SYMPOSIUM ON AMINO ACID ACTIVATION

BY INVITATION OF THE COMMITTEE ON ARRANGEMENTS OF THE AUTUMN MEETING

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CONTENTS

*CHAIRMAN'S INTRODUCTION: SOME FACTS AND PROBLEMS**

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In a very generalizing manner, one can describe biosynthetic processes as an interplay between group activation and group transfer. In the more recent past, the process of group activation has particularly caught our interest, since it is the initial phase of all biosynthetic mechanisms. In this laboratory we have, through the years, paid much attention to such processes of group activation where a primary carrier is created, around which an often quite diverse number of secondary acceptor reactions cluster, guiding the active group into a variety of biosynthetic products.[1] Against the background of these experiences, we were primarily interested in the initial group activation, hoping that, once the problem of amino acid activation was solved, the whole complex of protein synthesis would fall into line.

From the beginning, it seemed rather likely that the primary activation in the process of protein synthesis would occur by phosphorylation of the carboxyl end of the amino acid. In an attempt to rationalize this suggestion, I proposed quite a while ago a scheme of polypeptide synthesis[2] which, in its essentials, appears now to be confirmed, and, as it has some historical interest, it is here reproduced unchanged in Figure 1.

The initial step was visualized as activation by transfer of terminal phosphate from ATP to the amino acid. This was then, and remained for some time, the only form of group activation that we knew of. Only relatively recently, initiated by some work on the finer mechanism of acetate activation,[3] did we become aware that there exists another manner of utilizing the phosphate bond energy of nucleotide triphosphate. The two now known dominant pathways are illustrated in Figure 2. The "older" process, called there *Type 2*, represents an approach of the molecule which wants to be activated to the terminal P, causing activation by displacement of nucleotide diphosphate by X. The "newer" *Type 1* was recognized, through the appearance of pyrophosphate, as a product of reaction in various activation

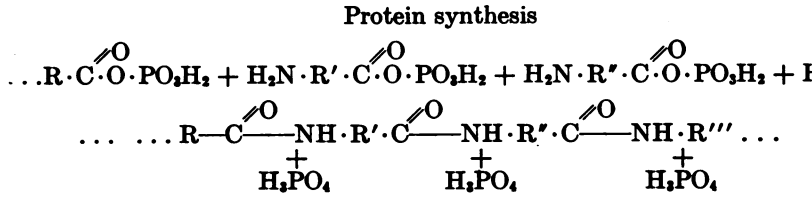
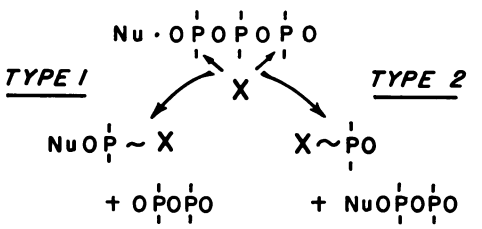


FIG. 1.—Scheme from F. Lipmann, *Advances in Enzymol.*, 1, 99, 1941.



PYROPHOSPHATE ELIMINATION

ACETYL ~ ; ACYL~
AMINOACYL ~
$(CHO)_X$-I-$PO_3^=$
CHOLINE-$PO_3^=$
SULFURYL~

NUCLEOTIDE-Di-PHOSPHATE ELIMINATION

γ GLUTAMYL ~
ACETYL~(MICROBIAL)
CARBAMYL ~
SUCCINYL
MYOSINE

FIG. 2.—This scheme describes the two most important types of group activation through reactions with nucleotide triphosphate. In many cases, activation and transfer are synchronized on the same enzyme and the phosphorylated intermediary is not well defined. This is the case with glutamate activation for glutamine synthesis, as well as succinate activation for syccinyl-CoA synthesis, shown on the right side of the scheme, and it certainly is true also for myosin in the same column.

processes;[3, 4] it subsequently emerged that, through PP-displacement, in such cases a Nu P ~X forms, and thus the activated molecule carries along the nucleotide.[5, 6] Since no evidence had come forward for a general *Type 2* activation of amino acids, but, on the other hand, the peptic bond in pantothenic acid was shown to follow a *Type 1* displacement,[4] in a recent scheme I then proposed a *Type 1* process for amino acid activation. This more elaborate scheme for amino acid activation and peptide synthesis[7, 8] furthermore, included some suggestions toward a mechanism for a specific lining-up of amino acids.

Some merit of this rather crude model was its switch to a *Type 1* reaction as the general means of amino acid activation. This proposition was taken up by Dr. Mahlon Hoagland. He, as most of you know, while working in Paul Zamecnik's laboratory, discovered the presence of such amino acid-activating enzymes in liver extract[9, 10] and, in particular, in those fractions which would complement amino acid incorporation into microsomes. Furthermore, DeMoss *et al.*[11] showed that such amino acid activation could be found in practically all bacteria and that lencyl

adenylate, which they synthesized, reacted as predicted with this fraction in reverse direction (cf. Fig. 3).

AMINO ACID ACTIVATION

HYDROXAMATE FORMATION

$$R\cdot CHNH_2\cdot C(=O)O^- + ATP + NH_2OH \longrightarrow$$

$$R\cdot CHNH_2\, C(=O)-NHOH + PP + AMP$$

PP-EXCHANGE

$$R\cdot CHNH_2\, C(=O)O^- + APP^{x\,x}P \longleftrightarrow (AP\sim COCHNH_2R) + P^{x\,x}P$$

FIG. 3.—Formulation of two alternate methods used for assay of amino acid activating enzymes.

Tests for this general amino acid activation may be made in two different ways; these are formulated in Figure 3. The first assay method makes use of hydroxylamine as a trapping reagent for activated carboxyls.[12] The second test depends on the reversibility of the reaction between ATP and amino acid.[9, 11] This may be measured by the amino acid–dependent exchange between adenosine triphosphate and radioactive pyrophosphate. I have to mention, however, that the forward reaction expected from the second equation in Figure 3, namely, formation of amino acid adenylate from ATP and amino acid, is proved largely indirectly by trapping the "active" amino acid with hydroxylamine. The requirement for a rather high concentration of hydroxylamine to get this trapping effect has been interpreted as indicating that the amino acyl adenylate is in some way buried in the enzyme.[10] The finer mechanism of the reaction still appears to be somewhat obscure.

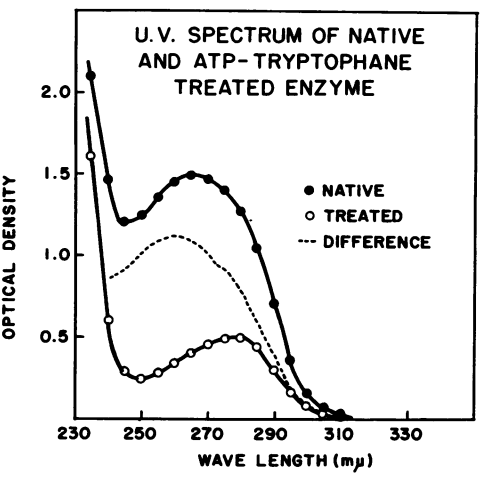


FIG. 4.—The tryptophan-activating enzyme used was approximately 50 per cent pure enzyme, as judged from ultracentrifuge versus activity data. Further details are found in Davie *et al.*, from whose work this figure is taken.

At this stage we became intensely interested in these amino acid-activating enzymes. Drs. Davie and Koningsberger in our laboratory succeeded in isolating a tryptophan-activating enzyme[13] which happens to be abundant in beef pancreas. The preparation obtained is specific for tryptophan and analogues, as shown in Table 1. A tyrosine specific activating enzyme has also been isolated from various sources.[14, 15] A specificity of such enzymes for a particular amino acid is therefore,

TABLE 1*

SUBSTRATE SPECIFICITY FOR HIGHLY PURIFIED TRYPTOPHAN-ACTIVATING ENZYME

Substrate	Hydroxylamine (μMoles/Mg/Hr)	Exchange (μMoles/Mg/Hr)
L-Tryptophan	86	400
DL-Tryptazan	48	64
N-Acetyl-DL-tryptophan	0	...
Glycyl-L-tryptophan	0	...
D-Tryptophan	0	...
L-Tyrosine	3	8
L-ϕ-Alanine	0	8
L-Leucine	0	0
Glycine	0	0
L-Cysteine	0	0

* From Davie *et al.*, *Arch. Biochem. and Biophys.*, **65**, 21, 1956.
The slight contamination with tyrosine and phenyl alanine–reactive enzyme was absent in other preparations. All preparations, however, showed reactivity with such tryptophan analogues as were incorporated into protein (cf. n. 18).

TABLE 2

AMINO ACID–DEPENDENT PP^{32}-ATP EXCHANGE: ACTIVATION OF ALL COMMON AMINO ACIDS BY PIGEON PANCREAS EXTRACTS

(Average of Three Experiments)

	Δ mμM PP^{32} per mg. Protein*		Δ mμM PP^{32} per mg. Protein*
Arginine	2.3 (1–3)	Lysine	44.0
Glutamic acid	4.0 (0–9)	Cysteine	46.0
Glutamine	4.4	Aspartic acid	53.0
Asparagine	8.1	Tyrosine	55.0
Glycine	12.0	Alanine	58.0
Methionine	19.0	Valine	65.0
Phenylalanine	26.0	Tryptophan	105.0
Isoleucine	26.0	Threonine	108.0
Serine	32.0	Histidine	142.0
Leucine	35.0	Proline	179.0

Amino acid–dependent PP^{32}-ATP exchange was measured as described by Davie *et al.* The nonparticulate supernate of 6–8-week-old pigeon pancreas was used, prepared as described by Weiss *et al.*
* Background exchange without amino acids: 18–20 mμM PP^{32}/mg protein.

TABLE 3*

TRANSFER OF CARBOXYL-LABELED LEUCINE TO POLYNUCLEOTIDE "FACTOR"

Incubation Mixture	Total Counts per Minute: Washed Acid-Alcohol ppt.	Total Counts per Minute: Phenol-Extracted Non-dialyzable
Heated supernatant + "factor"	92	103
Supernatant, 0.25 ml. alone	49	43
Supernatant, 0.25 ml. + 0.4 ml. "factor" (= 5 ml. super.†)	2,060	1,993
Same	26 (hot TCA wash)	...

* From Weiss *et al.*
† Concentrate after phenol extraction. Both the preparation from pigeon pancreas, and the assay methodology are described by Weiss *et al.*

TABLE 4

INCORPORATION OF LABELED TRYPTOPHAN INTO NUCLEOTIDE ON TRYPTOPHAN-ACTIVATING ENZYME FROM BEEF PANCREAS

Incubation Mixture	Total Counts/Min/Mg Acid-Ethanol Ppt.: Direct	Phenol-extracted
Boiled enzyme	825	...
Enzyme alone	29,400	29,400
"Factor" alone	910	...
Enzyme + "factor"	17,800	18,000
Enzyme + "factor," no ATP	1,280	...
Enzyme + "factor," no PP-ase + PP	1,260	...

A purified enzyme of the type described by Davie *et al.* was used. The two preparations used for counting are analogous to the ones used in the experiments described in Table 3 (cf. n. 17).

it seems, most likely. It also was shown in both cases that the same enzyme is responsible for amino acid hydroxamate synthesis and for the amino acid-dependent pyrophosphate exchange.

Very briefly I would like here to mention that, in most extracts from the various sources, only a certain number of amino acids were found to be activated. Our own experience with pancreas extract made us suspect that this was due to experimental difficulties, because the degree of activation of the different amino acids was variable not only with pancreas from different organisms but also from batch to batch. Lately, Drs. Acs and Weiss in this laboratory have been able to demonstrate with pigeon pancreas extract that all 20 amino acids are activated. This is shown in Table 2 and furnishes a welcome additional confirmation of the role of these enzymes in protein synthesis.

Returning now to the tryptophan-activating enzyme, we made observations in the course of its isolation which may now prove to be relevant. Although the enzyme could be highly purified, e.g., to judge by ultracentrifuge data, to about 70 per cent pure enzyme, during this purification it carried along about 4–5 per cent of an obviously tightly bound nucleotide which was not TCA-precipitable and was also largely dialyzable. It could not be removed by charcoal or Dowex 1 treatment. The only manner in which Dr. Koningsberger eventually succeeded in getting most of the nucleotide off the protein, as shown in Figure 4, was to incubate the enzyme with tryptophan and ATP. However, after such treatment, the activity of the enzyme in the hydroxamate or pyrophosphate exchange tests had not changed;[13]

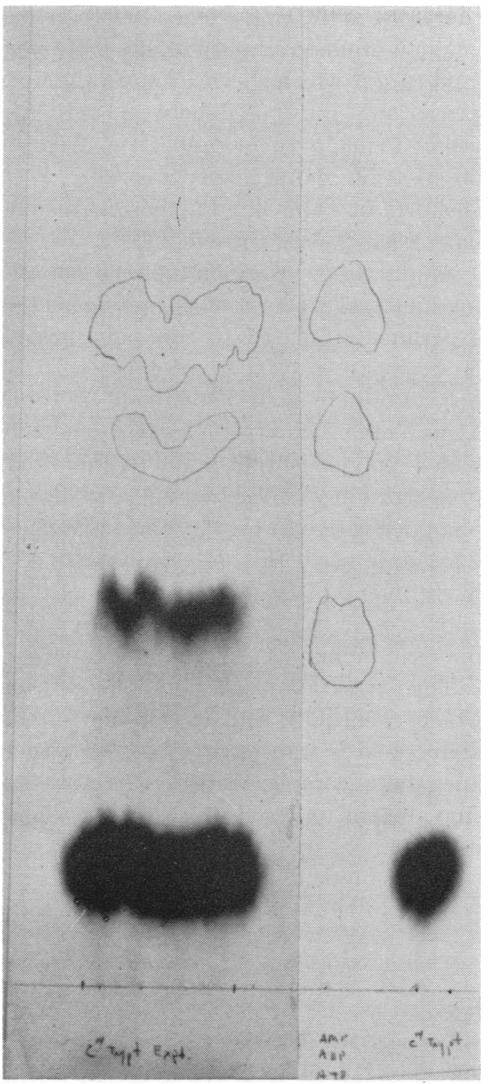


FIG. 5.—Radio autograph of pH 4.5 citrate buffer; paper electrogram made from incubate of tryptophan-activating enzyme + ATP + radioactive tryptophan. Enzyme was precipitated by acidification to pH 5, and supernate was used for paper electrogram. Radioactivity, indicating bound tryptophan, and quenching due to nucleotide, can be shown to be superimposed if the fluorescence of the tryptophan is not too strong. The tryptophan-carrying nucleotide migrates slightly above AMP; tryptophanyl-adenylate, which could be formed in the reaction, migrates in the opposite direction.

On the next to last place, markers were included for ATP, ADP, and AMP. On the last, a marker for radioactive tryptophan. The encircled areas indicate quenching as observed under Mineralight.

therefore, activation seemed not to be dependent on this nucleotide.

I now want to amplify briefly on the recent very revealing observations of Hoagland *et al.*[16] which showed that in the liver system a non-particulate polynucleotide carrier was interposed between activating enzyme and microsome. Dr. Weiss[17] isolated such a nucleotide carrier from pigeon pancreas extract and confirmed the observations on its carrier function. A transfer of the amino acid to this nucleotide is shown in Table 3. Further details on amino acid incorporation in the pancreas have been reported recently.[18]

All during our work on the tryptophan-activating enzyme, we had been searching for a natural acceptor of the amino acid. Now, obviously, the Hoagland-Zamecnik experiments pointed to this polynucleotide as an acceptor. When it was tried, however, to transfer tryptophan with the specific purified enzyme to the carrier polynucleotide of the experiment of Table 3, it appeared, as shown in Table 4, that a nucleotide-bound tryptophan was formed with activating enzyme alone, and added polynucleotide actually inhibited. This unexpected result is now under investigation. Since adenylic acid also appears to be incorporated into the tryptophan derivative formed by the activating enzyme, its relation to the enzyme-bound nucleotide needs further substantiation. It may, at present, be described as most likely representing an initial product of the interaction between ATP, tryptophan, and enzyme, more stable than tryptophanyl adenylate, and of opposite electrophoretic mobility.

The activation of the amino acid, then, appears to initiate a chain of reactions which eventually lead to peptide formation in the microsome. On its way, the amino acid is transferred first to a nucleotide and one may state with some assurance that a complex array of carry-overs is interposed between the initial activation step and the final acceptor reaction when the amino acid actually is connected with its neighbors in the protein. These obviously represent parts of the machinery that produces the sequential arranging of the amino acids and determines the specific and unique features of a particular protein.

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The following abbreviations have been used: ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; PP, pyrophosphate; P, phosphate; NuP, nucleotide monophosphate; A, adenosine.

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*INTERMEDIATE REACTIONS IN AMINO ACID INCORPORATION**

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As a result of work carried on in several laboratories, including our own, it has appeared until recently that the process of incorporation of labeled amino acids into protein could be divided into three parts.[1] At the present time it seems likely that another subdivision may be made, as illustrated in Figure 1. Evidence has

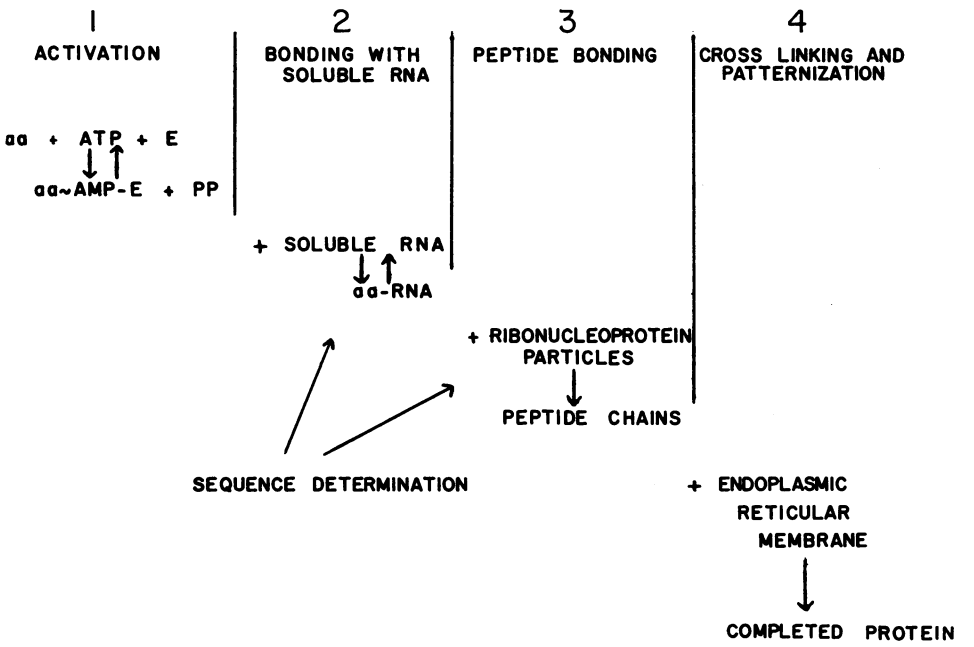

FIG. 1.—Postulated Steps in incorporation of labeled amino acids into proteins.

accumulated that a soluble RNA fraction of cell cytoplasm serves as an intermediate in the incorporation of labeled amino acid into protein.

Our interest in this soluble RNA fraction was aroused in a devious way. Some