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STUDIES ON PHOSPHATE TRANSPORT IN *ESCHERICHIA COLI*

II. EFFECTS OF METABOLIC INHIBITORS AND DIVALENT CATIONS

A. S. RAE and K. P. STRICKLAND

Department of Biochemistry, University of Western Ontario, London, N6A 5C1, Ontario (Canada)

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SUMMARY

1. Study has been made of the effects of a variety of metabolic inhibitors and divalent cations (Ni^{2+} and Mn^{2+}), normally after 5 min exposure, on the biphasic uptake of inorganic phosphate (P_i) exhibited by phosphate-deprived cells of *Escherichia coli*, strains AB3311 (Reeves *met*$^-$) and CBT302 (a ($Ca^{2+}+Mg^{2+}$)-ATPase-deficient mutant).

2. In AB3311 cells cyanide (1–10 mM) produced comparable reductions in phosphate uptake to anaerobiosis, but in both instances significant uptake was maintained. Examination of intracellular P_i concentrations showed that, despite these inhibitions, P_i is still concentrated 130 times compared to 394 times under aerobic conditions. Arsenate (100 μM) and iodoacetate (100 μM pre-exposed 15 min) both abolished anaerobic-supported uptake. Under aerobic conditions the former eliminated primary uptake while the latter reduced both phases of uptake 60 %. The uncouplers, dinitrophenol (100–1000 μM) and carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) (50 μM) produced very significant, but not complete inhibitions of both phases of uptake. Inhibitions by iodoacetate and dinitrophenol were additive while dithiothreitol protected against the effects of 50–250 μm CCCP. *N,N'*-Dicyclohexylcarbodiimide (DCCD), the potent inhibitor of membrane-bound (Ca^{2+} + Mg^{2+})-ATPase, at 10^{-3} M caused significant inhibitions of aerobic- (approx. 60 %) and anaerobic- (approx. 80 %) supported uptakes thus suggesting some obligatory requirement for this ATPase.

3. CBT302 cells, like AB3311, supported P_i transport both aerobically and anaerobically. CCCP (50 μM) reduced the primary uptake similarly to AB3311 cells, but the secondary uptake was less affected. DCCD (10^{-5}–10^{-3} M), as expected, showed no effects in contrast to AB3311 cells.

4. In AB3311 cells Ni^{2+} (10 mM) caused significant but different reductions of secondary (70 %) and primary (33 %) phases of phosphate uptake. Mn^{2+} (10 mM) showed a greater differential effect with the primary uptake being minimally affected

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone; DCCP, *N,N'*-dicyclohexylcarbodiimide.

and the secondary uptake being abolished (97 %). Partial relief of these inhibitions by Mg^{2+} (10 mM), suggested that these ions compete with Mg^{2+} transport. High voltage electrophoresis studies showed that Ni^{2+} cause intensification in the labelling from $^{32}P_i$ (i.e. during P_i uptake) of hexose phosphates and a reduction in the labelling of complex molecules left at the origin. With Mn^{2+}, labelling of fructose 1,6-diphosphate was reduced, the triose phosphate area was intensified and an unknown area (X) was intensely labelled. When Mn^{2+} was combined with anaerobiosis, phosphate uptake though diminished in rate exceeded after 16 min the plateau level of uptake under aerobic conditions with Mn^{2+} present.

INTRODUCTION

The current status of energy coupling in the active transport carried on by bacteria has been appropriately summarized recently [1] in the words "the molecular mechanism of energy coupling still remains an intriguing problem" despite the extensive studies done on a variety of solutes. Included amongst this group is the transport of inorganic phosphate (P_i) in *Escherichia coli* which has been under study in Rosenberg's laboratory [2–6] and more recently in our own laboratory [6, 7]. Medveczky and Rosenberg [3], in their studies on phosphate uptake in *E. coli* (strain AB3311) showed that this bacterium carries out the active transport of phosphate as judged by its dependency on oxidizable carbon sources and its severe impairment by a variety of metabolic inhibitors. In addition, the transport of P_i was found to be very sensitive to osmotic shock and moderately sensitive to cold shock [2]. Osmotic shock was shown to release, amongst a number of periplasmic proteins, a phosphate binding protein [2] which has been strongly implicated in the more rapid primary phase of P_i uptake [3].

Although in the initial studies of Medveczky and Rosenberg [3] on phosphate transport in *E. coli* the effects of quite a number of metabolic inhibitors were examined, the degree of detail in the study was inadequate for full elucidation of the mechanism of energy coupling in the transport of phosphate. Hence, a more intensive study was undertaken. Some of the results obtained, particularly those relating to the effects of (a) the powerful uncoupling agent carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), (b) anaerobiosis and (c) KCN have already been reported [7]. The latter study showed that in AB3311 cells there is a significant portion of phosphate transport not dependent on respiratory activity or conditions which normally are required for the generation of a transmembrane proton or electrochemical gradient (for discussion see refs 1 and 8). The further finding that significant phosphate uptake could be carried on under aerobic and anaerobic conditions in a mutant (CBT302) deficient in membrane-bound ($Ca^{2+}+Mg^{2+}$)-ATPase activity indicated that the latter is not obligatory for phosphate transport. This conclusion was also reached by Rosenberg et al. [4] in an independent study using mutants deficient in ($Ca^{2+}+Mg^{2+}$)-ATPase and/or electron transport.

The additional results obtained on the effect of inhibitors are now reported. Included are data not only from experiments done on the effects of chase by unlabelled ("cold") P_i, arsenate, iodoacetate, dinitrophenol and dicyclohexylcarbodiimide (DCCD), but also further experiments on the effects of anaerobiosis, cyanide and

CCCP. In addition, since in earlier studies Medveczky and Rosenberg [3] had reported that the Mg^{2+}-dependent secondary phase of P_i uptake is abolished by the addition of Ni^{2+} (10 mM), a number of experiments were done examining the effects of the divalent cations Ni^{2+} and Mn^{2+}. As will be shown and commented on, the evidence obtained from these and earlier studies [2, 3, 5–7] indicates that an appreciable portion of the phosphate uptake of *E. coli* AB3311 fulfills the characteristics described recently by Berger and Heppel [9] for what are called "shock-sensitive permeases".

MATERIALS AND METHODS

Chemicals. Carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), 2,4-dinitrophenol, iodoacetic acid, potassium arsenate and dithiothreitol were all obtained from the Sigma Chemical Co. (St. Louis, Mo.). Tris (THAM) was purchased from the Fischer Scientific Co. (Toronto, Ont.). *N*, *N'*-dicyclohexylcarbodiimide (DCCD) was obtained from Schwarz Bioresearch Inc. (Orangeburg, N.Y.). $^{32}P_i$ was supplied as carrier-free $H_3{}^{32}PO_4$ from the Atomic Energy of Canada Ltd. Commercial Products (Ottawa). All other inhibitors and chemicals used were of reagent grade or the highest purity available.

Bacterial strains. The two strains of *E. coli* (derived from K12), used previously [7], were also used in these studies. These were: AB3311 (Reeves *met*$^-$) kindly supplied by Dr. H. Rosenberg, John Curtin School of Medical Research, Australian National University and CBT302 (a ($Ca^{2+}+Mg^{2+}$)-ATPase-negative mutant) kindly supplied by Dr. B. D. Sanwal of this Department. Both strains were maintained on rich solid media (1 % tryptone, 1 % beef extract, 0.5 % NaCl, 0.3 % yeast extract and 2 % agar). Cells were grown overnight as described previously [3, 6] to the stationary phase at 37 °C in a gyrotory shaker. The cells were harvested by centrifugation, then resuspended in phosphate-free media [3, 6] and incubated for 2 h with shaking at 37 °C. These phosphate-starved cells were collected by centrifugation for use in subsequent phosphate uptake studies.

Phosphate uptakes. The cells above were suspended in uptake medium (phosphate-free Tris/salts/glucose medium) [3, 6] preparatory to doing P_i uptakes. Inhibitors or other factors were added as indicated later. $^{32}P_i$ stock solutions for uptake studies were prepared and filtered as described by Medveczky and Rosenberg [3]. Following preincubation at 37 °C, P_i uptakes were carried out as outlined previously [7] and the results expressed in nmol P_i uptake/10^9 cells (i.e. per ml of cells at 100 Klett). Final concentration of the $^{32}P_i$ added for uptake was 100 μM. Cell densities were measured in Klett units on a Klett-Summerson Colorimeter using the blue filter.

High voltage electrophoresis. In a number of experiments cells were used directly from the uptake flask for high voltage electrophoresis on Whatman 3 MM paper to provide information on the intracellular pool of $^{32}P_i$ and its radioactive phosphate ester metabolites present in *E. coli*. For this purpose the method of Medveczky and Rosenberg [3] as modified more recently by Rae and Strickland [7] was used. Tentative identification has been made, by comparison with appropriate markers and independent paper chromatographic separations, of a number of the bands seen under radioautography.

Inhibitor studies. Stock solutions of inhibitors were prepared, where possible, in aqueous solutions and added at precisely defined times to the uptake media prior to,

or after the uptake commenced. The stock solution for DCCD had to be prepared in ethanol in appropriate concentrations such that the added ethanol had no effect. Uptakes done under anaerobic conditions were carried out in the presence of N_2 as described by Rae and Strickland [7].

Ni^{2+} and Mn^{2+} inhibition. In these experiments the divalent cation (as $NiSO_4$ or $MnSO_4$) was added to give final concentrations ranging from 1 to 10 mM at 4 or 5 min prior to uptake. In certain experiments, the combined effects of Mn^{2+} and anaerobiosis (established as in ref. 7) were examined.

RESULTS

Phosphate uptake under anaerobic (i.e. N_2) conditions or equivalent (i.e. 1–10 mM *KCN*)

These experiments were undertaken to provide information on the accumulation of P_i that occurs under essentially anaerobic conditions (i.e. N_2 or 1–10 mM

TABLE 1

SUMMARY OF EFFECTS OF METABOLIC INHIBITORS ON PHOSPHATE UPTAKE IN *E. COLI* STRAINS AB3311 AND CBT302

Inhibitor(s) added 5 min prior to uptake (i.e. t, −5 min) unless otherwise indicated.

Inhibitor(s)	Percent inhibition of uptake		
	At 1 min	During 12th min	After 20 min
AB3311 Cells			
Control	0	0	0
N_2 (i.e. anaerobiosis)*	49	76	55
CN^- (1–10 mM)*	59	75	59
Arsenate (100 μM)**	89	13	57
Arsenate (100 μM) plus N_2**	97	94	97
Iodoacetate (100 μM)	28	45	30
Iodoacetate (100 μM, added at t, −15 min)	66	58	59
Iodoacetate as above plus N_2	91	86	88
Dinitrophenol (100 μM)	41	33	34
Dinitrophenol (400 μM–1 mM)	70	69	69
Dinitrophenol (100 μM) plus iodoacetate (100 μM)	73	77	71
CCCP (50 μM)	67	68	66
CCCP (50 μM) plus N_2 or CN^- (10 μM)	73	85	79
DCCD (1 mM, added at t, −12 h)***	64	—	—
DCCD as above plus N_2***	78	—	—
CBT 302 cells			
Control	0	0	0
N_2 (i.e. anaerobiosis)	66	66	51
CCCP (50 μM)	66	38	43
DCCD (1 mM, added at t, −12 h)***	5	—	—

* Calculations from data in earlier report [7] are included.

** As a competitive inhibitor of P_i utilization arsenate can inhibit not only P_i transport directly but intracellular reactions utilizing P_i.

*** Uptakes done for only 4 min.

KCN) as opposed to aerobic conditions. Under the former conditions any active accumulation of P_i must be dependent on the energy of anaerobic metabolism. For these and other conditions to be described later, the percent inhibitions compared to control aerobic conditions were calculated from typical biphasic uptake curves after 1 min (i.e. an index of effect on primary uptake phase) during the 12 min (i.e. an index of effect on secondary uptake phase) and after 20 min of uptake (i.e. an index of the effect on total uptake). These values are reported in Table I. Concentrations of 1 and 10 mM KCN which were found to produce respective inhibitions of respiration of 92 and 98 % each produced inhibitions (approx. 58 %) in phosphate uptake comparable to that obtained for anaerobiosis (approx. 53 %).

Examination was made by the high voltage electrophoresis technique of the pattern of labelling of phosphate compounds from $^{32}P_i$ for control (i.e. aerobic) and KCN (10 mM)-treated cells after times of 1 and 10 min. A radioautograph of the pattern obtained is shown in Fig. 1. Although, as expected, in the first minute of uptake there was rapid incorporation of $^{32}P_i$ into the P_i pool, significant $^{32}P_i$ (40–50 % of total) is esterified. This held true, albeit to a lesser extent, when uptake times as short as 10 s were examined. A progressive increase in the labelling at the origin (due to $^{32}P_i$ incorporation into nucleic acids and phospholipids) was found to occur with time. The quite intense labelling evident in the area(s), between what has been identified as the upper fructose 1,6-diphosphate (Fru-1,6-P_2) area and the P_i area, is attributable to triose phosphates which migrate to this region. Labelling of the adenine nucleotide (ATP and ADP) areas just below the P_i area, while evident was with few exceptions not very intense even as time progressed (see, however, Fig. 7 for exception). In KCN-treated cells, except for the Fru-1,6-P_2 and triose phosphate areas, all other areas were reduced in intensity compared to the control. Also little or no

TABLE II

INTRACELLULAR POOLS OF P_i IN CONTROL AND CYANIDE-TREATED CELLS OF *E. COLI* AB3311 DETERMINED FROM RADIOACTIVE ANALYSIS OF PRODUCTS LABELLED FROM $^{32}P_i$ AND SEPARATED BY HIGH VOLTAGE ELECTROPHORESIS

AB3311 cells were grown to stationary phase, deprived of phosphate and used in the phosphate uptake procedure described in Materials and Methods. KCN, to give a final concentration of 10 mM, was added 5 min prior to the commencement of uptake. At fixed intervals samples were taken for uptake estimations and for electrophoresis as described by Rae and Strickland [7]. After identification of ^{32}P-labelled products by radioautography, the radioactive areas were cut out and counted in a Nuclear Chicago automatic gas flow counting system. nmol of P_i were calculated from the counts obtained on the basis that 1 nmol equalled 1333 cpm. Intracellular concentrations are based on the assumption that 10^9 cells equal 0.6 μl in volume.

A	Intracellular P_i nmol at time (min)					Total uptake at 20 min
	1	2	4	10	20	
Control	6.25	6.75	7.45	8.50	14.3	40.0
KCN treated	3.25	3.75	4.28	5.00	6.75	13.3

B	Extracellular P_i (μM at 20 min)	Intracellular P_i (mM at 20 min)	Transmembrane gradient (intracellular/extracellular)
Control	60.0	23.6	394
KCN treated	86.7	11.3	130

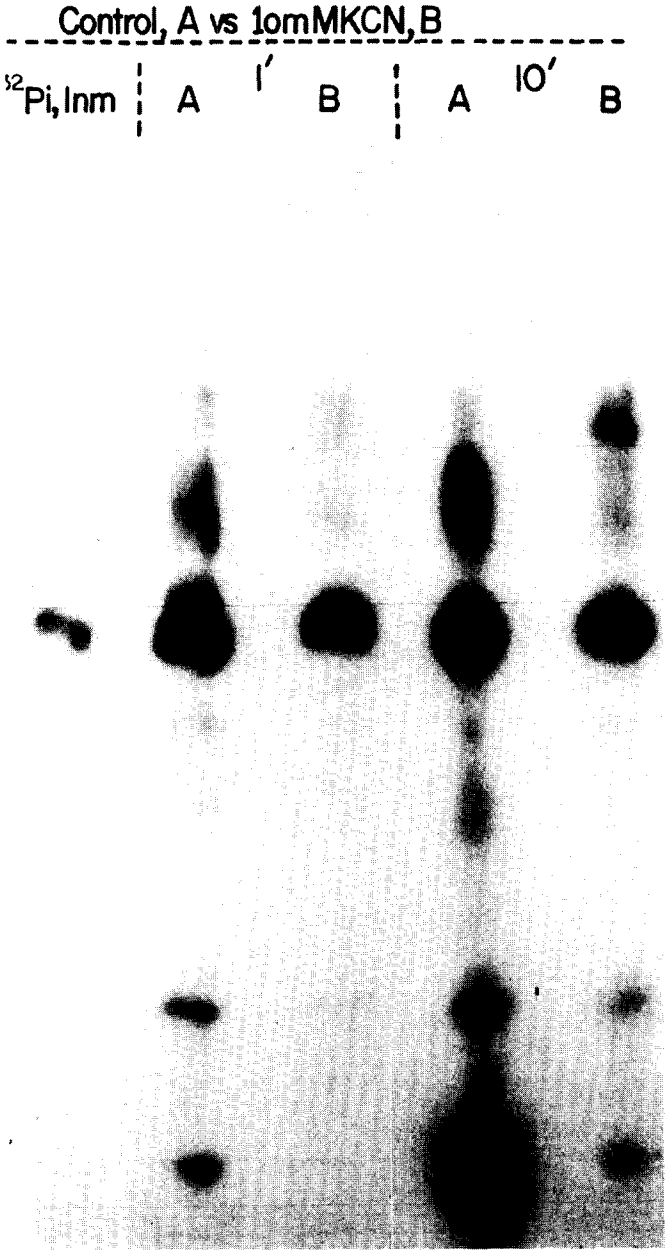

Fig. 1. Radioautograph of high voltage electrophoresis run on sample of AB3311 cells taken during phosphate uptake. Cells were grown to stationary phase, deprived of phosphate and used in phosphate uptakes as described in Materials and Methods. Samples were taken from control cells (A) and KCN (10 mM)-treated cells (B) at 1 and 10 min and directly subjected to high voltage electrophoresis as described by Rae and Strickland [7]. Radioactive areas were located by exposure to Kodak XG-14 X-ray film. $^{32}P_i$, 1 nmol corresponds to 1 nmol of phosphate.

incorporation occurred into the nucleic acids and phospholipids at the origin indicating presumably that relatively little labelled ATP was formed for incorporation of label into these complex molecules.

Since the high voltage electrophoresis technique, developed earlier [7] and applied here, migrates all of the P_i, as well as many of its simple organic phosphate metabolites, this procedure was used to determine intracellular P_i at various times of uptake in control and KCN-treated cells (Table II). After a rapid initial uptake of $^{32}P_i$ into the P_i pool in the first minute, it was found that there is a slow progressive increase in succeeding minutes in both groups of cells, but levels in the KCN-treated cells always remained at about one-half those of the control.

Calculation of the intracellular P_i concentration and comparison of the value obtained with the extracellular concentration gave ratios of 394 and 130 for the transmembrane gradient of control and KCN-treated cells, respectively. It should be noted that these gradients represent minimal values based on the assumption of an initial intracellular phosphate concentration of zero. These results indicate that P_i is, in both groups of cells, being actively accumulated against a concentration gradient.

Arsenate, P_i-preload, P_i-chase and iodoacetate

The experiments reported here (Figs. 2–4 and Table I) provide confirmation and extension of some of the studies of Medveczky and Rosenberg [3]. The addition of either 20 μM unlabelled P_i or 100 μM arsenate were found to be just sufficient to bring about the elimination of the primary uptake (Fig. 2). In experiments not reported, higher concentrations of arsenate (up to 1 mM) caused, in addition, significant inhibition of secondary uptake. Under anaerobic conditions 100 μM arsenate completely abolished the uptake of P_i. Thus arsenate, a competitive inhibitor of many

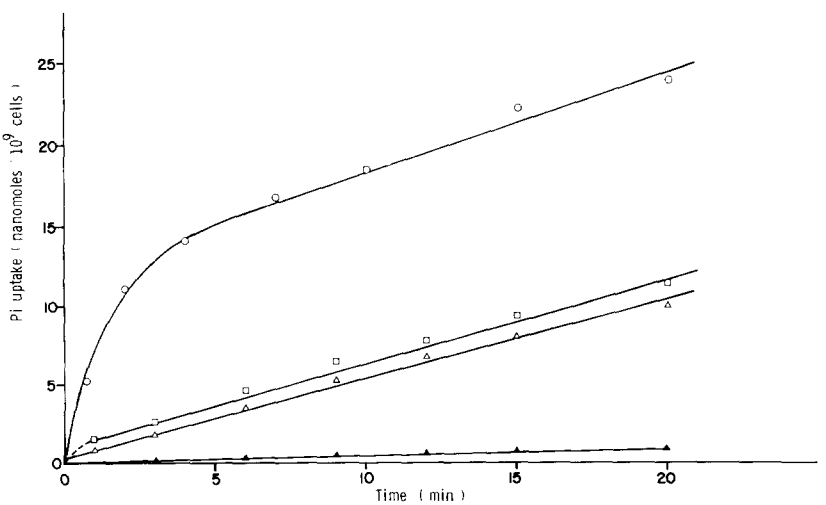

Fig. 2. Effect of unlabelled P_i preload and arsenate on phosphate uptake by AB3311 cells. Cells were prepared and used for uptake as described in Fig. 1. Uptakes are reported in nmol $P_i/10^9$ cells. (a) ○—○, control cells; (b) □···□, 20 μM unlabelled P_i 4 min before uptake; (c) △···△, 100 μM arsenate added 4 min before uptake and (d) ▲ ▲, flushed with N_2 5 min before uptake and 100 μM arsenate added 4 min before uptake.

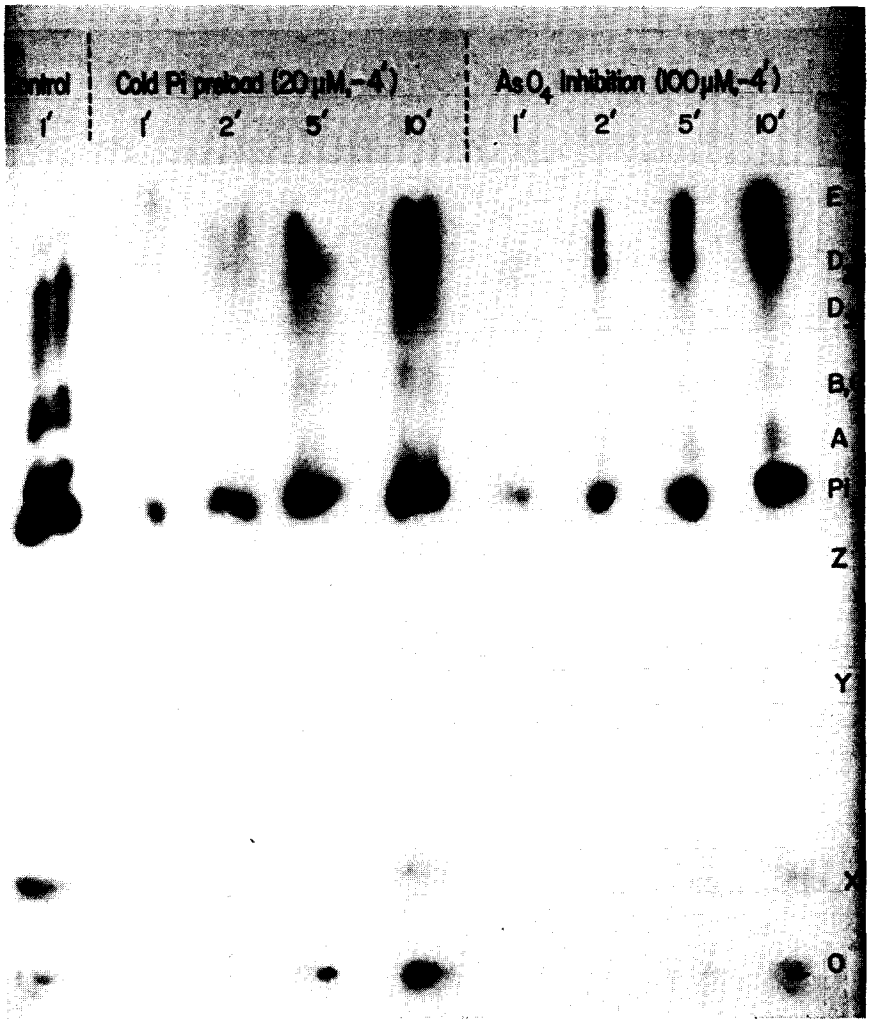

Fig. 3. Radioautograph of high voltage electrophoresis run on samples of AB3311 cells taken from phosphate uptakes described in Fig. 2. Samples were taken as follows: control cells, 1 min; cells exposed to 20 μM unlabelled P_I, 1, 2, 5 and 10 min and cells exposed to 100 μM arsenate, 1, 2, 5, and 10 min. Areas were tentatively identified (on the basis of comparisons with markers and separate chromatographic runs) as follows: X, Y, unknowns; Z, region of ATP; P_I, inorganic phosphate; A, hexose phosphates; B, C and D_1, triose phosphates; D_2, dihydroxyacetone phosphate; and E, fructose-1,6-diphosphate (Fru-1,6-P_2).

reactions involving P_i, is exhibiting at least two effects. It mimics unlabelled P_i in its elimination of the primary uptake and, under conditions where anaerobic metabolism maintains P_i uptake, it abolishes uptake presumably through its inhibition of anaerobic glycolysis.

Confirmation and amplification of the above views are provided by the results of an electrophoresis run that is depicted in the radioautograph shown in Fig. 3. As expected, the addition of unlabelled P_i 4 min prior to uptake (= preload) caused a

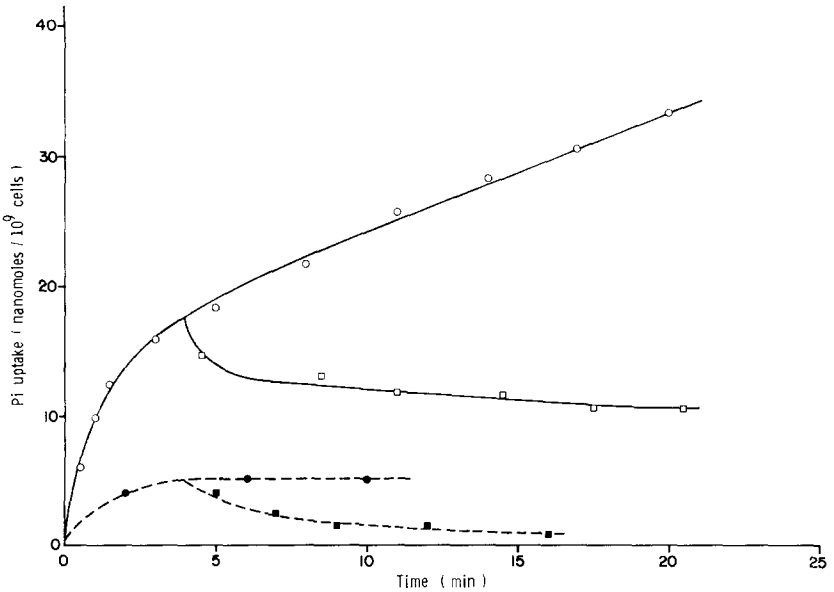

Fig. 4. Effect of chase with unlabelled P_i on phosphate uptake and intracellular P_i. Cells were prepared and used for uptake as described in Figs. 1 and 2. (a) ○—○, control cells; (b) □—□, 10 mM unlabelled P_i added after 4 min of uptake; (c) ●—●, intracellular $^{32}P_i$ of control cells determined by high voltage electrophoresis with the radioactive areas located by radioautography, cut out and counted as described in Table II and (d) ■—■, intracellular $^{32}P_i$ after addition of 10 mM unlabelled P_i at 4 min.

delayed appearance of label in all areas normally labelled. A similar examination of what happens with 100 μM arsenate showed a somewhat different pattern in certain of the areas labelled, whereas the overall accumulation of P_i was comparable and great enough to indicate the retention of some active transport. The areas tentatively identified as Fru-1,6-P_2 (area E) and dihydroxyacetone phosphate (area D_2) were relatively more intensely labelled in arsenate inhibition whereas the other triose phosphate (areas B, C and D_1) were only very slightly labelled. This pattern seems to fit well with that expected for the classic inhibition of arsenate at the glyceraldehyde-3-phosphate dehydrogenase step of glycolysis.

In view of the demonstration by Medveczky and Rosenberg [3] that unlabelled P_i can chase $^{32}P_i$, further examination was made of the effects of 10 mM unlabelled P_i, added 4 min after uptake commenced, on both total uptake and intracellular P_i. Very close to parallel decreases were observed in total uptake and intracellular P_i and almost all of the intracellular $^{32}P_i$ was chased after 15 min (Fig. 4). These results are comparable to those of Medveczky and Rosenberg [3] with the exception that the extent of efflux observed here was significantly less apparently because a smaller percentage of the primary uptake was comprised of the intracellular pool of $^{32}P_i$.

When 100 μM iodoacetate was added at 5 and 15 min prior to commencement of the P_i uptake to *E. coli* AB3311 cells under aerobic and anaerobic conditions, it was observed that at 5 min iodoacetate caused only a slight inhibition in both groups of cells with most of the effect being on the secondary uptake but that at 15 min (see

Table II) a much more striking inhibition was observed which affected mainly the primary phase of uptake. Under aerobic conditions about one-third of the original uptake was maintained whereas under anaerobiosis practically all of the uptake of both phases was abolished. Although uptake supported by either aerobic or anaerobic metabolism is greatly affected by iodoacetate, it is evident that anaerobic-supported uptake is the more sensitive, no doubt, due to the sensitivity of a glycolytic enzyme such as glyceraldehyde-3-phosphate dehydrogenase to iodoacetate.

The uncouplers, dinitrophenol and CCCP

The addition of dinitrophenol, at 5 min before uptake, in concentrations of 40–1000 μM caused reductions in uptake ranging from 25 to 70 % with about equal affects on both phases of uptake. Values obtained for two concentration groups are listed in Table I. On the assumptions that iodoacetate is primarily a glycolytic inhibitor whereas dinitrophenol is basically a respiratory uncoupler, a series of experiments were done in which the two inhibitors were added together. It is interesting to note (Table I) that, when concentrations (100 μM added 5 min prior to uptake) of each inhibitor are selected which are roughly equal but suboptimal in terms of their maximum effect, the effects of the inhibitors were additive as might be expected from the above assumptions. As concentrations of each inhibitor are increased further this additive pattern remains.

In our previous studies [7] with the uncoupler, CCCP we observed that a 50 μM concentration caused severe inhibitions of both the primary and secondary phases of phosphate uptake (listed in Table I) and that the thiol agent, dithiothreitol, added before, or even after the initiation of phosphate uptake could protect against or release this inhibition. This pattern applied not only to total phosphate uptake, but to the intracellular P_i pool. In additional unreported experiments we have observed that dithiothreitol (10 mM) also will protect against concentrations of CCCP reaching 250 μM. These results are in general agreement with and extend those of Kaback et al. [12] for lactose and serine transport in bacterial membrane vesicles. The effect of CCCP (50 μM) on P_i uptake by CBT302 cells (i.e. the $(Ca^{2+}+Mg^{2+})$-ATPase-deficient mutant) was also examined. From the uptake values reported in Table I it can be seen that the primary uptake (based on calculation at 1 min) is affected about the same as the wild type AB3311 cells whereas the secondary uptake (calculated at 12 min) and total uptake (calculated for 20 min) are much less affected.

DCCD inhibition

The effects of DCCD, a potent inhibitor of membrane-bound ATPase in bacteria and mitochondria [10, 11], have been examined in both wild type AB3311 and CBT302 *E. coli* cells (Table III). DCCD, over the concentration range 10^{-5}–10^{-3} M, caused progressive inhibitions in the uptake of AB3311 cells under both aerobic and anaerobic conditions, but the effect is greatest under the latter condition (80 % reduction as opposed to a 60 % reduction at 10^{-3} M). These results contrast with those obtained by Klein and Boyer [13] for proline transport where it was observed that only anaerobic-supported uptake was significantly affected by DCCD. In the CBT302 cells exposed to DCCD, there was no appreciable affect of this inhibitor at any of the concentrations studied (10^{-5}–10^{-3} M). On the assumption that the action of DCCD is exerted through its inhibition of membrane-bound ATPase, the above

TABLE III

THE EFFECT OF *N,N'*-DICYCLOHEXYLCARBODIIMIDE (DCCD) ON PHOSPHATE UPTAKE IN AB3311 AND CBT302 CELLS

Cells were prepared and used for uptake as described in Fig. 1. DCCD was added at the concentrations listed 12 h prior to uptake (cells stored at 4 °C for this period). Uptakes were carried out as described in Materials and Methods for 4 min under aerobic or anaerobic conditions (as in ref. 7). Uptakes relative to the control were calculated from uptake values obtained after 2 min.

Condition	DCCD (M)	Relative uptake	
AB3311			
Aerobic	0	1.00	
	$1 \cdot 10^{-5}$	0.87	
	$1 \cdot 10^{-4}$	0.65	
	$1 \cdot 10^{-3}$	0.40	
Anaerobic	0	0.50	1.0*
	$1 \cdot 10^{-5}$	0.36	0.72
	$1 \cdot 10^{-4}$	0.26	0.52
	$1 \cdot 10^{-3}$	0.10	0.20
CBT302			
Aerobic	0	1.00	
	$1 \cdot 10^{-5}$	0.88	
	$1 \cdot 10^{-4}$	1.04	
	$1 \cdot 10^{-3}$	0.98	

* Recalculated relative to control anaerobic uptake.

data would partially implicate the ATPase in support of uptake in wild type cells (AB3311), but confirm that uptake can go on perfectly well in mutant cells deficient in $(Ca^{2+} + Mg^{2+})$-ATPase. The fact that DCCD shows no inhibition where there is no membrane-bound ATPase is consistent with the action cited above [10].

Divalent cations, Ni^{2+} and Mn^{2+}

Fig. 5 shows the effect of Ni^{2+} at 2 and 10 mM concentrations on phosphate uptake by AB3311 cells. At both concentrations the secondary uptake is reduced (25 and 70 % for 2 and 10 mM, respectively) more than the primary uptake (15 and 33 %, respectively). However, despite many attempts it was not possible to produce such a great differential effect as that reported by Medveczky and Rosenberg [3]. They observed that 10 mM Ni^{2+} caused no appreciable affect on the primary uptake whereas the secondary uptake was practically abolished. Our findings indicated that there is a lesser but steady increase in inhibition of the primary uptake. The effects of adding Mn^{2+} in concentrations of 1, 2 and 5 mM on phosphate uptake in AB3311 cells is shown in Fig. 6. With Mn^{2+} a much more sharply defined differential effect is seen than for Ni^{2+}. For the three concentrations shown, inhibitions (as measured during the 12th min of uptake) of 75, 88 and 97 % were obtained at 1, 2 and 5 mM, respectively, whereas there was little ultimate effect on primary uptake.

Using the high voltage electrophoresis technique for the separation of P_i and organic phosphate esters from cells of *E. coli*, examination was made of the pattern of

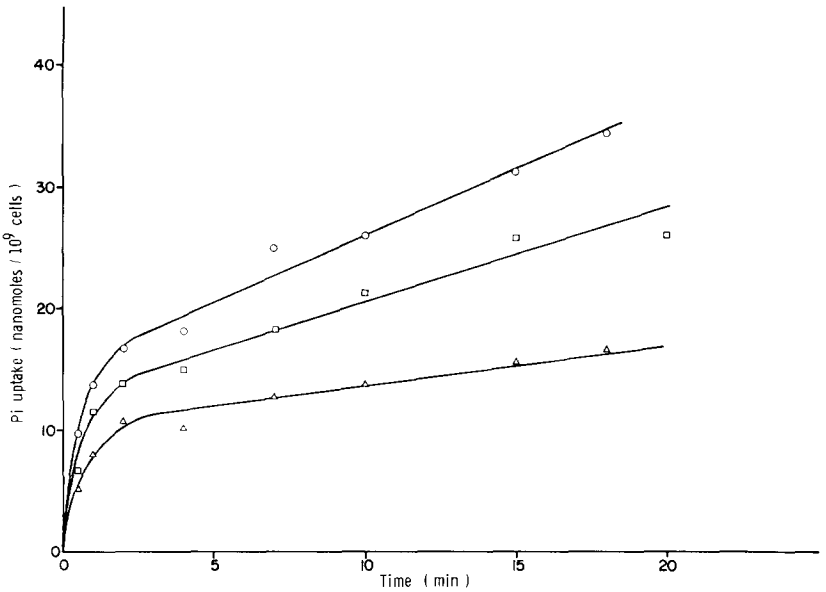

Fig. 5. The effect of Ni^{2+} on uptake of P_i by AB3311 cells. Cells were prepared and used for uptake as described in Figs. 1 and 2. $NiSO_4$ was added to give final concentrations shown at 5 min prior to uptake. (a) ○—○, control cells; (b) □—□, 2 mM $NiSO_4$ and (c) △—△, 10 mM $NiSO_4$.

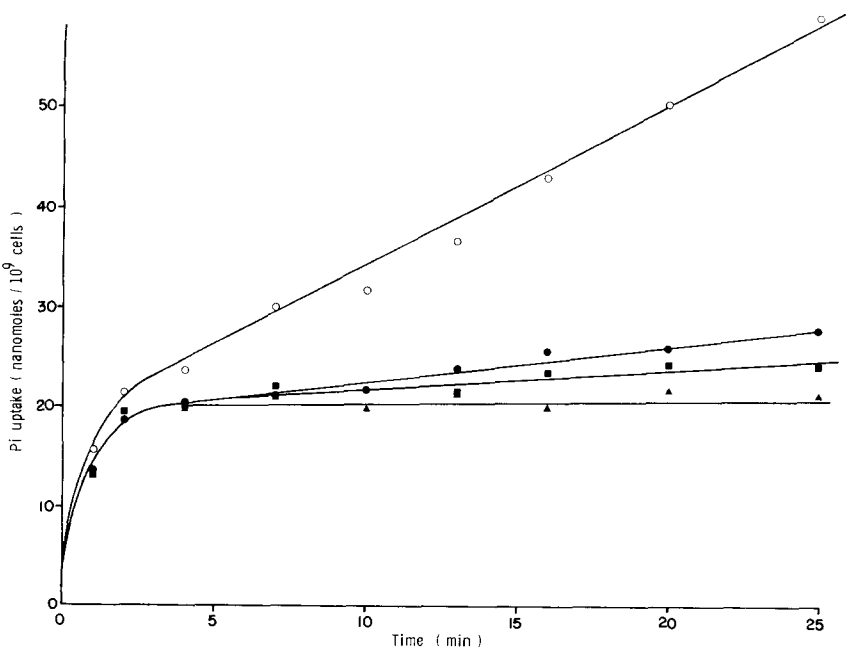

Fig. 6. The effect of Mn^{2+} on uptake of P_i by AB3311 cells. Cells were prepared and used for uptake as described in Figs. 1 and 2. $MnSO_4$ was added to give final concentrations shown at 5 min prior to uptake. (a) ○—○, control cells; (b) ●—●, 1 mM $MnSO_4$; (c) ■—■, 2 mM $MnSO_4$ and (d) ▲—▲, 5 mM $MnSO_4$.

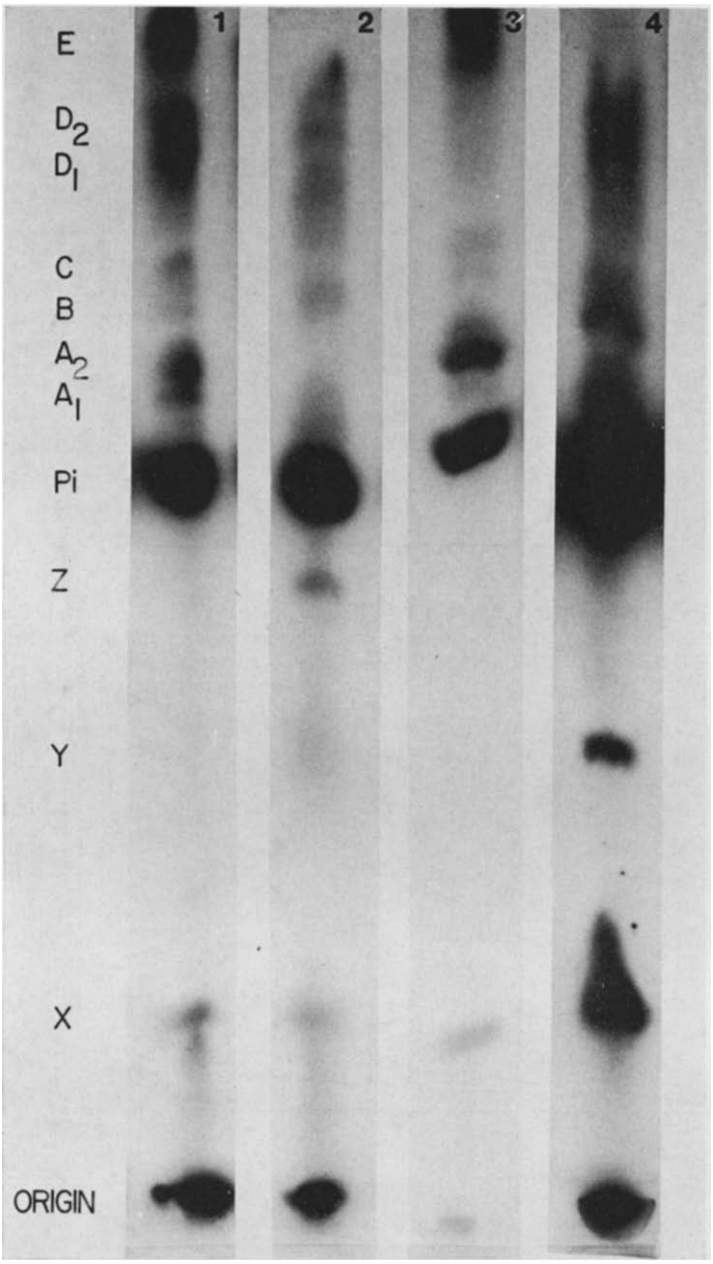

Fig. 7. Radioautograph of high voltage electrophoresis run on samples of AB3311 cells obtained after 12 min of P_i uptake. Cells were prepared and used for uptake as described in Fig. 1. Samples of cells were taken as shown and directly subjected to high voltage electrophoresis as described by Rae and Strickland [7]. Radioactive areas depicted were located by exposure to Kodak XG-14 X-ray film. Area designation and assignment as in Fig. 3.

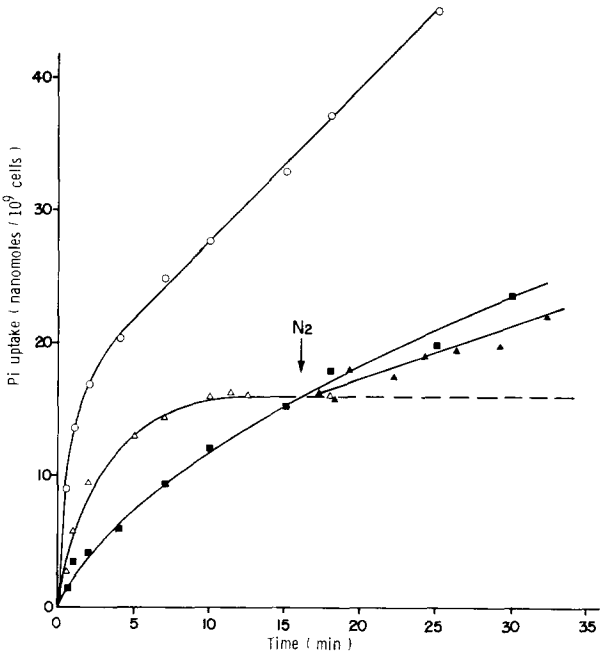

Fig. 8. The combined effects of Mn^{2+} and anaerobiosis on the uptake of P_i by AB3311 cells. Cells were prepared and used for uptake as described in Fig. 1. (a) ○ - ○, control cells; (b) ■ - ■, 10 mM $MnSO_4$ and N_2 flushing both at 5 min prior to uptake; (c) △ - △, $MnSO_4$, 10 mM added 5 min prior to uptake and (d) ▲ - ▲, as for (c) but N_2 flushing commenced at 16 min after uptake. The control for N_2 alone is not shown, but during its initial phases, it follows closely the curve described under (c) and after 10 min uptake proceeds at the same slope as the curve described under (d).

labelling from $^{32}P_i$ that occurs after 12 min of uptake in the presence of added Ni^{2+} or Mn^{2+} (10 mM). The radioautograph obtained from this examination is shown in Fig. 7. Also since some interesting changes in labelling patterns were observed as a result of post-uptake incubation at 37 °C of cells collected on membrane filters, a representative pattern is included. Compared to the control some differences in the relative intensities of labelling were noted. The Fru-1,6-P_2 (area E) and hexose phosphates (areas A_1 and A_2) seemed to be more intensely labelled with Ni^{2+} present and somewhat less labelling was evident at the origin. With Mn^{2+} added, the unidentified area X was intensely labelled and Fru-1,6-P_2 was labelled relatively less. The most interesting finding on post-uptake inhibition was that area Z, corresponding in all probability to ATP, was quite intensely labelled. This observation that cells allowed to incubate at 37 °C, once uptake is terminated, alter their metabolic pattern such as to build up and label area Z more than would normally occur should uptake be allowed to continue, would suggest that there is rapid turnover of labelled ATP during normal conditions of P_i uptake.

Fig. 8 shows the combined and sequential effects of Mn^{2+} (10 mM) and anaerobiosis on the uptake of phosphate by AB3311 cells. Under aerobic conditions Mn^{2+} produced the typical effect already described (Fig. 6). However, this ion rather surprisingly caused a more marked reduction of rate in respect to the primary uptake

than that normally observed for anaerobiosis alone. Despite this reduced rate the uptake continued steadily and surpassed after 16 min the total uptake of the aerobic system containing Mn^{2+}, which by this time had reached a plateau in phosphate uptake. What is interesting, but equally difficult to explain, is the observation that anaerobiosis when introduced after 16 min of aerobic uptake with Mn^{2+} present results in uptake commencing once again at the same rate as for the uptake under anaerobic conditions containing Mn^{2+}.

DISCUSSION

The results of this paper and our previous reports [6, 7] on phosphate transport in *E. coli* help considerably to extend the earlier findings of Medveczky and Rosenberg [2, 3]. The available evidence clearly indicates that *E. coli* AB3311 cells are capable of active uptake of phosphate, by as a minimum, a shock-sensitive system that is dependent on metabolic energy. The latter authors have shown that the P_i uptake occurs by two kinetically distinct components, a high affinity one which appears to require phosphate binding protein and a low affinity component which is highly dependent on the subsequent incorporation of P_i into more complex molecules [3]. However, as suggested by the results of our previous work [6, 7] and this study, *E. coli* is capable of transporting phosphate by more than one system. In fact, Willsky et al. [14] have indicated that "the ability to utilize P_i in *E. coli* is governed by at least four genetically distinct transport systems". Despite this latter complicating feature, considerable insight into the nature of P_i uptake and its energy coupling is provided by the above studies. In the ensuing discussion we shall outline the evidence which suggests that there is a major shock-sensitive component of phosphate transport in AB3311 which possesses energetics similar to those of the "shock-sensitive permeases" described by Berger and Heppel [9].

From the results reported here and elsewhere [7] a number of conclusions may be made. Thus, it is evident that both strains of *E. coli* (AB3311 and CBT302) take up P_i equally well under aerobic conditions, and that transport supported under anaerobic conditions (N_2, or 10 mM KCN) is in both instances reduced by about one-half. The latter was shown by examination of the intracellular P_i concentrations (Table II). The observation that the ATPase mutant (CBT302) takes up phosphate equally well compared to the wild type (AB3311) suggests that this membrane-bound enzyme is not essential under either aerobic or anaerobic conditions. The presence of anaerobic-supported transport in the ATPase mutant indicates that transport under these conditions must be energized by a mechanism other than a proton or an electrochemical gradient. As expected all of the anaerobic-supported uptake is inhibited by arsenate (100 μM) and iodoacetate (100 μM), two strong inhibitors of glycolysis (at glyceraldehyde-3-phosphate dehydrogenase step). However, under aerobic conditions only part of the uptake was sensitive to these inhibitors. Arsenate, presumably through competition, completely abolishes the primary uptake. On the other hand, electrophoretic data (e.g. Fig. 3) indicates that some active transport continues which must be contained in the residual uptake of the secondary phase. The less severe inhibition by iodoacetate under aerobic compared to anaerobic conditions may still be the result of action of this inhibitor on a glycolytic step rather than on a step in the electron transport chain or subsequent to it (e.g. phosphorylation, proton

gradient, "energized" membrane state). The latter suggestion is supported by the observation that the combined effects of dinitrophenol (which uncouples phosphorylation and dissipates the proton gradient) and iodoacetate are somewhat additive.

Results from studies with the two uncouplers, dinitrophenol and CCCP, reported here or earlier [7] provide convincing evidence that a large portion, but not all of the uptake of P_i is uncoupler sensitive. Thus with dinitrophenol in the concentration range of 0.4–1 mM and CCCP at 50 μM, inhibitions of both phases of uptake of the order of 70% are obtained (Table I). The remaining 30% of uptake under these conditions becomes all the more important when examination is made of the reduction in intracellular P_i. When this was done [7], the actual reduction proved to be less than 50%. This means that a significant portion of P_i accumulation is insensitive to the uncoupling effects of CCCP and presumably the dissipation of any electrochemical gradients [8].

In their original investigation of phosphate transport in *E. coli* AB3311, Medveczky and Rosenberg [3] observed that K^+ (or Rb^+) was necessary for primary uptake and Mg^{2+} for normal secondary uptake. In unreported experiments we have in general, confirmed these findings. The above authors also noted that Ni^{2+} abolished the Mg^{2+}-supported secondary uptake. Our experiments on Ni^{2+}, only in part, support this observation since it has not been possible to abolish completely the secondary uptake of phosphate without causing appreciable inhibition of the primary uptake phase. On the other hand, the addition of Mn^{2+} produces effects quite comparable to those reported by Medveczky and Rosenberg [3] for Ni^{2+}.

These inhibitory effects of Mn^{2+} and possibly of Ni^{2+} may be explainable in terms of the competitive effects of these divalent cations on Mg^{2+} transport which is essential for the maintenance of intracellular levels of Mg^{2+} for the support of a variety of metabolic reactions [15–18]. A system for the active transport of Mg^{2+} in *E. coli* has been studied independently in the laboratories of Kennedy [15, 18] and Silver [16, 17]. The former workers have described a system particularly sensitive to Co^{2+} while the latter workers have described a system that is competitively inhibited by Mn^{2+} ($K_i = 0.5$ mM Mn^{2+}). In respect to this inhibition Silver and Clark [17] noticed that 5–10 mM Mn^{2+} caused a rapid efflux of Mg^{2+}. It is reasonable to assume that Mn^{2+} is acting in this manner in our study on phosphate transport with the result that the cells become essentially Mg^{2+} depleted and in this condition cannot support secondary uptake. Although no definitive study is available for Ni^{2+}, it is possible that a somewhat similar effect is being exerted. In unreported experiments we observed some protective effect by Mg^{2+} which would also support the view that both Ni^{2+} and Mn^{2+} are antagonistic in a competitive manner towards Mg^{2+}.

Differences between the effects of Ni^{2+} and Mn^{2+} are evident in the patterns of labelling of the phosphate esters of the glycolytic intermediates and other unidentified phosphate compounds during P_i uptake. However, without further detailed study and analysis of such differences, one cannot define the independent inhibitory actions of these ions in terms of any precise effects on glycolysis or other cellular activities.

It is difficult to offer any consistent or complete explanation for the combined effects of anaerobiosis and Mn^{2+} on phosphate uptake. Under anaerobiosis there must be a change in the basic metabolism of the AB3311 cells to one mainly dependent on glycolysis where uptake, although slowed by Mn^{2+}, is not prevented but rather continues at an appreciable rate over the entire period studied, and even exceeds the

Mn^{2+}-inhibited aerobic uptake. It is possible in this system that anaerobic metabolism is less sensitive to Mg^{2+} depletion than its aerobic counterpart, or alternatively that less Mg^{2+} efflux occurs under anaerobiosis.

With few exceptions none of the inhibitors or conditions studied showed differential effects in respect to either the "high affinity" (primary) component or the "low affinity" (secondary) component of phosphate transport. The exceptions include (a) arsenate under aerobic conditions where little effect is seen on the secondary uptake, (b) N_2 and CN^- (10 mM) which show more pronounced effects on secondary uptake, (c) CCCP (50 μM) inhibition of CBT302 cells which showed less effect on secondary uptake and (d) Ni^{2+} and Mn^{2+} inhibition which affected mainly the secondary uptake. Since the secondary phase is highly dependent on the incorporation of P_i into more complex molecules such as phospholipids and nucleic acids, mediated through ATP, it is probable that in the above situations where secondary uptake is affected that this is directly related to the inhibition of ATP synthesis or its precursors.

As is to be expected from the study of a transport system as difficult as phosphate, where no non-metabolizable analogue is available and where multiple transport systems may exist, it is very difficult to fit all of our findings into any one mechanism for energy coupling of active transport. Two possibilities would seem to apply in respect to P_i transport in the two strains of *E. coli* studied. The first, and perhaps more plausible, possibility is that two separate mechanisms exist, one dependent on respiration which creates a proton or electrochemical gradient or "energized" state that can be utilized to drive P_i transport and a second dependent on the ATP generated from glycolysis, or alternatively an energy-rich intermediate of glycolysis. Most of the inhibitor effects are accountable by this proposal. Thus N_2, cyanide and the uncouplers, dinitrophenol and CCCP, do abolish the respiration-driven mechanism and arsenate, and iodoacetate the glycolytic-supported mechanism. The non-obligatory requirement for $(Ca^{2+}+Mg^{2+})$-ATPase shown by CBT302 cells would be expected. The second possibility, for which the proof is more tenuous, embodies a single mechanism in which ATP, or an intermediate (e.g. glycolytic) derived from it, is the immediate source of energy for P_i transport. This mechanism is essentially analogous to what Berger [19] has proposed for the transport of glutamine. With this proposal both respiration and glycolysis would support P_i transport through the generation of ATP (or the above unknown intermediate). The more efficient pathways of aerobic metabolism might be expected to produce more ATP and hence greater total transport. N_2, cyanide and the uncouplers, CCCP and dinitrophenol, would reduce the cell to one carrying on transport through ATP supplied by glycolysis. Arsenate and iodoacetate would block the contribution of the latter. This proposal would require the function of the $(Ca^{2+}+Mg^{2+})$-ATPase only for respiration-supported transport. The results obtained with DCCD, a potent inhibitor of $(Ca^{2+}+Mg^{2+})$-ATPase, on AB3311 cells provide support for such a requirement since under aerobic conditions this inhibitor caused up to a 60 % inhibition of primary uptake of P_i. Two observations are not accounted for in this mechanism. These relate to the $(Ca^{2+}+Mg^{2+})$-ATPase. The strong inhibition by DCCD on AB3311 cells under anaerobic conditions, where glycolysis is presumably forming ATP, provides equally convincing evidence that this ATPase is involved. Also the manner in which the ATPase-deficient mutant, CBT302 can bring about additional support of P_i transport aerobically is not accounted for. These discrepancies may be accountable on the basis that the AB3311 cells

which contain the ATPase have adapted their energetics of P_i transport to require this enzyme, or that DCCD under the conditions studied exerts additional effects.

Irregardless of which of the above proposals apply, either a significant portion or all of P_i transport in these strains of *E. coli* would appear to occur by a system dependent on ATP, or a glycolytic intermediate. This conclusion taken in conjunction with the observation that a large portion of P_i uptake is sensitive to osmotic shock [3, 6] would indicate that a significant segment of P_i transport fits reasonably well the criteria outlined by Berger and Heppel [9] for "shock-sensitive permeases". These workers [9, 19] and others [20–23] have demonstrated that a number of amino acids (glutamine, α, ξ-diaminopimelic acid, arginine, histidine, ornithine and isoleucine), glycylglycine, D-ribose and galactose are all transported by permease systems which are sensitive to osmotic shock, have an obligatory requirement for phosphate-bound energy and require participation of $(Ca^{2+}+Mg^{2+})$-ATPase in the utilization of energy derived by the electron transport chain.

While most of the discussion has related to the type of mechanism involved in P_i transport in *E. coli* and comparison of this with other systems that are sensitive to shock, it is appropriate in concluding to make some comparison with studies of P_i transport in other organisms or organelles. The most recent definitive study is that of Harold and Spitz [24] who have described experiments on arsenate, phosphate and aspartate transport in the anaerobic-dependent organism, *Streptococcus faecalis*. In their studies they have concluded that phosphate accumulation occurs by a P_i^-/OH^- antiport system which is driven by ATP, or by a metabolite derived from ATP. Thus, there is some general agreement on the immediate source of energy for transport in the above organism and that suggested in our studies on *E. coli*. We have no evidence which would bear on the question of an electroneutral exchange such as proposed for *S. faecalis*. In the future it will be necessary to carry out a variety of experiments to test this possibility and to evaluate the mechanisms for energy coupling proposed in this study. In respect to the latter a more intensive study of the ATPase mutant with regards to the metabolic inhibitors employed in this study for the wild type strain as well as the application of the electrophoresis technique described elsewhere [7] should provide exciting answers to many questions concerning P_i transport and energetics. An alternative approach would be to obtain and study mutants of *E. coli* which have been shown to possess a single transport system for P_i which is either shock sensitive or shock resistant.

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