SPECIFIC PERMEATION MECHANISMS IN NATURAL MEMBRANES.

by

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INTRODUCTION.

In living cells perhaps the most important structures, physiologically speaking, are the envelopes which surround the cell and which separate the cytoplasm of each cell from that of its neighbour or from the external medium. The nutrients required by the cell for its continued existence and growth have to pass inwards through these envelopes and the waste-products of metabolism must be eliminated by the cell through them. At the same time, the metabolic intermediates needed for the synthesis of new cell material must be retained within the cell. In the struggle for survival it would also be of advantage to the cell to be able to prevent the entry of toxic substances from the medium into its vulnerable cell interior. The apparent paradox of the ability of the cell envelopes to prevent the passage of certain substances whilst allowing the rapid permeation of other physically similar substances has not gone unnoticed. In the field of micro-biology particularly, where there is a very obvious conflict of low cell permeability

compared with very rapid metabolism of externally-added substrates, this paradox has been recognised for many years (see, for example, Topley and Wilson, 1929).

The investigation reported here is a study of the mechanism by which the cell envelopes show such selective permeability. In the choice of the most suitable biological material for experiment several factors were taken into consideration. These factors, which are summarised in Chapter II, led to the choice of the bacterial cell as being the most convenient biological material available. The work stems directly from Dr. Peter Mitchell's study of the transport of inorganic phosphate across the plasma-membrane of Staphylococcus aureus, and that of Dr. Mitchell in collaboration with the present author on the permeability properties of several species of bacteria to a large range of solutes of small molecular weight. These studies, to which detailed reference is made in the text of Chapter I, were carried out between 1950 and 1955 in the Biochemistry Department of the University of Cambridge and latterly in the Zoology Department of the University of Edinburgh.

The present investigation is principally concerned with the specific permeation mechanism for succinate in Micrococcus lysodeikticus but the principles involved in this particular mechanism may be found to be of general validity and applicable to the specific permeation of many substances through many different natural membranes.

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CHAPTER I.

HISTORICAL REVIEW.

The permeability properties of natural membranes have interested biologists for many years and studies on permeability problems extend over the whole of the first half of this century. Evidence has accumulated over the years that natural membranes are capable of transporting substances through them by processes ranging from free diffusion through facilitated diffusion (exchange-diffusion) to active transport in which transport of the solute is strictly coupled to cellular metabolism. The cells and tissues that have been used experimentally have been many but the studies using them have almost always been concerned with the movement of inorganic ions such as Na^+, K^+ and Cl^-. As the field of membrane-transport is so large and diverse it will not be possible to cover the whole of it in this historical review, so, since the original work to be reported here concerns the permeability of the bacterial plasma-membrane, only permeation studies on bacterial cells

will be described in this chapter. Davson and Danielli (1943) and, more recently, Harris (1956) have discussed very thoroughly the permeation of ions and other substances into erythrocytes, frog skin, muscle, nerve and plant and yeast cells. In addition, several symposia have been held in recent years and their reports underline various aspects of the subject; for example, the 8th Symposium of the Society for Experimental Biology on 'Active transport and secretion' (1954) and the 21st Discussion of the Faraday Society on 'Membrane phenomena' (1956).

Bacterial Permeability.

At the end of the last century, Fischer made a series of very careful observations on the effect of suspending Gram-negative bacteria in media of high solute concentration. He found that the cells plasmolysed in salt or sucrose solutions, that is to say the protoplasm shrank away from the rigid cell-wall; but did not plasmolyse in glycerol, urea or chloral hydrate solutions at the same concentration (Fischer, 1903). This was good evidence for the presence of a membrane covering

the surface of the protoplasm, distinct and morphologically separate from the rigid and porous cell-wall, and permeable to substances of high lipid solubility but impermeable to salts and sucrose. Fischer was unable to plasmolyse Gram-positive bacteria and, like many other workers (e.g. Brudny, 1908; Eisenberg, 1910; Baumgärtel, 1924), considered that the plasma-membrane of Gram-positive bacteria was much more permeable than that of Gram-negative bacteria. Some indication of the nature of the plasma-membrane of Gram-negative bacteria was provided by Eisenberg (1910) who treated cells with lipid solvents such as methanol, ethanol, phenol and chloroform and found that the cells could not subsequently be plasmolysed.

In 1930, Knaysi demonstrated an apparent plasmolysis in the Gram-positive bacilli by suspending them in solutions of very high salt concentration, but it is doubtful if this was a true plasmolysis for the very high salt concentration required may well have damaged the cells and ruptured the normal bonding between the cell-wall and the plasma-membrane in these organisms. Robinow and Murray (1953), for example, have produced

apparent plasmolysis of Gram-positive species by treatment of the cells with ether vapour. Normally, when they are placed in media of high salt concentration, Gram-positive cells contract as a whole, Bacillus megaterium contracting by 14-25 % on transfer from broth to broth containing 2M NaCl (Dutky, 1933). Staphylococcus aureus harvested near the stationary phase of growth contracts by 6-9 % by volume on transfer from 0.1M NaCl to 1.0M NaCl (Mitchell and Moyle, 1956a). From these observations it may be inferred that the cell-wall of some bacteria is elastic. This fact had been demonstrated directly by Wámoscher (1930) by micro-dissection.

As it was not possible to cause true plasmolysis in Gram-positive bacteria, Fischer (1903) suggested that the plasma-membrane of these organisms was permeable to solutes of small molecular weight. However, it was shown later that young cells of Staph. aureus, Micrococcus lysodeikticus, Sarcina lutea and B. megaterium viewed by phase contrast microscopy shrank in size when transferred from 0.1M NaCl to 0.1M NaCl containing 1.2M sucrose but not when transferred to 0.1M NaCl containing

1.2M glycerol (Mitchell and Moyle, 1956a). Thus the plasma-membrane of Gram-positive bacteria has, after all, been shown to be functionally analogous to that of Gram-negative bacteria. Structurally, however, there is a difference between the two groups of bacteria, for there is very strong adhesion between the cell-wall and the plasma-membrane in Gram-positive cells, preventing them from exhibiting the plasmolysis which occurs so readily in Gram-negative organisms. In large organisms such as Beggiatoa mirabilis it has been shown that the cell-wall actually buckles inwards when the cells are suspended in salt solutions that, because of their high osmotic pressure, cause a shrinkage of the cytoplasm (Ruhland and Hoffmann,1926) but that the cell-wall can be made to expand away from the plasma-membrane by treatment of the cells with reagents which destroy the adhesion between the two envelopes.

By 1930, the early work had resulted in a generally-accepted view of the bacterial plasma-membrane as impermeable to many small molecular weight solutes (see, for instance, Henrici, 1934) in spite of the anomaly of low permeability and yet

vigorous metabolism of externally-added substrates that this view created (Topley and Wilson, 1929).

However, Roberts and his colleagues at the Carnegie Institution in Washington produced evidence against such an osmotic barrier in Bacterium coli a few years ago. They reported that Bact. coli was permeable to a number of inorganic cations such as sodium and potassium (Cowie et al., 1949; Roberts et al., 1949) and to sugar phosphates (Roberts and Wolffe, 1952). This group of workers also found, using radioactive tracers, that the cells of Bact. coli were permeable to inorganic phosphate (Roberts and Roberts, 1950), the external labelled inorganic phosphate exchanging rapidly with inorganic phosphate in the cells. Roberts and Roberts proposed that the so-called 'inorganic phosphate' of the cytoplasm existed as a labile adsorption-complex which was capable of exchanging one phosphate group for another and which remained constant in amount because there was a fixed number of adsorption sites per cell. Another member of the group at the Carnegie Institution presented evidence to show that the plasma-membrane of Staph. aureus was permeable to glutamic acid (Britten, 1951-2).

In a review of the work of this group on the permeability of the cells of Bact. coli, Roberts et al. (1955) stated that the water-space of the cytoplasm was freely accessible to small molecular weight solutes in the medium and compared the cytoplasm to a sponge covered by a hairnet (the plasma-membrane).

At about this time, Gale and his group in Cambridge were making extensive studies on the assimilation of amino-acids by bacteria (Gale, 1953; 1954). It was found that Gram-positive bacteria were able to accumulate several amino-acids including glutamic acid. There was no accumulation of glutamic acid in Staph. aureus unless glucose metabolism occurred at the same time, but lysine accumulation occurred in the absence of an energy source and was unaffected by metabolic inhibitors. The accumulation of glutamic acid was prevented by sodium azide, 2,4-dinitrophenate and crystal violet although these substances were shown not to inhibit glucose metabolism. Gale referred to the accumulated amino-acids as 'free' inside the cell but he pointed out (Gale, 1953; 1954) that, at that time, there was very little

evidence to show that the glutamic acid was actually free to diffuse within the cytoplasm and enclosed within an osmotic barrier impermeable to glutamic acid rather than combined as some labile derivative with a substance of large molecular weight in cells of which the plasma-membrane was permeable to glutamic acid. The accumulation of glutamic acid was accompanied by an inflow of potassium ions into the cells, the ratio of glutamic acid molecules to potassium ions accumulated being very approximately one to one.

In order to clarify the position as far as the nature of the so-called 'free' internal solutes of small molecular weight in bacterial cells was concerned, Mitchell (1953) compared the total weight of solutes extracted from the cells of Staph. aureus by cold 5 % (w/v) trichloroacetic acid with the internal osmotic pressure of intact cells, for if the 'free' internal solutes existed as complexes with substances of large molecular weight they would not exert as great an osmotic pressure as would be expected if they were free in solution. The internal osmotic pressure was measured by a new vapour-pressure equilibrium method (Mitchell

and Moyle, 1956a) which gave a value of 20-25 atmospheres for the osmotic pressure of the cells. The solutes extractable by trichloroacetic acid represented about 18 % of the dry weight of the cells and, since the protoplasmic water volume was about 1.5 ml./g. cell dry weight when estimated by direct permeability measurements (Mitchell, 1953), the small molecular weight solutes should give a 12 % (w/v) solution in this organism. If a mean particle weight of 100 is assumed, then the internal osmotic pressure of Staph. aureus should be 20-30 atmospheres. The good agreement of the values for the osmotic pressure of the cells obtained by these two methods made it certain that a large proportion of the small molecular weight internal solutes must be free to diffuse within the cell, and that, therefore, the plasma-membrane of this organism must be impermeable to these solutes.

In the case of Bact. coli, about 6 % of the dry weight of the cell consisted of solutes of small molecular weight. Since the protoplasmic water volume was 2.4 ml./g. cell dry weight, it would have been expected that the internal osmotic

pressure would be about 5 atmospheres if the solutes were free in solution. Experiments on the plasmolysis threshold of this organism gave a value of 2-3 atmospheres for the internal osmotic pressure (Mitchell and Moyle, 1956a).

Further evidence for an osmotic barrier (Mitchell, 1949b) to small molecular weight solutes near the surface of bacterial cells was obtained by estimation, using a thick suspension, of the volume of the cells impermeable to externally-added solutes (Mitchell, 1953). This method, although technically difficult, is capable of giving unequivocal results. The cells of Staph. aureus were found to have a volume inaccessible to externally-added inorganic phosphate of about 2.4 ml./g. cell dry weight, and (since the specific volume of the cell contents lay between 0.7 and 1.0) the volume of water in the cells inaccessible to phosphate was approximately 1.5 ml./g. cell dry weight. When the cells had been treated either with 5 % butanol or 5 % trichloroacetic acid to destroy the plasma-membrane the phosphate-impermeable volume fell to only 0.76 ml./g. cell dry weight. There can be no doubt that intact

cells of Staph. aureus are impermeable to inorganic phosphate. The proportion of the volume of the centrifuged cell pad of the near-spherical staphylococci represented by the phosphate-impermeable volume of the cells was only 7 % less than would be expected from close-packed spheres (Mitchell, 1953). The osmotic barrier to phosphate was, therefore, near the external surface of the cells, and it was possible that the extra volume was occupied by water-filled interstices in the cell-wall. Using a dextran of molecular weight 10,000, Mitchell and Moyle (1956a) obtained a dextran-impermeable volume 8 % larger than the phosphate-impermeable volume implying that the extra volume was indeed occupied by the cell-wall, the pores of which were too small to be permeated by the large dextran molecule. When the cells were treated with 5 % butanol, the dextran-impermeable volume decreased only slightly, confirming that the osmotic barrier for large molecules such as the dextran is the cell-wall whereas the osmotic barrier for small molecular weight solutes is the plasma-membrane.

Estimations of the volume of the staphylococcal cell impermeable to glutamic acid and glutamine

(Mitchell and Moyle, 1956a) showed, using the thick suspension technique, that glutamic acid did not permeate the cells but that some of the added glutamine was apparently adsorbed by the cells. This result was in direct conflict with the finding of Britten (1951-2) that Staph. aureus was permeable to glutamic acid. However, Britten had taken no account of the interspace volume (26 %) for close-packed spheres when interpreting his experimental results. He found on recalculation of his data taking this factor into account (Britten, 1955) that his results were no longer in conflict with those of Mitchell and Moyle. A recent paper by Gale's group (Rowlands et al., 1957) confirms that the plasma-membrane of Staph. aureus is not freely permeable to glutamic acid. Weibull (1955) showed that the cells of B. megaterium resembled those of Staph. aureus and were impermeable to phosphate, sucrose and diphosphopyridine nucleotide.

It seemed unlikely that Bact. coli would differ so profoundly in permeability properties from Staph. aureus as suggested by the work of Roberts et al. (1955), but it was possible that Gram-positive and Gram-negative organisms did differ in this way.

Using the same organism and growth medium as Roberts and his collaborators, Mitchell and Moyle (1956a) found that the plasma-membrane of Bact. coli was impermeable to phosphate giving a phosphate-impermeable volume of about 3.2 ml./g. cell dry weight and an interspace volume of 22 %. It is not possible to reconcile the results of the two groups of workers. Davis (1956) has used a thick suspension technique to investigate the permeability to added citrate of an Aerobacter strain which was unable to metabolize citrate. He found that the citrate did not permeate the cells, but it is not possible to obtain from his data (owing to the relatively low concentrations of cells used) any accurate value for the citrate-impermeable volume in this organism.

By making use of changes in light scattering caused by the swelling and shrinkage of bacterial cells in dilute suspension, it is possible to determine whether a solute permeates the cells. For example, suspensions of Bact. coli in water buffered with 0.01M phosphate at pH 6.8 (or in solutions of penetrating solutes) scattered only 70 % of the light scattered by suspensions of the same organisms in non-penetrating 0.2M NaCl in which they did not

swell (Mitchell and Moyle, 1956a). Bact. coli was found to be impermeable to a large range of electrolytes including NaCl, KCl, NH_4Cl, (NaH_2PO_4 + Na_2HPO_4), NaCNS, KCNS, but permeable to NH_4 acetate, though not to Na acetate or K acetate at pH 6 or 7. It would seem that the plasma-membrane of Bact. coli was impermeable to Na^+, K^+, Cl^- and CNS^-. It was shown to be very permeable to glycerol, less permeable to erythritol and impermeable to lactose. Mitchell, Moyle and Stephen (unpublished) have confirmed and extended these results by following plasmolysis of the cells under phase contrast microscopy, for cells suspended in media of solute concentration above that corresponding to the plasmolysis threshold initially plasmolysed but de-plasmolysed if the solute entered the cells. Bact. coli was impermeable to sucrose, D-glucose, L-arabinose and D-xylose but permeable to D-ribose (Mitchell and Moyle, 1956c). The rate of entry of glucose into the cells in the absence of potassium ions or in the presence of inhibitors such as mercuric chloride, cyanide, 2,4-dinitrophenate or iodoacetate was less than 20 % of the rate of its metabolism in this organism, suggesting that glucose only entered the

cells by an active transport mechanism strictly coupled to metabolism.

In 1953, Weibull prepared protoplasts from the cells of B. megaterium by dissolving the cell-walls with lysozyme. The protoplasts were spherical in shape, and electron micrographs showed that the cytoplasm was surrounded by a membrane. They remained intact when suspended in a medium giving an osmotic pressure of about 5 atmospheres but were exploded in media of low osmotic pressure. It seemed that protoplasts might provide a useful tool for the investigation of the permeability properties of Gram-positive organisms in which the plasmolysis technique was not applicable. As Staph. aureus was practically insensitive to the action of lysozyme, Mitchell and Moyle (1956d) prepared protoplasts from M. lysodeikticus and Sarcina lutea by a method similar to that of Weibull (1953). It was known that the internal osmotic pressure of Staph. aureus was about 20 atmospheres and that Grula and Hartsell (1954) had failed to prepare stable protoplasts of M. lysodeikticus in 0.2M sucrose, so the cells of these organisms were supported by 1.2M sucrose during the action of lysozyme. Protoplasts were formed that were stable in 1.2M

sucrose or 1.2M NaCl, but burst immediately on transfer to 1.2M glycerol or water. The turbidity of suspensions of intact protoplasts was 60-70 % that for suspensions of intact cells, but the turbidity of burst protoplasts was negligible. The protoplasts of M. lysodeikticus and Sarcina lutea were found to remain intact when suspended in 1.5 molal solutions of many test substances (the estimation of turbidity of the protoplast suspension being made after subsequent transfer to 1.2M NaCl) indicating that they were impermeable to a large number of solutes including NaCl, KCl, NH_4Cl, $(KH_2PO_4 + K_2HPO_4)$, Na glutamate, D-glucose and sucrose. The protoplasts were very permeable to glycerol and NH_4 acetate, and quite permeable to D-ribose.

It was found that under suitable conditions Staph. aureus would form 'protoplasts' quite rapidly without the addition of lysozyme to the cell suspensions (Mitchell and Moyle, 1957). These 'protoplasts' were osmotically fragile and were used for direct permeability experiments. They were impermeable to NaCl, KCl, NH_4Cl, $(KH_2PO_4 + K_2HPO_4)$ and many other electrolytes but were permeable to NaCNS and

KCNS, particularly at low pH values (but not to NaCl at these pH values), indicating that Staph. aureus is permeable to Na^+ and K^+ (Mitchell and Moyle, 1956c). The 'protoplasts' were impermeable to glucose and sucrose but permeable to glycerol and D-ribose. The protoplasts of M. lysodeikticus and Sarcina lutea and the 'protoplasts' of Staph. aureus showed essentially the same permeability properties as the intact cells of these species.

In general, the plasma-membrane of bacteria is impermeable to particles carrying more than 4 water molecules, but, in addition, the configuration of the solute is an important factor in determining the rate of penetration of the solute, and suggests that factors other than lipid solubility are concerned in the passage of these solutes through the plasma-membrane (Mitchell and Moyle, 1956c). On the whole, the Gram-negative bacteria seem to be somewhat less permeable than Gram-positive bacteria.

In 1953, Mitchell and Moyle found that there was a rapid exchange of labelled inorganic phosphate added outside the cells of Staph. aureus with the internal inorganic phosphate, just as Roberts and Roberts (1950) had found in Bact. coli. This

exchange was strictly reciprocal in resting cells (Mitchell, 1953), one phosphate molecule moving outwards for every phosphate molecule moving inwards. When the cells oxidized glucose, there was a net flow of phosphate inwards caused by a cessation of the outward flow of phosphate, the inward flow rate remaining the same as before (Mitchell and Moyle, 1953). It, therefore, seemed likely that the same mechanism operated both the exchange and the uptake reactions. The fact that Staph. aureus was impermeable to phosphate and that most of the internal small molecular weight solutes exerted the expected osmotic pressure made the exchange-adsorption hypothesis for phosphate of Roberts and Roberts (1950) most unlikely. It was much more likely that the uptake of phosphate represented a true active transport across a phosphate-impermeable osmotic barrier, and that the resting cell phosphate exchange was by some sort of exchange-diffusion system such as Ussing (1947) described to explain the exchange of sodium across the muscle membrane. Confirmation of the validity of this view came from the study of the action of inhibitors on the phosphate exchange in Staph. aureus (Mitchell, 1953). It was found that

phenyl mercuric ions in number equivalent to only 3 % of the number of cell inorganic phosphate molecules was sufficient to block the phosphate exchange reaction indicating that the exchange was mediated by carrier groups in the plasma-membrane small in number compared with the number of internal phosphate molecules, and not by the adsorption of the internal phosphate on a large number of cytoplasmic adsorption sites. The specificity of the phosphate exchange was found to be very high (Mitchell, 1954a). Many anions tested did not interfere at all with the phosphate exchange but a few, such as chlorate, borate and arsenate, acted as inhibitors. Only arsenate was able to substitute for phosphate in the exchange and, in these circumstances, there was the same reciprocity between arsenate and phosphate as between phosphate and phosphate. It was, therefore, possible, by suspending the cells (which contain 100 mM inorganic phosphate) in 1 mM arsenate, to observe an accumulation of arsenate in the cells against a concentration gradient, the final concentration of internal arsenate approaching 100 mM. Heavy metals, 2,4-dinitrophenate, aureomycinate, iodine (at pH 5.5), and N-ethylmaleimide were all inhibitors of phosphate

exchange but iodoacetate did not inhibit. These results indicated a functional thiol group of low reactivity and, taken in conjunction with the effect of 'uncoupling' agents, might have implicated cell metabolism in the phosphate exchange mechanism. However, it was found that, even when residual metabolism was reduced to an extremely low level, the phosphate exchange rate remained unaltered, so it was presumed that the rate of phosphate exchange was determined by thermal movements of constituents of the plasma-membrane and not by some metabolic reaction associated with the exchange system.

Kinetic studies (Mitchell, 1954a; 1954b) suggested that it was the $H_2PO_4^-$ ion which was involved in the exchange reaction. It was also shown that the phosphate exchange system differed in one important respect from the exchange-diffusion system of Ussing (1949). Ussing pointed out that when the carrier of his exchange-diffusion system was unsaturated on one side of the membrane, there should be considerable leakage of the substrate on that side. In the case of the phosphate exchange system, Mitchell (1954a) could demonstrate no leakage outwards when the external phosphate concentration was 0.1 m$\underline{M}$, and yet

the K_m for phosphate exchange was 0.8 m<u>M</u>. A possible mechanism for the phosphate exchange was put forward (Mitchell, 1954b):

> Phosphorylated carriers are present in the osmotic barrier the phosphate groups of which, due to thermal movements of the carriers, come into contact with the media on either side where an enzyme-catalysed exchange may occur between the phosphate groups of the carriers and phosphate ions. The essential difference between Ussing's system and this one is that in the latter the free energy of formation of the carrier-ion complex (or compound) is assumed to be conserved during the enzyme-catalysed exchange of the ion, the carrier-ion complex thus not being in equilibrium with its dissociation (or hydrolysis) products on either side of the barrier as is the case in Ussing's system.

The thermodynamic characteristics of the phosphate exchange reaction suggested that the exchange could be regarded as a reversible denaturation of protein components of the plasma-membrane.

The study of the transport of substances through an osmotic barrier should be assisted by a knowledge of the chemical and biochemical composition of that barrier, and some work has already been done in this field in the case of bacteria. Mitchell and Moyle (1951) isolated a fraction from sonically disrupted staphylococci by differential centrifugation

which they suggested might represent the plasma-membrane of intact cells. The fraction consisted of small particles containing 41 % by weight protein and 22.5 % by weight lipid. This fraction represented 10 % of the dry weight of the cells. Recently, using an autolytic procedure to prepare 'protoplasts' of Staph. aureus (Mitchell and Moyle, 1957), it has been possible to obtain intact plasma-membranes of this organism. It was found that the plasma-membranes were very fragile and, on washing, broke up into particles which appeared to be identical to the particles isolated from mechanically-disintegrated cells when viewed in the electron microscope (Mitchell and Moyle, 1956b). The weights of the plasma-membranes and particles obtained by the two treatments were the same, confirming that they represented the same morphological entity in the intact cell. From the known size of the cells of Staph. aureus (0.7 μ in diameter) and the weight of the plasma-membrane fraction it could be calculated that this fraction would form a spherical shell about 50 Å thick when dry, consisting of one or two monolayers of lipid and one or two monolayers of protein. This would be in

accord with the classical concept of the lipo-protein membrane (see Davson and Danielli, 1943). Of course, the exact arrangement of the lipid and protein components of the plasma-membrane is not known. The calculated thickness agrees well with the thickness of the plasma-membrane seen in ultra-thin sections of bacteria in the electron micro-scope (Bradfield, 1956) and by staining techniques (Robinow and Murray, 1953).

Using protoplasts of M. lysodeikticus and collecting the plasma-membranes after osmotic ex-plosion it was possible to show that there were practically no small granules adhering to freshly-prepared membranes which appeared under Anoptral contrast (Reichert, Austria) as uniformly-dense spherical shells. However, one washing of this preparation of membranes was sufficient to cause many of the membranes to disintegrate into particles, some of which appeared to adhere to those membranes still left intact (Mitchell, 1957a).

Concerning the biochemical composition of the plasma-membrane, Weibull (1953) showed that the plasma-membrane of B. megaterium contained the cytochrome system of the cell. Mitchell

(1954c) found that the plasma-membrane of Staph. aureus also contained the bulk of the cytochrome system of the cell, almost all of an acid phosphatase and a very active lactic dehydrogenase. The plasma-membrane of Staph. aureus also contained more than 90 % of the cell succinic dehydrogenase, malic dehydrogenase, and formic dehydrogenase activity (Mitchell and Moyle, 1956b). α-glycerophosphate dehydrogenase activity was shared between the plasma-membrane and the cytoplasm, and other enzymes occurred almost exclusively in the cytoplasmic fraction.

Before the demonstration of the fragility of the plasma-membrane by Mitchell and Moyle (1956b) and Mitchell (1957a) there had been many reports of small respiratory particles containing the cytochrome system and related enzymes in bacterial cells fractionated after mechanical disintegration, many authors comparing these particles to the mitochondria of animal cells. Tissières (1952; 1954) and Tissières and Slater (1955) showed oxidative enzyme activities in particles obtained from Aerobacter aerogenes and Azotobacter vinelandii, and oxidative phosphorylation by particles from the

latter organism. Repaske (1954) found the succinic dehydrogenase activity of Azotobacter vinelandii in a particulate fraction. Millman and Youmans (1955) found respiratory enzymes in a particulate fraction of Mycobacterium tuberculosis, and Linnane and Still (1955) found succinoxidase activity in the particulate fraction of Serratia marcescens which they noted was a heterogeneous fraction. Woody and Lindstrom (1955) obtained succinic dehydrogenase activity in particles from Rhodospirillum rubrum. Stanier et al. (1953) found succinic, malic and mandelic dehydrogenase activities in particles from Pseudomonas fluorescens. These particles varied greatly in size but all possessed the same specific activity. Stanier (1954) suggested that the particles obtained in these fractionations might not exist as such in the cell but form part of the plasma-membrane. The terminal oxidation system of Azotobacter vinelandii was also investigated by Wilson and Wilson (1955) and Alexander and Wilson (1956) who found that, by breaking the cells sonically, particles of different sizes were produced. When these were fractionated by differential centrifugation all the

particulate fractions had the same specific activity whatever the size of the particles. Finally, Nossal et al. (1956) showed that when the particulate fractions obtained from several species of bacteria were re-fractionated more and more of the enzymic activity originally associated with the particulate fraction became 'solubilized' or, rather, no longer sedimentable at the relative centrifugal force used in the original fractionation.

It seems undeniable that the respiratory particles were artefacts caused by the rough treatment necessary to break the cells by mechanical methods, and that the material found as particles formed the plasma-membrane in the intact cell. Recently, Storck and Wachsman (1957) have confirmed the association of the cytochrome system of B. megaterium with the plasma-membrane, and have also found all the succinic, lactic and ∝-ketoglutaric dehydrogenase activities of the cell in this fraction.

It follows that part of the protein component of the plasma-membrane must be composed of enzyme protein molecules (Mitchell and Moyle, 1956b). The thickness of the plasma-membrane makes it possible for a protein molecule of medium size to be in

contact with the external medium on one side of the membrane and, at the same time, to be in contact with the cytoplasm on the other side of the membrane, but it cannot be taken for granted that the active centre of the enzyme is accessible to externally-added substrates. However, since the end-products of metabolism of the staphylococci are succinate, formate, and lactate, and succinic, formic and lactic dehydrogenases were found in the plasma-membrane of Staph. aureus, it was legitimate to suggest that the dehydrogenases might be the means of transporting these substances through the plasma-membrane. From consideration of the transport of inorganic phosphate through the plasma-membrane of Staph. aureus (Mitchell, 1954b) it would seem likely that the carriers for all the substances of small molecular weight which cannot traverse the plasma-membrane by free diffusion would be found to have enzyme-like specificity, and some of them would have enzymic as well as carrier function (Mitchell and Moyle, 1956a; 1956c).

The work of Monod and his group on the permeation of amino-acids (Cohen and Rickenberg, 1956) and sugars (Monod, 1956; Rickenberg et al., 1956)

into Bact. coli has contributed much interesting information about bacterial adaptation and solute transport systems. Cohen and Rickenberg found that the cells of Bact. coli were able to accumulate valine, but only in the presence of an energy source such as succinate. The accumulation was extremely rapid at 37°, equilibrium being reached in less than 1 min., but at 0° equilibrium was only reached after 2 hours. Isoleucine and leucine not only interfered with the accumulation of valine but could exchange rapidly with the valine already accumulated. Cohen and Rickenberg held the view that the valine was accumulated by the mediation of a 'permease' system.

Monod has studied the mechanism of induction of β-galactosidase in Bact. coli. Thiogalactosides proved useful in these investigations because some, for example methyl-β-D-thiogalactoside, were good inducers although not metabolized by the cells. Monod found that induced cells were able to concentrate the thiogalactoside intracellularly in the presence of succinate whereas non-induced cells could not. The internal thiogalactoside was removable from the cells by boiling water. The

accumulation was inhibited by 2,4-dinitrophenate and sodium azide, and the equilibrium point for the accumulation was reached in 5 min. at 37°, and in 2 hr. at 0°. There was a rapid exchange between the accumulated thiogalactoside and galactosidic analogues, but not with other glycosides (such as glucosides). The quantitative aspect of the accumulation process precluded the interpretation that the accumulated thiogalactoside was bound to specific acceptor sites, and it was therefore suggested that the accumulation was mediated by 'concentration catalysts'. The concentrating system (or 'permease') increased during the induction process and so did the galactosidase but they were not considered to be identical for the following reasons. Some mutants which could not be induced to produce galactosidase nevertheless could be induced to produce some 'permease'. Other mutants which produced normal amounts of galactosidase when induced with high concentrations of thiogalactoside could not be induced by lactose, and the induced galactosidase was poorly active on lactose in the intact cells. In this mutant only traces of 'permease'

were demonstrable.

The transport of organic acids of the Krebs' tricarboxylic acid cycle into bacteria has not yet been investigated very systematically, and yet these substances form one of the important groups of metabolites which have to be transported through the plasma-membrane since many of them are known to pass out of bacteria as end-products of metabolism. The work that has been done has usually merely involved a study, in general terms, of metabolic pathways and conditions for metabolism of the acids in different species. The work by other authors on transport of organic acids in bacteria is held over to Chapter IV where it is discussed in relation to the original work reported in this thesis as it is particularly relevant in that context.

All the studies of bacterial permeability so far accomplished have demonstrated specific permeability properties of the plasma-membrane which are in line with the concept of natural membranes in general, particularly neatly described by Rosenberg (1948). The detailed study of the phosphate transport mechanism in Staph. aureus, taken

in conjunction with the work of other authors quoted in this chapter and with the original work described in this thesis, formed the experimental basis for the general theory of membrane-transport which has been developed this year by Mitchell (1957b) and which, although based on bacterial permeability studies, it is expected will be found to be of general validity for all natural membranes. The theory states that:

> .....metabolic energy is generally converted to osmotic work by the formation and opening of covalent links between translocators in the membrane and the carried molecules exactly as in enzyme-catalysed group-transfer reactions. The translocation itself is supposed to be due either to a thermal movement of the translocator in the membrane, or to the simultaneous accessibility of the translocator from both sides; and this movement or availability may be determined by a normal enzyme (or perhaps by a special protein) which we may call a translocase.

It is suggested that active transport of nutrients inwards may be driven by metabolic end-products which are covalently-bound to the translocators and exchange for nutrient molecules at the outer surface of the membrane. Alternatively, it is proposed that the translocators may have internal bonds (such as the -S-S- form of ∝-lipoic acid)

which may be opened by the entry of the nutrient molecule on the outer surface of the membrane and closed again, with the release of the nutrient on the inner surface, by coupling with oxido-reduction reactions inside the cell. The formation of external peptides and internal protein from externally-added amino-acids without their passage through the internal free amino-acid pool (Gale, 1948) and the rapid exchange of analogues across the plasma-membrane (Monod, 1956; Cohen and Rickenberg, 1956; Umbarger and Brown, 1955) and the synthesis of cell-wall polypeptides and polysaccharides (Park and Strominger, 1957) may be explained on the basis of this theory.

The specific permeation of succinate into M. lysodeikticus is discussed in terms of Mitchell's general theory of membrane-transport (1957b) in Chapter IV.

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CHAPTER II.

MATERIALS AND METHODS.

Choice of Material.

There were many reasons for choosing bacteria rather than yeast, higher plant or animal cells as material for the study of permeation mechanisms in natural membranes although it must be admitted that, at the time when the studies began, many of the facts enumerated here in support of the choice of bacteria as the biological material were as yet unknown or, at best, unconfirmed.

1. Bacterial cells are morphologically very simple. The cell envelopes consist of an outer relatively rigid cell-wall and a thin delicate plasma-membrane which lies just inside the cell-wall. These envelopes enclose the cytoplasm which, unlike that of most animal and plant cells, is not compartmentalised (Bradfield, 1956; Maaloe and Birch-Andersen, 1956). There is no evidence for a membrane round the nuclear mass (Mason and Powelson, 1956), and there are no vacuoles or structures resembling mitochondria except perhaps

in a few species of very large bacteria. This is not really surprising for most bacteria are themselves slightly smaller than the mitochondria of animal or yeast cells. Electron micrographs of thin sections of the cells of Staphylococcus aureus (Bradfield, 1956) show no structures in the cytoplasm larger than 20 mμ. It is important in permeability studies to know the volume of the space accessible to solutes that have permeated the plasma-membrane. In cells containing vacuoles and mitochondria which are surrounded by membranes, it is often uncertain whether a solute which has permeated the plasma-membrane has permeated these other membranes as well and, since the proportion of the total cytoplasmic volume occupied by the vacuoles and mitochondria is often very large, errors in interpretation of the experimental data may result.

2. The small size of bacterial cells may be an advantage in kinetic studies since the time taken for solute molecules entering the cell to diffuse throughout the cytoplasm is very short indeed.

3. The ease of culture of bacteria particularly recommends them for experimental work. Large yields

may be obtained of cells which, individually, vary little one from another, and the reproducibility of cultures grown under the same conditions is very good indeed. So, although single bacterial cells are too small for chemical or biochemical analysis, suspensions consisting of a large number of like individuals may be successfully used. It is convenient that many bacteria can be grown in easily-prepared media, and that their rate of growth is high.

4. Most bacterial cells are reasonably robust and may be suspended in, and washed repeatedly with, distilled water. This allows the removal of growth (or other) medium quantitatively from the cells and, in chemical and biochemical work, this is an important consideration.

5. The biochemistry of many bacterial species has been investigated in great detail. In spite of their small size, bacteria have been shown to be capable of carrying out as great a range of biochemical reactions as any animal cell, and broadly speaking the main metabolic pathways have been found to be common to bacteria and animal cells (see Stephenson, 1949). A knowledge of the biochemical properties

of the cell under investigation would clearly be helpful in this work.

In selecting a species of bacterium for study, due weight was given to the fact that in this laboratory much of the previous work on this subject (reviewed by Mitchell and Moyle, 1956a; 1956c) had used cells of Staph. aureus. Since the osmotic barrier for small molecular weight solutes in bacteria had been shown to be the plasma-membrane it was likely to be important to be able to obtain intact plasma-membranes of the test organism uncontaminated with other cell fractions. It is not possible to separate the cell-wall from the plasma-membrane by gentle physical methods in the Gram-positive cocci, but lysozyme has been shown to dissolve away completely the cell-walls of sensitive organisms (Salton, 1954) and allow separation of the plasma-membranes. Unfortunately, Staph. aureus is insensitive to lysozyme action but another species of the same genus, Micrococcus lysodeikticus, is extremely sensitive to lysozyme. It was largely for this reason that it was decided to use M. lysodeikticus in this work.

The two main groups of bacteria (classified by their Gram-staining reaction) have already been shown to differ qualitatively from one another in their permeability properties (Mitchell and Moyle, 1956c), so some of the experiments using the Gram-positive coccus M. lysodeikticus were repeated using the Gram-negative rod Bacterium coli for comparison.

The choice of the solute whose permeation mechanism was to be studied in detail was simplified by certain considerations. Briefly, these were that the solute should be an organic intermediate of metabolism of fairly small molecular weight and, as such, be representative of an important group of solutes entering and leaving the cell. Since the solute chosen was to be a metabolic intermediate, it would clearly help interpretation of results very materially if its metabolic pathways were well-understood. In these circumstances, succinate seemed the obvious choice of a model solute for, being a direct participant in the Krebs' tricarboxylic acid cycle (as well as being concerned in other syntheses and degradations) succinate is,

at the same time, both a key substance of metabolism and one whose metabolic pathways have been studied in great detail by classical biochemical methods.

Growth of Organisms.

M. lysodeikticus (NCTC 2665) was grown aerobically at 25° in a tryptic digest medium of the following composition:- 3 % (w/v) Tryptone (Oxo), 1 % glucose, 0.1 % Marmite and 0.0005N H_3PO_4. One-litre lots of this medium were sterilized in 10 l. flasks by autoclaving at a pressure of 15 lb./sq. in. for 20 min., the glucose being autoclaved separately with the phosphoric acid in 50 ml. of distilled water and added to the medium just before inoculation with the organism. The inoculum for each flask was two 24-hr. cultures of M. lysodeikticus each in 4 ml. of the Tryptone medium. The flasks were rotated at 3 revolutions per sec. in an apparatus designed by Mitchell (1949a) to give good aeration of growing cultures with a minimum of frothing of the growth medium.

Bacterium coli (American strain B) was grown

aerobically at 25° using the rotated flask technique (Mitchell, 1949a) and inorganic medium (Roberts et al., 1955) containing 0.2 % NH_4Cl, 0.6 % Na_2HPO_4, 0.3 % KH_2PO_4, 0.5 % NaCl, 0.01 % $MgCl_2.6H_2O$, 0.015 % Na_2SO_4, and 0.4 % glucose. The glucose was autoclaved with 10 % of the KH_2PO_4 separately in one tenth of the total volume of the medium, and added to the rest of the medium just before inoculation. One litre of the autoclaved medium in a 10 l. flask was inoculated with a 24-hr. culture of Bact. coli in 4 ml. of the inorganic medium.

Determination of Dry Weight.

Relative values for the dry weight concentration of cells were obtained by a spectrophotometric technique based on that of Longsworth (1936). The optical density of 1 cm. depth of suspensions of M. lysodeikticus and Bact. coli was measured using a Unicam spectrophotometer (SP 500) at a wavelength of 700 mμ. Suspensions were diluted to give readings ($Log_{10}(I_o/I)$) between 0.1 and 0.2, over which range the extinction was known to be proportional to dry weight (see Mitchell and Moyle, 1957).

Corrections were made for extinction due to the growth medium. These turbidity measurements were calibrated against measurements of actual dry weight. Samples of bacterial suspension containing 50-100 mg. dry weight of cells were centrifuged, and the growth medium removed, and the cells washed on the centrifuge three times without loss of cells. The cells were weighed after drying to constant weight at 105°.

Owing to the changes in volume and reflecting power of the cells during the growth cycle, both of which affect turbidity (Mitchell, 1950), it was found necessary to calibrate the turbidity against dry weight at several cell suspension densities if the turbidity was to be used to estimate dry weight accurately. Table I shows the variation in turbidity relative to actual dry weight found during the growth of M. lysodeikticus.

Harvesting of Organisms.

A typical growth curve for M. lysodeikticus (NCTC 2665) grown under the conditions described above is shown in Figure 1. Routinely, this organism was harvested towards the end of the loga-

rithmic phase of growth, at an optical density of 3.50 corresponding to a dry weight concentration of 2.34 mg./ml. Organisms were harvested from the growth medium by centrifugation at 15° and washed once on the centrifuge with distilled water. The cells were then suspended in distilled water at a concentration of 30-35 mg. dry weight/ml. Samples of the suspension were taken for estimation of actual dry weight.

When harvested at the end of the logarithmic phase of growth, the cells of M. lysodeikticus were found to be somewhat fragile as evidenced by a marked rise in the viscosity of washed suspensions left either at room temperature or at 5° for 30 minutes. The increase in viscosity was presumably caused by deoxyribonucleic acid released into the medium from broken cells since it could be prevented or reversed by addition of deoxyribonuclease. Because of this fragility, washed cell suspensions were used for experiment immediately they had been prepared. However, it was found that suspensions could be stored at -15° without damage to the cells (see Chap. III). The suspension prepared from a

single culture was stored in several tubes, the volume of suspension in each tube arranged so as to be sufficient for one experiment. In this way, cells were frozen and thawed once only before use. In the experiments described in Chapter III, freshly-prepared cell suspensions were used except where it is stated that suspensions had been stored at -15°.

Bact. coli (American strain B) was also harvested towards the end of the logarithmic phase of growth, at an optical density of 1.10 corresponding to a dry weight concentration of 0.67 mg./ml. The cells were collected by centrifugation, and washed on the centrifuge at 15° in 0.17M NaCl. The cells were finally suspended in 0.17M NaCl at a concentration of 30-35 mg. dry weight/ml.

Preparation of Protoplasts.

Protoplasts of M. lysodeikticus were liberated from intact cells by the method of Mitchell and Moyle (1956d). The cells were suspended at a final concentration of 30-35 mg. dry weight/ml. in a medium containing 1.2M sucrose and 0.08M sodium phosphate

buffer at pH 6.8 (i.e. a 1:1 mixture of equimolar solutions of Na_2HPO_4 and NaH_2PO_4). Crystalline egg-white lysozyme was added at a final concentration of 100 µg./ml. The suspensions were incubated at 25°, and the progress of protoplast formation was followed by measurement of the turbidity (at a wavelength of 700 mµ) of samples of the suspension after dilution into 1.2M NaCl or distilled water. Intact cells remained intact in both diluents, but protoplasts were osmotically exploded in water with an almost total loss of turbidity although they were supported intact by the isotonic 1.2M NaCl (see Mitchell and Moyle, 1956d). Under the conditions given above, the action of lysozyme was very rapid with completion of protoplast release usually occurring in about half an hour. It was discovered that protoplasts could also be released in the same medium in the absence of lysozyme, presumably by an autolytic process similar to that described for Staph. aureus (Mitchell and Moyle, 1956a; 1957). In the case of M. lysodeikticus, however, the rate of this autolytic reaction was rather slow and protoplast release took

many hours. Protoplasts prepared by these two methods behaved identically, the long incubation period (usually 24 hr. at 25°) required in the preparation of protoplasts by the autolytic method apparently having no effect on metabolic activity (see Chap. III).

Broken Cells.

It was necessary to obtain cells of M. lysodeikticus in which the plasma-membrane was ruptured so that its inner surface was available to added substrates, but in which the plasma-membrane was otherwise undamaged. Owing to the extreme delicacy of the plasma-membrane, the method used had to be a gentle one or complete disintegration of the membrane would be likely to occur. The method chosen was to dissolve the rigid cell-wall by the action of lysozyme, and allow the resulting protoplast to explode osmotically in a hypotonic medium. The cells were suspended at a concentration of 30-35 mg. dry weight/ml. in 0.08M sodium phosphate buffer at pH 6.8 or in 0.08M NaCl when absence of phosphate was required. A small quantity (0.1 mg.

per 10 ml. reaction medium) of deoxyribonuclease (Streptodornase of the American Cyanamid Co.) was added to hydrolyse the deoxyribonucleic acid as it escaped from broken cells. Without the addition of deoxyribonuclease, the viscosity of the suspension became so great as to make subsequent manipulation impossible. After incubation with 100 µg. lysozyme/ml. at 25° for 1 hr., it was found that all the cells had ruptured, judging by turbidity measurements.

Cell Fractionation.

Fractionation of the cells of M. lysodeikticus was carried out by the method used by Mitchell and Moyle (1957) for the fractionation of 'protoplasts' of Staph. aureus. Protoplasts of M. lysodeikticus were prepared exactly as described above. The protoplast suspension was then added, with careful stirring, to 5 volumes of isotonic (1.2M) NaCl in which the protoplasts remained intact. The suspension was centrifuged for 1 hr. at 4,000 g at 15°, and the resulting supernatant containing the (now soluble) constituents of the inert cell-wall was discarded. The pellet was dispersed in

as small a volume of 1.2M NaCl as possible and then squirted, with vigorous stirring, into 10 times its volume of distilled water at 20° containing 0.1 mg. deoxyribonuclease per 100 ml. water. The sudden reduction in osmotic pressure of the medium caused the protoplasts to explode, the soluble cytoplasmic constituents escaping into the water. After 20 min., the suspension was cooled to 5° and centrifuged at 20,000 g for 1 hr. at 5°. The supernatant containing the cytoplasmic constituents was removed, and the orange-brown pellet consisting of punctured plasma-membranes was dispersed in a small volume of distilled water. Phase contrast microscopy of this fraction showed that it was composed of spherical envelopes of uniformly low contrast.

Metabolic Methods.

The activity of oxidative enzymes in intact cells, protoplasts, broken cells and in cell fractions of M. lysodeikticus and in intact cells of Bact. coli was measured either anaerobically by the Thunberg tube technique or aerobically by Warburg manometry.

(a) Thunberg Technique. The oxidative enzymes of cell fractions were assayed anaerobically using methylene blue as a hydrogen acceptor. The reciprocal of the time taken for the reduction (and decolorization) of a known amount of methylene blue gave a measure of the enzymic activity towards the added substrate and was compared with values for oxidation of other substrates. The reaction mixture consisted of 0.06M sodium phosphate buffer at pH 6.8, 0.07M substrate (sodium salt adjusted to pH 6.8), 0.1M KCN (adjusted to pH 6.8) added in the assay of lactic dehydrogenase in order to assist the forward reaction by trapping pyruvate as it was formed, and 1.3×10^{-5}M methylene blue which was placed in the hollow stopper of the Thunberg tube. Samples of cell fractions corresponding to 20 mg. dry weight of cells were added to the reaction mixture and the total volume of fluid in each tube adjusted to 3.5 ml. The Thunberg tubes were evacuated on a Hivac pump to exclude all oxygen from the system, care being taken to avoid excessive frothing of the reaction mixture, and then incubated at 25° in a water bath.

After 5 min. equilibration, the methylene blue was tipped in from the stopper and the time taken for the decolorization of the methylene blue in each tube noted. Controls were done in which the reaction mixture lacked substrate.

(b) Manometry. Here the oxidative enzyme activity was estimated in an aerobic system by direct measurement of oxygen uptake and, where a respiratory quotient (v_{CO_2}/v_{O_2}) was required, by measurement of carbon dioxide output as well. Warburg manometers were used as recommended by Dixon (1934) for experiments on intact cells, cell fractions and broken cells.

(i) The basic composition of the reaction mixture for intact cells of M. lysodeikticus was :- 1.0M KCl, 0.05M NaCl, 0.002M substrate (sodium salt adjusted to pH 6.8) placed in the side-bulb of the flask, and a sample of cell suspension to give a dry weight of organisms of 25-30 mg. per flask. The total volume of the reaction mixture in each flask was 4.0 ml. and the gas phase was air. When oxygen uptake was measured, carbon dioxide was removed by a 'KOH-paper' in the centre

cup of the flask — a rolled piece of filter paper (1.5 cm. x 3 cm.) soaked with 0.2 ml. 2M KOH. In estimations of carbon dioxide output, a parallel set of flasks was prepared as above except that the 'KOH-papers' were omitted so that the gas change caused by oxygen uptake and carbon dioxide output together could be measured. It was simple to calculate carbon dioxide output from the results obtained from the parallel sets of flasks.

The reaction mixture used for Bact. coli only differed from that for M. lysodeikticus in containing 0.2M KCl in place of 1.0M KCl.

The reason for using such high salt concentrations in the reaction mixtures was that it was important to preserve the intactness of cells during very prolonged experiments. The final salt concentrations were therefore arranged to be isotonic to the suspended cells so that, even if the cell-walls became weakened by autolysis, the cells should remain whole. This point is discussed in some detail in Chapter III.

It will be noticed that this reaction mixture was unbuffered, and it may be thought that this

was unwise. The chief reason for omitting buffer was that the only common buffer for the range of pH values 6-8 which was not either inhibitory to bacterial metabolism or metabolized by the bacteria themselves was phosphate. It was then discovered that phosphate was an inhibitor of the succinate permeation system. This being so, it was decided to see whether, in the absence of any added buffer, the cells' own buffering power was sufficient to keep the pH value of the reaction mixture constant during an experiment. The pH of the unbuffered reaction mixture during, and at the end of, manometric experiments was determined with a glass electrode. The pH remained constant at a value of 7.4 in the presence or absence of substrates and/ or inhibitors.

Where reaction mixtures differed in composition from that given above, mention is made of the fact in the text.

The Warburg flasks were incubated at 25° and shaken at 120 strokes/minute; and readings were taken once every 10 minutes. It was found necessary, owing to an exceedingly high rate of endogenous

respiration in freshly-harvested organisms, to incubate all suspensions for 5 hr. before tipping in substrates and/ or inhibitors from the side-bulbs. At the end of this pre-incubation period the endogenous respiration rate had fallen to a reasonably low rate (see Chap. III) so that the increase in oxidation rate due to oxidation of added substrates could then be accurately assessed.

(ii) The composition of the reaction mixture used for broken cells was as follows:- 0.04M sodium phosphate buffer at pH 6.8, 0.001M $MgCl_2$, 0.05M KCl, 0.002M substrate (sodium salt adjusted to pH 6.8) placed in the side-bulb, and an amount of broken cells corresponding to 60-70 mg. dry weight of intact cells. The total volume of the reaction mixture was 4 ml. It was necessary to pre-incubate the reaction mixture for 2-3 hr. before the endogenous respiration had fallen to a sufficiently low value to allow accurate evaluation of the oxidation of added substrates.

(iii) For cell fractions, the reaction mixture contained 0.04M sodium phosphate buffer at pH 6.8, 0.025M substrate (sodium salt adjusted to pH 6.8) placed in the side-bulb, 0.1M KCN (adjusted to pH 6.8), 5×10^{-5}M methylene blue and an amount of the cell

fraction corresponding to c. 60 mg. dry weight of cells, all in a final volume of 4 ml. per flask. No pre-incubation period was needed in experiments with cell fractions.

Estimation of Formic Acid.

The method of Warner and Raptis (1955) was modified to allow estimation of small amounts (10-60 µmole) of formic acid. The method makes use of the fact that chloroform forms azeotropes only with formic acid and water. Acetic and higher acids do not form azeotropes with chloroform and are not estimated by this method, nor do they interfere with the estimation of formic acid. Aqueous samples at pH 7.4 were placed in 25 ml. round-bottomed distillation flasks, frozen and then dried *in vacuo* over sulphuric acid. Chloroform (5 ml.) and salicylic acid (0.2 g.) and a small glass bead were added to each flask and a condenser, 15 cm. long, fitted. The chloroform was distilled off completely and collected in a small conical flask. Methanol (5 ml.) and water (1 ml.) were added to the distillate and 1 drop of 1 % chlorophenol red as indicator. The

formic acid content of the distillate was determined by titration with 0.1N methanolic NaOH (made by diluting 2N aqueous NaOH with methanol) delivered from a 1.0 ml. all-glass syringe. The titre for control samples containing no formic acid was small (0.02 ml.) and that for samples of formic acid was 97 % of the theoretical titre both in the presence or absence of 4 mmole KCl. It was found that 5 ml. chloroform was sufficient to distil only about 25 μmole of formic acid, but by adding further 5-ml. lots of chloroform to the distillation flask and performing further distillations, the theoretical yield of formic acid was obtained from larger samples. For example, four such distillations were required to complete the distillation of 70 μmole of formic acid, the titres being 1) 0.24 ml. 2) 0.24 ml. 3) 0.13 ml. 4) 0.06 ml. In estimation of formic acid in M. lysodeikticus, reaction mixtures in which 20 μmole succinate had been oxidized by the cells were compared with (a) reaction mixtures containing no substrate and (b) those to which 60 μmole of formate had been added. The reaction mixtures were carefully re-

moved from the Warburg flasks into ice-cold distillation flasks and the procedure described above followed.

Chemicals.

All the inorganic chemicals used in this work were of AnalaR grade, and the organic compounds were Research Reagent grade supplied by Hopkin and Williams or Light. Sir Rudolph Peters, F.R.S., very kindly gave a sample of fluoroacetic acid, and Dr. M. Dixon, F.R.S., a sample of D-malic acid.

Appendix.

Reprints of two papers by Dr. Peter Mitchell and the present author are appended at the end of this thesis for easy reference. The appended papers are particularly relevant to this study; not only to the methods described in this chapter, but also to some of the experimental results reported in Chapter III.

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CHAPTER III.

EXPERIMENTAL RESULTS.

Enzymic Composition of the Plasma-membrane of Micrococcus lysodeikticus.

Protoplasts of Micrococcus lysodeikticus were prepared and subsequently fractionated to give a plasma-membrane fraction and a cytoplasmic fraction. For a preliminary survey, the Thunberg technique was used to estimate the oxidative activity of the fractions towards several substrates and Table II shows the distribution of succinic, lactic, formic and α-glycerophosphate dehydrogenases between the plasma-membrane and the cytoplasm. Experiments 1 and 2 refer to fractions obtained from two separate cultures of M. lysodeikticus. The oxidative activity per tube is expressed in arbitrary units. One unit = $\frac{100}{T}$, where T is the time taken for decolorization of the methylene blue (in minutes). Formic dehydrogenase was absent in this organism, and there was only a trace of α-glycerophosphate dehydrogenase activity. However, succinic and lactic dehydrogenase activities were large; practically the whole

of the succinic dehydrogenase activity of the cell appearing in the plasma-membrane fraction. It will be noticed that the values for total activity of each enzyme per unit dry weight of cells was markedly different in the two experiments. This was attributed to a difference in the length of time that fractions were stored at 4° before the enzymic assays were performed. In the case of Experiment 1, five hours elapsed between completion of fractionation and the assays, whereas in Experiment 2, the assays were done immediately the fractionation had been completed. The fractions of Experiment 1 showed even lower activities when the enzymic assays were delayed by a further four hours. Lactic dehydrogenase was inactivated to a greater extent than succinic dehydrogenase, and it may well have been due to this gross inactivation that, in Experiment 1, this enzyme appeared to be distributed equally between the plasma-membrane and the cytoplasm. In the active preparations of Experiment 2, the lactic dehydrogenase was found, like the succinic dehydrogenase, almost exclusively in the plasma-membrane. It was possible to keep intact

cells of M. lysodeikticus at 25° for 24 hr. without any loss of activity of oxidative enzymes on fractionation, but it was clear that, once the cell structure had been destroyed, autolytic processes became very active. The cell fractions lost enzymic activity rapidly, even when kept at 0°.

The preliminary findings obtained by use of the Thunberg technique were confirmed and extended by following oxygen uptake in Warburg manometers. Table III shows the distribution of oxidative enzymes estimated in this way, and it will be seen that the results agree well with those obtained by the Thunberg method. Cell fractions from protoplasts liberated by the action of lysozyme were compared with fractions from protoplasts formed autolytically, both types of protoplast having been prepared from samples of the same culture of M. lysodeikticus. Although the formation of protoplasts by the autolytic process took 24 hr. at 25°, the activity of the cell fractions obtained from them was the same as the activity of cell fractions from protoplasts liberated in 1 hr. by lysozyme action.

During the fractionation of autolytically-prepared protoplasts that had been exploded osmotically, it was noticed that the appearance of the fractions was identical to that of fractions from lysozyme-treated cells. There was no fraction corresponding to the cell-wall (as found, for example, in the fractionation of autolytically-prepared 'protoplasts' from Staphylococcus aureus (Mitchell and Moyle, 1957)). It would seem, therefore, as if the autolytic system involved here were truly lysozyme-like, and capable of solubilizing the cell-wall completely.

The lack of oxidative enzyme activity in the cytoplasmic fraction might not, as it has been supposed so far, have been due to absence of the enzyme concerned, for it could equally well have been due to the action of an inhibitor residing in this fraction. The latter possibility was tested by comparing the oxygen uptake of a sample of the plasma-membrane fraction with the oxygen uptake of a similar sample to which a comparable amount of the cytoplasmic fraction had been added. The results of such an experiment are shown in Table IV, and it

will be seen that there was only slight inhibition of the oxidative activity of the plasma-membrane fraction by cytoplasmic factors. Thus, the lack of succinic dehydrogenase and lactic dehydrogenase activity in the cytoplasmic fraction must have been due to the absence of the enzymes in this fraction.

Permeability of M. lysodeikticus to Some Solutes.

The permeability of bacteria to an externally-added solute may conveniently be measured by studying the effect on osmotically-fragile protoplasts prepared from the bacteria of suspension in an isotonic solution of the solute. Where there is no free diffusion of the solute through the plasma-membrane, the osmotic pressure exerted on the protoplast preserves it intact, but where the plasma-membrane is freely permeable, the protoplast is osmotically exploded. Changes in turbidity of protoplast suspensions provide a means of following the permeation process with time, for the intact protoplasts of cocci scatter about 70 % as much light as intact organisms whereas after osmotic explosion they scatter practically none (Mitchell and Moyle, 1956a; 1956d).

Some data are already available for the permeability properties of the plasma-membrane of M. lysodeikticus (Mitchell and Moyle, 1956c; 1956d). They show the plasma-membrane to be impermeable to a large range of small molecular weight solutes. It was necessary, however, to determine the permeability of the plasma-membrane to the solutes involved in this investigation.

Samples (0.1 ml.) of protoplast suspensions were pipetted into 5 ml. of 1M solutions of the solutes adjusted to pH 6.8 and containing 0.02M sodium phosphate buffer at pH 6.8, at 20°. The turbidity was measured at a wavelength of 700 mμ and readings were taken up to 1 hr. after the initial mixing of the protoplast suspension with the test solution. Figure 2 shows the stability of protoplasts to suspension in several solutes. The values obtained for the various solutes have been plotted on a relative scale. The somewhat low 10-min. readings found in the case of protoplasts suspended in succinate and malonate were at least partly caused by differences in refractive index of the suspending media. Only ammonium acetate,

of the solutes tested, freely permeated the plasma-membrane so that the protoplasts were exploded almost at once. The plasma-membrane was impermeable to NaCl, KCl, NH_4Cl, Na malonate, NH_4 malonate, and almost completely impermeable to K malonate, Na succinate, and K succinate. The conditions of this experiment were only semi-anaerobic, so the slight permeation of K succinate through the plasma-membrane can almost certainly be put down to the effect of metabolism.

Effect of Oxido-reduction Potential on the Oxidation of Succinate by Intact Cells.

The oxidation of succinate by animal succinic dehydrogenase normally requires the participation of a cytochrome system, the molecular oxygen ultimately needed for the oxidation coming from the action of cytochrome oxidase. The bacterial succinoxidase system is similar to this, although the bacterial 'cytochrome oxidase' is not identical to the animal one (Smith, 1954). The cytochrome oxidase may be by-passed and replaced by the addition of cyanide and methylene blue (as H acceptor)

to the reaction mixture, the over-all reaction still consuming oxygen which is made available through the autoxidizable methylene blue. The oxido-reduction (O-R) potential (E'_o) of methylene blue is +11 mV at pH 7, whereas that of the succinate-fumarate system is -10 mV, so that oxidation of succinate successfully reduces oxidized methylene blue. At the same time, the state of the bulk of the methylene blue in a reaction mixture may be used as an indicator of the prevailing O-R potential relative to the O-R potential of the succinate-fumarate system since the O-R potentials of the two systems are so close in value. It would be expected that the O-R potential of the cell suspension would have to be positive for succinate oxidation to occur, and experiments were carried out to evaluate the O-R potential of suspensions under different conditions and to relate O-R potential to ability of the cells to oxidize succinate.

First of all, the effect of O-R conditions on the rate of the endogenous respiration of intact cells of M. lysodeikticus was determined. Oxygen uptake was measured manometrically in a reaction

mixture of the following composition:- 0.04M sodium phosphate buffer at pH 6.8, 0.1M KCN (adjusted to pH 6.8), 0.05 % (w/v) methylene blue, the total volume per flask being 4 ml. The dry weight of organisms added to each flask was varied, as was the rate of shaking of the flasks in a water bath at 25°. The results are shown in Figure 3. The experimental points were derived from estimations in which bacteria from a number of separate cultures were used. At low concentrations of cells the oxygen uptake was proportional to cell dry weight but at high cell concentrations the oxygen uptake was very nearly constant per flask and independent of cell concentration. At low rates of shaking of the manometer flasks, the asymptotic value for oxygen consumption was lower than at high rates of shaking. The state of oxidation of the methylene blue in each flask was judged by inspection and it will be seen that, where oxygen uptake was proportional to cell dry weight, the O-R potential was positive (at least +11 mV) but that, where oxygen uptake was not proportional to dry weight, the O-R potential was negative, probably less than -30 mV

(i.e. more than 95 % of the methylene blue was in the leuco reduced form).

There are two likely explanations for the asymptotic characteristics of oxygen uptake. The first is that, over the asymptotic range, the rate of solution and diffusion of gaseous oxygen into the reaction mixture was limiting. This could explain the higher asymptotic value at the higher rates of shaking of the flasks. However, this explanation cannot be correct for, under different conditions, it was possible to obtain oxygen consumptions of at least 600 µl./hr./flask at a shaking rate of 120 strokes/minute. The second possibility is that the autoxidation rate of methylene blue was limiting. If this were so, it should be possible to alter the asymptotic value by altering the amount of methylene blue in the flasks but Table V shows that the oxygen uptake in endogenous respiration was independent of methylene blue concentration.

Whatever the primary cause of the falling off of oxygen uptake per unit cell dry weight in flasks containing high cell densities (and it was

probably some cellular component for which the oxygen tension was limiting), the fact remained that merely by shaking flasks at different rates it was possible to obtain cells, suspended at the same concentration, at an O-R potential either more positive or more negative than the O-R potential of the succinate-fumarate system.

Using the same reaction mixture as in the previous set of experiments, but with the addition of 0.025M succinate (sodium salt adjusted to pH 6.8) to the side-bulbs of an otherwise duplicate set of flasks, the effect of O-R potential on succinate oxidation was investigated. Figure 4 shows the results of these experiments, using the same symbols as in Figure 3. The extent of succinate oxidation is indicated by the length of the arrows at each experimental point on the graph. Once again, the reproducibility of results from one bacterial culture to another was good, for the experimental points plotted in the Figure were taken from the results of a number of separate experiments. It will be seen that there was little, or no, succinate oxidation in reaction mixtures of negative O-R potential but that there was succinate oxidation,

quantitatively proportional to the dry weight concentration of cells, when the O-R potential was positive. Unfortunately, owing to the rather large endogenous respiration, the accuracy of the estimations of succinate oxidation was not very great.

The results described above indicated the importance of a sufficiently positive O-R potential for the oxidation of succinate. In order to make sure that the O-R potential of the reaction mixture in routine manometric experiments would be sufficiently positive, only 25-30 mg. dry weight of cells was used in each flask, and the flasks were always shaken at 120 strokes/minute.

Endogenous Respiration of M.lysodeikticus.

Freshly-prepared washed suspensions of M. lysodeikticus possessed a very large endogenous respiration. Indeed, it was many times greater than the expected oxidation rate for added succinate by the cells. It was essential, therefore, to find a way of reducing this oxygen uptake considerably before any quantitative work on succinate permeation and oxidation could be attempted. Luckily, it was

found possible to reduce the endogenous respiration of the cells very simply, merely by setting up the Warburg flasks with reaction mixture containing the freshly-harvested cells and incubating them at 25°. The flasks were shaken at 120 strokes/min. with the tap of the manometer left in the open position. As this pre-incubation continued, the endogenous respiration declined until, after 5 hr., it had become more or less steady at a fairly low rate. The endogenous respiration of cells from two bacterial cultures is plotted in Figure 5.

As mentioned in Chapter II, this pre-incubation procedure was, of necessity, carried out routinely in all manometric experiments on intact cells.

SPECIFIC PERMEATION OF SUCCINATE.

A very simple experimental approach was used to assist the elucidation of the mechanism of succinate permeation. A comparison was made of the conditions required for the oxidation of succinate by intact cells of M. lysodeikticus (in which permeation and metabolism were both involved) and by

broken cells (in which metabolism alone was involved).

Effect of pH and the Requirement for K^+.

In order to test the effect of pH on succinate oxidation by intact cells, it was necessary to supplement the usual manometric reaction mixture with 0.04M sodium phosphate buffers so as to hold the pH at the required value, although it was known that phosphate was somewhat inhibitory. The actual pH value of the flask contents was determined at the end of each experiment by means of a glass electrode. In varying the K^+ content, the usual 1.0M KCl of the reaction mixture was partially replaced by NaCl so that the final salt concentration remained the same. In the absence of K^+ there was no succinate oxidation at pH 7.4, although oxidation occurred under these conditions at pH 6.0. However, in 1.0M KCl, the oxidation at pH 7.4 was slightly greater than at pH 6.0 (see Table VI).

The oxidation of succinate by broken cells was 3.7 times more rapid at pH 7.4 than at pH 6.0 (thus resembling classical succinic dehydrogenase),

the Q_{O_2} for oxidation of succinate by broken cells at pH 7.4 being c. 1.0. Potassium ions did not affect the oxidation rate of broken cells.

The oxidation of fumarate and L-malate (or DL-malate) at pH 7.4 by intact cells required the presence of K^+, but acetate oxidation was only inhibited by 29 % by absence of K^+ (see Table VII). The fact that acetate could permeate the plasma-membrane of these cells and that its oxidation was much less affected by lack of K^+ than the oxidation of succinate was consistent with the idea that the specific permeation of succinate required potassium ions.

Extent and Probable Pathway of Succinate Oxidation.

It was most unlikely that the oxidation of succinate in intact cells of M. lysodeikticus would proceed as far as fumarate and that the oxidation would stop at that point. It was thought that it would help the interpretation of experiments if more were known about the fate of succinate after it had permeated into the cell. By adding sufficiently small quantities of substrates, oxidation

could be followed manometrically to completion and the total oxygen uptake determined. Figure 6 shows the results of such an experiment plotted from the moment of tipping in 8 μmole of substrate from the side-bulb. This experiment has been plotted graphically so that it may serve as an illustration of the many similar experiments which are described later in this chapter. It will be noticed that the endogenous respiration rate remained steady throughout the period of the experiment; that the rate of succinate oxidation was about five times that of the endogenous respiration so that the effect of inhibitors, etc., could be estimated accurately; that the rate of succinate oxidation was (after a slight initial lag) constant almost to the end of the oxidation of added substrate; and that, when the oxidation of added succinate was completed, the rate of oxygen uptake returned to a value exactly corresponding to that of the endogenous respiration.

Since

$$\text{succinate} + \tfrac{1}{2}O_2 = \text{fumarate} + H_2O,$$

the expected oxygen uptake could be calculated.

It was found that the oxidation of succinate consumed exactly 4 atoms instead of 1 atom of oxygen per mol. of succinate; that the oxidation of fumarate consumed 3 atoms of oxygen; and that the oxidation of acetate consumed 2.5 atoms of oxygen (see Table VIII for experimentally-found values). Clifton (1952) reported somewhat similar oxygen uptake values for the oxidation of tricarboxylic acid cycle substrates by a number of different species of bacteria. The fact that the oxygen consumption per mol. of added substrate was always exactly a whole number indicated that the oxidative pathway was a reasonably direct one without the complication of side-reactions or the piling up of intermediates. No doubt, by the end of the pre-incubation period, the cells had become depleted of metabolic intermediates which might otherwise have allowed the complication of side-reactions. Since fumarate consumed 1 less atom of oxygen per mol. than succinate, the Krebs' tricarboxylic acid cycle seemed a likely possibility as the metabolic path. The arguments for and against the operation of the Krebs' cycle as a major metabolic pathway in bacteria has

recently been reviewed by Wiame (1957).

Respiratory Quotient.

When a balance of oxygen uptake and carbon dioxide output was made for the endogenous respiration and for the oxidation of added succinate and acetate, an interesting fact emerged. The R.Q. (v_{CO_2}/v_{O_2}) for the endogenous respiration was 0.8 — a usual value indicating almost complete oxidation of the endogenous substrates. The R.Q. for succinate oxidation, however, was only 0.5, for only 1 mole of carbon dioxide was produced per mol. of succinate oxidized.

Returning to the problem of the metabolic pathway of succinate: the only way in which the Krebs' cycle can function cyclically is by the added substrate entering as 'acetate' and for there to be trace amounts of all the cycle intermediates present. Addition of a substance directly into the cycle sequence of intermediates will certainly cause oxidation, but not by a cycling of the Krebs' cycle, for the acetate required at each revolution of the cycle is not available. Very soon all the

intermediates oxidized before citrate will begin to pile up. The stoichiometry of succinate oxidation in M. lysodeikticus seems to preclude this possibility. A hypothetical pathway can be constructed, however, so that succinate and fumarate do enter the Krebs' cycle as 'acetate' (see Figure 7). On examination of this hypothetical pathway, it will be noticed that it would be expected that 4 mol. of carbon dioxide would be produced per mol. of succinate oxidized. In order that the hypothesis should fit the observed facts, Dr. Peter Mitchell suggested that, where there was simultaneous oxidation and decarboxylation, the CO_2 and 2H might condense to form HCOOH thus using carbon dioxide as an oxidizing agent. Neuberg (1914) was the first to describe a bacterial system which converted α-keto acids into formic acid and the corresponding fatty acid with one less C atom. More recently, Krebs (1937), Kalnitsky and Werkman (1943), Chantrenne and Lipmann (1950) and Novelli (1955) have demonstrated a system of this kind in Bacterium coli which, it is now known, converts pyruvate in the presence of CoA into formate and acetyl-CoA.

It was possible that such systems operated in M.lysodeikticus in the oxidative decarboxylation of pyruvate and ∝-ketoglutarate, and also of isocitrate which is oxidatively decarboxylated by a single enzyme (Moyle, 1956). If this were the case, oxidation of 1 mol. of succinate should require 4 atoms of oxygen and produce 1 mol. of carbon dioxide:-

$$HOOC.CH_2.CH_2.COOH + 4O = 3(H.COOH) + CO_2.$$

Similarly, an oxygen uptake of 3 atoms per mol. would be predicted for the oxidation of fumarate.

Recently, a glyoxylate cycle has been postulated for bacteria (Kornberg and Krebs, 1957). The two enzymes fundamental to the cycle are malic synthetase which condenses one molecule of acetate and one of glyoxylate to form malate (Wong and Ajl, 1956) and isocitritase which converts isocitrate to one molecule of succinate and one of glyoxylate (Campbell et al., 1953; Smith and Gunsalus, 1954; Olson, 1954). It is known that M. lysodeikticus contains a very active isocitritase (Smith and Gunsalus, 1955) and it seems likely that the

glyoxylate cycle is functional in this organism. If it were so, the values of oxygen uptake of 5 atoms and carbon dioxide output of 1 mol. for the oxidation of 2 mol. of acetate would be precisely what one would have anticipated (Fig. 7).

It was hoped that it might be possible to demonstrate the formation of formic acid by cells of M. lysodeikticus during the oxidation of succinate and so substantiate the theory concerning the condensation of (CO_2 + 2H) in oxidative decarboxylation. It was confirmed meanwhile that the cells were almost completely unable to oxidize formate. The specific method of Warner and Raptis (1955) was used for the estimation of formic acid in reaction mixtures in which a known amount of succinate had been oxidized by cells of M. lysodeikticus. No formic acid could be detected, although samples of formic acid added to a reaction mixture could be estimated accurately by this method. Presumably, the cells retained the (H.COOH) in some form more complex than free formic acid.

Michaelis Constant.

The effect of the concentration of substrates

on the rate of their oxidation by intact cells of M. lysodeikticus showed that the apparent Michaelis constant (K_m) for succinate and fumarate was low (Fig. 8). For succinate the K_m was less than 1.0×10^{-3} M. It cannot be decided from these results whether the K_m represented the affinity of the carrier in the plasma-membrane for the substrate or the affinity of one of the enzymes of the metabolic pathway for its substrate. The maximum Q_{O_2} (i.e. the rate of oxygen uptake expressed in µl./hr./mg. cell dry weight) for succinate, fumarate and L-malate was equivalent if the fact that the oxidation of 1 mol. of succinate took up 4 atoms of oxygen while oxidation of fumarate and L-malate took up 3 atoms was taken into account. This would be consistent with the idea that the K_m measured in the case of succinate and fumarate was that of some enzymic step beyond malate in the oxidative pathway, and that the K_m for L-malate largely represented the affinity of the carrier in the plasma-membrane for malate. On the other hand, the K_m in each instance might represent the affinity of the carrier for the substrate. The fact of the equivalent maximum Q_{O_2} would then

suggest that the same carrier might be responsible for the permeation of all three substrates. A third possibility to account for the equivalent maximum Q_{O_2} for the various substrates is that the rate of entry of the substrates on their specific carriers is limited by the rate of exit of a common exchanger for all the substrates (see Mitchell, 1957a; 1957b).

Effect of Malonate.

The classical competitive inhibitor for succinic dehydrogenase is sodium malonate, a structural analogue of succinate with a very high affinity for the enzyme (Quastel and Wooldridge, 1928). The effect of this non-penetrating inhibitor on the oxidation of succinate by intact cells would be expected to give information as to whether the carrier for succinate was identical in specificity to succinic dehydrogenase. Some exploratory experiments showed a variable inhibition by malonate in a reaction mixture containing 0.025M Na succinate and 0.05M Na malonate, a ratio of substrate to inhibitor which would be expected to give 90-100 % inhibition of

succinic dehydrogenase. The fragility of freshly-harvested cells of M. lysodeikticus had already been noticed but it was thought that the high concentration of salt in the manometric reaction mixture would prevent rupture of cells during an experiment. However, a check was made on this point, by comparing reaction mixtures before and after experiments.

The first method used was the estimation of the amount of free nucleotide (by extinction at 260 mμ) in the external medium. The values obtained were compared with the total nucleotide of the fresh cells (present in the supernatant after treatment of the cells with 5 % trichloroacetic acid). Even at the beginning of experiments (less than 30 min. after harvesting the cells) 25 % of the total cell nucleotide had leaked into the surrounding medium, and at the end of experiments 40 % of the total cell nucleotide had leaked out. There was little variation from one experiment to another, and yet these same experiments showed a variation in inhibition by malonate of between 0 % and 30 %. Clearly, this method did not

measure the proportion of cells ruptured. Rather, it was an interesting observation on the permeability of the plasma-membrane of bacteria to nucleotides.

The second method employed was a direct turbidity measurement at 700 mμ of the reaction mixtures, suitably diluted with water. Table IX shows the agreement between percentage inhibition of succinate oxidation by malonate and the percentage of cells ruptured when estimated in this way. Succinate oxidation by truly intact cells was, without doubt, not inhibited by malonate.

The above findings stress the danger of assuming that experiments performed on washed suspensions of bacteria are, in fact, experiments on intact organisms (see also Davis, 1956). Very rarely is it known for certain whether the bacteria remain whole throughout the time course of an experiment. It would be well for microbiologists not to overlook this factor if they wish to interpret their experimental results correctly.

In broken cells of M. lysodeikticus succinate oxidation was almost completely inhibited by malonate.

The experimental values for percentage inhibition at pH 6 and 7.4 varied between 85 and 100 %. These results are fully in agreement with those of Krampitz and Werkman (1941) and Saz and Krampitz (1955) on the inhibition of succinate oxidation by malonate in intact and crushed cells of this organism. The succinate oxidation of intact cells of Bact. coli was unaffected by malonate. Cells of M. lysodeikticus that had been stored at -15° were found to have exactly the same oxidation rate for succinate as freshly-harvested cells from the same culture, and the oxidation was not inhibited at all by malonate. It was judged from these results that the stored cells were undamaged from the point of view of the structure and function of the plasma-membrane, and this encouraged use of stored cells for some experiments.

Ammonium malonate was also used in experiments on intact cells of M. lysodeikticus for comparison with the non-penetrating Na malonate, for it was hoped that the plasma-membrane would be permeable to NH_4 malonate. The results indicated, however, that the NH_4 malonate did not permeate for

it did not inhibit succinate oxidation. This supposition was confirmed when it was found that protoplasts remained stable in 1.0M NH_4 malonate.

The results described above lead to the conclusion that the carrier for succinate in the plasma-membrane is at least as specific as the enzyme succinic dehydrogenase although its specificity is different.

In order to accumulate more information about the specificity of the carrier for succinate, the effect of a number of other structural analogues of succinate on the oxidation of succinate by intact cells was studied. Some of these substances were reported to be inhibitors of succinic dehydrogenase where it is probable that the spacing of the two -COOH groups in the substrate is largely responsible for enzymic specificity. Others were potential inhibitors of the succinate carrier for they contained the $-CH_2.CH_2-$ configuration which might well be involved in the specificity of the succinate carrier. The succinate analogues were tested at a molar concentration equal to, or double, that of the succinate to which they were added.

Effect of β-phenyl Propionate.

At a molar concentration double that of succinate, β-phenyl propionate was found to inhibit succinate oxidation in intact cells of M. lysodeikticus by only 3 %. The β-phenyl propionate was not itself oxidized. In tests with broken cells, the percentage inhibition of succinate oxidation rose to 14 %. Succinic dehydrogenase was reported to be inhibited by β-phenyl propionate (Quastel and Wooldridge, 1928).

Effect of Mesotartrate.

Mesotartrate did not inhibit the oxidation of succinate by intact cells when it was added at an equimolar concentration to the succinate. In fact, the oxidation rate of succinate in the presence of the mesotartrate was 105 % the rate in succinate alone. Mesotartrate was not oxidized by the cells. There was no inhibition of succinate oxidation by broken cells either, although succinic dehydrogenase was supposed to be somewhat inhibited by mesotartrate (Quastel and Wooldridge, 1928).

Effect of Succinimide.

Quastel and Wooldridge (1928) found no inhibition of succinic dehydrogenase by succinimide, but it was expected that it might affect succinate permeation in M. lysodeikticus. In fact, it only slightly inhibited the oxidation of succinate by intact cells when present at a molar concentration double that of the succinate, but was itself rapidly oxidized. For each mol. of succinimide oxidized, 1.5 atoms of oxygen was taken up. This result could be explained by the following hypothesis for succinimide oxidation:-

$$\begin{matrix} CH_2.CO. \\ CH_2.CO. \end{matrix} NH + 2H = \begin{matrix} CH_3.CO. \\ CH_3.CO. \end{matrix} NH + H_2O = CH_3.CO.NH_2 + CH_3.COOH$$

Effectively, each molecule of succinimide would be converted by one reductive step into one molecule of acetamide and one of acetate. Assuming that acetamide was not oxidized by the cells and that acetate followed the pathway already discussed (Fig. 7), it would be predicted that one mol. of succinimide should take up 1.5 atoms of oxygen. Experi-

mental evidence showed that acetamide was not oxidized by M. lysodeikticus. Mole for mole of substrate, the oxidation rate for succinimide in intact cells was 3 times greater than that for succinate, the Q_{O_2} values being 12 and 11 respectively in one experiment. In view of the probability that succinimide was free to diffuse through the plasma-membrane, this was interesting. The oxidation rate of succinate and succinimide together was 93 % of the sum of the rates of oxidation of the two substances separately. The implications of these results are discussed in Chapter IV.

In broken cells, succinimide was not oxidized nor did it affect the oxidation rate of succinate appreciably. This would, indeed, have been expected if succinimide were oxidized through the hypothetical pathway mentioned above, for acetate was not oxidized by broken cells (presumably because of dilution effects). Experiments by Utter et al. (1946) on oxidation by lysed cells and by what appear from their description to have been protoplasts of this organism showed that there was no oxidation of acetate by lysed cells.

Effect of Propionate.

Propionate did not greatly inhibit the oxidation of succinate by intact cells but was itself rapidly oxidized with a Q_{O_2} of 12. One mol. of propionate was oxidized at the expense of 4 atoms of oxygen. If propionate were oxidized to pyruvate -

$$CH_3.CH_2.COOH - 2H + O = CH_3.CO.COOH$$

and then by the hypothetical pathway, a consumption of 4 atoms of oxygen per mol. of propionate oxidized would be expected. The metabolic pathway of propionate is, however, not unequivocally proved to be through pyruvate. Flavin et al. (1955) have experimental evidence that propionyl-CoA is carboxylated to give methylmalonyl-CoA which is then converted to succinyl-CoA. Metabolism by this route would also be expected to require 4 atoms of oxygen per mol. propionate oxidized. The oxidation rate for propionate and succinate together was 82 % of the sum of the rates for succinate and propionate separately.

Effect of D-malate.

The 'unnatural' isomer of malic acid, D-malate, was the last of the series of structural

analogues tested for their efficacy as inhibitors of succinate permeation. Somewhat surprisingly, the non-penetrating D-malate was oxidized slowly by M. lysodeikticus with a Q_{O_2} of 1.4 at a concentration of 0.002M D-malate. The oxidation of succinate in the presence of this concentration of D-malate was inhibited by 14 %. It was discovered later that the K_m for D-malate was rather high (Fig. 8) so that, at the concentration used in this experiment, it would not have been expected that the D-malate would have competed very effectively with succinate.

The results of experiments on the effect of structural analogues of succinate on succinate permeation in M. lysodeikticus, and the concentration at which each analogue was tested, are summarised in Table X. None of these substances interfered with the succinate permeation mechanism so that a very high specificity must be inferred for the succinate carrier. The effect of a number of metabolic inhibitors on succinate permeation is described below. The inhibitors were added to reaction mixtures in solution at pH 6.8, as the sodium salt

or with the necessary adjustment of pH made by neutralization with NaOH or HCl.

Effect of Inorganic Orthophosphate and Arsenate.

It has already been mentioned that phosphate was found to be somewhat inhibitory to the oxidation of succinate by intact cells, and that it was for this reason that the routine manometric reaction mixture contained no buffer. The presence of externally-added phosphate had no effect on the rate of endogenous respiration in intact cells or in broken cells. Phosphate at a concentration of 0.05M inhibited the oxygen uptake due to the oxidation of succinate in intact cells by more than half, but at a concentration of 0.005M the inhibition was slight. Succinate oxidation in broken cells was unaffected, however, by phosphate. Oxidation of fumarate by intact cells was not inhibited by the presence of external 0.05M phosphate — in fact, it was stimulated by about 15 %. The inhibition of acetate oxidation was not large but was somewhat variable from one cell suspension to another. The oxidation of L-malate by intact cells

was greatly inhibited by 0.05M phosphate, but oxidation of DL-malate was rather less inhibited.

Arsenate (0.01M) inhibited succinate oxidation by intact cells of M. lysodeikticus by 10 %, but it had no effect at this concentration on oxidation of succinate by broken cells. It is known that arsenate, being structurally very similar to phosphate, is sometimes indistinguished from phosphate by metabolic systems, and it would appear probable, from the quantitative aspect of the results quoted above, that in this case arsenate and phosphate were acting in the same way as competitive inhibitors of succinate permeation (see Mitchell, 1954a). Table XI gives the experimental values found for the percentage inhibition of oxidation of substrates by arsenate and phosphate.

Effect of 2,4-dinitrophenate.

The 'uncoupling' reagent 2,4-dinitrophenate (Loomis and Lipmann, 1948) at a concentration of 0.001M was found to increase the endogenous respiration of intact cells of M. lysodeikticus and Bact. coli very markedly, as might have been

expected by inference from the effect of this reagent on respiration of animal tissues. In M. lysodeikticus there was an immediate rise in the rate of endogenous respiration on addition of the 2,4-dinitrophenate and this high rate was sustained for about one hour. During the next hour, the respiration rate slackened until it had returned to the same rate as in untreated cells. In Bact. coli the endogenous respiration rate rose slowly after addition of the reagent to a higher rate which was maintained for several hours. Endogenous respiration in broken cells of M. lysodeikticus was unaffected by 0.001M 2,4-dinitrophenate. Owing to the effect of 2,4-dinitrophenate on the endogenous respiration in intact cells, a concentration of 0.025M of each substrate was used so that it would allow oxidation by the cells at a constant rate for more than two hours. This enabled a more accurate evaluation of substrate oxidation rate in the presence of the inhibitor. As can be seen from Table XII, 2,4-dinitrophenate completely inhibited succinate oxidation in intact cells but not in broken cells, and almost completely inhibited oxidation

of fumarate and DL-malate by intact cells. In M. lysodeikticus acetate oxidation was not affected by 2,4-dinitrophenate, but in Bact. coli it was completely inhibited. This observation was in conformity with the idea that 2,4-dinitrophenate inhibited the specific permeation mechanism for succinate.

Effect of Iodoacetate.

The thiol-reactor iodoacetate, at a concentration of 0.01M, increased the endogenous respiration rate of intact cells but decreased the endogenous respiration rate of broken cells. Oxidation of succinate by both intact and broken cells was partially inhibited by this concentration of iodoacetate, indicating that any effect on the intact cells was more likely to be due to effect on the subsequent metabolism of the succinate than on the specific permeation mechanism (Table XIII). The oxidation of acetate by intact cells was initially unaffected by the presence of iodoacetate, but ultimately there was complete inhibition.

Effect of Phenyl Mercuric Acetate.

The endogenous respiration of intact cells of M. lysodeikticus was halved in rate by the presence of 1 x 10^{-4}M phenyl mercuric acetate, and the oxidation of succinate, acetate and fumarate was completely inhibited. However, the oxidation of succinate by broken cells was not inhibited by this concentration of phenyl mercuric acetate (Table XIV), indicating that the inhibition in intact cells was due to the action of this thiol-reactor on the succinate carrier mechanism.

Effect of Arsenite.

The addition of 0.01M arsenite to the reaction mixture reduced the rate of endogenous respiration in intact and broken cells of M. lysodeikticus to half the usual rate. Oxidation of succinate, acetate and fumarate was completely inhibited in intact cells by the presence of arsenite (Table XV). In broken cells, the oxidation rate for succinate was apparently slightly stimulated by the addition of 0.01M arsenite. These results confirm the involvement of a thiol grouping in the carrier for succinate, for the trivalent arsenicals are thiol-

reactors which are particularly reactive with dithiol groups.

Effect of Fluoroacetate.

Fluoroacetate at a concentration of 0.001M did not inhibit the endogenous respiration of intact cells of M. lysodeikticus — in fact, the fluoroacetate seemed to be oxidized with an oxygen uptake of 2.7 atoms per mol. of fluoroacetate. Of course, the temporary increase in oxygen uptake rate found may have reflected an alteration in the steady state proportions of all the endogenous substrates caused by the addition of the inhibitor rather than a direct oxidation of the fluoroacetate itself.

The rate of oxidation of succinate was not much affected by the presence of fluoroacetate, but that of acetate was inhibited 50 % (Table XVI). However, the stoichiometry of both succinate and acetate oxidation was altered by the presence of fluoroacetate. For each mol. of succinate oxidized 1.6 atoms of oxygen were taken up, and for each mol. of acetate oxidized 1.1 atoms of oxygen were

taken up. The mechanism of the *in vivo* inhibition of metabolism by fluoroacetate was, for many years, in doubt for, *in vitro* fluoroacetate did not inhibit any one of the Krebs' tricarboxylic acid cycle enzymes although it was a powerful inhibitor of intact systems *in vivo*. Recent work has established that it is fluorocitrate, which is synthesised from fluoroacetate by the condensing enzyme, which is a potent inhibitor of aconitase *in vivo* (Peters, 1955; 1957; and personal communication). If the hypothetical pathway for succinate in *M. lysodeikticus* (Fig. 7) were to be blocked at aconitase, it would be expected that each mol. of succinate oxidized should take up 2 atoms of oxygen, but only if the effective equilibrium of all the enzymic steps involved were 100 % towards the product (citrate). If the effective equilibrium were such as to allow less than 100 % oxidation of the substrate, then a somewhat lower value for oxygen uptake would be expected than the stoichiometric value — as was indeed found. On the basis of the hypothesis that acetate followed the same pathway as succinate after conversion of two molecules of acetate to one of succinate, it

would be predicted that the oxidation of 1 mol. of acetate would take up (somewhat less than) 1.5 atoms of oxygen. In fact an oxygen uptake of 1.1 atoms was found. This result would, of course, only be expected if the glyoxylate cycle reactions were unaffected by fluoroacetate. As postulated by Kornberg and Krebs (1957), the malic dehydrogenase, condensing enzyme and aconitase of Krebs' tricarboxylic acid cycle also form enzymic links in the glyoxylate cycle. If this is correct, acetate oxidation by this pathway should have given a maximum of 0.5 atoms of oxygen uptake per mol. in the presence of fluoroacetate. Perhaps it is permissible to postulate some alternative pathway between malate and isocitrate in the new glyoxylate cycle which is not susceptible to fluorocitrate inhibition. If this were so, it might resolve some of the complications of the effect of fluoroacetate on bacteria, mentioned by Peters (1957).

Effect of Semicarbazide.

Semicarbazide at a concentration of 0.1M had little effect on the endogenous respiration of intact

cells of M. lysodeikticus. Neither succinate nor fumarate oxidation was inhibited by semicarbazide, although acetate oxidation was almost completely inhibited (Table XVI). However, the addition of 2×10^{-4} M succinate (i.e. one tenth the amount of acetate used) to acetate in the presence of 0.1M semicarbazide restored the oxidation of acetate to its normal rate, and both these facts indicated that the action of the carbonyl-binding semicarbazide was on some enzymic step occurring in the metabolic pathway of acetate but not in the metabolic pathway of succinate. Stoichiometrically, semicarbazide had the effect of increasing the oxygen uptake during oxidation of succinate and fumarate each by one atom per mol. of substrate. For instance, in the presence of semicarbazide, succinate oxidation required 5.1 atoms of oxygen per mol., while fumarate oxidation required 3.7 atoms per mol. The reason for this effect is not known.

Effect of Hydroxylamine.

Because of their instability, hydroxylamine solutions were prepared immediately before use

from a stock solution of recrystallized hydroxylamine hydrochloride (which is stable in solution at 0°) by neutralization with NaOH to pH 7. The endogenous respiration of cells of M. lysodeikticus was reduced by a half in the presence of 0.1M hydroxylamine. Succinate oxidation was completely inhibited both in intact and broken cells by 0.1M hydroxylamine, but there was no inhibition of succinate oxidation by 0.001M hydroxylamine (see Table XVI). Hydroxylamine is a carbonyl-binding reagent, and a concentration of 0.001M is sufficient for complete inhibition of a sensitive enzyme (see, for example, Antia, Hoare and Work, 1957). For the formation of hydroxamic acids by the probably non-enzymically-catalysed action of hydroxylamine on 'activated' substrates the concentration of hydroxylamine required is high, and even at a concentration of 0.5M is still rate-limiting (Elliott, 1956). The results described above made it impossible to use this method to test whether the succinate was 'activated' at the carrier, but they made it clear that a carbonyl group was not involved in the permeation of succinate.

Effect of Carbon Dioxide.

In experiments in which oxygen uptake and carbon dioxide output were measured in order to determine the respiratory quotient for the oxidation of externally-added substrates, it was noticed that the time taken for completion of oxidation of succinate in flasks in which there were no 'KOH-papers' was almost twice as long as the time taken for the oxidation of the same amount of succinate in flasks containing 'KOH-papers' to remove carbon dioxide. In the case of acetate, there was a slight slowing of the rate of oxidation in the presence of carbon dioxide but the effect was much less marked (see Table XVI).

Competition between Substrates.

It had been shown in Figure 8 that the oxidation rate of succinate, fumarate and acetate by intact cells of M. lysodeikticus was constant and independent of concentration above a concentration of 0.002M. Table XVII (Expt. 1) shows a comparison of the oxidation rates of succinate, fumarate and acetate alone with those of succinate with fumarate

and succinate with acetate, all added at a concentration of 0.002M. There was no competition between succinate and acetate, the rate of oxidation of both together being almost equal to the sum of the rates of oxidation of each separately. However, there was a strict competition between succinate and fumarate, the oxidation rate of the two together being only equal to the rate of oxidation of succinate alone. Experiment 2 shows a similar comparison of succinate, fumarate and D-malate oxidation rates with those of succinate and D-malate together and fumarate and D-malate together. Once again, there was competition between the substrates but, since the concentration of D-malate used was below that required for saturation (see Fig. 8), the oxidation rate of combinations of substrates was nearer to that for succinate (or fumarate) than that of D-malate. Experiment 3 shows the effect of the addition of L-malate (at a concentration (0.05M) sufficient to saturate the system) to succinate at a concentration of 0.002M. The oxidation rate of the two substrates together was exactly intermediate between the oxidation rates of succinate and L-malate

separately, suggesting a strict competition between succinate and L-malate with the combined oxidation rate contributed equally by the two substrates under these conditions.

It cannot be decided from these results whether the competition described here was due to competition for the same plasma-membrane carrier sites, or for the same exchanger molecules (the substrates having separate carriers), or to some limiting reaction in the metabolic pathway of the substrates. It would appear to be unlikely to be the last-named for succinate and acetate did not compete with each other and yet it was probable that their metabolic pathways were the same.

Accumulation of Succinate.

Earlier in this chapter the effect of 2,4-dinitrophenate and arsenite on succinate oxidation by intact cells of M. lysodeikticus was described. Both of these substances were shown to inhibit the permeation of succinate while scarcely affecting its metabolism. It was possible, therefore, to find out whether succinate was accumulated to any

appreciable extent by the cells or whether it was metabolized as rapidly as it entered the cells, by adding the inhibitor of permeation some time later than the succinate and following the subsequent oxidation with the knowledge that permeation of more succinate molecules was prevented and that the observed oxidation must be that of 'internal' succinate which had accumulated in the cells before the addition of the inhibitor. Figure 9 shows the result of a typical experiment on these lines. Succinate (0.025M) was added to a manometric reaction mixture containing intact cells of M. lysodeikticus in the usual way and 0.001M 2,4-dinitrophenate added either simultaneously with the succinate (0 min.) or some time later (10 and 30 min.). In the Figure, oxygen uptake is plotted from the moment of adding the inhibitor and corrected for the oxygen uptake due to endogenous respiration in the presence of the inhibitor. The oxygen uptake found when substrate and inhibitor were added simultaneously was, of course, caused by the small proportion of the normal succinate permeation not inhibited by the 2,4-dinitrophenate. It will be

noticed that, where the inhibitor was added later than the substrate, there was an increase in the oxygen uptake of 96 µl./flask (24.2 mg. cell dry weight) over and above that found in the 0-min. sample. Assuming a stoichiometry as before, this is equivalent to 96/44.8 = 2.1 µmole succinate. If we assume an internal water space of about 50 µl. per 25 mg. cell dry weight (Mitchell, 1953), then, if the succinate accumulated were free inside the cells, it would make an approximately M/25 solution. Similar experiments using 0.01M arsenite as permeation inhibitor in place of 2,4-dinitrophenate gave the same results, as shown in Table XVIII. The amount of succinate accumulated per unit cell dry weight was the same whether 2,4-dinitrophenate or arsenite was used, but the rate of oxidation of the internally-accumulated succinate was slightly slower in the presence of arsenite. This was the reason for allowing a standard interval of 90 min. for the completion of the oxidation of the accumulated succinate in estimating the extent of the accumulation. The amount of succinate accumulated in the cells in experiments using an external concentration of 0.025M

succinate would give a concentration inside equivalent to that outside the cells. An experiment, the results of which are quoted in Table XVIII, was carried out to estimate the amount of succinate accumulated when the external concentration of succinate was reduced to 0.002M. The amount accumulated (per unit cell dry weight) was the same from the lower as from the higher external concentration, the succinate being accumulated from the lower concentration against a concentration gradient, the final internal concentration being 20 times greater than the external concentration. The accumulated succinate represented one quarter of the total succinate added (at the lower concentration).

In a series of experiments, the results of which appear in Table XVIII, the time interval between addition of substrate and inhibitor was reduced to discover the least time required for the completion of the accumulation process after the addition of the external substrate. It was found that succinate accumulation was complete within 2 min. at 25°. Since the rate of succinate oxidation was known, it could be calculated that during the accumu-

lation process at least 10 molecules of succinate were accumulated for every 1 molecule of succinate oxidized.

Conditions required for the accumulation of fumarate were the same as those for the accumulation of succinate. The amount of fumarate accumulated was equal to that of succinate accumulated when the stoichiometry of their oxidation was taken into account — the oxygen uptake in the oxidation of accumulated fumarate was ¾ that obtained in oxidation of accumulated succinate. This method of estimation did not disclose any accumulation of acetate by the cells. However, since acetate was able to permeate the plasma-membrane and there was no oxygen uptake at all in the 0-min. sample (cf. Fig. 9) in the presence of arsenite, the oxidation of acetate (unlike that of succinate and fumarate) must be inhibited by arsenite. It may be that one of the enzymes of the glyoxylate cycle is sensitive to this inhibitor.

The effect of the addition of acetate on the amount of succinate accumulated was studied by making use of the fact that acetate was not oxidized (and

possibly not accumulated) in the presence of arsenite. Succinate and acetate were added at the same concentration (0.002M), and the results of the experiments are shown in Table XIX. The presence of acetate prevented the accumulation of succinate more effectively initially than later during the course of the accumulation process. At 2 min., when accumulation of succinate was just completed, the acetate hardly affected the amount of succinate accumulated, but if the addition of arsenite was delayed up to 10 min. after addition of the substrate, the amount of succinate accumulated in the presence of acetate was double that accumulated in succinate alone. Presumably, acetate initially competed with succinate in the accumulation process even though the permeation of acetate through the plasma-membrane did not require specific carriers.

An attempt was made to discover whether the accumulated succinate was recoverable from the cells merely by destroying the plasma-membrane with organic solvents and releasing the soluble cytoplasmic constituents. In order to make manipulations easier,

instead of incubating 4 ml. of reaction mixture in each of 6 Warburg flasks, 24 ml. of reaction mixture of the usual composition was placed in each of two 100 ml. conical flasks plugged with cotton wool. These flasks were incubated at 25° with mechanical shaking for the standard 5-hr. pre-incubation period, and then 48 μmole succinate was added to the contents of one flask and 2 min. later the contents of both flasks were cooled to 0°, transferred to centrifuge tubes and centrifuged at 2°. The supernatants were withdrawn and the cells dispersed in 10 ml. ice-cold 1.0M KCl. Cold ether (5 ml.) was added to each tube which was then vigorously shaken for 5 minutes. The suspensions were centrifuged at 2° and the aqueous supernatants carefully removed. The succinate content of all the supernatants was assayed by oxidation using freshly-harvested cells of M. lysodeikticus in the usual way. It was found that all (114 %) of the succinate added was recoverable in the external medium, and none was found in the cytoplasmic fraction. On the basis of the experiments already described (see Fig. 9), it would have been expected that

one quarter of the external succinate would have disappeared from the medium in the 2 min. allowed before chilling the suspensions. Obviously, under these conditions, there was no accumulation of succinate. There was one fundamental way in which conditions in this experiment differed from those in experiments in which succinate accumulation had been demonstrated — carbon dioxide was present in the reaction mixture in the conical flasks as the flasks did not contain 'KOH-papers'. It was confirmed that the presence of carbon dioxide completely prevented accumulation of succinate by following oxygen uptake and (oxygen uptake and carbon dioxide output) manometrically in flasks with, and without, 'KOH-papers'. Arsenite was used to inhibit permeation as previously described. Where carbon dioxide was present there was no succinate accumulation in 2 min. (Fig. 10). Owing to the relatively small carbon dioxide output in succinate oxidation by M. lysodeikticus, the gas change in the flasks without 'KOH-papers' should have been 67 % the gas change in flasks with 'KOH-papers' to represent the same succinate accumulation. The difference

in gas change found between 0-min. and 2-min. samples in the former was only 6 % of the difference in gas change in the latter (see Fig. 10). It will be noticed that the gas change for 0-min. samples in flasks without 'KOH-papers' was equal to that for the 0-min. sample in a flask with a 'KOH-paper', when calculated as μl. O_2/flask and corrected for the endogenous respiration in the presence of the inhibitor. This implies that, in the presence of arsenite, the oxygen uptake due to incomplete inhibition of succinate permeation was not accompanied by any carbon dioxide output, nor was that oxidation rate inhibited at all by the presence of carbon dioxide. This finding throws further light on the involvement of carbon dioxide in the mechanism of the specific permeation of succinate and will be discussed later.

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CHAPTER IV.

DISCUSSION AND CONCLUSIONS.

Summary and Discussion of Succinate Permeation.

The permeation of organic acids into the cells of bacteria as exemplified by the permeation of succinate and related acids into Micrococcus lysodeikticus has been shown to be an exceedingly specific process, as had been expected, for the cells of M. lysodeikticus are not freely permeable to succinate at pH 7.4 and yet succinate is rapidly oxidized by them. Studies on the effects of structural analogues of succinate on the permeation process show that the carrier molecules which transport the succinate molecules through the plasma-membrane have a very high specificity, certainly comparable to enzymic specificity. As in the case of Staphylococcus aureus (Mitchell and Moyle, 1956b) and Bacillus megaterium (Storck and Wachsman, 1957), practically the whole of the succinic dehydrogenase of the cell forms part of the plasma-membrane in M. lysodeikticus and this enzyme would seem to be a

likely candidate as the carrier of the succinate molecules from the outer medium into the cytoplasm. The properties of succinic dehydrogenase, which has only recently been purified although it has been the subject of enzymological work for so many years, have been re-assessed by Singer et al. (1957) and been shown to be largely the same, as far as the action of inhibitors is concerned, as the Keilin and Hartree (1938) preparations on which the earlier work had been done (Singer et al., 1955). The succinate carrier, however, differs from classical succinic dehydrogenase for it is not inhibited at all by malonate added outside the cells although malonate completely inhibits the oxidation of succinate once the inner surface of the plasma-membrane is available to the inhibitor. The fact that the specificity of the succinate carrier is not quite the same as the specificity of the classical succinic dehydrogenase does not entirely exclude the possibility that succinic dehydrogenase is the carrier of succinate through the plasma-membrane. It is conceivable that the shape of the enzyme protein is such that the active centre is available to

succinate molecules but cannot be approached by malonate molecules from the outer surface of the plasma-membrane, but that both succinate and malonate can approach the active centre from inside the cell. The plasma-membrane is so thin (Mitchell and Moyle, 1951; 1956a) that a constituent protein molecule of medium size could be in direct contact with the medium on both sides of the plasma-membrane.

Succinate permeation at pH 7.4 has a positive requirement for potassium ions and is inhibited by phosphate and carbon dioxide. The mechanism involves a thiol, or possibly a di-thiol, group for there is inhibition of permeation but not of oxidation of succinate by thiol-reactors such as phenyl mercuric acetate and arsenite. In his recent general theory of membrane-transport, Mitchell (1957b) postulates that nutrients pass inwards through the plasma-membrane as covalent compounds with specific translocators in the membrane. This idea would be in accord with the findings here, and would suggest that the translocator for succinate might be a compound such as ∝-lipoic acid. It may be

relevant that Singer and Kearney (1955) have found ∝-lipoic acid in their preparations of purified succinic dehydrogenase, although they are not yet sure whether the ∝-lipoic acid is a functional part of the enzyme or should be considered an impurity. Dounce (1956) proposed that, if the ∝-lipoic acid were an integral part of the enzyme, succinate might form a covalent compound with the -S-S- form of ∝-lipoic acid through its two $-CH_2$ groups. This is an interesting speculation in view of the known specificity of the succinate carrier in M. lysodeikticus. The effect of the 'uncoupling' agent, 2,4-dinitrophenate, on succinate oxidation by intact cells is due to inhibition of the specific permeation reaction for succinate in this organism, for succinate metabolism and acetate permeation are not affected by this reagent. Dinitrophenate similarly inhibits the exchange and uptake of phosphate across the plasma-membrane of Staph. aureus (Mitchell, 1954a), and it is probable that its effect on amino-acid accumulation (Gale, 1954) and galactoside accumulation (Monod, 1956) may also be directly on the transport mechanism.

There is very strong circumstantial evidence that the metabolism of succinate, fumarate, L-malate and acetate follows the same pathway in M. lysodeikticus. This pathway is illustrated in a simplified manner in Figure 7. No attempt has been made in the Figure to show the participation of 'activated' substrates where these are known to be required for a certain enzymic reaction, but the Figure merely serves to indicate the oxidative and decarboxylative steps and the relation of one externally-added substrate to another in the metabolic scheme. The reasons for supposing that succinate must enter the Krebs' cycle as 'acetate' have already been discussed, and the stoichiometry of succinate, fumarate, succinimide, propionate and acetate oxidation of these washed and depleted organisms strongly suggests that the pathway illustrated in Figure 7 is followed by all these metabolites. If it is accepted that this is so, then the fact that the rates of oxidation of succinate and acetate (or succinate and succinimide) are additive when oxidized together, even though the concentrations of each are above the saturation concentration for oxidation of each

separately, must mean that the equivalent maximum Q_{O_2} for the permeation-oxidation of succinate, fumarate and L-malate does not reflect a limiting maximum rate for one of the later steps in the metabolic pathway. There are still two possible explanations of the equivalent maximum Q_{O_2} values. Firstly, they may represent a limiting rate of permeation. If so, the simplest explanation for the equivalence would be that there were the same number of carriers in the plasma-membrane for each of the substrates, but, because of the strict competition between succinate and fumarate (or succinate and L-malate), one would have to postulate that the same carriers were common to all three substrates. The very high specificity of the succinate carrier makes this seem rather an unlikely explanation. Alternatively, the equivalent Q_{O_2} values and the strict competition between substrates which need a specific permeation mechanism in order to traverse the plasma-membrane may be caused by a limiting rate of exit from the cell of some compound on the carrier which exchanges for succinate on the outside of the plasma-membrane, and which is

a common exchanger for all dicarboxylic acids entering the cell. If this were the case, the carriers carrying the dicarboxylic acids inwards could be specific for the individual acids. Mitchell (1957b) has suggested this method of driving the active transport of nutrients into the cell as part of his general theory of membrane-transport and he proposes that carbonic acid or some other metabolic end-product may be used as an exchanger in this way. The observations that have been made on the permeation of organic acids into *M. lysodeikticus* certainly favour the view that carbon dioxide is very intimately concerned in the permeation of succinate, and the equivalent maximum Q_{O_2} for all the related substrates and the competition between substrates makes it likely that there is a common exchanger for all of them.

From a study of the succinate accumulation process, it can be calculated that the entry of succinate into the cells under appropriate conditions (i.e. for accumulation) may be about 10 times faster than during normal permeation-oxidation. Therefore, the rate of succinate entry cannot be the

limiting factor in determining the maximum Q_{O_2} for the over-all process. The finding of a 50 % inhibition of the rate of succinate permeation-oxidation by the presence of carbon dioxide in the system makes it plausible to suggest that carbon dioxide may be the common exchanger. At least two types of 'activated' carbon dioxide have now been isolated and indicate the type of compound which may be directly or indirectly involved here (Coon, 1955; Flavin et al., 1956).

A further indication of the involvement of carbon dioxide in succinate permeation comes from experiments in which succinate and arsenite were added simultaneously to the cells. Under these conditions succinate permeation was almost completely inhibited. However, there was always a small oxygen uptake over and above that due to endogenous respiration. When a balance was made of oxygen uptake and carbon dioxide output it was found that the oxygen uptake in the presence of arsenite was not accompanied by any carbon dioxide output. It would, therefore, appear that arsenite acts on carbon dioxide output rather than on suc-

cinate entry. As might be expected, under these conditions the oxygen uptake was not inhibited in rate by the presence of carbon dioxide in the system, indicating again that it was the output of carbon dioxide that was inhibited by the presence of carbon dioxide in the system. The fact that there is a stoichiometric output of 1 mole of carbon dioxide for each mole of succinate entering the cells also strongly suggests that carbon dioxide may be the exchanger for succinate on the translocators in the plasma-membrane.

It is interesting that under conditions in which one quarter of the externally-added succinate is accumulated inside the cells within 2 min. the oxygen uptake by the cells remains constant in rate until all the succinate has been metabolized. The oxidation rate for succinate is only about 10 % of the accumulation rate, and even when there is no accumulation of succinate the oxidation rate only falls to half, the rate (as before) remaining constant until all the added succinate has been metabolized.

The involvement of carbon dioxide in the succinate accumulation process seems undoubted, for there is no accumulation whatsoever in presence of carbon dioxide, and succinate is oxidized as rapidly as it permeates the plasma-membrane. The function of the succinate accumulation process is unclear for accumulation may not occur under natural conditions, but the fact that it does occur in the absence of carbon dioxide makes it a useful tool in the investigation of the succinate permeation mechanism.

The remarkable speed with which succinate is accumulated would favour the proposal of some simple mechanism for the accumulation process, but the number of molecules of succinate accumulated by each cell is many orders of magnitude too great for the accumulation to represent the saturation of carrier molecules on the surface of the plasma-membrane. It can be calculated that there would not be space for that number of protein molecules (carrier sites) in the plasma-membrane. Most, at any rate, of the accumulated succinate must be inside the cell. It might exist either as free succinate in solution in

the cytoplasm or as a compound with some fairly small molecule such as a nucleotide (of which it is known that there is more than enough in the cytoplasm to form such a compound (Mitchell and Moyle, 1954)). The fact that fumarate accumulates to the same extent as succinate suggests that the two substrates exist as compounds with the same cytoplasmic component when they are accumulated. It may be relevant in this connection to note the recent isolation of adenylosuccinic acid by Joklik (1956) and Ballio and Serlupi-Crescenzi (1957).

Discussion of Some Recent Work by Other Authors.

It may be profitable to discuss some recent publications on the subject of metabolism of Krebs' cycle intermediates by various species of bacteria in the light of our present knowledge of the mechanism for the specific permeation of succinate in M. lysodeikticus.

In 1950, Barron et al. studied the metabolism of organic acids by Corynebacterium creatinovorans and found that there was no oxidation of succinate

at pH 7, but that there was oxidation of this acid at pH 5.5. Measurement of oxygen uptake was carried out in a reaction mixture containing phosphate buffer but it is not stated whether sodium or sodium-potassium phosphates were used. It seems likely, by inference from the present work, that the lack of succinate oxidation at pH 7 was due to lack of potassium ions. In experiments by Barron et al. on the effect of malonate, it was found that at pH 7 acetate oxidation was not affected by malonate, but that at pH 5.3 acetate and succinate oxidation was almost completely inhibited by malonate. In view of the observation that malonate is unable to permeate intact cells of M. lysodeikticus and does not inhibit succinate permeation-oxidation in these cells either at pH 6 or 7.4 it seems that C. creatinovorans may be very fragile at low pH, the damaged cells allowing malonate to permeate their plasma-membranes. In this connection it may be relevant to note that although acetate oxidation was still 80 % as rapid at pH 5.3 as at pH 7, at pH 4.9 there was no oxidation of acetate at all by these cells. These results also suggest that C. creati-

novorans becomes suddenly very much more fragile as the pH drops below 5, for it is known that acetate oxidation does not occur in broken cells of several species of bacteria in which the intact cells oxidize acetate rapidly (Utter et al., 1946; Kogut and Podoski, 1953; present work).

Campbell and Stokes (1951); Barrett et al. (1953); and Kogut and Podoski (1953) have studied the oxidation of Krebs' cycle intermediates in several species of Pseudomonas. When cells were grown with acetate as carbon source, Campbell and Stokes found that only acetate, pyruvate and malate were immediately oxidized by intact cells whereas all of the Krebs' cycle intermediates were oxidized by dried cells in which the plasma-membrane had been destroyed. Barrett et al. found that cells grown on fumarate oxidized fumarate rapidly, but citrate only slowly. However, after a lag period of up to 2 hr. the oxidation rate for citrate increased to a steady rate comparable quantitatively to the rate of oxidation of fumarate. Kogut and Podoski found that cells grown in a medium with succinate as carbon source oxidized only succinate,

fumarate, L-malate and oxaloacetate immediately. Oxidation of other Krebs' cycle intermediates only occurred after a lag period in washed suspensions of intact cells although all the oxidative enzymes required could be demonstrated in cell extracts. Kogut and Podoski favour the view that the 'adaptation' which occurs during the lag period may be a 'permeability adaptation' but Barrett et al. suggest that the 'adaptation' to externally-added substrates may be caused by the synthesis of carriers in the plasma-membrane.

Williams and Wilson (1954) have investigated the same phenomenon in Azotobacter vinelandii and have also come to the conclusion that the observed lag period may be due to the time needed for synthesis of a transportation system.

A mutant of Bacterium coli was shown by Gilvarg and Davis (1954) to contain enzymes necessary for the oxidation of citrate via the tricarboxylic acid cycle, but to be unable to oxidize externally-added citrate when the cells were intact.

Green (quoted by Davis, 1956) investigated the citrate utilization of an Aerobacter strain

which, when grown on glucose, showed no oxidation of citrate for a period of about 30 min. after addition of the citrate, but which, grown on citrate, oxidized citrate immediately. Cells grown on either substrate were shown to possess all the relevant oxidizing enzymes. The 'adaptation' to citrate in the glucose-grown cells required the presence of a nitrogen source, suggesting that net protein synthesis was necessary for the production of the new transport system.

There are two points concerning the 'adaptation' process which may help in the formulation of an explanation of the mechanism of the process. First, it has been found that the oxidative enzymes are present in the 'unadapted' cells in approximately the same amount as in the 'adapted' cells. Second, there is a very intimate genetic connection between the carriers for the organic acids and their respective oxidative enzymes. Taken together, these points suggest that 'adaptation' may be the manifestation of a rearrangement in space (and in accessibility to the external medium) of carriers (enzymes?) already present in the cells,

this rearrangement being triggered by the presence of the substrate outside the cells, rather than a synthesis of new material, particularly since, in many cases, adaptation occurs in the absence of an external nitrogen source in washed cells.

Green (see Davis, 1956) has also studied the diauxie found in cultures of Aerobacter growing on a mixture of glucose and citrate, but not in cultures grown on succinate and citrate, using radioactive citrate and following the production of radioactive carbon dioxide. Since citrate was an obligatory intermediate, this result was interpreted as the effect of the presence of glucose in blocking the appearance of a transport system for citrate. Davis (1956) and his collaborators measured directly the disappearance of citrate from the external medium in which cells of Aerobacter which had been grown on glucose were suspended, and confirmed Green's results obtained using tracer techniques. They noticed that in stationary phase cells the osmotic barrier to citrate appeared to break down spontaneously during the experiments, and Davis pointed out the dangers of unsuspected damage to cells

in permeability studies. Using thick suspensions of glucose-grown Aerobacter, these workers found that citrate did not permeate the cells, and they found that with citrate-adapted cells the metabolism of the citrate appeared to be as rapid as its permeation. In his summary, Davis commented on the resemblance of the transport mechanisms to enzymes.

All the published work on the metabolism of Krebs' cycle intermediates by different species of bacteria reported in this section is consistent with the view that the specific mechanism of permeation of succinate into M. lysodeikticus may be of general validity.

Future Developments.

It has already been shown that the specific permeation of succinate into the cells of M. lysodeikticus can be interpreted on the basis of Mitchell's general theory of membrane-transport (1957b). Nevertheless, the exact nature of the translocator for succinate is not yet known although it has been established that a thiol group is involved in the

transport of succinate across the plasma-membrane. More information is also required before it can be stated for certain whether the translocator for succinate is the same as the translocator for the related acids. The question of whether the translocase for succinate is actually succinic dehydrogenase or is some other protein with an equally specific combining centre is still unanswered, as is the question of the nature of the substance which, by passing out of the cell covalently-bound to the translocator, allows the entry of succinate molecules. It is clear that carbon dioxide is involved here, but whether directly or indirectly it requires further work to decide. Yet another question that remains unanswered is the form in which succinate is held in the cell when it accumulates in the absence of external carbon dioxide.

A programme of work designed to answer these outstanding questions unequivocally is already planned but, in the meantime, sufficient experimental results have been obtained to allow a clear description of the mechanism basically concerned in the specific permeation of succinate into the cells of *M. lysodeikticus*.

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TABLE I.

The relationship between turbidity ($Log_{10}(I_o/I)$) at 700 mμ and actual dry weight of cells of *Micrococcus lysodeikticus* during the growth cycle.

Cell suspension density ($Log_{10}(I_o/I)$)	Actual dry wt. of sample (mg.)	X (μg./ml.)
0.255	14.8	70
0.520	23.2	70
3.98	84.4	63
5.88	118.2	54
19.4	105.2	37
20.0	138.5	36

X represents the dry weight concentration of cells giving a turbidity ($Log_{10}(I_o/I)$) of 0.100.

TABLE II.

Distribution of some enzyme activities in fractions from cells of *Micrococcus lysodeikticus* estimated by Thunberg technique at 25° at pH 6.8.

	Expt. 1		Expt. 2	
Total cell dry wt. (mg.)	310		370	
Fraction	Plasma-membrane	Cyto-plasm	Plasma-membrane	Cyto-plasm
Vol. of fraction (ml.)	10	96	1.3	20
Vol. of sample (ml.)	0.6	2.0	0.2	2.0
Substrate added (0.07M)	Oxidative activity (100/T) per sample			
None	<1	2	<1	16
Succinate	47	2.6	300	25
Lactate	1.9	2.5	300	10
Formate	<1	<1	<1	16
α-glycerophosphate	<1	4.8	9	15

(T is the decolorization time in minutes.)

Enzyme	Percentage of total activity			
Succinic dehydrogenase	96	4	95	5
Lactic dehydrogenase	57	43	>95	<5
	Total activity/100 mg. cell dry weight			
Succinic dehydrogenase	260		535	
Lactic dehydrogenase	18		514	

TABLE III.

Distribution of some enzyme activities in fractions from cells of *Micrococcus lysodeikticus* estimated manometrically by oxygen uptake at 25° at pH 6.8.

	Lysozyme-prepared protoplasts		Autolytically-prepared protoplasts
Total cell dry wt. (mg.)	580		580
Fraction	Plasma-membrane	Cyto-plasm	Plasma-membrane
Vol. of fraction (ml.)	2.1	40	6.5
Vol. of sample (ml.)	0.2	2.0	0.6
Substrate added (0.025M)	**Oxygen uptake (μl. O_2/hr./flask)**		
None	18	19	15
Succinate	56	17	53
Lactate	30	19	36
Formate	20	17	15
α-glycerophosphate	18	17	15
Enzyme	**Percentage total activity**		
Succinic dehydrogenase	>95	<5	
Lactic dehydrogenase	>95	<5	
	Total activity (μl. O_2/hr./580 mg. cell dry wt.)		
Succinic dehydrogenase	400		410
Lactic dehydrogenase	186		195

TABLE IV

Oxidation of succinate and lactate by fractions and recombined fractions from the cells of Micrococcus lysodeikticus at 25° at pH 6.8.

Total cell dry wt. (mg.) 290

Fraction	Plasma-membrane	Cyto-plasm	Plasma-membrane + cytoplasm
Vol. of fraction (ml.)	1.1	20	
Vol. of sample (ml.)	0.2	2.0	
Substrate (0.025M)	Oxygen uptake (μl. O_2/hr./flask)		
None	13	17	29
Succinate	44	16	53
Lactate	29	17	44

TABLE V

Effect of concentration of methylene blue on the rate of endogenous respiration of intact cells of Micrococcus lysodeikticus in the presence of 0.1M KCN at 25° at pH 6.8.

Cell dry wt. (mg.)/flask	21	84
Concn. methylene blue	Oxygen uptake (µl. O_2/hr./flask)	
0.05 % (w/v)	56	205
0.002 % (w/v)	68	197
None	35	220

TABLE VI

Effect of pH and concentration of potassium ions on rate of oxidation of 0.025M succinate by intact cells of *Micrococcus lysodeikticus* in the presence of 0.04M sodium phosphate buffers at 25°.

K^+ concn.	Q_{O_2} (Succinate)	
	pH 6.0	pH 7.4
0	2.7	0.07
0.125M	5.2	2.8
1.0M	4.0	4.9

TABLE VII.

Requirement for potassium ions in the oxidation of substrates by intact cells of Micrococcus lysodeikticus at 25° at pH 7.4.

Substrate	Substrate oxidation rate in absence of K^+ expressed as a percentage of of that in presence of 1M K^+	
Succinate (0.025M)	2	
Fumarate (0.002M)	1	
Acetate (0.002M)	71	
L-malate (0.025M)	18	(cells stored at -15°)
DL-malate (0.025M)	2	

TABLE VIII

Oxygen uptake and carbon dioxide output during oxidation of 8 μmole of substrates by intact cells of *Micrococcus lysodeikticus* at 25° and at pH 7.4.

Substrate	Atoms oxygen/ mol. substrate	Mol. CO_2/ mol. substrate	R.Q.
(Endogenous respiration			0.80, 0.79, 0.79)
Succinate	3.9, 4.0, 3.9, 4.1	0.75, 0.80	0.4
Fumarate	3.0, 2.9		
Acetate	2.4, 2.5, 2.6	0.50, 0.53	

TABLE IX

Effect of 0.05M malonate on the rate of oxidation of 0.025M succinate at 25° at pH 7.4 by 'intact' cells of Micrococcus lysodeikticus.

Percentage of cells ruptured was estimated by comparison of extinction at 700 mμ of the suspensions at the beginning and the end of experiments.

Inhibition of succinate oxidation rate (%)	Cells ruptured (%)
0	4
30	25
25	26
19	11
15	8

TABLE X.

Effect of structural analogues as potential inhibitors of succinate permeation in *Micrococcus lysodeikticus* at 25° and at pH 7.4.

Analogue	Succinate concn.	Inhibition of succinate oxidation rate (%)	
		Intact cells	Broken cells
Malonate (0.05M)	0.025M	0-5	85-100
β-phenyl propionate (0.004M)	0.002M	3	14
Mesotartrate (0.002M)	0.002M	0	0
Succinimide (0.004M)	0.002M	16	10
Propionate (0.002M)	0.002M	28	
D-malate (0.002M)	0.002M	14	

TABLE XI.

Effect of inorganic orthophosphate and arsenate on oxidation of substrates by *Micrococcus lysodeikticus* at 25° at pH 7.4.

Substrate (0.002M)	Inhibitor concn.	Inhibition of substrate oxidation rate (%)	
		Intact cells	Broken cells
	Phosphate		
Succinate	0.005M	2	
Succinate	0.05M	61, 62	0
Acetate	0.05M	0, 22	
Fumarate	0.05M	0	
L-malate	0.05M	72 (cells stored at -15°)	
	Arsenate		
Succinate	0.01M	10	0

TABLE XII

Effect of 0.001M 2,4-dinitrophenate on oxidation of substrates by Micrococcus lysodeikticus and Bacterium coli at 25° at pH 7.4.

Substrate (0.025M)	Inhibition of substrate oxidation rate (%)	
	Intact cells	Broken cells
(a) M. lysodeikticus		
Succinate	93, 87	11
Acetate	0, 0, 3, 4	
Fumarate	76, 75	
DL-malate	95, 76	
(b) Bact. coli		
Succinate	100	
Acetate	100	

TABLE XIII

Effect of 0.01M iodoacetate on oxidation of substrates by *Micrococcus lysodeikticus* at 25° at pH 7.4.

Substrate (0.002M)	Inhibition of substrate oxidation rate (%)	
	Intact cells	Broken cells
Succinate	33	17
Acetate	0 (initially)	
Acetate	100 (after 1 hr.)	

TABLE XIV

Effect of 1 x 10^{-4} M phenyl mercuric acetate on oxidation of substrates by Micrococcus lysodeikticus at 25° at pH 7.4.

Substrate (0.002M)	Inhibition of substrate oxidation rate (%)	
	Intact cells	Broken cells
Succinate	98	5
Acetate	95	
Fumarate	94	

TABLE XV

Effect of 0.01M arsenite on oxidation of substrates by *Micrococcus lysodeikticus* at 25° at pH 7.4.

Substrate (0.002M)	Inhibition of substrate oxidation rate (%)	
	Intact cells	Broken cells
Succinate	98	0
Acetate	99	
Fumarate	98	

TABLE XVI

Effect of some inhibitors on the oxidation of 0.002M substrates by intact cells of Micrococcus lysodeikticus at 25° at pH 7.4.

Inhibitor	Inhibition of substrate oxidation rate (%)		
	Succinate	Acetate	Fumarate
Fluoroacetate (0.001M)	15	50	
Semicarbazide (0.1M)	0	92	0
Hydroxylamine (0.1M)	97		
Hydroxylamine (0.01M)	27		
Hydroxylamine (0.001M)	0		
Carbon dioxide	43, 50	24, 14	

TABLE XVII

Competition between succinate, fumarate, acetate and malate in intact cells of *Micrococcus lysodeikticus* at 25° at pH 7.4.

Substrate	Oxidation rate (µl. O_2/hr./flask) corrected for endogenous respiration.		
	Expt. 1	Expt. 2	Expt. 3
Succinate (0.004M)	475		376
Succinate (0.002M)	485	454	404
Acetate (0.002M)	175		
Fumarate (0.002M)	360	327	
Succinate (0.002M) + acetate (0.002M)	640		
Succinate (0.002M) + fumarate (0.002M)	490		
D-malate (0.002M)		42	
Succinate (0.002M) + D-malate (0.002M)		432	
Fumarate (0.002M) + D-malate (0.002M)		312	
L-malate (0.05M)			222
Succinate (0.002M) + L-malate (0.05M)			314

TABLE XVIII

Accumulation of substrates by intact cells of *Micrococcus lysodeikticus* at 25° at pH 7.4.
The amount of substrate accumulated is expressed as the difference in oxygen uptake (determined 90 min. after addition of inhibitor) between reaction mixtures in which the inhibitor was added after the substrate and reaction mixtures in which inhibitor and substrate were added simultaneously.

Inhibitor	Substrate	Cell dry wt./flask (mg.)	Time interval between addition of substrate and inhibitor	Substrate accumulated ($\mu l. O_2$/flask)
DNP (0.001M)	succinate (0.025M)	24.2	10 min.	89
			30 min.	96
Arsenite (0.01M)	succinate (0.025M)	30.8	10 min.	94
Arsenite (0.01M)	succinate (0.025M)	24.2	10 min.	58
	succinate (0.002M)		10 min.	81
			30 min.	61
Arsenite (0.01M)	succinate (0.002M)	29.6	2 min.	107
			5 min.	137
			10 min.	97
Arsenite (0.01M)	succinate (0.002M)	23.8 (stored at -15°)	10 sec.	7
			40 sec.	43
			2 min.	65
Arsenite (0.01M)	fumarate (0.002M)	24.0	2 min.	37
			10 min.	61
Arsenite (0.01M)	fumarate (0.002M)	23.8 (stored at -15°)	30 sec.	22
			2 min.	74
			10 min.	74
Arsenite (0.01M)	acetate (0.002M)	24.0 (stored at -15°)	2 min.	7
			10 min.	4

TABLE XIX

Effect of the presence of acetate on accumulation of succinate by intact cells of *Micrococcus lysodeikticus* at 25° at pH 7.4 .
The amount of substrate accumulated is expressed in the same units as in Table XVIII. Arsenite (0.01M) was used as the inhibitor of succinate permeation and of acetate oxidation. Cells used had been stored at -15°.

Substrate (0.002M)	Cell dry wt./flask (mg.)	Time interval between addition of substrate and inhibitor.	Substrate accumulated (μl. O_2/flask)	Succinate accumulated in presence of acetate as a percentage of that in succinate alone
Acetate			3	
Succinate	23.8	10 min.	87	
Succinate + acetate			191	220
Acetate			-	
Succinate	25.7	2 min.	90	
Succinate + acetate			84	94
Acetate			5	
Succinate	25.7	1 min.	54	
Succinate + acetate			34	63
Acetate			2	
Succinate	25.7	20 sec.	49	
Succinate + acetate			8	16

FIGURE 1.

Growth curve of *Micrococcus lysodeikticus* grown aerobically at 25° in a tryptic digest medium. Inset, part of the growth curve is plotted semi-logarithmically. Cultures were harvested routinely at the end of the logarithmic phase of growth at the cell suspension density indicated by the arrows.

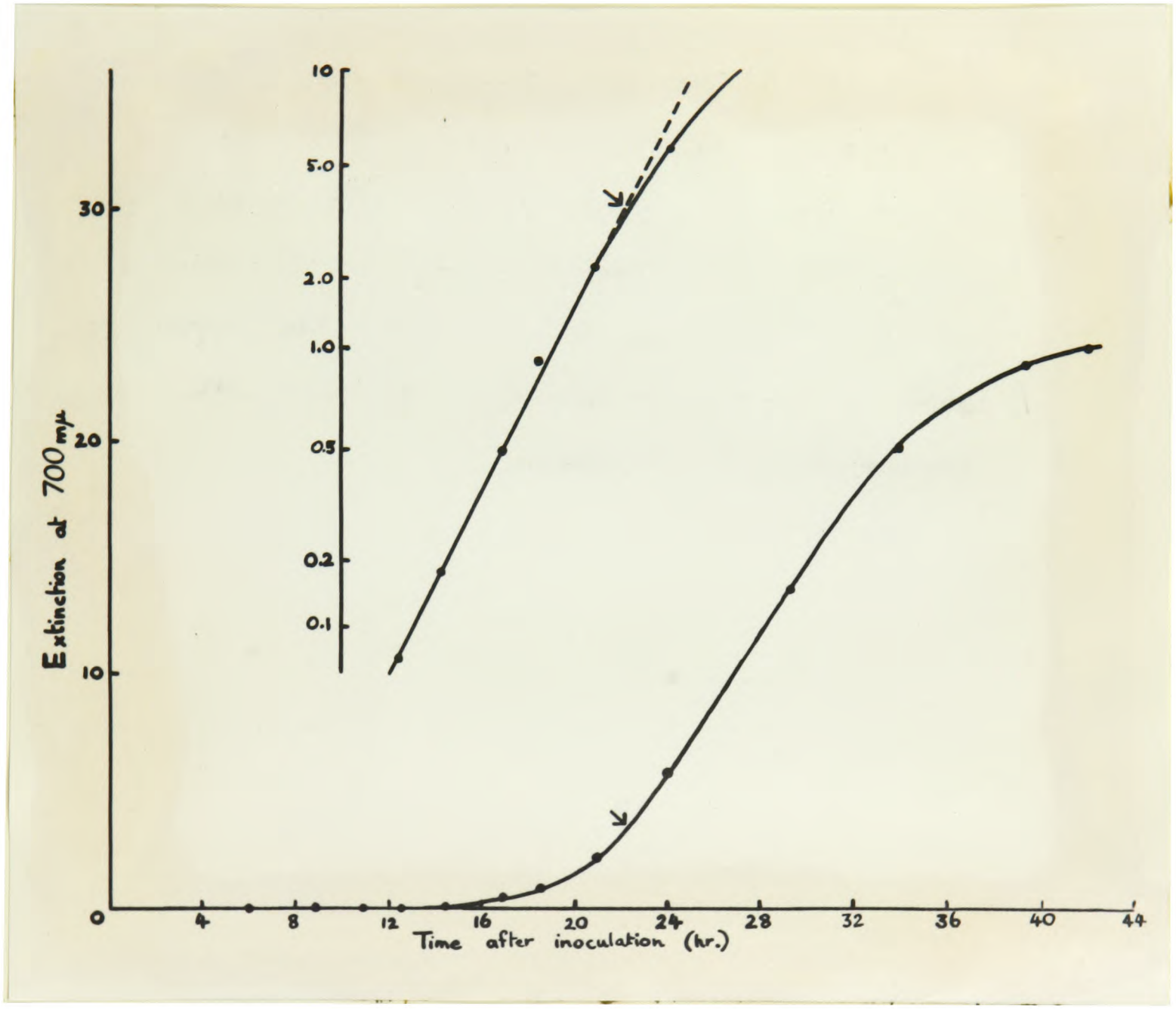
Extinction at 700 mμ
30
20
10
0
10
5.0
2.0
1.0
0.5
0.2
0.1
4
8
12
16
20
24
28
32
36
40
44
Time after inoculation (hr.)

FIGURE 1

FIGURE 2

Stability of protoplasts of Micrococcus lysodeikticus suspended at 20° in molar solutions of some solutes containing 0.02M sodium phosphate buffer at pH 6.8.

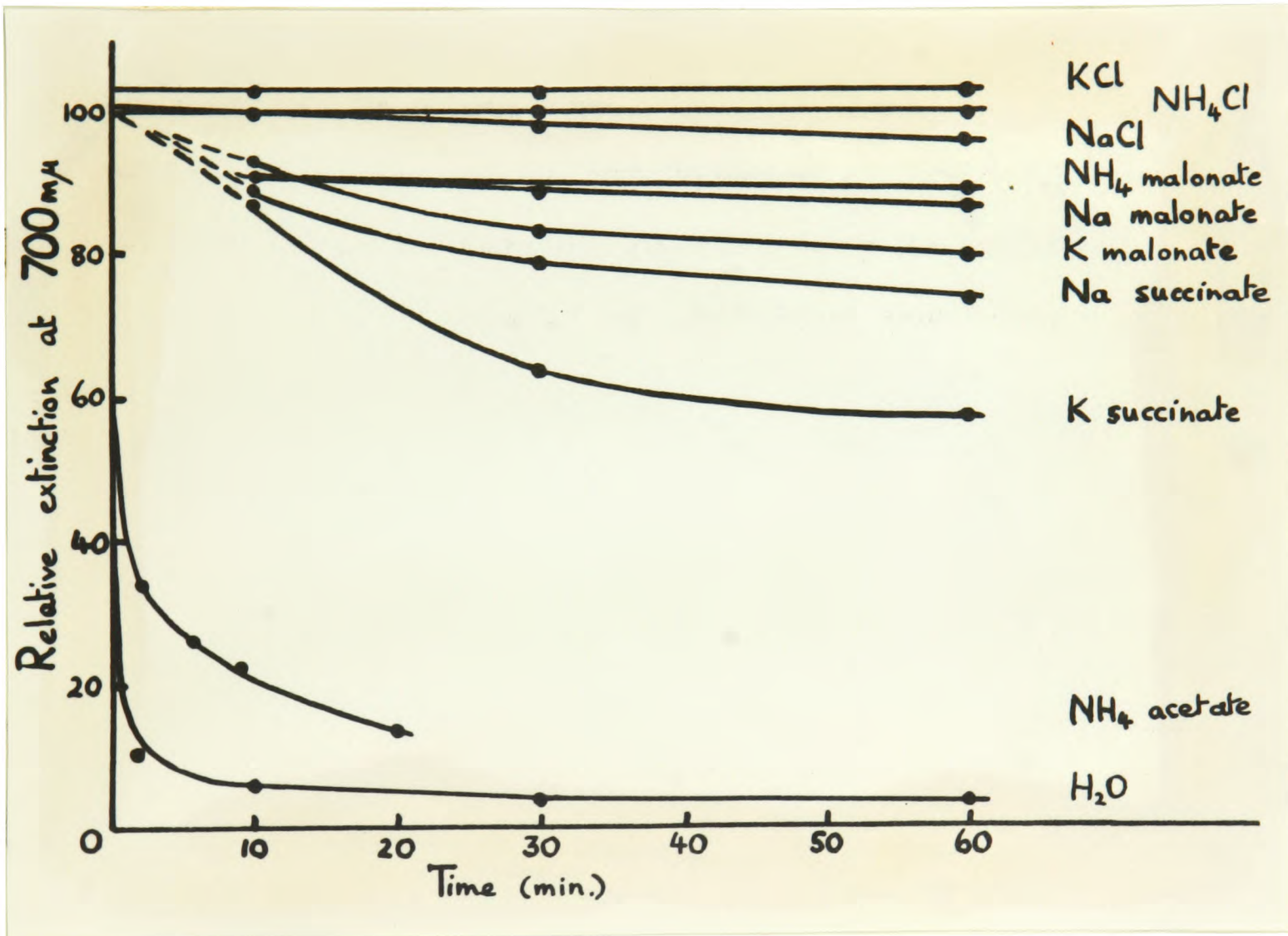
Relative extinction at 700 mμ
100
80
60
40
20
0
10
20
30
40
50
60
Time (min.)
KCl
NH4Cl
NaCl
NH4 malonate
Na malonate
K malonate
Na succinate
K succinate
NH4 acetate
H2O

FIGURE 2

FIGURE 3

Effect of the rate of shaking of Warburg flasks on the rate of endogenous respiration and on the O-R potential of suspensions of intact cells of Micrococcus lysodeikticus at 25° and at pH 6.8 in the presence of 0.1M KCN and 0.05 % (w/v) methylene blue.

Rate of shaking of flasks - • 120 strokes/min.
× 90 strokes/min.
+ 60 strokes/min.

O-R potential - • × + reaction mixtures in which it was judged that more than 50 % of the methylene blue was oxidized, i.e. O-R potential more positive than +11 mV.

⊙ ⊗ ⊕ reaction mixtures in which more than 95 % of the methylene blue was reduced (colourless), i.e. O-R potential more negative than -30 mV.

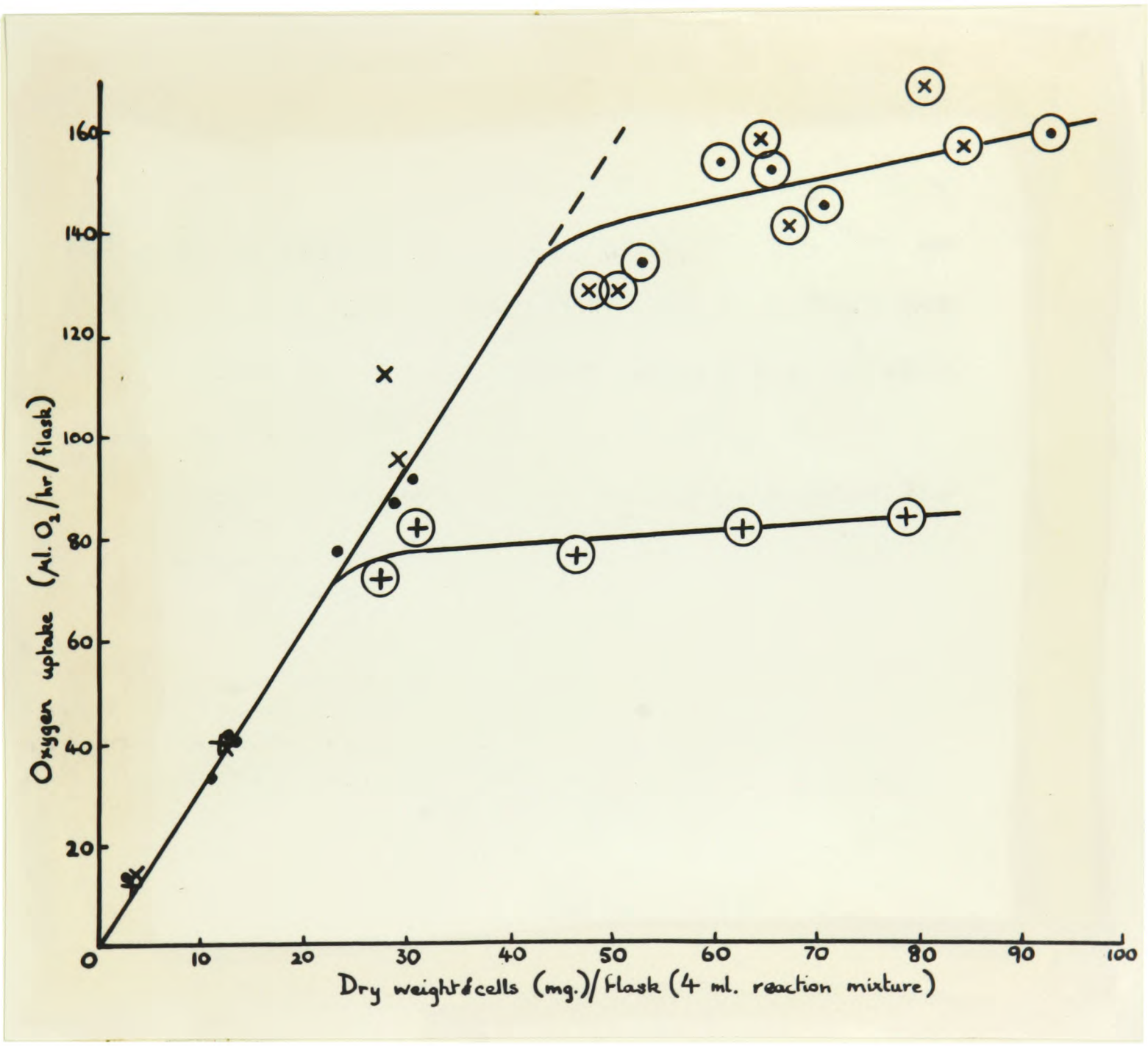
Oxygen uptake (µl. O2/hr/flask)
160
140
120
100
80
60
40
20
0
10
20
30
40
50
60
70
80
90
100
Dry weight of cells (mg.)/ Flask (4 ml. reaction mixture)

FIGURE 3

FIGURE 4

Effect of the O-R potential on the oxidation of succinate by intact cells of *Micrococcus lysodeikticus* at 25° and at pH 6.8 in the presence of 0.1M KCN and 0.05 % (w/v) methylene blue. Endogenous respiration rate is plotted using the same symbols as in Figure 3. Succinate oxidation is indicated by arrows, the length of each arrow representing the rate of oxygen uptake due to oxidation of added (0.025M) succinate.

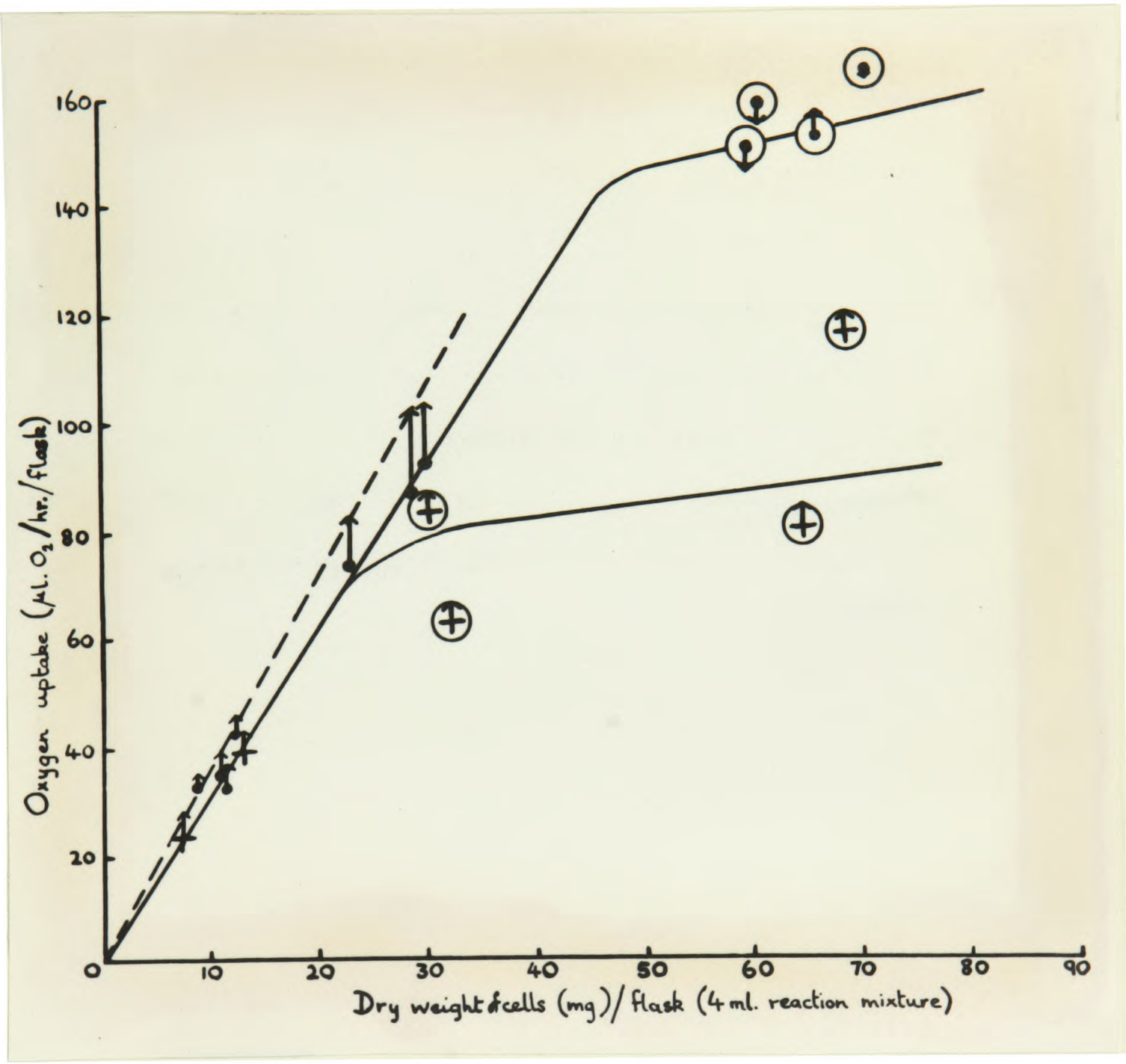
160
140
120
100
80
60
40
20
0
Oxygen uptake (μl. O2/hr./flask)
10
20
30
40
50
60
70
80
90
Dry weight of cells (mg)/ flask (4 ml. reaction mixture)

FIGURE 4

FIGURE 5

The rate of endogenous respiration of freshly-harvested intact cells of Micrococcus lysodeikticus at 25° and at pH 7.4 as a function of time. Warburg flasks were shaken throughout the 5-hr. period at 120 strokes/minute. Results from experiments on two bacterial cultures are plotted in the graph.

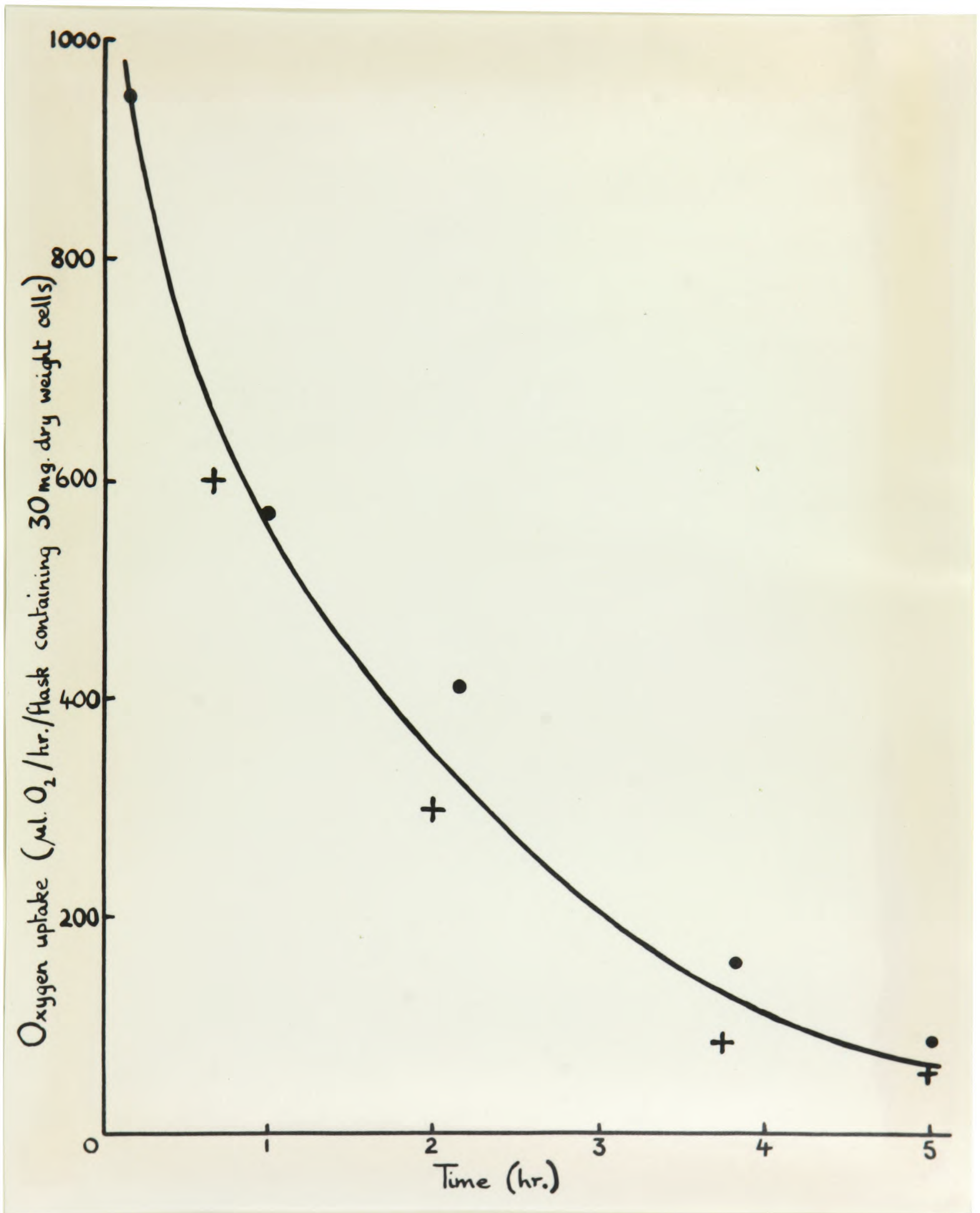
Oxygen uptake (µl. O2 /hr./flask containing 30 mg. dry weight cells)
1000
800
600
400
200
0
1
2
3
4
5
Time (hr.)

FIGURE 5

FIGURE 6

Time course of oxygen uptake after the addition of 8 μmole succinate or fumarate to intact cells of Micrococcus lysodeikticus at 25° and at pH 7.4. The 'control' represents endogenous respiration.

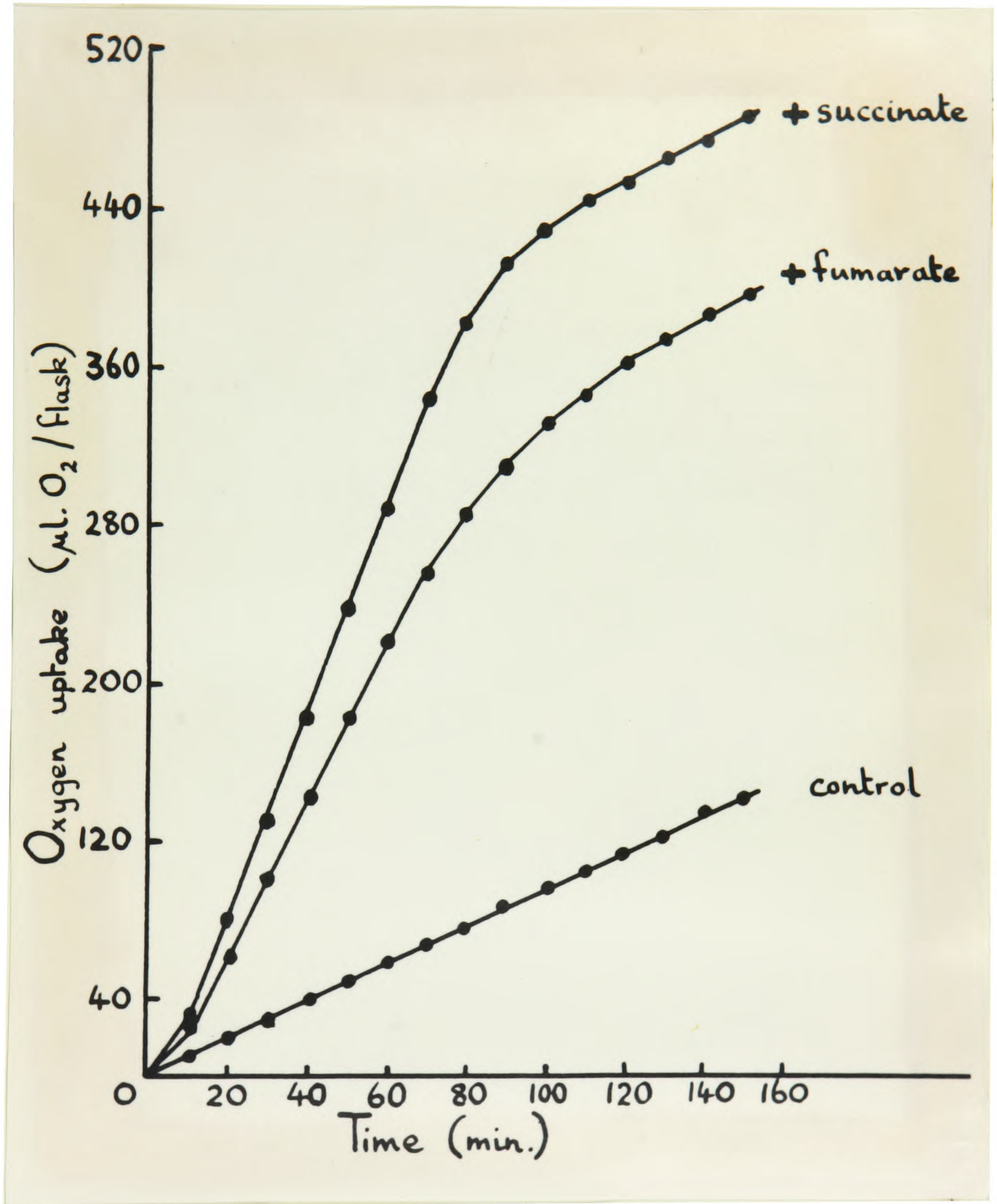
520
440
360
280
200
120
40
0
Oxygen uptake (μl. O2 / Flask)
20
40
60
80
100
120
140
160
Time (min.)
+ succinate
+ fumarate
control

FIGURE 6

FIGURE 7

Hypothetical pathway for the oxidative metabolism of externally-added succinate, fumarate, acetate and L-malate by Micrococcus lysodeikticus.

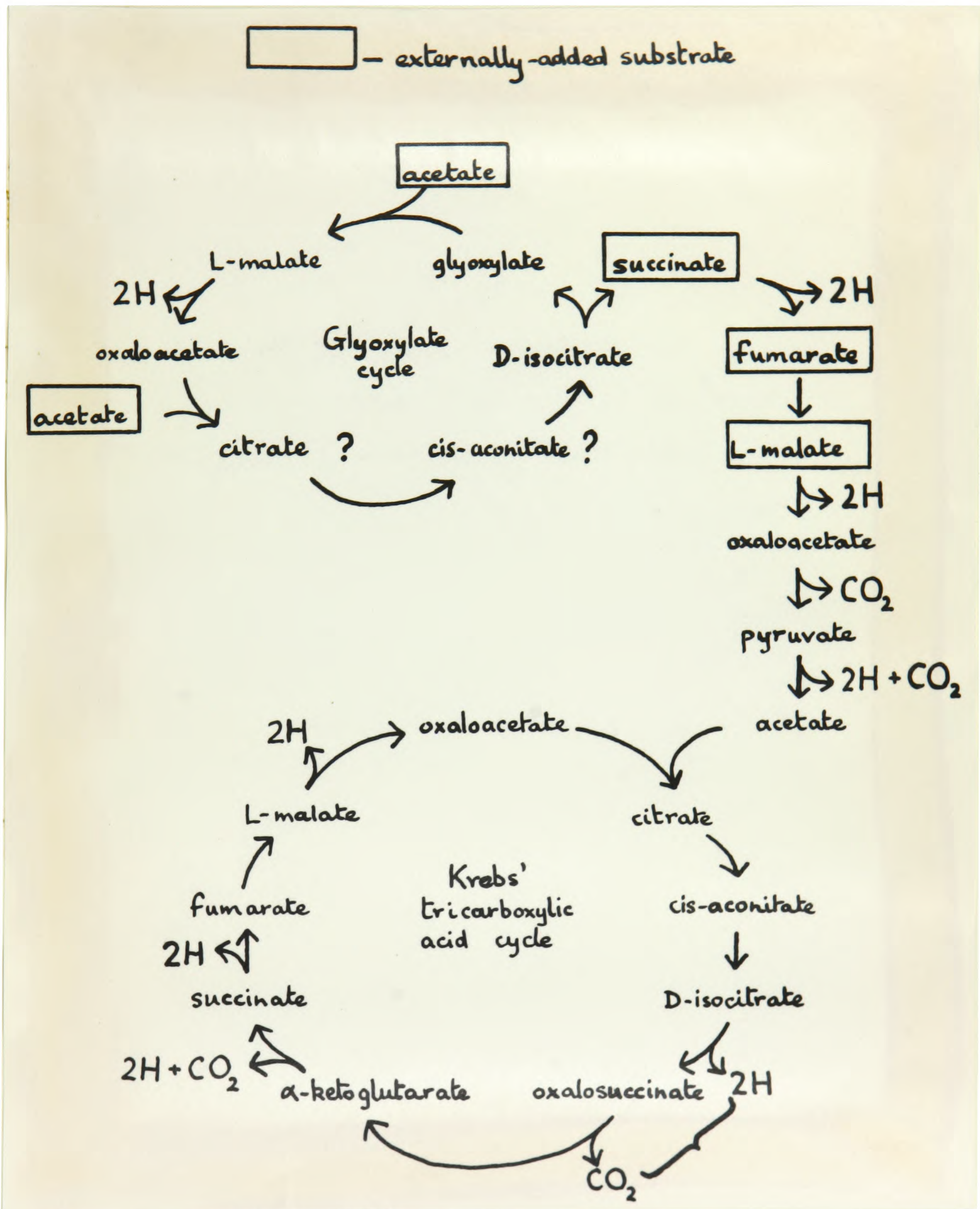
– externally-added substrate
acetate
L-malate
glyoxylate
succinate
2H
2H
oxaloacetate
Glyoxylate cycle
D-isocitrate
fumarate
acetate
citrate ?
cis-aconitate ?
L-malate
2H
oxaloacetate
CO2
pyruvate
2H + CO2
2H
oxaloacetate
acetate
L-malate
citrate
Krebs' tricarboxylic acid cycle
fumarate
cis-aconitate
2H
succinate
D-isocitrate
2H + CO2
α-ketoglutarate
oxalosuccinate
2H
CO2

FIGURE 7

FIGURE 8

Effect of substrate concentration on the rate of oxygen uptake due to oxidation of substrates by intact cells of *Micrococcus lysodeikticus* at 25° and at pH 7.4 .

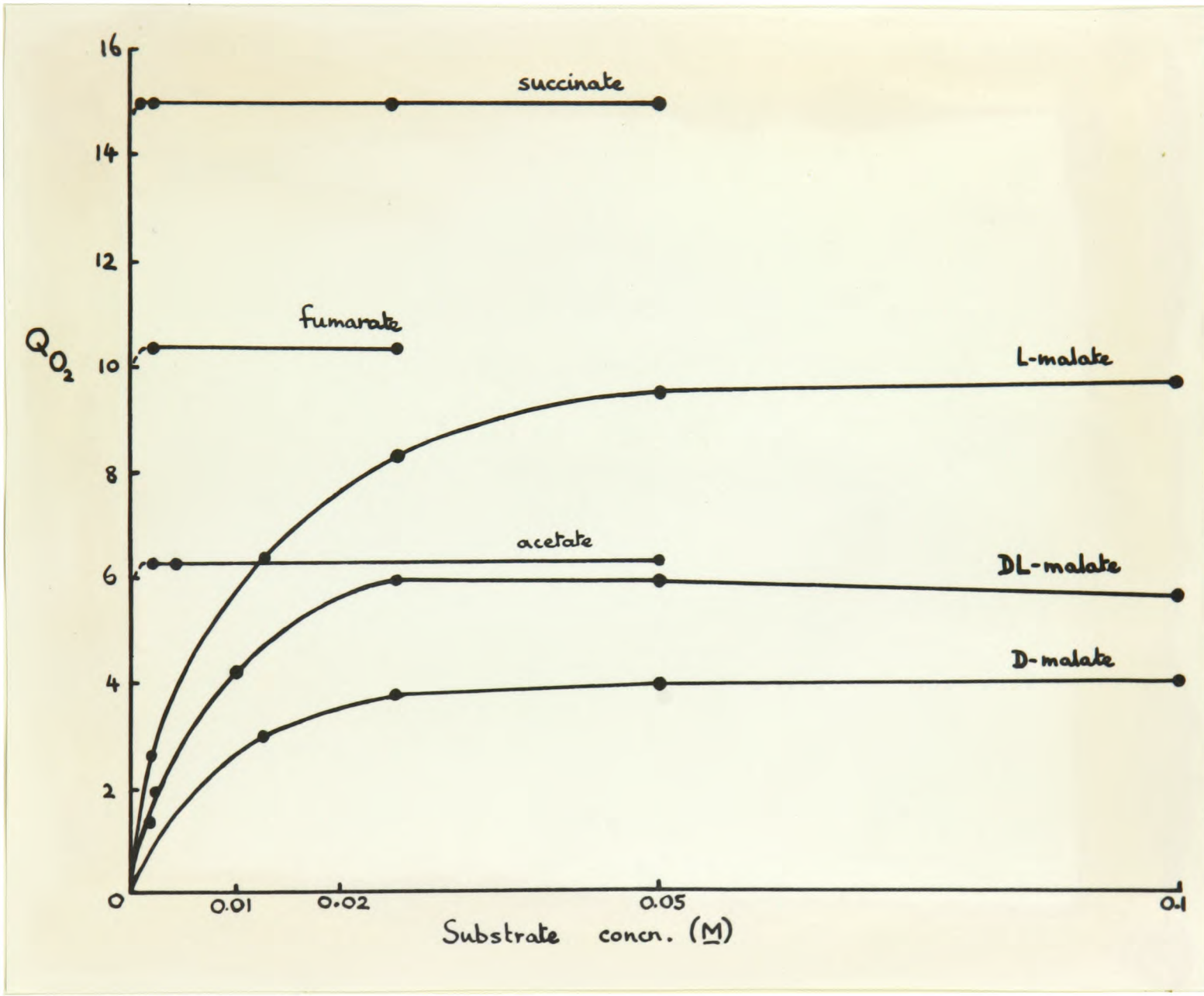
16
14
12
10
8
6
4
2
0
Q_{O_2}
succinate
fumarate
L-malate
acetate
DL-malate
D-malate
0.01
0.02
0.05
0.1
Substrate concn. (M)

FIGURE 8

FIGURE 9

Effect of addition of 0.001M 2,4-dinitrophenate (DNP) on the oxidation of 0.025M succinate by intact cells of *Micrococcus lysodeikticus* at 25° and at pH 7.4, when the substrate and inhibitor were added simultaneously (0 min.) and when the DNP was added 10 or 30 min. after the addition of succinate. Oxygen uptake is plotted from the time of addition of the inhibitor and is corrected for oxygen uptake due to endogenous respiration in presence of DNP.

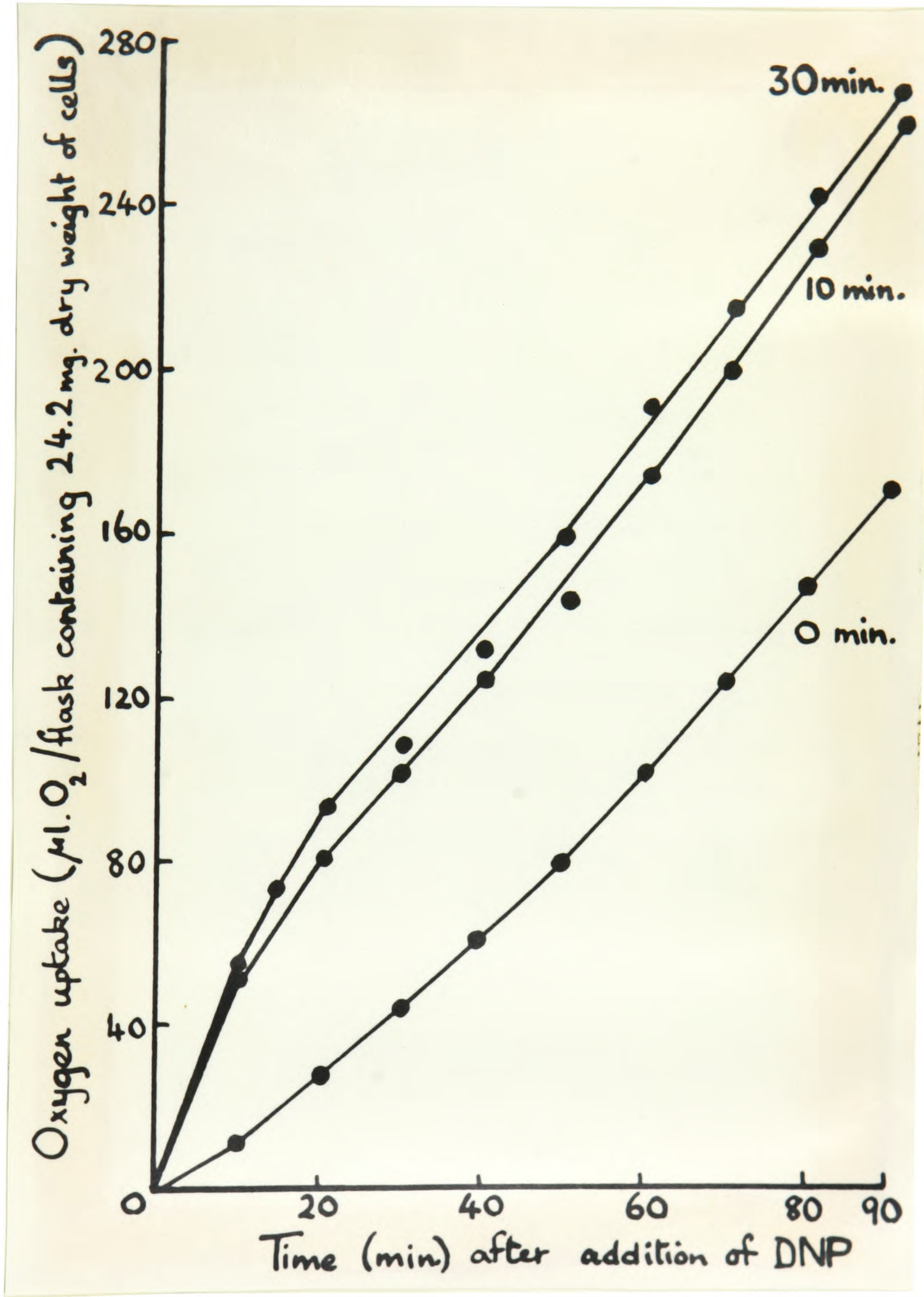
Oxygen uptake (µl. O_2 /flask containing 24.2 mg. dry weight of cells)
280
240
200
160
120
80
40
0
30 min.
10 min.
0 min.
0
20
40
60
80
90
Time (min) after addition of DNP

FIGURE 9

FIGURE 10

Effect of presence or absence of carbon dioxide on accumulation of succinate by intact cells of Micrococcus lysodeikticus at 25° and at pH 7.4, using 0.01M arsenite to inhibit succinate permeation. Arsenite was added simultaneously with (0 min.) or 2 min. after (2 min.) the addition of 0.002M succinate. Oxygen uptake is plotted from the time of addition of the inhibitor and is corrected for oxygen uptake due to endogenous respiration in presence of arsenite.

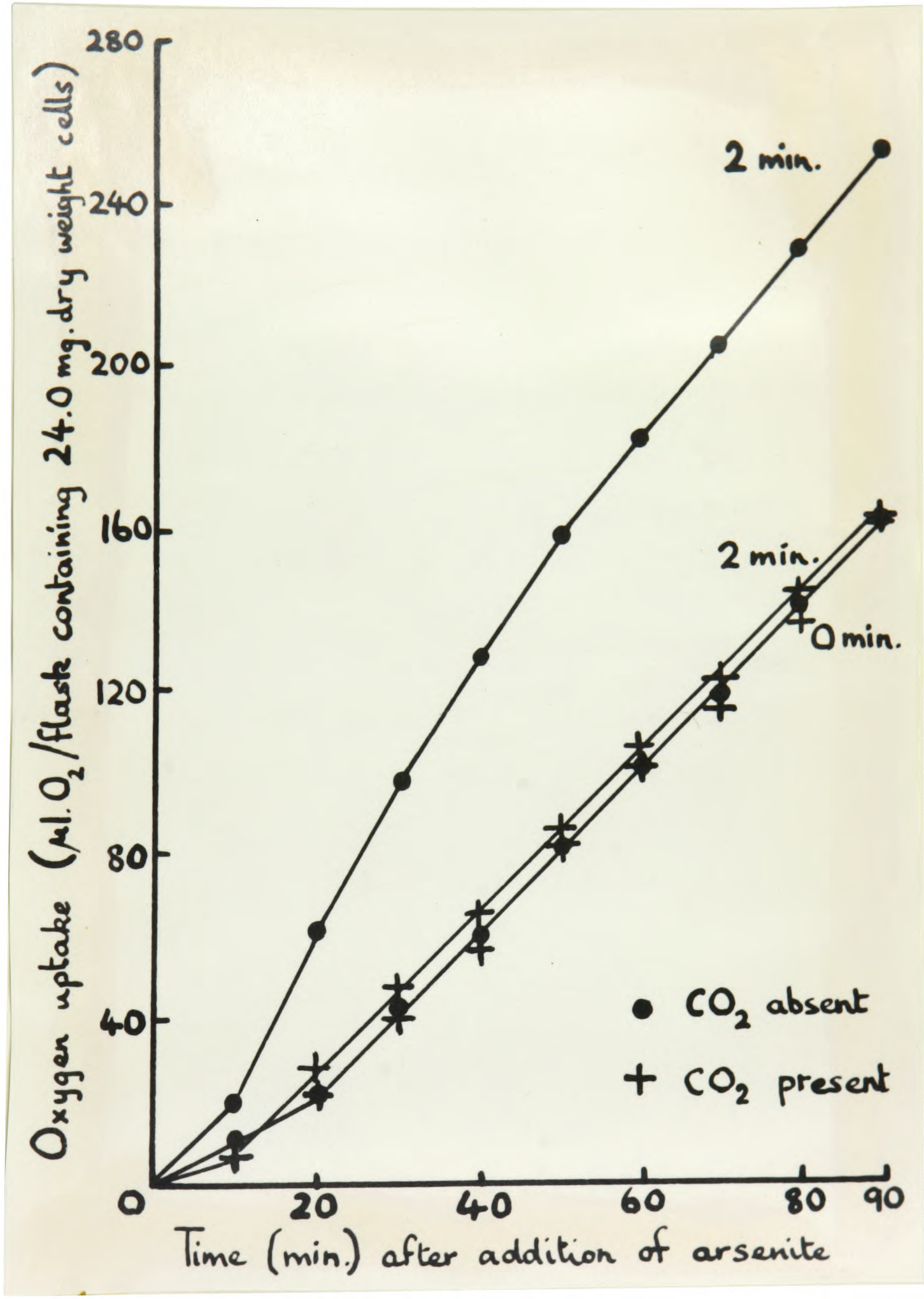
Oxygen uptake (µl. O_2 /flask containing 24.0 mg. dry weight cells)
280
240
200
160
120
80
40
0
20
40
60
80
90
Time (min.) after addition of arsenite
2 min.
2 min.
0 min.
• CO_2 absent
+ CO_2 present

FIGURE 10

PERMEATION MECHANISMS IN BACTERIAL MEMBRANES

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Bacteria possess a plasma-membrane which acts as an osmotic barrier between the internal medium of the cell and the external medium for many solutes, but establishes osmotic linkage between the internal and external media for the transport of nutrient and waste between the cell and its environment. The specificity and kinetics of phosphate transport in *Staph. aureus* resembles that of enzyme-linked reactions and the temperature characteristics suggest a complex movement of components of the plasma-membrane during the passage of phosphate from one side to the other. The material of the plasma-membrane is a complex lipo-protein, of which there is sufficient to form about one monolayer of lipid and one of protein. The protein component includes the cytochrome system and a number of enzymes. It is suggested that some of these enzymes may themselves be the carriers of the substrates which are found to pass through the membrane during metabolism and in some cases, where exchange diffusion occurs, also during rest.

The plasma-membrane of living organisms performs a dual function: it causes the separation of the internal medium of the cell from the external medium with respect to many solutes, but for other solutes (notably the nutrients and end-products of metabolism) it allows osmotic connection or acts as a specific osmotic link between the internal and external media. The two questions which we would like to answer are (i) what physical and chemical properties of the plasma-membrane determine its effectiveness as an osmotic barrier, and (ii) what is the molecular mechanism of osmotic linkage through the membrane?

1. Plasma membrane as an osmotic barrier

At the end of the last century, the botanist Alfred Fischer [1] carried out a carefully controlled series of studies on the penetration of salts and polyhydric alcohols into bacteria. He observed that the protoplasm of many bacteria was caused to retract from the rigid outer cell-wall by salt and sugar solutions of high osmotic pressure but not by glycerol, urea or chloral hydrate solutions of the same osmotic pressure, and obtained some evidence that the protoplasm did not possess intrinsic rigidity. He therefore suggested that although bacteria are so small—having a volume of the order of $1\mu^3$—the protoplasm is nevertheless covered by a delicate plasma-membrane which is an effective barrier to the free diffusion of salt and sugar molecules between the outer medium and the interior of the cell. Fischer's view met with considerable opposition [2] because the very nutrients which, according to his plasmolysis experiments, could not pass through the plasma-membrane into the cell, were rapidly metabolized under appropriate conditions. However, evidence in favour of the existence of a plasma-membrane of low permeability in bacteria gradually accumulated. Some of this evidence has been reviewed by Knaysi,[3] Mitchell,[4] Weibull [5] and Mitchell and Moyle.[6]

Six main methods have been employed to measure the passive permeability of bacterial plasma-membranes:

(i) Microscopic observation of the plasmolysis and rate of deplasmolysis of individual cells suspended in hypertonic solution.
(ii) Macroscopic measurement, by light scattering, of the dependence of the shrinkage and swelling of the cells on solute concentration and time.
(iii) Microscopic measurement of the change of volume of individual cells suspended at different solute concentrations.
(iv) Macroscopic measurement, by light scattering, of the rate of lysis of suspensions of naked bacterial protoplasts in initially isotonic solution.
(v) Measurement of the net rate of efflux of a solute by serial chemical analysis of the " internal medium " obtained by subsequent treatment of the cells with trichloroacetic acid, organic solvents, detergents or heat.
(vi) Measurement of the net rate of influx of a solute by serial chemical analysis of the suspension medium and/or " internal medium " in suspensions adjusted so that the total volumes of water on either side of the plasma-membrane are approximately equal.

Non-electrolytes. Methods (i) and (ii) supplemented by method (iii) have been applied to *Bacterium coli* (American strain B).[7] In suspension media buffered at neutral pH with 0·02 M (Na_2HPO_4 + NaH_2PO_4) at 20° C, the apparent permeability of the plasma-membrane to the following non-electrolytes is too low to be measured: sucrose, lactose, D-glucose, D-fructose, D-mannose, D-galactose, D-sorbose, D-sorbitol, L-rhamnose, L-arabinose, D-xylose. But for some other non-electrolytes the approximate times for half equilibration across the membrane are as follows: erythritol and pentaerythritol 15 min, D-ribose 5 min, glycerol less than 1 min. The rates of penetration of ribose and the polyhydric alcohols are not materially affected when carbohydrate metabolism is inhibited by mM mercuric chloride, mM sodium iodoacetate or mM potassium cyanide. In the presence of 7 mM K^+, however, the metabolism of D-glucose, D-galactose, D-mannose or D-ribose causes a limited rise in the internal osmotic pressure of the cells, which can be abolished by mM mercuric chloride, mM sodium iodoacetate, mM potassium cyanide or 10^{-1} mM sodium dinitrophenate.

Methods (iv), (v) and (vi) have been applied to *Staphylococcus aureus* (strain Duncan),[8, 9, 10] *Micrococcus lysodeikticus* (NCTC 2665) and *Sarcina lutea* (laboratory strain),[11] and methods (iii) and (vi) to *Bacillus megaterium* (strain KM).[12, 13] In *Staph. aureus*, *M. lysodeikticus*, and *S. lutea* the permeability of the plasma-membrane in media buffered at neutral pH with 0·02 M(Na_2HPO_4 + NaH_2PO_4) at 20° C is very low for sucrose, D-glucose, D-fructose, D-mannose, D-galactose and

D-sorbose. The permeability of *B. megaterium* to sucrose is also very low. The half equilibration times for the diffusion of some other non-electrolytes across the plasma-membrane of *Staph. aureus*, *M. lysodeiktus* and *S. lutea* are approximately as follows: D-sorbitol 60 min, L-arabinose 30 min, D-ribose 5 min, erythritol 20 sec, glycerol 3 sec. Urethane equilibrates rapidly across the membrane of *B. megaterium*.

It will be noted that in all the organisms studied, the pentoses, arabinose and xylose, penetrate more slowly than ribose although the only difference between the sugars is in the configuration of the OH groups. Also, while ribose penetrates at about the same rate in all the organisms studied, glycerol, erythritol, arabinose and sorbitol penetrate the plasma-membrane of the cocci more than an order of magnitude faster than they penetrate the membrane of *B. coli*, the ratio of the surface area to volume of these organisms being about the same (*ca.* $10\mu^{-1}$). A half equilibration time of 7 min corresponds to a permeability coefficient of *ca.* 1 Å/sec.

There is no doubt that the hexoses, glucose, mannose and galactose, which can be metabolized by all the organisms studied at a rate of about 1 mole/l. wet cell volume per hour, nevertheless do not approach osmotic equilibrium across the plasma-membrane to a significant extent (when present initially in the external medium at a concentration between 0·1 and 0·5 M) either in normal cells or in cells treated with the metabolic inhibitors, dinitrophenol, iodoacetate, cyanide or mercuric chloride. The passage of the hexoses through the plasma-membrane (either without chemical change or after chemical transformation on the outer surface of the plasma-membrane) must therefore be linked in some way to carbohydrate metabolism and cannot be due to an independent passive permeability of the plasma-membrane as appears to be the case for D-ribose.

We can suggest two distinct types of mechanism whereby linkage between the penetration of the hexoses and their metabolism might occur. (*a*) The membrane may be impermeable to the hexoses themselves, but permeable to a metabolic product of the hexoses which is formed on the outer surface of the plasma-membrane. (*b*) The membrane may be permeable to the hexoses through a specific carrier mechanism of the type studied by Rosenberg and Wilbrandt [14] in the red blood corpuscle membrane. If the sugar molecules pass through the membrane only as a specific carrier complex, as the concentration of the sugar on the side of the membrane towards which it is diffusing rises across the dissociation constant of the carrier complex, the net rate of diffusion drops rapidly and becomes effectively zero when the carrier is saturated on both sides of the membrane. If the dissociation constant of the carrier complex were in the region of that often observed for enzyme-substrate complexes, namely *ca.* 10^{-4}M, the entry of sugar might readily be controlled by its rate of metabolism within the cell. We should, perhaps, point out that in the permeability experiments described earlier, one measures the osmotic equilibration of solutes at a concentration of 0·1 to 0·5 M across the plasma-membrane. If specific carrier mechanisms exist for the diffusion of solutes across the plasma-membrane, we would expect to be able to observe them only when the concentrations of the solutes used were not much higher than the effective dissociation constants of the carriers. This poses a difficult practical problem It is, however, an important step to have become aware of it (see § 2).

ELECTROLYTES. Methods (i), (ii) and (vi) applied to *B. coli*,[7] and methods (iv), (v) and (vi) applied to *Staph. aureus*,[9, 10] *S. lutea* and *M. lysodeikticus* [11] have shown that in media buffered at neutral pH with 0.02 M(Na_2HPO_4 + NaH_2PO_4) at 20° C, the plasma-membranes of these organisms are practically impermeable to the following salts: NaCl, KCl, NH_4Cl, $MgCl_2$, KBr, Na acetate (pH 9). K acetate (pH 9), (Na_2HPO_4 + NaH_2PO_4), (K_2HPO_4 + KH_2PO_4), Na_2SO_4. We have observed that 0·01 M glucose causes a limited rise in the internal osmotic pressure of cells suspended in saline media containing 0·007 M K^+. When bacteria have been reported to be permeable to salt solutions on the grounds that deplasmolysis occurs in the course of about an hour, the " permeability " was

probably due to the presence of low concentrations of K^+ and glucose or other metabolites.

B. coli is practically impermeable to NaCNS, KCNS and NH_4CNS, but *Staph. aureus* is permeable to these salts. For KCNS at pH 6·8, pH 6·1 and pH 5·0 the times for half equilibration across the plasma-membrane are respectively about 10 min, 5 min and 1 min. The dependence of the rate of penetration of NaCNS on pH is similar to that of KCNS, the rate scale being reduced by a factor of 0·8. These observations show that the plasma-membrane of *Staph. aureus* is permeable to the cations Na^+ and K^+ in the presence of CNS^-. The pH dependence of the permeability to the alkali thiocyanates suggests that the anion and cation permeate separately and that the rate of penetration of NaCNS and KCNS is determined by the rate of CNS^- penetration through a titratable, charged plasma-membrane. However, although it is certain that the CNS^- ion does not cause a general rise in membrane permeability, there being no loss of internal inorganic phosphate under the conditions of our experiments, it is possible that the presence of CNS^- may cause an increase in the permeability to cations, or alternatively that the alkali and ammonium thiocyanates may penetrate in the unionized state.

Unlike *Staph. aureus*, *B. coli* is not significantly permeable to the alkali thiocyanates. It is also impermeable to NH_4Cl at pH 7 or 8 and to Na acetate and K acetate at pH 7 or 6, provided that traces of glucose or other metabolites are absent. On the other hand, NH_4 acetate at pH 6 to 8 equilibrates across the membrane in a few seconds. The NH_4 acetate does not damage the plasma-membrane, for the cells may subsequently be plasmolysed by the addition of NaCl to the NH_4-acetate-containing medium. These observations suggest that the plasma-membrane of *B. coli* is impermeable to the cations K^+ and Na^+, and to the anions Cl^- and CNS^-.

One may perhaps ask why we have attempted to measure the permeability of the membranes of bacteria by observing net solute transport instead of making use of up-to-date tracer techniques.[15] The reason for this is that the flux rates measured by tracers may include an exchange diffusion component of unknown magnitude. With phosphate, for example (with which we shall deal in more detail later) the flux rate measured with tracers is high, but the net flux of phosphate or the permeability of the membrane in the old sense is negligibly low. The net flux and the mutual exchange of solute molecules across a membrane may represent two quite different processes between which we wish to distinguish.

In order to determine whether the net flux of an ion through a membrane is possible, it is necessary to allow an electric charge to pass across the membrane. For membranes which are accessible from both sides, this may be done as Ussing has shown [27] by making a suitable electrical connection between the media on either side of the membrane. As the medium on the inside of the bacterial plasma-membrane is not accessible to electrode systems, the permeability of the membrane to one ion can only be measured in conjunction with that of another ion. As pointed out above, isotope exchange methods will not yield the information which we require. One therefore searches for a salt to both ions of which the membrane is permeable so that either ion may then be used to investigate the permeability of the membrane to other ions of opposite sign.

PARTICIPATION OF A ' LIPID ' PHASE IN THE PLASMA-MEMBRANE. The permeability properties of the plasma-membranes of the bacteria described above are generally in accord with the view that the obstacle to the free diffusion through the plasma-membrane may be a thin hydrophobic or ' lipid ' layer, for the rates of penetration of the solutes tend to decrease rapidly as the hydration is increased. However, there is no doubt from the results described that the configuration of the solutes also plays an important part in determining the rate of penetration, and that factors other than lipid solubility must be concerned in the interaction between solute and plasma-membrane during penetration.

CHEMICAL COMPOSITION OF THE PLASMA-MEMBRANE. Determinations of the weight, morphology and chemical composition of fragments of mechanically

disintegrated *Staph. aureus*, segregated into morphologically homogeneous fractions by differential centrifugation, indicated that the plasma-membrane of this organism, which readily disintegrates into small particles, is a complex lipo-protein containing 41 % by weight protein and 22·5 % lipid.[17] Semi-quantitative amino-acid analysis of the protein component, by paper chromatography, showed the presence of a high content of the non-polar amino acids, glycine and alanine, and of the acidic amino acid, glutamic acid. The lipid component contained 1·85 % P and 1·3 % N—about half the phosphorus content and rather more than half the nitrogen content of lecithin. The material of the " small particle fraction " has been found to account for some 10 to 15 % of the dry weight of the cells.[17, 18] Since the cells are *ca.* 0·7 μ in diameter and have a ratio of wet to dry weight of *ca.* 3, it can readily be calculated that the material of the " small particle fraction " would form a layer *ca.* 5 mμ thick if unhydrated. This would correspond to about a monolayer of lipid and a monolayer of protein. Work which is at present in progress has shown that the composition of morphologically intact protoplast membranes from *Staph. aureus* correspond fairly closely to that of the " small particle fraction ", and there can be little doubt that the material described above corresponds to that of the plasma-membrane of normal intact cells.

2. The plasma-membrane as an osmotic link

It has been suggested that exchange diffusion might represent the translocation reaction of active transport uncomplicated by the activity of the coupled reactions which normally drive it; and that the study of the characteristics of exchange-diffusion might shed light upon the mechanism of active transport.[19] We would like to add that when the permeability of the membrane to a particular solute is caused by a specific carrier, although net transport effectively ceases when the solute concentration is such as to saturate the carrier on both sides of the membrane, exchange diffusion would be expected to continue at its maximum rate, and to be very strictly coupled. Also, we suggest that if the passage of a solute through the plasma-membrane is facilitated by the *mutual* occupation of hydrophilic groups of a " carrier " component of the membrane and the solute, the movement of the " carrier " across the membrane may be as dependent upon the presence of the specifically carried solute as the movement of the solute is dependent upon the "carrier". The study of exchange diffusion reactions may therefore be expected to play an important part in research on the mechanisms of membrane permeability and active transport.

Transport of phosphate. Some of the evidence for the participation of an exchange diffusion-like reaction in the exchange of phosphate across the plasma-membrane of resting *Staph. aureus* has already been reviewed.[19] We shall summarize this evidence [8, 20, 21] and discuss it critically in the light of recent observations.[10, 22]

(i) The plasma-membrane of resting *Staph. aureus* is apparently impermeable to the $H_2PO_4^-$ and HPO_4^{2-} ions when they are present in solution as the alkali salts. This might be due either to impermeability of the membrane to cations or to impermeability to phosphate ions or both. However, the phosphate ions do not exchange across the membrane with acetate, arsenite, azide, bicarbonate, bromide, chloride, chromate, cyanide, fluoride, glutamate, iodide, molybdate, nitrate, nitrite, oxalate, pyroantimonate, succinate, sulphate, thiocyanate, thiosulphate, *p*-toluenesulphonate, tungstate or versenate: they exchange strictly with arsenate. The membrane may therefore be impermeable to all the above anions including phosphate, or be specifically permeable to phosphate and arsenate alone and impermeable to the associated cations. While there is a slow permeation of the membrane by NaCl estimated as chloride and a rapid permeation by NaCNS estimated as thiocyanate, there is no significant exchange of phosphate for Cl^- or CNS^-. We may therefore consider two possibilities: (*a*) The membrane is impermeable to $H_2PO_4^-$ and HPO_4^{2-} but slightly permeable to Cl^- and very permeable to CNS^-. (*b*) The strong electrolytes NaCl and NaCNS permeate

unionized and the membrane may be specifically permeable to $H_2PO_4^-$ and/or HPO_4^{2-} (but not to the unionized salts), and impermeable to the associated cations.

(ii) By labelling the inorganic phosphate of the medium internal or external to the plasma-membrane of *Staph. aureus* with ^{32}P, a mixing of the phosphate of the media on either side of the membrane is observed. The rate of this mixing varies with the salinity, the pH and the phosphate concentration of the external medium and with temperature.

The dependence of the rate of phosphate exchange on pH and external phosphate concentration indicates that the $H_2PO_4^-$ ion and not the HPO_4^{2-} ion takes part in the exchange. The rate of exchange ($\dot{P}$) may be described in terms of the external $H_2PO_4^-$ concentration ($[H_2PO_4^-]_E$) by the equation

$$1/\dot{P} = 1/\dot{P}_{max.} + K/[H_2PO_4^-]_E \dot{P}_{max.}, \quad (1)$$

in which $\dot{P}_{max.}$ stands for the maximum value of $\dot{P}$ and K is the value of $[H_2PO_4^-]_E$ at which $P = \dot{P}_{max.}/2$. When P is expressed in μ mole phosphate/g cell dry weight min, and $[H_2PO_4^-]_E$ is expressed in mM, K has a value of $0{\cdot}8 \pm 0{\cdot}1$ mM between pH 5·5 and 8·5; and $\dot{P}_{max.}$ has a value of *ca.* 10 μmole/g min at pH 7 and a positive slope of some 5 μmole/g min pH unit.

The hyperbolic form of eqn. (1)—which is formally identical to the enzyme kinetics equation of Michaelis and Menten [23]—shows that a saturation phenomenon occurs in phosphate exchange and implies that one stage in the movement of phosphate through the plasma-membrane involves a specific spatial or bonding relation between a component of the membrane and the phosphate molecule.

(iii) The rate of phosphate exchange is very sensitive to certain inhibitors, notably phenyl-Hg^+ and other compounds which combine with thiols of low reactivity. The relationship between the degree of inhibition of the exchange reaction and the concentration of phenyl-Hg^+ may be represented by

$$K' = Mn/(100 - n), \quad (2)$$

M being the amount of phenyl-Hg^+, n the percentage activity of $\dot{P}$ and K' a constant. This indicates a reaction of the type $M + X \rightleftharpoons MX$, X representing the sites which when combined with inhibitor (as MX), cause inactivation of a corresponding number of units controlling phosphate exchange. The number of these sites can be estimated to correspond to not more than 4·4 μ mole phenyl-Hg^+/g cell dry weight.

(iv) From the dependence of $\dot{P}$ on temperature it can be calculated that the total heat of activation for the exchange movement of phosphate across the membrane is 37,400 cal/mole. Using the above estimate of the number of exchange sites, the absolute value of $\dot{P}$ gave a maximum value for the free energy of activation of 19,700 cal/mole, leaving an entropy of at least 17,700 cal/mole. The thermodynamic data suggest that the movement of phosphate across the plasma-membrane is accompanied by a molecular disturbance quite out of proportion to that which would be expected unless the phosphate moves in relation to some larger molecule or molecules within the plasma-membrane.[21] The resemblance of the data to those of reversible protein denaturation is perhaps significant.

There can be no doubt that whether the plasma-membrane is permeable to phosphate ions ($H_2PO_4^-$ ions) or not, the movement of phosphate groups across it is dependent upon the existence of a highly specific reaction mechanism. There are three simple alternatives for this mechanism: (*a*) The membrane allows a net transport of $H_2PO_4^-$ through the specific reaction mechanism, and the distribution of phosphate ions across the membrane is determined by the distribution of cations, to which the membrane is supposed to be impermeable. The exchange mechanism may, under these conditions, be identical to that proposed by Ussing for Na^+ exchange.[16] (*b*) The membrane will not allow a net transport of $H_2PO_4^-$. Since there is negligible net movement of phosphate across the membrane even when the concentration on the outside is as low as 0·08 mM (an order of magnitude less

than the apparent dissociation constant K of the carrier implicit in eqn. (1)), the distribution of phosphate ions across the membrane in resting cells must be maintained by a strict one-to-one carriage of phosphate inwards and outwards by the exchange mechanism. This might be accomplished by the type of carrier considered by Ussing if it is assumed either that the carrier can move only with a phosphate passenger or that the carrier is always occupied with a phosphate group and that this group may exchange with phosphate ions in the media on either side of the membrane. (*c*) It is possible that no carrier is involved, but that the media on each side of the plasma-membrane are connected by a "pore" at either end of which there is an adsorption site accessible to phosphate ions in the medium on that side only. One of the adsorption sites is assumed to be always occupied by a phosphate group which is supposed to be able to move from one end of the pore to the other and to be able to exchange with a phosphate group in the medium at the appropriate end of the pore, but not to be able to leave the pore unoccupied.

Analysis of the kinetics will not distinguish between the alternative mechanisms proposed above. The fact that the membrane is only one or two molecules thick encourages one to consider that the carriage of solutes across it may occur by a thermal rotation of protein, lipid or other component in a manner similar to that visualized by Langmuir [24] in surface films and by Lundegardh [25] in plant cell membranes. The formal similarity of the kinetics of the phosphate exchange reaction to enzyme-linked reactions does not, of course, show that enzymes are involved. There are, however, other circumstances which suggest that the phosphate exchange reaction may be coupled to enzyme reactions. When glucose is present there is a net transport of phosphate inwards through the osmotic barrier. This occurs, not as a result of an increase in the rate of influx but as a result of a decrease in the rate of outflux below that of resting cells. It has therefore been suggested that the phosphate exchange reaction of resting cells represents the active transport reaction operating reversibly because it is not being driven by coupling with carbohydrate metabolism. This concept has been supported by the observation that all the inhibitors of phosphate exchange are also inhibitors of active phosphate uptake. *Staph. aureus* is not peculiar in possessing the phosphate exchange system, for a similar system has been demonstrated in *B. coli*. As might have been anticipated, exchange diffusion of phosphate does not occur across the plasma-membranes of the strict aerobes *S. lutea* and *M. lysodeikticus*, under semi-anaerobic conditions.

ENZYMES OF THE PLASMA-MEMBRANE. The material of the plasma-membrane of *Staph. aureus*, isolated as the " small particle fraction " described above, contains more than 90 % of the total activity of an acid phosphatase which acts at the outer side of the plasma membrane in intact cells. It also contains at least 90 % of the cytochrome, measured by the total extinction at a wavelength of 425 mμ, and some 90 % of the total succinic dehydrogenase activity, as well as potent lactic dehydrogenase activity, the latter showing the characteristics of the cytochrome *b*-linked enzyme.[22] The cytochrome spectrum shows the presence of components with extinction maxima at 604, 558 and 528 mμ. The plasma-membrane of *B. megaterium* has also been reported to contain the cytochrome system.[26]

These studies show that part of the protein of the plasma-membrane of *Staph. aureus* is constituted of enzymes, and we are at present investigating the possible participation of these enzymes in the reactions causing phosphate exchange and accumulation across the plasma-membrane. We suggest that the occurrence of the cytochrome *b*-linked enzymes in the plasma-membrane may be connected with the fact that they are the last members of the enzymic chain and deal with the end-products of metabolism, namely lactic, succinic and formic acids which must be specifically carried through the plasma-membrane.

We are indebted to the Scottish Hospital Endowments Research Trust for personal grants in support of this work, and to the Rockefeller Foundation for a grant for equipment.

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Liberation and Osmotic Properties of the Protoplasts of *Micrococcus lysodeikticus* and *Sarcina lutea*

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SUMMARY: Stable protoplasts may be released from *Micrococcus lysodeikticus* and *Sarcina lutea* by digestion of the cell-wall with lysozyme in sucrose or NaCl solutions having an osmotic pressure of some 25 atmospheres, but not in glycerol solutions of the same osmotic pressure. The stability of the protoplasts depends not only upon the depression of the water activity by the solute but upon an osmotic pressure exerted against the protoplast (plasma-) membrane. The permeability of the protoplast membrane to a number of solutes resembles that of the osmotic barrier of intact *Staphylococcus aureus*.

This paper describes work on the protoplasts of *Micrococcus lysodeikticus* and *Sarcina lutea* which was completed just over a year ago in the Department of Biochemistry, University of Cambridge. It was undertaken as part of a research programme designed to investigate the physiological role of the plasma-membrane in bacteria (Mitchell & Moyle, 1956*a*, *b*).

Three years ago, Weibull (1953) discovered that spherical bodies, which were thought to be intact protoplasts, emerged from *Bacillus megaterium* when the cell walls were digested by lysozyme in solutions of which the water activity had been depressed by the addition of polyethylene glycol, sucrose or certain other solutes. It was not known, however, whether the mechanism of the stabilizing action of the solutes was by the exertion of an osmotic pressure across the membrane, thus preventing osmotic explosion of the protoplast, or whether the solutes prevented the swelling of hydrated constituents of the protoplasm which would otherwise have ruptured the external membrane. It was therefore of interest to measure the permeability of isolated protoplasts to a range of solutes; for it would be expected that if the protoplasts were stabilized by the external osmotic pressure, only non-penetrating solutes would be effective as stabilizers, whereas if the mechanism of stabilization were by dehydration of components of the cytoplasm the effectiveness of a solute would depend only upon the lowering of the activity of the water. The lysozyme-sensitive cocci were chosen for study because we wished to obtain data which might usefully be compared with similar data on the internal osmotic pressure and permeability of *Staphylococcus aureus*.

Grula & Hartsell (1954) attempted to prepare protoplasts of *Micrococcus lysodeikticus* by the method used for *Bacillus megaterium* (Weibull, 1953), but most of the protoplasts swelled and disintegrated as soon as they were released from the cell-walls. Dr M. R. J. Salton (personal communication) attempted to obtain protoplasts from *M. lysodeikticus* in higher sucrose concentrations, but could observe no indication of protoplast formation from the changes in light-

scattering of the suspensions in sucrose. In view of our observations on *Staphylococcus aureus* which implied that the osmotic pressure was between 20 and 25 atmospheres (Mitchell & Moyle, 1956*a*), it was possible that the protoplasts of the cocci might require at least M-sucrose or M-saline solutions to preserve them from osmotic explosion. Moreover, it seemed possible that at very high sucrose concentrations, the depression of the light scattering which would result from the elevation of the refractive index of the medium might be sufficient to make the protoplasts invisible by light scattering unless they were subsequently transferred to a medium of lower refractive index.

METHODS

Growth and preparation of organisms. Cultures of *Micrococcus lysodeikticus* (NCTC 2665) and *Sarcina lutea* (laboratory strain) were grown aerobically at 25° in a medium containing 3 % (w/v) tryptic digest of casein, 1 % glucose and 0·1 % Marmite, using the rotated flask technique (Mitchell, 1949). The organisms were harvested at a concentration equivalent to 1·5–2 mg. dry weight/ml., washed twice with distilled water (for *M. lysodeikticus*) or 0·5 M-NaCl (for *S. lutea*) and suspended at a concentration corresponding to *c.* 100 mg. dry weight/ml. in distilled water (for *M. lysodeikticus*) or 0·5 M-NaCl (for *S. lutea*).

Measurement of turbidity. The extinction of 1 cm. depth of suspension was measured at a wavelength of 700 mμ., using the Beckman model DU spectrophotometer.

Microscopy. The intact organisms and protoplasts were examined with a Cooke Troughton and Simms binocular phase-contrast microscope in films of aqueous solution sealed between slide and coverslip with vaseline.

Protoplast release and stability

Method I. *Visibility of protoplasts and intact organisms in* NaCl *and sucrose solutions.* Samples of organisms were incubated for 2 hr. at 25° at a standard concentration corresponding to 200 μg. dry wt. organisms/ml., with a range of concentrations of NaCl or sucrose in 0·04 M-sodium phosphate buffer (pH 6·8), containing 10 μg. (for *Micrococcus lysodeikticus*) or 40 μg. (for *Sarcina lutea*) crystalline egg-white lysozyme/ml. The turbidities of the suspensions were then measured and compared with those of controls in which the lysozyme was omitted.

Method II. *Dependence of protoplast stability on osmotic pressure of release-medium.* Samples of suspensions were incubated as above but at a concentration corresponding to 10 mg. dry wt. organisms/ml. Samples (0·1 ml.) of the incubated suspensions were pipetted into 5 ml. samples of M-NaCl containing 0·01 M-sodium phosphate buffer at pH 6·8. After 20 min. at 20°, the turbidities of the suspensions were measured. Controls were done omitting lysozyme only.

Method III. *Dependence of protoplast stability on osmotic pressure of NaCl solutions to which protoplasts are transferred after release in NaCl or sucrose.* The protoplasts were released by incubating the organisms with lysozyme at 25° for 2 hr. at a concentration corresponding to 10 mg. dry wt. organisms/ml.

in 1·2 M-sucrose or 2 M-NaCl containing phosphate buffer as in (I). Samples (0·1 ml.) of the protoplast suspension were pipetted into 5 ml. samples of NaCl ranging from 0 to 2 M, containing 0·01 M-sodium phosphate buffer at pH 6·8. After 20 min. the turbidities of the suspensions were measured. Controls were done omitting lysozyme only.

Method IV. *Dependence of protoplast stability on osmotic pressure of sucrose,* NaCl *or glycerol solutions to which protoplasts are transferred after release in* 1·2 M-*sucrose.* The protoplasts were released in 1·2 M-sucrose as in method III. The incubated suspension was mixed with 5 vol. 1·2 M-NaCl (to decrease the specific gravity) and centrifuged at 20°; and the protoplasts were redispersed in 1·2 M-NaCl to give a suspension density corresponding to 100 mg. original dry wt. organisms/ml. Samples (0·5 ml.) of the protoplast suspension were pipetted into 4·5 ml. samples of NaCl, sucrose or glycerol arranged to give final concentrations ranging from 0 to 2 M in 0·01 M-sodium phosphate buffer at pH 6·8. After 30 min. incubation at 25°, samples (0·1 ml.) of the protoplast suspensions in the NaCl, sucrose and glycerol solutions were pipetted into 5 ml. samples of 1·2 M-NaCl containing 0·01 M-sodium phosphate buffer at pH 6·8, and after a further 20 min. the turbidities were measured.

Method V. *Determination of protoplast permeability.* The protoplasts were released in sucrose as in method III. The incubated suspension was mixed with 5 vol. 1·2 M-NaCl and centrifuged at 20°; and the protoplasts were redispersed in 1·2 M-NaCl to give a suspension density corresponding to 10 mg. original dry weight organisms/ml. Samples (0·1 ml.) of the protoplast suspension were pipetted into 1·5 molal solutions (in 0·01 M-sodium phosphate buffer at pH 6·8) of the solutes to which the permeability was to be measured. The rate of lysis of the protoplasts, measured by the change of light scattering, was taken as an approximate index of the rate of permeation of the solute.

RESULTS

'*Protoplast*' *release*

The effect of lysozyme treatment (Method I) on the turbidity of suspensions of *Micrococcus lysodeikticus* in concentrations of sucrose from 0·1 to 1·0 M is shown in Fig. 1. At the low sucrose concentrations the lysozyme-treated suspensions scattered very little light, but, although the amount of light scattered was still small, at concentrations of sucrose between 0·5 and 1·0 M they scattered between 10 and 20 % as much light as the corresponding untreated suspensions. This suggests that protoplasts were being liberated and were stable in sucrose concentrations approaching 1 M, but that their light scattering (like that of the intact organisms) was depressed by the high refractive index of the sucrose solutions. The lysozyme treatment was therefore done in NaCl solutions (method I) with the results shown in Fig. 2. At concentrations of NaCl above 0·5 M the lysozyme-treated organisms scattered between 50 and 60 % as much light as the untreated organisms, but below 0·5 M-NaCl the light scattered by the lysozyme-treated organisms fell towards some 5 % of that scattered by the untreated organisms. Similar results were

obtained with *Sarcina lutea*, the only significant difference being that *S. lutea* tended to lyse slowly in distilled water. The suspensions of *S. lutea* were therefore washed and made up in 0·5 M-NaCl in place of distilled water.

Phase-contrast microscopy showed that the lysozyme-treated suspensions of both organisms in concentrations of NaCl above 1·0 M contained spherical bodies of approximately the same diameter but of lower contrast than organisms of corresponding control suspensions, and whereas the organisms of the controls adhered to one another the spherical bodies in the lysozyme-treated suspensions were separate. In concentrations of NaCl below 1·0 M, the lysozyme-treated suspensions contained spherical bodies (which were larger than the cells of corresponding control suspensions) together with ghost membranes of extremely low contrast. The lower the NaCl concentration, the larger

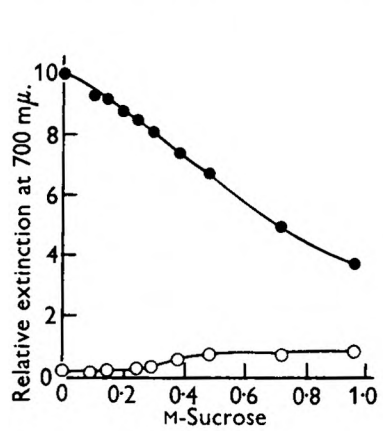

Fig. 1

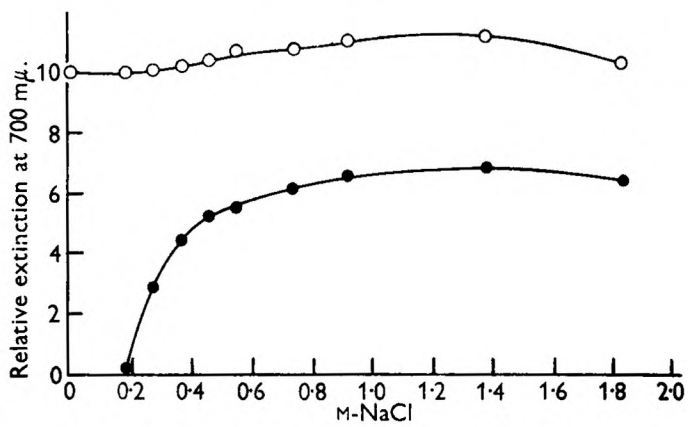

Fig. 2

Fig. 1. The dependence of the extinction of suspensions of *Micrococcus lysodeikticus* on the concentration of sucrose in the suspension medium after incubation with lysozyme (○) and without lysozyme (●) (see method I).

Fig. 2. The dependence of the extinction of suspensions of *Micrococcus lysodeikticus* on the concentration of NaCl in the suspension medium after incubation with lysozyme (●) and without lysozyme (○) (see method I).

was the ratio of ghosts to intact spherical bodies; and no intermediate forms were present. It was evident that the spherical bodies corresponded to the light-scattering units of the suspensions and that since they either remained intact or lysed completely, the amount of light scattered by a suspension was approximately proportional to the number of surviving spherical bodies. Microscopic examination of the suspensions treated with lysozyme in sucrose (corresponding to Fig. 1) was not successful because the contrast was much decreased by the high refractive index of the sucrose solutions; but by diluting the sucrose-containing suspensions with M-NaCl, the same general pattern as described above could be observed.

In order to ascertain quantitatively whether lysozyme treatment in NaCl solutions followed the same pattern as in sucrose solutions, samples of organisms were treated with lysozyme in a range of sucrose and NaCl concentrations, and subsequently transferred to M-NaCl to measure the turbidity (method II). Fig. 3 shows the results of such an experiment with *Micrococcus lysodeikticus*, harvested at a population density corresponding to *c.* 1·5 mg. dry weight/ml.;

similar results were obtained with *Sarcina lutea*. It is evident that to stabilize 50 % of the light-scattering units of the lysozyme-treated suspension requires the presence of 0·33 M-NaCl or 0·53 M-sucrose. From the vapour-pressure equilibrium data of Robinson & Sinclair (1934), it is found that 0·53 M-sucrose corresponds to 0·34 M-NaCl in water activity (or osmotic pressure for a membrane impermeable to sucrose and NaCl). Thus, the stabilization of the lysozyme-treated suspensions is practically the same whether the water activity is depressed (or osmotic pressure raised) by NaCl or by sucrose.

The stabilization of the lysozyme-treated organisms in the higher NaCl and sucrose concentrations might have been due either to stabilization of protoplasts which had been completely released from the cell walls, or to an inhibitory effect of the sucrose and NaCl on the lysozyme reaction. The latter

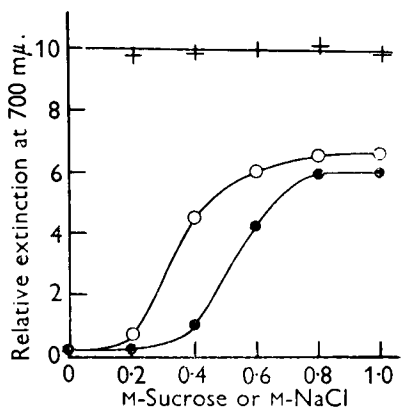

Fig. 3

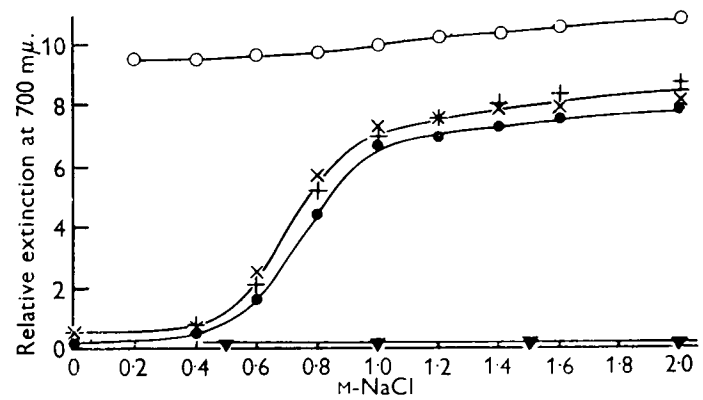

Fig. 4

Fig. 3. The dependence of the extinction of suspensions of *Micrococcus lysodeikticus* in M-NaCl on the concentration of NaCl or sucrose in which the cells were previously incubated with lysozyme (NaCl, ○; sucrose, ●) and without lysozyme (mean for NaCl and sucrose +) (see method II).

Fig. 4. The dependence of the extinction of suspensions of *Sarcina lutea* on the concentration of NaCl to which the cells are transferred after incubation in 2 M-glycerol, 1·2 M-sucrose, or M or 2 M-NaCl solutions with lysozyme (2M-glycerol, ▼; 1·2 M-sucrose, ●; M-NaCl, ×; 2 M-NaCl, +) and without lysozyme (mean for NaCl, sucrose and glycerol, ○) (see method III).

possibility was eliminated by showing that the organisms in the suspensions treated with lysozyme at high sucrose or NaCl concentrations lysed when transferred to solutions of lower concentration. The organisms were treated with lysozyme in 1·2 M-sucrose, or M or 2 M-NaCl solutions and subsequently transferred to a range of NaCl concentrations (method III). Fig. 4 shows such an experiment with *Sarcina lutea*. The turbidity of the suspensions that were treated with lysozyme in M or 2 M-NaCl was slightly higher than those treated with lysozyme in 1·2 M-sucrose over the whole range of NaCl concentrations to which they were transferred. However, the dependence of the stability of the light-scattering elements upon the NaCl concentration to which the suspensions were transferred was practically the same whether lysozyme treatment was done in 1·2 M-sucrose, M or 2 M-NaCl. It followed that the action of the high concentrations of sucrose or NaCl was not due to an inhibition of the lysozyme reaction, but could be attributed to stabilization of

'protoplasts' which had been completely released from the normal mechanical protection of the cell wall.

In order to determine whether the 'protoplasts' were stabilized by an osmotic pressure acting across a semi-permeable membrane, or whether the stabilization was due to dehydration of protoplasmic constituents which would otherwise swell and cause the 'protoplasts' to disintegrate, the lysozyme treatment was carried out in 2 M-glycerol in place of sucrose (method III); for it was shown by Fischer (1903) that many bacteria are permeable to glycerol. The results (Fig. 4), showed that the 'protoplasts' were not stabilized when released in 2 M-glycerol. The stabilization cannot therefore be due to lowering of the water activity alone: it must be caused by the substitution of an osmotic

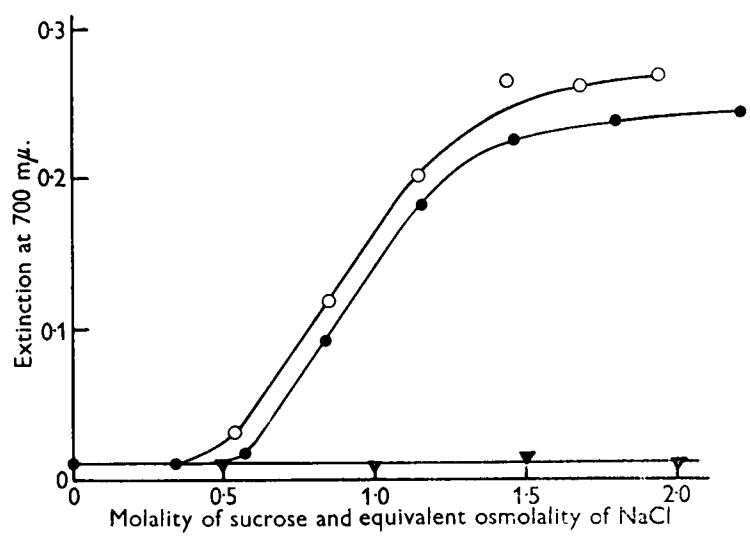

Fig. 5

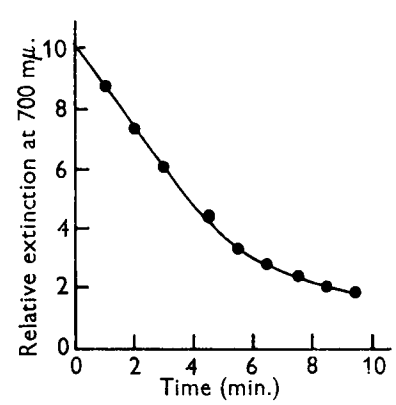

Fig. 6

Fig. 5. The dependence of the extinction of suspensions of 'protoplasts' of *Micrococcus lysodeikticus* in 1·2 M-NaCl on the concentration of glycerol (▼), sucrose (●) or NaCl (○) to which they are intermediately transferred after release by lysozyme in 1·2 M-sucrose (see method IV).

Fig. 6. The dependence of the extinction of 'protoplasts' of *Micrococcus lysodeikticus* suspended in 1·5 molal D-ribose on time at 20° (see method V).

pressure at the outer surface of the 'protoplast' membrane for the hydrostatic pressure exerted by the cell-wall of the intact organism. The molarity of NaCl at which half the 'protoplasts' of *Sarcina lutea* are stabilized is *c.* 0·7 M in the experiment of Fig. 4: it corresponds to an osmotic pressure of between 25 and 30 atmospheres. This probably represents about the mean osmotic pressure of the cells.

An alternative and perhaps preferable method of examining the dependence of the stability of the 'protoplasts' on the osmotic pressure and composition of the suspension medium is to release a large batch of 'protoplasts' in 1·2 M-sucrose, to transfer samples of these 'protoplasts' to ranges of concentration of NaCl, sucrose and glycerol, and then after a fixed time interval to determine the proportion of surviving 'protoplasts' by measuring their light-scattering when transferred to 1·2 M-NaCl (method IV). The results of such an experiment with *Micrococcus lysodeikticus* are plotted in Fig. 5. The scale of concentration has been converted to molality of sucrose and equivalent osmolality of NaCl by

means of the vapour pressure equilibrium data of Robinson & Sinclair (1934) and specific gravity tables. As with *Sarcina lutea*, sucrose and NaCl are about equally effective in stabilizing the 'protoplasts' from *M. lysodeikticus* while glycerol is not effective. The osmolality required to stabilize half the 'protoplasts' is *c.* 0·9, corresponding to an osmotic pressure of *c.* 20 atmospheres. The osmolality of sucrose or NaCl solutions required to stabilize half the 'protoplasts' of *M. lysodeikticus* and *S. lutea* varied from one culture to another about a mean of 1·0 osmolal, when harvested at a dry weight of 2·0 mg./ml.

'Protoplast' permeability

The rate of lysis of the 'protoplasts' of *Micrococcus lysodeikticus* and *Sarcina lutea* was less than 10 %/hr. at 20° in 1·5 molal solutions of the following solutes in 0·01 M-sodium phosphate buffer at pH 6·8 (except where otherwise stated): NaCl, KCl, NH_4Cl, $MgCl_2$, NaBr, KBr, Na-acetate (pH 9), K-acetate (pH 9), K_2SO_4, ($KH_2PO_4 + K_2HPO_4$), Na-glutamate, lysine-HCl, D-glucose, D-fructose, D-mannose, D-galactose, D-sorbose and sucrose (method V). Since it was possible that metabolism of the carbohydrates might affect the stability of the 'protoplasts', the effect of the presence of 10^{-2} M-KCN, 10^{-2} M-sodium iodoacetate, 10^{-3} M-sodium dinitrophenate or 10^{-5} M-$HgCl_2$ on the rate of lysis of the 'protoplasts' was determined. The rate of lysis was, as in the case of the untreated suspensions, less than 10 %/hr. The 'protoplast' membrane is therefore only slightly permeable to the above solutes under the conditions of these experiments. However, under the same conditions as above, the 'protoplasts' were *c.* 70 % lysed in 3 sec. in glycerol, 20 sec. in erythritol, 5 min. in D-ribose, 30 min. in L-arabinose and 1 hr. in D-sorbitol. The progress of lysis of 'protoplasts' of *Micrococcus lysodeikticus* in D-ribose is shown in Fig. 6.

It is not possible to estimate accurately the rate of equilibration of the solutes across the 'protoplast' membrane from measurements such as those of Fig. 6 without much additional information. However, an approximate estimate of the permeability of the membrane can be obtained since we know from the data of Figs. 4 and 5 that 70 % lysis of the 'protoplasts' corresponds to about an osmotic pressure difference of 0·75 osmolar across the 'protoplast' membrane, or to half equilibration of the 1·5 molal non-electrolyte solutions used in these experiments. Thus, we may say that the 70 % lysis times given above correspond approximately to the times for half equilibration of the solutes across the 'protoplast' membrane.

DISCUSSION

There is no doubt that the 'protoplasts' liberated from *Micrococcus lysodeikticus* and *Sarcina lutea* by lysozyme possess the properties that would be expected of the protoplasm of the cell covered by a plasma membrane. It is, however, important to emphasize that we do not yet know whether the composition of the membrane of the 'protoplasts' liberated by lysozyme is the

same as that of the plasma-membrane of the intact organism. Even so, we suggest that the removal of the inverted commas from the word protoplast when used to describe the bodies which are liberated from *M. lysodeikticus* and *S. lutea* by lysozyme in media of high osmotic pressure is justified on the following grounds: (i) Lysozyme is known to dissolve the cell wall of these organisms (Salton, 1954). (ii) The bodies are approximately the same size as the intact organisms, and of only slightly lower contrast in phase-contrast microscopy, when suspended in media in which the degree of lysis is small. (iii) The bodies scatter about two-thirds as much light as the intact organisms. (iv) The bodies are covered by a semipermeable membrane which retains the small molecular weight internal solutes of which the total osmotic pressure is some 20 atmospheres, and the bodies are osmotically exploded in media of low osmotic pressure. (v) After lysis in media of low osmotic pressure the empty membranes are visible by phase-contrast microscopy. (vi) In media containing a high concentration of solutes known to be unable to penetrate the plasma membrane of *Staphylococcus aureus* and *Escherichia coli* (Mitchell & Moyle, 1956*a*), the membrane of the bodies is subject to an external osmotic pressure which substitutes for the hydrostatic pressure normally exerted by the cell wall and prevents explosion of the bodies. (vii) When the osmotic pressure of the external medium is raised by glycerol or other solutes which are known to penetrate the plasma membrane of intact bacteria, the passage of the solute through the 'protoplast' membrane results in osmotic explosion.

Since this work was completed, Weibull (1955) has described experiments on the osmotic properties of the protoplasts of *Bacillus megaterium* with which our observations are essentially in agreement. He observed, however, that the total volume of the protoplasts of *B. megaterium* was independent of the osmotic pressure of the sucrose solution in which they were released, over a range from 0·125 to 0·4 M. In the work described here, there was no indication that the protoplasts of the cocci distinguished between the osmotic pressure of the medium in which they were released and that to which they were subsequently transferred. It may, perhaps, be relevant that whereas the protoplasts of *B. megaterium* must change shape from cylinders to spheres, there is little or no change of shape of the protoplasts of the cocci during release by lysozyme.

The aim of the work described in the present paper was to obtain a general view of the effectiveness of the membrane of *Micrococcus lysodeikticus* and *Sarcina lutea* as a barrier to the free diffusion of solutes between the cell interior and the environment. It is, perhaps, relevant to point out that the membrane does not act only as an osmotic barrier. Its most specific function is to link the internal and external media, allowing entry of specific metabolites and exit of end-products of metabolism. The rate of lysis of the protoplasts in 1·5 molal glucose and in the other hexose solutions shows that these sugars enter both the normal (semi-anaerobic) and the inhibitor-treated protoplasts at a rate of less than 0·1 mole/l. protoplast volume/hr. On the other hand, we have recently found that under aerobic conditions the intact cocci or the protoplasts oxidize glucose at a rate corresponding to 1·0 mole or more/l.

protoplast volume/hr. It follows that the movement of glucose through the plasma membrane is closely linked to its oxidation. The implication of this coupling has been discussed briefly elsewhere (Mitchell & Moyle, 1956*b*), and we have suggested that glucose is carried through the membrane on an enzyme or on a protein carrier of equivalent specificity and affinity. Further work on this problem, which is now in progress, will be described shortly in another paper.

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