Properties of Ribosomal Proteins from Two Mammalian Sources

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1. Ribosomal protein fractions from rabbit reticulocytes and rat liver were prepared by extracting ribosomes with 0.2N-hydrochloric acid or guanidinium chloride and subsequent dialysis. 2. Treatment for 2.5 hr. or less with 0.2N-hydrochloric acid dissolved 46–54% of the proteins, which were richer in arginine and lysine and in *N*-terminal alanine groups and poorer in aspartic acid and glutamic acid and in *N*-terminal glycine groups than the acid-insoluble proteins. 3. Protein fractions prepared from the guanidinium chloride extract of ribosomes from rat liver were usually more basic than those from rabbit reticulocytes. 4. The ratios lysine: arginine of fractions in the guanidinium chloride extracts were appreciably higher for proteins from rabbit reticulocytes than from rat liver. 5. The concentration of urea and the pH of the gel affected the rate of migration and number of bands in starch-gel electrophoresis.

Ribosomes from different sources all contain 40–60% of protein; overall amino acid composition of the proteins is similar (Ts'o, Bonner & Dintzis, 1958; Cohn, 1962; Spahr, 1962; Mathias & Williamson, 1964). However, this similarity is accompanied by the occurrence of different *N*-terminal amino acid residues in the proteins of different cells. Thus ribosomal proteins from *Escherichia coli* are characterized by the presence of alanine and methionine (Waller & Harris, 1961) and those from the buds of pea seedlings possess alanine and valine mainly (Setterfield, Neelin, Neelin & Bayley, 1960). In rat liver, four *N*-terminal groups have been detected: alanine, glycine, proline and serine (Butler, Cohn & Simson, 1960; Cohn, Simson & Butler, 1960); the presence of the same *N*-terminal amino acid residues has been observed in ribosomal proteins from another mammalian source: rabbit reticulocytes (Cohn, 1962). This common property would suggest a similarity of polypeptide chains. The possible existence of such a relationship has been examined by several techniques, the results of which are described below.

MATERIALS AND METHODS

All chemicals used were of analytical reagent quality where available. Solutions of guanidinium chloride were prepared by the action of conc. HCl on guanidinium carbonate A.R. (Cox & Arnstein, 1963). The preparation of subcellular fractions and the fractionation of proteins were carried out at 2–4°.

Induction of reticulocytosis. Rabbits weighing 2.0–3.5 kg.

were injected intermuscularly or intraperitoneally with a neutralized solution of 2.5% (w/v) phenylhydrazine hydrochloride (8.3 mg./kg. body wt.) daily five or six times during a period of 6 or 7 days.

Preparation of reticulocyte ribosomes. Reticulocytes were washed, lysed and ribosomes separated by differential centrifugation (Schweet, Lamfrom & Allen, 1958). The washed pellets were either used immediately or stored at –20°.

Preparation of ribosomes from rat liver. Ribosomes were isolated from the microsome fraction of starved albino rats by the action of sodium deoxycholate according to method 1 of Cohn & Simson (1963).

Preparation of Lubrol-deoxycholate pellet. The volumes employed in the method of Rendi & Hultin (1960) were adjusted in such a way that the mitochondrial supernatant liquid (16.6 ml.) was treated with solutions of 2.5M-KCl–0.01M-MgCl₂ (4.8 ml.), 10% (w/v) Lubrol W–0.01M-MgCl₂ (1.2 ml.) and 10% (w/v) sodium deoxycholate (2.4 ml.) and then layered over 50 ml. of a solution (placed in a tube for the no. 21 rotor of the Spinco model L centrifuge): 0.3M-sucrose, 0.6M-KCl, 0.01M-MgCl₂, 0.035M-tris adjusted to pH 7.8 with N-HCl. Ribosome pellets were obtained by centrifuging at 44 000 *g*_{av} for 2.5 hr.

Extraction of basic proteins. Ribosome pellets (5–12 mg. of ribonucleoprotein) were extracted with 0.2N-HCl and protein was isolated by centrifuging, dialysing the extract and precipitating with trichloroacetic acid (Cohn & Simson, 1963). When the period of extraction employed was 1.5 min. the pellets, together with 0.2N-HCl (2.0–2.5 ml. for each pellet), were placed in a Potter-Elvehjem-type homogenizer and homogenized by carrying out 15–20 up-and-down strokes during that period. The suspension was then centrifuged in the usual way (Cohn & Simson, 1963).

Treatment of ribosomes with guanidinium chloride. Ribosomes were extracted twice with guanidinium chloride

(Cox & Arnstein, 1962). Samples of ribosomes from rat liver required the addition of 0.5 vol. of ethanol to precipitate RNA. The two extracts from a sample were pooled and dialysed in Visking tubing (20/32 in.) against water at 3° on a rocking apparatus. A precipitate (GS) soon formed, which was separated from the soluble protein (GL) by centrifuging at 20000 g_{av} for 10 min. after the diffusate had become free from guanidinium chloride. Fraction GS was washed with acetone and dried *in vacuo*. The diffusate was either freeze-dried or treated with trichloroacetic acid (100%, w/v) to give a final concentration 15% (w/v) and the proteins (fraction G) were converted into their hydrochlorides.

Treatment of hydrochloric acid-insoluble residues with guanidinium chloride. The residue left after extraction of ribosomes with 0.2N-HCl was resuspended in a solution containing 0.05M-tris adjusted to pH 7.6 with N-HCl, 0.025M-KCl and 0.005M-MgCl₂. A sufficient volume of 0.1N-KOH was added to raise the pH to 7.6. The method of extraction of protein with guanidinium chloride and the

isolation of fractions HGL and HGS corresponded to the details given for the treatment of ribosomes.

Analysis of ribosomes and proteins. Estimation of RNA and total protein of pellets and HCl-insoluble residues and determination of nitrogen and phosphorus were performed by the techniques described previously (Cohn & Butler, 1958). Amino acid and N-terminal group analyses were carried out by a modification of the fluorodinitrobenzene method of Sanger (1945) as described by Phillips (1958) and Phillips & Johns (1959).

Starch-gel electrophoresis. A gel was prepared from starch hydrolysed according to the method of Smithies (1955) or obtained from the British Drug Houses Ltd. ('Starch, hydrolysed for starch-gel electrophoresis'). If urea was to be included in the gel it was added to the warm suspension (45–50°) just before gelling began. Solutions of 0.02M-sodium acetate were adjusted to pH 3.5 with N-HCl. The protein sample was dissolved in the same solution as that used for preparing the starch gel. Electrophoresis was

Table 1. *Amino acid composition of ribosomal proteins of rabbit reticulocytes*

The amino acids are expressed as moles/100 moles of all amino acids found and no correction has been made for the hydrolytic losses of amino acids. Amide and tryptophan were not determined. The proportions of N-terminal groups are molar percentages of all such groups found. The values represent the means of the stated number of samples analysed.

No. of preparations		...	...	3	1	1†	1‡	3‡		
Amino acids										
Aspartic acid	} acidic			19.5	{	8.6	8.2	10.0	—	
Glutamic acid						10.1	9.7	10.3	—	
Glycine						7.6	11.7	6.9	—	
Alanine						8.4	7.5	8.7	—	
Valine	}			7.0		6.7	7.6	7.6	—	
Leucine						{	8.2	8.4	—	
Isoleucine							5.4	5.1	—	
Serine						—	5.2	—		
Threonine						4.9	4.7	5.2	—	
Phenylalanine						3.9	3.0	3.3	—	
Tyrosine						2.5	1.9	4.4	3.0	—
Proline						5.3	4.9	5.0	4.8	—
Tryptophan				—	—	0.7	0.6	—		
Cystine/cysteic acid				—	0.6	0.6	2.1	—		
Histidine	} basic			{	2.1	2.5	2.2	2.4	—	
Lysine					11.0	11.1	10.7	9.4	—	
Arginine					7.9	8.6	8.4	6.1	—	
Basic: acidic ratio				1.08	1.19	1.19	0.88	—		
<i>N</i> -Terminal amino acids										
Alanine				—	15	—	—	13		
Glycine				—	27	—	—	9		
Methionine				—	—	—	—	56		
Proline				—	28	—	—	—		
Serine				—	14	—	—	—		
Threonine				—	4	—	—	—		
Valine				—	5	—	—	15		
Others*				—	7	—	—	—		
Unidentified				—	—	—	—	7		
Wt. (g./mole of <i>N</i> -terminal groups)				—	30000	—	—	12700		

* These include aspartic acid, glutamic acid, lysine and threonine.

† Ts'o *et al.* (1958). These authors stated that the values for glycine were probably too high and those for serine unreliable.

‡ Mathias & Williamson (1964).

carried out in a Perspex tray (Smithies, 1955). Strips of paper (Whatman 3MM) dipped into the electrode compartments containing 2l. of dilute hydrochloric acid or buffer. The potential difference across the starch gel in each channel (19mm. wide, 6mm. deep) was 5v/cm., producing a current of 4–10 ma. At the end of the experiment a fall in pH was noticed near the anode end and a rise near the cathode end. These changes were observed to be accompanied by an increase in the voltage and a decrease in the current. Gels were stained with a saturated solution of Naphthalene Black 10B or Nigrosine in methanol–water–acetic acid (5:5:1, by vol.).

RESULTS

In Table 1 the amino acid composition of ribosomal protein, the starting material used in the present investigation, is shown. In these samples the content of arginine and lysine is approximately equal to that of aspartic acid and glutamic acid. Cysteine and cystine are virtually absent. The

principal *N*-terminal groups found are alanine, glycine, proline and serine. The values for all amino acids except tyrosine agree with those obtained by Ts'o *et al.* (1958); but the analysis published by Mathias & Williamson (1964) contains higher figures for aspartic acid, glutamic acid and cystine/cysteic acid and lower figures for the basic amino acids, particularly arginine, than those presented in the first three columns of Table 1. It should be noted that Mathias & Williamson (1964) observed methionine to be the *N*-terminal group of more than half of the peptide chains present.

Table 2 shows the results of extracting ribosomal proteins with 0.2*N*-hydrochloric acid. The extracted proteins can be distinguished from the insoluble proteins by their increased content of basic amino acids, particularly lysine, and their reduced content of aspartic acid and glutamic acid. Also, the proportions of glycine, phenylalanine and

Table 2. *Amino acid composition of basic and hydrochloric acid-insoluble proteins from ribosomes of rabbit reticulocytes and rat liver*

The amino acids are expressed as moles/100 moles of all amino acids found and no correction has been made for the hydrolytic losses of amino acids. Amide was not determined. The proportions of *N*-terminal groups are molar percentages of all such groups found.

Period of extraction (hr.)	Rabbit reticulocytes						Rat liver	
	1	then 5	22	0.5	then 16		0.025	0.025
Protein fraction	Basic	Basic	HCl-insoluble	Basic	Basic	HCl-insoluble	Basic	Basic
Amino acids								
Aspartic acid	18.4	19.3	23.6	17.8	18.1	22.4	8.0	8.3
Glutamic acid								
Glycine	8.7	7.9	11.1	7.8	8.1	7.0	7.9	7.0
Alanine	9.2	8.6	7.8	8.2	9.0	7.8	9.0	8.3
Valine	8.4	8.6	7.1	19.8	20.8	7.6	8.2	8.4
Leucine and isoleucine	12.5	13.8	11.2					
Serine	6.0	4.5	6.3	5.9	5.9	6.4	5.7	5.7
Threonine	5.2	4.7	4.9	4.8	4.8	4.8	4.8	4.9
Phenylalanine	3.3	3.4	3.2	2.6	2.7	4.3	3.4	3.0
Tyrosine	1.3	1.1	2.1	1.7	1.4	2.2	1.8	2.7
Proline	5.3	4.9	3.9	4.8	5.2	5.1	5.7	4.8
Histidine	1.9	1.9	2.3	2.4	2.2	2.2	2.2	2.3
Lysine	12.8	12.9	8.4	13.5	12.2	8.5	11.9	13.7
Arginine								
Cystine (half)	—	—	—	0.5	0.1	0.3	0.6	0.3
Basic : acidic ratio	1.18	1.20	0.80	1.46	1.32	0.80	1.40	1.36
<i>N</i>-Terminal amino acids								
Alanine	—	—	4	24	—	6	23	28
Glycine	—	—	32	12	—	32	16	13
Proline	—	—	38	43	—	32	37	27
Serine	—	—	7	6	—	11	9	17
Valine	—	—	8†	5	—	6	7†	7†
Others*	—	—	11	10	—	13	8	8
Wt. (g./mole of <i>N</i> -terminal groups)	—	—	100 000	39 000	—	56 000	26 000	24 000

* These include aspartic acid, glutamic acid, leucine and isoleucine, lysine and threonine.

† Leucine and isoleucine did not separate from valine and are therefore included in this value.

tyrosine vary, but they do not assume a recognizable pattern. The two fractions are further characterized by the distribution among peptide chains of the principal *N*-terminal amino acids, alanine, glycine and proline. The basic proteins are poor in glycine whereas the hydrochloric acid-insoluble proteins have a low content of alanine *N*-terminal groups. In both fractions proline *N*-terminal groups are abundant. The last two columns of Table 2 show the similarities between the composition of amino acids and *N*-terminal groups of basic proteins from ribosomes of rat liver and of rabbit reticulocytes.

The results in Table 3 indicate that short periods of extraction yield a somewhat greater proportion of protein than long periods. The ultraviolet-absorption spectra of the extracted and dialysed proteins have maxima at 275 m μ and minima at about 250 m μ . The $E_{275}:E_{260}$ ratio is about 1.50 for the first extract but decreases in samples obtained by further extractions.

Treatment with guanidinium chloride solubilizes all the protein moiety of reticulocyte ribosomes and over 80% of the proteins of ribosomes of rat liver; RNA remains insoluble (Cox & Arnstein, 1962). The composition of the protein fractions obtained by extracting whole ribosomes and their hydrochloric acid-insoluble residue with guanidinium chloride is shown in Table 4. The fraction remaining soluble (GL) on completion of the dialysis amounts to 17–23% of the proteins extracted from ribosomes from both sources. Fraction GL from rabbit reticulocytes is richer in aspartic acid and glutamic acid but poorer in arginine and lysine than that from rat liver. In fraction GS the proportions of aspartic acid and glutamic acid are the same, but fraction GS as well as GL from reticulocytes possess similar, higher, lysine:arginine ratios than those from rat liver. When the fractions prepared from the hydrochloric acid-insoluble residue of reticulocyte ribo-

somes are examined the fraction soluble in guanidinium chloride but subsequently insoluble in water and also the final residue have the same ratio of basic to acidic amino acids, which is lower than that for the corresponding fractions from rat liver. The lysine:arginine ratios, although different for fractions from the same tissues, are yet again higher in rabbit reticulocytes than in rat liver. The principal *N*-terminal groups of the final residue are glycine and proline.

Electrophoresis in starch gel made up in dilute hydrochloric acid and urea gave patterns of bands that varied with the pH and the concentration of urea. The basic proteins from ribosomes of rat liver and rabbit reticulocytes migrated little in a gel containing 0.01*N*-hydrochloric acid (pH 2.3) only, whereas histone fractions from calf thymus separated well over a distance of 3.5 cm. (Fig. 1*a, b, c*). It was found necessary to include urea in the gel to cause migration of the fastest components to a distance of 5–7 cm. During the early part of this investigation gels were always prepared in 0.02*N*-hydrochloric acid–6*M*-urea (pH 3.1). Samples from both rat liver and rabbit reticulocytes gave 9–15 bands, the fastest of which ran approx. 5 cm. (Fig. 1*d, e*). When the pH of the gel was raised to 3.45 by reducing the concentration of hydrochloric acid (0.01*N*) not more than six bands were visible, which had moved to distances not greater than 3.5 cm. The pattern of bands observed in a gel containing 4*M*-urea and 0.02*N*-hydrochloric acid (pH 2.85) was very similar, though spread over an increased distance, to that in 6*M*-urea and 0.02*N*-hydrochloric acid (pH 3.1) (Fig. 1*f*). Under these conditions of high concentrations of urea electrophoresis produced bands and narrow lines which often appeared in pairs.

In contrast with those observations electrophoresis in 1–2*M*-urea revealed between four and eight bands, the fastest of which migrated 7.0–7.5 cm. at pH 2.0–2.5 (Fig. 1*g, h, i*). When 0.02*M*-sodium acetate buffer and 2*M*-urea were present in the gel to give pH 4.15 migration of the broadest band, which moved fastest, did not exceed 2 cm. (Fig. 1*j*). At concentrations of 1–2*M*-urea bands appeared to be broad and rarely present in pairs.

DISCUSSION

In the foregoing results some properties of ribosomal proteins from two related animal species are compared. Appreciable methodological differences stem from the fact that rabbit reticulocytes have no nucleus and little if any endoplasmic reticulum. The absence of a nucleus rules out the remote possibility that nuclear histones, which may have escaped into the cytoplasm during preparation of the homogenate, may have contaminated the

Table 3. *Proportion of protein extractable with 0.2*N*-hydrochloric acid from ribosomes of rabbit reticulocytes*

No. of preparations	Period of extraction with 0.2 <i>N</i> -HCl (hr.)	Amount of protein extracted (%) *	$\frac{E_{275}}{E_{260}}$
3	0.025	54	—
3	0.5	40	1.56
	then 16.0	6	1.19
1	16.75	30	1.48
	then 5.0	7	1.34
1	1.0	41	1.48
	then 0.5	6	1.06
	then 1.0	5	1.03

* RNA + protein = 100%.

Table 4. *Proportion of basic and acidic amino acids in ribosomal protein fractions soluble and insoluble in guanidinium chloride*

The amino acids are expressed as moles/100 moles of all amino acids found and no correction has been made for the hydrolytic losses of amino acids. Amide was not determined. The values for the basic and acidic amino acids only are given for the sake of clear presentation. Those omitted do not differ appreciably from the results of the analyses given in Tables 1 and 2. The proportions of *N*-terminal groups are molar percentages of all such groups found.

	Extraction of ribosomes					Extraction of HCl-insoluble fraction					Residue	
	Soluble		Insoluble		after dialysis	Soluble		Insoluble				
	after dialysis		GS	after dialysis		after dialysis						
	GL											
	Rabbit* reticulocytes	Rat liver	Rabbit reticulocytes	Rat liver		Rabbit reticulocytes	Rat liver	Rabbit reticulocytes	Rat liver		Rabbit reticulocytes	Rat liver
	23	17	75	66		12	6	42	30		6	24
Proportion of total ribosomal protein (%)												
Amino acids												
Aspartic acid	22.1	18.6	20.0	20.6		21.4	—	25.3	21.3		11.5	21.1
Glutamic acid											11.9	
Histidine	{ 2.1	2.2	1.8	2.0		2.0	—	1.2	1.9		1.5	1.9
Lysine	{ 11.1	13.0	10.6	8.4		9.4	—	7.7	8.7		6.0	6.4
Arginine	{ 6.7	10.9	6.8	6.7		5.9	—	3.9	6.4		4.6	6.0
Lysine:arginine ratio	1.66	1.19	1.56	1.25		1.59	—	1.98	1.36		1.30	1.07
Basic:acidic ratio	0.90	1.40	0.96	0.83		0.81	—	0.51	0.80		0.52	0.68
<i>N</i> -Terminal amino acids												
Alanine	—	—	—	—		—	—	—	—		—	9
Glycine	—	—	—	—		—	—	—	—		—	25
Proline	—	—	—	—		—	—	—	—		—	33
Serine	—	—	—	—		—	—	—	—		—	11
Others†	—	—	—	—		—	—	—	—		—	22
Wt. (g./mole of <i>N</i> -terminal groups)	—	—	—	—		—	—	—	—		—	140 000

* Mean of results from two preparations.
† These include aspartic acid, glutamic acid, lysine and threonine.
‡ Sample not suitable for analysis.

Fig. 1. Starch-gel electrophoresis of proteins. All samples (1.0–1.2mg. for electrophoresis) except (c) are basic proteins of ribosomes from: (a) rat liver in 0.01N-HCl (pH 2.3); (b) rabbit reticulocytes in 0.01N-HCl (pH 2.3); (c) total histone from calf thymus in 0.01N-HCl (pH 2.3); (d) rat liver in 6M-urea–0.02N-HCl (pH 3.1); (e) rabbit reticulocytes in 6M-urea–0.02N-HCl (pH 3.1); (f) rat liver in 4M-urea–0.02N-HCl (pH 2.85); (g) rabbit reticulocytes in 2M-urea–0.02N-HCl (pH 2.05); (h) rabbit reticulocytes in 1M-urea–0.02N-HCl (pH 2.05); (i) rat liver in 1M-urea–0.02N-HCl (pH 2.05); (j) rat liver in 2M-urea–0.02M-sodium acetate buffer (pH 4.15).

extract of basic proteins from ribosomes. Ribosomes from rabbit reticulocytes also need not be subjected to the action of a detergent to free them from membranes.

The composition of ribosomal proteins from rat liver (Cohn & Simson, 1963) differs slightly from

that of rabbit reticulocytes. The amino acid content of the basic proteins and the hydrochloric acid-insoluble residues from both animals are each similar, but the proportion of *N*-terminal proline groups is higher in these two fractions from rabbit reticulocytes than from rat liver. Another difference is revealed by the fractionation with guanidinium chloride: protein fractions from reticulocytes possess a higher ratio lysine:arginine than those from rat liver, whereas most of those from rat liver have a higher ratio basic:acidic amino acid residues than those from rabbit reticulocytes.

Starch-gel electrophoresis of soluble ribosomal proteins presented no clear picture either of the number of protein components present or of differences between the two animals. The effects of applying different pH values and various concentrations of urea indicate that proteins may occur in different stages of aggregation and that the polypeptide chains have different charges. However, Setterfield *et al.* (1960) found urea not to be necessary for the resolution into up to 15 bands by starch-gel electrophoresis of ribosomal proteins from pea seedlings. More recently, Cox & Flaks (1964) demonstrated that ribosomal proteins from *Escherichia coli* gave rise to 34 bands during disk electrophoresis in acrylamide gel. It is impossible to state whether these differences in electrophoretic behaviour can be ascribed to the properties of ribosomal proteins from these cells or are a result of changes of conformation or even of primary structure undergone during preparation.

The presence of methionine as *N*-terminal group in ribosomal proteins has been reported for *E. coli* (Waller & Harris, 1961) and in contrast with the present results also for rabbit reticulocytes (Mathias & Williamson, 1964). Webster, Engelhardt & Zinder (1966) have claimed *N*-formylmethionine to be the initial amino acid assembled during the synthesis of the polypeptide chains of the coat protein of some virus particles.

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