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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
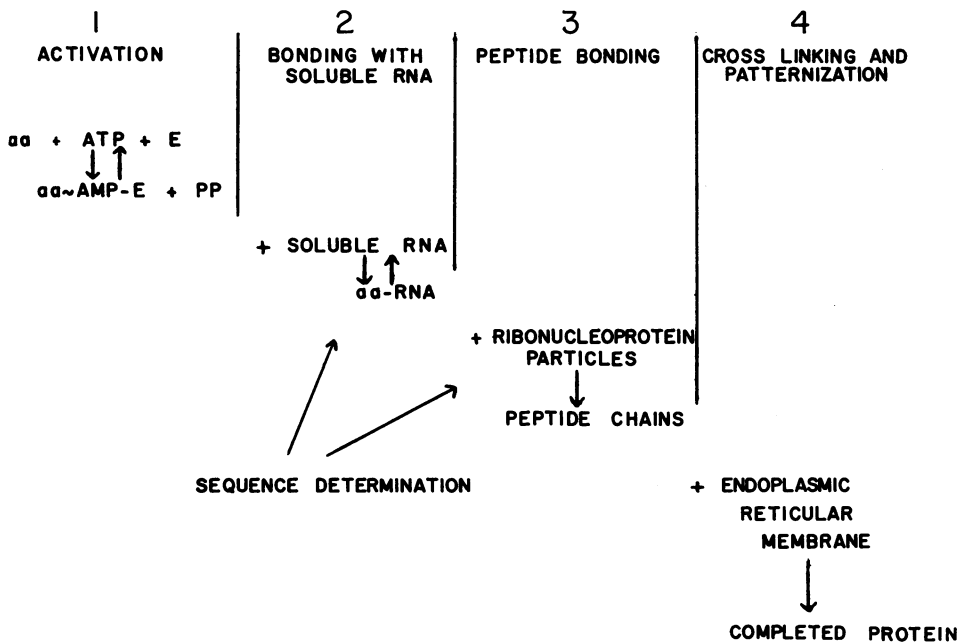

FIG. 1.—Postulated Steps in incorporation of labeled amino acids into proteins.

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

time ago we wished to inquire whether the same cell-free system in which amino acids were incorporated into protein would serve to incorporate nucleotide precursors into RNA. We found that 8-C[14]-ATP (but not 8-C[14]-ADP) served very well as a precursor for the formation of labeled RNA in this system. As a control, we added C[14]-leucine rather than C[14]-ATP. Isolation of the RNA in this case revealed that the C[14]-leucine had also become bound to the RNA. Fractionation of the cellular RNA in such a system indicated that the maximal labeling of RNA by the C[14]-leucine occurred in the pH 5 precipitable portion of the 105,000 × *g*. supernate.[2] This same cellular fraction is active in the incorporation of mononucleotides into the terminal residues of the RNA located therein, when C[14]-ATP, C[14]-UTP, and C[14]-CTP are employed as precursors,[3-5] as shown in Table 1. The

TABLE 1

MONONUCLEOTIDE TRIPHOSPHATES AS PRECURSORS OF INCORPORATION
OF MONONUCLEOTIDE RESIDUES INTO RNA*

	mμMoles per Mg. RNA
ATP-C[14]	14.3
ATP-C[14] + CTP	36.8
ATP-C[14] + CTP + GTP + UTP	39.4
CTP-C[14]	37.3
CTP-C[14] + ATP + GTP + UTP	38.0
UTP-C[14]	3.0

* The nucleotides (1 μmole/ml) were incubated for 10 minutes at 37° C. with 0.5 ml. of pH 5 fraction prepared from ascites cells. The medium contained 0.01 M phosphopyruvate; 0.004 M MgCl$_2$; 0.037 M Tris buffer, pH 7.6; 0.020 M KCl; and 10 μg. pyruvate kinase/ml. The specific activity of the labeled nucleotides was 243,000 c.p.m./μmole ATP; 58,000 c.p.m./μmole CTP; and 200,000 c.p.m./ μmole UTP.

mechanism of the mononucleotide incorporation reactions, as illustrated in Figure 2, involves a common pattern of pyrophosphate cleavage, thus differing from the polynucleotide phosphorylase reaction of Ochoa and co-workers.[6] Since these reactions of Figure 2 appear to be reversible, they suggest that the soluble RNA may

$$C^*RPPP + RNA \rightleftharpoons C^*RP\text{-}RNA + PP$$

$$U^*RPPP + RNA \rightleftharpoons U^*RP\text{-}RNA + PP$$

$$A^*RPPP + CRP\text{-}RNA \rightleftharpoons A^*RP\text{-}CRP\text{-}RNA + PP$$

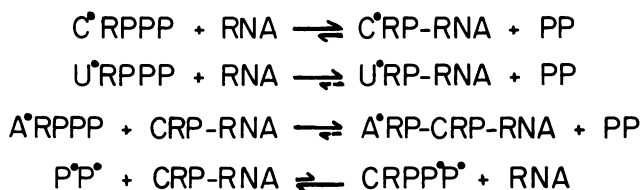$$P^*P^* + CRP\text{-}RNA \rightleftharpoons CRPP^*P^* + RNA$$

FIG. 2.—Mechanism of incorporation of mononucleotides into soluble RNA

serve as a storehouse for releasable nucleotide coenzymes, which play a directive role in regulation of the metabolism in a particular type of cell and logically also in its progeny, equipped with the same type of RNA. Whether there is a link between the nucleotide and amino acid incorporations into the soluble RNA of the pH 5 precipitable fraction remains a question for the future.

As to the nature of this soluble RNA, it has an apparent molecular weight of the order of 10,000, is polydisperse in the ultracentrifuge,[7] and needs more precise characterization.

Returning to the role of this soluble RNA in incorporation of amino acids into protein, our present impression is that the labeled amino acid–RNA compound is an intermediate on the path to completed protein, as illustrated in Figure 1. If such is the case, it becomes logical to inquire whether enzymes participate in this additional step, as illustrated in Figure 3. On this basis, one might postulate the presence of at least four separate enzymes in the over-all incorporation reaction. The first two steps in the incorporation reaction can be studied in the absence of microsomes. The first step involves activation of amino acids by way of the amino-acyl-adenylate intermediate postulated by Hoagland.[8, 9]
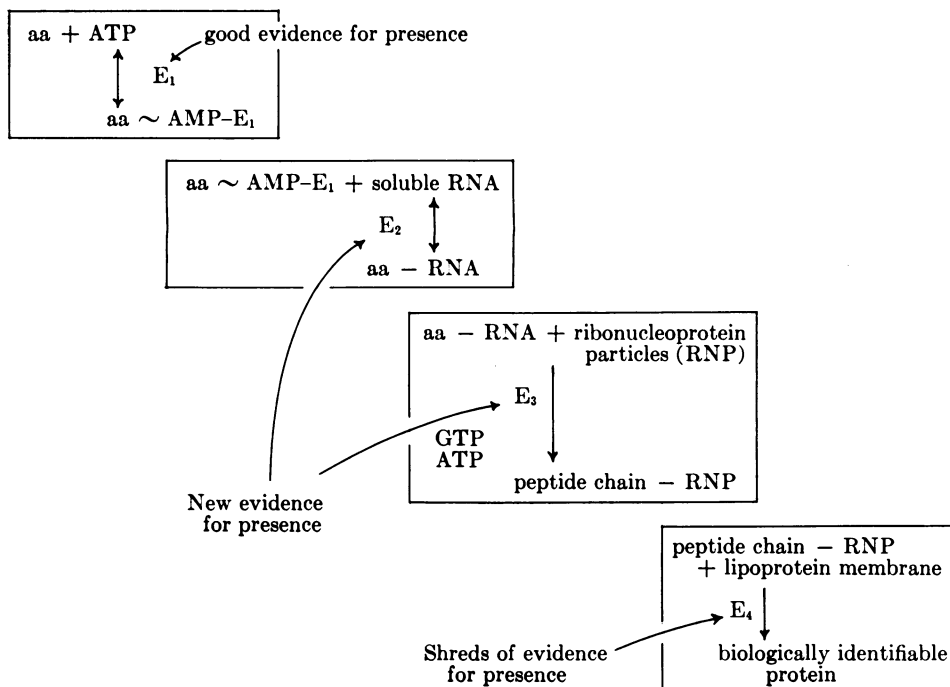

FIG. 3.—Possible enzymes involved in protein synthesis

Support for this aminoacyl-adenylate concept has come from the transfer of O^{18} from tryptophan, labeled with O^{18} in the carboxyl group, to AMP, in the presence of ATP, tryptophan-activating enzyme, and hydroxylamine.[10] This indicates a direct interaction of the tryptophan with the phosphate of AMP, in consonance with a mixed anhydride intermediate. Evidence for the presence of such a free aminoacyl-adenylate has been lacking, and it has therefore been considered to be tightly enzyme-bound. In recent experiments from this laboratory, it has, however, been found that if C^{14}-valine is incubated with ATP and the pH 5 fraction in the presence of a large excess of inert valyl-AMP as a trapping agent, labeled valyl-AMP may be isolated in small amount.[11]

These two pieces of evidence, plus the lack of sensitivity of the amino acid–activating reaction to ribonuclease, point toward a first step in the over-all incorporation reaction which does not involve RNA. This soluble RNA is, however,

closely associated with the pH 5–activating enzymes and may be part of a reaction sequence bound together in the intact cell.

For the present we may consider the interaction with soluble RNA to be a second step in the sequence. If the leucine-labeled RNA is to be considered as a true intermediate in the over-all scheme rather than as a storage locus for activated amino acids, it should satisfy certain criteria of intermediates. It should, for example, be more rapidly labeled than are the peptide chains in the ribonucleoprotein particles. Such was found to be the case in short time experiments carried out on intact ascites tumor cells.[7] The labeling of soluble RNA by the amino acid was such a rapid reaction that it was difficult to obtain a satisfactory time curve, even when the reduced temperature of 25° C. was employed. Another criterion for an intermediate is that it should when labeled, be, able to transfer its labeled amino acid to the ribonucleoprotein particles. Leucine-labeled soluble RNA was therefore prepared and isolated by a phenol extraction procedure. Addition of this labeled soluble RNA to microsomes in the presence of GTP and ATP and a small aliquot of a dilute supernatant fraction resulted in transfer of the labeled leucine to peptide bonding in the ribonucleoprotein particles.[7] The additional protein from the supernatant cell fraction required to mediate this transfer is designated E_3. In the crude separations presently employed, this enzyme is located both in the pH 5-precipitable and in the pH 5-supernatant (or S_4) fraction. GTP participates as a cofactor in this reaction sequence and cannot be replaced by UTP or CTP.[2] Another piece of evidence pointing to soluble RNA as an intermediate in the over-all incorporation reaction is shown in Table 2. In the

TABLE 2

INCORPORATION OF C^{14}-LEUCINE INTO RNA AND PROTEIN OF ASCITES CELL FRACTIONS AFTER INCUBATION OF FRACTIONS WITH C^{14}-LEUCINE*

Effect of Added pH 5 RNA on the Incorporation

	TOTAL COUNTS IN FRACTIONS			
	RNA-Amino Acid		Protein	
Added RNA	0	120 μg.	0	120 μg.
Complete system:				
S_4 + microsomes	136	927	136	357
S_4 + microsomes + pH 5 fraction	...	918	...	321
Partial systems:				
S_4	...	0	...	5
Microsomes	93	240	62	82
pH 5 fraction	0	210	4	12
pH 5 fraction + microsomes	255	584	137	224

* A 10 per cent lysate of ascites cells was prepared according to the method of Littlefield and Keller[12]. The fractions were separated by differential centrifugation. The fraction sedimenting after 10 minutes at 12,000 × *g.* was discarded, and the supernatant was centrifuged for 2 hours at 105,000 × *g.* in a Spinco preparative ultracentrifuge. The microsome pellet was resuspended in one-tenth the original volume of medium. The 105,000 × *g.* supernatant was brought to pH 5.2 with *N* acetic acid, and the resultant precipitate was isolated by centrifugation. This pH 5 fraction was resuspended to the original volume in the medium. The 105,000 × *g.* supernatant minus the pH 5 fraction (supernatant 4, or S_4) was readjusted to pH 7.4. The amount of each fraction incubated was as follows: microsomes 1.72 mg. dry protein, 438 μg. RNA; pH 5 fraction, 0.81 mg. protein, 30 μg. RNA; S_4, 1.08 mg. protein, and 5.2 μg. RNA. After incubation both RNA and protein were isolated and counted (see n. 7).

Each flask contained 1 μmole ATP; 0.1 μmole C^{14}-leucine containing 139,000 c.p.m.; 10 μmoles phosphopyruvate; 0.01 mg. pyruvate kinase; 0.25 μmole GTP plus the cell fractions containing 3.0 μmoles MgCl₂; 10 μmoles KCl; 35 μmoles Tris, pH 7.6; and 300 μmoles sucrose. The total volume was 1.5 ml. Where indicated, 120 μg. liver pH 5 RNA was added. The RNA was prepared from liver pH 5 fraction by a modification of the phenol methods (see nn. 7, 13, 14). Incubation was for 10 minutes at 37° C.

complete system (first line, Table 2), the addition of previously isolated soluble RNA produces a marked stimulation of the incorporation of leucine into protein, and likewise a striking increase in the total RNA-bound C^{14}-leucine. A similar type of stimulation of incorporation of amino acid into protein had already been

observed in unpublished experiments by Weiss and Lipmann.[15] In our case, it became possible to demonstrate this stimulating effect of RNA on amino acid incorporation into protein by employing the so-called S_4 fraction, which is relatively free from endogenous RNA. The S_4 fraction alone is low in leucine activating enzyme (E_1) and therefore is itself unable to catalyze the two-step leucine labeling of RNA. It is rich in E_2 and E_3, as evidenced by its stimulation of RNA-labeling and incorporation of labeled amino acids into protein in the presence of microsomes. The microsomes alone contain (or have not been freed from) sufficient amounts of activating enzymes (E_1) to catalyze maximal RNA and protein-labeling without addition of pH 5 fraction, provided that S_4 is present. The relationship of the postulated enzymes in the three cell fractions which concern us here is illustrated in Figure 4.
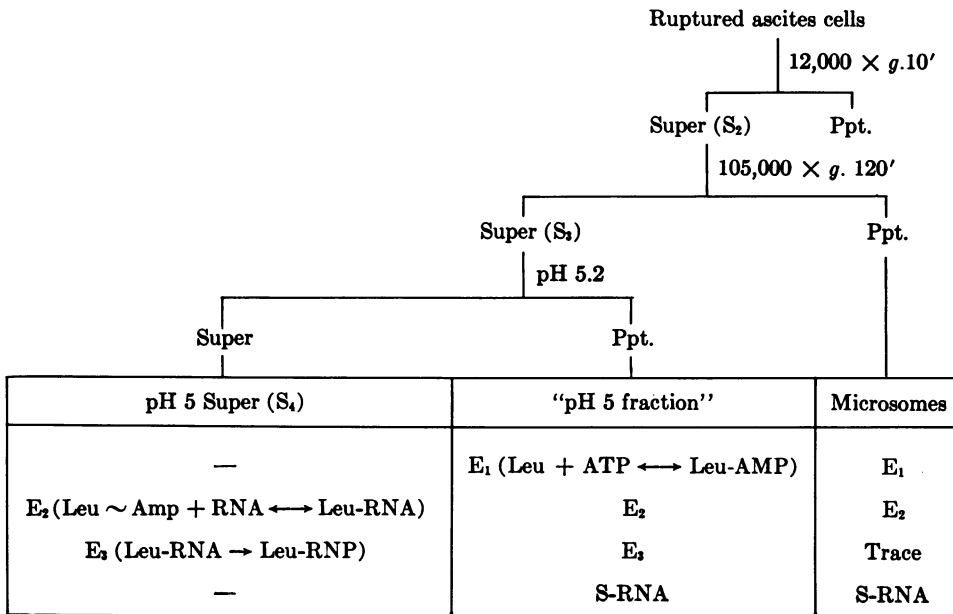

FIG. 4.—Location of enzymatic components of leucine incorporation system in ascites tumor fractions

It is also possible that determination of the amino acid sequence may be a two-step RNA-participating process.

Summary.—In summary, it may be said that further fractionation of cell-free systems from the mouse Ehrlich ascites tumor and the rat liver points toward the participation of soluble RNA in the incorporation of amino acid into protein and indicates that there may be at least three separate enzymatic steps from free amino acid to peptide bonding in the ribonucleoprotein particles.

uridine triphosphate; CTP or CRPPP, cytidine triphosphate; and GTP, guanosine triphosphate.

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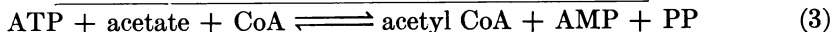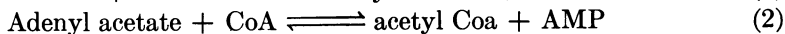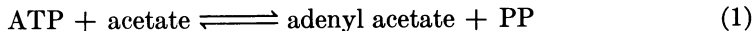
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AN ENZYMATIC MECHANISM FOR LINKING AMINO ACIDS TO RNA*

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INTRODUCTION

For the past several years we have been interested in the general problem of acyl group activation. Our initial studies were concerned primarily with the enzymatic mechanism of acetyl CoA formation.[1, 2] The results of these investigations demonstrated the existence of a new type of reaction in which the carboxyl group of acetate reacts with ATP and becomes linked to the phosphate of adenosine-5'-phosphate. This "activated" acetyl group is subsequently transferred, by the same enzyme, to coenzyme A (eqs. [1]–[3]).

$$\text{ATP} + \text{acetate} \rightleftharpoons \text{adenyl acetate} + \text{PP} \tag{1}$$

$$\text{Adenyl acetate} + \text{CoA} \rightleftharpoons \text{acetyl Coa} + \text{AMP} \tag{2}$$

$$\overline{\text{ATP} + \text{acetate} + \text{CoA} \rightleftharpoons \text{acetyl CoA} + \text{AMP} + \text{PP}} \tag{3}$$

The widespread occurrence of this type of reaction in the activation of higher fatty acids,[3–6] inorganic acids,[7–9] and other acyl compounds[10, 11] has been demonstrated by a number of investigators. Of particular interest was the finding by Hoagland et al.[12] and others[13–16] of enzymes which carry out a reaction analogous to that shown in equation (1) with amino acids. Purification and characterization of some of these enzymes showed, first, that the enzymes were specific for a single amino acid and, second, that the product of the reaction was the adenyl amino acid