L-SERINE TRANSPORT IN MEMBRANE VESICLES OF *BACILLUS SUBTILIS* ENERGIZED BY NADH OR REDUCED PHENAZINE METHOSULFATE

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1. Introduction

In a recent report, Kaback and Milner [1] showed that the transport of amino acids into membrane vesicles of *Escherichia coli* could be energized by D-lactate, whereas NADH was five times less effective. This result was surprising because NADH is necessary for any growing cell, whereas lactate, which was not added to the medium, usually is a fermentation product. We have observed a similar effect of L- (rather than D-) lactate in membrane vesicles of *Bacillus subtilis*, in which NADH showed only a transient and succinate almost no effect. However, we shall show that L-serine transport can be maintained at a high rate by NADH if oxygen, which is rapidly utilized by NADH-oxidase, is continuously supplied. Succinate can also energize if phenazine methosulfate (PMS) is added to couple succinic dehydrogenase to the electron transport chain.

2. Materials and methods

Growth: *B. subtilis* strain 60015 (requiring indole and methionine) was grown at 37° in 14 l medium containing per liter 14 g K_2HPO_4, 6 g KH_2PO_4, 0.25 g $MgSO_4 \times 7\ H_2O$, 2 g $(NH_4)_2SO_4$, 1 g ammonium citrate, 25 mg L-tryptophan, 1 g vitamin-free casein hydrolysate (Nutritional Biochemicals, Cleveland, Ohio) and 10 g D-glucose. The cells were harvested during exponential growth. Washed cells were suspended (80 ml/g wet weight) in 0.1 M K phosphate, pH 7.3, plus 0.5 M sucrose and incubated at 33° in the presence of 250 μg/ml lysozyme for about 45 min (continued for 15 min after at least 95% of the cells were spheroplasts). Membrane vesicles were prepared according to Kaback [2], suspended in 0.1 M K phosphate, pH 6.6, at 6–8 mg/ml, distributed in 2 ml aliquots into thin-walled plastic tubes and stored in liquid nitrogen. All experiments were performed within 2 hr after thawing.

Amino acid uptake into membrane vesicles: Per 50 μl membrane preparation, 20 μl water and 10 μl 0.1 M $MgSO_4$ were added and the mixture was incubated for 15 min at the temperature stated. Following this preincubation, ^{14}C-L-serine (128 mCi/mmole, 15.6 μM in the final reaction mixture) and the electron donors (20 mM) were rapidly added in this sequence. In the experiments using special oxygenation the tube contents were rapidly stirred by 7 mm long Teflon-coated magnetic stirring bars. Following addition of the electron donor, water-saturated oxygen was blown over the mixture. The reaction was terminated by the addition of 2 ml 0.1 M LiCl and filtered (Millipore filter HAWP, 25 mm diameter). The filters were washed with 2 ml 0.1 M LiCl, dried on planchets for 10 min at 105° and counted in a gas flow counter. For all experimental points the value obtained for zero time incubation (LiCl added before serine) was subtracted.

3. Results and discussion

The incorporation of ^{14}C-L-serine into membrane vesicles of *B. subtilis* was first measured in reaction mixtures standing in a water bath. With a temperature optimum of 33°, L-lactate produced the highest up-

take, which was sustained for 10 min or longer (fig. 1). NADH also produced a significant uptake but only for 1–2 min; after that time the uptake rate declined drastically. 5 min preincubation of the membranes with NADH prevented L-lactate from stimulating serine uptake (fig. 1). This deficiency resulted from the exhaustion of oxygen. Measurements of NADH-oxidase showed that the membrane preparations typically used for amino acid incorporation experiments would reduce the concentration of dissolved oxygen by 90% in 5 sec (table 1). Consequently, serine uptake energized by NADH, was measured under conditions of increased oxygen supply (fig. 2). The uptake continued at a high rate for about 8 min, at which time NADH was depleted (table 1). Thereafter, a rapid efflux of serine from the membrane vesicles took place at a rate of about 8%/min. When the amount of enzyme (membrane) was halved, the rate of serine

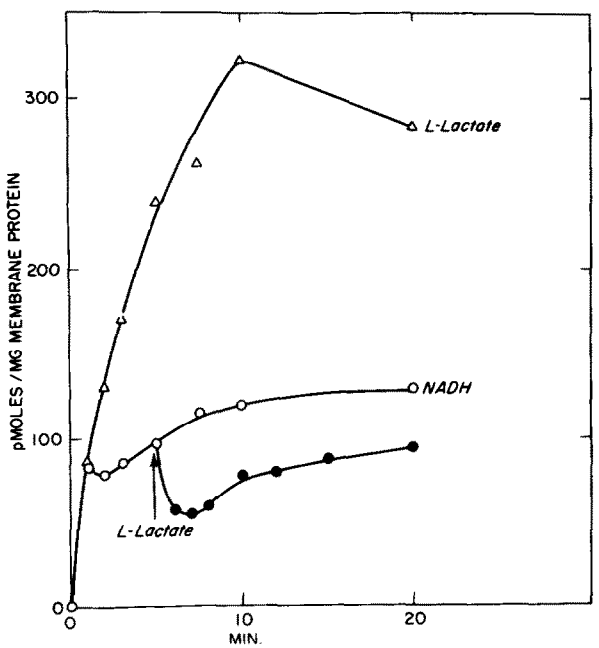

Fig. 1. Serine uptake at 33° into membrane vesicles (3 mg protein/ml) of *B. subtilis* 60015 in the presence of 10 mM Li-L(+)lactate (△), 20 mM NADH (○), and Li-L(+)lactate after 5 min incubation with NADH (●). The reaction mixtures of 800 μl stood in tubes in a 33° water bath. 100 μl samples were assayed as described in Materials and methods. Without energy source serine was taken up at a rate of less than 2 pmoles/min/mg.

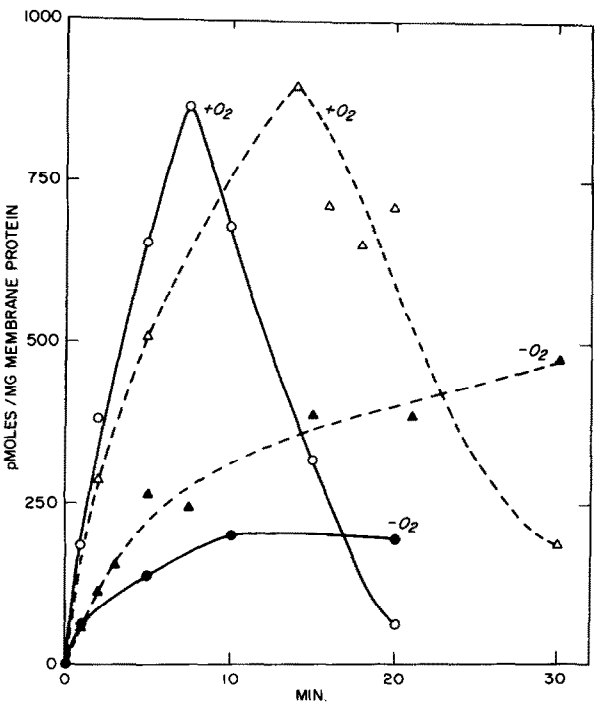

Fig. 2. Serine uptake into membrane vesicles in the presence of 20 mM NADH at 25°. The reaction mixtures of 100 μl were incubated in 10 mm internal diameter tubes standing at 25° (full symbols) or in 11 mm internal diameter tubes each containing a rapidly moving magnetic stirring bar and a tubing blowing oxygen over the mixture (open symbols). ○ and ● = 3 mg membrane protein/ml. △ and ▲ = 1.5 mg membrane protein/ml. The values without oxygenation were higher than in fig. 1 because the tubes contained a smaller liquid volume, allowing maintenance of a higher oxygen supply by diffusion.

uptake was reduced by almost one-half but the reaction lasted twice as long, indicating a longer availability of NADH (fig. 2). NADPH energized L-serine incorporation at about the same rate as NADH.

It is apparent that NADH or NADPH energized serine uptake at a higher rate than L-lactate, provided that oxygen was maintained at a high concentration. This result, which also holds for other amino acids, places the proper emphasis on the important pyridine coenzymes rather than lactate. It will be interesting to see whether also in other microorganisms, e.g. *E. coli*, special oxygenation can maintain amino acid incorporation in the presence of NADH at a higher rate in the presence of lactate.

Table 1
Specific activity of membrane-bound enzymes or enzyme systems.

Enzyme	Specific activity (nmoles/mg membrane protein/min)	Time* required for 90% oxygen consumption (sec)	Time* required for Substrate oxidation (min)
Succinate dehydrogenase	216	21	31
NADH oxidase	1042	4.3	6.4
NADPH oxidase	1042	4.3	6.4
Ascorbate-PMS oxidase	1111	3.8**	5.7**

Oxygen consumption was measured polarographically in a 2 ml reaction chamber at 25° with 20 mM substrate in 0.05 M K phosphate, pH 6.6, and with 200 μM PMS for ascorbate.

* Calculated from the specific activity in column 1 for a reaction mixture containing 3 mg membrane protein/ml and 20 mM substrate. The initial O_2 concentration was 258 μM; the substrate oxidation data was obtained under conditions of special oxygenation (see Materials and methods).

** Nonenzymic (167 nmoles/min) and enzymic reaction rates added.

Succinate energized L-serine transport only at a low rate (fig. 3) which could not be enhanced by oxygenation. Succinic dehydrogenase, which had a high activity, apparently was only loosely coupled to the electron transport chain because almost no succinic oxidase activity was found (table 1). Artificial coupling could be achieved by the addition of PMS (100 μM), which in the presence of 20 mM succinate strongly stimulated L-serine uptake. Addition of PMS 5 min after succinate also stimulated L-serine uptake, which proves that succinate (in contrast to NADH) did not exhaust a compound needed for transport. In the

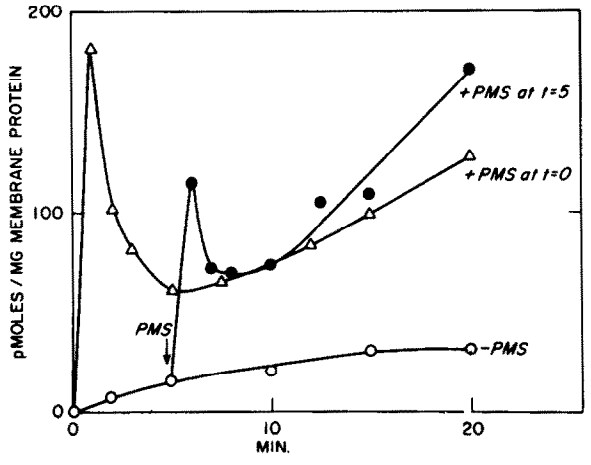

Fig. 3. Serine uptake at 25° into membrane vesicles (3 mg protein/ml) in the presence of 20 mM Na_2 succinate (○), Na_2 succinate + 100 μl PMS added at t = 0 (△) and Na_2 succinate with PMS added at t = 5 min (●). Conditions as in fig. 1.

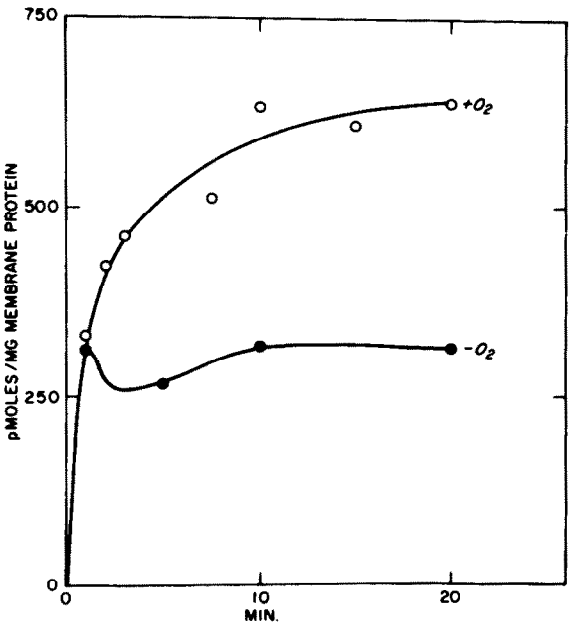

Fig. 4. Serine uptake into membrane vesicles (3 mg protein/ml) in the presence of 20 mM succinate and 200 μM PMS at 25°. For conditions, symbols and remarks see fig. 2.

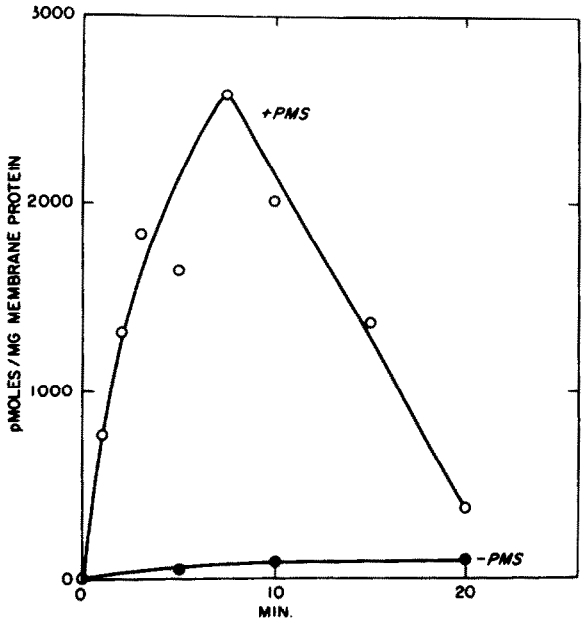

Fig. 5. Serine uptake at 25° into membrane vesicles (3 mg protein/ml) in the presence of 20 mM Na-ascorbate (•) and Na-ascorbate + 100 μM PMS (○). 100 μl reaction mixtures were incubated with special oxygenation as in fig. 2.

presence of ascorbate and PMS, oxygen was used up very rapidly (table 1), resulting in a decline of serine uptake after one min. With special oxygenation a high uptake rate could be maintained; later a high steady state level was obtained (fig. 4) because succinate was not used up as rapidly as NADH (table 1).

L-Serine uptake could also be energized by PMS that had been non-enzymatically reduced by ascorbate (fig. 5). The effect of L-acetate, however, could not be potentiated by PMS.

Acknowledgements

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References

[1] H.R.Kaback and L.Milner, Proc. Natl. Acad. Sci. U.S. 66 (1970) 1008.
[2] H.R.Kaback, in: Methods in Enzymology, ed. W.B.Jakoby (Academic Press, New York), in press.