Amino Acid Incorporation in Vitro by Ribonucleoprotein Particles Detached from Guinea Pig Liver Microsomes

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Since it has been shown that the recombined mitochondrial, microsomal, and supernatant fractions of rat liver are able to incorporate radioactive alanine into triehloroacetic acid-insoluble protein (1), many investigators have worked on the purification and characterization of the amino acid incorporation system of liver cells. The results of their efforts have been reviewed by W. Anderson (2) and more recently by Loftfield (2), Chantrenne (3), and Simkin (4). Zamecnik and Keller (5) showed that the microsomes by themselves were sufficient for incorporation if a soluble nondialyzable fraction, adenosine triphosphate, and an adenosine triphosphate-generating system were present. Littlefield et al. (6) focused attention on the attached ribonucleoprotein particles of the microsomes by demonstrating that after an injection in vivo of radioactive amino acid the deoxycholate-insoluble particles became labeled more rapidly than either the whole microsomes or the soluble cell protein. This finding pointed to the ribonucleoprotein particles as the most likely primary site of amino acid incorporation.

The experiments reported here were carried out to determine if ribonucleoprotein particles detached from the microsomal membranes still retain their ability to incorporate radioactive amino acids in vitro.

EXPERIMENTAL

Materials—dL-Leucine-1-C14 with a specific activity of 5.6 mc per mmole was obtained from Isotope Specialities Company. Uniformly labeled t-valine with a specific activity of 8.0 mc per mmole and uniformly labeled L-glutamic acid of specific activity 0.0 mc per mmole were purchased from Volk Radiochemical Company. The other radioactive amino acids were supplied by Mann Research Laboratories, Inc. Crystalline pyruvic kinase was obtained from C. F. Boehringer and Sons, Mannheim, Germany, and from Sigma Chemical Company. ATP and GTP were also purchased from Sigma. The pH of the nucleotide solutions was adjusted to approximately 7.6 with KOH. P-enolpyruvate, obtained as the silver-barium salt from Boehringer, was converted to the potassium salt at pH 7, lyophilized, and stored in the deep freeze. Fresh P-enolpyruvate was weighed out before each experiment as it was observed to lose most of its activity if kept frozen in solution for more than a few days. Deoxycholic acid was obtained from Wilson Laboratories and converted to the potassium salt at pH 7.8 before use. All other chemicals were of reagent grade.

Preparation of Particles and pH 5 Enzymes—In all experiments, 5- to 6-months-old albino guinea pigs, weighing from 450 to 500 g, were used. The livers were quickly excised, under ether anesthesia, and chilled immediately in ice cold Medium A (7) containing 0.35 M sucrose, 0.004 M MgCl2, 0.025 M KCl, and 0.05 M Tris buffer, pH 7.6. A total of 56 g was collected. All further operations were carried out at 4°. The livers were homogenized for 30 seconds, 8 g at a time, in 19 ml of Medium A in a Potter-Elvehjem homogenizer (8) fitted with a Teflon pestle. The nuclei and mitochondria were sedimented by centrifuging the homogenate for 10 minutes at 15,000 X g in a Spinco model L preparative ultracentrifuge. The pellet was discarded and the supernatant fluid centrifuged at 105,000 X g for 1 hour to sediment the microsomes. The microsomal supernatant was decanted off and saved for the preparation of the pH 5 enzymes. The microsomal pellets were combined and suspended in 56 ml of Medium A, i.e., 1 ml per g wet weight of original tissue. The microsomal suspension thus obtained contained about 15 mg of protein per ml.

To prepare the detached RNP-particles, the microsomal suspension was diluted 5 times to 250 ml with Medium B (9) which contains 0.00 M sucrose, 0.004 M MgCl2, and 0.025 M KCl. To the diluted suspension, 18.7 ml of 0.1 M MgCl2 were then added, followed by 26.5 ml of a 3% deoxycholate solution which was added 5 ml at a time with shaking after each addition. The final MgCl2 and deoxycholate concentrations in this suspension were 0.000 M and 0.25 g per 100 ml, respectively. The 325 ml of particulate suspension were immediately centrifuged at 105,000 X g for 10 minutes in three Spinco preparative ultracentrifuges. The precipitate was discarded and the supernatant fluid centrifuged for 1 hour at 105,000 X g in order to obtain the final pellets of RNP-particles. The total yield at this stage was usually about 30 mg of particulate protein from the original 56 g of liver. Each tube, and the surface of the pellet therein, was rinsed three times with Medium A to remove excess deoxycholate, and finally all the pellets were resuspended and collected in 2.6 ml of Medium A.

The pH 5 enzymes were prepared by diluting the microsomal supernatant three times with Medium B and proceeding according to Keller and Zamecnik (9). The final protein concentration of this preparation was about 10 mg per ml.

Electron Microscopy—The ribonucleoprotein particles were fixed for electron microscopy by overlaying the final 105,000 X g pellet with 5 to 10 ml of 1% OsO4 in 0.88 M sucrose and allowing it to stand for 2 to 10 hours at 4°. The fixed pellets were cut into small strips of known orientation, dehydrated with graded
The complete system contained 3 to 4 mg of detached particle protein, 1 mg of pH 5 enzymes, 0.1 μ mole of ATP, 0.25 μ mole of GTP; 0.1 μ mole of L-leucine-1-C¹⁴ (5.6 mc per mmole), 5.0 μ moles of MgCl₂, 10 μ moles of P-enolpyruvate, and 0.14 mg of pyruvic kinase in a final volume of 1 ml.

In Experiment II, 0.4 ml of microsomal supernatant containing about 16 mg of protein was substituted for the pH 5 enzymes, and the final volume was 1.2 ml.

The incubation time was 20 minutes at 37°.

### Table I

<table>
<thead>
<tr>
<th>Experiment</th>
<th>c.p.m. per mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td></td>
</tr>
<tr>
<td>complete system</td>
<td>81, 67, 66*</td>
</tr>
<tr>
<td>complete system minus P-enolpyruvate</td>
<td>8</td>
</tr>
<tr>
<td>complete system minus pyruvic kinase</td>
<td>7</td>
</tr>
<tr>
<td>complete system minus GTP</td>
<td>4, 3*</td>
</tr>
<tr>
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<tr>
<td>II</td>
<td></td>
</tr>
<tr>
<td>complete system</td>
<td>60</td>
</tr>
<tr>
<td>complete system minus supernatant</td>
<td>27, 6†</td>
</tr>
<tr>
<td>complete system minus particles</td>
<td>10</td>
</tr>
</tbody>
</table>

* Results are for multiple samples.
† The second figure is corrected for the reduced amount of protein present. See text.

### Incorporation Studies

The usual incubation mixture consisted of 0.3 ml of particulate suspension (containing 3 to 4 mg of protein); 0.1 ml of pH 5 suspension (containing approximately 1.0 mg of protein); 0.1 μ mole of L-leucine (5.6 mc per mmole); 5.0 μ moles of MgCl₂; 10 μ moles of P-enolpyruvate; 1.0 μ mole of ATP; 0.25 μ mole of GTP; and 0.14 mg of pyruvic kinase in a final volume of 1.0 ml. In certain experiments 0.4 ml of microsomal supernatant containing approximately 10 mg of protein was substituted for the pH 5 suspension.

After incubation the protein was precipitated in 5% trichloroacetic acid (final concentration) and allowed to stand at 4° for at least 2 hours. The precipitated protein was prepared for counting essentially as in (1). The planchets were counted in a Nuclear-Chicago gas flow counter with a counting efficiency of about 10%.

### Analyses

RNA and phospholipid were extracted essentially by the Schneider method (cf. 10). RNA was determined by the orcinol method of Mejbaum (12), protein by nesslerization after Kjeldahl digestion, and phospholipid phosphorus by the Fiske-SubbaRow method (13) after acid hydrolysis of the combined alcohol and alcohol-ether extracts.

### Results

When examined systematically from top to bottom in the electron microscope, the particulate pellet proved to consist almost entirely of small, dense particles. Plate 2 represents a typical field in the lower half of the pellet. The particles are identical, both in size and density, with the 100 to 150 A RNP-particles prepared from rat liver microsomes (10). Plate 1 is a representative area from the top half of the pellet. The main contaminant appears as large clumps of partly amorphous material, which probably represent aggregates of damaged particles. This clumped material is generally present throughout the top half of the pellet and takes from 10 to 25% of the area occupied by the structures present. In the incorporation studies described below, there was no correlation between the amino acid uptake and the amount of contamination.

There are no longer any recognizable microsomes in the preparation. A few vestigial membranes occur almost exclusively in a thin band at the top of the pellet, are swollen and have no attached particles.

The RNA to RNA + protein ratio of this fraction is about 0.43, varying between 0.41 and 0.46 in three different preparations. The microsomes by comparison have an RNA to RNA + protein ratio of about 0.1. Approximately 25% of the microsomal RNA is recovered in the particles. This low recovery is due to the preliminary 10-minute centrifugation at 105,000 x g, to the solubilizing effect of deoxycholate on RNA (6, 14), and to the relatively short (90 minutes) duration of the final spin. The particles are practically free of alcohol-ether extractable phospholipid as they contain about 0.1% phospholipid phosphorus as compared to 2% for the whole microsomes (10).

Table I shows the dependence of the incorporation of leucine upon the various components of the incubation mixture. The requirement for ATP, an ATP-generating system, and GTP is very clear as the omission of any one of these components results in a low incorporation. The GTP effect, in particular, is much more evident here than in the microsome system (9). The situation might be explained by the washing out of adsorbed GTP during the 3-fold dilution of the microsomes and the solubilizing of microsomal membranes involved in the preparation of the particles.

Experiment II in Table I shows that the particles are able to carry out a reduced but still significant incorporation (27 c.p.m. per mg protein) in the absence of supernatant. This figure was obtained by dividing the total number of counts (86 c.p.m.) by the amount of particle protein (3.5 mg) actually present in the system. To make this specific activity comparable to values given in the rest of this paper and in the literature (cf. 9), the total number of counts should be divided by the amount of particulate protein in the system plus the amount of supernatant protein used in the control (3.5 plus 10 mg, respectively). Under these conditions the much lower figure of 6 c.p.m. per mg of protein is obtained.

Although deoxycholate produces a very clean preparation of RNP-particles, it has the disadvantage of being a very potent inhibitor of the incorporation system. In one experiment in which the whole microsome system was used (9), the addition of deoxycholate inhibited the incorporation by 50 and 80% at the respective final concentrations of 0.03 and 0.06%. Because of this finding, an attempt was made to produce more active particles by the use of smaller concentrations of deoxycholate in more dilute tissue suspensions. A pellet prepared from a 10% (10 g wet weight original tissue equivalent per 100 ml of medium) suspension of microsomes and containing 0.1% deoxycholate as opposed to the 20% microsome suspension and 0.25% deoxycholate usually used, was active in incorporation. Chemical analysis of this preparation gave an RNA to RNA + protein
ratio of 0.40, but it was found to be grossly contaminated with swollen membranes when examined with the electron microscope. The high RNA content of such a contaminated preparation emphasized the necessity of careful electron microscopic examination in all further experiments.

In addition to leucine, other amino acids are actively incorporated into the proteins of the particle-pH 5 enzyme system as shown in Table II. The relative labeling by the various amino acids reflects neither the relative amino acid composition of liver proteins as listed by Block and Bolling (15), nor that of guinea pig liver supernatant as determined by the technique of Sackman et al. (16). Moreover, in different preparations, the relative order of labeling does not remain constant. For example, in an experiment other than that listed in Table II, leucine was incorporated to the extent of 87 c.p.m., glutamic acid 87 c.p.m., and valine 39 c.p.m. per mg of protein. Each figure represents an average of two determinations and all three specific activities are recalculated to 5.0 mc per mg, the specific activity of the leucine used.

The RNP-particles, unlike the microsomes, are able to continue the incorporation of radioactive leucine for at least 60 minutes as shown in Fig. 1. Zamecnik and Keller (5) found that the incorporation in rat liver microsomes ceases entirely after only 10 minutes, and we have found that guinea pig liver microsomes abruptly cease to incorporate after 20 minutes (Fig. 1). It is not clear at present whether this difference between the incorporation by microsomes and by particles is due to the presence of activating enzymes in the whole microsomes or to other circumstances. The gradual leveling off of the particulate incorporation curve is apparently not due to a lack of high energy compounds, as a "high energy supplement" consisting of 4.0 pmole of P-enolpyruvate, 0.5 pmole of ATP, and 0.25 mg of pyruvic kinase, added 30 minutes after the beginning of the incubation, failed to stimulate the incorporation.

Amino acid N-terminal analyses of the radioactive proteins obtained after 30-minute and 90-minute incubations of the particle-pH 5 enzyme system were carried out by the dinitrofluorobenzene method (17), in order to determine whether or not the incorporation represented a nonspecific amino acyl condensation with the protein such as reported by Castelfranco et al. (18), and by Zioudrou et al. (19). These investigators found that most of the radioactivity could be recovered as dinitrophenyl amino acids in experiments in which labeled synthetic amino acyl adenylates were used. In the particle pH 5 enzyme incorporation system, both the 30- and 90-minute points showed less than 1% of the recoverable radioactivity as ether-extractable dinitrophenyl compounds, indicating the leucine to be bound internally in the protein.

Table III gives the effect of storage of the unsuspended particulate pellets under various conditions. It is seen that the detached particles, unlike the microsomes (5), keep perfectly well in the deep freeze with no loss in activity for at least 4 weeks. Storage at higher temperatures results in an overnight loss in activity. Sachs (20) has previously obtained a stable incorporation system by lyophilizing the entire mitochondrial supernatant. He found that the microsomes were stabilized by the supernatant. The particles apparently are quite stable without it when prepared by the method described.

We wish to thank Dr. W. H. Stein for performing the quantitative amino acid analysis.
It has been reported (21, 27) that the incorporation of radioactive amino acids into pea seedling particles is stimulated by a supplement of nonradioactive amino acids. Bates and Simpson (23) have observed a similar effect in their studies on the synthesis of cytochrome c by calf heart mitochondria, as have Schweert et al. (27), in their work on hemoglobin biosynthesis by rabbit reticulocyte microsomes. To test this effect on the detached RNP-particles, three mixtures of 19 nonradioactive amino acids, excluding leucine, were prepared. Mixture I contained 0.1 μmole of each of the 19 amino acids, Mixture II had 0.25 μmole of each, and Mixture III was composed of the 19 amino acids in the relative amounts in which they occur in total microsomal supernatant protein based on the addition of 0.1 μmole of DL-leucine. All three mixtures not only failed to stimulate the incorporation but actually inhibited it from 20 to 70%. The reason for this inhibition is not known. As the amount of leucine actually incorporated into the particle-pH 5 enzyme protein is only of the order of 0.001 pmole per 5 mg of trichloroacetic acid-insoluble protein, it is quite possible that sufficient nonradioactive amino acids are present either in the pH 5 fraction or in the particles themselves to be incorporated along with the leucine.

**DISCUSSION**

From these experiments it can be seen that the microsomal membrane is not essential for amino acid incorporation in the liver system. A likely function of the membranous component of the microsomes is suggested by earlier work from this laboratory (24). In a study of the secretory process in the pancreatic exocrine cell, it was shown that the precursors of the zymogen granules first appear in the cavities of the endoplasmic reticulum (intracisternal granules), although the zymogens themselves are probably synthesized by the attached RNP particles. The membrane in this case seems to act as a site of segregation for that protein which is intended for later export from the cell.

Free RNP-particles have been isolated from many sources (cf. 25) and characterized to a varied extent. They have been shown to be capable of incorporation in vitro of radioactive amino acids when prepared from mouse ascites tumor (7), and yeast (22). Kornor has preliminarily reported incorporation in vitro by particles obtained by treating liver microsomes with 0.5% deoxycholate (29). The pea seedling system in some respects has been particularly fruitful as both Raecie (21) and Webster (26) have reported synthesis of an amount of new protein large enough to be measured gravimetrically. The synthesis of a specific protein has, however, not as yet been followed.

For the investigation of the intimate mechanisms of protein synthesis, the detached RNP-particles obtained from liver microsomes seem to offer some advantages over the pea seedling system by virtue of their relative cleanliness. They are free of membranes, and the omission of either the pH 5 fraction, ATP, or GTP reduces the incorporation essentially to zero. These considerations should make the particles particularly useful in studies on the transfer of amino acids from the soluble RNA to protein.

**SUMMARY**

Ribonucleoprotein particles have been isolated from deoxycholate-treated guinea pig liver microsomes and have been shown to incorporate amino acids into protein in vitro. This incorporation is dependent upon the same coenzymes that govern the incorporation by rat liver microsomes. The omission of pH 5 enzymes, adenosine triphosphate, or guanosine triphosphate almost completely abolishes the uptake.

Five different radioactive amino acids were studied and all of them were actively incorporated.

Incorporation continues at a high rate for at least 60 minutes, with a gradual approach to a plateau.

A dinitrofluorobenzene analysis of the end groups showed that incorporation was 75% of the total amino acids.

References

2. Lofthild, R. B., Prog. in Biophys. and Biophys. Chem., 6, 347 (1957).
PLATE 1. A field selected from the top half of the pellet to show the 100 to 150 A ribonucleoprotein particles, the clumped material (c) which forms the main contaminant, and the remnants of smooth-surfaced vesicles (v). Magnification: 96,000X.
Plate 2. A representative section from the lower half of the pellet, showing a homogeneous population of ribonucleoprotein particles. Magnification: 96,000X.