A PROPOSED ROLE FOR THE CA ION
IN THE
CHEMOTACTIC RESPONSE OF PHYSARUM POLYCEPHALUM

by

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A thesis presented in the fulfillment of the requirements

for the degree of

MASTER OF SCIENCE

of

UNIVERSITY OF CAPE TOWN

March, 1977

TABLE OF CONTENTS

TABLE OF CONTENTS

SUMMARY

Durham, in a review published in 1974, presented the following hypotheses concerning the factors that control amoeboid movement: (1) actin and myosin are present in all cells that exhibit amoeboid movement and, changes in the internal $[Ca^{++}]$ regulate contraction, (2) filaments of actin and myosin form an intimate association with the surface membrane and depending on the local $[Ca^{++}]$, the filaments can cause the membrane to relax or become rigid, (3) Ca^{++}fluxes across the external membrane (viz. efflux and influx) regulate the state of contraction in the proposed actinomyosin-surface membrane network and, (4) such Ca^{++} fluxes operating across the membrane manifest themselves (especially with slime mould plasmodia) as waves of adhesion running across the undersurface of a cell and aid in movement.

A working hypothesis, that encompasses the ideas of Durham, is that Ca^{++} entry and efflux across the external membrane control such cellular processes as extension of pseudopodia, exocytosis, endocytosis and the direction of movement (chemotactic response) of amoeboid cells. In the specific case of slime mould plasmodia, which best exemplify all of Durham's hypotheses, the simplest hypothesis to explain the control of chemotaxis is that attractants (sugars, food, organisms) cause a Ca^{++} efflux across the membrane and a subsequent movement forward. Repellents would act in a reverse manner by causing Ca^{++} entry. This hypothesis also allows for the existence of a Ca^{++}-accumulating organelle. This organelle might replace or act in concert with the proposed Ca^{++} fluxes across the external membrane. The investigations reported in this thesis were devised to examine experimentally this hypothesis. The results may be summarized as follows:

1. Plasmodia submerged in an aqueous environment have a normal Ca^{++} efflux that continues for the life of the plasmodia. Chemotactic attractants and other chemicals may evoke a sudden increase in the efflux rate but this event is transitory and considered to be an artifact. Beyond that, attractants do not produce any significant long-term changes in the Ca^{++} efflux rate. This observation disproves the hypothesis that attractants affect the external membrane and cause an increase in Ca^{++} efflux . Simultaneous optical recordings confirmed Durham's observation (1976) that attractants generally increased the internal motile oscillations. The recordings also disclosed the existence of a "shock" period, in which the oscillations ceased for 10-15 minutes following exposure to sugars as attractants.

An artificial increase of Ca^{++} efflux by leeching with a chelating agent (EGTA) did not affect the streaming except after 20-30 minutes exposure. The ability of the plasmodium to withstand the artificially high Ca^{++} efflux suggests that there is an internal source of calcium. Furthermore, since the mechanisms that regulate the internal $[Ca^{++}]$ in the case of contractile proteins are not associated with Ca^{++} transport across the external membrane, the proposed hypothesis that external Ca^{++} fluxes are involved in chemotaxis is also not substantiated.

2. Half plate assay experiments demonstrated that plasmodia were repelled when the external Ca^{++} or the EGTA concentrations in the agar substrate were at least 1 mM. EGTA is a repellent because of its leeching effect of Ca ions from the plasmodium's membrane. Plasmodia were unable to discriminate between 5 mM concentrations of Mg^{++} and Ca^{++} and were equally repelled by either. This confirmed the finding by Ueda _et al._ (1975) that there is a threshold concentration at which the cations, Ca^{++}, Mg^{++}, Mn^{++} and Ba^{++}, to an equal degree, begin to cause the plasmodial membrane to be depolarized and to alter the direction of the applied movtive force.

Migrating plasmodia show no preference or rejection towards different external $[Ca^{++}]$ in the range 10^{-4} to 10^{-7} M. The chemotactic response towards 1 mM glucose did not change when these different $[Ca^{++}]$ were present in the agar substrate. It appears therefore, that the Ca ion has no effect on the submembrane actinomyosin network, a conclusion that runs counter to the proposed hypothesis that Ca^{++} fluxes across the membrane control chemotaxis.

3. A technique is described for the isolation of mitochondria from shaker culture microplasmodia. Using rat liver mitochondria as a standard, isolated microplasmodia mitochondria showed a poor respiratory function and almost no oxidative phosphorylation capability. No increase in the rate of respiration occurred when Ca^{++} was added to the suspension. When studied by murexide dual-wavelength spectrophotometry, mitochondrial suspensions from microplasmodia did not show the active Ca^{++} uptake observed with rat liver mitochondria. The preliminary results from the polarography and murexide studies suggest that plasmodial mitochondria have no inherent energy-linked Ca^{++}-accumulating ability and that therefore, they could not participate in the chemotactic response by regulating the internal Ca^{++} concentration. However, the poor respiratory ability of the mitochondria may have been due to difficulties experienced during their isolation, and it does not preclude a real but impaired Ca^{++}-accumulating function.

CHAPTER ONE

INTRODUCTION

With the evolution of life, certain similar characteristics of sensory mechanisms have persisted in all life forms. This sensation, recognition and response are thus among the main characteristics of living cells. In order to understand the smell and taste reception of higher animals, current research is attempting to elucidate the molecular mechanisms behind chemotaxis in simpler, unicellular organisms. The most primitive example of chemotaxis can be seen in the response of flagellated bacteria to various sugars, amino acids and repellents (Adler, 1969, Berg & Brown, 1972). More advanced chemotaxis is seen in amoeboid movement by eukaryotic cells.

Cytoplasmic streaming in the myxomycete *Physarum polycephalum* is one of the best-studied forms of amoeboid movement (Kamiya, 1959, Stewart, 1964, Komnick *et al*. 1973). Yet until recently it has not been understood how a chemotactic substance initiates the sequence of events at the membrane leading to motion in the appropriate direction. It is also not known how the initial recognition of the substance is conveyed internally to the mechanisms for motion and how the membrane coordinates with the internal "shuttle" streaming to bring about movement.

A Physarum plasmodium is effectively a single enormous amoeba, with a large volume of cytoplasm (several cm^2 surface area), enclosed in one continuous external membrane. The cytoplasm streams in a singular, rhythmic, back and forth pattern through a finely dichotomously-branched "venous" network of channels. Hydrostatic pressure gradients throughout the plasmodium supply the motive force to drive the cytoplasm. These gradients act in concert to produce waves of alternative contraction and relaxation and time-lapse cine films vividly show these waves (cf. intestinal peristalsis) sweeping across a migrating plasmodium (Stewart, 1964). There is biochemical evidence showing that contractile proteins, homologous with muscle proteins are the basis for the waves of contraction.

Many large organisms move by propagating waves along their external surface. A similiar mechanism has often been suggested to underlie amoeboid movement. Durham and Ridgway, in 1976, gave experimental evidence supporting the hypothesis that the rate of the "shuttle" streaming (viz. frequency of the waves) can direct the movement of a plasmodium. In brief, they proposed that plasmodia migrate towards those situations (chemotactic attractants, warmth) which increase the frequency of the waves, and away from those which decrease the frequency (repellents).

Durham and Ridgway's hypothesis explains how a plasmodium can be seen as an efficient food seeking machine. It is useful to think of the plasmodium as being polyrhythmic, in the sense that different areas have faster and slower rates of oscillations in streaming. A major premise in their hypothesis is the existence of a membrane-linked actinomyosin network and they propose that attractants acted by reducing the $[Ca^{++}]$ in the region of the network. The membrane and the network would then relax at that region and the pressure of the internal cytoplasm would cause a "bulge" forward. This region now the front, will inherently develop an increase in the rate of oscillations and so doing entrain the "slower" areas of the plasmodium to move in that direction. And in the reverse situation, the hypothesis explains the retreat of a plasmodoium away from a repellent. This hypothesis will be explained in greater detail in the second chapter.

Various workers (Wohlfarth-Bottermann, 1964, Komnick et al. 1973) have pointed out the homology between amoeboid movement and more organized muscle movement. Both have the contractile proteins, actin and myosin, and these interact to supply the force. Until recently, there was only experimental evidence for Ca ions as being the regulating element for contraction in muscle. Ridgway and Durham (1976) presented evidence for a similiar role of Ca^{++} in amoeboid movement. They demonstrated that Ca^{++} concentrations inside a plasmodium changed synchronously with its oscillations. If Ca ions are the regulating element for the contractile proteins and these govern the rate of streaming, then this raises the question of what intracellular process regulates the $[Ca^{++}]$ in the cytoplasm.

The sarcoplasmic reticulum has been established as the intracellular organelle regulating local concentrations of Ca^{++} for muscle concentration. No similar organelle has been found in Physarum plasmodia. However Ettienne (1972) and Braatz and Komnick (1973), have shown by electron microscopy, a Ca^{++}-accumulating ability in certain membrane-bounded ATP-dependent vacuoles located near contractile fibrils in the ectoplasm. Ca^{++}-specific pumps have also been identified in cellular membranes and there is a range of cell types among uni- and multicellular organisms, from those not requiring Ca^{++} to those with a marked dependence on the presence of external Ca^{++}. It has not been determined whether Ca^{++} pumps exist in the external membrane in the case of acellular slime moulds.

A reasonable model for chemotaxis would begin with the assumption that there are inherent Ca^{++} pumps in the external membrane and the internal oscillations in the $[Ca^{++}]$, observed by Ridgway and Durham, reflect their activity. The pumps would act in waves across the plasmodium and account for the observed waves of contraction. Accompanying this is the basic assumption that the cytoplasmic $[Ca^{++}]$ is the regulatory element for the contractile proteins. Chemotactic attractants or their metabolic byproducts would have a two-fold effect. Agreeing with Durham and Ridgway's observation (1976), attractants will increase the frequency of streaming, but as the result of increased frequency of the waves of Ca^{++} pumping internally and across the external membrane. Secondly, attractants would act on the hypothesized membrane-actinomyosin network by lowering the $[Ca^{++}]$ and bring about localized relaxation. Internal vacuoles, mitochondria or Ca^{++} in the external membrane could do this and the subsequent relaxation, would cause a "bulge" forward.

Further, it is known from tissue cultures (Manery, 1966) that Ca^{++} is required for adhesion of cells to the substrate. Assuming the existence of waves of Ca^{++} effluxes across the plasmodium, these would have the effect of causing the underside of a plasmodium to act in waves of relaxation and adhesion. The sum total of these actions would result in the plasmodium migrating toward attractants and by reversing the effects, move away from repellents.

To test the above hypothesis and determine whether or not significant Ca^{++} fluxes occur and of what magnitude, Durham and the author designed a flow-through chamber device. The chamber enabled the sequential collection of the ^{45}Ca-bearing effluent, which previously flowed over a large surface area of a ^{45}Ca-labelled plasmodium. The latter plasmodium was perfused with a control solution, followed by changeovers to solutions of chemotactic attractants and repellents. The level of ^{45}Ca efflux was determined by scintillation counting. In order to better assess the response of the organism and determine if any causal relationship existed between the external Ca^{++} efflux and the streaming, the chamber was made transparent and simultaneous optical recordings were made of the motile streaming.

Although Braatz and Komnick (1973) have shown the existence of Ca^{++}-accumulating vacuoles, other organelles, notably the mitochondria, may be responsible for regulating the cytoplasmic Ca^{++} concentrations. Cheung et al. (1974) observed that mitochondria were primarily in the "peripheral cytoplasm of the advancing plasmodial fan" while Braatz and Komnick (1973) reported calcium deposits of calcium precipates could be seen in mitochondria. Both these two observations together with Durham's premise of attractants acting by reducing the $[Ca^{++}]$ in the advancing peripheral region, suggest that mitochondria might play a role in chemotaxis. In order to study whether slime mould mitochondria per se have an energy-linked Ca^{++}-accumulating ability, some preliminary experiments were done to measure the respiration and Ca^{++} accumulation of rat liver mitochondria. After proficiency in the relevant techniques had been obtained, this knowledge was then applied to the study of slime mould mitochondria.

CHAPTER TWO

REVIEW OF LITERATURE

A. General survey of calcium involvement in cellular metabolism

1. Extra- and intracellular roles of calcium

Calcium is a comparatively common element. Its basic involvement in the eukaryotic cell's metabolism is evidenced by the age of 2,7 X 10^9 years, attributed to calcareous algal limestones found in Rhodesia (Seifriz, see Kamiya, 1959). The beginning of cellular evolution most likely took place in the sea and the uni- and multicellular life forms which arose have ever since segregated the cations Na^+, K^+, and Ca^{++} either inside or outside cellular membranes.

Ionized calcium concentrations are often expressed logarithmically, thus molar concentrations of 10^{-2}, 10^{-5} and 10^{-9} correspond to pCa values of 2, 5 and 9 respectively. Using this convention, intracellular pCa is approximately 7, whereas in the interstitial fluid the pCa is closer to 3 (Rasmussen, 1970). This partitioning involves Ca^{++} fluxes between three regions; (1) the extracellular milieu, (2) the cell's plasma membrane, and (3) the intracellular region. With such a large Ca^{++} gradient there should be a significant inward net driving force. The passive entry of Ca ions is prevented by various cellular mechanisms which will be discussed later.

Besides bone formation, many tissues can form solid deposits of calcium salts. The list includes carapaces of invertebrates, egg shells, stem calcification in stonewart algae and higher plants. The potential for undergoing calcification is also inherently present in most tissues of higher animals. It is well known that certain human organs and tissues can undergo calcification under pathological conditions. Lehninger (1970) drew the conclusion that, because of this biological potential for calcification, there must be some ubiquitous process for the deposition of insoluble calcium salts, but also the necessary mechanisms for its regulation.

Apart from the structural function, the ionized form of calcium has a very important "mediator" function within cells. Nerve impulses and hormones communicate with target organs via the nervous and circulatory systems respectively. Neurons, secretory cells of glands, cardiac muscle, etc, all have in common the fact that each one receives an external stimulus (e.g. electrical, hormonal, mechanical). Superficially, it appears that each cell type carries out a specific function separate from that of the others. However, internally all may have similar basic mechanisms controlling their specific functions.

Cyclic AMP and Ca ions have interrelated roles as intracellular messengers (Rasmussen, 1970). The external stimulus reacts with the cell membrane of the cell types mentioned previously. It may result in an intracellular increase of cyclic AMP and Ca^{++}. The increase in cyclic AMP usually activates phorylating enzymes (protein kinases). The products of the phosphorylation reaction catalyzed by these enzymes are now sensitive to the increased Ca^{++} concentrations. Their activation by Ca^{++} may lead either to further enzymatic activation, to a change in protein structure (contractile proteins), regulation of packaged vesicle secretions (hormonal, neural), or to control of transport of Ca^{++} (Rasmussen, 1970).

The response of cardiac muscle to epinephrine, with the resultant glycogenolysis and contraction, illustrates the way in which the system functions in a specific case. Epinephrine stimulates the cell membrane, thereby inducing increased cyclic AMP production and raising internal Ca^{++} concentrations. The presence of cyclic AMP leads to the phosphorylation of a phosphorylase b kinase to its active form by a phosphorylase b kinase kinase. The product of that reaction, as a kinase now sensitive to Ca ions present, converts phosphorylase b to phosphorylase a. The latter form now is able to reduce glycogen to glucose-1-phosphate. Besides the activation of the protein kinase, the increased Ca^{++} also plays a part in cardiac actomyosin interaction.

Another example of the mutual reliance between cyclic AMP and Ca^{++} is thought to be the endocrine secretion of insulin from beta cells of the pancreas. It has been demonstrated that the release of insulin brought about by glucose requires the presence of Ca ions (Rasmussen, 1970). Cyclic AMP is also involved but its site of action is still unclear. Also of interest is that Lacy et al. (1968) showed that calchicine blocks glucose-induced secretion. He proposed that this indicated that calchicine interferes with the microtubules involved in intracellular transport of insulin-bearing vesicles to the cell's surface membrane for fusion and secretion. As microtubules are made of contractile proteins, Ca ions probably regulate their function and influence vesicle transport.

In summary the roles of Ca^{++} as an intracellular messenger (Rasmussen, 1970) are; (1) the specific activation of the phosphorylated protein (enzyme) produced as the result of protein kinase action, (2) activation of other enzymatic reaction, (3) possible action as a feedback inhibitor of the enzyme for cyclic AMP synthesis (e.g. membrane bound adenyl cyclase), and (4) the regulation of contractile proteins (muscle, microtubules, microfilaments).

A network of microfilaments underlying cell membranes has been implicated in cell division (cleavage furrow formation), cytokinesis, and non-muscle movement represented by amoeboid and cytoplasmic streaming (Chapman-Anderson, 1973, Durham, 1974). There is much evidence pointing to Ca^{++} as being vital to all cellular functions involving contractile mechanisms.

2. Factors regulating intracellular calcium (Ca^{++})

Many intracellular processes require that the intracellular $[Ca^{++}]$ be approximately 10^3 times less inside the cell than outside. The mechanisms responsible for maintaining the intracellular pCa fall into two groups: (1) intracellular sequestering organelles, and (2) membrane-involved transport. The cell membrane mechanisms for extruding Ca^{++} will be discussed after the Ca^{++} sequestering organelles have been dealt with.

The organelles of the first group consist of mitochondria and the sarcoplasmic reticulum of muscles. In most cells the uptake and release (buffering action) of Ca^{++} by mitochondria seems to be quantitatively the most important for maintaining the normal low pCa value of the cytoplasm. Both organelles are known for their Ca^{++} sequestering function. In the short term, either are capable of stabilizing the pCa in the region of 6 and 8, but this capacity is limited (Lehninger, 1970, Borle, 1973). Ultimately, if the total internal $[Ca^{++}]$ is not to rise, the Ca ions which enter the cell must be transported actively across the surface membrane.

The mitochondria of every tissue type of higher animals and plants examined have been shown to have the ability to accumulate large quantities of Ca^{++} by an energy-dependent process. Further, of all the common uni- and bivalent cations present in cells, only Ca^{++} is rapidly and stoichiometrically accumulated by mitochondria (Lehninger, 1970). The possible control that some mitochondria may have over the Ca^{++} involved in actomyosin contractile systems is a major part of this thesis. This relationship between mitochondria, Ca^{++} uptake and cytoplasm was best described by Borle (1973). His hypothesis, was that generally the calcium activity (concentration) of the cytoplasm (Ca_c) was a function of three independent variables: (1) calcium activity (concentration) in the mitochondria (Ca_m), (2) rate of calcium efflux (J_{mc}) and (3) rate of calcium influx (J_{cm}). For a steady state (homeostasis), the rate of calcium influx (J_{cm}) into the mitochondria is equal to its efflux rate (J_{mc}).

$$(1) \qquad J_{mc} = J_{cm}$$

The rates are determined by a specific rate constant and calcium activity for each.

$$J_{mc} = K_{mc} \times Ca_m \quad \text{and} \quad J_{cm} = K_{cm} \times Ca_m$$

$$(2) \quad \text{or} \quad K_{mc} \times Ca_m = K_{cm} \times Ca_c$$

Transposing gives the equation that sums up Borle's argument.

$$(3) \qquad Ca_c = \frac{Ca_m \times K_{mc}}{K_m}$$

Thus it is possible for the Ca^{++} level in the cytosol to be changed by these three variables. It can rise or fall by three different mechanisms: (1) by changing the Ca^{++} concentration in the mitochondria (Ca_m), (2) by changing the efflux rate of Ca^{++} from the mitochondria to cytoplasm (K_{mc}), or (3) by changing the rate of sequestration of Ca ions into the mitochondria (K_{cm}). Two examples of factors influencing cytoplasmic Ca^{++}: phosphate ions (PO_4^{3-}) and the parathyroid hormone, will be discussed in relation to Borle's equation (3).

The phosphate ion, as a permiant anion, will be precipitated when taken up with Ca^{++} by mitochondria. A rise in cellular phosphate will immediately increase the precipitation of calcium phosphate in the mitochondrial matrix and therefore reduce the free Ca^{++} concentration.

within the matrix. According to Borle's equation, since the cytoplasmic $[Ca^{++}]$ (Ca_c) is a direct function of the mitochondrial Ca^{++} (Ca_m) concentration, cytoplasmic Ca^{++} will drop accordingly. The converse occurs with a drop of cellular phosphate ions.

It is firmly established that parathyroid hormone acts by stimulating the enzyme adenyl cyclase thus raising intracellular cyclic AMP concentrations (Rasmussen, 1970). It also brings on a dramatic Ca^{++} efflux from mitochondria and the cell membrane into the cytoplasm. These results can be interpreted in terms of a stimulation by cyclic AMP of the rate of Ca^{++} efflux (K_{mc}) from mitochondria. Logically from the equation, such a stimulation would immediately increase the free Ca^{++} concentration (Ca_c) of the cytoplasm.

Although it has been easy to demonstrate mitochondrial Ca^{++} uptake to the extent of massive loading and the subsequent lowering of the Ca^{++} concentration in a cell free medium to 10^{-7}M, no one has successfully demonstrated a physiological process whereby mitochondria may release their hoarded calcium back into the cytoplasm. Borle (1974), using rat liver mitochondria, showed that cyclic AMP stimulated Ca^{++} release from isolated mitochondria. The cyclic AMP, in this instance, would be acting by influencing the efflux rate (K_{mc}) of Ca^{++} into the cytoplasm. Other investigators have commented that his results have not proven repeatable (Green, 1975).

The contraction-relaxation cycle of cardiac muscle, like that of skeletal muscle is controlled by the translocation of Ca^{++} among intracellular structures (Scarpa and Graziotti, 1973). The in vivo relaxation of the myofibrils requires a Ca^{++} concentration of about 10^{-7}M, certainly within the ability of cardiac mitochondria. The act of Ca^{++} removal occurs in about 200 ms during each beat. The sarcoplasmic reticulum, which is the source of Ca^{++} removal during the relaxation of skeletal muscle, is relatively scarce in heart muscle. Therefore, some investigators have suggested that cardiac mitochondria may regulate the contractile cycle of the heart by taking up and releasing significant amounts of Ca^{++} during the cycle. Scarpa and Graziotti (1973) showed that cardiac mitochondria, in vitro, could not transport Ca^{++} with sufficiently rapidity to participate in the Ca^{++} redistribution for each heart cycle.

The other factors controlling the internal $[Ca^{++}]$ involves the cell membrane, changes in its permeability to Ca ions, and the extrusion of Ca^{++} across it against the existing gradient. One mechanism is the active transport by ATP-dependent Ca^{++} carriers in the cellular and organelle membranes. The evidence of their action has been most clearly seen in erythrocytes and a variety of cultured cells including HeLa and L-cells (Schatzman, 1974). Another type of Ca^{++}-ATPase pump is known for certain vacuoles in slime mould plasmodia (Ettienne, 1972, Braatz and Komnick, 1973). It has been shown that these vacuoles accumulate Ca^{++} ions from very low concentrations and are dependent on the presence of ATP. Rasmussen (1970) showed that in many examples of excitation and subsequent hormone secretion or nerve transmission, Ca^{++} is required extracellularly. From that observation and results of other researchers, Rasmussen proposed that the increased cyclic AMP (brought about by hormone-adenyl cyclase interaction) acted by allowing Ca^{++} influx across the cell membrane (increased permeability) and also "mobilizing Ca^{++} from intracellular pools" (mitochondria, vacuoles, etc).

A more specialized transport mechanism, that of Ca^{++} extrusion in exchange for Na^{+} entry, has only been shown to exist in the giant squid axons and to a lesser extent for muscle and the gut (Kretsinger and Nelson,

1976) . The Na^+ gradient is maintained by a Na^+-ATPase pump. There is no evidence for a Ca^{++}-ATPase pump in the squid axon or any other "excitable" tissues. Ca^{++} extrusion can occur in the absence of ATP hydrolysis. It appears that one Ca ion passes outward with the influx of three Na ions. This mechanism evidently can keep the Ca^{++} level of the axon's axoplasm between $10^{-5.5}$ and 10^{-7}M. Ca^{++} is only one of the ions involved with the generation and propagation of nerve potentials. How Ca^{++} ions are involved with nerve potentials is still unclear and the actual function of the Ca^{++} gradient remains unknown.

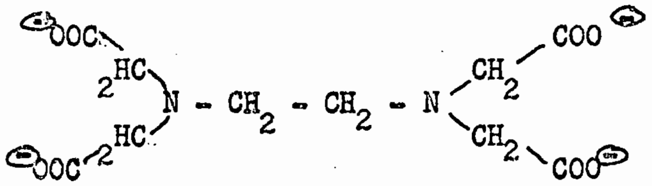
3. Current experimental approaches for determining changing calcium concentrations

Most cellular studies have used one of the following lines of thought. The changes of Ca^{++} influx or efflux from cells can be recorded while they are subjected to various conditions. Conversely, one can observe how the cells respond to predetermined manipulated changes in either the internal or external Ca^{++} concentrations. By circumstance these studies deal with the detection of changes in very low calcium concentrations. There is a limit therefore to the degree that such studies can become quantitative. Ca^{++} studies of intact cells represent a special difficulty because: (1) in most eukaryotic cells the concentrations of free Ca^{++} in the cytoplasm is 10^{-6} to 10^{-7}M, (2) membrane bound or non-ionized calcium may interfere with measurements and (3) the presence of other ions (e.g. Mg^{++}, Na^{+}) may also interfere.

All the reasons listed above explain why in vivo studies (e.g. tissue cultures, glycerinated models of muscles, amoebae, slime moulds, electron microscopy), for the large part, are qualitative in nature. Thus when one considers the many different approaches, he must consider how his particular approach may interfere with the cell's normal physiology and whether it suits the particular cell type and experimental situation. The pros and cons of six methods will be discussed below.

a. Calcium chelating buffers

In many studies it is essential to distinguish between the total calcium and the free ion. Further, in many systems the Ca^{++} concentrations are in the range of 10^{-5} - 10^{-8}M. Since it is extremely difficult to measure the free Ca^{++} versus bound Ca^{++}, one can resort to choosing a buffering agent that will "set" the concentration of free Ca^{++} just as one uses a H^{+} buffer. Using this analogy with pH buffers, one can control the pCa of any medium. One chemical agent used is EGTA (ethylene glycol bis (βaminoethyl ether)-N, N'-tetraacetate. It is a calcium chelator and as such its structure is so modified as to wrap its four carboxyl groups around the Ca ion and effectively remove it from solution.



Solutions containing mixtures of EGTA and its calcium complex have known calculated stabilized concentrations of free Ca^{++} which are changed only slightly by dilution at a given pH or by addition of extra Ca^{++} or Mg^{++}. Another factor in its favor is that its chelating capacity is most effective in the pCa range from 8 to 5. Ca^{++}-EGTA buffers have been used for determining the precise Ca^{++} concentration that will cause contraction in the large muscle fibers of the spider crab (Portzehl _et al._ 1964). By injecting into these cells successively lower buffered pCa solutions, the resting pCa was, in theory, determined by finding the buffer which caused no contraction (viz. 10^{-6} M). Similarly, EGTA was used in the medium to show up the Ca^{++} accumulating vacuoles in electron microscope studies of _Physarum_ (Braatz and Komnick, 1973).

b. _Calcium electrodes_

These electrodes measure Ca ion levels similarly to either oxygen or H^+ electrodes and have an effective pCa range of 1 to 5 (10^{-1} - 10^{-5}M (Kretsinger and Nelson, 1976). The usefulness of calcium determinations is limited therefore by its comparative lack of sensitivity towards the very low calcium concentrations found in cells. Carr (see Kretsinger and Nelson, 1976) pointed out that the reliability of the measurements is "strongely dependent" on careful calibration of the electrode with standard calcium solutions, maintaining a constant temperature and taking suitable precautions against possible interfering ions, such as Mg^{++}, Ba^{++} and Ni^{++}. Various researchers have expressed the hope that Ca^{++} electrodes will be miniaturized to allow insertion into cells and that the pCa range will be extended from 2 to 8.

c. Spectrophotometric indicators of calcium

Various chemical reagents will react colorimetrically to different metal ions. Perhaps the most commonly used is murexide (ammonium purpurate), a metallochromatic indicator with a high sensitivity for the calcium ion. Murexide binds with calcium forming a Ca^{++}-murexide complex which has a different absorption maximum from that of free murexide. Spectrophotometric measurements with murexide have the advantages of speed, relative accuracy and dependability. However it is unstable (has to be made fresh daily) and there can be interference from most divalent and trivalent cations excluding Mg^{++} (Kretsinger and Nelson, 1976).

The assay will detect changes of $[Ca^{++}]$ to as low as 2×10^{-7} M, and its speed has led to considerable success in kinetic studies of Ca^{++} uptake by rat liver mitochondria (Mela and Chance, 1968, Scarpa, 1970). Using a cuvette, murexide can be added (30-50 μM) to a cellular or subcellular suspension and with a double-beam spectrophotometer, absorbances at two wavelengths can be measured. Double-beam refers to having the absorbance of one wavelength (reference ~500 nm) substracted from a second one of a different wavelength (measuring ~540 nm)(Mela and Chance, 1968). The difference ($\Delta_A = \Delta_M - \Delta_R$) is then displayed on a chart recorder.

d. Aequorin spectroscopy

Whereas murexide is a chemical indicator, aequorin has a biological origin. It is a bioluminescent photoprotein and was isolated from the jellyfish Aequorin (Kretsinger and Nelson, 1976). The reaction involves two Ca ions and the rate of light emission is linear over the concentration range of 10^{-4} - 10^{-8}M. With such a range, it has obvious applications as a method for the determination of $[Ca^{++}]$ inside cells. No other factors besides Ca ions are required for the reaction to take place. Aequorin was successfully injected into the streaming network of a Physarum plasmodium and then by using a sensitive photomultiplier, the rate of light emitted led to oscillations that presumably arose from internal $[Ca^{++}]$ fluctuations (Ridgway and Durham, 1976).

e. <u>^{45}Ca radioactivity measurements</u>

The one isotope of calcium, ^{45}Ca, is easy to use because of a long half life of 162 days, it is a β emitter and is inexpensive. It is standard procedure to add the aqueous sample to a commercial scintillation "cocktail" and the vials are counted using the proper channels limits on the scintillation counter. One useful technique consists in incubating subcellular fractions (mitochondria, sarcoplasmic reticulum vesicles, etc) and at set times, using Eppendorf pipettes (viz. microliter amounts), rapidly filtering the aliquots with bacterial filters (Klingenberg and Pfaff, 1967). Such filters (Millepore, Nucleopore) can be chosen according to pore size, chemical resistance, and the extent to which it willdissolve in the scintillation cocktail and thus not hinder the counting of the vial.

Langer and Frank (1972) used ^{45}Ca because of its sensitivity and suitability for the in vivo study. They found a method which involved attaching and growing a single layer of contractile cardiac cells on a slide made of scintillation material. The slide was then set in a two part scintillation vial-flow cell. Half the apparatus was a scintillation vial which held the slide for counting and the top half (flow cell) had influx and efflux tubing to either introduce ^{45}Ca or wash off the isotope with the effluent. During the time the apparatus is in the scintillation counter, print outs show the isotope activity during calcium uptake or release.

The method of Langer and Frank is very similar to the one developed by A.C.H. Durham and the author and reported in the present thesis.

In our case, we were interested in the ^{45}Ca efflux from a plasmodium of Physarum spread on agar. The plasmodium was in a sealed rectangular chamber with an influx tubing at one end and opposite to it an efflux tubing. We used a fraction collector to collect 1 ml aliquots from the chamber efflux. More detail will be given in the Materials and Methods section.

f. Enzyme assays as calcium indicators

A last method based on enzyme assays will be mentioned. If an intracellular enzyme is sensitive to Ca ions, the activity of the enzyme in vivo may reflect the pCa to which it is normally exposed. Adenyl cyclase in certain membrane and protein kinases have been shown to be sensitive to Ca ions. The reduction of NAD is calcium dependent and enzyme reactions which use NAD as a cofactor can be very easily followed spectrophotometrically. Mitochondria, when actively respiring, take up Ca^{++} with the oxidation of $NADH_2$ and with consumption of oxygen, both of which can be monitored. Reed and Bygrave (1974) monitored the oxygen consumed by rat liver mitochondria. They showed by a decrease in oxygen respiration that the La^{3+} ion and ruthenium red competitively blocked calcium binding sites on the mitochondrial membrane.

B. Classification of cell motility

1. Mechanisms of motility among different cell types

Komnick et al. (1973), in an exhaustive review on cell motility, classified all "direct" movement into three groups: (1) protoplasmic movement, (2) ciliary and flagellar movement, and (3) muscle contraction. The unity of the three types of movement is indicated by the fact that all three are found in vertebrates. For example, leucocytes use amoeboid movement to creep through the lymphatic system, the sperm cells are flagellar and in the third category are the highly organized muscles (visceral and skeletal). The first of the three types of movement is the most important on account of its pervasiveness and diversity throughout the animal and plant kingdom. The other modes of motion are thought to have arisen phylogenetically from it.

Protoplasmic movement includes two separate phenomona: amoeboid movement and protoplasmic streaming. Protoplasmic streaming usually occurs within a rigid cell wall which plays no part in the cell's motility. The cell's nucleus remains stationary within the cytoplasm while all about it various patterns of protoplasmic movement occur. This phenomenon is best demonstrated by freshwater algae (Nitella) and marine coenocytic algae (Acetabularia). In both of these organisms, the streaming occurs in elongated stem cells (Kamiya, 1959). Streaming has also been observed in animal tissue cells and free-living cells (e.g. protozoa such as Stentor, Paramecium, etc).

Cells which utilize amoeboid movement, on the other hand, lack a cell wall and the protoplasmic streaming leads to distortions of the cell's membrane. This chacteristic ability of the membrane thus enables the cell to engulf food and expel wastes (viz. endocytosis and exocytosis) and affords both the means of attachment in an aqueous environment and locomotion. Amoebae, leucocytes, cultured fibroblasts and plasmodia of acellular slime moulds exhibit this type of movement.

For more than a century up until the 1950's, all experimentation on cell motility centered on observations and manual manipulations of any relatively large plant or animal cell that suited the experimenter's purpose (e.g. Nitella, Acetabularia, Physarum, etc). Progress towards understanding the cellular mechanisms actually producing the motive force underlying each type of protoplasmic streaming, came with modern physical and biochemical techniques, in conjunction with patient and astute microscopic observations. Such progress has shown that the molecular mechanisms of all types of movement previously discussed are basically the same. Therefore these are now termed "contractile movements" (Komnick et al. 1973). This term is also applicable to any cellular process that involves an actomyosin system and hence contraction and relaxation involving Ca ions (cell cleavage, morphogenesis, exocytosis and endocytosis, wound healing response)(Komnick et al. 1973, Durham, 1974). It is not possible here to include these other processes and the discussion will be limited to the causal mechanisms of amoeboid movement in the amoebae and slime moulds. Much of the material discussed concerning amoebae will also pertain to a large degree to slime moulds.

There are many species of amoebae with various patterns of amoeboid movement, but all have in common the motive force necessary to drive the cytoplasmic streaming which is ultimately derived from cytoplasmic contractile processes. Before the presently favored contractile theory, others were proposed involving surface tensions, membrane potential differences and "jet propulsions" as possible mechanisms. These theories have now been discarded, leaving the ectoplasmic theory (hydraulic pressure theory) as the one most widely accepted today.

Amoebae (e.g. Amoeba proteus) may extend pseudopodia over a surface by a "ballooning" forward of the cell's membrane due to internal sol-gel transformations (Komnick et al. 1973). The term sol-gel refers to an outer, more or less solid ectoplasm surrounding a more fluid inner endoplasm. Amoeboid movement may involve one or more mechanisms. The "ballooning" mentioned refers to a theory of Durham (1974), in which he proposed that within the ectoplasmic cortex were randomly arranged actin and myosin filaments. The myosin filaments are thought to be free, while the actin filaments are attached at one end to the underside of the plasma membrane. There are many electron microscope studies supporting this view (Pollard, 1973). Analogous to the conditions in muscle, a local decrease in Ca^{++} concentration underneath the membrane and in the area of the filaments would allow a relaxing of the microfilament network. Because of constant pressure (tension) of the membrane by the cytoplasm, there would be a subsequent bulging forward at the relaxed region. Continuation of this process would allow the extention of a pseudopod. Likewise, local increases in $[Ca^{++}]$ would tighten the actinomyosin network preventing any extension forward. A hypothetical model incorporating the above features goes far in explaining the mode of action of phagocytosis and amoeboid movement.

Such a network lying underneath the membrane has been implicated as the source of the contractions of the ectoplasmic cortex. The presently favoured hydraulic pressure theory proposes that such contractions in the rear (uroid) of the amoeba drives the central endoplasm forward when a pseudopod is extended. It appears that as the central endoplasm is pushed forward, the protoplasm at the front is brought to the rear and reverted to ectoplasm, which contracts and exerts pressure on the endoplasm and the cycle is repeated (Komnick et al. 1973).

A second theory, the frontal contraction theory, holds that the pressure is exerted at the front when a pseudopod is extended. Contraction at the front would tend to squeeze the endoplasm forward, so displacing the plasma membrane. After adhesion of the cell surface to the substratum, the cytoplasm near the point of contact would then gel, and subsequent contractions from this site would pull the cell body towards the new site of adhesion. The pattern of birefringence, which reveals fibrilar organization, supports this theory (Allen, 1961).

The first evidence connecting contractile processes with amoeboid movement came when Hoffmann-Berling (1956) demonstrated that glycerinated amoebae contract under the same conditions as glycerinated muscle fibrils. The amoebae were kept in 20% buffered glycerol at 0 °C for two days. They were then washed with a buffer solution to remove the glycerol. The procedure of glycerination increases the permeability of the cell membrane, preserves intracellular organelles but allows the gradual removal of ions and small molecules from the cell. Following this procedure, it was observed by Hoffmann-Berling that when a solution of 10^{-3} M $MgCl_2$ and 10^{-3} M ATP was added, a transient movement of the dead protoplasm occurred lasting about two minutes, but did not occur when cyclic AMP or pyrophosphate was added.

Akin to the procedure of glycerination and giving more support to the actinomyosin nature of amoeboid movement is the use of heavy meromyosin to decorate actin filaments in vivo. When cleaved enzymatically, myosin gives rise to two components, light and heavy meromyosin (HMM). The latter is considered to be the head region, to possess ATPase activity and to be responsible for binding to the actin filaments. Glycerination allows the penetration of HMM through the cell membrane. Using glycerinated amoebae, Pollard (1973) demonstrated that certain thin filaments, in situ as well as isolated from the cell, were able to bind HMM of rabbit skeletal muscle. The "decorated" filaments showed a characteristic arrowhead array indicating the binding of amoeba F-actin with the HMM of skeletal myosin.

Amoebae actin and myosin proteins have been isolated and shown to combine with their skeletal muscle counterparts. Schaefer-Danneel (see Komnick _et al_. 1973) have shown by electron microscopy, that the internal arrangements of thin and thick filaments, when favorably sectioned, showed a "lateral alignment with periodic cross connections." This "ladder" structure is also seen in muscle actomyosin and reflects the interaction between actin and myosin filaments. Schaefer-Danneel concluded that when the similarity in structure was taken into consideration as well as the observation that the length of the filaments did not change during contraction, pointed to a possible sliding mechanism akin to that found in muscle. All the evidence indicates that the underlying mechanisms of amoeboid movement and muscular contraction are fundamentally similar at the molecular level. This view is further supported when comparative studies on slime mould plasmodia are taken into consideration.

On a much larger scale, the cytoplasmic streaming of slime mould (myxomycete) plasmodia seems to resemble the observations made with amoebae. When growing on a petri dish (Fig. 1) a plasmodium of _Physarum polycephalum_ gives the impression of being effectively a single enormous amoeba, with a large volume of cytoplasm (surface area of several cm^2) enclosed in one continuous membrane. It accomplishes its movement and the distribution of cytoplasm by a singular mode of cytoplasmic movement termed "shuttle" streaming by Seifriz (see Kamiya, 1959). The cytoplasm streams rhythmically back and forth through a network of "venous"

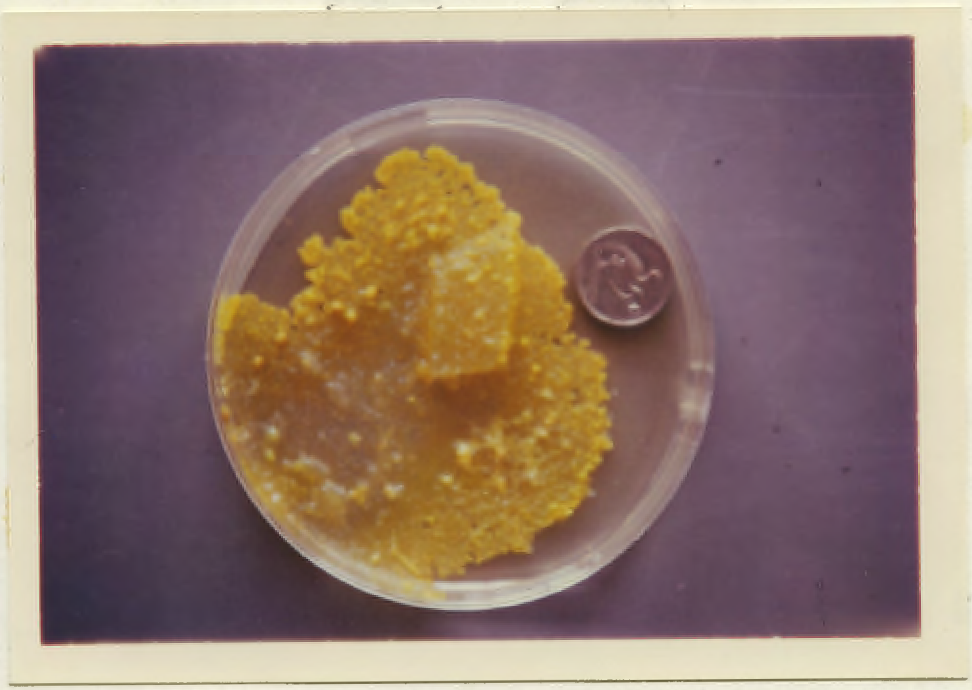


Fig. 1. A plasmodium of Physarum polycephalum grown as an axenic culture.

channels and is believed to be driven by hydrostatic pressure gradients. External influences (chemotactic agents, temperature, adverse conditions) can superimpose a slow purposeful net movement over the whole organism.

As in the case of amoebae, the motive force creating the hydrostatic pressure and thus the cytoplasmic streaming, is produced by contractile proteins organized as filaments (Komnick et al. 1973). These filaments (plasma fibrils) are capable of attaining a sufficient size to become visible under the light microscope and are observed in regions of the plasmodium, where an increased generation of motive force for cytoplasmic streaming must be assumed. Wohlfarth-Bottermann (1964), partly from the following evidence came to the conclusion that the fibrillar network is not permanent but forms (viz. assembles from precursor elements) during or prior to contraction and disappears during relaxation. He observed that if a 1 mm diameter venous channel was cut, the force of the cytoplasmic streaming extruded a drop of protoplasm. Within a minute, a new membrane will have formed and within 10 minutes, the drop will have receded back into the "veins."

Fixation and sectioning of these drops show that the reabsorption is brought about by contraction of an extensive fibrillar network in the peripheral ectoplasm of the drop. The resulting pressure to cause the droplet's protoplasm to recede must be higher than that within the plasmodial vein. Or if a plasmodial strand is suspended in the air, when the suttle streaming causes the endoplasm to flow against the pull of gravity, an extraordinary amount of work is involved. Wohlfarth-Bottermann clearly showed that this work involved the formation of fibrils.

Their ultrastructure by electron microscopy corresponds in a striking way to the actomyosin arrangement seen in vertebrate smooth muscle and striated muscle (Wohlfarth-Bottermann, 1964, Komnick et al. 1973). Combined histochemical and biochemical evidence shows that these filaments have ATPase activity and contain actomyosin. Taken as a whole, the evidence would suggest that the streaming occurs as an all pervasive hydrostatic-contractile process.

2. Physiology of myxomycete plasmodia

a. Life cycle and morphology

There are different facets of the life cycle of the slime moulds that give them unique animal and fungal affinities. Some early mycologists referred to them as mycetozoans (fungal animals), others also believing they had a fungal affinity used the term myxomycetes (slime fungi). Although the production of spores and spore bearing structures (sporangia) might have justified inclusion with the fungi, other features such as their phagotrophic mode of nutrition, type of flagellation, amoeboid movement, meiosis in developing spores and certain important biochemical differences between them and fungi make them look much more like protozoa.

The mycologists Martin and Alexopoulus (see Olive, 1975), after reviewing the available cytological and biochemical evidence, placed them in a special subdivision of fungi called Myxomycotina. The life cycle of slime moulds is usually a simple one and generally has distinct haploid and diploid phases (Fig. 2). Spores germinate to liberate either uninucleate flagellate cells or myxoamoebae. Both types feed by phagocytozing other unicellular organisms and undergo cell division. Syngamy and karogamy occur when two cells of compatible strains fuse to form a diploid zygote. By repeated nuclear divisions, a growing multinucleate plasmodium arises. The plasmodium continues to feed until adverse conditions occur (temperature, humidity, food supply) and it may either form a sclerotium (dormant state) or produce spore-bearing fruiting structures (Fig. 2). Meiosis occurs with spore formation completing the cycle (Alexopoulos, 1962).

The plasmodium is the stage of interest here in the life cycle. The most intensively studied plasmodium is that of <u>Physarum polycephalum</u>,

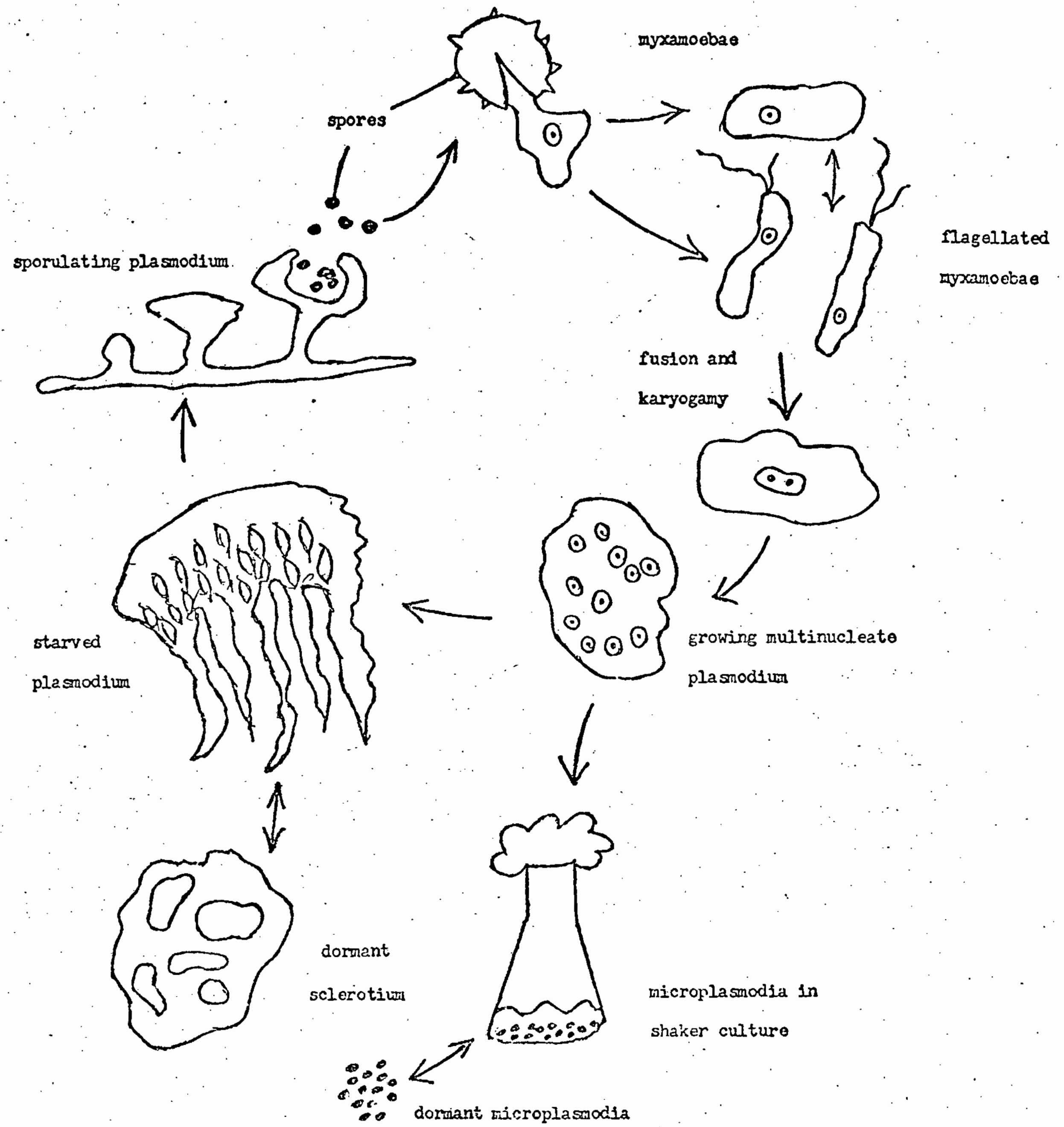


Fig. 2. A diagramatic representation of Physarum's life cycle. (Modified after Alexopoulos, 1962)

which can now be maintained in axenic culture (Daniel and Rusch, 1961). It is commonly observed in nature, growing on rotting logs and decaying organic matter on the forest floor. In the laboratory, grown as an axenic culture, it consists of a bright yellow, flattened, fan-shaped mass with a "venous" network or reticulum, leading a continuous advancing front (Fig. 1). It was given the common name "slime mould" because of an outer layer that can be felt and is termed a slime sheath. This slime sheath corresponds to the glycocalyx of amoebae. McCormick (1970) established that the slime was a sulfate galactose polymer containing traces of rhamnose. Underneath the sheath is the membrane proper of the cell.

Any edge of a plasmodium may become an advancing front or a retreating rear when a chemotactic agent (viz. food source, metabolic poison, etc) is encountered during its migration. The ability to respond is a result of the interdependence between its "venous" network and amoeboid movement. As it lacks individual cells (acellular), it can best be described as being a reticulated network of channels making up the mass of protoplasm spread over a firm substrate. The structural basis of the network consists of two phases of protoplasm that are interchangeable in form and differ in viscosity. As in amoebae, there is a gel-like ectoplasm which constitutes the walls of the network's channels, through which courses a fluid endoplasm (Fig. 3).

In P. polycephalum, the endoplasm flows through one more channels in a certain direction for about a minute, pauses and resumes streaming in the opposite direction (shuttle streaming). The streaming endoplasm contains numerous ill-defined granules, mitochondria, nuclei, ingested matter, etc, which serve as indices for observing the streaming. The "shuttle" streaming can readily be recorded by using a combination

Fig. 3. A 125X photomicrograph of a plasmodium showing the "venous" network which consist of ectoplasm and endoplasm.

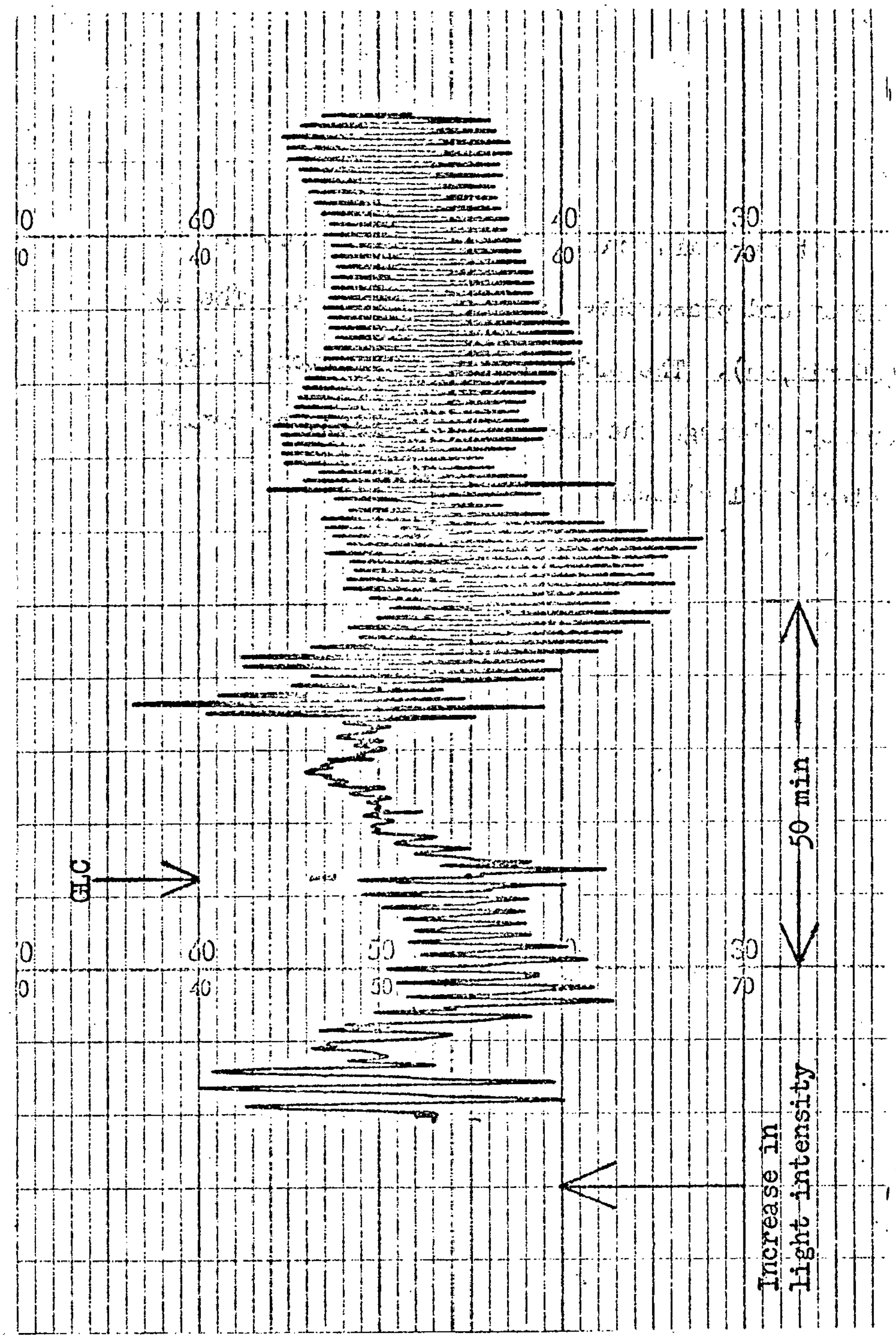
microscope, photocell and chart recorder apparatus that will convert changes in light entensity through the plasmodium into corresponding tracings on the chart recorder (Fig. 4).

The protoplasmic streaming in this organisms shares a certain characteristic with amoeboid movement, viz. that the internal streaming involves changes in the form of the organism and, as a rule, also causes locomotion. Hence it can be placed in the category of amoeboid movement. In addition to contributing to plasmodial migration, the movement of the protoplasm is undoubtedly involved in maintaining an even distribution of oxygen, nutrients and metabolites throughout the plasmodium. Because the streaming occurs in two opposite directions, it is the net difference between the quantities of protoplasm carried in each direction which contributes to its locomotion. In effect the plasmodium advances in that direction towards which a larger amount of protoplasm has been transported (Kamiya, 1959). The rate of streaming is a function of the pressure (motive force) exerted by the channel's walls, the protoplasmic viscosity, and the every-changing diameter of its dichotomously-branching channels.

The question central to the whole study of plasmodial movement is, What factors control the motive force, how is this force exerted and what directs the plasmodium's movement? The most important contributions in this regard have come from the experiments of Kamiya (1959), Ueda _et al_. (1975), Carlisle (1970) and Durham and Ridgway (1976). This section will be concluded with descriptions of the works by Kamiya, Ueda _et al_. and Carlisle. The section, concluding the literature review, will discuss the hypotheses of Durham (1974, 1976) with emphasis on the role of Ca^{++} in the plasmodium's chemotactic response.

b. Kamiya's experimental apparatus for studying cytoplasmic streaming in Physarum

Fig. 4. A chart recording tracing showing the "ebb and flow" streaming oscillations of endoplasm through a major channel. The abscicca indicates the time (10 min/cm). The ordinate, top to bottom, indicates decreasing light intensity, through the area of plasmodium, as monitered by the photocell-electrical circuit.


GLC
50 min
Increase in light intensity
60
40
30
40
60
70
60
50
30
40
50
70

To answer the previous question, Kamiya (1959) was the first to devise a "double chamber" method for measuring the pressure of the motive force. The chamber was divided into two compartments (Fig. 5). In each compartment there was a small mass of plasmodium which was connected to a corresponding portion in the other compartment by a strand of protoplasm breaching an air-tight divider between the two. Flow of protoplasm from one chamber to the next will increase the air pressure in the next chamber and depress the water-filled manometer. If however, there is also a device for exerting additional pressure into the system (rubber bulb and screw, Fig. 5), the experimenter can increase the pressure sufficiently to counterbalance the pressure exerted on the protoplasm and stop its flow.

If rapid successive readings of the counter-pressure are taken from the manometer and plotted against time as the abscissa, undulating curves which represent the changes in the pressure (motive force) are obtained (Fig. 6). Such a curve termed a "dynamoplasmogram" by Kamiya (1959) is helpful as a criterion for judging the physiological reaction of the protoplasm to various experimental situations.

Kamiya proceeded to use his method to test the effect of metabolic inhibitors, chloroform, ether, plant auxins, temperature and nucleotides (ATP and cyclic AMP) on oscillations of the motive force. Later his method was adopted to include simultaneous measurements of changes in the respiratory rate and electrical potential differences between the two protoplasmic masses. The potential differences between the anterior and posterior parts of the plasmodium in the double chamber were measured in conjunction with the streaming pressure (motive force) to establish whether there was a relationship between the two. The differences in the potential (viz. electromotive force) between the two

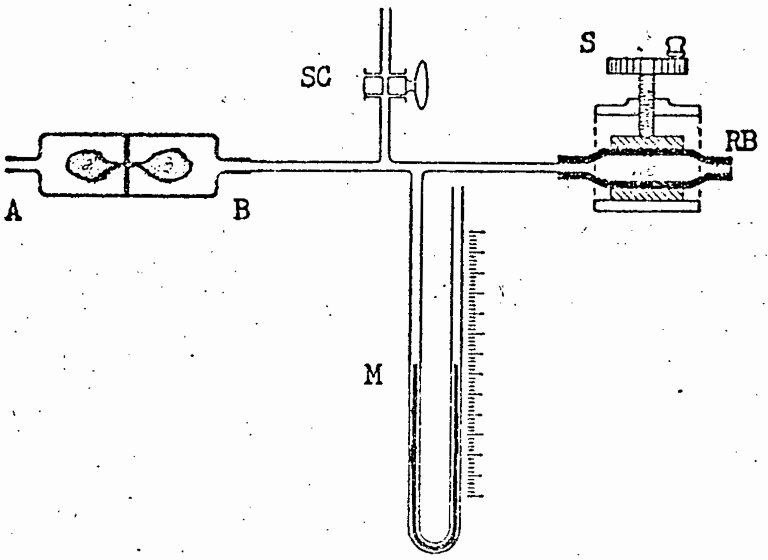
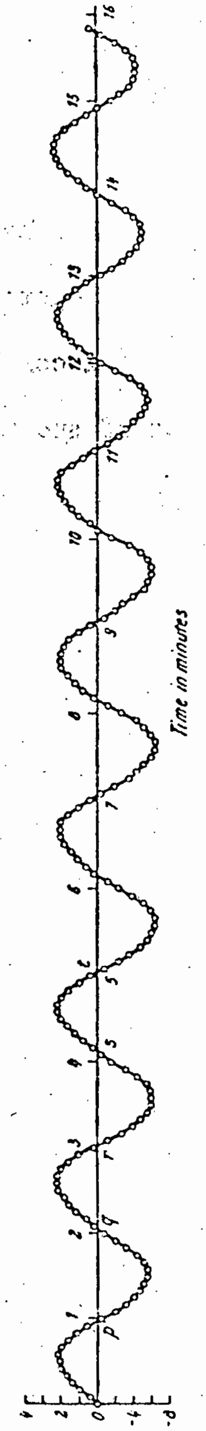
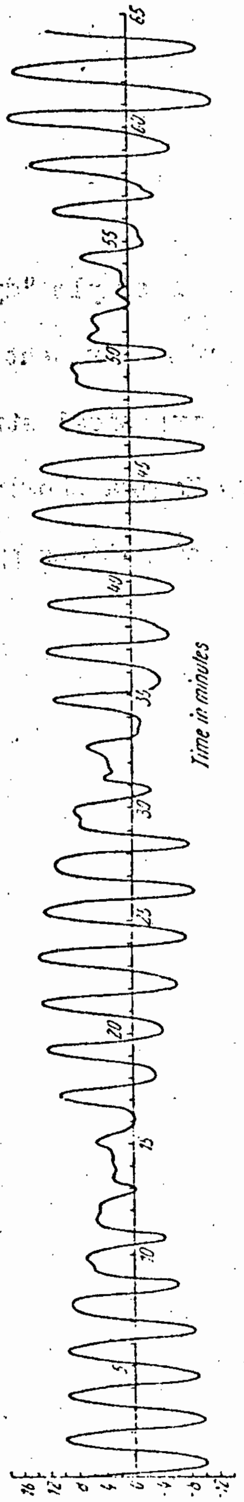


Fig. 5. Diagram showing the general arangement used by Kamiya (1959) for measuring the motive force of protoplasmic streaming in a plasmodium. The whole system consists of a double-chamber having compartments A and B, manometer M, stopcock SC and rubber bulb RB, the volume of which is controlled by screw S. The protoplasmic masses in compartments A and B exchange cytoplasmic streaming through an air-tight divider.

Fig. 6. a. A simple "dynamoplasmogram" recorded by Kamiya (1959). The plotted points represent cm of water moved (manometer) versus time. Note the simple synusoidal streaming pattern. b. A complex "dynamoplasmogram" recorded by Kamiya showing the introduction of spontaneous changes in the streaming oscillations by the plasmodium.



Motive force (cm of water)



Motive force (cm of water)

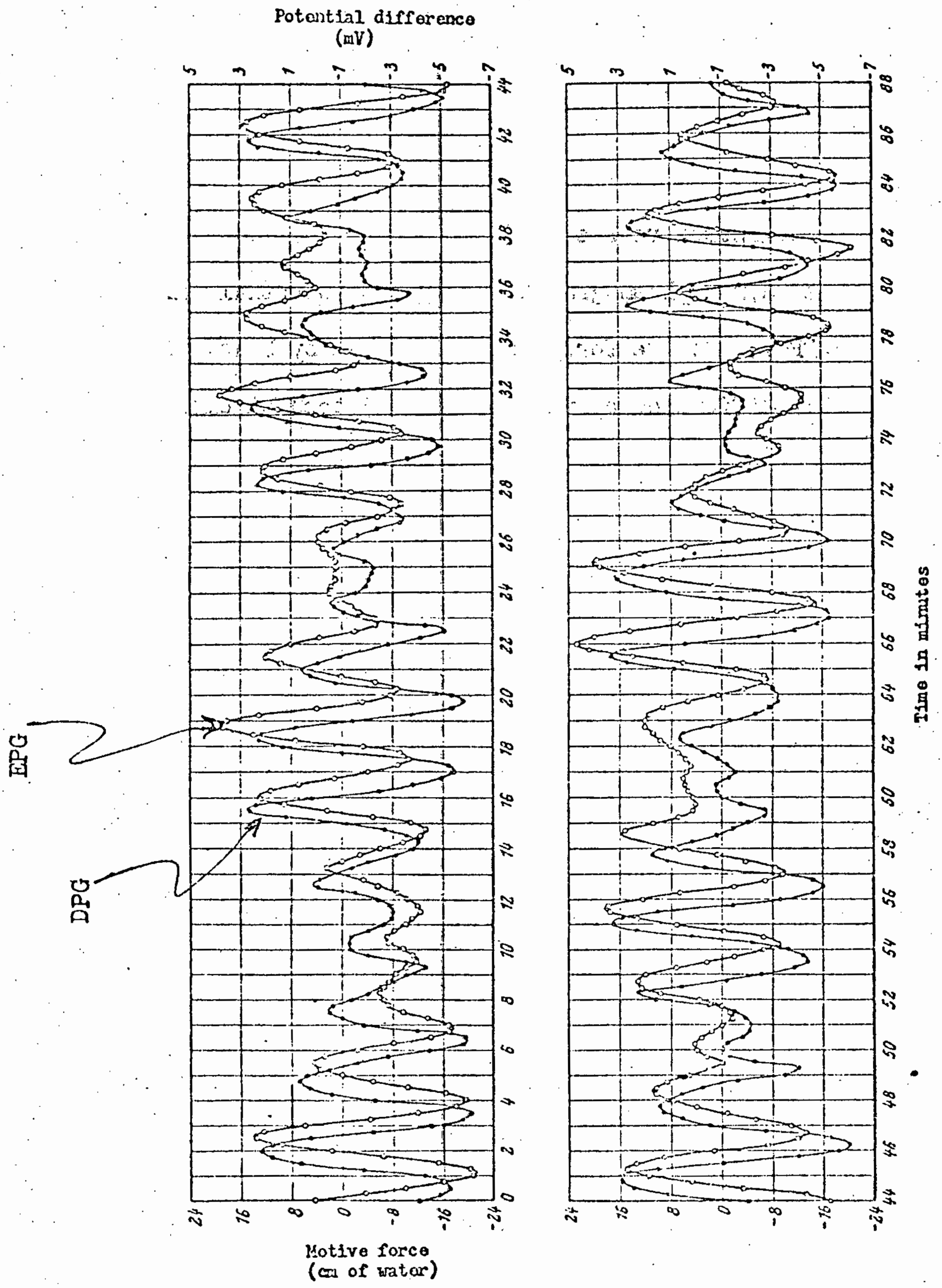
compartments was measured by a potentiometer conducted with a pair of calomel electrodes through salt bridges which were connected with the agar of each compartment. Whereas Kamiya simply read the changes in millivolts from the potentiometer, Ridgway and Durham (1976) used a high-impedance differential voltage amplifier which was linked to a chart recorder. Figure 7 shows two curves expressing the changes in the pressure which cause the streaming and the potential difference in the plasmodium.

Kamiya referred to the potential difference curve as the "electroplasmogram" (EPG) in analogy to the "dynamoplasmogram" (DPG). Figure 7 also shows that under normal conditions, both curves parallel to each other as regards wave form, amplitude and wavelength. However, the curve representing variations in pressure and hence streaming rate (DPG), precedes electrical potential variations (EPG) by up to a quarter of a cycle. As for an explanation of why the two curves do not coincide, Ridgway and Durham (1976) suggested that the EPG is an extracellular record of the voltage difference between the two ends, whereas the recorded motive force measurements, the plasmodium's internal response shows immediately the internal reactions. Therefore, they reasoned maximum electronegativity does not necessarily correspond to peak intracellular depolarization of the contracting end. As will be discussed later, Durham found that Ca^{++} concentrations increase at the end which is depolarizing, becoming relatively more electronegative and contracting.

The data of Kamiya (1959) obtained with the equipment described above, contain several pertinent observations that aid in the understanding of some aspects of the factors controlling the motive force and contractile process.

If the whole plasmodium within the double chamber is treated

Fig. 7. Superimposed representation of the oscillations of the recorded motive force (dynamoplasmogram-DPG) and the potential difference (electroplasmogram-EPG). Motive force curve is shown by black circles and precedes the potential difference curve indicated by open circles (Kamiya, 1959).


Potential difference
(mV)
5
3
1
-1
-3
-5
-7
Motive force
(cm of water)
24
16
8
0
-8
-16
-24
Time in minutes
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
38
40
42
44
46
48
50
52
54
56
58
60
62
64
66
68
70
72
74
76
78
80
82
84
86
88
EPG
DPG

with an air and CO_2 mixture, the streaming stops and gelation of the endoplasm ensues. As a consequence, the pressure (motive force) is reduced to zero and readings can no longer be taken. The phase relationship before and after gelation is conspicuous. It can be clearly shown that the waves that succeed gelation are in a phase which would suggest a "continued periodic activity during the gelated period." Kamiya surmized that the mechanisms controlling the periodic generation of the motive force must have been functioning no matter whether the protoplasm was free or in a congealed state.

In conjunction with the previous observation is another finding, namely that along with induced gelation, the periodic waves of the EPG curve continue despite the fact that there is no motive force. The EPG oscillations continue if the central connecting strand is solidified or if the strand linking the two protoplasmic masses is replaced entirely by a piece of wet cotton thread (Kamiya, 1959). Using the double-chamber volumetric method, if the streaming through the connecting strand is accelerated, or made to flow counter to its normal direction for several oscillations of streaming, the EPG curve continues unaffected. Kamiya concluded that the rhythmic potential variation proceeds independently of the protoplasmic streaming, although there is a high positive correlation between the two curves of the EPG and DPG under normal conditions.

c. <u>Carlisle's petri dish chemotactic assays</u>

Carlisle (1970) was the next worker after Kamiya (1959) to contribute to the investigation into the plasmodium's movement. Previously, researchers were hampered by lack of axenic cultures and chemically defined growth media. Measurements of the degree of chemotactic response (viz. to attractants and repellents) at a given concentration took days of patient observation and was dependent on subjective judgment. Even then, the lowest concentration exerting a chemotactic effect remained vague.

The experimentation for determining the degree of chemotactic response was quantitated by the use of axenically grown plasmodia and Carlisle's method of assaying migration. The results were assessed by means of statistical analysis. The tests on the chemotactic effectiveness of sugars were carried out on petri plates of sugar-free standard agar medium from which two wells and a central trough had been excised (Fig. 8). Distilled water was placed in one well and an equal volume of sugar solution in the other and the dish was left for 1 to 4 days for the sugar to diffuse through the agar to the edge of the trough. An excised square of starved plasmodium was placed in the centre of the trough so that it touched both halves of agar in the petri plate.

The extent of migration was observed daily, or more frequently if necessary. An individual test was recorded as positive if the plasmodium had reached the well containing the test solution but had not reached the control well, and negative if the control well was reached first. If the plasmodium reached both wells, or neither well, that particular test was considered unsuccessful and not included in the total count. If out of ten runs and totally random migration occurred, then a score of five positive results would be expected whereas using a strictly positive or negative chemotactic substance would yield ten or zero positive results respectively. The binomial distribution was used to determine the significance of a series of petri dish tests. Carlisle found that 1% solutions of glucose, galactose, mannose, maltose and peptone produced positive chemotaxis while sucrose, fructose or ribose did not. This paralleled their ability to support growth.

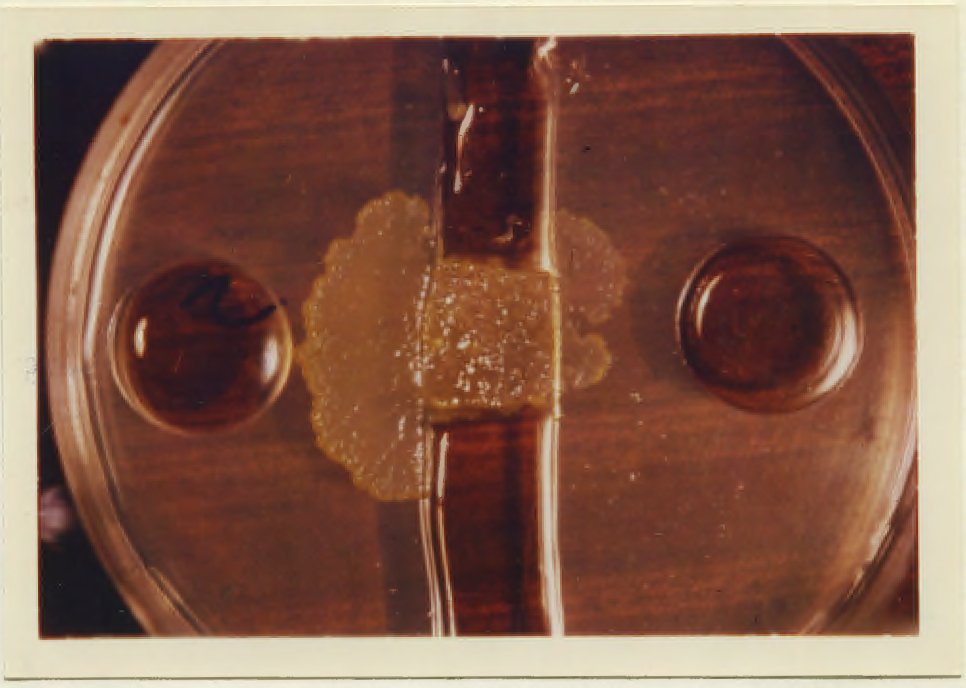


Fig. 8. A repeat of Carlisle's petri dish chemotactic assays. The left well has 1% glucose, the opposite distilled water and the test is 3 hours old.

d. Ueda's experiments to determine the threshold concentrations for ionic and chemotactic chemicals

Five years after Carlisle's paper, Ueda et al. (1975) reported a series of experiments showing the precise "threshold" concentration for recognition to certain chemotactic chemicals. Modification of Kamiya's double-chamber technique allowed Ueda and his coworkers to define the "tactic motive force" as the difference in pressure of streaming when the plasmodium is actually moving from one compartment to another during a chemotactic response to a chemical. This difference in pressure was determined by finding the base line of the DPG oscillations for a control and when a test solution of the chemical was added to a chamber (Fig. 9). Since the plasmodium migrates to the chamber containing an attr actant, the baseline of the DPG oscillations of the motive force (pressure) tracing would rise. A repellent would cause migration away and the base line would fall. The rise or fall of the DPG baseline was determined as the difference between the baseline of the control and that obtained under the influence of various chemicals was given in cm H_2O read from the water filled manometer and was defined as positive for attractants.

Various chemotactic agents and ions were added to one compartment (Fig. 9) via the tubing, at increasing concentrations, and it was observed that for a particular sugar, ion, nucleotide, etc, there was a threshold concentration at which a change in both the electrical potential and the motive force occurred. Glucose and galactose at a concentration of 10^{-4} M were attractants and produced a change in both parameters. The chloride salts of the ions tests: K^+, Na^+, NH^+, Ca^{++}, Mg^{++}, La^{3+}, acted as repellents.

Ueda et al. (1975) also discovered that there was some dependence of the cations' threshold concentrations (C_{th}) on the ion's valen-

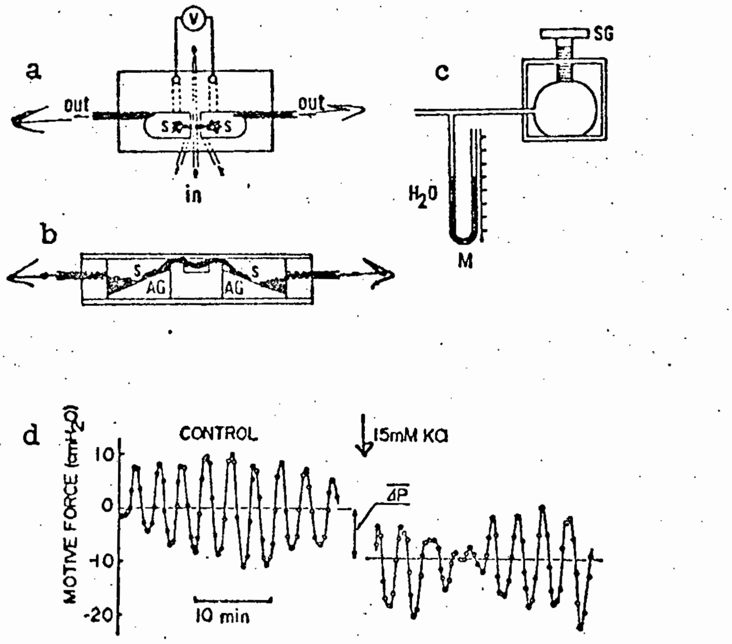


Fig. 9. Schematic diagram illustrating the general arrangement used by Ueda et al. (1975) for measuring membrane potential and motive force of protoplasmic streaming. a. Shows the double chamber used with enclosed slime moulds. V is the potentiometer and the solutions are exchanged by tubing. b. Sideview of the double chamber where AG signify the supporting agar gels. c. Water-filled manometer (M) and rubber bulb-screw (SC) assembly for supplying pressure to the chambers. d. Dynamoplasmogram: solution in one compartment was exchanged from water to 15 mM KCl at the arrow indicated in the figure. Motive force of taxis of the plasmodium is the difference between the baselines of the oscillations, as depicted in the figure.

cies (Z). When plotted as log Z versus log C_{th}, a linear relationship was found and this held true for all the cations studied. From that discovery, the authors concluded that the ionic effect followed a well-known law of colloid chemistry (Shultz-Hardy rule) and they suggested that "the effect of valencies of cations on the electrical phenomena at the plasmodium's membrane plays an indispensable rôle in the reception and discrimination of chemical substances."

C. Summary and thesis of Ca^{++} involvement in Physarum amoeboid movement

Durham, in a 1974 review, presented a comprehensive theory for nonmuscle movement of certain cells that gave several functions to the Ca ion. He focused particularly upon the control of movement by calcium ion fluxes across membranes as influenced by chemical and electrical processes at those membranes. He envisaged that waves of alternating net Ca^{++} entry and efflux can move along the external membranes of cells and that this was the controlling element in directing amoeboid movement. No experimental evidence has shown the existence of such waves in any cells that exhibit amoeboid movement. However, Durham presented indirect evidence from a wide range of cell types to support his theory.

An obvious parallel to the proposed role of Ca^{++} is the Na^{+} action potential that is propagated along nerve axons and leads to a sequence of opening and closing of specific ion channels and a change in membrane potential. Independently, Baker and Blaustein (see Durham, 1974) have shown that there is Ca^{++} pumping in the squid axon is voltage sensitive and that Ca^{++} entry continues at the normal resting potential of the cell at a rate that is sensitive to small fluctuations in the membrane potential.

Whereas the Na^{+} action potential predominates in importance over Ca^{++} entry for nerve axons, Durham (1974) cites evidence showing Ca^{+} action potentials for Ca^{++} crossing the external membrane and consequent stimulation in the case of the muscle cells for guinea pig taenia coli, barnacles, and snail and frog heart. According to Durham, the "mostly Ca^{++} action currents" (sic) differ from the more studied Na^{+} action currents by: (1) a more gradual rise and fall, (2) the amplitude is not dependent of stimulus intensity and (3), there is a lower resting potential of the membrane. External Ca^{++} is further implicated in controlling movement by the fact that Ca^{++} entry alters the membrane potential and thereby controls the movement and direction of the beating in the case of Paramecia cilia (Eckert, 1972).

Evidence from nerve action potentials and other cellular studies led Durham (1974) to proppse that a Ca^{++} current (wave) controls amoeboid movement in the amoeba and the much larger plasmodia of slime moulds. As Kamiya demonstrated (see Fig. 7), electrical potential differences between two points of the plasmodia fluctuate at the same frequency as the pressure differences (viz. motive force) with a similar waveform but different phase. The visible waves of luminescence that pass over the surface of large marine jellyfish (viz. Ca^{++}-aequorin interaction) may represent the propagation of waves of Ca^{++} entry into these organisms. Similar oscillations of the internal $[Ca^{++}]$ have been shown to exist in the cytoplasm of plasmodia by Ridgway and Durham (1976). They used a complex apparatus to measure the potential differences (viz. EPG) as explained before, and also simultaneously measured the light emitted by aequorin injected into the plasmodium's "venous" network.

It is known that aequorin combines specifically with Ca^{++} and emits light (Ridgway and Ashley, 1967). Ridgway and Durham found that the curves for the light emission closely followed the curves of the EPG recording. Also, as implied by the aequorin light emission, maximum ionized calcium (Ca^{++}) concentrations preceeded maximum external electrode electronegativity by a small fraction of a cycle. As Tasaki and Kamiya (1950) observed, Ridgway and Durham also found that a gentle mechanical shock (light tap) to the housing apparatus induced a sharp peak in the EPG but also induced a peak in the light emission tracing of aequorin. The close parallel of the EPG with the internal

[Ca^{++}] suggested to Durham a possible causal relationship between the membrane potential and the control of the free [Ca^{++}] in the cytoplasm.

Because an EPG of a plasmodium is an extracellular record, Durham (1974) suggested that the electrical waves (sic) may represent a manifestation of ion fluxes across the external membrane. If experimentally confirmed as being waves of Ca^{++} entry and efflux along the plasmodium's surface, it would satisfy two necessary requirements: (1) tangential movement along the membrane and (2) oscillating character. These two characteristics are prerequisites for Durham's theory, if such waves are to direct and control the plasmodium's movement. This aspect will be discussed in more detail later.

Intracellular Ca^{++} has been firmly established as the regulating factor for the actin and myosin proteins in amoeboid movement as well as in organized muscle fibres (Komnick <u>et al</u>. 1973, Durham, 1974). Wohlfarth-Bottermann (1964) has demonstrated the existence of contractile fibrils in the cortical or ectoplasmic layer of plasmodia. With sequential fixation and electron microscopy he showed the appearance and organizationof contractile fibrils at the periphery of a recently extruded droplet of cytoplasm. Wohlfarth-Bottermann also presented experimental evidence showing that it was the cortical contractile fibrils which exerted the pressure to force the cytoplasm back into the severed "vein." The existence of such a cortical contractile network is an important adjunct to the proposals of Durham (1974) and Durham and Ridgway (1976) for explaining the chemotactic action of attractants and repellents.

Durham and Ridgway (1976) found that chemical attractants and warmth generally brought about a higher frequency of streaming while repellents and lower temperature caused a lower rate. The plasmodium can be visualized as an efficient food-seeking organism, always migrating (creeping) over the substrate in a random fashion. The question can be asked, "What mechanism enables the plasmodium to bring its bulk around and move in the direction of a newly encountered food source (viz. attractant)?" Durham and Ridgway reasoned that if an extended part (i.e. a pseudopod for amoebae) of a plasmodium encountered an attractant, that particular area would develop a faster rate of streaming, become the front and entrain the rest of the plasmodium to follow. A repellent would cause a reverse action and a retreat.

With that simple concept in mind, one may think of an amoeba or much larger plasmodium as being similar to a flattened liquid droplet with a certain surface tension. Underlying the surface membrane is a hypothetical actinomyosin network with the ends of the actin filaments attached to the membrane and the myosin filaments randomly associated with them. The "droplet" study by Wohlfarth-Bottermann (1964) and numerous electron microscope studies (Pollard and Korn, 1971) tend to support but do not conclusively prove Durham's premise of such a network. The proposed network of filaments imparts a surface tension keeping the interior under slight pressure. Increases or decreases in the free $[Ca^{++}]$ would cause the actinomyosin network respectively to contract into a tight meshwork or to relax. Durham made a further assumption that attractants acted by causing a decrease in the internal free $[Ca^{++}]$, thereby relaxing the network and allowing the membrane to "bulge" forward from internal pressure.

Thus for the local region involved there will be a "bulging" forward of the plasmodium after encountering the attractant and a resulting faster frequency in streaming. This region of faster frequency will entrain the rest of the plasmodium which has a relatively slower frequency, to move in that direction. Kamiya's observation can now be included, namely that a net flow of cytoplasm in one direction can occur as the resultant of repeated cycles of streaming. Repellents would cause a contraction of the underlying actinomyosin network (viz. by reduced internal $[Ca^{++}]$), tighten the membrane, produce a slower frequency of streaming and be entrained by the "faster" regions of the plasmodium and thus be retracted.

The simplest model for chemotaxis would suggest that attractants increase the rate of pumping of calcium ions outwards across a cell's external membrane. The withdrawal would lower the cytoplasmic $[Ca^{++}]$ in the region of Durham's hypothetical membrane-actinomyosin network and begin the sequence of events described. Thus the free $[Ca^{++}]$ in the cytoplasm has two functions: (1) to regulate the hypothetical membrane-actinomyosin network and chemotactic acceptance or rejection and (2) to regulate the contractions of the contractile proteins responsible for the hydraulic pressure (motive force) generation.

A third possible function of the Ca ion may be to aid membrane adhesion to other cells and to the underlying substrate. Durham (1974) proposed that the internal oscillations in the $[Ca^{++}]$, may also be exhibited on the surface as was suggested by the external EPG measurements which were taken to mean ion fluxes. Taken with the fact that cells in tissue cultures require Ca^{++} in the medium for proper adhesion to their culture bottles (Manery, 1966), such fluxes of Ca^{++} from the plasmodial membrane would serve the function of repeated cycles of relaxation and adhesion of the membrane to the substrate.

Assuming the existence of waves of alternating net Ca^{++} entry and efflux moving along the membrane, there would also be an accompanying wave of contraction and relaxation travelling in the peripheral actinomyosin network near the surface of a cell. Durham (1974) in his hypothesis proceeded to explain that any one place on the cell surface would tend to experience an expansion and relaxation of the local surface area alternating in time. When such waves of contraction and relaxation operate in combination with cycles of adhesion, the bottom surface will tend to move sidewise relative to the underlying substrate. Whether the cell is an amoeba or a particular region of a plasmodium and whether it will move in the same direction as the travelling waves, or the opposite direction, will depend upon the exact phase relationship between the adhesion wave and the contraction wave. Durham cites the fact that many multicellular organisms use oscillations (ripples) of their undersurface for locomotion, such as slugs, earthworms, caterpillars and "slugs" of the cellular slime mould Dictyostelium discoideum.

It would be of interest and it is the object of this thesis to verify the three proposed functions of Ca^{++} discussed above. The first objective was to investigate whether the internal oscillations in $[Ca^{++}]$ for Physarum plasmodia propagate themselves to the surface and whether chemotactic attractants produce an increase in Ca^{++} efflux from the surface membrane as predicted by Durham's hypothesis. The experiments described in the following chapters were designed to test the above hypothesis and more specifically to determine whether a chemotactic response produces any significant Ca^{++} efflux from the slime mould into an aqueous environment, of what magnitude these are and whether there is a causal link between the changes in Ca^{++} efflux and the motile oscillations. Secondly, slime mould mitochondria were isolated and their functional integrity was tested by respiration measurements, similar to those used with standard rat liver mitochondria preparations. This was followed by an investigation of the in vivo energy-linked Ca^{++} accumulating ability so as to give an indication of whether plasmodial mitochondria could participate in regulating the cytoplasmic $[Ca^{++}]$.

CHAPTER THREE

MATERIALS AND METHODS

1. Axenic culture of *Physarum polycephalum*

Howard in 1931 (see Alexopoulos, 1962) reported a number of different food substrates for the culturing of the plasmodial phase of the myxomycete *Physarum polycephalum*. The plasmodia were first cultured on flesh agarics (*Agaricus sapidus*) and later on pure cultures of fungi growing on nutrient agar. Ettienne (1972) used an oat-meal agar medium when a well-defined medium was not needed and Cummins and Rusch (1968) used half-strength corn meal agar supplemented with a food bacterium, *Aerobacter aerogenes*.

The lack of pure cultures and defined media often hampered the assessment of chemotactic response and biochemical studies. As a result, the pure culture of the plasmodia of *P. polycephalum* on a semi-defined medium developed by Daniel and Rusch (1961) has been a boon to reseachers working with this organism. The complete growth medium for maximal growth is given below:

Component	Amount (g/l medium)	Substitutions
Tryptone (Difco)	9,0	Peptone (Oxoid) 10,0 g/l
Glucose, anhydrous	9,0	
Yeast extract	1,4	
Citric acid . H_2O	3,6	
K_2HPO_4	1,8	
$CaCl_2 \cdot H_2O$	0,54	
$MgSO_4 \cdot H_2O$	0,54	
$MnCl_2 \cdot 4H_2O$	0,076	
$FeSO_4 \cdot 7H_2O$	0,085	$FeCl_2 \cdot 4H_2O$- 0,06 g/l (N.B. All iron must be ferrous)
$ZnSO_4 \cdot 7H_2O$	0,03	
Hemin (Added separately- see below)		Hemoproteins and chick embryo extract

In three papers (Daniel and Rusch, 1961, Daniel *et al.* 1963, Daniel and Baldwin, 1964), Daniel gave details on the culture of Physarum for both semi-defined and fully defined media. With the semi-defined medium given above, it was found that for convenience, the metal salts (bracketed above) could be made up as a concentrated stock, usually 40X in a 0,5 liter volumetric flask. It was necessary to dissolve the citric acid beforehand to avoid the formation of insoluble precipitates. The medium was usually made up in liter quantities and adjusted to pH 4,6 with KOH pellets. To limit deterioration of the nutrients, the flasks were strictly autoclaved for 10 minutes at 15 lbs pressure.

Daniel *et al.* (1963) also discovered a unique requirement for an iron protoporphyrin (IX). This is supplied by making up a 0,05% hemin solution in 1,0% NaOH which is autoclaved separately from the nutrient media. The hemin is then kept refrigerated and transferred at the concentration of 1% v/v either to the shaker flasks before use or to the cooled agar medium prior to pouring into petri dishes. The hemin can be replaced by other hemoproteins or chick embryo extract. As a rule, for optimum aeration and fragmentation, the shaker flasks were kept one-fifth full (i.e. 50 ml and 200-300 ml for 250 and 2000 ml flasks respectively).

P. polycephalum, strain M3cV (obtained from A.C.H. Durham), was initially in the dormant sclerotium stage on sterile filter paper. Using aseptic technique, the conspicuous orange patches of the scerotium were cut from the paper in an elongated triangular shape. These were inserted into 250 ml shaker flasks, lying sideways with 50 ml medium, so that only the tip of the triangle touched the edge of the liquid medium. The paper acted as a wick drawing moisture to the sclerotium and, once revived, a slowly spreading plasmodium was observed within 48 hours.

Once the plasmodium had spread out on the surface in a thin "lace" pattern and reached approximately 2-3 cm^2 in size, the flask was turned upright. To obtain microplasmodia, the flask was shaken sufficiently to maintain aeration, to break up the initial mass into individual microplasmodia (m.p.) but not to the extent of producing froth (viz. 150 rpm setting- G10 Gyratory Shaker, New Brunswick Scientific Company, New Brunswick, New Jersey, U.S.A.). A two ml inoculum transferred to a 250 ml flask containing 50 ml reached a peak in growth in three days and was then transferred again. Alternatively, the whole flask may be flamed and the contents tipped into a 2 liter flask. Some flasks should be set aside in the refrigerator as insurance against future contamination. It can be estimated that 2-4% of the m.p. develop into dormant microsclerocia which can be revived later. For plasmodia on solid agar media, 2 ml of packed (settled) m.p. can be pipetted onto a plate and these will fuse within 12 hours and develop nuclear synchrony after about two mitotic divisions (Cummins and Rusch, 1968). Nuclear synchrony refers to a characteristic property of the multinucleate plasmodia, namely that all the nuclei undergo intranuclear division with the subsequent nuclear phases in strict synchrony.

Two autoclaved pipette cannisters for 1 and 5 ml pipettes were kept for adding hemin and transferring cultures. To avoid much needless time and effort removing bacterial or fungal contaminants, strict observance of aseptic technique is necessary when transferring cultures. Developing contaminants can usually be seen on a plate. If such is the case, it is a simple matter to either excise the contaminant or if it is overwhelming, to excise a 2-3 cm^2 piece of plasmodium free of the contaminant and transfer it to a new plate. If the contaminant is anintegral part of the plasmodium, then the plasmodium's speed of migration can be used to advantage. An excised section is transferred to a blank agar plate and in a starved state, it may migrate up to 4 cm in 6 hours; after repeated transfers this usually leaves the contaminant behind.

For shaker cultures, absence of contaminants is indicated by a clear medium and no ring around the flask (viz. surface-air interface) beyond that caused by the hemin. An indistinct cloudiness indicates bacterial or yeast contaminants, while mycelial fungi usually roll into small, gray spheres- "tennis balls." Such contaminants can never successfully be removed from a culture and therefore a new line has to be started from a refrigerated flask or an agar plate.

2. Technique of the flow-through chamber

Kamiya (1959) was the first to devise an apparatus in which part of a plasmodium was exposed to different solutions. The degree of chemotactic response to these solutions was determined by making manometric determinations and simultaneous measurements of the potential differences between the opposing ends of the plasmodium. Using Kamiya's basic idea of a chamber that could be perfused with a solution, equipment was designed to collect the ^{45}Ca efflux carried off by a continuous perfusion that initially flowed over a large surface area (several cm^2) of plasmodium. The surface area varied from run to run and this factor affected the level of ^{45}Ca efflux with each run. To assess the organism's physiological response, the motile streaming oscillations were optically recorded at the same time as the collection of the perfusing solution. For these purposes, the best form of plasmodium for our needs had to be considered, viz. how best to incorporate the ^{45}Ca isotope and design the transparent flow-through chamber. For the optical recording, the accompanying optical-electronic circuitry had to be devised.

P. polycephalum was maintained as microplasmodia in an axenic shaker culture. For the best optical recording the plasmodium had to be orientated in the length of the chamber (Fig. 10). Also it had to be in a starved condition and actively migrating. To meet these conditions, two methods were devised. Both began with the ^{45}Ca incorporation by a calcium-deprived plasmodium on a radioactive plate. To increase the incorporation, the microplasmodia were previously transferred to a flask containing the usual medium but lacking $CaCl_2$. After two days of logarithmic growth, in which their calcium reserves were depleted, 2 ml of settled microplasmodia were pipetted onto a thinly poured petri dish containing 1 nCi/ml of $^{45}CaCl_2$ (Radiochemical Centre, Amersham) in an other-

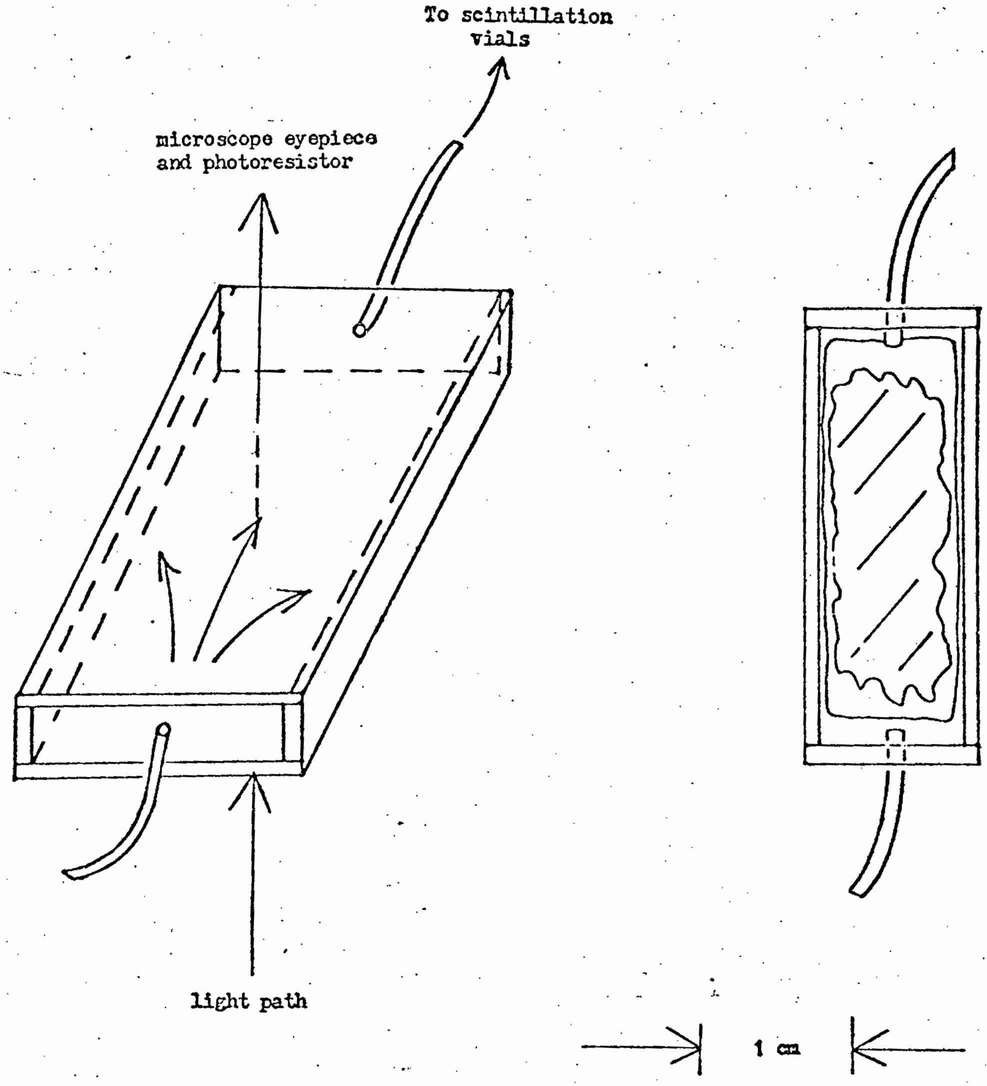


Fig. 10. A diagram showing two views of the flow-through chamber.

wise calcium-free nutrient agar medium.

These microplasmodia fused into one plasmodium and covered the radioactive plate. Characteristically, when they had exhausted the available nutrients, the migrating plasmodia formed large, lemon-yellow accumulations preparatory to sporulation. When the first of the two methods for orientating the plasmodia in the chamber was followed, these accumulations were transferred to a freshly poured 2% agar plate with 1 mM NaCl and 1 mM $CaCl_2$. They then reverted back to a plasmodial form and proceeded to migrate around the plate. Approximately 60% of those put down, migrated and orientated properly, viz. with a migrating front, behind which were major "veins" and a trailing posterior. These plasmodia were then cut out with enough supporting agar to fit the chamber. This method has its disadvantages because: (1) the time can be extremely variable (4-48 hours) as to when the plasmodium becomes orientated properly, (2) approximately 40% never become orientated properly and (3) after 24 hours, the plasmodium ages and has a dried appearance.

An alternative method was developed to avoid these disadvantages. Rather than have the plasmodia migrate haphazardly on a plate, a method was developed so that they would migrate within a chamber and cover it to the greatest possible extent. Using the blunt end of a spatula, Parafilm was molded around the inside of the chamber leaving enough Parafilm to project 1-2 cm outside. The bottom of the chamber was layered with 2% agar gel containing 1 mM NaCl and 1 mM $CaCl_2$. The plasmodial accumulations from the radioactive plate were then transferred to the chamber and their migration confined by the hydrophobic nature of the Parafilm. Several chambers can thus be made and incubated at 25 °C in a moist atmosphere while the accumulations migrate outwards over the agar, covering an area of several cm^2. Usually within 3-4 hours, the plasmodium develops "veins." The Parafilm can then be trimmed away and the chamber covered with a glass microscope slide, sealed with Vaseline, and placed on the objective stage of a binocular microscope.

Several chambers, 5cm X 2cm X 1cm in dimensions, were constructed with strips of plexiglass and epoxy glue. Two holes were drilled at the opposite ends and the tubes attached with epoxy. The tubing ran from the solution reservoir above the chamber and the efflux was carried by gravity to a fraction collector and a waste container below the chamber. Red light (760 filter) passed through the molded Parafilm, the supporting agar and finally through the small area of the plasmodium to a photoresistor set in one eyepiece (Fig. 10). A regular, sinusoidal optical tracing was produced when either a major "vein" or the periphery of the plasmodium was in the eyepiece's field of vision. Conversely, when there were many smaller veins, representing a complex combination of overlapping areas of contraction, a ragged chart recorder tracing resulted.

Changes in the organism's surface area or thickness related to its motile streaming oscillations influenced the light beam transmitted. The photoresistor transferred these fluctuations in light intensity, as electrical impulses, to an amplifier whose output was displayed on a chart recorder (REC 61 Servograph, Radiometer, Copenhagen). The electronic circuitry was constructed as shown in Fig. 11.

Simultaneously with the optical recording, a neutral solution of 1 mM NaCl, 1 mM $CaCl_2$ was made to flow through the chamber at a rate of fifteen drops per fraction collector vial or four vials per minute. These vials, containing ^{45}Ca leeched from the plasmodium were filled with 4 ml of a scintillation cocktail (Insta-gel, Packard Instruments), and their radioactive counts per minute (CPM) were measured using the ^{14}C preset energy channel of a Packard Tri-Carb Model 3385 scintillation counter.

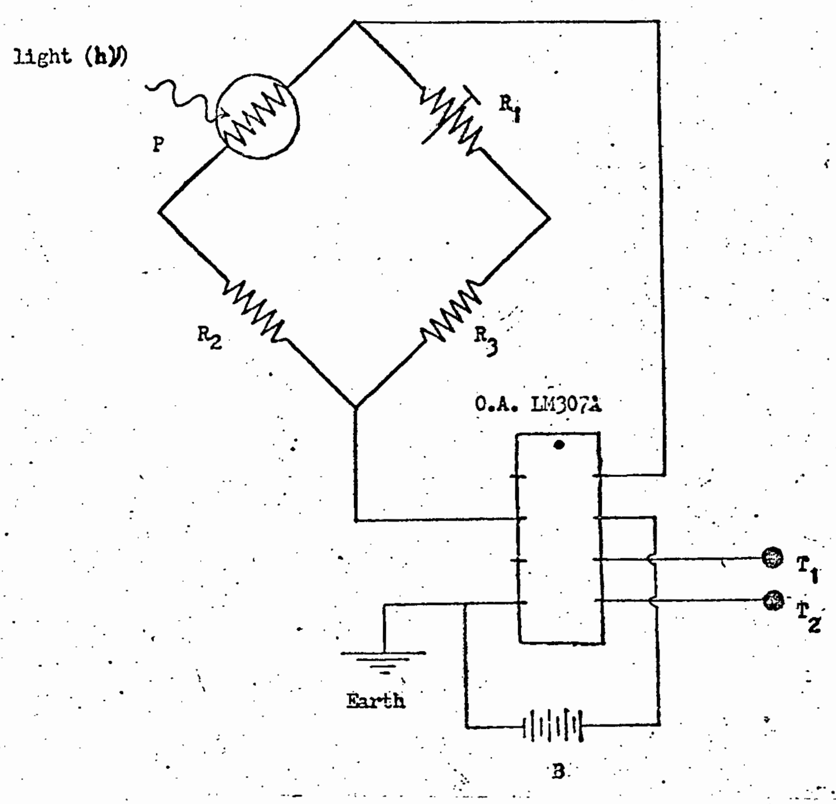


Fig. 11. A diagram of the electronic circuit devised for optically recording the motile oscillations in Physarum plasmodia. B, 9,0 V battery; P, photocell set in microscope eyepiece; R_1, 200 kohm preset resistor; R_{2-3}, 100 kohm resistors; T_{1-2}, terminals to carry output to chart recorder; O.A. LM307A, miniaturized operational amplifier.

A typical experiment consisted of preparing a chamber and allowing the neutral solution to flow over the plasmodium for 30 minutes. It was previously determined that the plasmodium suffered no ill effects by being submerged and would continue a streaming pattern for up to 6-8 hours. Thereafter, the streaming would become progressively weaker and finally stop. After the first 30 minutes, a regular streaming pattern ensued from which it was relatively easy to judge the organism's response to different solutions applied. Before changing solutions, the position was noted of several vials coinciding with the optical recording on the chart recorder. Thereafter, the CPM of each vial could be superimposed on the optical recordings.

3. Preparation of Ca^{++}-EGTA buffers for half plate assays

The Ca^{++}-EGTA buffer used in the following half plate assays were made up according to the calculations of Portzehl et al. (1964). The calculations and the preparation of media were done as follows.

EGTA (ethylene glycol bis (β-aminoethylether)-N, N'-tetra-acetate) from Sigma Chemicals, is specific for the binding and chelation of ionized calcium. The EGTA ligand (L) has four ionizable protons and each disassociates at a particular pH. Only the forms of HL^{3-} and L^{4-} are effective in the chelation of Ca^{++} and the association equations for each are given below.

$$(1) \qquad Ca^{++} + L^{4-} \rightleftarrows Ca\ L_{Tot} \qquad K_{Ca\ L} = 10^{6,88} = \frac{[Ca\ L\ Tot]}{[Ca^{++}][L^{4-}Tot]}$$

$$(2) \qquad Ca^{++} + HL^{3-} \rightleftarrows Ca\ HL_{Tot} \qquad K'_{Ca\ HL} = 10^{3,88} = \frac{[Ca\ HL_{Tot}]}{[Ca^{++}][HL^{3-}]}$$

As is seen, the protonated form (HL^{3-}) has a much lower association constant and as a consequence, that reaction can be deleted from further calculations. The constants given are for a pH of 7,1 (Portzehl et al. 1964). The bound Ca^{++} is effectively equal to the total $[Ca^{++}]$ as there is very little free Ca^{++} in comparison (equation 3).

$$(3) \qquad [Ca\ L_{Tot}] = [Ca^{++}{}_{Tot}] - [Ca^{++}{}_{free}]$$

Thus, substituting and rearranging the first equation gives the equation below for finding the molar concentration of EGTA needed in making the buffer, once the total $[Ca^{++}]$ and the desired pCa value are known.

$$(4) \qquad [L^{4-}_{Tot}] = \frac{[Ca^{++}{}_{Tot}]}{[K'_{Ca\ L}][Ca^{++}{}_{free}]}$$

One-tenth volume of 0,1 M Ca^{++}-EGTA buffer was added to the agar medium. A range of pCa values from 4 to 7 (viz. $[Ca^{++}_{free}]$, 10^{-4}, 10^{-5} M, etc) were obtained by using the molar concentrations of EGTA indicated in the table below:

Table 1 . The concentrations of Ca^{++} and EGTA required to achieve the indicated pCa value.

pCa	Ca_{Tot}	$EGTA_{Tot}$
4	0,1 M	0,13 mM
5	"	0,0013 M
6	"	0,013 M
7	"	0,131 M

The pCa values were maintained by adding 10% v/v of the Ca^{++}-EGTA buffer to all agar media. The pH was adjusted to 7,0 by the addition of 5 mM Tris and the medium was gelled with 1,5% v/v of Ionagar (Oxoid, Code L12 purity). The half plate assay method of Carlisle (1970) as modified by Durham (1976) was used here. Plastic disposable petri dishes were employed. The bottom plate was divided into two equal halves and the pCa value of the half containing glucose was indicated with a marker pen. The medium was autoclaved with a magnetic stirrer added until the agar was melted. Sterilization was not necessary, since the plates were used immediately and the results of the plasmodia's migration were usually known within 6 hours.

Once melted, the medium for a particular pCa was mixed thoroughly with the magnetic stirrer and poured into the dishes, allowed to gel and one half scooped out with a spatula following the drawn demarcation line. The medium of a differing composition (viz. pCa, $[Mg^{++}]$, added glucose) was then poured in and carefully brought up to the same height as that of the first half. To avoid diffusion of Ca ions across the interface (viz. blurring the sharp demarcation line of the two halves), the plates were made just prior to putting down the plasmodial accumulations on the interface line.

4. Isolation of mitochondria from rat liver and slime mould microplasmodia

This section is devoted to descriptions of methods for the isolation of two dissimilar types of mitochondria from rat liver and slime mould microplasmodia. The integrity of the isolated mitochondria (i.e. degree of respiratory control) was assessed by the polarographic "oxygen electrode" technique.

The mitochondrial protein was determined by the biuret method (Koch and Putnam, 1971). Crystalline bovine serum albumin (Miles Laboratory) was used to set up the standard curve. Koch and Putnam modified the biuret method to measure the protein content in impure systems such as bacterial cultures. Their method corrected for increased turbidity, partly because the absorption of the copper-peptide complex was made more sensitive by measurement in the ultraviolet region. When compared with the Lowry method, in which potassium ions and Tris buffer can adversely affect colour development, the biuret method is more accurate and shows better reproducibility.

Mitochondria are intracellular organelles characterized by certain well defined structural features and a narrow range of sedimentation coefficients and are the main sites for the phosphorylating electron transport chain. For studying respiration and electron transport, biochemists have developed various procedures for the isolation of mitochondria, the procedure varying with the type of tissue or cell.
The beginning of any isolation procedure starts with a decision as regards the manner of mechanical disruption (homogenization) of the tissues or cells in an appropriate medium. Preference should be given to the method gentlest in rupturing the cells and releasing their constituents, followed by the best degree of reproducibility and final yield. Differential centrifugation and filtration are then employed to separate the

mitochondria from the cell debris, red blood cells, microsomes and soluble components. The respective methods will be discussed for rat liver tissue and slime mould microplasmodia.

a. Rat liver

Rat liver mitochondria were prepared according to conventional methods (Vinogradov and Scarpa, 1973) and the initial addition of 1 mM EGTA was included in order to obtain mitochondria relatively free of endogenous calcium. A rat starved overnight was stunned by striking its head against a bench counter. It's liver was dissected out as quickly as was possible and extraneous tissue trimmed away. The isolation procedure was carried out throughout on ice.

The liver was minced in an ice-cold medium of 0,25 M sucrose, 5 mM Tris, 1 mM EGTA at a pH of 7,4. The sucrose-blood mixture was decanted off and fresh sucrose medium was added. Mincing was continued until the pieces were 1-2 mm in size. When most of the blood had been removed, the minced liver was transferred to a prechilled Braun Dounce homogenizer. The tube was filled to the 50 ml mark with cold sucrose medium. The homogenizer tube had a capacity of 50 ml and a clearance of 0,1 mm with the teflon pestle and the pestle was driven by a stirring motor with a shaft rotation of 600 rpm.

Using the stirring motor to drive the pestle, the homogenizer tube was passed up and down until only a few fragments of tissue remained. Usually this meant six strokes and it was found to be preferable that some tissue remain rather than risk damage to the mitochondria already released. The homogenate was divided between four 50 ml polypropelene tubes for the Sorval SS34 fixed angle rotor and centrifuged for 10 min at 600g on the Sorvall ultracentrifuge Model RC-2. After centrifugation (Fig. 12), the pellets (cell debris, blood cells) were discarded and the supernatant

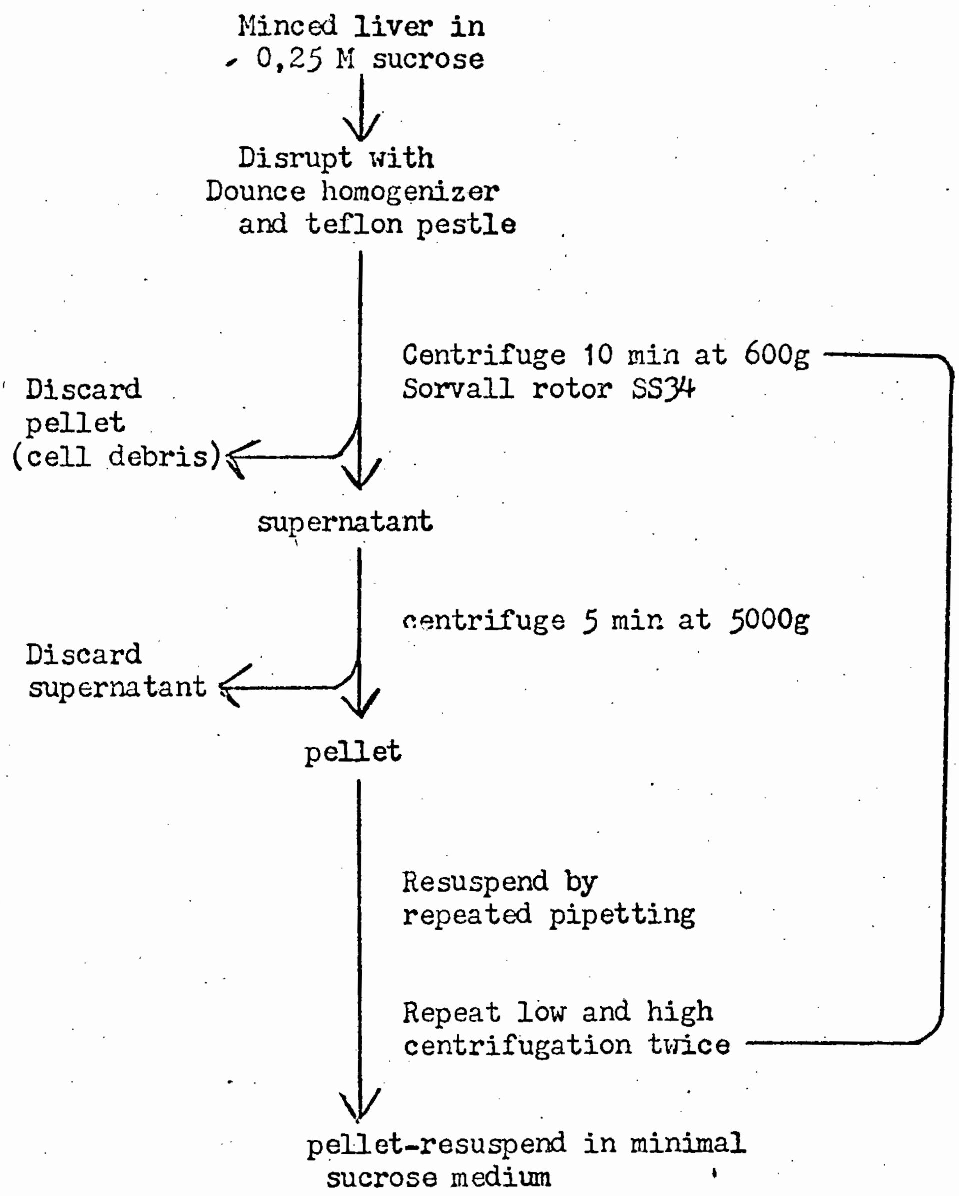


Fig. 12. A diagram showing the isolation procedure for rat liver mitochondria.

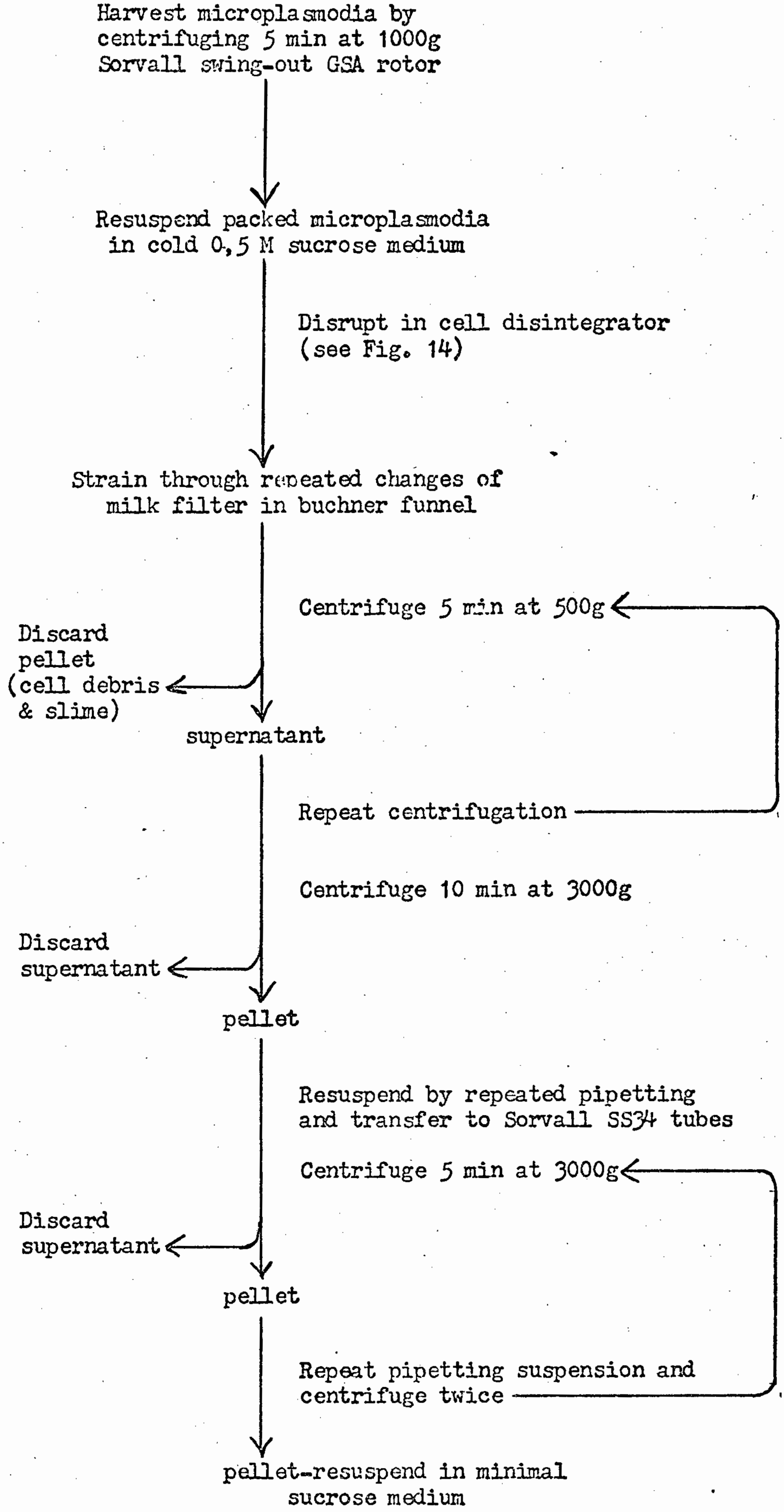
was centrifuged for 5 min at 5000g. The resulting pellets were resuspended by repeated pipetting with minimal medium and combined into one tube. The low and high speed centrifugations were repeated twice to remove as many contaminants as possible from the mitochondrial fraction. For the second washing and the final suspension, the medium was changed to 0,25 M sucrose, 5 mM K_2HPO_4, 2 mM $MgCl_2$ and 5 mM Tris at pH 7,4. After the final high speed spin, the pellet was resuspended in a minimal volume of sucrose and kept on ice.

b. Slime mould microplasmodia

The isolation procedure of Grant and Poulter (1973) was followed with minor changes. These were primarily to adapt the procedure from plasmodia to microplasmodia shaker cultures and to handle the larger amount of material. P. polycephalum microplasmodia were grown under sterile conditions and the isolation procedure was carried out throughout on ice. The microplasmodia were harvested from three 2-litre shaker flasks with 200 ml of growth media in each. After three days growth, the settled mass of microplasmodia in a measuring cylinder equalled 250-300 ml. These were decanted into four 200 ml cellulose nitrate tubes. The tubes were then placed into the swing-out holders of a Sorvall GSA rotor and centrifuged for 5 min at 500g (Fig. 13).

After centrifugation, each pair of microplasmodial pellets were broken up in a beaker of 200 ml of cold 0,5 M sucrose, 5 mM Tris and 1 mM EGTA at a pH 7,4. It was found to be important that the microplasmodia be suspended in a volume of at least 10 times the wet weight of the pellets used, as this allowed for a proper velocity through the disintegrator and a sufficiently thin homogenate for later filtration through the milk filters. Individually, each of the 200 ml beakers were decanted into the pre-chilled cylinder tissue disintegrator designed by

Fig. 13. A diagram showing the isolation procedure for slime mould mitochondria from microplasmia.


Harvest microplasmodia by centrifuging 5 min at 1000g Sorvall swing-out GSA rotor
Resuspend packed microplasmodia in cold 0.5 M sucrose medium
Disrupt in cell disintegrator (see Fig. 14)
Strain through repeated changes of milk filter in buchner funnel
Centrifuge 5 min at 500g
Discard pellet (cell debris & slime)
supernatant
Repeat centrifugation
Centrifuge 10 min at 3000g
Discard supernatant
pellet
Resuspend by repeated pipetting and transfer to Sorvall SS34 tubes
Centrifuge 5 min at 3000g
Discard supernatant
pellet
Repeat pipetting suspension and centrifuge twice
pellet-resuspend in minimal sucrose medium

Polson in 1973 (Fig. 14).

Polson's apparatus utilizes compressed air to force the microplasmodia-sucrose suspension through an adjustable space between a stationary solid cone and an adjustable concavity. It provided an efficient, reproducible method of homogenizing suspensions of single cells (especially since they are devoid of cell walls) and the extent of disintegration can be controlled. The factors favoring pressure homogenization are: (1) over 90% of the cells are disrupted, (2) microplasmodia and their liberated components are not subjected to prolonged exposure to disrupting forces, (3) a high degree of reproducibility of the homogenate and, (4) the parameters of the system-pressure (i.e. velocity), time, aperture width and suspending medium can be varied to suit the need of the operator.

The optimum setting for the disintegrator was with a conical space of 0,4 mm and an air pressure of 25 kg/cm^2. The resulting homogenate was filtered through a single cotton-wool milk filter pad (Springbok-190 mm mediums, National Dairy Equipment Ltd, Johannesburg) on a buchner funnel and a low enough suction applied so as to avoid perforating the filter. The filter was changed for every 100 ml of homogenate. After the first filtration, the homogenate was filtered again and the filter pad changed every 200 ml. This step proved very important in removing the slime from the preparation.

The filtered homogenate was divided between the four cellulose nitrate tubes of the GSA rotor and cold fresh sucrose medium added to each to make them 80% full. After twice centrifuging and discarding the pellet (Fig. 13), the supernatant was subjected to high speed centrifugation. The pellets from the four tubes were resuspended, combined and transferred to a 50 ml polypropylene tube for a Sorvall SS34 rotor. After the first of two high speed spins, to remove the slime from the

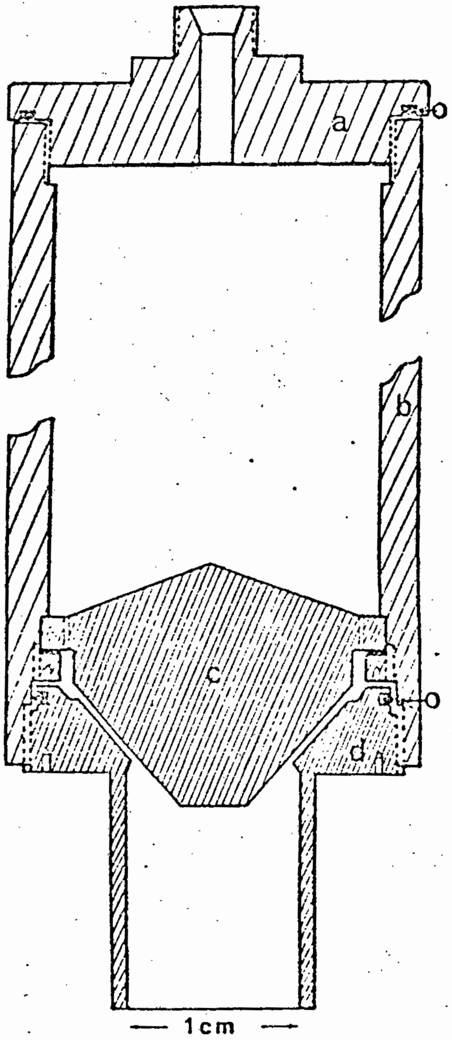


Fig. 14. A diagram of the cell distintegrator designed by Polson (1973). The microplasmodia were added to the chilled cylinder and the top screwed on. The top is connected to a compressed air cylinder and the O-ring provides the seal. The lower part of the appartus consists of a solid brass cone (c) designed to fit the underlying conical cavity. Screw threads on the sides of the conical cavity (d) allow for adjusting the width of the gap between the opposing convex and concave surfaces.

mitochondrial fraction, the pellet was resuspended in cold 0,5 M sucrose, 5 mM K_2HPO_4, 2 mM $MgCl_2$, and 5 mM Tris at pH 7,4 and the same medium was also used to resuspend the final pellet.

5. Polarography of mitochondria

The application of the oxygen electrode technique for the study of mitochondria respiration and oxidative phosphorylation was first developed and reported by Chance and Williams (1955). The technique provides a simple, direct and rapid means of determining the ADP:O (viz. P/O) ratio. Since it is a direct measure of the number of moles of ADP phosphorylated to ADP with each oxygen atom, the ratio is considered to be a major criterion of mitochondrial phosphorylative ability and function integrity. Several types of electrodesexist, but there are two basic types: the platinum wire electrode and the "Clark" type which is partially encased in glass and has the exposed Pt electrode immersed in saturated KCl and a polyethylene membrane separating the KCl phase from the reaction medium.

Both electrodes depend on a polarized electric current. The voltage applied between the anode and cathode parts of the electrode immersed in an oxygen-containing solution (Pt electrode negative to anode) causes the dissolved oxygen to undergo electrolytic reduction. With a current of -0,5 to -0,8 volts, the voltage across the anode and cathode is directly proportional to the oxygen concentration of the solution. Specifically for the Clark electrode used, a potential of -0,6 V was applied to the Pt electrode relative to the Ag-AgCl electrode and passed through a variable potentiometer in the O_2 meter. The current generated across the electrodes was displayed on a chart recorder (REA 160 Titrigraph Module, Radiometer, Copenhagen) with 1 mV full-scale deflection.

The bare platinum electrode fits very well into an optical cuvette, can be made to vibrate or rotate for mixing and consequently, is used in conjunction with spectrophotometric studies. Because of its direct contact with the solution, the electrode has a very fast response although the sensitivity can vary unexpectedly. This is mainly due to the fact that if uncoated, the electrode can be easily "poisoned" by reactive chemicals such as cyanide and iodide; even mitochondrial proteins cause a loss of reactivity that can affect future experiments (Hagihara, 1961). Therefore, the electrode must be coated with a protective film, usually collodium. This coating does however wear thin after repeated use and leads to spurious results.

Membrane-coated electrodes (e.g. Clark) largely avoid these hazards and are preferable in situations not needing a fast response. This type indirectly measures the free oxygen that diffuses across the membrane to the KCl phase and is electrolytically reduced by the current generated between the electrodes. Hence the voltage is a measure of the oxygen activity rather than the actual oxygen concentration. The activity coefficient of O_2 in solution varies in proportion to the concentration of electrolytes or ionic strength. For the reaction mixtures used in the respiration studies, an initial oxygen concentration of 240 µM O_2/l was used for the air saturated solutions. This value was taken from Chappell (1964) and Chance and Williams (1955) who used the values of 237 and 240 µM O_2/l, respectively.

A Clark electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio, U.S.A.) was used in the polarography experiments. The vessel used to hold the reaction mixture was a modified 5 ml glass beaker with a small projecting tube (spout-0,2 cm diam.) attached to one side. The spout was so situated as to be immediately adjacent to the surface of the reaction medium and was used to make additions with micropipettes (Eppendorf) of 5-50 µl capacity. The amount added never changed the total volume by more than 5%.

The small volume of the reaction vessel helped to extend the mitochondrial preparations, thus increasing the number of possible experiments per isolation and also facilitated mixing of the solution by a magnetic stirrer. The speed with which the electrode responded to changes in O_2 concentration induced by respiratory activity of the mitochondria appeared to be limited only by the rate of mixing. Back diffusion of oxygen at the air-medium interface was almost wholly prevented by the nearly perfect fit of the membrane-coated electrode into the beaker. The additions of small volumes of ADP solution led to an immediate increase in respiratory rate for rat liver mitochondria. Addition of neutralized KCN to produce a final concentration of 1 mM caused an immediate inhibition of respiration, "immediate" indicating the response after reaching a steady-state solution within 15 sec. All experiments were run at ambient temperature.

6. Dual-wavelength murexide spectrophotometry

Murexide is a divalent cation-sensitive dye and as such has been used to study Ca^{++} and Mn^{++} movements in biological systems (i.e. sarcoplasmic reticulum vesicles, mitochondria, intact cells). When used in a suspension, murexide remains in the external medium because of its insolubility in and permeability through biological membranes. Because of a high coefficient of absorption, low concentrations (20-50 µM) can be used. Murexide has a low affinity and specificity for Ca^{++} but the rate constant for the reaction of murexide with Ca^{++} is high enough to allow fast kinetic measurements of Ca^{++} binding or transport (Scarpa, 1972).

The change in absorbance when the complex is formed can be seen when Ca^{++} is added to a cuvette of murexide: there is an observable colour change from purple to orange. The Ca^{++}-murexide complex, as compared to murexide alone, exhibits a lower light absorbance at 540 nm and a higher absorbance at 470 nm and an equivalent absorption (isobestic point) for both at 507 nm. The production and disappearance of the Ca^{++}-murexide complex can therefore be measured through changes in absorbance at 540 or 470 nm. For the study of mitochondria, there is concomitant production of a turbid solution and consequent light scattering effect. This interference is minimized by taking absorbance readings at two close wavelengths. One wavelength is the reference and the second is the measuring wavelength and is substracted from the first.

Mela and Chance (1968), in their mitochondrial studies, used a dual-wavelength spectrophotometer to record absorbance measurements at 540-510 nm, the reference and measuring wavelength respectively. They demonstrated that murexide remained outside of mitochondria and did not affect mitochondrial respiratory control. In addition, the measured wavelengths of 540-510 nm were unaffected by the absorbance changes of the respiratory carriers (cytochromes).

Rat liver and slime mould mitochondria have been isolated as described previously in media including 1 mM EGTA (pH 7,4) to remove endogenous calcium. Thereafter, for the final washing and suspension of the pellets, media of 0,25 M sucrose-RLM (0,5 M sucrose-SMM), 5 mM K_2HPO_4, 5 mM Tris at pH 7,4 were used. The temperature of the reaction medium was 24 °C. Murexide was a gift of Dr. Bermann and the Aminco Chance dual wavelength spectrophotometer was used with the kind permission of Professor Kench. Murexide stock (5,25 mM) was made up the same day as the experiments and 20 µl was transferred to the 3 ml cuvettes to have a final concentration of 35 µM.

The spectrophotometer was used in the dual wavelength mode, in which the reference and measuring light beams (540 and 510 nm respectively) are alternately time-shared through a single cuvette. A tungsten-iodide (visible) lamp was used for the wavelengths needed. The optical chopper was set at normal and the absorbance range set at 0,02 in order to limit pen deflection to a reasonable height on the graph paper. The response speed of the recorder unit was set at slow (viz. absorbance readings every 2,5 seconds) to prevent the mixing of the mitochondrial suspension from resulting in a too "jerky" pen response. The speed of the pen was set at 50 sec/in which was felt to be satisfactory to separate absorbance changes to additions to the cuvette.

CHAPTER FOUR

RESULTS

1. ^{45}Ca efflux and chemotactic response, as measured with the flow-through chamber

The primary aim of the following experiments was to find if there was any correlation between the initial chemotactic response of the plasmodium and the calcium efflux from its surface. The flow-through chamber was used here to observe simultaneously the cytoplasmic movements and the rate of calcium extrusion from an organism labelled with ^{45}Ca. The radioactive counting rate of each fraction collector vial (CPM) gave the average relative rate per 15 seconds (approximately) of ^{45}Ca efflux from the plasmodium. No absolute figures for the ^{45}Ca efflux could be obtained as this was influenced by such uncontrollable factors as the area (cm^2) of the plasmodium within the chamber, the extent of migration (relative age) and the assimilation of ^{45}Ca from the radioactive plate. Over many hours, the rate of ^{45}Ca efflux remained approximately steady, being more affected by the changes in the surface area of the plasmodium than by depletion of the internal calcium stores. Random fluctuations between vials were usually less than 10% of the CPM readings of adjacent vials.

Before the experiments were done, it was deemed necessary to know to what extent the slime sheath contributed ^{45}Ca to the efflux counted, since this layer is in direct contact with the flowing (perfusing) solution. A preliminary experiment was devised to answer this question. A chamber containing a non-radioactive plasmodium (deprived of Ca^{++}) was filled with ^{45}Ca-labelled growth medium (1 nCi/ml) for 15 minutes. Afterwards, the chamber was perfused with a neutral solution of 1 mM NaCl and 1 mM $CaCl_2$ (pH 7,0) at a rate of 1 drop/sec or 15 drops/vial.

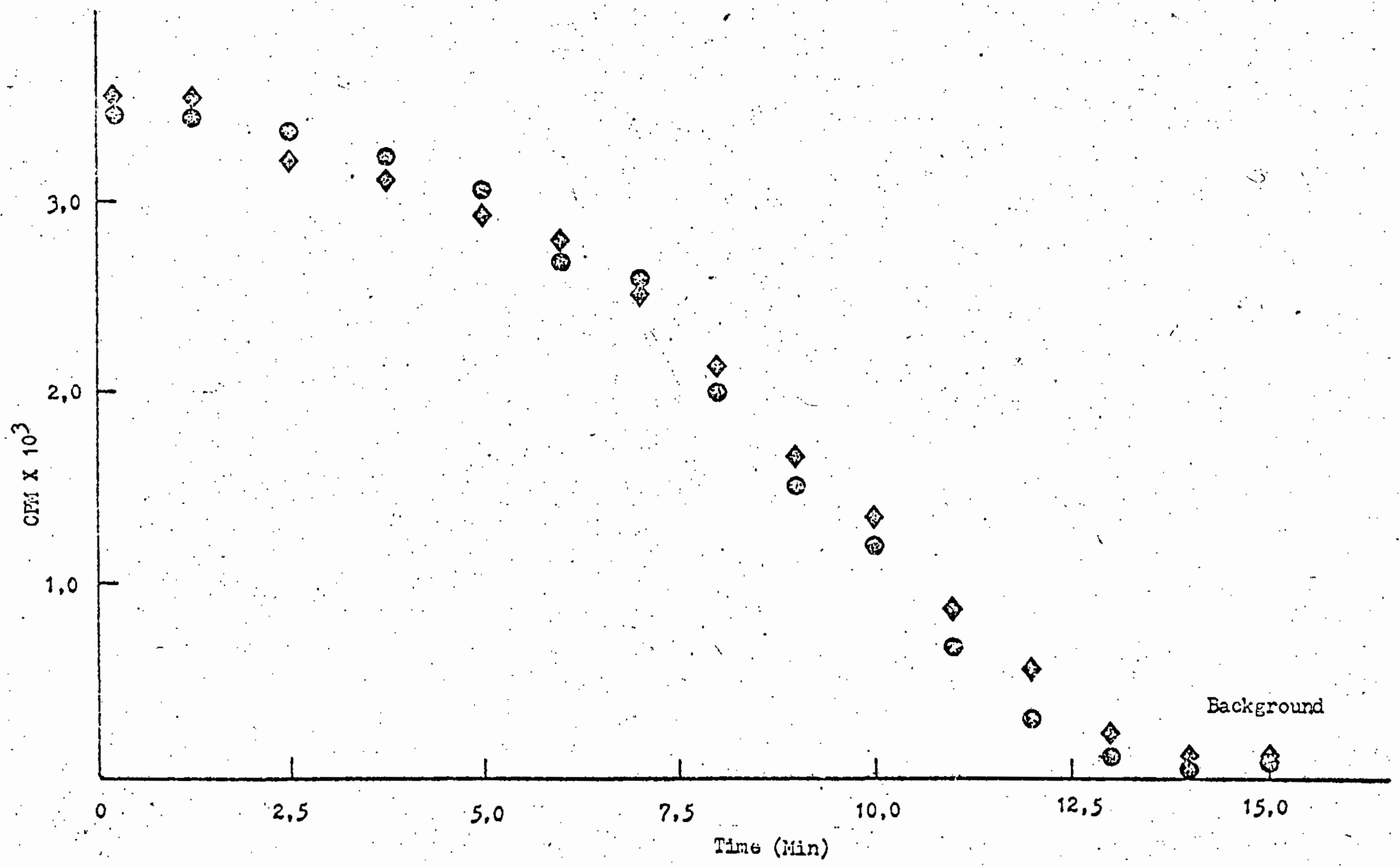
A second chamber was treated in the same manner but 0,5 mM EGTA (pH 7,0) was added to the neutral perfusing solution. As shown in Figure 16, the two curves (viz. CPM/vial vs time) for solutions with and without EGTA, closely approximate each other and both show that the efflux rate decreases within minutes to an insignificant level. Because the efflux rate decrease was short rather than prolonged and the perfusing solution with EGTA showed no greater increase in the Ca^{++} efflux over the first, it appears that the slime sheath, which is a galactose polysaccharide (McCormick et al. 1970), has little or no long term binding affinity for calcium.

In the following experiments, the sugars glucose, galactose and sucrose were used because of their known chemotactic effect (Carlisle, 1970, Ueda et al. 1975). Sucrose, in contrast to glucose and galactose has a mild repellent effect. Therefore it was of interest to compare the responses initiated by both types of sugars in terms of calcium efflux and physiological response (motile streaming).

a. Glucose

The first experiment was with glucose. The plasmodium was prepared as described in Section 2 of the Materials and Methods and allowed to establish a steady rhythm by being perfused for at least 30 minutes with the neutral solution. The view through the microscope's eyepiece had to be changed often in order to show a regular sinusoidal chart recorder tracing. Thereafter, the neutral solution was abruptly switched over to a solution of 1 mM glucose, 1 mM NaCl and 1 mM $CaCl_2$. The corresponding fraction collector vial and time was marked on the recorder paper. The chamber was perfused with the sugar solution for approximately 10-15 minutes and then returned to the neutral solution. If no changes in the streaming were observed, the experiment was terminated. The fraction collector vials were then prepared for scintillation counting and their

Fig. 16. ^{45}Ca efflux from the external slime sheath after radioactive incubation with ^{45}Ca for 15 minutes. (●) indicates the CPM/vial for the Ca^{++} efflux under the passive neutral solution of 1 mM NaCl and 1 mM $CaCl_2$. (⬢) indicates the CPM/vial for the Ca^{++} efflux from a second incubated plasmodium which underwent perfusion by a solution of 0,5 mM EGTA (pH 7,0), 1 mM NaCl and 1 mM $CaCl_2$. Note the close resemblance of the two curves and the sharp dropoff to the background level.


CPM X 10^3
3,0
2,0
1,0
0
2,5
5,0
7,5
10,0
12,5
15,0
Time (Min)
Background

counts per minute (^{45}Ca-CPM) were plotted over the chart recorder tracing.

After having repeated the glucose experiments 15 times, two short-term effects became apparent. The first was an abrupt cessation of streaming which marked the beginning of a period of "shock" usually lasting for 15 minutes (Fig. 17). The second short-term effect was a sudden transient ^{45}Ca efflux ("peak") followed by a gradual decline to the normal efflux level (viz. the baseline). The decline probably reflects the time taken to wash the transient radioactivity out of the chamber and obscures the point where the efflux peak ends. When using glucose and testing each vial with a urine sugar analysis paper (Lilly Tes-Tape), it was found that the first traces of sugar appeared in the third vial or about 45 seconds after the changeover to the glucose solution. The third vial in the majority of the runs, had the highest CPM in the "peak" and marked the onset of the "shock" period. Hence, it seems probable that both phenomena commence with the entrance of the sugars into the chamber.

It was difficult to define (i.e. assign percentages) the frequency of appearance for either of the events described above. Often the events were distinct, sharp and easy to recognize. However there were chart recorder tracings in which "ragged" or confused oscillations, preceding or following the event, made the particular tracing difficult to interpret. Such tracings were due to improper placement of the chamber under the microscope objective lens. Another possible cause would be that physical movement of the plasmodium during a run would shift the microscope's field of vision from that of a major "vein" to overlapping areas of contraction. The ^{45}Ca peaks were easily distinguished but did not appear with some runs.

Both events often appeared together or singly and very seldom, they were absent altogether (compare Fig. 17 and 18). These observations

Fig. 17. Simultaneous recordings of motile oscillations (continuous line) and ^{45}Ca efflux (points-CPM/vial), illustrating particularly a "shock" period following the start of exposure to 1 mM glucose (arrow). Notice the increased frequency when the oscillation restarts, and the very steady calcium efflux rate. For Figures 17-28, Abscissa: Time in minutes. Ordinates: Thousands of radioactive counts per minute per vial of effluent, and transmission of light through the plasmodium.

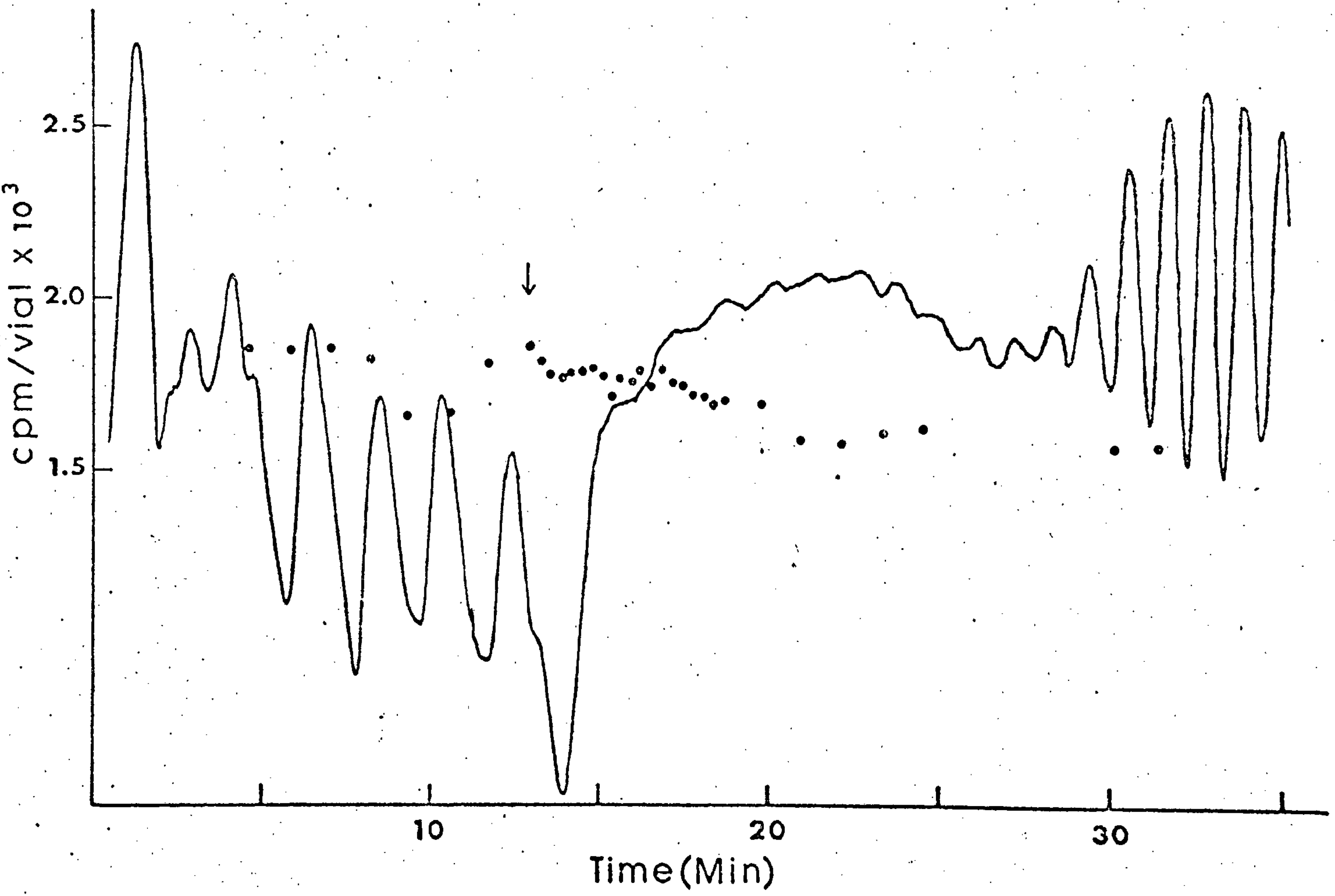
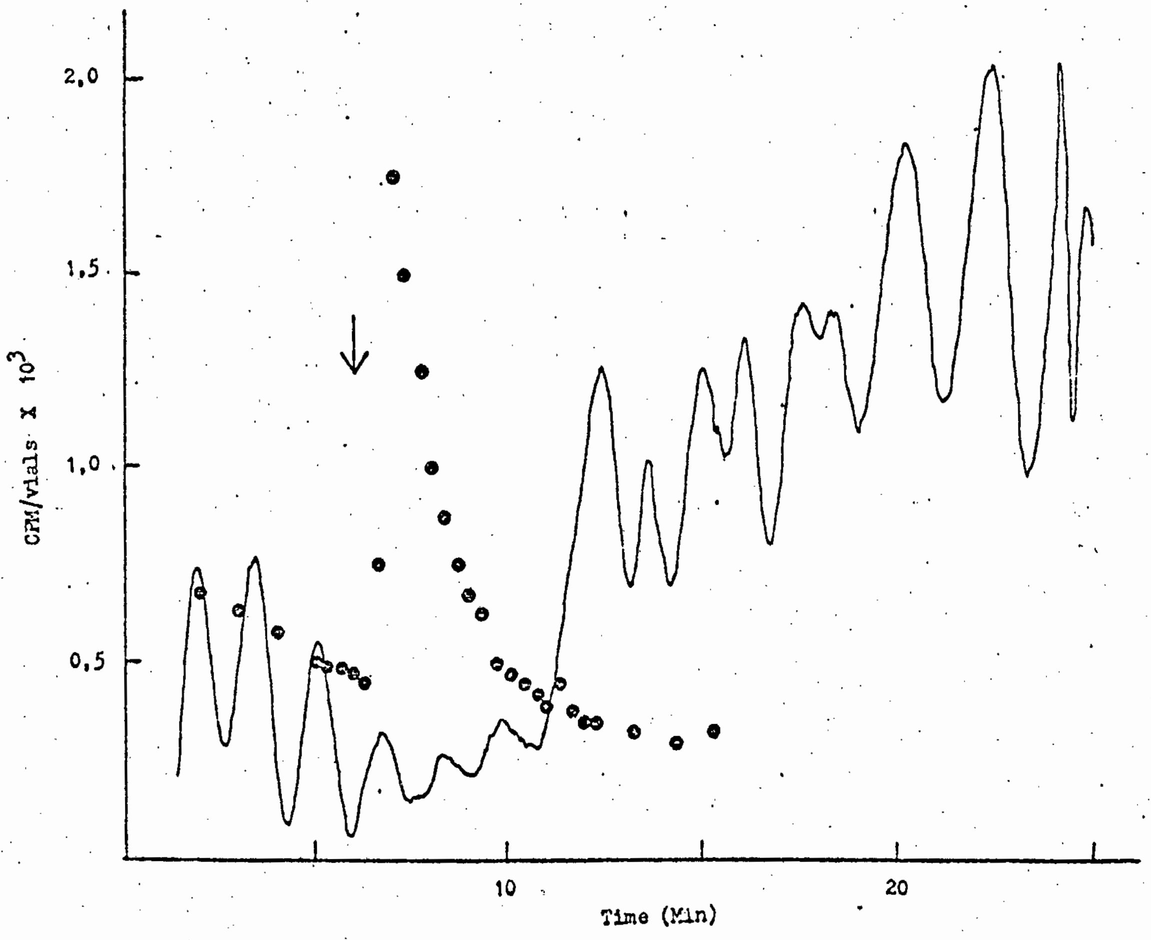

cpm/vial x 10^3
2.5
2.0
1.5
10
20
30
Time (Min)
↓

Fig. 18. A repeat of the experiment presented in Fig. 17, illustrating a ^{45}Ca efflux peak, the return of the efflux to a baseline and the absence of a period of "shock" as seen in Fig. 17.


2,0
1,5
1,0
0,5
CPM/vials X 10^3
10
20
Time (Min)

were not related to any obvious factor such as the age (freshness) of the plasmodium. Rose et al. (1972) reported that glucose would stop an actively migrating starved plasmodium on an agar plate. The period of shock described here demonstrates such inhibition of migration and shows graphically the physiological response induced by the added glucose solution.

b. Galactose

Ueda et al. (1975) reported that both glucose and galactose attracted plasmodia to the same extent when the concentrations exceeded about 10^{-4} M. Therefore it was of interest to test whether galactose, as an attractant, produced similar phenomena as mentioned for glucose.

Ten experimental runs were made using 1 mM galactose, following the same procedure as for glucose. Figure 19 shows the results of one such experiment in which both phenomena can be seen. However, under the exact same conditions, in another run (Fig. 20), the ^{45}Ca peak was absent. As was observed for glucose, those runs with "peaks" had the highest radioactivity (CPM) in the third vial after the changeover.

Of the ten runs with galactose, 8 showed a "shock", 6 showed a ^{45}Ca peak and 4 of those runs with "peaks" also had a discernable "shock" period. A satisfactory explanation for these variations is not available at present.

c. Sucrose

Ueda et al. (1975) also demonstrated that sucrose would begin to repel at 0,03 M concentration. This is considerably more than the concentrations of glucose and galactose in the previous experiments. Sucrose, while being a sugar, differs not only in being a repellent but also in requiring a higher concentration to initiate the chemotactic response. On the basis of Ueda's observation, five of the following exper-

Fig. 19. Simultaneous recordings of motile oscillations (continuous line) and ^{45}Ca efflux, illustrating a "shock" and ^{45}Ca efflux peak following the start of exposure to 1 mM galactose (arrow). Notice the increased frequency as with 1 mM glucose in Fig. 17.

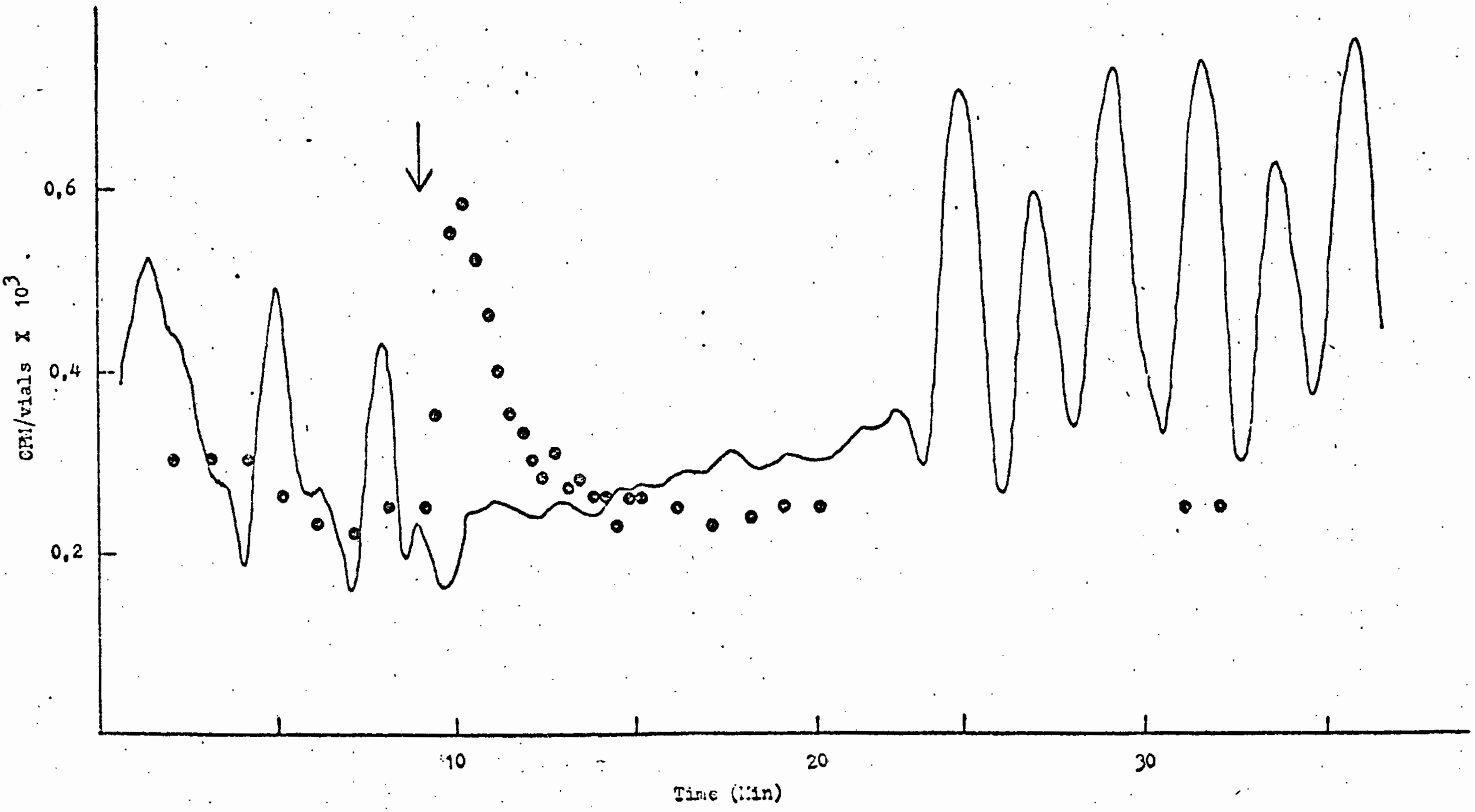
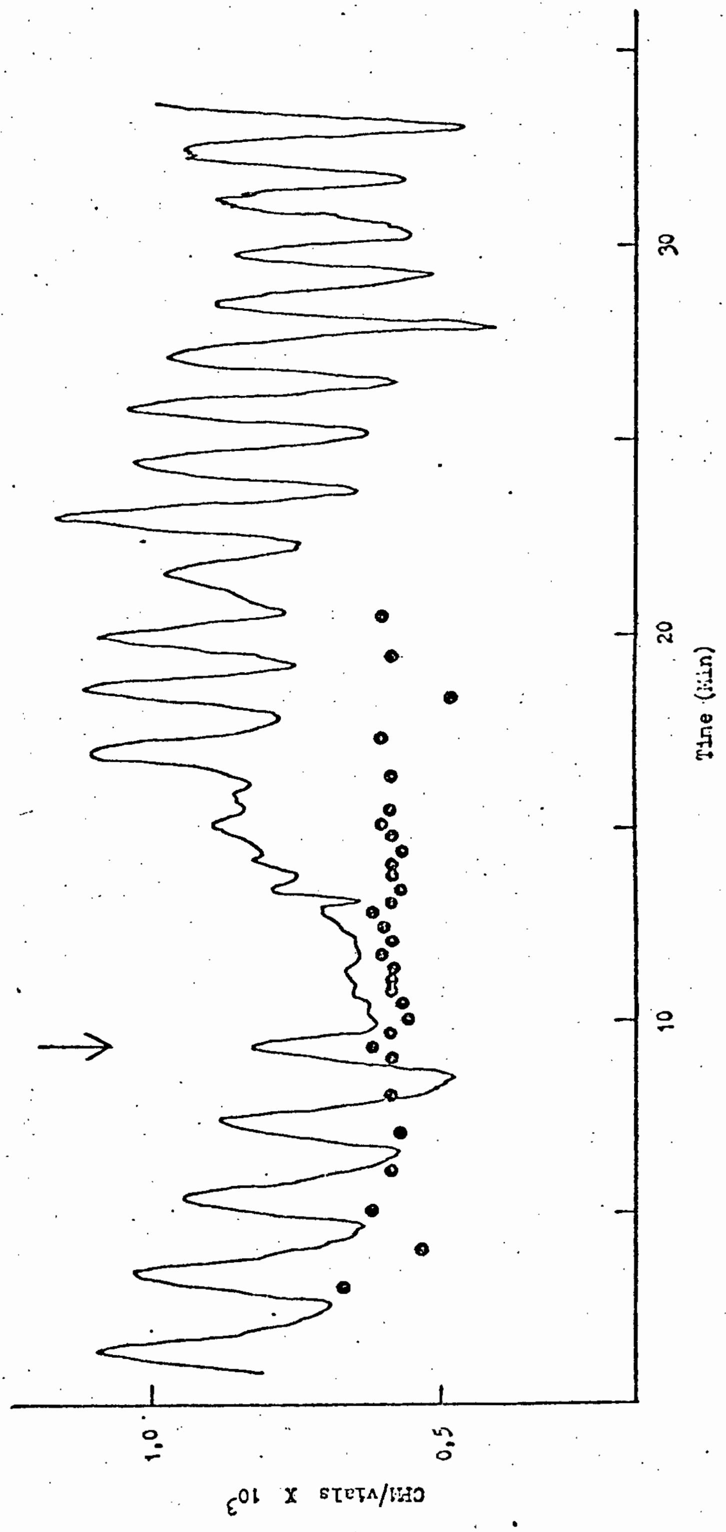

0,6
0,4
0,2
CPM/vials X 10^3
10
20
30
Time (Min)

Fig. 20. Simultaneous recordings of motile oscillations (continuous line) and the ^{45}Ca efflux, illustrating an absence of a ^{45}Ca efflux peak when 1 mM galactose was introduced.



1,0
0,5
CPM/vials X 10^3
10
20
30
Time (Min)

iments were done with 1 mM sucrose and the remaining five with 0,03 M sucrose.

Figure 21 shows an experiment with 1 mM sucrose in which no "shock" event can be discerned but a ^{45}Ca peak is present. None of the 1 mM experiments showed "shocks" but in three of them the ^{45}Ca peak was present. Figure 22 shows an experiment with 0,03 M sucrose in which both the ^{45}Ca peak and a period of "shock" can be seen. In contrast to the inconsistency seen with the glucose and galactose experiments, all the experiments with 0,03 M sucrose showed both phenomena to be present.

d. Valinomycin and EGTA

Two further experiments were also done: one with valinomycin to find if changing the membrane potential had any appreciable effect and the second, with EGTA, to determine the extent of the anticipated Ca^{++} withdrawal and its effect on the motile streaming. The potassium-specific ionophore valinomycin has been shown to transport potassium ions passively across biological and artifical membranes (Grant and Reynolds, 1972). Microelectrode measurements by Hato et al. (1976) gave values of -50 to -90 mv for the plasmodial membrane potential, depending on the external medium. In theory, outward transport of K^+ by valinomycin should hyperpolarize the membrane and thus have an effect on Ca^{++} transport if the transport is regulated by the membrane potential. It remains uncertain to what extent the slime sheath blocks the ionophore from crossing the membrane.

In the actual experiment, five runs were made in which 10 µg/ml valinomycin (Sigma Chemical) was perfused through as with the previous runs. In all five, there were no "shock" periods or changes in motile streaming pattern but in 2 runs the changeover did evoke similar ^{45}Ca efflux peaks as with the sugars (Fig. 23. This suggests that the effect

Fig. 21. Simultaneous recordings of motile oscillations and ^{45}Ca efflux, illustrating a prominent efflux peak and no apparent change in the motile streaming when a solution of 1 mM sucrose was introduced.

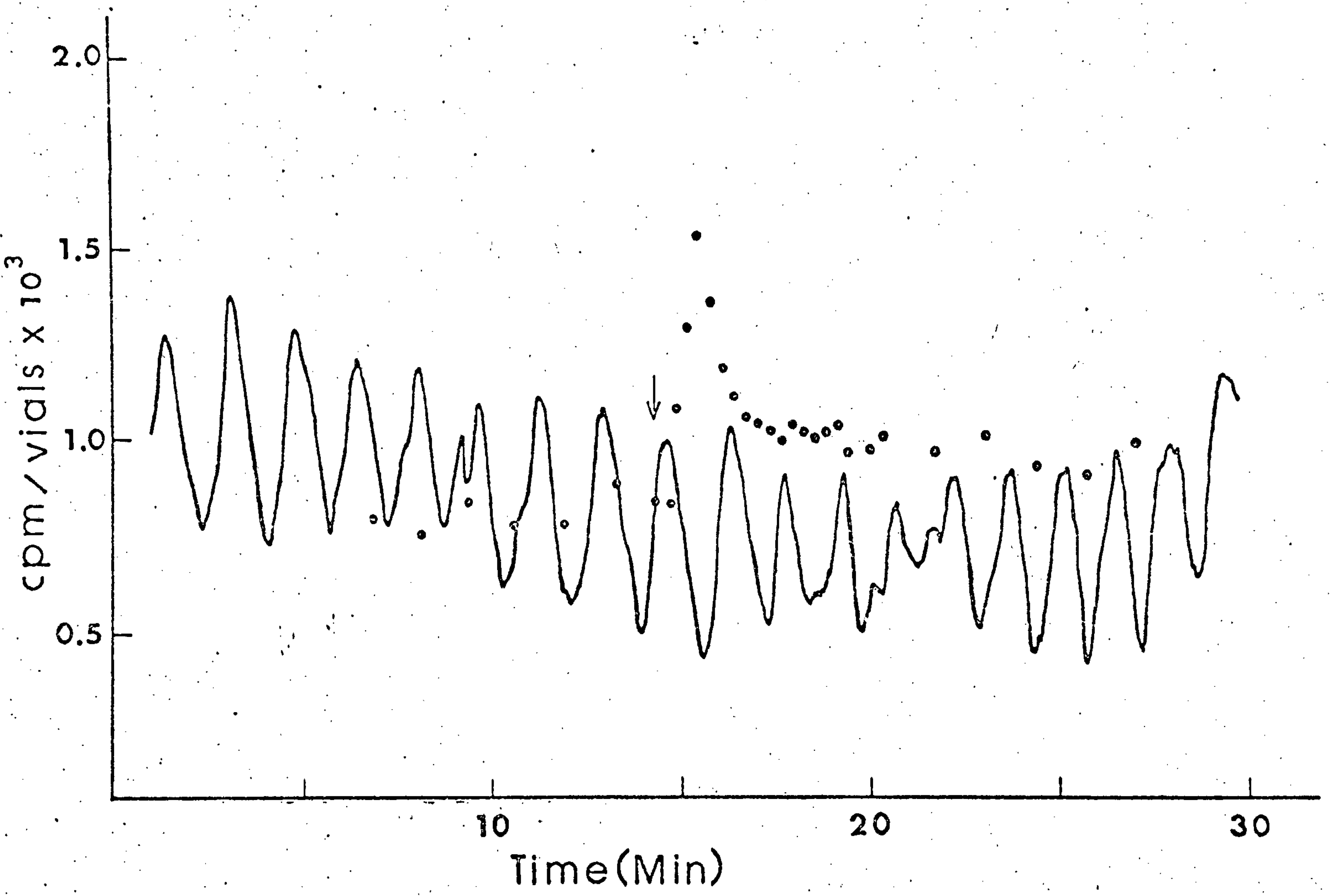

2.0
1.5
1.0
0.5
cpm / vials x 10^3
10
20
30
Time(Min)

Fig. 22. A repeat of the experiment in Fig. 21 but the perfusion solution contained 0,03 M sucrose. Note the presence of both the ^{45}Ca efflux peak, the start of a "shock" period when the sugar solution is introduced (arrow) and the lower frequency when the oscillation restarts.

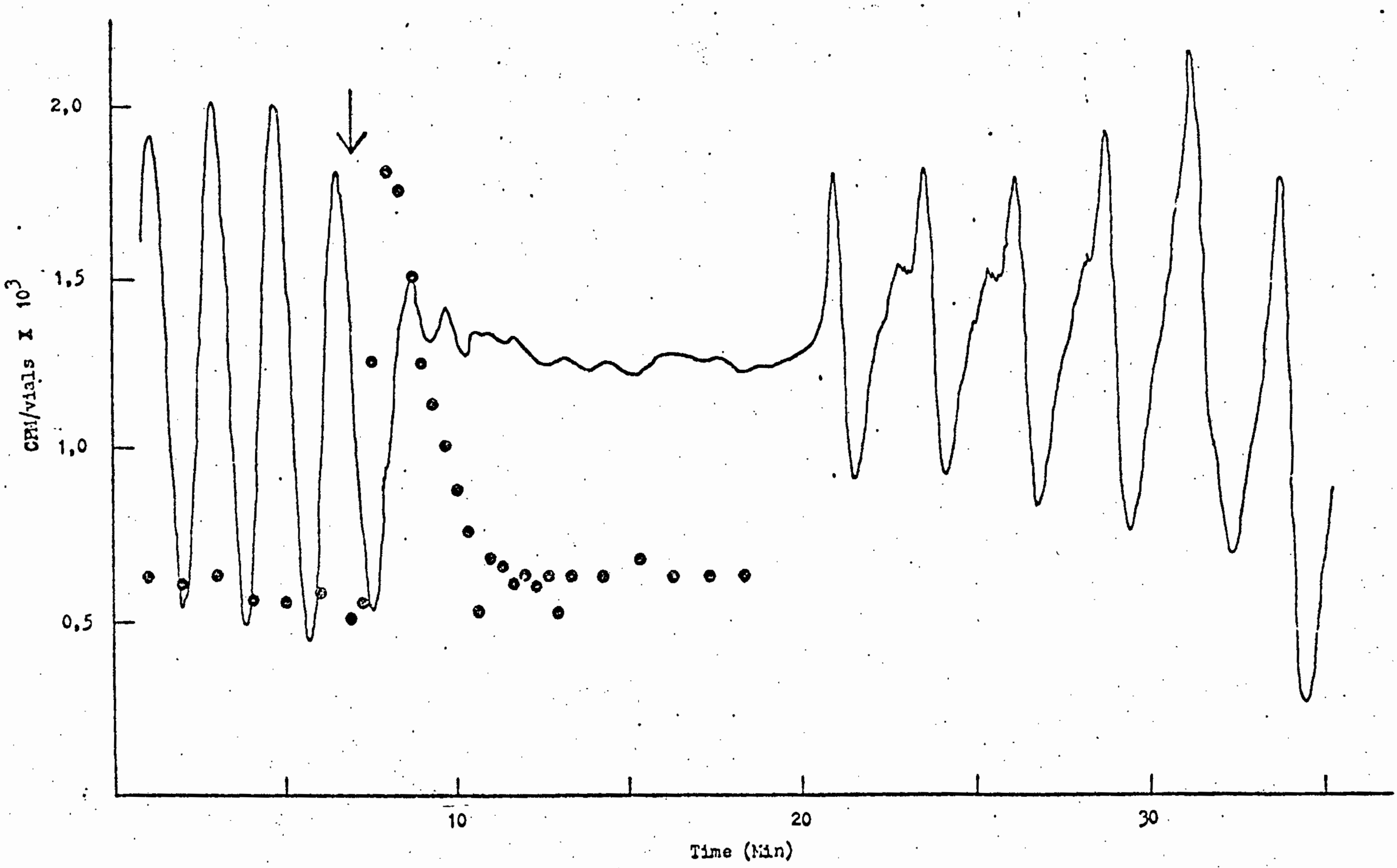

2,0
1,5
1,0
0,5
CPM/vials X 10^3
10
20
30
Time (Min)

Fig. 23. Simultaneous recordings of the motile oscillations and the ^{45}Ca efflux, illustrating the absence of change in the motile streaming when 10 µg/ml of valinomycin was introduced. Note at the point of introduction the beginning of a calcium efflux peak.

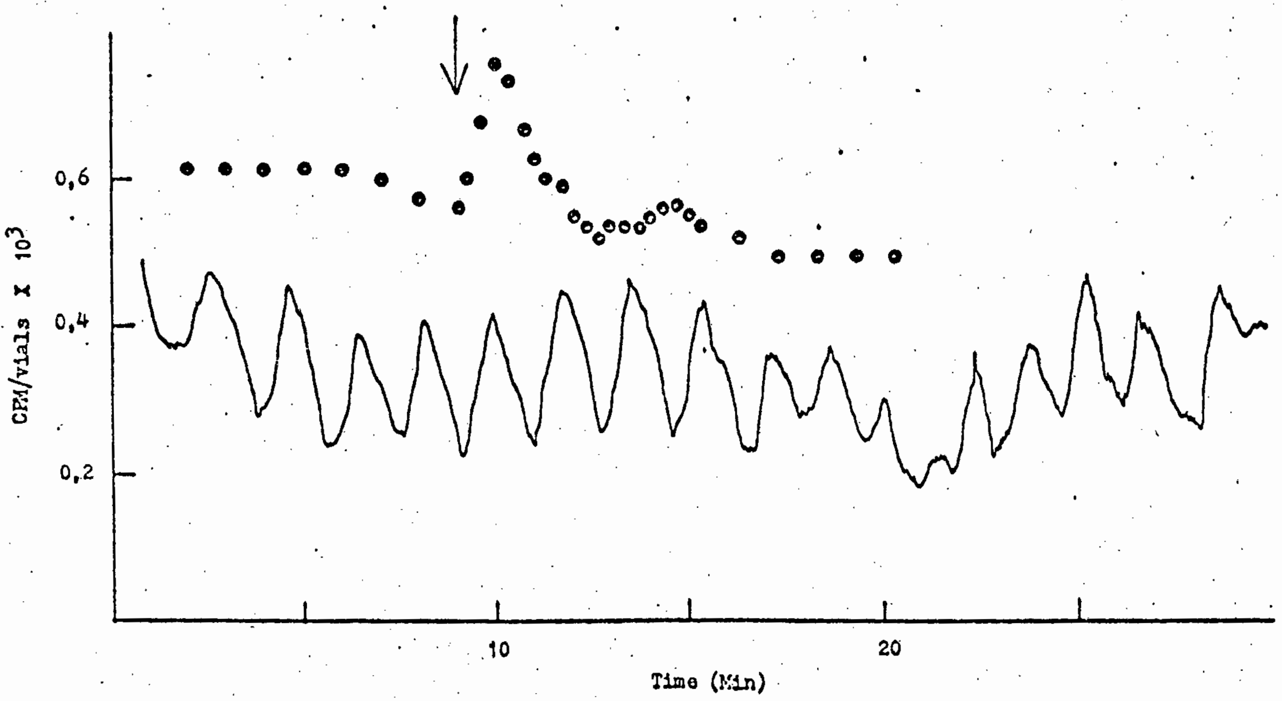
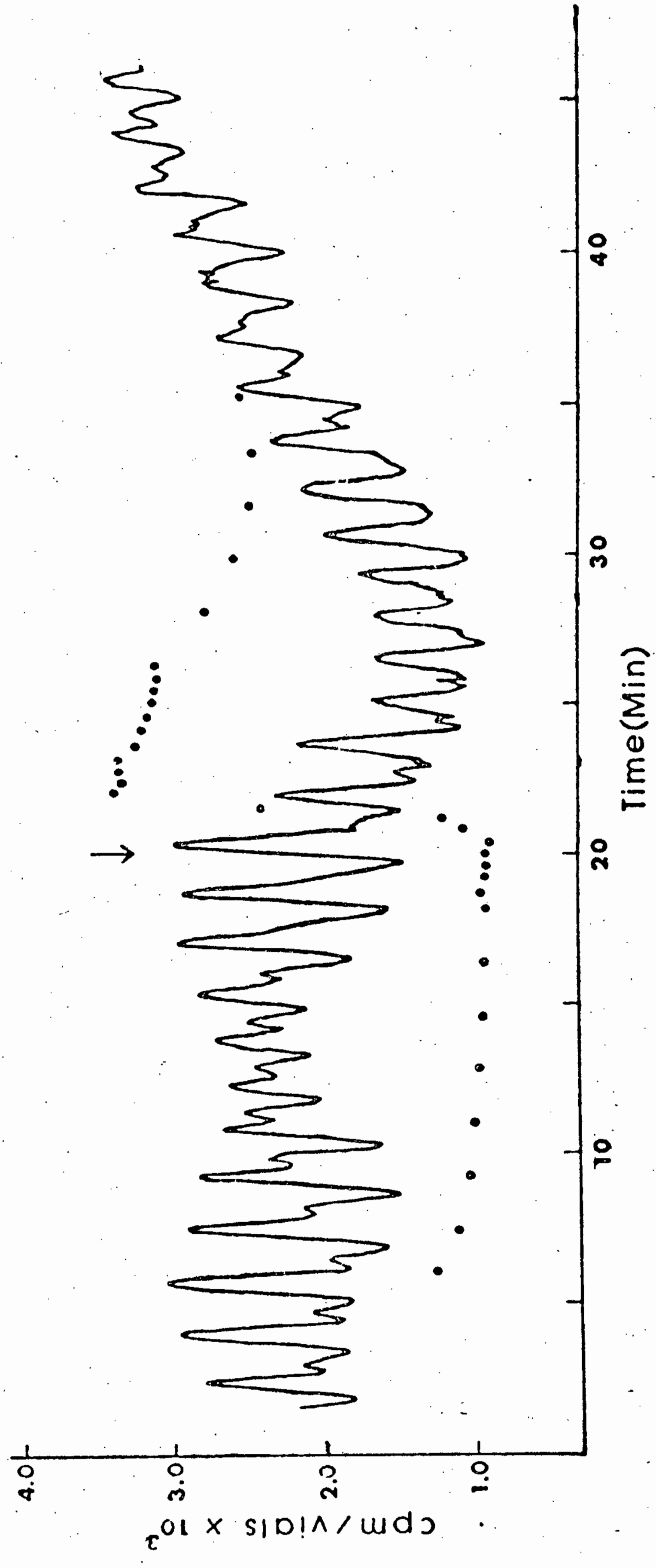

0,6
0,4
0,2
CPM/vials X 10^3
10
20
Time (Min)

Fig. 24. Simultaneous recordings of motile oscillations and calcium efflux, illustrating particularly a more than threefold increase in calcium efflux rate following the start of exposure to 0,5 mM EGTA (pH 7,0-arrow). Notice how the oscillations become ragged and gradually die away with continuous calcium chelation.



cpm / vials x 10²
4.0
3.0
2.0
1.0
10
20
30
40
Time(Min)

was not specific for the sugars tested.

The last experiment consisted of a changeover from the neutral solution to a solution of 0,5 mM EGTA (adjusted to pH 7,0 with NaOH). As expected, this induced a prolonged calcium efflux (Fig. 24). This experiment was repeated three times and during each run, the plasmodium survived at least 20 minutes of a threefold increased Ca^{++} efflux before there was a significant change in its movement. As seen in Fig. 24, after 15-20 minutes, the amplitude of the oscillations becomes shorter and ragged but they are not altered in frequency. The ability of the plasmodium to withstand the artifically high baseline (continued leeching by EGTA) suggests that there is an internal source for the calcium which is not originating from the slime sheath.

e. Summary

After performing more than forty such related experiments, the conclusion can be reached that there is a normal persistent baseline ^{45}Ca efflux into the perfusing solution. Table 2 below gives a synopsis of the observations made with the flow-through chamber.

Table 2. Occurrence of the "shock", ^{45}Ca efflux peak and other events.

Substance	Concentration	Observation
Glucose	1 mM	"Shock" and ^{45}Ca peak events present
Galactose	1 mM	" " " " " "
Sucrose	1 mM 30 mM	No "shock" but ^{45}peak event present "Shock" and ^{45}Ca peak present in every run
Valinomycin	10 ug/ml	No "shock" but ^{45}Ca peak event present
EGTA	0,5 mM	Neither event occurred, no change in streaming, sustained elevated ^{45}Ca efflux

As Table 2 shows, all the runs with sugars produced some chart recorder tracing with "shock" periods. In a minority of runs, for an unknown reason, the "shock" event did not appear. These periods of quiescence in the motile oscillations may represent the time needed by the organism to adjust internally to the newly encountered chemotactic substance. It seems reasonable to suggest that the "shock" event developed through the course of evolution because it aided in the phagotrophic process. After the initial chemotactic recognition, the plasmodium would expend needless energy in migrating in other directions when one part of if had already encountered a food source or favourable temperature (warmth vs cold). Section C of the literature review summarizes various hypotheses concerning the reorientation of a plasmodium in such situations. Valinomycin and EGTA (non-sugar solutions) produced no change in the streaming, thus supporting this effect of sugars.

Since, the peak of ^{45}Ca efflux observed with the sugars and valinomycin is of short duration and since the efflux returns to approximately the initial level,, it is possible that this phenomenon is an artifact and has no physiological basis. Another explanation for this effect would be that the introduction of the "test" solutions altered the osmotic pressure of the aqueous medium perfusing over the plasmodium. The change in pressure could conceivably detach Ca ions that were tenuously attached to negative charges of the external "slime" layer.

The significant result is that there was no measurable long term change in the Ca^{++} efflux rate in response to chemotactically attractant sugars, despite the clear change in oscillation frequency. From that observation and the fact that the elevated EGTA-induced Ca^{++} efflux did not alter the streaming, it may be hypothesized that the normal Ca^{++} is not related to the chemotactic recognition and response. These observations also argue against the original hypothesis that Ca^{++} fluxes control the chemotactic response, and indicate that the essential mechanisms for the regulation of the internal $[Ca^{++}]$ and of the contractile proteins does not lie with Ca^{++} transport across the external membrane.

2. Ca^{++}-stimulated respiration of rat liver and slime mould mitochondria by polarography

Mitochondrial fractions of rat liver and slime mould mitochondria were isolated as described in Section 4 of Materials and Methods. The aim of the following experiments in polarography were based on the fact that those mitochondria having an energy-linked ability to accumulate Ca^{++}, display this ability by having their respiration increased markedly during Ca^{++}-accumulation. Hence it can be a method of investigation into whether slime mould mitochondria possess a similar Ca^{++}-accumulating capability.

a. Determination of P/O ratios

It is first necessary to determine that the mitochondria are functionally intact and have not been injured by the isolation procedure. The best and most rapid criterion is by determining the P/O ratio that occurs during the process of oxidative phosphorylation.

Biochemists first observed that phosphate was taken up during the aerobic oxidation of citrate, pyruvate, glutamate, succinate and malate. It was later determined that phosphate was consumed by the process of phosphorylation of ADP to ATP during the passage of electrons and protons through the mitochondrial electron transport chain. The process of oxidative phosphorylation can be followed by determining the ratio of the moles of phosphate (viz. ADPO converted to ATP to the atoms of oxygen taken up in the reaction. These measurements, called P/O ratios, were found to vary with the substrate used.

Specifically, the mitochondrial oxidation of succinate produces fumarate with the concomitant production of a reduced coenzyme, $FADH_2$ (flavin adenine dinucleotide). This flavoprotein becomes oxidized as it passes its electrons and protons to the respiratory chain. As the electrons and protons pass through the chain, 2 moles of ADP are phosphorylated to ATP with the eventual reduction of atomic oxygen to water. Rotenone is added to mitochondrial suspensions to limit the observation, during a respiratory study, to only the oxidation of succinate and subsequent ATP production. Rotenone is a specific inhibitor of NAD-linked substrates and thus blocks the production of ATP from all other substrates excepting succinate.

When succinate is added with rotenone, the highest theoretical value for the P/O ratio is 2,0. The observed experimental value is usually less than 2, but the closer the value the greater the degree of intactness of the respiratory chain and citric acid cycle with all the necessary enzymes present for both. Such mitochondria have good respiratory control and are termed to be "tightly coupled."

The equipment for the experiments consisted of a Clark oxygen electrode linked to a chart recorder which had an adjustable time scale. The electrode was tested by adding a mixture of baker's yeast and glucose to an air-saturated water medium (Fig. 25). The resulting chart recorder tracing shows there was a nearly linear decrease in oxygen. This led eventually to amaerobic conditions brought on by the constant respiration of the yeast.

With the accuracy of the electrode established, the experiments for the P/O determination could begin. The experimentation began using rat liver mitochondria as a standard, to which later experiments on slime mould mitochondria could be compared. Figure 26 shows the tracing for the P/O ratio determination for the RLM. To 3 ml of the reaction medium, a 0,5 ml mitochondrial suspension was added to give a final concentration of 2,1 mg protein/ml. The reaction medium was air saturated and the same medium was used for the final washing and suspension of the mitochondria during centrifugation.

Fig. 25. A chart recording tracing showing the linear decrease in the oxygen content when a solution of baker's yeast and 5% glucose is added to 3 ml air-saturated water mixture. The immediate decrease in the oxygen content is due to the anaerobic conditions of the yeast-glucose solution.

Fig. 26. A tracing showing succinate and ADP stimulated respiration by rat liver mitochondria with 2,1 mg protein/ml present. The reaction medium and additions (viz. shown by arrows) are described in the text. For the P/O calculations, the respiration after the second ADP addition (viz. covered 17 vertical divisions) was used. For Fig. 26-29, the additions (arrows) indicate the final concentration for the chemical added.

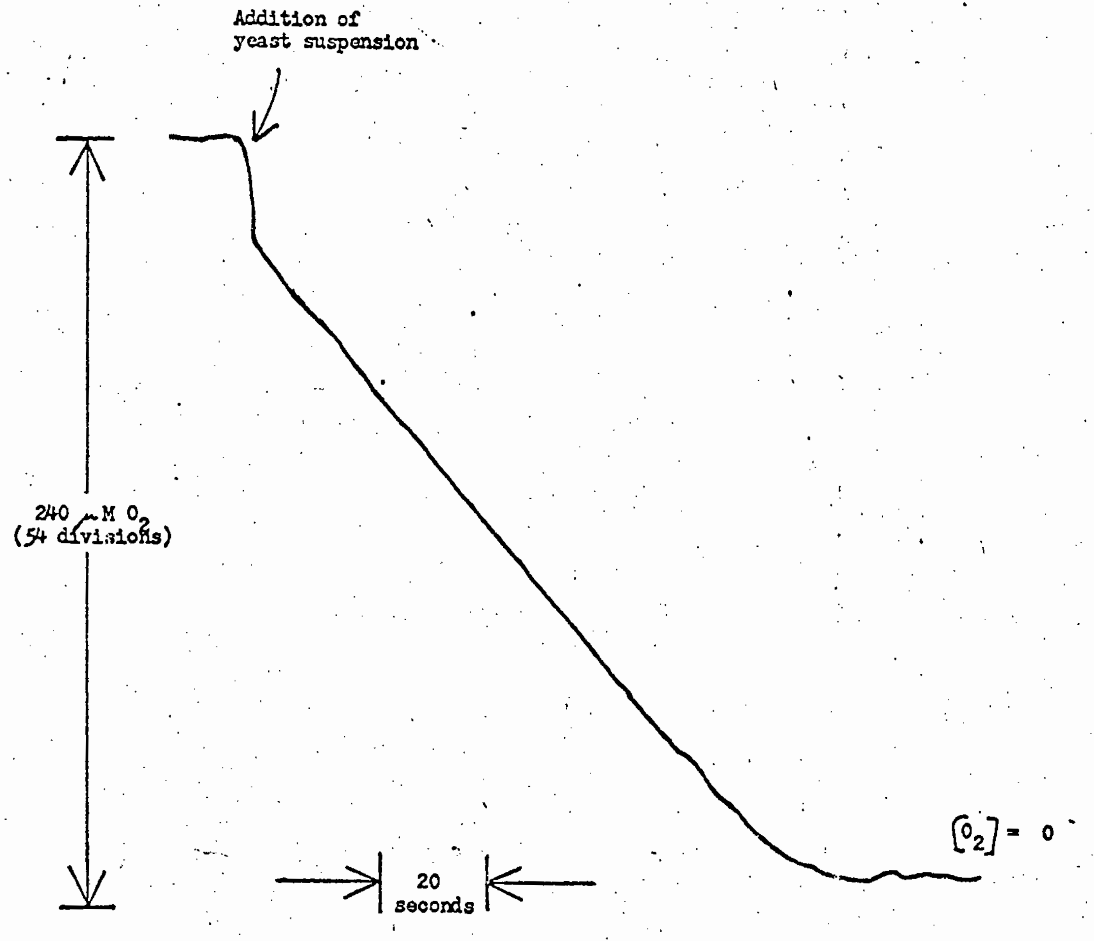


Figure 25

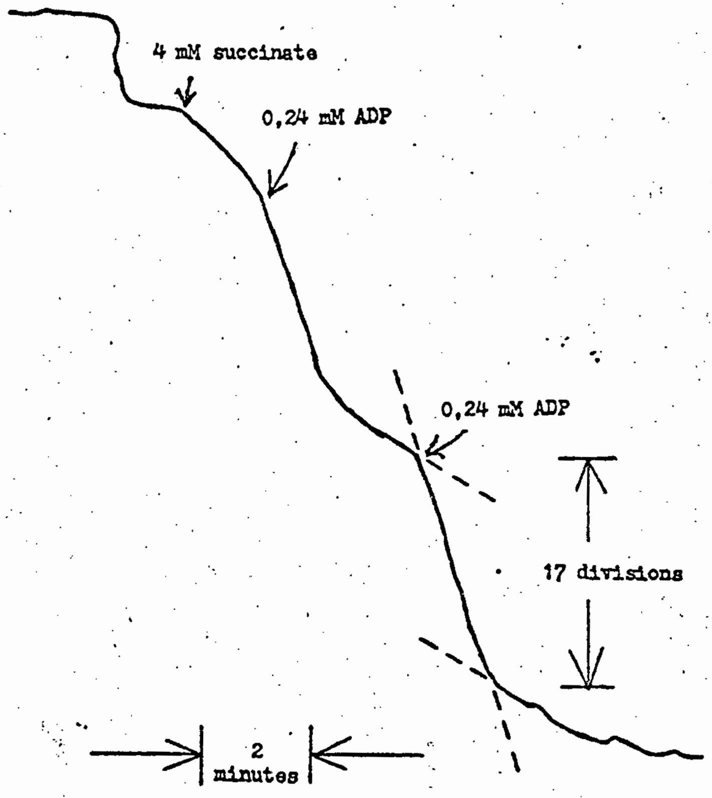


Figure 26

There is an initial drop in the oxygen measured as the anaerobic mitochondrial suspension was added, followed by nearly zero endogenous respiration. At the first arrow (see Fig. 26), 20 µl of 0,35 M succinate was added as substrate. The respiration increased and reached a steady rate. Following that, 50 µl of 0,17 M ADP was added and a very rapid rate of respiration ensued. The succinate respiration resumed after the ADP added was exhausted. The same amount of ADP was again added at the third arrow and that amount nearly reduced the oxygen level to zero. The second addition of ADP was used for determining the P/O ratio.

Since the divisions of the chart recorder paper reflect the oxygen consumed, it is necessary to find the percentage of the total oxygen consumed and multiply that figure by the actual oxygen content of the saturated medium at the start. There were a total of 54 divisions and the ADP respiration occurred during 17 divisions or covered 31% of the distance. If 240 µM of O_2 were in the reaction medium initially, then 31% of the oxygen consumed is 75,5 µM or 151 µ-atoms of oxygen. Adding 50 µl of 0,017 M ADP gave a final concentration of 0,24 mM or 240 µM or 240 µM of ADP. The ratio of ADP consumed to oxygen is found by dividing 240 µM by 151 µ-atoms of oxygen, giving the answer of 1,59. This figure compares favorably with the 1,80 value of Chance and Williams (1955) and the 1,72 one of Hagihara (1961).

After experience had been gained with RLM, similar experiments were performed on slime mould mitochondria (SMM). Figure 27 shows a tracing for SMM that followed the same pattern of additions as in Fig. 26. Because of the extreme slowness of the respiration, the chart recorder speed was set at 5 min/cm, as against 1 min/cm for rat liver. The addition of the mitochondrial suspension brought the total volume to 3,4 ml and a mitochondrial protein concentration of 2,0 mg/ml. Fol-

Fig. 27. A tracing showing succinate and ADP stimulated respiration by slime mould mitochondria with 2,0 mg protein.ml present. The reaction medium and additions (shown by arrows) are described in the text. For the P/O calculations, the respiration after the first ADP addition (covering 20 divisions) were used.

Fig. 28. Previous to the addition of the RLM suspension, 50 µl of 0,023 mM rotenone and 20 µl of 0,035 M succinate were added. RLM were added (2,2 mg protein/ml) and followed by the indicated additions of Ca^{++} and succinate. Note the increased respiration after the first calcium addition, indicating the stimulating effect of Ca^{++} on respiration during Ca^{++}-accumulation by the mitochondria.

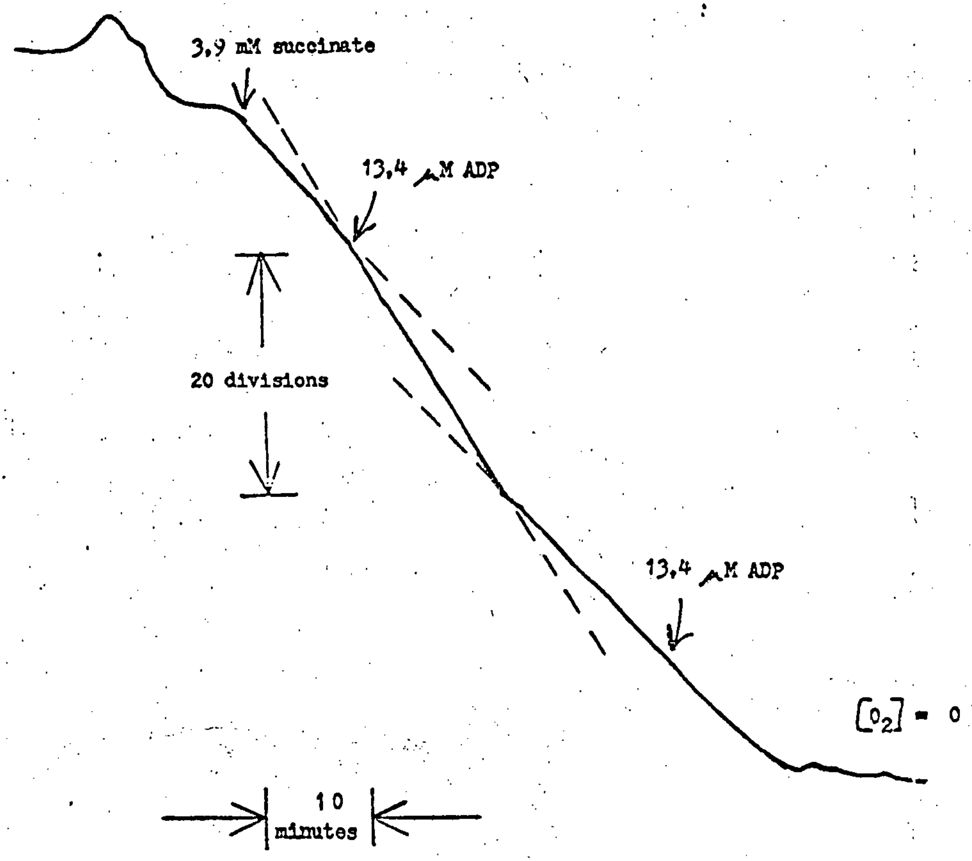


Figure 27

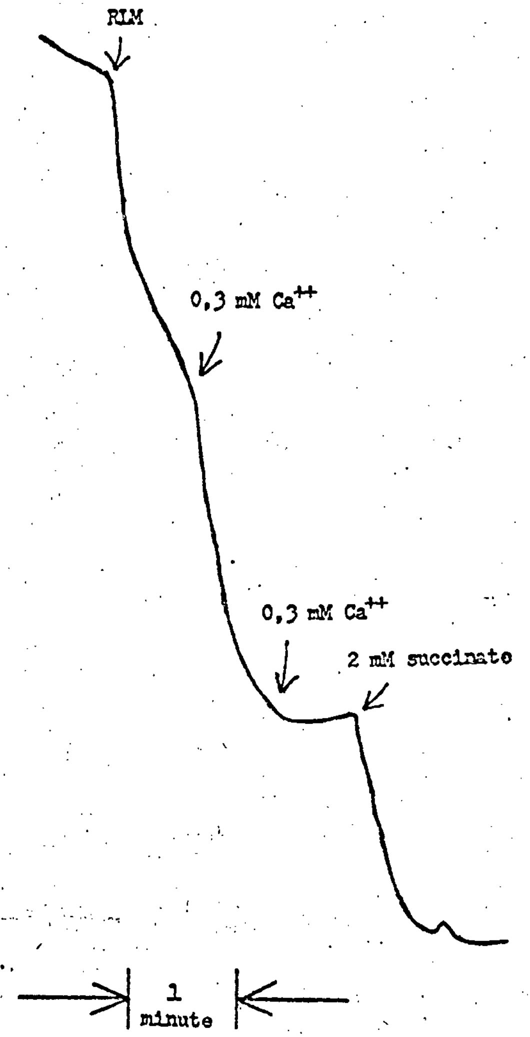


Figure 28

lowing a slight decrease in the oxygen content there was no endogenous respiration. Adding 20 µl of 0,67 M succinate brought a slow constant respiration indicating the slow oxidation of the succinate. Ten minutes later, 10 µl of 4,5 mM ADP was added and the mitochondria showed a slight increase in the respiration rate when oxidative phosphorylation was expected. Thirty minutes later, after a noticeable return to succinate respiration, 10 µl of ADP was again added but no change in rate of respiration was observed. Eventually, anaerobic conditions were reached.

The total vertical distance covered was 54 divisions and represented the 240 µM of oxygen consumed. The ADP respiration covered 20 divisions or 37% of the total. Therefore 37% of 240 µM gives 89 µM O_2 or 178 µ-atoms of oxygen taken up. Adding 10 µl of 4,5 mM ADP gave a final concentration of 0,0134 mM or 13,4 µM of ADP. Dividing 13,4 µM by 178 µ-atoms gives the answer of 0,075 for the ADP/O (viz. P/O) ratio.

This figure is twice the figure of 0,03 derived by Barnes _et al_. (1973) but both reflect the extremely poor phosporylation ability and the fact that the SMM were not "tightly coupled." Barnes and his coworkers interpreted the finding that NAD greatly stimulated the mitochondrial rate whereas mitochondria are normally impermeable to nicotinamide nucleotides, as meaning that the mitochondria were probably not intact. Five separate mitochondrial isolations and P/O determinations produced an average of 0,06. The increased oxidative phosphorylative ability over that reported by Barnes _et al_. (1973) may be interpreted as coming from the differences in the isolation procedure.

b. Ca^{++}-stimulated respiration by polarography

Rat liver and slime mould mitochondria have shown P/O values which conform approximately to published values. Increased respiration with the addition of Ca^{++} is a criterion that can be used for determining whether the ability for energy-linked Ca^{++} transport exists in the mitochondria. Chance (1963) and Reed and Bygrave (1974) used this method to assess Ca^{++} transport in RLM and to establish whether the respiration rate was affected by Ca^{++} and agents that interfere with Ca^{++} transport. Polarographic measurements of respiring RLM show this effect clearly and will be used to compare the behavior of RLM to that of SMM.

To a reaction medium of 0,25 M sucrose, 5 mM Tris and 5 mM K_2HPO_4 at a pH 7,4, 50 µl of a 0,023 mM ethanolic solution of rotenone (Sigma Chemical) and 20 µl of 0,35 M succinate were added. The rotenone inhibits the ATP production from any other substrates other than succinate and aids in the full oxidation of succinate. To 3 ml of the above medium, 0,5 ml of the RLM was added bringing the total concentration of mitochondrial protein to 2,2 mg/ml. The reaction was initiated by the addition of the mitochondria and after a steady rate of succinate respiration was achieved, 20 µl of 0,05 M $CaCl_2$ was added (see Fig. 28, first arrow). As can be seen, there was an immediate deflection indicating a marked increase in the respiration rate. With the exhaustion of the succinate, the respiration rate fell to a barely perceptible level. Another 20 µl of calcium induced no change but adding 20 µl of succinate resumed the respiration until it reached anaerobic conditions. This effect of increased oxygen consumption illustrates the stimulating effect of Ca^{++} on the respiration rate and shows when there is accompanying Ca^{++} transport into the mitochondria (Lehninger, 1970).

This property has been shown to exist in all mammalian mitochondria, those of higher plant cells and of the fungus *Neurospora crassa* (Lehninger, 1970, Carafoli, 1973). So far however, such activity has not been determined for slime mould plasmodial mitochondria. Admit-

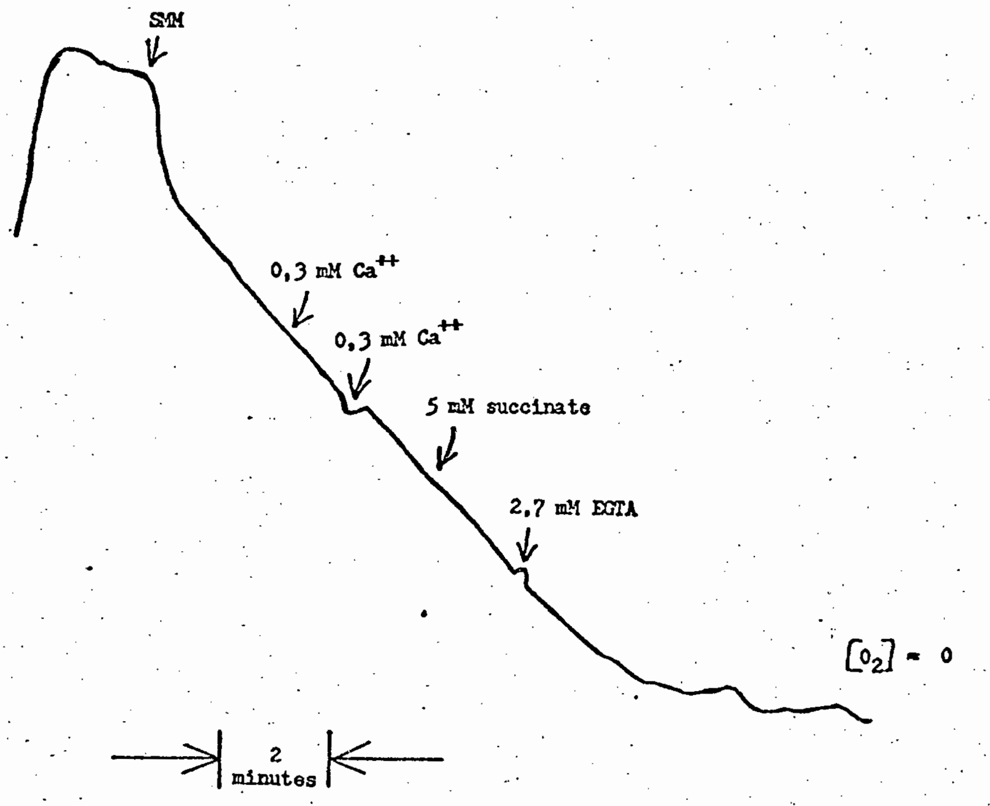


Figure 29. The same initial additions of rotenone and succinate were made to the reaction medium as in Fig. 28. SMM were added (1,8 mg protein/ml) and followed by the indicated additions of Ca^{++}, succinate and EGTA. Note the lack of any increased respiration when Ca^{++} is added and the lack of any decrease in respiration when Ca^{++} is removed when EGTA is added.

tedly, the isolation procedure is difficult and the low P/0 ratio suggests that a large percentage of the mitochondria may not be intact. However it was of interest to repeat the above experiment and try to determine if the respiration of slime mould mitochondria could be increased by adding Ca^{++}.

The same reaction medium was employed as with the RLM, except that 0,5 M sucrose was used. Barnes et al. (1973) had previously reported that succinate oxidation was not affected by the inhibitor rotenone and that it inhibited malate and NAD oxidation. Therefore the succinate respiration should be unaffected by the addition of rotenone. To 3 ml of the reaction medium 0,6 ml of mitochondrial suspension was added to make a total concentration of mitochondrial protein of 1,8 mg/ml. As can be seen in Figure 29, after the mitochondria were added a steady respiration rate ensued under succinate oxidation. At two separate points (arrows) 20 µl of 0,05 M Ca^{++} was added but there was no observable effect. Additional substrate (50 µl of succinate) produced no increase in the respiration and 20 µl of 0,5 M EGTA (pH 7,4) produced no effect.

The lack of any observable increase in respiration when Ca^{++} was added must be interpreted as meaning that SMM under these conditions, do not accumulate Ca^{++} to an appreciable extent. The same lack of stimulation was also observed where oxidative phosphorylation was expected upon addition of ADP. This was confirmed when an excess amount of the EGTA was added to the medium. The respiration rate did not change when the available calcium was suddenly removed from the medium.

3. Chemotactic response of plasmodia with changing external $[Ca^{++}]$ in half plate assays

The aim of the experiments was to determine whether: (1) plasmodial migration (viz. direction of movement) was affected by the predetermined pCa in the agar substrate, (2) plasmodia can distinguish between equal concentrations of Mg^{++} or Ca^{++}, and (3) the chemotaxis of plasmodia is affected by the external pCa of the agar substrate.

Ionagar (Oxoid, Code L12 purity) is widely used for electrophoretic studies because of its purity and gel strength and for these reasons it was employed in the present work. Although the Oxoid manual gives an "average" batch analysis of 150-200 ppm for the calcium content of their purified agar, it was felt that an analysis of the calcium content should be repeated, since excessive calcium and potential binding sites of the agar could be major interfering factors in maintaining the calculated pCa values. Therefore the calcium content of the agar was analyzed by flame atomic absorption and by murexide spectrophotometry.

For analysis by atomic absorption, the powdered agar was dessicated by storing with silica gel for two days. Three samples were prepared by mixing 1,5% w/v with: (1) twice distilled water, filtered and using the filtrate, (2) 0,1 M HCl and 0,1 M HNO_3 and boiled for 15 minutes and, (3) 1 mM EGTA (pH 7,0) for 15 minutes, filtered and using the filtrate.

The samples, free of solid matter, were analyzed with a Varian Techtran, Model AA6 and the calcium content (expressed in parts per million-PPM) was determined by comparison with appropriate standards. The results are shown in Table 3 for each respective treatment. As can be seen the dissolved acid treated agar had the greatest amount of calcium. The other two samples that were filtrates had approximately half the amount of calcium. The difference in analysis figures represents the free calcium of the filtrates as against the total inherent calcium in the agar. For the half plate assays, the total $[Ca^{++}]$ of the agar substrate was 10^{-2} M. The atomic absorption analysis showed that a mixture of 1,5% agar would contribute approximately 10^{-5} M of calcium as compared to the 10^{-2} M added in the preparations. From the calculations shown in Section 3 of the Materials and Methods, the amount of calcium inadvertently added becomes insignificant and does not alter the calculated pCa value of the agar.

Table 3. Atomic absorption analysis of agar or filtrate after acid and Ca^{++} chelation treatment.

Treatment	Calcium concentration	
	µg/g agar (ppm)	Molar (1,5% w/v agar)
(1) Distilled H_2O/filtered	325	$8,1 \times 10^{-6}$
(2) 0,1 M HCl, HNO_3 & boiled for 15 min	820	$20,5 \times 10^{-6}$
(3) 1 mM EGTA (pH 7,0) & mixed for 15 min/filtered	410	$10,3 \times 10^{-6}$

An alternative method to atomic absorption, which measures the total calcium, is murexide spectrophotometry. As discussed in Section 6 of the Materials and Methods, murexide readily complexes with free Ca ions and the consequent absorbance decrease is measured by using a dual wavelength spectrophotometer. This method is therefore able to determine the free Ca^{++} vs. the bound calcium in the unmelted agar preparation. The agar preparation can be added to a cuvette (1,5% w/v) and kept suspended by using a vibrating coiled platinum electrode. The effect of light scattering is largely avoided because of the use of a dual wavelength spectrophotometer.

Figure 30 shows the additions of 10 µl aliquots of 5 mM Ca^{++} to a blank. Each addition produced an almost identical absorbance decrease. After the third addition, an excess of EGTA was added and the absorbance returned to its starting level. In Fig. 32, 1,5% agar was added which caused a small drop in absorbance. This was followed by an addition of 10 µl of 5 mM Ca^{++} which alone would have produced a final concentration in the 3 ml cuvette of 16,6 µM. The decrease in absorbance was approximately 2,3 cm and is the same as the other additions in Fig. 30. The agar caused an absorbance lowering of 1 cm.

Since the length of the absorbance decrease is in direct proportion to the amount of Ca^{++} added, the decrease that occurred when the agar was added corresponds to a final $[Ca^{++}]$ of $7,2 \times 10^{-6}$ M (7,2 µM). This figure confirms the results of the atomic absorption analysis. The prompt rise to the initial absorbance level when the excess EGTA is added, indicates that the EGTA readily chelates all the available Ca^{++}, including the calcium added with the agar. This observation also suggests that the chemical groups in the agar bind Ca ions less strongly than EGTA and that these groups do not interfere appreciably with either the equilibrium reaction for the chelation of Ca^{++} by EGTA or the final pCa value of the agar. It may be concluded from these experiments that the Ca^{++} content of the agar will not interfere or alter adversely the pCa values used.

The next prerequisite was to determine the upper limits of $[Ca^{++}]$ and [EGTA] which would repel plasmodia. Concentrations of 1, 5 and 10 mM of Ca^{++} were used without adding EGTA for buffering the agar and likewise, concentrations of 0,5, 1 and 5 mM EGTA were used without the addition of calcium. Opposite the plate halves containing either Ca^{++} or EGTA, were "blanks" made with freshly distilled water, 5 mM

Fig. 30. Murexide spectrophotometry showing sequential additions of Ca^{++} to a distilled water blank containing 35 µM murexide. For Fig. 30-31, the additions (arrows) indicate the final concentration for the chemical added. Note the constant decrease in the absorbance with each Ca^{++} addition.

Fig. 31. Murexide spectrophotometry showing the drop in absorbance when 1,5% w/v of agar is added to the cuvette. This is followed by two additions of Ca^{++} and EGTA. The agar is kept suspended by a vibrating electrode and the interference from light scattering is avoided by the subtraction of the absorbance at 510 nm from that of 540 nm.

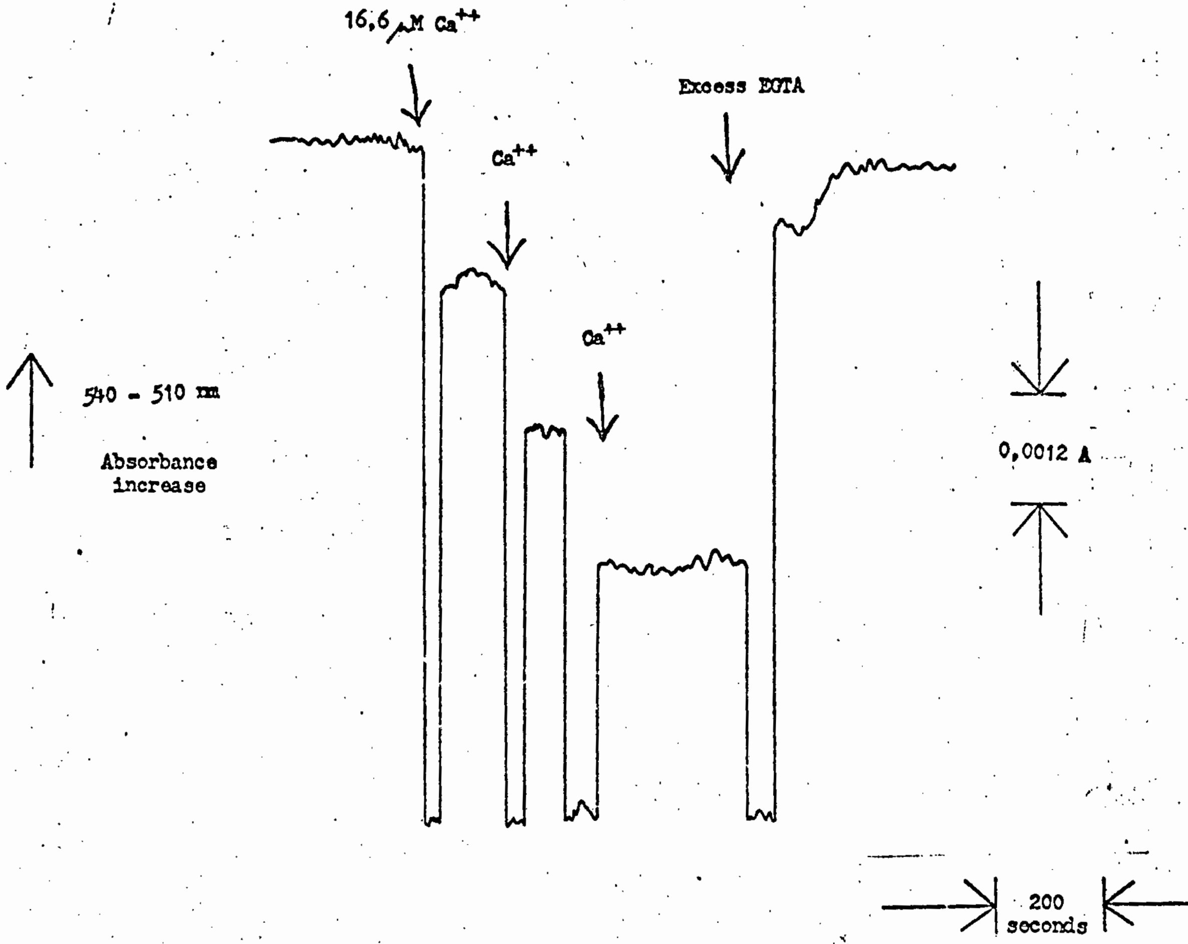


Figure 30

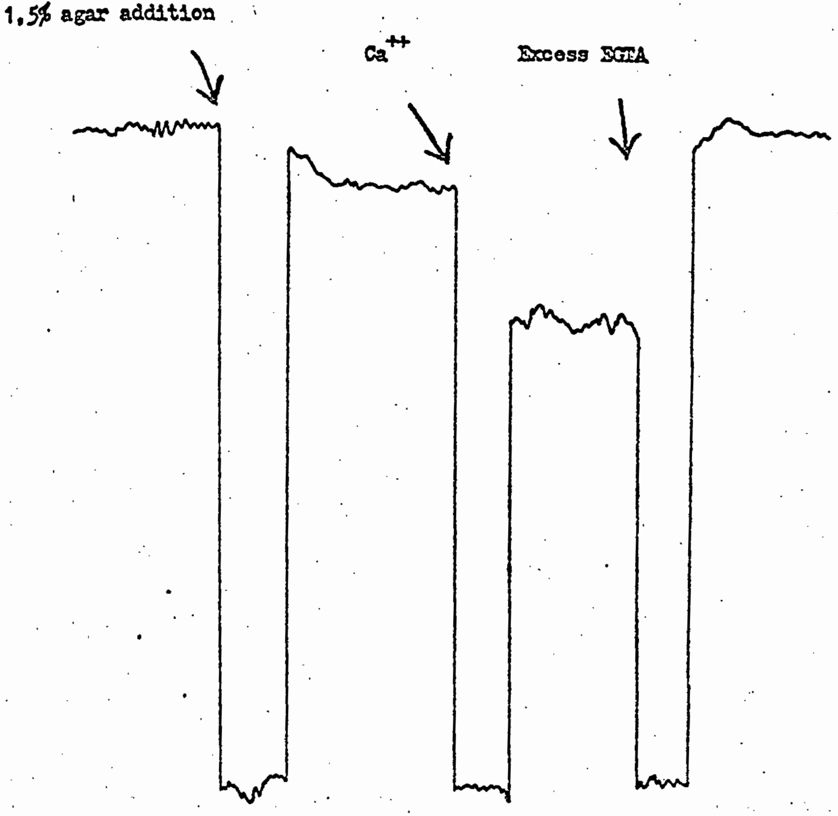


Figure 31

Tris (pH 7,0) and 1,5% agar. These tests were followed by a single experiment to test the plasmodia's response to either 5 mM Mg^{++} or Ca^{++}. Usually 20 plates were used for each half plate assay. For plates in which it was difficult to decide to which half the plasmodia has moved (i.e. when spread equally to both sides or had died), the results were classified as "Indeterminate" and counted separately from the other plates. The results are given in Table 4 below.

Table 4. Half plate assays demonstrating repelling concentrations of Ca^{++}, EGTA and Mg^{++}.

I Ca^{++}	Total No. plates	No. positive with 'taxis to Ca^{++} half	Percentage of plates counted	"Indeterminate" (Not counted)
1 mM	20	8	8/16X100 = 15%	4
5 mM	24	4	16%	0
10 mM	20	0	0%	0
II EGTA		No. positive with 'taxis to EGTA half		
0,5 mM	20	8	8/14X100 = 57%	6
1 mM	20	6	30%	0
5 mM	20	0	0%	0
III Mg^{++} & Ca^{++}		No. positive with 'taxis to Ca^{++} half		
5 mM apposing halves	20	6	6/13X100 = 46%	7

The results of these tests indicate that a Ca^{++} concentration between 1-5 mM has the effect of repelling plasmodia. The range of EGTA concentrations used leads to repelling because of the severe leeching of Ca ions from the underside of the plasmodia. There is no apparent effect on plasmodial migration with 0,5 mM EGTA, but some influence begins at

1 mM and there is a marked effect at 5 mM. The juxtaposition of media with respective concentrations of 5 mM for Mg^{++} and Ca^{++}, both equally buffered (pH 7,0) demonstrates that the plasmodia prefer neither.

Since 5 mM of Ca^{++} has been found to repell, this equality of action for Mg and Ca ions indicates that the plasmodium cannot distinguish between the two cations and is repelled by both. This inability to differentiate between the two cations was first shown by Ueda et al. (1975). Beginning at the threshold point (0,1 mM), the membrane depolarizes in a linear fashion with increasing concentrations of the cations: Ca^{++}, Mg^{++}, Mn^{++} and Ba^{++}. Ueda and his coworkers also showed, using changes in the motive force as the criterion, that the chloride salts of Ca^{++} and Mg^{++} among other ions acted as repellents. Thus the repelling effect of Ca^{++} is not specific, but has a common action with other cations, namely that it changes the membrane potential (depolarization). EGTA repells because it leeches Ca ions from the external membrane and thereby damages the membrane's integrity.

a. Effect of differing pCa on plasmodial migration

Four experiments were done to establish whether migrating plasmodia were influenced by the concentration of free Ca^{++} of the substrate. Fresh accumulations of plasmodia were used and once on new agar media, these readily reverted back to the active migrating form. The pCa values for each half of the petri dishes are shown in the first column of Table 5.

Except for the second series of plates tested (pCa 5-6), the percentages show that there was an overall tendency of the plasmodia to migrate towards the half with the lower $[Ca^{++}]$. This test was meant only as an attempt to discover obvious preferences and a large number of tests together with a statistical analysis would be required to determine if this tendency is significant.

Table 5. Half plate assays demonstrating plasmodia's lack of ability to distinguish external [Ca^{++}] of agar substrate.

Apposing pCa halves	Total No. plates	No. plates with 'taxis toward higher pCa	Percentage of plates counted	"Indeterminate" (Not counted)
4-5	22	6	6/17X100 = 35%	5
5-6	20	9	64%	6
6-7	21	6	43%	7
4-7	21	7	47%	6

b. Effect of differing pCa on plasmodial chemotaxis

These tests differ from those in the previous section in that 1 mM glucose is present in one of the two halves and the whole plate has the same pCa. They were designed to test whether the chemotactic movement of the plasmodia towards a known chemotactic substance (see example, Fig. 8) is altered by varying the [Ca^{++}] of the substrate.

Table 5. Half plate assays demonstrating chemotactic response towards 1 mM glucose with changing external Ca^{++} of the agar substrate.

pCa	Total No. plates	No. positive chemotaxis	Percentage of plates counted	"Indeterminate" (Not counted)
4	20	18	90%	2
5	20	17	85%	3
6	20	19	95%	1
7	21	18	85%	3
No Ca^{++} and 1 mM EGTA (pCa ~13)	22	12	75%	6

As Table 5 shows, for the pCa range tested, the chemotactic response was not altered by the different Ca^{++} concentrations. Only with a large excess of EGTA and no Ca^{++} (pCa ~13) did the plasmodia occasionally die ("Indeterminate" column) from the extreme leeching of Ca ions before it could reach the glucose half. In summary, the following conclusions can be made:

1) plasmodia were repelled by concentrations of 1-5 mM Ca^{++} and 5 mM EGTA,
2) plasmodia were repelled equally by, and hence could not distinguish between, the cations Mg^{++} and Ca^{++},
3) migration of plasmodia was not affected by changes to the Ca^{++} concentration of the agar in the pCa range of 4 to 7,
4) chemotaxis of plasmodia towards 1 mM glucose was not affected by changes in the pCa range of 4 to 7.

4. Murexide spectrophotometry of mitochondrial Ca^{++} fluxes

This section is devoted to the study of the extent of Ca^{++}-accumulation by rat liver and slime mould mitochondria (RLM and SMM respectively). The main reasons for the experiments with RLM were to gain experience working with mitochondria and to establish what the norm is for Ca^{++}-uptake as shown by murexide spectrophotometry. The later experiments with SMM stem from the fact that no research has been published on Ca^{++}-transport or accumulation by the mitochondria of plasmodia. There is indirect evidence, as discussed earlier in Section C of the Literature Review, to suggest that if such an ability was found to exist, the mitochondria could serve the important function of regulating the $[Ca^{++}]$ of the cytoplasm.

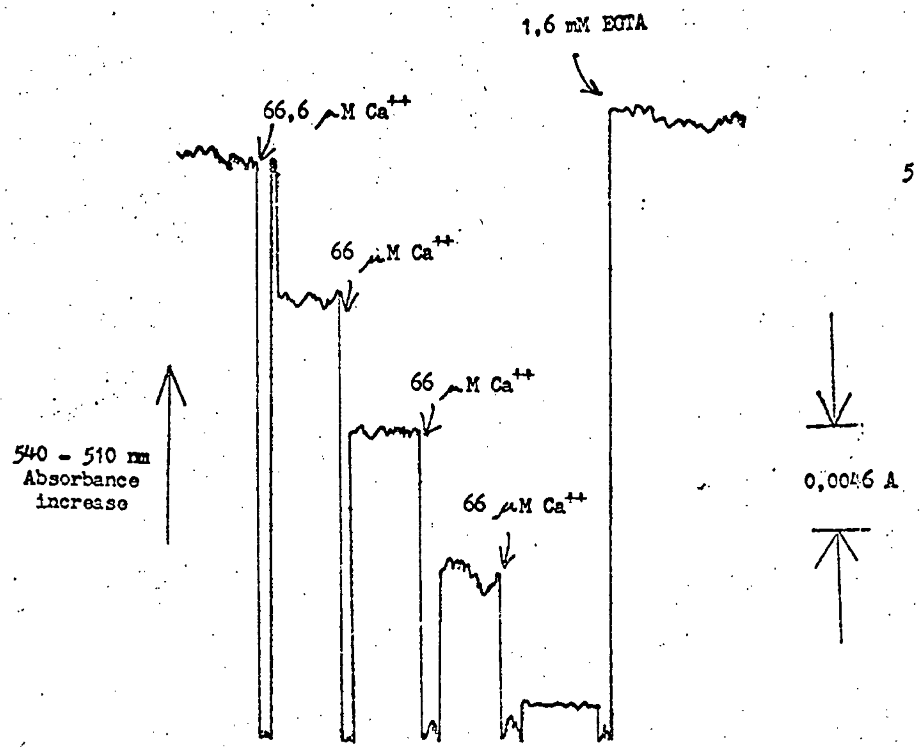
a. Rat liver

For the spectrophotometry, a uniform suspension of the mitochondria was maintained by constant agitation with a vibrating platinum wire electrode. The electrode did not interfere with the light beam path. Microliter amounts of the different concentrated chemicals were deposited on the end of a long-handled plastic "dip stick" which then stirred the contents of a cuvette inside the spectrophotometer. A few strokes of the stick assurred complete mixing and reduced the interruption of the absorbance reading to a minimum.

Figure 32 demonstrates the correlation between changes in Ca^{++} concentration and absorbance of murexide when repeated micromolar amounts (20 µl) of 10 mM Ca^{++} were added to a reaction medium containing 35 uM of murexide. With each 66 µM of Ca^{++} added, there is an absorbance decrease at 540 nm. This is because of the formation of the Ca^{++}-murexide complex which absorbs less than murexide alone. When 10 µl of 0,5 M EGTA were added, the absorbance returned to the initial level.

Fig. 32. Calibration of the murexide method for Ca^{++} determination in a medium devoid of RLM. The reaction medium (3 ml) contained 0,25 M sucrose, 5 mM K_2HPO_4, 5 mM Tris (pH 7,4) and 35 µM murexide. The subsequent additions of Ca^{++} and EGTA are reported in the figure. Arrows in Fig. 32-39 indicate the final concentrations for the additions made.

Fig. 33. Determination of the calcium content in successive additions of succinate, ADP and K_2HPO_4. The reaction medium (3 ml) contained 0,25 M sucrose, 5 mM Tris (pH 7,4) and 35 µM murexide. The additions made are as reported in the figure.



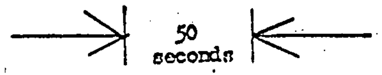


Figure 32

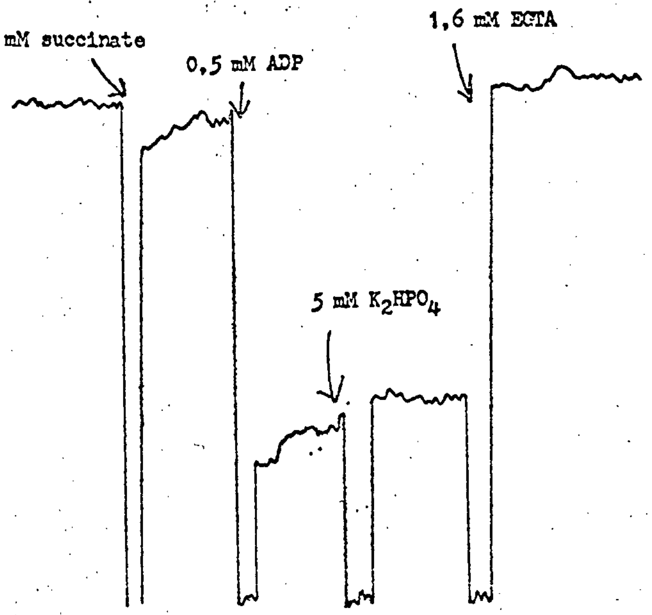


Figure 33

The speed of the return illustrates the ease with which the Ca^{++}-murexide complex dissociates.

It is an important prerequisite for the mitochondrial experiments to know the extent to which interference of the added reactants willaffect the Ca^{++}-murexide absorbance readings. The most significant interference would be the inadvertent addition of Ca^{++}. Therefore control tests were run with the reactants: succinate, ADP and K_2HPO_4. Figure 33 shows the sequential 40 µl additions of 0,37 M succinate, 37,5 mM ADP and 0,37 M K_2HPO_4. Of the three, only ADP produced a significant absorbance decrease, indicating the presence of increased Ca^{++} in the medium. The final addition of 10 µl of 0,5 M EGTA returned the absorbance to the level found before ADP was added.

Inesi and Scarpa (1972) pointed out that a major cause of error in such studies could be due to the fact that commercial preparations of ADP or ATP may contain appreciable amounts of calcium. This is what was observed in Fig. 33 and will have to be kept in consideration when ADP is used in later experiments on oxidative phosphorylation.

The main purpose of these experiments was to duplicate the conditions shown by polarography of energy-linked Ca^{++}-accumulation by mitochondria. Figure 34 shows a murexide absorbance tracing of Ca^{++}-accumulation by RLM during succinate-induced respiration. The total volume was 3 ml and there were 1,9 mg protein/ml present. The reaction medium consisted of 0,25 M sucrose, 5 mM Tris (pH 7,4) and 35 µM murexide. At the first arrow (see Fig. 34), 40 µl of 0,37 M succinate was added and followed by two later 50 and 40 µl additions of 10 mM Ca^{++} and 0,37 M K_2HPO_4 respectively.

The addition of succinate induces no change, but as is known from the polarographic studies, succinate initiates rapid respiration.

Fig. 34. Ca^{++}-accumulation by RLM under succinate-induced respiration. The reaction medium (3 ml) contained 0,25 M sucrose, 5 mM Tris (pH 7,4), 35 µM murexide and no phosphate was included. There is 1,9 mg protein/ml present. The sequential additions of succinate, Ca^{++} and K_2HPO_4 are as reported in the figure.

Fig. 35. The effect of rotenone on Ca^{++}-accumulation by RLM. The reaction medium (3 ml) contained 0,25 M sucrose, 5 mM K_2HPO_4, 5 mM succinate, 5 mM Tris (pH 7,4) and 35 µM murexide. There is 2,1 mg protein/ml present. The subsequent additions of Ca^{++} and rotenone are as reported in the figure.

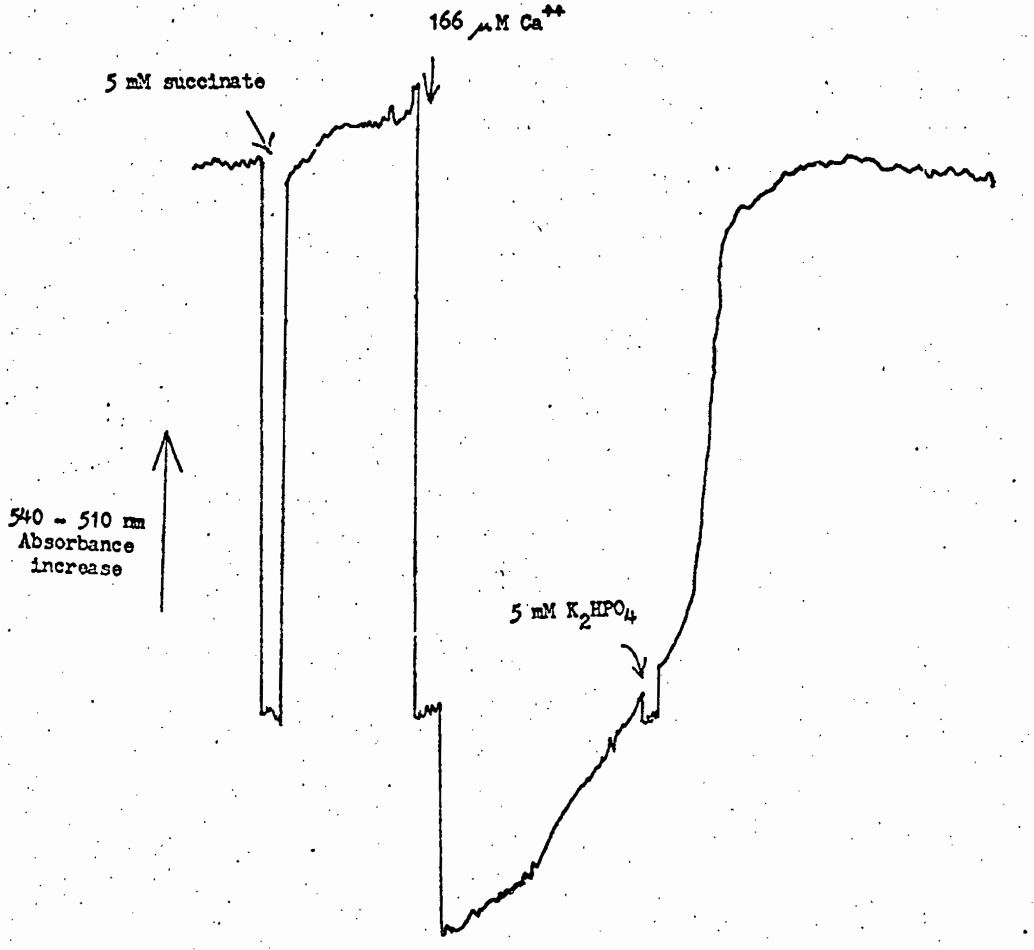


Figure 34

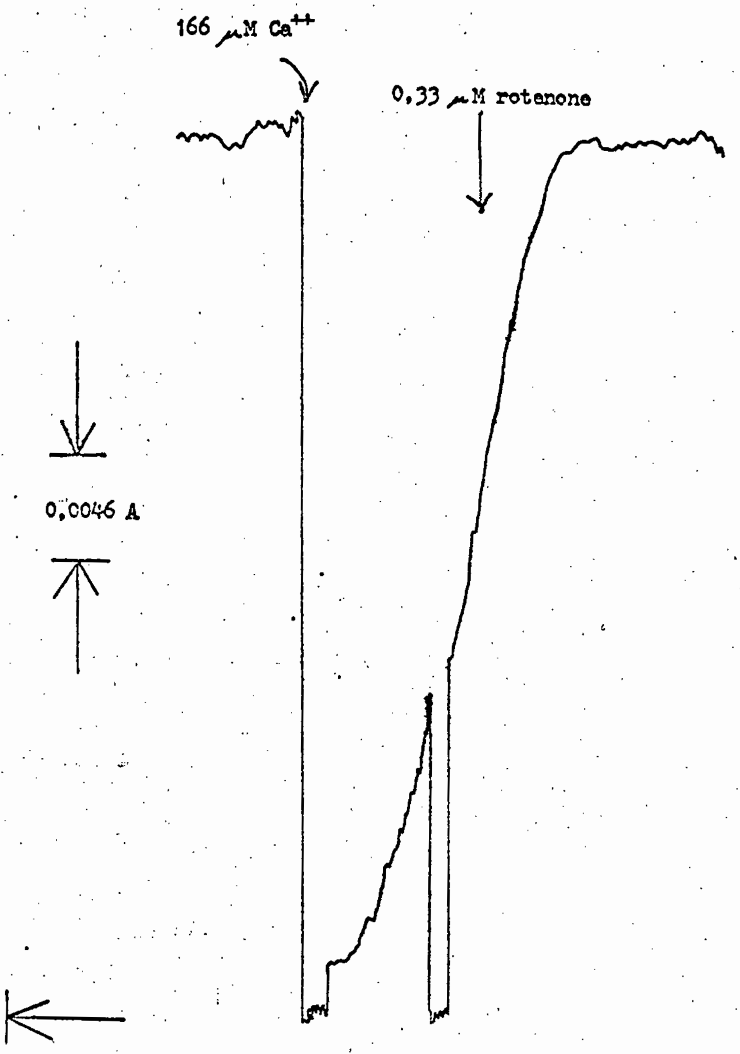


Figure 35

A minute later, 166 µM of Ca^{++} were added and caused an immediate deflection in absorbance. Immediately thereafter the mitochondria began to take up Ca^{++} at a constant rate. When phosphate ion (K_2HPO_4) was added, a sharp acceleration was seen until all the added Ca^{++} was sequestered.

It is known that phosphate may accompany the respiration-linked accumulation of Ca^{++} and that 1,7-2,0 Ca ions and about 1,0 phosphate ions are accumulated per pair of electrons traversing the respiratory chain (Lehninger, 1970). In the absence of phosphate or ATP or ADP, the succinate oxidation only allows limited Ca^{++} uptake (i.e. $\sim$80 nmol/mg mitochondrial protein)(Lehninger, 1970). In the presence of acetate or phosphate, the anion facilitates Ca^{++}-accumulation to the extent of 200-300 nmol/mg protein. This is what was observed in Fig. 34, viz. the accelerated Ca^{++}-accumulation because of the presence of a permeant anion (PO_4^{3-}).

Rotenone had no effect on succinate and Ca^{++}-accumulating respiration as shown in Fig. 35. The figure shows a tracing of an experiment in which rotenone was added during active Ca^{++}-accumulation by RLM. There was 1,8 mg protein/ml present and the total volume was 3,2 ml. The reaction medium was 0,25 M sucrose, 5 mM K_2HPO_4, 5 mM Tris (pH 7,4) and 35 µM murexide. At the first arrow, 50 µl of 10 mM Ca^{++} was added to the cuvette. Midway through the Ca^{++} accumulation, 50 µl of 0,023 mM rotenone was added. The addition of the rotenone caused no discernable change in the Ca^{++} -accumulation rate. This lack of interference with Ca^{++}-accumulation is the reason what Reed and Bygrave (1974) could use rotenone in their succinate Ca^{++}-stimulated respiration experiments.

As was seen in the previous polarographic studies (Fig. 26), when ADP was added and initiated oxidative phosphorylation, the respiration accelerated to a constant rate until all the ADP had been consumed. The addition of Ca^{++} was also seen to stimulate succinate-induced respiration. It would be of interest to know whether the rate of Ca^{++} uptake is influenced by the onset of oxidative phosphorylation. Figure 36 shows a repeat of the polarographic experiment in Fig. 26, in which, RLM under succinate respiration, undergo oxidative phosphorylation. The reaction medium is 0,25 M sucrose, 5 mM K_2HPO_4, 5 mM Tris (pH 7,4), 5 mM succinate, 0,33 µM rotenone and 35 µM murexide. The total volume is 3 ml and the total RLM concentration is 2,4 mg protein/ml. At the first and second arrows, 50 µl of 10 mM Ca^{++} and 50 µl of 17 mM ADP were added.

After the addition of Ca^{++}, the mitochondria began to accumulate the Ca^{++} actively. The ADP was added half way through the accumulation and it caused the absorbance to be reduced abruptly. Previous control experiments had shown that this was due to the unavoidable contamination of the purified commercial ADP preparation with calcium. The amount of deflection indicates that about 38,7 µM of Ca^{++} was added with the ADP and hence, the 17 mM ADP stock contained 2,3 µM Ca^{++}. Figure 36 demonstrates that the rate of Ca^{++} accumulation after the addition of ADP is the same as the initial uptake rate. Therefore the process of oxidative phosphorylation does not interrupt Ca^{++} uptake in rat liver mitochondria.

Lehninger (1970) showed that a third type of Ca^{++} transport could occur when phosphate and ATP or ADP are present. In this case, mitochondria become massively loaded (3000 nmol/mg protein) with precipitated calcium phosphate ($Ca_3(PO_4)_2$). No reason why ADP or ATP should bring about such a difference in Ca^{++} transport has been presented.

In this section, experience was gained in observing Ca^{++} accumulations by RLM using the murexide technique. The conditions shown

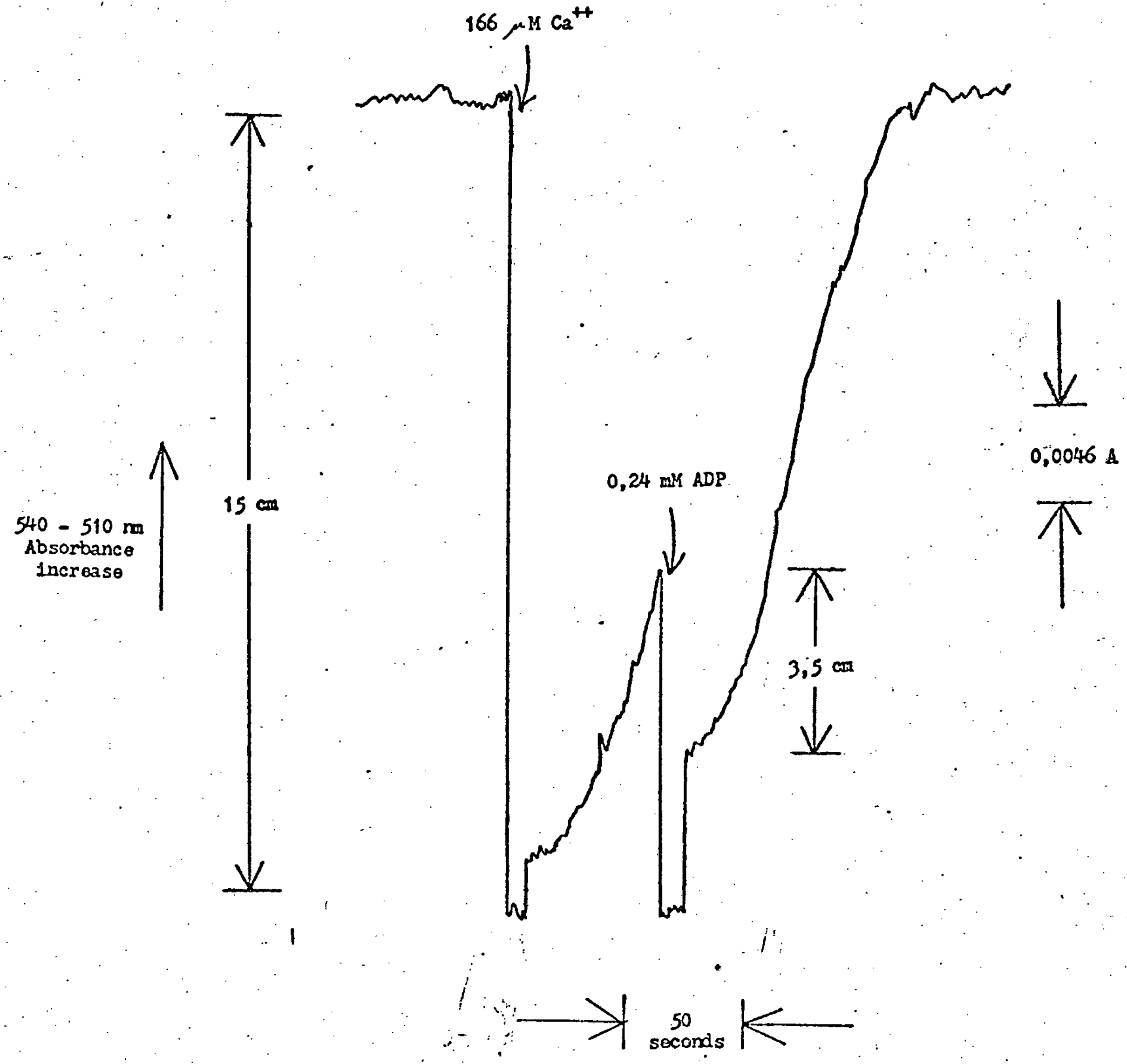


Fig. 36. Ca^{++}-accumulation by RLM during oxidative phosphorylation. The reaction medium is the same as in Fig. 35. There is 2,1 mg RLM protein/ml present. The subsequent additions of Ca^{++} and ADP are as reported in the figure.

to promote Ca^{++}-accumulation by RLM were then applied to SMM.

b. Slime mould

Slime mould mitochondria (SMM) of P. polycephalum were shown by polarography to have a slow respiration under succinate oxidation, a low P/O value and a rate of respiration which was unaffected by the addition of Ca^{++}. However, there have been no studies done to determine whether SMM have a Ca^{++}-accumulating ability akin to mammalian mitochondria. Although it must be assumed that the SMM have been damaged by the isolation procedure or naturally possess poor respiratory function, it would be of interest to know whether some potential for active Ca^{++}-transport exists during respiration. The existence of even a poor Ca^{++} uptake capability would suggest that the mitochondria could have a role in regulating the cytoplasmic $[Ca^{++}]$. As such, this proposed function of the ubiquitous mitochondria which were observed by Cheung et al. (1974) to be in the "peripheral cytoplasm of the advancing plasmodial fan" would concur with and fulfil the need for a regulating factor (i.e. cytoplasmic $[Ca^{++}]$, see Section C, Chap. 2).

To demonstrate whether a Ca^{++}-accumulating ability existed, SMM were subjected to the same succinate respiration and oxidative phosphorylation experiments as RLM. The mitochondria preparations were generally highly coloured owing to the presence of the characteristic yellow pigment produced by P. polycephalum. It is possible that these pigments and the "slime" (a galactose polysaccharide) are interfering factors in the absorbance readings of murexide. Sachsenmaier et al. (1973) reported that the living plasmodia and the extracted pigments had two absorption maxima, at 360 and 380 nm and little absorption in the region of murexide measurement (510 and 540 nM). The interference of the contaminating "slime" and pigments isolated with the mitochondrial fraction was determined in the following control test.

The SMM had previously been isolated in a medium of 0,5 M sucrose, 5 mM K_2HPO_4, 5 mM Tris (pH 7,4) and included 1 mM EGTA, which was omitted in the last wash and suspension. A thick mitochondrial suspension (4,4 mg protein/ml) was centrifuged briefly at 5000g to pellet the mitochondrial fraction. The yellow supernatant was transferred to a cuvette and murexide (35 µM) was added. Figure 37 shows the murexide absorbance tracing and to the cuvette were added four successive 10 µl aliquots of 10 mM Ca^{++} followed by a 10 µl addition of 0,5 M EGTA.

The first two additions of Ca^{++} caused the expected decrease in absorbance but was followed by a slight rise in the absorbance. The last two additions of Ca^{++} caused the absorbance to fall to nearly the same extent as the first two additions and there was no rise in the absorbance afterwards. When 1,6 mM EGTA (excess for chelation) was added, most of the added calcium was removed from the solution but some remained in the media as shown by the fact that the absorbance did not return to the initial level. The next control experiment shows more clearly the true absorbance changes that will occur without the interfering effect of the "slime" and pigments.

The reaction medium was the same as for the previous experiment but without the SMM preparation and with the same Ca^{++} and EGTA additions. The levels of the subsequent absorbance changes for the Ca^{++} and EGTA are indicated in Fig. 37 by regions of cross hatching. When the control and the experiment with the slime are compared, the presence of the slime and pigments appears to have two effects. The first is the sequestering of a small amount of the calcium that was added in the first two additions as shown when the absorbance did not decrease to the level without the contaminants (viz. the control). And when EGTA was added at the end of the experiment, the binding of the slime prevented the

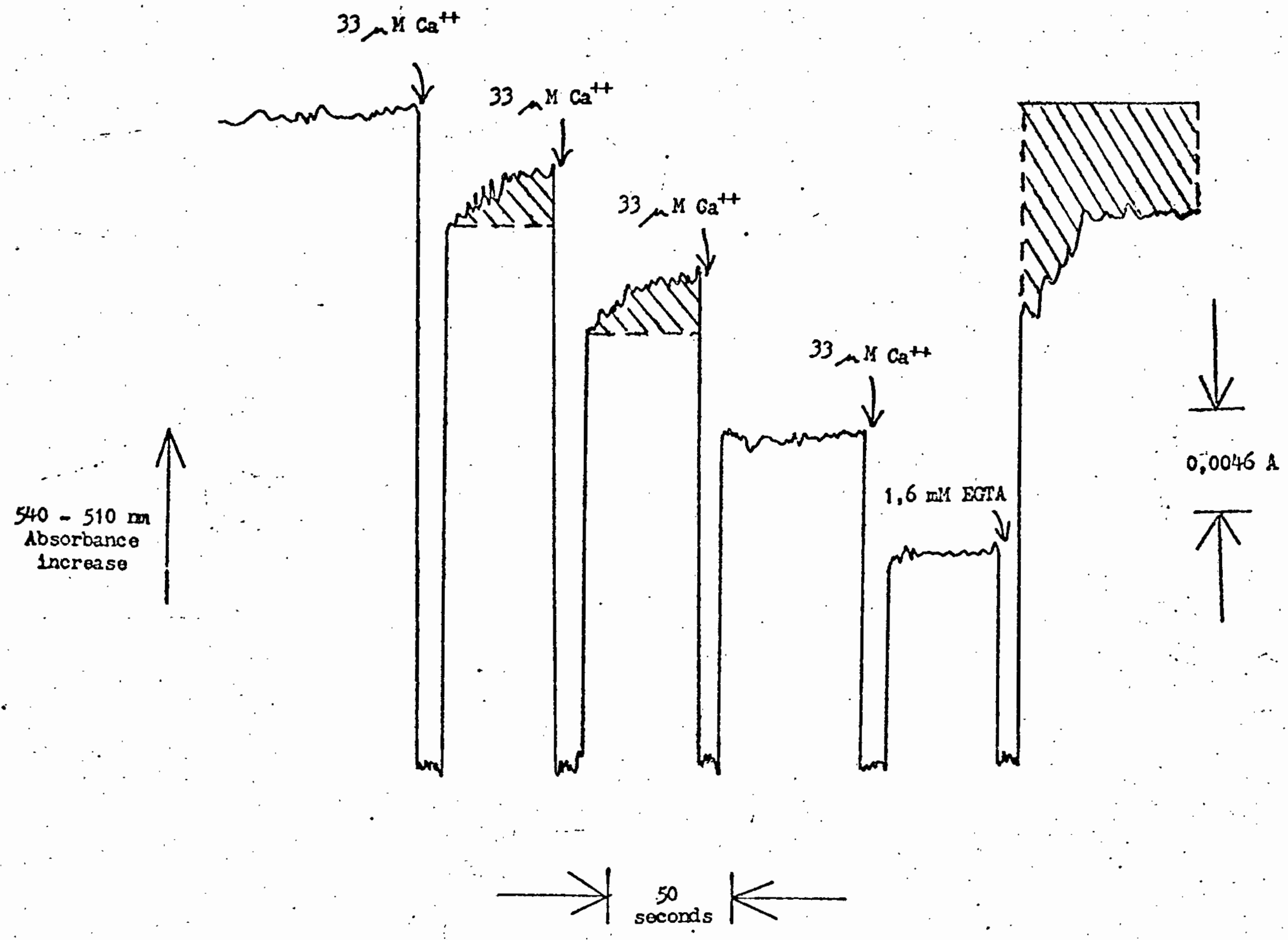


Fig. 37. Determination of the interference from the "slime" polysaccharide and pigments on murexide absorbance readings and Ca^{++} determination. Two experiments are presented: (1) main tracing indicates absorbance changes with "slime" supernatant present and, (2) a control without "slime" fraction present. The reaction medium in both experiments consisted of 0,5 M sucrose, 5 mM K_2HPO_4, 5 mM Tris (pH 7,0) and 35 µM murexide. The cross hatched area indicates differences in absorbance between the control and "slime" experiment.

removal of the Ca^{++} from the medium and the return of the absorbance to the initial level. These effects of the contaminants will have to be considered when interpreting the results of experiments with SMM respiration and Ca^{++} uptake.

To show possible Ca^{++} uptake under succinate respiration, a suspension of SMM was added to a cuvette with the reaction medium, to a total concentration of 2,2 mg protein/ml. The total volume was 3 ml and the reaction medium consisted of 0,5 M sucrose, 5 mM K_2HPO_4, 5 mM Tris (pH 7,4) and 35 µM murexide. As indicated in Fig. 38, successive additions were made of 40 µl of 0,37 uM succinate, 50 µl of 10 mM Ca^{++} and 10 µl of 0,5 M EGTA. Figure 38 shows that there was no absorbance change when succinate was added and a minute after the beginning of succinate respiration 166 µM of Ca^{++} was added. The calcium reduced the absorbance to a low level but the Ca^{++} was not taken up as rapidly as in the case of RLM (see Fig. 34 & 35). The absorbance rose slowly to 25% of the total absorbance decrease and leveled off. When 1,6 mM EGTA was added, the absorbance increased until almost all the calcium had been chelated.

The discrepancy between the Ca^{++} uptake ability of RLM and SMM may be due to the SMM having either a poor or non-existent Ca^{++}-accumulating ability. The contaminating slime and pigments bind Ca ions to a small and undetermined degree but most of the Ca^{++} is free to be accumulated by the mitochondria as is shown when EGTA is added at the end of the experiment (Fig. 38). The slow rise in absorbance seen after the calcium is added may indicate either slow binding of Ca ions by the slime or almost negligible accumulation by the damaged mitochondria.

Figure 39 shows an absorbance tracing of SMM undergoing oxidative phosphorylation. As indicated in the figure, the first and second

Fig. 38. Ca^{++}-accumulation by SMM under succinate-induced respiration. There is 2,2 mg protein/ml present. The reaction medium is the same as in Fig. 37 and the additions are as reported in the figure.

Fig. 39. The effect of rotenone and oxidative phosphorylation on Ca^{++}-accumulation by SMM. The reaction medium and SMM concentration were the same as in Fig. 38. The additions are as reported in the figure.

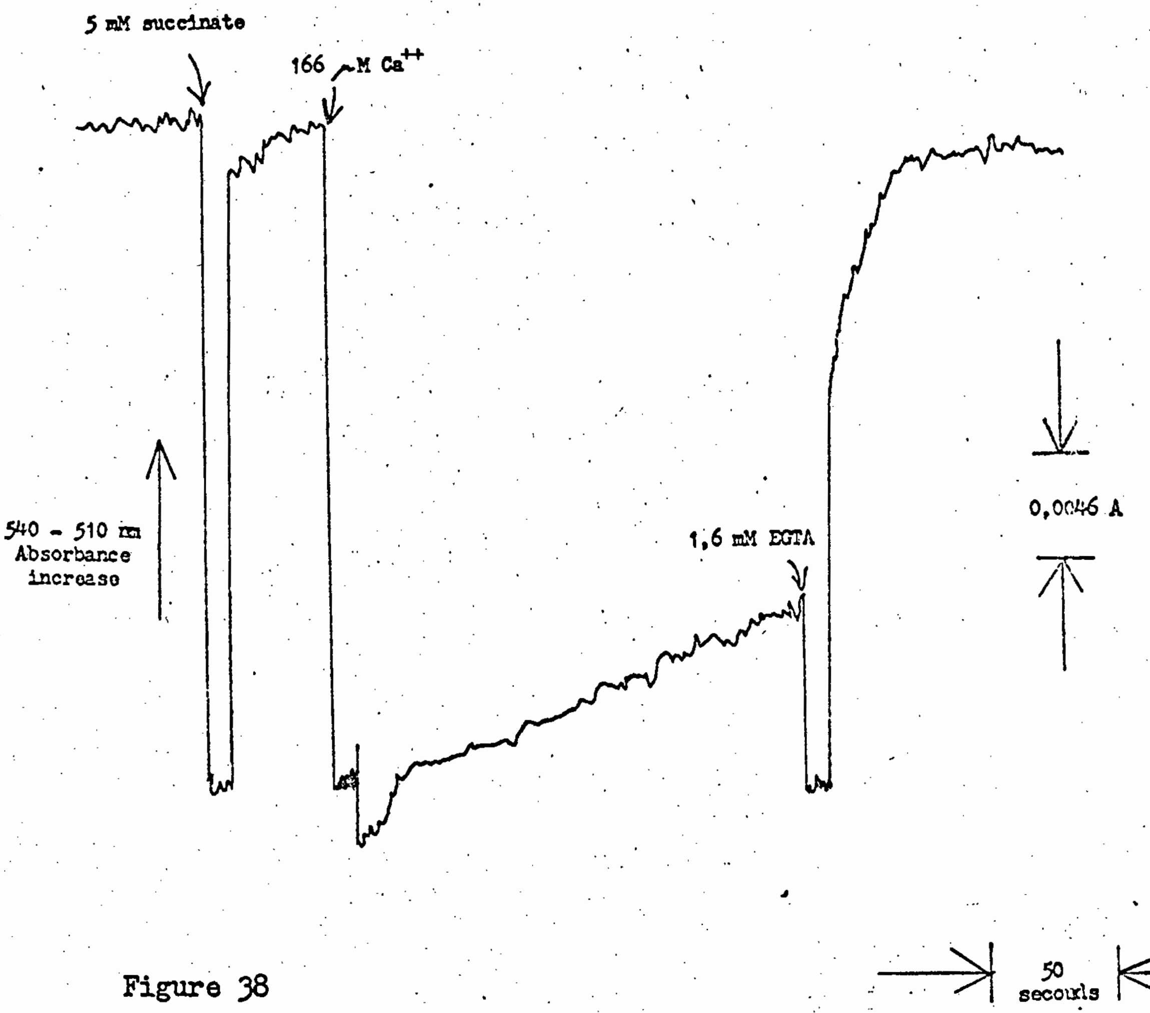


Figure 38

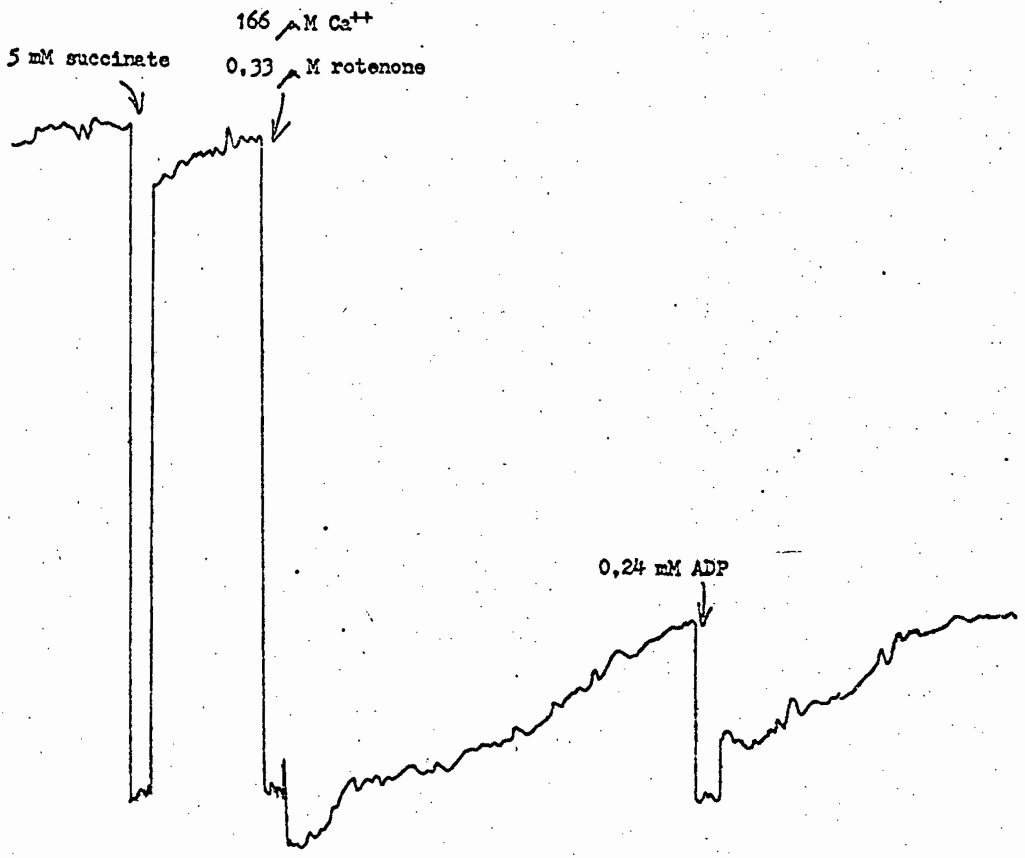


Figure 39

additions were 40 and 50 µl of succinate and Ca^{++} respectively, and the last was a combined addition of 50 µl of 20 uM rotenone and 50 µl of 14 mM ADP. The reaction medium and SMM concentration were the same as in the previous figure. The presence of rotenone had no effect on the apparent slow Ca^{++} accumulation. The ADP added reduced the absorbance because of the appreciable amount of calcium in the purified ADP as previously determined in a control experiment (see Fig. 36). The period of oxidative phosphorylation following the addition of ADP did not affect the slow disappearance of Ca^{++}.

The experiments described in Fig. 37-39 were chosen from five different trials, each using SMM preparations that varied because of the isolation procedure (viz. length of time for initial disintegration, filtration procedure, centrifugation). The murexide spectrophotometry confirmed the polarographic experiments in demonstrating that SMM do not actively accumulate Ca^{++} during respiration or oxidative phosphorylation. Further work will be necessary to see whether these conclusions should be quantified. Such work should involve a better isolation technique to obtain mitochondria with fully intact respiratory function and no "slime" contamination in the suspension.

The poor respiratory control of the mitochondria prevents one from completely ruling out the existence of any Ca^{++} transport. However, the above observations make it appear that SMM have no energy-linked Ca^{++} transport capability and that they are thus similar to fungal mitochondria. The results also do not support the idea that plasmodial mitochondria could have played a role in regulating the cytoplasmic $[Ca^{++}]$.

CHAPTER FIVE

DISCUSSION

For the reasons fully discussed in the literature review, the simplest model for chemotaxis would be to suggest that attractants increase the rate of pumping of calcium ions outwards across a cell's external membrane (Durham, 1974, 1976). This hypothesis would answer both questions of how the chemotactic substance affects the membrane locally and also how the recognition (chemoreception) is conveyed internally to the contractile mechanism for movement.

In order to test this model, the experiments in this thesis were designed to investigate: (1) the influence of chemotactic substances on the magnitude of Ca^{++} fluxes from the external membrane of a plasmodium, (2) the extent that the external $[Ca^{++}]$ affects plasmodial migration and, (3) whether plasmodial mitochondria possess the ability to regulate the internal $[Ca^{++}]$.

The results obtained with the flow-through chamber and half plate assays appear to rule out the importance of Ca^{++} fluxes in such a model. There was no appreciable long term change in Ca^{++} efflux in the presence of attractants, although the model predicted an increased sustained Ca^{++} efflux. Likewise, there was no change in the efflux in the presence of a repellent. From the results it may be concluded that any change in the Ca^{++} efflux that does take place during a chemotactic response represents less than 10% of the normal baseline efflux. However, a solution of 0,5 mM EGTA, perfused to chelate the calcium, caused up to a 200% change in efflux without noticeably affecting the motile oscillations. It was also observed that attractants could cause a brief physiological reaction (a "shock" period), while both attractants and repellents respectively increased and decreased the frequency of the motile oscillations.

The neutral (basal) medium used in the perfusion of the chamber (1 mM NaCl and 1 mM $CaCl_2$ at pH 7,0), was chosen to limit unspecific leeching of Ca ions from the plasmodium. It was assumed that the Ca^{++} present (pCa 3) in the solution would "cus the external surface from releasing its bound ^{45}Ca and the resultant counted ^{45}Ca efflux would reflect only physiologically linked fluxes.

The loss of Ca ions (^{45}Ca) may have either of two effects or both: if their origin is outside the external membrane, the ions lost are probably replaced by cations in the perfusion medium and thus there is no change in the surface charge. Hato <u>et al</u>. (1976) found that plasmodia have a membrane potential of -50 to -90 mv, being negative on the inside of the cell with respect to the outside. Hence, the other effect would be that if the Ca ions are from internal stores and are pumped out beyond the membrane, as indicated by the prolonged EGTA-induced efflux, then the membrane would become hyperpolarized. The change in potential would be counteracted to a certain extent since the existing surface charge (the sum of negative and positive charges) is thought to be reduced (viz. become less negative) because the Na^+ and Ca^{++} cations present in the perfusion medium tend to cancel the negative charges present. Since the membrane potential and surface charge are interdependent, this would tend to keep the membrane depolarized to a small but unknown extent.

Sugars are non-electrolytes and thus would not influence the membrane potential in this manner. The potassium ionophore valinomycin was used in conjunction with the flow-through chamber experiments to observe how the Ca^{++} efflux would change when the potential changed. In theory, the ionophore had the ability to transport K ions from the cytoplasm into the external medium, following the existing concentration gradient for potassium. Loss of the K^+ cations would result in increased negativity on the internal side of the membrane and thus cause the membrane to be hyperpolarized. The extent of the interference of the slime layer with the ionophore crossing the membrane remains unclear. If hyperpolarization is assumed, this had no observable effect on the Ca^{++} efflux or the motile oscillations (see Fig. 23). More will be said later about the action of sugars and cations on the membrane potential.

That Ca^{++} fluxes play an important role can also be ruled out by the lack of directed movement in response to changes in external Ca^{++} concentrations. This was illustrated by the experiments in which plasmodia migrated in the half plate Ca^{++}-EGTA buffer assays. These tests showed there was no preference for or avoidance of any of the pCa concentrations between 4 to 7. Furthermore, there was no preference for or ability to distinguish between 5 mM $MgCl_2$ and 5 mM $CaCl_2$, and plasmodia migrated towards 1 mM glucose just as readily in the presence as in the absence of 1 mM EGTA (pH 7,0).

Thus the outcome of these experiments supports the findings of Hatano (1970) and Ueda et al. (1975). Hatano found that cytoplasmic streaming in Physarum did not respond to changes in external Ca^{++} concentrations unless the external membrane's permeability to Ca ions was increased by initial treatment with caffeine. Ueda et al. observed that Ca^{++} affected the membrane potential and direction of movement in the same way as any other divalent cation. In contrast to the behaviour of Physarum, Chi and Francis (1971) found that Dictyostelium discoideum amoebae on a solid surface release calcium in response to the chemotactic attractant cyclic AMP. When released by individual amoebae, the secretion of cyclic AMP is known to cause aggregation of all the cells in the vicinity, leading eventually to sporulation. Durham (unpublished observations) found, however, that these amoebae in a liquid suspension showed no change in Ca^{++} efflux in response to cyclic AMP.

Our results have shown that the existing Ca^{++} fluxes are not linked to the chemotactic response and that the organism is unaffected by different external $[Ca^{++}]$. Stated differently, the organism's chemotactic recognition and response was shown not to be dependent on a specific concentration of Ca ions in the surrounding medium. It is clearly advantageous to an organism like Physarum to be independent of external sources of Ca ions.

Other cellular processes which are believed to be controlled by internal $[Ca^{++}]$ and to be independent of external $[Ca^{++}]$, include cleavage of sea urchin egg cells (Schroeder, 1972) and cytoplasmic streaming in the alga Chara (Pickard, 1972). It is possible that in a marine environment, in the early stages of cellular evolution, simple organisms did carry out their Ca^{++} fluxes across external surfaces. At a later stage, when organisms were faced with situations of limited calcium (rain water, solid dry surfaces), what little calcium obtained had to be conserved. Thus free-living organisms evolved cellular processes that were independent of fluctuations in the external $[Ca^{++}]$, whereas processes inside multicellular organisms, such as synaptic transmitter release (Katz and Miledi, 1966) and liver cell division rate (Whitfield et al. 1976) have remained extremely sensitive to the external Ca^{++} concentrations.

If one accepts that Ca^{++} is conserved and that the internal rhythmic and peristaltic contractions of contractile proteins occur because of oscillations in the cytoplasmic $[Ca^{++}]$, then the question which needs to be answered is: How is the membrane involved in the local recognition and the control of amoeboid movement via control of the internal $[Ca^{++}]$?

Durham's hypothesis that regions of lower and higher oscillations in streaming control the direction of the plasmodium, is the only reported hypothesis concerning that aspect of amoeboid movement. The regulatory role ascribed by Durham (1974) to the Ca ion and Ca^{++} concentrations (see literature review) is not supported by the results of the flow-through chamber and half plate assays. The question also remains as to which factors regulate the internal $[Ca^{++}]$. Ettienne (1972) and Braatz and Komnick (1973) have shown the existence of Ca^{++}-accumulating vacuoles which so far, are the only reported organelles capable of regulating the internal $[Ca^{++}]$.

The organelles known to accumulate Ca^{++} and the factors that regulate the internal $[Ca^{++}]$ have been listed in the literature review. The mitochondria appear to be the most important organelles that would have the ability to regulate the cytoplasmic $[Ca^{++}]$ in the plasmodium. Despite the large volume of research done involving slime mould mitochondria, no work has been published concerning ion transport or more specifically calcium transport capability.

The results of the polarography experiments reported in this work, showed that the plasmodial mitochondria have a poor respiratory function and a low oxidative phosphorylation capacity. These are prime indicators of mitochondrial integrity and together they indicate that the majority of the mitochondria were functionally impaired. This probably reflects the difficulty of the isolation procedure. In view of our hypothesis that an organelle is responsible for internal $[Ca^{++}]$ regulation, it was of interest to examine the mitochondria for Ca^{++} transport ability despite their apparent lack of integrity.

Chance (1963) and Scarpa and Graziottie (1973) defined the minimum requirements that cardiac mitochondria would have to fulfill if they were to have an active role in the contractile cycle. These are: (1) to be capable of accumulating high intra-extramitochondrial Ca^{++} ratios and, (2) kinetically the Ca^{++} transport should operate with sufficient rapidity to participate in the Ca^{++} redistribution required by the contractile cycle. The same requirements also apply to plasmodial mitochondria.

Along with evidence for poor respiratory control in the case of plasmodial mitochondria, the polarography experiments showed that, contrary to the abilities of mammalian mitochondria, the addition of Ca^{++} had no effect on the rate of respiration. Experiments with murexide spectrophotometry showed that Ca^{++} was taken up to a negligible extent. It seems, therefore, that plasmodial mitochondria do not meet the two requirements stated above and that they resemble those of yeasts and mycelial fungi in having no Ca^{++} transport capacity. However, until a better isolation procedure is devised and mitochondria can be obtained which have unimpaired respiratory function and the "slime" fully removed, the possibility cannot be ruled out completely that plasmodial mitochondria could have such a function. Thus, for this particular link in the chain of interrelated chemical processes between the initial recognition and the chemotactic response, the question has not been conclusively settled.

Evidence from Kamiya (1959) and recently from Ueda _et_ _al_. (1975) and Hato _et_ _al_. (1976) supports the hypothesis that the initial recognition (chemical stimulus) involves an induced change in the membrane potential. Kamiya first demonstrated that the two curves DPG and EPG (see Fig. 7, literature review), representing changes in the motive force generation and membrane potential, followed each other closely and seemed to be interdependent. The two papers of Ueda et al. (1975) and Hato et al. (1976) showed that the recognition and taxis, involving different concentrations of sugars and ions, varied directly with the degree of depolarization of the membrane and the reduction of the surface charge.

Both Ueda and Hato with their coworkers make the suggestion that the changes they observed are the result of a structural or conformational change in the membrane's structure which is transmitted to the motile system of the plasmodium. This suggestion provides some of the answer to the question of how the membrane could react locally and it could explain the extensions of pseudopodia and selected movement towards attractants.

Conceivably, the conformational change would be the trigger that switches on a membrane-bound enzyme and this would produce a "second messenger" (Rasmussen, 1970). This messenger would act as an intermediary in regulating mitochondrial or vesicular release of calcium or it could influence the concentration of an intermediary and hence the Ca^{++} stores and the contractile apparatus. A second alternative would be that the detected membrane depolarization would be that the detected membrane depolarization would be conveyed internally, akin to passage of a nerve action potential to the sarcoplasmic reticulum in muscle, and would regulate the Ca^{++} stores in this manner. What is notably absent and has not been shown to exist by any electron microscope study, is the required all-pervasive and ramifying plasma membrane system to convey the depolarization internally to each organelle and outwards in a ripple effect.

The evidence of a conformational change and the suggestion of a chemical "second messenger" should promote further research into determining the levels of cyclic nucleotides (i.e. cyclic AMP) before and after exposure to a chemotactic substance. The concept of membrane depolarizations regulating taxis becomes reasonable when it is considered that the sensory receptors of larger organisms (i.e. sight, smell, taste, etc) function by having the environmental signals stimulate the appropriate cells and the stimulus transduced into changes of ion movements. The complexity of chemotactic phenomenona and amoeboid movement in Physarum is clearly illustrated by the inconclusive nature of the results obtained in this thesis.

CHAPTER SIX

CONCLUSION

It was the purpose of this thesis to review the involvement of the Ca ion in amoeboid movement, especially pertaining to the chemotactic response of the plasmodial phase of P. polycephalum. The factors regulating Ca^{++} concentrations and current experimental approaches for determining changes in cellular $[Ca^{++}]$ were reviewed.

The study began with gaining familiarity and competence in the culturing of the plasmodium on solid and liquid media. A condition of a hypothesis put forth by Durham was reviewed and then tested with the constructed flow-through chamber device. The outcome of tests with attractants and repellents showed that no significant long term Ca^{++} fluxes occurred in a chemotactic situation. The direction of plasmodial migration was used as a criterion, in the half plate assays, and the results demonstrated that generally, external Ca^{++} concentrations held no special significance to Physarum. The cations Mg^{++} and Ca^{++} could not be differentiated and, the chemotactic recognition and attraction towards 1 mM glucose was not changed with varying Ca^{++} concentrations of the substrate.

It was reasoned therefore, that if Ca^{++} controlled the contractions of contractile proteins, in analogous a manner to muscle, there must be an internal specialized organelle regulating the cytoplasmic $[Ca^{++}]$. Mammalian mitochondria show a well established ability for Ca^{++} accumulation. The physiological significance or purpose of this function remains speculative because of the lack of knowledge of why this function should exist. On the strength of the hypothesis proposed by some researchers, that cardiac mitochondria function in the contractile cycle, it was decided to investigate if a parallel Ca^{++}-accumulating ability existed for slime mould mitochondria (SMM).

Contrary to the observations from standard rat liver mitochondria preparations, the addition of Ca^{++} induced no increase in respiration of plasmodial mitochondria. Using murexide in the suspension, in conjunction with a dual-wavelength spectrophotometer, an active Ca^{++} transport and accumulation ability for rat liver mitochondria could be demonstrated. Identical experimental conditions for slime mould mitochondria showed that they possessed no energy-linked Ca^{++} uptake capability. The inability may be genuine and if such is the case it would group the SMM together with those of yeasts and mycelial fungi in having no such inherent ability. The question is not conclusively decided since the lack of functional integrity, as evidenced by the poor respiratory control, may mask an existing ability for Ca^{++} transport.

Some suggested ideas for further work are as follows. More work is needed to devise a better isolation procedure for obtaining mitochondria with full respiratory control and oxidative phosphorylation ability. It would then be essential to reexamine their Ca^{++} uptake ability. The half plate assays of migrating plasmodia could be extended to the study of different concentrations of caffeine and lanthanum (La^{3+}). Hatano (1970) showed that the cytoplasmic streaming of fragmented plasmodia did respond to changes in the external $[Ca^{++}]$ if caffeine was present to modify the membrane's permeability. Lanthanum ions have been used as a "probe" in biological systems (i.e. muscle contraction) because the ion can often compete with and inhibit the site of action for Ca^{++}. Similarly, if the membrane potential is in some way an intermediary step between chemotactic recognition and the actual response, then chemical agents known to uncouple or prevent membrane depolarization should selectively interfere with chemotaxis.

If as proposed in the discussion, attractants bind to specific receptors on the external membrane and initiate the release of a "second messenger" which would then react with hypothesized organelles for Ca^{++} storage, it would be of interest to determine cyclic nucleotide levels before and after exposure to an attractant. Such sites of "recognition" on the membrane may prove to be soluble with detergents and an experiment could be devised whereby fresh accumulations of plasmodia would be agitated in various detergent solutions, washed free of the detergent and the chemotactic response tested by the half plate assays. Or the same procedure could be adapted (viz. using centrifugation) for microplasmodia by the procedure of Ueda _et_ _al_. (1975).

Two further methods can be envisaged for repeating the experiments with the flow-through chamber and external Ca^{++} fluxes. For the ^{45}Ca measurement, Langer and Frank (1972) described a novel apparatus for the attachment and growth of a layer of contractile cardiac cells on a slide overlaid with scintillation material. The slide is then mounted in a scintillation cell with accompanying influx and efflux tubing for perfusing solutions through. The tubing allows the introduction or washing out of theperfusing solution from the scintillation vial as the cell is counted in the scintillation counter. This method conceivably could be adapted for plasmodia.

Another apparatus can be envisaged for the murexide spectrophotometric measurement of aerated microplasmodia. An aerated chamber would be positioned above the cuvette compartment. A mesh would prevent the circulating microplasmodia from entering the adjoining cuvette and blocking the light path. The liquid medium would flow freely through the mesh and allow absorbance readings to be made in the cuvette compartment. Additions could be made in microliter amounts and these would result in the same final concentrations of the chemicals used in the flow-through chamber experiments. The results from either of the last two experiments could be used to confirm and further investigate the phenomena studied in this thesis.

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APPENDIX 1

ABBREVIATIONS AND SYMBOLS

ADP	Adenosin diphosphate
ATP	Adenosine triphosphate
^{o}C	Degrees centrigrade
C-AMP	Cyclic adenosine monophosphate
cm	Centimetre
CPM	Radioactive counts per minute
EGTA	Ethylene glycol bis (β-aminoethylether)-N, N'-tetraacetate
Fig.	Figure
g	Acceleration due to gravity
gm	Gram
HMM	Heavy meromyosin
M	Molar solution (containing 1 gm molecular weight per litre of solution)
min	Minute
mm	Millimetre
mg	Milligram
µl	Microlitre = 10^{-3} ml
µM	Micromolar = 10^{-6} M
nCi	Nanocurie = 10^{-9} Ci
No.	Number
pCa	Negative logarithm of the calcium ion concentration
pH	Negative logarithm of the hydrogen ion concentration
ppm	Parts per million
RLM	Rat liver mitochondria
SMM	Slime mould mitochondria
v/v	Volume by volume
w/v	Weight by volume

APPENDIX 2

The author has been associated with the research work reported in the following publication:

DURHAM, A.C.H. & RIDGWAY, E.B. (1976) Control of chemotaxis in *Physarum polycephalum*. J. Cell Biol. *69*, 218-223.

Some of the results obtained in this thesis have been published in the following paper:

LUDLOW, C.T. & DURHAM, A.C.H. (1977) Calcium ion fluxes across the external surface of *Physarum polycephalum*. Protoplasma, in press.

ACKNOWLEDGEMENTS

The author wishes to express his sincere appreciation to Professor M. H. V. van Regenmortel for his continuous guidance and encouragement throughout the execution of this work; to all the staff of the Microbiology Department for their generous assistance, in particular Dr. A. C. H. Durham and Dr. A. Polson for their advice and guidance with the experimental work and to Dr. W. Hardie for her patience in the editing and preparation of the manuscript; to Mr. Benjamin Klopper of the Geochemistry Department for certain analyses by atomic absorption; to Professor J. E. Kench of the Chemical Pathology Department, Groote Schuur, for the use of their dual-wavelength spectrophotometer and Dr. M. C. Berman for his assistance in the murexide-mitochondrial study.