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The Sedimentation of Rat Skeletal-Muscle Ribosomes

EFFECT OF HYDROCORTISONE, INSULIN AND DIET

BY V. R. YOUNG, S. C. CHEN* AND JANE MACDONALD
Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Mass. 02139, *U.S.A.*

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1. The influence of hydrocortisone, insulin and diet on the size distribution of ribosomes in a post-mitochondrial supernatant prepared from rat skeletal muscle was studied by sedimentation analysis with a linear 15–40% (w/v) sucrose gradient. 2. Within 4hr. after the injection of 5mg. of hydrocortisone to well-nourished rats, a decrease in the yield per g. of muscle and proportion of total RNA due to polyribosomes was observed. Similar results were obtained in rats given a protein-free diet for 3 days before administration of the hormone. 3. Insulin injection increased the yield and proportion of polyribosomes within 2hr. and decreased the proportion of the lighter ribosomal aggregates. Similar results were noted in rats given a protein-free diet for 3 days before injection. A protein-free diet given for 3 days decreased the yield and proportion of polyribosomes. Insulin did not increase the yield of polyribosomes if rats were starved for 52hr. before injection, but decreased the yield and proportion of the lighter ribosome species. 4. A 52hr. period of starvation or 2,4-dinitrophenol (15mg./kg. body wt.) given 1hr. before the rats were killed resulted in a decreased yield and proportion of polyribosomes, and, within 6hr. of re-feeding the rats with protein-free diets, an increased concentration of polyribosomes was noted. 5. The effects of a protein-free diet, hydrocortisone and insulin on the sedimentation of muscle ribosomes were found to be in accord with their net effects on muscle protein synthesis.

Muscle undergoes marked changes in composition as a result of feeding the animal with a diet deficient in protein or calories (Cabak, Dickerson & Widdowson, 1963). These changes presumably reflect in a direct way the protein-synthesizing capacity of the synthetic machinery of muscle, and Waterlow & Stephen (1966) have reported a decrease in the uptake of [^{14}C]lysine into muscle protein of rats given a low-protein diet for 5 weeks, although they reported no decrease in the amount or specific activity of the free lysine in the muscle. Muscle is also influenced by a range of hormones, and diet is known to affect the activities of many endocrine glands (Munro, 1964). Feeding starving animals with carbohydrate results in enhanced incorporation of labelled amino acids into muscle protein, a result that may be related to a stimulation of insulin secretion by changes in dietary carbohydrate. Insulin stimulates protein synthesis in muscle tissue (Wool, Rampersad & Moyer, 1966), and this effect does not appear to be secondary to an increase in glucose or amino acid transport in this tissue (Wool & Cavicchi, 1966). On the other hand, administration of cortisone over short periods results in a loss of protein from muscle (Kochakian & Robertson, 1951) and a decreased incorporation of labelled amino acids into muscle protein (Wool & Weinshelbaum, 1959; Wool, 1960). This decrease in muscle tissue is often associated with a stimulation of hepatic protein synthesis and an increase in the protein content of other viscera (Goodlad & Munro, 1959).

Polyribosomes play a central role in cytoplasmic protein synthesis in a large variety of tissues, and protein synthesis may be partly controlled by the formation and breakdown of the polyribosomes in response to the specific requirements of the cell. Tata (1967) has discussed the significant effects of several hormones on the formation and distribution of ribosomes in a number of tissues, and Wunner, Bell & Munro (1966) have examined the effects of giving a tryptophan-deficient diet to rats on the distribution and activity of hepatic ribosomes. We have studied the effects of diet, glucocorticoids and insulin on the size distribution of ribosomes prepared from rat skeletal muscle as a preliminary to our studies of the interaction of diet and infectious stress on muscle metabolism.

The distribution of ribosomes from rat skeletal

* Present address: Market Quality Research Division, U.S. Department of Agriculture, Beltsville, Md. 20705, U.S.A.

muscle was evaluated in the present study by analysis of ribosomes in a post-mitochondrial supernatant.

MATERIALS AND METHODS

Animals and diets. Male Sprague–Dawley rats (Charles River Breeding Laboratories, Wilmington, Mass., U.S.A.) were used in the studies. Their weights were in the range 40–90g. and they were housed in wire-bottom cages in air-conditioned quarters at 24°. Details of the diet and the treatment with hydrocortisone (solu-Cortef; Upjohn Co., Kalamazoo, Mich., U.S.A.) and insulin (Insulin U-40; Squibb Co., New York, N.Y., U.S.A.) are given in the Results section. The composition of the protein-free diet used was as follows (% by weight): sucrose, 28; dextrin, 56·5; corn oil, 10; salt mix, 5; vitamin mix, 0·5. The compositions of the salt and vitamin mixtures were identical with those used by Rogers & Harper (1965). In addition, choline was added to the diet as an aqueous solution at a level of 0·3g./100g. of dry diet. The 18%-casein diet was of a similar composition to the protein-free diet except that the casein and 0·3% L-methionine replaced sucrose and dextrin in equal amounts. All rats were given this latter diet for 3 or 4 days before use in the experiments and given the diet during the period of study unless stated otherwise.

Sucrose-gradient analysis of muscle ribosomes. Rats were decapitated by guillotine and muscle was removed from the rear legs. After the muscle had been cleaned quickly of visible fat, it was immersed in a chilled buffer of the following composition: 0·25M-KCl, 0·01M-$MgCl_2$, 0·01M-tris–HCl buffer, pH7·6. Preparation of the post-mitochondrial supernatant and method of sucrose-gradient analysis have already been described in detail (Chen & Young, 1968). The distribution of ribosomes in the gradient was determined by pumping the linear 15–40% (w/v) sucrose gradients from the bottom at the rate of 3·4ml./min. with a Sigmamotor pump and monitoring the gradient automatically at 260mμ through a 5mm. flow-cell with a Gilford Absorbance Recorder. As described by Chen & Young (1968), the present method allows preparation of muscle polyribosomes in a substantially higher yield than indicated in previous studies of skeletal muscle (Breuer, Davies & Florini, 1964; Wool & Cavicchi, 1967). Any aggregate larger than a dimer is considered as a polyribosome.

The distribution of monomers, dimers and polyribosomes was estimated by measurement of the area under the curve, by the method described by Wunner *et al.* (1966). Perpendicular lines were drawn by inspection from the trough between the monomer and dimer peaks and the dimer peak and the polyribosomes. The percentage contribution made by these species to the total area was calculated and used as a measure of the response of muscle ribosomes to the various dietary and hormonal treatments. The RNA of the ribosome pellet, obtained by centrifugation of the post-mitochondrial supernatant from the well-nourished control rats for 4hr. at 40000rev./min. (105000$g_{av.}$) in the Spinco rotor 40, represented 22% of total RNA in skeletal-muscle tissue. These estimates were not altered by giving rats a low-protein diet for 20 days (V. R. Young, unpublished work). Although the recovery of polyribosomes from skeletal muscle is incomplete, under the controlled conditions described by Chen & Young (1968), it appeared worth while to use the area under the tracing of the gradient as an approximation of the yield of the ultraviolet-absorbing material (ribosomal RNA) per unit weight of wet muscle and as a further means of comparing the effects of the dietary and hormonal treatments.

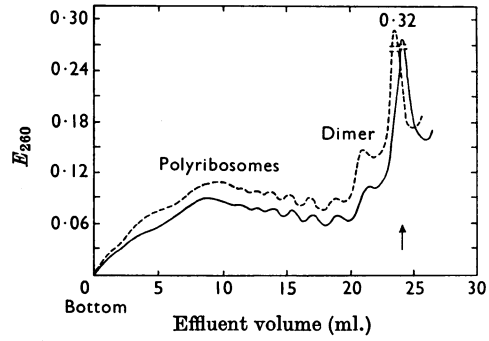

Fig. 1. Effect of hydrocortisone on the sedimentation of rat skeletal-muscle ribosomes. A detergent-treated post-mitochondrial supernatant (0·7ml.) prepared from skeletal muscle of control rats or rats injected (intraperitoneally) with 5mg. of hydrocortisone succinate 4hr. before they were killed was layered over a linear 15–40% (w/v) sucrose gradient in 0·25M-KCl, 0·01M-$MgCl_2$ and 0·01M-tris–HCl buffer, pH7·6. The gradients were centrifuged at 25000rev./min. (63000$g_{av.}$) in a Spinco SW25.1 rotor for 2hr. Each curve represents the profile obtained with 0·4g. of muscle equivalent prepared from a pooled muscle sample from four rats. The gradient profile was monitored at 260mμ (i.e. E_{260}) and recorded automatically by using a flow-cell with a 5mm. light-path and a Gilford Absorbance Recorder: ——, hydrocortisone-treated rats; – – – –, control rats. The arrow indicates the position of the monomeric (80s) ribosomes.

RESULTS

Response to hydrocortisone. A sucrose-gradient pattern of the ribosomes of skeletal muscle obtained with control rats and rats given (intraperitoneal injection) 5mg. of hydrocortisone succinate 4hr. before they were killed is shown in Fig. 1. The ribosome profile of the rats injected with the glucocorticoid is characterized by a decrease of heavy polyribosomes and a slight decrease in the lighter ribosome species (monomers and dimers). This experiment was repeated on six different occasions. The consistent finding was a decrease in the polyribosome concentration per unit weight of muscle tissue. Although the proportions of the monomers and dimers to the total ribosome population were consistently increased, a variability in response to hydrocortisone was noted since in a number of experiments the monomer and dimer peak heights were increased; as shown in Fig. 1, a slight decrease in this fraction of the gradient

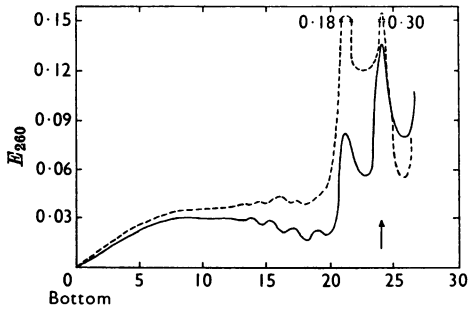

Fig. 2. Effect of hydrocortisone on skeletal-muscle ribosomes in rats given a protein-free diet. The rats were given a protein-free diet for 3 days and then injected (intraperitoneally) with 5mg. of hydrocortisone succinate 4hr. before they were killed. The equivalent of 0·26g. of muscle, prepared from a pooled sample from four rats, was layered on each gradient: ——, hydrocortisone-treated rats; ----, protein-free control rats. Determination of the ribosome profile and explanation of the arrow are described in Fig. 1.

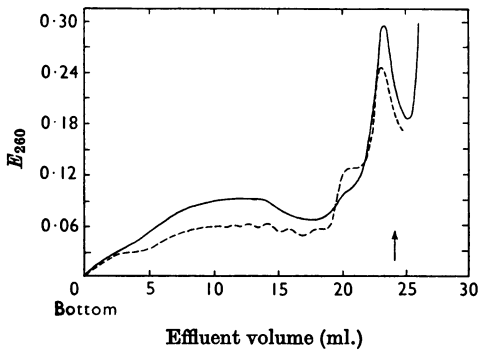

Fig. 3. Effect of insulin on skeletal-muscle ribosomes. Rats were injected (intraperitoneally) with 2 units of insulin and killed 2hr. later. Post-mitochondrial supernatant equivalent to 0·4g. of muscle, prepared from a pooled sample from four rats, was layered on each gradient: ——, insulin-treated rats; ----, control rats. Determination of the ribosome profile and explanation of the arrow are described in Fig. 1.

followed hydrocortisone injection, as was found in other experiments. We have not been able to resolve this latter inconsistency.

A decreased yield of total ribosomes was noted in four experiments, and in the remaining two experiments the yield was similar in control and hydrocortisone-injected rats.

Since the protein catabolic response to cortisone is independent of protein intake (Goodlad & Munro, 1959), and the increased nitrogen in the urine appears to derive from muscle tissue (Munro, 1964), the influence of pre-feeding weanling rats with a protein-free diet for 3 days on the response to hydrocortisone injection was studied. The results obtained in one experiment are given in Fig. 2. Hydrocortisone injection resulted in a decrease in the yield of heavy polyribosomes and of the lighter ribosome species. Consequently, there appeared to be a decrease in the total ribosome yield from muscle of protein-depleted rats injected with hydrocortisone. However, the percentage distribution of the ribosome species after hydrocortisone injection in rats pre-fed with the protein-free diet approached that obtained with well-nourished controls (compare Figs. 1 and 2).

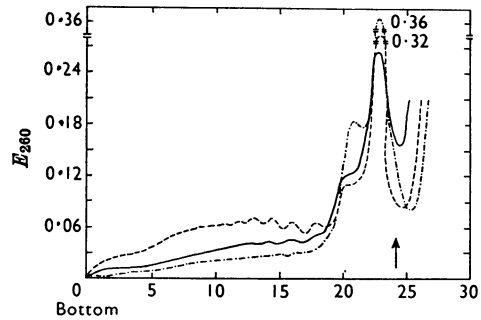

Fig. 4. Effect of insulin on skeletal-muscle ribosomes in rats given a protein-free diet. The rats were given a protein-free diet for 3 days and then injected (intraperitoneally) with 2 units of insulin and killed 2hr. later. The equivalent of 0·4g. of muscle, prepared from a pooled sample from four rats, was layered on each gradient: ----, well-nourished control rats; -·-·-, rats given protein-free diet; ——, rats given protein-free diet plus insulin. The sucrose-gradient analysis and explanation of the arrow are described in Fig. 1.

Response to insulin. Two units of insulin given 2hr. before the rats were killed consistently resulted in an increased yield and proportion of larger ribosomal aggregates above control values. The results of one typical experiment are shown in Fig. 3. The resolution of the gradient just below the dimer region in the insulin-treated rats was not as clear as that obtained in most of these experiments. The yields of the monomers and dimers were unchanged or slightly decreased in our experiments, and their proportions to the total ribosome population were consistently decreased. The total yield of ribosomes, by estimation of the total area under the gradient curve, was consistently increased after insulin injection. The increases were in the range 107–128% in a series of four separate experiments.

Pre-feeding weanling rats with a protein-free

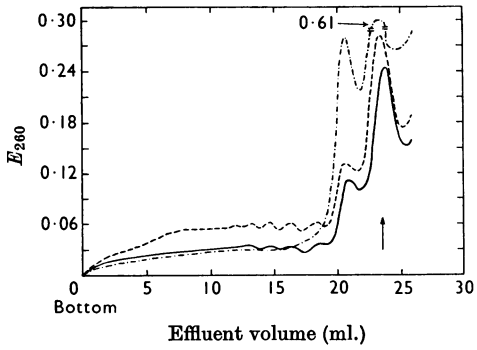

Fig. 5. Effect of insulin and starvation on skeletal-muscle ribosomes. Rats were starved for 52hr. and then injected (intraperitoneally) with 2 units of insulin and killed 2hr. later. The control rats were fed on 18%-casein diet throughout. The equivalent of 0·4g. of muscle, prepared from a pooled muscle sample from three or four rats, was layered on each gradient: ----, well-nourished control rats; ——, rats starved for 52hr.; -•-•-, rats starved for 52hr. plus insulin. The sucrose-gradient analysis and explanation of the arrow are described in Fig. 1.

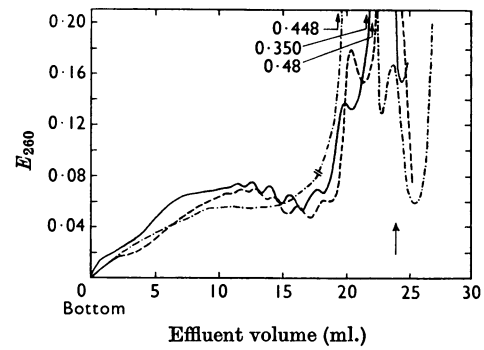

Fig. 6. Effects of starvation and 2,4-dinitrophenol on skeletal-muscle ribosomes. Rats were starved for 32hr. or adequately nourished rats were injected (intraperitoneally) with 2,4-dinitrophenol (15mg./kg. body wt.) 1hr. before they were killed. The equivalent of 0·3g. of muscle, prepared from a pooled sample from four rats, was layered on each gradient: ——, well-nourished control rats; -•-•-, rats starved for 32hr.; ----, well-nourished rats given 2,4-dinitrophenol. Determination of the ribosome profile and explanation of the arrow are described in Fig. 1.

diet for 3 days before insulin injection did not change the pattern of response to the hormone. These results are shown in Fig. 4. The polyribosome concentration was increased by insulin and the proportion of polyribosomes to the total ribosome population was greater than in the rats given the protein-free diet. The monomer and dimer ribosome species were decreased in yield, and the proportion of these smaller ribosomal aggregates decreased after insulin administration. These results are summarized in Table 1. This experiment also illustrates the influence of feeding the rats with a protein-free diet on the size distribution of skeletal-muscle ribosomes. A decreased proportion and yield of the heavy polyribosomes followed 3 days of protein depletion and an increase in the proportions of the monomer and dimer species.

In contrast with the results obtained with rats given adequate or protein-free diets, insulin did not bring about an increase in the heavy polyribosomes if rats were starved for 50hr. before injection. These results are illustrated in Fig. 5. However, the yields of the monomer and dimer ribosome species were markedly decreased after insulin administration to starved rats; consequently, the percentages of the ribosome population as polyribosomes and small ribosomal aggregates were comparable with those obtained in well-fed non-injected control rats.

Response to starvation and re-feeding. Starvation for 32 or 52hr. produced a decrease in the yield of polyribosomes and a shift in the size distribution of the ribosome species towards the lighter aggregates (monomer and dimer). Measurement of the total area under the sucrose-gradient curve suggested that a 32hr. starvation period did not significantly decrease the total yield of ribosomes per unit weight of muscle; but a decrease followed a 52hr. starvation period. The results obtained after starvation for 32hr. are shown in Fig. 6. It can also be noted that the resolution of the gradient appears to be less precise than that obtained with well-nourished rats. This has been a consistent finding in our experiments involving a starvation period of 32hr. or longer.

Since 2,4-dinitrophenol uncouples oxidative phosphorylation and decreases the ATP concentration in the cell, the influence of 2,4-dinitrophenol on the size distribution of muscle ribosomes was investigated as well. Fig. 6 shows that 2,4-dinitrophenol (intraperitoneal injection, 15mg./kg. body wt.) given 1hr. before the rats were killed decreased the yield of heavy polyribosomes and increased the yield and proportions of the monomer and dimer ribosome species.

The effects of short-term re-feeding with protein-free or adequate protein diets after starvation for 52hr. are shown in Fig. 7. Within 6hr. after feeding each rat with 4g. of either diet, the polyribosomes increased and the monomer and dimer proportions were decreased. The protein-free diet resulted in a slightly greater increase in the lighter ribosome species compared with that obtained after re-feeding the rats with an adequate protein diet. Both diets caused comparable increases in the heavy polyribosome region of the gradient. Short-term re-feeding did not bring about a complete return to the

control yield of polyribosomes found in the muscle of well-nourished rats. The areas under the curves represented by the ribosome species of larger aggregations than dimers were 26% for starved rats, 50% for rats re-fed with the protein-free diet and 45% for rats re-fed with the adequate protein diet; each is compared with adequately fed controls.

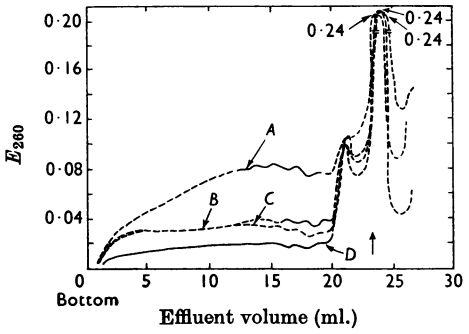

Fig. 7. Effects of starvation and re-feeding on skeletal-muscle ribosomes. Rats were starved for 52hr. and then given 4g. of 18%-casein diet or casein-free diet and killed 6hr. later. Control rats were fed on 18%-casein diet throughout the experimental period. The equivalent of 0·4g. of muscle, prepared from a pooled sample from four rats, was layered on each gradient. Curve *A*, well-nourished control rats; curve *B*, rats given 18%-casein diet; curve *C*, rats given casein-free diet; curve *D*, rats starved for 52hr. The sucrose-gradient analysis and explanation of the arrow are described in Fig. 1.

DISCUSSION

The results obtained in the present experiments are summarized in Table 1 and demonstrate that the yield of polyribosomes and the size distribution of muscle ribosomes change in response to a number of hormonal and dietary situations.

The present results on the approximate yield of polyribosomes after hydrocortisone or insulin injection are based on the wet weight of muscle tissue and do not take into account hormonal changes in the water content of muscle. However, in support of the present conclusions, D'Arcy & Howard (1965) demonstrated a marked diuretic effect of cortisol in rats, which was maximal 4hr. after administration of the steroid, and Palmer (1966) has reported a small decrease in the percentage of muscle water in rats after a single injection of cortisol. Scharff & Wool (1965) found that the water content of the perfused rat heart was not affected by insulin. These findings suggest that the differences in yield of muscle polyribosomes observed in the present studies were probably not due to opposite changes in the water content of the tissue.

A decreased yield of polyribosomes in rat skeletal muscle occurred 4hr. after hydrocortisone injection. The decreased rate of protein synthesis that occurs in skeletal muscle after glucocorticoid administration (Wool, 1960) may be the consequence of a decreased polyribosome concentration in muscle. Palmer (1966) has suggested that the failure of rats

Table 1. *Summary of mean results obtained in sucrose-gradient experiments on the influence of diet, hydrocortisone and insulin on the yield and distribution of ribosomes in rat skeletal muscle*

The yield and distribution of the ribosome species were estimated from the area under the curve as described in the Materials and Methods section. The estimate of yield for control rats in the experiments was arbitrarily assigned a rating of 100. Details of experimental treatments are given in the Results section.

	Yield				% of total population		
Group	Monomer	Dimer	Polysome	Total	Monomer	Dimer	Polysome
Control	100	100	100	100	27	17	58
Hydrocortisone	107	98	57	80	38	21	41
Protein-free	157	63	56	79	41	24	35
Control	100	100	100	100	49	13	48
Insulin	92	91	132	112	32	11	57
Control: protein-free	100	100	100	100	41	24	35
Hydrocortisone	50	45	74	58	36	18	46
Control: protein-free	100	100	100	100	46	24	30
Insulin	76	63	153	93	44	17	39
Control: starved	100	100	100	100	51	28	21
Insulin	62	38	100	62	50	17	33
Control	100	100	100	100	34	11	55
Starved for 52hr.	70	77	26	46	52	18	30
Starved, re-fed with protein-free diet	88	68	50	53	40	13	47
Starved, re-fed with adequate protein diet	90	68	45	56	38	13	49

to gain body weight after a single injection of cortisol may be due to an inhibition of the synthetic mechanisms in growing tissues such as muscle. The present findings appear to support this view.

Insulin administration was found to cause an increase in the concentration of muscle polyribosomes. This effect correlates with the stimulation of amino acid uptake and protein synthesis in muscle caused by injection of insulin (Wool & Krahl, 1959) and the increased activity *in vitro* of ribosomes prepared from diabetic rats treated with insulin (Wool & Cavicchi, 1966). Stirewalt, Wool & Cavicchi (1967) reported that diabetes decreased the number of larger ribosomal assemblies, and that insulin restored the sedimentation characteristics of the ribosomes to normal. Our results with intact rats support those obtained by Stirewalt *et al.* (1967), although the yield of muscle polyribosomes obtained by these workers would be substantially lower than that obtained in the present studies (Chen & Young, 1968).

Insulin increased the concentration of muscle polyribosomes in rats given protein-free diets for 3 days, but this effect was not observed in rats starved for 52hr. When starved rats were injected with insulin, the hormone produced a marked decrease in the concentration of the lighter ribosome species. It is noteworthy that insulin results in an increased nitrogen output in the urine when injected into starved animals (Sokhey & Allen, 1924; Reid, 1936).

Feeding weanling rats with a protein-free diet caused a decreased yield of polyribosomes in skeletal muscle. Waterlow & Stephen (1966) have reported that a low-protein diet leads to a decrease in the rate of amino acid uptake into muscle protein, and our results suggest that this may in part be related to the decreased yield of polyribosomes in this tissue. A decrease in the concentration of polyribosomes may be expected to decrease the rate of protein synthesis in the tissue, and this suggestion is supported by the results of Fleck, Shepherd & Munro (1965) and Wunner *et al.* (1966), who demonstrated that feeding rats with a tryptophan-deficient diet decreased the yield of polyribosomes and amino acid incorporation activity *in vitro* of polyribosomes from rat liver. Sidransky & Verney (1967) have also shown that the activity *in vitro* of muscle ribosomes is decreased after feeding rats with a threonine-devoid diet.

A 52hr. starvation period caused a decrease in the total ribosomal population and a decrease in the proportion of heavy to light ribosome species. The response to prolonged starvation may be related to a decrease in the concentration of cellular ATP, since 6hr. after re-feeding rats with protein-free or adequate protein diets the polyribosome concentration was found to increase. 2,4-Dinitrophenol injection, which results in a diminished content of cellular ATP, mimicked the influence of starvation, again lending support to the suggestion of the importance of an adequate supply of ATP. Webb, Blobel & Potter (1966) have shown that a similar response occurs in the rat liver polyribosomes.

The present results suggest that skeletal-muscle polyribosomes respond less rapidly to re-feeding than do those in liver. In this respect, our findings parallel those of Mendes & Waterlow (1958), who found that muscle protein content increased less rapidly than liver protein after re-feeding of protein-depleted rats with an adequate protein diet.

The present findings of changes in polyribosome concentrations are in accord with the established net effects of the hormones and diet on muscle protein content. However, our results do not afford an opportunity to describe the mechanism(s) of the diet- and hormone-induced changes in muscle ribosomes. Further, in view of the findings of Sox & Hoagland (1966) and Decken (1967) demonstrating that the amino acid incorporation activity *in vitro* of hepatic polyribosomes from the same region of the sucrose gradient may differ according to the nutritional state of the donor rats, it will be necessary to establish the influence of protein intake on the synthetic capacity of the ribosomes of skeletal muscle.

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