ASPARTATE TRANSPORT IN BACTERIUM CADAVERIS WITH SPECIAL REFERENCE TO THE PHOSPHATE AGING EFFECT

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Few bacterial enzymes have been the subject of as much contention with regard to cofactor requirements as has aspartase. Since Gale’s (1938) proposal of the two aspartases, it has become increasingly clear that much of the difficulty has arisen from the extrapolation of resting cell effects, based on ammonia data, to cell-free enzyme. The demonstration by Smith and Lichstein (1954) and by Trudinger (1951, 1954, 1955) that a miscellany of cofactors including glucose, adenosine, and inorganic salts were producing membrane permeability effects emphasized the need for more definitive studies on resting cells. Conspicuously absent have been experiments in which uptake of substrate was measured, or attempts made to examine an uptake system divorced from the deaminase. It has been assumed unjustifiably that ammonia production by resting cells was a true index of intracellular aspartase activity. This fallacy has been pointed out by Ellfolk (1956) and emphasized by recent experimental findings (Williams, 1957).

Clarification of the role of aspartase cofactors of indeterminate status would be significantly advanced by thoroughly investigating transport in resting cells. Of these remaining substances, biotin is by far the most important and most intriguing. The acid-hydrolyzed glucose factor reported by Christman and Williams (1952) and Williams and Christman (1953) was found on analysis to be a mixture of succinic and formic acids, which did not stimulate the cell-free enzyme.

The case for biotin rests almost entirely on the phosphate aging technique as developed by Lichstein and Umbreit (1947), although Smith and Lichstein (1954) have also presented some data for dried cells. An unequivocal demonstration of a cofactor role for biotin with cell-free enzyme, either crude or purified, has not been made despite intensive effort in this laboratory (Williams and McIntyre, 1955a) and others.

Phosphate aging is an empirical treatment of washed cells with molar primary phosphate which appears to render certain of their enzymes sensitive to specific stimulants. It has been proposed that phosphate aging effects the resolution of certain intracellular enzymes but this has never been proved for aspartase.

The purpose of the present communication was to (a) investigate the nature of phosphate aging in the resting cell, (b) study the transport of aspartate by resting cell preparations, and (c) discuss the interpretation of existing data in the light of this information.

MATERIALS AND METHODS

Organism. The strain of Bacterium cadaveris used is apparently identical with B. cadaveris strain ATCC 9760.

Media and cultural conditions. B. cadaveris was grown aerobically at 30°C on a medium consisting of 1 per cent yeast extract, 1 per cent tryptone, and 0.5 per cent KH2PO4. Cells were harvested and washed once with distilled water preparatory to resting cell experiments.

Phosphate aging technique. Cells were phosphate aged by standing in molar phosphate, pH 4.0, at room temperature for 30 min to 1 hr. Care was taken to adjust buffer systems to the same pH and ionic strength for phosphate aged and control cells at the time of incubation with substrate. Phosphate aged and control cells were always prepared from the same flask of growing culture and always diluted to the same cell density.

Analyses for substrates and products. (a) Aspartic acid was determined with ninhydrin by a modification of the method of Troll and Cannan (1953). The pH was buffered to 5.0 so that ammonia would not interfere in the determination. (b) Adenosine was determined by measuring its absorbancy at 262.5 mμ in the Beckman model DU spectrophotometer. Before analysis, adenosine was separated from its reaction products by one-dimensional paper chromatography as de-
scribed by Williams and McIntyre (1955b). (c) Ammonia was determined by Nesslerization and colorimetry in a Klett-Summerson colorimeter equipped with a no. 50 filter.

Resting cell experiments. Resting cell studies were performed in air at 37 C in 0.05 m phosphate buffer, pH 7.0 or 6.8. The usual final substrate concentration was either 0.001 or 0.002 m. Cell densities were always carefully equated. The reaction was stopped at the desired time by adding 1/20 volume of N HCl, rapidly chilling the tubes in an ice bath, followed by centrifugation and decanting the supernatant solution into clean tubes for analysis. The data presented were all determined from analyses of such supernatant solutions. Preliminary experiments established that there was little or no diffusion of substrate or interfering materials from the suspended cells. Since neither aspartic acid nor adenosine accumulates in the cells (as will be shown) the problem of diffusion from inactivated cells could be dismissed.

L-threo-β-Methylaspartic acid was supplied through the generosity of Dr. H. Albert Barker.

**EXPERIMENTAL RESULTS**

Phosphate aging studies. Figure 1 gives both aspartic acid and ammonia data for supernatant solutions obtained from resting cell experiments with normal and phosphate aged cells. These data reveal the dramatic effect of the aging treatment on substrate uptake. This finding was in accord with the previous report of this laboratory that severe phosphate aging rendered *B. cadaveris* completely cryptic to aspartate but did not destroy the intracellular aspartase (Williams, 1957). Figure 1 also shows that the aspartate may be accounted for as either unused substrate or product at any stage in the experiment. Aspartate, therefore, does not accumulate in the cells and the rate of deamination is at least equal to the rate of uptake.

A kinetic analysis of substrate uptake by resting cells would be based in part on the usual velocity-concentration curves. Figure 2 shows a comparison of such data for control and phosphate aged cells examined under otherwise identical conditions. These rates were calculated from uptake of substrate from the incubation mixtures. Whereas the control cells display diphasic kinetics, the curve for phosphate aged cells is atypical and shows a different type of response of the cells to increased concentration of substrate. An interpretation of these findings will be offered in the discussion.

The fact that phosphate aging reduces the ability of the resting cell to take up substrate does not explain the nature of phosphate aging. The deleterious effect of dilute solutions of phos-
phosphate was studied by Goucher et al. (1955), who presented data to support their interpretation that magnesium deprivation was responsible for the diminished oxidative activity of Azotobacter agilis. More directly pertinent is the report by Smith and Lichstein (1954) that phosphate aged B. cadaveris is stimulated by glucose. It seemed desirable, therefore, to examine phosphate aged cells in the presence of both glucose and magnesium. Freshly harvested cells were washed once and divided into two equal parts. One portion of the cells was phosphate aged as described earlier and the second portion was suspended in distilled water and allowed to stand at room temperature for the same length of time. At the end of the aging period the cells were added to tubes containing the desired additions with the exception of substrate. The additions were made so as to adjust all tubes to the same pH and ionic strength. A recovery period of 45 min was allowed so that the aged cells might have adequate time to adjust to a pH of 6.8 before the addition of substrate. It was realized at the time that the control cells would show deterioration from the 30-min water treatment and subsequent 45-min phosphate buffer treatment, the water-aging effect having been described and studied by Gale (1938) and the phosphate buffer effect having been described by Goucher et al. (1955). The deamination was carried out at pH 6.8 with the following concentrations of additions: magnesium sulfate, 0.002 M; glucose, 0.0005 M. Figure 3 shows the effect of magnesium, glucose, and the two combined on phosphate aged cells as compared with the control cells to which no stimulants were added. Figure 7 gives the data for the effect of glucose and magnesium on the control cells (which served for a parallel experiment on 2,4-dichlorophenoxyacetic acid to be described later). The effect of magnesium and glucose on the control cells was not included in figure 3 because it was readily discernible from figure 7. The data presented in figures 3 and 7 were calculated from both ammonia and aspartic acid determinations to insure that substrate uptake was being measured. A comparison of the control cells with the phosphate aged cells reveals that both have undergone deterioration, the difference appearing to be one of degree and not kind. With phosphate aged cells, magnesium was slightly effective, glucose quite effective, and the combination more than additive in restoring the ability of the cells to take up the substrate. Full restoration of activity was not obtained, however,
aging affects cellular permeability another enzyme system was examined. The adenosine deaminase of *B. cadaveris* was chosen because it has been studied previously (Williams and McIntyre, 1955b) and has never been associated with a biotin requirement, as have certain other deaminases. The phosphate aging and resting cell experiments were performed exactly as for aspartase and the results are shown in figure 4. Phosphate aging proved to be highly deleterious to adenosine uptake. Ammonia data are also given to show that adenosine does not accumulate in the cells. These experiments are being extended to other enzyme systems to determine the scope of the aging effect.

**Aspartate transport system.** The phosphate aging experiments had focused our attention on uptake of substrate by the cells and pointed up the necessity of obtaining more information about whatever mechanism might be involved. Experiments were performed, therefore, to de-

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**Figure 5.** Substrate specificity of the aspartate transport system in *Bacterium cadaveris*.

**Figure 6.** Effect of 0.004 M 2,4-dichlorophenoxyacetic acid on aspartic acid uptake in *Bacterium cadaveris*.

**Figure 7.** Effect of glucose and magnesium on aspartic acid uptake by normal and 2,4-dichlorophenoxyacetic acid (2,4-D) inhibited cells of *Bacterium cadaveris*. 

but simply to the level of the control cells having no additions. With the control cells (figure 7) magnesium was more effective than glucose and the combination roughly additive.

To strengthen the contention that phosphate
termine substrate specificity and to study the action of an inhibitor, 2,4-dichlorophenoxyacetic acid.

(1) Specificity of the transport system for substrate.—The specificity of cell-free aspartase for L-aspartate is well established (Williams and McIntyre, 1955a; Ellfolk, 1956). Figure 5 shows the parallel specificity of B. cadaveris resting cells in the transport or uptake of substrate. Whereas the concentrations of D-aspartate, L-glutamate, and β-methylaspartate remain essentially constant in their respective incubation mixtures, L-aspartate rapidly disappears. It is unfortunate that the system does not respond to β-methylaspartate as it does to L-aspartate since this would have provided a means of studying the transport system independently of the deaminase. It is assumed that the curve for L-aspartate would be essentially the same even if the intracellular deaminase were inactive. These findings will be discussed in connection with the kinetic study.

(2) Inhibition by 2,4-dichlorophenoxyacetic acid (2,4-D):—The inhibiting effect of 2,4-D on permeases is well established (Cohen and Monod, 1957), although the mode of action of 2,4-D in these systems is not precisely known. It has been variously described as a phosphorylation uncoupler and as an essential metal ion chelating agent. Despite the fact that the mechanism whereby B. cadaveris resting cells take up aspartate is not known, it was felt worthwhile to determine whether or not the mechanism was sensitive to 2,4-D. Preliminary experiments in this laboratory showed that 0.004 m 2,4-D was without inhibitory action on cell-free aspartase.

Inhibition of aspartate transport. The substrate uptake process in resting cells, however, was found to be strongly inhibited by 2,4-D (figure 6). From these data it may be seen that aspartate uptake is inhibited 50 to 70 per cent.

Johnson and Colmer (1957) successfully reversed 2,4-D inhibition of carbohydrate metabolism in Azotobacter with stoichiometric quantities of magnesium and calcium ions. For the 2,4-D inhibition of aspartate uptake, however, we found these additions (0.002m) to be ineffective, even when the cells were preincubated with the metal salts and the 2,4-D added 30 min after the addition of substrate. The preincubated cells were no more resistant to the immediate inhibitory action of 2,4-D than were the control cells. Negative findings were also obtained for the addition of biotin, adenosine triphosphate, formate, adenosine monophosphate, and combinations of these. The fact that biotin does stimulate phosphate aged cells suggests that 2,4-D inhibition of transport may be different from phosphate inhibition. The data presented in figure 7 show, however, that 2,4-D inhibited cells respond to glucose and the combination of glucose and magnesium in a manner similar to that exhibited by phosphate aged cells (figure 3). As has already been stated, the experiments shown in figures 3 and 7 were performed with the same set of controls so as to be fully comparable. The final concentration of 2,4-D was 0.004 m and as previously given for glucose and magnesium. As with phosphate aged cells, the major effect is from glucose. It is of interest to note that with the “water aged” control cells magnesium alone was more effective than glucose in restoring activity. In all cases, the combination of glucose and magnesium was at least additive and usually more so.

**DISCUSSION**

Phosphate aging. Regardless of what other effects may be produced by the phosphate aging technique, the data presented in figures 1, 3, and 4 show that there is a primary and severe effect on the uptake of substrate by resting cells. This effect is not restricted to aspartate uptake or to systems which respond to biotin following phosphate aging, but can be demonstrated for an enzyme such as adenosine deaminase and probably others. This is an area in which additional investigation will be carried out.

Phosphate aging appears to differ in degree only from the type of deterioration already known to occur for B. cadaveris and Escherichia coli (Gale, 1938) when cells are allowed to stand for prolonged periods in water or phosphate buffer. The response of these aged cells to magnesium, glucose, and combinations of these could be explained in several ways. The addition of magnesium and glucose may simply make the membrane more permeable to the substrate. An alternate hypothesis would propose the existence of active transport in which the uptake of aspartate is exergonically linked to carbohydrate metabolism. Thus, the addition of glucose and magnesium to the damaged cells would either supply a factor removed by the phosphate treatment (e. g., magnesium) or permit the system to function more efficiently. Whether an exergonically linked transport system exists in these cells cannot be
determined from the present data. There is, however, considerable evidence for the existence of a carrier mechanism of some type.

Aspartate transport. The existence of a carrier mechanism is supported by three lines of evidence: (a) the stereospecificity of the uptake system for L-aspartate; (b) the kinetics of the uptake process; and (c) the sensitivity of the uptake system to 2,4-D.

The entrance of substrates into cells is mediated by two processes: nonspecific diffusion due to permeability of the cell membrane and selective permeation resulting from the operation of a carrier mechanism. If only diffusion is operating, then the membrane will exhibit permeability to substances of similar solubility, molecular weight, charge, etc. The fact that resting cells of B. cadaveris are impermeable to D-aspartate, L-glutamate, and L-threo-β-methylaspartate makes it unlikely that little if any L-aspartate enters the cell by simple diffusion. In any event, the amounts of these substances which disappear because of diffusion are too small to be detected by the analytical methods used in these experiments.

The kinetics of a diffusion process follow a first order rate law whereas the operation of a carrier mechanism results in diphasic kinetics of the type exhibited by enzyme systems (Reiner, 1959). The carrier acceptor site need not be an enzyme for it to behave in the same way as the concentration of substrate is increased. Figure 2 shows that with control cells the rate, measured either as substrate disappearance or ammonia produced, shows saturation of acceptor sites at higher concentrations of substrate. Phosphate aged cells, on the other hand, display atypical response to increasing concentration of substrate. No explanation is available at present for the unusual shape of this curve, other than to say that the selective permeation system has been damaged. The permeation rate may be determined by measuring either aspartate uptake or ammonia production, since the two sets of data will be fully corroborative and it may be shown that the ingress process is rate controlling. The failure to recognize this fact has led to much confusion in the interpretation of studies based on resting cells. Ammonia production was long accepted as a valid measure of aspartase activity in resting cells. Identification of the rate controlling step must be made indirectly since it has not been possible, thus far, to examine the permeation system removed from the deaminase. An analysis of this type is possible, however, and a detailed discussion has been given by Reiner (1959). For the system under consideration here, a summary of $Q_{\text{NH}_3}$ and $K_M$ data from various laboratories has been given by Williams and McIntyre (1955a) and additional information may be found in theses by Smith (1955) and Bagala (1957). Crude uncentrifuged sonic lysates and washed resting cells from the same culture exhibit vastly different deamination velocities when equated as to protein. The $Q_{\text{NH}_3}$ for the former is about 1200 and for the latter, about 360. If the deamination step were rate controlling, aspartate would accumulate in the cells (which it does not) and the $Q_{\text{NH}_3}$ for deamination in the resting cells would be as large as that for cell-free crude lysates. Trudinger (1955) arrived at a similar conclusion from studies of Proteus X-19. Furthermore, the Michaelis constant for cell-free aspartase is known to be about $1 \times 10^{-2}$ moles per L, whereas that for resting cells is about $5 \times 10^{-3}$ moles per L. This difference has not been regarded as noteworthy. The writer feels, however, considering the reproducibility of these values in the hands of different workers, that it is a clear indication that different systems are being measured, i.e., the $K_M$ for resting cells is the catalysis constant for the transport system, whereas with cell-free enzyme, the true $K_M$ for aspartase is obtained.

The stimulation of the transport system by glucose and magnesium could be explained as altered permeability of the cell membrane, assuming that a diffusion process is operating. If this were true, however, one would expect the addition of glucose and magnesium to increase the permeability of the cells to D-aspartate, and this is not the case. The mode of action of glucose and magnesium in stimulating the transport system in B. cadaveris is not known. Suffice it to say that other permeation systems in related organisms (E. coli) have been shown to be linked to the metabolic activity of the cell, and this possibility cannot be ignored in B. cadaveris. The inhibition of the carrier mechanism by 2,4-D presents a second case of a phenomenon which has frequently been observed with other transport systems (Cohen and Monod, 1957).

The final decision on these points will be made when an aspartate analogue is found which can
be transported by the cells but not metabolized by the deaminase. The data which have been presented support the contention that aspartate enters the cells of B. cadaveris by catalytic transport and that this system displays a sensitivity to certain inhibitors and stimulants known to be effective in other transport systems. That phosphate aging likewise exerts a marked effect on the permeation system is clearly shown. This permeation system may not be the only site damaged, but the writer feels that the case for enzyme resolution in aspartase by phosphate aging will have to be strengthened by a new approach to the problem.

SUMMARY

The phosphate aging treatment of resting cell suspensions of Bacterium cadaveris results in diminished ability of the cells to transport aspartic acid. Similar results were obtained for adenosine, thus demonstrating that the effect is not specific for aspartate transport. Aspartate is believed to enter the cell by a carrier mechanism since the uptake process is specific for L-aspartate, exhibits catalysis kinetics, and is sensitive to 2,4-dichlorophenoxyacetic acid (2,4-D). Of the three processes, ingress of aspartic acid, deamination, and egress of ammonia—the first is rate controlling. Both 2,4-D inhibition and phosphate aging are appreciably reversed by glucose, and the combination of glucose and magnesium is even more effective. Whereas phosphate aging is known to be reversed by biotin, 2,4-D inhibition is not.

REFERENCES


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