Effect of Insulin Deficiency on Hepatic Ribosomal Aggregation

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SUMMARY

Ribosomes were isolated from liver homogenates of normal and alloxan diabetic rats. Sedimentation of these ribosomes through a continuous sucrose gradient indicated that insulin deficiency was associated with an increase in the proportion of ribosomes existing as the 80 S monomer. In a second series of experiments ribosomes from diabetic animals were also shown to bind more avidly to ^{3}H-polyuridylic acid (polyU). When hepatic ribosomes from normal and diabetic rats were incubated for forty-five minutes at 37° C. in a complete amino acid-incorporating system they underwent essentially total disaggregation into single ribosomes, and the previously noted difference between normal and diabetic ribosome binding to polyU was abolished. The sedimentation characteristics of ribosomes from normal and diabetic rats indicate that insulin deficiency results in an increase in the proportion of ribosomes free of mRNA, and, since they are free of mRNA, a greater than normal proportion of ribosomes from diabetic animals are capable of complexing with polyU. Obviously, producing equal numbers of monosomes by prior incubation in an amino acid-incorporating system would be expected to obliterate the difference in polyU binding between ribosomes from normal and diabetic animals. Thus, both lines of experimental evidence are consistent with the hypothesis that insulin deficiency produces a disaggregation of hepatic polysomes, leading to an increase in the proportion of ribosomes that are not bound to mRNA. DIABETES *21*:84-88, February, 1972.

Previous studies have indicated that the production of experimental diabetes mellitus in the rat leads to a decrease in the protein synthetic capacity of isolated liver ribosomes.[1,2] This effect of insulin deficiency can only be demonstrated when protein synthesis is directed by endogenous mRNA, and differences in activity between hepatic ribosomes from normal and diabetic rats are abolished when polyuridylic acid is used to direct protein synthesis.[2] On the basis of these findings we concluded that the defect in hepatic protein synthesis resulting from insulin deficiency was most likely due to a decrease in the amount of mRNA bound to ribosomes, leading to a decrease in the hepatic polysome population. In the present experiments we have utilized two different experimental approaches to more directly assess the effect of diabetes mellitus on the binding of mRNA to hepatic ribosomes. In the first series of experiments hepatic ribosomes were sedimented in linear sucrose gradients, and polysome profiles were obtained from normal and diabetic rats. In a second series of experiments we compared the ability of hepatic ribosomes from normal and diabetic animals to combine with polyuridylic acid. In both instances the results of these studies provided further support for the hypothesis that insulin deficiency results in a decrease in the proportion of hepatic ribosomes bound to mRNA.

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MATERIALS AND METHODS

A. Animal treatment. Experiments were performed on female Sprague-Dawley rats, weighing between 180 and 200 gm. Experimental diabetes was produced by intravenous injection of alloxan (75 mg./kg. body weight) after an eighteen-hour fast. The animals were killed by decapitation seventy-two hours after receiving alloxan, and only animals with a blood glucose greater than 250 mg./100 ml.* were used. Control animals, injected with saline,

* Dextrostix, Ames Co., Elkhart, Ind.

were kept under identical conditions and killed at the same time.

B. Ribosomal isolation. After decapitation, livers were removed and placed in ice-cold buffer containing 0.35 M sucrose, 0.07 M KCl, 0.004 M $MgCl_2$, 0.05 M Tris-HCl (pH 7.8), and 0.006 M β-mercaptoethanol (Medium A). The following procedures were then performed at 4° C. Livers were homogenized for 30 seconds with a Virtis A Homogenizer at a setting of 50 in 2 vol. of a medium containing 0.25 M sucrose, 0.025 M KCl, 0.005 M $MgCl_2$, and 0.05 M Tris-HCl (pH 7.6).[3] After centrifugation at 600 x g for ten minutes, the supernatant fluid was decanted and centrifuged at 20,000 x g for ten minutes. The supernatant fluid was removed and 0.15 ml. of 10 per cent deoxycholate was added per milliliter supernate. After standing for twenty minutes the preparation was centrifuged in a Spinco preparative ultracentrifuge at 10,000 x g for two hours. The ribosomal pellet was then resuspended in a medium containing 0.02 M Tris-HCl (pH 7.5) and 0.005 M $MgCl_2$, and treated in the following manner to remove RNase. The ribosomal suspension was adjusted to approximately 20 $O.D._{260}$ units/0.1 ml. of solution and 30 $O.D._{260}$ units were layered over a 10 to 30 per cent linear sucrose gradient. The gradients were centrifuged for fifteen hours in an SW 40 rotor at 35,000 rpm. After centrifugation the supernatant fluid was removed, the pellets rinsed slightly with the original medium, resuspended, and centrifuged for ten minutes at 3,000 rpm. The supernatant fluid, as the final source of the ribosomes, was removed, and its absorbance ($O.D._{260}$) measured on a Beckman Model DB spectrophotometer.

C. Determination of polysome profiles. Ribosomes were suspended in 0.01 M $MgCl_2$, 0.08 M KCl, 0.05 M Tris-HCl (pH 7.6) at a final concentration of 10 $O.D._{260}$ units/ml.[4] A 0.4 ml. aliquot of this suspension was layered on 12.0 ml. linear sucrose gradient (10 to 40 per cent) containing the same medium. The preparation of the gradient was done with a sucrose solution which was first heated with 7.2 per cent Norit A for one hour at 80° C. and then passed through a Whatman No. 3 filter in order to remove material absorbing at 260 mμ. The gradients were centrifuged at 35,000 rpm for ninety minutes in an SW 40 rotor of a Spinco Model L ultracentrifuge. The sedimentation of the ribosomes in the gradient was determined by displacing the gradient from the top at a rate of 0.3 ml./min. and analyzing the effluent at 260 mμ with a Beckman Model DB spectrophotometer. The effluent of the gradient was also analyzed at 320 mμ and this ferritin absorbance was used to correct the absorbance determined at 260 mμ.

D. Measurement of ribosomal binding to ^{3}H-polyuridylic acid (polyU). Ribosomes were combined with ^{3}H-polyU* in a buffer solution containing 0.01 M Tris-HCl (pH 7.5), 0.05 M KCl, 0.001 M β-mercaptoethanol, and concentrations of $MgCl_2$ varying between 0.005 and 0.03 M $MgCl_2$. The ratio of ^{3}H-polyU to ribosomes (expressed as $O.D._{260}$ units) was kept at 1:7, and 0.5 ml. of the above was applied to Bio Gel columns prepared in the following manner. Bio Gel (Bio Rad Labs), with a porosity of P-30, was allowed to swell overnight at 4° C. in the buffer solution described above. The columns (1.0 x 5.5 cm.) were prepared the next morning and rinsed with the buffer solution. No column was ever used for more than two experiments in order to avoid aging of the polyacrylamide. The ^{3}H-polyU ribosome complexes were eluted from the column with 10 ml. of the same buffer, and aliquots taken for determination of absorbance ($O.D._{260}$) and radioactivity (Packard Liquid Scintillation Counter).

RESULTS

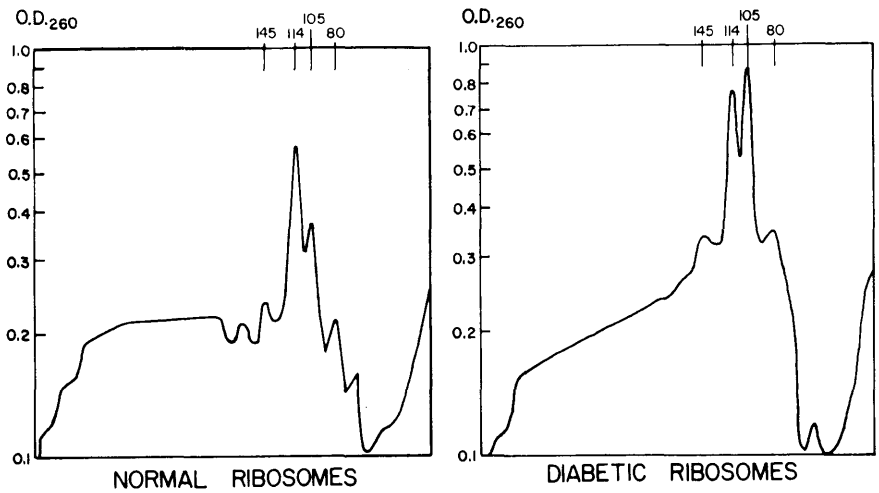
A. Polysome profiles. Typical results obtained when ribosomes from normal and diabetic livers are sedimented through a continuous sucrose gradient are illustrated in figure 1. These patterns are strikingly similar to those previously reported for normal and diabetic muscle ribosomes by Stirewalt et al.,[4] and we have employed the sediment coefficients they obtained using the analytical centrifuge to describe the results. These polysome profiles indicate that experimental diabetes is associated with a relative increase in the proportion of hepatic ribosomes existing as the monomer (80 S), and a corresponding decrease in ribosomal aggregates with sedimentation coefficients of 145 or greater (polyribosomes). In an effort to provide a semi-quantitative estimate of the effect of experimental diabetes on hepatic ribosomal aggregation we have used the technic of Palmiter, Christensen, and Schimke,[5] in which the areas under the various ribosomal peaks were replotted on linear graph paper, cut out, and weighed. We compared hepatic ribosomal preparation from normal and diabetic animals in this manner on five occasions, and found that the average proportion of ribosomes existing as monosomes increased from 9 to 21 per cent with the advent of insulin deficiency.

B. Binding of ribosomes to ^{3}H-polyU. If insulin deficiency results in a decrease in the relative proportion of hepatic ribosomes bound to endogenous mRNA, a greater proportion of ribosomes should then be available

* Schwarz Bioresearch, 7.76 mc/mM.

FIGURE 1

Polysome profiles (continuous sucrose gradient) obtained from livers of normal and alloxan diabetic rats. The numbers at the top of the figure represent an effort to relate the sedimentation of liver ribosomes on linear sucrose gradients to sedimentation coefficients calculated from schlieren patterns obtained in the analytical centrifuge, and are adapted from the data of Stirewalt et al.[4] Stirewalt et al. indicated that the 80 S particle is most likely the monomer and that aggregates having sedimentation coefficients greater than 145 S are polyribosomes. Using these criteria it is apparent that the effect of alloxan diabetes on the ribosome population of the liver is to decrease the relative proportion of polysomes to monosomes.



to bind to a synthetic messenger. In order to pursue this line of reasoning we have taken advantage of the observations of Logan and Singer,[6] who indicated that free polyU will not pass through a polyacrylamide column

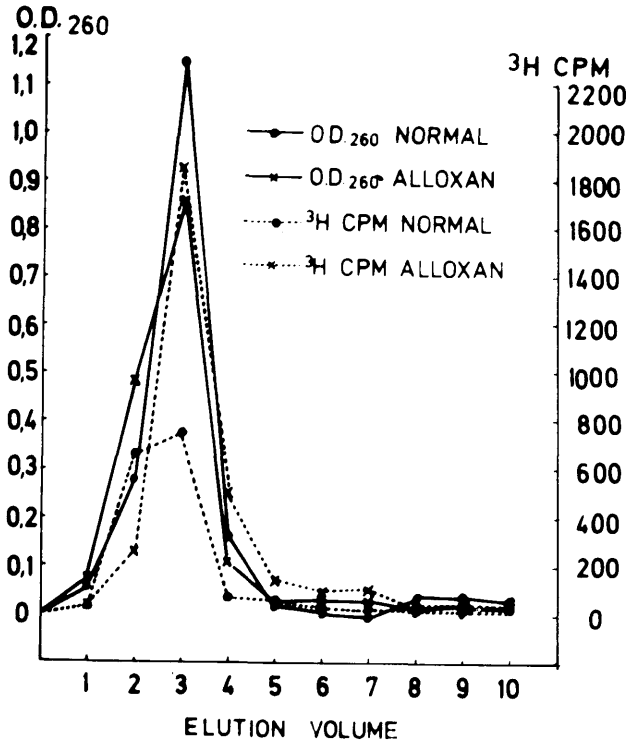


FIG. 2. The effect of insulin deficiency on the binding of ribosomes to ^{3}H-polyU. Isolated hepatic ribosomes from normal and diabetic animals were combined with ^{3}H-polyU and applied to acrylamide columns. These elution patterns indicate that ribosomes from insulin-deficient animals bound significantly greater amounts of ^{3}H-polyU.

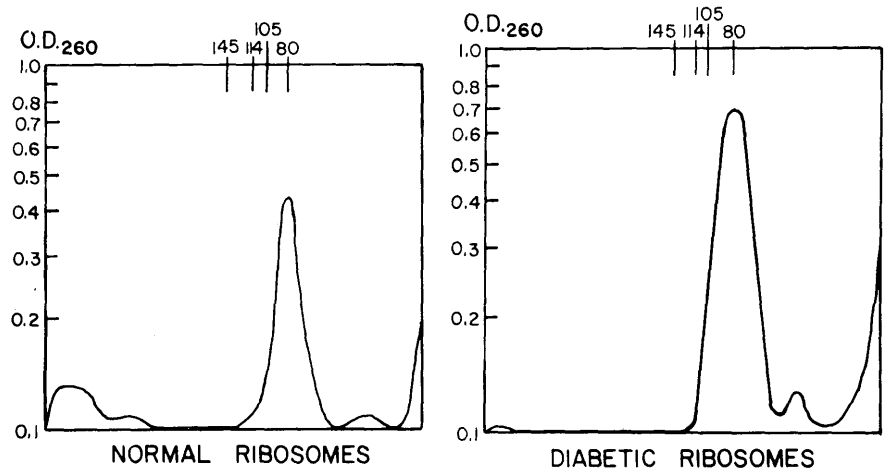
whereas the ribosome-polyU complex can be eluted. When hepatic ribosomes from normal and diabetic animals are combined with ^{3}H-polyU and applied to an acrylamide column the results are as seen in figure 2. Significantly more radioactivity was eluted from the column when ribosomes from diabetic animals were applied, indicating that diabetic ribosomes have a greater avidity for polyU. These results can be explained on the basis that a greater proportion of diabetic ribosomes are not bound to endogenous mRNA, and therefore free to bind to ^{3}H-polyU. Alternatively, these results may simply reflect an increase in the intrinsic ability of diabetic ribosomes to bind to ^{3}H-polyU. In order to differentiate between these two possibilities hepatic ribosomes from normal and diabetic rats were incubated for forty-five minutes at 37° C. in a complete amino acid-incorporating system[2] prior to being sedimented through a continuous sucrose gradient. Previous studies[7,8] had indicated that such treatment results in essentially total disaggregation of polysomes into single ribosomes, and this is confirmed by our results (figure 3). The results of adding preincubated ribosomes from normal and diabetic rats to ^{3}H-polyU are seen in figure 4, and indicate that the previously observed increase (figure 2) in binding of ^{3}H-polyU to diabetic ribosomes has been obliterated. Thus, there appears to be no intrinsic difference in the avidity of diabetic ribosomes for polyU, and the increase in binding of diabetic ribosomes to polyU seen in figure 2 is most likely due to the fact that a greater proportion of diabetic ribosomes are free of mRNA and more available to form complexes with synthetic messenger.

DISCUSSION

Previous studies have indicated that experimental dia-

FIGURE 3

The effect of prior incubation in a complete amino acid-incorporating system on the sedimentation of hepatic ribosomes through a continuous sucrose gradient. Ribosomes from normal and diabetic animals were incubated as previously described,[2] and their subsequent sedimentation in sucrose gradients clearly indicates that the entire ribosomal population has been converted to the 80 S monomer. As before, the numbers at the top of the figure represent an effort to relate the sedimentation of liver ribosomes on linear sucrose gradients to sedimentation coefficients calculated from schlieren patterns obtained in the analytical centrifuge, and are adapted from the data of Stirewalt et al.[4]



betes mellitus leads to a decrease in hepatic protein synthesis,[1,2] and we have suggested that this change was due to a decrease in the amount of mRNA bound to ribosomes.[2] The current studies offer additional support, based upon two lines of evidence, for the original hypothesis. The polysome profiles provide direct evidence that acute insulin deficiency leads to a disaggregation of hepatic polysomes, a result similar to that previously described in rats with more chronic alloxan diabetes.[9] Further evidence that diabetes results in a decrease in the proportion of hepatic ribosomes bound to mRNA is derived from the studies of ribosomal binding to polyU. Hepatic ribsosomes from diabetic animals bound more polyU than did ribosomes from control rats, and this difference was abolished when ribosomes were preincubated in an amino acid-incorporating system, and converted from polysomes to monosomes. The most direct explanation of these findings is that a greater proportion of diabetic ribosomes are free of mRNA, and available to combine in greater quantities with polyU. Thus, both lines of new experimental evidence lend support to the hypothesis that diabetes leads to a decrease in the proportion of hepatic ribosomes bound to mRNA.

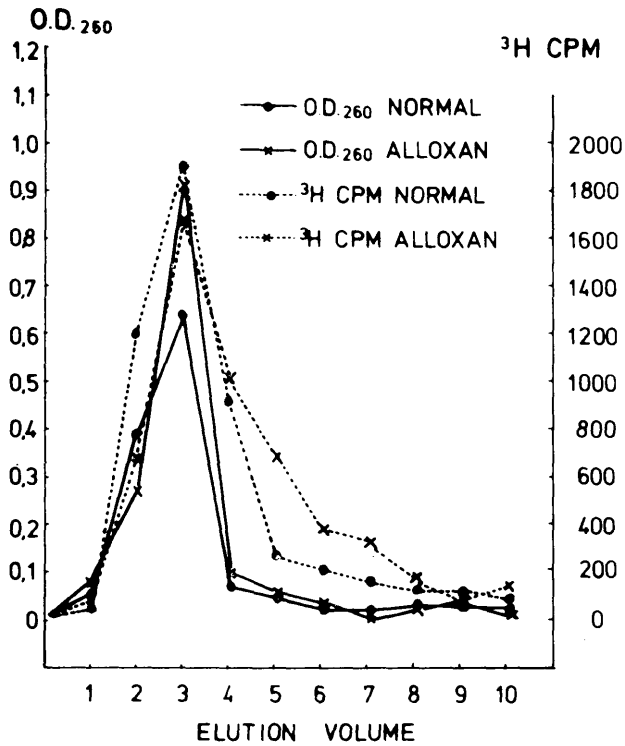


FIG. 4. The effect of insulin deficiency on the binding of preincubated ribosomes to ^{3}H-polyU. Isolated hepatic ribosomes from normal and diabetic rats were incubated in an amino acid-incorporating system,[2] combined with ^{3}H-polyU, and applied to acrylamide columns. These elution patterns indicate that prior incubation obliterates the previously demonstrated increase (figure 2) in binding of ^{3}H-polyU to diabetic ribosomes.

The simplest explanation for the associated changes in hepatic ribosomal aggregation and function that occur in diabetes would be that insulin deficiency leads to a decrease in the amount of mRNA. Thus, the decrease in protein synthetic capacity of diabetic ribosomes, and associated decrease in ribosomal aggregation, would be assumed to be due to a defect at the level of transcription. However, recent recognition of several factors which function in the binding of ribosomes to mRNA[10] makes this simple explanation no longer tenable, and a decrease in the proportion of ribosomes bound to mRNA can also result from a defect in the normal functioning of any one of several binding factors in the presence of normal amounts of mRNA.

Finally, in all these studies ribosomes have been isolated from liver homogenates, and our attempts to formu-

late a molecular mechanism by which insulin deficiency affects hepatic protein synthesis has neglected to consider the additional variable of ribosomal compartmentalization. For example, does diabetes result in a general disaggregation of hepatic polysomes, or does it only affect ribosomes in certain anatomical areas? In an effort to answer these questions we have begun to carry on simultaneous studies of the effect of experimental diabetes on hepatic ultrastructure and protein synthesis. Preliminary results have indicated that insulin deficiency results in a marked decrease in the endoplasmic reticulum, with an associated disappearance of membrane-bound polysomes. These findings are also compatible with the thesis that insulin deficiency results in a decrease in the proportion of hepatic polysomes, and we are currently engaged in an effort to confirm these preliminary findings.

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