The author wishes to express his gratitude to Professor C. C. Lucas, of the Banting and Best Department of Medical Research, for helpful suggestions with respect to the design of the microdialysis cell, and to Mrs D. Parrott for her able technical help.

This work was carried out during tenure of a Senior Medical Research Fellowship awarded by the National Research Council of Canada. It was facilitated by the able technical assistance rendered by Mrs Dorothy Parrot.

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The Free Amino Acids in Growing and Non-Growing Populations of *Escherichia coli*

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(*Received* 21 *August* 1957)

It was reported by Taylor (1947) that there were no free amino acids in *Escherichia coli* and other Gram-negative bacteria, and this was considered to represent a fundamental distinction between them and the Gram-positive organisms. Proom & Woiwod (1949), using the more sensitive method of paper chromatography, showed that extracts of coliform bacteria contained a large selection of amino acids. From the conditions of their experiments it was difficult to be sure that the free amino acids they found were not the result of contamination from the hydrolysed casein of the growth medium. More recently free amino acids have been demonstrated in Gram-negative bacteria grown in glucose and ammonium salts, where there is no possibility of contamination by the medium (see Mandelstam, 1955, 1956*a*; Britten, Roberts & French, 1955; Markovitz & Klein, 1955).

Although it seems likely that the free amino acids are on the pathway of protein synthesis (Britten *et al.* 1955), there has not yet been any detailed study of their behaviour in *E. coli*. In the present paper the level of the free amino acids during growth and during nitrogen or carbon starvation is described. Experiments were done on several coliform strains in an attempt to ensure that the conclusions should be generally valid and not due to the peculiarities of a single strain.

MATERIALS AND METHODS

Organisms. A coliform organism called *Bacterium cadaveris* by Gale & Epps (1944) (NCTC 6578) and the following strains of *E. coli* were used: ML30, K12, NCTC1433, ML328c (leucine-requiring), 160–37 (requiring arginine or ornithine), NCTC 4139 (proline-requiring). The organisms were grown with shaking at 35° in the following medium: NH_4Cl, 0·5 g.; $(NH_4)_2SO_4$, 0·5 g.; KH_2PO_4, 13·6 g.; $MgSO_4$, 20 mg.; $Fe(NH_4)_2(SO_4)_2,6H_2O$, 15·6 mg.; glucose, 20 g.; water to 1 l. The pH was adjusted to 7·2 with NaOH. For the amino acid-requiring strains the medium was supplemented with L-arginine HCl (100 μg./ml.) or DL-leucine (300 μg./ml.) or L-proline (150 μg./ml.). The bacteria were generally harvested towards the end of the exponential phase of growth, when the culture contained about 0·8mg.

dry wt. of bacteria/ml. The cells were washed twice with 0·05M-phosphate buffer (prepared from KH_2PO_4 brought to pH 7 with NaOH) and then incubated with shaking in the same buffer at 35° under specified conditions at a bacterial density of about 1 mg. dry wt. of bacteria/ml.

Analysis of amino acids. For analysis of the free amino acids a sample of the suspension, 30–40 ml., was removed and centrifuged. The supernatant solution was treated separately, and amino acids found in it will be referred to as the extracellular amino acids. The bacteria were suspended in 10 ml. of water and heated at 100° for 20 min. to liberate the intracellular amino acids. The suspension was centrifuged and the clear extract poured off. This extract and the supernatant were put on to columns (3 cm.^{2} cross-section) of Zeo-Karb 225 (5 ml.) in the H^+ form. The columns were washed with about 50 ml. of water and the amino acids were then eluted with an excess (25 ml.) of aq. 1·5N-NH_3 soln. The eluates were evaporated to dryness and the residue was dissolved in 0·2 ml. of water and transferred with two washings of 0·1 ml. to Whatman no. 3 paper. In most experiments the chromatograms were developed with butanol–acetic acid–water (63:10:27) for 18–24 hr. For one-dimensional separation of glycine, serine and threonine, *m*-cresol–water was used. Leucine and isoleucine were separated from each other with *tert.*-amyl alcohol (Work, 1949). Two-dimensional chromatograms were run on Whatman no. 1 paper, phenol–ammonia followed by butanol–acetic acid–water being used.

The amount of amino acid in each spot was determined by a method essentially similar to that of Kay, Harris & Entenman (1956). In our experience the method was accurate to within about 10% for amounts of amino acid exceeding 10 μg.; below this level the error increased considerably. It was necessary to run standards for every estimation because the intensity of the ninhydrin colour varied somewhat from day to day. Results are expressed as μg. of amino acid/100 mg. dry wt. of bacteria. The total amino acid content was obtained by summing the individual fractions.

The complete isolation and estimation of an amino acid was not carried out unless it was necessary for determination of specific radioactivity or for some other special reason. In general, the one-dimensional chromatogram obtained with butanol–acetic acid was used, and the amino acids were grouped into several fractions for estimation. Two-dimensional chromatograms and the special solvents referred to above showed that these fractions had the following composition. The 'leucine' fraction consisted of leucine and isoleucine; the 'valine' fraction consisted mainly of valine but also contained some methionine; the 'glycine' fraction contained glycine, serine and aspartic acid (the last being present in very small amounts in comparison with the other two components); the 'glutamic' fraction contained mostly glutamic acid and threonine; 'tyrosine' and 'alanine' fractions consisted almost entirely of tyrosine and alanine but contained small amounts of other amino acids which were not identified; the 'arginine' fraction contained basic amino acids and possibly glutathione. To avoid confusion, quotation marks will be used when referring to a fraction.

Specific radioactivity of leucine. The specific radioactivity of [1-^{14}C]leucine was determined by separating the leucine as described above. The radioactivity was measured on the chromatogram with the scanning instrument described by Piper & Arnstein (1956), and the amount of leucine was then determined by the method of Kay *et al.* (1956). Radioactive standards containing 25 and 50 μg. of leucine were run on each sheet.

EXPERIMENTAL

Free amino acids of Escherichia coli

Extracts from freshly harvested *E. coli* yielded at least a dozen distinct ninhydrin-reacting spots in one-dimensional chromatograms, and more were obtained by two-dimensional analysis. The following amino acids have been identified in wild-type strains (ML 30 and 6578): glycine, alanine, serine, threonine, aspartic acid, glutamic acid, arginine, lysine, methionine, valine, phenylalanine, tyrosine, isoleucine, leucine. In addition, most extracts contained several unidentified amino acids or peptides.

Although the same amino acids were found in all the strains examined, the relative concentration of any particular amino acid not only varied from one strain of bacteria to another, but also depended upon the stage of growth (see below). Similar variations were encountered in the total pool, which varied from about 0·25 to over 1% of the bacterial dry weight.

The amino acid content was not significantly decreased by repeated washing in phosphate buffer or by short periods of incubation in the presence of glucose. Analysis of the washings in such experiments showed that only negligible traces of amino acids were being removed.

Free amino acids during growth

When *E. coli* (ML 30) was grown in 500 ml. of medium in a 5 l. conical flask with the normal shaking rate of 90 oscillations/min. with a horizontal excursion of 8 cm., growth remained exponential until the bacterial density had reached 1 mg./ml. With a horizontal excursion of only 4 cm. the aeration was insufficient and the growth rate began to fall slowly at about 0·3 mg./ml. The variation in the intracellular free amino acid of such a culture was followed by removing samples, each containing 40 mg. of bacteria, and analysing them as described. Contrary to the findings of Dagley & Johnson (1956), no extracellular amino acids could be found.

As growth proceeded the content of intracellular free amino acids fell more or less linearly (Fig. 1*a*). The relationship between the level of the free amino acids and growth was roughly linear as shown in Fig. 1 (*b*), where the specific rate of growth calculated from the data of Fig. 1 (*a*) has been plotted against the size of the pool, the values for the first sample being taken as 100 in both cases. An arbitrary measure of the specific growth

rate was obtained by calculating the percentage increase in unit time at various points on the growth curve of Fig. 1 (*a*).

The proportionality between growth rate and the free amino acid content held also for most of the amino acid fractions that were examined: 'leucine', 'valine', 'alanine' and 'glutamic'. Exceptions were 'tyrosine', whose concentration remained unchanged throughout, and the 'glycine' fraction whose concentration fell slightly at the last sample.

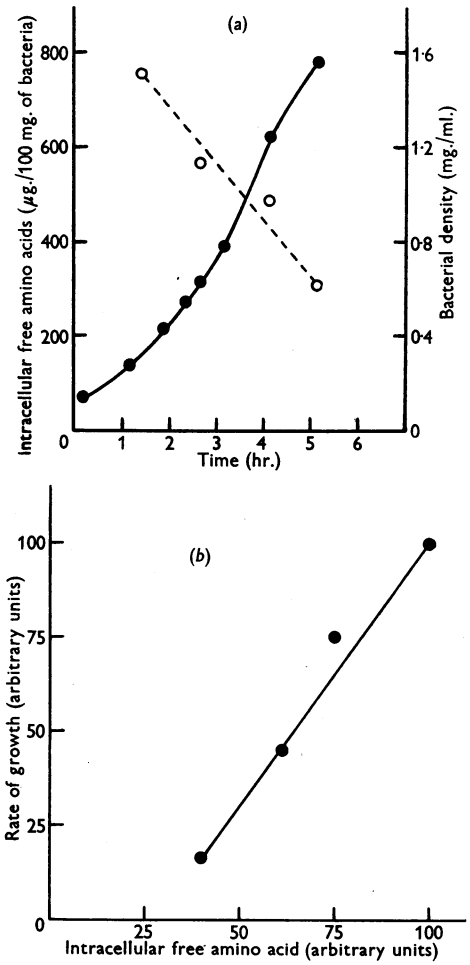

Fig. 1. (*a*) Free amino acid content of *E. coli* (ML30) during growth under sub-optimum conditions of aeration (see text). ●, Bacterial density; ○, total intracellular free amino acids. (*b*) Linear relationship between growth rate and intracellular amino acid content in *E. coli* (ML30). Specific growth rate, calculated from the data of (*a*), is plotted against the free amino acid content. Values at 1 hr. 25 min. (see *a*) were arbitrarily taken as 100.

Maximum content of intracellular free amino acids

An attempt was made to measure the maximum amount of free amino acid that could accumulate intracellularly by allowing bacteria to synthesize amino acids but not to incorporate them into protein. This was done in two ways. The first method consisted of incubating wild-type *E. coli* at 1 mg./ml. in the complete growth medium containing chloramphenicol (20 μg./ml.) to inhibit protein synthesis. In the second method, an amino acid-requiring strain was used, and protein synthesis was prevented by omitting the essential amino acids from the medium.

Both procedures yielded results that were similar in all respects and a typical example of amino acid accumulation is shown in Fig. 2. At the end of 40 min., when the first sample was taken, the total intracellular amino acid content had risen from 475 to 710 μg./100 mg. and 400 μg./100 mg. had been secreted into the external medium. Further incubation did not lead to any increase in the intracellular amino acids, but amino acids continued to accumulate in the external medium.

The behaviour of the various amino acid fractions closely resembled that of the total amino acid in that each had reached its maximum at the end of 40 min. and did not change significantly during the ensuing 2 hr.

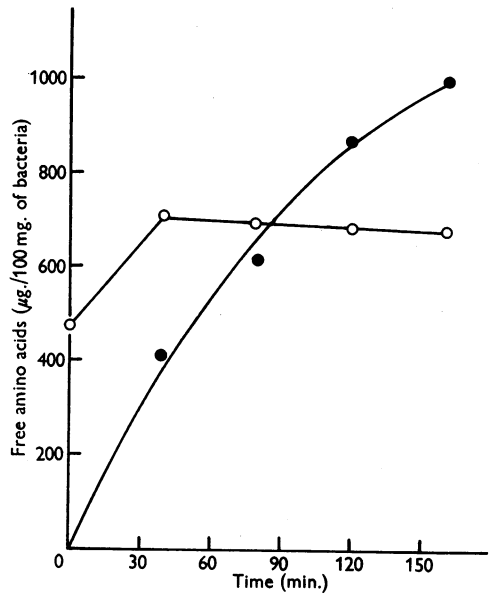

Fig. 2. Accumulation of free amino acids under conditions of blocked protein synthesis. Arginine-requiring bacteria were incubated with glucose and ammonium salts, arginine being omitted from the medium to prevent protein synthesis. ○, Intracellular amino acids; ●, extracellular amino acids.

The fixed levels reached by the total intracellular amino acids and by each of the constituent fractions were characteristic of the bacterial strain. In Table 1 the maximum values are shown for four strains of bacteria: two wild-type (ML 30 and 6578) and two amino acid-requiring strains (160–37 and 4139). There is considerable variation between the strains, not only in the total amount of amino acid (0·3–1·5 % of the bacterial mass) but also in the relative distribution of the different fractions.

It is perhaps significant that the strain with a very low free amino acid content (6578) grew much more slowly than any of the other strains. It had a generation time of 90 min., whereas the generation time for the other strains varied from 55 to 65 min.

Effect of azide and of 2:4-dinitrophenol upon the level of intracellular free amino acids

The effect of azide and 2:4-dinitrophenol was tested to determine whether normal energy metabolism was required to maintain the level of the intracellular amino acids.

Washed bacteria (ML 30) were incubated either with 1 % (w/v) glucose or with 1 % (w/v) glucose and inhibitor. The final concentration of sodium azide was 12 mM and that of the dinitrophenol was 0·6 mM. The results appear in Table 2.

Incubation with glucose alone caused the intracellular amino acids to increase from 425 to 574 μg./100 mg. in 20 min. This effect will be considered in detail in the next section. In contrast with this, treatment with azide caused the level to fall from 425 to 355 μg./100 mg. Most of the material lost was derived from the 'valine', 'alanine' and 'glutamic' fractions. The 'glycine' fraction was not significantly affected, and the 'arginine' fraction increased.

The effect of dinitrophenol was more marked and more general. The total amino acids decreased by about 50 % (425–215 μg./100 mg.) and all the constituent fractions of the pool except 'glycine' were affected. Some of the fractions such as 'leucine' and 'valine' were lost altogether.

Table 1. *The capacity of four strains of* Escherichia coli *to accumulate intracellular free amino acids*

Accumulation was caused by allowing amino acid synthesis from glucose and ammonium salts to proceed for 160 min. under conditions of blocked protein synthesis. The amino acids have been divided into various fractions (see Methods).

	Amino acid content (μg. of amino acid/100 mg. of bacteria)			
Strain	ML 30	6578	160–37	4139
Amino acid fraction				
'Leucine'	33	17	13	16
'Valine'	220	13	165	161
'Tyrosine'	20	34	25	43
'Alanine'	440	33	163	250
'Glutamic'	571	81	259	635
'Glycine'	130	93	65	105
'Arginine'	135	28	140	166
Total	1549	299	830	1376

Free amino acids during nitrogen starvation

In the previous section it was noted that when washed bacteria were incubated with glucose the free amino acid content increased. This effect was followed by incubating a similar suspension and taking samples for analysis over a longer period. In addition, the external medium was also examined for the presence of amino acids.

In a typical experiment (see Fig. 3*a*) the intracellular amino acids increased steadily from 376 to 608 μg./100 mg. in the first 80 min. and then remained constant. In addition 166 μg./100 mg. was found in the external medium. The appearance of amino acids in the external medium under these conditions is essentially similar to that reported by Dagley & Johnson (1956). In these experiments there was always some loss of material to the medium even though the maximum internal level

Table 2. *Effect of azide and of 2:4-dinitrophenol upon the intracellular free amino acids of* Escherichia coli

Washed cells (ML30) were incubated for 20 min. with glucose alone (control) or with glucose and inhibitor. Concentrations: 2:4-dinitrophenol, 0·6 mM; azide, 2 mM.

	Amino acid content (μg. of amino acid/100 mg. of bacteria)			
Amino acid fraction	Before treatment	Control	Azide	2:4-Dinitrophenol
'Leucine'	15	24	9	0
'Phenylalanine'	5	18	0	0
'Valine'	42	42	15	0
'Tyrosine'	15	4	10	8
'Alanine'	81	156	54	18
'Glutamic'	99	192	87	30
'Glycine'	93	90	90	108
'Arginine'	75	48	90	51
Total	425	574	355	215

had not been attained. Nevertheless, the greater proportion of extra amino acid that appeared in the course of the experiment was retained by the cells.

A rather different result was obtained by omitting glucose, i.e. by shaking the bacteria in phosphate buffer. It will be seen (Fig. 3*b*) that, whereas there was, as before, an increase in the total free amino acids, all the extra material was lost to the external medium and the prolonged carbon starvation produced a slow decline (586–476 μg./100 mg.) in the level of the intracellular amino acids.

Although nitrogen starvation almost invariably produced an increase in the total free amino acids, it was difficult to obtain quantitatively reproducible results from one experiment to another even with the same strain of bacteria. The probable reason for this will be discussed below.

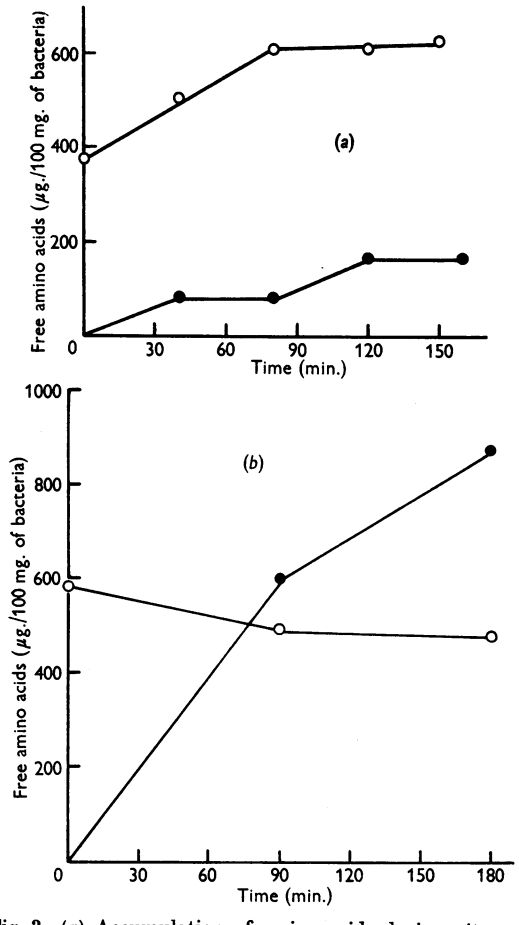

Fig. 3. (*a*) Accumulation of amino acids during nitrogen starvation. Washed bacteria (*E. coli* ML30) were incubated with glucose in the absence of a nitrogen source. ○, Intracellular amino acids; ●, extracellular amino acids. (*b*) Accumulation of amino acids during carbon and nitrogen starvation. Washed bacteria (*E. coli* ML30) were incubated in buffer in the absence of any exogenous source of carbon or nitrogen. ○, Intracellular amino acids; ●, extracellular amino acids.

Origin of the free amino acids released during nitrogen starvation. While protein was the most obvious source for the amino acids released during nitrogen starvation, they might also have been synthesized from traces of ammonium salts carried over from the growth medium, or alternatively through the utilization of nitrogen from non-protein metabolites.

An attempt was made to determine the origin of the released amino acids by using a strain of bacteria (ML 328c) which was unable to synthesize leucine (Hirsch & Cohen, 1953), and carrying out the same type of nitrogen starvation procedure. If this strain liberated leucine in the same way as the wild type (ML 30), breakdown of protein would be indicated. If, on the other hand, leucine did not appear together with the other amino acids it would indicate that the increase was due to production of amino acids from a non-protein source. In preliminary experiments it was found that leucine appeared together with the other amino acids in the same way as it had done in the wild type.

But there still remained the possibility that the block in the synthesis of leucine was not absolute, and that the bacteria were able to synthesize a small amount of leucine, insufficient for growth, but sufficient to account for the increase observed in the experiment. The experiment was therefore carried out with labelled leucine as follows (see also Mandelstam, 1956*a*). The bacteria were grown from a small inoculum (0·04 mg. dry wt. of bacteria) in 500 ml. of synthetic medium supplemented with 100 μg./ml. of DL-[1-^{14}C]leucine (approx. 0·5 μc/mg.). They were harvested at a density of 0·80–0·85 mg./ml., washed twice in phosphate buffer and suspended in 410 ml. of the same buffer containing 1 % of glucose. Duplicate samples, each containing 100 mg. of bacteria, were taken at the beginning of the experiment and after 2 hr. To conserve radioactive leucine, the extracellular and intracellular samples were pooled and estimated together.

The increase in total free amino acid was 255 μg./100 mg. of bacteria (Table 3), which is equivalent to about 0·45 % of the bacterial protein. Leucine accounted for 6·5 % of the total increase, which would be expected since leucine constitutes about 6 % of the protein in *E. coli* (Polson, 1948).

Quadruplicate estimations of specific radioactivity showed that the leucine before incorporation had an activity of 36·4–38·6 counts/min./μg. of leucine; the values for the leucine released by the bacteria during the incubation were 35·4 and 36·7 counts/min./μg. This was within the error of the method, and demonstrated that the released leucine had not been synthesized by the bacteria.

Table 3. *Production of free amino acids by* Escherichia coli *during nitrogen starvation*

Leucine-requiring bacteria were washed and incubated in phosphate buffer with 1% glucose for 2 hr., and samples were taken at the beginning and end of the incubation. Leucine was estimated separately; the other amino acids were pooled and estimated together. In the last column the amount of amino acid produced is expressed as a percentage of the amino acid in the cell protein.

	Amino acid content (μg. of amino acid/100 mg. of bacteria)		
	Before incubation	After 2 hr.	% produced
Leucine	Trace	16·5	0·47
All other amino acids	228	483	0·45

DISCUSSION

The experiments on the capacity of the bacteria to hold amino acids indicate that there is a level for the amino acids beyond which no more material can be retained in the cell. This finding could be explained by assuming that there are fairly specific adsorption sites for the amino acids and that these become saturated under conditions where protein synthesis is blocked and amino acids accumulate.

A theory of specific adsorption sites was put forward by Cohen & Rickenberg (1955) to account for their observations on the concentration of [^{14}C]valine. They had found that the valine was rapidly taken up by *E. coli* in the presence of a source of energy. The valine could be recovered by heating the cells at 100°. Alternatively, it could be displaced rapidly by unlabelled valine and also by leucine or isoleucine. The addition of azide or 2:4-dinitrophenol at the beginning of the experiment prevented the uptake of labelled valine, and the addition of azide after uptake of the valine led to a slow loss of it from the cells.

In a later communication Cohen & Rickenberg (1956) abandoned their earlier theory, although they admitted that it accounted satisfactorily for these phenomena. They preferred, however, the theory of a specific catalytic concentrating mechanism ('permease'), thus bringing their interpretation into conformity with that advanced for the concentration of galactosides in *E. coli* (Rickenberg, Cohen, Buttin & Monod, 1956).

The 'permease' theory is as follows:

$$\text{External valine} \xrightarrow{\text{Permease}} \text{Internal valine} \xrightarrow{\Delta} \text{External valine.}$$

'Permease' is considered to be a catalytic concentrating mechanism which transports valine into the cell and whose action is competitively inhibited by similar molecules such as leucine and isoleucine. The transport system requires energy and is inhibited by azide and dinitrophenol. The valine which has been concentrated is expelled from the bacteria by another mechanism, Δ. According to this model there is thus a rapid circulation of valine through the cell so that [^{14}C]valine is rapidly displaced from the bacteria by addition of valine (or leucine or isoleucine). If azide is added, however, the loss of valine is much slower, and to explain this fact the authors find it necessary to assume that azide inhibits Δ as well as permease. They also suggested that the concentration of amino acids generally is effected by means of specific permeases.

This scheme appears to contain a number of unnecessary postulates. First, there seems to be insufficient justification for assuming the existence of a catalytic concentrating mechanism, and if this assumption is made it becomes necessary to postulate the system Δ and to postulate further that it is inhibited by azide. Even with these additional assumptions the theory fails to provide a satisfactory explanation for our present findings that all amino acid above a certain fixed level is lost from the cells whereas below this level it is firmly retained. This is particularly noteworthy with lysine which, in the absence of an energy source, appears to diffuse fairly freely in coliform bacteria (Mandelstam, 1956*b*) so that its concentration is the same inside and outside the cells. Nevertheless, a small amount of intracellular lysine does not seem to be able to equilibrate in this manner since it is always found in the cells. It cannot be removed even by repeated washings, nor (in *Bacterium cadaveris*) is it destroyed by lysine decarboxylase which is always present in this strain (Mandelstam, 1954).

It seems that the present experimental findings can be more simply and more adequately explained in terms of Cohen & Rickenberg's (1955) original theory that there are specific adsorption sites for the amino acids and that a good supply of energy is necessary for rapid equilibration between added and adsorbed amino acid. This follows from the fact that very little labelled valine is taken up by the cells without a source of energy, whereas in the presence of a source of energy there is rapid exchange between the labelled valine and the unlabelled valine which the bacteria contain when they are harvested. However, the maintenance of the pool seems to require much less energy, because prolonged carbon starvation produces only a slight fall in the intracellular pool (Fig. 3*b*). That some energy is needed is indicated by the fact that azide and dinitrophenol increase the rate of loss of amino acids from the cells.

The increase found in free amino acids during nitrogen starvation is similar to that observed

when nitrogen-starved yeast cells were incubated with alcohol instead of glucose (Halvorson, Fry & Schwemmin, 1955). In *E. coli* the increase in free amino acids is obtained equally in the presence of glucose or of succinate or, indeed, with no exogenous carbon source at all. The experiments with labelled leucine show that this amino acid, at least, is not synthesized by the bacteria from non-protein nitrogen. This does not formally prove that all the amino acids thus released arise from the breakdown of protein, but, in the next paper, independent evidence is presented to show that there is a fairly well-balanced degradation and resynthesis of protein in nitrogen-starved bacteria. The process continues for 3–4 hr. at a rate equivalent to about 5% of the total protein/hr. (Mandelstam, 1957). Since the amino acid pool is equivalent to only about 0·5–2% of the total protein, the amount of material passing through the pool is large in comparison with what is already there. It is clear that a relatively small imbalance in the rates of synthesis and degradation of protein could lead to appreciable changes in the size of the pool. Thus the change in free amino acid reported in Table 3 could be accounted for if the rates of degradation and resynthesis of protein were 5 and 4·7%/hr. respectively. This may explain the difficulty experienced in getting quantitatively reproducible results in the nitrogen-starvation experiments.

The relationship of the intracellular free amino acids to protein synthesis may now be considered. Britten *et al.* (1955) showed that ^{14}C-labelled proline was apparently adsorbed by suspensions of *E. coli*, and that the radioactivity incorporated into protein during growth was directly proportional to the specific activity of the proline in the adsorbed state. They stated that even if only a small fraction of the proline entering the protein had by-passed the adsorbed pool it would have been detected by their method. The fact that the rate of growth is proportional to the total free amino acid content (Fig. 1*b*) suggests that the findings from the experiments with proline may apply to the other amino acids as well, and that the adsorbed pool as a whole is on the pathway of protein synthesis.

There is no evidence to indicate the manner of adsorption or the nature of the adsorbing surface, but Britten *et al.* (1955) suggested hydrogen-bonding on a surface of ribonucleic acid. Now the amino acid pool when 'full' contains 1000–1500 μg. of amino acid/100 mg. dry wt. of bacteria, i.e. about 10 μmoles of amino acid/100 mg. The amount of ribonucleic acid in the cells is roughly 50 μmoles of ribonucleic acid nucleotide residues/100 mg. (Britten *et al.* 1955), and thus there is ample nucleotide material to account for the adsorption of the amino acid pool. While this calculation shows that nucleic acid could be the adsorbing surface, there is no evidence to prove that it is, and it could be argued with equal force that the amino acids are adsorbed on protein. There are roughly 100 amino acid residues in protein for every amino acid molecule in the free state, so that again there seems to be ample protein to provide the adsorbing surface.

If it is accepted tentatively that adsorption at a specific site is an essential preliminary step in protein synthesis, it is of interest to calculate the rate of flow of material through the adsorption sites during growth. With a pool equivalent to 1% of the bacterial protein, and a generation time of 1 hr., the turnover of amino acids in the pool would be 100% in 0·77 min., i.e. each adsorption site would, on average, take up a fresh amino acid molecule about once every 45 sec. This result may be compared with one derived from the penicillinase-forming system of *Bacillus cereus* (M. R. Pollock, personal communication). The rate of induced penicillinase formation is dependent upon the number of penicillin molecules specifically fixed by the bacteria (about 200/cell). If it is assumed that each molecule allows one enzyme-forming site to become functional, it can be calculated that the rate of formation of the enzyme is of the order of one molecule/site/min., i.e. the enzyme-forming site has about the same turnover-time as the amino acid-adsorbing sites. This agreement may be considered additional support for the view of Britten *et al.* (1955) that the amino acids are adsorbed at the actual sites of protein synthesis.

SUMMARY

1. Coliform bacteria grown in a medium containing glucose and inorganic salts contained at least 15 amino acids in the free state. Together these constituted 0·25–1·5% of the dry weight, depending upon the type of bacteria.

2. The total free amino acid content varied directly with the specific growth rate of the bacteria.

3. The results support the theory that there is a limited number of adsorption sites for each amino acid.

4. Retention of the free amino acids within the cell was linked with energy metabolism.

5. When bacteria were starved of nitrogen or carbon, free amino acids accumulated and this has been shown to be due to protein degradation.

6. The possible role of the free amino acids in protein synthesis is discussed.

I wish to thank Dr T. S. Work for advice on chromatographic procedure and I am greatly indebted to Miss R. Coyle for invaluable technical assistance.

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Turnover of Protein in Growing and Non-Growing Populations of *Escherichia coli*

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(*Received* 21 *August* 1957)

The classical theory of the dynamic state of proteins was based upon isotopic experiments with mammals. Recently the concept has been questioned on the basis of experiments with bacterial systems. Three independent groups of workers reported that the proteins of *Escherichia coli* were stable and that the rate of turnover of protein in growing suspensions was negligible (Rotman & Spiegelman, 1954; Hogness, Cohn & Monod, 1955; Koch & Levy, 1955). Hogness *et al.* (1955) went on to suggest that proteins in the mammalian cell also were stable and that the observed turnover was not true intracellular turnover but was due to replacement of material released by secretion or cell lysis.

However, a number of facts pointed to the occurrence of degradation and synthesis of protein in non-growing populations, and suggested that it might not be valid to extrapolate from growing bacteria to mammalian systems where the cell population is, by comparison, virtually static. Thus Podolsky (1953) had reported a slow rate of protein degradation (about 0·25%/hr.) and Melchior, Klioze & Klotz (1951) had found that [^{35}S]-methionine was incorporated into the proteins of washed *E. coli*. Further evidence for the synthesis of protein is to be found in the fact that washed, or even nitrogen-starved, bacteria can synthesize inducible enzymes (for examples see Mandelstam, 1956; Pollock, 1958).

The present paper is a report of experiments designed to measure the extent of protein turnover in non-growing populations of *E. coli* by separate determination of the rates of degradation and of synthesis. Factors affecting both processes have been studied and, in addition, the rates of degradation in growing and non-growing populations have been compared. In this paper the term non-growing will be used to denote suspensions of bacteria in which there is no net synthesis of protein.

The experiments were carried out with mutant strains of *E. coli* with specific amino acid requirements. For the measurement of protein synthesis in non-growing suspensions, leucine- or arginine-requiring strains were used. The bacteria were first starved of the essential amino acid and then incubated with an excess of labelled glycine. The conditions were therefore such that protein synthesis, with concomitant incorporation of glycine, could not take place until leucine or arginine was released by the degradation of existing protein. The validity of this method as a measure of protein synthesis will be considered in the Discussion.

For investigation of protein breakdown a strain of *E. coli* was used which required both leucine and threonine for growth. The proteins were labelled by growing the bacteria in the presence of labelled leucine. The bacteria were then washed and incubated with an excess of unlabelled leucine to trap labelled leucine liberated from the proteins. In the experiments with non-growing suspensions, only a carbon source was added; when a growing population was required the medium was supplemented with ammonium salts and threonine.