Effects of Anti-insulin Serum, Insulin, and Glucose on Output of Triglycerides and on Ketogenesis by the Perfused Rat Liver*

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The rate of change of the concentration of various metabolites in blood in vivo and of the metabolism of free fatty acids by the perfused liver in vitro was studied as a function of time after the induction of acute insulin deficiency in rats by administration of guinea pig anti-insulin serum; the rate of reversal of these changes after treatment of the anti-insulin serum diabetic rats with insulin was also investigated. The concentrations of blood glucose and ketone bodies, and plasma free fatty acids increased rapidly after injection of anti-insulin serum, while plasma triglycerides increased more slowly. These alterations were restored rapidly toward normal after treatment of the diabetic animals with insulin. The rate of ketogenesis by the isolated liver, perfused with oleic acid, was accelerated, while secretion of triglyceride was suppressed after injection of anti-insulin serum; maximal changes were observed 10 hours after treatment of animals with anti-insulin serum. The rate of hepatic catabolism of free fatty acids increased rapidly as a function of time after induction of insulin deficiency until a maximum was attained. Treatment of 10-hour anti-insulin serum diabetic rats with insulin for 2 or 4 hours prior to perfusion of the liver failed to alter rates of secretion of triglyceride or ketogenesis, treatment for 10 hours produced partial correction while treatment for 20 hours resulted in complete correction of these alterations. It can be concluded from these results that deficiency of insulin probably induces certain secondary changes in hepatic pathways involved in the metabolism of fatty acid (e.g. alterations in enzyme activity), which require a considerable period of time to be corrected after administration of insulin in vivo. Insulin added to the medium in vitro did not affect secretion of triglyceride or ketogenesis by livers from 10-hour anti-insulin serum diabetic rats, even when the duration of the experiment was increased to 8 hours. Insulin added in vitro depressed the rate of ketogenesis by livers from rats pretreated with anti-insulin serum for 3 hours, but did not significantly stimulate secretion of triglyceride.

Glucose added to the perfusate in vitro depressed ketogenesis and stimulated secretion of triglyceride by livers from 10-hour anti-insulin serum diabetic animals, but the rates of these processes were still quite different from those observed with livers from normal fed rats. By inference, therefore, decreased availability of carbohydrate in liver may be an important factor contributing to the increase in the rate of hepatic catabolism of fatty acid in insulin deficiency. When insulin and glucose were added together, the rate of ketogenesis was depressed below that observed in glucose alone, but, paradoxically, output of triglyceride was depressed simultaneously. It is concluded that deficiency of insulin per se and decreased concentration of carbohydrate in the liver contribute partially to the alterations in the metabolism of triglycerides and ketogenesis by the liver in diabetes.

It is known that hepatic metabolism of triglyceride and ketogenesis are altered in the diabetic state. Secretion of triglyceride is depressed (4–7) and ketogenesis is accelerated (3, 4, 6–8) in perfused livers obtained from rats made diabetic by pretreatment with alloxan. Increased rates of ketogenesis have also been observed in perfused livers from pancreatectomized rats (9, 10). Analogous changes in these metabolic pathways have been observed in perfused livers from fasted rats (11–14). Clearly, hepatic catabolism of free fatty acids is accelerated in the fasting or diabetic state. These findings have been supported generally by studies with diabetic animals in

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Hepatic Lipid Metabolism in Experimental Diabetes

Vivo and with juvenile diabetic humans (15, 16). The alterations in hepatic lipid metabolism induced by insulin deficiency and the mechanisms involved in their production, however, need yet to be characterized more clearly. The changes in hepatic metabolism of free fatty acids induced by the diabetic condition may be attributable to deficiency of insulin per se, and/or to secondary hormonal and metabolic changes. Ketogenesis by perfused livers from normal fed rats has been reported to be depressed by addition of insulin to the medium in vitro (7, 17, 18). The secretion of triglyceride by perfused livers from fed rats, however, has variously been reported to be increased (17), decreased (19), or unaffected (7) by addition of insulin in vitro. It was reported, moreover, that insulin added in vitro did not influence ketogenesis in perfused livers from fasted (8, 13) or pancreaticectomized rats (9, 10), or ketogenesis and secretion of triglyceride by perfused livers from alloxan diabetic rats (7).

In an attempt to answer certain of these questions about the effects of insulin, we investigated hepatic metabolism of free fatty acids in the rat made diabetic by treatment with guinea pig anti-insulin serum rather than with alloxan or pancreaticectomy. Conventionally, 48 hours have been allowed to elapse after injection of alloxan (20), and 17 hours after pancreaticectomy (9), to induce a stable acute diabetic state in rats, during which time certain secondary changes may be produced in the liver and other tissues. It may be difficult, therefore, to differentiate with these latter procedures, between primary and secondary changes associated with insulin deficiency. Acute insulin deficiency, however, can be induced almost immediately after injection of anti-insulin serum into rats (21). Thus, alterations in hepatic lipid metabolism induced by insulin deficiency can be examined as a function of time after treatment of rats with anti-insulin serum. Similarly, the rates of reversion of these changes in lipid metabolism by treatment of anti-insulin serum diabetic animals with insulin can also be studied, and might provide some insight into the interrelationships among hypertriglyceridemia, severe ketosis, fatty liver, and hepatic metabolism of free fatty acids in insulin deficiency. Furthermore, it was considered that insulin added in vitro might reverse the increased hepatic catabolism of free fatty acids induced by deficiency of short duration. Accordingly, we investigated (a) the sequential changes induced in the secretion of triglyceride and in ketogenesis by the isolated perfused liver following treatment of rats with anti-insulin serum; (b) the sequential changes following treatment of anti-insulin serum diabetic rats with insulin in vivo (i.e., the onset and reversal of those changes in metabolism associated with insulin deficiency); and (c) the variable capacity of insulin and glucose added in vitro to reverse the actions of anti-insulin serum. Preliminary reports of this work have appeared (1-2).

MATERIALS AND METHODS

Animals-Male Sprague-Dawley rats (Holtzman Co., Madison, Wis.) weighing 200 to 275 g, were used for the experiments. The animals were injected intravenously or intraperitoneally with anti-insulin serum at various times prior to surgical removal of the livers (Table I). Animals within control groups were either untreated or were injected with normal guinea pig serum for 1 or 10 hours prior to hepatectomy and perfusion of the liver. In certain experiments, rats were pretreated with anti-insulin serum for 10 hours and then were treated with insulin in vivo for varying periods of time prior to study (Table I). All animals were allowed laboratory chow and tap water ad libitum throughout the periods of study.

Guinea Pig Anti-Insulin Serum—Male albino guinea pigs (Albino Farms, Red Bank, N. J.), weighing 350 to 500 g, were used for production of anti-insulin serum. The anti-insulin serum was assayed with 125I insulin, according to the procedures for radioimmunoassay described by Wright and associates (22). Potency of the anti-insulin serum is expressed as units of insulin bound/ml of serum. Normal serum was obtained from untreated guinea pigs. All sera were stored in the frozen state at -20°.

Perfusion of Livers—Livers were isolated surgically from rats anesthetized with ethyl ether and were perfused in vitro using methods (23) and procedures (11) described previously. Anti-insulin serum was not used. When required, 2 to 3 ml of blood were withdrawn from the abdominal inferior vena cava during the operative procedure for removal of the liver, and were analyzed for various metabolites. Blood for the perfusate was obtained from the abdominal aorta of large fed male rats, and was defibrinated immediately. The perfusate medium consisted of 70 ml of defibrinated rat blood diluted to 70 ml with Krebs-Henseleit bicarbonate buffer, pH 7.4 (24). The liver was placed in the perfusion chamber and was allowed to equilibrate for 20 min with the recycling perfusate; thereafter, the liver was usually perfused for 4 hours. In certain experiments, the period of perfusion was increased to 8 hours. For the experiments lasting 4 hours, a complex of oleic acid (14.2 μmol/ml) and bovine serum albumin (1.45 μmol/ml) was prepared in 0.15 M NaCl (3), filtered through a Millipore filter (pore size 3 μ), and infused into the medium at the rate of 0.20 ml/min (106 μmol of oleate/hour). For the experiments lasting 8 hours, a complex was prepared similarly, but contained oleic acid in a concentration of 21.2 μmol/ml; it was infused at a rate of 0.13 ml/min (166 μmol of oleate/hour).

When desired, insulin and/or glucose were added to the initial volume of perfusate and to the oleate-albumin solution. When indicated, 25, 250, or 2500 milliunits of insulin were added to the basal medium; in addition, sufficient hormone was added to the oleate-albumin solution to allow 25, 250, or 2500 milliunits to be infused per hour. Glucose, as a 0.28 M solution, was added to the original medium and to the oleate-albumin solution such that the respective concentrations of glucose were 16.7, 25.0, or 41.7 mM.

Aliquots of perfusate were removed for chemical analysis immediately before insertion of the liver into the perfusion apparatus. Aliquots of perfusate were then separated from phospholipids by elution with chloroform. The lipid extracts were taken to dryness in a vacuum, dissolved in a small amount of CHCl₃, and added to columns centrifuged to sediment the erythrocytes, and 1-ml aliquots of the plasma were extracted for total lipids with 20 ml of chloroform/methanol, 2/1, v/v (29). The lipid extracts were taken to dryness in a vacuum, dissolved in a small amount of CHCl₃, and added to columns containing 3.0 g of silica gel. Throughout the experiments, the free fatty acids were then separated from phospholipids by elution with chloroform.

Aliquots of the cholesterol esters were analyzed for triglyceride by a modification of the method of Van Handel and Zilversmit (30) described by Newman and associates (31); mannitol was used as the primary standard for triglyceride analysis (32). Free fatty acids were analyzed by the method of Duncumb (33). Livers were homogenized in ethanol, and the total lipids were extracted with ethyl ether using a Soxhlet apparatus (34). Aliquots of the hepatic lipid extracts were then added to silicic acid columns as described above, and aliquots of the cholesterol esters were analyzed for triglyceride.

Chemicals-Oleic acid (>99% pure) was obtained from Mann Research Laboratories (New York), from Supelco, Inc. (Bellefonte, Pa.), or from Nu-Chek Prep (Elysian, Minn.). The bovine serum albumin (Fraction V powder) was purchased from Pentex, Inc. (Kankakee, Ill.), and was further purified by extraction with isocaproic-glyceric acid, dialysis, and lyophilization (35). Crystalline bovine insulin was supplied by Dr. M. Root, Eli Lilly and Co., Indianapolis, Ind. This insulin, Lot PJ-4905, assaying 23.6 units/mg and containing less than 0.0003% glucuron, was used for liver perfusion studies in vitro and for the radioimmunoassay of the insulin antisera. Crystalline bovine insulin (also supplied by Dr. Root, Lot 7935472), assaying 24.2 units/mg and containing less than 0.1% glucuron, was used to inactivate guinea pigs for the preparation of anti-insulin serum. All chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.).
Table 1

Effects of anti-insulin serum and insulin administered in vivo on body weight and liver weight

| Experimental group and treatment | Body weight | Liver (wet weight) |
|----------------------------------|-------------|-------------------|
|                                  | Initial     | After treatment   |
| Control                          |             | 9.31 ± 0.45       |
| No treatment (7)                 | 238 ± 9     |                   |
| 1 hour normal serum (3)*         | 246 ± 11    | 9.24 ± 0.65       |
| 10 hours normal serum (5)*       | 236 ± 5     | 8.42 ± 0.31       |
| Diabetic                         |             |                   |
| 1 hour anti-insulin serum (5)*   | 230 ± 5     | 8.60 ± 0.36       |
| 3 hours anti-insulin serum (4)*  | 227 ± 3     | 7.76 ± 0.14       |
| 6 hours anti-insulin serum (4)*  | 230 ± 6     | 7.94 ± 0.24       |
| 8 hours anti-insulin serum (4)*  | 232 ± 2     | 7.11 ± 0.12       |
| 10 hours anti-insulin serum (4)* | 238 ± 5     | 7.20 ± 0.23       |
| 24 hours anti-insulin serum (3)* | 230 ± 1     | 6.50 ± 0.39       |
| 30 hours anti-insulin serum (4)* | 232 ± 1     | 6.54 ± 0.32       |

10 hours anti-insulin serum* plus insulin in vivo

|            | Initial | Before insulin | After insulin |
|------------|---------|---------------|--------------|
| 2 hours (3)* | 228 ± 2 | 214 ± 2 | 217 ± 2 |
| 4 hours (3)* | 231 ± 2 | 217 ± 2 | 220 ± 2 |
| 10 hours (4)* | 228 ± 1 | 214 ± 2 | 220 ± 2 |
| 20 hours (3)* | 236 ± 4 | 224 ± 4 | 227 ± 6 |

*The number of perfusion experiments are indicated in parentheses.
†0.75 to 1.0 ml of normal serum/100 g of body weight, intravenously.
‡0.5 ml of normal serum/100 g of body weight, intraperitoneally.
§2.5 units of anti-insulin serum/100 g of body weight, intravenously.
¶2.5 units of anti-insulin serum/100 g of body weight, intraperitoneally and repeated after 90 min.
‖6.5 to 7.5 units of anti-insulin serum/100 g of body weight, intraperitoneally.
**6.5 to 7.5 units of anti-insulin serum/100 g of body weight, intraperitoneally initially and repeated after 12 hours.
A A single dose only of anti insulin serum was given to this group on
were of reagent grade and all solvents were redistilled in glass before
use.

In the calculations for net changes of the various metabolites in the
medium during perfusion, corrections were made for losses due to
correcting, for the addition of volumes infused, and for changes in the
hematocrit of the medium. Data are expressed per g wet weight of liver,
or per total liver. Mean values in the tables and figures are presented ±
S.E.M. The Student t-test (double tailed) was employed to evaluate
the significance of differences between two treatment means (36). In
all cases, the 5% level was used as the limit for statistical
significance.

RESULTS

The objectives of the first series of experiments were to study the
onset of metabolic alterations in the intact animals and in the
isolated liver as a function of time following administration
of anti-insulin serum. A primary objective was to determine
the time required after treatment of the animals with anti-
insulin serum to obtain maximal changes in triglyceride
metabolism and ketogenesis in the perfused liver (this required
10 hours, as discussed below). The subsequent aim was to
determine the rates at which these metabolic changes were
restored to control values after treatment of 10-hour anti-
insulin serum diabetic rats with insulin.

The body weights of the animals, and of the livers after
perfusion, decreased following administration of anti-insulin
serum, and approached normal values again after treatment
with insulin (Table 1). It was, therefore, essential that differences
in liver mass between groups be considered in the
expression of the data in order to compare accurately hepatic
metabolic processes in the fasted state with those in the diabetic
condition. It is known that liver mass decreases during fasting,
as in diabetes; the total hepatic content of deoxxyribonucleic
acid, however, is unchanged during prolonged starvation (37).
It is probable, therefore, that the total number of cells in the
liver remains relatively constant in fasting or diabetes. We
have expressed some of the data per g of liver, but to compare
specifically the results observed in livers from rats pretreated
with anti-insulin serum with those obtained with organs from
normal animals, the data are expressed per total liver. Presen-
tation of the data per total liver as reported here is equivalent
to expression per 100 g of original body weight, since initial
body weights of the animals were similar in all experimental
groups. The effects of anti insulin serum and insulin on various
metabolites in the blood are presented in Fig. 1. The blood
glucose, plasma triglycerides, and blood ketone bodies were
measured in the blood of the untreated normal fed rats under our conditions is somewhat higher
than that reported by certain other investigators (38, 39). However, in
our experiments, the sample of blood was obtained during the surgical
procedure for removal of the liver, several minutes after the animal was
anesthetized with ether. When samples of blood were obtained more
rapidly (e.g., by heart puncture) from rats anesthetized briefly with
ether, the concentration of blood ketone bodies was 0.96 ± 0.15
mM/100 ml (N = 4), moreover, when the blood was obtained from
unanesthetized rats by decapitation, the concentration was 0.51 ± 0.03
mM/100 ml (N = 4). These data suggest that ether anesthesia increases
the blood concentration of ketone bodies. Bates and co-workers (39)
observed that blood concentrations of ketone bodies increased in rats
anesthetized with ether, but not in animals anesthetized with pento-
barbital.
The effects of anti-insulin serum and insulin, administered to the intact animal, on the output of triglyceride by the liver in diabetic rats, the concentration of all of the metabolites measured in the blood decreased to levels which were similar to or less than the corresponding concentrations in the untreated normal fed rats. The concentration of ketone bodies in the blood of the diabetic rats treated with insulin for 20 hours, however, was significantly greater than the concentration in normal fed animals (p < 0.005).

The concentration of triglyceride in livers that were not perfused was determined only for normal fed untreated rats and for rats pretreated with anti-insulin serum for 10 hours (Table II). The concentration of triglyceride in livers from 10-hour anti-insulin serum diabetic rats, expressed per g of liver, was almost twice that in livers from control fed rats (p < 0.005). The total amount of triglyceride per liver for the diabetic tissue, however, was only 30% more than that of the control, and this difference was not statistically significant. For reasons which were discussed earlier, the data expressed per liver probably have greater physiological meaning.

The concentration of free fatty acids in the erythrocyte-free perfusate and the cumulative uptake of free fatty acids by liver from normal fed rats are presented in Fig. 2. The concentration of free fatty acids in the perfusate increased gradually, approaching near maximal levels after 3 hours. Uptake of free fatty acids by the liver was linear throughout the experiment, even though the concentration of free fatty acids increased slowly; this probably is attributable to the high rate of flow of perfusate through the liver. The concentration of free fatty acids in the perfusate at the end of the 4-hour perfusion period was similar in all groups indicated in Table I (approximately 1 mM). The rate of hepatic uptake of free fatty acids appeared to be more rapid after administration of control serum when the data were expressed per g of liver (e.g., 62 μmol of free fatty acids/g of liver/4 hours for control fed rats, and 80 μmol of free fatty acids/g of liver/4 hours for 10-hour anti-insulin serum diabetic rats); these differences between normal and anti-insulin serum-treated groups disappeared, however, when expressed per total liver. The mean uptake of free fatty acids for all groups indicated in Table I was 572 ± 2 μmol/liver/4 hours, which is 86% of the total quantity of oleate infused.

The effects of anti-insulin serum and insulin, administered to the intact animal, on the output of triglyceride by the liver probably have greater physiological meaning.
isolated perfused liver are shown in Fig. 3. Output of triglyceride by livers from untreated fed rats or from rats treated with normal serum was similar and was relatively linear during the period of perfusion. Output of triglyceride per liver from animals pretreated with anti-insulin serum for 1, 3, or 6 hours was similar to that of the control groups, although generally not linear with time. A decrease in secretion of triglyceride was observed with livers from rats treated with anti-insulin serum for 8 hours or more before killing. Maximal depression of output, to less than 8% of that obtained with livers from fed animals (p < 0.001), was observed 10 hours after administration anti-insulin serum. Output of triglyceride by livers from diabetic rats pretreated with insulin for 2 or 4 hours was not different from that of livers from untreated diabetic rats. Treatment of the diabetic animals with insulin for 10 hours partially corrected partially the depressed output of triglyceride, whereas treatment for 20 hours restored the output to rates similar to those observed in livers from control fed rats.

Rates of ketogenesis by livers from control, diabetic, and insulin-treated diabetic rats were relatively linear during the experiment. Total production of ketone bodies by livers from rats treated with normal serum for 1 or 10 hours exceeded that of livers from untreated animals (p < 0.02) (Fig. 4). The rate of ketogenesis increased rapidly with time after administration of anti-insulin serum; maximal rates were attained 10 hours after administration of the antiserum. Total output of ketone bodies by livers from 10-hour anti-insulin serum diabetic rats with insulin for 2 or 4 hours failed to decrease the elevated rates of ketogenesis (Fig. 4). After 10 hours of treatment with insulin, total output of ketone bodies was approximately one-third of the rate observed in livers from rats treated with anti-insulin serum for 30 hours, but was still twice that of the untreated control group (p < 0.01). Twenty hours after administration of insulin, the total output of ketone bodies was approximately one-third of the rate observed in livers from rats treated with anti-insulin serum for 30 hours, but was still twice that of the untreated control group (p < 0.01).

The concentrations of triglyceride in the livers at the termination of the experiments are shown in Table III. The total quantity of triglyceride was similar in livers from the untreated control group and in livers from rats treated with
Hepatic Lipid Metabolism in Experimental Diabetes

### Table III

**Effects of anti-insulin serum and insulin administered in vivo on concentration of triglyceride in liver**

Details of the experiments are described in Table I and in the text. Values represent the concentration of triglyceride present in the liver at the end of perfusion. Data are presented as amount of triglyceride/mg of liver and per total liver ± S.E. Figures in parentheses indicate number of observations.

| Treatment                  | Micromoles of triglyceride/mg of liver | Micromoles of triglyceride/mg of liver |
|----------------------------|----------------------------------------|----------------------------------------|
| Control                    |                                        |                                        |
| No treatment (7)           | 16.2 ± 1.9                             | 148.0 ± 14.9                           |
| 1-hour normal serum (5)    | 13.8 ± 1.3                             | 123.6 ± 6.9                            |
| 10-hour normal serum (5)   | 14.7 ± 1.2                             | 130.4 ± 14.0                           |
| Diabetic                   |                                        |                                        |
| 1-hour anti-insulin serum (5) | 14.1 ± 1.1                             | 121.3 ± 11.7                           |
| 3-hour (4)                 | 18.6 ± 2.1                             | 143.9 ± 15.2                           |
| 6-hour (5)                 | 12.3 ± 1.9                             | 92.7 ± 13.2                            |
| 8-hour (4)                 | 7.3 ± 0.6                              | 51.6 ± 3.4                             |
| 10-hour (4)                | 10.7 ± 0.8                             | 76.9 ± 5.0                             |
| 24-hour (3)                | 6.7 ± 0.8                              | 43.1 ± 5.1                             |
| 30-hour (4)                | 10.4 ± 1.7                             | 66.9 ± 13.8                            |
| 10-hour anti-insulin serum diabetic plus insulin in vivo | 6.2 ± 0.5 | 42.7 ± 3.4 |
| 2-hour (3)                 | 10.1 ± 0.8                             | 80.7 ± 15.0                            |
| 4-hour (3)                 | 8.9 ± 0.9                              | 76.0 ± 11.6                            |
| 10-hour (4)                | 19.9 ± 2.4                             | 201.7 ± 22.2                           |

The total quantity of triglyceride in livers from rats pretreated with anti-insulin serum for 1 and 3 hours was not different from that of the control groups, whereas the amount in livers from rats treated with anti-insulin serum for 6 hours or longer was less than the control (p < 0.05). The quantity of triglyceride in the perfused livers from 10-hour anti-insulin serum diabetic rats treated with insulin remained similar to that of livers from untreated diabetic animals until after 20 hours of treatment; the concentration of triglyceride in the latter group of perfused livers was similar to that of livers from normal fed rats.

The effects of anti-insulin serum on ureogenesis are presented in Table IV. Production of urea by livers from all treatment groups was relatively proportional with time during the experiment. The effects of anti-insulin serum were variable and inconsistent; indeed, pretreatment of rats with anti-insulin serum for more than 1 hour may have decreased slightly total hepatic production of urea. It was reported previously that ureogenesis is increased in perfused livers obtained from alloxan diabetic rats (4). Jefferson et al. (43) reported that output of urea was increased in perfused livers from rats pretreated with anti-insulin serum for 1 hour, whereas Haft (44) failed to observe any increase in ureogenesis in perfused livers from rats pretreated with anti-insulin serum for 90 to 120 min. We observed recently that ureogenesis by perfused livers from 10-hour anti-insulin serum diabetic rats was suppressed by addition of free fatty acids to the medium in vitro. The presence of free fatty acids in the medium, as well as differences in techniques of perfusion may have accounted, in part, for the discrepancy between our results and those of Jefferson et al. (43).

The effects of anti-insulin serum on output of glucose by the perfused liver are shown in Table IV. A large output of glucose is observed consistently during the 20-min period of equilibration under our experimental conditions. Output of glucose by livers from rats treated with normal serum for 1 or 10 hours was approximately the same as that by livers from untreated animals. Total output of glucose per 4 hours by livers from rats pretreated with anti-insulin serum for all periods of time was less than that of the untreated control (p < 0.02), and decreased with time after injection of anti-insulin serum, but considerable improvement was observed in the group treated with anti-insulin serum 30 hours earlier. The decrease in output of glucose under our experimental conditions by livers from animals pretreated with anti-insulin serum probably is attributable to the rapid depletion of glycogen in vivo prior to perfusion (43). Jefferson et al. (43) reported that the concentration of glycogen in the liver was reduced approximately 25% after treatment of rats with anti-insulin serum for only 1 hour; they observed further that output of glucose by livers from rats treated with anti-insulin serum 1 hour previously increased about 75% during the first 30 min of perfusion, compared with livers from rats treated with normal serum. In the experiments reported here, however, total output of glucose during the 20-min period of equilibration by livers from rats treated with anti-insulin serum 1 hour earlier did not differ significantly from that of livers from untreated control rats or from animals treated with normal serum for 1 hour. The difference between our results and those of Jefferson et al. may be attributable to differences in procedures for initiation of perfusion (i.e., perfusion in situ immediately after cannulation of the portal vein versus isolation of the liver prior to perfusion). The output of...
glucose measured under our conditions would be less than under those of Jefferson et al. (43), since the loss of glucose during the time required for isolation of the liver would not be measured with our procedures. A gradual restoration in the hepatic output of glucose was observed following administration of insulin to the 10-hour anti-insulin serum diabetic rats (Table IV). The total output of glucose for the 4-hour period by perfused livers from 10-hour anti-insulin serum diabetic rats treated with insulin for 20 hours, however, was still significantly less than that of the untreated normal control (p < 0.02).

Since output of triglyceride by the perfused liver was not depressed significantly until 8 to 10 hours after administration of anti-insulin serum, and since variability in output was extensive in livers from rats treated with anti-insulin serum for 8 hours, livers from rats pretreated with anti-insulin serum for 10 hours were selected as the model for subsequent studies of insulin deficiency. We investigated effects of insulin added to the medium in experiments lasting either 4 or 8 hours, and secondly, effects of glucose added to the perfusate alone, or in combination with insulin.

Insulin, added without exogenous glucose to the medium-perfusing livers from 10-hour anti-insulin serum diabetic rats, had no significant effects on any of the parameters investigated in experiments lasting for 4 hours (Figs. 5 and 6). The results obtained with 25 or 2500 milliunits of insulin were identical with those obtained with the dose of 250 milliunits. In a number of experiments, livers were perfused for 8 hours to ascertain whether insulin might correct the effects of treatment with anti-insulin serum over a longer period of perfusion. The results of these additional studies were also negative, except that production of urea/g of liver/8 hours was depressed significantly by insulin (p < 0.05); the magnitude of the change, however, was small (Table V). Total uptake of free fatty acids per liver by livers from diabetic rats was not altered by addition of insulin to the medium, and was similar to that of the normal control perfused for a period of 8 hours. The concentration of free fatty acids approached 1.1 mM after 4 hours, and remained relatively constant during the remainder of the experiment (Fig. 1). Uptake of free fatty acids was linear with time during the entire 8-hour experiment (Fig. 1).

Output of triglyceride by livers from normal fed rats during the first 4 hours was about one-half of that during the second 4 hours, in agreement with observations of other workers (45, 46) (Table V). Similarly, output of triglyceride by livers from 10-hour anti-insulin serum diabetic rats was also larger during the second 4 hours in comparison to the first 4 hours of perfusion; total output (per liver) by the diabetic livers for the entire 8-hour period, however, was only 14% of that by livers from normal fed animals (Table V).

Production of ketone bodies by livers from normal fed rats was almost 1.7 times greater during the last 4 hours of perfusion than during the first 4-hour period (Table V). Although total production of ketone bodies (per liver) over the 8-hour period by diabetic livers was approximately 3 times greater than that for the control livers, the output by livers from diabetic rats in the last 4 hours was only 56% of that observed during the first 4 hours (Table V).

Triglyceride accumulated in livers from normal fed rats during the 8 hours of perfusion, when compared to the

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**Fig. 5.** Effects of insulin and glucose added in vitro on ketogenesis, output of triglyceride, and hepatic concentration of triglyceride by livers from 10-hour anti-insulin serum diabetic rats. Rats were pretreated with anti-insulin serum for 10 hours as described in Table I. Oleate was infused at the rate of 166 mM/hour. When required, glucose was added to the perfusate and to the oleate-albumin solution to produce the concentration required. Insulin, 250 milliunits, was added to the perfusate initially and 250 milliunits were infused/hour. Data for secretion of triglyceride and ketogenesis are expressed as total mol/liver for the 4-hour period. The hepatic concentration of triglyceride is the total amount present in the liver at the end of perfusion. Data are means ± S.E. for at least three observations.

**Fig. 6.** Effects of insulin and glucose added in vitro on production of urea by livers from 10-hour anti-insulin serum diabetic rats. Details of the experiments are described in Fig. 5. At least four experiments were performed in each group. Symbols indicate means ± S.E.
Hepatic Lipid Metabolism in Experimental Diabetes

TABLE V

Rates of output of triglyceride, ketogenesis, output of glucose, and ureogenesis by perfused livers from normal fed and 10-hour anti-insulin serum diabetic rats during perfusion for 8 hours.

Details of the experiments are described in the text and in Fig. 2.

| Experiment | Hours of perfusion | Output of triglyceride | Ketogenesis | Output of glucose | Ureogenesis |
|------------|--------------------|------------------------|-------------|------------------|-------------|
|            | 4                  | 6                      | 8           | 8                |             |
|            | µmol/g of liver    | µmol/g liver           | µmol/g liver| µmol/g liver    | µmol/g liver|
| Normal fed (4) | 4.66 ± 0.63        | 8.15 ± 1.40            | 19.7 ± 1.6  | 104 ± 10        |             |
| 10-hour anti-insulin serum (4) | -0.66 ± 0.28        | 0.62 ± 0.36            | 1.98 ± 0.27 | 14.6 ± 1.9      |             |
| 10-hour anti-insulin serum + insulin in vitro (4) | -1.05 ± 0.12        | 0.34 ± 0.31            | 2.22 ± 0.59 | 15.6 ± 4.1      |             |
| Normal fed (4) | 33.9 ± 8.3         | 62.6 ± 17.7            | 90.2 ± 19.8 | 757 ± 186       |             |
| 10-hour anti-insulin serum (4) | 192.0 ± 5.0         | 245.0 ± 4.0            | 300.0 ± 1.0 | 2218 ± 28       |             |
| 10-hour anti-insulin serum + insulin in vitro (4) | 170.0 ± 17.0        | 230.0 ± 19.0           | 267.0 ± 24.0| 1867 ± 143      |             |
| Normal fed (4) | 92.6 ± 11.4        | 144.0 ± 12.0           | 173.0 ± 14.0| 1434 ± 131      |             |
| 10-hour anti-insulin serum (4) | 12.8 ± 4.7          | 25.4 ± 5.4             | 42.0 ± 6.1  | 312 ± 48        |             |
| 10-hour anti-insulin serum + insulin in vitro (4) | 18.5 ± 1.9          | 30.0 ± 3.2             | 37.0 ± 5.1  | 258 ± 31        |             |
| Normal fed (4) | 40.3 ± 2.1         | 54.9 ± 3.6             | 70.3 ± 5.0  | 539 ± 75        |             |
| 10-hour anti-insulin serum (4) | 34.2 ± 2.1          | 50.6 ± 5.0             | 67.1 ± 6.8  | 492 ± 46        |             |
| 10-hour anti-insulin serum + insulin in vitro (4) | 30.0 ± 1.8          | 42.8 ± 3.6             | 53.9 ± 4.3  | 364 ± 36        |             |

Concentration present in livers that were not perfused (Table II). In contrast, the amount of triglyceride in livers from diabetic animals actually was reduced during perfusion (Table II).

Since the addition of insulin to the medium in vitro failed to alter the depressed secretion of triglyceride or the elevated rate of ketogenesis by livers from diabetic rats, we decided to determine whether the hormone might influence these processes in the presence of glucose. Details of the experiments are described in the legend to Fig. 5. The concentration of free fatty acids in the perfusate and the total uptake of free fatty acids by livers from diabetic animals were not affected by the addition of glucose, either alone or in combination with insulin; these data, therefore, are not presented. Output of triglyceride by livers from the diabetic animals, in the presence or absence of added insulin, increased in an approximately linear fashion until maximal rates were observed at a glucose concentration of 25 mM (Fig. 5). Total output of triglyceride by livers from diabetic rats in the presence of 25 or 41.6 mM glucose was only 26 to 27% of that by livers from normal fed rats perfused with oleate alone (42.1 µmol/liver/4 hours). Even large amounts of glucose added to the medium, therefore, were unable to restore the output of triglyceride by livers from diabetic rats to rates comparable to those of livers from normal fed rats. The addition in vitro of glucose 41.6 mM alone or in combination with insulin (250 milliunits) to the medium-perfusing livers from normal fed animals did not alter hepatic output of triglyceride. Paradoxically, for reasons which remain obscure, insulin, in the presence of added glucose, depressed rather than stimulated secretion of triglyceride by the diabetic livers (p < 0.001) by analysis of variance, factorial design (35)). The rate of ketogenesis, in the presence of 25 mM glucose, by livers from diabetic rats was reduced to a minimum which was 62% of that observed with diabetic livers perfused with oleate alone (Fig. 5). When insulin and glucose were added together, the rates of ketogenesis at each concentration of glucose were reduced on the average 21% below the rates obtained with glucose alone (p < 0.001) by analysis of variance, factorial design (36)). Glucose, alone or in combination with insulin, did not affect the hepatic concentration of triglyceride in perfused livers from the diabetic rats (Fig. 5).

Glucose alone failed to suppress output of urea by perfused livers from diabetic rats (Fig. 6). When insulin was added together with glucose, however, production of urea was decreased below the rates observed with glucose alone (p < 0.001) by analysis of variance, factorial design (36)). In contrast to these results, it was reported that insulin, in the presence of glucose, did not suppress ureogenesis by perfused livers from alloxan diabetic rats (7) These latter divergent results may be related to differences in the duration and/or severity of diabetes produced by the two methods.

DISCUSSION

Insulin deficiency produced by administration of anti-insulin serum has a particular advantage for study of hepatic fatty acid metabolism in experimental diabetes. Under our
experimental conditions, triglyceride in the liver did not increase significantly in livers from rats pretreated with anti-insulin serum for 10 hours, although the hepatic rate of ketogenesis was increased maximally under these conditions. The quantity of triglyceride observed in livers from rats pretreated with anti-insulin serum for relatively short periods of time is much less than that observed in livers from alloxan diabetic rats (3, 5, 6). Ketogenesis by perfused livers from anti-insulin serum diabetic animals is proportional to the amount of free fatty acids added to the medium; in contrast, ketogenesis by perfused livers from alloxan diabetic rats is independent of added free fatty acid because hydrolysis of intrahepatic triglyceride provides more than sufficient substrate to saturate ketogenesis (3, 8).

The rate of hepatic catabolism of free fatty acids accelerates rapidly after induction of insulin deficiency with anti-insulin serum. Although the rate of ketogenesis was maximal and the rate of secretion of triglyceride minimal 10 hours after treatment of normal fed rats with anti-insulin serum, the rate of change of these reaction rates did not parallel one another, i.e. the rate of ketogenesis increased proportionately with time after administration of anti-insulin serum whereas the output of triglyceride did not differ significantly from that of the control livers until 8 hours after injection of the antiserum. One might have anticipated immediate reciprocal changes between ketogenesis and output of triglyceride as a consequence of increased hepatic catabolism of fatty acid. An explanation for this apparent discrepancy was suggested by the results of subsequent studies on the effects of concentration of free fatty acids on the metabolism of free fatty acids by livers from normal fed rats and from animals treated with anti-insulin serum 10 hours earlier (2). In those studies, maximal output of triglyceride by perfused livers from normal fed rats was observed when uptake of free fatty acids was 75 to 100 μmol/liver/hour. The uptake of free fatty acids, when 168 μmol/hour were infused as reported here, was 138 to 150 μmol/liver/hour, which exceeds that required for maximal rates of output of triglyceride by livers from normal fed male animals under these experimental conditions. It is probable, therefore, that although catabolism of free fatty acids was increased in livers from rats pretreated with anti-insulin serum for 6 hours or less, the quantity of free fatty acids taken up was sufficient to induce rates of output of triglyceride similar to those observed with livers from normal fed rats. It is evident, however, that 8 hours after treatment of rats with anti-insulin serum, hepatic catabolism of fatty acid was so accelerated that the quantity of free fatty acids available to the liver was insufficient to maintain rates of output of triglyceride equal to those of the controls. From this reasoning, it might be predicted that, if livers from rats pretreated with anti-insulin serum for 6 hours or less were perfused with an amount of oleate sufficient to produce an uptake of approximately 75 to 100 μmol/hour, the rate of output of triglyceride would decrease in parallel with an increase in the rate of ketogenesis. The data presented in Table VI agree with this postulate. Rats were pretreated with anti-insulin serum for 3 hours, and the livers were perfused with sufficient oleate (83 μmol infused/hour) to produce an uptake of 70 to 75 μmol of free fatty acids/liver/hour; output of triglyceride by the livers from 3 hour anti-insulin serum diabetic rats was reduced by 38%, and the rate of ketogenesis was increased by 39% in comparison with control values.

1 W. F. Woodside and M. Heimberg, unpublished observation.

| Experiment                        | Output of triglyceride | Ketogenesis |
|-----------------------------------|------------------------|-------------|
|                                   | μmol/                      | μmol/liver/4 hours |
| A. Normal fed (4)                 | 27.9 ± 1.1               | 537 ± 25     |
| B. 3-hour anti-insulin serum (3)  | 17.2 ± 4.3               | 854 ± 23     |
| C. 3-hour anti-insulin serum +    | 20.0 ± 0.9               | 354 ± 65     |
| insulin in vitro (5)              |                        |              |

Statistical significance:

- A versus B                        p < 0.05
- A versus C                        p < 0.01
- B versus C                        Not significant
- p < 0.05

It is of interest to compare our data on the sequence of changes in the concentration of blood ketone bodies and free fatty acids in vivo and rates of ketogenesis by livers from rats pretreated with anti-insulin serum with those comparable data reported recently by McGarry et al. (14) for rats fasted for different periods of time. McGarry et al. reported that the concentration of free fatty acids in plasma increased after only 6 hours of starvation. The blood concentration of ketone bodies in vivo and the rate of ketogenesis by the isolated perfused liver were unchanged after 8 hours of starvation, but, with further fasting, these parameters increased simultaneously. The concentrations of ketone bodies and free fatty acids in the blood and the rate of ketogenesis by the liver attained maximal values after 12 hours of starvation. Similar observations have been made in this laboratory. It is clear, then, that the ketogenesis and free fatty acids in blood increased more rapidly and reached higher maxima after treatment of rats with anti-insulin serum than by fasting. Maximal rates of ketogenesis, however, were similar in perfused livers from rats fasted for 24 hours or from animals made diabetic by pretreatment with alloxan (8). We observed that the rate of ketogenesis by perfused livers from rats fasted for 10 to 12 hours was similar to that by livers from 10-hour anti-insulin serum diabetic rats. It is probable, therefore, that although insulin deficiency produced in rats by administration of anti-insulin serum increased the rate of ketogenesis by the liver more rapidly than did deprivation of food, the maximal changes induced by either procedure are similar. It is possible, therefore, that the primary immediate cause of severe ketosis in diabetes compared with the moderate ketosis in fasting is the excessive mobilization of free fatty acids in insulin deficiency. Evidence in support of this contention has been obtained in our laboratory (2).

McGarry et al. (8) further observed that, when rats which had been fasted previously for 24 hours were re-fed for only 3 hours, the blood concentrations of free fatty acids and ketone bodies and the rate of ketogenesis by the perfused livers were...
decreased to values similar to those of normal fed animals. Under these circumstances, the simultaneous decline in the plasma concentration of free fatty acids (the primary substrate for ketogenesis by the liver) and of hepatic rates of ketogenesis both contributed to decreased production of ketone bodies by the liver and, subsequently, to the decreased concentration of blood ketone bodies in vivo. In our studies, the decline in the blood concentration of ketone bodies in 10-hour anti-insulin serum diabetic rats treated with insulin for 2 or 4 hours could only have been attributed to the decline in the plasma concentration of free fatty acids, since rates of ketogenesis by perfused livers isolated from animals treated with insulin for this period of time did not differ from those of livers from untreated diabetic animals.

Since we did not observe any change resulting from addition of insulin to the medium in the output of triglyceride or ketone bodies by perfused livers from 10-hour anti-insulin serum diabetic animals, it is probable that deficiency of insulin per se is not the only factor contributing to the alterations induced in hepatic lipid metabolism by anti-insulin serum. It has been reported that insulin added in vitro can suppress ketogenesis by perfused livers from normal fed rats (7, 17, 18). Topping and Mayoe (17) also reported that insulin stimulated the secretion of triglyceride by perfused livers from such animals. Once hepatic catabolism of fatty acid becomes accelerated maximally, as in the 10-hour anti-insulin serum diabetic animal, and secondary processes become affected, insulin may no longer be effective in short term experiments in vitro. Insulin, however, may effectively suppress hepatic catabolism of free fatty acids when this process is only partially accelerated. The following experiments support this contention. Livers from rats pretreated with anti-insulin serum 3 hours earlier were perfused such that uptake of free fatty acids was approximately 70 to 75 \( \mu \text{mol/liver/hour} \) (Table VI). Under these conditions, insulin added in vitro decreased the rate of ketogenesis by 35\% to that of livers from normal fed rats, but did not alter output of triglyceride.

The observations that rates of ketogenesis, ureogenesis, and secretion of triglyceride by livers from 10-hour anti-insulin serum diabetic rats were reduced by addition of glucose plus insulin below those rates obtained with glucose alone, and that insulin alone had no effect on these processes, suggest that glucose has a permissive effect on the action of insulin. It had been reported previously that the addition of insulin plus glucose to the perfusate did not affect ketogenesis or secretion of triglyceride by livers from alloxan diabetic rats (7), or ketogenesis by livers from pancreatectomized rats (9, 10). These differences may have resulted, in part, from variations in the severity and duration of the diabetic states produced with these several methods. It is of related interest that the addition of insulin plus glucose to the perfusate did not alter the rates of ketogenesis by livers from rats treated for 24 hours (8, 13). The concentrations of insulin used in the experiments reported here were considerably greater than the physiological levels in vivo. This caution should be kept in mind in interpretation of the data.

It has been suggested by several laboratories (12, 13, 17, 38, 47, 48) that decreased quantities of carbohydrate in the liver resulting from depletion of glycogen contribute to the alterations in hepatic metabolism of fatty acids induced by fasting or by deficiency of insulin. These conclusions are supported by our observations that glucose added in vitro decreased ketogenesis and stimulated secretion of triglyceride by perfused livers from 10-hour anti-insulin serum diabetic rats. Wieland and Matschinsky reported that, whereas fructose and glycerol suppressed ketogenesis by livers from fasted rats when perfused with oleate, glucose did not have any antiketogenic effect even when present in a concentration of 55.5 \( \text{mM} \) (38, 47). Krebs and Hems (49) reported, however, that glucose (10 \( \text{mM} \)) suppressed endogenous ketogenesis by perfused livers from fasted rats as effectively as did fructose and glycerol; in their experiment, though, fatty acid had not been added to the medium. They observed that when 2 \( \text{mM} \) oleate was added to the medium, ketogenesis was not suppressed by addition of any carbohydrate. The concentration of free fatty acids present in the medium in the experiments of Wieland and Matschinsky (38, 47) was not stated explicitly, but from the quantity of oleate added, was estimated to approximately 1.5 to 2.0 \( \text{mM} \). The failure of Wieland and Matschinsky to observe a reduction in ketogenesis by glucose, therefore, may have been the result of the concentration of oleate in the medium.

In the experiments reported here, output of glucose by the perfused liver decreased approximately in parallel with the increase in ketogenesis, as a function of time, with maximal effects observed 10 hours after injection of anti-insulin serum. A similar reciprocal relationship was observed by McGarry and associates (14), who reported that the rate of ketogenesis by the perfused liver increased with length of time that the rats were deprived of food, concomitant with a decrease in the concentration of glycogen in the liver. It should be noted, however, that output of glucose by livers from rats pretreated with anti-insulin serum for 30 hours was considerably larger than that by livers from rats pretreated for 10 or 24 hours, yet the rate of ketogenesis was similar in each of these groups (Fig. 4). It is probable, therefore, that a simple reciprocal causal relationship does not exist between availability of carbohydrate in the liver and the rate of hepatic ketogenesis, as has been considered previously. It appears reasonable to conclude that deficiency of insulin, increased concentration of glucagon in blood (50, 51), and decreased quantities of carbohydrate in the liver all contribute in an additive manner to the alterations in hepatic metabolism of fatty acids produced by diabetes.

The biochemical mechanisms by which hepatic ketogenesis and secretion of triglyceride are regulated by insulin are unknown. It has been proposed that a hormone sensitive lipase activated by cyclic AMP\(^1\) is operative in the liver (17, 52-54), analogous to the enzyme present in adipose tissue (55). Moreover, ketogenesis is stimulated and secretion of triglyceride is depressed by livers from normal fed rats by the addition of \( \text{N}^\text{O}^2 \text{H} \text{dibutyryl adenosine 3':5'-monophosphate} \) to the medium (56). Activation of a lipase would presumably result in increased availability of fatty acid for \( \beta \) oxidation and ketogenesis, and thereby decrease the pool of triglyceride in the liver. The rapid utilization of endogenous hepatic triglyceride by livers from alloxan diabetic rats was reported previously (3, 6). It has been observed that hepatic levels of cyclic AMP are increased in alloxan diabetic rats, and are reduced to control levels 30 min after treatment of the animals with insulin (43). Other workers reported that hepatic lipolytic activity is increased in alloxan diabetic rats, and is reduced to control levels after treatment of these animals with insulin for 90 min (53). It has also been reported that hepatic levels of cyclic AMP (43) and hepatic lipolytic activity (52) are both increased in rats pretreated with anti-insulin serum. Yet, in the experiments

\(^1\)The abbreviation used is: cyclic AMP, cyclic adenosine 3':5'-monophosphate.
reported here, the elevated rate of ketogenesis and depressed rate of secretion of triglyceride by livers from 10-hour anti-insulin serum diabetic rats were unaffected by treatment with insulin in vivo 2 or 4 hours prior to study. If one makes the assumption that the treatment of 10-hour anti-insulin serum diabetic rats with insulin in vivo for 2 or 4 hours resulted in a rapid decrease in hepatic concentrations of cyclic AMP and a hepatic lipolytic activity, as obtained with alloxan diabetic rats, the quantitative role of increased hepatic lipolytic activity and hepatic concentrations of cyclic AMP in regulation of alterations in hepatic triglyceride metabolism and ketogenesis must be questioned. Other mechanisms, as changes in the levels of certain hepatic enzymes, may be of greater significance (57).

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W F Woodside and M Heimberg

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