THE EFFECT OF SILVER IONS ON THE TRANSPORT OF PHOSPHATE IN *ESCHERICHIA COLI* K12

by

Wilhelmus Jacobus Antonius Schreurs

A thesis submitted for the degree of

Doctor of Philosophy

at the Australian National University.

April 1982

"A question in your nerves is lit ..."

Bob Dylan

STATEMENT

The work described in this thesis is the candidate's own, except where otherwise stated (see Chapter 5).

Willie Schreurs

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr Harry Rosenberg, for his enthusiastic support and guidance during the course of my studies, for that second chance, and for allowing me to look after his house while he was away on leave. Please note that lightning has not yet struck! Thanks also to all the members of, and visitors to, the Department of Biochemistry, J.C.S.M.R., who helped in so many ways with these studies. In particular Dr Simon Silver for his help with the silver-resistant mutants and for having such a delightfully apposite name, and to Dr Graeme Cox for explaining the uncs and for all those strains. John Gourley's technical assistance was also appreciated.

For advice on the PDP computer, and for his apparently never-ending fund of stories, I would like to thank Howard Kinns, and thanks to Tony Szeto for his help with the UNIVAC and the Processor.

My proof readers, soon-to-be Drs Anthony Braithwaite and Tony Szeto for their overnight service, and Robyn Cassidy for her while-you-wait service, deserve my grateful thanks.

To all my friends, for helping me to retain (mostly) the requisite blend of sanity and insanity and for making sure that biochemistry wasn't the only, or even the most important, thing I learned; among others, to Tony, Robyn, Roger, Elzbieta, Anthony, Anne, Chandra, Carla, Alan, and to Susan, my deepest gratitude, thanks, and love. You know, it was better than a slap in the belly with a wet fish!

And thanks to my family, for simply being there, behind it all.

Willie Schreurs

Easter, 1982

PREFACE

The work described in this thesis was carried out between June 1979 and March 1982 in the Department of Biochemistry, John Curtin School of Medical Research, The Australian National University, under the supervision of Dr Harry Rosenberg. Financial support between February 1978 and March 1982 was by an Australian National University Postgraduate Research Award.

The literature search for the Introduction (Chapter 1) to this thesis was concluded in July 1981. Relevant publications which appeared after this date have been included in later chapters.

Abbreviations commonly used:

A_{660}	absorbancy at 660nm
ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
ATPase	adenosine triphosphatase
CCCP	carbonyl cyanide m-chlorophenylhydrazone
DCCD	N,N'-dicyclohexylcarbodiimide
DMO	5,5-dimethyloxazolidine-2,4-dione
DNA	deoxyribonucleic acid
DNP	2,4-dinitrophenol
DTT	dithiothreitol
EDTA	ethylene diamine tetraacetic acid
enzyme II^{sugar}	enzyme II complex of the PTS which exhibits specificity towards the named sugar
ester-P	esterified phosphate
FCCP	carbonyl cyanide-p-trifluoromethoxy-phenylhydrazone
gluc	glucose
HQNO	2-n-heptyl-4-hydroxyquinoline-N-oxide
MES	2-(N-morpholino)ethanesulphonic acid
αMG	α-methyl-D-glucopyranoside
mtl	D-mannitol
$\Delta\tilde{\mu}_{H^+}$	electrochemical potential of hydrogen ions across the cell membrane
NADH	reduced nicotinamide adenine dinucleotide

NEM	N-ethylmaleimide
Δp	protonmotive force
ΔpH	pH difference across the cell membrane
P_i	inorganic phosphate
Pit	phosphate (inorganic) transport system
pmf	protonmotive force
$\Delta\psi$	electrical potential difference across the cell membrane
Pst	phosphate (specific) transport system
PTS	phosphoenolpyruvate:sugar phosphotransferase system
RNA	ribonucleic acid
succ	succinate
TBT	tributyltin
TCS	tetrachlorosalicylanilide
TEA	triethanolamine
TMG	methyl-β-D-thiogalactopyranoside
$TPMP^+$	triphenylmethylphosphonium ion
Tris	tris(hydroxymethyl)aminomethane

ABSTRACT

The hypothesis of energy coupling by anhydrous intramembrane protons, transported by a small, lipophilic carrier such as chloride, was tested by examining the effects of silver ions on the transport of phosphate and other metabolites. Silver ions inhibited phosphate uptake and exchange in Escherichia coli and caused efflux of accumulated phosphate from cells which remained intact and viable. Ag^+ also caused efflux of proline, glutamine, succinate, mannitol, and potassium ions. Proton efflux also occurred, followed by a "fallback" of the released protons. Ag^+-induced efflux was more pronounced in protonmotive force-coupled systems than in "phosphate-bond energy"-coupled systems. Thiols, N-ethylmaleimide, and carbonyl cyanide phenylhydrazone uncouplers prevented Ag^+-induced efflux. Neither uncouplers of oxidative phosphorylation nor inhibitors of the respiratory chain caused efflux of P_i, but tributyltin chloride, like Ag^+, caused efflux of phosphate and other metabolites. Although Ag^+ collapsed ΔpH and partially collapsed $\Delta\psi$, Ag^+-induced efflux was not mediated by its effect on the protonmotive force. Plasmid-bearing strains resistant to Ag^+ were studied, and these studies suggested that resistance may have been due to detoxification by reduction of Ag^+. Examination of strains carrying mutations affecting the proton-translocating ATPase showed that, in strains which were capable of phosphorylation of ADP to ATP, Ag^+-induced phosphate efflux rates were comparable to those in wild-type strains, whereas strains which were incapable of phosphorylation showed reduced rates of Ag^+-induced efflux of phosphate.

The results imply that Ag^+ interfered with energy coupling to an extent which was not achieved by uncouplers or respiratory chain inhibitors, abolishing the cell's ability to maintain concentration gradients of transported metabolites, and these observations were consistent with the above-mentioned hypothesis.

TABLE OF CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

EARLY STUDIES ON BACTERIAL TRANSPORT

> "In the course of our observations of Escherichia coli we have been led to the simple hypothesis that the cell wall of this organism is highly permeable to many molecules. Furthermore, it does not appear that any mechanism other than simple diffusion through a highly permeable membrane is involved in the transport of material across the membrane."
> (Roberts et al., 1955)

These words indicate the view, still widely held at the time, that the cytoplasm was exposed to the outside environment and that nutrients, waste products and ions could freely diffuse in and out of the cell as required.

There are a number of things, however, which this model cannot easily account for. One is the phenomenon of 'crypticity' - the inability of cells to metabolise a given substance even though they have the relevant enzyme systems. A good example of this is given by Doudoroff et al. (1949), who found a strain of Escherichia coli which could metabolise maltose but not glucose. Since maltose is broken down to glucose, which is then further metabolised, the above result can best be explained if these cells are permeable to maltose, but not to glucose. 'Crypticity' can also be demonstrated by showing that cell-free preparations can metabolise a compound which cannot be used by the intact cell (Barrett et al., 1953; Kogut and Podoski, 1953). Also, the accumulation of high concentrations inside bacterial cells of, for example, free amino acids (Gale, 1954) and phosphate (Mitchell, 1954), is difficult to explain if the bacterial cell membrane is highly permeable.

Soon it was generally accepted that the membrane had an important role to play in the transport of substances into, and out of, the cell, and was not just a leaky bag surrounding the protoplasm.

> "Since it now appears that the cells are neither

> completely permeable nor completely impermeable, the whole situation must be reconsidered."
> (Roberts et al., 1957)

Much descriptive work followed, establishing that there are a large number of systems which can be differentiated on the basis of their specificity and kinetic parameters. Work on the transport of lactose into E. coli and its metabolism led to the elucidation of the lac operon and the beginnings of a genetic basis for the study of transport systems (Cohen and Monod, 1957; Kepes and Cohen, 1962). This led to the idea of 'permeases', which were proteins, specific for the substrate being transported, and distinguished from the metabolic enzymes, e.g. the β-galactoside permease is genetically distinct from the enzyme β-galactosidase.

In studying the assimilation of amino acids Gale (1951,1954; Gale and Paine, 1951) found that the accumulation of amino acids requires energy supplied by the fermentation of glucose. This accumulation can be inhibited by sodium azide and 2,4-dinitrophenol at concentrations where fermentation is unaffected, suggesting that the energy does not necessarily come from glycolysis, but could be derived in some way from oxidative phosphorylation.

Progress in the field over the last twenty-five years has been aided by many advances in technique. For example, the use of membrane filters for the separation of cells from the medium (Atkinson and McFadden, 1956) was more reliable and more rapid than centrifugation. However, progress is due largely to two major advances, one technical, the other theoretical. The technical advance was the development of methods for producing membrane vesicles (Kaback, 1960; Kaback and Stadtman, 1966). These vesicles overcome many of the difficulties of working with intact cells, such as endogenous energy reserves, and metabolism of the transport

substrate after translocation, while retaining the essential characteristic of transport: the transfer of the substrate from one compartment to another across a membrane. Thus vesicles allow the study of phenomena associated with the plasma membrane, freed from any complications produced by events occurring in the cytoplasm. The other advance was the proposal by Mitchell (1961) of the chemiosmotic hypothesis.

THE CHEMIOSMOTIC HYPOTHESIS

Mitchell's early studies on the exchange and uptake of inorganic phosphate through the plasma membrane led him to consider mechanisms for this transport (Mitchell, 1954,1972). One such mechanism, for example, involves an enzyme which spans the membrane and transports phosphate by covalent attachment on one side of the membrane and release on the other (Mitchell, 1957).

The proposal of group translocation followed from enzymological ideas of group transfer, with the addition of a vectorial component (Mitchell and Moyle, 1958). Thus, if an enzyme is embedded in a membrane separating two compartments, substrate can interact with the enzyme on one side, and products can leave from the other (see the description of the phosphoenolpyruvate: sugar phosphotransferase system in the next section).

The two ideas that led to the proposal of the chemiosmotic hypothesis were: (i) that electron transfer in the respiratory chain or in the photosystems of photosynthetic organisms leads to a separation of hydrogen and hydroxyl ions across the membrane , and (ii) that there is an anisotropic ATPase associated with the membrane that can make use of this gradient of hydrogen (or hydroxyl) ions to synthesise ATP from ADP and inorganic phosphate

(Mitchell, 1961, 1961a). Thus respiration (or photosynthesis) and phosphorylation of ATP can be chemiosmotically coupled via the gradient of hydrogen ions across the membrane. This gradient of hydrogen ions gives rise to the electrochemical potential of hydrogen ions across the membrane, $\Delta\tilde{\mu}_{H}+$, which has two components. These are ΔpH, the pH difference across the membrane, and $\Delta\psi$, the electrical potential difference across the membrane caused by the separation of charge. $\Delta\tilde{\mu}_{H}+$, ΔpH and $\Delta\psi$ are related in the following way (Rosen and Kashket, 1978):

$$\Delta\tilde{\mu}_{H}+ = F\Delta\psi - 2.3RT\Delta pH$$

where F is the Faraday, R is the gas constant, and T is the temperature in kelvins.

Mitchell, by analogy with the electronmotive force, emf, refers to the electrochemical potential difference of protons across the membrane as the protonmotive force, pmf, or p, as follows (Mitchell, 1966):

$$\Delta p = \Delta\tilde{\mu}_{H}+/F = \Delta\psi - 2.3RT\Delta pH/F$$

$$= \Delta\psi - Z\Delta pH$$

MODELS OF ENERGY COUPLING

The simplest form of transport across a bacterial cell membrane is facilitated diffusion, where the substrate moves down its concentration gradient, the equilibration being mediated by a porter protein in the membrane. This type of system is not coupled to metabolic energy. In E. coli, uptake of glycerol occurs by facilitated diffusion (Richey and Lin, 1972).

Most systems, however, accumulate the substrate at concentrations considerably higher than those in the external medium. This is active transport, and energy is required for this process. One way of achieving this is by group translocation, as mentioned in the previous section. The phosphoenolpyruvate:sugar phosphotransferase system (Kundig et al., 1964; Kaback, 1970; Saier, 1977) is probably the best known system of this type. The substrate is modified during transport (e.g. in the phosphoenolpyruvate:sugar phosphotransferase system the sugar is phosphorylated) and thus the compound that appears in the cytoplasm is not the same as the one that disappears from the external medium, ensuring that it cannot leave via the same substrate-specific carrier protein. Note that these sugars are phosphorylated as they enter the cell, whereas glycerol is phosphorylated by glycerol kinase after it enters the cell via the glycerol facilitator (Hayashi and Lin, 1965). The net result, however, is the same since in neither case is a gradient of the transported substance established.

In the 'true' active transport systems, the substrate is unaltered during the translocation step and it is these systems which are most interesting to study with respect to energy coupling.

Membrane vesicles have been very useful tools in the study of energy coupling to active transport, but the initial results were somewhat misleading. These vesicles cannot use glucose to drive active transport, but require the addition of an oxidisable energy source. Different species of bacteria differ in the energy source they can best use (Rosen and Kashket, 1978). In E. coli D-(-)-lactate is quite effective in energising active transport (Kaback and Milner, 1970). There is no relationship between the efficacy of electron donors at stimulating transport and their rate

of oxidation (Kaback and Barnes, 1971). ATP added outside the vesicles is unable to support transport (Hamilton, 1977). These observations led to the hypothesis that active transport is obligatorily linked to the respiratory chain (Kaback and Barnes, 1971) and that the site of coupling, i.e. the location of the transport proteins, is between D-lactate dehydrogenase and cytochrome b_1 (Kaback, 1972). The carrier can exist in two forms, with -SH groups being alternately reduced and oxidised by the electron flow in the respiratory chain. The oxidised form binds substrate from the external medium. Upon reduction of the carrier, the binding site faces the inside of the cell, its affinity for the substrate is decreased, and the substrate is released (Boos, 1974; Rosen and Kashket, 1978). The action of uncouplers of oxidative phosphorylation (Barnes and Kaback, 1971) and mutants defective in active transport but not respiration (Hong and Kaback, 1972), were explained by proposing that the carriers are components of shunts from the main portion of the electron transfer chain. In this way the carriers could be inhibited by uncouplers or affected by mutations, thus inhibiting active transport, without any effect on respiration.

However, this model cannot explain energy coupling in anaerobic organisms such as Streptococcus faecalis and Streptococcus lactis which do not have a respiratory chain. Also, ATPase-deficient mutants were isolated which cannot accumulate amino acids even though D-lactate oxidation is unimpaired (Simoni and Shallenberger, 1972), and vesicles pre-loaded with ATP can support the uptake of serine (van Thienen and Postma, 1973). These observations, and the mounting evidence that membrane vesicles could generate a membrane potential (Hirata et al., 1973; Altendorf et al., 1975) led to this respiratory chain model being abandoned in favour of chemiosmotic

coupling (Schuldiner and Kaback, 1975; Ramos et al., 1976). This hypothesis was useful, nevertheless, since it had promoted a great deal of work on active transport, both in vesicles and intact cells, and had shown that there were many active transport systems which did not depend on the hydrolysis of ATP or other high-energy phosphate compounds (Kaback and Milner, 1970; Rosen and Kashket, 1978).

The chemiosmotic hypothesis evolved from studies on microbial active transport (Mitchell, 1972) and was applied to the coupling of phosphorylation to oxidation and photosynthesis (Mitchell, 1961, 1961a, 1966), where the emphasis remained for some time. The ability of chemiosmotic ideas to explain translocation of substrates across membranes was noted (Mitchell, 1967) and applied to transport and energy transformations in bacteria (Pavlasova and Harold, 1969; Harold, 1972). Two classes of chemiosmotically coupled transport are recognised, primary and secondary (Mitchell,1967; Harold, 1977a).

Primary translocations are those in which movement is linked to chemical processes (e.g. breaking and making of covalent bonds) carried out by enzymes. It is in these reactions that 'chemical bond energy' is transferred to osmotic potential: they are the 'chemiosmotic reactions'. Examples of primary transport systems are the proton-translocating ATPases, respiratory chains, photosynthetic systems, and bacteriorhodopsin (Harold, 1977). Thus, oxidative phosphorylation can be said to occur by the chemiosmotic coupling of two primary transport systems, the respiratory chain and the proton-translocating ATPase.

Secondary translocations are those in which the movements are not directly linked to a chemical reaction but to an electrical potential, a concentration gradient, or both. The catalysts for these reactions are known as translocators, or porters, to distinguish them from the enzymes of primary transport systems (Mitchell, 1967). Secondary transport systems can be:

(i) uniports, which catalyse the transport of the substrate across the cell membrane in response to its own concentration gradient if it is uncharged (facilitated diffusion), or the membrane potential if the substrate is charged;

(ii) symports, where the transport of the substrate is coupled to the movement of another substrate in the same direction, the second substrate moving down its potential gradient; and

(iii) antiports, where the transport of the substrate is coupled to the movement of a second substrate moving in the opposite direction down its potential gradient.

Symports and antiports can be electrogenic or neutral, depending upon whether or not there is a net transfer of charge across the membrane during the transport step (Mitchell, 1967; Harold, 1977; Rosen and Kashket, 1978).

These secondary transport systems can make use of the proton gradient that is produced by the primary systems of the respiratory chain and photosynthesis. Under anaerobic conditions, or in organisms like S. faecalis which lack a respiratory chain, the proton gradient is established by the ATPase complex, which hydrolyses ATP (produced during the fermentation of glucose) with the simultaneous extrusion of protons from the cell. When the protons move back into the cell down their electrochemical gradient, they can be coupled to the various secondary transport systems to do useful work.

Many such coupled systems are now recognised: e.g. electrogenic symport of sugars and amino acids with protons, electroneutral symport of anionic compounds with protons, and antiport of protons with cations such as sodium and calcium to remove them from the cell (Simoni and Postma, 1975; Harold, 1977a; Rosen and Kashket, 1978).

There is yet another form of energy coupling to active transport, which cannot be seen if vesicles are used, although recent developments in the use of vesicles may soon make it possible to do so (Hugenholtz et al., 1981). This form of energy coupling occurs in transport systems which are sensitive to osmotic shock. When cells are subjected to osmotic shock, they lose proteins from the periplasm, the space between the plasma membrane and the cell wall (Neu and Heppel, 1965). Shock-sensitive systems have an associated periplasmic binding protein, which is lost during the shock procedure, whereas shock-resistant systems do not have a binding protein and usually retain their activity in membrane vesicles, where their energetics can be studied without the complications produced by the endogenous energy reserves in intact cells.

As shock-sensitive systems only function in intact cells, it is necessary to eliminate these endogenous energy reserves. Koch (1971) achieved this by forcing the cells to transport α-methylglucoside in the presence of azide, which stimulates this transport (Kepes and Cohen, 1962), until the reserves are eliminated. Berger (1973) used a modification of this procedure, incubating the cells with 2,4-dinitrophenol, uncoupling oxidative phosphorylation, and forcing the cells to use up their endogenous reserves in an attempt to maintain an energised membrane. After this treatment, the cells are dependent on externally added energy sources and the ability of

these to drive transport can be examined. By comparing the effects of D-lactate and glucose in normal cells and ATPase-deficient mutants, and by the use of uncouplers, Berger (1973; Berger and Heppel, 1974) showed that glutamine transport, a shock-sensitive system, is energised by phosphate-bond energy; but proline transport, which is shock-resistant, can be driven by the protonmotive force. This phosphate-bond energy could be provided by acetylphosphate rather than ATP (Hong et al., 1979).

Other work on these systems indicates that phosphate-bond energy may be necessary, but not sufficient, for uptake to occur through shock-sensitive systems (Plate et al., 1974; Lieberman and Hong, 1976; Plate, 1979). Treatments which reduce membrane potential without affecting ATP levels (e.g. colicin K or valinomycin-plus-potassium added to ATPase-deficient cells), inhibit the uptake of glutamine (Plate et al., 1974; Plate, 1979) and uptake in other shock-sensitive systems (Lieberman and Hong, 1976). Results indicate that protonmotive force is not the immediate energy source, so it is possible that an energised membrane is necessary to maintain a functional system while phosphate-bond energy is the driving force for the actual translocation (Silhavy et al., 1978). This appears to be the converse of a recently described model for potassium transport in S. faecalis which requires both ATP and the protonmotive force. In this case, it is proposed that the porter is activated by ATP and energised by the pmf (Bakker and Harold, 1980).

ENERGY TRANSMISSION: LOCALISED OR DELOCALISED PROTON GRADIENTS?

It is now commonly accepted that the chemiosmotic hypothesis provides a logical basis for the description of energy coupling to oxidative phosphorylation (Boyer *et al.*, 1977) and bacterial transport (Harold, 1977; Rosen and Kashket, 1978).

However, the molecular mechanism by which the electrochemical proton gradient links electron transfer to phosphorylation and transport is still uncertain. Most of the current discussion is centred on two differing views of the proton gradient. The chemiosmotic view first proposed by Mitchell (1961) is that the protons are delocalised *across* the membrane as a gradient between the aqueous phases on either side. A 'localised proton' model, proposed by Williams (1961) and subsequently modified to include the lipid membrane (Williams, 1962), postulates that the protons produced by electron transfer remain *within* the membrane. There are many versions of the localised proton model, but all share the view that the electrochemical proton gradient exists within the membrane or at the membrane/water interface (Wikstrom, 1981).

Since I have already discussed the chemiosmotic hypothesis, this section will be largely concerned with a consideration of the evidence which suggests that a localised proton model may be feasible.

Major support for the chemiosmotic hypothesis comes from the ability to measure membrane potentials and pH gradients, both in whole cells and in vesicles (Rottenberg, 1975). However, it has been observed in many cases that the measured protonmotive force is too small to sustain phosphorylation (Boyer *et al.*, 1977; Williams, 1978). This may be due to technical difficulties such that the

probes are not measuring the full extent of the membrane potential or pH gradient, although in the case of membrane potential direct measurements with microelectrodes in giant cells of E. coli have confirmed previous results (Felle et al., 1980). To explain the inability of the pmf to account for solute accumulation at higher external pH values (at an external pH of 5.5 the pmf is sufficient for accumulation), Kaback (1976) proposed that the ratio of H^+ translocated per solute molecule varies with the external pH, being 1 at pH_e 5.5, and rising to 2 at pH_e 7.5. Under limiting conditions, with alanine as the limiting nutrient and the sole source of carbon, Collins et al. (1976) showed a selection in favour of higher H^+/alanine uptake ratios. However, an explanation of the above-mentioned shortfall by proposing that there is a more direct link between electron transfer and ATP synthesis than that of a proton current through the aqueous phases on either side of the membrane cannot be ruled out (Boyer et al., 1977).

Experiments in chloroplasts on the relationship between photophosphorylation and internal acidification as a function of illumination time, and differing sensitivities of ATP synthesis to uncouplers, have shown that photophosphorylation can start long before there is any significant acidification of the inner aqueous phase of the lamellar system. This has led Ort (1978; Ort et al., 1976) to suggest that protons produced by electron transfer may be used directly for phosphorylation without entering the bulk of the inner aqueous phase. The imposition of an external electrical field on thylakoid vesicles gives rise to a transmembrane potential difference without a pH gradient, and ATP synthesis is possible under these conditions (Witt et al., 1976). Witt suggested that any protons driven by this field may be taken, not from the inner aqueous phase but from the membrane near the inner surface.

Boguslavsky et al. (1975) performed an interesting series of experiments in two phase water/octane systems. If the soluble F_1 component of the mitochondrial ATPase is added to the aqueous phase, the presence of ATP causes the appearance of an electrical potential difference across the water/octane interface, positive in the octane phase. There is an absolute requirement for a lipid-soluble proton acceptor such as 2,4-dinitrophenol in the octane phase. Bacteriorhodopsin in the presence of light also forms an electrical potential positive in the octane phase, provided there is a lipid-soluble proton acceptor in the organic phase. Boguslavsky et al. (1975) deduced that the F_1 ATPase, at the water/octane interface, injects protons into the octane phase during ATP hydrolysis.

NADH oxidation by ferricyanide in submitochondrial particles at the water/octane interface leads to the appearance of a potential difference, positive in the octane phase, provided again that the lipid-soluble proton acceptor is present. Addition of F_1 ATPase causes this potential to reverse in sign, and there is some evidence of ATP synthesis (Yaguzhinsky et al., 1976). The authors conclude that "the energy of proton solvation can be used for ATP synthesis".

Mitchell (1977) has stated that these results are not inconsistent with the predictions of the chemiosmotic hypothesis, but they can more easily be seen as supporting the idea that protons diffuse within the membrane.

If protons are to diffuse within the lipophilic region of the membrane a proton carrier must be postulated, since it is highly unlikely that the free ion will be able to move in this environment. Note that in the above experiments, a potential is not generated unless a lipid-soluble proton acceptor is present in the octane phase. The natural proton acceptor must be anionic, so that it can

bind the proton, lipophilic once it has accepted the proton, and small, to allow rapid diffusion. Robertson and Boardman (1975) suggested that under some natural conditions this small lipophilic anion may be chloride, so that hydrogen chloride becomes the proton carrier. As a mechanism for injecting protons into the membrane they propose that ubiquinone, accepting electrons from the electron transport chain, becomes negatively charged at its polar group and moves towards the polar portion of the lipid bilayer. At the surface of the membrane it accepts hydrogen ions, thereby losing charge and sinking back into the lipophilic region. Losing electrons to the next member of the respiratory chain, it releases the protons. These protons combine with the chloride ion which has been held in the vicinity by the non-haem Fe^{3+} near the cytochrome. When the Fe^{3+} is reduced, the chloride ion is released to interact with the proton, forming hydrogen chloride. This is described as the 'bobbing-up-and-down' hypothesis.

Robertson and Thompson (1977) explained an electrically silent $^{36}Cl^-$ flux across artificial membranes which is 1000 times larger than that calculated from electrical parameters by suggesting that it is due to the permeation of $H^{36}Cl$. Partitioning into the membrane as HCl from H^+ and Cl^- would be a very rare event, so they postulate a mechanism involving the 'bobbing up and down' of phosphatidylcholine for introducing HCl into the lipophilic region, a process termed 'activated partitioning'.

The uncoupling effects of oligomycin require chloride ions, and are not shown when chloride is replaced by bromide or nitrate ions (Ariel and Avi-Dor, 1973). In chloride-containing media, trialkyltins act as uncouplers at concentrations which produce a chloride-hydroxyl exchange, and in KCl the rates of oxidation in

rat-liver mitochondria are two to three times higher than they are in sucrose (Stockdale et al., 1970). This and other evidence indicates that chloride ions may have an important role to play in the cell membrane.

Recently, submitochondrial particles have been prepared which are 'open' fragments of the inner mitochondrial membrane, i.e. they are not sealed vesicles (Storey et al., 1980; Storey and Lee, 1981). These fragments show an energy-linked decrease in quinacrine fluorescence, which is enhanced by thiocyanate. This is interpreted as showing that these fragments, in which transmembrane ion gradients cannot be sustained, have the capacity for energy coupling and that therefore the protons involved in energy coupling must be localised in the membrane.

Wikstrom (1981) advises caution, however, asking for more rigorous checks on whether or not the fragments are really 'open', and stating that the only real proof would be to demonstrate oxidative phosphorylation at a P/O ratio comparable to that in intact mitochondria.

In the light of all the evidence in favour of both the chemiosmotic delocalised proton gradient and the intramembrane localised proton model, the comment made by Harold (1972) still seems appropriate:

> "There is much to recommend a constructive synthesis, which includes chemical intermediates on the direct path to ATP, and ΔpH and $\Delta\psi$ as the energy donors for transport."

INORGANIC PHOSPHATE TRANSPORT IN ESCHERICHIA COLI

In Escherichia coli there are two major systems for the uptake of inorganic phosphate (P_i) (Willsky et al., 1973; Rosenberg et al., 1977). These systems have been designated Pit (for 'P_i transport') and Pst (for 'phosphate specific transport'). Uptake of P_i has also been reported through two other systems (Silver, 1978): the GlpT system (Cozzarelli et al., 1968), which is inducible by glycerol phosphate and glycerol (Hayashi and Lin, 1965); and the Uhp system for the transport of hexose phosphates. They are not deemed to be important since neither system can transport enough P_i to permit growth (Sprague et al., 1975).

The Pit and Pst systems can be distinguished from each other in a number of ways. They are genetically distinct, the Pit system being coded by a single known gene, pit (Sprague et al., 1975), at minute 76 on the E. coli linkage map (Bachman et al., 1976). The Pst system requires the phoT gene (minute 82) for activity (Willsky et al., 1973; Rosenberg et al., 1977), and the phoS gene, which is closely linked to phoT, codes for the periplasmic binding protein (Gerdes and Rosenberg, 1974; Willsky and Malamy, 1974, 1976). A third gene, pst, is also involved but its function remains unknown (Sprague et al., 1975).

The Pit system is constitutive (Rosenberg et al., 1977) but, although the Pst system does operate in cells grown at high (1mM or greater) concentrations of P_i, its rate of uptake increases about 5-fold during phosphate deprivation (Rosenberg et al., 1977). The concentration of phosphate-binding protein increases about 100-fold under these conditions (Willsky and Malamy, 1976). This binding protein has an affinity constant for P_i of about 10^{-6}M (Gerdes and Rosenberg, 1974). Estimations of molecular weight vary from 42,000

(Medveczky and Rosenberg, 1970) to 32,000 (calculated from amino acid composition in Gerdes and Rosenberg, 1974). With osmotic shock, or in the formation of spheroplasts, this binding protein is lost and transport of phosphate through the Pst system is severely inhibited (Medveczky and Rosenberg, 1970; Gerdes et al., 1977). Uptake is restored in spheroplasts by the addition of purified binding protein (Gerdes et al., 1977). The Pit system is fully functional in spheroplasts (Rosenberg et al., 1977) and in vesicles (Konings and Rosenberg, 1978).

The maximal velocity values are the same in both systems, at 60μmol per min. per gram (dry weight of cells). A phoS mutation reduces the maximal velocity of the Pst system about 15-fold. The affinity for phosphate (K_m) is 25μM in the Pit system and 0.2μM in Pst. Loss of the binding protein by mutation has no effect on the affinity in the Pst system (Rosenberg et al., 1977).

The Pit system catalyses a rapid exchange of intracellular and extracellular P_i (Medveczky and Rosenberg, 1971), which is repressed in glucose-grown cells (Rosenberg et al., 1977).

The Pit and Pst systems have the characteristics of shock-resistant and shock-sensitive transport systems (Berger and Heppel, 1974) respectively (Rosenberg et al., 1977). Studies on energy coupling in these systems show that the Pit system is coupled to the protonmotive force and the Pst system is energised by phosphate-bond energy (Rosenberg et al., 1977, 1979), as expected if they conform to the scheme proposed by Berger and Heppel (1974).

Uptake of P_i has an absolute requirement for the presence of K^+, and *vice versa* (Russell and Rosenberg, 1979). The link between P_i and K^+ transport is permissive, i.e. there is no direct link between any pair of potassium and phosphate transport systems (Russell and Rosenberg, 1980). These results were taken to indicate that P_i enters the cell in symport with protons and that the role of potassium ions is the control of cytoplasmic pH by antiport with protons as suggested by Harold (1977a) (Russell and Rosenberg, 1980).

THE EFFECT OF SILVER IONS ON THE RESPIRATORY CHAIN AND ON TRANSPORT

As long ago as 1937 it was noted that silver ions inhibit the respiratory chain of *E. coli* (Yudkin, 1937). Uncoupler-like effects were observed by Chappell and Greville (1954), using low concentrations (5-10μM) of $AgNO_3$.

More recently, Rainnie and Bragg (1971) noticed a loss of silver ions from uncoated oxygen electrodes into the surrounding medium and found that the respiration of *E. coli* was inhibited by Ag^+, after a brief initial stimulation. This led to a more detailed study in an attempt to find the site or sites of inhibition by silver ions in the respiratory chain (Bragg and Rainnie, 1974). They found two levels of inhibition by Ag^+ of the respiratory chain. The more sensitive site is located between the b-cytrochromes and cytochrome d. Inhibition at this site is not completely abolished in the presence of reduced glutathione, which prevents inhibition by silver ions at the other, less sensitive site. This site is near the NADH and succinate dehydrogenases, before 'flavoprotein' (Bragg and Rainnie, 1974), and is probably that which is inhibited by zinc ions

(Kasahara and Anraku, 1972). Sulphydryl groups are implicated at this site (Kasahara and Anraku, 1972; Bragg and Rainnie, 1974).

Juan *et al.* (1979) found that purified NADP-linked glutamate dehydrogenase from *Trypanosoma cruzi* is strongly inhibited by Ag^+ and that this inhibition is reversed by reduced glutathione.

Silver ions are potent inhibitors of the transport of succinate into *E. coli* membrane vesicles (Rayman *et al.*, 1972). There is no inhibition by N-ethylmaleimide in this system, although other thiol reagents, like p-chloromercuribenzoate do inhibit uptake.

AIMS

In view of the observed inhibitory effects of silver ions on the respiratory chain and on active transport, it was of interest to examine the consequences of the treatment with Ag^+ of the transport systems for inorganic phosphate in *E. coli* in an attempt both to elucidate the mechanism of action of Ag^+ and to use silver ions as a tool to explore the nature of the coupling of energy to active transport.

This would involve:

i) an examination of the effects of silver ions on the transport process and on the retention of the transported substances,

ii) testing of other inhibitors, such as uncouplers of oxidative phosphorylation, inhibitors of the respiratory chain, heavy metal ions other than Ag^+, thiol-reactive reagents, and tributyltin, examining their effects on transport and on Ag^+ action,

iii) testing the effects of Ag^+ on the components of the protonmotive force, i.e. membrane potential and pH gradient, and

iv) examination of cells which are resistant in some way to the

effects of silver ions, to see whether elucidation of the mechanism of resistance could give any information on the mode of Ag^+ action.

CHAPTER 2

GENERAL MATERIALS AND METHODS

A. CHEMICALS

All chemicals were of the highest purity commercially available.

Carbonylcyanide m-chlorophenylhydrazone (CCCP), N-ethylmaleimide (NEM), polyvinyl polypyrrolidone, valinomycin, and kanamycin were obtained from Sigma, St. Louis, Missouri, USA. Tetrachlorosalicylanilide (TCS) was a gift from Dr F.M. Harold of the Division of Molecular and Cellular Biology, National Jewish Hospital and Research Center, Denver, Colorado, USA. Carbonylcyanide-p-trifluoromethoxyphenylhydrazone (FCCP) was a gift from Dr P.G. Heytler of E.I Du Pont de Nemours and Co., Wilmington, Delaware, USA.

Tributyltin chloride (TBT) was from Tokyo Kasei, Tokyo, Japan. When necessary it was converted to tributyltin acetate by passing through Dowex 1 in the acetate form.

Ribonuclease was from Worthington Biochemical Corporation, New Jersey. Deoxyribonuclease I was from Calbiochem-Behring Corporation, La Jolla, California. Lysozyme was from Sigma.

Triphenylphosphine and triethanolamine (TEA) were from Fluka A.G., Buchs, Switzerland.

B. RADIOISOTOPES

Carrier-free [^{32}P]orthophosphate (8GBq/mmol) was initially obtained from the Australian Atomic Energy Commission, Lucas Heights, New South Wales, and later from The Radiochemical Centre, Amersham, U.K.

L-[U-^{14}C]proline (10GBq/mmol), L-[U-^{14}C]glutamine (1.8GBq/mmol), [2,3-^{14}C]succinic acid (0.8GBq/mmol), D-[1-^{14}C]mannitol (2GBq/mmol), [^{110m}Ag]$AgNO_3$ (3.4GBq/mmol), [^{3}H]H_2O (185GBq/ml), [^{3}H]methyl iodide (92GBq/mmol), [^{14}C]acetic acid (2GBq/mmol), 5,5-dimethyl [2-^{14}C]oxazolidine-2,4-dione (DMO) (2GBq/mmol), and [^{14}C]methylamine hydrochloride (2GBq/mmol) were all obtained from The Radiochemical Centre.

[^{14}C]methoxy inulin (3GBq/mmol) was from New England Nuclear, Boston, Massachusetts.

C. BACTERIAL STRAINS

Details of the bacterial strains used are given in Table 2.1.

D. MEDIA, BUFFERS, AND SOLUTIONS

All pH values were measured at 37°C.

1. Growth Medium

Cells were grown in medium which contained:

K_2HPO_4	61mM
NaH_2PO_4	39mM
$(NH_4)_2SO_4$	15mM
$MgSO_4$	1mM

and was sterilised by autoclaving. The pH after autoclaving was 6.8-7.0. This medium was supplemented with either glucose (20mM) or succinate (20mM). Thiamine (3μM), 2,3-dihydroxybenzoate (10μM), arginine (1mM), proline (1.5mM), histidine (0.3mM), tryptophan (0.4mM), uracil (0.4mM), or acid-hydrolysed casein (Difco, Detroit, Michigan, USA) (0.1%) were added where required as sterile solutions to the growth medium.

Table 2.1 Strains of Escherichia coli and plasmids used.

Strain	Relevant genotype/phenotype[a]	Source/reference
AN249[b]	uncA401, argH, entA	Gift from G. Cox. (Cox et al., 1973)
AN259	argH, entA	Gift from G.Cox. (Butlin et al., 1973)
AN346	F^-, ilvC, argH, pyrE, entA	Gift from G. Cox. (Gibson et al., 1977)
AN710	phoT101, argH, entA	Rosenberg et al., 1977
AN719	uncB402, argH, pyrE, entA	Gift from G. Cox. (Gibson et al., 1977a)
AN781	uncC424, argH, pyrE, entA	Gift from G. Cox. (Gibson et al., 1977a)
AN845	uncE408, argH, pyrE, entA, recA	Gift from G. Cox. (Cox et al., 1982)
AN955	uncE410, argH, pyrE, entA	Gift from G. Cox. (Downie et al., 1979a)
AN1088	pit, argH, entA	Russell and Rosenberg, 1980
AN1156	uncB454, argH, pyrE, entA	Gift from G. Cox. (Downie et al., 1981)

Table 2.1 (cont.)

Strain	Relevant genotype/phenotype[a]	Source/reference
AN1326	*uncB434*, *argH*, *pyrE*, *entA*	Gift from G. Cox.
AN1332	F'(pAN10), *uncE408*, *argH*, *pyrE*, *entA*, *recA*	Gift from G. Cox. (Cox *et al.*, 1982)
AN1419	*uncF469*, *argH*, *pyrE*, *entA*	Gift from G. Cox. (Downie *et al.*, 1981)
AN1510	*uncC473*, *argH*, *pyrE*, *entA*	Gift from G. Cox.
AN1515	*uncF476*, *argH*, *pyrE*, *entA*	Gift from G. Cox. (Downie *et al.*, 1981)
AN1871	(pAN128), *unc-416::MuB*$^{+}$*EFHAGDC*, *argH*, *pyrE*, *entA*, *recA*	Gift from G. Cox. (Cox *et al.*, 1982)
E15	*pit*, *phoA8*	Gift from M. Schlesinger. (Derivative of K10 Cavalli, Fan *et al.*, 1966)
J62.2	*lac28*, *his51*, *trp30*, *proC23*	Gift from R. Hedges. (Bachman, 1972)

Table 2.1 (cont.)

Strain	Relevant genotype/phenotype[a]	Source/reference
J62.2(pCS35)	(pCS35), *lac28*, *his51*, *trp30*, *proC23*	Gift from R. Hedges. (see footnote c)
HR16	(pCS35), *phoT101*, *argH*, *entA*	Isolated after conjugation between J62.2(pCS35) and AN710, and selected for resistance to kanamycin and silver.
HR17	(pCS35), *pit*, *argH*, *entA*	Isolated after conjugation between J62.2(pCS35) and AN1088, and selected for resistance to kanamycin and silver.
Plasmids		
pAN10	*uncE408*, *pyrE*$^+$, *ilvC*$^+$, *argH*$^+$	Downie *et al.*, 1979a
pAN128	Cm^r, Tc^s, *uncB*$^+$*E408F*$^+$*H*$^+$*A*$^+$*G*$^+$*D*$^+$*C*$^+$	Cox *et al.*, 1982
pCS35	Km^r, Ag^r	See footnote c

Table 2.1 (cont.)

[a] The gene designations follow those of Bachman *et al.* (1976) and the plasmid nomenclature is that of Novick *et al.* (1976).

[b] The "AN" and "HR" strains in this table were derived from strain AN248, which has the following genotype: *entA403*, *ilvC*, *xyl5*, *rpsL109*, λ^-, *supE44*.

[c] This silver-resistance plasmid arose in Citrobacter strain S35 and was mobilised using the plasmid R1 *drd16*, Km^r, and the recipient strain contained a 94 Mdal plasmid with Ag^r and Km^r as markers (R. W. Hedges; S. Silver, personal communication). The recipient strain is designated J62.2(pCS35), and the 94Mdal plasmid is called pCS35.

2. Solid Growth Media

'Meat infusion agar' contained:

agar	2.0%
tryptone	1.0%
beef extract	1.0%
NaCl	0.5%
yeast extract	0.3%
glucose	2mM

All other media contained 2% agar (Difco) in growth medium (Section D.1), with growth supplements and selective agents added as required.

3. Phosphate-free Medium

This medium, used to deplete cells of P_i, and for P_i uptake measurement, contained:

Triethanolamine (TEA)	50mM
KCl	15mM
$(NH_4)_2SO_4$	10mM
$MgSO_4$	1mM

The pH was adjusted to 6.5 with citric acid. In some cases, citric acid was replaced with HCl, since citrate was found to interfere with the assay of inorganic phosphate.

4. Chloride-free Phosphate-free Medium

This medium was used to replace the standard phosphate-free medium in all experiments where silver ions were involved, unless otherwise stated. It was the same as the phosphate-free buffer (section D.3) except that K_2SO_4 (7.5mM) replaced KCl, and H_2SO_4

replaced HCl. In some cases, where mentioned in the text, the uptake buffer used contained 50mM 2-(N-morpholino)ethanesulphonic acid (MES), adjusted to pH 6.5 with TEA base, but otherwise the same as that described above.

5. Potassium-free Medium

This medium was used for measurements of potassium movements. It contained:

TEA	50mM
Li_2SO_4	20mM
$MgSO_4$	1mM

The pH was adjusted to 6.5 with citric acid.

6. Lightly Buffered Medium

This medium was used for measurements of proton movements. It contained:

TEA	5mM
Li_2SO_4	50mM
$MgSO_4$	1mM

The pH was adjusted as required with H_2SO_4.

7. 'Low Phosphate' Medium

This medium was used when defined chloride-free medium containing phosphate was required, for the measurement of uptake of ^{14}C-substrates (Chapter 5), for flow dialysis experiments (Chapter 6), and for measurement of cell growth (Chapter 7). It was the chloride-free phosphate-free medium (Section D.4) supplemented with 1mM P_i.

8. Wash Solutions

This solution was used for washing the filtration membranes during uptake measurements. It contained:

TEA	10mM
KCl	100mM
$(NH_4)_2SO_4$	10mM
$MgSO_4$	1mM

The pH was adjusted to 6.5 with HCl.

Chloride-free wash solution, used for $^{110}Ag^+$ uptake studies, was the same as that described above, except that KCl was replaced by K_2SO_4 (50mM) and HCl was replaced by H_2SO_4.

9. Scintillant

The scintillant used for counting ^3H and ^{14}C contained:

2,5-diphenyloxazole (PPO)	0.5%
xylene/Triton X114	2:1 by volume

E. GROWTH OF CELLS AND PREPARATION FOR UPTAKE STUDIES

Cells were grown to stationary phase overnight (16 hours) by shaking at 37°C in Erlenmeyer flasks of appropriate size filled to 25-30% of capacity.

Cell densities were measured on a Gilford 300 spectrophotometer at 660nm and converted to mg dry weight of cells/ml, using the experimentally derived factor: A_{660} of 1.0 = 0.43mg dry weight/ml.

Cells were harvested by centrifugation, washed twice with the appropriate uptake medium, and suspended in the same medium at A_{660} of 0.35. Except where otherwise stated, cells were then supplemented with 5mM succinate or 1mM glucose, plus other growth supplements as required, and shaken for 1-1.5 hours at 37°C to deplete the cells of P_i and to induce maximal uptake in the Pst system.

After washing the cells twice in uptake medium, they were suspended at A_{660} of 0.35 and shaken for 1 hour at 37°C with 1mM 2,4-dinitrophenol (DNP) and without an energy source.

The cells were then washed three times in uptake medium, suspended at A_{660} of 0.35, and stored at 4°C for up to 4 hours until required for uptake assays.

F. MEASUREMENT OF UPTAKE

Cell suspensions were shaken at 37°C in a water bath for 5 minutes, in the presence of a carbon source (20mM) unless otherwise stated. Uptake was initiated by adding radioactively labelled substrate to the required concentration and specific radioactivity. Samples (0.5ml) of cell suspension were withdrawn at the appropriate times and filtered through cellulose nitrate membranes (pore size 0.45 μm), which were then washed with two 2ml portions of the wash solution. The filters were removed from the filtration apparatus, and membranes with ^{32}P- or ^{110}Ag-labelled cells were pressed upside-down onto aluminium planchets, dried, and counted for radioactivity in a Nuclear Chicago gas-flow counter. Membranes containing ^{14}C-labelled cells were dried and placed into scintillation vials. Scintillant was added, and radioactivity was determined in a Packard Tri-Carb 460CD liquid scintillation spectrometer, with facilities for quench and chemiluminescence correction and for calculation of

dpm values for a single isotope, or for two isotopes counted simultaneously, according to pre-set programs and calibration.

Standards for specific radioactivity and blanks (filtrations without cells) were counted at the same time. Uptake was expressed as nmol substrate/mg dry weight of cells.

G. EDTA TREATMENT OF CELLS

Cells were treated with ethylenediamine tetra-acetic acid (EDTA), to increase their permeability, as described by Lieve (1968).

In the actual procedure, cells prepared for uptake were washed twice with 50mM TEA-H_2SO_4 (pH 8.0) and suspended at A_{660} of about 5.0 in the same buffer. The cell suspension was gently swirled at 37°C for 5 minutes, EDTA was added at a final concentration of 1mM, and swirling was continued for a further 2 minutes. The cell suspension was then centrifuged, washed three times, and suspended in the appropriate uptake buffer. This suspension was stored at 4°C in the absence of a carbon source to prevent the repair of the permeability barrier, and were used within 2 hours.

H. PREPARATION OF SPHEROPLASTS

Cells were grown and prepared in the usual way, and spheroplasts were prepared essentially as described by Gerdes *et al.* (1977). After centrifuging and washing twice with 50mM TEA-H_2SO_4 (pH 8.0), cells were suspended at A_{660} of 1.6 in 50mM TEA-H_2SO_4-30% sucrose (pH 8.0). After swirling the suspension gently for 2 minutes at 24°C, EDTA was added to a final concentration of 600µM, and swirling was continued for a further 2 minutes. At this time, lysozyme

solution (1mg/ml in water) was added to a final concentration of 30µg/ml. Deoxyribonuclease I and ribonuclease were added to 5µg/ml each to remove any DNA and RNA which had attached to the cell membranes (Kaback, 1971). This made the pellet easier to suspend.

Spheroplast formation was checked by examination under the microscope, by release of periplasmic alkaline phosphatase, or by susceptibility to osmotic shock. The last-mentioned procedure was found to be rapid and reliable and involved dilution of the spheroplast suspension into water, followed by measurement of the absorbance at 660nm using a Gilford 300 spectrophotometer. This absorbance was compared with that of an identical dilution of a control suspension of cells which had not been treated with lysozyme and EDTA.

The spheroplasts were recovered by centrifugation at 20,000g for 3 minutes, suspended at A_{660} of 0.8 in chloride-free uptake medium containing 20% sucrose, and stored at $4^{o}C$ until required.

I. MEASUREMENT OF PHOSPHATE UPTAKE IN SPHEROPLASTS

Uptake was measured as described for whole cells, with the following differences:

i)uptake and wash solution contained 20% sucrose,and

ii) 25µl samples were withdrawn from the incubation mixture at the desired times and mixed with 1ml of wash solution/20% sucrose before filtration.

J. ANALYTICAL PROCEDURES

1. Protein was determined by the method of Lowry et al. (1951) with the modification (Bailey, 1962) that sodium citrate was used instead of sodium tartrate to give a more stable reagent.

2. Alkaline phosphatase was assayed by the method of Bracha and Yagil (1969).

3. Inorganic phosphate was estimated by the method of Harris and Popat (1954).

4. Analysis of inorganic and esterified phosphate was carried out using a modification (Rosenberg et al., 1982) of the method of Ohnishi (1978) as follows:

Cell samples were prepared by the extraction of filtered cells with 0.25M perchloric acid, followed by mixing 4 parts (by volume) of extract with 1 part sodium acetate (1M) and 5 parts ammonium molybdate (5%). Liquid samples of cell filtrate or supernatant were acidified with perchloric acid to 0.25M, and then prepared as described above.

A micro-column, with a total holding volume of 0.5ml, was made from a disposable pipette tip plugged with cotton wool. The stationary phase bed of the column was made with 0.2ml of a 10% suspension of polyvinyl polypyrrolidone. The column was pre-treated and the samples washed through as originally described (Ohnishi, 1978), except that the sample and wash volumes were 0.2ml. Elution was with two 0.2ml portions of 1.25M NH_4OH. The column was suitably supported to allow the collection of the effluent and eluate into separate microfuge tubes of 0.5ml capacity, which were than capped and placed into empty scintillation vials. The radioactivity was

determined by Cerenkov radiation in a Packard Tri-Carb 460CD counter.

5. Assay of total phosphate

Sample, in a volume of 0.2ml, was added to 2.0ml of 8.6% sodium persulphate, autoclaved in a sealed tube, and cooled. The P_i liberated by hydrolysis was assayed in the same tube according to the method of Harris and Popat (1954) after the addition of appropriate reagents.

K. PREPARATION OF DIALYSIS TUBING

Visking dialysis tubing (Scientific Instrument Centre Ltd., London, England) was boiled for 5 minutes in a solution of Na_2CO_3 (50g/1) and Na_2EDTA (50mM), washed thoroughly with distilled water, and stored at 4^oC in 50% ethanol. This tubing was hydrated before use by soaking overnight in distilled water.

CHAPTER 3

THE EFFECTS OF SILVER IONS ON PHOSPHATE TRANSPORT

INTRODUCTION

The Pit and Pst systems for the transport of P_i in Escherichia coli, and the effects of silver ions on the respiratory chain of E. coli have already been discussed in Chapter 1.

Exchange of intracellular and extracellular P_i is catalysed by the Pit system, but not by the Pst system (Rosenberg et al., 1977), and appears to be a facilitated process depending on the product of the pit gene (Rosenberg et al., 1982). Evidence supporting this comes from observations that exchange occurs in wild-type strains containing both the Pit and Pst systems and in a strain carrying only the Pit system, but does not occur in strains carrying only the Pst system (Rosenberg et al., 1977; 1982). Also, both exchange and transport of P_i are reversibly inactivated at low temperatures, and both are blocked by mercuric ions (Rosenberg et al., 1982). This exchange can occur under conditions of energy depletion such that cells cannot carry out net phosphate uptake, although it was pointed out by Rosenberg et al. (1982) that the cells are not totally depleted of energy.

Phosphate is rapidly esterified after it enters the cell (Medveczky and Rosenberg, 1971), and the ester-P also exchanges with extracellular P_i, but it must flow through the intracellular P_i pool (Rosenberg et al., 1982).

In this chapter I show that silver ions inhibited the uptake of phosphate and caused efflux of accumulated phosphate. This efflux was not due to a damaged cell membrane. Thiols or, to a lesser extent, bromide mitigated the effects of Ag^+. The concentration-dependence of the Ag^+ effect, and uptake experiments using radioactively labelled silver, indicated that there were many sites

of interaction with silver ions. Silver ions inhibited exchange, and caused efflux of phosphate at low temperatures.

METHODS

The materials and basic methods used have been described in Chapter 2.

Measurement of inorganic and esterified phosphate

i) Intracellular: Cells which had taken up ^{32}P were filtered through cellulose nitrate membranes (pore size 0.45μm) and washed as usual. Each membrane filter was then extracted with 1.0ml of 0.25M $HClO_4$ at 0°C. The extract was filtered through cellulose nitrate membranes (pore size 0.45μm) to remove any cells and debris which had been dislodged from the filter. The filtered extract was then assayed for inorganic and esterified phosphate as described in Chapter 2.

ii) Extracellular: Cells which had taken up ^{32}P were centrifuged and re-suspended in fresh uptake medium to remove $^{32}P_i$ which had not been taken up from the medium. After the cell suspension had been treated as required, a sample was removed and filtered through a cellulose nitrate membrane (pore size 0.45μm). The membrane was not washed, and the filtrate was acidified with $HClO_4$ to 0.25M and assayed for inorganic and esterified phosphate as described in Chapter 2.

RESULTS

3.1 Inhibition of phosphate uptake by silver ions.

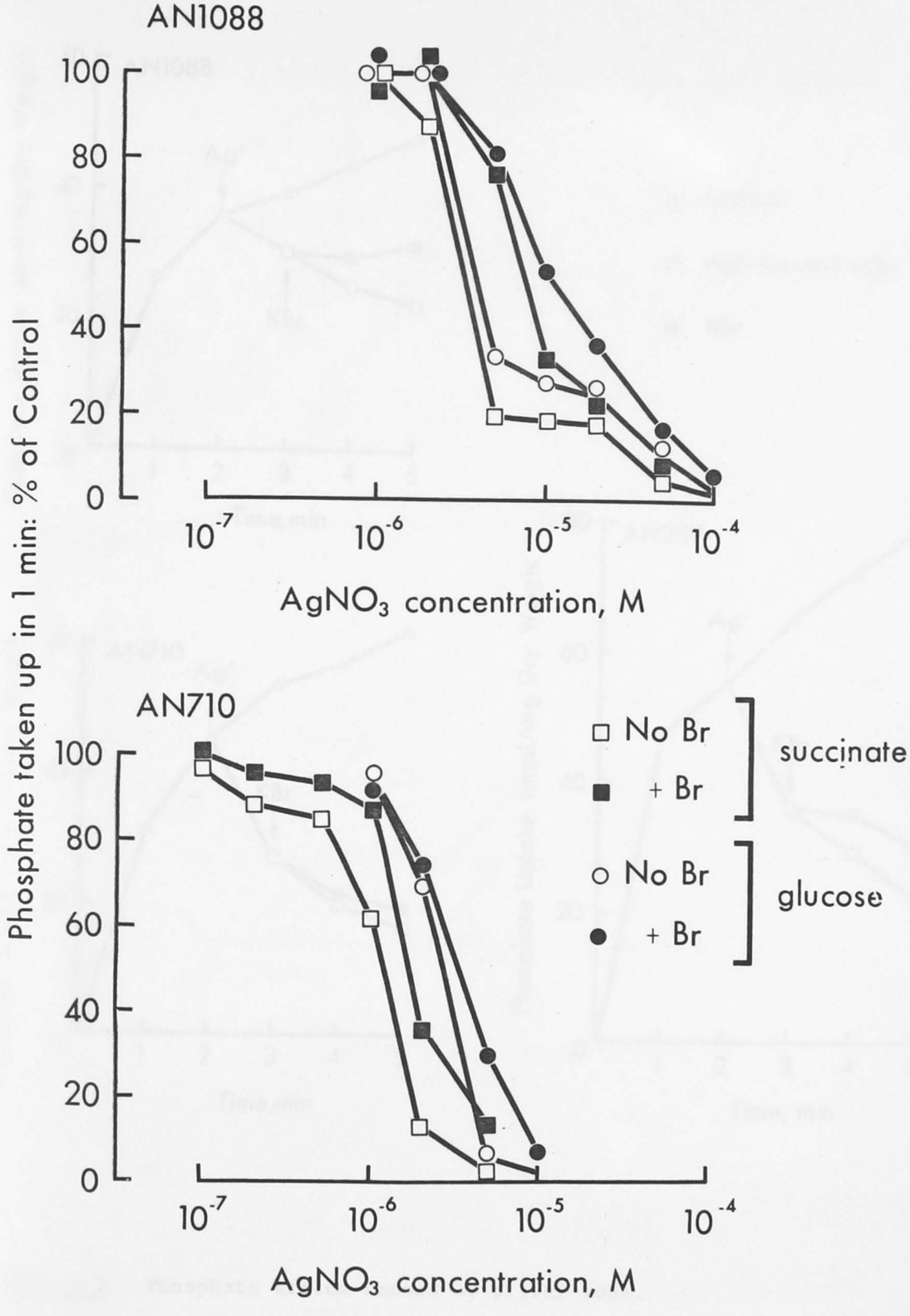
Treatment of cells with $AgNO_3$ inhibited P_i transport in both the Pit and Pst systems (Fig. 3.1). The systems were affected differently, the Pst system requiring a higher Ag^+ concentration than the Pit system for the same effect. Cells grown and assayed with glucose as the sole carbon source were more resistant to the effect of Ag^+ than cells grown and assayed with succinate. This difference was more pronounced in strain AN710, which contains only the Pit system. Addition of KBr after $AgNO_3$ to remove excess Ag^+ relieved the inhibition to some extent, and this effect of KBr was more noticeable in strain AN1088, which carries only the Pst system. In the presence of 2mM bromide, the free Ag^+ concentration is about 10^{-10}M, calculated using the solubility product for AgBr of $5 \times 10^{-13} mol^2 l^{-2}$. The degree of inhibition did not depend on the length of time of preincubation with $AgNO_3$ (data not shown).

3.2 Effect of silver ions on accumulated phosphate.

When $AgNO_3$ was added to a suspension of cells which had taken up $^{32}P_i$, an efflux of radioactivity was observed (Fig. 3.2). The effect on the Pit system was more pronounced than that on the Pst system (Fig. 3.2, Fig. 3.3). The addition of KBr to remove excess Ag^+ caused uptake to resume in cells containing only the Pst system, an effect never observed in cells carrying only the Pit system. Intermediate results were observed in AN259, a strain containing both the Pit and Pst systems (Fig. 3.2). The effect of Ag^+ on the Pit system was less severe when glucose, rather than succinate, was the source of energy (Fig. 3.3). The carbon source made little

Fig. 3.1 Inhibition of P_i uptake by silver ions.

The cell suspensions were incubated at 37°C for 5 minutes in the presence of the energy source shown before the addition of $^{32}P_i$. In the control suspension, $^{32}P_i$ was added and uptake followed for 1 minute. Silver-treated suspensions received $AgNO_3$ 2 minutes before $^{32}P_i$. KBr was added 1 minute after the $AgNO_3$ (1 minute before the $^{32}P_i$). In those suspensions $^{32}P_i$ uptake was also followed for 1 minute. Results are expressed as % of $^{32}P_i$ taken up in 1 minute in the control suspensions.


AN1088
100
80
60
40
20
0
10^{-7}
10^{-6}
10^{-5}
10^{-4}
$AgNO_3$ concentration, M
Phosphate taken up in 1 min: % of Control
AN710
100
80
60
40
20
0
10^{-7}
10^{-6}
10^{-5}
10^{-4}
$AgNO_3$ concentration, M
Phosphate taken up in 1 min: % of Control
□ No Br
■ + Br
succinate
○ No Br
● + Br
glucose

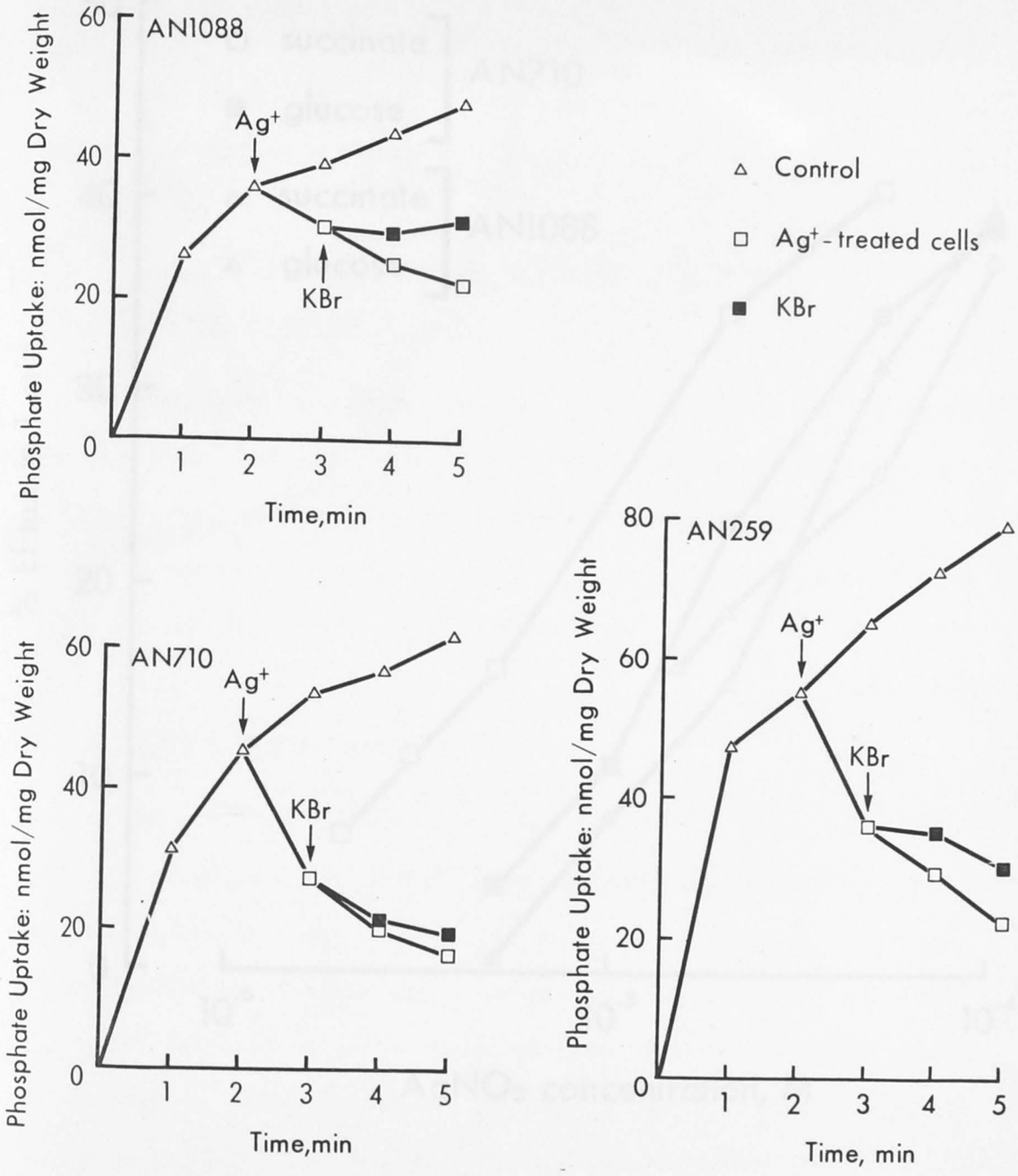


Fig. 3.2 Phosphate efflux caused by silver ions.

Phosphate uptake was assayed as described in Chapter 2. Additions of $AgNO_3$ (20μM) and KBr (2mM) as shown. The source of energy was succinate.

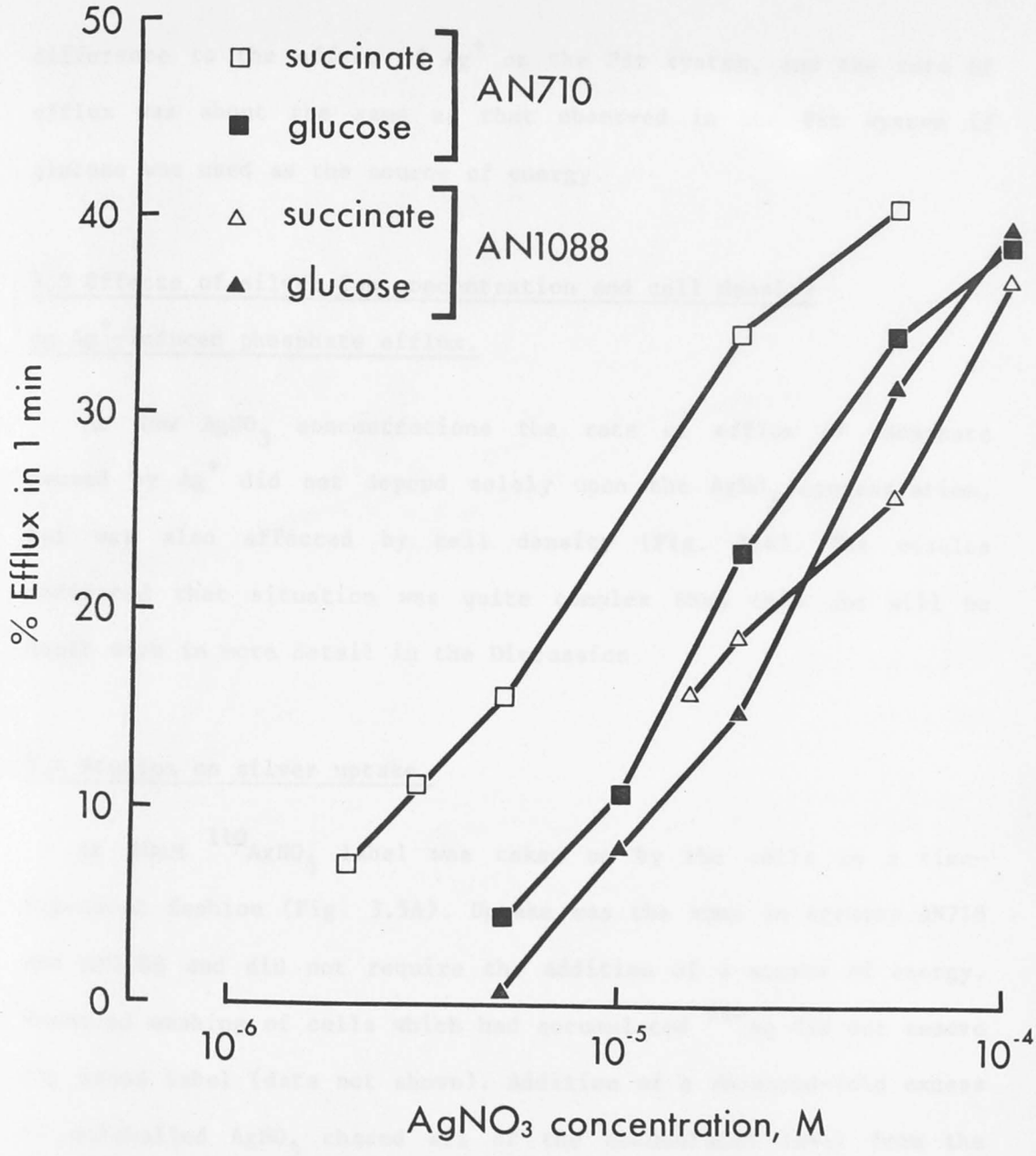


Fig. 3.3 Effect of $AgNO_3$ concentration on phosphate efflux.

The experiment was performed as described in Fig. 3.2. $AgNO_3$ was added 2 minutes after $^{32}P_i$ and efflux was followed for 1 minute. The data were calculated as % radioactivity lost from the cells during that minute.

difference to the effect of Ag^+ on the Pst system, and the rate of efflux was about the same as that observed in the Pit system if glucose was used as the source of energy.

3.3 Effects of silver ion concentration and cell density on Ag^+-induced phosphate efflux.

At low $AgNO_3$ concentrations the rate of efflux of phosphate caused by Ag^+ did not depend solely upon the $AgNO_3$ concentration, but was also affected by cell density (Fig. 3.4). The results indicated that situation was quite complex than this and will be dealt with in more detail in the Discussion.

3.4 Studies on silver uptake.

At 20μM $^{110}AgNO_3$ label was taken up by the cells in a time-dependent fashion (Fig. 3.5A). Uptake was the same in strains AN710 and AN1088 and did not require the addition of a source of energy. Repeated washing of cells which had accumulated ^{110}Ag did not remove the bound label (data not shown). Addition of a thousand-fold excess of unlabelled $AgNO_3$ chased all of the accumulated label from the cells (Fig. 3.5A). At 0.2μM $^{110}AgNO_3$, the situation was different (Fig. 3.5B). In this case, the label bound rapidly to the cells (25-35% of the $^{110}Ag^+$ added bound within the first minute in the absence of a carbon source) and there was no further increase in uptake with time. In the presence of a source of energy, this initial binding was reduced by 2- to 4-fold. Carbon source added after the initial uptake of $^{110}Ag^+$ had no effect on the retention of label.

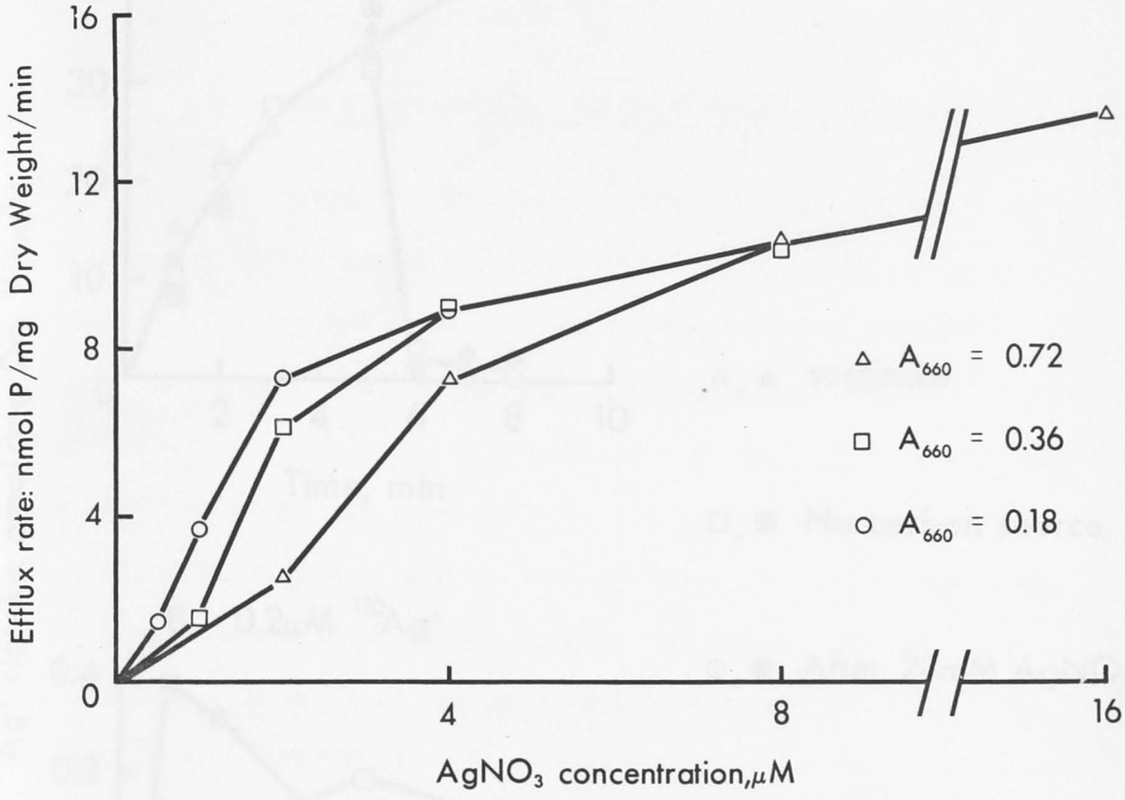


Fig. 3.4 Effects of Ag^+ concentration and cell density on Ag^+-induced phosphate efflux.

Cells (strain AN710) were grown on succinate and prepared for phosphate uptake assay as described in Chapter 2. After suspension of the cells at the A_{660} shown, phosphate uptake was followed for 5 minutes, the cell suspension was centrifuged, resuspended at the appropriate A_{660} in fresh uptake medium containing succinate and shaken at 37°C. After 3 minutes $AgNO_3$ was added at the concentration shown and efflux of ^{32}P was monitored for a further 2 minutes.

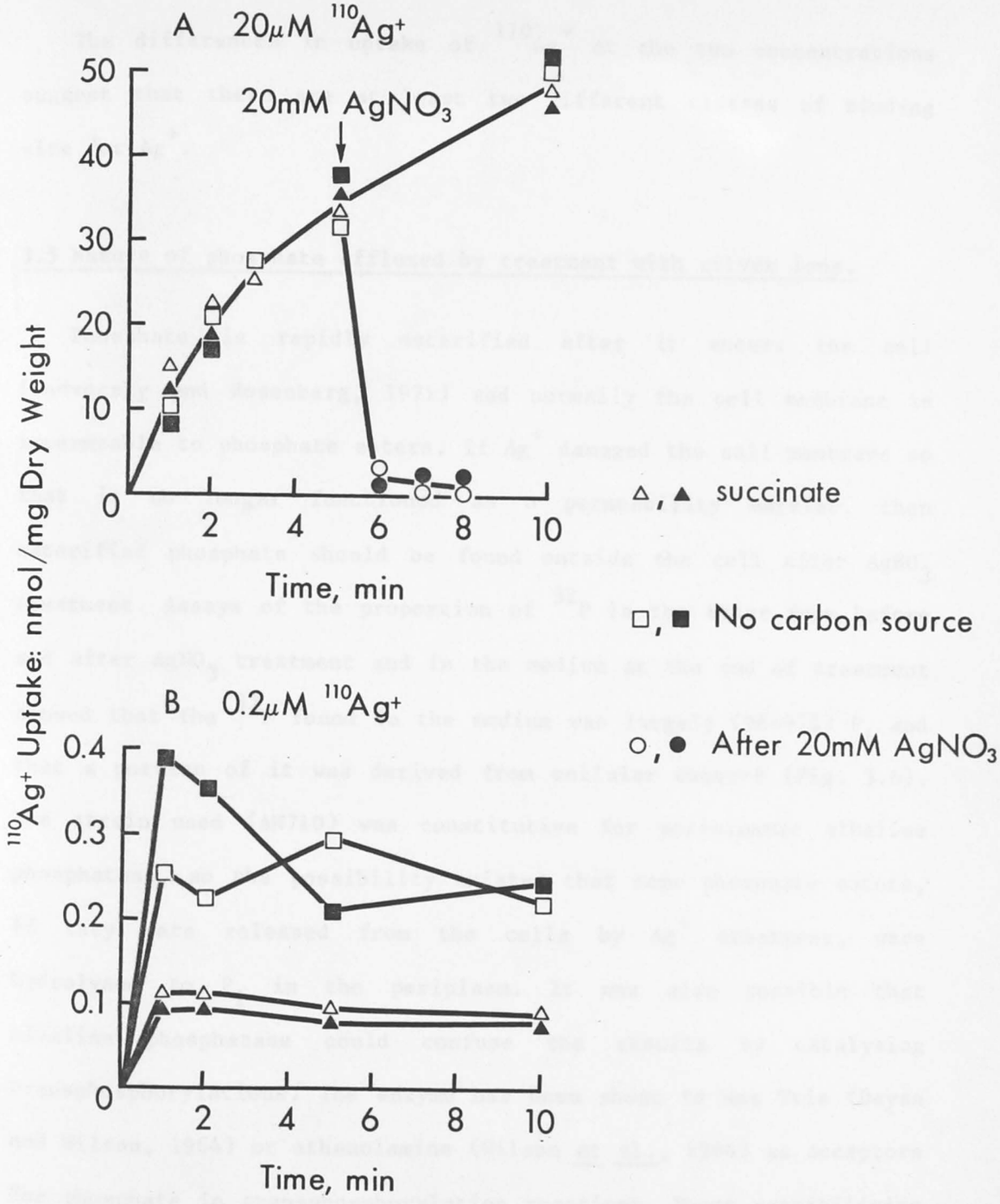


Fig. 3.5 Uptake of $^{110}Ag^+$.

Cells were grown and prepared as for P_i uptake (Chapter 2). Chloride-free uptake medium and chloride-free wash solution were used, uptake was initiated by the addition of 20μM (A) or 0.2μM (B) $^{110}AgNO_3$ and measured as described in Chapter 2. Addition of 20mM $AgNO_3$ (non-radioactive) was as shown.

Open symbols - strain AN710; Filled symbols - strain AN1088

The differences in uptake of $^{110}Ag^+$ at the two concentrations suggest that there are at least two different classes of binding site for Ag^+.

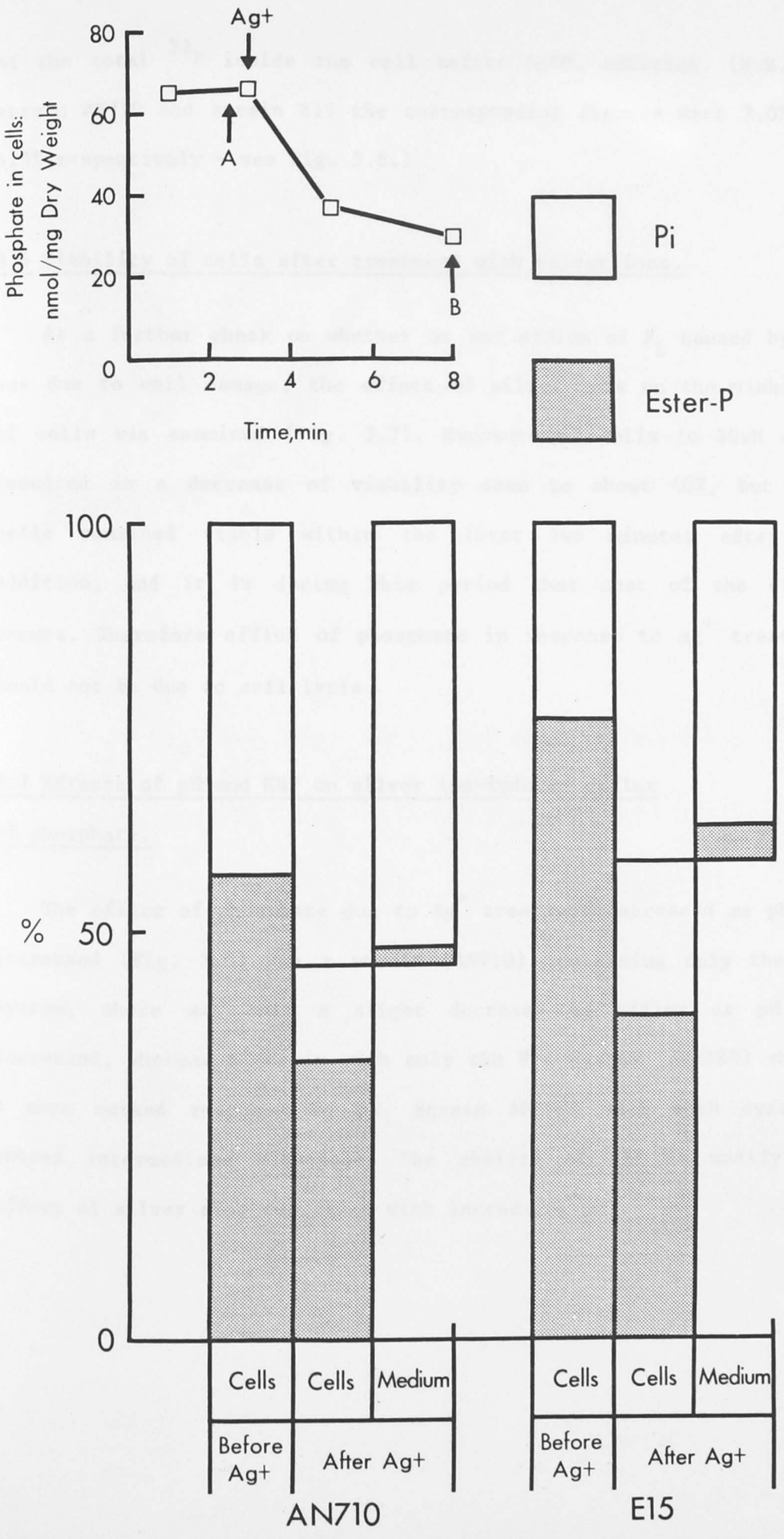
3.5 Nature of phosphate effluxed by treatment with silver ions.

Phosphate is rapidly esterified after it enters the cell (Medveczky and Rosenberg, 1971) and normally the cell membrane is impermeable to phosphate esters. If Ag^+ damaged the cell membrane so that it no longer functioned as a permeability barrier, then esterified phosphate should be found outside the cell after $AgNO_3$ treatment. Assays of the proportion of ^{32}P in the ester form before and after $AgNO_3$ treatment and in the medium at the end of treatment showed that the ^{32}P found in the medium was largely (96-97%) P_i and that a portion of it was derived from cellular ester-P (Fig. 3.6). The strain used (AN710) was constitutive for periplasmic alkaline phosphatase, so the possibility existed that some phosphate esters, if they were released from the cells by Ag^+ treatment, were hydrolysed to P_i in the periplasm. It was also possible that alkaline phosphatase could confuse the results by catalysing transphosphorylations. The enzyme has been shown to use Tris (Dayan and Wilson, 1964) or ethanolamine (Wilson et al., 1964) as acceptors for phosphate in transphosphorylation reactions. These possibilities did not exist in the phosphatase-negative mutant strain E15, and when the experiment was repeated using this strain the result (Fig. 3.6) was similar to that observed with strain AN710.

The experiment was also performed using spheroplasts of strain AN710, which lose the outer membrane and the periplasmic proteins, including alkaline phosphatase, during the spheroplasting procedure. Ester-P in the medium outside the cells after Ag^+ treatment was 7.4%

Fig. 3.6 Nature of phosphate effluxed by Ag^+ treatment.

Cells (A_{660} = 0.35) were allowed to take up $^{32}P_i$ for 5 minutes. The cell suspension was then centrifuged, resuspended in fresh uptake buffer at A_{660} of 1.75 and shaken at 37°C. $AgNO_3$ (25μM to strain AN710, 100μM to strain E15) was added 3 minutes after this, as shown in the insert. 1 ml samples were taken at 'A' and 'B', filtered, extracted with 0.25M $HClO_4$ and assayed for inorganic and esterified phosphate as described in Chapter 2. The filtrate from sample 'B' was acidified with $HClO_4$ to 0.25M and assayed for inorganic and esterified phosphate as described. Results are expressed as percentages of the total ^{32}P inside the cell before $AgNO_3$ addition.


Ag+
A
B
80
60
40
20
0
2
4
6
8
Phosphate in cells:
nmol/mg Dry Weight
Time,min
Pi
Ester-P
100
%
50
0
Cells
Cells
Medium
Before Ag+
After Ag+
AN710
Cells
Cells
Medium
Before Ag+
After Ag+
E15

of the total ^{32}P inside the cell before $AgNO_3$ addition. (N.B. For strain AN710 and strain E15 the corresponding figures were 2.0% and 4.5% respectively - see Fig. 3.6.)

3.6 Viability of cells after treatment with silver ions.

As a further check on whether or not efflux of P_i caused by Ag^+ was due to cell damage, the effect of silver ions on the viability of cells was examined (Fig. 3.7). Exposure of cells to 20μM $AgNO_3$ resulted in a decrease of viability down to about 40%, but most cells remained viable within the first two minutes after Ag^+ addition, and it is during this period that most of the efflux occurs. Therefore efflux of phosphate in response to Ag^+ treatment could not be due to cell lysis.

3.7 Effects of pH and KBr on silver ion-induced efflux of phosphate.

The efflux of phosphate due to Ag^+ treatment decreased as pH was increased (Fig. 3.8). In a strain (AN710) containing only the Pit system, there was only a slight decrease in efflux as pH was increased, whereas a strain with only the Pst system (AN1088) showed a more marked response to pH. Strain AN259, with both systems, showed intermediate responses. The ability of KBr to modify the effect of silver also decreased with increasing pH.

Fig. 3.7 Viability of cells after treatment with Ag^+.

The cell suspension was preincubated at 37°C for 5 min in the presence of 20mM succinate. At zero time, P_i was added to a final concentration of 50μM. After 2 minutes, $AgNO_3$ (20μM) was added. Samples were taken at the times shown and diluted 100-fold into uptake buffer containing 2mM KBr. Samples were further diluted and spread onto meat infusion agar medium, and colonies were counted after overnight growth at 37°C. Each point is a mean from three experiments. Within each experiment, '100% viability' for each strain was defined by the mean of the counts from all plates of that strain at 1 minute (☆) (i.e. before addition of Ag^+).

Open symbols - No Ag^+; Closed symbols - $AgNO_3$ at 2 minutes.

% Viable Cells

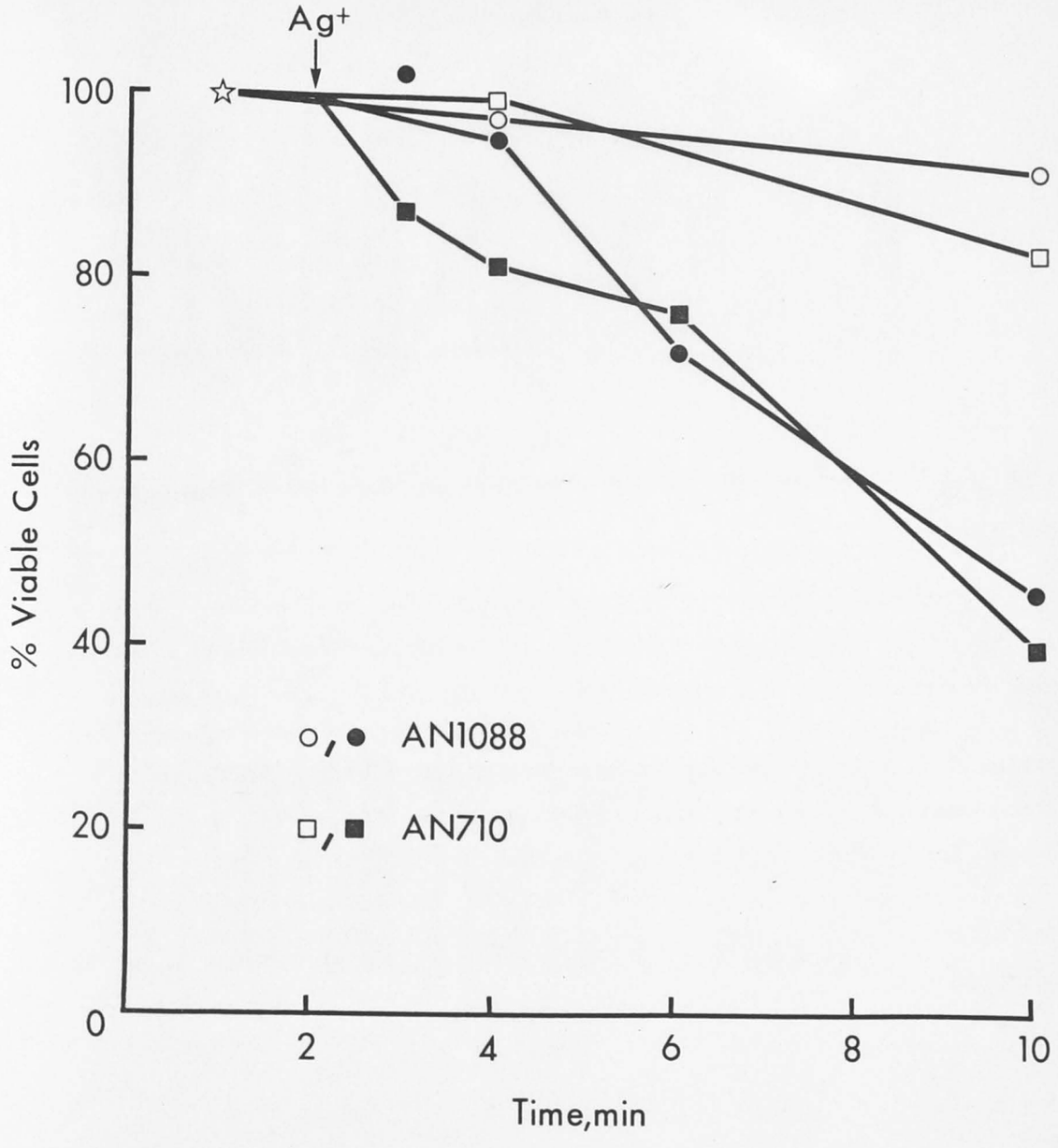
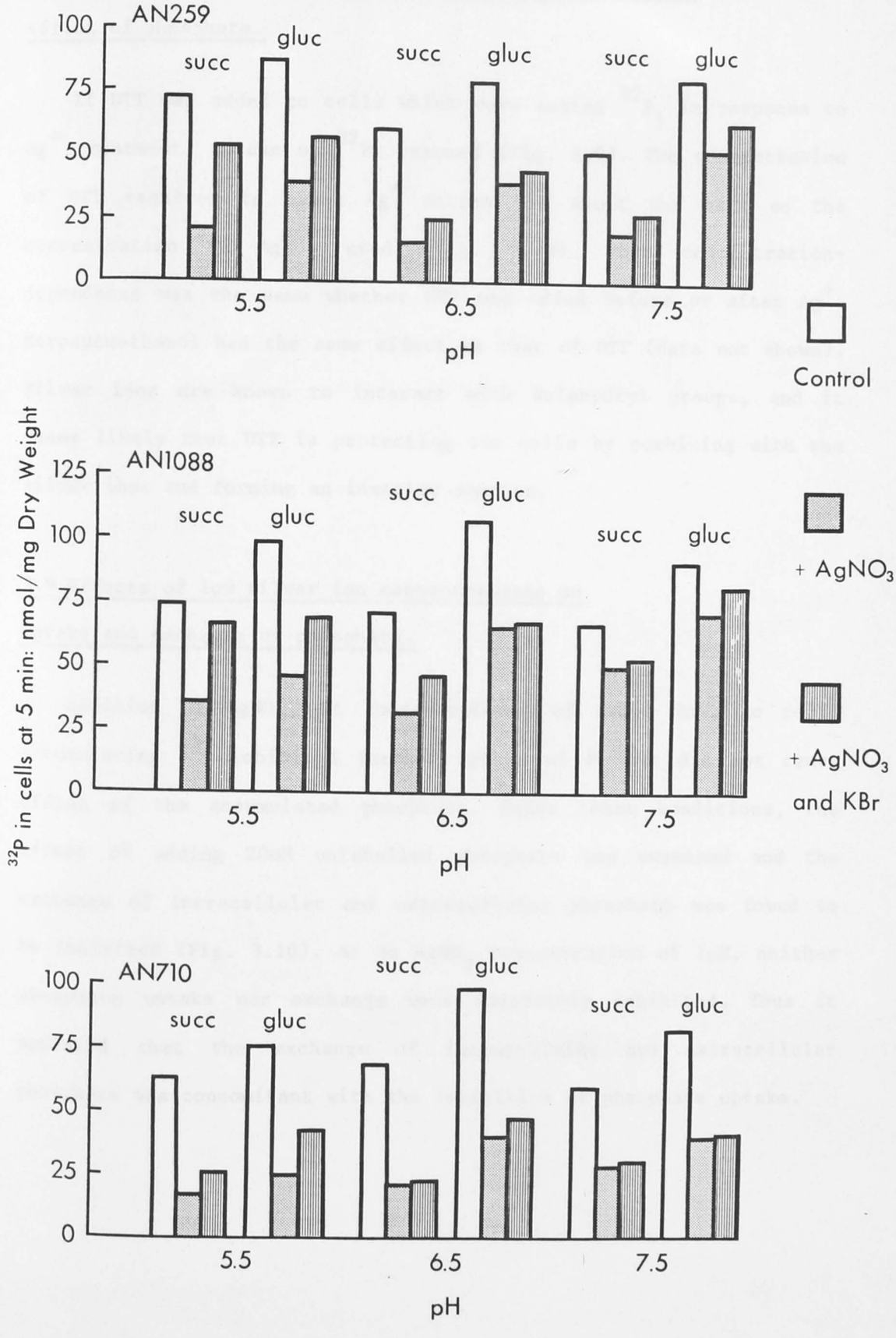

Ag+
100
80
60
40
20
0
% Viable Cells
○, ● AN1088
□, ■ AN710
2
4
6
8
10
Time,min

Fig. 3.8 Effect of pH and KBr on Ag^+-induced efflux of phosphate.

Cells were prepared for uptake as described in Chapter 2 and suspended at the appropriate pH in the chloride-free phosphate-free uptake buffer containing MES. The experiment was performed as described in Fig. 3.3. i.e. $AgNO_3$ (20μM) was added 2 minutes after $^{32}P_i$, and if KBr was used it was added 1 minute after Ag^+. The amount of ^{32}P in the cell at 5 minutes was measured.


AN259
succ
gluc
succ
gluc
succ
gluc
100
75
50
25
0
5.5
6.5
7.5
pH
Control
AN1088
succ
gluc
succ
gluc
succ
gluc
125
100
75
50
25
0
5.5
6.5
7.5
pH
+ AgNO3
+ AgNO3 and KBr
32P in cells at 5 min: nmol/mg Dry Weight
AN710
succ
gluc
succ
gluc
succ
gluc
100
75
50
25
0
5.5
6.5
7.5
pH

3.8 Effect of dithiothreitol (DTT) on silver ion-induced efflux of phosphate.

If DTT was added to cells which were losing $^{32}P_i$ in response to Ag^+ treatment, uptake of $^{32}P_i$ resumed (Fig. 3.9). The concentration of DTT required to block Ag^+ action was about the same as the concentration of $AgNO_3$ used (Fig. 3.9B). This concentration-dependence was the same whether DTT was added before or after Ag^+. Mercaptoethanol had the same effect as that of DTT (data not shown). Silver ions are known to interact with sulphydryl groups, and it seems likely that DTT is protecting the cells by combining with the silver ions and forming an inactive complex.

3.9 Effects of low silver ion concentrations on uptake and exchange of phosphate.

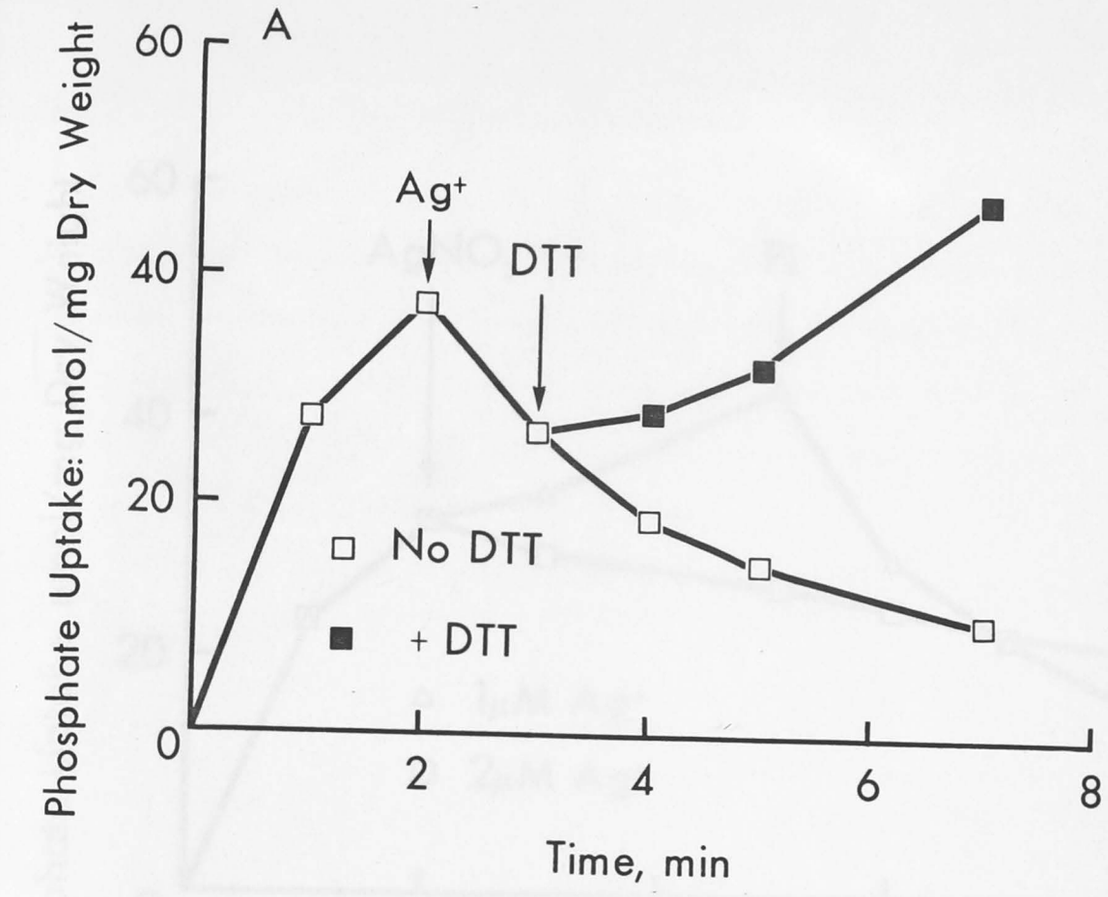
Addition of $AgNO_3$, at concentrations of about 2μM, to cells accumulating ^{32}P inhibited further uptake of P_i but did not cause efflux of the accumulated phosphate. Under these conditions, the effect of adding 20mM unlabelled phosphate was examined and the exchange of intracellular and extracellular phosphate was found to be inhibited (Fig. 3.10). At an $AgNO_3$ concentration of 1μM, neither phosphate uptake nor exchange were completely inhibited. Thus it appeared that the exchange of intracellular and extracellular phosphate was concomitant with the inhibition of phosphate uptake.

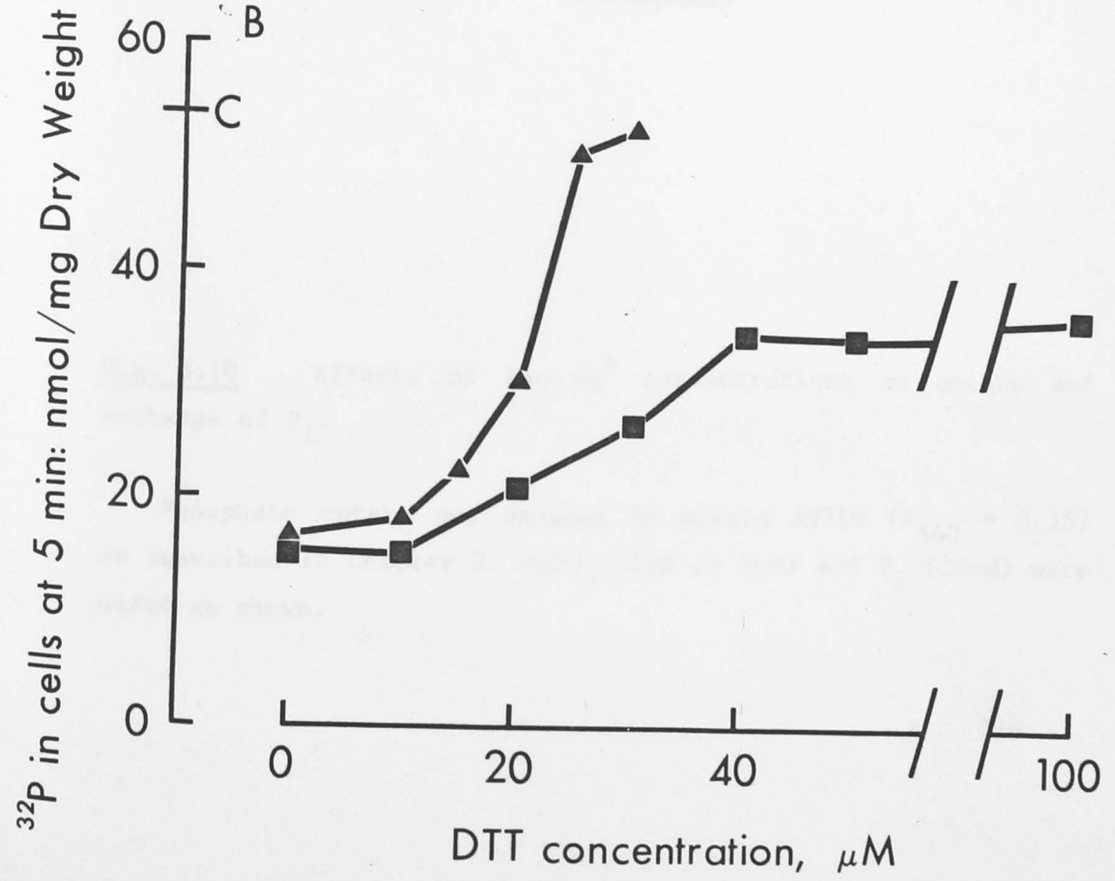
Fig. 3.9 Effect of dithiothreitol on Ag^+-induced efflux of phosphate.

Phosphate uptake was measured in strain AN710 as described in Chapter 2. The carbon source was succinate.

A) $AgNO_3$ (20μM) and DTT (50μM) were added as shown.

B) DTT was tested over a range of concentrations, either added after $AgNO_3$, at 3 minutes (■) (as in (A)), or before $AgNO_3$, at 1 minute (▲). Results are expressed as the amount of ^{32}P in the cell at 5 minutes. The point marked 'C' on the vertical axis indicates the level of ^{32}P in the cells at 5 minutes in control suspensions.


A
60
40
20
0
Ag+
DTT
No DTT
+ DTT
2
4
6
8
Time, min
Phosphate Uptake: nmol/mg Dry Weight


B
C
60
40
20
0
0
20
40
100
DTT concentration, μM
32P in cells at 5 min: nmol/mg Dry Weight

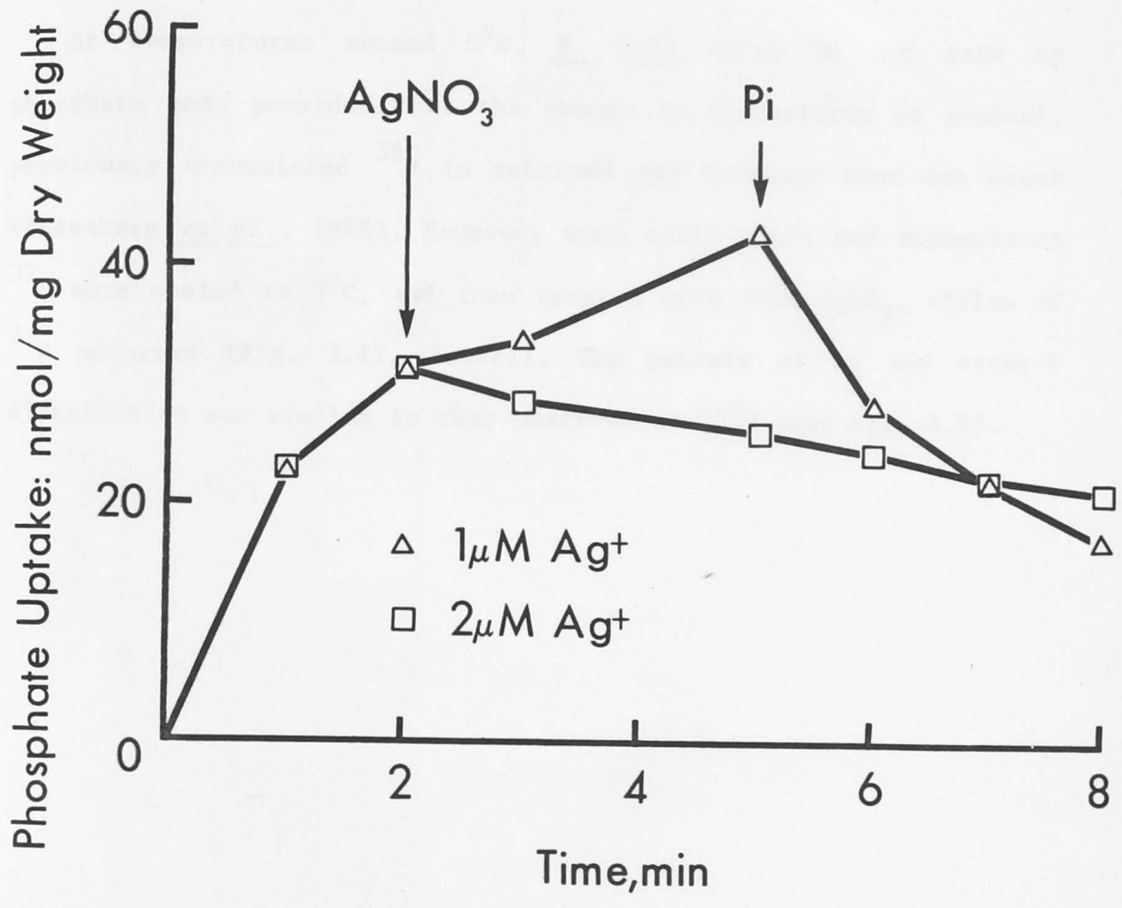


Fig. 3.10 Effects of low Ag^+ concentrations on uptake and exchange of P_i.

Phosphate uptake was assayed in strain AN710 (A_{660} = 0.35) as described in Chapter 2. $AgNO_3$ (1μM or 2μM) and P_i (20mM) were added as shown.

3.10 The effect of silver ions at low temperature.

At temperatures around 0°C, E. coli cells do not take up phosphate and, provided that the change in temperature is gradual, previously accumulated ^{32}P is retained and exchange does not occur (Rosenberg et al., 1982). However, when cells which had accumulated ^{32}P were cooled to 0°C, and then treated with 20μM $AgNO_3$, efflux of ^{32}P occurred (Fig. 3.11, insert). The pattern of P_i and ester-P distribution was similar to that observed at 37°C (see Fig. 3.6).

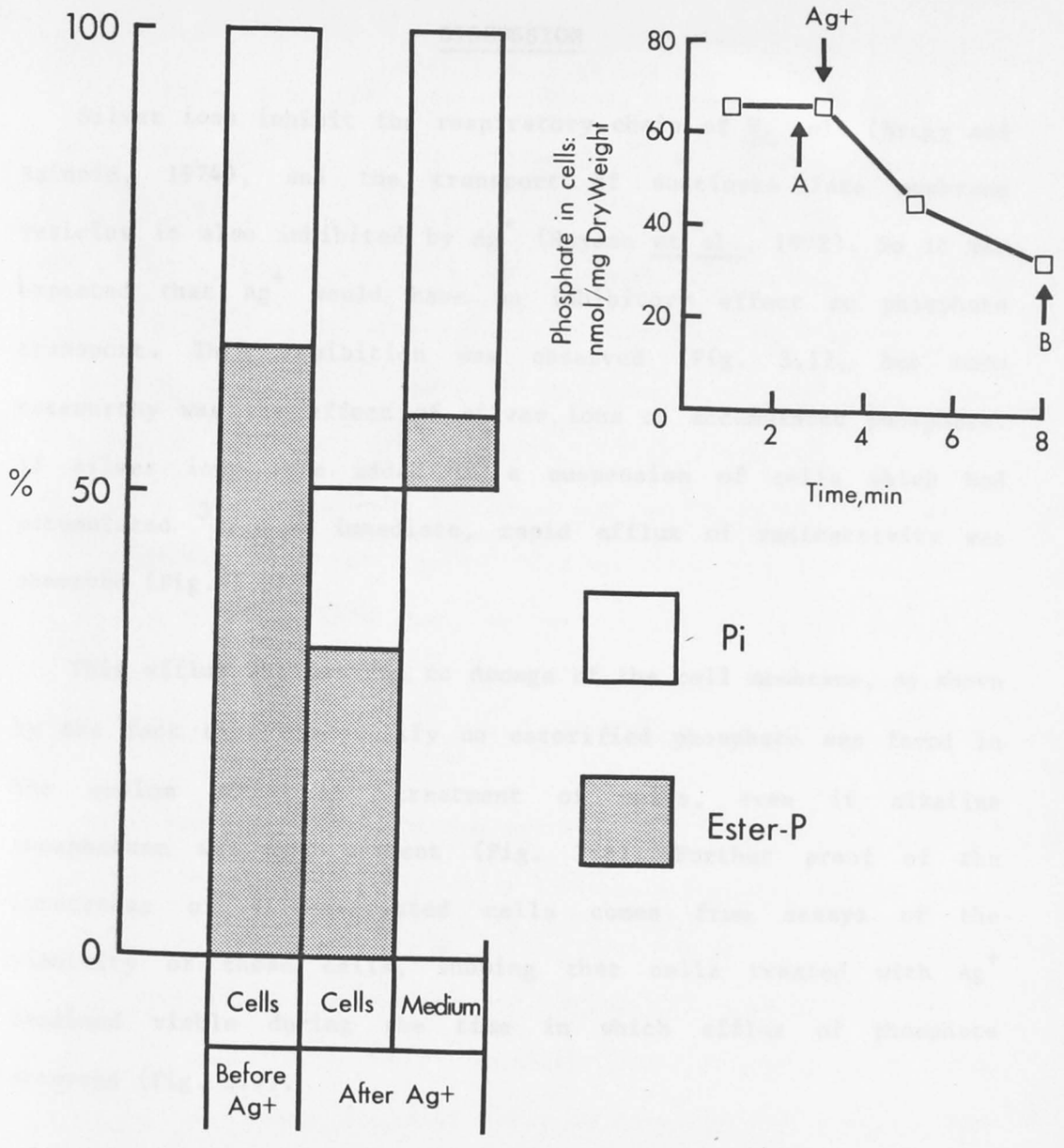


Fig. 3.11 Silver ion-induced exit of phosphate at low temperature.

Phosphate uptake was monitored in strain AN710 for 5 minutes. The cell suspension was then centrifuged, resuspended in fresh uptake medium at A_{660} of 1.75, then cooled gradually to $0^{o}C$. 25μM $AgNO_3$ was added as shown in the insert, and samples were taken and assayed for inorganic and esterified phosphate as described in Fig. 3.6. The source of energy was succinate. Results are expressed as percentages of the total ^{32}P inside the cell before $AgNO_3$ addition.

DISCUSSION

Silver ions inhibit the respiratory chain of E. coli (Bragg and Rainnie, 1974), and the transport of succinate into membrane vesicles is also inhibited by Ag^+ (Rayman et al., 1972). So it was expected that Ag^+ would have an inhibitory effect on phosphate transport. This inhibition was observed (Fig. 3.1), but more noteworthy was the effect of silver ions on accumulated phosphate. If silver ions were added to a suspension of cells which had accumulated ^{32}P, an immediate, rapid efflux of radioactivity was observed (Fig. 3.2).

This efflux was not due to damage of the cell membrane, as shown by the fact that essentially no esterified phosphate was found in the medium after Ag^+ treatment of cells, even if alkaline phosphatase was not present (Fig. 3.6). Further proof of the intactness of silver-treated cells comes from assays of the viability of these cells, showing that cells treated with Ag^+ remained viable during the time in which efflux of phosphate occurred (Fig. 3.7).

If silver ions interacted reversibly with a single site to cause the efflux of phosphate, then the response of the cells to increasing concentrations of Ag^+ should show standard Michaelis-Menten-type kinetics. Fig. 3.4 shows that this is not the case, but the curve is not that which one would expect if silver ions reacted stoichiometrically with a single site to cause efflux of phosphate. Rather, the curve exhibits characteristics of many types of interaction, indicating a heterogeneity of binding sites related to the efflux of phosphate. No clear division between different types of binding site could be seen in the curves in Fig. 3.4. There could be a single complex class or a series of many different classes of

binding sites. Results from $^{110}Ag^+$ uptake experiments (Fig. 3.5) also indicated that there were different classes of binding site, with binding at lower concentrations of $^{110}Ag^+$ apparently occurring at a limited number of high affinity sites. At the low $^{110}Ag^+$ concentration, energised cells took up less silver. Energy-dependent pumping of Ag^+ out of the cells, as described by Tynecka et al. (1981a) for Cd^{2+}, is one possible explanation of this, but carbon source added after the initial rapid binding phase had no effect on the amount of $^{110}Ag^+$ bound to the cells, so it is possible that in energised cells some of the Ag^+-binding sites are inaccessible to Ag^+. It is also possible that, in the absence of energy, enough Ag^+ accumulated within the cell, or cell membrane, to interfere with energy coupling to the pump, or to inhibit the pump directly.

Cells carrying only the Pst system were less sensitive to the effects of silver ions than cells with only the Pit system (Fig. 3.1, Fig. 3.2, Fig. 3.3, Fig. 3.8). The effects of Ag^+ in cells using glucose as the sole source of carbon were less severe than the effects in cells using only succinate (Fig. 3.1, Fig. 3.3). This difference was larger in cells carrying only the Pst system. These differences indicate that silver ions may be exerting their effects by interfering with the coupling of energy to the transport of phosphate and that coupling via the pmf is more sensitive to this inhibition than coupling by way of 'high-energy phosphate'. Note that the Pst system is energised by 'high-energy phosphate' whereas the Pit system is coupled to the protonmotive force (Rosenberg et al., 1977; 1979). Also, succinate can only enter the cell's energy metabolism through the respiratory chain, whereas glucose has the alternative route of glycolysis and substrate-level phosphorylation. So it appears that silver ions have a greater effect on systems which couple to energy directly through the cell membrane than on

those which rely on phosphate-bond energy.

As external pH increases, ΔpH decreases in magnitude from -110mV at pH5.5 until it reaches zero at pH7.5, while $\Delta\psi$ in intact cells increases from -100mV at pH5.5 to -140mV at pH7.5 (Felle et al., 1980). Silver ions had a more severe effect on ΔpH than on $\Delta\psi$ (see Chapter 6), so the decrease in Ag^+-induced phosphate efflux in the Pit system due to increasing pH may be due to the decreasing contribution of ΔpH to the pmf. Since the Pst system is energised by phosphate-bond energy (Rosenberg et al., 1977; 1979), the effect of pH on Ag^+-induced efflux in the Pst system cannot be explained as above for the Pit system, unless the pmf is necessary to maintain a functional Pst system, as has been found for other shock-sensitive transport systems (Plate et al., 1974; Lieberman and Hong, 1976; Plate, 1979). Another possibility is that the efflux observed in cells carrying the Pst system is due to interaction of Ag^+ at a different site, a site which may become less accessible to Ag^+ as the pH increases, the accessibility perhaps being mediated by the energisation of the membrane.

When added to cells after Ag^+, bromide inhibited the Ag^+-induced efflux of phosphate (Fig. 3.1, Fig. 3.2, Fig. 3.8). The effect of bromide was more pronounced in cells carrying the Pst system. The decreasing effect of bromide on efflux at increasing pH (Fig. 3.8) was probably due simply to the reduction of efflux itself as pH increased. The different effects of bromide in the Pit and Pst systems is again suggestive of different sites of interaction with Ag^+, the site leading to efflux in cells with the Pst system having a lower affinity for Ag^+ than the site giving efflux in cells with the Pst system.

Dithiothreitol completely restored $^{32}P_i$ uptake through the Pit system (Fig. 3.9) under conditions where bromide had practically no effect (Fig. 3.2). Since silver ions react readily with thiols, the likely mechanism of DTT action would be the interaction of DTT with Ag^+ leading to the removal of silver ions from the medium. The dependence of this 'rescue' on DTT concentration (Fig. 3.9B) shows that it appears to be due to a titration of the Ag^+ with DTT. A comparison of the effects of DTT and bromide shows that DTT was a much more effective rescuing reagent than bromide. One possible reason for this is the higher affinity of Ag^+ for sulphur, indicated by the solubility products for AgBr and Ag_2S, which are $5 \times 10^{-13} mol^2 l^{-2}$ and $6 \times 10^{-50} mol^3 l^{-3}$ respectively. In addition, the lipophilic nature of thiols (compared to Br^-) would facilitate their access to any site of action of Ag^+ that may be located within the cell membrane. The fact that phosphate uptake can be restored to silver-treated cells is further proof that these cells remained intact, and indicates that the interaction of Ag^+ with cells was reversible.

Silver ions inhibited the exchange of intracellular and extracellular phosphate through the Pit system (Fig. 3.10). This inhibition of exchange was observed under the same conditions as the inhibition of phosphate uptake. Exchange can occur under conditions of energy depletion, but Rosenberg *et al.* (1982) state that a limited amount of energy may be required for exchange to occur. If that is the case then Ag^+, by interfering drastically with the energy supply, may inhibit both exchange and uptake of phosphate, with higher concentrations of Ag^+ inhibiting energy coupling to such an extent that the concentration gradient of phosphate cannot be maintained.

At temperatures near $0^{o}C$, E. coli cannot take up or exchange phosphate (Rosenberg et al., 1982), so the phosphate carrier requires a fluid membrane to be able to function. When $AgNO_3$ was added to cells at $0^{o}C$, efflux of phosphate still occurred (Fig. 3.11). Examination of the proportions of ester-P and inorganic phosphate outside the cell after Ag^+ treatment indicates that the cell membrane remained intact. This result implies that phosphate efflux did not occur through the carrier that catalyses phosphate uptake and exchange. In this context, it is interesting to note that Meury et al. (1980) have observed efflux of potassium ions from cells after treatment with N-ethylmaleimide and have proposed K^+ channels distinct from the uptake systems.

The results show that Ag^+ had a severe effect on the ability of cells to accumulate and retain phosphate. The effect of Ag^+ appeared to be on energy coupling and there was some indication that the site of action related to efflux was within the cell membrane. In view of these effects, and of the postulated function for chloride within the membrane as a carrier for anhydrous intramembrane protons (Robertson and Boardman, 1975), one possible mechanism of Ag^+ action could be interaction with chloride, precipitating as AgCl and effectively removing the proton carrier. In this way the coupling of energy via intramembrane protons would be blocked.

CHAPTER 4

OTHER INHIBITORS OF PHOSPHATE UPTAKE

INTRODUCTION

It is now fairly well established that uncouplers of oxidative phosphorylation inhibit active transport by conducting protons across the lipid membrane, thereby collapsing the protonmotive force (Harold et al., 1974; Heytler, 1979). Both the protonated acid form and the anion can diffuse through the lipid phase of the membrane, the protonated form carrying H^+ down its concentration gradient and the anion diffusing back through the membrane to accept another proton. It must be remembered, however, that in addition to their 'proton porter' function, individual uncouplers may have other effects on cells. For example, the action of CCCP is prevented by thiols (Heytler, 1963; Kaback et al., 1974), indicating a possible interaction of the uncoupler with sulphydryl groups, and molecular shape may best account for the observed activity of the salicylanilide series (Storey et al., 1975).

N-ethylmaleimide (NEM), a specific reagent for sulphydryl groups (Riordan and Vallee, 1972), inhibits energised uptake of phosphate through the Pit system (Rosenberg et al., 1982). It has no effect on phosphate exchange per se, but inhibits the hydrolysis and formation of ester-P within the cell. Its effect is to decrease the amount of ^{32}P chased from the cell by excess non-radioactive P_i by inhibiting turnover between the ester-P and P_i pools.

Trialkyltin compounds, in addition to effects similar to oligomycin in mitochondria, catalyse a Cl^-/OH^- exchange across membranes in media containing chloride (Selwyn et al., 1970; Stockdale et al., 1970). Singh and Bragg (1979) observed an inhibition by tributyltin chloride of the uptake of proline and glutamine by E. coli. The inhibitor also caused efflux of previously accumulated proline and glutamine. These effects required the

presence of chloride, bromide or iodide ions. Tributyltin (TBT) also mediated a chloride-dependent dissipation of a pH gradient in intact cells and everted membrane vesicles. They concluded that the OH^-/anion exchange catalysed by tributyltin chloride dissipated the ΔpH component of the pmf, and was responsible for the action of the inhibitor on proline and glutamine uptake and retention. Triphenyltin inhibition of transmembrane proton transfer in chloroplasts (Gould,1976; 1978) does not involve chloride/hydroxyl exchange, but the interaction of triphenyltin with the membrane-bound component of the chloroplast ATPase, the CF_0 proton pore. The evidence indicated that triphenyltin blocked the proton pore by reaction with vicinal dithiols. The inhibition by TBT of the energy-dependent transhydrogenation of $NADP^+$ by NADH in E. coli membranes (Singh and Bragg, 1979a) also appears to involve sulphydryl groups. This inhibition does not require the presence of a permeant anion, is reversed by thiol compounds, especially dithiols, and occurs at concentrations of TBT that are 10-fold higher than those inhibiting proton translocation.

In this chapter I show that uncouplers of oxidative phosphorylation blocked uptake of phosphate through the Pit system but did not cause efflux of accumulated phosphate. Their effects on silver action varied: carbonyl cyanide phenylhydrazones blocked Ag^+-induced efflux of phosphate and partially inhibited exchange, but did not prevent the total inhibition of exchange by Ag^+. They also increased the binding of ^{110}Ag to cells, and caused partitioning of ^{110}Ag into octanol; DNP did not block exchange or Ag^+-induced efflux of phosphate, and TCS blocked both exchange and Ag^+-induced phosphate efflux. Neither of the latter two uncouplers affected the partitioning of ^{110}Ag between aqueous and organic phases. NEM caused some efflux of phosphate, blocked Ag^+-induced

efflux of phosphate but not the inhibition of exchange by Ag^+, and slightly inhibited exchange. Mercuric ions inhibited phosphate uptake, exchange, and Ag^+-induced efflux. Tributyltin caused efflux of phosphate, appearing to have two modes of action, one requiring chloride. TBT and uncouplers acted synergistically, causing an increased, chloride-independent, efflux of phosphate. Inhibitors of the respiratory chain did not cause efflux of phosphate. Prevention of the operation of the respiratory chain by anaerobiosis did not affect the ability of silver ions to cause efflux of phosphate.

METHODS

The materials and methods used have been described in Chapter 2.

RESULTS

4.1 Effects of uncouplers on phosphate exchange and silver ion-induced efflux of phosphate.

When CCCP was added to cells accumulating P_i, further uptake was blocked and Ag^+ no longer caused P_i efflux from these cells (Fig. 4.1a). CCCP-treated cells still showed phosphate exchange (Fig. 4.1b), although at a lower rate than in untreated cells (see Fig. 3.10), but this exchange was blocked by Ag^+ (Fig. 4.1). Cells treated with FCCP showed the same result (Fig. 4.2A,B). DNP inhibited neither exchange nor Ag^+-induced efflux (Fig. 4.2C), and TCS blocked both phosphate exchange and the efflux of phosphate caused by Ag^+ (Fig. 4.2D). The uncoupler concentrations used in these experiments were the lowest which still blocked uptake. The higher concentrations tested did not cause efflux of phosphate (data not shown).

Since all uncouplers did not inhibit Ag^+-induced efflux, the inhibition of this efflux by CCCP and FCCP may not be due to the uncoupling action of these compounds and could possibly involve an interaction with Ag^+. This could also be the case with TCS, although the concomitant inhibition of exchange, and the apparent lack of interaction of TCS with Ag^+ (see Section 4.2), may indicate another mechanism.

Fig. 4.1 Effects of CCCP and Ag^+ on phosphate uptake and exchange.

Phosphate uptake was assayed in strain AN710 as described in Chapter 2.

a) Ag^+ added before 20mM P_i

b) Ag^+ added during exchange

CCCP (10μM), KBr (2mM), P_i (20mM) and $AgNO_3$ (20μM) added as shown.

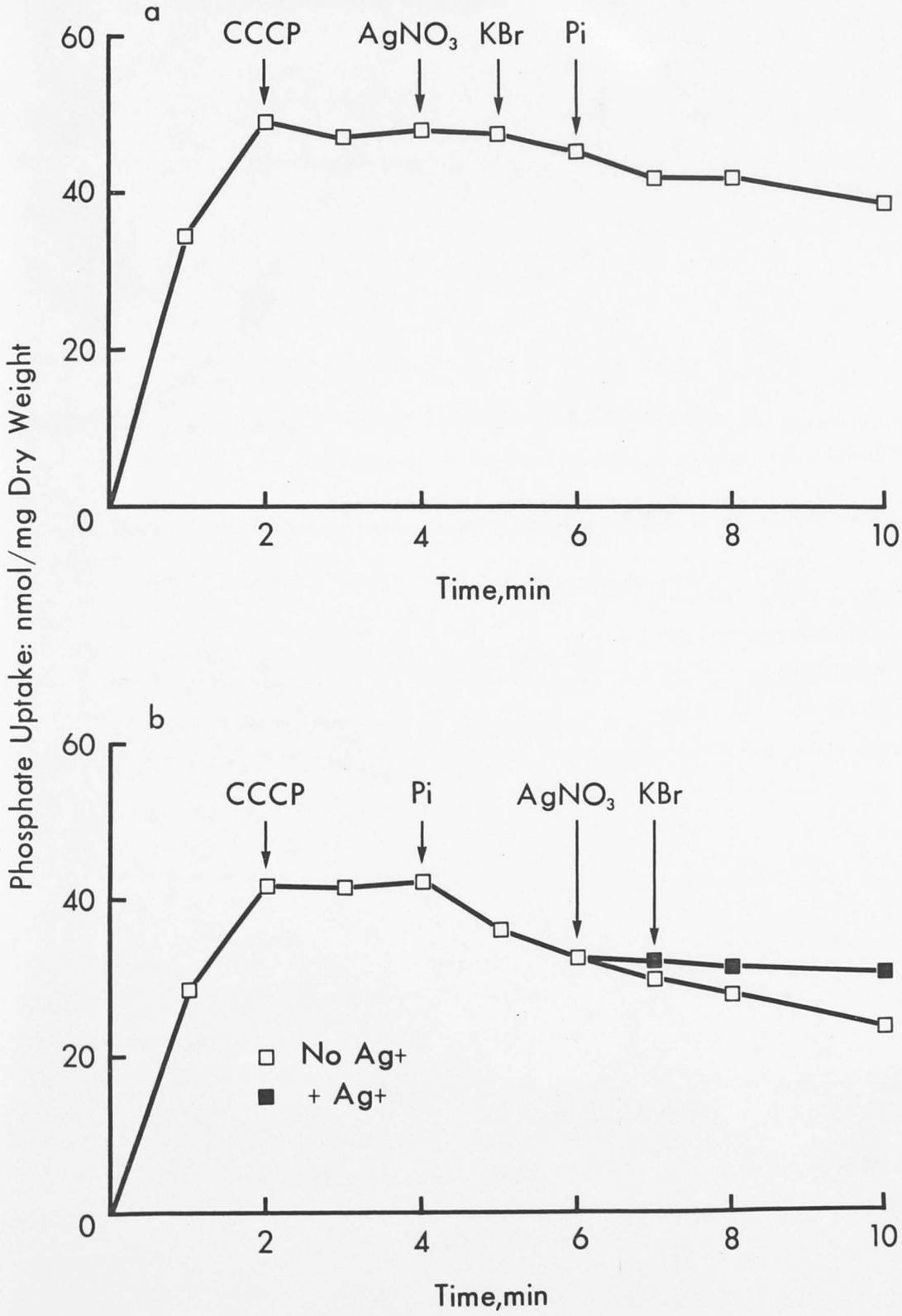

a
CCCP
AgNO3
KBr
Pi
60
40
20
0
2
4
6
8
10
Time,min
Phosphate Uptake: nmol/mg Dry Weight
b
CCCP
Pi
AgNO3
KBr
No Ag+
+ Ag+
60
40
20
0
2
4
6
8
10
Time,min

Fig. 4.2 The effects of FCCP, TCS and DNP on phosphate uptake, exchange, and the Ag^{+}-induced efflux of phosphate.

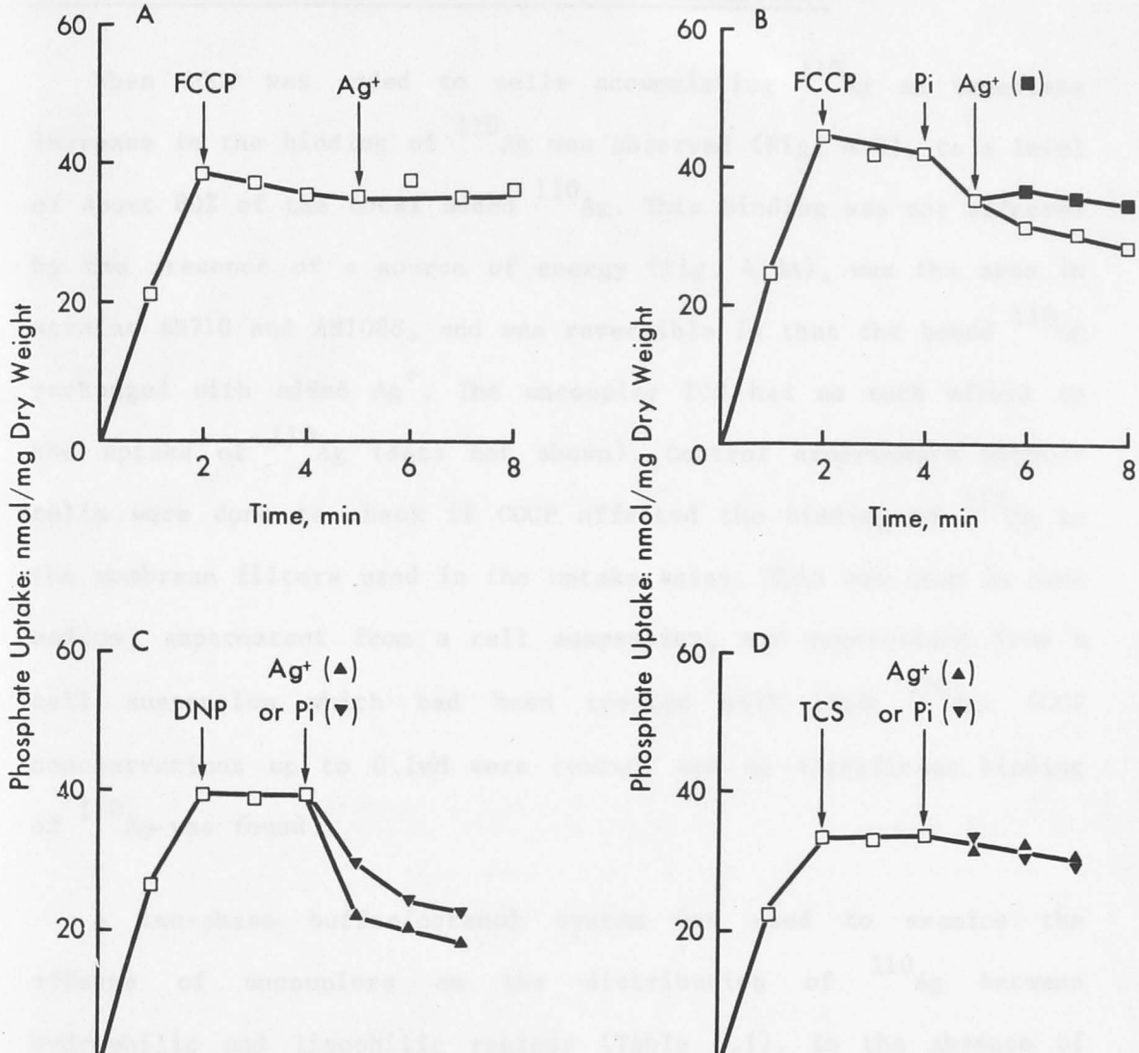
The experiments were performed using strain AN710 as in Fig. 4.1.

A) and B) FCCP effects

C) DNP effects

D) TCS effects

FCCP (20μM), TCS (50μM), or DNP (1mM), and $AgNO_3$ (20μM) and P_i (20mM) were added as shown.


A
FCCP
Ag+
B
FCCP
Pi
Ag+ (■)
C
DNP
Ag+ (▲)
or Pi (▼)
D
TCS
Ag+ (▲)
or Pi (▼)
Phosphate Uptake: nmol/mg Dry Weight
Time, min
0
20
40
60
2
4
6
8

4.2 Interactions between silver ions and uncouplers.

When CCCP was added to cells accumulating ^{110}Ag an immediate increase in the binding of ^{110}Ag was observed (Fig. 4.3), to a level of about 80% of the total added ^{110}Ag. This binding was not affected by the presence of a source of energy (Fig. 4.3A), was the same in strains AN710 and AN1088, and was reversible in that the bound ^{110}Ag exchanged with added Ag^{+}. The uncoupler TCS had no such effect on the uptake of ^{110}Ag (data not shown). Control experiments without cells were done to check if CCCP affected the binding of ^{110}Ag to the membrane filters used in the uptake assay. This was done in pure medium, supernatant from a cell suspension, and supernatant from a cell suspension which had been treated with 20μM ^{110}Ag. CCCP concentrations up to 0.1mM were tested, and no significant binding of ^{110}Ag was found.

A two-phase buffer/octanol system was used to examine the effects of uncouplers on the distribution of ^{110}Ag between hydrophilic and lipophilic regions (Table 4.1). In the absence of uncoupler, ^{110}Ag remained totally within the aqueous phase. TCS and DNP had little effect on the distribution of ^{110}Ag, but CCCP and FCCP caused significant amounts of ^{110}Ag to appear in the octanol phase.

4.3 Effects of N-ethylmaleimide on silver action.

The sulphydryl reagent N-ethylmaleimide (NEM) has been shown to inhibit the uptake of phosphate without interfering with exchange (Rosenberg *et al.*, 1982). When added to cells which had accumulated $^{32}P_i$ NEM caused a slow, steady efflux of ^{32}P (Fig. 4.4). The efflux rate was low and did not markedly increase with increasing NEM concentration (3.0 nmol ^{32}P/min/mg dry weight of cells at 0.05mM

Fig. 4.3 Effects of CCCP on ^{110}Ag uptake.

$^{110}Ag^{+}$ uptake was measured as described in Fig. 3.5. $^{110}AgNO_3$ concentration was 20μM. Under these conditions (A_{660} = 0.35), 100% ^{110}Ag binding (10nmol ^{110}Ag bound/0.5ml sample) is equivalent to 134 nmol ^{110}Ag/mg Dry Weight.

A) Uptake measured in strain AN710. CCCP (10μM) added where shown. Open symbols - no carbon source. Closed symbols - 20mM succinate.

B) CCCP (10μM) and non-radioactive $AgNO_3$ (20mM) added where shown. No carbon source.

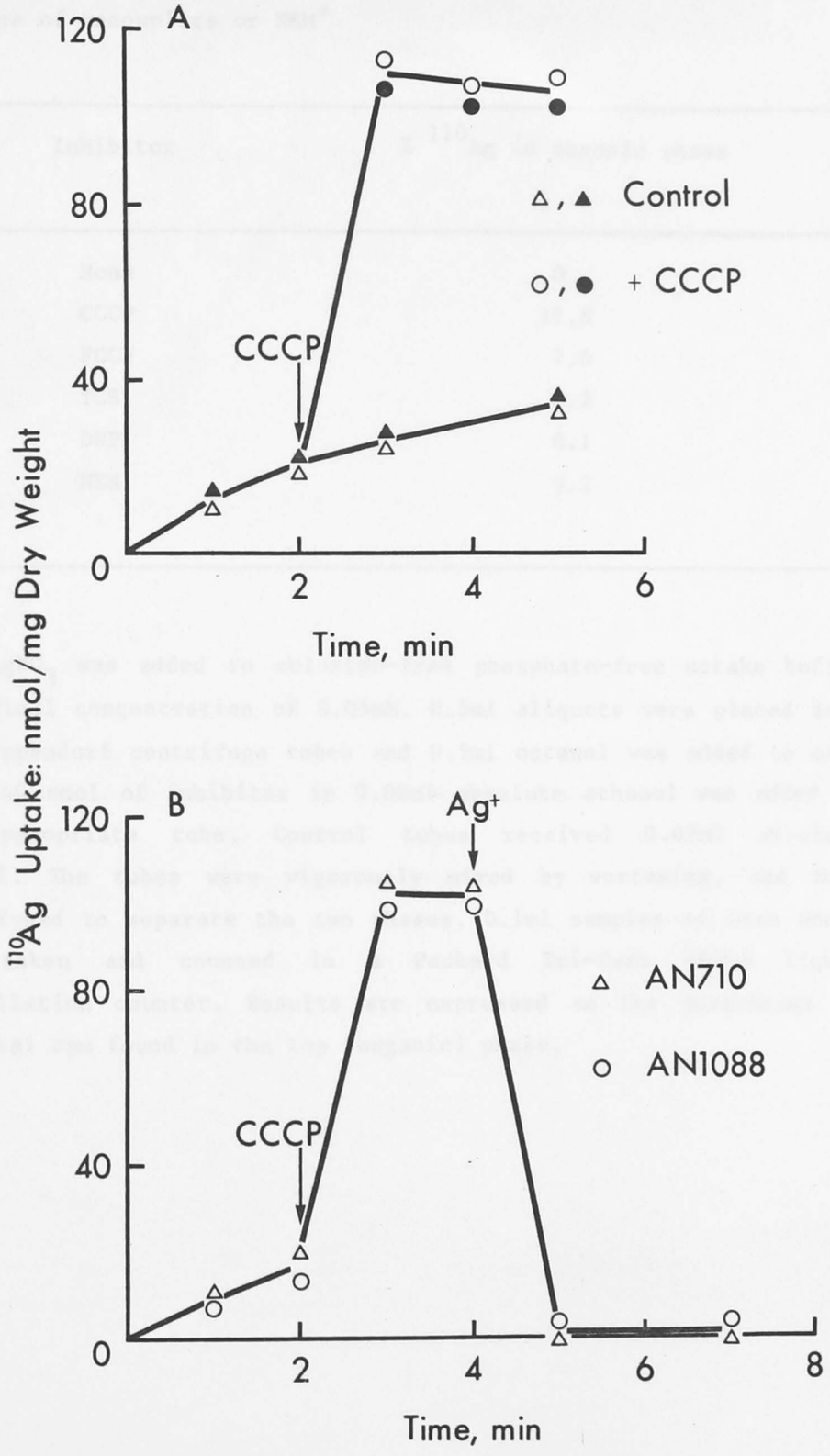

A
120
80
40
0
2
4
6
Time, min
△, ▲ Control
○, ● + CCCP
CCCP
^{110}Ag Uptake: nmol/mg Dry Weight
B
120
80
40
0
2
4
6
8
Time, min
Ag+
CCCP
△ AN710
○ AN1088

Table 4.1 Distribution of ^{110}Ag between water and octanol in the presence of uncouplers or NEM[a]

Inhibitor	% ^{110}Ag in organic phase
None	0
CCCP	11.8
FCCP	7.6
TCS	0.2
DNP	0.1
NEM	0.2

a $^{110}AgNO_3$ was added to chloride-free phosphate-free uptake buffer to a final concentration of 0.05mM. 0.5ml aliquots were placed into 2 ml Eppendorf centrifuge tubes and 0.5ml octanol was added to each tube. 40 nmol of inhibitor in 0.02ml absolute ethanol was added to the appropriate tube. Control tubes received 0.02ml absolute ethanol. The tubes were vigorously mixed by vortexing, and then centrifuged to separate the two phases. 0.1ml samples of each phase were taken and counted in a Packard Tri-Carb 460CD liquid scintillation counter. Results are expressed as the percentage of the total cpm found in the top (organic) phase.

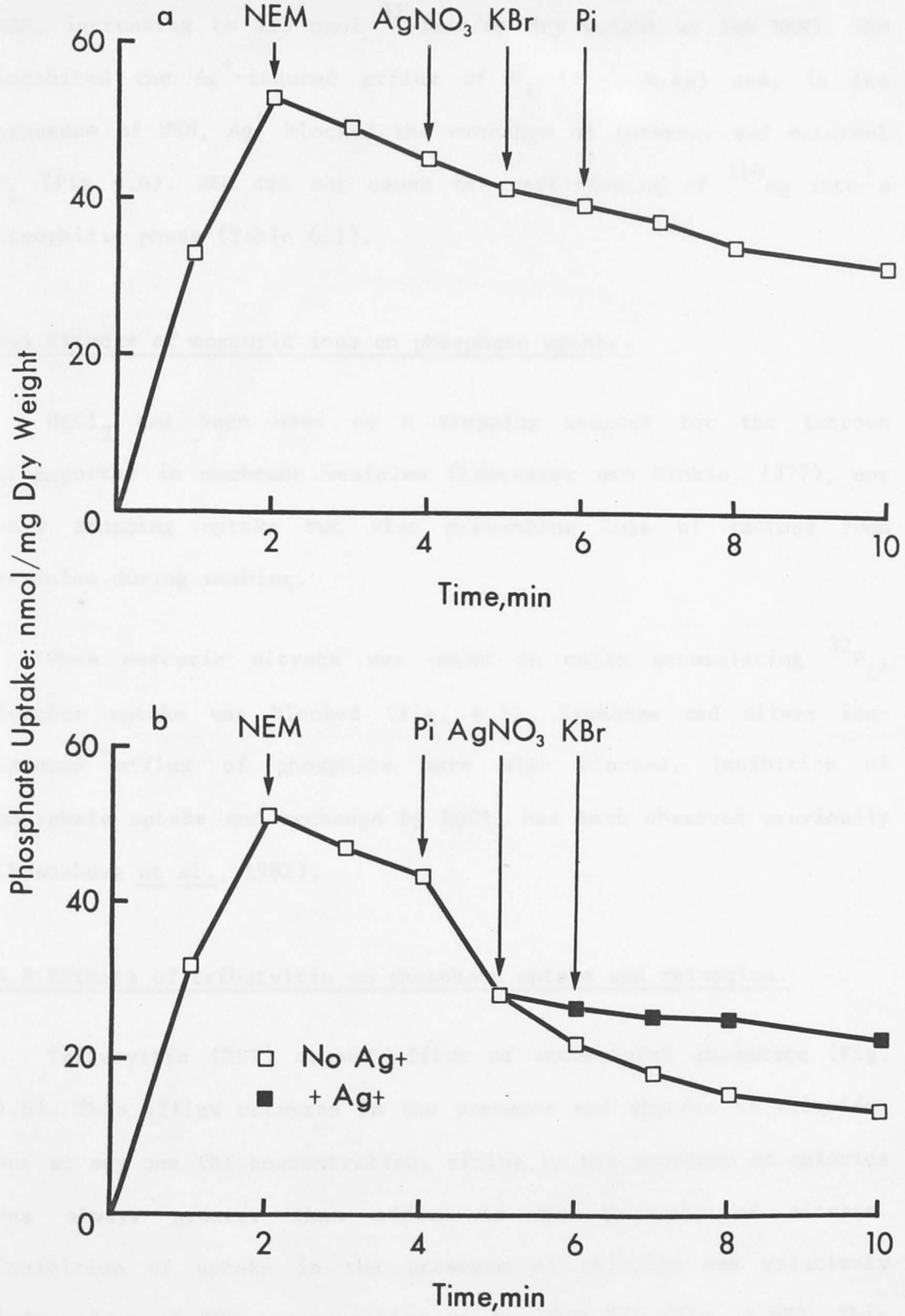
Fig. 4.4 Effects of NEM and Ag^+ on phosphate uptake and exchange.

Phosphate uptake was assayed in strain AN710 as described in Chapter 2.

a) Ag^+ added before 20mM P_i

b) Ag^+ added during exchange

NEM (50μM), KBr (2mM), $AgNO_3$ (20μM) and P_i (20mM) added as shown.


a
NEM
AgNO3
KBr
Pi
60
40
20
0
2
4
6
8
10
Time,min
Phosphate Uptake: nmol/mg Dry Weight
b
NEM
Pi
AgNO3
KBr
No Ag+
+ Ag+
60
40
20
0
2
4
6
8
10
Time,min

NEM, increasing to 5.3 nmol ^{32}P/min/mg dry weight at 1mM NEM). NEM inhibited the Ag^+-induced efflux of P_i (Fig. 4.4a) and, in the presence of NEM, Ag^+ blocked the exchange of internal and external P_i (Fig 4.4). NEM did not cause the partitioning of ^{110}Ag into a lipophilic phase (Table 4.1).

4.4 Effects of mercuric ions on phosphate uptake.

$HgCl_2$ has been used as a stopping reagent for the lactose transporter in membrane vesicles (Lancaster and Hinkle, 1977), not only stopping uptake but also preventing loss of lactose from vesicles during washing.

When mercuric nitrate was added to cells accumulating $^{32}P_i$, further uptake was blocked (Fig. 4.5). Exchange and silver ion-induced efflux of phosphate were also blocked. Inhibition of phosphate uptake and exchange by $HgCl_2$ has been observed previously (Rosenberg et al., 1982).

4.5 Effects of tributyltin on phosphate uptake and retention.

Tributyltin (TBT) caused efflux of accumulated phosphate (Fig. 4.6). This efflux occurred in the presence and absence of chloride, but at any one TBT concentration, efflux in the presence of chloride was always greater than efflux in the presence of nitrate. Inhibition of uptake in the presence of chloride was relatively independent of TBT concentration up to 10μM TBT (Fig. 4.6B). This may indicate a catalytic function for TBT, most likely chloride/hydroxyl exchange, already saturated at the lowest TBT concentration examined. The break in the curve, occurring both in the presence and absence of chloride, suggests that a second site of action for TBT is involved, possibly an interaction of TBT with

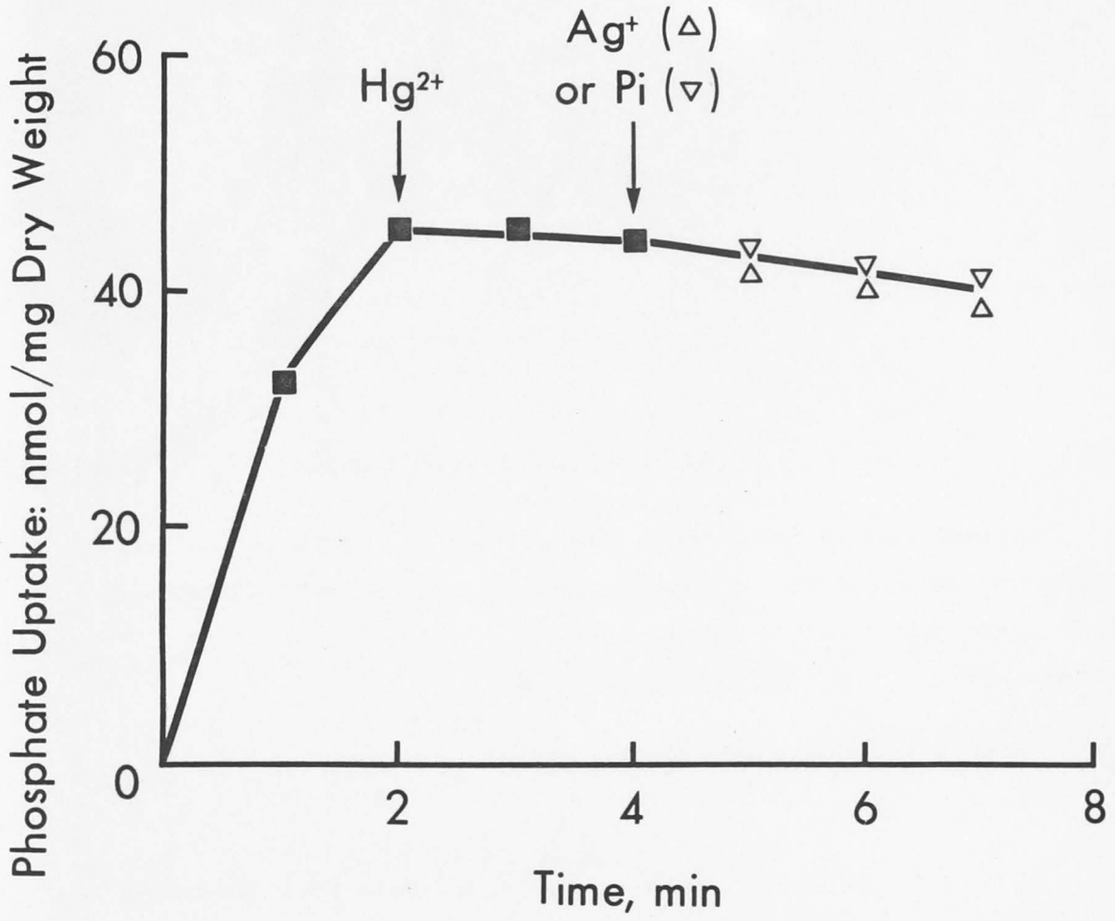


Fig. 4.5 Effects of mercuric ions on phosphate uptake.

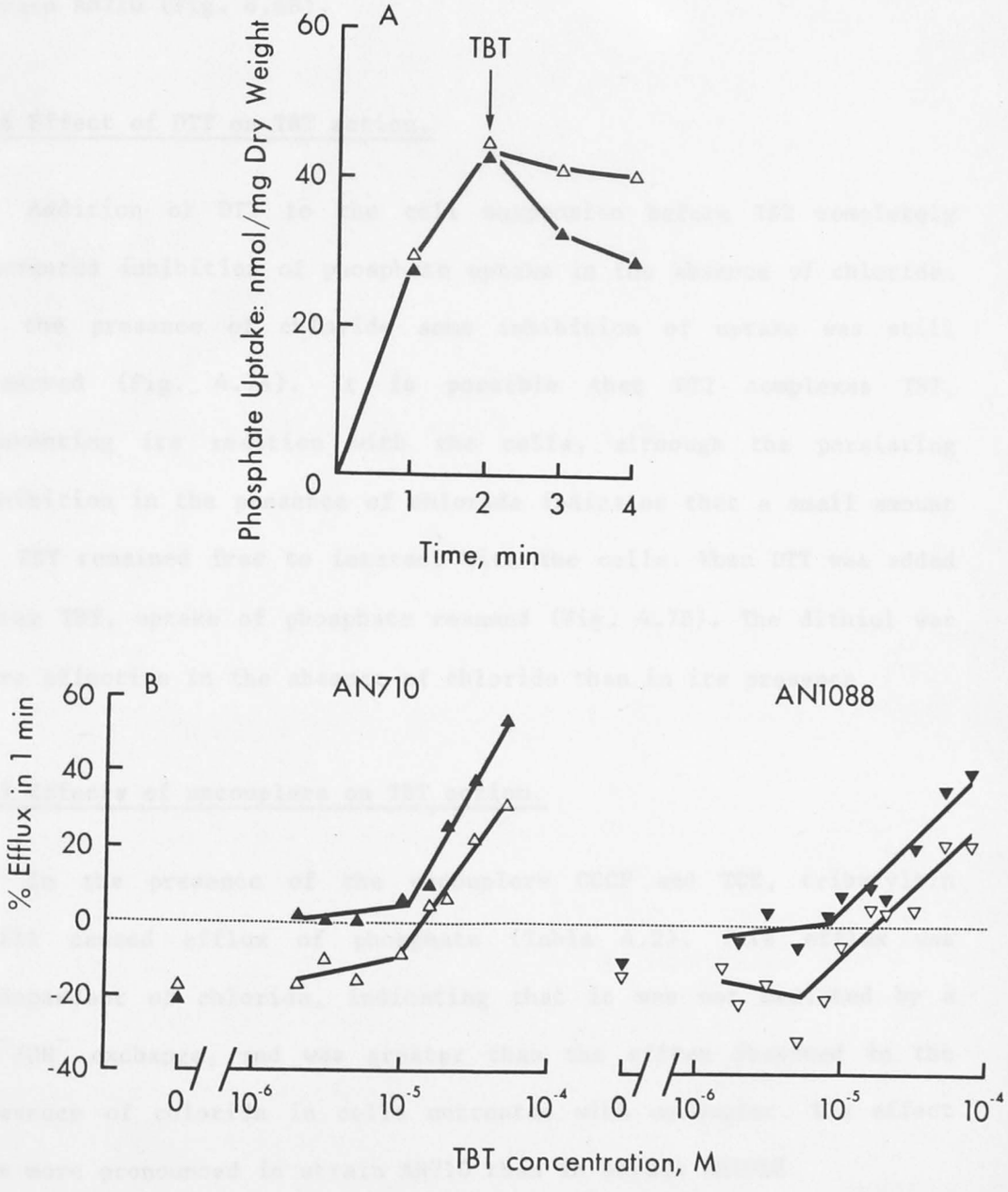
Phosphate uptake was measured in strain AN710 as described in Chapter 2. $Hg(NO_3)_2$ (12μM), $AgNO_3$ (20μM) and P_i (20mM) were added as shown.

Fig. 4.6 Effects of tributyltin on phosphate uptake.

Succinate-grown cells were prepared and phosphate uptake was measured as described in Chapter 2 with either KNO_3 (25mM) or KCl (25mM) added to the uptake medium.
Open symbols - KNO_3. Filled symbols - KCl.

A) Strain AN710. TBT acetate (20μM) was added at the time shown.

B) Strains AN710 and AN1088. The experiments were performed as in (A). ^{32}P in the cell was monitored at 2 and 3 minutes. Results are expressed as the percentage of ^{32}P lost from the cells in the first minute after TBT addition. Points below zero indicate that uptake still continued.


A
TBT
60
40
20
0
1
2
3
4
Phosphate Uptake: nmol/mg Dry Weight
Time, min
B
AN710
AN1088
60
40
20
0
-20
-40
% Efflux in 1 min
0
10^{-6}
10^{-5}
10^{-4}
TBT concentration, M

sulphydryl groups. Strain AN1088 was less sensitive to TBT than strain AN710 (Fig. 4.6B).

4.6 Effect of DTT on TBT action.

Addition of DTT to the cell suspension before TBT completely prevented inhibition of phosphate uptake in the absence of chloride. In the presence of chloride some inhibition of uptake was still observed (Fig. 4.7A). It is possible that DTT complexes TBT, preventing its reaction with the cells, although the persisting inhibition in the presence of chloride indicates that a small amount of TBT remained free to interact with the cells. When DTT was added after TBT, uptake of phosphate resumed (Fig. 4.7B). The dithiol was more effective in the absence of chloride than in its presence.

4.7 Effects of uncouplers on TBT action.

In the presence of the uncouplers CCCP and TCS, tributyltin still caused efflux of phosphate (Table 4.2). This efflux was independent of chloride, indicating that it was not mediated by a Cl^-/OH^- exchange, and was greater than the efflux observed in the presence of chloride in cells untreated with uncoupler. The effect was more pronounced in strain AN710 than in strain AN1088.

4.8 The effects of respiratory chain inhibitors on phosphate uptake.

Zinc ions inhibit the respiratory chain of *E. coli* (Kasahara and Anraku, 1972), probably at the less sensitive of the two Ag^+ sites (Bragg and Rainnie, 1974). Zn^{2+} inhibited phosphate uptake (Fig. 4.8) at concentrations about 1000 times higher than those of silver ions. Strain AN710 was more sensitive to the effects of Zn^{2+} than

Fig. 4.7 Effects of DTT on TBT-induced efflux of phosphate.

Phosphate was measured in two cell suspensions of strain AN710, one with KNO_3 (20mM) (Δ) and the other with KCl (20mM) (▲). The source of carbon was succinate.

A) DTT (50μM) was added 1 minute after $^{32}P_i$, followed 1 minute later by TBT acetate (20μM). ^{32}P was monitored for a further 2 minutes.

B) Two minutes after $^{32}P_i$ addition TBT acetate (20μM) was added, followed 1 minute later by DTT (50μM). ^{32}P was monitored for a further 2 minutes.

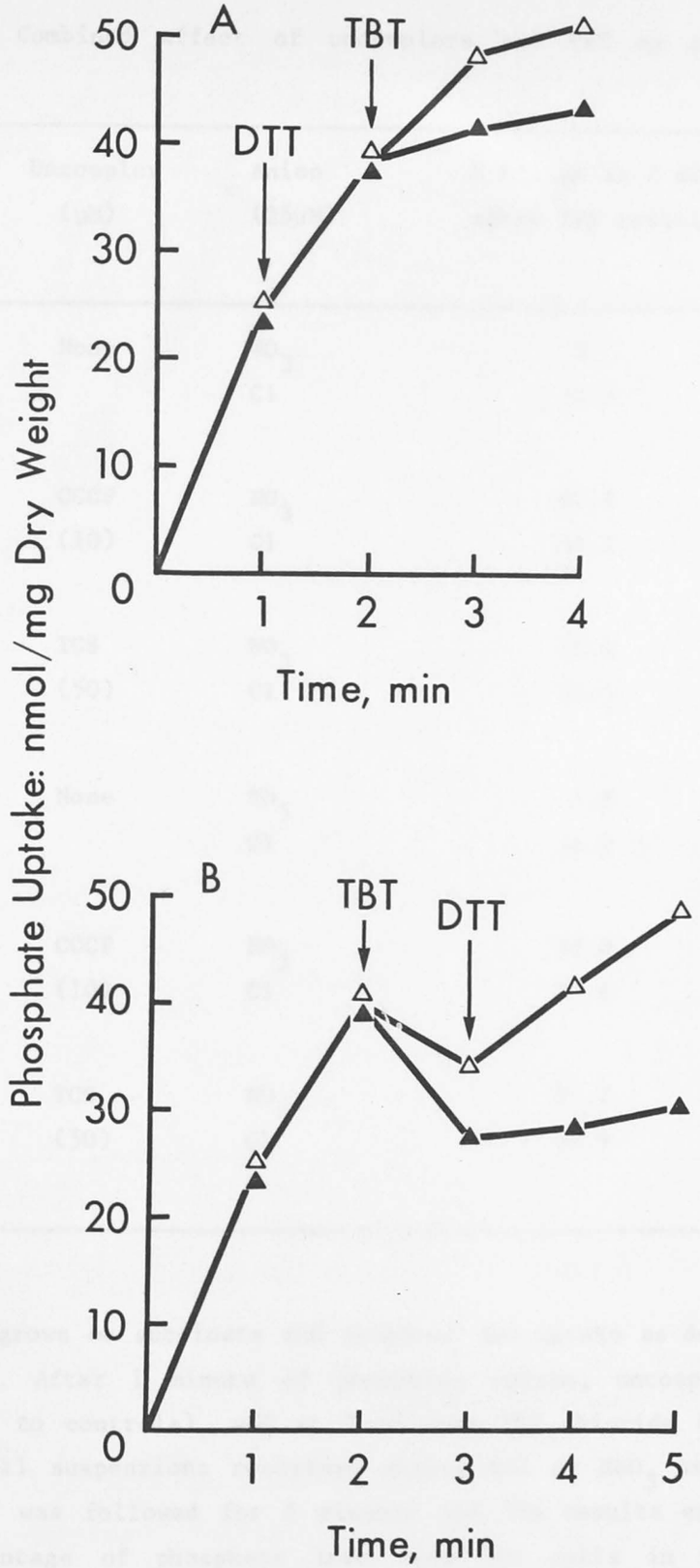

A
TBT
DTT
50
40
30
20
10
0
1
2
3
4
Time, min
Phosphate Uptake: nmol/mg Dry Weight
B
TBT
DTT
50
40
30
20
10
0
1
2
3
4
5
Time, min

Table 4.2 Combined effect of uncouplers and TBT on phosphate retention[a]

Strain	Uncoupler (μM)	Anion (25μM)	% Efflux in 2 min after TBT addition
AN710	None	NO_3	0
		Cl	22.7
	CCCP	NO_3	47.2
	(10)	Cl	47.1
	TCS	NO_3	36.4
	(50)	Cl	33.5
AN1088	None	NO_3	1.2
		Cl	11.1
	CCCP	NO_3	32.0
	(10)	Cl	35.6
	TCS	NO_3	23.7
	(50)	Cl	20.9

[a] Cells were grown on succinate and prepared for uptake as described in Chapter 2. After 1 minute of phosphate uptake, uncoupler was added (except to controls), and at 2 minutes TBT chloride (6.25μM) was added. Cell suspensions contained either KCl or KNO_3 as shown. Efflux of ^{32}P was followed for 2 minutes and the results expressed as the percentage of phosphate lost from the cells in those 2 minutes. The amounts of ^{32}P in the cells prior to TBT addition were 34.5 and 43 nmol/mg dry weight of cells for strains AN710 and AN1088 respectively.

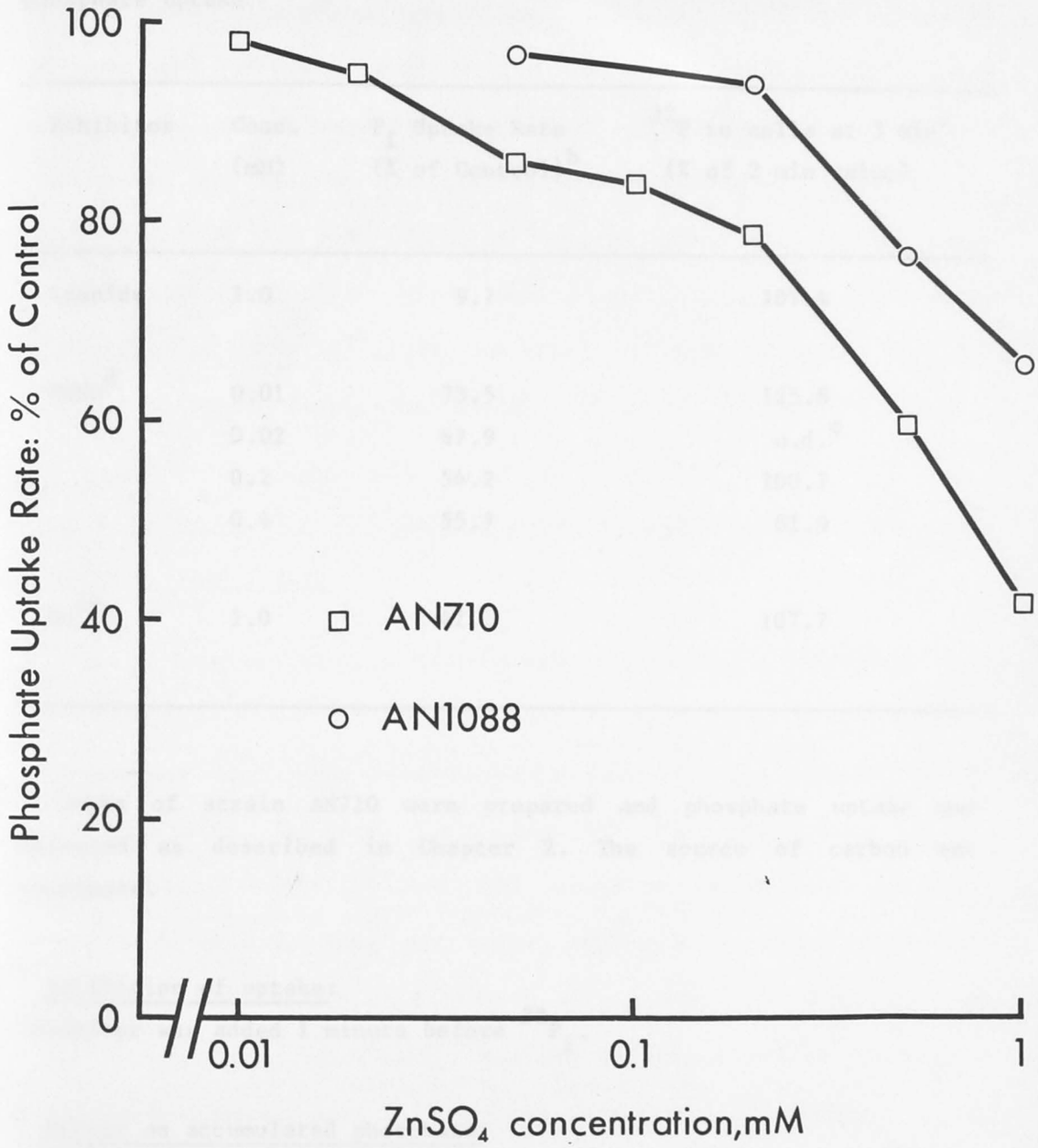


Fig. 4.8 Inhibition of phosphate uptake by zinc ions.

The experiment was carried out as described in Fig. 3.1, except that the chloride-free phosphate-free uptake buffer containing MES was used. Results are expressed as the percentage of the uptake rate observed with untreated cells.

Table 4.3 Effects of inhibitors of the respiratory chain on phosphate uptake[a]

Inhibitor	Conc. (mM)	P_i Uptake Rate (% of Control)[b]	^{32}P in cells at 3 min[c] (% of 2 min value)
Cyanide	1.0	9.7	107.4
HQNO[d]	0.01	73.5	125.8
	0.02	67.9	n.d.[e]
	0.2	54.2	100.7
	0.4	55.7	81.9
Zn^{2+}	1.0	42.1	107.7

[a] Cells of strain AN710 were prepared and phosphate uptake was measured as described in Chapter 2. The source of carbon was succinate.

[b] Inhibition of uptake:
Inhibitor was added 1 minute before $^{32}P_i$.

[c] Effect on accumulated phosphate:
Inhibitor was added after 2 minutes of phosphate uptake, and ^{32}P was monitored for a further minute. Results are expressed as % ^{32}P present in cells at 3 minutes, relative to 100% at 2 minutes. Control suspensions showed 125% at 3 minutes.

[d] Cell suspensions receiving HQNO had been treated with EDTA as described in Chapter 2.

[e] n.d. - not determined.

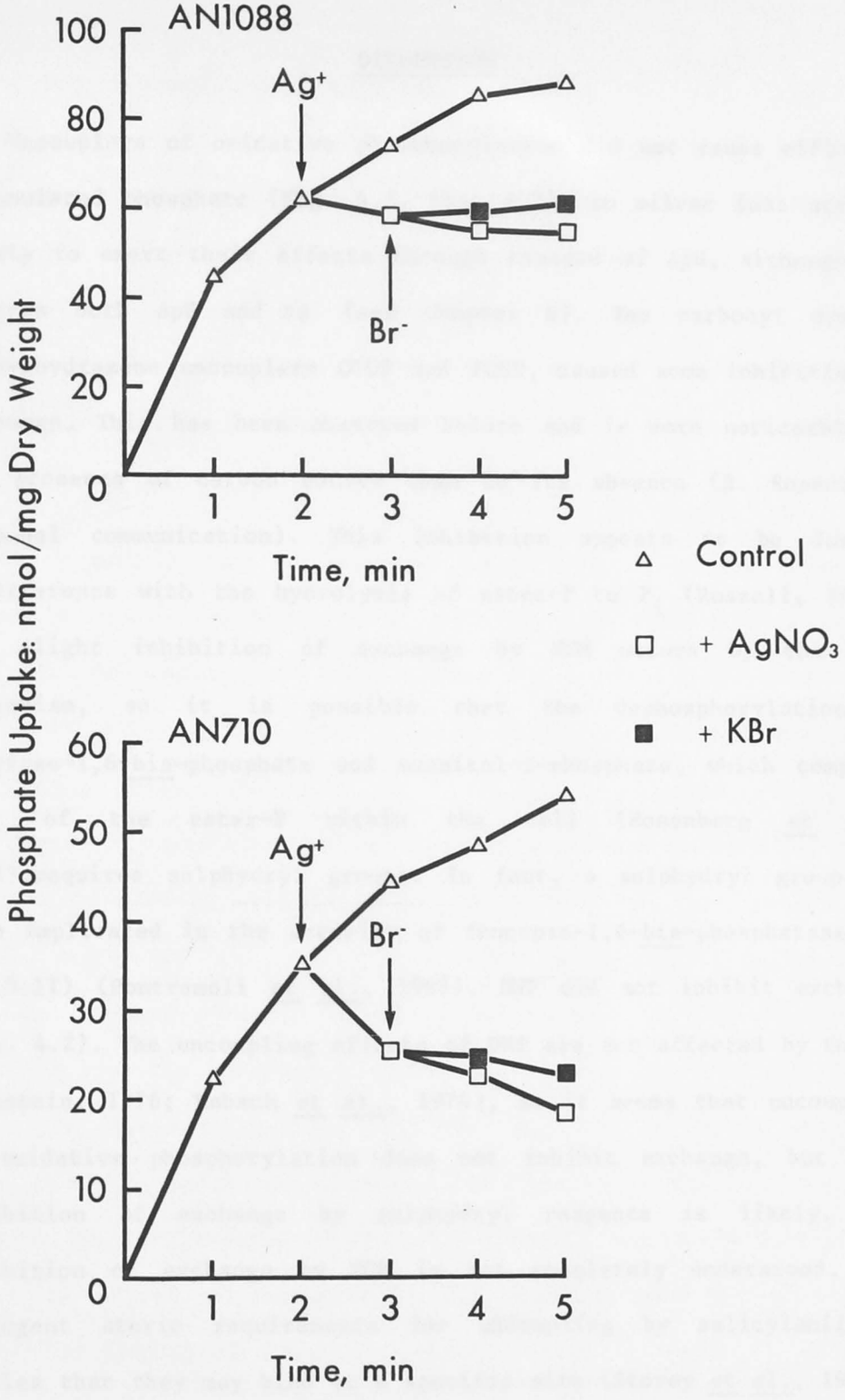
strain AN1088. Concentrations of Zn^{2+} above 1mM could not be tested because zinc phosphate precipitated and was trapped on the filters used in the uptake assay. Zn^{2+} did not cause efflux of accumulated phosphate, and neither did cyanide at concentrations where uptake was inhibited by 90% (Table 4.3). The inhibitor 2-n-heptyl-4-hydroxyquinoline-N-oxide (HQNO) did cause efflux, but only at a concentration (4×10^{-4}M) far in excess of those normally used for inhibition of the respiratory chain (10^{-8} to 10^{-5}M) (Appleby, 1969).

4.9 The effects of silver ions on phosphate uptake under anaerobic conditions.

As a further check on the involvement of the respiratory chain in the effects of silver on phosphate transport, anaerobic cell suspensions were treated with silver ions. If the effects of Ag^{+} were mediated by its inhibition of the respiratory chain, silver-induced efflux of phosphate should not occur under anaerobic conditions. Silver ions did cause efflux under these conditions (Fig. 4.9). The extent of efflux in strain AN710 was about the same as that observed aerobically, whereas efflux in strain AN1088 was about half (compare Fig. 3.3, glucose as a source of carbon).

Fig. 4.9 The effects of silver ions on phosphate uptake under anaerobic conditions.

Cells were grown using glucose as a source of carbon and prepared for uptake as described in Chapter 2. For each assay 5ml of washed cells were placed into 10ml Kimax tubes and purged of oxygen by bubbling for 5 minutes with N_2 which had been passed through a double NILOX scrubber (from Jencons, Hemel Hempstead, U.K.). Bubbling with N_2 continued, $^{32}P_i$ (50μM) was added, and samples (0.5ml) were taken, filtered, washed and counted as described previously. $AgNO_3$ (20μM) and KBr (2mM) were added as shown. The source of energy was glucose.


AN1088
100
80
60
40
20
0
Ag+
Br-
1
2
3
4
5
Time, min
Phosphate Uptake: nmol/mg Dry Weight
Control
+ AgNO3
+ KBr
AN710
60
50
40
30
20
10
0
Ag+
Br-
1
2
3
4
5
Time, min

DISCUSSION

Uncouplers of oxidative phosphorylation did not cause efflux of accumulated phosphate (Fig. 4.1, Fig. 4.2), so silver ions are not likely to exert their effects through changes of ΔpH, although Ag^+ affects both ΔpH and $\Delta\psi$ (see Chapter 6). The carbonyl cyanide phenylhydrazone uncouplers CCCP and FCCP, caused some inhibition of exchange. This has been observed before and is more noticeable in the presence of carbon source than in its absence (H. Rosenberg, personal communication). This inhibition appears to be due to interference with the hydrolysis of ester-P to P_i (Russell, 1979). The slight inhibition of exchange by NEM occurs by the same mechanism, so it is possible that the dephosphorylation of fructose-1,6-bis-phosphate and mannitol-1-phosphate, which comprise most of the ester-P within the cell (Rosenberg et al., 1982),requires sulphydryl groups. In fact, a sulphydryl group has been implicated in the activity of fructose-1,6-bis-phosphatase (EC 3.1.3.11) (Pontremoli et al., 1965). DNP did not inhibit exchange (Fig. 4.2). The uncoupling effects of DNP are not affected by thiols (Hanstein, 1976; Kaback et al., 1974), so it seems that uncoupling of oxidative phosphorylation does not inhibit exchange, but some inhibition of exchange by sulphydryl reagents is likely. The inhibition of exchange by TCS is not completely understood. The stringent steric requirements for uncoupling by salicylanilides implies that they may bind at a specific site (Storey et al., 1975), and this site may be involved in some way with exchange of phosphate.

In the presence of FCCP and CCCP, Ag^+-induced efflux of phosphate was completely inhibited, but the inhibition of exchange by Ag^+ was not prevented (Fig. 4.2). DNP did not prevent Ag^+-induced efflux, and TCS blocked it, so the effects of these four uncouplers on Ag^+ action is probably not due to their function as proton porters, but to individual chemical effects. For example, the carbonyl cyanide chlorophenylhydrazones could have reacted with sulphydryl groups (Heytler, 1963; Kaback et al., 1974).

CCCP caused a massive increase in binding of silver ions to cells (Fig. 4.3). The bound label could be chased out of the cells by excess non-radioactive Ag^+, indicating that the binding was reversible. CCCP and FCCP also facilitated partitioning of Ag^+ into a lipophilic phase (Table 4.1), so it is likely that the effects of this class of uncoupler on Ag^+ action can be explained by the formation of CCCP-Ag^+ complexes in the hydrophobic environment of the cell membrane. Ag^+ could still inhibit exchange, so either the free Ag^+ concentration remained sufficient to effect this inhibition, or the CCCP-Ag^+ complex itself was effective in this regard. DNP did not interact with Ag^+ and had no effect on Ag^+ action, but TCS, while not affecting the binding of Ag^+ to the cell or aiding the partitioning of Ag^+ into octanol, completely inhibited Ag^+-induced efflux of phosphate. It is possible that TCS competes with Ag^+ for binding to a specific site.

The effects of NEM on Ag^+ action were similar to those of CCCP (Fig. 4.4), although, unlike CCCP it did not partition ^{110}Ag into a lipophilic phase (Table 4.1). The fact that NEM caused some efflux of phosphate, but no inhibition of exchange, may be due to an interaction of NEM at the same site as Ag^+ but with less drastic consequences. Competition between NEM and Ag^+ may be sufficient to

prevent Ag^+ from causing efflux in the presence of NEM, while still allowing enough Ag^+ to bind to inhibit exchange. Alternatively, the inhibition of exchange by Ag^+ may be mediated by another site, not sensitive to NEM.

Mercuric ions bind to the sulphydryl groups of proteins in membranes, interfering with membrane structure and function (Berlin, 1979). Mercuric chloride, when used as a stopping agent for the lactose transporter (Lancaster and Hinkle, 1977), stopped both uptake of lactose and its loss from vesicles, suggesting that the membrane, while remaining intact, was so 'frozen' that nothing could pass through it.

When added to cells taking up phosphate, mercuric nitrate inhibited uptake of phosphate, as well as phosphate exchange and Ag^+-induced efflux of phosphate (Fig. 4.5). Comparison of this result with the effect of low temperature on Ag^+ action (Section 3.10) shows that mercuric ions, in addition to preventing uptake and exchange of phosphate, caused enough disruption of the cell membrane to close the putative channel through which phosphate exits in response to Ag^+ treatment.

Tributyltin caused the efflux of previously accumulated phospate (Fig.4.6). This efflux was greater in the presence of chloride, but the requirement for chloride was not absolute. The biphasic nature of the dependence of efflux on the TBT concentration (Fig. 4.6B) suggests that TBT caused efflux of accumulated phosphate by two different mechanisms. Below 10μM TBT the effect, relatively independent of TBT concentration, and requiring chloride, may involve Cl^-/OH^- exchange. The effect above 10μM TBT may be due to the interaction of TBT with sulphydryl groups, as has been found by Singh and Bragg (1979a) for TBT and by Gould (1976; 1978) for

triphenyltin. Note that chloride-independent inhibition of phosphate uptake was prevented (Fig. 4.7A) and reversed (Fig. 4.7B) more readily by dithiothreitol than the chloride-dependent effects. Strain AN1088 required higher TBT concentrations than strain AN710 for the same effect, indicating that TBT may have been interfering with some component of the energy coupling system, affecting pmf-coupled transport systems more than "phosphate-bond energy"-coupled systems.

Efflux of phosphate caused by TBT was independent of chloride when TBT was added to cells treated with uncouplers (Table 4.2). It was also 1.5- to 3-fold greater than that observed with chloride in the absence of uncouplers. This synergistic effect of uncouplers and TBT may be due to the unmasking by uncouplers of TBT-sensitive sites. These sites may not be readily available to TBT when the membrane is energised, and the collapse of the pH gradient caused by uncouplers may expose these sites to TBT. The action of TBT in this case was not mediated by Cl^-/OH^- exchange and may involve sulphydryl groups.

Since Ag^+ inhibits the respiratory chain (Yudkin, 1937; Bragg and Rainnie, 1974) it was possible that its effects were due to this inhibition. However, other inhibitors of the respiratory chain, while inhibiting uptake of phosphate, did not elicit this efflux (Table 4.3). This was the case with cyanide, inhibiting cytochrome d; HQNO, which inhibits near cytochrome b (Cox *et al.*, 1970), where Ag^+ also acts (Bragg and Rainnie, 1974); or Zn^{2+}, which is thought to inhibit between NADH or succinate and 'flavoprotein', at the other site for Ag^+ (Bragg and Rainnie, 1974). Also, silver ions caused efflux of previously accumulated phosphate under anaerobic conditions with glucose as a source of energy (Fig. 4.9). It

therefore seems likely that the effects of Ag^+ are not mediated solely by its inhibition of the respiratory chain.

Plate and Suit (1981) have recently described mutants of E. coli which were able to maintain a normal pmf but could not couple it to the transport of proline and methyl-β-D-thiogalactopyranoside (TMG), and which could not transport TMG in response to an artificially imposed ΔpH. The mutations were in a genetic locus which they designated eup, for energy uncoupled phenotype, and they postulated a role for the gene product of this locus in H^+/solute symport in the lactose and proline transport systems. It is possible that the eup product is the target for Ag^+. The above model for H^+/solute symport requires at least two polypeptides, the products of the eup and the lacY loci, for energy-coupled transport. Newman et al. (1981), however, postulate that a single polypeptide, the product of the lacY gene, is sufficient for energy-coupled lactose transport. This conclusion is based on successful experiments with proteoliposomes reconstituted from the pure lacY gene product and E. coli lipid. Reconstitution experiments using asolectin rather than E. coli lipid were not as successful, however (Newman and Wilson, 1980), even though a less purified preparation of the lacY product was used, so the possibility that the eup gene product forms a factor which is present in the E. coli lipid used in the reconstitution cannot be excluded.

Since neither uncouplers of oxidative phosphorylation nor inhibitors of the respiratory chain caused efflux of accumulated phosphate, the actions of Ag^+ leading to the efflux of phosphate are unlikely to be mediated by interference with the pH gradient or by inhibtion of the respiratory chain. The interactions of Ag^+ with uncouplers are complex and seem to be related to individual chemical

effects of the uncouplers rather than to their protonophoric action. Tributyltin acted similarly to Ag^+, the results suggesting two sites of action, one involving chloride and the other implicating sulphydryl groups. I conclude that a link in the energy transduction system, affected by both Ag^+ and TBT, may be responsible for sustaining a level of membrane energisation which maintains the concentration gradient of transported phosphate. Bearing in mind the chloride-dependence of tributyltin and the known effects of Ag^+ on chloride, it is possible that intramembrane anhydrous protons, transported within the lipid bilayer as covalent HCl (Robertson and Boardman, 1975), form part of this link. The possibility that Ag^+ acts on a protein responsible for coupling the proton gradient to transport in H^+-symport systems (Plate and Suit, 1981) cannot, however, be ruled out.

CHAPTER 5

EFFECTS ON OTHER TRANSPORT SYSTEMS

INTRODUCTION

The proline and glutamine transport systems in E. coli were the archetypal examples of shock-resistant, pmf-coupled transport, and shock-sensitive, P-coupled transport, respectively (Berger, 1973). The list of examples of both types of system has been expanded (Berger and Heppel, 1974; Rosen and Kashket, 1978) and in general the distinctions between the two types of system still hold true. However, "high-energy phosphate" linkage and coupling to the pmf need not be mutually exclusive (discussed in Chapter 1).

E. coli has an inducible transport system for succinate and other C_4-dicarboxylic acids which is saturable, specific and capable of concentrative uptake. This system is coded for by the genes dctA and dctB which may code for two membrane proteins, and cbt, which has been shown to code for a low molecular weight periplasmic binding protein (Kay and Kornberg, 1969; Lo et al., 1972). Succinate transport is sensitive to osmotic shock, and cells carrying a mutation in the cbt gene are unable to take up dicarboxylic acid (Lo et al., 1972), but vesicles prepared from these mutant cells are capable of uptake (Rayman et al., 1972). These data indicate that the succinate binding protein is not directly involved in passage of the solutes through the inner membrane, but may be required to facilitate passage through the outer membrane. Succinate transport is coupled to proton uptake such that the complex is electroneutral (Gutowski and Rosenberg, 1975) indicating that energisation of succinate uptake is by the pmf. Succinate transport into E. coli membrane vesicles (Rayman et al., 1972) and intact cells (Gutowski and Rosenberg, 1975) is severely inhibited by silver ions. Uncoupler (CCCP) causes efflux of accumulated succinate (Gutowski and Rosenberg, 1975).

Mannitol enters *E. coli* via the phosphoenolpyruvate:mannitol phosphotransferase system (Solomon and Lin, 1972; Lengeler, 1975; 1975a). Specificity for mannitol is conferred by the membrane-bound enzyme II^{mtl} coded for by the *mtlA* gene. As happens in all PTS systems, the transport substrate (in this case mannitol) is phosphorylated as it enters the cell, appearing in the cytoplasm as mannitol-1-phosphate.

Potassium transport in *E. coli* is performed by two systems, designated Kdp and TrkA (Epstein and Laimins, 1980).

The Kdp system is a high affinity system, with a K_m for K^+ of 2μM and V_{max} of 150 μmol/g/min, repressible by growth in high (10mM) K^+ concentrations (Rhoads *et al.*, 1976). This system is ATP-driven and was initially thought to have a periplasmic protein component (Rhoads and Epstein, 1977), but later investigations found no evidence for such a component or for an outer membrane component of the Kdp system (Laimins *et al.*, 1978; Rhoads *et al.*, 1978).

The TrkA system, which is constitutive, is the major potassium transport system of *E. coli*, with a V_{max} of 550 μmol/g/min and K_m for K^+ of 1.5mM (Rhoads *et al.*, 1976). This system requires both the pmf and ATP for activity (Rhoads and Epstein, 1977) and it has been proposed that this dual requirement indicates an ATP-driven system which is regulated by the pmf (Rhoads and Epstein, 1977; 1978). The genetics of this system are quite complex, with six *trk* genes widely scattered over the chromosome (Epstein and Kim, 1971). This complexity originally led to the belief that there were two additional systems, which were called TrkD and TrkF (Rhoads *et al.*, 1976). The evidence now suggests that there is a single system, TrkA, affected in many different ways by mutations in the *trk* genes (Epstein and Laimins, 1980).

Internal concentrations of K^+ are regulated in response to the osmotic strength of the suspension medium, from 150mM in 100mosM medium to 600mM in 1200mosM medium, essentially independently of the available external K^+ (Epstein and Schultz, 1965).

Potassium transport in Streptococcus faecalis, while less well characterised genetically than in E. coli, has been more amenable to studies of the effects of ionophores and uncouplers (Harold and Baarda, 1967; 1968; Harold and Altendorf, 1974). The basic mechanisms for K^+ transport in S. faecalis appear similar to those in E. coli (Harold and Altendorf, 1974). Uptake of K^+ appears to be in response to the pmf and occurs by exchange of K^+ for Na^+ or H^+ (Harold and Papineau, 1972). More recent work shows a requirement for both the pmf and ATP and the favoured explanation involves a secondary porter, modulated by ATP but energetically coupled to the proton circulation, mediating symport of K^+ with H^+ (Bakker and Harold, 1980).

The requirement of phosphate uptake for the presence of K^+ has been explained by proposing that P_i enters the cell by symport with protons and that potassium maintains the intracellular pH by antiport with protons (Russell and Rosenberg, 1980).

The work described in this chapter shows that silver ions caused efflux of accumulated substrate in a variety of different uptake systems. The results in the proline and glutamine uptake systems were analogous to those in the Pit and Pst systems. Efflux of accumulated succinate, mannitol and potassium ions was also observed. CCCP and TCS caused efflux of K^+ in glycolysing cells but failed to do this in cell suspensions where all the glucose had already been used. Iodoacetate inhibited this efflux. Tributyltin did not cause extensive efflux of K^+. Ag^+ caused efflux of K^+ under

anaerobic conditions, and the extent of K^+ efflux depended on the amount of K^+ which had previously accumulated.

METHODS

The materials, buffers and general methods used have been described in Chapter 2.

1. Preparation of cells for the measurement of potassium movements.

Cells were grown and depleted of phosphate as described in Chapter 2. The cells were then washed, resuspended in potassium-free medium at A_{660} of 0.35, and depleted of K^+ by shaking for 1 hour at 37°C without an energy source and in the presence of 1mM DNP. The cells were then washed three times, suspended in the potassium-free medium at A_{660} of about 5.0, and stored at 4°C until required. On some occasions, when proton and K^+ movements were to be measured simultaneously, the final resuspension was in the lightly buffered medium described in Chapter 2.

2. Measurement of potassium movements.

Potassium movements were measured at 37°C.

K^+ movements were monitored using a miniature K^+-sensitive electrode developed in this laboratory (Rosenberg, 1979). This electrode was inserted into a water-jacketed vessel, the contents of which were aerated and stirred. A separate reference electrode was connected to the main electrode vessel through a salt bridge of 1% agarose in a support medium containing: TEA-H_2SO_4 (20mM with respect to TEA), Li_2SO_4 (70mM), and $MgSO_4$ (1mM); pH was 6.5.

The electrode was connected to one input of a dual-pen Rikadenki recorder through a Radiometer PHM64 meter and a voltage back-up device, set so that full-scale deflection equalled a change of 20mV, representing about $0.3\log[K^+]$. The response (in mV) was converted to concentrations of K^+ by the use of standards.

RESULTS

5.1 Effects of silver ions on proline and glutamine uptake

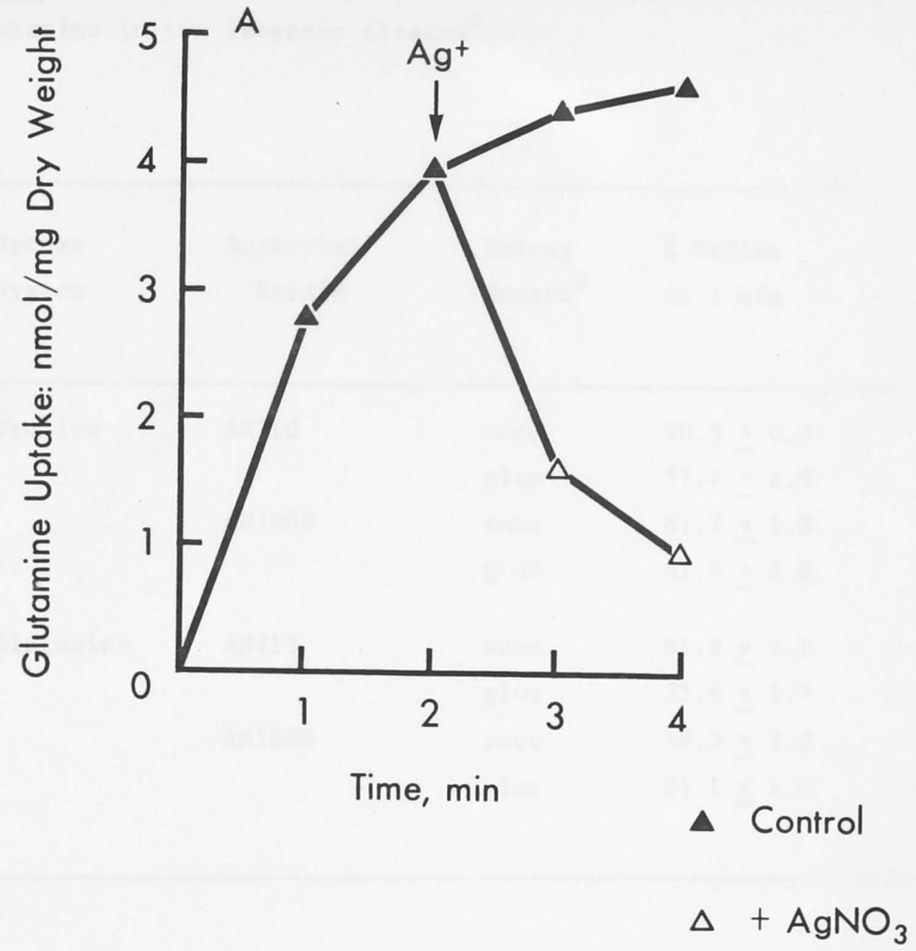
When silver nitrate was added to cell suspensions taking up proline or glutamine, efflux of the respective amino acids was observed (Fig. 5.1). Proline efflux was greater than glutamine efflux, and the use of glucose as a source of energy resulted in less Ag^+-induced efflux than was found if succinate was the source of energy (Table 5.1). The choice of strain used for the uptake assay had a minor effect on Ag^+-induced efflux compared to that due to the transport system tested. This suggests that there were no side effects of the *pit* and *phoT* mutations which affected the response of the cells to Ag^+, and that the response was entirely dependent upon the transport system present.

5.2 Effects of TBT on proline and glutamine uptake

Tributyltin caused efflux of accumulated proline and glutamine (Fig. 5.2). In the presence of chloride, the percentages of proline and glutamine effluxed were similar, but in the absence of chloride there was still considerable efflux of glutamine whereas efflux of proline was minimal. If the difference in efflux between "plus Cl^-" and "minus Cl^-" cell suspensions is taken, the chloride-specific efflux of amino acid due to TBT is seen to be greater for proline than for glutamine.

Fig. 5.1 Effects of Ag^+ on proline and glutamine uptake.

Cells of strain AN710 were grown on succinate and prepared for uptake in 'low phosphate' medium (Chapter 2, Section D.7) as described in Chapter 2. ^{14}C-glutamine (10μM)(A) or ^{14}C-proline (10μM)(B) was added and ^{14}C uptake was monitored. $AgNO_3$ (20μM) was added where shown. Chloramphenicol (50μg/ml) was present in the uptake medium. The source of energy was succinate.


A
Ag+
Glutamine Uptake: nmol/mg Dry Weight
0
1
2
3
4
5
1
2
3
4
Time, min
▲ Control
△ + AgNO3

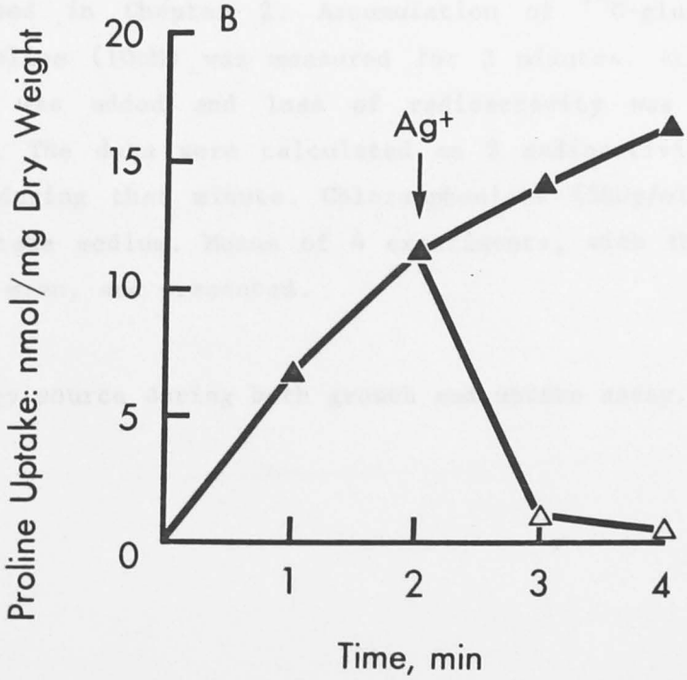

B
Ag+
Proline Uptake: nmol/mg Dry Weight
0
5
10
15
20
1
2
3
4
Time, min

Table 5.1 Effects of energy source on Ag^+-induced efflux of proline and glutamine in two isogenic strains[a].

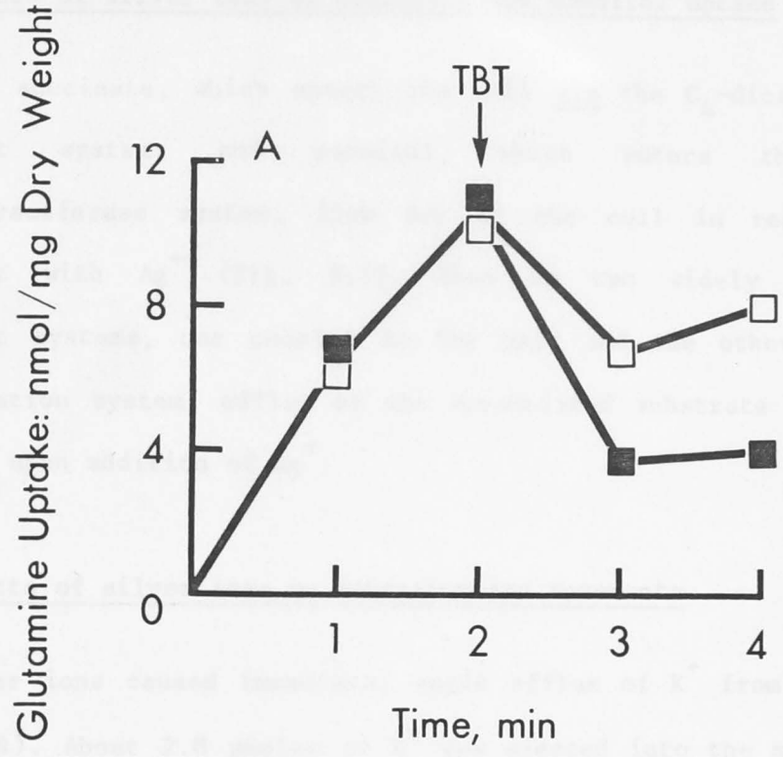
Uptake System	Bacterial Strain	Energy Source[b]	% Efflux in 1 min
Proline	AN710	succ	90.5 ± 0.3
		gluc	57.4 ± 2.2
	AN1088	succ	81.7 ± 1.8
		gluc	61.5 ± 2.0
Glutamine	AN710	succ	61.9 ± 2.2
		gluc	32.6 ± 1.7
	AN1088	succ	58.9 ± 2.2
		gluc	24.1 ± 1.6

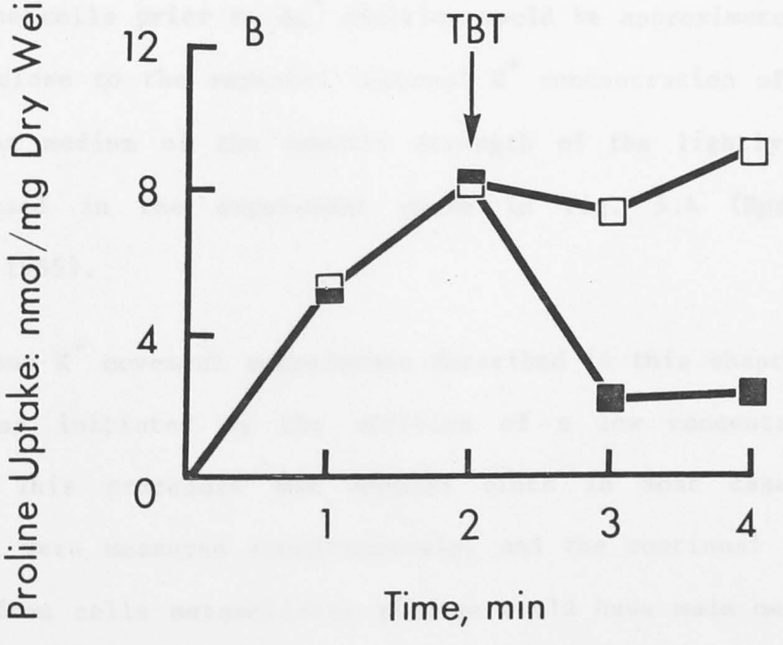
[a] Cells were grown and prepared in 'low phosphate' medium as described in Chapter 2. Accumulation of ^{14}C-glutamine (10μM) or ^{14}C-proline (10μM) was measured for 2 minutes. At 2 minutes $AgNO_3$ (20μM) was added and loss of radioactivity was monitored for 1 minute. The data were calculated as % radioactivity lost from the cells during that minute. Chloramphenicol (50μg/ml) was present in the uptake medium. Means of 4 experiments, with the standard error of the mean, are presented.

[b] Energy source during both growth and uptake assay.

Fig. 5.2 Effects of TBT on proline and glutamine uptake.

Cells of strain AN710 were grown, prepared and uptake was measured as described in Fig. 5.1. TBT acetate (10μM) was added where shown. Uptake medium contained either 20mM KNO_3 (open symbols) or 20mM KCl (filled symbols). Carbon source was succinate.


A
TBT
12
8
4
0
1
2
3
4
Time, min
Glutamine Uptake: nmol/mg Dry Weight


B
TBT
12
8
4
0
1
2
3
4
Time, min
Proline Uptake: nmol/mg Dry Weight

5.3 Effects of silver ions on succinate and mannitol uptake

Both succinate, which enters the cell via the C_4-dicarboxylate transport system, and mannitol, which enters through a phosphotransferase system, flow out of the cell in response to treatment with Ag^+ (Fig. 5.3). Thus in two widely different transport systems, one coupled to the pmf, and the other a group translocation system, efflux of the accumulated substrate was still observed upon addition of Ag^+.

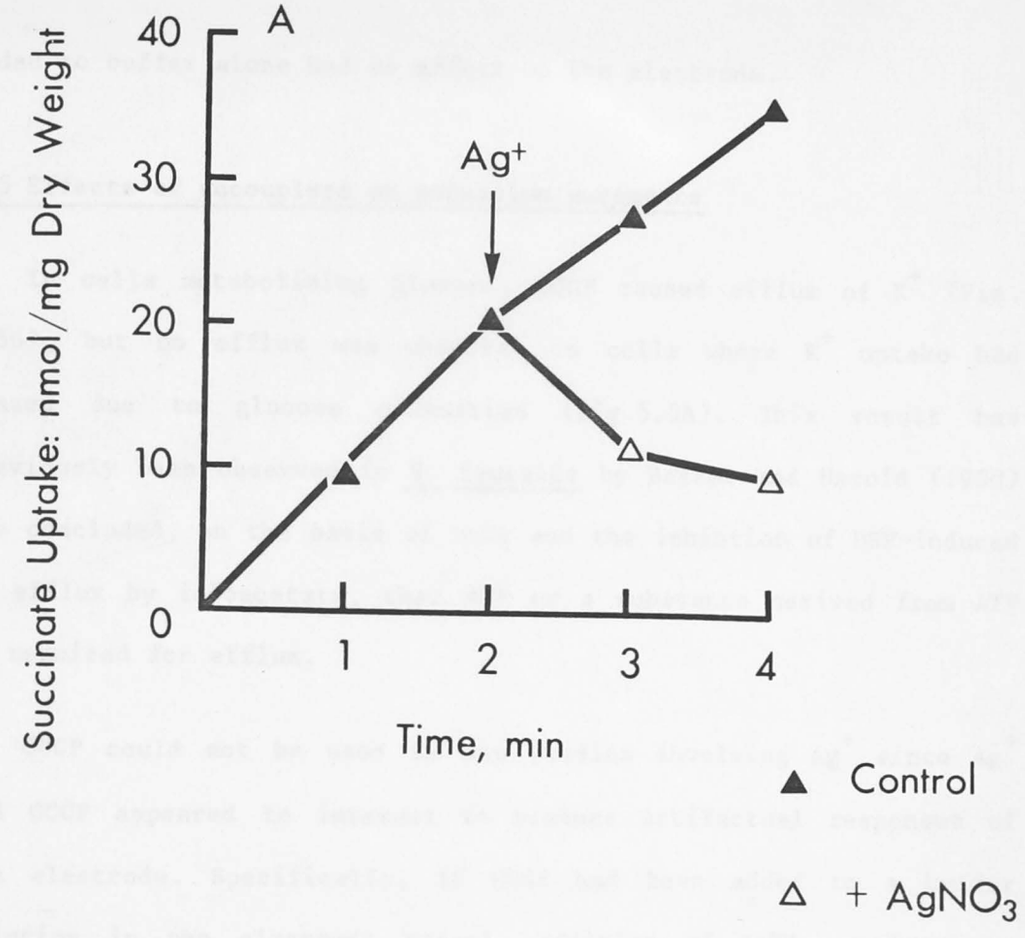
5.4 Effects of silver ions on potassium ion movements

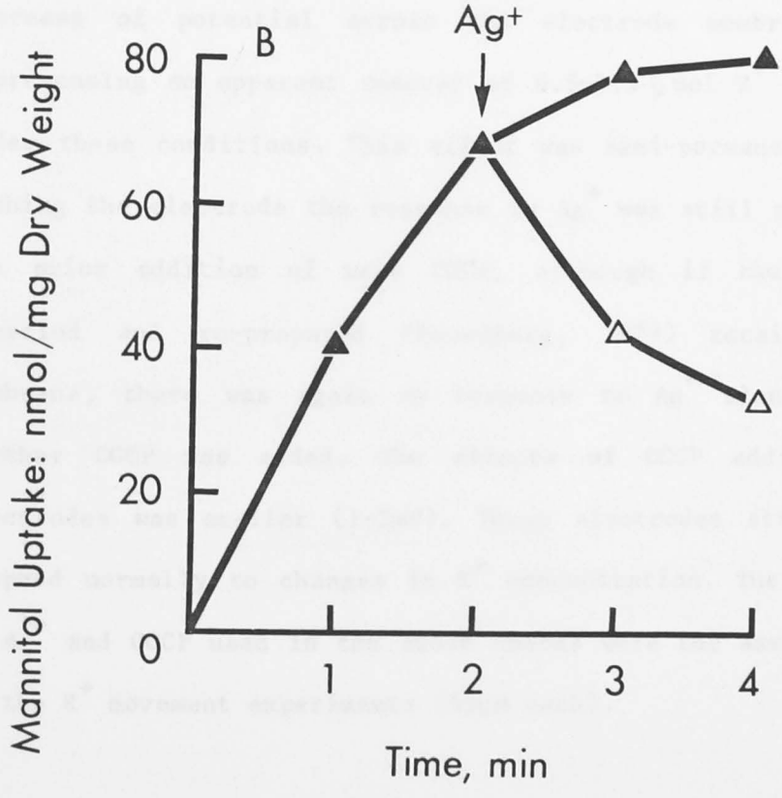
Silver ions caused immediate, rapid efflux of K^+ from the cell (Fig. 5.4). About 2.8 µmoles of K^+ was ejected into the medium. If this represented all the K^+ in the cell, then, assuming 4µl cell water/ml of cell suspension at A_{660} = 6.8 (see Appendix III for estimation of internal volume of cell), the concentration of K^+ within the cells prior to Ag^+ addition would be approximately 175mM. This is close to the expected internal K^+ concentration of 200mM in suspension medium of the osmotic strength of the lightly buffered medium used in the experiment shown in Fig. 5.4 (Epstein and Schultz, 1965).

In many K^+ movement experiments described in this chapter uptake of K^+ was initiated by the addition of a low concentration of glucose. This procedure was adopted since in most cases proton movements were measured simultaneously, and the continual efflux of protons from cells metabolising glucose would have made measurement of proton movements difficult. K^+ uptake proceeded rapidly and ceased after about 1 minute. This cessation was due to glucose exhaustion, since successive additions of glucose caused K^+ uptake to resume (result not shown). Control experiments showed that $AgNO_3$

Fig. 5.3 Effects of Ag^+ on succinate and mannitol uptake.

Cells of strain AN710 were grown overnight and prepared for uptake in 'low phosphate' medium (Chapter 2, Section D.7) as described in Chapter 2. Cells used for mannitol uptake were shaken in the presence of 5mM mannitol during the phosphate depletion stage. ^{14}C-succinate (10μM)(A) or ^{14}C-mannitol (20μM)(B) was added and ^{14}C uptake was monitored. $AgNO_3$ (20μM) was added where shown. Succinate uptake: no energy source. Mannitol uptake: succinate was used as a source of energy.


A
40
30
20
10
0
Ag+
1
2
3
4
Succinate Uptake: nmol/mg Dry Weight
Time, min
▲ Control
△ + AgNO3


B
80
60
40
20
0
Ag+
1
2
3
4
Mannitol Uptake: nmol/mg Dry Weight
Time, min

added to buffer alone had no effect on the electrode.

5.5 Effects of uncouplers on potassium movements

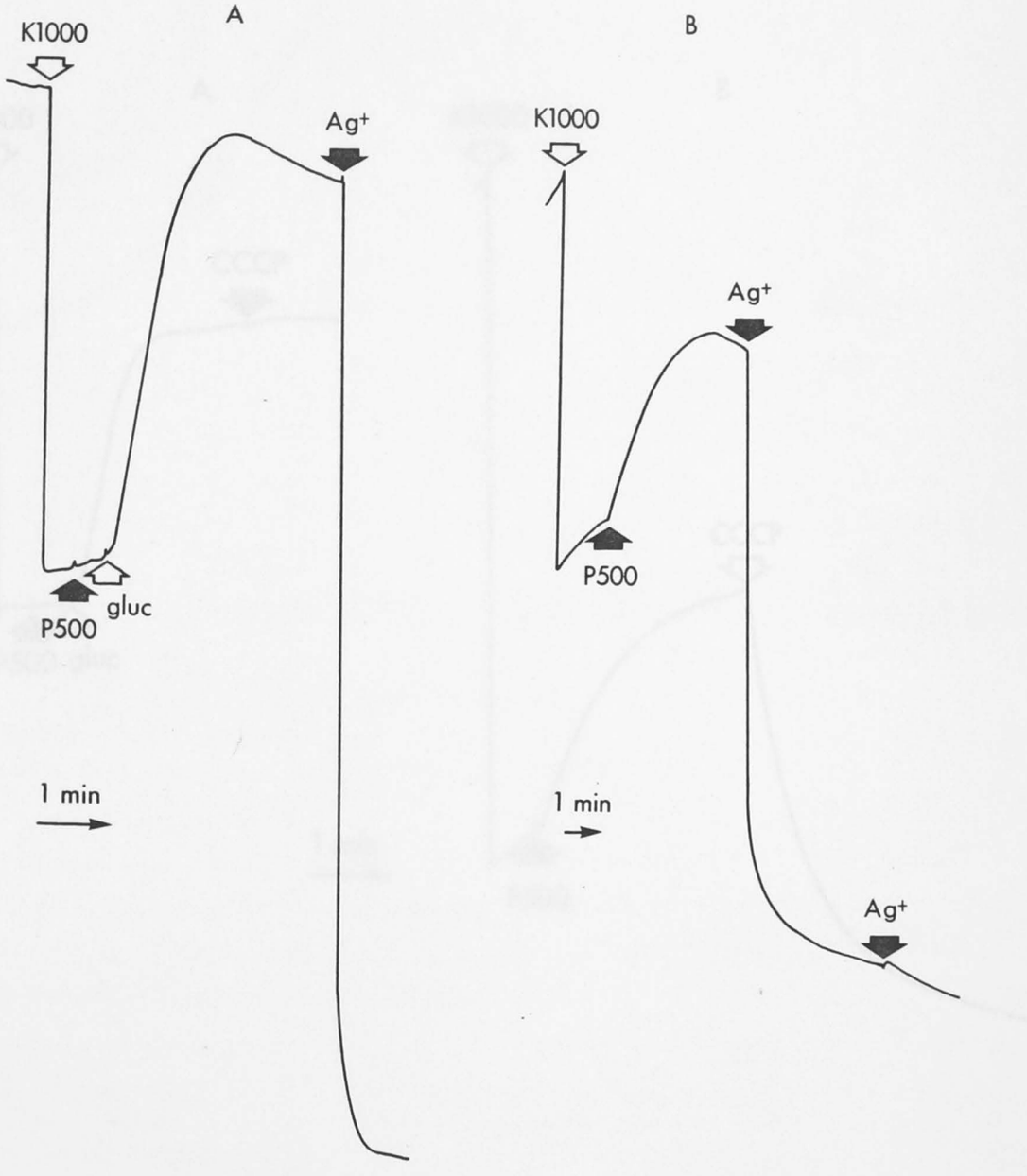
In cells metabolising glucose, CCCP caused efflux of K^+ (Fig. 5.5B), but no efflux was observed in cells where K^+ uptake had ceased due to glucose exhaustion (Fig.5.5A). This result has previously been observed in S. faecalis by Bakker and Harold (1980) who concluded, on the basis of this and the inhibtion of DNP-induced K^+ efflux by iodoacetate, that ATP or a substance derived from ATP is required for efflux.

CCCP could not be used in any studies involving Ag^+ since Ag^+ and CCCP appeared to interact to produce artifactual responses of the electrode. Specifically, if CCCP had been added to a buffer solution in the electrode vessel, addition of $AgNO_3$ produced an increase of potential across the electrode membrane of 5-15mV, representing an apparent removal of 0.5-1.5 μmol K^+ from the medium under these conditions. This effect was semi-permanent, since after washing the electrode the response to Ag^+ was still observed without the prior addition of more CCCP, although if the electrode was recycled and re-prepared (Rosenberg, 1979) retaining the same membrane, there was again no response to Ag^+ alone, provided no further CCCP was added. The effects of CCCP addition to these electrodes was smaller (1-2mV). These electrodes still appeared to respond normally to changes in K^+ concentration. The concentrations of Ag^+ and CCCP used in the above checks were the same as those used in the K^+ movement experiments (50μM each).

Fig. 5.4 Effect of Ag^+ on K^+ movements.

A) Cells of strain AN710 were prepared as described in 'Methods'. 4ml of cell suspension (A_{660} = 6.8) was placed in the electrode vessel and K_2SO_4 was added to about 0.5mM (with respect to K^+) so that the potential of the K^+ electrode was about 140mV. When cells reached ionic equilibrium a standard addition of 500nmol of K_2SO_4 (i.e. 1µmol K^+) was made to calibrate the apparatus. Then 500nmol Na phosphate was added, followed by glucose to 50µM. $AgNO_3$ (50µM) was added where shown.

B) Cells of strain AN710 (A_{660} = 5.6) were prepared and K^+ movements measured as described in 'A' above, except that glucose (5mM) was added at the start of the run. $AgNO_3$ was added twice, the first addition to 50µM, the second addition taking the concentration to 100µM.


A
K1000
Ag+
P500
gluc
1 min
B
K1000
Ag+
P500
1 min
Ag+

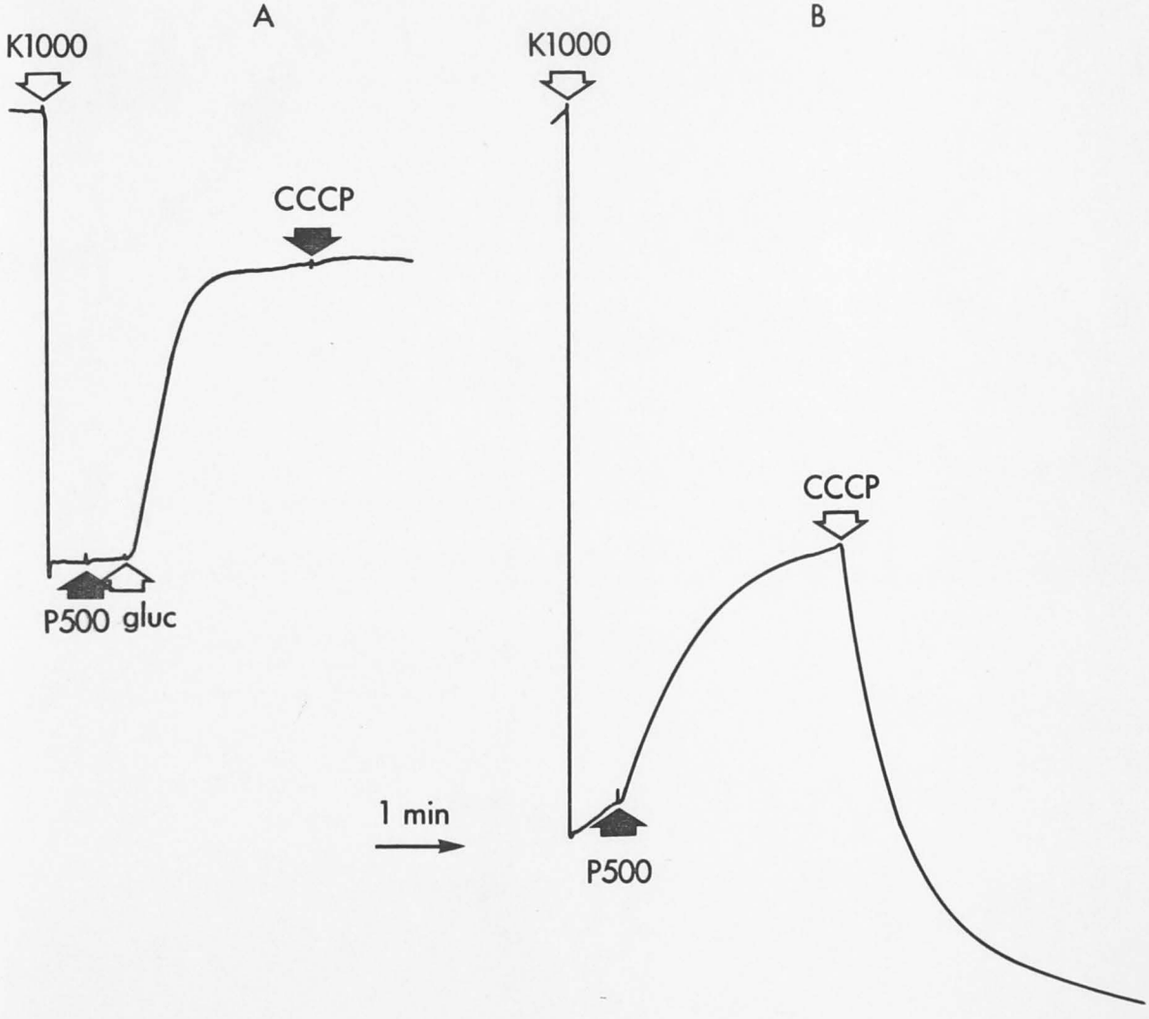


Fig. 5.5 Effects of CCCP on K^+ movements.

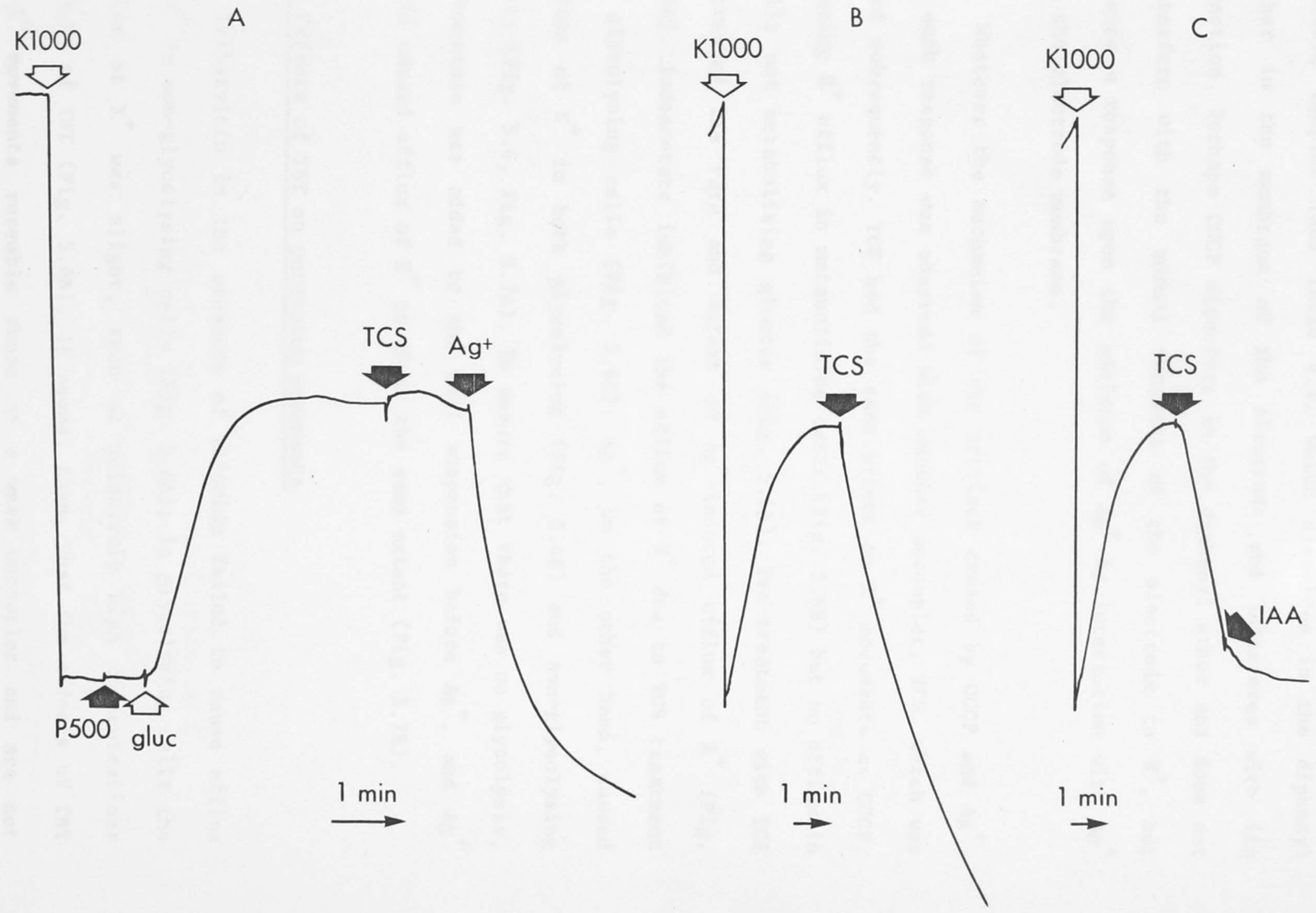
A) K^+ movements were measured in strain AN710 (A_{660} = 6.7) as described in Fig. 5.4A. CCCP (50μM) was added where shown.

B) K^+ movements were measured in strain AN710 (A_{660} = 7.2) as described in Fig. 5.4B. CCCP (50μM) was added where shown.

Fig. 5.6 Effects of TCS on Ag^+-induced K^+ efflux.

A) K^+ movements were measured in strain AN710 (A_{660} = 5.1) as described in Fig. 5.4A. TCS (50μM) and $AgNO_3$ (50μM) were added as shown.

B & C) K^+ movements were measured in strain AN710 (A_{660} = 4.9) as described in Fig. 5.4B, except that Na phosphate (500nmol) was added at the start of the run. TCS (50μM) and lithium iodoacetate (IAA, 2mM) were added where shown.


A
K1000
TCS
Ag+
P500
gluc
1 min
B
K1000
TCS
1 min
C
K1000
TCS
IAA
1 min

It is possible that Ag^+ and CCCP interact to form a lipid-soluble complex (see Table 4.1) which dissolves in the diphenyl ether in the membrane of the electrode and interferes with its function. Perhaps CCCP dissolves in the diphenyl ether and does not interfere with the normal response of the electrode to K^+, but causes a response upon the addition of Ag^+ by interaction with Ag^+ in the electrode membrane.

Whatever the mechanism of the artifact caused by CCCP and Ag^+, no such response was observed with another uncoupler, TCS, which was used subsequently. TCS had the same effect on K^+ movements as CCCP, causing K^+ efflux in metabolising cells (Fig. 5.6B) but no efflux in cells not metabolising glucose (Fig. 5.6A). Pre-treatment with TCS decreased the rate and extent of Ag^+-induced efflux of K^+ (Fig. 5.6A). Iodoacetate inhibited the efflux of K^+ due to TCS treatment of glycolysing cells (Fig. 5.6C). Ag^+, on the other hand, caused efflux of K^+ in both glycolysing (Fig. 5.4B) and non-glycolysing cells (Fig. 5.4, Fig. 5.7A). To ensure that there was no glycolysis, iodoacetate was added to the cell suspension before Ag^+, and Ag^+ still caused efflux of K^+ to about the same extent (Fig. 5.7B).

5.6 Effects of TBT on potassium movements

Tributyltin in the presence of chloride failed to cause efflux of K^+ in non-glycolysing cells (Fig. 5.8A); in glycolysing cells the efflux of K^+ was slight, even at relatively high concentrations (100μM) of TBT (Fig. 5.8B). It seems, then, that the effects of TBT on K^+ movements resemble those of a weak uncoupler and are not similar to the actions of silver ions.

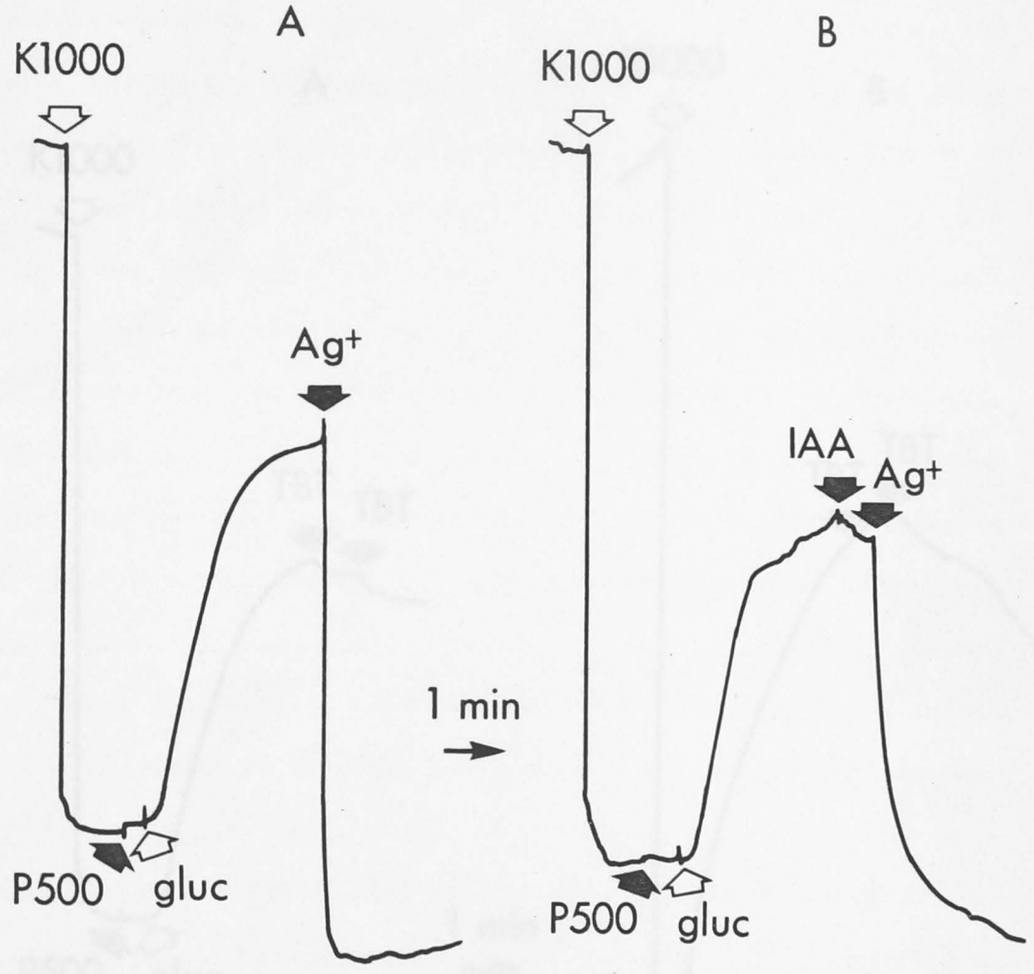


Fig. 5.7 Effect of iodoacetate on Ag^+-induced efflux of K^+.

K^+ movements were measured in strain AN710 (A_{660} = 4.9) as described in Fig. 5.4A. Lithium iodoacetate (IAA, 2mM) and $AgNO_3$ (50μM) were added where shown.

A) No iodoacetate

B) Iodoacetate added before $AgNO_3$.

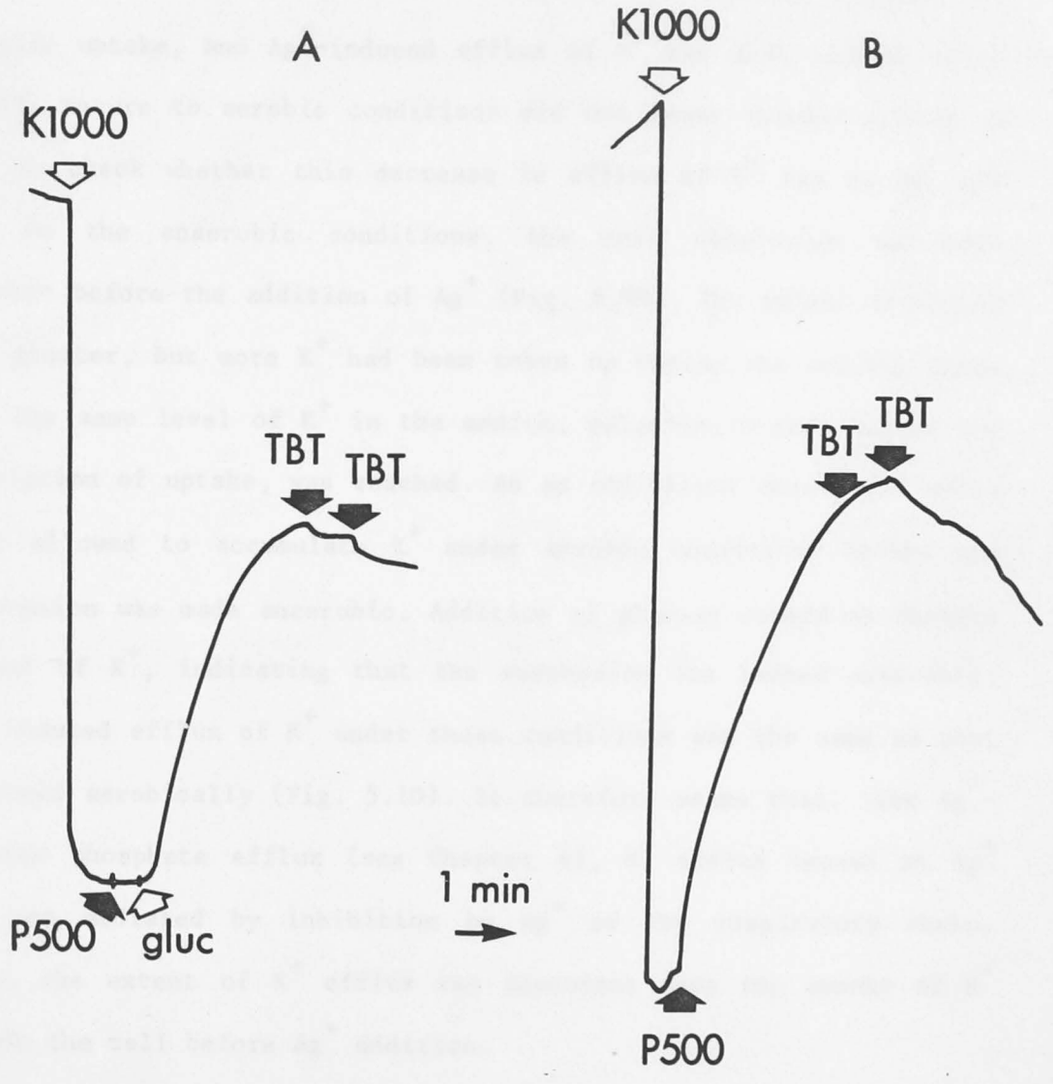


Fig. 5.8 Effects of TBT on K^+ movements.

K^+ movements were measured in strain AN710 (A_{660} = 4.9). The assay medium contained 25mM LiCl.

A) K^+ movements were measured as described in Fig. 5.4A. TBT acetate (50μM) was added where shown. A second addition of TBT took the concentration to 100μM.

B) K^+ movements were measured as described in Fig. 5.4B. TBT additions were as described in 'A' above.

5.7 Silver ion-induced potassium efflux under anaerobic conditions

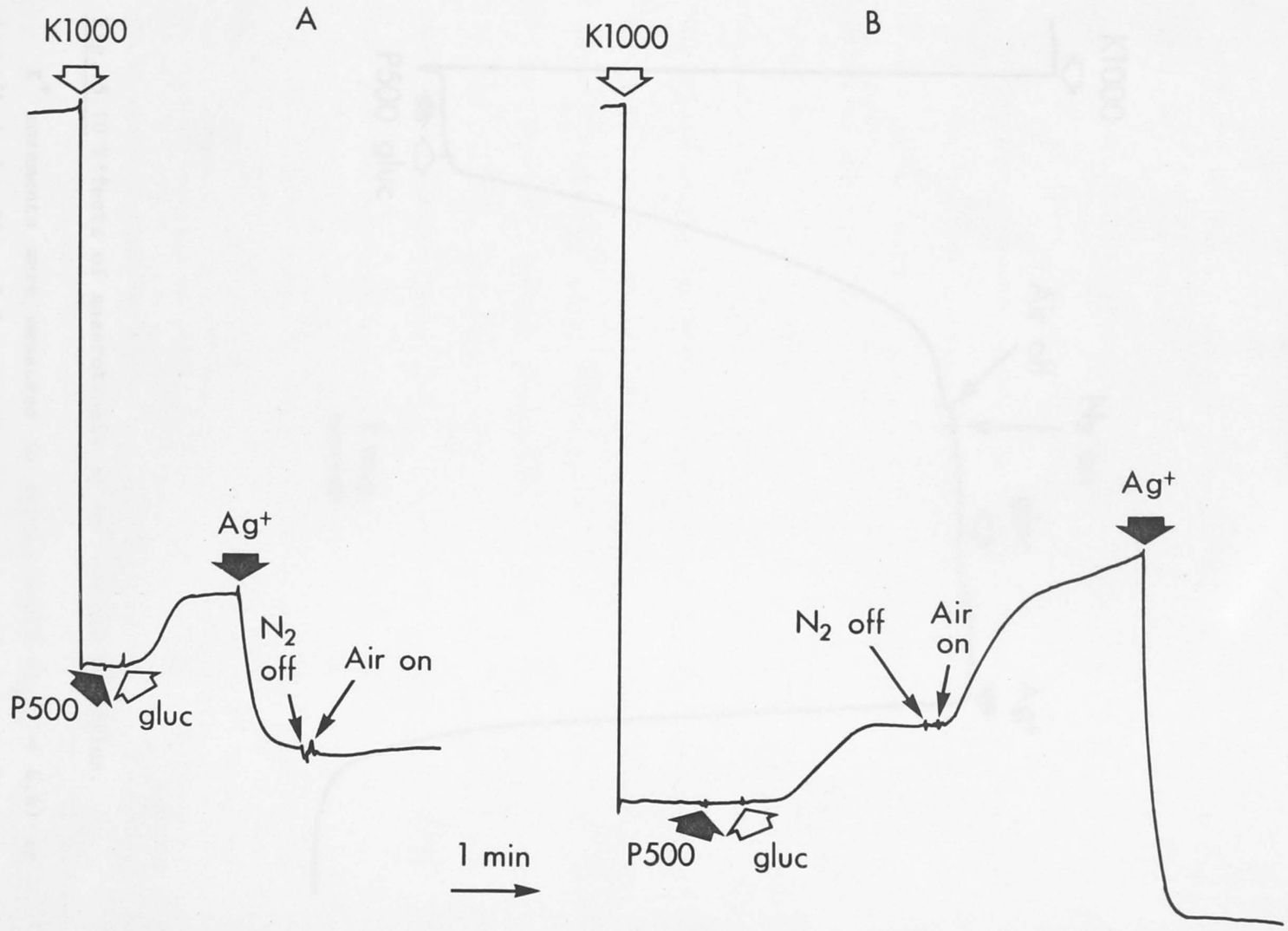
Under anaerobic conditions K^+ uptake was reduced compared to aerobic uptake, and Ag^+-induced efflux of K^+ was also reduced (Fig. 5.9A). Return to aerobic conditions did not cause further efflux of K^+. To check whether this decrease in efflux of K^+ due to Ag^+ was due to the anaerobic conditions, the cell suspension was made aerobic before the addition of Ag^+ (Fig. 5.9B). The extent of efflux was greater, but more K^+ had been taken up during the aerobic phase and the same level of K^+ in the medium, relative to that before the initiation of uptake, was reached. As an additional check, the cells were allowed to accumulate K^+ under aerobic conditions before the suspension was made anaerobic. Addition of glucose caused no further uptake of K^+, indicating that the suspension was indeed anaerobic. Ag^+-induced efflux of K^+ under these conditions was the same as that observed aerobically (Fig. 5.10). It therefore seems that, like Ag^+-induced phosphate efflux (see Chapter 4), K^+ efflux caused by Ag^+ was not mediated by inhibition by Ag^+ of the respiratory chain. Also, the extent of K^+ efflux was dependent upon the amount of K^+ inside the cell before Ag^+ addition.

Fig. 5.9 Effects of anaerobiosis on K^+ uptake and Ag^+-induced K^+ efflux.

K^+ movements were measured in strain AN710 (A_{660} = 4.6) as described in Fig. 5.4A except that, where shown, N_2 (which had been passed through KOH-pyrogallol) instead of air was bubbled through the suspension in the electrode vessel. N_2 was replaced with air, and $AgNO_3$ (50μM) was added, where shown

A) $AgNO_3$ added under anaerobic conditions.

B) $AgNO_3$ added after aeration was started.


A
K1000
Ag+
N2 off
Air on
P500
gluc
B
K1000
Ag+
N2 off
Air on
P500
gluc
1 min

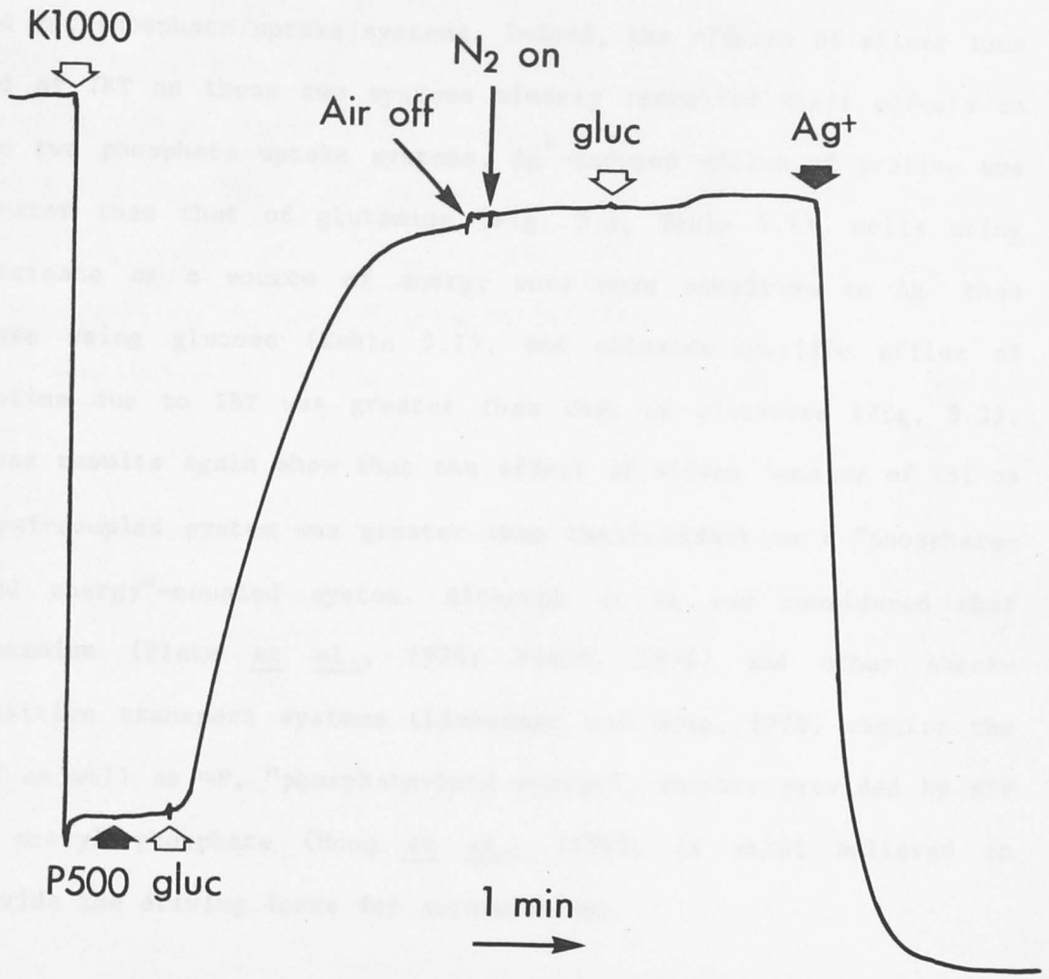


Fig. 5.10 Effects of anaerobiosis on Ag^+-induced K^+ efflux.

K^+ movements were measured in strain AN710 (A_{660} = 4.6) as described in Fig. 5.8, except that the cell suspension was initially aerobic. At the time shown, aeration was stopped and bubbling with N_2 commenced. Glucose (50μM) was added twice (as shown). $AgNO_3$ (50μM) was added where shown.

DISCUSSION

In terms of energy coupling, the proline and glutamine uptake systems can be considered to be analogues, respectively, of the Pit and Pst phosphate uptake systems. Indeed, the effects of silver ions and of TBT on these two systems closely resembled their effects on the two phosphate uptake systems. Ag^+-induced efflux of proline was greater than that of glutamine (Fig. 5.1, Table 5.1), cells using succinate as a source of energy were more sensitive to Ag^+ than those using glucose (Table 5.1), and chloride-specific efflux of proline due to TBT was greater than that of glutamine (Fig. 5.2). These results again show that the effect of silver ions or of TBT on a pmf-coupled system was greater than their effect on a "phosphate-bond energy"-coupled system. Although it is now considered that glutamine (Plate *et al.*, 1974; Plate, 1979) and other shock-sensitive transport systems (Lieberman and Hong, 1976) require the pmf as well as ∿P, "phosphate-bond energy", whether provided by ATP or acetyl phosphate (Hong *et al.*, 1979), is still believed to provide the driving force for accumulation.

In the results with TBT it should be noted that TBT-induced efflux of proline and glutamine in the presence of chloride was about the same and the chloride-specific effect of TBT was different in the two systems only by virtue of the larger chloride-independent efflux of glutamine compared to proline. This chloride-independent effect is unlikely to be due to direct inhibition by TBT of the ATPase complex since, like oligomycin, TBT does not inhibit the ATPase complex of *E. coli* although it does inhibit the ATPase complex of mitochondria (Singh and Bragg, 1979). Possibly TBT has a direct effect on glutamine transport *per se*.

The succinate transport system is shock-sensitive and has a binding protein for succinate (Lo et al., 1972), but is coupled to the pmf (Gutowski and Rosenberg, 1975). CCCP causes efflux of about 40% of the accumulated radioactive label from cells pre-loaded with ^{14}C-succinate (Gutowski and Rosenberg, 1975). Failure to cause complete efflux was stated to be due to the metabolism of succinate during the experiment. Ag^+ caused a similar efflux of ^{14}C from cells which had accumulated ^{14}C-succinate (Fig. 5.3A). In this case, efflux caused bv Ag^+ could be explained by the effects of Ag^+ on the pmf (see Chapter 6) since an uncoupler is also capable of causing efflux.

This explanation is not sufficient, however, to explain the efflux of ^{14}C from cells which had accumulated ^{14}C-mannitol (Fig. 5.3B). Uncouplers of oxidative phosphorylation do not inhibit PTS systems and in fact have been found to increase the steady-state accumulation of α-methyl-D- glucopyranoside (αMG) in the presence of an exogenous oxidisable energy source and to have little or no effect in the absence of an exogenous oxidisable energy source (Hoffee et al., 1964). ATPase mutants starved by incubation in the absence of a carbon source and in the presence of DNP have greatly reduced levels of TMG and proline uptake, indicating a very low membrane potential, while uptake of αMG and mannitol is largely unaffected (Moczydlowski, E.G. and Wilson, D.B., cited by Saier and Moczydlowski, 1978). Mannitol enters E. coli by group translocation, becoming phosphorylated upon entry to the cell and unable to leave via the PTS system. However, Solomon et al. (1973) showed an apparent exit of free mannitol from a mutant strain lacking enzyme of the PTS system if the mannitol system was induced. Mannitol did not exit from uninduced cells.

In this system also, efflux of accumulated radioactivity was observed upon treatment of the cells with Ag^+ (Fig. 5.3B). The nature of the ^{14}C-containing compound(s) effluxed was not examined, but it is unlikely to be mannitol-1-phosphate since, although mannitol-1-phosphate and fructose-1,6-bis-phosphate contain more than 60% of the ^{32}P in the cell after $^{32}P_i$ accumulation (Rosenberg et al., 1982), I have shown (Fig. 3.6, Section 3.5) that no esterified phosphate was found outside the cell after Ag^+ treatment. If Ag^+ had the same effect on the mannitol-specific PTS system as loss of enzyme I, and if free mannitol was generated inside the cell, as it must be if P_i is to be liberated (see Chapter 3), then this is a possible mechanism to account for the observed efflux of radioactivity after Ag^+ treatment of cells which had accumulated ^{14}C-mannitol.

Potassium uptake under the conditions used in the experiments described in this chapter occurs mostly through the TrkA system, which requires both ATP and the pmf (Rhoads and Epstein, 1978). The Kdp system is repressed by growth in the presence of high concentrations of K^+.

Uncouplers caused efflux of K^+ (Fig 5.5, Fig. 5.6), but only if the cells were glycolysing. If glycolysis was stopped, either by exhaustion of glucose or by addition of iodoacetate, uncouplers did not cause K^+ efflux. Iodoacetate blocks glycolysis by inhibiting glyceraldehyde-3-phosphate dehydrogenase, so this block occurs prior to any steps on the glycolytic pathway generating ATP. Bakker and Harold (1980), on the basis of similar results in S. faecalis, postulated that ATP, or a substance derived from ATP, is required for K^+ efflux. The same conclusion can be reached for E. coli. Silver ions, on the other hand, caused efflux of K^+ in both

glycolysing and non-glycolysing cells (Fig. 5.4, Fig. 5.7). This result supports the conclusion reached in Chapter 4 that the effects of Ag^+ are not mediated solely by interference of Ag^+ with the pmf, and also implies that K^+ efflux in Ag^+-treated cells is different from that in uncoupler-treated cells in that it does not require ATP.

The interaction of CCCP and Ag^+ to cause artifactual potential changes in the K^+ electrode may be explained by reference to the observation (Table 4.1) that CCCP facilitated partitioning of Ag^+ into a lipophilic phase. The formation of a lipid-soluble CCCP-Ag^+ complex in the diphenyl ether in the electrode membrane is very likely to interfere with the functioning of the electrode. There appeared to be no movement of K^+ from the electrode fluid into the assay vessel, since the potential increased rather than decreased. The combined action of Ag^+ and CCCP on the bacterial was to inhibit the movement of phosphate across the membrane (Fig. 4.1) and it is possible that the CCCP-Ag^+ complex has the same effect on K^+ movements across the electrode membrane.

TCS decreased the rate and extent of Ag^+-induced K^+ efflux (Fig. 5.6), possibly by competition with Ag^+ for binding to a specific site, as was suggested in Chapter 4.

Tributyltin, even at relatively high concentrations in the presence of chloride, caused no K^+ efflux from non-glycolysing cells and only very slow efflux from glycolysing cells (Fig. 5.8). These effects are probably due to the uncoupling action of TBT by the Cl^-/OH^- exchange. That the effects of TBT were not as marked as those of other uncouplers may be due to the nature of the uncoupling action. The protonophoric uncouplers CCCP and TCS cause H^+ influx down the pH gradient and K^+ may efflux to maintain

electroneutrality, whereas TBT uncouples by electroneutral Cl^-/OH^- exchange, obviating this reason for K^+ efflux. That TBT did not cause efflux like that observed in Ag^+-treated cells is puzzling, since in the proline, glutamine, and both phosphate transport systems the TBT effects closely resembled those of silver ions. Perhaps TBT has some effect on the K^+ carrier leading to an inhibition of K^+ exit.

Silver ions still caused efflux of K^+ under anaerobic conditions, with glucose as a source of energy for the cells (Fig. 5.9, Fig. 5.10). Under these conditions the respiratory chain cannot function, so the effects of Ag^+ do not seem to be mediated by its inhibition of the respiratory chain, reinforcing the conclusion reached in Chapter 4. Fig. 5.9 and Fig. 5.10 also show that the extent of K^+ efflux depended upon the amount of K^+ that the cells had accumulated. It seems likely, therefore, that Ag^+ treatment leads to a total depletion of intracellular K^+ until the K^+ concentration gradient is completely collapsed, as suggested in Section 5.4.

The pleiotropic nature of the effects of silver ions on all transport systems examined so far suggests that Ag^+ interferes with some basic process which is common to all the transport systems. The differences between the effects of Ag^+ on the Pit and Pst systems (see Chapter 3), on the proline and glutamine uptake systems (Fig. 5.1, Table 5.1),and between succinate and glucose as sources of energy (Table 5.1; also Chapter 3) indicate that this process may be the coupling of energy to transport. Uncouplers did not cause the same effects (Fig. 5.5, Fig.5.6; also Chapter 4) but tributyltin in the presence of chloride was similar in action to Ag^+ (Fig. 5.2; also Chapter 4), except in the K^+ transport system (Fig. 5.8).

Although differences in the Ag^+ effect correlated with different forms of energy coupling, the overall result, efflux of the uptake substrate, was the same in all systems, indicating that an essential basic level of membrane energisation underlies all these transport systems and is responsible for maintaining the concentration gradients of the transported metabolites. Ag^+ and tributyltin affect this, whereas uncouplers do not, so some function, most probably membrane-associated, must be postulated which is accessible to Ag^+ and TBT but not to uncouplers of oxidative phosphorylation. I suggest that anhydrous protons within the membrane, carried by lipophilic anions such as chloride, are involved with this function.

CHAPTER 6

EFFECTS OF SILVER IONS AND OTHER INHIBITORS ON PROTON MOVEMENTS AND THE PROTONMOTIVE FORCE.

INTRODUCTION

Interest in testing the chemiosmotic hypothesis (Mitchell, 1961; 1966) has led to many attempts to measure the components of the protonmotive force. The protonmotive force (Δp) is related to its components, the membrane potential ($\Delta\psi$) and the pH difference (ΔpH) in the following way:

$$\Delta p = \Delta\psi - 2.3RT\Delta pH/F$$
$$= \Delta\psi - Z\Delta pH$$

where Δp, $\Delta\psi$ and ΔpH have the meanings described above and Z ($= 2.3RT/F$) has a value of 58.2mV at $20^{o}C$.

Attempts to measure ΔpH have included titrametric methods (Gear et al., 1967; Mitchell and Moyle, 1969); pH indicators, like bromothymol blue, supposedly located solely inside the particles being measured (Chance and Mela, 1966); and the accumulation of weak acids (Waddell and Butler, 1959) or weak amines (Rottenberg et al., 1972). Problems with the first two of these methods have been pointed out by Rottenberg (1975). Titrametric methods for mitochondria involved either centrifugation followed by dissolving of the pellet with detergent and measuring the pH of the solution obtained (Gear et al., 1967), which cannot be very accurate, or titrations in the presence and absence of detergent to determine the buffering capacities within, and outside of, the matrix (Mitchell and Moyle, 1969). This assumes that the detergent treatment does not change the buffering capacity, which is not likely to be so. Internal pH indicators must be exclusively located within the particles, and no known indicators have this property (Rottenberg, 1975). One exception is phosphate, as used in ^{31}P nuclear magnetic resonance (nmr) (Navon et al., 1977). The existence of a ΔpH caused a split in the phosphate resonance peak, with positions

corresponding to internal and external pH. Results using ^{31}P nmr have confirmed measurements of acid or base distributions (Navon et al., 1977; Padan et al., 1981). The most commonly used methods to date have been those which measure the distribution of weak acids (for measurements within compartments which are more alkaline internally than the surrounding medium) or weak bases (for measurements in acidic vesicles). A weak acid which permeates the membrane only when neutral will be in equilibrium when the concentrations of the neutral form on both sides of the membrane are equal. If the internal space is more alkaline than the surrounding medium, the concentration of the charged form, and hence the total concentration, of the weak acid will be higher in the internal space than in the medium. Thus the weak acid will accumulate on the more alkaline side of the membrane. The same argument holds true for weak bases and acidic vesicles. Useful weak acids or bases must permeate the membrane only when neutral, must not bind to membranes or proteins, must not be metabolised, and must not by itself affect ΔpH. DMO (5,5-dimethyloxazolidine-2,4-dione) (Waddell and Butler, 1959; Addanki et al., 1968) is one weak acid that satisfies these criteria. Acetate is useful in isolated mitochondria (Rottenberg, 1973) and membrane vesicles (Ramos et al., 1976), where it is not metabolised. The weak base methylamine has proved useful in chloroplasts (Rottenberg et al., 1972) and measurement of the quenching of fluorescent amines such as 9-aminoacridine upon uptake has also proved useful (Schuldiner et al., 1972).

Measurement of $\Delta\psi$ has involved the use of microelectrodes (Tupper and Tedeschi, 1969); changes in the spectroscopic properties of naturally occurring (Witt, 1971) or artificial (Azzi, 1969; Waggoner, 1979) chromophores within, or bound to, the membrane; or the distribution of permeating ions (Rottenberg, 1979). The use of

microelectrodes has been questionable until recently due to uncertainty of the exact location of the electrode tip and damage to the cell or organelle being examined. Tupper and Tedeschi (1969), while using microelectrodes for the measurement of membrane potential in mitochondria, state that the mitochondria collapsed within seconds after impalement with the electrode. Recently, however, Felle et al. (1980) have successfully used hydrophobically coated microelectrodes to measure membrane potential in giant cells of E. coli. The recorded potential remained high for about 5 minutes after puncture and the $\Delta\psi$ values obtained agreed closely with those obtained from the distribution of a permeant cation. A postulated naturally occurring membrane probe, the changes in the speed of absorbance decay at 515nm observed by Witt (1971), may not reflect changes in total membrane potential but instead reflect perturbations of a local field within the membrane (Rottenberg, 1975). It has recently been shown that changes in fluorescence of cyanine dyes correlate with changes in membrane potential (Waggoner, 1979) and these changes have been calibrated in terms of $\Delta\psi$ by comparison of fluorescence values with $\Delta\psi$ values obtained by measurements of the distribution of Rb^+ (Ghazi et al., 1981). For quantitative estimations of $\Delta\psi$, fluorescence changes must be calibrated by reference to independently measured $\Delta\psi$ values since there is no theoretical method of quantitatively relating the level of fluorescence to the membrane potential (Waggoner, 1979).

Permeating ions used for the measurement of $\Delta\psi$ include K^+ or Rb^+ in the presence of valinomycin (Rottenberg, 1975; Altendorf et al., 1975) and organic, lipid-soluble cations such as dibenzyldimethyl ammonium ion (DDA^+) (Skulachev, 1971; Harold and Papineau, 1972; Altendorf et al., 1975), triphenylmethylphosphonium ion ($TPMP^+$) (Harold and Papineau; 1972; Ramos et al., 1976), and

tetraphenylphosphonium ion (TPP^+) (Felle et al., 1980). For an ion to be useful for the measurement of $\Delta\psi$, it must freely permeate the membrane in its charged form, establish a true equilibrium distribution, and it must not bind significantly to proteins or accumulate within the membrane. It must also not be metabolised and must not by itself affect the $\Delta\psi$. The distribution of the ion across the membrane should then be determined solely by the membrane potential, with cations accumulating inside vesicles which are more negative than the external medium, and anions accumulating in inside-positive vesicles. The above-mentioned ions satisfy these criteria to a large extent, although it has recently been suggested that $TPMP^+$ overestimates the membrane potential at low $\Delta\psi$ values, due to non-specific binding of $TPMP^+$ to cells (Ghazi et al., 1981). Also, at high concentrations most cations depolarise the membranes to some extent. This is easily avoided by using concentrations low enough so that this does not occur. Thallous ions do not satisfy the above criteria, since they are actively transported (Kashket, 1979) and thus do not establish a true equilibrium distribution.

Techniques which have been used for the measurement of the distribution of permeant cations are: centrifugation (Padan et al., 1976) or filtration (Altendorf et al., 1975; Schuldiner and Kaback, 1975) of cell suspensions; flow dialysis (Schuldiner et al., 1976; Ramos et al., 1976); and ion-selective electrodes (Kamo et al., 1979; Hosoi et al., 1980). Flow dialysis has the advantage over centrifugation and filtration methods in that the distribution can be monitored continuously without the requirement for the separation of cells from the media, but it can only measure distributions in the steady-state, with response times of the order of minutes. Electrodes sensitive to the permeant ion being examined are potentially more sensitive to rapid changes in the distribution of

the permeant ion.

The experiments described in this chapter show that protons flowed out of the cell in response to the addition of $AgNO_3$. Uncouplers inhibited this efflux of protons and caused extruded protons to re-enter the cell. Tributyltin, in the presence of chloride but not in its absence, caused an increase of the pH of the external medium. Studies under anaerobic conditions showed that only a small proportion of the H^+ efflux occurred under these conditions, but H^+ efflux resumed when aeration was started. Flow dialysis experiments using ^3H-TPMP$^+$ to measure $\Delta\psi$ and ^{14}C-DMO to measure ΔpH showed that Ag^+ collapsed ΔpH and partially collapsed $\Delta\psi$. Tributyltin had no effect on $\Delta\psi$, but collapsed ΔpH. This collapse of ΔpH required chloride.

METHODS

The materials, buffers and general methods used have been described in Chapter 2. The bacterial strain used in the work described in this chapter was AN710.

1. Measurement of proton movements

Cells were prepared as described in Chapter 5 (Methods, Section 1), with the final resuspension at A_{660} of about 5.0 in the lightly bufferred medium described in Chapter 2.

Proton movements were measured at 37°C using a Radiometer G 202 C electrode. This was placed in the same vessel as the K^+-sensitive electrode (see Chapter 5) and connected to the other input of a dual-pen Rikadenki recorder through a Radiometer PHM62 meter and a voltage back-up device, set so that full-scale deflection equalled a change of 20mV, representing 0.4 pH units. A separate reference electrode was connected to the main electrode vessel through a chloride-free salt bridge as described in Chapter 5. Standards were used to calibrate the apparatus in terms of molar H^+ movements.

2. Flow dialysis

Cells were grown and depleted of phosphate as described in Chapter 2. After washing, cells were suspended in chloride-free phosphate-free medium at A_{660} of about 200 and kept at 4°C until required. Cells for $\Delta\psi$ measurement were EDTA-treated as described in Chapter 2 to make the cells permeable to $TPMP^+$. This was done before the final suspension and the cells were used within 2 hours.

The flow dialysis cell used in these studies was made by Mr D. Abigail of this Department, according to the specifications of Colowick and Womack (1969). The upper and lower chambers were separated by Visking dialysis membrane which had been prepared as described in Chapter 2, and both chambers were stirred with Teflon-coated magnetic bars. Chloride-free phosphate-free buffer, from a flask fitted with a Mariotte tube to maintain a constant head, was gravity-fed through the lower chamber at a rate of 3.5ml/min. Fractions were collected at 1 minute intervals and aliquots were counted in a Packard Tri-Carb 460CD liquid scintillation spectrometer. Chloride-free phosphate-free buffer was placed in the top chamber and isotopically labelled permeant anion (for $\Delta\psi$ measurement) or weak acid or weak base (for ΔpH measurements) were added. After equilibrium was established, cell suspension and glucose were added to give a total volume of 0.8ml. Controls received buffer instead of cell suspension. Other reagents were added as described in the figure legends. Note that all additions were made to the top chamber. All flow dialysis experiments were carried out at room temperature. The initial steady-state period before the addition of the cell suspension (fractions 5-15) was used to equalise the experimental and control curves. This was done by taking the means of the fraction 5-15 dpm values for each curve, obtaining the ratio of the means (control/experimental), and applying the correction factor thus derived to the experimental curve.

3. Measurement of membrane potential

The electrical potential across the membrane ($\Delta\psi$) was determined by measuring the accumulation of ^{3}H-TPMP^{+} by flow dialysis. $\Delta\psi$ was calculated by substitution of the experimental data into an equation derived from the Nernst equation (see Appendix II). A value of 5.9µl of intracellular water per ml of cell suspension of A_{660} = 5.0 was used (see Appendix III for measurement of intracellular water space).

4. Measurement of pH gradients

Internal pH was determined by measuring the accumulation of ^{14}C-DMO or ^{14}C-methylamine by flow dialysis and calculation as described by Waddell and Butler (1959) (Appendix II). The external pH of the cell suspension was measured in a parallel experiment where buffer, cells, and other reagents were added at the same times and in the same concentrations in a total volume of 2.0ml in a stirred vessel. pH was measured with a Philips C14/02 pH electrode connected to a Radiometer PHM62 meter and through this to one input of a dual-pen Rikadenki recorder, set so that full-scale deflection represented 1 pH unit. ΔpH was determined by the difference between the internal and external pH (Appendix II).

RESULTS

6.1 The effect of silver ions on proton movements

In the presence of K^+ and P_i, addition of glucose resulted in a rapid net extrusion of protons from the cells, followed by net H^+ removal from the medium as the glucose was exhausted (Fig. 6.1). That this "fallback" was due to cessation of glycolysis was shown by the addition of iodoacetate to a call suspension which was metabolising glucose and rapidly extruding protons. This addition caused H^+ efflux to cease, immediately followed by the commencement of H^+ influx (Fig. 6.1, insert). Addition of $AgNO_3$ to the cell suspension caused an immediate, rapid efflux of protons, followed by a "fallback" of most of the effluxed H^+ (Fig. 6.1).

6.2 Effects of uncouplers on silver ion-induced proton efflux

The uncoupler CCCP caused a slight increase in the rate of proton "fallback" (Fig. 6.2A) whereas TCS had no effect (Fig. 6.3A). Addition of the uncouplers earlier in the "fallback" phase showed a greater effect of CCCP, but still no effect with TCS (data not shown). Both uncouplers markedly decreased the extent of the H^+ efflux due to $AgNO_3$ addition and caused the eventual re-uptake of all the effluxed protons (Fig. 6.2A, Fig. 6.3A). When added after silver nitrate, the uncouplers caused the immediate removal of H^+ from the medium until the H^+ concentration was about the same as that before $AgNO_3$ addition (Fig. 6.2B, Fig. 6.3B).

Fig. 6.1 Effect of Ag^+ on proton movements.

Cells were prepared as described in 'Methods'. 4 ml of cell suspension (A_{660} = 5.1) was placed in the electrode vessel and K_2SO_4, to a final concentration of about 0.75mM (with respect to K^+), and Na phosphate (500 nmol) were added at the beginning of the run. Glucose (50μM) and $AgNO_3$ (50μM) were added where shown. The apparatus was calibrated by the addition of appropriate amounts of H_2SO_4.

Insert: Glucose (5mM), P_i (500 nmol) and K^+ (0.75mM) were added at the start of the run and H^+ extrusion was monitored. Lithium iodoacetate (2mM) was added where shown.

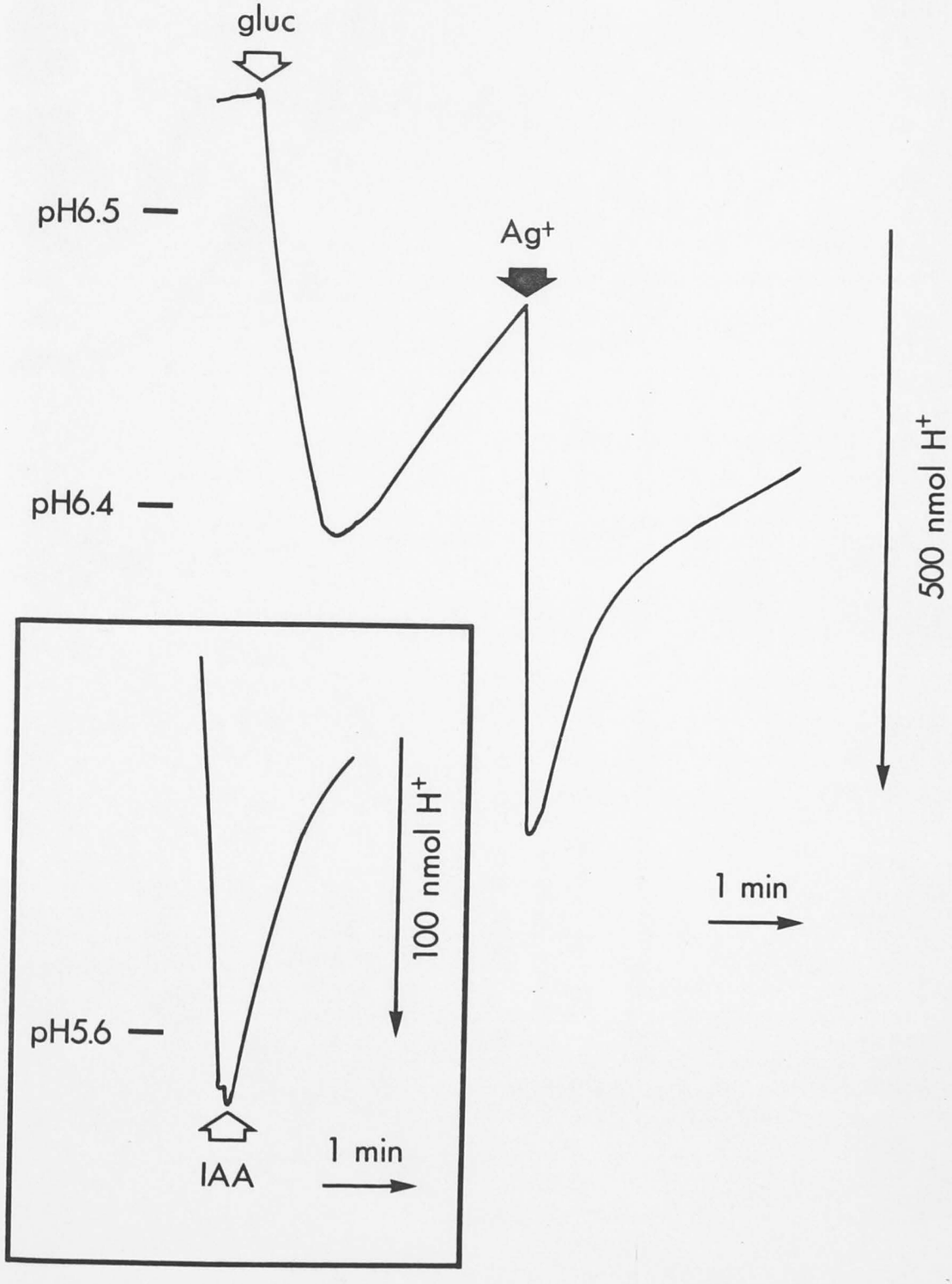

gluc
pH6.5
Ag+
pH6.4
500 nmol H+
1 min
100 nmol H+
pH5.6
IAA
1 min

Fig. 6.2 Effects of CCCP and Ag^+ on proton movements.

Proton movements were measured as described in Fig. 6.1 (Cell suspension A_{660} = 6.6). Glucose (50μM), CCCP (50μM) and $AgNO_3$ (50μM) were added as shown.

A

gluc

B

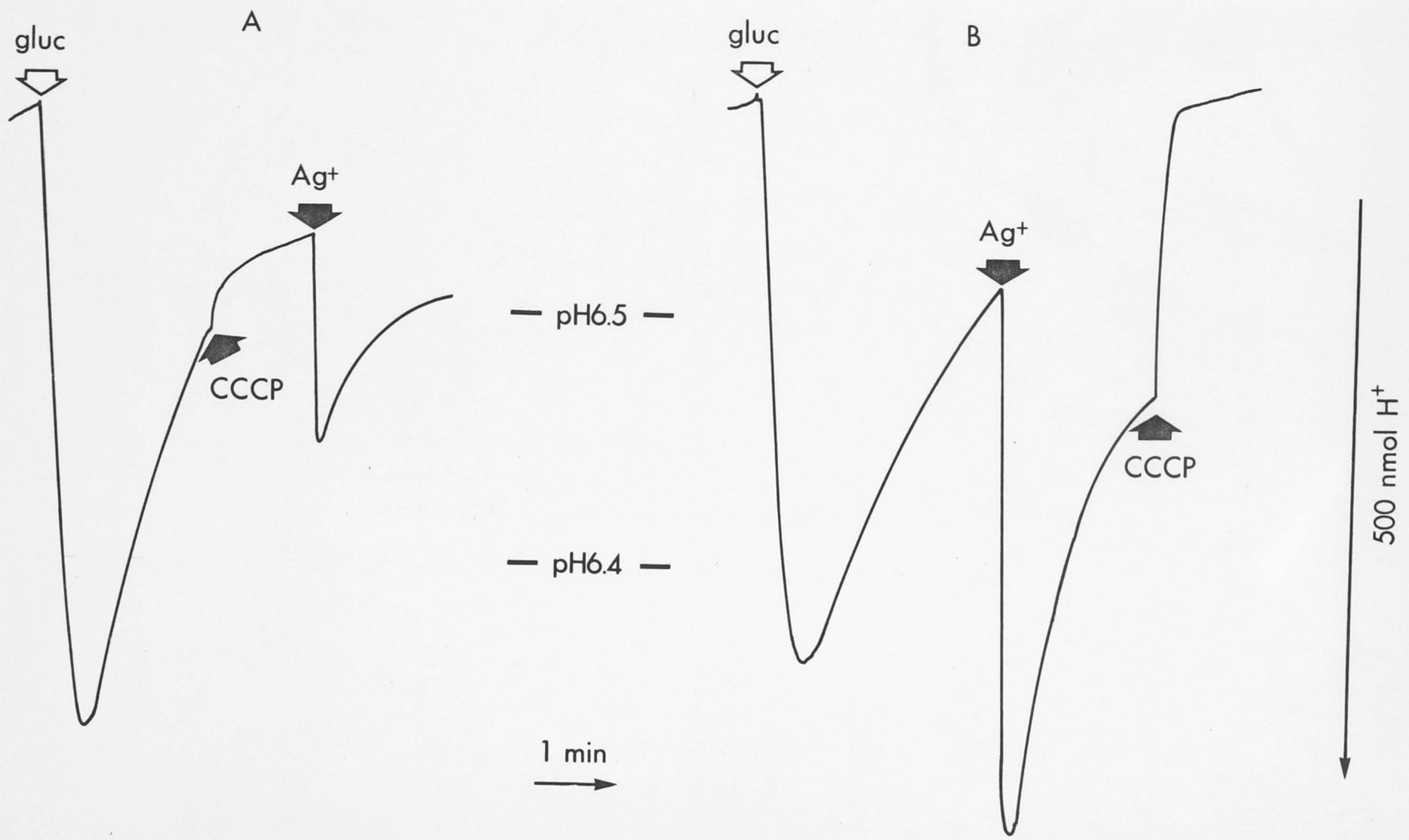
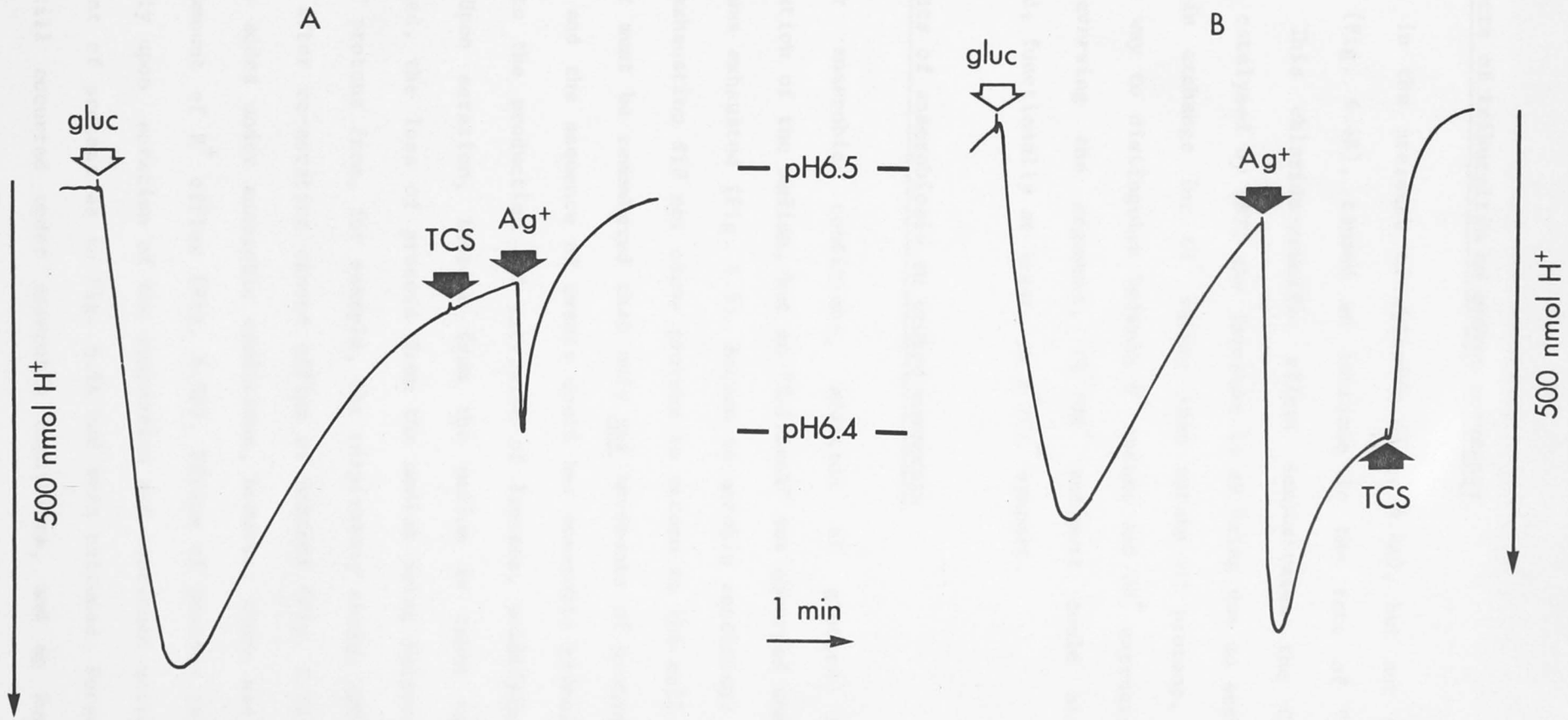

A
gluc
Ag+
CCCP
pH6.5
pH6.4
1 min
B
gluc
Ag+
CCCP
500 nmol H+

Fig. 6.3 Effects of TCS and Ag^+ on proton movements.

Proton movements were measured as described in Fig. 6.1 (Cell suspension A_{660} = 5.1). Glucose (50μM), TCS (10μM) and $AgNO_3$ (50μM) were added as shown.


A
gluc
TCS
Ag+
500 nmol H+
pH6.5
pH6.4
1 min
B
gluc
Ag+
TCS
500 nmol H+

6.3 Effects of tributyltin on proton movements

TBT, in the presence of chloride (Fig. 6.4A), but not in its absence (Fig. 6.4B), caused an increase in the rate of "proton uptake". This chloride-specific effect demonstrates the Cl^-/OH^- exchange catalysed by TBT, the increase in pH being due to extrusion of OH^- in exchange for Cl^- rather than uptake of protons, there being no way to distinguish between H^+ uptake and OH^+ extrusion. In fact, reversing the argument, Cl^-/OH^- antiport could also be described, functionally at least, as H^+/Cl^- symport.

6.4 Effects of anaerobiosis on proton movements

Under anaerobic conditions, addition of glucose caused acidification of the medium, but no "fallback" was observed when the glucose was exhausted (Fig. 6.5). Return to aerobic conditions after glucose exhaustion did not cause protons to return to the cell (Fig. 6.5A). It must be remembered that only net movements of protons are observed and the sequence of events could be: anaerobic glycolysis, leading to the production and extrusion of lactate, acidifying the medium. Upon aeration, lactate from the medium is taken up and metebolised, the loss of protons from the medium being balanced by efflux of protons from, for example, the respiratory chain. Addition of $AgNO_3$ after re-aeration caused efflux of protons (Fig. 6.5A). If $AgNO_3$ was added under anaerobic conditions, however, there was only a small amount of H^+ efflux (Fig. 6.5B). Efflux of protons resumed immediately upon aeration of the suspension and continued until the same amount of protons as in Fig. 6.5A had been extruded. Potassium efflux still occurred under anaerobic conditions, and no further efflux was observed upon re-aeration (see Fig. 5.9A), showing that H^+ efflux was not obligatorily tied to the efflux of K^+.

Fig. 6.4 Effects of TBT on proton movements.

Proton movements were measured as described in Fig. 6.1 (Cell suspension A_{660} = 6.4(A) or 6.6(B)). Glucose (50µM) was added where shown.

A) LiCl (25mM) was present in the assay medium. TBT was added twice, first to 5µM, with a second addition taking the concentration to 10µM.

B) Li_2SO_4 (12.5mM) was present in the assay medium. TBT (5µM) was added where shown.

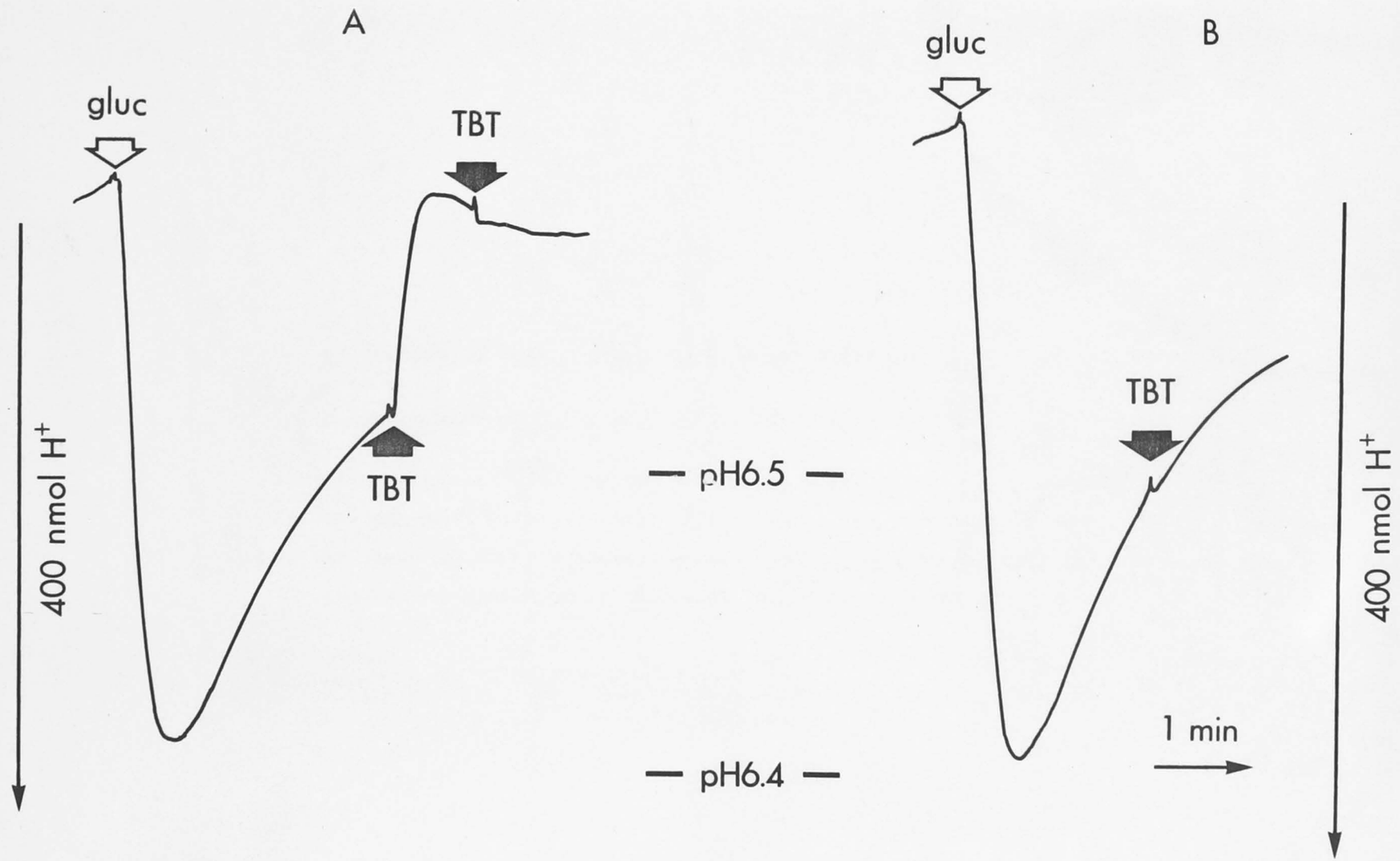

A
gluc
TBT
TBT
400 nmol H+
pH6.5
pH6.4
B
gluc
TBT
1 min
400 nmol H+

Fig. 6.5 Effects of anaerobiosis on Ag^+-induced proton movements.

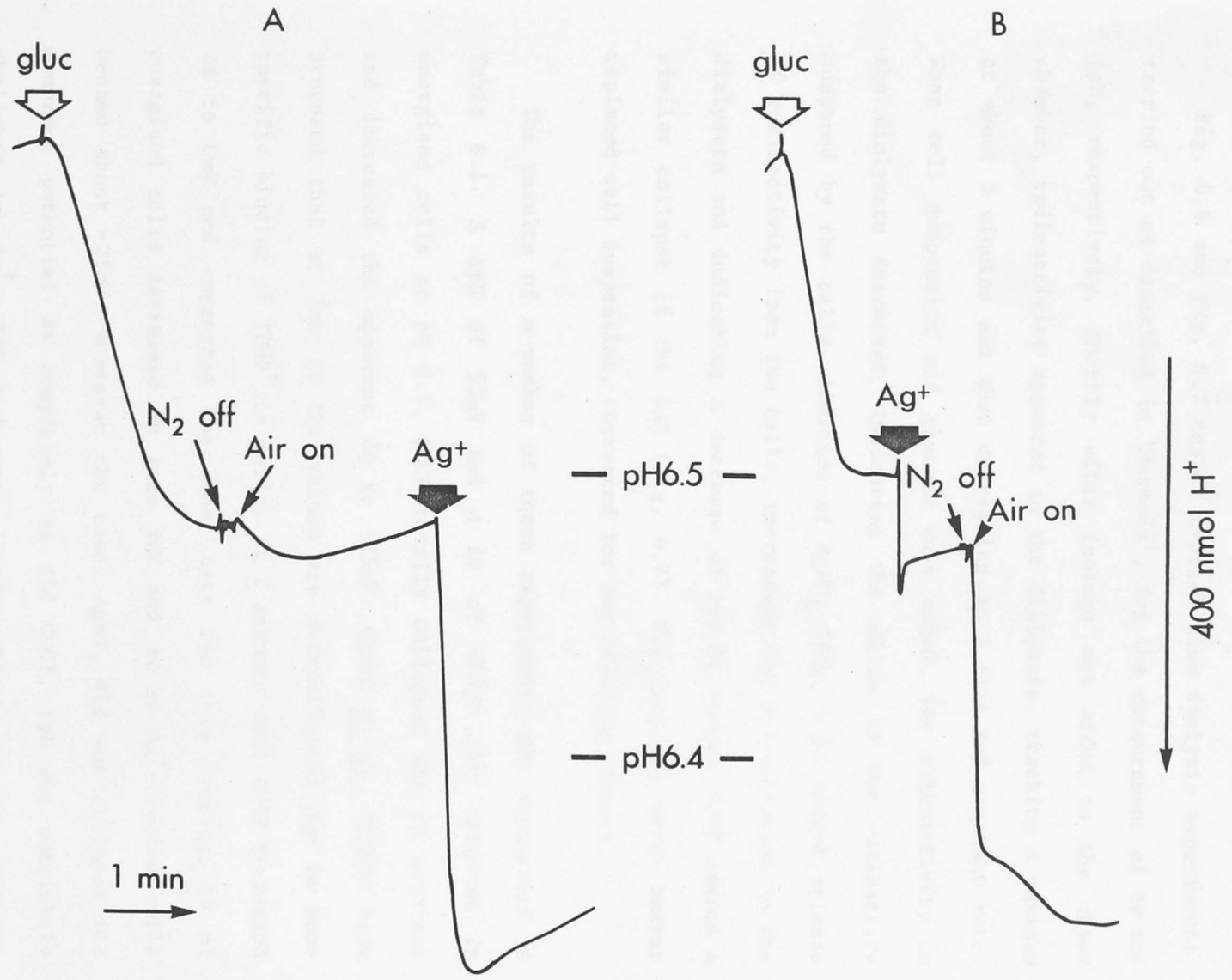
Proton movements were measured as described in Fig. 6.1 except that the cell suspensions were initially bubbled with N_2 (which had been passed through pyrogallol). Where shown, N_2 was replaced with air. $AgNO_3$ (50μM) was added where shown.

A) Suspension made aerobic before $AgNO_3$ addition.

B) Suspension made aerobic after $AgNO_3$ addition.

A

B


A
gluc
N2 off
Air on
Ag+
1 min
pH6.5
pH6.4
B
gluc
Ag+
N2 off
Air on
400 nmol H+

6.5 Effects of silver ions, CCCP, and TBT on the components of the pmf

Fig. 6.6 and Fig. 6.7 depict typical flow dialysis experiments, carried out as described in 'Methods', for the measurement of $\Delta\psi$ and ΔpH, respectively. Shortly after isotope was added to the upper chamber, radioactivity appeared in the dialysate, reaching a maximum at about 5 minutes and then decreasing at a slow and constant rate. When cell suspension and glucose were added, the radioactivity in the dialysate decreased, indicating the uptake of the radioactive compound by the cells. Addition of $AgNO_3$ (Fig. 6.6) caused release of radioactivity from the cells, increasing the concentration in the dialysate and indicating a decrease of the $\Delta\psi$ value. CCCP caused a similar collapse of the ΔpH (Fig. 6.7). The control, where buffer replaced cell suspension, corrected for any dilution effects.

The results of a number of these experiments are summarised in Table 6.1. A ΔpH of 53mV and a $\Delta\psi$ of -85mV were attained in energised cells at pH 6.5. CCCP totally collapsed the pH gradient and decreased the apparent $\Delta\psi$ to -25mV. Ghazi *et al.* (1981) have proposed that at low $\Delta\psi$ the values are overestimated due to non-specific binding of $TPMP^+$ to cells. If I assumed that CCCP decreased $\Delta\psi$ to 0mV and corrected the other data for this binding, $\Delta\psi$ of energised cells decreased by some 3mV and $\Delta\psi$ of Ag^+-treated cells became about -25mV. Whatever the case, $AgNO_3$ did not collapse the membrane potential as completely as did CCCP. ΔpH was completely abolished by Ag^+. TBT had very little effect on the membrane potential, but abolished the ΔpH in the presence of chloride. In the absence of chloride, there was very little effect on ΔpH, showing that the uncoupling effect was most probably due to Cl^-/OH^- exchange. As noted in Appendix III, if the cytoplasmic volume rather

Fig. 6.6 Uptake of $^{3}H\text{-}TPMP^{+}$ determined by flow dialysis, calculated $\Delta\psi$ derived from $TPMP^{+}$ uptake, and effect of Ag^{+} on $\Delta\psi$.

Cells were grown and prepared, and flow dialysis was carried out, as described in 'Methods'. 0.73ml of buffer ('low phosphate', Chapter 2, Section D.7) and 20µl of $^{3}H\text{-}TPMP^{+}$ (0.25mM) were added and collection of fractions was started. Cell suspension (50µl) was added where shown to give A_{660} of 12.80, followed 30 seconds later by glucose (5mM). $AgNO_3$ (100µM) was added where shown. $\Delta\psi$ was calculated as described in Appendix II.

• experimental curve, 50µl of cell suspension added at fraction 15.

o control curve, 50µl of buffer added at fraction 15.

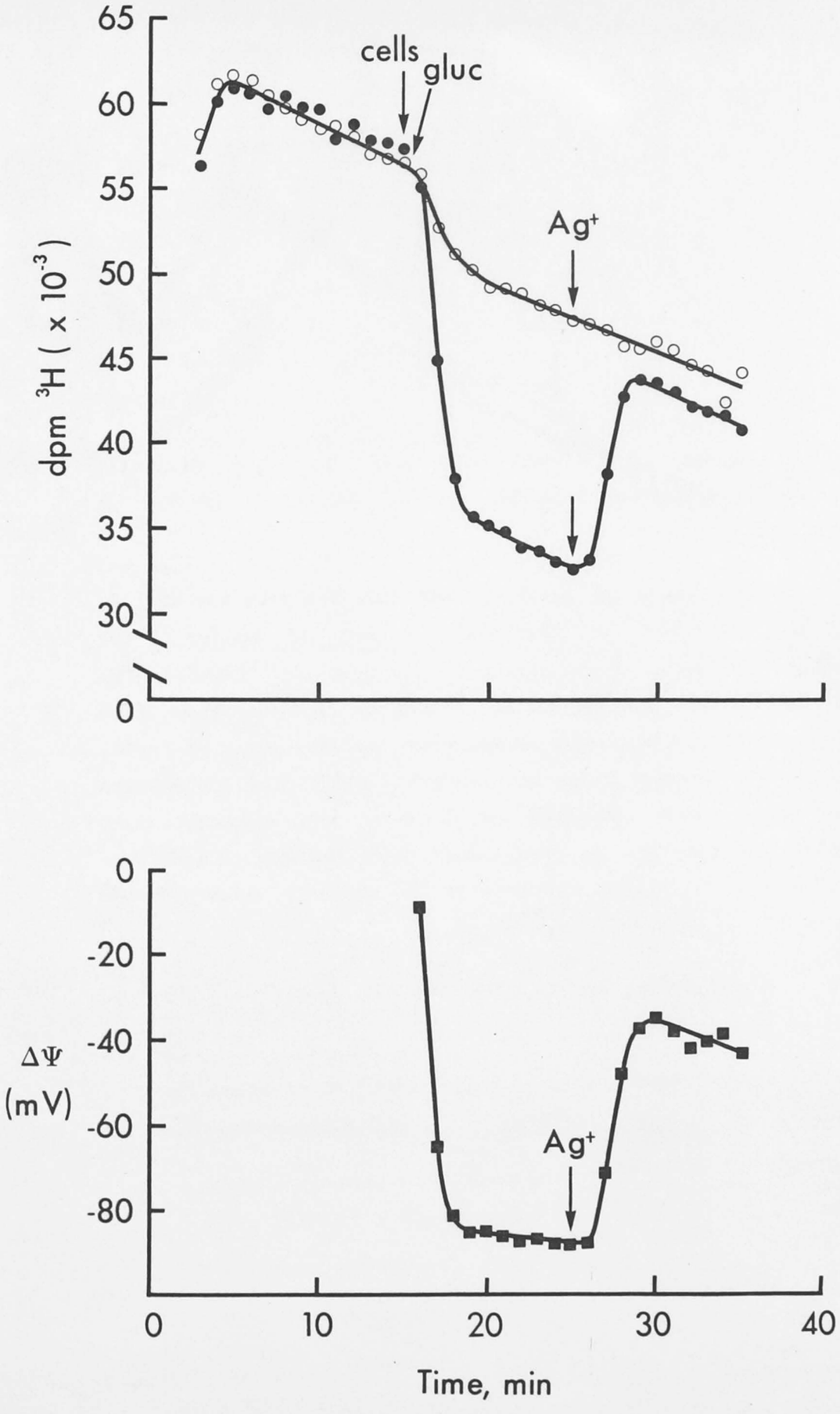

65
60
55
50
45
40
35
30
0
cells
gluc
Ag+
dpm ³H (x 10⁻³)
0
-20
-40
-60
-80
ΔΨ
(mV)
Ag+
0
10
20
30
40
Time, min

Fig. 6.7 Uptake of ^{14}C-DMO determined by flow dialysis, calculated ΔpH derived from DMO uptake, and effect of CCCP on ΔpH.

Cells were grown and prepared, and flow dialysis was carried out, as described in 'Methods'. 0.74ml of buffer ('low phosphate', Chapter 2, Section D.7) and 10µl of ^{14}C-DMO (16µM) were added and collection of fractions was started. Cell suspension (50µl) was added where shown to give A_{660} of 16.64, followed 30 seconds later by glucose (5mM). CCCP (50µM) was added where shown. External pH (------) was measured in a parallel experiment as described in 'Methods'. ΔpH was calculated as described in Appendix II. The pK_a value for DMO used in the calculation was 6.3.

• experimental curve, 50µl of cell suspension added at fraction 15.

o control curve, 50µl of buffer added at fraction 15.

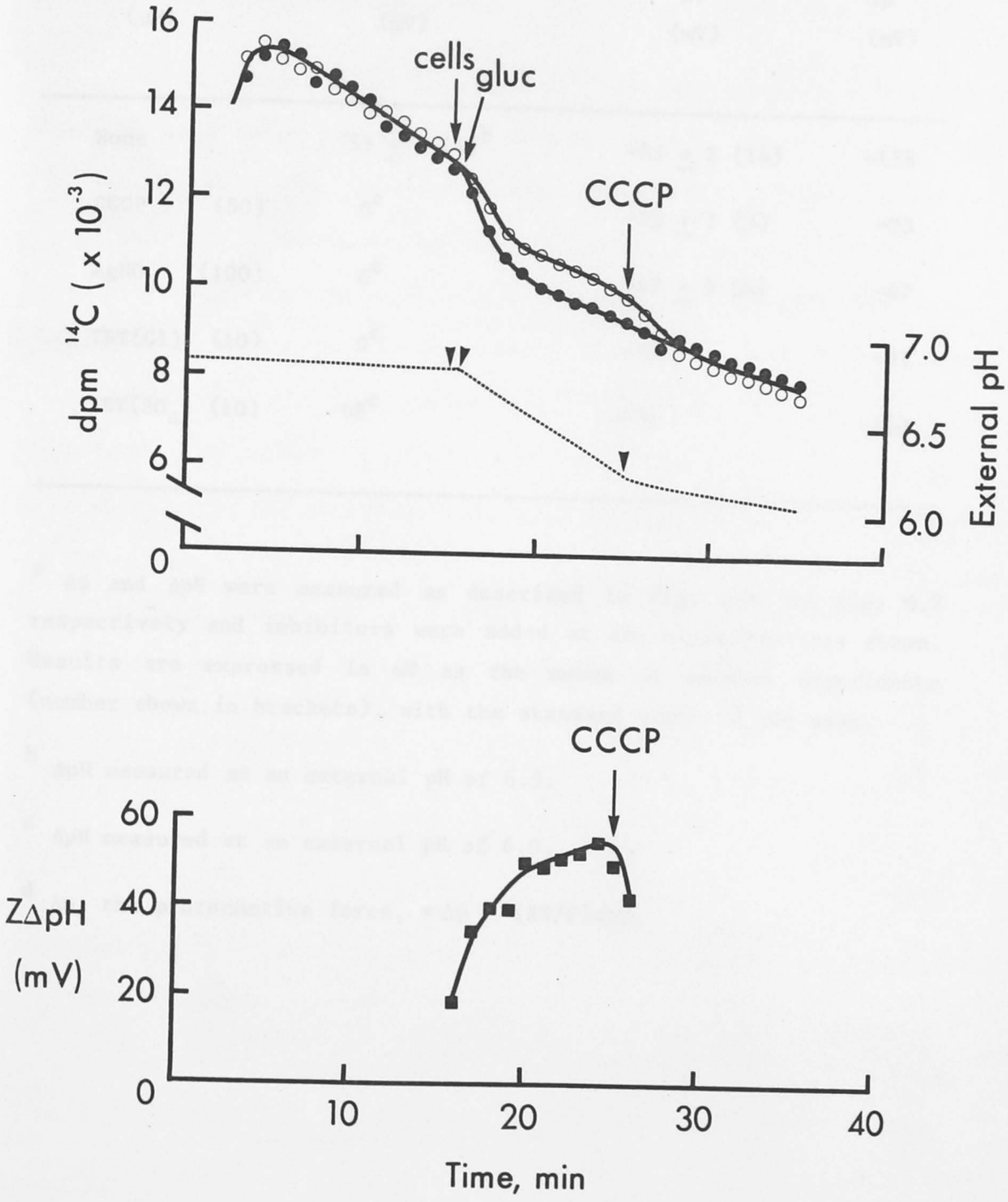

16
14
12
10
8
6
0
dpm ^{14}C (x 10^{-3})
cells
gluc
CCCP
7.0
6.5
6.0
External pH
CCCP
60
40
20
0
ZΔpH
(mV)
10
20
30
40
Time, min

Table 6.1 Effects of inhibitors on ΔpH, $\Delta\psi$ and pmf[a].

Inhibitor (μM)	$(RT/F)\Delta pH$ (mV)	$\Delta\psi$ (mV)	Δp[d] (mV)
None	53 ± 4 (4)[b]	-85 ± 2 (14)	-138
CCCP (50)	0[c]	-25 ± 7 (3)	-25
$AgNO_3$ (100)	0[c]	-47 ± 3 (4)	-47
TBT(Cl) (10)	0[c]	-73	-73
$TBT(SO_4)$ (10)	68[c]	-72	-140

[a] $\Delta\psi$ and ΔpH were measured as described in Fig. 6.6 and Fig. 6.7 respectively and inhibitors were added at the concentrations shown. Results are expressed in mV as the means of several experiments (number shown in brackets), with the standard error of the mean.

[b] ΔpH measured at an external pH of 6.5.

[c] ΔpH measured at an external pH of 6.0.

[d] Δp, the protonmotive force, $= \Delta\psi - (RT/F)\Delta pH$.

than the total cell volume (cytoplasm plus periplasm) was used in the calculation of ΔpH and $\Delta\psi$, the values increased by about 15mV, except for the ΔpH values of 0mV.

To check the possibility that Ag^+ may have caused the interior of the cell to become more acid than the medium, the distribution of ^{14}C-methylamine was measured. Being a weak base, methylamine would accumulate in the more acidic compartment. No such accumulation within cells was observed after treatment of the cell suspension with $AgNO_3$ (Data not shown). Energised cells appeared to exclude methylamine, indicating that these cells were more alkaline than the medium. ΔpH cannot accurately be quantified from data of this nature, where the concentration inside the cell is less than that in the medium.

DISCUSSION

Immediate efflux of protons from E. coli, followed by re-uptake of protons, is observed upon the addition of $AgNO_3$ to a suspension of bacteria (Fig. 6.1). However, when ΔpH was examined, $AgNO_3$ addition caused the collapse of the pH gradient (Table 6.1), and for this to occur one would expect to see an influx of protons into the more alkaline intracellular space. An explanation of this apparent discrepancy may be the difference in response time between the two experiments. The H^+ electrode responds within seconds whereas the flow dialysis apparatus essentially averages the results over a few minutes, and so was not able to detect the transient outflow of protons from the cell. A way of resolving this may be to use an ion-selective electrode sensitive to an appropriate permeant cation (Kamo et al., 1979; Hosoi et al., 1980). The response time would be 2-5 seconds rather than a few minutes, and a transient increase in ΔpH may be observed.

The observed efflux is most likely to be due to symport of protons with many of the metabolites which flow out of the cell in response to Ag^+ treatment. In all cases examined so far, treatment with Ag^+ caused efflux from the cell of the metabolite being examined, and it must be assumed that many others will do likewise. Some, such as lactate (Brink and Konings, 1980; Otto et al., 1980) and phosphate, may leave by symport with protons and others, K^+ for example, may exit via an antiporter. The summation of all these proton movements need not necessarily balance to zero, and what was observed in the H^+ electrode experiments would have been this net movement, composed of both entry and exit but with efflux predominating. The "fallback" of protons after this net efflux would be due to movement down their concentration gradient through the de-

energised membrane, although it could be seen that the membrane was not completely permeable since uncouplers increased the rate of "fallback" (Fig. 6.2B, Fig. 6.3B). This slight impermeability which was retained after Ag^+ treatment could also be seen in the small remaining membrane potential after Ag^+ treatment (-25mV if corrected for $TPMP^+$ binding by assuming total collapse of $\Delta\psi$ by CCCP; Table 6.1). The decrease by uncouplers of the extent of Ag^+-induced H^+ efflux (Fig. 6.2A, Fig. 6.3A) can be explained by the proton-conducting actions of the uncouplers, such that any significant proton gradient could not be maintained, however briefly.

Tributyltin showed effects on proton movements (Fig. 6.4) and ΔpH (Table 6.1) which required chloride. In the presence of chloride, TBT caused proton influx and collapsed the proton gradient, but in the absence of chloride no effect was observed. This result supports previous reports that tributyltin catalyses a Cl^-/OH^- exchange across membranes (Selwyn et al., 1970; Stockdale et al., 1970). TBT collapsed the pH gradient by means of this Cl^-/OH^- exchange, but, since this exchange is electroneutral, there was no effect of TBT on the membrane potential, in the presence or absence of chloride (Table 6.1).

Under anaerobic conditions, Ag^+ caused only a small amount of H^+ efflux (Fig. 6.5B). Phosphate (Fig. 4.9) and potassium (Fig. 5.10) efflux due to Ag^+ treatment still occurred under anaerobic conditions, but other uptake systems were not tested anaerobically. The pattern of efflux may have been different under anaerobic conditions, with some compounds not leaving the cell until aeration was started. This may explain the result if the proton movements were the result of symport or antiport with effluxing metabolites. If the pattern of metabolite efflux under anaerobic conditions was

essentially the same as that under aerobic conditions. The H^+ efflux observed anaerobically would be the summation of the proton entry and exit due to antiport or symport with these metabolites, and the H^+ efflux, from cells treated with Ag^+ while under N_2, observed upon commencement of aeration occurred by a process which is not as yet understood. $AgNO_3$ abolished the proton gradient and markedly reduced the membrane potential, but its effects on the retention of metabolites are not likely to be mediated by these changes since CCCP caused similar, more severe in the case of $\Delta\psi$, changes and yet did not cause efflux of metabolites as $AgNO_3$ did. Tributyltin chloride, which abolished ΔpH and had no effect on $\Delta\psi$, mimicked the effects of Ag^+ in many cases.

Unless the small remaining $\Delta\psi$ is required for Ag^+-induced efflux to occur, I must conclude that Ag^+ does not exert its effects on metabolite retention through changes in the electrochemical gradient of protons between the two bulk aqueous phases on either side of the membrane. The effects of Ag^+ are still likely to be membrane-associated, however, and could be due either to effects on the postulated small, lipophilic carrier of anhydrous protons within the membrane (Robertson and Boardman, 1975) or on the equally hypothetical eup gene product (Plate and Suit, 1981).

CHAPTER 7

THE ATTENUATION OF THE TOXIC EFFECTS OF SILVER IN SOME CLASSES OF MUTANTS OF E. COLI

INTRODUCTION

1. Resistance to metals

Resistance to the toxic effects of metals, or other compounds toxic to the cell, such as antibiotics, occurs by three major mechanisms: (1) alteration of the target site; (2) interference with transport into the cell; or (3) detoxification.

Plasmid-determined resistance to erythromycin is due to methylation of its target, the 23S RNA of the large ribosome subunit (Davies and Smith, 1978). Resistance to cadmium ions, on the other hand, is due to an alteration in its transport.Cells which carry the resistance plasmid show lower rates of net Cd^{2+} entry (Chopra, 1975; Tynecka et al., 1981). This is due to plasmid-coded energy-dependent efflux of Cd^{2+}, probably by a cadmium/proton antiporter (Tynecka et al., 1981a). The third mechanism of resistance, detoxification, is exemplified by microbial resistance to mercury (Summers and Silver, 1972) and organomercurials (Schottel et al., 1974; Schottel, 1978). Organomercurials are cleaved to form Hg^{2+} by an organomercurial lyase (Schottel, 1978; Summers and Silver, 1978). The resistance plasmid also codes for a mercuric reductase, which reduces Hg^{2+} to Hg^{0}, which is volatile (Summers and Silver, 1972; 1978).

Argyria, the black pigmentation of tissues after exposure to Ag^{+}, may be the result of a specific detoxification mechanism for silver. The colour is caused by the deposition of insoluble silver-protein complexes, or silver sulphide (Luckey and Venugopal, 1977).

Bacteria resistant to Ag^+ have been isolated from hospital patients who were being treated for burns with the topically applied antibiotic silver-sulphadiazine (Hendry and Stewart, 1979). Resistance was found to be due to the presence of a plasmid, which has since been mobilised and transferred to E. coli (Dr S. Silver, personal communication).

2. The proton-translocating ATPase of E. coli

The membrane-bound, proton-translocating ATPase (EC 3.6.1.3) is an important link in energy transduction, so studies of the effects of Ag^+ on strains carrying mutations in this system may lead to an increased understanding of the mechanism of action of Ag^+. The ATPase can generally be divided into two parts. One part, the F_1-ATPase, is extrinsic to the membrane and can be readily separated from it. This portion carries the ATP hydrolytic activity. The second part, the F_0-component, is located within the membrane. It has been suggested that F_0, as well as providing a base for the attachment of F_1 to the membrane, forms a proton pore between the external medium and the site of ATP synthesis on the F_1-ATPase (Altendorf et al., 1974). When the F_1 "knob" is stripped from membranes, they become proton-permeable, as judged by direct measurement of proton conductance (Okamoto et al., 1977) or by impairment of the ability of NADH to induce quenching of atebrin (quinacrine) fluorescence (Rosen and Adler, 1975). Treatment of these membranes with DCCD (Altendorf et al., 1974) or F_1-ATPase (Okamoto et al., 1977) restores proton impermeability.

It is possible to obtain mutants, referred to as unc mutants, in which oxidative phosphorylation is uncoupled from electron transport. These mutants cannot grow on non-fermentable carbon sources, such as succinate, show low growth yields on limiting glucose, cannot perform oxidative phosphorylation, and are unable to energise membranes by hydrolysis of ATP (Butlin et al., 1971). Generally, unc mutants fall into two groups, those that retain ATPase activity, and those that do not. Those lacking ATPase activity have been shown to be affected in the F_1 portion of the ATPase, whereas those which retain the ability to hydrolyse ATP have a defective F_0 sector (Downie et al., 1979). Of the genes so far identified, uncA, uncC, uncD, uncG, and uncH code for proteins in F_1 (Downie et al., 1979; 1980; Gunsalus et al., 1982), and uncB, uncE and uncF code for subunits of the F_0 sector (Downie et al., 1981).

Gene-polypeptide relationships are as follows: uncA codes for the α subunit, uncD for the β subunit (Downie et al., 1979), uncC for the ε subunit, uncG for the γ subunit (Downie et al., 1980), and uncH for the δ subunit (Gunsalus et al., 1982), all in the F_1 portion. In the F_0 sector, uncE codes for the DCCD-binding protein, which may be directly involved in H^+ translocation (Wachter et al., 1980). The alleles uncB and uncF code for the 24,000 and 18,000 dalton subunits, respectively, of the F_0 (Downie et al., 1981).

In general, in strains with mutations in either component of the ATPase, there is no ATP-dependent membrane energisation as judged by the absence of ATP-driven atebrin fluorescence quenching. In F_0 mutants, NADH-dependent atebrin fluorescence quenching in membranes which have been stripped of F_1 is near normal, indicating that the membranes are proton-impermeable. Energy-dependent quenching of atebrin fluorescence is usually taken to indicate that atebrin is

taken up in response to a pH gradient across the membrane (Schuldiner and Avron, 1971), but there is increasing evidence that atebrin is monitoring an intramembrane H^+ transfer associated with the energisation of the membrane (Lee, 1974; Huang et al., 1977; Storey et al, 1980; Storey and Lee, 1981).

In cells carrying the uncE408 allele, the ATPase does not assemble properly (Cox et al., 1982). However, in a homoallelic partial diploid strain which carries the mutant uncE408 allele on an F' plasmid, or in a strain which carries the mutant uncE408 allele on a multi-copy plasmid, the fault is rectified, a phenomenon known as self-complementation (Ash, 1981; Cox et al., 1982). An abnormal, but functional, DCCD-binding protein is shown to be incorporated into the membrane (Cox et al., 1982). In strains carrying the unc410 allele, an abnormal DCCD-binding protein is incorporated, assembly is normal, and strains do not exhibit the property of self-complementation. No phosphorylation of ADP is observed, whether energy is provided by electron transport or by an artificially imposed pH gradient. In a strain carrying multiple copies of the uncE408 allele, however, phosphorylation could be driven by electron transport, but not by an artificial pH gradient. Cox et al. (1982) suggest that protons involved in oxidative phosphorylation either enter F_0 from within the membrane (when supplied by electron transport) or from the external medium (when driven by pH), and that both mechanisms may operate in vivo.

Mutants of Bacillus megaterium (Decker and Lang, 1977; 1977a; Guffanti et al., 1981) and of E. coli (Ito and Ohnishi, 1981) have been isolated which are resistant to uncouplers of oxidative phosphorylation. ATP synthesis was less sensitive to uncoupler in these strains than in the wild-type, but only when energisation was

by respiration and not when an artificially imposed gradient was used (Guffanti et al., 1981). It was proposed that ATP synthesis was coupled to a "microscopic gradient" of protons, which was present when energisation was by respiration but not when an artificial gradient was established, when the bulk gradient is likely to be the predominant form. This so-called "microscopic gradient" may in fact be intramembrane anhydrous H^+.

In an attempt to gain information about the mode of action of Ag^+ I have examined mutants which showed resistance to the effects of silver ions. Plasmid-determined resistance to silver, expressed as an ability to grow in the presence of Ag^+, was shown to be expressed only in the presence of chloride. Resistance could only be seen clearly in the long term, when growth in the presence of Ag^+, or uptake of phosphate after prolonged exposure to Ag^+, was examined. Measurements of P_i uptake and oxygen uptake in the presence and absence of Ag^+, and of ^{110}Ag uptake, were not able to unequivocally demonstrate a difference between resistant and sensitive strains. I concluded that resistance to Ag^+ in this case was due to a detoxification process.

A different type of "resistance" was encountered upon examination of a series of strains of E. coli carrying mutations in the unc region of the genome. Strains fell into two classes, one in which Ag^+ caused efflux of P_i to about the same extent as that found in wild strains, and the other in which efflux was significantly reduced. Strains which were capable of phosphorylation of ADP comprised the first class, and the second class contained strains which were incapable of phosphorylation. Most of the strains in the second class carried mutations affecting the F_0 sector.

METHODS

The materials, bacterial strains, buffers, and general methods used have been described in Chapter 2.

1. Measurement of growth in liquid media

Growth was measured in "low phosphate" medium (Chapter 2, Section D.7), supplemented according to the requirements of the strain, and containing either no chloride, or 15mM KCl. A known amount of a fresh liquid culture of cells was inoculated into sterile side-arm flasks, which were then shaken at 37°C. Turbidity was measured at intervals using a Klett-Summerson colorimeter and recorded as arbitrary Klett units (Klett 300 is approximately equivalent to 10^9 cells/ml).

2. Measurement of oxygen uptake

Oxygen uptake was measured at 37°C with a Clark-type oxygen electrode (Titron Instruments, Melbourne, Victoria, Australia) as described by Cox *et al.* (1970). The apparatus was calibrated to give a pen deflection of 100% for an air-saturated solution, and zero for a cell suspension which had been supplied with glucose and left to consume all the oxygen in the vessel. A value for the solubility of oxygen of 375ng atoms of O/ml at 37°C was used (derived from Chappell (1964) and corrected for the decreased atmospheric pressure at Canberra, 600m above sea level).

3. Transfer of plasmid

Plasmid transfer from strain J62.2(pCS35) to strains AN710 and AN1088 was by conjugation at 37°C for 5.5 hours, followed by selection on minimal agar plates supplemented with glucose (20mM), thiamine (3μM), 2,3-dihydroxybenzoate (10μM), arginine (1mM), and kanamycin (30μg/ml). The above conjugations and initial selection were performed by Dr S. Silver. Further selection on the above medium supplemented with $AgNO_3$ (50μM) was followed by checking the growth, in liquid medium containing $AgNO_3$ (10μM) and KCl (15mM), of selected kanamycin-resistant, silver-resistant colonies. Care was taken to exclude light from the media containing $AgNO_3$. Single-colony isolates of kanamycin-resistant, silver-resistant transconjugants derived from AN710 and AN1088 were called, respectively, HR16 and HR17.

RESULTS

7.1 Growth in the presence of silver ions and the effect of chloride ions.

The silver resistance plasmid was originally identified by the ability of the strain carrying it to grow in the presence of silver (Hendry and Stewart, 1979; S. Silver, personal communication) (Fig. 7.1). Plasmid-carrying strains grew in media containing silver ions only when chloride was also present (Fig. 7.2). In the absence of KCl the plasmid-carrying strain was as susceptible to silver ions as the wild strain.

7.2 Effect of silver ions on oxygen uptake.

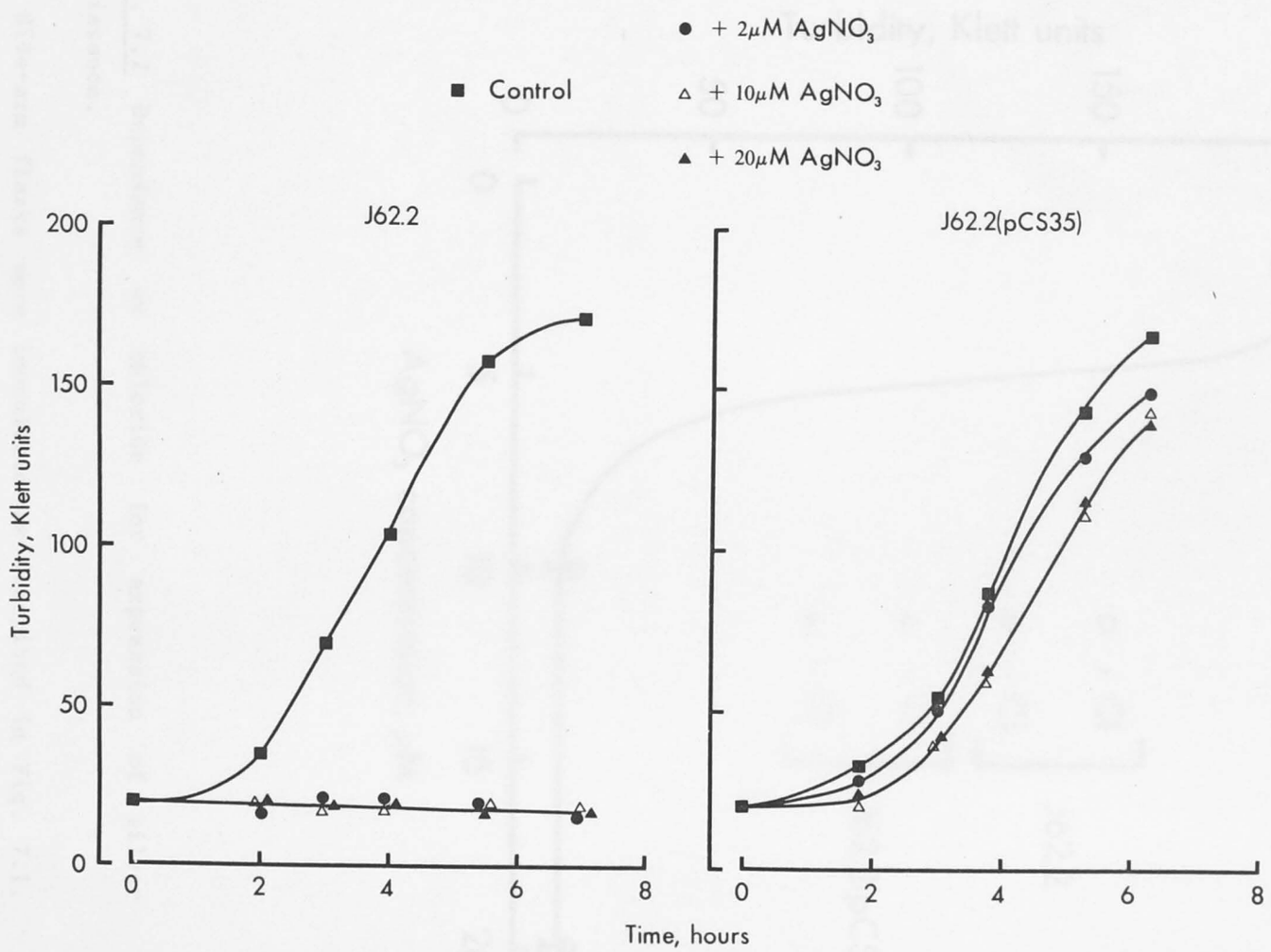
When $AgNO_3$ was added to respiring cells, oxygen uptake was first stimulated briefly, followed by inhibition (Fig. 7.3). This initial stimulation has been noted before (Yudkin, 1937; Bragg and Rainnie, 1974). Control experiments showed that, in the absence of silver ions, oxygen uptake proceeded linearly until the oxygen was exhausted (Fig. 7.3A). There appeared to be no significant difference in the degree of inhibition between the sensitive and resistant strains (Fig. 7.3).

Since growth of Ag^+-resistant strains in the presence of Ag^+ occurred only in the presence of chloride (Fig. 7.2), oxygen uptake was measured in medium containing 15mM KCl. Again no difference between the strains was observed (Fig. 7.4). The sharp drop in oxygen uptake, with a mid-point at 15mM $AgNO_3$, indicates that the result was due to a titration of the Cl^- by Ag^+, emphasised by the similarity between the shapes and positions of the inhibition curves and the "titration curve" for free Ag^+ (Fig. 7.4).

Fig. 7.1 Growth of J62.2 and J62.2(pCS35) in the presence of silver ions.

Cells from overnight cultures were inoculated as described in 'Methods'. The growth medium contained 15mM KCl and was supplemented with proline (1.5mM), histidine (0.25mM), tryptophan (0.2mM), and glucose (20mM). $AgNO_3$ was added at the concentrations shown, and the flasks were shaken at 37°C. Turbidity was measured at intervals and recorded as Klett units.

This figure was re-drawn from data provided by Dr S. Silver


● + 2μM AgNO3
■ Control
△ + 10μM AgNO3
▲ + 20μM AgNO3
J62.2
J62.2(pCS35)
Turbidity, Klett units
200
150
100
50
0
0
2
4
6
8
Time, hours

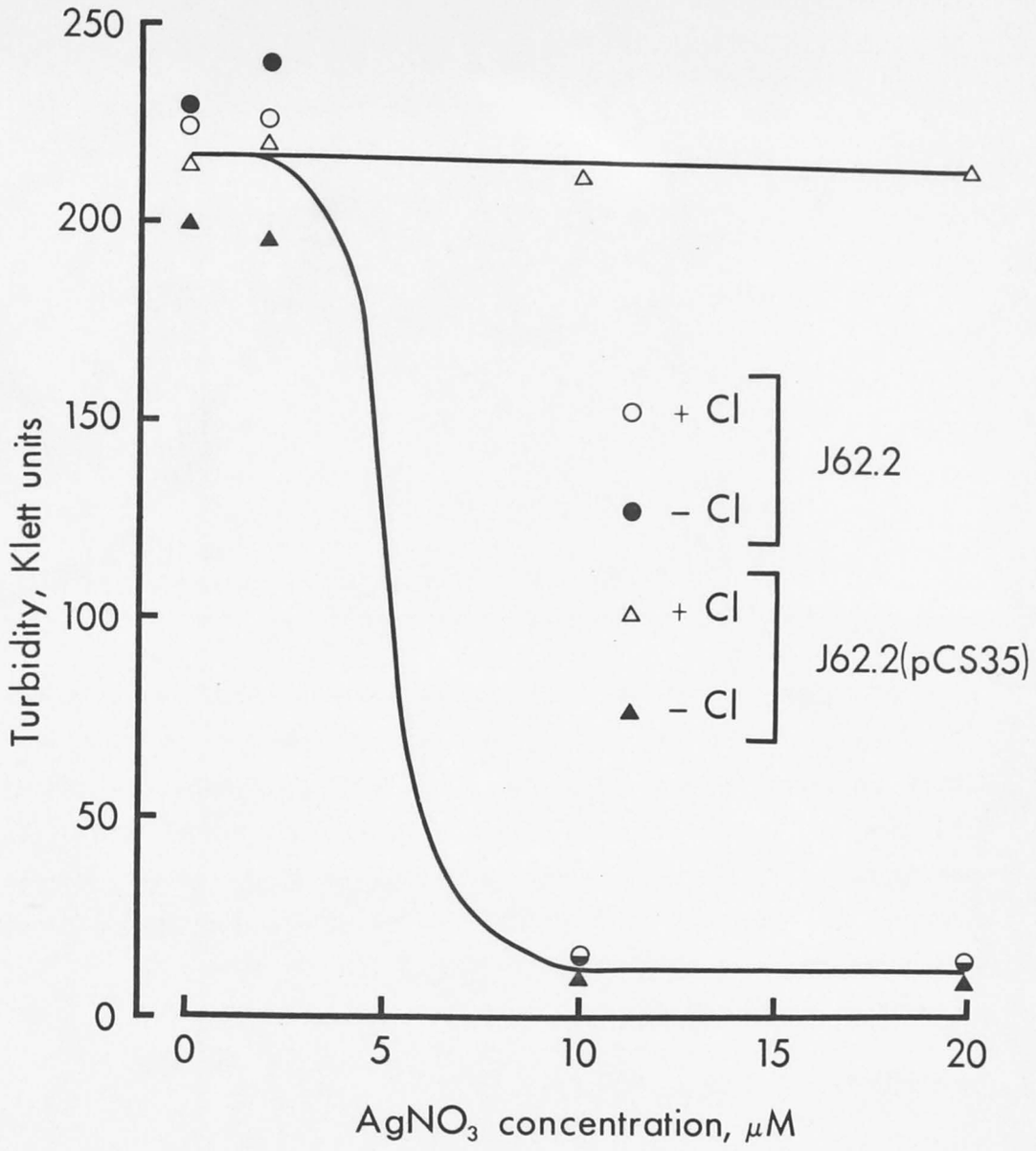


Fig. 7.2 Dependence on chloride for expression of silver resistance.

Side-arm flasks were inoculated as described in Fig. 7.1. Chloride-free medium was used where shown. Turbidity was measured after 20 hours of growth at 37°C.

This figure was re-drawn from data provided by Dr S. Silver

Fig. 7.3 Effect of silver ions on oxygen uptake.

Cells were grown overnight using glucose (20mM) as a source of carbon, harvested, and washed and resuspended in chloride-free phosphate-free buffer. The same buffer (2.4ml) was placed in the electrode vessel, glucose added to 10mM, and cell suspension (50µl), and $AgNO_3$ (80µM) added where shown. Oxygen uptake was measured as described in 'Methods'. The oxygen level is expressed as % saturation.

(----) No $AgNO_3$ added.

A) Strain J62.2 (A_{660} = 1.65)

B) Strain J62.2(pCS35) (A_{660} = 1.60)

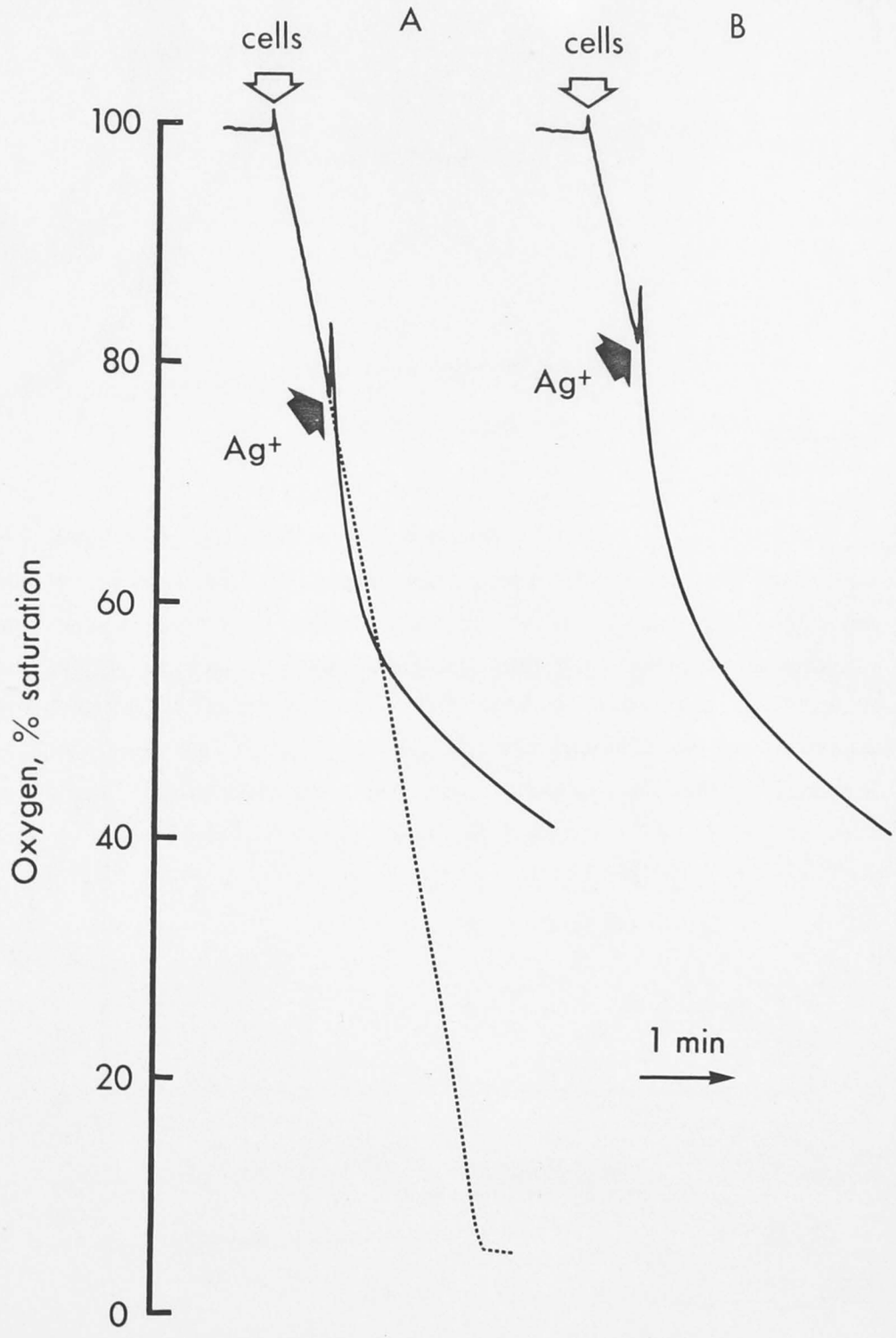
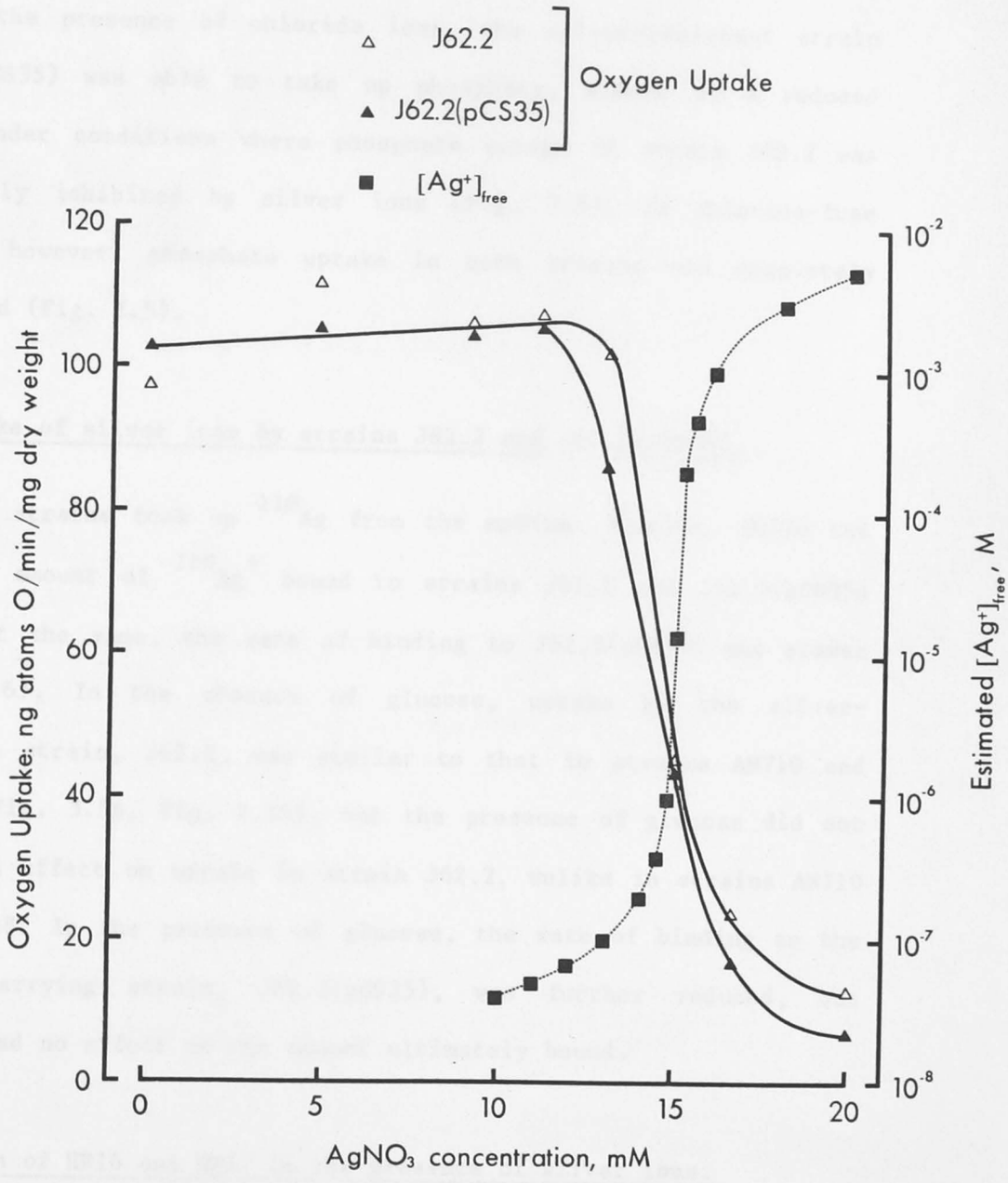

A
B
cells
cells
100
80
60
40
20
0
Oxygen, % saturation
Ag+
Ag+
1 min

Fig. 7.4 Effect on oxygen uptake of silver ions in the presence of chloride.

Cells were prepared, in medium containing 15mM KCl, as described in Fig. 7.3. $AgNO_3$ was added, at the concentrations shown, to the concentrated cell suspension 5 minutes before the suspension was added to the electrode vessel. Oxygen uptake was measured as described in Fig. 7.3 and calculated in terms of ng atoms of oxygen/min/mg dry weight of cells, as described in 'Methods'. The approximate free Ag^+ concentration, estimated from the solubility product for AgCl ($2 \times 10^{-10} mol^2 l^{-2}$), is also plotted.


J62.2
J62.2(pCS35)
Oxygen Uptake
[Ag+]free
Oxygen Uptake: ng atoms O/min/mg dry weight
Estimated [Ag+]free, M
AgNO3 concentration, mM
0
20
40
60
80
100
120
0
5
10
15
20
10-2
10-3
10-4
10-5
10-6
10-7
10-8

7.3 Phosphate uptake in strains J62.2 and J62.2(pCS35): effects of silver ions.

In the presence of chloride ions, the silver-resistant strain J62.2(pCS35) was able to take up phosphate, albeit at a reduced rate, under conditions where phosphate uptake in strain J62.2 was completely inhibited by silver ions (Fig. 7.5). In chloride-free medium, however, phosphate uptake in both strains was completely inhibited (Fig. 7.5).

7.4 Uptake of silver ions by strains J62.2 and J62.2(pCS35).

Both strains took up ^{110}Ag from the medium. However, while the eventual amount of $^{110}Ag^{+}$ bound to strains J62.2 and J62.2(pCS35) was about the same, the rate of binding to J62.2(pCS35) was slower (Fig. 7.6). In the absence of glucose, uptake by the silver-sensitive strain, J62.2, was similar to that in strains AN710 and AN1088 (Fig. 3.5B, Fig. 7.10), but the presence of glucose did not have much effect on uptake in strain J62.2, unlike in strains AN710 and AN1088. In the presence of glucose, the rate of binding to the plasmid-carrying strain, J62.2(pCS35), was further reduced, but glucose had no effect on the amount ultimately bound.

7.5 Growth of HR16 and HR17 in the presence of silver ions.

Because previous work on the action of silver ions was done with the defined isogenic strains AN710 and AN1088, the plasmid was transferred into these strains as described in 'Methods'. The resulting plasmid-containing isolates grew in the presence of silver ions in media containing chloride (Fig. 7.7). Strains AN710 and AN1088 did not grow under these conditions.

Fig. 7.5 Inhibition of phosphate uptake by Ag^+: J62.2 and J62.2(pCS35).

Cells were grown and prepared for uptake as described in Chapter 2. The source of energy was succinate (20mM). When present, $AgNO_3$ was added 4 minutes before $^{32}P_i$, at a concentration of 5μM.

Open symbols - medium containing 15mM KCl.

Filled symbols - chloride-free medium.

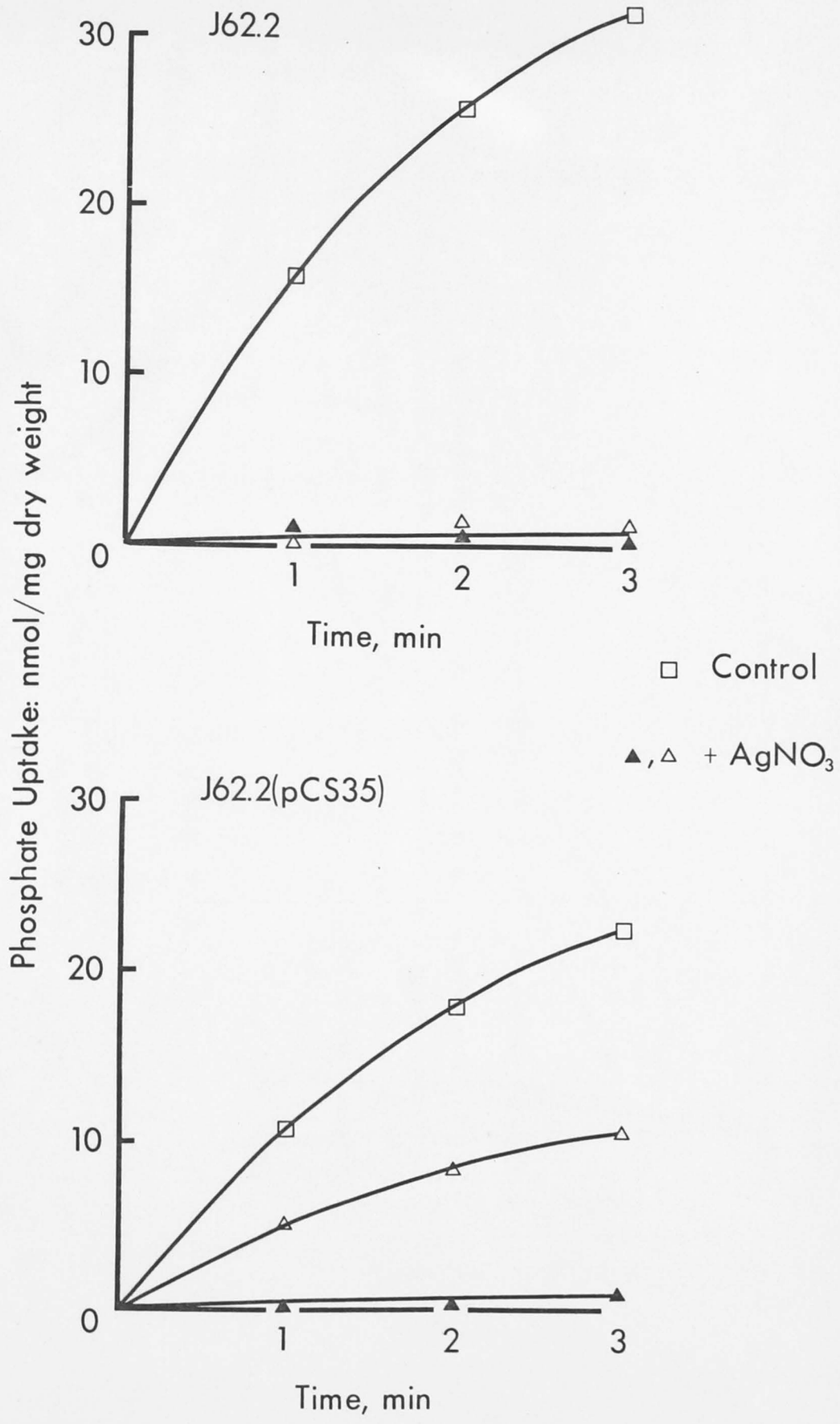

J62.2
30
20
10
0
1
2
3
Time, min
Phosphate Uptake: nmol/mg dry weight
□ Control
▲, △ + AgNO3
J62.2(pCS35)
30
20
10
0
1
2
3
Time, min

Fig. 7.6 Uptake of $^{110}Ag^{+}$ by J62.2 and J62.2(pCS35).

Cells were grown and prepared for uptake assay as described in Chapter 2. Cell suspensions were pre-incubated for 5 minutes at 37°C before the addition of 0.2μM $^{110}AgNO_3$. Glucose, where present, was at 20mM.

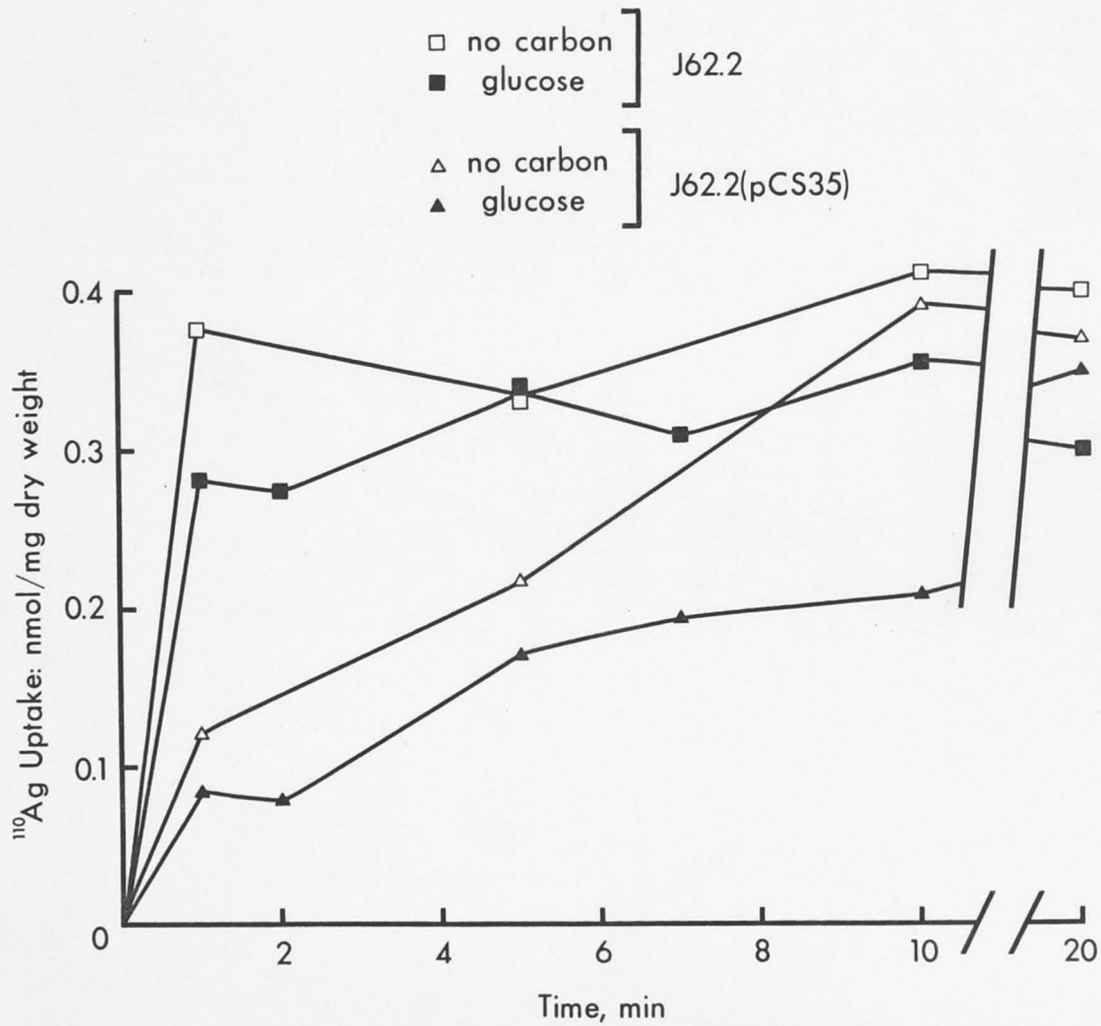
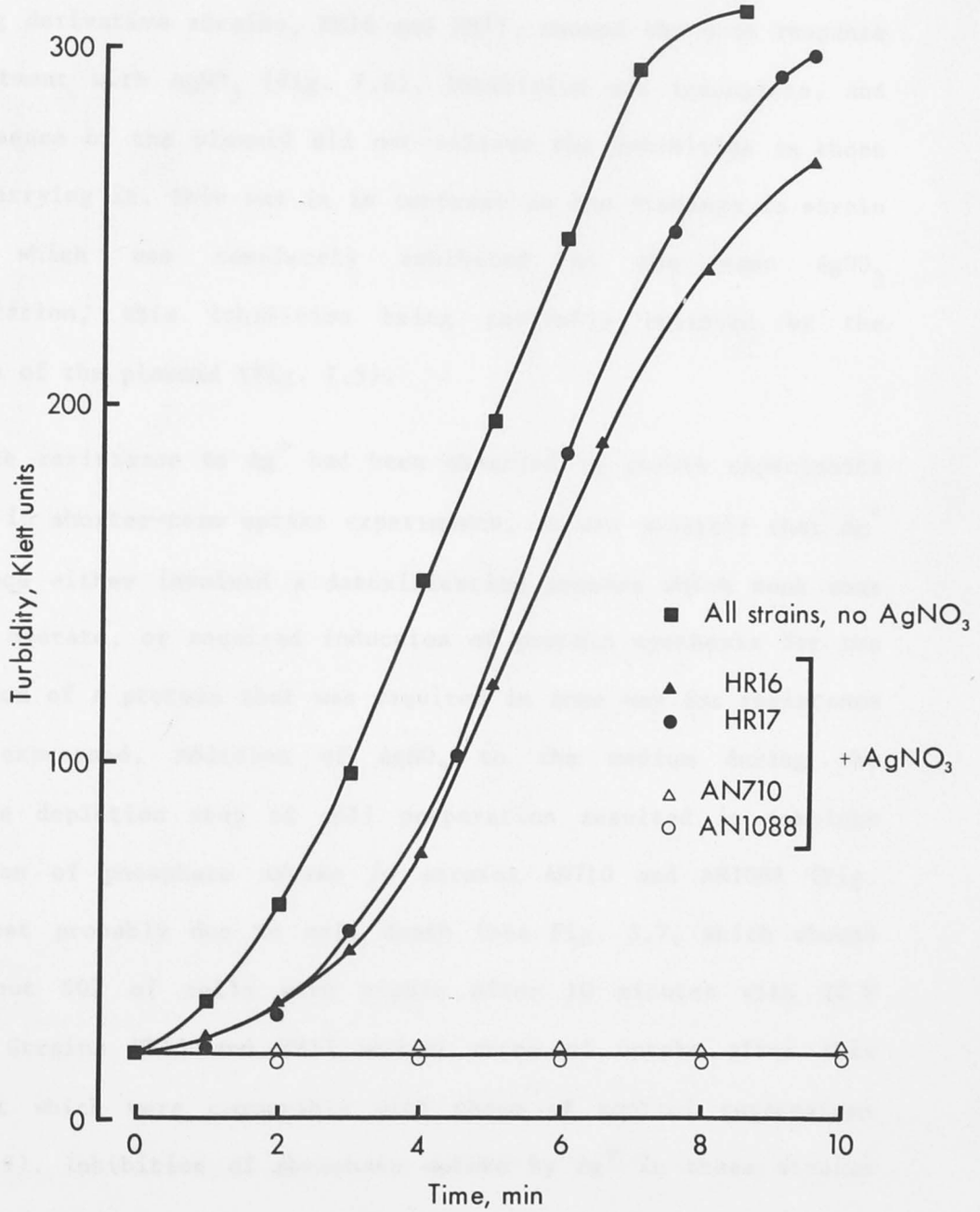

□ no carbon
■ glucose
J62.2
△ no carbon
▲ glucose
J62.2(pCS35)
^{110}Ag Uptake: nmol/mg dry weight
0.4
0.3
0.2
0.1
0
2
4
6
8
10
20
Time, min

Fig. 7.7 Growth of HR16 and HR17 in the presence of silver ions.

The experiment was carried out, in medium containing 15mM KCl, as described in Fig. 7.1, except that the growth supplements were arginine (1mM), thiamine (3μM), 2,3-dihydroxybenzoate (10μM), and succinate (20mM). The concentration of $AgNO_3$ was 10μM.


300
200
100
0
Turbidity, Klett units
0
2
4
6
8
10
Time, min
All strains, no AgNO3
HR16
HR17
AN710
AN1088
+ AgNO3

7.6 Phosphate uptake in strains HR16 and HR17: effects of silver ions.

Strains AN710 and AN1088 and their corresponding plasmid-carrying derivative strains, HR16 and HR17, showed the same response to treatment with $AgNO_3$ (Fig. 7.8). Inhibition was incomplete, and the presence of the plasmid did not relieve the inhibition in those cells carrying it. This was is in contrast to the findings in strain J62.2, which was completely inhibited at the same $AgNO_3$ concentration, this inhibition being partially relieved by the presence of the plasmid (Fig. 7.5).

Since resistance to Ag^+ had been observed in growth experiments but not in shorter-term uptake experiments, it was possible that Ag^+ resistance either involved a detoxification process which took some time to operate, or required induction of protein synthesis for the production of a protein that was required in some way for resistance to be expressed. Addition of $AgNO_3$ to the medium during the phosphate depletion step of cell preparation resulted in complete prevention of phosphate uptake in strains AN710 and AN1088 (Fig. 7.9), most probably due to cell death (see Fig. 3.7, which showed that about 50% of cells were viable after 10 minutes with 20 M $AgNO_3$). Strains HR16 and HR17 showed rates of uptake after this treatment which were comparable with those of control suspensions (Fig. 7.9). Inhibition of phosphate uptake by Ag^+ in these strains after prolonged exposure to $AgNO_3$ was, if anything, more severe (Fig. 7.9) than that in cells which had not been exposed to Ag^+ (Fig. 7.8), indicating that silver resistance, at least in terms of phosphate uptake, was not an inducible phenomenon. Similar results were obtained after overnight growth in the presence of Ag^+.

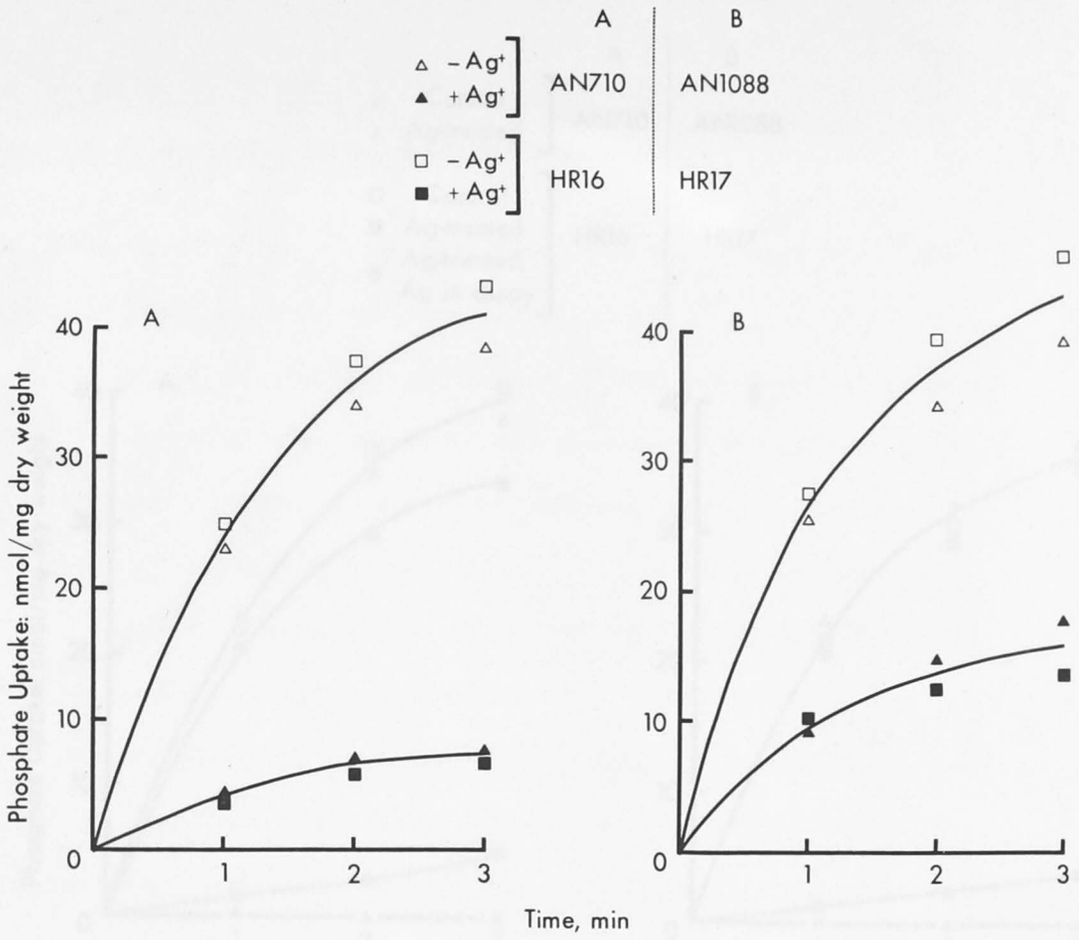


Fig. 7.8 Inhibition of phosphate uptake by Ag^+: HR16 and HR17 compared with AN710 and AN1088.

The experiment was carried out, in medium containing 15mM KCl, as described in Fig. 7.5. $AgNO_3$ (5μM) was added 4 minutes before $^{32}P_i$.

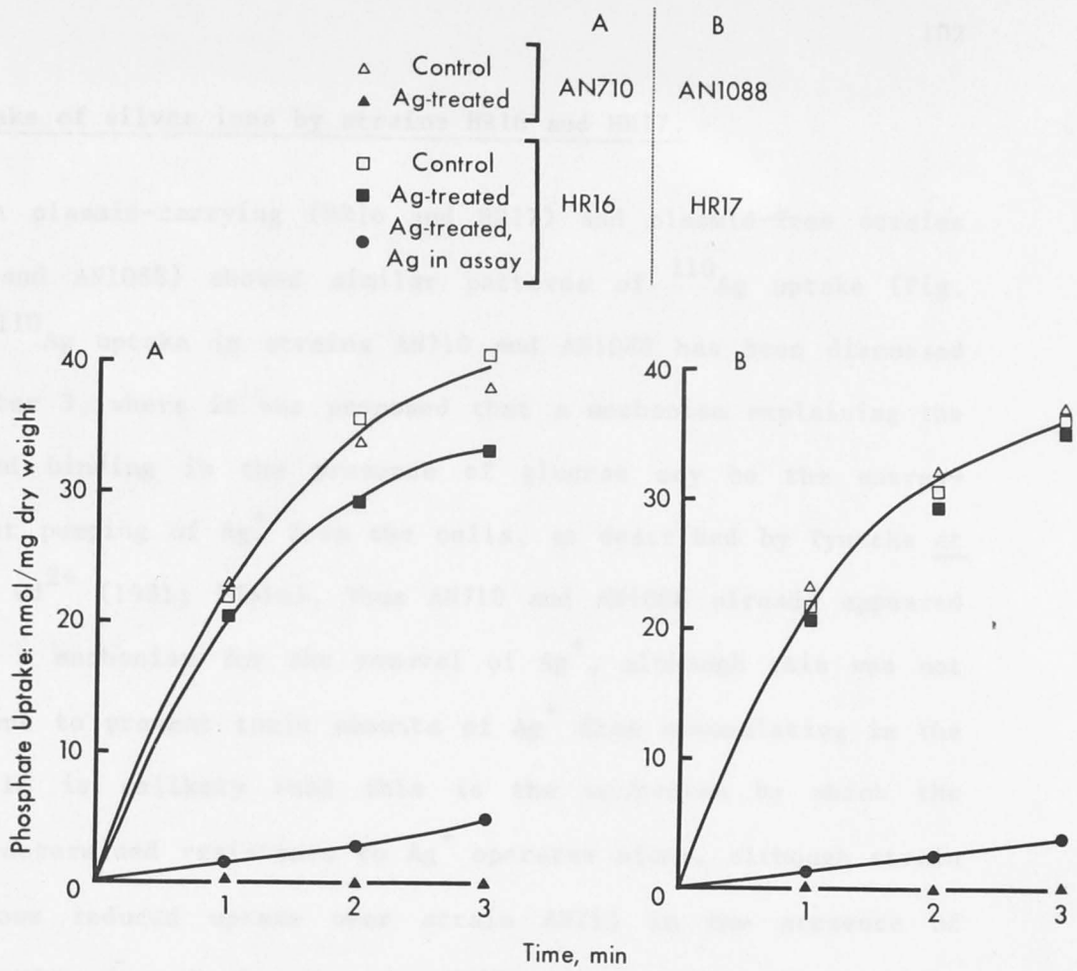


Fig. 7.9 Effects on phosphate uptake of pre-treatment with Ag^+.

Cells were grown overnight, and prepared in medium containing 15mM KCl, as described in Chapter 2, except that, where shown, $AgNO_3$ (10μM) was added to the medium during the phosphate depletion step. Phosphate uptake was then assayed in treated and untreated cells. When $AgNO_3$ was present in the assay, it was added 4 minutes before $^{32}P_i$, at a concentration of 5μM.

7.7 Uptake of silver ions by strains HR16 and HR17.

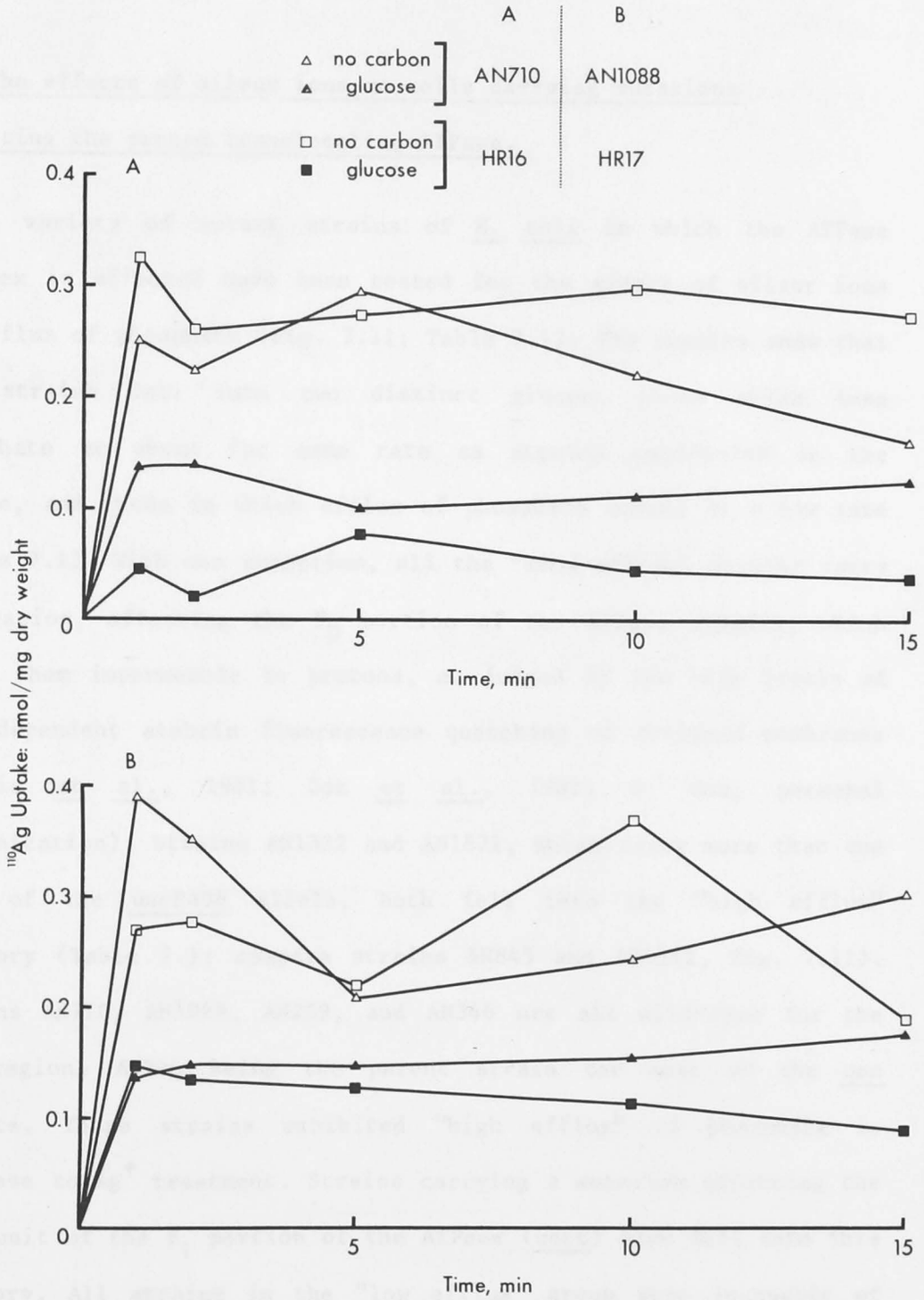
Both plasmid-carrying (HR16 and HR17) and plasmid-free strains (AN710 and AN1088) showed similar patterns of ^{110}Ag uptake (Fig. 7.10). ^{110}Ag uptake in strains AN710 and AN1088 has been discussed in Chapter 3, where it was proposed that a mechanism explaining the decreased binding in the presence of glucose may be the energy-dependent pumping of Ag^+ from the cells, as described by Tynecka *et al.* for Cd^{2+} (1981; 1981a). Thus AN710 and AN1088 already appeared to have a mechanism for the removal of Ag^+, although this was not sufficient to prevent toxic amounts of Ag^+ from accumulating in the cells. It is unlikely that this is the mechanism by which the plasmid-determined resistance to Ag^+ operates since, although strain HR16 shows reduced uptake over strain AN710 in the presence of glucose, this is not the case for HR17. Also, strain J62.2(pCS35), which is resistant to Ag^+, eventually binds as much ^{110}Ag as the plasmid-free strain J62.2 (Fig. 7.6).

7.8 The discolouration of the cell pellet after treatment with silver nitrate.

When the plasmid-carrying strains HR16 and HR17 were grown for some time in the presence of $AgNO_3$ in chloride-containing medium and then centrifuged, the cell pellet was consistently observed to contain brown-black coloured material, most often forming a "horseshoe" shape. This was not seen in plasmid-free cells, or if plasmid-containing cells were grown in chloride-free medium in the presence of $AgNO_3$. this dark material was very prominent in precipitates from cell suspensions which had been treated with Ag^+ for 4 or 6 hours, and was less noticeable in 1 and 2 hour treatments. These observations suggest that the plasmid-carrying

Fig. 7.10 Uptake of $^{110}Ag^{+}$ by HR16 and HR17.

The experiment was performed as described in Fig. 7.6. For comparison purposes, the data of Fig. 3.5B for AN710 and AN1088 were re-plotted in this figure.


A
B
no carbon
glucose
AN710
AN1088
no carbon
glucose
HR16
HR17
A
B
0.4
0.3
0.2
0.1
0
5
10
15
Time, min
^{110}Ag Uptake: nmol/mg dry weight

cells produced something which interacted with ionic silver and promoted its chemical or photochemical reduction.

7.9 The effects of silver ions on cells carrying mutations affecting the proton translocating ATPase.

A variety of mutant strains of E. coli in which the ATPase complex is affected have been tested for the effect of silver ions on efflux of phosphate (Fig. 7.11; Table 7.1). The results show that the strains fall into two distinct groups: those which lose phosphate at about the same rate as strains unaffected in the ATPase, and those in which efflux of phosphate occurs at a low rate (Table 7.1). With one exception, all the "slow efflux" strains carry a mutation, affecting the F_0 portion of the ATPase complex, which makes them impermeable to protons, as judged by the high levels of NADH-dependent atebrin fluorescence quenching of stripped membranes (Downie et al., 1981; Cox et al., 1982; G. Cox, personal communication). Strains AN1332 and AN1871, which carry more than one copy of the uncE408 allele, both fell into the "high efflux" category (Table 7.1; compare strains AN845 and AN1332, Fig. 7.11). Strains AN710, AN1088, AN259, and AN346 are all wild-type for the unc region, AN346 being the parent strain for most of the unc mutants. These strains exhibited "high efflux" of phosphate in response to Ag^+ treatment. Strains carrying a mutation affecting the ε subunit of the F_1 portion of the ATPase (uncC) also fell into this category. All strains in the "low efflux" group were incapable of phosphorylation, and strains able to phosphorylate fell into the "high efflux" group.

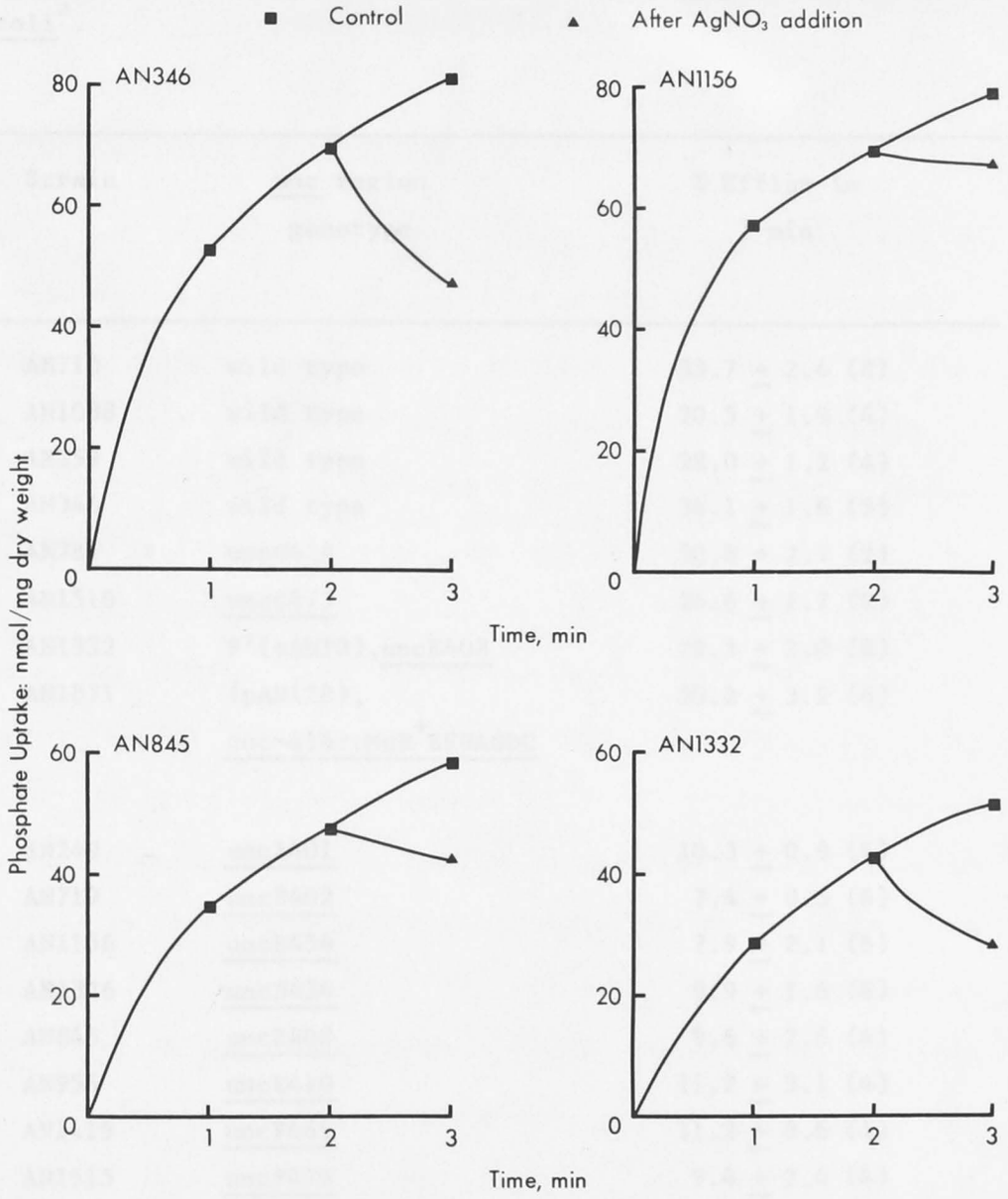


Fig. 7.11 Effects of unc mutations on Ag^+-induced P_i efflux.

Cells were grown on glucose in growth medium supplemented as required, prepared, and phosphate uptake was assayed in chloride-free medium as described in Chapter 2. $AgNO_3$ (20μM) was added where shown.

Table 7.1 Effects of Ag^+ treatment on various unc mutant strains of E. coli[a].

Strain	unc region genotype	% Efflux in 1 min
AN710	wild type	33.7 ± 2.4 (8)
AN1088	wild type	20.5 ± 1.9 (4)
AN259	wild type	28.0 ± 1.2 (4)
AN346	wild type	34.1 ± 1.6 (2)
AN781	uncC424	30.8 ± 2.7 (2)
AN1510	uncC473	26.6 ± 1.7 (2)
AN1332	F'(pAN10),uncE408	22.3 ± 3.0 (8)
AN1871	(pAN128), unc-416::MuB^+EFHAGDC	30.2 ± 3.2 (6)
AN249	uncA401	10.3 ± 0.9 (6)
AN719	uncB402	7.4 ± 0.5 (6)
AN1156	uncB454	7.9 ± 2.1 (6)
AN1326	uncB434	9.9 ± 1.6 (6)
AN845	uncE408	9.6 ± 2.5 (4)
AN955	uncE410	11.2 ± 3.1 (4)
AN1419	uncF469	11.2 ± 0.6 (4)
AN1515	uncF476	9.4 ± 2.4 (4)

[a] The experiments were carried out as described in Fig. 7.10. Results are expressed as the percentage of ^{32}P lost from the cells during the first minute after $AgNO_3$ addition. Means of several experiments (number shown in brackets), with the standard error of the mean, are presented.

DISCUSSION

1. Plasmid-determined resistance to silver ions.

Resistance to the effects of silver ions in plasmid-carrying cells was most clearly expressed as the ability of the plasmid-containing strains to grow in the presence of Ag^+ (Fig. 7.1, Fig. 7.7). Resistance to Ag^+ was only expressed in the presence of chloride (Fig. 7.2). It is not likely that chloride played a direct part in the resistance process, but served to maintain the free silver ion concentration at a very low level while the resistance mechanism was brought into operation. High levels of silver ion presumably killed the cell before resistance could be fully expressed, as in the plasmid-carrying cells in chloride-free medium (Fig. 7.2). Sensitive cells, on the other hand, while no more susceptible to short-term exposure to these low Ag^+ concentrations (Figs. 7.3, 7.4, 7.6, 7.8, 7.10), may have, in the absence of detoxification of Ag^+, accumulated a lethal amount of Ag, the AgCl acting, in effect, as a "silver buffer".

Oxygen uptake was inhibited to a similar degree in both sensitive and resistant strains (Fig. 7.3, Fig. 7.4). Chloride was required for the expression of resistance when this was measured in terms of growth, but, even in the presence of chloride, no difference between sensitive and resistant strains was observed when oxygen uptake was measured after pre-incubation with various concentratons of Ag^+ (Fig. 7.4). Both strains showed a sharp decrease in oxygen uptake as the concentration of added $AgNO_3$ approached that of the KCl present in the medium. This decrease mirrored the calculated rise in free silver ion concentration, in effect monitoring the titration of the Cl^- by Ag^+.

When phosphate uptake was monitored in the sensitive and resistant strains, some difference between the "J" strains and the AN710 and AN1088 derivatives was observed. Strain J62.2 was completely sensitive to pre-incubation with 5μM Ag^+, and Ag^+-resistant P_i uptake by strain J62.2(pCS35) could be observed (Fig. 7.5). Strains AN710 and AN1088 showed some uptake in the presence of Ag^+, and this basal level was unchanged in the plasmid-carrying strains HR16 and HR17 (Fig. 7.8). Note that these experiments were performed in the presence of 15mM KCl, so the free Ag^+ concentration was very low. Incubation of cell suspensions with Ag^+ for 1 hour killed strains AN710 and AN1088, which precluded uptake of phosphate (Fig. 7.9). Strains HR16 and HR17, on the other hand, were practically unaffected with respect to phosphate uptake. This treatment did not, however, enhance silver-resistant phosphate uptake in strains HR16 and HR17, so resistance to the short-term effects of Ag^+ could not be induced by previous exposure to Ag^+.

Uptake of $^{110}Ag^+$ was also different in the "J" strains compared to strains AN710 and AN1088 and their plasmid-carrying derivatives. Strain J62.2 took up the maximal amount of $^{110}Ag^+$ within the first minute, whether glucose was present or not (Fig. 7.6). Strain J62.2(pCS35) eventually took up the same amount of $^{110}Ag^+$ as strain J62.2, but only after 10 minutes in the absence, and 20 minutes in the presence, of glucose. On the other hand, strains AN710 and AN1088 were similar to J62.2 in the absence of glucose, but in the presence of glucose, they took up about 30% to 50% as much $^{110}Ag^+$ within the first minute, and this amount never increased (Fig. 7.10). The presence of the plasmid made no substantial difference.

These differences between the strains, in terms of $^{32}P_i$ and $^{110}Ag^+$ uptake, indicate that AN710 and AN1088 have some inherent mechanism for an energy-dependent partial removal of Ag^+ from the cell, a mechanism which strain J62.2 does not possess. This, however, cannot be the mechanism for plasmid-determined silver resistance since strains AN710 and AN1088 were still susceptible in the long term, as shown by the inability of these strains to grow at silver ion concentrations which allowed the growth of their plasmid-carrying derivatives.

The results, particularly in strains AN710 and AN1088, indicated that plasmid-determined resistance to Ag^+ occurred by a process which takes some time for the full effect to become apparent. The appearance of precipitable dark material in the cell suspension after prolonged treatment of resistant, but not sensitive, strains with Ag^+ suggests that the mechanism of resistance involved the formation of silver-protein complexes or silver sulphide (Luckey and Venugopal, 1977) or perhaps, since chloride was required, the formation of AgCl followed by the chemical or photochemical reduction of the silver. The requirement for chloride may have been to maintain the free Ag^+ at a low concentration while the detoxification mechanism was operating. Maintenance of low free Ag^+ levels was not of itself sufficient to prevent poisoning of sensitive cells (Fig. 7.2).

2. Effects of silver ions on unc mutants.

Strains containing mutant unc genes affecting the three different components of the F_0 sector have been tested for the effects of silver ions on the efflux of phosphate from these strains, and all showed the "low P_i efflux" characteristic (Table

7.1).

Of particular interest among these F_0 mutants are the uncE mutants, especially the uncE408 allele, which exhibits the unusual property of self-complementation (Ash, 1981; Cox et al., 1982). Strains carrying a single mutant allele belonged to the "low efflux" category, whereas in strains with more than one copy of the allele, Ag^+-induced efflux of phosphate was high (Table 7.1). This change in P_i efflux characteristics with the increase in copy number of the allele coincided with the occurrence of self-complementation. Strain AN781, with the mutant uncC424 allele, apparently does not assemble properly. There is partial assembly, however, leading to slow growth on succinate, and those cells in which the ATPase does assemble are capable of phosphorylation, albeit at a low rate (G. Cox, personal communication). Not much is known about the uncC473 allele (strain AN1510). Both of these strains efflux phosphate at the high rate (Table 7.1). In strain AN249, there may be a modification of the F_0 such that the proton pore is blocked (G. Cox, personal communication).

The general pattern that emerges from these results, then, is as follows:

i)strains which are incapable of phosphorylation show low Ag^+-induced phosphate efflux,

ii)strains which have a functionally normal ATPase show high Ag^+-induced phosphate efflux, and

iii)strains which can catalyse electron transport-driven, but not pH-driven, phosphorylation also show high Ag^+-induced phosphate efflux.

If, as postulated (Cox et al., 1982), protons involved in oxidative phosphorylation can either enter F_0 from within the membrane, or

from the external medium, and if Ag^+ interferes with intramembrane protons in some way, then strains which cannot use intramembrane protons would be expected to be less susceptible to Ag^+ than those which could. If intramembrane protons cannot enter the F_0, they will probably be trapped within the membrane. This may be what is shown by NADH-dependent atebrin fluorescence quenching (Lee, 1974). If they are indeed not able to leave the membrane, then they may not be readily displaced from the postulated carrier, chloride (Robertson and Boardman, 1975), by Ag^+ in strains where the putative intramembrane channel in the F_0 sector was blocked. An alternative mechanism, whereby Ag^+ gains entry to the intramembrane space *via* this proposed channel, and cannot do so when the channel is blocked, is less likely since it would be expected that this channel would communicate with the F_1 portion of the ATPase rather than with the external medium.

The mode of energisation which is resistant to uncoupler in uncoupler-resistant mutants (Decker and Lang, 1977; 1977a; Guffanti *et al.*, 1981) could involve intramembrane protons, as described above, and the mutation could have made the strain more tightly coupled to intramembrane protons than to the pH gradient. It would be of interest to see what the effect of Ag^+ was in these mutants.

CHAPTER 8

CONCLUDING DISCUSSION

CONCLUDING DISCUSSION

The hypothesis of energy coupling by intramembrane protons, transported by a small, lipophilic carrier, as proposed by Robertson and Boardman (1975), is, in general, supported by the results described in this thesis. If the proposed lipophilic carrier for intramembrane protons is chloride (Robertson and Boardman, 1975), then Ag^+ would have some effect on energy coupling, simply by precipitating chloride as AgCl, effectively removing the carrier. Detailed arguments have been presented in each chapter, but, briefly summarised, the major results are as follows:

i) silver ions caused efflux of phosphate (Chapter 3) and of a variety of other metabolites (Chapter 5) from intact *E. coli* cells;

ii) Ag^+-induced efflux was more pronounced in pmf-coupled systems than in "phosphate-bond energy"-coupled systems (Chapter 3, Chapter 5);

iii)tributyltin, in the presence of chloride, mimicked many of the effects of Ag^+ (Chapter 4, Chapter 5);

iv) uncouplers of oxidative phosphorylation, while decreasing the protonmotive force to a greater extent than did Ag^+ (Chapter 6), did not cause efflux of accumulated phosphate (Chapter 4);

v) respiratory chain inhibitors did not cause efflux of phosphate (Chapter 4);

vi) strains which are incapable of phosphorylation of ADP by the proton-translocating ATPase, and which are suspected to be incapable of using intramembrane protons (Cox *et al.*, 1982), showed reduced Ag^+-induced efflux of P_i (Chapter 7);

vii) plasmid-determined resistance to Ag^+ probably occurred by detoxification (Chapter 7). While interesting in its own right, this could give no further information on the mode of action of Ag^+ on energy coupling.

These results show that silver ions did indeed appear to have a marked effect on energy coupling such that cells treated with Ag^+ could not maintain gradients of accumulated substrates. The pleiotropic nature of the effect indicated that Ag^+ affected a basic process common to all transport systems. The effect was not mediated by the inhibition by Ag^+ of the respiratory chain, or by interference by Ag^+ with the pmf, since inhibitors of these systems did not have the same effect on accumulated substrates as Ag^+ did. The involvement of chloride ions was suggested by the similarity between the effects of Ag^+ and the chloride-dependent effects of TBT. Studies by Cox *et al.* (1982) on strains carrying mutations affecting the F_0 portion of the proton-translocating ATPase suggest that the ATPase may use protons from within the membrane as well as a transmembrane proton gradient. The results presented here are compatible with an effect of Ag^+ on energy coupling *via* intramembrane protons but not coupling to a pH gradient between the bulk aqueous phases on either side of the membrane. The possibility that Ag^+ interacts with the postulated energy-coupling gene product of the *eup* locus (Plate and Suit, 1981) has been discussed in Chapter 4.

The above results are consistent with a hypothesis for energy coupling which has been termed "localised chemiosmosis" (Williams, 1962). In a recent critique of localised chemiosmosis, Westerhoff *et al.* (1981), on the basis of measurements in mitochondria of the dependence of the phosphorylation potential on $\Delta\tilde{\mu}_{H^+}$ under various conditions, stated that the hypothesis of localised chemiosmosis was unsatisfactory. On closer examination, however, it can be seen that, whereas their results contradict the hypothesis whereby $\Delta\tilde{\mu}_{H^+}$ is formed solely as a by-product of the formation of an energised membrane state by "localised protons", they are consistent with a

hypothesis which allows two types of energy transduction, derived independently from each other, to occur in parallel. Such a "parallel coupling" hypothesis has been proposed by Rottenberg and his co-workers (Rottenberg et al., 1967; Padan and Rottenberg, 1973), who state that both $\Delta\tilde{\mu}_{H^+}$ and a more direct form of coupling, which is unspecified, operate in mitochondria. Intramembrane anhydrous protons are candidates for this more direct role in energy coupling, a role which does not replace chemiosmotic coupling, but is additional to it. Thus, intramembrane anhydrous protons can give rise to a direct form of coupling, they may be what is measured by atebrin fluorescence quenching (Lee, 1974), they could operate in "open" fragments of the inner mitochondrial membrane (Storey and Lee, 1981), and may be inaccessible to uncouplers of oxidative phosphorylation. This may then explain the existence of uncoupler-resistant mutants (Decker and Lang, 1977; 1977a; Guffanti et al., 1981) and the mechanism of resistance in such mutants. This is not the only explanation for resistance to uncouplers. Ito and Ohnishi (1981) propose that uncouplers interact with the ATPase and that resistance is brought about by mutations affecting the binding of uncouplers to subunits of the ATPase.

Extreme alkalophiles, such as Bacillus alcalophilus, have a low pmf (10-20mV) at the optimum pH for growth (Guffanti et al., 1978; Padan et al., 1981). Electrogenic transport systems are still active under these conditions, but synthesis of ATP by oxidative phosphorylation is more difficult to explain using a purely chemiosmotic argument. This difficulty may be overcome by postulating that intramembrane protons, not dependent on the protonmotive force, provide energy for ATP synthesis, and perhaps also for some transport systems.

The conclusions are still mostly speculative and further investigations will be necessary to elucidate the exact nature of the coupling of energy to active transport. If intramembrane protons, with chloride as a carrier, are involved, then silver ions may be a useful tool in these studies.

APPENDICES

APPENDIX I

Synthesis of tritiated triphenylmethylphosphonium

^{3}H-TPMP was synthesised from triphenylphosphine and methyl iodide according to the method described by Hong (1977).

Triphenylphosphine (0.37mmol) dissolved in toluene was added to $C^{3}H_{3}I$ (4.3GBq/mmol, 0.21mmol), in toluene, to a final volume of 1ml. The solution was shaken at room temperature for 45 minutes, then a further 0.28mmol of $CH_{3}I$ was added and shaking was continued for a total of 2 hours. The reaction flask was stored overnight at 4°C.

The ^{3}H-TPMP$^{+}I^{-}$ was crystallised by evaporation of the toluene using a Buchi rotary evaporator, and the crystals were dissolved in absolute ethanol. The ^{3}H-TPMP$^{+}I^{-}$ solution was then added to a column of Dowex-1 ion exchange resin, SO_{4}^{2-} form. The column was eluted with distilled water. Fractions were collected, and aliquots were counted by liquid scintillation spectrometry, and checked for iodide by the addition of starch and periodic acid. Most of the radioactivity eluted in the initial fractions, and no iodide was detected. The fractions were pooled and taken to dryness on a Buchi rotary evaporator. The residue was dissolved in chloroform and filtered through glass fibre. Crystallisation was induced by the dropwise addition of toluene. The crystals were harvested by centrifugation, dissolved in chloroform and recrystallised. The crystals were collected and dried *in vacuo* over paraffin wax. Elemental analysis of carbon and hydrogen was carried out by the Microanalysis Section of the Medical Chemistry Group, John Curtin School of Medical Research, and phosphate was assayed as described in Chapter 2 (Section G 5). The following results were obtained:

Calculated ($TPMP^+HSO_4^-$): C 60.96, H 5.08, P 8.29

Found : C 61.17, H 5.32, P 8.59

The specific radioactivity was 2.3GBq/mmol. The crystals were dissolved in absolute ethanol, filtered, and stored at -15^oC.

APPENDIX II

The calculation of $\Delta\psi$ and ΔpH

$\Delta\psi$

The distribution of $TPMP^+$ across the membrane is in accord with the Nernst equation:

$$\Delta\psi = -(RT/F)\log([TPMP]_{in}/[TPMP]_{out})$$

(Ramos *et al.*, 1976)

In the steady state, the concentration of isotope in the effluent from the lower chamber of the flow dialysis apparatus is a true measure of the concentration in the upper chamber (Colowick and Womack, 1969).

Let

T_e = $[TPMP]_{out}$

T_i = $[TPMP]_{in}$

T_t = $T_i + T_e$

V_i = volume of intracellular water

V_e = volume of external water

V_t = $V_i + V_e$

Then $T_i = (T_t V_t - T_e V_e)/V_i$

and $T_i/T_e = (1/V_i)(T_t V_t/T_e - V_e)$

Let e be the dpm/ml in a fraction from the experimental curve and c be the dpm/ml in the corresponding fraction from the control curve (Refer to Fig. 6.6).

Then:

$e = kT_e$

$c = kT_t$

where k is a constant depending on the rate of diffusion of $TPMP^+$ from the upper to the lower chamber and the rate of flow through the lower chamber.

Then $T_i/T_e = (1/V_i)(cV_t/e - V_e)$

$= (1/V_i)(c(V_e + V_i)/e - V_e)$

Rearranging:

$T_i/T_e = (c/e)(1 + V_e/V_i) - V_e/V_i$

Therefore

$\Delta\psi(mV) = -(RT/F)\log[(c/e)(1 + V_e/V_i) - V_e/V_i]$

<u>ΔpH</u>

Internal pH was calculated from the distribution of a weak acid, using the equation derived by Waddell and Butler (1959):

$$pH_i = pK_a + \log[<(c_t/c_e)(1 + V_e/V_i) - V_e/V_i> \times (10^{(pH_e - pK_a)} + 1) - 1] \quad (1)$$

where

pH_i = internal pH

pH_e = external pH

pK_a = dissociation constant of the weak acid

c_t = weak acid concentration in the total water

c_e = weak acid concentration in the extracellular water

V_e = volume of external water

V_i = volume of intracellular water

Since, as described above in the Δψ section:

$c = kc_t$ and $e = kc_e$,

pH_i is calculated by equation (1) with c and e substituted for c_t and c_e respectively.

If a weak base is used, the equation is:

$$pH_i = pK_a - \log[<(c_t/c_e)(1 + V_e/V_i) - V_e/V_i> \times (10^{(pK_a - pH_e)} + 1) - 1]$$

where pK_a is now the dissociation constant of the conjugate acid of

the weak base and the other values are as previously defined.

Now

$$\Delta pH(mV) = (RT/F)(pH_i - pH_e)$$

Thus ΔpH and $\Delta\psi$ can be calculated from the measured values of T, c, e, V_e, V_i, and pH_e and the known values of pK_a, R, and F.

APPENDIX III

Measurement of E. coli internal water space

The internal water space of the bacterial was measured with $^{3}H_2O$, using ^{14}C-inulin to correct for the excluded volume.

$^{3}H_2O$ was added to a cell suspension of A_{660} = 5.0, mixed, and left for 10 minutes to equilibrate. ^{14}C-inulin was then added, mixed, and aliquots were centrifuged in an Eppendorf microfuge. The supernatants were carefully removed, and samples taken for counting. The precipitates were resuspended and quantitatively transferred into scintillation vials for counting. All samples were counted for ^{3}H and ^{14}C simultaneously, and dpm calculated, with a Packard Tri-Carb 460CD liquid scintillation spectrometer. The internal water space was calculated as follows:

Let

total dpm ^{3}H in supernatant = T

total dpm ^{14}C in supernatant = I

total dpm ^{3}H in pellet = t

total dpm ^{14}C in pellet = i

Then dpm ^{3}H outside cells in pellet = iT/I

Therefore dpm ^{3}H inside cells = t - iT/I

Thus internal volume (ml) = (t -iT/I)/T

= t/T - i/I

This volume was calculated to be 5.85 $\pm$ 0.16µl/ml of suspension of A_{660} = 5.00. This value converts to 2.72µl/mg dry weight, using the experimentally derived factor A_{660} of 1.0 = 0.43mg dry weight/ml. This compares well with values of 2.7 (Winkler and Wilson, 1966), 2.60 (Bakker, 1978), and 2.68µl/mg dry weight (Booth *et al.*, 1979) for *E. coli*, and 2.74 (Harold *et al.*, 1970), 2.64 (Kashket and

Barker, 1977), and 2.60µl/mg dry weight (Bakker, 1978) for streptococci. This volume, however, represents both the cytoplasm and the periplasm, since inulin does not penetrate the outer cell wall. If probes of the excluded volume such as sucrose or sorbitol are used, lower values of the excluded volume are obtained. Probes like this, which penetrate the outer, but not the inner, membrane give values of about 1µl/mg dry weight (Booth et al., 1979; Bakker 1978) for E. coli and about 1.5µl/mg dry weight for streptococci (Kashket and Barker, 1977; Bakker, 1978). In the presence of glucose, this space increases in volume, becoming 1.40µl/mg dry weight in E. coli (Bakker, 1978).

In Chapter 6 I have used the value of 2.72µl/mg dry weight which I had measured using inulin. If the value of 1.40µl/mg dry weight was substituted in the equations for the calculation of $\Delta\psi$ and ΔpH, the values of $\Delta\psi$ or ΔpH obtained were only about 15mV higher than those obtained when 2.72µl/mg dry weight had been used. Values for the internal volume determined with inulin or hydroxymethyl inulin are still used by many workers for the calculation of ΔpH and $\Delta\psi$ (Felle et al., 1980; Ghazi et al., 1981).

REFERENCES

REFERENCES

Addanki, S., Cahill, F.D., and Sotos, J.F. (1968). J. Biol. Chem. 243 2337-2348. Determination of intramitochondrial pH and intramitochondrial-extramitochondrial pH gradient of isolated heart mitochondria by the use of 5,5-dimethyl-2,4-oxazolidinedione.

Altendorf, K., Harold, F.M., and Simoni, R.D. (1974). J. Biol. Chem. 249 4587-4593. Impairment and restoration of the energised state in membrane vesicles of a mutant of Escherichia coli lacking adenosine triphosphatase.

Altendorf, K., Hirata, H., and Harold, F.M. (1975). J. Biol. Chem. 250 1405-1412. Accumulation of lipid-soluble ions and of rubidium as indicators of the electrical potential in membrane vesicles of Escherichia coli.

Appleby, C.A. (1969) in Data for Biochemical Research (Dawson, R.M.C., Elliott, D.C. Elliott, W.H., and Jones, K.M., eds.), 2nd edition, pp 380-387, University Press, Oxford.

Ariel, N. and Avi-Dor, Y. (1973). Biochem. J. 136 911-917. Chloride-dependent uncoupling mediated by oligomycin in rat-liver mitochondria.

Ash, G.R. (1981). Ph.D. Thesis, Australian National University. Studies on the F_0-sector of the Escherichia coli membrane-bound adenosinetriphosphatase.

Atkinson, D.E. and McFadden, B.A. (1956). J. Bacteriol. 71 123-124. Use of membrane filters in the measurement of biological incorporation of radioactive isotopes.

Azzi, A. (1969). Biochem. Biophys. Res. Commun. 37 254-260. Redistribution of the electrical charge of the mitochondrial membrane during energy conservation.

Bachman, B.J. (1972). Bacteriol. Rev. 36 525-557. Pedigrees of some mutant strains of Escherichia coli K-12.

Bachman, B.J., Low, K.B., and Taylor, A.L. (1976). Bacteriol. Rev. 40 116-167. Recalibrated linkage map of Escherichia coli K12.

Bailey, J.L. (1962). Techniques in Protein Chemistry, p 293, Elsevier, Amsterdam.

Bakker, E.P. (1978). Biochemistry 17 2899-2904. Accumulation of thallous ions (Tl^+) as a measure of the electrical potential difference across the cytoplasmic membrane of bacteria.

Bakker, E.P. and Harold, F.M. (1980). J. Biol. Chem. 255 433-440. Energy coupling to potassium transport in Streptococcus faecalis.

Barnes, E.M. and Kaback, H.R. (1971). J. Biol. Chem. 246 5518-5522. Mechanisms of active transport in isolated membrane vesicles. I. The site of energy coupling between D-lactic dehydrogenase and β-galactoside transport in Escherichia coli membrane vesicles.

Barrett, J.T., Larson, A.D., and Kallio, R.E. (1953). J. Bacteriol. 65 187-192. The nature of the adaptive lag of Pseudomonas fluorescens toward citrate.

Berger, E.A. (1973). Proc. Natl. Acad. Sci. 70 1514-1518. Different mechanisms of energy coupling for the active transport of proline and glutamine in Escherichia coli.

Berger, E.A. and Heppel, L.A. (1974). J. Biol. Chem. 249 7747-7755. Different mechanisms of energy coupling for the shock-sensitive and shock-resistant amino acid permeases of Escherichia coli.

Berlin, M. (1979) in Handbook on the Toxicology of Metals (Friberg, L., Nordberg, G.F., and Vouk, V.B., eds.), pp 503-530, Elsevier, Amsterdam. Mercury.

Boguslavsky, L.I., Kondrashin, A.A., Kozlov, I.A., Metelsky, S.T., Skulachev, V.P., and Volkov, A.G. (1975). FEBS Lett. 50 223-226. Charge transfer between water and octane phases by soluble mitochondrial ATPase (F_1), bacteriorhodopsin, and respiratory chain enzymes.

Boos, W. (1974). Annu. Rev. Biochem. 43 123-146. Bacterial transport.

Booth, I.R., Mitchell, W.J., and Hamilton, W.A. (1979). Biochem. J. 182 687-696. Quantitative analysis of proton-linked transport systems. The lactose permease of Escherichia coli.

Boyer, P.D., Chance, B., Ernster, L., Mitchell, P., Racker, E., and Slater, E.C. (1977). Annu. Rev. Biochem. 46 955-1026. Oxidative phosphorylation and photophosphorylation.

Bracha, M. and Yagil, E. (1969). J. Gen. Microbiol. 59 77-81. Genetic mapping of the phoR regulator gene of alkaline phosphatase in Escherichia coli.

Bragg, P.D. and Rainnie, D.J. (1974). Can. J. Microbiol. 20 883-889. The effect of silver ions on the respiratory chain of Escherichia coli.

Brink, B.J. and Konings, W.N. (1980). Eur. J. Biochem. 111 59-66. Generation of an electrochemical proton gradient by lactate efflux in membrane vesicles of Escherichia coli.

Butlin, J.D., Cox, G.B., and Gibson, F. (1971). Biochem. J. 124 75-81. Oxidative phosphorylation in Escherichia coli K12. Mutations affecting magnesium ion or calcium ion stimulated adenosine triphosphatase.

Butlin, J.D., Cox, G.B., and Gibson, F. (1973). Biochim. Biophys. Acta 292 366-375. Oxidative phosphorylation in Escherichia coli K12: the genetic and biochemical characterization of a strain carrying a mutation in the uncB gene.

Chance, B. and Mela, A. (1966). J. Biol. Chem. 241 4588-4599. Hydrogen ion concentration changes in mitochondrial membranes.

Chappell, J.B. (1964). Biochem. J. 90 225-237. The oxidation of citrate, isocitrate, and cis-aconitate by isolated mitochondria.

Chappell, J.B. and Greville, G.D. (1954). Nature 174 930-931. Effect of silver ions on mitochondrial adenosine triphosphatase.

Chopra, I. (1975). Antimicrob. Agents Chemother. 7 8-14. Mechanism of plasmid-mediated resistance to cadmium in Staphylococcus aureus.

Cohen, G.N. and Monod, J. (1957). Bacteriol. Rev. 21 169-194. Bacterial permeases.

Collins, S.H., Jarvis, A.W., Lindsay, R.J., and Hamilton, W.A. (1976). J. Bacteriol. 126 1232-1244. Proton movements coupled to lactate and alanine transport in Escherichia coli: isolation of mutants with altered stoichiometry of alanine transport

Colowick, S.P. and Womack, F.C. (1969). J. Biol. Chem. 244 774-777. Binding of diffusable molecules by macromolecules: rapid measurement by rate of dialysis.

Cox, G.B., Gibson, F., and McCann, L. (1973). Biochem. J. 134 1015-1021. Reconstitution of oxidative phosphorylation and the adenosine triphosphate-dependent transhydrogenase activity by a combination of membrane fractions from uncA$^-$ and uncB$^-$ mutant strains of Escherichia coli K12.

Cox, G.B., Langman, L.P., Jans, D.A., Downie, J.A., Senior, A.E., Gibson, F., Fimmel, A.L., James, L.B., and Ash, G. (1982). J. Bacteriol. submitted manuscript. Oxidative phosphorylation in Escherichia coli: Mutations in the uncE gene causing unusual effects on assembly and function of the adenosine triphosphatase.

Cox, G.B., Newton, N.A., Gibson, F., Snoswell, A.M., and Hamilton, J.A. (1970). Biochem. J. 117 551-562. The function of ubiquinone in Escherichia coli.

Cozzarelli, N.R., Freedberg, W.B., and Lin, E.C.C. (1968). J. Molec. Biol. 31 371-387. Genetic control of the L-α-glycerophosphate system in Escherichia coli.

Davies, J. and Smith, D.I. (1978). Annu. Rev. Microbiol. 32 469-518. Plasmid-determined resistance to antimicrobial agents.

Dayan, J. and Wilson, I.B. (1964). Biochim. Biophys. Acta 81 620-623. The phosphorylation of Tris by alkaline phosphatase.

Decker, S.J. and Lang, D.R. (1977). J. Biol. Chem. 252 5936-5938. Mutants of Bacillus megaterium resistant to uncouplers of oxidative phosphorylation.

Decker, S.J. and Lang, D.R. (1977a). J. Biol. Chem. 253 6738-6743. Membrane bioenergetic parameters in uncoupler-resistant mutants of Bacillus megaterium.

Doudoroff, M., Hassid, W.Z., Putnam, E.W., Potter, A.L., and Lederberg, J. (1949). J. Biol. Chem. 179 921-934. Direct utilization of maltose by Escherichia coli.

Downie, J.A., Cox, G.B., Langman, L., Ash, G.R., Becker, M., and Gibson, F. (1981). J. Bacteriol. 145 200-210. The three genes coding for the subunits of the membrane sector (F_0) of the adenosine triphosphatase complex from Escherichia coli.

Downie, J.A., Gibson, F., and Cox, G.B. (1979). Annu. Rev. Biochem. 48 103-131. Membrane adenosine triphosphatases of prokaryotic cells.

Downie, J.A., Langman, L., Cox, G.B., Yanofsky, C., and Gibson,F. (1980). J. Bacteriol. 143 8-17. Subunits of the adenosine triphosphatase complex translated in vitro from the Escherichia coli unc operon.

Downie, J.A., Senior, A.E., Gibson,F., and Cox, G.B. (1979a). J. Bacteriol. 137 711-718. A fifth gene (uncE) in the operon concerned with oxidative phosphorylation in Escherichia coli.

Epstein, W. and Kim, B.S. (1971). J. Bacteriol. 108 639-644. Potassium transport in Escherichia coli K-12.

Epstein, W. and Laimins, L. (1980). Trends in Biochem. Sci. 5 21-23. Potassium transport in Escherichia coli: diverse systems with common control by osmotic forces.

Epstein, W. and Schultz, S.G. (1965). J. Gen. Physiol. 49 221-234. Cation transport in Escherichia coli. V. Regulation of cation content.

Fan, D.P., Schlesinger, M.J., Torriani, A., Barrett, K.J., and Levinthal, C. (1966). J. Molec. Biol. 15 32-48. Isolation and characterization of complementation products of Escherichia coli alkaline phosphatase.

Felle, H., Porter, J.S., Slayman, C.L., and Kaback, H.R. (1980). Biochemistry 19 3585-3590. Quantitative measurements of membrane potential in Escherichia coli.

Gale, E.F. (1951). Biochem. J. 48 286-290. The assimilation of amino acids by bacteria. 10. Action of inhibitors on the accumulation of free glutamic acid in Staphylococcus aureus and Streptococcus faecalis.

Gale, E.F. (1954) in Active Transport and Secretion (Symposia of the Society for Experimental Biology, No. 8), pp 242-253, University Press, Cambridge. The accumulation of amino acids within staphylococcal cells.

Gale, E.F. and Paine, T.F. (1951). Biochem. J. 48 298-301. The assimilation of amino acids by bacteria. 12. The action of inhibitors and antibiotics on the accumulation of free glutamic acid and the formation of combined glutamate in Staphylococcus aureus.

Gear, A.R.L., Rossi, C.S., Reynafarje, B., and Lehninger, A.L. (1967). J. Biol. Chem. 242 3403-3413. Acid-base exchanges in mitochondria and suspending medium during respiration-linked accumulation of bivalent cations.

Gerdes, R.G. and Rosenberg, H. (1974). Biochim. Biophys. Acta 351 77-86. The relationship between the phosphate binding protein and a regulator gene product from Escherichia coli.

Gerdes, R.G., Strickland, K.P. and Rosenberg, H. (1977). J. Bacteriol. 131 512-518. Restoration of phosphate transport by the phosphate-binding protein in spheroplasts of Escherichia coli.

Ghazi, A., Schechter, E., Letellier, L., and Labedan, B. (1981). FEBS Lett. 125 197-200. Probes of membrane potential in Escherichia coli cells.

Gibson, F., Cox, G.B., Downie, J.A., and Radik, J. (1977). Biochem. J. 162 665-670. Partial diploids of Escherichia coli carrying normal and mutant alleles affecting oxidative phosphorylation.

Gibson, F., Cox, G.B., Downie, J.A., and Radik, J. (1977a). Biochem. J. 164 193-198. A mutation affecting a second component of the F_0 portion of the magnesium ion-stimulated adenosine triphosphatase of Escherichia coli K12.

Gould, J.M. (1976). Eur. J. Biochem. 62 567-575. Inhibition by triphenyltin chloride of a tightly-bound membrane component involved in photophosphorylation.

Gould, J.M. (1978). FEBS Lett. 94 90-94. Dithiol-specific reversal of triphenyltin inhibition of CF_0-catalyzed transmembrane proton transfer in chloroplasts.

Guffanti, A.A., Blumenfield, H., and Krulwich, T.A. (1981). J. Biol. Chem. 256 8416-8421. ATP synthesis by an uncoupler-resistant mutant of Bacillus megaterium.

Guffanti, A.A., Susman, P., Blanco, R., and Krulwich, T.A. (1978). J. Biol. Chem. 253 708-715. The protonmotive force and α-aminoisobutyric acid transport in an obligately alkalophilic bacterium.

Gunsalus, R.P., Brusilow, W.S.A., and Simoni, R.D. (1982). Proc. Natl. Acad. Sci. 79 320-324. Gene order and gene-polypeptide relationships of the proton-translocating ATPase operon (unc) of Escherichia coli.

Gutowski, S.J. and Rosenberg, H. (1975). Biochem. J. 152 647-654. Succinate uptake and related proton movements in Escherichia coli K12.

Hamilton, W.A. (1977) in Microbial Energetics (Haddock, B.A. and Hamilton,W.A., eds.), pp 185-216, University Press, Cambridge. Energy coupling in substrate and group translocation.

Hanstein, W.G. (1976). Biochim. Biophys. Acta 456 130-148. Uncoupling of oxidative phosphorylation.

Harold, F.M. (1972). Bacteriol. Rev. 36 172-230. Conservation and transformation of energy by bacterial membranes.

Harold, F.M. (1977). in Current Topics in Bioenergetics (Sanadi, D.R., ed.), Vol. 6, pp 83-149, Academic Press, New York. Membranes and energy transduction in bacteria.

Harold, F.M. (1977a). Annu. Rev. Microbiol. 31 181-203. Ion currents and physiological functions in microorganisms.

Harold, F.M. and Altendorf, K. (1974) in Current Topics in Membranes and Transport (Bronner, F. and Kleinzeller, A., eds.), Vol. 5, pp 1-50, Academic Press, New York. Cation transport in bacteria: K^+, Na^+, and H^+.

Harold, F.M., Altendorf, K., and Hirata, H. (1974) Ann. N.Y. Acad. Sci. 235 149-160. Probing membrane transport mechanisms with ionophores.

Harold, F.M. and Baarda, J.R. (1967). J. Bacteriol. 94 53-60. Gramicidin, valinomycin, and cation permeability of Streptococcus faecalis.

Harold, F.M. and Baarda, J.R. (1968). J. Bacteriol. 96 2025-2034. Inhibition of membrane transport in Streptococcus faecalis by uncouplers of oxidative phosphorylation and its relationship to proton conduction.

Harold, F.M. and Papineau, D. (1972). J. Membrane Biol. 8 27-44. Cation transport and electrogenesis by Streptococcus faecalis. I. The membrane potential.

Harold, F.M., Pavlasova, E., and Baarda, J.R. (1970). Biochim. Biophys. Acta 196 235-244. A transmembrane pH gradient in Streptococcus faecalis: Origin, and dissipation by proton conductors and N,N'-dicyclohexylcarbodiimide.

Harris, W.D. and Popat, P. (1954). J. Amer. Oil. Chem. Soc. 31 124-127. Determination of the phosphorus content of lipids.

Hayashi, S. and Lin, E.C.C. (1965). Biochim. Biophys. Acta 94 479-487. Capture of glycerol by cells of Escherichia coli.

Hayashi, S. and Lin, E.C.C. (1965a). J. Molec. Biol. 14 515-521. Product induction of glycerol kinase in Escherichia coli.

Hendry, A.T. and Stewart, I.O. (1979). Can. J. Microbiol. 25 915-921. Silver-resistant enterobacteriaceae from hospital patients.

Heytler, P.G. (1963). Biochemistry 2 357-361. Uncoupling of oxidative phosphorylation by carbonyl cyanide phenylhydrazones. I. Some characteristics of m-Cl-CCP action on mitochondria and chloroplasts.

Heytler, P.G. (1979). Meth. Enzymol. 55 462-472. Uncouplers of oxidative phosphorylation.

Hirata, H., Altendorf,K., and Harold, F.M. (1973). Proc. Natl. Acad. Sci. 70 1804-1808. Role of an electrical potential in the coupling of metabolic energy to active transport by membrane vesicles of Escherichia coli.

Hoffee, P., Englesberg, E., and Lamy, F. (1964). Biochim. Biophys. Acta 79 337-350. The glucose permease system in bacteria.

Hong, J.-S. (1977). J. Biol. Chem. 252 8582-8588. An ecf mutation in Escherichia coli pleiotropically affecting energy coupling in active transport but not generation or maintenance of membrane potential.

Hong, J.-S., Hunt, A.G., Masters, P.S., and Lieberman, M.A. (1979). Proc. Natl. Acad. Sci. 76 1213-1217. Requirement of acetyl phosphate for the binding protein-dependent transport systems in Escherichia coli.

Hong, J.-S. and Kaback, H.R. (1972). Proc. Natl. Acad. Sci. 69 3336-3340. Mutants of Salmonella typhimurium and Escherichia coli pleiotropically defective in active transport.

Hosoi, S., Mochizuki, N., Hayashi, S., and Kasai, M. (1980). Biochim. Biophys. Acta 600 844-852. Control of membrane potential by external H^+ concentration in Bacillus subtilis as determined by an ion-selective electrode.

Huang, C.S., Kopacz, S.J., and Lee, C.P. (1977). Biochim. Biophys. Acta 459 241-249. Energy-linked protonation of quinacrine in beef heart submitochondrial membranes.

Hugenholtz, J., Hong, J.-S., and Kaback, H.R. (1981). Proc. Natl. Acad. Sci. 78 3446-3449. ATP-driven transport in right-side-out bacterial membrane vesicles.

Ito, M. and Ohnishi, Y. (1981). FEBS Lett. 136 225-230. Isolation of Escherichia coli mutants which are resistant to an inhibitor of H^+-ATPase, tributyltin, and also to uncouplers of oxidative phosphorylation.

Juan, S.M., Segura, E.L., and Cazzulo, J.J. (1979). Experienta 35 1139-1140. Inhibition of the NADP-linked glutamate dehydrogenase from Trypanosoma cruzi by silver nitrate.

Kaback, H.R. (1960). Fed. Proc. 19 130. Uptake of amino acids by "ghosts" of mutant strains of Escherichia coli.

Kaback, H.R. (1970). Annu. Rev. Biochem. 39 561-598. Transport.

Kaback, H.R. (1971). Meth. Enzymol. 22 99-120. Bacterial membranes.

Kaback, H.R. (1972). Biochim. Biophys. Acta 265 367-416. Transport across isolated bacterial cytoplasmic membranes.

Kaback, H.R. (1976). J. Cell Physiol. 89 575-594. Molecular biology and energetics of membrane transport.

Kaback, H.R. and Barnes, E.M. (1971). J. Biol. Chem. 246 5523-5531. Mechanisms of active transport in isolated membrane vesicles. II. The mechanism of energy coupling between D-lactic dehydrogenase and β-galactoside transport in membrane preparations from Escherichia coli.

Kaback, H.R. and Milner, L.S. (1970). Proc. Natl. Acad. Sci. 66 1008-1015. Relationship of a membrane-bound D(-)lactic dehydrogenase to amino acid transport in isolated bacterial membrane preparations.

Kaback, H.R., Reeves, J.P., Short, S.A., and Lombardi, F.J. (1974). Arch. Biochem. Biophys. 160 215-222. Mechanisms of active transport in isolated bacterial membrane vesicles. XVIII. The mechanism of action of carbonylcyanide m-chlorophenylhydrazone.

Kaback, H.R. and Stadtman, E.R. (1966). Proc. Natl. Acad. Sci. 55 920-927. Proline uptake by an intact cytoplasmic membrane preparation of Escherichia coli.

Kamo, N., Muratsugu, M., Hongoh, R., Kobatake, Y. (1979). J. Membrane Biol. 49 105-121. Membrane potential of mitochondria measured with an electrode sensitive to tetraphenyl phosphonium and relationship between proton electrochemical potential and phosphorylation potential in steady state.

Kasahara, M. and Anraku, Y. (1972). J. Biochem. 72 777-781. Inhibition of the respiratory chain of Escherichia coli by zinc ions.

Kashket, E.R. (1979). J. Biol. Chem. 254 8129-8131. Active transport of thallous ions by Streptococcus lactis.

Kashket, E.R. and Barker, S.L. (1977). J. Bacteriol. 130 1017-1023. Effects of potassium ions on the electrical and pH gradients across the membrane of Streptococcus lactis cells.

Kay, W.W. and Kornberg, H.L. (1969). FEBS Lett. 3 93-96. Genetic control of the uptake of C_4-dicarboxylic acids by Escherichia coli.

Kepes, A. and Cohen, G.N. (1962) in The Bacteria (Gunsalus, I.C. and Stanier, R.Y., eds.), Vol. 4, pp 179-221, Academic Press, New York. Permeation.

Koch, A.I. (1971). J. Molec. Biol. 59 447-459. Energy expenditure is obligatory for the downhill transport of galactosides.

Kogut, M. and Podoski, E.P. (1953). Biochem. J. 55 800-811. Oxidative pathways in a fluorescent Pseudomonas.

Konings, W.N. and Rosenberg, H. (1978). Biochim. Biophys. Acta 508 370-378. Phosphate transport in membrane vesicles from Escherichia coli.

Kundig, W., Ghosh, S., and Roseman, S. (1964). Proc. Natl. Acad. Sci. 52 1067-1074. Phosphate bound to histidine in a protein as an intermediate in a novel phospho-transferase system.

Laimins, L.A., Rhoads, D.B., Altendorf, K., and Epstein, W. (1978). Proc. Natl. Acad. Sci. 75 3216-3219. Identification of the structural proteins of an ATP-driven potassium transport system in Escherichia coli.

Lancaster, J.R. and Hinkle, P.C. (1977). J. Biol. Chem. 252 7657-7661. Studies of the β-galactoside transporter in inverted membrane vesicles of Escherichia coli. I. Symmetrical facilitated diffusion and proton gradient-coupled transport.

Lee, C.P. (1974) in Dynamics of Energy-Transducing Membranes (Ernster, L., Estabrook, R.W., and Slater, E.C., eds.), BBA Library, Vol. 13, pp 337-353, Elsevier, Amsterdam. Reaction mechanism of the respiratory chain-linked energy conservation.

Leive, L. (1968). J. Biol. Chem. 243 2373-2380. Studies on the permeability change produced in coliform bacteria by ethylenediaminetetraacetate.

Lengeler, J. (1975). J. Bacteriol. 124, 26-38. Mutations affecting transport of the hexitols D-mannitol, D-glucitol, and galactitol in Escherichia coli K-12: isolation and mapping.

Lengeler, J. (1975a). J. Bacteriol. 124 39-47. Nature and properties of hexitol transport systems in Escherichia coli.

Lieberman, M.A. and Hong, J.-S. (1976). Arch. Biochem. Biophys. 172 312-315. Energisation of osmotic shock-sensitive transport systems in Escherichia coli requires more than ATP.

Lo, T.C.Y., Rayman, M.K., and Sanwal, B.D. (1972). J. Biol. Chem. 247 6323-6331. Transport of succinate in Escherichia coli. I. Biochemical and genetic studies of transport in whole cells.

Lowry, O.H., Rosebrough, N.J., Farr, A.J., and Randall, R.J. (1951). J. Biol. Chem. 193 265-275. Protein measurement with the Folin phenol reagent.

Luckey, T.D. and Venugopal, B. (1977). Metal Toxicity in Mammals. 1. Physiologic and Chemical Basis for Metal Toxicity, pp 95 & 167, Plenum Press, New York.

Medveczky, N. and Rosenberg, H. (1970). Biochim. Biophys. Acta 211 158-168. The phosphate binding protein of Escherichia coli.

Medveczky, N. and Rosenberg, H. (1971). Biochim. Biophys. Acta 241 494-506. Phosphate transport in Escherichia coli.

Meury, J., Lebail, S., and Kepes, A. (1980). Eur. J. Biochem. 113 33-38. Opening of potassium channels in Escherichia coli membranes by thiol reagents and recovery of potassium tightness.

Mitchell, P. (1954) in Active Transport and Secretion (Symposia of the Society for Experimental Biology, No. 8), pp 242-253, University Press, Cambridge. Transport of phosphate through an osmotic barrier.

Mitchell, P. (1957). Nature 180 134-136. A general theory of membrane transport from studies of bacteria.

Mitchell, P. (1961). Nature 191 144-148. Coupling of phosphorylation to electron and hydrogen transfer by a chemi-osmotic type of mechanism.

Mitchell, P. (1961a). Biochem. J. 79 23P-24P. Chemiosmotic coupling in oxidative and photosynthetic phosphorylation.

Mitchell, P. (1966). Biol. Rev. 41 445-502. Chemiosmotic coupling in oxidative and photosynthetic phosphorylation.

Mitchell, P. (1967). Adv. Enzymol. 29 33-87. Translocations through natural membranes.

Mitchell, P. (1972). J. Bioenerg. 3 5-24. Chemiosmotic coupling in energy transduction: A logical development of biochemical knowledge.

Mitchell, P. (1977). FEBS Lett. 78 1-20. A commentary on alternative hypotheses of protonic coupling in the membrane systems catalysing oxidative and photosynthetic phosphorylation.

Mitchell, P. and Moyle, J. (1958). Nature 182 372-373. Group translocation: a consequence of enzyme-catalysed group transfer.

Mitchell, P. and Moyle, J. (1969). Eur. J. Biochem. 7 471-484. Estimation of membrane potential and pH difference across the cristae membrane of rat liver mitochondria.

Navon, G., Ogawa, S., Shulman, R.G., and Yamane, T. (1977). Proc. Natl. Acad. Sci. 74 888-891. High-resolution phosphorus-31 nuclear magnetic resonance studies of metabolism in aerobic Escherichia coli cells.

Neu, H.C. and Heppel, L.A. (1965). J. Biol. Chem. 240 3685-3692. The release of enzymes from Escherichia coli by osmotic shock and during the formation of spheroplasts.

Newman, M.J., Foster, D.L., Wilson, T.H., and Kaback, H.R. (1981). J. Biol. Chem. 256 11804-11808. Purification and reconstitution of functional lactose carrier from Escherichia coli.

Newman, M.J. and Wilson, T.H. (1980). J. Biol. Chem. 255 10583-10586. Solubilization and reconstitution of the lactose transport system from Escherichia coli.

Novick, R.P., Clowes, R.C., Cohen, S.N., Curtiss,R., Datta, N., and Falkows, S. (1976). Bacteriol. Rev. 40 168-189. Uniform nomenclature for bacterial plasmids: a proposal.

Ohnishi, S.T. (1978). Anal. Biochem. 86 201-213. A new method of separating inorganic orthophosphate from phosphoric esters and anhydrides by an immobilized catalyst column.

Okamoto, H., Sone, N., Hirata, H., Yoshida, M., and Kagawa, Y. (1977). J. Biol. Chem. 252 6125-6131. Purified proton conductor in proton-translocating adenosine triphosphatase of a thermophilic bacterium.

Ort, D.R. (1978). Eur. J. Biochem. 85 479-485. Different sensitivities of chloroplasts to uncouplers when ATP formation is induced by continuous illumination, by brief illumination, by pre-illumination, or by acid-base transitions.

Ort, D.R., Dilley, R.A., and Good, N.E. (1976). Biochim. Biophys. Acta 449 108-124. Photophosphorylation as a function of illumination time. II. Effect of permeant buffers.

Otto, R., Sonnenberg, A.S.M., Veldkamp, H., and Konings, W.N. (1980). Proc. Natl. Acad. Sci. 77 5502-5506. Generation of an electrochemical proton gradient in Streptococcus cremoris by lactate efflux.

Padan, E. and Rottenberg, H. (1973). Eur. J. Biochem. 40 431-437. Respiratory control and the proton electrochemical gradient in mitochondria.

Padan, E., Zilberstein, D., and Rottenberg, H. (1976). Eur. J. Biochem. 63 533-541. The proton electrochemical gradient in Escherichia coli.

Padan, E., Zilberstein, D., and Schuldiner, S. (1981). Biochim. Biophys. Acta 650 151-166. pH homeostasis in bacteria.

Pavlasova, E. and Harold, F.M. (1969). J. Bacteriol. 98 198-204. Energy coupling in the transport of β-galactosides by Escherichia coli: Effect of proton conductors.

Plate, C.A. (1979). J. Bacteriol. 137 221-225. Requirement for membrane potential in active transport of glutamine by Escherichia coli.

Plate, C.A. and Suit, J.L. (1981). J. Biol. Chem. 256 12974-12980. The eup genetic locus of Escherichia coli and its role in H^+/solute symport.

Plate, C.A., Suit, J.L., Jetten, A.M., and Luria, S.E. (1974). J. Biol. Chem. 249 6138-6143. Effects of colicin K on a mutant of Escherichia coli deficient in Ca^{2+},Mg^{2+}-activated adenosine triphosphatase.

Pontremoli, S., Luppis, B., Traniello, S., Wood, W.A., and Horecker, B.L. (1965). J. Biol. Chem. 240 3469-3472. Fructose diphosphatase from rabbit liver. III. Nature of the groups reactive with dinitrofluorobenzene.

Rainnie, D.J. and Bragg, P.D. (1971). Anal. Biochem. 44 392-396. Loss of silver ions from oxygen electrode.

Ramos, S., Schuldiner, S., and Kaback, H.R. (1976). Proc. Natl. Acad. Sci. 73 1892-1896. The electrochemical gradient of protons and its relationship to active transport in Escherichia coli.

Rayman, M.K., Lo, T.C.Y., and Sanwal, B.D. (1972). J. Biol. Chem. 247 6332-6339. Transport of succinate in Escherichia coli. II. Characteristics of uptake and energy coupling in Escherichia coli.

Rhoads, D.B. and Epstein, W. (1977). J. Biol. Chem. 252 1394-1401. Energy coupling to net K^+ transport in Escherichia coli K-12.

Rhoads, D.B. and Epstein, W. (1978). J. Gen. Physiol. 72 283-295. Cation transport in Escherichia coli. IX. Regulation of K^+ transport.

Rhoads, D.B., Laimins, L., and Epstein, W. (1978). J. Bacteriol. 135 445-452. Functional organization of the kdp genes of Escherichia coli K-12.

Rhoads, D.B., Waters, F.B., and Epstein, W. (1976). J. Gen. Physiol. 67 325-341. Cation transport in Escherichia coli. VIII. Potassium transport mutants.

Richey, D.P. and Lin, E.C.C. (1972). J. Bacteriol. 112 784-790. Importance of facilitated diffusion for effective utilization of glycerol by Escherichia coli.

Riordan, J.F. and Vallee, B.L. (1972). Meth. Enzymol. 25 449-456. Reactions with N-ethylmaleimide and p-mercuribenzoate.

Roberts, R.B., Abelson, P.H., Cowie, D.B., Bolton, E.T., and Britten, R.J. (1955). Studies of Biosynthesis in Escherichia coli, p 58, Carnegie Institute of Washington, Washington, D.C.

Roberts, R.B., Abelson, P.H., Cowie, D.B., Bolton, E.T., and Britten, R.J. (1957). Studies of Biosynthesis in Escherichia coli, 2nd edition,p 94c, Carnegie Institute of Washington, Washington, D.C.

Robertson, R.N. and Boardman, N.K. (1975). FEBS Lett. 60 1-6. The link between charge separation, proton movement, and ATPase reactions.

Robertson, R.N. and Thompson, T.E. (1977). FEBS Lett. 76 16-19. The function of phospholipid polar groups in membranes.

Rosen, B.P. and Adler, L.W. (1975). Biochim. Biophys. Acta 387 23-36. The maintenance of the energised membrane potential and its relation to active transport in Escherichia coli.

Rosen, B.P. and Kashket, E.R. (1978) in Bacterial Transport (Rosen, B.P., ed.), pp 559-620, Marcel Dekker, New York. Energetics of active transport.

Rosenberg, H. (1979). Anal. Biochem. 96 90-93. An inexpensive, miniature potassium-ion-selective electrode.

Rosenberg, H., Cox, G.B., Butlin, J.D., and Gutowski, S.J. (1975). Biochem. J. 146 417-423. Metabolite transport in mutants of Escherichia coli K12 defective in electron transport and coupled phosphorylation.

Rosenberg, H., Gerdes, R.G., and Chegwidden, K. (1977). J. Bacteriol. 131 505-511. Two systems for the uptake of phosphate in Escherichia coli.

Rosenberg, H., Gerdes, R.G., and Harold, F.M. (1979). Biochem. J. 178 133-137. Energy coupling to the transport of inorganic phosphate in Escherichia coli K12.

Rosenberg, H., Russell, L.M., Jacomb, P.A., and Chegwidden, K. (1982). J. Bacteriol. in press. Phosphate exchange in the Pit transport system in Escherichia coli K-12.

Rottenberg, H. (1973). J. Membrane Biol. 11 117-137. The mechanism of energy-dependent ion transport in mitochondria.

Rottenberg, H. (1975). Bioenergetics 7 61-74. The measurement of transmembrane electrochemical proton gradients.

Rottenberg, H. (1979). Meth. Enzymol. 55 547-569. The measurement of membrane potential and ΔpH in cells, organelles, and vesicles.

Rottenberg, H., Grunwald, T., and Avron, M. (1972). Eur. J. Biochem. 25 54-63. Determination of ΔpH in chloroplasts. 1. Distribution of [^{14}C]-methylamine.

Rottenberg, H., Caplan, S.R., and Essig, A. (1967). Nature 216 610-611. Stoichiometry and coupling: theories of oxidative phosphorylation.

Russell, L.M. (1979). Ph.D. Thesis, Australian National University. Studies on the transport of phosphate into Escherichia coli K-12.

Russell, L.M. and Rosenberg, H. (1979). Biochem. J. 184 13-21. Linked transport of phosphate, potassium ions and protons in Escherichia coli.

Russell, L.M. and Rosenberg, H. (1980). Biochem. J. 188 715-723. The nature of the link between potassium transport and phosphate transport in Escherichia coli.

Saier, M.H. (1977). Bacteriol. Rev. 41 856-871. Bacterial phosphoenolpyruvate:sugar phosphotransferase systems: structural, functional, and evolutionary interrelationships.

Saier, M.H. and Moczydlowski, E.G. (1978) in Bacterial Transport (Rosen, B.P., ed.), pp 103-125, Marcel Dekker, New York. The regulation of carbohydrate transport in Escherichia coli and Salmonella typhimurium.

Schottel, J.L. (1978). J. Biol. Chem. 253 4341-4349. The mercuric and organomercurial detoxifying enzymes from a plasmid-bearing strain of Escherichia coli.

Schottel, J.L., Mandal, A., Clark, D., Silver, S., and Hedges, R.W. (1974). Nature 251 335-337. Volatilisation of mercury and organomercurials determined by inducible R-factor systems in enteric bacteria.

Schuldiner, S. and Avron, M. (1971). FEBS Lett. 14 233-236. On the mechanism of the energy-dependent quenching of atebrin fluorescence in isolated chloroplasts.

Schuldiner, S. and Kaback, H.R. (1975). Biochemistry 14 5451-5460. Membrane potential and active transport in membrane vesicles from Escherichia coli.

Schuldiner, S., Rottenberg, H., and Avron, M. (1972). Eur. J. Biochem. 25 64-70. Determination of ΔpH in chloroplasts. 2. Fluorescent amines as a probe for the determination of ΔpH in chloroplasts.

Schuldiner, S., Weil, R., and Kaback, H.R. (1976). Proc. Natl. Acad. Sci. 73 109-112. Energy-dependent binding of dansylgalactoside to the lac carrier protein: direct binding measurements.

Selwyn, M.J., Dawson, A.P., Stockdale, M., and Gains, N. (1970). Eur. J. Biochem. 14 120-126. Chloride-hydroxide exchange across mitochondrial, erythrocyte and artificial lipid membranes mediated by trialkyl- and triphenyltin compounds.

Silhavy, T.J., Ferenci, T., and Boos, W. (1978) in Bacterial Transport (Rosen, B.P., ed.), pp 127-219, Marcel Dekker, New York. Sugar transport systems in Escherichia coli.

Silver, S. (1978) in Bacterial Transport (Rosen, B.P., ed.), pp 221-324, Marcel Dekker, New York. Transport of cations and anions.

Simoni, R.D. and Postma, P.W. (1975). Annu. Rev. Biochem. 43 523-554. The energetics of bacterial active transport.

Simoni, R.D. and Shallenberger, M.K. (1972). Proc. Natl. Acad. Sci. 69 2663-2667. Coupling of energy to active transport of amino acids in Escherichia coli.

Singh, A.P. and Bragg, P.D. (1979). Can. J. Biochem. 57 1376-1383. The action of tributyltin chloride on the uptake of proline and glutamine by intact cells of Escherichia coli.

Singh, A.P. and Bragg, P.D. (1979a). Can. J. Biochem. 57 1384-1391. The action of tributyltin chloride on energy-dependent transhydrogenation of $NADP^+$ by NADH in membranes of Escherichia coli.

Skulachev, V.P. (1971) in Current Topics in Bioenergetics (Sanadi, D.R., ed.),Vol. 4, pp 127-190, Academic Press, New York. Energy transformations in the respiratory chain.

Solomon, E. and Lin, E.C.C. (1972). J. Bacteriol. 111 566-574. Mutations affecting the dissimilation of mannitol by Escherichia coli K-12.

Solomon, E., Miyai, K., and Lin, E.C.C. (1973). J. Bacteriol. 114 723-728. Membrane translocation of mannitol in Escherichia coli without phosphorylation.

Sprague, G.F., Bell, R.M., and Cronan, J.E. (1975). Mol. Gen. Genet. 143 71-77. A mutant of Escherichia coli auxotrophic for organic phosphates: evidence for two defects in inorganic phosphate transport.

Stockdale, M., Dawson, A.P., and Selwyn, M.J. (1970). Eur. J. Biochem. 15 342-351. Effects of trialkyltin and triphenyltin compounds on mitochondrial respiration.

Storey, B.T. and Lee, C.P. (1981). Trends in Biochem. Sci. 6 166-170. Is transmembrane $\Delta\tilde{\mu}_{H^+}$ essential to mitochondrial energy coupling? --Transmembrane $\Delta\tilde{\mu}_{H^+}$ is not obligatory.

Storey, B.T., Scott,D.M., and Lee, C.P. (1980). J. Biol. Chem. 255 5224-5229. Energy-linked quinacrine fluorescence changes in submitochondrial particles from skeletal muscle mitochondria: evidence for intramembrane proton transfer as a primary reaction of energy coupling.

Storey, B.T., Wilson, D.F., Bracey, A., Rosen, S.L., and Stephenson, S. (1975). FEBS Lett. 49 338-341. Steric and electronic effects on the uncoupling activity of substituted 3,5 dichlorosalicylanilides.

Summers, A.O. and Silver, S. (1972). J. Bacteriol. 112 1228-1236. Mercury resistance in a plasmid-bearing strain of Escherichia coli.

Summers, A.O. and Silver, S. (1978). Annu. Rev. Microbiol. 32 637-672. Microbial transformations of metals.

Tupper, J.T. and Tedeschi, H. (1969). Proc. Natl. Acad. Sci. 63 370-377. Microelectrode studies on the membrane properties of isolated mitochondria.

Tynecka, Z., Gos, Z., and Zajac, J. (1981). J. Bacteriol. 147 305-312. Reduced cadmium transport determined by a resistance plasmid in Staphylococcus aureus.

Tynecka, Z., Gos, Z., and Zajac, J. (1981a). J. Bacteriol. 147 313-319. Energy-dependent efflux of cadmium coded by a plasmid resistance determinant in Staphylococcus aureus.

van Thienen, G. and Postma, P.W. (1973). Biochim. Biophys. Acta 323 429-440. Coupling between energy conservation and active transport of serine in Escherichia coli.

Wachter, E., Schmid, R., Deckers, G., and Altendorf, K. (1980). FEBS Lett. 113 265-270. Amino acid replacement in dicyclohexylcarbodiimide-reactive proteins from mutant strains of Escherichia coli defective in the energy-transducing ATPase complex.

Waddell, W.J. and Butler, T.C. (1959). J. Clin. Invest. 38 720-729. Calculation of intracellular pH from the distribution of 5,5-dimethyl-2,4-oxazolidinedione (DMO). Application to skeletal muscle of the dog.

Waggoner, A.S. (1979). Meth. Enzymol. 55 689-695. The use of cyanine dyes for the determination of membrane potentials in cells, organelles, and vesicles.

Westerhoff, H.V., Simonetti, A.L.M., and van Dam, K. (1981). Biochem. J. 200 193-202. The hypothesis of localised chemiosmosis is unsatisfactory.

Wikström, M. (1981). Trends in Biochem. Sci. 6 166-170. Is transmembrane $\Delta\tilde{\mu}_{H^+}$ essential to mitochondrial energy coupling? --The question of localized or delocalized proton circuits.

Williams, R.J.P. (1961). J. Theoret. Biol. 1 1-17. Possible functions of chains of catalysts.

Williams, R.J.P. (1962). J. Theoret. Biol. 3 209-229. Possible functions of chains of catalysts II.

Williams, R.J.P. (1978). Biochim. Biophys. Acta 505 1-44. The multifarious couplings of energy transduction.

Willsky, G.R., Bennett, R.L., and Malamy, M.H. (1973). J. Bacteriol. 113 529-539. Inorganic phosphate transport in Escherichia coli: Involvement of two genes which play a role in alkaline phosphatase regulation.

Willsky, G.R. and Malamy, M.H. (1974). Biochem. Biophys. Res. Commun. 60 226-233. The loss of the phoS periplasmic protein leads to a change in the specificity of a constitutive inorganic phosphate transport system in Escherichia coli.

Willsky, G.R. and Malamy, M.H. (1976). J. Bacteriol. 127 595-609. Control of the synthesis of alkaline phosphatase and the phosphate binding protein in Escherichia coli.

Wilson, I.B., Dayan, J., and Cyr, K. (1964). J. Biol. Chem. 239 4182-4185. Some properties of alkaline phosphatase from Escherichia coli.

Winkler, H.H. and Wilson, T.H. (1966). J. Biol. Chem. 241 2200-2211. The role of energy coupling in the transport of β-galactosides by Escherichia coli.

Witt, H.T. (1971). Q. Rev. Biophys. 4 365-477. Coupling of quanta, electrons, fields, ions and phosphorylation in the functional membrane of photosynthesis.

Witt, H.T., Schlodder, E., and Gräber, P. (1976). FEBS Lett. 69 272-276. Membrane-bound ATP synthesis generated by an external electrical field.

Yaguzhinsky, L.S., Boguslavsky, L.I., Volkov, A.G., and Rakhmaninova, A.B. (1976). Nature 259 494-496. Synthesis of ATP coupled with the action of membrane protonic pumps at the octane/water interface.

Yudkin, J. (1937). Enzymologia 2 161-170. The effect of silver ions on some enzymes of Bacterium coli.