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Correlation of albumin production rates and albumin mRNA levels in livers of normal, diabetic, and insulin-treated diabetic rats

(protein synthesis/liver perfusion/cell-free translation/cDNA hybridization)

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ABSTRACT **We have studied the effects of alloxan-induced diabetes and subsequent insulin replacement on albumin and total hepatic protein synthesis. Diabetes resulted in a reduction to approximately 20% of normal in albumin synthesis relative to the rate of total protein synthesis *in vivo* and a reduction to 10% in the absolute rate of albumin secretion by perfused livers. In contrast, the synthesis of total secretory protein and retained hepatic protein was affected to a lesser extent by diabetes. Treatment of diabetic rats with insulin restored rates of albumin and total hepatic protein synthesis to normal levels. The molecular basis of these alterations in albumin synthesis was investigated by examining albumin mRNA levels in livers of normal, diabetic, and insulin-treated diabetic animals. The level of albumin mRNA, whether assayed by cell-free translation or by hybridization to a specific complementary DNA probe, was markedly decreased in livers of diabetic animals and was restored to normal by insulin treatment. These changes occurred in parallel with changes in the rates of albumin secretion observed in perfused liver, suggesting that albumin mRNA content is the primary factor responsible for altering rates of albumin synthesis under these conditions.**

A role for insulin in regulating protein synthesis in liver has not been clearly established. In rats, experimentally induced diabetes caused no change in liver protein synthesis rates *in vivo*, while synthesis rates in skeletal muscle and heart were markedly impaired (1). These findings are supported by studies using perfused tissues from normal rats in which measurements of protein synthesis showed that addition of insulin had no effect on the synthesis of hepatic proteins (2) but stimulated the synthesis of skeletal muscle (3) and heart (4) proteins. While some workers have reported that insulin stimulates liver protein synthesis (5), rates of protein synthesis were not determined rigorously in these experiments.

Studies of protein synthesis in liver are complicated by the fact that this tissue synthesizes both proteins for export and intracellular proteins. The studies mentioned above measured primarily the synthesis of intracellular proteins. Other studies with perfused livers (6) or isolated hepatocytes (7) indicated an impaired secretion of proteins in diabetic rats. Such a defect may relate to the marked disruption and disorganization of the rough endoplasmic reticulum observed in liver of diabetic rats (8–10), as export proteins are synthesized almost exclusively on the rough endoplasmic reticulum (11).

In the studies presented here, we have investigated the effect of alloxan-induced diabetes and subsequent insulin replacement on albumin and total protein production *in vivo* and in perfused livers, and have correlated the observed changes in albumin synthesis with changes in albumin mRNA levels. Diabetes resulted in a marked decrease in albumin synthesis and secretion *in vivo* and in perfused livers, with a corresponding decrease in the albumin mRNA level. Treatment of diabetic rats with insulin restored both the albumin synthesis rate and the albumin mRNA level to normal. A preliminary report of these observations has been presented (12).

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MATERIALS AND METHODS

Animals. Male Sprague–Dawley rats were purchased from Charles River Breeding Laboratories. Animals were allowed free access to food (Purina lab chow) and water at all times. At the beginning of an experiment, animals weighed 100–120 g.

Alloxan Injection and Insulin Treatment. Diabetes was induced after an overnight fast by the intravenous injection of 60 mg/kg of body weight of alloxan (Eastman) dissolved in 0.15 M NaCl. Control animals received injections of 0.15 M NaCl only. Untreated diabetic animals typically had blood glucose values in excess of 500 mg/dl. Insulin treatment of short duration (12 or 24 hr) consisted of a single subcutaneous injection of 3 units (U) of Lente insulin (Squibb). Longer term treatment consisted of a daily subcutaneous injection of 1.6 U of protamine/zinc insulin (Lilly) per 100 g of body weight. Insulin treatment of either type significantly reduced blood glucose values and reduced glucosuria. In addition, diabetic animals treated with multiple doses of protamine/zinc insulin grew at a rate equivalent to normal rats, in contrast to untreated diabetic animals, which generally lost weight. There was no difference in liver size relative to body weight between normal and untreated diabetic rats (4.6 ± 0.1 vs. 4.7 ± 0.1 g of liver per 100 g of body weight, mean ± SEM), but in insulin-treated animals there was a marked enlargement of the liver relative to body size (6.8 ± 0.2 g of liver per 100 g of body weight). This increase in size has been noted previously (13, 14) and is due to a transitory accumulation of glycogen and fat in the liver. Because of these differences in liver weights between the different groups, rates of protein synthesis (see below) are expressed on the basis of the body weight of the donor animals.

Determination of the Relative Rate of Albumin Synthesis. The relative amount of albumin synthesis *in vivo* was determined as described previously (15, 16). This determination involved the intraperitoneal injection of 500 μCi/100 g of body weight of [^{3}H]leucine (New England Nuclear, Boston, MA, 60 Ci/mmol) (1 Ci = 3.7×10^{10} becquerels) into rats, followed 10 min later by removal and homogenization of the liver, detergent treatment of the homogenate, and brief centrifugation to remove tissue debris. Radioactivity present in total protein was determined by trichloroacetic acid precipitation on filter paper discs (17), and that present in albumin was determined by immunoprecipitation with a specific antibody (18). Immunoprecipitation reactions were performed with several different

Abbreviations: U, units; R_0t, product of RNA concentration in moles of nucleotides per liter and time in seconds.

ratios of liver homogenate to antialbumin to ensure that precipitation of albumin was quantitative. Correction for nonspecific background absorption was determined by ovalbumin/antiovalbumin precipitation as described previously (16, 18). Radioactivity present in albumin is expressed as a percentage of that present in trichloroacetic acid-precipitable protein.

Liver Perfusion. Livers were perfused *in situ* as described previously (16). The basic perfusion medium consisted of Krebs–Henseleit bicarbonate buffer (pH 7.4) containing 10 mM glucose, 3% bovine serum albumin (Miles, fraction V), and sufficient washed bovine erythrocytes to give a hematocrit of 30%. Amino acids were added at 5 times the concentrations found in normal rat plasma (19), with the exception of leucine, which was 5 mM in all cases. The advantages of using high concentrations of leucine in determining rates of liver protein synthesis were discussed previously (16).

The liver was first perfused for an initial period of 30 min before the addition of radioactive substrate. Then 250 μCi of [^{3}H]leucine was added to the 100 ml of recirculating perfusate. Samples of perfusate were collected during the course of perfusion and centrifuged to remove erythrocytes, and the supernatant fluid was analyzed for radioactivity present in albumin and in total secreted protein as described above. At the end of perfusion, livers were rapidly removed from the carcass, blotted, weighed, and frozen in Wollenberger clamps cooled to the temperature of liquid nitrogen. Samples of frozen liver tissue were analyzed for radioactivity in retained protein‡ by trichloroacetic acid precipitation.

Isolation of Liver Poly(A)-Containing RNA. Total liver poly(A)-containing RNA was isolated from unfractionated liver homogenates by phenol/chloroform extraction (15) and subsequent poly(U)-Sepharose affinity chromatography (20), as described previously. Recovery of poly(A)-containing RNA was essentially the same from each of the treatment groups, and accounted for approximately 1% of the total liver RNA. A value of $A_{1\,cm}^{50\,\mu g/ml} = 1$ at 260 nm was employed to calculate concentration for all RNA samples.

Translational Assay of Albumin mRNA Activity. Total poly(A)-containing RNA was examined for albumin mRNA translational activity in a cell-free protein synthesizing system utilizing mRNA-dependent rabbit reticulocyte lysates prepared according to Pelham and Jackson (21). The translational assay was performed essentially as described for an untreated lysate system (18). Various amounts of unfractionated poly(A)-containing RNA (1–6 μg/ml) were incubated for 30 min in the lysate reaction mixture containing 200 μCi of [^{3}H]leucine (New England Nuclear) per ml; the radioactivity incorporated into albumin and total protein was assayed as described above (18).

Hybridization Assay of Albumin mRNA. Specific ^{3}H-labeled complementary DNA (cDNA) to rat albumin mRNA was prepared and characterized as previously described (22), except that [^{3}H]dCTP was the only radioactive precursor employed. Albumin mRNA levels were assayed by RNA-excess hybridization, with hybrids determined by S_1 nuclease resistance, according to Tse *et al.* (23).

RESULTS

Incorporation of [^{3}H]leucine into albumin and total protein after a 10-min pulse *in vivo* was determined in normal, diabetic, and insulin-treated diabetic animals (Fig. 1). For this time interval, radioactivity present in albumin can be expressed as a percentage of radioactivity present in total protein because newly synthesized albumin molecules are not secreted in less than about 15 min (24), and the leucine content of albumin is approximately the same as that of total liver protein (25, 26). Albumin synthesis accounted for about 12% of total liver protein synthesis in normal control animals. After alloxan injection, there was a marked, progressive decrease in the relative rate of albumin synthesis, reaching a value of 2–3% of total protein synthesis by 3 days (approximately 20% of normal) and thereafter remaining constant. Treatment of 72-hr diabetic rats 3

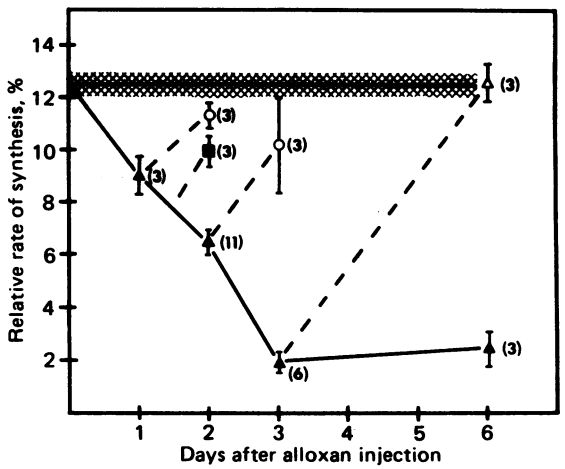

FIG. 1. Relative rate of albumin synthesis in normal, diabetic, and insulin-treated diabetic rats. Radioactivity in albumin and in total protein was measured after a 10-min pulse *in vivo*. Radioactivity in albumin is expressed as a percentage of that in total protein. The horizontal shaded line represents the mean ± 1 SEM of values from 18 normal control animals; ▲, untreated diabetic animals; ○, diabetic animals injected with 3 U of Lente insulin 24 hr before sacrifice; ■, diabetic animals injected with 3 U of Lente insulin 12 hr before sacrifice; △, diabetic animals injected with 1.6 U of protamine/zinc insulin per 100 g of body weight on days 3, 4, and 5 after alloxan injection. Numbers in parentheses are the number of animals per group, and vertical bars represent 2 SEM.

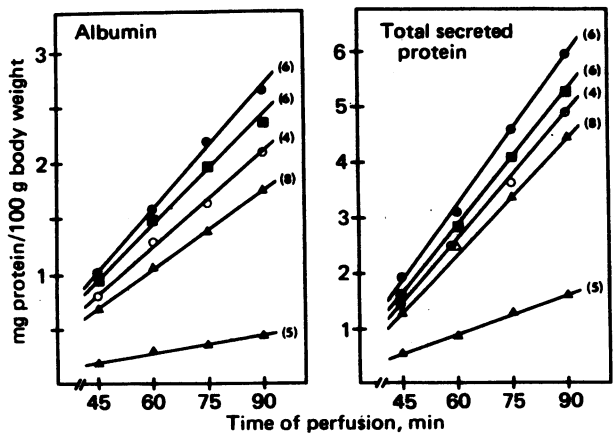

FIG. 2. Secretion of albumin and total protein by livers perfused *in situ*. At zero time, [^{3}H]leucine was added to a final concentration of 2.5 μCi/ml. At the times indicated, samples of perfusate were collected for determination of radioactivity present in albumin and in total protein and for subsequent quantitation of the rates of protein synthesis. ●, Livers from normal rats; △, livers from untreated diabetic rats 48 hr after alloxan; ▲, livers from untreated diabetic rats 72 hr after alloxan; ■, livers from diabetic rats 48 hr after alloxan, treated with 3 U of Lente insulin 24 hr before perfusion; ○, livers from diabetic rats 72 hr after alloxan, treated with insulin 24 hr before perfusion.

‡ "Retained protein" is used to designate nonexport or cellular proteins synthesized and retained by the liver, in contrast to secretory proteins, which are not retained by the liver.

Table 1. Albumin and total protein secretion by livers of normal, diabetic, and insulin-treated diabetic rats

Liver donor*	Albumin mg/100 g per hr†	Albumin % of normal	Total secreted protein mg/100 g per hr†	Total secreted protein % of normal
Normal (6)	2.14 ± 0.1	100	5.45 ± 0.4	100
48-hr diabetic (8)	1.33 ± 0.1‡	62	4.09 ± 0.3‡	75
48-hr diabetic + insulin§ (6)	2.00 ± 0.2¶	93	4.79 ± 0.3	88
72-hr diabetic (5)	0.18 ± 0.02‡	8	1.35 ± 0.07‡	25
72-hr diabetic + insulin‖ (4)	1.70 ± 0.07‡¶	79	4.61 ± 0.1¶	84

* Numbers in parentheses are the number of livers perfused.
† Rates of secretion are per 100 g of body weight, mean ± SEM.
‡ Significantly different from normal, $P < 0.01$.
§ Livers from animals perfused 48 hr after alloxan injection. Animals were treated with 3 U of Lente insulin 24 hr before perfusion.
¶ Significantly different from corresponding diabetic, $P < 0.01$.
‖ Livers from animals perfused 72 hr after alloxan injection. Animals were treated with 3 U of Lente insulin 24 hr before perfusion.

days after alloxan injection with protamine/zinc insulin for 3 consecutive days completely restored the relative rate of synthesis to normal (Fig. 1). Treatment of diabetic rats with a single injection of Lente insulin 12 or 24 hr before sacrifice (24, 36, or 48 hr after alloxan) prevented any further decrease in the relative amount of albumin synthesis and restored the values to near normal.

Albumin and total protein secretion rates were determined in liver perfusion experiments, as were the rates of synthesis of retained liver proteins. Secretion of both albumin and total protein remained linear for 90 min of perfusion (Fig. 2). Livers of rats treated with alloxan 48 and 72 hr prior to perfusion secreted albumin at 62% and 8% of the rate of control livers, respectively (Table 1). Total secretory protein production was also reduced, but to a somewhat lesser extent, being 75% and 25% of normal at 48 and 72 hr after alloxan, respectively. Similar changes have also been observed with isolated hepatocytes from normal and diabetic animals (unpublished observations). The synthesis of retained liver proteins was relatively less affected by the diabetic state of the animal (Fig. 3). Perfused livers from 48- and 72-hr diabetic animals synthesized retained proteins at 85% and 60% of the rate of livers from control rats, respectively. Thus it would appear that the production of secretory proteins, particularly albumin, is affected to the greatest extent by the insulin status of the animal.

Calculations based on the data shown in Fig. 2 and Table 1 show that albumin accounts for about 40% of liver protein secretion in normal rats and decreases to as low as 13% in livers of 72-hr diabetic animals (Fig. 4). Treatment of diabetic animals with a single dose of Lente insulin 24 hr prior to perfusion markedly improved liver protein production whether measured 48 or 72 hr after alloxan injection. The secretion of albumin and total protein (Fig. 2 and Table 1), as well as the synthesis of retained proteins (Fig. 3), was restored to normal or near-normal levels by insulin treatment.

To investigate the molecular basis by which diabetes reduced hepatic albumin production, albumin mRNA levels were determined in livers of normal, diabetic, and insulin-treated di-

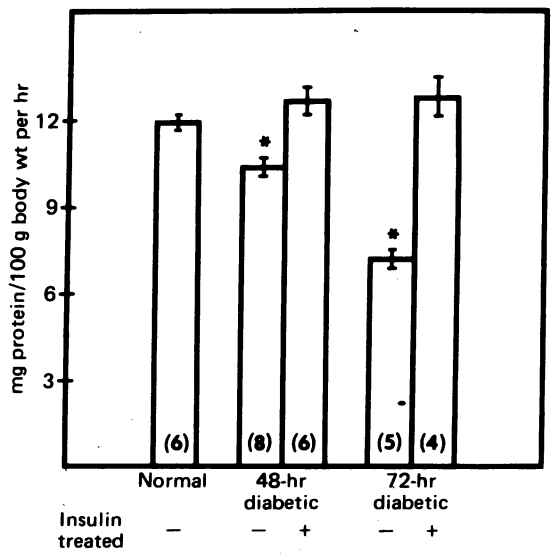

FIG. 3. Synthesis of total retained protein by perfused livers. After perfusion, livers were removed, weighed, and frozen for analysis by trichloroacetic acid precipitation of radioactivity present in nonexport proteins. No correction was made for radioactivity present in secretory proteins within the liver at the time the liver sample was taken because this would amount to less than 10% of the total labeled proteins. Insulin-treated animals (+) were given a single subcutaneous injection of 3 U of Lente insulin 24 hr before perfusion. The ordinate axis shows the rate of retained protein synthesis per 100 g of body weight of donor animals.
*Significant difference ($P < 0.05$) from normal.

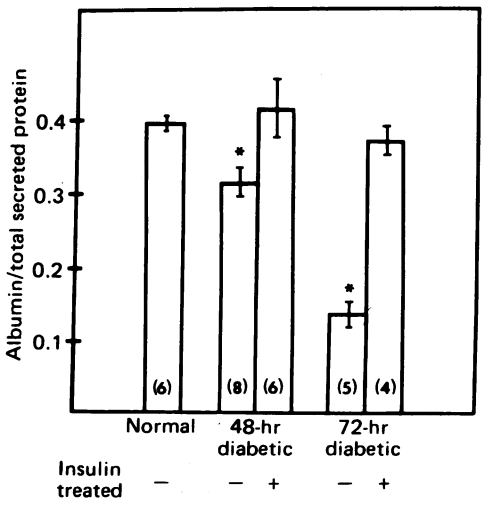

FIG. 4. Albumin secretion as a fraction of total protein secretion by perfused livers. The ordinate axis shows values calculated from data presented in Table 1.
*Significant difference ($P < 0.01$) from normal.

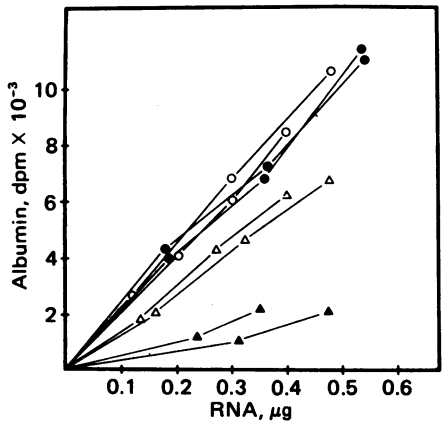

FIG. 5. Translation assays of albumin mRNA activity. Total liver poly(A)- containing RNA was assayed for its albumin mRNA translational activity by using the mRNA-dependent reticulocyte lysate system. The radioactivity present in albumin is expressed relative to the amount of total poly(A)-containing mRNA added. Counting efficiency was 40%. ●, RNA derived from normal rat liver; △, RNA from livers of rats 48 hr after alloxan injection; ▲, RNA from livers of rats 72 hr after alloxan injection; ○, RNA from livers of rats 72 hr after alloxan injection, treated with 3 U of Lente insulin 24 hr before sacrifice.

abetic animals. Total liver RNA was isolated by phenol/chloroform extraction, and the poly(A)-containing RNA was separated by affinity chromatography on poly(U)-Sepharose. The RNA preparations were assayed for albumin mRNA activity and content by translation and hybridization assays. Data from the translation assays are shown in Fig. 5. In the mRNA-dependent reticulocyte lysate system utilized, albumin synthesis (Fig. 5) and total protein synthesis (data not shown) were linear with respect to the amount of mRNA added over the range shown, and all RNA samples showed equivalent total translational capacities. There was a progressive decrease in the amount of translatable albumin mRNA relative to total mRNA

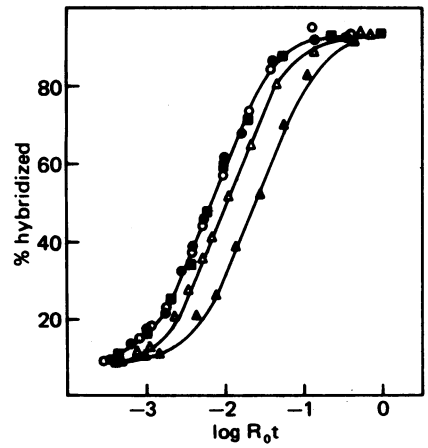

FIG. 6. Hybridization assay of total liver poly(A)-containing RNA with albumin cDNA. Total poly(A)-containing RNA was hybridized up to 2500-fold weight excess to 100 pg of albumin cDNA. Hybrid formation was assayed by resistance to S_1 nuclease. R_0t is the product of RNA concentration in moles of nucleotides per liter and the time in seconds. ●, RNA derived from normal rat liver; △, RNA from livers of rats 48 hr after alloxan injection; ▲, RNA from livers of rats 72 hr after alloxan injection; ○, RNA from livers of rats 72 hr after alloxan injection, treated with 3 U of Lente insulin 24 hr before sacrifice; ■, RNA from livers of rats 6 days after alloxan injection, treated with 1.6 U of protamine/zinc insulin per 100 g of body weight on days 3, 4, and 5 after alloxan injection.

Table 2. Quantitation of albumin mRNA in rat liver RNA by cDNA hybridization

RNA sample	$R_0t_{1/2}$, mol·s·liter^{-1}	Albumin mRNA, % of total*	% of normal
Pure albumin mRNA	5.34×10^{-4}	—	—
Total poly(A)-containing RNA			
Normal	5.62×10^{-3}	9.5	100
48-hr diabetic	8.91×10^{-3}	6.0	63
72-hr diabetic	2.00×10^{-2}	2.7	28
72-hr diabetic + insulin†	5.62×10^{-3}	9.5	100
6-day diabetic + insulin‡	5.62×10^{-3}	9.5	100

Hybridization reactions were performed on samples of RNA pooled from three or four rats, and each experiment was performed in duplicate or triplicate. Mean values are presented.

* Determination was made by comparing the $R_0t_{1/2}$ values of total poly(A)-containing RNA samples with the $R_0t_{1/2}$ value of pure albumin mRNA hybridized to an albumin cDNA of about 470 nucleotides in length.

† RNA was extracted from livers of animals 72 hr after alloxan injection. Animals were treated with 3 U of Lente insulin 24 hr before sacrifice.

‡ RNA was extracted from livers of animals 6 days after alloxan injection. Animals were treated with 1.6 U of protamine/zinc insulin per 100 g of body weight on days 3, 4, and 5.

in livers of animals after alloxan injection, such that by 48 hr albumin mRNA activity was 70% of normal and by 72 hr it was reduced to 30% of normal. Treatment of 48-hr diabetic animals with 3 U of Lente insulin 24 hr prior to sacrifice prevented the further drop in translatable albumin mRNA that would have occurred between 48 and 72 hr in the absence of insulin, and the treatment restored albumin mRNA activity to normal.

Hybridization assays were also performed on total liver poly(A)-containing RNA preparations from livers of normal, diabetic, and insulin-treated diabetic animals. The results of these assays are presented in Fig. 6 and Table 2. Hybridization reactions were observed to be complete over a range of 2 log R_0t units, and they went to approximately 95% completion. By comparing rates of hybridization of albumin cDNA with pure albumin mRNA and with total liver poly(A)-containing RNA, an estimate was made of the relative albumin mRNA content of each of the poly(A)-containing RNA preparations. In livers of normal animals, albumin mRNA accounted for 9.5% of total mRNA. After alloxan treatment, the amount of albumin mRNA relative to total mRNA was significantly reduced. In 48- and 72-hr diabetic animals, albumin mRNA accounted for only 6.0% and 2.7% of the total mRNA, respectively; this represents a reduction to 63% and 28% of normal (Table 2). Treatment of diabetic rats with insulin, using either of the two treatment regimens indicated in Table 2, completely restored albumin mRNA levels to normal, as indicated by the nearly identical hybridization curves (Fig. 6).

DISCUSSION

This study demonstrates a marked effect of diabetes on hepatic albumin production, and correlates the reduced rates of albumin synthesis in livers of diabetic animals with a reduction in albumin mRNA levels. The effect of diabetes appeared to be especially marked for albumin, because the production of total secreted protein and total retained protein was relatively less affected by the diabetic state of the animal. Insulin treatment of diabetic animals restored both albumin production and albumin mRNA to normal levels. The effect of insulin in restoring hepatic protein synthesis to normal levels suggests that

the defect in the protein synthetic process in livers of alloxan diabetic rats was primarily due to the insulin-deficient state of the animals, rather than to a direct cytotoxic effect of alloxan on the liver.

In livers of normal rats, albumin synthesis accounted for about 12% of total liver protein synthesis, whether measured *in vivo* (Fig. 1) or in perfused livers (Figs. 2 and 3, Table 1). This value agrees with previous measurements made *in vivo* (15, 16, 25) and in perfused livers (16). By 72 hr after alloxan injection, the relative level of albumin synthesis decreased to a minimum value of about 3% of total protein synthesis (Fig. 1). This value is considerably lower than that observed in hypophysectomized (15, 16) or thyroidectomized (27) animals or in rats fed a protein-deficient diet (25, 28). In spite of the marked reduction in the rate of albumin synthesis, the serum albumin level in rats 6 days after the induction of diabetes was unchanged from the normal level (29), suggesting a concomitant decrease in the rate of albumin degradation.

A reduction in the synthesis of albumin (29) and total secreted protein (6) by perfused livers of diabetic rats has previously been reported. However, the molecular mechanism by which protein synthesis is reduced in livers of diabetic animals has not been resolved. Ribosome preparations from diabetic rats showed reduced protein synthetic activity in cell-free translation systems (30–32). Tragl and Reaven (31) suggested that the reduction in hepatic protein synthesis in diabetic animals was due to a reduction in the amount or availability of mRNA, but provided no direct evidence of such a change. In support of such a possibility, Pain (32) reported an effect of diabetes at the level of peptide chain initiation, an observation that might be explained by decreased mRNA levels. The results of our studies reported here indicate that parallel changes occurred in albumin production and albumin mRNA levels. Therefore, it would appear that the reduction in albumin synthesis observed in livers of diabetic rats is mainly the result of a decrease in albumin mRNA levels. However, the possibility exists that a translational defect may also be present or that the decrease in albumin mRNA levels may occur subsequent to a more direct effect of diabetes on albumin synthesis. The relatively good agreement between the results of the translational and hybridization assays indicates that there is an actual decrease in albumin mRNA levels in livers of diabetic animals, rather than conversion of active mRNA to a nontranslatable form. The reduction in albumin mRNA in diabetic rat liver could be the result of either an alteration in transcription of the albumin gene or an alteration in metabolism of the mRNA or mRNA precursor once transcribed.

To our knowledge, a decrease in the level of a specific mRNA in livers of diabetic animals has not been demonstrated previously. Also, diabetes resulted in one of the largest reductions in albumin production that has been reported for a hormone-deficient state. Thus, the diabetic model may prove useful in deciphering the molecular mechanisms by which a specific eukaryotic structural gene is regulated. Furthermore, the marked response of albumin mRNA and protein synthetic levels to diabetes and insulin treatment may prove to be especially useful in examining the molecular mechanisms of insulin action.

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