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Biochem. J. (1962) **83**, 69

Incorporation of Radioactive Amino Acids into Serum Albumin by Isolated Rat-Liver Ribosomes

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(Received 15 September 1961)

It is generally accepted that protein synthesis occurs in the ribosome part of the microsomes of cells and, indeed, that much of the intracellular synthesis of protein takes place in these ribonucleo-protein particles (see Hoagland, 1960, for review). Ribosomes can now be isolated from rat liver (as well as from other tissues) in a state in which they will incorporate amino acids into protein (Korner, 1959*a*, 1960*a*, 1961*a*; Takamami, 1960; Rendi & Hultin, 1960). Amino acid incorporation into protein is not, however, synonymous with synthesis of protein and, in the hope of providing some support for the idea that isolated ribosomes can synthesize protein, it was decided to attempt to demonstrate the radioactive labelling of a specific protein by isolated rat-liver ribosomes.

Serum albumin is synthesized in the liver in considerable quantity (Miller, Bly, Watson & Bale, 1951; Jensen & Tarver, 1956; Gordon & Humphrey, 1960). The net synthesis of albumin has been studied with perfused rat liver (Miller *et al.* 1951), with slices of chick liver (Peters & Anfinsen, 1950) and with slices of rat liver (Campbell & Stone, 1957). Ogata, Hirokawa & Omori (1960) and Campbell, Greengard & Kernot (1960) showed that serum albumin becomes radioactively labelled when rat-liver microsomes are incubated *in vitro* with radioactive amino acids, although in these experiments net synthesis of albumin could not be demonstrated. Further, serum albumin can be isolated by chemical and immunological means, and therefore the ability of isolated ribosomes to incorporate radioactive

amino acids into this protein was studied. The successful result has been reported briefly (Korner, 1960*b*), and this paper gives fuller details of the methods used and the results achieved. Lingrel & Webster (1961) have reported the labelling of albumin by isolated microsomes and ribosomes, and Decken & Campbell (1962) have obtained similar results with ribosomes prepared by the method of Rendi & Hultin (1960).

METHODS

Radioactive amino acids. Generally labelled L-leucine (7.1 mc/m-mole) and DL-[1-¹⁴C]leucine (5.4 mc/m-mole) were obtained from The Radiochemical Centre, Amersham, Bucks.

Chemicals. The sodium salts of ATP and GTP were obtained from Sigma Chemical Co. The sodium salt of creatine phosphate was made by the method of Ennor & Stocken (1948) and creatine phosphokinase was prepared by the method of Kuby, Noda & Lardy (1954).

Preparation of albumin. Rat-serum albumin was separated from rat serum by the method of Korner & Debro (1956) and Debro, Tarver & Korner (1957). Rat-serum proteins were precipitated by adding to the serum an equal volume of cold 10% (w/v) trichloroacetic acid. The proteins were spun down and the albumin was removed from the precipitate by three extractions each with a volume of cold 96% (v/v) ethanol-1% (w/v) trichloroacetic acid equal to the original volume of serum. Material that did not dissolve was removed by centrifuging, the alcohol-acid extract was dialysed at 4° against several changes of water to remove alcohol and acid, and the albumin was freeze-dried. Chicken-serum albumin was prepared similarly.

Albumin prepared from serum in this way is free from other serum proteins (except for insulin) and appears to be unchanged by the preparative procedure. Its behaviour during electrophoresis on paper or in the Tiselius apparatus at various pH values is the same as that of albumin in serum and of albumin separated by the procedure of Cohn *et al.* (1946) (Schwert, 1957; Delaville, Delaville & Delaville, 1954; Levine, 1954; Debro *et al.* 1957). No contamination of rat-serum albumin prepared by the method of Debro *et al.* (1957) could be detected by Campbell *et al.* (1960) during electrophoresis on cellulose acetate paper, a method which is known to achieve the separation of α -globulin and albumin (Kohn, 1957; Campbell, Kernot & Roitt, 1959). The immunological behaviour of albumin prepared in this manner shows it to be identical with native serum albumin (Korner & Debro, 1956, and unpublished experiments) and no evidence of its heterogeneity was detected by Campbell *et al.* (1960) by the techniques of Udin (1948) and Ouchterlony (1953). Finally, its viscosity and its solubility under various conditions are identical with those of native albumin (A. Korner, unpublished work; Schwert, 1957).

Preparation of albumin antisera. Rabbits were injected subcutaneously with rat- or chick-serum albumin, together with Freund's adjuvant (Freund, Thomson, Hough, Sommer & Pisani, 1948); the injection was repeated a week later and in some experiments for a third time after another 2 weeks. Two or 3 weeks after the last injection a sample of blood, withdrawn from the ear vein of the rabbit, was assayed for antiserum activity by serial dilution. If the activity was reasonably high the rabbit was killed, the blood collected and the serum distributed in small samples, which were frozen. Each sample was thawed immediately before use and any left over was not used in subsequent experiments.

Preparation of ribosomes. Young adult male or female albino rats (150–180 g. body wt.), starved overnight to deplete the liver of much of its glycogen, were decapitated and the liver was removed rapidly, plunged into ice-cold 0.44M-sucrose and cut into small pieces with scissors. The method of isolating ribosomes from liver was described by Korner (1961a); a summary is given here. The liver was lightly homogenized in 0.44M-sucrose, centrifuged at 15 000g at 0° for 10 min., and the supernatant fluid removed and treated with one-ninth of its volume of freshly made ice-cold 5% (w/v) sodium deoxycholate solution in 0.03M-tris buffer, pH 8.2. The ribosomes were centrifuged at 105 000g for 2 hr. at 0°, the supernatant fluid was removed, the pellet rinsed with ice-cold 0.44M-sucrose and the inside walls of the tube were wiped with tissue paper. The ribosome pellet was washed by suspending it by gentle homogenization in 12.5 ml. of ice-cold medium A (0.03M-tris buffer at pH 7.5 which was 5 mM with respect to $MgCl_2$, 0.05M with respect to NaCl and 0.08M with respect to KCl). The washed ribosomes were sedimented again by recentrifuging at 105 000g for 90 min. and the washings were carefully removed.

Preparation of cell sap. Cell sap was obtained from an homogenate of rat liver in 0.44M-sucrose by centrifuging at 15 000g for 10 min., followed by spinning at 105 000g for 2 hr. at 0°. The supernatant fluid was pipetted off.

In some experiments a pH 5 enzyme preparation (Hoagland, Keller & Zamecnik, 1956) was used in place of the whole cell sap. It was prepared by the drop-by-drop addition of ice-cold 0.1M-acetic acid to rat-liver cell sap

kept in an ice bath until the pH had reached 5.0. The precipitate was spun off and dissolved in medium A, and any insoluble material was removed by centrifuging. The precipitation at pH 5.0 and redissolving in medium A were repeated.

Method of incubation. Samples (0.5 ml.) of ribosomes suspended in medium B, 50% more concentrated than medium A (0.045M-tris at pH 7.5 which was 7.5 mM with respect to $MgCl_2$, 75 mM with respect to NaCl and 0.12M with respect to KCl) were pipetted into ice-cold 15 ml. centrifuge tubes, containing 0.25 ml. of cell sap in 0.44M-sucrose and 0.25 ml. of medium A which was 0.02M with respect to ATP and 2.4 mM with respect to GTP, and contained 0.5 μ C of radioactive amino acid, 100 units of penicillin and 40 μ g. of chloramphenicol. Thus the final incubation mixture of 1 ml. contained about 2 mg. of ribosome protein and 3 mg. of cell-sap protein, 0.5 μ C of radioactive amino acid, 100 units of penicillin and 40 μ g. of chloramphenicol; it was 0.03M with respect to tris, 5 mM with respect to $MgCl_2$, 0.05M with respect to NaCl, 0.08M with respect to KCl, 0.11M with respect to sucrose, 5 mM with respect to ATP and 0.6 mM with respect to GTP.

In those experiments where pH 5 enzymes dissolved in medium A were used in place of cell sap in sucrose, the final concentration of salts was maintained at the same level by suspending the ribosomes in medium A instead of medium B. An ATP-generating system is not essential in this system (Korner, 1961a), but in those experiments where it was used creatine phosphate was added at a final concentration of 0.02M, together with 0.03 mg. of creatine phosphokinase, and the final ATP concentration was 1 mM instead of 5 mM.

The tubes were shaken at 37° for various times and the reaction stopped by the addition of 10% trichloroacetic acid, or, if the ribosomes were to be re-separated from the cell sap, by the addition of 10 ml. of ice-cold 0.44M-sucrose containing excess of unlabelled amino acid corresponding to the radioactive one used. The ribosomes were then separated from the rest of the reaction mixture by centrifuging at 105 000g for 1 hr. The supernatant fluid was removed and kept and the ribosomes were washed by suspending them by gentle homogenization in ice-cold 0.44M-sucrose and then centrifuged again. The washings were added to the supernatant fluid and the ribosomes were suspended again in water.

Isolation of albumin from the incubation mixture. Albumin was isolated from the whole incubation mixture, or separately from the resuspended ribosomes and from the supernatant fluid, by adding an equal volume of 10% trichloroacetic acid and extracting the albumin from the precipitated protein pellet with three washes of 1% (w/v) trichloroacetic acid–96% ethanol. The protein not extracted by acid-ethanol was collected and termed non-albumin protein. The extract was dialysed overnight at 4° against several changes of water and finally against aq. 0.9% NaCl. This procedure, which extracts only albumin from serum (Korner & Debro, 1956), removes all the albumin together with some other proteins from rat liver or fractions of liver. The extract was cleared of material which might be precipitated along with the rat albumin when antiserum to rat albumin is added, by treatment with antiserum to chick-serum albumin after addition of chick-serum albumin in 0.9% NaCl (Campbell & Stone, 1957). The cleared extract was then treated with antiserum to rat-serum albumin at

37° for 15 min. and at 4° overnight, and the precipitated rat-serum albumin-antibody complex was collected by centrifuging. The supernatant fluid was added to the non-albumin protein.

In most experiments the amount of serum albumin to be isolated was so small that known amounts of rat serum were added to the incubation mixture, or to the separated parts of it, before the addition of the trichloroacetic acid, so that the albumin in the serum could act as a carrier for the radioactive albumin.

In most experiments the albumin associated with the ribosomes was extracted directly by the acid-ethanol method in the same manner as the albumin in the soluble part of the incubation mixture, but in some experiments attempts were made to remove albumin from the particles in a way that did not denature it before precipitation with trichloroacetic acid. Several methods were tried and partial success was achieved, but in no case could all the radioactive material, nor all the proteins, be removed from the ribosomes. The methods used included attempts to disrupt the ribosomes by ultrasonic vibration or by incubation of the ribosomes at 37° in water, 0.1M-EDTA, 4M- and 8M-urea, or in the presence of ribonuclease. After vibration or incubation the particulate matter was spun off by centrifuging at 105 000g for 60 min. and the soluble portion treated with acid-ethanol in the way described above.

Measurement of radioactivity. The serum albumin-antibody complex and the non-albumin protein were precipitated with 0.5N-HClO₄, washed three times with 0.5N-HClO₄ containing unlabelled amino acid corresponding to the radioactive one used and then heated in 0.5N-HClO₄ to 95° for 20 min. to remove RNA and to coagulate the albumin. The protein was then dissolved in N-NaOH containing unlabelled amino acid and reprecipitated with 6N-HCl 1 hr. later and washed twice with chloroform-ether-ethanol (1:2:2, by vol.) and dried with ether. The protein was plated and its radioactivity assessed as described by Korner (1959b, 1961b).

RESULTS

Fig. 1 illustrates the results of an experiment in which the amount of incorporation of radioactive leucine into albumin extracted from the whole incubation mixture was measured after various times of incubation. In this experiment 0.1 ml. of rat serum was added to each tube just before the addition of the 10% trichloroacetic acid (used to stop the reaction), so as to provide carrier albumin. In addition, the precipitated albumin-antibody complex was worked up for counting without re-separating the albumin, so that the specific activities reported are for the albumin extracted from the incubation mixture plus carrier albumin plus antibody to albumin. Essentially similar results were achieved in an experiment in which no carrier albumin was added and in which the albumin was released from the antigen-antibody complex with ethanol at pH 2.4 (Peters, 1958) before the protein was prepared for counting.

It is clear that amino acid incorporation into albumin continues for at least 2 hr. Korner (1961a) found that carefully prepared rat-liver ribosomes usually continue to incorporate amino acids into total protein for 2 hr. and sometimes for 3 hr. or more. The inability of Campbell (1961) to obtain incorporation of amino acids into ribosome protein for more than 30 min. may reflect damage to the ribosomes during preparation.

The incorporation of amino acids into albumin, like the incorporation into total protein of the ribosome-cell-sap system (Korner, 1961a), depends on the availability of amino acid-activating enzymes and soluble RNA of the cell sap and on the availability of ATP. These conclusions are supported by the results shown in Table 1: omission of the cell

Fig. 1. Time course of incorporation of L-[¹⁴C]leucine into albumin in a ribosome-cell-sap system. The radioactivities are expressed as counts/min./mg. of albumin-antibody protein.

Table 1. *Conditions necessary for incorporation of DL-[1-¹⁴C]leucine into albumin*

The complete system is described in the Methods section.

	Specific activity of albumin (counts/min./mg.)
Complete system	186
ATP omitted	9
ATP at 1 mM instead of at 5 mM	47
ATP at 1 mM instead of at 5 mM and 0.02M-creatine phosphate and 0.03 mg. of creatine phosphokinase added	153
GTP omitted	131
Cell sap omitted	12
pH 5 enzymes in place of cell sap	159
Puromycin (600 μM) added	19

Table 2. *Comparison of incorporation of L-[¹⁴C]leucine into albumin and non-albumin protein in ribosomes and supernatant fluid*

For conditions, see the Methods section. The figures in the columns headed 'Albumin' and 'Non-albumin' are total counts/min.

Time (min.)	Ribosomes			Supernatant fluid		
	Albumin	Non- albumin	Percentage of total counts in albumin	Albumin	Non- albumin	Percentage of total counts in albumin
0	1	6	—	2	7	—
60	49	845	5.5	11	125	8.1
120	132	1550	7.8	27	236	10.3

sap results in almost complete inhibition of incorporation of amino acids into albumin, and ATP is essential for incorporation. An ATP-generating system is not necessary (cf. Korner, 1961*a*), provided that more ATP and Mg^{2+} ions are present than is usual when a generating system is added (5 or 10 mM ATP instead of 1 mM). The cell sap can be replaced by a pH 5 enzyme preparation (Hoagland *et al.* 1956). Puromycin inhibits amino acid incorporation into albumin, suggesting that soluble RNA is involved as an intermediary in this process (Yarmolinsky & de la Haba, 1959). In general the optimum conditions (concentration of salts and ATP, or the optimum pH) for amino acid incorporation into albumin were similar to those for amino acid incorporation into total ribosome and cell-sap protein (Korner, 1961*a*). Addition of NAD or NADP to the incubation mixture caused no consistent stimulation of amino acid incorporation into total ribosome protein or total cell-sap protein, or into albumin. This result is in contradiction to the large NAD-stimulation of amino acid incorporation into albumin in a microsome-cell-sap system claimed by Lingrel & Webster (1961).

Table 2 gives the results of an experiment in which the ribosomes were re-separated from the supernatant fluid after incubation; the albumin from both fractions was separately isolated and its radioactivity measured and compared with that of the non-albumin protein of each fraction. The results are reported as total counts in each fraction, since 0.1 ml. of serum was added as carrier; the albumin fraction is the antibody-antigen complex. The albumin contains up to 10 % of the total counts in each fraction.

DISCUSSION

The results of this paper confirm the preliminary report (Korner, 1960*b*) that isolated rat-liver ribosomes in the presence of cell sap can incorporate radioactive amino acids into a specific protein, serum albumin. Rat liver synthesizes considerable quantities of albumin (Miller *et al.* 1951; Jensen & Tarver, 1956; Gordon & Humphrey, 1960), and

there is good evidence that ribosomes are the cellular site at which a considerable amount of protein is synthesized (Littlefield, Keller, Gross & Zamecnik, 1955; Hoagland, 1960). Our results show that isolated ribosomes can incorporate amino acids into a specific protein. The results do not, however, offer any evidence that the net synthesis of albumin can occur in this system.

The preliminary report of Decken & Campbell (1962) supports the results reported here. Lingrel & Webster (1961) have also obtained labelling of albumin by rat-liver ribosomes, but they report that 50 % or more of the radioactivity of the supernatant fluid can be attributed to the radioactivity in albumin. In the experiments reported above, the proportion of radioactivity that could be ascribed to albumin is much less than that reported by Lingrel & Webster (1961). No explanation of this difference can be offered. If the radioactivity of the material that is soluble in trichloroacetic acid-ethanol is assessed without further purification by precipitation with antiserum to rat albumin, about 50–60 % of the radioactivity in the total protein is found to be present in it. Although the acid-ethanol method removes only albumin (and insulin) from serum (Korner & Debro, 1956; Debro *et al.* 1957), it removes proteins other than albumin from liver, and these other proteins are highly radioactive. The protein cleared by the treatment with chick albumin and chick-albumin antiserum often contained radioactivity.

Precipitation of the albumin from proteins soluble in trichloroacetic acid-ethanol by antiserum to rat albumin after clearing with chick albumin gives good grounds for believing that the material called albumin is indeed albumin. Jungblut, Heimbürger & Turba (1959) have indeed shown that, from microsomes, four proteins can be obtained which are precipitated by antiserum to rat albumin and that only one of them has the electrophoretic properties of albumin. Their results may mean that precursors of albumin, perhaps incomplete polypeptide chains, could be precipitated by antiserum to completed albumin. However, it is possible to

remove the albumin from its antigen-antibody complex (Peters, 1958) and then to examine it electrophoretically. When this was done over 80 % of the radioactivity of the albumin-antigen complex was removed as material that ran as a single peak during electrophoresis on paper with the characteristics of freshly prepared rat-serum albumin.

Amino acid incorporation into albumin requires the amino acid-activating enzymes of the cell sap and the intermediary action of soluble RNA. In addition to the correct complement of ions, a supply of ATP is required; an ATP-generating system is not necessary, in agreement with the finding (Korner, 1961*a*) that the ribosome system does not require an ATP-generating system in order to incorporate amino acids into protein. In contradiction to the findings of Lingrel & Webster (1961) with microsomes, NAD had no effect on the extent of incorporation in the ribosome system.

Some of the albumin that is radioactively labelled in the ribosomes is released from the ribosomes to the supernatant fluid. This may be the result of breakage of ribosomes during incubation, but in view of the relative stability of ribosomes (Korner, 1961*a*) and of the results of Hultin (1961) this explanation seems incorrect. Campbell *et al.* (1960) state that albumin which is labelled in rat-liver microsomes cannot be released to the supernatant fluid. The conditions required for the release of finished protein from the ribosome templates are critical (Lamfrom, 1961; Hultin, 1961). Enzymes stimulating release exist, and the releasing activity may vary from tissue to tissue and with the way in which the tissue has been treated. It seems likely that the correct conditions for release of labelled protein were not achieved by Campbell *et al.* (1960). Indeed, Decken & Campbell (1962) found incorporation of amino acids into the albumin of the soluble fraction as well as into the albumin of rat-liver ribosomes, thus confirming my own result (Korner, 1960*b*). Lingrel & Webster (1961) have also reported the appearance of labelled albumin in supernatant fluid incubated with rat-liver ribosomes.

The amount of albumin present in subcellular fractions of chick liver has been assessed by Peters (1957), and it can be expected from the results he reports that only a small percentage of the protein of the rat-liver cell sap will be albumin. Still less albumin is associated with the ribosomes; so that the radioactivity found in albumin of cell sap and, even more, that of albumin from ribosomes reported in Table 2 of this paper, must represent very high specific activities of albumin greatly diluted by carrier albumin. These results are consistent with the idea that albumin is synthesized in the ribosomes and is then released to the cell sap. It is realized that these experiments offer no evidence

that ribosomes can bring about net synthesis of albumin *in vitro*.

SUMMARY

1. Isolated rat-liver ribosomes incubated with adenosine triphosphate and cell sap incorporate amino acids into serum albumin for at least 2 hr. The conditions required for incorporation are described.

2. Albumin associated with the ribosomes attains a high specific activity, and albumin labelled with amino acids is progressively transferred from the ribosomes to the soluble part of the cell-free system.

I wish to thank the Medical Research Council for an expenses grant; the British Drug Houses Ltd. for a grant for technical assistance; Mrs B. Jones, B.Sc., for highly skilled assistance and Professor F. G. Young, F.R.S., for his constant encouragement.

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Biochem. J. (1962) **83**, 74

The Formation of Glycerylphosphorylcholine during the Clotting of Plasma

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(Received 22 September 1961)

It has been known since the experiments of Wooldridge (1886) that tissue juices are capable of accelerating the coagulation of blood. The active principle in these extracts is a complex of phospholipid and protein (Howell, 1935), and the phospholipid portion of the complex is essential to the activity of the protein (Cohen & Chargaff, 1940). Since then much work has been carried out on the effect of various added phospholipids on the coagulation of plasma.

The purpose of the work described in this paper was to see whether the plasma phospholipids themselves undergo any chemical change during clotting. In most of the experiments Russell's viper venom has been used to coagulate the plasma. It has been shown (Macfarlane & Barnett, 1934) that when this venom was added to plasma in the presence of calcium ions there is a rapid conversion of prothrombin into thrombin.

taining sodium citrate (final concn. 0.38%). The supernatant plasma was centrifuged at 25 000g for 15 min. to remove most of the platelets. This plasma was first dialysed for 2 hr. against Krebs-Ringer phosphate (Krebs & Henseleit, 1932), which contained sodium citrate (final concn. 0.38%), to reduce the specific activity of the exchangeable phosphate. The plasma was then dialysed for 18 hr. in the cold against two changes of a modified Krebs-Ringer bicarbonate solution. The solution had the following composition (μ moles/l.): NaCl, 119; KCl, 4.8; CaCl_2 , 2.6; MgSO_4 , 1.2; NaHCO_3 , 25; sodium citrate, 1.3; $\text{N}_2 + \text{CO}_2$ (95:5) was bubbled through the solution, which had a pH of 7.5.

Platelets. These were obtained from freshly drawn venous blood. The blood was centrifuged at 500g for 20 min. at 0–2° to sediment erythrocytes and white cells. The plasma was then centrifuged at 25 000g to obtain a pellet of platelets. The platelets were resuspended in the dialysed plasma.

Russell's viper venom. The dried venom was a gift from the Wellcome Physiological Research Laboratories.

Methods

Clotting of the plasma. The dialysed plasma (usually 5 ml.) was mixed with fresh platelets derived from the same volume of non-radioactive plasma, and incubated in roughened glass tubes for 10 min. at 37°. The plasma was then transferred to plastic tubes and incubated at 37° with Ca^{2+} ions (0.025 ml. of m-CaCl_2 /ml. of plasma). The plasma clotted about 135 sec. after the addition of the Ca^{2+} ions. In the experiments in which viper venom was used, platelets were not added to plasma but, except where stated, the plasma was incubated with venom (10 μ g./ml. of plasma) which was added at the same time as the Ca^{2+} ions. The plasma usually clotted 30 sec. after the addition of the venom.

Determination of phosphorus. The acid-soluble phosphate

MATERIALS AND METHODS

Materials

Plasma. Blood was withdrawn from patients with polycythaemia vera who had received a single injection of 5–10 mc of [32 P]phosphate. Preliminary experiments showed that plasma phospholipids reached their maximum specific activity about 50 hr. after the injection of the labelled phosphate. The blood was obtained by venepuncture and collected in plastic centrifuge tubes con-

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