Studies of the Metabolic Effects of Acute Insulin Deficiency

II. Changes in Hepatic Glycolytic and Krebs-Cycle Intermediates and Pyridine Nucleotides

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SUMMARY

(1) Various glycolytic and Krebs-cycle intermediates and pyridine nucleotides were measured in the livers of anti-insulin serum treated (i.e., acute insulin deprivation) and alloxan diabetic rats (i.e., chronic insulin deprivation).

(2) Acute insulin deprivation of three-hour duration resulted in significant changes in the concentration profile of the various glycolytic intermediates suggesting marked impairment of glycolysis at the phosphofructokinase step, activation of glycogenolysis, and decreased hexosemonophosphate shunt activity. The tissue levels of citrate and malate were not elevated under these conditions.

(3) Insulin deficiency of forty-eight hours' duration produced changes in the hepatic levels of glycolytic and Krebs-cycle intermediates which differed in several important aspects with those seen in acute insulin deficiency. The accumulation of P-pyruvate and 3-P-glycerate in forty-eight hour alloxan diabetic rats, when gluconeogenesis is accelerated, suggests that the rate limiting step in this system resides at some step between the conversion of 3-P-glycerate and dihydroxyacetone-P.

(4) Acute insulin deficiency of three-hour duration produced no significant changes in the levels of oxidized and reduced pyridine nucleotides. Chronic insulin deficiency (48-hr. alloxan diabetic rats) did not alter the content of TPN, TPNH or DPN but did cause a significant decrease (32 per cent) in the level of DPNH. DIABETES *15*:451-56, July, 1966.

The parenteral administration of anti-insulin serum (AIS) produces an acute state of diabetes mellitus and is associated with the progressive impairment of hepatic protein and fatty acid synthesis.[1,2] In the previous paper, it was shown that the factors responsible for these metabolic changes differ from those observed in the chronic insulin deficient alloxan diabetic rat. In the present study, the effect of acute insulin deficiency on the hepatic levels of several glycolytic and Krebs cycle intermediates and pyridine nucleotides was examined. Similar studies were also performed on 48-hr. alloxan diabetic rats. The results demonstrate that the metabolic response of the liver to acute insulin deprivation differs significantly from its ultimate adaptation to a more prolonged diabetic state and help to clarify the rate limiting steps involved in gluconeogenesis.

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METHODS

Animals. Male Sprague-Dawley rats (Holtzman Co., Madison, Wisconsin) weighing 175-200 gm. were matched according to age and weight (±5 gm.) and fed Purina laboratory chow ad libitum. Sucrose (10 per cent) was added to the drinking water 24 hrs. before the animals were killed.

Anti-insulin serum. Guinea pig anti-insulin serum of high titer was prepared by a modification of the method of Moloney and Coval.[3]

Experimental procedures. One animal of a pair received 2.0 ml. of guinea pig anti-insulin serum (AIS) intravenously in the tail vein at 0 time and at 90 min., and the control partner received normal guinea pig serum at the same time intervals. At 180 min. the animals were placed under light ether anesthesia and the abdomen was opened. A lobe of liver was isolated with a cup fashioned from aluminum foil and frozen *in situ* by continuous pouring of Freon 12 (CCl_2F_2) previously cooled to —150° C.[4] The frozen lobe was stored at —70° C. until analyses were performed. Heparinized blood samples for plasma glucose[6] and nonesterified fatty acids (NEFA) determinations were withdrawn from the inferior vena cava. NEFA was determined by the colorimetric method of Duncombe[7] using the Dole extraction procedure.[8] The remaining unfrozen portion of liver was utilized for the determination of hepatic protein and fatty acid synthesis already reported.[2]

Another group of rats of comparable age and weight was injected intravenously with alloxan monohydrate, 40 mg. per 100 gm. body weight, after an overnight fast. They were subsequently fed Purina chow and water ad libitum. After forty-eight hours, the rats were anesthetized and frozen liver and blood specimens obtained as described above. None of the AIS injected rats exhibited either ketonemia or ketonuria. Three of the alloxan diabetic animals demonstrated moderate ketonuria when tested by Acetest tablets.

Pyridine nucleotide and substrate analyses on frozen liver specimens. Samples of frozen liver (50 to 100 mg.) from control and diabetic rats were weighed at —20° C. Samples were homogenized at 0° C. in 0.02 M NaOH containing 0.5 mM cysteine for determination of reduced pyridine nucleotides and in 0.02 N H_2SO_4 with 0.1 M Na_2SO_4 for assay of the oxidized forms and heated at 60° C. Pyridine nucleotide content in these homogenates was measured by the enzymatic cycling and fluorometric technics of Lowry et al.[4,9,10] Glycolytic intermediates were determined by fluorometric methods on 2 M $HClO_4$ extracts of frozen liver after neutralization to *p*H 7.0 with $KHCO_3$.[11] When the anticipated level of a substrate was low (e.g., in the case of fructose diphosphate and dihydroxyacetone-P), the neutralized liver extracts as well as reagent blanks and standards were pretreated with washed Fluorisil, 60 mg./ml., to remove fluorescent materials such as flavins.

The assay for citrate employed a stabilized preparation of aconitase from pig heart. Since the enzyme was contaminated with glucose-6-P dehydrogenase and isocitrate dehydrogenase, glucose-6-P and isocitrate were removed first by preincubation in Tris buffer, *p*H 8.5, with an excess of purified dehydrogenases and TPN^+. The TPNH formed in these reactions was determined directly in a fluorometer. After the addition of aconitase, the TPNH subsequently formed represented the amount of citrate present.*

Malate was determined by its reaction with acetyl-DPN in the presence of malic dehydrogenase at *p*H 9 in hydrazine-hydrochloride buffer. The fluorescence of the acetyl-DPNH formed was used as a measure of this substrate.†

Glycogen was determined by modification of the procedures of Bueding and Hawkins[16] and Gatfield and Lowry.[17] The sample was first homogenized in 0.2 M HCl (fortyfold dilution) at 0° C. and then further homogenized after the addition of 5 mM EDTA (two hundredfold dilution). After heating for 10 min. at 100° C., aliquots of the suspension were assayed fluorometrically for glycogen at a forty thousandfold dilution in 20 mM potassium phosphate buffer, *p*H 7.2, containing AMP, $MgCl_2$ and TPN^+ in the presence of P-glucomutase, phosphorylase containing amylo-1-6-glucosidase and glucose-6-P dehydrogenase. The fluorescence of TPNH formed was a measure of glucose-6-P produced from glycogen. Standardized rabbit liver glycogen (Mann Research Laboratories, Inc., N.Y., N.Y.) treated similarly was used as the basis for calculations.

RESULTS

Effect of AIS and alloxan diabetes on plasma glucose and NEFA Levels (table 1). Plasma glucose and NEFA levels increased approximately threefold within three hours after the initial injection of AIS. Animals receiving nonimmune guinea pig serum at the same dosage schedule showed no significant change in either plasma glucose or NEFA content. The highest concentrations of plasma glucose and NEFA were observed in the 48-hr. alloxan diabetic rats.

TABLE 1

Plasma glucose and nonesterified fatty acid (NEFA) concentrations in rats following intravenous administration of anti-insulin serum (AIS) or alloxan. Numbers in parentheses indicate number of animals. Values are mean ± SEM

	Plasma glucose (mg. per 100 ml.)	Plasma NEFA (μEq./L.)
Control (9)	148± 7	432±34
AIS (9)	460±27	1,379±65
AIS + acute adrenex (5)	426±32	1,266±110
Alloxan (5)	857±12	1,635±80

Effect of AIS and alloxan diabetes on hepatic content of glycolytic and Krebs cycle intermediates (table 2). Hepatic glycogen content decreased 43 per cent from control levels of 5.5 gm. per 100 gm. wet wt. (304 μmoles glucose equivalent/gm. wet wt.) to 3.1 gm. per 100 gm. (173 μmoles glucose equivalent/gm. wet wt.) within three hours after the injection of AIS serum. To exclude variations in epinephrine secretion as a causative factor for the changes observed in these studies, rats were adrenalectomized 30 min. before beginning AIS treatment. This did not affect the response of the blood glucose, plasma NEFA or hepatic glycogen level to AIS injection (table 1 and 2).

Although the free glucose content of liver was

*Goldberg, N., Passoneau, J. V., and Lowry, O. H., unpublished method.

† Fleming, M., Passoneau, J. V., and Lowry, O. H., unpublished method. A modification of the spectrophotometric technic of Hohorst[14] and Holzer and Soling.[15]

TABLE 2

Glycolytic intermediates in livers of rats following the administration of anti-insulin serum or alloxan. All values are expressed in millimicromoles/gm. wet wt., except glycogen and glucose which are expressed as micromoles glucose/gm. wet wt., and represent mean ± SEM. Numbers in parentheses indicate number of animals.

Intermediate	Control (9)	AIS (9)	Alloxan (5)
Glycogen	304± 3.0	173± 30.0* (160± 47.0)*†	66± 16.0*
Glucose-6-P	195± 22.0	329± 34.0*	209± 13.0
Glucose	7.1± 0.4	16.7± 1.6*	35.4± 4.7*
Fructose diphosphate	38± 5.0	17± 2.0*	21± 1.0*
Dihydroxyacetone-P	44± 3.0	26± 2.0*	20± 1.0*
α-Glycerol-P	404± 42.0	246± 23.0*	303± 41.0
3-P-glycerate	296± 40	277± 52.0	424± 44.0
P-pyruvate	113± 12.0	153± 31.0	189± 22.0*
Pyruvate	252± 23.0	137± 24.0*	88± 9.0*
Lactate	2,640±220.0	1,800±230.0*	3,010±220.0*

*p value < 0.01.
†Five rats were adrenalectomized 30 min. before AIS treatment was begun.

markedly increased after AIS injection, its distribution ratio between the plasma and tissue water was not affected. In control animals, plasma glucose content was 8.2 ± 0.4 μmoles/ml. and in liver, 8.8 ± 0.5 μmoles/ml. tissue water (based on a total tissue water content of liver of 0.80 ± 0.06 ml./gm. wet wt. as determined by drying to constant weight at 100° C.). A similar distribution equilibrium was seen in AIS treated (plasma glucose, 25.6 ± 1.5 μmoles/ml.; hepatic glucose, 21.0± 2.1 μmoles/ml.) and alloxan diabetic rats (plasma glucose 47.5 ± 0.7 μmoles/ml.; hepatic glucose, 44.2 ± 5.0 μmoles/ml.). These results indicate that glucose penetration across the cell membrane is not a limiting event in hepatic glucose metabolism nor is this process influenced by either the acute or chronic deprivation of insulin.

The concentration profile of the glycolytic intermediates (table 2) in the livers of AIS treated rats differed significantly from that seen in control animals. Glucose-6-P increased 70 per cent whereas fructose diphosphate, dihydroxyacetone-P, α-glycerol-P, pyruvate and lactate decreased to levels ranging from 40 to 65 per cent of control values $p < 0.01$. In spite of these changes, the lactate/pyruvate and α-glycerol-P/dihydroxyacetone-P did not differ from control ratios (table 3). Although the P-pyruvate level also tended to increase in AIS treated rats (35 per cent greater than control levels), the content of 3-P-glycerate was not significantly altered. Furthermore, neither the citrate nor malate content was significantly increased in AIS treated rats (table 4).

Similar measurements in alloxan diabetic rats revealed several striking differences with the results obtained with AIS treated animals. Glycogen depletion was more marked, the content being less than 22 per cent of control animals. Although the fructose diphosphate and dihydroxyacetone-P levels were similar to those seen in animals receiving AIS, glucose-6-P was not increased. P-pyruvate, on the other hand, was increased 68 per cent, 3-P-glycerate 43 per cent, citrate 100 per cent and malate 61 per cent above control values (in each case $p < 0.01$). Another distinguishing feature of the alloxan diabetic liver was the fall in pyruvate and dihydroxyacetone-P relative to lactate and α-glycerol-P respectively, resulting in significantly greater lactate/pyruvate and α-glycerol-P/dihydroxyacetone-P ratios than noted in control and AIS treated animals (table 3).

Effect of AIS and alloxan diabetes on hepatic pyridine nucleotide levels. The pyridine nucleotide values

TABLE 3

Substrate concentration ratios in liver of AIS and alloxan diabetic animals. Substrate levels obtained from table 1. Numbers in parentheses indicate number of animals. All values represent mean ± SEM

	Lactate pyruvate	α-glycerol-P dihydroxyacetone-P
Control (9)	11±0.9	9±0.6
AIS (9)	14±0.7	9±0.6
Alloxan (5)	34±2.0*	15±1*

*p value < 0.01.

TABLE 4

Tissue levels of citrate and malate in livers of AIS and alloxan diabetic rats. Values expressed in millimicromoles/gm. wet wt., ± SEM. Asterisk denotes p value <0.01. Numbers in parentheses indicate number of values.

Intermediate	Control (9)	AIS (9)	Alloxan (5)
Citrate	196±21.0	222±23.0	392±22.0*
Malate	382±33.0	400±21.0	612±72.0*

obtained in the control group of this study (table 5) are in agreement with the levels previously reported for normal rat liver using the same analytical procedures.[4] AIS treatment did not affect the hepatic content of either DPN or DPNH. The DPNH level of 48-hr. alloxan diabetic rats, however, was significantly decreased ($p < 0.05 > 0.01$) resulting in a slight increase in the DPN/DPNH ratio. No significant differences (p 0.1) were observed in the levels of oxidized and reduced TPN in the livers of either AIS or alloxan diabetic rats.

TABLE 5

Oxidized and reduced pyridine nucleotides in livers of rats given anti-insulin serum or alloxan. Number of animals indicated in parentheses. Values are mean ± SEM

Value	Control (5)	AIS (5)	Alloxan (5)
	(millimicromoles/gm. fresh weight)		
DPN	846±18	808±25	878±35
DPNH	78± 7	85±14	53± 5*
Total DPN	924±11	893±33	930±31
DPN/DPNH	11.2± 1.0	10.5± 1.7	17± 2
TPN	367±15	400±33	362±16
TPNH	372±15	396±16	324±15
Total TPN	739±11	796±38	686±29
TPN/TPNH	1.0± .07	1.0± 0.1	1.1± .01

*$p < 0.05 > 0.01$

DISCUSSION

The rapid development of hyperglycemia in AIS-treated rats reflects both impaired peripheral utilization of glucose as well as an accelerated rate of hepatic glycogenolysis. Previous studies in this laboratory have shown that glucose utilization by striated muscle is reduced to the level observed in chronic alloxan diabetic animals within 90 min. following the intravenous injection of anti-insulin serum.[16] Approximately 250 mg. of glucose is released from the liver of a 200 gm. rat during this period as calculated from the data in table 2. The addition of this quantity of sugar to the glucose pool, assuming a glucose volume of distribution of 30 per cent of total body weight,[17] would increase the plasma level approximately 400 mg. per 100 ml. Since the observed plasma level increased 310 mg. per 100 ml., this would indicate that 90 to 100 mg. were either excreted in the urine or utilized during this period, representing a maximal utilization rate of 10-20 mg. per hr. per 100 gm. body wt., a level of utilization previously reported for the chronically insulin deprived animal.[17,18]

One of the most striking features of the acutely insulin deficient rat is the rapid increase in the hepatic level of glucose-6-P. This change can be attributed to both rapid glycogenolysis and concomitant impairment of utilization of this substrate by the glycolytic and hexosemonophosphate pathways. Inhibition of glycolysis occurs at the P-fructokinase step as is evident from the marked decrease in the level of fructose diphosphate. A similar change in fructose diphosphate has also been observed in cardiac muscle of the alloxan diabetic rat.[20] The decreased levels of the triose phosphates, although consistent with impaired glycolysis, might also reflect decreased activity of the hexose monophosphate shunt. This possibility deserves further attention in view of the recent demonstration by Eger-Neufeldt et al.[19] that glucose-6-P dehydrogenase is competitively inhibited by long chain acyl CoA derivatives, substrates which are markedly increased in liver following insulin deprivation.[2] A depression in both the glycolytic and hexosemonophosphate pathways in association with decreased glycogen synthetase activity[21] would all contribute to the decreased rate of hepatic glucose utilization typical of the insulin deficient state. The normal levels of glucose-6-P seen in the chronically insulin deficient alloxan diabetic animal, even in the presence of increased rates of gluconeogenesis, can be accounted for by the increase in glucose-6-phosphatase activity known to occur in this preparation.[22-24]

Recently Passoneau and Lowry reported that citrate is a potent inhibitor of P-fructokinase.[25] Parmeggiani and Bowman observed that citrate levels were increased significantly in the liver of the 48-hr. alloxan diabetic rat and suggested that the accumulation of this intermediate was the cause of impaired glycolysis.[26] The present study also demonstrates an increase of both citrate and malate in the liver of 48-hr. alloxan diabetic rats. However, in AIS-treated animals, at a time when P-fructokinase activity is decreased, hepatic citrate levels remained unchanged. Although these data question the role of citrate as a primary factor responsible for impaired glycolysis, it should be emphasized that citrate is compartmentalized in the liver cell[27] and consequently small changes in one compartment sufficient to influence glycolysis may be masked in whole tissue determinations. Furthermore, the possibility that changes in inorganic phosphate, AMP, ATP, or other factors known to influence P-fructokinase activity[25] may be responsible for the changes seen following acute insulin deficiency cannot be excluded.

The decreased levels of pyruvate and the rise in P-pyruvate seen in both the acutely and chronically insulin deficient rat suggest a marked acceleration of the pyruvate carboxylase-phosphoenolpyruvate carboxykinase system. This is consistent with recent reports of the rapid appearance of increased levels of the latter enzyme fol-

lowing acute insulin privation[28] and the activation of the pyruvate carboxylase enzyme by acetyl CoA,[29] a substrate known to be increased in the alloxan diabetic liver.[30] Although increased gluconeogenesis has not been observed in liver slices obtained from AIS treated rats[22] accelerated precursor incorporation into plasma glucose has been observed in vivo in the AIS treated rat. It would appear, therefore, that acute changes in the activity of the pyruvate carboxylase-phosphoenolpyruvate carboxykinase system are causally related to concomitant changes in gluconeogenesis.

The hepatic content of oxidized and reduced DPN and TPN in alloxan diabetic rats has been reported to rise, fall or remain unchanged.[31-36] In the present study, utilizing a rapid freezing technic and procedures designed to minimize artifactual changes during extraction[4,5,9,10] no significant change in pyridine nucleotide content was observed in AIS-treated rats. A slight but significant decrease in DNPH level was noted in 48-hr. alloxan diabetic rats. Hohorst et al.[37,38] have reported that the reduction state of the DPN system in the soluble compartment of the liver cell may be calculated from the concentration ratios of lactate/pyruvate and α-glycerol-P/dihydroxyacetone-P. In AIS-treated animals, the failure to demonstrate any alteration in these substrate concentration ratios would indicate that the DPN/DPNH ratio in the soluble compartment remained unchanged. The marked increase in lactate/pyruvate and α-glycerol-P/dihydroxyacetone-P ratios observed in chronic alloxan diabetic rats would indicate an increased state of reduction of the soluble pyridine nucleotide system. Yet the total DPN/DPNH tissue ratio, if anything, increased slightly. This emphasizes the fact that the determination of total tissue pyridine nucleotide levels does not necessarily reflect their oxidation-reduction state in the various subcellular compartments. The failure to demonstrate any significant changes in absolute or relative TPN/TPNH levels also supports the conclusions of others[2,39-41] that decreased TPNH availability is not responsible for the impaired lipogenesis observed under these conditions.

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