The Assimilation of Amino-acids by Bacteria

1. The Passage of certain Amino-acids across the Cell wall and their concentration in the internal environment of *Streptococcus faecalis*

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SUMMARY: By a method for determining the free amino-acid content of bacterial cells, *Streptococcus faecalis* (Lancefield Group D) was found to have free lysine, glutamic acid, ornithine and histidine in its internal environment, the amount depending to a certain extent upon the growth medium. The internal lysine and glutamic acid are unable to diffuse out of the cells when these are suspended in water or suitable salt solutions.

When the cells are grown in a medium deficient in amino-acids, the internal environment is also deficient and those cells can be used to study the conditions governing the passage of certain amino-acids into the cell. Lysine enters the cell by diffusion, probably passing the cell wall in the isoelectric state. Glutamic acid, glutamine and histidine cannot pass across the cell wall unless energy is supplied by some exergonic metabolism such as the simultaneous fermentation of glucose.

Under equilibrium conditions the internal concentration of free lysine or glutamic acid within the cell is markedly greater than that outside; the lower the external concentration, the higher the concentration difference across the cell wall.

Glutamic acid inside the cell can pass out into the external environment if fermentation is taking place. The final equilibrium conditions are the same whether the glutamic acid is entering or leaving the cell. Lysine cannot diffuse freely out of the cell, but outward migration takes place if fermentation occurs simultaneously. The uptake of lysine by deficient cells is decreased by the presence of glucose and this effect is, in turn, partially abolished by the uptake of glutamic acid in the presence of glucose.

Recent studies of bacterial metabolism have thrown considerable light on the amino-acid requirements of bacteria, and of the mechanisms utilized for the breakdown of amino-acids. Generally speaking, the organisms appear to fall into two groups. Members of the first group, which includes many organisms loosely classified as Gram-negative, are able to synthesize most if not all of their amino-acid requirements and also to produce a variety of catabolic mechanisms. Members of the second group, which includes many Gram-positive organisms, are often able to synthesize but few of their amino-acid requirements and have very restricted catabolic activities towards amino-acids. Moreover, the growth processes of the second group (e.g. *Strep. haemolyticus*) are markedly more susceptible to interference by bacteriostatic agents such as penicillin, the sulphonamides, certain dyes, etc., than are those of the first group (e.g. *Bacterium coli*). There would appear to be some fundamental difference between the two groups the elucidation of which should add materially to our knowledge of bacterial growth processes and their inhibition by chemical means. It is possible that this difference lies somewhere in the synthesis of protein by the

two groups, but little is known, as yet, of the biochemical changes that intervene between the synthesis of amino-acids and their eventual appearance as bacterial protein. This series of papers represents an attempt to investigate the processes involved in the assimilation of amino-acids by bacteria, the effect thereon of bacteriostatic agents, and the differences displayed by Gram-positive and Gram-negative organisms.

EXPERIMENTAL

Methods

Organism and growth media. The work described in this paper was carried out with a non-haemolytic Group D *Streptococcus* (N.C.T.C. no. 6782).

Two media were used for growth purposes:

Medium A consisted of a tryptic digest of casein + 0.1 % Marmite + 1.0 % glucose.

Medium B consisted of a simplified medium similar to that previously described (Gale, 1945*a*) with the difference that the Marmite was not hydrolysed and no cysteine was added.

For diffusion experiments, etc. the cells were suspended in a salt solution containing: 0.1 % KH_2PO_4 ; 0.33 % Na_2HPO_4 ; 0.1 % NaCl ; 0.07 % $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; the pH was adjusted to 7.2. Dry weights of organism in cultures and washed suspensions were estimated turbidimetrically by the use of a Hilger absorptiometer previously calibrated against suspensions of the organism of known dry weight.

Estimation of amino-acids. The estimations of the amino-acids described in the paper were carried out manometrically by the use of specific amino-acid decarboxylase preparations (Gale, 1945*b*, 1946*a*). These preparations estimate the free unsubstituted 'natural' amino-acids only.

Preliminary work

In the course of some investigations into the amino-acid constitution of various bacteria, it seemed probable that the method of analysis of acid hydrolysates of thick washed suspensions might give inaccurate results if significant amounts of free amino-acid were carried down from the medium either on the surface of the washed organisms or in their internal environments. To test this possibility, a thick suspension of streptococcal cells, grown in medium A and washed twice in water, was divided into two equal parts. One was hydrolysed in *c.* 5*N*-HCl for 24 hr. and the HCl then removed *in vacuo*, and the other was shaken with glass beads on the shaker (Curran & Evans, 1942) for 7 hr. after which the viable count was reduced to 0.001 % of the initial value. The two preparations were then made up to the same volume and their free amino-acid composition determined. The results are shown in Table 1; 31.0 % of the total glutamic acid estimated in the acid hydrolysate was found in the free state in the disintegrated cell suspension; 25.5 % of the total lysine, 25.0 % of the total histidine and 92.1 % of the total ornithine were also estimated in the cell disintegrate. No free arginine or tyrosine was found in the disintegrate.

The free amino-acids of the cell disintegrate might arise in a number of ways. First, it is possible that the cell protein becomes broken down during the disintegration of the bacterial cells; if this were the case it is difficult to see why no free arginine or tyrosine should be found and repetition of the disintegration

at 0° or in the presence of toluene or caprylic alcohol had no effect on the result. Secondly, the free amino-acid might be carried down from the medium absorbed on the surface of the organism. Thirdly, the amino-acids might exist in a free state in the internal environment of the cells. Using the decarboxylase method of estimating amino-acids it is possible to distinguish between these three possibilities. If a decarboxylase preparation is added to a suspension of bacterial cells the enzyme cannot penetrate the cell wall and any amino-acid estimated must therefore be present outside the cell. Fig. 1 shows the effect of

Table 1. *The amino-acid composition of Strep. faecalis cells*

Results expressed as amino-acid N as % total N.

Amino-acid	Acid hydrolysate (a)	Disintegrate (b)	Combined amino-acid (a-b)
<i>l</i> -(+)-Lysine	11.30	2.90	8.40
<i>l</i> -(+)-Arginine	5.19	0.10	5.09
<i>l</i> -(+)-Glutamic acid	5.60	1.74	3.86
<i>l</i> -(-)-Histidine	2.84	0.70	2.14
<i>l</i> -(+)-Ornithine	1.48	1.27	0.21
<i>l</i> -(-)-Tyrosine	0.81	Nil	0.81

adding glutamic acid decarboxylase from the side-bulb of a manometer to a thick suspension of washed streptococcal cells in M/5 acetate buffer pH 4.5 (Gale, 1945*b*). A small CO₂ output occurred, representing the decarboxylation of glutamic acid carried down with the cells during washing. The amount of this glutamic acid varied with the degree of washing of the cells prior to estimation. If an equivalent amount of the cell suspension is now disintegrated by shaking, the amount of glutamic acid estimated is greatly increased. The same result is obtained if the cell suspension is placed in boiling water for 10 min. before estimation. If the cells are disintegrated by mechanical means or disrupted by heat, glutamic acid is released in the free state and this free amino-acid must presumably be held within the internal environment of the cells prior to disruption. This is confirmed, as shown later (Gale & Taylor, 1946), by the fact that the same amount of free amino-acid is released from the cells if the permeability of the cell wall is altered by the action of detergent substances.

It is possible to estimate the free amino-acid content of the internal environment of the cells by carrying out decarboxylase estimations (1) on the intact cells, and (2) on disrupted cells, and so determining the amount of amino-acid released on disruption. Since placing the cell suspension in boiling water gives the same result as mechanical disintegration but in a much shorter time (10 min., instead of 6 hr.), the cells have usually been disrupted in this way in the work to be described. Fig. 1 gives representative results obtained with the glutamic acid decarboxylase and a thick suspension of *Strep. faecalis* cells. Using the other specific decarboxylase preparations, it was possible to show the existence of lysine, histidine and ornithine, in a free state in the internal environment of these cells. No free arginine or tyrosine was found probably because the organism possesses a very active arginine dihydrolase (Hills, 1940) and tyrosine decarboxylase (Gale, 1940) so that the growth

medium contains no free arginine or tyrosine at the time of harvesting (Gale, 1945*a*). The action of arginine dihydrolase gives rise to the formation of ornithine in the medium and this probably accounts for the ornithine content of the organism. Table 1 shows that 92.1 % of the ornithine estimated in the hydrolysed organism is found free in the disintegrate; allowing for the experimental errors involved in the various procedures it seems probable that all the hydrolysate ornithine arises from free ornithine carried down in the internal environment of the cells and that this amino-acid does not exist in a combined form in the protein of the organism.

By the use of this method for the estimation of the free amino-acid in the internal environment of the cells, it is possible to study the factors involved in the accumulation of such amino-acids within the internal environment and in their passage across the cell wall.

Diffusion of amino-acids out of the streptococcal cell

General method. Organisms grown in medium A, cells washed once, the internal amino-acid assayed, the cells then suspended in water or salt solution at a final cell suspension strength of *c.* 1 mg. dry weight cells/ml.; after a suitable interval the cells are centrifuged down and made up into a thick suspension for comparative assay.

When *Strep. faecalis* is grown in medium A the cells can be shown to possess free lysine and glutamic acid in their internal environment. To investigate whether this free amino-acid is able to diffuse out of the cells, a thick suspension of washed cells was taken, the internal lysine and glutamic acid assayed as described, and the cells then poured into distilled water at 0° to give a final cell suspension of approx. 1 mg. dry weight/ml. After 24 hr. the cells were centrifuged down and the internal amino-acid contents again determined. There appeared to be little or no loss of either lysine or glutamic acid from inside the cells. It was possible that the cells had been damaged by suspension in distilled water so the experiment was repeated, using the salt solution described as suspending medium. Samples of the cell suspension were centrifuged down at intervals and the internal lysine and glutamic acid estimated. Table 2 shows that over a period of 48 hr. at 2° there was no loss of internal lysine and about 25 % loss of internal glutamic acid. It would seem that there is no free diffusion of the amino-acids out of the cells. Table 2 also shows that the effect is not dependent on external pH within physiological limits although there is some loss of lysine when the external pH is 4.5.

Variation of internal amino-acid content with age of culture

The amount of free amino-acid existing in the internal environment of the cell at any moment must represent the balance between the amount passing through the cell wall from the external environment and the amount being metabolized either by breakdown or by synthesis into bacterial protein. To determine how the amount of free lysine or glutamic acid within the cell varied with the growth of the cell, samples of culture were taken during growth from a large-scale culture in medium A, the organisms centrifuged down, washed and the internal amino-acid content estimated. Table 3 shows the results expressed

as μ l. lysine or glutamic acid in the internal environment of 100 mg. dry weight of cells. Cells taken after the culture had been growing at 37° for 2½ hr. showed no lysine but a high concentration of internal glutamic acid. Cells harvested during the growth of the culture showed an increasing internal

Table 2. (a) *Loss of lysine and glutamic acid from the internal environment of Strep. faecalis cells on standing in amino-acid-free salt solution*

Temp.	Time (hr.)	μ l. amino-acid in internal environment assayed on 95 mg. dry weight of cells	
		Lysine	Glutamic acid
2°	0	152	208
	3	158	224
	6	158	200
	24	152	176
	48	161	152
37°	0	152	208
	6	143	198

(b) *Effect of external pH on loss of amino-acids as above at 2° for 24 hr.*

Cells suspended in salt solution so that final concentration = approx. 1 mg./ml. during incubation.

External pH	μ l. amino-acid in internal environment assayed on 78.4 mg. dry weight of cells	
	Lysine	Glutamic acid
Initial content	145	102
4.5	74	88
5.5	118	108
6.5	126	108
7.5	129	110
8.5	102	93
9.5	98	100

Table 3. *Variation of internal concentration of free amino-acids with age of culture*

Age of culture (hr.)	Growth mg. cells/ml. medium	Cell sample (mg.)	Internal concentration of l-(+)-lysine		Internal concentration of l-(+)-glutamic acid	
			Per sample (μ l.)	Per 100 mg. cells (μ l.)	Per sample (μ l.)	Per 100 mg. cells (μ l.)
2½	0.052	19.5	Nil	Nil	36	185
4	0.316	22.4	12	54	41	184
6	0.440	20.8	45	216	41	197
8	0.530	22.2	50	225	57	256
10	0.530	28.6	61	214	58	203
24	—	21.2	39	184	18	57

content of both lysine and glutamic acid, reaching a maximum value at the period of cessation of active cell division. After growth had ceased there was a small loss but this was not marked until after 24 hr.; this loss may be due to autolysis of a proportion of the cells. When it is desired to produce cells containing a high internal content of free amino-acid (Gale & Taylor, 1947) the cells should therefore be harvested soon after the cessation of growth in medium A.

Passage of amino-acids into streptococcal cells

The cells used hitherto were grown in the amino-acid-rich medium A and were found to possess a high internal content of certain free amino-acids. When the cells were grown in an adequate medium in which the amino-acid content was just sufficient to supply growth needs, e.g. medium B, the internal content of lysine, glutamic acid and histidine was greatly decreased (Table 4).

Table 4. *Variation of internal concentration of free amino-acid with nature of growth medium*

Amino-acid	Growth medium A		Growth medium B	
	$\mu\text{l./ml.}$ medium	$\mu\text{l. in internal}$ environment of 100 mg. cells	$\mu\text{l./ml.}$ medium	$\mu\text{l. in internal}$ environment of 100 mg. cells
<i>l</i> -(+)-Lysine	150-200	170-200	2-3	15-25
<i>l</i> -(+)-Glutamic acid	150-180	200-300	2-5	9-15
<i>l</i> -(-)-Histidine	45-60	20-35	1-2	3-5

Such cells are deficient in internal amino-acids as compared with those harvested from medium A and will be referred to below as 'deficient cells'. By suspending such deficient cells, after washing, in solutions of various amino-acids it is possible to follow the appearance of the amino-acids in the internal environment and to study the factors involved in their passage across the cell wall. The results vary with the age of the culture and the best effects of the type described below are again obtained with cells harvested at about the end of active cell division. In medium B cells should be harvested after about 8-10 hr. growth at 37°.

General method. Organisms grown in medium B, cells washed once, the internal amino-acid assayed, the cells suspended in salt solution containing known concentrations of amino-acid with or without glucose (final suspension strength = approx. 1 mg./ml.), after suitable treatment the cells are centrifuged down and made up into thick suspension for comparative assay.

Lysine

When a thick washed suspension of *Strep. faecalis* cells grown in medium B is assayed for internal lysine, the cells suspended in a solution of lysine (100 $\mu\text{l./ml.}$, as above) and then centrifuged out of solution at intervals for assay, it is found that the internal lysine content begins to increase immediately the deficient cells come into contact with the amino-acid. Fig. 2 shows that the internal lysine increases rapidly and reaches an equilibrium concentration within 20-30 min. of contact at 10° and pH 7.2. This rapid rate of passage of lysine into the cell is in marked contrast to the complete absence of outward diffusion previously studied. The position of the experimental points on the time scale is difficult to determine with accuracy as the organisms have to be centrifuged out of the lysine solution before assay is possible. The cells are usually centrifuged at *c.* 1500 $\times$ g. for 6 min. in the cold and then washed in ice-cold water before assay so that the abscissae which represent the time for which the cells were held in lysine solution prior to centrifuging should probably be increased by 6 min.

Fig. 1. Action of glutamic acid decarboxylase on intact and disrupted cells (*Strep. faecalis* grown in medium A). Each estimation carried out on 60 mg. dry weight of cells.

Fig. 2. Rate of appearance of lysine in internal environment of deficient *Strep. faecalis* cells standing in lysine solution (300 μl./ml.) at 10°. External pH=7.2. Internal lysine assayed on 32.0 mg. dry weight of cells.

The equilibrium concentration of lysine reached in the cell is dependent upon the lysine concentration in the external environment. This can be shown by suspending deficient cells in solutions of lysine of different concentrations and centrifuging the cells down after allowing a sufficient time (3 hr.) for equilibrium to be reached in all cases. Fig. 3 shows the variation of the internal concentration at equilibrium with increasing external lysine concentrations.

Effect of external lysine concentration on rate of entry of lysine. Fig. 2 shows that lysine enters the cell rapidly during the first 20 min. of contact. If samples are taken after 5 or 10 min. of contact it is possible to study the influence of various environmental factors on the rate at which lysine is entering the cell. Fig. 4 shows the effect of increasing lysine concentration on the rate of entry into the deficient cell. Within the concentration range studied the rate of entry increases with increasing external concentration.

Effect of pH on the rate of entry of lysine. At pH 4.5 lysine enters the cell slowly and the rate increases with increasing pH up to 9.5 which represents the limit of alkali tolerance for this organism (Fig. 5). The isoelectric point for lysine is 9.47 and the results shown in Fig. 5 suggest that lysine passes most easily into the cell in the isoelectric form. The final equilibrium concentration reached inside the cell after 24 hr. at 2° is independent of the external pH within experimental error.

Effect of temperature on rate of lysine entry. Lysine enters the cells at an appreciable rate at 0° and the rate increases with temperature (Fig. 6), the temperature coefficient over the range 20–30° being 1.40, which is only slightly greater than that which would be expected for the free diffusion of lysine (Davson & Danielli, 1943). It is possible that the pH effects described are due to an alteration in the permeability of the cell wall with pH, in which case the temperature coefficient should also alter with pH. The value was determined with cells suspended at an external pH of 5.5 and the value obtained, 1.35, is not significantly different from that determined at pH 7.4.

Concentration of lysine within the cell. A knowledge of the volume available for amino-acids in solution in the internal environment of the cell (the internal 'free-space') is necessary for calculation of the actual concentration of lysine inside the cell. An attempt to determine this volume has been made as follows.

The wet volume was measured directly by preparing a thick suspension of washed streptococcal cells and placing measured amounts into accurately graduated centrifuge tubes made from sealed pipettes. These were then embedded in rubber and centrifuged on an Ecco-Blitz supercentrifuge at 7000 g. until there was no further diminution in the volume of the solid material. After 1½ hr. the meniscus had ceased to move in the tubes and the volume of the intact cells was read off. The dry weight of the original suspension was determined as usual so that it was then possible to calculate the volume occupied by a known weight of intact cells.

It is reasonable to assume that the cells are composed largely of protein and since the specific volume of proteins lies between 0.70 and 0.75 it is possible to calculate the approximate volume occupied by the solid material of the cells. In order not to minimize the volume of the internal free space, the value of 0.70 was taken for the specific volume of the solid material. A series of experi-

mental determinations give the volume occupied by 100 mg. intact cells = 0.347 ml., volume of solid material = 0.07 ml., consequently volume of internal

Fig. 3. Effect of external lysine concentration on (1) internal lysine concentration ●—●, (2) and (3) ratio of internal/external lysine concentrations. (2) △—△, calculation based on volume of intact cell, (3) ▲—▲, calculation based on volume of (intact cell less solid debris). Cells suspended in lysine solutions as shown for 3 hr. at 4°. Lysine assayed on 28.0 mg. dry weight of cells in each case.

Fig. 4. Effect of external concentration of lysine or glutamic acid on rate of appearance of these amino-acids in the internal environment of deficient cells. ●—●, lysine: temperature = 10°; lysine assayed on 30.8 mg. dry weight cells. ○—○, glutamic acid: temperature = 37° in pressure 0.5% glucose; glutamic acid assayed on 27.2 mg. dry weight of cells. Initial pH = 7.2 in all cases.

'free-space' = 0.277 ml. On the basis of these calculations, the internal lysine concentrations are represented by the values given in Fig. 3. Curves 2 and 3 show the variation with the external lysine concentration of the ratio of the

internal to the external concentration. In curve 2 values are calculated on a basis of the total volume of the intact cell (0.347 ml./100 mg.); in curve 3 on a basis of the volume of the free space (0.277 ml./100 mg.). The actual value in each case will presumably lie between the two curves. It can be seen that the internal concentration is three to four times greater than the external when the latter is of the value of 300 μ l./ml. and the ratio rises to fifteen to twenty times

Fig. 5

Fig. 6

Fig. 5. Effect of pH on the rate of appearance of lysine in the internal environment of deficient cells standing in lysine, temperature = 10°. External lysine concentration = 100 μ l./ml. Lysine assayed on 21.2 mg. dry weight of cells in each case.

Fig. 6. Effect of temperature on rate of entry of: $\times - \times$, lysine—amount entering 33.2 mg. cells in 5 min.; $\bullet - \bullet$, glutamine—amount entering 25.2 mg. cells in 15 min.; $\circ - \circ$, glutamic acid—amount entering 25.2 mg. cells in 15 min. External concentration = 100 μ l. amino-acid/ml.

when the external concentration is 25 μ l./ml. Thus although the absolute concentration within the cell is lower for small external concentrations than for high, there is an actual concentrating action across the cell wall which increases as the external lysine decreases. The actual concentration of the internal lysine when that in the external environment is equal to 300 μ l./ml. is 0.035–0.05 M.

Glutamic acid and glutamine

The results obtained in the experiment illustrated in Fig. 4 suggest that lysine passes into the cell most easily at its isoelectric point. The passage of glutamic acid into the cell was next studied as the isoelectric point of this amino-acid lies at 3.22.

Suspensions of deficient cells were therefore diluted with solutions of glutamic acid the pH values of which were adjusted to cover the range 4.0–9.5. The suspensions were then incubated for 3 hr. at 37° and also at 2°, the organisms then centrifuged down and the internal glutamic acid content assayed.

In no case was there any significant increase in the internal glutamic acid content. McIlwain, Fildes, Gladstone & Knight (1939) showed that certain haemolytic streptococci were unable to grow in the absence of glutamine although glutamic acid is present. It is possible that glutamine (isoelectric point 5.56) passes the bacterial cell wall more easily than glutamic acid so the experiment was repeated with glutamine; the use of the decarboxylase preparation on boiled cells does not distinguish between glutamine and glutamic acid. Although there was a small increase in the glutamine content of the cells under the experimental conditions used, this was scarcely significant. When, however, deficient cells were placed in casein digest, with or without glucose, there was a marked increase in the internal glutamic acid concentration, this increase being greater in the presence of glucose than in its absence. Finally, it was found that when deficient cells were incubated in glutamic acid solution containing glucose there was a rapid increase of internal glutamic acid. There was, however, no increase if the incubation was carried out at 0°; this fact enables the rate of entry of glutamic acid into the deficient cell to be studied with greater accuracy than was possible with the lysine system, as the reaction can be checked in this case by lowering the temperature to 0°.

The rate of glutamic acid entry. A series of centrifuge tubes were put up each containing 25 ml. of glutamic acid (200 μ l./ml.) in salt solution and incubated at 37°. When a steady temperature had been attained, 2.5 ml. of thick suspension of deficient cells (20–30 mg. dry weight of cells per ml.) were pipetted into each tube. At the end of 5, 10, 20 and 30 min. approx. 20 g. of frozen distilled water was dropped into one tube and the organisms immediately centrifuged down and washed once in ice-cold water before assay.

The results are shown in Fig. 7. There was no increase in internal glutamic acid or glutamine in the absence of glucose. In the presence of glutamine and glucose in the external environment, glutamine (or glutamic acid—the decarboxylase preparation does not distinguish) entered the cell from the time of contact and the internal concentration reached an equilibrium value within 20–30 min. In the presence of glutamic acid and glucose in the external environment, glutamic acid entered the cell but there appeared to be a short lag phase before glutamic acid entered and the final equilibrium was reached in 30 min. after contact. Using equivalent external concentrations of glutamic acid and glutamine, the final internal concentration reached was the same in both cases. The lag period could not be demonstrated with cells harvested earlier in the growth period.

In the preliminary tests it was shown that the internal glutamic acid content of deficient cells was raised by incubation in casein digest whether or not glucose was present. This suggested that glucose acts as a source of energy necessary for the transfer of glutamic acid across the cell wall and that glucose was not the only available energy source, some other substance in casein digest being able to act as such. Casein digest consists mainly of amino-acids and the amino-acid catabolism of these streptococci is limited to the breakdown of arginine by arginine dihydrolase (Hills, 1940) and of tyrosine by tyrosine decarboxylase (Gale, 1940). The former is an exothermic reaction and it is

possible that arginine may therefore act as energy source for the transfer of glutamic acid into the cell. Fig. 7 shows the increase in internal glutamic acid that resulted from suspension of the deficient cells in glutamic acid + 0.1% arginine. It can be seen that glutamic acid entered the cell under these conditions although the rate of entry was considerably less than that achieved when glucose was present as energy source.

Concentration of glutamic acid within the cell. When deficient cells were incubated at 37° in glutamic acid-glucose-salt solutions, the final concentration of glutamic acid attained inside the cell was dependent upon the external glutamic acid concentration (Fig. 8). Knowing the volume of the internal environment (determined as described above) the ratio of the internal to external concentration can be calculated. The curves show that this ratio rises

Table 5. *Gradient in glutamic acid concentration across the cell wall*

Internal glutamic acid concentrations calculated on the basis that 100 mg. intact cells occupy a volume of 0.347 ml.

External concentration of glutamic acid (μ l./ml.)	Internal concentration of glutamic acid (μ l./ml.)	Gradient across cell wall (μ l.)
10	506	496
25	556	531
50	730	680
100	836	736
200	927	727
300	1058	758
400	1190	790

from 3–4 for external values of 300 μ l. glutamic acid/ml. to 50–60 for external values of 10 μ l./ml. The internal concentration when the external value = 300 μ l./ml. is approx. 0.04–0.06M. It appears that, again, a concentration mechanism is brought into play as glutamic acid passes across the cell wall into the cell which is highly effective when the external concentration is low.

Further light on this concentration mechanism is obtained when the data are presented in a different form. When the internal concentration of glutamic acid and the difference between the internal and external concentrations are calculated the values shown in Table 5 are obtained. It can be seen that the gradient from external to internal concentrations across the cell wall is approximately constant for external values greater than 100 μ l./ml. Below this there is a fall in the gradient although this may be less than that recorded, as experimental error increases with decreasing external concentration. It is possible that the cell wall maintains a constant gradient between internal and external concentrations and that this gradient is maintained by some sort of secretory mechanism. If the same treatment is applied to the values obtained for lysine (Fig. 3), where the passage of the amino-acid across the cell wall appears to be one of diffusion, values for the gradient across the cell wall are obtained which are not even approximately constant.

Effect of pH on the rate of entry of glutamic acid into the cell. By taking samples of deficient cells within 10 min. of contact with standard glutamic acid glucose salt solutions, it is possible to study the effect of variation of the

external environment on the rate of entry of glutamic acid. All these experiments were carried out in duplicate, one set with glutamic acid in the external environment, and the other with an equimolar concentration of glutamine. In

Fig. 7. Rate of entry of glutamine or glutamic acid into deficient cells in presence and absence of glucose or arginine. Temperature = 37°. External concentration of glutamine/glutamic acid = 200 μl./ml. Initial pH = 7.2. Dry weight of cells assayed = 20.0 mg.

Fig. 8. Effect of external glutamic acid concentration on (a) internal glutamic acid concentration ●—●, (b) ratio of internal/external glutamic acid concentrations. △—△, calculation based on volume of intact cell; ▲—▲, calculation based on volume of (intact cell — solid debris). Cells suspended in glutamic acid solutions (containing salts and 0.5% glucose) as shown for 3 hr. at 37°. Initial pH = 7.4. Glutamic acid assayed on 22.8 mg. dry weight of cells (14 hr. culture).

no case was there any significant difference in the results obtained; the glutamic acid values are quoted in the figures. Fig. 9 shows the effect of the external pH on rate of entry of glutamic acid. The various solutions were buffered with phosphate buffers and pH determinations made at the beginning and end of

each experiment. When glucose was present as energy source, the rate of glutamic acid entry was small in the acid range, rose to an optimum at *c.* pH 6.5 and fell less steeply in the alkaline range, the rate still being rapid at pH 8.5. The shape of the curve was essentially a reflexion of the effect of pH on the glucose fermentation system and did not give any information about the variation of cell wall permeability with pH towards glutamic acid. With arginine as energy source again the results seemed to depend largely upon the sensitivity of the energy-providing system to pH variation (Fig. 9, lower curve), although there was a suggestion that the complete system was more effective as the limit of alkali tolerance was approached. In general, the use of arginine as energy source was unsatisfactory and gave variable results; in later studies glucose was used as energy source and the external environment initially adjusted to *c.* pH 7.5.

Effect of temperature on rate of entry of glutamic acid. At 2° there was no significant increase in the internal glutamic acid concentration over a period of 3 hr. The rate increased rapidly with a rise of temperature, the temperature coefficient over the range 20–30° being 1.94. This temperature curve (Fig. 6) is typical of an enzyme system (presumably the controlling factor being the rate of glucolysis) and is markedly different from that given by the lysine entry curve which represents the effect of temperature on simple diffusion. Glutamine gave a similar temperature curve (temp. coefficient 2.35 though the experimental error was so great that this difference may not be significant).

Effect of external glutamic concentration on rate of entry. The curve for glutamic acid (Fig. 6) is markedly different from that given for lysine in that the rate rapidly reached a maximum value above which increasing external concentrations had no effect on the rate of entry, suggesting the relation existing between the rate of an enzyme action and its substrate concentration rather than that of diffusion across a membrane as shown by lysine.

Histidine

Table 6 shows that a Group D *Streptococcus* grown in medium A had free histidine within the internal environment although the concentration was less than that of lysine or glutamic acid. Cells grown in medium B contained little or no free histidine. Experiments similar to those described were therefore made on the histidine content of the internal environment of deficient cells incubated in histidine solutions. Table 6 shows that there was no increase in the internal environment when the cells are incubated at 0° or 37° in histidine-salt solutions but a small increase when glucose was also present in the suspension phase. Histidine therefore resembles glutamic acid in that it cannot pass into the cell in the absence of a source of energy. Table 6 also shows the effect of external histidine concentration on the internal concentration, and the ratio of the internal to external concentration calculated as previously described. It can be seen that this ratio varied between 2 and 0.5 with external concentration, suggesting that, if allowance be made for experimental error, the internal concentration was approximately equal to that in the external environment, so that there was no concentration effect across the cell membrane as there was in

the case of glutamic acid. The magnitude of the effect is so small in this case that no further attempts were made to study the factors involved.

Aspartic acid

No aspartic decarboxylase has yet been discovered so that it has not been possible to investigate whether the passage of aspartic acid across the cell wall occurs under conditions similar to those required by glutamic acid. An attempt

Table 6. (a) *Uptake of l-(–)-histidine in the internal environment of deficient cells*

Dry weight of cells assayed = 33.4 mg. Cells suspended for 3½ hr. in salt solution containing 300 µl. l-(–)-histidine/ml., initial pH = 7.2, other conditions as below.

Treatment	Histidine in internal environment	
	(a)	(b)
Initial	0	2
Incubation at 4°	2	—
Incubation at 37°	4	6
Incubation at 37° in presence of 0.5 % glucose	7	13
Incubation at 37° in presence of 0.2 % arginine	—	5

(b) *Effect of external histidine concentration on concentration within internal environment. Concentrations calculated on a basis of (1) volume of intact cell; (2) volume of (intact cell – solid debris) (see text)*

Dry weight of cells assayed 39.2 mg. Conditions of incubation: cells suspended in salt solution (about 1.5 mg. cells/ml.) containing 0.5 % glucose and histidine as below for 3½ hr. at 37°.

External histidine concentration µl./ml.	Internal histidine concentration µl./ml.		Ratio internal/external histidine concentrations	
	Basis 1	Basis 2	Basis 1	Basis 2
300	119	109	0.4	0.56
200	112	158	0.56	0.8
100	90	127	0.9	1.27
50	82	116	1.64	2.3
25	52	74	2.08	2.98

was made to determine whether the process took place in the presence or absence of glucose by the use of chloramine T as a means of estimating aspartic acid.

Dakin (1917) showed that chloramine T reacts with amino-acids in such a way as to liberate CO₂ from the carboxyl group. In the case of aspartic acid, both carboxyl groups are thus attacked and Cohen (1940) used this fact to estimate aspartic acid manometrically although the method is non-specific. Deficient cells were therefore suspended in salt solutions containing aspartic acid with and without glucose under the usual conditions, incubated for 1 hr. at 37°, the organism centrifuged down, washed and taken up in 10 % H₂SO₄. After standing 0.5 ml. 10 % sodium tungstate solution was added and the protein precipitate centrifuged down. The supernatant liquid was collected in each case, neutralized to pH 4.5 and 'aspartic acid' estimated according to the method of Cohen (1940).

There was no increase in attacked —COOH groups in the organism incubated with aspartic acid alone but cells incubated in the presence of aspartic acid and glucose showed approx. 350 % increase in —COOH which probably represented aspartic acid. It is not possible to differentiate between internal and external aspartic acid by this method but the results suggest that aspartic acid resembles glutamic acid in that it passes into the cell only in the presence of a source of energy such as glucose.

Effect of ions in suspension medium

The experiments described above were carried out in the presence of a salt solution containing Na^+ , K^+ , Mg^{++} , $\text{PO}_4^{''}$, Cl^- , $\text{SO}_4^{''}$ and traces of Ca^{++} . Diffusion experiments conducted with other cells are often markedly affected by the presence of certain ions in the medium; consequently experiments were carried out to determine whether the uptake of lysine or glutamic acid was dependent upon the presence of any of the ionic constituents used. The effect of Ca^{++} was tested by investigating the uptake of glutamic acid in a medium carefully freed from traces of Ca^{++} , and in the same medium saturated with Ca^{++} to the point of the precipitation of calcium phosphate. To test the effect of omission of other ions parallel experiments were carried out in the usual salt mixture and in a medium containing 0.9 % NaCl and buffered by $\text{M}/80\text{—NaHCO}_3$ in the presence of 5 % CO_2 + 95 % N_2 gas mixture to give pH 7.2 under the experimental conditions. The presence or absence of Ca , Mg or $\text{SO}_4^{''}$ had no significant effect on the uptake of glutamic acid by deficient cells; analysis of the simplified medium showed that it contained 4.6 μg . phosphorus/ml.

The uptake of lysine was independent of the presence of Mg^{++} or $\text{SO}_4^{''}$ (Ca^{++} was not tested) and was unaffected by 0.01 M - HCN or iodoacetate, while the uptake of glutamic acid was inhibited by any substance interfering with glucolysis.

Passage of amino-acids out of the cells and attainment of equilibrium between internal and external environments

General method. The organisms were grown in medium A, the cells washed once and the internal amino-acid assayed, the cells suspended in salt solution + 1 % glucose to a final suspension strength of *c.* 1 mg./ml. , after suitable treatment the cells were centrifuged down and made up into thick suspension for comparative assay.

Glutamic acid

The work previously described showed that glutamic acid passed from the external environment into deficient streptococcal cells until the internal concentration was considerably greater than that outside the cell, and that this internal amino-acid was not then free to diffuse out through the cell wall when the cells were incubated in distilled water. When, however, cells grown in medium A and containing a high internal concentration of glutamic acid were incubated in a salt solution + glucose, the internal concentration decreased (Fig. 10). The glutamic content of the cells dropped rapidly at first and then the rate decreased until, in the case quoted, the internal concentration had fallen to 34 $\mu\text{l./47 mg.}$ cells assayed from an initial content of 105 $\mu\text{l./47 mg.}$ cells.

No further decrease in internal content could be shown over a period of 4 hr. at 37°. In the absence of glucose in the external medium, no significant decrease in the glutamic content of the cells occurred.

Fig. 9. Effect of external pH on rate of entry of glutamic acid. ●—● in 10 min. in presence of 0.5% glucose, ○—○ in 30 min. in presence of 0.1% arginine. External glutamic acid concentration = 100 μ l./ml. Temperature = 37°. Dry weight of cells assayed = 25.0 mg.

Fig. 10. Loss of lysine (■) and glutamic acid (●) from cells incubated at 37° in salt solution alone (□ ○) or with 1% glucose (■ ●). Assays on 47 mg. dry weight of cells.

The shape of the curve in Fig. 10 suggests that the glutamic acid concentration was approaching an equilibrium, probably by the passage of glutamic acid back through the cell wall until equilibrium was restored between internal and

external environments. To determine whether this is the case or whether the glutamic acid disappearing is being metabolized, the experiment was carried out on a larger scale, the organisms centrifuged down after 3 hr., the supernatant solution concentrated *in vacuo* and assayed for glutamic acid. Table 7 shows that when the cells were suspended in salt solution alone under these

Table 7. *Passage of internal glutamic acid out of cell*

The cells were grown in medium A and made up into washed suspension of dry weight 40.5 mg./ml. One ml. was used for assay of initial glutamic content. The suspension was then diluted in salt solution as below to a final suspension strength of 4.05 mg./ml. and incubated for 3 hr. at 37°. The cells were then centrifuged out of suspension, washed once and made up into thick suspension of 40.5 mg./ml. for glutamic assay. The supernatant (external medium during incubation) was evaporated *in vacuo* and assayed for glutamic acid.

Treatment	Internal glutamic acid (μ l./40.5 mg.)	Vol. of suspension diluted (ml.)	Glutamic acid in external medium (μ l.)	Glutamic acid	
				Lost from organism (μ l.)	Gain in supernatant (μ l.)
Initial	87	—	0	—	—
Incubated in salt alone	79	3	0	24	—
Incubated in salt + 1 % glucose (1)	20	5	189	335	189
Incubated in salt + 1 % glucose (2)	24	5	139	315	139

Table 8. *Equilibrium between internal and external concentrations of glutamic acid*

Type of experiment	External concentration of glutamic acid μ l./ml.	Internal concentration of glutamic acid μ l./100 mg. cells	Ratio <i>b/a</i> (<i>c</i>)
	(<i>a</i>)	(<i>b</i>)	
<i>Series A.</i> Deficient cells incubated in glutamic acid + glucose solutions until equilibrium is reached at 37°	10	134	13.4
	25	162	6.5
	50	239	4.8
	100	274	2.7
	200	304	1.5
<i>Series B.</i> Cells grown in full medium (A) and assayed while in equilibrium with medium at harvesting	121	211	1.7
	165	230	1.4
<i>Series C.</i> Cells grown in full medium (A) and then incubated in glucose or in glucose + glutamic acid solutions until internal value drops to equilibrium at 37°	3	60	20.0
	4.5	50	11.2
	5.5	61	11.0
	6.5	81	12.5
	21	83	4.0
	130	113	0.9

conditions, no glutamic acid was found in the supernatant but when glucose was present during the incubation, glutamic acid passed out of the cells and could be found in the supernatant. The amount of glutamic acid in the supernatant accounted for 50–60 % of that disappearing from the internal environment during the incubation; it seems probable that the remainder was metabolized (this will be dealt with in a later paper). The presence of a small amount of glutamic acid in the suspending medium resulted in a greater

retention of glutamic acid in the internal environment. Table 8 shows the amount of glutamic acid held in the internal environment of 100 mg. cells when in equilibrium with three different external environments. In the cases quoted in series C the values of the external concentration of glutamic acid were obtained by direct assay of the external environment after incubation. The ratio of the internal concentration to external concentration varied as described in the previous section. For external concentrations of approx. 10 μ l. glutamic acid/ml. the empirical ratio calculated in column (c) is approx. 13 for experiments carried out as in series A; for the reverse type of experiment as in series C the ratio is approx. 12. The ratio fell with rising external concentration, being approx. 6 for 25 μ l. external (series A) and approx. 4 for 21 μ l. external (series C). The agreement between these values, whether approached as in series A or C, gives convincing evidence that for any given external glutamic acid concentration there is an equilibrium internal concentration and that the internal concentration will adjust to this value whether it is initially higher or lower and as long as glucose (or some other source of energy) is available.

The ratio values given in series B are in good agreement with those obtained in series A for similar concentrations, showing that the equilibrium conditions are the same whether the external environment is a complicated one as in the growth medium or a simple one as in the experiments of series A.

Early in these investigations it was found that satisfactory results, either with regard to a high internal amino-acid content of cells grown in medium A (Table 3) or for satisfactory amounts of lysine or glutamic acid taken up by deficient cells, were only obtained with cells harvested early in the growth period. This suggested that the equilibrium between internal and external environments altered as the cultures aged.

A large-scale culture was grown in medium B and samples taken at intervals throughout the growth period. The cells were centrifuged out of each sample, washed once and then incubated for 40 min. at a suspension strength of *c.* 1 mg./ml. in salt solution containing 200 μ l. glutamic acid/ml. and 0.5 % glucose. After this treatment, the cells were centrifuged down, washed once and the internal glutamic acid content assayed as usual.

Table 9 shows that the amount of glutamic acid taken up per 100 mg. cells under these conditions varied greatly with the age of culture, being very high with cultures taken as soon as possible after inoculation and then decreasing steadily throughout the growth period and after. Since the external concentration of glutamic acid was the same in all these experiments, it follows that the efficiency of the concentrating effect across the cell membrane is greatest in rapidly growing cells and decreases when growth ceases. This must also mean that the internal concentration of glutamic acid in equilibrium with a given external concentration is very high in cells early in the growth phase.

Lysine

As shown previously there was no diffusion of lysine out of streptococcal cells grown in medium A. Since glutamic acid passed out of the cell during glycolysis it was of interest to determine whether lysine would also pass out of

the cell under these conditions. The experiments described in the previous section were therefore repeated and the internal lysine content of the cells followed. Incubation of cells harvested from medium A for 3 hr. in salt solution at 37° resulted in no significant loss of lysine from the internal environment but when glucose was added to the external medium, there was a marked disappearance of lysine within the cells. The internal lysine did not fall to negligible proportions and about 60 % of the lysine disappearing from the internal environment could be recovered from the external medium at the end

Table 9. *Effect of the age of culture on the amount of internal glutamic acid in equilibrium with a given external concentration*

Organisms grown in medium B and sampled at intervals. Temperature = 37°. Organisms centrifuged out of culture, washed once, and then incubated at 37° for 40 min. in salt solution + 0.5 % glucose + glutamic acid (200 μ l./ml.). Cells then centrifuged down, washed and assayed for internal glutamic acid.

Age of culture (hr.)	Growth (mg./ml. medium)	Dry wt. of cells assayed (mg.)	μ l. glutamic acid at equilibrium	
			Per sample (μ l.)	Per 100 mg. cells (μ l.)
2.5	0.050	6.5	39	600
3	0.15	12.4	58	460
4	0.222	21.0	80	380
6	0.300	31.4	76	241
8	0.306	26.4	66	260
10	0.306	25.4	81	320
11	0.306	25.0	68	212
12	0.306	30.0	32	107
14	0.306	23.2	28	121
18	0.300	19.6	36	132
24	—	31.6	34	108

of the experiment. The loss of internal lysine from the cells in the presence of glucose was (Fig. 10) considerably more rapid than the corresponding loss of glutamic acid. The shape of the curve suggests that the final condition represented some state of equilibrium.

Relation between glutamic acid and lysine uptake by deficient cells

The facts discovered so far can be summarized as follows:

1. Glutamic acid cannot diffuse across the cell wall in either direction except in the presence of a source of energy (e.g. glucose); glutamic acid then passes across the cell wall in either direction until an equilibrium is set up between the internal and external concentrations, the former being greater than the latter.

2. Lysine will diffuse into the deficient cell but is unable to leave the 'saturated' cell suspended in lysine-free salt solution.

3. Lysine will pass out of the 'saturated' cell into an environment deficient in lysine but only when energy is supplied to the system from glycolysis, etc.

The passage of lysine across the cell wall therefore appears at first sight to involve some unsymmetrical arrangement which allows diffusion across the cell wall in an inward direction only. The question therefore arises whether the

inward diffusion of the lysine is, despite its kinetics, due to some residual metabolism. To test this the effect of the presence of glucose on (a) the rate of inward passage of lysine, and (b) the final equilibrium conditions existing between the internal and external lysine concentrations was examined. The first point was difficult to investigate because the rate of lysine entry was so rapid at temperatures at which glycolysis would take place at a significant

Table 10. *Effect of glucose on internal concentration of lysine attained in deficient cells suspended in lysine solutions*

Cells grown in medium B and lysine assayed on samples of 32.5 mg. dry weight. Cells suspended in salt solutions containing lysine and glucose as below, final suspension strength = 3.25 mg./ml. Incubation period = 3 hr. at 25°.

External concentration of lysine (μ l./ml.)	Internal lysine concentration at equilibrium (μ l./32.5 mg. cells)	
	(a) Glucose absent	(b) Glucose present (0.5 %)
25	23	3
50	38	4
100	42	4
400	45	3

Table 11. *Effect of glucose and glutamic acid on the uptake of lysine by deficient cells*

Cells grown in medium B and made up into washed suspension of strength 33.0 mg. dry weight of cells/ml. Cells diluted in salt solution with additions as below to final suspension strength = 3.3 mg./ml. and incubated for 1 hr. at 37° (first treatment) followed by 2 hr. at 25° (second treatment). Cells centrifuged out of suspension and lysine assayed on 33.0 mg. Concentrations: lysine 200 μ l./ml.; glutamic acid 200 μ l./ml.; glucose 0.5 %; salt mixture as usual.

First treatment		Second treatment	Internal lysine μ l./33.0 mg. cells
1.	—	Lysine alone	50
2.	—	Lysine + glucose	12
3.	—	Lysine, glucose, glutamic acid	35
4.	Glucose	Lysine alone	14
5.	Glutamic acid	Lysine alone	42
6.	Glucose + glutamic acid	Lysine alone	33
7.	Glucose + glutamic acid	Lysine + glucose	14

rate; it was not possible to determine any effect of glucose on the rate of lysine entry into deficient cells at 25°. The presence of glucose greatly decreased the amount of lysine that the cell could take up (Table 10).

These results were obtained with a simple system consisting of salts, lysine and glucose and do not accord with those obtained in the more complex system of the full-growth medium since cells harvested from this medium contained approximately equimolar amounts of both lysine and glutamic acid although growth had taken place in the presence of excess glucose. The presence of glucose decreased the uptake of lysine by the cells but the presence of both glucose and glutamic acid restored, although not completely, the lysine uptake (Table 11). The system was further analysed by treatment of the cells with glutamic acid and/or glucose prior to incubation with lysine and Table 11 shows that treatment with glucose alone decreased the internal lysine uptake,

glutamic acid alone had no effect, while pretreatment with glucose and glutamic acid partially abolished the depressant action of glucose alone.

These results suggest that whatever the system is that is responsible for the uptake of lysine by the deficient cells it was inactivated or abolished by the presence of glucose but protected from this inactivation by a simultaneous or previous assimilation of glutamic acid.

DISCUSSION

It has been shown that: (a) the streptococcal cells used had an internal environment in which certain amino-acids existed in a 'free' state; (b) some mechanism exists whereby lysine and glutamic acid may exist in a more concentrated solution inside the cell than outside under equilibrium conditions; (c) the passage of lysine into the cell across the cell wall appears to be one of simple diffusion; (d) the passage of glutamic acid, glutamine, aspartic acid and, probably, histidine into the cell requires energy which can be supplied by some exergonic reaction such as fermentation or the breakdown of arginine.

The conditions under which a substance can exist in a more concentrated solution on one side of a membrane than on the other are not clearly understood. Where the substance can pass across the membrane by diffusion as appears to be the case with lysine three conditions may hold: (1) where there are several forms in equilibrium on either side of the membrane then the concentration of the form which actually passes through the membrane must be the same on both sides of the membrane, (2) the substance must enter into combination with some other component of the system on one side of the membrane or (3) the substance must be held in some electrostatic combination on one side of the membrane. Until more is known about the conditions of ionization across the membrane, it is not possible to give a decision on the first possibility. Since, however, the equilibrium concentration of lysine reached within the cell when the external concentration was constant was independent of the external pH, it seems improbable that this could be the explanation. There remains the possibility that the internal lysine is combined chemically with some internal component of the cell. Since the amino-acid decarboxylases cannot react with their amino-acid substrates unless the three polar groups of the substrate molecule are in a free unsubstituted condition (Gale, 1946*b*) any chemical combination must be of a very labile nature or the amino-acids would not be estimated by the method used.

There remains the possibility that the lysine is held in the cell by electrostatic forces. Bacteria in general are negatively charged (Winslow, Falk & Caulfield, 1924) and the lysine molecule carries a positive charge under the conditions of these experiments. It seems probable that the diffusion of lysine into the cell is due to electrostatic attraction. The effect of glucose in decreasing the lysine uptake could thus be explained as a decrease in electro-negative charge in the cell as a result of glucolysis. It is well known that glucolysis is accompanied by the migration of various ions across the cell wall. Thus Leibowitz & Kupermintz (1942) showed that K ions enter *Bact. coli* cells when glucolysis begins. Conway (1942) explains this as follows: the cell membrane is

permeable to K or smaller ions but impermeable to Na or larger ions, permeable to phosphate but not to phosphate esters of glucose, etc. When glucose enters the cell, it is esterified and the effective product of K and phosphate ions falls so that both ions then enter the cell. The esterification of phosphate within the cell may lead to a decrease in the negative charge and consequent decreased attraction for lysine ions. This attraction would be increased again if there were an inward migration of negative ions at the same time as glucolysis occurs; this happens when glutamic acid is present in the external medium. The explanation of the decreased uptake of lysine in the presence of glucolysis and the partial abolition of this decrease by the further addition of glutamic acid probably lies in ionic transfers of this nature. If the lysine passes into the cell as a result of electrostatic attraction, it follows that it will not diffuse out unless modification of the internal charge occurs. When cells containing high concentrations of both lysine and glutamic acid are incubated in an amino-acid-free medium containing glucose, then a further series of migrations takes place in the course of which both lysine and glutamic acid pass out of the cell. The attainment of glutamic acid equilibrium across the cell wall will alter the charge in the cell and the capacity to fix lysine, although comparison of the rates at which lysine and glutamic acid pass out of the cell (Fig. 10) would appear to indicate that the loss of glutamic acid itself is not the controlling factor in lysine loss or retention in this case.

It is clear that neither glutamic acid nor glutamine can pass across the cell wall unless energy is supplied by some other metabolism of the cell such as glucolysis. Whether the decrease in negative charge of the cell during glucolysis is such as to cause a general uptake of negative ions into the cell or whether the inward migration of glutamic acid is accompanied by an equivalent outward migration of some other negative ion has yet to be determined. Whatever the mechanism, it is clear that the metabolism of substances such as glutamic acid must depend upon the presence of an energy system. Thus Hills (1940) records no glutaminase action with washed suspensions of streptococci while McIlwain (1946) has found that washed suspensions of streptococci will show highly active glutaminase action if glucose is added to the cell-glutamine mixture. This has been confirmed by the author with the organisms used in the present work. McIlwain suggests a linkage between glucolysis and glutaminase action but, in view of the above work, it seems probable that glutaminase is an intracellular enzyme and that the glutamine cannot cross the cell wall and so gain access to the enzyme surface in the absence of a source of energy such as glucose. It is possible that micro-organisms have enzymes both on the outside of the cell and within the cell so that whether epi- or endo-metabolism of a given substrate takes place will depend on the presence of energy-giving substrates in the environment.

The concentration gradient across the cell membrane will have the effect that the bacterial cells are able to pick out amino-acids from an environment which is deficient in such nutrients. It would seem that this mechanism is more important from a biological point of view in nutritionally exacting organisms than in those which can synthesize their own amino-acids and are therefore

independent of the external environment in this respect. It can be seen from Table 1 that there is a definite correlation, in the cases of lysine, glutamic acid, histidine and tyrosine, between the amounts of free amino-acid within the internal environment and the proportion of those amino-acids in the cell protein.

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