PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 149, 494–499 (1975)

The Effect of 3,3′,5-Triiodo-L-Thyronine on the Renal Handling of Citrate (38835)

ARTHUR M. FELDMAN[1] AND SIGMUND GROLLMAN

(Introduced by H. C. Harrison)

Department of Zoology, University of Maryland, College Park, Maryland 20742

In previous studies Nieth and Schollmeyer (1) demonstrated that under normal conditions only 10% of the metabolic activity of the kidney was fueled by citrate and that free fatty acids (FFA) were the major substrate oxidized. However, the kidney can metabolize citrate to a much greater extent as demonstrated by several physiological as well as metabolic studies (2–10); however, neither the mechanisms responsible for the changes nor the functional significances are known.

Since the early work of Krebs and Johnson (11) it has been shown that the isocitrate dehydrogenase isozymes (NADP-IDH and NAD-IDH) catalyze the catabolism of citrate while citrate synthase (CS) catalyzes its synthesis from carbohydrate, free fatty acid (FFA), and amino acid byproducts. NAD-IDH was considered to be the rate-limiting reaction of the tricarboxylic acid cycle (TCA). However, recent studies by Srere *et al.* (12) using purified rat kidney CS have shown the enzyme to be allosteric in nature being inhibited by ADP, NADH, NADPH, and several carboxylic acids. Therefore, CS could control the entry of metabolites into the TCA cycle. Citrate has also been shown to be broken down by citrate lyase to provide the primary source of acetyl CoA for extramitochondrial fatty acid biosynthesis.

Kadenbach *et al.* (13, 14) found that hyperthyroidism increased NAD-IDH and oxidative activity and decreased NADP-IDH and biosynthetic activity in the rat kidney. It has been assumed that this increased TCA flux was fed by increased fat metabolism as has been demonstrated in several other organs (15). These results were consistent with increased basal metabolic rate and oxidative enzyme synthesis associated with thyroid treatment (16).

Since FFA is in great demand by other organs during hyperthyroidism, it would be advantageous for the kidney to metabolize increased quantities of a secondary substrate. This study was designed to determine whether citrate metabolism by the kidney could be regulated by 3,3′,5-triiodo-L-thyronine (T_3). By measuring renal, urine, and plasma citrate levels as well as CS activities at various times after T_3 injection, we have demonstrated an increased utilization of citrate in the kidney of the hyperthyroid rat.

Methods. Sprague–Dawley rats (Charles River Co., Lexington, VA) weighing 200–250 g were given a sham injection of 0.8 ml of physiological saline or saline containing 2.2 μg T_3/g body wt. After injection, the animals were placed in metabolic cages and were sacrificed 12, 18, 24, 36, and 48 hr after injection. They were allowed free access to water but no food. Each experimental group consisted of both control and treated animals. After administration of ether anesthesia, a midventral incision was made the length of the body of the rat. The pleural cavity was exposed and 4 ml of blood were drawn in a heparin-coated syringe (20 USP units, Eli Lilly Co.) by way of a ventricular puncture. The samples were kept cold. One kidney, to be used for substrate and nucleotide studies, was removed, quickly cleaned of fat, frozen in a Dry Ice–acetone solution, and stored at −70°C. The remaining kidney was used for the enzyme studies. It was quickly excised and placed in a solution of cold .25 *M* sucrose.

Enzyme assays. All enzyme assays were done on the same day the animals were sacrificed. Assays were performed on mitochondrial extracts prepared according to Williams

[1] Present address: Department of Pediatrics, Johns Hopkins University School of Medicine, Baltimore, MD 21205.

(17) from kidneys homogenized individually in 10 vol of .25 *M* sucrose in 20 m*M* Tris–Cl buffer, pH 7.3, with a Potter and Elvehjem-type homogenizer. The mitochondrial suspension, in a final volume of 3 ml of .1 *M* Tris–Cl buffer, pH 7.5, was sonified for 30 sec with a Biosonik IV sonifier (Bronwill, Co.) equipped with a cooling collar maintained at −5°C. All tissue preparations were carried out at 3°C and the efficiency of the sonification was assessed microscopically. Activities were measured at 22°C in a Hitachi Perkin–Elmer UV-Vis spectrophotometer Model 139, equipped with a Beckman Model 1005 recorder.

Citrate synthase activity was assayed by a modification of Srere, Brazil, and Gonen's method (18). The reaction mixture consisted of 1.84×10^{-3} *M* oxaloacetate, 6.0×10^{-4} *M* DTNB, 1.8×10^{-4} *M* acetyl coenzyme A (Calbiochem), and .1 *M* Tris–Cl buffer (pH 7.5), and enzyme solution in a total volume of 1.0 ml.

NADP-IDH activity was measured in order to compare previously demonstrated hyperthyroid states with our own and to correlate IDH changes with CS changes. The assay used was that of Goebell and Klingenberg (19). All enzyme assays were performed in duplicate and were linear with respect to time and protein concentration. Protein concentrations were measured by the method of Lowry *et al.* (20).

Citrate analysis. Plasma samples were deproteinized by the addition of 7% trichloroacetic acid, and citrate was assayed from the resulting supernatant. Kidney samples were homogenized in 12 vol of 7% trichloroacetic acid, and citrate was assayed from the supernatant while urine samples were diluted in 20 m*M* Tris–Cl buffer and assayed directly. Endogenous fluorescence in the urine and kidney extracts was removed by the addition of magnesium silica gel (Florisil). Citrate concentrations were determined using the fluoroenzymatic method of Costello and O'Neill (21) (personal communication, 1972). Plasma citrate is expressed as nmoles citrate/ml plasma, kidney citrate as nmoles citrate/gram wet weight, and urine citrate as μmoles creatinine/ml/μmoles citrate/ml. Creatinine levels were assayed by a modification of the spectrophotometric method of Folin (22).

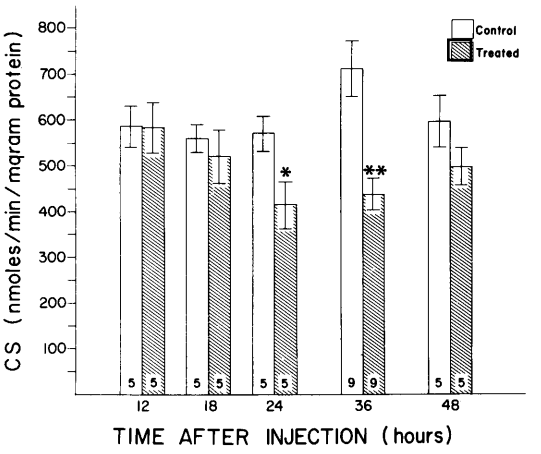

FIG. 1. Citrate synthase (CS) activities in the kidney of control and T_3-treated rats. Rats were sacrificed 12, 18, 24, 36, and 48 hr after intraperitoneal injection. Activities were expressed as nmoles/min/mg protein. Bars represent means ±SE and number of rats is indicated at base of each bar. Statistical comparisons were made between control and treated rats of individual experiments and significant differences are indicated: $*P < 0.05$, $**P < 0.01$.

Because variations were observed between the control animals sacrificed at different times, all statistical comparisons were made between control and treated animals of the same experimental group.

Results. Figure 1 shows the mean renal citrate synthase values of rats sacrificed at varying times after an identical injection of T_3 or of a saline placebo. Initially there was no significant difference between control and experimental animals; however, in the group sacrificed 24 hr after treatment the activity of the treated group was 39% of the controls. There was a greater difference between the activity means of the treated and control rats sacrificed 36 hr after injection (Fig. 1, $P < 0.01$). This was correlated with a 30% decrease in NADP-IDH activity in the same rats (Fig. 2). This change is identical with that demonstrated by Kadenbach (13) using a similar protocol.

The injection of T_3 resulted in a significant hypercitricemia in the 12-, 18-, and 48-hr experimental animals while there was no significant change in citrate between the con-

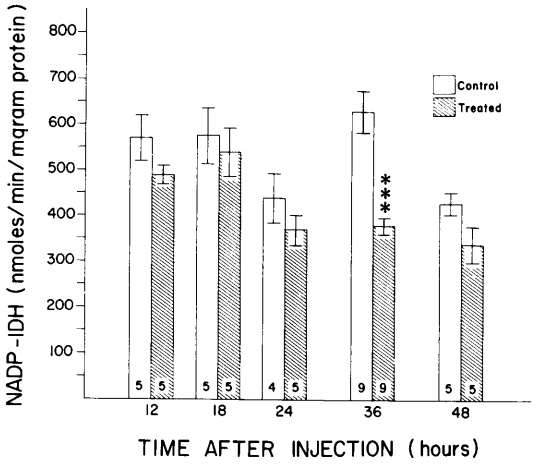

FIG. 2. NADP-Isocitrate dehydrogenase (NADP-IDH) activities in the kidney of control and T_3-treated rats. Rats were sacrificed 12, 18, 24, 36, and 48 hr after intraperitoneal injection. Activities were expressed as nmoles/min/mg protein. Bars represent means ±SE and number of rats is indicated at base of each bar. Statistical comparisons were made between control and treated rats of individual experiments and significant differences are indicated: *** $P < 0.001$.

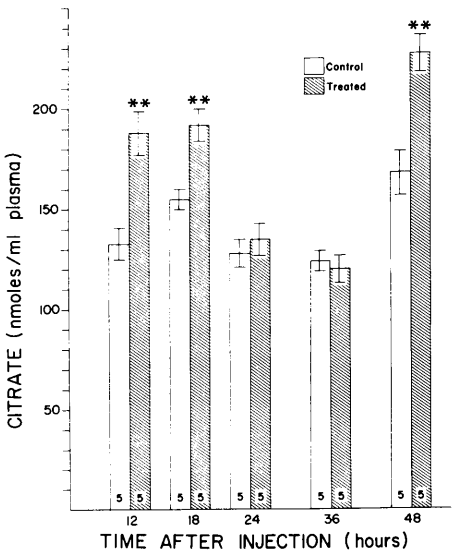

FIG. 3. Citrate levels in the plasma of control and T_3-treated rats. Rats were sacrificed 12, 18, 24, 36, and 48 hr after intraperitoneal injection. Citrate concentrations were expressed as nmoles citrate/ml plasma. Bars represent means ±SE and number of rats is indicated at base of each bar. Statistical comparisons were made between control and treated rats of individual experiments and significant differences are indicated: ** $P < 0.01$.

trol values and the treated values in the 24- and 36-hr experiments (Fig. 3). The variation between control and treated means was greatest in the 12-hr and less in the 48- and 18-hr experimental groups. There was no significant rise in renal citrate concentration (Table I). T_3 did not alter urine citrate levels in the 12-, 18-, 24-, 36-, or 48-hr experimental groups (Table I) indicating that the T_3-induced changes in plasma citrate were caused by factors influencing release, synthesis, or metabolism, and not increased excretion.

Discussion. Enzyme activities. While increases in kidney catabolic enzyme activities after T_3 injection have been well studied (13, 14), the effects of T_3 on citrate synthase have not received as much attention. This study demonstrated an alteration in citrate synthase activity 24 and 36 hr after T_3 injection. There was a 28% drop in the CS levels in the rats treated with T_3 and sacrificed 24 hr after treatment and a 39% drop in those sacrificed 36 hr after treatment (Table I). It should be noted that all comparisons were made between the control rats and the treated rats sacrificed at a given time. Thus, it appears that while there is an increase in oxidative activity of the TCA cycle after T_3 treatment, there is a decrease in the ability of fuel substrates to enter the cycle as an acetyl CoA derivative through the citrate synthase step.

Comparisons with renal citrate synthase levels found in other mammalian systems or from other renal studies are difficult since little work has been done on citrate synthase from crude homogenates. Our control values are generally higher than those observed in previous studies using similar mitochondrial preparations (23). This may be explained by preparation techniques. Structural protein cleavage and enzyme denaturation were small due to minimal sonication, resulting in higher values.

While the effects of T_3 on CS activity are significant, the mechanism responsible for these changes is unclear. Three compounds, all known to exist in increased quantities in the mitochondria of hyperthyroid kidneys, have been experimentally implicated in citrate synthase inhibition: nicotinamide nu-

TABLE I. KIDNEY AND URINE CITRATE LEVELS IN RATS SACRIFICED AT VARYING TIMES AFTER THE INJECTION OF T_3[a].

	Time sacrificed after injection (hr)			
	12	24	36	48
Kidney citrate				
Control	253.33 ± 22	263.66 ± 34	260.14 ± 36	202.03 ± 15
Treated	302.57 ± 22	342.71 ± 21	250.05 ± 29	224.32 ± 32
P	NS	NS	NS	NS
Urine citrate				
Control	375.65 ± 45	390.48 ± 35	342.47 ± 43	470.00 ± 42
Treated	400.25 ± 52	377.85 ± 25	319.58 ± 22	445.45 ± 73
P	NS	NS	NS	NS

[a] Kidney values are expressed as nmoles/g wet weight while urine values were expressed as μmoles creatinine/μmole citrate/ml. Values are ±SE, $N = 10$.

cleotides, adenine nucleotides, and carboxylate compounds (2, 24). Therefore, we cannot resolve whether metabolic changes govern cellular permeability and substrate utilization or whether substrate availability controls metabolic selectivity and cellular absorption.

NADP-IDH activity was measured to demonstrate that the metabolic condition noted in previous studies (13) had been duplicated and that IDH changes could be correlated with CS changes. The 39% drop in NADP-IDH activity accompanied the maximum CS change and the values were consistent with those seen by Kadenbach (13). However, the activity change was observed 36 rather than 24 hr after the injection of T_3. The TPN-specific isozyme was chosen because the assay lent itself to mitochondrial preparations from small quantities of tissue and was sensitive and accurate. Also, the citrate synthase activity could be determined from the same pellet on the same day as the animals were sacrificed.

Effects of T_3 on citrate levels. T_3 resulted in a marked hypercitricemia in the rats sacrificed 12, 18, and 48 hr after injection while citrate levels were unchanged 24 and 36 hr after injection (Fig. 3). It is proposed that the high circulating citrate levels (+50 nm/ml), reduced at 24 and 36 hr during citrate synthase inhibition, and increased oxidative activity, may be utilized by the kidney in place of substrates which normally enter the TCA cycle at the CS step. This interpretation requires the assumptions that the kidney is the predominant organ responsible for citrate metabolism, and that the kidney has the ability to metabolize increased amounts of circulating citrate. Selective uptake and utilization of citrate by the kidney has been well documented. In experiments with rats, Simpson (5) found that serum citrate levels increased within 2 hr after nephrectomy. While normal values were within the range of this study, the increase after 2 hr was close to 10 times the control values. Herndon and Freeman (6) found that when circulating citrate levels were increased as much as four times endogenous values by citrate infusion, far greater than the 35% rise seen in Fig. 3, the kidney was able to metabolize the increased circulating citrate. Hence, the increased circulating citrate seen in this study is well within the metabolic capacity of the kidney for citrate.

The similarity between the control and treated citrate urine levels is consistent with the hypothesis that the changes in plasma citrate levels at various times after T_3 injection are not the result of changes in renal citrate excretion but in metabolism. This is also consistent with previous experimental evidence that only a small portion of endogenous citrate is excreted in the urine (25).

No significant differences in renal citrate levels were observed between the control and experimental group. The exact mechanism of citrate transport into the cell is unknown, but Cohen and Barac-Nieto (10) proposed that it is similar to the antiluminal mechanism elucidated for α-ketoglutarate. Peritubular citrate would be transported into the

proximal tubules, and rapidly catabolized unaccompanied by an increase in intracellular citrate level.

The hypercitricemia and CS changes demonstrated after a T_3 injection in the present study are quite interesting. However, the exact nature of the cause–effect relationship between T_3, hypercitricemia, and citrate synthase has yet to be determined.

The ability of the kidney to selectively metabolize increased quantities of citrate during hyperthyroidism is advantageous to the organ. During hyperthyroidism, increased metabolic rate results in rapid utilization of glucose and increased metabolism of FFA by many organs (15). Since the kidney metabolizes FFA at a greater rate than any other substrate (1), and FFA is in great demand in numerous organs, it would be beneficial to the kidney to be able to metabolize increased concentrations of a secondary substrate. Citrate performs this function well. It is readily metabolized, has a high Tm allowing easy access into the proximal cell (26), has large storage pools in the bone, enters the TCA cycle at the initial step resulting in maximum production of H^+ and its principal fate is CO_2 utilization (27). Furthermore, as we have demonstrated, its circulating levels are raised after T_3 injection, and its use facilitated by a decrease in CS activity.

Summary. The effects of a 500-μg injection of T_3 on the renal handling of citrate by the albino rat was studied by measuring citrate synthase activity, NADP-isocitrate dehydrogenase activity, and plasma, kidney, and urine citrate concentrations 12, 18, 24, 36, and 48 hr after injection. Kidney citrate synthase activity of the T_3-injected rats was significantly lower than the controls in the 24- and 36-hr treatment groups, while NADP-IDH activity was significantly lowered only in the 36-hr treatment group. The injection of T_3 resulted in hypercitricemia in the 12-, 18-, and 48-hr experimental animals while there was no significant change in citrate between the control values and treated values in the 24- and 36-hr experiments. There was no significant change in renal citrate levels in any of the treatment groups, and hypercitrauria was not observed.

The results of the present study suggest that T_3 can control citrate utilization by increasing the levels of circulating citrate and then increasing the utilization of citrate by the kidney. This is facilitated by a decrease in NADP-IDH activity resulting in a decrease in biosynthesis and a decrease in citrate synthase activity resulting in a decrease in FFA metabolism. It is proposed that this system functions in providing fuel (citrate) for the increased Krebs cycle flux occurring in hyperthyroidism.

1. Nieth, N., and Schollmeyer, P., Nature (London) **205,** 1244 (1966).
2. Freeman, S., and Chang, T. A., Amer. J. Physiol. **160,** 334 (1950).
3. Freeman, S., and Chang, T. A., Amer. J. Physiol. **160,** 341 (1950).
4. Freeman, S., and Elliott, J. R., Endocrinology **59,** 190 (1956).
5. Simpson, D. P., Amer. J. Physiol. **205,** 1049 (1963).
6. Herndon, R. F., and Freeman, S., Amer. J. Physiol. **192,** 369 (1958).
7. Cohen, R. D., and Prout, R. E. S., Clin. Sci. **28,** 487 (1965).
8. Martensson, J., Scand. Arch. Physiol. **83,** 113 (1939).
9. Martensson, J., Acta Physiol. Scand. Suppl. **11,** 1 (1940).
10. Cohen, J. J., and Barac-Nieto, M., *in* "Handbook of Physiology—Renal Physiology" (J. Orloff and R. W. Berliner, eds.), p. 909, Amer. Physiol. Soc. (1974).
11. Krebs, H. A., and Johnson, W. A., Enzymologia **4,** 148 (1937).
12. Srere, P. A., Matsuoka, Y., and Mukherjee, A., J. Biol. Chem. **248,** 8031 (1973).
13. Kadenbach, B., Biochem. Z. **344,** 49 (1966).
14. Kadenbach, B., Goebell, H., and Klingenberg, M., Biochem. Biophys. Res. Commun. **14,** 335 (1964).
15. Gold, M., Scott, J. L., and Spitzer, J. J., Amer. J. Physiol. **213,** 239 (1967).
16. Tata, J. R., Gen. Comp. Endocrinol. Suppl. **2,** 385 (1969).
17. Williams, J. N., Jr., J. Biol. Chem. **197,** 709 (1952).
18. Srere, P. A., Brazil, H., and Gonen, L., Acta Chem. Scand. **17,** 129 (1963).
19. Goebell, H., and Klingenberg, M., Biochem. Z. **340,** 441 (1964).
20. Lowry, H., Rosebrough, N. J., Farr, A. L., Randall, R. J., J. Biol. Chem. **193,** 265 (1951).

21. Costello, L. C., and O'Neill, J. J., J. Appl. Physiol. **27,** 120 (1969).
22. Folin, O., J. Biol. Chem. **17,** 469 (1914).
23. Cuestas, R., and Dixit, P. K., Proc. Soc. Exp. Biol. Med. **142,** 889 (1973).
24. Srere, P. A., Matsuoka, Y., and Mukherjee, A., J. Biol. Chem. **248,** 8031 (1973).
25. Kuyper, A. C., and Matill, H. A., J. Biol. Chem. **103,** 51 (1933).
26. Crawford, M. A., Biochem. J. **88,** 115 (1963).
27. Pashley, D. H., and Cohen, J. J., Amer. J. Physiol. **225,** 1519 (1973).

Received Jan. 20, 1975. P.S.E.B.M., 1975, Vol. 149.