STUDIES ON THE NATURE OF THE AMINO ACID INCORPORATION PROCESS OF HEN OVIDUCT TISSUE

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A number of recent investigations have been concerned with the relative localization of ribonucleic acid and of protein-synthetic processes within specific structural components of cells (1-9). The results thus far have shown that, after incorporation of radioactive amino acids in vivo or in vitro, the radioactivity is concentrated mainly in the small cytoplasmic particles called microsomes. Preparations of isolated microsomes have also been shown to be most active in incorporating labeled amino acids. The microsomes also account for most of the ribonucleic acid (10-13) of the cells, and this correspondence of ribonucleic acid content and amino acid-incorporating ability is in accord with a proposed relationship between these two properties (1, 2).

The present paper describes experiments performed with a tissue highly specialized with respect to protein synthesis, the oviduct of the laying hen. These results also demonstrate a relationship between amino acid-incorporating ability and ribonucleic acid content, but, in contrast to other tissues studied thus far, microsome-like material in this tissue has been found to sediment in low centrifugal fields (<600 X g in less than 10 minutes as opposed to 20,000 X g or greater for 1 hour or longer (13)). Chemical and cytochemical examinations have shown that the bulk of the cytoplasmic basophilia of the cells is contained in this sedimenting fraction.

It has been determined further that this fraction has the greatest tendency to lose previously incorporated amino acids when the specific activity of the free amino acid pool is lowered during the incubation. In contrast, the more soluble proteins continue to increase in specific activity during the period of incubation in the medium in which the specific activity is lowered.

EXPERIMENTAL

Tissue System, Medium, and Incubation—Hen oviducts obtained from laying hens were minced with scissors in the cold and incubated in a gas-

* Part of this work was performed as a Fellow of The National Foundation for Infantile Paralysis.
equilibrated medium (95 per cent O₂-5 per cent CO₂) (14) which had the following final concentrations: NaCl 0.033 m; KCl 0.072 m; NaHCO₃-NaH₂CO₃ 0.040 m; glucose 2 gm. per liter; and phenol red indicator solution 2 ml. per liter. C¹⁴O₂ was added to the gas-equilibrated medium as NaH¹⁴CO₃. 5 gm. of tissue were gently shaken with 12.5 ml. of medium at 37° for 2 hours unless otherwise stated.

Fractionation without Sucrose—After incubation, the suspension was centrifuged at about 1000 to 2000 r.p.m. and washed once with water. The soluble extracellular proteins were precipitated from the supernatant fluid by the addition of alcohol to 70 per cent. The washed tissue mince was homogenized for two 2 minute periods with half its volume of distilled water in a Potter-Elvehjem glass homogenizer which was immersed in an ice bath. Microscopic examination has shown that at least 90 per cent of the cells were broken by this procedure.¹ The homogenate was centrifuged at about 1000 to 2000 r.p.m. for 2 minutes. The pellet was washed three times with distilled water and is referred to here as the cell debris (CD). The supernatant fluid obtained by centrifuging the homogenate was adjusted to pH 5 with 1 M acetic acid and brought to 40 per cent saturation with solid ammonium sulfate (AMS) (40 per cent AMS precipitate). The proteins soluble in 40 per cent ammonium sulfate were precipitated by the addition of trichloroacetic acid (TCA) to about 8 per cent final concentration. Pure egg albumin was isolated by isoelectric crystallization from the 40 per cent AMS-soluble fraction (14). This protein was then crystallized three times after the addition of carrier. Plakalbumin was obtained from the egg albumin by enzymatic degradation with Bacillus subtilis enzyme (14).

Fractionation with Sucrose—The tissue was minced either in the buffer used for incubation or in ice-cold 0.25 M sucrose. In the former case, excess buffer was removed by centrifugation, and in the latter case a separate sucrose wash was used to exclude all traces of external salts. The tissue obtained in both instances was homogenized in about 5 volumes of ice-cold 0.25 M sucrose and fractionated by differential centrifugation as described by Schneider (13). For the tissue fractionated after incubation, the same procedure was followed with and without first washing in 0.25 M sucrose.

Determinations of Ribonucleic Acid—1 ml. aliquots of suitably diluted homogenate and of the various protein fractions described above were precipitated with 1 ml. of cold 1 N HClO₄ and washed once with 2 ml. of cold 1 N HClO₄. After the above precipitation and extraction, 2 ml. of 1 N HClO₄ and 2 ml. of orcinol reagent were added to each sample, and the suspensions were boiled for 30 minutes in a water bath. The reagent was

¹ Hendler, R. W., and Glenner, G., unpublished data.
prepared before use by dissolving 10 mg. per ml. of orcinol in a stock solution of 0.4 per cent ferric ammonium sulfate in concentrated HCl (15). After removal of precipitated protein by centrifugation, the optical density was determined at 660 m\(\mu\), and the values obtained were corrected for the reagent blank.

**Preparation of Proteins for Determinations of Radioactivity**—The proteins were precipitated by adding a 10 per cent TCA solution to a final concentration of about 8 per cent, and the precipitate was washed four times in 5 per cent TCA and the second wash heated at 95° for 15 minutes. The proteins were then washed once with absolute alcohol and three times with a 3:1 mixture of alcohol and ether, with 5 minutes heating at 65° during each alcohol-ether wash. Finally, the proteins were washed once with absolute ether and then plated from an absolute ether suspension on previously weighed planchets of 1.54 sq. cm. area. The radioactivity of the planchet dried in air was determined with a thin window Geiger-Müller counter, sufficient counts being taken to give less than 5 per cent statistical error. Corrections were applied for self-absorption by using an empirical curve obtained for BaCO\(_3\). In several cases it was further determined that the proteins so treated retained their radioactivity after being dialyzed 24 hours at room temperature in a solution of 1 m NH\(_4\)OH made up in 60 per cent dimethyl formamide.\(^2\)

**Removal of Radioactivity before Continued Incubation to Lower Specific Activity of Amino Acid. By Gas Exchange**—After 2 hours of incubation, the well agitated flasks were flushed with 95 per cent O\(_2\)-5 per cent CO\(_2\), and flushing was continued throughout the rest of the experiment.

**By Medium Exchange**—After 2 hours of incubation, the contents of the flasks were gently centrifuged (about 1000 r.p.m.), washed in buffer, and resuspended in either unlabeled buffer, radioactive buffer, or unlabeled buffer in which hen oviduct mince had been incubated for 2 hours.

**Results**

**Sedimentation of Ribonucleic Acid and Incorporated Radioactivity**—It has been consistently observed (see Tables I to III) that, in incubations of hen oviduct mince with radioactive CO\(_2\), which mainly labels glutamic and aspartic acids, and with radioactive glycine, phenylalanine, valine, and alanine, the cell debris accounts for the major fraction of incorporated amino acid. The specific activity of this material is usually appreciably higher than that of the other fractions. Since it is known that this fraction represents a mixture of proteins, it might be expected that subfrac-
### Table I

Comparison of Fractions* Obtained by Water and Sucrose Fractionation

<table>
<thead>
<tr>
<th>Fraction</th>
<th>C.p.m. per mg.</th>
<th>Total mg.</th>
<th>Per cent homogenate by weight</th>
<th>Total c.p.m. (\times 10^4)</th>
<th>Per cent homogenate by c.p.m.</th>
<th>Per cent distribution of total RNA†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose‡</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD</td>
<td>30</td>
<td>363</td>
<td>45</td>
<td>10.9</td>
<td>68</td>
<td>72</td>
</tr>
<tr>
<td>Mitochondria§</td>
<td>32</td>
<td>42</td>
<td>5</td>
<td>1.34</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>Microsomes§</td>
<td>33</td>
<td>21</td>
<td>3</td>
<td>0.68</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Supernatant</td>
<td>8.2</td>
<td>385</td>
<td>47</td>
<td>3.16</td>
<td>20</td>
<td>4</td>
</tr>
<tr>
<td>CD</td>
<td>33</td>
<td>304</td>
<td>46</td>
<td>10.0</td>
<td>61</td>
<td>70</td>
</tr>
<tr>
<td>40% AMS ppt.</td>
<td>17</td>
<td>348</td>
<td>52</td>
<td>6.0</td>
<td>37</td>
<td>17</td>
</tr>
<tr>
<td>40% AMS-soluble</td>
<td>18.6</td>
<td>15</td>
<td>2</td>
<td>0.28</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Water</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* All incubations were carried out with intact cells, the fractionation being accomplished after incubation.
† The ribonucleic acid (RNA) distributions were made with a portion of the mince which was not incubated.
‡ The sucrose preparation was minced in a salt buffer and then, after the buffer was removed by centrifugation, was fractionated in sucrose as described in the text.
§ These terms refer to the material which sedimented in fractions which, by analogy to other tissues, would be so classified, but do not imply identity to these structures from other tissues.

### Table II

Fractions* Obtained from Sucrose† with Complete Exclusion of External Salt

<table>
<thead>
<tr>
<th>Fraction</th>
<th>C.p.m. per mg.</th>
<th>Total mg.</th>
<th>Per cent homogenate by weight</th>
<th>Total c.p.m. (\times 10^4)</th>
<th>Per cent homogenate by c.p.m.</th>
<th>Per cent distribution of total ribonucleic acid†</th>
</tr>
</thead>
<tbody>
<tr>
<td>C(^{14}O)₂-incubated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD</td>
<td>16</td>
<td>245</td>
<td>50</td>
<td>3.92</td>
<td>66</td>
<td>69§</td>
</tr>
<tr>
<td>Mitochondria</td>
<td></td>
<td></td>
<td>26</td>
<td>36</td>
<td>7</td>
<td>0.94</td>
</tr>
<tr>
<td>Microsomes</td>
<td></td>
<td></td>
<td>34</td>
<td>26</td>
<td>5</td>
<td>0.88</td>
</tr>
<tr>
<td>Supernatant</td>
<td>6</td>
<td>186</td>
<td>38</td>
<td>0.23</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>CD</td>
<td>40</td>
<td>245</td>
<td>50</td>
<td>9.80</td>
<td>60</td>
<td>69§</td>
</tr>
<tr>
<td>Mitochondria</td>
<td></td>
<td></td>
<td>38</td>
<td>36</td>
<td>7</td>
<td>3.13</td>
</tr>
<tr>
<td>Microsomes</td>
<td></td>
<td></td>
<td>92</td>
<td>26</td>
<td>5</td>
<td>2.39</td>
</tr>
<tr>
<td>Supernatant</td>
<td>19</td>
<td>186</td>
<td>38</td>
<td>0.72</td>
<td>4</td>
<td>6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Glycine-C(^{14}) incu-bated</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
</table>

* All incubations were carried out with intact cells, the fractionations being accomplished after incubation.
† Tissue was minced, washed, homogenized, and fractionated in 0.25 M sucrose as described in the text.
‡ The ribonucleic acid distributions were made with a portion of the mince which was not incubated.
§ After 2 hours incubation, 76 per cent appears in this fraction.
|| These terms refer to the material which sedimented in fractions which, by analogy to other tissues, would be so classified, but do not imply identity to these structures from other tissues.
### TABLE III

*Generality of Amino Acid-Incorporating Ability of Cell Debris*

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Glycine</th>
<th>Phenylalanine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C.p.m. per mg.</td>
<td>Total c.p.m.</td>
</tr>
<tr>
<td>CD</td>
<td>224</td>
<td>17,090</td>
</tr>
<tr>
<td>40% AMS ppt.</td>
<td>131</td>
<td>7,070</td>
</tr>
<tr>
<td>40% AMS-soluble</td>
<td>94</td>
<td>4,230</td>
</tr>
<tr>
<td>Soluble extracellular</td>
<td>2.9</td>
<td>50</td>
</tr>
<tr>
<td>Valine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD</td>
<td>594</td>
<td>45,000</td>
</tr>
<tr>
<td>40% AMS ppt.</td>
<td>450</td>
<td>24,300</td>
</tr>
<tr>
<td>40% AMS-soluble</td>
<td>315</td>
<td>14,200</td>
</tr>
<tr>
<td>Soluble extracellular</td>
<td>6.7</td>
<td>120</td>
</tr>
<tr>
<td>Alanine</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Based on the average weight of each fraction in this experiment, CD, 76 mg.; 40 per cent AMS precipitate, 54 mg.; 40 per cent AMS-soluble, 45 mg.; soluble extracellular, 18 mg.

**Fig. 1.** Specific activity of free glutamic (lighter lines) and aspartic acids (heavier lines) as a function of time of incubation of hen oviduct mince with C¹⁴O₂. The dotted lines, which start at 2 hours, represent the specific activities of free glutamic (lighter lines) and aspartic acids (heavier lines) as a function of time during which unlabeled 95 per cent O₂-5 per cent CO₂ was flushed through the medium. These data correspond to the experiments of Fig. 4.
tionation would yield certain components having a markedly higher specific activity than that determined for the whole fraction. It is interesting to note that, when rigid steps were taken to exclude all external salt, the material sedimenting under high speed centrifugation (microsomes, by analogy to liver) showed a higher specific activity than the average for the cell debris fraction (Table II). This microsome-like fraction, however, represented only a small portion of the total incorporated radioactivity. These results suggest that the high specific activity of the cell debris fraction may be due in part to potentially separable "microsome-like" material.

Contrary to results obtained with other tissues, it may be seen that, in the tissues of the hen oviduct, material which corresponds in ribonucleic acid content and amino acid-incorporating ability to "microsomes" sediments easily in low centrifugal fields. The cell debris fraction contains nearly all of the ribonucleic acid, as determined by the orcinol method and

**Fig. 2.** Specific activities of free glutamic (○) and aspartic acids (●) as a function of time of incubation of hen oviduct mince with C\(^{14}\)O\(_2\). The dotted lines, which start at 2 hours, represent the specific activities of free glutamic (lighter line) and aspartic acids (heavier line) as a function of time after the medium was replaced at 2 hours with a fresh labeled medium for the control, and a fresh unlabeled medium for the experiment. These data correspond to the experiment of Fig. 5.
cytochemically\textsuperscript{1} by affinity for basic dyes before and after ribonuclease treatment. These results were essentially independent of the method of fractionation.

The radioactivity of the cell debris proteins, obtained after incubation with C\textsuperscript{14}O\textsubscript{2}, was completely localized in the amino acids, principally glutamic and aspartic acids, isolated from the hydrolyzed protein by chromatography on Dowex 50.\textsuperscript{3} This radioactivity was not removed by hot TCA or warm alcohol-ether, or by dialysis for 24 hours against 60 per cent dimethyl formamide in 1 N NH\textsubscript{4}OH.

That the radioactive amino acids in the protein fraction were not merely physically adsorbed was further indicated by the observation that, in the free amino acid pool, the total and specific radioactivity of glutamic acid was greater than that of aspartic acid, whereas in the protein hydrolysate this situation was reversed.

\textit{Amino Acid-Incorporating Activity of Cell Debris Fraction in Relation to}
That of Other Fractions—The rates of labeling of the free glutamic and aspartic acids during the incubations are shown in Figs. 1, 2, and 3. Figs. 4 and 5 and Table IV show the corresponding kinetics of labeling for each protein fraction.

An initial lag period in the rate of labeling is generally noticeable for the ovalbumin (or plakalbumin) and extracellular protein fraction. This lag resembles that described by Peters (16) and attributed by him to the possible passage of label through precursors before entering these proteins. A lag is not observed for the cell debris proteins and only slightly for the fraction precipitable by 40 per cent of ammonium sulfate saturation. In those cases in which incubation was continued after the specific activity of the amino acid pool had been lowered, it may be seen (Figs. 4 and 5 and Table IV) that the cell debris proteins were affected the most, and that they actually decreased in specific activity in four out of the five experimental cases studied. On the other hand, amino acid incorporation continued at an appreciable rate into the ovalbumin and extracellular

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4 See also Ziegler and Melchior (8).
protein fraction, as reflected by the increase in specific activity of these fractions during incubation in the medium of lowered specific radioactivity.

![Graph](https://example.com/graph.png)

**FIG. 5.** Four protein fractions, of which three are identical to the ones in Fig. 4 and the fourth is pure crystalline egg albumin (Ea). At 2 hours the specific activity of the free amino acid pool was altered by replacing the medium with a fresh C\(^{14}\)O\(_2\)-containing medium for the control and a fresh C\(^{14}\)O\(_2\)-containing medium for the experimental fraction. See Fig. 2 for the specific activities of the free amino acids. The curves swing abruptly up at 2 hours and the AMS precipitate and CD curves cross at 5 hours because the diluting bulk of soluble proteins was removed at 2 hours. The branching at 2 hours represents a control (upper branch) and an experimental (lower branch) fraction obtained after incubation in fully radioactive medium and medium of reduced specific activity, respectively.

**DISCUSSION**

The correlation between the ribonucleic acid content and the ability to incorporate radioactive amino acids, which has been found for other tissues, is also apparent in the oviduct. However, it is shown that, although the material previously demonstrated to account for these characteristics was sedimented only with difficulty in relatively high centrifugal fields
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 (>20,000 X g), corresponding material in this tissue sediments quite easily (<600 X g in a few minutes).

The possibility cannot be eliminated that, in this tissue, some hitherto undescribed process is responsible for the artificial clumping of microsomes out of solution. External salt was eliminated as a possible clumping agent by the fractionations in sucrose. Removal of the connective tissue before fractionation did not alter the basic distribution pattern of ribonucleic acid and radioactivity. The pH of the albumin-secreting region of the oviduct

TABLE IV

Rate of Incorporation of Radioactivity into Different Protein Fractions and Effect of Reducing Specific Activities of Precursors

<table>
<thead>
<tr>
<th>Time</th>
<th>Cell debris</th>
<th>40% AMS ppt.</th>
<th>Plakalbumin</th>
<th>Extracellular supernatant proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>min.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>45</td>
<td>44*</td>
<td>38</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>hrs.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2†</td>
<td>145</td>
<td>128</td>
<td>35</td>
<td>4</td>
</tr>
<tr>
<td>5</td>
<td>355</td>
<td>273</td>
<td>110</td>
<td>17</td>
</tr>
<tr>
<td>10</td>
<td>624</td>
<td>588</td>
<td>246</td>
<td>53</td>
</tr>
<tr>
<td>5† (Gas)</td>
<td>267</td>
<td>248</td>
<td>73</td>
<td>21</td>
</tr>
<tr>
<td>10</td>
<td>205</td>
<td>369</td>
<td>102</td>
<td>46</td>
</tr>
<tr>
<td>5† (Medium A)</td>
<td>147</td>
<td>142</td>
<td>52</td>
<td>15</td>
</tr>
<tr>
<td>10</td>
<td>170</td>
<td>200</td>
<td>61</td>
<td>25</td>
</tr>
<tr>
<td>5† (Medium B)</td>
<td>140</td>
<td>134</td>
<td>62</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>132</td>
<td>229</td>
<td>80</td>
<td></td>
</tr>
</tbody>
</table>

* All the figures are in counts per minute per mg. of protein.
† Starting at 2 hours, unlabeled (95 per cent O2-5 per cent CO2) gas was passed through the remaining flasks in the gas exchange experiment. In the experiment of Medium A the mince was centrifuged and resuspended in a 2 hour incubated unlabeled medium and in Medium B the mince was resuspended in fresh unlabeled medium. These procedures are more fully described under “Experimental.”

has been reported to be 6.3 to 6.6 (17); thus, clumping due to the effects of extreme pH appears to be eliminated.

Recent papers have described a lace-like continuum found in the cytoplasm of many cells (18–21). This network has associated with it intensely basophilic small particles of microsomal nature, and appears to account for the basophilic properties of the cytoplasm. The endoplasmic reticulum, as this network has been called, provides a basis for considering the results of this paper and for reconciling them with previous work on the freely floating submicroscopic microsomes. Microusomes may normally be a part of, or in association with, this network. In the case of liver and other
tissues, in which free microsome preparations were readily obtained, they
could have been dissociated from other structural elements during frac-
tionation. Alternatively, the degree of association between "microsomes"
and network may vary for different cells, with hepatic cells representing a
type with a greater degree of dissociation. Microscopic examination of
the preparations studied in this paper has revealed a peripheralization of
basophilic material around adjacent intracellular protein globules rather
than an accumulation at their interstices, and is consistent with the asso-
ciation of this basophilic material with a pliable matrix. The size of some
of the larger discernible basophilic structures is of the order of 1 μm. If
these were freely floating bodies, they might not be expected to sediment
as easily as has been observed. Their observed sedimentation behavior
suggests that they may be strongly associated with some larger easily
sedimentable structure such as the endoplasmic reticulum. If the early
stages of protein synthesis are associated with such a fixed structure, it
might be possible to explain the absence to date of findings of precursor
stages such as peptides in the soluble part of the cell.

The relative behavior of the protein fractions discussed here is consistent
with the possibility that free amino acids are first incorporated into some
part of the cell debris proteins, and that there then occurs a subsequent
transfer of labeled precursor substances to the other proteins of the cell.
The high activity of the cell debris fraction with respect to the incorpo-
ration of amino acids has been observed with all six of the amino acids
studied in this work.

Gale and his coworkers at Cambridge (22) have studied a protein frac-
tion obtained after subjecting *Staphylococcus aureus* to sonic vibration.
This preparation contains the gross cell structure and is active in incor-
porating amino acids under conditions favorable for protein synthesis and
also under conditions favorable for amino acid exchange. Although
their system is different from the one reported here, some analogy may
be drawn which might link the structural part of the cell to protein-syn-
thetic reactions.

**SUMMARY**

1. The cytoplasmic ribonucleic acid-containing basophilia and the
material of the highest amino acid-incorporating ability in hen oviduct
tissue are easily sedimentable.

2. The specific radioactivities of the dicarboxylic amino acids contained
in this easily sedimentable fraction are markedly decreased when incuba-
tions are continued after the addition of non-isotopic glutamic and aspartic
acids, or the replacement of C14O2 by C12O2. The more soluble proteins
continue to increase in radioactivity after such dilution.
3. These results are discussed in relation to the endoplasmic reticulum and the mechanism of protein synthesis.

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