Studies on Mechanism of Glucose Synthesis in Diabetic and Normal Rat Liver

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SUMMARY

Glucose synthesis and enzymatic activities of various CO_2-fixing enzymes have been studied in normal and diabetic liver preparations. A three-fold increase in pyruvate carboxylase and α-ketoglutarate carboxylase has been observed in alloxan-diabetic, anti-insulin serum and cortisol treated liver preparations as compared to normal. The increased glutarate carboxylase activity was found to be localized in the mitochondrial fraction. In addition a six-fold increase in phosphoenolpyruvate carboxykinase was observed. Studies on net glucose synthesis show that combination of pyruvate and glutamate are far more effective than any single substrate. Furthermore, oxaloacetate utilization was found to be increased in diabetic and cortisol treated rat livers. Based on these results a pathway for gluconeogenesis has been formulated. It is suggested that increased gluconeogenesis observed in diabetic animals involves CO_2 fixation with pyruvate and glutamate giving rise to increased formation and utilization of oxaloacetate. DIABETES *15*:19-23, January, 1966.

It has been recognized for many years that the hormones of the adrenal cortex promote hepatic gluconeogenesis[1,2] and that hepatic glucose production is increased in diabetes mellitus.[3,4] A systematic study[5-10] has been undertaken in this laboratory over the past few years to investigate the mechanisms of gluconeogenesis under dietary and hormonal treatment. Livers from diabetic rats show an increase in C-14 glucose formation when incubated with a variety of labeled substrates including radioactive bicarbonate.[5,6] Since reversal of glycolysis from pyruvate involves CO_2 fixation, attention has been focused on activities of several enzymes involved in the formation of phosphoenolpyruvate (PEP) from pyruvate. PEP formation in liver is mediated by an enzyme phosphoenolpyruvate carboxykinase and oxalacetate and inosine triphosphate or guanosine triphosphate are essential cofactors. The properties of this enzyme were first described by Utter and Kurahashi,[11] and it has recently been found that PEP carboxykinase activity increases rapidly in acute insulin insufficiency[7] or treatment with adrenal glucocorticoids.[12] It now appears that in liver pyruvate may be directly carboxylated to form oxalacetate. The properties of this enzyme, pyruvate carboxylase, have been described by Utter and associates.[13,14] Another reaction involving CO_2 fixation that may also contribute to glucose formation involves carboxylation of α-ketoglutarate and reversal of the citric acid cycle with subsequent cleavage of citrate to form oxalacetate and actyl CoA.[15,16] The present study reports observations on glucose formation from various metabolic precursors and enzymatic reactions involving carbon dioxide fixation leading to increased gluconeogenesis in livers of normal and alloxan-diabetic rats.

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EXPERIMENTAL

A. *Animals.* Male albino rats of the Wistar strain, weighing between 100-150 gm., were used. They were fed ad libitum on Purina Laboratory Chow. Alloxan diabetes was produced by intravenous injection of alloxan monohydrate (40 mg. per kilogram of body weight) in a manner described previously.[5]

B. *Studies on enzymatic activities involving CO_2-fixation enzymes in diabetic and normal rats.* Normal and diabetic rats were killed by decapitation, their livers removed, homogenized and various subcellular fractions obtained by differential centrifugation were assayed for enzymatic activities. Phosphoenolpyruvate carboxykinase activity was assayed[8,11] in 105,000 × g supernatant (equivalent to 150 mg. liver tissue) with 5 μc bicarbonate (specific activity 0.1 μc per μmole), 20 μmoles of oxaloacetate, 2 μmoles of $MnCl_2$ and with or without inosine triphosphate (2.0 μmoles). At the end of ten minutes, incubation mixtures were deproteinized with 0.5 ml. of 10 per cent trichloroacetic acid and gassed with CO_2 for fifteen minutes. All steps were done at 5° C. C-14-O_2 fixed was counted from an aliquot of filtrate in an anthracene packed cuvette in a Packard Tri-carb Scintillation counter. Pyruvate carboxylase was also assayed in the 105,000 × gm. supernatant fraction but liver homogenates were prepared at room

temperature.[9] The enzymatic activity was measured by incubating an aliquot of the supernatant fraction (equivalent to 150 mg. fresh liver) with 20 μmoles of K-pyruvate, 50 μmoles $NaHCO_3$ (5 μc), 3.3 μmoles $MgCl_2$, 1.25 μmoles ATP, 0.38 μmoles acetyl CoA and 50 μmoles Tris HCl *p*H 7.6. At the end of ten minutes, incubation mixtures were deproteinized and the CO_2 fixed was assayed as described earlier. Glucose-6-phosphatase activity was assayed in whole homogenates incubated for thirty minutes in citrate buffer with glucose 6-phosphate as the substrate.[8] The results of these various studies are given in table 1. CO_2 fixation stimulated by αketoglutarate was assayed[17] in mitochondria (equivalent to 50 mg. of liver tissue) with 50 μmoles of $NaHCO_3$ (5 μc), 3.0 μmoles of $MnCl_2$, 1.25 μmoles ATP, 2.0 μmoles DPNH and TPNH and 40 μmoles of αketoglutarate in phosphate buffer *p*H 7.6. In addition effect of succinate, malonate and aniline hydrochloride was also studied. CO_2 fixed was assayed as ascribed earlier. The results of these studies are given in table 2.

C. *Studies on net glucose synthesis using pyruvate, glutamate and glutarate.* Rats were fasted overnight, and glucose production by liver slices incubated in a Ringer-bicarbonate medium using different substrates was studied. In these experiments duplicate samples were employed for the determination of initial and final glucose concentrations. Approximately 400 mg. of liver slices were incubated in 6.0 ml. of Ringer-bicarbonate medium with pyruvate, glutamate and α-ketoglutarate to give an initial concentration of 1 mg. per milliliter of medium. At the end of ninety minutes of incubation, homogenates were prepared of slices in media, and total carbohydrates were determined by the anthrone method.[18] Net glucose production was determined by the difference in initial and final carbohydrate values. The results are given in figure 1.

D. *Studies on utilization of oxaloacetate.* Increases in pyruvate carboxylation observed in diabetic liver preparations may be influenced by the rate of removal of oxaloacetate formed. In order to exclude this possibility the removal of oxaloacetate by normal and diabetic liver preparations was studied. Homogenates and 105,000 × g supernatant fractions from normal and diabetic rats were incubated with oxaloacetate for ten minutes. At the end of the incubation unused oxaloacetate was measured by the procedure of Tonhazy et al.[19] The results of this study are given in table 3.

TABLE 1

Activities of various enzymes in normal and experimental animals*

Type of animals used	PEP carboxy-kinase	Pyruvate carboxylase	Glucose G-phosphatase
Normal fed	100	100	100
Normal fasted (12 hrs.)	128±15	130±14	136±14
Normal serum treated (12 hrs.)	108±12	102±6	133±14
Anti-insulin serum treated (12 hrs.)	520±46	230±22	128±12
Cortisol treated (five days)	230±28	240±28	180±21
Alloxan-diabetic	720±68	320±32	215±23

*PEP carboxykinase and pyruvate activity was expressed as μmoles CO_2 fixed per gram liver. Glucose 6-phosphate activity was expressed as μmoles Pi released from glucose 6-phosphate per gram wet liver. The values for normal rat livers were 5.72±0.56 μmoles CO_2 fixed/gm. for PEP carboxy-kinase; 12.8±1.1 μmoles CO_2 fixed/gm. for pyruvate carboxylase and 147.0±9 μmoles Pi/gm. for glucose 6-phosphatase. These values were taken as 100 per cent. Each figure is the per cent of normal and is an average of six values.

TABLE 2

Fixation of C-14-O_2 by mitochondria from normal and diabetic preparations*

	Normal	Diabetic
Complete system — glutarate	2.70±0.18	4.56±0.36
Complete system + glutarate	16.80±0.18	67.92±7.20
Complete system + glutarate + malonate	8.16±0.60	52.80±4.56
Complete system + glutarate + aniline • HCl†	10.38±0.72	18.6±1.56
Complete system — succinate	0.72±0.06	1.56±0.06
Complete system + succinate	7.38±0.54	16.08±1.26
Complete system + succinate + malonate	2.88±0.18	3.84±0.24

*Complete system contained mitochondrial preparation equivalent to 50 mg. liver, 50 μmoles of $NaHCO_3$ (5 uC), 3.0 μmoles of $MnCl_2$, 1.25 μmoles of ATP and 2.0 μmoles of DPHN and TPNH; 40 μmoles of α-ketoglutarate or succinate was added per incubation. All values are expressed as μmoles C-14-O_2 incorporated per gram wet liver per hour.

†Aniline • HCl was added after the incubation.

RESULTS

Studies on various enzymes in livers of diabetic and normal rats are summarized in table 1. It can be seen that pyruvate carboxylase activity is increased three to fourfold, whereas phosphoenolpyruvate carboxylinase activity is increased six to sevenfold. Similar increases in enzymatic activities were observed in cortisol and anti-insulin treated animals. Fasting or administration of normal serum for twelve hours had no effect on these enzymatic activities. The results (table 2) clearly show

TABLE 3

Utilization of oxaloacetate by liver preparation from normal and experimental animals*

Type of animal used	Oxaloacetate utilized Homogenate (μmoles/gm./10 min.)	Supernatant (μmoles/gm./10 min.)
Normal (fed)	40.0±3.0	23.6±3.2
Normal fasted (12 hrs.)	46.0±3.2	27.2±2.8
Normal serum treated (12 hrs.)	36.6±4.2	21.3±2.8
Anti-insulin serum treated (12 hrs.)	56.6±6.3	28.5±2.8
Cortisol treated (5 days)	88.0±6.0	34.5±4.0
Alloxan diabetic	126.0±8.0	48.2±6.8

*Homogenate or supernatant fraction equivalent to 50 mg. of liver tissue was incubated with 200 μmoles of oxaloacetate, 10 μmoles inosine triphosphate and 10 μmoles of $MnCl_2$. At the end of ten minutes of incubation, unused oxaloacetate was determined. Each figure is an average of six values.

a three-fold increase in carboxylation of α-ketoglutarate by diabetic liver preparations as compared to normal. Addition of malonate inhibited CO_2 fixation by 50 per cent in normal whereas it had little effect in diabetic preparations with glutarate as the substrate. Furthermore, with succinate as the substrate very little CO_2 was fixed, and this was completely inhibited by the addition of malonate in both normal and diabetic liver preparations. Loss of radioactivity with aniline hydrochloride addition would suggest that this CO_2 fixed was incorporated into a β-keto acid (i.e. oxaloacetate or oxalosuccinate).

Liver slices show net glucose synthesis (figure 1) from pyruvate, glutamate and α-ketoglutarate and an additive effect is observed with the combination of substrates. In all cases, more glucose was formed by diabetic liver preparation as compared to normal. Because of high liver glycogen level it was difficult to demonstrate net glucose production by liver slices from fed rats and hence twelve-hour fasted rats were used.

Table 3 summarizes the data on oxaloacetate utilization in diabetic and normal liver preparations. Both homogenates and supernatant fractions from diabetic liver preparations utilize more oxaloacetate than normal liver preparations. There was a three-fold increase in oxaloacetate utilization in diabetic rat liver and two-fold increase in liver from cortisol treated animals when compared to normal liver. It was further observed that homogenates were more effective than supernatant fractions.

DISCUSSION

The diabetic state has been characterized as involving both overproduction and underutilization of glucose. In previous studies the metabolism of several labeled amino acids by normal and diabetic rat liver preparations[5,6] showed a greater incorporation of amino acids and other substrates into glucose in diabetic livers than in normal livers. These results suggested a metabolic pattern reflecting preferential utilization of nonglycogen carbons for glucose formation. These studies with the liver of diabetic and cortisol-treated rats suggest that both insulin and adrenal steroids may be involved in the regulation of CO_2 fixation. This concept is in harmony with the increased activities of the hepatic enzymes phosphoenolpyruvate carboxykinase[7,12] and pyruvate carboxylase,[9,20-22] which are involved in CO_2 fixation and gluconeogenesis in diabetic or cortisol treated rats.

Reversal of glycolysis from pyruvate to phosphoenolpyruvate appears to proceed primarily via oxaloacetate as an intermediate. Topper and Hastings[23] found that the dicarboxylic acid shuttle was a very important pathway in the synthesis of glucose from pyruvate. Increased conversion of pyruvate and glutamate to glucose would therefore result in an increase in CO_2 fixation which indeed has been found to be increased in the diabetic rats.[10] The present studies indicate two possible mechanisms for the increase in CO_2 fixation observed in diabetic and cortisol treated rats.

The pathway for oxaloacetate formation from pyruvate[13,14] and α-ketoglutarate[17,24] through CO_2 fixation has been established. In the present studies, a three-fold increase in CO_2 fixation in diabetic animal preparations in the presence of pyruvate and α-ketoglutarate and an additive effect observed on net glucose synthesis with glutamate and pyruvate and accumulation of acetyl CoA[25] in the diabetic liver preparation would suggest the following pathway for gluconeogenesis in diabetes (figure 2). Similar pathway for lipid synthesis through the reversal of tricarboxylic acid cycle in epididymal fat pad has been recently suggested.[16]

The mechanism of glucose synthesis may fall into two parts. First formations of oxaloacetate from various precursors and second conversion of this oxaloacetate to phosphoenolpyruvate for the conversion to glucose. This may constitute a self-regulating mechanism for glucose synthesis which in turn depends on the supply of oxaloacetate formed from precursors such as pyruvate and glutamate by CO_2 fixation in the mitochondrial compartment and to be transferred from the mitochondria

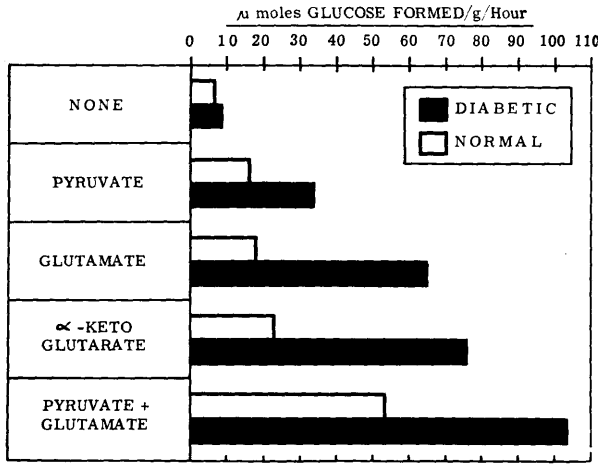

FIG. 1. Studies on net glucose formation with various substrates in diabetic and normal rat liver slices. Approximately 400 mg. of liver slices were incubated in 6.0 ml. of Ringer-bicarbonate medium with various substrates (8mM). All values are expressed in μmoles glucose formed/gm. per hour.

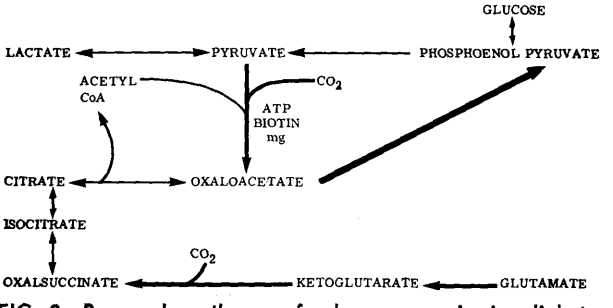

FIG. 2. Proposed pathway of gluconeogenesis in diabetes.

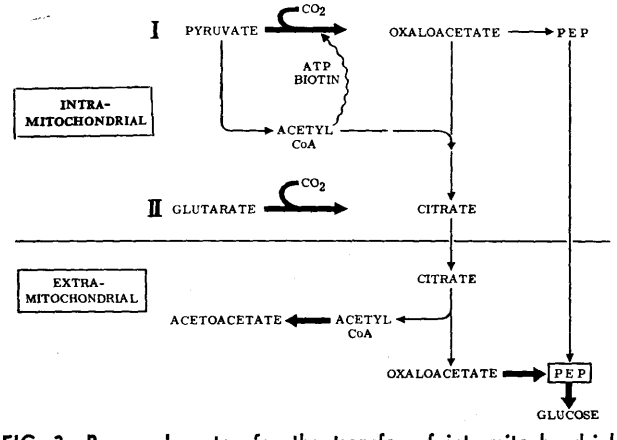

FIG. 3. Proposed routes for the transfer of intramitochondrial precursors to the extramitochondrial compartment of the cell for the conversion to glucose. Thick lines indicate the pathways that are stimulated in diabetic animals.

into the extramitochondrial compartment of the cell where enzymes synthesizing glucose from oxaloacetate are located. Figure 3 shows alternative routes that may be involved in the supply of various precursors for glucose synthesis in the cell. These routes involve formation of oxaloacetate from pyruvate and CO_2 in mitochondria, and part of this oxaloacetate formed is further converted to phosphoenolpyruvate and part is condensed with acetyl CoA to form citrate. In addition, citrate is also being formed primarily by reversal of tricarboxylic acid cycle by CO_2 fixation with glutarate. Citrate formed is then diffused out of the mitochondria which is cleaved by cleavage enzyme to give oxaloacetate which is further converted to phosphoenolpyruvate to glucose. Various increases in enzymatic activities observed in mitochondrial and supernatant fraction are agreeable with the present scheme.

ACKNOWLEDGMENT

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REFERENCES

[1] Long, C. N. H., Katzin, B., and Fry, E. G.: The adrenal cortex and carbohydrate metabolism. Endocrinology *26*:309-44, 1940.

[2] Lewis, R. A., Kuhlman, D., Delbue, C., Koepf, F. G., and Thorn, G. W.: The effects of the adrenal cortex on carbohydrate metabolism. Endocrinology *27*:971-82, 1940.

[3] Bondy, P. K., Bloom, W. L., Whitner, V. S., and Farrar, B.: Studies on the role of the liver in human carbohydrate metabolism by the venous catheter technique. II Patients with diabetic ketosis before and after administration of insulin. J. Clin. Invest. *28*:1126-33, 1949.

[4] Feller, D. D., Strisower, E. H., and Chaikoff, I. L.: Turnover and oxidation of body glucose in normal and alloxan diabetic rats. J. Biol. Chem. *187*:571-88, 1950.

[5] Wagle, S. R., and Ashmore, J.: Interrelationships between amino acid metabolism and carbohydrate formation in insulin deficiency. J. Biol. Chem. *236*:2868-71, 1961.

[6] Wagle, S. R., and Ashmore, J.: Studies on experimental diabetes. II. Carbon dioxide fixation. J. Biol. Chem. *238*:17-20, 1963.

[7] Wagle, S. R., and Ashmore, J.: Studies on carbon dioxide fixation in normal and alloxan-diabetic animals. Biochim. Biophys. Acta *74*:564-65, 1963.

[8] Wagle, S. R., and Ashmore, J.: Studies on experimental diabetes III. Effects of acute insulin insufficiency on C-14-glucose formation from labeled substrates. J. Biol. Chem. *239*: 1289-91, 1964.

[9] Wagle, S. R.: Studies on pyruvate carboxylase activity in alloxan diabetes and normal animals. Biochim. Biophys. Res. Comm. *14*:533-36, 1964.

[10] Wagle, S. R.: Studies on mechanism of gluconeogenesis in

diabetes. Biochim. Biophys. Acta 97:142-44, 1965.

[11] Utter, M. F., and Kurahashi, K.: Oxalacetate synthesizing enzyme, in Methods in Enzymology. S. P. Colowick and N. O. Kaplan, Vol. 1. New York, Academic Press, p. 758.

[12] Shrago, B., Lardy, H. A., Nordlie, R. C., and Foster, D.: Metabolic and hormonal control of phosphoenol pyruvate carboxykinase and malic enzyme in rat liver. J. Biol. Chem. 238:3188-92, 1963.

[13] Utter, M. F., and Keech, D. B.: Pyruvate carboxylase. I. Nature of the reaction. J. Biol. Chem. 238:2603-08, 1963.

[14] Keech, D. B., and Utter, M. F.: Pyruvate carboxylase II. Properties. J. Biol. Chem. 238:2609-14, 1963.

[15] Spencer, A. F., and Lowenstein, J. M.: The supply of precursors for the synthesis of fatty acids. J. Biol. Chem. 237:3640-48, 1962.

[16] Madsen, J., Abraham, S., and Chaikoff, I. L.: Conversion of glutamate carbon to fatty acid carbon via citrate in rat epididymal fat pads. J. Lipid Res. 5:548-53, 1964.

[17] Ochoa, S.: Isocitric dehydrogenase and carbon dioxide fixation. J. Biol. Chem. 159:243-44, 1945.

[18] Roe, J. H.: The determination of sugar in blood and spinal fluid with anthrone reagent. J. Biol. Chem. 212:335-43.

[19] Tonhazy, N. E., White, N. G., and Umbreit, W. W.: A rapid method for the estimation of the glutamic-aspartic transaminase in tissues and its application to radiation sickness. Arch. Biochem. 28:36-42, 1950.

[20] Henning, H. V., Seiffert, I., and Seubert, W.: Cortisol Induzierter ansteig der Pyruvatcarboxylase-Aktivitat in der Rattenleber. Biochim. Biophys. Acta 77:345-48, 1963.

[21] Prinz, W., and Seubert, W.: Effect of insulin on pyruvate carboxylase in alloxan diabetic animals. Biochem. Biophys. Res. Comm. 16:582-85, 1964.

[22] Freedman, A. D., and Kohn, L.: Pyruvate metabolism and control: factors affecting pyruvic carboxylase activity. Science 145:58-60, 1964.

[23] Topper, Y. J., and Hastings, A. B.: A study of the chemical origins of glycogen by use of C-14-labeled carbon dioxide, acetate and pyruvate. J. Biol. Chem. 179:1255-64, 1949.

[24] Ochoa, S.: Biosynthesis of tricarboxylic acids by carbon dioxide fixation. J. Biol. Chem. 174:133-57, 1948.

[25] Wieland, O., and Weiss, L.: Increase in liver acetyl-coenzyme A during ketosis. Biochim. Biophys. Res. Comm. 10: 333-39, 1963.

The Status of Artificial Sweeteners

A review of recent studies on artificial sweeteners shows they are safe as presently used, the Food and Drug Administration, Department of Health, Education, and Welfare, announced recently.

FDA said the review was made because of recent questions regarding the safety of artificial sweeteners—specifically sodium cyclamate. The agency added that, as is the case with many substances in foods, scientific studies of the extended use of artificial sweeteners in various amounts will continue, and these studies will all be evaluated in light of current uses.

FDA said that artificial sweeteners—saccharin and the cyclamates—have been used in foods for many years to provide a reduced caloric intake by replacing sugar in the diet. Such products are labeled as foods for special dietary use.

The Food and Nutrition Board of the National Academy of Sciences—National Research Council in 1955 recommended that artificial sweeteners be used in special purpose foods for those who must restrict their intake of sugar and total food energy. It cited, however, evidence that cyclamates may produce a mild laxative effect at intakes of five grams or more per day and suggested the need for additional studies of the safety of cyclamates when used at the higher levels. In 1962 the Food and Nutrition Board republished its recommendations in a similar fashion.

Within the past year, FDA has received new experimental data on the safety of cyclamates, including animal studies, tests involving ingestion by children and other data. FDA's Bureau of Medicine and Division of Toxicological Evaluation have reviewed these studies and concluded that there is no evidence that cyclamates at present use levels are a hazard to health.

U. S. Department of Health, Education, and Welfare