Effect of Insulin and Denervation on the Activity of Ribosomes of Rat Diaphragm Muscle†

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ABSTRACT: A procedure is described for the isolation of ribosomes from 0.5- to 1.0-g quantities of diaphragm muscle. The ribosomes have an A_{260}/A_{280} ratio greater than 1.8 and an A_{260}/A_{235} ratio greater than 1.5. Probably 60–70% of the ribosomes available are collected. Ribosomes prepared from muscle first incubated *in vitro* show a diminished capacity for incorporation of leucine and phenylalanine into protein by comparison with ribosomes from fresh tissue. When insulin is present during incubation, incorporating capacity of the ribosomes declines less rapidly. The long established effect of insulin to promote incorporation of amino acids into protein of muscle *in vitro* can thus be seen to be associated with an influence of the hormone on the ribosomes of the tissue. Insulin maintains the activity of the ribosomes during incubation because it reduces the extent of polysome disaggregation during incubation. By use of [^{3}H]puromycin it can be seen that in the presence of insulin a larger proportion of the ribosomes carry nascent peptide chains. Ribosomes from incubated tissue remain active in translation of poly(U)—a greater response to poly(U) being seen at 5 mM Mg the greater the extent of polysomal disaggregation. The presence of a mixture of amino acids during incubation reproduces the effects of insulin. During denervation hypertrophy of diaphragm muscle, although there is a large increase in the number of ribosomes, their specific activity in incorporation is little changed nor is there obvious difference in polysome profile or proportion of ribosomes bearing nascent chains. The denervated tissue is known to respond poorly to insulin and this is reflected in the finding that polysome disaggregation during incubation is less and the incorporating capacity of the ribosomes of the denervated tissue declines less than does that of normal tissue even when the latter is incubated in the presence of insulin.

The action of insulin on protein synthesis in muscle has been the subject of many investigations. For the most part these have been concerned in recent years with subcellular preparations from muscles taken from animals which were either diabetic or treated with insulin *in vivo*. The increased rate of protein synthesis brought about by insulin under these conditions was associated with a rise in the proportion of ribosomes attached to messenger to form polysomes (Wool *et al.*, 1968) and in the proportion of ribosomes bearing nascent polypeptide chains (Wool and Kurihara, 1967). The experiments with whole animals do not exclude the possibility that insulin acts by first increasing the uptake of glucose or amino acids into muscle. Perfusion of the rat heart and a hemicorpus preparation with insulin diminishes the content of native ribosomal subunits, presumably as a result of an increase in their rate of attachment to messenger to form polysomes (Morgan *et al.*, 1971; Jefferson *et al.*, 1972). Activity in protein synthesis of ribosomes isolated from the tissues perfused in this manner was not investigated.

In earlier work on the actions of insulin on protein synthesis by rat diaphragm, insulin was added to the muscle *in vitro* and this made it possible to show that the increased amino acid incorporation by the intact muscle was independent of the presence of glucose or amino acids in the ambient medium (Manchester and Young, 1961). In order to extend these findings and because there are no previous reports on subcellular preparations from skeletal muscle treated with insulin *in vitro*, the present paper describes changes in ribosomes isolated from hemidiaphragms incubated with and without insulin. In addition, diaphragm was studied after denervation because the increased capacity for protein synthesis during the unique denervation hypertrophy of diaphragm (Manchester and Harris, 1968) may show certain features in common with insulin stimulation During the course of the work it became necessary to find methods of improving the yield of ribosomes obtainable from the relatively small amounts of muscle available in such experiments.

Experimental Section

Materials. L-[U-^{14}C]leucine, 331 Ci/mol, L-[U-^{14}C]phenylalanine, 531 Ci/mol, and [8-^{3}H]puromycin, 2 Ci/mmol, were obtained from Amersham-Searle, Chicago, Ill. Hepes,[1] ATP, GTP, EGTA, and creatine phosphate were from Sigma Chemical Co., St. Louis, Mo. Other chemicals were from reputable suppliers and of high grade.

Methods. Diaphragm muscle was usually taken from rats that were about 120 g weight and which had access to food and water. The whole of the diaphragm septum was dissected out, laid on filter paper, and divided into the two lateral halves and posterior third. The lateral halves were incubated with shaking for various periods, usually 30 min, at 37° in Krebs Ringer bicarbonate buffer gassed with 95% oxygen and 5% CO_2 and containing the additions stated. The posterior third of the tissue was kept in saline at 0° and used as the unincubated control.

Left unilateral phrenicectomy was performed when appropriate under ether anesthesia by a thoracotomy incision 3 days prior to removal of the diaphragm.

PREPARATION OF MUSCLE RIBOSOMES. Two methods, one a slight modification of the other, were used. In the first, after incubation, the muscle, usually 0.5–0.7 g, was placed at

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[1] Abbreviations used are: poly(U), poly(uridylic acid); Hepes, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)tetraacetic acid.

0° in 10 ml of homogenizing medium consisting of 250 mM KCl–15 mM $MgCl_2$–5 mM EGTA–0.1 mM EDTA–20 mM Hepes adjusted to pH 7.6 with KOH–10 mM mercaptoethanol (buffer A). The tissue was minced finely with scissors, then homogenized for about 10 sec at a medium speed setting in a fluted 100-ml glass vessel of the VirTis 45 instrument. The homogenate was centrifuged at 10,000*g* for 10 min, and the supernatant was poured into 0.5 ml of 10% Lubrol WX in water. The mixture was left about 1 hr at 0°, then centrifuged at 30,000*g* for 20 min when a white precipitate usually appeared. The supernatant was layered over 3 ml of homogenizing medium containing 1 M sucrose and centrifuged for 2 hr at 105,000*g*. The supernatant above the sucrose step was aspirated off, the sucrose step was poured off, and the remaining pellet was drained and gently washed with a few milliliters of buffer B (100 mM KCl–5 mM $MgCl_2$–0.1 mM EDTA–20 mM Hepes (pH 7.6)–10 mM MSH).

In the modified version of this method, used in most of the experiments described, the precipitate appearing after centrifugation at 30,000*g* was dispersed in 1 ml of buffer A, warmed for 5 min at 37°, then centrifuged for 5 min at 5000*g* and the supernatant was added to the original supernatant.

Ribosomal pellets were dispersed in 0.5–0.75 ml of buffer B by agitation, warmed at 37° for 5 min, and clarified by centrifugation at 5000*g* for 10 min. An 0.1-ml sample of supernatant was diluted with water to 3 ml and its $A_{260,280,235}$ determined. Dilution of the ribosome solution was then made if thought desirable to equalize the amount of material in the different samples. The solution used for the optical density measurements was made 0.1 M with respect to KOH and incubated at 37° for 1 hr; 1 ml of 1 N $HClO_4$ was then added and after standing and centrifugation the solution was read at A_{260} and A_{275} and the RNA content was calculated according to Fleck and Munro (1962). RNA-P content was usually about 5% of the concentration of ribosomes estimated on the basis that A_{260} of 1.0 = 90 μg of ribosomes.

MEASUREMENT OF AMINO ACID INCORPORATION. Measurement of incorporation was carried out in 0.25 ml total volume in buffer B fortified with 1 mM MgATP, 0.1 mM GTP, 5 mM creatine phosphate, 19 nonlabeled amino acids each 10 μM, [^{14}C]phenylalanine or leucine at about 0.5 μM (0.06 μCi/tube), 50 μl of hepatic cytoplasm prepared as below, and about 40 μg of ribosomes. Poly(U) when present was at a concentration of 400 μg/ml. The usual procedure was to run for each ribosomal pellet tubes containing [^{14}C]phenylalanine for 20 and 40 min and additional tubes with poly(U) as described in the results. Simultaneously, tubes containing [^{14}C]leucine were run containing the same quantity of ribosomes as with [^{14}C]phenylalanine and double the quantity of ribosomes to ensure that incorporation rates were proportional to the concentration of ribosomes, which they invariably were under the conditions employed. Incubation was for 20 min at 37°, after which the tubes were chilled in ice and duplicate 0.1-ml aliquots spotted onto Whatman 3 MM filter paper discs which were placed in 7% Cl_3CCOOH containing 0.1% phenylalanine and leucine. After soaking, the discs were transferred to fresh Cl_3CCOOH solution in which they were heated to 90° for 20 min, then transferred to fresh Cl_3CCOOH solution and successively to ethanol and ether. After air drying, the discs were placed in 10 ml of toluene containing 0.5% 2,5-diphenyloxazole and counted in a Beckman LS-100 scintillation counter. Efficiency of counting was 50–60%.

PREPARATION OF LIVER CELL SAP. Cell sap was prepared from the livers of the diaphragm donors. Each gram of liver was homogenized in 3 ml of buffer B containing 0.25 M sucrose. The homogenate was centrifuged for 10 min at 10,000*g* and the supernatant further centrifuged 2 hr at 105,000*g*. The surface layer of lipid-containing fluid was discarded and only the clear supernatant collected. Shortly before use aliquots were run through a Sephadex G-25 column equilibrated with buffer B. The treated sap had a protein content of about 5 mg/ml.

SUCROSE DENSITY GRADIENT PROFILES. An aliquot, usually 100 μl of the ribosome suspension, was layered over a linear 10–40% sucrose gradient made up in buffer B. The gradients were centrifuged at 0° for 30 min at 50,000 rpm (334,000*g*) in a Beckman SW 50.1 rotor and monitored by upwards displacement with 55% sucrose through a Uvicord flow cell assembly.

RELEASE OF NASCENT PEPTIDES BY PUROMYCIN. The procedure was a modification of that of Pestka *et al.* (1972). Samples (50 μl) of ribosomes were mixed with 50 μl of buffer B with extra KCl added to 0.9 M and containing approximately 6 μM [^{3}H]puromycin (2 Ci/mmol). They were incubated for 30 min at 37°, then duplicate 50-μl aliquots were spotted out onto glass fiber disks and placed in 7% Cl_3CCOOH. The disks were washed at room temperature with three changes of Cl_3CCOOH solution, two of ethanol, and finally ether, then allowed to dry, and placed in 10 ml of scintillation fluid as above. Efficiency of counting was about 20%. Preliminary experiments showed slightly higher counting efficiency with glass fiber as opposed to paper disks. The formation of acid-insoluble radioactivity rose rapidly in the first 5 min and reached a steady level by 30 min. If incubation was carried out at 0° counts obtained were 50–60% of those after incubation at 37° (Earl and Hindley, 1971).

Results

The yield of ribosomes obtained by the first procedure described ranged from 0.45–1.10 mg/g of normal muscle. Some preparations clearly were more efficient than others, and it is also possible that the RNA content of the muscle used (previously found to be about 1 mg/g) varied to some extent. The $A_{260/280}$ was consistently in the range 1.93–2.00 and the $A_{260/235}$ usually 1.5–1.6. Yields and optical density ratios tended to be consistent for different samples in any one experiment. When it was realized that an appreciable quantity of RNA was contained in the 30,000*g* precipitate, the additional step involving extraction of this material was introduced. Gradients indicated the material extracted to be mainly larger oligosomes and polysomes. As a result of this step the yield of ribosomes has risen to 1–1.5 mg/g of tissue, but there seems to be some slight drop in the A_{260}/A_{280} ratio to about 1.8–1.9 and the A_{260}/A_{235} ratio to 1.4–1.5.

The method has been largely empirical in development. Use of EGTA to complex Ca^{2+} (suggested to the author by I. G. Wool and in Zak *et al.*, 1972) increases the yield of ribosomes possibly by relaxing the fibers. The most critical step in order to obtain ribosomes with reasonable $A_{260/280}$ and $A_{260/235}$ ratios is the centrifugation at 30,000*g* for 20 min after keeping the post-mitochondrial supernatant at 0° for a period. If centrifugation is omitted this precipitate comes down with the ribosomes. If the period at 0° before the centrifugation is omitted the material is not usually sedimentable at 30,000*g* and again comes down with the ribosomes. The nature of the precipitate, other than the fact that it contains ribosomes and polysomes, is not known.

Lubrol has been used in preference to Triton X 100 or deoxycholate because it has a much lower optical density in the 260-nm region. Trace contamination is therefore less likely to

TABLE I: Incorporation of Labeled Leucine and Phenylalanine by Ribosomes Isolated from Diaphragm Muscle Incubated for Various Periods in the Presence or Absence of Insulin.[a]

	Incorporation of Radioactivity (cpm/μg of ribosomes) Min of Incubation			
	0	10	30	60
Leucine				
Incubation without insulin		79	40	31
	92			
Incubation with insulin		81	71	59
Phenylalanine				
Incubation without insulin		50	25	23
	57			
Incubation with insulin		55	48	34

[a] The figures shown are of experiments in which incorporation at each time interval was studied simultaneously. Many experiments at one time interval only, *e.g.*, 20 or 40 min, as well as the times stated confirm the general pattern. Concentration of insulin used was 0.1 unit/ml.

influence optical density readings. However, it is not necessary to have any detergent present and very similar results have been obtained in other experiments without use of Lubrol.

Extent of incorporation by the ribosomes ranged from 0.5 to 1 leucine/ribosome and 0.25–0.5 phenylalanine/ribosome. In a separate series of experiments it was estimated that the specific activity of the labeled amino acid is probably halved by dilution by amino acids in the Sephadex-treated liver sap. Whether this represents incomplete extraction of free amino acids by the Sephadex (Wannemacher *et al.*, 1970) or results from amino acids arising from proteolysis in the sap after Sephadex filtration is not known.

Effect of Insulin. Ribosomes isolated from diaphragm muscle after incubation for various periods ranging from 10 to 60 min invariably show greater incorporating capacity if incubation has been in the presence of insulin. Figures for incorporation are shown in Table I for both leucine and phenylalanine. They demonstrate that the enhanced rate of incorporation of amino acids into protein exhibited by isolated diaphragm muscle in the presence of insulin results at least in part from greater activity of the ribosome–polysome complexes.

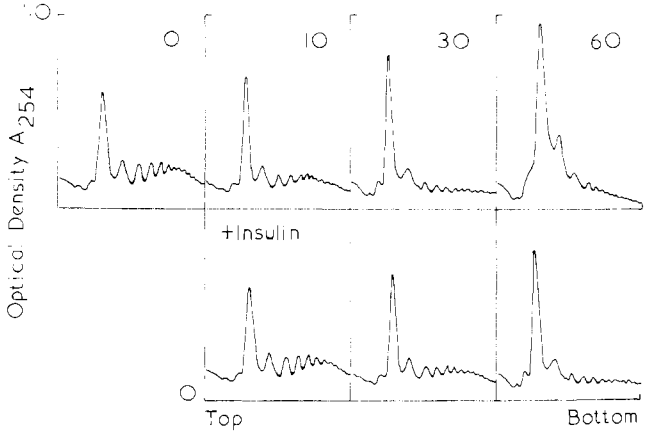

FIGURE 1: Effect of incubation in the presence and absence of insulin on the sucrose density gradient profiles of ribosomes from diaphragm muscle. Numbers in the upper panels indicate minutes of incubation for both upper and lower panels. In the upper panels incubation was in the absence of insulin; in the lower panels in its presence. Each profile is produced by 0.9–1.0 A_{260} unit of ribosomes.

TABLE II: Effect of Incubation of Diaphragm Muscle in the Presence and Absence of Insulin on the Number of Ribosomes Carrying Nascent Peptide Chains.

	Proportion of Ribosomes Carrying Peptide Chains (%) Min of Incubation			
	0	10	30	60
Incubation without insulin [a]		21	17	11
	37			
Incubation with insulin		29	25	22

[a] Figures are the average of two separate experiments.

Table I also demonstrates that during incubation there is a progressive decline in the activity of the isolated ribosomes by comparison with preparations from the unincubated tissue. Indeed the effect of insulin in this context is now seen to be one of retarding the extent of the decline in activity of the ribosomes, as opposed to providing stimulation above the base line level of activity.

Activity of ribosomal preparations to incorporate amino acids depends *inter alia* on (a) the proportion of ribosomes in the preparation bound to mRNA and therefore potentially capable of protein synthesis as opposed to being subunits, monomers, or dimers, and (b) the efficiency of the polysome-contained ribosomes in catalyzing elongation. Figure 1 shows sucrose density gradient profiles of the ribosomes prepared from muscle which has been incubated for various periods of time with or without addition of insulin. The quantity of polysomal material appears relatively small with respect to the monomer peak but as incubation of the tissue proceeds there is a progressive decrease in the amount of polysomal material extractable and a rise in the monomer and to a less extent dimer peak. While a similar change is seen even in tissue incubated in the presence of insulin, the disaggregation of the polysomes is less rapid than in the absence of insulin. These results are consistent with the findings of Morgan *et al.* (1971) and Jefferson *et al.* (1972) that insulin suppresses the appearance of ribosomal subunits in muscle on perfusion. The results suggest that during incubation or perfusion the rate of initiation in muscle declines with respect to the rate of elongation and this decline is less in muscle treated with insulin.

An additional and alternative method of estimating the extent of disaggregation of protein synthesizing ribosome-mRNA complexes during incubation is by determining by labeling with [^{3}H]puromycin the number of nascent peptide chains in the various preparations. Table II shows that in the ribosomes extracted from unincubated tissue 30–40% of the ribosomes appear to carry nascent peptides. Incubation of the tissue for various periods prior to preparation of the ribosomes leads to progressive loss of the number of detectable chains, and, again, the presence of insulin during incubation decreases the rate of the decline in the number of chains.

Effects of Amino Acids and Inhibitors of Protein Synthesis. In the studies of Jefferson and Korner (1969), Morgan *et al.* (1971), and Jefferson *et al.* (1972) the run down of polysomes and rise in quantity of subunits during perfusion are lessened by addition to the perfusate of amino acids at several times plasma concentrations. In a like manner addition to the medi-

TABLE III: Effect of Incubation of Diaphragm Muscle in the Presence of Amino Acids, Cycloheximide, or Puromycin on the Incorporation of Labeled Leucine and Phenylalanine by Ribosomes after Isolation and on the Proportion of Ribosomes Carrying Nascent Peptide Chains.

Expt No.	Incorporation of Radioactivity from Leucine (cpm/μg of ribosomes)	Incorporation of Radioactivity from Phenylalanine (cpm/μg of ribosomes)	Proportion of Ribosomes Carrying Peptide Chains (%)
1. Incubation without amino acids	37	26	15
Incubation with amino acids	55	38	23
Unincubated tissue	72	48	31
2. Incubation without cycloheximide	54	24	14
Incubation with cycloheximide (100 μg/ml)	92	39	22
Unincubated tissue	94	41	28
3. Incubation without puromycin	57	29	15
Incubation with puromycin (1 mM)	24	13	2
Unincubated tissue	113	68	40
4. Incubation without actinomycin	34	15	16
Incubation with actinomycin (10 μg/ml)	38	18	16
Unincubated tissue	91	48	38

um containing diaphragm muscle of a complete mixture of amino acids at approximately five times the normal plasma concentration enhanced the synthetic capacity of the isolated ribosomes (Table III). It also diminished the rate of disaggregation of polysomes on incubation (Figure 2) and decrease in number of nascent peptide chains (Table III).

The two inhibitors of protein synthesis, cycloheximide and puromycin, having quite different sites of action, might be expected to have markedly different effects on the extent of changes in polysomal aggregation. Cycloheximide by virtue of its capacity to inhibit elongation (Baliga *et al.*, 1970) restrains polysome disaggregation during incubation (Figure 2) and thus retains amino acid incorporating capacity by the isolated ribosomes due to preservation of nascent peptide chains (Table III). Puromycin by contrast causes marked reduction in capacity for amino acid incorporation (Table III), with very marked apparent decrease in number of nascent peptide chains, but with a less obvious shift of the polysome profile toward the monomer region than might have been anticipated (Figure 2). Actinomycin was without obvious effect on any of these parameters and did not interfere with the effect of insulin (not shown).

Poly(U) Primed Incorporation. Polysomal disaggregation may result *inter alia* from development of a defect in the ribosomes in their capacity to attach to and to translate mRNA or for other reasons. One measure of the functional capacity of isolated ribosomes is their ability to translate poly(U). Addition of poly(U) stimulated incorporation of [^{14}C]phenylalanine—the increase in incorporation in the presence of poly(U) being up to as much as 100-fold in extreme instances that seen in its absence. Addition of poly(U) was made either at the beginning of the incubation, or after incubation had proceeded for 20 min.

When poly(U) was added at the start of the incubation of the isolated particles incorporation was less with ribosomes from fresh tissue, was greatest with those of the tissue showing the greatest extent of polysomal disaggregation, for example, after incubation in the absence of insulin, and intermediate with ribosomes from tissue incubated with insulin or amino acids (Table IV). This result is consistent with the probability that the response to poly(U) is proportional to the number of ribosomes not already primed with endogenous messenger.

When poly(U) was added after a period of "run-off" the activity of the ribosomes from the incubated tissue was slightly greater (Table IV), as is consistent with run-off leading to an increase in the number of free ribosomes. With ribosomes from incubated tissue the response to poly(U) after run-off was less

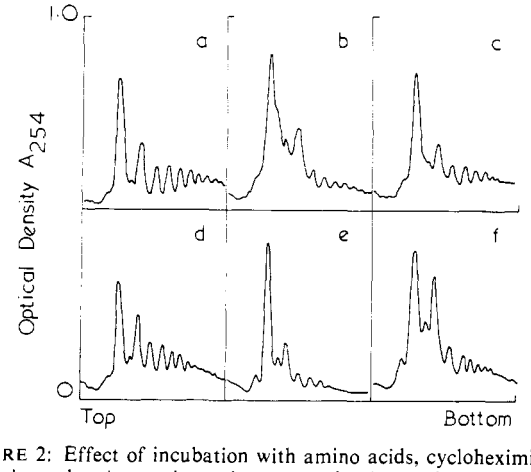

FIGURE 2: Effect of incubation with amino acids, cycloheximide, puromycin, and actinomycin on the sucrose density gradient profiles of ribosomes from diaphragm muscle: (a) unincubated tissue; (b) after incubation for 30 min in buffer without additions; (c–f) in buffer plus amino acids, cycloheximide, puromycin, or actinomycin, respectively. Each profile is produced by 0.9–1.0 A_{260} unit of ribosomes.

TABLE IV: Incorporation of [^{14}C]Phenylalanine in the Presence of Poly(U) by Ribosomes Isolated from Diaphragm Muscle before and after Incubation with Insulin or Amino Acids.

Expt no.	Incorporation of Radioactivity Poly(U) Added at Zero Time (cpm/μg of ribosomes)	Incorporation of Radioactivity Poly(U) Added After 20 Min (cpm/μg of ribosomes)
1. Incubation without insulin	1052	917
Incubation with insulin[a]	921	1041
Unincubated tissue	827	878
2. Incubation without amino acids	1180	841
Incubation with amino acids	1022	963
Unincubated tissue	763	797

[a] Concentration of insulin was 0.1 unit/ml.

TABLE V: Effect of Denervation and Incubation on the Incorporation of Labeled Leucine and Phenylalanine by Ribosomes after Isolation and on the Proportion of Ribosomes Carrying Nascent Chains.[a]

	Incorporation of Radioactivity from (cpm/μg of ribosomes)		Proportion of Ribosomes Carrying Peptide Chains (%)
	Leucine	Phenylalanine	
Unincubated tissue			
Innervated	120	59	37
Denervated	119	56	35
Incubation without insulin			
Innervated hemidiaphragm	41	27	21
Denervated hemidiaphragm	67	44	24
Incubation with insulin			
Innervated hemidiaphragm	59	46	26
Denervated hemidiaphragm	67	48	25

[a] The data for the unincubated tissue are the results of four independent experiments. The data for the incubated tissue are from an experiment in which all four parameters were run simultaneously. Similar results for activity of ribosomes from normal *vs.* denervated muscle incubated in the absence or in the presence of insulin have been obtained in other experiments studying one or other parameter separately.

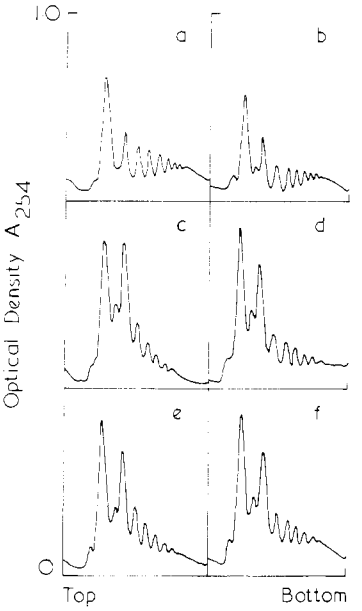

FIGURE 3: Effect of denervation and of incubation with and without insulin on the sucrose density gradient profile and ribosomes from diaphragm muscle: (a and b) unincubated tissue; (c and d) incubated without insulin; (e and f) incubated with insulin; (a, c, and e) innervated muscle; (b, d, and f) denervated muscle. Each profile is produced by 1.2–1.3 A_{260} unit of ribosomes.

than before run-off, particularly in the case of ribosomes from diaphragm incubated in the absence of insulin or amino acids. Where polysomes had disaggregated in the tissue during incubation, run-off is unlikely to lead to much increase in the number of free ribosomes and therefore of any potential for increase in response to poly(U). Why there should be any decrease is not obvious. It could arise if proteolysis were taking place during run-off, so leading to dilution of the high specific activity [^{14}C]phenylalanine.

Incorporation in the presence of poly(U) was optimal under the conditions used without addition of extra magnesium (*i.e.*, the concentration of magnesium in excess of ATP and GTP was 5 mM—consistent with a concentration of the free ion of about 3.5 mM (Manchester, 1970a)). In fact, in the present system extra magnesium inhibited polyphenylalanine synthesis, as noted by others (Williamson, 1969; Pilkis and Korner 1971; Clemens, 1972), probably because of decreasing activity of the components of the soluble fraction (unpublished observations). Since the quantity of labeled phenylalanine added is low, and the amount incorporated in the presence of poly(U) much increased, the possibility was considered that in the assay system used differences in response to poly(U) might have been obscured because the ability to form phenylalanine-tRNA had become rate limiting. Although decreasing the quantity of ribosomes added raised slightly their specific activity in phenylalanine incorporation, there was no change in the relative activity of samples either with smaller aliquots of ribosomes or if the quantity of phenylalanine present was increased.

Effects of Denervation. Following unilateral nerve section the denervated hemidiaphragm undergoes a transient hypertrophy. At the height of this hypertrophy the RNA content of the denervated tissue is almost double the normal value (Manchester and Harris, 1968). Ribosomes extracted from the denervated tissue at this period were found in an earlier study to be somewhat more active in amino acid incorporation than those extracted from the innervated tissue (Turner and Manchester, 1973a). A similar result has not been seen in the present work (Table V), possible reasons for which are discussed later. It is not possible to see any significant difference in the polysome profile in the denervated muscle (Figure 3), nor do the ribosomes appear to carry a larger number of growing peptide chains (Table V).

During the period of hypertrophy the denervated diaphragm shows diminished sensitivity toward insulin, both as regards glucose metabolism (Buse and Buse, 1959, 1961) and incorporation of amino acids into protein (Buse *et al.*, 1965; Harris and Manchester, 1966). In view of the influence of insulin described above on polysome disaggregation and ribosome activity it was of obvious interest to see in what way the ribosomes of the denervated tissue would behave during incubation, in particular whether polysomes of the denervated muscle might resist disaggregation to a greater extent than those of the normal tissue.

During incubation there was undoubtedly disaggregation of the polysomes in the denervated tissue as with the innervated muscle but the amount of polysomal material remaining after 30-min incubation appeared to be greater in the denervated tissue (Figure 3). This was reflected in a greater rate of incorporation of leucine and phenylalanine by ribosomes from the denervated muscle (Table V). It will be seen that incorporation of these amino acids by the denervated muscle was greater than that of the normal tissue even when the latter was incubated in the presence of insulin and that the presence of insulin did not influence the behavior of the ribosomes from denervated muscle. Likewise though the number of nascent chains declined on incubation they did not reach as low levels as found in tissue incubated in the absence of insulin (Table V). In general a smaller response to poly(U) was also shown by ribosomes from the denervated tissue after incubation (not shown).

Discussion

The procedure used in the present work seems to provide a reliable method for preparing ribosomes from small quantities

of muscle of an adequate degree of purity suitable for amino acid incorporation and other studies. Probably the yields in the present work are as high as any hitherto reported (listed in Turner and Manchester, 1973a). The critical features of the method used seem to be (a) use of EGTA, (b) degree of homogenization attained with the VirTis homogenizer, (c) removal of material sedimenting at 30,000*g*, and (d) recovery from the 30,000*g* precipitate of RNA also sedimenting at this point. It still remains the case, however, that only about 60–70% of the total ribosome population is being recovered. There may be some loss of subunits due to failure to sediment in the centrifugal forces used, but the greater loss is likely to be of the larger polysomes that are not extracted, as suggested by the findings of Morgan *et al.* (1971) with cardiac muscle. It is likely therefore that the ribosome preparations contain higher proportions of monomers than exist *in vivo*—it would indeed be surprising if in muscle of well-nourished rats only 30–40% of the ribosomes were carrying nascent peptide chains. It is possible of course that the method used for estimating the number of chains detects only a proportion. It can, however, be argued first that 30–40% of ribosomes in polysomes in the ribosomal preparations is reasonably consistent with the polysome profiles presented, secondly that since incubation with puromycin at 0° labels only about half as many chains as at 37° there is translocase activity and peptides in both the donor and acceptor sites are detected, and thirdly the figures presented here are as great or greater than those found by Wool *et al.* (1968), Baliga *et al.* (1970), and Pestka *et al.* (1972) for mammalian ribosomes from other sources—a figure as high as 70% can be achieved with hepatic polysomes (not shown). When gradients are run in media in which potassium is raised to 0.5 M and magnesium reduced to 2.5 mM large 40S and 60S peaks appear. The monomers and dimers therefore do not seem to be stabilized by possession of mRNA. This suggests that they are unlikely to have resulted from sheering of polysomes during preparation.

Effects of Insulin. The results with insulin suggest that the hormone in some manner enhances initiation in the tissue. This is consistent with similar findings in diabetes (Wool *et al.*, 1968), malnutrition (Young and Alexis, 1968; von der Decken and Omstedt, 1970), and with other muscle preparations (Morgan *et al.*, 1971; Jefferson *et al.*, 1972). The existence of a rough proportionality between the rates of incorporation and numbers of nascent chains suggests that there is little if any effect of insulin under these conditions on elongation. The fact that incubation of the tissue in the absence of insulin did not lessen the response of the ribosomes to poly(U) suggests that lack of insulin does not directly affect the functional capacity of the ribosomes and that the initiation defect is likely to be due to some other cause. The results with poly(U) are of interest with respect to the findings of Castles *et al.* (1971) that at low magnesium concentrations poly(U) primes [^{14}C]phenylalanine incorporation onto nascent peptide chains. In the present study, where the magnesium concentration in excess of nucleoside triphosphate was 5 mM, there was an inverse correlation between phenylalanine incorporation in the presence of poly(U) and the number of nascent chains carried by the ribosomes, more typical of results of Wool *et al.* (1968) with ribosomes from normal and diabetic muscle at higher magnesium concentrations.

How insulin may affect rates of initiation either in the diabetic muscle or during incubation or perfusion is not understood. Insulin is known to assist retention of potassium and to diminish inbibition of water and sodium by isolated tissues (Mortimore, 1961; Creese, 1968). It also diminishes release of amino acids and protein by the isolated diaphragm and change in weight during incubation (Manchester, 1961; Manchester and Wulwick, 1969), and affects the distribution of the intracellular pH indicator DMO (Manchester, 1970b). While it is not known how any of these changed are mediated, the overall influence of the hormone is to preserve the condition or tone of the tissue, and a greater capacity for protein synthesis as controlled through initiation may be one consequence of this. Increased rates of incorporation of amino acids into protein in the intact tissue are therefore not directly dependent on any stimulation of transport of amino acids into the cell or any subcellular compartments. When hearts are perfused in the absence of insulin large membrane-lined vacuoles slowly develop in the perinuclear region and in the rows of mitochondria (Morgan *et al.*, 1972). Addition of insulin prevents the development of vacuoles. Similar information is not at present available for diaphragm muscle, but the inference is that insulin influences the form and rate of morphological change in the tissue during incubation and these changes are very likely to influence protein synthesis (and breakdown).

Effects of Denervation. In the present experiments, unlike earlier work, the ribosomes from diaphragm 3 days after denervation did not show greater specific activity than in the innervated tissue. Neither did they show a significantly different polysome profile, nor difference in number of nascent chains. In the denervated muscle at this stage there is a very much larger quantity of RNA (Manchester and Harris, 1968) and the yield of ribosomes was around 80% increased per gram of tissue. It is conceivable, given the fact that it is at present impossible to extract ribosomes from muscle completely, that in the earlier experiments (Turner and Manchester, 1973a), using a slightly different method of extraction, a different profile of ribosomes was prepared and that this is why differences in activity were seen between normal and denervated muscle. It remains the case in the present work that the ribosomes of the denervated muscle remained more active after incubation because the rate of disaggregation of the polysomes of the tissue appears to be slower. Moreover the influence of insulin, as with the intact denervated tissue, is minimal, but in this particular circumstance it could be argued that the tissue is in some manner less in need of the supportive effect of the hormone. Whether the denervated muscle is richer in initiation factors, whether its higher concentration of free amino acids (Turner and Manchester, 1973b) is of importance, or for what other cause it can resist the disruption in protein synthesizing capacity otherwise occurring on *in vitro* incubation remains to be determined.

References

Baliga, B. S., Cohen, S. A., and Munro, H. N. (1970), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 8,* 249.

Buse, M. G., and Buse, J. (1959), *Diabetes 8,* 218.

Buse, M. G., and Buse, J. (1961), *Diabetes 10,* 134.

Buse, M. G., McMaster, J., and Buse, J. (1965), *Metabolism 14,* 1220.

Castles, J. J., Rolleston, F. S., and Wool, I. G. (1971), *J. Biol. Chem. 246,* 1799.

Clemens, M. J. (1972), *Proc. Nutr. Soc. 31,* 273.

Creese, R. (1968), *J. Physiol. 197,* 255.

Earl, D. C. N., and Hindley, S. T. (1971), *Biochem. J. 122,* 267.

Fleck, A., and Munro, H. N. (1962), *Biochim. Biophys. Acta 55,* 571.

Harris, E. J., and Manchester, K. L. (1966), *Biochem. J. 101,* 135.

Jefferson, L. S., Koehler, J. O., and Morgan, H. E. (1972), *Proc. Nat. Acad. Sci. U. S. 69,* 816.
Jefferson, L. S., and Korner, A. (1969), *Biochem. J. 111,* 703.
Manchester, K. L. (1961), *Biochem. J. 81,* 135.
Manchester, K. L. (1970a), *Biochim. Biophys. Acta 213,* 532.
Manchester, K. L. (1970b), *Hormones 1,* 342.
Manchester, K. L., and Harris, E. J. (1968), *Biochem. J. 108,* 177.
Manchester, K. L., and Wulwick, C. (1969), *Protein Polypeptide Horm., Proc. Int. Symp., 1968,* 828.
Manchester, K. L., and Young, F. G. (1961), *Vitam. Horm. (New York) 19,* 95.
Morgan, H. E., Jefferson, L. S., Wolpert, E. B., and Rannels, D. E. (1971), *J. Biol. Chem. 246,* 2163.
Morgan, H. E., Rannels, D. E., Wolpert, E. B., Giger, K. E., Robertson, J. W., and Jefferson, L. S. (1972), *in* Insulin Action. Fritz, I. B., Ed., New York, N. Y., Academic Press, p 437.
Mortimore, G. E. (1961), *Amer. J. Physiol. 200,* 1315.
Pestka, S., Goorha, R., Rosenfeld, H., Neurath, C., and Hintikka, H. (1972), *J. Biol. Chem. 247,* 4258.
Pilkis, S. J., and Korner, A. (1971), *Biochim. Biophys. Acta 247,* 597.
Turner, L. V., and Manchester, K. L. (1973a), *Biochim. Biophys. Acta 299,* 612.
Turner, L. V., and Manchester, K. L., (1937b), *Biochim. Biophys. Acta 320,* 352.
von der Decken, A., and Omstedt, P. T. (1970), *J. Nutr. 100,* 623.
Wannemacher, R. W., Cooper, W. K. C., and Muramatsu, K. (1970), *Proc. Soc. Exp. Biol. Med. 135,* 180.
Williamson, A. R. (1969), *Biochem. J. 111,* 515.
Wool, I. G., and Kurihara, K. (1967), *Proc. Nat. Acad. Sci. U. S. 58,* 2401.
Wool, I. G., Stirewalt, W. S., Kurihara, K., Low, R. B., Bailey, P., and Oyer, D. (1968), *Recent Progr. Horm. Res. 24,* 139.
Young, V. R., and Alexis, S. D. (1968), *J. Nutr. 96,* 255.
Zak, R., Etlinger, J., and Fischman, D. A. (1972), *Excerpta Med. Found., Int. Congr. Ser. No. 240,* 163.

Structure of 11S Acetylcholinesterase. Subunit Composition†

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ABSTRACT: The subunit compositions of several 11S acetylcholinesterase (EC 3.1.1.7) preparations from the electric eel were investigated. Data from polyacrylamide gel electrophoresis in 1% sodium dodecyl sulfate and gel exclusion chromatography in 6 M guanidine hydrochloride indicated that the active enzyme is a tetramer composed of subunits with molecular weights of 70,000. From analyses of ^{32}P-phosphorylation patterns and cyanogen bromide fragment compositions, the subunits were shown to contain one active site each and to be identical within the limits imposed by these techniques, except for a variability in manifesting two sites of cleavage, probably caused by proteolytic or glycolytic agents. One cleavage occurs at a site A and splits the intact subunit (I) into a major fragment (II) of 50,000 molecular weight and a minor fragment (III) of 20,000–22,000 molecular weight. A second cleavage at a site B generates a second minor fragment (IV), with a molecular weight of 18,000–20,000, from III. In the absence of disulfide reduction these cleavages did not appear to result in the release of measurable polypeptides from the subunit structures. In the native enzyme both cleaved and intact subunits appear to exist as subunit dimers with a covalent intersubunit linkage which involves disulfide bonding. Hence the subunits in the native tetramer are arranged as a dimer of dimers $((\alpha)_2)_2$, where α is either the cleaved or intact subunit containing the catalytic site.

Acetylcholinesterase (EC 3.1.1.7) from the electric eel *Electrophorus electricus* has been isolated in each of several molecular species. Species isolated from extracts of fresh electric organ tissue are characterized by sedimentation coefficients of 8 S, 14 S, and 18 S and appear in electron micrographs as clusters of respectively 4, 6–8, and 10 or more subunits attached to an elongated "tail" (Rieger *et al.*, 1973; Dudai *et al.*, 1973). All three of these species may be converted to an 11S form either by treatment with trypsin or other purified proteolytic enzymes, or by an apparent "autolysis" on storage of crude enzyme solutions (Massoulié and Rieger, 1969; Rieger *et al.*, 1972a,b). Electron micrographs of the 11S species made in parallel with the other forms show globular structures composed of four subunits without the "tail."

The acetylcholinesterase properties reported here refer to an 11S enzyme purified by affinity chromatography from extracts of toluene-stored electric eel tissue (Rosenberry *et al.*, 1972; Chen *et al.*, 1974). Several purified preparations free of detectable protein contaminants have been obtained, and the physical

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[1] Abbreviations used are SDS, sodium dodecyl sulfate; DFP, diisopropylphosphoryl fluoride; DIP-acetylcholinesterase, diisopropylphosphorylated acetylcholinesterase; NEM, *N*-ethylmaleimide; IAA, iodoacetamide; PAS, periodic acid–Schiff reagent; DTT, dithiothreitol.