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EFFECT OF BIOTIN, PANTOTHENIC ACID AND NICOTINIC ACID DEFICIENCIES ON AMINO ACID TRANSPORT IN *LACTOBACILLUS PLANTARUM*

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SUMMARY

1. The effects of biotin, pantothenic acid, nicotinic acid and folic acid deficiencies and of physiological age on the rate and amount of glutamic acid accumulation in *Lactobacillus plantarum* have been investigated.

2. The time-course of glutamic acid uptake was markedly affected by biotin and pantothenic acid deficiencies. After several minutes of uptake at a normal rate, accumulation ceased or continued slowly for 20 to 40 min, after which it resumed at a more rapid though generally inferior rate resulting in submaximal pool levels.

3. High concentrations of sucrose restored uptake by these cells to a normal level and pattern. Acetate and the respective vitamin individually stimulated uptake by biotin and pantothenate-deficient cells and together they allowed normal uptake. With biotin-deficient cells, the stimulatory effect of biotin, but not of acetate, was inhibited slightly by chloramphenicol. With pantothenic acid-deficient cells high concentrations of KCl in contrast to sucrose failed to restore uptake to fully normal levels.

4. In contrast, nicotinic acid-deficient cells accumulated glutamate and glycolyzed at markedly reduced rates. Sucrose and acetate failed to stimulate uptake, while nicotinic acid greatly improved the accumulation rate. The effects of this deficiency on uptake appear to be related primarily to energy metabolism.

5. Early exponential phase, nutritionally normal cells also showed a slight dip in the accumulation curve which could be corrected by acetate and usually, although not consistently, by sucrose. Folic acid-limited cells accumulated as well as early exponential phase control cells indicating that this deficiency does not create any disturbance in the transport system.

6. Penicillin inhibited amino acid uptake by early exponential phase cells much more than by early stationary phase cells and sucrose protected against this inhibition.

7. The adverse effects of biotin and pantothenic acid deficiencies on amino acid transport may arise from structural changes in the cell. The reversal of these effects by high extracellular osmotic pressure indicates that these vitamins probably do not participate directly in the transport step. These findings are discussed in terms of the

probable effects of biotin and pantothenate deficiencies on lipid synthesis and consequently on cell membrane structure. The facile reversal by osmotic means of the effects of these presumed membrane changes on transport suggests that membrane permeability properties and the activity of transport catalysts may not be strictly dependent on a specific membrane composition. An alternate possibility also is considered, *viz.*, that the aberrations in accumulation activity occur primarily as a result of cell wall changes.

INTRODUCTION

Attempts to identify the catalysts involved in amino acid transport so far have proven unsuccessful. In an effort to determine whether nutritional deficiencies would provide clues to the identity of components of these systems, previous studies in this laboratory have dealt with the relation of vitamin B_6 to amino acid transport in *L. plantarum*[1,2]. Although a deficiency of this vitamin markedly reduced the amount of amino acid which could be accumulated by this organism, the initial rate was not affected, and the capacity for glutamate accumulation could be restored essentially to normal levels either by carrying out the experiments in the presence of high concentrations of sucrose or KCl (ref. 3), or by adding acetate, ammonium and vitamin B_6 to the accumulation buffer[4]. These observations suggested that the principal defect which limited transport in vitamin B_6-deficient cells was a structural change, most likely an abnormality in the cell wall, which adversely affected its tensile properties. These conclusions are supported by experiments showing that walls from vitamin B_6-deficient cells are markedly deficient in the muramylpeptide component[5]. Since the transport systems of severely vitamin B_6-deficient cells could function in an essentially normal manner when the extracellular osmotic pressue was appropriately adjusted, it seems very unlikely that the vitamin is a component of these catalytic systems.

During these studies it was observed that biotin, pantothenic acid and nicotinic acid deficiencies also markedly affected the time-course of amino acid uptake[2]. The involvement of biotin and pantothenic acid in fatty acid synthesis would lead one to expect changes in lipid composition and membrane structure in deficient cells, possibly resulting in changes in the operation of transport systems. The involvement of pantothenic acid in acetylation reactions would also be expected to produce limitations on acetylaminosugar and, therefore, on cell wall biosynthesis, possibly with a consequent reduction in the ability of deficient cells to restrain internal osmotic pressures. A nicotinic acid deficiency might limit transport by adversely affecting energy metabolism. In view of the manifold ways in which these deficiencies could influence the transport process, their observed effects on amino acid accumulation were investigated further. The findings, presented below, demonstrate that the adverse effects of biotin and pantothenic acid deficiencies can be reversed by high concentrations of sucrose, or by acetate and the respective vitamin. Preliminary accounts of some of these experiments have been reported[6–8]. During and after the completion of this study several investigations were reported showing a relation between biotin deficiency and changes in cell permeability properties[9–12].

METHODS AND MATERIALS

The organism and general procedures for culturing cells have been described previously[1-4]. Vitamin-deficient cultures contained greatly reduced amounts of one of the vitamins which was supplied at a level just sufficient to allow growth to densities of 0.1 to 0.2 mg/ml (dry weight). This contrasts with nutritionally normal, control cultures, designated control (L), which grew much more rapidly and at the end of the logarithmic growth phase had attained a density of 0.7 to 0.9 mg/ml. Several experiments will be described using nutritionally normal cells harvested in the early portion (0.13 to 0.20 mg/ml) of the logarithmic growth phase (control (E)). Vitamin-limited cultures were harvested just when growth (as judged by absorbance changes) had reached the stationary or nearly stationary phase. The amounts of vitamin required to achieve the indicated growth yields varied slightly from one batch of medium to the next, but fell within the following ranges: low biotin cultures (Lo Bi), 45–65 mμg/l; low pantothenic acid cultures (Lo P. A.), 3–8 μg/l; low nicotinic acid cultures (Lo N. A.) 7–14 μg/l; low folic acid cultures (Lo F. A.), folic acid omitted from medium and *p*-aminobenzoic acid at 14 mμg/l. Inocula for all vitamin-limited cultures were grown overnight in complete medium. They were collected by centrifugation, resuspended in water and dispensed to the growth culture at 0.25 mg/l.

After all the experiments reported here had been completed, the strain of *L. plantarum* which we have used (or possibly one of the medium constituents) underwent a change which consistently resulted in a 30% lower accumulation capacity. This effect could be reversed by increasing the nicotinic acid content of the growth medium 10-fold. Most of the experiments subsequently have been repeated using cells cultured in the high nicotinic acid medium with essentially the same results as were obtained originally.

Amino acid uptake was determined as described previously[1,3] by measuring the accumulated radioactivity in extracts of cells collected by centrifugation. Rates of glycolysis were determined using a Radiometer titragraph. Cells at 0.16 mg/ml were incubated at 37° in 7.6 ml of water containing 5 μmoles of Tris phosphate and 35 μmoles of glucose. The acid produced was titrated using 0.02 M Tris.

All chemicals were obtained from commercial sources.

RESULTS

The effects of physiological age and of several vitamin deficiencies on glutamic acid accumulation by *L. plantarum* are summarized in Fig. 1A and B. Cells taken from cultures near the end of the exponential phase or later accumulated glutamic acid at a relatively constant rate for over 40 min and then at a gradually diminishing rate until the saturation capacity was attained. Earlier studies have demonstrated that an increase in intracellular L-glutamic acid accounts for 85 to 90% of the accumulated radioactivity. Cells taken from early exponential phase cultures accumulated nearly equal amounts of glutamate, but the time-course of uptake was altered. Following a brief period of normal uptake, the rate decreased at pool levels far below the maximum attainable. After 10 to 20 min of relatively slow accumulation, the rate increased gradually and was maintained at nearly maximal levels until the saturation capacity was attained.

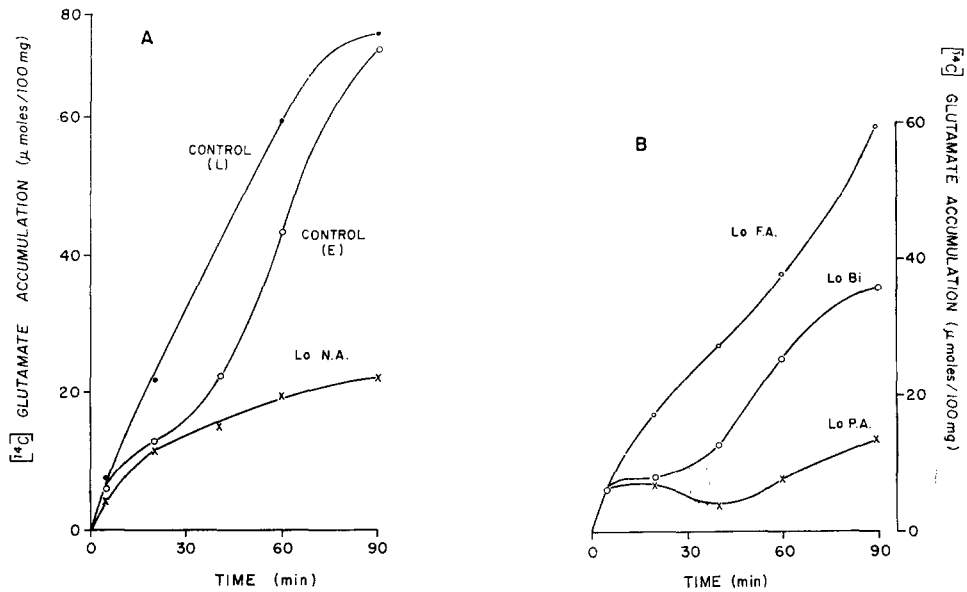

Fig. 1. Uptake of L-[^{14}C]glutamic acid by nutritional variants of *L. plantarum*. The extracellular glutamate concentration was 3 mM and the amount of amino acid accumulated was calculated from the radioactivity of cell extracts. All values in this and subsequent figures are corrected for a small amount of radioactivity taken up by cells exposed briefly to glutamic acid at 2°. The cells were cultured under the conditions and harvested at the densities and times indicated below. A, Control (L), complete medium, 0.65 mg/ml, 14.5 h; control (E), complete medium, 0.15 mg/ml, 11 h; Lo N.A., nicotinic acid at 14 μg/l, 0.18 mg/ml, 16.5 h. B, Lo F. A., folic acid omitted and *p*-aminobenzoic acid at 14 mμg/l, 0.10 mg/ml, 15 h; Lo Bi, biotin at 65 mμg/l, 0.17 mg/ml, 15.5 h; Lo P.A., pantothenic acid at 8 μg/l, 0.13 mg/ml, 19 h.

Biotin and pantothenic acid deficiencies markedly extended the duration of the intermediate period during which the accumulation rate was greatly reduced. In many, although not in all batches of cells, the initial rate was not significantly altered, suggesting that the availability of effective accumulation catalysts at least during the first few minutes of transport was not reduced by these deficiencies. Enzymatic analyses of biotin- and pantothenate-limited cell extracts for L-glutamic acid demonstrated that most of the increase in pool glutamate predicted from the accumulation of radioactivity could be accounted for as an increase in L-glutamic acid at all periods of incubation. A nicotinic acid deficiency produced a pronounced decrease in the accumulation rate throughout the entire incubation period. In contrast, a folic acid deficiency affected neither the accumulation rate nor the time-course of uptake to any significant extent in comparison to the behavior of nutritionally normal cells harvested at comparable cell densities. This result shows that the unusual accumulation pattern observed with biotin- and pantothenate-deficient cells is not a non-specific effect which can be observed in any cell type whose growth has been curtailed at grossly suboptimal densities by a nutrient deficiency.

Restoration of normal accumulation in biotin-deficient cells

Sucrose at high concentrations restored the time-course of uptake by biotin-deficient cells to the pattern observed in late exponential phase nutritionally normal

cells (Fig. 2). Under these conditions, the initial rate consistently fell in the range observed with control cultures indicating strongly that this vitamin does not directly participate in the rate-limiting step of the transport event. The similarity of this response to that observed with vitamin B_6-deficient cells[3] prompted a study of the effects of acetate, NH_4^+, vitamin B_6 and biotin on glutamate uptake. As shown in Fig. 3, acetate and biotin individually stimulated uptake when they were added separately to the buffer, and together they restored uptake during the affected phase to levels observed with nutritionally normal cells. Pyridoxamine and NH_4^+ had no effect alone or in the presence of acetate and biotin. The cell count and total amount of RNA were only slightly affected by these supplements. For example, in the experiment illustrated in Fig. 3, after 90 min, there was only a 4% increase in cell numbers

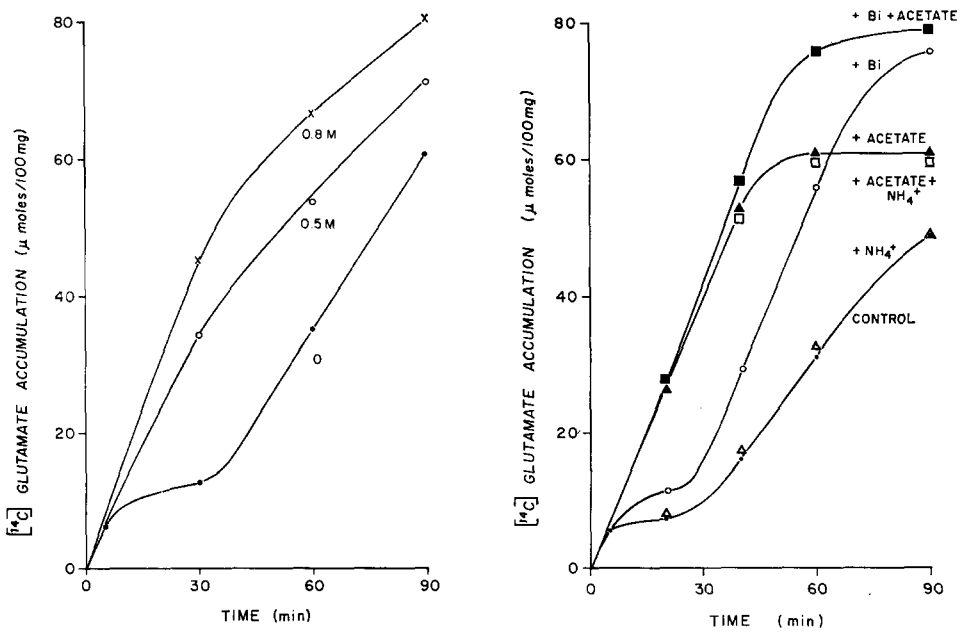

Fig. 2. Stimulation of glutamic acid accumulation in biotin-deficient cells by high concentrations of sucrose. The standard uptake buffer was supplemented with sucrose to obtain the indicated molar concentrations. Biotin-deficient cells were harvested at 0.15 mg/ml after 14 h incubation in medium containing 65 mμg/l of biotin.

Fig. 3. Stimulation of glutamate uptake in biotin-deficient cells by acetate and biotin. The standard uptake buffer was modified to contain the following substances at the indicated concentrations: ●—●, control, no additions; ○—○, biotin, 1.2 μg/ml; ▲—▲, potassium acetate, 5.8 mM; △—△, NH_4Cl, 3.0 mM; □—□, potassium acetate and NH_4Cl; ■—■, biotin and potassium acetate. Biotin-deficient cells were harvested at 0.11 mg/ml after 14.3 h incubation in medium containing 50 mμg/l of biotin.

in the presence or absence of biotin. When both biotin and acetate were provided, cell numbers increased only 15% in 90 min. In contrast, at 20 min there was a 200% increase in the amount of glutamate accumulated, indicating that these supplements are effective largely because they increase the accumulation activity of the cells originally added to the incubation buffer. In the experiment shown, a large excess of

biotin was used. Reducing the amount of biotin by a factor of one thousand (to 1.2 mμg/ml) did not reduce the extent of stimulation. It should also be noted that there was some variability in the extent to which a biotin deficiency (and also a pantothenate deficiency) affected amino acid uptake and the responsiveness of these cells to the stimulatory substances. This appears to be related partly to a heightened sensitivity of these cells to experimental manipulations, a phenomenon which was encountered earlier and to a much greater extent with vitamin B_6-deficient cells[2].

Examination of Figs. 2 and 3 suggests that during incubation for 20 to 30 min, in the absence of supplements, biotin-deficient cells undergo a change which then allows them to accumulate glutamate at nearly normal rates. It was of interest to determine whether preincubation in the absence of the transport substrate, glutamic acid, would allow this change to occur. Vitamin B_6-deficient cells, in which the accu-

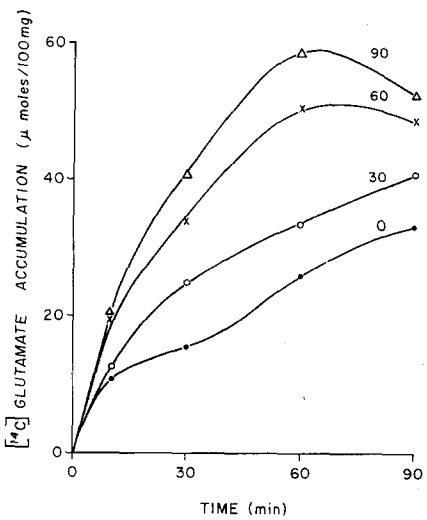

Fig. 4. Effect of preincubation in buffer on the uptake of glutamic acid by biotin-limited cells. Biotin-deficient cells at 1.6 mg/ml were preincubated at 37° in the standard uptake buffer containing glucose (28 mM) for 30, 60 and 90 min. Control cells (0 preincubation) were suspended momentarily in this buffer at 2°, and then treated the same as the preincubated cells. At the end of the pretreatment period the cells were collected by centrifugation at 3 800 × *g* for 10 min. The pellets were gently resuspended in cold uptake buffer to a density of 20 mg/ml and added to the standard uptake buffer containing L-[^{14}C]glutamic acid and glucose to determine their ability to accumulate this amino acid. The numbers on the curves refer to the minutes of pretreatment. Biotin-deficient cells were harvested at 0.14 mg/ml after 17 h growth in a medium containing 65 mμg/l of biotin.

mulation capacity appears to be limited by a reduced cell wall content, could be made to enlarge their accumulation capacity significantly only by pretreatment in a buffer of high osmotic pressure supplemented with acetate, NH_4^+, vitamin B_6 and glutamic acid[4]. Although the behavior of biotin-deficient cells was somewhat variable, generally pretreatment in a phosphate buffer containing only glucose sufficed to correct the accumulation defect, *i.e.* cells pretreated at 37° in this manner when used subsequently in an uptake experiment no longer exhibited a diphasic accumulation pattern (Fig. 4). It appears, therefore, that except for an energy source, biotin-limited cells do

not have an absolute requirement for exogenous supplements to carry out the reactions on which the improvement in accumulation rate depend.

These reactions, however, do appear to be slightly sensitive to chloramphenicol. In the absence of any other additions to the uptake test system, chloramphenicol at low levels (50 μg/ml) caused a slight inhibition and at high levels (1000 μg/ml) a moderate stimulation of glutamate uptake by biotin-limited cells (Fig. 5). The stimulation observed when acetate was added to the uptake test system was not inhibited and possibly was improved slightly at high levels of chloramphenicol. In contrast, the stimulatory effect of biotin was inhibited at all levels of chloramphenicol. A provisional interpretation of these findings is that acetate may be a limiting metabolite in biotin-deficient cells and that it can be used in the restoration process without the involvement of protein synthesis. Biotin may act either by making additional amounts

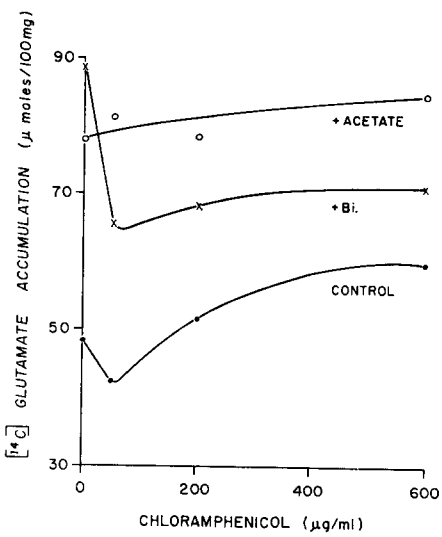

Fig. 5. Effect of chloramphenicol on glutamic acid accumulation by biotin-deficient cells. Standard uptake conditions were modified to include chloramphenicol at the indicated concentrations and potassium acetate (5.8 mM) and biotin (1.3 μg/ml) as shown on the respective curves. The curves show the amounts of [^{14}C]glutamic acid accumulated after 90 min. Biotin-deficient cells were grown with 60 mμg/l of biotin and harvested at 0.14 mg/ml after 16.5 h.

of acetate available or by increasing its utilization rate; in either case protein synthesis appears to be involved. The endogenous recovery probably involves the production of acetate utilizing existing catalysts and, therefore, there is no substantial inhibition by chloramphenicol. The stimulation of uptake observed with large amounts of the inhibitor is not readily explained. There is not sufficient incorporation of glutamate into the cell residue to account for the appearance of this much additional glutamate in the pool when protein synthesis is inhibited by chloramphenicol.

The possibility was considered that a reduction in the glycolytic rate and, therefore, the availability of energy was the direct cause of the aberrant uptake pattern. Titrimetric determinations showed that while acid production from glucose was decreased slightly by a biotin deficiency[25], the reduction in the glycolytic rate was

much smaller than the reduction in the uptake rate. For example, in the period when glutamic acid uptake was reduced 75% the glycolytic rate was decreased only 20%. The most likely explanation of these findings is that the changes which affect glutamate uptake also alter the glycolytic pathway slightly. Interestingly, analysis of residual glucose in the uptake buffer failed to disclose any effect of biotin limitation on the rate of glucose removal. While the small effects on the glycolytic rate may exaggerate the effects of a biotin deficiency on the glutamate uptake system, they do not appear to be directly causative of this unusual behavior.

An examination of the ability of a large number of organic acids to substitute for acetate in stimulating glutamate uptake, failed to disclose any which approached acetate in activity. This result corresponds to the specificity of acetate in restoring the glutamate accumulation capacity of vitamin B_6-deficient cells[4].

Restoration of normal accumulation in pantothenic acid-deficient cells

A pantothenate deficiency imposes a more severe restraint than a biotin deficiency on glutamate uptake. In unsupplemented buffer, uptake was as poor as that observed with vitamin B_6-deficient cells, showing only a slight tendency towards self-reactivation after a long incubation period (Fig. 1B). Both acetate and pantothenic acid individually stimulated uptake but only after pronounced lag periods. When both substances were provided, uptake was essentially normal (indistinguishable in rate and amount from that observed with late exponential phase control cultures).

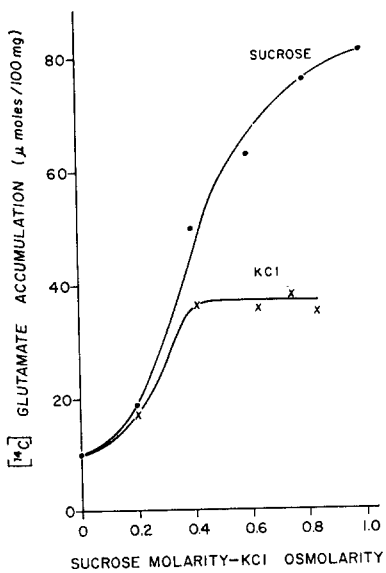

Fig. 6. Comparative stimulatory effects of sucrose and potassium chloride on glutamate accumulation by pantothenate-deficient cells. The standard uptake buffer was modified to contain sucrose at the indicated molar concentrations or potassium chloride at graded concentrations between 0.1 and 0.6 M. The amounts of glutamate accumulated in the presence of potassium chloride are plotted for comparative purposes at the osmotically equivalent sucrose concentrations which were calculated from published values for water activities[13,14]. Pantothenic acid-deficient cells were harvested at 0.22 mg/ml after 18.5 h growth in medium containing 3 μg/l of pantothenic acid.

In this regard these cells behaved similarly to biotin-deficient cells. NH_4^+ had little or no effect on the reactivation of pantothenate-deficient cells. Changes in cell numbers during these incubations with or without supplements were minor and of too small magnitude to account for the large changes in accumulation capacity.

As shown in Fig. 6 high concentrations of sucrose restored the glutamate accumulation capacity to normal levels in the absence of added pantothenic acid. With vitamin B_6-deficient cells, KCl and sucrose over a 10-fold range of iso-osmotic concentrations produced essentially identical improvements in glutamate uptake[3]. In contrast, pantothenic acid-deficient cells responded to low levels of KCl, but above 0.2 M this salt failed to improve uptake as well as osmotically equivalent amounts of sucrose, and there was no further improvement above 0.25 M KCl (equivalent to 0.4 M sucrose) (Fig. 6).

As with biotin-deficient cells, a pantothenic acid deficiency reduced the rate of acid production from glucose approximately 10 to a maximum of 20% without any significant change in the rate of removal of this sugar from the buffer. No stimulation of acid production was observed using sucrose at concentrations which markedly improved glutamate accumulation. It is unlikely that these relatively small changes in glycolytic rate produce the large fluctuations in glutamate accumulation activity observed in these experiments.

Effects of a nicotinic acid deficiency on glutamate accumulation

The marked reduction in the initial accumulation rate of nicotinic acid-deficient cells is consistent with the dependence of this process on high-energy compounds which, in this microaerophilic bacterium, are provided by the pyridine nucleotide-dependent glycolytic pathway. Nicotinic acid alone was capable of greatly stimulating the accumulation rate (Fig. 7), although restoration to completely normal levels usually was not observed without pretreatment. In contrast to vitamin B_6, biotin and

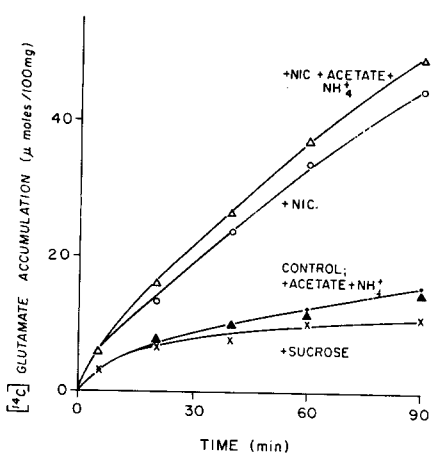

Fig. 7. Stimulation of glutamate uptake in nicotinic acid-deficient cells by nicotinic acid. The standard uptake buffer was modified to contain the following substances at the indicated concentrations: ●—●, control, no additions; ×—×, sucrose, 0.5 M; ▲—▲, potassium acetate, 5.8 mM and NH_4Cl, 3.0 mM; ○—○, nicotinic acid, 13.3 μg/ml; △—△, nicotinic acid, potassium acetate and NH_4Cl. Nicotinic acid-deficient cells were harvested at 0.29 mg/ml after 24 h growth in a medium containing 14 μg/l of nicotinic acid.

pantothenate-deficient cells, nicotinic acid-deficient cells failed to respond to sucrose or to acetate and NH_4^+ in the absence of the vitamin. In the presence of nicotinic acid, acetate and NH_4^+ stimulated slightly suggesting that there might be a structural or metabolic abnormality in these cells comparable to that seen in control (E) cells (see below) which is favorably influenced by these substances.

Glycolytic studies which showed that a nicotinic acid deficiency reduced the rate of glycolysis approx. 60% support the proposal that the uptake process is primarily limited in these cells by a limitation in the energy supply. The addition of nicotinic acid to the uptake buffer increased the rates of both processes approximately equally.

Effect of physiological age on glutamate accumulation

Cells harvested from a nutritionally complete medium in the early portion of the exponential growth phase also showed a slight abnormality in the time-course of glutamate accumulation (Fig. 1A). This behavior was seldom observed in cells from cultures which had passed the midpoint of the exponential growth phase. Acetate stimulated uptake in the intermediate period, so that accumulation corresponded closely in amount and rate to that observed with late exponential phase cultures. Ammonium, pyridoxamine and other B-vitamins did not promote a further stimulation. The response of such cultures to sucrose has been variable. Usually sucrose restored the accumulation time-course to a normal pattern; occasionally no stimulation was observed. This lack of constancy may be related to the equally variable behavior

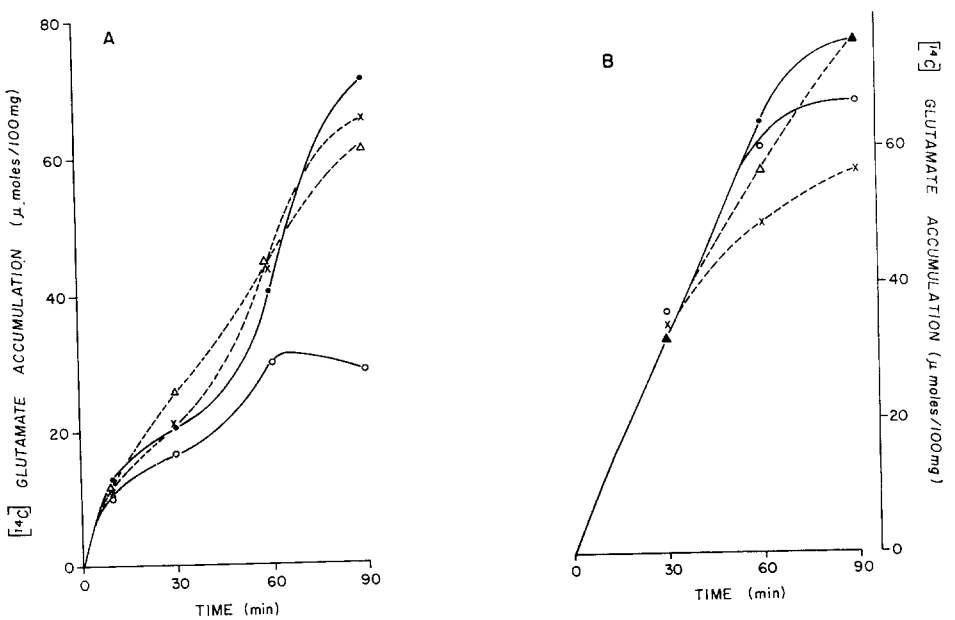

Fig. 8. Effect of penicillin on glutamate accumulation in early exponential and early stationary phase, nutritionally normal cells. The standard uptake buffer was modified to contain the following substances: ●—● control, no additions; ○—○, penicillin, 100 units/ml; ×- - -×, sucrose, 0.6 M; △- - -△, penicillin and sucrose. A, early exponential phase cells harvested at 0.18 mg/ml after growth for 12.5 h in complete medium. B, early stationary phase cells, harvested at 0.80 mg/ml after growth for 18.5 h.

of late exponential and early stationary phase cells which occasionally were not affected and in other experiments were markedly *inhibited* by sucrose concentrations which consistently increased uptake by vitamin B_6-, biotin- or pantothenate-deficient cells. It is possible that the factors which occasionally make late exponential phase cells susceptible to inhibition by sucrose also render early exponential phase cells incapable of being stimulated by this substance. The origin of this inconstant behavior has not been established.

Nutritionally normal, early exponential and early stationary phase cells also differed from each other in their responses to penicillin. Early exponential phase cells were distinctly more sensitive to the antibiotic than were early stationary phase cells (*cf.* Fig. 8A and B). The inhibitory effect was largely avoided in early exponential phase cells by adding sucrose to the uptake buffer (Fig. 8A; note that in the absence of penicillin these cells failed to respond to sucrose). In early stationary phase cells penicillin inhibited only slightly and appeared in fact to reverse an inhibitory effect of sucrose (Fig. 8B). The very minor inhibitory effect of penicillin on uptake by early stationary phase cells except at extended periods of incubation (where there was still only a 10% reduction of capacity) indicates that this substance does not directly interfere with the operation of the transport catalysts.

Effect of a biotin deficiency on alanine and proline accumulation

The experimental findings shown in Fig. 9 demonstrate that a biotin deficiency also distorts the time-course of alanine accumulation. As with glutamic acid accumulation, acetate and biotin individually stimulated accumulation of alanine. Entirely comparable results were obtained when proline uptake was studied. This is distinctly different from the behavior of vitamin B_6-deficient cells which in addition to NH_4^+,

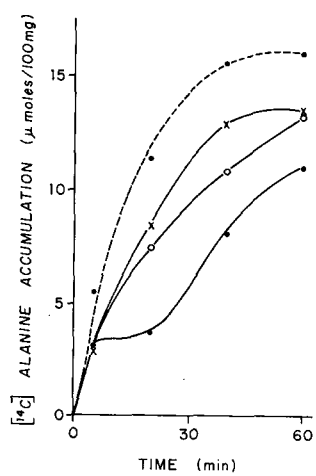

Fig. 9. Effect of biotin deficiency on alanine accumulation. The standard uptake buffer was used with uniformly labeled L-[^{14}C]alanine in place of glutamic acid. Biotin-deficient cells (—) were harvested at 0.16 mg/ml after growth for 17.5 h in a medium containing 55 mμg/l of biotin. Control cells (- - - -) were grown in complete medium (biotin at 2 μg/l) and harvested at 0.73 mg/ml after 16.5 h. The following additions were made to the standard uptake buffer: ●—●, no additions: ○—○, biotin, 1.3 μg/ml; ×—×, potassium acetate, 5.8 mM; ●- - -●, control biotin-sufficient cells, no additions.

acetate and vitamin B_6 also required glutamic acid to improve alanine or proline accumulation[4]. It should be noted that as with vitamin B_6-deficient cells, alanine, proline and glutamate accumulations diverge to an abnormal time-course after approx. 5 min of accumulation despite the fact that the amounts of these amino acids and, therefore, their contributions to the intracellular osmotic pressure differ greatly. This suggests that the change in accumulation rate is not caused solely by an increase of intracellular osmotic pressure which the cell is structurally incapable of restraining, and that other metabolic factors very likely contribute to this phenomenon.

DISCUSSION

The possibility that biotin, pantothenic acid or folic acid react directly in the amino acid transport process as a functional part of the catalytic systems is inconsistent with the failure of deficiencies of these vitamins, especially when sucrose is present in the extracellular buffer, to modify the initial uptake rates. However, as indicated previously[2], regardless of how completely other catalysts known to be dependent on a cofactor, are shown to be inactivated by a deficiency of this substance, one must note the theoretical possibility that the catalytic system under study may have an extremely high affinity for the cofactor which prevents its effective desaturation. The clearcut effect of nicotinic acid on the uptake rate seems almost certainly to be related primarily to an effect of this deficiency on the rate of energy production, although additional modes of action are not excluded. We must also conclude that the synthesis of the transport catalysts cannot have been significantly reduced by the biotin or pantothenate deficiencies, again because there is no other way to account for the essentially normal accumulation observed with such cells in the presence of sucrose. It appears, therefore, that these cells contain a normal complement of effective transport catalysts and it is their ability to function which is impeded by a metabolic or structural consequence of these deficiencies.

Considering the very likely possibility that biotin and pantothenic acid deficiencies affect lipid synthesis which in turn would be expected to affect membrane structure, it is surprising that the profound effects of these deficiencies on transport are reversed by simple osmotic adjustments in the external buffer. This suggests that the principal effect of the deficiencies, if they do alter the membrane, may be on its permeability properties, and specifically on the ability to retain amino acids and possibly other low molecular weight solutes at high concentrations within the cell. The occurrence of a permeability change in the membrane of pantothenate-limited cells also is suggested by the pronounced inequality of sucrose and KCl in stimulating amino acid accumulation. Such a change in permeability might be largely compensated for by causing a retraction and presumably a contraction of the cell membrane by osmotic removal of water from the cell. (We have directly demonstrated by electron microscopy that cells exposed to sucrose contain a contracted cytoplasm surrounded by a membrane which has separated from the cell wall[15].) If true, this would indicate that under favorable osmotic conditions, a wide latitude exists in the composition and structure of membrane lipids with regard to the effectiveness of the structure in controlling the diffusion of amino acids out of the cell. These observations also suggest that the transport catalysts, presumably located in the cell membrane, probably do not have strict requirements for specific lipids.

These are admittedly speculative conclusions and we are presently studying the lipid constituents of vitamin-deficient cells in an effort to determine whether the membrane composition is altered by these deficiencies. There are several reports which suggest that such changes will be found. CROOM, MCNEILL AND TOVE[16], using the same strain (17–5) of *L. plantarum* that was used in our study, found a change in fatty acid composition and a 20% reduction in the lipid content of cells grown in a biotin-deficient medium. It should be noted that the cells used in our study were grown with much lower levels of the vitamin and, presumably, were in an even more depleted state. In *Escherichia coli* a biotin deficiency altered the distribution of fatty acids among cell fractions causing a notable reduction in their occurrence in the cell wall–cell membrane fraction[17]. In the yeast *Hanseniaspora valbyensis* both pantothenic acid and vitamin B_6 deficiencies have been shown to reduce the total amount of lipid and its content of the unsaturated fatty acid, palmitoleic acid[18].

Furthermore, several investigators have described effects of biotin deficiency on the permeability and structure of various microorganisms. In the glutamic acid-forming *Brevibacterium flavum* SHIIO and coll.[9–11] have concluded that the enhanced production of this amino acid when the organism is grown in biotin-limited media is caused by an increased permeability of the membrane to amino acids including glutamate which allows them to diffuse out of the cell thereby minimizing a limitation on glutamate synthesis by feedback control. AHMAD AND ROSE[19] reported a heightened fragility in biotin-deficient cells of *Saccharomyces cerevisiae*. The permeability of the peripheral membrane of this organism to chloride, phosphate and protein also appeared to be increased by this deficiency[12]. The rates of alanine uptake and incorporation into protein were reduced by biotin deficiency in *Neurospora crassa*[20]. In *Arthrobacter globiformis* CHAN[21] has described a morphological aberration caused by biotin deficiency which may derive from cell wall changes. Alterations in cell wall composition or structure, of course, may arise as secondary effects of membrane changes. We are unaware of comparable findings with microorganisms that can be attributed to pantothenate deficiency except for a report from this laboratory[22] and by DAS, FENG AND TOENNIES[23] that *Streptococcus faecalis* growing in pantothenate-deficient medium undergoes extensive lysis. In any case, there is considerable support for the premise that changes in membrane composition accompany biotin and pantothenic acid deficiencies. It may be instructive to establish what these changes are and how their effects on the transport systems are reversed by osmotic means.

It is possible to entertain an alternate view of these findings, namely that biotin and pantothenic acid deficiencies principally affect the structure of the cell wall thereby reducing its tensile strength and indirectly causing a reversible deactivation of the membrane-localized accumulation process. The superficial similarity of the findings with biotin- and pantothenate-deficient cells to those with vitamin B_6-deficient cells, where this appears in fact to be the correct explanation, together with the greater probability that a wall defect rather than a membrane defect might be readily reversed by osmotic means, initially led us to an extensive investigation of the effects of these vitamin deficiencies on cell wall composition. As yet we have not encountered compositional changes which would be expected to affect the tensile properties of this structure. The amount of wall substance if anything is increased in biotin- and pantothenate-deficient cells, and the relative amounts of the muramylpeptide and teichoic acid components are not significantly changed[5]. A pantothenate deficiency

does reduce the O-ester content and greatly increase the sensitivity of the wall to muramidase lysis[24], but a relation of these changes to the strength of the wall is not apparent. Electron micrographs do not indicate any reduction in the thickness of the wall layer in biotin- and pantothenate-deficient cells[26]. We are encouraged by these findings to believe that the changes relevant to an explanation of the abnormal behavior of the transport systems will be encountered in the cell membrane.

The activity of acetate as a participant in the reversal of biotin- and pantothenate deficiency effects as well as those of a vitamin B_6 deficiency deserves comment. It appears that under the conditions of growth used in this study, *L. plantarum* has a marginal ability to synthesize acetate. (It should be noted that a biotin deficiency in addition appears to reduce the ability of *S. faecalis* to form acetate from pyruvate[22].) Acetate can be utilized for fatty acid and acetylaminosugar biosynthesis which could result in membrane and cell wall changes respectively. In vitamin B_6-deficient cells we have demonstrated that stimulation of amino acid accumulation by acetate is accompanied by its incorporation into cell wall substance[7]. Preliminary studies with biotin- and pantothenic acid-deficient cells indicate that acetate incorporation into the lipids of this organism is reduced by these deficiencies and markedly stimulated by the respective vitamin. The relation of this observation to the activity of acetate and the vitamins in stimulating the accumulation process has not yet been established.

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