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DEPENDENCE OF AMINO ACID TRANSPORT AND ACCUMULATION ON OSMOTIC FACTORS IN VITAMIN B_6-DEFICIENT *LACTOBACILLUS ARABINOSUS*

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SUMMARY

A vitamin B_6 deficiency markedly reduces the total amount of extracellular L-glutamic acid which can be taken up and accumulated intracellularly by *Lactobacillus arabinosus*. Sucrose at 0.6–0.9 M restores the glutamate accumulation capacity to normal levels. Glucose, galactose, lysine, KCl, KBr, KNO_3 and NH_4Cl at high concentrations also greatly improve the accumulation capacity of B_6-deficient cells. Sucrose and KCl at iso-osmotic concentrations have exactly equal effects on accumulation. Sucrose is confined largely to the extracellular space in nutritionally normal and vitamin B_6-deficient cells whereas glycerol, which does not stimulate accumulation, penetrates most of the intracellular water. In contrast to findings with nutritionally normal cells, large pools accumulated by deficient cells in the presence of sucrose are lost instantaneously by washing with dilute buffers. Alanine and proline accumulation also are depressed by the deficiency and restored to normal by high concentrations of sucrose. Interaction between alanine and glutamate during accumulation by B_6-deficient cells is more pronounced than in normal cells suggesting that the amino acids may contribute to the intracellular osmotic load.

These findings demonstrate that the accumulation process in vitamin B_6-deficient cells can function at its maximum level so long as the extracellular water activity is reduced. Therefore, in this bacterium vitamin B_6 does not play a catalytic role directly in the accumulation process. It is proposed that the primary effect of this deficiency is a reduction in cell-wall rigidity. In the absence of sucrose or other protecting substances this leads to an excessive influx of water with a consequent impairment of accumulation which becomes prominent only as the intracellular pool fills. Some of the data suggest that the pool is osmotically active, and, therefore, that accumulation involves active transport.

INTRODUCTION

Lactobacillus arabinosus 17-5 takes up and accumulates intracellularly large amounts of glutamic acid during incubation in buffers containing this amino acid and glucose[1]. A vitamin B_6 deficiency greatly reduces the total amount of glutamate which can be

accumulated[2]. The failure of the deficiency to modify the initial rate of uptake, the inability of the vitamin alone to improve the capacity of vitamin B_6-deficient cells, and the lack of significant inhibition by various vitamin B_6 antagonists, suggested that the vitamin does not function as a catalytic component in the uptake process itself and that under the conditions used, the deficiency influences accumulation indirectly.

In the course of these studies it was observed that vitamin B_6-deficient cells of *L. arabinosus* are morphologically abnormal, having a swollen, bulged appearance[3]. Since the cell wall appears to be responsible for maintaining the morphology of rod-shaped bacteria[4], these results indicated that this structure might be less rigid than normal in vitamin B_6-deficient cells. It was also observed that these deficient cells leaked unusually large amounts of intracellular constituents including nucleotides and protein[5]. Together, these observations suggested that the amount of amino acid taken up by vitamin B_6-deficient cells of *L. arabinosus* might be limited primarily by a change in the structural stability of the cell and only indirectly by a reduction in the activity of the amino acid transport system. Guided by this premise, the effects of washing and incubation conditions were reinvestigated with the finding, described here, that the extracellular osmotic pressure has a profound effect on the uptake of amino acids by vitamin B_6-deficient *L. arabinosus*. Preliminary reports of portions of this work have appeared[6–8]. A separate report[9] will describe a metabolic procedure for elevating amino acid accumulation capacity in such cells which also is consistent with the suggestion that a structural defect limits the capacity for glutamate accumulation[6,10,11].

METHODS

Procedures for the growth of cells, their preparation for use in uptake experiments, and the incubation conditions and analytical procedures used in these studies have been described[1,2]. Three cell types distinguished by the amount of pyridoxamine supplied in the growth medium were utilized. These are: cells grown without addition of the vitamin to the growth medium (LB_6), cells grown in the presence of trace amounts of pyridoxamine ($LB_6(+)$), and cells grown with an excess of pyridoxamine (HB_6). The two vitamin B_6-deficient cell types were always harvested at the end of, or very shortly after, the period of active growth. Their intracellular vitamin B_6 levels and responses in most experiments were closely similar, and, therefore, $LB_6(+)$ cells were used more often to take advantage of higher cell yields obtained in these cultures. The concentrations of cells, phosphate, glucose, uniformly labeled L-[^{14}C]-glutamic acid and trace salts were those of the standard uptake condition described previously[1], but most experiments were performed with 6 mg* of cells in a total volume of 3.75 ml. In most of the experiments described here, the release of glutamate accumulated within the cells was achieved using a modified extraction procedure. Following an uptake experiment, cells were frozen and then thawed, macerated in the cold and suspended in a total of 3.8 ml of boiling water. In the process the cells were transferred to 12-ml graduated glass centrifuge tubes. The tubes were placed in a boiling-water bath for 15 min. After cooling, the volume was adjusted to 4.0 ml and the tubes were centrifuged. The supernatants were poured off, plated to determine

* In all instances cell mass refers to the dry weight.

their content of isotope, and in some experiments assayed for L-glutamic acid by enzymic decarboxylation. Comparative experiments have shown that this procedure and the extraction methods described previously[1] give identical results.

For the determination of solute penetration into intra- and extracellular spaces, washed cells were adjusted to a density of approximately 100 mg/ml in water. A sample was dried to constant weight at 98° to determine the actual dry-cell concentration. A measured volume was placed in a plastic centrifuge tube and the cells were sedimented at 25000 × *g* for 10 min. The supernatant was withdrawn and its volume measured using a graduated pipette having a finely-drawn tip. The pellet volume was calculated by difference. In most experiments this first cell pellet was resuspended in 0.8 ml of the buffered salts solution used in uptake experiments, and the centrifugation and measurement of supernatant volume were repeated prior to resuspension in the test solution. The cell pellet was macerated gently and then mixed with a pellet volume or less of solutions containing dextran, inulin, [^{14}C]sucrose or [^{14}C]glycerol. These substances were dissolved in the buffered salt solutions used in uptake experiments. The suspensions were incubated for various times at 37° or in an ice bath and recentrifuged as before. The supernatant again was withdrawn and its volume measured. The isotope concentration in the supernatant was determined by plating 50-μl aliquots and counting as described previously[1]. The dilution of isotope by the water available in the cell pellet was determined by comparison to standards prepared by diluting the stock solution over the expected range of concentration changes. In addition, sucrose and inulin were determined by a modification of the method of Roe[12], and dextran and inulin by a modified anthrone method[13].

RESULTS

Previously described experiments have established that cells of *L. arabinosus* take up and accumulate large amounts of glutamic acid (approx. 10 mg; 70 μmoles/100 mg cells) when incubated at 37° in phosphate buffers containing glucose. The intracellular glutamate is readily extractable and there is little metabolic loss of the amino acid. Accumulation occurs in opposition to large apparent concentration gradients, shows a high degree of structural specificity and continues only while glucose is metabolized. It was also found that cells grown in vitamin B_6-deficient medium accumulate much smaller amounts of L-glutamic acid than do cells grown with an excess of the vitamin[2] (Fig. 1). With LB_6 cells, uptake proceeds normally for a few minutes and then terminates abruptly at unusually low levels. In view of the possibility that these cells might have defective cell walls and thus be incapable of resisting large increments in intracellular osmotic pressure, the effect on uptake of raising the extracellular osmotic pressure was studied. The effect of high concentrations of sucrose on glutamate accumulation by LB_6 cells is illustrated in Fig. 1. As the sucrose concentration was raised, the initial rate of uptake was maintained for longer periods with the result that increasingly large amounts of glutamate were accumulated. In most experiments, sucrose at 0.6–0.9 M in the buffer permitted LB_6 cells to accumulate approximately as much glutamate in a 90-min experiment as do HB_6 cells incubated under standard uptake conditions. Control experiments established that this additional accumulation did not occur at 4°, or at 37° in the absence of glucose and that the amount of intracellular glutamate predicted by the isotope content of the cell extract agreed closely

with the L-glutamate content as determined by enzymic assay. Previous studies[2] revealed that a vitamin B_6-deficiency does not modify the initial rate of amino acid uptake indicating, therefore, that the vitamin very likely is not involved as a mandatory catalyst directly in the accumulation process. This conclusion is strengthened by the present observations which show that in the presence of sucrose, cells depleted of all but traces of the vitamin also accumulate essentially normal total amounts of amino acids at rates comparable to those found with HB_6 cells.

As shown in Table I, a large number of structurally unrelated substances stimulate accumulation at high concentrations. In addition to sucrose, glucose, galactose, lysine, KCl, NH_4Cl and a number of other inorganic salts permitted the accumulation of markedly larger amounts of glutamate. A number of other substances such as sorbitol and fructose were much less effective, possibly because they penetrate the cell more rapidly. In keeping with this view, glycerol, a substance known to enter many cell types rapidly, failed to stimulate uptake. Ribose and rhamnose also showed

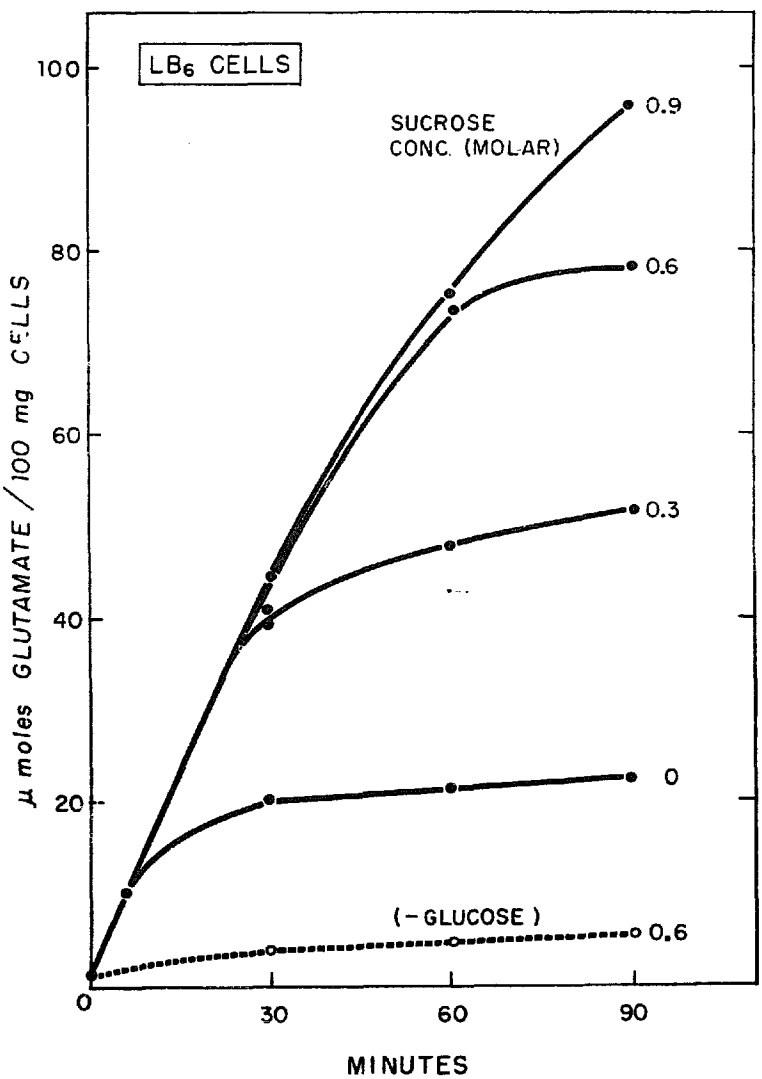

Fig. 1. Stimulation of glutamic acid accumulation in vitamin B_6-deficient *L. arabinosus* by addition of sucrose to the incubation buffer. The numbers next to the curves show the sucrose concentrations used. Otherwise, incubation was under standard uptake conditions except for the lowest curve which shows the effect of omitting glucose.

neither a stimulatory or inhibitory effect. A number of substances were markedly inhibitory. Of interest is the behavior of NaCl which became inhibitory abruptly as the concentration was raised from 0.6 to 0.9 M. The diverse nature of the substances

TABLE I

STIMULATION OF GLUTAMATE ACCUMULATION BY VARIOUS SUBSTANCES

L. arabinosus ($LB_6(+)$) was incubated for 90 min under standard uptake conditions modified to include the test substance at the stated concentration. The results were taken from a number of experiments in all of which sucrose was used as a reference stimulatory compound. I, inhibition.

Degree of activity	*Compound*	*Percent of maximum stimulation*		
		0.3 M	*0.6 M*	*0.9 M*
High stimulation	Sucrose	47	92	100
High stimulation	Glucose	32	66	72
High stimulation	Galactose	16	45	57
High stimulation	Lysine	50	74	74
High stimulation	KCl	58	97	91
High stimulation	KBr	46	85	76
High stimulation	KNO_3	50	80	61
High stimulation	NH_4Cl	70	85	71
Moderate stimulation	Sorbitol	10	34	48
Moderate stimulation	Fructose	7	13	24
Moderate stimulation	Potassium acetate	22	30	I
Moderate stimulation	NaCl	40	51	I
Moderate stimulation	$MgCl_2$	71	22	I
No stimulation	Glycerol	0	0	0
No stimulation	Rhamnose	0	0	0
No stimulation	Ribose	9	0	0
Inhibition	Potassium lactate	15	I	I
Inhibition	Glycine	I	I	I
Inhibition	KH_2PO_4	I	I	I
Inhibition	KCNS	I	I	I

TABLE II

PERMEABILITY OF *L. arabinosus* TO SUCROSE AND GLYCEROL

Approx. 100 mg of centrifuged cells were resuspended and incubated at 37° for 15 or 45 min in an equal volume of the test substance dissolved in 0.12 M phosphate buffer. Dextran (av. mol. wt. 60000–90000) was used at 100 mg/ml, inulin at 50 mg/ml, sucrose at 0.2 or 0.3 M, and glycerol at 0.1 M. The cells were centrifuged and the supernatant analyzed as described under METHODS. All incubations were carried out in duplicate. The values shown are averages of 4 experiments with each cell type. The indicated spaces were obtained by subtracting the volume accessible to the test substance from the total pellet volume.

Space measured	*Test substance*	*HB_6 cells*	*LB_6 cells*
		Volume (ml/g dry wt.)	
Total pellet	—	4.16	4.43
Total cell	Dextran	3.15	3.13
Total cell	Inulin	3.04	3.19
Sucrose-impermeable	Sucrose	2.88	2.71
Sucrose-impermeable	[^{14}C]Sucrose	2.50	2.33
Glycerol-impermeable	[^{14}C]Glycerol	1.15	1.28

possessing stimulatory activity and the high concentrations required indicate that these effects are osmotic in nature, *i.e.*, substances which reduce extracellular water activity can stimulate accumulation in LB_6 cells.

Further evidence favoring this interpretation is presented in Fig. 2 which shows that KCl and sucrose have precisely equal stimulatory effects on glutamate uptake over a wide range of iso-osmotic concentrations. At high concentrations sucrose has a greater tendency than KCl to inhibit uptake. This may be related to an unequal tendency of these substances to enter the cell during prolonged incubation.

A possible explanation of these findings is that intracellular solutes and/or accumulated glutamate occur in an osmotically active form. In nutritionally normal cells the resultant stress attendant upon the influx of water can be accommodated without reduction in activity of the accumulation catalysts. However, in vitamin B_6-deficient cells because the wall is less rigid the influx of water induced by these substances might distend the membrane sufficiently to modify the activity of the transport catalysts contained therein and permit equilibration of the entry and exit rates at a lower than normal extracellular concentration. The ability of sucrose to restore normal uptake, therefore, would require that it be confined largely to the extracellular phase where it could retard the movement of water into the cell.

Consequently, measurements of the cell space available to various substances were carried out to determine whether their activity in stimulating accumulation

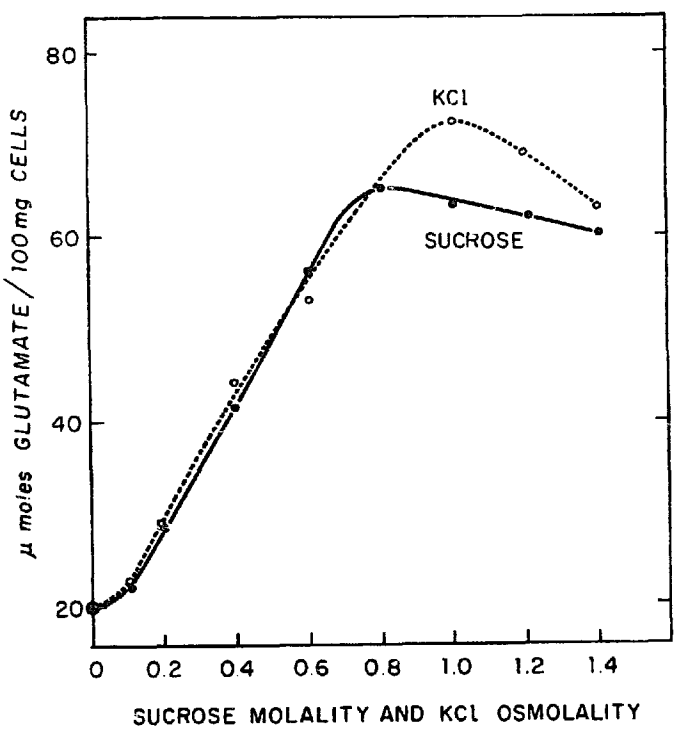

Fig. 2. Comparative effectiveness of sucrose and KCl in stimulating glutamate accumulation by LB_6 cells. In order to maintain equal cell concentrations at equivalent levels of sucrose and KCl, the amount of these substances required to give the indicated molal concentration was added to the usual volume (3.45 ml) of uptake buffer containing glucose and L-[^{14}C]glutamic acid. Thus the total volumes increased as the molality was increased, particularly in the set of tubes containing sucrose. However, the volume of water, and hence the concentration of cells, remained constant. Osmotically equivalent concentrations of sucrose and KCl were calculated using published values for water activities[14,15]. The amount of glutamate was calculated from the isotope content of cell extracts. Incubation was for 100 min at 37°.

correlated with an inability to penetrate cell water. Most of these experiments were performed using the thick cell suspension technique as described above (see METHODS). The results of incubations performed in the absence of glucose (to minimize pH changes) are summarized in Table II. Dextran and inulin were used to measure the intercellular water space and, therefore, the total cell volume (*i.e.*, the difference between the total pellet volume and the pellet volume penetrated by these substances). Sucrose was used as a typical accumulation stimulatory substance and glycerol as a typical non-stimulatory substance. The total pellet and intracellular volumes of LB_6 cells were slightly higher than those found with HB_6 cells. This may be related to the slight bulging which is seen in these cells[3] and the possibility that the cell wall may be more porous than normal. The intercellular volume constitutes, in tightly packed pellets of this rod-shaped organism, approx. 27 % of the pellet volume. The size of of this space varied with the molecular weight of the test material, being smallest for high-molecular-weight dextrans. This suggests that part of the extracellular water space includes cell wall interstices which can be penetrated by substances of smaller molecular dimensions such as raffinose and most likely also sucrose. Glycerol rapidly entered all but 28 % of the total pellet volume. The glycerol-impermeable volume is somewhat larger than expected (assuming a specific volume for cell solids of 0.8 ml/g) indicating that a small fraction of cell water is not accessible to glycerol. Sucrose was excluded from a much larger portion of the cell volume. The sucrose-impermeable volume in HB_6 cells is only slightly smaller than the dextran- and inulin-impermeable volumes indicating that there is a barrier to sucrose in the region of the cell membrane. With LB_6 cells the sucrose-impermeable volume was slightly smaller and more variable. In some cases, it approached that found with HB_6 cells, in others it was distinctly lower. In both cell types the sucrose-impermeable volume measured isotopically was consistently lower than that measured by the colorimetric assay. This appears to be caused by the elution of material from the cells which increased the isotope self-adsorption error and, therefore, the apparent dilution of sucrose. Control suspensions in buffer also showed that only insignificant amounts of material which interfered in the colorimetric assays were eluted from the cells. The colorimetric determination, therefore, appears to be more reliable.

It is clear that in HB_6 cells sucrose does not equilibrate with most of the cell volume accessible to glycerol. In LB_6 cells there is a possibility that sucrose enters a small portion of the glycerol-permeable volume. Since LB_6-cell pellets frequently contain somewhat larger numbers of non-viable cells, a more likely possibility is the occurrence of a small population of cells which is completely penetrated by sucrose. Amino acid uptake by LB_6 cells in the presence of sucrose occasionally is slightly lower than the maximal levels attained by HB_6 cells, again suggesting the presence of totally inactive and possibly completely permeable cells.

These experiments were performed in the absence of glucose to avoid the large changes in pH which would result from glycolysis by such concentrated masses of cells. Thus, the experiments described above did not exclude the possibility that sucrose might equilibrate more completely with cell water in the presence of glucose if its entry was dependent on the expenditure of energy. The experiments of ABRAMS[16], which were published after the completion of these studies, demonstrated an energy-dependent entry of sucrose into *S. faecalis* protoplasts. Accordingly, the entry of ^{14}C-labeled sucrose into LB_6 cells was studied in the presence of glucose under con-

ditions closely similar to those of amino acid uptake experiments. In these experiments the sucrose-impermeable cell volume measured in the absence of glucose was essentially the same as that found previously in the thick cell suspension studies.

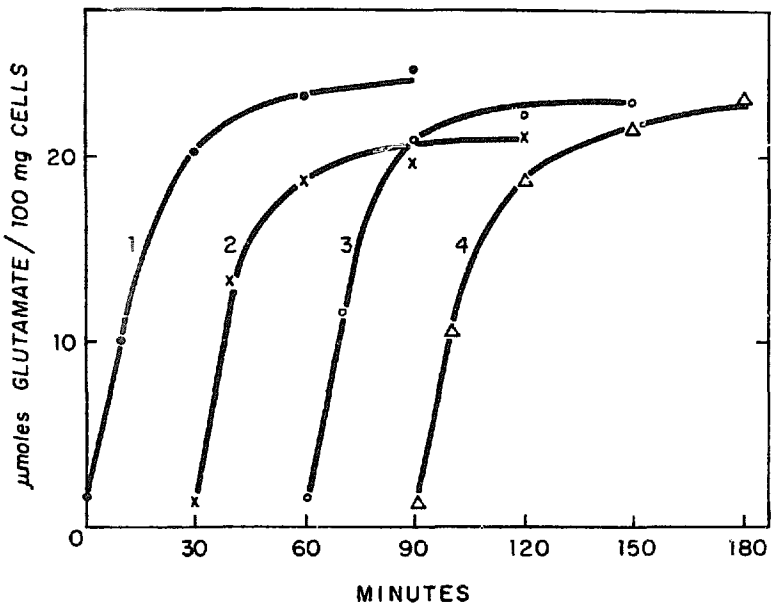

Fig. 3. Effect of preincubation in buffer on accumulation of glutamate by vitamin B_6-deficient *L. arabinosus*. Cells were incubated under standard uptake conditions except that glutamic acid was omitted and added after the following periods of preincubation at 37°: 1, 0 min (control); 2, 30 min; 3, 60 min; 4, 90 min.

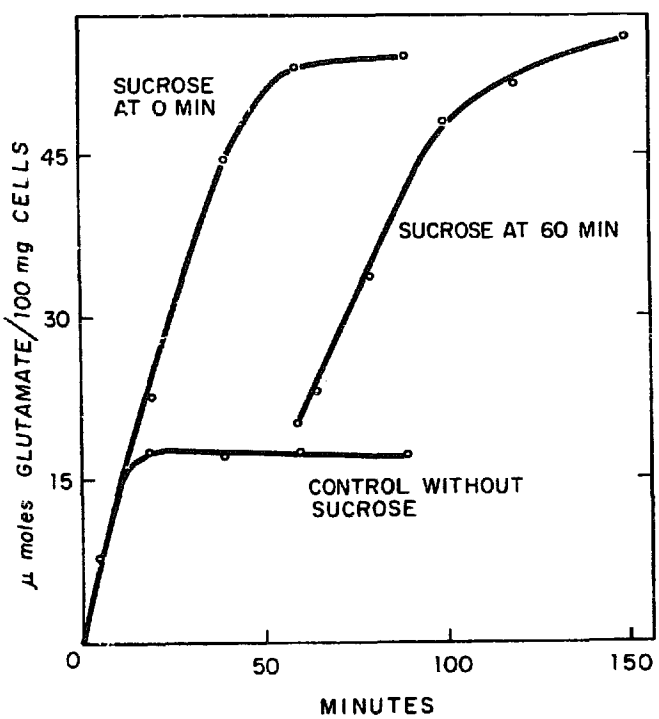

Fig. 4. Response of *L. arabinosus* (LB_6) to sucrose following accumulation of glutamic acid. Three sets of tubes were incubated as follows: under standard uptake conditions (control), with sucrose (0.4 M) added at zero time, and with sucrose added after 60 min of accumulation. The preincubation of the latter set was carried out at 3.0 ml making the cell and glutamate concentrations somewhat higher than in the controls. The addition after 60 min of 0.75 ml of sucrose at 2.0 M brought the constituents of all incubation mixtures to the same concentrations.

This volume did not change significantly during incubation for 45 min in the presence of glucose showing that glycolysis did not modify the distribution of sucrose. It appears likely, therefore, that sucrose exerts a beneficial effect on amino acid accumulation even though it is exluded from most of the cell volume, thus supporting the proposal that it acts by preventing the influx of water into the cell.

Additional characteristics of sucrose-stimulated uptake

Exposure of $LB_6(+)$ cells in the absence of sucrose to buffer containing glucose for extended periods of time before the addition of glutamate did not significantly alter the rate or amount of uptake (Fig. 3). The stability of the initial rate even after 90 min of preincubation shows that exposure to the buffer alone does not cause abnormal accumulation. The data are consistent with the possibility that accumulation itself may be a condition required for expression of the defect in this process. On the other hand, as shown in Fig. 4, the accumulation of a small glutamate pool by LB_6 cells in the absence of sucrose does not modify the subsequent response of these cells to sucrose even after 60 min preincubation. These results indicate that cells in which net accumulation has stopped at submaximal levels have not sustained irreversible damage to the accumulation system.

Although uptake of amino acid in HB_6 cells requires the metabolism of glucose, the accumulated amino acid is only slowly lost from the cell during subsequent incubation in water or buffer[1]. As shown in Fig. 5 the amino acid accumulated by $LB_6(+)$ cells in the presence of sucrose is retained only so long as sucrose is present in the

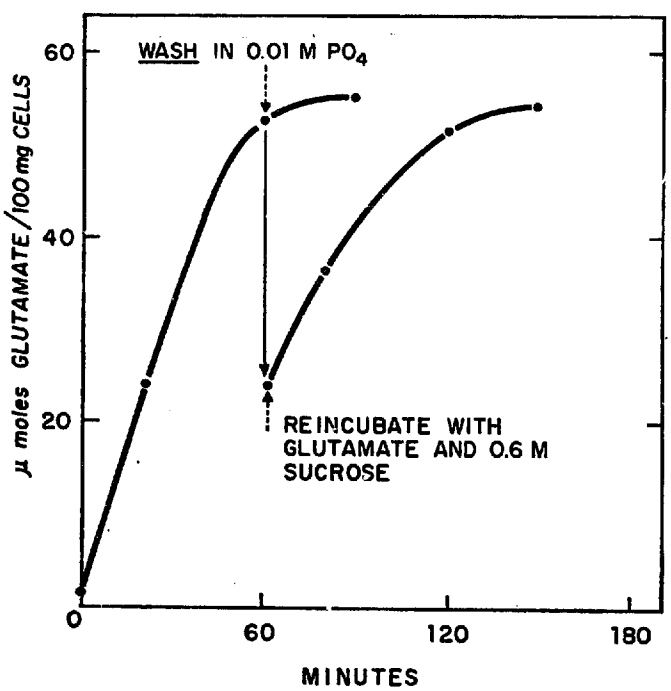

Fig. 5. Effect of resuspending *L. arabinosus* ($LB_6(+)$) in dilute buffer on the retention and reaccumulation of a large glutamic acid pool. Cells were incubated under standard uptake conditions with 0.6 M sucrose. At 60 min the cells were centrifuged at 5000 × *g* for 7 min and the supernatant was discarded. A control aliquot was resuspended in a series of tubes containing glucose, [^{14}C]glutamate and 0.6 M sucrose as in the original incubation. The other aliquot was rapidly resuspended in 0.01 M phosphate buffer (pH 6.8) at 4° and immediately recentrifuged at 5000 × *g* for 7 min. A cell sample was taken to determine the [^{14}C]glutamate content and the remainder was resuspended in tubes containing glucose, L-[^{14}C]glutamate and 0.6 M sucrose and incubated at 37°. Cell glutamate levels were calculated from ^{14}C contents of cell extracts.

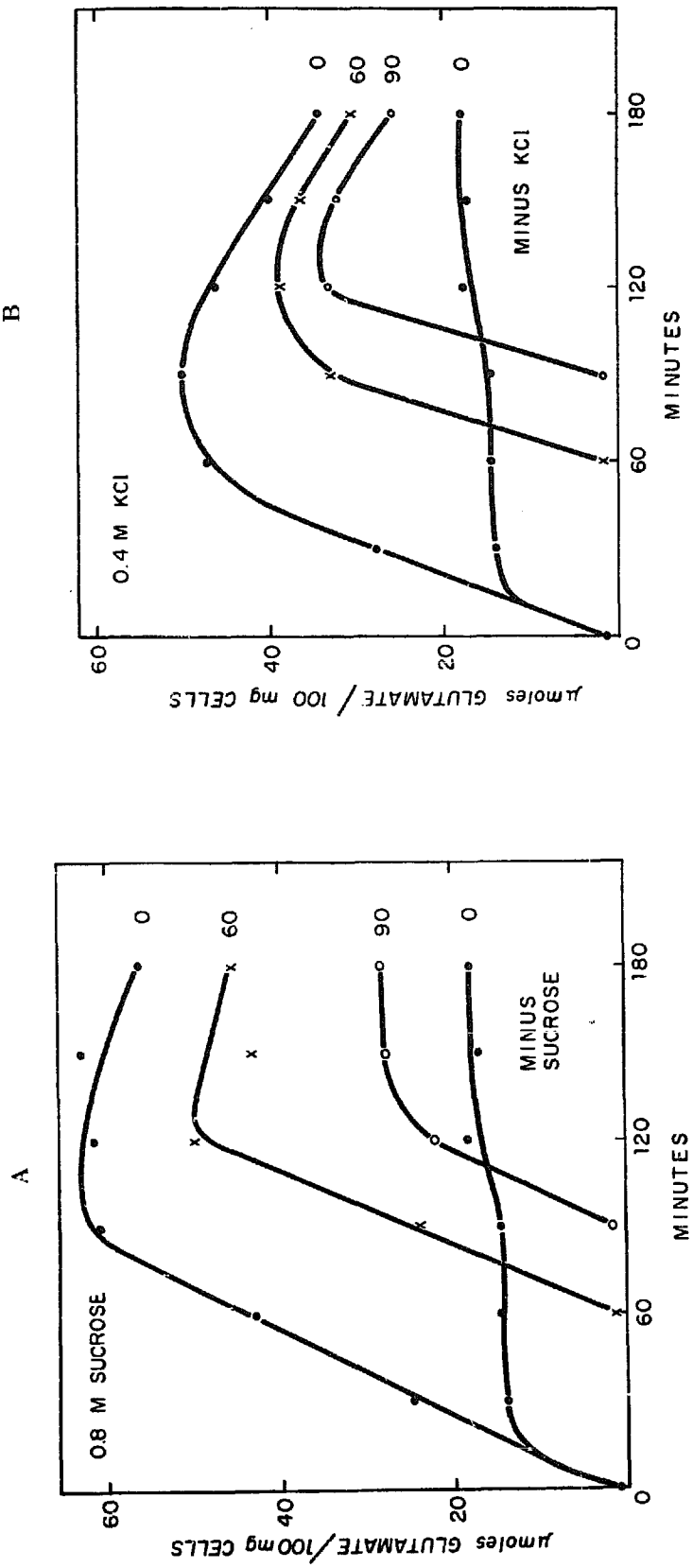

Fig. 6. Effect of preincubation with (A) 0.8 M sucrose, or (B) 0.4 M KCl, on glutamate accumulation by *L. arabinosus* ($LB_6(+)$). Cells were incubated at 37° in buffer and glucose with the indicated compounds. At the times shown on the curves, L-[^{14}C]glutamic acid was added and the incubation was continued. To ensure an adequate supply of glucose, two-thirds the usual level (0.018 M) was provided in all tubes at the start of the experiment, and a second equal supplement was added at 60 min to all remaining tubes.

buffer. When it is removed, there is a very rapid loss of amino acid from the cell. This loss, however, appears to occur with little damage to the accumulation system since subsequent addition of sucrose and labeled amino acid to these cells is followed by reaccumulation of the amino acid.

Extended incubation of glutamate-loaded cells in sucrose-containing buffer does lead to a slow loss of amino acid from the cell. A comparable decline in the amount of amino acid which can be accumulated also is seen when cells are preincubated with sucrose or KCl prior to the addition of labeled amino acid (Figs. 6 A and B). This suggests that these substances do in fact enter the cell slowly or that there is a reduction in the activity of the accumulation process under these conditions. It should be noted that this decline becomes prominent at 90 min and later, which is longer than the incubation periods used to establish the exclusion of sucrose from most of the intracellular water (Table II).

Proline and alanine uptake

L. arabinosus accumulates a number of amino acids in large amounts*. In all cases, accumulation depends on the simultaneous metabolism of glucose. Although some amino acids (*e.g.*, alanine, glycine, threonine) compete with one another and may, therefore, interact with the same catalytic site, accumulation of other amino acids appears to occur by independent routes. Rate studies, for example, have revealed little or no competitive interactions between alanine, proline or glutamic acid. It was of interest, therefore, to determine whether the uptake of alanine and proline also would be reduced by a vitamin B_6 deficiency and if so, whether normal accumulation could be restored by raising the extracellular osmotic pressure. As shown in Figs. 7 A and B, a vitamin B_6 deficiency markedly reduces the amount of alanine or proline which *L. arabinosus* can accumulate. In both instances, 0.8 M sucrose in the buffer permits essentially normal accumulation by LB_6 cells. Occasionally proline accumulation by $LB_6(+)$ cells incubated with sucrose has exceeded the amounts retained by HB_6 cells.

Vitamin B_6-deficient cells accumulate reduced amounts of proline and alanine even though the maximum capacity for these amino acids (as observed in HB_6 cells) does not exceed the level of glutamic acid which they can accumulate. The levels of proline and alanine retained in LB_6 cells should have been altered only slightly if the intracellular osmotic activity of these amino acids alone controlled their further accumulation. These findings suggest, therefore, that the need for osmotic protection is imposed by factors other than, or in addition to, the accumulated amino acid.

Fig. 8, which compares the increases in glutamate, proline and alanine accumulation as the extracellular sucrose concentration is raised, also shows that the pattern of amino acid uptake in B_6-deficient cells cannot be understood solely in terms of the osmotic activity of sucrose outside and of the amino acid uniformly distributed inside the cell. The increases in alanine and proline accumulation do fall along straight lines having the same slope. The alanine capacity is higher and maximum stimulation, therefore, requires higher concentrations of sucrose. Both properties suggest that the amino acids play a role in determining the need for osmotic protection. However, the relationship is not a simple one. Approx. 4 μmoles/ml of extracellular sucrose enable the cells to retain an additional load of alanine or proline, which if they were uniformly

* J. T. HOLDEN, unpublished observations.

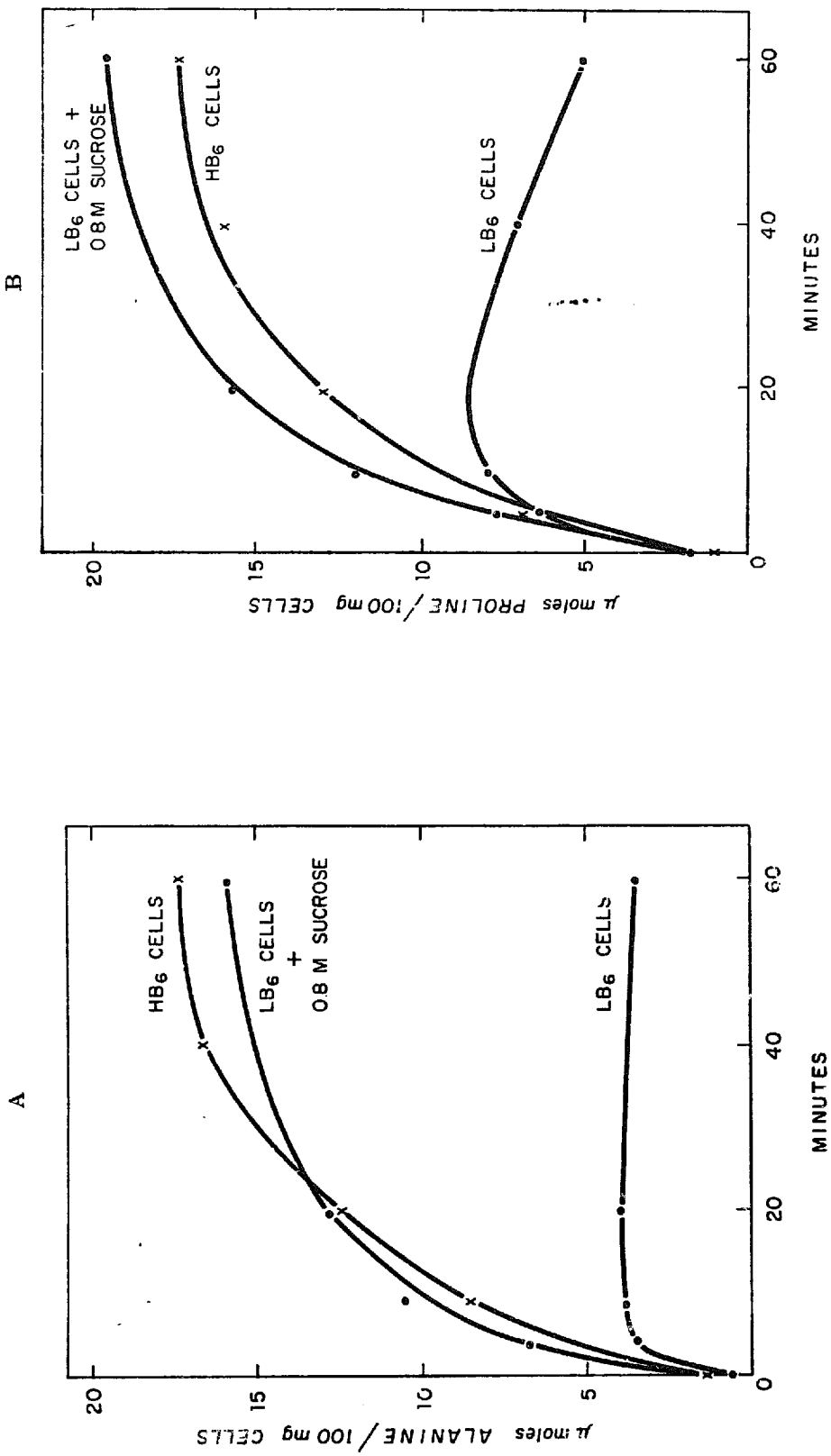

Fig. 7. Effect of a vitamin B_6 deficiency on the accumulation of (A) alanine, and (B) proline, by *L. arabinosus*. Incubation was under standard uptake conditions except for the use of uniformly labeled L-[^{14}C]alanine or uniformly labeled L-[^{14}C]proline at 0.002 M in place of glutamic acid. When used with LB_6 cells, sucrose was present at 0.8 M. The intracellular amino acid content was calculated from the isotope content of cell extracts.

distributed in the total available cell water (approx. 2.0 ml/g, *cf.* Table II) would increase their intracellular concentration by only 1 μmole/ml. Assuming that these amino acids have activity coefficients of approx. 1, it would seem that other sub-substances are contributing to the osmotic load, or that the amino acids are sequestered in a fraction of the cell water with a corresponding increase in concentration in this region.

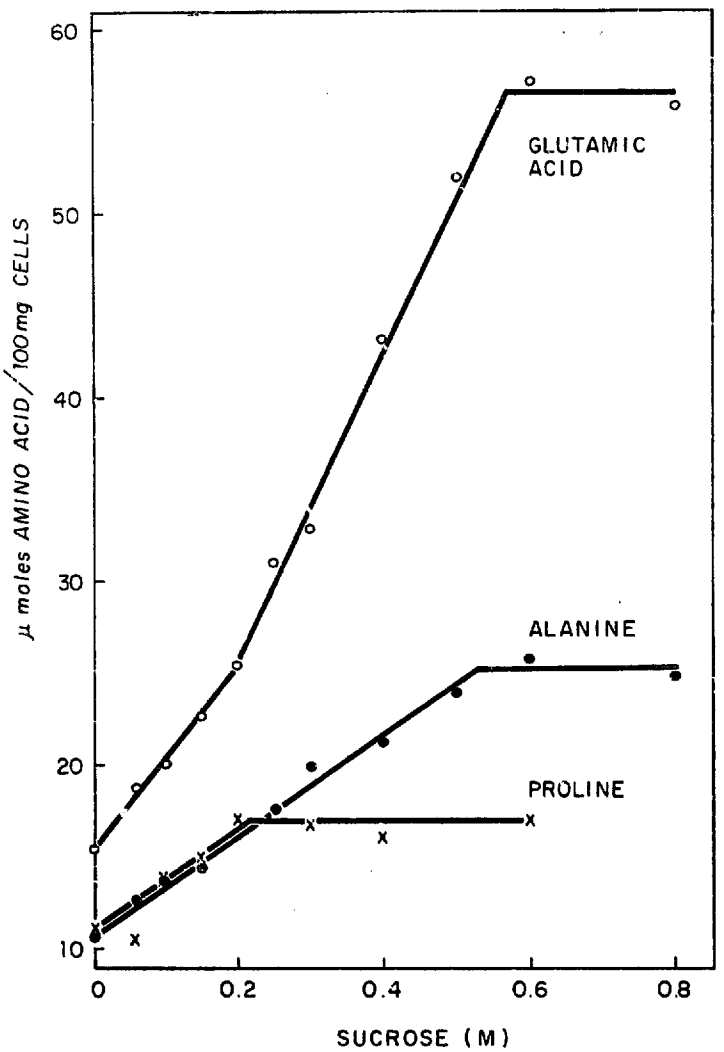

Fig. 8. Stimulation of alanine, proline and glutamic acid accumulation in $LB_8(+)$ cells of *L. arabinosus* at various extracellular sucrose concentrations. Incubation was at 37° for 60 min using the indicated concentration of sucrose in the standard uptake buffer containing the L-[^{14}C]amino acid at 0.003 M. Accumulation was calculated from the amount of isotope found in cell extracts.

The increases in glutamate accumulation fall on two lines having different slopes at low and high extracellular sucrose (or KCl) concentrations. It is only in the range 0.20 to 0.50 M sucrose that the increases in intracellular glutamate concentration approach levels suggesting an osmotic interaction. In this range an increase of 2 μmoles per ml in extracellular sucrose concentration leads to an apparent increase of 0.84 μmoles/ml in intracellular glutamate concentration. These values are reasonably close to the expected ratio (somewhat less than 2) assuming that at the intracellular pH glutamate behaves as a uni-univalent ion of the monobasic salt. Water activities of

TABLE III

EFFECT OF VITAMIN B_6 DEFICIENCY ON THE SIMULTANEOUS ACCUMULATION OF ALANINE AND GLUTAMATE

Both amino acids were used at 0.003 M. Sucrose concentration, 0.3 M.

Cell type	Glutamate accumulation			Alanine accumulation		
	Alone	Presence alanine	Reduction (%)	Alone	Presence glutamate	Reduction (%)
	(μmoles/100 mg)			(μmoles/100 mg)		
HB_6	80.0	75.0	6.2	17.9	15.8	11.8
LB_6	19.7	17.0	13.7	11.2	8.0	28.6
LB_6 (+ sucrose)	50.5	44.3	12.3	25.6	14.0	45.3

TABLE IV

RELATION BETWEEN GLUTAMATE ACCUMULATION AND LEAKAGE OF 260 mμ-ABSORBING MATERIAL

Test substance	Concn. (M)	Glutamate accumulation			Leakage of 260 mμ-absorbing material*		
		(a) Control	(b) + Test substance	(c) Percent stimulation**	(d) Control	(e) + Test substance	(f) Percent reduction***
		(μmoles/100 mg cells)			Absorbancy		
Sucrose	0.5	21.1	69.2	228	1.86	1.49	20
Sucrose	0.5	19.4	58.5	201	1.95	0.91	53
Sucrose	0.6	31.3	89.6	186	1.40	0.53	62
Sucrose	0.6	16.9	63.6	276	2.33	0.98	58
Galactose	0.9	15.8	47.8	202	2.40	2.09	13
Lysine	0.6	15.8	39.7	151	2.40	2.21	8
KCl	0.6	31.3	87.9	180	1.40	1.26	10
KCl	0.6	16.9	62.6	270	2.33	1.81	22
KBr	0.6	15.8	40.2	154	2.10	1.93	8
NH_4Cl	0.6	15.8	44.6	182	2.10	0.91	57

* Values shown are the absorbancies of the buffer after incubation of LB_6 cells at 1.6 mg/ml under standard uptake conditions (control) or in the presence of the indicated compound for 60 or 90 min. See ref. 5 for additional experimental details. The values shown for sucrose are from separate experiments.

** $\frac{(b) - (a)}{(a)} \times 100.$

*** $\frac{(d) - (e)}{(d)} \times 100.$

the monosodium salt of glutamic acid have been calculated from freezing-point-depression data[17].

Studies on the simultaneous accumulation of two amino acids revealed interactions in LB_6 cells also suggesting that the amino acids may contribute significantly to the osmotic load (Table III). With HB_6 cells, alanine or glutamic acid accumulation are reduced only slightly when the other amino acid is simultaneously being taken up. This mutual inhibition is greatly increased in LB_6 cells, the effect being more pronounced on alanine than on glutamate accumulation. It is of interest that 30.8 μmoles/100 mg additional glutamate were accumulated by LB_6 cells incubated with this amino acid alone and 0.3 M sucrose. When both amino acids were accumulated

simultaneously the sucrose-stimulated increment was 27.3 μmoles of glutamate and 6.0 μmoles of alanine. Assuming that intracellularly alanine has approximately one-half the osmotic effect of glutamate, the total increment expressed in terms of the equivalent glutamate concentration would be 30.3 μmoles/100 mg; *i.e.*, the combined load did not exceed the amount of glutamate accumulated by itself.

Leakage of 260 mμ-absorbing material

In addition to their impaired amino acid accumulation, LB_6 release abnormally large amounts of 260 mμ-absorbing substances and protein during incubation in uptake buffer[5]. It was of interest, therefore, to determine whether the osmotically stimulated increase in amino acid retention was accompanied by a reduction in nucleotide leakage. The observations recorded in Table IV show a lack of correlation between these effects. For example, KCl, which consistently stimulated amino acid accumulation never significantly decreased and sometimes increased the leakage of 260 mμ-absorbing substances. The effect of sucrose itself on nucleotide leakage was variable. In some cases, there was a marked reduction in leakage, in others there was only a small change. It is apparent, therefore, that conditions which permit normal amino acid retention in LB_6 cells do not always prevent the loss of nucleotides from the cell. Additional studies have shown that sucrose decreases leakage primarily by reducing RNA degradation rather than by improving the retention of nucleotides in the intracellular pool. Thus, even in those cases where leakage is reduced there is no clear evidence that permeability to nucleotides has been modified.

A number of the experiments reported here also provide information bearing on the recurrent suggestion that intracellular polymers such as RNA may be involved in retaining amino acid pools. In Fig. 4, for example, the cells incubated in buffer without sucrose for 60 min degraded 25 % of the cellular RNA. The cells diluted out of sucrose-containing buffer in Fig. 5 sustained a similar loss. Nevertheless, in both cases the subsequent addition of sucrose permitted accumulation of an amount of glutamate equal to that attained in cells which had been incubated from the start of the experiment in sucrose-containing buffer and which had not suffered as extensive a loss of RNA.

DISCUSSION

The studies described show clearly that the reduced amino acid accumulation activity imposed by a vitamin B_6 deficiency in *L. arabinosus* can be restored to normal by increasing the extracellular osmotic pressure. Besides demonstrating that vitamin B_6 does not function directly in the accumulation mechanism, these findings suggest that excessive water influx or overhydration of the cell interior interferes with the normal operation of this process. The relevance of this finding to the mechanism of amino acid accumulation in microorganisms over which there is still considerable disagreement (*cf.* HOLDEN[8], BRITTEN AND MCCLURE[18] for detailed discussion) deserves comment. While many investigators view this process as one involving active transport, it is clear that decisive proof on this point is lacking. In particular, it has not been excluded that a significant portion of the amino acid pool, internally synthesized or accumulated from external sources, is retained in association with intracellular binding sites. Interpreting the present findings in terms of the active-transport hypothesis in which accumulation would depend on the operation of membrane

catalysts, the influx of water in LB_6 cells attendant upon the accumulation of amino acid in the free form could distend the membrane, thereby modifying the relative rates of amino acid influx and efflux most likely bringing them into balance at a lower than normal intracellular concentration. It is equally plausible to propose, however, that an excessive concentration of water within the cell might also distort intracellular binding sites, thereby reducing their effectiveness in retaining amino acids. The extraction of cell water by increasing the tonicity of the extracellular fluid might reverse this process and restore site activity. Thus, the observation that normal accumulation capacity in vitamin B_6-deficient cells is dependent on osmotic protectants, by itself does not permit a choice between these alternate mechanisms of accumulation. However, it should be noted that the site theory predicts that extracellular sucrose would assist accumulation by extracting water already present in the cell, whereas the active-transport mechanism predicts that this stimulation would result from an inhibition of water influx which occurs while the cell was accumulating osmotically active amino acid.

An unequivocal demonstration that the accumulated amino acids contribute to the osmotic stress would favor the active-transport hypothesis. The experiments reported here tend in this direction but do not give a clear-cut answer to this question. Some of the evidence, in fact, indicates that much of the requirement for extracellular sucrose is not related to the amount of amino acid accumulated. For example, alanine and proline uptake by LB_6 cells are reduced markedly even though the maximum amount of these amino acids accumulated by HB_6 cells never exceeds the capacity of LB_6 cell to accumulate an osmotically equivalent amount of glutamate. In addition, the increments in alanine and proline accumulation as the extracellular sucrose concentration is raised are much lower than expected on the basis of a simple osmotic relationship. On the other hand, glutamate increments appear to be determined by an osmotic relation to the extracellular sucrose concentration. Furthermore, when two amino acids are accumulated simultaneously, there is a much more pronounced competition, for example, between alanine and glutamate in LB_6 than in HB_6 cells. This could result if both amino acids promoted water influx into a cell having a greatly limited capacity to oppose intracellular osmotic pressure. More convincing evidence in this direction has come recently from studies on amino acid uptake by *S. faecalis* protoplasts which show that the entry of amino acid in opposition to a concentration gradient results in swelling of the protoplast[8]. A determination of the quantitative relation between amino acid accumulation and protoplast swelling as in the studies of SISTROM[19] on galactoside accumulation by *E. coli* spheroplasts will allow a more conclusive statement regarding the mechanism of amino acid accumulation.

Investigation of another aspect of amino acid accumulation by vitamin B_6-deficient *L. arabinosus* may also facilitate a choice in this question of mechanism. Studies on the mechanism by which acetate, NH_4^+ and vitamin B_6 restore nearly normal accumulation capacity in LB_6 cells have revealed no major alterations in the composition of cells treated with these substances other than an increase in cell-wall substance[9,11]. Increasing the mass and most likely the rigidity of the cell wall would be expected to reduce membrane distensibility under the stress of water influx. Therefore, the favorable effect of wall synthesis on accumulation by B_6-deficient cells is readily understood in terms of a membrane-localized transport mechanism. On the other hand, it seems less plausible that the synthesis of wall

would modify the existing distribution of water within the cell sufficiently to effect a reactivation of adsorption sites. Thus, we are led again, on the basis of this evidence, to conclude that the membrane plays a crucial role in the accumulation process and to favor the active transport mechanism. Still, it must be recognized that this evidence is only permissive. It does not, for example, exclude the association of some amino acid with an internal retention site following uphill entrance catalyzed by a membrane component. Clearly, the final resolution of this question will require incontrovertible quantitative evidence concerning the intracellular state of the accumulated amino acid.

With regard to the identity of catalysts operating in the accumulation process, our present findings are clearly inconsistent with the suggested participation of vitamin B_6 in an entry reaction, thus confirming the conclusion reached in earlier studies[2]. OXENDER AND ROYER[20], and CHRISTENSEN[21], also have concluded recently that the effect of pyridoxal in stimulating amino acid accumulation in Ehrlich ascites-tumor cells is not explicable in terms of an increase in the entry rate, but may arise from a decrease in the rate of exit. In contrast to the present agreement of the findings with unicellular systems, evidence continues to appear which suggests that vitamin B_6 may have an effect on transport in organized tissues such as the intestine[22].

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