Role of Adenosine 3',5'-Monophosphate in the Effects of Insulin and Anti-insulin Serum on Liver Metabolism*

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SUMMARY

Injection of guinea pig anti-insulin serum into rats decreased the concentration of glycogen in the liver and increased the level of nonesterified fatty acid and glucose in the blood and the concentration of citrate in the heart. The uptake of glucose was reduced in hearts removed from antiserum-treated rats and perfused in vitro.

Glucose production by perfused livers from rats treated with antiserum in vivo was increased as a result of accelerated glycogenolysis and gluconeogenesis. Urea production and K+ efflux were also increased. The rates of these processes could be reduced to or below control values by addition of insulin to the perfusion medium. Insulin added in vitro also decreased these processes and the loss of organic phosphate in livers from normal rats. Antiserum added to the medium perfusing normal hearts or livers was without effect except that it abolished the action of insulin.

Administration of anti-insulin serum in vivo caused a progressive rise in the liver cyclic AMP concentration. Diabetes induced by alloxan also resulted in an increase in liver cyclic AMP, which was rapidly abolished by insulin treatment. Insulin added to the medium perfusing livers from normal or antiserum-treated rats caused a small decrease in liver cyclic AMP. Insulin antagonized the effects of epinephrine and glucagon on glucose output by the perfused liver and reduced the level of cyclic AMP in the presence of glucagon.

The hypothesis is advanced that changes in cyclic AMP levels may be partly responsible for the alterations in liver metabolism caused by insulin and diabetes.

As has been well known since the work of Moloney and Coval (2), acute insulin deficiency induced by intravenous injection of

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insulin antiserum causes an abrupt increase in blood glucose. There is much evidence that impaired utilization of glucose by insulin-sensitive tissues contributes to the rise in blood glucose. For example, the utilization of glucose is substantially reduced in diaphragm and epididymal fat pads removed from antiserum-treated rats (3), and antiserum neutralizes the effects of insulin on muscle (4) and adipose tissue in vitro (5-8). The hyperglycemia following injection of antiserum may also be due to increased glucose output by the liver. Antiserum administered in vivo has been reported to decrease liver glycogen in intact animals (9) and to stimulate gluconeogenesis in liver slices (10).

In this study we have used an isolated, perfused liver preparation to confirm that treatment of the rat with insulin antiserum causes increased glucose output by the liver. This is due to increased glycogenolysis and gluconeogenesis, which appear to be brought about by a rise in the tissue level of adenosine 3',5'-monophosphate (cyclic AMP). We have also confirmed the observation of Mortimore (11) that insulin directly suppresses glucose production by the liver, and have shown that insulin can lower cyclic AMP and antagonize the action of glucagon or epinephrine. It seems likely that some of the effects of insulin and diabetes on the liver are due to changes in cyclic AMP concentration.

MATERIALS AND METHODS

Animals—Male Sprague-Dawley rats maintained on Purina laboratory chow were used. Where indicated, rats received 1 ml of anti-insulin or normal serum in a tail vein. At various times thereafter, perfusion experiments were begun or tissue and blood samples were taken. Samples of liver and heart were rapidly removed from animals anesthetized by intraperitoneal injection of 60 mg of sodium pentobarbital per kg, and were quick frozen at the temperature of liquid nitrogen (12). They were stored at -70° until analyzed. Samples of blood were collected from the neck after decapitation, placed in tubes at 0°, and centrifuged. The serum was analyzed immediately or stored at -15°. Alloxan diabetes was induced by intravenous injection of alloxan monohydrate (90 mg per kg of body weight).

Anti-insulin Serum—Pooled anti-insulin serum with an insulin-binding capacity of 2 to 3 units per ml was assayed by the method of Berson et al. (13) was harvested from guinea pigs.
that had received monthly injections of either crystalline bovine insulin in adjuvant (14) or protamine zinc porcine insulin. The latter was injected subcutaneously as an initial dose of 10 units, followed by gradually increasing doses to a maintenance level of 20 units. No differences in metabolic responses were noted between the two lots of Al-serum. Normal serum obtained from untreated guinea pigs was devoid of insulin-neutralizing activity. All sera were stored at -20°C until used.

Perfusion of Hearts—Hearts form Al-serum- or normal serum-treated rats weighing 200 to 250 g and fasted for 18 to 22 hours were perfused in the manner described by Morgan et al. (15) with Krebs-Henseleit bicarbonate buffer gassed with O₂ : CO₂ (95:5). After a 3-min "wash-through" to remove blood and to establish a regular rate of contraction, hearts were perfused with recirculation for 20 min with 15 ml of buffer containing 7.5 mg of glucose and 15 mg of bovine serum albumin. Samples of the perfusion medium were removed at intervals and stored at -15°C until analyzed.

Perfusion of Livers—Fed rats, weighing 110 to 140 g, were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg per kg), and the livers were perfused in situ for 90 min at 37°C by a modification (17) of the technique described by Mortimore (18). The perfusion medium was Krebs-Henseleit bicarbonate buffer containing 4 g of bovine serum albumin per 100 ml and sufficient 0.9% NaCl-washed rat erythrocytes to give a hematocrit of 20 to 22%. In some experiments, 14C-glucose or 14C-lactate was added to the medium. The flow rate was maintained constant at 7 ml per min, which was sufficient to provide adequate oxygenation. The initial volume of recirculating medium was 40 or 50 ml. Samples of the medium were removed at 30-min intervals and promptly centrifuged at 4°C, and the supernatant perfusate plasma was analyzed immediately or stored at 15°C. On completion of the perfusion, the liver was rapidly frozen as described above and stored at -70°C until analyzed.

Analytical Methods—For the determination of tissue glycogen (19) portions (about 0.5 g) of frozen liver were rapidly weighed and digested in KOH (30 g/100 ml) at 100°C. The glucose yielded by hydrolysis was estimated by the ferricyanide method with a Technicon AutoAnalyzer. Glucose in serum and perfusate plasma was also measured in this way; values obtained agreed closely with those found by the glucose oxidase method (20).

To determine 14C-glucose in perfusate plasma, samples were deproteinized (21) and treated with Dowex 50-X8 (H⁺ form) and Duolite A-4 (OH⁻ form) to remove charged metabolites (17). Portions (0.5-ml) of the resin-free supernatant fluid were added to 10 ml of p-dioxane containing 100 g of naphthalene, 7 g of 2,5-diphenyloxazole (PPO), and 0.3 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene per liter, and the radioactivity was determined in a Packard Tri-Carb liquid scintillation spectrometer. Values were corrected for quenching by the use of internal standards. As described by Eston and Park (17), the method is satisfactory for determining 14C-glucose in this system. The serum nonesterified fatty acid concentration was determined by the method of Dole (22), the heart citrate concentration by the method of Stern (23), and the liver inorganic phosphate concentration by the method of Wabr and Wollenberger (24). The perfusate plasma was analyzed for potassium with a Carl Zeiss flame photometer (model PMQ II), and for urea nitrogen and inorganic phosphate with the AutoAnalyzer. The cyclic AMP content of the frozen liver samples was measured as described by Butcher et al. (25).

Calculation of Glucose Utilization and Production—Hepatic glucose balance was estimated by an isotope dilution technique with 14C-glucose. As a first approximation, it was assumed that the disappearance of glucose radioactivity from the perfusate, when corrected for sequential changes in specific activity, was equivalent to glucose utilization by the liver. Thus between two sampling times,

$$\text{Glucose utilization, } \mu\text{moles}$$

$$= \frac{\text{mean } 14C\text{-glucose specific activity, cpm/mole}}{\text{(decrease in } 14C\text{-glucose, cpm/ml})(\text{glucose space, ml})}$$

where glucose activity and specific activity are those of the perfusate plasma and the glucose space is that of the perfusate and liver calculated as described previously (17). This method of calculating glucose uptake by the liver probably underestimates the process, since it does not account for 14C-glucose incorporated into and then released from glycogen, or for 14C-glucose resynthesized from the products of 14C-glucose breakdown.

Although glucose disappearance measured in this way does not distinguish between glucose consumption by the liver and by the erythrocytes, any changes in overall glucose utilization due to the action of hormones must result from alterations in liver function, since glucose consumption by erythrocytes is unaffected by hormones (26). In any case, observations in livers from fasted rats have indicated that any lactate derived from glucose breakdown by rat erythrocytes is largely reconverted to glucose by the liver (17). That is, the utilization of 14C-glucose by the erythrocytes probably does not result in much over-all disappearance of 14C-glucose from the system.

The net output of glucose by the liver between two sampling times was calculated from the change in the glucose concentration of the perfusate plasma multiplied by the glucose space of the medium and liver calculated as described previously (17). A correction for glucose utilization by the erythrocytes was applied (17).

Glucose production by the liver was calculated as the sum of net glucose output and glucose utilization.

Chemicals—Uniformly labeled d-glucose-14C was obtained from New England Nuclear, and sodium D-lactate-2-14C, from Nucler-Chicago. Materials used for the determination of radioactivity were obtained from the Packard Instrument Company. Alloxan monohydrate was obtained from Eastman Kodak; t-epinephrine hydrochloride from Parke, Davis; and Fraction V bovine serum albumin from Nutritional Biochemicals. Insulin (10 times recrystallized) was kindly supplied by Novo Laboratorium, Copenhagen; other insulin samples (used to produce anti-insulin serum) and glucagon were generous gifts from Eli Lilly and Company.

Expression of Results—In the tables, mean values are followed by the standard error of the mean, while in the figures, mean values are plotted with standard errors represented by vertical bars. The number of observations is indicated in parentheses. Values are expressed per g of liver (wet weight) or per 100 g of body weight. The significance of differences between means was established by the Student's t test. Values for p which were greater than 0.05 were taken to indicate nonsignificant changes.

1 The abbreviation used is: AI-serum, anti-insulin serum.
RESULTS

Effects of Anti-insulin Serum in Intact Rats—Rats were given an injection of AI-serum or normal serum, and samples of serum, liver, and heart were taken 1 hour later. Whereas normal serum produced no significant changes, AI-serum decreased liver glycogen from 294 ± 22 to 217 ± 17 μmoles per g, wet weight (p < 0.025), and increased serum nonesterified fatty acids from 277 ± 29 to 650 ± 70 μeq per liter and blood glucose from 8.4 ± 0.2 to 16.7 ± 0.3 mm. These changes are similar to those reported by other workers (9, 27, 28) and establish the biological potency of the AI-serum used in this study. It was also found that AI-serum increased the heart citrate concentration from 1.7 ± 0.2 to 3.1 ± 0.3 μmoles per g, dry weight.

Effects of Anti-insulin Serum Administered in Vivo and in Vitro on Glucose Uptake by Perfused Rat Heart—Perfused hearts obtained from rats that had received AI-serum 1 hour previously showed a 75% reduction in glucose uptake (Table I). Addition of AI-serum to the perfusion medium was without effect in hearts from normal rats but it abolished the increased glucose uptake found in hearts from rats that had received insulin prior to perfusion (Table I). Normal guinea pig serum injected in vivo or added in vitro produced no significant changes in glucose uptake.

Effects of Anti-insulin Serum and Insulin on Glucose Production by Perfused Rat Liver—The changes in net glucose output and glucose balance in perfused livers from rats treated with AI-serum or normal serum 1 hour previously are shown in Fig. 1. The antiserum caused a substantial increase in net glucose output, whereas normal serum produced no significant change. The increase produced by anti-insulin serum could be reduced to below the control by addition of excess insulin to the perfusion medium. The bar graph on the right side of the figure indicates that these changes were due to effects on glucose production rather than utilization. Glycogen is the major source of glucose released from the livers in these experiments. In livers from rats given normal serum, the final glycogen level was 136 ± 11 μmoles per g, whereas in those from rats treated with AI-serum it was 93 ± 6 μmoles per g (p < 0.0025).

In contrast to its effects when injected in vivo, AI-serum added to the medium produced no significant increase in glucose production (p > 0.05) (Fig. 2). In livers from normal rats, insulin in vitro reduced glucose production by 43% (Fig. 2), confirming the reports of Mortimore (11, 29) and others (30–32). The bar graph indicates that this effect was due to diminished glucose production, as concluded by Mortimore (29) on other grounds. Insulin was effective at a level of 100 micromolts per ml, and anti-insulin serum added in vitro neutralized its action (Fig. 2). Insulin increased the incorporation of 14C from 14C-glucose into glycogen by 2.5-fold, and into fatty acids by 1.95-fold. These changes were greater than those attributable to the increased specific activity of the medium 14C-glucose.

Effects of Anti-insulin Serum and Insulin on Glucose Synthesis from Lactate—By measuring the incorporation of 14C from 14C-lactate into glucose, it was possible to determine whether the AI-serum treatment in vivo had stimulated gluconeogenesis as well as glycogenolysis. With a saturating concentration of lactate (20 mM) in the medium, net output of glucose in the control livers was much increased, as can be seen by comparing Fig. 3 with Fig. 1. Treatment with AI-serum caused a substantial further increase in glucose production (Fig. 3). The bars on the right of the figure show that part of this effect was due to a greater conversion of lactate to glucose; the remainder was presumably due to increased glycogenolysis. Insulin added in vitro abolished the increased glucose output due to AI-serum (Fig. 3), and this reduction could again be partly ascribed to suppression of gluconeogenesis from lactate. There was a small incorporation of label into glycogen which was not significantly affected by treatment with AI-serum in vivo or insulin in vitro. The absence of significant changes is not surprising, since glycogen labelling reflects both the conversion of 14C lactate to glucose or glucose-6-P and the balance between glycogen synthesis and breakdown.

In livers from normal rats perfused for 30 min with 20 mM 14C-lactate, insulin reduced glucose production from 175 ± 12 to 128 ± 14 μmoles/100 g of body weight (p < 0.025) and decreased the incorporation of label into glucose from 57,800 ± 5,500 to 41,000 ± 2,900 cpm/100 g of body weight (p < 0.0125). The labeling of glycogen was not significantly increased by insulin.

**Table I**

<table>
<thead>
<tr>
<th>Treatment of rats</th>
<th>Additions to perfusion medium</th>
<th>Glucose uptake μmoles/g heart/hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal serum (4)</td>
<td>None</td>
<td>29 ± 3</td>
</tr>
<tr>
<td>AI-serum (6)</td>
<td>None</td>
<td>7 ± 2</td>
</tr>
<tr>
<td>None (10)</td>
<td>AI-serum</td>
<td>26 ± 2</td>
</tr>
<tr>
<td>None (9)</td>
<td>Normal serum</td>
<td>26 ± 2</td>
</tr>
<tr>
<td>Insulin (15)</td>
<td>AI-serum</td>
<td>58 ± 3</td>
</tr>
<tr>
<td>Insulin (16)</td>
<td>AI-serum</td>
<td>33 ± 3</td>
</tr>
</tbody>
</table>

**Fig. 1.** Effect of anti-insulin serum (AIS) in vivo and in vitro on glucose uptake by isolated, perfused rat hearts.
FIG. 2. Anti-insulin serum (AIS) neutralization of the insulin effect on glucose production by perfused livers of fed rats. Insulin was added to the perfusing medium as a 50-milliunit priming dose and a 60-milliunit-per-hour continuous infusion. Anti-insulin serum (2 ml) was added to the perfusate at the start of perfusion. Normal serum (2 ml) added to the control livers did not affect the production of glucose.

FIG. 3. Effect of anti-insulin serum (AIS) in vivo and the counteracting effect of insulin in vitro on glucose production and lactate gluconeogenesis by perfused livers of fed rats. The experiment was as described for Fig. 1, except that the perfusing medium contained 20 mM L-lactate and sodium D(-)-2-14C-lactate (1 μCi/100 ml). NS, normal serum.

Liver—Urea production reflects the catabolism of protein by the liver under the conditions of these experiments. As shown in Fig. 4, injection of AIS-serum stimulated the production of urea, and addition of insulin in vitro returned urea production to the control level. In agreement with the observations of Miller (33) and Mondon and Mortimore (11, 34), insulin also decreased by 25% the amount of urea produced by the perfused livers of normal rats. Excess anti-insulin serum added to the perfusion medium produced no changes other than neutralization of the effect of insulin.

Effects of Anti-insulin Serum and Insulin on Release of Potassium and Inorganic Phosphate by Perfused Liver—As shown in Fig. 5, potassium release by the perfused liver was increased.

FIG. 4. Effect of anti-insulin serum (AIS) in vivo and the counteracting effect of insulin in vitro on urea production by perfused livers of fed rats. Details of the experiment were the same as those described from Fig. 1. NS, normal serum.

FIG. 5. Effect of anti-insulin serum (AIS) in vivo and the counteracting effect of insulin in vitro on potassium exchange by perfused livers of fed rats. Details of the experiment were the same as those described for Fig. 1. NS, normal serum.
significantly when the liver donor rats were treated with AI- serum. At the end of 90 min, livers from anti-insulin serum- treated animals had released 70% more potassium into the medium than control livers. No changes were observed when AI-serum was added to the medium. Adding excess insulin to the perfusion medium not only counteracted the effect of injected AI-serum, but resulted in a substantial uptake of potassium during the first 30 min of perfusion. Lambotte and Shoemaker had noted earlier (35) that AI-serum stimulated potassium release from the canine liver in situ. Workers in several laboratories (18, 36-38) have reported stimulatory effects of insulin on potassium uptake by the liver in vitro.

**Table II**

*Effect of insulin on inorganic phosphate release by perfused livers of fed rats*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Inorganic phosphate released (μmoles/100 g liver/90 min)</th>
<th>Liver inorganic phosphate (μmoles/g)</th>
<th>Cyclic AMP level change from control (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (10)</td>
<td>38.9 ± 3.0</td>
<td>0.99 ± 0.00</td>
<td>+70%</td>
</tr>
<tr>
<td>Insulin (10)</td>
<td>30.6 ± 3.0</td>
<td>1.62 ± 0.08</td>
<td>+40%</td>
</tr>
</tbody>
</table>

In agreement with previous observations (36), livers released inorganic phosphate into the medium during perfusion (Table II); insulin added *in vitro* reduced this by 21%. The level of inorganic phosphate in livers perfused with insulin was 64% higher than in the control livers.

**Effects of Insulin Deficiency on Liver Cyclic AMP Level—**

Glycogenolysis (39, 40), gluconeogenesis (1, 41), protein catabolism (33, 42), and potassium release (43, 44) are stimulated in liver in *vivo*. Effects of insulin deficiency on liver cyclic AMP levels in *vivo* were examined at intervals after injection of AI-serum or normal serum. As shown in Fig. 6, anti-insulin serum caused a progressive rise in the cyclic AMP level over 60 min, whereas normal serum caused no significant change. The figure also shows that livers from anti-insulin serum-treated rats still had an increased cyclic AMP content after 90 min of perfusion.

In a second series of experiments, insulin deficiency was produced by injection of alloxan in a dosage giving a form of diabetes which is well developed at 48 hours and fatal between 72 and 96 hours. In these animals, the level of cyclic AMP in the liver in *vivo* was examined at intervals after injection of AI-serum or normal serum. A significant reduction was also observed 30 min after intravenous injection of crystalline insulin (Table III). A significant reduction was also observed 30 min after intravenous injection of crystalline insulin (Table III).

**Effects of Insulin in Vitro on Liver Cyclic AMP—** To determine whether insulin can act directly to reduce the liver level of cyclic AMP, livers were perfused for 20 min with insulin *in vitro*. Insulin caused a 40% decrease in net glucose production and a 22% fall in the cyclic AMP level (Table IV). The hormone also caused a barely significant decrease in the cyclic nucleotide level of livers from rats treated with anti-insulin serum. These results indicate that insulin in *vivo* can lower the level of cyclic AMP but that the changes are relatively small. To determine whether insulin could counteract the effects of hormones which act by elevating the tissue cyclic AMP concentration, livers from fed rats were perfused with epinephrine in the presence or absence of insulin. As shown in Table V, insulin caused a greater inhibition of the stimulatory effect of epinephrine on glucose output than...
TABLE IV
Effects of insulin and AI-serum on glucose production and cyclic AMP levels of perfused livers

Livers from fed rats were perfused for 20 min. Insulin was added as an initial dose of 1 unit, followed by an infusion of 0.6 unit per hour. Anti-insulin serum (1 ml) was injected intravenously 1 hour prior to perfusion.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Glucose output</th>
<th>Cyclic AMP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μmol/g liver/hr</td>
<td>mmol/g liver</td>
</tr>
<tr>
<td>Control (12)</td>
<td>66.3 ± 2.1</td>
<td>0.65 ± 0.03</td>
</tr>
<tr>
<td>Insulin in vitro (12)</td>
<td>39.9 ± 1.5</td>
<td>0.52 ± 0.02</td>
</tr>
<tr>
<td>AI-serum in vitro (5)</td>
<td>120.0 ± 13.2</td>
<td>1.01 ± 0.08</td>
</tr>
<tr>
<td>AI-serum in vivo + insulin in vitro (5)</td>
<td>86.1 ± 7.1</td>
<td>0.79 ± 0.09</td>
</tr>
</tbody>
</table>

Statistical significance:
- Insulin versus control: *p < 0.0005*
- AI-serum versus control: *p < 0.0005*
- AI-serum versus AI-serum + insulin: *p < 0.05*

TABLE V
Effect of insulin on stimulation of hepatic glucose output by epinephrine

Livers from fed rats were perfused for 20 min. L-Epinephrine bitartrate was added to the reservoir to produce an initial concentration of 2 × 10^{-3} M and then infused at the rate of 0.06 μmole per hour. Insulin was added as an initial dose of 0.5 unit, followed by an infusion of 0.6 unit per hour.

<table>
<thead>
<tr>
<th>Epinephrine concentration</th>
<th>Glucose production</th>
</tr>
</thead>
<tbody>
<tr>
<td>μmol/100 g rat/20 min</td>
<td>μmol/100 g rat/20 min</td>
</tr>
<tr>
<td>0</td>
<td>72 ± 7 (10)</td>
</tr>
<tr>
<td>2 × 10^{-7}</td>
<td>182 ± 17 (8)</td>
</tr>
</tbody>
</table>

TABLE VI
Effects of insulin on stimulation of glucose and cyclic AMP formation by glucagon

Livers from fed rats were perfused for 1 hour, and then infusions of glucagon and insulin were commenced at the designated rates. Liver cyclic AMP levels were determined 4 min after the start of the infusions; glucose production was measured over a 60-min period from the start of the infusions.

<table>
<thead>
<tr>
<th>Glucagon infusion</th>
<th>Insulin infusion</th>
<th>Glucose production</th>
<th>Cyclic AMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>μmol/min</td>
<td>milliunits/min</td>
<td>μmol/g liver/hr</td>
<td>mmol/g liver</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>41 ± 4 (6)</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>2.5</td>
<td>31 ± 6 (6)</td>
<td></td>
</tr>
<tr>
<td>0.25</td>
<td>0</td>
<td>197 ± 26 (10)</td>
<td></td>
</tr>
<tr>
<td>0.25</td>
<td>2.5</td>
<td>60 ± 0 (10)</td>
<td></td>
</tr>
<tr>
<td>5.0</td>
<td>0</td>
<td>2.27 ± 0.17 (9)</td>
<td></td>
</tr>
<tr>
<td>5.0</td>
<td>50</td>
<td>1.53 ± 0.17 (9)</td>
<td></td>
</tr>
</tbody>
</table>

could be attributed to its effect in the absence of epinephrine. Measurement of cyclic AMP in these livers showed a more than 2-fold increase with epinephrine, but the decrease with insulin was not significant.

Glucagon at 1 × 10^{-7} M concentration produces an 80-fold elevation of cyclic AMP in the perfused liver, whereas 1 × 10^{-4} M epinephrine causes only a 2- to 3-fold increase (45). Consequently, it is necessary to use much lower concentrations of glucagon to demonstrate antagonism of its glycogenolytic activity by insulin. In initial experiments there was considerable variation in the response to glucagon, and insulin did not produce consistent results. However, significant effects were obtained in livers perfused for 1 hour prior to infusion of hormones. Under these conditions, insulin almost abolished the effect of low levels of glucagon on glucose output (Table VI) and decreased the cyclic AMP concentration found with higher doses of glucagon (*p < 0.005*). Cyclic AMP was not measured at the lower levels of glucagon; at the higher levels of glucagon, no effect of insulin on glucose production was observed, since the level of cyclic AMP was not reduced below that causing maximum stimulation of glucose output (see Reference 45). It is likely that insulin also antagonizes the effects of epinephrine in previously perfused livers, but this was not tested.

**DISCUSSION**

The rapid rise in blood glucose following anti-insulin serum administration to the rat is well documented (3, 9, 27, 40, 47) and is thought to be due, in part, to impaired glucose utilization. This was reflected in our experiments by the very low rate of glucose uptake in hearts taken from antisem-r-treated animals. Other workers (3, 7) have observed low rates of glucose metabolism in the epididymal fat pad and diaphragm from antisem-r-treated rats. Since antiserum in vitro was without effect (except to neutralize added insulin), the depression of glucose uptake must have been caused by inhibitory factors present in vivo. Presumably one of these factors was the rise in free fatty acids in the blood, since fatty acids have been shown to inhibit the utilization of glucose by the isolated, perfused rat heart (48-51). Fatty acids reduce the sensitivity of glucose transport to insulin (52) and raise citrate levels (53, 54), as seen in this study. Citrate may depress glucose uptake as a consequence of its inhibitory effect on phosphofructokinase (53, 55).

The rise in blood glucose following anti-insulin serum in vivo is also the consequence of increased glucose production by hepatic glycogenolysis, as observed originally by Stern et al. (9). In our studies, the glycogen content of the liver fell by an amount that could itself account for the total increase in blood sugar. Some contribution to blood glucose was presumably also made by increased gluconeogenesis. Stimulation of gluconeogenesis from endogenous amino acids would account for the increase in urea production. As was the case with glucose uptake by the heart, the hepatic effects of anti-insulin serum, including those on potassium, were only obtained when the antibody was administered in vivo.

Many of the effects of AI-serum on hepatic function appear to be explained by the rise in tissue cyclic AMP. For example, stimulation of glycogenolysis (56), gluconeogenesis (41), urea production (45), and potassium loss (45) can be induced in the perfused liver by exogenous cyclic AMP and by glucagon or catecholamines (1, 33, 41, 42, 45, 56-59), which presumably act by increasing intracellular cyclic AMP (60). Although the effects of AI-serum on hepatic lipid metabolism may be related to the increase in cyclic AMP, it is more likely that they result from the increased mobilization of fatty acids from adipose tissue (27, 28). It is probable that AI-serum exerts other direct or indirect effects on the liver not involving cyclic AMP.
It seems clear that insulin deficiency alone does not induce the rise in hepatic cyclic AMP. If this were the case, anti-insulin serum would have been as effective in vitro as in vivo. It is probable that the factors controlling the cyclic AMP content of the liver in vivo are subject at all times to opposing hormonal influences. On this basis, the following explanation is proposed.

Glucagon from the pancreas and catecholamine from hepatic nerve endings exert tonic stimulatory effects on adenyl cyclase, tending to raise cyclic AMP levels. This tendency is continuously opposed by the action of endogenous insulin. Neutralization of insulin by antiserum removes this restraint, and cyclic AMP levels increase. Anti-insulin serum is not effective in vitro, since the continuous input of glucagon and catecholamines has been interrupted. Some of these hormones are doubtless carried over from the intact animal into the isolated organ preparation, but are rapidly destroyed by the liver.

Just as the elevation of cyclic AMP can account for all the above effects of anti-insulin serum, it seems likely that a lowering of cyclic adeylnucleotides accounts for a part, at least, of the inhibitory action of insulin on glycogenolysis, gluconeogenesis, potassium release, and urea output. While we favor this view, there are some unresolved difficulties in this proposal, and other explanations remain possible.

One problem relates to the cyclic AMP content of the control tissue, which, expressed as a mean concentration in cell water, is about $1 \times 10^{-6}$ M (Table III and Fig. 6). This is at least an order of magnitude greater than the concentration which can fully activate the canine liver phosphorylase system in extracts (60), yet phosphorylase and other cyclic AMP-sensitive systems in the rat liver are not activated. In the absence of a gross species difference in sensitivity, one must assume that the action of cyclic AMP is in some way inhibited or that the compound is largely bound or compartmentalized so that it does not reach its target enzymes. A naturally occurring inhibitor has been described by Murad (61), but its physiological role, if any, is unknown. Tight binding of cyclic AMP to phosphofructokinase in muscle has been described by Kemp and Krebs (62), and preliminary studies in our laboratory indicate that about 60% of the cyclic AMP in homogenates of normal liver is in particulate fractions.

A second problem is how the relatively small change in cyclic AMP induced by insulin can be effective. There is no certain answer to this question, but it can be postulated, in line with the foregoing considerations, that the small changes in total nucleotide may be in fact large changes in that fraction of cyclic AMP which is in contact with the sensitive enzyme systems. In other words, the changes appear small only because of a large background of inhibited, bound, or sequestered nucleotide.

The question arises whether the hepatic effects of insulin which appear to be mediated by a fall in cyclic AMP can occur without prior or concomitant stimulation by glucagon or catecholamines. In other words, must cyclic AMP be elevated before a suppression by insulin is demonstrable? This is probably the case, and may account for some of the difficulties encountered in the past in obtaining consistent insulin effects on liver tissue. We have observed (Table VI) that insulin effects are greater in the presence of a low level of glucagon or epinephrine and are not observed in untreated livers when the hormone is administered late in the perfusion period. There is also evidence that cyclic AMP is elevated at the start of the perfusion and that the falling off of the nucleotide content requires some time, as indicated by Fig. 6. The period of insulin sensitivity appears to correspond to the time when the cyclic AMP is still elevated.

The rise of cyclic AMP in the liver of the diabetic rat is of interest in several respects. It can account for the depletion of liver glycogen in severe diabetes and for the stimulation of gluconeogenesis (1).

Other changes beside the elevation of cyclic AMP appear to be responsible for the accelerated rate of gluconeogenesis in the diabetic liver. In studies to be reported separately, it has been found that the rates of gluconeogenesis are substantially higher in livers from alloxan-diabetic rats than in normal livers in which high or higher cyclic AMP levels have been induced acutely by glucagon. The elevation of cyclic nucleotide in the normal, and presumably also in the diabetic, tissue activates a rate-limiting step in the gluconeogenic pathway, and gluconeogenesis rises 2- to 3-fold (1, 41, 42). Since the elevation of cyclic AMP is maintained in the diabetic tissue, the flow in the gluconeogenic pathway presumably remains high and may lead to the observed increases in the quantity of gluconeogenic enzymes (63), resulting over a period of hours or days in a substantial further rise in the gluconeogenic rate. Alternatively, the increase in cyclic AMP may affect the process of enzyme synthesis more directly.

A second point of interest relates to the striking fall in hepatic cyclic AMP 30 min after insulin administration in vivo to the alloxan-diabetic rat. Preliminary studies indicate that this effect of insulin in vivo is larger than any seen in vitro. Although it is difficult to make valid comparisons, it is our impression that the activity of insulin on hepatic glucose metabolism is generally not as great in vitro as in vivo. It is possible that important physiological components are still missing from the system in vitro.

While the effect of insulin on cyclic AMP may account for many and possibly all of the rapid direct actions of insulin in the liver, it is uncertain how it will relate to the relatively slow changes in enzyme content or activity (or both) with administration of the hormone in vivo. The extent to which changes produced by insulin on other tissues affect liver metabolism also remains to be worked out. It seems likely, for example, that diminished release of glycerol from adipose tissue, and of amino acids, results in decreased gluconeogenesis per se. It has also been proposed that the reduction in gluconeogenesis by insulin in vivo is due to a diminished supply of fatty acid to the liver (64), although this view has been questioned (17).

The present work suggests that the minute-to-minute glucose output by the liver is determined by the balance between glucagon and catecholamine activities, on the one hand, and insulin activity, on the other hand, acting on the systems synthesizing or degrading cyclic AMP. It seems likely that an analogous balance involving principally the catecholamines and insulin controls the cyclic AMP content of adipose tissue, and hence lipase activity and the output of free fatty acids. The effect of insulin on cyclic AMP in fat cells has been presented elsewhere (65).

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Role of Adenosine 3',5'-Monophosphate in the Effects of Insulin and Anti-insulin Serum on Liver Metabolism


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