Dietary Protein Intake and Skeletal-Muscle Protein Metabolism in Rats

STUDIES WITH THE AMMONIUM CHLORIDE-WASH FRACTION FROM CRUDE POLYRIBOSOMES OF WELL-NOURISHED AND PROTEIN-DEPLETED RATS

By S. DANIEL ALEXIS and VERNON R. YOUNG

Department of Nutrition and Food Science, Massachusetts Institute of Technology,
Cambridge, Mass. 02139, U.S.A.

(Received 3 July 1973)

1. Crude polyribosomes from skeletal muscle of the hind leg of rats fed on a low-protein diet for 10 days are less active in cell-free protein synthesis than are polyribosomes obtained from well-nourished control rats. 2. The polyribosomes were salt-washed (0.5M-NH₄Cl) and the wash extract was examined for its amino acid incorporating activity and for EF (elongation factor) 1 and EF 2 activities. 3. Compared with preparations from control rats, the salt-wash fraction from protein-depleted rats was less active and showed lower EF 1 and EF 2 activity. 4. The ribosomes were rendered equal in activity by salt-washing, but no inhibitor was detected in the salt wash. 5. Differences in the incorporating activity of crude polyribosomes from the diet groups persisted in the presence of saturating amounts of partially purified EF 1 and EF 2. 6. It is concluded that the lowered protein-synthetic activity of crude polyribosomes caused by restricted protein intake is not causally related to the lower activities of EF 1 and EF 2 in the polyribosome preparations. 7. The possible nature of the change in crude polyribosome activity due to low-protein feeding is discussed.

It is not known which cellular mechanism is responsible for the lower rates of protein synthesis *in vivo* in skeletal muscle when rats are given inadequate dietary protein (Waterlow & Stephen, 1968; Young *et al.*, 1971; Millward, 1970). Previously we have found that the cell-free protein-synthetic activity of crude preparations of skeletal-muscle polyribosomes was decreased when young rats were fed on a protein-deficient diet (Young & Alexis, 1968; Alexis *et al.*, 1972); however, salt-washed polyribosomes obtained from muscles of well-nourished and protein-depleted rats were not significantly different in their capacity to promote amino acid incorporation *in vitro* (Alexis *et al.*, 1972). Hence the dietary-induced change in crude polyribosome activity might be associated with alterations in non-ribosomal factors, which can be removed by salt treatment of polyribosomes. Washing eukaryote ribosomes with high concentrations of salt removes factors required for polypeptide-chain initiation *in vitro* (Miller & Schweet, 1968; Pritchard *et al.*, 1970; Heywood, 1970; Comstock *et al.*, 1972), elongation (Skogerson & Moldave, 1967; Moldave *et al.*, 1969) and termination (Lawford *et al.*, 1971; Lubsen & Davis, 1972); these factors also include aminoacyl-tRNA synthetases (Gilbert & Anderson, 1970; Irwin & Hardesty, 1972; Roberts & Coleman, 1972) and tRNA (Gilbert & Anderson, 1970).

We compared the protein-synthetic activity of a salt-wash fraction obtained with crude polyribosomes from skeletal muscle of young rats fed on either adequate or low-protein diets for 10 days. Our results show that the activity of the ribosome-wash fraction is diminished in rats fed on a low-protein diet; this decrease is associated with lowered elongation-factor (EF 1 and EF 2) activity in the fraction.

Materials and Methods

Animals and diets

Male rats (CD strain; Charles River Breeding Laboratories, Wilmington, Mass., U.S.A.) weighing 80–90g were received approx. 3 days before the diet experiments began. During this time they were fed on an adequate diet and were then continued either on the adequate-protein diet or a low-protein (0.5% lactalbumin) diet for 10 days. They were offered food and water *ad libitum*. The compositions of the experimental diets are described by Alexis *et al.* (1972). Rats were housed in individual cages at a room temperature of about 24°C and with light from 07:00h to 19:00h. All rats were killed between 08:00h and 09:00h after having had access to food during the night.

Preparation of subcellular fractions

Crude and purified (NH₄Cl-washed) polyribosomes from a mince of mixed hind-leg muscles, a muscle pH 5-enzyme fraction, and the resolved, partially purified elongation factors (EF 1 and EF 2) were prepared by methods described by Alexis *et al.* (1972).

To isolate the ribosome-wash fraction, the crude polyribosome pellet (2–3 mg of RNA) was gently resuspended in 60 ml of buffer [0.5 M-NH₄Cl; 0.01 M-Tris-HCl (pH 7.6); 0.025 M-KCl; 0.01 M-MgCl₂; 0.3 M-sucrose] by using an all-glass homogenizer with a loose-fitting pestle. The suspension was stirred for 3 h at 4°C and centrifuged in the Spinco rotor 40 at 105 000 *g* for 4 h. The supernatant was removed and dialysed overnight against buffer [0.05 M Tris-HCl (pH 8.0); 1 mM-dithiothreitol; 5% (w/v) glycerol]. The dialysate was concentrated by vacuum dialysis; portions of the ribosome-wash fraction were stored at -70°C.

The protein concentration of the ribosome wash was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard. The RNA of the polyribosome preparations was assayed as previously described by Young & Alexis (1968).

Cell-free protein synthesis

The incubation mixtures for assay of crude and purified polyribosome preparations and determination of incorporation of radioactivity were described by Alexis *et al.* (1972). The incubations were performed in duplicate with differences of usually less than 5%; counting efficiency was about 83% for ¹⁴C. When salt-washed polyribosomes were used in the

incubations the Mg²⁺ concentration was 5 mM; the Mg²⁺ concentration was raised to 10 mM for assay of crude or unwashed polyribosomes.

Materials and chemicals

Details of chemicals and labelled compounds are described by Alexis *et al.* (1972). Trypsin (B-grade) and soya-bean trypsin inhibitor (B-grade) were obtained from Calbiochem, Los Angeles, Calif., U.S.A.

Results

As summarized in Table 1, crude polyribosomes obtained from skeletal muscles of rats given the low-protein diet for 10 days show about 60% of the incorporating activity compared with those from well-nourished age-control rats. The cell-free protein synthetic capacity of purified (salt-washed) polyribosomes from each diet group is similar; polyribosomes obtained from protein-depleted rats showed 90% of the activity obtained from well-nourished control rats. These findings served to confirm our previous observations (Alexis *et al.*, 1972). Studies were then carried out with the ribosome-wash fractions.

The capacity of the ribosome-wash fraction to promote transfer of radioactivity from [¹⁴C]leucyl-tRNA into hot-trichloroacetic acid-precipitable material was studied for effects of incubation time and Mg²⁺ concentration of the cell-free system. Purified polyribosomes were used in the cell-free system but elongation factors were omitted. Maximum incorporation of radioactivity occurred by 20 min at an optimum magnesium concentration of 5–6 mM.

Table 1. *Effect of a low-protein diet on the cell-free protein-synthetic activity of crude and purified (salt-washed) polyribosomes from rat skeletal muscle*

Crude polyribosomes (40 µg of rRNA) were assayed in the presence of pH 5-enzyme protein (500 µg) and [U-¹⁴C]leucine and purified polyribosomes (18–25 µg of rRNA), with [¹⁴C]leucyl-tRNA and non-limiting amounts of muscle elongation factors, as described previously (Alexis *et al.*, 1972). The Mg²⁺ concentrations of the incubation systems were 10 mM for crude polyribosomes and 5 mM for purified polyribosomes. Each experiment was based on duplicate determinations with pooled samples prepared from three to five rats, or 12 rats for the crude polyribosomes of rats fed on the low-protein diet. Numbers in parentheses represent the percentage of the value obtained with the well-nourished control group.

	Diet group	
	Adequate protein	Low protein
Crude polyribosomes	583 ± 51*	332 ± 29 (57 ± 5)
Purified polyribosomes	527 ± 172†	479 ± 48 (91 ± 10)

* Mean ± S.D. for five experiments. Results are expressed as 10⁻³ × c.p.m. incorporated from [¹⁴C]leucine/mg rRNA.

† Mean ± S.D. for seven experiments. Results are expressed as 10⁻² × c.p.m. incorporated from [¹⁴C]leucyl-tRNA/mg rRNA.

Table 2. *Effect of a low-protein diet on the yield and cell-free protein-synthetic activity of a ribosome-wash fraction prepared from crude polyribosomes of rat skeletal muscle*

The cell-free system was supplied with purified (salt-washed) polyribosomes (18–25µg of rRNA) from well-nourished control rats and [¹⁴C]leucyl-tRNA (as described in the Materials and Methods section), but did not contain added muscle elongation factors. The ribosome wash was extracted from a pooled crude polyribosome preparation obtained from five control rats and 12 protein-depleted rats for each experiment. Incubations were performed in duplicate, containing the amounts of ribosome wash (µg of protein) indicated in parentheses. —, Not determined.

Expt. no.	10 ⁻² × Incorporation (c.p.m./µg of rRNA)		Yield of wash (µg of protein/µg of rRNA)	
	Adequate protein	Low protein	Adequate protein	Low protein
1	374 (63)	147 (60)	3.0	2.9
2	763 (68)	441 (67)	3.6	4.0
3	633 (60)	319 (60)	—	—
4	183 (37)	84 (35)	1.1	0.5
5	319 (40)	195 (40)	2.5	3.4
6	417 (60)	242 (60)	1.5	2.4

Fig. 1. *Time-course of incorporation of radioactivity from [¹⁴C]leucyl-tRNA into protein with ribosome-wash fractions extracted from muscle crude polyribosome preparations of rats given adequate (● and ■) or low-protein (○ and □) diets*

The wash fractions were incubated with salt-washed ribosomes in the absence (○, ●) and presence (□, ■) of saturating amounts of elongation factors. Incubation conditions are described in Table 5.

Most of the comparative studies on the protein synthetic capacity of ribosome-wash fractions were conducted under these conditions.

The results of a number of experiments with wash fractions from the two diet groups are summarized in Table 2. Although the yield of ribosome wash varied among experiments, the specific and total activity of the fraction from protein-deprived rats was lower than that prepared with well-nourished rats.

Fig. 1 shows the rate of incorporation of radioactivity from [¹⁴C]leucyl-tRNA into systems containing the wash fractions. Throughout the entire incubation period the wash fraction obtained from the low-protein group showed a lower total incorporation than did the fraction from well-nourished rats. Fig. 1 also shows the rate of incorporation of radioactivity with the cell-free system containing ribosome wash in the presence of muscle elongation factors. The results indicate that addition of the ribosome-wash fraction does not alter the rate of radioactivity incorporation of salt-washed polyribosomes incubated in the presence of saturating amounts of EF 1 and EF 2.

The possible presence of an inhibitor of elongation-factor activity in the wash fractions was tested by assaying them with purified polyribosomes and limiting amounts of elongation factors (Table 3). The wash fractions did not decrease incorporation of radioactivity at these amounts of added EF 1 and EF 2. Therefore it appears that the lowered activity of the ribosome wash from protein-deprived animals is not caused by an inhibitor of the elongation reaction.

Heat treatment [60°C for 15 min in 0.05M-Tris-HCl (pH7.6), 1mM-dithiothreitol, 5% (w/v) glycerol] abolished the capacity of the ribosome-wash fractions to promote the amino-acid incorporation. Table 4 summarizes the results obtained by pre-incubating a ribosome wash with trypsin and shows

Table 3. *Effect of ribosome-wash fractions, prepared from crude polyribosomes isolated from skeletal muscle of rats fed on an adequate or low-protein diet, on the activity of a cell-free system containing limiting amounts of added elongation factors*

The cell-free system contained purified polyribosomes (22 μ g of rRNA), [14 C]leucyl-tRNA and limiting amounts of elongation factors. The ribosome-wash fraction was extracted from a pooled crude polyribosome preparation obtained from five control rats and 12 protein-depleted rats. The amount of ribosome-wash protein added in each case was 60 μ g.

Elongation factors added (μ g of protein) ...	$10^{-2} \times$ Incorporation (c.p.m./mg of rRNA)						
	None	EF 1		EF 2		EF 1 + EF 2	
		4 μ g	8 μ g	4 μ g	8 μ g	4 μ g	8 μ g
Source							
None	16	46	94	32	53	355	484
Adequate protein	417	496	537	396	445	531	566
Low protein	242	404	508	259	283	474	531

Table 4. *Effect of trypsin on the activity of a ribosome-wash fraction prepared from crude muscle polyribosomes of rats fed on a low-protein diet for 10 days*

The ribosome wash or elongation factors were preincubated in the incubation medium for 10 min at 37°C. Salt washed polyribosomes and [14 C]leucyl-tRNA were added and incubation was continued for 20 min. The amount of trypsin and trypsin inhibitor added to each incubation was 0.3 and 0.5 μ g respectively.

Condition and addition	Incorporation (c.p.m./incubation)
Wash, preincubated	259
Wash, preincubated with trypsin	50
Wash, preincubated with trypsin and inhibitor	218
Wash, trypsin and inhibitor	209
EF 1 and 2 preincubated	934
EF 1 and 2 preincubated with trypsin	53
EF 1 and 2 preincubated with trypsin and inhibitor	996
EF 1 and 2, trypsin and inhibitor	932

that the activity of the wash was eliminated by this treatment. From these findings we conclude that protein is the active component of the ribosome-wash fraction, although these experiments do not identify the nature of the protein(s) involved.

The cell-free activity of the wash fractions was next examined in the presence of saturating amounts of elongation factors; these results are given in Table 5. Addition of EF 1 alone to the ribosome wash from either group stimulated greater incorporation of radioactivity than did addition of EF 2 alone. Hence, the ribosome wash from both diet groups appeared to be limiting in EF 1 activity. However, the ribosome wash prepared from protein-deprived rats showed a lower incorporating activity in the presence of excess of either EF 1 or EF 2 than did the wash from adequately-fed rats.

The effects of various amounts of wash protein on the incorporation of radioactivity were tested (Fig. 2). At all amounts added, the fractions obtained from

rats fed on the low-protein diet showed a decreased activity compared with well-nourished rats. The highest incorporation was achieved within the system containing EF 1 and ribosome wash obtained from well-nourished rats. Because EF 1 was added in excess, the lower rate of incorporation obtained with the ribosome wash from protein-deprived rats appears to be due, in part, to a lower EF 2 activity in the fraction.

The effect of addition of various amounts of EF 1 to the ribosome wash collected from polyribosomes of protein-deprived rats is summarized in Table 6. Addition of 16 μ g of EF 1 protein was required to achieve the same total radioactivity incorporation with this fraction, compared with an equivalent amount of ribosome wash from control animals. Hence the ribosome wash from the protein-deprived group has a lower content of EF 1 activity.

In an attempt to determine if the lower activity of crude polyribosomes from protein-deprived rats was caused by limited availability of EF 1 and/or EF 2 in

Table 5. Activity of a ribosome-wash fraction, prepared from crude muscle polyribosomes of rats fed on an adequate or low-protein diet, in a cell-free system containing saturating amounts of muscle elongation factors

The cell-free systems contained a common source of purified polyribosomes (20μg of rRNA). Poly(U) (20μg) was added when the [¹⁴C]phenylalanyl-tRNA was used: 32μg of EF 1 and 16μg of EF 2 were used. The wash fractions were prepared from a mince of pooled muscle from five control rats and 12 protein-depleted rats. The amount of wash protein added in each assay system was 40μg. Each value is the mean of duplicate determinations. Values are c.p.m.

Addition	Incorporation from	
	[¹⁴ C]Leucyl-tRNA	[¹⁴ C]Phenylalanyl-tRNA + poly(U)
None	58	159
EF 1	175	733
EF 2	350	271
EF 1 + EF 2	2138	3894
Ribosome wash		
(adequate protein)		
+EF 1	633	353
+EF 2	1377	1659
+EF 1 + EF 2	981	763
Ribosome wash		
(low protein)		
+EF 1	2152	3918
+EF 2	319	231
+EF 1 + EF 2	896	1328
	696	499
	2148	4007

Fig. 2. Incorporation of [¹⁴C]leucine into protein from [¹⁴C]leucyl-tRNA as a function of the amount of the ribosome-wash fraction prepared from muscle crude polyribosomes of rats given adequate (● and ○) or low-protein (■ and □) diets

Ribosomes were incubated either with the wash fraction (●, ■) or with the wash fraction plus a saturating amount of EF 1 (○, □). Details of incubation conditions are described in Table 5.

the cell-free system, the crude polyribosome-pH5-enzyme system was supplemented with elongation factors and/or ribosome wash. The results of these studies are summarized in Table 7. Addition of EF 1 and/or EF 2 did not change the incorporation obtained with polyribosomes in the presence of excess of pH5 enzyme. Studies on the effects of addition of the wash fraction to various combinations of elongation factors and pH5 enzyme are summarized in Table 8. These results show that the ribosome wash did not further stimulate incorporation by crude polyribosomes in either diet group when they were incubated with the pH5-enzyme fraction.

Discussion

The amount and/or activity of elongation factors may act to control rates of polypeptide-chain elongation *in vivo* in various mammalian tissues (Henshaw *et al.*, 1971; Willis & Starr, 1971; Girigis & Nicholls, 1971, 1972), although Earl & Hindley (1971) concluded that aminoacyl-tRNA is freely available in the liver and muscle of starved rats, and that neither its rate of binding nor the translocation of peptidyl-tRNA is rate limiting in protein synthesis.

Observation of the activity of the NH₄Cl-wash fraction from crude polyribosomes of skeletal muscle

Table 6. *Effect of addition of various amounts of EF 1 on the activity of a ribosome-wash fraction prepared from crude muscle polyribosomes of rats fed on a low-protein diet*

A common source of purified ribosomes (19 μ g of rRNA) was used throughout. The wash fractions (40 μ g of protein) were prepared from a pooled muscle sample from five control rats and 12 protein-depleted rats. The cell-free system containing [14 C]leucyl-tRNA is described in the Materials and Methods section; it did not contain added EF 2.

Addition and source of ribosome wash	Added EF 1 (μ g of protein)	Incorporation (c.p.m./incubation)					
		0	4	8	12	16	32
None				113		136	
Adequate protein		763					
Low protein		441	542	625	698	736	789

Table 7. *Effect of addition of elongation factors to a pH5-enzyme-crude-ribosome system on incorporation of radioactivity from [14 C]leucine and [14 C]leucyl-tRNA*

The cell-free system is described in Table 1 and in the Materials and Methods section. The Mg^{2+} concentration of the medium was 10mM. Where appropriate, 500 μ g of pH5 enzymes, 32 μ g of EF 1 and 16 μ g of EF 2 were added to the incubations containing 16–20 μ g of rRNA from crude polyribosomes. Values are $10^{-2} \times$ incorporation (c.p.m./mg of rRNA).

Source of ribosomes (diet group) ...	Incorporation			
	Adequate protein		Low protein	
	[14 C]Leucine	[14 C]Leucyl-tRNA	[14 C]Leucine	[14 C]Leucyl-tRNA
None	1432	318	753	420
pH5	4946	1577	3523	1203
EF 1	1463	935	1304	807
EF 2	—	751	—	506
pH 5+EF 1	4929	1415	3348	1134
pH 5+EF 2	4573	1569	3622	1235
EF 1+EF 2	1684	1116	1141	964
pH5+EF 1+EF 2	4779	1418	3305	1041

revealed that the EF 1 and EF 2 activities were lower in the fraction obtained from protein-depleted rats than from well-nourished rats. However, supplementation of the cell-free system, which contains crude polyribosomes from protein-depleted rats and muscle pH5 enzyme fraction, with partially purified elongation factors or the ribosome-wash extracts, did not enhance total incorporation of labelled amino acid. An enhancement of incorporation would be expected if the difference in the protein-synthetic activity of crude polyribosomes from well-nourished and protein-deprived animals was caused by a limitation in amount of bound EF 1 and/or EF 2. Hence from these observations we conclude that the decreased protein-synthetic activity of muscle crude polyribosomes due to dietary protein deprivation is

probably not causally related to a decrease in the amount of bound elongation-factor activity.

There are a number of alternative explanations that should be discussed in evaluating the significance of our findings. Salt-washed polyribosomes require the addition of both EF 1 and EF 2 to the cell-free system for significant incorporation of radioactivity from 14 C-labelled aminoacyl-tRNA (Alexis *et al.*, 1972). Low incorporation of radioactivity is obtained when salt-washed polyribosomes are incubated with either elongation-factor fraction separately. However, this so-called 'complementary' effect of EF 1 and EF 2 on amino acid incorporation was not observed when crude polyribosomes were studied for their response to elongation-factor addition (Table 8). It is possible, therefore, that crude and salt-washed polyribosomes

Table 8. *Effects of addition of the ribosome-wash fraction and elongation factors on [¹⁴C]leucine incorporation by crude ribosomes from muscle of well-nourished and protein-depleted rats*

Muscle pH5 enzyme and EF 1 and EF 2 were prepared from a common source. Incubation conditions are described in Table 1. Where appropriate, 500 µg of pH5 enzyme protein, 32 µg of EF 1, 16 µg of EF 2, and 40 µg of wash fraction were added to the system containing 16–20 µg of rRNA from crude polyribosomes. Values are 10⁻² × incorporation (c.p.m./mg of rRNA).

Addition	Source of ribosomes (diet group)	Incorporation	
		Adequate protein	Low protein
None		1698	1827
pH5 enzyme		6097	4462
Wash (adequate protein)		2385	2129
Wash (low protein)		2119	2006
EF 1		2278	2325
EF 2		2236	2053
EF 1+EF 2		3218	2506
pH5 enzyme+wash (adequate protein)		5798	4264
pH5 enzyme+wash (low protein)		6054	4307
pH5 enzyme+wash (adequate protein)+EF 1		5652	4110
pH5 enzyme+wash (low protein)+EF 1		5817	4312
pH5 enzyme+wash (adequate protein)+EF 2		6258	4613
pH5 enzyme+wash (low protein)+EF 2		5932	4466

differ in their cell-free protein-synthetic response to supplementation with exogenous elongation factors. Our observations with the crude polyribosomes might also be explained by the presence of non-limiting amounts of bound EF 2 on the polyribosomes of both diet groups. The latter possibility supported by Gill & Dinius (1973), who have shown that most of the cellular EF 2 in most tissues is associated with the ribosomes. Also, we have observed that the ratio of cellular EF 2 to ribosomes does not decrease in muscle when rats consume inadequate amounts of protein (S. D. Alexis, V. R. Young & D. M. Gill, unpublished work).

Dietary protein deprivation might alter the capacity of crude polyribosomes to interact with the EF 1–aminoacyl-tRNA–GTP complex (Moon & Weissbach, 1972), resulting in lower amino acid incorporation *in vitro*. We found a variable degree of transfer of radioactivity from labelled aminoacyl-tRNA to trichloroacetic acid-precipitable material when crude polyribosomes were incubated in the presence of EF 1 alone. Hence, we cannot say whether the above mechanism accounts for the effects of an inadequate protein intake on the protein-synthetic activity of crude polyribosomes.

Differences in the amounts of initiation factors in the crude polyribosome preparations or in the initiation capacity of the ribosomes could also explain why dietary protein restriction decreases the protein-synthetic activity of the cell-free muscle system. However, optimum initiation-factor activity, as assessed with washed ribosomes, is usually observed

at lower concentrations of Mg²⁺ than were used in our incubation system with crude polyribosomes (e.g., Schafritz & Anderson, 1970; Grummt & Bielka, 1971; Comstock *et al.*, 1972).

Wool *et al.* (1972) have suggested that diabetes and insulin action may result in an alteration of the structure of muscle ribosomes and an ability of the particle to promote cell-free protein synthesis. A similar situation may arise from dietary protein restriction, as circulating insulin concentrations are decreased in rats within 1 week of being given a low-protein diet (Young *et al.*, 1973). Therefore treatment of crude polyribosomes with high concentrations of salt may tend to eliminate alteration of ribosomal structure, and so account for the similar protein-synthetic activity of washed polyribosomes from both well-fed and protein-deprived rats.

Thus the nature of the dietary-induced change of the unwashed polyribosome and its physiological significance in relation to the regulation of the rate of muscle protein synthesis remain to be determined.

This study was supported in part by Grant no. AM13432 from the U.S. National Institutes of Health. This is Contribution no. 1926 from the Department of Nutrition and Food Science, Massachusetts Institute of Technology.

References

- Alexis, S. D., Basta, S. S. & Young, V. R. (1972) *Biochem. J.* **128**, 521–530
- Comstock, J. P., O'Malley, B. W. & Means, A. R. (1972) *Biochemistry* **11**, 646–652

- Earl, D. C. N. & Hindley, S. T. (1971) *Biochem. J.* **122**, 267–276
- Gilbert, J. M. & Anderson, W. F. (1970) *J. Biol. Chem.* **245**, 2342–2344
- Gill, D. M. & Dinius, L. L. (1973) *J. Biol. Chem.* **248**, 654–658
- Girigis, G. R. & Nicholls, D. M. (1971) *Biochim. Biophys. Acta* **247**, 335–347
- Girigis, G. R. & Nicholls, D. M. (1972) *Biochim. Biophys. Acta* **269**, 465–476
- Grummt, F. & Bielka, H. (1971) *Eur. J. Biochem.* **21**, 210–216
- Henshaw, E. C., Hirsch, C. A., Morton, B. E. & Hiatt, H. H. (1971) *J. Biol. Chem.* **246**, 436–446
- Heywood, S. M. (1970) *Nature (London)* **225**, 696–698
- Irwin, J. D. & Hardesty, B. (1972) *Biochemistry* **11**, 1915–1920
- Lawford, G. R., Kaiser, J. & Hey, W. C. (1971) *Can. J. Biochem. Physiol.* **49**, 1301–1306
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Lubsen, N. H. & Davis, B. D. (1972) *Proc. Nat. Acad. Sci. U.S.* **69**, 353–357
- Miller, R. L. & Schweet, R. (1968) *Arch. Biochem. Biophys.* **125**, 632–646
- Millward, D. J. (1970) *Clin. Sci.* **39**, 591–603
- Moldave, K., Galasinski, W., Rao, P. & Siler, J. (1969) *Cold Spring Harbor Symp. Quart. Biol.* **34**, 347–356
- Moon, H. M. & Weissbach, H. (1972) *Biochem. Biophys. Res. Commun.* **46**, 254–262
- Pritchard, P. M., Gilbert, J. M., Schafritz, D. A. & Anderson, W. F. (1970) *Nature (London)* **226**, 511–514
- Roberts, K. & Coleman, W. H. (1972) *Biochem. Biophys. Res. Commun.* **46**, 206–214
- Schafritz, D. A. & Anderson, W. F. (1970) *J. Biol. Chem.* **245**, 5553–5559
- Skogerson, L. & Moldave, K. (1967) *Biochem. Biophys. Res. Commun.* **27**, 568–572
- Waterlow, J. C. & Stephen, J. M. L. (1968) *Clin. Sci.* **35**, 287–305
- Willis, D. B. & Starr, J. L. (1971) *J. Biol. Chem.* **246**, 2828–2834
- Wool, I. G., Castles, J. J., Leader, D. P. & Fox, A. (1972) *Handb. Physiol. Sect. 7: Endocrinol.* **1**, 385–394
- Young, V. R. & Alexis, S. D. (1968) *J. Nutr.* **96**, 255–262
- Young, V. R., Stothers, S. C. & Vilare, G. (1971) *J. Nutr.* **101**, 1379–1390
- Young, V. R., Vilare, G., Newberne, P. M. & Wilson, R. B. (1973) *J. Nutr.* **103**, 720–729