considerable part of it, is not important in determining the physicochemical properties of hyaluronic acid in solution. Although it remains true that no sample free from protein appears to have been isolated with so high an intrinsic viscosity as that of the complex it seems likely that this is because methods powerful enough to remove all the protein can also cause depolymerization of the polysaccharide.

SUMMARY

1. Ethylenediaminetetra-acetic acid aids the degradation of hyaluronic acid complex under various conditions.

2. Papain has a proteolytic activity on the complex but appears, as a reducing agent, to cause more general degradation as well.

3. Trypsin and chymotrypsin together can remove 65% of the protein from the complex without any loss of viscosity by the complex.

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Factors Influencing the Incorporation of Amino Acid into the Protein of Microsome and Mitochondria Preparations of Rat Liver and Liver Tumour

BY OLGA GREENGARD AND P. N. CAMPBELL

Courtauld Institute of Biochemistry, Middlesex Hospital Medical School, London, W. 1

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The requirement of the soluble cytoplasm (cell sap) for the incorporation of amino acids into the protein of isolated liver microsomes has been demonstrated by Zamecnik & Keller (1954). Both for microsomes derived from rat liver and mouse Ehrlich ascites tumour cells, the active components of the cell sap are precipitated when the pH is adjusted to 5.2 (pH 5 fraction) (Keller & Zamecnik, 1956; Littlefield & Keller, 1957). It has previously been observed that microsome preparations from rat-liver tumours are considerably less active for amino acid incorporation than are similar preparations from normal liver (Campbell & Greengard, 1959). Further, it appeared that not only

were the microsomes deficient in this respect, but that the cell sap contributed to this difference. The extent to which the pH 5 fraction from the two tissues reflects the differences found with the whole cell sap has now been investigated.

For amino acid incorporation into isolated mitochondria, the role of the cell sap seems to differ from that with microsomes. In the presence of a suitable source of energy, there is no absolute requirement for cell sap (McLean, Cohn, Brandt & Simpson, 1958; Greengard, 1959), although its inclusion in the reaction mixture enhances the incorporation of amino acids. Other factors influencing the extent of incorporation of amino

acids into mitochondrial protein have now been studied. It is shown that the properties of tumour mitochondria are qualitatively similar to those derived from normal liver.

MATERIALS AND METHODS

Chemicals. Adenosine triphosphate (dipotassium salt; ATP) and guanosine triphosphate (sodium salt; GTP) were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. Solutions of these substances were brought to pH 7.3 (indicator papers) with KOH before use. The barium-silver salt of phosphoenolpyruvic acid was prepared by the method of Ohlmeyer (1951), and the barium salt of fructose 1:6-diphosphoric acid was obtained from C. F. Boehringer und Soehne, Mannheim, Germany. Solutions of the free acids were prepared shortly before use and adjusted to pH 7.3 (indicator papers) with KOH. Crystalline ribonuclease was obtained from Armour and Co. Ltd., Eastbourne, Sussex and from Worthington Biochemical Corp., New Jersey, U.S.A. Pyruvic kinase was that of C. F. Boehringer und Soehne, Mannheim.

Radioactive amino acids. Uniformly labelled L-[¹⁴C]-leucine (100 μ C = 1.49 mg.) and L-[¹⁴C]lysine (100 μ C = 1.13 mg.) were obtained from The Radiochemical Centre, Amersham, Bucks.

Tissues. (1) Normal liver. This was obtained from rats (150 g. body wt.) fed on stock diet. (2) Liver tumours. Tumours were induced in rats by feeding them on a diet containing 0.06% of 4-dimethylaminoazobenzene as described by Campbell (1955). All rats bearing tumours were kept on a normal diet free of the carcinogen for at least 4 weeks before use.

Tissue preparations. (1) Microsomes. The method used followed closely that described by Zamecnik & Keller (1954) and was exactly as described previously (Campbell & Greengard, 1959). (2) Mitochondria. The method was based on that of Schneider & Hogeboom (1950) and was the same as that previously described (Campbell & Greengard, 1959), except that the mitochondria were washed twice instead of once. (3) pH 5 fraction. The cell sap (105 000 g for 50 min. supernatant) was brought to pH 4.9-5.1 (glass electrode) by the addition of *m*-acetic acid. The precipitate was centrifuged down, washed with 0.25 *M*-sucrose and resuspended in a volume of medium equivalent to one-tenth of the original volume of cell sap. The pH 5 fraction prepared thus had a protein content of about 1.5% (w/v).

Method of incubation. Microsomes or mitochondria (7-10 mg. of protein in each tube) were incubated in a total

volume of 1 ml. at 37° for 50 min. under a continuous stream of N₂ + CO₂ (95:5) or O₂ + CO₂ (95:5), with 0.5 μ C of ¹⁴C-labelled amino acid, together with a suitable substrate as explained in the legends to the Tables. When studying the incorporation into the pH 5 fraction the preparation was incubated for 10 min. in air, with or without ATP.

Isolation of protein from the particulate fractions. After incubation, the microsomes or mitochondria were separated from the soluble constituents by centrifuging. The proteins were precipitated in aqueous 5% (w/v) trichloroacetic acid and were then treated with hot trichloroacetic acid and organic solvents according to a modified Schneider procedure (Zamecnik, Loftfield, Stephenson & Steele, 1951).

Treatment of the pH 5 fraction and estimation of ribonucleic acid. The pH 5 fraction was treated after incubation with 5 vol. of 0.4 *N*-perchloric acid and the precipitate washed with cold perchloric acid and organic solvents as described by Hoagland, Stephenson, Scott, Hecht & Zamecnik (1958). After determination of the specific radioactivity of the washed and dried pH 5 fraction, the ribonucleic acid (RNA) was extracted by heating for 45 min. in 5% (w/v) trichloroacetic acid and its quantity estimated by the orcinol method of Meijbaum (1939). The protein residue from the trichloroacetic acid extraction was washed with organic solvents, dried and weighed and its specific radioactivity measured.

Measurement of radioactivity. The material was compressed on a 0.28 cm.² stainless-steel disk with a stainless-steel pestle, as described by Busch, Simbonis, Anderson & Greene (1956), and counted at infinite thickness in a Tracerlab SC-16 windowless flow counter. The accuracy of counting was as described previously (Campbell & Greengard, 1959).

RESULTS

Incorporation of [¹⁴C]leucine into the microsomal protein of liver and liver tumour

In the presence of a source of energy-rich phosphate, microsomes of liver and liver tumour incorporate amino acid when the pH 5 fraction of either of these tissues is present in the incubation mixture. In Table 1 the effectiveness of the pH 5 fraction derived from the two hepatic tissues is compared. It can be seen that the results are not influenced by increasing the quantity of pH 5 fraction present. The highest extent of incorporation of leucine occurs when liver microsomes are incubated with liver pH 5 fraction. Neither the

Table 1. *Incorporation of [¹⁴C]leucine into microsomal protein of liver and liver-tumour preparations*

Microsomes and pH 5 fraction (in quantities given under Methods) were incubated under N₂ + CO₂ (95:5) for 50 min. with 2 μ moles of ATP, 10 μ moles of phosphoenolpyruvate (PEP), PEP-kinase, 0.25 μ mole of GTP and 0.5 μ C of [¹⁴C]-leucine. The microsomes were collected by centrifuging after incubation. Radioactivity is expressed as counts/min. at infinite thickness/0.28 cm.² in a windowless flow counter. Control values, obtained by incubating the same preparations in the absence of PEP and ATP, were less than 10% of the experimental results.

Origin of pH 5 fraction	Liver microsomes				Tumour microsomes			
	Liver		Tumour		Liver		Tumour	
Amount of pH 5 fraction (ml.)	0.3	0.6	0.3	0.6	0.3	0.6	0.3	0.6
Radioactivity of microsomal protein	432	428	74	80	86	76	98	99

Table 2. *Incorporation of amino acid into the pH 5 ribonucleic acid of liver and liver tumour*

The pH 5 fraction (0.7 ml.) was incubated in air for 10 min. at 37° and was then treated with perchloric acid and organic solvents as described under Methods. Radioactivity is expressed as in Table 1.

Amino acid	Radioactivity			
	Liver pH 5 fraction		Liver-tumour pH 5 fraction	
	Control	Plus ATP (5 μ moles)	Control	Plus ATP (5 μ moles)
[¹⁴ C]Leucine	2	719	10	460
[¹⁴ C]Lysine	38	344	63	132

Table 3. *Incorporation of [¹⁴C]leucine into and the ribonucleic acid/protein content of pH 5 fractions from liver and liver tumour*

The pH 5 fractions were incubated in air for 10 min. at 37° with 5 μ moles of ATP, then treated with perchloric acid (PCA). After treatment of the precipitate with organic solvents the radioactivity was determined and is expressed as in Table 1. The RNA was extracted from the precipitate with trichloroacetic acid and the amount estimated by the orcinol method of Mejbaum (1939). The radioactivity of the remaining protein was less than 10% of that before extraction.

Origin of pH 5 fraction	Expt. no.	Radioactivity	Amount of material incubated [PCA ppt. (mg.)]	RNA Protein $\times 100$
Liver	1	691	11.7	5.4
	2	976	10.7	6.5
Liver tumour	1	284	9.4	6.5
	2	408	11.3	6.4

combination of liver pH 5 fraction with tumour microsomes nor that of liver microsomes with tumour pH 5 fraction raises the extent of incorporation above that obtained with tumour constituents alone.

Incorporation of amino acid into the pH 5 ribonucleic acid of liver and liver tumour

It has been demonstrated that amino acids are incorporated into the RNA moiety of the pH 5 fraction (pH 5 RNA) before their transfer to microsomal protein (Hoagland *et al.* 1958). It was therefore of interest to determine the extent of incorporation of amino acids into the pH 5 RNA derived from the two different tissues. The results shown in Table 2 indicate that, in the presence of an ATP-generating system, incorporation into the extract from the tumour tissue does occur, although the extent of this incorporation is lower than that for the extract of normal tissue. This is so both for [¹⁴C]leucine and [¹⁴C]lysine. Since treatment of the perchloric acid precipitate with hot trichloroacetic acid in all cases reduced the radioactivity of the precipitates to that of the control, lacking ATP, it is concluded that the incorporation was associated with the pH 5 RNA. These results are in accord with those of Hoagland *et al.* (1958).

Provided that the ratio of the amount of material incubated (i.e. weight of perchloric acid precipitate) to the amount of radioactive leucine

was similar, two reasons for the lower extent of labelling of the tumour pH 5 fraction compared with that of normal liver may be considered: (i) a difference in the proportion of RNA to protein in the extracts; (ii) a lower catalytic activity of the extract. A further series of experiments were carried out in which the incorporation of [¹⁴C]leucine was studied and the RNA content of each extract determined. The RNA/protein ratio of the fractions prepared from different tumours showed considerable variation, but in the examples given in Table 3 it will be seen that the fractions of the two tissues were of similar composition. In spite of this a marked difference in the extent of the incorporation into the pH 5 RNA was found.

Incorporation of amino acid by mitochondria from liver and liver tumour

The mitochondria used in these studies were prepared in such a way as to minimize contamination by microsomes. In order to obtain a measure of such contamination the RNA content was estimated, and was found to be 1.5–2.0 mg./100 mg. of protein both in liver and liver-tumour mitochondria.

In the presence of a suitable ATP-generating system mitochondria from both liver and liver tumour incorporated amino acid into their proteins, as shown in Table 4. In both cases, the substrate-free control values were higher in the presence of O₂ in the gas phase and mitochondria

Table 4. *Incorporation of [^{14}C]leucine into liver and liver-tumour mitochondrial protein*

Mitochondria from liver or liver tumour were incubated with [^{14}C]leucine in the presence of cell sap from the same tissue for 50 min. at 37°. PEP refers to phosphoenolpyruvate and FDP to fructose diphosphate. The mitochondria were collected after incubation by centrifuging and the protein was precipitated with trichloroacetic acid. Radioactivity is expressed as in Table 1. Nicotinamide was added to inhibit the breakdown of diphosphopyridine nucleotide.

Additions	Gas phase	Radioactivity of protein	
		Liver	Liver tumour
None	$\text{N}_2 + \text{CO}_2$ (95:5)	20	15
10 μmoles of PEP + 2 μmoles of ATP		181	31
10 μmoles of FDP + 1 μmole of nicotinamide		132	39
None	$\text{O}_2 + \text{CO}_2$ (95:5)	44	33
10 μmoles of FDP + 1 μmole of nicotinamide		229	66

Table 5. *Effect of fractions of the soluble cytoplasm on the incorporation of [^{14}C]leucine into mitochondria from normal liver and liver tumour*

Mitochondria were incubated with [^{14}C]leucine at 37° in $\text{N}_2 + \text{CO}_2$ (95:5) for 50 min. The energy source was 10 μmoles of phosphoenolpyruvate (PEP) and 2 μmoles of ATP. Where the pH 5 fraction was used, PEP-kinase and 0.25 μmole of GTP were also included. In the absence of soluble fraction PEP-kinase was added. Radioactivity is expressed as in Table 1. Incorporation into cell sap alone is insignificant.

Energy source ...	Radioactivity of mitochondrial protein			
	Liver		Liver tumour	
	Absent	Present	Absent	Present
Soluble fraction present				
None	8	48	0	15
Liver whole cell sap	12	140	6	39
Tumour whole cell sap	11	153	9	43
Liver pH 5 fraction	6	88	—	—
Tumour pH 5 fraction	5	46	—	—

from both tissues exhibited the highest activity under aerobic conditions, with fructose diphosphate as substrate. The absolute values, however, were always lower in tumour preparations.

The results of the experiments on the effect of fractions of the cell sap on the incorporation of [^{14}C]leucine into hepatic mitochondria are summarized in Table 5. It can be seen that an energy-dependent incorporation of amino acid into the mitochondrial protein of both tissues occurred in the absence of cell sap, but the addition of cell sap to the incubation mixture stimulates this process. Moreover, the stimulating effect of tumour cell sap was at least as great as that of normal liver cell sap. The addition of the pH 5 fraction to liver mitochondria also results in an increased uptake of [^{14}C]leucine, but the pH 5 fraction of the tumour tissue is less active in this respect. The stimulating action of the pH 5 fraction was apparent only in the presence of GTP. It was observed, however, that the extent of incorporation which occurred in the absence of soluble fractions was not influenced by the presence of GTP.

Effect of ribonuclease on the amino acid incorporation into mitochondrial protein

Ribonuclease has been shown to inhibit the uptake of amino acids by microsomal preparations

Table 6. *Effect of ribonuclease on the incorporation of [^{14}C]leucine into liver mitochondria in the presence and absence of cell sap*

Mitochondria were incubated under $\text{N}_2 + \text{CO}_2$ (95:5) at 37° for 50 min. with [^{14}C]leucine, 10 μmoles of phosphoenolpyruvate (PEP) and 2 μmoles of ATP, in the presence and absence of cell sap in the presence of different concentrations of ribonuclease. The mitochondria were then collected by centrifuging. The radioactivity of the mitochondrial protein is expressed as in Table 1. The control values, obtained in the absence of the PEP and ATP, did not exceed 8 counts/min.

Concn. of ribonuclease (mg./ml.)	Cell sap absent	Cell sap present
None	29	70
0.1	40	28
0.25	29	26

(Zamecnik & Keller, 1954) but not that into mitochondrial preparations (McLean *et al.* 1958). The effect of this enzyme on the incorporation of amino acids in mitochondria when incubated in the presence and absence of cell sap has now been studied. The results of a typical experiment are shown in Table 6. It can be seen that the incorporation in the absence of cell sap is stimulated by the lower concentration of ribonuclease, but is

unaffected by the higher concentration. The incorporation of leucine into mitochondrial protein in the presence of cell sap, on the other hand, is reduced by both concentrations to the level obtained when cell sap is not included in the reaction mixture. Both in the presence and absence of cell sap the results obtained with 0.5 mg. of ribonuclease/ml. were the same as those with 0.25 mg./ml. Similar results were obtained with a different preparation of ribonuclease (Armour and Co. Ltd.).

DISCUSSION

Liver-tumour tissue is capable of a rapid rate of protein synthesis both *in vivo* and in the form of slices (Zamecnik *et al.* 1951; Campbell & Stone, 1957). The extent of the incorporation of amino acids into the microsomes of such slices is somewhat greater than that into the corresponding fraction of slices of normal liver (Campbell, Greengard & Jones, 1957). In spite of this, when microsomes were isolated from liver tumour and were incubated with radioactive amino acid and cell sap, the incorporation of radioactivity was much less than that in a similar system from normal liver. Efforts have been made to locate the reasons for this deficiency.

It was thought that the cell sap from the tumour might contain an inhibitor for the incorporation of amino acids into microsomes. Since the pH 5 fraction has been shown to replace the whole cell sap for incorporation of amino acids into normal liver microsomes, it was of interest to determine the effect of a similar fraction from tumour cell sap. However, the previously observed difference in the incorporation between the tumour and normal liver microsomes in the presence of whole cell sap was not altered by the substitution of pH 5 fraction.

On the basis that the ribonucleic acid in the pH 5 fraction, the so-called pH 5 RNA, plays a role in the transfer of amino acids to the microsomal protein, the incorporation of amino acids into pH 5 RNA from tumour cell sap was also studied. Less amino acid was incorporated into the pH 5 RNA derived from tumour than into that derived from liver, but the difference between the two ribonucleic acids was much less than the difference in activity between the pH 5 fractions for the incorporation of amino acids into the microsomes. It has previously been observed, in a comparison of regenerating and normal liver, that the ability of the pH 5 RNA to bind amino acid does not correlate with the ability of the pH 5 fraction to bring about incorporation into microsomes (Rendi, 1959). Thus it appears that the rate of transfer of amino acid from pH 5 RNA to the microsomes is more important than is the number of binding sites for amino acid on the pH 5 RNA.

Although the microsomes are the most active organelles of the liver cell for the incorporation of amino acid, and presumably for protein synthesis, an incorporation of amino acid dependent on an ATP-generating system has been obtained with mitochondrial preparations (Bates, Craddock & Simpson, 1958; Campbell & Greengard, 1959). It has been shown (Greengard, 1959) that even if the ribonucleic acid content of the mitochondria used in the present studies was due to contamination by microsomes, only a negligible fraction of the incorporated amino acid would have been due to the presence of microsomal protein. Similar considerations apply to the tumour mitochondria.

It will be seen from Table 4 that, in confirmation of the results of McLean *et al.* (1958) and Greengard (1959), normal liver mitochondria incorporate amino acids when supplied with a suitable source of energy. Liver-tumour mitochondria behave similarly, although the similarity is only qualitative, since the extent of incorporation of [^{14}C]leucine is lower than into the corresponding fraction of normal liver.

For the incorporation of amino acids into microsomal protein the cell sap is required (Zamecnik & Keller, 1954). However, for a similar incorporation into mitochondrial protein there appears to be no such requirement. Unless it is assumed that the constituents of the cell sap required for incorporation are retained within the membrane of the mitochondria, a different mechanism for the incorporation in the two types of organelle is indicated. The latter view is supported by the enhancement of the incorporation into mitochondria, in the absence of cell sap, by ribonuclease and the lack of effect of GTP, results which are contrary to experience with microsomes.

The increase in the incorporation of amino acids into mitochondria which is effected by the cell sap is reduced by the presence of ribonuclease (Table 6). The contrast between the effect of ribonuclease in the absence and presence of cell sap suggested that two mechanisms for the incorporation of amino acid might be operating. Although the second mechanism bears a certain resemblance to that which occurs with microsomes there do seem to be some differences. For mitochondria, pH 5 fraction (+GTP) probably does not include all the factors responsible for the stimulatory effect of cell sap (Greengard, 1959). The present studies with liver tumour are also of interest in this respect.

Liver-tumour cell sap or pH 5 fraction are much less effective than their normal counterparts in stimulating amino acid incorporation into microsomal protein; therefore if the same cell sap constituents were responsible for stimulating the process in both organelles, a similar quantitative difference would be expected in their effect on

mitochondrial incorporation. This, however, is not so (Table 5), since the cell sap derived from liver tumour was at least as effective as that derived from normal liver for the incorporation of amino acids into normal liver mitochondria. In contrast the tumour pH 5 fraction was considerably less effective than a similar fraction from normal liver in this respect. It thus appears that there are factors present in the cell sap which are not concerned with the incorporation of amino acids into microsomes, but which promote incorporation into mitochondrial protein.

SUMMARY

1. After the incubation of microsomes and pH 5 fraction of liver and liver tumour with [^{14}C]leucine, the radioactivity of the tumour-microsomal protein is lower than that of liver. When liver microsomes were incubated with tumour pH 5 fraction, instead of liver pH 5 fraction, a lower extent of incorporation resulted.

2. The adenosine triphosphate-dependent incorporation of leucine and lysine into the ribonucleic acid of pH 5 fraction of liver tumour was lower than into that of normal liver.

3. The adenosine triphosphate-dependent incorporation of [^{14}C]leucine into preparations of liver and tumour mitochondria was studied. Incorporation into the mitochondrial protein of both tissues took place in the absence and presence of cell sap, but was higher in its presence.

4. Under all experimental conditions the amino acid incorporation into tumour mitochondria was lower than into liver mitochondria. The stimulating action of tumour cell sap, however, on liver mitochondria was as great as that of liver cell sap. The

tumour pH 5 fraction, on the other hand, was less effective than that of liver in stimulating mitochondrial incorporation.

5. Ribonuclease stimulates the incorporation into liver-mitochondrial protein in the absence of cell sap, but reduces the incorporation stimulated by cell sap.

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A Quantitative Method for the Separation of Thyroid Hormones and Related Compounds from Serum and Tissues with an Anion-Exchange Resin*

BY VALERIE A. GALTON AND ROSALIND PITT-RIVERS
National Institute for Medical Research, Mill Hill, London, N.W. 7

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The use of paper chromatography and radioautography in the study of the metabolism of ^{131}I -labelled thyroid hormone is well known. Some concentration of the iodinated compounds present in biological material is usually necessary and the most widely used technique is that of extraction with butanol. However, the amount of material

that can be extracted in this way is limited; this method is therefore not suitable when the amount of radioactivity is small, as chromatographic analysis of the extracted compounds is virtually impossible.

Blanquet, Dunn & Tobias (1955) have employed the anion-exchange resin Dowex-2 for the separation of organic and inorganic iodide present in hydrolysed thyroid gland or blood plasma; the

* This work forms part of a Ph.D. thesis submitted to the University of London.