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Research Article

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Characteristics of Membrane-Bound and Free Hepatic Ribosomes from Insulin-Deficient Rats

I. ACUTE EXPERIMENTAL DIABETES MELLITUS

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ABSTRACT Membrane-bound and free ribosomes were prepared by discontinuous density gradient centrifugation from livers of rats 2–3 days after receiving alloxan (75 mg/kg) or streptozotocin (100 mg/kg). Hepatocytes from these animals were also examined by electron microscopy and subjected to quantitative morphometric analysis. The results indicated that the two populations of hepatic ribosomes respond differently to acute insulin deficiency. There was an overall reduction ($P < 0.001$) in total number of bound ribosomes per volume cytoplasm; the remaining bound ribosomes underwent a shift to smaller-sized ribosomal messenger RNA (mRNA) aggregates ($P < 0.02$); and the protein-synthetic activity of these bound ribosomes was less than normal ($P < 0.02$) when protein synthesis was directed by endogenous mRNA. However, there was no difference between bound ribosomes from livers of normal and diabetic rats when protein synthesis was directed by polyuridylic acid. In contrast, free ribosomes were unchanged in number and degree of ribosomal mRNA aggregation, but displayed a significantly increased rate of in vitro protein synthesis ($P < 0.01$) as compared to normal controls. This increased protein-synthetic activity occurred when amino acid incorporation was directed by endogenous mRNA or polyuridylic acid. These changes in structure and function of bound and free hepatic ribosomes were prevented by the concomitant administration of insulin. The decrease in protein-synthetic activity of bound hepatic ribosomes from acutely diabetic rats seems to be secondary to marked disruption and disaggregation of the rough endoplasmic reticulum (RER) with production of smaller ribosomal mRNA aggregates which incorporate less amino acids into protein. Increased protein synthetic activity of free ribosome appears to be related to the ability of these ribosomes to copy mRNA more efficiently.

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INTRODUCTION

Recent studies from our laboratory have indicated that the decrease in hepatic ribosomal protein synthesis that accompanies acute experimental diabetes mellitus (1, 2) is associated with marked ultrastructural changes in the membrane-bound population of hepatic ribosomes (2). This led us to postulate that the observed reduction in total hepatic protein synthesis was the result of an effect of insulin lack on the structure and function of the membrane-bound polyribosomes (2). In the current experiments we have attempted to provide further support for this hypothesis by separating bound and free hepatic ribosomes (3, 4) and independently evaluating the ability of the two ribosomal fractions to synthesize protein in vitro. The clarification of this issue seemed important in view of the fact that hepatic membrane-bound ribosomes have been shown to synthesize proteins which differ qualitatively from those synthesized by ribosomes free in the cytosol (5–8), and this difference may have special relevance in the diabetic state. The results to be reported indicate that bound polyribosomes from diabetic rat liver were less active than bound polyribosomes from normal rat livers in carrying out protein synthesis. In contrast, the protein-synthetic activity of free ribosomes from livers of acutely diabetic

rats appeared to be greater than that of normal controls. These conclusions were also supported by findings from electron microscopy. Thus, acute and severe experimental diabetes exerts a widely divergent effect on the protein synthetic behavior of the two hepatic ribosomal populations.

METHODS

Experimental protocol. Female Sprague-Dawley rats weighing 180–200 g were used for all studies. Diabetes mellitus was produced by intravenous injection of alloxan monohydrate (75 mg/kg body wt) in saline or streptozotocin[1] (100 mg/kg) in pH 4.5 citrate buffer after an 18 h overnight fast. Paired control rats were given equal volumes of isotonic saline. 48 h after alloxan administration, and 72 h after streptozotocin administration, the rats were killed by decapitation and the livers rapidly removed. Streptozotocin-treated rats required 72 h to reach the degree of hyperglycemia achieved in 48 h in alloxan-treated rats. Because previous studies had indicated that diabetic animals ate little food on the last day of the experiment, all rats were fasted for 18 h before death. Before this 18-h period rats ate 10–18 g of rat chow/day, and no significant difference was observed in food consumption between normal and diabetic animals. Only animals with blood glucose[2] levels greater than 650 mg/100 ml were used in diabetic experiments. Blood glucose levels of control rats were 88–105%. Some streptozotocin-treated rats received insulin in order to prevent the development of insulin deficiency. Subcutaneous lente insulin injections were begun 18 h after streptozotocin administration when blood glucose was already greater than 200 mg/100 ml, and 8–10 U of insulin were given per 24 h in divided doses, to keep blood glucose levels below 200 mg/100 ml. For some experiments, ^{3}H-labeled ribosomes were produced by injecting 100 μCi [^{3}H]phenylalanine (6.5 Ci/mm) intraperitoneally 12 h before sacrifice.

Comparisons between individual data and group means were carried out by paired and unpaired Student *t* tests.

Preparation of membrane-bound and free ribosomes. Excised livers were immediately weighed and homogenized in 2 vol of 7.6 TKM-S0.25 (0.05 M Tris-HCl, pH 7.6, 0.025 M KCl, 0.004 M $MgCl_2$, 0.25 M sucrose[3]) with six strokes of a Teflon pestle (approximately 1,000 rpm) in a ground glass homogenizer. 500 and 10,000 *g* supernates were prepared in parallel by centrifuging aliquots of homogenate for 10 min at appropriate speeds (Sorvall SS4; Ivan Sorvall Inc., Newtown, Conn.). The 500 and 10,000 *g* supernates were then layered over a discontinuous sucrose gradient for isolation of the ribosome fractions.

Sucrose gradients for the separation of free and membrane-bound ribosomes were made by a modification of the technique of Blobel and Potter (3, 9). 4 ml of supernate, either 500 or 10,000 *g*, was layered over 3 ml 0.5 M sucrose solution, in turn layered over 3 ml 2.0 M sucrose solution, both containing ribonuclease inhibitor prepared from rat liver cytosol (10, 11) diluted 1:1 with 7.5 TKM (0.05 M Tris-HCl, pH 7.5, 0.025 M KCl, 0.005 M $MgCl_2$). Mg^{++} concentration in the resulting sucrose gradient solutions was measured as 4.3 mM; the K^+ concentration was 36 mM.

The discontinuous gradients were centrifuged 18 h at 105,000 *g*. After the centrifugation, the top 2 ml (clear and colorless) was discarded. The turbid layer (about 5 ml) above the 2.0 M sucrose solution was removed. This layer contained the membrane-bound ribosomes. The 2.0 M solution and the pellet contained the free ribosomes. The free and bound fractions were each diluted to 8 ml with 7.6 TKM-S0.25. 10% wt/vol sodium deoxycholate (DOC)[4] was added to 1.3% final concentration to the bound fraction. Both fractions were then centrifuged 4 h at 105,000 *g*. The supernates were discarded and the pellets were suspended in 7.6 TKM-S0.25 and centrifuged for 3 min at 3,000 *g*. Aliquots of the clear suspension were placed into small tubes and stored at − 20°C.

Evaluation of separation technique. Free and bound ribosomes ^{3}H-labeled in vivo were harvested by sucrose gradient centrifugation of postmitochondrial supernate from rats given [^{3}H]phenylalanine before sacrifice. The turbid layer above the 2.0 M sucrose layer contained labeled bound ribosomes. The pellet contained labeled free-ribosomes and the 2.0 M layer was discarded. The distribution of labeled bound and free ribosomes was studied by combining aliquots of each labeled fraction with post-mitochondrial supernate from a rat which had not received [^{3}H]phenylalanine and centrifuging over discontinuous sucrose gradients as described above. After centrifugation, the contents of the tubes were carefully removed in aliquots of 1 ml or less. The pellets remaining in the tube were suspended in 1 ml 7.6 TKM-S0.25. Radioactivity of the aliquots was measured by liquid scintillation with Aquasol[5] with [^{3}H]-phenylalanine internal standards.

Recovery of bound and free hepatic ribosomes. Livers were weighed, homogenized in known volumes of buffer, centrifuged at either 500 or 10,000 *g*, and 4 ml of the resulting supernate used to prepare bound and free ribosomes. The total RNA content of the various ribosomal preparations was determined by the method of Schneider (12), and recovery of bound and free ribosomal RNA from livers of normal and diabetic rats was then calculated and expressed as milligrams of ribosomal RNA per gram of wet weight liver.

Amino acid incorporation directed by endogenous mRNA. Protein-synthetic activity of the isolated ribosomes was estimated by in vitro incorporation of [^{3}H]phenylalanine into TCA-precipitable radioactivity. The basic details of the assay system have appeared in earlier publications (2, 13). The conditions of the assay are such that the incorporation of labeled amino acid into protein is linearly related to the amount of ribosomal RNA incubated and the amount of ribosomes is the rate-limiting factor for protein synthesis. Ribosome suspension containing 100–300 μg ribosomal RNA were added to 1 ml of an incubation solution containing 1.5 mg pH 5 fraction protein (derived from pooled normal rat liver), 1.9 μmol ATP, 0.37 μmol GTP, 8.2 μmol phosphoenolpyruvate, 35 μg pyruvate kinase (Sigma, Type II; Sigma Chemical Co., St. Louis, Mo.), 0.17 μmol for each of 19 unlabeled amino acids, 0.57 μmol [^{3}H]phenyl-

[1] Streptozotocin was a gift of the Upjohn Company, Kalamazoo, Mich.

[2] Ames Dextrostix/Reflectance Meter, Ames Co., Div. of Miles Lab, Inc., Elkhart, Ind.

[3] Schwarz/Mann ultrapure ribonuclease-free sucrose was used for all sucrose solutions. Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.

[4] *Abbreviations used in this paper:* DOC, sodium deoxycholate; mRNA, messenger RNA; polyU, polyuridylic acid; RER, rough endoplasmic reticulum; TKM, solution of Tris-HCl, KCl, $MgCl_2$; TKM-SO.25, solution of Tris-HCl, KCl, $MgCl_2$, and sucrose.

[5] New England Nuclear Corp., Boston, Mass.

alanine (7 Ci/mm, Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.), 0.7 μmol dithiothreitol, and 300 μmol sucrose. The solution was buffered with 0.04 M Tris-HCl, pH 7.8. The Mg^{++} concentration was adjusted to 11 mM and the K^+ concentration was 55 mM.

Samples were incubated at 37°C and duplicate 100-μl aliquots were withdrawn at 0, 10, 20, and 30 min and placed on filter papers (14). Nonprotein radioactivity was extracted with hot 5% TCA and remaining TCA-precipitable radioactivity was counted by liquid scintillation. Protein-synthetic activity was expressed as counts per minute of [^{3}H]phenylalanine incorporated per milligram ribosomal RNA in the incubated sample. All comparisons between normal and diabetic ribosomes were made with samples isolated on the same day in the same assay. Optimal amino acid incorporation for both normal and diabetic bound and free ribosomes occurred at 11 mM Mg^{++} concentration. Relative differences in amino acid incorporation found at 11 mM Mg^{++} persisted for Mg^{++} concentrations between 3 and 18 mM.

Amino acid incorporation directed by polyuridylic acid. Protein synthesis directed by polyuridylic acid was assayed for all pairs of normal and alloxan-diabetic ribosomes preparations. 0.05–0.08 mg ribosomal RNA was preincubated at 37°C for 60 min in a total volume of 600 μl with composition as for endogenous mRNA incubations except that [^{3}H]phenylalanine was omitted. Polysome profiles of preincubated samples showed that over 95% of the polysomes had been converted to monosomes. After the preincubations, samples were divided into duplicate 300μl aliquots. 75 μl of incubation solution (2.85 μmol/ml ATP, 1.1 μmol/ml GTP, 12.8 μmol/ml phosphoenolpyruvate, 100 μg/ml pyruvate kinase, 7.0 mM [^{3}H]phenylalanine, 0.3 mM dithiothreitol, 0.1 M sucrose, and 0.015 M Tris-HCl, pH 7.8, Mg^{++} and K^+ concentration 18 and 23 mM, respectively) containing polyuridylic (polyU) acid (1.4 mg/ml) was added to one aliquot, and 75 μl of incubation solution without polyU was added to the other.

Duplicate 75-μl aliquots of the incubating samples were taken after 0 and 120 min of 37°C incubation and placed on filter papers to be processed and counted by liquid scintillation as described above. Protein-synthetic activity was expressed as counts per minute incorporated into TCA-precipitable radioactivity per milligram RNA for each incubated sample. Net activity was calculated by subtracting counts per minute for the sample without polyU from counts per minute for the sample with polyU. Background radioactivity, calculated from a tube with polyU but no ribosomes, was subtracted from all samples. Incubations were discarded if the sample without polyU incorporated more than 5% as much radioactivity as the sample with polyU. Under the conditions of this assay, radioactivity incorporated into protein was linearly related to the amount of ribosomes incubated up to 0.15 mg RNA. Peak amino acid incorporation for both normal and diabetic ribosome samples in this assay system was at 18 mM Mg^{++} concentration. Relative differences persisted from 8–26 mM Mg^{++}.

Preparation of polysome profiles. Aliquots of ribosome suspensions (about 0.3 mg ribosomal RNA) were layered on a 13-ml 10–40% chilled linear sucrose gradient in 0.05 M Tris-HCl, pH 7.5, 0.025 M KCl, and 0.005 M $MgCl_2$. The gradients were centrifuged 45 min at 40,000 rpm and 4°C in a Spinco SW-40 rotor (Beckman Instruments Inc., Spinco Div., Palo Alto, Calif.). The gradients were pumped through an 80μl flow cell[6] at a rate of 0.4 ml/min and absorbance was continuously recorded at 260 nm with a Beckman DU spectrophotometer and chart recorder (Beckman Instruments Inc., Electronic Instruments Div., Schiller Park, Ill. (15, 16).

Preparation of electron micrographs. Ultrastructural morphometric analyses were carried out on the same livers used for the study in vitro protein synthesis and polysome profiles. Tissue was taken from the central area of the left hepatic lobe, fixed 4 h in 1% osmium tetroxide in Millonig's phosphate buffer (pH 7.2), dehydrated in graded alcohols, and finally embedded in a mixture of epon and araldite plastic. Thin sections (300–500 Å) were obtained from areas within six hepatocytes from a given portal triad. The thin sections were collected on Formvar-coated grids, stained with uranyl acetate and Reynold's lead citrate, and inspected with a JEM 100-B electron microscope.

Tissues were examined for general ultrastructural characteristics. In addition, two separate quantitative morphometric analyses were performed. In the first instance, the area of rough endoplasmic reticulum (RER) per cytoplasmic volume was estimated by the methods of Loud (17) and Weibel (18) in which the surface density of RER (S_{RER}) is quantitatively represented by the contour length density of RER profiles. This latter is estimated by placing a grid of test lines (l) on the electron micrographs and counting the intersections (C) formed by these lines with the RER profile borders. This relationship is given by the equation:

$$S_{RER} = 4C\,M/1{,}000\,l$$

where: S_{RER} = area of RER/volume of cytoplasm (μ^2RER/μ^3 cytoplasm); C = number of intersections of RER profiles ($\times 2$) with sampling lines; l = sum of length of sampling lines measured in millimeters; M = total magnification of photographic print. Very low magnification (3,000 ×) electron micrographs were prepared of four nucleated cells from each of four rats from each experimental group. A random selection of cells was assured by the fact that the microscopist could not distinguish the details of the cells at the magnification chosen and focusing for photographs was done electronically. The electron micrograph of each cell was photographically enlarged on double sheets of 11 × 14 inch paper to a final magnification of 16,380 ×. The amount of RER present was evaluated at an additional threefold magnification by a technician unaware of the origin of the samples.

In the second quantitative method the number of ribosomes per area of endoplasmic reticulum membrane was quantitated (18). Higher magnification electron micrographs (13,300 ×) of portions of three cells from each of four animals for each experimental group were selected randomly. The micrographs were printed on 11 × 14 inch paper, bringing the final magnification of the area to 56,600 ×. In photographs in which small amounts of RER were present, every strand was evaluated. For larger complexes of RER, the pictures were divided into quadrants (by a random number system on the reverse side of the photograph) and all RER strands within the selected quadrant were examined. The length of the designated RER (L) which lay perpendicular to the plane of the section was traced with a map-measuring device in each photograph. The sum of the lengths of closely opposed RER

[6] Lux Scientific Instrument Corp., New York.

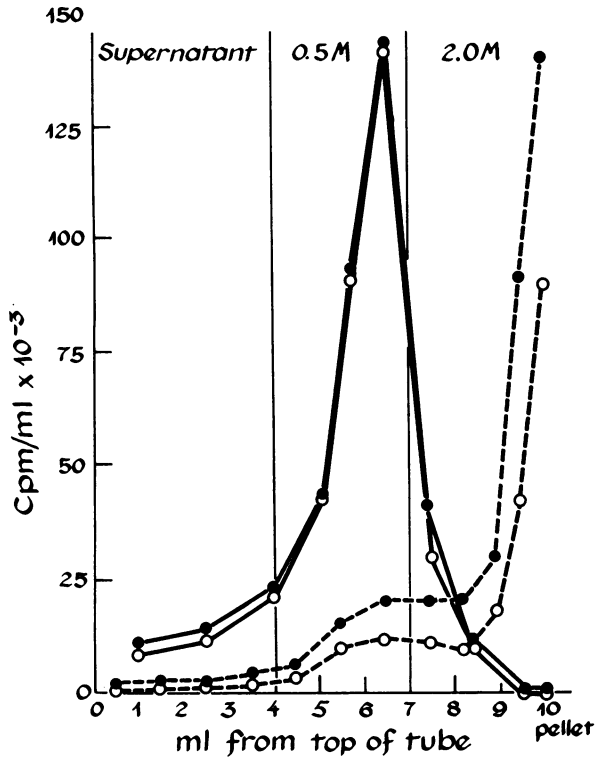

FIGURE 1 Distribution of radioactivity from labeled bound and free ribosome fractions centrifuged through a sucrose gradient. The distribution of bound ribosomes is indicated by solid lines (——) and free ribosomes are represented by interrupted lines (- - - -). Ribosomes from normal animals are represented by open circles while ribosomes from alloxan diabetic rats are shown with closed circles. The displayed data were obtained using 10,000 *g* supernates from two separate labeling studies, one free and one bound.

profiles was doubled (to account for both sides of the organelle), while the circumference of circular or irregular RER profiles was directly traced and recorded without doubling. It must be assumed that ribosomes are attached to a portion of the endoplasmic reticulum sheet extending through the entire thickness of the section; in this study the width of the membrane sheet is estimated to be 1.2 times the section thickness of 400 Å. The number of ribosomes can thus be related to an area of membrane ($L \times 400$ Å $\times 1.2$) which permits calculation of the number of ribosomes per square micron of membrane (18). The estimation of section thickness was obtained by the interference color method of Peachey (19), and the uniformity of the section thickness can be attested to by the low standard error (less than 5% of the mean) within the control group (column 2, Table II). As before, measurements were made by individuals who were not aware of the identity of the photographs.

RESULTS

Separation of bound and free hepatic ribosomes. Bound ribosomes were operationally defined as those ribosomes contained in the 0.5 M sucrose layer after centrifugation of a postmitochondrial supernate for 18 h at 105,000 *g*. When [^{3}H]phenylalanine-labeled bound ribosomes were combined with normal carrier postmitochondrial supernate, layered over a sucrose gradient, and centrifuged, the radioactive bound fractions were distributed as shown in Fig. 1 (solid lines). 83±1%[7] of the radioactivity is found above the 2.0 M sucrose interface of the gradient, while only 17% of the radioactivity is found in the 2.0 M sucrose layer. The distributions of labeled normal and diabetic bound ribosome fractions were the same. Furthermore, when labeled control or diabetic bound ribosomes were combined with diabetic carrier postmitochondrial supernate, the results were the same as those displayed in Fig. 1. Finally, results were identical when the carrier supernate is prepared by either a 500 *g* or 10,000 *g* centrifugation.

To estimate the purity of these bound ribosomes, the 0.5 M sucrose layer was removed and recentrifuged for 4 h at 105,000 *g*, and the pellet was fixed and examined by electron microscopy. Purity of the bound ribosome fraction was a function of the original supernate from which the ribosomes were prepared. Bound fractions prepared from an original 500 *g* supernate contained mitochondria, lysosomes, and other membranes. On the other hand, lysosomes and mitochondria were only rarely seen in preparations from the 10,000 *g* supernate, and there appeared to be smaller fragments of RER than were seen in the 500 *g* supernate.

Free ribosomes were defined operationally as those labeled ribosomes contained in the pellet after the initial centrifugation of the postmitochondrial supernate through a discontinuous sucrose gradient. When [^{3}H]phenylalanine-labeled free ribosomes from either normal or diabetic rat livers were combined with carrier postmitochondrial supernate and centrifuged through a discontinuous sucrose gradient, the distribution of radioactivity was as shown in Fig. 1 (interrupted lines). Although a good fraction of the labeled free ribosomes were contained in the pellet (43%), a highly significant fraction of the total radioactivity was found in the 2.0 M sucrose layer and would be discarded if just the pellet were taken to represent the free ribosome population as is conventionally done. On the other hand, a total of 82±2%[8] of the radioactivity is found in the pellet and the 2.0 M sucrose layer, and only 18% of the radioactivity of free ribosomes is found above the 2.0 M interface. The percentage of radioactivity below the 2.0 M interface is the same for normal and diabetic free ribosomes (Fig. 1) and the distribution of radioactivity was the same when

[7] Mean ±SEM of six experiments, three using labeled normal and three using labeled diabetic bound ribosomes.

[8] Mean ±SEM of six experiments, three using labeled normal free ribosomes and three using labeled free ribosomes from diabetic rat liver.

TABLE I

Mean±SE Recovery of Membrane-Bound and Free Ribosomes from Livers of Normal and Alloxan Diabetic Rats

Bound ribosomes			Free ribosomes		
Supernate	Control (n = 8)	Diabetic (n = 8)	Supernate	Control (n = 8)	Diabetic (n = 8)
g	*mg RNA/g liver*		*g*	*mg RNA/g liver*	
500	1.64±0.15	1.15±0.22*	500	2.36±0.19	2.44±0.22
10,000	1.09±0.24	0.80±0.19*	10,000	2.61±0.23	2.29±0.31

* $P < 0.01$ (Student t test).

carrier postmitochondrial supernate was obtained either from normal or diabetic rats, or from a 500 *g* or 10,000 *g* centrifugation. The fact that free ribosomes were present in substantial numbers in the 2.0 M layer made it essential that free ribosomes be harvested from both pellet and 2.0 M layer. Not only does the 2.0 M sucrose layer contain a significant portion of free ribosomes, but it selectively contains monosomes, making the pellet unrepresentative of the total free ribosome population.[9]

In order to evaluate the purity of the free ribosomal population, the pellet of free ribosomes was resuspended in the 2.0 M sucrose layer, diluted to 0.8 M sucrose, repelleted, and examined by electron microscopy. The ribosomes were uncontaminated by membrane structures, mitochondria, or nuclear fragments, and this was true of preparations from both 500 and 10,000 *g* supernates.

From these experiments, it appears that membrane-bound and free ribosomes are each prepared in relative purity by the method employed in this paper and that both ribosome populations are prepared with equal efficiency from normal and acutely diabetic rats.

Recovery of bound and free hepatic ribosomes. Recoveries of bound and free hepatic ribosomes are shown in Table I. Recovery of bound ribosomes from livers of diabetic animals was significantly decreased as compared to controls. This was true of pellets obtained from both 500 and 10,000 *g* supernate. As expected, ribosomal recovery was greater from 500 *g* than 10,000 *g* supernate, due to the fact that a larger fraction of the RER centrifuges down with nuclei and mitochondria at the higher sedimentation rate (3). Free ribosomes were recovered in equal amounts from livers of normal and diabetic rats, irrespective of the supernate from which they were derived. The measured decrease in recovery of bound ribosomes in diabetes is consistent with our previous observation that the amount of RER is decreased in severe insulin deficiency (2).

Effect of acute insulin deficiency on bound hepatic ribosomes. The morphological abnormalities seen after streptozotocin are essentially identical with those reported earlier (2) after the production of acute insulin deficiency by alloxan, and consist primarily of a loss of glycogen, and accumulation of neutral fat, an enlargement of mitochondria, proliferation of the smooth endoplasmic reticulum, and marked disruption and disaggregation of the RER and its attendant population of bound polyribosomes. It is this last abnormality which we feel is responsible for the decrease in hepatic protein synthesis that occurs in acute diabetes. The development of these changes can be prevented by insulin replacement after the administration of streptozotocin or alloxan (2).

To quantitatively assess the effect of acute insulin deficiency on the amount of RER, we have estimated the amount of RER present in hepatocytes. These results appear in Table II, column 1, and indicate that the area of RER per volume of hepatocyte cytoplasm is significantly reduced in rats with streptozotocin-induced diabetes. This decrease in RER can be prevented by the concomitant administration of insulin. The decrease in RER is quite comparable to that we have earlier described in alloxan-treated rats (2) except that each value has been doubled to account for the fact that profiles of RER actually represent double membranes.

TABLE II

Morphometric Analysis of the Effect of Untreated and Insulin-treated streptozotocin Diabetes on the Ribosome and Membrane Components of the RER of Rat Hepatocytes (Mean±SEM)

	1 Area RER per volume cytoplasm	2 Number ribosomes per area RER	3 Bound ribosomes per volume cytoplasm
	μm^2 *RER/μm^3 cytoplasm*	μm^2 *RER*$^{-1}$	μm^3 *cytoplasm*$^{-1}$
Control (n = 4)	5.26±0.18	369±15	1941±133.2
Streptozotocin (n = 4)	2.34±0.09*	240±22*	562±56.2*
Streptozotocin plus insulin (n = 4)	5.32±0.33	462±40	2458±266.1

* $P < 0.001$ compared to control.

[9] D. T. Peterson, unpublished observations.

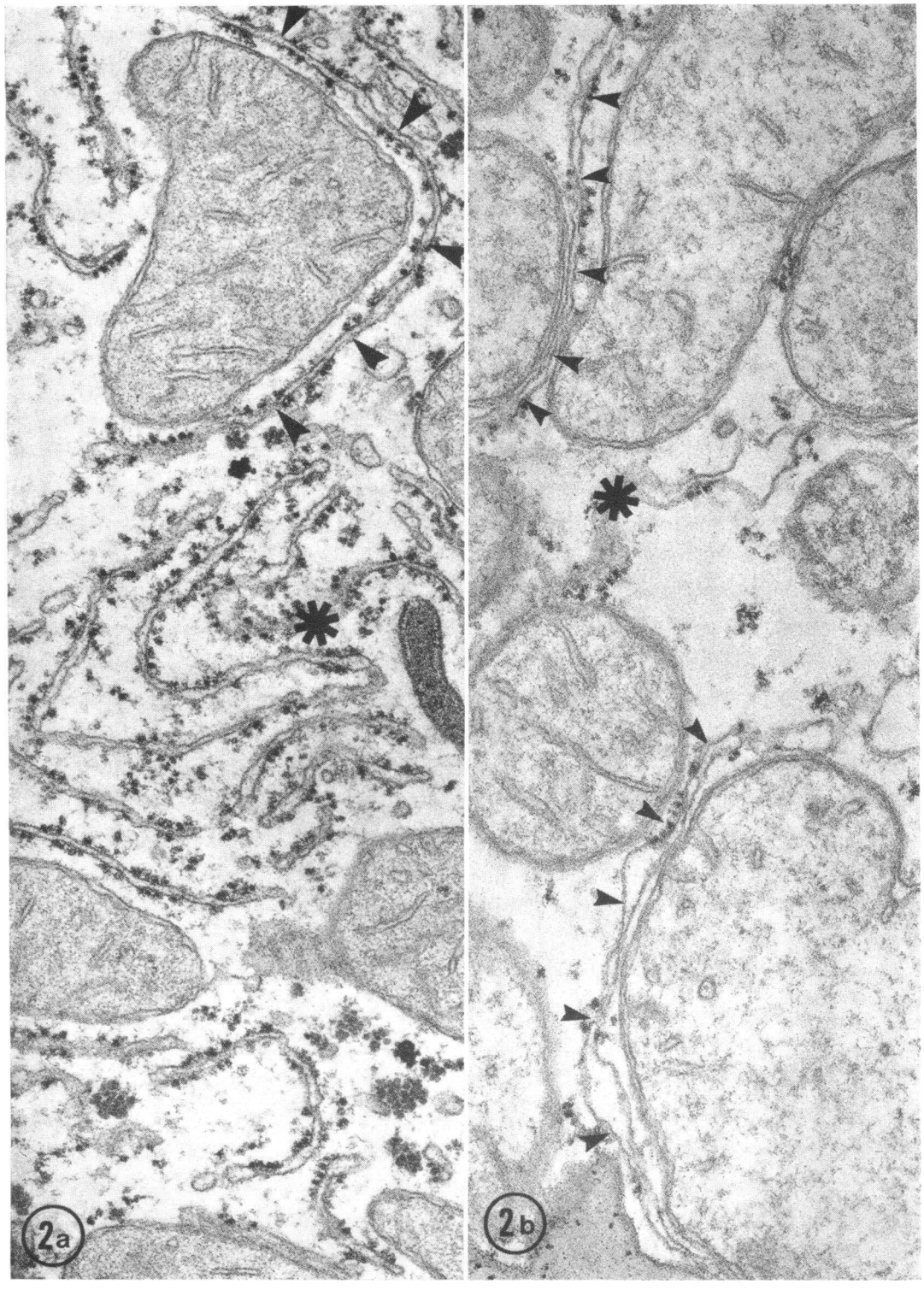

FIGURE 2 Enlarged view of typical profiles of hepatocyte rough endoplasmic reticulum from control rat (*a*) and streptozotocin-treated rat (*b*). Measurements of ribosomes per area RER were made from RER profiles with a clear discernible trace as indicated by arrowheads. Tangentially-sectioned membranes (asterisks) were not included in the measurements. × 56,600.

In an effort to assess quantitatively the effect of acute insulin deficiency on the structural integrity of the membrane:ribosome unit we have estimated the number of bound ribosomes per area of RER membrane from hepatocytes of control rats and rats made diabetic with streptozotocin. Representative micrographs of RER from normal and diabetic rat liver are seen in Fig. 2. In the diabetic hepatocyte (*2b*) it is noted that the groups of ribosomes are separated by large gaps of unoccupied membrane. The quantitative data are seen in Table II, column 2, and demonstrate that acute, severe insulin deficiency results in a significant decrease in the number of ribosomes per unit area of membrane. Similar results are obtained when alloxan is used to produce acute insulin deficiency. Insulin replacement prevents this decrease. The proportion of ribosomes per unit volume of cytoplasm can be derived from the data of columns 1 and 2. In diabetic hepatocytes this figure is seen to be approximately 30% of normal (Table II, column 3) and this decrease is also prevented by the concomitant administration of insulin.

Effect of acute insulin deficiency on polysome profiles of bound hepatic ribosomes. The size of the mRNA-bound ribosomal aggregates can be estimated by studying their distribution on linear sucrose gradients. The results of such polysome profiles are seen in Fig. 3. The peaks at the left of the profile represent smaller mRNA-ribosomal aggregates and those to the right are indicative of large mRNA-ribosomal aggregates. In order to evaluate quantitatively these polysome profiles the area under the tracings for several paired samples of normal

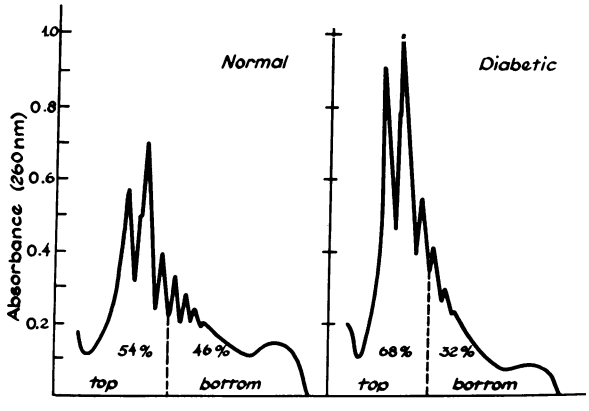

FIGURE 3 Polysome profiles of membrane-bound ribosomes prepared from 500 *g* supernates from livers of normal and alloxan diabetic rats. Percentage figures under the left half of each tracing express the total area under the profile as small ribosomal aggregates while percentages at the right refer to the proportion of total area under the tracing as larger ribosomal aggregates. Planimetry of four paired samples showed ribosomes from alloxan diabetic rats to have a significantly greater proportion of small aggregates compared to normal controls ($P < 0.02$).

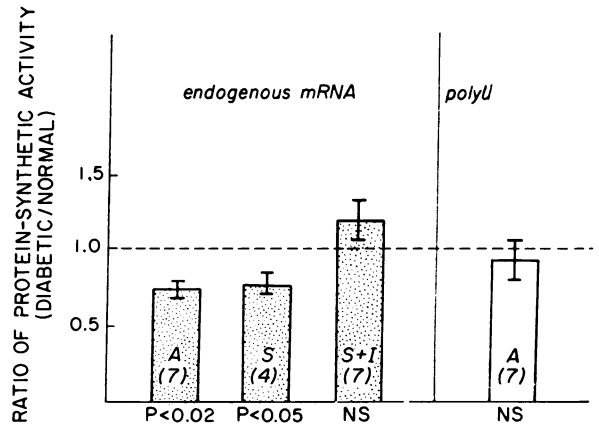

FIGURE 4 Protein-synthetic activity of bound ribosomes from livers of normal and diabetic rats. Results shown are the mean ratios of diabetic/normal paired samples from 500 *g* supernates. The three bars (mean±SEM) on the left are obtained from 30-min incubations in which protein synthesis was directed by endogenous mRNA while the bar (Mean±SEM) on the extreme right is for 120-min incubations directed by polyU. The numbers in parentheses refer to the number of pairs in each experimental group. *A*, alloxan diabetes; *S*, streptozotocin diabetes; $S + I$, streptozotocin diabetes treated with insulin.

and diabetic ribosome preparations was measured by planimetry. The first three peaks of the polysome profile were arbitrarily taken to represent smaller ribosome aggregates (to the left of the dotted lines in Fig. 3). The remainder of the polysome profile was taken to represent larger polysome aggregates (greater than trimers). Area under the "small" and "large" aggregate peaks was separately measured by planimetry and expressed as a percentage of the total area. The data indicate that the effect of acute diabetes on the bound ribosomal population is to produce a decrease in proportion of larger mRNA-ribosomal aggregates, with concomitant increase of mono-, di-, and trisomes. This was true when bound ribosomes were prepared from 500 *g* or 10,000 *g* supernates, and when acute insulin deficiency was produced by streptozotocin or alloxan.

Effect of acute insulin deficiency on protein synthesis by bound hepatic ribosomes. The effect of acute alloxan or streptozotocin diabetes on in vitro protein synthesis by isolated bound polyribosomes is seen in Fig. 4. Results are expressed as the ratio of protein-synthetic activity of diabetic to normal bound polyribosomes, and are based upon comparing normal and diabetic bound polyribosomes prepared and incubated on the same day. The data indicate that in vitro protein synthesis directed by endogenous mRNA is significantly decreased in bound polyribosomes from livers of alloxan and streptozotocin-treated rats. The decrease in protein synthesis was prevented by the concomitant administration of insulin. Fi-

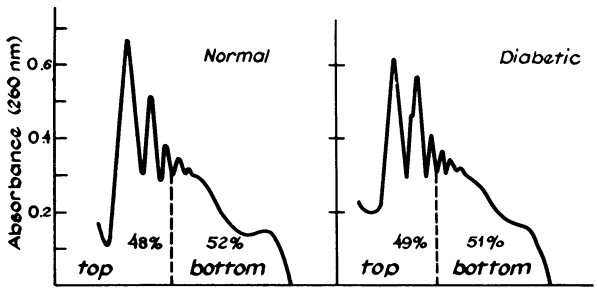

FIGURE 5 Polysome profiles of free ribosomes prepared from 500 *g* supernates from livers of normal and alloxan diabetic rats. Percentage figures under the left half of each tracing express the percentage of total area under the profile of small ribosomal aggregates, while percentages at the right refer to the proportion of total area under the tracing of larger ribosomal aggregates. Planimetry of five paired samples from normal and diabetic rat livers revealed no significant difference.

nally, the right-hand panel in Fig. 4 indicates that the difference in protein-synthetic capacity of control and diabetic bound polyribosomes is abolished when endogenous mRNA is removed and artificial messenger polyU substituted in its place. The results described were all based upon study of bound polyribosomes derived from the 500 *g* supernate. Comparable results were obtained from an equal number of observations using 10,000 *g* supernate from the alloxan-treated animals.

Effect of acute insulin deficiency on free hepatic ribosomes. There was no detectable effect of acute diabetes on the electron microscopic appearance or on the polysome profiles of free ribosomes (Fig. 5). This was true regardless of the manner in which the diabetes was produced, or whether the free ribosomes were prepared from 500 or 10,000 *g* supernates.

However, acute diabetes did have a significant effect on in vitro protein synthesis of isolated free hepatic ribosomes. These results are shown in Fig. 6, and indicate that in vitro protein synthetic activity was increased in free polyribosomes from animals made diabetic with either alloxan or streptozotocin. As before, the change in protein synthesis could be prevented by the simultaneous administration of insulin. Since there was no striking change in the ultrastructure or the polysome profiles of the free ribosome population, the possibility arose that the increase in protein synthesis by the free ribosomal population might be due to a direct effect of acute insulin deficiency to make ribosome more efficient at copying mRNA. Furthermore, this increased efficiency of translation might also be seen when free ribosomes are copying an artificial messenger. This possibility was tested by comparing polyU-stimulated amino acid incorporation of normal and diabetic free ribosomes, and the results appear in the right panel of Fig. 6. The ratio of the protein-synthetic capacity of diabetic/normal free ribosomes remained greater than unity, indicating that diabetic free ribosomes remain more active than control ribosomes even when amino acid incorporation is being directed by artificial mRNA. The results displayed were obtained with use of the 500 *g* supernate, but comparable results were seen when 10,000 *g* supernate served as the source of the free ribosomes.

DISCUSSION

The data presented in this paper suggest that the two ribosomal populations in the liver are not only morphologically distinct, but respond to experimental diabetes mellitus in fundamentally different ways. The effects of acute insulin deficiency on the membrane-bound ribosomes can be summarized as follows. There is marked disaggregation and disruption of the RER, resulting in a marked reduction in the total number of bound ribosomes per volume of cytoplasm.The decrease in the number of bound ribosomes is due to both a loss of RER and to a decrease in the number of bound ribosomes per area of RER membrane. Polysome profiles indicate that the bound ribosomes remaining in the livers of acutely diabetic rats have undergone a shift to smaller-sized ribosomal mRNA aggregates. Furthermore, the protein-synthetic activity of these bound ribosomes is decreased per milligram of ribosomal RNA. Since smaller ribosomal aggregates copy less message and incorporate less amino acid into protein in vitro (15, 20),

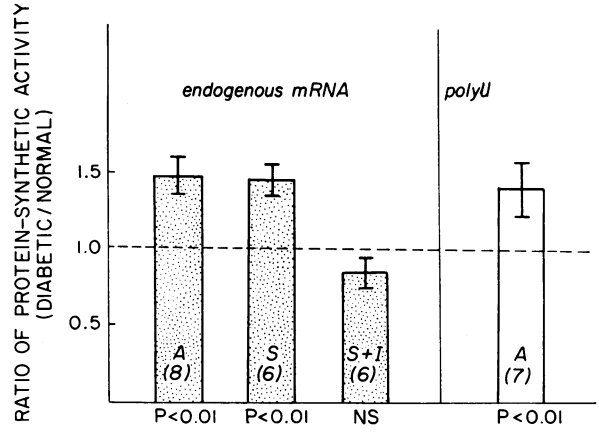

FIGURE 6 Protein synthetic activity of free hepatic ribosomes from liver of normal and diabetic rats. Results show the mean ratios of diabetic/normal paired samples from 500 *g* supernates. The three bars (mean±SEM) on the left are obtained from 30-min incubations in which protein synthesis was directed by endogenous mRNA while the bar (mean±SEM) on the extreme right is for 120-min incubations directed by polyU. The numbers in parentheses refer to the number of pairs in each experimental group. *A*, alloxan diabetes; *S*, streptozotocin diabetes; *S* + *I*, streptozotocin diabetes treated with insulin.

the shift to smaller-sized ribosomal mRNA aggregates provides an explanation for the observed reduction in protein-synthetic activity of the bound ribosomes. Finally, when endogenous mRNA was removed, and protein synthesis directed by artificial messenger, there was no longer any difference in the protein-synthetic activity of bound ribosomes from livers of normal and diabetic rats. These experimental findings are internally consistent and strongly suggest that the decrease in protein-synthetic activity of bound hepatic ribosomes from acutely diabetic rats is secondary to marked disruption of the RER, with production of smaller ribosomal mRNA aggregates which are less active in protein synthesis.

In contrast are the effects of acute insulin deficiency on ribosomes free in the cytosol. The free ribosomes do not appear to be reduced in quantity, they do not seem to be broken down into smaller aggregates, and they are not less active in incorporating amino acid into protein. Indeed, free hepatic ribosomes from diabetic animals are more active than normal in amino acid incorporation, and this is true when protein synthesis was directed by either natural mRNA or polyU. Since the polysome profiles indicate that there is no difference in the degree of aggregation of ribosomes and mRNA, the increased protein synthetic activity of free ribosomes prepared from diabetic liver may not be due to changes in the amount of mRNA. The finding that diabetic free ribosomes are more active than normal in amino acid incorporation directed by artificial mRNA (polyU) raises the possibility that diabetic free ribosomes are intrinsically more efficient in copying messenger. This hypothesis also provides an explanation for the increased amino acid incorporation of diabetic ribosomes directed by endogenous mRNA.

Before dealing with the physiological implications of these studies, a methodological problem inherent in these experiments must be considered. The method of isolating free and bound ribosomes from homogenized liver does not quantitatively recover all ribosomes. While free ribosomes are quantitatively recovered by this method, the recovery of bound ribosomes is reduced (3). Less than quantitative recovery means that the population of ribosomes studied may not be representative of the total population in vivo. For this reason, free and bound ribosomes were isolated from both a 500 *g* supernate and 10,000 *g* supernate. The 500 *g* supernate allows a larger recovery of bound ribosomes, but the preparation is contaminated by mitochondria and lysosomes. A 10,000 *g* supernate contains a smaller fraction of the bound ribosomes, but the sample is much more pure, with no evidence of contaminating mitochondria or lysosomes. The fact that the experimental results are identical with ribosomes isolated from either a 500 *g* or a 10,000 *g* supernate increases the likelihood that the results are applicable to the in vivo population of ribosomes. In addition, the ultrastructural changes observed in hepatocytes obtained from intact animals before the isolation procedure are consistent with the in vitro results. However, considerable caution must be exercised in applying the results of in vitro studies to events in the living cell. The level of protein synthesis in vivo obviously reflects the interplay of many factors; ribosomes, transfer RNA, amino acids, soluble cytosol factors, and membrane phenomena, among others. In view of the pronounced effects of these factors (21, 22) on protein synthesis, it will be necessary to evaluate their changes before the in vivo status of protein synthesis in diabetic liver can be fully understood. On the other hand, these studies do demonstrate that membrane-bound and free hepatic ribosomes respond in opposite directions by different mechanisms to the altered physiologic state of acute insulin deficiency, and it appears that these two ribosomal populations are separately controlled. We feel it is highly likely that these changes represent a direct effect of insulin lack on hepatic ultrastructure. However, it should be noted that although food intake in the two groups was similar, we did not correct for the calorie loss via glycosuria in the diabetic rats and the degree of calorie loss could conceivably effect the results.

Regardless of the specific mechanisms invoked to explain the divergent effects of acute diabetes on bound and free ribosomes, the question as to the potential physiological significance of these disparate effects remains to be considered. It seems reasonable to assume that the overall impact of acute diabetes on hepatic protein synthesis will be related to the manner in which insulin deficiency directly affects bound and free ribosomes, to the different roles fulfilled by the two ribosomal populations under normal conditions, and to the metabolic response of the liver to the acute diabetic syndrome. The major effect of acute diabetes on the bound ribosomal population seems to be directly related to the extensive destruction of the RER. The ensuing decrease in protein synthesis would be expected to primarily involve proteins synthesized for export (5–8), and a loss of this function might not be excessively detrimental to the rat during the short time course of these studies. In contrast, the increase in protein-synthetic capacity of the free ribosomal population is possibly best explained by viewing it as a response to the development of acute insulin deficiency. Specifically, if the free ribosomal population normally synthesizes protein for intracellular use, it is possible that the accelerated gluconeogenesis required for survival (23, 24) would lead

to increased synthetic activity of free ribosomes in order to meet the demand for new intracellular protein, e.g., gluconeogenic enzymes.

The complex nature of the effect of acute insulin deficiency on hepatic protein synthesis should not be too surprising, as it is already apparent that acute diabetes modifies protein synthesis differently in different organs. For example, muscle ribosomes from diabetic rats, which are essentially all free, shown decreased protein-synthetic activity (25). In contrast, kidney ribosomes from acutely diabetic rats, which are also predominantly free in the cytosol, show increased protein-synthetic activity (13). Thus, at this time there does not appear to be a consistent effect of acute diabetes on protein synthesis, nor does there appear to be any unifying hypothesis to explain the divergent effects.

Finally, it must be emphasized that these results were seen after 2–3 days of severe insulin deficiency. They were not dependent upon the mode of producing experimental diabetes, and all the changes could be prevented by administering insulin concomitantly with the diabetogenic agent. Thus, the results seem to be a specific reflection of acute insulin deficiency. However, the degree of insulin deficiency was extremely severe, and these animals barely survived the experimental period. Therefore, quite different results might be seen after the production of more chronic, and less severe, insulin deficiency. This seems to be the case in the studies of Pilkis and Korner (26), in which there was a decrease in protein synthesis by isolated free ribosomes after alloxan diabetes of greater than 1 mo duration. It should be noted, however, that bound ribosomes were not prepared separately in that study. The apparent discrepancy between the effects of acute and chronic insulin deficiency on heaptic protein synthesis requires further elucidation, and we are currently studying this problem.

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