figure 2.3).

The parameters of the least squares regression line fitted to their relationship was used to calculate average molecular weights of 6 000, 2 200 and 880, for chemotactic peaks I, II and III respectively.

As can be seen from Figure 2.4, similar peaks of chemotactic activity were found for neutrophils and macrophages although the response of the latter was not as strong as that of the former.

CHARACTERIZATION OF LOW MOLECULAR WEIGHT CASEIN CYTOTAXINS

Further rational attempts to isolate the chemotactic materials present in casein required some notion of the chemical characteristics of the compounds concerned. The following experiments were performed to obtain this information.

Starting material

The experiments discussed in the preceding pages had indicated that the cytotaxins had relatively low molecular weights and might, therefore, be amenable to preliminary purification by rapid ultrafiltration through commercially available "hollow fibres". This would have a technical advantage over the acid precipitation procedure in that the need for centrifuging large volumes would be obviated.

Accordingly, a model CH3 Amicon ultrafiltration cell[1] fitted with a hollow fibre cartridge with a nominal molecular weight cut-off of

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(1) Amicon Corporation, Lexington, Massachusetts, U.S.A.

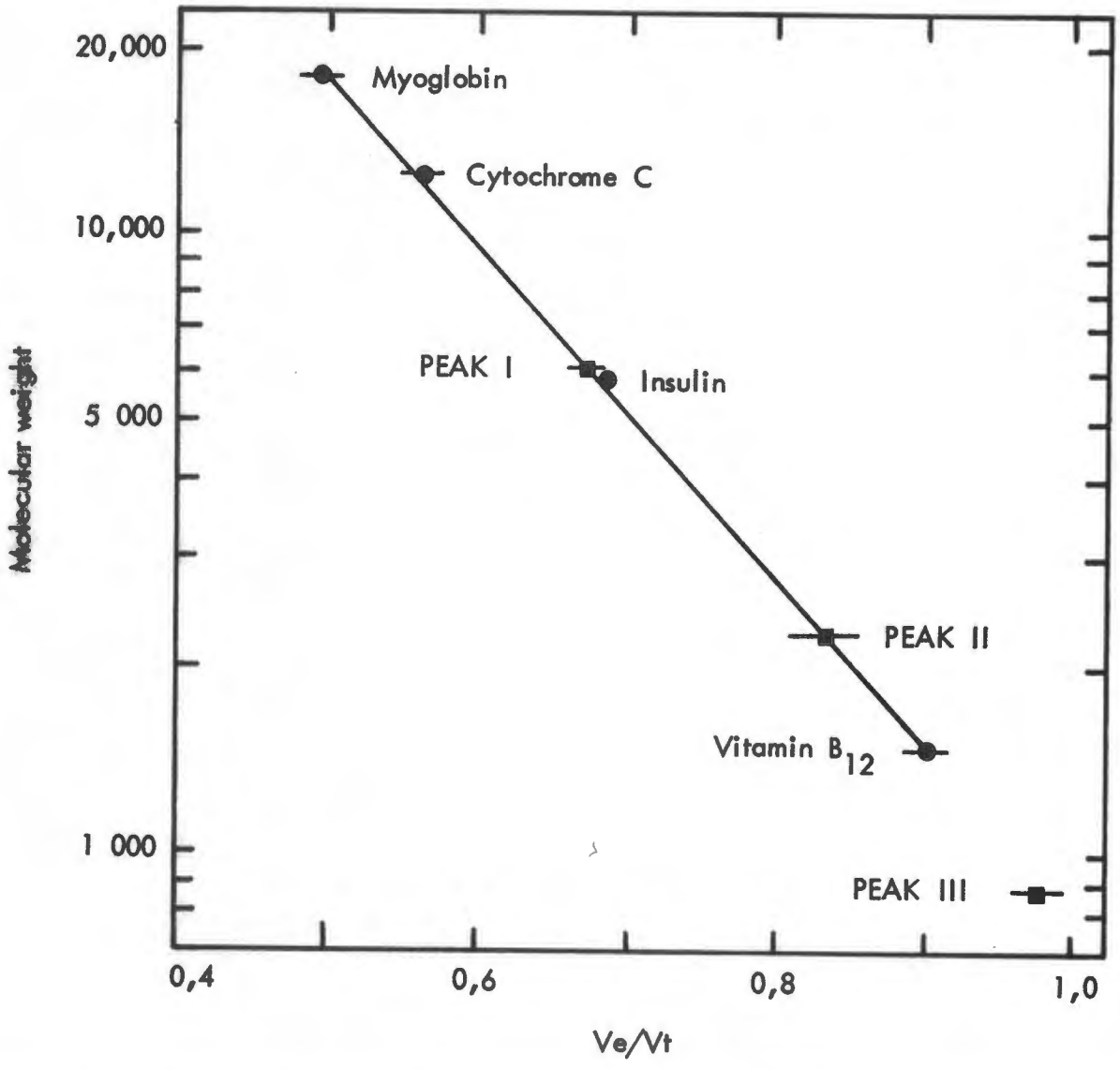

Figure 2.3 Calibrated Biogel P-10 chromatography of casein cytotaxin (SNF).

The diagram summarizes the results of 6 identical runs in which semi-purified cytotaxin (SNF) was chromatographed in a 2,5x92 cm column of Biogel P-10 with the marker proteins indicated.

The results have been analysed by the method of Andrews (5) in which $\ln$ molecular weight is plotted as a function of the ratio of elution volume to total colume (Ve/Vt). The elution volume of sucrose was taken as the total column volume.

The points and horizontal lines represent the means and ranges, respectively, of the Ve/Vt values observed. Molecular weights of 6 000 and 2 200 were calibrated for peak I and peak II respectively by interpolation. A molecular weight of 880 was calibrated for peak III by extrapolation.

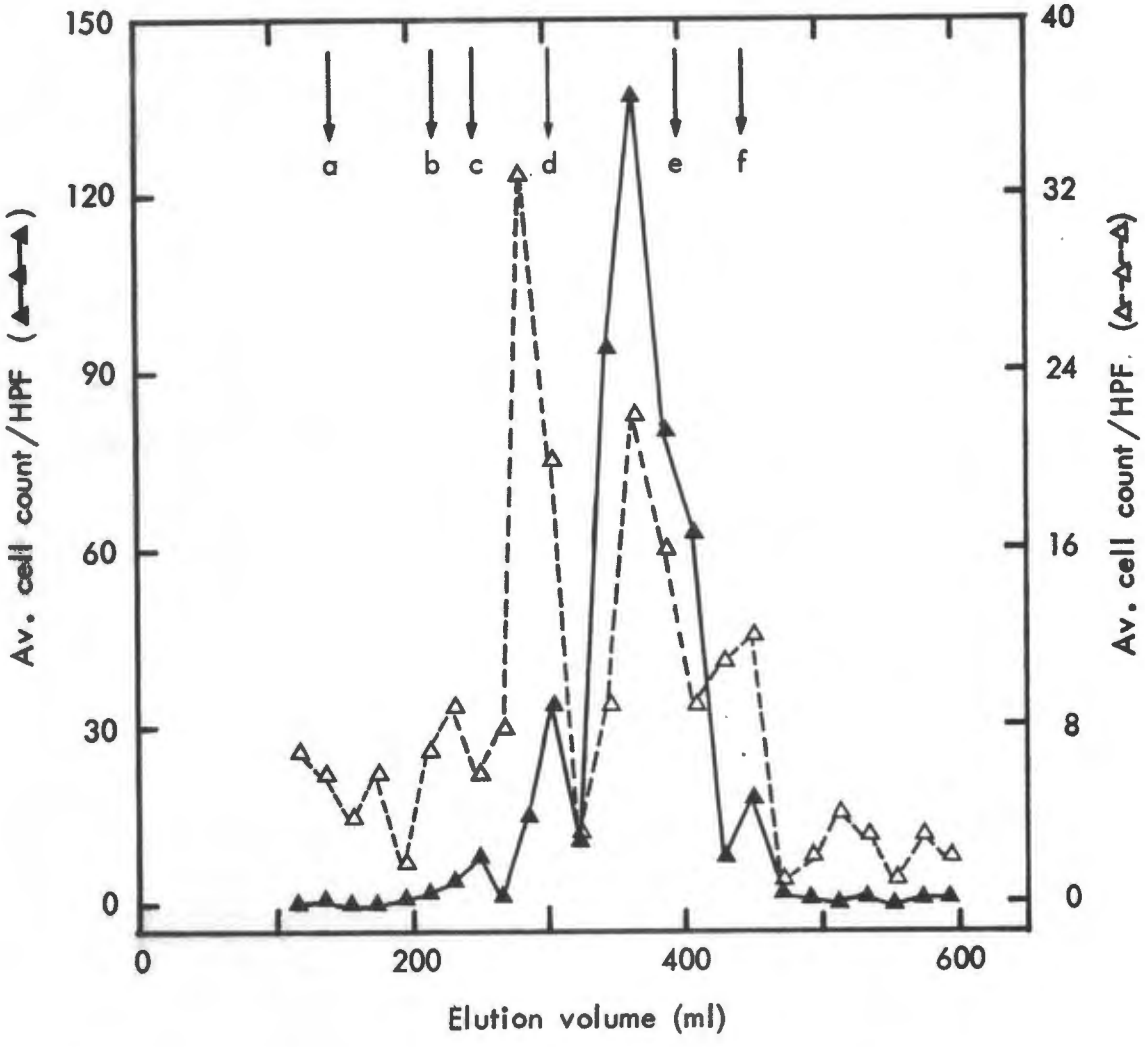

Figure 2.4 Gel chromatography of casein cytotaxin (SNF)

Elution profile of chemotactic activity present in 2,0ml of casein-SNF preparation chromatographed on a 2,5x92cm column of Biogel P-10 equilibrated with TBS and eluted with the same buffer at a flow rate of 20ml/hr at 4°C.

Ten millilitre fractions were collected and assayed directly for macrophage chemotaxis (△--△) and neutrophil chemotaxis (▲—▲).

The lettered arrows indicate the volumes at which blue dextran (a); myoglobin (b); cytochrome C (c); insulin (d); vitamin B_{12} (e) and sucrose (f) were eluted.

10 000 Daltons was used, at room temperature, to process 5 litres of 1% casein, pH 7,1 dissolved in 0,9% NaCl. As ultrafiltration proceeded, the retained casein solution became more concentrated and its viscosity increased considerably with a concomitant rise in the filtration pressure. To avoid damage to the fibres, the cell was modified by the inclusion of a safety valve in the retentate circuit, set to release at a pressure in excess of 22 psi. Distilled water was added to the retentate as ultrafiltration proceeded. This served to reduce viscosity and to "wash" additional cytotaxin into the ultrafiltrate.

With this method, *10 l* of diluted ultrafiltrate could be prepared from *5 l* of 1% casein solution in approximately 3h.

The ultrafiltrate was sterilized by membrane filtration and concentrated to an approximate volume of 100 ml in a sterile model 404 Amicon stirred-cell ultrafiltration apparatus[(1)] using a UM05 filter and operating under a nitrogen pressure of 75 psi at 4°C. The UM05 retentate was assayed for chloride content, adjusted to isotonicity with additional sodium chloride and tested for chemotactic activity.

The results of three such preparations are summarized in Table 2.9 from which can be seen that hollow fibre ultrafiltration rapidly and effectively passed low molecular weight chemotactic material into the ultrafiltrate. As with the acid precipitation method, a certain amount of chemotactic activity remained with the casein in the retentate. Despite this loss of retained chemotactic activity, a considerable degree of purification of the cytotaxin was obtained as measured approximately by the chemotactic activity observed (in cell count/HPF/mg of protein) in

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(1) Amicon Corporation, Lexington, Massachusetts, U.S.A.

Table 2.9

Preparation of low molecular weight casein cytotaxin : casein ultrafiltrates.

Preparation	Fraction	Protein (mg/ml)	Chemotaxis[(a)] (Av. cell count/HPF)	Specific[(b)] activity ($\frac{\text{Cell count/HPF}}{\text{mg protein/ml}}$)
CUF-2	1% w/v casein: initial	8,43	143	17
	1% w/v casein: hollow fibre retentate	8,43	97	12
	Hollow fibre ultrafiltrate	-	-	-
	UM05 retentate (1/100)	0,25	111	444
	UM05 ultra-filtrate (1/1)	0,03	2	-
CUF-3	1% w/v casein: initial	7,96	460	58
	1% w/v casein; hollow fibre retentate	7,96	431	54
	Hollow fibre ultrafiltrate	0,48	469	977
	UM05 retentate (1/500)	0,08	305	3813
	UM05 ultra-filtrate (1/1)	0,02	3	-
CUF-4	1% w/v casein: initial	8,15	337	41
	1% w/v casein; hollow fibre retentate	8,15	304	37
	Hollow fibre ultrafiltrate	0,067	222	3313
	UM05 retentate (1/50)	0,04	152	3800
	UM05 ultra-filtrate(1/1)	0,022	7	-

(a) Chemotaxis assays were performed as described in the Appendix. Each fraction was tested by adding 2,5 ml of the undiluted fraction directly to the lower compartment of the Boyden chamber.

(b) Values given under the heading "Specific activity" are not valid in the strict conventional sense since the assay for biological activity is not linear with time or with protein concentration. I have included them to indicate that chemotactic activity was enriched with respect to protein concentration and to provide a very approximate estimate of the extent of this enrichment.

the ultrafiltrate. The UM05 filter effectively retained all chemotactic activity.

The chemotactic material prepared by hollow fibre ultrafiltration of casein contained molecules of molecular weight less than 20 000 and was shown by polyacrylamide gel electrophoresis to be completely free of the large molecular weight contaminants always present in the acid supernatant fraction.

Chemotactic material used for all the following experiments was prepared in this manner and will be referred to as CUF-N where N denotes the preparation batch number.

Susceptibility to treatment with acid or alkali.

A concentrated hollow fibre ultrafiltrate of casein (CUF-3) containing 38,9 mg protein/ml was diluted 50-fold or 250-fold with sterile distilled water. Six millilitre aliquots of the dilutions of CUF-3 were adjusted to pH 12,75; pH 2,09 and pH 1,12 by the addition of 60µl of 10M NaOH; 1M HCl and 10M HCl respectively. Acid-treated samples were then incubated at 37°C for 1 or 20h. Alkaline samples were incubated for 1 or 20h both at 37°C and at 60°C. After incubation the pH was restored to 7,4 (0,001% phenol red) with measured volumes of HCl or NaOH solution. The ionic strength was adjusted to isotonicity by the addition of calculated volumes of 5M NaCl and the samples were assayed for residual chemotactic activity. Control samples received water instead of acid or alkali and were incubated in parallel with test samples at a final pH of 7,4.

The results of these experiments are given in Table 2.10. As can be seen some chemotactic activity was lost with mild acid or alkaline hydrolysis, but in no case was chemotactic activity completely destroyed.

Table 2.10

The effects of mild acid and alkali hydrolysis on the chemotactic activity of low molecular weight casein ultrafiltrate (CUF-3).

No.	Cytotaxin (mg protein/ ml)	Incubation	Treatment	pH	Chemotaxis (Av. cell count/HPF)
1	0,16	37°C; 1h	-	7,4	215
			0,01NHCl	2,1	112
			0,1NHCl	1,1	73
			0,1N NaOH	12,8	59
		37°C;20h	-	7,4	273
			0,01N HCl	2,1	137
			0,1N HCl	1,1	91
			0,1N NaOH	12,8	59
		60°C; 1h	-	7,4	209
			0,1N NaOH	12,8	115
		60°C;20h	-	7,4	158
			0,1N NaOH	12,8	59
2	0,78	37°C; 1h	-	7,4	335
			0,01N HCl	2,1	276
			0,1N HCl	1,1	167
			0,1N NaOH	12,8	222
		37°C; 20h	-	7,4	370
			0,01N HCl	2,1	324
			0,1N HCl	1,1	206
			0,1N NaOH	12,8	176
		60°C; 1h	-	7,4	259
			0,1N NaOH	12,8	121
		60°C; 20h	-	7,4	328
			0,1N NaOH	12,8	185

Susceptibility to perchloric acid treatment.

CUF-3 was diluted 1:50 and 1:250 with distilled water and 5,5ml aliquots of these dilutions were added, in sterile tubes, to 0,6 ml of 5,0M $HClO_4$ to give a final pH of approximately 0,2 and a final concentration of $HClO_4$ of approximately 0,5M. The tubes were then incubated at 60°C for 1 or 20h during which time a small precipitate formed.

At the end of incubation, samples were treated in one of two ways.

(a) The pH was restored to neutrality (0,001% phenol red) and perchlorate ions were removed by the addition of a previously determined requisite volume of saturated KOH. The samples were centrifuged to remove insoluble $KClO_4$. No prior attempt was made to separate the material that had precipitated during incubation.

(b) The tubes were centrifuged (2000xg; 15 min; 4°C) and the supernatant fluid was decanted. The supernate was treated with KOH to remove ClO_4^- ions and to restore neutrality. The pellet was dissolved in 6 ml of 0,1M NaOH and adjusted to pH 7,4 with 1M HCl.

In all cases the ionic strength was adjusted to isotonicity with a calculated volume of 5M NaCl before assay.

Control samples were incubated without $HClO_4$. To these were added, before assay, the supernatant fluids obtained after centrifuging 0,6ml of 5M $HClO_4$ to which neutralizing volumes of saturated KOH had been added. The pH and isotonicity were then adjusted as for test samples.

The results of two such experiments are given in Table 2.11, from which it can be seen that chemotactic activity was moderately susceptible to perchloric acid treatment and could be recovered in the

Table 2.11

The effects of perchloric acid treatment on the chemotactic activity of casein ultrafiltrates (CUF-3).

Expt no.	Cytotaxin (mg protein/ml)	Incubation	Treatment	pH	Fraction	Chemotaxis (Av.cell count/HPF)
I	0,16	60°C; 1h	-	7,4		209
			0,5N $HClO_4$	0,2		71
		60°C;20h	-	7,4		158
			-	7,4	+$KClO_4$ supernate	205
			0,5N $HClO_4$	0,2		54
	0,78	60°C; 1h	-	7,4		259
			0,5N $HClO_4$	0,2		153
		60°C;20h	-	7,4		328
			-	7,4	+$KClO_4$ supernate	318
			0,5N $HClO_4$	0,2		* 18
II	0,16	60°C; 1h	-	7,4		357
			0,5N $HClO_4$	0,2	supernate	1
					pellet	209
		60°C; 24h	-	7,4		†N.D.
			0,5N $HClO_4$	0,2	supernate	1
					pellet	94
	0,78	60°C; 1h	-	7,4		355
			0,5N $HClO_4$	0,2	supernate	3
					pellet	†N.D.
		60°C; 24h	-	7,4		†N.D.
			0,5N $HClO_4$	0,2	supernate	2
					pellet	212

*Some of the acid precipitated material was removed together with the $KClO_4$ and not redissolved.

†Samples not suitable for chemotaxis assay due to decantation errors.

pellet after precipitation with this compound.

Susceptibility to enzyme digestion.

An attempt was made to identify the nature of the chemotactic compounds in terms of their susceptibility to degradation by specific enzymes.

In essence, these experiments were designed to see whether or not a particular enzyme would digest the cytotaxin and so destroy biological activity. Although seemingly straightforward, the valid interpretation of these experiments required that the following important conditions be fulfilled.

(a) It was necessary, at the end of incubation with the enzyme, to separate the enzyme from the intact or degraded cytotaxin. This was achieved in two ways. Firstly, immobilized enzymes were used that could be removed by the simple expedient of centrifugation. In this case, the supernatant fluid could be tested for residual chemotactic activity. Secondly, enzymes could be separated from the incubation medium by ultrafiltration with cellophane membranes, relying on the fact that the enzyme would be retained and the low molecular weight cytotaxin would pass into the ultrafiltrate. In both cases it was necessary to ensure that the removal of the enzyme from the test solution was complete.

I found, when the appropriate tests were done, that the immobilized enzymes were, in fact, firmly bound to the matrices to which they had been fixed and could be removed completely from the test solution. In the case of the soluble enzymes trypsin,

chymotrypsin and pronase however, enzymatic activity could be detected in the ultrafiltrate.

(b) It was necessary to show that the enzyme preparations used did not, either by autocatalysis or by virtue of impurities they contained, generate chemotactic activity. This could be tested by inclusion of the appropriate control tubes.

(c) It was necessary to show that the enzymes, by their action upon irrelevant, contaminating substrates in the chemotactic preparation, did not generate *inhibitors* of chemotaxis. In all cases, therefore, the enzyme-free products of digestion were tested for their ability to inhibit the chemotactic response to fresh cytotaxin.

(d) It was necessary to show that the enzymes were specific in their action and free of contaminating hydrolytic activity on other substrates. These tests were all done with the range of substrates given in the Appendix. The phosphodiesterase preparation contained significant amounts of tryptic activity that could not be completely inhibited by treatment with 0,01M diisopropylfluorophosphonate and dialysis.

With a need for these controls in mind, the following experiments were performed in which CUF-3 cytotaxin was dissolved, at a final concentration of 0,78 mg protein/ml, in Gey's BSS without antibiotics or bicarbonate, and incubated, under sterile conditions, with the various enzyme preparations at 37°C for 20h. Brief details of the incubating conditions are given in the legends to Figures 2.5 (a to d). Complete details of the enzymes used are given in the Appendix.

The results of these experiments are summarized in Figures 2.5(a) to (d). These show that all of the proteolytic enzymes tested caused loss of chemotactic activity. Pronase abolished activity completely;

Figure 2.5(a)

Figure 2.5(a) Effect of enzyme digestion on chemotactic activity of cytotaxin (CUF-3)

Cytotaxin was assayed for chemotactic activity following incubation at 37°C for 20h without (control) and with the following enzymes:-

A	-	Trypsin (250 u/ml)
A'	-	Immobilized trypsin (28,1 u/ml)
B	-	Chymotrypsin (4,8 u/ml)
B'	-	Immobilized chymotrypsin (20,3 u/ml)
C-HP	-	Hog pancreatic lipase (26,5 u/ml)
C-C	-	*Candida* lipase (73,0 u/ml)
D	-	Phosphodiesterase I (DFP-treated; 1,0 u/ml)
E	-	Activated leucine aminopeptidase (10,0 u/ml)
F	-	Pronase
G	-	Immobilized carboxypeptidase A.

In each case, cytotaxin was present at a concentration of 0,78 mg protein/ml Gey's BSS without $NaHCO_3$. Each point represents the mean of 8 counts on 2 filters. The interrupted parallel lines indicate the range of the control counts.

For experimental details see text and Appendix; A.9.

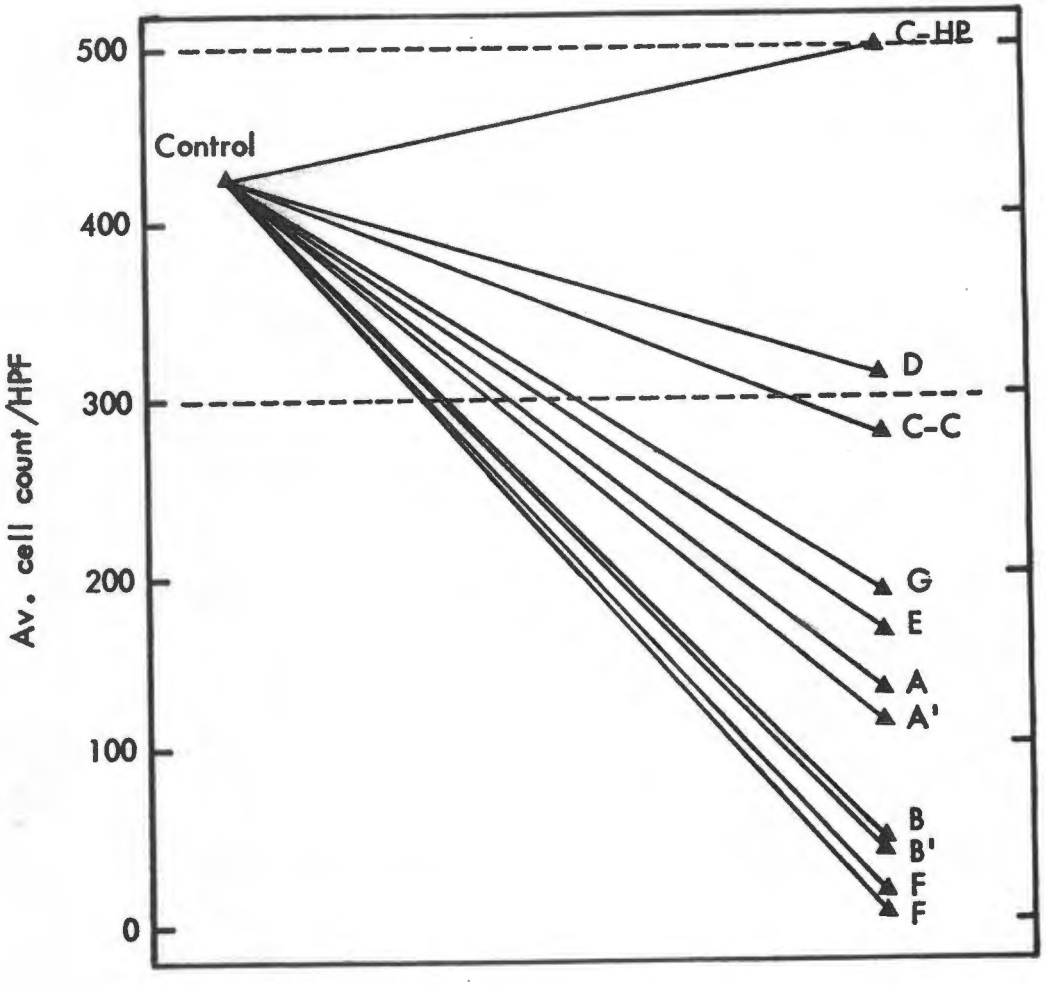
500
400
300
200
100
0
Av. cell count/HPF
Control
C-HP
D
C-C
G
E
A
A'
B
B'
F
F

Figure 2.5(a)

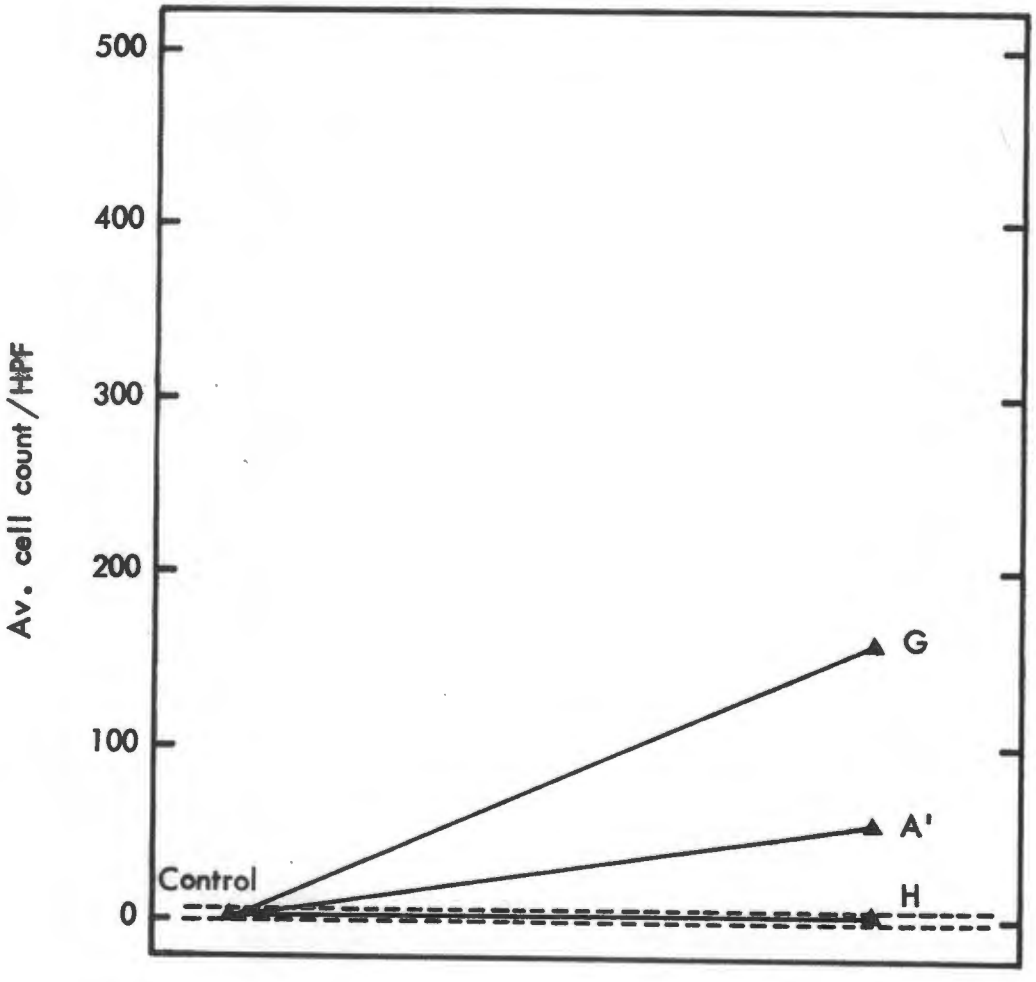

Figure 2.5(b) Chemotactic activity of enzyme preparations used.

Control experiment performed to test for chemotactic activity present in, or generated by, the various enzyme preparations used after incubation in Gey's BSS (without $NaHCO_3$) for 20h at 37°C without added cytotaxin.

The point labelled "control" indicates the chemotactic activity of Gey's BSS alone after 20h incubation.

Point G represents the activity observed with carboxypeptidase A and Point A' that found with the immobilized trypsin preparation.

Point H (showing zero chemotactic activity) indicates the average cell count observed with all other enzymes tested in the legend to Figure 2.5(a).

Figure 2.5(c)

Figure 2.5 (c) Effect of enzyme reaction product on cytotaxin (CUF-3)

Control experiment performed to test for the presence of inhibitors in the enzyme preparation or reaction products upon freshly added cytotaxin (0,19mg protein/ml) and/or failure to separate enzyme from reaction products before assay.

Note that ultrafiltrates of reaction mixtures containing soluble trypsin (A), chymotrypsin (B) and pronase (F) inhibited chemotactic responses to fresh cytotaxin. This could be ascribed to the passage of active enzyme into the ultrafiltrate. The ultrafiltrate of hog pancreas lipase (C-HP) was also inhibitory - presumably due to the generation of chemotactic inhibitors as a result of the action of this enzyme on components present in CUF-3 or the presence of inhibitors in the enzyme preparation itself.

Enzyme-free, reaction products of other enzymes - immobilized trypsin (A'); immobilized chymotrypsin (B'); *Candida* lipase (C-C); phosphodiesterase I (D); leucine aminopeptidase (E) and immobilized carboxypeptidase A (G) were without effect.

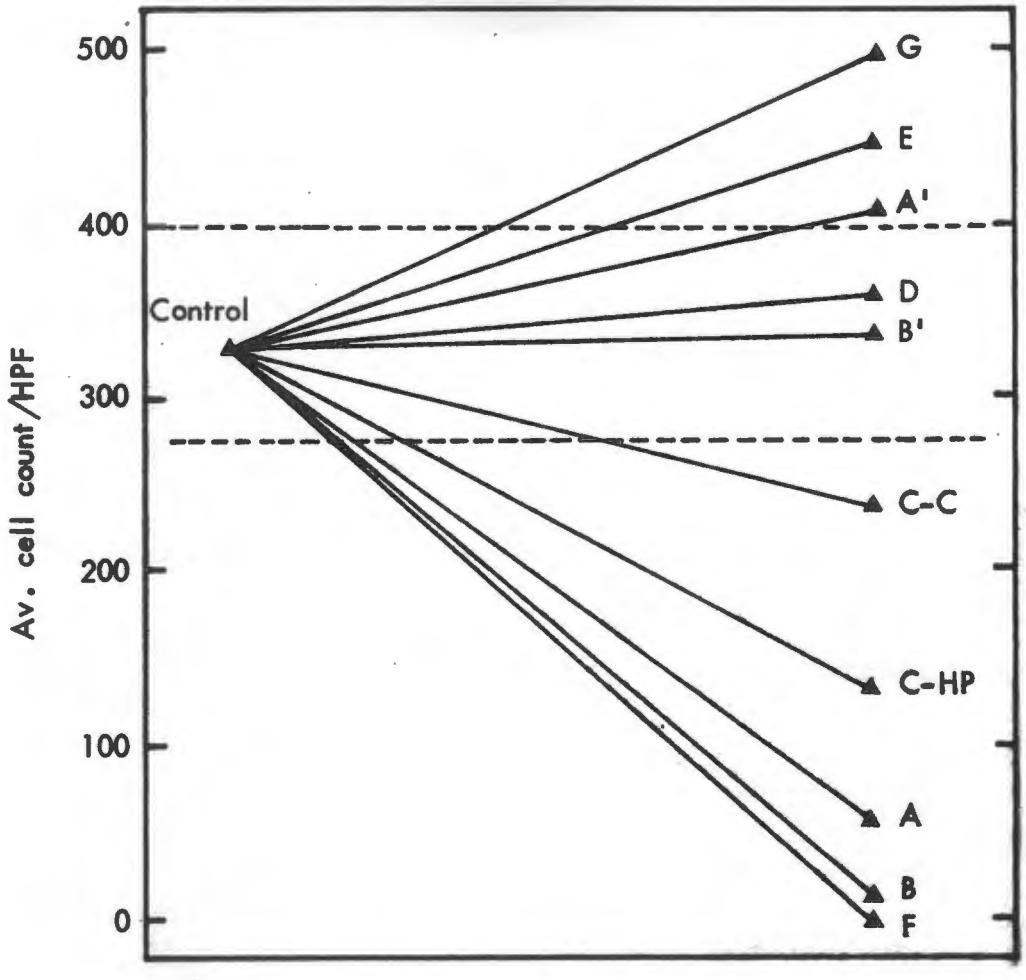
500
400
300
200
100
0
Av. cell count/HPF
Control
G
E
A'
D
B'
C-C
C-HP
A
B
F

Figure 2.5 (c)

Figure 2.5(d)

Figure 2.5 (d) Effect of enzyme digestion on chemotactic activity of 1% casein.

The ultrafiltrates of solutions of 1% casein in Gey's BSS (without $NaHCO_3$) were assayed for chemotactic activity following incubation of the casein at 37°C for 20h without (control) or with the following enzymes:

A	-	Trypsin (25,0 u/ml)
A'	-	Immobilized trypsin (28,1 u/ml)
B	-	Chymotrypsin (4,8 u/ml)
B'	-	Immobilized chymotrypsin (20,3 u/ml)
C-HP	-	Hog pancreatic lipase (26,5 u/ml)
C-C	-	*Candida* lipase (73,0 u/ml)
D	-	Phosphodiesterase I (DFP-treated; 1,0 u/ml)
E	-	Activated leucine aminopeptidase (10,0 u/ml)
F	-	Pronase
G	-	Immobilized carboxypeptidase A.

Each point represents the mean of four counts in two filters. The interrupted parallel lines indicate the range of the control counts.

For experimental details see text and Appendix; A.9.

Note that chemotactic activity of 1% casein was only diminished to a significant extent by chymotrypsin and hog pancreas lipase.

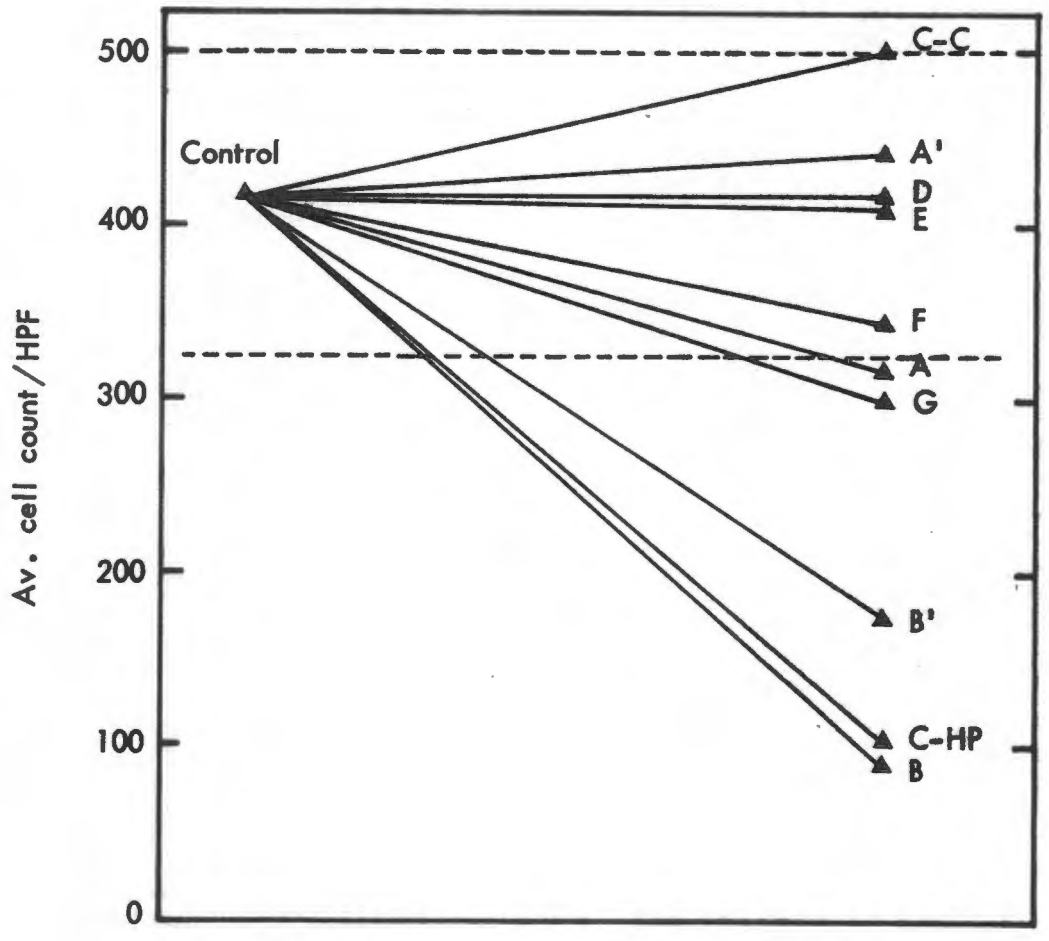
500
400
300
200
100
0
Av. cell count/HPF
Control
C=C
A'
D
E
F
A
G
B'
C-HP
B

Figure 2.5 (d)

chymotrypsin was very nearly as effective as pronase in this regard and was the only proteolytic enzyme that caused a significant decrease in the chemotactic activity of a 1% casein solution; carboxypeptidase A and immobilized trypsin generated small amounts of chemotactic activity when incubated alone. If these "background" values were subtracted from the values observed for trypsin- or carboxypeptidase-treated cytotaxin, it would appear that they too, were effective in degrading the cytotaxin.

It seemed, at first sight, that the action of soluble trypsin, chymotrypsin and pronase had generated inhibitors of chemotactic activity since the ultrafiltrates of the incubation mixtures containing these enzymes reduced the activity of fresh cytotaxin. It transpired, however, that sufficient active enzyme had escaped with the ultrafiltrate to degrade the fresh cytotaxin added.

One can, therefore, on the basis of these experiments, conclude that the chemotactic materials found in casein are susceptible to hydrolytic degradation by the action of proteolytic enzymes.

Neither of the lipase preparations tested, nor the DFP-treated phosphodiesterase had any significant effect upon the low molecular weight cytotaxin; they did not generate chemotactic activity and, with the exception of hog pancreatic lipase, did not generate inhibitors. The action of hog pancreatic lipase was interesting in that it had no effect upon the chemotactic activity of CUF-3 at a dilution of 1:50. The ultrafiltrate from this preparation, however, was able to inhibit the chemotactic response to a 1:200 dilution of CUF-3. Furthermore, hog pancreatic lipase also inhibited the chemotactic action of 1% casein. These results are most readily interpreted as indicating that the hog pancreatic lipase has no direct effect upon the chemotactic compounds, but generated, from other substrates present in crude casein (and, to a lesser extent in the relatively pure CUF-3) inhibitors of chemotaxis.

Adsorption of cytotaxin to DEAE-cellulose, hydroxylapatite and activated charcoal.

(a) DEAE-cellulose

One millilitre of CUF-3 containing 38,9mg of protein was added to a 2,0ml column of Whatman's DE-52 DEAE-cellulose[1] pre-equilibrated with 0,01M Na_2HPO_4/KH_2PO_4 buffer, pH 7,4 and eluted with the same buffer.

The effluent was collected and assayed for chemotactic activity after the ionic strength had been brought to isotonicity and the protein concentration had been adjusted to correspond to that of a 1:50 dilution of the CUF-3. When compared with the CUF-3, the DEAE treatment appeared to have removed approximately 76% of the protein present in the sample; relatively insignificant amounts of chemotactic activity were removed by this procedure (Table 2.12).

Table 2.12

The effects of DEAE-cellulose ion exchange chromatography on casein (Hammarsten) ultrafiltrates (CUF-3)

No.	Sample (mg protein)	Treatment	Sample (mg protein)	Chemotaxis (Av.cell count/HPF)	Protein (mg/ml)
1	38,9	-	38,9	500	0,78
2	38,9	DEAE cellulose chromatography	10,4	341	0,20
				294	0,78

(1) W & R Balston Ltd., Maidstone, England.

(b) Hydroxylapatite

Biorad HTP hydroxylapatite[1] was slurried in, and equilibrated with 0,01M Na_2HPO_4/KH_2PO_4 buffer pH 7,4 and packed into a 0,5 ml column in a disposable plastic syringe. One millilitre of CUF-3 (38,9mg of protein) was added and the column eluted with the same buffer. The ionic strength of the effluent was adjusted to isotonicity and the resulting solution was diluted to compare, in terms of volume or protein concentration, to a 1:50 dilution of CUF-3. The hydroxylapatite column removed 27% of the original protein with a negligible effect upon the chemotactic activity (Table 2.13).

Table 2.13

The effects of hydroxyl apatite (HTP) adsorption on casein (Hammarsten) ultrafiltrates (CUF-3)

No.	Sample (mg protein)	Treatment	Sample (mg protein)	Chemotaxis (Av. cell count/HPF)	Protein (mg/ml)
1	38,9	-	38,9	500	0,78
2	38,9	HTP adsorption	28,5	298	0,61
				347	0,78

(c) Activated charcoal

Norit A[2] was added as 0,1g of dry powder per ml of CUF-3 diluted to 3,89 mg protein/ml with distilled water. After incubation at room temperature for 1h the charcoal was

(1) Biorad Laboratories, Richmond, California, U.S.A.

(2) Sigma Chemical Company, St. Louis, Missouri, U.S.A.

removed by centrifugation (2 000xg; 15 min; 4°C) and membrane filtration (0,45µm filter). The clarified solution was adjusted to pH 7,4 with 0,1N NaOH and to isotonicity with 5M NaCl. The solution was then assayed for chemotactic activity after diluting 1:5 with normal saline (equivalent to 1:50 dilution of CUF-3). As can be seen from Table 2.14 charcoal treatment effectively removed all chemotactic activity and 85% of the protein.

Attempts to elute the chemotactic activity with 15% aqueous pyridine were unsuccessful. Other elution systems have not yet been investigated.

Table 2.14

The effects of charcoal adsorption on casein (Hammarsten) ultrafiltrates (CUF-3)

No.	Sample (mg protein)	Treatment	pH	Protein (mg)	Chemotaxis (Av. cell count/HPF)
1	38,9	-	7,4	38,9	306
2	38,9	charcoal adsorption	7,4	5,0	12

Extraction into chloroform-methanol

Attempts to purify cytotaxin from CUF-3 by extraction from an aqueous solution with buffered phenol resulted in an emulsion that resisted efforts to achieve phase separation with common techniques such as low temperature or the addition of iso-amyl alcohol. This suggested that the cytotaxin might have detergent-like properties and would be extractable into organic solvents. Accordingly, the Folsch chloroform/

methanol extraction procedure was attempted.

Redistilled chloroform and methanol (2:1 v:v) were used to pre-extract cellulose, single thickness Whatman's thimbles[1] in a Soxhlet apparatus for 3h. The thimbles were then dried to free them of organic solvents, saturated with distilled water and plunged into a dry ice-acetone bath to seal the pores with ice.

One millilitre of CUF-3 (or water in the case of the control thimble) was then shell-frozen into the thimble and the contents lyophilized. The thimbles (one containing cytotaxin and the other serving as a control) were then extracted individually in a Soxhlet apparatus with two changes of 200ml of chloroform:methanol (2:1) for a total of 4h.

The extracted, dry residue remaining in the thimble was dissolved in 1ml 1N NaOH and diluted to 10 ml with distilled water. The pH was adjusted to 7,1 (0,001% phenol red) with 4N HCl and the ionic strength adjusted to isotonicity by the addition of 10µl/ml of 5M NaCl.

The chloroform-methanol solvents were removed from the extract by rotary evaporation under water-tap vacuum at 37°C. The material recovered from the extract in this way was dissolved in 10 ml of 0,1M NaOH, neutralized and brought to isotonicity as above.

The extract and the residue were assayed for chemotactic activity after dilution with saline to correspond to a 1/50 dilution of the starting material. The results are summarized in Table 2.15.

The material remaining in the thimble after extraction was found to be completely free of chemotactic activity. Most of the cytotaxin was recovered from the chloroform-methanol extract. The control extract had no activity.

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(1) W & R Balston Ltd., Maidstone, England.

Table 2.15

Chloroform-methanol extraction of the cytotaxins in casein (Hammarsten) ultrafiltrates.

No.	Sample	Protein (mg)	*Chemotaxis (Av. cell count/HPF)
1	†CUF-3; extracted residue	38,0	7
2	Control; chloroform-methanol extract		
	sterile	1,9	2
	non sterile	2,2	2
3	CUF-3; chloroform-methanol extract		
	sterile	6,3	243
	non sterile	6,7	283

*Three millilitre volumes of a $1x10^6$ cells/ml suspension were added to the upper compartments of the Boyden chambers instead of the standard $2x10^6$ cell/ml concentration.

†The original sample of casein cytotaxin used for extraction contained 38,9 mg protein in 1 ml.

Discussion

Some of the cytotaxins present in casein have the following characteristics:

(i) They are heat stable compounds with molecular weights ranging from 6 000 to 880 that pass through cellophane membranes and through commercially available hollow-fibre filters with a nominal molecular weight exclusion cut-off of 10 000. They are retained by Amicon UM05 filters with a nominal molecular weight cut-off of approximately 500.

(ii) They are relatively resistant to mild acid or alkaline hydrolysis and to treatment with perchloric acid.

(iii) They are degraded by proteolytic enzymes indicating that they are polypeptides. Their susceptibility to exopeptidases (leucine aminopeptidase and carboxypeptidase A) indicates that their C- and N- terminal residues are freely accessible. The effective degradation by chymotrypsin suggests that aromatic amino acids are prominent.

(iv) They are not obviously anionic at neutral pH since they pass freely through a DEAE-cellulose column. They are completely removed from solution by activated charcoal - a procedure that normally removes compounds of an aromatic nature.

(v) They are extractable into chloroform:methanol, providing further evidence for the presence of hydrophobic residues in the polypeptide chain.

On the basis of these results I conclude that the cytotaxins present in casein are polypeptide in nature and contain hydrophobic amino acid residues.

It is tempting to speculate on the possibility that, in addition to hydrophobic residues, the chemotactic molecules also possess regions of hydrophilicity conferring upon them detergent characteristics. If this were so, the biological activity of these compounds might reside in their simultaneous ability to attach, by hydrophobic interactions, to the lipid membrane of the cell and, by hydrophilic interactions, to a wettable

substrate. This would provide a means whereby increased cell adhesiveness might mediate directional movement as discussed in Chapter IV. It should be noted, however, that the detergent effect referred to earlier was observed with relatively crude CUF-3 and not with purified cytotaxin. Any attempts to correlate chemotactic activity with molecular structure must await definitive identification of the active compounds. It is my hope that the experiments reported here will provide me with the basis to pursue this matter further.

The literature contains relatively few reports of attempts to isolate and identify compounds with attractant properties for leucocytes. Menkin in 1938 (115) obtained a crystalline, water-soluble dialysable material by pyridine:acetone extraction of pleural exudates. This material, which gave a positive indole test, he referred to as "Leukotaxin", since it was able to elicit leucocyte emigration from capillaries when tested *in vivo* (118,119). An amorphous, heat stable, water-soluble, ninhydrin-positive, dialysable material obtained by pyridine:acetone extraction of pleural exudates followed by charcoal adsorption and elution was later described by Cullumbine and Rydon (39). This material was also chemotactic by *in vivo* assay. There are a number of respects in which these compounds and the cytotaxins I have studied are similar.

Duthie and Chain (43) described a dialysable trypsin-sensitive and biuret-positive chemotactic compound that could be isolated from incomplete proteolytic digests of fibrin, albumin or globulin. Spector (146) isolated a brown, amorphous, water-soluble material by extracting pepsin digests of fibrin with ethyl alcohol:ether (1:1). This material could be precipitated with 75% $(NH_4)_2SO_4$ and had leucotactic activity when tested *in vivo*. Spector, in this paper speculated on the importance of surfactant properties for chemotactic activity. The findings of these

authors are entirely consistent with my own.

The origin of the chemotactic peptides present in milk is obscure. There would appear, from the results of column chromatography, to be at least three such compounds and one is not, on the basis of the evidence available, able to say whether these represent multimeric forms of a single polypeptide; different stages of degradation of a common precursor; or biochemically unrelated species. As previously discussed, milk is an extremely complex fluid which contains, apart from the major casein fraction, lactalabumins, lactoglobulin, serum proteins, tissue debris and, in all probability, the products of inflammatory mastitis, bacterial growth or enzymatic activity. The reason for the presence of the compounds in milk is not, in itself, of such intrinsic interest as is their molecular structure and the relationship that they bear to other chemotactic peptides. For if all chemotactic compounds were found to possess some common physical or biochemical characteristic, this would contribute greatly to a unifying hypothesis to explain the striking biological phenomenon of chemotaxis. It is my sincere intention to pursue the chemical studies initiated in this work with this ultimate goal in view.

Chapter III

Biochemical aspects of the chemotactic response

It can be taken as a valid and general tenet, that any attempt to apply *in vitro* techniques to the understanding of a biological phenomenon should start with those experiments that define the basic requirements of the experimental system that is to be used. Such factors, for example, as composition and pH of the medium, substrate requirements and conditions of incubation, although seemingly mundane and "technical", are clearly of importance, not only for establishing the validity of the experimental approach, but equally for the insight they give into the biological phenomenon itself.

This chapter describes a number of experiments in this category and includes a discussion of the conclusions that can be drawn from the results.

pH

To determine the effect of variations in the hydrogen ion concentration of solutions of cytotaxin obtained from casein, the following experiments were undertaken.

Concentrated SNF-cytotaxin was diluted with TBS to give, as indicated by pilot experiments, an optimal chemotactic response with rabbit polymorphonuclear leucocytes. The pH of various aliquots of this diluted cytotaxin solution was either raised or lowered by the addition of 1N NaOH or 1N HCl respectively to cover the pH range 4,0 to 9,0 (due to the low concentration of triethanolamine in the buffer system, only small amounts of these solutions were necessary to effect the pH alterations required, thus changes in ionic strength were minimal and insignificant). The chemotactic activity of the various solutions were assayed using a modified Boyden chamber technique as detailed in the Appendix.

As can be seen from the results of three experiments summarized in Figure 3.1 the chemotactic response diminished rapidly above pH 7,5 and was unaffected by pH values in the range 4,0 to 7,5.

I repeated the experiment using 0,005M sodium acetate-acetic acid (pKa = 4,7) buffered 0,154M NaCl to cover the pH range 4,0 to 6,5. Triethanolamine buffer was still used over the range pH 7,0 to pH 10,0. The pH values of the chemotactic solutions were adjusted with 1N NaOH or 1N HCl as before and the samples assayed for their ability to elicit a chemotactic response from neutrophils, as described in the Appendix.

The results summarized in Figure 3.2 once again showed that chemotaxis was markedly depressed by alkaline conditions while pH values below pH 7,5 were without effect.

When the pH of the Gey's BSS ($NaHCO_3$-free)-2% HSA medium in which the cells were suspended was varied over the pH range 4,0 to 10,0 by the addition of 1N HCl or 1N NaOH (Figure 3.3) a definite pH effect was evident, with optimal responses at neutrality.

The depression of the chemotactic response observed with pH values above neutrality could have been due to an effect upon the cytotaxin. Alternatively, since the filters separating the cell suspensions from the chemotactic test solutions were permeable to the hydrogen ion, the effect may have been upon the responding polymorphonuclear leucocytes.

The susceptibility of cells to alkaline or acid conditions has been well documented and it would appear that they are, generally, better able to withstand hydrogen ion concentrations on the acid side of neutrality, than on the alkaline side (126). I suspect therefore that the depression of chemotactic responsiveness at alkaline pH values was due to a non-specific effect on cell viability.

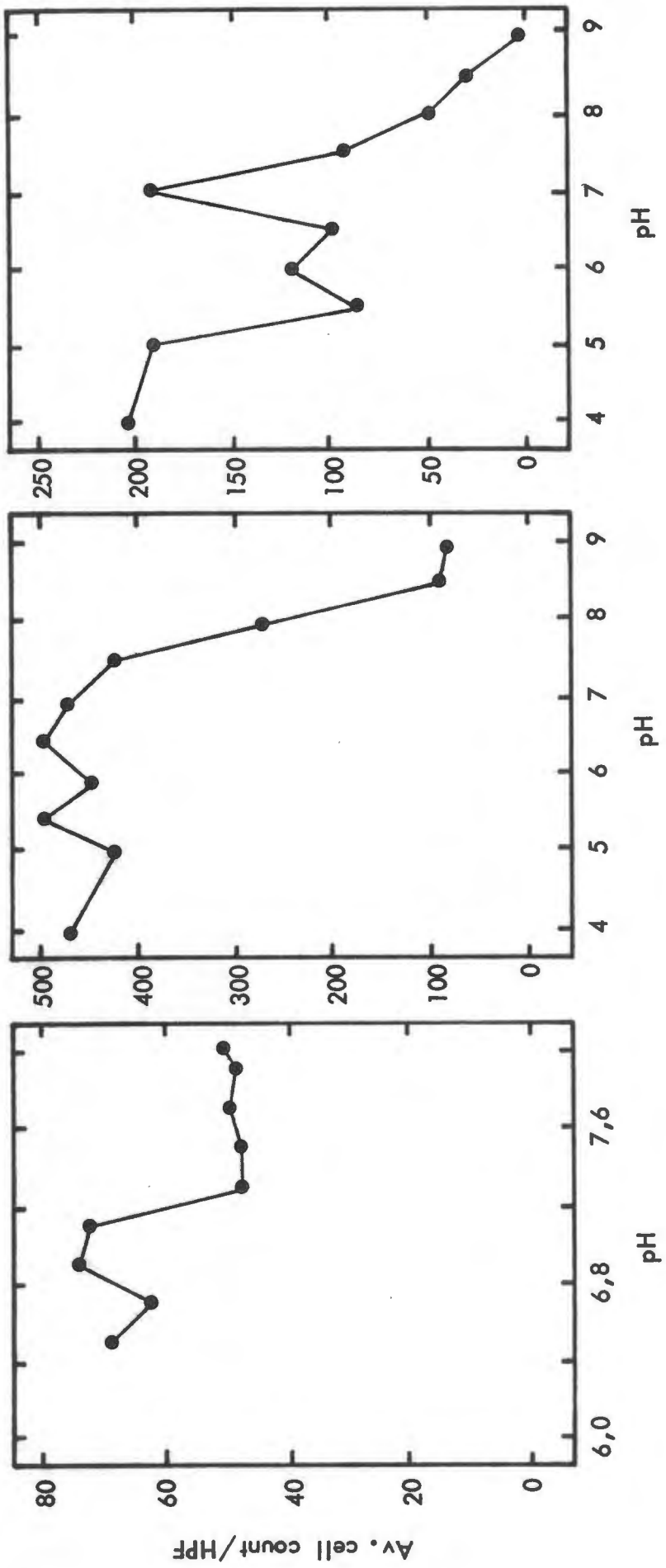

Figure 3.1 Neutrophil chemotactic response to cytotaxin at different pH values

Results of three similar experiments in which cells suspended in medium of pH 7,2 responded to cytotaxin (SNF) dissolved in TBS of the different pH values indicated.

Note that cells are relatively insensitive to acid deviations from neutrality in the cytotaxin compartment. The response to alkaline cytotaxin solutions is diminished.

Menkin, in developing his thesis that the cellular composition of inflammatory exudates is conditioned by the hydrogen ion concentration, performed experiments to show that polymorphonuclear leucocytes in canine pleural inflammatory exudates were damaged at pH 6,6 and were unaffected at pH 7,5 (117). He did not study the effect of higher pH values.

The acid conditions I employed did not inhibit the ability of the cells to respond to the cytotaxin from casein in the *in vitro* assay. This would be in keeping with the *in vivo* observations that cells migrate actively towards inflammatory sites where pH values as low as pH 5,5 have been recorded (159).

Casein is quoted as being susceptible to alkaline denaturation and hydrolysis (35,36,45,46). Since the low molecular weight, chemotactic material might have been a product of casein degradation, it was conceivable that it, too, would have been affected by alkaline conditions. It appears, from preliminary experiments, that this was not the case, since increasing the pH of the SNF above that of neutrality and then readjusting it, after a period of incubation at room temperature or 37°C to pH 7,1, did not affect the cytotaxin/s in an irreversible manner. The possibility remains that the cytotaxin/s being polypeptides, might be basic in nature and only active in a cationic form. Until the isoelectric point of the cytotaxin is known, this must remain speculative.

The relevance of the pH effect that I observed *in vitro* to chemotaxis *in vivo*, or to the mechanism of polymorphonuclear leucocytes migration is uncertain.

The effect of local hydrogen ion concentration on the accumulation of leucocytes at the site of injury has long been a subject of debate. The production of lactic acid (155) and other acid metabolites at the site of inflammation has led various workers to suggest that the hydrogen ion gradient itself provides the directional chemotactic stimulus. (Reviewed by McCutcheon (110) and Harris (63).)

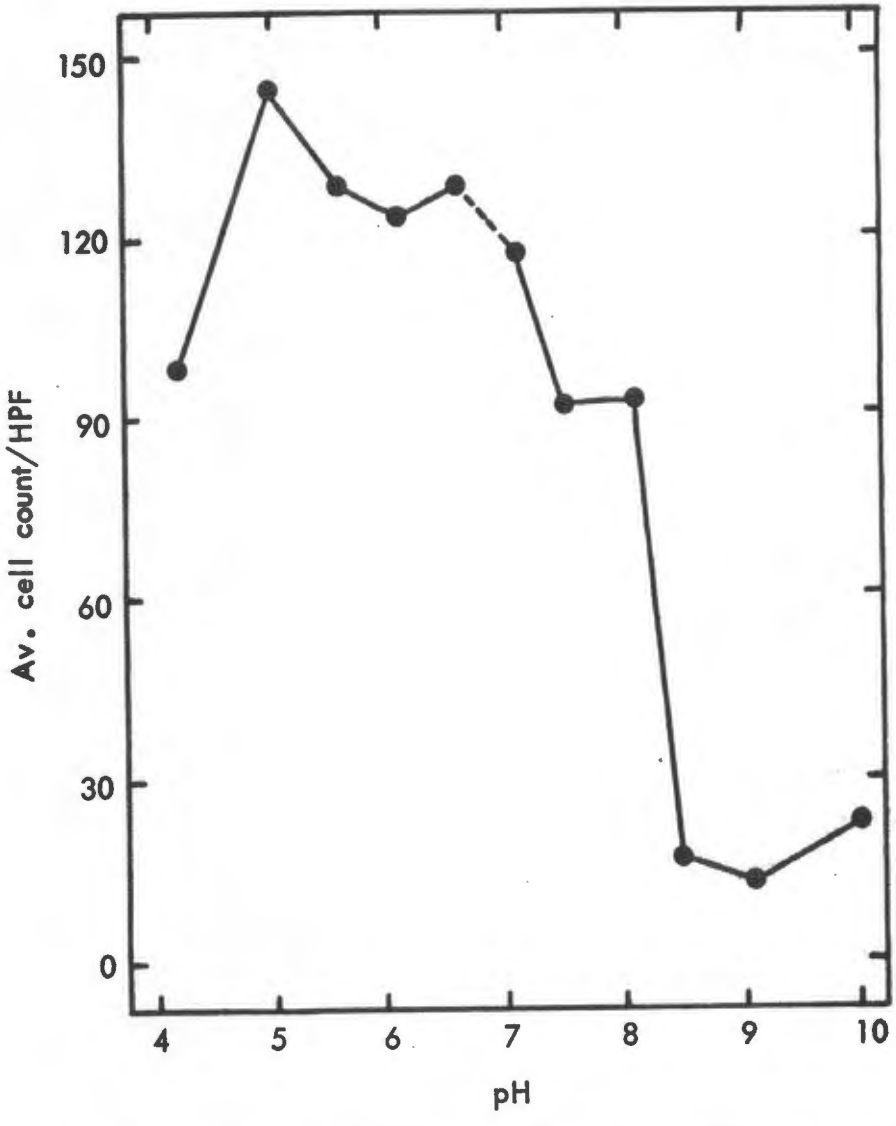

Figure 3.2 Neutrophil chemotactic response to cytotaxin solutions buffered at different pH values with acetate or triethanolamine buffer.

Cells were suspended in Gey's BSS with 2% HSA pH 7,2 and their chemotactic response to cytotaxin dissolved in saline of different pH values was assayed. Acetate buffer (0,005M) was used to buffer cytotaxin solution between pH 4 and pH 6,5. Triethanolamine (0,005M) was used to buffer cytotaxin solutions between pH 7,0 and pH 10,0.

The switch from acetate to triethanolamine buffer is indicated by the interrupted line.

Results similar to those depicted in Figure 3.1 were obtained.

The results of 2 experiments, using bicarbonate-free Gey's BSS-2% HSA over the pH range 3,0 to 10,0 are summarized diagrammatically in Figure 3.3. They do not support this hypothesis.

Abramson (1) suggested that the lower pH of injured tissues relative to normal tissues or blood, might render the injured area relatively more electropositive with the result that migration of leucocytes to a point of injury would be favoured by the electrokinetic potential of the negatively charged migrating cell membrane.

Bryant, des Prez, van Way and Rogers (26) found that the random migration of human polymorphonuclear leucocytes against gravity, in glass capillary tubes, was not affected by pH changes between 6,8 and 7,6 when he used hydrochloric acid, lactic acid or beta-hydroxybutyric acid to adjust the pH. Silverman (144), who assessed chemotactic responses by microscopic observation of cells migrating towards solidified agar fragments, found that the phenomenon was unaffected by the pH of the agar over the range 5,5 to 8,5. Baum, Mowat and Kirk (12) using a modification of the Boyden chamber technique to assay chemotaxis, found that pH gradients between the chemotactic solution and cell medium had no effect on chemotaxis and cells migrated in response to a casein chemotactic stimulus despite cell medium pH values as low as 5,2. Zigmond (191) studied the effect of pH changes in the cell suspending media over the range 6,5 to 7,9 on chemotaxis. Marked inhibition of chemotaxis was evident at pH values of 7,9 or greater. Little effect of pH was evident between 6,5 and 7,6 except for a slight peak at pH 7,3 to 7,4. These results are in general agreement with those of Nahas, Tannieres and Lennon (121) who studied individual cell locomotion rates and found little pH effect between 6,5 and 7,5 but a marked drop in locomotion above pH 7,6.

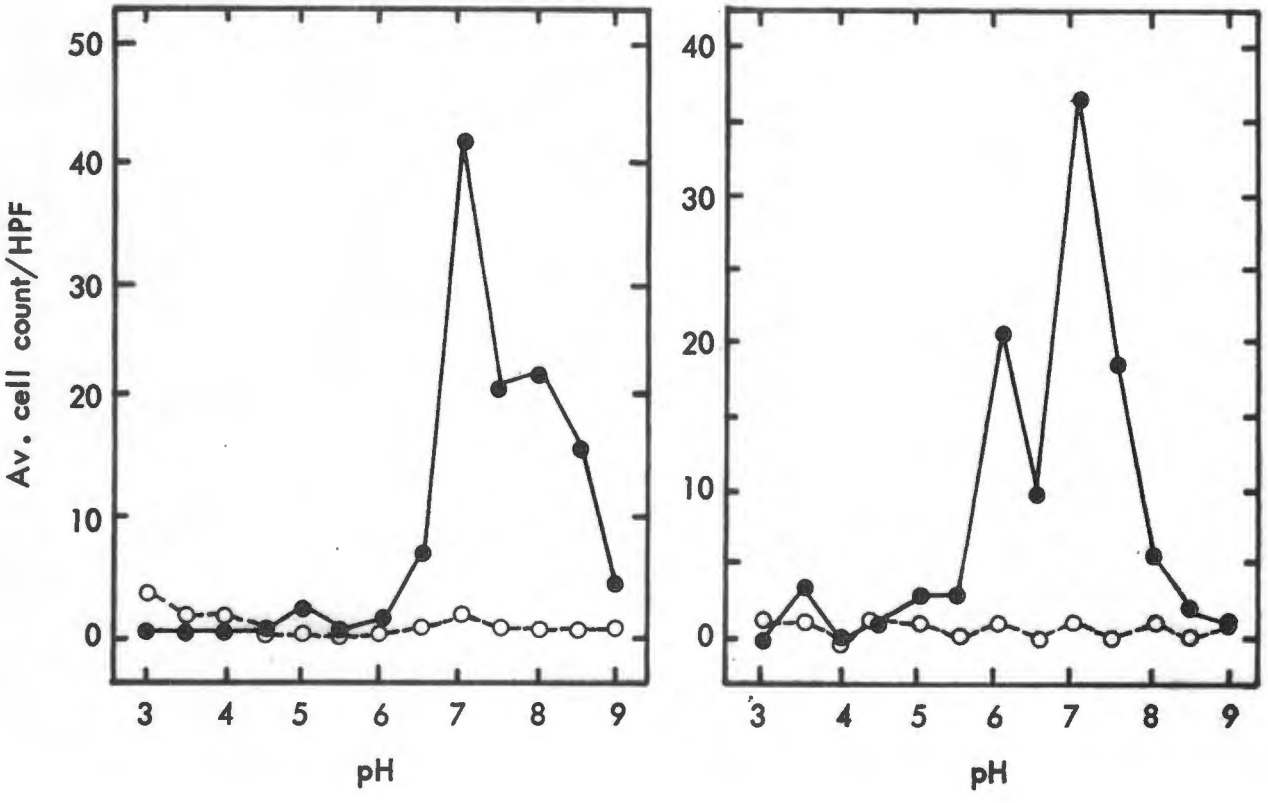

Figure 3.3. Effect of pH on neutrophil chemotactic response.

Results of two identical experiments in which (a) cells, suspended in medium of the varying pH values indicated, responded to cytotaxin (CUF-3) at constant pH in the lower chamber (●—●) and (b) cells, suspended in medium of constant pH responded to Gey's BSS + 2% HSA, at different pH's, *without* cytotaxin (O--O).

Note that the pH of the immediate environment of the cells (i.e. the cell suspending medium) had a pronounced effect upon chemotaxis with an optimal response at neutral pH. Cells did not respond to pH gradients in the absence of cytotaxin.

Phelps and Stanislaw (129), using a modified Boyden chamber technique, demonstrated a critical dependence of chemotaxis on pH with a definite peak response over the pH range 7,2 to 7,4 when the cells themselves were suspended in media of various pH. This observation was verified by Wilkinson (185) although the data he presented diagrammatically in Figure 3.4 of his publication were not entirely convincing.

The possibility that the hydrogen ion concentration gradient across the filter provided the chemotactic stimulus is unlikely in view of the work of Baum et al. (12) and in the light of my own observations testing this hypothesis (Figure 3.3). There was also no observable effect of pH acting to increase chemotactic responses to casein cytotaxins at lower pH values.

The optimal pH range of 7,2 to 7,4 observed by myself and some workers (Figure 3.3) when the pH of the medium bathing the neutrophils was varied, might have *in vivo* relevance for possible membrane receptor activation or for the inhibition of leucocyte migration away from an inflamed area. The obvious lack of any definite inhibition when the pH of the test solution was lowered below physiological neutrality, might indicate that the leucocyte response to a chemotactic stimulus *in vivo* is undiminished by acidosis at the site of injury.

Ionic strength

The effect of varying the ionic strength of the cytotaxin solution on the chemotactic response of rabbit peritoneal exudate cells was examined by diluting concentrated SNF-cytotaxin to a concentration that was known to give an optimal chemotactic response under ordinary conditions of *in vitro* assay.

Dilutions were made in 0,005M triethanolamine HCl buffer pH 7,1. The tonicity of the solution was adjusted with 2M NaCl to cover the range 0,0045M NaCl to 0,4M NaCl. The pH of all solutions was adjusted to pH 7,1 and the effect of varying the ionic strength on the chemotactic response of rabbit neutrophils was determined. In all cases the responding cells were suspended in isotonic Gey's BSS-2% HSA.

As can be seen from the three experiments summarized in Figure 3.4 there was a maximum response of polymorphonuclear leucocytés when the whole assay system was carried out under isotonic conditions of 0,15M NaCl. Increases in the tonicity of the cytotaxin solution by 50µmole/ml of sodium chloride caused a dramatic decrease in the chemotactic response and complete inhibition was observed with increases of 100µmole/ml of sodium chloride or greater.

Deviation from isotonicity towards hypotonic conditions were not nearly as dramatic and complete inhibition was only observed when sodium chloride was omitted from the cytotaxin solution altogether.

It is common knowledge that cells require physiological, balanced salt solutions for normal growth and function and the significance of these findings to usual *in vivo* circumstances is doubtful.

It is, however, known that renal concentrating mechanisms provide a hypertonic *milieu* in the medulla of the kidney that might compromise neutrophil recruitment to this region and hence render it particularly susceptible to infection.

It is perhaps important, in considering the relevance of these studies to *in vivo* neutrophil function, to draw a distinction between the behaviour of cells that are bathed in an unphysiological osmolar environment on the one hand, and on the other hand, the

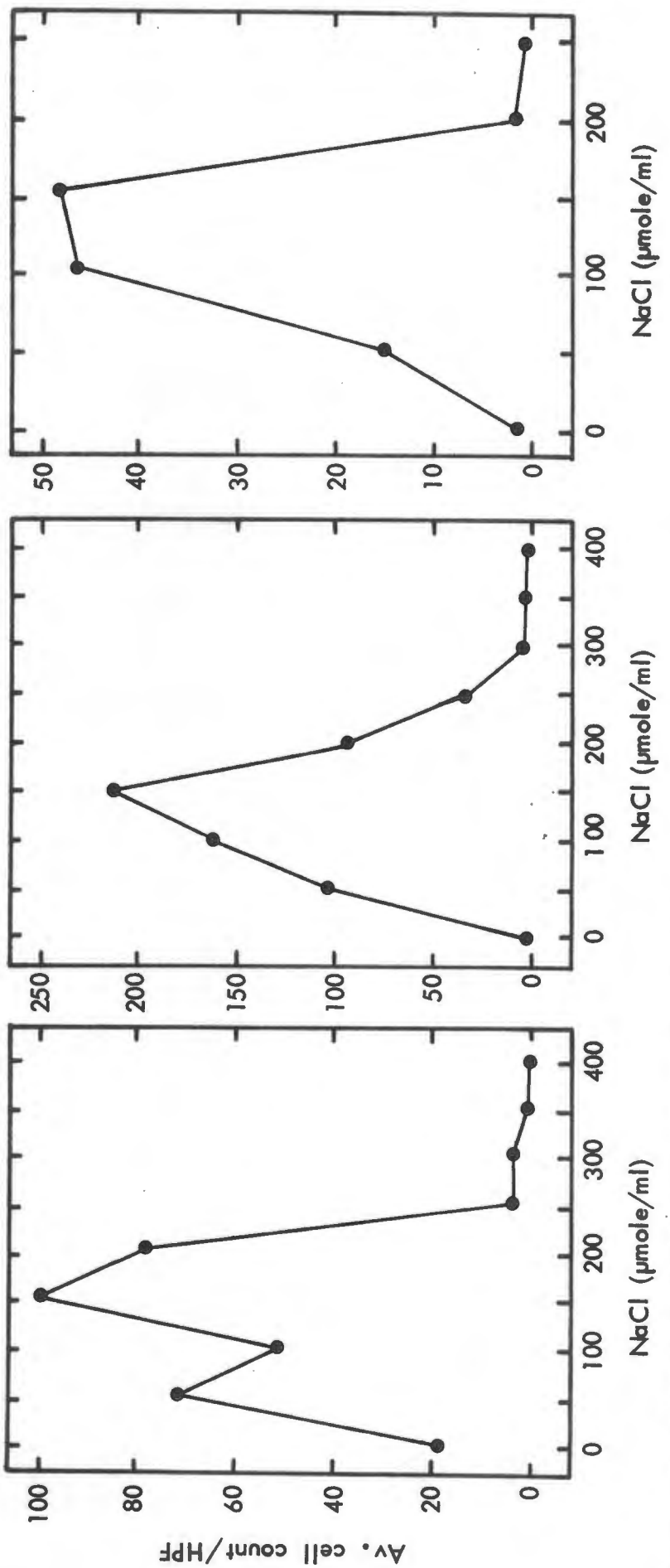

Figure 3.4 Effect of ionic strength on chemotactic response.

Results of three separate experiments in which cells, suspended in Gey's BSS with 2% HSA responded to a constant concentration of cytotaxin (SNF) dissolved in NaCl solutions of varying ionic strength.

Note that, in each case, optimal response occurred in response to cytotaxin dissolved in saline of physiological (0,15) ionic strength.

response of cells in a normal environment to a chemotactic substance in a region of altered tonicity.

The results of duplicate filters from two experiments designed to investigate any differences in the pattern of the cellular response of a single cell population in both these situations to casein cytotaxin are shown in Figure 3.5. In these experiments changes in tonicity were produced by making dilutions of 3x concentrated Gey's BSS with distilled water to cover the ionic strength range 0 to 400. Cells suspended in these solutions of differing tonicity, in the presence and absence of 2% added HSA, were assayed in Boyden chambers for their response to optimum concentrations of CUF-3 in Gey's BSS. At the same time, cells suspended in Gey's BSS-2% HSA were tested for their response to the same cytotaxin stimulus, except that the tonicity of the cytotaxin solution was varied over the same ionic strength range.

As before, hypotonic conditions were more readily tolerated by the cells than were hypertonic conditions irrespective of whether the cells were bathed in the media themselves or responding to cytotaxins dissolved in the media. The presence of added protein in the cell suspending medium preserved the cells and allowed relatively normal chemotaxis. Poor responses were obtained when cells were assayed in Gey's BSS alone. Cells subjected to repeated centrifugation and handling showed reduced chemotactic responses under physiological conditions when compared to untreated cells. This was most obvious in the poorer responding cell population.

Lotz and Harris (102) using direct microscopic observations of cells in media of varying osmolarity observed that cells could withstand hypotonic and hypertonic solutions. Decreases in tonicity by 125 milliosmols had little effect on the cell chemotaxis but below this level, the cells survived only a few minutes and showed no chemotaxis. The degree of hypertonicity which the cells were able to withstand depended

Figure 3.5

Figure 3.5 Effect of varying ionic strength on chemotactic response of neutrophils incubated under different conditions.

Results of two separate experiments depicting the chemotactic response of neutrophils to cytotaxin (CUF-3) dissolved in Gey's BSS. Incubation conditions as follows:-

(i) ●—●—● ; Cells suspended in Gey's BSS + 2% HSA of constant ionic strength.
Ionic strength of Gey's BSS in *cytotaxin compartment* varied.

(ii) o--o--o ; Cells suspended in Gey's BSS + 2% HSA;
ionic strength of *cell suspending medium* varied.
Ionic strength in cytotaxin compartment constant.

(iii) o—o—o; Cells suspended in Gey's BSS of *varying ionic strength* without HSA. Ionic strength in cytotaxin compartment constant.

Note (a) Poor responses were observed in the absence of HSA.

(b) Cells responded optimally at an ionic strength of approximately 0,15 (i.e. physiological conditions).

(c) The poorer response of cells, at an ionic strength of 0,15, when those incubated under condition (ii) are compared with those incubated under condition (i) reflects the effects of subjecting the cells to repeated centrifugation to adjust the ionic strength of the cell suspending medium.

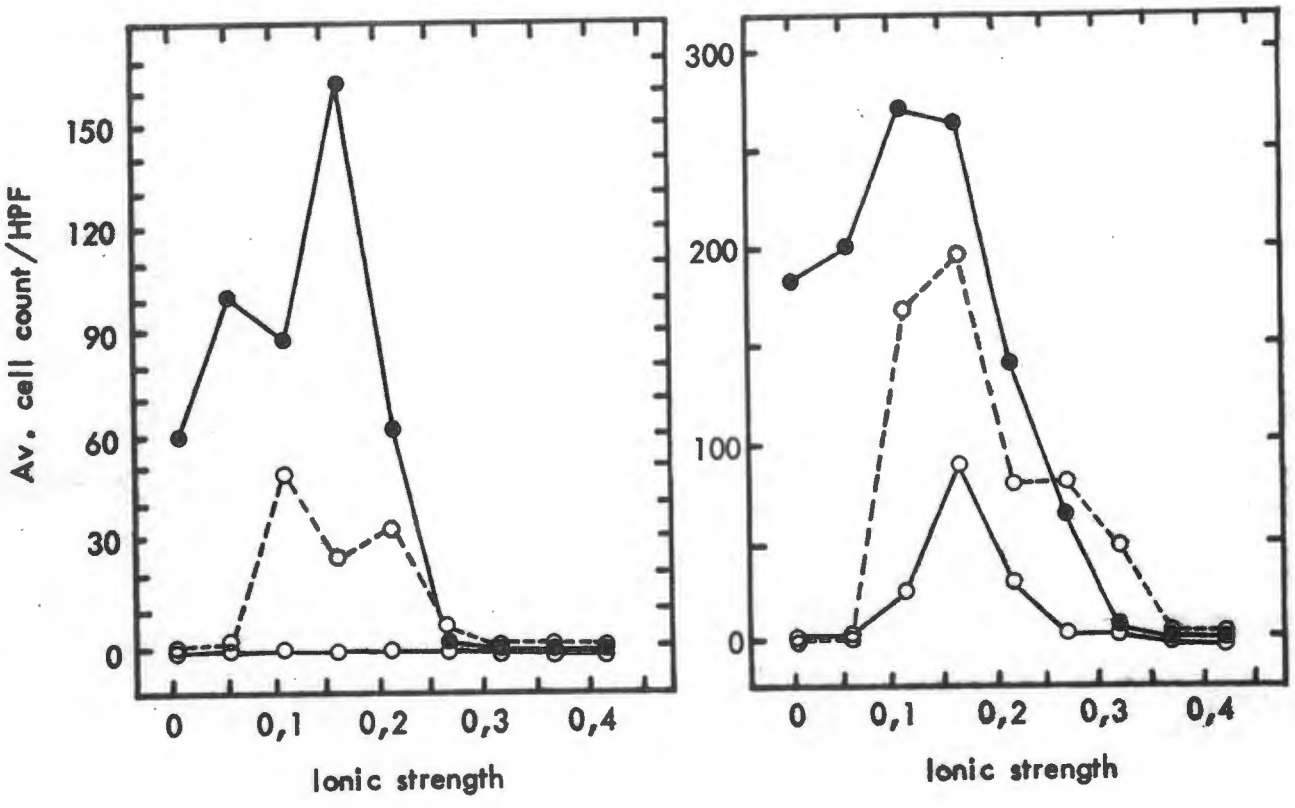
Av. cell count/HPF
150
120
90
60
30
0
0
0,1
0,2
0,3
0,4
Ionic strength
300
200
100
0
0
0,1
0,2
0,3
0,4
Ionic strength

Figure 3.5

on the solute added. They showed that increases with urea by 500 milliosmols; with glucose by 200 milliosmols; and with mannitol, sucrose or sodium chloride by 100 to 125 milliosmols had little effect on the cells and they inferred that osmolar effects could be correlated with membrane permeability for the solute in question. This work was later verified by Zigmond (191) using a modified Boyden chamber technique for assaying normal random cell locomotion and not chemotaxis.

It is highly improbable that an *in vivo* situation could arise in which neutrophils in the blood were exposed to such osmolar extremes. It is, however, entirely conceivable that the accumulation of metabolites and other products at inflammatory sites could result in marked local changes in tonicity. The studies that I have performed to investigate the response of cells in physiological conditions to cytotaxins present in environments of different tonicity would, therefore, seem *a priori* to be more relevant in their design.

Calcium and magnesium

Gey's BSS was made up with the omission of calcium chloride and magnesium chloride. Peritoneal exudate cells were harvested in heparinized 0,9% w/v NaCl, centrifuged and resuspended in Ca^{++}- and Mg^{++}-free Gey's BSS-2% HSA to give a final cell suspension containing $2x10^6$ cells/ml. The Ca^{++} and Mg^{++} concentrations of separate 10 ml volumes of this suspension were adjusted by the addition of aqueous 0,35M $CaCl_2$ or 0,41M $MgCl_2$ to give final concentrations ranging from 0 to $8,8x10^{-4}$M for Ca^{++} and 0 to $10,3x10^{-4}$M for Mg^{++}. Isotonicity was maintained by substituting equi-osmolar amounts of sodium chloride for

calcium chloride or magnesium chloride where appropriate. In addition, cell suspensions were prepared containing neither calcium nor magnesium with and without 1mM disodium ethylenediamine tetra acetic acid (EDTA.2Na). The concentrations of Ca^{++} and Mg^{++} normally present in Gey's BSS are $8,8x10^{-4}M$ and $10,3x10^{-4}M$ respectively. These concentrations were taken as optimal and the experimental protocol was so arranged as to vary the concentration of one ion in the presence of a standard concentration of the other. Casein (1% w/v) in 0,9% NaCl adjusted to pH 7,1 with 1N NaOH was used as the cytotaxin in five separate experiments. The results are summarized in Table 3.1.

Although chemotactic responses varied considerably from one experiment to the next, with each experiment an optimal response was reproducibly observed with $3,5x10^{-4}M$ $CaCl_2$ in the cell medium. Cell counts fell off on either side of the optimum, but not dramatically. No consistent optimal concentration could be found for Mg^{++}. Omission of both Ca^{++} and Mg^{++} from the medium and addition of 0,001M EDTA.2Na to chelate any divalent cations that might have diffused through the filter from the casein ($0,4x10^{-4}M$ Ca^{++}; $0,5x10^{-4}M$ Mg^{++} as determined by atomic adsorption spectroscopy) had a pronounced inhibitory effect on chemotaxis.

The experiments, although somewhat inconclusive, have indicated that a requirement for divalent cations exists. In the case of calcium ions, an optimal concentration can be defined. The situation as far as magnesium ions is concerned is less clear.

Zigmond (191) using a modified Boyden chamber technique, found that the complete absence of calcium from the medium used to suspend horse neutrophils that had been washed with 0,001M EDTA.2Na saline only slightly depressed random locomotion. Optimum locomotion

Table 3.1

The role of calcium and magnesium ions in chemotaxis

	Chemotactic response: Average cell count per high-power field												
Ca^{++} (mM)	,00	,18	,35	,53	,71	,88	,88	,88	,88	,88	,88	,88	,00*
Mg^{++} (mM)	1,03	1,03	1,03	1,03	1,03	1,03	,00	,21	,41	,62	,83	1,03	,00*
Expt no.													
1	218	140	425	242	184	317	276	264	437	340	170	164	-
2	29	39	75	32	17	25	43	59	46	21	9	25	9
3	35	84	88	59	57	30	72	48	71	93	87	82	21
4	16	23	50	39	24	22	83	54	35	40	32	22	29
5	32	52	90	87	49	42	64	201	292	219	49	110	58

*The chemotactic response with no added calcium and magnesium in the presence of 0,001M EDTA.2Na.

Rabbit peritoneal exudate cells were suspended in calcium-and magnesium-free Gey's BSS containing 2% w/v HSA. Calcium and magnesium ions were added from sterile stock solutions (0,35M and 0,42M respectively) and the chemotatactic response to 1% w/v casein measured in Boyden chambers assembled with 3μm Millipore filters. Each entry in the table represents the mean of 8 counts (4 high-power fields on two filters).

was observed, at a calcium concentration of $3,2x10^{-4}M$. No similar effect with either magnesium or manganese was found.

The requirement of divalent metal ions for leucocyte locomotion and adherence has been studied by several workers.

Lotz and Harris (102) found that human neutrophils, observed microscopically, moved slowly towards *Staphylococcus albus* in media free of divalent ions and containing 0,04M EDTA.2Na. The inhibitory effect I found could similarly be due to a reduction in the overall rate of cell migration, as such an effect would result in fewer cells traversing the entire filter width in the three hour incubation period when compared with the controls.

Bryant, des Prez, van Way and Rogers (26) found that human leucocyte migration in capillary tubes, against gravity, was completely inhibited by 0,012M EDTA.2Na. This was due to the inability of the cells to adhere to glass. Adherence to glass was found to be dependent on the presence of magnesium rather than calcium.

Garvin (54) has reported a combined calcium ($2,5x10^{-3}M$) and magnesium ($1x10^{-3}M$) requirement for human neutrophil adherence to siliconised glass wool columns although the latter ions were fairly effective alone. Cells observed microscopically in the presence of EDTA.2Na (0,01M) although unable to adhere to the glass slides, were able to change shape within their own axes.

Zigmond (191) found that calcium ions moderately increased neutrophil adherence to glass coverslips while calcium ions ($6,4x10^{-4}M$) in the presence of manganese ions ($1,6$ to $3,2x10^{-4}M$) caused increases in adherence of up to 200% of the controls.

The obviously close relationship between cell-substrate adherence and cellular locomotion and the implications of the divalent metal ion requirement in terms of cell-cell and cell-substrate interactions are discussed fully in Chapter IV.

Metabolic inhibitors

The effects of a variety of enzyme and metabolic inhibitors on the chemotactic response of rabbit peritoneal neutrophils was investigated in an attempt to obtain, in a general fashion, some idea of the major metabolic pathways of importance to the responding cells. The inhibitors used, the commercial sources from which they were obtained and the generally accepted major site of inhibition are summarized in Table 3.2.

Rabbit peritoneal exudate cells were harvested as usual in 0,9% NaCl containing 15 units of heparin/ml. Following centrifugation the cells were resuspended in Gey's BSS-2% HSA to give a final suspension of $2,2x10^6$ cells/ml. To various 9 ml aliquots of the cell suspension, containing $20x10^6$ cells, 1 ml aliquots of different dilutions of the various inhibitors in Gey's BSS were added. When necessary the pH was adjusted to 7,2 with either 1N NaOH or 1N HCl. The final cell concentration was $2x10^6$ cells/ml. The cells were preincubated in the presence of the various inhibitors at 37^oC, with gentle rocking, for 30 to 60 min as indicated in Table 3.3. After preincubation, the treated cells were pelleted by centrifugation at 4^oC for 10 min at 300xg. The supernatant fluid was decanted and replaced with 10 ml of Gey's BSS-2% HSA. The chemotactic responsiveness of these treated cells was tested using the modified Boyden chamber technique as described in the Appendix with 1% w/v casein in saline, pH 7,1, in the lower compartment. The viability of the treated cells was assessed by their ability to exclude trypan blue dye. Control cells, incubated and centrifuged as for the various inhibitors, were included in each experiment. The results of the experiments together with the concentrations of inhibitors used are summarized in tabular form in Table 3.3.

Table 3.2

Table 3.2

Enzyme and metabolic inhibitors used

No.	Inhibitor	Action	Reference	Supplier
1	Iodoacètate	Inhibits a large number of enzymes by alkylation of cysteine residues. Usually regarded as an inhibitor of glycolysis by virtue of its predominant effect on glyceraldehyde-phosphate dehydrogenase (1.2.1.12)	104, 173	E. Merck, Dramstadt, Western Germany *pro analysi;* catalogue number 374
2	Sodium Fluoride	Complexes with metallo-enzymes or inactivates metal ions. Major metabolic effects on glycolysis by inhibition of phospho-pyruvate hydratase (Enolase)(4.2.1.11) in the presence of inorganic phosphorous. Also acts upon cytochrome oxidase (cytochrome a_3) (1.9.3.1)	104, 173	British Drug House Chemicals Ltd. Poole, England. *analar;* catalogue number 10246
3	Potassium cyanide	Major effect upon respiration by inhibition of cytochrome oxidase (cytochrome a_3). Complexes with metals, metal enzymes, metallo-porphyrins and cuproproteins.	104, 173	-
4	Antimycin A	Inhibits respiration by blocking the transfer of electrons from cytochrome b to cytochrome c_1	104, 173	Calbiochem. Inc., Los Angeles, California U.S.A. chromatographically homogenous (>99%), grade B catalogue number 1782.

5	2,4(α)-dinitrophenol	Uncouples oxidative phosphorylation	104, 173	E. Merck, Dramstadt, West Germany *pro analysi;* catalogue number 3464
6	Sodium arsenate	Uncouples oxidative phosphorylation	104	British Drug House Chemicals Ltd., Poole, England *analar;* catalogue number 10237
7	Puromycin dihydrochloride	A structural analogue of amino-acyl t-RNA that inhibits protein synthesis by blocking translation of m-RNA	40, 104	Sigma Chemical Company Ltd., Saint Louis, Missouri, U.S.A. dihydrochloride; crystalline; catalogue number p 7255
8	Cycloheximide (Actidione)	Inhibits translation, probably by preventing interaction between ribosomes and m-RNA	104	Nutritional Biochemicals Corporation, Cleveland, Ohio, U.S.A. catalogue number 33A
9	Actinomycin D	Combines with guanine residues on DNA and so inhibits DNA-dependent nucleic acid synthesis and hence transcription of m-RNA	40, 104	Calbiochem Inc., Los Angeles, California U.S.A. catalogue number 114666
10	Mitomycin C	Causes covalent cross-linking and fragmentation of DNA strands with consequent inhibition of m-RNA transcription	40, 104	Kyowa Hakko Kogyo Company Ltd., Tokyo, Japan.

All of the inhibitors produced some degree of depression of chemotaxis relative to untreated control cells. Iodoacetate ($1x10^{-6}$M to $1x10^{-1}$M), sodium fluoride ($1x10^{-6}$M to $1x10^{-1}$M), actinomycin D ($2x10^{-8}$M to $2x10^{-4}$M) and 2,4(α)-dinitrophenol ($1x10^{-6}$M to $1x10^{-2}$M) showed convincing effects inasmuch as inhibition became progressively more severe as the concentration of inhibitor was increased. In all other cases moderate and erratically similar degrees of inhibition were seen over all the various concentration ranges used, indicating that the inhibitors were not exerting specific effects on biochemical events of crucial importance to directional cell movement.

When significant inhibition was encountered, this could not be attributed to cellular death since, by the criterion of trypan blue exclusion, cellular viability was not affected by the inhibitors.

My finding that iodoacetate and sodium fluoride had a profound inhibitory effect upon chemotaxis whereas potassium cyanide ($1x10^{-1}$M), antimycin A ($2,5x10^{-5}$M) and sodium arsenate ($1x10^{-1}$M) were relatively ineffectual, is in accord with the work of others and with the view that glycolysis rather than oxidative metabolism provides the energy source for directional cell movement. Lotz and Harris (102) showed that inhibition of respiration had little effect upon chemotaxis. Carruthers (28,29) found that $1x10^{-4}$M iodoacetate, glucose deprivation and $6x10^{-3}$M 2-deoxyglucose inhibited *in vitro* chemotactic responses by human peripheral blood neutrophils. Zigmond (191) working with equine peripheral blood neutrophils, showed similar effects with $2x10^{-4}$M iodoacetate, $1x10^{-2}$M sodium fluoride and $1x10^{-3}$M 2-deoxyglucose when studying random cell locomotion and not chemotaxis. She also reported insignificant effects of antimycin A ($1x10^{-4}$M), 2,4(α)-dinitrophenol ($1x10^{-3}$M), potassium cyanide ($1x10^{-3}$M) and oligomycin ($1x10^{-4}$M).

Table 3.3

Table 3.3

Effect of metabolic inhibitors on chemotactic responsiveness of rabbit neutrophils and cell viability

Rabbit peritoneal exudate cells in Gey's BSS-2% HSA were preincubated at 37°C with the inhibitors, washed by pelleting and resuspended in Gey's BSS-2% HSA for assay of chemotactic responsiveness to 1% casein. The cell counts given are means of 4 counts on each of 2 filters. Cell viability was estimated by trypan blue exclusion.

Inhibitor	Concentration (M)	Av.cell count per HPF	Inhibition %	Viability %	Inhibitor	Concentration (M)	Av.cell count per HPF	Inhibition %	Viability %
Iodoacetate (30min pre-incubation)	0	385	0	97	Sodium fluoride (30min pre-incubation)	0	385	0	97
	10^{-6}	138	64	91		10^{-6}	222	42	98
	10^{-5}	217	44	N.D.		10^{-5}	332	14	N.D.
	10^{-4}	9	98	95		10^{-4}	216	44	96
	10^{-3}	1	100	N.D.		10^{-3}	167	57	N.D.
	10^{-2}	0	100	90		10^{-2}	198	49	98
	10^{-1}	1	100	N.D.		10^{-1}	3	99	N.D.
Potassium cyanide (30min pre-incubation)	0	385	0	97	Antimycin A (60min pre-incubation)	0	252	0	84
	10^{-6}	248	36	97		$2,5\times10^{-8}$	120	52	78
	10^{-5}	285	26	N.D.		$2,5\times10^{-7}$	156	38	N.D.
	10^{-4}	307	20	99		$2,5\times10^{-6}$	116	14	82
	10^{-3}	247	36	N.D.		$2,5\times10^{-5}$	229	9	N.D.
	10^{-2}	198	49	98					
	10^{-1}	127	67	N.D.					

2,4(α)-dinitro-phenol (30 min pre-incubation)	0	385	0	97	Sodium arsenate (30min pre-incubation)	0	385	0	97
	10^{-6}	291	24	99		10^{-6}	109	72	97
	10^{-5}	237	38	N.D.		10^{-5}	173	55	N.D.
	10^{-4}	309	20	98		10^{-4}	157	59	98
	10^{-3}	130	66	N.D.		10^{-3}	320	17	N.D.
	10^{-2}	55	86	90		10^{-2}	177	54	96
	10^{-1}		N.D.	N.D.		10^{-1}	198	49	N.D.
Puromycin dihydro-chloride (60min pre-incubation)	0	252	0	84	Cycloheximide (Actidione) (60min pre-incubation)	0	252	0	84
	5×10^{-8}	185	27	90		5×10^{-8}	207	18	76
	5×10^{-7}	172	32	N.D.		5×10^{-7}	182	28	N.D.
	5×10^{-6}	191	24	92		5×10^{-6}	89	65	89
	5×10^{-5}	219	13	N.D.		5×10^{-5}	143	43	N.D.
	5×10^{-4}	180	29	84		5×10^{-4}	116	54	71
Mitomycin C (60min pre-incubation)	0	252	0	84	Actinomycin D (60min pre-incubation)	0	52	0	84
	5×10^{-8}	136	46	89		1×10^{-8}	246	2	88
	5×10^{-7}	120	52	N.D.		1×10^{-7}	171	32	N.D.
	5×10^{-6}	139	45	72		1×10^{-6}	109	57	85
	5×10^{-5}	179	29	N.D.		1×10^{-5}	3	99	N.D.
	5×10^{-4}	150	40	68		1×10^{-4}	0	100	69

The relative paucity of neutrophil mitochondria and the abundant glycogen reserves found in these cells (Figure 3.6) provide further circumstantial evidence to support the general notion that energy requirements for neutrophil locomotion are drawn from anaerobic metabolic sources.

It is appropriate in drawing this conclusion to focus attention on two facts. Firstly, 2,4 (α)-dinitrophenol, which is generally believed to act principally by uncoupling oxidative phosphorylation, inhibited chemotaxis significantly, particularly at the relatively high concentration of $1x10^{-2}M$ (Zigmond (191) it will be recalled found no inhibition at $1x10^{-3}M$ for random cell locomotion, not chemotaxis). It is difficult to reconcile this finding with the general view that respiratory generation of high-energy phosphate bonds does not appear to be required for chemotaxis, without postulating some other mode of action for 2,4(α)-dinitrophenol at these concentrations.

There is good experimental precedent for such a postulate in the observations of Webb and Hollander (174) who were able to distinguish two actions of 2,4(α)-dinitrophenol upon the contractile apparatus of myocardial cells. The first and almost instantaneous effect was basically a direct one upon the membrane with consequent disturbances of myocardial ionic fluxes. The second, and more "conventional" effect was upon oxidative phosphorylation with cardiac arrest due to depletion of ATP stores. It is possible, therefore, that the effects of 2,4(α)-dinitrophenol on chemotaxis may have been mediated by direct action on neutrophil membranes. (Unfortunately I have no experimental results that examine this possibility.) It is also conceivable that, by the effect of 2,4(α)-dinitrophenol upon oxidative phosphorylation, neutrophil ATP-stores may have become depleted with secondary effects consequent upon the lack of an important biosynthetic energy source.

Figure 3.6

Figure 3.6 Electron microphotograph of a thin section through a 3μm Millipore filter through which cells were migrating in response to chemotactic stimulus (1% casein).

The neutrophil shows numerous electron-dense granules; fine deposits of glycogen; sparse mitochondria; and phagocytic vesicles, some of which have fused to form phagolysosomes. Active pseudopodial extrusion is evident. The intimate contact between regions of the cell surface and the fibres of the filter is clearly shown.

The direction of the chemotactic gradient is indicated by the arrow.

(Magnification 14 000x; glutaraldehyde fixation with osmium tetroxide staining).

I am indebted to Professor C.J. Uys and Mr. J. Dale of the Department of Pathology for this, and other electron microscopic studies.

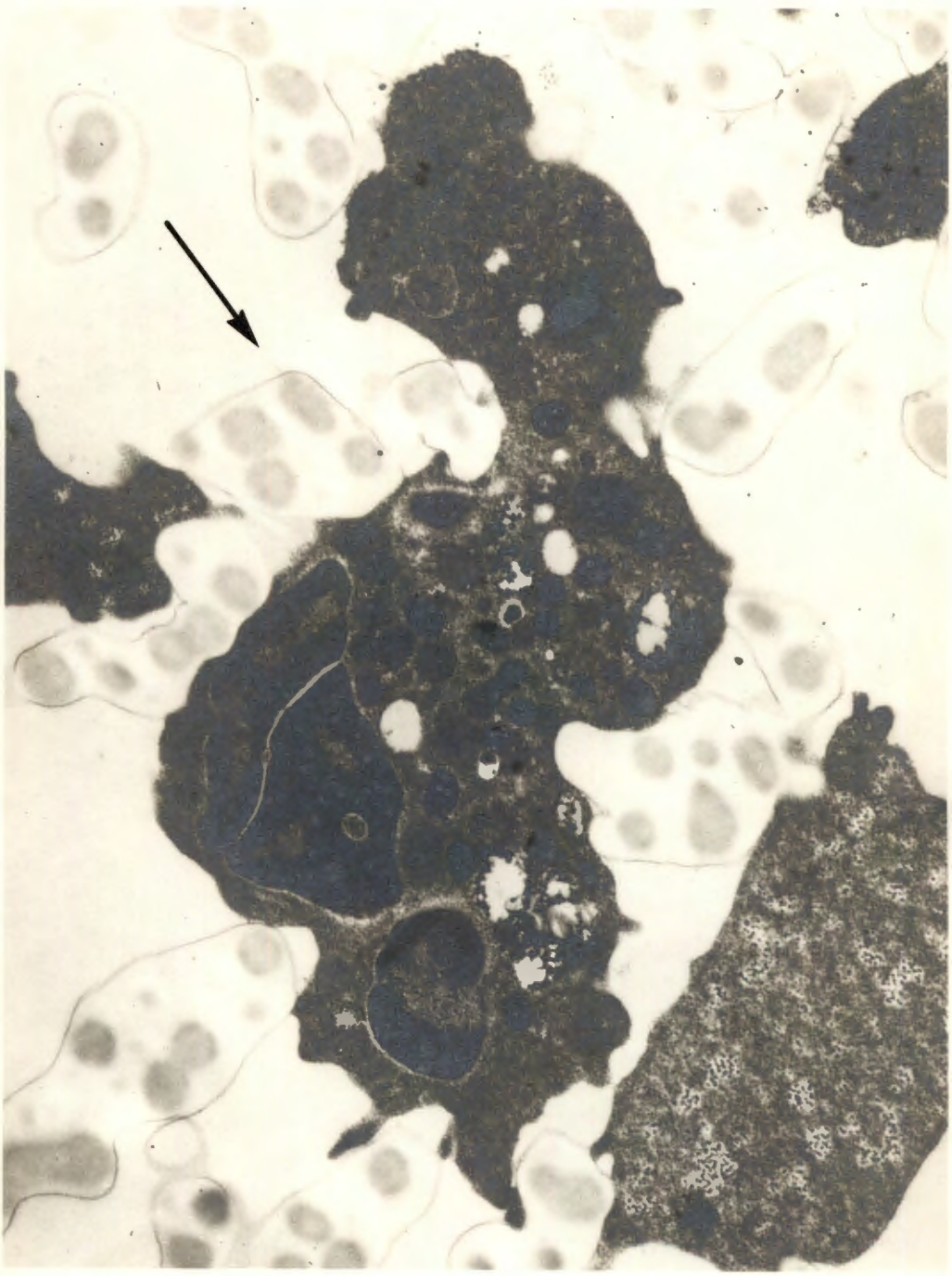

Figure 3.6

Secondly, it is important to note that neither iodoacetate nor sodium fluoride are entirely specific in their metabolic effects. Iodoacetate, for example, is known to cause significant inhibition of at least 55 enzymes at concentrations of $1x10^{-3}M$ (173). It is nevertheless also the case that glyceraldehyde phosphate dehydrogenase (and consequently glycolysis) is particularly sensitive to this compound, being completely inhibited by concentrations of $2x10^{-4}M$. Hence a measure of specificity can be obtained by working at low concentrations, although keto acid oxidation is also affected by these concentrations.

One would not, *a priori*, have expected new DNA synthesis to be a requirement for chemotaxis since neutrophils are "end cells" locked in the G_o phase of the cell cycle. It was, however, conceivable that the mobilisation of migrating cells might require induction of enzyme synthesis and that membrane repair during locomotion might require the intact machinery for protein synthesis. Jacoby (68), Rosenberg (135), Taylor (154), Little and Edwards (101), Weiss and Coombs (177), Weiss and Lachmann (178) and Weiss (176) have shown, for example, that macrophages and other cell lines that move across a solid surface separate from points of adherence to the substrate at an *intramembranous* plane of cleavage with the result that a "trail" of detached outer membrane surface is left by the moving cell. It was, therefore, of interest to study the effects of inhibitors of transcription and translation upon the chemotactic response. The results of these experiments proved most interesting. Puromycin ($5x10^{-8}M$ to $5x10^{-4}M$) and cycloheximide ($5x10^{-8}M$ to $5x10^{-4}M$) both inhibitors of translation (Table 3.2) had insignificant, dose-independent effects on chemotaxis, indicating that requirements for new protein synthesis are not critical for this phenomenon. Similarly, the inhibitor of transcription, mitomycin C ($5x10^{-8}M$ to $5x10^{-4}M$), showed little effect on chemotactic responsiveness,

indicating that neither new DNA or m-RNA synthesis was required for cell locomotion to take place. Actinomycin D, however, which is generally regarded as a potent inhibitor of transcription that acts by coupling to guanine residues in DNA (57), completely inhibited chemotaxis at concentrations above $2x10^{-5}$M. Carruthers (29) and Ward (165) have also demonstrated the inhibitory effects of actinomycin D upon chemotaxis. The latter author found that mitomycin C was not inhibitory.

One can only explain this seemingly analogous action of actinomycin D by accepting that it executes its inhibitory effects by some mechanism other than one which involves interference with protein synthesis. Actinomycin D has been reported as having depressive effects upon respiration and glycolysis of leukaemic lymphocytes and granulocytes (94) hence its action on chemotaxis might have been effected by this mechanism. Lipid binding characteristics have also been described for actinomycin D (37), hence it may have operated by way of some subtle effect on the neutrophil membrane. Finally, it is eminently possible that actinomycin D, by virtue of its affinity for guanine residues, might interact with cGMP or GTP and so inactivate the intracellular "second messenger" for chemotaxis or compromise an important intracellular energy source. Some evidence for the antagonistic roles of cAMP and cGMP in the modulation of leucocyte chemotaxis has been published by Estensen, Quie and Hogan (47) and will be discussed fully when considering my results concerning cAMP levels in cytotaxin stimulated and control cells. Further indirect evidence that actinomycin D has some effect on cells other than the inhibition of transcription comes from the findings of Pieroni and Broderick (130) that the simultaneous intraperitoneal injection of 25µg of actinomycin D with *Salmonella typhosa* endotoxin produced a $5x10^{4}$ to $1x10^{5}$ fold decrease in the LD_{50} of the toxin.

Although the judicious use of pharmacological inhibitors has been of considerable value in dissecting metabolic events of importance to the co-ordinated function of intact cells, it is nevertheless salutary to remember that extrapolation from results obtained with isolated and relatively pure enzyme systems to the complex situation that exists in intact cells is seldom entirely justifiable. Certain inhibitors may, for example, require to be in an uncharged form to pass through the barrier of the cell membrane while their biochemical effect requires that they be in a charged form. The facility with which inhibitors penetrate membranes - clearly a prerequisite for their intracellular activity - is yet another variable which affects their action on living cells. The questionable specificity of many inhibitors and the secondary effects that result from metabolic consequences far removed from the primary site of action have already been alluded to and constitute further reasons for hesitating to ascribe too much significance to conclusions drawn from inhibitor studies.

With these reservations, therefore, it would appear that chemotaxis depends mainly on anaerobic glycolysis for energy needs and is independent of requirements for substantial amounts of new protein synthesis.

In his report published on the biological effects of cytochalasin B, Carter (32) stated that this metabolic product of the mould *Helminthosporium dermatiodeum* inhibited motility and cytoplasmic cleavage in cultured cells at concentrations (0,5µg/ml to 1,0µg/ml) that did not affect cell viability. This paper appeared as a companion to Carter's important publication on haptotaxis (31), and in it he set forward a plausible theory to reconcile the effects of cytochalasin B with his theory of shifting substrate adherence as the basis for cell movement.

Schroeder (140) subsequently reported the disappearance of microfilaments from the cleavage furrows of dividing cells in the presence of cytotachalasin B. As a result of these and similar observations (for review see Wessels, Spooner, Ash, Bradley, Luduena, Taylor, Wrenn and Yamada (182)), the intuitively appealing belief developed that cells, in order to move, must possess some "contractile apparatus" that was susceptible to disruption by cytochalasin B. Microfilaments in a quaint, anthropomorphic view, were the "muscles" of this contracile mechanism. It is now recognized that cytochalasin B does not act in this specific way.

One of the many intriguing observations made by Carter (32) concerned the lack of any effect of cytochalasin B, even at concentrations as high as 50µg/ml, on the motility of a number of fresh water ciliates or flagellates and on the movement of murine spermatozoa. This invites the generalization that the compound acts upon cells that "crawl", but not upon those that are equipped with appendages that enable them to swim. Since polymorphonuclear leucocytes belong to the category of cells that crawl, and since they appear to contain microfilaments (Figure 3.7) I felt that it would be of interest to study the effects of cytochalasin B on directional cell movement.

Cytochalasin B[1] was dissolved in dimethylsulfoxide[2] (DMSO) to give a 4mg/ml stock solution which was stored at -20°C. Dilutions of this stock preparation were prepared freshly, just before use, using dimethylsulfoxide, DMSO, as the diluent. An equal volume (15µl) of each dilution was then added to a corresponding 6ml aliquot of

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(1) Imperial Chemical Industries Ltd., Cheshire, England.

(2) E. Merck, Därmstadt, West Germany.

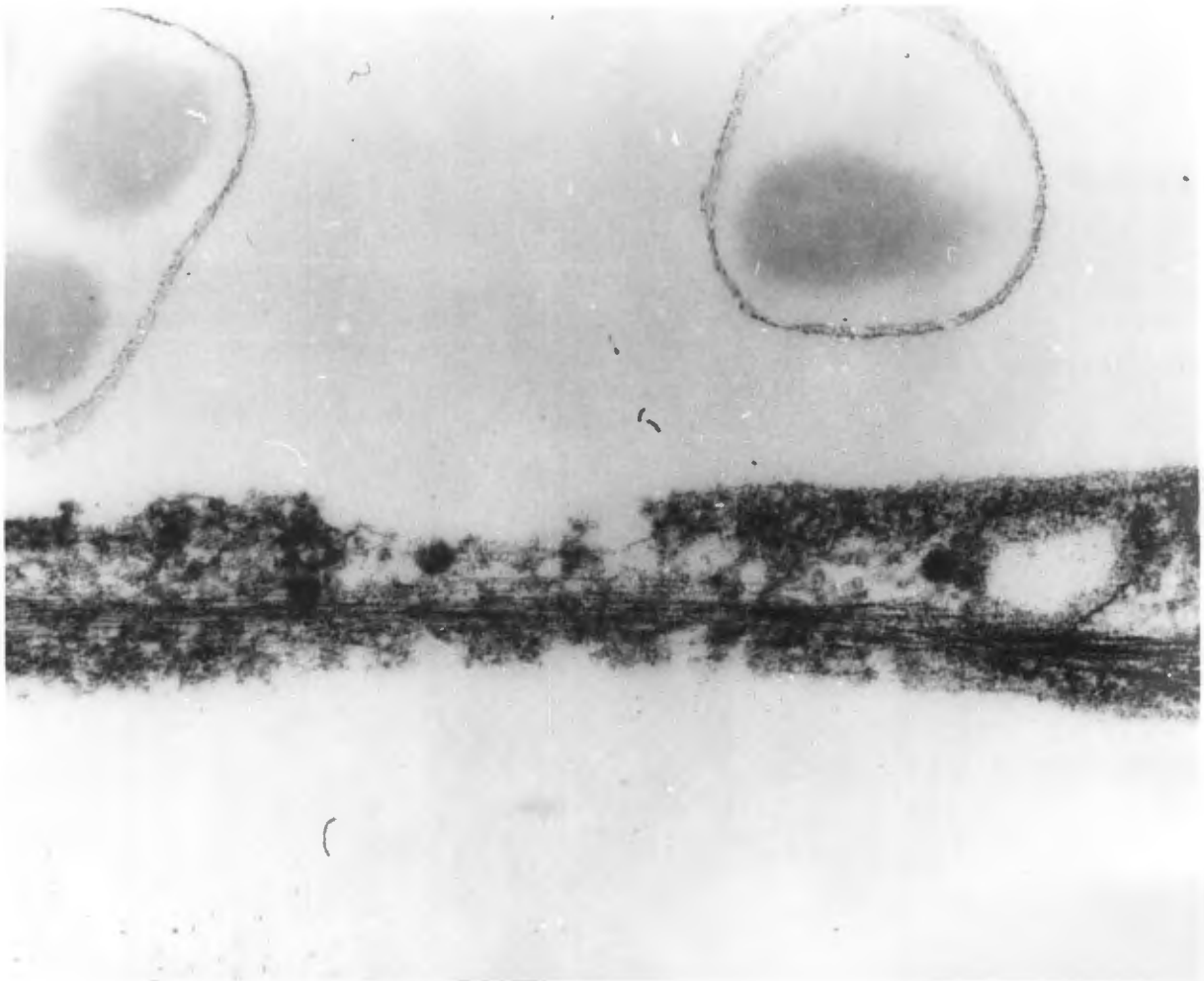

Figure 3.7 Electron microphotograph of extruded neutrophil pseudopod showing axially dispersed, fine microfilaments.

(Neutrophils, within the substance of a 3μm Millipore filter, responding to a 1% casein chemotactic stimulus; magnification 104 000x).

rabbit polymorphonuclear leucocytes suspended in Gey's BSS-2% HSA, at a concentration of $2x10^6$ cells/ml, to cover the range of 0,01µg/ml to 10µg/ml cytochalasin B. In all cases the final concentration of the dimethylsulfoxide DMSO was 0,25% v/v. A control cell suspension contained only 0,25% v/v dimethylsulfoxide DMSO. After thorough mixing, the cell-inhibitor suspensions were added to the Boyden chambers. Prior incubation of the cells in the presence of the inhibitor was not deemed necessary since the effects of cytochalasin B are known to be rapid (182,192). The chemotactic material used to stimulate the cells was 1% w/v casein in 0,9% NaCl, pH 7,1. The experiment was repeated on two separate occasions. The results are summarized in graphic form in Figure 3.8.

On both occasions the presence of cytochalasin B had a dramatic, dose-dependent inhibitory effect on the chemotactic responsiveness of rabbit neutrophils with complete inhibition at concentrations of inhibitor greater than 1µg/ml.

In his original paper Carter (32) ascribes the immobilizing effect of cytochalasin B on murine fibroblasts to an overall increase in adhesion between the cells and the solid support on which they rested. This, the author suggested, disorganized the "adhesion gradient" required for haptotactic movement. He also noted that cells that had been treated with cytochalasin B and washed before plating, adhered more rapidly than control cells but took longer to spread and suggested, to explain this phenomenon, an additional effect of cytochalasin B upon the viscosity of the cell membrane.

Other workers have studied the effects of cytochalasin B on neutrophil motility. Zigmond and Hirsch (192) using phase contrast microscopy of moving cells and their modification of the Boyden chamber

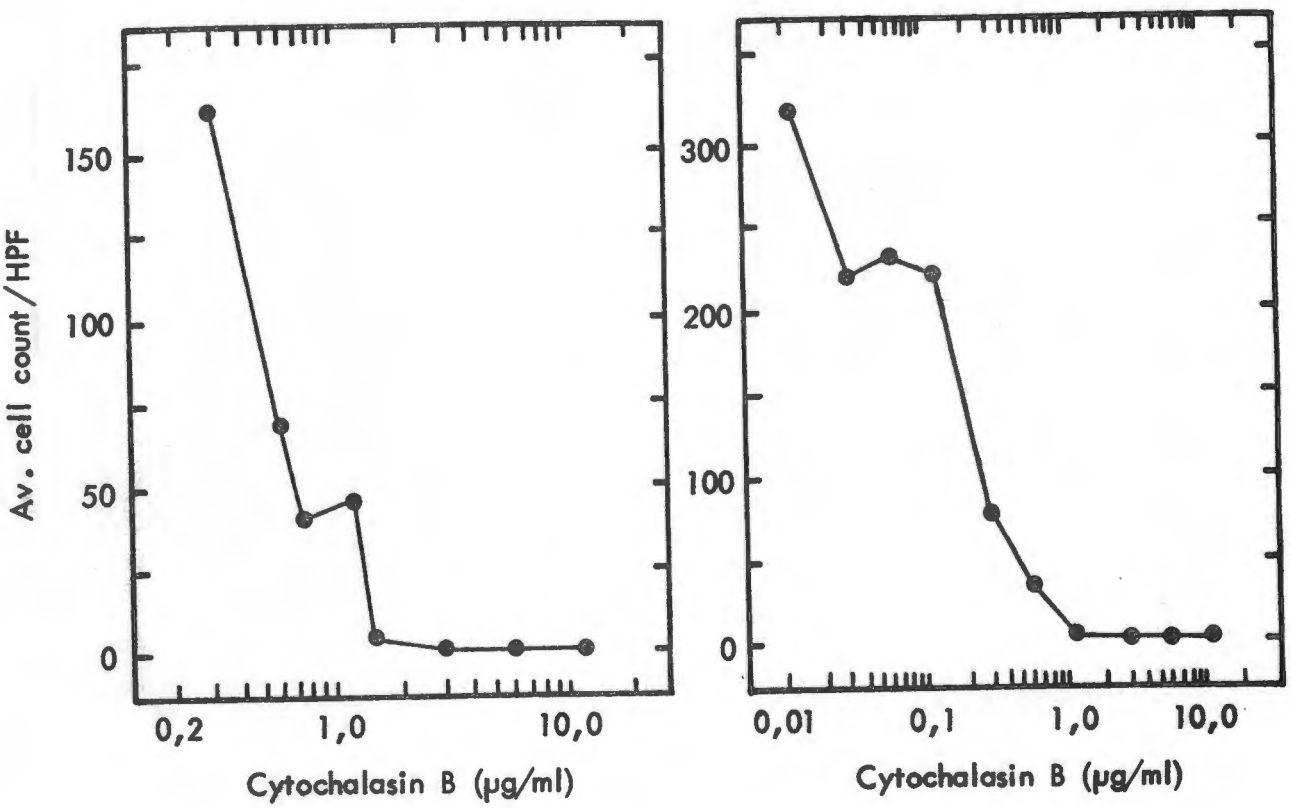

Figure 3.8 Effect of cytochalasin B on neutrophil chemotaxis

Results of two experiments in which the chemotactic response of neutrophils ($2x10^6$/ml) to 1% casein was studied. The upper (cell) compartment contained cytochalasin B at the concentrations indicated and DMSO at a final concentration of 0,25% v/v. This concentration of DMSO was shown to have no effect on chemotaxis.

system showed that random movement of equine, rabbit and human neutrophils was completely inhibited by 3µg/ml in the cell suspending medium. This effect was not mediated by inhibition of glycolysis since, although the inhibitor impeded cellular uptake of tritiated-2-deoxyglucose (193), concentrations that stopped locomotion allowed more than sufficient glycolysis to support cell movement under ordinary circumstances. These authors made the significant observation that cellular "contractile" and "saltatory" movements were visible even at 10µg/ml, indicating that cytochalasin B does not act primarily by its effect on a putative microfilamentous network responsible for co-ordinated cellular motility. Finally, they noted that neutrophils exposed to 5µg/ml of cytochalasin B lost their firm attachment to the substrate and "rounded up". They used this observation to discount Carter's haptotactic model without commenting on the differences in concentration used by Carter and themselves. These authors did not study the effect of cytochalasin B on chemotaxis.

Becker, Davis, Estensen and Quie (15) published a paper in 1972 in which they claimed to have demonstrated an inhibitory effect of cytochalasin B on both random and directional cell movement. Concentrations of cytochalasin B, tenfold lower than those required for inhibition, caused *stimulation* of chemotaxis without affecting haphazard locomotion. The authors gave considerable emphasis to this observation and implied, in their speculative discussions, that these distinctive dose-effect relationships distinguished the two processes of directional and random migration.

Neither Zigmond and Hirsch (192) nor I were able to document a stimulatory effect of low doses of cytochalasin B on cell movement, and I am hesitant to accept the findings of Becker et al. (15) as

evidence to indicate that directional and random movement involve fundamentally distinct basic processes without confirmatory evidence.

While the inhibitory effect of cytochalasin B on cell migration seems unequivocal, the mechanism by which this effect is produced is less clear. If one considers the accumulating circumstantial evidence, there would seem to be some to implicate a direct effect of the mould metabolite on cell membranes rather than upon microfilaments. Thus inhibition of transport of glucose (48,87,192,193), 2-deoxyglucose (48,87,193), glucosamine (48,87,137,193), thymidine (48) into certain cells has been well documented.

It is of interest to note that although the above transport systems are affected, there is some degree of selectivity inasmuch as leucine (137,193) and choline (48) uptake into certain treated cells appear not to be affected.

In one particularly interesting report, the effects of cytochalasin B on intracellular energy stores of chick fibroblasts was investigated (172). Normal levels of ATP were found in control and cytochalasin B treated cells indicating that the morphological cell changes observed were not due to depletion of intracellular reserves of this energy source. Iodoacetate-treated cells showed a decrease in intracellular ATP levels to about 1/5 of the control. If cells pretreated with cytochalasin B were then exposed to the iodoacetate, no significant decrease in intracellular ATP concentrations were noted, indicating that the cell membrane permeability had been altered or the cells had changed to an iodoacetate-insensitive, ATP generating system.

It would thus appear that what was once considered an inhibitor of fairly specific action is now regarded as a compound with more complex effects that have as yet to be defined. In my own view, Carter's experiments are some of the most elegant that have yet been done on the subject of cell movement and I am intuitively inclined

to accept his view that membrane-substrate interaction is the principal target for the action of cytochalasin B. As originally formulated, his theory required that the inhibitor should immobilize cells by increasing cell-substrate adhesion and spreading. Experimental evidence on this point is conflicting. Krishan (89) and Warner and Perdue (172) have demonstrated such increased adherence whereas Zigmond and Hirsch (192) and Sanger and Holtzer (137) have shown a contrary effect.

The fact remains that effects of cytochalasin B upon membrane-associated processes are pronounced and adherence alone may be irrelevant to more fundamental derangement of co-ordinated membrane functions that lead to inhibition of cell motility.

A number of recent studies have indicated that certain biologically effective cellular events in leucocytes are related to an organized system of colchicine-sensitive microtubular structures of the sort described by Borisy and Taylor (20,21) situated in close proximity to the cell membrane. The drug has been shown to inhibit concanavalin A receptor mobility on lymphocyte membranes (44) and appears to have some effect in the same receptor mobility of mobile human polymorphonuclear leucocytes (136). It is also reputed to interfere with concanavalin A induced agglutination of neutrophils (18). In an elegant series of experiments, Ukena and Berlin (160) showed that when colchicine-treated cells phagocytosed latex particles, they incorporated into the phagocytic vacuole, membrane receptors for adenine and lysine that would ordinarily have escaped internalization. One thus has a concept of co-ordinated lateral movement of membrane receptors whose dynamic topography is controlled by micro-tubular structures.

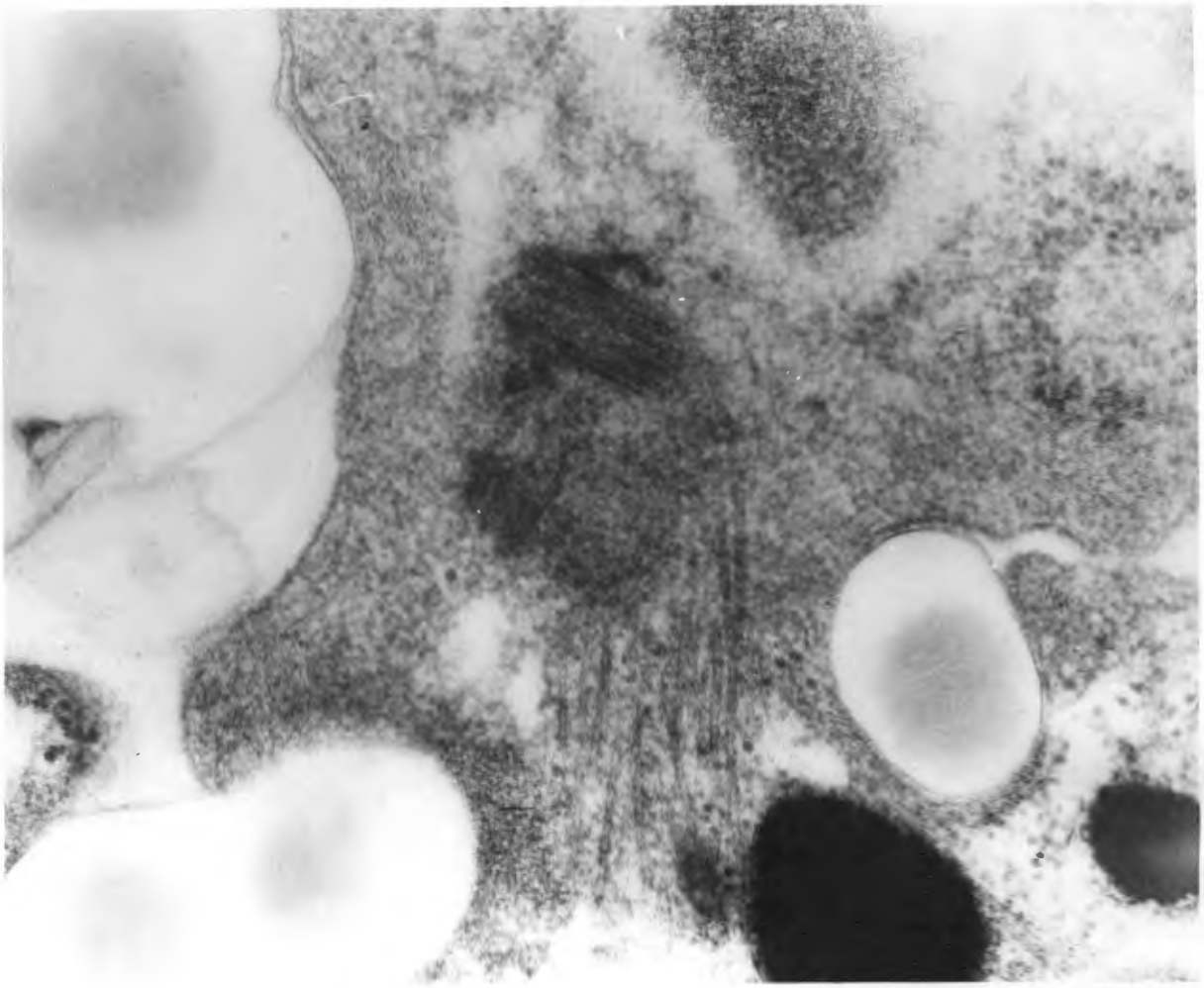

Figure 3.9 Electron microphotograph of a portion of a neutrophil showing microtubules leaving a centriole.

(Neutrophils, within the substance of a 3μm Millipore filter, responding to a 1% casein chemotactic stimulus; magnification 130 000x).

More direct evidence for the effect of colchicine on directional cell movement is quoted by Wessels (181) and indicates that the stabilized "tails" of migrating embryonic cardiac and nerve cells are disrupted by the drug, leaving the cell free to move in any direction that the now-circumferential undulating membrane may take.

In view of these observations and since microtubules have been demonstrated in mature neutrophils by Malawista and Bench (106) and myself (Figure 3.9) it was clearly of interest to study the effect of colchicine upon chemotaxis.

Colchicine(1) was dissolved in TBS pH 7,2 containing 0,001% w/v chlorhexidine as a stock solution of 40mg/ml and kept at -20°C. Dilutions of the stock solution were prepared freshly, before use, using TBS as the diluent. Twenty five microlitres of each dilution were added to 10ml samples of cell suspension ($2x10^6$ cells/ml of Gey's BSS-2% HSA to give a final concentration of colchicine ranging from 0,01µg/ml to 10µg/ml ($2,5x10^{-8}$M to $2,5x10^{-5}$M).

Three experiments were performed in all. In the first two experiments the cell-inhibitor suspensions were added to the Boyden chambers without pre-incubation. In the third experiment half the cells were pre-incubated for 60 min at 37°C before they were added to the chambers and the other half were added to the chambers immediately, without pre-incubation. The effects of different concentrations of colchicine on chemotaxis are summarized diagrammatically in Figures 3.10 and 3.11.

The results show that, apart from an erratic variation about the chemotactic response of control cells, within the limits of the

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(1) Hopkins and Williams Ltd., Chadwell Heath, Essex, England or Sigma Chemical Co. Ltd., St. Louis, Missouri, U.S.A.

in vitro assay system there appeared to be no progressive overall trend either towards inhibition or stimulation with changing colchicine concentrations over the range 0,01µg/ml to 10µg/ml ($2,5x10^{-8}$M to $2,5x10^{-5}$M) tested.

The results I obtained are essentially similar to those obtained by Zigmond (191) who was similarly unable to detect any convincing inhibitory effect of colchicine on neutrophil locomotion. Using her particular assay system she was able to demonstrate a stimulatory effect of low colchicine concentrations (0,1µg/ml; $2,5x10^{-7}$M) on leucocyte motility for which she offered no explanation. Ryan, Borysenko and Karnovsky (136) were unable to show a detectable effect of colchicine on human neutrophil movement or chemotactic polarization towards *Staphylococcus epidermidis* at concentrations of 10^{-4}M. The same concentration of alkaloid had no effect on the redistribution of concanavalin A receptors on the cell membrane. The drug did however inhibit capping in immobilized cells (using either cytochalasin B or serum free media for immobilization).

In a brief report, Caner (27) indicated that he had observed inhibition of human neutrophil chemotactic responses to suspensions of *Staphylococcus albus* with concentrations of colchicine ranging from 0,02µg/ml to 20µg/ml ($5x10^{-8}$M to $5x10^{-5}$M). Detailed methodology and results were not given in the paper thus it is difficult to evaluate his conclusions. It is, however, noteworthy that he reports with concentrations of colchicine varying from 0,03µg/ml to 0,3µg/ml and has based his conclusions upon differences between 227 and 421 cells/high-power field without any indication of the filter variations he observed.

Ward (166) in a review of his experiences in the field, reports, without supporting data or references, to having observed 50% inhibition of chemotaxis with 0,5mM (200µg/ml) colchicine.

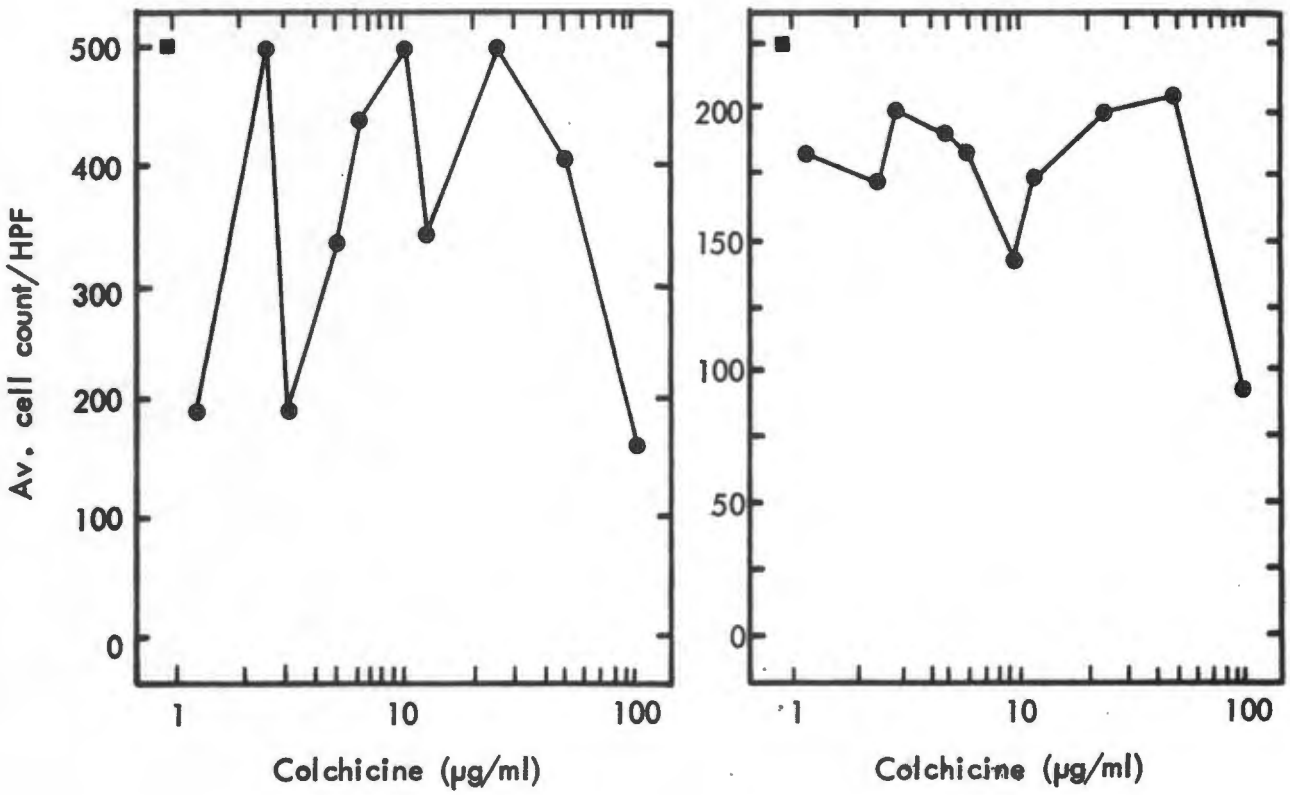

Figure 3.10 Effect of colchicine on neutrophil chemotaxis.

Results of two similar experiments in which the effects of the indicated concentration of colchicine upon neutrophil chemotactic responses to 1% casein were studied.

Note (a) Despite the fact that, in each experiment, $6x10^6$ cells were added to each chamber, different maximal chemotactic responses were observed in the two experiments.

(b) The between chamber variation in the first experiment (*left*) where more intense responses were observed, was high.

(c) No well-defined effect of colchicine could be detected.

In the experiments inhibitor was present throughout the incubation period and was added to the cell suspending medium only.

Closed squares (■) indicate counts observed without added colchicine.

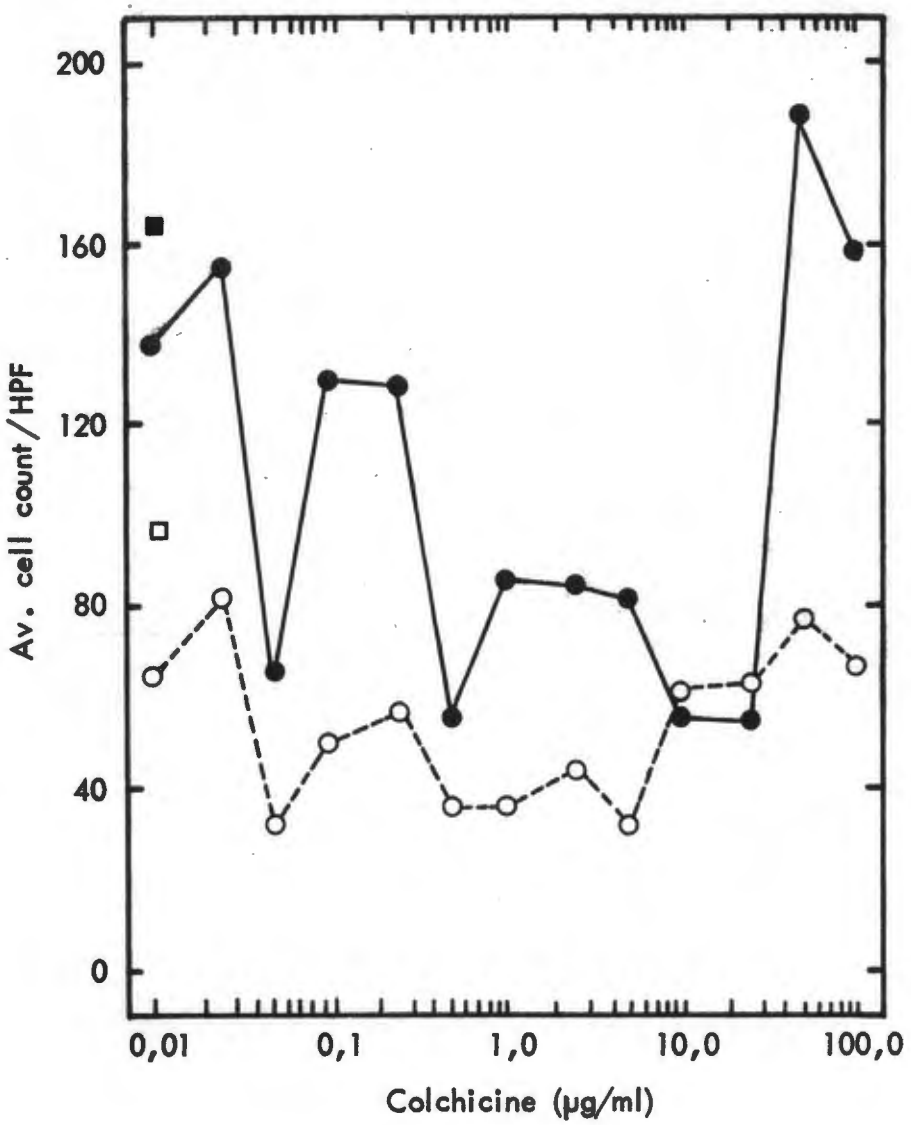

Figure 3.11 Effects of colchicine on neutrophil chemotaxis.

In this experiment cells from a common pool were divided into two lots. The first lot tested directly in the absence (■) or presence (●—●) of colchicine at the indicated concentration. The second lot was preincubated for 60 min at 37°C in the absence (□) or presence (○--○) of colchicine at the indicated concentration before testing for chemotaxis at the same colchicine concentration. Casein (1%) was used as the chemo-attractant in all chambers.

Colchicine had no clearly defined effect upon chemotaxis.

On balance, therefore, I am inclined to favour the view that chemotaxis is not significantly affected by this alkaloid.

Although the results of these experiments were, in a sense, negative, they do nevertheless indicate fairly convincingly that whatever the receptors and other membrane components of the chemotactic response may be, they are probably distinct from those that serve other functions such as receptor movement or lectin binding.

Energy requirements

A good deal of evidence has accumulated to indicate that chemotactic substances increase intrinsic cellular motility and that the energy for this increase is derived from active cellular metabolic sources. In this section I examine some of the metabolic consequences of adding a chemotactic compound to a suspension of viable neutrophils incubated *in vitro*.

It is well known that neutrophils are able to metabolise glucose using both the pathways of aerobic and anaerobic metabolism (49,138,139,192). In order to study the effect of a chemotactic substance on the metabolic processes involved, I investigated the following phenomena in the presence and absence of SNF-cytotaxin.

Materials and Methods

Oxygen Consumption

This was measured by two procedures.

(a) Warburg constant volume manometry (161).

Cells were suspended at a known concentration in a medium of the following composition (NaCl 0,137M; KCl 5,10mM; $CaCl_2$ 0,88mM; $MgCl_2$ 1,03mM; KH_2PO_4 0,18mM; Na_2HPO_4 0,8mM;

glucose 11,10mM; streptomycin sulphate 50µg/ml; sodium benzylpenicillin 200 units/ml; phenol red 0,01mg/ml and human serum albumin 20mg/ml adjusted with 0,03M Na_2HPO_4 to pH 7,2). Aliquots (2,7ml) of this suspension were added to the main compartments of 15 ml Warburg flasks containing strips of fluted filter paper spotted with 20% w/v KOH in the centre wells. The side arms contained 0,5ml of either TBS or TBS containing approximately 2mg of SNF-protein. After equilibration for 15 min at 37°C with air, the stopcocks were closed and manometer readings were taken at 10 min intervals. When the thermobarometer and oxygen uptake rates had stabilized, the contents of the side arms were added to the flasks by tipping and further readings were taken for approximately 3h in two experiments. Flask and manometer constants were supplied by the manufacturer(1)

In a further series of 7 experiments contaminating erythrocytes were lysed and the cells washed and finally suspended in a medium of the same composition as that described above except that glucose was present at a concentration of 5,55mM. Aliquots (2,5ml) were added to Warburg flasks prepared with fluted filter paper fans as above. The side arms contained 500µl of 0,067M radioactive glucose dissolved in either TBS or TBS containing approximately 1mg SNF-protein. Following a 15 min equilibration period at 37°C in air, the manometer stopcocks were closed and the side arm contents were added to the flasks by tipping. Readings were taken immediately

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(1) B. Braum, Melsungen, West Germany.

and after 60 min incubation. Flask and manometer constants were supplied by the manufacturers as above.

(b) Electrometric procedure

Cell suspensions of known cell concentrations in Gey's BSS(-$NaHCO_3$)-2% HSA were equilibrated with air at 37°C for 15 min and loaded into a chamber fitted with an oxygen electrode and equipped with oxygen monitor(1) with care to avoid entrapment of bubbles below the electrode membrane. The chamber assembly was maintained at 37°C in a water bath and oxygen consumption was recorded as a function of time on a recorder calibrated to give a full scale deflection with balanced salt solution equilibrated with air saturated with water vapour at 37°C (i.e. pO_2 = 149,33mmHg). A solubility coefficient of 0,0239 for oxygen in balanced salt solution at 37°C (161) was used to correct recorder readings into µl of O_2. Gentle agitation of the cell suspension was maintained throughout with a magnetic stirrer. In one experiment, cells were pre-treated with either TBS or TBS containing approximately 0,7mg of SNF-protein before adding them to the electrode chamber. In another three experiments 0,15ml of TBS or TBS containing 0,7mg of SNF-protein was injected into the chamber after oxygen consumption had been monitored for 5 to 10 min.

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(1) Oxygen electrode model 5301; Yellow Springs Instrument Co. Inc., Yellow Springs, Ohio, U.S.A.

Metabolic conversion of glucose to carbon dioxide

Rabbit peritoneal exudate cells were harvested as described in the Appendix, exposed to brief hypotonic shock to lyse contaminating erythrocytes and washed to remove cell debris. Warburg flasks were charged by adding 2,5ml of a suspension of cells in a medium of the following composition (NaCl 0,137M; KCl 5,10mM; $CaCl_2$ 0,88mM; $MgCl_2$ 1,03mM; KH_2PO_4 0,18mM; Na_2HPO_4 0,84mM; glucose 5,55mM; streptomycin sulphate 50µg/ml; sodium benzylpenicillin 200 units/ml; phenol red 0,01mg/ml and human serum albumin 20mg/ml) to the main compartment. Cell counts were performed on each suspension. The side arms were filled with 0,25ml of a solution containing 0,067M radioactive glucose,either (4-^{14}C)glucose (0,143mCi/mmole), (1-^{14}C)glucose (0,143mCi/mmole) or (6-^{14}C)glucose (0,149mCi/mmole) and 0,25ml of either TBS or TBS containing approximately 1 mg of SNF-protein. The centre wells contained fluted filter paper strips spotted with 20% w/v KOH to trap ^{14}C-labelled CO_2. After equilibration at 37°C in air, the stopcocks were closed and the contents of the side arms were tipped into the main compartments to give a final glucose concentration of 10,18mM (1,84mg/ml). Incubation was terminated at the end of 60 min and the filter paper flutes were removed for radioassay by adding them to vials containing Unogel[1] for counting on a liquid scintillation spectrometer.[1] Measured aliquots of radioactive substrates were counted in the same manner. Counts per minute were corrected to disintegrations per minute by recounting the vials after the addition

(1) Packard Instrument Company Incorporated, Downers Grove, Illinois, U.S.A.

of standardized (n-^{14}C)hexadecane.[1] Conversion of labelled glucose to CO_2 was calculated and expressed as nmoles of glucose converted to CO_2 per 10^7 cells. A control flask contained radioactive substrate without cells. Medium pH and cell viability were determined before and after incubation.

Results and Discussion

Oxygen consumption

The results of two experiments in which the effect of cytotaxin on oxygen consumption by neutrophils was studied by Warburg respirometry are summarized diagrammatically in Figure 3.12. The raw data from which these graphs were drawn is represented in tabular form in the Appendix. Each line represents the average value of three flasks. No significant effect of cytotaxin on oxygen consumption was evident from the experiments.

Seven further experiments were performed in which oxygen consumption was measured manometrically over a period of 1h. The results of these experiments are presented in Table 3.4 from which it can be seen that respiratory activity varied widely between experiments. The ratio of oxygen consumed by cytotaxin-stimulated cells : control cells for each individual experiment varied from 0,97 to 1,14 with a mean of 1,04. This was clearly not significantly different from 1,0 indicating, once more, that stimulation by cytotaxin had no effect upon oxygen consumption.

Electrometric measurement of oxygen uptake gave essentially

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(1) Radiochemical Centre, Amersham, Buckinghamshire, England.

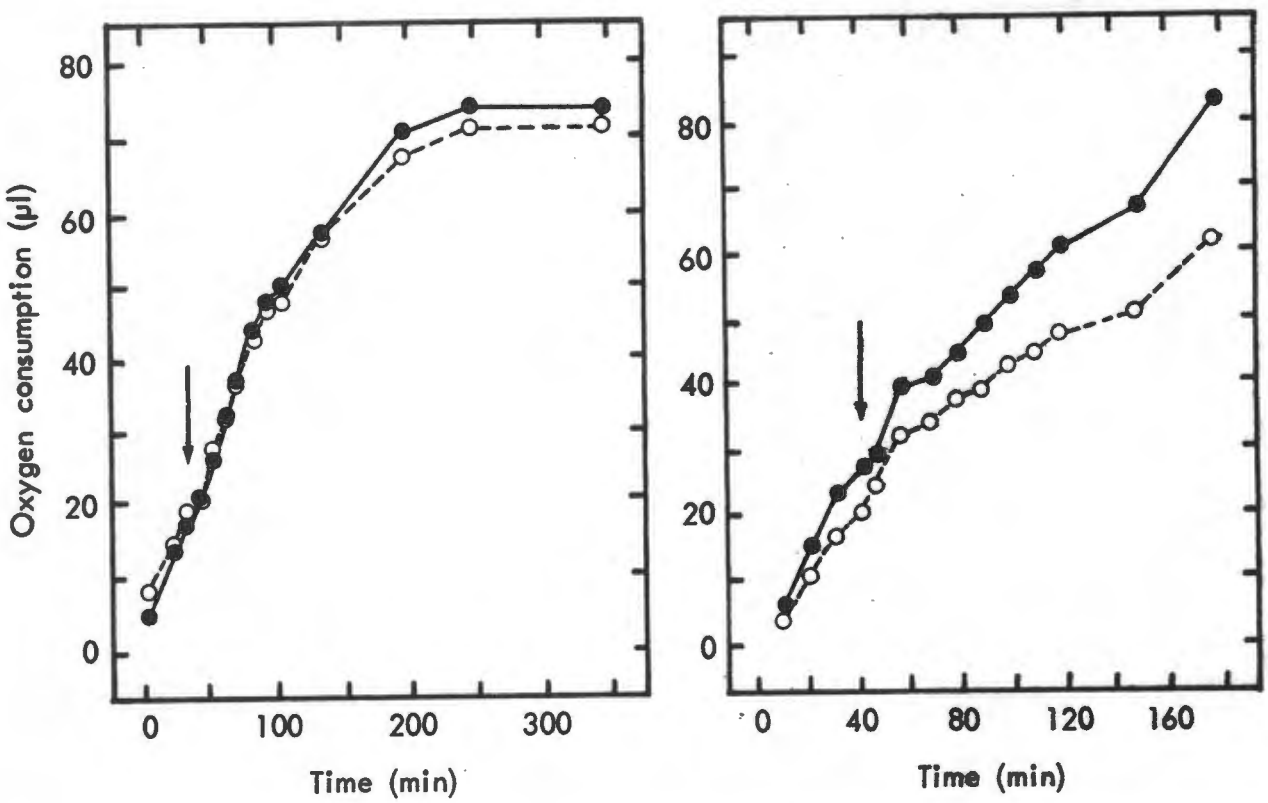

Figure 3.12 Effect of cytotaxin on oxygen uptake by neutrophils incubated *in vitro*.

The diagrams depict the results of two separate experiments in which $1,21x10^8$ neutrophils (*left*) and $1,49x10^8$ neutrophils (*right*) were incubated at 37°C in Warburg flasks in 2,7ml of Gey's BSS (without bicarbonate) containing 20mg/ml HSA and adjusted to pH 7,2 with 0,03M Na_2HPO_4. The centre wells contained filter paper flutes spotted with 20% KOH. Cytotaxin (SNF; 0,5ml; 2mg protein) or TBS (0,5ml) was tipped from the side arm at the times indicated by the arrows. Oxygen consumption was measured by the interval uptake method (161) and was plotted cumulatively as a function of time.

Note that cells receiving cytotaxin (●—●) respired at the same rate as control cells receiving TBS (o---o).

Table 3.4

Effect of cytotaxin on leucocyte oxygen consumption

Expt no.	Cells ($x10^6$)	Oxygen consumption ($\mu l/10^9$ cells/min)								Ratio SNF/Control
		1	2	3	Mean	1	2	3	Mean	
1	125	2,55	2,52	-	2,54	2,86	2,56	-	2,71	1,07
2	95	6,44	-	-	6,44	6,65	6,12	-	6,39	0,99
3	90	3,53	2,82	2,57	2,97	3,50	3,74	2,62	3,29	1,11
4	109	7,70	7,47	8,76	7,98	7,52	8,30	8,39	8,07	1,01
5	105	8,39	7,42	7,91	7,91	7,80	8,43	7,31	7,85	0,99
6	143	7,35	7,56	8,02	7,64	9,61	7,93	8,54	8,69	1,14
7	109	7,18	6,56	8,50	7,41	6,90	6,51	8,22	7,21	0,97
				Mean	6,13				Mean 6,32	Mean 1,04

Oxygen consumption was measured by Warburg manometry over a period of 60 min. The cells were suspended in 3,0ml of phosphate buffered Gey's BSS-2% HSA containing TBS (control) or 1,0mg SNF-protein in TBS (test).

Table 3.5

Oxygen consumption (µl $O_2/10^9$ cells/min)

Expt no.	Rabbit	Control (TBS treated) cells			SNF treated cells		
		Before	After	Ratio	Before	After	Ratio
1	258	-	5,95	-	-	5,95	-
2	263	7,68	8,98	1,17	9,02	9,76	1,08
3	258	7,05	6,52	0,92	-	-	-
		6,32	5,34	0,84	5,69	5,61	0,99
4	282	N.D.	5,30	-	5,52	5,07	0,92

Oxygen consumption in control (TBS treated) or cytotaxin-stimulated (0.7 mg SNF-protein in TBS) rabbit peritoneal exudate cells suspended in phosphate buffered Gey's BSS containing 2% w/v HSA. The oxygen concentration in a fixed volume of cell suspension was monitored with time by an oxygen electrode and recorded. The slope of the tracing recorded was used to calculate the oxygen uptake of the cells.

similar results. These are presented in Table 3.5. Whichever experimental protocol was followed, no consistent effect of adding cytotaxin upon oxygen uptake could be detected.

In the straightforward experiment in which oxygen consumption was measured in TBS or TBS containing SNF-protein pretreated cell populations, identical oxygen consumption rates were observed (Table 3.5; Experiment 1). In the experiments in which TBS or TBS containing SNF-protein were added to cells where oxygen consumption rates had been measured for a period of time before the addition, the effects of these additions were measured. The oxygen consumption rates after adding TBS containing SNF-protein varied from 0,92 to 1,08 of those before the addition while the corresponding ratios after adding TBS alone varied from 0,84 to 1,12 (Table 3.5; Experiments 2 to 4).

These results are in accord with those showing negligible effects of inhibitors of oxidative metabolism on chemotaxis and lend support to the belief that the effects of cytotaxin do not involve major demands upon aerobic energy sources.

Conversion of (^{14}C)glucose to $^{14}CO_2$

Ten experiments were performed in which the amount of radioactive glucose converted to carbon dioxide in one hour was measured in the presence and absence of SNF-protein. The results, presented in Table 3.6, sustain the following general observations and conclusions.

(i) Approximately 5 nmoles of uniformly labelled glucose carbon were converted, on average, to CO_2 by 10^7 cells in one hour. This was clearly a weighted rather than a uniform average, since simultaneous estimates of CO_2 derived from the first

and sixth carbon atoms of the glucose molecules showed that approximately 12 atoms of C-1, but only approximately 1,3 atoms of C-6, were oxidized to CO_2.

At the present time three major pathways of glucose metabolism are generally recognized. These are glycolysis, the pentose phosphate pathway and the 2-oxy-3-deoxy-6-phosphogluconate cleavage pathway. Only the first two need concern us here since the latter pathway has been found only in micro-organisms.

In terms of the rate at which $^{14}CO_2$ is released from radioactive glucose, carbon atoms 1 and 6 are equivalent by the glycolytic route since they both become the methyl carbon of pyruvate. These carbon atoms are not equivalent when metabolism proceeds via the pentose phosphate cycle since there is a preferential loss of C-1 as CO_2 when glucose-6-phosphate is oxidatively decarboxylated via 6-phosphogluconate; C-6 cannot yield CO_2 until it has traversed the tricarboxylic acid cycle twice or it has appeared in the C-1 position of hexose reformed from two molecules of glyceraldehyde-3-phosphate by aldol condensation or it has entered glycolysis by way of aldo- and ketotriose phosphate interconversions catalysed by D-glyceraldehyde-3-P-ketol isomerase.

Although there are many practical and theoretical obstacles to setting too much store by the disproportionate oxidation of glucose-1-C to carbon dioxide, the ratio

$$\frac{\text{glucose-1-C} \rightarrow CO_2}{\text{glucose-6-C} \rightarrow CO_2}$$

(C1/C6 ratio) does provide an indication of the extent to which the pentose phosphate cycle

is active in living cells (see Axelrod (9) for a review). In these experiments I found a C1/C6 ratio of approximately 12 in control neutrophils. Others who have studied the metabolism of neutrophils have similarly found evidence for an active pentose phosphate cycle in these cells (49,138,139,192).

(ii) In any individual experiment, neutrophils incubated in the presence of cytotaxin invariably converted more (1-^{14}C)glucose to CO_2 than did control cells incubated in the presence of buffer alone. This is shown in Table 3.6, by a positive value in the column showing differences in the amount of (1-^{14}C)glucose converted to CO_2 between SNF-treated and control cells. The mean difference of 2,72 was significantly different from zero at less than the 0,5% level.

A similar effect of cytotaxin on (6-^{14}C)glucose oxidation was not seen. The net effect of cytotaxin, therefore, was to increase the C1/C6 ratio and hence, by inference, the activity of the pentose phosphate pathway.

(iii) The SNF-cytotaxin preparations had no effect upon cell viability or upon the final pH of the incubation medium. The latter observation may be taken as indicating that lactic acid production, and hence glycolysis, was not unduly stimulated by cytotaxin.

Although a stimulatory effect of cytotaxin upon pentose phosphate cycle activity seems highly probable, the biochemical relevance of this effect to increased intrinsic cellular

Table 3.6

Effect of cytotaxin (SNF) on conversion of glucose to CO_2 by rabbit polymorphonuclear leucocytes*

Expt no.	(U-^{14}C)Glucose→ $^{14}CO_2$			(1-^{14}C)Glucose→ $^{14}CO_2$			(6-^{14}C)Glucose→ $^{14}CO_2$			C1/C6 Ratio		
	SNF	TBS	Δ†	SNF	TBS	Δ†	SNF	TBS	Δ†	SNF	TBS	Δ†
1	N.D.	N.D.	-	13,97	9,39	4,58	0,76	0,75	0,01	18,38	12,52	5,86
2	5,36	3,60	1,76	11,11	7,93	3,18	0,67	0,61	0,06	16,58	13,00	3,58
3	5,03	4,67	0,36	11,88	10,20	1,68	1,25	1,23	0,02	9,50	8,29	1,21
4	9,72	7,43	2,29	25,65	20,72	4,93	2,74	2,33	0,41	9,36	8,89	0,47
5	4,19	3,71	0,48	12,44	11,71	0,73	0,46	0,43	0,03	27,04	27,23	-0,19
6	6,98	6,52	0,46	19,95	15,13	4,82	2,00	1,95	0,05	9,98	7,76	2,22
7	2,02	2,88	-0,86	8,23	6,83	1,40	0,39	0,47	-0,08	21,10	14,53	6,57
8	7,07	7,57	-0,50	19,12	15,50	3,62	2,56	2,48	0,08	7,47	6,25	1,22
9	4,11	3,70	0,41	13,53	11,64	1,89	N.D.	N.D.	-	N.D.	N.D.	-
10	3,90	3,90	0,00	10,26	9,88	0,38	1,09	0,99	0,10	9,41	9,98	-0,57
Mean	5,38	4,89	0,49	14,61	11,89	2,72	1,32	1,25	0,08	14,31	12,05	2,26
p			N.S.			<0,005			N.S.			<0,025

*Values are given as nmoles glucose converted to CO_2 per hour by 10^7 cells.

†Δ= difference between cytotaxin-treated (SNF) and control (TBS) cells.

motility is entirely obscure. This metabolic pathway, in addition to providing substrates for oxidative aerobic energy metabolism, also yields important intermediates and co-factors for cellular biosynthesis (9). The products of these synthetic processes may play an important role in the control of cell movement and hence in the ability of neutrophils to respond to chemotactic substances. Sbarra and Karnovsky (139) have shown that pentose phosphate cycle activity was increased during phagocytosis by neutrophils and that this was associated with increased incorporation of radioactive precursors into cellular lipid.

I am encouraged by the fact that, to the best of my knowledge, the reproducible demonstration of increased C1/C6 ratios on exposure of neutrophils to cytotaxin, represents the first clearly defined metabolic change associated with the phenomenon of chemotaxis. Hopefully it will provide the starting point for a fruitful area of future research.

Intracellular Adenosine 5' - triphosphate.

Since, as is generally believed to be the case, one of the principal effects of chemotactic substances is to stimulate random cell movement, it seemed reasonable to investigate the effects of a known cytotaxin on the synthesis and accumulation of intracellular ATP. This compound is well known for its role as a source of chemical energy that is immediately available for a variety of cellular functions and I therefore felt it appropriate to study the effects of SNF-cytotaxin on its synthesis from radioactive adenine as a function of time.

For my experimental approach to this problem, I used the knowledge that one can readily achieve extremely satisfactory separation of adenosine 5'-triphosphate (ATP), adenosine 5'-diphosphate (ADP), adenosine 5'-monophosphate (AMP) and adenine by chromatography on cellulose anion exchange paper. This can be seen from Figure 3.13 which shows a typical radiochromatogram scan of a mixture of (^{14}C)labelled ATP, ADP, AMP and adenine resolved by ascending chromatography on Whatman's DE-81 paper with 0,25M triethylamine in 16% acetic acid pH 3,5.

Neutrophil cell membranes are, moreover, readily permeable to (^{14}C)adenine and this base is rapidly incorporated into intracellular adenine nucleotides.

I therefore decided to incubate neutrophils in the presence of ^{14}C-labelled adenine and in the presence or absence of SNF-protein. At varying times after the start of incubation the cells were washed and treated with ice cold perchloric acid. Radioactive adenine nucleotides were isolated from the protein-free cell lysates and separated by chromatography for quantitation. Tritium-labelled ATP was added with the perchloric acid to correct for losses of the ^{14}C-nucleotide during isolation.

Using this procedure I was able to obtain satisfactory data in three experiments defining the accumulation of (^{14}C)ATP as a function of time in cytotaxin-treated and control neutrophils. No effect of cytotaxin could be demonstrated.

Materials and Methods

Rabbit peritoneal cells were harvested as described in the appendix and contaminating erythrocytes were lysed by brief hypotonic

Figure 3.13

Figure 3.13 Radiochromatography of adenine compounds

$6,7x10^7$ peritoneal exudate cells were incubated for 90 min at 37°C in 1,0ml of Gey's BSS containing 2% HSA and 1µCi of (8-^{14}C) adenine. After incubation, the washed cells were extracted with perchloric acid-EDTA as described in the text. A 25µl sample of the extract (representing $5,2x10^6$ cells) was analysed by ascending ion-exchange chromatography on Whatman DE-81 paper with 0,25M triethylamine in 16% acetic acid pH 3,5 for 15h at room temperature.

The dried chromatograms were then scanned for radioactivity using a radioactive chromatograph scanner[1] to obtain the tracing depicted opposite.

Radioactive peaks were identified as adenine, AMP, ADP and ATP by co-chromatography of unlabelled, pure compounds. The markers were identified under ultraviolet illumination as quenched spots against the pale blue, autofluorescent background of the filter paper.

ATP could be satisfactorily separated from the other compounds by this procedure.

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(1) Actigraph Model 1006; Nuclear Chicago, Des Plaines, U.S.A.

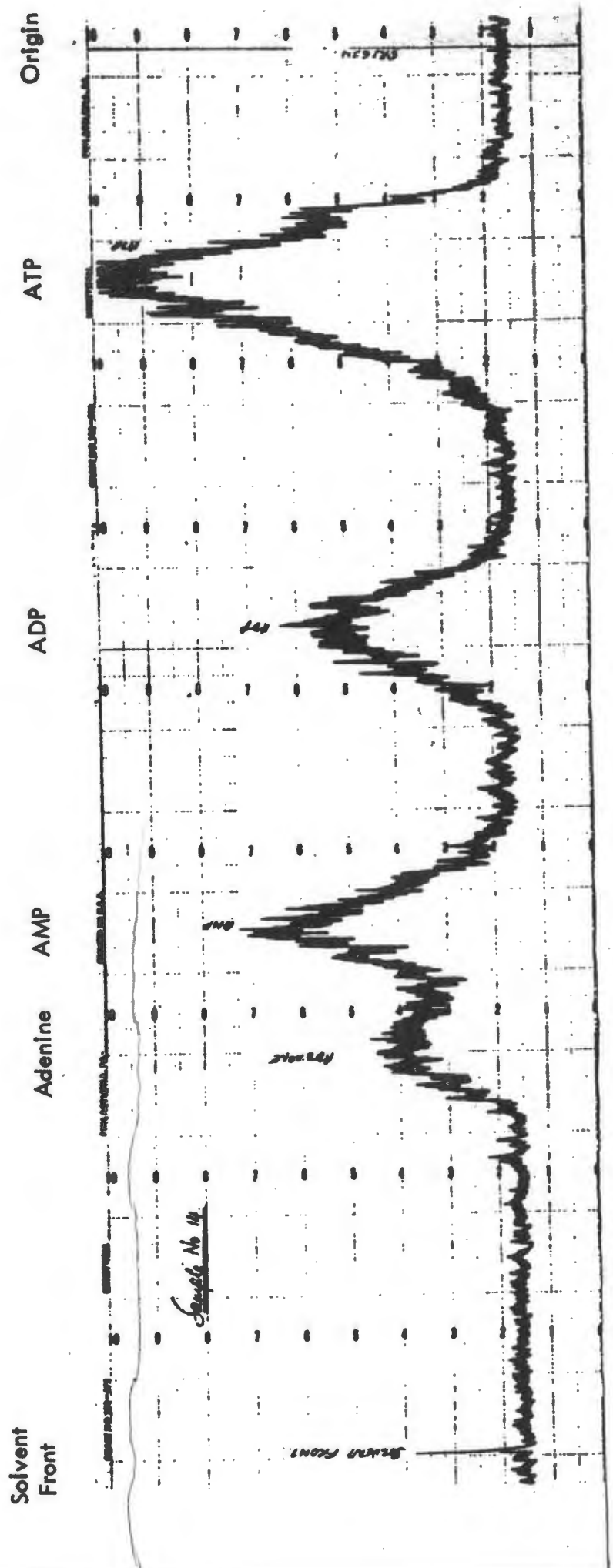

Figure 3.13

shock. The cells were then washed free of cellular debris, resuspended in Gey's BSS-2% HSA and counted.

In the first experiment, the cell suspension was adjusted to 44×10^6 cells/ml. Nine millilitres of this suspension were added to each of two sterile vials containing 90µCi of purified (8-^{14}C)adenine. One of the flasks contained a predetermined stimulatory amount of SNF-cytotaxin dissolved in 0,9ml of TBS; the control flask contained 0,9ml of TBS alone. The flasks were then incubated with gentle shaking at 37°C in a water-bath and accurately measured 1,0ml samples of well dispersed cell suspensions were removed for measurement of intracellular (^{14}C)ATP at the time intervals indicated in Figure 3.14*(left)*. This bulk method of incubation suffered from the disadvantage that cells tended to clump, necessitating vigorous dispersal before removal of each aliquot. To avoid this complication the following procedure was adopted in the subsequent two experiments. Purified (8-^{14}C) adenine (approximately 1µCi/ml cells) was added to the total cell suspension prepared as before but containing 67×10^6 cells/ml and 78×10^6 cells for experiments 1 and 2 respectively. This radioactive suspension was then divided into two portions, one of which received a stimulatory amount of SNF-protein dissolved in TBS while the other received an equivalent volume of buffer alone. One millilitre volumes of these two suspensions were then accurately pipetted into a series of sterile plastic tubes(1) which were sealed and incubated at 37°C with shaking. Individual tubes were removed at various intervals of time (Figure 3.14*(middle and right)*).

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(1) Catalogue number 2054; Falcon Plastics, Los Angeles, California, U.S.A.

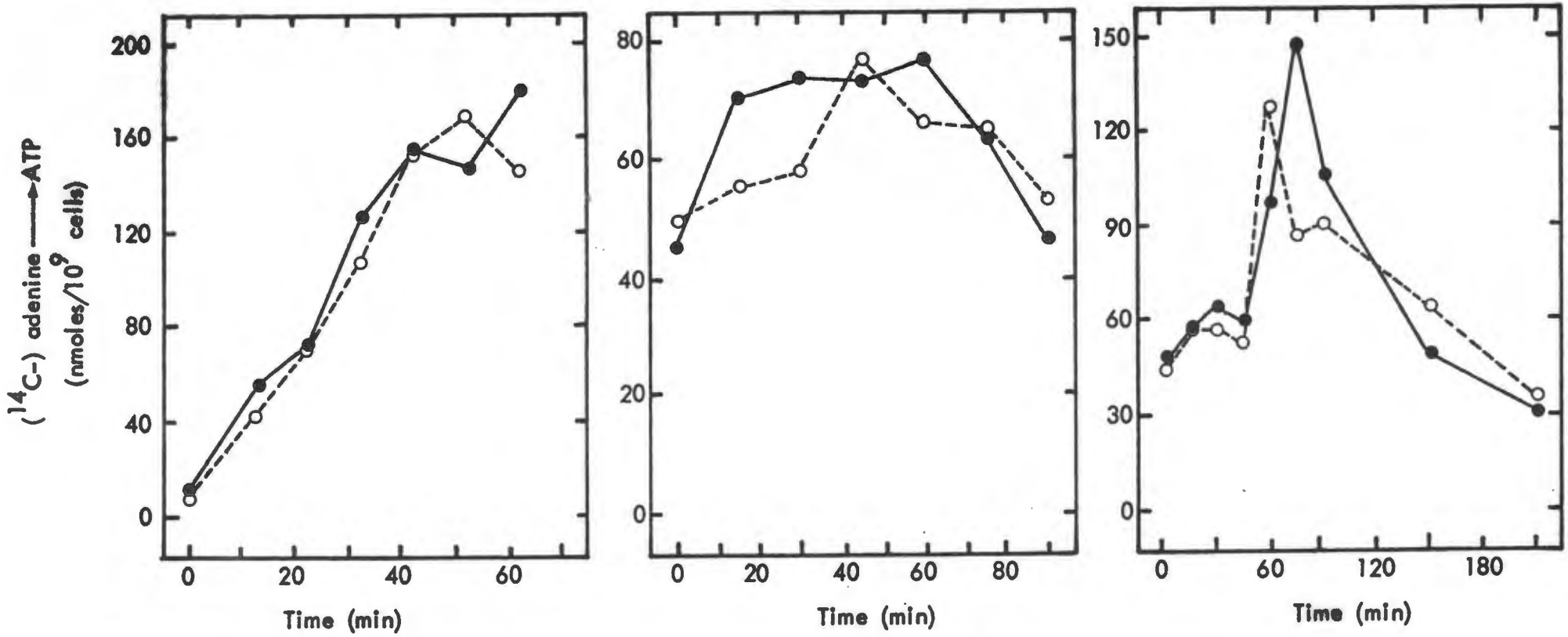

Figure 3.14 Effect of cytotaxin on incorporation of (8-^{14}C) adenine into ATP by neutrophils incubated *in vitro*.

Results of three separate experiments in which neutrophils were incubated in bulk (*left*) or in separate 1 ml volume aliquots (*middle and right*) in the presence of (8-^{14}C) adenine, with (o---o) or without (●——●) cytotaxin (SNF). At the times indicated, cell suspensions ($44x10^6$ cells/ml *left*; $67x10^6$ cells/ml *middle* and $78x10^6$ cells/ml *right*) were harvested and their contents of ATP-^{14}C assayed.

For experimental details see text.

Note that there was no significant effect of stimulation with cytotaxin upon incorporation of (^{14}C) adenine into ATP.

In both procedures incubation was terminated by centrifuging the aliquots at 300xg for 15 min at 4°C. The supernatant fluid was decanted and the cell pellet washed once with cold 0,9% NaCl (1 ml) to remove excess free (^{14}C)adenine. Five hundred microlitres of 20% w/v perchloric acid containing 0,05M disodium EDTA, $1x10^{-3}$M "cold" carrier ATP and $94,6x10^{3}$ cpm of (^{3}H)ATP were added to each pellet. The tube contents were mixed thoroughly on a vortex and kept on ice for at least 60 min to ensure complete precipitation of protein.

The precipitated protein was removed by centrifugation (2 200xg; 30 min; 4°C) and the supernatant fluid, containing the nucleotides, was decanted and kept on chipped ice.

The perchloric acid was removed by titration of the supernatant fluid to pH 7,4 with 20% w/v KOH containing disodium EDTA. Precipitated potassium perchlorate was removed by centrifugation (1 100xg; 20 min; 4°C) and the supernatant fluid was either stored overnight at 4°C or used immediately.

Whatman's DE-81 cellulose anion exchange chromatography paper(1) was cut into 50x26 cm sheets and 25 1 of solution of "cold" ATP (0,02M), ADP (0,02M), AMP (0,02M) and adenine (0,02M) was added to each spot to provide sufficient carrier nucleotides for easy detection following chromatography.

The chromatograms were developed according to the method of Gevers, Kleinkauf and Lipmann (55) for approximately 16h at room temperature by ascending chromatography with 0,25M triethylanine in 16% acetic acid; pH 3,5 and dried in air.

The separated nucleotides were identified under ultra-violet

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(1) W & R Balston Ltd., Kent, England

illumination as dark spots against a pale blue fluorescent background. An area of the developed chromatogram measuring 2x3,6cm was cut from the centre of the ATP spot and placed in a counting vial containing 10ml of Unogel for simultaneous radioassay of ^{3}H and ^{14}C by dual channel counting on a liquid scintillation spectrometer. The gain and discriminator levels were set so that there was negligible spill-over of tritium counts from the ^{3}H channel into the ^{14}C channel. When ^{14}C was counted alone a reproducible 52% of the count rate recorded in the ^{14}C channel was detected in the ^{3}H channel. Thus, when two isotopes were counted simultaneously, tritium counts were corrected by subtracting 52% of the counts in the ^{14}C channel. Recoveries were based upon count rates of measured volumes of tritiated-ATP counted under identical conditions.

The efficiency with which ^{14}C-adenine was measured with this technique was determined by counting an accurate volume of the adenine solution that had been dried on a 2x3,6cm DE-81 cellulose strip. The absolute activity present on the strip was found by counting the same volume of the ^{14}C-adenine solution in 10 ml of Unogel and recounting after addition of standardized (n-^{14}C)hexadecane as an internal standard. With the machine settings used, (^{14}C)adenine was counted with an efficiency of 25,9% in the ^{14}C channel. This value was used to convert cpm to dpm. The specific activity given by the supplier[1] was 42µCi/µmole. Chromatography revealed the presence of trace amounts of radiochemical impurities. The adenine was therefore purified by adding approximately 250µCi in aqueous solution, to a 6x1,1 cm column of AG 50W-X8 (200 - 400 mesh)

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(1) Schwarz Mann, Orangeburg, New York, U.S.A.

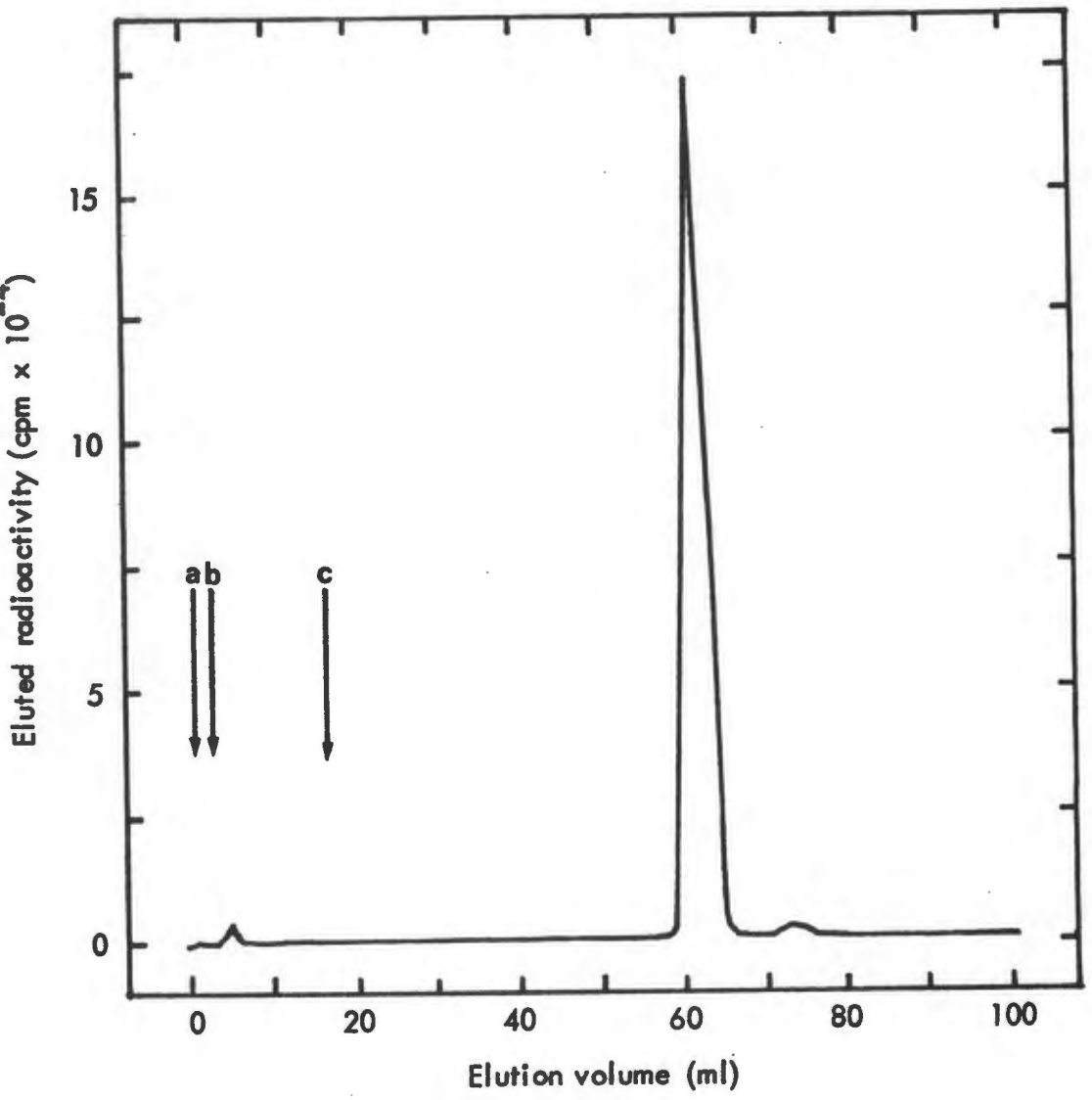

Figure 3.15 Purification of (8-^{14}C) adenine

Elution profile of radioactivity from a 6x1,1cm column of AG50W-X8 cation exchange resin equilibrated with water. Sample introduced at a in 2,5ml. Distilled water (14,4ml) started at b. (8-^{14}C) adenine eluted as a single peak of radioactivity after 45,5ml of 0,1M NH_4OH (started at c) had passed through the column.

Note that this procedure removed two small contaminating peaks of radioactivity.

cation exchange resin[1] in the H^+ form. The column was washed with distilled water (approximately 15 ml) until no further radioactivity could be detected in the effluent. The adenine was eluted as a sharp, single, radiochemically pure peak with 0,1M NH_4OH. A typical elution profile is shown in Figure 3.15. The pooled column fractions containing the (^{14}C)adenine were lyophilized. The white residue was dissolved in 2,5ml distilled water for use. This final solution contained 1,07µCi of ^{14}C in 10µl.

Results and Discussion

The results from the three experiments described above are shown diagrammatically in Figure 3.14. In all cases the cells showed rapid incorporation of (^{14}C)adenine into intracellular ATP. In the first experiment (Figure 3.14 *left*) this was approximately linear with time for 60 min. Although dissimilar in shape, the patterns of incorporation of radioactivity into intracellular ATP in the other two experiments conducted for longer times, showed the same overall tendancy to an initial rise with subsequent fall. In no case could a significant effect of cytotaxin be demonstrated with this procedure.

Unfortunately a negative experiment of this sort does not allow any definite conclusions regarding the participation of ATP as a source of chemical energy for cytotaxin-induced motility. Not only might the method have been insufficiently sensitive to detect minor changes in the size of the intracellular ATP pool, but the rate of synthesis and degradation of the terminal phosphate bonds were clearly not measurable with this approach.

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(1) Biorad Laboratories, Richmond, California, U.S.A.

Intracellular Adenosine 3':5'-cyclic-phosphate

The simultaneous discovery of cyclic adenosine monophosphate (cAMP) by Sutherland and Rall (151) and Cook, Lipkin and Markham (38) in 1957 provoked a spate of research with publications increasing at an exponential rate over the following decade. All of this work has testified to the validity of the "second messenger" hypothesis put forward by Sutherland, Øye and Butcher (152b) in which it was suggested that this cyclic nucleotide served as an intracellular mediator for a variety of hormones and other biologically active compounds. The ubiquitous presence of cAMP and of adenyl cyclase, the membrane bound enzyme that generates cAMP from ATP, in all living cells has been well established and reviewed (71,98,114,125,133,152a). The diversity of biological effects that are regulated by mechanisms involving the mediation of cAMP has been so well documented that one is virtually compelled to investigate the possibility that cAMP may play some role in *any* cellular response elicited by a biologically active substance. In the case of chemotaxis, the grounds for investigating participation of the adenyl cyclase - cAMP system seemed reasonable. Neutrophils possess glycogen granules (Figure 3.6) and the regulatory role of cAMP in stimulating glycogenolysis to mobilize potential reserves of substrate for glycolysis and energy is well known (163). Furthermore, the intimate association of adenyl cyclase with cell membranes made this enzyme an intuitively appealing mediator for the consequences of interaction between cytotaxin and putative membrane receptors.

I therefore performed a series of experiments designed to study the effects of SNF-protein upon the rate of incorporation of (^{14}C)adenine into intracellular cAMP and on intracellular levels of cyclic nucleotide.

In both cases, neutrophils were incubated in the presence or

absence of cytotaxin and sampled after various intervals of time. The rate of (^{14}C)adenine incorporation into cAMP in the presence of the phosphodiesterase inhibitor, theophylline, provided a measure of adenyl cyclase activity while cellular levels of cAMP gave an indication of the pool size, and hence the overall cellular economy, of the cyclic nucleotide.

Although ATP is the immediate substrate for adenyl cyclase action, limited cellular permeability for this compound made labelled adenine a more suitable substrate for the radioactive assay of the enzyme in living cells. The (^{14}C)adenine assay required isolation of pure cAMP from the mixture of labelled nucleotides in the protein-free cell lysate after incubation. This was achieved by column ion-exchange chromatography, chromatography on alumina and selective removal of contaminating nucleotides by adsorption with nascent $BaSO_4$ and $Zn(OH)_2$. Tritium-labelled cAMP was used to correct for losses during the isolation procedure.

Cellular cAMP concentrations were determined by lysing the cells and measuring the cAMP in the protein-free lysate with a competitive protein binding assay.

Satisfactory data were obtained from a total of 4 experiments to indicate the stimulation of living neutrophils with cytotaxin had no effect upon the activity of adenyl cyclase nor upon intracellular concentrations of cAMP.

Materials and Methods

Rabbit neutrophils were harvested from caseinate-induced peritoneal exudates as described in the Appendix. The cells were collected by centrifugation (300xg; 4°C; 10min) and resuspended in Gey's BSS-2% HSA at a final concentration of $5x10^7$ cells/ml.

(a) The method used to measure the incorporation of (^{14}C)adenine into cellular cAMP was based upon procedures described by Humes, Rounbehler and Kuehl (67) and White and Zenser (183).

Ten to twenty ml of cell suspension (depending upon the cell yield) prepared as above was added to each of two 25 ml sterile conical flasks containing approximately 10 to 20µCi of purified (8-^{14}C)adenine(1) (specific activity 42µCi/µmole); and sufficient theophylline(1) to give a final concentration of 1mM. To one of the flasks I added SNF-cytotaxin dissolved in TBS to give a concentration of cytotaxin known to be stimulatory. The conical flasks received an equivalent volume of TBS alone.

The flasks were then incubated at 37°C with shaking. At varying times after the start of incubation, aliquots of the well dispersed cell suspension were removed and added to an equal volume of ice-cold 0,6N perchloric acid. A known amount of (^{3}H)cAMP(1) (specific activity 12,8Ci/mmole); was added immediately after the cell suspension to measure subsequent recoveries of (^{14}C)cAMP. After thorough mixing the tubes

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(1) Schwarz Mann, Orangeburg, New York, U.S.A.

were left for 1h at 4°C for complete protein precipitation. The protein precipitate was removed by centrifugation (1 100xg; 15min; 4°C) and washed once with 1,0 ml of 0,6N perchloric acid. The supernatants and wash fluids were pooled and added to 7x0,7cm columns of AG50W - X8 cation exchange resin in the H+ form equilibarated with distilled water.

The columns were eluted with distilled water and fractions of approximately 2ml volume were collected. A measured aliquot (usually 0,2ml) of each fraction was added to vials containing 6ml Unogel for radioassay in a tricarb liquid scintillation spectrometer. Gain and discriminator levels were set for negligible spillover of ^{3}H-channel counts into the ^{14}C-channel; 39,6% of the ^{14}C-channel counts were detected in the ^{3}H-channel. A typical elution profile is shown in Figure 3.16. Adenosine triphosphate (ATP) and adenosine diphosphate (ADP) eluted first followed by cyclic-3':5'-adenosine monophosphate (cAMP) and finally adenosine monophosphate (AMP; not shown). The major fraction of free adenine and thophylline remained bound to the column.

Those fractions with high $^{3}H/^{14}C$ ratios (e.g. fractions 6 to 16 in Figure 3.16) were pooled for each column and lyophilized. The dry residue was dissolved in 0,5 to 1,0 ml of 0,05M tris/HCl pH 7,5 buffer and the whole sample was washed onto 7x0,7cm column of aluminium oxide[1] pre-equilibrated with 0,05M tris/HCl

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(1) Research Specialities Company, California, U.S.A.

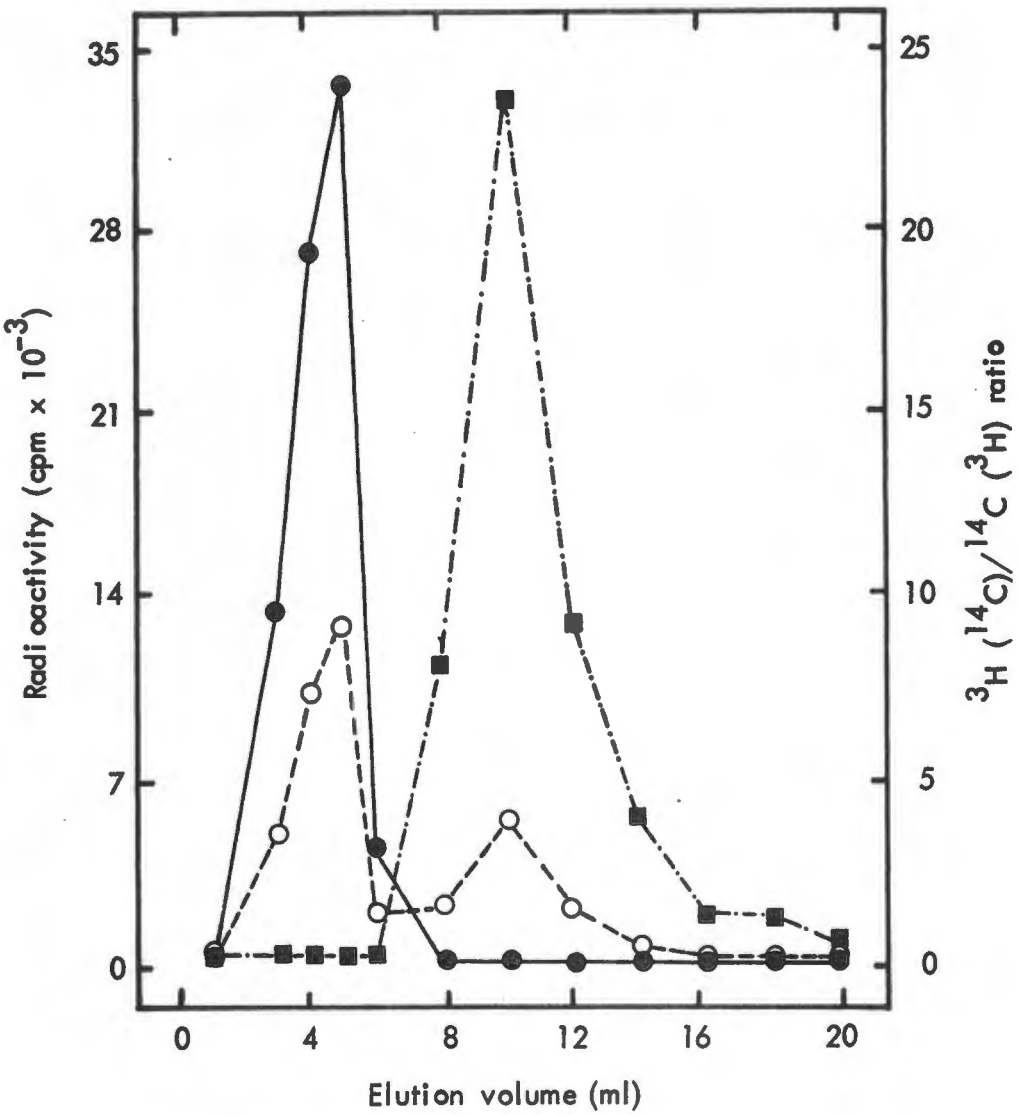

Figure 3.16 Chromatographic separation of cAMP.

Peritoneal cells ($3,7x10^7$) were incubated for 30 min at 37°C in the presence of 1µCi(8-^{14}C) adenine and 10^{-3}M theophyllinein Gey's BSS/2% HSA. After incubation, cellular proteins were precipitated with an equal volume of 0,6N $HClO_4$. A trace amount ($1,1x10^5$ dpm) of (^{3}H)cAMP was added to the acidified extract. The acid-soluble material was chromatographed on a 7x0,7cm column of Biorad Ag50W-X8 cation exchange resin equilibrated and eluted with the distilled water.

Radioactivity in 0,2ml aliquots of each 2ml fraction recorded in the ^{14}C-channel (●—●); the ^{3}H-channel (O--O); and the ratio of ^{3}H-channel cpm/^{14}C-channel cpm (■-·-■); of the liquid scintillation counts are plotted as a function of elution volume.

(^{3}H)cAMP eluted between approximately 6 and 16 ml and was well separated from other components.

Figure 3.17

Figure 3.17 Chromatographic separation of cAMP

Peritoneal cells ($2,6x10^6$/ml) were incubated for 150 min at 37°C in the presence of 1µCi(8-^{14}C)adenine in Gey's BSS/2% HSA. After incubation, 100µl 10^{-4}M theophylline in TBS together with 10µl (8-^{14}C)cAMP ($1,1x10^4$ dpm) was added. Proteins were precipitated by heating at 100°C for 3 min. A trace amount ($5,5x10^4$ dpm) of (^{3}H)cAMP was added to the supernatant fluid which was then chromatographed on a 7x0,7cm column of Biorad Ag50W-X8 cation exchange resin equilibrated and eluted with distilled water. Eluted fractions with ratios of ^{3}H(^{14}C)-channel cpm/^{14}C(^{3}H)-channel cpm greater than 1,0 were pooled and lyophilized.

The residue was reconstituted with 0,5ml of 0,05M tris-HCl buffer pH 7,5 and added to a 3,5g column of aluminium oxide equilibrated and eluted with the same buffer.

Radioactivity in the ^{3}H(^{14}C)-channel (●—●); ^{14}C(^{3}H)-channel (O--O) and the ratio of ^{3}H(^{14}C)/^{14}C(^{3}H) (■·-·■) are plotted as a function of elution volume.

Each point represents the cpm in 0,2ml aliquots of collected fractions.

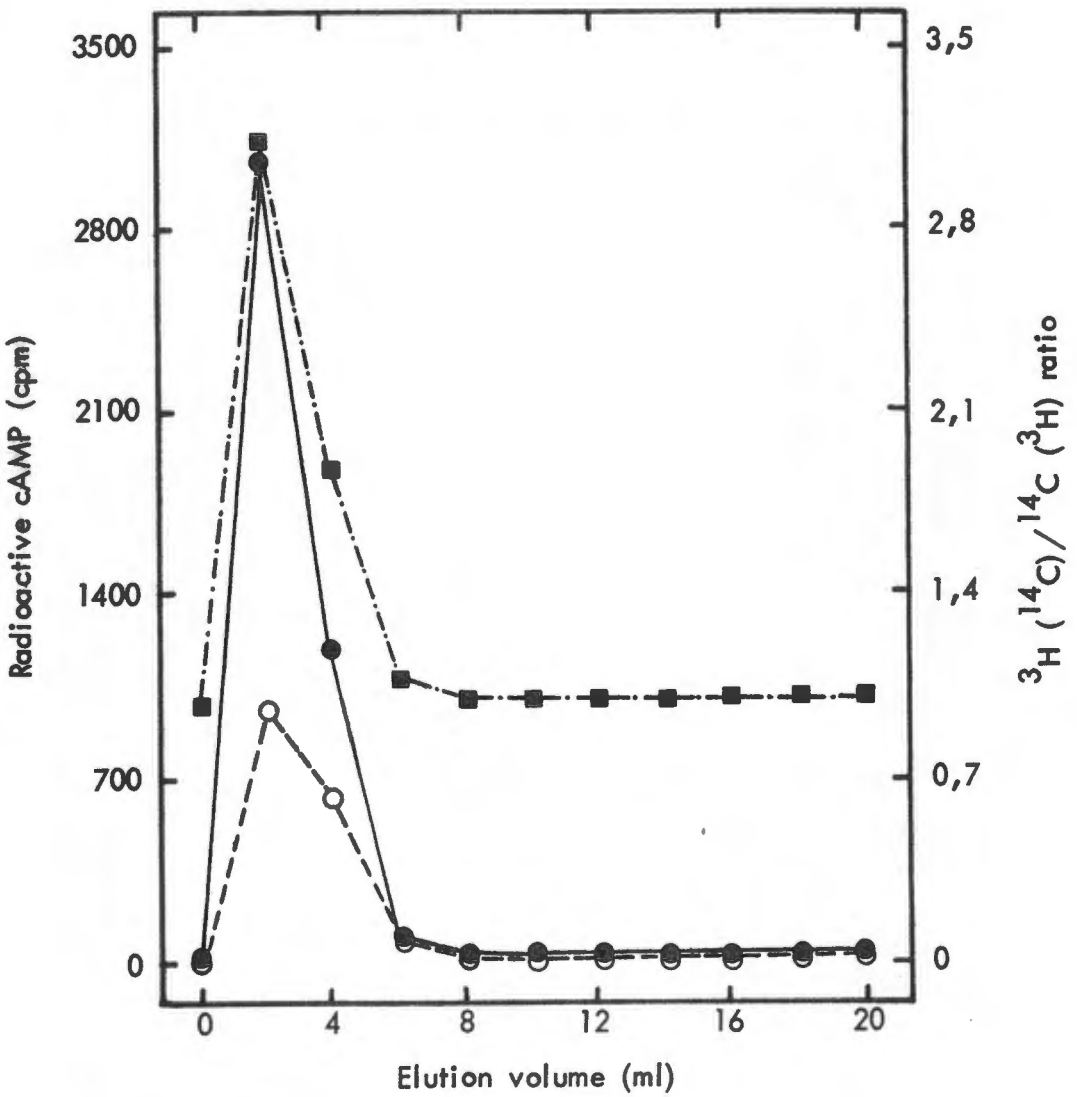
3500
2800
2100
1400
700
0
Radioactive cAMP (cpm)
3,5
2,8
2,1
1,4
0,7
0
^3H (^{14}C)/^{14}C (^3H) ratio
0
4
8
12
16
20
Elution volume (ml)

Figure 3.17

pH 7,5 and eluted with the same buffer. The unbound nucleotide was collected as a 10 ml effluent pool. (The elution profile of a mixture of (^{14}C)cAMP and (^{3}H)cAMP on an aluminium oxide column is shown in Figure 3.17 where all the radioactivity was recovered in the first 7,5 ml). Two samples of 1,0 ml each were taken for liquid scintillation counting of ^{14}C and ^{3}H.

To the remaining sample I then added 0,3N $ZnSO_4$ and the exact volume of approximately 0,3N $Ba(OH)_2$ that had been established, by accurate pre-titration, to be required to neutralize the $ZnSO_4$ to pH 7,6. The 0,3N $ZnSO_4$ was added to the supernate in the ratio of 0,5ml to 10ml. (Since pH control is critical for success in this procedure, the $ZnSO_4$ and $Ba(OH)_2$ solutions were prepared and stored under a nitrogen atmosphere). The flocculent $BaSO_4/Zn(OH)_2$ precipitate, with adsorbed contaminating nucleotides, was separated by centrifugation. The supernatant fluid was decanted into a second tube and the $ZnSO_4/Ba(OH)_2$ adsorption procedure repeated.

One millilitre samples of the twice-adsorbed supernatants were added to scintillation counting vials containing 10ml of Unogel for simultaneous liquid scintillation counting of ^{3}H and ^{14}C as before. Tritium counts were corrected for ^{14}C-spillover and used to correct for losses on the basis of (^{3}H)cAMP added initially and counted under identical conditions.

The results of the three experiments are summarized diagrammatically in Figure 3.18.

(8-^{14}C)Adenine (specific activity 42μCi/μmole) was purified and standardized as described previously. It was used at a final concentration of 0,107μCi/ml.

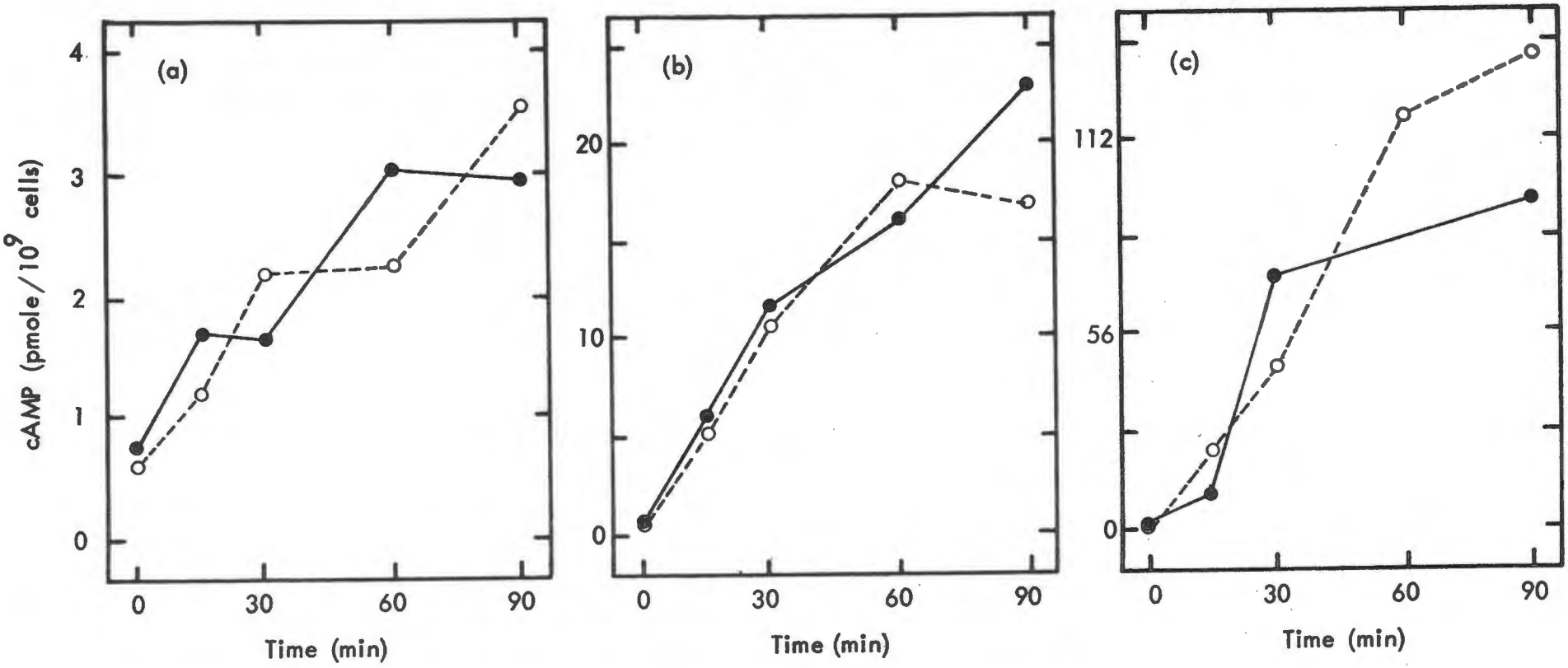

Figure 3.18 Effect of cytotaxin treatment on incorporation of (8-^{14}C)adenine into cyclic AMP.

Results of three experiments in which $6{,}0 \times 10^7$ (a); $2{,}3 \times 10^7$ (b); and $3{,}7 \times 10^7$ (c) cells were incubated in the presence of 1µCi of (8-^{14}C)adenine and 10^{-3}M theophylline with (●—●) or without (○--○) cytotaxin (SNF).

At the times indicated, cells were removed and assayed for their content of (^{14}C)cAMP as described in the text. Cytotaxin treatment had no effect upon the rate of incorporation of (^{14}C)adenine into cAMP.

(b) The effect of cytotaxin on cellular cAMP was measured using a protein binding inhibition assay. Cell suspensions were prepared as described above. For each experiment the suspension was divided into two equal portions. A stimulatory amount of SNF-cytotaxin in TBS was added to one portion. The control portion received an equivalent volume of TBS alone. The suspensions were mixed and each was dispensed in 1,0ml volumes into different sets of plastic tubes. The tubes were then incubated at 37°C with constant shaking and duplicates were removed for cAMP measurement at the times indicated in the results section. Incubation was terminated by plunging the tubes into a dry ice-alcohol bath and lysing the cells by several cycles of freezing and thawing. Protein was precipitated by the addition of 2ml of absolute ethanol to each tube. After 10 min at 4°C, the precipitated protein was separated by centrifugation and washed once with 1,0ml of 66,7% ethanol. Supernatant and wash solutions were combined and taken to dryness at 55°C under a stream of nitrogen. The residue was dissolved in 0,5ml of 0,05M tris buffer pH 7,5 containing 0,16M EDTA and used for the assay.

Control tubes containing Gey's BSS-2% HSA, with or without SNF-cytotaxin, but without cells were incubated and treated identically.

For the cAMP assay I used a commercially available assay kit[1]

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(1) Radiochemical Centre, Amersham, Buckinghamshire, England.

based upon the method of Gilman (56). Briefly, this technique depends upon competition between cAMP in the test sample and added (^{3}H)cAMP, for binding to a protein kinase with high specific affinity for cAMP. Bound and free cyclic nucleotides are separated by adsorption of the latter with coated charcoal. A standard curve is constructed by plotting the ratio of counts bound in the presence of known amounts of added cAMP to counts bound in the absence of added cAMP (C_x/C_o) as a function of the amount of added unlabelled cAMP. As can be seen from Figure 3.19a an excellent linear relationship was observed over the range 1 to 20 pmoles of added cAMP. Concentrations of cAMP in unknown solutions were calculated from the parameters of the least squares regression line fitted to the points of the standard curve.

The results obtained in this experiment are shown in Figures 3.19b and c.

Results

As can be seen in Figure 3.18 there was no significant difference in the rate of incorporation of (8-^{14}C)adenine into cAMP for cytotaxin-treated compared to control, untreated cells in any one cell population. The actual concentration (pmoles/10^9 cells) of labelled cAMP found after a specific period of incubation varied widely from one cell population to the next.

The intracellular concentrations of cAMP found in cytotaxin-treated and untreated peritoneal cells were similar (Figure 3.19c).

The level of cyclic nucleotide remained relatively constant over the entire 120 min assay period. No noticeable increase in the intracellular cAMP concentration was detected after cytotaxin treatment. It should be noted, however, that this assay was not suitable for my purpose as some compound in the SNF-cytotaxin preparation interfered with the binding of free, labelled cAMP to the charcoal adsorbent. As has been shown previously (Chapter II), the low molecular weight cytotaxin present in CUF-3 binds avidly to charcoal at neutral pH and could thus compete with the free nucleotide. After correction for the amount of cAMP present in Gey's BSS-2% HSA containing TBS or SNF-cytotaxin, there was no obvious difference in the cAMP levels of the two cell populations.

Based on these findings, it would appear that the adenyl cyclase-adenosine-3';5'-cyclic monophosphate "second messenger" system is not important in the mechanism of cytotaxin-cell stimulation.

That this nucleotide is present in neutrophils is not surprising in view of the role it plays in the activation of phosphorylase and hence glycogen mobilization. The cyclic nucleotide has been isolated from leucocytes but in very low concentrations by various other workers (22,23,107,141).

The roles of cAMP and adenyl cyclase in the chemotactic response have been studied by various workers in terms of

(a) The effects of cAMP; dibutyryl cAMP, stimulators of adenyl cyclase and inhibitors of phosphodiesterase on the chemotactic response of neutrophils and

(b) the chemotactic activity of solutions of the above mentioned substances.

Stossel, Murad, Mason and Vaughan (148) showed that leucocyte glycogen phosphorylase and glycogen synthetase levels were influenced

Figure 3.19

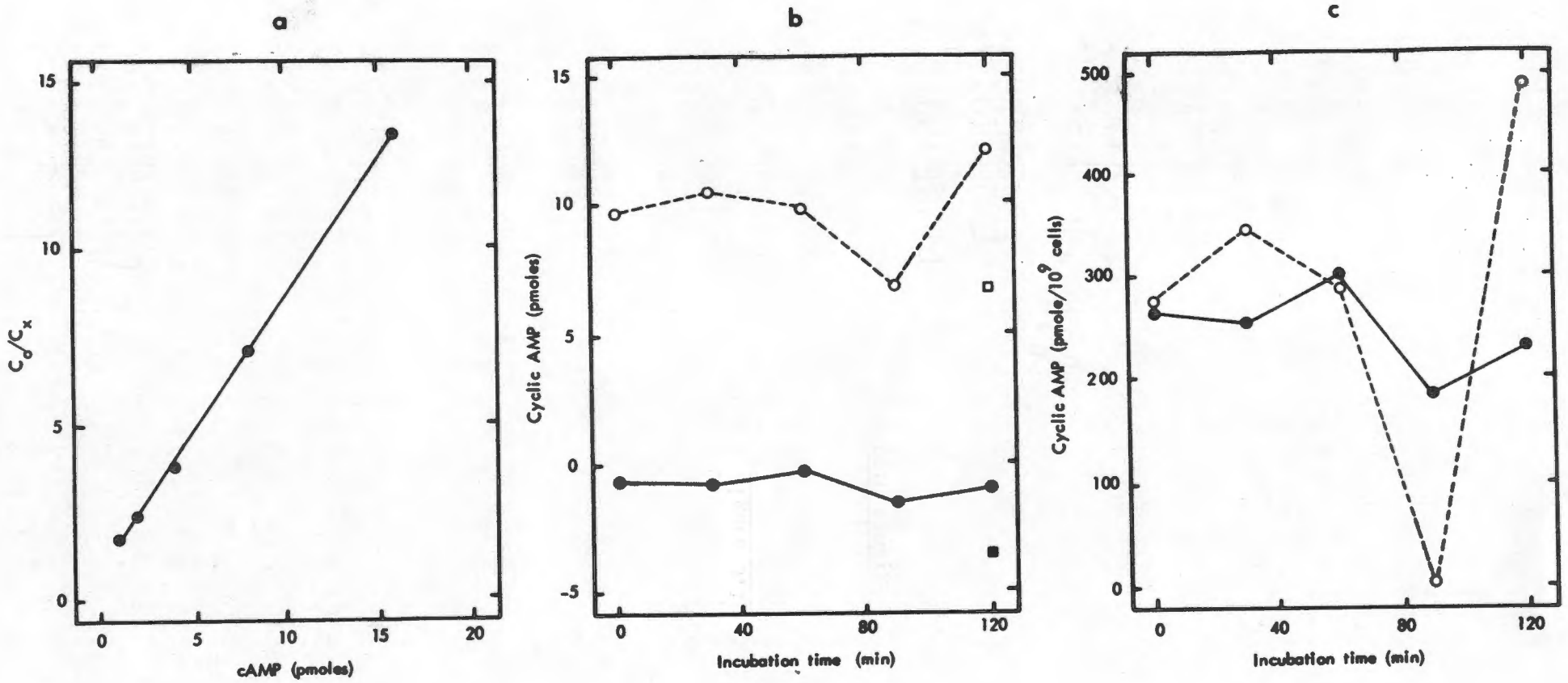
a
b
c
C_o/C_x
cAMP (pmoles)
Cyclic AMP (pmoles)
Incubation time (min)
Cyclic AMP (pmole/10^9 cells)
Incubation time (min)

Figure 3.19

Figure 3.19 Competitive binding assay for cellular cAMP. Effects of cytotaxin (SNF) on assay and cellular concentration.

A commercially available "kit" for cAMP assay was used to construct the standard curve shown in Figure 3.19a. In this system, stable cAMP competes with (^{3}H)cAMP for binding to a bovine muscle cAMP-dependent protein kinase (56). Competition is manifest by the increasing ratio of bound radioactivity in the absence of added stable cAMP (Co) to bound radioactivity in the presence of added stable cAMP(Cx). The standard curve was satisfactorily linear and sensitive when constructed in buffer alone, without added cytotaxin or other components.

When this technique was used to measure amounts of cAMP at various times in incubation mixtures containing $1,1x10^7$ cells with (●—●) or without (○--○) cytotaxin (SNF), cytotaxin-containing incubation mixtures appeared to contain less cAMP than did controls (Figure 3.19b). It can be seen, however, that cytotaxin alone (■) interfered with the assay, giving spuriously high values for "bound" counts. This resulted in anomalous "negative" values for detected cAMP.

When appropriate blanks (■ & □) were subtracted from experimental values erratic results were obtained that showed no significant effect of cytotaxin on cellular cAMP concentrations (Figure 3.19c).

by agents acting through the cAMP system as well as exogenous glucose which does not effect cAMP levels.

Symon, McKay and Wilkinson (153) noted, without comment, that cAMP showed no chemotactic activity over the concentration range 10^{-6} to 10^{-3}M. Similarly, Kaley and Weiner (73) found that cGMP, cAMP and dibutyryl cAMP were not chemotactic over the concentration range 10^{-4} to 10^{-5}M, although prostaglandin E_1 was chemotactic in the absence of serum. They thus concluded that the chemotactic activity of prostaglandin E_1 was not dependent on its ability to activate adenyl cyclase in leucocytes.

In contrast to these findings, Leahy, McLean and Bonner (96) have claimed that concentrations of 10^{-5}M cAMP were significantly chemotactic. This was based on their findings that 14 of 24 rabbit peritoneal neutrophil preparations responded to 10^{-5}M cAMP present in the lower compartment of a Boyden chamber. The significance of this observation is somewhat vitiated by their simultaneous observation that only 14 of 18 similar preparations showed chemotactic responses to 0,2% casein. While the chemotaxis assay is, admittedly, only marginally adequate for quantitating minor differences in chemo-attractant activity, I have had little difficulty with its use in distinguishing chemotactic activity on a "yes-or-no" basis. The fact that these authors were only able to detect a response to cAMP in approximately 50% of their experiments suggests that their experimental conditions may not have been optimal.

The findings of Leahy et al. (196) have been confirmed to some extent by others. Gamow, Böttger and Barnes (53) have reported that $4x10^{-3}$M cAMP is chemotactic for horse neutrophils as assessed by the ability of this compound to alter the distribution of cells migrating out of plasma clots. Wissler, Stecher and Sorkin (187) have reported

unpublished observations to indicate that cAMP, amongst other nucleotides, although not chemotactic *per se*, can replace cocytotaxin in the anaphylatoxin-cocytotaxin binary serum peptide system.

Rivkin and Becker (132) have reported inhibition of the chemotactic response of neutrophils assayed in the presence of added cAMP or dibutyryl cAMP. Substances causing intracellular accumulations of the cyclic nucleotide also resulted in an inhibition of neutrophil chemotactic response if present in the cell compartment during their assay. They have confirmed the findings of Leahy et al. (96) to show that cAMP is chemotactic, and suggested that increases in intracellular cAMP concentration may inhibit chemotaxis by decreasing the ability of neutrophils to move in response to a chemotactic stimulus.

Tse, Phelps and Urban (158) noted a dose-dependent inhibition of both random and directional mobility of leucocytes by cAMP.

Estensen, Hill, Que, Hogan and Goldberg (47) found that cells treated with dibutyryl cAMP (10^{-4} to 10^{-2}M) or agents causing intracellular accumulations of cAMP inhibited neutrophil chemotaxis. Dibutyryl cGMP (10^{-5}M), and agents stimulating increases in intracellular concentrations of cGMP, resulted in increased cell chemotactic responsiveness. The authors suggest that cell motility may be modulated by cyclic nucleotides. Although certainly an interesting suggestion it should be noted that the concentrations of dibutyryl cAMP used to pretreat the cells (10^{-2} to 10^{-4}M) in these experiments, were higher than those of the dibutyryl cGMP used (10^{-5}M).

In summary, the role of cyclic nucleotides in cell movement, especially neutrophil chemotaxis, is uncertain. The studies in which the effects of added nucleotides or their analogues on cellular behaviour

were investigated have used concentrations orders of magnitude in excess of the concentrations normally present in these cells or serum. The sequence of events following cell-cytotaxin interaction are poorly understood, and consequently the observed effects of added pharmacologically active compounds might be of a secondary nature rather than having a direct effect on the sequence of events under study. My inability to detect obvious differences in cAMP levels between control and cytotaxin-treated cells would imply that the cAMP-adenyl cyclase system does not have a major role in the cytotaxin-cell interaction.

Chapter IV

The effect of cytotaxin on cell adherence and cell surface charge

Cell Adherence

Introduction

The adherence of circulating leucocytes to vascular endothelium at the site of an inflammatory lesion is a well documented phenomenon (3,33,34) that appears to be associated with changes in the surface properties of both the endothelial cells lining the small blood vessels (3,33,34) and the adherent inflammatory cells (3). Considered teleologically, this interaction between leucocyte and vessel wall could provide the means for the initial entrapment of cells in the vasculature of an injured area prior to their emigration by diapedesis, into local perivascular spaces. The fact that attractive intermolecular forces between components of the neutrophil membrane and the surfaces of neighbouring structural tissue components may also play an important part in determining the *direction* of cellular movement was emphasized by the observations of Carter (30,31). In these elegant studies on *haptotaxis*, he observed a striking tendency for motile cells to move preferentially from a less adhesive to a more adhesive surface.

With this information available, it seemed to me entirely conceivable that directional neutrophil movement across a Millipore filter in the presence of a chemotactic gradient might be related to an effect of the chemotaxin upon the adherence of cells to the cellulose ester fibres of the filter. If chemotaxin were to enhance adherence in a concentration-dependent manner, the chemotactic gradient could, in effect, produce a gradient of adhesivity analagous to that used by Carter (30) in his experiments. I therefore decided to investigate the effects of casein cytotaxin upon neutrophil adherence to foreign surfaces. This chapter records the results of the experiments performed with this purpose in view.

An elementary consideration of the sequential course of events taking place when a cell adheres to a foreign surface would suggest that the process occurs in two phases (188).

Firstly, during the phase of *attachment*, the cell would approximate the surface and establish initial contact at one or more topographically limited points. In considering this phenomenon one may make the reasonable assumption that the cell is in a constant state of thermal and intrinsic movement, extruding and retracting pseudopodia. Extrusion of a pseudopod in the direction of the surface would increase the probability of contact; retraction of such a pseudopod would diminish this probability. One may further assume that the likelihood of durable initial contact being made would be influenced by translational movement of the cell relative to the substratum; by mutually repulsive and attractive short-range, non-covalent, intermolecular forces between cell membrane and substrate; and by shear forces tending to dislodge the attached cell. This first phase, therefore, would be characterized by the balanced interplay of several factors tending to favour, inhibit or disrupt cellular contact. One can reasonably imagine that time would be an important dimension during this phase with rate-constants giving the probability of association or dissociation.

Secondly, there would be a phase of *anchorage* during which the cells establish broader, more intimate contact with the surface. This is visible, under normal tissue culture conditions, as "spreading".

If these two phases be accepted, it is clear that they suggest two approaches to the technical quantitation of cell adherence. Firstly, one might, in a "kinetic" approach, attempt to measure the rate at which cells attach to a foreign surface under constant conditions of incubation, relative translational movement and shear. Secondly, in a more static

approach, one might attempt to quantitate the amount of force required to dislodge cells that have already entered the second, stable, phase of anchorage.

Few techniques have been published for the assay of cell adherence, and those that are available in the literature have tended to use the second approach - i.e. the measurement of the force required for *detachment* (26,51,175).

I felt that it would be more illuminating to use the first approach, for the intuitive reason that such a technique would detect subtle differences in the kinetics of cell adherence that might be lost in the grosser, conventional techniques. I therefore devised a procedure in which cell suspensions were incubated in stoppered, semi-filled test tubes that were set horizontally and "rolled" about their horizontal axes at a constant, low, angular velocity of one revolution per min. With this arrangement cells were able to settle by gravity and come into contact with the constantly moving substrate produced by the inner surface of the tube. Adherence was quantitated, for comparative purposes, by estimating the number of cells adhering to the inner surface of the tube as a function of time.

Materials and Methods

Apparatus

Six experiments were performed in which cell suspensions were incubated in screw-capped serum bottles set in a 37°C incubator upon the rolling apparatus shown in Figure 4.1. The dimensions of the roller wheels and the synchronous motor were chosen so that each 35x14mm(6ml)

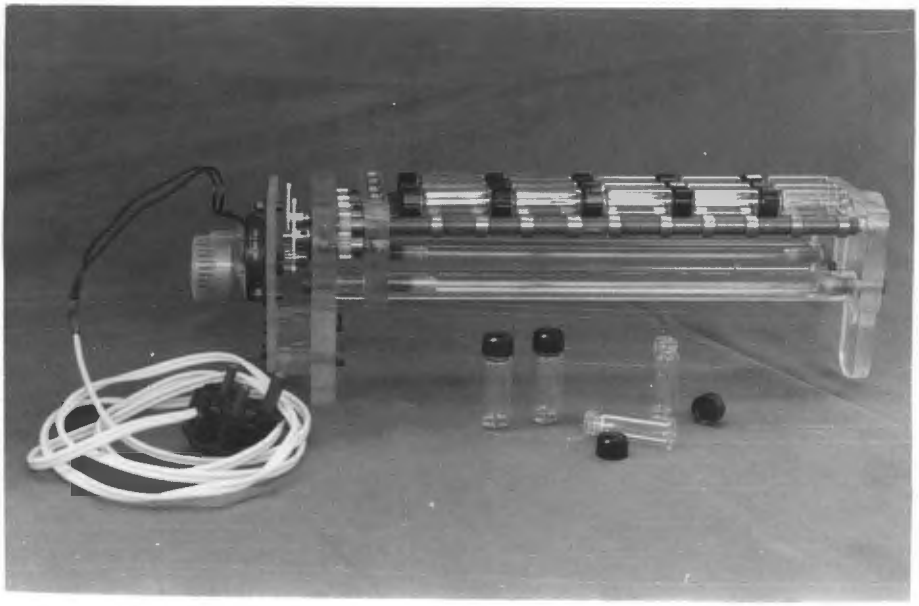

Figure 4.1 Apparatus used for rolling the small, screw-capped, sterile serum bottles shown. The bottles rested on parallel rollers that were rotated by the synchronous motor and gear train on the left of the apparatus.

serum bottle would rotate at an angular velocity of one revolution per min.

Five experiments were performed in which cell suspensions were incubated at 37°C in 35x8,5mm stoppered cellulose-ester coated test tubes set in the apparatus shown in Figure 4.2. This apparatus was designed to rotate each tube about its horizontal axis at one revolution per min.

Incubation vessels

Bakelite, screw-capped, neutral borosilicate-type glass serum vials(1) or polyethylene-stoppered, borosilicate glass test tubes(2) were cleaned with chromic acid or detergent(3) and washed extensively with distilled water. Caps were washed with the same detergent and rinsed well with water. Serum bottles were dried at 40°C in air and used without further treatment following sterilization with ethylene oxide. Borosilicate tubes were coated with cellulose ester by a minor modification of the method described by Carter (30). Briefly, fragments of 3µm Millipore filters were dissolved, at a final concentration of 0,5% w/v, in 1:4 dioxane. Dissolution, in tight stoppered containers, took approximately two weeks to complete. Residual particulate matter was removed from the solution by filtration through glass wool and the tubes were filled with a cellulose ester solution. The solution was decanted and the tubes were drained by inverting and standing vertically, under vacuum, to avoid clouding by atmospheric humidity. Immediately before use the tubes were placed in a 280°C oven for 10 sec to melt the ester layer and to fix it firmly to the inner surface of the tube. Polyethylene stoppers were sterilized by ethylene oxide treatment.

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(1) Glassblowing Industries (Pty) Ltd., Johannesburg, South Africa

(2) Scientific Glassblowers (Pty) Ltd., Cape Town, South Africa.

(3) RBS; Chemical Concentrates Ltd., London, United Kingdom or Decon 75; Atomic Export Import Corporation (Pty) Ltd., Johannesburg South Africa.

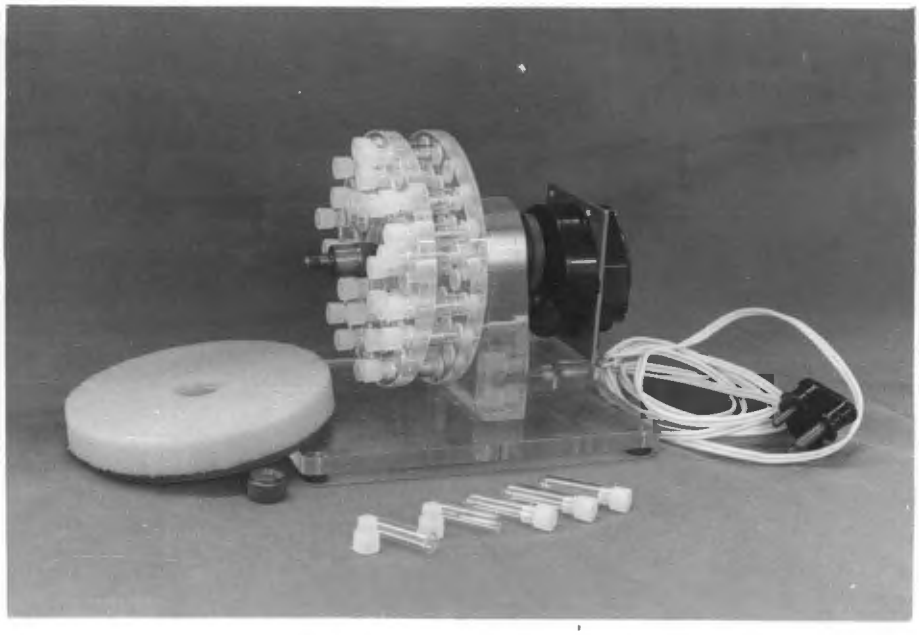

Figure 4.2 Apparatus used for rotating the small, capped, sterile test tubes shown. When in use, the tubes were retained by the sponge pad clamped in position with the knurled nut.

Cytotaxin preparation

SNF-cytotaxin in TBS was concentrated in Amicon stirred cells fitted with UM2 membranes designed to retain >85% of molecules with molecular weight >1 400 for solutions of relatively pure solutes. (With admixtures containing higher molecular weight species, the membrane retentivity for lower molecular weight species greatly increases.)

Cells

Rabbit peritoneal exudate cells were harvested in the usual manner and suspended in Gey's BSS-2% HSA to a final concentration of $2x10^6$ cells/ml. To a 10 ml sample of this suspension was added from 0,2 to 0,5 ml of concentrated SNF-cytotaxin. The final concentration of cytotaxin was known to be maximally effective in the Boyden chamber assay. A second 10 ml sample received an equal volume of TBS. The two cell suspensions were equilibrated at 37°C for 15 min with constant shaking, after which 1,0ml volumes were dispensed into incubation tubes. The tubes were then stoppered and rolled at 37°C.

At selected time points (from 5 to 180 min) duplicate or triplicate test (i.e. cytotaxin-containing) and control vessels were removed from the incubator; emptied of medium and non-adherent cells by inversion and allowed to drain dry at room temperature in this inverted position for 60 min. Cells adherent to the walls of the incubation vessels were fixed with absolute ethanol for 10 min and stained. Freshly prepared Giemsa stain was used for the serum bottles; Weigert's iron haematoxylin was used for the cells adherent to cellulose-ester coated tubes. Stained tubes and bottles were allowed to dry overnight at 37°C

in an inverted position and then coded for "blind" counting.

Counting of adherent cells.

Cells were counted microscopically with an 8x eyepiece and a 40x objective. The serum bottles or tubes were held in a specially constructed holder (Figure 4.3) on the mechanical stage of the microscope. All cells in the microscopic field of view in focus on the inner concave surface of the vessel nearest the objective were counted in a 30mm strip along the length of the vessel as measured on the vernier of the mechanical stage. Two counts were performed on each bottle or tube; the second on the face opposite to the first.

After counting the two counts were averaged and decoded.

Results

As can be seen from the data summarized in Table 4.1 adherence of cells to uncoated glass proceeded, with time, to reach a maximum at the 120 min time point after which no further cells adhered, or detachment occurred. In three experiments, the presence of cytotaxin had the predicted effect upon cell adherences; in two experiments treated and control cells were similar in their tendency to adhere and in one experiment the adherence of control cells was slightly superior to that of cytotaxin-treated cells.

Cellular attachment to cellulose-ester coated tubes showed a similar tendency to increase to a maximum with time and then to diminish (Table 4.2). In three cases maximal counts were recorded at 120 min and in two cases at 60 min in these experiments. In two experiments (Nos. 1 and 3) the presence of cytotaxin appeared to exert a pronounced enhancing

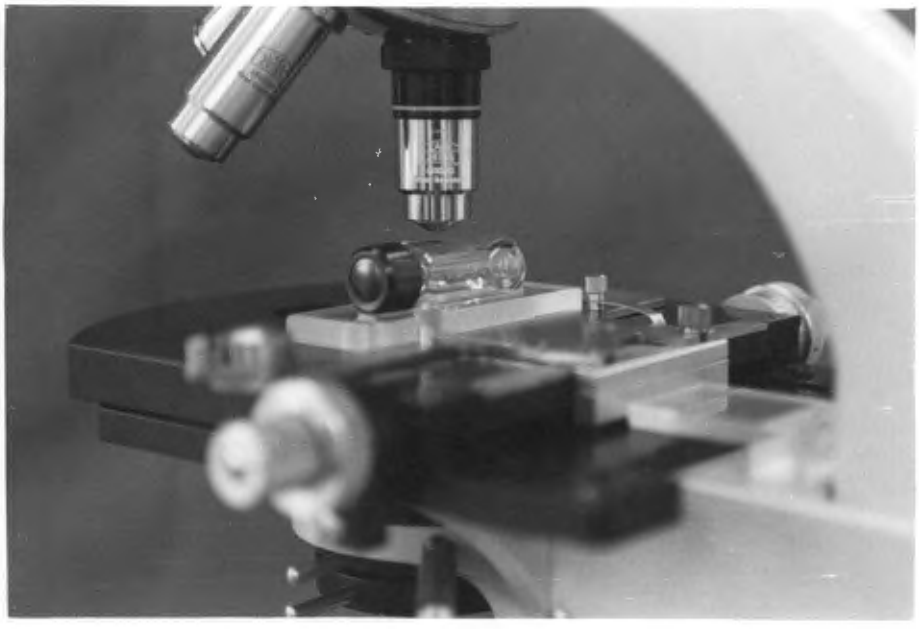

Figure 4.3 Microscope stage attachment for holding the bottles and tubes used for cell adherence studies.

effect upon cellular adherence. In the other three experiments, superior adherence was observed in the presence of cytotaxin, but was of minor degree and of doubtful significance.

Table 4.1

Polymorphonuclear leucocyte adherence to glass (average cell count/30mm).

Expt no.	Rabbit	Treatment	Incubation time (min) 0	60	120	180
1	229	TBS	54	156	313	233
		SNF/TBS	57	270	513	532
3	237	TBS	95	428	521	507
		SNF/TBS	72	376	476	458
4	236	TBS	128	123	173	156
		SNF/TBS	138	344	427	271
5	187	TBS	28	100	221	127
		SNF/TBS	36	225	331	234
6	237	TBS	93	539	688	469
		SNF/TBS	92	435	502	354
7	188	TBS	44	145	298	135
		SNF/TBS	37	203	213	151

Table 4.2

Polymorphonuclear leucocyte adherence to cellulose ester (average cell count/30mm)

Expt no	Rabbit	Treatment	Incubation time (min) 0	60	120	180
1	186	TBS	112	268	391	N.D.
		SNF/TBS	45	856	1762	N.D
2	189	TBS	52	469	478	345
		SNF/TBS	128	457	620	537
3	181	TBS	136	383	169	276
		SNF/TBS	168	1165	823	639
4	186	TBS	91	2160	2510	508
		SNF/TBS	85	2367	2617	844
5	182	TBS	54	472	352	269
		SNF/TBS	47	512	243	267

Discussion

The results of the experiments reported in this Chapter were not sufficiently reproducible to bear any definite conclusions. Taking the data at their face value, one could claim that in five experiments with cellulose-ester coated tubes, cytotaxin treatment gave enhanced peak counts relative to the controls in every case. The likelihood of such an outcome arising by chance alone would be $(\frac{1}{2})^5 = 0{,}031$ and one might, therefore, ascribe this level of "significance" to the assumption that cytotaxin does increase cellular adherence. I should prefer, however, to be more tentative in my interpretation of the results and to claim justification for salvaging no more than the following conclusions from the work.

(i) In both the case of uncoated and coated vessels, adherent cell counts tended to rise and fall with time in a consistent pattern, indicating that this experimental approach to the quantitation of adherence has some potential in that it is, at least, qualitatively reproducible.

(ii) Coating of the tubes with cellulose ester should, *a priori*, have provided a substrate for cellular adherence more comparable to the surface offered by the cellulose-ester fibres in the filter assay. Using these tubes, two experiments (Nos. 1 and 3) yielded results that gave most encouraging support to the notion that cell-substrate adhesive interactions are intensified by cytotaxin. I suspect that technical refinements might give more uniformly positive results. It was, for example, difficult to standardise the procedure for removing non-adherent cells; the coating procedure could be improved upon; the number of cells introduced into the tube might be

optimized; and various other technical improvements might be introduced to validate the assay. Unfortunately, circumstances have thus far precluded my taking this work to its proper conclusion.

It is, for a number of reasons, my serious intention to devote further time and effort to the study of cell adhesivity as a determining mechanism for directional movement. My commitment to this notion stems from the following observations and considerations.

(i) Carter (30,31) whose work I have already quoted, has shown that the direction in which cells move can be influenced by variations in the physico-chemical nature of the surface to which they are adherent. Carter (30) showed that mammalian cells in culture would not adhere to surfaces of cellulose acetate. If the surface is metallized *in vacuo* with palladium or one of several other metals, the cells adhere and spread. By varying the amount of metal deposited, surfaces allowing different degrees of cell adherence could be obtained. Carter achieved a gradient of adhesivity by placing a stainless steel rod onto a slide coated with a cellulose-acetate film and then shadowing the film with vapourised palladium. Using mouse fibroblasts (Earle's L strain), he showed a uniformity of directional movement up the gradient in the direction of increasing substrate adherence, which contrasted with the apparently random movement of cells on evenly metallized cellulose acetate.

(ii) A large number of biological compounds have been described that have chemotactic properties. Although the biochemical

characterization of these materials has not been completed, I suspect that they will be found to differ widely in their chemical composition and steric structure. It is, therefore, unlikely that the information contained in a chemotactic gradient could be relayed to the motile cell by a system of membrane receptors specific (in the sense of steric complementarity) for the diverse chemical compounds that are chemotactic. My prediction would be that such substances owe their common chemotactic properties to some shared characteristic of a grosser order.

(iii) The elementary nature of the neutrophil with its paucity of defined inducible functions, inclines me to the deterministic view that the cell is equipped, constitutively, for random movement only. Any coordinated, directional movement that it might exhibit is extrinsically determined by the physico-chemical nature of its micro-environment.

(iv) As an adherent neutrophil moves across a surface to take up a new position, separation from the surface at its previous position takes place at a plane of cleavage *within* the cell membrane and *not* at the membrane-surface interface. This is evident in the "tracks" of membrane-derived material left by moving cells (68,101,135,154,176,177,178,179). This implies that the advancing regions of the cell establish fresh points of contact to which they adhere. Contraction of the cell must then result in intramembranous cleavage at the trailing edge while the advancing regions remain fixed. If this did not occur, translocation of the cell would not take place.

Clearly, the adhesive forces between the advancing edge and the substrate must be greater than those between the trailing edge and the substrate.

(v) My own observations, although not strikingly confirmative, are at least consistent with the "adhesion" hypothesis.

If, therefore, one accepts the view that chemotaxis is the result of non-specific, environmental effects upon the attachment of neutrophils to the substrate, one may proceed to speculate on the nature of the mechanism responsible for the trans-cellular disparity in adhesion forces in the presence of a chemotactic gradient.

As a first approximation, it is reasonable to assume that the adhesive forces at the trailing edge of the cell are determined (a) by the nature of the hydrophobic interaction between the two laminae of the lipid bilayer of the cell membrane and (b) by the area over which these forces are applied. Zigmond and Hirsch (194) have shown that the "tail" of the migrating cell assumes a pointed shape, so diminishing the area available for attachment. More recent studies (136) with fluorescein-labelled concanavalin A have shown that the tail of the migrating cell is relatively rich in lectin-binding receptors, indicating that a redistribution of membrane glycoprotein accompanies cellular translocation. The accumulation of such components at the trailing region may operate to diminish the hydrophobic forces discussed above.

At the leading edge of the cell, two obvious interactions require consideration. Firstly, both the external cell membrane and the surface (for example cellulose acetate film) bear net negative charges. One would imagine, therefore, that mutually *repulsive* electrostatic forces

would operate. My own observations, reported later in this Chapter indicate that, if anything, the effect of cytotaxin is to *increase* the net negative charge on a cell. It may be that binding of the negative cell surface to a negatively charged substrate is mediated by divalent cations such as calcium. If this were the case, chemotactic factors, by binding non-specifically to the cell membrane and increasing the negative charge density, might enhance electrostatic binding by providing additional sites for the secondary attachment of divalent, cation ligands.

Secondly, for the cell to attach to a fresh point of contact, it is clear that its membrane must displace molecular constituents of the medium from that site. Whether or not it is able to do so will depend upon the success with which membrane-surface interactive forces compete with interactive forces between the medium and the surface. A simple analogy is provided by the behaviour of an oil droplet placed upon a glass slide. If the slide is in air, the droplet will spread to "wet" the surface - i.e. oil-glass interactions are stronger than air-glass interaction. If the slide is immersed in water, the droplet will remain confined, oil-glass interaction being weaker than water-glass adhesive forces. Any agent with surface active properties, that was able to favour membrane-surface interaction might, therefore, reasonably be expected to favour adherence at the advancing cell edge.

This argument raises the possibility that chemotactic substances might owe their biological effects to surfactant properties. Becker (13) has reported that the detergent "Lubrol W" has a marked effect on the chemotactic responsiveness of neutrophils. My own experience has been that a chemotactic ultrafiltrate of a casein solution will stabilize a phenol-water emulsion indefinitely. These tenuous observations suggest that chemotaxins may, indeed, exert their effect by virtue of their surface

active properties.

I must, unfortunately, conclude this section of my thesis with speculations rather than conclusions. I hope that my future work based upon these ideas will yield definite results.

The charge on the cell surface

Introduction

The possibility that cell surface charge might influence directional movement has been suggested by a number of observations.

Firstly, in 1927, Abramson (1) made the direct suggestion that migration of neutrophils to an area of injury *in vivo* could be explained by the action of an electrical field generated by potential differences between injured and healthy tissue, upon migrating charged leucocytes. His argument was based upon the fact, known at the time, that the external surface of the healthy cell is anionic at physiological pH. The accumulation of acid products of cell death and inflammation would tend to suppress proton dissociation at the site of injury with the result that a potential difference would be established between blood and lesion, the latter being electropositive relative to the former. A potential difference of 10mV between capillary wall and inflammatory zone (the distance of ≃ 50μm) would produce a voltage gradient of 2V/cm. This was sufficient, in Abramson's experiment to produce appreciable annodal movement of the neutrophil. Although the significance of Abramson's observations is diminished by the fact that chemotaxis can be observed *in vitro* under circumstances where similar electrical potential gradients do not exist, he must be credited for attempts to formulate an explanation for directional cell movement based upon electrostatic interactions.

Secondly, and perhaps of more significance for our present purposes, we have the observations I have discussed, pertaining to the importance of cell adherence for chemotaxis. At the pH at which the Boyden chamber incubations were performed, the external surface of the

neutrophil and the surface of the cellulose ester fibres both have a net negative charge. One would imagine, therefore, that these surfaces would be mutually repulsive. If chemotaxin were to operate by increasing cellular adhesion it might do so by neutralizing the negative charge on the cell membrane or by functioning as a ligand for a divalent cation to serve as a bridge between cell and filter. In either case one would expect chemotaxin to bind to the cell and, in so doing, to alter its net surface charge.

The experiments reported in this section were accordingly developed to examine the effect of cytotaxin on the surface charge of the neutrophil.

The method for measuring cellular surface charge was based upon its relationship to electrophoretic mobility of the cell in a uniform electric field. The formal relationship between the surface charge and zeta potential (ζ) is given by the Helmholtz-Smoluchowski equation

$$\zeta = \frac{4\pi\eta}{D} \cdot V$$

where η = relative viscosity of the medium

D = dielectric constant of the medium

V = electrophoretic mobility in $\mu m.volt^{-1}.cm.sec^{-1}$

Inspection of the equation shows that, for constant viscosity and dielectric constant of the suspending medium, zeta potential is directly proportional to electrophoretic mobility. The experimental approach that I used compared the electrophoretic mobility of cytotaxin-treated and untreated cells suspended in identical media so that consistency of viscosity and dielectric constant, could be assured although neither of these were measured directly. The results are accordingly given in terms of cell displacement or electrophoretic mobility; zeta potentials were not calculated.

Materials and Methods

Cells

Rabbit peritoneal exudate cells were harvested 18h after a provocative intraperitoneal injection of sodium caseinate as described in the Appendix. The cells were suspended in Gey's BSS-2% HSA* and adjusted to the concentration ($5x10^6$ to $1x10^8$ cells/ml) required for each experiment. Details are given for each experiment in the Results section. Suspensions were divided into two equal volumes, one of which received SNF-cytotaxin at a final concentration established, by previous assay, to the optimally chemotactic; the other received an equal volume of TBS.

The cell suspensions were then incubated, with gentle agitation at 37°C for 60min in stoppered conical flasks in a shaking incubator. Following incubation the cells were pelleted by gentle centrifugation (300xg; 7min; 20°C) and resuspended at the requisit concentration in electrophoresis buffer.

Cell electrophoresis

Cell electrophoresis was performed using two techniques requiring commercially available apparatus of different kinds

(a) Continuous particle electrophoresis (CPE) experiments used the Beckman CPE system(1). This apparatus, shown in Figure 4.4 consisted essentially of a cooled electrophoretic cell in which a "curtain" of buffer 540mm tall, 48mm wide and 2mm deep,

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(1) Beckman Instruments Incorporated, Fullerton, California, U.S.A.

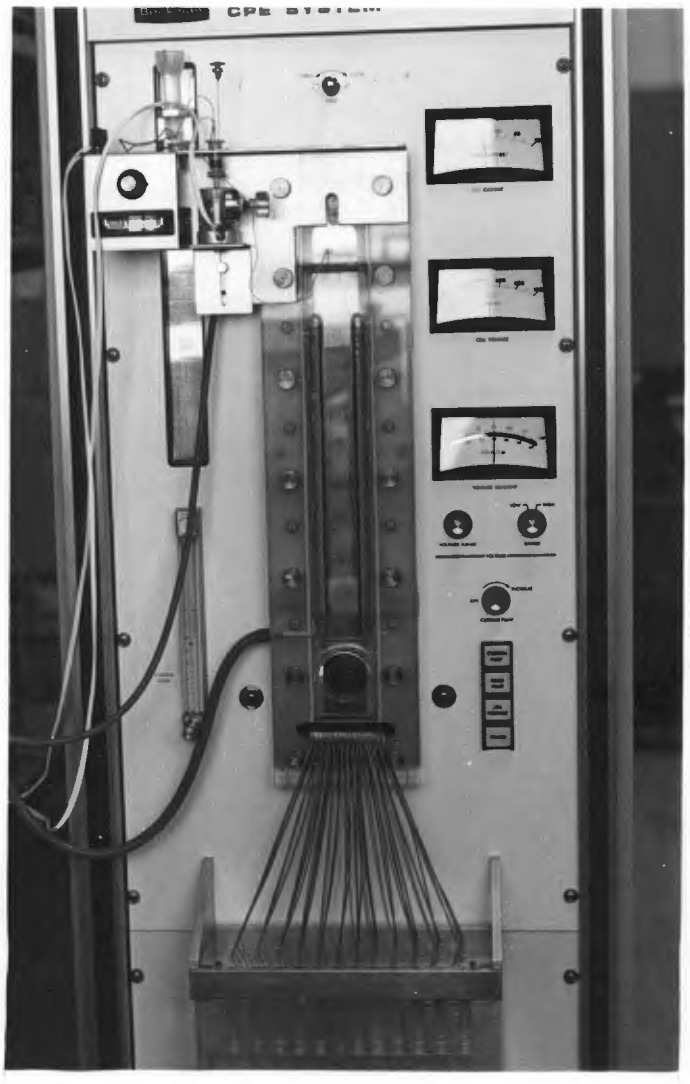

Figure 4.4 Beckman Cell-Particle Electrophoresis Apparatus.

Cells, deflected by the lateral electrical field, emerge from the orderly array of tubes at the bottom of the electrophoresis chamber and are collected, as individual fractions, in test tubes as shown.

The circular viewing window (enlarged in Figure 4.5) is seen immediately above the tube manifold outlet.

flowed at constant velocity from top to bottom. The suspension of the particles was introduced, by way of a downwardly directed, bent tube, as a fine steady stream into this laminar, vertically flowing curtain. Electrodes, situated vertically along each lateral edge of the cell, were used to apply a horizontal d.c. electrical field to the curtain and the stream of cells. Each particle in the suspension had two components of motion; a vertical component (V_v) which was the same as the velocity of the curtain, and a horizontal component (V_h) which was linearly related to the electrophoretic mobility of the cell. Under equilibrium conditions, the resultant velocity vector of each particle would be directed away from the vertical by an angle (arctan V_h/V_v) whose magnitude was directly proportional to the electrophoretic mobility of the cell under conditions of constant V_v and electric field. The bottom edge of the cell was equipped with 48 evenly spaced collecting ports through which the buffer left the chamber. The cells could be collected and counted and a histogram prepared showing cell counts as a function of exit port number; port number 1 being situated closest to the cathode and port number 48 closest to the anode.

In addition, the lower portion of the cell was equipped with a viewing window through which the particle stream could be viewed or photographed. The viewing window was inscribed with a scale graduated in mm. My experience has confirmed the precise correspondence between the position of the vertical particle stream indicated on the graduated scale and the number of the exit port through which the particle emerged. For example, the broad stream of cells seen crossing the scale between graduations 28 and 33 and 31 and 37 in Figure 4.5 emerged from the exit port

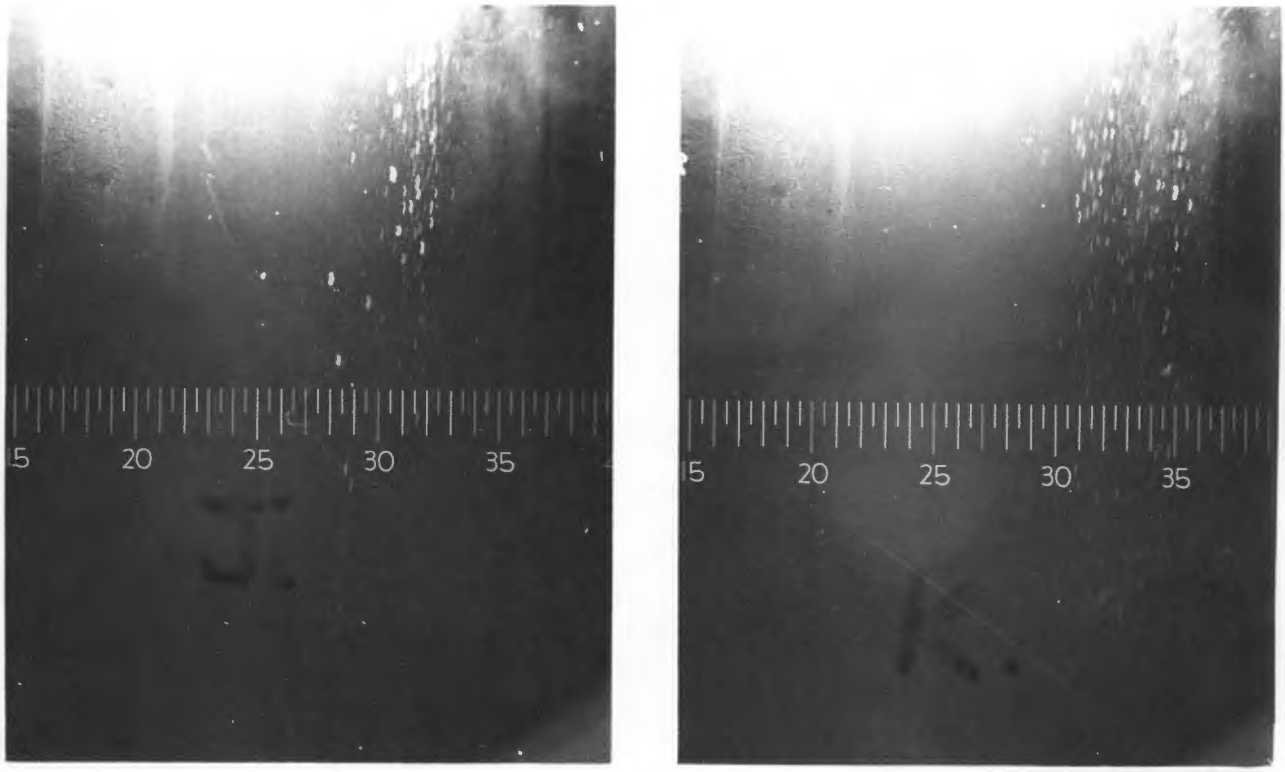

Figure 4.5 Effect of cytotaxin on cell charge

These photographs of the viewing window of the CPE apparatus were taken during electrophoresis of untreated cells (*left*) and cytotaxin (SNF)-treated cells (*right*). The cells, which were moving downwards during the exposure, appear as blurred white dots.

Note that untreated cells emerged at a scale reading of approximately 31 whereas cytotaxin-treated cells emerged at an average scale reading of approximately 34.

Anode to the right.

with modes at 31 and 34 respectively.

The operation of the apparatus, the precautions necessary to obtain valid results and the interpretation of the results obtained are discussed in detail in papers by Strickler, Kaplan and Vigh (150), Strickler (149) and Hubener and Lawson (66) and in the manufacturer's manual. The recommended operating procedure was modified by incorporating a magnetic stirrer into the cell reservoir to maintain the cells in a state of even suspension. Instead of allowing the cell suspension to enter the electrophoretic cell by gravity, it was injected slowly into the curtain with the gas pressure generated by the electrolysis of 0,1M phosphate buffer pH 7,4 with a current of 20-30μA. The pressure could be regulated by varying the electrolytic current.

Neutrophils for continuous particle electrophoresis were resuspended in 10^{-3}M veronal buffer at pH 8,6 containing 0,001% chlorhexidine. Samples were individually collected from the collection ports and cell counts were performed either manually, with a modified Neubauer haemocytometer stage, or electronically with a Coulter counter.(1)

In all experiments reported in this section, the cells traversed a vertical distance of 290mm through a horizontal electric field of 100V/cm. The time (t) taken in seconds to traverse this distance was calculated from the curtain buffer flow rate

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(1) Coulter Electronics Incorporated, Hileah, Florida, U.S.A.

(F;ml/sec), the cross sectional area (cm^2) of the chamber between the electrodes (A) and the vertical length (cm) of the field (l) from the equation

$$t = \frac{A.l}{F}$$

The vertical position (P_o) of the stream of cells in the absence of applied voltage, was measured on the graduated scale of the viewing window.

The voltage was then applied and the horizontal displacement of the cell stream monitored visually at the viewing window. When equilibrium had been reached (usually after 10 min), as indicated by no further lateral displacement of the stream, fractions were collected through the exit ports for cell counting. Histograms of the displaced cell population were plotted and the position of the mode (P_m) recorded as the exit port number through which the mode emerged. Owing to spreading of the deflected stream, horizontal displacement of the mode of the cell population by the electrical field could not be determined satisfactorily from the viewing window scale. The procedure adopted - i.e. plotting histograms - gave a more accurate estimate of this value and provided information regarding the distribution of displaced cells.

Lateral displacement (s) of the cells was calculated from the equation

$$s = P_m - P_o$$

Electrophoretic mobility (V) was calculated from the equation

$$V = \frac{s \times 1\,000}{t \times vg}$$

V has the dimension $\mu m. volt^{-1}. cm. sec^{-1}$

The factor 1 000 was introduced into the numerator to convert mm to μm.

s and t are as defined above

vg is the voltage gradient - approximately 100 volts.

(b) A cylindrical microelectrophoresis apparatus[1] described by Bangham, Flemans, Heard and Seaman (10) and Seaman (142) was used to measure cell mobility in an electric field in 7 experiments.

In this apparatus the cell suspension was introduced into a thick-walled capillary tube 150mm long and 2mm in internal diameter which formed a bridge between two electrode vessels containing excess cell suspension. The capillary wall was ground with one optically flat surface through which a microscope could be focussed upon the suspended cells. The electrophoresis assembly and microscope objective were immersed in a constant temperature water bath. The eyepiece was equipped with a calibrated graticule so that the lateral movement of any particular cell in focus at the stationary level could be followed across a measured distance in the cell and timed with a stop watch. In each experiment the anodal rate of movement of 20 cells was recorded. The electrophoretic mobility of each cell was measured in both directions by reversing the polarity of the

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(1) Rank Brothers, Bottisham, Cambridge, England.

electrodes after recording a measurement in one direction.

For these experiments, cells were suspended in Gey's BSS-2% HSA to a final concentration of 1 to 20×10^6 cells/ml depending on cell yield.

Results

The results of three representative experiments in which cytotaxin-treated and untreated cells were subjected to continuous particle electrophoresis are depicted in histogram form in Figure 4.6. These diagrams show that the cell distribution, as they left the chamber, could be described by Gaussian curves with an approximately symmetrical distribution about a mode for each population. In each of the experiments the distribution of the entire cytotaxin-treated cell population was shifted anodally relative to that of the corresponding control cell population indicating that the effect of cytotaxin treatment was to increase the net negative surface charge upon the cells.

The results obtained from 12 experiments are summarized in tabular form in Table 4.3. As can be seen from these results, the electrophoretic mobility of control neutrophils varied from 1,09 to 1,80 with a mean of 1,48 $\mu m.volts^{-1}. cm.sec^{-1}$. Corresponding values for cytotaxin-treated cells were : range 1,64 to 2,51; mean 1,98 $\mu m.volts^{-1}. cm. sec^{-1}$.

In 11 experiments the cytotaxin-treated cells showed greater anodal electrophoretic mobility than did the control cells. In the 12th experiment, no obvious difference was evident.

Trypan blue exclusion tests for viability of the cells showed, in 4 cases, that 93% of the cells that were able to exclude trypan blue before electrophoresis were still viable by this criteria thereafter.

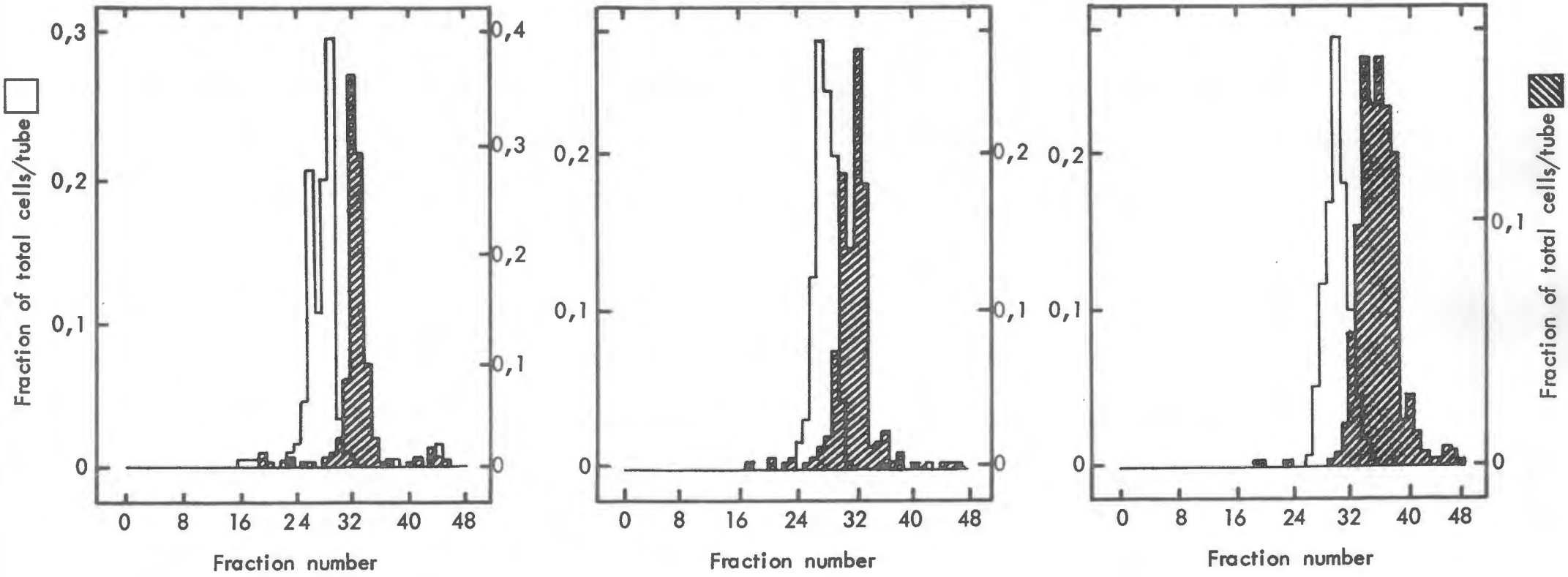


Figure 4.6 Effect of cytotaxin treatment upon cell charge.

Results of three experiments in which untreated cells (*clear histograms*) and cytotaxin (SNF)-treated cells (*hatched histograms*) were subjected to cell-electrophoresis (100V/cm) in the Beckman CPE system. Cell counts were performed on fractions emerging from the bottom of the electrophoresis chamber. As plotted, the anode is to the right.

Note that in each case, cytotaxin treatment shifted the distribution of the emerging cells anodally, indicating that these cells acquired an increased negative charge as a result of contact with crude cytotaxin.

Table 4.3

Table 4.3

The effect of partially purified cytotaxin (SNF) on electrophoretic mobility of rabbit peritoneal exudate cells as measured by cell particle electrophoresis.

Expt no.	Cells ($x10^{-6}$/ml)	Experimental conditions						Displacement (mm)	Electrophoretic mobility ($\mu m.volt^{-1}.cm.sec^{-1}$)	Cytotaxin / TBS
		Treatment	Voltage gradient (V/cm)	Current (mA)	Buffer curtain flow (ml/min)	Sample feed (μA)	Temperature (^{o}C)			
1	57	TBS	100,0	40	23	20,0	11,0	10,40	1,53	1,24
		Cytotaxin		45				12,88	1,89	
2	44	TBS	100,0	32	20,0	20,0	11,0	11,0	1,40	1,34
		Cytotaxin		32				14,75	1,88	
3	39	TBS	100,0	33	20,5	20,0	4,0	11,75	1,54	1,13
		Cytotaxin		32				13,25	1,73	
4	5	TBS	100,0	37,5	23,5	20,0	11,5	12,0	1,80	1,40
		Cytotaxin		37,5				16,75	2,51	
5	27	TBS	100,0	32,5	23,0	20,0	10,0	9,85	1,45	1,42
		Cytotaxin		30,5				14,0	2,06	

6	27	TBS	100,0	33,5	23,0	20,0	10,0	9,65	1,42	1,42
		Cytotaxin		33,5				13,75	2,02	
7	68	TBS	100,0	34,0	22,5	20,0	15,0	10,88	1,56	1,34
		Cytotaxin		34,0				14,53	2,09	
8	53	TBS	100,25	45,0	17,5	30,0	7,0	9,75	1,09	1,72
		Cytotaxin		45,0				16,75	1,87	
9	15	TBS	100,25	41,0	17,5	30,0	8,5	14,75	1,64	1,00
		Cytotaxin		41,0				14,75	1,64	
10	97	TBS	100,25	40,5	17,5	30,0	7,0	11,75	1,31	1,43
		Cytotaxin		45,5				16,74	1,87	
11	97	TBS	100,25	40,5	17,5	30,0	7,0	11,75	1,31	1,51
		Cytotaxin		49,0				17,75	1,98	
12	39	TBS	100,25	41,5	17,5	30,0	8,5	15,75	1,76	1,25
		Cytotaxin		44,0				19,75	2,20	

Table 4.4

The effect of partially purified cytotaxin (SNF) on electrophoretic mobility of rabbit peritoneal exudate cells.

Expt no.	Rabbit	Cells ($x10^{-6}$/ml)	Voltage gradient (volts/cm)	Electrophoretic mobility* ($\mu m.volt^{-1}.cm.sec^{-1}$)	
				+ Cytotaxin	Control
1	279	11	3,2	1,33 (0,55-2,91)	0,77 (0,23-1,59)
2	280	22,5	3,2	1,11 (0,47-4,00)	0,97 (0,35-2,04)
3	334	3	2,90	1,63 (0,65-2,69)	2,05 (0,66-3,34)
4	277	8	6,67	1,14 (0,58-2,09)	0,94 (0,23-1,92)
5	277	8	5,50	1,25 (0,77-2,13)	1,59 (0,48-3,20)
6	284	4	3,2	1,41 (0,59-3,13)	1,23 (0,55-2,63)
7	336	5	3,67	1,05 (0,53-1,90)	1,31 (0,78-2,18)
			Grand mean†	1,27 (1,05-1,63)	1,27 (0,77-2,05)

*Mean values. Range in parenthesis.

†Grand mean. Range of means in parenthesis.

The results of 7 experiments in which cell electrophoretic mobilities were measured in the cylindrical microelectrophoresis apparatus are summarized in Table 4.4. As measured with the apparatus, control neutrophils had electrophoretic mobilities ranging from 0,77 to 2,05 (mean 1,27) $\mu m.\ volts^{-1}.\ cm.\ sec^{-1}$. Corresponding values for cytotaxin-treated cells were: range 1,05 to 1,63 (mean 1,27) $\mu m.\ volts^{-1}.\ cm.\ sec^{-1}$. As is evident from these data mobilities of cytotaxin-treated cells did not differ significantly from those observed for corresponding control cells in any individual experiment.

Discussion

The data presented in this section indicate that the treatment of cells with chemotactic material increased their net negative surface

charge when measured on a CPE apparatus. This effect was not apparent when the cylindrical microelectrophoresis apparatus was used. This discrepancy is readily explicable by the fact that the ionic strength, and hence conductivity, of the suspending medium in the cylindrical apparatus was too high to permit voltage gradients higher than 6 volts/cm without excessive heating. The buffer used in the CPE apparatus (10^{-3}M veronal buffer) had a much lower conductivity so that gradients of 100 volts/cm could easily be sustained. Since there is no reason, *a priori*, to believe that the two techniques differ in any other fundamental, physical manner, it is reasonable to presume that the effect of the casein supernatant fluid upon cell surface charge was real, but required a relatively high voltage gradient for its detection.

It is, therefore, justifiable to conclude that the incubation of cells with a chemotactic solution derived from casein in some way increased the net negative surface charge density of these cells. There are, however, a number of reasons that preclude interpreting this observation as having major relevance for the phenomenon of chemotaxis. Firstly, this effect of cytotaxin was observed under highly unphysiological circumstances, even by *in vitro* standards! The cells were studied in a grossly hypotonic medium and, despite the fact that viability studies indicated reasonable survival, it would be inappropriate to assume that the results obtained could necessarily have been valid for isotonic media.

Secondly, the cytotaxin used was not by any means a pure solution of a single chemotactic compound. It almost certainly contained contaminating, biologically inactive impurities that might well have been responsible for inducing the charge difference observed. It would, therefore, be unjustified to claim, on the basis of these findings, that it is characteristic of chemotactic compounds that they induce a negative charge on responding cell surfaces as part of their mode of action.

If the results reported in this section contribute anything to the understanding of cytotaxin action, it is in the neutral sense that they are consistent with the hypothesis that chemotaxins alter the net charge on the cell surface. The implications of this hypothesis for chemotaxis and cell adherence have been discussed.

Chapter V

Esterase activation and the chemotactic response

Introduction

Leucocyte chemotaxis is readily demonstrable *in vitro* by a number of reproducible and reliable techniques that should, by established biological precedent, have provided the basis for definitive studies on the molecular mechanisms involved in this phenomenon. Yet most of the work that has been published on this subject has been descriptive or correlative. Relatively few serious attempts have been made to define the sequence of biochemical processes whereby the stimulus of the chemotactic gradient is transduced into effective, directional cellular movement.

Perhaps the most notable (and most often quoted) of those who have addressed their experimental efforts to the fundamental aspects of the chemotactic response have been the American workers, Dr. E.L. Becker and Dr. P.A. Ward. This section of my thesis is largely concerned with a discussion of their work and with a report of my own attempts to confirm and develop their observations.

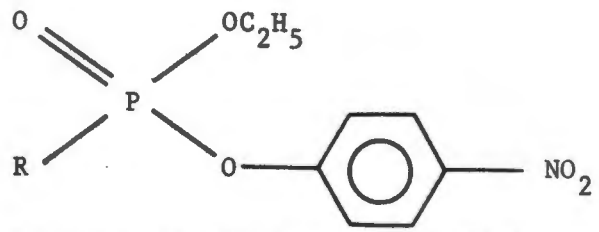
In formulating their experimental approach to this subject, it is apparent that Becker and Ward assumed that chemotaxis might be an "activatable" phenomenon (in the sense that resting cells could be stimulated to directional movement) and that enzymatic processes of one sort or another should be involved in this activation. Since Ward's first major paper on the mechanisms of chemotaxis (170) was published while he was working in Dr. H.J. Müller-Eberhard's laboratories it is not surprising that esterases, - well recognized for their activatable function as complement components, - were selected as likely enzymes for further study in chemotaxis.

It is also apparent that Becker and Ward decided that inhibitors, with presumably specific pharmacological actions, could be used to establish

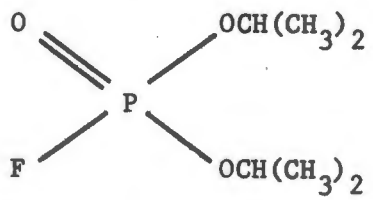
the role of the esterases concerned and to achieve their characterization.

In a series of papers (14,16,17,167,168,169) published between 1967 and 1972, these workers made and interpreted the following observations using the trimolecular, activated complement-component complex, $\overline{C567}$, as the chemotaxin:-

(i) When neutrophils were pre-incubated in the presence of para-nitrophenyl ethyl phosphonate esters, washed free of unbound inhibitor and then tested in Sykes-Moore type chambers, it was found that their chemotactic responsiveness was diminished. These inhibitors have the general formula



where R represents an alkyl, a phenylalkyl, an ω-aminoalkyl or an ω-chloroalkyl group, and they belong to the group of organophosphorous compounds of which diisopropylphosphofluoridate (DFP)



is perhaps the best known example. These compounds are potent inhibitors of those proteases and esterases that possess a serine residue at the active site and there is good reason to believe that they act by binding to the hydroxyl carbon of the serine. Ward and Becker therefore concluded that chemotaxis required the activity of a cellular esterase that was already present in an active state since it was available for organophosphorous inhibition.

The inhibition they observed with this experimental protocol - i.e. pre-incubation with inhibitor, wash and then test - they referred to as "cell-dependent inhibition".

(ii) They also used a modified experimental protocol, in which cells were pre-incubated in medium; centrifuged and resuspended (to simulate the washing procedure) and then tested for chemotaxis in the *presence* of phosphonate esters in the cell compartment and chemotaxin in the lower compartment. With this protocol inhibition of chemotaxis was also demonstrable. However, (and this is the crucial point upon which their entire theme rests) the inhibition observed with this protocol differed from that seen with the first in that, with certain esters (e.g. R = amino propyl or R = 5-aminopentyl) inhibition was encountered when the inhibitor was present during the assay but not when it was present only during pre-incubation. They therefore referred to the inhibition seen with this experimental procedure as "chemotactic factor-dependent inhibition" and ascribed it to the action of the phosphonate ester on an esterase, usually present in the inactive form (and hence inaccessible to the inhibitor) that was activated by interaction with the chemotaxin and hence rendered susceptible to inhibition. This esterase they referred to as the "activatable esterase of chemotaxis".

(iii) Neutrophils pre-incubated with chemotaxin, washed and then tested for chemotaxis showed diminished responsiveness - a phenomenon referred to by Ward and Becker as "deactivation". Certain phosphonate esters when present during pre-incubation protected against this deactivation. Those esters that

protected were the same - both structurally and kinetically - as those that gave rise to "chemotactic factor-dependent inhibition". They concluded, therefore, that the "activatable esterase" was involved in "deactivation".

(iv) Derivatives of aromatic amino acids also protected against deactivation whereas non-aromatic amino acids, carboxylic acid and phosphonate esters did not. They, therefore, concluded that the activatable esterase was a serine esterase with specificity for aromatic substrates.

(v) Using a spectrophotometric assay, with synthetic esterase substrates, the authors then showed that rabbit neutrophils possess esterases capable of catalysing the hydrolysis of acetyl DL-phenylalanine-β-naphthyl ester. Inhibition studies with para-nitrophenyl ethyl 5-chloropentyl phosphonate revealed the presence of one esterase, susceptible to inhibition over the range $2x10^{-9}$M to $6x10^{-9}$M and a second, inhibited over the range $2x10^{-7}$M to $6x10^{-6}$M. These they referred to as "esterase 1" and "esterase 2" respectively.

(vi) "Esterase 1" showed "inhibition profiles" (i.e. percentage inhibition of activity as a function of carbon chain length within any homologous series of phosphonate esters) similar to those seen with the "activatable esterase".

(vii) A third esterase could be demonstrated which was not inhibited by prolonged incubation with high concentrations of any of the inhibitors. This third type of esterase they referred to as "esterase 3".

(viii) Acetate esters, they found, could protect against phosphonate-ester induced "cell-dependent inhibition" and inhibited chemotaxis when present during the assay. The "activated esterase", they concluded, was an acetate-esterase.

In summary, then, Becker and Ward had suggested that an effective chemotactic response required the functional integrity of two serine esterases - "activated" and "activatable".

It is reasonable for investigational purposes to presume that the chemotactic response involves three mechanisms:

(i) *An afferent mechanism* by means of which the information contained in the chemotactic gradient is received and relayed to the cell.

(ii) *A central mechanism* by means of which the information is interpreted and the response coordinated.

(iii) *An efferent mechanism*, involving the motile apparatus of the cell, by means of which directional movement takes place.

Although there was little in the Ward and Becker experiments to indicate whether the esterase was involved in the afferent, central or the efferent phases of the response, the very fact that there was a proesterase that could be activated by specific interaction with chemotactic material indicated the likelihood that it would fit in to an afferent - central - efferent scheme as an important component. I, therefore, decided to enlarge upon the Ward and Becker experiments using slightly different technical procedures to characterize the activatable esterase.

My approach was based upon the prediction that unstimulated and stimulated cells should show differences in the amount of the "activatable

esterase/s" on or in the cell. I chose to use two techniques.

Firstly, I used conventional enzymatic and analytical techniques to compare, both qualitatively and quantitatively, the esterase content of cytotaxin-treated and untreated cells.

Secondly, since the enzymes concerned were serine esterases, I expected them to bind irreversibly to radioactive DFP. By polyacrylamide gel electrophoresis and autoradiography or section-counting, I then hoped to define the molecular weights and isoelectric points of any esterases, labelled in this manner, that were present in greater amounts on or in neutrophils that had been stimulated with chemotaxin. By appropriate competition with para-nitro-phenylguanidinobenzoate (NPGB), I hoped to distinguish (^{3}H)DFP-binding proteins as esterases or proteases. The experimental justification for this manoeuvre has been reviewed by Shaw (143). In essence, this approach depends upon the fact that NPGB has a higher association constant for proteases than does DFP and it inhibits the binding of (^{3}H)DFP. This is not the case with esterases.

Materials and Methods

Cells

Rabbit peritoneal exudate cells were harvested 18h after intraperitoneal injection of 50 ml of 7% w/v sodium caseinate as described in the Appendix. The cells were pelleted by light centrifugation (300xg; 10 min; 20^{o}C) and resuspended as indicated below.

Histochemical identification of neutrophil esterases

Cells were resuspended in Gey's BSS-2% HSA to a final concentration of approximately 2x10^6 cells/ml. Boyden chambers were assembled with 3μm

filters. The lower compartments contained either 2,5 ml of 1% w/v casein in saline (test) or Gey's BSS-2% HSA (unstimulated). Three millilitres ($6x10^6$ cells) of the cell suspension was added to the upper compartment and the chambers were incubated at 37°C for 3h.

After incubation, the filters were removed from the chambers; rinsed briefly at 4°C in formol-calcium fixative; and incubated individually at 4°C in 5 ml volumes of the same fixative for 4h.

After fixation each filter was rinsed briefly in gum-sucrose solution, transferred individually to a 5 ml volume of the same solution and soaked at 4°C for 24h.

Excess gum-sucrose solution was removed by taking each filter through six sequential changes of distilled water to a covered Wheaton dish[1] containing 5 ml of incubation medium containing the desired substrate. The filters were allowed to incubate at 37°C for various periods of time up to 1½h.

Excess incubation medium was removed by taking each filter through six sequential changes of distilled water. The cells were then counterstained with Mayer's carmalum or haematoxylin.

Excess stain was removed by rinsing the filters in distilled water. The stain was then allowed to differentiate in distilled or tap water. Filters were then dehydrated and mounted for histochemical evaluation.

Frozen tissue sections with well documented, positive enzyme activity were used as positive controls for the staining procedure.

Details of the histochemical procedures are given in the Appendix.

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(1) Arthur H. Thomas Company, Philadelphia, Pennsylvania, U.S.A. catalogue number 3836-F22.

EFFECTS OF CYTOTAXIN ON NEUTROPHIL ESTERASES.

Three experimental protocols were followed to study the effects of cytotaxin on cellular esterases.

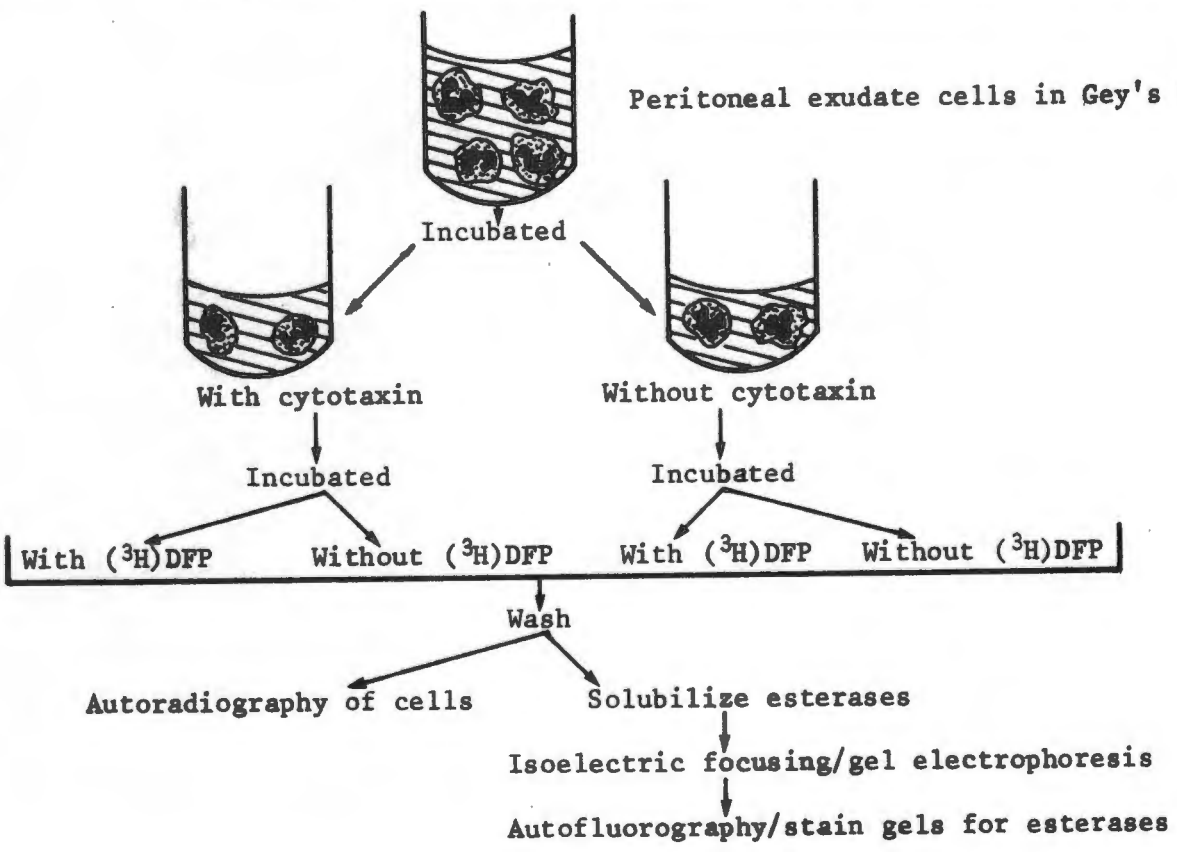
Protocol A distinguished patterns of esterase activity on the basis of isoelectric focusing or polyacrylamide gel electrophoresis. Esterases were detected as (^{3}H)DFP-binding proteins or by a staining procedure for esterase enzymes.

Protocol B distinguished (^{3}H)DFP-binding proteins on the basis of NPGB competition.

Protocol C used a spectrophotometric technique that measured the ability of cellular esterases to catalyse the hydrolysis of an aromatic synthetic substrate.

Protocol A: Identification of esterases as DFP-binding proteins and as enzymes in isoelectric focusing gels.

The protocol followed may be summarized diagrammatically as follows:-



Pelleted cells were gently resuspended in Gey's BSS-2% HSA to a final concentration of $1x10^7$ to $2x10^7$ cells/ml. This suspension was divided into two equal portions, each containing approximately 10^7 to 10^8 cells, depending on yield. A measured volume of CUF-cytotaxin solution was added to one portion to give a final concentration of cytotaxin established, by Boyden chamber assay, to be stimulatory. The other portion received an equivalent volume of saline and served as the unstimulated control.

Stimulated and control suspensions were then incubated at 37°C for 60 min with constant shaking. Following incubation, each portion was again divided into two equal samples; one sample was used for (^{3}H)DFP labelling and the other reserved for biochemical identification of the esterases.

(^{3}H)DFP[1] (specific activity 1 to 10 Ci/mmole) dissolved in propylene glycol was added to stimulated and control cell samples (each now representing one fourth of the original cell suspension) to give 0,5 to 5,0 µCi/10^7 cells. These samples were then incubated for a further 30 min at 37°C with constant shaking. After incubation the cells were washed three times with measured volumes of Gey's BSS-2% HSA. Scintillation counting of aliquots of the wash fluids indicated that this procedure removed greater than 99,0% of the unbound (^{3}H)DFP. Trypan blue exclusion revealed no significant loss of viability at this stage.

A small aliquot (approximately 10^6 cells) was removed for autoradiography and the remaining cells were then pelleted by centrifugation (300xg; 10 min; 20°C); drained; vigorously resuspended on a vortex mixer in 1 ml per 5 to $10x10^7$ cells of 0,3% v/v Triton X-100 in 0,03M EDTA.2Na pH 7,0; and left to stand at 4°C for 18h. Undissolved material was

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(1) The Radiochemical Centre, Amersham, England

removed by centrifugation and the supernatant fluid, containing more than 80% of the original cell-bound radioactivity, was examined, without further treatment, by isoelectric focusing in polyacrylamide gels according to the method of Young and Bittar (190) as detailed in the Appendix. For most experiments 0,5x7 cm rod gels were used and samples (0,1 to 2 mg protein; $5x10^3$ to $1x10^5$ cpm) were included in the gels before polymerization. With this technique the distribution of radioactivity in the gels was determined by slicing the rod into 1 mm length discs with a gel slicer(1) and counting the radioactivity in each segment after combustion to ^3HOH in an automatic combustion apparatus(2).

In other experiments, isoelectric focusing and SDS gel electrophoresis were performed in 130x200x1,6 mm slab gels using the apparatus described in the Appendix. When vertical slab gels were used, samples were layered into individual wells at the top of each gel after polymerization. Each well contained 100 to 500 µg of protein and 10^4 to 10^5 cpm. The distribution of radioactivity in the gel was determined by autofluorography using the method of Bonner and Laskey (19) details of which are given in the Appendix. Running conditions for the individual isoelectric focusing experiments - i.e. Ampholine pH range, gel concentration etc. - are given in the legends to the Figures in the results section.

Samples of cell suspension for the biochemical identification of esterases in isoelectric focusing gels were treated and electrophoresed in exactly the same manner as described for the (^3H)DFP labelled cells with the exception that (^3H)DFP was not added. Esterases in the focused gels were identified by the method of Young and Bittar (190)

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(1) The Mickle Laboratory Engineering Company, Gomshall, Surrey, England

(2) Packard Instrument Company Incorporated, Downers Grove, Illinois, U.S.A

described in the Appendix. Briefly, this involved incubating the gels in substrate-containing medium and identifying enzymatically active bands by the characteristic, coloured reaction products released by the hydrolysis of synthetic substrate. When inhibitors were used to define the esterases in terms of their susceptibility to diisopropylfluorophosphate (DFP), para-chloromercuribenzoate (PCMB) and silver nitrate ($AgNO_3$), the gels were incubated in 1,0 mM or 10,0 mM concentrations of the inhibitors in 0,01M tris/H_2SO_4 pH 7,4 for 30 min at room temperature. The gels were then removed and stained for esterases, in the presence of inhibitor, with substrate dissolved in tris/H_2SO_4 buffer instead of McIlvaine's buffer. Details are given in the Appendix. Experimental details are given in the legend to the Figures in the Results section.

Cells taken for autoradiography were streaked onto clean slides with a camel hair paint brush. The slides were air-dried; fixed in 100% methanol for 10 min and processed for autoradiography by the dipping technique (88) with Kodak NTB/2 liquid emulsion(1). After 7 to 14 days exposure at -20°C in dessicated containers the slides were developed, fixed and stained with Giemsa stain for microscopic evaluation of the (^3H)DFP binding.

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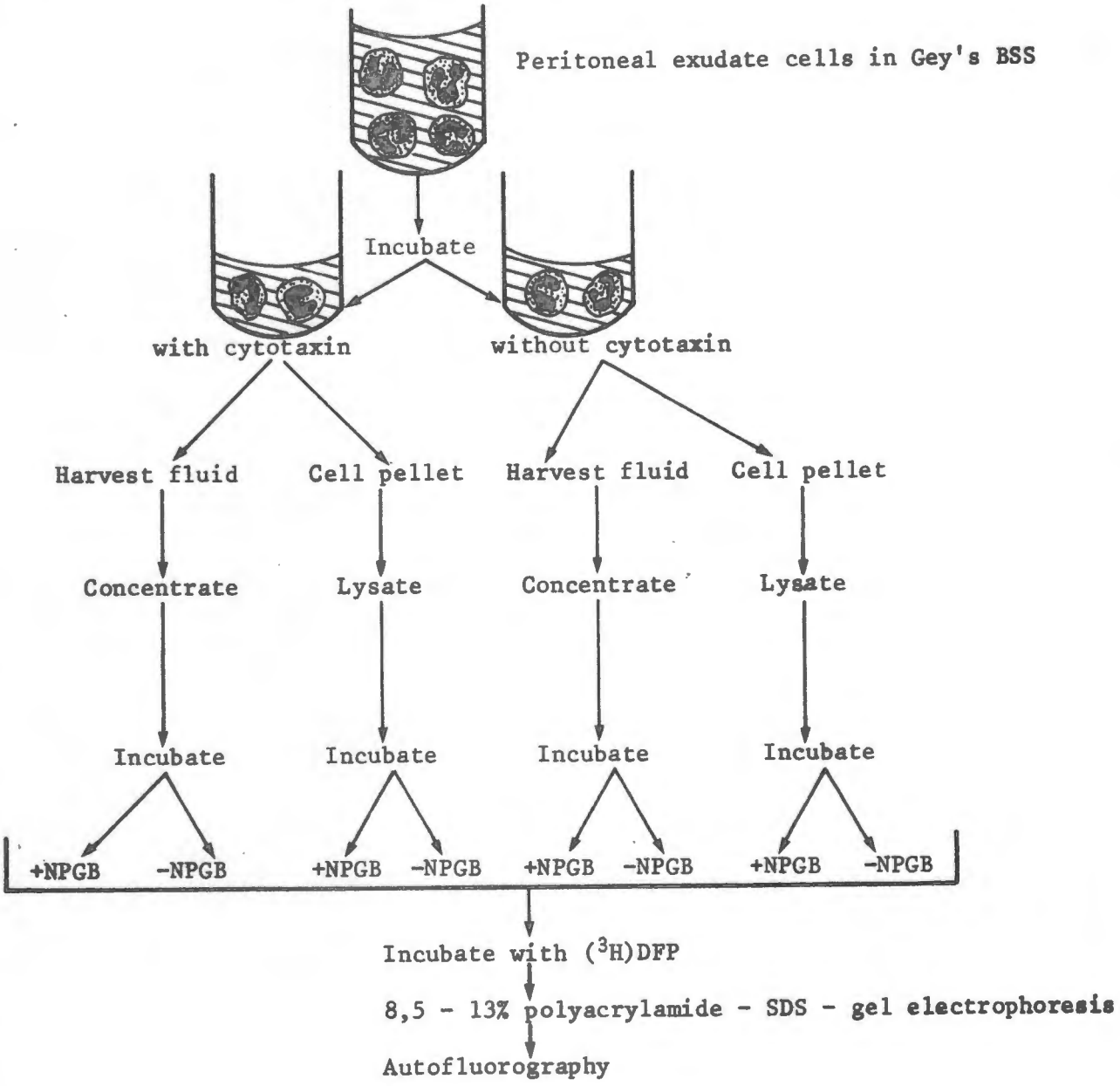
(1) Eastman Kodak Company, Rochester, New York. U.S.A.

Protocol B: *Identification of DFP-binding proteins with and without NPGB competition in polyacrylamide gels.*

The experimental protocol may be summarized diagrammatically as follows:-



Cells were resuspended in Gey's BSS (without human serum albumin) to a final concentration of 10^7 cells/ml and incubated with or without CUF-cytotaxin as described under Protocol A. At the end of the 60 min

incubation at 37°C the cells were centrifuged to give "stimulated" and "unstimulated" cells and "stimulated" and "unstimulated" supernatant fluids (referred to as harvest fluids, HF).

The cell pellets were extracted for 24h at 4°C or 2h at 37°C with 1,5 ml of extraction buffer per 10^7 cells. The extraction buffers had the following composition: 0,3% v/v Triton X-100; 0,03M EDTA.2Na pH 7,0; or 0,5% v/v Nonidet P_{40}; 0,15M NaCl; 0,05M tris/HCl pH 8,0. The extraction mixture was centrifuged to remove cellular debris and the supernatant fluids were retained as "stimulated" and "unstimulated" cell extracts.

To each of the harvest fluids and the cell extracts was added one tenth volume of 1,0M tris/H_2SO_4 pH 7,3. The harvest fluids were then placed in dialysis bags and concentrated to approximately one tenth of the required volume by covering with dry Sephadex G150. After concentration the harvest fluids and extracts were dialysed at 4°C for 72h against 3x100 volume changes of 0,1M tris/H_2SO_4 pH 7,3. The protein concentrations of the dialysed samples were determined spectrophotometrically from the 260 nm/28 nm absorbance values and adjusted with 0,1M tris/H_2SO_4 pH 7,3 to 500 µg/1,5 ml. At this stage two 1,5 ml aliquots of each of the four samples were taken. To one aliquot from each pair was added 15 µl of 0,01M NPGB[(1)] in dimethylsulfoxide (DMSO); the other aliquot received 15 µl of DMSO. The samples were incubated at room temperature in the dark for 2h after which 10 µCi of (^{3}H)DFP dissolved in 15 µl of propylene glycol was added to each sample. Incubation was continued for 18h at room temperature in the dark. The final concentration of DFP achieved

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(1) A gift from the Drug Development Branch, Division of Cancer Treatment, National Cancer Institute, National Institute of Health, Bethesda, Maryland, U.S.A.

at this stage varied from $0,7x10^{-6}$M to $3x10^{-6}$M, depending on the specific activity of the (^{3}H)DFP supplied.

After incubation a 25 µl volume of aqueous 10% w/v recrystallized sodium dodecylsulphate (SDS) was added to each sample. A 25 µl aliquot was then removed for radioassay and the samples were dialysed at room temperature for 48h against 2x250 volume changes of 0,167% w/v SDS. After dialysis, a 25 µl aliquot of the retentate was removed for radioassay to determine the degree of labelling. The samples were then lyophilized in individual conical test tubes. Each residue was dissolved in 50 µl of distilled water and 10 µl of 1,5M tris/HCl pH 8,8 followed by 10 µl of a solution containing glycerol and 0,4% w/v bromophenol blue in the ratio 4:1, were then added. After thorough mixing each 70 µl sample was added to a sample well on a 130x180x1,6 mm slab gel containing 0,1% SDS and a linear polyacrylamide gradient from 8,5 to 13% w/v. A 20 mm 5% polyacrylamide spacer gel containing 0,1% SDS was used to concentrate the samples. The samples were electrophoresed at a constant current of 8 mA for 16h at room temperature. The slab gels were fixed and stained in 0,05% w/v brilliant comasie blue in methanol : acetic acid : water (30:10:60) for 18h at room temperature and destained in several changes of methanol : acetic acid : water (30:10:60). The gels were then dehydrated and processed for autofluorography as described by Bonner and Laskey (19) and detailed in the Appendix.

Protocol C: The spectrophotometric esterase assay using the aromatic amino acid ester, acetyl-DL-phenylalanine-β-naphthyl-ester as substrate.

The method I used was essentially similar to that described by Ward and Becker (169). Rabbit peritoneal neutrophils were harvested in the usual manner, pelleted by centrifugation and resuspended to a final

concentration of $2x10^7$ cells/ml in 0,1% w/v gelatin containing 0,15M NaCl buffered to pH 7,3 with 0,005M tris/HCl (TGS).

Duplicate tubes were prepared containing 0,1 ml of the cell suspension ($2x10^6$ cells), 0,4 ml of substrate solution and 50 µl of a predetermined stimulatory amount of CUF-cytotaxin or an equivalent volume of saline. The substrate solution was prepared freshly for use, by diluting one part of a stock solution (0,016 gm acetyl-DL-phenylalanine-β-naphthyl ester[1] dissolved in 6 ml N'N-dimethylformamide and 12 ml acetone) with 9 parts of sterile TGS.

The reaction was initiated by the addition of cells and the tubes were incubated at 22°C or 37°C, with occasional agitation, for various periods of time. After removal of an aliquot (25 to 50 µl) of the suspension for viability studies, 1 ml volumes of acetone were added to each tube to precipitate the protein which was removed by centrifugation after not less than 15 min at 22°C. The supernatant fluid was decanted and 150 µl volumes of the diazonium coupler, fast red[2] (0,016 gm/10 ml distilled water) were added to each tube. The tubes were then left at room temperature for at least 30 min before the colour intensity of the resultant orange-yellow complex was read at 485 nm against a distilled water blank.

In all experiments, control tubes containing no cells but only the cytotaxin or an equivalent volume of saline were included for the minimum and maximum incubation times. This was done to determine the spontaneous hydrolysis of the ester in the presence or absence of the cytotaxin.

Cell viability was estimated by trypan blue exclusion according to the method given in the Appendix.

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(1) Schwarz Mann, Orangeburg, New York, U.S.A.

(2) Koch Light Laboratories Ltd., Colnbrook, Buckinghamshire, England.

Results

Histochemical identification of esterases.

The use of indoxyl acetate as a non-specific esterase substrate and Mayer's carmalum as counter stain, gave the most satisfactory results in that localized enzyme activity could readily be identified as intense blue regions against a generally pink cellular background. As is shown in Figure 5.1 neutrophils showed well defined, discrete regions of esterase activity randomly superimposed upon the cytoplasmic area. The degree of staining seen over each cell varied from a few isolated sites to fairly intense, almost confluent, stippled staining. This same gradation was evident both in cells that had migrated into or through the filter in response to cytotaxin and in cells that had migrated randomly without exposure to cytotaxin; there was no discernible difference between the overall intensity or pattern of staining shown by these two populations of cells (Figure 5.1). In comparison with neutrophils, macrophages, as evidenced by the deeper and more confluent staining they exhibited, appeared to contain larger amounts of esterases (Figure 5.1). Once again, there was nothing in the appearance of stimulated or randomly migrating cells to suggest that cytotaxin in any way affected the overall quantity or distribution of esterases in these cells.

The results obtained with α-naphthyl acetate or acetyl-DL-phenylalanine-β-naphthyl ester as substrates and hexazotized pararosanilin as the coupling reagent were somewhat disappointing in that uniform, brick-red staining of all macrophages and neutrophils was seen (Figures 5.2 and 5.3) without well-defined discrete sites of enzyme activity that might have localized the enzyme to regions of the cell and so facilitated detection

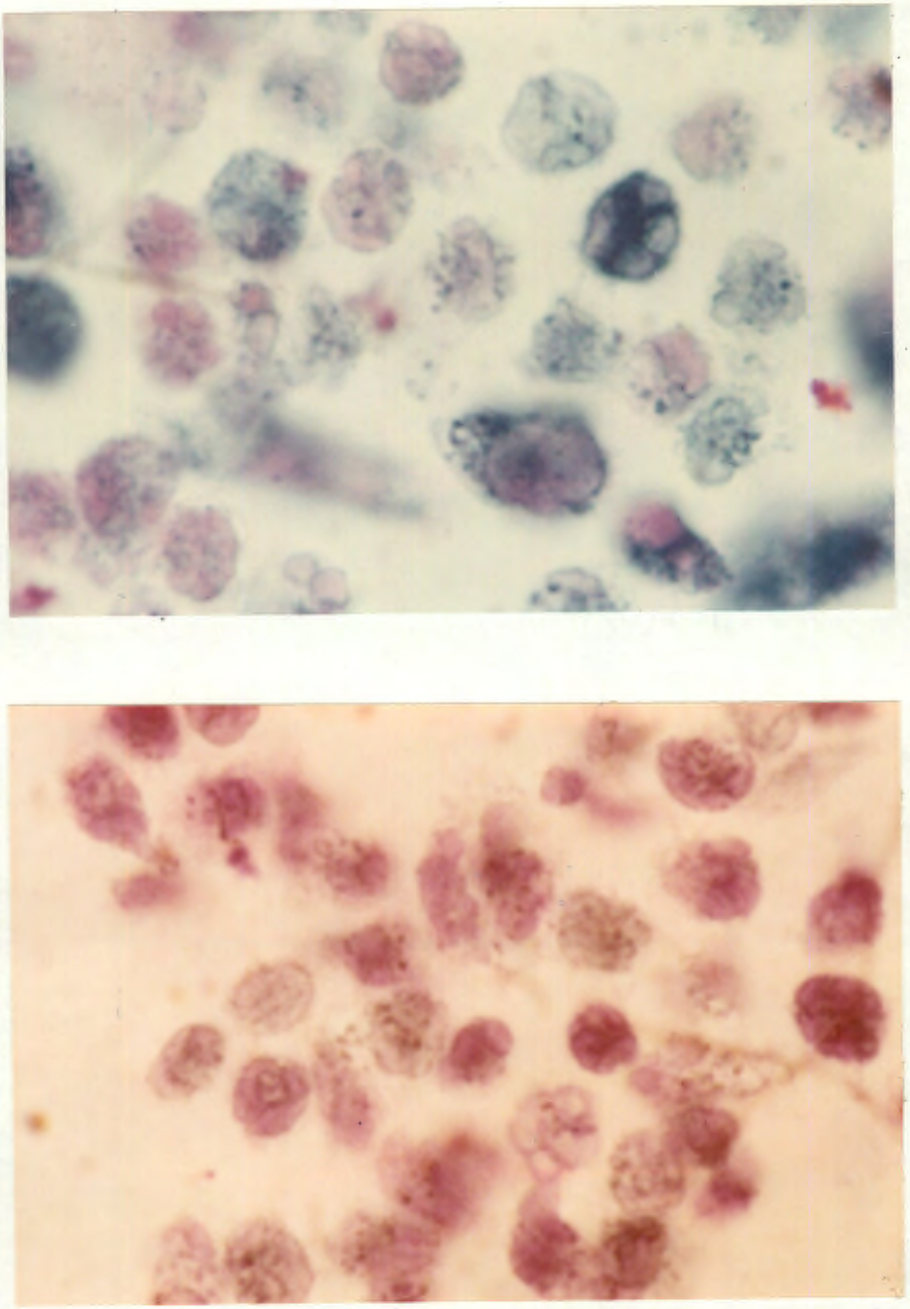


Figure 5.1 Esterase content of migrating and stationary cells.

Photomicrographs showing cells at the upper surface of a negative filter (i.e. stationary cells) (*above*); and cells at the lower surface of a positive filter (i.e. cells that have migrated through the filter in response to 1% casein) (*below*).

The cells have been stained with 5-bromo-indoxyl-acetate for non-specific esterases. With this stain, neutrophils show fine punctate spots of enzyme activity whereas macrophages (visible in the upper photographs) show intense uniform cytoplasmic staining.

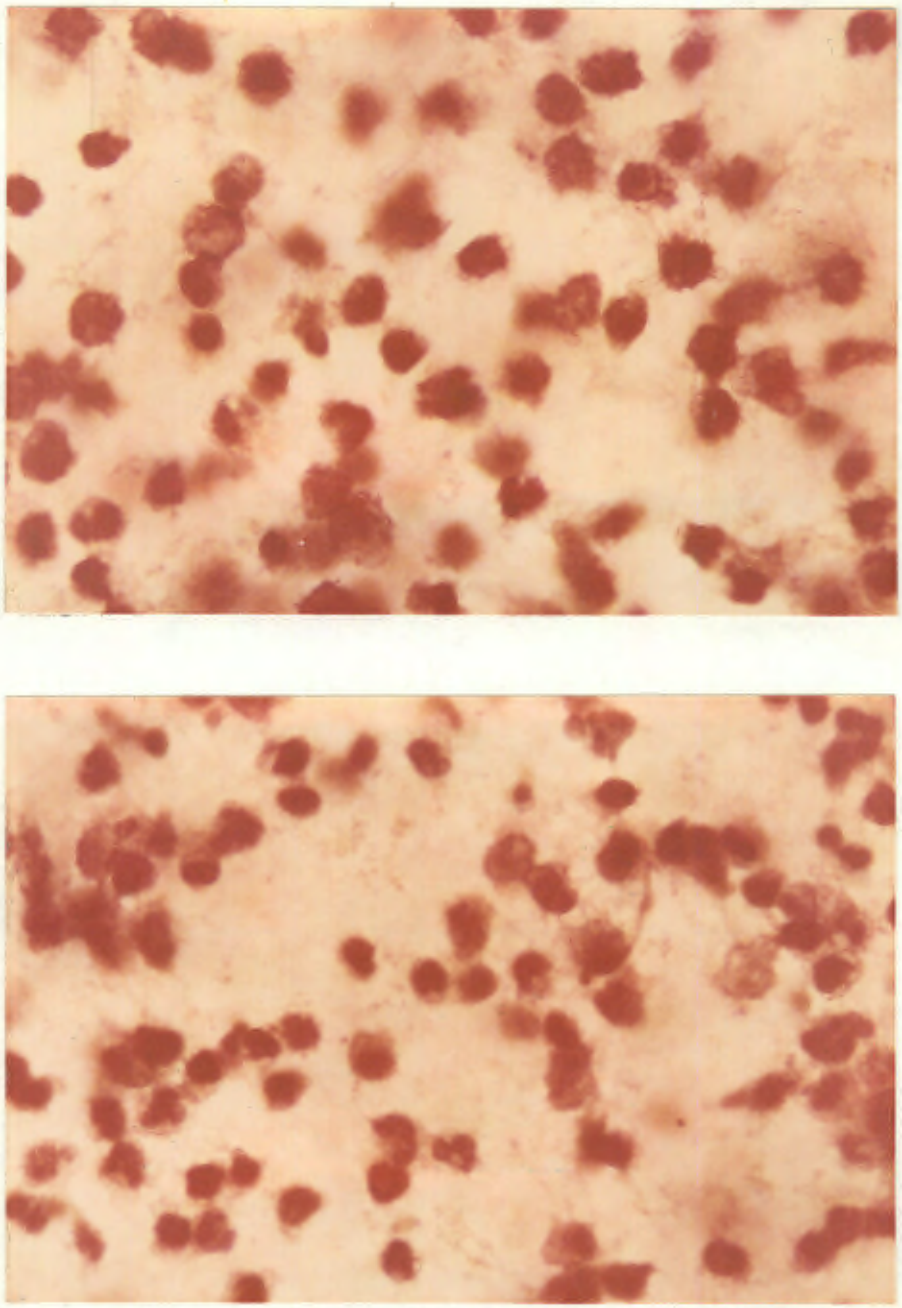


Figure 5.2 Esterase content of migrating and stationary cells.

Photomicrographs showing cells at the upper surface of a negative filter (i.e. stationary cells)(*above*); and cells at the lower surface of a positive filter (i.e. cells that have migrated through the filter in response to 1% casein) (*below*).

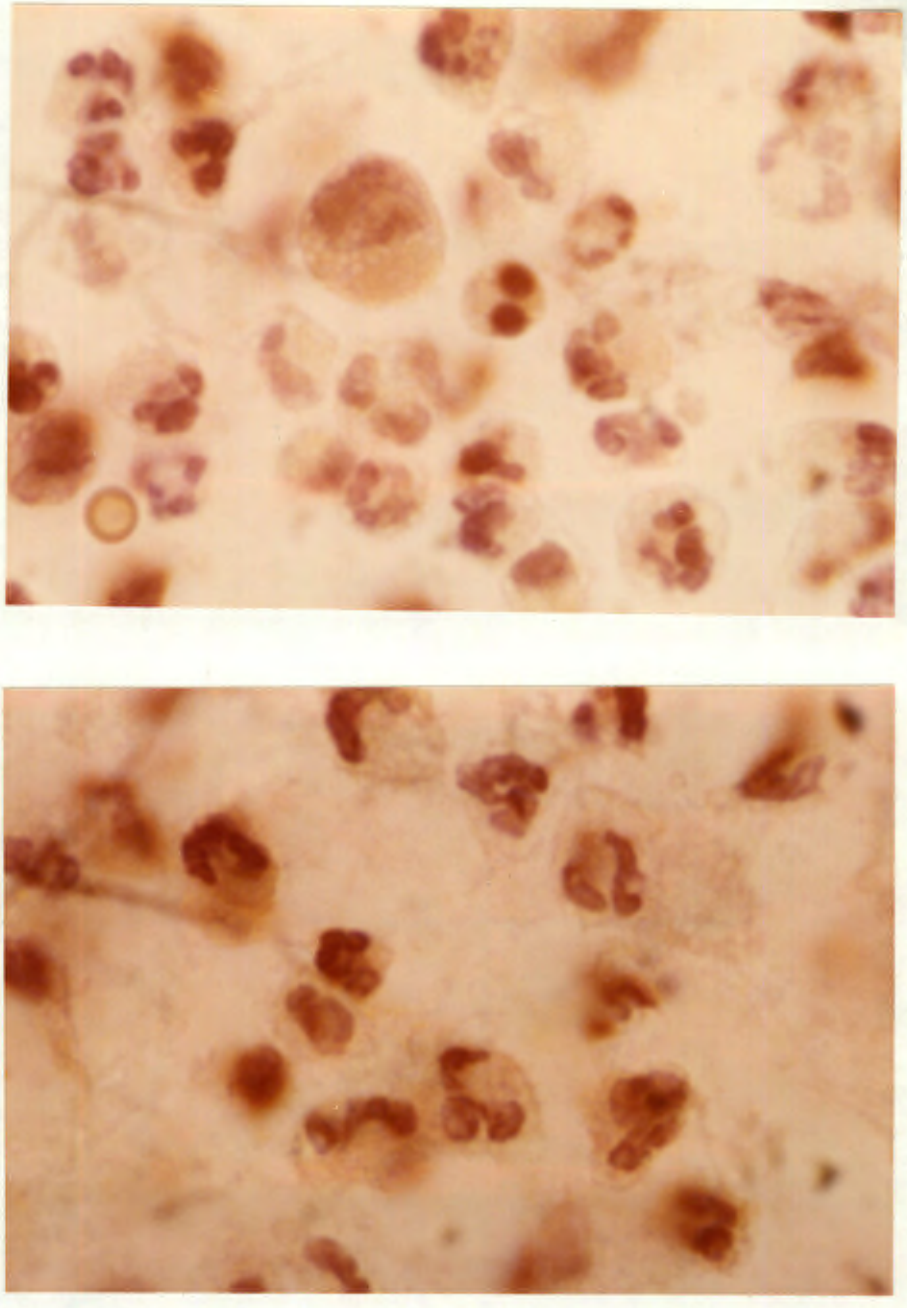


Figure 5.3 Esterase content of migrating and stationary cells.

Photomicrographs showing cells at the upper surface of a negative filter (i.e. stationary cells) (*above*); and cells at the lower surface of a positive filter (i.e. cells that have migrated through the filter in response to 1% casein) (*below*).

The cells have been stained with acetyl-DL-phenylalanine-β-naphthyl and hexazotized pararosanalin for aromatic esterases.

Note that stationary and migrating cells show similar staining intensities.

of any effect of cytotaxin. This pattern appeared to be unrelated to the time of incubation with substrate (from 5 to 60 min) and was similar in chemotactically stimulated and randomly migrating cells.

These experiments, therefore, failed to reveal any visibly obvious differences in the esterase activity of stimulated and control cells.

Incorporation of (^{3}H)DFP into cell-associated macromolecules.

When stimulated and control cells were exposed to radioactive DFP according to Protocol A, the extent to which radioactivity was incorporated into cell-associated macromolecules varied, between experiments, from approximately 0,6% to 27% (Table 5.1). I am unable to offer a satisfactory explanation for this variability. The amount of incorporation obtained did not correlate with cell number, nor could it be attributed to varying degrees of contamination with cell species such as mononuclear cells or erythrocytes that might have contained larger amounts of esterases.

Within any individual experiments, however, the amount of (^{3}H)DFP incorporated into cellular macromolecules in the presence or absence of cytotaxin was the same. To the extent, therefore, that (^{3}H)DFP labelling can be used to obtain a quantitative estimate of cellular serine esterases, one may conclude that chemotactic stimulation had no measurable effects upon these enzymes.

Isoelectric focusing of DFP-binding proteins and cellular esterases.

When (^{3}H)DFP labelled cellular extracts from a total of four experiments were prepared according to Protocol A and subjected to isoelectric focusing on polyacrylamide gel rods containing Ampholines designed to provide different pH ranges, the results summarized diagrammatically

in Figures 5.4 to 5.7 were obtained. It should be noted in interpreting these results that the gel slicing procedure introduced three technical difficulties that I was unable to overcome entirely satisfactorily. Firstly, the gels required freezing onto the slicer stage, both to immobilize them and to provide a suitable consistency for slicing; since solid carbon dioxide snow was used for this purpose, reliable pH measurements of the segments could not be obtained. Secondly, despite care in casting and handling the rods, it proved impossible to achieve or maintain the precise uniformity of gel dimensions required for perfect register between different gel rods from the same experiment; in two cases (Figure 5.6) the radioactive profiles have been adjusted, for presentation, so as to bring the main peaks into register. Thirdly, since the slicing procedure proceeded in sequential 1 mm steps from the cathodal or the anodal end of the rod some degree of resolution of separated peaks was inevitably lost.

Table 5.1

(^{3}H)DFP incorporation into rabbit peritoneal exudate cells treated with saline or cytotaxin (CUF-2 and CUF-3)

Expt no.	Rabbit	No. of cells (x 10^{-6})	(^{3}H)DFP added (μCi/10^7 cells)	% Incorporation of ^{3}H (+ saline)	(+cytotaxin)
1	872	53	3,8	0,80	0,62
2	189	138	1,5	4,88	5,54
3	864	59	0,3	5,31	5,67
4	866	56	0,8	2,08	1,88
5	865	32	3,4	12,71	13,16
6	891	91	2,8	17,15	17,54
7	*892	201	1,2	27,22	25,38
8	894	328	0,8	14,83	14,38

*The peritoneal exudate cells were heavily contaminated with erythrocytes.

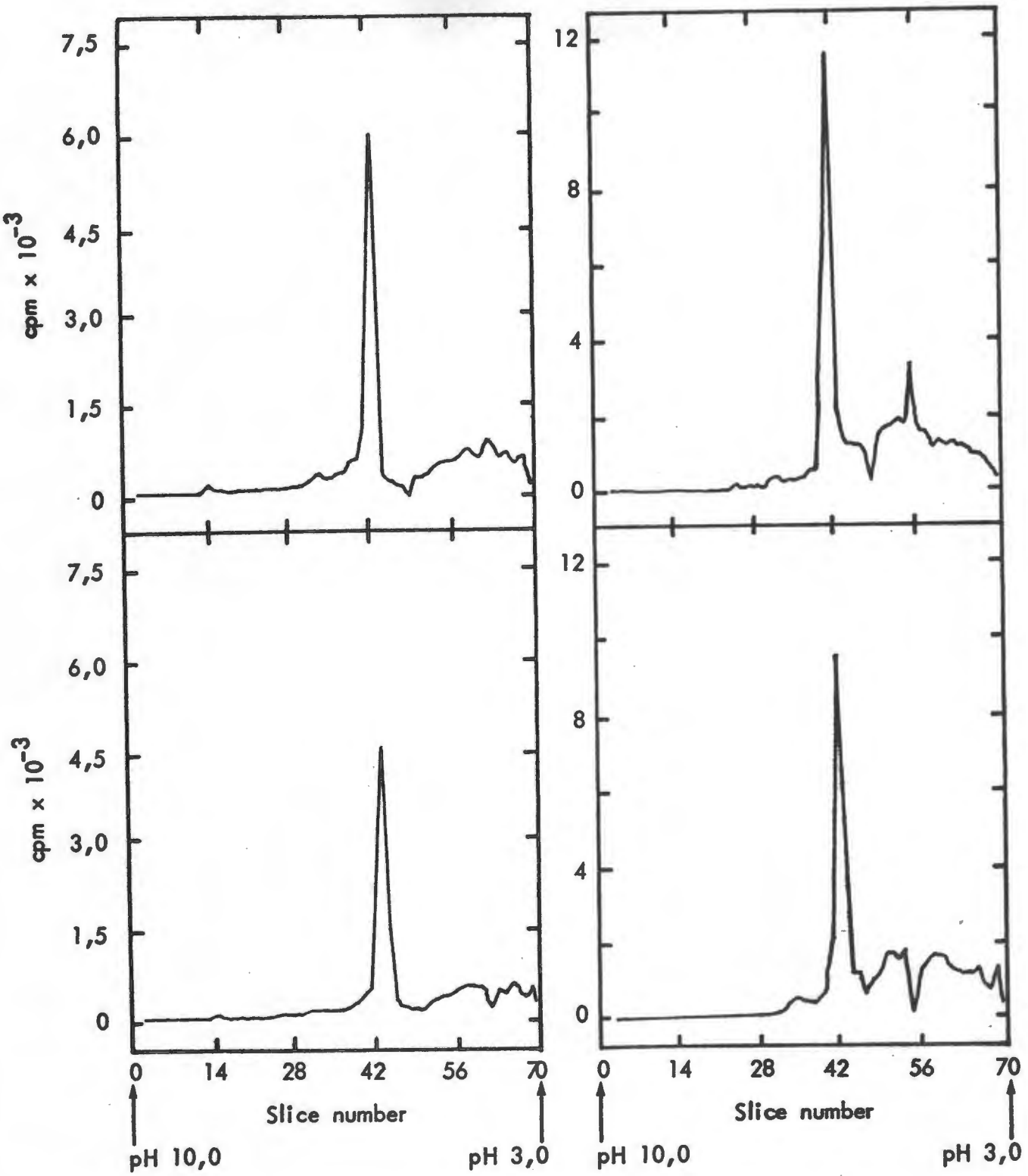


Figure 5.4 Effect of cytotaxin on (^{3}H)DFP-binding proteins in cell lysates.

Results from a single experiment in which untreated cells (*above*) and cytotaxin (CUF-2)-treated cells (*below*) were incubated with (^{3}H)DFP according to Protocol A. The cells were lysed and radioactive proteins (1mg -*left*; 2mg - *right*) were electrophoresed in 5% polyacrylamide isoelectric focusing rod gels containing 1,4% Ampholines, pH 3 to 10.

After electrophoresis for 22h at 4°C gels were sliced and counted to obtain the radioactivity profiles shown.

No significant qualitative or quantitative effects of cytotaxin treatment upon (^{3}H)DFP binding proteins could be detected.

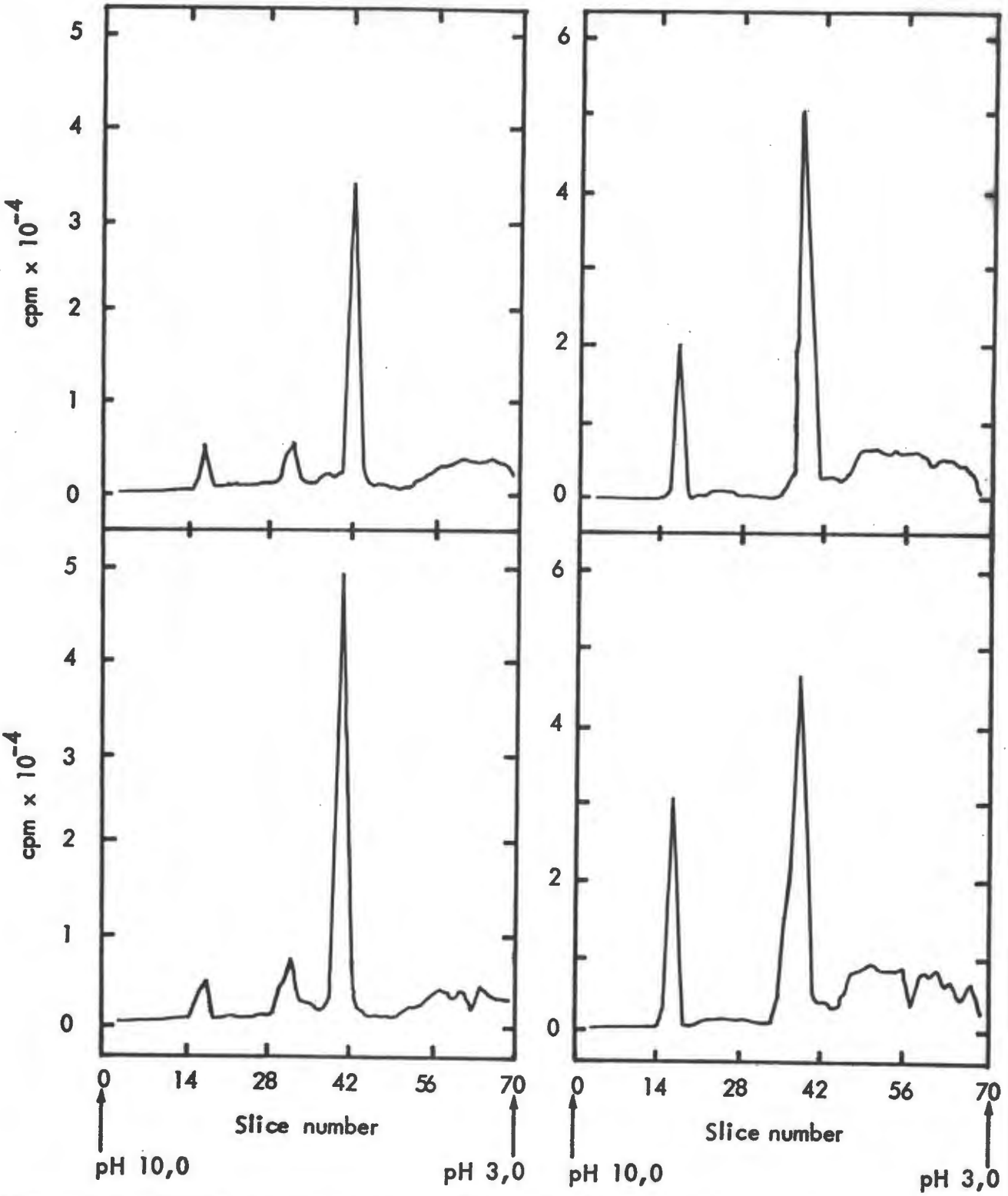


Figure 5.5 Effect of cytotaxin on (^{3}H)DFP-binding proteins in cell lysates.

Results from a single experiment in which untreated (*above*) and cytotaxin (CUF-2)-treated cells (*below*) were incubated with (^{3}H)DFP according to Protocol A. The cells were lysed and radioactive proteins (0,5mg - *left*; 1mg - *right*) were electrophoresed in 5% polyacrylamide isoelectric focusing rod gels containing 1,4% Ampholines, pH 3 to 10. After electrophoresis for 22h at 4°C gels were sliced and counted to obtain the radioactivity profiles shown.

No significant qualitative or quantitative effect of cytotaxin treatment upon (^{3}H)DFP-binding proteins could be detected.

Figure 5.6

Figure 5.6 Effect of cytotaxin on (^{3}H)DFP-binding proteins in cell lysates.

Results from a single experiment in which untreated cells (*above*) and cytotaxin (CUF-2)-treated cells (*below*) were incubated with (^{3}H)DFP according to Protocol A. Radioactive lysate proteins (2mg - *left*; 1mg - *right*) were electrophoresed in 5% polyacrylamide isoelectric focusing rod gels containing 1,5% Ampholines, pH 3 to 10. After electrophoresis for 21h at 4°C gels were sliced and counted to obtain radioactivity profiles shown. (Due to technical difficulties during slicing, the profiles had to be adjusted to bring the main peaks into register for presentation.)

No significant qualitative or quantitative effects of cytotaxin treatment upon (^{3}H)DFP-binding proteins could be detected.

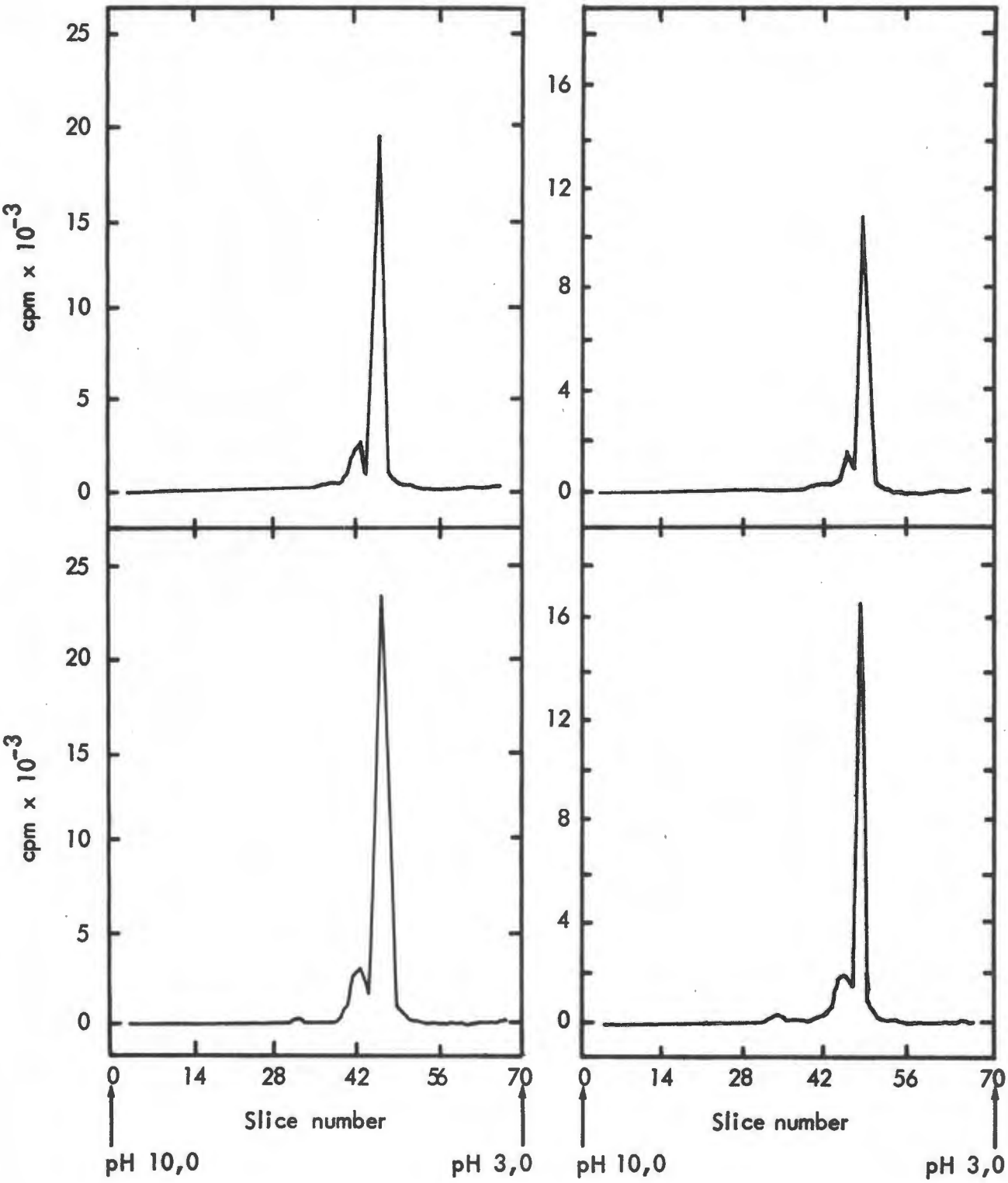

cpm x 10^{-3}
25
20
15
10
5
0
16
12
8
4
0
14
28
42
56
70
Slice number
pH 10,0
pH 3,0

Figure 5.6

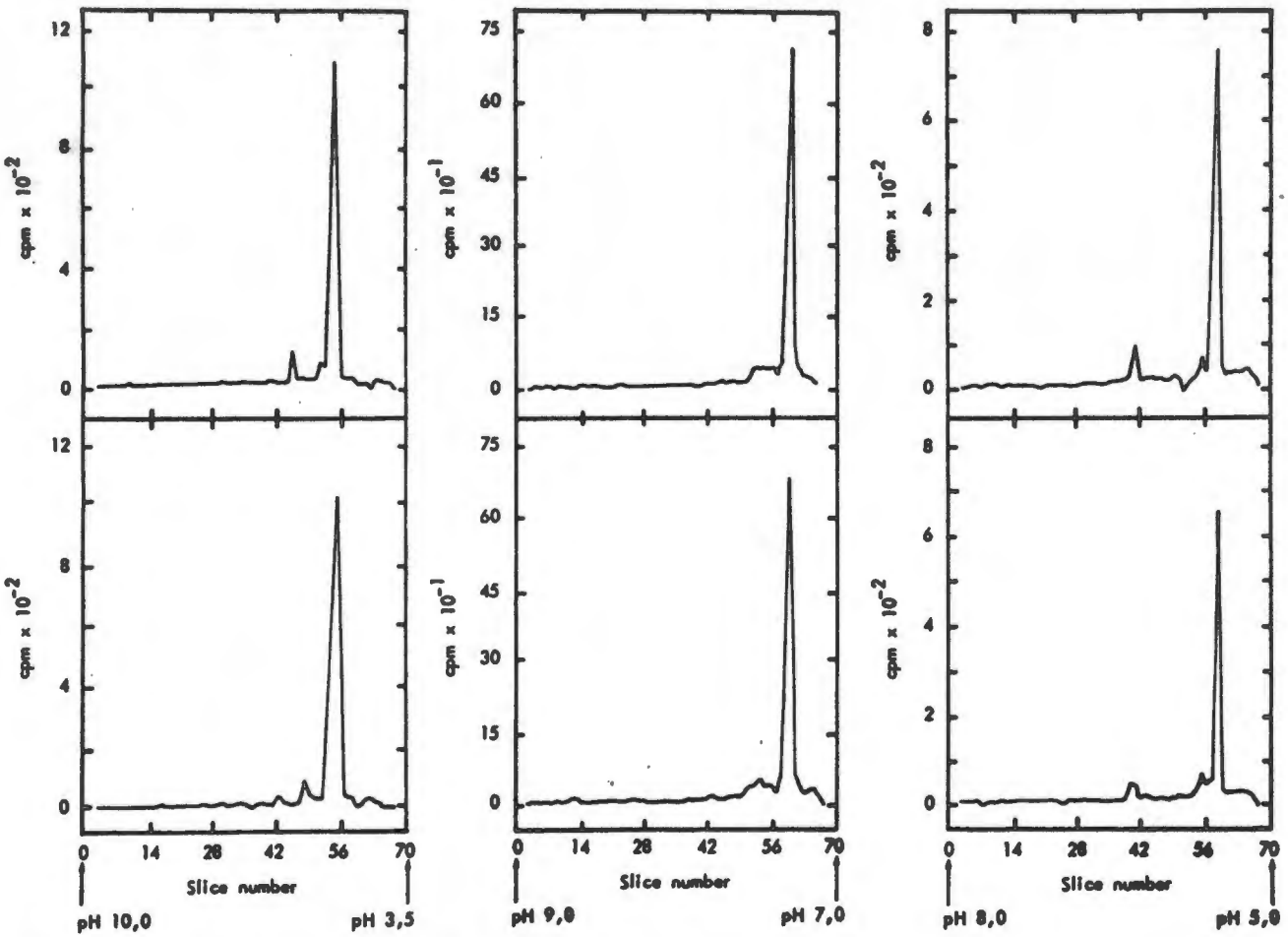


Figure 5.7 Effect of cytotaxin on (^{3}H)DFP-binding proteins in cell lysates.

Results from a single experiment in which untreated (*above*) and cytotaxin (CUF-2)-treated cells (*below*) were incubated with (^{3}H)DFP according to Protocol A. The cells were lysed and radioactive proteins (0,25mg in each case) were electrophoresed in 5% polyacrylamide gels containing 1,3% Ampholines, pH 3,5 to 10 (*left*); pH 7 to 9 (*centre*) and pH 5 to 8 (*right*). After isoelectric focusing for 20h at 4°C, gels were fixed and sliced to obtain the radioactivity profiles shown.

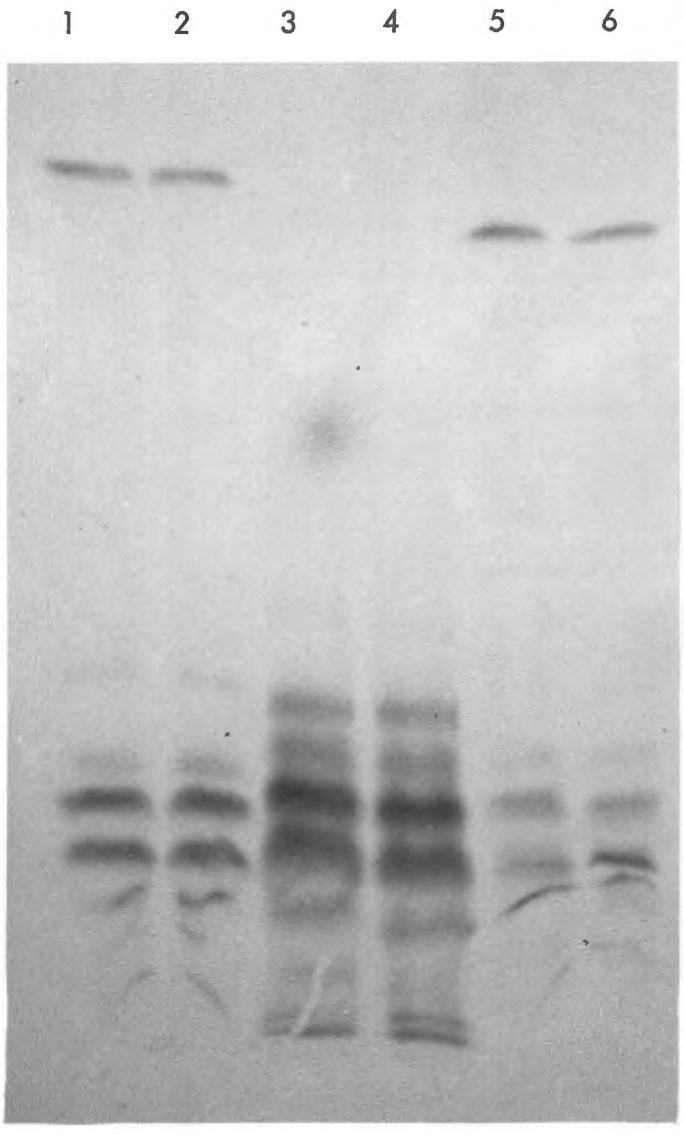
Once again, no significant qualitative or quantitative effects of cytotaxin treatment on cellular DFP-binding proteins could be demonstrated.

Despite these reservations, it is evident from the radioactivity profiles depicted in Figures 5.4 to 5.7 that all cellular extracts contained DFP-binding protein with a major peak corresponding to a pI of approximately 5 - 6 on the pH 3,0 - 10,0 Ampholine gels. This peak, as was to be expected, was situated nearer the anode when pH 7,0 to 9,0 or pH 5,0 to 8,0 Ampholines were used (Figure 5.7). On one occasion (Figure 5.5) an additional, clearly defined peak was located in the more basic region of the gel. Since the cells for this experiment came from a rabbit of a genetically distinct strain, I have assumed, without further investigation and for lack of an obvious alternative explanation, that this basic DFP-binding protein reflected a genetically controlled charge difference in a serine esterase or protease that was not present in the other animals used.

It is apparent, from the data depicted in Figures 5.4 to 5.7, that incubation of the cells with cytotaxins had no significant or consistent effect upon either the pattern or the amounts of esterase detected by this technique.

Slab gel isoelectric focusing of (^{3}H)DFP-labelled cell extracts prepared according to Protocol A, followed by autofluorography revealed the presence of at least five radioactive bands that were similar in number, isoelectric points and intensities in cytotaxin-treated and control cells. These results are presented in Figure 5.8. Electrophoresis of these extracts on 8,5 to 13% polyacrylamide gel slabs in the presence of 0,1% SDS with subsequent autofluorography revealed two major (^{3}H)DFP-binding bands and a number of minor bands. No differences between cytotaxin-treated and control cell extracts were apparent (Figure 5.9). The isoelectric focusing technique in slab gels provided superior resolution of radioactive bands and, by virtue of the fact that different samples could be electrophoresed in adjacent slots, afforded a better visual comparison of these bands.

Figure 5.8 Effect of cytotaxin treatment on cellular (^{3}H)DFP binding proteins



Autofluorograph of (^{3}H) DFP-labelled protein prepared from three rabbits according to Protocol A and electrophoresed in a 5% polyacrylamide vertical slab gel containing 3,3% pH 6 to 8 Ampholines; 0,6% pH 5 to 7 Ampholines and 0,6% pH 3,5 to 10 Ampholines.

Tracks 1 and 2: Rabbit 891; without and with cytotaxin (CUF-3)-treatment respectively.

Tracks 3 and 4: Rabbit 892; without and with cytotaxin (CUF-3)-treatment respectively.

Tracks 5 and 6: Rabbit 894; without and with cytotaxin (CUF-3)-treatment respectively.

Cytotaxin treatment had no apparent effect upon the number or intensity of bands capable of binding radioactive DFP.

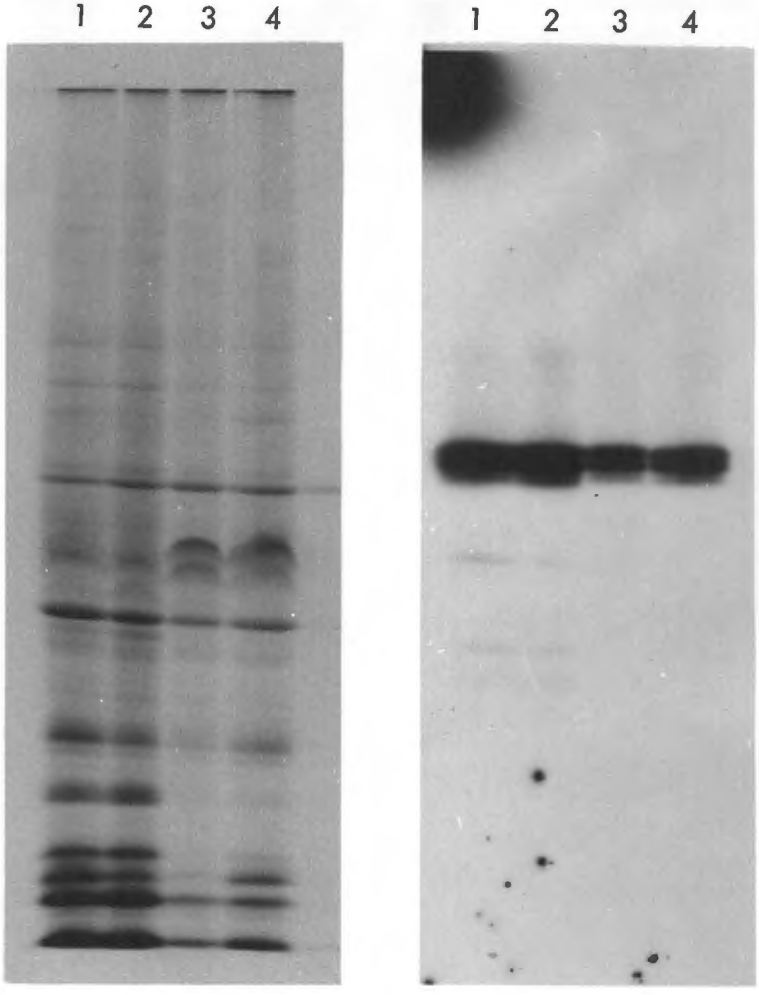


Figure 5.9 Effect of cytotaxin treatment on(^{3}H)DFP binding protein.

(^{3}H)DFP-labelled proteins were prepared from peritoneal cells from two rabbits according to Protocol A, and electrophoresed in a vertical slab gel containing a linear 8,5 to 13% polyacrylamide gradient and 0,1% SDS. The gel was fixed and stained for protein (*left*) after which autofluorography was prepared (*right*).

Tracks 1 and 2: Rabbit 865; with and without cytotaxin (CUF-2) respectively.

Tracks 3 and 4: Rabbit 864; without and with cytotaxin (CUF-2) respectively.

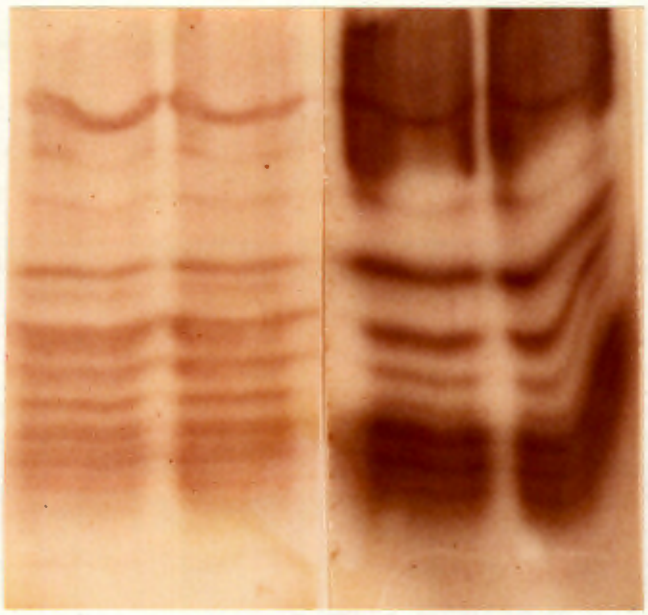
Note that cytotaxin-treatment had no effect upon the pattern of DFP-binding protein shown in the autofluorograph.

When rabbit peritoneal cell exudates were examined by isoelectric focusing and stained for esterases with α-naphthyl acetate or indoxyl acetate as substrate, as many as 15 bands of enzyme activity could be detected (Figure 5.10).

The results obtained merit comment in the following respects:

(i) Cells obtained from different rabbits contained esterases that showed different banding patterns when focused in the same gel. Furthermore, the patterns seen with cells from the same rabbit but taken on different occasions also varied. There are many possible explanations for the differences observed. Phenotypic differences between rabbits; variation in cell composition from one exudate to the next; minor degrees of proteolytic or oxidative degradation of the enzymes during preparation; or the formation of coacervates between enzymes and contaminating charged proteins - any or all of these may have contributed to the apparent charge heterogeneity of the enzymes and to the variations observed.

(ii) Despite the variability and complexity of the banding patterns encountered, one or two major bands were consistently found in rod gels with apparent isoelectric points between pH 4,8 and 5,4 (Figure 5.11). These bands were resolved into at least 4 major bands by isoelectric focusing in slab gels with isoelectric points between 5,8 and 6,6 (Figure 5.12). I have concluded that the enzymes focusing between pH 4,8 and 5,4 on rod gels and between 5,8 and 6,6 on slab gels are the same on the basis of their obvious quantitative preponderance in each case. It is strange that the major fraction should have focused at different pH's in the rod and slab systems and I have no entirely satisfactory explanation for this discrepancy. It was, however, consistently observed



1 2 3 4

Figure 5.10 Effect of cytotaxin treatment on peritoneal cell esterases. Comparison of staining methods.

Results of a single experiment in which the esterases were extracted from cells treated according to Protocol A and electrophoresed in 6% polyacrylamide vertical slab gels containing 2,5% pH 5 to 7 Ampholines; 1,25% pH 4 to 6 Ampholines and 1,25% pH 3,5 to 10 Ampholines.

Tracks are numbered as follows:

1 and 3 : The same extract from untreated cells

2 and 4 : The same extract from cytotaxin (CUF-3) treated cells.

Tracks 1 and 2 were stained with α-naphthyl acetate/fast garnet GBC; tracks 3 and 4 with indoxyl acetate/fast garnet GBC.

Apart from minor differences, both substrates identified the same esterase bands. Cytotaxin-treated and untreated cells showed identical bands of enzyme activity.

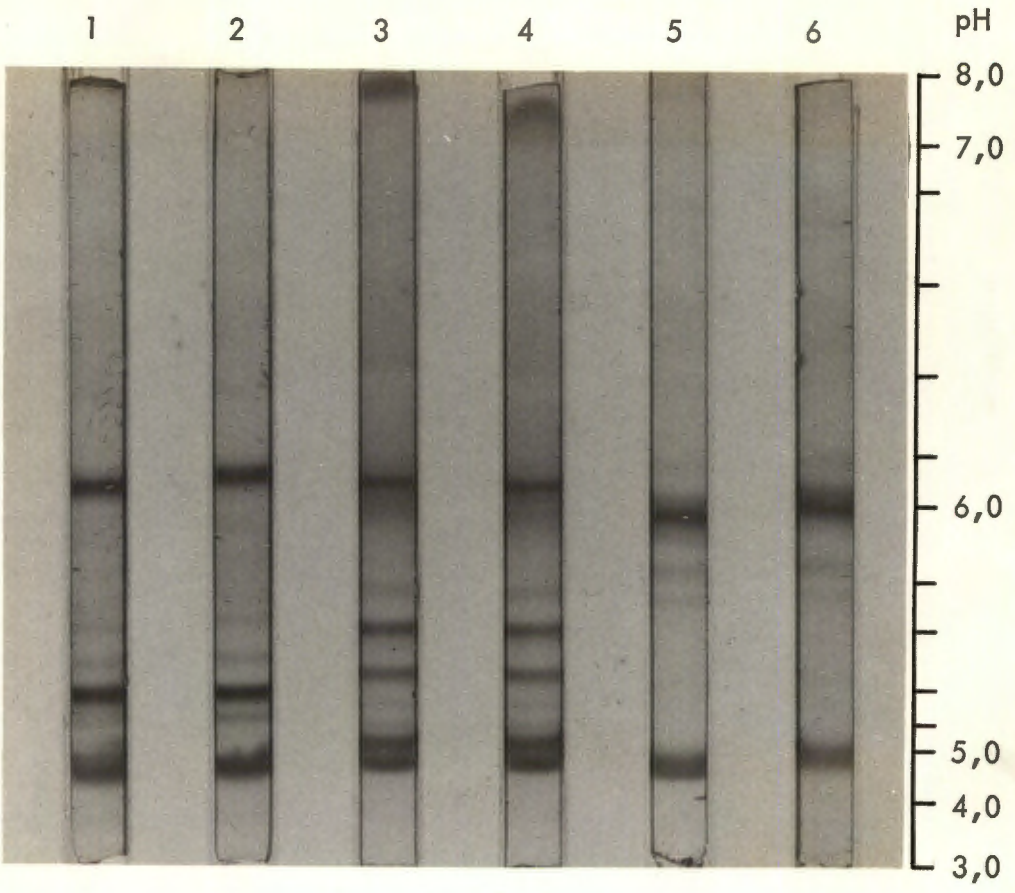


Figure 5.11 Effect of cytotaxin treatment on cell esterases.

Peritoneal exudate cells from three rabbits were treated with or without cytotaxin (CUF-3) according to Protocol A. Triton X-100 cell extracts were electrophoresed in 5% polyacrylamide rod gels containing 1,4% pH 5 to 7 Ampholines, and stained for non-specific esterases with α-naphthyl acetate/fast garnet GBC.

Gels are numbered as follows:

Gels 1 and 2: Rabbit 891; with and without cytotaxin treatment respectively.

Gels 3 and 4: Rabbit 892; with and without cytotaxin treatment respectively.

Gels 5 and 6: Rabbit 894; with and without cytotaxin treatment respectively.

Different esterase banding patterns were observed for each rabbit. Cytotaxin treatment had no effect upon the patterns observed.

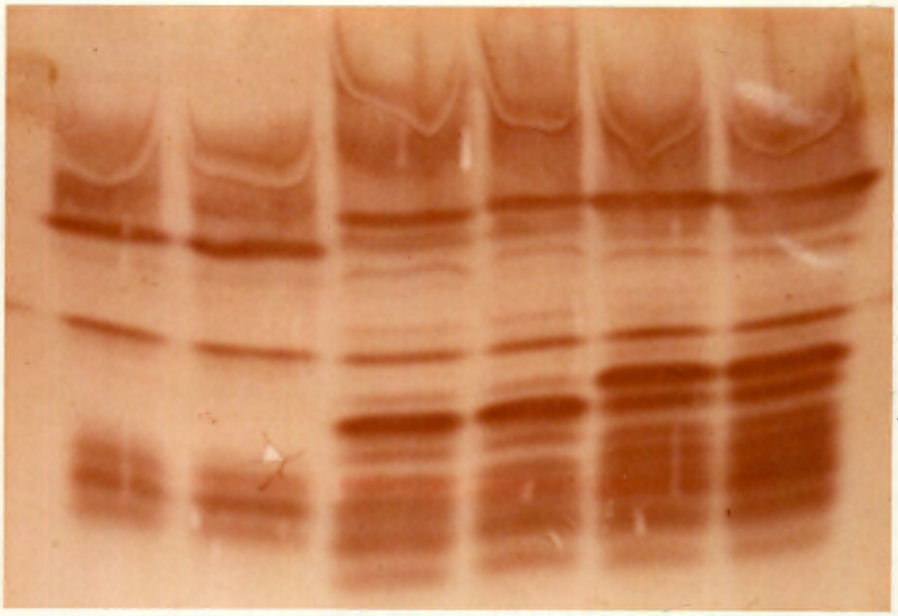


Figure 5.12 Effects of cytotaxin treatment on cell esterases.

Peritoneal exudate cells from three rabbits were treated with or without cytotaxin (CUF-3) according to Protocol A. Triton X-100 cell extracts were electrophoresed in 6% polyacrylamide vertical slab gels containing 2% pH 5 to 7 Ampholines; 1% pH 4 to 6 Ampholines; 0,5% pH 5 to 8 Ampholines and 0,5% pH 3,5 to 10 Ampholines. The gel was stained for non-specific esterases with α-naphthyl acetate/ fast garnet GBC.

Tracks are numbered as follows:

Tracks 1 and 2: Rabbit 976; without and with cytotaxin treatment respectively.

Tracks 3 and 4: Rabbit 865; without and with cytotaxin treatment respectively.

Tracks 5 and 6: Rabbit 871; without and with cytotaxin treatment respectively.

Different esterase banding patterns were observed for each rabbit. Cytotaxin treatment had no effect upon the patterns observed.

and relates, I suspect, to technical differences, either in the introduction of the sample or in the procedures necessary to achieve stable pH gradients in the two systems.

(iii) Histochemical procedures for categorizing different esterases present in cells or tissue sections rely on the different susceptibility of these enzymes to various inhibitors. The following table (Table 5.2) abbreviated from Pearse's textbook on histochemistry (127) summarizes generally accepted criteria for the interpretation of inhibitor studies. When slab gels were treated with inhibitors before staining for esterases, the results depicted in Figures 5.13 (a) to (c) were obtained.

Table 5.2

Esterase inhibitor susceptibility.† (modified from Pearse (127)).

No.	Esterase	Complete inhibition $AgNO_3$	PCMB	DFP
1	A-esterase Arom-esterase Organophosphate resistant esterase I	+(10mM)	+(1mM)	o
2	B-esterase Ali-esterase Organophosphate sensitive esterase	+(10mM)	o	+(0,1mM)
3	C-esterase Organophosphate resistant esterase II	+(10mM)	s(1mM)	o
4	Cholinesterases	o	o	+(0,1mM)
	Acetylcholinesterases	o	o	+(0,1mM)
	Pseudocholinesterases	o	o	+(0,1mM)

†The use of symbols +; o and s denote complete inhibition; no effect and enzyme stimulation respectively.

Figure 5.13 (a) to (c)

Figure 5.13 (a) to (c) Effect of cytotaxin treatment and various inhibitors on cellular esterases.

The photographs on the following three pages portray the results of three separate identical experiments in which esterases were extracted from cytotaxin (CUF-3) treated and untreated cells according to Protocol A.

The extracted enzymes were electrophoresed in 6% polyacrylamide vertical slab gels containing 3% Ampholines pH 5 to 8; 0,5% Ampholines pH 5 to 7; and 0,5% Ampholines pH 3,5 to 10.

After electrophoresis each gel was divided longitudinally into 4 strips, each strip encompassing two adjacent tracks.

The strips were then incubated with PCMB (1mM (a); 10mM (b) and (c)); DFP (1mM (a); 10mM (b) and (c)); $AgNO_3$ (1mM (a); 10mM (b) and (c)) or tris buffer(control). After incubation in the various inhibitors, strips were stained for non-specific esterase activity with α-naphthyl acetate/fast garnet GBC.

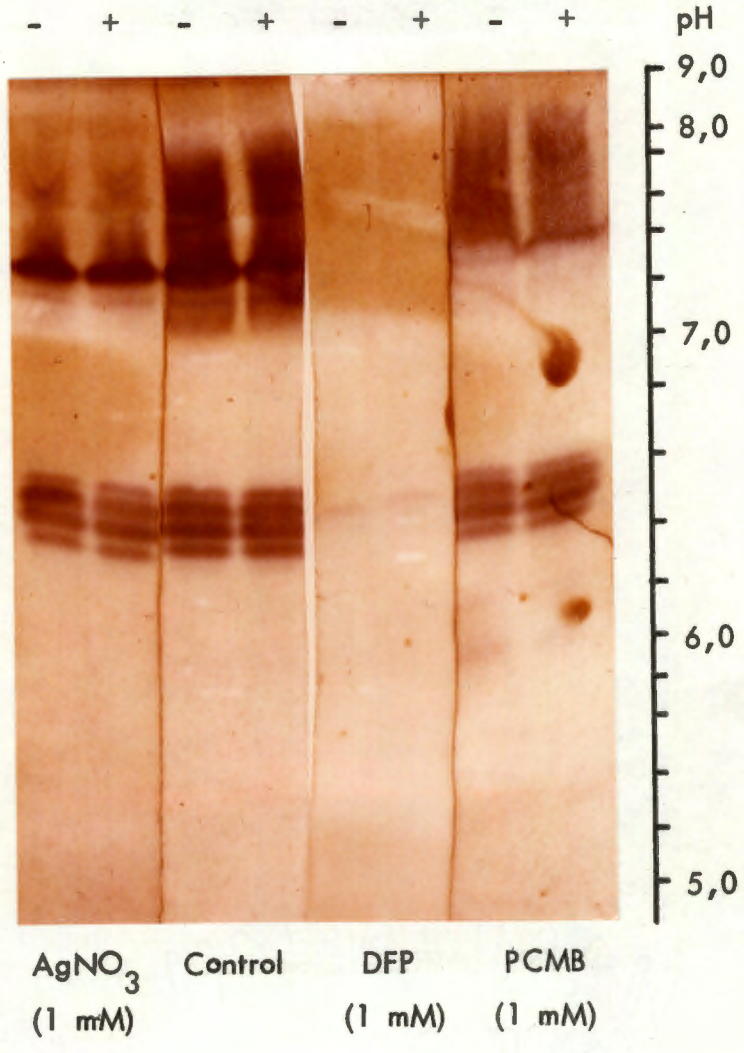
The symbols (+) or (-) above the tracks signify samples obtained from cytotaxin-treated or control cells respectively.

The following comments are appropriate:

(i) In all cases, DFP markedly inhibited enzymatic activity.

(ii) PCMB treatment revealed the presence of minor esterase bands that focused at a pI of approximately pH 5,4 to 6,2. These reproduced best in the photograph shown in Figure 5.13(b).

(iii) Cytotaxin treatment had no effect upon the pattern of enzymatically active bands seen. The apparent exception (Figure 5.13(b)) is discussed in the legend to that figure.



Rabbit 870

Figure 5.13 (a)

Figure 5.13 (b)

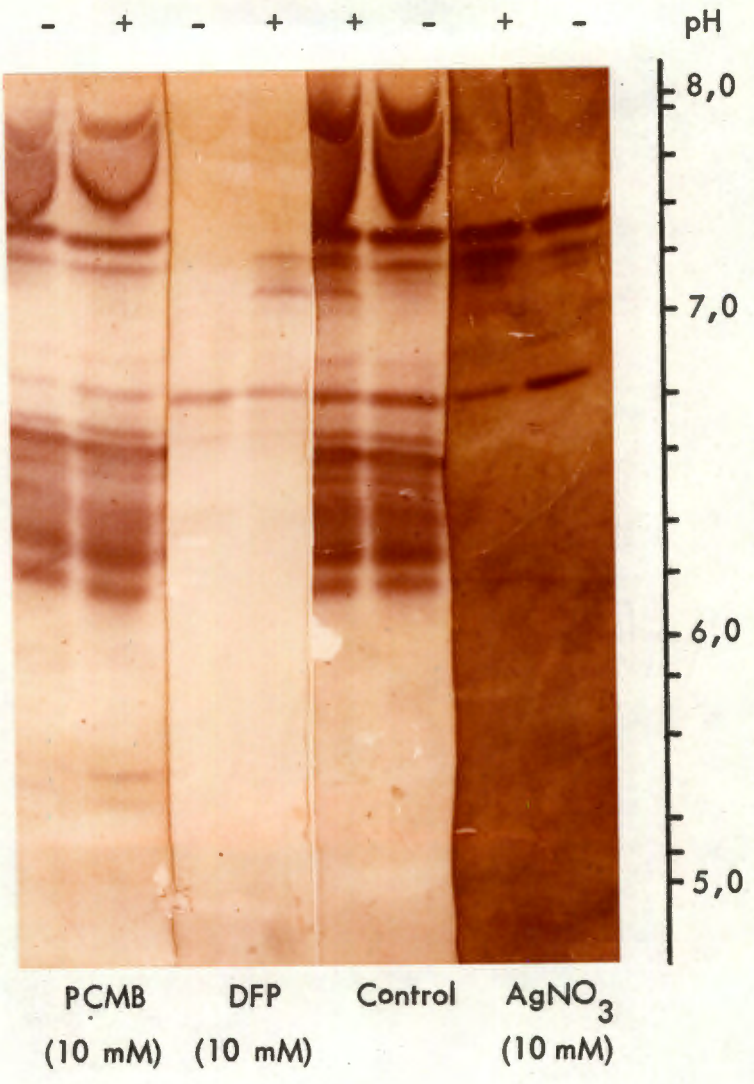
Figure 5.13 (b)

In this experiment, cytotaxin treatment apparently led to the appearance of two DFP-resistant esterase bands that were not present in the extract from cells that had not been exposed to cytotaxin.

These bands, most evident in the DFP-treated strip, can also be seen in the control strip.

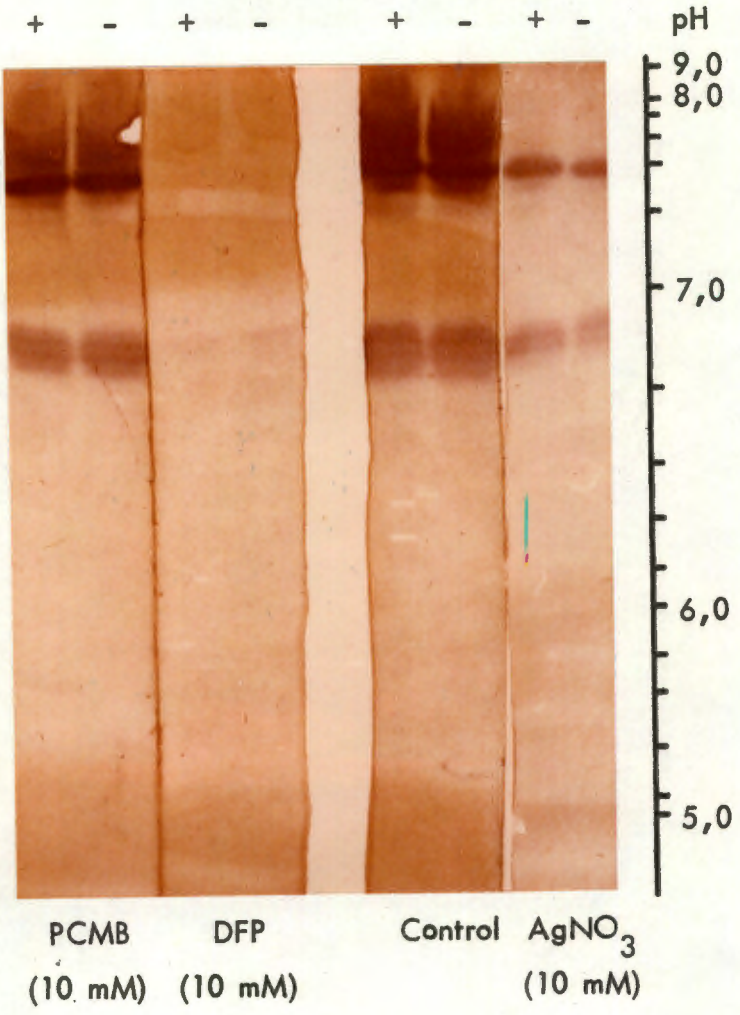
The significance of these findings is dubious, since, when these same extracts were electrophoresed on a previous occasion (Figure 5.12; tracks 3 and 4) the differences were not seen.

It is unlikely that these bands could represent the "activatable esterase" of Ward and Becker (167) since the enzyme they described was sensitive to DFP.



Rabbit 865

Figure 5.13 (b)



Rabbit 869

Figure 5.13 (c)

As can be seen, all esterases were completely or markedly inhibited by 1,0 or 10,0 mM DFP. Silver nitrate (10mM) generally showed overall inhibition although the extent of this varied between experiments. Incubation with 1,0 or 10,0 mM PCMB (a) caused very little, if any, inhibition and (b) activated at least two esterases that focuse- at a pH of approximately 5,4 to 6,2.

(iv) Despite the considerable variation in the isoelectric focusing patterns seen between animals and experiments, extracts prepared from untreated and cytotaxin-treated cells showed identical patterns, provided the cells used were from the same batch. In other words, there were no discernible effects - either quantitative or qualitative - of cytotaxin treatment upon the activity or number of cellular esterases observed.

(v) Comparison of esterase activities from cytotaxin-treated cells and their appropriate untreated controls after exposure of the gels to DFP, $AgnO_3$, or PCMB similarly showed no differences with the one exception illustrated in Figure 5.13(b). In this experiment two bands of DFP-resistant, PCMB-sensitive activity were evident in the cytotaxin-stimulated cell extract that were absent in the control. The significance of these two bands is dubious, since previous isoelectric focusing runs of the *same extract* had failed to reveal their presence (Figure 5.12). In only this one instance did I find any evidence to indicate that cytotaxin treatment led to the appearance or "activation" of esterase activity. It is important to note that the "new" bands were DFP-resistant whereas the "activatable proesterase" of Ward and Becker was allegedly DFP-sensitive.

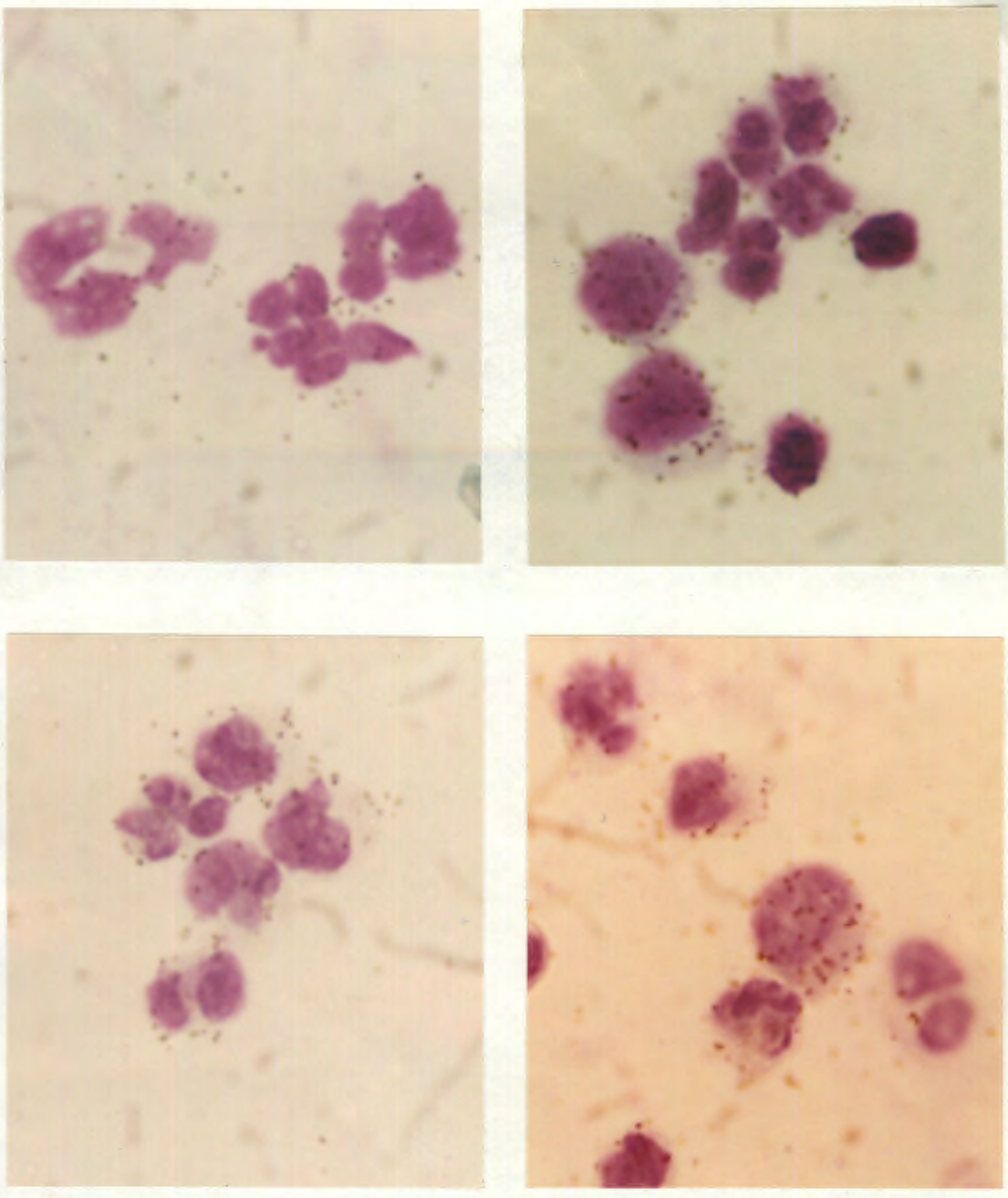


Figure 5.14 Effect of cytotaxin treatment on cell-binding of (^{3}H)DFP

Cells were incubated with saline (*above*) or cytotaxin (CUF-2) (*below*) and subsequently treated with (^{3}H)DFP. The cells were washed and prepared for autoradiography by the method of Kopriwa and Leblond (88). After exposure the cells were stained with Giemsa stain.

Sites of (^{3}H)DFP binding appear as sparse, punctate silver grains that appeared similar, in distribution and intensity, in cytotaxin-treated and untreated cells.

When autoradiographs of (^{3}H)DFP labelled cells were examined microscopically, neutrophils exhibited varying degrees of labelling from approximately 5 - 50 grains per cell. Macrophages showed more intense, evenly distributed labelling. This pattern of labelling appeared to be the same as that encountered with indoxyl acetate in the histochemical procedure and similarly showed no difference between stimulated and control cells (Figure 5.14).

Effects of chemotactic stimulation on cellular (^{3}H)DFP-labelling patterns with and without NPGB competition.

The procedure detailed in the Methods section under Protocol B was designed (a) to detect serine esterases or proteases secreted by cells into the medium and those within or closely associated with the cells; (b) to define those enzymes whose labelling with (^{3}H)DFP could be inhibited by NPGB (thus signifying that they were proteases rather than esterases) and (c) to note the effects of chemotactic stimulation upon the enzymes defined by these techniques.

In all cases, (^{3}H)DFP-binding proteins were detected in SDS-polyacrylamide gradient slab gels by the autofluorographic method of Bonner and Laskey (19). Electrophoresis in SDS-polyacrylamide gradient gels separates proteins on the basis of molecular weight rather than charge and provides less resolution of cellular (^{3}H)DFP-binding proteins than does isoelectric focusing.

A total of 9 experiments were performed with experimental Protocol B. As can be seen from the results of representative experiments shown in Figures 5.15 and 5.16, two major bands of radioactivity were detected in cellular extracts; the position of these bands corresponded to molecular weights of approximately 60 000 and 65 000. Neither band was influenced

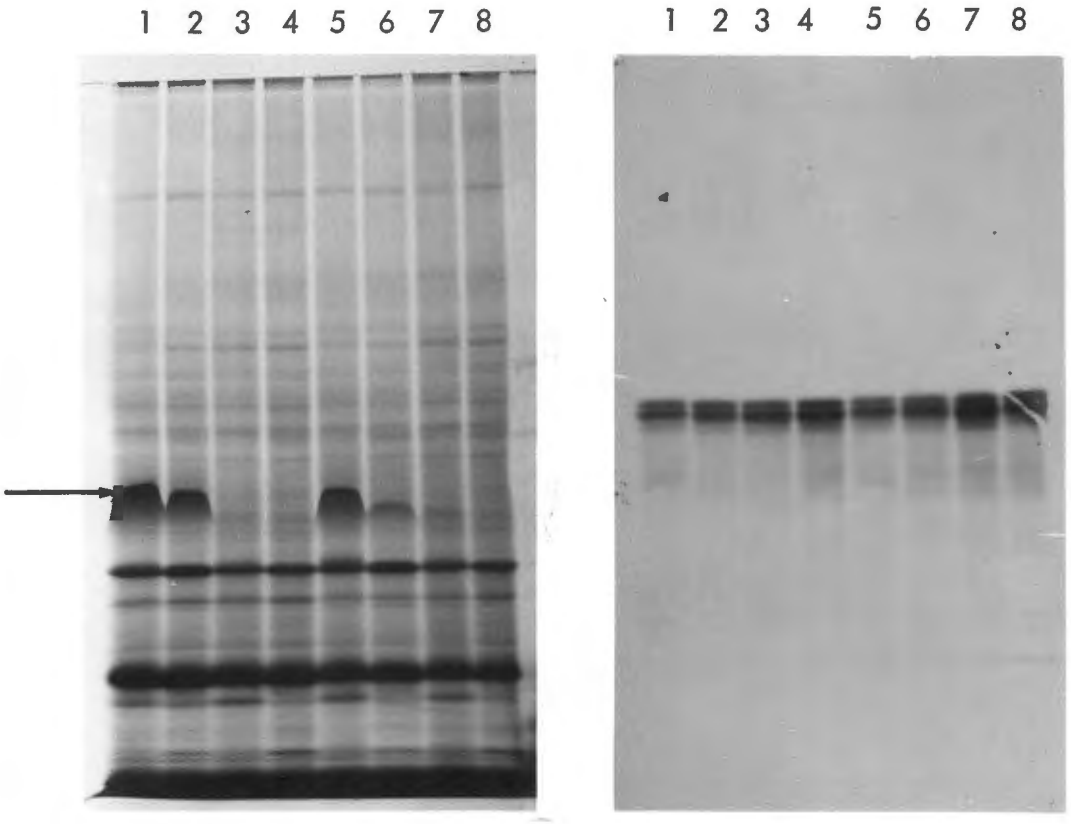


Figure 5.15 Effect of cytotaxin treatment and NPGB on (^{3}H)DFP binding proteins.

Vertical slab gel (8,5 to 13% polyacrylamide; 0,1% SDS) electrophoresis of peritoneal cells from a single rabbit treated with cytotaxin (CUF-3) and NPGB according to Protocol B.

Fixed and stained for protein (*left*); corresponding autofluorograph (*right*).

Tracks 1,2, 5 and 6 - not treated with cytotaxin
Tracks 3,4, 7 and 8 - treated with CUF-3
Tracks 1,3, 5 and 7 - treated with NPGB
Tracks 2,4, 6 and 8 - not treated with NPGB
Tracks 1,2, 3 and 4 - extracted with NP_{40} lysis buffer
Tracks 5,6, 7 and 8 - extracted with Triton X-100 lysis buffer.

Note:

(i) DFP-labelling was not inhibited by NPGB
(ii) Cytotaxin treatment had no effect upon (^{3}H)DFP binding
(iii) The intense protein band (arrow) seen in untreated cells but not in cytotaxin-treated cells, was not encountered in other experiments.

Figure 5.16

Figure 5.16 Effect of cytotaxin-treatment and NPGB on (^{3}H)DFP binding proteins. Results of an experiment on cells from a single rabbit : Protocol B.

The four photographs on the opposite page portray two 8,5 to 13% gradient polyacrylamide-SDS gels that have been fixed and stained for proteins (*left*) and their corresponding autofluorographs (*right*).

The upper gel contained electrophoresed protein from cells that were treated with cytotaxin (CUF-3). The lower gel contained electrophoresed proteins from untreated cells.

Numbered gel tracks were as follows:

1 and 7: Gey's BSS alone (*lower gel*) or with CUF-3 (*upper gel*). These were included to demonstrate that CUF-3 does not contain (^{3}H)DFP-binding activity.

6: Partially reduced IgG marker.

2 and 3: Harvest fluid. Treated with NPGB.

8 and 9: Harvest fluid. Not treated with NPGB.

The presence of human serum albumin in the harvest fluids resulted in these tracks being grossly overloaded with this protein. In can be seen from the autofluorograph that the albumin possessed (^{3}H)DFP-binding activity (probably due to a contaminant) that was inhibited by NPGB.

4 and 5: Triton X-100 cell-free extracts, treated with NPGB

10 and 11: Triton X-100 cell-free extracts; not treated with NPGB.

The Triton extracts contained two clearly visible, (^{3}H)DFP-binding bands that migrated slightly behind IgG heavy chain. These bands were not affected by NPGB.

Cytotaxin treatment had no effect upon the number or intensity of the (^{3}H)DFP-binding protein bands seen.

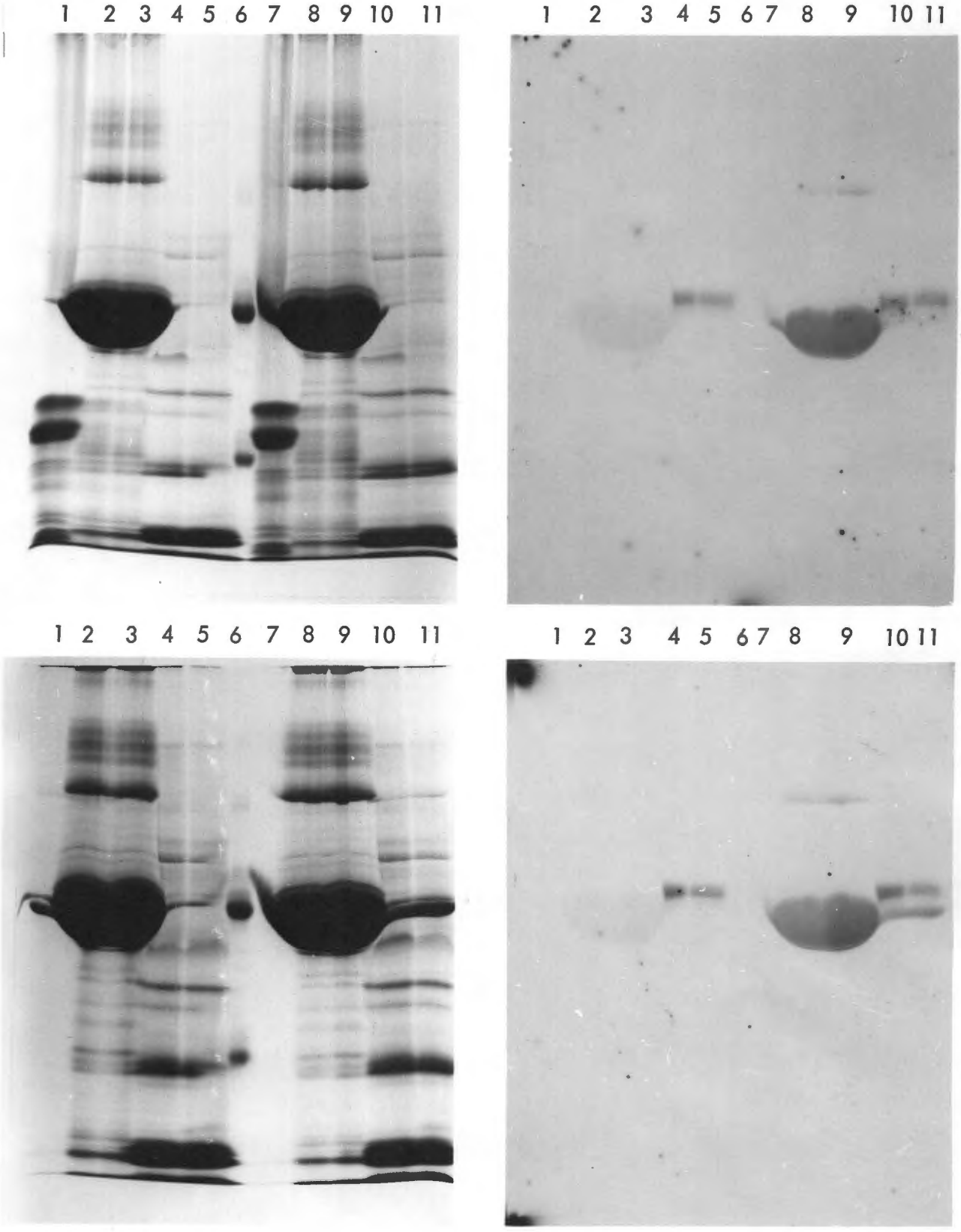

1 2 3 4 5 6 7 8 9 10 11
1 2 3 4 5 6 7 8 9 10 11
1 2 3 4 5 6 7 8 9 10 11
1 2 3 4 5 6 7 8 9 10 11

Figure 5.16

by labelling with (^{3}H)DFP in the presence of NPGB, indicating that they were both esterases rather than proteases. The validity of the NPGB inhibition procedure could be established from the fortuitous presence of an NPGB-inhibitable, (^{3}H)DFP-binding protein contaminating the human serum albumin present in one set of harvest fluid preparation (Figure 5.16). Incubation in the presence of cytotaxin did not influence the number, intensity or molecular weights of the radioactive bands found in the cellular extracts.

Electrophoresis and autoradiographs of concentrates of the harvest fluids gave technically unsatisfactory results for two reasons. Firstly, the amounts of (^{3}H)DFP labelled protein present were so small that they could barely be detected, even with prolonged autofluorographic exposure (up to 3 months). Secondly, the presence of cytotaxin components in the stimulated cell harvest fluids interfered with the electrophoretic separation and gave rise to distorted and smudged patterns. No conclusions could, therefore, be drawn from this study of the harvest fluids.

Effect of cyotaxin on cellular esterases hydrolyzing acetyl-DL-phenylalanine-β-naphthyl ester.

Spectrophotometric assay of cellular esterases capable of hydrolyzing n-acetyl-DL-phenylalanine-β-naphthyl ester gave the results summarized diagrammatically in Figures 5.17 and 5.18. Two experiments were performed from which it can be seen that both control and stimulated cell populations caused hydrolysis that proceeded more or less linearly with time for 30 min.

At all time points at which samples were taken, cytotaxin-treated cells gave higher optical density readings at 485nm than did control cells.

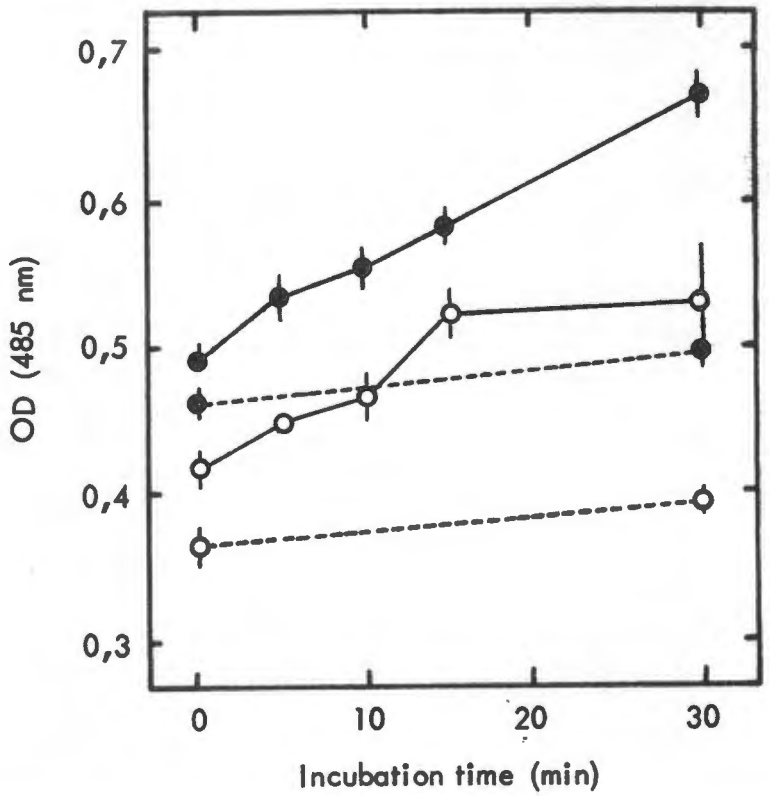


Figure 5.17 Spectrophotometric esterase assay: hydrolysis of acetyl-DL-phenylalanine-β-naphthyl ester.

The time course of hydrolysis of acetyl-DL-phenylalanine-β-naphthyl ester was followed by measuring the coloured reaction product of free β-naphthyl and fast red at 485nm at the times indicated. The curves indicate the extent of hydrolysis of substrate (a) in the presence of cytotaxin (CUF-2)-treated cells (●—●); (b) in the presence of untreated cells (○—○); (c) in the presence of cytotaxin (CUF-2) alone (●--●); or, (d) in the absence of cells and cytotaxins (○--○). The cells were 98% viable by trypan blue exclusion.

Points represent the mean of two readings. The vertical lines indicate the range of each duplicate.

Note that both cells and cytotaxin contributed to the OD_{485nm} at t=0 and that the rate of hydrolysis by cytotaxin-treated and untreated cells was the same.

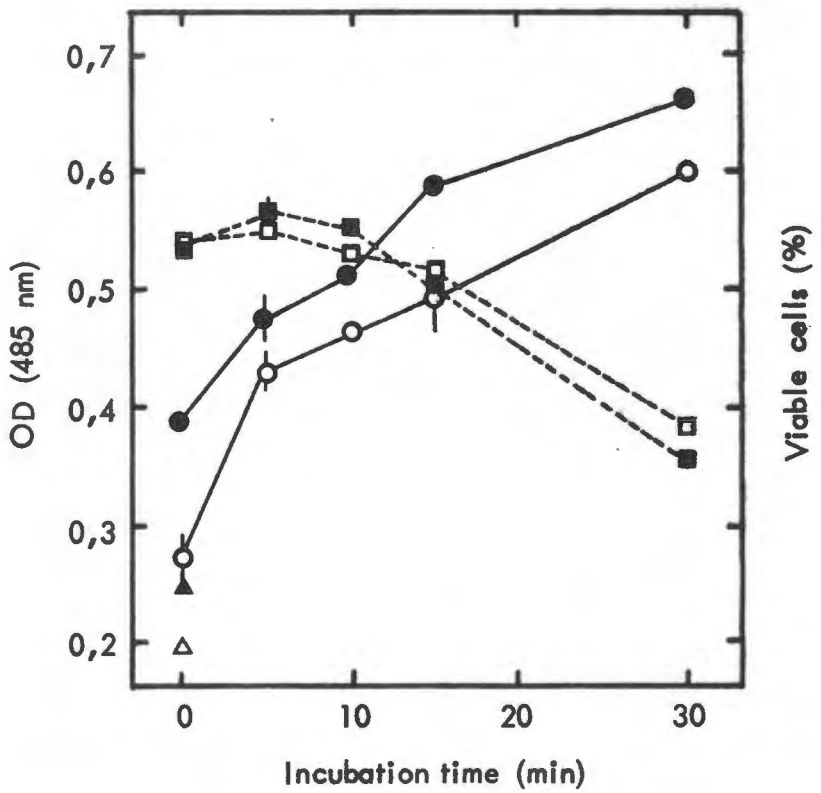


Figure 5.18 Spectrophotometric esterase assay: hydrolysis of acetyl-DL-phenylalanine-β-naphthyl ester and cell viability as a function of time.

Cumulative substrate hydrolysis was determined at the time points indicated in the presence of (CUF-3)-cytotaxin-treated cells (●—●) or untreated cells (○—○). Cytotaxin (▲) and saline (△) controls were measured at t=0.

Substrate hydrolysis proceeded at the same rate with cytotaxin-treated and untreated cells.

Trypan blue estimates of cell viability were similar in cytotaxin-treated (■--■) and untreated (□--□) cells.

Points and vertical lines represent duplicate means and ranges respectively. For experimental details see text.

This, however, could be attributed to the effects of cytotaxin on the assay. When allowances were made for this contribution of cytotaxin to the blank value, it was apparent that there were no significant differences between control and stimulated cells in terms of their esterase activity for this substrate. Indeed, the curves showing optical density at 485nm as a function of time for control and stimulated cells were essentially parallel.

Discussion

In this section of the thesis I have demonstrated esterase activity in suspensions of rabbit peritoneal exudate cells comprising neutrophils as the predominant cell type. Enzymatic activity could be detected in histological preparations by histochemical techniques or by autoradiography, following incubation with (^{3}H)DFP and was found to be associated with neutrophils and macrophages. Although macrophages, by virtue of their lesser numbers, probably contributed only fractionally to the total esterase content of the cell suspensions, it was visibly evident that they possessed more enzymatic activity per cell than did neutrophils.

Analysis of cellular extracts by isoelectric focusing in polyacrylamide gels revealed the presence of at least 5 DFP-binding proteins all of which coincided with bands of esterase activity. Separation of cellular extracts on the basis of molecular weight by electrophoresis in gradient gels containing SDS revealed the presence of two major DFP-binding proteins, neither of which reacted with para-nitro-phenol-guanadinobenzoate to any appreciable extent, indicating that they possessed esterolytic rather than proteolytic activity.

The majority of the esterases detectable by isoelectric focusing were inhibited by DFP, indicating, by conventional criteria, that they were "non-specific, aliphatic, B-esterases" or cholinesterases (127). The effects of silver nitrate, although variable, were consistent with this classification. Two faint esterase bands could be discerned that focused at pH 5,4 to 6,2 and were only detectable after incubation with PCMB, classifying them as "non-specific, C-esterases". It is clear, from these studies, that peritoneal exudate cell esterases constitute a complex set of hydrolytic enzymes. A good deal of work remains to be done to achieve their complete biochemical characterization.

With none of the techniques that I employed was I able to find any evidence to support the notion that chemotactic stimulation affects, either qualitatively or quantitatively, the esterase content of responsive cells. These results contradict the conclusions of Becker and Ward regarding the part played by cellular esterases in the chemotactic response.

It may be argued that the substrates (indoxyl acetate and α-naphthyl acetate) that I used to best effect in the histochemical procedures were inappropriate for examining the "activatable esterase" hypothesis, since this enzyme was alleged to have a measure of specificity for aromatic esters. On the other hand, when acetyl-DL-phenylalanine-β-naphthyl ester was used as the substrate, neither the histochemical procedure nor the spectrophotometric assay (performed exactly as reported by Ward and Becker (169) showed any effects of chemotactic stimulation on esterase activity. Furthermore, since all of the organophosphorus inhibitor studies on which the esterase hypothesis rests were predicated upon the basic assumption that the enzymes concerned contained serine at the active site, (^{3}H)DFP binding studies should have shown qualitative or quantitative differences between

the patterns of protein-bound radioactivity extracted from control and stimulated cells. I should at least have expected to have found an additional or more intense radioactive band in the cytotaxin-treated cells as evidence for the existence of the "activated" form of Becker and Ward's "activatable esterase".

In evaluating any bearing that my results might have on the conclusions of Becker and Ward, it should be emphasized (a) that they used, for most of their studies, a chemotaxin derived from complement activation ($\overline{C567}$) whereas I used casein cytotaxin and (b) that their speculations were based largely upon the results of organophosphorus inhibition studies whereas I used an entirely different experimental approach. My findings,therefore, do not negate theirs; they do, however, cast doubt upon the validity of the general proposition that effective chemotactic stimulation invariably involves esterase activation in responsive cells. For if this were true, the phenomenon of chemotaxis should be associated with consistently demonstrable esterase activation, irrespective of the nature of the chemotactic stimulus or the source of the neutrophils (i.e. from peripheral blood or peritoneal exudates). The authors recognized this difficulty when they reported their inability to demonstrate increased "esterase I" activity in peritoneal exudate cells after stimulation with $\overline{C567}$ (17) or in peripheral blood neutrophils after exposure to chemotactic "bacterial factor" (14).

My reluctance to accept esterase activation as a significant and invariable concomitant of the chemotaxic response stems not only from my own inability to demonstrate this enzymatic phenomenon, but also from the following considerations of the Ward and Becker papers.

In their first paper on the mechanisms of inhibition of chemotaxis by phosphonate esters (167), Ward and Becker used the following basic

protocol to distinguish "cell-dependent inhibition" and "chemotactic factor-dependent inhibition". Cells were preincubated for a period of time, P, washed and introduced into the upper compartment of a Sykes-Moore chamber and incubated for a period of time, I. The cells were exposed to a chemotactic stimulus during period I, at the end of which the chemotactic response was assayed by counting cells that had migrated. Inhibitors were added according to one of two protocols:

(a) Inhibitor present during P and absent during I (P+I-)

(b) Inhibitor absent during P and present during I (P-I+)

Inhibition of chemotaxis with P+I- was referred to as "cell-dependent inhibition" (CDI). If inhibition was found with P-I+ but *not* with P+I-, this was referred to as "chemotactic factor-dependent inhibition" (CFDI). Inhibitors that caused both CDI and CFDI were those in which inhibition with protocol P+I- was *less than* inhibition with P-I+. Reduced to its essential logic, the argument used by the authors in interpreting this observation was developed as follows: Active serine esterases are inhibited by organophosphorus compounds; proesterases are not. This inhibition varies with the chemical structure of the inhibitor and with the nature of the esterase. By the judicious choice of experimental conditions, different inhibitors could be shown to inhibit chemotaxis in different ways, namely "cell-dependent inhibition" or "chemotactic factor-dependent inhibition". Therefore two distinct esterases are involved in chemotaxis; one, susceptible to CDI, exists in an activated form on or in the neutrophil; the other, susceptible to CFDI, is only inhibited when activated by chemotaxin.

There are a number of respects in which this work and the interpretation can be criticized. These I enumerate as follows:

(i) The argument for the existence of CFDI as a biochemically distinct phenomenon from CDI rests heavily upon the assumption that inhibition by organophosphonates is invariably *irreversible*. This assumption is demonstrably false. The reversibility of organophosphate inhibition of esterases is determined, among other things, by the affinity of the enzyme for the inhibitor and the rate of hydrolysis of the enzyme-inhibitor complex. Both of these characteristics are markedly affected by the nature of the alkyl group (42). It is entirely conceivable that in the protocol P+I-, a reversible inhibitor of a single esterase may have been removed by the wash between period P and I so that no inhibition would have been detected during the chemotaxis assay. With the protocol P-I+, however, inhibitor was constantly present during period I, when cell migration was being assayed. Thus, even if esterase activity were specifically related to chemotactic responsiveness, the experimental protocol alone did not necessarily identify more than one esterase.

(ii) In Figure 2 of their paper (167), Ward and Becker present the results of kinetic studies in which para-nitrophenyl ethyl propyl phosphonate, para-nitrophenyl ethyl phenyl phosphonate and para-nitrophenyl ethyl 5-aminopentyl phosphonate caused progressive CFDI with time in the presence of these inhibitors, yet little or no CDI when duplicate samples were treated with protocol P+I-. We are given no indication of how the time studies are performed. Consider the time points at 30 min. For the result of CDI and CFDI to be experimentally comparable, these could only have been obtained by making periods P and I, for both protocols P+I- and P-I+, 30 min. One sees from their Figure 1 (167), that at

30 min, approximately 50% of the maximal migration at 90 min was achieved. It would seem, from Figure 5 and Table 1 (167) that approximately 100 cells represented maximal migration. One must therefore presume that the differences between CDI and CFDI at 30 min represented differences in cell counts of approximately 20 cells! The variability inherent in the chemotaxis assay technique is such as to make differences of this magnitude very unreliable.

(iii) As the authors themselves stated, they were unable to define CFDI when P+I- gave inhibition that was greater than or equal to P-I+. In other words, by their own arguments it is not permissable to speak of CDI greater than CFDI. Yet in Figure 6 of their paper (167) they show CDI greater than CFDI with $4x10^{-4}$M para-nitrophenyl ethyl hexyl phosphonate.

(iv) Had CFDI been attributable to inhibition of "chemotaxin-activated esterase", preliminary incubation of the cells with chemotaxin and "chemotactic factor-dependent inhibitors" should have diminished subsequent chemotaxis. The results of such an experiment were not reported in their first paper. When this experiment was done, it did not give the expected result. It was found that the presence of chemotaxin alone during period P *inhibited* chemotaxis during period I. The presence of "chemotactic factor-dependent inhibitor" *prevented* this inhibition instead of aggravating it! In a subsequent paper (168) the authors accommodated this result by suggesting that preliminary incubation with chemotaxin "deactivated" the leucocytes and that phosphonate inhibitors *prevented* this deactivation in a manner suggesting that the

"chemotactic factor-dependent, activatable esterase" was involved in neutrophil deactivation. From this the inference was drawn that activation of the proesterase, once initiated by chemotaxin, proceeded autocatalytically; phosphonate esters prevented total autocatalytic destruction of the esterase, leaving sufficient residual enzyme for subsequent chemotactic responsiveness to be retained. This ingenious interpretation was supported, for the most part, by the results of experiments with different inhibitors in which the extent of CFDI or protection against "deactivation" was plotted as a function of alkyl chain length to obtain a series of "inhibitor profiles". Similar profiles were observed for CFDI and for protection, indicating that the same enzyme was involved in both phenomena. Although neutrophil "deactivation" as a function of time in contact with chemotaxin was described, no direct experiments were reported to indicate that autocatalytic inactivation did, in fact, occur. These would have been straightforward experiments to do and would have contributed considerably more to the validity of their general hypothesis than did the presentation of "profiles" depicting statistically dubious differences in cell counts.

(v) In subsequent work (17) Becker and Ward assayed neutrophil esterases spectrophotometrically and showed, on the basis of the kinetics of phosphonate inhibition, that at least three esterases were present. My own results confirm and enlarge upon this observation. By isoelectric focusing and staining for esterases, as many as 15 esterases could be demonstrated (Figure 5.10 to 5.13). The authors concluded, once again with recourse to "inhibition profiles", that the esterases

were inhibited by organophosphonates in such a manner as to suggest that their "esterase I" (inhibited by 10^{-9}M phosphonate) was the "activatable esterase" of chemotaxis. It should be emphasized, however, that they were unable to demonstrate activation of the esterase on incubation with chemotaxin. This they suggested, was due to the fact that peritoneal neutrophils had already responded to a chemotactic stimulus by virtue of the technique used to obtain them. Most of the enzyme in this cellular population would, therefore, have been in the activated form. In support of this explanation they noted that only 5 - 40% of peritoneal cells were capable of showing a chemotactic response. They therefore turned to the study of circulating peripheral blood neutrophils. In a paper describing their results with these cells (169) the authors report a "statistically highly significant" difference between the chemotactic responsiveness of peritoneal and peripheral blood neutrophils. The latter, in accord with their theory, were more active; they also possessed more esterase activity. "Esterase I" (inhibitable by $1x10^{-7}$M phosphonate) was the predominant esterase in neutrophils; no difference in the activity of this esterase between peritoneal and peripheral blood neutrophils could be detected. Esterase activity could be induced by incubating neutrophils with activated $\overline{C567}$. This phase of the Ward and Becker work reported in the two papers published in 1969 and 1970 (17, 169) is inadequate and unconvincing in many important respects:

(a) It is incorrect to say that only 5-40% of peritoneal neutrophils will respond to a chemotactic stimulus. Provided a 3μm filter is used, it is not at all difficult

to induce virtually all of the neutrophils to migrate. As I have indicated previously, neutrophils simply do not migrate through a 0,65μm filter (Chapter 1).

(b) The data presented in Table II of their 1970 paper (169) have been incorrectly analysed. Using the correct arithmetic, one finds that there was, in fact, no significant difference between the chemotactic responsiveness of peripheral blood and peritoneal neutrophils.

(c) Applying the authors own reasoning, one would have expected the esterase in peripheral blood (i.e. unstimulated) neutrophils to be largely in the proesterase form and hence the measurable esterase to be *lower* in these cells than in peritoneal cells. The authors found that it was higher!

(d) Incubation of intact cells with $1x10^{-8}$M para-nitrophenyl ethyl 5-chloropentyl phosphonate inhibited esterase activation by $C\overline{567}$ by 80%, yet concentration of $5x10^{-4}$M inhibitor were required in Figure 2 (167) to show an effect on CFDI of approximately 50%.

This considerable disparity between the concentration of phosphonate compound required to inhibit esterase activation and that required to achieve CFDI raises the question of whether or not these two effects were in any way related. It is of interest to note, in this regard, that Woodin and Wieneke (189) found that DFP and tri-alkyl phosphates

exerted a "detergent-like" action upon the leucocyte membrane at the site of the "potassium pump". This effect enhanced leucocyte susceptibility to leucocidin and was entirely unrelated to esterase inactivation.

(e) One is invited to accept (169) that a mean difference of 0,037 ΔOD units is significant reason for believing that $\overline{C567}$ activates cellular proesterases. The data from which the values were calculated are not given, but, from elsewhere in the paper it is evident that this represents the mean of differences between ΔOD values of 0,5 to 0,7. My own experience with this assay makes me reluctant to set much store by differences of this order of magnitude.

It may justifiably be argued that there were two respects in which the electrophoretic techniques and protocols that I used were inadequate to deny the existence of a cytotaxin-activatable esterase. Firstly, the techniques may have been insufficiently sensitive to detect the "activated" esterase in cytotaxin-treated cells if this enzyme were present in very small amounts. Secondly, the cytotaxin-activated enzyme may have had an isoelectric point or electrophoretic mobility that coincided with one of the major esterases in control cells. It would, in this case, have been difficult or impossible to distinguish differences between gel patterns of esterases from cytotaxin-treated and control cells. Furthermore, this problem of "high background" levels may have been particularly severe with rabbit peritoneal neutrophils that came from caseinate-induced exudate since much of the potentially activatable

esterase in these cells may have been in the activated state.

I must concede that I have not, at the time of writing, a definitive answer to either of these arguments. The question of sensitivity can, to a certain extent, be answered by the following considerations:-

(i) Bonner and Laskey (19) estimate that autofluorography will detect 500 dpm of ^{3}H in a 10x1mm band on a polyacrylamide gel slab with one week's exposure.

(ii) The specific activity of the DFP that I used was, in the highest case, 10Ci/mmole or $3,686x10^{-8}$ dpm/molecule. Therefore, I should have been able to detect, with one week's exposure, $1,356x10^{10}$ molecules of ^{3}H in any band.

(iii) The samples that I electrophoresed represented the total extract from approximately $6,7x10^{6}$ cells.

Providing, therefore, that the activatable esterase had an electrophoretic mobility that differed from that of other (^{3}H)DFP binding proteins, comparative electrophoresis and autofluorography should, in any band, have revealed as little as $1,356x10^{10} \div 6,7x10^{6} = 2,025x10^{3}$ molecules/cell of such an enzyme. Since I exposed the gel slabs for 3 months, it is likely that I would have detected even lesser amounts of activatable enzyme.

Moreover, it is evident, from simple inspection of the results of autofluorographs or radioactive measurement on the one hand and staining for enzymes on the other, that specific staining methods are more sensitive than radioactive techniques. If this is so, I should have been able, with isoelectric focusing and with α-naphthyl acetate/fast garnet GBC, to have detected well below 1000 "new esterase" molecules per cytotaxin-treated cell had these been present.

The second argument - that of electrophoretic superposition of (^{3}H)DFP-binding proteins or esterase bands from control and stimulated cells - requires experimental answers that I am not yet in a position to provide. Firstly, I should repeat the experiments with "chemotactically virgin" neutrophils isolated from the peripheral blood. Secondly, I should eliminate background enzyme activity by incubating the cells with DFP *before* cytotaxin stimulation and subsequent esterase detection. The activatable proesterase would not, in theory, be susceptible to inhibition by preincubation with DFP and should be readily detectable after cytotaxin treatement in the absence of DFP. It is my intention to complete these experiments as soon as circumstances permit.

I feel justified in concluding this chapter by stating that impure preparations of rabbit peritoneal neutrophils possess numerous esterases. There is no completely convincing evidence to indicate that activation of a proesterase is involved in mediating the chemotactic response.

Appendix

Section A.1 : Rabbit peritoneal exudates

Cells for the studies reported in this thesis were obtained from rabbit peritoneal exudates. This technique for obtaining large numbers of viable inflammatory cells for experimental purposes has been well documented (64,79,83,147,186) and depends upon the fact that one is able to induce a sterile peritonitis by the intraperitoneal injection of glycogen, sodium caseinate or liquid paraffin.

Exudates harvested 16 to 18h after intraperitoneal administration of glycogen or sodium caseinate contained polymorphonuclear leucocytes as the predominant (>85%) cell type; those obtained 96h after the injection of liquid paraffin had a preponderance of macrophages (>70%).

The following is a detailed account of the procedures used to obtain neutrophils. The modification necessary to get macrophage-rich exudates are described subsequently.

Animals

Randomly outbred Californian and New Zealand White rabbits were purchased from local commercial suppliers(1). Young adults of either sex were used since there appeared to be no obvious differences in the quality or quantity of cells obtained from males or females. The abdominal walls of older male rabbits were more difficult to penetrate with a trochar and cannula, so such animals were avoided if possible.

Animals could be used as cell donors repeatedly at monthly intervals, in many cases for as long as two years, without apparent ill effect upon the animal or the yield of cells.

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(1) Coney Protein (Pty) Ltd., Cape Town, South Africa.

Polymorphonuclear leucocytes (147,186)

Procedure

Fur was shaved from the ventral abdominal skin and the injection site cleaned with 70% ethanol. Approximately 40 ml of sterile 7% w/v sodium caseinate pH 7,4 was injected into the peritoneal cavity through a 1½"15 gauge, long bevel needle inserted through the mid-line without anaesthesia. An all-glass sterile 50 ml syringe was used to deliver the injectate. (Figure A.1.1(a)).

Sodium caseinate(1) was prepared for injection by the gradual addition, in divided portions, of 70 g of the dry powder to a stirred solution of 9 g of NaCl in 700 ml of double-distilled water. A 1,0M solution of NaOH was added after each addition of sodium caseinate in an amount just sufficient to maintain the pH of the mixture between 8 and 9 and so to facilitate solution. It was important to avoid the excessive addition of alkali, since restoration of the pH with acid resulted in the separation of insoluble casein around the acid droplets. When all of the caseinate had been added the pH was carefully adjusted to pH 7,4 with 0,1M HCl or NaOH and the solution made up to a final volume of 1 litre with double-distilled water. The final, slightly opalescent, viscous solution was dispensed into glass bottles in 100 ml volumes and sterilized by autoclaving at 10 psi for 10 min. Exposure to higher pressures or longer times resulted in a brownish discolouration of the solution. The sterile solutions were stored at -15°C and thawed at 37°C immediately before use.

Sixteen to eighteen hours after the intraperitoneal injections

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(1) Nutritional Biochemical Corporation, Cleveland, Ohio, U.S.A.

the animals were anaesthetized by the slow intravenous injection of a solution (30mg/ml) of pentobarbitone sodium[1]. A satisfactory level of light, general anaesthesia was indicated by the appearance of nystagmoid eye movements and loss of pedal reflexes. This was usually secured with a dose ranging from 90 to 120 mg of the drug. In most cases the general anaesthesia was supplemented by infiltrating the cannulation site with a solution of lignocaine hydrochloride (10mg/ml) containing 1:100 000 Adrenaline. This prevented jerking during the subsequent cannulation, and the local vasoconstriction minimized red cell contamination from abdominal wall bleeding.

With the rabbit lying supine, the abdominal wall was cleaned with 70% ethanol and the peritoneal cavity was cannulated with a 3mm trochar and cannula[2] inserted through the ventral abdominal midline approximately 2 cm below the umbilicus. The cannula used was modified by drilling 4 sets of 3x1mm holes in 90° sections of the terminal 1,5 cm (Figure A.1.1(b)).

The trochar was removed and 250 ml of sterile 0,9% NaCl containing 15 units of preservative-free heparin/ml was introduced into the peritoneal cavity through the cannula connected, by sterile plastic tubing, to an infusion bottle (Figure A.1.1(c)).

The peritoneal contents were distributed by gentle massage and siphoned back into the lowered infusion bottle (Figure A.1.1(d)).

The saline-exudate mixture was cleared of adipose fragments, fibrinous strands and other particulate debris by filtration through sterile gauze pads. Inflammatory cells were collected by centrifuging

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(1) Sagatal Veterinary; May and Baker Ltd., Dagenham, England or Nembutal Veterinary; Abbott Laboratories Ltd., Queensborough, Kent, England.

(2) Aesculap; Jetter and Schearer Corporation, Tuttlingen, West Germany; Catalogue number B-9710.

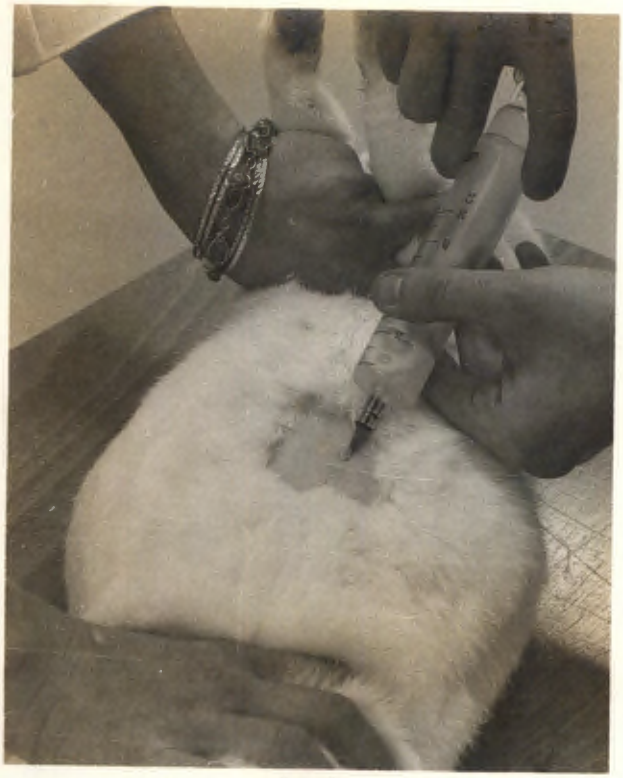
Figure A.1.1 (a to d)

Figure A.1.1(a)
Fifty millilitres of sterile 7% sodium caseinate is injected intraperitoneally into the restrained non-anaesthetized rabbit.

Figure A.1.1(b)
To harvest the exudate, the peritoneal cavity of the anaesthetized rabbit is cannulated.

Figure A.1.1(c)
Sterile heparinized saline is infused into the peritoneal cavity through the cannula.

Figure A.1.1(d)
The peritoneal exudate is removed by siphonage into the same bottle that was used for infusion.



(a)

(b)

(c)

(d)

Figure A.1.1

(500xg; 10 min; 20°C) the filtrate at room temperature. The pelleted cells were gently resuspended in the requisite media for each experiment.

Notes and Comments

In most instances the technique described for obtaining viable neutrophils gave very satisfactory results. In Figures A.1.2 and A.1.3 I have summarized diagrammatically the cell yields obtained and the results of differential cell counts on the exudates. It is evident from these data, that I was routinely able to get from $1x10^8$ to $2x10^9$ cells for each experiment. These comprised 68% to 99% neutrophils (mean 85%); the major contaminating species were mononuclear cells that had the appearance of macrophages. Attempts to separate neutrophils and macrophages by density gradient centrifugation (25) were unsuccessful.

I have made the following observations during my experience with the method. None of them did I document formally.

(i) Neutrophils suspended in Gey's BSS-2% HSA are remarkably hardy cells and are able to survive for many hours at room temperature without appreciable loss of viability or chemotactic performance.

(ii) These cells react poorly to pelleting by high-speed centrifugation, becoming clumped and very difficult to resuspend. I found that I was not able to use centrifugal fields in excess of 500xg or to pellet and resuspend the cells more than twice without considerable losses.

(iii) Although the induction and harvesting of peritoneal exudates was not done under strictly sterile conditions, all precautions were taken to prevent bacterial or fungal contamination of the

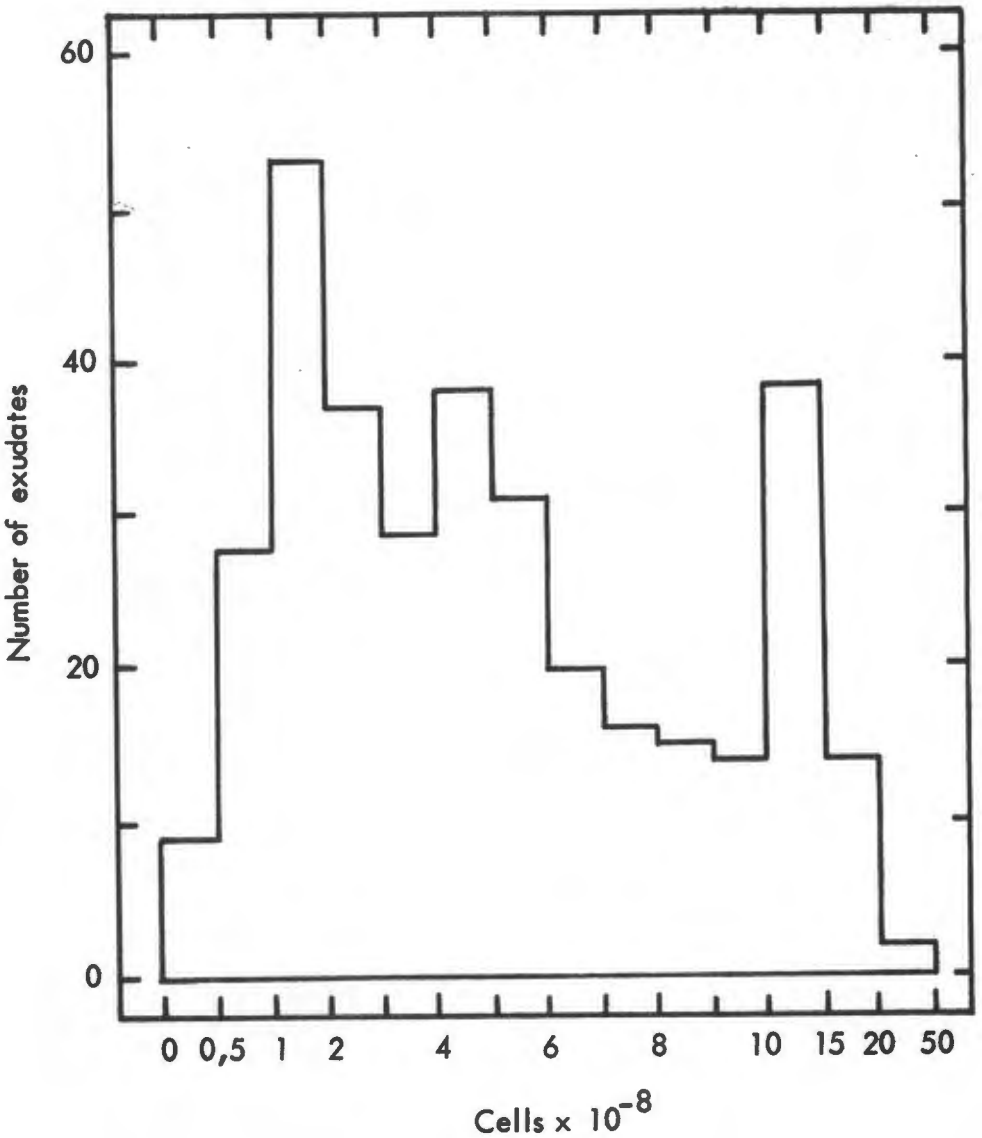


Figure A.1.2 Cellular response to intraperitoneal sodium caseinate.

Histogram showing numbers of cells obtained by peritoneal lavage 18h after intraperitoneal injection of 50 ml of 7% sodium caseinate. A total of 344 exudates is included in the analysis.

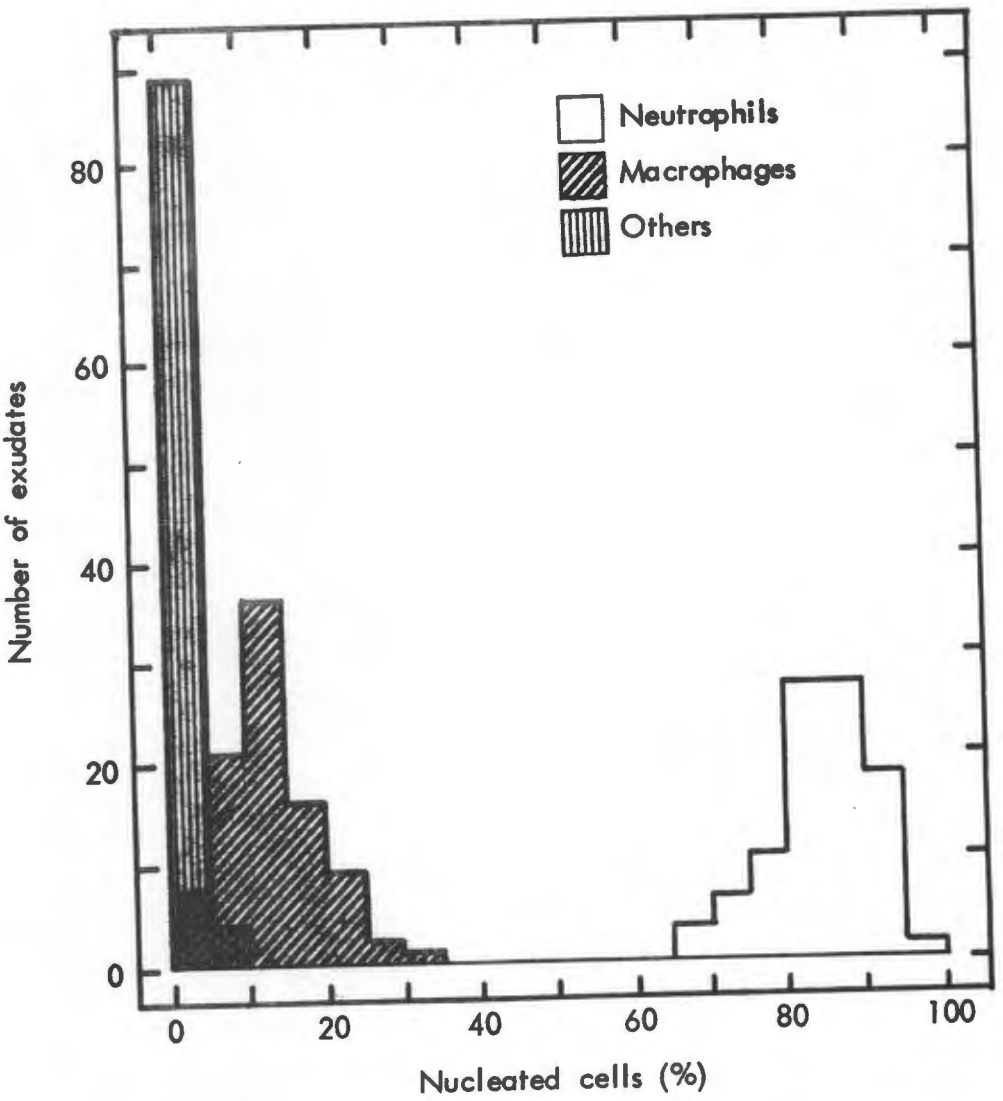


Figure A.1.3 Distribution of cell types observed in 92 peritoneal exudates

Histogram showing frequency distribution of various cell types observed in 92 peritoneal exudates elicited by injection of 50 ml of 7% sodium caseinate. In all cases, neutrophils constituted the predominant cell type.

exudates and infections in the donors.

(iv) The rabbit abdomens were clean-shaven the day before harvesting the exudates as loose hairs made it difficult to maintain sterility.

(v) I found that small animal clippers fitted with angora blades[1] gave the closest shave. Various other makes tested could not handle the thick, fine rabbit fur and easily became jammed.

Macrophages (83)

Procedure

The procedures for inducing and harvesting macrophage-rich peritoneal exudates were similar to those used for neutrophils with the following exceptions.

(a) Sterile liquid paraffin (50 ml) instead of sodium caseinate was injected intraperitoneally to stimulate exudates.

(b) Exudates were harvested 96h after liquid paraffin injection.

(c) Contaminating liquid paraffin was removed from the cell suspensions by flotation at 500xg with two washes with 0,9% NaCl containing 15 units/ml of preservative-free heparin.

(d) Rabbits could only be used once owing to the adhesive peritonitis produced by the liquid paraffin.

Notes and Comments

On the three occasions on which the technique was used I obtained

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(1) John Oster Manufacturing Company, Milwaukee, Wisconsin, U.S.A.

cell yields ranging from $1x10^8$ to $3x10^8$, 70% of which were macrophages and the rest predominantly neutrophils.

Section A.2: The Boyden chamber chemotaxis assay.

The assay I used was a modification of that originally described by Boyden (24) and adapted by Keller (75).

This assay involved the incubation of cells in one compartment of a Boyden chamber, separated by a filter from a second compartment containing a chemotactic solution. Cells, attracted by the chemotactic agent, migrated into and through the filter. After incubation the filters were processed for histological examination and cell migration was evaluated histologically.

Chambers

The Boyden chambers (Figure A.2.1) were made of Perspex and consisted of an upper cell-containing compartment separated from a lower test-substance-containing compartment by a filter membrane held in place by a tightly fitting nylon sealing bung. The design of the chambers was such that when the cell-compartment and test-compartment were charged with 3,0 ml and 2,5 ml respectively, hydrostatic pressures across the filter were balanced. Chambers were manufactured by Celloplex, Basel, Switzerland. Before use the chambers and sealing bungs were washed in warm 2,5M ammonia and rinsed thoroughly in distilled water. Air-dried chambers and bungs were sterilized by exposure to ethylene oxide.

Chambers were assembled immediately before use on a clean working surface. Strict sterile precautions were not observed at this stage; sterile filters were not used.

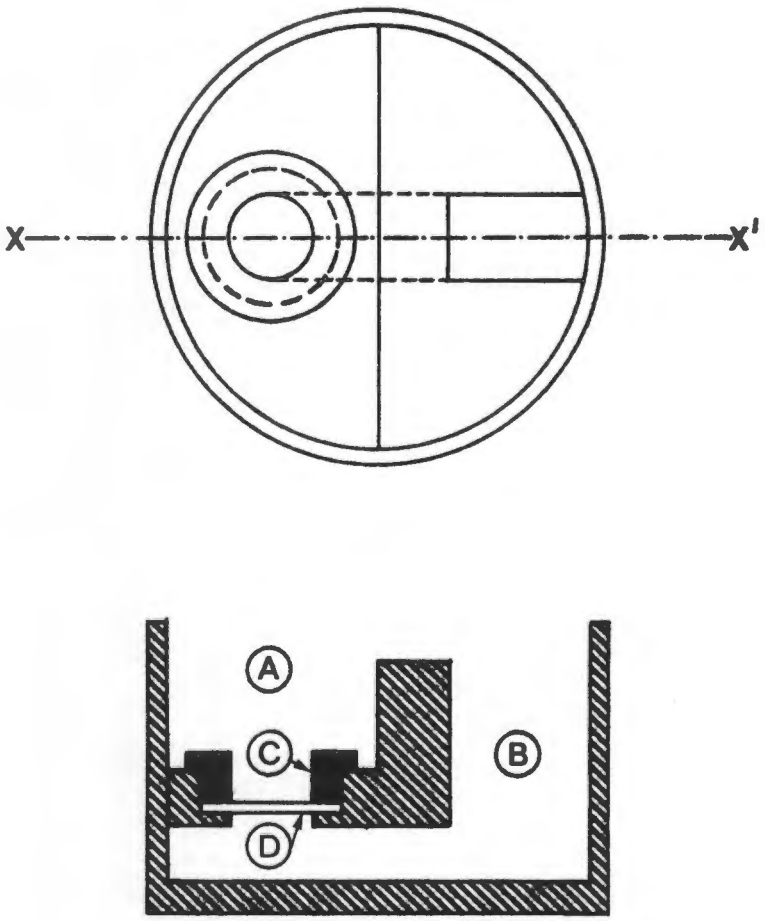

Figure A.2.1

Diagram of Boyden chamber used for chemotaxis assays shown (*above*) in plan and (*below*) in side elevation through the sagittal plane (Scale: 1,5cm = 1cm). The chamber was machined from Perspex (hatched in lower figure) and consisted of a cell compartment (A) and a chemotaxin compartment (B). These were separated by a Millipore filter (D) held in place by a tightly fitting, annular nylon bung (C) that retained the filter by vertical compression. It was necessary that the bung should effect a seal that effectively precluded passage of liquid between compartments A and B by any route other than via the filter. The dimension of the compartments were such that when 3ml were added to A and 2,5ml to B the liquid levels in the two compartments were the same and hydrostatic flow of liquid across the filter did not occur. The chambers used were designed according to Boyden (24) and were manufactured by Celloplex, Basel,Switzerland.

Filters

Filters were purchased as 293mm diameter discs from which 12mm diameter discs (the size required for the Boyden chambers) were cut using a leather punch.

Millipore 3µm cellulose ester filters(1) were used for neutrophil chemotaxis studies.

Sartorius 8µm cellulose nitrate filters(2) were used for studying macrophage chemotaxis.

Filters were handled with gloves or clean forceps and were punched with the protective separating paper sheets in position. Randomly selected discs were checked for even wettability when floated on water.

Procedure

In most cases cells were suspended in Gey's BSS-2% HSA to a final concentration of $2x10^6$ cells/ml.

Chambers were filled individually by two people. First, 2,5ml of test solution were pipetted by one worker into the lower compartment with the Boyden chamber angled slightly to prevent entrapment of air bubbles below the filter. Immediately the filter was seen to be wet, 3,0ml of cell suspension were introduced rapidly into the upper cell compartment by the second worker and the Boyden chamber was set level. The volumes added and the timing of the sequential additions were important to achieve satisfactory trans-membrane concentration gradients of the chemotactic compound.

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(1) Millipore Corporation, Bedford, Massachusetts, U.S.A.; catalogue number SSWP 29300.

(2) Sartorius-Membranfilter GmbH, Göttingen, West Germany; catalogue number SM 11301.293.

Charged chambers were incubated at 37°C in a humid atmosphere of air or 5% carbon dioxide in air.

The duration of incubation varied with the experiment or the cell type being studied. In general, incubation periods of 3h were used for studying neutrophil chemotaxis; macrophage studies involved 5h incubation periods.

All manipulations were performed with sterile glassware and reagents, but "clean" rather than rigorous aseptic precautions were used for handling the chambers and apparatus.

At the end of the incubation period, sterility of the solution in the chambers was checked by inoculating samples into 5 ml of Difco Nutrient Broth(1) and incubating these cultures for 48h at 37°C.

With the filters still in position, individual chambers were emptied sequentially by decantation of cell suspensions and test solutions. The filters were washed twice *in situ* with 0,9% NaCl and fixed by the addition of 100% ethanol for 10 min followed by a distilled water wash.

The filters were then removed from the chambers. Each filter was stained individually for two min in 2 ml of Weigert's iron-haematoxylin. Excess stain was removed by rinsing the filters in distilled water after which they were transferred to tap water for a minimum of 10 min for the colour to develop and intensify.

The filters were then dehydrated by sequential 2 minute-treatments with each of the following solutions in the order given.

Twice with 70% ethanol.

Four times with 95% ethanol.

Four times with ethanol : butanol (80:20)

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(1) Difco Laboratories, Detroit, Michigan, U.S.A.

The filters were then cleared by transferring them to xylene.

After at least 45 min of clearing in the xylene the filters were mounted on numbered glass slides under coverslips in a drop of Canada balsam : xylene (2:1) with the under surface of the filter facing upwards.

Care was taken to preserve the orientation of the filters through the fixing, staining, clearing and mounting procedures.

Filters were examined with a 40x objective and an 8x eye piece. The eye piece incorporated a grid graticule to facilitate counting. In general, four fields of the under surface of each filter was selected for counting; only those cells in the same plane of focus as the fibres of the under surface of the filter were counted - i.e. those cells that had traversed the entire filter thickness. The mean of the four field counts in the same filter was taken as a measure of the cell response for any particular cell sample.

Notes and Comments

(i) Cellulose derivative membrane filters show batch variations in their suitability for chemotaxis work. I have used filters from two commercial suppliers, Messrs. Millipore Corporation, Bedford, Massachusetts, U.S.A. (mixed cellulose ester filters) and Messrs. Sartorious-Membranfilter GmbH, Göttingen, West Germany (cellulose nitrate filters).

Both manufacturers were most cooperative in supplying me with samples of several batches for preliminary testing before an order was placed.

Chamber-to-chamber variations could be minimized by punching filter discs for a particular experiment from a single large sheet rather than using pre-cut filter discs supplied in lots that had been machine cut from stacked sheets.

The reason for this batch variability is obscure. Most manufacturers maintain close control over the quality of filters to be used for the usual specified purposes of filtration, electrophoresis, nucleic acid binding etc. It is thus probable that the unsuitability of certain batches for chemotaxis experiments is the result of slight physical, chemical or contaminant variations, control of which is not commercially necessary. It is not generally realized, for example, that Millipore filters, as supplied, contain traces of detergents (4) and are capable of binding substantial amounts of protein (56). Both of these characteristics may have relevance to the problems encountered with the use of certain batches for chemotaxis research.

(ii) The filter technique assumes the establishment of a chemotactic gradient across the thickness of the filter. A number of factors may interfere with this gradient.

Firstly, leaks in the filter or its seating, will allow chemotactic material to diffuse rapidly into the cell chamber and equilibrate across the filter.

Secondly, if the apparatus is assembled and charged in such a way that a hydrostatic pressure exists across the filter, bulk flow of liquid across the filter will result. In the Boyden chamber, for example, it is of great importance that the fluid levels in the two compartments be the same.

Thirdly, air bubbles may become entrapped between the filter and the chemotactic solution. The usual procedure adopted to avoid this error involves two operators to fill the chamber. The first pipettes the test solution into the lower chamber

and the second, pipettes the cell suspension into the upper chamber immediately, *but not before*, the filter is visibly wet. This requires nice timing and adroit cooperation!

Finally, traces of grease on filters cause hydrophobic barriers to the establishment of the gradient. Filters should therefore be kept scrupulously clean and handled only with clean, flattened curved forceps.

(iii) With all filter systems, cell suspensions are delivered into an upper chamber and allowed to settle by gravity on to the upper surface of the filter. For subsequent evaluation of the filter, it is desirable that the cells should settle in such a manner that the number of cells per unit area is the same across the plane of the filter. It has been my common experience that cells, presumably because they do not adhere to the filter instantly upon contact, distribute unevenly if the filter is not flat or set level in the incubator, tending to congregate in depressions and to be more sparse on ridges or elevated areas. It is difficult to avoid a slight "sag" in most circular filters, with the result that subsequent cell counts over the centre tend to be higher than those over the periphery. To avoid excessive variations, cells in similar regions of different filters should be counted.

(iv) In most techniques, filters are evaluated by fixing, staining and mounting them for counting of cells under high-power magnification. As I have shown (Chapter I) cells are by no means evenly distributed through the thickness of the filter so that filters that are not

mounted flat may give rise to error since different filter planes may be included in a single high-power field of view.

(v) Heparin is used to prevent clotting of peritoneal exudate cells. It is important that it be free of preservatives.

(vi) Forty millimetre diameter watch glasses were convenient vessels for the staining solutions. Wheaton biological preparation dishes with moulded covers[1] were ideal containers for the dehydration and clearing solution.

(vii) In transferring the filters from one solution to the next during the dehydrating and clearing procedure it was essential to rinse the tips of the forceps in 100% ethanol before the transfer to xylene. This prevented contamination of the xylene with water. Water in the xylene resulted in uneven clearing which made cell counting in the opaque regions difficult.

(viii) I found it necessary to dehydrate the filters slowly, allowing a minimum of two minutes in each dehydration solution before transfer to the next. Inadequate dehydration resulted in poor clearing and opaque filters.

(ix) Butanol was added to the final dehydration solution as it was found to increase the filter wet strength.

(x) Canada balsam was, in my hands, the most satisfactory mountant as it dried slowly allowing adequate time for the filter preparations to stabilize and flatten. The material used was purchased as Canada balsam, glass hard, dissolved in xylene (2:1) for microscopy[2] and used as such.

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(1) 37x25mm; Arthur H. Thomas Company, Philadelphia, Pennsylvania, U.S.A.

(2) E. Merck, Darmstadt, West Germany; catalogue number 1693.

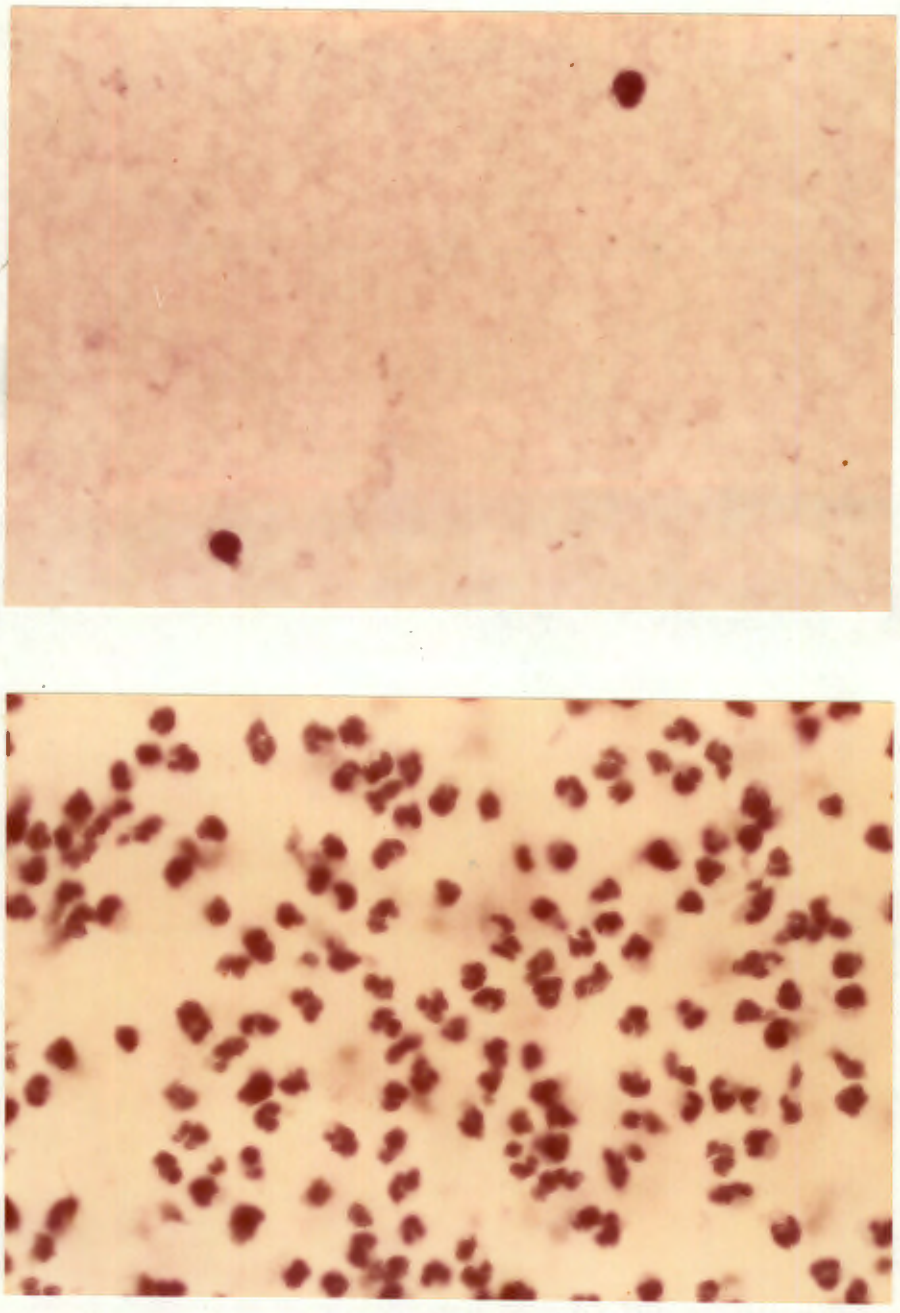

Figure A.2.2 Photomicrograph of under surfaces of filters showing negative (*above*) and positive (*below*) chemotaxis.
Microscope magnification 400x.

(xi) Bacterial contamination of the chambers occurred only on very rare occasions. Such filters were discarded.

(xii) Microphotographs of test and control filters are shown in Figure A.2.2.

Section A.3: Preparation of test and reference casein chemotactic solutions

The quantitative study of neutrophil chemotaxis is beset with a number of difficulties that stem from the biological and technical variables inherent in the assay. Firstly, one has the problem of wide variation in the chemotactic responsiveness of cells obtained from different animals and of cells obtained from the same animal on different occasions. Secondly, different commercially available preparations of casein vary in their chemo-attractant properties. Thirdly, the kinetics of the assay are generally such that non-linearity obtains with respect to cell concentration, time and concentration of chemotactic material. Finally, crude casein contains, in addition to chemotactic material/s, compound/s that inhibit neutrophil movement when present at high concentration.

As a result of this combination of factors, one cannot justifiably compare cellular or chemotaxin activities in absolute terms. These can only be measured semi-quantitatively by reference to some arbitrary but constant standard that is included in each experimental assay.

In this section I describe the way in which I prepared

(i) *1% w/v Casein reference standard*

This was prepared in large amounts from a single batch of casein and stored as a sterile, frozen solution. In all experiments this material was used in at least one chamber to serve as a standard and a "positive control".

(ii) *Acid-precipitated casein supernatant fraction (SNF)*

The bulk of the casein protein was precipitated from a 7% w/v crude casein solution at a pH of 4,5. The supernatant fluid, containing a large proportion of the chemotactic material,

was concentrated, sterilized and stored frozen.

(iii) *Concentrated ultrafiltrate of casein low molecular weight cytotaxin (CUF)*

A 1% w/v solution of crude casein was processed in a hollow-fibre ultra-filtration apparatus designed to pass molecules of molecular weight less than 10 000. The ultrafiltrate, containing a large proportion of the low molecular weight chemotactic material, was concentrated, sterilized and frozen.

The chemotactic preparations SNF and CUF were prepared in an attempt to remove inhibiting material and to serve as starting material for the purification procedures reported in Chapter II.

The following is a detailed account of the technique used to prepare the three solutions.

Casein

Casein was purchased as Casein (Hammarsten) from two commercial sources[1].

For convenience and standardisation a single large batch of material was purchased from the former supplier and was supplied as a dry, white powder packed in polyethylene bags in 10 kg amounts. It could be kept at room temperature for many months without apparent deterioration.

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(1) British Drug Houses Chemical Limited, Poole, England and E. Merck, Darmstadt, West Germany.

1% w/v casein reference standard.

Ten grams of casein were dissolved in a solution of 9,0g NaCl in 700ml distilled water. Solution was effected by adding small portions of the 10g casein sample to the stirred solution and adjusting the pH to 7 to 8 after each addition with 4,0N or 1,0N NaOH. Care was taken to avoid adding excess alkali. By the judicious addition of 0,1N NaOH after the last of the 10g of casein had been added, a final pH of 7,4 could be attained. If necessary, the pH of the final solution was adjusted to 7,4 with 0,1N HCl or NaOH. The solution was then made up to one litre with distilled water, dispensed in 100ml volumes into screw cap bottles, and sterilized by autoclaving at 10 psi for 10 min.

The sterile solution was stored in 100ml volumes at -20°C until required. Thawing was done rapidly at 37°C. To prevent denaturation and maintain sterility the solutions were not frozen and thawed more than twice. If necessary the 100ml lots were dispensed into more convenient smaller volumes.

Acid precipitated casein supernatant fraction (SNF)

Casein (140g) was dissolved, by piecemeal addition, in approximately 1 500 ml of 0,9% NaCl. A solution of NaOH (4N; 1N or 0,1N) was added after each addition to maintain the pH in the range 7 to 8. Care was taken to ensure that this range was not exceeded. The final pH was adjusted to 7,4 and the volume made up to 2 000 ml with 0,9% NaCl. The solution was kept at room temperature with constant stirring for approximately 1½h.

Casein was precipitated from solution by the slow, dropwise addition of 1N HCl. To ensure a fine, particulate precipitate with minimal entrapment of the mother-liquor, the acid was added slowly with constant stirring.

The acid was added to a final pH of 4,5 to 4,6 - the isoelectric point of casein. At this pH the major portion of the casein was allowed to precipitate at room temperature for 2h while the solution was constantly stirred. The mother liquor was collected by centrifugation (2 000xg; 30 min; 4°C) and decantation.

The acid supernatant fluid was filtered (Whatmans 541) and was then brought to a pH of 7,4 with NaOH. Triethanolamine (final concentration 0,005M) and chlorhexidine (final concentration 0,001% w/v) were then added and the pH was checked (7,2 to 7,4). The supernatant fluid was then sterilized by filtration through 0,45µm Millipore filters and concentrated in a sterile Amicon stirrer cell system equipped with a stainless steel reservoir and fitted with a UM2 Diaflo membrane(1). The UM2 membrane had a specified retention of >95% for pure solutes of molecular weight greater than 10 000, although retentivity of the membrane was greatly increased for lower molecular weight compounds in the presence of higher molecular weight species. An approximate forty-fold concentration was achieved after approximately one week under constant pressure of 70 psi of nitrogen. The concentrate was re-sterilized by membrane filtration and stored frozen at -20°C in convenient volumes. It was thawed rapidly at 37°C when required for use.

The relative chemotactic activities of all preparations used in this thesis, together with any minor differences in their respective preparations are given in Table A.3.1.

Notes and Comments

(i) The susceptibility of casein to alkaline hydrolysis has been

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(1) Amicon Corporation, Lexington, Massachusetts, U.S.A.

Table A.3.1

Table A.3.1

Chemotactic activities of acid-precipitated casein (Hammarsten) supernatant fluid concentrates.

SNF	Source	Protein Concentration (mg/ml)	Chemotaxis (average cell count/HPF) Retentate[x]	Ultrafiltrate	1% casein[†]
I	BDH lot 0868390	7,5	500 (1/100)	5	500
II(a)	BDH lot 0868390	1,5	500 (1/10)	1	500
			500 (1/20)		
II(b)	BDH lot 0868390	2,31	377 (1/30)	2	-
III	BDH lot 0868390	1,87	500 (1/20)	22	500
IV	Merck	2,25	282 (1/30)	1	-
V	Merck	4,50	350 (1/60)	N.D.	-
VI	Merck	discarded	-	-	-
VII	Merck	discarded	-	-	-
VIII	BDH lot 249680	4,50	500 (1/30)	198	-
IX	BDH lot 2337110	2,19	259 (1/25)	N.D.	-
			285 (1/30)	313	-
X(a)	BDH lot 2894030; 3012760	2,21	393 (1/25)	N.D.	-
			299 (1/30)	77	-
X(b)	BDH lots 2894030; 3012760	3,07	389 (1/40)	17	-
			289 (1/40)	N.D.	-
XI(a)	BDH lot 0868390	2,44	301 (1/15)	1	-
			281 (1/30)	0	-
XI(b)	BDH lot 0868390	1,76	412 (1/25)	0	-
XII(a)	BDH lot 300200	5,81	421 (1/35)	2	276
			262 (1/70)		
XII(b)*	BDH lot 300200	5,75	500 (1/35)	4	-
			317 (1/70)		
			475 (1/40)	N.D.	348
			500 (1/80)		
XIII	BDH lot 300200 (reppt)	4,87	232 (1/5)	N.D.	132
			176 (1/10)		
			146 (1/25)		
			50 (1/50)		

XIV	BDH lot 300200	4,26	359 (1/5) 490 (1/10) 500 (1/50) 500 (1/75)	4	500
XV	BDH lot 300200	3,54	500 (1/20) 500 (1/30) 500 (1/40)	33	500
XVI	BDH lot 300200	4,02	352 (1/10) 500 (1/20) 500 (1/40) 500 (1/50)	4	500
XVII	BDH lot 300200	5,67	not used	-	-
XVIII	BDH lot 300200	4,27	54 (1/5) 59 (1/10) 65 (1/20) 65 (1/50)	3	125

x Numbers in parenthesis represent the dilution at which the SNF-cytotaxin retentate was assayed.

† Chemotactic assays were done in conjunction with Dr. V.J. Stecher for SNF-preparations I to XI and the chemotactic responses recorded for 1% w/v casein controls are not available.

* The casein precipitate obtained in preparing SNF XII(a) was dissolved and the supernatant from the casein obtained on reprecipitation was concentrated and used as SNF XII (b).

reported (35,36,45,46). Care was thus taken to ensure that the pH of the solution did not exceed 8,0 at any stage.

(ii) During the acid precipitation step, care was taken to avoid overshooting the isoelectric point as the precipitated casein went back into solution at pH's below the pI. I found that I could judge the amount of acid to add at the end-point by inspection of the mother-liquor. This had an opalescent appearance until the pI was reached when it cleared abruptly.

(iii) Filtration of the supernatant before adjusting the pH to 7,4 was necessary to remove fine particles of precipitated casein which decanted with the supernate and would have redissolved at the neutral pH.

(iv) Sterility was not a problem in the initial stages and only became so during the concentration period which took days to complete. The Amicon system was sterilized with ethylene oxide except for the membrane which was sterilized with 25% ethanol. It was important to avoid bacterial or fungal contamination of the extract as their products are known to be chemotactic (62,82,171).

(v) The UM2 membrane filtrate was routinely collected and tested for chemotactic activity relative to the initial neutral supernatant fluid before concentration. The chemotactic response of cells to the filtrate was essentially the same as their background response to saline, indicating that the major chemotactic activity was retained by the UM2 filter. The UM2 filter concentrate contained a proportion of high molecular weight compounds which probably increased retentivity for the small molecular weight

cytotaxins found in the casein supernatant fluid. As I have shown in Chapter II these had molecular weights in the range 1 000 to 6 000 by gel chromatography and should, in theory, have passed through the membrane.

(vi) The precipitated casein, if redissolved, evoked a chemotactic response from neutrophils of the same order of magnitude as that shown by the original starting material. A substantial amount of chemotactic material thus co-precipitated with the casein at pH 4,5.

Concentrated ultrafiltrates of casein low molecular weight cytotaxins (CUF)

A 10% w/v stock solution of casein in 0,9% NaCl, pH 7,4, was prepared and sterilized as described in the preceding section. This stock solution was diluted 1:10 with sterile saline and subjected to ultrafiltration in an Amicon Model CH3 hollow fibre concentrator(1). A hollow fibre assembly with a molecular weight exclusion limit of approximately 10 000 was used to concentrate the casein. A 0,9% NaCl solution was added continuously to the concentrate to replace the volume removed by ultrafiltration. By so doing the concentration of casein was maintained at approximately 1% w/v and excessive pressure build-up prevented.

The ultrafiltrate was collected and sterilized by membrane filtration. It was then introduced into an Amicon stirrer cell concentrator connected to a stainless steel reservoir and equipped with a UM05 Diaflo membrane. The ultrafiltrate was concentrated under sterile conditions at 75 psi of nitrogen. Ten litres of ultrafiltrate took approximately

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(1) Amicon Corporation, Lexington, Massachusetts, U.S.A

three weeks to concentrate 50 to 100 times.

The concentrated hollow-fibre ultrafiltrate (CUF) was stored at -20°C in 0,5 to 2,0 ml volumes after re-sterilization. It was thawed rapidly at 37°C before use.

Notes and Comments

(i) Since, in this procedure, the majority of the large molecular weight components had been removed from the casein solution by ultrafiltration on the hollow fibre device, it was necessary to use a UM05 Diaflo membrane in the concentration stage. This membrane has a specified retentivity of greater than 90% for molecules of molecular weight greater than 1 000. The material passing through the UM05 membrane lacked chemotactic activity.

(ii) Both the retentate and the ultrafiltrate of the hollow fibre procedure contained considerable chemotactic activity. This may have been due to the presence of large molecular weight cytotaxins in the original 1% w/v casein solution. Alternatively, the lower molecular weight cytotaxins may have aggregated or combined with large molecular weight components in the casein solution. The reason for the retention of so much chemotactic activity in the casein was not further investigated.

(iii) In those experiments where it was felt desirable to prepare CUF free of salts, the 10% w/v casein stock solution was diluted with distilled water and the hollow fibre concentrate was replenished with distilled water.

(iv) As in the preparation of SNF, sterility only became a problem during the concentration period and the Amicon apparatus used was sterilized as for SNF concentration.

Table A.3.2

Chemotactic activities of low molecular weight concentrates of ultrafiltrates from casein (Hammarsten).

CUF	Source	Protein Concentration (mg/ml)	Chemotaxis (average cell count/HPF) Retentate†	Ultrafiltrate	1% casein
1*	BDH lot 300200 UM2 concentrate	1,42	4 (1/10) 0 (1/50) 0 (1/100) 0 (1/200)	0	139
2	BDH lot 300200 UM05 concentrate	24,8	111 (1/10) 117 (1/50) 111 (1/100) 6 (1/500)	2	143
3	BDH lot 300200 UM05 concentrate	38,9	373 (1/10) 502 (1/50) 527 (1/100) 305 (1/500)	3	460
4	BDH lot 300200 UM05 concentrate	2,0	385 (1/10) 152 (1/50) 33 (1/100) 10 (1/250)	7	337

†Numbers in parenthesis represent the dilution at which the CUF-cytotaxin retentate was assayed.

*Discarded and not used.

(v) The chemotactic activities of the various preparations used in this thesis are given in Table A.3.2, in terms of the minimum dilution capable of evoking a maximal chemotactic response from $2x10^6$ cells/ml.

Section A.4: Histological and histochemical procedures.

A.4.1 *Chrome-gelatin slides*

Two hundred millilitres of distilled water were heated in a boiling water bath. To this was added 1g of gelatin and the mixture was swirled. When the gelatin had dissolved, the solution was cooled to approximately 60°C and 0,1g of finely ground, powdered chrome alum was added. When the chrome alum had dissolved, particulate matter was removed by filtration through a Millipore AP2004700 glass fibre prefilter. The solution was stored in dark bottles at 4°C or used immediately.

Glass microscope slides were cleaned with chromic acid and rinsed thoroughly in distilled water. The slides were allowed to drain dry in a dust-free atmosphere. The slides were coated by dipping in the chrome-gelatin solution and air-drying at room temperature. Coated slides could be stored at room temperature indefinitely.

Notes

(i) When ideally coated, glass slides should wet evenly and maintain a film of water over their entire surface. Patches of hydrophobicity gave unsatisfactory results.

(ii) The chrome-gelatin coat provided a suitable surface for making smears of cells suspended in protein-free media. The addition of protein to the media did much to preserve cell morphology and was desirable, even when chrome-gelatin slides were used.

(iii) During the preparation of the reagents and coating of the slides all precautions were taken to avoid contamination with dust. The presence of particulate matter in the chrome-gelatin or on the slide surfaces resulted in uneven coating.

(iv) The chrome-gelatin could be used repeatedly provided it was free of particulate material.

A.4.2 *Fixation and storage for esterase detection.*

Cold formol-calcium fixation with storage in gum-sucrose was used for enzyme histochemistry.

Formol-calcium solution was prepared as follows:

$CaCl_2 2H_2O$	...	1 gm
Concentrated formaldehyde (approximately 35%)	...	10 ml
Distilled water up to	...	100 ml

The pH was adjusted to 7,0 with 1N NaOH.

After filtration, the fixative was stored at 4°C.

Gum-sucrose solution was prepared as follows:

Gum acacia (dry material)	...	2,0 g
Sucrose	...	50,0 g
Distilled water up to	...	200 ml

The solution was filtered and stored at 4°C for no longer than one month.

Cell preparations were fixed in formol-calcium for 4h at 4°C, rinsed in gum-sucrose and stored in gum-sucrose for 24h at 4°C. Gum-sucrose was removed by rinsing in distilled water before applying the histochemical procedure.

A.4.3 *Cell stains*

The two modified Romanowsky stains that I used routinely for staining rabbit peritoneal exudate cell smears were those developed in 1902 by Giemsa and May and Grünewald (59). The former gives the standard 3 colour staining reaction (purple nuclei; blue cytoplasm and red erythrocytes); with the latter only red and blue staining reactions are observed.

Cell-containing filters removed from Boyden chambers were stained with Weigert's iron haematoxylin stain. This stain colours cell nuclei an intense dark blue. It withstood the dehydration and clearing procedures which I used and had the advantage that the filter fibres also took on a faint blue stain. This did not detract from the ease with which cells could be identified but helped to define the precise focal plane of the undersurface of the filter. The haematoxylin stains described by Harris and Mayer (59) are more subtle and are preferred for histological work. They were not, in my experience, as well suited for staining cells within the filter.

Giemsa Stain

Stock stain solution was prepared by dissolving 1,0g of compounded Giemsa stain[1] in 54 ml of warm glycerol. After cooling, 54 ml of methanol was added. The solution was filtered and stored in dark bottles at room temperature.

Stock diluent was prepared by titrating 0,15M Na_2HPO_4 to pH 6,8 with 0,15M KH_2PO_4.

Smears were air dried and fixed in methanol for 10 min. The

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(1) E. Merck, Darmstadt, West Germany. Catalogue number 9203.

Giemsa stock solution was diluted 1:10 with diluent or with tap water.

The fixed smears were stained for 10 min with the diluted Giemsa at room temperature. Excess dye was removed by rinsing with diluent or with tap water.

Notes

(i) Water could be used as a diluent for the dye and for rinsing, but better colour differentiation was obtained with the buffer.

(ii) The diluted Giemsa was prepared just before use and used only once.

May-Grünewald/Methylene blue

Working solutions were prepared by (a) dissolving 0,2g of May-Grünewald stain[1] in 100 ml methanol and (b) dissolving 1,0g of methylene-blue[2] in 100 ml distilled water. Both solutions were filtered and stored at room temperature.

Air-dried, unfixed cell smears were flooded with undiluted May-Grünewald stain for 3 to 5 min, preferably under cover to avoid evaporation of the solvent. Excess dye was shaken off and the films counter-stained with methylene blue for ½ to 1 min. Finally, the smears were rinsed in distilled water and drained dry.

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(1) George T. Gurr, Searle Scientific Services, High Wycombe, Buckinghamshire, England. Catalogue number 17300.

(2) George T. Gurr, Searle Scientific Services, High Wycombe, Buckinghamshire, England. Catalogue number 18400; colour index number 52015.

Notes

(i) Since the May-Grünewald solution contained 100% methanol, fixing and staining were achieved simultaneously.

(ii) The May-Grünewald stock could be used in more dilute form using methanol as the diluent.

(iii) The smears were not rinsed between stains.

(iv) The distilled water rinse could be replaced with a 0,15M Na/K phosphate buffer pH 6,8 rinse with similar, if not better, colour differentiation.

(v) The May-Grünewald/methylene blue differential stain gave satisfactory results allowing easy identification of the various cells. The polymorphonuclear leucocytes with their pale pink cytoplasm and blue nuclei were easily distinguished from the blue-staining monocytes.

(vi) This technique required careful attention to detail as the rabbit cells did not readily take up the stain.

Weigert's iron-haematoxylin

Stock solutions were prepared by (a) dissolving 10,0g haematoxylin[1] in 1 000 ml of 95% ethanol and (b) dissolving 11,6g ferric chloride hexahydrate ($FeCl_3.6H_2O$) in 980 ml of distilled water and 10 ml of concentrated hydrochloric acid. Both stock solutions were filtered and allowed to "mature" at room temperature in dark bottles, for a minimum of 2 weeks before use.

The solutions were mixed in equal proportions and aged for

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(1) George T. Gurr, Searle Scientific Services, High Wycombe, Buckinghamshire, England; catalogue number 14500; colour index number 75290.

approximately 10 min before use. The stain was used without further dilution. Cell preparations were exposed to the stain for 2 min, rinsed free of excess stain in distilled water and "blued" by immersion in tap water for 10-15 min.

A.4.4 *Trypan blue exclusion test for cell viability.*

This assay is based upon the fact that viable cells are able to exclude trypan blue from the cell cytoplasm whereas damaged cells are not. Cytoplasmic staining with the dye is therefore taken as an indication of cell death.

Trypan blue powder[1] was prepared as a 0,125% w/v solution in double-distilled water and sterilized and cleared by filtration through a 0,45µm membrane filter. This reagent was stable indefinitely in the dark at room temperature.

Immediately before use four volumes of 0,125% trypan blue were combined with one volume of 4,5% NaCl to give a working isotonic solution (0,1% trypan blue; 0,9% NaCl).

The cell suspensions were mixed with an equal volume of the working solution of trypan blue and incubated at room temperature for 10 to 15 min. The cells were then examined microscopically on a Neubauer counting chamber. Four hundred cells were examined and cell viability was estimated as the percentage of cells that were unstained.

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(1) George T. Gurr, Searle Scientific Services, High Wycombe, Buckinghamshire, England; catalogue number V-30350; colour index number 23850.

Notes

(i) Trypan blue has a high affinity for protein with the result that any protein supplements present in the cell-suspending medium bind the dyes. If, therefore, viability assays are done in the presence of large amounts of protein, artificially high estimates of cell viability may be obtained.

(ii) Polymorphonuclear leucocytes, being phagocytic cells often appeared to have discrete spots of stained material within the cytoplasm. These I took to be pinocytic or phagocytic vesicles and such cells were not scored as "dead". Dead cells appeared as large amorphous blue discs with indistinct nuclei.

(iii) Incubation of the cells with trypan blue at 37°C or room temperature appeared to make no difference to the viable cell count.

(iv) I found that the number of viable cells dropped progressively with exposure to the tungsten illumination of the microscope. This was particularly pronounced in the case of protein-free cell suspensions and could be controlled by inserting a heat absorbing filter into the light path below the microscope sub-stage condenser.

A.4.5 *Esterase stains*

The histochemical stains I used to detect esterases in rabbit peritoneal exudate cells were based, in principle, on the ability of the enzymes to hydrolyse synthetic substrates with the formation of insoluble, coloured reaction products at the sites of enzymatic hydrolysis.

Theoretical and practical aspects of these procedures have been discussed in detail by Pearse (127).

Indoxyl acetate

Hydrolysis of the synthetic substrate, 5-bromoindoxyl-acetate liberated free indoxyl groups which were oxidized by atmospheric oxygen to give insoluble, bright blue indigo dye.

The following stock reagents were prepared:-

(a) 0,1M tris/HCl pH 6,8

(b) 0,05M potassium ferricyanide; $K_3\left[Fe(CN)_6\right]$

(c) 0,05M potassium ferrocyanide; $K_4\left[Fe(CN)_6\right]$

(d) 0,1M $CaCl_2$

(e) The substrate, 5-bromoindoxyl-acetate[1] was dissolved in ethanol immediately before use at a concentration of 1,3mg/ml.

(f) Counter stain: Mayer's carmalum was prepared by dissolving 10g of carmalum powder[2] in approximately 180ml distilled water containing 5ml glacial acetic acid by boiling for 1h. After cooling the volume was adjusted to 200ml with distilled water and the solution was filtered into dark bottles for storage at room temperature.

The working solution was prepared immediately before use from the stock reagents by preparing the following mixture.

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(1) Koch Light Laboratories Limited, Colnbrook, Buckinghamshire, England; catalogue number 0747h.

(2) Hopkins and Williams Limited, Chadwell Heath, Essex, England; catalogue number 958600.

0,1M tris/HCl pH 6,8	...	20,0 ml
0,05M $K_3\left[Fe(CN)_6\right]$	...	10,0 ml
0,05M $K_4\left[Fe(CN)_6\right]$	...	10,0 ml
0,1M $CaCl_2$	...	10,0 ml
0,13% w/v 5-bromoindoxyl-acetate in ethanol	...	1,0 ml
Distilled water	...	49,0 ml

The 99ml of salt solution was prepared initially after which the 1ml substrate stock was added rapidly as a sharp jet.

The incubation medium was used immediately, without further treatment, with the substrate at a final concentration of 0,0013% w/v.

Notes

(i) The blue enzyme reaction products and carmalum counterstain were stable to alcohol dehydration, xylene clearing and permanent mounting.

(ii) Any suitable diazonium salt could be used for coupling to the enzymatically liberated indoxyl group to form insoluble coloured complexes at the site of enzyme action.

α-Naphthyl acetate/hexazotized pararosanilin

Hydrolysis of the synthetic substrate α-naphthyl acetate liberated free naphthol which combined with the diazonium salt to form an insoluble, brick-red complex at the site of enzymatic activity.

The following reagents were prepared:

(a) 0,15M Na_2HPO_4/KH_2PO_4 buffer pH 7,6.
Filtered and stored at 4°C.

(b) 4% w/v sodium nitrite
Prepared freshly just before use in distilled water.

(c) 4% w/v pararosanilin hydrochloride.
One gram of pararosanilin[1] was dissolved in a mixture of 20 ml distilled water and 5 ml concentrated HCl by warming at 37°C for about 30 min. After cooling, the stock was filtered and the filtrate stored at room temperature in the dark.

(d) Substrate stock; (0,6% w/v α-naphthyl acetate). Thirty milligrams of α-naphthyl acetate[2] was dissolved in 5 ml 2-ethoxyethanol (ethylene glycol monomethyl ester) immediately before use.

(e) Coupler; Hexazotised pararosanilin.
Ten ml of 4% pararosanilin hydrochloride solution (c) was mixed with 2,0 ml freshly prepared 4% sodium nitrite (b). The mixture was shaken for 1 min at room temperature and the gold-brown solution used immediately.

(f) Counter stain: Harris' haematoxylin.
Ten grams of ammonia alum were dissolved in 500 ml distilled water with warming. To this was added 25 ml of a freshly prepared 10% w/v solution of haematoxylin[3] in ethyl alcohol.

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(1) Edward Gurr Limited, London, England; catalogue number 722

(2) Koch Light Laboratories Limited, Colnbrook, Buckinghamshire, England; catalogue number 4139h.

(3) George T. Gurr, Searle Scientific Services, High Wycombe, Buckinghamshire, England; catalogue number 14500, colour index no. 752

The mixture was brought to the boil and, while still boiling, 1,25 g mercuric oxide were added and dissolved with stirring. The solution was cooled rapidly by immersion in cold water, filtered and stored at room temperature in the dark.

For use, 53,4ml of phosphate buffer (a) was mixed with 3,6 ml of hexazotized pararosanilin (e) and brought to pH 6,1 with 1N NaOH. The stock substrate solution (d) was then added as a 3,0ml volume. Any precipitate was removed by filtration and the medium used immediately.

Notes

(i) I found it more convenient to adjust the final pH of the incubation medium before the addition of the substrate stock as a fairly large adjustment from approximately 4,0 to 6,1 was necessary. If the substrate was present in the acid medium, a brown flocculent precipitate formed on adjustment.

(ii) Any counterstain which would provide a contrasting background to the enzyme sites could be used. I used Harris' haematoxylin, Weigert's iron haematoxylin (which gave blue backgrounds) or 1% w/v aqueous methyl green (which gave a green background). All three counterstains were satisfactory.

(iii) Any diazonium salt could be used to couple free naphthol liberated as a product of enzyme hydrolysis, provided the final complex was insoluble and easily localised by its characteristic colour. Other standard couplers used were fast red, fast blue and fast garnet GBC.

I used hexazotised pararosanilin as the coupler in those

experiments where dehydration and clearing of the specimens before preparing permanent mounts was intended. The α-naphthol/hexazotised pararosanilin complex was stable to dehydration and mounting whereas the other couplers mentioned did not give stable complexes.

N-acetyl-DL-phenylalanine-β-naphthyl ester/fast red

The substrate was hydrolysed by the enzyme to liberate naphthol which coupled with the diazonium salt to give a red-yellow insoluble complex at the site of the enzyme.

The following reagents were prepared:-

(a) Tris-gelatin-saline (TGS)

One half gram of gelatin was dissolved in 0,15M NaCl containing 5mM tris/HCl buffer, pH 7,3 by heating to 56°C in a water bath. The solution was cooled to room temperature, sterilized by membrane filtration and stored at room temperature.

(b) Coupler solution (0,009% fast red).

A solution of 8mg of fast red(1) in 90 ml of TGS was prepared immediately before use.

(c) Substrate stock solution was prepared immediately before use by dissolving 1,6mg of N-acetyl-DL-phenylalanine-β-naphthyl

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(1) diazonium salt of 2-amino-5-chlorotoluene; Koch Light Laboratories Limited, Colnbrook, Buckinghamshire, England; catalogue number 1588h.

ester[1] in 18ml of a mixture of 12 parts acetone and 6 parts of N,N'-dimethylformamide.

To prepare the incubation medium, 10 ml of substrate stock solution was added to 90ml of the coupler solution. Any precipitate which formed was removed by filtration.

Notes

(i) The fast red diazonium coupler was replaced with fast garnet GBC in some experiments. Where dehydration and clearing were required, hexazotised pararosanilin was used at the same concentration.

(ii) The substrate appeared to be very labile in aqueous solution with rapid spontaneous hydrolysis and the formation of large amounts of precipitate.

(iii) When hexazotised pararosanilin was used in place of fast red, the pH of the TGS/coupler solution was adjusted to pH 7,3 before the addition of the substrate.

α-Naphthyl acetate/fast garnet GBC

The synthetic substrate α-naphthyl acetate was hydrolysed by the enzymes to liberate free naphthol which was coupled by the diazonium salt to form an insoluble magenta/burgundy coloured complex at the site of enzymatic activity.

The following reagents were prepared:

(a) McIlvaine's buffer (Disodium phosphate citric acid buffer pH 7,0) was prepared by mixing 86,3 ml 0,1M citric acid with 453,7 ml

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(1) Schwarz Mann, Orangeburg, New York, U.S.A.; catalogue number 631.

0,2M disodium phosphate, adjusting the pH to 7,0 with the relevant solution and making the volume up to 1 000 ml with distilled water. The buffer was filtered and stored at 4°C.

(b) Coupler solution (0,5% w/v fast garnet GBC).
Sixty millilitres of McIlvaine's buffer pH 7,0 was used to dissolve 0,03g fast garnet GBC[1] immediately before use.

(c) Substrate (1% w/v α-naphthyl acetate). Five millilitres of acetone were used to dissolve 0,05g α-naphthyl acetate[2] immediately before use.

One millilitre of the freshly prepared substrate solution was added as a jet to the 60 ml fast garnet solution. Any precipitate was removed by filtration and the medium was used immediately.

Notes

(i) This histochemical stain was used mainly for staining bands of esterase activity in polyacrylamide gels.

(ii) The solubility of the coloured complex in alcohol precluded the use of this technique with histochemical procedures requiring dehydration, clearing and mounting.

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(1) Edward Gurr Limited, London, England; catalogue number 297.

(2) Koch Light Laboratories Limited, Colnbrook, Buckinghamshire, England; catalogue number 4139h.

Section A.5: Protein determination

Rough estimates of protein concentration were obtained from measurement of the optical density of the solution at 260nm and 280nm. Protein concentration was then calculated from the empirically established relationship (72).

$$\text{Protein concentration (mg/ml)} = 1{,}45\ OD_{280nm} - 0{,}75\ OD_{260nm}$$

The 280nm chromophores in proteins are principally aromatic amino acid residues, and the relationship given above would obviously depend upon the abundance of tyrosine, phenylalanine and tryptophan in the protein concerned. Although subject to this inherent source of error, the method was extremely useful for rapid approximate use.

More precise determinations were done with Folin's phenol reagent using a minor modification of the method of Lowry, Rosebrough, Farr and Randall (103) and crystalline bovine serum albumin (BSA) as a standard. Briefly, the procedure was as follows:-

The following reagents were prepared:

(a) Na_2CO_3 20g
Sodium potassium tartrate 0,2g
0,1N NaOH to 1 litre

(b) $CuSO_4.5H_2O$ 0,5g
Water to 100 ml.

(c) Folin-Ciocalteau phenol reagent was purchased from British Drug Houses Chemicals Limited, Poole, England as a solution in approximately 2N HCl. The precise molarity of the HCl was determined by titration with NaOH.

(d) Shortly before use, alkaline copper tartrate reagent was prepared by mixing 50 parts of (a) with one part of (b).

(e) Shortly before use, working Folin-Ciocalteau reagent was prepared by diluting (c) with distilled water to make it 1N with respect to HCl.

The assay was performed as follows: To 0,4ml of sample containing less than 400µg of protein was added, with immediate mixing, 2ml of alkaline copper tartrate (reagent d). After 30 min 0,2ml of working Folin-Ciocalteau solution (reagent e) was added with mixing on the vortex. The blue colour that developed was read after 120 min at 500mn. Blank tubes (containing no protein) and standard tubes containing from 50 to 400µg of BSA) were run simultaneously.

A standard curve of OD_{500nm} vs µg BSA added,was constructed by fitting a second degree polynomial equation to the observed points by the method of least squares. The unknown protein concentrationswere then computed by interpolation using the parameters of the quadratic equation.

Occasionally, protein solutions in 1N NaOH were assayed. In this case, BSA standards were dissolved in 1N NaOH and 0,1N NaOH was omitted from reagent (a).

The sensitivity of the method could be extended to cover the range 10 to 50µg of protein by halving the volume of the reagents and working with a final reaction volume of 1,3 ml, or reading optical densities at 730nm instead of 500nm.

Section A.6 : Preparation of dialysis tubing.

Dialysis tubing was prepared for use by boiling it in a neutral solution of 3,72g of disodium EDTA and 4,86g of $NaHCO_3$ in 2ℓ of distilled water.

The tubing was then washed extensively by soaking in several changes of distilled water and stored, in distilled water, at $4^{o}C$.

Section A.7: Polyacrylamide gel electrophoresis.

Electrophoresis in polyacrylamide gels has become a standard procedure in the technical repertoire of macro-molecular analytical biochemistry, and there are innumerable references in the literature that testify to its usefulness for the resolution of complex protein mixtures and for the identification of components of such mixtures in terms of molecular size and charge.

A number of papers have been published in which the theoretical and practical aspects of polyacrylamide gel electrophoresis have been discussed at length (41,58,90,105,123). Of these, I have personally found the article by Maizel (105) most helpful. This detailed review and the paper by Young and Bittar (190) dealing specifically with isoelectric focusing techniques for the identification of tissue esterases, have had considerable influence upon my choice of technical procedures. For the most part I used either (a) electrophoresis in slab gels consisting of linear gradients of polyacrylamide and containing sodium dodecyl sulphate (SDS) or, (b) isoelectric focusing in rod or slab gels containing Ampholines. The former technique separated known proteins on the basis of size, so that linear relationships were found between electrophoretic mobility and the logarithm of the molecular weight. Isoelectric focusing separated proteins on the basis of isoelectric points. The following is a detailed account of the technique that I employed.

Reagents

Since the reagents and apparatus used were common, in most instances, to both SDS-gradient gel electrophoresis and the isoelectric focusing technique I shall describe these first and give technical details for each procedure separately.

(R.A) Acrylamide

Acrylamide[1] was recrystallized by dissolving 70g in 1ℓ of chloroform at 50°C and filtering, at the same temperature, without suction, to remove undissolved material. The filtrate was cooled gradually by stepwise reduction of temperature to room temperature, 4°C and finally -15°C. The flat, clear crystals were harvested in a sintered glass funnel and washed with -15°C chloroform, dried under vacuum and stored in dark bottles at room temperature.

(R.B) *bis*-Acrylamide

N,N'-*bis*-methylene acrylamide[2] was recrystallized from acetone by dissolving 12g of the powder in 1 000 ml of acetone at 37°C and filtering, without suction, at the same temperature. White, needle-like crystals were harvested after gradual cooling with stepwise reduction of temperature to room temperature, 4°C and -15°C. The crystals were washed with -15°C acetone, dried under vacuum and stored at room temperature in dark bottles.

(R.C) Temed

N,N,N',N' tetramethylenediamine[3] was stored undiluted, without further preparation, in dark bottles at 4°C.

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(1) E. Merck, Darmstadt, West Germany; catalogue number 830 or Eastman Kodak, Rochester, New York, U.S.A.; catalogue number 5221

(2) Eastman Kodak, Rochester, New York, U.S.A.; catalogue number 8383 or Pfleuger, Brussels, Belgium.

(3) Eastman Kodak, Rochester, New York, U.S.A.; catalogue number 8178

(R.D) Sodium Dodecyl Sulphate (SDS)

SDS[1] was recrystallized from ethanol by dissolving 15g in 300ml of 95% ethanol at 60°C and filtering, without suction, at this temperature. The filtrate was cooled to 35°C by allowing the container and the waterbath in which it was immersed to cool slowly to this temperature. Contaminants that separate at 35°C were removed by filtration at this temperature and the filtrate was cooled to 4°C for 16h. The crystals were harvested on a sintered funnel, washed with ice-cold 95% ethanol, dried under vacuum and stored in dark bottles at room temperature.

(R.E) Ampholyte Solutions

Carrier ampholytes[2] were supplied as 40% w/v sterile aqueous solutions in multidose vials to produce the following pH gradients, and stored under sterile conditions, at 4°C.

(i) pH 3,5 - 10,0 (batch 40 or batch 21)

(ii) pH 5 - 8 (batch 21)

(iii) pH 5 - 7 (batch 8)

(iv) pH 7 - 9 (batch 7)

All other reagents were analytical grade and used without further purification.

Stock and Working Solutions

(S.1a) Resolving gel buffer for SDS gels (1,5M tris/HCl, pH 8,8)

18,171g tris (hydroxymethyl)aminomethane were dissolved in approximately 80ml double distilled water; brought to pH 8,8 with 4N HCl (should require approximately 6ml);

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(1) British Drug House Chemicals Limited, Poole, England; catalogue number 30176.

(2) LKB Produkter AB; Bromma, Sweden.

and made up to 100ml. Stored at 4°C.

(S.1b) Spacer gel buffer for SDS gels (0,5M tris/HCl pH 6,8)

6,057g tris(hydroxymethyl)aminomethane were dissolved in approximately 70ml of double-distilled water; brought to pH 6,8 with 4N HCl (requires approximately 12 ml); and made up to 100ml. Stored at 4°C.

(S.2) Acrylamide-*bis*-acrylamide stock

30g of acrylamide (R.A.) and 0,8g of *bis*-acrylamide (R.B) were dissolved in water and made up to 100ml.

Reservoir Buffers

(S.2a) Anode and cathode buffer for SDS gradient gels (0,025M tris; 0,192M glycine pH 8,3). 15,1425g tris and 67,2673 glycine were dissolved in double distilled water and made up to 5ℓ with water. Stored at 4°C.

(S.2b) Anode buffer for isoelectric focusing (0,01M phosphoric acid)

Prepared freshly before use.

(S.2c) Cathode buffer for isoelectric focusing (0,02M NaOH)

Prepared freshly before use.

Other Solutions and Reagents

(S.3) Initiator (1%w/v ammonium persulphate)

0,1g $(NH_4)_2S_2O_8$ were dissolved in 10ml double distilled water freshly before use.

(S.4) Stock SDS solution (10% w/v SDS)

10g dry, recrystallized SDS (R.D) were dissolved in water and made up to 100ml. Stored in dark bottles at room temperature.

(S.5) Stock bromophenol blue (0,4% w/v in distilled water)

400mg bromophenol blue were dissolved in 100ml distilled water and brought to pH 7,0 with 1N NaOH.

(S.6) Coomassie brilliant blue R250 (1% w/v aqueous solution).

1g of the dye[1] was dissolved in 100ml distilled water. The solution was filtered and stored at room temperature.

(S.7a) Fixing and staining solutions for protein in SDS gels

Methanol, 30ml; glacial acetic acid, 10ml; water, 55ml; and Coomassie brilliant blue (S.6), 5ml were mixed freshly before use.

(S.7b) Destaining solution

For use after S.7a. Prepared as S.7a with 5ml water substituting for 5ml S.6.

(S.8) Fixing and staining solutions for proteins in isoelectric focusing gels containing Ampholines. (3,5% Perchloric acid with or without 0,04% Coomassie brilliant blue G250).

Gels were fixed in 3,5% aqueous perchloric acid (PCA); stained with 3,5% PCA containing 0,04% Coomassie brilliant blue G250[2] and destained with 3,5% aqueous PCA. At the pH of 3,5% PCA, Coomassie brilliant blue G250 has an orange colour when free and a blue colour when complexed to protein. 3,5% PCA was prepared by diluting 70% PCA 20-fold with distilled water.

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(1) Schwarz Mann, Orangeburg, New York, U.S.A.; catalogue number 9207.

(2) Serva Feinbiochemica GmbH and Company, Heidelberg, West Germany; catalogue number 17524.

(S.9) Requirements for Auto-fluorography

(S.9a) Dimethylsulfoxide[1] (DMSO). Used as supplied.

(S.9b) DMSO containing 22% w/v 2,5-diphenyloxazole[2] (PPO). 22g PPO were dissolved in DMSO and made up to 100ml. Stored at room temperature in dark bottles. Avoid skin contact.

(S.9c) Osray M3 no-screen 8"x10" x-ray film[3].

(S.9d) Kodak x-ray liquid developer DX-80[4]. Prepared and used according to the accompanying manufacturer's instructions.

(S.9e) Kodak x-ray liquid fixer FX-40[4] prepared and used according to the accompanying manufacturer's instructions.

(S.10) Agarose sealing mixture (1,5% agarose in 0,1% sodium azide) 1,5g of agarose[5] were dissolved in 70ml of distilled water with boiling. When the agarose had dissolved the solution was made up to 100ml with distilled water at 60°C and 0,1g sodium azide was added. The solution was then dispensed in 2,5ml volumes into glass test tubes and allowed to cool and solidify. The solidified agarose gel in one tube was molted at 100°C just before use and used to seal the electrophoresis chambers. Unused agarose was discarded as it lost its effective sealing capacity after re-melting.

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(1) E. Merck, Darmstadt, West Germany; catalogue number 2931

(2) Packard Instrument Company Incorporated, Downer's Grove, Illinois, U.S.A.; catalogue number 6002023

(3) Agfa-Gevaert, Tontgen, Belgium.

(4) Eastman Kodak Company, Rochester, New York, U.S.A.

(5) Miles Laboratories (Pty) Limited, Goodwood, Cape Province, South Africa.

Apparatus

Rod gel electrophoresis

Three types of apparatus were used.

(i) A commercially available disc electrophoresis apparatus(1) in which eight 75mmx5mm diameter gel tubes were held vertically by waterproof rubber grommets between cylindrical upper and lower electrode reservoirs. The tubes were set in circular array equidistant from centrally placed circular electrodes.

(ii) A modification of the above apparatus in which eight 130mmx2,3mm diameter tubes were held, in the same configuration as the Shandon design. In this model, however, the lengths of the tubes protruding from the upper reservoir were cooled by almost complete immersion in the buffer in a much deeper lower reservoir.

(iii) An apparatus constructed in our own workshops, in which tubes were held vertically between upper and lower electrode vessels in linear array. The sections of the gel tubes not covered by buffer were cooled by circulating water at $4^{o}C$ through an intervening compartment.

Vertical slab gel electrophoresis.

The apparatus used for this procedure was similar, in basic design, to that described by Reid and Bieleski (131). It was made in our own workshops and is shown diagrammatically in Figures A.7.1 and

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(1) Shandon Scientific Company, Willesden, England.

Figure A.7.1

Figure A.7.1 Exploded view of gel chamber of vertical slab gel electrophoresis.

The gel chamber was assembled before from two glass plates (H and I) separated by Perspex side spacers (J) measuring 220mmx10mmx1,6mm. The front plate (I) was rectangular and measured 220mmx150mmx3mm. The rear plate (H) was identical in outside dimensions and was provided, at its upper edge, with a centrally situated notch 130mm wide and 20mm deep.

A spacer comb (L) was fashioned from Perspex and was equipped with "teeth" of the same thickness (1,6mm) as the side and bottom spacers. The comb was inserted into the gel before polymerization to serve as a mould for the castellated sample wells at the top of the gel.

The assembled chamber was clamped in the electrophoresis apparatus with the notched plate (H) against the face plate (A in Figure A.7.2) and separated from it by a silicone rubber gasket.

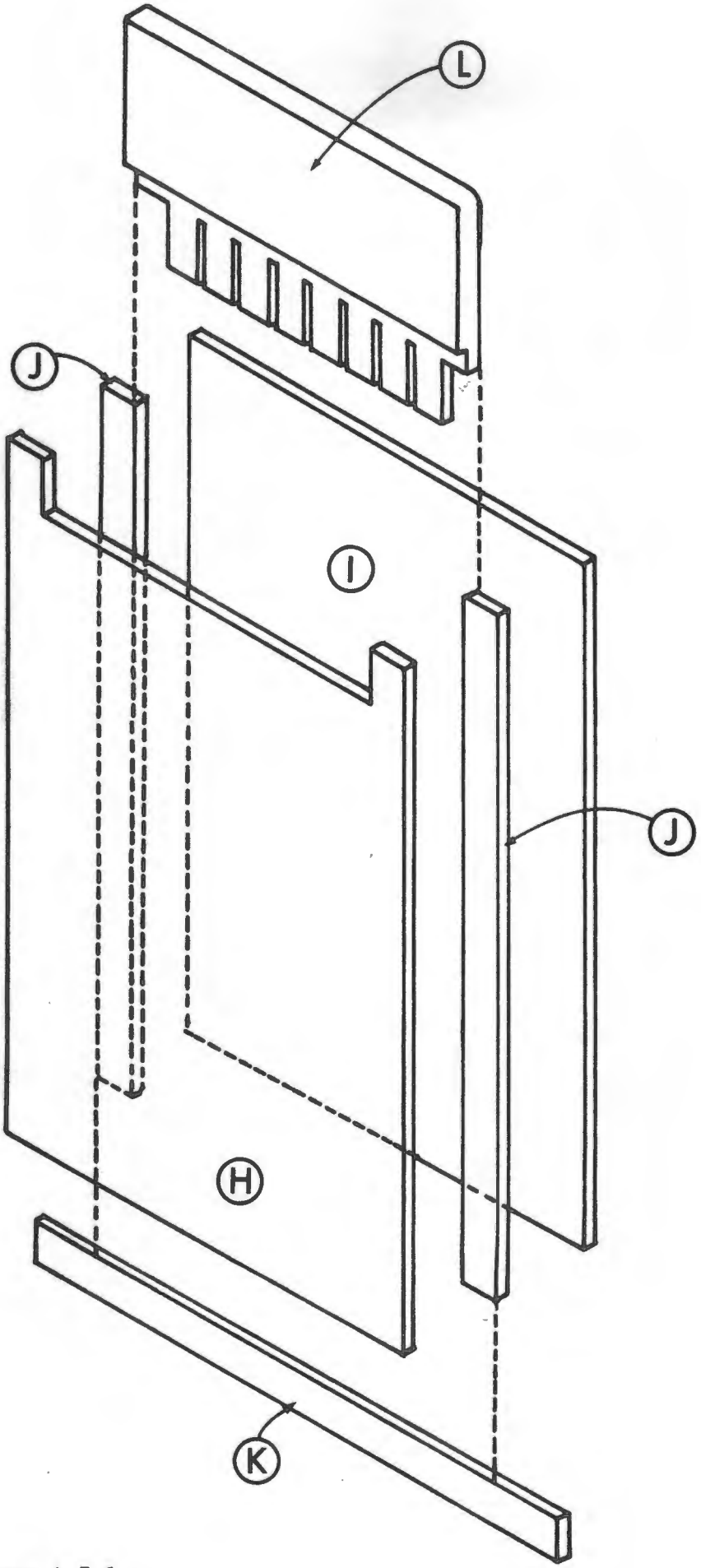

Figure A.7.1

Figure A.7.2

Figure A.7.2 Apparatus used for vertical slab gel electrophoresis.

The apparatus was constructed of Perspex sheet and consisted of a face plate (A) that formed the front side of the upper buffer reservoir (B) and the rear side of the lower buffer reservoir (C). The assembled electrophoresis chamber shown in Figure A.7.1. was clamped to the face plate by means of large stationary clamps that gripped the chamber and the protruding edges (D) of the face plate.

The chamber was separated from the face plate by means of a 2,0mm thick sheet of silicone rubber (not shown) cut to the precise pattern of the notched rear wall of the chamber (H in Figure A.7.1). This secured a leak-proof seal between upper and lower reservoirs.

The assembled chamber rested on blocks (E) set in the rear corners of the lower reservoir.

Platinum electrodes (F) were connected to the power supply with crocodile clips.

The entire assembly was mounted on a Perspex base plate (G) measuring 180x190cm.

Dimensions

The dimensions were not critical. The face plate (A) was cut to the exact same width as the chamber and was of a height such that the notch in the rear plate (H) of the chamber coincided exactly with the notch at the top of the face plate. The level of the buffer in the reservoir was above that of the notch during electrophoresis.

Reservoir dimensions (inside)

	Side to side	Front to rear	Vertical depth
Upper reservoir	130mm	70mm	25mm
Lower reservoir	150mm	70mm	35mm

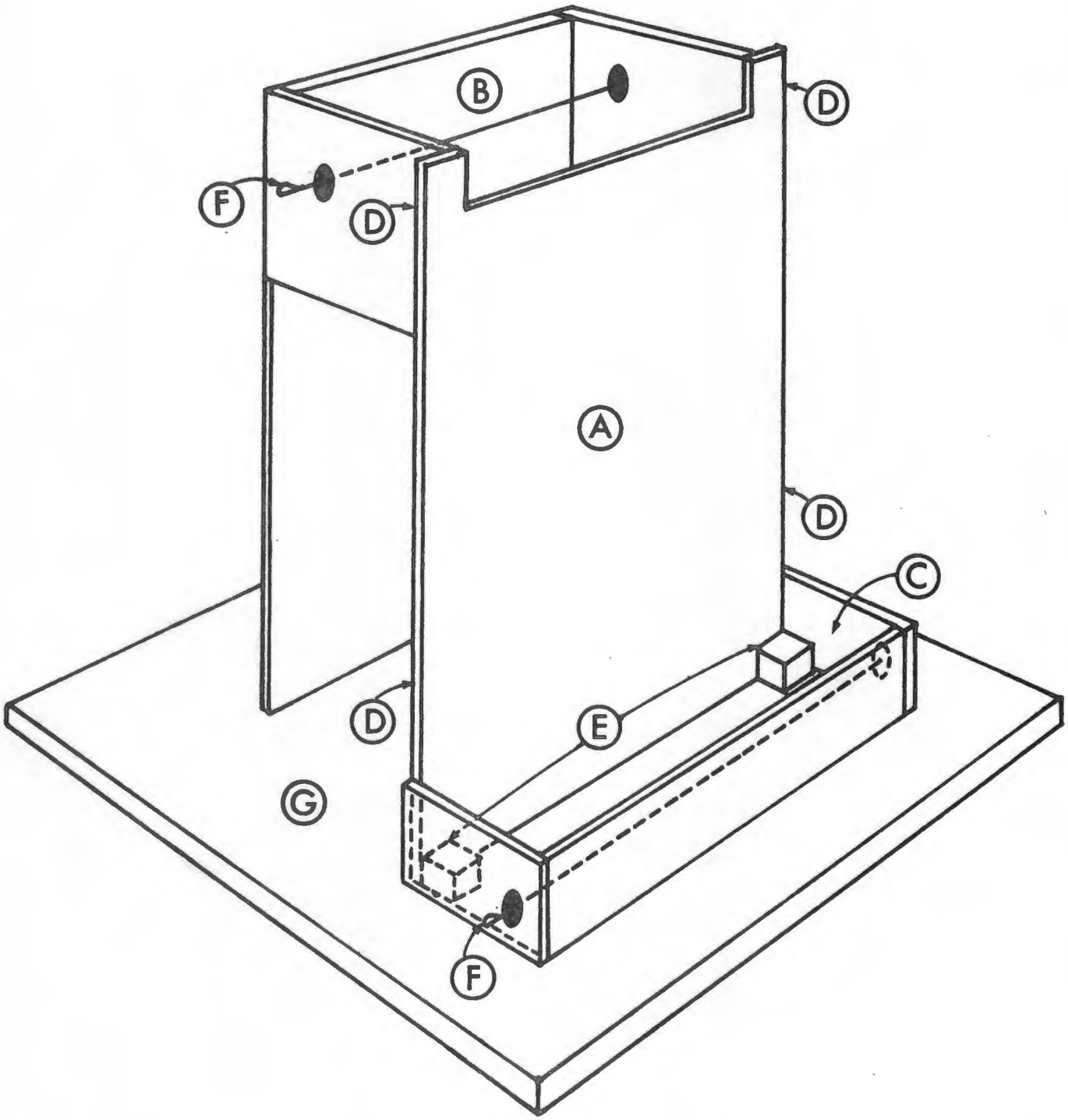

Figure A.7 .2

A.7.2. It consisted, essentially, of (a) a gel chamber assembled from flat glass plates separated by Perspex spacers and sealed with agarose (S.10) and (b) an electrophoresis tank made of Perspex and equipped with upper and lower electrode reservoirs and platinum wire electrodes.

Gradient former

The apparatus used for forming linear gradients of polyacrylamide consisted of two vertical, 30ml, cylindrical vessels that communicated at their bases by means of a narrow tubular orifice that could be sealed with a stopcock. One vessel was filled with low-concentration polyacrylamide solution; the other (the mixing vessel) was filled with high-concentration polyacrylamide solution whose density had been increased by the addition of glycerol or sucrose. The mixing vessel contained a magnetic stirring bar and was equipped with an outlet at the base controlled by a second stopcock. From this outlet a length of silicone rubber tubing led through a roller pump to a 15 gauge hypodermic needle that was clamped to the upper, inside edge of the assembled electrophoresis chamber. After the vessels had been charged, the stopcocks were opened and the pump set to deliver approximately 3ml/min. The chamber was thus filled, from below upwards, with a thin stream of polyacrylamide solution of progressively decreasing density and monomer concentration.

Drying Apparatus

Slab gels were dried, for storage or contact autofluorography, by layering them on a wet sheet of Whatman's 3MM filter paper resting on a sheet of proour polyethylene. The assembled layers of gel-filter paper-porous polyethylene were then placed upon a perforated stainless steel plate that formed the roof of a shallow stainless steel chamber fitted with

an attachment for a vacuum line. The whole assembly was then enveloped in domestic plastic wrap to effect an air-tight seal and vacuum was applied. Gentle, even heating by means of a stream of hot air directed at the gel through the plastic wrap accelerated drying and attached the dried gel as an adherent, firm, thin film to the filter paper.

Gel slicer

Two gel slicers were used. The first, a commercially available device[1] involved freezing the gel with dry ice on to a platform. Frozen serial sections were then cut with a blade that moved, in "chopper" fashion, advancing automatically, a set distance (usually 1mm) between each section. This had the disadvantage that the dry ice used to freeze the gel on to the platform interfered with subsequent pH measurements in the sections.

A much superior device was constructed in our own workshops and consisted of 90 safety razor blades threaded, with 1,6mm spacing washers, on brass rods. The rods were threaded at each end to receive clamping nuts that held the assembly of razor blades firmly aligned. Gels, either as rods or as strips of suitable width, were, after freezing in a -80°C freezer in Perspex troughs, cut into 1,6mm slices with a single vertical movement of the hand-held, pre-cooled (-15°C) slicer.

Power supply

A VoKam power supply[2] was used. This apparatus could be operated in either the "constant current" or "constant voltage" mode.

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(1) Mickle Laboratory Engineering Company, Surrey, England.

(2) Shandon Scientific Company, Willesden, England

It was capable of delivering 0-80mA of direct current at a stabilized voltage from 0-300V.

Techniques

SDS gradient gel electrophoresis in vertical slab gels

The slab gel apparatus depicted in Figure A.7.1 was assembled, sealed along the three spacer edges with agarose (S.10) and filled, while clamped vertically, with an 8,5% - 13% polyacrylamide gradient. The two limiting solutions were prepared as follows:-

	8,5%	13%
Acrylamide/*bis*-acrylamide(S.2)	6,82ml	10,43ml
1,5M tris/HCl (S.1a)	6,00ml	6,00ml
Glycerol	-	7,33ml
10% SDS (S.4)	0,24ml	0,24ml
Water	10,94ml	-
	24,00ml	24,00ml

12µl of temed (R.C) and 50µl of initiator (S.3) were added to each solution before loading 18ml of the 13% solution into the mixing vessel and the same volume of 8,5% solution to the reservoir of the gradient former.

The formed gradient was overlaid with distilled water and polymerization allowed to proceed in the chamber for 2h after which the water layer was replaced with 0,375M tris/HCl pH 8,8 containing 0,1% SDS. The gel was then left overnight at room temperature.

A spacer, 3% gel, was prepared from the following solution:

Acrylamide/*bis*-acrylamide (S.2)	1,00ml
0,5M tris/HCl, pH 6,8 (S.1b)	2,50ml
10% SDS (S.4)	0,10ml
Water	6,4ml
	10,00ml

The solution was degassed at the water-pump for 2 to 5 min. Polymerization was inititated by the addition of 10µl of temed (R.C) and 300µl of initiator (S.3). The mixture was added to the gel chamber, the spacer comb added with care to avoid entrapped air bubbles and polymerization allowed to proceed for 1h. The chamber was then clamped into position in the tank after removal of the lower spacer (Figure A.7.1; K). Electrode buffer (S.2a), made 0,1% in SDS by the addition of 5ml of 10% SDS (S.4) to 495ml of buffer (S.2a), was added to the upper, cathodal reservoir (200ml) and to the lower, anodal reservoir (300ml).

Samples were prepared in aqueous 1% SDS. To 50µl of sample was added 10µl of 1,5M tris/HCl (S.1a) and 10µl of a mixture of 4 parts of glycerol to 1 part of 0,4% bromophenol blue (S.5). Fifty microlitres of each sample were added to each well in the spacer gel by layering the heavy sample solution under the electrode buffer in the wells.

Partially reduced human IgG was routinely electrophoresed simultaneously with the samples to provide molecular weight markers. Intact IgG (M.W. 160 000) heavy chains (M.W. 55 000) and light chains (M.W. 22 500) were readily detectable in the fixed and stained gel.

Gels were electrophoresed at a constant current of 6 to 10mA at room temperature until the tracking dye had migrated to 0,5 to 1 cm from the lower gel edge. This took approximately 16h, by which time final voltages of 120 to 180V were recorded.

Gels were fixed and stained in S.7a and destained in S.7b

Isoelectric focusing in rod gels

The glass tubes that were to contain the gels were thoroughly cleaned, dried, sealed at the bottom with a layer of Parafilm[1] and set vertically.

An acrylamide/*bis*-acrylamide stock mixture was prepared by dissolving 2,0g of sucrose[2]; 1,25g of acrylamide (R.A) and 0,05g of *bis*-acrylamide (R.B) to a final volume of 20ml with distilled water.

Samples were polymerized into 5% acrylamide gels containing ampholytes of the desired pH range by mixing (for example)

6,00ml of Acrylamide/*bis*-acrylamide stock described above

0,25ml of 40% ampholyte solution (R.E)

and 0,50ml of sample.

The mixture was cooled on chipped ice and polymerization was initiated by the addition of 350µl of initiator (S.3) and 15µl of temed (R.C).

Tubes were then filled with the cold mixture with care to avoid air bubbles. This could most conveniently be done with a hypodermic syringe fitted with a needle and a length of fine polythene tubing that would reach the bottom of the tube. The gel mixture was overlaid with a drop of distilled water to provide a flat surface and polymerization allowed to proceed for 1h at room temperature.

The filled tubes were then introduced into the apparatus described for rod gels. The upper, cathodal, electrode reservoir received 0,02M NaOH (S.2c) and the lower, anodal reservoir received 0,01M phosphoric acid (S.2b).

Electrophoresis proceeded at 4°C with a direct current of 1mA

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(1) American Can Company, Neenah, Wisconsin, U.S.A.

(2) Schwarz Mann, Orangeburg, New York, U.S.A.; catalogue number 54 0009530.

per gel under "constant current" control until the voltage had reached 150V. At this point the power supply was switched to "constant voltage" operation and electrophoresis continued until the current had dropped below 0,5mA. Gels were then extruded from the rods under pressure and either stained for esterases, fixed and stained for protein or frozen and sliced for radioactivity measurements.

Isoelectric focusing in vertical slab gels.

Slab gel electrophoresis offered the considerable advantage over techniques with the rod gels in that it allowed several samples to be run side by side on the same gel. Comparisons could thus be made with greater confidence and minor differences in the electrophoretic behaviour detected with improved reliability.

My attempts to achieve satisfactory isoelectric focusing in horizontal slab gels were consistently unsuccessful and I was simply unable to reproduce the results reported by others who have used this technique (8,69,95,162).

I eventually developed a method for isoelectric focusing in vertical slab gels that has given consistently good results in my hands. The major obstacle I encountered in the early stages of developing this method stemmed from a simple technical step. Under normal circumstances, the electrophoresis chamber depicted in Figure A.7.1 was filled with the liquid gel mixture after sealing along the edges with Perspex spacers and molten agarose; the assembly being held together with stationery clamps. After polymerization the spacer along the lower edge was removed and the *clamps were released* in order to clamp the chamber to the tank. It was at this stage that the difficulty arose. When the clamps were released, the minute elastic recoil of the glass plates was sufficient to disrupt

the close bonds between the edges of the gel and the side spacers, with the result that electrode buffers could penetrate between the edges of the gel and the Perspex spacers during electrophoresis, so disturbing the pH gradient and producing bizarre "edge effects" in the separation patterns. This problem was overcome by omitting the lower edge spacer and sealing the bottom of the chamber with Parafilm. A short (approximately 1,5cm) "wick" of gel was then cast in the bottom of the free-standing chamber. After this had polymerized, the parafilm was removed and the chamber was clamped to the tank. In this way the remainder of the chamber could be filled *without disturbing the side clamps* until electrophoresis was complete.

The following is a detailed account of the procedure.

The electrophoresis chamber (Figure A.7.1) was assembled, omitting the bottom spacer, by clamping together the two glass plates separated by the side spacers only. Agarose was not used to seal the side edges. The lower 5 to 10 cm of the chamber was encased in Parafilm, so sealing the bottom. With the chamber vertical, a 3ml, 10% polyacrylamide "wick" was cast using the following solution:

Acrylamide/*bis*-acrylamide (S.2)	1 ml
Ampholyte solution (R.E)	0,075ml
Distilled water	2 ml

Polymerization was initiated with 7,5µl of temed (R.C) and 175µl of initiator (S.3). The mixture was added to the bottom of the chamber and carefully overlaid with distilled water containing ampholytes (R.E) at the same concentration as present in the wick.

After the gel had polymerized (approximately 30 min) the water overlay was aspirated, the Parafilm removed and the gel chamber clamped firmly to the tank.

Filling of the chamber was then completed as follows: An acrylamide/*bis* acrylamide stock was prepared by dissolving 10ml glycerol; 2,5g of acrylamide (R.A) and 0,1g *bis*-acrylamide (R.B) to a final volume of 40ml with distilled water. To 36ml of this stock solution was added 4ml of ampholyte solution (R.E); 0,1ml of temed (R.C) and 2,1ml of initiator (S.3). The chamber, now attached to the tank, was filled from above by tilting it to an angle of 10 to 20 degrees from the horizontal. By this manoeuvre, hydrostatic pressure on the side spacers was reduced and leakage was no problem. When filled, the spacer comb was inserted and the gel chamber set horizontally for polymerization to take place.

When gelling had occurred (after approximately 60 min) the spacer comb was removed and the tank-chamber assembly set vertically. Anode buffer (S.2c; 300ml) was added to the lower reservoir and cathode buffer (S2b; 200ml) to the upper reservoir.

The samples were introduced into the wells by mixing 100µl of sample solution (prepared in distilled water or very dilute buffer); 20µl of a mixture of 4 parts of glycerol to 1 part of 0,4% bromophenol blue; and 13µl of ampholytes solution (R.E). This was then layered under the anode buffer in the wells with a micropipette. In some cases the sample layer was separated for the anode solution by a "protective" layer of a solution made by mixing 100µl of distilled water; 10µl of glycerol : bromophenol blue (4:1) and 12µl of ampholyte (R.E).

Gels were electrofocused for 24h at $4^{o}C$ at a constant voltage of 300V.

After electrofocusing was complete the gels were removed for fixing and staining with perchloric acid : Coomassie brilliant blue (S.8); for autofluorography; or for staining for esterases.

To define the pH gradients in the gels, direct readings from the wet gel surface were taken with a flat, moist-surface combination electrode[1] at 5mm intervals along the longitudinal axis of the gels. Alternatively, a 2mm longitudinal strip was cut from the gel, frozen and sliced transversely into 1,6mm slices. Each slice was then equilibrated with 2ml of 0,9% NaCl and the pH read with an immersion combination electrode.[2]

Autofluography of slab gels

The method used was essentially that of Bonner and Laskey (19) who were the first to exploit, in a systematic way, the advantage of incorporating the primary scintillator, 2,5-diphenyloxozole (PPO) in the gel and exposing the film at -80°C. Both of these procedures enhance the sensitivity of autoradiographic detection of weak β-emitting isotopes in gels very considerably. Briefly, the method used was as follows:- Stained gels were dehydrated by incubating them, in flat trays, in 3 changes of DMSO (S.9a). The volume of DMSO used for each change was 6 times that of the gel volume. The gels were then impregnated with PPO by incubating in 3 to 4 volumes of 22% PPO in DMSO (S.9b) for 3h at room temperature. The gels were then rehydrated with running tap water for 60min and dried on to Whatman's 3MM filter paper.

Indian ink containing a trace amount of ^{14}C-glycine was used to spot register marks on the dried gel films. The dried gels were then placed in apposition to the emulsion surface of x-ray film (S.9c) in metal cassettes and exposed at -80°C.

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(1) Dr. W. Ingold A.G., Urdorf, Switzerland; type 403-30-M8

(2) Radiometer A/S, Copenhagen N.V., Denmark; type GK 2321C

After exposure for periods of time varying from 3 weeks to 3 months (depending on the radioactivity in the gel) the films were developed in DX-80 (S.9d) and fixed in FX-40 (S.9e).

Notes

(i) It should be noted that acrylamide and *bis*-acrylamide are toxic and care should be taken to avoid skin contact or ingestion.

(ii) It was important to enxure that the glass plates for the electrophoresis chamber were scrupulously clean. Coating with silicone was not found to be necessary.

(iii) Although 5% polyacrylamide gels formed a very suitable matrix for stabilizing pH gradients in isoelectric focusing, molecular sieving effects were evident with high molecular weight proteins when the sample was applied at the top of the vertical gels. I found, for example, that myoglobin and haemoglobin focused to sharp bands at their isoelectric points in 24h, whereas horse spleen ferritin (M.W. 440 000; pI 4,2 - 4,6) failed to reach its isoelectric point after 72h. Since the esterases I studied had molecular weights of the order of 50 000 to 60 000 Daltons by SDS gradient gel electrophoresis, I doubt that molecular sieving effects were very prominent in this case.

(iv) There was poor correlation between the pH gradients established in rod gels and those found in slab gels when the same Ampholines were used. For example, 2% pH 5-7 Ampholines resolved esterase bands in rod gels whereas 4% pH 6-8 Ampholines were required to give the same resolution in slab gels. Furthermore, the isoelectric points of esterases measured in slab gels were consistently higher than those found in rod gels. I have no really satisfactory

explanation for this and can only presume the effects of coacervate formation or free-radical action were more pronounced in rod gels where the sample was incorporated into the monomer solution before polymerization.

(v) Occasionally the acrylamide/*bis*-acrylamide stock solution (S.2) was slightly discoloured. This could be decolorized by treatment with activated charcoal.

(vi) Protein bands in isoelectric focusing gels could be stained with 0,05% Coomassie brilliant blue in 12,5% trichloracetic acid (TCA), but several "washes" with 12,5% TCA were required, before staining, to remove unfixed ampholines and avoid a dark background. The PCA technique described was generally more satisfactory.

Section A.8: Physiological solutions

A.8.1 *Gey's balanced salt solution supplemented with human serum albumin (Gey's BSS-2% HSA)*

The Gey's balanced salt solution that I used had the following final composition when supplemented with human serum albumin.

NaCl	$1{,}369 \times 10^{-1}$ M
KCl	$5{,}1 \times 10^{-3}$ M
$CaCl_2$	9×10^{-4} M
$MgCl_2$	1×10^{-3} M
Na_2HPO_4	8×10^{-4} M
KH_2PO_4	2×10^{-4} M
$NaHCO_3$	$5{,}4 \times 10^{-3}$ M
D(+) glucose	$1{,}11 \times 10^{-2}$ M
Phenol red	0,001% w/v
Penicillin	200 u/ml
Streptomycin	50µg/ml
Human serum albumin	2% w/v

Stock solutions

(a) Five-fold concentrated Gey's balanced salt solution (5x Gey's BSS)

A stock solution was prepared from the following ingredients:

NaCl	80,0 g
KCl	3,8 g
$CaCl_2.2H_2O$	1,3 g
$MgCl_2.6H_2O$	2,1 g
$Na_2HPO_4.2H_2O$	1,5 g
KH_2PO_4	0,25 g
D(+) glucose	20,0 g

Each solute was dissolved separately in a minimum volume of double-distilled water. The solutions were then combined and rinsed into a volumetric flask and made up to 2ℓ with double-distilled water. The stock solution was sterilized by filtration through 0,45µm Millipore filters and stored at 4°C in sterile 50 ml bottles.

(b) 7,5% w/v $NaHCO_3$ in double-distilled water

The solution was sterilized by membrane filtration and stored at 4°C in 10 ml amounts in sterile bottles.

(c) 0,5% w/v Phenol red[1]

The sterile solution was dispensed in 2 ml volumes into sterile one ounce Bijou bottles and stored at 4°C.

(d) Penicillin

Preservative-free sodium benzyl penicillin was purchased as Crystapen from Glaxo-Allenbury South Africa (Pty) Limited, Johannesburg, South Africa as 600 mg amounts ($1x10^6$ units) in multi-dose vials. The powder was dissolved in 5 ml sterile double-distilled water immediately before use.

(e) Streptomycin

Preservative-free streptomycin was purchased in 1 g sterile multi-dose vials as streptomycin sulphate B.P. from Glaxo-Allenbury South Africa (Pty) Limited, Johannesburg, South Africa.

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(1) Grand Island Biological Company, New York, U.S.A.; catalogue number 510.

and Nova Industries (Pharmaceuticals)(Pty) Limited, Johannesburg, South Africa. The dry powder was dissolved in 5 ml of sterile double-distilled water for use.

(f) Human serum albumin (HSA)

Preservative-free human serum albumin was obtained in sterile multi-dose vials as a 20% w/v solution in pyrogen free water stabilized with 0,02M sodium caprylate and 0,02M mandelic acid from the Natal Blood Transfusion Service, Durban, South Africa.

Working Solution

The following stock solutions were combined in a sterile stoppered measuring cylinder and made up to a total volume of 100ml with sterile double-distilled water to give Gey's BSS-2% HSA.

20 ml	5x Gey's BSS
10 ml	20% w/v HSA
0,6 ml	7,5% w/v $NaHCO_3$
0,2 ml	0,5% w/v phenol red
0,1 ml	$2x10^5$ units penicillin/ml
0,05 ml	200 mg streptomycin/ml

This solution was mixed by inversion and the pH adjusted to pH 7,2 to 7,4 with 7,5% w/v $NaHCO_3$ if necessary.

If there was any reason to believe there had been a breach in sterile procedure, the medium was membrane-sterilized immediately before use.

Notes and Comments

(i) I found it essential to dissolve the ingredients for the five-fold concentrated Gey's BSS stock solution separately and combine them as solutions. If the dry salts were combined first, complete solution of the mixture was not obtained.

(ii) Sodium bicarbonate was stored as a separate stock solution to prevent precipitation of salts from solution with storage.

(iii) The addition of caprylic acid and mandelic acid as stabilizers to the human serum albumin occasionally required additional sodium bicarbonate to adjust the pH.

(iv) All chemicals used were of the highest purity available, Merck guaranteed reagents(1): British Drug House analar reagents(2) and Fluka purissimum reagents(3).

A.8.2 *Triethanolamine buffered saline pH 7,2 (TBS)*

Stock solution: 1,0M triethanolamine; 0,9% w/v NaCl; 0,01% w/v chlorhexidine.

Nine grams of NaCl and 185,7g triethanolamine white crystalline powder(4) were dissolved in approximately 800ml double-distilled water. To this solution was added dropwise, with stirring, 1 ml of 10% w/v chlorhexidine. The pH was adjusted to pH 7,2 ± 0,1 with 4N NaOH and the volume made up to 1 000 ml with double-distilled water. The stock was sterile filtered and stored at 4°C.

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(1) E. Merck, Darmstadt, West Germany.

(2) British Drug House Chemicals Limited, Poole, England

(3) Fluka AG Chemische Fabrik, Buchs, Switzerland

(4) Boehringer, Mannheim GmbH, Germany.

Working solution: 0,005M triethanolamine; 0,9% w/v NaCl; 0,001% w/v chlorhexidine.

The stock triethanolamine was diluted 1/200 with 0,9% w/v NaCl.

Aqueous 10% w/v chlorhexidine preservative was added to give a final 0,001% w/v concentration.

After checking the pH, the buffer was sterilized by membrane filtration, and stored at $4^{o}C$.

Notes and Comments

(i) Triethanolamine interferes with the Folin-Ciocalteau protein assay. It has negligible absorbance at 260 nm and 280 nm at a concentration of 0,005M, so the direct photometric method (72) was used for routine measurement of protein concentration in the presence of this compound.

(ii) The preservative, chlorhexidine, may form insoluble complexes with Cl^{-} and SO_4^{--} ions. This can be avoided by reducing the concentration of the preservative in the final mixture. It is a cationic substance that is not suitable for use with cation exchange resins. It is a poor fungistatic agent (50).

Section A.9: Enzymes used in cytotaxin inactivation studies.

Upper case letters in parenthesis - e.g. (A), (A'), (C-HP) etc. refer to the enzyme codes used in the figures and tables in Chapter II.

Trypsin (E.C. 3.4.4.4)

Specificity: Endopeptidase, preferentially catalysing the hydrolysis of peptide bonds between the carboxyl group of arginine and lysine and the amino groups of other amino acids. It also acts as an esterase and amidase and acts in other non-specific catalyses.

Assay: The rate of hydrolysis of para-toluene-sulfonyl-L-arginine methyl ester at pH 8,1 in the presence of $CaCl_2$ was measured by the increase in absorbance at 247nm at 25°C.

Unit of activity: That amount that will hydrolyze 1µmole of para-toluene-sulfonyl-L-arginine methyl ester per minute at 25°C and pH 8,1 in the presence of 0,01M calcium.

Two forms of trypsin were used - the soluble enzyme (A) and the immobilized enzyme (A').

Soluble Trypsin (A)

Source: Bovine pancreas

Form: Lyophilized, salt-free, 2x crystallized.

Supplier: Worthington Biochemical Corporation, Freehold, New Jersey, U.S.A. Code number TRL lot number 35E645.

Enzyme specifications: 250 units/mg

Digestion Conditions

Enzyme preparation: The powder was dissolved in 0,001N HCl immediately prior to use at a concentration of 10mg/ml (2 500 units/ml).

Sample preparation:

Sample	Enzyme concentration
(i) Gey's BSS; pH 8,0	0,1mg/ml; 25u/ml
(ii) Gey's BSS + 0,78mg/ml CUF-3 protein; pH 8,0	0,1mg/ml; 0,13mg/mg CUF-3 32,1u/mg CUF-3
(iii) Gey's BSS + 10mg/ml casein; pH 8,0	0,1mg/ml; 0,01mg/mg casein 2,5u/mg casein.

Incubations:

Incubated under sterile conditions at 37°C for 20h.

Termination:

Enzyme was separated from reaction products by negative pressure ultrafiltration through cellophane membranes at 4°C.

Assay:

Ultrafiltrates were adjusted to pH 7,4, sterilized by membrane filtration and assayed for chemotactic activity using a modified Boyden chamber technique.

Comments:

(a) The enzyme preparation was free of other enzymes used in this study.

(b) Cytotaxin, CUF-3, at a stimulatory concentration of 0,19mg protein/ml was inactivated by ultrafiltrates of trypsin incubated without substrate. This was found to be due to ultrafilterable trypsin (approximately 5% of that added to the samples originally).

Immobilized Trypsin (A')

Source: Bovine pancreas

Form: Insoluble, covalently bound to Sepharose 4B by the cyanogen bromide method, 2x crystallized gel suspension in 0,1M acetate buffer pH 4,5 containing 0,02% NaN_3 and 0,04% EDTA.

Supplier: Worthington Biochemical Corporation, Freehold, New Jersey, U.S.A. Code number IEA-TRL, lot number 35E631.

Enzyme specifications: 23,4 units/mg protein
1,8 units/mg gel dry weight.

Digestion Conditions

Enzyme preparations: The immobilized enzyme preparation was washed free of preservatives and acetate buffer with 3x10ml changes of Gey's BSS pH 7,4 per 5 ml settled bed volume.
The washed gel was resuspended in Gey's BSS pH 7,4 to give an enzyme concentration of 12mg/ml (280,8 units/ml; i.e. 0,63: 0,37:: settled bed volume: solvent).

Sample preparation:

Sample	Enzyme concentration
(i) Gey's BSS; pH 8,0	1,2mg/ml; 28,1 u/ml
(ii) Gey's BSS + 0,78mg/ml CUF-3 protein; pH 8,0	1,2mg/ml; 1,54mg/mg CUF-3 36u/mg CUF-3
(iii) Gey's BSS + 10mg/ml casein; pH 8,0	1,2mg/ml; 0,12mg/mg casein 2,8u/mg casein

Incubations:

Incubated under sterile conditions at 37°C for 20h with constant agitation.

Termination:

Immobilized enzyme was separated from reaction products by centrifugation.

Assay:

Supernatants were adjusted to pH 7,4 sterilized by membrane filtration and assayed for chemotactic activity using a modified Boyden chamber technique.

Comments:

(a) The enzyme preparation was free of other enzymes used in this study.

(b) The supernatant fluid of trypsin incubated alone was found to contain slight chemotactic activity.

(c) The supernatant fluid fractions were free of measurable tryptic activity.

α-Chymotrypsin (E.C. 3.4.4.5)

Two forms of chymotrypsin were used - the soluble enzyme (B) and the immobilized enzyme (B')

Specificity: Endopeptidase, preferentially catalysing the hydrolysis of peptide bonds involving the L-isomers of tryptophan, phenylalanine and tyrosine. It also readily acts on the amides and esters of susceptible amino acids. In addition to bonds involving aromatic amino acids it also catalyses, at a high rate, bonds involving leucine, methionine, arginine and glutamine residues.

Assay: The rate of hydrolysis of benzyl-L-tyrosine ethyl ester at pH 7,8 in the presence of $CaCl_2$ is measured by the increase in absorbance at 256nm at 25°C.

Unit of activity: That amount which will hydrolyze 1µmole of substrate per minute at 25°C and pH 7,8 in the presence of 0,05M calcium.

Soluble chymotrypsin (B)

Source: Bovine pancreas

Form: Lyophilized, salt-free, 3x-crystallized.

Supplier: Worthington Biochemical Corporation, Freehold, New Jersey, U.S.A. Code number CDI, lot number 35A630.

Specifications: 48,1 units/mg

Digestion conditions

Enzyme preparation: The powder was dissolved in 0,001N HCl immediately before use at a concentration of 10mg/ml (481u/ml)

Sample preparation:

Sample	Enzyme concentration
(i) Gey's BSS; pH 8,0	0,1mg/ml; 4,8u/ml
(ii) Gey's BSS + 0,78mg/ml CUF-3 protein; pH 8,0	0,1mg/ml; 0,13mg/mg CUF-3 6,2u/mg CUF-3
(iii) Gey's BSS + 10mg/ml casein; pH 8,0	0,1mg/ml; 0,01mg/mg casein 0,48u/mg casein

Incubations:

Incubated under sterile conditions at 37°C for 20h.

Termination:

Enzyme was separated from reaction products by negative pressure ultrafiltration at 4°C.

Assay:

Ultrafiltrates were adjusted to pH 7,4, sterilized by membrane filtration and assayed for chemotactic activity using a modified Boyden chamber technique.

Comments:

(a) The enzyme preparation was free of other enzymes used in this study.

(b) Cytotaxin, CUF-3, at a stimulatory concentration of 0,19mg protein/ml was inactivated when added to ultrafiltrates of chymotrypsin incubated alone. This was found to be due to ultrafilterable chymotrypsin (approximately 5% of that added to the samples originally).

Immobilized α-chymotrypsin (B')

Source: Bovine pancreas

Form: Insoluble, covalently bound to Sepharose 4B by the cyanogen bromide method: 3x crystallised: Gel suspension in 0,1M acetate buffer pH 4,5 containing 0,02% NaN_3 and 0,04% EDTA.

Supplier: Worthington Biochemical Corporation, Freehold, New Jersey, U.S.A. Code number IEA-CDI, lot number 95A001.

Specifications: 13,1 units/mg protein
1,3 units/mg gel dry weight.

Digestion conditions

Enzyme preparation: The immobilized enzyme preparation was washed free of preservatives and acetate buffer with 3x10ml changes of Gey's BSS (pH 7,4) per 5ml settled bed volume. The washed gel was resuspended in Gey's BSS pH 7,4 to give an enzyme concentration of 15,5mg/ml (203,1u/mg).

Sample preparation:

Sample	Enzyme concentration
(i) Gey's BSS; pH 8,0	1,6mg/ml; 20,3u/ml
(ii) Gey's BSS + 0,78mg/ml CUF-3 protein; pH 8,0	1,6mg/ml; 1,99mg/mg CUF-3 26,03u/mg CUF-3
(iii) Gey's BSS + 10mg/ml casein pH 8,0	1,6mg/ml; 0,16mg/mg casein 2,0u/mg casein

Incubations:

Incubated under sterile conditions at 37°C for 20h with constant agitation.

Termination:

Immobilized enzyme was separated from reaction products by centrifugation.

Assay:

Supernatants were adjusted to pH 7,4 sterilized by membrane filtration and assayed for chemotactic activity using a modified Boyden chamber technique.

Comments:

(a) The enzyme preparation was free of other enzymes used in this study.

(b) The supernatant fluid of chymotrypsin autodigestion was found to be free of chemotactic activity.

(c) The supernatant fluid fractions were free of measurable chymotryptic activity.

Lipase (Glycerol ester hydrolase) (E.C.3.1.1.3) (C-C)

Specificity: Relatively non-specific lipase catalysing the hydrolysis of emulsified water-insoluble esters of glycerol containing long chain fatty acids. The activity of the enzyme decreases with increasing water solubility of the esters. Mono-, di- and tri-glycerides hydrolized at rates increasing in that order.

Assay: The rate of hydrolysis of olive oil, emulsified in a solution containing sodium taurocholate, gum acacia, NaCl and $CaCl_2$, is measured by titration of the liberated fatty acids with NaOH at 25°C.

Unit of activity: That amount that will liberate 1µmole of acid per minute at 25°C under specified conditions.

Source: *Candida albicans*

Form: Lyophilised, highly purified, salt free.

Supplier: Worthington Biochemical Corporation, Freehold, New Jersey, U.S.A.
Code number CLP, lot number 55H357.

Specifications: 730 units/mg protein

Digestion conditions

Enzyme preparation: The powder was dissolved in Gey's BSS pH 7,4 immediately before use at a concentration of 10mg/ml (7300u/ml).

Sample preparation:

Sample	Enzyme concentration
(i) Gey's BSS; pH 8,0	0,1mg/ml; 73u/ml
(ii) Gey's BSS + 0,78mg/ml CUF-3 protein; pH 8,0	0,1mg/ml; 0,13mg/mg CUF-3 93,6u/mg CUF-3
(iii) Gey's BSS + 10mg/ml casein; pH 8,0	0,1mg/ml; 0,01mg/mg casein 7,3u/mg casein

Incubations:

Incubated under sterile conditions at 37°C for 20h.

Termination:

Enzyme was separated from low molecular weight materials and salt solutions by negative pressure ultrafiltration at 4°C.

Assay:

Ultrafiltrates were adjusted to pH 7,4, sterilized by membrane filtration and assayed for chemotactic activity using a modified Boyden chamber technique.

Comments:

(a) The enzyme preparation was free of other enzymes used in this study.

(b) The ultrafiltrates were free of any measurable enzyme activity.

(c) The enzyme was as active towards olive oil emulsions prepared in the absence of added sodium taurocholate as towards those prepared in the presence of the bile salt. (The enzyme digestions were performed in the absence of bile salts.)

Lipase (Glycerol ester hydrolase) (E.C.3.1.1.3) (C-HP)

Specificity: True lipase, catalysing the hydrolysis of emulsified water-insoluble esters of glycerol containing long chain fatty acids. The hog pancreas lipase has almost absolute specificity for the α and α' chains of triglycerides. The activity of the enzyme decreases with increasing water solubility of the esters. Mono-, di- and tri-glycerides hydrolyzed at rates increasing in that order.

Assay: The rate of hydrolysis of olive oil emulsified in a solution containing sodium taurocholate, gum acacia, NaCl and $CaCl_2$, is measured by titration of the liberated fatty acids with NaOH at 25°C.

Unit of activity: That amount which will liberate 1μmole of acid per minute at 25°C under specified conditions.

Source: Hog pancreas

Form: Lyophilized, partially purified.

Supplier: Worthington Biochemical Corporation, Freehold, New Jersey, U.S.A.
Code number PLI, lot number 55S587

Specifications: 265 units/mg protein.

Digestion conditions

Enzyme preparation: The powder was dissolved in Gey's BSS pH 7,4 immediately before use at a concentration of 10mg/ml (2650u/ml).

Sample preparation:

Sample	Enzyme concentration
(i) Gey's BSS; pH 8,0	0,1mg/ml; 26,5u/ml
(ii) Gey's BSS + 0,78mg/ml CUF-3 protein; pH 8,0	0,1mg/ml; 0,13mg/mg CUF-3 34,0u/mg CUF-3
(iii) Gey's BSS + 10mg/ml casein; pH 8,0	0,1mg/ml; 0,01u/mg casein 2,65u/mg casein

Incubations:

Incubated under sterile conditions at 37°C for 20h.

Termination:

Enzyme was separated from reaction products by negative pressure ultrafiltration at 4°C.

Assay:

Ultrafiltrates were adjusted to pH 7,4, sterilized by membrane filtration and assayed for chemotactic activity using a modified Boyden chamber technique.

Comments:

(a) The enzyme preparation was free of other enzymes used in this study.

(b) The ultrafiltrates were free of any measurable enzyme activity.

(c) The enzyme was as active towards olive oil emulsions prepared in the absence of added sodium taurocholate as towards those prepared in the presence of the bile salt. (The enzyme digestions were performed in the absence of bile salts).

(d) The ultrafiltrates of lipase incubated alone contained a minor inhibitor of chemotaxis.

(e) Ultrafiltrates of enzyme incubated with 1% casein contained appreciable amounts of inhibitors of chemotaxis.

Phosphodiesterase I (Orthophosphoric diester phosphohydrolase) (E.C.3.1.4.1) (D)

Specificity: Catalyses the hydrolysis of oligonucleotides (ribo and deoxyribo-) with free 3'-hydroxyl end groups liberating mononucleoside-5'-phosphates.

Assay: The rate of hydrolysis of calcium bis-(para-nitrophenyl) phosphate, in tris-acetate buffer pH 8,8 containing magnesium acetate, is measured at 25°C by the increase in absorbance at 440nm due to liberated para-nitrophenol.

Unit of activity: That amount which will liberate 1µmole of para-nitrophenol per minute at 25°C under specified conditions.

Source: *Crotalus adamanteus* venom

Form: Lyophilized; partially purified acetone fraction.

Supplier: Worthington Biochemical Corporation, Freehold, New Jersey, U.S.A. Code number VPH, lot number 35H797

Specifications: 25 units/mg protein.

Digestion conditions

Enzyme preparation: The powder was dissolved in distilled water containing 10^{-2}M di-isopropylfluorophosphate and incubated at 20°C for 3h. The unreacted inhibitor was removed by dialysis against distilled water at 4°C for 20h. Final enzyme concentration was 4mg/ml (100u/ml).

Sample preparation:

Sample	Enzyme concentration
(i) Gey's BSS; pH 8,0	0,04mg/ml; 1u/ml
(ii) Gey's BSS + 0,78mg/ml CUF-3 protein; pH 8,0	0,04mg/ml; 0,05mg/mg CUF-3 1,28u/mg CUF-3
(iii) Gey's BSS + 10mg/ml casein; pH 8,0	0,04mg/ml; 0,004mg/mg casein 0,1u/mg casein

Incubation:

Incubated under sterile conditions at 37°C for 20h.

Termination:

Enzyme was separated from reaction products by negative pressure ultrafiltration at 4°C.

Assay:

Ultrafiltrates were adjusted to pH 7,4, sterilized by membrane filtration and assayed for chemotactic activity using a modified Boyden chamber technique.

Comments:

(a) The phosphodiesterase preparation as supplied contained substantial catalytic activity for the hydrolysis of para-toluenesulfonyl-L-arginine methyl ester. Before use

this preparation was treated with the serine protease/ esterase inhibitor diisopropylfluorophosphate. The treated preparation showed reduced, but not complete, inhibition of "tryptic-activity" with a similar reduction in activity for its own substrate.

(b) The ultrafiltrates were free of any measurable enzyme activity.

Leucine aminopeptidase (E.C.3.4.1.1.) (E)

Specificity: Exopeptidase, catalysing the hydrolysis of the peptide bond adjacent to a free amino group. It reacts most rapidly with leucine compounds. Many aliphatic amides are also hydrolysed.

Assay: The rate of hydrolysis of the peptide bond of leucinamide at pH 8,5 in the presence of $MnCl_2$ and $MgCl_2$ is measured as the decrease in absorbance at 238nm at 25°C relative to a standard amount of leucine containing $MgCl_2$ and enzyme.

Unit of activity: That amount which will hydrolyze 1µmole of L-leucinamide per minute under specified conditions.

Source: Hog kidney

Form: Suspension in 0,1M tris; 0,005M $MgCl_2$, 75% $(NH_4)_2SO_4$ pH 8,0. Chromatographic fraction free of detectable tryptic or chymotryptic activity.

Supplier: Worthington Biochemical Corporation, Freehold, New Jersey, U.S.A. Code number LAPC, lot number 55B411.

Specifications: 100 units/mg protein

3,54 mg protein/ml

Digestion conditions

Enzyme preparation: The $(NH_4)_2SO_4$ enzyme suspension was activated in the presence of 0,01M $MnCl_2$/mg protein at pH 8,5 and 40°C for 2 to 3h. The ammonium sulphate and manganese chloride were removed by dialysis against 0,025M tris/HCl pH 8,5 buffer containing 0,005M $MgCl_2$ for 20h at 4°C. Final enzyme concentration 1,7mg/ml (1700u/ml).

Sample preparation:

Sample	Enzyme concentration
(i) Gey's BSS; pH 8,0	0,1mg/ml; 10u/ml
(ii) Gey's BSS + 0,78mg/ml CUF-3 protein; pH 8,0	0,1mg/ml; 0,13mg/mg CUF-3 12,8u/mg CUF-3
(iii) Gey's BSS + 10mg/ml casein; pH 8,0	0,1mg/ml; 0,01mg/mg casein 1,0 u/mg casein

Incubation:

Incubated under sterile conditions at 37°C for 20h.

Termination:

Enzyme was separated from low molecular weight materials and salt solutions by negative pressure ultrafiltration at 4°C.

Assay:

Ultrafiltrates were adjusted to pH 7,4, sterilized by membrane filtration and assayed for chemotactic activity using a modified Boyden chamber technique.

Comments:

(a) The enzyme preparation was free of other enzymes used in this study.

(b) The ultrafiltrates were free of any measurable enzyme activity.

Pronase (F)

Specificity: Non-specific protease digesting most proteins/peptides almost completely to amino acids.

Assay: The rate of hydrolysis of denatured haemoglobin at pH 7,4 is measured at 37°C as an increase in adsorbance at 280nm of trichloroacetic acid-soluble hydrolysis products.

Unit of activity: Not defined.

Source: *Streptomyces griseus*

Form: Lyophilised, enzyme mixture, Grade B.

Supplier: Calbiochem Incorporated, Los Angeles, California, U.S.A. Code number 53702, lot number 400325.

Specifications: Not defined.

Digestion conditions

Enzyme preparation: The powder was dissolved in 0.001N HCl immediately before use at a concentration of 10mg/ml.

Sample preparation:

Sample	Enzyme concentration
(i) Gey's BSS; pH 7,4	0,1mg/ml
(ii) Gey's BSS + 0,78mg/ml CUF-3 protein; pH 7,4	0,1mg/ml; 0,13mg/mg CUF-3
(iii) Gey's BSS + 10mg/ml casein pH 7,4	0,1mg/ml; 0,01mg/mg casein

Incubations:

Incubated under sterile conditions at 37°C for 20h.

Termination:

Enzyme was separated from reaction products by negative pressure ultrafiltration at 4°C.

Assay:

Ultrafiltrates were adjusted to pH 7,4, sterilized by membrane filtration and assayed for chemotactic activity using a modified Boyden chamber technique.

Comments:

(a) The enzyme preparation was free of other enzymes used in this study.

(b) Cytotaxin, CUF-3, at a stimulatory concentration of 0,19mg protein/ml was inactive when added to ultrafiltrates of pronase incubated alone. This was found to be due to ultrafilterable pronase (approximately 10 to 20% of that added to the samples originally).

Carboxypeptidase A (E.C.3.4.2.1.)(G)

Specificity: Exopeptidase, catalysing the hydrolysis of the peptide bond adjacent to a free carboxyl group in proteins and peptides.

Assay: The rate of hydrolysis of N-benzoylglycyl-L-Phenylaline at pH 7,5 in the presence of LiCl is measured as an increase in adsorbance at 254nm at 25°C.

Unit of activity: That amount which will hydrolyze 1µmole of substrate per minute under specified conditions.

Source: Bovine pancreas

Form: Insoluble, covalently bound to Sepharose 4B by the cyanogen bromide method.

Supplier: Worthington Biochemical Corporation, Freehold, New Jersey, U.S.A. Code number IEA-COA, lot number 35P720

Digestion conditions

Enzyme preparation: The immobilized enzyme preparation was washed free of preservatives and acetate buffer with 3x10ml changes of Gey's BSS (pH 7,4) per 5ml settled bed volume and resuspended in Gey's BSS pH 7,4 to give a settled bed voluem: solvent ratio of 0,67:0,33

Sample preparation:

Sample	Enzyme concentration
(i) Gey's BSS; pH 7,4	0,67 ml gel/10 ml
(ii) Gey's BSS + 0,78mg/ml CUF-3 protein; pH 7,4	0,67 ml gel/10ml
(iii) Gey's BSS + 10mg/ml casein pH 7,4	0,67 ml gel/10ml

Incubation:

Incubated under sterile conditions at 37°C for 20h with constant agitation.

Termination:

Immobilized enzyme was separated from soluble material by centrifugation.

Assay:

Supernatants were adjusted to pH 7,4, sterilized by membrane filtration and assayed for chemotactic activity using a modified Boyden chamber technique.

Comments:

(a) The enzyme preparation was free of other enzymes used in this study.

(b) The supernatant fluid of carboxypeptidase incubated alone was found to contain slight chemotactic activity.

(c) The supernatant fluid fractions were free of measurable enzyme activity.

Section A.10: Respirometric data.

The following 12 tables present the results of the oxygen uptake studies discussed in Chapter III. Data recorded in the first 6 tables (Flasks A through F) were used to construct the diagram on the left in Figure 3.12; the second 6 tables (Flasks G through L) refer to the diagram on the right of Figure 3.12.

Standard interval-uptake Warburg respirometry was used to measure oxygen uptake (161).

Cells (121×10^6 in the first 6 tables; 149×10^6 in the second 6 tables) were suspended in 2,7ml of Gey's BSS-2% HSA, (buffered with sodium phosphate instead of bicarbonate) in the main compartment of each flask. Centre wells contained filter paper flutes spotted with 20% KOH.

The 37°C flask constant (K_e) for each manometer-flask combination and the contents of the side-arm are given above each table.

Manometer readings (in mm of Brodie's solution) for the thermobarometer (TB) and the flasks are given under the corresponding column headings in each table.

Oxygen consumption (µl O_2) was calculated for each interval as the product of the flask constant and the difference between flask and thermobarometer readings for that interval.

e.g. µl O_2 consumed in Flask A = K_e ($\Delta A - \Delta TB$)

K_e = 1,433 ; side arm 500µl TBS

Time (min)	TB (mm)	ΔTB (mm)	A (mm)	ΔA (mm)	ΔA-ΔTB (mm)	µl O_2	µl O_2 (cumulative)
0	201,8	0	226,4	-	-	-	-
10	201,8	0	219,8	-6,6	-6,6	-9,46	-9,46
20	201,8	0	218,0	-1,8	-1,8	-2,58	-12,04
30	201,8	0	211,0	-7,0	-7,0	-10,03	-22,07
40	201,8	0	211,0	0,0	0,0	0,00	-22,07
50	201,8	0	206,0	-5,0	-5,0	-7,17	-29,24
60	201,8	0	202,5	-3,5	-3,5	-5,02	-34,26
70	201,8	0	198,9	-3,6	-3,6	-5,16	-39,42
80	201,8	0	195,0	-3,9	-3,9	-5,59	-45,01
90	201,8	0	192,0	-3,0	-3,0	-4,30	-49,31
100	201,8	0	191,0	-1,0	-1,0	-1,43	-50,74
130	201,8	0	185,0	-6,0	-6,0	-8,60	-59,34
190	201,8	0	177,0	-8,0	-8,0	-11,47	-70,81
240	201,8	0	175,0	-2,0	-2,0	-2,87	-73,68
340	201,8	0	174,8	-0,2	-0,2	-0,29	-73,97

K_e = 1,552; side arm 500µl TBS

Time (min)	TB (mm)	ΔTB (mm)	B (mm)	ΔB (mm)	ΔB-ΔTB (mm)	µl O_2	µl O_2 (cumulative)
0	201,8	0	203,0	-	-	-	-
10	201,8	0	198,9	-4,1	-4,1	-6,36	-6,36
20	201,8	0	193,8	-5,1	-5,1	-7,92	-14,28
30	201,8	0	192,5	-1,3	-1,3	-2,02	-16,30
40	201,8	0	191,0	-1,5	-1,5	-2,33	-18,63
50	201,8	0	187,0	-4,0	-4,0	-6,21	-24,84
60	201,8	0	184,0	-3,0	-3,0	-4,66	-29,50
70	201,8	0	181,2	-2,8	-2,8	-4,35	-33,85
80	201,8	0	177,2	-4,0	-4,0	-6,21	-40,06
90	201,8	0	175,0	-2,2	-2,2	-3,41	-43,47
100	201,8	0	174,5	-0,5	-0,5	-0,78	-44,25
130	201,8	0	168,0	-6,5	-6,5	-10,09	-54,34
190	201,8	0	161,7	-6,3	-6,3	-9,78	-64,12
240	201,8	0	159,5	-2,2	-2,2	-3,41	-67,53
340	201,8	0	159,5	0,0	0,0	0,0	-67,53

K_e = 1,326; side arm 500µl TBS

Time (min)	TB (mm)	ΔTB (mm)	C (mm)	ΔC (mm)	ΔC-ΔTB (mm)	µl O_2	µl O_2 (cumulative)
0	201,8	0	246,0	-	-	-	-
10	201,8	0	240,0	-6,0	-6,0	-7,96	-7,96
20	201,8	0	232,0	-8,0	-8,0	-10,61	-18,57
30	201,8	0	230,8	-1,2	-1,2	-1,60	-20,17
40	201,8	0	229,0	-1,8	-1,8	-2,39	-22,56
50	201,8	0	223,8	-5,2	-5,2	-6,90	-29,46
60	201,8	0	219,7	-4,1	-4,1	-5,44	-34,90
70	201,8	0	216,7	-3,0	-3,0	-3,98	-38,88
80	201,8	0	212,7	-4,0	-4,0	-5,30	-44,18
90	201,8	0	209,3	-3,4	-3,4	-4,51	-48,69
100	201,8	0	208,9	-0,4	-0,4	-0,53	-49,22
130	201,8	0	202,5	-6,4	-6,4	-8,49	-57,71
190	201,8	0	193,7	-8,8	-8,8	-11,67	-69,38
240	201,8	0	189,5	-4,2	-4,2	-5,57	-74,95
340	201,8	0	188,5	-1,0	-1,0	-1,33	-76,28

K_e = 1,444; side arm 500µl SNF

Time (min)	TB (mm)	ΔTB (mm)	D (mm)	ΔD (mm)	ΔD-ΔTB (mm)	µl O_2	µl O_2 (cumulative)
0	201,8	0	207,8	-	-	-	-
10	201,8	0	203,8	-4,0	-4,0	-5,78	-5,78
20	201,8	0	198,0	-5,8	-5,8	-8,38	-14,16
30	201,8	0	195,8	-2,2	-2,2	-3,18	-17,34
40	201,8	0	193,0	-2,8	-2,8	-4,04	-21,38
50	201,8	0	188,9	-4,1	-4,1	-5,92	-27,30
60	201,8	0	185,2	-3,7	-3,7	-5,34	-32,64
70	201,8	0	182,2	-3,0	-3,0	-4,33	-36,97
80	201,8	0	177,2	-5,0	-5,0	-7,22	-44,19
90	201,8	0	174,5	-2,7	-2,7	-3,90	-48,09
100	201,8	0	173,2	-1,3	-1,3	-1,88	-49,97
130	201,8	0	167,0	-6,2	-6,2	-8,95	-58,92
190	201,8	0	158,0	-9,0	-9,0	-13,00	-71,92
240	201,8	0	157,0	-1,0	-1,0	-1,44	-73,36
340	201,8	0	155,5	-1,5	-1,5	-2,17	-75,53

K_e = 1,522; side arm 500µl SNF

Time (min)	TB (mm)	ΔTB (mm)	E (mm)	ΔE (mm)	ΔE-ΔTB (mm)	µl O_2	µl O_2 (cumulative)
0	201,8	0	220,0	-	-	-	-
10	201,8	0	216,0	-4,0	-4,0	-6,09	-6,09
20	201,8	0	210,0	-6,0	-6,0	-9,13	-15,22
30	201,8	0	207,8	-2,2	-2,2	-3,35	-18,57
40	201,8	0	204,0	-3,8	-3,8	-5,78	-24,35
50	201,8	0	200,7	-3,3	-3,3	-5,02	-29,37
60	201,8	0	196,7	-4,0	-4,0	-6,09	-35,46
70	201,8	0	194,5	-2,2	-2,2	-3,35	-38,81
80	201,8	0	190,0	-4,5	-4,5	-6,85	-45,66
90	201,8	0	187,0	-3,0	-3,0	-4,57	-50,23
100	201,8	0	186,0	-1,0	-1,0	-1,52	-51,75
130	201,8	0	181,5	-4,5	-4,5	-6,85	-58,60
190	201,8	0	173,5	-8,0	-8,0	-12,18	-70,78
240	201,8	0	170,5	-3,0	-3,0	-4,57	-75,35
340	201,8	0	171,0	+0,5	+0,5	+0,76	-74,59

K_e = 1,398; side arm 500µl SNF

Time (min)	TB (mm)	ΔTB (mm)	F (mm)	ΔF (mm)	ΔF-ΔTB (mm)	µl O_2	µl O_2 (cumulative)
0	201,8	0	230,0	-	-	-	-
10	201,8	0	228,0	-2,0	-2,0	-2,80	-2,80
20	201,8	0	221,2	-6,8	-6,8	-9,51	-12,31
30	201,8	0	218,0	-3,2	-3,2	-4,47	-16,78
40	201,8	0	216,8	-1,2	-1,2	-1,68	-18,46
50	201,8	0	214,0	-2,8	-2,8	-3,91	-22,37
60	201,8	0	209,1	-4,9	-4,9	-6,88	-29,22
70	201,8	0	206,0	-3,1	-3,1	-4,33	-33,55
80	201,8	0	199,2	-6,8	-6,8	-9,51	-43,06
90	201,8	0	197,2	-2,0	-2,0	-2,80	-45,86
100	201,8	0	194,9	-2,3	-2,3	-3,22	-49,08
130	201,8	0	190,9	-4,0	-4,0	-5,59	-54,67
190	201,8	0	179,5	-11,4	-11,4	-15,94	-70,61
240	201,8	0	175,8	-3,7	-3,7	-15,17	-75,78
340	201,8	0	176,5	+0,7	+0,7	+0,98	-74,80

K_e = 1,433; side arm 500µl TBS

Time (min)	TB (mm)	ΔTB (mm)	G (mm)	ΔG (mm)	ΔG-ΔTB (mm)	µl O_2	µl O_2 (cumulative)
0	254,0	-	227,5	-	-	-	-
10	252,0	-02,0	222,0	-05,5	-03,5	-5,02	-5,02
20	250,8	-01,2	216,0	-06,0	-04,8	-6,88	-11,90
30	251,7	00,9	213,0	-03,0	-03,9	-5,59	-17,49
40	250,5	-01,2	208,9	-04,1	-02,9	-4,16	-21,65
45	287,5	seal lost	207,0	-01,9	-01,9	-2,72	-24,37
55	287,0	-00,5	200,5	-06,5	-06,5	-8,60	-32,97
66	284,9	-02,1	196,5	-04,0	-01,9	-2,72	-35,69
76	282,7	-02,2	192,5	-04,0	-01,8	-2,58	-38,27
86	283,0	00,3	191,5	-01,0	-01,3	-1,86	-40,13
96	284,0	01,0	190,2	-01,3	-02,3	-3,30	-43,43
106	283,5	-00,5	188,8	-01,4	-00,9	-1,29	-44,72
116	283,5	00,0	187,0	-01,8	-01,8	-2,58	-47,30
146	282,2	-01,3	183,8	-03,2	-01,9	-2,72	-50,02
176	280,9	-01,3	176,0	-07,8	-06,5	-9,31	-59,33

K_e = 1,324; side arm 500µl TBS

Time (min)	TB (mm)	ΔTB (mm)	H (mm)	ΔH (mm)	ΔH-ΔTB (mm)	µl O_2	µl O_2 (cumulative)
0	254,0	-	261,0	-	-	-	-
10	252,0	-02,0	256,0	-05,0	-03,0	-3,97	-3,97
20	250,8	-01,2	251,0	-05,0	-03,8	-5,03	-9.00
30	251,7	00,9	247,2	-03,8	-04,7	-6,22	-15,22
40	250,5	-01,2	244,0	-03,2	-02,0	-2,65	-17,87
45	287,5	seal lost	241,8	-02,2	-02,2	-2,91	-20,78
55	287,0	-00,5	236,0	-05,8	-05,3	-7,02	-27,80
66	284,9	-02,1	233,9	-02,1	00,0	-0,00	-27,80
76	282,7	-02,2	228,5	-05,4	-03,2	-4,24	-32,04
86	283,0	00,3	227,7	-00,8	-01,1	-1,46	-33,50
96	284,0	01,0	226,5	-01,2	-02,2	-2,91	-36,41
106	283,5	-00,5	224,0	-02,5	-02,0	-2,65	-39,06
116	283,5	00,0	222,5	-01,5	-01,5	-1,99	-41,05
146	282,2	-01,3	219,0	-03,5	-02,2	-2,91	-43,96
176	280,9	-01,3	209,0	-10,0	-08,7	-11,52	-55,48

K_e = 1,552; side arm 500µl TBS

Time (min)	TB (mm)	ΔTB (mm)	I (mm)	ΔI (mm)	ΔI-ΔTB (mm)	µl O_2	µl O_2 (cumulative)
0	254,0	-	233,5	-	-	-	-
10	252,0	-02,0	230,0	-03,5	-01,5	-2,33	-2,33
20	250,8	-01,2	223,0	-07,0	-05,8	-9,00	-11,33
30	251,7	00,9	219,8	-03,2	-04,1	-6,36	-17,69
40	250,5	-01,2	215,9	-03,9	-02,7	-4,19	-21,88
45	287,5	seal lost	212,0	-03,9	-03,9	-6,05	-27,93
55	287,0	-00,5	206,0	-06,0	-05,5	-8,54	-36,47
66	284,9	-02,1	201,5	-04,5	-02,4	-3,72	-40,19
76	282,7	-02,2	197,5	-04,0	-01,8	-2,79	-42,98
86	283,0	00,3	196,8	-00,7	-01,0	-1,55	-44,53
96	284,0	01,0	194,7	-02,1	-03,1	-4,81	-49,34
106	283,5	-00,5	192,9	-01,8	-01,3	-2,02	-57,36
116	283,5	00,0	190,5	-02,4	-02,4	-3,72	-55,08
146	282,2	-01,3	186,0	-04,5	-03,2	-4,97	-60,05
176	280,9	-01,3	176,5	-09,5	-08,5	-12,73	-72,78

K_e = 1,515; side arm 500µl SNF

Time (min)	TB (mm)	ΔTB (mm)	J (mm)	ΔJ (mm)	ΔJ-ΔTB (mm)	µl O_2	µl O_2 (cumulative)
0	254,0	-	228,0	-	-	-	-
10	252,0	-02,0	222,5	-05,5	-03,5	-5,30	-5,30
20	250,8	-01,2	214,0	-08,5	-07,3	-11,06	-16,36
30	251,7	00,9	210,0	-04,0	-04,9	-7,42	-23,78
40	250,5	-01,2	205,9	-04,1	-02,9	-4,39	-28,17
45	287,5	seal lost	205,2	-00,7	-00,7	-1,06	-29,23
55	287,0	-00,5	196,9	-08,3	-07,8	-11,82	-41,05
66	284,9	-02,1	193,1	-03,8	-01,7	-2,58	-43,63
76	282,7	-02,2	188,8	-04,3	-02,1	-3,18	-46,81
86	283,0	00,3	186,5	-02,3	-02,6	-3,94	-50,75
96	284,0	01,0	185,2	-01,3	-02,3	-3,48	-54,23
106	283,5	-00,5	182,9	-02,3	-01,8	-2,93	-56,96
116	283,5	00,0	179,0	-03,9	-03,9	-5,91	-62,87
146	282,2	-01,3	174,0	-05,0	-03,7	-5,61	-68,48
176	280,9	-01,3	163,7	-10,3	-09,0	-13,64	-82,12

K_e = 1,394; side arm 500µl SNF

Time (min)	TB (mm)	ΔTB (mm)	K (mm)	ΔK (mm)	ΔK-ΔTB (mm)	µl O_2	µl O_2 (cumulative)
0	254,0	-	228,0	-	-	-	-
10	252,0	-02,0	221,0	-07,0	-05,0	-6,97	-6,97
20	250,8	-01,2	213,0	-08,0	-06,8	-9,48	-16,45
30	251,7	0,09	208,0	-05,0	-05,9	-8,22	-24,67
40	250,5	-01,2	203,8	-04,2	-03,0	-4,18	-28,85
45	287,5	seal lost	199,8	-04,0	-04,0	-5,58	-34,43
55	287,0	-00,5	193,1	-06,7	-06,2	-8,64	-43,07
66	284,9	-02,1	191,0	-02,1	00,0	0,00	-43,07
76	282,7	-02,2	185,7	-05,3	-03,1	-4,32	-47,39
86	283,0	00,3	183,0	-02,7	-03,0	-4,18	-51,57
96	284,0	01,0	180,2	-02,8	-03,8	-5,30	-56,87
106	283,5	-00,5	177,2	-03,0	-02,5	-3,49	-60,36
116	283,5	00,0	175,2	-02,0	-02,0	-2,79	-63,15
146	282,2	-01,3	169,2	-06,0	-04,7	-6,55	-69,70
176	280,9	-01,3	154,2	-15,0	-13,7	-19,10	-88,80

K_e = 1,422; side arm 500µl SNF

Time (min)	TB (mm)	ΔTB (mm)	L (mm)	ΔL (mm)	ΔL-ΔTB (mm)	µl O_2	µl O_2 (cumulative)
0	254,0	-	228,0	-	-	-	-
10	252,0	-02,0	222,0	-06,0	-04,0	-5,69	-5,69
20	250,8	-01,2	215,8	-06,2	-05,0	-7,11	-12,80
30	251,7	00,9	211,1	-04,7	-05,6	-7,96	-20,76
40	250,5	-01,2	207,0	-04,1	-02,9	-4,12	-24,88
45	287,5	seal lost	207,8	00,8	00,8	-11,4	-26,02
55	287,0	-00,5	199,2	-08,6	-08,1	-11,52	-37,54
66	284,9	-02,1	196,0	-03,2	-01,1	-1,52	-39,10
76	282,7	-02,2	191,2	-04,8	-02,6	-3,70	-42,80
86	283,0	00,3	188,0	-03,2	-03,5	-4,98	-47,78
96	284,0	01,0	185,9	-02,1	-03,1	-4,41	-52,19
106	283,5	-00,5	182,0	-03,9	-03,4	-4,83	-57,02
116	283,5	00,0	180,0	-02,0	-2,00	-2,84	-59,86
146	282,2	-01,3	173,8	-06,2	-04,9	-6,97	-66,83
176	280,9	-01,3	160,9	-12,9	-11,6	-16,50	-83,33

References

1. Abramson, H.A. 1927. The mechanism of the inflammatory process. I. The electrophoresis of the blood cells of the horse and its relation to leucocyte emigration. *Journal of Experimental Medicine*.XLVI : 987-1002.

2. Alais, C. et Jollés, P. 1961. Etude comparée des caséino-glycopeptides formés par action de la présure sur les caséines de vache, de brebis et de chèvre. II. Etude de la partie non-peptidique. *Biochimica et Biophysica Acta*.51: 315-322.

3. Allison, F., Smith, M.R. and Wood, W.B. 1955. Studies on the pathogenesis of acute inflammation. I. The inflammatory reaction to thermal injury as observed in the rabbit ear chamber. *Journal of Experimental Medicine*. 102: 655-668.

4. Andrews, E.J. 1970. Toxicity of Millipore filter detergents to diffusion chamber cultures. *Transplantation* 10: 267-270.

5. Andrews, P.A. 1970. Estimation of molecular size and molecular weights of biological compounds by gel filtration. *Methods of Biochemical Analysis*. 18: 1-54.

6. Aschaffenburg, R. 1961. Inherited casein variants in cow's milk. *Nature*. 192: 431-432.

7. Aschaffenburg, R. and Drewry, J. 1957. Genetics of the β-lactoglobulins of cow's milk. *Nature*. 180: 376-378.

8. Awdeh, Z.L., Williamson, A.R. and Askonas, B.A. 1968. Isoelectric focusing in polyacrylamide gel and its application to immunoglobulins. *Nature*. 219: 66-67.

9. Axelrod, B. 1968. Other pathways of carbohydrate metabolism. *Metabolic pathways. Energetics, tricarboxylic acid cycle and carbohydrates*.1: 271-306. Edited by D.M. Greenberg. Third edition. Published by Academic Press Incorporated (New York and London).

10. Bangham, A.D., Flemans, R., Heard, D.H. and Seaman, G.V.F. 1958. An apparatus for micro-electrophoresis of small particles. *Nature*. 182: 642-644.

11. Barth, W.F., Willerson, J.T., Asofsky, R., Sheagren, J.N. and Wolff, S.M. 1969. Experimental murine amyloid. III. Amyloidosis induced with endotoxins. *Arthritis and Rheumatism*. 12: 615-626.

12. Baum, J., Mowat, A.G. and Kirk, J.A. 1971. A simplified method for the measurement of chemotaxis of polymorphonuclear leucocytes from human blood. *Journal of Laboratory and Clinical Medicine*. 77: 501-509.

13. Becker, E.L. 1971. Phosphonate inhibition of the accumulation and retention of K^+ by rabbit neutrophils in relation to chemotaxis. *Journal of Immunology*.106: 689-697.

14. Becker, E.L. 1972. The relationship of the chemotactic behaviour of the complement-derived factors, C3a, C5a and $\overline{C567}$, and a bacterial chemotactic factor to their ability to activate the proesterase 1 of rabbit polymorphonuclear leucocytes. *Journal of Experimental Medicine*. 135: 376-387.

15. Becker, E.L., Davis, A.T., Estensen, R.D. and Quie, P.G. 1972. Cytochalasin B. IV. Inhibition and stimulation of chemotaxis of rabbit and human polymorphonuclear leucocytes. *Journal of Immunology*.108: 396-402.

16. Becker, E.L. and Ward, P.A. 1967. Partial biochemical characterisation of the activated esterase required in the complement-dependent chemotaxis of rabbit polymorphonuclear leucocytes. *Journal of Experimental Medicine*. 125: 1021-1030.

17. Becker, E.L. and Ward P.A. 1969. Esterases of the polymorphonuclear leucocyte capable of hydrolysing acetyl-DL-phenylalanine-β-naphthyl ester. *Journal of Experimental Medicine.* 129: 569-584.

18. Berlin, R.D. and Ukena, T.E. 1972. Effect of colchicine and vinblastine on the agglutination of polymorphonuclear leucocytes by concanavalin A. *Nature New Biology.* 238: 120-122.

19. Bonner, W.M. and Laskey, R.A. 1974. A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. *European Journal of Biochemistry.* 46: 83-88.

20. Borisy, G.G. and Taylor, E.W. 1967. The mechanism of action of colchicine. Binding of colchicine-^{3}H to cellular protein. *Journal of Cell Biology.* 34: 525-533.

21. Broisy, G.G. and Taylor, E.W. 1967. The mechanism of action of colchicine. Colchicine binding to sea urchin eggs and mitotic apparatus. *Journal of Cell Biology.* 34: 535-548.

22. Bourne, H.R., Lehrer, R.I., Cline, M.J. and Melmon, K.L. 1971. Cyclic 3',5'-adenosine monophosphate in the human leukocyte. Synthesis, degradation, and effects on neutrophil candidacidal activity. *Journal of Clinical Investigation.* 50: 920-929.

23. Bourne, H.R., Lehrer, R.I., Melmon, K.L. and Cline, M.J. 1970. Cyclic adenosine-3',5'-monophosphate and the regulation of human granulocyte function. *Journal of Clinical Investigation.* 49: 11a-12a.

24. Boyden, S. 1962. The chemotactic effect of mixtures of antibody and antigen on polymorphonuclear leucocytes. *Journal of Experimental Medicine.* 115: 453-466.

25. Boyum, A. 1968. Isolation of mononuclear cells and granulocytes from human blood. *Scandinavian Journal of Clinical and Laboratory Investigation (Supplement)*. 97: 77-89.

26. Bryant, R.E., des Pres, R.M., van Way, M.H. and Rogers, D.E. 1966. Studies on human leukocyte motility. I. Effects of alterations in pH, electrolyte concentration and phagocytosis on leukocyte migration, adhesiveness and aggregation. *Journal of Experimental Medicine*. 124: 483-499.

27. Caner, J.E.Z. 1964. Colchicine inhibition of chemotactic migration of human polymorphonuclear leucocytes. *Arthritis and Rheumatism*. 7: 297-298.

28. Carruthers, B.M. 1966. Leucocyte motility. I. Methods of study, normal variations, effect of physical alterations in environment, and effect of iodoacetate. *Canadian Journal of Physiology and Pharmacology*. 44: 475-485.

29. Carruthers, B.M. 1967. Leucocyte motility. II. Effect of absence of glucose in medium; effect of presence of deoxyglucose, dinitrophenol, puromycin, actinomycin D and trypsin on the response to chemotactic substances; effect of segregation of cells from chemotactic substances. *Canadian Journal of Physiology and Pharmacology*. 45: 269-280.

30. Carter, S.B. 1965. Principles of cell motility. The direction of cell movement and cancer invasion. *Nature*. 208: 1183-1187.

31. Carter, S.B. 1967. Haptotaxis and the mechanism of cell motility. *Nature*. 213: 256-260.

32. Carter, S.B. 1967. Effects of cytochalasins on mammalian cells. *Nature*. 213: 261-264.

33. Clark, E.R. and Clark, E.L. 1935. Observations on changes in blood vascular endothelium in the living animal. *American Journal of Anatomy*. 57: 385-438.

34. Cliff, W.J. 1966. The acute inflammatory reaction in the rabbit ear chamber with particular reference to the phenomenon of leucocyte migration. *Journal of Experimental Medicine*. 124: 543-556.

35. Cohn, E.J. and Berggren, R.E.L. 1925. Studies in the physical chemistry of the protein. III. The relation between the amino acid composition of casein and its capacity to combine with base. *Journal of General Physiology*. VII: 45-79.

36. Cohn, E.J. and Hendry, J.L. 1923. Studies in the physical chemistry of the proteins. II. The relation between the solubility of casein and its capacity to combine with base. The solubility of casein in systems containing the protein and sodium hydroxide. *Journal of General Physiology* V: 521-554.

37. Cohn, Z.A. 1966. The regulation of pinocytosis in mouse macrophages. I. Metabolic requirements as defined by the use of inhibitors. *Journal of Experimental Medicine*. 124: 557-571.

38. Cook, W.H., Lipkin, D. and Markham, R. 1957. The formation of a cyclic dianhydrodiadenylic acid by the alkaline degradation of adenosine-5'-triphosphosphoric acid. *Journal of the American Chemical Society*. 79: 3607-3608.

39. Cullumbine, H. and Rydon, H.N. 1946. A study of the formation, properties and partial purification of leukotaxine. *British Journal of Experimental Pathology*. 27: 33-46.

40. Davidson, J.N. 1965. *In* The biochemistry of the nucleic acids. Fifth edition. Published by Methuen & Co. Limited (London) and John Wiley and Son Incorporated (New York).

41. Davis, B.J. 1964. Disc electrophoresis. II. Method and application to human serum proteins. *Annals of the New York Academy of Sciences.* 121: 404-427.

42. Dixon, M. and Webb, E.C. 1964. *In* Enzymes. Second edition. Published by Longmans, Green and Co. Limited (London).

43. Duthie, E.S. and Chain, E. 1939. A polypeptide responsible for some of the phenomena of acute inflammation. *British Journal of Experimental Pathology.* 20: 417-429.

44. Edelman, G.M., Yahara, I. and Wang, J.L. 1973. Receptor mobility and receptor-cytoplasmic interactions in lymphocytes. *Proceedings of the National Academy of Sciences.* 70: 1442-1446.

45. El-Negoumy, A.M. 1963. Effect of method of preparing isoelectric casein on its electrophoretic properties. *Journal of Dairy Science.* 46: 768-773.

46. El-Negoumy, A.M. and Bird, E.W. 1963. Lability of sulfur in casein during peptidization with sodium hydroxide. *Journal of Dairy Science.* 45: 650.

47. Estensen, R.D., Hill, H.R., Quie, P.G., Hogan, N. and Goldberg, N.D. 1973. Cyclic GMP and cell movement. *Nature.* 245: 458-460.

48. Estensen, R.D. and Plagemann, P.G.W. 1972. Cytochalasin B. Inhibition of glucose and glucosamine transport. *Proceedings of the National Academy of Sciences.* 69: 1430-1434.

49. Evans, W.A. and Karnovsky, M.L. 1961. A possible mechanism for the stimulation of some metabolic functions during phagocytosis. *Journal of Biological Chemistry.* 236: PC30-PC32.

50. Fischer, L. 1969. An introduction to gel chromatography. *Laboratory techniques in biochemistry and molecular biology.* 1: 157-396. Edited by T.S. Work and E. Work. Published by North-Holland Publishing Co. (Amsterdam and London).

51. Gail, M.H. and Boone, Ch.W. 1972. Cell-substrate adhesivity. A determinant of cell motility. *Experimental Cell Research.* 70: 33-40.

52. Gallin, J.I., Clark, R.A. and Kimball, H.R. 1973. Granulocyte chemotaxis. An improved *in vitro* assay employing ^{51}Cr-labelled granulocytes. *Journal of Immunology.* 110: 233-240.

53. Gamow, R.I., Böttger, B. and Barnes, F.S. 1971. Analysis of chemotaxis in white blood cells. *Biophysical Journal.* 11: 860-867.

54. Garvin, J.E. 1961. Factors affecting the adhesiveness of human leucocytes and platelets *in vitro.* *Journal of Experimental Medicine.* 114: 51-73.

55. Gevers, W., Kleinkauf, H. and Lipmann, F. 1968. The activation of amino acids for biosynthesis of gramicidin S. *Proceedings of the National Academy of Sciences.* 60: 269-276.

56. Gilman, A.G. 1970. A protein binding assay for adenosine 3':5'-cyclic monophosphate. *Proceedings of the National Academy of Sciences.* 67: 305-312.

57. Goldberg, I.H., Rabinowitz, M. and Reich, E. 1962. Basis of actinomycin action. I. DNA binding and inhibition of RNA-polymerase synthetic reactions by actinomycin. *Proceedings of the National Academy of Sciences.* 48: 2094-2101.

58. Gordon, A.H. 1969. Electrophoresis of proteins in polyacrylamide and starch gels. *Laboratory techniques in biochemistry and molecular biology.* 1: 1-149. Edited by T.S. Work and E. Work. Published by North-Holland Publishing Co. (Amsterdam and London).

59. Gurr, George T. 1963. *In* Biological staining methods. Seventh edition. Published by George T. Gurr Limited (London).

60. Hammarsten, O. 1873-1874. Om det chemiska förloppet vid caseinets coagulation med löpe. *Uppsala Läkarenförenings Förhandlingar.* IX: 363-389.

61. Hammarsten, O. 1873-1874. Om det chemiska förloppet vid caseinets coagulation med löpe. *Uppsala Läkarenförenings Förhandlingar.* IX: 451-486.

62. Harris, H. 1953. Chemotaxis of granulocytes. *Journal of Pathology and Bacteriology.* LXVI: 135-146.

63. Harris, H. 1954. Role of chemotaxis in inflammation. *Physiological Reviews.* 34: 529-562.

64. Hirsch, J.G. and Church, A.B. 1960. Studies of phagocytosis of group A *Streptococci* by polymorphonuclear leucocytes *in vitro*. *Journal of Experimental Medicine.* 111: 309-322.

65. Horwitz, D.A. and Garrett, M.A. 1971. Use of leukocyte chemotaxis *in vitro* to assay mediators generated by immune reactions. I. Quantitation of mononuclear and polymorphonuclear leukocyte chemotaxis with polycarbonate (Nuclepore) filters. *Journal of Immunology.* 106: 649-655.

66. Huebner, V.R. and Lawson, R.H. 1968. Performance characteristics of a new continuous-flow electrophoresis instrument. *Separation Science.* 3: 265-277.

67. Humes, J.L., Rounbehler, M. and Kuehl, F.A. 1969. A new assay for measuring adenyl cyclase activity in intact cells. *Analytical Biochemistry.* 32: 210-217.

68. Jacoby, F. 1965. Macrophages. *Cells and tissues in culture. Methods, biology and physiology.* 2: 1-93. Edited by E.N. Willmer. Published by Academic Press Incorporated (London and New York).

69. Jeppsson, J.O. and Berglund, S. 1972. Thin-layer isoelectric focusing for haemoglobin screening and its application to haemoglobin Malmö. *Clinica Chimica Acta.* 40: 153-158.

70. Jollès, P., Alais, C. et Jollès, J. 1961. Etude comparée des caséino-glycopeptides formés par action de la présure sur les caséines de vache, de brebis et de chèvre. I. Etude de la partie peptidique. *Biochimica et Biophysica Acta.* 51: 309-314.

71. Jost, J.P. and Rickenberg, H.V. 1971. Cyclic AMP. *Annual Review of Biochemistry.* 40: 741-774.

72. Kalchar, H.M. 1947. Differential spectrophotometry of purine compounds by means of specific enzymes. III. Studies of the enzymes of purine metabolism. *Journal of Biological Chemistry.* 167: 461-475.

73. Kaley, G. and Weiner, R. 1971. Effect of prostaglandin E_1 on leucocyte migration. *Nature New Biology.* 234: 114-115.

74. Kaplan, M.M., Abdussalam, M. and Bijlenga, G. 1962. Diseases transmitted through milk. *In* Milk Hygiene. Hygiene in milk production, processing and distribution. Published under the auspices of the Food and Agriculture Organisation of the United Nations and the World Health Organisation. World Health Organisation (Geneva).

75. Keller, H.U. 1966. Studies on chemotaxis. III. Modification of Boyden's technique for the evaluation of chemotactic agents. *Immunology.* 10: 225-230.

76. Keller, H.U. Personal communications.

77. Keller, H.U., Borel, J.F., Wilkinson, P.C., Hess, M.W. and Cottier, H. 1972. Reassessment of Boyden's technique for measuring chemotaxis. *Journal of Immunological Methods.* 1: 165-168.

78. Keller, H.U., Hess, M.W. and Cottier, H. 1974. Inhibiting effect of human plasma and serum on neutrophil random migration and chemotaxis. *Blood.* 44: 843-848.

79. Keller, H.U. and Sorkin, E. 1965. Studies on chemotaxis. I. On the chemotactic and complement-fixing activity of γ-globulins. *Immunology.* 9: 241-247.

80. Keller, H.U. and Sorkin, E. 1965. Studies on chemotaxis. II. The significance of normal sera for chemotaxis induced by various agents. *Immunology.* 9: 441-447.

81. Keller, H.U. and Sorkin, E. 1966. Studies on chemotaxis. IV. The influence of serum factors on granulocyte locomotion. *Immunology.* 10: 409-416.

82. Keller, H.U. and Sorkin, E. 1967. Studies on chemotaxis. V. On the chemotactic effect of bacteria. *International Archives of Allergy and Applied Immunology.* 31: 505-517.

83. Keller, H.U. and Sorkin, E. 1967. Studies on chemotaxis. VI. Specific chemotaxis in rabbit polymorphonuclear leucocytes and mononuclear cells. *International Archives of Allergy and Applied Immunology.* 31: 575-586.

84. Keller, H.U. and Sorkin, E. 1967. Studies on chemotaxis. IX. Migration of rabbit leucocytes through filter membranes. *Proceedings of the Society for Experimental Biology and Medicine.* 126: 677-680.

85. Kiddy, C.A., Johnston, J.O. and Thompson, M.P. 1964. Genetic polymorphism in caseins of cow's milk. I. Genetic control of α_s-casein variation. *Journal of Dairy Science.* 47: 147-151.

86. Kiddy, C.A., Thompson, M.P., Johnston, J.O. and Pepper, L. 1963. Genetic control of α_s- and β-casein. *Journal of Dairy Science* 46: 626-627.

87. Kletzien, R.F., Perdue, J.F. and Springer, A. 1972. Cytochalasin A and B. Inhibition of sugar uptake in cultured cells. *Journal of Biological Chemistry.* 247: 2964-2966.

88. Kopriwa, B.M. and Leblond, C.P. 1962. Improvements in the coating technique of radioautography. *Journal of Histochemistry and Cytochemistry.* 10: 269-284.

89. Krishan, A. 1972. Cytochalasin B. Time-lapse cinematographic studies on its effects on cytokinesis. *Journal of Cell Biology.* 54: 657-664.

90. Kuchler, R.J. 1972. Polyacrylamide gel electrophoresis. *In* Manometric and biochemical techniques. A manual describing methods applicable to the study of tissue metabolism. Edited by W.W. Umbreit, R.H. Burris and J.F. Stauffer. Fifth edition. Published by Burgess Publishing Company (Minneapolis, Minnesota).

91. Larson, B.L. and Kendall, K.A. 1957. Protein production in the bovine. Daily production of the specific milk proteins during the lactation period. *Journal of Dairy Science.* 40: 377-386.

92. Larson, B.L., Rolleri, G.D. and Kendall, K.A. 1956. Protein production in the bovine. Comparison of daily protein, fat and milk production during the entire lactation period. *Journal of Dairy Science.* 39: 204-213.

93. Larson, B.L., Rolleri, G.D. and Kendall, K.A. 1956. Appearance of the specific blood serum proteins in the lacteal secretion near parturition. *Journal of Dairy Science.* 39: 926.

94. Laszlo, J., Miller, D.S., McCarty, K.S. and Hochstein, P. 1966. Actinomycin D. Inhibition of respiration and glycolysis. *Science*. 151: 1007-1010.

95. Leaback,D.H. and Rutter, A.C. 1968. Polyacrylamide-isoelectric-focusing. A new technique for the electrophoresis of proteins. *Biochemical and Biophysical Research Communications*. 32: 447-453.

96. Leahy, D.R., McLean,E.R. and Bonner, J.T. 1970. Evidence for cyclic-3',5'-adenosine monophosphate as a chemotactic agent for polymorphonuclear leucocytes. *Blood*. 36: 52-54.

97. Lengemann, F.W., Swanson, E.W. and Monroe, R.A. 1957. Effects of season on secretion of iodine in milk. *Journal of Dairy Science*. 40: 387-393.

98. Liddle, G.W. and Hardman, J.G. 1971. Cyclic adenosine monophosphate as a mediator of hormone action. *The New England Journal of Medicine*. 285: 560-566.

99. Lindquist, B. 1963. Casein and the action of rennin. Part I. *Dairy Science Abstracts*. 25: 257-264.

100. Lindquist, B. 1963. Casein and the action of rennin. Part II. *Dairy Science Abstracts*. 25: 299-308.

101. Little, K. and Edwards, J.H. 1962. Electron microscope examination of whole fibroblasts. *Journal of the Royal Microscopical Society*. 81: 23-24.

102. Lotz, M. and Harris, H. 1956. Factors influencing chemotaxis of polymorphonuclear leucocytes. *British Journal of Experimental Pathology*. 37: 477-480.

103. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. 1951. Protein measurement with the phenol reagent. *Journal of Biological Chemistry*. 193: 265-275.

104. Mahler, H.R. and Cordes, E.H. 1966. *In* Biological chemistry. Harper international edition. Jointly published by Harper and Row (New York, Evanston and London) and John Weatherhill Incorporated (Tokyo).

105. Maizel, J.V. 1971. Polyacrylamide gel electrophoresis of viral proteins. *Methods in virology.* 5: 179-246. Edited by K. Maramorosch and H. Kobrowski. Published by Academic Press Incorporated (New York and London).

106. Malawista, S.E. and Bensch, K.G. 1967. Human polymorphonuclear leucocytes. Demonstration of microtubules and effect of colchicine. *Science.* 156: 521-522.

107. Manganiello, V., Evans, W.H., Stossel, T.P., Mason, R.J. and Vaughan, M. 1971. The effect of polystyrene beads on cyclic 3',5'-adenosine monophosphate concentration in leucocytes. *Journal of Clinical Investigation.* 50: 2941-2944.

108. Massart, J. 1892. Le chimiotaxisme des leucocytes et l'immunité. *Annales de LiInstitut Pasteur.* 6: 321-327.

109. Massart, J. et Bordet, Ch. 1891. Le chimiotaxisme des leucocytes et l'infection microbienne. *Annales de L'Institut Pasteur.* 5: 417-444.

110. McCutcheon, M. 1946. Chemotaxis in leucocytes. *Physiological Reviews.* 26: 319-336.

111. McKenzie, H.A. 1967. Milk proteins. *Advances in Protein Chemistry.* 22: 55-234.

112. McKenzie, H.A. (*Editor*).1970. *Milk proteins: Chemistry and molecular biology.* Volume 1. New York Academic Press.

113. McKenzie, H.A. (*Editor*). 1971. *Milk proteins: Chemistry and molecular biology.* Volume II. New York Academic Press.

114. McMahon, D. 1974. Chemical messengers in development. A hypothesis. *Science.* 185: 1012-1021.

115. Menkin, V. 1938. Studies on inflammation. XIV. Isolation of the factor concerned with increased permeability in injury. *Journal of Experimental Medicine.* LXVII : 129-144.

116. Menkin, V. 1938. Studies on inflammation. XVI. On the formation of chemotactic substances by enzyme action. *Journal of Experimental Medicine.* LXVII : 153-158.

117. Menkin, V. 1939. Inflammation. XVII. Direct effect of changes in the hydrogen ion concentration on leucocytes. *Archives of Pathology.* 27: 117-121.

118. Menkin, V. 1940. *In* Dynamics of inflammation. An inquiry into the mechanism of infectious processes. Published by MacMillan Company (New York).

119. Menkin, V. 1955. Factors concerned in the mobilization of leucocytes in inflammation. *Annals of the New York Academy of Sciences.* 59: 956-985.

120. Moon, V.H., Grand, C.G. and Tershakovec, G.A. 1954. Dynamics of inflammation and of repair. IV. Chemotactic substances in normal tissue. *Archives of Pathology.* 57: 44-50.

121. Nahas, G.G., Tannieres, M.L. and Lennon, J.F. 1971. Direct measurement of leucocyte motility. Effects of pH and temperature. *Proceedings of the Society for Experimental Biology and Medicine.* 138: 350-352.

122. Nuclepore Corporation. 1975. Leukocyte chemotaxis. Nuclepore Corporation, 7035 Commerce Circle, Pleasanton, California, U.S.A.

123. Ornstein, L. 1964. Disc electrophoresis. I. Background and theory. *Annals of the New York Academy of Sciences.* 121: 321-349.

124. Oser, B.L. (*Editor*). 1965. Milk. *In* Hawk's physiological chemistry p. 368-393. Fourteenth edition. Published by McGraw-Hill Book Company (New York, Toronto, Sydney and London).

125. Pastan, I. and Perlman, R.L. 1972. Cyclic AMP in metabolism. *Nature New Biology.* 229: 5-7 plus 32.

126. Paul, J. 1972. *In* Cell and tissue culture. Fourth edition. Published by Churchill Livingston (Edinburgh and London).

127. Pearce, Everson A.G. 1972. *Histochemistry theoretical and applied.* Volume II. Third edition. Published by Churchill Livingston (Edinburgh and London).

128. Phelps, P. 1970. Polymorphonuclear leucocyte motility *in vitro*. IV. Colchicine inhibition of the formation of chemotactic activity after phagocytosis of urate crystals. *Arthritis and Rheumatism.* 13: 1-9.

129. Phelps, P. and Stanislaw, D. 1969. Polymorphonuclear leucocyte motility *in vitro*. I. Effect of pH, temperature, ethyl alcohol and caffeine using a modified Boyden chamber technique. *Arthritis and Rheumatism.* 12: 181-188

130. Pieroni, R.E. and Broderick, E.J. 1969. A simple method for the quantitation of submicrogram amounts of bacterial endotoxin. *Federation Proceedings.* 28: 307.

131. Reid, M.S. and Bieleski, R.L. 1968. A simple apparatus for vertical flat-sheet polyacrylamide gel electrophoresis. *Analytical Biochemistry.* 22: 374-381.

132. Rivkin, I. and Becker, E.L. 1972. Possible implications of cyclic 3',5'-adenosine monophosphate in the chemotaxis of rabbit peritoneal polymorphonuclear leucocytes (PMN's). *Federation Proceedings*. 31: 657.

133. Robinson, G.A., Butcher, R.W. and Sutherland, E.W. 1969. Cyclic AMP. *Annual Review of Biochemistry*. 37: 149-174.

134. Rolleri, G.D., Larson, B.L. and Touchberry, R.W. 1956. Protein production in the bovine. Breed and individual variations in the specific protein constituents of milk. *Journal of Dairy Science*. 39: 1683-1689.

135. Rosenberg, M.D. 1960. Microexudates from cells grown in tissue culture. *Biophysical Journal*. 1: 137-159.

136. Ryan, G.B., Borysenko, J.Z. and Karnovsky, M.J. 1974. Factors affecting the redistribution of surface-bound concanavalin A on human polymorphonuclear leucocytes. *Journal of Cell Biology*. 62: 351-365

137. Sanger, J.W. and Holtzer, H. 1972. Cytochalasin B. Effect on cell morphology, cell adhesion, and mucopolysaccharide synthesis. *Proceedings of the National Academy of Sciences*. 69: 253-257.

138. Sbarra, A.J. and Karnovsky, M.L. 1959. The biochemical basis of phagocytosis. I. Metabolic changes during the injestion of particles by polymorphonuclear leucocytes. *Journal of Biological Chemistry*. 234: 1355-1362.

139. Sbarra, A.J. and Karnovsky, M.L. 1960. The biochemical basis of phagocytosis. II. Incorporation of ^{14}C-labelled building blocks into lipid, protein and glycogen of leucocytes during phagocytosis. *Journal of Biological Chemistry*. 235: 2224-2229.

140. Schroeder, T.E. 1969. The role of "contractile ring" filaments in dividing *Arbacia* egg. *The Biological Bulletin.* 137: 413-414.

141. Scott, R.E. 1970. Effects of prostaglandins, epinephrine and NaF on human leucocyte, platelet and liver adenyl cyclase. *Blood.* 35: 514-516.

142. Seaman, G.V.F. 1965. Electrophoresis using a cylindrical chamber. *In* Cell electrophoresis. p. 4-21. Edited by E.J. Ambrose. Published by Little, Brown and Co. (Boston).

143. Shaw, E. 1970. Selective chemical modification of proteins. *Physiological Reviews.* 50: 244-296.

144. Silverman, D. 1938. A chemotropic substance derived from normal tissue. *Archives of Pathology.* 25: 40-45.

145. Sorkin, E., Stecher, V.J. and Borel, J.F. 1970. Chemotaxis of leucocytes and inflammation. *Series Haematologica* 3: 131-162.

146. Spector, W.G. 1951. The role of some higher peptides in inflammation. *Journal of Pathology and Bacteriology.* 63: 93-110.

147. Stecher, V.J. and Sorkin, E. 1969. Studies on chemotaxis. XII. Generation of chemotactic activity for polymorphonuclear leucocytes in sera with complement deficiencies. *Immunology.* 16: 231-239.

148. Stossel, T.P., Murad, F., Mason, R.J. and Vaughan, M. 1970. Regulation of glycogen metabolism in polymorphonuclear leucocytes. *Journal of Biological Chemistry.* 245: 6228-6234.

149. Strickler, A. 1967. Continuous particle electrophoresis. A new analytical and preparative capability. *Separation Science.* 2: 335-355.

150. Strickler, A., Kaplan, A. and Vigh, E. 1966. Continuous microfractionation of particle mixtures by electrophoresis. *Microchemical Journal.* 10: 529-544.

151. Sutherland, E.W. and Rall, T.W. 1957. The properties of an adenine ribonucleotide produced with cellular particles, ATP, Mg^{++} and epinephrine or glucagon. *Journal of the American Chemical Society.* 79: 3608.

152a. Sutherland, E.W., Robinson, G.A. and Butcher, R.W. 1968. Some aspects of the biological role of adenosine 3',5'-monophosphate (cyclic AMP). *Circulation.* XXXVII: 279-306.

152b. Sutherland, E.W., Øye, I. and Butcher, R.W. 1965. The action of epinephrine and the role of the adenyl cyclase system in hormone action. *Recent Progress in Hormone Research.* 21: 623-646.

153. Symon, D.N.K., McKay, I.C. and Wilkinson, P.C. 1972. Plasma-dependent chemotaxis of macrophages towards *Mycobacterium tuberculosis* and other organisms. *Immunology.* 22: 267-276.

154. Taylor, A.C. 1961. Attachment and spreading of cells in culture. *Experimental Cell Research (Supplement).* 8: 154-173.

155. Thomas, L. and Stetson, C.A. 1949. Studies on the mechanism of the Schwartzmann phenomenon. *Journal of Experimental Medicine.* LXXXIX: 461-477.

156. Thompson, M.P., Kiddy, C.A., Pepper, L. and Zittle, C.A. 1962. Casein variants in milk from individual cows. *Journal of Dairy Science.* 45 : 650-651.

157. Thompson, M.P., Kiddy, C.A., Pepper, L. and Zittle, C.A. 1962. Variations in the α_s-casein fraction of individual cow's milk. *Nature.* 195: 1001-1002.

158. Tse, R.L., Phelps, P. and Urban, D. 1972. Polymorphonuclear leucocyte motility *in vitro*. VI. Effect of purine and pyrimidine analogues. Possible role for cyclic AMP. *Journal of Laboratory and Clinical Medicine*. 80: 264-274.

159. Tucker, D.N. 1963. The effect of pH on phagocytosis by rabbit mononuclear phagocytes. *Journal of Infectious Diseases*. 112: 47-52.

160. Ukena, T.E. and Berlin, R.D. 1972. Effect of colchicine and vinblastine on the topographical separation of membrane functions. *Journal of Experimental Medicine*. 136: 1-7.

161. Umbreit, W.W. 1972. Constant volume manometry - the "Warburg" *In* Manometric and biochemical techniques. A manual describing methods applicable to the study of tissue metabolism. Fifth edition. Edited by W.W. Umbreit, R.H. Burris and J.F. Stauffer. Published by Burgess Publishing Company (Minneapolis, Minnesota).

162. Vesterburg, O. 1972. Isoelectric focusing of proteins in polyacrylamide gels. *Biochemica et Biophysica Acta*. 257: 11-19.

163. Villar-Palasi, C. and Larner, J. 1970. Glycogen metabolism and glycolytic enzymes. *Annual Review of Biochemistry*. 39: 639-672.

164. Wake, R.G. and Baldwin, R.L. 1961. Analysis of casein fractions by zone electrophoresis in concentrated urea. *Biochemica et Biophysica Acta*. 47: 225-239.

165. Ward, P.A. 1968. Chemotaxis of polymorphonuclear leucocytes. *Biochemical Pharmacology (Supplement)*. p. 99-105.

166. Ward, P.A. 1971. Leukotactic factors in health and disease. *American Journal of Pathology*. 64: 521-530.

167. Ward, P.A. and Becker, E.L. 1967. Mechanisms of the inhibition of chemotaxis by phosphonate esters. *Journal of Experimental Medicine.* 125: 1001-1020.

168. Ward, P.A. and Becker, E.L. 1968. The deactivation of rabbit neutrophils by chemotactic factor and the nature of the activatable esterase. *Journal of Experimental Medicine.* 127: 693-709.

169. Ward, P.A. and Becker, E.L. 1970. Biochemical demonstration of the activatable esterase of the rabbit neutrophil involved in the chemotactic response. *Journal of Immunology.* 105: 1057-1067.

170. Ward, P.A., Cochrane, C.G. and Müller-Eberhard, H.J. 1965. The role of serum complement in chemotaxis of leucocytes *in vitro*. *Journal of Experimental Medicine.* 122: 327-340.

171. Ward, P.A., Lepow, I.H. and Newman, L.J. 1968. Bacterial factors chemotactic for polymorphonuclear leucocytes. *American Journal of Pathology.* 52: 725-736.

172. Warner, D.A. and Perdue, J.F. 1972. Cytochalasin B and the adenosine triphosphate content of treated fibroblasts. *Journal of Cell Biology.* 55: 242-244.

173. Webb, J.L. 1963. *Enzyme and metabolic inhibitors. I. General principles of inhibition.* Published by Academic Press Incorporated (New York and London).

174. Webb, J.L. and Hollander, P.B. 1956. Metabolic aspects of the relationship between the contractility and membrane potentials of the rat atrium. *Circulation Research.* 4: 618-626.

175. Weiss, L. 1961. The measurement of cell adhesion. *Experimental Cell Research (Supplement).* 8: 141-153.

176. Weiss, L. 1961. Studies on cellular adhesion in tissue culture. IV. The alteration of substrate by cell surfaces. *Experimental Cell Research.* 25: 504-517.

177. Weiss, L. and Coombs, R.R.A. 1963. The demonstration of rupture of cell surfaces by an immunological technique. *Experimental Cell Research.* 30: 331-338.

178. Weiss, L. and Lachmann, P.J. 1964. The origin of an antigenic zone surrounding HeLa cells cultured on glass. *Experimental Cell Research.* 36: 86-91.

179. Weiss, L. and Mayhew, E. 1966. The presence of ribonucleic acid within the peripheral zones of two types of mammalian cell. *Journal of Cellular Physiology.* 68: 345-360.

180. Weksler, B.B. and Hill, M.J. 1969. Inhibition of leucocyte migration by a staphylococcal factor. *Journal of Bacteriology.* 98: 1030-1035.

181. Wessels, N.K. 1971. How living cells change shape. All embryonic cells and many mature ones bend, bulge, stretch and even travel from place to place. In doing so the cells use two distinct kinds of filaments that seem to act as skeleton and muscle. *Scientific American.* 225: 77-82.

182. Wessels, N.K., Spooner, B.S., Ash, J.F., Bradley, M.O., Luduena, M.A., Taylor, E.L., Wrenn, J.T. and Yamada, K.M. 1971. Microfilaments in cellular and developmental processes. Contractile microfilament machinery of many cell types is reversibly inhibited by cytochalasin B. *Science.* 171: 135-143.

183. White, A.A. and Zenser, T.V. 1971. Separation of cyclic 3',5'-nucleoside monophosphates from other nucleotides on aluminium oxide columns. Application to the assay of adenyl cyclase and guanyl cyclase. *Analytical Biochemistry.* 41: 372-396.

184. Wilkinson, P.C. 1972. Characterization of the chemotactic activity of casein for neutrophil leucocytes and macrophages. *Experientia.* 28: 1051-1052.

185. Wilkinson, P.C. 1974. *In* Chemotaxis and inflammation. Published by Churchill Livingston (Edinburgh and London).

186. Wissler, J.H., Stecher, V.J. and Sorkin, E. 1972. Chemistry and biology of the anaphylatoxin related serum peptide system. III. Evaluation of leucotactic activity as a property of a new peptide system with classical anaphylatoxin and cocytotaxin as components. *European Journal of Immunology.* 2: 90-96.

187. Wissler, J.H., Stecher, V.J. and Sorkin, E. 1972. Biochemistry and biology of a leucotactic binary peptide system related to anaphylatoxin. *International Archives of Allergy and Applied Immunology.* 42: 722-747.

188. Witkowski, J.A. and Brighton, W.D. 1972. Influence of serum on attachment of tissue cells to glass surfaces. *Experimental Cell Research.* 70: 41-48.

189. Woodin, A.M. and Weineke, A.A. 1970. Action of DFP on the leucocyte and the axon. *Nature.* 227: 460-463.

190. Young, C.W. and Bittar, E.S. 1973. Analysis of tissue esterases from patients with Hodgkin's disease and other types of advanced cancer by isoelectric focusing in acrylamide gel. *Cancer Research.* 33: 2692-2700.

191. Zigmond, S.H. 1972. *In* Studies on polymorphonuclear leucocyte locomotion and chemotaxis. Ph.D thesis, Rockefeller University, New York, U.S.A.

192. Zigmond, S.H. and Hirsch, J.G. 1972. Effects of cytochalasin B on polymorphonuclear leucocyte locomotion, phagocytosis and glycolysis. *Experimental Cell Research.* 73: 383-393

193. Zigmond, S.H. and Hirsch, J.G. 1972. Cytochalasin B. Inhibition of D-2-deoxyglucose transport into leucocytes and fibroblasts. *Science*. 176: 1432-1434.

194. Zigmond, S.H. and Hirsch, J.G. 1973. Leucocyte locomotion and chemotaxis. New methods for evaluation and demonstration of a cell-derived chemotactic factor. *Journal of Experimental Medicine*. 137: 387-410.