INCORPORATION OF C\textsuperscript{14}-AMINO ACIDS INTO RIBONUCLEOPROTEIN PARTICLES FROM THE EHRLICH MOUSE ASCITES TUMOR*

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There have been many indications from earlier studies that ribonucleic acid is involved in protein synthesis, perhaps as the template for the formation of specific proteins (see Brachet (2) for a recent review). In studies on the mechanism of protein synthesis we have found that, when a C\textsuperscript{14}-amino acid is injected intravenously into a rat, the initial incorporation into the cytoplasmic ribonucleoprotein particles of the liver is several times greater than into other cell fractions (3). The rapid initial incorporation into these particles could represent the formation of new polypeptide chains on the surface of the RNA. In these experiments \textit{in vivo}, the C\textsuperscript{14} in the ribonucleoprotein declined after the first few minutes if the specific activity of the free intracellular amino acid decreased. If, however, the latter was kept constant, the labeling of the nucleoprotein remained constant, suggesting a steady state in which the rate of formation and release of polypeptide chains were equal. Only 1 per cent of the protein in the ribonucleoprotein particles became labeled in this process.

Earlier, Petermann \textit{et al.} had shown by ultracentrifugal and electrophoretic analyses that there is in the cytoplasm of liver and other cells a variety of ribonucleoprotein particles containing equal amounts of RNA and protein (4). The major type of particle in liver (Component B) has a sedimentation rate of 50 S. In tumors and other rapidly growing tissues another type of particle (Component C, 40 S) is prominent, as if it were somehow connected with cell division (5). Perhaps Component C contains the templates for the structural and enzymatic proteins of the cell formed during growth. These several ribonucleoprotein particles were presumably identi-

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1 The following abbreviations have been used: RNA, ribonucleic acid; DNA, deoxyribonucleic acid; ATP, adenosine triphosphate; GTP and GDP, guanosine triphosphate and guanosine diphosphate; PEP, phosphoenolpyruvate; Tris, tris(hydroxymethyl)aminomethane.
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cal with particles 150 A in diameter seen in electron micrographs of microsomes (6) and of thin sections of various tissues (7, 8).

For our incorporation experiments in vivo the ribonucleoprotein particles were isolated from the microsome fraction of rat liver by use of sodium deoxycholate (3), which renders soluble the membranous (non-ribonucleoprotein) component of the microsomes (9). The deoxycholate insoluble particles contained an equal amount of RNA and protein, had a major peak with a sedimentation rate of 47 S, and appeared as uniform particles 240 A in diameter in electron micrographs of unfixed, unstained preparations. Palade and Siekevitz have also reported the isolation of ribonucleoprotein particles by the use of deoxycholate (10). Petermann and Hamilton have shown that deoxycholate does not affect the major nucleoprotein particle peaks (11).

To explore further the role of these particles in the protein synthesis of normal and neoplastic cells, we have studied the Ehrlich mouse ascites tumor (12). The cytoplasm of these homogeneous, rapidly growing cells is rich in RNA (13) and relatively deficient in the membranous component of the endoplasmic reticulum (14). Suspensions of free tumor cells are easily handled and incorporate C\textsuperscript{14}-amino acids into protein at a high rate in vitro (15, 16). In part, the present paper deals with the isolation and characterization of ribonucleoprotein particles from this tumor, and presents evidence that they are important in the protein synthesis of the tumor cells, as are the corresponding particles in liver.

A cell-free system has been obtained from rat liver, which catalyzes the incorporation of C\textsuperscript{14}-amino acids into protein in the presence of adenosine triphosphate (ATP), an ATP-generating system, and either guanosine triphosphate (GTP) or guanosine diphosphate (GDP) (17, 18). The cell fractions required are the microsomes and the proteins precipitated from the soluble cell fraction at pH 5 (the "pH 5 enzymes"). The ribonucleoprotein particles present in the microsomes are labeled up to 8 times more rapidly than the other components of the cell-free system (3). The pH 5 enzymes catalyze the carboxyl activation of amino acids by ATP (19). No direct evidence has been obtained concerning the mode of action of the guanosine nucleotide in this system.

With the liver system it was not possible to determine whether the membranous component of the microsomes was essential for the incorporation. The purified particles isolated by the use of deoxycholate were inactive, but deoxycholate was inhibitory to the incorporation system. It has now been found that ribonucleoprotein particles can be isolated from the microsomes of Ehrlich ascites tumor by a procedure similar to that of Jeener (20),\textsuperscript{2} by using 0.5 M NaCl, and that these particles, when combined with

\textsuperscript{2} We are grateful to Dr. J. L. Simkin for stimulating our interest in the use of NaCl solutions in microsome fractionation.
pH 5 enzymes of tumor, ATP, and GTP, give active incorporation of C\textsuperscript{14}-amino acids into the ribonucleoprotein.

**Materials and Methods**

**Materials**—L-leucine-1-C\textsuperscript{14}, L-valine-1-C\textsuperscript{14}, L-isoleucine-1-C\textsuperscript{14}, DL-leucine-1-C\textsuperscript{14}, DL-alanine-1-C\textsuperscript{14}, and DL-phenylalanine-1-C\textsuperscript{14} were synthesized and kindly furnished by Dr. R. B. Loftfield (21) from BaC\textsuperscript{14}O\textsubscript{4} obtained on allocation from the United States Atomic Energy Commission.

We are indebted to Dr. J. D. Gregory for the pyruvate kinase, and Dr. S. A. Morell kindly supplied a preparation of mixed nucleotides from yeast. Crystalline disodium ATP and GDP were obtained from the Sigma Chemical Company, and GTP was obtained from the Pabst Laboratories. Other materials were the same as those used previously (3, 18).

All solutions used in incubation mixtures were adjusted to approximately pH 7.4 before use. All the solutions of nucleoside di- or triphosphates were made up with 1 mole of MgCl\textsubscript{2} per mole of nucleotide, so that addition of these solutions would not lower the Mg\textsuperscript{++} concentration of the incubation mixture.

**Preparation of Washed Tumor Cells**—The Ehrlich mouse ascites tumor was obtained from Dr. G. Klein in 1950, and has been maintained in this laboratory by weekly transfers made by the intraperitoneal injection of 0.2 ml. of ascitic fluid into young male and female mice of strain A. In the present work, mice were selected for prominent ascites 7 to 11 days after transplantation of the tumor. After decapitation of the animal, the ascitic fluid was drained through a large abdominal incision into a flask surrounded by ice. Subsequent operations were performed at 0-4\textdegree. The tumor cells were collected by centrifugation and washed by suspension in ice-cold medium (0.14 M NaCl, 0.02 M glucose, and 0.04 M tris(hydroxy-methyl)aminomethane (Tris) buffer at pH 8.5), followed by centrifugation. This washing procedure was repeated two to three times until the supernatant fluid was clear and only faintly colored. Red blood cells which tend to pack below the tumor cells were not separated from the tumor cells, since adult red blood cells contain very little RNA and do not incorporate labeled amino acids into proteins (22). The tared tubes containing the washed cellular pellets were weighed to determine the wet weight of the cells. About 1.5 gm. of wet cells were obtained from each mouse. Roughly, 85 per cent of the cell mass was tumor cells, or about $8 \times 10^8$ tumor cells per gm. of wet cells.

**Tumor Cell Lysis and Fractionation**—The cellular pellets were resuspended with a large, loose homogenizer in 10 volumes of ice-cold distilled water. After 5 minutes, concentrated solutions were added to give a final concentration of 0.25 M sucrose, 0.025 M KCl, and 0.005 M MgCl\textsubscript{2}. The lysate was centrifuged for 10 minutes at 15,000 $\times g$. The supernatant
fluid was aspirated carefully and centrifuged for 2 hours at 105,000 × g (Raverage) in the No. 40 rotor of the Spinco model L ultracentrifuge. (In experiments on cofactor requirements, the lysate was centrifuged at 15,000 × g for 10 minutes and the supernatant fluid treated with 15 to 20 per cent of its weight of moist Dowex 1-X8 acetate; after 2 to 3 minutes, the resin was removed by filtration, and concentrated solutions were added as above to the filtrate and the filtrate was centrifuged at 105,000 × g.)

The supernatant fluid containing the soluble cell fraction was collected by aspiration. This was diluted with 2 to 3 volumes of 0.025 M KCl when cofactor requirements were under study. The pH of the solution was then brought to 5 with 1 N acetic acid to cause precipitation of protein. After standing for 5 minutes, the suspension was centrifuged and the precipitate, containing about one-third of the soluble cell proteins, was washed with cold water and dissolved in ice-cold Medium A (0.25 M sucrose, 0.025 M KCl, 0.005 M MgCl₂, and 0.05 M Tris buffer, pH 7.6) to give a solution with about 10 mg. of protein per ml. A small amount of insoluble material was removed by centrifugation. The term “pH 5 enzymes” is used to refer to this preparation, which is similar to the “pH 5 enzymes” from liver. The tumor pH 5 enzymes catalyze the carboxyl activation of amino acids by ATP (19) and are essential in the cell-free incorporation system. These enzymes remain active for several days if kept frozen.

The small pink microsome pellets, well packed by centrifugation for 2 hours at 105,000 × g, were rinsed with ice-cold distilled water and homogenized in Medium A with a cold glass pestle, closely fitting the Lusteroid centrifuge tube, to give a suspension with about 10 mg. of protein per ml. A small amount of aggregated material was removed by centrifugation at 15,000 × g.

When separation of microsomes and pH 5 enzymes was not necessary, both could be concentrated conveniently from the lysate by acidifying the 15,000 × g supernatant fluid to pH 5 and collecting the precipitate by centrifugation.

Liver microsomes and pH 5 enzymes were obtained from young rats as described previously (18), except that 0.05 M Tris buffer, pH 7.6, was substituted for bicarbonate buffer in the homogenizing medium. Livers from the tumor-bearing mice were not used.

Isolation of “Deoxycholate-Insoluble Particles” from Tumor Microsomes—Pooled microsome pellets were homogenized in a 12.5 ml. Lusteroid centrifuge tube with 0.5 to 1.0 ml. of cold, freshly prepared 2.5 per cent solution of sodium deoxycholate in 0.2 M glycylglycine buffer, pH 8.0. An

We wish to thank Dr. Mahlon B. Hoagland for determining the amino acid activation by pH 5 enzymes of the tumor.
amount of deoxycholate approximately equal to the weight of microsome protein was used. After a few minutes, the tube was filled with ice-cold distilled water and the suspension centrifuged for 2 hours at 105,000 × g. Then the supernatant fluid containing the deoxycholate-soluble fraction of the microsomes was aspirated carefully to leave the loosely packed “deoxycholate-insoluble particles” behind (3).

Isolation of “NaCl-Insoluble Particles” from Tumor Microsomes—The microsome pellets were homogenized in an ice-cold medium containing 0.5 mM NaCl, 0.005 mM MgCl₂, and 0.01 mM Tris buffer, pH 7.6. After standing 10 minutes, the suspension was centrifuged for 2 hours at 105,000 × g. The supernatant fluid was discarded, and the well packed, nearly colorless pellet of “NaCl-insoluble particles” was rinsed with ice-cold distilled water and suspended in Medium A as above.

To reduce the centrifugation time, this procedure was abbreviated as follows. Concentrated solutions were added to the water lysate of the tumor cells to give a final concentration of 0.5 mM NaCl, 0.005 mM MgCl₂, and 0.01 mM Tris buffer, pH 7.6. After standing 10 minutes, the gelatinous material, including nuclei and whole cells, was removed by centrifugation at 15,000 × g for 10 minutes. The supernatant fluid was centrifuged at 105,000 × g for 2 hours to give a pellet of NaCl-insoluble particles similar to that described above. The supernatant fluid containing the soluble cell fraction in 0.5 mM NaCl was discarded, as it was not found possible to obtain satisfactory pH 5 enzymes from it.

Incorporation of C¹⁴-Amino Acids into Proteins of Whole Tumor Cells—Warm ascitic fluid was collected from nine to twelve mice. Enough 1 mM Tris buffer, pH 7.6, and 1 mM glucose were added to give a final concentration of 0.02 mM Tris buffer and 0.04 mM glucose, and the mixture was shaken in air in a large Erlenmeyer flask for 1 minute at 37° for temperature equilibration. 0.11 volume of 0.05 to 0.10 mM L-leucine-C¹⁴ or 0.05 to 0.10 mM L-valine-C¹⁴ was added rapidly and the incubation continued. Aliquots were removed at intervals. The period of incorporation was regarded as the time from the addition of the C¹⁴-amino acid to the time the tube containing the aliquot entered an ice bath. The tumor cells in each aliquot were washed and lysed, a sample was taken for analysis of whole cell protein, and the remainder fractionated to give deoxycholate-insoluble particles, the deoxycholate-soluble fraction of the microsomes, and pH 5 enzymes.

Incorporation of C¹⁴-Amino Acids into Proteins of Cell-Free Systems from Tumor—The 1.0 ml. incubation mixture contained a suspension of microsomes or NaCl-insoluble particles, plus pH 5 enzymes, C¹⁴-amino acid, ATP, GTP, phosphoenol pyruvate (PEP), and pyruvate kinase in the amounts shown in Tables II to IV. After incubation for 15 minutes at
37° in air, the reaction was stopped by addition of 10 ml. of cold 0.5 N perchloric acid and the precipitate treated as described below.

**Analyses**—Samples were precipitated and washed with cold 0.5 N perchloric acid, extracted twice with a mixture of alcohol, ether, and chloroform (2:2:1), and treated with 1 N NaOH for 1 hour at room temperature. The suspension was acidified with 6 N HCl, centrifuged, and the supernatant fluid saved for RNA analysis. The precipitate was extracted with 0.5 N perchloric acid at 70° for 15 minutes to remove deoxyribonucleic acid (DNA) in the case of whole cell material. The protein was then washed once with cold 0.5 N perchloric acid, twice with acetone, and dried at 110° for 5 hours in the tared centrifuge tubes. After the protein dry weight was obtained, the protein was ground, plated, and assayed for C¹⁴ (23). Six samples of dried tumor protein prepared in this way were analyzed by the Kjeldahl method and found to contain 14.9 per cent nitrogen. Representative dried protein samples from whole cell and cell-free incorporation experiments were analyzed for adsorbed radioactivity by the ninhydrin-CO₂ method, with inert alanine added as carrier (24). No C¹⁴ appeared in the evolved CO₂.

RNA was determined from the absorbance at 260 mμ of the acidified NaOH extract by using a Beckman model DU spectrophotometer (25). An extinction coefficient of 36.0 per mg. per ml. per cm. for Ehrlich ascites tumor RNA was used. This coefficient was obtained by analyzing the extracts from four tumors by the orcinol method (26) and for absorbance at 260 mμ. The standard for the orcinol method was a sample of yeast RNA of known phosphorus content. DNA was determined on the hot 0.5 N perchloric acid extract by the diphenylamine reaction (27).

The protein concentration of the solution of pH 5 enzymes was determined by the turbidimetric method (19).

**RESULTS AND DISCUSSION**

**Isolation and Characterization of Ribonucleoprotein Particles**—When the washed Ehrlich ascites tumor cells were suspended in 10 volumes of ice-cold distilled water for 5 minutes, microscopic examination showed lysis of most of the cells and the appearance of free nuclei. When the lysate was centrifuged for 10 minutes at 15,000 X g after the addition of sucrose to a concentration of 0.25 M, neither whole cells nor DNA was present in the supernatant fluid. Approximately 45 per cent of the RNA and 39 per cent of the protein of the whole cells remained in this supernatant fluid (Table I). If 90 per cent of the RNA in these tumor cells is in the cytoplasm (13), then one-half of this cytoplasmic RNA has been extracted by the lysis procedure. More protein but no more RNA was extracted by a longer period of lysis. The incorporation of C¹⁴-amino acids into protein
by the cell-free extract was neither increased nor decreased by longer lysis. The extraction of RNA and protein was reduced if less than 10 volumes of water was used.

When the $15,000 \times g$ supernatant fluid was centrifuged at $105,000 \times g$ for 2 hours, 36 per cent of the RNA and 7.6 per cent of the protein of the whole cells were found in the microsome pellet. This tumor microsome fraction contained 32 per cent RNA in the RNA plus protein, in contrast with liver microsomes which contained 11 per cent RNA (3). The high RNA to protein ratio indicates the relatively low content of membranous material in the tumor microsomes. Centrifugation for only 1 hour at $105,000 \times g$ or at a lower speed gave pellets with less total RNA but with the same RNA to protein ratio.

The tumor microsomes were fractionated further by treatment with either deoxycholate or $0.5 \ M \ NaCl$, followed by centrifugation again at $105,000 \times g$ for 2 hours. The pellet of deoxycholate-insoluble particles or NaCl-insoluble particles contained about 33 per cent of the RNA and 3 per cent of the protein of the whole cells. The per cent RNA was 50 to 54 in these ribonucleoprotein particles. Deoxycholate usually gave the higher values in this range.

The NaCl-insoluble particles were faintly colored, whereas the tumor microsomes were pink. The absorption spectrum in the visible range of tumor microsomes made soluble with deoxycholate, and of the NaCl-soluble material of tumor microsomes, resembled that of the cytochrome $b_1$ of liver microsomes (9) both before and after reduction with dithionite. The NaCl-insoluble particles showed no absorption peaks in the visible range.

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**Table I**

<table>
<thead>
<tr>
<th>Recovery of RNA and Protein in Tumor Cell Fractions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Per cent recovery</td>
</tr>
<tr>
<td>-------------------</td>
</tr>
<tr>
<td>Whole cells</td>
</tr>
<tr>
<td>$15,000 \times g$ supernatant fluid</td>
</tr>
<tr>
<td>Microsomes</td>
</tr>
<tr>
<td>Deoxycholate-insoluble particles</td>
</tr>
<tr>
<td>NaCl-insoluble particles</td>
</tr>
</tbody>
</table>

The values are average values from several experiments.

* See the text, footnote 4.

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* Per cent RNA in RNA plus protein in this paper means per cent RNA by weight in the combined RNA plus protein. Lipide, glycogen, salts, etc., that may be present in the preparation are not considered.
range. It appears that cytochrome b₁, or a similar cytochrome, exists in the tumor microsomes, and that it is made soluble by 0.5 M NaCl, just as it is by deoxycholate in the case of liver microsomes.

The tumor microsomes, deoxycholate-insoluble, and NaCl-insoluble particles were compared in the analytical ultracentrifuge (Fig. 1). The tumor microsomes showed a prominent B peak (50 S) and C peak (43 S), in agreement with the results reported by Petermann on this and other tumors (5). There was a small amount of more rapidly sedimenting inhomogeneous material apparent as a shoulder on the B peak in the 5 minute microsome pattern. The small size of this shoulder again indicates the low proportion of membranous component in the tumor microsomes. In the 5 minute patterns of the deoxycholate- and NaCl-insoluble particles (not seen in Fig. 1), this shoulder was absent.

The deoxycholate-insoluble particles showed three major peaks with sedimentation rates of 57, 54, and 50 S. Thus, deoxycholate has caused

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**Dr. Karl Schmid kindly performed the ultracentrifugal analyses.**
the separation of a third major peak in this range. The NaCl-insoluble particles also showed three major peaks, since the one with the sedimentation rate of 21 S (Fig. 1) separated into two peaks before reaching the bottom of the cell. The sedimentation rates of these NaCl-insoluble particles are only about one-half the values above. NaCl may have altered the size or shape of the particles. It seems unlikely that these low rates are due to extraction of protein, since the RNA to protein ratio is no higher than in the deoxycholate-insoluble particles. Furthermore, the NaCl-insoluble particles had the same specific activity after incorporation of labeled amino acids in whole tumor cells as the deoxycholate-insoluble particles, and were active in the cell-free incorporation system (see below).

Electron micrographs were taken of unstained, unshadowed preparations of tumor microsomes and particles (3). The deoxycholate-insoluble particles appeared quite similar in density, size, and uniformity to those from liver. The NaCl-insoluble particles varied in electron density but were otherwise similar. Tumor microsomes contained, in addition to many free particles, poorly defined, less dense material which did not take the form of flattened vesicles as in liver microsomes.

Incorporation of C\textsuperscript{14}-Amino Acids into Proteins of Whole Tumor Cells—Incorporation of C\textsuperscript{14}-amino acids into tumor cell proteins occurred at a rapid rate when whole ascitic fluid was fortified with glucose and buffer and incubated in air with the C\textsuperscript{14}-amino acid immediately after the fluid was obtained from tumor-bearing mice. An average rate of 4 per cent labeling of whole cell protein-leucine per hour\footnote{Per cent labeling of protein-leucine = counts per minute per mg. of protein X 100 divided by counts per minute per mg. of added leucine-C\textsuperscript{14} X 0.1 mg. of leucine per mg. of protein. This calculation assumes that the proteins concerned contained 10 per cent leucine (28). Per cent labeling of protein-valine was calculated in the same way by assuming 5 per cent valine in the protein.} (33 \textmu moles of leucine per gm. of protein per hour), calculated from the initial 10 minute incorporation, was obtained with tumor cells 7 days after transplantation, and by using a saturation level of L-leucine-C\textsuperscript{14} (10 \textmu moles per ml.). Older tumors gave slower rates. Such incorporation is more than enough to account for the rate of division of these cells \textit{in vivo} (29). It is several times higher than any incorporation so far reported for normal tissues of higher organisms (30), and than the values obtained with Ehrlich ascites tumor cells which had been washed and suspended in a saline medium (15, 16). Warburg and Hiepler have reported that ascites tumor cells show the highest metabolic rates when incubated in their own ascitic fluid (31).

Aliquots of the whole ascitic fluid were removed at several times during the incubation, and the tumor cells of each aliquot fractionated (Fig. 2). The incorporation into the ribonucleoprotein particles (deoxycholate-in-
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soluble) was much faster than that into the other cell fractions during the first 3 minutes. Thereafter, incorporation into the particles did not increase, as if a steady state had been reached as in liver. Incorporation into the other cell proteins continued at a nearly linear rate for the duration of the experiment. Identical results were obtained when L-leucine-C14 was used instead of L-valine-C14, and when the ribonucleoprotein particles were isolated by the use of NaCl instead of deoxycholate. Thus, when in one experiment NaCl-insoluble particles were isolated from one-half the cells and deoxycholate-insoluble particles from the other half, both preparations showed the same specific activity.

The soluble cell proteins not precipitated at pH 5 have been found, in other experiments, to have a lower rate of incorporation than whole cell protein. Nuclear and mitochondrial cell fractions have not been studied in this work. An independent synthesis of protein within the nucleus has been indicated by the recent experiments of Allfrey, Mirsky, and Osawa (32).

The maximal per cent labeling of the tumor ribonucleoprotein particles...
is similar to that obtained with liver particles in vivo (3). In the experiment on 11 day-old tumor cells (Fig. 2), 0.73 per cent of the ribonucleoprotein valine was labeled when the steady state was reached. In an experiment with leucine-$^{14}$C on 7 day-old cells, 1.3 per cent of the ribonucleoprotein-leucine was labeled in the steady state.

That the steady state in the whole cells is due to the release of labeled polypeptide chains rather than to the release of labeled amino acids by a reversal of incorporation is suggested by the following experiment. Labeled tumor microsomes, obtained from whole tumor cells incubated in ascitic fluid for 5 minutes with L-leucine-$^{14}$C, were then incubated for 15 minutes with non-radioactive L-leucine (10 μmoles per ml.) and seventeen other L-amino acids (each 1 μmole per ml.), plus ATP, GTP, PEP, pyruvate kinase, and pH 5 enzymes at the concentrations used for cell-free incorporation. The protein after incubation had the same specific activity as before, showing that the incorporation into the particles is not reversible under the conditions of active cell-free incorporation.

These experiments in vitro with ascites tumor cells provide an opportunity to compare the initial incorporation into ribonucleoprotein particles and into whole cell proteins. In the experiment in Fig. 3, the tumor cells contained 8.3 per cent RNA or 9.1 mg. of RNA per 100 mg. of whole cell proteins. According to Goldberg, Klein, and Klein (13) 90 per cent of the RNA in the ascites tumor cell is cytoplasmic, or in this case 8.2 mg. of cytoplasmic RNA per 100 mg. of whole cell proteins. If this was all in ribonucleoprotein particles which contain equal amounts of RNA and protein, 8.2 per cent of the whole cell proteins was in the particles. In Fig. 3, the observed initial rate of incorporation into the particles (from 1 to 2 minutes) was 9 times as rapid as the average rate of incorporation into whole cell proteins throughout the experiment. If all the $^{14}$C-amino acid incorporated into whole cell proteins had to pass through the particles, the initial rate of labeling of particles in Fig. 3 should have been about 12 times, i.e., 100 divided by 8.2, the rate of labeling of whole cell proteins. The observed results are consistent with the concept that most of the amino acids incorporated into whole cell proteins pass through the ribonucleoprotein particles. Even more of the total incorporation could occur by this pathway if labeled protein is released from the ribonucleoprotein particles before the steady state is reached.

Cell-Free Incorporation of $^{14}$C-Amino Acids by Tumor Microsome-pH 5 Enzyme System—A cell-free system which incorporates $^{14}$C-amino acids into protein can be prepared from the Ehrlich ascites tumor (Table II). This system is quite similar to that from liver (18), and the microsomes or pH 5 enzymes from the tumor are interchangeable with the corresponding fraction from liver without loss of activity. Microsomes and soluble cell
fractions from butter-yellow hepatomas have also been found to be inter-
changeable with those from liver (unpublished experiments of P. C. Za-
mecknik). It seems likely that there is an identical mechanism for amino
acid activation by soluble enzymes in these tissues.

The incorporation in this tumor system depended on the addition of
PEP, but it was not necessary to add pyruvate kinase (Table II). Con-
siderable activity occurred in the absence of added GTP, perhaps because
of residual GTP or GDP in the cell fractions used.

Various substances were added to this system in an effort to increase the
activity. No stimulation was obtained by adding a crude mixture of
nucleotides from yeast or a mixture of seventeen L-amino acids (each 0.1
µmole per ml.) in addition to the one C\textsuperscript{14}-amino acid. Substitution of the
whole soluble cell fraction of the tumor, concentrated by lyophilization, or
the whole soluble cell fraction of liver for the pH 5 enzymes of tumor did
not increase the activity.

Cell-Free Incorporation of C\textsuperscript{14}-Amino Acids by Tumor Particle-pH 5 En-
zyme System—When the tumor NaCl-insoluble particles were substitut-
ed for the tumor microsomes in the cell-free system, active incorporation into
protein occurred (Table III). Thus, the membranous component of the
microsomes is not essential for incorporation. Approximately equal
amounts of protein of pH 5 enzymes and particles gave the highest activity.
This system differed from the previous cell-free systems in the low ac-
tivity in the absence of added pH 5 enzymes. (It is therefore the most
appropriate system for any study of the soluble enzymes required for in-

\begin{table}[h]
\centering
\begin{tabular}{|c|c|}
\hline
Requirements & C.p.m. per mg. protein \\
\hline
Complete system & 120, 124 \\
\hline
\hline
\" minus microsomes & 3 \\
\hline
\hline
\" pH 5 enzymes & 36 \\
\hline
\hline
\" pyruvate kinase & 109 \\
\hline
\hline
\" PEP & 5 \\
\hline
\hline
\" ATP & 28 \\
\hline
\hline
\" GTP & 65 \\
\hline
\end{tabular}
\caption{Requirements for Incorporation of L-Leucine-C\textsuperscript{14} into Protein in Tumor Microsome-pH 5 Enzyme System}
\end{table}

The complete system contained microsomes (3.0 mg. of protein), 4.4 mg. of pH 5
enzymes, 0.05 mg. of pyruvate kinase, 10 µmoles of PEP, 1.0 µmole of ATP, 0.25
µmole of GTP, and 0.1 µmole of L-leucine-C\textsuperscript{14} (4.9 \times 10^6 c.p.m. per mg.), in a final
volume of 0.95 ml. Incubated 15 minutes at 37° in air.

* Added after incubation to give the same amount of protein in all the flasks for
C\textsuperscript{14} assay.
corporation.) Furthermore, an ATP-generating system was not required, as full activity was obtained with 10 μmoles of ATP per ml. (Fig. 4). This may be due to the low adenosinetriphosphatase activity in the tumor particles and pH 5 enzymes. When a suspension of NaCl-insoluble particles was incubated with 10 μmoles of ATP per ml. for 15 minutes at 37°, the phosphate liberated was one-half that liberated by tumor microsomes containing an equal amount of RNA. There was also apparently less adenosinetriphosphatase activity in the pH 5 enzymes from the tumor than in those from liver, since, when the latter was combined with tumor particles, the incorporation was active only in the presence of an ATP-generating system.

The tumor particle-pH 5 enzyme system was saturated by a very low concentration of GTP, 0.05 μmole per ml. (Fig. 5). This was demonstrated only after treating the tumor cell lysate with Dowex 1-X8 acetate to reduce the nucleotides in the preparation. Just as in the liver system (18), ATP was required, even when a large amount of GTP was added. 10 μmoles of GTP per ml. in the absence of ATP gave only 22 per cent of full activity. The combination of 10 μmoles of GTP per ml. and 1 μmole of

### Table III

**Requirements for Incorporation of L-Leucine-C\(^{14}\) into Protein in Tumor Particle-pH 5 Enzyme System**

The values are given in counts per minute per mg. of protein.

<table>
<thead>
<tr>
<th>Requirement</th>
<th>ATP-generating system</th>
<th>ATP system</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete system</td>
<td>112</td>
<td>120</td>
</tr>
<tr>
<td>&quot; &quot; treated with Dowex 1</td>
<td>89</td>
<td>90</td>
</tr>
<tr>
<td>&quot; &quot; 1 minus particles*</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>&quot; pH 5 enzymes*</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>&quot; &quot; GTP</td>
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<td></td>
</tr>
<tr>
<td>&quot; &quot; ATP</td>
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</tbody>
</table>

The complete system contained, in a final volume of 1.0 ml., NaCl-insoluble particles, pH 5 enzymes, 0.1 μmole of L-leucine-C\(^{14}\) (4.9 × 10\(^6\) c.p.m. per mg.), and either the ATP-generating system (10 μmoles of PEP, 0.1 mg. of pyruvate kinase, 1.0 μmole of ATP, and 0.25 μmole of GTP) or the ATP system (10 μmoles of ATP and 0.1 μmole of GTP). Incubated 15 minutes at 37° in air. For the Dowex 1-X8 acetate treatment, 90 ml. of the 15,000 X g supernatant fluid of a water lysate were treated with 15 gm. of Dowex 1-X8 acetate for 3 minutes at 4°. After removal of the resin by filtration, NaCl-insoluble particles were prepared from one-half of the filtrate, and pH 5 enzymes from the other.

* Added after incubation, to give the same amount of protein in all the flasks for C\(^{14}\) assay.
ATP per ml. gave only 52 per cent of full activity. GDP was nearly as active as GTP at low concentrations (Fig. 5).

The addition of cytidine triphosphate and uridine triphosphate (each 0.25 μmole per ml.) to the ATP and GTP did not increase the activity. There was no stimulation from the addition of 0.5 μmole per ml. of the diphosphates of adenosine, guanosine, cytidine, and uridine, although these compounds may be precursors of RNA (33). There was no stimulation from a mixture of seventeen L-amino acids (each 0.2 μmole per ml.) in addition to the one C14-amino acid. Substitution of the whole soluble fraction of liver for the pH 5 enzymes of tumor did not increase the activity. This suggests that the tumor system is not deficient in any of the cofactors, nucleotides, or amino acids which are present in relatively high concentration in the liver-soluble fraction.

Valine-C14, isoleucine-C14, alanine-C14, and phenylalanine-C14 were each incorporated at a rate comparable to that of leucine-C14. When DL-leucine-C14 at 0.25 μmole per ml. was used, the incorporation was reduced 97 per cent by the addition of 10 μmoles of non-isotopic L-leucine per ml., indicating the specificity of this system for the L isomer.

The activity of the system was inhibited 28 per cent by the addition of...
0.1 γ of crystalline ribonuclease per ml. to the incubation mixture, and 100 per cent by 1 γ per ml. On the other hand, the labeling of proteins in whole tumor cells described above was not affected by the addition of 200 γ of ribonuclease per ml. to the whole ascitic fluid.

**Table IV**

*Fractionation after Incorporation of Leucine-C\(^{14}\) into Protein in Liver and Tumor Cell-Free Systems*

<table>
<thead>
<tr>
<th>Cell-free system</th>
<th>Fractions isolated after incubation</th>
<th>Protein</th>
<th>RNA</th>
<th>Per cent RNA in RNA + protein*</th>
<th>Protein</th>
<th>Per cent labeling of ribonuclease-protein-leucine†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver microsomes-soluble proteins (3)</td>
<td>Microsomes, deoxycholate-insoluble</td>
<td>5</td>
<td>3.8</td>
<td>44</td>
<td>945</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Microsomes, deoxycholate-soluble</td>
<td>30</td>
<td>1.0</td>
<td>3</td>
<td>708</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>Soluble proteins</td>
<td>145</td>
<td>3.7</td>
<td>3</td>
<td>910</td>
<td>0</td>
</tr>
<tr>
<td>Liver microsomes-soluble proteins (3)</td>
<td>Microsomes, deoxycholate-insoluble</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor microsomes-pH 5 enzymes</td>
<td>Microsomes, deoxycholate-insoluble</td>
<td>10</td>
<td>8.3</td>
<td>45</td>
<td>3300</td>
<td>340</td>
</tr>
<tr>
<td></td>
<td>Microsomes, deoxycholate-soluble</td>
<td>7</td>
<td>0.5</td>
<td>7</td>
<td>450</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>pH 5 enzymes</td>
<td>33</td>
<td>1.0</td>
<td>3</td>
<td>1850</td>
<td>56</td>
</tr>
<tr>
<td>Tumor particles-pH 5 enzymes</td>
<td>NaCl-insoluble particles</td>
<td>9</td>
<td>7.4</td>
<td>45</td>
<td>1960</td>
<td>220</td>
</tr>
<tr>
<td></td>
<td>pH 5 enzymes</td>
<td>11</td>
<td>0.9</td>
<td>8</td>
<td>455</td>
<td>41</td>
</tr>
</tbody>
</table>

*See the text, footnote 4.
†See the text, footnote 7.

The distribution of labeled protein in cell fractions separated from this system after incubation is given in Table IV. For comparison, the tumor microsome-pH 5 enzyme system and the liver system (3) are included. The particles showed the highest specific activity in each case. Furthermore, they contained over 80 per cent of the labeled protein in the tumor particle-pH 5 enzyme system.
The incorporation in the tumor cell-free system, as in that from liver, was most rapid during the first 10 minutes of incubation at 37°, but some activity continued beyond this time (Fig. 6). If fresh particles were added to the system after 15 minutes of incubation, there was additional incorporation, suggesting that it is the particles which rapidly become inactive at 37°. In this regard Petermann has reported that the ribonucleoprotein particles, particularly Component C, are unstable on dialysis or standing at 4° (5), and that liver contains a dialyzable factor which tends to stabilize the particles (11). We have compared the ultracentrifugal patterns of tumor microsomes and NaCl-insoluble particles, each incubated 15 minutes at 37°, and non-incubated controls. Incubation reduced the size of Component C of the microsomes, and almost abolished the more slowly sedimenting peak of the NaCl-insoluble particles, which may correspond to Component C. For these reasons we feel that the slowing of incorporation in the cell-free system after 10 minutes of incubation at 37° is due to changes in the particles during this time.

The instability of the particles may also account for differences in percent labeling of ribonucleoprotein particles in the liver and tumor cell-free systems. The last column of Table IV shows that the percent labeling of ribonucleoprotein-leucine was greater in the liver system than in either tumor system. We have been unable to obtain more rapid or prolonged incorporation in the tumor systems by various modifications of the fractionation technique or additions to the incubation mixture. Since the liver cell-free system contains more of the membranous component of the microsomes and is more active, this membranous component may contribute, in some way, to the incorporation into the particles under cell-free conditions. Yet whole tumor cells, which contain relatively small amounts

Fig. 6. Time curve of incorporation of L-leucine-C\(^{14}\) into protein by tumor particle-pH 5 enzyme system. Each tube contained NaCl-insoluble particles (3.4 mg. of protein), 2.1 mg. of pH 5 enzymes, 10 \(\mu\)moles of ATP, 0.25 \(\mu\)mole of GTP, and 0.1 \(\mu\)mole of L-leucine-C\(^{14}\) (4.9 \(\times\) 10\(^4\) c.p.m. per mg.), in a final volume of 1.0 ml. Incubated at 37° in air for the periods indicated.
of membranous component (14), incorporate C\textsuperscript{14}-amino acids into protein at a higher rate than liver \textit{in vivo}. We are inclined to attribute the lower activity of the tumor cell-free systems to the different cell fractionation techniques used for liver and the tumor, and to the instability of the particles, especially Component C. Clarification of the mechanisms of amino acid incorporation into and protein release from the ribonucleoprotein particles may well require better conditions for their preparation and maintenance. Bacteria (34), yeast (35), or plants (36) may be a preferable source of particles for such studies.

**SUMMARY**

A cell-free system which incorporates C\textsuperscript{14}-amino acids into protein can be prepared from the Ehrlich mouse ascites tumor. The cell fractions required are ribonucleoprotein particles, containing equal amounts of ribonucleic acid (RNA) and protein, which can be isolated from the microsomes of the tumor by the use of 0.5 M NaCl, plus enzymes precipitated at pH 5 from the soluble proteins of the tumor cell. The soluble enzymes catalyze the carboxyl activation of free amino acids, while the ribonucleoprotein particles are the locus of over 80 per cent of the total incorporation. The energy requirement for the incorporation is provided by 10 \mu moles of adenosine triphosphate (ATP) per ml. Guanosine triphosphate or di-phosphate, 0.1 \mu mole per ml., and the C\textsuperscript{14}-amino acid complete the system.

This cell-free amino acid incorporation system represents a simplification of that previously described from liver. Active incorporation into ribonucleoprotein occurs without the membranous component of the microsomes. It is entirely dependent on the addition of soluble enzymes. An ATP-generating system is not required.

When whole tumor cells are incubated in ascitic fluid with a C\textsuperscript{14}-amino acid, the initial incorporation into whole cell protein is at a rate, e.g. 33 \mu moles of leucine per gm. of protein per hour, which is more than adequate to account for the known rate of division of such cells \textit{in vivo}. The ribonucleoprotein particles, which are estimated to contain 8 to 9 per cent of the whole cell proteins, are labeled up to 9 times more rapidly than whole cell proteins. This is consistent with the concept that most of the amino acids incorporated into whole cell proteins pass through the ribonucleoprotein particles.

We wish to thank Dr. Joseph C. Aub for all he has done to make this work possible, Dr. Paul C. Zamecnik for most valuable advice and interest throughout, Dr. Robert B. Loftfield for the C\textsuperscript{14}-amino acids and helpful discussions, Dr. Jesse F. Scott for advice on the RNA analyses, Mrs. Meredith A. Hannon for technical assistance, and Mr. Fred E. Mapplebeck for transplantations of the tumor and care of the animals.
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