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Studies on Incorporation of Amino Acids into Protein in Isolated Rat-Liver Ribosomes

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(Received 28 March 1961)

Cells contain microsomal particles, or ribosomes (Roberts, 1958), 10–25 m μ in diameter, which contain ribonucleoprotein on which, it is generally believed, amino acids, activated by reactions occurring in the cell sap (Hoagland, Keller & Zamecnik, 1956; Hoagland, Zamecnik & Stephenson, 1957), are assembled into polypeptide chains during the biosynthesis of proteins (see Hoagland 1960, for review). This view is greatly strengthened by the results obtained by Littlefield, Keller, Gross & Zamecnik (1955) in experiments with whole animals, which are consistent with the hypothesis that proteins are formed in the ribosomes and are transferred, via the lipoprotein of the endoplasmic reticulum, to the soluble fraction of the cell.

In the experiments of Littlefield *et al.* (1955) ribosomes were prepared from microsomes by treatment of the latter with sodium deoxycholate (Palade & Siekevitz, 1956), which solubilizes the lipoprotein part of the microsomes (Strittmatter & Ball, 1952). The ribosomes were reported (Littlefield & Keller, 1957) to be incapable of incorporating radioactive amino acids into protein *in vitro* in a system of the type described by Zamecnik & Keller (1954). The implication was that treatment with deoxycholate may have damaged the ribosomes.

Ribosomes from many tissues have been intensively studied recently and their ability to aggregate and disperse in a manner dependent on the pH and composition of the suspending medium and in particular on the concentration of magnesium ions in the medium has excited much attention (Tissière & Watson, 1958; Gilchrist & Bock, 1958; Wagman & Trawick, 1958; Bolton, Hoyer & Ritter, 1958; Petermann, Hamilton, Balis, Samarth & Pecora, 1958; Tissière, Watson, Schlessinger & Hollingsworth, 1959; Huxley & Zubay, 1960; Petermann & Hamilton, 1961). In view of these reports it was thought that deoxycholate could be used to prepare ribosomes from rat liver in a form capable of incorporating amino acids into protein *in vitro*, if care were taken to use the optimum concentration of magnesium ions during the preparation. It was soon found that ribosomes could be prepared from rat liver by the normal deoxycholate method, in the absence of magnesium, and that they would incorporate amino acids into protein *in vitro*. The result has been briefly reported (Korner, 1959*a*, 1960*a*), and this paper records the methods used to prepare the ribosomes, reports on their properties and gives the results of some studies carried out with them. Since the earlier

report (Korner, 1959a) other workers have obtained amino acid incorporation into protein with ribosomes isolated from mammalian tissues (Takamami, 1960; Rendi & Hultin, 1960; Kirsch, Siekevitz & Palade, 1960) and from *E. coli* (Tissière, Schlessinger & Gros, 1960).

METHODS

Radioactive amino acids. Generally labelled L-leucine (L-[G- 14 C]leucine), DL-[1- 14 C]leucine and DL-[1- 14 C]valine were used and 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 prepared by the method of Ennor & Stocken (1948) and creatine phosphokinase was prepared by the method of Kuby, Noda & Lardy (1954).

Preparation of microsomes and ribosomes. Young adult male and female albino rats were used. They were starved overnight to deplete the liver of most of its glycogen and were killed by decapitation. The liver was removed as rapidly as possible into ice-cold 0.44M-sucrose and was cut into small pieces with scissors. The liver was blotted with filter paper, weighed by displacement in fresh, ice-cold 0.44M-sucrose and homogenized in 5 vol. of ice-cold 0.44M-sucrose in a hand-operated homogenizer of the type described by Dounce, Witter, Monty, Pate & Cottone (1955), which was kept cold in an ice bath. Only three strokes (up and down) of the plunger were used since severe damage to the tissues diminishes its capacity to incorporate amino acids into protein *in vitro* (Zamecnik & Keller, 1954). Mitochondria, nuclei and cell debris were removed from this brei by centrifuging for 10 min. at 15 000g at 0° in a refrigerated centrifuge, and most of the supernatant fluid was carefully removed.

Two techniques were used for the preparation of ribosomes from this supernatant fluid. The first was based on that described by Littlefield *et al.* (1955), which was, in its turn, based on the observations of Palade & Siekevitz (1956) and Strittmatter & Ball (1952). The microsomes were separated from the soluble fraction of the cell by centrifuging at 105 000g for 1 hr. at 0° in a Spinco model L preparative ultracentrifuge. The soluble fraction of the cell was removed from the pellets, which were rinsed with sucrose, and the walls of the tubes were dried with tissue paper. Half of the microsomal pellets obtained were used directly for incorporation experiments, and the rest were used to prepare the ribosomes by suspending them by gentle homogenization in a freshly made, ice-cold 5% solution of sodium deoxycholate in 0.2M-glycylglycine buffer at pH 8.2. The volume of the sodium deoxycholate solution added was that which contained about 0.75 mg. of deoxycholate/estimated mg. of microsomal protein. After standing in ice for 10 min., ice-cold water (or, in some experiments, ice-cold 0.03M-tris buffer at pH 8.2 containing 5 mM-MgCl₂) was added to give a final concentration of deoxycholate of 0.5%, and the ribosomes were separated from the solubilized lipoprotein of the endoplasmic reticulum by centrifuging at 105 000g for 2 hr.

The second method used was to treat the supernatant fluid after the centrifuging at 15 000g with one-ninth of its volume of an ice-cold, freshly prepared 5% sodium deoxy-

cholate solution in 0.03M-tris buffer at pH 8.2 and to separate the ribosomes by centrifuging at 105 000g for 2 hr. (Hall & Doty, 1958). At this stage in both methods, the supernatant was removed, the tubes were drained carefully of as much of the supernatant as possible without loss of pellet material, and the walls wiped with tissue paper. In the earlier experiments the pellet was often loose, which made it difficult to remove all the supernatant fluid, but later it was found that if the rats were starved overnight the pellets obtained were more tightly packed, possibly because of the absence of liver glycogen, which spins down with the ribosomes.

It soon became apparent that the particles could be washed with buffer without loss of incorporating ability, and such a wash became part of the preparative routine. The pellets were suspended by gentle homogenization in as large a volume as possible (usually 12.5 ml./pellet) of 0.03M-tris buffer at pH 7.5 containing 5 mM-MgCl₂, 0.08M-KCl and 0.05M-NaCl and were sedimented again by centrifuging, and the washings carefully removed.

Conditions of incubation. The pellets were finally suspended by gentle homogenization in ice-cold 0.045M-tris buffer at pH 7.5 containing 7.5 mM-MgCl₂, 0.12M-KCl and 0.075M-NaCl. Samples (0.5 ml.) of the suspended ribosomes were pipetted into ice-cold 15 ml. centrifuge tubes containing 0.25 ml. of the cell sap in 0.44M-sucrose, or 0.25 ml. of a preparation of pH 5 enzymes (Hoagland *et al.* 1956) in 0.44M-sucrose, or 0.25 ml. of 0.44M-sucrose alone. To this was added 0.25 ml. of 0.03M-tris buffer at pH 7.5 containing 5 mM-MgCl₂, 0.08M-KCl and 0.05M-NaCl, in which was dissolved ATP, GTP, creatine phosphate, creatine phosphokinase, radioactive amino acid and, in some experiments, glutathione, in concentration to give 1 mM-ATP, 0.02M-creatine phosphate, 0.6 mM-GTP, 2.5 mM-glutathione, 0.03 mg. of creatine phosphokinase and 0.5 μ C of radioactive amino acid in the 1 ml. final vol. of incubation mixture. The tubes were shaken for various lengths of time at 37° in a Dubnoff bath. In most experiments penicillin (100 units) and chloramphenicol (40 μ g.) were added to the incubation mixture. The reaction was stopped by the addition of 0.5N-HClO₄ or, if the subcellular particles were to be re-separated from the incubation mixture, by adding ice-cold 0.44M-sucrose containing an excess of unlabelled amino acid, corresponding to the radioactive amino acid used, and centrifuging at 105 000g for 2 hr. at 0°. The microsomes or ribosomes were then resuspended in water and their proteins and those of the soluble fraction precipitated with 0.5N-HClO₄.

Preparation of radioactive proteins. The precipitated proteins were repeatedly washed with 0.5N-HClO₄ containing unlabelled amino acid to remove radioactive amino acids, RNA and lipids were extracted, and the residual protein was dried, plated on weighed aluminium planchets and counted in a Geiger-Müller gas-flow counter as described by Korner (1959b). Sufficient counts were recorded to reduce the standard error to 3% or less, and corrections were applied for the background counts. The results are given as counts/min./mg. of protein or counts/min./mg. of RNA.

Determination of ribonucleic acid. RNA was determined on samples of microsomes and ribosomes by the adaptation of the method of Scott, Fraccastoro & Taft (1956) described by Korner (1959b). The protein content of the residue after extraction of the RNA was estimated and the RNA:protein ratio calculated.

Determination of nitrogen. The N content of samples of the microsomes and ribosomes, cell sap, pH 5 enzymes and of the residual protein left after the RNA had been extracted in the samples used for RNA determination was determined by the micro-Kjeldahl technique. Corrections were applied for the N content of any tris buffer and of any RNA present, and the corrected value of N was used in calculation of the protein content of the samples by using the factor 6.25.

Measurements in the analytical ultracentrifuge. Samples of the ribosomes and of the microsomes were examined during centrifuging at 37 020 rev./min. or at lower speeds in the Spinco model E analytical ultracentrifuge in 0.03M tris buffer at pH 7.5 containing various concentrations of Mg^{2+} ions. The sedimentation coefficients (*S*) were calculated and were corrected to water and 20°.

The relative proportions of the particles with different *S* values was obtained by estimation of the area of each peak of the schlieren diagram. This estimation was done in two ways: by counting squares on enlarged photographs of the peaks or by weighing the peaks cut out from the enlarged photographs.

Determination of amino acids. The free amino acid content of the fractions was determined by a modification of the ninhydrin method of Moore & Stein (1948) which has been described by Korner (1959b).

RESULTS

The ribosomes prepared by either of the methods described are a yellowish straw colour in suspension possibly because of contamination by ferritin, which can be removed by precipitating the ribosomes with barium (Hamilton & Petermann, 1959; Petermann & Hamilton, 1961). That the preparations are essentially free of contaminating microsomes and endoplasmic reticulum is indicated by the high RNA:protein ratio (mean about 0.8:1 ranging from 0.65:1 to 0.9:1) and by the observation that a second treatment with 0.5% deoxycholate caused little alteration in the RNA:protein ratio of the ribosomes and in their amino acid-incorporating ability.

The sedimentation patterns obtained with schlieren optics when the ribosomes were centrifuged in the analytical ultracentrifuge and the effect of various concentrations of magnesium ions on these patterns were broadly similar to those obtained by other workers using ribosomes from liver and other tissues (Hamilton & Petermann, 1959; Petermann & Hamilton, 1961). Thus peaks with approximate *S* values of 45, 55, 60, 80, 110 and 140s were noted and the addition of higher magnesium ion concentrations resulted in more of the ribosome preparation sedimenting with higher *S* values.

Comparison of amino acid incorporation into ribosomes and into microsomes. Ribosomes have been prepared many times by both the methods described, and on each occasion they have shown

active incorporation of amino acids into protein when incubated *in vitro* under suitable conditions. Deoxycholate is inhibitory to the system and in order to achieve high rates of incorporation it is important to remove as much as possible of the deoxycholate before suspending the pellet in the incubation medium. Ribosomes can be washed by suspension in the tris buffer-salts solution followed by centrifuging without loss of incorporating activity, and, indeed, with some enhancement of activity probably because of the removal of last traces of deoxycholate. Table 1 records some data on the incorporation shown by microsomes and by washed and unwashed ribosomes during a 60 min. incubation experiment.

Ribosomes incorporate more amino acid/mg. of protein than microsomes. Washed ribosomes show a slightly greater specific activity than unwashed microsomes even when this is calculated per milligram of RNA incubated, which is a fairer method of comparing the activities of the ribosomes and microsomes. In earlier experiments (Korner, 1959a), before it was realized that it was important to remove as much as possible of the deoxycholate during preparation of the ribosomes even at the expense of losing some of them, the incorporation found per milligram of RNA was less in ribosomes than in microsomes. This position is reversed when washed particles are used, especially if long incorporation times are employed since ribosomes continue to incorporate for longer periods than microsomes (see below).

The radioactivity of the ribosome protein is not washed out by reincubation of the labelled ribosomes in the presence of excess of unlabelled amino acid.

Washed ribosomes resemble microsomes in requiring the addition of cell sap before full incorporation occurs and the whole cell sap can be replaced by a pH 5 enzyme preparation (Table 2). Boiled cell sap is no substitute for the fresh material. Table 2 shows that full incorporation

Table 1. *Comparison of the incorporation of DL-[1-¹⁴C]leucine into the protein of microsomes and of washed and unwashed ribosomes*

Incubation was carried out for 60 min. as described in the Methods section. Washing was by resuspension in the tris-buffered salts medium followed by centrifuging.

	Counts/min. of microsomal or ribosomal protein	Counts/min. incorporated into protein/mg. of microsomal or ribosomal RNA incubated
Microsomes	214	960
Ribosomes	632	840
Washed ribosomes	848	1085

Table 2. *Requirements for incorporation of DL-[1-¹⁴C]leucine into the protein of washed ribosomes*

The complete system is described in the Methods section. Incubation was carried out for 2 hr.

	Counts/min./mg. of ribosomal protein
Complete system	716
Soluble fraction omitted	67
pH 5 Enzyme in place of soluble fraction	655
Boiled soluble fraction in place of soluble fraction	89
GTP omitted	489
ATP omitted	309
ATP-generating system omitted	289
ATP and ATP-generating system omitted	92
Ribonuclease added (0.5 mg./ml.)	48
Deoxycholate added (0.5 %)	19
(0.1 %)	142
Puromycin added (2 μ M)	472
(20 μ M)	101
(600 μ M)	19

requires the addition of GTP and ATP but that the washed ribosomes show less dependence on an ATP-generating system than microsomes (Zamecnik & Keller, 1954) or unwashed ribosomes (Korner, 1959*a*). Further, it is possible to obtain high levels of incorporation when the ATP-generating system is omitted provided that higher ATP concentrations than the usual 1 mM are used. This point is discussed below.

Puromycin at a concentration of 10 μ M caused 80 % inhibition of incorporation (cf. Yarmolinsky & de la Haba, 1959) and 98 % inhibition was obtained with a concentration of 600 μ M. The presence of ribonuclease is, as was expected, inhibitory to the system and so is the presence of deoxycholate (Table 2).

Time course of labelling. Fig. 1 (a) compares the increase in specific activity with time of incubation of the proteins of microsomes and of washed ribosomes incubated under identical conditions. The microsomes incorporate amino acids into protein for about 30 min. and the specific activity subsequent to this time remains at a plateau (cf. Hoagland *et al.* 1956; Zamecnik & Keller, 1954), but the specific activity of ribosome protein continues to rise for 2 or 3 hr. Ribosomes prepared without the precautions designed to remove all the deoxycholate stop incorporating much earlier (Korner, 1959*a*). If the results depicted in Fig. 1 (a) are expressed as counts/min./mg. of RNA instead of counts/min./mg. of protein then it becomes clear (Fig. 1*b*) that microsomes are more rapidly labelled with radioactive amino acids than ribosomes but that they stop incorporating amino acids very much earlier than ribosomes.

During the incubation procedure labelled protein appeared in the supernatant fluid. A typical

experiment is illustrated in Table 3, which shows that when ribosomes are incubated with cell sap or with a pH 5 enzyme preparation, the protein of the supernatant fluid became progressively labelled with amino acid until about 20 % or more of the total counts were present in non-ribosomal protein. That the incorporation of amino acids into ribosomal protein and into the supernatant fluid

Fig. 1. Time course of incorporation of L-[G-¹⁴C]leucine into protein of ribosomes (●, ○) and microsomes (▲, △). (a), Expressed as counts/min./mg. of protein; (b), expressed as counts/min./mg. of RNA.

Table 3. *Appearance of radioactive proteins in the supernatant fluid during incubation of ribosomes (1.6 mg. of protein) with DL-[1-¹⁴C]leucine in the presence of either cell sap (2.2 mg. of protein) or a pH 5 enzyme preparation (2.0 mg. of protein)*

Incubation time (min.)	Incubation with cell sap			Incubation with pH 5 enzymes		
	Total counts in		Counts in cell-sap protein (% of total)	Total counts in		Counts in pH 5 enzyme protein (% of total)
	Ribosome protein	Cell-sap protein		Ribosome protein	pH 5 enzyme protein	
0	12	4.5	—	28	2	—
30	291	42	12.6	167	29	14.8
60	570	129	18.4	311	84	21.3
120	795	198	19.8	520	157	23.2

Table 4. *Effect of preincubation of ribosomes and microsomes at 37° on their subsequent ability to incorporate DL-[1-¹⁴C]leucine into protein in vitro*

Preincubation was carried out in the tris-buffered medium containing salts described in the Methods section.

Time of preincubation at 37° (min.)	Counts/min./mg.	
	Ribosome protein	Microsome protein
0	525	298
15	537	141
30	502	79
45	487	47
60	442	36
Ribosomes prepared from microsomes which had been preincubated for 30 min.	168	

protein was larger in the experiment in which cell sap was used than in the one when pH 5 enzyme was used is probably not significant.

All experiments in which long incubation times were used were carried out in the presence of 100 units of penicillin and 40 µg. of chloramphenicol/1 ml. of incubation mixture, and the absence of serious bacterial contamination was noted when samples of the incubation mixture after incubation were plated on a tryptic digest of casein supplemented with Marmite and glucose.

Stability of ribosomes. That incorporation of amino acids into protein occurs in ribosomes for longer periods of time than in microsomes argues that ribosomes are much more stable than microsomes. Preincubation experiments confirm this suggestion. Ribosomes, or microsomes, suspended in the buffered tris-salts medium were shaken at 37° for various periods before adding the cell sap, ATP, ATP-generating system, GTP and radioactive amino acid. The mixture was then incubated for 2 hr. and the results are shown in Table 4. Microsomes are extremely sensitive to exposure to 37° and a big fall in incorporating ability is found within 15 min. of preincubation, but the ribosomes show little decrease in incorporating ability until

after an hour of preincubation. Ribosomes prepared from preincubated microsomes show diminished ability to incorporate amino acids into protein.

Washed ribosomes can be stored at 0° (in tubes standing in crushed ice in a vacuum flask kept in the refrigerator) for at least a week without substantial fall in their ability to incorporate amino acids into protein (cf. Kirsch *et al.* 1960).

Freezing the ribosomes in acetone-carbon dioxide and then thawing them resulted in a variable fall in their ability to incorporate amino acids into protein which ranged from 10 to 40% of that of the unfrozen ribosomes. It appeared that rapid freezing is less damaging than slow freezing and some further loss in incorporating ability occurs on storage at -15°. Addition of the soluble fraction before freezing does not, to any substantial extent, protect the ribosomes against the damage caused by freezing, although it has been reported that microsomes are protected by this means (Sachs, 1957). Ribosomes can be dialysed in the cold for 24 hr. with variable (5-30%) loss of activity.

Effect of magnesium ion concentration on the S values of ribosomes and on amino acid incorporation into protein of ribosomes. Washed rat-liver ribosomes were suspended in 0.03M-tris buffer, pH 7.5, and various concentrations of magnesium acetate added to separate samples of it, which were then examined in the analytical ultracentrifuge. The S values of the peaks in the schlieren diagrams were determined and the area under each peak was estimated. As expected (Hamilton & Petermann, 1959; Petermann *et al.* 1958; Petermann & Hamilton, 1961), more of the larger sizes of ribosomes were noted in the presence of high concentrations of magnesium ions and when no magnesium was added more of the smaller sizes and less of the larger sizes of ribosomes were apparent. Ribosomes bind considerable amounts of magnesium (Petermann & Hamilton, 1961) so that, even when no magnesium had been added to the suspending medium, some was still present.

The extent of amino acid incorporation into ribosomes under the usual conditions but at different magnesium ion concentrations was determined and the results (Fig. 2) showed a marked dependence on the concentration of this ion with a sharp optimum at an added concentration of 5 mM. The experiment depicted in Fig. 2 was carried out in the presence of an ATP-generating system and at only one ATP concentration (1 mM). This magnesium ion concentration is one at which the largest proportion of the ribosomes appeared in the 80s form. It is tempting to use this rough correlation between extent of incorporation and 80s form to suggest that this is the incorporating size of ribosome but several factors counsel against the acceptance of this facile assumption (see Discussion).

Concentration of adenosine triphosphate and amino acid incorporation into ribosomes. As mentioned above, the ATP-generating system is not obligatory for amino acid incorporation into ribosomes as it is for incorporation into microsomes. Fig. 3 shows the results of incorporation experiments in which the creatine phosphate and creatine phosphokinase were omitted and in which various concentrations of ATP containing equimolar magnesium chloride were present. The incubation mixture had in addition the usual amount of magnesium (5 mM). Incorporation at a rate which was at least as high as that obtained with the complete incubation mixture could be obtained in the absence of an ATP-generating system provided that about five times the usual ATP concentration (plus equimolar magnesium) was used (i.e. 5 mM instead of 1 mM). This concentration of ATP appeared to be optimum, higher concentrations causing inhibition of incorporation.

However, the excess of ATP might be causing inhibition because the magnesium added with it, together with the magnesium in the incubation mixture, brings about the inhibition (cf. Fig. 2). To test this possibility, incorporation of amino acids into ribosomes was measured at a variety of ATP concentrations each at various magnesium ion concentrations and the results are shown in Fig. 4. At each magnesium concentration, the amount of ATP that gives the highest incorporation is approximately equimolar and even small deviations either side of equimolarity cause large changes in ability to incorporate amino acids into protein. It is clear that optimum incorporation is obtained with 0.01M-ATP and 0.01M-magnesium ions. Although higher concentrations of ATP (plus equimolar magnesium) are inhibitory, very high concentrations of ATP, which are by themselves almost completely inhibitory (see Fig. 3), can be tolerated to some extent if equimolar magnesium is present.

A series of curves comparable with those of Fig. 4 can be drawn in which incorporation at various magnesium concentrations is plotted, each at a variety of ATP concentrations. They show that ATP can protect to some extent against the inhibition of incorporation caused by high magnesium concentrations (cf. Fig. 2).

Need for other amino acids before incorporation can occur. One of the criteria that must be satisfied before amino acid incorporation in a cell-free system can be regarded as an indication of protein biosynthesis is that the system must be shown to need a supply of amino acids other than the radioactive one. Some systems satisfy this condition, notably the disrupted bacterial system (Gale & Folkes, 1955) the pea-seedling-ribosome system (Webster, 1959) and the mammalian mitochondrial system (McLean, Cohn, Brandt & Simpson, 1958). This requirement has not been met for the liver microsomal system, and it has been assumed that this system contains sufficient free amino acid to supply the materials needed for protein synthesis.

Although boiled cell sap was no substitute for fresh cell sap, addition of boiled cell sap to the complete system resulted in enhanced incorporation of the radioactive amino acid, especially if only

Fig. 2. Effect of concentration of magnesium ions on the incorporation of DL-[1-¹⁴C]leucine into ribosome protein.

Fig. 3. Effect of concentration of ATP on incorporation of DL-[1-¹⁴C]valine into ribosome protein. The complete system (see Methods section) minus the ATP-generating components was used. ATP and an equimolar amount of magnesium chloride were added. Final concentrations shown (see text).

small concentrations of pH 5 enzyme or of fresh cell-sap protein were present. The rise may possibly be caused by the addition of amino acids present in the boiled cell sap to the incubation mixture.

In an attempt to demonstrate more directly the need for amino acids other than the radioactive one in the ribosome system, ribosomes and pH 5 enzyme preparations were dialysed overnight at 4° to deplete them of free amino acids. The incorporation obtained with such systems was about 60–90 % of that of the fresh system and in some experiments addition of an amino acid mixture at a concentration of 0.01–2 mg./ml. and lacking the labelled amino acid did cause some enhancement of incorporation (see Table 5). In other experiments, however, especially when incorporation after dialysis was quite high, little or no enhancement of incorporation was obtained on adding unlabelled amino acids. An estimation of the free amino acid content of the ribosomes and pH 5 enzymes after dialysis revealed that during dialysis the free amino acid content fell and then rose again. Presumably as amino acids are dialysed out of the ribosomes or cell-sap suspensions, protein breaks down to replace the free amino acids.

Attempts were made to free concentrated preparations of ribosomes and pH 5 enzymes from amino acids by treatment with various ion-exchange resins and also with Sephadex (G25, from Pharmacia, Uppsala, Sweden). After filtering off the resin or Sephadex, the ribosomes and pH 5 enzyme effluents were diluted with buffered tris medium containing salts at concentrations calculated to give the molarities in the final incubation mixture which are usually used. Little incorpora-

Fig. 4. Effect of concentration of ATP on incorporation of DL-[1-¹⁴C]leucine into ribosome protein at different concentrations of magnesium ions. The complete system minus the ATP-generating components was used. Concentrations of magnesium ions (mM): ○—○, 2; ●, 5; □, 10; ■, 15; △, 20; ▲, 30; ○—○, 40.

Table 5. Effect of adding an amino acid mixture on incorporation of L-[G-¹⁴C]leucine into ribosome protein in a ribosome-pH 5 enzyme system which has been dialysed

The amino acid mixture consisted of 1 part of histidine, hydroxyproline, cysteine and tryptophan; 2 parts of methionine, arginine, tyrosine, isoleucine, aspartic acid, glycine, alanine, proline, threonine, glutamine and asparagine; 4 parts of lysine, serine, valine and glutamic acid; all in the L-form.

Amount of amino acid mixture added (mg./ml.)	Counts/min./mg. of ribosome protein
0	312
0.01	419
0.1	432
1.0	369
2.5	218
5.0	94
10.0	32

tion of radioactive amino acid occurred after this treatment, and, although some enhancement of incorporation was observed when an amino acid mixture was added, it was clear that inactivation of the ribosomes and pH 5 enzymes had occurred during treatment with the resins, for, even when optimum concentrations of amino acids were added, the incorporation obtained was small compared with that of the untreated systems.

High concentrations of the amino acid mixture (lacking the radioactive amino acid) were inhibitory to the system (see Table 5). The degree of inhibition reached 90% in some experiments, but the amount of amino acid mixture needed to cause inhibition to this extent varied from experiment to experiment in a way that is related to the amount of cell-sap protein or pH 5 enzyme protein used. Inhibition of incorporation of L-[G-¹⁴C]-leucine was also obtained with high concentrations of mixtures containing only eight of the amino acids, and in one experiment 1.0 mg. of isoleucine/ml. alone was sufficient to cause 40% inhibition, but a similar concentration of valine did not cause any inhibition of incorporation.

DISCUSSION

The results presented in this paper confirm the earlier reports (Korner, 1959*a*, 1960*a*) that it is possible to prepare particles from rat liver which are identical with the morphological entities, microsomal particles (Palade, 1955, 1958) or ribosomes (Roberts, 1958), and that these particles will incorporate amino acid into protein *in vitro*. Since the earlier report (Korner, 1959*a*), Takanami (1960) has described the preparation of ribosomes from rat liver by precipitation with bivalent cations and the ribosomes were shown to incorporate amino acids into protein. Rendi & Hultin (1960) used deoxycholate containing magnesium to prepare ribosomes from rat liver and Kirsch *et al.* (1960) report the separation of ribosomes from guinea-pig liver. Both of these preparations of ribosomes can incorporate amino acids into protein *in vitro*. In addition, incorporation of amino acids into protein *in vitro* has been obtained with ribosomes from *E. coli* (Tissière *et al.* 1960) and pea seedlings (Webster, 1959).

Preparation of ribosomes. In order to obtain incorporation of amino acids into ribosome protein it is important to remove as much as possible of the deoxycholate from the ribosome pellet before incubation because of the inhibitory action of the detergent. Littlefield *et al.* (1955) appear to have been in the practice of taking up the loose ribosome pellet together with some of the supernatant fluid, and the 0.5% deoxycholate contained in the fluid would probably account for their finding that the

particles were inactive (Littlefield & Keller, 1957). These workers were clearly aware of the inhibitory nature of deoxycholate and themselves suggest that this inhibition might account for their inability to find incorporation of amino acids into microsomal particle protein.

The addition of magnesium ions at a concentration of 5 mM to the sucrose used to homogenize the liver, and the presence of this amount of magnesium throughout the course of preparation of ribosomes, did not appear to raise the amount of amino acid incorporated into ribosomes in the subsequent experiments. The yield of ribosomes did, however, appear to be somewhat greater when magnesium was present and this is understandable in view of the fall in the proportion of ribosomes that are of low *S* values with increasing magnesium ion concentration (Petermann *et al.* 1958; Petermann & Hamilton, 1961). It is to be expected that a little less of the ribosomes would spin down during the preparative procedures in the absence of magnesium.

Stability of ribosomes. Ribosomes are more stable than microsomes. They can be preincubated, stored at 0°, and can be dialysed without substantial loss of incorporating ability, and they continue to incorporate amino acid into protein at 37° for a much longer time than the microsomes from which they are prepared. Kirsch *et al.* (1960) report that guinea-pig-liver ribosomes can be stored at -10° without loss of activity but that they lose most of their activity if stored overnight at 0°. This is opposite to the findings with rat-liver ribosomes reported in this paper.

Requirements for incorporation. Ribosomes are like microsomes in requiring for full incorporation the presence of GTP, ATP and either the cell sap or pH 5 enzymes. They are very sensitive to the presence of the correct concentration of ions and are especially sensitive to the concentration of magnesium. The optimum pH is between 7.3 and 8.0 (cf. Takanami, 1960). Unlike the microsome system, however, an ATP-generating system is not necessary provided extra ATP and magnesium, over and above that usually used in experiments with microsomes and ribosomes, is added. It is possible that adenosine-triphosphatase activity is removed from microsomes along with the lipoprotein of the endoplasmic reticulum when ribosomes are prepared. Takanami (1960), Rendi & Hultin (1960), Kirsch *et al.* (1960) and Tissière *et al.* (1960) all report the need for an ATP-generating system in their various ribosome systems, but in each case they used 1 mM-ATP in the experiments in which an ATP-generating system was omitted. It would be of interest to learn if higher ATP concentrations (together with, of course, the correct amount of magnesium) would show that a ATP-

generating system was not needed in their ribosome systems. Littlefield & Keller (1957) found that ribosomes isolated from ascites-tumour cells did not require an ATP-generating system.

Cohn (1959) reports that addition of cell sap is not necessary for amino acid incorporation into protein of particles prepared from microsomes with a non-ionic detergent, Lubrol-W. He suggests that the amino acid-activating enzymes are attached to microsomes and are made more accessible by the Lubrol treatment. It is further suggested that soluble RNA is not essential as an intermediate between the activated amino acids and the Lubrol or perfluoro-octanoate pellet (Cohn, 1959), or between activated amino acids and microsomes (Rendi & Campbell, 1959). The experiments reported in this paper make it clear that washed ribosomes do require added cell sap and do not have activating enzymes attached to them. The inhibition of incorporation by puromycin suggests that soluble RNA is an obligatory intermediate for incorporation into protein in this system (Yarmolinsky & de la Haba, 1959).

It is disappointing that a complete dependence of incorporation on the presence of a full complement of amino acids could not be demonstrated, but the evidence that was obtained was suggestive of such a dependence. Kirsch *et al.* (1960) report a similar failure to show stimulation of incorporation on adding an amino acid mixture to ribosomes from guinea-pig liver. The inhibition of incorporation by high concentrations of amino acid mixture, and even by one amino acid, is intriguing and is supported by similar observations of Kirsch *et al.* (1960) and Tissière *et al.* (1960).

Transfer of protein from ribosomes. Radioactively labelled protein could be detected in the supernatant fluid during incubation and the supernatant protein became progressively more highly labelled throughout the incubation. It is possible that it arises because breakdown of the ribosomes occurs during incubation, but the stability of ribosomes suggests that the labelled protein is transferred from the ribosomes to the supernatant fluid.

Effect of concentration of adenosine triphosphate and magnesium on incorporation. In the experiment in which various ATP and magnesium concentrations were added, the dual role of magnesium and of ATP becomes clear (Figs. 2-4). With 1 mM-ATP (Fig. 2) concentrations of magnesium below 5 mM are less efficient than the optimum concentration in causing amino acid incorporation, possibly because magnesium is needed for amino acid activation (cf. Kubovitz & Ott, 1944). Above 5 mM-magnesium ions inhibition occurs unless extra ATP is added to use up some of the magnesium. By the same token, in the absence of an

ATP-generating system at least 5 mM-ATP is required (Fig. 3), for this concentration was optimum even though what would appear to be inhibitory amounts of magnesium ions were added with it. In this experiment the magnesium concentration was greater than equimolar and so adding concentrations of ATP + magnesium ions greater than 5 mM were inhibitory possibly because of the addition of the extra magnesium. Where the ATP and magnesium concentrations keep pace with one another (Fig. 4), 0.01 M of each is optimum. High ATP and magnesium concentrations can be added in the presence of the other without completely inhibiting incorporation showing the protection each affords against the other. They are, however, both inhibitory at very high concentrations even in the presence of the other for reasons which can be speculated about but are not clear.

S of ribosomes and incorporation. It is generally agreed that the state of aggregation of ribosomes from many sources is dependent on the ionic strength, composition and pH of the suspending medium (Petermann *et al.* 1958; Hamilton & Petermann, 1959; Petermann & Hamilton, 1961). The role of magnesium ion concentration is very marked. In rat-liver ribosomes, 80s particles can be aggregated in high magnesium ion concentration to 110s and larger particles and 80s ribosomes break down on lowering the magnesium concentrations to 60s, 55s, 45s and smaller particles. Somewhat similar results were obtained by measuring *S* values at different magnesium concentrations in experiments described in this paper. It is, however, difficult to say what sizes of ribosome are actually present during incorporation from experiments of this type, since the other components of the incubation mixture, especially the salts, ATP, GTP and soluble RNA, will effect the state of ribosome aggregation. In addition the concentration of ribosomes used, with the attached magnesium ions (Petermann & Hamilton, 1961), is an important variable. It can be calculated from the work of Petermann & Hamilton (1961) that the ribosomes in a 5 mg./ml. suspension will carry enough magnesium with them to produce an approximately 1 mM concentration.

It is for these reasons that care must be taken not to jump to the conclusion that, because at 5 mM-magnesium ion concentration, good amino acid incorporation is obtained and a high proportion of 80s particles are present that the 80s particle is the incorporating form. At this magnesium concentration in the full incubation mixture ultracentrifugal studies of the whole incubation mixture reveal a variety of particles and the change in the proportion of those present is small when the magnesium concentration is altered

sufficiently to give a very big fall in incorporation. It is possible that incorporation is carried out by a small number of particles of a particular size (Tissière *et al.* 1960) the presence of which is masked by other non-incorporating particles of the same S.

Identity of the particles. That the particles studied in the experiments described here are indeed ribosomes seems certain from their RNA:protein ratios, their sedimentation characteristics and from the differences in behaviour between them and the microsomes from which they are derived. Their stability and their ability to transfer a fair proportion of labelled protein to the supernatant during incubation suggest that this system may prove a useful one for study of the mechanism of protein biosynthesis in mammalian tissues.

It is of course realized that no proof that ribosomes can synthesize protein *in vitro* has been presented but preliminary studies have demonstrated their ability to incorporate amino acid into a specific protein (Korner, 1960*b*).

SUMMARY

1. Ribosomes with a high ribonucleic acid:protein ratio have been prepared from rat liver by two methods which are described. They have been shown to incorporate radioactive amino acids into protein *in vitro*.

2. Ribosomes incorporate more amino acid into protein (calculated per mg. of protein or per mg. of ribonucleic acid) than microsomes and they continue to incorporate amino acids for 2 or 3 hr.

3. Adenosine triphosphate, guanosine triphosphate and the soluble fraction of the cell (or a pH 5 enzyme preparation) are required for incorporation but an adenosine triphosphate-generating system is not needed.

4. Ribosomes are more stable than microsomes, being able to withstand preincubation at 37° for an hour, dialysis, and storage at 0° for at least a week without loss of activity.

5. Labelled protein is transferred from the ribosome to the supernatant fluid during incubation.

6. The concentrations of magnesium ions and adenosine triphosphate that give maximal incorporation are very critical, but large amounts of either, which in themselves are inhibitory, can be tolerated to some extent if equimolar amounts of the other are present.

I thank the Medical Research Council for a grant towards the costs of this work, The British Drug Houses Ltd. for a grant for technical assistance and Mrs B. Jones, B.Sc., for highly skilled assistance. It is a pleasure to acknowledge the interest and encouragement of Professor F. G. Young, F.R.S.

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The Chemistry of Xanthine Oxidase

7. THE ANAEROBIC REDUCTION OF XANTHINE OXIDASE STUDIED BY ELECTRON-SPIN RESONANCE AND MAGNETIC SUSCEPTIBILITY*

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(Received 9 January 1961)

The reduction of xanthine oxidase by substrates and other reducing agents is accompanied by complex changes in the visible absorption spectrum. Part of the change occurs rapidly and can be correlated with the overall kinetics of the enzyme-catalysed reaction (Gutfreund & Sturtevant, 1959), whereas part occurs much more slowly and appears to be due to the reduction of 'inactive' xanthine oxidase (Morell, 1952). The stoichiometry of the optical changes was studied by Totter & Comar (1956). Bray, Malmström & Vänngård (1959) showed that electron-spin-resonance signals arise when the enzyme is treated with xanthine or sodium dithionite, and the present work consists of a quantitative extension of these studies. It is shown that the development of the signals on reduction of the enzyme consists of rapid and slow phases, whose rate and extent can be correlated with the nature and concentration of the substrate or other reducing agent and the composition of the xanthine-oxidase sample. The results provide information on the chemical nature of the reduced forms of the enzyme that may participate in the xanthine-oxidase reaction and in differences between the 'active' and 'inactive' modifications. Electron-spin-resonance and magnetic-susceptibility measurements have provided additional information on the state and function of the iron of the enzyme.

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EXPERIMENTAL

Reagents and buffers. The purity of the substrates, xanthine (from F. Hoffmann-La Roche and Co. Ltd., Basle, Switzerland), purine (from L. Light and Co. Ltd.) and salicylaldehyde, was checked by absorption measurements before and after complete oxidation by xanthine oxidase. The concentration of reduced diphosphopyridine nucleotide (DPNH; from Sigma Chemical Co., U.S.A.) was determined from the absorption at 340 m μ . Sodium dithionite solutions (1–10 mM) were prepared by dissolving the solid in pyrophosphate buffer, while bubbling nitrogen. The solution was immediately drawn into an all-glass micro-meter syringe and standardized by titration with approx. 30 μ m-moles of riboflavin phosphate (F. Hoffmann-La Roche and Co. Ltd.) in 2 ml. total volume, the end point being detected by the sharp disappearance of fluorescence under an ultraviolet lamp. Nitrogen was bubbled during the titration and a drifting end point, due to reoxidation, was corrected for by extrapolating to zero time. The concentration of riboflavin phosphate was determined from $\epsilon_{450} = 12\,200$ (Whitby, 1953). Molybdenum pentachloride and molybdenum trichloride dibenzoate were obtained from the Climax Molybdenum Co., New York. A solution of potassium octacyanomolybdenum (v) $[K_2Mo(CN)_8]$ was prepared by oxidizing potassium octacyanomolybdenum (rv) $[K_4Mo(CN)_8]$ with ceric sulphate (Weissman & Cohn, 1957) and a solution of oxotrichiocyanatomolybdenum $[MoO(SCN)_3]$ by dissolving $MoCl_5$ in a mixture of hydrochloric acid, acetone and KSCN (Perrin, 1958). Pyrophosphate buffer (0.05M, pH 8.0–8.2) was prepared by adjusting the pH of the tetrasodium salt with HCl.

Xanthine-oxidase solutions. These were prepared by the ammonium sulphate–butanol procedure, followed by calcium phosphate chromatography and in some cases by electrophoresis or low-temperature ethanol fractionation, as outlined by Haddow, de Lamirande, Bergel, Bray &