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INCORPORATION AND ACTIVATION OF AMINO ACIDS BY DISRUPTED PROTOPLASTS OF *ESCHERICHIA COLI*

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SUMMARY

Osmotically lysed protoplasts of *E. coli* have been examined for synthetic activity. They have been found capable of activating and incorporating a large variety of amino acids. Activation has been observed with virtually every amino acid tested. The L-amino acid is the preferred substrate, the presence of the D-isomer being inhibitory.

Optimal incorporating activity requires the presence of: (1) a complete and balanced mixture of amino acids; (2) Mn^{++} at a level of $8 \cdot 10^{-3} M$; (3) ATP; (4) a mixture of the four ribonucleoside diphosphates.

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INTRODUCTION

In recent years a start has been made on the study of protein synthesis in subcellular systems[1–3]. There is strong evidence that the first step in the process is an activation of the L-amino acids resulting from a reaction with ATP to yield an adenyl-amino acid derivative[4]. Enzymes capable of the specific activation of several amino acids have been prepared from a wide variety of biological sources[4–7,9]. There are preliminary indications from work with a liver microsomal system[10] that the amino acids moiety can be transferred in the presence of ATP to an RNA of low molecular weight (RNAs) and subsequently incorporated into microsomal proteins. The incorporation step is specifically dependent on the presence of GTP[11].

Experiments carried out with pea microsomes[3] reveal a somewhat different picture with respect to the specific requirements for amino acid incorporation. In this system, maximal incorporation requires the presence of ATP and all four ribonucleosides or even unspecific RNA.

The following tentative scheme can account for the steps involved:

$$\text{L-AA} + \text{ATP} \xrightarrow{\text{Activation enzymes}} \text{Adenyl-AA} + \text{PP}$$

$$\text{Adenyl-AA} + \text{RNAs} \longrightarrow \text{AA-RNAs}$$

$$\text{AA-RNAs} \xrightarrow[\text{microsomes}]{\text{GTP}} \text{proteins}$$

It is the purpose of the present work to detail the properties of amino acid incorporation in a bacterial subcellular system[8] derived from the *Escherichia coli* penicillin protoplasts. Optimal incorporation is observed in the presence of ATP and all four ribonucleoside diphosphates.

Further, maximal activity is attained only when amino acids are present at high concentrations in ratios corresponding to those found in the proteins of *E. coli*. All eighteen natural amino acids are activated by the system[9]. Preliminary studies suggest that the activation reaction may be the first enzymic step in protein synthesis.

METHODS

Preparation of the enzyme material

The protoplasts were prepared by the method of LEDERBERG[12]. Strain W-1485 of *E. coli* (K 12) was grown overnight on Pennassay medium, and transferred to twice the volume of the same fresh sterile medium containing $MgSO_4$ at a final concentration of 0.01 *M*, sucrose 0.5 *M* and 1000 U/ml of the K salt of penicillin. The bacteria were shaken for 120 min at 30°. Complete conversion to protoplasts was obtained, as shown by direct examination with the phase contrast microscope. The protoplasts were collected by centrifugation at 7000 rev./min for 15 min in the Sorvall SS1 centrifuge. They were then suspended in a medium containing sucrose (0.25 *M*), $MgSO_4$ (0.02 *M*), penicillin (5000 U/ml), TRIS buffer (pH 7.3, 0.02 *M*). The protoplasts

Abbrevations used in this paper: adenosine triphosphate, ATP; pyrophosphate, PP; ribonucleic acid, RNA; guanosine triphosphate, GTP; tris(hydroxymethyl)aminomethane buffer, TRIS; trichloroacetic acid, TCA; amino acid, AA; adenosine diphosphate, ADP; cytidine diphosphate, CDP; uridine diphosphate, UDP; adenosine monophosphate, AMP; cytidine monophosphate, CMP; uridine monophosphate, UMP; guanosine monophosphate, GMP; desoxyribonucleic acid, DNA; desoxyribonuclease, DNase.

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were osmotically disrupted by dilution of the protoplast suspension in 5–6 vol. of ice-cold water, and the lysate was stirred for 30–40 min. The disrupted protoplast material was centrifuged for 10 min in the refrigerated International centrifuge at 18,000 rev./min and the viscous supernatant was discarded. The sedimented pellet contains the systems responsible for incorporation and amino acid activation. Reactions were run by resuspending the pellet in a medium consisting of sucrose (0.25 *M*), $MgSO_4$ (0.012 *M*), TRIS buffer (pH 7.3, 0.04 *M*) and penicillin (5000 U/ml). Viable counts performed by diluting out the penicillin and making pour plates on nutrient agar, supplemented with 0.25 *M* sucrose, indicated that there were less than $1 \cdot 10^3$ per ml viable cells or protoplasts capable of reverting to bacteria. Furthermore, direct examination under the phase contrast microscope confirmed the virtual absence of unlysed protoplasts or bacteria.

Certain of our earlier preparations were made by precipitating the lysate with 50% saturated ammonium sulfate. The precipitate collected by centrifugation contained the active material. This type of preparation will be referred to as enzyme preparation "A", to distinguish it from the fraction described in the preceding paragraph.

[^{32}P]PP incorporation into ATP was measured according to BERG[7].

Measurement of amino acid incorporation

The incubated samples were precipitated with 10% TCA, washed twice with 10% TCA and extracted for 15 min with 4% TCA in a boiling water bath.

In reactions containing less than 100 μg protein, protein carrier (about 500 μg per sample) was added in order to facilitate sedimentation of the pellets. The extracted protein pellets were dissolved in 1.4 *N* ammonium hydroxide and aliquots were dried on aluminum planchets. The radioactivity was counted in a Nuclear gas flow counter. The amino acid incorporation after correction for isotopic dilution in the case of single or multiple radioactive amino acids was usually expressed as disintegrations per min per mg protein, and in some cases, in mμmoles/mg protein.

In order to increase the sensitivity of amino acid incorporation, several ^{14}C-labeled amino acids were used simultaneously in certain experiments.

The data (see Table II) indicated that the incorporation was additive. Consequently, the isotopic dilution could be easily estimated by summation.

Labeled amino acids

For the incorporation studies two types of radioactive amino acids were used throughout this work. These are noted with their specific activities in parentheses. Commercially available radioactive amino acids (Nuclear): DL-[1-^{14}C]leucine (5 μC/μmole); DL-[3-^{14}C]phenylalanine (1.1 μC/μmole); [1-^{14}C]glycine (1 μC/μmole); L-glutamic acid, uniformly ^{14}C-labeled, (6 μC/μmole). DL-[3-^{14}C]phenylalanine (0.45 μC/μmole) was prepared by Dr. G. WOLFE, University of Illinois, and a uniformly ^{14}C-labeled protein hydrolysate of *Chromatium D* (with a specific activity of 5 mC/μmole amino acids) by Dr. P. BERG.

ATP and the four ribonucleoside diphosphates were obtained from Sigma.

Non-radioactive L-amino acids were products of the California Foundation for Biochemical Research.

Two amino acid mixtures were used:

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1. An *equimolar* mixture containing 8 μmoles/ml of each of the 18 natural amino acids.

2. An *equilibrated* mixture containing the L-amino acids in the ratios in which they occur in the proteins of *E. coli*[12] (listed with their concentrations in μmoles/ml):

alanine	15	glutamate	12.5	leucine	9.5	proline	5.5
arginine	6.3	glycine	9.5	lysine	9.3	serine	7.2
aspartate	11.7	histidine	1.2	methionine	4	threonine	5.5
cysteine	2	isoleucine	5.5	phenylalanine	3.9	tryptophane	1.2
		valine	6.5	tyrosine	2.5		

ANALYTICAL PROCEDURES

Protein was estimated according to LOWRY *et al.*[14], DNA according to BURTON[15] and RNA according to CHARGAFF *et al.*[16].

RESULTS

Establishment of optimal conditions for incorporation

ATP requirement. Fig. 1 shows that when the *E. coli* enzyme preparation is incubated in the presence of the equilibrated mixture of amino acids, one of which is labeled, incorporation is stimulated 6–7-fold by the presence of ATP. An identical

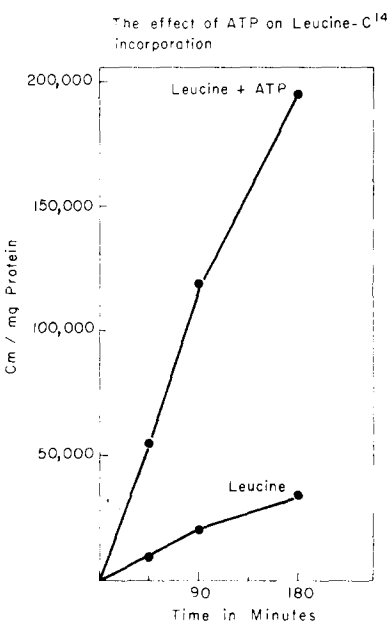

Fig. 1. The system was incubated without shaking in presence of the following constituents in amounts per 0.5 ml: 29 mμmoles DL-[^{14}C]leucine (spec. act. $5.6 \cdot 10^3$ counts/min/mμmole); 0.02 ml equilibrated amino acid mixture; 2 μmoles ATP; 4 μmoles $MnCl_2$; 1.1 μmoles $MgSO_4$; 25 μmoles TRIS buffer pH 7.3; 85 μg protein enzyme preparation VIII.

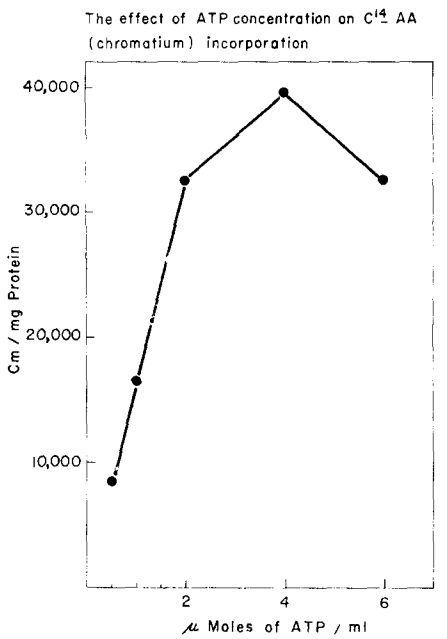

Fig. 2. The system was incubated without shaking for 45 min at 30° in presence of the following constituents in amounts per 0.5 ml: 0.01 ml of uniformly ^{14}C-labeled amino acids ($4.5 \cdot 10^5$ counts/min total); 2 μmoles $MnCl_2$; 1.1 μmoles $MgSO_4$; 25 μmoles TRIS buffer pH 7.3; 98 μg protein enzyme preparation III.

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type of stimulation is observed when all the amino acids are ^{14}C-labeled[26]. The optimal ATP concentration for the amino acid incorporation is $4 \cdot 10^{-3}$ *M* (Fig. 2). Attempts to replace ATP by an ATP-generating system, such as pyruvate kinase, have been thus far unsuccessful.

Effect of enzyme concentration. Fig. 3 shows that the incorporation is proportional to the enzyme concentration over a range of 160–200 μg protein/ml. Higher concentrations of the particulate material inhibits incorporation.

Effect of nucleotides. When all four ribonucleoside-5-diphosphates are present together with ATP (Table I, Fig. 4), an additional increase of the incorporational activity is observed. The same graph shows that the ribonucleoside diphosphates have some stimulatory effect *per se*.

Summation effect. When [^{14}C]phenylalanine, [^{14}C]glycine, and [^{14}C]leucine are used simultaneously, the total amount of labeled material incorporated corresponds to the sum of the individual labeled amino acids incorporated (Table II).

Requirement for amino acids. The incorporation of one or several labeled amino acids is increased by the presence of all the natural amino acids, and is optimal at high amino acid concentrations (Fig. 5). In the absence of all other amino acids the amount of incorporation is proportional to the total concentration of leucine, phenylalanine, and glycine. Fig. 6 shows that the incorporation is greater in the presence of an equilibrated amino acid mixture than in the presence of an equimolar mixture. Experiments to be discussed below suggest that the superior incorporation observed in the presence of the equilibrated amino acid mixture may be referable to competitive relations at the level of amino acid activation.

Mn^{++} requirement. SPIEGELMAN AND WOLIN[18] found that protein and enzyme synthesis in osmotic lysates requires the presence of high levels of Mn^{++}. Fig. 7 shows

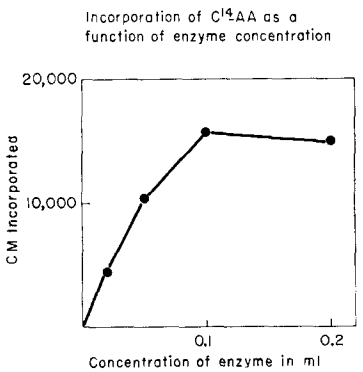

Fig. 3. The system was incubated without shaking for 45 min at 30° in presence of the following constituents in amounts per 0.5 ml: 2 μmoles ATP; 2 μmoles $MnCl_2$; 1.1 μmoles $MgSO_4$; 25 μmoles TRIS buffer pH 7.3; 98 μg protein enzyme preparation III.

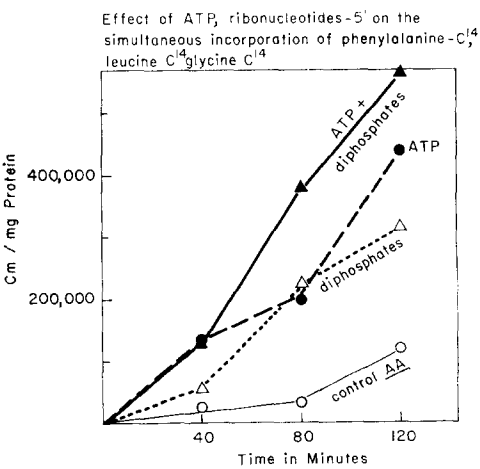

Fig. 4. The system was incubated without shaking at 30° in presence of the following constituents in amounts per 0.5 ml: 148 mμmoles DL-[^{14}C]phenylalanine ($1.1 \cdot 10^3$ counts/min/mμmole); 260 mμmoles [^{14}C]glycine (10^3 counts/min/mμmole); 14.5 mμmoles DL-[^{14}C]leucine ($5.6 \cdot 10^3$ counts/min/mμmole); 0.02 ml equilibrated amino acid mixture; 2 μmoles ATP; 80 μg of each of nucleoside diphosphates; 2 μmoles $MnCl_2$; 1.1 μmoles $MgSO_4$; 92 μg protein enzyme preparation IV.

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TABLE I

EFFECT OF NUCLEOSIDE DIPHOSPHATES ON ^{14}C-AMINO ACIDS

The system was incubated without shaking 120 min at 30°. Further, specific activities of amino acids refer to the L-isomer where a racemic mixture was used. Contents in 0.5 ml of reaction mixture: 1.1 μmoles $MgSO_4$; 2 μmoles $MnCl_2$; 25 μmoles TRIS buffer pH 7.3; 2 μmoles ATP; 80 μg of each nucleoside diphosphate; 100 μg protein of enzyme preparation VIII; 148 mμmoles DL-[^{14}C]phenylalanine ($1.1 \cdot 10^3$ counts/min/mμmole); 29 mμmoles DL-[^{14}C]leucine ($5.6 \cdot 10^3$ counts/min/mμmole); 260 mμmoles [^{14}C]glycine ($1 \cdot 10^3$ counts/min/mμmole); 0.02 ml equilibrated amino acid mixture.

Addition	*Incorporation counts/min/mg protein*
None	9,828
ATP	35,042
ADP, GDP, UDP, CDP	25,340
ADP, GDP, UDP, CDP + ATP	45,290
GDP, UDP, CDP + ATP	44,160
GDP, UDP + ATP	35,100
GDP, CDP + ATP	36,200
GDP + ATP	35,720
UDP + ATP	34,880

TABLE II

ADDITIVE EFFECTS OF AMINO ACIDS ON INCORPORATION

Incubation without shaking at 30° for 180 min. Contents per 0.5 ml of reaction mixture: 125 mμmoles glycine ($1 \cdot 10^3$ counts/min/mμmole); 28.5 mμmole DL-leucine ($5.6 \cdot 10^3$ counts/min/mμmole); 148 mμmoles DL-phenylalanine ($1.1 \cdot 10^3$ counts/min/mμmole); 1.1 μmoles $MgSO_4$; 4 μmoles $MnCl_2$; 25 μmoles TRIS buffer pH 7.3; 0.02 ml ^{14}C equilibrated amino acid mixture; 85 μg protein of enzyme preparation VII.

Additions	*Counts/min/mg protein*	
	Observed	*Corrected for isotopic dilution*
Glycine	11,250	7,000
DL-Phenylalanine	10,000	16,680
DL-Leucine	25,000	194,000
Glycine + phenylalanine + leucine	42,062	237,000

that a similar situation obtains for amino acid incorporation. The incorporation is increased 20-fold over the basal level when $8 \cdot 10^{-3}$ *M* Mn^{++} is added.

Amino acid activation. The activation of several amino acids by crude and purified preparations of mammalian tissues[4,6] and of microbial extracts has already been reported[5,7,9]. The over-all reaction for the activation can be described as:

$$\text{ATP} + \text{L-AA} \rightleftharpoons \text{adenyl-L-AA} + \text{PP}$$

The assumed active intermediate, the adenyl-AA, has never been directly isolated from the enzymatic reaction, therefore, the phenomenon of activation can be followed only indirectly. Pyrophosphate exchange with ATP in the presence of amino acids and the activation enzymes was employed in the present studies. Table V shows that the optimal enzyme concentration for the activation of L-valine by the particulate system is about 80–100 μg protein/ml. Table IV shows that freshly prepared material

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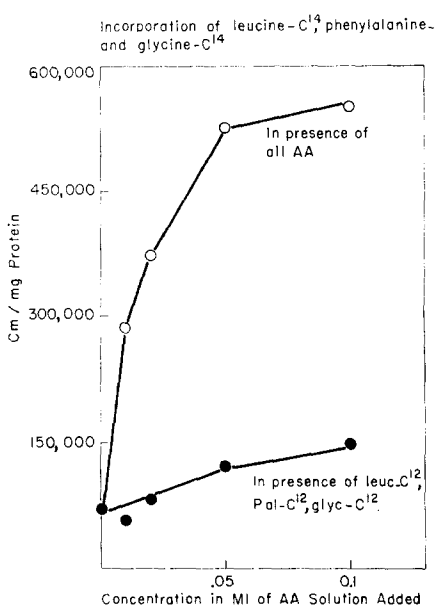

Fig. 5. The systems were incubated without shaking for 180 min at 30° in presence of the following constituents in amounts per 0.5 ml: 148 mμmoles DL-[^{14}C]phenylalanine, 260 mμmoles [^{14}C]glycine, 29 mμmoles DL-[^{14}C]leucine. The specific activities of the ^{14}C-amino acids are those indicated. 2 μmoles ATP, 1.1 μmoles $MgSO_4$ 2 μmoles $MnCl_2$, 25 μmoles TRIS buffer pH 7.3, 92 μg protein enzyme preparation IV. a. In the upper curve the ^{14}C-amino acids used were those of the equilibrated mixture. b. In the lower curve the [^{14}C]phenylalanine, [^{14}C]leucine, [^{14}C]glycine used were in the concentration they are in the equilibrated amino acid mixture.

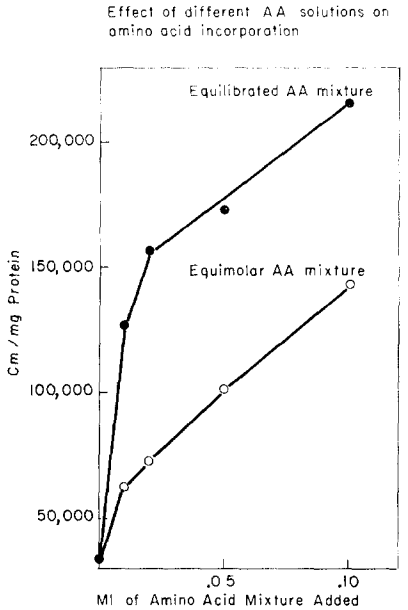

Fig. 6. The systems were incubated without shaking for 120 min at 30° in presence of the following constituents in amounts per 0.5 ml: DL-[^{14}C]leucine, DL-[^{14}C]phenylalanine, ^{14}C-glycine at the final concentration and specific activities indicated in Fig. 5. a. Upper curve: equilibrated amino acid mixture; b. Lower curve: equimolar amino acid mixture. The other constituents are identical to and in the same concentrations as indicated in Fig. 5; 98 μg protein of enzyme preparation VI.

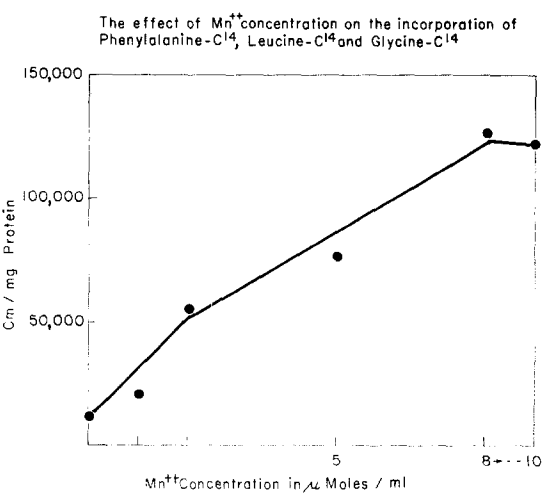

Fig. 7. The system was incubated without shaking for 120 min at 30° in presence of the following constituents in amounts per 0.5 ml: 2 μmoles $MnCl_2$; 1.1 μmoles $MgSO_4$; 25 μmoles TRIS buffer pH 7.3. The labeled ^{14}C-amino acids are of the same specific activities and concentrations as in Fig. 6. 0.02 ml equilibrated amino acid mixture, 98 μg protein enzyme preparation VI.

TABLE III

AMINO ACID ACTIVATION MEASURED BY ATP-[^{32}P]PP EXCHANGE

Preparations I and III were fresh and tested within 1 to 2 h after lysis of the protoplasts. Preparation IA was stored for 24 h at 0° and then tested. Preparation II is an aliquot of IA subjected to sonic treatment for 10 min in a 10 kc Raytheon oscillator. 1 ml reaction mixture contained: 2 μmoles of the indicated amino acid; 100 μmoles tris buffer pH 8.0; 5 μmoles $MgCl_2$; 2 μmoles ATP; 2 μmoles sodium pyrophosphate ($2 \cdot 10^5$ counts/min/μmole for I, IA and II, and $3.5 \cdot 10^4$ counts/min/μmole for III); 5 μmoles KF; 100 μg of enzyme preparation in terms of protein. All figures corrected for the control (minus AA).

	mμmoles [^{32}P]PP incorporated into ATP per mg protein in 15 min. Preparations			
	I	*IA*	*II*	*III*
Controls				
No amino acids	70	80	50	80
No ATP (with 0.02 ml of equimolar mixture of AA)	50	—	20	40
No ATP (with valine)	—	40	—	—
No enzyme (with ATP and valine)	40	30	—	—
Additions to complete system				
L-valine	910	1090	900	2348
Glycine	24	27.6	3.6	74
L-glutamate	326	348		110
Glutamine	—	—		139
L-serine	91	13	52	190
L-threonine	239	140	96	390
L-leucine	920	1360	912	876
L-isoleucine	702	660	552	1087
L-arginine	274	43	54	196
L-aspartate	229	120	4	226
L-asparagine	—	—	—	257
L-methionine	506	516	288	518
L-histidine	257	309	204	414
L-phenylalanine	180	143	132	336
L-tryptophane	500	725	600	996
L-tyrosine	619	440	396	680
L-proline	196	88	48	243
L-lysine	576	420	419	606
L-alanine	233	50	84	208
L-cysteine	282	222	156	516
All AA equimolar (0.2 ml)	2863	—	—	—
All AA equilibrated (0.2 ml	4356	—	—	—

TABLE IV

L-VALINE ACTIVATION AS A FUNCTION OF ENZYME CONCENTRATION

Incubation for 15 min at 30° without shaking. All constituents at concns. detailed in Table III. 1 ml of enzyme contained 2 mg protein. Specific activity of [^{32}P]PP was $8 \cdot 10^4$ counts/min/μmole; 12 μmoles/ml L-valine.

Concn. of enzyme ml	*Incorporation into ATP as mμmoles [^{32}P]PP*
0.02	27
0.03	43
0.04	57
0.05	60
0.04 (no amino acid)	3
No enzyme	0

activated all 18 natural amino acids. Asparagine and glutamine (BERG AND NISMAN, unpublished observations) (Table VI) have also been found to be activated by the same preparation. Whether each amino acid is activated by a distinct enzyme is not resolved by the data available.

Activation of glycine, glutamic acid, aspartic acid, serine, threonine, and arginine is inferior to the other amino acids tested and the activity on this group is lost more rapidly on aging or by sonic treatment of the enzyme preparations[9] (Table III). As may be seen from Table III, the sedimentable material activates the equilibrated amino acid mixture some 40 % better than the equimolar amino acids. This finding could explain the higher incorporation observed with the equilibrated mixture.

Table V shows that the L-isomers are activated. D-isomers inhibit to a certain degree the activation of the L-amino acids.

TABLE V

ACTIVATION OF D-, L- AND DL-AMINO ACIDS

All conditions identical to those of Table III; [^{32}P]PP: $3.5 \cdot 10^4$ counts/min/μmole PP.

	Incorporation into ATP in mμmoles [^{32}P]PP
L-valine	1260
D-valine	7.5
DL-valine	810
L-leucine	435
D-leucine	7.5
DL-leucine	261
L-isoleucine	420
D-isoleucine	30
DL-isoleucine	261

In view of the steric similarities between certain pairs of amino acids, it seemed possible that both members would be activated by a single enzyme. Accordingly, some preliminary experiments were conducted to determine whether or not competition exists for activation in the following cases (Table VI): serine and threonine; glutamic acid and glutamine; aspartic acid and asparagine. As shown in Table VI, there is a competition between the members of the first two pairs but not between the members of the last pair. The competition for the activation reaction could, nevertheless, be also a reflection of competitive inhibition of enzyme by the reciprocal substrates. For example, serine might have an affinity for the threonine enzyme and *vice versa*.

In view of the well-known valine–leucine antagonism in *E. coli* K 12 strains[17], it is interesting to note that the activation is not additive when both of these amino acids are used together. The figures for simultaneous activation of L-valine + L-leucine and L-valine + L-isoleucine are always lower than the corresponding figure for the activation of L-valine alone (Table VI). The same table shows that D-valine is not activated and, in addition, strongly inhibits the activation of L-leucine when present. This may explain the antagonisms between these amino acids observed in growth experiments[17].

Kinetics of the incorporation reaction. The kinetics of incorporation by the present enzymic system are different from those of the other systems which have been

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TABLE VI

COMPETITION BETWEEN AMINO ACIDS FOR THE ACTIVATION REACTION

All conditions identical to that of Table III. Enzyme preparation III is the same as described in Table III and used at the same level; 100 μg protein of enzyme preparation IV.

Substrate	*mμmoles [^{32}P]PP incorporated in ATP in 15 min Preparations*	
	III	*IV*
L-serine	190	—
L-threonine	390	—
L-serine + threonine	313	—
L-glutamic	110	—
L-glutamine	139	—
L-glutamic + glutamine	84	—
L-aspartic	226	—
L-asparagine	257	—
L-aspartic + asparagine	363	—
L-valine	—	1260
D-valine	—	7.5
L-leucine	—	435
L-isoleucine	—	420
L-valine + L-leucine	—	915
D-valine + L-leucine	—	75
L-valine + L-isoleucine	—	1200
D-valine + L-isoleucine	—	405

[^{32}P]PP in preparation IV: $3.5 \cdot 10^4$ counts/min/μmole.

TABLE VII

EFFECT OF SONIC TREATMENT ON THE AA INCORPORATION SYSTEM

9 kc Raytheon sonic oscillator. The incubation mixture was shaken at 30° for 120 min; contents of 1 ml of reaction mixture: *Experiment 1.* 200 μg protein of enzyme preparation I; 80 μg each of the four nucleoside diphosphates; 2 μmoles ATP; 0.1 mequiv. AA; 2.2 μmoles $MgSO_4$; 4 μmoles $MnCl_2$; 25 μmoles TRIS buffer pH 7.3; 100 μmoles [^{14}C]phenylalanine (1 μC/μmole). *Experiment 2.* Same as Experiment I except for use of 85 μg protein of enzyme preparation VIII.

Sonication time min		*Counts/min/incorp./mg protein*	
Expt. 1	*Expt. 2*	*Expt. 1*	*Expt. 2*
0	0	22,355	32,000
3	6	15,750	800
4		14,063	

previously studied. Fig. 8 shows that the rate of incorporation of the labeled amino acids rises with time resembling in this respect the induced synthesis of β-galactosidase in a somewhat analogous system described by SPIEGELMAN AND WOLIN[18]. (This type of kinetics could reflect the multistep reaction nature of protein synthesis.)

The initial lag phase can be suppressed or shortened by pre-incubating freshly prepared enzymic material with riboside diphosphates and ATP for 45 min[26].

Effect of sonic treatment of the particulate system. Preliminary experiments indicate that some incorporation activity of the enzymic preparations persists if sonic treatment does not exceed 4 min (Table VII).

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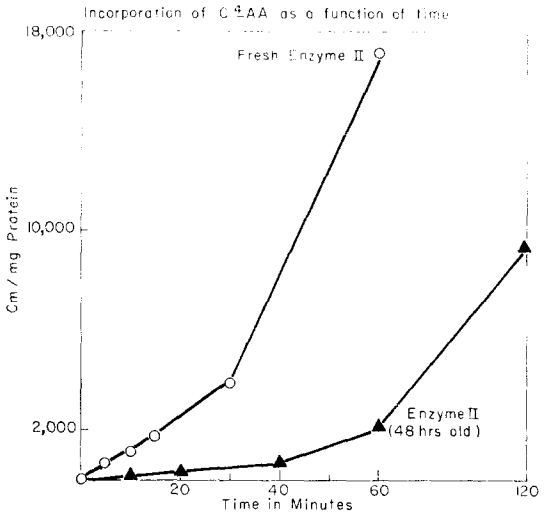

Fig. 8. The systems were incubated without shaking at 30° in presence of the following constituents in amounts per 0.5 ml: 0.01 ml ^{14}C uniformly labeled amino acids ($4.5 \cdot 10^5$ counts/min total); 2 μmoles ATP; 80 μg of each of the ribonucleoside diphosphates; 2 μmoles $MnCl_2$; 1.1 μmoles $MgSO_4$; 25 μmoles TRIS buffer pH_I 7.3; 100 μg protein enzyme preparation II.

Prolonged sonic treatment leads to a loss in the total activity of about 95%. The experiments on sonic treatment suggest that a certain degree of solubilization or fragmentation of the system can occur without loss of incorporation activity; but critical constituents of the system such as activating enzymes and the RNA may be destroyed by harsher treatment.

Effect of inorganic phosphate on the incorporation activity. Inorganic phosphate (0.05 *M*) almost completely abolishes the incorporation activity if added to the preparation prior to incubation (Fig. 9). A similar destructive effect of orthophosphate on the ability to synthesize enzyme in somewhat analogous lysates was observed by SPIEGELMAN AND WOLIN[18].

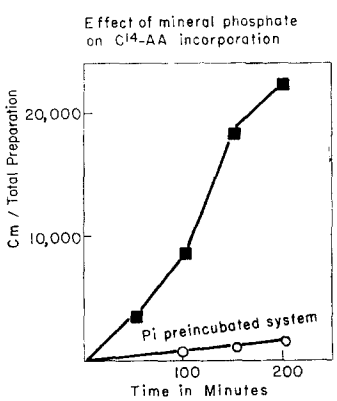

Fig. 9. The systems were incubated without shaking at 30° in precense of the following constituents in amounts per 0.5 ml: DL-[^{14}C]phenylalanine; DL-[^{14}C]leucine; [^{14}C]glycine in the same concentration and specific activity as indicated in Fig. 6. 0.02 ml equilibrated amino acids mixture; 2 μmoles ATP; 80 μg of each of all four ribonucleoside diphosphates; 2 μmoles $MnCl_2$; 1.1 μmoles $MgSO_4$; 25 μmoles TRIS buffer pH 7.3; 98 μg protein enzyme preparation VI. The phosphate inactivated enzyme was prepared by stirring preparation VI in presence of 0.05 *M* phosphate buffer pH 7.3 at 0° for 20 min and thereafter sedimenting the enzyme preparation by centrifugation in the International refrigerated centrifuge at 18,000/rev./min for 10 min.

Effect of strictly aerobic conditions. Most of the experiments were conducted in the presence of air but without shaking (Figs. 1, 2, 3 and 5). Under these conditions incorporation is very markedly stimulated by the addition of ATP. However, when

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experiments were conducted in shaken flasks, ATP was markedly less effective in stimulating incorporation. Indeed, under these conditions, a mixture of the nucleoside diphosphates stimulated incorporation as effectively as ATP alone and the addition of ATP to the mixed diphosphates did not increase incorporation further (Table VIII).

TABLE VIII

EFFECT OF STRICTLY AEROBIC CONDITIONS ON AMINO ACID INCORPORATION

The system was incubated aerobically with shaking at 30° and contained the following constituents in amounts per ml: 10 mμmoles DL-[^{14}C]phenylalanine (0.9 μC/1.5 μmole); 0.3 ml equilibrated AA; 200 μg protein of enzyme preparation I; 80 μg of each of the four nuclesiode diphosphates; 2.2 μmoles $MgSO_4$; 4 μmoles $MnCl_2$; 25 μmoles TRIS buffer pH 7.3.

Additions	*Counts/min/mg protein*	*Incubation time min*
None	500	60
None	1725	120
2 μmoles ATP	975	60
2 μmoles ATP	2525	120
Nucleoside diphosphates	997	60
Nucleoside diphosphates	3363	120
Nucleoside diphosphates + ATP	1182	60
Nucleoside diphosphates + ATP	2942	120

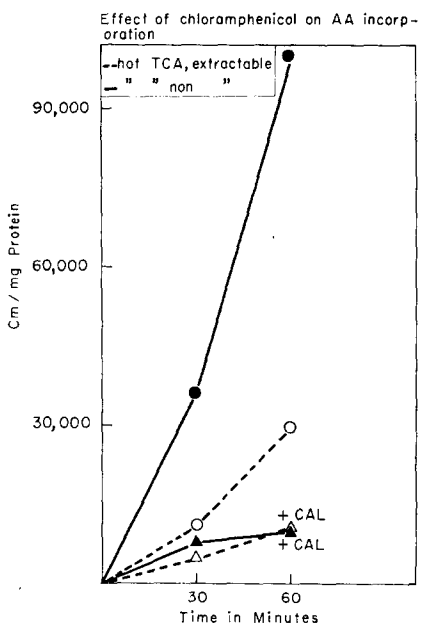

Fig. 10. The systems were incubated without shaking at 30° in presence of the following constituents in amounts per 0.5 ml: 0.02 ml of uniformly ^{14}C-labeled amino acids ($9 \cdot 10^5$ counts/min total); 2 μmoles ATP; 4 μmoles $MnCl_2$; 1.1 μmoles $MgSO_4$; 80 μg of each of all four ribonucleoside diphosphates; 20 μg chloramphenicol; 25 μmoles TRIS buffer pH 7.3. (The extractable radioactivity from the hot TCA extracts was counted after neutralization of the TCA with NH_4OH). 85 μg protein enzyme preparation VIII.

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This observation suggests that under aerobic conditions the preparation contains a systems which is capable of generating ATP by oxidative phosphorylation.

Effect of chloramphenicol on amino acid incorporation. The ^{14}C amino acids incorporated into the sedimentable material of *E. coli* occur in two forms: a hot TCA extractable fraction and a hot TCA unextractable one (Fig. 10). The radioactivity of both fractions increases with time. In the microsomal system from liver, HOAGLAND[10] has recently shown that the radioactivity extractable with hot TCA or perchloric acid consists of an RNA-amino acid fraction which is presumably an intermediate in protein synthesis. Experiments carried out by BERG *et al.*[25] suggest that the fraction in *E. coli* ML extracts is of similar nature. Chloramphenicol (CAL) inhibits the incorporation of the radioactivity of the ^{14}C-amino acids into both the TCA extractable and unextractable fractions (Fig. 10). The abolition of incorporation into the TCA unextractable fraction is not surprising since CAL has long been known to inhibit specifically protein synthesis[20]. Since CAL does not inhibit amino acid activation[21] nor the transfer to the RNAs, its inhibitory effect on the incorporation into the TCA extractable fraction suggests that it influences the synthesized RNA in such a way as to make the fixation of the amino acid impossible. In other words the RNA formed in presence of the inhibitor may be physiologically inert[22,23].

DISCUSSION

The *E. coli* system differs from the others by (a) its high saturation level for amino acids; (b) the requirement of a balanced mixture of amino acids for optimal incorporation; (c) a virtually absolute requirement for Mn^{++}; (d) an extreme lability to inorganic phosphate.

The *E. coli* system prepared as described contains all the amino acid activating enzymes and the incorporation system in the same pelletable fraction. A striking difference from the liver microsomal system is the lack of response of the *E. coli* system to GTP.

The experiments reported here provide evidence in favor of the following sequence of events outlined in the introductory part in protein synthesis:

1. L-AA + ATP $\rightleftharpoons$ Adenyl-AA + PP
2. Nucleosidediphosphates $\rightleftharpoons$ RNA
3. Adenyl-AA + RNA $\longrightarrow$ RNA-AA (Hot TCA extractable fraction)
4. RNA-AA $\longrightarrow$ Protein (Hot TCA unextractable fraction)

Reactions 1 and 4 are implied by the experiments demonstrating that all amino acids are activated and that the activation and incorporation reactions are optimal under the same conditions (equilibrated amino acid mixture and presence of ATP). Reaction 2 is inferred from the fact that the nucleoside diphosphates are most effective in promoting RNA synthesis[26] and amino acid incorporation. This could mean that these compounds are direct intermediates, serving as substrates for the polynucleotide phosphorylase of GRUNBERG-MANAGO AND OCHOA[24].

The transfer of amino acids to RNA (step 3) has been observed. The fate of the adenyl-moiety as well as the role of the RNA-AA are not as yet clear.

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