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SUMMARY

The work in this thesis is presented in three chapters, the contents of which may be summarised as follows:-

Chapter I

Approximately 16% of randomly selected blood donors resident in the Western Cape showed immediate cutaneous hypersensitivity reactions to Bermuda grass pollen. This material is, therefore, an important allergen in this area and attempts were made to define the nature of the antigens it contained.

Using gel chromatography and ion-exchange chromatography, two antigens, BGP_1 and BGP_2, could be identified in a crude extract of Bermuda grass pollen. BGP_1, the major antigen, elicited a positive skin reaction in all subjects sensitive to the crude extract. BGP_2, the minor antigen, gave positive skin test results in 75% of sensitive subjects.

Calibrated column chromatography, velocity sedimentation on sucrose density gradients and iso-electric focussion were used to define the following characteristics of the two antigens:-

	BGP_1	BGP_2
Molecular weight (Daltons)	30,000	14,400
Stokes radius (x 10^{-8} cm)	24.32	
Diffusion coefficient ($10^{-7} cm^2 sec^{-1}$)	8.86	11.88
$S_{20,w}$ (Svedberg units)	3.1	
Iso-electric points (3)	6.6,5.4,5.0	

There is a possibility that BGP_1 is a dimeric form of BGP_2.

Chapter II

A radio radial immunodiffusion assay was used to measure the serum IgE concentrations of 4650 randomly selected blood donors,

representing the three racial groups living in the Western Cape. Analysis of the results showed significant race and sex differences in serum IgE concentrations. Concentrations tended to increase in the order Whites, Coloureds, Bantu, and, within any race group, they tended to be higher in males than in females. Median serum IgE concentrations were as follows:-

White males	85 u/ml	White females	70 u/ml
Coloured males	226 u/ml	Coloured females	180 u/ml
Bantu males	642 u/ml	Bantu females	289 u/ml

Analysis by age showed that, in Whites, there was a significant tendency for serum IgE concentrations to be lower in the older age groups. The sex difference in serum IgE concentrations was most apparent in the middle years of life (30-49 years) and serum IgE concentrations in the elderly tended to be uniformly low.

Chapter III

A study of the interrelationships between allergy, cutaneous hypersensitivity to common allergens, helminthic infestation, and serum IgE concentrations in 268 individuals revealed that the race and sex differences in serum IgE concentrations could not be explained on the basis of race and sex differences in the prevalence of allergic symptoms. Only in Whites could a significant association between allergic symptoms and elevated serum IgE concentrations be demonstrated.

The Whites, and to a lesser extent the Coloureds, showed positive correlations between skin hypersensitivity to common allergens and serum IgE concentrations. Bantu males had a high prevalence of positive skin tests to common allergens, but showed no correlation between degree of skin hypersensitivity and serum IgE concentrations.

Within the White group the associations between allergy and elevated serum IgE concentrations and between cutaneous hypersensitivity and elevated serum IgE concentrations were far more significant in

males than in females. Further analysis showed that the sex difference in serum IgE concentrations was confined to those subjects who showed clinical or cutaneous evidence of hypersensitivity.

Both the Coloureds and the Bantu had a relatively high prevalence of helminthic infestation. Although within population groups there was no evidence that persons with proven helminthic infestation had higher serum IgE concentrations than persons without, there was good circumstantial evidence to suggest that helminthic infestation was at least partially responsible (a) for the elevated serum IgE concentrations found in these people, and (b) for the high prevalence of positive skin tests found in the Bantu.

The Coloureds and the Bantu, particularly the latter, had, by Western standards, not only elevated serum IgE concentrations but also elevated serum IgG and IgA concentrations. In the Bantu there were significant correlations between serum IgE concentrations and the serum concentrations of IgG and IgA. The factors responsible for elevated immunoglobulin levels in African populations appear to be very complex.

INTRODUCTION

Those diseases which are now recognised as being due to immediate or type I hypersensitivity constitute some of the commonest afflictions of man. In 1906 Von Pirquet first recognised that hypersensitivity represented the manifestation of a particular type of immune reaction and coined the word *allergy* (from the Greek ἄλλη ἔργεια meaning "altered capacity to react") to describe the abnormal responsiveness present in hypersensitivity diseases. Since that time there has been considerable progress in the understanding of the mechanisms involved in the allergic response. It is now generally accepted that allergy represents a state of immunological reactivity in which relatively low doses of immunogen induce an immune response that is characterized by the synthesis of homocytotropic, or reaginic antibodies. These antibodies, of which Immunoglobulin E (IgE) is the best defined, have an affinity for surface receptors on those cells (mast cells and basophils) which are endowed with the capacity for synthesis and release of pharmacologically active substances such as histamine. The engagement of the specific binding sites of the fixed antibody by complementary antigen initiates a sequence of cellular events that lead to the release of histamine and other compounds responsible for the vaso-dilatation and increased vascular permeability characteristic of the immediate allergic response. However, within the framework of this consensus regarding the pathogenesis of the allergic reaction, there are many respects in which knowledge is incomplete or lacking. What factors for example determine the induction of reaginic antibody synthesis rather than conventional antibody synthesis? Do these factors reside primarily in the nature of the stimulus, or in the host, or in some subtle inter-

action between the two? Are there genetic factors that predispose to reaginic antibody synthesis? Are there chemical or physical features that are common to allergens as a class? Is the route of immunization important? Is cellular collaboration necessary for IgE synthesis? These are a few of the many questions that may be asked.

In forming a realistic experimental approach to the study of allergy, one tends, for obvious reasons, to emphasize those aspects of the problem that seem important and at the same time amenable to solutions with the facilities available. When, at the beginning of 1971, I started the work that I report in this thesis, the state of knowledge on the one hand, and my particular circumstances on the other, were such that I felt it would be profitable to approach the study of human allergy with two broad objectives in view.

Firstly, I aimed to purify and characterize a common local allergen with a view to adding to the knowledge of the biochemical properties of allergens in general, and, with the use of tracer techniques, to studying their fate in normal and sensitive subjects. For this purpose I chose Bermuda grass pollen as a source of material. This choice was dictated by the fact that Bermuda grass is ubiquitous in warm temperate climates and its pollen was known to contain potent allergenic material that had been only partially characterized. There are no species differences between the Bermuda grass found in South Africa and that growing in Europe and the United States, and therefore Bermuda grass pollen is an allergen of more than local significance. Moreover, my experience at the Allergy Clinic, Groote Schuur Hospital, had indicated that hypersensitivity to Bermuda grass was common in the local population so ensuring an adequate supply of clinical material for study.

Chapter I of this thesis describes my work on the Bermuda grass pollen.

Secondly, no data were available on serum IgE concentrations in South African populations. In the Western Cape there are three population groups with relatively distinct genetic, cultural and socio-economic backgrounds. I felt it would be useful to establish a set of values for the serum IgE concentration encountered in generally healthy members of these three population groups and to explore the reasons for any differences discovered. Chapters II and III of this thesis describe these aspects of the work.

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Figure 1.1.
Bermuda grass (Cynodon dactylon) in flower.

CHAPTER 1.

Characterization of two of the antigens found in Bermuda grass pollen.

1.1 Introduction

Grasses belong to the order Gluminiflorae, family Gramineae. There are approximately 4,500 varieties in the world, only a few of which are important in clinical allergy. Bermuda grass (Cynodon dactylon, Figure 1.1) belongs to the tribe Chlorideae. It has a variety of trivial names such as Fine Kweek, Scutch grass, Dog's Tooth grass, Wire grass, Cane grass, Bahama grass and Indian Doob and is commonly encountered in South Africa where it is extensively cultivated as domestic lawn. It is also found in other parts of the world and is particularly abundant in the southern regions of the United States where, owing to its ability to withstand drought, it is popular both as domestic lawn and pasture.

In the Cape, Bermuda grass pollinates from November to March; in the warmer regions of the United States it is known to pollinate throughout the year. The inflorescence consists of tiny clusters of flowers carried on four or five short branches that arise at the termination of the main stem. The pollen occurs in anthers shaped like pea pods. These burst in the early hours of the morning, allowing the pollen to escape and to be disseminated by the wind. Early morning pollination may account for the fact that a number of allergic patients complain of exacerbation of their symptoms at that time of day. The pollen grain (Figure 1.2) is approximately 35 μ in diameter and is similar in structure to other grass pollens. The exine is smooth and there is only one germinal pore covered with an operculum.

Although Bermuda grass pollen is referred to in the literature as the source of clinically important allergenic material (Vaughan

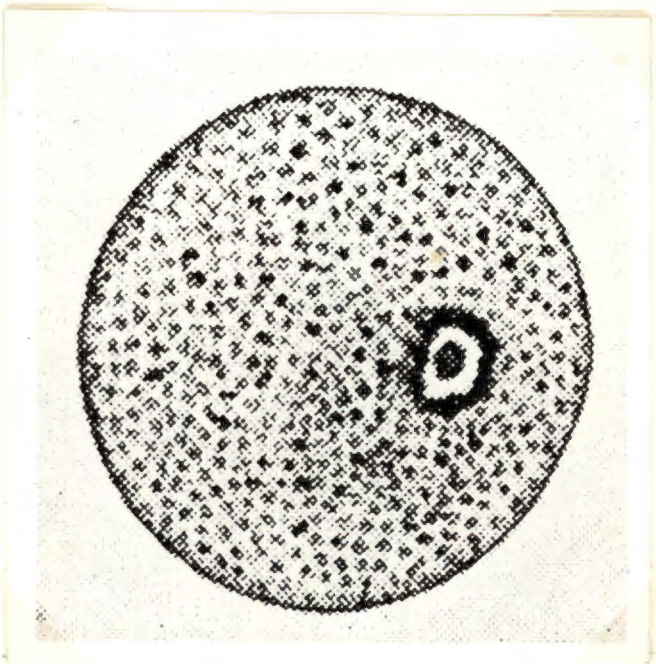


Figure 1.2

Bermuda grass pollen. The single germinal pore is visible in the photograph.

and Black (1948), Solomon, Durham and McKay (1967), Meyers, Berns-Mason, Thayer, Feldman and Rosengren (1971), no attempts appear to have been made to purify or characterize the offending substances. Little more is known of the immunochemistry of these substances than that there is little antigenic cross-reaction between Bermuda grass antigens and other grass antigens (Wodehouse (1955), Augustin (1959b), Marsh, Haddad and Campbell (1970)) and that a "hapten-like" dialysable substance can be detected in Bermuda grass pollen extract (Meyers et al (1971), Meyers, Berns-Mason, Thayer, Seda, Tokrisna and Caldwell (1972)). I felt, therefore, that purification and characterization of the whole Bermuda grass pollen antigens would fill a gap in our knowledge of the immunochemistry of pollen antigens.

My own experience (Chapter 3 of this thesis) has confirmed the importance of Bermuda grass pollen components as allergens in humans in as much as 16% of 268 randomly selected blood donors in the Western Cape showed cutaneous hypersensitivity to an extract of the material. In view of this I felt that a preparation of a purified Bermuda grass pollen antigen would provide a valuable tool for the study of allergy in this part of the world. At the commencement of this work, I hoped to be able to label a purified preparation with radioactive iodine in order to investigate, both *in vivo* and *in vitro*, the role that a specific antigen played in initiating a hypersensitivity reaction. This latter hope was unfortunately unfulfilled because I was unable to prepare an unaltered, immuno-reactive labelled antigen.

There is some confusion as to the precise meaning of some of the terms used by workers in the field of hypersensitivity. To avoid such confusion I will define here the sense in which I have used certain words. In doing so I am not implying that these terms cannot

be used with broader or different meanings.

Immuno-reactive is the term used to describe a substance in relation to a given *in vivo* or *in vitro* system, and indicates that the substance participates in an immune reaction in that system. It, therefore, indicates that the system contains antibodies or sensitized cells that react with the substance. In the context of this chapter the word *immuno-reactive* has generally been used to denote that a substance is capable of combining with skin sensitizing antibodies and so of eliciting a positive skin test in a given individual.

The term *reactive* when applied to a substance is a shortened form of the word immuno-reactive and it has the same meaning. When the term is applied to a human or animal subject, it is used in relation to a given substance and indicates that the subject will react with an immune reaction to that substance.

Immunogen is the term applied to a substance capable of eliciting an immune response. Such a substance is said to be *immunogenic*.

Allergen is the term applied to an immunogen that is capable of inducing the formation of antibodies that sensitize mast cells and basophils. Sensitized cells when exposed to an appropriate antigen will react with a Type I immediate hypersensitivity reaction *in vivo* or with degranulation and histamine release *in vitro*. Many heterogeneous substances such as pollens are known to be *allergenic*; that is they elicit reaginic antibody formation in certain subjects who are environmentally exposed. The antibodies formed are directed against certain specific antigens contained in that substance. The specific requirements for a substance to be allergenic are at present unknown, nor do we know whether the constituents of heterogeneous allergenic substances

retain the same degree of allergenicity when they are isolated from their parent substances. Therefore, although these heterogeneous substances contain several antigens and may contain several distinct *allergens* it is both convenient and scientifically justified to refer to these substances as *allergens* and I will adhere to this practice.

Antigen is the term applied to a substance that is capable of binding with specific antibodies or reacting with sensitized cells. The word *antigen* is often used to describe a substance that is capable of eliciting an immune response - in other words, no distinction is drawn between the terms immunogen and antigen. The state of immunological knowledge is now such that distinction between the meanings of these terms is undoubtedly warranted, and I feel that failure to restrict the meaning of the word antigen diminishes clarity and precision. Unfortunately, in the context of allergy there is no term in conventional usage that denotes specifically an antigen that elicits a Type I immediate hypersensitivity response *in vivo* or mast cell degranulation and histamine release *in vitro*. The term *allergen* is frequently used to denote this type of antigen but its use in this context results in a loss of distinction between immuno-reactive and immunogenic substances in the field of allergy. Therefore, I have elected to use the word *allergen* only in the restricted sense (that is to denote a particular type of immunogen) and to use the word *antigen* to denote an immuno-reactive substance in any immunological system. As I only investigated the constituents of Bermuda grass pollen in terms of their immuno-reactivity and not their immunogenicity, these pollen constituents will be referred to as antigens and not as allergens. Further work is required to establish whether those substances that I found to be immuno-reactive are the ones that elicit

the immune response in atopic subjects who are exposed to the pollen.

Antigenic determinant is the term applied to that part of an antigen that specifically binds with the antigen binding site present on an antibody or cell bound antigen receptor.

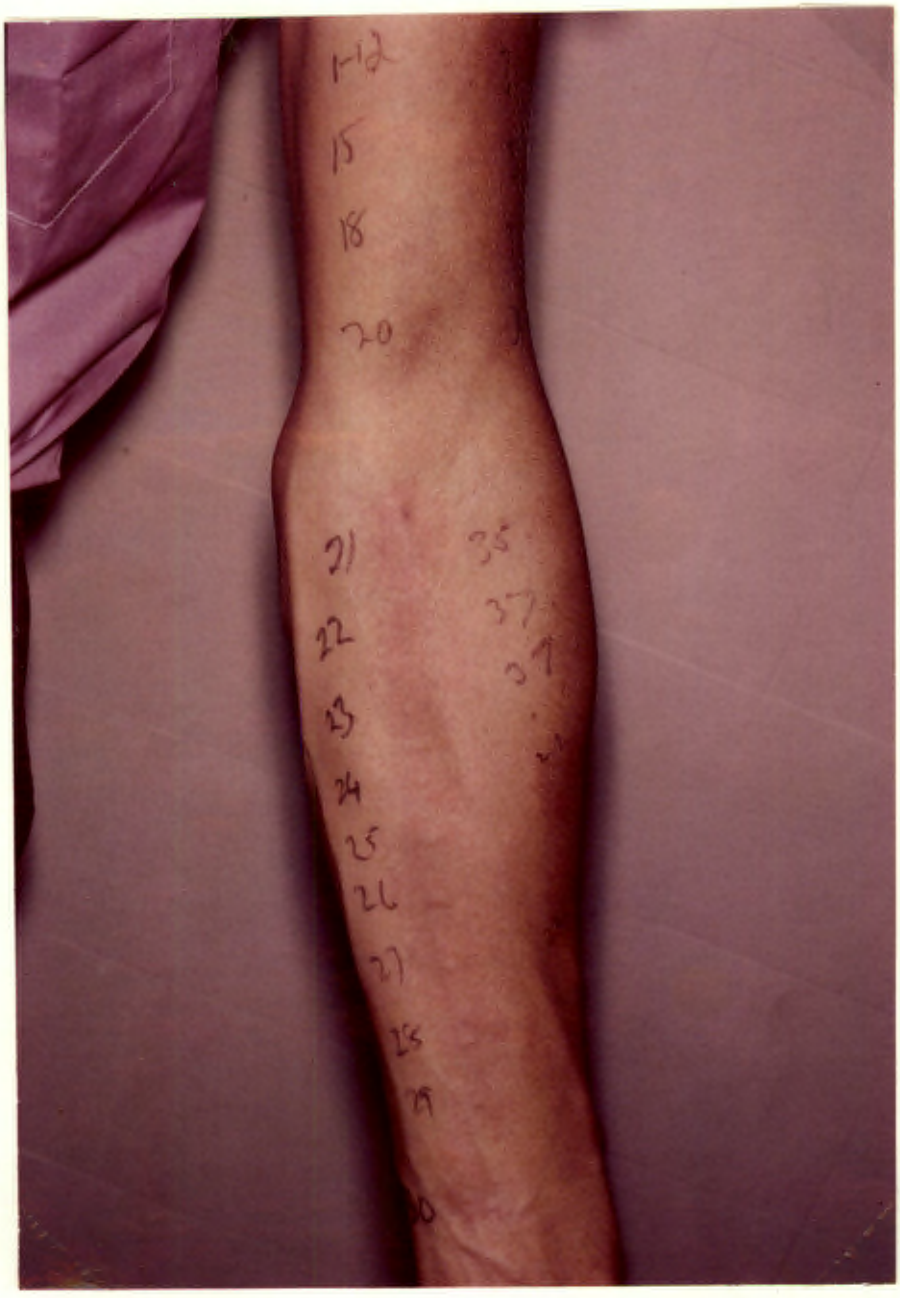


Figure 1.3

The prick test. Photograph of the response shown by a Bermuda grass sensitive subject to partially purified fractions of the extract.

1.2 Materials and Methods

1.2.a. Test subjects

The only way I had of establishing the presence of antigenic material in analytical or preparative samples was the use of these samples in the conventional skin prick test; therefore it was necessary for me to secure the co-operation of allergic subjects. For this purpose 29 subjects were selected, all of whom showed immediate cutaneous hypersensitivity to crude Bermuda grass pollen extract, and most of whom were sensitive to other common allergens. All had symptoms suggesting mucous membrane or bronchial allergy, and most gave a history of seasonal exacerbation characteristic of inhalant pollen allergy. Some of the subjects had received "desensitization" therapy but none were on antihistaminic drugs at the time of testing. Informed consent was obtained from all subjects. No untoward reaction was observed to any test.

In some experiments skin tests were done using solutions containing tracer amounts of ^{131}I or ^{125}I. Subjects tested with these solutions received intradermally not more than 3×10^{-4} nC of either, representing the infinitesimal maximal dose of approximately 3×10^{-4} millirads to the thyroid.

1.2.b. Skin testing

The presence of antigenic material was detected by direct skin testing using the prick test method (Figure 1.3). In performing or interpreting prick tests a number of factors should be considered. These are discussed in detail in Appendix I and will not be considered further at this stage, beyond stating that the various shortcomings inherent in the technique have been taken into account and have not substantially compromised the validity of the conclusions I wish to draw in this section.

Results were graded according to the diameter of the wheal and flare reaction, whichever was greatest, as follows:-

	Wheal	Flare
0	< 3mm	< 5mm
+	≥ 3mm, < 5mm	≥ 5mm, < 10mm.
++	≥ 5mm, < 8mm	≥ 10mm, < 20mm.
+++	≥ 8mm, < 10mm	≥ 20mm, < 26mm.
++++	≥ 10mm.	≥ 26mm.

Control tests to buffer solution were included. If it occurred at all, the transient wheal and flare reaction of the non-specific "triple-response" to skin trauma had subsided to less than a + reaction within 20 minutes of challenge and did not interfere with the interpretation of the specific response.

Prausnitz-Küstner (P-K) tests were performed by injecting 50 µl of neat or diluted reaginic serum intradermally into a non-sensitive individual and testing the site 24 hours later by means of a prick test. The reaginic serum used for the P-K tests came from a blood donor who had no history suggestive of hepatitis and who was known to be hepatitis associated antigen negative. Serum samples were sterilized by millipore filtration (Millipore, Bedford, Massachusetts). P-K reactions were graded in the same way as direct skin test reactions.

1.2.c. Bermuda grass pollen extract.

Crude Bermuda grass pollen extract was obtained through the kind courtesy of Dr. Milner of the Bencard Allergy Unit, Worthing, England. The extract was supplied as an aqueous solution containing 0.45% NaCl, 50% glycerol and 0.5% phenol. The majority of fractionation experiments

were carried out using a "6%" extract, but in some preliminary experiments the "2.5%" extract intended for prick testing was used. The percentages quoted here are those given by the manufacturers and refer to the weight of the pollen originally used to prepare the extract. One millilitre of the 6% extract yielded approximately 50 μg protein of the major antigenic fraction after dialysis, Sephadex G100 and DEAE cellulose chromatography. As the extract was available in only very limited quantities this low yield of partially purified material meant that only limited studies could be performed on the antigens.

Experiments were performed to determine the best method for storing the solutions after fractionation. A purified sample was divided into aliquots, and these were stored at -10°C in buffer alone or with 20% sucrose or 1% human serum albumin (HSA, Miles Seravac, Cape Town) added to the buffer. Initial skin reactivity and reactivity after one and two months storage were determined on one sensitive individual, JS. Results indicated that there was loss of skin reactivity with time but this loss was minimized if the sample was kept in 20% sucrose or 1% HSA. As sucrose is readily removable by dialysis all samples were stored in 20% sucrose at -10°C.

1.2.d. Preliminary purification of crude pollen extract

In the early experiments Sephadex G25 chromatography was used to remove the glycerol and phenol present in the pollen extract, and to equilibrate with the appropriate buffer for subsequent chromatography. Sephadex G25 medium (Pharmacia Fine Chemicals, Uppsala, Sweden) was equilibrated with 0.1M sodium phosphate buffer pH 8 and used to pack a small column 1 x 20 cm. The sample was applied to the column,

and eluted with the same buffer at a flow rate of 40 ml/hr. Transmission at 280 nm of the eluate was monitored continuously with an LKB Uvicord. Chromatography of the crude extract showed two distinct peaks of absorbance. Skin tests showed that the majority of antigenic activity eluted with the first peak. The fractions in the second peak elicited only very small skin reactions, and the absorbance at 280 nm was no doubt due to the presence of the phenol.

Sephadex G25 chromatography resulted in some dilution of the sample; therefore I decided to attempt to use dialysis for equilibration. Dialysis tubing size 8/32 (Union Carbide Co., Chicago, U.S.A.) was prepared for use by washing it in 1% sodium bicarbonate and distilled water. While Sephadex G25 chromatography will fractionate molecules down to a molecular weight of approximately 1000, molecules with a molecular weight of approximately 10,000 can pass through a dialysis membrane. Therefore in order to determine whether the first Sephadex G25 chromatography peak contained dialysable antigens, 6 ml of the 2.5% Bermuda grass pollen extract was passed through the Sephadex G25 column and the first peak dialysed against 50 ml of 0.1M sodium phosphate buffer pH 8 for 72 hours. The non-dialysable contents of the bag gave a +++ reaction on testing a sensitive subject while the dialysable material that had passed through the membrane (diffusate) elicited no reaction. On the basis of this result equilibration of samples with starting buffer was carried out by dialysing against the buffer for at least 24 hours and with at least three buffer changes. When necessary samples were concentrated by negative pressure dialysis using the same type of dialysis tubing.

These experiments did not prove conclusively that dialysable material was non-reactive in all Bermuda grass sensitive subjects. To do this it would have been necessary to test a large number of such subjects. Furthermore, potentially immuno-reactive material with a molecular weight of less than 1000 may have been removed together with the phenol and glycerol during Sephadex G25 chromatography. Therefore, for one set of experiments the 6% extract was fractionated by Sephadex G100 chromatography without preliminary purification. This had the disadvantage that the antigens were loaded on to the Sephadex G100 column in the glycerol phenol solution, but the advantage that all potential antigens present in the extract were available for skin testing.

1.2.e Quantitative Column Chromatography.

For fractionation of the pollen extract a column 1 x 150 cm was siliconized, packed with Sephadex G100 (Pharmacia Fine Chemicals, Uppsala, Sweden), equilibrated with 0.005M sodium phosphate buffer pH8 and run at 4°C with a flow rate of 4 ml per hour. This buffer was chosen because it was the starting buffer for subsequent Di-ethyl-aminoethyl (DEAE) cellulose chromatography and its use meant that minimal sample handling was required. This was important since, as discussed later, antigens were labile even under mild conditions. Andrews (1970) recommends that if buffers of low ionic strength are used with a Sephadex column, such a column should be used for analytical work only after saturation of any binding sites for the substances to be chromatographed. This was accomplished by running markers and pollen extract through the column before the analytical experiments were started.

For all analytical experiments the pollen extract was dialysed against starting buffer before chromatography. For the experiments designed to achieve fractionation of the complete extract, I felt that dialysis might result in the loss of low molecular weight antigens and it was, therefore, omitted. In these experiments a buffer of higher ionic strength (0.05M phosphate) was used to minimize the adverse effects that the glycerol might have on the chromatographic resolution.

For estimation of the Stokes radii, diffusion coefficients, and molecular weights of the antigens, the column was calibrated with markers with known physical characteristics. Markers used were 50 mg HSA, 10 mg horse skeletal muscle myoglobin, and 10 mg horse heart cytochrome C (both from Miles Seravac, Cape Town). The void volume of the column was determined with 10 mg Blue Dextran 2000 (Pharmacia Fine Chemicals, Uppsala, Sweden), and the elution volume at which fractionation of small molecules ceased was obtained by measuring the elution volume of tyrosine.

Samples were loaded in a volume of 2 ml or less. The presence of marker substances in the eluate was detected by measuring transmission at 280 nm with an LKB Uvicord. Using an automatic fraction collector 2 ml fractions were collected into siliconized glass test tubes.

1.2.f. Ion exchange cellulose chromatography.

In order to determine the best conditions for ion exchange cellulose chromatography preliminary electrophoresis and batch experiments were performed.

Crude pollen extract was electrophoresed for three hours on cellulose acetate strips using 0.05 M barbitone buffer pH 8.6 and a constant current of 5 mA. The cellulose acetate strips were cut into sections and they were eluted with 0.1M sodium phosphate buffer pH8. Skin tests results showed that the major antigens behaved as anions under these conditions.

Batch experiments were carried out with DEAE cellulose and carboxymethyl (CME) cellulose (Whatmans, Kent, England). The ion exchange celluloses were equilibrated with 0.005M sodium phosphate buffer pH 7. Pollen extract, after having been partially purified on the Sephadex G100, was concentrated to 38 µg/ml and dialysed against the same buffer. Under these conditions no protein or antigen bound to the CME but most of the protein and all of the immuno-reactive material bound to DEAE. The major antigen was preferentially eluted with 0.05M sodium phosphate buffer pH 8, whereas the majority of the protein came off at a higher molarity.

As a result of these experiments the major antigen containing peak from Sephadex G100 chromatography was fractionated on a 1.5 x 40 cm Whatmans column (Whatmans, Kent, England) packed with DEAE cellulose equilibrated with 0.005M sodium phosphate buffer pH 8. The sample had been equilibrated with the same buffer prior to Sephadex G100 chromatography. The column was developed at $4^{\circ}C$ using a linear gradient from 0.005 M to 0.2 M sodium phosphate buffer pH 8.0 (150 ml of each). Fractions of approximately 5 ml were collected in siliconised glass test tubes. The molarities of the fractions were ascertained by determining their sodium concentration with a Perkin Elmer Model 303 atomic absorption spectrometer. This was done by the kind courtesy of the Geochemistry Department,

University of Cape Town. Immuno-reactive fractions were detected by skin testing; the major antigen was eluted with a buffer of about 0.05 M.

1.2.g Protein determination.

Protein concentrations were determined by the method of Lowry, Rosebough, Farr and Randall (1951). This is a colourimetric technique that depends on the tyrosine and tryptophane contents of protein for colour development; therefore, as the amino-acid content of the pollen protein was unknown, the validity of the method was checked by determining the total nitrogen contents of a control sample of crude dialysed extract. Total nitrogen was determined by Kjeldahal digestion, followed by ammonia titration using Conway micro-diffusion units. Using a factor of 6.2 to convert micrograms nitrogen to micrograms protein, I found the protein content of the sample to be 142 µg/ml. This compared very favourably with the result, 141 µg/ml, obtained by the Lowry method, and therefore the Lowry method was used for all subsequent determinations.

All pollen antigens so far studied have been shown to consist mainly of protein (Herbertson, Porath and Colldahl (1958), Augustin (1959c), King, Norman and Connell (1964), Johnson and Marsh (1966a)) therefore I have made the assumptions that Bermuda grass antigens contain protein and that at least some of the protein detected represented antigenic material.

1.2.h. Labelling with radioactive iodine.

Protein iodination is discussed in Appendix II where details of the methods used are given. The method used for labelling the purified antigenic material was an adaption of Hunter and Greenwood's chloramine T method (Hunter and Greenwood (1962)). This procedure

subjects the sample to oxidation and reduction and it was found that the iodinated material tended to disintegrate into fragments under these conditions. Therefore, labelling of the antigenic material using the iodine monochloride method (McFarlane (1958)) was attempted; however this method resulted in extremely poor iodination and the labelled material still tended to disintegrate. The iodine monochloride method was successfully used for the trace labelling of HSA.

1.2.i. Ultracentrifugation in sucrose density gradients

Sucrose density gradient ultracentrifugation was used to estimate the sedimentation coefficient in water at 20°C ($S_{20,w}$) of the major antigen.

Five to twenty percent W/V sucrose gradients in 0.05M sodium phosphate buffer pH 7.5 were prepared in 4.4 ml Beckman cellulose nitrate tubes; the final depth of the gradients from tip to meniscus was 3.86 cm. The tubes were allowed to stand for 2 hours at 4°C before the samples (in 150 µl buffer) were layered on top. Tubes were centrifuged at 4°C in a No. 39 SW rotor in a Beckman Model L centrifuge for 16 hours at 35,000 rpm (145,000 x g). The tubes were drained by puncturing the bottoms and collecting fractions of 8 drops (approximately 150 µl) each. Assays for the presence of markers and antigens were then carried out on the fractions.

Protein markers of known $S_{20,w}$ were spun simultaneously with the sample. Two hundred µg yeast alcohol dehydrogenase (Sigma Chemical Co., St. Louis, U.S.A.) in 0.05M Tris-HCl buffer pH 7.5 was used as a heavy marker. Its presence was detected by measuring the rate of increase in absorption at 340 nm of a 1 ml reaction mixture containing 170 µmoles ethanol, 50 µmoles Tris pH 8.5, 15 µmoles NAD^+ and 5 to 20 µl test fraction. ^{131}I labelled HSA

(0.2 to 0.5 μC) was used as another heavy marker. Radioactivity in the fractions was detected using a Packard Autogamma scintillation detector. Markers lighter than the major antigen were 200 μg horse heart cytochrome C and 200 μg horse skeletal muscle myoglobin. They were not used together as they have nearly identical sedimentation coefficients. Their presence was detected by measuring absorption at 400 nm. Fractions containing myoglobin or cytochrome C tended to overlap with those containing the major antigen, and therefore these light markers were not spun in the same tube as the unknown. The difficulty of overlapping of samples was overcome by spinning, concurrently, one tube with the unknown and the heavy markers and one tube with heavy and light markers. The distance between the skin reactivity peak and the heavy markers in the first tube was used to calculate the distance the antigen would have been expected to move in the gradient of the second tube. Using this method markers with similar sedimentation coefficients to the antigens could be used.

1.2.j. Iso-electric focussing

Iso-electric focussing was performed on a 110 ml capacity LKB iso-electric focussing column. The LKB gradient former manufactured for use with the column was used to establish a 0 - 50% sucrose density gradient in the column. Ampholyte solution (L.K.B. Produkter AB, Sweden) was incorporated into the gradient at a final concentration of 1%. After dialysis overnight against 1% glycine the sample was

incorporated into the gradient by substituting it for an equal volume of the light solution. The cathode solution at the bottom of the column was 0.4 ml ethanolamine and 12 g sucrose in 14 ml distilled water. The anode solution at the top was 1% phosphoric acid.

Three hundred volts were applied across the column for about 48 hours after which time the current stabilized. The column was then drained and 4.5 ml fractions were collected. The pH of each was measured immediately using a Radiometer pH meter 26.

Fractions were dialysed individually for 36 hours against four changes of 0.005 M sodium phosphate buffer pH 8, with the fourth buffer change containing 20% sucrose. Dialysed fractions were used for skin testing.

1.2.k Reagents and Glassware.

All reagents used for the preparation of buffers and other working solutions were ANALAR grade chemicals and were obtained from commercial suppliers. They were not further purified.

As it has been shown that some grass antigens adsorb to glass (Malley, Reed and Leitz (1962)) all glassware that was used for storing or handling the pollen extract was siliconized.

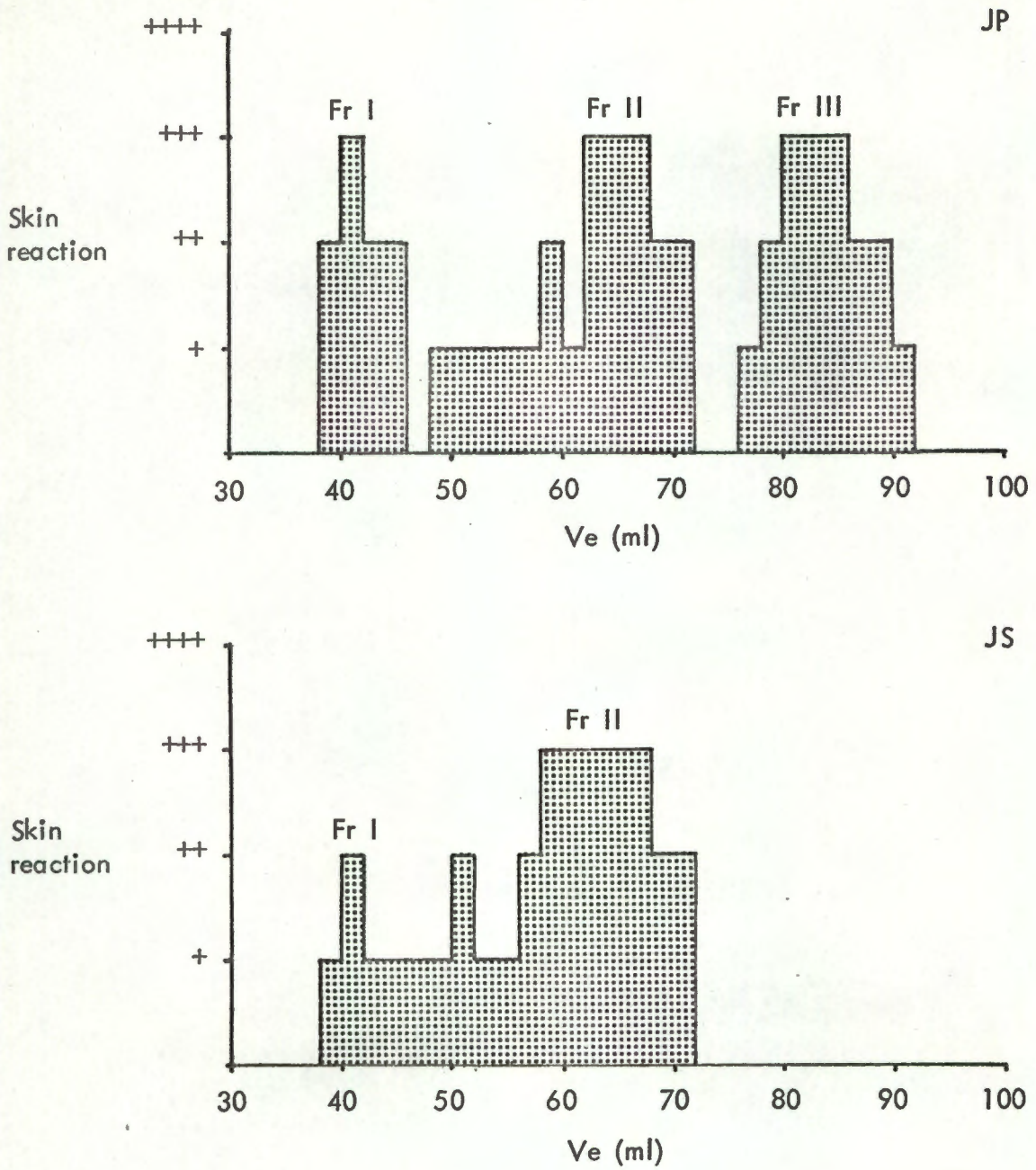


Figure 1.4

Sephadex G100 chromatography.
Elution profiles of skin reactivity of the antigens present Bermuda grass pollen extract. The figure shows the results of testing one set of chromatography fractions on two Bermuda grass pollen sensitive individuals, JP and JS.

1.3 Results

1.3.a. Identification of major and minor antigens in Bermuda grass pollen and determination of the pattern of sensitivity to these antigens.

Dialysed Bermuda grass pollen extract was fractionated by means of Sephadex G100 chromatography and DEAE cellulose chromatography. In order to ascertain the pattern of sensitivity to the different components so obtained the fractions were tested on members of the panel of Bermuda grass sensitive subjects.

For chromatography on the 1 x 150 cm Sephadex G100 column a maximum of either 6 ml of the 2.5% extract or 4 ml of 6% extract was equilibrated with starting buffer and concentrated by negative pressure dialysis to 2 ml or less before application to the column. This represented a protein load of approximately 400 - 600 µg. Two millilitre fractions were collected and used for skin testing. Elution profiles obtained from two of these tests are shown in Figure 1.4. These two subjects were tested on the same day with the same set of fractions. Subject JP showed three clearly separated peaks of reactivity eluting at approximately 40 ml, 65 ml and 85 ml and these will be referred to as Fraction I, Fraction II and Fraction III respectively. Subject JS, like subject JP, showed a large reaction to Fraction II but had a smaller reaction to Fraction I and none to Fraction III. Eight subjects were tested with Sephadex G100 chromatography fractions of the dialysed extract and their results are summarised in Table 1.1.

The results of testing eight other subjects with the chromatographic fractions of undialysed extract are also shown in Table 1.1.

Subject	Skin test result.		
	Fr. I	Fr. II	Fr. III
WA	+	+++	-
SR	-	+++	+
JP	+++	+++	+++
JS	++	+++	-
MS	++	++++	++++
DO	+++	++++	++++
CJ	+++	++++	++++
VS	++	+++	++++
HA*	+	++++	++++
HR*	++	+++	-
CM*	++	++++	+++
AC*	++	++++	++++
PB*	+++	++++	++++
JS*	+++	++++	++++
LN*	++	++	-
DB*	+	++++	++++
Number positive	15	16	12
Number tested	16	16	16

Table 1.1

Skin test reactions obtained on testing sixteen Bermuda grass sensitive individuals with the immuno-reactive material obtained by fractionating crude Bermuda grass pollen extract on Sephadex G100. The grade of skin test result is the maximum result obtained to any fraction of a particular peak.

*Indicates that undialysed extract was used for fractionation.

Subject	Testing solutions	Fr. I	Fr. II	Fr. III
CJ	Dialysed fractions	+++	++++	++++
	Undialysed fractions	++	++++	++++
DO	Dialysed fractions	+++	++++	++++
	Undialysed fractions	+	++++	++++
VS	Dialysed fractions	++	+++	++++
	Undialysed fractions	++++	++++	+++

Table 1.2

Results of testing three subjects with Sephadex G100 chromatography fractions of both dialysed and undialysed crude Bermuda grass pollen extract.

Because of the possibility that loss of some of the smaller antigens occurred during dialysis, the two sets of results shown in Table 1.1 may not have been strictly comparable. I, therefore, tested on three individuals, fractions obtained by chromatography of both dialysed and undialysed extract. Two 500 µl aliquots of 6% extract,only one of which had been dialysed, were chromatographed, and both sets of fractions were used for testing. Results are shown in Table 1.2; while there was some variation in the size of reactions to Fraction I, reactions to Fraction II and Fraction III were virtually identical, and the occasional differences observed were well within the error of the method. I, therefore, concluded as far as Fraction II and Fraction III were concerned, that it was acceptable to group together all the results presented in Table 1.1.

Skin tests in some subjects showed broader peaks of reactivity than others and this was possibly due to the presence of additional minor antigens to which some subjects were sensitive. Thus Fraction I, Fraction II and Fraction III do not necessarily represent all the antigenic material in the extract but they do contain the most commonly reactive antigens.

Fraction I eluted with the approximate void volume of the column and its activity may have been due to polymerization of low molecular weight antigens. King et al (1964) have demonstrated that Ragweed Antigen E tends to undergo polymer formation. All except one of the subjects tested gave some reaction to Fraction I, but reactions to Fraction I were almost invariably smaller than those to Fraction II.

Fraction II was reactive in all individuals tested and almost always elicited a response at least as great as that obtained with any other fraction.

Fraction III elicited a response in 12 out of 16 subjects. The reactions to Fraction III, when they did occur, tended to be as large as those to Fraction II. The apparent failure of four subjects to react to Fraction III may have been absolute or relative, inasmuch as, in the latter case, the concentration of the antigen in Fraction III may have been inadequate to elicit a response in some subjects. Paucity of material precluded any attempts to investigate this further, by challenging the unresponsive subjects with higher and varying concentration of Fraction III.

Whatever the explanation, the data in Table 1.1 indicate quite clearly that Bermuda grass-sensitive subjects differ in their spectrum of reactivity to the component antigens and that Fraction II in the present series, can be considered the major antigenic fraction.

Fraction II was chromatographed on DEAE cellulose and the fractions obtained used for skin testing eleven sensitive subjects. The elution profiles obtained with two of these tests are shown in Figure 1.5.

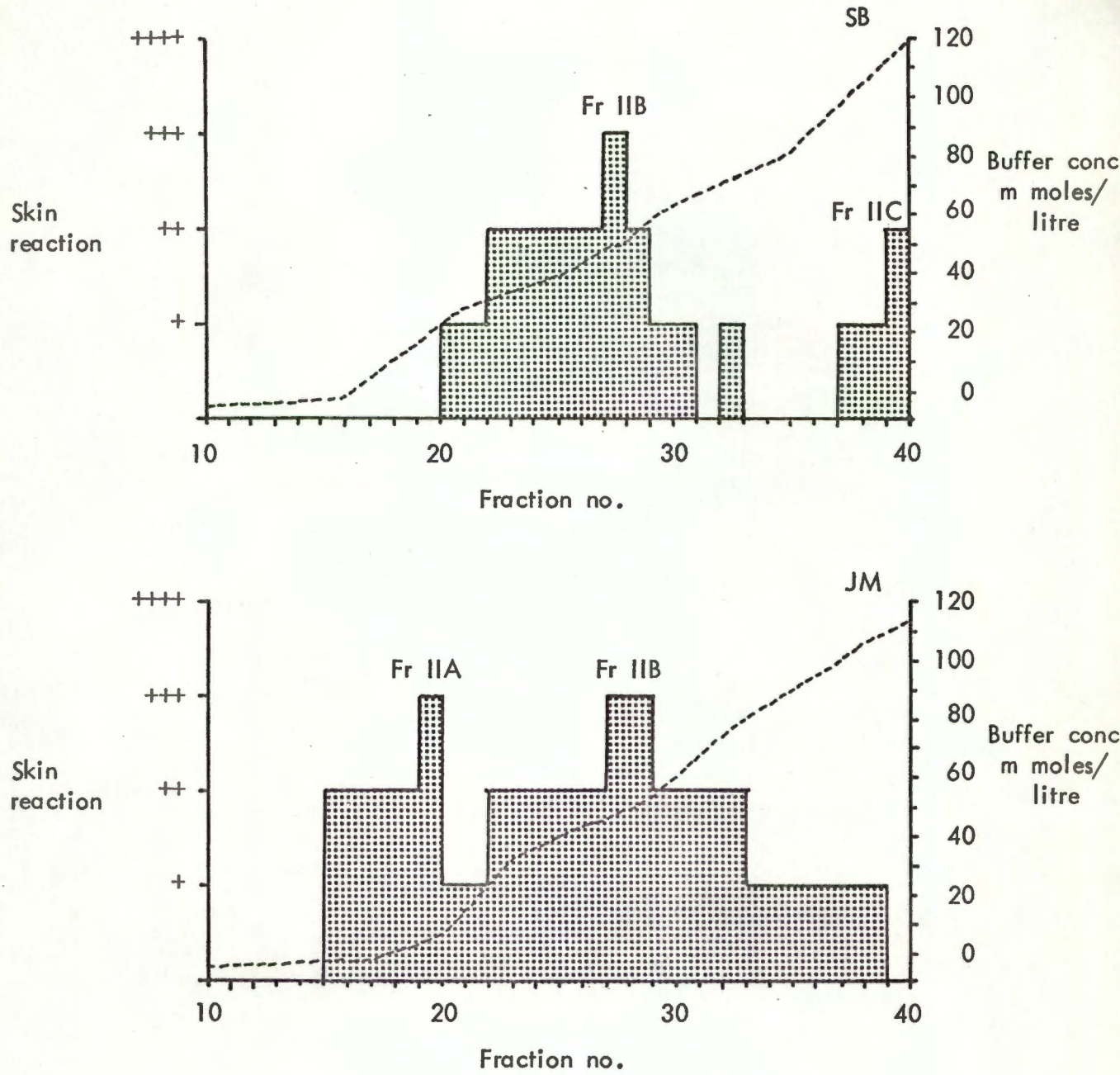


Figure 1.5

DEAE cellulose chromatography.
Elution profiles of skin reactivity and buffer concentration of the samples obtained with an elution gradient of 0.005M - 0.2M phosphate buffer pH 8. Fraction II from Sephadex G100 chromatography was used for these experiments.

------ indicates buffer concentration.

Tests on subject SB showed the presence, in Fraction II, of two definite antigen containing peaks, one eluting with a buffer concentration between 0.04 and 0.08M, and one with a buffer concentration of 0.1M. These two peaks I have called Fraction IIB and Fraction IIC respectively. Some subjects showed reactions to the material eluting between these two peaks. These reactions were so slight and variable that it is doubtful whether they represent the presence of separate antigens and I have chosen to ignore them. Subject JM reacted to Fraction IIB and not to Fraction IIC. She did, however, react in addition to material that eluted from the column with the starting buffer. This antigen containing peak I have called Fraction IIA. Table 1.3 summarises the results of skin testing eleven sensitive subjects with fractions obtained from DEAE chromatography.

All subjects reacted to Fraction IIB whereas only one reacted to Fraction IIA and seven to Fraction IIC. Since not all subjects reacted to Fraction IIA and Fraction IIC, these were regarded as containing minor antigens which were not characterized further.

Fraction IIB was re-chromatographed on Sephadex G100 and the fractions tested on three sensitive individuals. All three showed skin reaction peaks to material eluting with the elution volume of

Subject	Skin test result		
	Fr. IIA	Fr. IIB	Fr. IIC
NP	-	+++	-
NR	-	++++	+++
IM	-	++++	++
SB	-	+++	+
GH	-	+++	++
AB	-	+	-
EW	-	++	-
JS	-	++++	++
ED	-	++++	+
JM	++	+++	-
SS	-	++	++
Number positive	1	11	7
Number tested	11	11	11

Table 1.3

Skin test reactions obtained on testing eleven Bermuda grass sensitive individuals with the immuno-reactive material obtained by fractionating crude Bermuda grass pollen extract on Sephadex G100. The grade of skin test result is the maximum obtained to any particular peak.

Fraction II, and one subject, GC, showed in addition, a peak of reactivity to material having the elution volume of Fraction III. This result was unexpected since the lower molecular weight antigenic material should have been removed from Fraction II by the first passage through the Sephadex G100 column; its presence in Fraction IIB indicated that dissociation of material in Fraction II had occurred.

1.3.b. Investigation of Bermuda grass pollen extract for low molecular weight immuno-reactive components.

Preliminary investigations using the fractions obtained from Sephadex G25 chromatography had indicated that the low molecular weight components of Bermuda grass pollen extract were non-reactive or only weakly reactive on direct skin testing. To investigate the reactivity of these components further, 0.5 - 2 ml of undialysed 6% Bermuda grass pollen extract was fractionated on the 1 x 150 cm Sephadex G100 column. Fractions obtained were tested on eleven sensitive individuals. Some of the results of these tests are shown in Tables 1.1 and 1.2 and have already been discussed. Tables 1.1 and 1.2, however, do not show the results of testing with the low molecular weight fractions. The results of these tests were difficult to interpret because, while six of the eleven subjects did show a positive reaction to some of the low molecular weight fractions, different individuals reacted to different fractions.

In certain circumstances, which vary with the subject being challenged or with the pollen in question, low molecular weight antigenic components may fail to elicit an immediate response, yet reveal their immuno-reactive properties by indirect "inhibition tests". Such indirect tests include inhibition of the precipitin reaction (Malley and Campbell (1963)), inhibition of the direct skin reaction (Marsh, Milner and Johnson (1966)) and inhibition of the P-K reaction (Atallah and Sehon (1969)). If such indirect tests are positive they provide evidence for an immunochemical relationship between

the low molecular weight components concerned and the antigen used in the test system. As indicated in the previous paragraph, some of the allergic subjects in this series were reactive to low molecular weight components of crude pollen extract. In an attempt to investigate the relationship between the immunoreactive low molecular weight components and the antigen in Fraction II, tests for inhibition of both the direct skin test and the P-K test were performed. The experiments failed to reveal the presence of any factors capable of inhibiting either of these tests.

To test for inhibition of the direct skin test I used a Bermuda grass sensitive subject, HR, who was unreactive to any of the low molecular weight components. The low molecular weight fraction examined was obtained by Sephadex G100 chromatography of the undialysed extract and elicited a direct skin reaction in three of the eleven subjects tested. A control solution with equivalent glycerol and phenol concentrations was obtained by fractionating Bencard control prick test solution on the Sephadex G100 column. Fivefold dilutions of Fraction II were made using both the low molecular weight fraction and the control solution. Skin tests were performed in duplicate on HR in such a way that dilutions in the control solution were placed on symmetrical sites on the opposite arm to the equivalent dilutions in the test solution. Squire (1950) has shown that this is the preferential way of performing prick tests with two solutions that are to be compared for relative potency. At all dilutions the reactions to Fraction II diluted in the low molecular weight fraction were as large as those to Fraction II diluted to the control solution.

	Inhibitor				
	None (serum control)	Low molecular weight fraction 1st fraction	Control for 1st fraction	Low molecular weight fraction 2nd fraction	Control for 2nd fraction
Skin reactions	++++	++++	++	+++	+
	++	+++	++	+++	+++
	++	+++	+++	+++	+++

Table 1.4

Results of P-K inhibition studies using two low molecular weight fractions obtained by Sephadex G100 chromatography. All sites were challenged with Fraction II. Control tests on unsensitised sites with Fraction II and buffer were negative.

Tests for blocking of the P-K reaction were performed using two of the fractions obtained by chromatography of the undialysed extract. Both had been shown to be immuno-reactive in some of the individuals tested by direct skin testing. One hundred µl of each fraction or appropriate control was incubated with 200 µl of reaginic serum at 37°C for 1 hour and at room temperature overnight. Fifty µl aliquots of the samples and an undiluted serum control were used to sensitise the skin of a non-sensitive individual; the sites were challenged 24 hours later with Fraction II. Each test was performed in triplicate. The results are shown in Table 1.4 and indicate that neither of the fractions tested inhibited the P-K reaction to Fraction II.

Although the above tests were negative, the possibility remained that certain other fractions contained material capable of blocking a P-K response to Fraction II. As it was impractical to test all the low molecular weight fractions obtained by chromatography, and as all low molecular weight material present in the crude extract was also present in the diffusate of the extract, this diffusate was used in a further experiment to test for blocking of the P-K response. Five hundred µl of 6% Bencard extract and 500 µl of Bencard control were each dialysed for 72 hours against 5 ml of 0.5 M phosphate buffer pH8. Two hundred µl of each diffusate was incubated with 400 µl of reaginic serum at 37°C for one hour and then at room temperature overnight. P-K sites were sensitized with 50 µl aliquots of these mixtures. Sites were challenged 24 hours later with either Fraction II or whole dialysed extract. All tests were performed in duplicate. In each case the size of the reactions at the sites sensitized with the serum plus pollen diffusate were as large as those at the sites sensitized with serum plus control diffusate, indicating that no inhibition of the P-K reaction had taken place.

1.3.c Characterization of the antigens.

The antigens present in Fraction IIB were chosen for further characterization. The potency of Fraction IIB was demonstrated by the fact that less than 1 µg/ml protein could elicit a positive reaction. Squire (1950) has calculated that only 3 nl are injected intra-dermally during a prick test. This means that approximately 10^{-12} g protein from Fraction IIB elicited a positive reaction. The paucity of available material precluded the performance of rigorous experiments to establish the purity of the antigenic material in

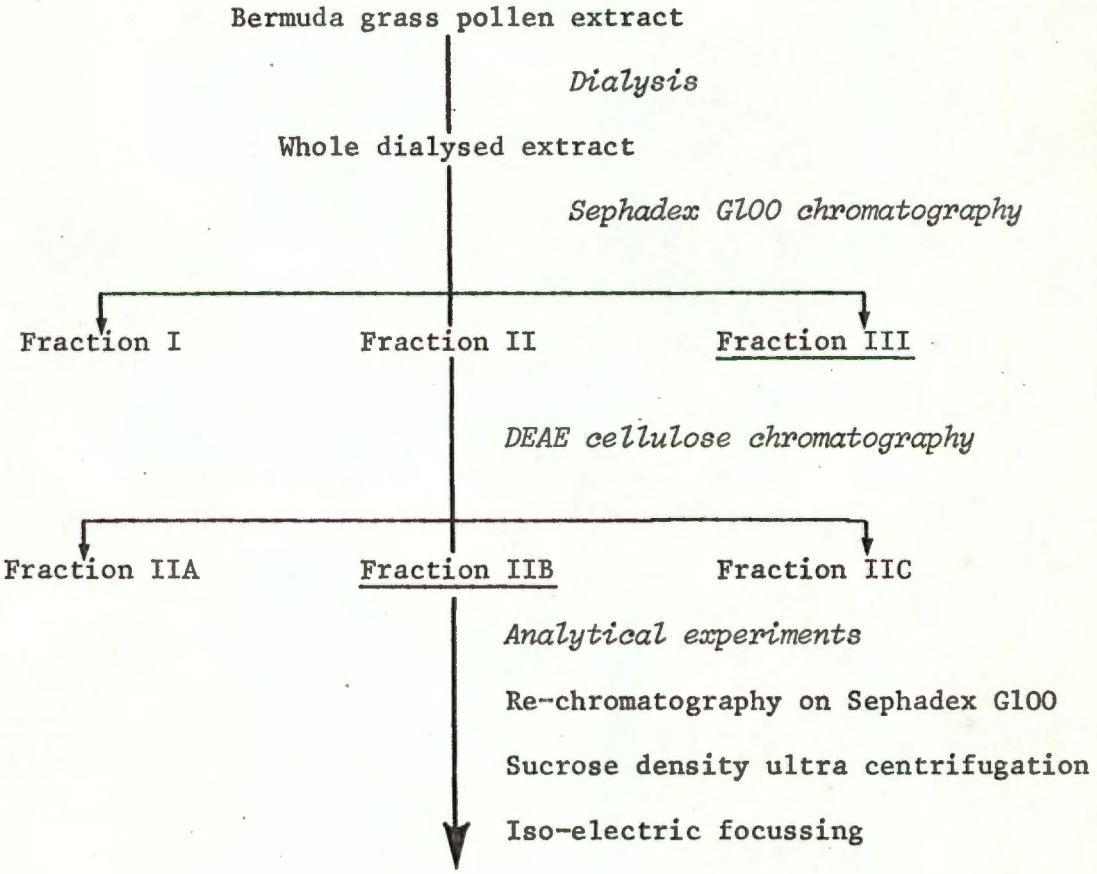


Figure 1.6

Flow diagram of the steps involved in the purification and characterization of the antigens in Bermuda grass pollen. All fractions were immuno-reactive in some individuals but only the antigens in Fraction IIB and Fraction III were chosen for characterizations. These two fractions have been underlined in the figure.

Fraction IIB or attempts to characterize the antigen chemically. However, any antigens present in Fraction IIB were homogeneous by the operational criteria of Sephadex G100 and DEAE cellulose chromatography, and therefore, I have regarded them as belonging to one antigenic group. The antigens present in Fraction III belong to another antigenic group. The steps involved in the purifaction and characterization of these antigens are shown in Figure 1.6.

1.3.c(i) Molecular weight, Stokes radius and diffusion coefficient determinations.

The results of gel filtration through the calibrated Sephadex G100 column were used to estimate the molecular weights, Stokes radii and diffusion coefficients of the antigenic groups in Fraction IIB and Fraction III.

The principles and techniques involved in the use of gel filtration columns for analytical work have been discussed by Fisher (1969) and Andrews (1970). Calibration of a column involves the determination of certain properties of the gel bed as well as the measurements of elution volumes of markers with known physical characteristics. The two characteristics of the column that I have used in the calculations are the void volume and the inner volume.

The void volume (V_o) is the volume of the solvent in the interstitial spaces between the beads in the gel bed. Blue Dextran 2000 (Pharmacia Fine Chemicals) has an average molecular weight of two million and is completely excluded from Sephadex gel beads. This means that its elution volume on a particular column is equal to the void volume of that column.

The inner volume (V_i) is the volume of solvent within the gel pores. This I determined by taking the difference between the elution volume at which separation of small molecules ceases and V_o. The former I determined by measuring the elution volume of tyrosine.

The behaviour of each solute passed through the column was characterized by measuring its elution volume and calculating its distribution coefficient. The elution volume (V_e) of a solute is the volume of eluant that is, on the average, required to carry the

Sample	Molecular Weight Daltons	Stokes radius a cm x 10^{-8}	Elution volume Ve ml	Distribution Coefficient Kd
Blue dextran 2000	$\pm$ 2 x 10^6		36*	-
Tyrosine	< 1000		110+	-
Cytochrome	12,4000	16.4	90	.7297
Myoglobin	17,800	20.7	76	.54054
HSA	69,000	35.5	46	.13514
Immunoreactive peak Fraction II			66	.4054
Fraction IIB			66	.4054
Fraction III			84	.6487

Table 1.5

Calibrated Sephadex G100 chromatography. The table gives the known molecular weights in Stokes radii of the markers and the elution volumes and distribution coefficients of the markers and antigens. These results were used to calculate the molecular weights and Stokes radii of the antigens (see Figure 1.7 and 1.8).

*Vo + Vi = Ve (tyrosine) - Vo

molecules of the solute through the column. The distribution coefficient (Kd) is the fraction of the internal volume, Vi, that is accessible to the solute molecules.

$$Ve = Vo + Kd\ Vi$$

therefore

$$Kd = (Ve - Vo) / Vi$$

The elution volume of the major antigen was obtained by re-chromatographing 75 µg protein from Fraction IIB through the 1 x 150 cm Sephadex G100 column. Immuno-reactive material eluted in a single narrow peak and its elution volume was measured to the nearest fraction (that is to the nearest 2 ml). As expected, this elution

volume was equal to the mean elution volume obtained for Fraction II. Table 1.5 gives the data that I used to characterise the column and the results that I obtained for the elution volumes and distribution coefficients of the antigens.

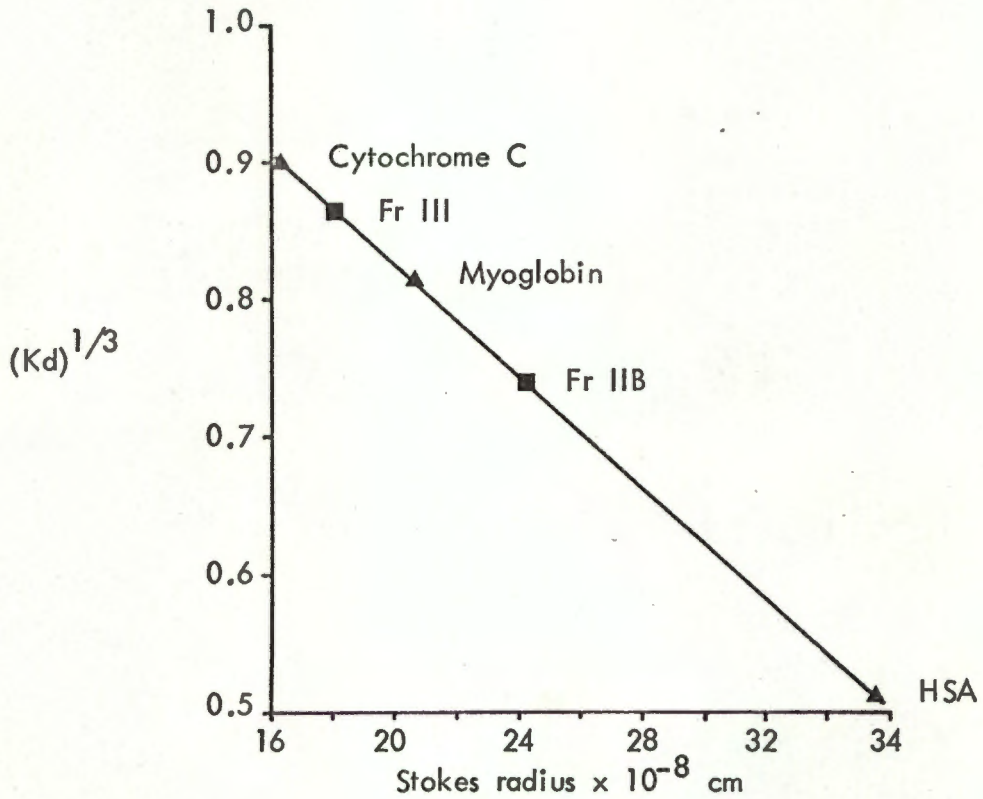


Figure 1.7

Calibrated Sephadex G100 chromatography.
Values of $(Kd)^{1/3}$ for the markers were plotted against the known values for their Stokes radii

▲ represents marker points

■ represents antigen points

From the parameters of the regression line through the marker points the Stokes radii of the antigens were calculated.

Stokes radius major antigen in Fraction IIB 24.32×10^{-8}cm.

Stokes radius antigen group in Fraction III 18.13×10^{-8}cm.

Porath (1963) has described the relationship

$(Kd)^{1/3} = n - ma$

where n and m are constants

and a is the Stokes radius.

The values of m and n were obtained by plotting the $(Kd)^{1/3}$ for each marker against its Stokes radius (Figure 1.7) and calculating the parameters (m and n) of the least squares line through the points. Using m and n the values of the Stokes radii of the antigens were calculated.

The diffusion coefficients were calculated from the values of the Stokes radii using the formula

$$D = \frac{KT}{6\Pi\eta a}$$

where D = diffusion coefficient

a = Stokes radius

T = absolute temperature

K = Boltzmans constant (1.386×10^{-16} erg/degree)

η = coefficient of viscosity of the medium

(1.00 centipoise for water or dilute aqueous solutions).

The diffusion coefficients for the antigenic groups in Fraction IIB and Fraction III were found to be $8.86 \times 10^{-7} cm^2\ sec^{-1}$ and $11.88 \times 10^{-7} cm^2\ sec^{-1}$ respectively.

The molecular weights of the antigens were estimated from their elution volumes. Granath and Flodin (1961), and Andrews (1962) have shown empirically that there is an approximately linear relationship between the molecular weights of macro-molecules and their elution volumes on a given gel filtration column. This relationship holds good for substances that have elution volumes that occur in the middle region of the working range of the column. The Bermuda grass pollen antigens were found to have elution volumes in this range on the Sephadex G100

column and therefore this column was suitable for estimating their molecular weights. The elution volume for each marker was plotted against the natural logarithm of its molecular weight and a least squares regression line was fitted through the points (Figure 1.8). The parameters of the line were used to calculate the approximate molecular weights of the antigens.

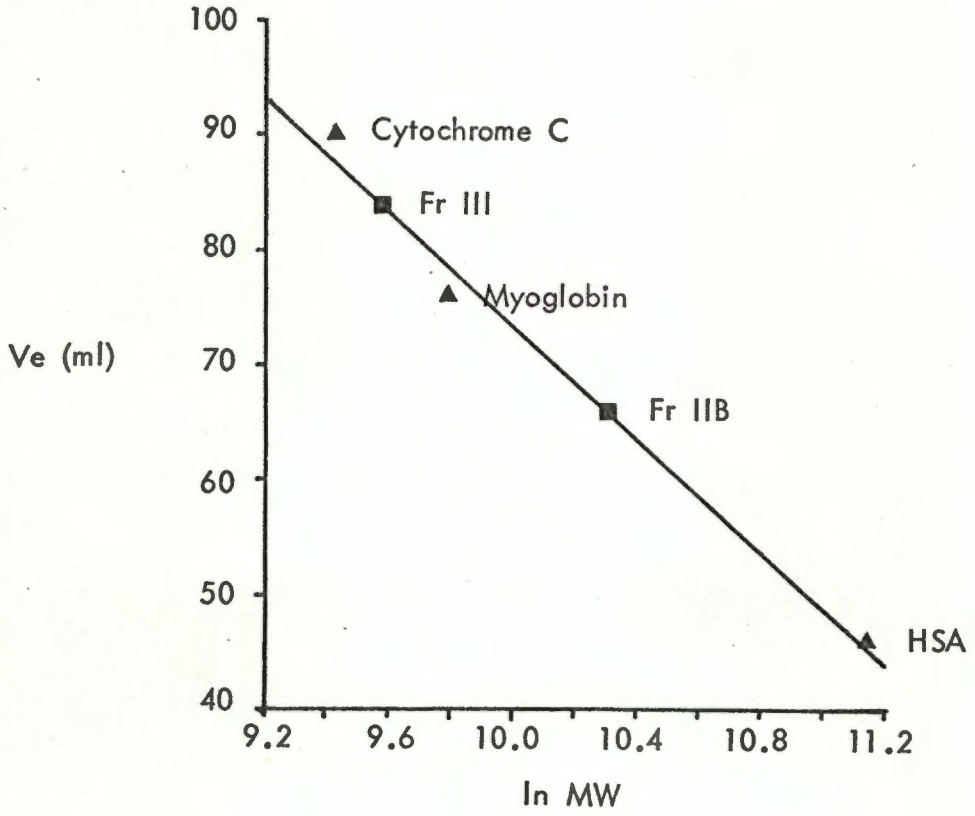


Figure 1.8

Calibrated Sephadex G100 chromatography.
Values of Ve for the markers were plotted against the natural logarithm of the known molecular weights (MW).
▲ represents marker points ■ represents antigen points.
From the parameters of the regression line through the marker points the MW of the antigens were estimated.
MW major antigen in Fraction IIB 29,950 Daltons
MW antigenic group in Fraction III 14,440 Daltons

From its theory, it is to be expected that gel filtration would be a more accurate method for the determination of molecular size than molecular weight. Unfortunately, owing to lack of material, the partial specific volumes of the antigens could not be determined. Therefore, although both the Stokes radii and the sedimentation coefficients were known, I could not calculate the molecular weights using the formula

$$M = 6\Pi\eta \; Na \; S \; (1 - \nu\rho)$$

where S = sedimentation coefficient

ν = partial specific volume

ρ = density of the medium

η = coefficient of viscosity of the medium.

The values for the physical characteristics of the antigens were as follows:

The major antigenic group in Fraction IIB.

Molecular weight	30,000 Daltons (to the nearest 100)
Stokes radius	24.32×10^{-8} cm
Diffusion coefficient	8.86×10^{-7} cm^2 sec^{-1}

The antigenic group in Fraction III.

Molecular weight	14,400 Daltons (to the nearest 100)
Stokes radius	18.13×10^{-8} cm
Diffusion coefficient	11.88×10^{-7} cm^2 sec^{-1}

1.3.c (ii) Sedimentation coefficient determination.

The sedimentation coefficient in water at 20°C ($S_{20,w}$) of the major antigenic group was determined by sucrose density ultracentrifugation.

Martin and Ames (1961) developed this method for the determination of the $S_{20,w}$ of a substance present in a solution in an impure form. The principle of the method is based on the relationship

$$\omega^2 S_{20,w}\, dt = \frac{(\rho_p - \rho_{20,w})\,\eta_{T,m}}{(\rho_p - \rho_{T,m})\,\eta_{20,w}} \cdot \frac{1}{x}\, dx \qquad (1)$$

where ω = angular velocity of the rotor in radians per second

x = distance from the rotor centre to the boundary

dx/dt = velocity of movement of the boundary

$\eta_{T,m}$ = viscosity of the medium at the temperature of centrifugation

$\eta_{20,w}$ = viscosity of water at 20°C

ρ_p = density of the unknown (reciprocal of the partial specific volume)

$\rho_{T,m}$ = density of the medium at the temperature of centrifugation

$\rho_{20,w}$ = density of water at 20°C

Therefore, if the partial specific volume of a substance is known its $S_{20,w}$ can be calculated directly from the distance it travels into a gradient under very carefully controlled conditions. The use of this equation, however, not only involves numerical integration of the right hand side of the equation but it also means that the angular velocity and temperature of centrifugation must be very accurately measured. Such measurements are subject to machine error. In the same paper Martin and Ames showed that the $S_{20,w}$ could be

measured more readily by spinning, concurrently, a standard of known $S_{20,w}$ and using the equation

$$\frac{\text{Distance travelled by the unknown from the meniscus}}{\text{Distance travelled by standard from the meniscus}} = \frac{S_{20,w}\ \text{unknown}}{S_{20,w}\ \text{standard}} \quad (2)$$

to calculate the $S_{20,w}$ of the unknown.
This was the method I used for the determination of the major antigenic group.

Equation (1) indicates that there is a relationship between the $S_{20,w}$ of a substance and its partial specific volume. The use of Equation (2) assumes that standards and unknowns all have the same partial specific volumes. If they are all proteins this assumption is fairly valid, as most proteins have a partial specific volume of 0.700 - 0.750 $cm^3\ g^{-1}$ (Edsall (1953)); Martin and Ames (1961) have indicated that the assumption of a partial specific volume of 0.725 $cm^3\ g^{-1}$ in equation (1) gives rise to only a 3% error for the $S_{20,w}$ of most proteins. Knowing that pollen antigens have been shown to consist mainly of protein (Herbertson et al (1958), Augustin (1959c), King et al (1964), Johnson and Marsh 1966a and b)), I assumed that the antigens in Bermuda grass pollen were proteins and chose protein standards. The error due to this assumption would not be great unless the antigens contained a significant amount of lipid and had partial specific volumes considerably greater than the protein markers. In that case the $S_{20,w}$ obtained would be too high. To check that the partial specific volume was not greater than 0.800 $cm^3\ g^{-1}$ a sample of Fraction IIB was layered on a 54% sucrose solution and spun at 35,000 rpm for 48 hours. Under these conditions the antigenic activity moved 0.68 cm into the sucrose solution.

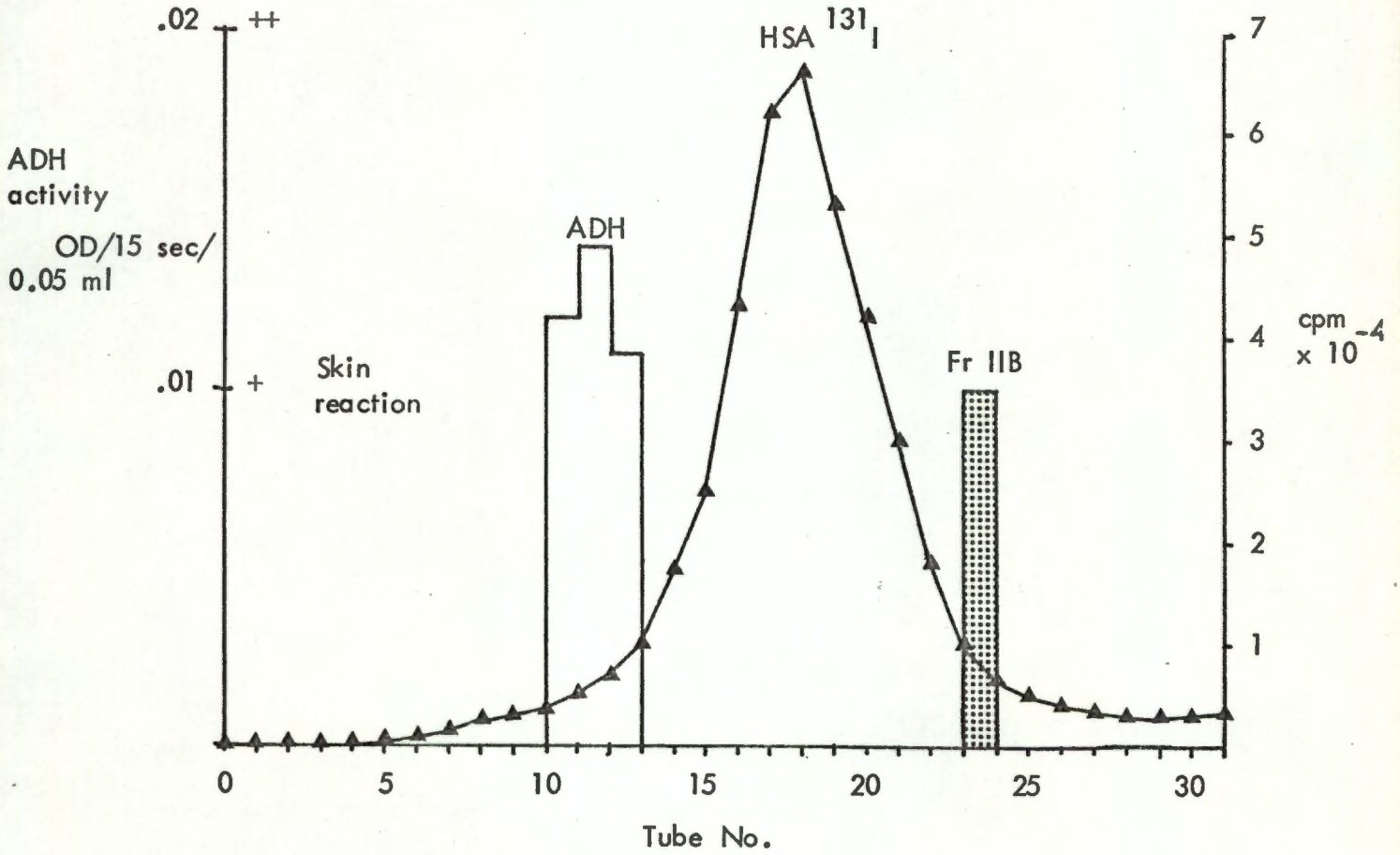


Figure 1.9

Sucrose density gradient ultracentrifugation. Sedimentation profile of Tube 1, Run 2 showing the distance travelled by ADH and ^{131}I labelled HSA relative to the skin reactivity peak of the antigen in Fraction IIB.

▲——▲ represents radioactivity

——— represents enzyme activity.

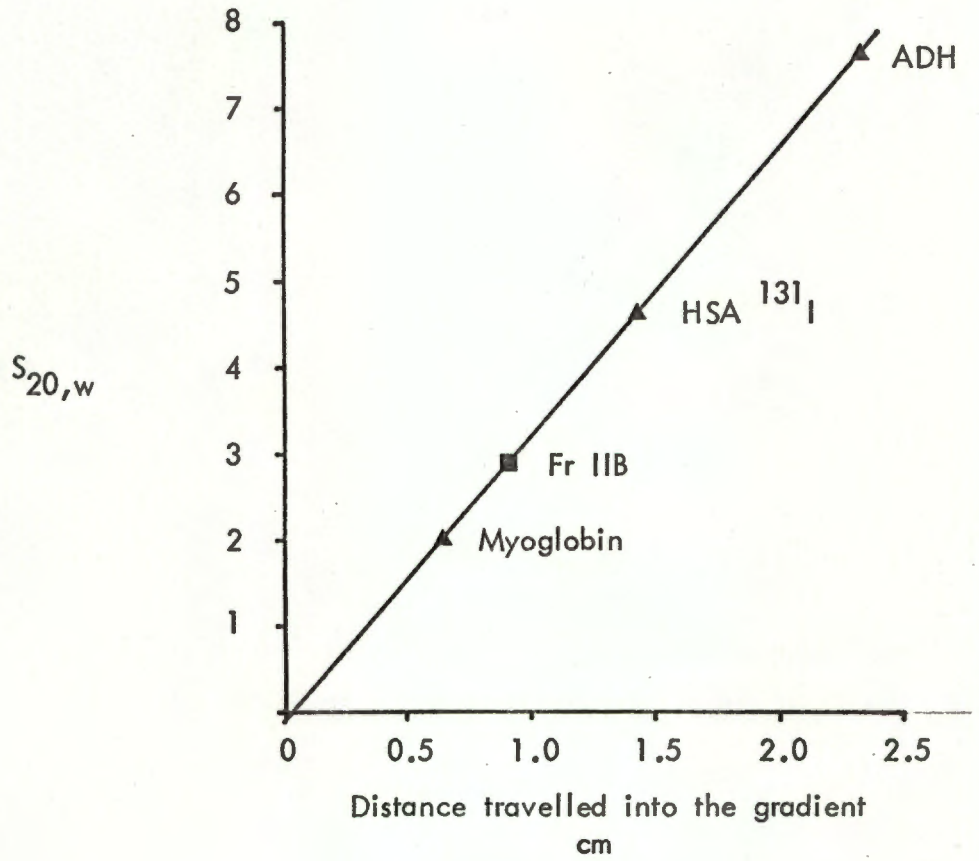


Figure 1.10

Sucrose density gradient ultracentrifugation. The sedimentation coefficients ($S_{20,w}$) of the markers were plotted against the distance they travelled into the density gradient.

▲ represents marker points

■ represents antigen point.

From the parameters of the least squares regression line through the marker points the $S_{20,w}$ of the antigen was calculated. The data used for this diagram were obtained from Tube 2, Run 2.

A control sample was layered on a 54% sucrose solution and left to stand at $4^{o}C$ for 48 hours; the antigenic activity in this control sample diffused only 0.28 cm into the sucrose solution. As 54% sucrose has a density of 1.25 g/cm^3 at $4^{o}C$ these results indicate that the antigen, in the hydrated form, had a density of at least 1.25 g/cm^3 corresponding to a maximal hydrated partial specific volume of 0.800 $cm^3\ g^{-1}$ (Siegel and Monty (1966)).

For each determination at least two standards were used to provide a check on the linearity and bleeding of the gradient. Approximately 10 µg protein Fraction IIB was used per tube; this resulted in a narrow well defined peak of immuno-reactivity. Figure 1.9 shows the sedimentation pattern of a sample containing ADH, ^{131}I labelled HSA and Fraction IIB.

The linear relationship between distance travelled and $S_{20,w}$, implicit in Equation (2) above, was used to calculate the $S_{20,w}$ of the unknowns from the results obtained for the distances travelled by the standards and unknowns. Figure 1.10 illustrates a least squares regression line through three of the standards that were spun concurrently in one of the experiments.

The results used for the calculation of the sedimentation coefficients of the antigens are given in Table 1.6. The result for the major antigen in Fraction IIB was 3.1 Svedberg units. This result was the mean of four determinations obtained during three separate ultracentrifugation experiments. While the fractions from the first two experiments (Runs 1 and 2) were tested on subject HAd, who was known

	Marker	$S_{20,w}$	Distance travelled cm	Distance travelled by antigen cm	$S_{20,w}$ antigen
Run I					
Tube 2	ADH	7.6	2.43	1.002*	3.2
	$HSA^{131}I$	4.6	1.54		
	Cytochrome	1.9	.555		
Run 2					
Tube 1	ADH	7.6	2.41	.97*	3.1
	$HSA^{131}I$	4.6	1.45		
Tube 2	ADH	7.6	2.32	.91*	2.9
	$HSA^{131}I$	4.6	1.42		
	Myoglobin	2.0	.6		
Run 3					
Tube 3	ADH	7.6	2.14	.93*	3.1
	HSA	4.6	1.43	.68+	2.2
	Cytochrome	1.9	.57		

Table 1.6

Results of sucrose density gradient ultracentrifugation. The table gives the known $S_{20,w}$ values of the markers and, for each experiment, the distance they travelled into gradient. From these results the $S_{20,w}$ values for the antigens were calculated. Four experiments were performed to determine the $S_{20,w}$ of the major antigen in Fraction IIB. In Run 3 another minor antigen with a lower $S_{20,w}$ was detected.

* Major antigen in Fraction IIB

\+ Minor antigen.

to be sensitive to only the major antigenic group in Fraction II, the fractions from the third experiment (Run 3) were tested on subject JP, who had previously been shown to be sensitive to both Fraction II and Fraction III (see Figure 1.4). Despite the fact that small molecules had been excluded from Fraction IIB by preceding Sephadex G100 chromatography, subject JP showed two peaks of reactivity to the fractions obtained from Run 3. One had an $S_{20,w}$ identical to that calculated for the major antigenic peaks in Runs 1 and 2. The other had an $S_{20,w}$ of 2.2 Svedberg units. This is further evidence that material in Fraction II tends to break down into small active components. Whether or not these components are the same as the antigen in Fraction III has not been established, but knowing that JP was sensitive to Fraction III makes this a distinct possibility. Furthermore, the observed $S_{20,w}$ of 2.2 would be in the range expected for a substance with a molecular weight about 15,000 Daltons.

1.3.c(iii) Iso-electric point determination

Iso-electric focussing was used for determination of the iso-electric points of the antigenic components of Fraction IIB.

The principles of iso-electric focussing in natural pH gradients have been described by Svensson and Vesterberg (Svensson (1961), Svensson (1962), Vesterberg and Svensson (1966), Vesterberg (1967)). Proteins are naturally occurring ampholytes; their iso-electric points can be determined by iso-electric focussing because the pH at the concentration maximum of an ampholyte in a natural pH gradient is its iso-electric point (Svensson (1962)).

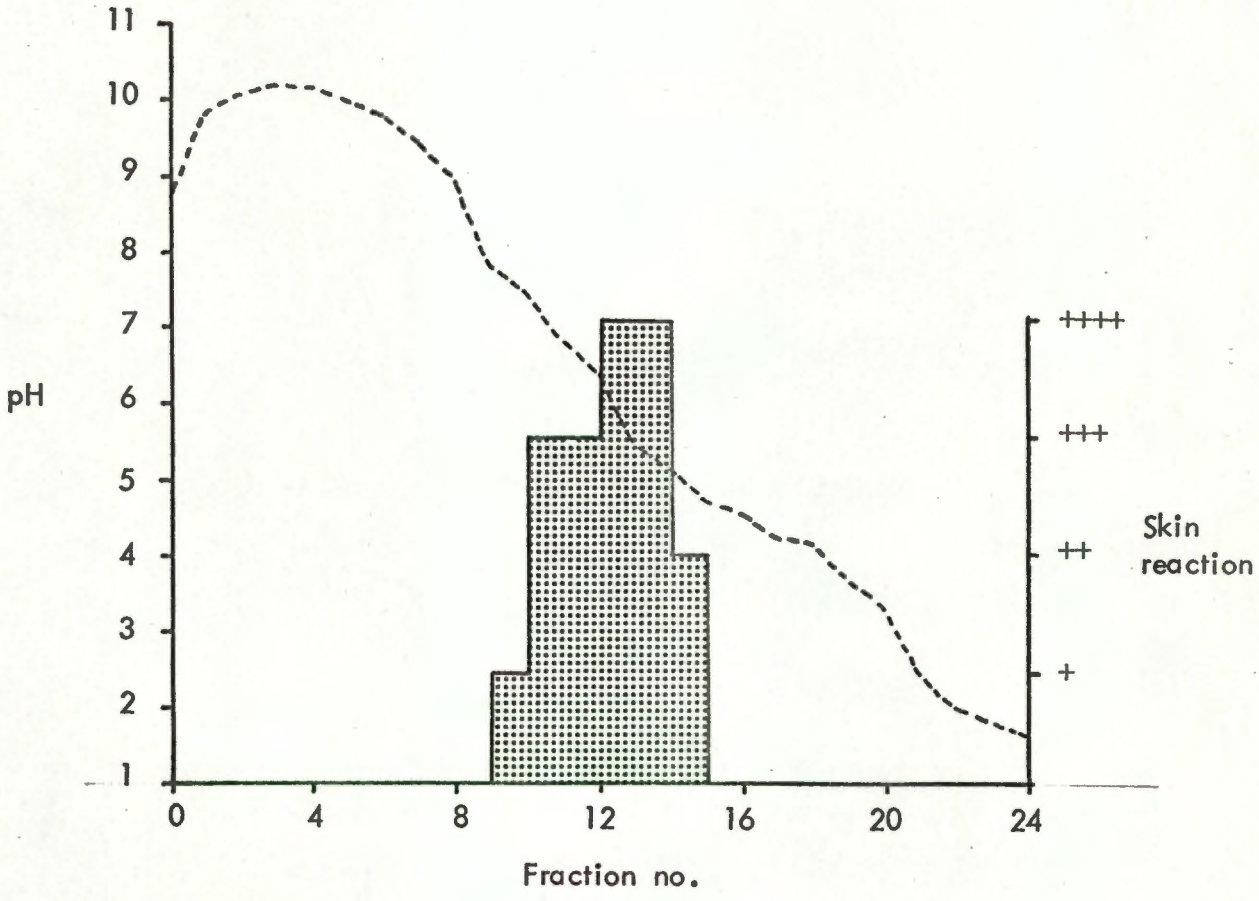


Figure 1.11

Iso-electric focussing.

Elution profile of skin reactivity and pH of samples obtained after iso-electric focussing in the presence of ampholytes pH range 3 - 10.

------ indicates pH.

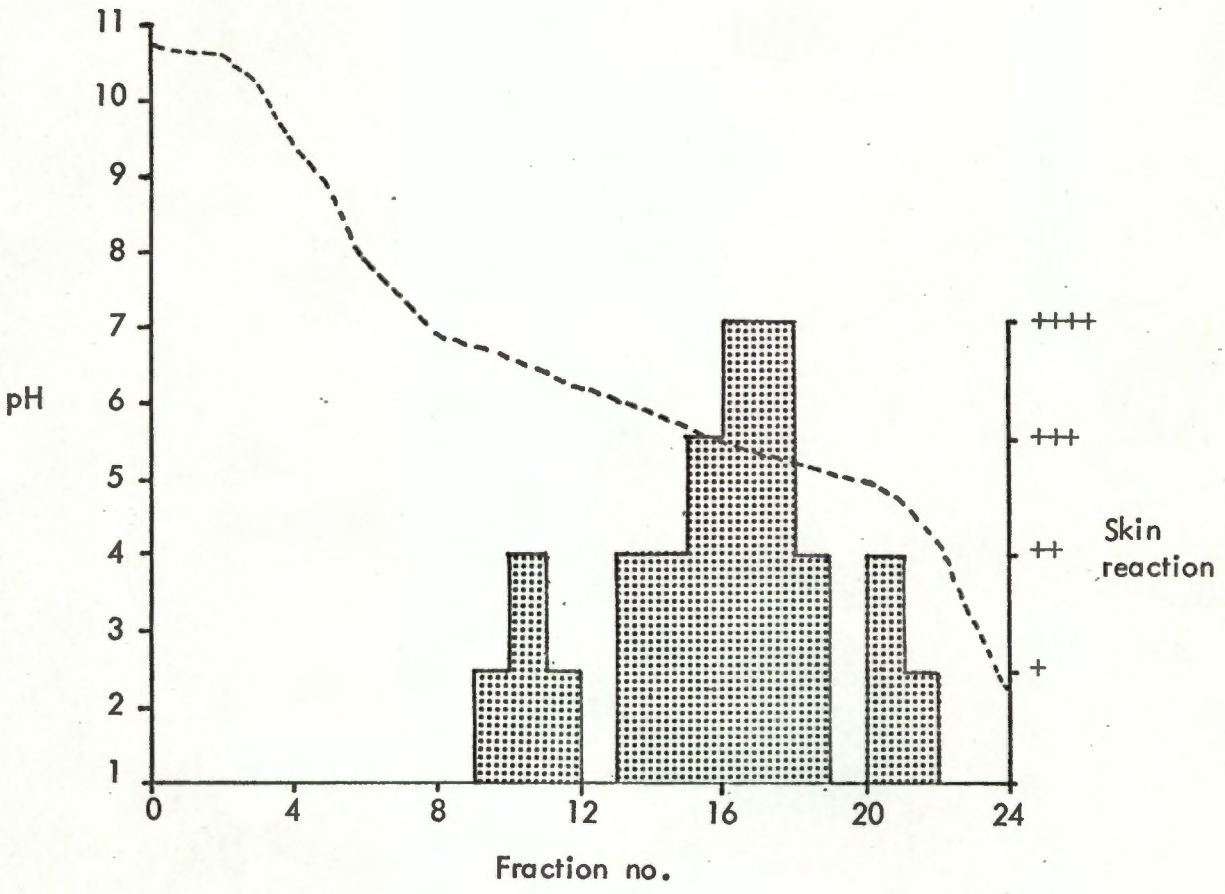


Figure 1.12

Iso-electric focussing.

Elution profile of skin reactivity and pH of samples obtained after iso-electric focussing in the presence of ampholytes pH range 5 - 8.

----- indicates pH.

For the initial experiment 10 ml of 17 µg/ml solution of Fraction IIB was dialysed overnight against 1% glycine, mixed with 2.5 ml of a 40% ampholyte solution pH range 3 - 10 (L.K.B. Produkter A.B., Sweden) and incorporated in the sucrose density gradient in the column. Subsequent to electrophoresis, the column was drained and the pH of the fractions measured. These fractions, after dialysis, were used for skin testing a sensitive individual PE. The elution profile (Fig. 1.11) showed only one peak of reactivity with maximum activity in the pH range 5.4 - 6.4.

In a subsequent experiment ampholytes with a narrow pH range 5 - 8 were used. Fractions were again tested on PE and Figure 1.12 shows that under these conditions the major antigenic group in Fraction IIB was resolved into three active components with iso-electric points of 6.6, 5.4 and 5.0. The component with an iso-electric point of 5.4 appeared to be the most active or the most abundant component.

The fractions obtained from each of these two experiments were tested on one other individual and in each case results similar to those found in subject PE were obtained.

These results suggest that the antigenic activity in Fraction IIB does not reside in one homogeneous protein. In view of the hetero-geneity in iso-electric charge that has been demonstrated in Antigen E (King and Norman (1962)) and Rye grass Group I Allergens (Johnson and Marsh (1965a)), this result was not surprising.

1.3.d. Radio-iodination of major antigen and experiments with the labelled material.

An attempt was made to label the protein in Fraction IIB with ^{125}I. Two millicuries of ^{125}I was used to label 20 µg protein of Fraction IIB using the chloramine T method (Hunter and Greenwood (1962)) as adapted for labelling Ragweed Antigen E by Yagi et al (1962) (see Appendix II).

While the material in Fraction IIB was not completely pure it was very immuno-reactive by skin testing and it was homogeneous by the criteria of Sephadex G100 and DEAE cellulose chromatography. Only 6% labelling was obtained but the specific activity achieved was approximately 6 µC/mg. Initially, the iodinated material was kept in a 1% solution of Bovine serum albumin (BSA, Miles Seravac, Cape Town) in an attempt to protect the protein from self-irradiation.

The immuno-chemical integrity of the labelled product was assessed, in the first instance, by radio-immuno-electrophoresis using the method suggested by Yagi et al (1963). Serum samples from a normal subject AO and a Bermuda grass pollen sensitive subject WA were electrophoresed in agarose, and polyvalent goat anti-human serum (Miles-Seravac, Kankakee, U.S.A.) was used to develop the immuno-precipitin arcs (see Appendix III). The gels were washed free of unreacted protein and soaked for one hour, at room temperature, in a solution of labelled Fraction IIB containing approximately 4 µg (24 µC) of ^{125}I labelled protein per ml. They were then washed exhaustively, dried and placed in direct contact with Kodirex X-ray film for three days. The auto-radiographs were then developed and fixed and the slides stained with Amido-Black. As can be seen from

the picture of the stained gel and its companion auto-radiograph shown in Figure 1.13 the radioactivity was not found exclusively in association with the immunoglobulin components of the allergic serum. Instead, labelled material bound to multiple serum components, including albumin. This result was found using both normal and sensitive sera.

Figure 1.13

Radio-immuno-electrophoresis: serum from a Bermuda grass sensitive subject was employed and the radioactively labelled antigenic material was labelled Fraction IIB. The slide in the left shows the stained immuno-electrophoretic pattern and the one on the right is an auto-radiograph of the same slide. The cathode is on the right. The auto-radiograph shows that radioactive material became bound to multiple serum components.

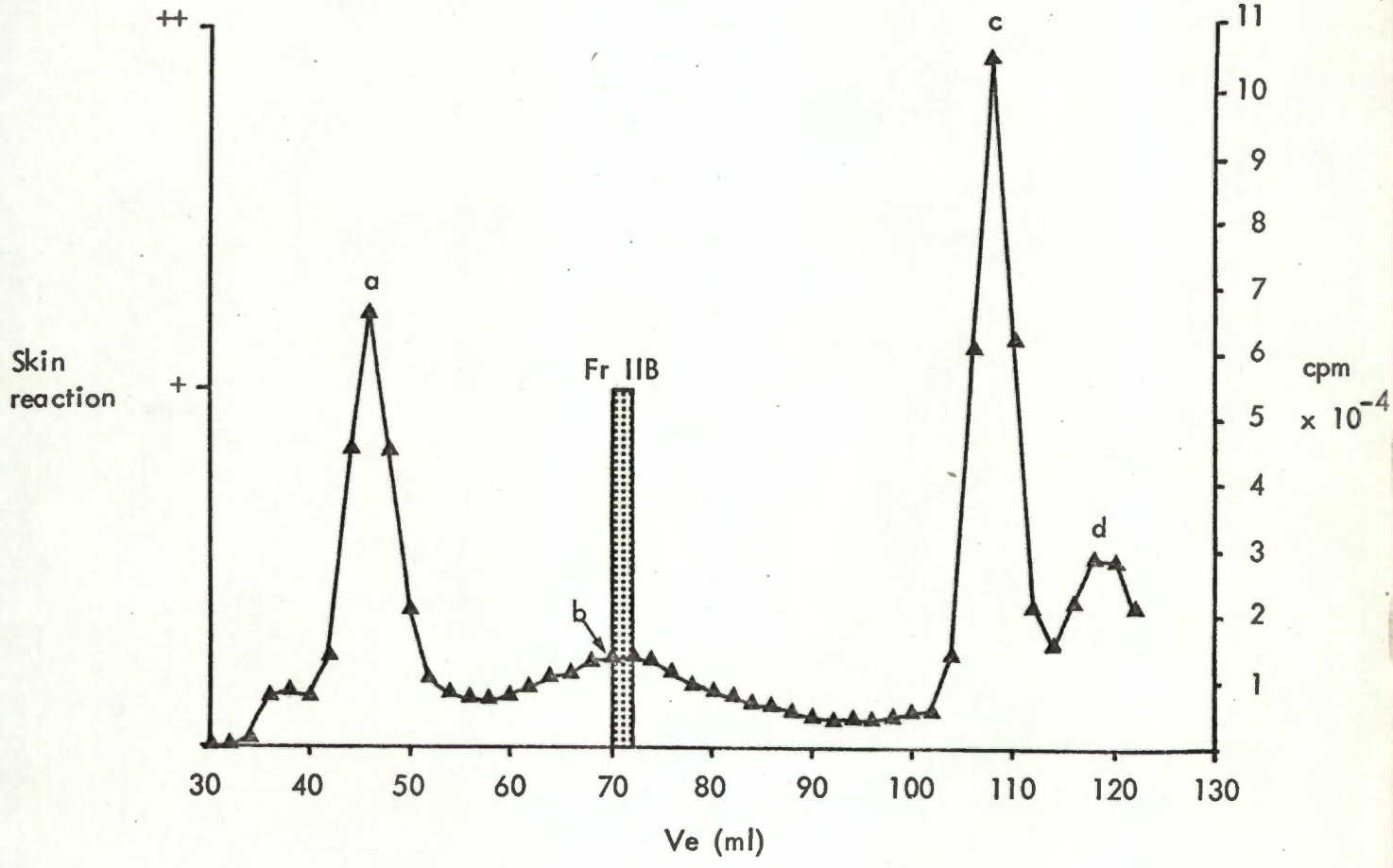


Figure 1.14

The effect of iodination on the physical integrity of the antigen in Fraction IIB.

The elution profile of skin reactivity and radio-activity of 50 µg Fraction IIB mixed with 0.2 µC ^{131}I labelled Fraction IIB and chromatographed by Sephadex G100 chromatography.

▲——▲ represents radio-activity.

The effect of iodination on the physical integrity of the material in Fraction IIB was investigated by chromatographing 0.2 µC of labelled Fraction IIB in 1% BSA together with 50 µg unlabelled Fraction IIB, on the 1 x 150 cm Sephadex G100 column. Fractions were counted in a Packard auto-gamma counter and used for skin testing on a sensitive subject HAd. The elution profile of these results is shown in Figure 1.14. Only a small broad peak of radioactivity eluted together with the peak of immuno-reactivity and the major radioactive peak eluted late, close to the free iodine peak.

Thus iodination had resulted in instability of the molecules in Fraction IIB which must have disintegrated subsequent to the original separation of the free iodine from the labelled protein. There was in addition a peak of radioactivity eluting early together with the BSA (peak (a) in Figure 1.14) indicating that at least some of the fragmented radioactive material had a tendency to bind to serum albumin. Binding to human serum albumin was confirmed by incubating 100 µl of the major radioactive peak (peak (c) in Figure 1.14) with 50 mg HSA for one hour at room temperature. The mixture was chromatographed on the 1 x 150 cm Sephadex G100 column and the results again showed radioactive material eluting with the albumin peak. A control experiment showed that free ^{125}I did not bind significantly to albumin.

To investigate the effect of iodination on the immuno-reactivity of Fraction IIB, 75 µg was labelled in the usual manner except that 10 µC of ^{131}I was used instead of 2 mC ^{125}I. Immediately after the addition of sodium metabisulphite the mixture was passed through the 1 x 150 cm Sephadex G100 column without preliminary removal of the free iodine. In a control experiment 75 µg unlabelled Fraction IIB

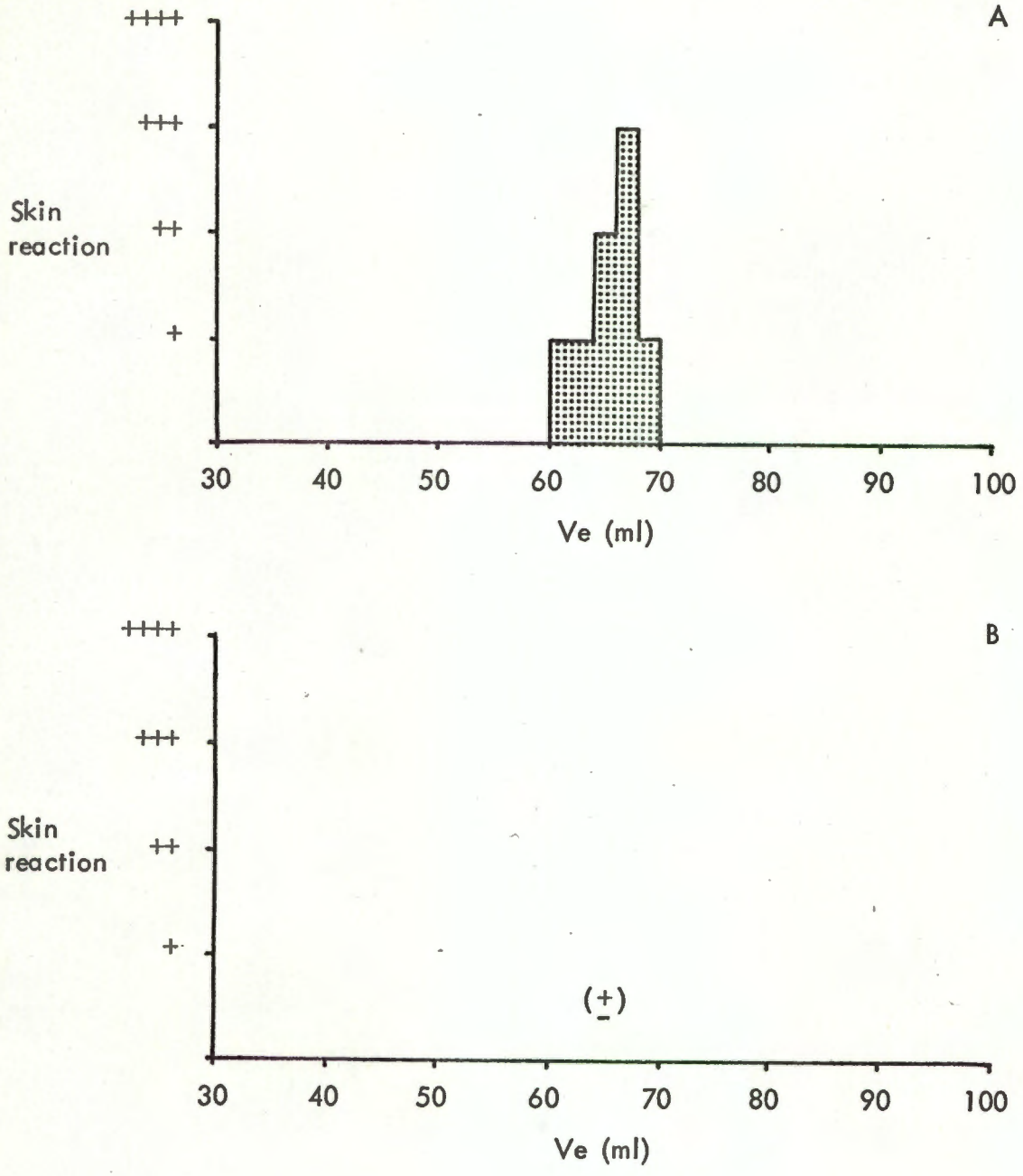


Figure 1.15

The effect of iodination on the immunological integrity of the antigen in Fraction IIB.

A shows the elution profile of skin reactivity in 75 µg Fraction IIB re-chromatographed on Sephadex G100, and B shows the elution profile of an equal quantity of Fraction IIB that had been subjected to the chloramine T iodination procedure just prior to chromatography.

was passed through the same column. Fractions from both experiments were tested simultaneously on one individual NR. Figure 1.15 shows that iodination had resulted in almost complete loss of immuno-reactivity of Fraction IIB. The control material elicited a peak response of +++, whereas, only a minimal (less than +) response was elicited by the radioactivity labelled material. Fractions containing low molecular weight material and eluting late from the column also showed some minimal reactivity, both in the test and in the control experiment. This indicated that there had been some tendency for Fraction IIB to disintegrate even prior to iodination and that subject NR was mildly sensitive to those breakdown products.

An attempt was made to label 25 µg of Fraction IIB using the iodine monochloride method of McFarlane (1958) (see Appendix II). Extremely poor iodination was achieved (< 1%) and, once again, the iodinated material obtained tended to break down.

As a result of the above experiment I concluded that the antigen in Fraction IIB was relatively unstable and unable to withstand the iodination procedure. This was disappointing since it precluded further experiments using the labelled antigen.

1.4 Discussion

Allergens are found in a wide variety of sources such as plant pollens, house dust, animal danders and certain foods and drugs. The concentration of specific antigens in these substances is usually very low and in sensitive individuals they represent some of the most biologically active substances known to man (Augustin and Hayward (1962)).

In this chapter I have reported my investigations into the nature of one of the antigens present in one particular allergenic substance - Bermuda grass pollen. Many workers have commented on the differences between Bermuda grass pollen antigens and those derived from other pollens. Wodehouse (1955) studied the antigens derived from the pollens of six common American hay fever grasses by means of gel diffusion against rabbit antisera and found that all except those from Bermuda grass showed marked cross-reactivity. Augustin (1959b) confirmed the antigenic uniqueness of Bermuda grass pollen antigens, again using precipitin tests. Frankland (1955) commented on the fact that while subjects sensitive to Cocksfoot pollen inevitably reacted to Timothy pollen and vice versa, 44 out of 152 such sensitive subjects were completely non-reactive to Bermuda grass pollen. Marsh et al (1970) used starch gel electrophoresis to purify the antigens present in pollens from seven grasses found commonly in the United States. Antigenic cross-reactivity was detected *in vitro* using rabbit antisera. The antigens in Bermuda grass pollen did not cross-react with those present in any of the other pollens, whereas, antigens from the six other grasses cross-reacted with one another.

Fractionation of pollen material has enabled workers to demonstrate more than one antigen in all allergenic pollens so far investigated

(Augustin and Hayward (1962), Malley, Reed and Lietze (1962), King and Norman (1962), Johnson and Marsh (1965a),Marsh, Haddad and Campbell (1970)); and in this work I have shown that Bermuda grass pollen is no exception. Owing to individual variation in the pattern of reactivity to antigens of any particular pollen (Augustin and Hayward (1962), Malley et al (1962), King and Norman (1962), Marsh, Milner and Johnson (1966), Marsh, Bias, Hsu and Goodfriend (1973)); it is convenient to use the terms *major antigen* and *minor antigen*. *Major antigens* are antigens that are immuno-reactive in almost all individuals sensitive to the parent unpurified material, whereas, *minor antigens* are as reactive as the *major antigens* only in a limited proportion of such subjects. In other subjects they are either non-reactive or,reactive only if they are used in much higher concentrations than those required to elicit a positive response to the major antigens. Johnson and Marsh (1965a and b) used the terms *major allergens* and *minor allergens* with exactly the same meanings that I am using for the terms *major antigen* and *minor antigen*. I have not adhered strictly to their terminology because, as discussed in the introduction, I have reserved the use of the word *allergen* for substances that are known to be immunogenic.

By the above criteria, the material in Fraction IIB that was responsible for positive skin tests in all 24 subjects tested was a major antigen. It proved to be an extremely potent antigen, some subjects reacted to less than 10^{-12} g of the substance measured as protein. It represents the major antigenic substance in Bermuda grass pollen and I will designate it BGP_1. It had a molecular weight of approximately 30,000 and a sedimentation coefficient of approximately

3.1. It was homogeneous by the operational criteria of Sephadex G100 chromatography and DEAE chromatography. Iso-electric focussing split it into three component parts; this finding will be discussed later.

A minor Bermuda grass pollen antigen was present in Fraction III. At the concentration used for testing it was reactive in only 12 of the 16 subjects tested. This I will designate as BGP_2. It had a molecular weight of approximately 14,400 and a sedimentation coefficient of 2.2.

A few individuals reacted in addition to non-dialysable material that was not present in either Fraction IIB or Fraction III, and therefore other minor antigens must have been present in the crude extract. Sensitivity to these antigens was relatively rare and I did not attempt to characterize these substances in any way.

A moderate amount of work has already been done on the physico-chemical properties of certain other pollen antigens and, in view of the antigenic uniqueness of Bermuda grass antigens, it is interesting to compare these results with my own. Johnson and March (1965a) showed the major Rye antigens (which they called Group I Allergens) to have molecular weights in the range of 32,000 to 34,000 and sedimentation coefficients in the range of $2.89 \pm .03$ and a group of minor Rye antigens (Group II Allergens) to have molecular weights around 10,000 and sedimentation coefficients in the range $1.36 \pm .03$. King, Norman and Connell (1964) found the major antigenic group in Ragweed, Antigen E, to have a molecular weight of 37,000 with an $S_{20,w}$ of $3.05 \pm .1$. Both groups of investigators used an analytical ultracentrifuge to determine their results. In 1967 another Ragweed antigen, Antigen K, was identified (King, Norman and Lichtenstein

(1967a and b)). Antigen K was found to be slightly less active than Antigen E and had a different amino acid composition but a similar molecular weight. The two antigens showed some antigenic cross-reactivity. Underdown and Goodfriend (1969) isolated a smaller minor Ragweed antigen, Ra3. It had a molecular weight of 15,000 and an $S_{20,w}$ of 1.18. Its amino acid composition was distinct from Ragweed Antigen E, but it too showed some antigenic cross-reactivity. When King and Norman (1962) first isolated Antigen E, they noted the presence of another antigen in whole Ragweed extract. This antigen was reactive in four out of 32 subjects tested; they estimated its molecular weight to be around 10,000 but they did not characterize it further. Recently Goodfriend and Lapkoff (1972) isolated a Ragweed antigen, Ra5, with a molecular weight of only 5,200; Marsh, Bias and Hsu (1973) found it to be active in 17% of Ragweed sensitive subjects. Despite the reported differences in molecular weight, Ra5 and the minor allergen reported by King and Norman are possibly identical. Two groups of investigators, Malley et al (1962) and Augustin and Hayward (1962), described two antigens present in the non-dialysable fraction of Timothy grass pollen extract. Malley and Dobson (1966) referred to these as Allergen A and Allergen B and found them to have sedimentation coefficients of 3.0 and 2.0 respectively. Malley and Harris (1967) refer to Allergen B as having a molecular weight of 10,500 as determined by sedimentation diffusion analysis in an analytical ultracentrifuge. The material, Antigen D, that the same workers isolated from the dialysable fraction of Timothy pollen extract (Malley and Campbell (1963), Malley, Campbell and Heimlich (1964)) had an estimated molecular weight of approximately 5,000, was immuno-reactive in indirect test systems,

Pollen	No. of allergen groups	Name of allergen (if any)	Molecular weight	$S_{20,w}$	Relative importance	References
Bermuda grass	2	BGP_1	30,000[a]	3.1	Major	
		BGP_2	14,400[a]	2.2	Minor	
Rye grass	2	Group I	32,000[b] ±1,000	2,89 ±0.03	Major	Johnson and Marsh (1965a)
		Group II	9,300[b] ± 700	1.36 ±0.03	Minor	
Ragweed	5(?4)	Antigen E	37,800[b] ±1,000	3.05 ±0.1	Major	King et al (1964)
		Antigen K	38,200 ±1,500		Minor	King et al (1967)
		A minor allergen	10,000[a]		Minor	King and Norman (1962)
		Ra3	15,000[b]	1.8	Minor	Underdown and Goodfriend (1969
		Ra5	5,200		Minor	Goodfriend and Lapkoff (1972)
Timothy grass	2	A	?	3.0	?	Malley and Dobson (1966)
		B	10,500[b]	2.0	?	
Cocksfoot grass	2		30,000	?	?	Augustin et al (1971)
			17,000	?	?	

Table 1.7

Summary of data available on the physical characteristics and relative importance of some important pollen antigens. "Major" and "Minor" are used as defined in the text and as used by Johnson and Marsh (1965b). Only the most relevant references are given - other references are given in the text.

a,b, indicate method determining molecular weight.

a. Calibrated column chromatography.

b. Sedimentation studies using an analytical ultracentrifuge.

and elicited in rabbits the synthesis of antibody that cross-reacted with Allergen B. Augustin, O'Sullivan and Davies (1971) refer to O'Sullivan's Ph.D Thesis where he determined the molecular weights of Cocksfoot antigens to be 30,000 and 17,000. Table 1.7 summarizes these results.

Johnson and Marsh (1965a) have drawn attention to the similarities that exist between Rye grass pollen and Ragweed pollen in that both contain at least two groups of antigens, one group containing antigens with molecular weights between 30,000 and 40,000, and the other group comprising very much smaller antigens with molecular weights in the region of 10,000. In both pollens the most immuno-reactive antigens were found in the group of antigens with the higher molecular weights. More information is now available and Johnson and Marsh's observation can be extended to include other pollen antigens. Table 1.7 indicates that all allergenic pollens, for which data are available, contain an antigenic component with a molecular weight in the range of 30,000 to 40,000. Although I have been unable to locate a reference to the molecular weight of Timothy Allergen A, it has an S value of 3.0 (Malley and Dobson (1966)) which is almost identical to the S values of the major antigens in Ragweed, Rye grass and Bermuda grass pollen. Furthermore, all the pollens contain smaller antigens which, in the case of Bermuda grass pollen, Ragweed pollen, and Rye grass pollen, have been shown to be less immuno-reactive than the larger antigens. It is interesting to note that Bermuda grass pollen contains antigens which are similar to other pollen antigens, both in molecular size and in the relative importance of the larger component. This similarity exists despite the antigenic uniqueness of the Bermuda antigens. If, as seems

highly likely, the antigens detected on skin testing are identical to the substances that elicit the immune response in exposed individuals, one may draw the circumstantial inference that molecular size plays some role in determining allergenicity.

Johnson and Marsh (1965a and b) introduced the term *iso-allergens* to describe groups of closely related "allergens". These are groups of substances that are antigenically cross-reactive and have similar molecular weights and amino-acid composition. They differ from each other in that they carry slightly different charges at alkaline pH so that they can be separated by ion exchange cellulose chromatography or electrophoresis. Cross-reactivity can be demonstrated by immuno-precipitation techniques *in vitro* and by inhibition of the P-K reaction *in vivo*. By the criteria that I have used in this thesis such substances are not necessarily allergens, nevertheless, the concept of *iso-allergens* is a useful one in that it serves to emphasize the similarity that sometimes exists between antigens from different allergenic sources as well as those co-existing in one source.

Both Ragweed Antigen E and Rye Group I Allergens have been shown to comprise more than one antigenic substance (King et al (1964), Johnson and Marsh (1965a and b)), and by the above criteria both are groups of iso-allergens. It is possible that some iso-allergens can be converted into other members of the same group by mild chemical means. Thus Callaghan and Goldfarb (1962) demonstrated that an antigen isolated from Dwarf Ragweed could be split into two electrophoretically separable components by oxidation or reduction, with each component retaining the ability to elicit a direct skin reaction.

King et al (1964) indicated that treatment with mercapto-ethanol can convert one component (IV-D) of Ragweed Antigen E into another (IV-C). Johnson and Marsh (1965a) have speculated that Rye grass Allergen α (I-B) is converted into Allergen β (I-C) by loss of an amide group. Unfortunately I did not have enough material for chemical and immunological characterization of the different antigenic fractions obtained by means of DEAE chromatography and iso-electric focussing of Bermuda grass pollen Fraction II. These were fractions of supposedly similar molecular weights, separable on the basis of electrical charge differences. It is, therefore, not unreasonable to speculate that at least some of them fulfil Johnson and Marsh's criteria for iso-allergens.

Iso-electric focussing is a relatively new technique that has not yet been fully exploited in the study of allergenic substances. It has a resolving power of 0.01 - 0.02 pH units if a narrow pH gradient and a suitable pH meter are used (Vesterberg (1967)), and it may well prove eminently suitable for the study of closely related substances such as iso-allergens. However, Jacobs (1973a and b) has very recently shown that iso-electric focussing may, under certain chemical conditions, result in a minor degree of protein oxidation that can give an artefactual appearance of heterogeneity. My own experiments were performed prior to 1973 and I did not take any precautions to prevent oxidation of the antigens during iso-electric focussing. It may well be that the major antigen BGP_1 is, in fact, a homogeneous single substance with an iso-electric point of 5.4. Similarly, some of the other "iso-allergens" may be figments of technical procedures rather than true natural compounds.

Elsayed and Aas (1971a) have successfully used iso-electric focussing to purify the antigenic components in the white muscle myogen of cod. The major component had an iso-electric point of 4.75. Goldfarb, Bhattatharya and Koerner (1958) using the Kolin iso-electric technique found an iso-electric point between 4.0 and 4.2 for the major antigen that they isolated from Giant Ragweed. Varga and Ceska (1972) used iso-electric focussing on acrylamide gel and the radio-immunosorbent allergen assay to analyse components of Timothy pollen. They found that the principal antigenic components had iso-electric points of 4.5 to 5.6 and that there were additional components with iso-electric points covering a very wide range. The method was valuable for demonstrating the heterogeneity of the pollen extract rather than for iso-electric point determination. This is probably because specific IgE will bind denatured antigenic components that no longer have the iso-electric point of the original antigen and are not capable of inducing an immediate hypersensitivity response *in vivo*. My own method of using direct skin testing to locate the antigen ensured that the iso-electric points of only immuno-reactive components were determined. Many workers (Augustin (1959c),King and Norman (1962), Callaghan and Goldfarb (1962), Marsh et al (1970)) have used electrophoresis to separate and identify pollen antigens and have assessed their relative electrophoretic mobilities at various pHs. Thus, most pollen antigens have been shown to have iso-electric points on the acidic side of neutral. Iso-electric focussing, if used with the proper precautions, should give more precise values and better separation of the component parts than have been obtained up to now.

The minor antigen, BGP_2, that I detected in Bermuda grass pollen had a molecular weight that was, within the limits of experimental error, half that of the major antigen, BGP_1. Re-chromatography of a purified preparation of BGP_1, that is Fraction IIB, showed it to contain antigenic material having an elution volume equal to that of BGP_2. This indicates that material in the extract had dissociated to give rise to immunologically active fragments that were, in all probability, identical with the minor antigen BGP_2. Although by no means conclusive, the relationship between the molecular weights of BGP_1 and BGP_2 suggest that the former is a dimeric form of the latter. This suggests further that BGP_2 arose in the purified preparation of BGP_1 because of the tendency of the dimer to split into its two component parts. Experiments in the presence of sodium dodecyl sulphate (SDS) have demonstrated that some other antigens are composed of sub-units that can dissociate under appropriate conditions. Johnson and Marsh (1966b) have demonstrated dissociation of Rye Group I Allergens during sedimentation in SDS solution. Hussain, Bradbury and Strejan (1973) have shown the presence of two sub-units of an Ascaris antigen (Asc-I) by polyacrylamide gel electrophoresis in the presence of SDS.

It is not possible with the information now available to offer a completely satisfactory explanation for the observation that persons who have been sensitized by a particular antigenic substance, include, among their number, a proportion that are non-reactive to minor antigens present in that substance.

As I tested subjects with only one concentration of each of the fractions obtained during the purification of the Bermuda grass pollen antigens, the findings that some individuals were unreactive to

those fractions that contained the minor antigens, may have been due to a concentration effect. In other words, they might have reacted to the minor antigens if higher concentrations had been used for testing. Nevertheless, my results do show that individuals sensitive to BGP_1 differed in their reactivity to BGP_2. As the positive reactions to BGP_1 indicated that all subjects were capable of releasing and reacting to mediators, the differences noted must have been differences in the immunological responsiveness of the subjects.

If BGP_2 is indeed a dissociation product it may not be present, as such, in the intact pollen to which subjects are naturally exposed. It is possible that people who do not react to the minor antigens lack the ability to degrade the parent material. Sensitivity to degradation products has been reported by Cooke (1942) who found that some sufferers from milk allergy were unreactive to whole milk but were reactive to their tryptic digestion products. This appears to have been an isolated finding and I do not know of any subsequent similar reports. Wide variations in the patterns of sensitivity to whole milk proteins have been reported (Goldman (1963)), indicating that individual to individual variation occurs primarily at the immune response stage rather than at the stage of enzymatic digestion.

McDevitt and Benacerraf (1969) have reviewed animal experiments showing that immune responses to several distinct antigens are under the control of specific autosomal dominant genes. More recently the same authors (Benacerraf and McDevitt (1972)) have reviewed the evidence that the ability of mice and guinea pigs to respond immunologically to certain synthetic polypeptides is controlled by genes that are closely linked to the major histocompatibility linkage group of the species. Vaz and Levine (1970) demonstrated that the

ability of mice to respond to minute doses of complex hapten protein conjugates was related to the histocompatibility type of the strain and, moreover, that those strains which responded to the antigens, responded with both an IgG_1 and a reaginic antibody response (Levine and Vaz (1970)). The reaginic response was long-lived, boosterable and a prominent part of the total response and thus may be analogous to the IgE response occurring naturally in man. Another type of genetic control of the reaginic response has been demonstrated (Levine (1971)). It is not antigen specific and is not linked to the histo-compatibility locus.

The results of these experiments have led workers to speculate that at least part of the genetic control of the allergic response in man may be found to reside in genes occurring near the histocompatibility loci.

Marsh et al (1973) concluded, on the basis of a study of Ragweed sensitive individuals, that skin sensitivity to the minor allergen Ra5 was significantly associated with the possession of a histo-compatibility antigen belonging to the HL-A7 "Greg" cross-reacting group. The authors, in analysing their data, fell into the trap that McDevitt and Bodmer (1972) warned investigators in the field of disease-antigen associations to avoid, in that they overestimated the statistical significance of their findings by selecting the most extreme association from the many associations that they calculated. In this type of situation it is not valid to use normal criteria of significance. For instance, by definition, of twenty comparisons, on the average, one of them is expected to be significant at the 5% level. McDevitt and Bodmer (1972) suggest that a possible way to compensate for this difficulty is to multiply the significance level found by the number of associations tested. Marsh et al (1973)

tested for association of Ra5 skin sensitivity with 24 individual HL-A antigens and 15 HL-A antigen groups. They found a positive association of p = .02 with the HL-A7 antigen and p = .006 with the HL-A7 "Greg" antigen group. If one uses the stratagem recommended by McDevitt and Bodmer (1972) the calculated levels of significance become p = .48 and p = .09. Thus the associations found by Marsh et al (1973) are not necessarily significant. An independent study of the relationship between the possession of an antigen in the HL-A7 "Greg" group and sensitivity to Ra5 is required to prove or disprove the conclusions of Marsh et al (1973).

Levine, Stember and Fotino (1972) reported the results of HL-A typing and allergy studies on seven families containing Ragweed sensitive individuals and concluded that clinical Ragweed hayfever and immediate skin hypersensitivity to Ragweed Antigen E were inherited together with a certain HL-A haplotype in any given family. They tested 46 individuals; complete details are given for only one family containing sixteen individuals. The remaining 30 individuals belonged to six different families and in these six families the average number of individuals tested was, therefore, five. A total of twenty individuals were reported as having Ragweed hayfever and four of these belonged to the large family of sixteen individuals. Thus, in each of the remaining six families an average of 2.5 individuals had Ragweed hayfever. Therefore, in some families the hayfever associated haplotype must have been chosen on the basis of data obtained from

only two individuals with hay-fever. Unless cross-over occurs all parents share at least one haplotype with their children and 75% of siblings share at least one haplotype with one another. To suggest linkage on the basis of these data is consequently not justified.

Therefore, the question of why certain individuals become allergic and why allergic individuals react to different antigens contained in the same allergenic substance remains completely open. The question is very interesting and one can only hope that the sound work that has been done on the genetic control of the immune response in animals will eventually be followed by equally sound and carefully thought out work in man.

Several workers have reported finding immuno-reactive substances in the low molecular weight, dialysable components of pollen extracts. These substances have, in some instances, been detected by direct skin testing. Richter et al (1958) showed that dialysable components of Ragweed could elicit a direct response. Similarly my experiments with the low molecular weight fractions obtained by Sephadex G100 chromatography of Bermuda grass pollen extract showed some of them to be capable of eliciting a direct response in certain subjects.

Some workers have used indirect or "inhibition" tests to demonstrate the immuno-reactivity of certain low molecular weight components. The fraction isolated by Attallah and Sehon (1969) from the diffusate of Ragweed pollen was inactive by direct tests but specifically inhibited P-K reactions elicited with the serum of sensitive subjects and whole aqueous Ragweed extract. I performed similar experiments using both the diffusate of whole Bermuda grass pollen extract and low molecular weight fractions obtained by Sephadex G100 chromatography; I was unable to demonstrate any inhibition of the P-K reaction; inhibition

of the direct skin test, likewise, could not be demonstrated.

Inhibition of a P-K reaction or of a direct skin test can only occur if the low molecular weight, "blocking" components carry antigenic determinants that cross-react with the determinants present on those antigens that are responsible for eliciting the P-K response or the positive skin test. Breakdown of high molecular weight antigens may lead to exposure of new determinants that were hidden in the intact molecule. Tests for inhibition of the P-K reaction or of the direct skin response would fail to detect molecules carrying such determinants. This may be the explanation for my finding that low molecular weight components that were capable of eliciting a direct skin response, were incapable of inhibiting the P-K reaction or the direct skin test.

The low molecular weight pollen component that has been investigated most fully, is the one that Malley and co-workers (1963, 1964) isolated from Timothy pollen. This component, which they called Antigen D, inhibited the precipitation of Timothy pollen antigens by rabbit anti-Timothy pollen. It did not induce the formation of skin sensitizing or other antibodies against itself in rabbits (Malley and Harris (1967)); but did induce reagin and blocking antibody formation in man (Malley and Perlman (1969)). It also induced the formation of anti-Timothy pollen Allergen B antibodies in man and rabbits. Malley and Perlman (1970) indicate that they found it more beneficial than crude pollen extract in the treatment of Timothy pollen hay-fever.

Meyers et al (1970, 1972) reported a similar type of investigation of low molecular weight components of Bermuda grass pollen. Using Sephadex G50 chromatography, they separated the diffusate of the whole pollen into two components. The component eluting first from the column elicited positive skin reactions in sensitive subjects, whereas,

the other component was unreactive or only minimally reactive. The inactive fraction inhibited the precipitation of antigens in whole pollen extract by rabbit anti-serum to the whole pollen extract. It also inhibited the PCA reaction of guinea pigs sensitized with anti-whole pollen extract and then challenged with whole-pollen extract. Because some of their experiments involved the use of animal anti-sera and animal subjects as opposed to human immune sera and human subjects, and because details of their experimental procedure are not available, it is not possible to compare their results with my own.

My attempts to label BGP_1 with ^{125}I were disappointing in that the labelling procedure resulted in loss of skin reactivity and fragmentation of the antigen. The chloramine T method of iodination subjects proteins to both oxidation and reduction. Pollen antigens seem to differ in their ability to withstand such procedures. Johnson and Marsh (1966a) found that the skin reactivity of Rye Group I Allergens was destroyed by oxidation and reduction. Lee, Markinovich and Robertson (1971) claim to have successfully labelled a Timothy antigen but they did only limited electrophoretic studies on the relationship of the labelled antigen to the original one. Callaghan and Goldfarb (1962) reported that, despite changes in the electrophoretic mobility of their Ragweed antigen after oxidation and reduction, the reactivity was not significantly affected. However, King et al (1967b) found that reduction and alkylation of Ragweed Antigen E reduced skin reactivity 10,000 fold. Their experiments on the chemical modification of Antigen E led them to the conclusion that disruption

of tertiary structure was as important as disruption of primary structure in the inactivation of the immunological integrity of the antigen. In contrast, Elsayed and Aas (1971b), who investigated an allergic food, cod, found it to possess a major antigen that withstood denaturation, oxidation and reduction; they suggested that the immuno-reactivity of this antigen was dependent on the sequence of certain amino acids rather than its stereo-chemical configuration.

As Antigen E has been successfully iodinated it is evident that it is not impossible to label those pollen antigens that require an intact stereo-chemical configuration for their activity; however, it is obvious that for studies involving labelled antigens to be meaningful the labelled material must behave exactly as its unlabelled counterpart in immunological systems. As I was unable to achieve this type of labelling with BGP_1,I decided not to proceed further with this line of investigation.

CHAPTER II

Studies on serum IgE concentrations in 4650 blood donors living in the Western Cape.

2.1 Introduction

A humoral factor associated with allergy has been recognised since 1921 when Prausnitz demonstrated passive transfer, to himself, of immediate, local skin hypersensitivity to fish, with an intradermal injection of serum from a fish-sensitive individual, Küstner. This phenomenon has since been known as the Prausnitz-Küstner (P-K) reaction. In 1925 Coca and Grove used the term "atopic reagin" for this factor and demonstrated its presence in the sera of persons with hayfever and asthma. It was only in 1966 that the Ishizakas and co-workers (Ishizaka and Ishizaka (1966a, b), Ishizaka, Ishizaka and Lee (1966), Ishizaka, Ishizaka and Hornbrook (1966a,b)) were able to demonstrate conclusively that the factor was an immunoglobulin and, furthermore that it belonged to an immunochemically distinct class of immunoglobulins. They first detected this immunoglobulin in the serum of a Ragweed sensitive individual and demonstrated that it had the ability to bind ^{131}I labelled antigen E. Working independently, Johannson and Bennich (1967a) described a myeloma protein (myeloma IgND) that likewise did not belong to any of the known immunoglobulin classes. They purified this protein and used it to develop a radio-immunoassay to measure serum concentrations of the corresponding normal protein (IgND). IgND was found to be a constituent of normal human serum (Johannson, Bennich and Wide (1968)), present in extremely low concentrations compared with the concentrations of the recognised immunoglobulins. Serum concentrations were found to be raised in persons with asthma and hayfever (Johannson (1967), Johannson and Bennich (1967b)); myeloma IgND was found to be able to

inhibit the P-K reaction (Stanworth, Humphrey, Bennich and Johannson (1967)). Johannson, Mellbin and Vahlquist (1968b) found that the serum concentrations of IgND were raised in Ethiopian children as compared to Swedish children and suggested that Ascaris infestation was one of the causative factors. Anti-sera were exchanged between workers in the United States and Sweden and it was found that both were working with an immunoglobulin bearing the same class specific antigenic determinant. In 1968 this immunoglobulin was officially designated by the World Health Organisation as IgE or γE (Bull. W.H.O. (1968)).

Since the discovery of IgE many of its physical and chemical properties have been documented and progress has been made in understanding the mechanism of its action. Frequency distributions of serum IgE concentrations in Caucasian populations with and without allergic diseases been reported (see section 2.4 b, and Table 2.15). Some of the physico-chemical properties of immunoglobulin E are summarised in Table 2.1. These, and other properties of IgE, are discussed in detail in a comprehensive recent review (Bennich and Johannson (1971)).

Molecular weight	190,000	Bennich & Johannson (1971)
Sedimentation coefficient, $S_{20,w}$	8.20	Bennich (1968)
Diffusion coefficient, $D_{20,w}$	3.71	Bennich (1968)
Carbohydrate content	11.7%	Bennich (1968)
Electrophoretic mobility	fast δ to slow δ	Bennich & Johannson (1971)

Table 2.1

Physico-chemical characteristics of Immunoglobulin E. (The data for this table was obtained from experiments on a myeloma IgE - immunoglobulin E(ND)).

In the Western Cape there are three racial groups living in close proximity. These three racial groups differ from each other not only genetically but in their cultural background and the socio-economic conditions under which they live. When I started to investigate the problem of allergy in 1971, no data were available on serum IgE concentrations in these local population groups. I considered the collection and analysis of such data both an important study in its own right and necessary background work for further research into allergic disease in this part of the world.

I also envisaged that the results of such a study might indicate some of the factors responsible for influencing serum IgE concentrations in the local population groups. With these ends in view I undertook the survey of serum IgE concentrations in 4650 blood donors, the results of which are presented in this Chapter.

2.2 Materials and Methods

2.2a Subjects

Subjects were all blood donors who attended clinics held by the Western Province Blood Transfusion Service. These donors were all between 18 and 65 years of age and represented a broad spectrum of cultural, social and racial backgrounds. The Western Province Blood Transfusion Service serves the whole of the Western Cape Province of South Africa, covering an area of approximately 40,000 square miles. Approximately 60,000 people donate blood each year of whom about 30,000 are Whites. Although clinics are held in many of the country towns that are scattered over the entire area, the majority of donors live in the vicinity of Cape Town. For practical reasons serum IgE concentrations were determined on batches of samples obtained at particular clinics. The selection of clinics for testing was completely random. Apart from serum IgE concentrations no information was obtained from or about donors other than that obtained routinely by the Blood Transfusion personnel.

The Blood Transfusion Service divides its donors into four racial groups - White, Coloured, Bantu and Asiatic. Only four out of the 4,650 blood donors tested were classed as Asiatic. These were included with the Coloured group for the purposes of my study. The majority of White donors belonged to one of the two major Caucasian groups living in South Africa. These are the Afrikaans-speaking and the English speaking (Gentile) groups. Blood group gene frequencies (Botha (1972)) have shown only minor genetic differences between these two groups, however both show statistically significant differences from Western European populations. Botha suggested that these differences exist because both groups possess a minor proportion of Bushman and Hottentot (Southern African) genes

and in addition the Afrikaans speaking group possesses a minor proportion of Asiatic genes. The White group includes, in addition, a small proportion of recent immigrants from various European countries as well as the Jewish population. This latter group Bronté-Stewart, Botha and Krut (1962) showed, by blood group gene frequencies, to be a homogeneous group clearly defined genetically from the rest of the White population. Members of the White group follow a Western way of life and as a group have a high standard of living by Western European criteria.

The Cape Coloured people have arisen due to inbreeding of White settlers, indigenous Hottentot and Bushmen (Southern African) peoples and Asian immigrants who came mainly as slaves imported from the Dutch possessions in the East. Botha (1972) has indicated, on the basis of blood group gene frequencies of Cape Coloureds, that they could be regarded as having approximately 34% Western European, 36% Southern African and 30% Asian genes. The Cape Malays are distinguished from the rest of the Cape Coloured population by virtue of their adherence to the Moslem faith. Blood group gene frequencies show them to be a group who are genetically distinct from the non-Malay Cape Coloureds. Although the Cape Coloureds follow a Western way of life, their standard of living is considerably lower than that of the White group.

Relatively few blood donors included in this survey were Bantu. Most of them belonged to the Xhosa tribe and were probably the most homogeneous group studied. Donors consisted both of urban Bantu living permanently in Cape Town and following an almost completely Westernised way of life and migratory labourers resident in the Transkei and earning a living temporarily in the Cape. Such people would, at home, have lived a semi-tribalised way of life.

The Bantu are the lowest income group studied and by Western criteria have a poor standard of living.

It might justifiably be argued that blood donors do not constitute a completely random sample of the population. On the other hand, I do not feel that any other method of sampling, on a volunteer basis, would have been any more satisfactory. Blood donors self-select for good health so that there is possibly a lower incidence of severe allergic disease amongst them than amongst the general population. Although, at enrolment, donors are questioned by Blood Transfusion personnel regarding allergic disease, they are not rejected on this account. Thus the values that I quote for serum IgE concentrations in the different population groups are values that are representative of serum IgE concentrations in the whole of that population group and not specifically for the non-allergic section.

I measured serum IgE concentrations of a total of 4650 blood donors but as some of the additional data were not available for some individuals, the number of results available for statistical analysis varied from test to test and was always slightly less than 4650. Because of the large number of subjects involved I have been unable to include in this thesis a table of all the data used for the analyses. A computer printout of all the data will be sent to any interested person on request.

2.2b Samples

Immunoglobulin E determinations were performed on serum samples that were obtained from blood taken at the time of venesection. Occasionally samples were kept at 4°C for two or three days before the red blood cells were removed. Preliminary experiments showed that such storage had no effect on IgE values observed. All serum samples were stored at -20°C.

2.2c Immunoglobulin E determinations

2.2c(i) Method

Immunoglobulin E was measured using a modification of the Rowe-modification of the Mancini technique (Rowe (1969)), (Mancini, Carbonara and Heremans (1965)). This method uses a radioactive "sandwich" technique (Figure 2.1) and autoradiography to increase the sensitivity of single radial immuno-diffusion. The further modification that I introduced used labelled specific antibody instead of a labelled γ-globulin fraction of immune serum as recommended by Rowe.

Moulds for the immuno-diffusion plates were prepared as follows:-

two pieces of glass 116mm x 97mm (one of these was siliconised) were separated by a "U" shaped brass spacer 1.0mm in thickness; the mould was held together with bulldog clips and masking tape. The use of the moulds resulted in agrose plates 10.2cm x 9cm and 1mm deep.

To prepare the plates a 1 in 2000 dilution of ε chain specific sheep anti-human IgE (Pharmacia, Uppsala, Sweden) in 1% BSA in phosphate buffered saline (PBS - 0.1M NaCl and 0.005M sodium phosphate buffer) was made; 8ml aliquots were warmed to 56°C and then mixed thoroughly with 8ml of melted 3% agrose (prepared in

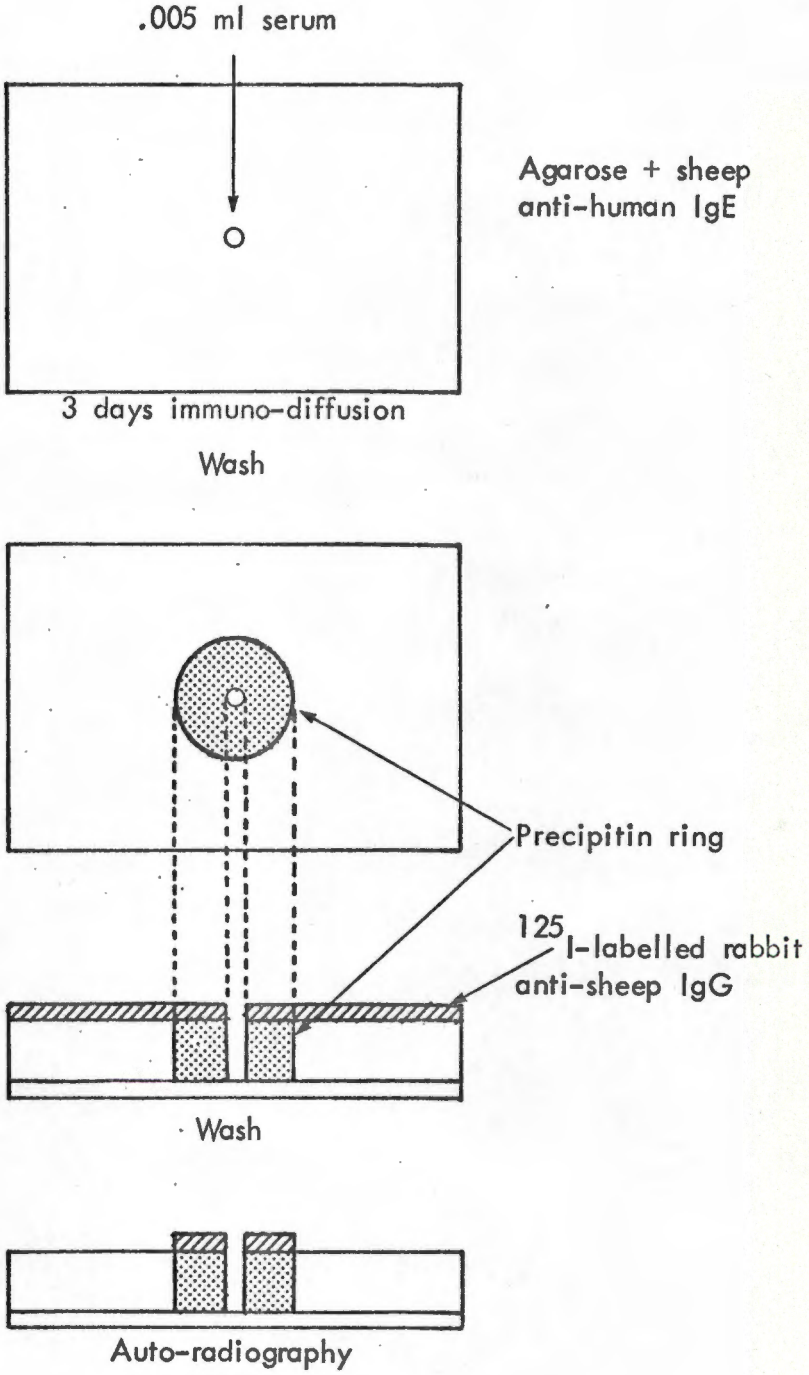


Figure 2.1

Diagramatic representation of the radio-radial immuno-diffusion method of human IgE determination.

0.1M sodium phosphate buffer pH 7.5 with 0.1% sodium azide present). The antibody-agrose mixtures were carefully poured into moulds that had been pre-warmed to about 40°C. After cooling the siliconised pieces of glass were removed and twenty 2.5mm diameter holes were cut per plate. Five microlitre samples of test serum were added to each well using a 10 µl Hamilton syringe (Hamilton Micromesure, The Hague, The Netherlands). Nearly all samples were run in duplicate and six standards in varying dilution were run per plate.

Immuno-diffusion was allowed to take place in a moist atmosphere at room temperature for three days. Great care was taken to ensure that the plates rested on a level surface. Plates were then washed for two days with PBS with at least four buffer changes.

The immuno-precipitin rings were labelled by allowing the sheep IgG in the precipitin rings to react with ^{131}I labelled anti-sheep IgG. Anti-sheep IgG had been prepared in rabbits (see Appendix IV) and specific antibody had been obtained from the anti-serum with the use of a glutaraldehyde immunoadsorbent (see Appendix V). Two hundred micrograms of this specific antibody was labelled with 2 mC ^{131}I using the method of McConahey and Dixon (see Appendix II). Plates were immersed overnight at room temperature in radioactive antibody solution. A dilution of 100 µC in 200ml of 1% BSA in PBS was sufficient to cover ten plates.

The plates were then washed for two to three days in PBS, for a variable period (1 hour to overnight) in 1M NaCl and finally, for half-an-hour with distilled water. In developing the method, it was established that this working schedule was adequate to remove unreacted ^{131}I labelled antibody.

After the plates had been dried autoradiographs were made by

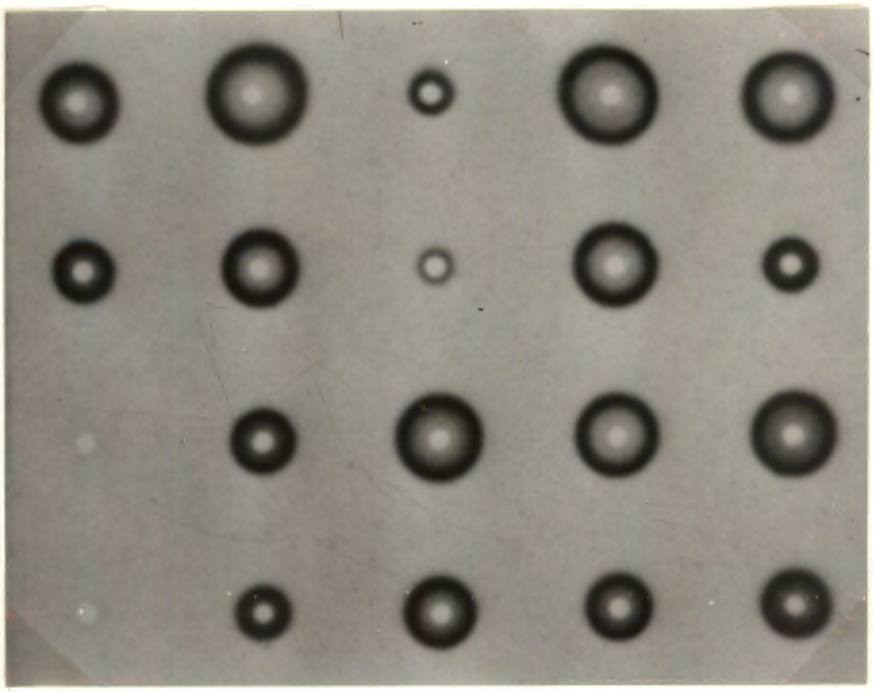


Figure 2.2

Auto-radiograph of a typical IgE plate. Standard dilutions are in the second column from the left and in the upper two holes of the middle column.

sticking the plates, face downward on to the outside of envelopes containing non-screen X-ray film (Kodirex film, Kodak, London). Plates were left firmly pressed onto the film for 48-72 hours and the films were then developed. Figure 2.2 shows a typical plate and demonstrates how clearly the immunoprecipitin rings appeared using this method. For measuring the rings, films were trans-illuminated and the diameters of the rings measured using a trans-parent plastic ruler. The final diameter used was the mean of the longitudinal and transverse diameters of the rings.

2.2c (ii) Calculations

The six standards on each plate covered the approximate range of 40-1600 u/ml. A standard curve for a particular plate was obtained by plotting, for each standard, the ring diameter squared against the known IgE concentration. Mancini et al (1965) have shown that if immuno-diffusion is allowed to proceed to completion the final area of the precipitate (and therefore the square of the ring diameter) is proportional to the amount of antigen inoculated into the well.I found, in the system that I used, that only for samples with an IgE concentration of 500 u/ml or less, and having a ring diameter of approximately 9mm or less, did this linear relationship hold true. Thus, the standard curve consisted of two portions: a linear portion and a non-linear portion. This indicated that for samples with a high IgE con-centration three days was insufficient for complete immuno-diffusion. Preliminary experiments indicated that four or five days immuno-diffusion made little difference to the shape of the standard curve, and it seemed impractical to wait until immuno-diffusion was complete for all six standards. Although most samples had IgE concentrations falling within the linear portion

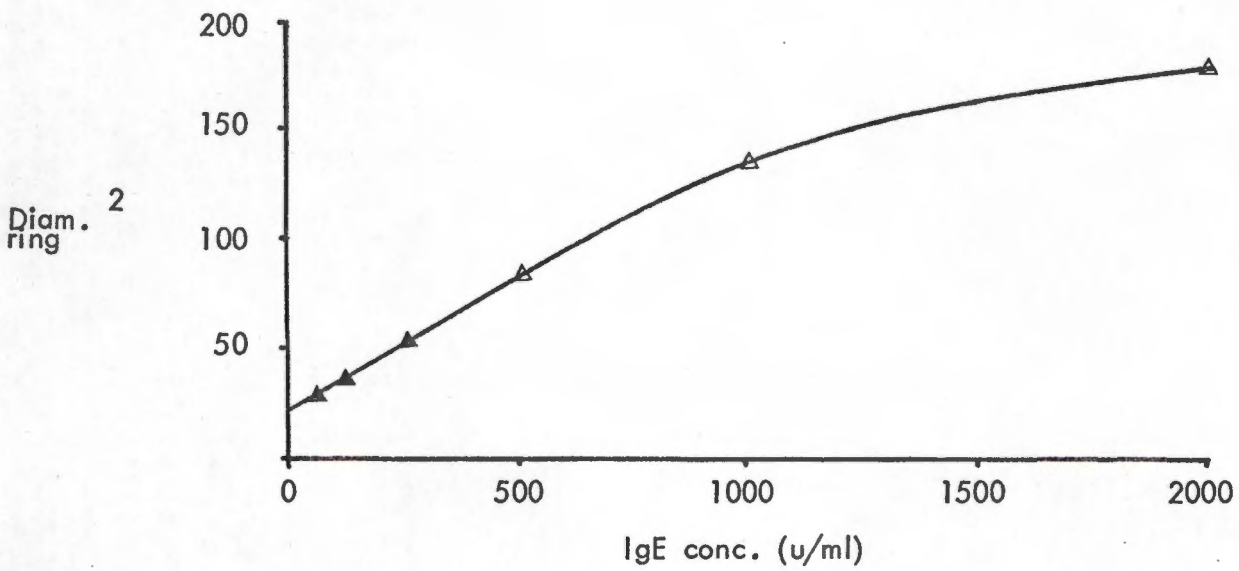


Figure 2.3

Calculated IgE standard curve for a typical IgE plate.

▲ represents points used to calculate the linear portion of the curve.

△ represents points used to calculate the logarithmic portion of the curve.

of the curve, many did not. Therefore, in an attempt to use the full range of the standard curve for calculating the IgE results, a programme was written for the Hewlett Packard Model 10 calculator and plotter that used a logarithmic correction to compensate for the deviation from linearity in the upper portion of the curve. It was found that values calculated by this method correlated extremely well with the values read directly from the standard curve. Figure 2.3 shows a plot of standard points and the calculated standard curve.

The programme first plotted all the standard points and calculated the parameters (a and b) for a least squares regression line through those points that fell within the linear portion of the curve. Thus for any sample with an IgE concentration x

$$y = ax + b \quad (1)$$

where y = square of the ring diameter

a = slope of the line

b = intercept

For each standard whose IgE concentration fell outside the linear portion of the curve, Equation (1) was used to calculate the square of the ring diameter that would have occurred had immunodiffusion proceeded to completion. The programme then calculated the difference Δy, between this extrapolated square of the diameter and the square of the measured diameter. Δy is the deviation of the curve from linearity at any particular value of y. Empirically, I found that this deviation increased logarithmically with increasing values of y. Thus for every $\Delta y > 0$ a linear relationship existed between the y and log Δy. The programme calculated the parameters (a' and b') of the least squares regression line describing this relationship so that

$y = a' \log \Delta y + b'$ (2)

where a'= slope of line

b'= intercept

The programme stored the constants a, b, a' and b' so that they could be used for calculations of the IgE concentrations of the unknowns.

The IgE concentration of any unknown whose ring diameter fell within the linear portion of the standard curve was calculated directly using Equation I.

In order to calculate the IgE concentration of any sample producing a ring diameter falling above the linear range of the standard curve it was necessary first to calculate Δy using Equation (2). As $y + \Delta y$ was the square of the diameter that would have been produced had immuno-diffusion been allowed to proceed to completion, $(y + \Delta y)$ could be substituted into Equation I. Thus

$(y + \Delta y) = ax + b$ (3)

and the IgE concentration, x, of the unknown could be calculated from (3).

The programme was written so that after the standard points had been plotted the operator decided, by inspection, on the number of standard coordinates that were to be included in the linear portion of the curve. This value was keyed into and stored in the calculator so that when the ring diameters of the unknowns were keyed in, the programme selected either Equation (1), or Equations (2) and (3), whichever was appropriate.

While acceptable for serum IgE levels in an intermediate range, the method of analysis gave very unreliable results for samples with high IgE levels. This was because of the logarithmic

nature of the relationship, over the extended curvilinear portion of the curve, between the square of the ring diameter and the IgE concentration. Wherever possible, IgE estimations were therefore repeated on dilutions of the original sample whenever the IgE concentration was greater than 800 u/ml. Dilutions were made in 1% BSA in PBS in such a way that the diluted samples had IgE concentrations that fell within the linear range of the standard curve.

A check on the validity of this method of calculation was made by comparing calculated results with the results obtained graphically using a standard curve drawn through the standard points. The results of 132 readings using nine different standard curves were compared. All readings were within the range of the standard curve but one-third of them fell outside the linear portion. The two methods of calculation yielded almost identical results with a correlation coefficient of .998 between the two sets of readings.

2.2c (iii) Calibration of IgE standards

A local standard was prepared by pooling sera from subjects with moderately raised IgE levels. Five standard dilutions were prepared by making doubling dilutions in 1% BSA in PBS. These were stored in 500 µl aliquots at -20°C until required. Each aliquot was used on only three occasions. During the survey three such standard serum pools were prepared.

The standard sera were calibrated against the W.H.O. International standard 68/341. Doubling dilutions from 1/4 to 1/64 of the international standard were run in duplicate on each of nine IgE plates. The other ten holes on each plate were used for samples of standard sera or their dilutions. A standard curve was drawn for every plate using the results obtained for the international standard and from this curve the IgE concentrations in u/ml

of the standard sera were obtained.

The mean IgE concentrations of the three local standards used were

Std. 1 1471 u/ml (S.E. of mean 28 u/ml)

Std. 2 1125 u/ml (S.E. of mean 49 u/ml)

Std. 3 1623 u/ml (S.E. of mean 40 u/ml)

2.2c (iv) Evaluation of the method

An estimation of the error of the method was made by performing twenty-six determinations of the IgE concentration of one sample; the concentration of this sample was such that it fell within the curvilinear range of the standard curve. Determinations were performed using nine different plates, and using the sample both undiluted and at appropriate dilutions. Fourteen of these dilutions had IgE concentrations within the linear range of the standard curve; for these determinations the coefficient of variation was 14%. Twelve of the dilutions had IgE concentrations that fell within the non-linear range of the standard curve; for these the coefficient of variation was 10%. The overall coefficient of variation for all twenty-six determinations was 12%. This error is considerably greater than that quoted for single radial immuno-diffusion by Mancini et al (1965). This may be partly explained by the increased sensitivity of the method. These results show that, provided the IgE levels of samples fall within the range of the standard curve, the coefficient of variation is no higher for the determinations on those samples with IgE concentrations falling in the curvilinear portion of the standard curve than those with IgE concentrations falling in the linear portion.

The lower limit of sensitivity of the method was found to be approximately 25 u/ml. This value was obtained by making doubling

dilutions of an IgE standard and determing the IgE concentration of the greatest dilution that resulted in a visible, measurable ring.

A possible source of error in this method is the occurrence, in some subjects, of heterophile antibodies that combine with components of normal sheep serum. Johannson, Bennich, Foucard and Lundkvist (1970), using bovine gammaglobulin as antigen, and a sensitive radio-immunoassay technique, detected heterophile antibodies in the serum of approximately 40% of blood donors. They found these heterophile antibodies to cross-react to a high degree with bovine, sheep, and horse gammaglobulin, less with dog, cat, pig and guinea pig gammaglobulin, and little, but significantly, with rabbit gammaglobulin. To estimate the importance of such antibodies in my own test system I prepared immuno-diffusion plates in the conventional manner except that a 1 in 2000 dilution of normal sheep plasma was used to substitute for the 1 in 2000 dilution of Pharmacia sheep anti-human IgE anti-serum. Following inoculation these plates were treated in an identical manner to the normal IgE plates. Rings would be present on the auto-radiographs of such plates only if the samples contained hetero-phile antibodies reacting with the components of sheep serum.

I used this system to estimate the occurrence of heterophile antibodies in normal blood donors. Samples from 251 blood donors were used (52 White males, 50 White females, 54 Coloured males, 50 Coloured females, 41 Bantu males and 4 Bantu females). Of these subjects 3 (2 White females and 1 Bantu male) had heterophile antibodies detectable; this represents an incidence of 1.2%. In each case the size of the ring was equal to that representing an IgE concentration of 100-200 u/ml. Unfortunately I only

considered the possibility of heterophile antibodies after I had completed my survey of serum IgE concentrations, and therefore controls were not run on all samples. This means that probably 1% of my results were falsely positive or falsely elevated.

It is apparent that the radio-radial immuno-diffusion method of IgE estimation had several disadvantages, the most serious of which was that it was insensitive to low IgE concentrations and unable to detect low normal levels. The error was quite high with a coefficient of variation of approximately 12%, and there was a 1% incidence of falsely elevated results. It had the technical disadvantage that it required two and a half weeks to complete a series of estimations.

Despite the above disadvantages, the radio-radial immuno-diffusion method was very well suited to my purpose. The method depended on direct binding of human IgE by anti IgE and was not subject to the same difficulties of non-specific inhibition that occur in the inhibition methods (Stokes, Hosking, Turner and Johannson (1973)). It had the very important practical advantage of requiring no purified human IgE. In addition it used only small quantities of anti-human IgE and each test required only a 5 µl sample of the unknown. Despite the long turn round time, the method was eminently suitable for determining IgE concentrations in large batches of samples. It was because of these factors that I was able to determine the IgE concentrations of such a large number of subjects. The difficulty of the high coefficient of variation was largely overcome by the use of non-parametric statistical tests which required only an ordinal level of measurement. Because of the lack of sensitivity of the method, I made no attempt to determine the prevalence of IgE deficiency in the populations I studied.

There was one observation that I made during the course of the survey which is of biochemical interest. Almost all rings seen on the autoradiographs of the plates were single rings. However, I very occasionally (< 0.4% of all rings) observed double rings with one ring having a diameter smaller than the other and so producing a "target-like" effect (Figure 2.4). In such cases I considered the outer ring to reflect the IgE concentrations of the sample. The reason for the double rings is not clear, certainly heterophile antibody to sheep serum was not responsible in the majority of cases. Bennich and Johannson (1971) described minor IgE components in myeloma IgE (meyloma ND) and in normal IgE present in allergic serum. They showed the minor myeloma component to have a molecular weight of 250 000. It would be of interest to establish is such a component was responsible for the additional inner rings that I observed on the autoradiographs of some samples.

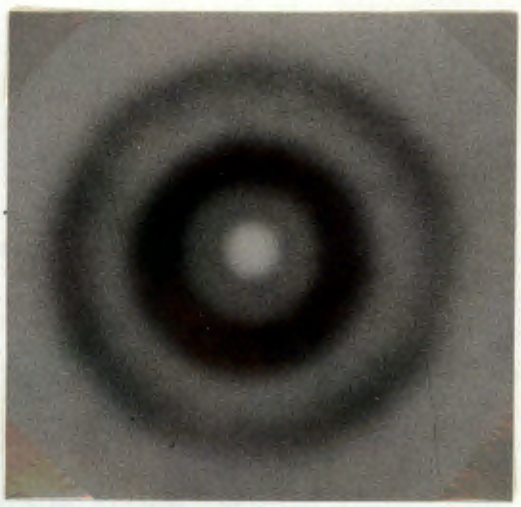


Figure 2.4

Picture of an autoradiograph of a double ring showing the "target-like" effect.

2.2.d. Blood group determinations and screening tests for syphilis and hepatitis B associated antigen.

These were done by the Western Province Blood Transfusion Service as part of their routine investigations of all blood samples.

The screening test for syphilis was a qualitative V.D.R.L. test (Bull. W.H.O. (1951)).

The test for hepatitis B associated antigen was an electro-immunodiffusion test that detected both antigen and antibody.

2.2.e. Statistical methods

The conclusions that I draw in this thesis, regarding the influence of various factors on serum IgE concentrations, rely on the results of non-parametric statistical tests. The processing of the data for these tests required the use of the University of Cape Town's Univac 1106 computer. The programmes that I wrote for the computer and the statistical tests that I employed are discussed in detail in Appendix VI. Nevertheless, since non-parametric statistical tests are less frequently used than parametric tests, I shall briefly discuss here those aspects of my methods of analysis which are particularly relevant to this section.

The frequency distributions of serum IgE concentrations in the three racial groups are shown in Figure 2.5. It is clear from this figure that in none of the population groups studied did serum IgE concentrations follow a normal curve. Also a normal distribution could not be achieved by logarithmic transformation of the data. For these reasons, and because I wished only to assume an ordinal level of measurement (that is that differences in IgE concentrations were determined only qualitatively - see Appendix VI) I used distribution-free, non-parametric statistics.

The median is the non-parametric measure of central tendency; I have used it to express the tendencies of the various population groups to have low or high serum IgE concentrations. Because the median is a measure of central tendency and not of scatter it is not entirely satisfactory for this purpose; the tendencies are best demonstrated by frequency distribution diagrams. Nevertheless, the tabulation of median serum IgE concentrations together with the results of the statistical tests, enabled me to show concisely in what population groups statistically elevated serum IgE levels occurred.

To test whether there were statistically significant differences between the serum IgE concentrations in the various population groups I used the Kruskal-Wallis one way analysis of variance by ranks. This test allows any number, k, independent samples to be compared simultaneously. The test involves calculating a statistic, H, the value of which determines whether the ranks of the variable under test are so disparate in the different samples that they are not likely to have been drawn from the same population. If the number of individuals in each sample is greater than five, and if there is no significant difference between the ranks of the variables in the k samples, then H follows an approximately Chi-square distribution with degrees of freedom (DF) equal to k-1.

The value of p that I chose to indicate significance was $p < .01$. This is a conservatively low value of p, and therefore in the tables of my results, I recorded, in brackets, values of p when they fell in the range $.01 < p < .1$. This information is occasionally useful in that it indicates trends in the differences that occurred. Although I looked for significant associations of serum IgE levels with several factors by repeatedly analysing

results that I had obtained from one population sample, grouping of individuals for each test was independent of grouping for other tests. For instance if a subject was White this did not militate against his/her being male or female and visa versa. For this reason I did not consider it necessary to choose a level of significance any lower than the one I had chosen.

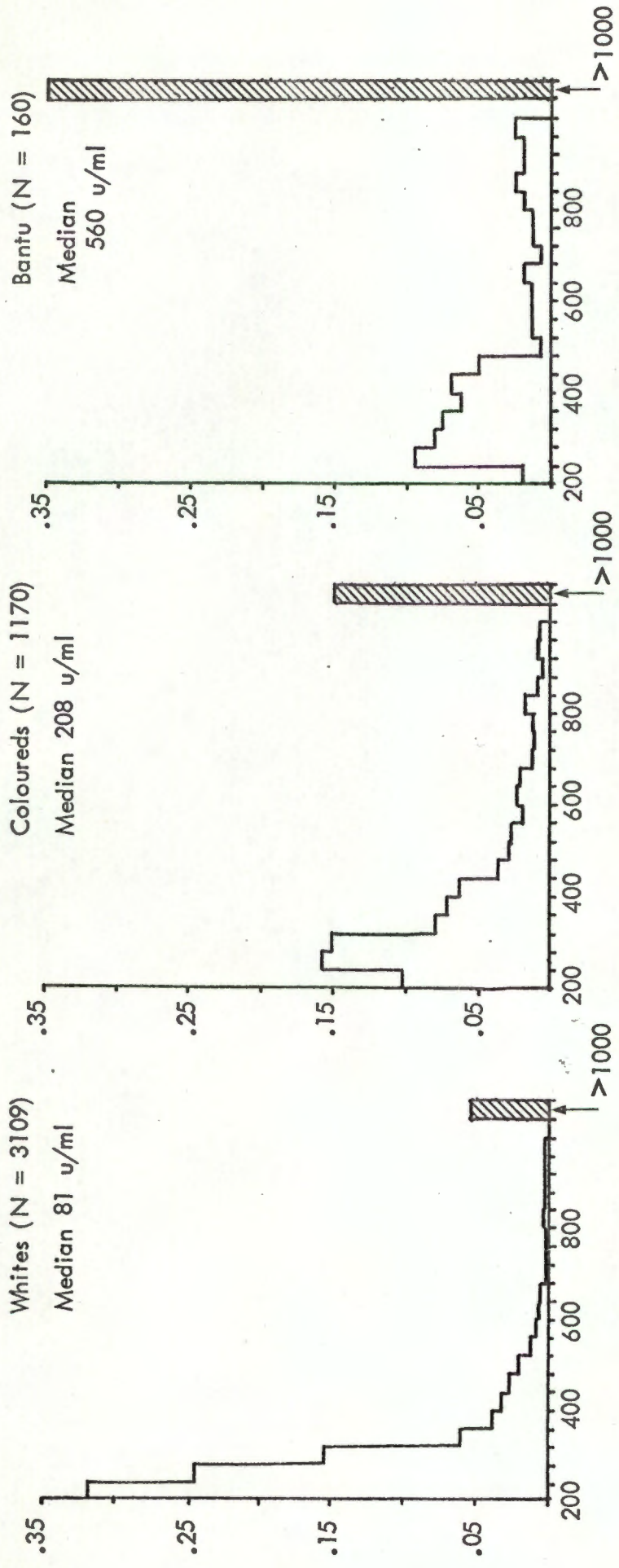


Figure 2.5

Frequency distribution patterns of serum IgE concentrations in members of the three racial groups living in the Western Cape.

2.3. Results

2.3.a. The distribution patterns of serum IgE concentrations in the three racial groups.

The frequency distribution patterns of serum IgE concentrations in the three racial groups living in the Western Cape are shown in Figure 2.5. It is evident that there was a tendency for serum IgE concentrations to increase in the order Whites - Coloureds - Bantu. Thus White blood donors (3019) had a median serum IgE concentration of 81 u/ml, while the corresponding values for Coloureds (1171) and Bantu (160) were 208 u/ml and 560 u/ml respectively. Analysis of the ranked data by the Kruskal-Wallis one way analysis of variance showed IgE levels were significantly ($p < .0005$) different in the three racial groups (Table 2.2). Moreover, as can be seen in Table 2.3, the conclusion that racial differences in serum IgE concentrations exist remained valid when males and females were considered separately.

	Whites	Coloureds	Bantu
Number	3019	1171	160
Median IgE (u/ml)	81	208	560
		N = 4350	
		H = 671 DF = 2	
		p < .0005	

Table 2.2

Results of the Kruskal-Wallis analysis of the effect of race on serum IgE concentrations in 4350 blood donors.
In this and subsequent tables

N = the total number of results analysed.
H = The statistic calculated by the Kruskal-Wallis test
DF = degrees of freedom
p = the probability that the value of the statistic presented in the table could have occurred by chance.
NS = not significant, that is $p > .01$

	Males (N=3075)			Females (N=1366)		
	Whites	Coloureds	Bantu	Whites	Coloureds	Bantu
Number	2159	772	144	950	400	16
Median IgE u/ml	85	226	642	70	180	289
	H = 463 DF = 2			H = 218 DF= 2		
	p < .0005			p < .0005		

Table 2.3

Results of the Kruskal-Wallis analysis of the effect of race on serum IgE concentrations in 3075 male blood donors and 1366 female blood donors.

2.3.b. Serum IgE concentrations in males and females.

The effect of sex on serum IgE concentrations is analysed in Table 2.4. In all races serum IgE concentrations tended to be lower in females than in males; the difference being highly significant in Whites ($p < .0005$). Frequency distributions (Figure 2.6) show that the principal difference between the distribution patterns of White males and White females lay in the relatively high incidence of very low serum IgE concentrations that occurred in females. In Coloureds and Bantu, the significance of the sex difference was only marginal ($p < .05$ and $p < .01$ respectively).

When, as shown in Figure 2.6, I plotted the frequency distributions of White males and White females over incremental intervals of 25 μ/ml, males appeared to have a bimodal distribution of serum IgE concentrations whereas females did not. Although the trough responsible for the bimodal distribution in males represents only one interval and occurred where IgE measurements

WHITES

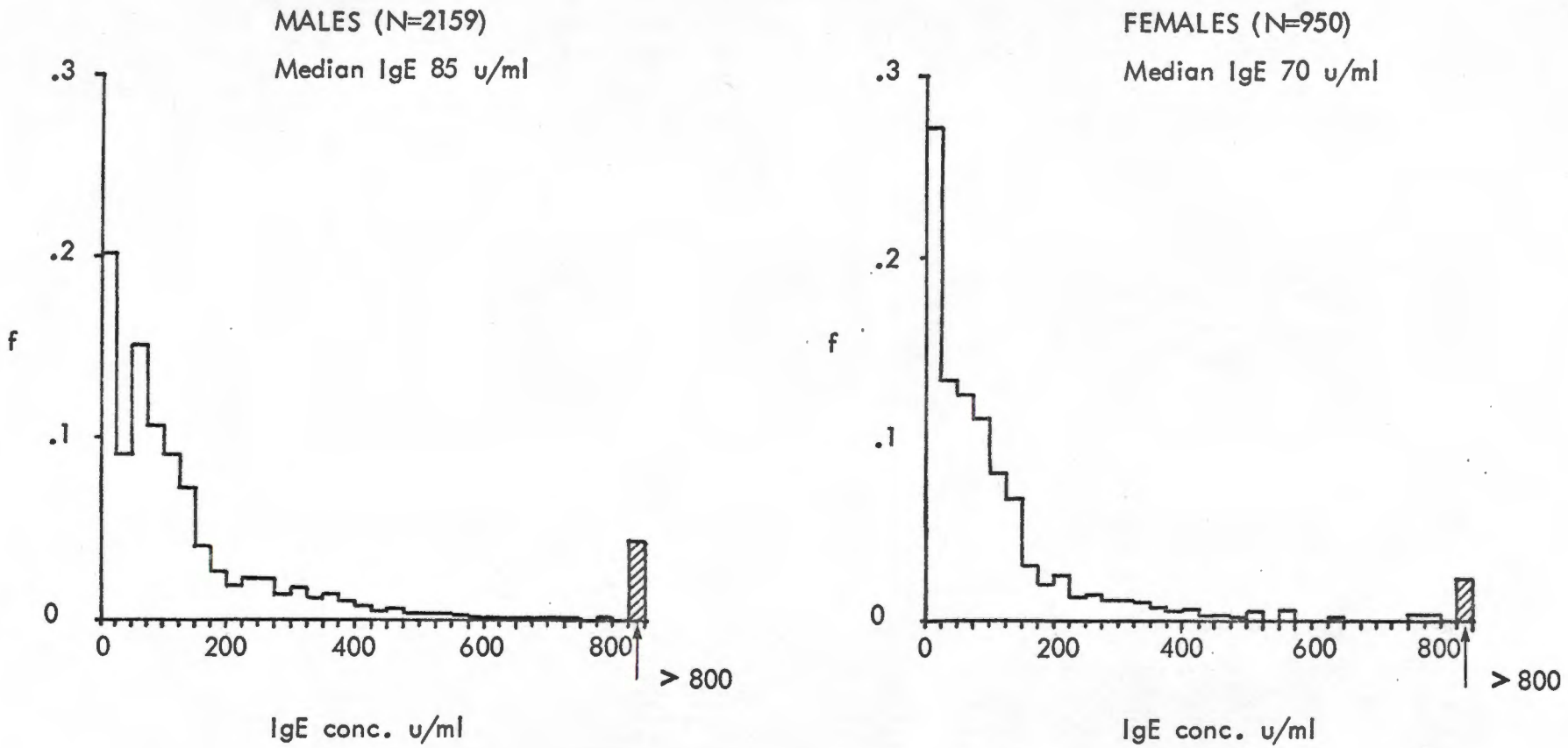


Figure 2.6

Frequency distribution diagrams of serum IgE concentrations in males and females in the White group. These diagrams have been drawn with an interval of 25 u/ml.

are technically uncertain; I feel that the observation has sufficient validity to warrant its further study using more refined and sensitive techniques. For this reason I propose to measure serum IgE concentrations in another group of blood donors using a sensitive double antibody technique that has a lower level of sensitivity in the range 1 - 5 ng/ml.

	Whites (N=3109)		Coloureds (N=1170)		Bantu (N=160)	
	Males	Females	Males	Females	Males	Females
Number	2159	950	772	398	144	16
Median IgE u/ml	85	70	226	180	642	289
	H=31.80 DF=1		H=5.00 DF=1		H=2.73 DF=1	
	$p < .0005$		NS($p < .05$)		NS($p < .1$)	

Table 2.4

Results of the Kruskal-Wallis analysis of the effect of sex on serum IgE concentrations of 3109 Whites, 1170 Coloureds and 160 Bantu blood donors.

2.3.c. Serum IgE concentrations in different age groups.

The Kruskal-Wallis analysis of variance was used to study the effect of age on serum IgE concentrations. All the subjects that I studied were blood donors aged between 18 and 65 years; this age span includes six decades and for the analysis I grouped subjects into six age groups, one for each decade.

Table 2.5 shows that serum IgE levels tended to be lower in the older age groups; this effect, although small, was statistically highly significant.

When I studied the effect of age in groups of individuals separated according to their race and sex, I found that the drop in serum IgE concentrations with advancing age was significant only in the Whites - both in males and females (Table 2.6).

Age in years	Under 20	20-29	30-39	40-49	50-59	Over 60
Number	383	1732	925	686	409	78
Median IgE u/ml	119	114	108	92	81	69

N = 4213

H = 41.57 DF = 5

p < .0005

Table 2.5

Results of the Kruskal-Wallis analysis of the effect of age on the serum IgE concentrations of 4213 blood donors.

Bantu males in the age group 40-49 years appeared to have higher IgE concentrations than individuals in the other age groups, but the significance was only $p < .02$.

2.3.d. Serum IgE concentrations in males and females in different age groups.

In the White population the difference in serum IgE concentrations between the two sexes was only significant in certain age groups, being minimally significant in those under 30, highly significant in those in their thirties and forties, minimally significant in those in their fifties, and not significant at all in people sixty and over (Table 2.7). Also I found that a bimodal distribution of serum IgE concentrations did occur in White females in those age groups in which there was no significant sex difference in serum IgE concentrations. Figures 2.7 and 2.8 show the frequency distributions in each sex in the two age groups 20-29 and 40-49 years respectively. The frequency distributions have been plotted using intervals of 25 u/ml. Figure 2.7 shows that in the 20-29 age group there was little difference between serum IgE concentrations in males and females and that in both groups

White Males (N = 2050)						
Age in years	Under 20	20-29	30-39	40-49	50-59	Over 60
Number	172	824	452	329	229	44
Median IgE u/ml	108	91	89	79	72	57
		H = 26.09 DF = 5 p < .0005				
White Females (N = 895)						
Age in years	Under 20	20-29	30-39	40-49	50-59	Over 60
Number	99	355	150	160	106	25
Median IgE u/ml	78	84	60	48	46	64
		H = 25.40 DF = 5 p < .0005				
Coloured Males (N = 724)						
Age in years	Under 20	20-29	30-39	40-49	Over 50	
Number	60	323	182	115	44	
Median IgE u/ml	181	214	242	262	417	
		H = 6.05 DF = 4 NS				
Coloured Females (N = 381)						
Age in years	Under 20	20-29	30-39	40-49	Over 50	
Number	41	184	87	48	21	
Median IgE u/ml	225	170	156	163	236	
		H = 4.04 DF = 4 NS				
Bantu Males (N = 133)						
Age in years	Under 20	20-29	30-39	40-49	Over 50	
Number	7	39	45	31	11	
Median IgE u/ml	848	312	481	4207	839	
		H = 13.19 DF = 4 NS (p < .02)				

Table 2.6

Results of the Kruskal-Wallis analysis of the effect of age on the serum IgE concentrations in blood donors in both sexes and in each race group. There were insufficient Bantu females for analysis.

Whites

Age in years	Under 20 (N=271)		20-29 (N=1179)		30-39 (N=602)	
	Males	Females	Males	Females	Males	Females
Number	172	99	824	355	452	150
Median IgE u/ml	108	78	91	84	89	60
	H=5.87 DF=1		H=4.57 DF=1		H=17.17 DF=1	
	NS($p < .02$)		NS($p < .05$)		$p < .0005$	

Age in years	40-49 (N=489)		50-59 (N=335)		Over 60 (N=69)	
	Males	Females	Males	Females	Males	Females
Number	329	160	229	106	44	25
Median IgE u/ml	79	48	72	46	57	64
	H=14.96 DF=1		H=8.58 DF=1		H=0.03 DF=1	
	$p < .0005$		$p < .005$		NS	

Table 2.7

The effect of age on the sex difference in serum IgE concentrations in White blood donors.

serum IgE concentrations followed a bimodal distribution. In the 40-49 age group, however, there was a highly significant difference in serum IgE concentrations with no bimodal distribution in females (Figure 2.8).

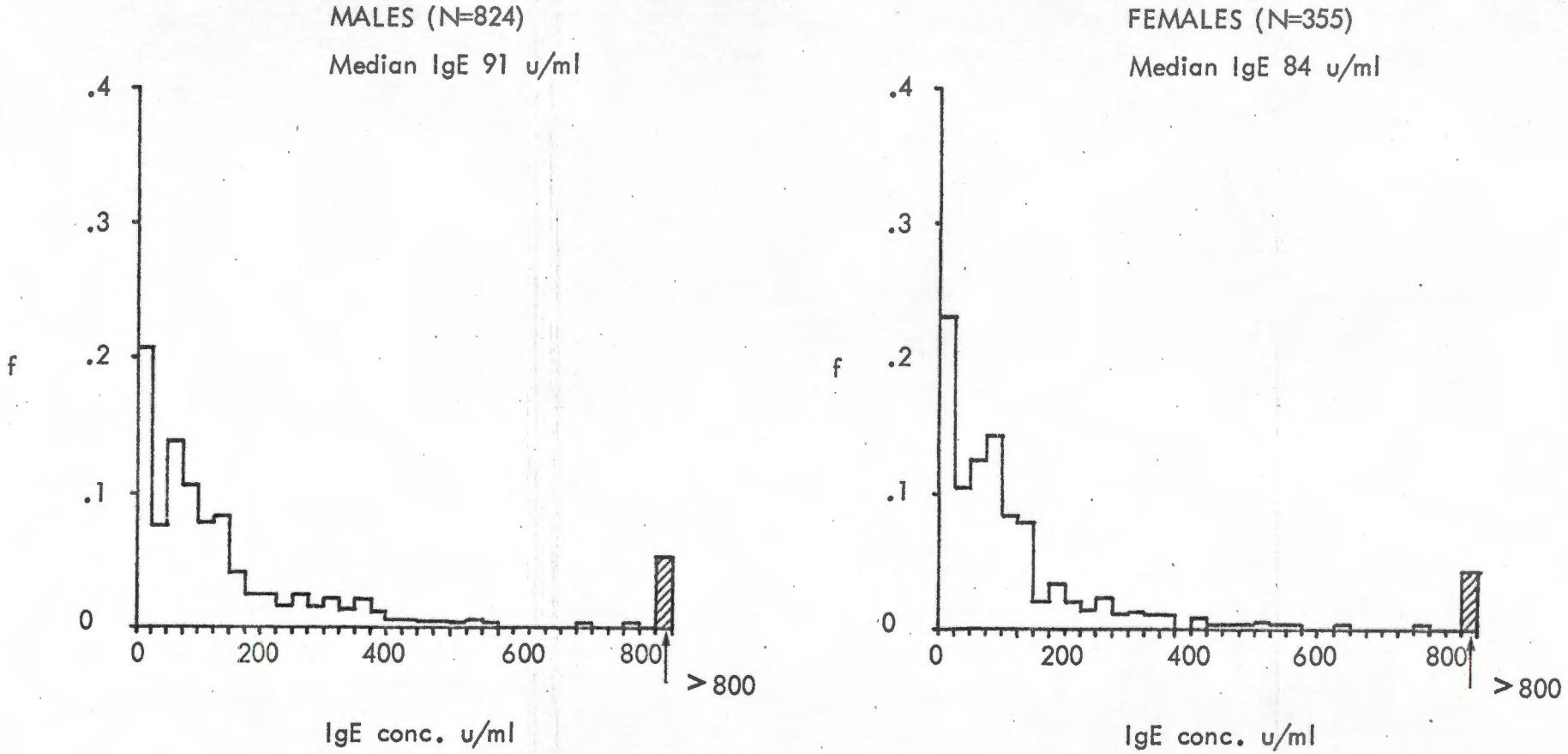


Figure 2.7

Frequency distribution diagrams of serum IgE concentrations in White males and White females in the age group 20-29 years.
These diagrams have been drawn with an interval of 25 u/ml.

WHITES AGE 40-49

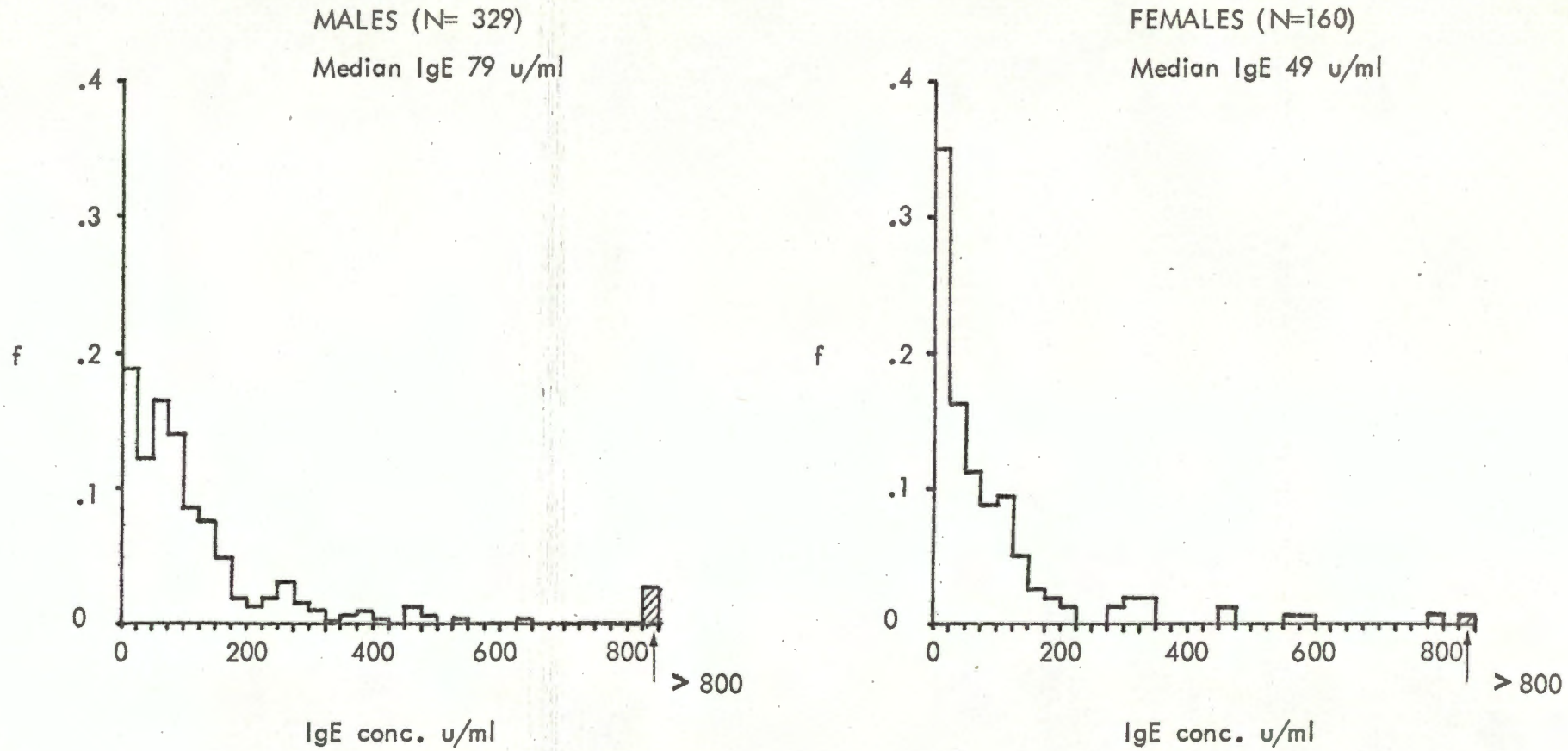


Figure 2.8

Frequency distribution diagrams of serum IgE concentrations in White males and White females in the age group 40-49 years.

These diagrams have been drawn with an interval of 25 u/ml.

	White Females (N = 949)		Coloured Males (N = 722)	
	VDRL-ve	VDRL+ve	VDRL-ve	VDRL+ve
Number	942	7	740	32
Median IgE u/ml	70	91	223	334
	H = 1.80	DF = 1	H = 3.75	DF = 1
	NS		NS	

	Coloured Females (N = 400)		Bantu Males (N = 144)	
	VDRL-ve	VDRL+ve	VDRL-ve	VDRL+ve
Number	388	12	134	10
Median IgE u/ml	177	390	742	206
	H = 3.07	DF = 1	H = 4.84	DF = 1
	NS		NS ($p < .05$)	

Table 2.8

The Kruskal-Wallis analysis of the relationship between serum IgE concentrations and the results of the VDRL test. There were insufficient White males and Bantu females with positive tests for the results to be analysed in these groups.

2.3.e. The relationship between serum IgE concentrations and the results of V.D.R.L. and Hepatitis B antigen tests.

Syphilis and viral hepatitis are both diseases in which the immunity of the host is believed to play an important role in the disease process. The Western Province Blood Transfusion Service routinely screens all sera for evidence of these diseases and this afforded the opportunity to determine whether there were any associations between these particular infections and elevated serum IgE levels (Tables 2.8, 2.9 and 2.10).

	White Males (N = 2151)	
	HB.Ag-ve	HB.Ag+ve
Number	2141	10
Median IgE u/ml	85	118
	H = 0.01 DF = 1	
	NS	
	Coloured Males (N = 767)	
	HB.Ag-ve	HB.Ag+ve
Number	745	22
Median IgE u/ml	223	303
	H = 2.23 DF = 1	
	NS	
	Bantu Males (N = 141)	
	HB.Ag-ve	HB.Ag+ve
Number	131	10
Median IgE u/ml	557	1113
	H = 2.478 DF = 1	
	NS	

Table 2.9

The Kruskal-Wallis analysis of the association of serum IgE levels with the results of the test for hepatitis B associated antigen (HB.Ag). All sera were negative for antibody to HB.Ag (HB.Ab-ve). In all race groups there were less than six HB.Ag+ve females and therefore results were analysed in males only.

	White males		White males	
	HB.Ag-ve Ab-ve*	HB.Ag-ve Ab+ve+	HB.Ag+ve Ab-ve∇	HB.Ag-ve Ab+ve+
Number	2141	7	10	7
Median IgE u/ml.	85	320	118	320
	N = 2148		N = 17	
	H = 6.81 DF = 1		H = 5.50 DF = 1	
	p < .01		NS (.01 < p < .02)	

Table 2.10

Kruskal-Wallis analysis of the association between elevated serum IgE levels and the presence of antibody to hepatitis associated antigen.

* HB.Ag-ve Ab-ve = hepatitis B associated antigen negative/antibody negative.

\+ HB.Ag-ve Ab+ve = hepatitis B associated antigen negative/antibody positive.

∇ HB.Ag+ve Ab-ve = hepatitis B associated antigen positive/antibody negative.

I failed to find any such association except in the case of White males where a significant association ($.005 < p < .01$) between elevated serum IgE levels and the presence of antibody to hepatitis B associated antigen was found (Table 2.10). None of the other population groups had more than five individuals with this antibody in their serum; therefore the analysis had to be restricted to White males and unfortunately even in this group there were only seven antibody positive subjects.

Nevertheless, when I compared the serum IgE concentrations of these antibody positive subjects with those of a group of White males who were antibody negative but hepatitis associated antigen positive, the results again showed a tendency ($p < .02$) for serum IgE concentrations to be elevated in the antibody positive group.

	White Males (N = 2150)			
	A	B	AB	O
Number	819	230	103	998
Median IgE u/ml	79	94	82	91
	H = 5.816 DF = 3 NS			
	White Females (N = 946)			
	A	B	AB	O
Number	322	106	49	469
Median IgE u/ml	68	70	71	70
	H = 0.289 DF = 3 NS			
	Coloured Males (N = 771)			
	A	B	AB	O
Number	235	188	50	298
Median IgE u/ml	214	243	264	226
	H = 0.559 DF = 3 NS			
	Coloured Females (N = 398)			
	A	B	AB	O
Number	142	89	35	132
Median IgE u/ml	183	162	286	157
	H = 6.112 DF = 3 NS			
	Bantu Males (N = 144)			
	A	B	AB	O
Number	57	25	11	51
Median IgE u/ml	325	557	564	999
	H = 5.842 DF = 3 NS			

Table 2.11

Kruskal-Wallis analysis of the relationship between serum IgE concentrations and the presence of the different ABO blood group antigens. For this analysis the sub-groups of A were grouped with A.

	White Males (N = 2150)		White Females (N = 946)	
	Rh+ve	Rh-ve	Rh+ve	Rh-ve
Number	1832	318	806	140
Median IgE u/ml	86	85	71	59
	H = 0.72 DF = 1		H = 2.81 DF = 1	
	NS		NS	

	Coloured Males (N = 771)		Coloured Females (N=399)	
	Rh+ve	Rh-ve	Rh+ve	Rh-ve
Number	748	23	387	12
Median IgE u/ml	226	291	182	148
	H = 0.25 DF = 1		H = 0.23 DF = 1	
	NS		NS	

Table 2.12

Kruskal-Wallis analysis of the relationships between serum IgE concentrations and the presence of the Rh blood group antigen. There were insufficient Rh-ve Bantu subjects for the results of the tests in the Bantu to be analysed.

2.3.f. Serum IgE concentrations in persons with different ABO and Rh blood groups.

It is well recognised that the presence or absence of certain of the blood group antigens is related to the susceptibility of individuals to certain diseases. I accordingly compared serum IgE concentrations in individuals belonging to the different ABO or Rh blood groups. Results are shown in Tables 2.11 and 2.12. In no instance did I find any association between the blood groups of the individuals tested and their serum IgE concentrations.

2.4 Discussion

The epidemiological survey reported in this Chapter was undertaken with two broad objectives in mind. Firstly, it was my intention to establish, empirically, the range and distribution of serum IgE concentrations that occurred in adult inhabitants of the Western Cape. Secondly, I hoped that the results of the survey would, on analysis, expose population differences that might contribute to an understanding of the factors that regulate the IgE immune response, or provide the basis for further study in this regard.

These aims were realised to the extent that IgE measurements were performed on serum samples obtained from 4650 randomly selected blood donors, so providing an indication of the levels of this immunoglobulin one may regard as "normal" and the way in which they are distributed. Analysis of the data showed that the values obtained were significantly influenced by race, sex and age.

2.4.a. Serum IgE concentrations : normal values.

Before considering the information presented in this Chapter, I think it appropriate to examine in medical terms, the conceptual meaning of the word "normal". When discussing the values for a particular constituent of human serum, use of the word "normal" usually implies:

(i) that measurements of the concentration of the constituent have been made on serum obtained from a representative sample of the healthy population. This in turn implies that the population sample studies was judged, *a priori*, and by independent criteria, to be free of relevant disease. In most instances, questions relating to the acceptability of these criteria, the relevance of associated diseases, and the importance of effects such as sex, age and environment

are straightforward issues that are easily resolved by the consensus of knowledge at the time. Altitude, for example, is known to influence the haematocrit so that results obtained on blood samples from persons living at high altitude could not be used to establish normal values for individuals living at sea level.

On occasion it may be necessary to define the circumstances prevailing at the time of taking the blood sample; this places a constraint on the meaning of "normal" - for example one may speak of normal *fasting* blood sugar or normal antibody titre *three weeks after injection of antigen.*

(ii) that the values are distributed in a manner that is amenable to description either in parametric (e.g. mean $\pm$ standard deviation) or non-parametric (median and percentile) terms, that a mode exists, and that both upper and lower values can be defined between which the values of most (e.g. 67% or 95%) of the values obtained for normal people fall.

The further implication here is that the method used for measurement was sufficiently sensitive to quantitate, with reasonable accuracy, those values that fall near the lower limit of the range and hence isolate and define the mode. In some cases, particularly where the accuracy of the technique varies with concentration in a proportional rather than an absolute manner, logarithmic transformation of the data is used to approximate a Gaussian distribution and the geometric mean is used.

In light of these considerations it would be misleading to present the data in this Chapter as valid for the establishment of normal values without qualifying discussion.

The first question that arises concerns the acceptability

of regarding the sample as representative of the normal healthy population. Most blood donors self select for good health, but, although at the Western Province Blood Transfusion clinics they are questioned, on enrolment, for a history of concurrent or past illness, jaundice is the only illness that automatically excludes them from donating blood. Thus blood donors include among their number both a proportion who suffer from allergic symptoms and a proportion who are, or who have recently been, infested with helminths (see Chapter III). Both these conditions are believed to influence serum IgE concentrations so that my results can only be used to establish the normal levels for the population as a whole, and not the normal levels for the non-allergic, non-infested section of the population. The age of all subjects was in the range 18 - 65 years; Johannson (1968) has shown that adult serum IgE concentrations are attained at approximately seven years of age; therefore my subjects fell in a range suitable both for establishing normal adult levels, and for studying the effect of advancing age on serum IgE concentrations.

The method that I used to determine serum IgE concentrations was insensitive and imprecise below 25 u/ml with the result that a lower limit of normal could not be defined. The method was relatively inaccurate for high values; the scatter of the upper values was, in all groups, extremely wide; and neither the data nor the log-transformed data followed a normal distribution or identified a clearly defined mode. For these reasons the usual parameters used to define population characteristics such as arithmetic or geometric mean and standard deviation would have little meaning.

Despite the problems involved, I felt that there was merit in attempting to formulate some idea of serum IgE concentrations one

might regard as "normal". This information would have clinical usefulness if elevated levels could be used to support the diagnosis of allergy or the allergic "diathesis"; to suggest that an individual had been exposed to an immunological stimulus, such as helminthic infestation, that would be particularly liable to evoke an IgE response; or to assist in the diagnosis and management of other diseases where an empirical association with elevated IgE levels has been observed (e.g. Wiskott-Aldrich Syndrome). Furthermore, if a lower limit of normal serum IgE concentration could be established, this limit might be considered to define a particular form of immuno-deficiency. Polmar, Waldmann, Balestra, Jost and Terry (1972), using a sensitive double antibody technique, attempted to define such a limit by selecting the tenth percentile (15 ng/ml) as the lower limit of normal. They found that in control subjects IgE deficiency, defined in this way, did not have any particularly striking clinical associations; it was, however, found with increased frequency in subjects with IgA deficiency and ataxia telangectasia.

In view of the technical difficulties in measuring low serum IgE concentrations, I have confined my consideration to the upper limits of the normal range. In other words, I have used my data to provide a tentative answer to the question, "when does one consider a serum IgE concentration to be elevated?". I have arbitrarily chosen to consider individuals with serum IgE concentrations above the 75th percentile as having moderately elevated levels, and those with IgE concentrations above the 95th percentile as having elevated levels. The values for the 75th and 95th percentiles, together with the medians, for both sexes and in each race group are given in Table 2.13. Thus, considering only the results I obtained

in Whites, males with serum IgE concentrations in the range 172 - 648 u/ml, and females with serum IgE concentrations in the range 136 - 460 u/ml, can be considered as having moderately elevated levels; individuals with serum IgE concentrations greater than these limits can be considered as having definitely elevated levels.

	Whites		Coloureds		Bantu	
	Males	Females	Males	Females	Males	Females
Number	2159	950	773	398	144	16
Median IgE u/ml	85	70	226	180	642	289
Upper 75th percentile u/ml	172	136	564	446	1765	715
Upper 95th percentile u/ml	648	460	4096	2331	18519	2885

Table 2.13

Values of the median, upper 75th and upper 95th percentile serum IgE concentrations found in male and female blood donors belonging to the White, Coloured and Bantu racial groups.

2.4.b. Values of normal serum IgE concentrations as reported by others.

Table 2.14 presents some of the data on normal serum IgE concentrations found by other workers, all of whom studied Caucasian subjects. In most cases results have been expressed in ng/ml. The number of nanograms per unit has not yet been definitively established. Johannson (1973) considers that, using a solid phase radio-immunoassay - the radio-immunosorbent technique (RIST)- nanograms and units are equivalent; whereas Bazeral and Hamburger (1972), using a different solid phase radio-immunoassay, found that there were 2.4 ng per unit.

The selection of subjects and the methods used for performing the determinations are included in Table 2.14. Despite the difficulties

Reference	Type of subjects and number of subjects	Mean or Median IgE	Range	Type of assay used
Johansson, Bennich and Wide (1968)	Unselected blood donors (62)	Mean 430 ng/ml.	110 ng/ml. to 5900 ng/ml.	Solid phase radio-immuno-assay (RIST)
Johansson (1968)	Non-allergic adults (125)	Mean 248 ng/ml.	66 ng/ml. to 1830 ng/ml.	Solid phase radio-immuno-assay (RIST)
Rowe and Wood (1970)	Adult blood donors (125)	-	64% undectable i.e. < 1% Standard 68/341 i.e. < ± 93u/ml to ±1500 u/ml.	Radio-active single radio-immuno-diffusion.
Hogarth-Scott, Howlett, McNicol, Simons and Williams (1971)	Non-asthmatic 10 year old children (37)	Mean 113 ng/ml.	50% less than 50 ng/ml. to 1000-1499 ng/ml.	Radio-active single radio-immuno-diffusion.
Bazeral, Orgel and Hamburger (1971)	Unselected adult females post-partum (35)	Mean 205 ng/ml.	19 ng/ml. to 810 ng/ml.	Solid phase radio-immuno-assay with low levels determined by a special absorption technique (see text).
Gleich, Averbeck & Swedland (1971)	Non-allergic adults	Mean 179 ng/ml. Median 80 ng/ml.	1 ng/ml. to 2700 ng/ml.	Double antibody i.e. liquid phase radio-immuno-assay.

Table 2.14

Reported serum IgE concentrations in non-allergic or unselected groups of individuals.

referred to earlier most authors have used either the arithmetic mean or the geometric mean to express the central tendency of their results. Although differences in technique and the way in which results are expressed make absolute comparisons difficult, it is interesting to compare the differences in the distribution patterns obtained using the various methods; the differences being most obvious in the determination of low IgE concentrations. Johannson and co-workers (Johannson, Bennich and Wide (1968a), Johannson (1967), Johannson (1968)) have used the solid phase radio-immunoassay extensively, and have found the lower limit of serum IgE concentrations to vary between 66 and 110 ng/ml. Allowing for the possibility that one unit of IgE is equivalent to two nanograms, and taking into consideration the lack of sensitivity of the method I used, the distribution patterns found by Johannson and co-workers show clearly that they did not find the high incidence of very low serum IgE concentrations that I found (25% of Whites had IgE concentrations of less than 25 u/ml). There is some evidence (Bazeral, Orgel and Hamburger (1971), Stokes et al (1973)) that there are non-specific blocking factors in certain biological fluids that interfere with solid phase radio-immunoassay at low IgE concentrations. Bazeral et al (1971) dealt with this difficulty by radio-immunoassay before and after specific immuno-absorption with anti-IgE and regarded the difference between the two readings as the true measurement of the IgE content of the sample. The non-specific blocking factors give rise to falsely elevated IgE values so that their presence would explain the difference between Johannson's data and mine. Rowe and Wood (1970) and Hogarth-Scott, Howlett, McNicol, Simons and Williams (1971), who used the radio-radial diffusion method, and Gleich, Averbeck and Swedlund (1971), who used a double antibody technique, found distribution patterns of serum IgE concentrations

that were similar to those that I observed in Caucasians.

2.4.c. The influence of race on serum IgE concentrations.

The elevation of serum IgE concentrations that I found in the Coloureds and the Bantu was due to both a broadening of the distribution pattern and to an increased frequency of high concentrations (Table 2.2, Figure 2.5); these effects were most pronounced in the Bantu. A similar elevation of serum IgE concentrations in an African population was reported by Johannson et al (1968b) who found that serum IgE concentrations were sixteen to twenty times higher in Ethiopian pre-school children than Swedish pre-school children. Although the subjects who I studied lived in close geographical proximity to one another, the differences between the environmental circumstances of the Whites and the Bantu were, in all likelihood, as great as those between the Ethiopian and Swedish children studied by Johannson et al (1968b). Other forms of hypergammaglobulinaemia have been reported in African populations (Andes, Kampeir and Adams (1935), Michaux (1966), Turner and Voller (1966) and Rowe, McGregor, Smith, Hall and Williams (1968)) and have been ascribed to the development of immunity to various bacterial, viral, protozoal and helminthic diseases. Johannson et al (1968b) suggested that the elevated serum IgE levels that they found in Ethiopians reflected a high incidence of helminthic infestation, particularly with Ascaris.

The pronounced racial differences in serum IgE concentrations that I observed could not adequately be explained with the information that I had available at the termination of this survey. To elucidate the contribution of allergy and parasitic infestation to these racial differences, and to investigate the inter-relationships between the various forms of hypergammaglobulinaemia in Africans, I undertook a

further survey in which subjects were questioned for symptoms of allergy, stools were examined, and serum levels of IgG, IgA and IgM were determined in addition to IgE levels. I will present and discuss the findings of that survey in Chapter III.

2.4.d. The influence of sex on serum IgE concentrations.

Analysis of the data obtained from White blood donors showed a highly significant tendency for the serum IgE concentrations of males to be higher than those of females. I know of no other similar reports. Other workers (Johannson (1968) and Gleich et al (1971)) looked for a sex difference in serum IgE concentrations but failed to find one. The difference, in terms of absolute values, was small, and would probably have been undetectable without the analysis of a large number of results.

Previous reports have indicated that the human allergic immune response is not identical in males and females. Thus Davidson, Baron and Walzer (1947) actively sensitized individuals with an extract of Ascaris lumbricoides and measured reagin formation by direct skin testing. They found that although 90% of their subjects became sensitive, this sensitivity developed more rapidly in males than females. Morrison-Smith (1961) carried out a survey of the occurrence of asthma in 49,000 school-children and found the prevalence of asthma in five year old boys to be twice that in five year old girls (2.58% and 1.02% respectively). Although they found that the difference tended to diminish with age, and although Williams (1959) found that figures for adults from various parts of the world did not indicate any sex difference in the prevalence of asthma, Morrison-Smith's data does demonstrate that at some stages of life males have a greater tendency to this type of allergic response than females.

In contrast to my finding with IgE concentrations, IgM

concentrations have been found to be higher in females than males (Butterworth, McCellan and Allensmith (1967)). Although these workers found no sex difference in IgG concentrations, other workers, such as Berg and Johannson (1969) report finding higher serum IgG concentrations in females than males. It would seem that at least one of the factors responsible for the sex difference in IgM concentrations is genetic. Two groups of workers have reported findings which strongly suggest that the X chromosome in man carries genes which influence IgM concentrations. Rhodes, Markham, Maxwell and Monk-Jones (1969) found that persons possessing three X chromosomes had higher IgM concentrations than normal females, and Grundbacher (1972) measured IgM concentrations in members of family groups and found that the serum IgE concentrations of boys correlated more closely with those of their mothers than with those of their fathers.

My tentative finding of a bimodal distribution of serum IgE concentrations in White males but not in White females (Figure 2.6) suggested to me that genetic factors may be responsible for determining the sex difference in serum IgE concentrations. Closer inspection of my results, however, indicates that other factors were involved. Thus the sex difference depended on age, inasmuch as, it was most marked in the middle years of life (30-49 years). Moreover, the bimodal distribution of serum IgE concentrations was exclusive to males only in those age groups in which serum IgE concentrations were significantly higher in males than females. The finding that females in certain age groups did show a bimodal distribution (Figure 2.7) indicates that the bimodal distribution observed in males was not due to a simple case of a sex linked gene coding for "enhanced" IgE production.

It seems unlikely that environmental factors alone were responsible for the sex difference in serum IgE concentrations. In

Whites, parasitic infestation was too infrequent to play a major role in determining IgE concentrations (see Chapter III) and exposure to inhalant allergens must have been nearly identical in the two sexes. If anything females were more exposed as they were often in contact with the potent allergens in house dust.

The immune system in man is highly complex and the initiation and control of immune responses are influenced by many factors. Humoral factors, either by direct action, or by their influence on the internal environment, probably play a role. There have been reports that females develop higher specific antibody titres after immunization than do males; Rowely and Mackay (1969) immunized individuals with Salmonella adelaide and found higher titres in females than in males. In this regard feedback control may be important; thus high levels of specific IgG or IgM may influence IgE production. Strannegård and Belin (1970), working in rabbits, and Ishizaka and Okudaira (1972), working in mice, found that passively administered specific IgG suppressed reagin production, whereas passively administered specific IgM sometimes enhanced reagin production (Strannegård and Belin (1971)). Thus the sex difference in serum IgE levels is probably part of a general difference in immune responsiveness that exists between males and females.

2.4.e. <u>The influence of advancing age on serum IgE concentrations.</u>

To the best of my knowledge, the results of this survey provide the first documented evidence of a significant tendency for serum IgE concentrations to be lower in older age groups. Johannson (1968) noted this tendency, but on analysing his data was unable to establish its statistical significance.

There is a certain amount of circumstantial evidence to suggest that immunological function is subject to decay with advancing age, and it may be that the general lowering of serum IgE concentrations

in the elderly is part of this general and non-specific process of aging. Human lymphoid tissues develop rapidly in early life and involute earlier than many other body tissues (Krumbhaar (1942)). Studies of the natural iso- and hetero-agglutinins have shown that titres rise to a maximum at approximately 10 years of age and then gradually decline (Thomsen and Kettel (1929)). Makinodan and Petersen (1964) stressed that age-associated changes in antibody-forming capacity do not necessarily reflect changes in either the number or efficiency of antibody-forming cells; changes in those factors which maintain homeostasis may be equally important. Nevertheless, from their results with adoptive transfer experiments in mice, they concluded that changes in the primary antibody-forming potential of the spleen were due mainly to changes in the number of antibody-forming cell precursors. Price and Makinodan (1972 a and b), once again on the basis of adoptive transfer experiments, suggested that the internal environment of older animals was relatively unfavourable for antibody production and that both T and B cells from old animals had a decreased ability to undergo growth and proliferation. Heidrick and Makinodan (1973) showed that there was no impairment in the ability of adherent spleen cells from old mice to co-operate with non-adherent cells during the initiation of an immune response to sheep red blood cells.

The effect of age on total immunoglobulin levels was studied by Buckley and Dorsey (1970). They measured immunoglobulin levels in U.S. residents who were mainly, but not exclusively, Caucasians, and found that, after the third decade, serum IgG and IgM concentrations tended to be lower, whereas mean IgA concentrations were unchanged. Age-associated changes in immunoglobulin levels appear to differ in different race groups; thus Rowe et al (1968) found that, in Gambians, IgG, IgM and IgA concentrations were higher in older age

groups. My observations were similar inasmuch as I found that the striking inverse relationship between age and serum IgE concentrations observed in Whites was not apparent in the Coloureds or the Bantu.

The complex inter-relationships between age and other factors influencing serum IgE concentrations are illustrated by my finding that the sex difference that I observed in the serum IgE concentrations of Whites was only significant in certain age groups and disappeared altogether in the older age groups. Thus elderly White subjects tended to have uniformly low IgE levels.

Unfortunately blood donors over the age of sixty are relatively rare with the result that I had insufficient elderly subjects in the follow-up survey (see Chapter III) to investigate the inter-relationship between serum IgE concentrations and allergy in the elderly.

Since the survey reported in this Chapter was "cross-sectional" rather than "longitudinal" in design, the findings with regard to age might be interpreted as suggestive that elevated serum IgE levels during young adulthood militate against longevity, or that state of health, in later years, which is required of blood donors. In other words, the older groups in the sample I analysed may have been selectively depleted of individuals with raised serum IgE concentrations.

Whatever the reasons may be, the data that I have accumulated are insufficient to bear the weight of any definite conclusion regarding the factors responsible for the tendency of older subjects to have generally lower serum IgE concentrations. Until the appropriate prospective studies have been completed, and a more direct approach to the regulation of IgE responses has been explored, the cause of the age effect must remain speculative.

2.4.f. The relationship between elevated serum IgE levels and the presence of antibody to hepatitis B associated antigen.

Antibody to hepatitis B associated antigen was detected in 18 of the 4436 blood donors tested; this represents a prevalence of approximately 0.4%. It was only in White males that sufficient numbers of antibody positive subjects were available for analysis. In this group, the seven antibody positive subjects had significantly elevated ($p < 0.1$) serum IgE levels compared with the 2141 who were antigen negative/antibody negative, and elevated levels ($p < 0.2$) compared with the 10 subjects who were antigen positive/antibody negative. The number of subjects with antibody to hepatitis B associated antigen was small and therefore I would like to confirm these results on a larger sample of the population. Nevertheless, the results do raise the question of whether persons who have the tendency to produce relatively large quantities of IgE also have the tendency, on antigenic stimulation, to produce relatively large amounts of other classes of immunoglobulin. Levine and Vaz (1970) found that those strains of mice that could mount a humoral immune response to low antigen dose responded with both IgG and reagin production. They suggested (Vaz and Levine (1970)) that the ability to respond to low antigen dose depended on the avidity with which cellular receptors could bind antigen and that animals that were able to recognise low doses of antigen responded with the production of more than one class of antibody. Although the situation in blood donors is not quite analogous, my findings are consistent with the hypothesis implicit in this work - that a relationship exists between IgE responsiveness and immune responsiveness as a whole.

CHAPTER III

Factors influencing allergic disease and serum IgE concentrations in persons living in the Western Cape.

3.1 Introduction

The results of the survey described in Chapter II showed significant tendencies for serum IgE concentrations to be higher in Bantu and Coloureds than in Whites; and higher in males than in females. Recent information on the biological function of IgE and its association with allergic and parasitic diseases (for reviews see Stanworth (1972) and Johannson, Bennich and Berg (1972)) possibly explain some of the population differences I observed, but this information, taken in conjunction with the results of my own experiments, raised further questions on the nature of the allergic response which I felt should be considered. These questions could be summarised as follows:-

(a) Given that IgE is known to be a cytophilic antibody capable of mediating immediate hypersensitivity, do those groups showing high total serum IgE concentrations show a high prevalence of allergic symptoms or cutaneous hypersensitivity, and if so, does the relationship between elevated serum IgE concentrations and allergic symptoms hold in all race and sex groups regardless of the "normal" serum IgE concentration for that group?

(b) Given that immediate skin hypersensitivity to common allergens frequently occurs in persons without any allergic symptoms; do serum IgE concentrations more closely reflect the skin reactivity of an individual or the tendency of an individual to develop allergic symptoms? Is there any relationship between the degree of cutaneous hypersensitivity (as estimated by the intensity and/or number of positive skin tests to common allergens) and the total serum IgE

concentration of an individual?

(c) If, as has been suggested by others (Johannson et al (1968b)), intestinal infestation results in elevated serum IgE concentrations, could this factor be invoked to explain the population differences I observed? If so, was it the only factor responsible for the elevated serum IgE concentrations in the Coloureds and the Bantu?

(d) Given that helminthic infestation in animals can induce the formation of antibody directed not only against worm antigens but also against unrelated antigens (Jarret (1972), Jarret, Henderson, Riley and White (1972)) is there any evidence to suggest that a similar phenomenon occurs in man? In other words, do individuals exposed to helminthic infestation have not only high total serum IgE concentrations but also evidence of increased sensitivity to unrelated allergens?

(e) Several workers have reported the general tendency for serum immunoglobulin levels in Africans to be elevated (Michaux (1966), Turner and Voller (1966), Rowe, McGregor, Smith, Hall and Williams (1968)). Were the raised serum IgE concentrations that I observed part of such a "general hypergammaglobulinaemia" rather than isolated immune events in their own right?

(f) Could the sex difference in serum IgE concentrations be explained on the basis of a difference in allergic responsiveness of males and females?

In an attempt to answer some of these questions and to examine the possibilities they suggested, I studied a further 268 blood donors. The results of this survey, which included an assessment of the allergic status of the subject, a test for immediate skin hypersensitivity to common allergens, a faecal specimen examination for evidence of parasitic infestation, an eosinophil count, and total serum IgE, IgM, IgA and IgE measurements, are presented in this Chapter.

3.2 Materials and Methods

3.2.a. Subjects used and protocol for the survey.

As in the previous survey, subjects were drawn from the panel of blood donors registered with the Western Province Blood Transfusion Service. Contact was established at the time of blood donation when the nature of the survey was explained and their co-operation solicited. Those who agreed to participate were questioned, according to a standard questionnaire, and by an experienced interviewer, for a history of allergic symptoms. Arrangements were made for them to provide a fresh stool specimen for microscopic examination, and to present for skin testing. Serum immunoglobulins and blood eosinophils were measured in the blood sample taken at the initial donation. Generally speaking, the history, skin tests, stool examination and blood sampling, in any individual, were completed within a period of two to three weeks. The survey was conducted during the months of October to February, that is in those months when seasonal allergic symptoms occur most commonly.

Not all tests were performed on all individuals; while most were willing to give a history, some subjects failed to keep their appointments for skin tests and some were unwilling to give stool specimens. One of the clinics visited was held at the Cape Divisional Council Disposal Works where the work performed involves collecting refuse and night soil. These subjects were found to have a particularly high prevalence of helminthic infestation and because I felt that this was due to occupational exposure I have not included either the results of their serum IgE determinations or the results of their stool examinations in my analyses. Thus more data were available for some of the statistical tests than for others. As I used all the available data for each test, the tables in Section 3.3 reflect the differences in population sampling for each investigation referred to above.

3.2.b. Histories of allergic symptoms.

In deciding whether or not symptoms could be ascribed to immediate hypersensitivity, reliance was placed primarily upon an assessment of the nature of the symptoms and their congruence with generally accepted clinical description. In addition, such factors as seasonal exacerbation, relief from anti-histamine drugs or di-sodium cromoglycate, or a positive family history of allergy were taken into account.

On the basis of the extent to which symptoms caused social, economic, or physical disability, or the extent to which they obtruded into the daily life of the individual, I graded the subjects as being clinically allergic, mildly allergic or non-allergic. Although seemingly arbitrary, this distinction could, in many cases, be made relatively easily. Thus subjects who had required or sought medical assistance for their symptoms, who took pains to avoid provocative stimuli or who were, when affected, acutely conscious of their symptoms, were classified as clinically allergic. Subjects who admitted, on direct questioning, to occasional symptoms were classified as mildly allergic, while those who denied relevant symptoms were classified as non-allergic. It should, however, be recognised that the diagnosis of allergy or its severity rests almost entirely upon the interpretation by the clinician of the patient's subjective account of his or her symptoms. There are few, if any, objective criteria which can be relied on in cases of doubt. Syndromes such as vernal conjunctivitis, allergic rhinitis, or childhood asthma with eczema, are usually typical and can be ascribed to allergy without much uncertainty; on the other hand one encounters syndromes such as "food allergy", urticaria, migraine, anaphylaxis and contact dermatitis, where either the clinical picture is vague and/or the pathogenetic role of the IgE-mediated type I hypersensitivity reaction

is uncertain. Here considerable difficulties arise. When confronted with such cases as these, I assigned the subject to one or the other category with less secure grounds for doing so. In every case, however, the assignation was made without prior knowledge of the skin test result or the serum IgE concentration.

A further problem was encountered in the case of many of the Bantu and some of the Coloured subjects. This arose largely from difficulties in communication that were not entirely due to language difficulties. In many instances I was left with the impression that their cultural background and social circumstances so modified their awareness of, or reaction to, minor symptoms that they did not complain of them or admit to their presence. The prevalence of allergy in these subjects, therefore, may well have been underestimated by the interview I conducted.

A copy of the questionnaire that was used in interviewing subjects appears in Appendix VII. This served as a "check test" for the interviewer; it was not completed by the subject. In subsequent sections of this Chapter I have, on occasion, grouped together as "allergic" subjects classified as "mildly allergic" and "moderately allergic". Since, *a priori*, similar immunopathological mechanisms are believed to operate in type I hypersensitivity of any grade of severity, I feel justified in having done so where distinctions of degrees were not called for. In all cases, my assignation of subjects to clinical groups should be considered in the light of the difficulties outlined above.

3.2.c. Examination of the stools

This was very kindly done by the staff of the Bacteriology Department, Medical School, University of Cape Town. Only one stool per individual was collected. A wet specimen from each stool was examined microscopically for the presence of parasites or ova.

3.2.d. Skin tests

Tests for immediate skin hypersensitivity to common allergens were performed using the prick test (for the method and discussion of the method see Appendix I).

Each individual was tested with prick test solutions of seventeen allergens and a control solution (Bencard, Brentford, England). All tests were done on the anterior surface of one forearm laid out as follows:-

Going from distal to proximal

Lateral	Medial
1. Control	10. Moulds A13
2. House dust	11. Moulds M10
3. House dust mite	12. Moulds M11
4. Group B 51 - S.A. Flower pollens	13. Bacteria F1
5. Group B 52 - S.A. Grass pollens	14. Bacteria F2
6. Group B 53 - S.A. Tree pollens	15. Candida albicans
7. Bermuda grass pollen	16. Aspergillus fumigatus
8. Group A_1 Mixed inhalants (Animal danders & dust)	17. Talc
9. Ascaris	18. Dry rot

Manufacturer's details regarding the allergens used to make up the prick test solutions and their concentration are given at the end of Appendix I. Subjects were only tested with one concentration of each solution as received from the manufacturer.

Before skin testing it was ascertained that subjects were not taking anti-histaminic drugs.

Each individual reaction was graded in the same way that reactions to the Bermuda grass pollen antigens were graded, that is:-

	Wheal	flare
0	< 3mm	< 5mm
+	≥ 3mm, <5mm	≥ 5mm, <10mm

++	≧ 5mm, <8mm	≧10mm, <20mm
+++	≧ 8mm, <10mm	≧20mm, <26mm
++++	≧ 10mm	≧26mm

An individual was considered to be skin test positive if he gave a positive reaction to any of the test solutions.

The results of the complete set of tests on any one individual were graded in more detail as follows:-

0 No reaction
1 One + reaction
2 More than one + reaction
3 One ++ reaction
4 One ++ reaction with one or more + reactions
5 More than one ++ reaction with or without + reactions
6 One +++ reaction
7 One +++ reaction with one or more + or ++ reactions
8 More than one +++ reaction with or without smaller reactions
9 One ++++ reaction
10 One ++++ reaction with smaller reactions
11 More than one ++++ reaction with or without smaller reactions.

By grading in this way, skin test results were influenced by both the size of the reactions and the number of positive reactions. The size of the largest reaction influenced the final grade more than the total number of positive reactions.

3.2.e. Immunoglobulin determinations.

IgE determinations were performed as described in Chapter II. In this survey samples containing more than 800 u IgE/ml were re-assayed after dilution.

Serum IgG, IgM and IgA concentrations were measured by radial immuno-diffusion (Mancini et al (1965)). IgM and IgA were determined using commercially available Tri-Partigen plates (Behringwerke, Marburg-Lahn, Germany). A few of the IgG determinations were also

done using Tri-Partigen plates; the majority were done on immuno-diffusion plates prepared in the laboratory.

The Tri-Partigen plates were used according to the manufacturer's instructions. Three of the wells on each plate contained 5 ul each of the three dilutions of the appropriate standard immunoglobulin solutions (Behringwerke) and the other nine wells contained 5 ul test samples. Immuno-diffusion was allowed to proceed to completion at room temperature. The diameters of the precipitin rings were measured directly without staining.

The IgG plates were prepared in exactly the same way as the IgE plates except that the antiserum incorporated in the agarose was a 1:10 dilution of anti-human IgG (see Appendix IV). Three dilutions of IgG standard immunoglobulin solution were used as standards. Test samples were diluted 1:20 with physiological saline. Immuno-diffusion was allowed to proceed to completion at room temperature (three to four days). The plates were then washed in physiological saline, dried and stained with amido-black.

The concentration of immunoglobulin in each test sample was calculated from the square of the diameter (D^2) of the precipitin ring by interpretation on a graph defining the linear relationship between D^2 and the immunoglobulin concentration of the standard solution. When the immunoglobulin concentration of the unknown was greater than that of the highest standard, the determination was repeated on a dilution of the unknown.

3.2.f. Peripheral eosinophil counts.

The majority of the peripheral eosinophil counts were obtained by determining the differential counts of white blood cells in stained smears (Leishmans stain) of whole blood.

A more reliable method, which I adopted during the course of

the survey, is the specific eosinophil count. This method is exactly the same as the method for a total white blood cell count except that eosinophil diluting fluid replaces white blood cell diluting fluid; eosinophil diluting fluid is made up of 5 ml of 1% eosin, 5 ml acetone, and 100 ml distilled water.

For the analyses I considered all subjects with > 3% eosinophils or > 250 eosinophils per cu.mm as having high peripheral eosinophil counts.

3.2.g. Statistical tests

The remarks I made in Section 2.2.e. regarding my reasons for using non-parametric statistical tests apply equally to the tests used in the present section; details of the principal tests I used are presented in Appendix VII.

The population sample used for this survey was considerably smaller than the one used for the earlier survey (presented in Chapter II) and therefore I have used a p value of $p < .02$ instead of $p < .01$ to indicate significance; I have again included in brackets p values when they fell in the range $.02 < p < .1$.

Although the Chi-square test and the Kruskal-Wallis one way analysis of variance were used for the majority of the analyses, the small numbers of subjects in certain categories occasionally precluded their use. In these cases I used the Fisher exact probability test or the Mann-Whitney U test respectively. This latter test only allows two independent samples to be compared, but, unlike the Kruskal-Wallis test, it does not have the restraint that when the total population examined is large, each of the samples must contain more than five members.

3.3. Results

The primary objects of the experiments reported in this Chapter were to investigate the relationship between allergy and serum IgE concentrations, and to examine some of the population differences in serum IgE concentrations that I had found during the initial survey (Chapter II). These objectives required estimates of the prevalence* of allergic symptoms, immediate hypersensitivity to common allergens, and intestinal helminthic infestation in the different population groups, and an assessment of the inter-relationships of those factors which I believed might influence either the prevalence of allergy or serum IgE concentrations. The results of this epidemiological study are shown in Tables 3.1 - 3.7 and Figures 3.1 - 3.10. The results of the analyses of the various factors influencing serum IgE concentrations are shown in Tables 3.8 - 3.26 and Figures 3.11 - 3.14. A computer printout of serum IgE concentrations and all the associated data used in these analyses is given at the end of Appendix VI.

* Throughout this thesis I have drawn a distinction between the concepts of *prevalence* and *incidence*. The word *prevalence* in the statistical context, connotes frequency of occurrence as estimated at a *particular point in time; incidence*, on the other hand, connotes frequency of occurrence as estimated *over a period of time*. It is important to draw this distinction in the case of diseases, such as allergy, that show a tendency to remission and exacerbation. Thus, if one had complete records on a sample of children from birth to puberty one might justifiably claim to have an estimate of the "incidence of allergy in childhood". If one has only an estimate based on results of a single assessment of the frequency of allergy in a group of children, a number of children might well have been included in the sample who, although not exhibiting evidence of allergy at the time of sampling, might previously have been or subsequently have become allergic. In this case one should refer to "prevalence of allergy" in a sample of children. As I only

assessed the allergic status of my subjects once, even although I took past histories of allergy into consideration, I consider it appropriate to refer to my findings as prevalence of allergy, prevalence of positive skin tests, etc. Obviously *prevalence*, in situations such as these, under-estimates *incidence*.

3.3.a. The relationship of sex to the prevalence of allergic symptoms, immediate skin hypersensitivity to common allergens and intestinal helminthic infestation.

I found no sex difference in the prevalence of allergic symptoms (Figure 3.1). Similarly in Whites and in Coloureds I found no sex difference in the prevalence of immediate skin hypersensitivity to common allergens (Figure 3.2). However, in the Bantu, females had a very much lower prevalence of positive skin tests than males. The reason for this finding is not clear, and because I investigated results from only nine Bantu females I am reluctant to draw any conclusions. Nevertheless, in view of this finding, I confined subsequent analyses of the results of the skin tests in the Bantu to the results I had obtained in males.

In each race the prevalence of helminthic infestation in the two sexes (Table 3.1) was the same.

3.3.b. The prevalence of allergy, immediate skin hypersensitivity to common allergens, and intestinal helminthic infestation in the Whites, the Coloureds and the Bantu.

As can be seen from Figure 3.3 Whites more frequently reported symptoms that could be attributed to allergy than did Coloureds or Bantu. Although highly significant, this finding might well be more apparent than real. This point is taken up again in the discussion.

The results of the skin tests showed that, as far as skin hyper-

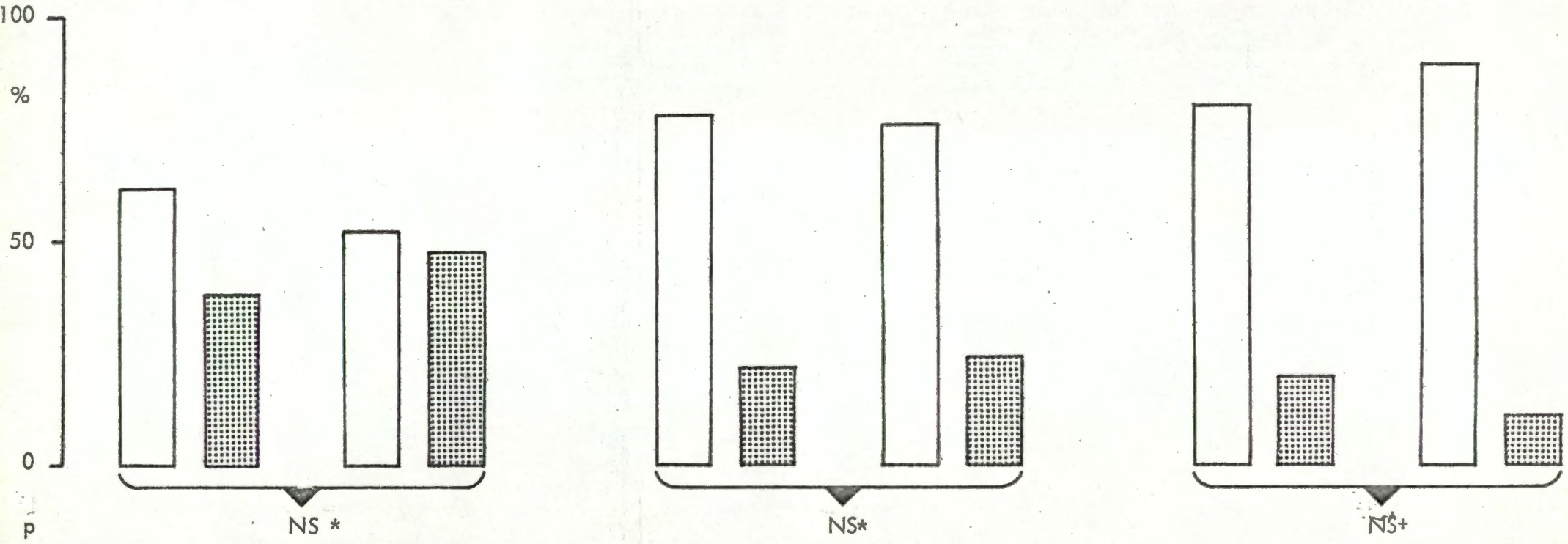


Figure 3.1

The prevalence of positive allergic histories analysed by sex in members of the three racial groups.

The analyses of the data summarised above indicated that there were no sex differences in the prevalence of allergic symptoms.

* Chi-square analysis + Fisher exact probability test.

∇ No distinction was drawn between symptoms of mild and clinical allergy.

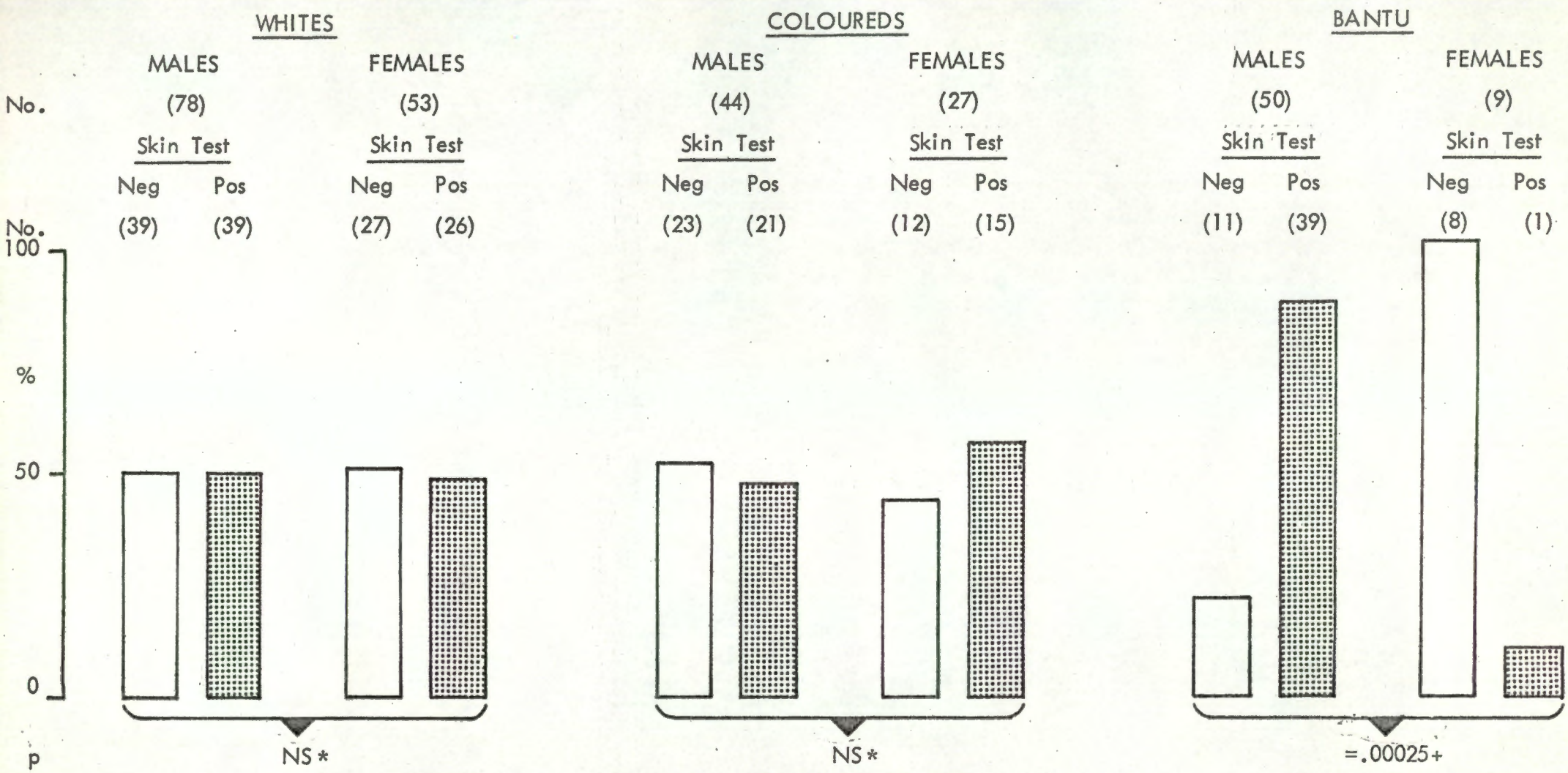


Figure 3.2

The prevalence of positive skin tests analysed by sex.

The analyses of the data summarised above indicated that, in the Whites and the Coloureds, there was no sex difference in the prevalence of positive tests, whereas, in the Bantu, males had a significantly (p = .00025) higher prevalence of positive tests than females.

* Chi-square analysis. + Fisher exact probability test.

Whites

	Parasites in the stools		
	Neg.	Pos.	Total
Males	75	2	77
Females	45	0	45
	120	2	122

NS+

Coloureds

	Parasites in the stools		
	Neg.	Pos.	Total
	29	11	40
	11	14	25
	40	25	65

NS * (.02 < p < .05)

Bantu

	Parasites in the stools		
	Neg.	Pos.	Total
Males	20	15	35
Females	5	4	9
	25	19	44

NS +

Table 3.1

Analysis of the relationship between sex and the prevalence of intestinal helminthic infestation as diagnosed by stool examination.

* Chi-square test

\+ Fishers extract probability test.

sensitivity was concerned, the Bantu were more responsive than the Whites or the Coloureds (Figure 3.4). Furthermore, the high prevalence of skin hypersensitivity in the Bantu could not be explained on the basis of an increased prevalence of skin hypersensitivity to Ascaris; Figure 3.5 demonstrates that even when individuals sensitive to Ascaris alone were excluded from the analysis, the Bantu still had a significantly elevated ($p < .02$) prevalence of positive skin tests.

As a corollary to my work on Bermuda grass pollen antigens, I determined the prevalence of sensitivity to Bermuda grass pollen extract in Whites, Coloureds and Bantu, and found skin hypersensitivity to this allergen to occur in 20%, 12% and 10% of subjects respectively (Table 3.2). Thus in this instance hypersensitivity occurred less frequently in the Bantu than in the Whites.

Skin test to Bermuda grass pollen extract

	Neg.	Pos.
Whites	80% (105)	20% (27)
Coloureds	88% (68)	12% (9)
Bantu	90% (53)	10% (6)

Table 3.2

Prevalence of immediate skin hypersensitivity to Bermuda grass pollen extract in Whites, Coloureds and Bantu.

Racial differences in the frequencies of positive tests to three different types of allergen - Ascaris, Bermuda grass pollen, and house dust mite - are shown in Table 3.3. The differences in the frequencies were not all statistically significant, but they did suggest that the patterns of positive tests tended to differ between subjects belonging to different racial groups.

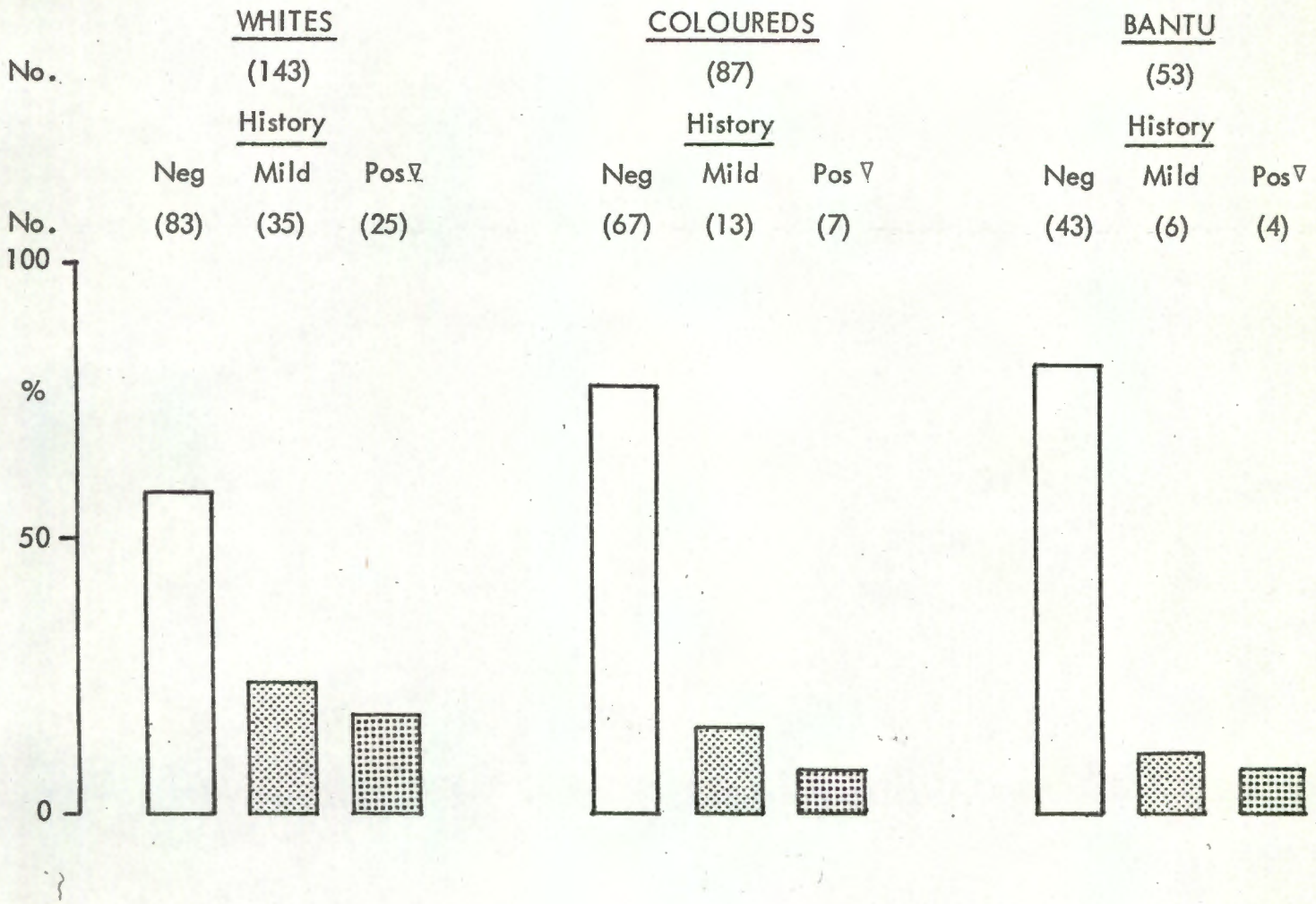


Figure 3.3

The prevalence of positive allergic histories analysed by race.

Chi-square analysis of the data summarised above indicated significant ($p < .005$) race differences in the prevalence of allergy.

∇ Pos indicates clinical allergy.

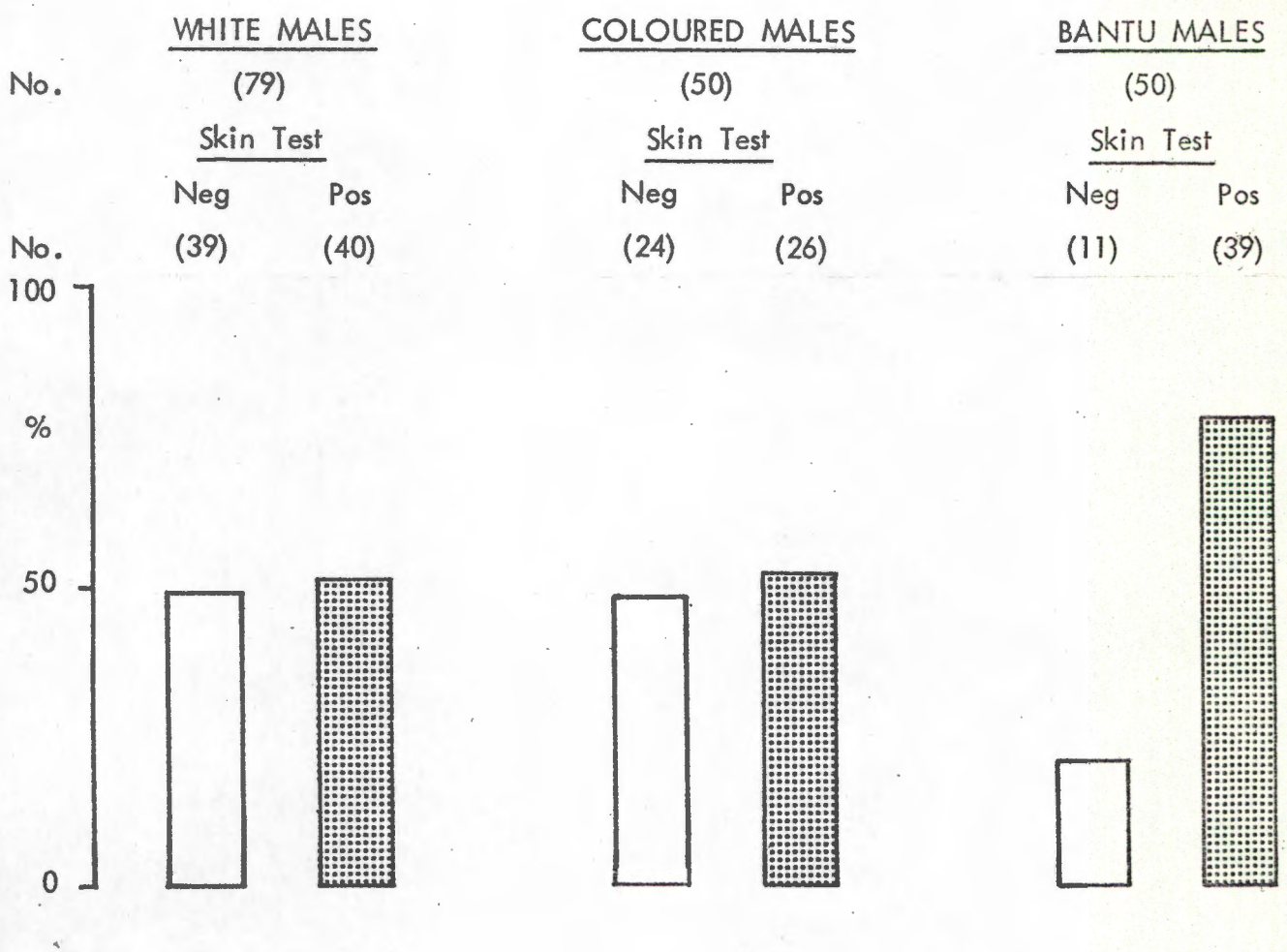


Figure 3.4

The prevalence of positive skin tests to common allergens analysed by race in males.

Chi-square analysis of the data summarised above indicated that the Bantu males had a significantly ($p < .005$) higher prevalence of positive skin tests than White males or Coloured males.

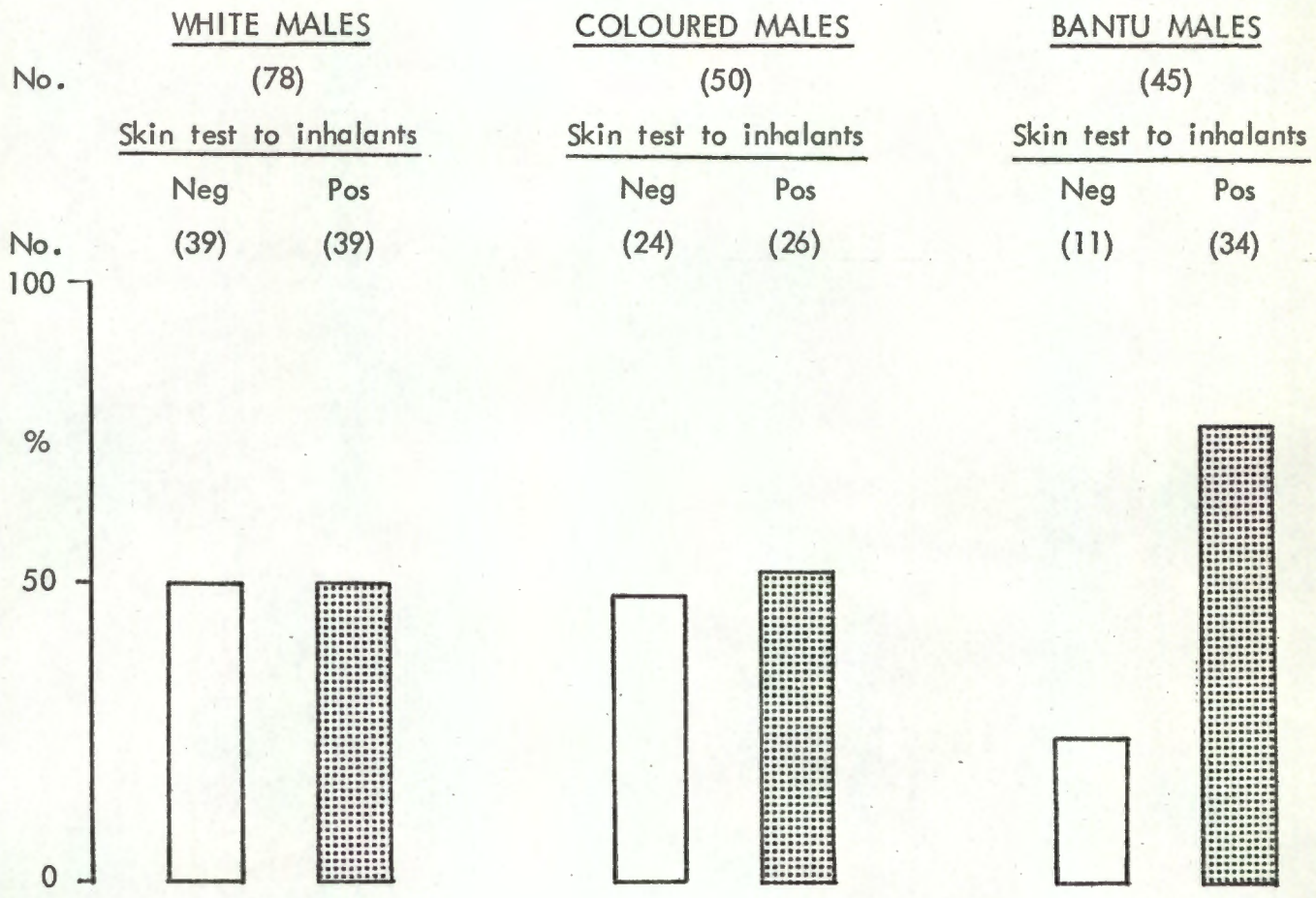


Figure 3.5

The prevalence of positive skin tests to common inhalant allergens analysed by race in males. All subjects sensitive to Ascaris extract alone were excluded from the analysis.

Chi-square analysis of the data summarised above indicated that Bantu males had a significantly ($p < .02$) higher prevalence of positive tests than White males or Coloured males.

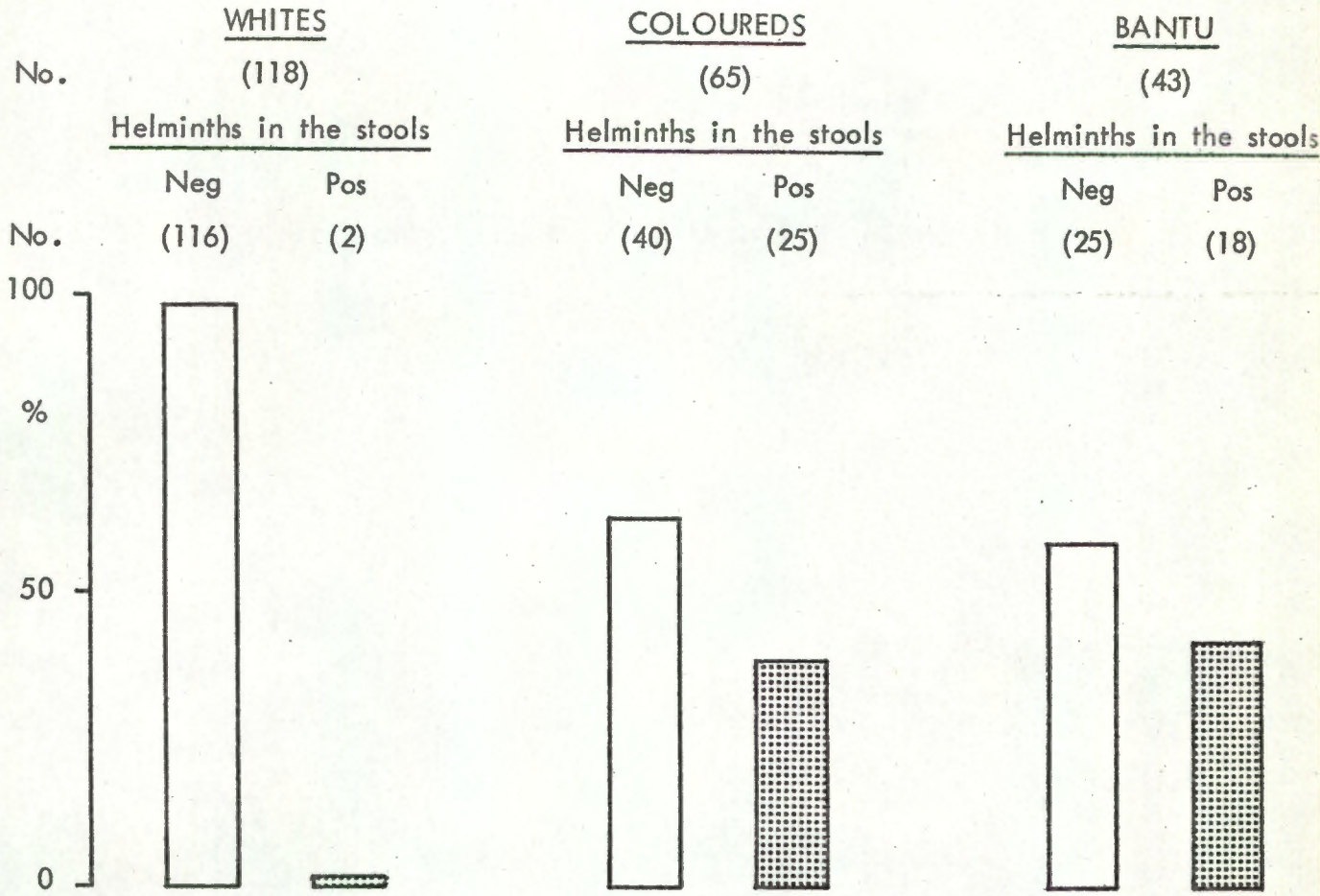


Figure 3.6

The prevalence of intestinal helminthic infestation analysed by race.

Chi-square analysis of the data presented above indicated significant ($p < .0005$) race differences in the prevalence of helminthic infestation.

Skin test

	Ascaris	Bermuda grass pollen	House dust mite
White males (79)	13% (10)	23% (18)	25% (20)
Coloured Males (50)	16% (8)	14% (7)	26% (13)
Bantu males (50)	46% (23)	12% (6)	32% (16)

Table 3.3

Prevalence of hypersensitivity to Ascaris extract, Bermuda grass pollen extract, and house dust mite extract in White males, Coloured males and Bantu males. Percentages of positive tests in each group are given with the actual number of positive tests in brackets.

Figure 3.6 shows that the prevalence of helminthic infestation, as indicated by faecal specimen examination, was much lower in the Whites than in the Coloureds or the Bantu. Table 3.4 shows the frequencies with which different genera of helminths were detected in the stool specimens; Trichuris trichuria was by far the most common helminth detected.

Ova in the stools

	Ascaris	Taenia	Trichuris
Whites (118)	1% (1)	-	1% (1)
Coloureds (65)	11% (7)	-	35% (23)
Bantu (43)	14% (6)	2% (1)	42% (18)

Table 3.4

Prevalence of infestation by Ascaris, Taenia and Trichuris in members of the three racial groups.

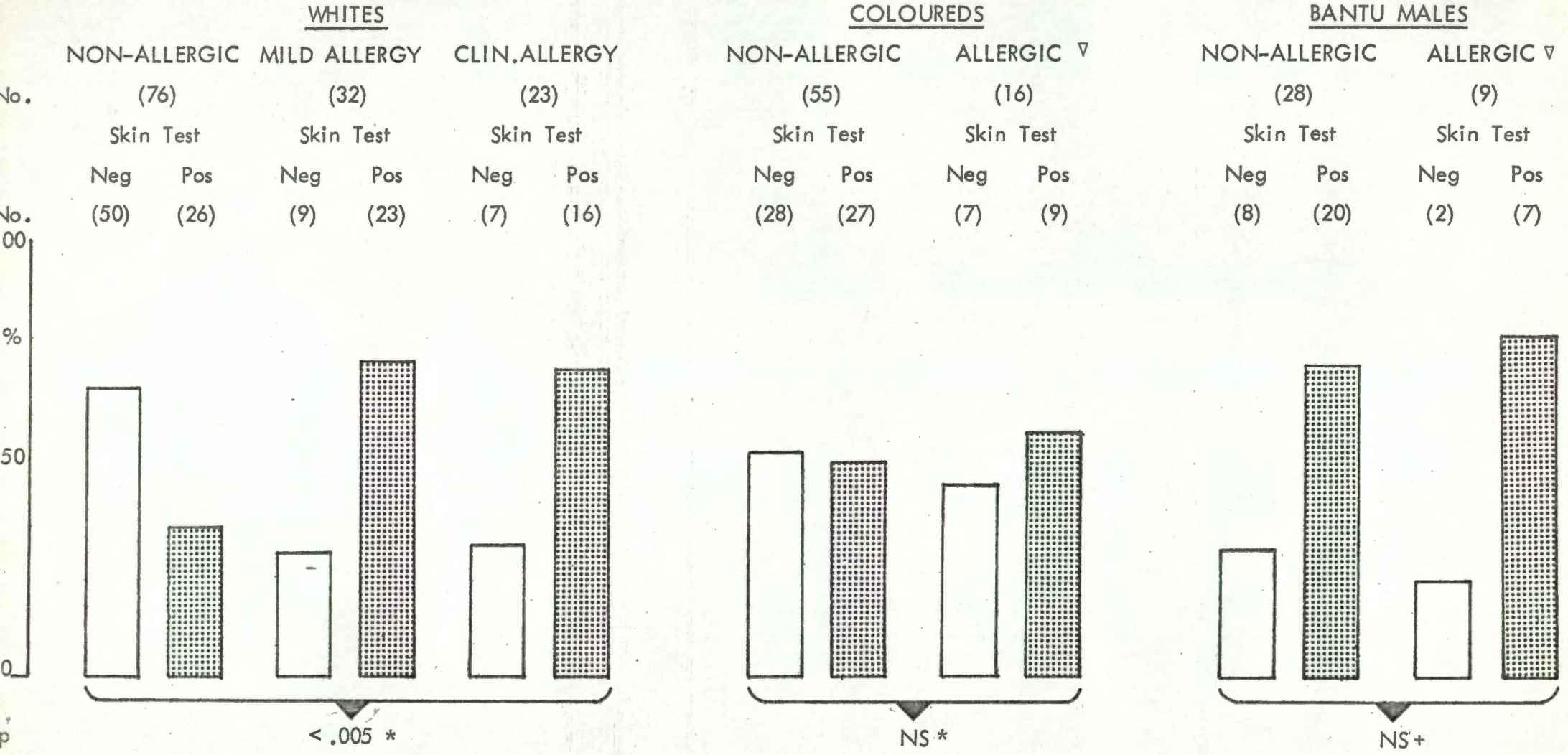


Figure 3.7

The prevalence of positive skin tests analysed according to allergic history in members of the three racial groups.
The analyses of the data summarised above indicated that only in Whites was there a significant ($p < .005$) association between positive allergic histories and positive skin tests.
* Chi-square analysis + Fisher exact probability test.
∇ No distinction was drawn, in the Coloureds and Bantu, between mild allergy and clinical allergy.

3.3.c. The relationship between allergic symptoms and immediate skin hypersensitivity to common allergens.

Figure 3.7 shows that in Whites there was a highly significant ($p < .0005$) relationship between the presence of allergic symptoms and positive skin tests to common allergens. The data showed that persons with mild allergy had the same high prevalence of skin hypersensitivity as persons with clinical allergy. Table 3.5 shows that while, in Whites, allergic symptoms occurred frequently in individuals with large skin test reactions, this was not invariably so and ten out of 33 individuals with a +++ reaction or greater had no allergic symptoms.

Whites

		History		
		Neg.	Pos.$^{\nabla}$	Total
Largest skin test reaction	Neg.	50	16	66
	+	8	6	14
	++	8	10	18
	+++ or ++++	10	23	33
		76	54	131

$p < .0005$*

Table 3.5

Analysis of the relationship between skin test results and positive allergic histories in the Whites. For this analysis the skin test results were graded according to the size of the maximum skin test result.

* Chi-square analysis.

∇ Pos. indiciates mild or clinical allergy.

In the Coloured and the Bantu, I found it necessary to group results from persons with mild allergy and those from persons with clinical allergy. In neither of these race groups was there any

association between positive allergic symptoms and immediate skin hypersensitivity to common allergens (Figure 3.7). As discussed earlier, Bantu males had a high prevalence of positive skin tests, Figure 3.7 shows that this high prevalence was found irrespective of whether allergic symptoms were present or not.

3.3.d. The relationship between intestinal helminthic infestation, allergic symptoms, and immediate skin hypersensitivity to common allergens.

The number of subjects was such that only in Coloureds was I able to use the Chi-square test to test for associations between helminthic infestation and allergic symptoms or immediate skin hypersensitivity to common allergens; no such associations were found (Table 3.6).

Coloureds

		Helminths in the stools		
		Neg.	Pos.	Total
History	Neg.	33	16	49
	Pos.∇	7	9	16
		40	25	65
		NS*		

		Helminths in the stools		
		Neg.	Pos.	Total
Skin test	Neg.	19	13	32
	Pos.	23	15	38
		42	28	70
		NS*		

Table 3.6

The relationship between helminthic infestation and allergic symptoms, and between helminthic infestation and immediate skin hypersensitivity to common allergens in the Coloureds.

* Chi-square analysis

∇ Pos includes persons with mild and clinical allergy.

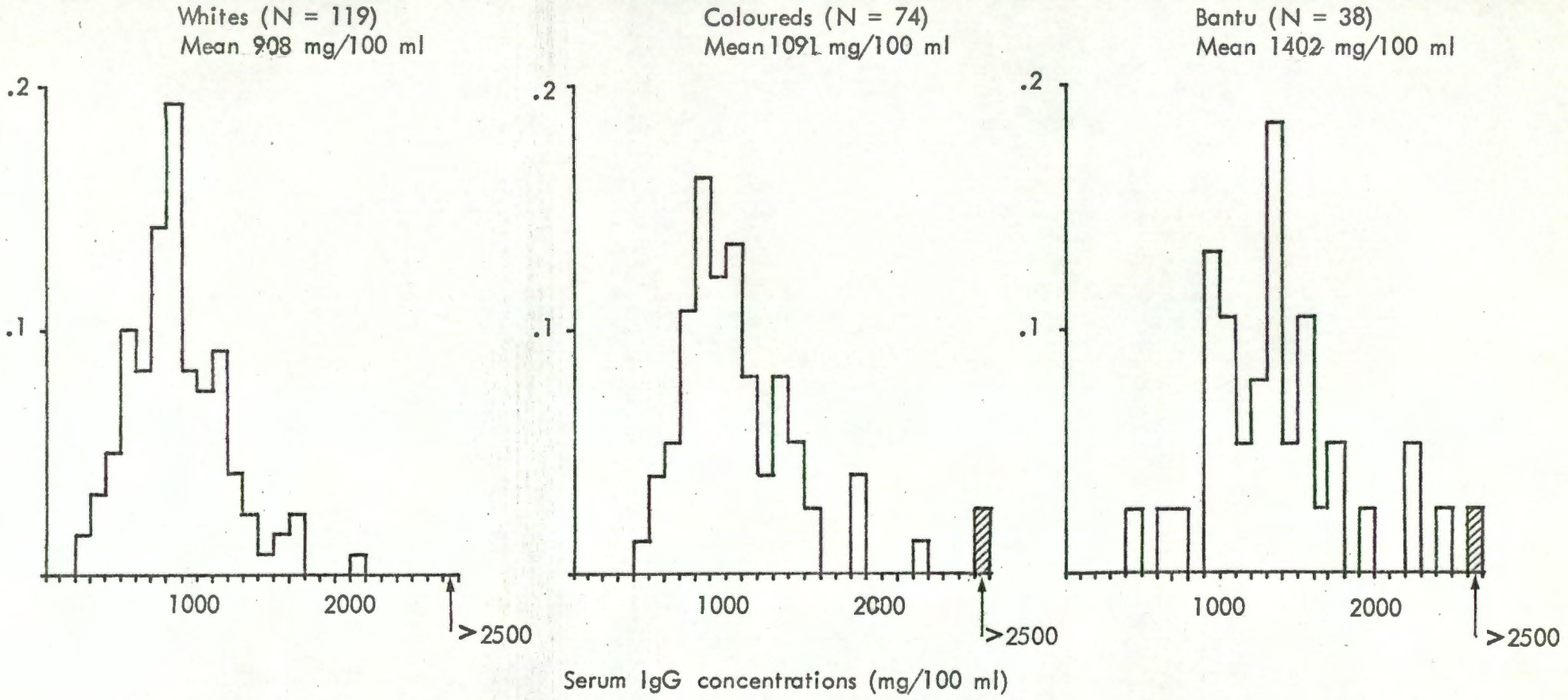


Figure 3.8

Frequency distribution patterns of serum IgG concentrations in members of the three racial groups living in the Western Cape.

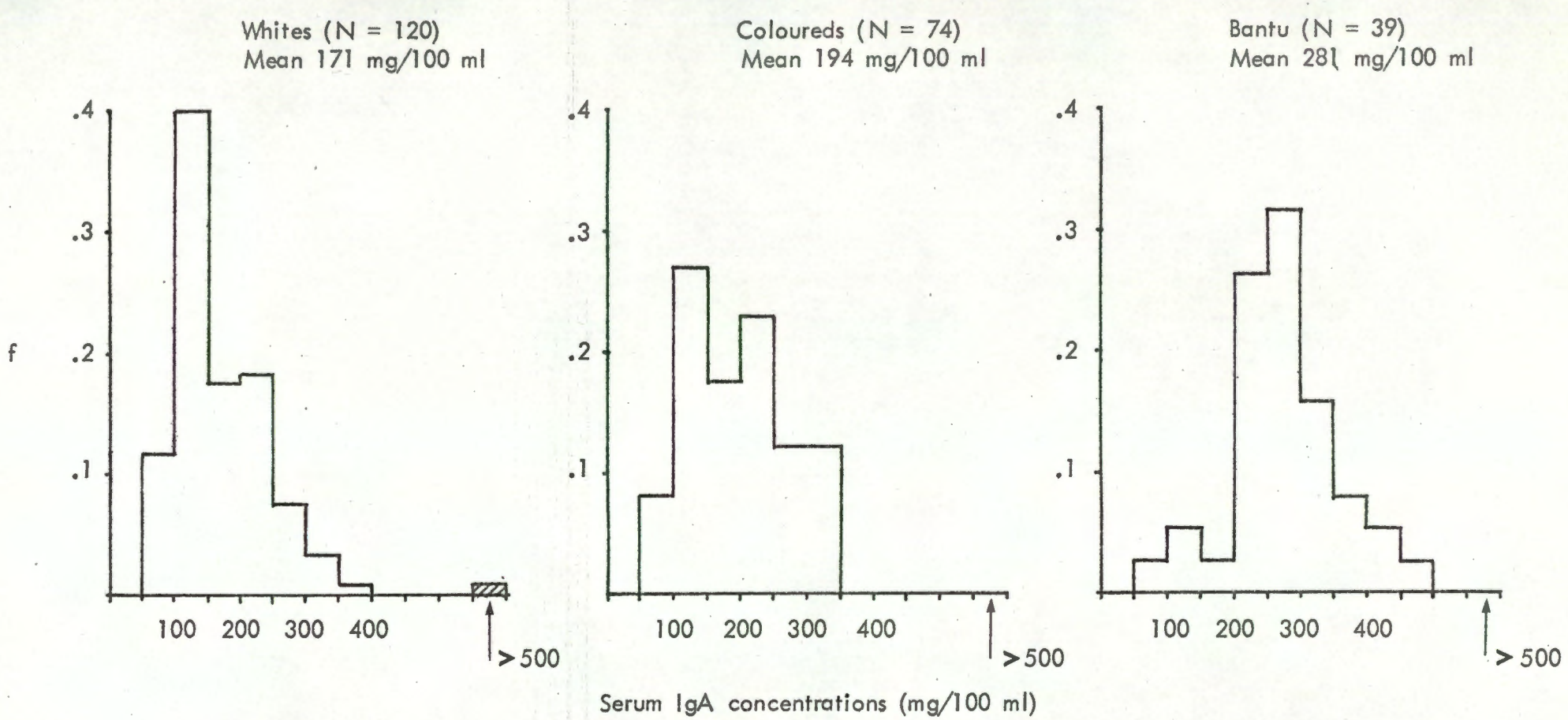


Figure 3.9

Frequency distribution patterns of serum IgA concentrations in members of the three racial groups living in the Western Cape.

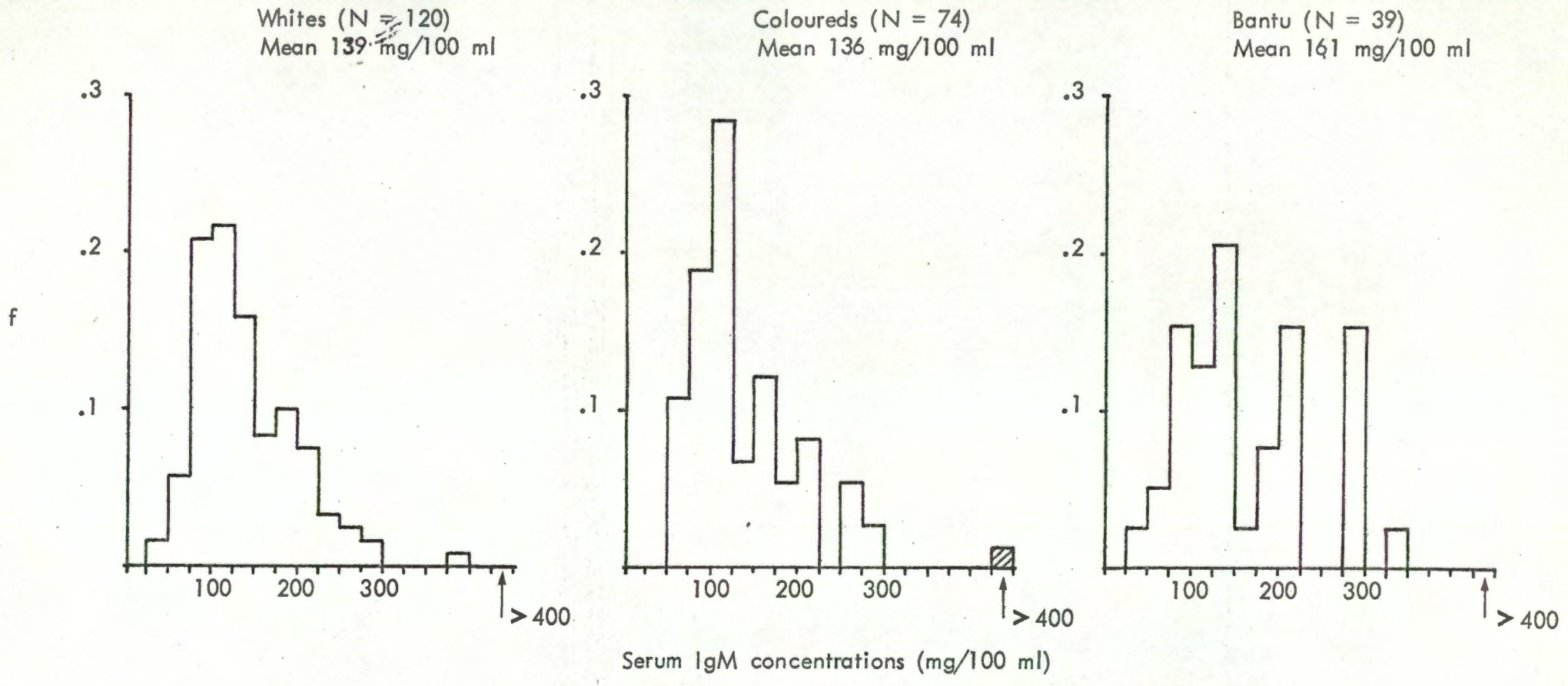


Figure 3.10

Frequency distribution patterns of serum IgM concentrations in members of the three racial groups living in the Western Cape.

	IgG (N = 231)			IgA (N = 233)		
	Whites	Coloureds	Bantu	Whites	Coloureds	Bantu
Number	119	74	38	120	74	39
Mean mg/100 ml.	908	1091	1402	171	194	281
	H = 42.59		DF = 2	H = 43.65		DF = 2
	p < .0005			p < .0005		

	IgM (N = 158)			IgM (N = 74)	
	White males	Coloured males	Bantu males	White females	Coloured females
Number	70	54	34	52	22
Mean mg/100 ml.	133	125	162	145	169
	H = 4.94		DF = 2	H = 1.55	DF = 1
	NS (p < .1)			NS	

Table 3.7

Kruskal-Wallis analysis of serum IgG, IgA and IgM concentrations in the three racial groups. Serum IgM concentrations were analysed in each sex separately; analysis in this way resulted in my having too few Bantu females for their IgM results to be included in this analysis.

The p values given above reflect the most extreme differences between the groups, in other words the differences between Whites and Bantu. Therefore I calculated p values for differences in serum IgG and IgA concentrations between Whites and Coloureds and between Coloureds and Bantu. The results were as follows:-

between Whites and Coloureds $p < .0005$ for serum IgG concentrations
$p < .01$ for serum IgA concentrations

between Coloureds and Bantu $p < .0005$ for serum IgG concentrations
$p < .0005$ for serum IgA concentrations.

3.3.e. Serum IgG, IgA and IgM concentrations in the Whites, the Coloureds and the Bantu.

Figures 3.8 , 3.9 and 3.10 show frequency distribution patterns for serum IgG, IgA and IgM concentrations in the three racial groups. Table 3.7 shows that there were significant racial differences in serum IgG and IgA concentrations, whereas there were no racial differences in serum IgM concentrations. Butterworth et al (1967) reported serum IgM concentrations to be higher in females than males, and on analysing my own results I found the same tendency ($p < .025$ for Whites and $p < .01$ for Coloureds). Therefore I analysed the influence of race on IgM concentrations in each sex separately. In neither males nor females could I detect significant racial differences in serum IgM concentrations (there were too few Bantu females for their results to be included in my analysis). The frequency distribution patterns of these three immunoglobulins approached normal distributions and therefore, in Table 3.7, I have given mean immunoglobulin levels and not median levels.

3.3.f. The influence of sex and race on the serum IgE concentrations of the present population sample.

As can be seen from Table 3.8, racial differences in serum IgE concentrations in this smaller population sample were again very highly significant ($p < .0005$). Median serum IgE concentrations in females were, as previously, lower than those in males. In the case of Whites and Coloureds, the differences were not statistically significant. I suspect that the requisite level of significance would have been achieved had this sample been larger. In the case of Bantu males and females, the Mann-Whitney U test showed a highly significant ($p < .005$) tendency for serum IgE concentrations to be higher in males.

	Race		
	Whites	Coloureds	Bantu
Number	120	73	39
Median IgE u/ml.	93	191	577

N = 232

H = 57.93 DF = 2

p < .0005

	Sex					
	Whites (N=120)		Coloureds (N=73)		Bantu (N=39)	
	Males	Females	Males	Females	Males	Females
Number	69	51	53	20	34	5
Median IgE u/ml.	101	59	200	144	784	150
	H=2.04 DF=1		H=1.37 DF=1		(Mann-Whitney U test)	
	NS		NS		p < .005	

Table 3.8

Analyses of the influence of race and sex on the serum IgE concentrations of the second population sample. Because of the small number of Bantu females, the Mann-Whitney U test was used to analyse for the sex difference in the Bantu.

	Whites (N = 116)	
History	Negative	Mild/Clin.Allergy
Number	72	44
Median IgE u/ml	42	113
	H = 11.772	DF = 1
	p < .001	

	Coloureds (N=73)	
History	Negative	Mild/Clin.Allergy
Number	53	20
Median IgE u/ml	202	153
	H = 1.411	DF = 1
	NS	

	Bantu (N = 39)	
History	Negative	Mild/Clin.Allergy
Number	31	8
Median IgE u/ml	739	511
	H = 0.001	DF = 1
	NS	

Table 3.9

Results of the Kruskal-Wallis analysis of the influence of allergy on serum IgE concentrations in the three racial groups.

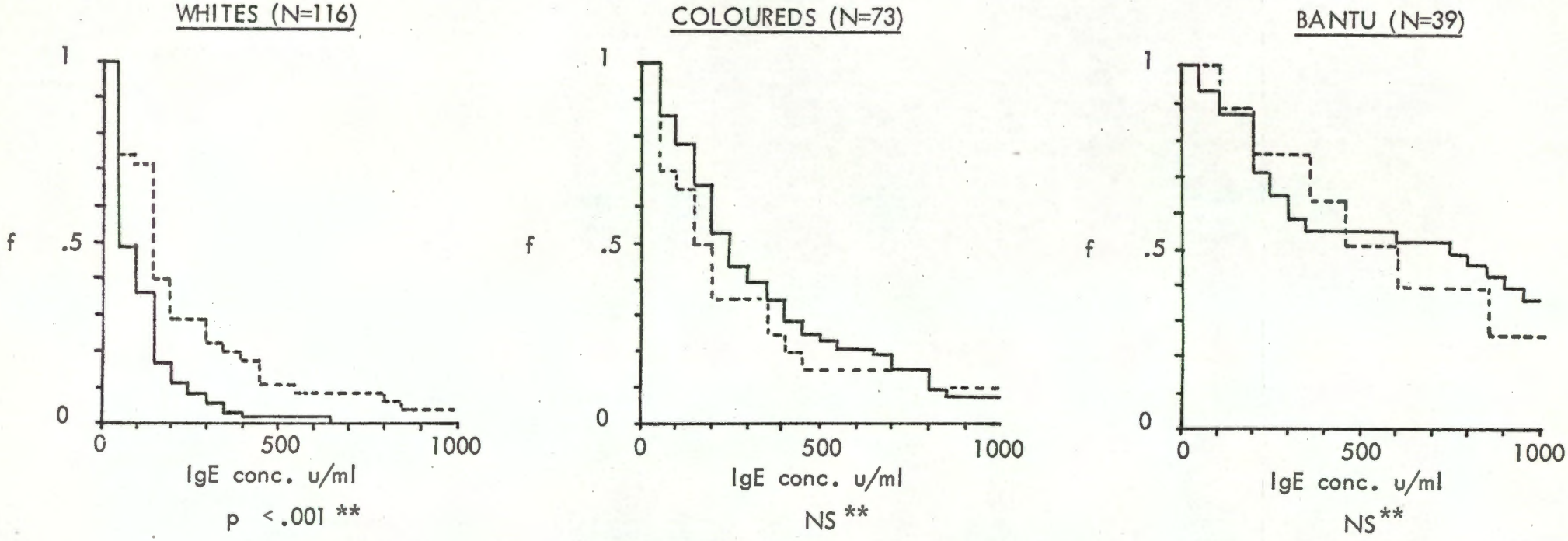


Figure 3.11

Cumulative frequency diagrams of serum IgE concentrations in allergic and non-allergic individuals belonging to the three racial groups with

(i) ——— a negative history of allergy

(ii) ---- a positive history of allergy (either mild or clinical allergy)

** Kruskal-Wallis analysis of the significance of the difference in serum IgE concentrations. These data are summarised in tabular form in Table 3.9.

History	White males (N = 65) Negative	White males (N = 65) Mild/Clin.Allergy	White females (N = 51) Negative	White females (N = 51) Mild/Clin.Allergy
Number	44	21	28	23
Median IgE u/ml	50	198	42	112
	H = 16.07 DF = 1		H = 1.22 DF = 1	
	p < .0005		NS	

History	Coloured males (N = 53) Negative	Coloured males (N = 53) Mild/Clin.Allergy	Coloured females (N = 20) Negative	Coloured females (N = 20) Mild/Clin.Allergy
Number	40	13	13	7
Median IgE u/ml	216	172	176	101
	H = 1.67 DF = 1		H = 0.03 DF = 1	
	NS		NS	

History	Bantu males (N = 34) Negative	Bantu males (N = 34) Mild/Clin. Allergy
Number	27	7
Median IgE u/ml	806	577
	H = 0.04 DF = 1	
	NS	

Table 3.10

Results of the Kruskal-Wallis analysis of the relationships between serum IgE concentrations and allergic histories in each sex and in members of the three racial groups. (There were insufficient Bantu females for me to analyse the results in that group.)

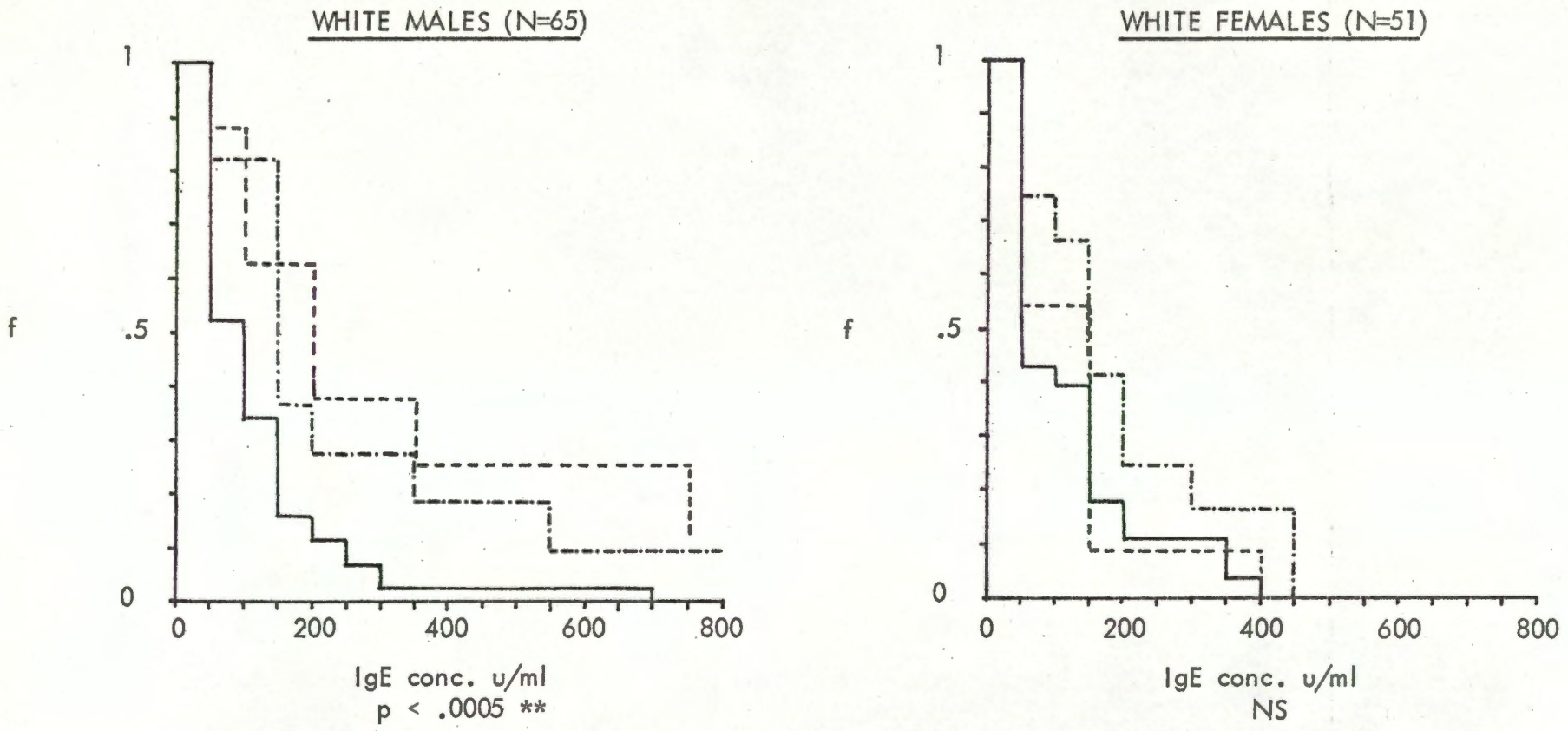


Figure 3.12

Cumulative frequency diagrams of serum IgE concentrations in White males and White females with

(i) ——— negative history

(ii) -·-·-·- mild allergic history

(iii) - - - - - clinical allergy.

** Kruskal-Wallis analysis of the significance of the difference in serum IgE concentrations. (p values for comparisons between mild and clinical allergy were in each case non-significant).

These data are summarised in tabular form in Table 3.11.

3.3.g. The relationships between positive allergic histories and serum IgE concentrations in the different population groups.

As can be seen from Table 3.9 and Figure 3.11 there was a highly significant association between elevated serum IgE concentrations and positive allergic histories in White subjects, whereas in Coloureds and Bantu this association was not evident. Furthermore, when males and females were considered separately, the association between allergy and elevated serum IgE concentrations in Whites could be accounted for by the highly significant ($p < .0005$) association between allergy and elevated serum IgE concentrations in White males; in White females the association was not significant (Table 3.10, Figure 3.12).

Only in White males and White females were there sufficient allergic subjects to allow me to analyse the relationships between serum IgE concentrations and the different grades of allergy. Table 3.11 and Figure 3.12 show that in neither White males nor White females were serum IgE concentrations significantly higher in subjects considered to be clinically allergic when compared with those considered to be mildly allergic.

	White males (N = 21)		White females (N = 23)	
History	Mild Allergy	Clin. allergy	Mild allergy	Clin.allergy
Number	13	8	12	11
Median IgE u/ml	114	232	116	101
	H = 0.223	DF = 1	H = 2.005	DF = 1
	NS		NS	

Table 3.11

Kruskal-Wallis analysis of the difference in serum IgE concentrations between subjects with mild allergy and subjects with clinical allergy.

I found that symptoms such as rhinoconjunctivitis or bronchospasm could be diagnosed and ascribed to allergy with greater diagnostic confidence than symptoms induced by food, drug or contact allergens. In Table 3.12 I have grouped together, for the purpose of analysis, all subjects with the former type of symptoms as having Inhalant allergy, and all subjects with no history of Inhalant allergy but a history of gastro-intestinal, drug or dermatological allergy as having Non-inhalant allergy. I found, in White males and White females (the only groups with adequate numbers for analysis), that there was no apparent tendency for serum IgE concentrations to differ in persons with the different types of allergy.

	White males (N = 21)		White females (N = 23)	
Type of allergy	Inhalant	Non-inhalant	Inhalant	Non-inhalant
Number	14	7	14	9
Median IgE u/ml	198	198	112	112
	H = 0.274 DF=1 NS		H = 0.045 DF=1 NS	

Table 3.12

Kruskal-Wallis analysis of the difference in serum IgE concentrations between subjects with inhalant allergy and subjects with non-inhalant (gastro-intestinal, drug or dermatological) allergy. All subjects with symptoms of inhalant allergy were classed in the inhalant allergy group whether or not they had symptoms of non-inhalant allergy.

3.3.h. The relationships between immediate skin hypersensitivity to common allergens and serum IgE concentrations in the different population groups.

When the serum IgE concentrations of individuals showing positive skin test reactions to one or more allergens were compared

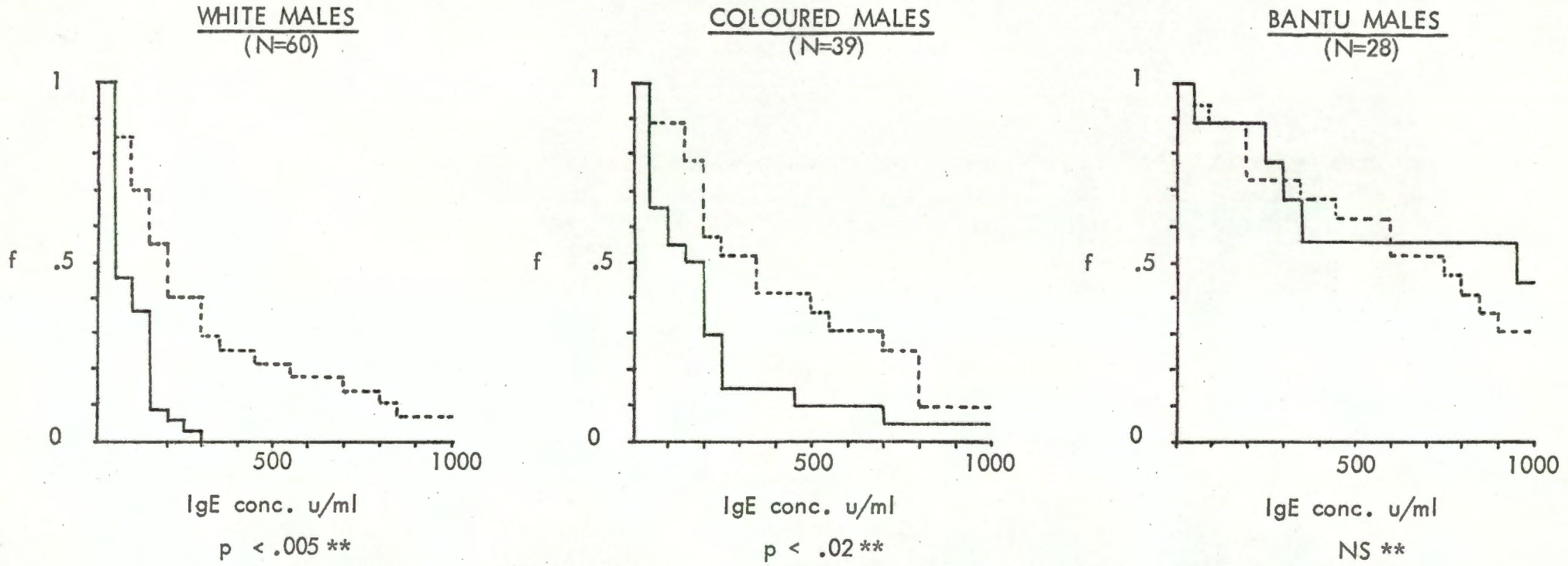


Figure 3.13

Cumulative frequency diagrams of serum IgE concentrations in males belonging to the three racial groups with

(i) ———— no positive skin tests

(ii) ------ at least one positive skin test.

** Kruskal-Wallis analysis of the significance of the difference in serum IgE concentrations.

These data are summarised in tabular form in Table 3.13.

	IgE with skin tests					
	White males	White females	Coloured males	Coloured females	Bantu males	Bantu females
Number	60	46	39	18	28	5
S*	629	207	162	38	-17	-2.0
Tau*	0.451	0.258	0.261	0.288	-0.049	-0.316
Z*	5.092	2.531	2.335	1.671	-0.369	
	$p < .00003$	$p < .006$	$p < .01$	NS ($p < .05$)	NS	NS

Table 3.14

Results of determinations of associations between skin test grades and serum IgE concentrations in the different population groups using the Kendall rank correlation coefficient Tau.

* S, Tau and Z are statistics defined in Appendix VI.

with those of individuals who were skin test negative to all allergens used, White males showed a highly significant ($p < .0005$) association between serum IgE concentrations and evidence of cutaneous hypersensitivity. This association was less prominent but nevertheless significantly demonstrable ($p < .02$) in Coloured males, and evident but not significant in White and Coloured females. Bantu males showed no association between serum IgE concentrations and the presence of cutaneous hypersensitivity. These results are summarised in Table 3.13 and Figure 3.13.

When the analysis was refined to consider, within each group, the ranked correlation between serum IgE concentrations and the graded hypersensitivity response, significant results were obtained in the case of White males, White females and Coloured males (Table 3.14). Thus, positive association between cutaneous hypersensitivity and elevated serum IgE concentrations could be detected in certain race and sex groups. In these groups, the correlation was more significant if cutaneous hypersensitivity was considered as an ordinal rather than a nominal variable.

3.3.i. The relationships between helminthic infestation and serum IgE, IgG, IgA and IgM concentrations in the different population groups.

In Coloured males and Bantu males I found no significant association between serum IgE concentrations and the presence of intestinal helminthic infestation (Table 3.15, Figure 3.14). I had too few Whites with detectable helminths, too few Coloured females without helminths, and too few Bantu females to permit similar analyses in these groups.

In Coloured females and Bantu males I used the Mann-Whitney U test to analyse the relationship between Ascaris infestation and serum

Helminths in the stools	Coloured males (N=36)		Bantu males (N=28)	
	-ve	+ve	-ve	+ve
Number	26	10	16	12
Median IgE u/ml.	191	146	750	816
	H = 0.04	DF = 1	H = 1.46	DF = 1
	NS		NS	

Table 3.15

Kruskal-Wallis analysis of the relationship between serum IgE concentrations and helminthic infestation in Coloured males and Bantu males.

Ascaris in the stools	Coloured females (N=17)		Bantu males (N=28)	
	-ve	+ve	-ve	+ve
Number	13	4	23	5
Median IgE u/ml.	101	371	555	1866
	(Mann-Whitney U test)		(Mann-Whitney U test)	
	NS (p = .025)		p < .02	

Table 3.16

Analysis of the relationships between serum IgE concentrations and Ascaris lumbricoides infestation in Coloured females and Bantu males; because of the small numbers involved the Mann-Whitney U test was used.

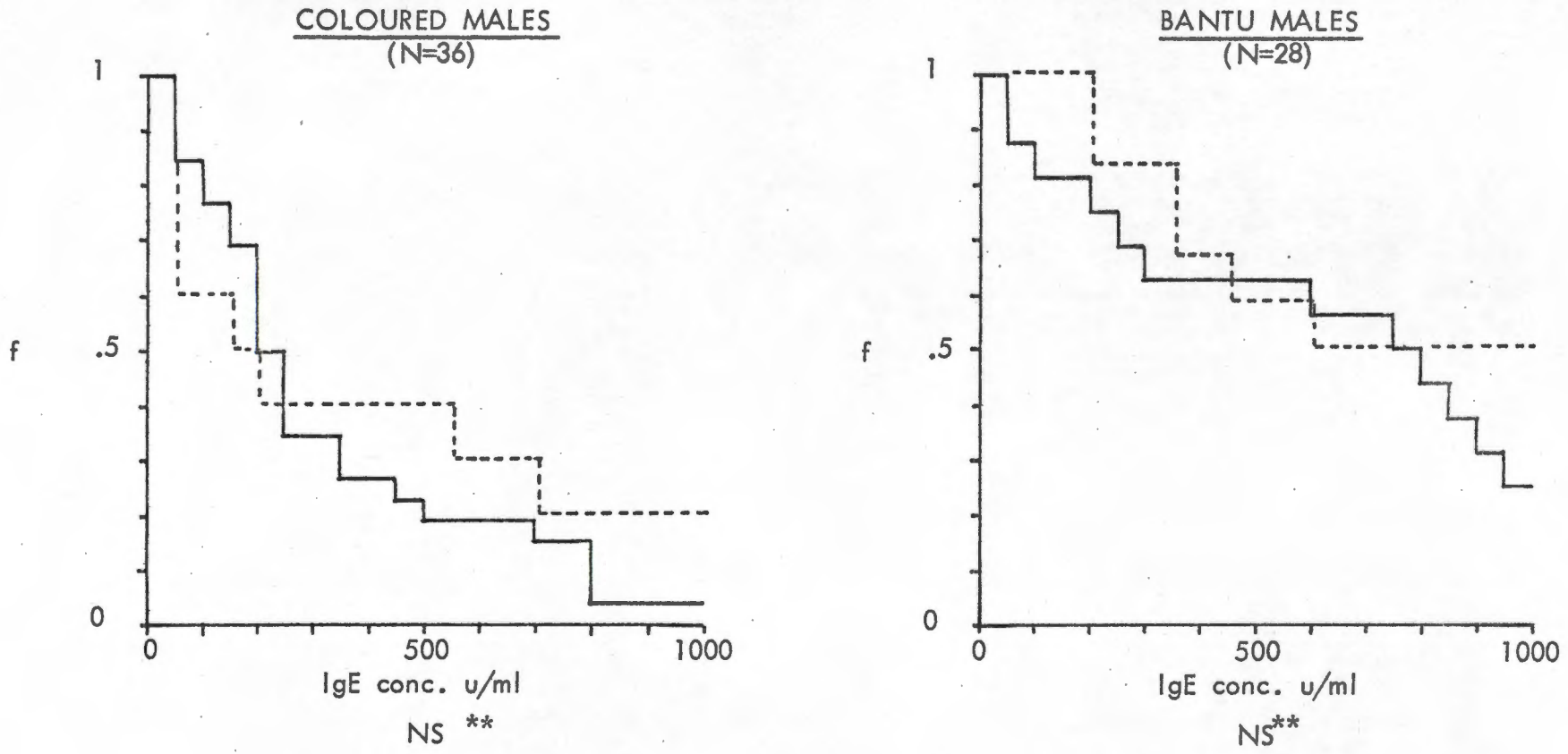


Figure 3.14

Cumulative frequency diagrams of serum IgE concentrations in Coloured males and Bantu males.

(i) ——— with no helminths detected

(ii) - - - - - - helminths detected

** Kruskal-Wallis analysis of the significance of the difference in serum IgE concentrations.

These data are summarised in tabular form in Table 3.15.

IgE concentrations; despite the small number of subjects the results do show the tendency for serum IgE concentrations to be elevated in subjects with Ascariasis (Table 3.16).

In the case of the Coloured and Bantu groups, I could find no association between serum IgG and IgA concentrations and either helminthic infestation or, specifically, overt Ascaris infestation (Tables 3.17, 3.18, 3.19 and 3.20). Because of the sex difference in serum IgM concentrations (see Section 3.3.e.) I examined the relationship between intestinal infestation and serum IgM concentrations in groups separated according to both race and sex; grouping the population in this way resulted in my having sufficient numbers of results for analysis only in the Coloured male and the Bantu male groups. Coloured males did show a significant tendency ($p < .01$) for serum IgM concentrations to be higher in subjects with intestinal helminthic infestation (Table 3.21), however, this isolated finding was not confirmed in Bantu males, and I am reluctant to draw any definite conclusions. I examined the relationship between Ascaris infestation and serum IgM concentrations in Coloured females and Bantu males using the Mann-Whitney U test - no significant association was detected (Table 3.22).

Helminths in the stools	Coloureds (N = 54) -ve	Coloureds (N = 54) +ve	Bantu (N = 32) -ve	Bantu (N = 32) +ve
Number	32	22	18	14
Mean IgG mg/100 ml	1046	1095	1427	1373
	H=0.48	DF=1	H=0.28	DF=1
	NS		NS	

Table 3.17

Kruskal-Wallis analysis of the relationship between helminthic infestation and serum IgG concentrations in the Coloureds and the Bantu.

Ascaris in the stools	Coloureds (N = 54) -ve	Coloureds (N = 54) +ve	Bantu (N = 32) -ve	Bantu (N = 32) +ve
Number	48	6	26	6
Mean IgG mg/100 ml	1140	923	1391	1457
	H=2.86	DF=1	H=0.76	DF=1
	NS		NS	

Table 3.18

Kruskal-Wallis analysis of the relationships between serum IgG concentrations and Ascaris infestation in the Coloureds and the Bantu.

Helminths in the stools	Coloureds (N = 54)		Bantu (N = 32)	
	-ve	+ve	-ve	+ve
Number	32	22	18	14
Mean IgA mg/100 ml	188	179	1427	1373
	H = 0.10	DF=1	H = 0.28	DF=1
	NS		NS	

Table 3.19

Kruskal-Wallis analysis of the relationship between serum IgA concentrations and helminthic infestation in the Coloureds and the Bantu.

Ascaris in the stools	Coloureds (N = 54)		Bantu (N = 32)	
	-ve	+ve	-ve	+ve
Number	48	6	26	6
Mean IgA mg/100 ml.	184	182	1391	1457
	H = 0.023	DF=1	H = 0.76	DF=1
	NS		NS	

Table 3.20

Kruskal-Wallis analysis of the relationships between serum IgA concentrations and Ascaris infestation in the Coloureds and the Bantu.

Helminths in the stools	Coloured males (N=37) -ve	Coloured males (N=37) +ve	Bantu males (N=28) -ve	Bantu males (N=28) +ve
Number	27	10	16	12
Mean IgM mg/100 ml	98	167	174	148
	H = 7.49	DF = 1	H = 0.82	DF = 1
	p < .01		NS	

Table 3.21

Kruskal-Wallis analysis of the relationships between serum IgM concentrations and helminthic infestation in Coloured males and Bantu males.

Ascaris in the stools	Coloured females (N=17) -ve	Coloured females (N=17) +ve	Bantu males (N=28) -ve	Bantu males (N=28) +ve
Number	13	4	23	5
Mean IgM mg/100 ml.	178	177	147	112
	(Mann-Whitney U test)		(Mann-Whitney U test)	
	NS		NS	

Table 3.22

Analysis of the relationships between serum IgM concentrations and Ascaris infestation in Coloured females and Bantu males; because of the small numbers involved the Mann-Whitney U test was used.

Eosinophils*	White males (N = 61) Normal	High	White females (N=44) Normal	High
Number	42	19	33	11
Median IgE u/ml	93	112	50	112
	H = 0.11	DF = 1	H = 0.12	DF = 1
	NS		NS	

Eosinophils*	Coloured males (N = 50) Normal	High	Bantu males (N = 31) Normal	High
Number	35	15	15	16
Median IgE u/ml	172	308	555	761/809
	H = 3.20	DF = 1	H = 0.79	DF = 1
	NS ($p < .1$)		NS	

Table 3.23

Kruskal-Wallis analysis of the relationship between high peripheral eosinophil counts and serum IgE concentrations.

* Normal eosinophil counts were considered as being < 4% on the differential count or $\leq$ 250 per cu.mm on the specific eosinophil count.

3.3.j. The relationship between serum IgE concentrations and specific eosinophil counts.

As shown in Table 3.23 in none of the groups that I analysed, White males, White females, Coloured males and Bantu males, did I detect a significant association between elevated serum IgE concentrations and high peripheral eosinophil counts.

3.3.k. Correlations between serum IgE concentrations and serum IgG, IgA and IgM concentrations.

In Tables 3.24 to 3.26 I have summarised the results of the Kendall rank correlation analysis designed to test for correlation between serum IgE concentrations and concentrations of other immunoglobulins. No such correlation could be found, except in the case of the Bantu, where serum IgE concentrations correlated significantly with serum concentrations of IgG and IgA.

	IgE with IgG		
	Whites	Coloureds	Bantu
Number	118	73	38
S	598	3	225
Tau	0.092	0.001	0.324
Z	1.474	0.014	2.861
	NS	NS	p<.0025

Table 3.24

Results of determinations of associations between serum IgE concentrations and serum IgG concentrations in the three racial groups using the Kendall rank correlation coefficient, Tau.

	IgE with IgA		
	Whites	Coloureds	Bantu
Number	119	73	39
S	-236	403	211
Tau	-0.035	0.155	0.290
Z	-0.571	1.935	2.602
	NS	NS	p<.005

Table 3.25

Results of determinations of associations between serum IgE concentrations and serum IgA concentrations in the three racial groups using the Kendall rank correlation coefficient, Tau.

	IgE with IgM					
	White males	White females	Coloured males	Coloured females	Bantu males	Bantu females
Number	69	51	53	20	34	5
S	316	-22	-29	-6	77	2
Tau	0.141	-0.018	-0.021	-0.032	0.139	0.2
Z	1.710	-0.191	-0.224	-0.198	1.154	
	NS($p<.05$)	NS	NS	NS	NS	NS

Table 3.26

Results of determinations of associations between serum IgE concentrations and IgM concentrations in the different population groups using the Kendall rank correlation coefficient, Tau.

3.4. Discussion

The work reported in this Chapter was undertaken to explore, in greater depth, some of the population differences exposed by the previous survey of 4650 blood donors.

The data obtained in this study enabled me to draw certain general conclusions regarding the prevalence of allergic symptoms, cutaneous hypersensitivity, and intestinal helminthic infestation in the various population groups and to relate these to one another, to serum IgE concentrations, and to serum concentrations of other immunoglobulins. Since these interrelationships are somewhat complex, I have chosen to present this discussion under interrogative headings.

3.4.a. Do population groups with high serum IgE concentrations show a correspondingly high prevalence of allergic symptoms?

It will be recalled, from the data presented thus far, that serum IgE concentrations tended to be higher in Coloureds and Bantu than in Whites, and in males than in females. Any suggestion that these population differences could be ascribed to corresponding differences in the prevalence of allergic disease would require, in substantiation, evidence to indicate that allergic symptoms occur with relatively greater frequency or severity in Coloureds and Bantu than in Whites, and in males than in females.

For the reasons discussed earlier (Section 3.2.b.) my estimates of the prevalence of allergic disease are subject to errors derived both from difficulties in eliciting symptoms and from ascribing these symptoms to IgE-mediated hypersensitivity. Despite these difficulties, my findings in Whites were in good accord with those reported for similar population groups by other workers. Thus Williams (1959) concluded that 10% of individuals

in the United States suffered from major allergic illness and that a total of 40% to 60% suffered allergic symptoms of some kind. More recently Davis (1972) estimated that 31 million residents of the United States (corresponding to approximately 15% of the population) suffered from allergic disease. My own data indicated that 18% of White blood donors had symptoms of allergy severe enough to warrant treatment or were "clinically allergic", and that a total of 42% of the population suffered from symptoms which could be ascribed to allergy.

Analysis of the data by race (Figure 3.3) showed that, if anything, the prevalence of allergic symptoms in the Bantu was lower than that in the Whites. This may be a spurious finding due to difficulties in ascertaining the true prevalence of allergic disease in the Coloureds and Bantu (see Section 3.2.b.). Nevertheless my finding of a low prevalence of allergic symptoms in the Bantu tends to refute the hypothesis that allergy is responsible for the elevated serum IgE concentrations found in these people. Johannson et al (1968b),the first group to report elevated serum IgE concentrations in Africans, did not specifically look for allergy in their subjects, but they did comment that allergic symptoms were not recognised as being particularly common in the population they were studying (Ethiopians).

My finding of a low prevalence of allergy in the Bantu also tends to refute the hypothesis of Tullis (1970) that asthma is associated with Ascaris infestation; I found the Bantu not only to have a low prevalence of allergic symptoms but also a much higher prevalence of helminthic infestation, including Ascaris infestation, than the Whites (Table 3.4, Figure 3.6). Also Table 3.6 shows that in the Coloureds (there were too few allergic Bantu to permit a similar analysis in the Bantu) there was no significant association

between the presence of helminths in the stools and positive histories of allergy. In refuting the work of Tullis (1970), Van Dellen and Thompson (1971) examined 258 stool specimens from 123 asthmatics and found none to contain helminths or helminthic ova.

Analysis of the data by sex (Figure 3.1) showed no sex difference in the prevalence of allergic symptoms. This is in keeping with the observations made in adults by most other workers (for review see Williams (1959)). The situation in children appears to be rather different, Logan (1953) examined the records kept by a number of general practitioners in England and Wales and extracted the following data on the prevalence of allergy:

Prevalence of asthma analysed by sex and age

Ages	Males	Females
0-14	0.97%	0.51%
15-44	0.64%	0.70%
45-64	1.07%	1.25%
65 plus	1.00%	1.18%

Thus, while he found asthma to be more prevalent in boys than girls, he also found that, in the middle-aged and elderly, asthma was more prevalent in women than men. Similarly, Morrison-Smith (1961) found that the prevalence of asthma in 5 year old boys was twice that found in 5 year old girls. This sex difference became far less obvious in children than in older age groups.

There would appear to be no grounds for believing that inter-racial differences in serum IgE concentrations are related to differences in the prevalence of allergy. The situation with the sex difference in serum IgE concentrations appears to be somewhat different; the good evidence for a sex difference in the prevalence of allergy in children but not in young adults does suggest a constitutional predisposition towards immediate hypersensitivity in males, the

manifestations of this sexual predisposition being obscured clinically, in adults, by environmental, humoral or other factors, but remaining detectable by the tendency for serum IgE concentrations to be higher in adult males than adult females.

3.4.b. Within population groups do persons with allergic symptoms tend to have higher serum IgE concentrations than asymptomatic individuals?

In 1967 Johannson first described elevated concentrations of IgND in the serum of persons with asthma. Since then, numerous reports have appeared indicating that serum levels of this immunoglobulin (now known as IgE) tend to be raised in patients with allergic disease (Berg and Johannson (1969), Juhlin, Johannson, Bennich, Högman and Thyresson (1969), Rowe and Wood (1970), Gleich, Averbeck and Swedlund (1971), and Hogarth-Scott, Howlett, McNicol, Sims and Williams (1971)). In most instances, workers who have reported on the relationship between allergic disease and serum IgE levels, have studied Caucasian patients with allergy of sufficient severity to warrant their seeking medical advice, whereas in this study, I derived all my data from blood donors who were not selected, *a priori*, for the presence or absence of allergic symptoms. Those who gave positive histories were judged sufficiently healthy to be blood donors and were probably less severely affected than allergic subjects studied by others. Despite this difference in population sampling, my results, in Whites, confirmed the results found by others (Table 3.9), inasmuch as I found Whites with allergic symptoms to have significantly elevated serum IgE concentrations. However, looking at the results in each sex (Table 3.10, Figure 3.12) I found that only in White males was there a significant ($p < .0005$) difference between the serum IgE concentrations of allergic and non-allergic subjects. The tendency for serum IgE concentrations

to be higher in allergic subjects was present in females but this was not statistically significant; in the Coloureds and the Bantu this tendency was not evident at all (Table 3.9, Table 3.10).

If the elevated serum IgE concentrations found in Caucasian allergic subjects reflect the presence of functionally active IgE capable of mediating allergic symptoms, one would expect to find a relationship between the severity of symptoms and the amount of IgE present in the serum of such allergic individuals. Although there are indications that such a relationship exists, it has not yet been definitely established. Berg and Johannson (1969) found that allergic children with asthma had higher levels than children with hayfever and suggested that this was associated with the more prolonged and severe symptoms that occur in asthma. They also found higher levels in children with perennial asthma than in children with seasonal asthma, and in children with inhalant allergy associated with atopic eczema than in children with inhalant allergy alone. Hogarth-Scott et al (1971) found a positive correlation between serum IgE concentrations and the clinical severity of the disease in asthmatic children. On the other hand, Juhlin et al (1969) investigating high serum IgE concentrations in adults with atopic dermatitis, found no significant association between serum IgE levels and severity of disease, or the presence of concomitant hayfever or asthma. Stenius, Wide and Seymore (1972) found no association between severity of allergic symptoms and total serum IgE levels. Kumar, Newcomb, Ishizaka, Middleton and Hornbrook (1971), commenting upon their failure to detect particularly high levels of serum IgE in children with severe asthma, suggested that steroid therapy might have obscured the expected association. My own results did not show any difference between the serum IgE concentrations of individuals I classed as clinically allergic and those I classed as mildly allergic. However, because none of my

subjects had what could be termed severe symptoms I do not feel that my findings are contrary to those reported by Berg et al (1969) and Hogarth-Scott et al (1971). Also, if their observations are correct, and serum IgE concentrations are related to the severity of allergic disease, these observations may partially explain my failure to find elevated serum IgE concentrations in allergic White female blood donors.

3.4.c. What is the clinical significance of positive skin tests?

One might reasonably assume that the direct skin test procedure would offer certain advantages, for estimating the prevalence of allergy, that are not provided by clinical interview. Prick-testing is objective, it gives information regarding the specificity of the allergic response, and it is generally agreed that a positive skin test reflects the presence of reaginic antibody. There are, however, a number of factors in regard to skin tests that need to be taken into account when considering their value in this context.

Firstly, the results obtained with direct tests depend, *inter alia*, upon the concentration of the allergen in the test solution (cf. Appendix I). The importance of concentration was clearly demonstrated by Bruce Pearson (1937) who tested non-allergic individuals with 20mg%, 2mg% and 0.2mg% solutions of horse dander protein and found the frequency of positive tests to be 37%, 12% and 2% respectively. Allergen containing solutions are notoriously variable in potency, and, as no completely satisfactory way of standardizing them is yet available, results can only be compared if identical solutions are used for tests.

Secondly, there is a measure of uncertainty regarding the criteria for calling a result "positive". Gottlieb, Stupniker and Askovitz (1960) investigated the reproducibility of intradermal skin tests and concluded that small reactions were less reproducible than

large ones, and should therefore be ignored. They did not take into consideration the linear-log relationship between wheal size and concentration of test solution described by Squire (1950)(cf. Appendix I). It follows from this relationship that small reactions are far more dose-dependent than large ones and, therefore, more subject to experimental error. Nevertheless a small positive reaction is as indicative of the presence of skin sensitizing antibody as a large one; its significance as an indication of the sensitivity of a subject obviously depends upon the dose of allergen required to elicit the reaction. Pepys (1971) points out that a carefully performed prick test causes minimal trauma so that a negative test shows only the site of the prick, if anything, and a very small reaction can be read as unequivocally positive. For these reasons I chose to consider small reactions as positive and accordingly I graded all persons with at least one reaction with a wheal greater than 2mm or a flare greater than 4mm as skin test positive.

Thirdly, there is considerable doubt regarding the relevance of a positive skin test to the clinical diagnosis of allergy. Aas (1969) reports cases showing poor correlations between skin tests, provocation tests and history of asthma, including some cases that had negative skin tests yet proved sensitive on bronchial challenge. I found that while in Whites there was a highly significant correlation between positive tests and allergic symptoms, no such associations were present in the Coloureds and the Bantu (Figure 3.7). Even in the Whites I found that 34% of individuals who gave a negative history of allergy had positive skin tests (Table 3.5) and 13% had at least one +++ reaction (a wheal greater than equal to 8mm or a flare greater than equal to 20mm).

Although, for reasons given above I cannot compare my results

directly with those obtained from other surveys, it is interesting to note that all workers who have performed tests on symptom-free individuals have found a considerable number of them gave positive reactions. Rackemann and Simon (1935) used intradermal injection of nine allergen extracts to test 60 patients who presented at hospital for reasons other than allergy and found that 50% of them showed at least one positive result. Grow and Herman (1936), again using intradermal tests, tested 150 symptom-free individuals with 13 allergen extracts and found 55.5% had positive tests. Forty of this group gave a history of some allergic symptoms in the past but the prevalence of positive tests in these subjects was no higher than in those subjects with completely negative histories. Bruce Pearson (1937) compared the prevalence of positive tests in asthmatic and control groups. Although tests were more frequently positive in asthmatic subjects, they were also positive in some subjects with completely negative personal and family histories. Curran and Goldmen (1961) stressed the need for excluding individuals with a past or family history of allergy from control groups of "normal" individuals. After careful history taking they selected 100 subjects who they considered "normal" and tested them with nine inhalant allergens. They found that 5% gave positive scratch tests and 9% gave positive reactions when intradermal tests were used to supplement doubtful or negative scratch tests. Fontana, Wittig and Holt (1963) assessed the prevalence of positive skin tests in children and found that sensitivity to house dust was particularly common; 88% of allergic children and 42% of non-allergic children reacted to it. It thus appears that skin-sensitivity to at least some of the antigens that occur in the environment can be considered "normal" in that such hypersensitivity frequently occurs in completely symptom-free individuals.

A number of workers have actively immunized human subjects for

skin-sensitizing antibody production. The results of these experiments show clearly that almost all individuals have the ability to synthesize skin-sensitizing antibody. Two groups of workers; Davidson, Baron and Walzer (1947) using multiple doses of Ascaris extract; and Greenert, Bernstein and Michael (1971) using multiple doses of short ragweed extract; were able to induce skin hypersensitivity in approximately 90% of non-sensitive individuals. The subjects studied by Greenert et al (1971) were carefully selected as being non-atopic, and, although they developed skin hypersensitivity, the results of provocative tests and tests for leucocyte histamine release were both negative. Nevertheless, there is good evidence that subjects with allergic symptoms more readily produce skin sensitizing antibody than do non-allergic subjects. Salvaggio and co-workers (Salvaggio, Cavanaugh, Lowell and Leskowitz (1964); Salvaggio, Keyman and Leskowitz (1966); Salvaggio, Castro-Murillo and Kundur (1969)) have shown that although both non-allergic and allergic individuals develop skin sensitivity after exposure to certain allergens, this sensitivity is induced more rapidly in allergic subjects. They found that the difference between allergic and normal subjects was particularly evident when intranasal immunization using aerosolized antigens was employed, and that atopic subjects immunized this way occasionally developed symptoms during the latter part of the immunization regimes. Kuhns and Pappenheimer (1952) found that allergic individuals more readily develop skin hypersensitivity to Diptheria toxoid than non-allergic individuals.

Thus the development of autologous skin sensitizing antibody is not, in itself, sufficient for the development of allergic symptoms, and allergic and non-allergic individuals do not differ from one another in their absolute ability to synthesize this anti-

body but rather in their rate of synthesis and possibly the amount they synthesize following a given antigenic stimulation. Direct skin tests with common allergens show which individuals have the tendency to develop this type of antibody following exposure to naturally occurring allergens and therefore these tests can be considered as measures of one aspect of the immune responsiveness of these individuals.

3.4.d. Do population groups with high serum IgE concentrations show a high prevalence of immediate cutaneous hypersensitivity?

As part of my analysis of the relationship between serum IgE concentrations and immediate cutaneous hypersensitivity in local population groups, I determined whether those population groups with relatively high serum IgE concentrations (that is males as opposed to females and Coloureds and Bantu as opposed to Whites) had correspondingly high prevalences of positive tests.

Analysis of the data by sex (Figure 3.2) demonstrated an equal prevalence of positive tests in each sex in the Whites and the Coloureds. In the Bantu, on the other hand, males were considerably more reactive than females. As I have commented previously, the group of Bantu females was small, and I do not feel justified in drawing conclusions from this result; however, owing to this finding my further discussion on skin test results in the Bantu refers specifically to the results I obtained in males.

Analysis of the data by race (Figure 3.4) showed that Bantu males had a high prevalence of positive tests compared to White males and Coloured males. Furthermore, although sensitivity to Ascaris was frequent in the Bantu this alone was insufficient to explain the high prevalence of positive tests in this group; thus when individuals who were sensitive to Ascaris alone were excluded from the analysis, the Bantu showed a significantly higher prevalence of

positive tests to inhalant allergens than did the Coloureds or the Whites (Figure 3.5). As noted earlier the Bantu had a low prevalence of allergic symptoms and in addition showed no relationship between positive allergic histories and positive skin tests. Thus allergy is not the reason for their high prevalence of positive tests. There is, however, some good circumstantial evidence to indicate that helminthic infestation is one of the factors responsible. As I shall discuss later, the stool examinations were more valuable in showing which groups were exposed to helminths than in diagnosing infestation in individual subjects, and, although I did not find subjects with proven helminths in their stools to have a high prevalence of positive tests (Table 3.6, only in Coloureds did I have sufficient subjects for analysis), I did find the Bantu to have a high prevalence of infestation (Figure 3.6). The work of Jarret and co-workers (Jarret (1972), Jarret, Henderson, Riley and White (1972)) has indicated that helminthic infestation in rats can promote the formation of both anti-worm reagin and reagin directed against antigens to which the animals were previously exposed. These results suggest the possibility that persons who have been exposed to helminths in such a way as to develop anti-worm hypersensitivity, may, due to the adjuvant like effect of helminthic infestation, develop hypersensitivity to other allergens. To examine the implications of this suggestion, I compared the frequency of positive skin tests to inhalant allergens in persons with and without positive tests to Ascaris extract (Table 3.27). Although I found Ascaris sensitive individuals did have a high prevalence of positive tests to other allergens, I found that the same was true of sensitivity to House Dust mite. Thus constitutional factors that predispose some individuals to synthesize reagin more easily than others appear to have been operative, and this analysis did not contribute

		Ascaris skin test		
		Neg.	Pos.	Total
Skin test to other allergens	Neg.	120	8	128
	Pos.	94	46	140
		214	54	268

$p < .0005^{*}$

		House Dust mite skin test		
		Neg.	Pos.	Total
Skin test to other allergens	Neg.	108	12	120
	Pos.	97	51	148
		205	63	268

$p < .0005^{*}$

Table 3.27

Analysis of the prevalence of skin hypersensitivity to other allergens in individuals sensitive to Ascaris extract and House Dust mite extract.

* Chi-square analysis.

to the question of whether Ascaris infestation promotes reagin synthesis to unrelated allergens. The fact remains, however, that elevated serum IgE concentrations, helminthic infestation, and positive skin tests occur with greater relative frequency in the Bantu and it is tempting to suggest that these three factors are immunologically related.

3.4.e. Within population groups do persons showing evidence of immediate skin hypersensitivity to common allergens have elevated serum IgE concentrations?

Reaginic antibody has the fundamental property of being able to sensitize tissue mast cells. Subsequent exposure of these cells to appropriate antigen results in the release of soluble

mediators and a type I hypersensitivity reaction. The skin test provides a semiquantitative way of measuring cell-bound specific reaginic antibody. The relationship between cell-bound and circulating antibody is not yet fully understood. Stanworth and Kuhns (1965) showed a linear relationship between wheal size and the logarithm of the concentration of serum (and therefore antibody) used to sensitize sites for P.K reactions; it seems reasonable to predict that a similar type of relationship exists between circulating autologous antibody and skin reactivity. A complicating factor is the possibility that reaginic antibodies of different specificities, including locally produced antibodies, compete with one another for cellular binding sites. Jarret, Orr and Riley (1971) demonstrated that passive sensitization of rat skin was inhibited if large amounts of reaginic antibody of unrelated specificity were present. Ishizaka, Soto and Ishizaka (1973) have developed a complicated method of measuring cell-bound IgE that involves the fixation and subsequent transfer of C1 molecules. They found no relationship between total serum IgE concentrations and the number of IgE molecules bound to circulating basophils. Their results did suggest that basophil binding sites tend to be more fully saturated in allergic rather than non-allergic individuals. Yet another factor to consider is that all human reaginic antibody may not be IgE; Parish (1970) has shown that some IgG antibodies have the ability to sensitize monkey skin; Ishizaka, De Bernado, Tomioka, Lichtenstein and Ishizaka (1972) demonstrated that anti-human IgG can mediate histamine release from human basophils in a similar type of reaction to that mediated by anti-human IgE. Nevertheless, they also showed that IgE was the principal antibody involved. In previous papers these authors had shown the reaginic activity of serum from a Ragweed-sensitive subject to be almost exclusively IgE (Ishizaka, Ishizaka and Hornbrook (1966b), and that IgE was

present on the basophils of both normal and allergic subjects (Ishizaka, Tomioka and Ishizaka (1970)).

If skin sensitivity to common allergens and total serum IgE concentrations are both measures of ability to mount an IgE response to environmental allergens, high serum IgE concentrations should be associated with immediate skin hypersensitivity to common allergens. However, comparing the serum IgE concentrations of skin test negative and skin test positive individuals, I found only White males and Coloured males to have significant associations in this regard (Table 3.13). Moreover, the association was far more significant in White males than Coloured males ($p < .0005$ against $p < .02$). White females and Coloured females did show the same trend but analysis of the data failed to show it significant. In Bantu males, the group which tended to have the highest serum IgE concentrations and which had the highest prevalence of positive tests, I found no relationship at all. Possible explanations for this are that the Bantu possessed antibodies directed against allergens which were not included in the skin test regime; or that they possessed antibodies that carried ε chain determinants, but that were incapable of mediating a type I immediate hypersensitivity response.

Some workers have reported significant associations between the intensity of positive skin tests to given allergens and levels of specific IgE in the serum. Stenius, Wide, Seymour, Holford-Strevens and Pepys (1971) demonstrated significant correlations between concentrations of specific IgE to Dermatophagoides pteronyssinus, Dermatophagoides farinae and grass pollen and the size of prick test reactions to the same allergens. Norman, Lichtenstein and Ishizaka (1973) found a similar relationship between specific serum IgE concentrations and the titres of Ragweed pollen

required to elicit a positive test. The relationship between the degree of skin reactivity and total serum IgE concentrations is obviously more complex, nevertheless Stenius et al (1972) found highly significant correlations between total serum IgE concentrations and the number of positive tests to common allergens and the size of these reactions. The method of skin test grading that I used to assess degree of skin hypersensitivity incorporated both the size of the largest reaction and the number of positive reactions. My results showed significant associations between the degree of skin hypersensitivity and total serum IgE concentrations in White males, White females and Coloured males (Table 3.14), the result being very highly significant ($p < .00003$) in White males. The trend was also present in Coloured females but it did not reach significance ($p < .05$), and in Bantu, of both sexes, there was no association. Thus although skin test positive White females as a group did not have significantly elevated serum IgE concentrations, there was a significant ($p < .01$) association between the grades of their reactions and their serum IgE concentrations.

3.4.f. Do serum IgE concentrations reflect primarily the tendency for individuals to develop skin hypersensitivity or the tendency for individuals to develop allergic symptoms?

It is generally accepted that the immunological reactions that initiate the wheal and flare response in the skin are the same as those that initiate symptoms in allergic disease. However, as I have discussed earlier (Section 3.4.c.), many subjects show evidence of cutaneous hypersensitivity without symptoms of systemic allergy. There are several possible explanations for this. Firstly, non-immunological factors, such as the ability of end organs to respond to mediators, may be of prime importance in

determining whether symptoms will develop. Secondly, locally produced IgE and local tissue mast cell concentration may be of importance in determining the reactivity of end organs. Thirdly, more IgE may be necessary to mediate symptomatic allergic reactions than is required to mediate cutaneous wheal and flare reactions. My own data could not be used to examine either of the first two explanations. I felt, however, that if I could show that serum IgE concentrations were higher in subjects with allergic symptoms and positive tests than in subjects with positive skin tests alone, this would provide supporting evidence in favour of the third explanation. For reasons discussed elsewhere (Sections 3.4.b. and 3.4.e.) White males were the only group to show significant associations between serum IgE concentrations and either positive allergic histories or positive skin tests and therefore my analysis was confined to this group. Figure 3.15 shows cumulative frequency diagrams of serum IgE concentrations in subjects who were (i) skin test negative, (ii) skin test positive and history negative, and (iii) skin test positive and history positive.

	White males (N = 60)		
	Skin test neg.	Skin test Pos./ History negative	Skin test Pos./ History positive
Number	33	11	16
Median IgE u/ml	42	75	265/294

H = 20.93 DF = 2

p < .0005

Table 3.28

Kruskal-Wallis analysis of serum IgE concentrations in White males who were (i) skin test negative, (ii) skin test positive and history negative, and (iii)skin test positive and history positive. The skin test negative group did contain two individuals who were skin test negative and history positive.

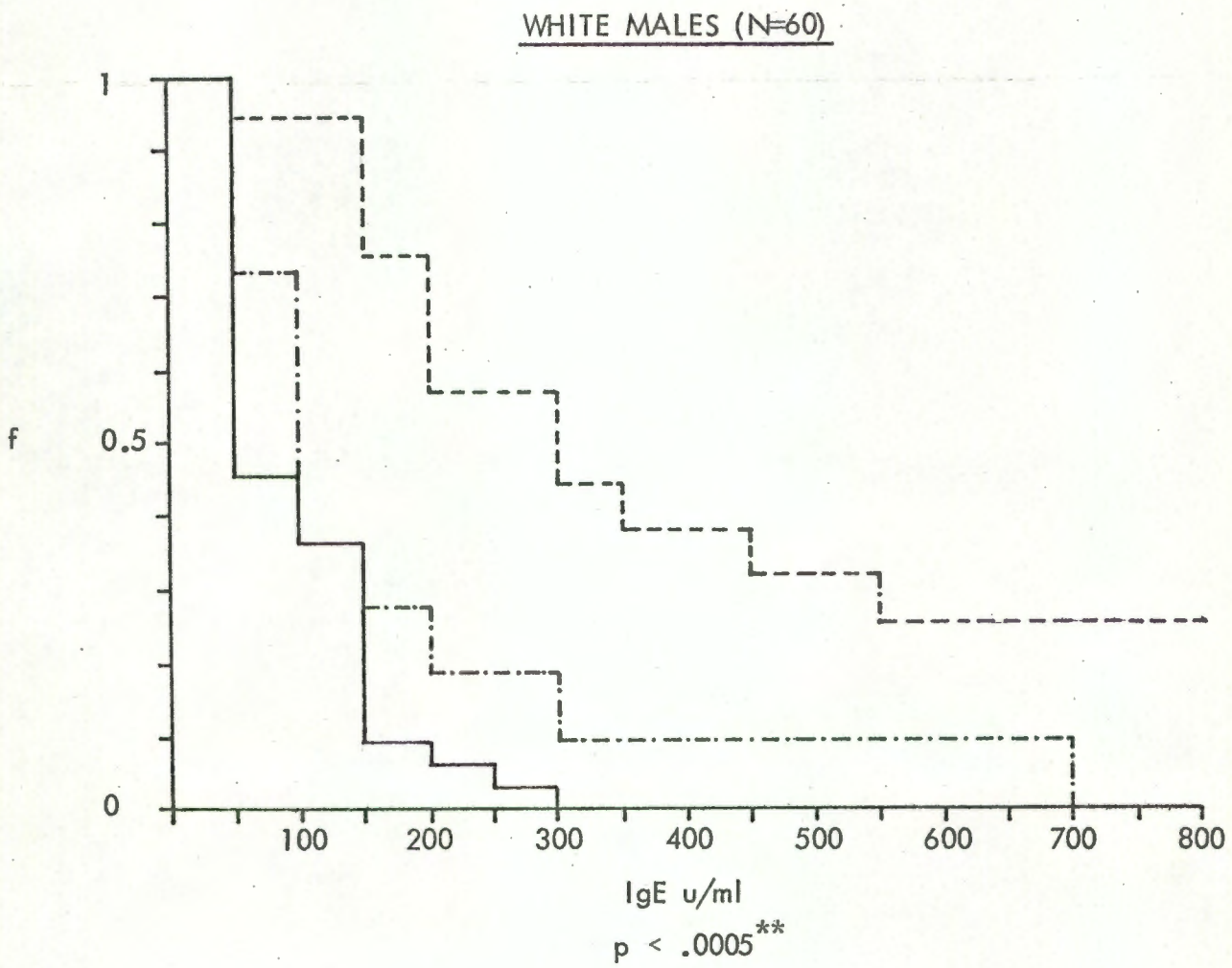


Figure 3.15

Cumulative frequency diagrams of serum IgE concentrations in White males.

(i) ——— skin test negative

(ii) -·-·-·- skin test positive/history negative

(iii) ------ skin test positive/history positive

** Kruskal-Wallis analysis of the significance of the difference in serum IgE concentrations between the groups. These data are summarised in tabular form in Table 3.28.

These results, summarised in Table 3.28, show that serum IgE concentrations were not raised in individuals with positive skin tests alone, whereas they were very significantly ($p < .0005$) elevated in persons with positive skin tests and positive allergic histories. Both positive skin tests and symptomatic allergic reactions depend on cell-bound rather than circulating IgE; therefore until it is possible to quantitate the amount of IgE bound to tissues it will not be possible to prove conclusively, at a cellular level, that more IgE is involved in mediating symptomatic allergic reactions than is involved in mediating wheal and flare reactions, nevertheless, my findings do strongly support this hypothesis.

3.4.g. Does the tendency for males to have higher serum IgE concentrations than females reflect an effect of the interaction between sex and atopy on the serum IgE concentration?

The results of the survey presented in Chapter II showed that serum IgE concentrations of White males tended to be significantly higher than those of White females, whereas the results of the present survey indicated that symptoms of allergy and cutaneous hypersensitivity were encountered with approximately equal frequencies in the two groups (Figure 3.1, Figure 3.2). Furthermore, in White males there were highly significant associations between both positive allergic histories and positive skin tests to common allergens, whereas in White females this was less obvious (Table 3.10, Figure 3.12, Table 3.13). Atopy may be regarded as a tendency towards hypersensitivity which may be manifest by either positive allergic symptoms, evidence of cutaneous hypersensitivity, or both. The observations discussed above, taken in conjunction, suggested a possible interaction between sex and atopy in their effects upon

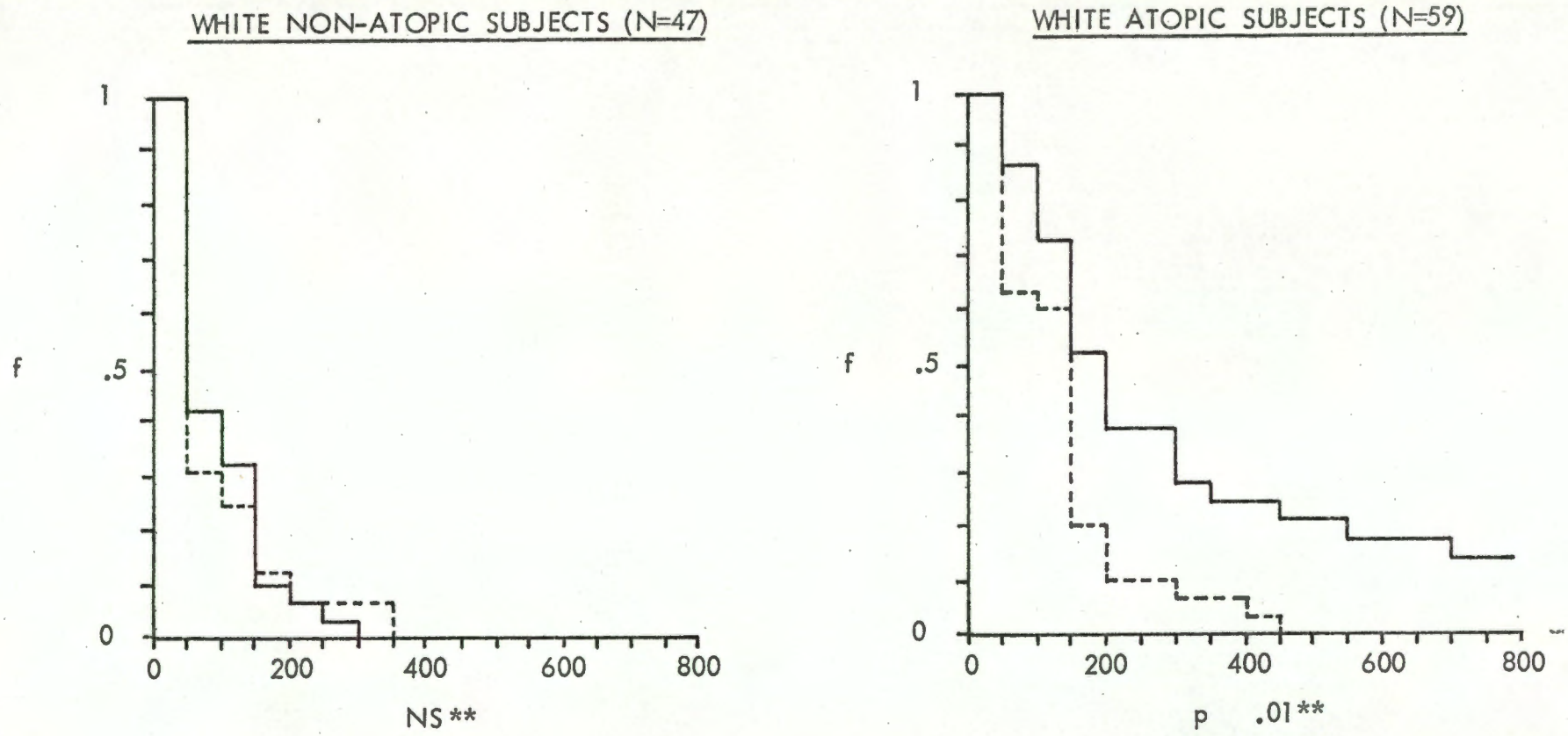


Figure 3.16

Cumulative frequency diagrams of serum IgE concentrations in non-atopic and atopic individuals. The data have been analysed by sex.

(i) ——— indicates males

(ii) - - - - - - - indicates females

** Kruskal-Wallis analysis of the significance of the difference in serum IgE concentrations. These data have been summarised in tabular form in Table 3.29.

serum IgE concentrations. In other words maleness and the atopic diathesis in combination might have a more pronounced effect upon serum IgE concentrations than the sum of the two effects individually. Analysis of the results to expose such an interaction showed that this was, indeed, the case.

As can be seen from the data summarised as cumulative frequency diagrams in Figure 3.16 and Table 3.29, atopic males tended to have significantly higher serum IgE concentrations than atopic females ($p < .01$) whereas non-atopic males and females showed no difference in this respect. It seems highly probable, therefore, that differences in serum IgE concentrations between males and females reflect a masculine tendency for hypersensitivity to be associated with increased serum IgE levels. Current knowledge of the relationship between serum IgE concentrations and allergic symptoms is such that I cannot draw any conclusions as to whether this tendency to elevated serum IgE concentrations in atopic males is in any way responsible for the high prevalence of allergy - particularly asthma - in boys as opposed to girls (Logan (1953), Morrison-Smith (1961)).

	White Non-atopic subjects (N = 47)		White atopic subjects (N = 59)	
	Males	Females	Males	Females
Number	31	16	29	30
Median IgE u/ml	42	42	167	112
	H = 0.44 DF = 1 NS		H = 7.27 DF = 1 p < .01	

Table 3.29

Analysis by sex of serum IgE concentrations in non-atopic and atopic White subjects. Atopic subjects were subjects who were either allergy history positive, skin test positive or both.

As a corollary to the results in Table 3.29 and to my finding higher serum IgE concentrations in skin test positive White males than skin test positive White females (Table 3.13) I examined the results of the skin tests to see if White males showed any evidence of greater skin hypersensitivity than White females. To do this I used the Kruskal-Wallis technique to analyse, by sex, graded skin test results obtained in Whites. Table 3.30 shows that, although the difference was not significant by my criteria ($p < .05$), there was a tendency for skin test grades to be higher in skin test positive White males than in skin test positive White females; thus the median grade for males was 8 (that is the maximum skin test was +++ and there was more than one +++ reaction) while the median grade for females was 5 (that is the median skin test was ++ and there was more than one ++ reaction).

	Whites - Skin test positive (N = 65)	
	Males	Females
Number	39	26
Median skin test grade	8	5
	H = 5.34	DF = 1
	NS ($p < .05$)	

Table 3.30

Analysis by sex of the graded skin test results of skin test positive Whites. Skin test results were graded according to the scheme given in Section 3.2.d.

My finding that the sex difference in serum IgE concentrations was confined to atopic subjects may offer an explanation for the fact that Johannson (1968) and Gleich et al (1971) failed to find a sex difference in serum IgE concentrations. Both groups investigated subjects whom they had established as being free of

allergic symptoms. The data I presented in Chapter II were obtained from an unselected group of blood donors (see Section 2.4.a.).

Two groups of workers have reported on the relationship between allergy and serum IgE concentrations in females. Johannson (1968) found that serum IgE concentrations of a group of allergic postpartum mothers were not significantly higher than those found in a group of healthy adults; on the other hand Bazeral (1973) found allergic postpartum mothers had higher serum IgE concentrations than a similar group of non-allergic subjects. As I discussed earlier, it is possible that I would have found a more significant rise in serum IgE concentrations of allergic females if I had investigated subjects with more severe allergy.

Also regarding the influence of sex, allergy, and the interactions of these two on serum IgE concentrations, it seems intuitively probable that these influence total serum IgE concentrations to a slight and subtle extent that is likely to be obscured by grosser factors, such as parasitic infestation, that lead, relatively speaking, to far more pronounced elevation in serum IgE concentrations. If this is so, it is scarcely surprising that the effect of hypersensitivity and sex were not as apparent in the Coloureds and the Bantu, as in the Whites.

3.4.h. Do population groups with high serum IgE concentrations show a correspondingly increased prevalence of helminthic infestation?

Johannson et al (1968b) considered that helminthic (particularly Ascaris) infestation was responsible for the elevated serum IgE concentrations that they encountered in Africans. Subsequent work has tended to support this hypothesis, in that, elevated serum IgE concentrations have been found in association with viseral larva

migrans (Hogarth-Scott, Johannson and Bennich (1969)), capillariasis (Rosenberg, Whalen, Bennich and Johannson (1970)), and trichinosis (Rosenberg, Polmar and Whalen (1971)). Phills, Harrold, Whiteman and Perelmutter (1972) found considerably elevated serum IgE concentrations in four Canadian students who, as victims of a prank, had eaten large numbers of ripe Ascaris ova. Thus, although the immunological mechanisms involved are obscure, there is a well documented relationship between helminthic infestation and elevated serum IgE concentrations in man. My own results are consistent with this conclusion, in that, I found that those race groups with high serum IgE concentrations, that is the Coloureds and the Bantu, had a high prevalence of helminthic infestation (Figure 3.6) and that Ascaris lumbricoides was one of the parasites involved (Table 3.4).

3.4.i. Within population groups do those individuals with helminthic infestation have higher serum IgE concentrations than those individuals in whom no such infestation can be demonstrated?

If helminthic infestation were responsible for the elevated serum IgE levels found in the Coloured and the Bantu, one might expect to find that, within these groups, individuals with proven intestinal helminthic infestation would have higher serum IgE levels than those in whom such parasites could not be detected. On analysis of my data (Table 3.15, Figure 3.14) I could find nothing to substantiate this expectation.

The significance of my failure to find the expected association is diminished by a number of factors. Firstly, the inability to detect ova or parasites in a single, random faecal specimen is by no means conclusive evidence of freedom from helminthic infestation. This is not only because the examiner may fail to detect ova that are being excreted erratically, and in small numbers, but also because

ova are not produced at all during the early stages of infestation; in the case of Ascaris, ova are only excreted two months after infestation. This is an important drawback to stool examination; the work of Phils et al (1972) on acute Ascaris infestation and that of Rosenberg et al (1971) on acute Trichinella infestation have shown that serum IgE concentrations are elevated two to three weeks after infestation. Secondly, elevated serum IgE levels may persist after elimination of the parasite. Thus Rosenberg et al (1970), for example, found that serum IgE levels of Filipinos treated 18 months previously for capillariasis, although lower than they were during the acute stage of the disease, were nevertheless considerably higher than those of uninfested controls. Thirdly, the nature of the offending parasite may be an important factor in determining whether an IgE response is invoked or not. Thus elevated serum IgE levels do not appear to be a feature of trichiuriasis (Rosenberg (1971)), or enterobiasis (Jarret and Kerr (1973)). In order to obtain sufficient numbers for analysis, I considered all cases of helminthic infestation as a group without regard for the species of helminth involved. By including, in this group, a preponderance of subjects with Trichuris trichuria infestation (Table 3.4), I may have obscured the effect of infestation with relevant parasites. Although the numbers involved are small, the results obtained when only cases of Ascaris infestation were considered (Bantu males and Coloured females, Table 3.16) do suggest that Ascaris infestation is, within any group, associated with elevated serum IgE concentrations. Fourthly, although it is fairly well established that certain helminthic infestations lead to elevated serum IgE concentrations there is no reason to believe that these are the only agents responsible for the elevated serum IgE concentrations in the Coloureds and Bantu.

3.4.j. Do those population groups who tend to have elevated serum IgE concentrations tend also to have elevated serum levels of other immunoglobulins?

It has been recognised since 1936 that Africans tend to have higher serum immunoglobulin concentrations than Caucasians (Andes, Kampier and Adams (1936)). This phenomenon has been speculatively ascribed to social and other factors that predispose these subjects to a high intensity of antigenic stimulation by various bacterial, viral, protozoal and helminthic diseases. In order to establish whether the raised serum IgE concentrations I had observed in the Coloureds and Bantu were manifestations of this type of general hypergammaglobulinaemia, I determined whether those population groups with elevated serum IgE concentrations had elevated serum concentrations of other classes of immunoglobulin. I found that both the Bantu and the Coloureds had elevated serum IgG and IgA concentrations compared to the Whites, this being most marked in the Bantu, and that there were no racial differences in IgM concentrations (Table 3.7). A number of workers in various parts of Africa have measured IgG, IgA, IgM and IgD concentrations in local population groups, Michaux (1966) working in the Congo, found only serum IgG concentrations to be elevated; Turner and Voller (1966) in Nigerians and Rowe, McGregor, Smith, Hall and Williams (1968) in Gambians found elevated levels of IgG and IgM, the latter group also found elevated serum IgD concentrations in children. Johannson et al (1968_b) in their studies of Ethiopian pre-school children found significantly elevated serum IgG and IgD concentrations but only slight elevation of serum IgA and IgM concentrations. One may conclude from these results that Africans seem, by Western standards, invariably to have elevated serum IgG concentrations, whereas the concentrations of IgA and IgM vary from one African population group to another. It

would appear from the work of Rowe et al (1968) and Johannson et al (1968b) that elevated serum IgD concentrations occur in African children rather than adults.

Most workers, including myself, who have investigated the hypergammaglobulinaemia that occurs in Africans have measured immunoglobulin levels in a control group of Caucasian subjects. This direct comparison obviates some of the problems of standardization. Shulman (1973) has pointed out that commercially available standards are not always satisfactory and that their use can lead to erroneous results in terms of absolute values. To overcome this difficulty the World Health Organisation provides International standards which can be used for calibrating local standards. Not realising, at the time I embarked upon this survey, the difficulties associated with the use of commercial standards, I used an International standard only for the IgE estimations. I shall, therefore, not attempt to compare the absolute values that I obtained for serum IgG, IgM and IgA concentrations with those reported by other workers.

My data show that not only were both serum IgG and IgE concentrations elevated in the Bantu, but also that in terms of absolute amounts of immunoglobulin present, the elevation of IgG concentrations was very much the greatest. "Normal" Caucasian IgE and IgG concentrations are approximately 100 ng/ml and 10 mg/ml respectively. An increase of 1µg/ml of either would lead to a tenfold increase in the IgE concentration but only to an undetectable (0.01%) change in the IgG concentration.

It is still speculative as to whether those factors that lead to increased levels of other immunoglobulins also lead to elevated IgE concentrations. Although it is recognised that bacterial infections do lead to increased immunoglobulin levels,

there is some evidence that this only occurs when the infection is severe and long standing. Belfrage (1963) found that increased gammaglobulin concentrations in subjects with acute pneumonia only occurred if fever had lasted 10 days or more. Nordbring, Högman and Johannson (1969) also studying subjects with pneumonia, found acute bacterial and Mycoplasma infections did not influence serum IgE levels, and that serum IgG and IgM levels became elevated only in cases of clinically severe bacterial infection and Mycoplasma infection respectively. On the other hand, chronic bacterial and fungal infections are consistently associated with elevated IgG concentrations, and certain protozoal infections such as trypanosomiasis are consistently associated with elevated IgM levels and increased IgM/IgG ratios (McKlvey and Fahey (1965)). It is thus apparent that long standing rather than transient infections affect total serum immunoglobulin concentrations and that the class of antibody produced depends to a certain extent on the infective agent involved.

How helminthic infestation, in particular, influences total serum IgG, IgM and IgA concentrations is also unknown. Higashi and Chowdhury (1971) found that levels of all three were high in Indian subjects compared with Caucasian subjects living in India, but they were unable to demonstrate that Indians with proven helminthic infestation had higher levels than those who were believed to be free of infestation. My own results, apart from my finding of high IgM concentrations in Coloured males with helminthic infestation, were very similar (Table 3.17 - 3.22). Unfortunately, both Higashi and Chowdhury's results and my own are compromised by the difficulty involved in establishing that an individual is free of parasitic infestation.

The change in total serum immunoglobulin level that occurs following strong antigenic stimulation is only partially explained

by increased amounts of circulating antibody specific for the antigen concerned. Humphrey (1963) observed that rabbits injected with killed tubercle bacilli (in the form of Freunds complete adjuvant) showed a much greater rise in total IgG levels than could be accounted for by antibody specific for the tubercle bacilli. Examination for antibodies that the animals were known to have been producing previously and for standard "natural" antibodies showed trivial increases at the most. Despite this, Freund's complete adjuvant, when given with antigen markedly enhances production of antigen-specific antibody. Thus certain agents have the ability to:

(1) promote specific antibody formation - in other words promote the formation of antibody that has the capacity for combining specifically with antigenic determinants on the agent itself;

(2) promote non-specific antibody formation - in other words promote the formation of antibody that does not combine specifically with determinants either on the agent itself or on antigens to which the animal has previously been exposed;

(3) act as an adjuvant - in other words promote the formation of antibody that is directed against antigenic determinants which are unrelated to the agent but to which the animal has at some time been exposed.

As indicated above infective agents can promote antibody formation in all three ways. However, the role of infective agents, other than certain helminths, in promoting specific reaginic antibody formation, and the role of infective agents in promoting non-specific reaginic antibody formation is unknown. It is possible that a proportion of the relatively large amounts of IgE found circulating in the Bantu is, in this sense, non-specific. The role of infective agents as adjuvants for reagin production in animals has been well

documented. Jarret's finding (Jarret (1972)) that Nippostrongylus brasiliensis infestation in rats promoted anti-ovalbumin reagin production has already been discussed (see Section 3.4.d.). Mota (1964) found that rats immunized with antigens and killed Bordetella pertussis produced reaginic antibody. Since then B. pertussis has been used by several workers (Tada and Okumura (1970), Jarret et al (1972)) to promote reagin production. Certain strains of killed Corynebacterium parvum have been shown to promote reagin production in rabbits (Pinckard and Halonen (1971)). Infective agents may well have the same type of adjuvant action in man. My finding that the Bantu had an increased prevalence of positive skin tests to common inhalant allergens (Figure 3.5) is consistent with the hypothesis that they had been exposed to certain infections which had enhanced their production of reaginic antibody directed against these inhalant allergens (see Section 3.4.d.).

Analysing my data to determine if, within population groups, there were correlations between serum IgE levels and levels of the other immunoglobulins, I found that there were no correlations in the Whites and in the Coloureds, whereas there were highly significant correlations between serum IgE levels and serum concentrations of IgG and IgA in the Bantu (Table 3.24 and Table 3.25). Thus I found that correlations between immunoglobulin levels were present in the race group that showed the most marked tendency to have elevated serum immunoglobulin levels. In no group did I find a significant correlation between IgE levels and IgM levels, nor could I demonstrate significant inter-racial differences in serum IgM levels.

It is not possible, with the information at present available, to draw any conclusions about the reasons for the associations between serum IgE, IgG and IgA concentrations in the Bantu. Certain of the Bantu may, owing to constitutional factors, have had a

heightened immune responsiveness to common antigens, or they may have been exposed to potent antigenic stimuli that tend to elicit the production of all three types of immunoglobulin. On the other hand the associations may have been fortuitous in that those stimuli leading to increased IgG and IgA production may have tended to occur, for sociological reasons, in those individuals particularly exposed to helminthic infestation. There is no evidence, either circumstantial or direct to support any one of these alternatives. There is clearly a need for information regarding the specificities of the immunoglobulins produced in excess.

APPENDIX I

The prick test

The prick test is a commonly used clinical test for immediate cutaneous hypersensitivity. By testing Bermuda grass sensitive subjects I was able to use this test to detect immuno-reactive material in the fractions of Bermuda grass pollen extract. Also I employed the prick test method to assess the prevalence of cutaneous hypersensitivity to common allergens in an unselected population of "normal" blood donors.

The tests were performed on the skin of the volar aspects of the arms and forearms. After cleaning the test area with water, drops of test solution were placed on the skin at least 3cm away from one another. The epidermis was pricked through the drops by gently lifting the superficial layers with the tips of sterile blood lancets in such a manner as not to draw blood. The solutions were wiped off after five minutes with care to avoid cross-contamination of adjacent prick test sites. The tests were read at the time of maximum immediate response - that is about 20 minutes after the initial challenge. The diameters of both wheal and flare were read using a transparent ruler.

In 1873 Blackley, using himself as a subject, performed the first skin test. He himself suffered from hayfever and by means of direct skin tests and inhalation tests he was able to show that certain pollens were the responsible agents. Cooke (1911) first used intracutaneous tests for clinical diagnosis and in the same year Noon (1911) introduced desensitization with offending pollens as a means of treatment for hayfever. Since then the various forms of skin tests for immediate hypersensitivity (the intracutaneous test, the scratch test, and the prick test) have been widely used in the practice of clinical allergy.

The accuracy of the prick test and the relationship between wheal size and antigen concentration were investigated by Squire (1950). Using solutions of histamine, he found that, provided the wheal size fell in the range 1mm - 6mm, there was an approximately linear relationship between wheal size and the logarithm of the histamine concentration. He also found a 16% coefficient of variation in wheal size when symmetrical body sites were tested on the same day, and a 22% coefficient of variation when the latter two precautions were not observed. He subsequently demonstrated that the relationship between wheal size and concentration of test solution applied equally when horse protein solutions were used for testing horse-sensitive subjects, and he used prick tests to identify and quantitate antigens in horse dandruff and horse serum. Johnson and Marsh (Johnson and Marsh (1965a),Marsh, Milner and Johnson (1966)) used this method which they called "quantitative prick testing" to estimate the relative potencies of their Rye grass pollen solutions. My own results with the fractions of the Bermuda grass pollen extract were based on the relationship described by Squire (1950), inasmuch as I assumed that the fractions which elicited the largest reactions contained the highest concentration of antigen or the most active antigen.

Although prick testing is a very satisfactory method for identifying antigen containing solutions, quantitative prick testing gives at least an ordinal level of measurement. There are several reasons why, despite the relationship described by Squire (1950), the method is not satisfactory for quantitating the amount of antigen present. The prick test is, in fact, a very superficial intradermal test and Squire (1950) calculated that the volume injected during a test is 3×10^{-6} ml. Therefore observations on the accuracy of intradermal tests are, for the most part, relevant to

the results obtained from prick tests. There is general agreement that the sizes of positive intradermal reactions vary with the body site of testing. By 1920 Schloss had shown that the sizes of positive reactions were smaller on the distal than on the proximal end of the arm; Alexander and McConnell (1930) extended this observation by testing on various body sites; they found that reactions on the trunk were consistently larger than those on the extremities. They also demonstrated that the variation that occurred was independent of whether testing was done with histamine on the skin of a normal individual, or with Ragweed pollen extract on the skin of a Ragweed sensitive individual. They considered that this result indicated that the size variation of positive tests was due to differences in the reactivity of skin sites and not to unequal distribution of skin-sensitizing antibody. Bowman (1935), Gottlieb et al (1960), and Becker and Rappaport (1948) all confirmed that there is a decrease of sensitivity at the distal as opposed to the proximal end of the arm; the latter group showed that this decrease was equivalent to an approximate 55% decrease in the strength of the skin testing solution. It is evident that it is possible to overcome the difficulty of anatomical variation in skin reactivity by testing symmetrical body sites, however this means that the relative potencies of only two solutions can be compared. Another problem with using prick tests quantitatively was pointed out by Johnson and Marsh (1965a), who found that although their prick test results confirmed the linear relationship between wheal size and log antigen concentration described by Squire (1950), the rate of variation of wheal size with antigen concentration varied for the different antigens in Rye grass pollen. For these reasons I made no attempt to determine the relative potencies of the different antigens in the Bermuda grass pollen extract.

Some authors (Herbertson et al (1958), Malley et al (1962), Augustin and Hayward (1962) and King and Norman (1962)) used "end point" skin test methods to detect the most active fractions of the allergens they were investigating; in other words they made dilutions (usually tenfold) of active fractions and recorded the greatest dilution giving a positive reaction. This method suffers from the difficulty, pointed out by Johnson and Marsh (1965a), that there is a tendency for active fractions to continue to produce small reactions even at extreme dilutions so that the end point is sometimes difficult to assess. For my purposes the end point method was not practical because of the large number of tests that it entailed per subject. I used prick testing to monitor samples for antigen activity and often had as many as 40 solutions to test per subject. Even the most co-operative of test subjects disliked having more tests than necessary.

As a method of detecting peaks of activity I found the prick test method very satisfactory. It was reproduceable in detecting Bermuda grass pollen antigens when identical sets of fractions were tested on different individuals. As discussed earlier, the relationship between wheal size and antigen concentration was linear/logarithmic; therefore, provided wheals were large enough for accurate measurement, the tests were most sensitive at low antigen dose. For the experiments performed to determine the physical constants of the antigens it was important to locate peak activity as accurately as possible, and therefore for these experiments I used small antigen loads, which resulted in small well defined peaks of activity. In the experiments performed to look for blocking of the direct skin response to the major antigen by low molecular weight material, it was necessary to accurately compare the reactions of paired samples. For these experiments symmetrical

body sites were used and tests were performed in duplicate on the same day.

Although investigations into the accuracy of direct skin tests have almost all been done measuring wheal diameters only, it is customary in evaluating prick tests clinically to measure both wheals and flares. I observed after testing a number of individuals that some tended to react with large wheals and relatively small surrounding flares and some to react with relatively large flares and small wheals. Therefore, maximum information can be obtained from skin tests if both wheals and flares are measured. This was done and reactions were graded, according to whether wheals or flares were relatively larger, following the grading scheme given in Chapter I (Section 1.2.b.).

Skin tests performed to detect cutaneous hypersensitivity to common allergens followed exactly the same procedure. As these tests were performed to determine the reactivity of subjects rather than the relative strength of antigenic solutions, the same problems of "quantitative prick testing" did not apply. Results were graded not only by the size of reactions but also by the number of positive reactions as outlined in Chapter III (section 3.2.d.). Due to their dark skin, flares could not be read in the Bantu and some of the Coloured subjects, and in these subjects reactions were graded on wheal size only.

All reactions with a wheal greater than 2mm or a flare greater than 4mm I classed as positive. These criteria may perhaps be considered as low. However, Pepys (1971) has indicated that a carefully performed prick test causes minimal trauma so that a negative test shows only the mark of the prick, if anything, and a very small wheal can be considered as unequivocably positive.

A small reaction is as indicative of the presence of skin sensitising antibody as a large one, and therefore I feel it should be considered positive. The clinical significance of small reactions is another question altogether, which I will discuss in Chapter III (Section 3.4.c.).

Details of the compositions and concentrations of the prick test solutions used in the epidemiological survey presented in Chapter III.

1. Control
2. House dust 150%
3. House dust mite (Dermatophagoides farinae) 1%
4. South African pollen Group B51 (flowers and shrubs) 2.5%
   - Cosmos
   - Hawthorn
   - Lupin
   - Golden rod
   - Heather
   - Michaelmas daisy
   - Nettle
   - Plantain
   - Privet
   - Rose
5. South African pollen Group B52 (grasses) 2.5%
   - Bermuda grass
   - Brome
   - Red top
   - Canary grass
   - Cocksfoot
   - Fescue
   - Meadow grass
   - Rye grass
   - Sorghum (Sudan grass)
   - Yorkshire Fog
6. South African pollen Group B53 (trees) 2.5%
   - Acacia
   - Oak

Plane

Poplar

Syringa

Willow

Cypress

Larch

Pine

Yew

7. Bermuda grass (Cynodon dactylon) 2.5%

8. A_1 Mixed inhalants 150%

Cat fur

Dog hair

Feathers

Horse hair and dander

House dust

Orris powder

9. Ascaris lumbricoides 5%

10. Moulds A13 5%

Alternaria tenuis

Aspergillis amstelodami

Aspergillis niger

Aspergillis terreus

Cladosporium herbarum

Neurospora sitophila

Mucor mucedo

Mucor racemosus

Mucor spinosus

Rhizopus nigricans

Penicillium brevicompactum

Penicillium expansum

Penicillium notatum

11. Moulds M10 5%

    Chaetomium globosum

    Epicoccum purpurascens

    Fusarium spp.

    Pullularia pullulans

12. Moulds M11 10%

    Helminthosporium halodes

    Paecilomyces marquandii

    Phoma betae

    Sporobolomyces roseus

13. Bacteria F1

    Streptococcus Lancefield Group C

    Streptococcus Lancefield Group G

    Streptococcus pneumonia Type I

    Streptococcus salivarius

14. Bacteria F2

    Haemophilus influenzae

    Klebsiella pneumoniae

    Neisseria catarrhalis

    Staphylococcus aureus

15. Candida albicans 5%

16. Aspergillus fumigatus 5%

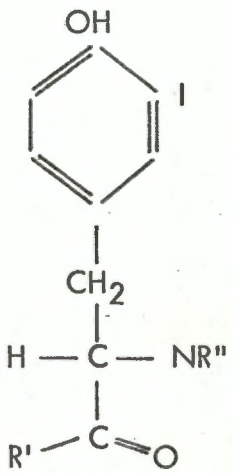
17. Talc 5%

18. Dry rot (Merulius lacrymans) 5%

APPENDIX II

Radio-iodination of proteins

Several of the experimental procedures that I employed involved the use of proteins trace labelled with radio-iodine. Although under certain circumstances iodine, when exposed to protein, may react with sulphydril groups and enter tryptophane or histadine, the chemistry of radio-iodination of proteins is essentially the chemistry of substitution of iodine into tyrosine groups (Hughs (1957)). Cationic iodine (I^+) is produced by oxidation of iodide and this is substituted into a tyrosine ring to form mono-iodotyrosine.



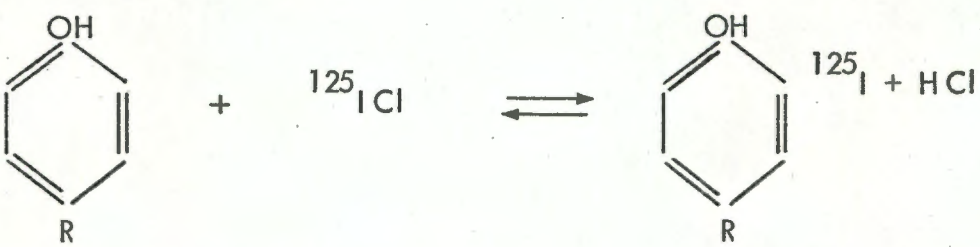
Efficient iodination therefore depends upon an efficient oxidizing agent. At the same time the oxidizing agent should not damage the protein in any way. Different proteins contain different numbers of readily available reducing groups which are capable of interfering with iodination and therefore the amount of oxidizing agent required varies from protein to protein. The conditions of iodination also depend on the amount of material available and the final specific activity required. Therefore for every protein that I wished to label I attempted to choose those conditions that were best suited to the material I had available and the final result I wished to achieve.

The chloramine T method was developed by Hunter and Greenwood (1962) for labelling small quantities of protein and producing high specific activity material suitable for radio-immunoassay. I hoped that by its use I would be able to label the small amount of protein that I had available in the purified antigenic fraction of Bermuda grass pollen extract (Fraction IIB). The method has the disadvantage that it subjects protein to exposure to a strong oxidizing agent and a reducing agent; nevertheless, Yagi et al (1963) successfully used a modification of this method to label Ragweed Antigen E. The procedure I followed was essentially the same as that of Yagi et al (1963). The antigenic fraction I wished to label (Fraction IIB) was dialysed against 0.0125 M borate buffer pH9 and approximately 20 µg protein in 200 ul buffer was added to a multidose vial containing 2mC Na ^{125}I in 500 µl (Radiochemical Centre, Amersham, England). This was immediately followed by 200 µl of a 1.41 mg/ml solution of chloramine T in the same buffer. After shaking thoroughly the mixture was allowed to stand for five minutes at room temperature and then 200 µl of a 1.26 mg/ml solution of sodium metabisulphate was added to reduce the unreacted iodine and the chloramine T. The mixture was passed through a 0.8 x 20 cm Sephadex G100 column equilibrated with borate buffer; by this means unreacted ^{125}I and low molecular weight compounds were separated from the labelled protein. Percentage iodination was calculated by determining the percentage of total radio-activity present in the protein fraction. Labelling of the Bermuda grass pollen antigen was not satisfactory; poor labelling was achieved (always < 7%) and the labelled material tended to disintegrate.

The method that I used for labelling the specific rabbit anti-sheep IgG antibodies was the McConahey and Dixon (1966) modification of the chloramine T method. They developed this method to expose proteins to less rigorous conditions than those originally

employed by Hunter and Greenwood (1962); this they achieved by using low concentrations of chloramine T and prolonging the reaction time. The rabbit antibody was dialysed against 0.05 M sodium phosphate buffer pH 7.5 and 200 µg protein in 250 ul buffer was added to a multidose vial containing 2 mC Na ^{131}I in 100 µl (Radiochemical Centre, Amersham, England). This was followed by 500 µl of a 0.2 mg/ml solution of chloramine T in water (freshly prepared). After shaking thoroughly the mixture was allowed to stand for five minutes and then 500 µl of a 0.2 mg/ml solution of sodium metabisulphate was added to stop the reaction. Separation of bound from free material was again by Sephadex G100 chromatography. The method was very satisfactory for labelling the rabbit antibody; it gave 50% or better iodination and the labelled material was functionally active in that it specifically bound sheep IgG *in vitro*.

Owing to my lack of success in labelling the Bermuda grass pollen antigen using the chloramine T method, I attempted to use McFarlane's (1958) iodine monochloride method. This method does not subject protein to direct oxidization by chloramine T but depends on the reaction



^{125}I Cl was produced by the addition of I Cl in glycine buffer pH 8.5 to a multidose vial containing Na ^{125}I. Once again bound and free material were separated on a Sephadex G100 column. This method, when used with the Bermuda grass pollen antigen, yielded an even lower percentage iodination than the chloramine T method and the labelled material still tended to disintegrate. However, I found the iodine monochloride method very satisfactory for labelling

human serum albumin that I used as a marker in the sucrose density gradient ulctracentrifugation experiments.

APPENDIX III

Immuno-electrophoresis

Microscope slides for immuno-electrophoresis were prepared with 1% agarose made up in 0.05M sodium barbitone buffer pH 8.4 with 0.1% sodium azide added. Wells and troughs were cut with a Shandon Pattern Cutter (Shandon Scientific Co., London, England) and the wells were filled with sample with care being taken to avoid any spilling over the edge of the well. The slides were electrophoresed in a Shandon Electrophoresis tank using 0.05M sodium barbitone buffer pH 8.4 in the buffer compartments; 100 volts were applied across the slides for 100 minutes. The troughs were then filled with appropriate antibody and immunodiffusion was allowed to take place for 24 hours at room temperature in moist conditions. The slides were then washed exhaustively in physiological saline. When necessary they were dried and stained with Amido Black.

APPENDIX IV

Preparation of rabbit anti-sheep IgG

Sheep γ globulins were precipitated from sheep serum with ammonium sulphate at 50% saturation and then dissolved in 0.9% Na Cl and dialysed against 0.005M sodium phosphate buffer pH 8. The γ globulin solution was then passed through a DEAE column that had been equilibrated with the 0.005M sodium phosphate buffer pH 8. This buffer was also used as the eluting fluid and the fraction that was not retained on the column under these conditions contained exclusively IgG as judged by immuno-electrophoresis (Appendix III).

Rabbits were given three subcutaneous injections of 1 mg sheep IgG in 1 ml buffer emulsified with 1 ml Freunds Complete Adjuvant (FCA)(Difco Laboratories, Detroit, Michigan) and then bled two weeks after the third injection.

Immuno-electrophoresis was used to establish the mono-specificity of the anti-serum. Only one immuno-precipitin arc was obtained when the anti-serum was diffused against electrophoresed whole sheep serum.

The procedures followed in the production of human IgG and rabbit anti-human IgG anti-serum were exactly the same as those described above for sheep IgG and rabbit anti-sheep IgG anti-serum.

APPENDIX V

The preparation and use of a glutaraldehyde immunoadsorbent

I used the method of Avrameas and Ternynck (1969) to prepare an immunoadsorbent from sheep IgG. This immunoadsorbent was then used to isolate the rabbit anti-sheep IgG antibodies from whole immune rabbit serum.

A solution of sheep IgG was prepared as outlined in Appendix IV and its concentration adjusted to approximately 25 mg protein per ml. Two ml of this solution was dialysed at 4°C for 18 hours against 0.1M sodium phosphate buffer pH 7.0. The immunoadsorbent was prepared by adding, slowly with stirring, 200 µl of a 2.5% aqueous solution of glutaraldehyde. The mixture was allowed to stand for three hours and during this time a gel formed. This gel was homogenized in a Potter homogenizer, washed twice in 0.2M sodium phosphate buffer pH 7.2, twice in 0.1M glycine Hcl buffer pH 2.8, and twice again in 0.2M sodium phosphate buffer pH 7.2.

Five ml undiluted anti-serum was added to packed immunoadsorbent and the mixture was stirred slowly at room temperature for 30 minutes. The supernatant serum was removed and the immunoadsorbent washed three times in 0.2M phosphate buffer in 0.1M NaCl (PBS). The adsorbed antibodies were eluted by adding 4 ml 0.1M glycine HCl buffer pH 2.8 to the packed wet immunoadsorbent, this mixture was stirred for five minutes at room temperature and after the supernatant had been removed the procedure was repeated once. The two supernatants were pooled, passed through a milipore (0.45µ), and dialysed against 0.05M phosphate buffer pH 7.5. Immunoelectrophoresis (see Appendix III) was used to establish that this solution contained rabbit immunoglobulins and that these

reacted specifically with sheep IgG. The purified antibody solution was stored in 250 ug aliquots at -20°C. The immuno-adsorbent, after washing, could be re-used. It was stored in 0.0001M merthiolate in 0.9% Na Cl.

APPENDIX VI

Statistical methods and computer programmes.

Appropriate statistical tests for any set of data can only be chosen after the scale of measurement used to obtain the data has been taken into consideration. Normal arithmetic manipulations and parametric statistical tests can only be used if the scale of measurement is such that not only the qualitative, but also the quantitative difference between any two values on the scale is known (interval scale). When this level of measurement is not achieved then non-parametric tests should be used.

The scale of measurement achieved depends both on the nature of the subjects under investigation and the precision of the method used to obtain the data. Consider the results of two measurements, A and B; if an experimenter can say that A and B are either different or identical but he is unable to define any other relationship between them, then he has achieved only a nominal scale; if he can say A>B, A=B or A<B but he does not know how great any difference is, then he has used an ordinal scale; if he can quantitate any difference between measurements, he has achieved an interval scale; if in addition he can locate the zero on his scale, then he has used a ratio scale.

Theoretically it should be possible to measure serum IgE concentrations on a ratio scale; however, the method I used was insensitive at low IgE concentrations and inaccurate at high concentrations; I therefore elected not to assume a level of measurement that I had not uniformly achieved. Moreover, neither the data, nor the log transformed data, followed a normal distribution. The non-parametric statistical tests that I used were suitable for my purpose in that they made no assumptions about the distribution

of the data and also assumed only an ordinal level of measurement. All tests that I used are presented and discussed by Siegel (1956).

The Kruskal-Wallis Test

In order to test whether there were any significant differences in the serum IgE concentrations of the various sections of the population, I used the Kruskal-Wallis one-way analysis of variance by ranks. This test allows results from any number, k, independent samples of the population to be compared; it tests the null hypothesis that the k samples come from identical populations as far as the variable under test is concerned. It assumes that these variables have an underlying continuous distribution and requires at least an ordinal level of measurement.

In the computation of the Kruskal-Wallis test for a total of N observations in k samples of the population, each of the N results is replaced by its rank. That is the smallest result in the combined series is given rank 1, the next smallest is given rank 2 and so on; when ties occur between two or more results, each result is given the mean of the ranks for which the results are tied. The sum of the ranks in each of the k samples is calculated. The test determines whether these ranks are so disparate that they are not likely to have come from samples which were all drawn from the same population.

To do this the statistic H is calculated from the formula

$$H = \frac{12}{N(N+1)} \sum_{j=1}^{k} \frac{Rj^2}{nj} - 3(N+1) \qquad (1)$$

where k = number of samples

nj = number of observations in the j th sample.

N = Σnj, the number of cases in all samples combined.

Rj = sum of ranks in the j th sample.

If ties are present, a correction for ties can be applied by dividing the calculated value of H by

$$1 - \frac{\Sigma T}{N^3 - N}$$

where $T = t^3 - t$ and t is the number of tied observations in a tied group of results.

Statistical tables are available that give the probability of finding an H as large as the one calculated. If the total number of cases is large, and each nj > 5 then the table of critical values of chi square can be used with degrees of freedom (DF) = k - 1.

The Kendall rank correlation coefficient, Tau.

To test the association of serum IgE concentrations with other variables for which I had also achieved at least an ordinal level of measurement, I used the Kendall rank correlation coefficient Tau. This is a non-parametric test that measures the degree of association between the ranks of two sets of variables.

To compute Tau, for two sets of variables, X and Y, results are arranged in increasing order of one of them, X, and then the number of inversions of the Y variables (or ranks of the Y variables) are determined as follows: let I be the number of times a Y result is followed, in the ordered sequence, by a smaller Y result, and let J be the number of times a Y result is followed by a larger Y result. The total score, S, for the inversions of the variables is J - I. The statistic Tau, if no observations are tied, is given by

$$\text{Tau} = \frac{\text{actual score}}{\text{maximum possible score}}$$

$$= \frac{S}{\binom{N}{2}}$$

$$= \frac{S}{\frac{1}{2}N(N-1)} \qquad (2)$$

where N = the total number of X or Y results.

If results are tied on the Y variable no inversion can occur and so they cannot contribute to S. If results are tied on the X variable then the corresponding results on the Y variable are not compared and so again they do not contribute to S. The formula for Tau then becomes

$$\text{Tau} = \frac{S}{\sqrt{\frac{1}{2}N(N-1) - Tx}\sqrt{\frac{1}{2}N(N-1) - Ty}} \qquad (3)$$

where $Tx = \frac{1}{2}\Sigma t\,(t-1)$, t being the number of tied observations in each group of ties on the X variable, and where $Ty = \frac{1}{2}\Sigma t\,(t-1)$, t being the number of tied observations in each group of ties on the Y variable. If N is greater than 10, Tau may be considered to be normally distributed with

$$\text{Mean} = \mu r = 0$$

$$\text{and Standard error} = \sigma r = \sqrt{\frac{2\,(2N+5)}{9N(N-1)}} \qquad (4)$$

$$\text{that is } Z = \frac{\text{Tau} - \mu r}{\sigma r} = \frac{\text{Tau}}{\sqrt{\frac{2(2N+5)}{9N\,(N-1)}}}$$

is approximately normally distributed with zero mean and unit variance, and statistical tables of the normal distribution can be used. If $N \leq 10$ special tables of probabilities associated with the observed values of S are available.

The Chi-square test

The chi-square test assumes only a nominal level of measurement and I used it to analyse the inter-relationships of those sets of data which I could ascribe to discrete categories. I applied the Yates correction for continuity for all the analyses in which the data fell into two by two contingency tables. The test is so frequently used and so amenable to computation that I shall not discuss it in detail here.

One limitation of the chi-square test is that the test should not be used for a two by two contingency table if the expected frequency of any of the cells is less than five, or for a contingency table of greater than two by two if the expected frequencies of 20% or more of the cells are less than five or any of the cells have an expected frequency of less than one.

The Fisher exact probability test.

The Fisher exact probability test, like the chi-square test, assumes only a nominal level of measurement. It is used for analysing data, from two independent samples, that can be assigned to one or other mutually exclusive categories. It is particularly useful for small samples and I used it in the analysis of two by two contingency tables when the data I had available was insufficient for chi-square analysis. This test is a very frequently used non-parametric test and I shall not discuss it in detail here.

The Mann-Whitney U test

The Mann-Whitney U test, like the Kruskal-Wallis one way analysis of variance, is a non-parametric test that assumes an ordinal level of measurement. As it can only be used for testing whether two independent groups have been drawn from the same population, and as I frequently wished to test more than two groups simultaneously, I did not use it for the majority of the analyses. However, when the total population in large, the Kruskal-Wallis test can only be used if each group in the population contains more than five observations; the Mann-Whitney U test, on the other hand, does not have this restraint. Occasionally, in analysing the data presented in Chapter III, I felt that it was important to detect trends in serum IgE concentrations even when the data I had

available contained very few observations in one of the groups, and in these circumstances I used the Mann-Whitney U test. The test is a very useful and frequently used non-parametric test; full details of its use are given in Siegel (1956).

Calculations

I used the Univac 1106 computer at the University of Cape Town to process the data for the majority of the statistical tests that I employed. I used, Subroutine ORDER, a package programme available to users of the computer, to rank the data; all other programmes I wrote myself. Print-outs of examples of these programmes are given at the end of this Appendix.

Briefly the programmes used were as follows:

For the Kruskal-Wallis analysis

Main programme - to read in the data and to call the Subroutines.

Subroutine SRT - to sort the data into separate arrays for

White males

White females

Coloured males

Coloured females

Bantu males

Bantu females

Subroutine ORDER - to rank data.

Subroutine TIES - to replace the ranks of tied data by the mean rank of all the data having the same result, and to calculate correction factors for ties for use in subsequent statistical tests.

Subroutine KUWAB - to calculate the statistic H for the Kruskal-Wallis test and to calculate the mean result of the variable under test in each of the population samples tested.

To calculate the frequency distributions and medians

Subroutine HIS2 (this subroutine could only be used after the data had been ranked).

For the Kendall Tau analysis

Main Programme - to read in the data, call Subroutines ORDER and TIES and calculate the statistics S, Tau and Z.

For the Chi-square test

The Univac 1106 was only used to sort the data for this test - the Hewlett Packard Model 10 desk top computer was used to calculate the values of chi-square.

For the Fisher exact probability test

Calculations for this test were done using factorial tables.

For the Mann-Whitney U test

A programme for this test was available for the Hewlett-Packard Model 10 calculator.

Computer Programmes

Variables used.

In order to facilitate the reading of the programme print-outs I will give the names of the principal variables that I used in the computations. The list does not include the names of all the variables that were generated during the course of the computations and whose relationships with the other variables are clear in the print-outs.

The majority of the data were loaded on disc, prior to computation, with the final IgE results already calculated from the original raw data. That is calculations to allow for duplicates, standards used, dilutions made and repeat determinations, had already been performed. The data for the Kendall Tau analysis were on cards in the original form, the variables E1, E2, F1, F2, ST, III and Y refer to the raw IgE data and the factors required to calculate the final IgE results from this data. These variables are not listed below.

Arrays of variables

E - set of all IgE results

JACE - set of codes giving the race of each donor

where 1 = White

2 = Coloured

3 = Bantu

4 = Asiatic

JEX - set of codes giving the sex of each donor

where 1 = male

2 = female

M - set of codes identifying subjects as blood donors

0 = blood donor

J (and A in KUWAB) - set of codes for the factor used to sample the population for the statistical test. Thus in the sample of the Kruskal-Wallis programme given J(and A) give the ABO blood group of each donor.

1 = A

2 = B

3 = AB

4 = O

5 = Sub A

7 = Sub AB

S - set of results (such as IgM concentrations) with which IgE results are compared in the Kendall Tau.

EORD - set of ordered IgE results

NR - set of ranks of IgE results

SORD - set of ordered results of the variable S.

NSR (and A in Kendall Tau) - set of ranks of the variables in S

EMM - set of IgE results in White males

EMF = set of IgE results in White females

CMM = set of IgE results in Coloured males

CMF = set of IgE results in Coloured females

BMM = set of IgE results in Bantu males

BMF = set of IgE results in Bantu females

EMMO = set of ordered IgE results in White males

EMFO = set of ordered IgE results in White females

CMMO = set of ordered IgE results in Coloured males

CMFO = set of ordered IgE results in Coloured females

BMMO = set of ordered IgE results in Bantu males

BMFO = set of ordered IgE results in Bantu females

LEM = set of ranks of IgE results in White males

LEF = set of ranks of IgE results in White females

LCM = set of ranks of IgE results in Coloured males

LCF = set of ranks of IgE results in Coloured females

LBM = set of ranks of IgE results in Bantu males

LBF = set of ranks of IgE results in Bantu females

L = set of frequencies of IgE results with an interval of 50.

LAC = set of accumulated frequencies of IgE results with an interval of 50.

L25 = set of frequencies of IgE results with an interval of 25

LA5 = set of accumulated frequencies of IgE results with an interval of 25.

Variables

N = total number of results read by the computer

NC = total number of results for which all information was available

MRM = total number of White males

MRF = total number of White females

KRM = total number of Coloured males

KRF = total number of Coloured females

NRM = total number of Bantu males

NRF = total number of Bantu females

K1, K2, K3, K4, K5, K7 = number of observations in each of k samples tested by the Kruskal-Wallis test.

R1, R2, R3, R4, R5, R7 = sum of the ranks of the variable under test in each of the k samples tested by the Kruskal-Wallis test.

AV1, AV2, AV3, AV4, AV5, AV7 = mean value of the variable under test in each of the k samples.

H = Kruskal-Wallis statistic H.

MED1, MED2 = Median results of the variable under test when an even number of observations have been analysed.

MED3 = Median result of the variable under test when an odd number of observations have been analysed.

MAIN PROGRAM - KRUSKAL WALLIS

```
1*          COMMON JACE(4700),JEX(4700),E(4700)
2*          DIMENSION M(4700),J(4700)
3*          EQUIVALENCE(M(1),JACE(2)),(J(1),JEX(2))
4*          N=4650
5*          DO 40 I=1,N
6*       44 READ(16,41)JACE(I),M(I),JEX(I),J(I),E(I)
7*       41 FORMAT(13X,I1,3X,I1,1X,I1,1X,I1,17X,F9.0)
8*          IF(M(I).GT.0)GO TO 42
9*          IF(J(I).EQ.0)GO TO 42
10*         IF(J(I).EQ.6)GO TO 42
11*         IF(J(I).GT.7)GO TO 42
12*         IF((E(I)-INT(E(I)+.00))GO TO 42
13*         E(I)=ABS(E(I))
14*         E(I)=E(I)+J(I)/10.
15*         GO TO 40
16*      42 N=N-1
17*         GO TO 44
18*      40 CONTINUE
19*         DIMENSION EMM(3500),EMF(1800),CMM(1800),CMF(1500),BMM(200),BMF(200
20*        1)
21*         CALL SRT(EMM,EMF,CMM,CMF,BMM,BMF,MRM,MRF,KRM,KRF,NRM,NRF,NC,N)
22*         DIMENSION EMMO(3500),EMFO(1800),CMMO(1800),CMFO(1500),BMMO(200),BM
23*        1FO(200),LEM(3500),LEF(1800),LCM(1800),LCF(1500),LBM(200),LBF(200)
24*         EQUIVALENCE(EMMO(1),EMFO(1),CMMO(1),CMFO(1),BMMO(1),BMFO(1)),(LEM(
25*        1),LEF(1),LCM(1),LCF(1),LBM(1),LBF(1))
26*          CALL ORDER(EMM,MRM,EMMO,LEM)
27*          CALL TIES(MRM,EMMO,LEM,MEM)
28*          CALL HIST(EMMO,MRM)
29*          CALL KUWAB(EMM,MRM,LEM,HEM,MEM)
30*          CALL ORDER(EMF,MRF,EMFO,LEF)
31*          CALL TIES(MRF,EMFO,LEF,MEF)
32*          CALL HIST(EMFO,MRF)
33*          CALL KUWAB(EMF,MRF,LEF,HEF,MEF)
34*          CALL ORDER(CMM,KRM,CMMO,LCM)
35*          CALL TIES(KRM,CMMO,LCM,MCM)
36*          CALL HIST(CMMO,KRM)
37*          CALL KUWAB(CMM,KRM,LCM,HCM,MCM)
38*          CALL ORDER(CMF,KRF,CMFO,LCF)
39*          CALL TIES(KRF,CMFO,LCF,MCF)
40*          CALL HIST(CMFO,KRF)
41*          CALL KUWAB(CMF,KRF,LCF,HCF,MCF)
42*          CALL ORDER(BMM,NRM,BMMO,LBM)
43*          CALL TIES(NRM,BMMO,LBM,MBM)
44*          CALL HIST(BMMO,NRM)
45*          CALL KUWAB(BMM,NRM,LBM,HBM,MBM)
46*          CALL ORDER(BMF,NRF,BMFO,LBF)
47*          CALL TIES(NRF,BMFO,LBF,MBF)
48*          CALL HIST(BMFO,NRF)
49*          CALL KUWAB(BMF,NRF,LBF,HBF,MBF)
50*          WRITE(5,15)N
51*          WRITE(5,15)NC
52*          WRITE(5,15)MRM
53*          WRITE(5,15)MRF
54*          WRITE(5,15)KRM
55*          WRITE(5,15)KRF
56*          WRITE(5,15)NRM
57*          WRITE(5,15)NRF
58*       15 FORMAT(' ',I6)
59*          END
```

SUBROUTINE SRT

```
      SUBROUTINE SRT(EMM,EMF,CMM,CMF,BMM,BMF,MRM,MRF,KRM,KRF,NRM,NRF,NC,
     1N)
      COMMON JACE(4700),JEX(4700),E(4700)
      DIMENSION EMM(MRM),EMF(MRF),CMM(KRM),CMF(KRF),BMM(NRM),BMF(NRF)
      MR=0
      KR=0
      NR=0
      MRM=0
      MRF=0
      KRM=0
      KRF=0
      NRM=0
      NRF=0
      DO 30 I=1,N
      IF(JACE(I).EQ.1)GO TO 31
      IF(JACE(I).EQ.2)GO TO 32
      IF(JACE(I).EQ.4)GO TO 32
      IF(JACE(I).EQ.3)GO TO 33
      GO TO 30
   31 MR=MR+1
      IF(JEX(I).EQ.1)GO TO 34
      IF(JEX(I).EQ.2)GO TO 35
      GO TO 30
   32 KR=KR+1
      IF(JEX(I).EQ.1)GO TO 36
      IF(JEX(I).EQ.2)GO TO 37
      GO TO 30
   33 NR=NR+1
      IF(JEX(I).EQ.1 )GO TO 38
      IF(JEX(I).EQ.2)GO TO 39
      GO TO 30
   34 MRM=MRM+1
      EMM(MRM)=E(I)
      GO TO 30
   35 MRF=MRF+1
      EMF(MRF)=E(I)
      GO TO 30
   36 KRM=KRM+1
      CMM(KRM)=E(I)
      GO TO 30
   37 KRF=KRF+1
      CMF(KRF)=E(I)
      GO TO 30
   38 NRM=NRM+1
      BMM(NRM)=E(I)
      GO TO 30
   39 NRF=NRF+1
      BMF(NRF)=E(I)
      GO TO 30
   30 CONTINUE
      NC=NRM+MRF+KRM+KRF+NRM+NRF
      RETURN
      END
```

OF COMPILATION: NO DIAGNOSTICS.

SUBROUTINE TIES

```
1*          SUBROUTINE TIES(N,EORD,NR,M)
2*          DIMENSION EORD(N),NR(N)
3*          DO 20 L=1,N
4*       20 NR(L)=NR(L)*10
5*          JT=1
6*          KUM=10
7*          M=0
8*          IG=INT(EORD(1))
9*          IC=0
10*         DO 21 I=2,N
11*         IC=IC+10
12*         IF(IG.EQ.INT(EORD(I)))GO TO 22
13*         IF(JT.EQ.1)GO TO 23
14*      27 M=M+(JT**3-JT)
15*         MEAN=KUM/JT
16*         DO 24 L=1,N
17*         IF(NR(L).GE.0)GO TO 24
18*         NR(L)=MEAN
19*      24 CONTINUE
20*         JT=1
21*         GO TO 23
22*      22 JT=JT+1
23*         KUM=KUM+10*I
24*         DO 25 K=1,N
25*         IF(NR(K).EQ.IC)GO TO 26
26*         IF(NR(K).EQ.(IC+10))GO TO 26
27*         GO TO 25
28*      26 NR(K)=-NR(K)
29*      25 CONTINUE
30*         IF(I.EQ.N)GO TO 27
31*         GO TO 21
32*      23 KUM=10*I
33*      21 IG=INT(EORD(I))
34*         RETURN
35*         END
```

ND OF COMPILATION: NO DIAGNOSTICS.

Note: This TIES subroutine replaced the ranks of tied observations with the mean rank of the tied observations and calculated the correction factor for ties for the Kruskal-Wallis test.

SUBROUTINE KUWAB

```
1*          SUBROUTINE KUWAB(E,N,NR,H,M)
2*          DIMENSION E(N),NR(N)
3*          NK=N
4*          K1=0
5*          K2=0
6*          K3=0
7*          K4=0
8*          K5=0
9*          K7=0
10*         R1=0
11*         R2=0
12*         R3=0
13*         R4=0
14*         R5=0
15*         R7=0
16*         I1=0
17*         I2=0
18*         I3=0
19*         I4=0
20*         I5=0
21*         I7=0
22*         DO 60 I=1,N
23*         A=10*(E(I)-INT(E(I)))
24*         IF(A.LT..5)GO TO 68
25*         IF(A.LT.1.5)GO TO 61
26*         IF(A.LT.2.5)GO TO 62
27*         IF(A.LT.3.5)GO TO 63
28*         IF(A.LT.4.5)GO TO 64
29*         IF(A.LT.5.5)GO TO 65
30*         IF(A.LT.6.5)GO TO 67
31*      68 NK=NK-1
32*         GO TO 60
33*      61 K1=K1+1
34*         R1=R1+NR(I)/10.
35*         I1=I1+INT(E(I))
36*         GO TO 60
37*      62 K2=K2+1
38*         R2=R2+NR(I)/10.
39*         I2=I2+INT(E(I))
40*         GO TO 60
41*      63 K3=K3+1
42*         R3=R3+NR(I)/10.
43*         I3=I3+INT(E(I))
44*         GO TO 60
45*      64 K4=K4+1
46*         R4=R4+NR(I)/10.
47*         I4=I4+INT(E(I))
48*         GO TO 60
49*      65 K5=K5+1
```

KUWAB (CNT)

```
50*             R5=R5+NR(I)/10.
51*             T5=T5+INT(E(I))
52*             GO TO 60
53*          67 K7=K7+1
54*             R7=R7+NR(I)/10.
55*             T7=T7+INT(E(I))
56*             GO TO 60
57*          60 CONTINUE
58*             AV1=T1/K1
59*             AV2=T2/K2
60*             AV3=T3/K3
61*             AV4=T4/K4
62*             AV5=T5/K5
63*             AV7=T7/K7
64*             SIG1=(R1**2)/K1
65*             SIG2=(R2**2)/K2
66*             SIG3=(R3**2)/K3
67*             SIG4=(R4**2)/K4
68*             SIG5= (R5**2)/K5
69*             SIG7=(R7**2)/K7
70*             SIGMA=SIG1+SIG2+SIG3+SIG4+SIG5+SIG7
71*             COR=1.-(FLOAT(M)/(N**3-N))
72*             HI=(12./(NK*(NK+1)))*SIGMA-3.*(NK+1)
73*             H=HI/COR
74*             WRITE(5,601)K1,K2,K3,K4,K5,K7,R1,R2,R3,R4,R5,R7,NK,HI,COR,H,SIGMA
75*          -  WRITE(5,603)AV1,AV2,AV3,AV4,AV5,AV7
76*       601 FORMAT(' ','     1     2     3     4     5     6',/' '6I6/' '6F
77*          1I5.3/' ','NK '
78*          1,I6,' HI ',F10.5,' COR',F10.5/' ',' H EQUALS ',F12.5/' ',' SIGMA
79*          1',F14.0)
80*       603 FORMAT(' ',' MEAN A ',F9.0,' MEAN B ',F9.0,' MEAN AB ',F9.0,' MEAN
81*          1 0 ',F9.0,' MEAN SUBA ',F9.0,' MEAN SUBAB ',F9.0)
82*             RETURN
83*             END

NO OF COMPILATION:        NO  DIAGNOSTICS.
```

Note: This KUWAB subroutine calculated Kruskal-Wallis statistic H for a population of 6 independent groups.

SUBROUTINE HIS2

```
1*              SUBROUTINE HIS2(EORD,N,MED1,MED2,MED3)
2*              DIMENSION EORD(N)
3*              DIMENSION L(202),LAC(202)
4*              DIMENSION L25(201),LA5(201)
5*              DIMENSION P25(201),PA5(201)
6*              DO 83 I=1,201
7*              L25(I)=0
8*           83 LA5(I)=0
9*              DO 80 I=1,202
10*             L(I)=0
11*          80 LAC(I)=0
12*             LL=0
13*             LLL=0
14*             DO 81 I=25,5000,25
15*             II=I/25
16*             DO 82 J=1,N
17*             IF(INT(EORD(J)).LT.I)GO TO 82
18*             L25(II)=LA5(II)-LL
19*             LL=LA5(II)
20*             IF(MOD(II,2).GT.0)GO TO 81
21*             L(II)=L25(II)+L25(II-1)
22*             LAC(II)=LLL+L(II)
23*             LLL=LAC(II)
24*             GO TO 81
25*          82 LA5(II)=LA5(II)+1
26*          81 CONTINUE
27*             LA5(201)=N
28*             L25(201)=N-LA5(200)
29*             LAC(202)=N
30*             L(202)=N-LAC(200)
31*             DO 85 I=1,201
32*             P25(I)=(FLOAT(L25(I)))/(FLOAT(N))
33*          85 PA5(I)=(FLOAT(LA5(I)))/(FLOAT(N))
34*             IF(MOD(N,2).GT.0)GO TO 802
35*             NRMED1=N/2
36*             NRMED2=N/2+1
37*             MED1=INT(EORD(NRMED1))
38*             MED2=INT(EORD(NRMED2))
39*             PRINT 803,MED1,N
40*             PRINT 805,MED2
41*             PRINT 804,(L25(I),LA5(I),P25(I),PA5(I),I,I=1,111)
42*             RETURN
43*          802 NRMED3=(N-1)/2+1
44*             MED3=INT(EORD(NRMED3))
45*             PRINT 803,MED3,N
46*          803 FORMAT('1',' MEDIAN 'I6,' NUMBER ',I6)
47*             PRINT 804,(L25(I),LA5(I),P25(I),PA5(I),I,I=1,112)
48*          805 FORMAT(' ','          'I6)
49*          804 FORMAT(' ','HISTOGRAM INTERVAL 25 ',/' '(2I6,2F11.7,I6))
50*             RETURN
51*             END
```

```
ND OF COMPILATION:        NO  DIAGNOSTICS.
```

MAIN PROGRAM - KENDALL TAU

```
1*          DIMENSION ST(500),III(500),Y(500)
2*          DIMENSION E(500),S(500),EORD(500),SORD(500),NR(500),NSR(500)
3*          DIMENSION A(500)
4*          DIMENSION JACE(500)
5*          DIMENSION IE0(500)
6*          DIMENSION E1(500),E2(500),F1(500),F2(500)
7*          DIMENSION JEX(500)
8*          DIMENSION NRORD(500),NNR(500)
9*          N=0
10*           DO 40 I=1,500
11*        44 READ(8,41,END=48)JACE(I),JEX(I),ST(I),III(I),E1(I),E2(I),F1(I),
12*         1F2(I),Y(I),S(I)
13*        41 FORMAT(T13,I1,I1,T35,F1.0,I1,F9.0,1X,F9.0,F2.0,1X,
14*         1F2.0,T65,F1.0,F4.0)
15*           N=N+1
16*         IF(Y(I).LT.2)GO TO 42
17*         IF(III(I).LT.1)GO TO 42
18*         IF(S(I).LT.1)GO TO 42
19*         IF(JACE(I).LT.3)GO TO 42
20*         GO TO 40
21*        42 N=N-1
22*         GO TO 44
23*        40 CONTINUE
24*        48 DO 46 I=1,N
25*         IF(ST(I).GT.0)GO TO 50
26*         GO TO 51
27*        50 E1(I)=E1(I)*1623/2000
28*         E2(I)=E2(I)*1623/2000
29*        51 IF(III(I).LT.2)E(I)=E1(I)
30*         IF(III(I).EQ.2)E(I)=(E1(I)+E2(I))/2
31*         IF(III(I).EQ.5)E(I)=E1(I)*F1(I)
32*         IF(III(I).EQ.4)GO TO 49
33*         GO TO 46
34*        49 IF(E1(I).GT.811.AND.E2(I).LT.812)GO TO 52
35*         IF(E2(I).LT.42.AND.E1(I).GT.41)GO TO 53
36*         E(I)=(E1(I)*F1(I)+E2(I)*F2(I))/2
37*         GO TO 46
38*        52 E(I)=E2(I)*F2(I)
39*         GO TO 46
40*        53 E(I)=E1(I)*F1(I)
41*        46 CONTINUE
42*         CALL ORDER(S,N,SORD,NSR)
43*         CALL TIES(N,SORD,NSR,TS)
44*         DO 45 I=1,N
45*        45 E(I)=1.0*(INT(E(I)))+(FLOAT(NSR(I))/10000)
46*         CALL ORDER(E,N,EORD,NR)
47*         CALL TIES(N,EORD,NR,TE)
48*         DO 60 I=1,N
49*        60 A(I)=1000*(EORD(I)-INT(EORD(I)))
```

KENDALL TAU (CNT)

```
50*           DO 64 I=1,N
51*        64 IEO(I)=INT(EORD(I))
52*           NN=N-1
53*           SK=0
54*           DO61 I=1,NN
55*           K=I+1
56*           DO 61 J=K,N
57*           IF(IEO(I).EQ.IEO(J))GO TO 61
58*           A(J)=A(J)+.40000
59*           IF(A(I).GT.A(J))SK=SK-1
60*           A(J)=A(J)-.80000
61*           IF(A(I).LT.A(J))SK=SK+1
62*           A(J)=A(J)+.40000
63*        61 CONTINUE
64*           TAU=SK/((SQRT((N*(N-1))/2-TE))*(SQRT((N*(N-1))/2-TS)))
65*           WRITE(5,62)N,TAU,SK,TE,TS
66*        62 FORMAT('1','N ',I5/' ',' TAU  ',F9.5/' ',' S  ',F9.4/' ',' TE  ',F
67*          19.4,' TS  ',F9.4)
68*           AN=FLOAT(N)
69*           Z=TAU/(SQRT((2*(2*AN+5))/(9*AN*(AN-1))))
70*           WRITE(5,66)Z
71*        66 FORMAT(' ',' Z ',F10.6)
72*            CALL ORDER(NR,N,NRORD,NNR)
73*           WRITE(5,65)(IEO(I),NRORD(I),A(I),E(I),S(I),NSR(I),I=1,N)
74*        65 FORMAT(' ',I7,I7,F10.2,F11.2,F7.1,I8)
75*           END
```

IgE DATA FOR THE EPIDEMIOLOGICAL SURVEY (Chapter III)

WHITE MALES

NO	IGE U/ML	AGE DECADE	HISTORY GRADE	HISTORY TYPE	SKINTEST GRADE	SKINTEST ASC	PARASITES TRI	PARASITES ASC	PARASITES TEA	E/PHIL COUNT	IGG	IGA	IGM
1	0	5TH	NEG		0			ND		0%	1120	88	318
2	75	3RD	NEG		2		-VE	-VE	-VE	ND	820	80	168
3	219	5TH	NEG		0		-VE	-VE	-VE	2%	960	82	212
4	85	5TH	NEG		5		-VE	-VE	-VE	ND	1050	60	253
5	18	5TH	NEG		2	+VE	-VE	-VE	-VE	ND	740	88	216
6	102	2ND	NEG		0		-VE	-VE	-VE	ND	1120	133	94
7	298	4TH	NEG		0		-VE	-VE	-VE	ND	980	91	157
8	1063	5TH	MILD	OTHER	11		-VE	-VE	-VE	1%	880	204	210
9	198	4TH	NEG		0		-VE	-VE	-VE	9%	880	70	224
10	112	3RD	MILD	OTHER	0		-VE	-VE	-VE	ND	1090	144	131
11	51	4TH	NEG		0		-VE	-VE	-VE	1%	720	86	216
12	114	4TH	MILD	OTHER	0		-VE	-VE	-VE	7%	520	176	162
13	42	2ND	NEG		0		-VE	-VE	-VE	4%	600	81	136
14	0	5TH	NEG		0		-VE	-VE	-VE	2%	220	121	136
15	42		ND		ND			ND		5%	580	84	216
16	0	3RD	NEG		0		-VE	-VE	-VE	0%	1660	251	216
17	670	3RD	NEG		11		-VE	-VE	-VE	3%	1100	297	120
18	533	3RD	MILD	OTHER	8		-VE	-VE	-VE	4%	820	31	137
19	198	3RD	CL.ALL	INHAL	11		-VE	-VE	-VE	5%	340	79	120
20	265	4TH	CL.ALL	BOTH	11	+VE	-VE	-VE	-VE	0%	1600	133	344
21	407	3RD	CL.ALL	INHAL	11		-VE	-VE	-VE	2%	960	290	120
22	803	4TH	CL.ALL	INHAL	11	+VE	-VE	-VE	-VE	3%	760	136	124
23	113	3RD	CL.ALL	INHAL	8		-VE	-VE	-VE	4%	640	397	88
24	42	3RD	NEG		0		-VE	-VE	-VE	2%	1560	108	132
25	42	3RD	NEG		0		-VE	-VE	-VE	4%	460	75	200
26	42	3RD	NEG		7		-VE	-VE	-VE	0%	1560	270	291
27	10163	4TH	MILD	INHAL	11	+VE	-VE	-VE	-VE	3%	1160	248	90
28	42	5TH	NEG		0		-VE	-VE	-VE	1%	ND	86	241
29	0	5TH	NEG		0		-VE	-VE	-VE	3%	800	155	206
30	112	3RD	NEG		0		-VE	-VE	-VE	1%	1160	122	110
31	50	3RD	NEG		0		-VE	-VE	-VE	5%	380	138	143
32	50	4TH	NEG		8		-VE	-VE	-VE	1%	880	115	141
33	101	3RD	NEG		0		-VE	-VE	-VE	3%	1000	49	217
34	42	3RD	NEG		7	+VE		ND		6%	680	84	133
35	42	4TH	NEG		0		-VE	-VE	-VE	2%	480	102	148
36	0	2ND	NEG		0		-VE	+VE	-VE	2%	900	57	123
37	0	5TH	NEG		0		-VE	-VE	-VE	2%	560	81	266
38	113	4TH	NEG		0		-VE	-VE	-VE	5%	760	86	98
39	198	4TH	CL.ALL	INHAL	10	+VE	+VE	-VE	-VE	3%	480	116	124
40	0	3RD	NEG		0		-VE	-VE	-VE	0%	720	126	118
41	273	4TH	NEG		2		-VE	-VE	-VE	1%	1240	121	322
42	167	3RD	NEG		5		-VE	-VE	-VE	1%	800	182	306
43	198	3RD	MILD	OTHER	1		-VE	-VE	-VE	16%	860	133	76
44	112	3RD	NEG		0		-VE	-VE	-VE	3%	1100	202	97
45	112	3RD	NEG		3			ND		0%	960	140	51
46	42	3RD	NEG		0			ND		5%	800	73	171
47	27	3RD	MILD	INHAL	11		-VE	-VE	-VE	2%	840	75	204
48	42		ND		ND			ND		2%	2040	84	57
49	112	3RD	NEG		0		-VE	-VE	-VE	2%	700	102	168
50	772	4TH	MILD	INHAL	11		-VE	-VE	-VE	3%	1210	170	265
51	42	3RD	NEG		0		-VE	-VE	-VE	4%	980	153	113
52	42	4TH	CL.ALL	INHAL	ND			ND		2%	880	122	121
53	0	4TH	NEG		0		-VE	-VE	-VE	0%	580	112	133
54	42	3RD	NEG		0		-VE	-VE	-VE	5%	280	112	247
55	42	3RD	MILD	INHAL	ND			ND		0%	840	210	190

WHITE MALES (Contd.)

NO	IGE U/ML	AGE DECADE	HISTORY GRADE	HISTORY TYPE	SKINTEST GRADE	SKINTEST ASC	PARASITES TRI	PARASITES ASC	PARASITES TEA	E/PHIL COUNT	IGG	IGA	IGM
56	306		ND		ND			ND		1%	460	110	185
57	50	4TH	NEG		ND			ND		ND	580	193	103
58	0	3RD	NEG		0		-VE	-VE	-VE	5%	6700	161	140
59	101	3RD	MILD	INHAL	11		-VE	-VE	-VE	2%	320	86	86
60	229	2ND	NEG		ND			ND		4%	1210	70	196
61	335	4TH	MILD	OTHER	6		-VE	-VE	-VE	400T	600	158	162
62	72	3RD	NEG		10		-VE	-VE	-VE	100T	740	128	100
63	85	3RD	NEG		0		-VE	-VE	-VE	100T	1060	122	112
64	113	3RD	NEG		0		-VE	-VE	-VE	100T	800	108	172
65	112	4TH	MILD	OTHER	1	+VE		ND		1%	520	93	163
66	294	3RD	CL.ALL	INHAL	11		-VE	-VE	-VE	1%	760	183	204
67	42	4TH	NEG		0		-VE	-VE	-VE	0%	800	212	239
68	112	3RD	MILD	INHAL	ND			ND		4%	760	260	200

IN THIS & SUSEQUENT PRINT-OUTS

ND=NOT DONE
AGE IS GIVEN IN DECADES
CL.ALL=CLINICAL ALLERGY
INHAL=INHALANT
OTHER=NON-INHALANT
BOTH=BOTH
ASC=ASCARIS
TRI=TRICHURIS
TEA=TEANIA
T=TOTAL EOSINOPHIL COUNT
IGG, IGA & IGM ARE GIVEN IN MG/100ML

WHITE FEMALES

NO	IGE U/ML	AGE DECADE	HISTORY GRADE	HISTORY TYPE	SKINTEST GRADE	SKINTEST ASC	PARASITES TRI	PARASITES ASC	PARASITES TEA	E/PHIL COUNT	IGG	IGA	IGM
1	136	6TH	NEG		5	+VE	-VE	-VE	-VE	5%	1400	130	134
2	356	3RD	NEG		3		-VE	-VE	-VE	ND	930	194	104
3	59	2ND	NEG		0		-VE	-VE	-VE	1%	1000	245	90
4	35	3RD	NEG		5	+VE	-VE	-VE	-VE	ND	1000	185	128
5	120	3RD	MILD	OTHER	4		-VE	-VE	-VE	ND	1380	116	206
6	0	6TH	NEG		ND			ND		1%	520	94	294
7	0	5TH	NEG		0		-VE	-VE	-VE	2%	740	86	118
8	0	6TH	CL.ALL	OTHER	1		-VE	-VE	-VE	2%	720	64	118
9	143	3RD	CL.ALL	INHAL	0			ND		3%	700	180	780
10	112	4TH	NEG		3		-VE	-VE	-VE	1%	600	142	175
11	309	3RD	NEG		ND			ND		2%	520	72	118
12	398	5TH	CL.ALL	BOTH	ND			ND		3%	880	90	98
13	198	2ND	MILD	INHAL	8	+VE		ND		4%	800	122	88
14	112	3RD	CL.ALL	INHAL	0			ND		0%	880	219	173
15	198	2ND	NEG		3		-VE	-VE	-VE	2%	1120	107	370
16	112	2ND	CL.ALL	INHAL	3			ND		ND	1280	212	136
17	0	6TH	MILD	INHAL	2		-VE	-VE	-VE	2%	1640	96	169
18	305	2ND	NEG		0		-VE	-VE	-VE	0%	1360	173	120
19	178	3RD	NEG		0		-VE	-VE	-VE	3%	840	111	181
20	406	3RD	MILD	INHAL	ND			ND		ND	580	113	256
21	112	3RD	NEG		6			ND		0%	820	217	142
22	112	5TH	NEG		10	+VE	-VE	-VE	-VE	1%	1180	189	176
23	0	3RD	CL.ALL	INHAL	0		-VE	-VE	-VE	1%	1080	140	113
24	112	2ND	NEG		0		-VE	-VE	-VE	0%	740	154	144
25	153	5TH	MILD	INHAL	2		-VE	-VE	-VE	8%	800	146	236
26	202		ND		ND			ND		1%	920	96	244
27	10	5TH	MILD	INHAL	0		-VE	-VE	-VE	0%	320	106	118
28	0	3RD	CL.ALL	OTHER	0		-VE	-VE	-VE	1%	560	233	169
29	0	3RD	NEG		0		-VE	-VE	-VE	5%	1120	141	186
30	0	3RD	MILD	INHAL	8		-VE	-VE	-VE	5%	820	126	130
31	0	2ND	NEG		0		-VE	-VE	-VE	0%	980	217	185
32	0	5TH	CL.ALL	OTHER	5		-VE	-VE	-VE	1%	640	123	114
33	112	3RD	MILD	OTHER	0		-VE	-VE	-VE	6%	520	127	140
34	0	5TH	NEG		0		-VE	-VE	-VE	5%	1320	240	266
35	273	3RD	MILD	INHAL	11	+VE	-VE	-VE	-VE	2%	1120	195	108
36	42	5TH	NEG		0		-VE	-VE	-VE	2%	1000	145	142
37	112	2ND	MILD	OTHER	0		-VE	-VE	-VE	0%	800	202	143
38	42	6TH	NEG		ND			ND		6%	1000	82	234
39	42	4TH	NEG		10		-VE	-VE	-VE	2%	960	139	218
40	42	3RD	CL.ALL	BOTH	0		-VE	-VE	-VE	1%	740	182	138
41	0	3RD	NEG		1		-VE	-VE	-VE	1%	1100	176	263
42	407	3RD	MILD	OTHER	10			ND		13%	1200	122	144
43	42	3RD	NEG		0			ND		ND	720	176	68
44	42	5TH	NEG		0		-VE	-VE	-VE	0%	880	118	108
45	42	5TH	NEG		0		-VE	-VE	-VE	0%	640	133	174
46	0	5TH	NEG		0		-VE	-VE	-VE	1%	620	121	142
47	112	4TH	CL.ALL	OTHER	1		-VE	-VE	-VE	8%	600	110	228
48	50	4TH	MILD	OTHER	0		-VE	-VE	-VE	2%	600	153	280
49	101	3RD	CL.ALL	INHAL	10		-VE	-VE	-VE	1%	460	153	98
50	101	3RD	NEG		0		-VE	-VE	-VE	0%	780	76	118
51	0	3RD	NEG		0		-VE	-VE	-VE	5%	580	114	158
52	42	3RD	NEG		0			ND		ND	ND	ND	ND

COLOURED MALES

NO	IGE U/ML	AGE DECADE	HISTORY GRADE	HISTORY TYPE	SKINTEST GRADE	SKINTEST ASC	PARASITES TRI	PARASITES ASC	PARASITES TEA	E/PHIL COUNT	IGG	IGA	IGM
1	166	3RD	NEG		0		-VE	-VE	-VE	0%	820	140	111
2	166	3RD	NEG		3		-VE	-VE	-VE	3%	1160	104	138
3	46	4TH	MILD	OTHER	0		+VE	-VE	-VE	3%	1020	91	222
4	248	3RD	NEG		0		-VE	-VE	-VE	5%	1440	88	284
5	339	3RD	MILD	INHAL	3		-VE	-VE	-VE	0%	2260	138	108
6	206	3RD	NEG		ND			ND		3%	1010	122	144
7	684	3RD	NEG		10		-VE	-VE	-VE	1%	1360	82	222
8	0	5TH	NEG		0		-VE	-VE	-VE	3%	1180	90	128
9	661	3RD	NEG		0		+VE	-VE	-VE	6%	760	76	170
10	182	4TH	CL.ALL	OTHER	0		-VE	-VE	-VE	1%	1240	70	195
11	115	5TH	NEG		ND			ND		0%	660	106	249
12	779	3RD	NEG		11		-VE	-VE	-VE	1%	1590	66	330
13	468	5TH	NEG		10		-VE	-VE	-VE	0%	780	156	309
14	346	3RD	NEG		1		-VE	-VE	-VE	0%	740	106	217
15	406	4TH	NEG		0		-VE	-VE	-VE	4%	820	70	134
16	38	4TH	NEG		0		-VE	-VE	-VE	ND	1090	54	157
17	225	3RD	NEG		0		-VE	-VE	-VE	ND	1440	85	263
18	762	3RD	NEG		10		-VE	-VE	-VE	6%	820	70	134
19	172	3RD	NEG		0			ND		1%	1500	160	279
20	172	4TH	MILD	OTHER	8		-VE	-VE	-VE	1%	1840	105	204
21	172	4TH	NEG		0		+VE	-VE	-VE	8%	820	110	200
22	59	3RD	NEG		0		-VE	-VE	-VE	4%	1860	119	122
23	172	4TH	CL.ALL	INHAL	11	+VE	-VE	-VE	-VE	4%	920	115	310
24	111	4TH	NEG		ND			ND		3%	1300	170	200
25	3116	3RD	NEG		1		+VE	+VE	-VE	ND	1400	215	309
26	119	3RD	NEG		0		+VE	-VE	-VE	1%	580	281	290
27	133	4TH	CL.ALL	INHAL	10		-VE	-VE	-VE	3%	1210	106	127
28	1	7TH	NEG		0		-VE	-VE	-VE	2%	500	54	144
29	166	3RD	NEG		0		-VE	-VE	-VE	0%	820	140	111
30	46	4TH	NEG		0		-VE	-VE	-VE	2%	1000	104	196
31	0	4TH	NEG		0			ND		2%	720	102	301
32	42	4TH	MILD	OTHER	7	+VE	+VE	+VE	-VE	2%	860	204	192
33	112	3RD	NEG		7		-VE	-VE	-VE	1%	880	118	58
34	198	3RD	NEG		3			ND		3%	940	208	140
35	615	2ND	NEG		ND			ND		3%	1180	281	103
36	549	4TH	NEG		7	+VE	+VE	-VE	-VE	12%	860	190	80
37	43	3RD	CL.ALL	OTHER	0		+VE	-VE	-VE	1%	920	101	160
38	43	4TH	MILD	OTHER	2		+VE	-VE	-VE	1%	680	180	124
39	398		NEG		ND			ND		9%	960	149	224
40	9738	5TH	NEG		1		+VE	-VE	-VE	6%	680	220	224
41	1457	2ND	NEG		ND			ND		2%	840	172	164
42	96	4TH	MILD	OTHER	0		-VE	-VE	-VE	1%	760	89	216
43	95	3RD	NEG		ND			ND		4%	900	250	196
44	772	4TH	NEG		1		-VE	-VE	-VE	150T	520	73	306
45	1054	3RD	MILD	OTHER	ND			ND		1%	1220	54	316
46	760	4TH	MILD	OTHER	ND			ND		6%	1840	106	285
47	308	2ND	CL.ALL	INHAL	ND			ND		8%	1340	164	175
48	421	4TH	NEG		ND			ND		2%	3020	83	260
49	1759	5TH	NEG		0		-VE	-VE	-VE	200T	620	80	216
50	202	3RD	NEG		7		-VE	-VE	-VE	100T	1020	93	262
51	200	3RD	NEG		0		-VE	-VE	-VE	300T	920	162	220
52	145	3RD	NEG		ND			ND		200T	880	93	247
53	278	3RD	NEG		ND			ND		350T	1060	126	316
54	305	3RD	NEG	—	ND			ND		100T	1060	100	257

COLOURED FEMALES

NO	IGE U/ML	AGE DECADE	HISTORY GRADE	HISTORY TYPE	SKINTEST GRADE	SKINTEST ASC	PARASITES TRI	PARASITES ASC	PARASITES TEA	E/PHIL COUNT	IGG	IGA	IGM
1	46	3RD	NEG		ND			ND		0%	1130	110	144
2	403	5TH	MILD	OTHER	ND			ND		3%	1360	80	310
3	375	3RD	NEG		2	+VE	-VE	-VE	-VE	1%	940	160	277
4	95	3RD	NEG		0		-VE	-VE	-VE	ND	920	122	157
5	0	5TH	NEG		2		-VE	-VE	-VE	1%	840	261	96
6	351	5TH	MILD	OTHER	3	+VE	-VE	+VE	-VE	1%	760	220	88
7	0	3RD	MILD	INHAL	0		+VE	-VE	-VE	4%	1140	160	246
8	257	5TH	NEG		1		+VE	-VE	-VE	2%	820	132	120
9	112	5TH	NEG		0		+VE	-VE	-VE	2%	760	304	246
10	101	2ND	CL.ALL	OTHER	0		+VE	-VE	-VE	6%	1120	118	152
11	176	2ND	NEG		0		+VE	+VE	-VE	5%	1390	190	132
12	74	2ND	NEG		0		+VE	-VE	-VE	3%	1000	190	173
13	42	4TH	NEG		5		-VE	-VE	-VE	2%	920	262	120
14	333	3RD	NEG		3		+VE	-VE	-VE	3%	740	162	92
15	191	4TH	NEG		0			ND		2%	1040	118	136
16	101	3RD	MILD	INHAL	0		-VE	-VE	-VE	1%	3020	105	140
17	391	4TH	NEG		5	+VE	+VE	+VE	-VE	1%	860	220	164
18	43	3RD	CL.ALL	INHAL	5		+VE	-VE	-VE	1%	1400	86	246
19	1752	3RD	MILD	INHAL	3	+VE	+VE	-VE	-VE	7%	440	250	96
20	848	3RD	NEG		3	+VE	+VE	+VE	-VE	1%	1300	77	210

BANTU MALES

NO	IGE U/ML	AGE DECADE	HISTORY GRADE	HISTORY TYPE	SKINTEST GRADE	SKINTEST ASC	PARASITES TRI	PARASITES ASC	PARASITES TEA	E/PHIL COUNT	IGG	IGA	IgM
1	891	4TH	NEG		6	+VE	-VE	-VE	-VE	100T	1400	178	280
2	215	4TH	NEG		0		-VE	-VE	-VE	50T	980	126	266
3	1311	5TH	NEG		0		-VE	-VE	-VE	250T	1380	138	280
4	555	5TH	NEG		7	+VE	-VE	-VE	-VE	50T	940	276	470
5	444	5TH	CL.ALL	INHAL	10	+VE	+VE	-VE	-VE	400T	980	204	222
6	311	7TH	NEG		2	+VE	+VE	-VE	-VE	100T	420	138	160
7	1051	3RD	NEG		0		-VE	-VE	-VE	50T	2400	290	312
8	280	3RD	NEG		0		-VE	-VE	-VE	100T	1180	28	216
9	170	4TH	NEG		2		-VE	-VE	-VE	650T	780	64	212
10	53	4TH	MILD	INHAL	1		-VE	-VE	-VE	100T	620	204	120
11	912	4TH	NEG		0		-VE	-VE	-VE	0T	1300	210	280
12	1411	4TH	NEG		10	+VE	+VE	-VE	-VE	50T	1000	80	550
13	809	4TH	CL.ALL	INHAL	8	+VE	-VE	-VE	-VE	400T	1360	74	212
14	739	4TH	NEG		10	+VE	-VE	-VE	-VE	300T	1360	180	258
15	7472	5TH	NEG		0		-VE	-VE	-VE	650T	1680	278	448
16	308	5TH	MILD	INHAL	0		+VE	-VE	-VE	100T	1780	120	342
17	1866	5TH	NEG		0		+VE	+VE	-VE	13%	1360	112	285
18	577	4TH	MILD	OTHER	11	+VE	+VE	+VE	-VE	14%	1080	131	114
19	1054	5TH	CL.ALL	INHAL	2	+VE	+VE	+VE	-VE	12%	1440	84	378
20	806	3RD	NEG		ND			ND		2%	1980	106	312
21	43	3RD	NEG		0		-VE	-VE	-VE	2%	ND	112	285
22	9940	5TH	MILD	INHAL	2		+VE	-VE	-VE	6%	2200	293	424
23	2327	6TH	NEG		3	+VE	+VE	+VE	+VE	6%	1560	276	285
24	43	4TH	NEG		4		-VE	-VE	-VE	7%	1200	213	233
25	761	3RD	NEG		9	+VE	-VE	-VE	-VE	12%	960	276	223
26	2814	4TH	NEG		ND			ND		3%	1380	125	208
27	198	3RD	NEG		10		+VE	-VE	-VE	ND	1080	117	322
28	198	3RD	NEG		ND			ND		8%	1500	77	82
29	198	5TH	NEG		2	+VE	+VE	-VE	-VE	5%	1720	147	284
30	1136	4TH	NEG		2		-VE	-VE	-VE	4%	4140	141	377
31	5135	6TH	NEG		2	+VE	+VE	+VE	-VE	ND	2200	79	285
32	1433	3RD	NEG		ND			ND		450T	1200	86	244
33	293	3RD	NEG		ND			ND		0T	1060	204	338
34	1720	3RD	NEG		ND			ND		ND	1240	330	360

BANTU FEMALES

NO	IGE U/ML	AGE DECADE	HISTORY GRADE	HISTORY TYPE	SKINTEST GRADE	SKINTEST ASC	PARASITES TRI	PARASITES ASC	PARASITES TEA	E/PHIL COUNT	IGG	IGA	IGM
1	150	5TH	NEG		0		-VE	-VE	-VE	0T	920	224	280
2	55	4TH	NEG		0		+VE	+VE	-VE	50T	1100	88	200
3	93	4TH	NEG		1	+VE	-VE	-VE	-VE	150T	1540	180	280
4	198	4TH	MILD	OTHER	0		+VE	-VE	-VE	9%	1300	147	312
5	246	3RD	NEG		0		-VE	-VE	-VE	2%	1540	150	222

APPENDIX VII

Copy of the Allergy History Questionnaire

Occupation

1. Suffer now from (i.e. within last 12 calendar months)

   A. Asthma

   Chest goes "tight"

   Acute attacks needing immediate treatment

   On any anti-spasmodic drugs

   What drugs

   Interferes with daily

   B. Hay Fever

   Runny nose

   Blocked nose

   Itchy eyes

   On any drugs

   What drugs

   Interferes with daily life

   C. Eczema or Dermatitis

   Location

   Skin discolouration

   palpable rash

   vesicles

   ulceration

   Discomfort - None

   Itchy

   Painful

   Any drugs

   What drugs

   Interferes with daily life

For any of the above

Seasonal or non-seasonal

Symptoms precipitated by dust

animals

plants

other know offenders (i.e. at work)

emotion

Improving

Remaining static (over past year)

Getting worse

Desensitisation

D. Drug Allergy or Food Allergy

Type reactions

Known offenders

E. Other Allergy

2. Previously suffered from

A. Asthma

Chest going tight

Acute attacks needing immediate treatment

On any anti-spasmodic drugs

What drugs

Interfering with daily life

B. Hay Fever

Runny nose

Blocked nose

Runny eyes

Itchy eyes

Needing drugs

What drugs

Interfering with daily life

C. Eczema or Dermatitis

Location

Skin discolouration

palpable rash

vesicles

ulceration

flaking

Discomfort - None

Itchy

Painful

Needing drugs

What drugs

Interfering with daily life

For any of the above

Seasonal or non-seasonal

Symptoms precipitated by dust
animals
plants
other known offenders (i.e. at work)
emotion

Duration of Symptoms

Desensitisation

D. Drug Allergy or Food Allergy

Type reaction

Known offenders

E. Other Allergy

3. Family History Allergy

4. Previous or present other illness

5. Worms in the stools - ever seen?
How recently
Treatment

6. At present on any drugs.

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