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The Inhibitory Effect of the Artificial Electron Donor System, Phenazine Methosulfate-Ascorbate, on Bacterial Transport Mechanisms

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The artificial electron donor system, phenazine methosulfate (PMS)-ascorbate, inhibited active transort of solutes in Pseudomonas aeruginosa irrespective of whether the active transport systems were shock sensitive or shock resistant. N,N,N′,N′-tetramethylphenylenediamine could be substituted for PMS but a higher concentration was required. PMS-ascorbate also inhibited active transport in several other bacterial species with the exception of Escherichia coli and of a nonpigmented strain of Serratia marcescens. PMS-ascorbate previously has been shown to energize active transport in isolated membrane vesicles, even those prepared from the same bacterial species in whose intact cells active transport was inhibited. The apparent K_m of glucose active transport in untreated cells of P. aeruginosa was 40 μM while the K_m of glucose transport in cells incubated with PMS-ascorbate was 25 mM, and PMS-ascorbate had no effect on efflux of accumulated glucose. These results strongly suggested that facilitated diffusion resulted upon exposure of the cells to PMS-ascorbate. Thus, PMS-ascorbate appeared to have an uncoupler-like effect on cells of P. aeruginosa. The experimental data also pointed out that there are fundamental differences between the response of intact cells and membrane vesicles to exogenous electron donors.

Key words: pseudomonas, transport, phenazinemethosulfate

In attempting to apply the experimental procedure of Berger (1) and Berger and Heppel (2) to Pseudomonas aeruginosa, we noted that active transport of a variety of substrates was inhibited by phenazine methosulfate (PMS)-ascorbate irrespective of whether the cells were normal or starved and irrespective of whether the active transport systems were shock sensitive or shock resistant. This was curious because the artificial electron donor system of PMS-ascorbate energizes active transport by membrane vesicles prepared from P. aeruginosa (3, 4) and from a wide variety of other bacterial species including E. coli (5), and because PMS-ascorbate energizes both shock-sensitive and shock-resistant active transport systems in starved cells of E. coli (2). Thus, the work presented herein was carried out in an effort to characterize and elucidate the inhibitory effect of PMS-ascorbate on active transport systems of intact cells of P. aeruginosa. Evidence will also be presented showing that active transport systems of bacterial species in addition to P. aeruginosa are inhibited similarly by PMS-ascorbate.

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MATERIALS AND METHODS

Cultivation of Organisms

The organisms used for these studies were: P. aeruginosa PAO (formerly Holloway strain 1); P. aeruginosa PAO 57, a mutant derived from PAO which was unable to grow on glucose but which formed the inducible glucose transport system when grown in media containing pyruvate plus glucose (3); P. aeruginosa OSU 64; P. aeruginosa A1466; P. fluorescens ATCC 13525; P. putida ATCC 12633; Escherichia coli ML 308-225; Enterobacter aerogenes UGA; Aeromonas hydrophila (an aerogenic strain); A. hydrophila (an anaerogenic strain); Serratia marcescens Sm7 (a pigmented strain); S. marcescens Sm39 (a nonpigmented strain); Bacillus subtilis SP491; and Staphylococcus aureus 832S-Y.

The strains of S. marcescens and P. aeruginosa A1466 were obtained from Dr. J. J. Farmer, Center for Disease Control, Atlanta, Georgia. The A. hydrophila strains were obtained from Dr. E. B. Shotts, College of Veterinary Medicine, University of Georgia. All other bacterial species and strains were from the culture collection of the Department of Microbiology, University of Georgia.

The Pseudomonas species were cultivated in a chemically defined basal medium and under the cultural conditions previously defined (3, 6). The final concentration of the carbon source was 11 mM glucose for all species except P. aeruginosa PAO 57. The latter was cultivated in 30 mM pyruvate plus 5 mM glucose. All other bacterial species, except S. aureus, were cultivated in the basal salts medium described by Tanaka, Lerner, and Lin (7) to which was added 11 mM glucose in final concentration and, in the case of B. subtilis, S. marcescens, and A. hydrophila, proline was also added to a final concentration of 87 μM. S. aureus was cultivated in tryptic soy broth (Difco Laboratories, Detroit, Michigan).

The basal salts solutions (200 ml) were dispensed in 1-liter Erlenmeyer flasks and sterilized by autoclaving. Concentrated, filter-sterilized solutions of the carbon sources were added aseptically to the sterilized basal salts solutions. In the case of S. aureus, tryptic soy broth was used instead and the flasks were sterilized by autoclaving. Each flask was inoculated with organisms washed from an agar slant composed of the same medium and incubated on a rotary shaker at 30 or 37°C according to the species being cultivated. The cells were allowed to grow to late exponential phase, harvested by centifuging, washed twice in basal salts solution, and then suspended in the basal salts solution to a density of 1 g wet weight per 20 ml. The suspensions were used directly for transport assays.

Transport Assays

The accumulation of radioactivity from [^{14}C]glucose, [^{14}C]gluconate, and [^{14}C]-proline was determined at 30 or 37°C on a reciprocal shaking water bath. Incubation mixtures were held in 10-ml Erlenmeyer flasks and, in final concentration as appropriate, they consisted of: 0.2 ml of cell suspension, 0.1 mM [^{14}C]glucose (7.88 mCi/mmol), 0.1 mM [^{14}C]gluconate (39 mCi/mmol), 10 μM [^{14}C]proline (20 mCi/mmol), 20 mM ascorbate, 140 μM PMS, and basal salts solution to a final volume of 1 ml. The cells were normally preincubated for 10 min in PMS-ascorbate when these reagents were used.

The reactions were started by the addition of substrate. At time intervals, 50-μl samples were withdrawn and delivered over a membrane filter (Millipore Corporation, Bedford, Massachusetts, 25-mm diameter, 0.45-μm pore size) previously overlayed with 1 ml of 0.1 M LiCl, filtered instantaneously, and immediately washed with an additional 5 ml of 0.1 M LiCl.

The filters bearing the cells were removed immediately from the suction apparatus and transferred to vials containing 10 ml of scintillation fluid, and radioactivity was determined as previously described (3, 6).

Osmotic Shock

Cells of A. hydrophila were harvested, washed, and subjected to the cold osmotic shock procedure as described by Nossal and Heppel (8). Cells of P. aeruginosa were subjected to the cold shock and osmotic shock techniques according to the procedure of Gilleland and Murray (9).

Efflux Experiments

The rate efflux of [^{14}C]glucose from P. aeruginosa PAO 57 was measured according to the technique of Lagarde, Pouysségur, and Stoeber (10).

Protein Determination

Protein was measured by the modified buiret procedure as described by King (11).

Chemicals

[U-^{14}C]Glucose and [U-^{14}C] potassium gluconate were purchased from Amersham-Searle (Arlington Heights, Illinois). [U-^{14}C] Proline was obtained from New England Nuclear Corporation (Boston, Massachusetts). PMS and N,N,N′,N′-tetramethylphenylenediamine dihydrochloride (TMPD) were purchased from the Sigma Chemical Company (St. Louis, Missouri). All other reagents were purchased from commercial sources in the highest state of purity.

The PMS solutions used in these studies were prepared at frequent intervals. They were stored for short intervals at 0–4°C in flasks wrapped in foil to exclude light.

RESULTS

Inhibitory Effect of PMS-Ascorbate on Active Transport Systems

Transport of proline by P. aeruginosa was extensively inhibited by PMS-ascorbate (Fig. 1). Data from several experiments consistently established that both the rate and extent of proline transport by P. aeruginosa was inhibited 85–90% by PMS-ascorbate. In contrast, PMS-ascorbate stimulated active transport of proline by E. coli (data not shown) and the effect ranged from 10% stimulation of the rate of transport to double the rate.

Active transport of proline by E. coli is a shock-resistant system (2) but it has not been established whether P. aeruginosa transports proline by a shock-resistant or shock-sensitive system. Active transport of glucose by P. aeruginosa, on the other hand, apparently is a shock-sensitive system because a glucose-binding protein has been detected (12). and because membrane vesicles prepared from P. aeruginosa were unable to actively transport glucose but they retained the ability to transport gluconate (3,4) and serine (unpublished observations). On the other hand, gluconate transport by P. aeruginosa apparently is shock resistant because membrane vesicles retained the ability to actively transport gluconate (3, 4). When PMS-ascorbate was tested against these 2 transport systems of P. aeruginosa, both glucose and gluconate transport were found to be as extensively inhibited as proline transport. Results for the PMS-ascorbate inhibition of glucose transport are

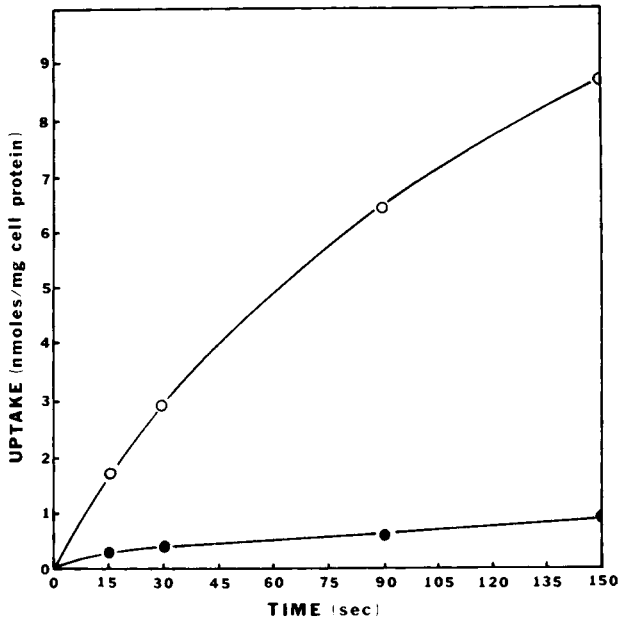

Fig. 1. Uptake of [^{14}C] proline by P. aeruginosa PAO in the absence (open circles) and presence (filled circles) of PMS-ascorbate.

shown in Fig. 2. Thus, both shock-resistant and shock-sensitive transport systems of P. aeruginosa were clearly inhibited by PMS-ascorbate.

Data in Fig. 2. also show that ascorbate had no effect on glucose transport by P. aeruginosa, that PMS alone inhibited glucose transport by about 50%, and that the combination of PMS-ascorbate was the most effective inhibitor. We interpreted these data to indicate that the reduced form of PMS was responsible for the inhibitory effect. We envision that cellular metabolites could reduce PMS when the latter was used alone, but PMS was reduced more effectively when ascorbate was added to the system.

The effect of PMS-ascorbate on the active transport of proline and glucose by a variety of bacterial species is shown in Table I. PMS-ascorbate inhibited proline transport by all species tested except E. coli and S. marcescens Sm39, a nonpigmented isolate. Glucose transport was inhibited in all species tested. These results are especially interesting because PMS-ascorbate has been demonstrated to energize active transport by membrane vesicles prepared from many of these same microorganisms [e.g., P. aeruginosa (3, 4), P. putida, E. coli, S. typhimurium, B. subtilis, and S. aureus (5)].

Inhibitory Concentration of PMS

While maintaining ascorbate at 20 mM, various concentrations of PMS were tested to determine the minimal level required to inhibit active transport of glucose by P. aeruginosa. The experimental results indicated that as little as 7 μM PMS had a pronounced inhibitory effect while the maximal inhibitory effect of PMS was attained at 40 μM (data not shown).

Inhibitory Effect of TMPD

Experiments were done to determine whether PMS could be replaced by TMPD, an alternate artificial electron carrier. It was found that TMPD-ascorbate inhibited glucose transport by P. aeruginosa, but a higher concentration of TMPD than of PMS was required for maximal inhibition (300 μM TMPD vs 40 μM PMS), and TMPD-ascorbate did not inhibit the rate and extent of glucose transport as effectively as PMS-ascorbate (data not shown).

Immediate Inhibitory Effect of PMS-Ascorbate

Incubation mixtures normally were preincubated with PMS-ascorbate for 10 min prior to starting the reaction by the addition of labeled substrate. In order to determine whether the inhibitory effect of PMS-ascorbate was immediate or delayed, we added PMS-ascorbate to incubation mixtures at time zero and after 30 sec of incubation. The results, which are shown in Fig. 3, indicated that the inhibitory effect of PMS-ascorbate was immediate, i.e., no demonstrable lag in inhibition was noted from the time of addition of PMS-ascorbate until the onset of inhibition.

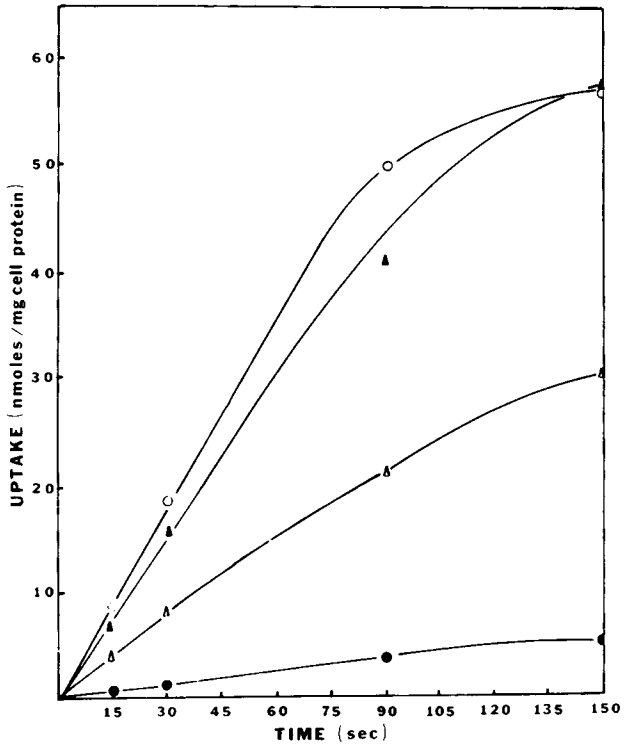

Fig. 2. Uptake of [^{14}C]glucose by P. aeruginosa PAO in the absence and in the presence of PMS, of ascorbate, and of PMS-ascorbate. ○) No additions; ▲) 20 mM ascorbate; △) 140 μM PMS; ●) 140 μM PMS plus 20 mM ascorbate.

TABLE I. Effect of PMS-Ascorbate on Transport of Glucose and Proline in Several Bacterial Species[a]

Microorganism	Percent inhibition			
	Rate of transport		Total uptake	
	Proline	Glucose	Proline	Glucose
P. aeruginosa PAO	85	90	89	94
P. aeruginosa OSU 64	ND	89	ND	87
P. aeruginosa A1466	ND	82	ND	74
P. putida ATTCC 12633	ND	88	ND	89
P. fluorescens ATCC 13525	ND	65	ND	90
E. coli ML 308-225	Stimulation	50	Stimulation	50
S. marcescens Sm7 (pigmented)	58	ND	42	ND
S. marcescens Sm39 (nonpigmented)	0	ND	15	ND
A. hydrophila (aerogenic)	97	ND	98	ND
A. hydrophila (anaerogenic)	91	ND	98	ND
E. aerogenes UGA	50	ND	52	ND
B. subtilis SP491	57	ND	55	ND
S. aureus 832S-7	43	ND	57	ND

[a]Rate of transport was determined from the linear portion of the uptake curve while total uptake represents the steady state level. ND – not done.

Effect of PMS-Ascorbate on Growth of P. aeruginosa

When PMS and ascorbate (final concentration 14 μM and 20 mM, respectively) were added to the culture medium, a marked increase in the lag phase was observed (Fig. 4). There was also a slightly reduced growth rate after growth was initiated. These data are consistent with the observation that glucose uptake is inhibited by PMS-ascorbate. The results also suggest that growth occurred after the ascorbate had been oxidized and/or after the PMS had been degraded. Moreover, when the concentration of PMS in the culture medium was increased 10-fold, no growth occurred.

Effect of PMS-Ascorbate on Kinetics of Glucose Transport

The apparent K_m for glucose transport by P. aeruginosa PAO 57 in the absence of PMS-ascorbate was 40 μM (Fig. 5). In the presence of PMS-ascorbate, however, the apparent K_m was increased to 25 mM (Fig. 6). The nearly 2,000-fold increase in K_m as a direct effect of PMS-ascorbate suggests that, in the presence of this artificial electron donor system, the active transport system for glucose was changed to a facilitated diffusion system.

Lack of Effect by PMS-Ascorbate on Glucose Efflux

Using P. aeruginosa PAO 57, the rate of efflux of glucose from preloaded cells was measured in the presence and absence of PMS-ascorbate. The rate of efflux of glucose was identical in the 2 systems (data not shown). Thus, since PMS-ascorbate did not effect the rate of efflux of glucose, this indicated that PMS-ascorbate did not alter the permeability of the cytoplasmic membrane to glucose nor change the affinity of the carrier for glucose on the inside of the cytoplasmic membrane. These data are also consistent with the observation that facilitated diffusion resulted on exposure to PMS-ascorbate.

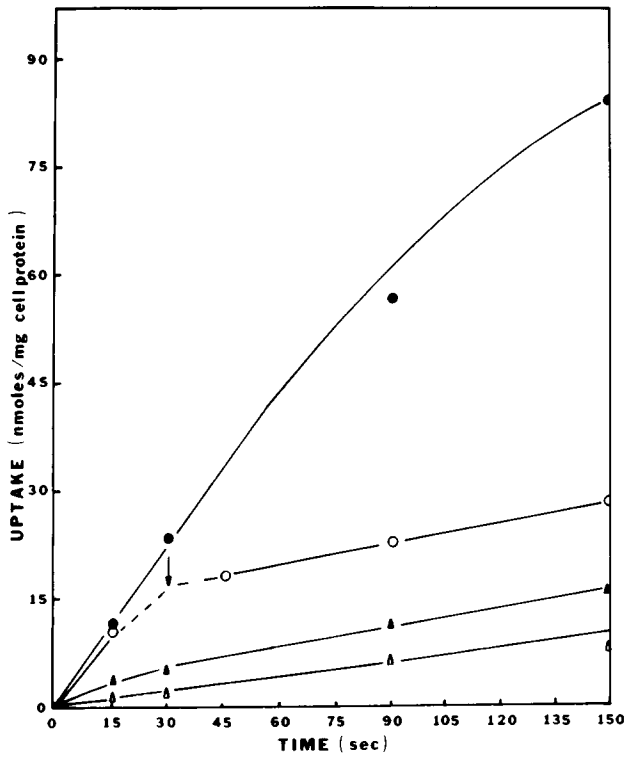

Fig. 3. The immediate inhibitory effect of PMS-ascorbate on the uptake of [^{14}C]glucose by P. aeruginosa PAO. •) No additions; △) cells were incubated with PMS-ascorbate for 10 min prior to starting the reaction by addition of substrate; ▲) PMS-ascorbate was added to the cells simultaneously with substrate to start the reaction; ○) PMS-ascorbate was added 30 sec after the start of the reaction as indicated by the arrow.

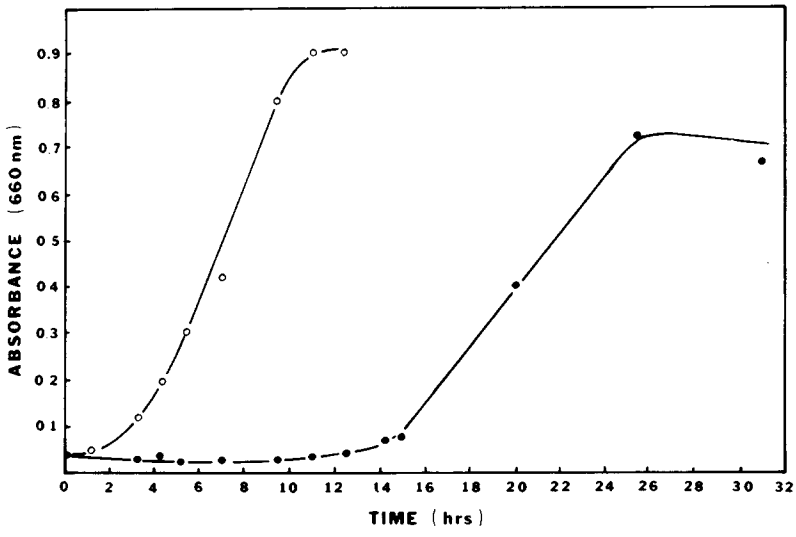

Fig. 4. Growth of P. aeruginosa PAO in the absence (open circles) and in the presence (filled circles) of PMS-ascorbate.

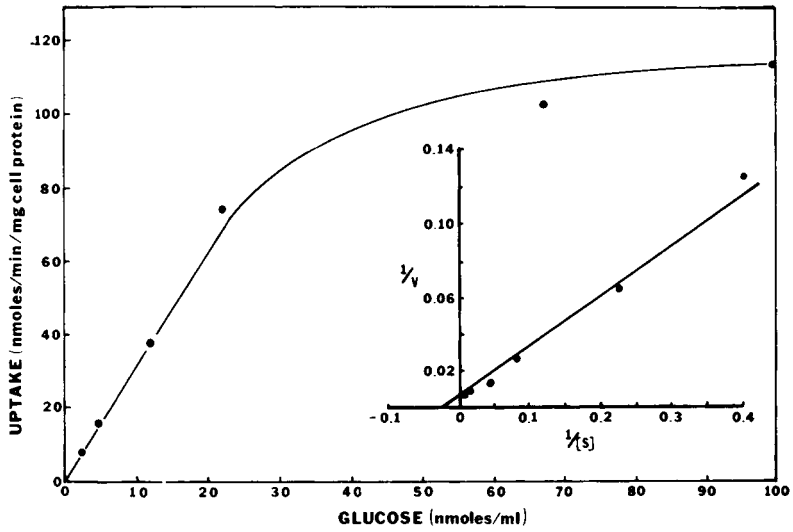

Fig. 5. Kinetics of [^{14}C]glucose uptake in the absence of PMS-ascorbate by P. aeruginosa PAO 57 as a function of exogenous substrate concentration. The reactions were initiated by the addition of cells. Initial rates were approximated by measuring glucose uptake after incubation for 15 sec. The inset is a Lineweaver-Burk plot of the kinetic data.

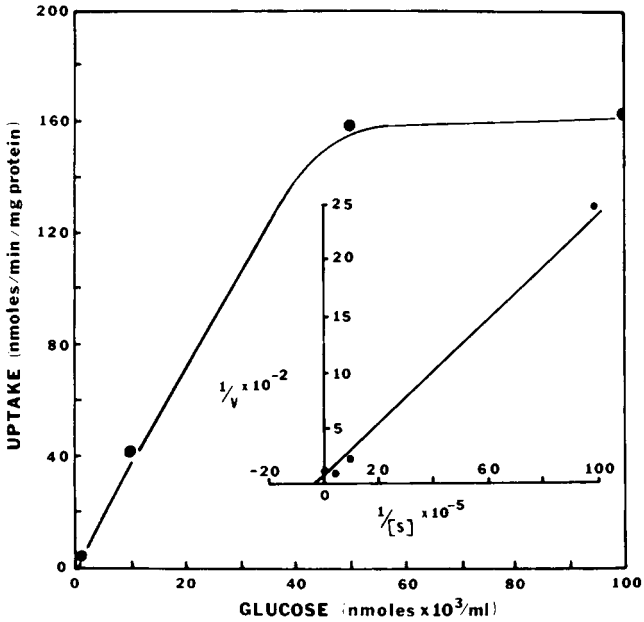

Fig. 6. Kinetics of [^{14}C]glucose uptake in the presence of PMS-ascorbate by P. aeruginosa PAO 57 as a function of exogenous substrate concentration. The reactions were initiated by addition of cells. Initial rates were approximated by measuring glucose uptake after incubation for 15 sec. The inset is

Osmotic Shock Experiments

Active transport by membrane vesicles prepared from P. aeruginosa (3, 4) and from a wide variety of other bacteria (5) is energized by PMS-ascorbate. Thus, in order to determine whether there was a shock-sensitive component responsible for PMS-ascorbate sensitivity, we subjected A. hydrophila to the cold osmotic procedure as described by Nossal and Heppel (8), and P. aeruginosa was subjected to the cold shock and osmotic shock procedures reported by Gilleland and Murray (9). These shocked bacteria exhibited unchanged sensitivity to PMS-ascorbate with respect to proline transport (data not shown), indicating that PMS-ascorbate sensitivity was not due to a shock releasable component.

Effect of PMS-Ascorbate on the Phosphoenopyruvate (PEP) Phosphotransferase System

A curious phenomenon is that PMS-ascorbate inhibited glucose uptake by E. coli (Table I, Fig. 7). Glucose is taken up by E. coli by group translocation via the PEP phosphotransferase system and not by active transport (13). Thus, PMS-ascorbate inhibition of this system was unexpected. We interpreted these data, however, to indicate that PMS-ascorbate stimulated respiration and that the PEP:glucose phosphotransferase system was inhibited by respiration. Other authors similarly have concluded that respiration, or an energized state of the membrane, inhibits glucoside transport by E. coli (14–16).

DISCUSSION

Our experimental results showed that the artificial electron donor system of PMS-ascorbate (and of TMPD-ascorbate as well) inhibited active transport of solutes in a variety of bacterial species. E. coli was a notable exception since PMS-ascorbate stimulated uptake

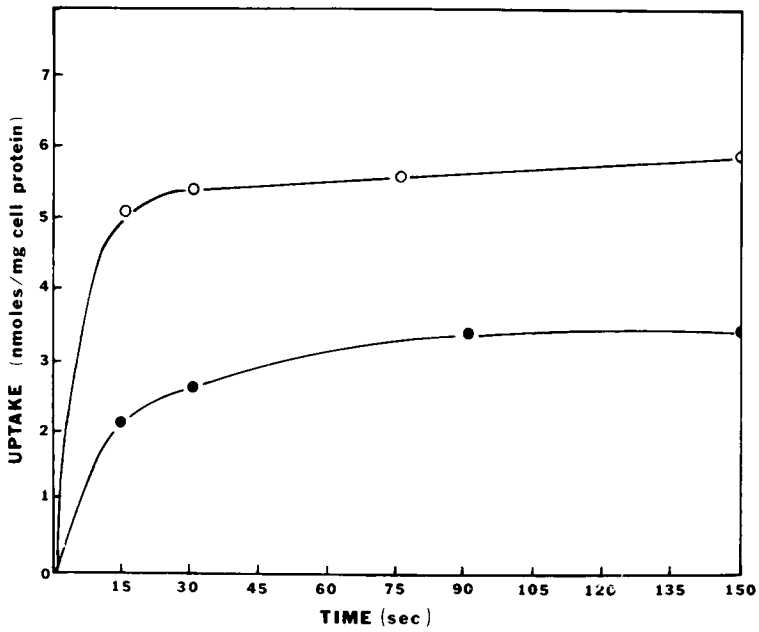

Fig. 7. Uptake of [^{14}C]glucose by E. coli ML 308-225 in the absence (open circles) and presence (filled circles) of PMS-ascorbate. Chloramphenicol (80 μg/ml) was added to the reaction mixtures.

of proline instead. Similarly, other authors have reported that glutamine and serine uptake by cells of E. coli was also stimulated by PMS-ascorbate (2). Why active transport systems in cells of E. coli should respond differently than other bacterial species is not immediately clear.

Another curious phenomenon which became evident from these studies was that intact cells responded differently to the artificial electron donor system, PMS-ascorbate, than membrane vesicles isolated from the same bacterial species (3, 5, 17, 18), and to TMPD-ascorbate, which has been shown to be a satisfactory substitute for PMS (19, 20). Thus, on the one hand, PMS-ascorbate energizes active transport in isolated membrane vesicles while, on the other hand, PMS-ascorbate inhibits active transport by intact cells from which the membrane vesicles were prepared.

Other workers have noted that exogenous electron donors such as pyruvate, succinate, and glycerol (14, 15) and D-lactate (16) inhibited α-methylglucoside (α-MG) uptake by cells of E. coli. α-MG is taken up in E. coli by group translocation via the PEP:glucose phosphotransferase system and not by active transport (13). It was concluded that α-MG uptake was inhibited by the energized membrane state resulting from electron flow (14–16). One group of workers argued against a role of ATP in formation of the energized membrane state (14, 15) while other workers presented evidence that the energized membrane state could also be formed from adenosine triphosphate (ATP) hydrolysis (16). The latter conclusion was based on the observation that galactose inhibited α-MG uptake in E. coli, presumably by formation of ATP via substrate-level phosphorylation. Be that as it may, it is probably that PMS-ascorbate inhibited glucose uptake by E. coli in a manner analogous to the inhibition of α-MG uptake which resulted form the presence of exogenous natural electron donors as reported by other workers (14–16).

By analogy to the inhibition of the E. coli PEP:glucose phosphotransferase system by the energized membrane as discussed above, at first thought it might be suspected that PMS-ascorbate, or TMPD-ascorbate, inhibited active transport of external solutes in P. aeruginosa and other species by formation of an energized membrane state. We think that this cannot be the case, however, because according to current concepts active transport is driven by the energized membrane state (19, 21–24). Thus, it is not likely that the energized membrane could function both to energize and to inhibit active transport.

Similarly, we do not believe that PMS-ascorbate had a specific effect on specific protein carriers because of the generalized effect of electron donors other than PMS ascorbate (i.e., TMPD-ascorbate), and because transport mechanisms in general were inhibited in intact cells by these exogenous electron donors.

It has been shown by Ramos, Schuldiner, and Kaback (23) that at pH 7.5 the electrochemical potential of protons across the membrane of isolated vesicles of E. coli was due almost solely to the electric potential across the membrane and that internal pH was nearly equal to external pH. Thus, since our transport measurements were conducted at pH 7, we presume that the various effects that we noted were due chiefly to the electric potential (or perhaps to its absence in the presence of PMS-ascorbate) across the cytoplasmic membrane and not to a pH gradient.

A distinct possibility in explanation of our observations is that PMS-ascorbate may have fed electrons into the electron transport chain distal to an energy-coupling site for active transport which has been postulated by Kaback and co-workers (24). Electron donors that have been shown to energize active transport in membrane vesicles characteristically donate electrons to the electron transport chain at the cytochrome b or cytochrome c level (3, 17, 19, 20). Thus, if PMS-ascorbate donated electrons distal to the energy-coupling site for active transport, or distal to the terminal energy conservation site, it can

be envisioned that the flow of electrons anterior to this site(s) would be impeded. This, in turn, would prevent the formation of the energized membrane and active transport, but not facilitated diffusion, would be inhibited. In support of this hypothesis is our recent observation that PMS-ascorbate has an uncoupler-like effect on P. aeruginosa because, upon exposure to PMS-ascorbate, there was a rapid depletion of intracellular ATP (unpublished preliminary observations).

The difference in response between intact cells and membrane vesicles of P. aeruginosa and other species to PMS-ascorbate cannot be explained at the present time. [The exception is E. coli since intact cells and membrane vesicles appear to respond similarly to exogenous electron donors and membrane vesicles are considered to retain the same orientation as the membrane in the intact cell (23, 24).] PMS-ascorbate, however, energizes active transport in isolated membrane vesicles, and the energy-coupling sites and components of the electron transport chain would be expected to be the same in membrane vesicles as in intact cells. Membrane vesicles, however, are perturbed systems. The possibility exists therefore, that certain electron transport components that are involved in the PMS-ascorbate effect are lost when membrane vesicles are prepared; or, that the electron transport components in isolated membrane vesicles are perturbed in such a manner that the effects of PMS-ascorbate are overcome. An explanation will have to await further experimentation to compare such factors as the electron transport components of intact cells vs membrane vesicles. Similarly, comparative studies between E. coli and other species, such as P. aeruginosa, will also be required.

ACKNOWLEDGMENTS

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