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Oxidation, Accumulation, and Turnover of Citrate in Normal and Diabetic Rats (38308)

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In a previous publication (1) we reported *in vitro* changes in the activity of certain enzymes of the citric acid cycle. We found decreased utilization of isocitrate by muscle and elevated production of citrate by liver in diabetic rats. Although production of citrate was depressed in diabetic rat muscle, this decrease was smaller than the decrease in isocitrate utilization and this may have resulted in an increased concentration of citric acid in plasma and tissues of diabetic animals and patients as described by earlier workers (2–5). The significance of our findings, however, could not be ascertained until studies of citrate metabolism were carried out in living animals. In this paper we report rate of oxidation, accumulation, and turnover of citrate in normal and alloxan diabetic rats.

Methods. Animals. The procedure for the alloxanization of rats has been described previously (1).

Determination of citric acid. The assay for citrate is based on the procedure used by Moellering and Gruber (6). The citric acid present in the sample is converted to acetate and oxaloacetate by the addition of an excess of citrate lyase, and the resulting oxaloacetate is reduced to malate by NADH in the presence of malate dehydrogenase. The decrease in optical density at 340 nm is proportional to the amount of citrate present. Citrate determinations were run in duplicates and the values obtained agreed within 5%.

Accumulation of citrate in vivo. The accumulation of citrate *in vivo* was studied by blocking aconitase activity with intraperitoneal injections of *trans*-aconitate. This substance has been shown (7) to competitively inhibit aconitase in muscle, kidney, and liver, resulting in the accumulation of citrate. When citrate utilization was thus inhibited, the rate of increase of plasma citrate reflected the rate of citrate production by the animals, provided that the channeling of citrate to routes other than oxidation (namely urinary excretion, cleavage and deposition into bone) is negligible. Six to seven month old male rats were anesthetized with ether and one ml of blood was withdrawn by cardiac puncture with a heparinized syringe. *Trans*-aconitic acid was dissolved in an equivalent solution of Na_2CO_3 (4.3 *N*) to a final concentration of 200 mg/ml and the resulting CO_2 removed by aeration. The freshly prepared solution was injected intraperitoneally in doses of 2 g of the free acid/kg body wt. In another set of rats a larger dose (3 g/kg) was administered as follows: 2 g/kg followed by two 0.5 g/kg doses separated by 40 min intervals. One ml blood samples were obtained by cardiac puncture at predetermined intervals after the injection of *trans*-aconitate. Three to five samples were obtained from each rat, the plasma separated by centrifugation. Plasma citrate concentrations were plotted against the time after injection.

The oxidation of citric acid in vivo. Seven to nine weeks old or 1 yr old male rats were anesthetized with ether and blood samples were obtained by cardiac puncture. Forty microliters of a solution containing 6-^{14}C sodium citrate (New England Nuclear, Boston, MA. Specific activity 10 μCi/ml, 0.324 mg/ml) were diluted in 0.5 ml of isotonic saline and injected peritoneally. The animal was immediately kept in the CO_2 collecting system consisting of a large, air-tight desiccator jar containing a stainless steel wire platform (0.5 in. mesh) on which the animal was placed. At the bottom of the desiccator 3 ml of 0.1 *N* H_2SO_4 were placed in order to acidify the voided urine. The jar was connected to a water trap consisting of glass tubes placed in ice containing 5 ml of anhydrous methanol. The water trap was in turn connected to CO_2 collecting

vials containing 11 ml of a mixture of ethanolamine and anhydrous methanol (3:7 vol/vol). Such a mixture can absorb up to 600 mg CO_2 and collect 97–99% of the radioactivity passed during any collection interval. The water trap was connected to the vials by means of a 12 outlet glass manifold equipped with valves that permitted easy switching of the air stream from one pair of vials to the next without opening or disconnecting any part of the system. After the animal was placed in the jar and the system was closed a compressed air source was connected to the jar and air flow was started at a rate of 100–200 ml/min. Collection of radioactive CO_2 was made at 20 and 30 min after the injection and at 5 min intervals thereafter. Radioactivity was determined at 30% efficiency in a Nuclear Chicago scintillation counter. Five microliters of the radioactive citrate solution were then added to vials containing ethanolamine–methanol and 0.4% Omnifluor [98% PPO and 2% Bis-MSB (*p*-bis [*o*-methylstyryl]-benzene) (purchased from New England Nuclear, Boston, MA) in toluene mixtures, from which the amount of radioactivity injected in each rat was calculated. A cumulative curve of the radioactivity collected against post injection time was plotted for each rat. The slope of the rectilinear portion of the curve was determined graphically and recorded as the rate of collection of radioactivity (cpm/min). Plasma citrate concentration was determined by the citrate lyase method in the blood sample drawn at the beginning of the experiment and the total plasma volume was estimated at 4.5% of the body wt; the product of the two represented total plasma citrate in the rat. The specific radioactivity of the plasma citrate immediately after injection equalled the cpm injected divided by the total plasma citrate. The rate of oxidation of plasma citrate was calculated from the equation:

$$\text{Rate of oxidation of plasma citric acid } (\mu g/\text{min}) = \frac{\text{Rate of collection of radioactivity (cpm/min)}}{\text{Specific radioactivity of the plasma citrate (cpm}/\mu g)} \quad (1)$$

The rate of oxidation was calculated as μg of citric acid oxidized/min/kg body wt. The rate of oxidation can be calculated from the equation:

$$\text{Rate of citrate oxidation } (\mu g/\text{min} \times \text{kg}) = \frac{\text{Rate of } {}^{14}C \text{ collection (cpm/min)} \times \text{plasma citrate concentration (mg/100 ml)} \times 450}{{}^{14}C \text{ injected (cpm)}} \quad (2)$$

Determination of citrate turnover. The "turnover rate" of citrate was defined as the total amount of citrate being added to or removed from the plasma citrate pool per unit time, and the "turnover constant" as the proportionality constant between the turnover rate and the total amount of citrate present in the pool; that is, the fraction of the pool being replaced or withdrawn per unit time. The turnover constants in this experiment were determined by measuring the decay in plasma radioactivity following the injection of radioactive citrate. When the isotope disappears from the plasma citrate pool without being replaced, its rate of disappearance is proportional to the amount of isotope present at any given time and therefore, it must follow the first order kinetics (8) described by an equation of the form: $\log A_t = \log A_0 - kt$ where A_t = specific radioactivity at time t, A_0 = specific radioactivity at time zero, and k = turnover constant (not to be confused with K, the proportionality constant between the rate of citrate oxidation and plasma citrate concentration, to be discussed later). A plot of the logarithm of the specific radioactivity against time yields a straight line of slope equal to the turnover constant. Multiplication of this constant by the total plasma citrate would give the turnover rate of plasma citrate.

In our experiments citric acid labeled at carbon-6 was used in order to prevent recycling of the radioactivity. The 6-carboxyl group is decarboxylated during the first step of oxidation by the enzyme isocitric dehydrogenase and is released as CO_2. Hence, any radioactivity —other than that evolved as $^{14}CO_2$ or present as $H^{14}CO_3$—will be found in citrate, *cis*-aconitate or isocitrate. All three of these compounds are in equilibrium with each other and hereafter will be termed "citrate pool." Rapidity of the distribution of the radioisotope was assured by intravenous injection.

Seventy-five microliters of the 6-^{14}C citrate solution in 0.5 ml of isotonic saline were injected into the tail vein of ether anesthetized rats (17–19 weeks old). Blood samples were collected by cardiac puncture at 15, 30, 45, and 60 min after the injection of the isotope. Plasma was sepa-

rated, acidified to release any free $^{14}CO_2$ and its radioactivity measured in a scintillation counter. The plasma citrate was then measured and the log of its specific radioactivity (cpm/μg citric acid) was plotted against time after injection of the isotope. The slope of the line obtained was recorded as the turnover constant, k. The mean plasma citrate concentration was calculated for all the plasma samples obtained from each rat and this value, multiplied by k, yielded the turnover rate of the plasma citrate pool for that animal.

Results. Accumulation of citrate in vivo. Increased plasma citrate concentration was consistently observed after the injection of *trans*-aconitate. The dose of *trans*-aconitate used was determined in a series of preliminary experiments. Significant increases in plasma citrate were observed when *trans*-aconitate doses of 2 g/kg or larger were used. Under these conditions plasma citrate began to increase within 15 min following the injection and continued to increase in a linear manner for at least 40 min. When a dose of 3 g/kg was used, the rate of increase of plasma citrate was about four times higher. This indicated that the inhibition of aconitase by *trans*-aconitate was dose dependent and perhaps with larger doses complete inhibition of the enzyme might occur.

The rates of increase in plasma citrate in diabetic and normal rats were compared by plotting the plasma citrate concentrations against the time after injection. For each group the best fitting line was calculated using the least squares method and the slope of the line was recorded as the mean rate of increase in plasma citrate concentration for that group. The results presented in Table I indicate that the rate of increase in plasma citrate was significantly greater in the normal, than in the diabetic rats.

The oxidation of citrate in vivo. A typical record of $^{14}CO_2$ collection after the 6-^{14}C citrate injection is shown in Fig. 1. The rate of collection of carbon dioxide between 30 and 60 min after the injection was remarkably constant indicating that a steady state had been attained. The calculations were based on this part of the curve. During the pilot experiments collections were made for 3 hr after the injection, after which time no further increase in the collection of radioactivity was noticed and a plateau was reached.

The oxidation of citric acid was measured in young (7–9 week old, group I) and old (51–63 week old, group II) normal and diabetic rats. The mean oxidation rates for the rats in group I were 17.8 ± 1.7 and 22.4 ± 5.2 μg/min/kg body wt in the control and the diabetic rats, respectively. In group II the corresponding values were 7.6 ± 0.6 and 8.7 ± 0.9 μg/min/kg. The differences between controls and diabetics were not statistically significant.

From equation 2 (see Methods) it would seem that for a given animal the rate of oxidation of citrate is proportional to the plasma citrate concentration. This relationship is also applicable for all *in vitro* enzyme systems that follow Michaelis–Menten kinetics unless the enzyme is fully saturated with substrate. Equation (2) suggests that there is a direct correlationship or proportionality factor between plasma citrate concentration and plasma citrate oxidation rate. This factor, termed K, may be defined by the equation:

$$K = \frac{\text{Rate of collection of } ^{14}CO_2}{\text{Amount of } ^{14}C \text{ injected}} \times 450. \quad (3)$$

TABLE I. The Mean Rate of Increase in Plasma Citric Acid in Diabetic and Normal Rats after the Injection of *Trans*-Aconitate.[a]

Conditions	Normal	*N*	*n*	Diabetic	*N*	*n*	*P*
Group I 2 g/kg single dose	0.0759	30	9	0.0218	31	7	< 0.001
Group II 3 g/kg fractionated dose	0.1205	22	6	0.0354	21	6	< 0.001

[a] The units are mg citric acid per 100 ml of plasma/min. N = number of plasma citric acid determinations, n = number of rats in each group; p represents the statistical significance of difference between the mean values of the two groups of animals.

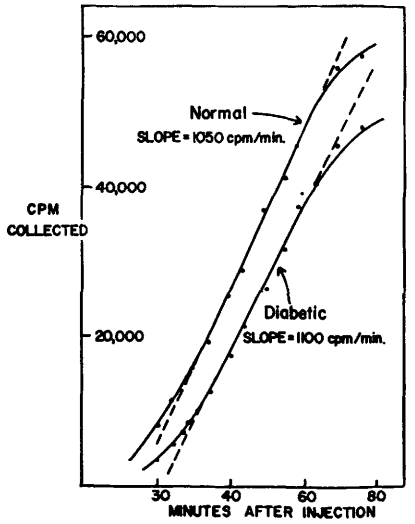

FIG. 1. Collection of $^{14}CO_2$ after the injection of 6-^{14}C citric acid into a normal and diabetic rat.

K may be expressed in units as follows: μg of citrate × 100 ml/min × mg citrate × kg body wt. Therefore, it follows that: (4)

$$\text{Rate of citrate oxidation} = K \times \text{plasma citrate concentration.}$$

By definition, then, the factor K should be independent of the citrate concentration and would be a measure of the *capacity* of the animal to metabolize citrate, regardless of the amount of citrate available for oxidation. Determination of this factor for the various metabolic states would be of more interest than the determination of the rate of citrate oxidation by the animal. The latter parameter obviously depends on the availability of citrate and hence may be affected by factors such as endogenous production and dietary sources of citrate. We felt it important to answer two questions at this point: (a) Whether the proportionality factor, K, is different in diabetes, and (b) whether this factor actually remains constant during changes in the plasma citrate concentration for a given rat.

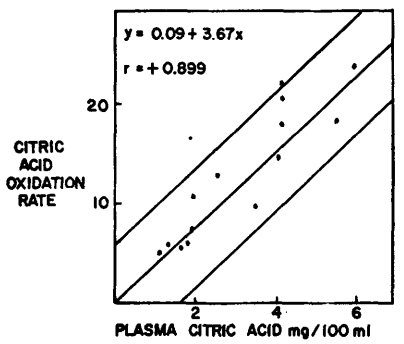

FIG. 2. Correlation diagram for the rate of oxidation of citric acid (μg/min/kg body wt) and the plasma citric acid concentration in normal rats. The animals were from groups I and II (7–9 and 51–63 weeks old, respectively). The best fitting line (least-squares method) and its equation are shown, as well as two standard deviation intervals on each side and the correlation coefficient, r.

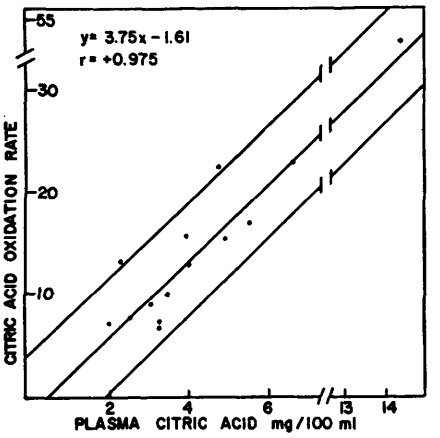

FIG. 3. See legend for Fig. 2. Data for the diabetic rats of groups I and II.

The first question was answered by calculating K from the data collected already; the results of these calculations are shown in Table II. There was about 27% difference in the K value between the controls and the diabetics in group II, but this was not found to be significant.

To answer the second question, we altered the plasma citrate concentration by fasting the animals and studying the changes in the rate of citrate oxidation. Animals of group I were chosen at random and citrate oxidation rates were determined before and after a 48-hr fasting period during which the animals were deprived of food but had access to water. Plasma citrate, citrate oxidation, and K value were calculated for each animal prior to and after the fasting period. The effect of fasting on citrate oxidation, plasma citrate and K is summarized in Table III. Citrate oxidation rates in the two groups of rats decreased significantly with fasting, and the relative decrease was much more marked in the diabetic group. This was consistent with the greater decrease in plasma citrate concentrations in the diabetics and the fact that oxidation rate

TABLE II. Proportionality Factor, K, Between Plasma Citrate Concentration and Plasma Citrate Oxidation Rate in Normal and Diabetic Rats.[a]

Group	Mean K ± SEM Normal	Diabetic	N
I	3.95 ± 0.32	3.55 ± 0.22	7
II	4.29 ± 0.34	3.15 ± 0.42	7
I and II	4.11 ± 0.23	3.35 ± 0.23	14

[a] The difference between normal and diabetic mean values was not statistically significant for any of the groups.

$$K = \frac{\mu g \text{ citric acid oxidized/min/kg body wt}}{\text{mg citric acid/100 ml plasma}}$$

should be proportional to citrate concentration. The proportionality factor, K, however, was not significantly changed in either the control or the diabetic group in spite of the changes in plasma citrate concentration, again emphasizing that K is independent of the latter. However, the value of K was slightly lower in the diabetic rats (see Table II) when compared with the controls.

As is the case with the aconitase inhibition experiments, the data from the citrate oxidation studies are useful only for comparative purposes and are subject to the following variables which may affect the rate of collection of radioactive CO_2: (a) the respiratory rate of the rats; (b) the plasma volume of the animals (dehydration in the diabetic rats would result in hemoconcentration with subsequent changes in plasma CO_2 and bicarbonate concentration, and hence, in the rate of CO_2 excretion through the lungs); (c) the rate of absorption of 6-^{14}C citrate from the peritoneal surfaces; (d) the amount of adipose tissue in the rats (CO_2 is lipid soluble and its exchange will be slower in the presence of large fat depots); (e) any factors affecting the metabolic rate of the animals, such as surface area and body temperature.

Plasma citrate turnover. The logarithm of the plasma citrate specific radioactivity was plotted against time after the injection of labeled citrate and when a steady state had been achieved. The plots obtained were in most cases linear indicating that the disappearance of radioactive citrate from the blood followed first order kinetics.

The mean turnover rate was approximately the same in both groups (Table IV). The values obtained were 1057 and 960 μg/kg body wt/min for the normal and the diabetic rats respectively. The turnover constant was significantly lower in the diabetic group; a value of 0.558/hr was obtained in comparison with 0.798/hr for normal rats. There was a significant elevation (40%) in the plasma citrate levels of the diabetic rats (4.2 ± 0.3 mg/100 ml) compared to the control group (3.0 ± 0.2 mg/100 ml).

TABLE III. Plasma Citrate Oxidation Rate and K in Normal and Diabetic Rat Before and After a 48 hr Fast.

	Citrate oxidation (μg citric acid/min/kg body wt)		K (μg/min/kg)/(mg/100 ml)	
	Fed	Fasted	Fed	Fasted
Normal	16.5 ± 2.8 (5)	10.4 ± 1.4 (5)	3.8 ± 0.5 (5)	3.2 ± 0.5 (5)
	< 0.05		N.S.	
Diabetic	19.7 ± 1.5 (5)	5.6 ± 0.9 (6)	4.7 ± 1.1 (5)	4.2 ± 0.9 (6)
	< 0.001		N.S.	
	N.S.	< 0.05	N.S.	N.S.

N.S., not significant. The parentheses show the number of rats in each group.

TABLE IV. Citrate Turnover Constant, Turnover Rate, and Plasma Concentration in Normal and Diabetic Rats.

Condition of animals	Citrate turnover constant (hr^{-1}) Mean SEM	No. of rats	Citrate turnover rate (μg citric acid/hr/kg body wt) Mean SEM	No. of rats	Plasma citrate concentration (mg citric acid/100 ml) Mean SEM	No. of rats
Normal	0.798 ± 0.094	8	1057 ± 137	8	3.0 ± 0.2	8
Diabetic	0.558 ± 0.055	7	960 ± 98	7	4.2 ± 0.3	7
P =	< 0.05		N.S.		< 0.05	

P for the difference between diabetics and normals.
N.S., not significant.

Discussion. Our data indicate that the rate of citrate accumulation in diabetic rats is lower than in normal; this is in accord with the earlier observation that the citrate condensing enzyme (CCE) activity in muscle homogenates of diabetic rats is lower than in normal animals (1). The mass of muscle in the body is considerably greater than that of liver tissue and the total body citrate perhaps reflects the citrate condensing enzyme activity of muscle rather than that of liver; this assumes that there is no increased citrate production in other tissues that might counterbalance the decreased synthesis observed in muscle. The assumption seems justifiable because the CCE activity of the other tissues studied was considerably lower than that of muscle (1).

Decreased endogenous production of citrate in diabetic animals is not incompatible with the observed elevated plasma citrate levels in these animals, because the latter may be due to an increased dietary ingestion of citrate. De Villiers (9) found a mean citric acid intake of 101 mg/100 g body wt/24 hr in the diabetic rats, compared to 25 mg/100 g body wt/24 hr in the normal rats. This increase of 300% in citrate intake can more than compensate for a decrease of 72% or less in the rate of citrate production. Our observations in vivo as well as the results from the enzyme studies seem to indicate that the hypercitremia of diabetes is perhaps not due to increased endogenous production of citric acid.

Our experiments show that in diabetes, in the presence of higher amounts of citrate available for oxidation, the ability of the organism to oxidize citrate is low. The mean plasma citrate concentration in diabetic animals is 40% higher than in normals (Table IV), but the rate of citrate oxidation in the diabetic rats increased only 22%.

The constancy of the factor, K, after fasting 48 hr (Table III) indicates that it is not affected by decreases in the amount of available citrate. This is true in both diabetic and normal rats. In the fasting experiments, however, the variability of K was much greater. During a fast of more than 24 hr the glycogen reserves are depleted and the organism begins to utilize lipid reserves for energy. These reserves are nearly absent in the already emaciated diabetic animals which must then break down tissue proteins for oxidizable substrates; thus, the sources of calories are different in starving normal and diabetic rats. In spite of this difference the value of K was unaltered after fasting, suggesting that it is also independent of short term changes in the composition of the substrates being oxidized.

When the citrate turnover rate and turnover constant were determined, the turnover rate was found to be slightly diminished whereas the turnover constant was significantly lowered in the diabetic rats (see Table IV). This decrease was similar to that observed for K (proportionality factor) in the diabetic rats (Table II) perhaps indicating a close relationship between the turnover constant and the capacity to oxidize citrate.

The purpose of our reseach was to determine whether changes in the enzymatic and metabolic machinery during diabetes can account for the observed increases in plasma and tissue citrate. The changes observed *in vitro* in the activity of the enzymes reported earlier would result in increased citrate concentrations, however, changes in the production and utilization of citrate *in vivo* reported in the present study would tend to lower the citrate levels. The results obtained on live animals are, however, closer indi-

cators of the events that occur in physiological conditions and should be accorded primary importance. These observations therefore suggest that hypercitremia of diabetes may not be of endogenous origin. Whether dietary citrate or fat content contributes to the elevation of plasma citrate levels needs to be investigated.

Summary. We compared the rate of accumulation, oxidation and turnover of citrate in normal and alloxan-diabetic rats, in order to explain the elevations of citric acid reported in diabetic states. Citrate utilization was blocked by intraperitoneal injections of sodium *trans*-aconitate (a competitive inhibitor of the enzyme aconitase); the rate of accumulation of citrate was found significantly lower in the diabetic rats. This suggested a decreased synthesis of citrate *in vivo*, and correlated with earlier findings of decreased *in vitro* citrate synthesis by diabetic rat tissues. Normal and diabetic rats were injected intraperitoneally with 6-^{14}C citrate, and expired $^{14}CO_2$ was collected at definite time periods to determine the rate of oxidation of injected citrate. In other groups of rats, the plasma citrate specific radioactivity and turnover were calculated following intravenous injection of 6-^{14}C citrate. No significant difference was found in the oxidation or turnover rate of plasma citrate between normal and alloxan-diabetic rats. On the basis of these studies it seems that the hypercitremia in diabetes is not solely of endogenous origin.

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