Incorporation of Amino Acids into Microsomal Proteins of Normal and Malignant Tissues

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SUMMARY
Rapidly proliferating normal and malignant cells had a greater initial rate of amino acid incorporation into microsomal proteins than did normal cells. The rate of microsomal protein renewal in Novikoff hepatoma was greater than that of liver cells. The initial rates of amino acid incorporation into hepatoma and liver ribonucleoprotein particles were approximately equal. The soluble fractions of the liver and hepatoma cells were essentially interchangeable. Hepatoma microsome and pH 5 enzymes had a greater proportion of ribonucleic acid (RNA) in RNA plus proteins than did normal liver microsomes.

Zamecnik and Keller demonstrated that the amino acid-incorporating system of rat liver cells, consisting of two subcellular fractions, the soluble and the microsomal, requires the presence of certain cofactors (8, 18). It has also been shown that the portion of the soluble cell fraction precipitable at pH 5.2 contains the enzymes that catalyze the carboxyl activation of amino acids (5) and that the ribonucleoprotein granules of the microsomes are the actual sites of protein formation (10). Littlefield and Keller have observed a qualitatively similar mechanism for microsomal protein synthesis in Ehrlich mouse ascites tumor cells (11).

In an attempt to elucidate further the differences or similarities in the over-all amino acid-incorporating processes of normal, rapidly growing normal, and malignant tissues, a series of isotopic experiments were conducted with normal and regenerating rat livers, spleen, Novikoff hepatoma, and Murphy-Sturm lymphosarcoma. The relative proportion of microsomal ribonucleic acid in the various tissues and their possible role in protein formation were also investigated.

MATERIALS AND METHODS

Chemicals.—Adenosine triphosphate (ATP) (disodium salt), 3-phosphoglycerate1 (barium salt, converted to potassium salt with K2SO4), and pyruvate kinase were obtained from the Sigma Chemical Company; guanosine triphosphate (sodium salt) from Pabst Laboratories; and phosphoenolpyruvate (barium-silver salt, converted to potassium salt with Dowex 50-X8) from C. F. Boehringer and Soehne. Solutions of ATP, 3-phosphoglycerate, and guanosine triphosphate were adjusted to about pH 7.5 (pH paper), and pyruvate kinase was dialyzed against several changes of cold distilled water for 3-4 hours. Uniformly labeled L-leucine-C14, L-valine-C14, and L-lysine-C14 were purchased from Nuclear-Chicago. All reagents used were of chemically pure grade.

Tissues.—Normal liver and spleen were obtained from 200-250-gm. male Holtzman rats, and regenerating liver from similar rats that were 70 per cent hepatectomized 42-45 hours prior to the experiments. Novikoff hepatoma tissues were used 4-5 days after intraperitoneal transplantation, and blood clots adhering to the tumor were removed before homogenization. Older tumors were found to be much less active with regard to amino acid incorporation. To obtain reproducible results, it was essential that only young, necrosis-free tumor be used. It was observed during our earlier experiments that indiscriminate selection of tumors with respect to age and appearance resulted in considerable daily variation in tumor activity. In the case of liver and regenerating liver, consistent results can be obtained if the incubation experiments are performed within a prescribed time after the animals are killed.

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Murphy-Sturm lymphosarcoma was used 10–12 days after transplantation subcutaneously into the thigh, and necrotic areas of the tumor were avoided. This tumor was not investigated in as great detail as was the Novikoff hepatoma. The animals were maintained on stock diet at all times.

Preparation of cell fractions.—The method used followed essentially the procedure described by Keller and Zamecnik (8), with minor changes as specified. The animals were killed by decapitation and the liver, spleen, or tumor excised quickly and chilled in ice-cold medium A (11) containing 0.35 m sucrose, 0.025 m KCl, 0.008 m MgCl₂, and 0.05 m Tris A buffer (pH 7.6). All subsequent operations were performed at 0°-4° C. The tissues after being blotted and weighed were homogenized gently in 2.5 volumes of medium A in a Teflon homogenizer and centrifuged at 20,000 X g for 10 minutes. The supernatant fluid (hereafter referred to as 20,000 X g supernatant fluid) in which the fluffy layer immediately above the firm pellet was avoided, was then centrifuged in a Model L Spinco centrifuge at 150,000 X g for 90 minutes. The supernatant fluid (designated as cell sap) was aspirated carefully, avoiding the fluffy layer, and the microsome pellets were washed twice by carefully overlaying the pellets with medium B (8) containing 0.90 m sucrose, 0.008 m MgCl₂, and 0.025 m KCl, drained, and the inner walls of the tubes were wiped with a clean absorbent paper.

To prepare liver fractions containing an equal quantity of microsomal protein as a corresponding hepatoma fraction, one volume of 20,000 X g supernatant fluid was mixed with three volumes of liver cell sap. A gram of liver (wet weight) was found to contain approximately 4 times as much microsomal protein as an equal wet weight of hepatoma. The corresponding ratio in the case of regenerating liver as compared with normal animals was found to be approximately 1:2. Samples prepared in this manner provided comparable 20,000 X g supernatant fluids containing equal dry weight of microsomal protein suspended in an equal volume of their respective cell sap fractions.

To study the effect of various combinations of cell sap fractions on the incorporating capacity of microsomes, the required amount of microsome pellet was suspended gently in an appropriate volume of cell sap in a loosely fitting all-glass homogenizer.

The pH 5 enzyme fraction was prepared as described by Keller and Zamecnik (8).

Method of incubation.—Since it was observed that the incorporating capacity of the microsomes decreased considerably upon aging even at 0–4° C., the experiments were done as soon as possible after the tissues were removed from the animals. The time elapsed after killing was usually 4–5 hours for the experiments in which pH 5 enzyme fraction was used and 2–3 hours when the 20,000 X g supernatant fluid was employed.

A 125-ml Erlenmeyer flask containing an appropriate volume of 20,000 X g supernatant fluid and a similar flask containing a mixture of cofactors were equilibrated separately in a Dubnoff incubator at 37° C. for 1½–2 minutes. Longer periods of preincubation resulted in considerable loss of activity in both liver and hepatoma systems. The concentrations of Mg⁺⁺ and phosphoglycerate used were optimal for both systems, and omission of either component resulted in negligible incorporation. At zero time, the contents of the two flasks were mixed and incubated aerobically at 37° C. with constant shaking. In preliminary experiments, no significant differences were found with anaerobic incubations. Aliquots were withdrawn at specified intervals and transferred to chilled lusteroid tubes containing NaF sufficient to give a final concentration of approximately 0.01 m. Each tube was then made up to 12 ml with ice-cold medium B and centrifuged at 105,000 X g for 90 minutes to separate the microsome and cell sap fractions. The separation of the ribonucleoprotein particles from the deoxycholate-soluble fraction when required was carried out according to a procedure previously described (9).

For the experiments with pH 5 enzyme-microsome system, the procedure of Keller and Zamecnik (8) was followed.

Purification of proteins and radioactivity determinations.—The various fractions, after incubation and centrifugation, were precipitated in cold 5 per cent trichloroacetic acid (TCA) and centrifuged. The precipitates were subsequently treated as follows: 5 per cent TCA at 95°–100° C. for 20–30 minutes, 5 per cent TCA at room temperature (3 times), drained and placed into boiling water bath for 10 minutes (16), 95 per cent ethanol, alcohol-ether (3:1) once at 50°–55° C. and once at 25° C., and ether. The protein residue was dried overnight in a desiccator over P₂O₅ followed by further drying in vacuo at 80° C. for 4 hours. The dried samples were placed on 5-sq. cm. stainless steel planchets, weighed, distributed evenly by suspension in 0.5 m NH₄OH, dried under an infrared lamp, and counted under standard conditions in a gas-flow counter (Nuclear-Chicago) equipped with a micromil window. Under these conditions, "infinitely thin" samples...
could be prepared in which specific activity remained constant when up to 4 mg. of protein was plated. Two to 4-mg. samples were plated as described above, and radioactivity is reported as counts/min/mg protein. Further washings of the protein residue with thioglycollate and dilute alkali did not alter the specific activity of the proteins.

Nucleic acid and protein determinations.—The cold TCA precipitates, after being washed and defatted, were hydrolyzed overnight in 0.5 N NaOH at 30°C. After acidification with cold 0.5 N perchloric acid and centrifugation, the clear supernatant was adjusted to pH 7, and nucleic acid was determined by its absorption at 260 mμ in a Beckman DU spectrophotometer (2) and by phosphate analysis (4). Purified rat liver microsomal RNA was used as standard.

The protein concentrations of the microsomal and activating enzyme suspensions were determined by a modified biuret with the use of turbidity blanks (3) and Lowry (12) methods. Bovine serum albumin was used as standard.

RESULTS

Experiments with 20,000 × g supernatant fluids.—Preliminary studies on the effects of the quantity of microsomal protein incubated and the total length of incubation period on the relative specific activities of the liver and Novikoff hepatoma systems were made, and the results are shown in Table 1.

TABLE 1

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Cell fraction</th>
<th>Wet weight of tissue (g)</th>
<th>Total microsomal protein (mg)</th>
<th>Total microsomal RNA (mg)</th>
<th>Specific activity* of microsomal protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Leucine C14 After 3 min.</td>
</tr>
<tr>
<td>1. Novikoff hepatoma</td>
<td>8 cc. 20,000 × g supernatant fluid</td>
<td>0.71</td>
<td>3.2</td>
<td>0.86</td>
<td>665</td>
</tr>
<tr>
<td>2. Rat liver</td>
<td>8 cc. 20,000 × g supernatant fluid</td>
<td>0.72</td>
<td>15.0</td>
<td>1.60</td>
<td>200</td>
</tr>
<tr>
<td>3. Rat liver</td>
<td>2 cc. 20,000 × g supernatant fluid and 6 cc. liver cell sap</td>
<td>0.18</td>
<td>3.7</td>
<td>0.41</td>
<td>190</td>
</tr>
</tbody>
</table>

* Counts/min/mg protein.

Each fraction contained either 8 ml. 20,000 × g supernatant fluid or a mixture of 2 ml. 20,000 × g supernatant fluid plus 6 ml. cell sap, 40 μmoles ATP, 40 μmoles MgCl2, 160 μmoles 3-phosphoglycerate, and 0.4 μmoles L-leucine-C14 (1.2 × 104 counts/min) or 0.4 μmoles L-valine-C14 (1.0 × 104 counts/min), in a total volume of 0.2 ml. Incubated in air in Dubnoff metabolic shaker at 37°C.

It can be seen that the specific activity of liver microsomes is dependent upon the quantity of microsomal protein incubated (tissues 2 and 3); and the relative specific activity of hepatoma, when compared with that of liver on an equal wet weight basis (1 and 2) or on an equal dry weight basis (1 and 3), varied considerably with the length of incubation period.

It should be stressed that, since both normal and neoplastic tissues were homogenized in 2.5 volumes of buffered medium, the cell sap as existing in the living cell was in effect diluted 3.5-fold for both tissues. This proportion of microsomes to cell sap was maintained in hepatoma 20,000 × g supernatant fluid. However, since the comparative activities of the tissues were based upon equal dry weight of microsomal protein, the concentration of microsomal protein in liver 20,000 × g supernatant fluid was necessarily reduced by a factor of 4, as described earlier (Table 1, Tissue 3).

Another point to be emphasized is that, although the total incubation volumes in all the experiments were not equal, the concentrations of the various components were kept constant. In some experiments, larger volumes were employed in order to obtain sufficient microsomal proteins for radioactivity determinations.

In subsequent experiments, as noted above, to compare the amino acid-incorporating capacity of malignant cells with that of normal cells, equal dry weight of microsomal protein was used as
the basis for comparison, and the relative rates of incorporation were determined over a period of 20 minutes.

The time courses of incorporation of leucine-C\(^{14}\) into liver microsomes, ribonucleoprotein particles, and deoxycholate-soluble fraction are shown in Chart 1A. The incorporation at zero time was negligible. A slight but distinct lag phase was consistently observed for the amino acid uptake by the microsomes, with the maximum uptake occurring between 10 and 20 minutes. The rate of incorporation into the ribonucleoprotein particles is reproduced in Chart 1A to illustrate the effect of duration of incubation on the relative specific activities of the liver and hepatoma microsomes. Similar studies were done with l-valine-C\(^{14}\) and l-lysine-C\(^{14}\), and the results are shown in Charts 2B and 2C, respectively. These results seem to indicate that the initial rate of amino acid incorporation into microsomal protein and the rate of renewal of this fraction are considerably greater in the malignant cell than in the normal hepatic cell.

It has been reported recently that a microsome-
incorporation was again much greater in the more rapidly growing regenerating tissue than in the corresponding normal tissue, with the peak occurring at 6 minutes. The rate of renewal of microsomal protein was, however, not as great as in the case of malignant hepatic tissue. It has been previously shown that regenerating liver is more active than normal resting liver with respect to protein synthesis (1, 6). The initial rate of incorporation of leucine-C\textsuperscript{14} into Murphy-Sturm lymphosarcoma microsomes was also greater than that of spleen but considerably less than that of hepatoma.

To investigate the relative amino acid-incorporating capacities of the microsome and cell sap fractions, time curve studies of various combinations of liver and hepatoma microsomes and cell sap fractions were made, and the results are shown in Chart 5. When hepatoma microsomes were incubated with either hepatoma or liver cell sap,
CHART 4.—Comparative rates of L-leucine-C\textsubscript{14} incorporation into Murphy lymphosarcoma and spleen microsomes. Flask contained either 30 ml. spleen or lymphosarcoma 20,000 X g supernatant fluid and 100 \( \mu \) moles ATP, 100 \( \mu \) moles MgCl\(_2\), 400 \( \mu \) moles 3-phosphoglycerate, and 1 \( \mu \) mole L-leucine-C\textsubscript{14} (3 \( \times \) 10\(^8\) counts/min) in a total volume of 23 ml. Incubated in air at 37° C.

CHART 5.—Effects of various combinations of cell sap and microsomes on amino acid incorporation.

A. Flask contained liver microsome (LM) obtained from 6 ml. of 30,000 X g supernatant fluid after centrifugation at 105,000 X g for 90 minutes, suspended in either 24 ml. of liver (L) cell sap or 24 ml. of hepatoma (H) cell sap, 120 \( \mu \) moles ATP, 120 \( \mu \) moles MgCl\(_2\), 480 \( \mu \) moles 3-phosphoglycerate, and 0.6 \( \mu \) moles L-leucine-C\textsubscript{14} (1.8 \( \times \) 10\(^6\) counts/min).

B. Flask contained hepatoma microsomes (HM), obtained from 24 ml. of 20,000 X g supernatant fluid after centrifugation at 105,000 X g for 90 minutes, suspended in either 24 ml. of liver (L) cell sap or 24 ml. of hepatoma (H) cell sap, 120 \( \mu \) moles ATP, 120 \( \mu \) moles MgCl\(_2\), 480 \( \mu \) moles 3-phosphoglycerate, and 0.6 \( \mu \) moles L-leucine-C\textsubscript{14} (1.8 \( \times \) 10\(^6\) counts/min). Incubated in air at 37° C.
there was no significant change in activity. When liver microsomes were used with liver or hepatoma cell sap, the lag phase associated with the liver microsome-liver cell sap system was abolished by the substitution of hepatoma cell sap, although the rates of incorporation after 4 minutes were equal. In view of the earlier observation that the initial rates of amino acid incorporation into the liver and hepatoma RNP particles are approximately equal, the characteristic lag phase seems to be a property peculiar to the liver microsome rather than to the liver cell sap. It has been shown by Littlefield and Keller (11) that both microsomes and pH 5 enzymes from Ehrlich ascites cells are interchangeable with the corresponding fractions from liver without loss of activity. The results reported here also seem to indicate that, aside from the ability of the hepatoma cell sap to abolish the lag phase, the soluble fractions of the liver and hepatoma are similar with respect to amino acid incorporation.

Microsome-pH 5 enzyme systems.—The requirement of guanosine triphosphate and a source of energy by the liver microsome-pH 5 enzyme system has been shown by Keller and Zamecnik (8). In Table 2, the effects on the incorporation of leucine-C\(^14\) into regenerating and hepatoma microsomes caused by the omission of any one of the constituents required by the normal system are given, and it can be seen that the cofactor requirement of these rapidly proliferating normal and neoplastic tissues were similar to those of the normal liver. The relatively high activities of these systems in the absence of pyruvate kinase and activating enzymes may be due to contaminations of the microsome fraction with these enzymes.

The effect of adding hepatoma microsomal RNA purified by the phenol method (9) on leucine-C\(^14\) incorporation into hepatoma microsomes was also investigated, and it was found that activity was not enhanced by additional RNA.

RNA content in microsome and activating enzymes.—With the purpose of obtaining some information with regard to the role of microsomal RNA in the processes of protein synthesis, the concentrations of this polymer in the microsomes of the various tissues were determined. As shown in Table 3, the per cent of RNA to RNA plus protein in the hepatoma microsomes was greater than those of the regenerating and normal liver microsomes, indicating a relatively low content of endoplasmic reticular membranous portion in the tumor cells. It has been shown that the microsomes of the ascites tumor cells also have a higher RNA to protein ratio (11). Similar results have been obtained by von der Decken and Hultin with microsomes from regenerating liver (17). In the case of the activating enzymes, the same ratios were again higher in the hepatoma enzyme than in the corresponding regenerating and normal liver enzymes.

**DISCUSSION**

It was shown several years ago that primary p-dimethylaminoazobenzene-induced rat hepatoma incorporated amino acids more rapidly into total cellular proteins than did normal resting protein\(^1\) in the hepatoma microsomes was greater than those of the regenerating and normal liver microsomes, indicating a relatively low content of endoplasmic reticular membranous portion in the tumor cells. It has been shown that the microsomes of the ascites tumor cells also have a higher RNA to protein ratio (11). Similar results have been obtained by von der Decken and Hultin with microsomes from regenerating liver (17). In the case of the activating enzymes, the same ratios were again higher in the hepatoma enzyme than in the corresponding regenerating and normal liver enzymes.

**TABLE 3**

**PER CENT RNA IN RNA PLUS PROTEIN OF THE MICROSONES AND pH 5 ENZYMES**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Per cent RNA*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microsomes from:</td>
<td></td>
</tr>
<tr>
<td>Normal liver</td>
<td>9.0</td>
</tr>
<tr>
<td>Regenerating liver</td>
<td>12.0</td>
</tr>
<tr>
<td>Hepatoma</td>
<td>24.0</td>
</tr>
<tr>
<td>pH 5 enzymes from:</td>
<td></td>
</tr>
<tr>
<td>Normal liver</td>
<td>4.2</td>
</tr>
<tr>
<td>Regenerating liver</td>
<td>4.5</td>
</tr>
<tr>
<td>Hepatoma</td>
<td>8.1</td>
</tr>
</tbody>
</table>

* Per cent RNA in RNA plus protein (see footnote 2).

RNA and protein were determined as described under "Materials and Methods."

\(^1\) Per cent RNA in RNA plus protein means RNA by weight in combined RNA plus protein, as defined by Littlefield and Keller (5).
adult liver (19). The experiments reported in this communication were performed with the intent of elucidating further the possible differences in the over-all mechanism of protein synthesis in normal, rapidly proliferating normal, and neoplastic tissues. The data presented in Table 1 indicate that, when relative specific activity values are used as a measure of the rate of protein formation, it is necessary not only to specify the common denominator employed, i.e., expression of rate per unit of equal wet weight or dry weight of microsomal protein, but it is also essential to determine the time course of the incorporation.

The results obtained indicate that the initial rate of amino acid incorporation into microsomal protein and the rate of protein renewal were considerably greater in the hepatoma cell than in the normal hepatic cell. The corresponding rates associated with the ribonucleoprotein particles were, however, quite similar in the two types of tissues, suggesting that the processes of amino acid activation and the subsequent transfer of the activated amino acids to the ribonucleoprotein particles of the microsomes may be similar in both normal and tumor cells. This is in accord with the observations that the soluble fractions of the two types of cells are essentially interchangeable and that the cofactor requirement of the malignant cell is similar to that of the normal cell.

It is also of interest to note that, although the specific activity of hepatoma microsomes exceeds that of liver, the total activity of liver microsomes was considerably greater at 6 minutes when the comparison was made on equal wet weight basis (Table 1), indicating a greater over-all capacity of liver cells to synthesize proteins.

The striking features of the liver cell system are the presence of the lag phase and the conspicuously slower rates of amino acid incorporation into microsomal proteins and turnover of these proteins. That the lag phase of the microsome curve is paralleled by a similar type of curve for the deoxycholate-soluble fraction suggests that the “lag” may be attributable to the delay in the transfer of radioactivity from the ribonucleoprotein particles to the membranous portion of the microsomes. Such a “lag” was not observed with hepatoma ribonucleoprotein and deoxycholate-soluble fractions. A contributing factor accounting for the “lag” may also be the relatively larger amount of endoplasmic reticular membrane present in normal liver cell in contrast to the paucity of this structure in tumor cells—i.e., the initial high radioactivity in the liver ribonucleoprotein particles is masked by the extremely large amount of nonradioactive membranous proteins when the specific activities of the whole microsomes are measured. Peters (18) has observed a “lag” in the appearance of radioactivity in serum albumin and has shown that it was due not to the time required for the synthesis of the protein molecule but to the time required for the physiological release of albumin by the ribonucleoprotein particles.

The relatively more rapid uptake of amino acids by the microsomes is a property also evident in regenerating rat liver and Murphy-Sturm lymphosarcoma. A similar rapid incorporation of phenylalanine into the microsomes of rabbit reticulocytes has been shown recently by Rabinovitz and Olson (15).

Although tumor microsomes contain a greater proportion of RNA per unit weight of microsomal protein and, as observed, a greater rate of protein renewal, the precise role of the microsomal RNA in protein synthesis is not yet clear. Since the addition of purified RNA does not enhance the incorporating capacity of hepatoma microsomes, the quantity of RNA in the microsomes may not be a significant factor. It is possible, however, that a simple addition of purified RNA to a microsomal system may not be equivalent to a functionally active RNA presumably present in physiologically intact microsomes. It has been shown that part of the soluble RNA is transferred into the microsome proper during protein formation (7). However, the exact relationship of the soluble and microsomal RNA is not known.

Since the initial rates of amino acid incorporation into the ribonucleoprotein particles of normal and malignant cells are similar, the observed difference in the renewal of microsomal proteins appears to lie in the rates at which the newly synthesized proteins are released into the soluble fractions. The fact that the hepatoma microsomes are deficient in membranous component may account for the greater renewal of microsomal proteins and indicates that this difference in the rates of protein renewal is due to structural variations in the normal and tumor microsomes rather than to a basic biochemical difference in the mechanism of protein synthesis. In this respect, Porter and Bruni have recently shown, by means of electron microscopic examinations, the occurrence of drastic structural changes in the hepatic endoplasmic reticulum of rats treated with 3'-methyl-4-dimethylaminoazobenzene (14). It has been suggested by these authors that such an alteration in the morphological structure of microsomes could lead to some changes in the normal processes of protein synthesis.
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