Dietary Protein Intake and Skeletal-Muscle Protein Metabolism in Rats

STUDIES WITH SALT-WASHED RIBOSOMES AND TRANSFER FACTORS

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(Received 1 November 1971)

1. Aspects of skeletal muscle protein synthesis *in vitro* were studied in young rats given a low-protein diet for up to 10 days and during re-feeding with an adequate diet.
2. Partially purified muscle transfer factors (transferases I and II), crude and purified (NH₄Cl-washed) ribosomes and a pH5 enzyme fraction were prepared for this purpose.
3. A marked decrease in the capacity of crude ribosomes to carry out cell-free polypeptide synthesis occurred within 4 days of feeding the low-protein diet.
4. The capacity of salt-washed ribosomes to promote amino acid polymerization, in the presence of added transfer factors and aminoacyl-tRNA, was only slightly decreased by the dietary treatment.
5. However, the capacity of salt-washed ribosomes to bind ¹⁴C-labelled aminoacyl-tRNA was decreased by feeding the low-protein diet.
6. The capacity of the pH5 enzyme fraction to promote amino acid incorporation in a complete cell-free system was decreased within 2 days of feeding the low-protein diet. There is no evidence that the change is associated with aminoacyl-tRNA synthetase or binding enzyme activities of the pH5 fractions.
7. These changes are discussed in relation to the diminished rate of protein synthesis in the intact muscle cell when rats are given a low-protein diet.

Protein metabolism in skeletal muscle is sensitive to changes in the adequacy of dietary protein intake (Waterlow & Stephen, 1966, 1968; Young *et al.*, 1968a; Young & Alexis, 1968; von der Decken & Omstedt, 1970; Millward, 1970). Associated with the loss of sarcoplasmic and fibrillar proteins in the skeletal muscle of protein-calorie malnourished animals (Young, 1970) is a decreased rate of protein synthesis *in vivo* in the muscle cell (Waterlow & Stephen, 1966; Young *et al.*, 1971). In previous studies (Young & Alexis, 1968) we observed that preparations of crude ribosomes obtained from skeletal muscle of rats fed with a low-protein diet for 6 to 30 days showed about half the capacity of ribosomes obtained from well-nourished rats to carry out cell-free protein synthesis. Similar observations have been reported by von der Decken & Omstedt (1970). However, the nature of the diet-induced alterations in the ribosomal protein synthetic activity has not been studied in detail.

It is known that various protein factors are associated with crude ribosome preparations, and these include the initiation (Miller & Schweet, 1968; Pritchard *et al.*, 1970; Shafritz & Anderson, 1970; Heywood, 1970) and elongation (Moldave *et al.*, 1969; Smulson & Rideau, 1970) factors. Hence, a variable concentration of these factors in crude ribosome preparations may account in part for the differences observed by Young & Alexis (1968) in

the protein synthetic activity of muscle ribosomes of protein-depleted and well-nourished rats.

From studies with Ehrlich ascites cells, van Venrooij *et al.* (1970) suggested that the rate of protein synthesis may be modulated, by nutritional factors, at the level of peptide-chain initiation and elongation, in addition to substrate limitation. Morgan *et al.* (1971) and Jefferson *et al.* (1971), by using a perfused heart preparation, have also concluded that peptide-chain initiation and elongation are likely to be points of control in the regulation of protein synthesis. Vaughan *et al.* (1971) found that with HeLa cells a prompt inhibition of translation initiation occurred when the cells were deprived of single essential amino acids.

In view of the above findings, we have now studied the effect of acute dietary protein restriction and subsequent re-feeding in rats on the capacity of muscle ribosomes for the binding of aminoacyl-tRNA and for polypeptide-chain elongation *in vitro*. For this purpose, partially purified muscle transfer factors, aminoacyl-tRNA transferase (transferase I, binding enzyme) and peptidyl-tRNA translocase (transferase II), and salt-washed ribosomes were prepared from mixed hind-leg muscles.

The studies show that salt-washed ribosomes bind aminoacyl-tRNA less efficiently when they are prepared from muscle of protein-deprived rats. Although the capacity of crude ribosomes for polypeptide

synthesis was markedly decreased below that of controls within 4 days of feeding a low-protein diet, there was little difference between the diet groups when the cell-free activities of salt-washed ribosomes were compared. It was also observed that the pH5 enzyme fraction from protein-deprived rats promoted the transfer of radioactivity from amino acids and aminoacyl-tRNA to proteins less efficiently than the pH5 enzyme fraction from control animals.

Materials and Methods

Animals and diets

Male rats (Charles River Breeding Laboratories, Wilmington, Mass., U.S.A.) weighing 90–100g were received 3 days before the experiments were begun. During this adaptation period, they were fed with an adequate protein (18% lactalbumin) diet and then randomly allocated to either the low- or adequate-protein diet (Table 1). The rats were fed *ad libitum* and water was available at all times.

Preparation of subcellular fractions

Crude ribosomes from a mince of mixed hind-leg muscles were prepared by a modification of the method of Heywood *et al.* (1968) as previously described (Alexis *et al.*, 1971). For preparation of salt-washed ribosomes a modification of the method of Skogerson & Moldave (1967) was followed. An equal volume of 1.6M-sucrose in buffer A [0.5M-NH₄Cl, 0.01M-tris-HCl (pH7.6), 0.025M-KCl, 0.01M-MgCl₂] was added to the detergent-treated post-mitochondrial supernatant (Alexis *et al.*, 1971) of muscle, and the preparation was mixed and left at 4°C for 4h. The ribosomes were collected by centrifugation at 105000g_{av} for 3h in the Spinco no. 40 rotor and the pellet was stored at -20°C for 2 days

under 0.5ml of buffer T [0.625M-tris-HCl (pH7.6), 0.1M-NH₄Cl, 0.25mM-MgCl₂]. The pellets were then gently resuspended in buffer A by using an all-glass homogenizer. The suspension was clarified by a 10min centrifugation at 2000rev./min, and 5ml portions of the suspension were layered over a discontinuous NH₄Cl-sucrose gradient consisting of 2ml of 1.0M-sucrose in buffer A carefully layered over 3ml of 2M-sucrose in buffer A. The gradients were spun at 105000g_{av} for 4h in the no. 40 rotor and the ribosome pellets were stored at -80°C under 0.5ml of buffer T until required for the experiments.

The pH5 enzyme and cell-sap fractions were obtained from a muscle homogenate prepared in 2vol. of 0.375M-sucrose in TKM buffer [0.05M-tris-HCl (pH7.6), 0.025M-KCl, 5mM-MgCl₂]. A post-mitochondrial supernatant was obtained by centrifugation at 19000g_{av} for 15min and the supernatant was filtered through nylon cloth and then again centrifuged at 105000g_{av} for 3h in the no. 40 rotor of the Spinco model L ultracentrifuge, to obtain the cell sap. Factors inhibiting amino acid incorporation were removed from the cell sap by passing 20ml of this fraction through a column (2cm×34cm) of Sephadex G-25 which had been previously equilibrated with TEG buffer [0.2M-tris-HCl (pH7.6), 0.1mM-EDTA, 1mM-GSH]. The pH5 fraction, containing tRNA and aminoacyl ligases, was then obtained from the Sephadex-treated cell sap by adjusting it to pH5.0 with 1M-acetic acid (Young *et al.*, 1970). The pH5 enzyme fraction was pelleted by centrifugation in the no. 30 rotor at 19000g_{av} for 20min. The pellet was finally suspended in a small volume of TEG buffer and stored at -40°C (usually for 1 day) until used for the experiments.

Rat skeletal muscle transferases (transferase I and transferase II) were isolated and partially purified by the method of Gasior & Moldave (1965). Cell sap was prepared and the pH5 enzyme fraction removed as described above. The supernatant was readjusted to pH7.0 with aq. NH₃ and then dithiothreitol was added to a final concentration of 1mM. The adjusted supernatant was passed through a Sephadex G-25 column (4cm×56cm), previously equilibrated with buffer [0.05M-tris-HCl (pH8.0), 1mM-dithiothreitol, 5% (v/v) glycerol]. To the column effluent, 0.5vol. of hydroxyapatite gel suspended in 1mM-potassium phosphate buffer (pH6.8) was added and the mixture was stirred for 1h at 4°C. The transferases were extracted by elution from the gel successively with 120ml of 0.125M-, 60ml of 0.150M-, 60ml of 0.175M- and 120ml of 0.250M-potassium phosphate buffers (pH6.8). The 0.15M- and 0.175M-phosphate extracts were discarded.

The method of Galasinski & Moldave (1969) was used to purify transferase II. The 0.125M-phosphate buffer extract from above was first made 70% saturated with respect to ammonium sulphate by

Table 1. Composition of the diets used for studies of the effects of protein intake on muscle protein synthesis

The compositions of the vitamin mix and the salt mix are described by Young *et al.* (1968b). Choline was added to diet as an aqueous solution containing 1g/5ml. Entries are given as % by weight.

Ingredients	Low-protein diet	Adequate-protein diet
Lactalbumin	0.5	18.0
Sucrose	27.9	22.1
Dextrin	55.9	44.2
Cottonseed oil	10.0	10.0
Vitamin mix	0.5	0.5
Salt mix	5.0	5.0
Choline	0.2	0.2

addition of the fine powder and the precipitate was dissolved in a small volume of buffer P [0.05M-potassium phosphate (pH 6.8), 1mM-dithiothreitol, 5% (v/v) glycerol] and then dialysed against 1 litre of the same buffer for 12–16h. The dialysate was concentrated, by vacuum dialysis, to a volume of

Fig. 1. Elution profile on cellulose phosphate of the 0.125M-phosphate extract from hydroxyapatite gel

The column was eluted with (1) phosphate buffer, (2) 0.05M-KCl in phosphate buffer, (3) 0.5M-KCl in phosphate buffer. The peak fractions eluted with the 0.5M-KCl buffer were combined and used as the transferase II fraction. Each fraction was 4ml.

8–10ml and then layered on a cellulose phosphate column which had been previously equilibrated with buffer P. The protein was successively eluted with 210ml of buffer P, and 230ml of 0.05M-KCl in buffer P and 130ml of 0.5M-KCl in buffer P. The eluate was monitored at 280nm and the elution medium was changed when the E_{280} was less than 0.03 unit. The fractions eluted with 0.5M-KCl giving the highest E_{280} were combined for use as the transferase II preparation and concentrated by vacuum dialysis. Fig. 1 shows a typical elution pattern for the muscle preparation as obtained with the cellulose phosphate column.

The 0.250M-phosphate buffer extract obtained from the hydroxyapatite gel was further purified for transferase I by the method of Schneir & Moldave (1968) as follows. Finely powdered ammonium sulphate was added to 70% saturation and the precipitate was collected by centrifugation as described above. It was resuspended in tris-dithiothreitol buffer [0.05M-tris-HCl (pH 8.0), 1mM-dithiothreitol, 5% (v/v) glycerol], then it was dialysed against the same buffer for 10–16h and concentrated to a volume of 8–10ml by using a Diaflo (Amicon Corp., Cambridge, Mass., U.S.A.) ultrafiltration device with a UM-10 membrane. The transferase I protein was then further purified by passage through a Sephadex G-200 column (2cm×34cm), previously equilibrated with the tris-dithiothreitol buffer. Fractions containing primarily transferase I activity (Fig. 2) were pooled and concentrated by vacuum dialysis. The transferase I fraction was stored in small portions at -80°C until required for the experiments. Rat liver transfer factors, kindly supplied by Dr. B. S. Baliga, were used initially to aid in the isolation and resolution of the muscle transfer factors.

Fig. 2. Elution profile on Sephadex G-200 of the 0.25M-phosphate extract from hydroxyapatite

For experimental details see the text. (a) Fractions (1 ml) 10–16 were taken for concentration and used as the partially purified muscle transferase I fraction. (b) Dependence of fractions 10–16 for transferase II to promote cell-free amino acid incorporation. Incorporation with fraction (o) alone and (●) plus transferase II.

Cell-free systems

The reaction mixture containing the pH5 enzyme and crude ribosomes was similar to that described by Alexis *et al.* (1971). The incubation mixture (total volume 1 ml) contained 80 μ mol of NH_4Cl , 5 μ mol of MgCl_2 , 10 μ mol of GSH, 5 μ mol of ATP, 1 μ mol of GTP, 80 μ mol of tris-HCl (buffer, pH 7.6), 1 mg of pH5 enzyme protein, 40–110 μ g of rRNA, 2 nmol of L-[U- ^{14}C]leucine (0.5 μ Ci) and 0.02 μ mol each of the following unlabelled L-amino acids: arginine, alanine, aspartic acid, asparagine, cystine, glutamic acid, glutamine, glycine, histidine, isoleucine, lysine, methionine, proline, serine, threonine, tryptophan, tyrosine, valine and phenylalanine.

Incubations were performed at 37°C for 60 min, and the reaction was stopped by addition of an equal volume of 10% (w/v) trichloroacetic acid. The incubation tubes were then heated at 90°C for 15 min. The hot-acid-insoluble material was collected on glass-fibre filters and thoroughly washed with 5% (w/v) trichloroacetic acid and 95% (v/v) ethanol. The filters were placed in scintillation vials and dried under an i.r. lamp, and the protein was dissolved by incubation with 0.5 ml of NCS solubilizer at 55°C for 15 min. The counting fluid was 10 ml of toluene containing 0.7% 2,5-diphenyloxazole and the radioactivity was measured in a Packard model 2000 liquid-scintillation counter. Incubations were done in duplicate throughout, with a mean difference of usually less than 5%. Counting efficiency was 83%.

For assay of the purified (salt-washed) ribosomes with the muscle transfer factors the following incubation system was used: 80 mM- NH_4Cl , 6 mM- MgCl_2 , 4 mM-dithiothreitol, 0.2 mM-GTP, 40 mM-tris-HCl buffer, pH 7.6, 20–40 μ g of rRNA, 21 μ g of transferase I and of transferase II, leucyl- or phenylalanyl-tRNA (3000 c.p.m.) and 40 μ g of poly(U), in a final volume of 0.5 ml. The Mg^{2+} , NH_4^+ and GTP

concentrations for maximum incorporation of radioactivity in the cell-free system were 5–6 mM, 80–100 mM and 0.2 mM respectively. The amount of leucine incorporated reached a peak at between 20 and 30 min and was proportional to the quantity of rRNA added up to about 50 μ g/incubation mixture. Incubations were conducted for 20 min at 37°C and the reaction was terminated by addition of an equal volume of 10% (w/v) trichloroacetic acid. The reaction mixture was then prepared for radioactivity counting as described above.

The binding of aminoacyl-tRNA by muscle ribosomes was measured essentially by the method of Nirenberg & Leder (1964). The incubation system used contained the following materials in a total volume of 0.5 ml: salt-washed ribosomes, 35 μ g of rRNA; tris-HCl buffer, pH 7.6, 50 mM; NH_4Cl , 80 mM; GTP, 0.2 mM; *N*-ethylmaleimide, 0.4 mM; transferase I, 20 μ g; magnesium acetate, 6 mM; and either [^{14}C]leucyl-tRNA, 6 μ g, or [^{14}C]phenylalanyl-tRNA, 100 μ g in the presence of poly(U), 25 μ g. The amount of tRNA refers to the mixture of [^{14}C]leucyl-tRNA and non-radioactive aminoacyl-tRNA species taken for incubation. The reaction was carried out at 37°C for 25 min and stopped by the addition of 3 ml of ice-cold (4°C) buffer containing tris-HCl (50 mM), pH 7.6, NH_4Cl (80 mM) and KCl (25 mM). The contents of each tube were filtered through a Millipore disc (type HA; 0.45 μ m, 25 mm diameter) by gentle water-suction. Each reaction tube was washed three times with 6 ml of the ice-cold buffer, and the washings were poured over the filter disc. The disc was then dried under an i.r. lamp and counted for radioactivity as described above.

The requirements for binding of aminoacyl-tRNA in the system are given in Table 2. Binding was only moderately dependent on the addition of GTP, which may be explained by the possible presence of

Table 2. Requirements for the binding of aminoacyl-tRNA by salt-washed muscle ribosomes

The assays were performed with 35 μ g of rRNA as described in the Materials and Methods section. In the assay 20 μ g of transferase I protein was used and, where indicated, 25 μ g of poly(U).

Labelled substrate	Omissions	Incorporation (c.p.m. per incubation mixture)
Leucyl-tRNA	None	108
	Mg^{2+}	71
	GTP	92
	NH_4^+	70
	Transferase I	43
Phenylalanyl-tRNA	None	165
	Transferase I	55
	Poly(U)	63
	Poly(U), transferase I	42

Table 3. Binding of [14 C]leucyl-tRNA by muscle ribosomes in the presence of *N*-ethylmaleimide, by using cold-buffer or hot-trichloroacetic acid treatments

Conditions of the assay system are described in the Materials and Methods section. After incubation the hot-acid tubes were heated at 90°C for 15min in the presence of 10% trichloroacetic acid, filtered and prepared for radioactivity counting. The cold-buffer system was prepared exactly as described in the Materials and Methods section.

Omission or addition	Incorporation (c.p.m. per incubation mixture)	
	Hot-acid treatment	Cold-buffer treatment
-Transferase I	62	68
+Transferase I	48	140
+Transferase I + <i>N</i> -ethylmaleimide	50	158
+Transferases I and II	770	820
+Transferases I and II + <i>N</i> -ethylmaleimide	265	380

Fig. 3. Effect of Mg^{2+} concentration of the incubation medium on the enzymic binding of leucyl-tRNA by salt-washed muscle ribosomes in the presence of transferase I

For experimental details see the text. ●, Plus transferase I; ○, no transferase I.

GTP in the aminoacyl-tRNA utilized. Table 3 shows that inclusion of *N*-ethylmaleimide, an inhibitor of transferase II, in the incubation system did not significantly decrease binding of aminoacyl-tRNA radioactivity and that the addition of transferase I alone did not increase the radioactivity of hot-acid-insoluble material. These findings indicate that the salt-washed ribosomes were not significantly contaminated with transferase II. *N*-Ethylmaleimide was included in the binding assay used for the experiments in case the diet treatment differentially affected the extent to which transferase II was removed from the ribosomes during passage through the ammonium

chloride gradient. The effect of Mg^{2+} concentration on the binding reaction is shown in Fig. 3. Binding of [14 C]leucyl-tRNA was linear in the system for up to about 120 μ g of rRNA added per incubation mixture and reached maximum incorporation after 10min of incubation. However, a disadvantage of the assay is the relatively large amount of radioactivity retained on the filters in the absence of transferase I, amounting to about 30–50% of the radioactivity retained for the complete system. Previous studies with liver (Ibuki & Moldave, 1968) and reticulocyte systems (McKeehan & Hardesty, 1969) have generally utilized a significantly greater amount of ribosomes than was utilized in the present incubation system. It was not feasible to use larger quantities of ribosomes in our system in view of the lower concentration of RNA, and consequently of ribosomes in rat skeletal muscle, especially with protein-depleted animals.

Quantitative analysis

Protein concentration was measured by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard. The RNA content of ribosome preparations was determined as described by Alexis *et al.* (1971).

Materials

[U- 14 C]Leucine (262mCi/mmol) and [14 C]leucyl-tRNA (0.355 μ Ci/mg of mixed aminoacyl-tRNA species) and [14 C]phenylalanyl-tRNA (0.156 μ Ci/mg of mixed aminoacyl-tRNA species), prepared from *Escherichia coli* B, and 2,5-diphenyloxazole were purchased from New England Nuclear Corp., Boston, Mass., U.S.A. Poly(U), ATP, GTP, dithiothreitol

and *N*-ethylmaleimide were obtained from Calbiochem, Los Angeles, Calif., U.S.A. Hydroxyapatite was a product of Clarkson Chemical Co., Williamsport, Pa., U.S.A. Sephadex G-25 and G-200 were purchased from Pharmacia Fine Chemicals Inc., Piscataway, N.J., U.S.A. The cellulose phosphate (Whatman P11) and glass-fibre filters were purchased from Reeve Angel, Clifton, N.J., U.S.A. and the Millipore filters from Millipore Corp., Bedford, Mass., U.S.A. The non-radioactive amino acids were obtained from General Biochemicals Inc., Chagrin Falls, Ohio, U.S.A. and NCS solubilizer was obtained from Amersham/Searle, Des Plaines, Ill., U.S.A.

Results and Discussion

The findings for the cell-free activity of crude ribosomes prepared from muscle of rats after 2, 4 or 10 days of feeding the low-protein diet are summarized in Table 4. The protein-synthetic activity of these ribosomes was decreased within 4 days of feeding the low-protein diet and after 10 days their activity was 60% lower than for ribosomes prepared from muscle of well-nourished rats. Re-feeding for 1 day with the adequate diet, after either a 4 or 10 day depletion period, completely reversed these changes. Because the ribosomes were assayed within 1 or 2 days after preparation, the activity of ribosomes *in vitro* from the 2-, 4- and 10-day groups was determined at different times. There is no clear reason why the ribosomes for the adequate- and low-protein groups at 10 days showed an overall lower activity as compared with those for the earlier groups. However, duplicate determinations in all assays agreed within 5% and so it is considered valid to compare results of assays performed at the same time.

The present results agree with our previous findings (Young & Alexis, 1968), which showed that cell-free amino acid incorporation by ribosomes prepared from skeletal muscle of rats fed on a low-protein diet was about half that obtained with ribosomes prepared

from well-nourished rats. The changes occurred within 6 days of feeding the low-protein diet and persisted throughout the 4-week feeding period.

The decreased protein-synthetic activity can be partly correlated with a decrease in the proportion of large polyribosome aggregates obtained from the muscle of rats on low-protein or protein-free diets (Young *et al.*, 1968a; Young & Alexis, 1968). Similar observations have been reported by von der Decken & Omstedt (1970) for rats given a protein-free diet for 4–6 days, and Howarth & Baldwin (1971) have also shown that a 3-week period of calorie restriction in rats decreases the capacity of muscle microsomal fractions for cell-free protein synthesis.

It was thought that a variable contamination of protein factors (Miller & Schweet, 1968; Shafritz & Anderson, 1970; Heywood, 1970; Moldave *et al.*, 1969; Smulson & Rideau, 1970) in preparations of crude ribosomes from the adequate-protein and low-protein groups may account for the above findings. This possibility is supported by the observation that the enhanced protein-synthetic activity of ribosomes prepared from spleens of immunized rats is due to higher contamination of these ribosomes with bound transfer factors as compared with ribosomes obtained from control spleens (Willis & Starr, 1971). Therefore, salt-washed (purified) ribosomes were examined for their capacity to bind aminoacyl-tRNA and to participate in the elongation phase of polypeptide synthesis. These studies utilized a cell-free system supplied with the partially-purified muscle-transfer factors.

As summarized in Table 5, the binding of [¹⁴C]-leucyl-tRNA to purified ribosomes obtained from the muscle of rats given the low-protein diet was consistently less efficient than for ribosomes prepared from control rats. Similar results were obtained with phenylalanyl-tRNA in the presence of poly(U), except that the differences tended to be greater with this latter system. Re-feeding with the adequate diet for 1 day after 4 and 10 days of low-protein feeding

Table 4. *Effect of a low-protein diet on the protein-synthetic activity of crude muscle ribosomes*

Ribosomes were prepared from a pooled muscle sample of 10 rats per group. They were incubated, as described in the Materials and Methods section, in the presence of muscle pH5 enzyme prepared from well-nourished rats.

Period on diet (days)	Period of re-feeding (days)	Source of ribosomes	...	10 ⁻³ × Incorporation (c.p.m./mg of rRNA)	
				Adequate-protein diet rats	Low-protein diet rats
2	—			144	159
4	—			143	103
4	1				183
10	—			65	39
10	1				69

Table 5. *Capacity of salt-washed ribosomes, prepared from muscle of rats given adequate- or low-protein diets, to bind ^{14}C -labelled aminoacyl-tRNA in the presence of transferase I*

Values are means of duplicate determinations made on ribosome preparations obtained from a pooled muscle sample of 10 rats per group and are corrected for non-enzymic binding as measured in the absence of transferase I. Conditions of the assay are described in the Materials and Methods section. Abbreviations: AP, rats given the adequate-protein diet, and LP, rats given the low-protein diet for the number of days indicated.

Sources of ribosomes (diet group)	Incorporation (c.p.m./mg of rRNA)	
	$[^{14}\text{C}]\text{Leucyl-tRNA}$	$[^{14}\text{C}]\text{Phenylalanyl-tRNA}$
AP-2	1000	1228
LP-2	514	743
AP-4	1000	1314
LP-4	428	457
LP-4, AP-1	485	285
AP-10	1085	2514
LP-10	771	1514
LP-10, AP-1	571	1828
LP-10, AP-2	971	4228

Table 6. *Effect of a low-protein diet on the protein-synthetic activity of purified (salt-washed) ribosomes from skeletal muscle, in the presence of muscle transfer factors*

Details of the incubation system are given in the Materials and Methods section. Each value represents the mean of duplicate determinations performed with a ribosome preparation from a pooled muscle sample, 10 rats for each group. AP and LP are defined in Table 5.

Source of ribosomes		10 ⁻² × Incorporation (c.p.m./mg of rRNA)						
		[¹⁴ C]Leucyl-tRNA			[¹⁴ C]Phenylalanyl-tRNA			
		Expt. no.	...	1	2	3	2	
No poly(U)	+Poly(U)						No poly(U)	+Poly(U)

AP-2			355		148	587		
LP-2			318		130	331		
AP-4			324		270	525		
LP-4			314		237	440		
LP-4, AP-1			320		367	603		
AP-10	296	298	544		343	624	217	455
LP-10	229	237	492		224	498	211	431
LP-10, AP-1	310	294			221	653		
LP-10, AP-2	392	316			401	772		

did not increase the binding of aminoacyl-tRNA by the ribosomes, but 2 days of re-feeding after 10 days on the low-protein diet enhanced the binding of aminoacyl-tRNA to the ribosomes. The efficiency of the binding reaction applied in these studies was low and, therefore, caution should be taken in the interpretation of these results.

The differences in the enzymic binding of aminoacyl-tRNA by ribosomes from protein-deprived and control rats were expected to be paralleled by differ-

ences in their capacity to carry out polypeptide synthesis *in vitro*. However, the results of three experiments, summarized in Table 6, indicate that the activity of salt-washed ribosomes, assayed in the presence of $[^{14}\text{C}]\text{leucyl-tRNA}$ and transfer factors from normal muscle, is only slightly decreased by the dietary protein restriction. These findings were independent of the Mg^{2+} concentration of the incubation medium (Fig. 4).

The salt-washed ribosomes from the various dietary

Fig. 4. Effects of Mg^{2+} concentration of the incubation medium on the protein-synthetic activity of salt-washed ribosomes from muscle of rats fed on a low-protein (■) or adequate-protein (●) diet for 10 days

Ribosomes were prepared from a pooled muscle sample of 10 rats per dietary group. For other experimental details see the text.

groups, when assayed in the absence of added transfer factors or in the presence of transferase I alone or transferase II alone, showed little protein-synthesizing activity (results not shown), demonstrating that most of the bound transferases were removed from the ribosomes during the purification procedure.

These studies show that the cell-free protein-synthetic capacity of a preparation of crude ribosomes is decreased to a greater extent than that of salt-washed ribosomes after dietary protein deprivation. Assuming that little initiation occurs with the present incubation system (see, e.g., Falvey & Staehelin, 1970) and that the incorporation of radioactivity represented the elongation of existing polypeptide chains, our findings suggest that the greater differences observed with the crude ribosome preparations from rats given the adequate and low-protein diets could be due to a relatively higher content of bound transfer factors, particularly transferase II (Moldave *et al.*, 1969), associated with the crude ribosomes obtained from muscle of well-nourished rats. This possibility is of interest in view of the suggestion of Gill *et al.* (1969) that transferase II may normally be rate limiting in kidney, intestinal mucosa and muscular tissues. However, it is recognized that the presence or absence of other factors in crude ribosome preparations may be responsible for the diminished

activity of ribosomes from rats deprived of dietary protein.

The capacity of salt-washed ribosomes prepared from the low-protein groups to bind aminoacyl-tRNA, in the presence of transferase I, was about half of that for ribosomes from control rats, although as discussed above, the capacity of the ribosomes for amino acid polymerization did not differ to this extent. This discrepancy may be explained in several, though not mutually exclusive, ways; it is possible that the binding of aminoacyl-tRNA is not rate limiting in the overall elongation-phase polypeptide synthesis *in vitro* (Earl & Hindley, 1971). Further, studies with ribosomes from muscle of diabetic rats (Castles *et al.*, 1971) suggest that the binding of phenylalanyl-tRNA by ribosomes at low Mg^{2+} concentrations was diminished to a greater extent than was the synthesis of polyphenylalanine by the ribosome preparations. A difference in the relative amount of peptidyl-tRNA located at the 'A' (acceptor) and 'P' (donor) sites of the ribosome would also be expected to alter the extent to which aminoacyl-tRNA is enzymically retained by ribosomes on Millipore filters. Although the findings of Earl & Hindley (1971) do not support the latter possibility, use of the puromycin reaction for the determination of the distribution of peptidyl-tRNA between the P and A sites (Skogerson & Moldave, 1968; Baliga *et al.*, 1970) should help to resolve this question.

We also examined the muscle pH 5 enzyme fraction from rats given the low-protein diet for 2, 4 or 10 days. The fractions were assayed in the presence of [^{14}C]leucine and crude ribosomes from normal muscle and also in a second incubation system with [^{14}C]leucyl-tRNA and salt-washed ribosomes. As shown in Table 7, the pH 5 enzyme preparation from rats given the low-protein diet was 40–60% less active in catalysing cell-free amino acid polymerization, compared with the pH 5 enzyme fraction from control rats. Because the assay system with aminoacyl-tRNA does not involve the aminoacyl-tRNA synthetases for the charging of tRNA and did not contain added ATP, these results suggest that differences between the pH 5 enzyme fractions were not due to differences in the aminoacyl-tRNA synthetase activity of these fractions. This conclusion is consistent with the results of Stephen (1968) showing that the activities of these enzymes in muscle do not decrease during protein depletion, although enzyme activity increases in liver under these dietary conditions (Gaetani *et al.*, 1964; Stephen, 1968).

Because the pH 5 enzyme fraction may contain transfer factors (Willis & Starr, 1971) the binding-enzyme (transferase I) activity of the pH 5 enzyme fractions was also measured and the results of these studies are shown in Fig. 5. Little consistent difference was observed in the binding-enzyme activity of the crude pH 5 enzyme fractions obtained from muscle

Table 7. Capacity for transfer of radioactivity from [¹⁴C]leucine and [¹⁴C]leucyl-tRNA into proteins in a cell-free system by the pH5 enzyme fraction from rats fed adequate- or low-protein diets

Values are based on duplicate determinations made on the pH5 fraction obtained from a pooled muscle sample of 10 rats per group. The conditions of the assay are described in the Materials and Methods section. The system with [¹⁴C]leucine as the labelled substrate was identical with that used for assay of the crude ribosomes. The cell-free system with [¹⁴C]leucyl-tRNA as the labelled substrate was identical with that used for assay of purified ribosomes except that 1 mg of pH5 enzyme protein was substituted for the transfer factors. AP and LP are defined in Table 5.

Source of pH5 enzyme	Substrate	...	Incorporation (c.p.m./mg of rRNA)	
			10 ⁻³ × [¹⁴ C]Leucine	10 ⁻² × [¹⁴ C]Leucyl-tRNA
AP-2			144	—
LP-2			92	—
AP-4			143	145
LP-4			81	62
AP-10			65	135
LP-10			31	79

Fig. 5. Binding-enzyme activity of the pH5 enzyme fraction prepared from muscle of rats fed on the adequate- or low-protein diet for 2(a), 4(b) or 10(c) days

Conditions of the binding-enzyme assay are similar to those described in the Materials and Methods section for the binding of aminoacyl-tRNA by ribosomes, except that the crude pH5 enzyme fraction replaced the transferase I in the incubation system. Each fraction was prepared from a pooled muscle sample of 10 rats per dietary group. ●, control (adequate-protein diet); ○, low-protein diet.

of the various dietary groups, indicating that the decreased activity of the pH5 fraction in cell-free protein synthesis is not due to a lower content of transferase I in the preparation from protein-deprived rats.

The tRNA portion of pH5 enzyme fraction may likewise be unaffected because the decrease in the cell-free activity of the pH5 enzyme preparation was also evident when the ¹⁴C-labelled aminoacyl-tRNA was used as a substrate. However, an alteration in the tRNA component caused by diet is not ruled out by the present studies for the following reasons: (a) if leucyl-tRNA was more highly charged in the pH5 fraction isolated from the muscle of depleted rats, this would lead to a decreased specific radio-

activity of the [¹⁴C]leucyl-tRNA substrate and so lower the extent of total radioactivity incorporated into trichloroacetic acid-insoluble material; (b) the decreased activity of the pH5 fraction from protein-depleted rats *in vitro* could be explained by the existence of an aminoacyl-tRNA species, other than leucyl-tRNA, which is inactive or rate limiting in the assay system.

Further work will be required to establish the basis for the diminished activity of the pH5 enzyme fraction after a low protein intake and whether it is associated with the protein and/or the tRNA component.

In conclusion, dietary protein restriction decreased the cell-free protein synthetic activity of the

pH5 enzyme fraction and crude muscle ribosomes. The lower activity of crude ribosomes *in vitro* from muscle of the protein-deprived rats appears to be related to a difference in the content and/or activity of non-ribosomal factors associated with the ribosome preparations rather than being due to an alteration in the ribosomes themselves. However, the significance of the present findings remains to be established in relation to the physiological mechanism(s) responsible for diminished muscle protein synthesis *in vivo* under these dietary conditions. It might be speculated that inhibitory or stimulatory factors may act to control the activity of the protein-synthesizing apparatus in skeletal muscle *in vivo* when the dietary protein supply is restricted.

This study was supported by grant no. AM 13432 from the U.S. National Institutes of Health. This is Contribution no. 1882 from the Department of Nutrition and Food Science, Massachusetts Institute of Technology. We thank Mr. Gaston Vilaire for his assistance in these studies.

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