Protein Synthesis by Skeletal Muscle Ribosomes. Effect of Diabetes and Insulin*

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ABSTRACT: The exact conditions necessary for optimum incorporation of radioactivity from [^{14}C]phenylalanyl-transfer ribonucleic acid into protein by ribosomes from skeletal muscle were determined, and the assay system was used to investigate the effect of diabetes and insulin on protein synthesis. In the assay system we adopted, protein synthesis could be increased by adding more ribosomes, or by increasing the amount of [^{14}C]phenylalanyl-tRNA. But if the amount of [^{14}C]phenylalanyl-tRNA was increased to the point where it was no longer limiting for protein synthesis, incorporation was still proportional to the number of ribosomes. Ribosomes from muscle of diabetic animals catalyzed protein synthesis far less efficiently than did ribosomes from normal animals. The defect in protein synthesis that follows administration of alloxan takes 48 hr to develop. However, the defect in ribosomal protein synthesis occurred in 3 hr when animals were made acutely diabetic by administration of antiinsulin serum. When diabetic animals were treated with insulin before they were killed, their ribosomes carried out increased amounts of protein synthesis; generally insulin restored synthesis to normal or near normal. The minimal amount of insulin required was 0.1 unit (4 μg)/animal; 0.1 unit would increase ribosomal protein synthesis in 5 min. Ribosomes from diabetic animals catalyzed fewer aminoacyl transfers no matter what amount of [^{14}C]phenylalanyl-tRNA was added to the reaction mixture. Ribosomes from diabetic animals were less efficient than ribosomes from normal animals irrespective of ribosome concentration. We found no evidence that decreased protein synthesis due to diabetes is the result of an inhibitor associated with the ribosome. Addition of polyuridylic acid increased incorporation of radioactivity from [^{14}C]phenylalanyl-tRNA into protein by skeletal muscle ribosomes from normal and from diabetic animals; the synthetic polyribonucleotide restored to normal the efficiency of diabetic ribosomes. Moreover, at every concentration of polyuridylic acid tested, the increase in protein synthesis due to the homopolymer was greater for diabetic ribosomes than for normal. We do not know why diabetic ribosomes are more sensitive to stimulation by polyuridylic acid. There is evidence, however, that a deficiency of template RNA is not the sole basis of the defect in protein synthesis due to insulin lack, for it has been shown before that the hormone can repair the defect in circumstances in which the total amount of template available to the ribosome is unlikely to have been changed.

When it was found that ribosomes from heart muscle of rats would catalyze the transfer of significant amounts of radioactivity from [^{14}C]phenylalanyl-tRNA to protein (Rampersad *et al.*, 1965), we used the reaction to analyze how insulin stimulates protein synthesis in muscle. We found (Rampersad and Wool, 1965; Wool *et al.*, 1966) that adding insulin to heart muscle ribosomes *in vitro* did not alter their ability to carry out protein synthesis; that ribosomes from animals made diabetic with alloxan incorporated less amino acid into protein; and that when insulin was given to normal or diabetic animals 1 hr before they were killed, increased ribosomal protein synthesis resulted.

A disadvantage of using ribosomes from heart muscle is that only about 0.5 g of that tissue can be obtained from a 150-g rat. The yield of ribosomes (125 μg of ribosomal RNA/g of tissue) is so small that large numbers of animals are required for each experiment. Florini and Breuer (Breuer *et al.*, 1964; Florini and Breuer, 1965, 1966; Breuer and Florini, 1965) have shown that ribosomes isolated from skeletal muscle (the hind limbs) of rats are efficient in carrying out protein synthesis *in vitro*. The amount of skeletal muscle that can be obtained from each rat is of course far greater than the amount of heart muscle, and skeletal muscle yields almost as large a proportion of ribosomes. Hence we prepared and assayed the activity of ribosomes from that tissue. Although the characteristics of the skeletal muscle ribosomal system have already been described in great detail (Breuer *et al.*, 1964), in the first part of this report we specify the exact conditions necessary for optimum incorporation of radioactivity from [^{14}C]phenylalanyl-tRNA into protein. We feel justified in doing so, because our purposes require conditions somewhat different from those described by Florini and Breuer, and the precise con-

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ditions for optimal incorporation are critical for our analysis of how insulin influences ribosomal protein synthesis. The second part of this paper describes the effect of diabetes and insulin on the synthesis of protein by skeletal muscle ribosomes.

Experimental Procedure

Materials. The uniformly labeled [^{14}C]phenylalanine (297 or 366 mc/mmole), [^{14}C]leucine (274 mc/mmole), and a ^{14}C-labeled mixture of 15 amino acids (216.5 mc/mmole) were purchased from New England Nuclear Corp.; pyruvate kinase (type II), from Sigma Chemical Co.; glucose oxidase, electrophoretically purified deoxyribonuclease (type I), and ribonuclease B, from Worthington Biochemicals Corp.; phosphoenolpyruvic acid (trisodium salt), adenosine 5-triphosphate (dipotassium salt), cytidine 5-triphosphate, and guanosine 5-triphosphate (sodium salt), from Sigma Chemical Co.; polyuridylic acid, from Miles Laboratories, Inc.; stripped soluble ribonucleic acid from *Escherichia coli* strain B, and Lubrol WX, from General Biochemicals; [^{12}C]amino acids, puromycin dihydrochloride, and cycloheximide, from Nutritional Biochemicals Corp.; alloxan monohydrate and 2-mercaptoethanol, from Eastman Kodak Co.; bovine plasma albumin, fraction V, from Armour Pharmaceutical Co.; sodium deoxycholate, from Fisher Scientific Co.; BBOT,[1] scintillation grade, from Packard Instrument Co. The *E. coli* strain B, grown on a minimum medium and harvested in early log phase, was obtained from Grain Processing Co. Insulin (beef zinc insulin crystals, lot PJ-4609) assaying 24 units/mg and containing less than 0.003% glucagon was a gift of Dr. O. Behrens of Eli Lilly Co.; it was dissolved in 0.003 N hydrochloric acid to form a stock solution of 20 units/ml. In some experiments we used the Eli Lilly and Co. commercial preparation of regular insulin (Iletin). Antiinsulin serum prepared by immunization of guinea pigs with bovine insulin (Robinson and Wright, 1961) was a gift of Dr. P. H. Wright; each 1 ml was assayed to be capable of neutralizing 1–1.5 units of insulin. Serum for treatment of control animals was obtained from guinea pigs that had not been injected with insulin. The MS-2 RNA was a gift of Dr. T. Nakimoto and D. Kolakofsky.

Animals. Male Sprague–Dawley rats were maintained under standard conditions (Wool and Krahl, 1959) and allowed free access to food and water. Diabetes was induced in most rats by rapid intravenous injection of 60 mg/kg of alloxan monohydrate after they were starved overnight; the animals were used 2–4 days later (*cf.* Table IV). One group of rats was made diabetic by intraperitoneal injections of 1.5 ml of antiinsulin serum at 0 and 90 min and the animals were killed at 180 min. Control animals received a similar amount of serum from guinea pigs that had not been injected with insulin. The plasma glucose concentration (in mg/100 ml) of the diabetic animals (not starved) was: alloxan diabetic, 500–700; alloxan diabetic, treated 1 hr before with 5 units of insulin intraperitoneally, 250–350; and treated with antiinsulin serum, 390.

Preparation of Skeletal Muscle Ribosomes. The skeletal muscle ribosomes were prepared by a method modified only in minor ways from that developed by Florini and Breuer (1966). The ribosomes were 51.3% RNA and 48.7% protein. The yield was approximately 80 μg of ribosomal RNA/g of tissue (wet weight), and generally 8 g of hind limb skeletal muscle could be obtained from a rat if the animal weighed between 100 and 120 g, a total of 640 μg of ribosomal RNA/rat. The yield from heart muscle is 125 μg/g (Rampersad *et al.*, 1965), and for a rat of the same weight (100–120 g) the heart will weigh approximately 400 mg, to give a total of 50 μg of ribosomal RNA/animal. Thus, using skeletal muscle instead of heart muscle increases the yield of ribosomes from each animal by 10- to 13-fold. The ribosomes from normal and diabetic animals were stored for 3 months at $-20°$ without change in their activity.

Preparation of [^{14}C]Phenylalanyl-tRNA. Supernatant enzyme was prepared by a modification of the method of Nirenberg and Matthaei (1961) from *E. coli* strain B cells grown on a minimum medium and harvested in early log phase and used to charge tRNA from *E. coli* with one ^{14}C-labeled amino acid and 19 additional [^{14}C]amino acids (von Ehrenstein and Lipmann, 1961). The specific activity of the ^{14}C-labeled aminoacyl-tRNA was determined from its absorption at 260 mμ (μg of RNA/ml = $OD_{260}/0.0221$) and its radioactivity. Generally the specific activity was in the range 10–30 $\times$ 10^3 cpm/100 μg of tRNA; the exact specific activity was contingent on the specific activity of the ^{14}C-labeled amino acid and the amount of tRNA used in the aminoacylation reaction. We do not know the percentage of the tRNA that was actually charged with ^{14}C-labeled amino acid. The [^{14}C]phenylalanyl-tRNA was stored at $-20°$ in 1-ml aliquots.

Preparation of Superantant Protein ("Transfer Enzyme Preparation"). The "transfer enzyme" (105,000*g* supernatant protein) was prepared from the soluble fraction of a homogenate of rat liver (Rampersad and Wool, 1965). The concentration of protein was calculated from the formula given by Warburg and Christian (1942). The supernatant protein was stored in 3-ml aliquots at $-20°$.

Conditions for the Assay of Protein Synthesis. Prior to the assay a 1:100 dilution of the ribosomal suspension was made and its absorption at 235, 260, and 280 mμ was determined. The absorption ratios, 260:235 and 260:280, were calculated and used to assess the purity of the particles (Petermann, 1964). The ribosome preparation was not used if the ratio at 260:280 did not exceed 1.75, and that at 260:235, 1.45. Most frequently the ratios at 260:280 and 260:235 were 1.85–1.95 and 1.50–1.60, respectively.

The concentration of ribosomes (actually ribosomal

[1] Abbreviations used: BBOT, 2,5-bis[2-(5-*t*-butylbenzoxazolyl)]thiophene; ADP and ATP, adenosine di- and triphosphates; GTP, guanosine triphosphate.

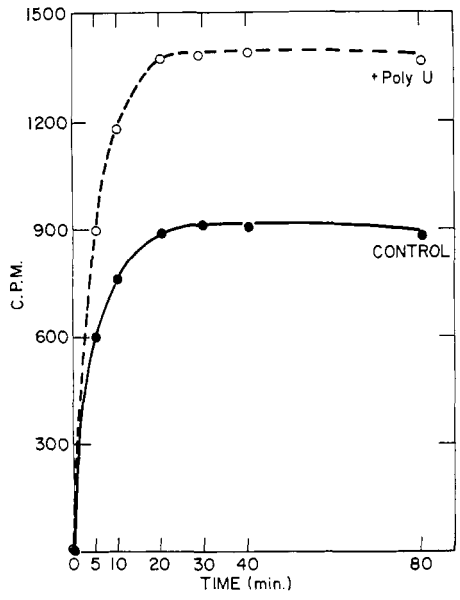

FIGURE 1: Time course of incorporation into protein of radioactivity from [^{14}C]phenylalanyl-tRNA by ribosomes from skeletal muscle. The ribosomes (65 μg of ribosomal RNA) were incubated for different periods of time with 125 μg of [^{14}C]phenylalanyl-tRNA (11 $\times$ 10^3 cpm) as described in Experimental Procedure. The amount of polyuridylic acid when present was 100 μg. Each 100 cpm of radioactivity in protein was the result of the incorporation of 3.73 $\times$ 10^{-1} $\mu\mu$mole of phenylalanine.

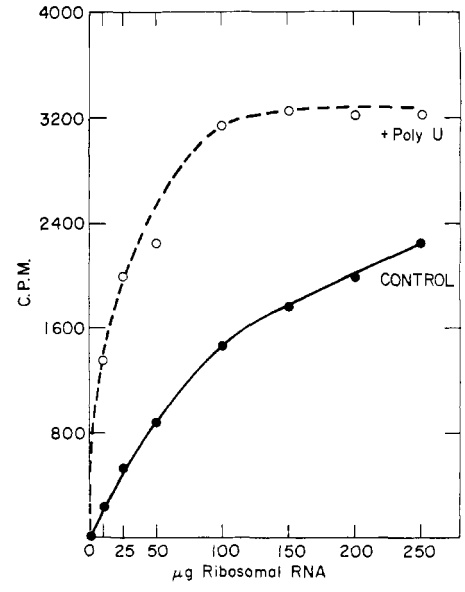

FIGURE 2: Effect of the concentration of ribosomes from skeletal muscle on the incorporation into proteins of radioactivity from [^{14}C]phenylalanyl-tRNA. Different concentrations of ribosomes were incubated with 125 μg of [^{14}C]phenylalanyl-tRNA (11 $\times$ 10^3 cpm) as described in Experimental Procedure. The amount of polyuridylic acid when present was 100 μg. Each 100 cpm of radioactivity in protein was the result of the incorporation of 3.73 $\times$ 10^{-1} $\mu\mu$mole of phenylalanine.

RNA) was determined before they were diluted for assay by a modification of the method of Fleck and Munro (1962). To 0.1 ml of the ribosomal suspension we added 0.9 ml of 0.3 N potassium hydroxide; the blank contained 0.1 ml of 0.25 M sucrose in medium A. If the concentration of the ribosomes exceeded 1.5 mg/ml, 0.05 ml of the suspension was analyzed. The sample was stirred vigorously and then incubated for 1 hr at 37° in a water-bath shaker. The sample was cooled on ice and 2 ml of 1.0 N perchloric acid was added; the sample was stirred and allowed to stand in ice for 5–10 min, then centrifuged in the cold (4°) at 1600*g* in an International centrifuge. The supernatant (2 ml) was diluted 1:1 with water and the absorption at 260 mμ was determined. The concentration of ribosomes (as ribosomal RNA) was calculated from the following formula: $OD_{260} \times 218 = \mu g$ of ribosomal RNA/0.1 ml of original suspension. The ribosomes were then diluted with 0.25 M sucrose in medium A to the concentration required. When simultaneous comparison was made of protein synthesis by ribosomes from different groups of animals, the dilution was adjusted so the concentration of ribosomes used in each assay was the same. The concentration was redetermined after dilution and any small difference between groups compensated for by an appropriate correction in the results.

The assay was carried out in 1.02 ml containing 50 μmoles of Tris-HCl (pH 7.8), 80 μmoles of KCl, 15 μmoles of $MgCl_2$, 10 μmoles of 2-mercaptoethanol, 1 μmole of ATP, 0.4 μmole of GTP, 10 μmoles of phosphoenolpyruvic acid, 0.1 mg of pyruvate kinase, 100 μg of tRNA charged with a ^{14}C-labeled amino acid and 19 [^{12}C]amino acids, and 2.5 mg of supernatant protein. The reaction was initiated by adding 0.1 ml of the ribosome suspension; incubation was for 30 min (unless another time is specifically noted) at 37° in a water-bath shaker. When the conditions were altered (different amounts of the basic components, addition of other materials, etc.) the specific details of the change are given in the text or in the legends to the figures and tables. The reaction was stopped by adding 3 ml of cold 10% trichloroacetic acid.

The assays were carried out in triplicate, and the results in the tables and figures are the mean of the three determinations.

Preparation and Counting of Radioactive Protein. After incubation the protein samples were prepared and their radioactivity was determined as described by Wool and Cavicchi (1966).

Determination of Ribosomal Protein. The protein (0.1–2 mg) in a suspension of ribosomes was determined by the method described by Goa (1953).

Determination of Blood Glucose Concentration. The blood glucose concentration was determined by the glucose oxidase method (Huggett and Nixon, 1957).

Results

General Characteristics of the System. TIME COURSE OF INCORPORATION OF RADIOACTIVITY FROM [^{14}C]PHENYLALANYL-tRNA INTO PROTEIN BY SKELETAL MUSCLE RIBOSOMES. Assay of ribosomal activity *in vitro* showed that [^{14}C]phenylalanine was incorporated very rapidly for the first 5 min; thereafter the rate declined until after 20 min no further synthesis occurred (Figure 1). The addition of polyuridylic acid increased the amount of [^{14}C]phenylalanine incorporated into protein without altering the time required to reach maximum incorporation.

RIBOSOME CONCENTRATION. Incorporation of [^{14}C]-phenylalanine into protein was directly proportional to the concentration of ribosomes in the absence of polyuridylic acid but not in its presence (Figure 2). Incorporation then was proportional to the concentration of ribosomes only if the amount of ribosomal RNA did not exceed 100 μg. In most of the experiments that follow we used for each assay less than 100 μg of ribosomal RNA, usually 30 μg, to ensure that protein synthesis would be proportional to the concentration of ribosomes in both the presence and absence of polyuridylic acid.

REQUIREMENTS FOR INCORPORATION OF AMINO ACIDS INTO PROTEIN BY SKELETAL MUSCLE RIBOSOMES. Incorporation of radioactivity from [^{14}C]phenylalanyl-tRNA into protein by skeletal muscle ribosomes required a source of energy (ATP, phosphoenolpyruvate, and pyruvate kinase), transfer enzyme, magnesium, and, to a lesser extent, GTP (Table I). There was no clear requirement for 2-mercaptoethanol in the circumstances of the assay. Polyuridylic acid, once again, stimulated the incorporation of [^{14}C]phenylalanine into protein but without markedly changing the requirements for protein synthesis (Table I). Omission of an energy generating system did result in greater relative inhibition of synthesis, and in the presence of polyuridylic acid, a requirement for GTP was clearly evident. Ribonuclease (100 μg) abolished protein synthesis whether polyuridylic acid was present or not.

In the experiment summarized in Table I, in the absence of polyuridylic acid, 12% or 4.25 $\mu\mu$moles of the [^{14}C]phenylalanine present initially in the assay mixture was incorporated into protein; in the presence of polyuridylic, incorporation of phenylalanine was increased to 28% or 10 $\mu\mu$moles.

OPTIMUM CONDITIONS FOR INCORPORATION OF AMINO ACIDS INTO PROTEINS BY SKELETAL MUSCLE RIBOSOMES. The optimum concentration of GTP for the incorporation of amino acid into protein by skeletal muscle ribosomes was found to be 0.2 μmole/ml. If an energy-generating system (phosphoenolpyruvate and pyruvate kinase) had been added to the reaction mixture, ATP was not required, due, we presume, to the presence in the preparation of supernatant protein of ADP. In those circumstances, ATP was inhibitory even when added in small amounts (1 μmole). The optimum amount of magnesium was 15 μmoles and of transfer enzyme (*i.e.*, dialyzed supernatant protein) 2 mg. The addition of the transfer enzyme preparation in an amount exceeding 2 mg inhibited protein synthesis. The nature of the inhibitor is not known to us, but it might be ribonuclease. The optimum concentrations of GTP, magnesium, and transfer enzyme (supernatant protein) were not changed by addition of polyuridylic acid.

In the first test to determine the optimum concentration of [^{14}C]phenylalanyl-tRNA, 65 μg of ribosomal RNA was incubated with 0, 125, 250, and 500 μg of aminoacyl-tRNA containing respectively 0, 9800, 19,600, and 39,200 cpm, both with and without polyuridylic acid. We were surprised to find that incorporation of radioactivity into protein was not saturated even with the largest amount of [^{14}C]phenylalanyl-tRNA tested. The finding was not the result of exchange of free phenylalanine with the phenylalanine acylated to tRNA, for little or no exchange took place. For example, addition to the reaction mixture of 30 μmoles of [^{12}C]phenylalanine, an amount that could dilute the [^{14}C]phenylalanyl-tRNA 300-fold if exchange took place, decreased incorporation by only 28%. Addition of 0.1 μc of [^{14}C]phenylalanine (0.035 μc of [^{14}C]phenylalanyl-tRNA was present) did not increase incorporation of radioactivity into protein at all, and addition of 1 and 10 μc did so by only 21 and 52%, respectively. The results indicate that little exchange takes place; the altered incorporation that did occur was most likely the result of a limited capacity of the system to acylate tRNA due to the presence in the supernatant protein preparation of amino acid activating enzymes. A separate experiment confirmed that incorpo-

TABLE I: Incorporation into Protein of Radioactivity from [^{14}C]Phenylalanyl-tRNA by Ribosomes from Skeletal Muscle. Characteristics and Requirements.[a]

Additions	Incorporation (cpm) −Polyuridylic Acid	Incorporation (cpm) +Polyuridylic Acid
Complete system	1192	2699
−"Transfer enzyme"	17	32
−ATP, phosphoenolpyruvate, and pyruvate kinase	197	280
−ATP, phosphoenolpyruvate, pyruvate kinase, and GTP	123	264
−GTP	833	1404
$-MgCl_2$	12	20
−2-Mercaptoethanol	1223	3076
+100 μg of ribonuclease	16	24

[a] The assay was carried out as described in Experimental Procedure; 60 μg of ribosomal RNA and 125 μg of tRNA charged with [^{14}C]phenylalanine (9.56×10^3 cpm) were added. Each 100 cpm of radioactivity in protein was the result of incorporation of 3.73×10^{-1} $\mu\mu$mole of phenylalanine.

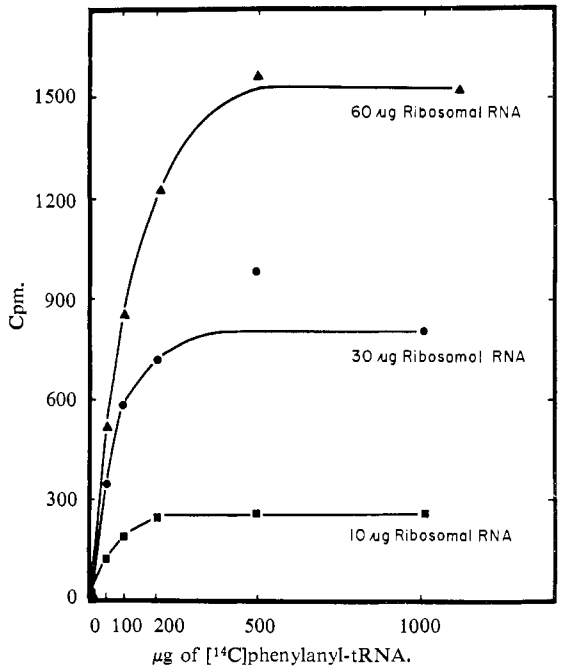

FIGURE 3: Effect of the concentration of [^{14}C]phenylalanyl-tRNA on the incorporation of radioactivity into protein by ribosomes from skeletal muscle. The ribosomes (either 10, 30, or 60 μg of ribosomal RNA) were incubated with differentc oncentrations of [^{14}C]phenylalanyl-tRNA, without polyuridylic acid, as described in Experimental Procedures. Each 100 μg of [^{14}C]-phenylalanyl-tRNA contained 14.4 $\times$ 10^3 cpm; each 100 cpm of radioactivity in protein was the result of the incorporation of 2.2 $\times$ 10^{-1} $\mu\mu$mole of phenylalanine.

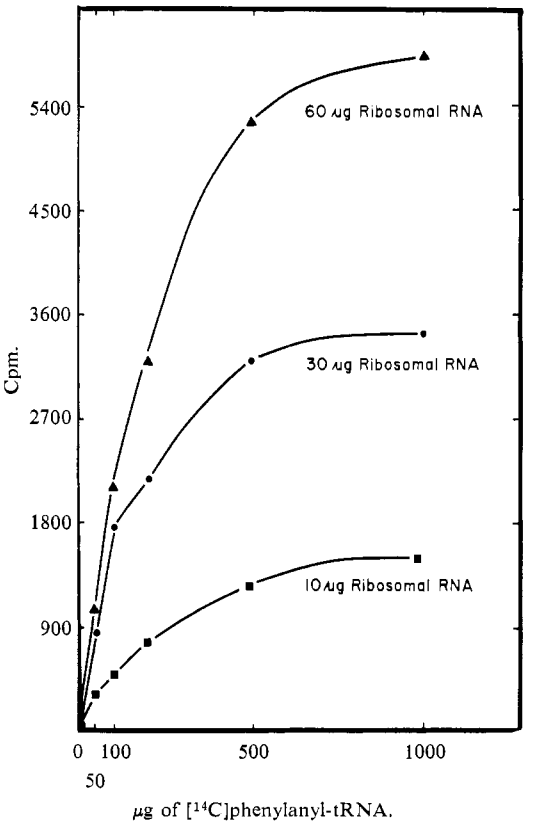

FIGURE 4: Effect of the concentration of [^{14}C]phenylalanyl-tRNA in the presence of polyuridylic acid on the incorporation of radioactivity into protein by ribosomes from skeletal muscle. The ribosomes (either 10, 30, or 60 μg of ribosomal RNA) were incubated with different concentrations of [^{14}C]phenylalanyl-tRNA and with 100 μg of polyuridylic acid as described in Experimental Procedure. Each 100 μg of [^{14}C]phenylalanyl-tRNA contained 14.4 $\times$ 10^3 cpm; each 100 cpm of radioactivity in protein was the result of the incorporation of 2.2 $\times$ 10^{-1} $\mu\mu$mole of phenylalanine.

ration was indeed from [^{14}C]phenylalanyl-tRNA: [^{12}C]phenylalanyl-tRNA was found to inhibit incorporation of radioactivity from [^{14}C]phenylalanyl-tRNA into protein by an amount that was in reasonable agreement with that predicted from the dilution of the latter by the former. Finally, it was possible to show that deacylated tRNA itself was, if anything, slightly inhibitory. We do not know why, but it may be that the commercial preparations of tRNA are contaminated with ribonuclease.

Having satisfied ourselves that aminoacyl-tRNA was the precursor for protein synthesis by skeletal muscle ribosomes, and that no significant exchange took place, we again attempted to determine the optimum amount of [^{14}C]phenylalanyl-tRNA (Figures 3 and 4). For that purpose amounts of [^{14}C]phenylalanyl-tRNA ranging up to 1000 μg (144,000 cpm) were incubated with 10, 30, or 60 μg of ribosomal RNA, with and without polyuridylic acid. The minimum amount of aminoacyl-tRNA required to saturate incorporation in the absence of polyuridylic acid was found, as expected, to be dependent on the concentration of ribosomes in the reaction mixture (Figure 3). The estimated amounts were: with 10 μg of ribosomal RNA, 230 μg of [^{14}C]phenylalanyl-tRNA; with 30 μg of ribosomal RNA, 400 μg of [^{14}C]phenylalanyl-tRNA; and with 60 μg of ribosomal RNA, 500 μg of [^{14}C]phenylalanyl-tRNA. When protein synthesis was assayed in the presence of polyuridylic acid, there was no evidence that even the largest amount tested, 1 mg of [^{14}C]phenylalanyl-tRNA, was sufficient for saturation (Figure 4). It is not practical or economical to use such a large amount of aminoacyl-tRNA, so we have generally used 30 μg of ribosomal RNA and 100 μg of [^{14}C]phenylalanyl-tRNA. We recognize that in those circumstances the amount of aminoacyl-tRNA is less than optimum.

EFFECT OF PUROMYCIN AND CYCLOHEXIMIDE. Protein synthesis by skeletal muscle ribosomes was inhibited 94% by addition of 50 μg of puromycin. Cycloheximide was not nearly so effective an inhibitor; 100 μg inhibited protein synthesis by only 9% and even 1 mg by only 55%. Why it was less effective *in vitro* is not known.

Cycloheximide administered *in vivo* inhibits protein synthesis in skeletal muscle as efficiently as puromycin (Wool and Cavicchi, 1966).

EFFECT OF DIABETES AND INSULIN ON PROTEIN SYNTHESIS BY SKELETAL MUSCLE RIBOSOMES. Skeletal muscle ribosomes from alloxan diabetic animals transferred radioactivity from [^{14}C]phenylalanyl-tRNA into protein about one-half as effectively as did ribosomes from control animals (Table II). When diabetic animals were treated with insulin 1 hr before they were killed, their ribosomes carried out 105% more protein synthesis than did those from untreated diabetic animals; generally insulin restored synthesis to normal or near normal. The results are similar to those we have obtained before with heart muscle ribosomes (Rampersad and Wool, 1965) and with skeletal muscle ribosomes (Wool and Cavicchi, 1966).

TABLE II: Incorporation into Protein of Radioactivity from [^{14}C]Phenylalanyl-tRNA by Ribosomes from Skeletal Muscle. Effect of Diabetes and Insulin.[a]

Source of Ribosomes	Poly U (100 μg)	Incorp (cpm)	Change (%)
Normal	0	892	
	+	4108	
Diabetic	0	439	−50[b]
	+	4179	
Diabetic + insulin	0	901	+105[b]
	+	3653	

[a] The insulin (5 units) was administered intraperitoneally 1 hr before the animals were killed and the ribosomes were assayed. The average blood glucose concentration (mg/100 ml) was diabetic (543) and diabetic treated with insulin (298). The assay was carried out as described in Experimental Procedure; 95 μg of ribosomal RNA and 125 μg of tRNA charged with [^{14}C]phenylalanine (11.5 × 10^3 cpm) were added. Each 100 cpm of radioactivity in protein was the result of the incorporation of 3.73 × 10^{-1} $\mu\mu$mole of phenylalanine. [b] For calculation of the per cent change diabetic were compared with normal and the diabetic plus insulin with diabetic.

Decreased protein synthesis owing to insulin deficiency was not peculiar to assays that had [^{14}C]phenylalanyl-tRNA as substrate. A similar inhibition was observed when incorporation of radioactivity into protein was from tRNA acylated with [^{14}C]leucine or with a mixture of 15 ^{14}C-labeled amino acids (Table III). The per cent decrease associated with diabetes was nearly the same in each case.

Most frequently we found that the difference in effectiveness between skeletal muscle ribosomes from normal and from diabetic animals was abolished in the presence of 100 μg of polyuridylic acid (Table II). That was not the case with ribosomes from heart muscle (Rampersad and Wool, 1965); the difference persisted even in the presence of saturating concentrations of polyuridylic acid. What is more, the difference was still manifest when ribosomes from heart muscle of normal and diabetic animals were preincubated to remove endogenous mRNA and their response to added polyuridylic acid was titrated (Wool *et al.*, 1966). We do not know why skeletal muscle and heart muscle ribosomes respond differently in the presence of polyuridylic acid.

TABLE III: Incorporation into Protein of Radioactivity from tRNA Acylated with ^{14}C-Labeled Amino Acids by Ribosomes from Skeletal Muscle. Effect of Diabetes.[a]

Aminoacyl-tRNA	Incorp (cpm) Source of Ribosomes: Normal	Incorp (cpm) Source of Ribosomes: Diabetic	Difference (%)
tRNA–[^{14}C]phenylalanine	938	588	−37
tRNA–[^{14}C]amino acid mixture	1460	849	−41
tRNA–[^{14}C]leucine	2260	1477	−34

[a] The assay was carried out as described in Experimental Procedure; 30 μg of ribosomal RNA was added. The amount and radioactivity of the aminoacyl-tRNA was: [^{14}C]phenylalanyl-tRNA, 100 μg and 14,866 cpm; tRNA–[^{14}C]amino acid mixture, 50 μg and 19,107 cpm; [^{14}C]leucyl-tRNA, 100 μg and 50,376 cpm. Each 100 cpm of radioactivity in protein was the result of incorporation of 3.73 × 10^{-1} $\mu\mu$mole of phenylalanine, 3.71 × 10^{-1} $\mu\mu$mole of the amino acid mixture, or 2.94 × 10^{-1} $\mu\mu$mole of leucine.

To determine the reproducibility of the insulin effect ribosomes were prepared from three separate groups of diabetic animals treated with the hormone and assayed for their ability to catalyze protein synthesis. The variability was only ±6%.

The defect in protein synthesis that follows administration of alloxan takes between 24 and 48 hr to develop (Table IV); no further change occurs between 48 and 96 hr. The rats may be used to prepare ribosomes at any time between the second and fourth day after receiving alloxan. Diabetes, as judged from the blood sugar, occurred in all animals treated with alloxan. Generally we used the animals 48 hr after they received alloxan, for at that time they appeared in better health and the mortality rate was far less than when we waited until 96 hr had passed.

The minimal amount of insulin that had to be administered to diabetic animals to increase protein

TABLE IV: Incorporation into Protein of Radioactivity from [^{14}C]Phenylalanyl-tRNA by Ribosomes from Skeletal Muscle. Induction of the Defect Owing to Alloxan Diabetes.[a]

Source of Ribosomes	Days after Alloxan	Incorp (cpm)	Change (%)
Normal	1	779	
Diabetic		786	−
Normal		1752	
Diabetic	2	943	−46
Normal		1049	
Diabetic	3	455	−47
Normal		1012	
Diabetic	4	474	−53

[a] The assay was carried out as described in Experimental Procedure; 30 μg of ribosomal RNA and 100 μg of tRNA charged with [^{14}C]phenylalanine (19.0×10^3 cpm) was added. Each 100 cpm of radioactivity in protein was the result of the incorporation of 2.2×10^{-1} $\mu\mu$mole of phenylalanine.

TABLE V: Incorporation into Protein of Radioactivity from [^{14}C]Phenylalanyl-tRNA by Ribosomes from Skeletal Muscle. Minimal Effective Dose of Insulin.[a]

Source of Ribosomes	Insulin (units)	Incorp (cpm)	Change (%)
Normal	0	899	
Diabetic	0	386	−57
Diabetic	0.0001	352	
Diabetic	0.001	379	
Diabetic	0.01	320	
Diabetic	0.1	774	+101
Diabetic	5	781	+102

[a] The insulin was administered intraperitoneally 1 hr before the animals were killed and the ribosomes were isolated. The assay was carried out as described in Experimental Procedure; 25 μg of ribosomal RNA and 100 μg of tRNA charged with [^{14}C]phenylalanine (15.0×10^3 cpm) were added. Each 100 cpm of radioactivity in protein was the result of the incorporation of 2.2×10^{-1} $\mu\mu$mole of phenylalanine.

synthesis (assayed *in vitro*) was 0.1 unit (Table V). Quite remarkably, 0.1 unit (4 μg) was as effective as 5 units, and 0.01 unit had no effect at all. While the response was all or none insofar as we tested it, we do not know that it would not be linear with dosages between 0.1 and 0.01 unit of insulin.

TABLE VI: Incorporation into Protein of Radioactivity from [^{14}C]Phenylalanyl-tRNA by Ribosomes from Skeletal Muscle. Time Required for Insulin Reversal of the Defect Owing to Diabetes.[a]

Source of Ribosomes	Incorp (cpm)	Change (%)
Normal	1031	
Diabetic	450	−56
Diabetic + insulin	730	+62

[a] The insulin (0.1 unit) was administered intraperitoneally 5 min before the animals were killed and the ribosomes were isolated. The assay was carried out as described in Experimental Procedure; 30 μg of ribosomal RNA and 100 μg of tRNA charged with [^{14}C]-phenylalanine (16.88×10^3 cpm) were added. Each 100 cpm of radioactivity in protein was the result of the incorporation of 2.2×10^{-1} $\mu\mu$mole of phenylalanine.

Not only was 0.1 unit of insulin sufficient to increase protein synthesis, it would do so in 5 min (Table VI). We do not know, however, that the response is maximal in 5 min. The same amount of insulin did not change the blood glucose concentration in so short a time (Table VII). Even after 60 min, 0.1 unit of insulin had appreciably lowered the blood glucose in only two of four animals tested. The defect in ribosomal protein synthesis appears to be due to insulin deficiency rather than to toxic action of the alloxan, for the same defect is manifest when the animals are made acutely diabetic by administration of antiinsulin serum (Table VIII).

Characteristics of Incorporation of Radioactivity from [^{14}C]Phenylalanyl-tRNA into Protein by Skeletal Muscle Ribosomes from Diabetic Animals. TIME COURSE OF INCORPORATION. Ribosomes from diabetic and normal animals incorporated radioactivity into protein with exactly the same time course (Figure 5); the rate was rapid for 5 min, then decelerated, and after 20 min all but ceased. Diabetes influenced the magnitude but not the time required to reach maximum ribosomal protein synthesis.

EFFECT OF CONCENTRATION OF [^{14}C]PHENYLALANYL-tRNA. When the concentration of skeletal muscle ribosomes used in the assay of protein synthesis is 30 μg/ml, the saturating amount of [^{14}C]phenylalanyl-tRNA is approximately 250 μg. Since it is not economically feasible to use so much radioactive aminoacyl-tRNA for each assay, we generally used 100 μg. But the amount of [^{14}C]phenylalanyl-tRNA is one of the two factors limiting protein synthesis in the *in vitro* assay (the other is the amount of ribosomes). It is, therefore, important to determine whether this factor is responsible for the difference in effectiveness of ribosomes from normal and from diabetic animals.

TABLE VII: Effect of Insulin (0.1 unit) on the Blood Glucose Concentration of Diabetic Rats.[a]

Treatment			Blood Glucose Concentration (mg/100 ml)		
Saline	Insulin	Time (min)	Initial	Final	Change (%)
+		5	782	744	−5
	+	5	713	703	−1
	+	5	830	858	+3
+		60	830	835	+1
+		60	455	415	−9
	+	60	420	498	+19
	+	60	755	358	−53
	+	60	670	720	+7
	+	60	755	538	−29

[a] An initial sample of blood for determination of the glucose concentration was obtained by cutting off the end of the rat's tail. The animals, who weighed about 130 g, were then injected (intraperitoneally) with either 0.1 unit of insulin in 0.5 ml of saline or an equal volume of saline alone. After either 5 or 60 min the rats were decapitated, and a second sample of blood was obtained for the final blood glucose determination.

To test the possibility, we assayed protein synthesis by ribosomes from normal and diabetic animals in the presence of amounts of [^{14}C]phenylalanyl-tRNA ranging from 125 to 1000 μg (Figure 6). Ribosomes from diabetic animals catalyzed fewer aminoacyl transfers no matter what amount of [^{14}C]phenylalanyl-tRNA was added. The decrease due to the diabetes was 69, 63, 65, and 63%, respectively, when 125 250, 500, and 1000 μg of [^{14}C]phenylalanyl-tRNA was present in the assay mixture. Even when the amount of [^{14}C]phenylalanyl-tRNA exceeded that required for optimum incorporation, ribosomes from diabetic animals synthesized less protein.

RIBOSOME CONCENTRATION. Various concentrations of ribosomes from diabetic and from normal animals were compared for their ability to catalyze protein synthesis in the presence and in the absence of polyuridylic acid (Figure 7). In the absence of polyuridylic acid, ribosomes from diabetic animals were less efficient than ribosomes from normal animals irrespective of ribosomal concentration. In the presence of poly-

TABLE VIII: Incorporation into Protein of Radioactivity from [^{14}C]Phenylalanyl-tRNA by Ribosomes from Skeletal Muscle. Effect of Antiinsulin Serum.[a]

Source of Ribosomes	Incorp (cpm)	Change (%)
Control	584	
Antiinsulin serum	355	−39

[a] One group of rats was given 1.5 ml of antiinsulin serum intraperitoneally at 0 min and again at 90 min and the animals were killed at 180 min and the ribosomes isolated. The controls received the same volume of serum from guinea pigs which had not been immunized. The average blood glucose concentration (mg/100 ml) was control, 110; antiinsulin serum treated, 390. The assay was carried out as described in Experimental Procedure; 30 μg of ribosomal RNA and 100 μg of tRNA charged with [^{14}C]phenylalanine (9.2×10^3 cpm) were added. Each 100 cpm of radioactivity in protein was the result of the incorporation of 3.06×10^{-1} $\mu\mu$mole of phenylalanine.

TABLE IX: Incorporation into Protein of Radioactivity from [^{14}C]Phenylalanyl-tRNA by Ribosomes from Skeletal Muscle. Effect of Polyuridylic Acid.[a]

Polyuridylic Acid (μg)	Increase Owing to Poly U			
	Cpm		Ratio[b]	
	Normal	Diabetic	Normal	Diabetic
10	266	489	0.19	1.30
25	1278	1545	0.91	6.71
50	2224	3905	1.58	12.92
100	3037	4912	2.16	15.57
200	4300	7172	3.05	21.52
500	4452	7463	3.16	22.58

[a] The assay was carried out as described in Experimental Procedure; 30 μg of ribosomal RNA and 100 μg of tRNA charged with [^{14}C]phenylalanine (16.28×10^3 cpm) were added. Each 100 cpm in protein was the result of the incorporation of 2.2×10^{-1} $\mu\mu$mole of phenylalanine. [b] Counts per minute with polyuridylic acid to counts per minute without polyuridylic acid. The data used to calculate the ratio are in Figure 8.

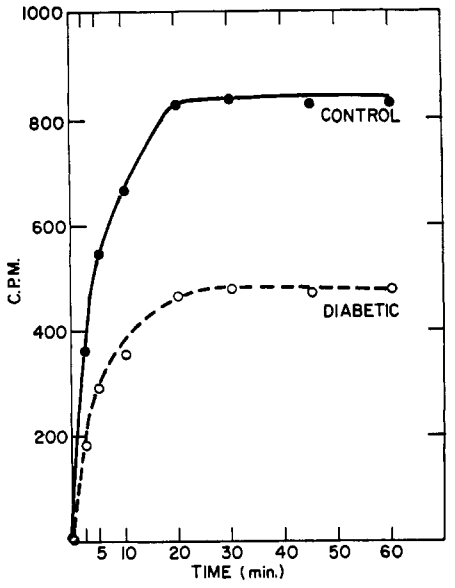

FIGURE 5: Comparison of the time course of incorporation into protein of radioactivity from [^{14}C]phenylalanyl-tRNA by ribosomes from skeletal muscle of diabetic and normal animals. The ribosomes (60 μg of ribosomal RNA) were incubated for different periods of time with 125 μg of [^{14}C]phenylalanyl-tRNA (4.08 $\times$ 10^3 cpm) as described in Experimental Procedure. Each 100 cpm in protein was the result of the incorporation of 3.73 $\times$ 10^{-1} $\mu\mu$mole of phenylalanine.

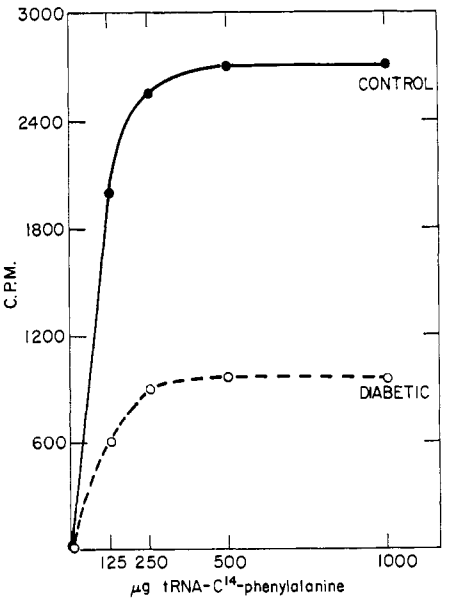

FIGURE 6: Comparison of the effect of the concentration of [^{14}C]phenylalanyl-tRNA on the incorporation of radioactivity into protein by ribosomes from skeletal muscle of normal and diabetic animals. The ribosomes (60 μg of ribosomal RNA) were incubated with different concentrations of [^{14}C]phenylalanyl-tRNA as described in Experimental Procedures. Each 125 μg of tRNA-[^{14}C]phenylalanine contained 17.8 $\times$ 10^3 cpm; each 100 cpm of radioactivity in protein was the result of the incorporation of 3.73 $\times$ 10^{-1} $\mu\mu$mole of phenylalanine.

uridylic acid, the difference between normal and diabetic ribosomes seemed to disappear, at least with smaller amounts of ribosomes (10–50 μg of ribosomal RNA); with large amounts (100–250 μg of ribosomal RNA), the diabetic ribosomes were in this experiment a little less efficient. In the absence of polyuridylic acid, the difference between normal and diabetic ribosomes was relatively constant; with 10, 25, 50, 100, 150, 200, and 250 μg of ribosomal RNA the per cent decrease in protein synthesis due to the diabetes was, respectively, 44, 58, 62, 57, 52, 45, and 47.

POLYURIDYLIC ACID CONCENTRATION. In the presence of lesser amounts of polyuridylic acid (10 or 25 μg), ribosomes from diabetic animals incorporated less radioactivity from [^{14}C]phenylalanyl-tRNA into protein than did ribosomes of normal controls; with larger amounts of polyuridylic acid (50–500 μg), just the reverse was true (Figure 8). When the increased protein synthesis due to polyuridylic acid was compared with the lesser synthesis in the absence of the polyribonucleotide, it was apparent that at every concentration the difference, reckoned either as per cent or in counts incorporated into protein, was always greater for diabetic ribosomes than for normal (Table IX). We do not know why the diabetic ribosomes were more sensitive to stimulation by polyuridylic acid. It cannot be merely that, having less natural mRNA, they bind more polyuridylic acid, for this greater sensitivity is apparent even when the comparison is made at a concentration of polyuridylic acid (10 μg/ml) far less than is saturating for ribosomes from normal animals.

These anomalous results might be partially reconciled if the synthetic polyribonucleotide were displacing endogenous mRNA from ribosomes. Polyuridylic acid can completely displace polycytidylic acid from ribosomes (Moore, 1966). If both normal and diabetic ribosomes bind only, or almost only, polyuridylic acid, which has displaced all, or almost all, natural mRNA, we would expect just such results as were observed. Ribosomes both from normal and from diabetic animals incorporated nearly the same amounts of amino acid into protein in the presence, but not in the absence, of polyuridylic acid. The possibility is easily tested. All that is required is that ribosomes be incubated in the usual system for assaying protein synthesis but with [^{14}C]leucyl-tRNA rather than [^{14}C]phenylalanyl-tRNA, and with and without polyuridylic acid. If the prediction is correct, polyuridylic acid should inhibit incorporation of [^{14}C]-leucine into protein by displacing endogenous mRNA, which, of course, contains the codons for leucine.

The results of that experiment were as follows. In the absence of polyuridylic acid, 3019 cpm of radio-

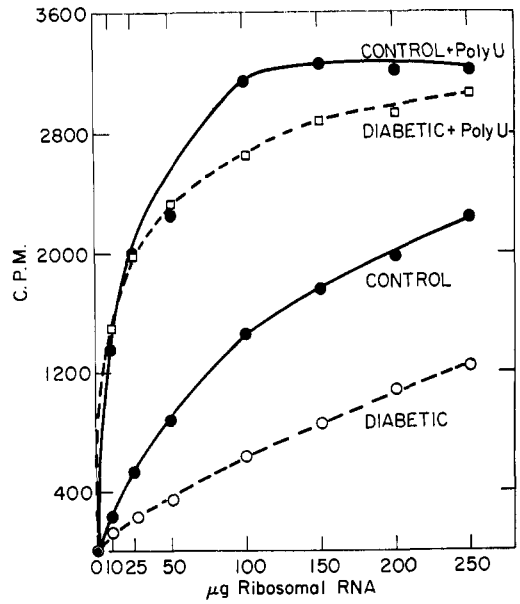

FIGURE 7: Comparison of the effect of the concentration of ribosomes from skeletal muscle of normal and diabetic animals on the incorporation into protein of radioactivity from [^{14}C]phenylalanyl-tRNA. Different concentrations of ribosomes were incubated with 125 μg of [^{14}C]phenylalanyl-tRNA (9.3 $\times$ 10^3 cpm) as described in Experimental Procedure. The amount of polyuridylic acid when present was 100 μg. Each 100 cpm of radioactivity in protein was the result of the incorporation of 3.73 $\times$ 10^{-1} $\mu\mu$mole of phenylalanine.

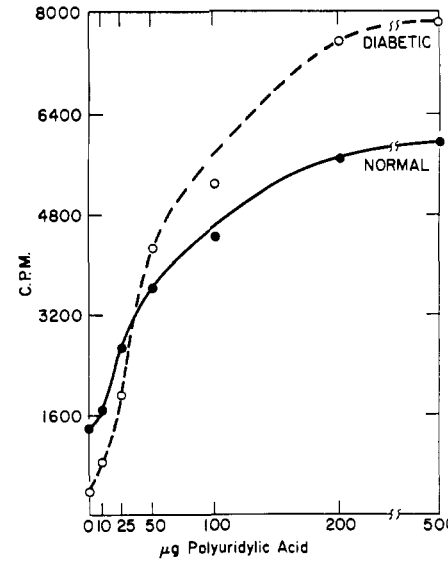

FIGURE 8: Comparison of the effect of the concentration of polyuridylic acid on the incorporation into protein of radioactivity from [^{14}C]phenylalanyl-tRNA by skeletal muscle ribosomes from normal and from diabetic animals. The ribosomes (30 μg of ribosomal RNA) were incubated with 100 μg of [^{14}C]phenylalanyl-tRNA (16.3 $\times$ 10^3 cpm) as described in Experimental Procedure. Each 100 cpm of radioactivity in protein was the result of the incorporation of 2.2 $\times$ 10^{-1} $\mu\mu$mole of phenylalanine.

activity from [^{14}C]leucyl-tRNA was incorporated into protein; in the presence of 200 μg of polyuridylic acid, incorporation was 2826 cpm, a decrease of 6%. When the ribosomes were incubated with [^{14}C]phenylalanyl-tRNA, the same amount of polyuridylic acid increased incorporation from 1421 to 5366 cpm. The polyuridylic acid does not appear to displace significant amounts of endogenous mRNA from skeletal muscle ribosomes. Moreover, the results indicate that there is no phenylalanine–leucine ambiguity in the reading of the UUU triplet by skeletal muscle ribosomes, as there is when *E. coli* ribosomes are used (Bretscher and Grunberg-Manago, 1962; Jones and Nirenberg, 1962).

In one experiment a natural messenger, the RNA from MS-2 virus, was assayed; the addition of up to 200 μg failed to stimulate protein synthesis by ribosomes from either normal or from diabetic animals.

PROTEIN SYNTHESIS BY A MIXTURE OF RIBOSOMES FROM NORMAL AND DIABETIC ANIMALS. The decreased ribosomal protein synthesis due to diabetes might be accounted for by the presence of an inhibitor, although that does not seem likely, since the supernatant factors come from either *E. coli* (tRNA) or the liver of normal rats ("transfer enzyme," *i.e.*, dialyzed 105,000*g* supernatant). Nonetheless, it might be argued that the inhibitor is associated with, or bound to, the ribosomes. To test the possibility we mixed equal amounts of ribosomes from normal and from diabetic animals and compared the same amounts of the mixture, of solely normal, and of solely diabetic ribosomes for their capacity to catalyze the transfer of radioactivity from [^{14}C]phenylalanyl-tRNA to protein (Table X). The mixture carried out an amount of protein synthesis only slightly greater than the mean between the amount carried out by ribosomes from normal and from diabetic animals. The results are incompatible with the idea of an inhibitor associated with the ribosomes from diabetic animals, at least an inhibitor capable of dissociation from the ribosomes.

Discussion

Breuer *et al.* (1964) were the first to demonstrate that ribosomes prepared from rat skeletal muscle would catalyze the incorporation of significant amounts of amino acid into protein when assayed *in vitro*. We confirm their findings. In the circumstances of our experiments the transfer of aminoacyl groups from [^{14}C]phenylalanyl-tRNA to protein by the skeletal muscle ribosomes depended on a source of energy (we used ATP, phosphoenolpyruvate, and pyruvate kinase), supernatant protein, GTP, and the proper concentration of magnesium. Incorporation was stimulated by addition of the synthetic polyribonucleotide poly-

TABLE X: Incorporation into Protein of Radioactivity from [^{14}C]Phenylalanyl-tRNA by Ribosomes from Skeletal Muscle. Incorporation by a Mixture of Ribosomes from Normal and Diabetic Rats.[a]

Source of Ribosomes	Incorp (cpm)	Change (%)
Normal	1213	
Diabetic	612	−49
0.5 normal + 0.5 diabetic	994	−18

[a] The assay was carried out as described in Experimental Procedure; 126 μg of ribosomal RNA and 125 μg of tRNA charged with [^{14}C]phenylalanine (11×10^3 cpm) were added. Each 100 cpm of radioactivity in protein was the result of the incorporation of 3.73×10^{-1} $\mu\mu$mole of phenylalanine.

uridylic acid; it was markedly inhibited by ribonuclease and puromycin. (Cycloheximide was a less effective inhibitor when added *in vitro* to skeletal muscle ribosomes.) Transfer of [^{14}C]phenylalanine to protein by ribosomes was rapid during the first 5 min of incubation; thereafter the rate declined, and after 20 min no further synthesis of protein occurred. The general characteristics of the reaction catalyzed by skeletal muscle ribosomes accord very closely with the features of protein synthesis by ribosomes from heart muscle, liver, and other mammalian tissues.

When ribosomes are used to study the regulation of protein synthesis, it is imperative to know which component or components in the complex reaction mixture limit incorporation. Ideally, synthesis should be dependent only on the number of ribosomes; that is to say, all the necessary cofactors should be present in optimal or excess amounts. The conditions of our experiments were such that protein synthesis could be increased not only by adding more ribosomes, but also by increasing the amount of [^{14}C]phenylalanyl-tRNA. But if the amount of [^{14}C]phenylalanyl-tRNA was increased to the point where it was no longer limiting for protein synthesis, incorporation was still proportional to the number of ribosomes. For practical reasons our assays were generally carried out with less than saturating amounts of [^{14}C]phenylalanyl-tRNA, but a separate experiment showed that the results obtained with ribosomes from diabetic animals were not affected by the amount of [^{14}C]phenylalanyl-tRNA.

Ribosomes from muscle of diabetic animals catalyze protein synthesis far less efficiently than do ribosomes from normal animals. When the ribosomes are from heart muscle, the defective protein synthesis due to insulin deficiency cannot be corrected by adding insulin to the ribosomes *in vitro;* it is corrected, however, by administering insulin before the animal is killed and the ribosomes are isolated (Rampersad and Wool, 1965).

The conclusion that diabetes leads to a loss in the efficiency with which ribosomes from heart muscle carry out protein synthesis was based on the following observations. Ribosomes from diabetic animals incorporated less radioactivity into protein than did an equal number of ribosomes from normal animals even when the concentrations of all cofactors necessary for protein synthesis were the same; and the difference between the two classes of ribosomes (normal and diabetic) persisted even in the presence of near-saturating amounts of polyuridylic acid (Rampersad and Wool, 1965). The latter finding suggested that the difference was not due merely to a disparity in the amount of endogenous mRNA associated with the ribosomes. This inference was considerably reinforced when we added polyuridylic acid to ribosomes from which endogenous mRNA had been removed by preincubation and found that the diabetic ribosomes carried out far less protein synthesis than did the normal (Wool *et al.*, 1966). The results of the experiments with preincubated ribosomes from heart muscle supported the possibility that insulin regulated protein synthesis by conditioning the translation of preformed mRNA.

The results obtained with skeletal muscle and heart muscle ribosomes from diabetic animals are in general qualitatively similar. However, the decreased protein synthesis due to diabetes and the stimulation that follows insulin administration are both consistently greater when skeletal muscle ribosomes are used. But there is one conspicuous difference. Addition of polyuridylic acid normalizes the efficiency of diabetic skeletal muscle ribosomes and not of diabetic heart muscle ribosomes. We cannot account for the difference in response of ribosomes from the two tissues.

We would expect diabetic ribosomes to be more sensitive than normal ribosomes to stimulation by polyuridylic acid if differing amounts of natural mRNA are associated with the two classes of ribosomes. If the ribosomes from diabetic animals were deficient in mRNA, then they should bind greater amounts of synthetic template RNA (polyuridylic acid) and hence incorporate more phenylalanine into protein than ribosomes from normal animals. Although that was exactly what we observed, nonetheless there is evidence that a deficiency of template RNA is not the basis of the primary defect in protein synthesis. The defective ribosomal protein synthesis can be corrected by the administration of insulin to diabetic animals pretreated with sufficient actinomycin to all but completely suppress RNA synthesis (Wool and Cavicchi, 1966). That is to say, the defect can be repaired by insulin in circumstances where it is most unlikely that the hormone has changed the total amount of template available to the ribosomes.

In interpreting the results of experiments with polyuridylic acid, it is well to keep in mind that we are by no means certain that this synthetic polyribonucleotide is a good model for natural mRNA. For example, the binding of polyuridylic acid to ribosomes seems not to have the same characteristics as the binding of natural mRNA (Okamoto and Takanami, 1963),

which may be due to the random coil configuration of polyuridylic acid; binding is favored by a lack of secondary structure (Takanami and Okamoto, 1963). At any rate, the difference in response to polyuridylic acid observed between skeletal muscle ribosomes from normal and from diabetic animals needs to be tested with stripped ribosomes, as has been done with ribosomes from heart muscle (Wool *et al.*, 1966).

The exact nature of the biochemical fault in ribosomal protein synthesis owing to diabetes still cannot be specified. A fundamental problem that needs to be resolved is the nature of the population of "diabetic ribosomes." Are all the ribosomes one-half as efficient as those from normal animals or are one-half the ribosomes fully active while the remainder are inactive? The answer to this question may provide an important clue to how insulin participates in the regulation of protein synthesis in muscle.

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