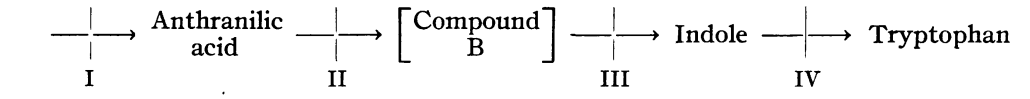
mutations in separate loci. A comparison of the present findings with those of Demerec has shown that the four phenotypic groups are identical with the four transduction classes, which suggests that each group is characterized by a block in one of the steps of tryptophan biosynthesis and that each block is produced by mutation in one gene locus. The data are therefore consistent with the following biosynthetic pathway in which each step is controlled by a single gene:



The precise role of compound B as an intermediate has not yet been determined. It possesses no growth-promoting activity for mutants blocked in earlier stages and may, in fact, be produced as a side reaction of the normal intermediate. This problem is at present under investigation.

The author wishes to express his gratitude to Dr. M. Demerec for providing facilities for this work.

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*AMINO ACID ADSORPTION AND PROTEIN SYNTHESIS IN ESCHERICHIA COLI**

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Two quite distinct theories of protein synthesis are currently popular. One holds that amino acids are linked into small peptides which then serve as building blocks for the proteins. The other postulates that amino acids are individually adsorbed on a large template molecule and are then linked together into the polypeptide chains. This paper reports the results of studies of amino acid incorporation by growing *Escherichia coli*. The results furnish experimental evidence in favor of the template theory.

When *E. coli* cells (grown in a medium[1] containing glucose, salts, and ammonia) are washed and treated with cold 5 per cent trichloroacetic acid (TCA), a number of amino acids are extracted. These amino acids are transient intermediates of synthesis. When C^{14}-glucose is added to the medium, their specific radioactivity

rises more rapidly than that of the cell as a whole. They equally rapidly lose their radioactivity when the C^{14}-glucose is replaced by C^{12}-glucose. The radioactivity thus lost by the TCA-soluble fraction of the cells is transferred to the protein fraction. It therefore appeared that a study of the TCA-soluble fraction and its kinetic relationship to the protein fractions of the cell might throw some light on the mechanisms of protein synthesis.

The quantity of individual amino acids occurring in the TCA-soluble fraction depends on the conditions of growth of these cells. In the absence of supplements, glutamic acid, alanine, and valine are major components, while traces of other amino acids are present, and proline and methionine cannot be detected. When amino acids (including proline and methionine) are added as supplements to the medium, they appear in the TCA-soluble fraction.

Since *E. coli* is highly permeable to amino acids, it appears that the amino acids extracted by TCA are held within the cell by a loose binding which can be broken by 5 per cent TCA. For simplicity in discussion these transient loosely bound amino acids will be referred to as "adsorbed" even though neither the site nor the nature of the binding has been identified.

The time course of adsorption and incorporation of amino acids into protein can be measured accurately by adding to growing cultures of bacteria small quantities of highly radioactive amino acids (25 per cent C^{14}). To measure the total uptake of radioactivity, samples of bacteria were harvested by passing the suspension through porous collodion membrane filters, a process requiring only 5–10 seconds. To measure the incorporation into the protein, samples of the cell suspension were injected into an equal volume of 10 per cent TCA and after 15 minutes at room temperature were filtered as described above. The incorporation of radioactivity into the whole cell and into the TCA-precipitable fraction could thus be measured under identical conditions. The difference gives the uptake into the TCA-soluble fraction of the cell and measures the adsorbed quantity of amino acid. Measurements have been made of the quantity of amino acid adsorbed on the filter in the absence of cells, and, where necessary, appropriate corrections have been made.

A typical experiment proceeds as follows: The tracer amino acid dissolved in the culture medium is injected with a hypodermic syringe into a suspension of growing cells, giving complete mixing within 1 or 2 seconds. Samples are withdrawn with a small hypodermic syringe (arranged with a stop so that it delivers a constant sample size) and squirted onto the filter or into TCA.

Figure 1 shows the results of an experiment measuring the uptake of C^{14}-proline. After a lag of less than 10 seconds the proline is taken up into the compounds of the TCA precipitate at a constant rate until the supply in the medium approaches exhaustion. The total quantity taken up into the cell rises rapidly at first and then parallels the uptake into the protein. The difference between these two curves measures the quantity of adsorbed proline. This quantity rises rapidly at first, then remains constant for a period, and finally decreases as the proline is transferred to the protein after the supply in the medium is exhausted. The concentration of TCA-soluble proline per milliliter of cells is 500–1,000 times the concentration of proline in the medium, showing that the proline is bound in a nondiffusible form.

The radioactive material extracted from the cells with TCA after a 1-minute exposure to C^{14}-proline in a similar experiment has been shown to be authentic proline by paper-chromatographic fingerprinting.[1] We have not yet succeeded in removing the adsorbed proline from the cell and still preserving its association with other cellular components.

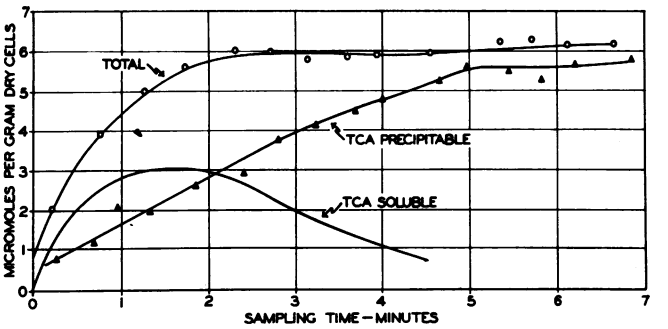

FIG. 1.—Incorporation of C^{14}-proline by a suspension of growing *E. coli* cells. The temperature was 24° C. and the generation time about $2^1/_4$ hours. The suspension contained glucose, ammonia, mineral salts, 1(−)C^{14}-proline at 1.2×10^{-6} molar and 0.2 mg. (dry weight) of cells per ml.

The cold TCA precipitate has been further fractionated,[1] and the results are shown in Table 1. The distribution among the fractions of the cold TCA precipitate is similar to that observed for other tracers which label cellular proteins. Chromatography of these fractions before hydrolysis shows no separation of the radioactivity from the proteins. Chromatography after hydrolysis shows that the radioactivity is almost entirely in proline, with traces in glutamic acid and arginine.

TABLE 1*

FRACTIONATION OF COLD TCA PRECIPITATE

Fraction	Per Cent of Radioactivity	Fraction	Per Cent of Radioactivity
Cold TCA precipitate	100	Hot TCA–soluble	5
Hot ethanol–soluble	15	Hot TCA precipitate	80

* In an experiment similar to that shown in Fig. 1 a sample of the suspension was injected into TCA at 1 minute. After 15 minutes the TCA precipitate was harvested and extracted with 80 per cent ethanol for one-half hour at 80° C. This precipitate was then extracted with 5 per cent TCA at 90° C. for one half hour, leaving nearly pure protein as the final precipitate.

The adsorption of proline is highly specific. Figure 2 shows the uptake of C^{14}-proline under the same conditions as those in the experiment of Figure 1, except that fifteen other amino acids were added, each at 100 times the concentration of the proline. A proline contamination of 0.2 per cent in any of the other amino acids is sufficient to account for the slight difference between Figure 1 and Figure 2. If the adsorption of proline were not specific, the quantity adsorbed would have been reduced several hundredfold. When the proline is supplied at high concentrations (10^{-4} *M*), a greater adsorption (20 μM/gm dry) is observed. In this case the adsorption is reduced by the presence of other amino acids. It thus appears that there are two types of sites: specific sites which are the only ones involved

at the low concentrations used in most of these experiments and nonspecific sites which become prominent at higher concentrations.

The adsorption process also requires energy. Figure 3 shows the results of an experiment in which cells which had exhausted the supply of glucose several hours previously were supplied C^{14}-proline. The rate of adsorption is reduced by approximately a factor of 20. This residual rate is probably due to endogenous reserves of energy which slowly become available. When glucose is added along with the C^{14}-proline, adsorption begins instantly and the protein synthesis seems to be delayed for less than a minute.

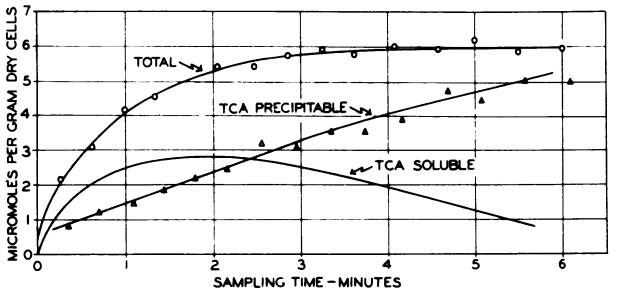

FIG. 2.—Incorporation of C^{14}-proline in the presence of other amino acids. The suspension was identical to that of Figure 1 with the addition of 0.013 mg per ml (about 10^{-4} molar) of each of the following: alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, serine, threonine, tyrosine and valine.

On the other hand, adsorption can proceed under certain conditions in which protein synthesis is blocked. A methionine-requiring mutant rapidly adsorbed proline when growth was prevented by the absence of methionine. The adsorbed proline was not transferred to the protein until methionine was added to the medium. Similarly, nitrogen-deficient cells or cells treated with chloramphenicol (50 μg/ml) could adsorb proline even though its incorporation into protein was blocked.

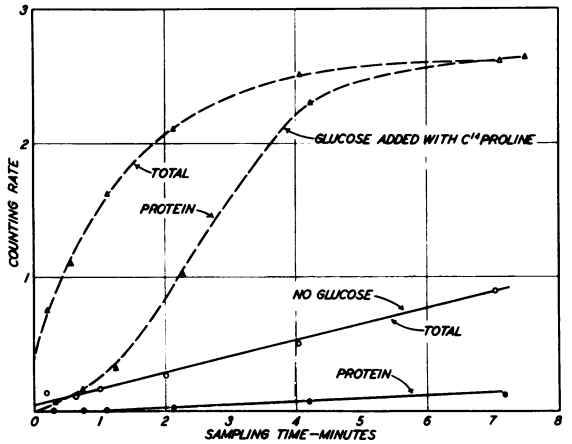

FIG. 3.—Effect of glucose on the incorporation of C^{14}-proline. Lower curves (solid line) show the incorporation in the absence of glucose. The upper curves (dotted line) show the incorporation when 0.1 per cent glucose was added with the C^{14}-proline. C^{14} proline concentration, 0.28×10^{-6} molar; cell concentration, 0.07 mg. (dry weight) per ml.; temperature, 37° C.

The experiments described above show that the adsorbed materials pass on into the protein and that the adsorption is a specific process suggesting sites involved in protein synthesis. They do not tell whether it is necessary for the amino acid to pass through the adsorbed pool to be utilized for protein synthesis or whether the adsorption is merely a storage mechanism.

To distinguish between these alternatives, a suspension of cells was pretreated for 1 minute in a solution of nonradioactive proline to build up within the cell a pool of unlabeled proline; then labeled proline was added and a series of samples withdrawn.

Figure 4 shows that there is a delay in the incorporation into the protein. The

shape of the incorporation curve agrees remarkably well with a curve calculated on the assumption that the slope of the protein-incorporation curve is proportional to the radioactivity of the adsorbed pool. Since the cells were pretreated with unlabeled proline, the total amount of proline in the adsorbed state should be approximately constant until the exogenous proline approaches exhaustion. Thus, if the adsorbed proline is the only source of proline for the protein and supplies proline at a constant rate, the rate of entry of radioactive proline into the protein will be proportional to the radioactivity of the adsorbed pool. If even a few per cent of the proline entering the protein had by-passed the adsorbed state, it would have caused a detectable initial rise in the protein-incorporation curve. Thus it is clear that the adsorption process is a necessary step in the incorporation of exogenous proline into the protein.

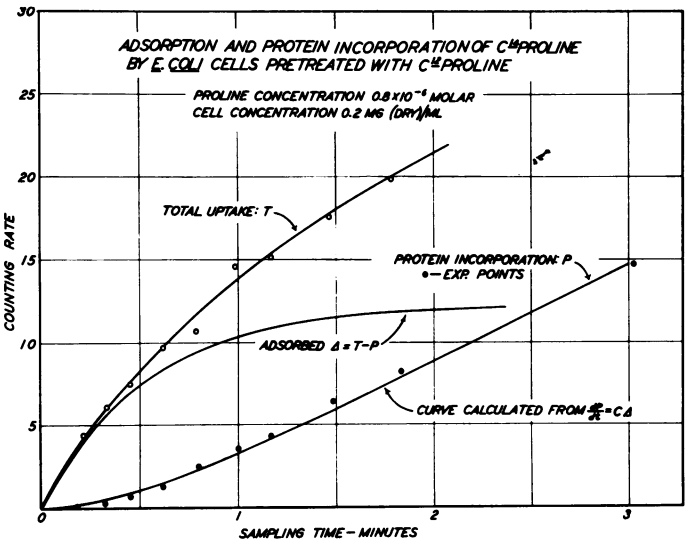

FIG. 4.—C^{12}-proline (0.8×10^{-6} molar) was added 1 minute before the carrier-free C^{14}-proline. An amount of medium was added with the C^{14}-proline so that there was no change in proline concentration.

At 0° C. the adsorption is negligible, as might be expected in an energy-requiring process. However, exchange can occur. Growing cells were suspended in unlabeled proline for 2 minutes at 24° C. and then chilled to 0° C., the chilling process taking about 5 minutes. C^{14}-proline was then added to the system and a series of samples taken. Figure 5 shows that the labeled proline entered the TCA-soluble fraction of the cell, but almost no incorporation into the TCA precipitate occurred. It appears that the external C^{14}-proline exchanged with the C^{12}-proline that was previously adsorbed at 24° C. and had remained on the sites during the chilling process. To show that exchange was occurring, a small amount of C^{12}-proline was added after equilibrium had been approached. The amount of C^{14}-proline adsorbed then fell as a new exchange equilibrium was approached. Specificity for proline adsorption was again shown, since the equilibrium was not displaced by the addition of a hundredfold excess of each of fifteen other amino acids.

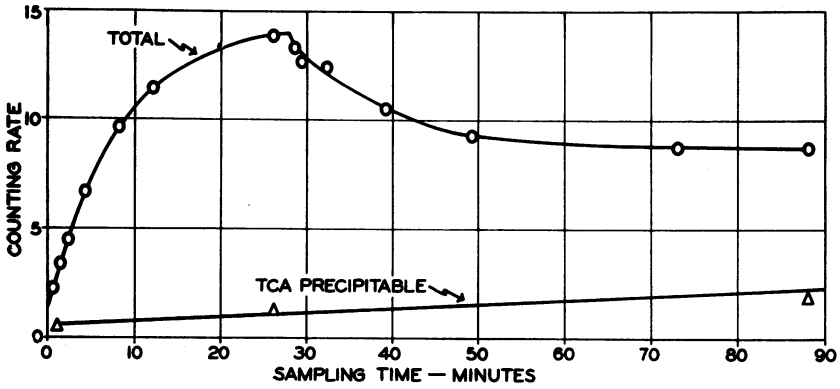

FIG. 5 —Exchange between adsorbed and exogenous proline at 0° C. At 28 minutes C^{12}-proline was added. Circles represent total incorporation, triangles incorporation into TCA precipitate.

In order to study the stability of the binding of the proline, a suspension was prepared as above. After exchange equilibrium at 0° C. had been achieved, the cells were exposed to various reagents at 0° C. The data of Table 2 show that the adsorbed proline is freed from the cells by mild treatment—small shifts in pH or moderate concentrations of ethanol. It is indicated that the proline is not held by stable chemical bonds.

TABLE 2*

EXTRACTION OF ADSORBED PROLINE

Added Reagent	Final Concentration	Per Cent of TCA-soluble Proline Extracted
TCA	5%	100
TCA	0.25%	20
Ethanol	10%	0
Ethanol	20%	47
Ethanol	30%	95
Ethanol	40%	107
Butanol	10%	90
Toluene	Saturated	20
Pyridine	1%	0
Roccal	0.5%	100
Dinitrophenol	0.002 *M*	40
Glucose	10%	0
NaCl	10%	35
NaOH	pH 10.5	116
NaOH	pH 8.1	40
NaOH	pH 7.7	10
HCl	pH 6.5	0
HCl	pH 5.5	26
HCl	pH 4.7	50
HCl	pH 4.3	60
HCl	pH 2.8	47
HCl	pH 1.8	101
HCl	pH 1.0	100
Chill to −80° C. and thaw	Once	25
	Twice	37
Sonic disintegration to reduce optical density at 650 mμ by 70 per cent		80

* Samples of a suspension in exchange equilibrium were added to tubes at 0° C. containing reagents in the proper amounts to bring the final suspension to the condition described in the second column. After 10 minutes these suspensions were filtered, and the fraction of the TCA-soluble proline that had been extracted was calculated from the radioactivity of the precipitate.

These studies have been conducted chiefly with proline, but the principal points have also been checked with methionine. These two amino acids are particularly suited, as they are end products rather than intermediates of amino acid synthesis and are not extensively degraded by the cells. Furthermore, they are not present in growing cells (in the adsorbed state) in any appreciable quantity unless they are added as supplements to the medium. Some observations have been made using other amino acids, including glutamic acid, alanine, valine, tyrosine, phenylalanine, arginine, and lysine. In all cases the adsorption can be observed and distinguished from incorporation into proteins by kinetic measurements of the type described above. In addition, mixtures of fifteen labeled amino acids have been added and chromatograms made of the TCA-soluble fractions of successive samples. Those amino acids which are normally present in appreciable quantities when the cells grow in the glucose-ammonia medium (glutamic acid, alanine, and valine) show radioactivity in the TCA-soluble fraction for some time, whereas the radioactivity of the others is more rapidly incorporated into the protein.

No radioactive compounds in addition to the amino acids supplied were observed in the TCA-soluble fraction. These conditions would be very favorable for the detection of small peptides. The combination of the high specific radioactivity with short periods of observation forms what might be termed a "chemical microscope" which can focus on the newly incorporated material and ignore the much larger quantities of the other material already present in the cell. Neither were any transient intermediates found in the various fractions of the cold TCA-insoluble material. Accordingly, we find no evidence for peptides as intermediates of protein synthesis.

As shown above, the adsorption of proline and methionine is highly specific and requires energy. If the amino acids were converted into an "active" form, receiving energy from a common source before being adsorbed, it would be expected that an excess of other amino acids would compete for the energy source and would thereby interfere with the adsorption of proline and methionine. Since this does not occur, it seems reasonable to believe that the energy is utilized to prepare the site for subsequent adsorption of the amino acid. A part of the energy could remain in the amino acid site complex to supply energy for the synthesis of peptide bonds.

The kinetic studies show that this adsorption is a necessary step in the process of protein synthesis. However, adsorption is not necessarily followed by peptide-bond formation. No peptide fragments have been observed as transient intermediates. Neither was adsorbed proline bound into a peptide linkage in a mutant blocked by the lack of a single amino acid. A model of protein synthesis wherein the peptide bonds were formed in a process like knocking down a row of dominoes would be consistent with these observations.

We have not been able to determine what type of bond holds the amino acid; hydrogen bonding seems likely in view of the ease of extraction. Neither have we any indication as to what molecules provide the binding sites. Ribose nucleic acid is of course an attractive possibility in view of its long-known association with protein synthesis. The quantity of RNA in these cells is roughly 500 μM of RNA nucleotides per gram of dry cells. Accordingly, if 5 per cent of the nucleotides provided binding sites for proline (proline makes up 5 mol per cent of the protein),

there should be 25 μM of sites available. This is more than adequate to account for the 3–5 μM of specific binding observed.

The formulation of a general theory of amino acid incorporation into proteins on the basis of these observations with proline and methionine would not be justified. However, any model of protein synthesis must provide a means of selectively locating amino acids and must provide the energy to form the peptide bonds. These studies of proline and methionine incorporation give an experimental demonstration of the operation of these two important processes in the synthesis of protein.

* Note added in proof: A report of similar studies carried out nearly simultaneously by G. N. Cohen and H. V. Rickenburg appears in *Comptes rendus des seances de l'Academie des sciences*, **240**, 2086.

[1] Various technical details such as culture media, chromatographic solvents, special chromatographic methods, and procedures for chemical fractionation of the cells are described in Roberts, Abelson, Cowie, Bolton, and Britten, "Studies of Biosynthesis in *Escherichia coli*," *Carnegie Inst. Washington Publ.*, No. 607, 1955.

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*VIRULENT-AVIRULENT CONVERSIONS OF RICKETTSIA RICKETTSII IN VITRO**

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Communicated by K. F. Maxcy, July 26, 1955

Earlier reports[1, 2] from this laboratory have demonstrated that virulent strains of *Rickettsia rickettsii*, the etiological agent of Rocky Mountain spotted fever (RMSF), exist under certain conditions in an avirulent phase in its arthropod vector, *Dermacentor andersoni*. This phase is virulent for chick embryos but avirulent for guinea pigs and many other laboratory animals.[2] Previous experiments also showed that temperature[1, 3] and the molting process[2] of the arthropod were important in controlling the virulence of the rickettsiae in the arthropod vector. The correlation between the virulent-to-avirulent changes and the epidemiological behavior of *R. rickettsii* has been discussed previously.[2]

In this paper is reported (*a*) the in vitro conversion of virulent *R. rickettsii* to an avirulent state by para-aminobenzoic acid (PABA); (*b*) the in vitro reactivation or conversion of the PABA-avirulent and arthropod-avirulent rickettsiae[1] to the virulent form by the addition of Coenzyme I (CoI) or Coenzyme A (CoA); and (*c*) the observation that the state of virulence for guinea pigs parallels the adsorptivity of *R. rickettsii* to minced guinea pig tunica.

RESULTS

In Vitro Conversion of Virulent R. rickettsii to an Avirulent State by PABA.— In Table 1 are given the results of a typical experiment in which a 10 per cent virulent yolk sac suspension of *R. rickettsii*[1] in sucrose-phosphate-glutamate (SPG) solution[4] was incubated at 25° C. for 60 hours with 5 mg/ml of PABA. Although