realization of this situation should be the possession of every member of modern society, and I, for myself, would not be willing to call a man properly educated who did not instinctively realize this, nor would I call any system of education adequate which did not impart this realization. The second stage, after the appreciation of the situation, is to devise methods of coping with it. This is, of course, a much more difficult matter, and something that we do not yet know how to do. Whatever the final answer, we can make one pronouncement now which we can anticipate that we will not have to take back. This is that the situation is *not* to be dealt with by succumbing to discouragement and turning back to the outlooks of the past. This procedure is, however, so often advocated that there is occasion here for active alarm. The situation that confronts us is a situation brand new in the history of the human race, both in our vision of the nature of the crisis and in our vision of possible ways of meeting it.

The scientist is in a position to play an important role here. In fact, I would be tempted to say that the scientist has a special responsibility, were it not that I regard the concept of responsibility as loaded and to be avoided. It must be admitted that the scientist, because of his special experience, is in a strategic position to see what the nature of the situation is. This is a simple statement of fact that may be made without immodesty, in the realization that the situations of the humanist are incomparably more complex than those of the scientist and that it is inevitable that the simple situations be apprehended first. What is more, the scientist, fortified by the spectacular success which he has admittedly attained in meeting the limited objectives of science, is in a unique position to confront the difficulties ahead without discouragement, confident of final success. The weariness and discouragement which too often impel the humanist to advocate a return to the past are symptoms of loss of intellectual morale. The modern world has lost its intellectual morale; the scientist can help to recover it. And it seems to me that a little more militancy on the part of the scientist would not be out of place.

THE ENZYMATIC ACTIVATION OF AMINO ACIDS VIA THEIR ACYL-ADENYLATE DERIVATIVES

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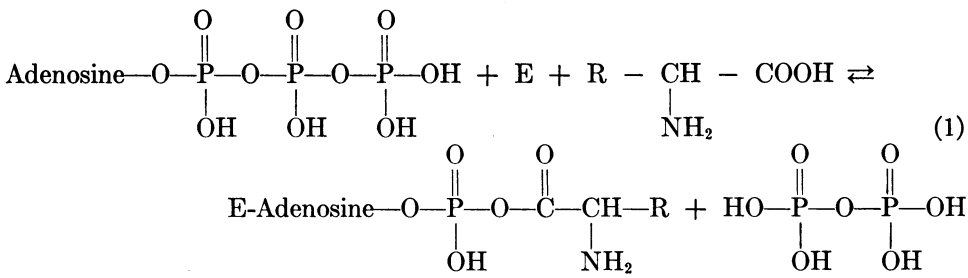
Although it has been known for a long time that energy is necessary for protein synthesis, there is little information available to suggest in which reaction energy is utilized for the eventual formation of the peptide bonds in the protein molecule. Recently several systems for the activation of amino acids have been described which may cast some light on the manner in which energy is utilized in protein synthesis.

An amino acid–dependent exchange between P^{32}-labeled inorganic pyrophosphate (PP) and adenosine triphosphate (ATP) was discovered by Hoagland[1] to be catalyzed by an extract of rat liver. We have found a similar enzyme system to be

distributed ubiquitously in microörganisms.[2, 3] Berg[4] reported the occurrence of a methionine-specific enzyme in yeast extracts which catalyzes the PP^{32}-ATP exchange reaction.

The reaction in each case appears to be a simple exchange on an enzyme surface, since careful balance studies have failed to indicate a net reaction. A clue to the nature of the reaction was given by the finding that the presence of high concentrations of hydroxylamine during the exchange led to a breakdown of ATP and the formation of PP, of adenosine monophosphate (AMP), and of the corresponding amino hydroxamates.[1] This observation suggested that amino acids are activated through their carboxyl groups during the exchange reaction. The fact that AMP does not undergo exchange under conditions where PP exchanges rapidly with ATP led Hoagland[5] to postulate the following mechanism for the exchange reaction:



(1)

It is visualized that the enzyme interacts with ATP and the amino acid, to split out PP and leave the intermediate, amino acyl–AMP, attached to the enzyme. This formulation is analogous to that postulated by Berg[4] for the activation of acetate by ATP in yeast extracts. In the latter case, acetyl-AMP was chemically synthesized and shown to be converted to ATP in the presence of PP or to acetyl-CoA in the presence of Coenzyme A. Equation (1) suggests that the carboxyl group of the amino acid undergoes reaction with ATP to form an anhydride with AMP through the elimination of PP. The amino acyl–AMP, being a substituted acyl phosphate, would be expected to be in the class of energy-rich compounds similar to acetyl phosphate,[6] acetyl-AMP,[4] and amino acyl phosphates[7] and might be expected to react with hydroxylamine at neutral pH to give the corresponding hydroxamate.

We have now obtained evidence in strong support of equation (1) by synthesizing L-leucyl-AMP (LAMP)[8] and demonstrating its ready conversion to ATP by a purified enzyme which catalyzes a leucine-dependent exchange between PP and ATP, apparently by reversal of reaction (1). The purpose of the present communication is to describe the synthesis of leucyl-AMP and its enzymatic conversion to ATP.

METHODS

The enzyme extracts used in these experiments were prepared by grinding cells of *Escherichia coli* with alumina and extracting with phosphate buffer, as previously described.[9] In certain cases the extracts were purified about 20-fold with respect to leucine-dependent exchange by treatment with ribonuclease, fractionation with ammonium sulfate, and adsorption and elution from calcium phosphate gel. Such preparations are free from inorganic pyrophosphatase and ATPase. The activity of the extracts was determined by measuring their ability to catalyze the amino

acid-dependent exchange between PP^{32} and ATP, as previously described.[9] An enzyme unit is defined as that amount which catalyzes the exchange of 1 μM of PP under defined conditions.[9] Radioactivity in ATP was measured by hydrolyzing ATP adsorbed to charcoal with 1.0 *N* HCl and counting an aliquot of the supernatant.[10] In some experiments, ATP was estimated as acid-labile phosphate[11] after elution from charcoal with 0.1 per cent ammonia in 50 per cent ethanol. In other experiments, ATP was measured by the decrease in acid-labile phosphate after incubation with an excess of yeast hexokinase and glucose. Inorganic pyrophosphate was measured by acid-labile phosphate or by the colorimetric method of Flynn *et al.*[12] Adenine was measured by optical density at 260 mμ and adenylic acid by using 5′ adenylic acid deaminase according to the method of Kalckar.[13]

Amino acyl-AMP was measured by the following modification of the original hydroxamic acid method of Lipmann and Tuttle.[14] The sample, containing 0.5–3.0 μM, is mixed with 1.0 ml. of 2 *M* hydroxylamine solution (prepared by mixing equal volumes of 4 *M* hydroxylamine hydrochloride and 3.5 ml. *N* NaOH). The volume of the reaction mixture is brought to 2.0 ml. with distilled water and allowed to stand at room temperature for 5 minutes. Then 2.0 ml. of a ferric chloride solution (1 part 3.5 *N* HCl plus 3 parts 20 per cent $FeCl_3 \cdot 6H_2O$ in 0.1 *N* HCl) are added. The reagents are mixed quickly and read immediately in a Klett colorimeter at 540 mμ. The readings must be taken quickly, since the color fades rapidly. Under these conditions 1.0 μM of leucine hydroxamate reads 130 Klett units. In some cases leucine hydroxamate was identified by one-dimensional paper chromatography using the solvent system 2-butanol:formic acid:H_2O (75:15:10). Leucine was estimated by the ninhydrin method of Troll and Cannon.[15]

RESULTS

Synthesis of L-*Leucyl-AMP.*—Several methods for the preparation of the amino acyl-AMP compounds were first explored without success. Attempts to acylate AMP in aqueous pyridine by the method of Avison,[16] using N-carboxy amino acid anhydrides, apparently led to the formation of amino acid polymers. When the amino group is protected with an acetyl, carboethoxy, or carbobenzoxy group, AMP can be smoothly acylated by this method with a mixed anhydride prepared according to Wieland and Rueff.[17] However, all compounds prepared with a blocked amino group were inactive in our test system, and we have as yet been unable to remove the protecting group under conditions sufficiently mild to avoid destruction of the compounds. Since Berg[4] had successfully prepared acetyl-AMP by treating the silver salt of AMP with acetyl chloride, a method first introduced by Lipmann[18] in the synthesis of acetyl phosphate, we tried this method with the acid chlorides of amino acids and succeeded in preparing active compounds. The synthesis of L-leucyl-AMP is detailed below.

To 25.0 ml. of glacial acetic acid are added 2.0 mM of L-leucine acid chloride (HCl salt) prepared according to Levine[19] and 2.0 mM of disilver AMP; this material is shaken mechanically for 30 minutes at 30° C. The acid chloride is soluble in glacial acetic acid, while disilver AMP is not. At the end of the reaction time, 1.25 ml. of H_2O is added, and the suspension is allowed to stand at room temperature for 5 minutes. This procedure results in the decomposition of any unreacted acid chloride. During this time any free AMP, leucine hydrochloride, and leucyl-

AMP pass into solution in the glacial acetic acid. The remaining silver salt is removed by centrifugation. The supernatant is chilled to 0°, and 1 volume of cold absolute ethanol is added, followed by 2 volumes of cold ether. The resulting white precipitate is collected by centrifugation in the cold and then washed once with cold ether-ethanol (75:25), once with ether-ethanol (85:15), once with ether-ethanol (95:5), and finally with 100 per cent ether. The material is quickly dried by blowing a current of air into the tube. The product at this stage generally contains 7–8 μM of leucyl-AMP, approximately 13–15 μM of free AMP, and about 8 μM of free leucine per 10 mg. The over-all yield is about 9 per cent. We have found that this product, although contaminated with both AMP and free amino acid, is satisfactory for most purposes. We have also successfully prepared L-alanyl-AMP, D-leucyl-AMP, and DL-phenylalanyl-AMP by this method.

The compound may be further purified by passage through a Dowex column as follows: 50 μM of crude L-leucyl-AMP was dissolved in water and brought to pH 4.8 with solid $KHCO_3$. The solution was passed over a column of Dowex-1, X-10 (chloride form), 0.8 cm. in diameter and 4.0 cm. long. Leucyl-AMP passed through the column while free AMP was retained. This procedure resulted in the removal of almost all of the free AMP.

Characterization of Leucyl-AMP.—The proposed structure of the amino acyl–AMP compounds is presented in Figure 1. We have obtained evidence in support

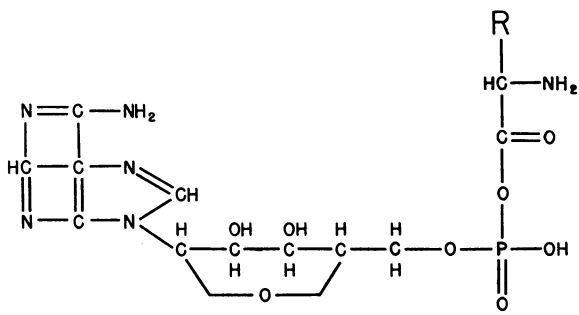


FIG. 1.—Amino Acyl-AMP

of this formulation in the case of synthetic leucyi-AMP. When the compound is treated with hydroxylamine in neutral solution, it rapidly reacts with hydroxylamine to give *only* leucine hydroxamate which has been estimated colorimetrically and identified by paper chromatography with an authentic sample of leucine hydroxamate prepared from the acid chloride. This is presumptive evidence that the carboxyl group of leucine exists in an anhydride linkage in the compound. The amount of adenine present in the compound was measured by its optical density at 260 mμ and expressed as 5′ adenylic acid. When a purified preparation of the compound is treated with 5′ adenylic acid deaminase, there is little or no change in optical density at 265 mμ, indicating that the 5′ adenylic acid in the compound is chemically bound in such a way that it was not attacked by the deaminase. When, however, a 5-fold excess of hydroxylamine is added to the cuvette containing the sample and deaminase, there is an immediate liberation of 5′-AMP, as evidenced by a rapid decrease in absorption at 265 mμ. Since hydroxylamine gives rise to

leucyl hydroxamate simultaneously with the liberation of 5'-AMP, it is clear that the carboxyl group of leucine is bound in an anhydride linkage with the phosphate group of AMP. A summary of these data is presented in Table 1.

TABLE 1

COMPOSITION OF LEUCYL-AMP

	μM*		μM*
Total adenine	1.06	Bound 5'-AMP	0.92
Free 5'-AMP	0.14	Leucyl hydroxamate	1.12

* For details of analysis see text.

By paper electrophoresis in 0.015 *M* potassium citrate buffer at pH 3.8 the compound was found to migrate with a positive charge, as would be expected from the structure given in Figure 1. Under these conditions, 5'-AMP migrated with a negative charge. All these facts are consistent with the formulation of the compound given in Figure 1.

Leucyl-AMP is quite stable at 37° in aqueous solution at acid pH. As the pH is increased, the rate of hydrolysis increases, and at pH 8.0 the compound has a half-life of about 4 minutes. The rate of hydrolysis at different pH values at 37° is plotted in Figure 2. The rate of hydrolysis appears to be increased by imidazole

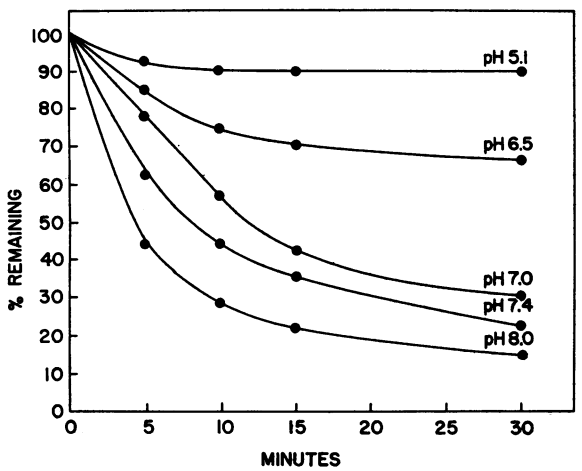


FIG. 2.—Effect of pH on rate of hydrolysis of Leucyl-AMP

uffer. For example, at pH 6.5 in phosphate buffer the half-life of the compound is greater than 30 minutes, whereas at the same pH in imidazole buffer the half-life is only 3 minutes.

Enzymatic Conversion of L-*Leucyl-AMP to ATP.*—In Table 2 are presented data which demonstrate the enzymatic conversion of L-leucyl-AMP to ATP. For routine analysis of reaction mixtures, it is more convenient to determine labeled ATP as counts per minute (c.p.m.) adsorbed to charcoal, as is routinely carried out in our exchange experiments. It was, therefore, important to compare the radioactivity method with more specific methods for measuring ATP. Accordingly, the reaction mixture described in Table 2 was divided into three aliquots, and ATP

was determined as counts per minute adsorbed to charcoal and compared with material eluted from the charcoal and assayed as acid-labile phosphate and as phosphate transferable to glucose by hexokinase. It can be seen that there is reasonably good agreement among the three methods. Therefore, radioactivity measurements were used in all subsequent experiments, thereby permitting considerable conservation of materials.

In the experiment described in Table 2, determination of residual leucyl-AMP indicated that the compound had largely disappeared in the control sample as well as in the complete system. These data were insufficient to make a balance study of the conversion of the compound to ATP. It was of interest, therefore, to compare the rate of disappearance of leucyl-AMP with the rate of formation of ATP. The results of such an experiment are presented in Table 3.

TABLE 2

FORMATION OF ATP FROM LEUCYL-AMP

	μM ATP DETERMINED BY		
	C.p.m. Adsorbed on Charcoal	Heat-labile P_i	Hexokinase Assay
Complete system	1.4	1.5	1.2
Enzyme omitted	0.03	0.01	..
LAMP omitted	0.07	0.02	..

* Conditions: 200 μM phosphate buffer, pH 6.8; 60μM $MgSO_4$; 20 μM PP^{32}; 6.8 μM LAMP; 0.4 ml. *E. coli* extract (200 units) in a final volume of 1.4 ml. Incubated 15 minutes at 37° C.

TABLE 3*

RATE OF FORMATION OF ATP FROM LAMP

TIME (Min.)	No Enzyme		No PP		Complete	
	LAMP (μM)	ATP† (μM)	LAMP (μM)	ATP† (μM)	LAMP (μM)	ATP† (μM)
0	13.5	..	13.5	..	13.5	..
1	13.4	0.2	13.5	0.0	8.7	4.3
3	10.5	0.1	10.7	0.0	4.5	6.1
5	7.8	..	7.6	..	4.0	..

* Conditions: 300 μM phosphate buffer, pH 6.8; 90 μM $MgSO_4$; 30 μM PP^{32}; 13.5 μM LAMP; 0.8 ml. purified *E. coli* extract (600 units) in a final volume of 3.0 ml. Incubation was at 37° C.
† Determined by counts per minute adsorbed on charcoal.

In 1 minute of incubation at 37° C., 4.8 μM of compound disappeared and 4.3 μM of ATP was formed. After 3 minutes of incubation, 8.9 μM of compound had disappeared, of which 6.1 μM had been converted to ATP. During the same time interval the controls had lost 2.8 μM of compound, presumably through hydrolysis, since no ATP was formed. Thus, the leucyl-AMP which is not spontaneously hydrolyzed is quantitatively converted to ATP. The data of Table 3 also indicate that the conversion is dependent both upon the enzyme and upon the presence of inorganic pyrophosphate.

Since we had found previously[3] that the exchange reaction was specific for L-isomer of the amino acids and that, furthermore, crude extracts catalyzed the exchange in the presence of only eight active amino acids (leucine, isoleucine, valine, histidine, methionine, tryptophan, tyrosine, and phenylalanine), it became of interest to see whether the formation of ATP from the synthetic compounds had similar specificities.

In Table 4 are presented data showing that with a purified extract (purified about 20-fold with respect to leucine) only L-leucyl-AMP is active in forming ATP,

whereas D-leucyl-AMP has little or no activity. With L-alanyl-AMP, the ATP formed in the crude extract is significant, and this activity is lost as the enzyme is

TABLE 4*

SPECIFICITY OF THE CONVERSION OF LEUCYL-AMP TO ATP

ADDITIONS	μM ATP FORMED†			ADDITIONS	μM ATP FORMED†		
	No Extract	Crude Extract	Purified Extract		No Extract	Crude Extract	Purified Extract
None	0.1	0.1	0.1	D-Leucyl-AMP	..	..	0.3
L-Leucyl-AMP	..	1.5	1.3	L-Alanyl-AMP	..	0.6	0.2

* Conditions: 100 μM phosphate buffer, pH 6.8; 30 μM $MgSO_4$; 10 μM PP^{32}; 8 μM amino acyl-AMP; 200 units *E. coli* extract in a final volume of 1.0 ml. Incubated 5 minutes at 37° C.

† Determined by counts per minute adsorbed on charcoal.

purified. That fact that L-alanyl-AMP does form some ATP in the crude extract is of interest, since alanine is one of the amino acids which are inactive in the exchange reaction.

DISCUSSION

The fact that L-leucyl-AMP is converted to ATP by a purified extract which catalyzes a leucine-dependent exchange between PP^{32} and ATP and that the formation of ATP exhibits specificities similar to the exchange reaction suggests that amino acyl–AMP compounds are intermediates in the exchange reaction. Since no net breakdown of ATP can be observed during a reaction between ATP and leucine, even in the presence of a potent inorganic pyrophosphatase, whereas the intermediate compound, leucyl-AMP, is rapidly converted to ATP, it appears that the intermediate has little or no tendency to dissociate from the enzyme surface during the exchange reaction. All attempts to demonstrate net formation of leucyl-AMP, either isotopically with C^{14}-leucine or colorimetrically, gave negative results. Furthermore, no net reaction can be obtained from ATP and amino acid at pH levels where the intermediate would spontaneously hydrolyze rapidly, again indicating that essentially no dissociation of the intermediate occurs.

This situation is completely analogous to results obtained by Berg[20] with purified yeast aceto-CoA-kinase. In this case, also, exchange data indicate that acetate and ATP react with the enzyme to split out PP and that synthetic acetyl-AMP is converted rapidly to ATP in the presence of PP or to acetyl-CoA in the presence of CoA. However, here again all attempts to demonstrate the net formation of acetyl-AMP have proved negative.

The property of negligible dissociation would be beneficial in the case of the amino acyl derivatives, if these compounds are intermediates in protein synthesis, since the compounds would thus be protected from spontaneous breakdown or interaction until a natural acceptor was available to remove them from the enzyme surface. In the case of aceto-CoA-kinase, the natural acceptor of acetyl-AMP is CoA. It is reasonable to assume, therefore, that an analogous system may occur for the amino acyl–AMP compounds.

That the amino acyl–AMP compounds are indeed protected from spontaneous breakdown while they are on the enzyme surface is evident from the fact that in free solution the amino acyl–AMP compounds react immediately with hydroxylamine when the latter is present in low concentration, whereas a concentration of 1.0–2.0

M hydroxylamine is required to trap the activated amino acid during the exchange reaction.

The hydrolysis data indicate the great reactivity of these compounds, and since the breakdown of the compounds appears to be catalyzed by amino groups, it would appear that under appropriate conditions the amino acyl–AMP compounds could easily polymerize to form peptides or proteins.

Although we have not yet obtained direct evidence for the participation of this amino acid–activating system in protein synthesis, Hoagland *et al.*[5] have presented evidence that a similar system in rat liver may play a part in the incorporation of amino acids into microsomal proteins. Work is now in progress which is designed to obtain direct evidence for the participation of this activating system in protein synthesis. We are also attempting to determine whether the apparently inactive amino acids may be activated through a transacylation system with one of the active amino acyl–AMP compounds.

SUMMARY

The synthesis of certain amino acyl–AMP compounds has been described, and some of the properties of such compounds have been indicated. The rapid formation of ATP from leucyl-AMP and inorganic pyrophosphate by a purified extract of *E. coli* which catalyzes the leucine-dependent exchange of PP^{32} and ATP is reported. This finding suggests that amino acyl–AMP compounds are intermediates in the exchange reaction. The properties of these compounds as well as those of the enzyme system are discussed in relation to their possible participation in protein synthesis.

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