The Effects of Fluoroacetate, Malonate and Acid-Base Balance on the Renal Disposal of Citrate

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Ostberg (1931) showed that sodium hydrogen carbonate could increase the urinary excretion of citric acid. Orten & Smith (1937) demonstrated that a number of salts of organic acids could also increase citrate excretion. Krebs, Salvin & Johnson (1938) observed an increase in urinary citrate, α -oxoglutarate and succinate after administration of the succinic-dehydrogenase inhibitor, sodium malonate. They claimed that these acids were excreted in excessive amounts owing to their accumulation in the blocked metabolism. Orten & Smith (1939) demonstrated a large increase in citrate of renal tissue and urinary citrate after the administration of sodium malate; they found no increase in blood, liver or muscle citrate and therefore concluded that the kidney was the site of citrate synthesis.

Evidence has been accumulating that citrate excretion by the kidney is dependent on the intracellular pH of the renal tubular cell (Cooke *et al.* 1954; Clarke, Evans, MacIntyre & Milne, 1955; Crawford, Milne & Scribner, 1959). During an investigation on the effects of acid-base balance on citrate excretion, Crawford *et al.* (1959) showed that injection of potassium or sodium hydrogen carbonate produced an increase of citrate in renal tissue and urine but not in blood concentration. This appeared to be identical with the effect of sodium malate found by Orten & Smith (1939), and it was postulated that urinary citrate was a function of citrate within the renal tubular cell.

At first sight it would appear that the hydrogen carbonate was increasing citrate synthesis, but the pH-dependence of citrate excretion must be borne in mind. It was therefore decided to test further for a relationship between renal-tissue citrate and urinary citrate by use of the aconitase inhibitor, fluoroacetate (Peters, 1957), and to compare the effects of carbonate and malate. Part of this work has been reported in abstract (Crawford & Milne, 1960).

METHODS

Albino rats were used throughout. In a separate series of experiments it was found that control concentrations of renal-tissue citrate differed in two different colonies. This was attributed to dietary and possibly age factors. Consequently, in an effort to obtain uniform and comparable results in a given experiment rats only from the one

colony were used at a time. The rats were kept in groups born within a period of 2 weeks. This enabled rats of approximately the same weight and age to be used for each experiment. Control data were obtained for each experiment. All dosages are expressed per kg. body wt. The methods used were essentially those employed by Crawford *et al.* (1959). Citric acid was determined by McArdle's (1955) modified pentabromoacetone method. It was, however, found necessary to retain the precaution in the original method (Natelson, Lugovoy & Pincus, 1947) of reading the final colour at two different wavelengths (435 m μ for total extinction and 620 m μ for background opalescence, which causes interference in this method).

α -Oxoglutarate was determined by the formation of the 2,4-dinitrophenylhydrazone, chromatographic isolation and colorimetry. The procedure of McArdle (1957) was followed except that the separation of the dinitrophenylhydrazone was found to be more satisfactory with the chromatographic techniques of De Groote, Deschepper & Vandenbroucke (1955).

Blood and plasma samples were deproteinized immediately. Kidney tissue was excised from the ether-anesthetized rat and immediately weighed in cold 10% trichloroacetic acid before homogenization. Urine samples and protein-free centrifuged samples were stored at -15° . Citrate analyses were started within 24 hr. Analysis for α -oxoglutarate was commenced as soon as possible after completion of the experiment.

When urine specimens were being collected for keto acid analysis, 2 ml. of 2,4-dinitrophenylhydrazine-HCl reagent was pipetted into the collecting flask of the metabolism cage before commencement of the experiment. This acted as a trap for the relatively unstable keto acids.

All experiments were carried out under conditions of water loading (50–60 ml. of water/kg. body wt.) to ensure frequent micturition and accuracy of urine collection. Injections were given intraperitoneally. The solutions were warmed to 37° because a large volume of cold liquid appeared to suppress urine formation, possibly because of shock.

The dose injected varied with the experiment. However, 8 m-moles/kg. was the maximum dose of KHCO_3 tolerated and was administered slowly in 60 ml. of water/kg. Otherwise, salts were injected dissolved in 50 ml. of water/kg. Accuracy of urine collection was checked by estimation of urinary creatinine during a control collection and the subsequent experimental period (Crawford *et al.* 1959).

RESULTS

The injection of fluoroacetate produced no increase in urinary citrate despite a large increase in renal-tissue citrate (Tables 1 and 2). When

administered together with potassium hydrogen carbonate, fluoroacetate did not materially alter the effect of the hydrogen carbonate on the urinary citrate although the increases of kidney citrate were approximately additive (Table 2).

The results obtained in similar experiments, when α -oxoglutarate was estimated in place of citrate, were essentially the same, except that kidney α -oxoglutarate did not increase during treatment with fluoroacetate alone. Fluoroacetate did not alter either the normal urinary concentration or the urinary response to hydrogen carbonate during 4 hr. Furthermore, an increase in kidney α -oxoglutarate occurred in animals treated with both hydrogen carbonate and fluoroacetate and

was similar to the kidney concentrations of citrate found after treatment with hydrogen carbonate alone (Table 3).

The sodium hydrogen salts of succinate, malate and malonate gave quantitatively similar increases in citrate excretion, during the 4 hr. collection period, to that found for equimolar sodium hydrogen carbonate (Table 4). Similar increases were also found in kidney citrate. The dose was the same as that used initially by Krebs *et al.* (1938) to demonstrate the inhibition of succinic dehydrogenase by malonate *in vivo*.

It was intended to compare the effects of the free acids with those of the salts. Unfortunately at this high dose the free acids were toxic when ad-

Table 1. *Effect of injection of fluoroacetate on urinary and renal-tissue citrate*

Six rats were used in each group and were killed at the end of the urine collection for kidney analysis; the dose of fluoroacetate was 0.5 mg./kg.

Treatment	Duration of expt. (hr.)	Urinary citrate (μ moles/kg./hr. $\pm$ s.d.)	Renal-tissue citrate (μ mole/g. $\pm$ s.d.)
Control	1	1.83 $\pm$ 0.45	0.226 $\pm$ 0.050
	4	1.72 $\pm$ 0.68	0.213 $\pm$ 0.043
Fluoroacetate	1	1.62 $\pm$ 0.31	0.830 $\pm$ 0.128
	4	1.78 $\pm$ 0.59	0.935 $\pm$ 0.199

Table 2. *Effect of injection of fluoroacetate and potassium hydrogen carbonate on urinary and renal-tissue citrate*

Six rats of approximately the same weight were used in each group. Urine was collected for 4 hr.

Treatment	Urinary citrate (μ moles/kg./hr. $\pm$ s.d.)	Renal-tissue citrate (μ moles/g. $\pm$ s.d.)
Control	1.63 $\pm$ 0.42	0.230 $\pm$ 0.060
Fluoroacetate (0.33 mg./kg.)	1.58 $\pm$ 0.32	0.559 $\pm$ 0.090
Fluoroacetate (0.66 mg./kg.)	1.59 $\pm$ 0.28	1.55 $\pm$ 0.37
KHCO ₃ (8 m-moles/kg.)	46.5 $\pm$ 6.3	1.60 $\pm$ 0.30
KHCO ₃ (8 m-moles/kg.) + fluoroacetate (0.33 mg./kg.)	45.9 $\pm$ 8.2	1.91 $\pm$ 0.32
KHCO ₃ (8 m-moles/kg.) + fluoroacetate (0.66 mg./kg.)	46.1 $\pm$ 5.1	3.04 $\pm$ 0.67

Table 3. *Effect of injection of fluoroacetate and potassium hydrogen carbonate on urinary and renal-tissue α -oxoglutarate*

Treatment	α -Oxoglutarate		
	In renal tissue (μ mole/g.)	In urine (μ moles/kg./hr.)	In blood (μ mole/ml.)
Control	0.086	0.30	0.038
	0.092	0.39	0.042
	0.061	0.26	0.035
Fluoroacetate (0.66 mg./kg.)	0.043	0.25	0.035
	0.031	0.30	0.036
	0.072	0.19	0.036
KHCO ₃ (6 m-moles/kg.)	0.189	2.3	0.028
	0.236	3.4	0.034
	0.288	4.4	0.037
Fluoroacetate (0.66 mg./kg.) + KHCO ₃ (6 m-moles/kg.)	0.175	4.1	0.039
	0.256	2.6	0.040
	0.197	3.9	0.025

ministered intraperitoneally. The free acids and the salts were therefore compared at 5 m-moles/kg. At this dose the free acids did not significantly increase urinary citrate although at the same dose the salts caused marked increases. The free acids increased the kidney concentration of citrate but the increases were not as great as those given by the salts (Table 5).

Table 4. *Comparison of effects of sodium hydrogen carbonate and sodium salts of organic acids on excretion of citrate*

Dose used was 10 m-moles/kg. body wt. and there were six rats in each group. One group of rats was used for collection of urine for 4 hr.; another group was killed 1 hr. after injection for blood analysis.

Treatment	Urinary citrate (μ moles/kg./hr. $\pm$ s.d.)	Blood citrate (μ mole/ml. $\pm$ s.d.)
Sodium hydrogen succinate	54.0 $\pm$ 7.0	0.139 $\pm$ 0.030
Sodium hydrogen malate	59.0 $\pm$ 8.0	0.147 $\pm$ 0.044
Sodium hydrogen malonate	47.0 $\pm$ 9.9	0.123 $\pm$ 0.033
NaHCO ₃	51.0 $\pm$ 7.8	0.109 $\pm$ 0.027
Control (water)	1.7 $\pm$ 0.4	0.099 $\pm$ 0.021

Table 6 shows that the excretion of α -oxoglutarate is also increased by the sodium hydrogen malate but not by the free acids. Fluoroacetate did not alter the excretion of α -oxoglutarate in response to sodium hydrogen malate. Indeed, after pre-treatment with both sodium malonate and fluoroacetate the urinary response to sodium hydrogen malate was not reduced.

Finally the effects of sodium hydrogen carbonate and of fluoroacetate were compared on the citrate contents of several tissues (Table 7). Injection of sodium hydrogen carbonate significantly increased the citrate content only in the kidney but, as expected, fluoroacetate caused an increase in virtually every tissue studied (Peters, 1957).

DISCUSSION

The experiments with fluoroacetate were designed to test further for a relationship between citrate of renal tissue and urinary citrate as proposed by Crawford *et al.* (1959). Fluoroacetate causes mitochondrial accumulation of citrate (Peters, 1957; Schneider, Striebig & Hogeboom, 1956) but it did not increase urinary citrate (Table 1). Karem, Harrison, Hartog & Fraser (1961) have reported similar findings for the effect

Table 5. *Comparison of effects of free acids and their sodium salts on excretion of citrate*

Dose used was 5 m-moles/kg. body wt. and there were six rats in each group. One group of rats was used for collection of urine for 4 hr.; a second group was killed 2 hr. after injection for tissue and blood analyses.

Treatment	Citrate		
	In renal tissue (μ mole/g. of fresh tissue $\pm$ s.d.)	In urine (m-moles/kg./hr. $\pm$ s.d.)	In plasma (μ mole/ml. $\pm$ s.d.)
Control	0.268 $\pm$ 0.066	2.0 $\pm$ 0.5	0.098 $\pm$ 0.017
Succinic acid	0.368 $\pm$ 0.075	4.1 $\pm$ 0.7	0.184 $\pm$ 0.047
L-Malic acid	0.402 $\pm$ 0.098	5.5 $\pm$ 1.0	0.125 $\pm$ 0.069
Na ₂ CO ₃	0.780 $\pm$ 0.111	45.4 $\pm$ 9.6	0.200 $\pm$ 0.067
Sodium succinate	0.691 $\pm$ 0.164	38.1 $\pm$ 9.1	0.228 $\pm$ 0.070
Sodium malate	0.830 $\pm$ 0.141	39.7 $\pm$ 7.6	0.265 $\pm$ 0.081

Table 6. *Use of substrates and inhibitors of the tricarboxylic acid cycle to demonstrate the pH-dependent excretion of α -oxoglutaric acid*

Dose was 5 m-moles/kg. body wt. unless otherwise stated. The four values given are from individual rats.

Treatment	Urinary α -oxoglutarate (μ moles/kg./hr.)			
	0.4	0.4	0.91	0.3
Water (40 ml./kg.)	5.7	4.7	4.8	3.1
NaHCO ₃	4.6	5.7	4.2	6.3
Sodium hydrogen malate	0.5	0.8	0.5	0.6
Malic acid	5.7	6.3	5.9	4.3
Sodium hydrogen malonate	6.4	6.9	7.1	6.8
Pre-treatment with 5 m-moles of sodium hydrogen malonate/kg. and 0.66 mg. of fluoroacetate/kg.; 5 m-moles of sodium hydrogen malate/kg. was given 1 hr. later	3.8	6.4	4.9	4.8
Sodium hydrogen malate + 0.66 mg. of fluoroacetate/kg.				

Table 7. Comparison of effects of potassium hydrogen carbonate and fluoroacetate on tissue citrate in the rat

Results are means obtained from five rats in each experiment. The fluoroacetate analyses are presented as controls and demonstrate the presence of the condensing enzyme.

Tissue	Citrate (μ mole/g. of fresh tissue)		
	Control	KHCO ₃ (6 m-moles/kg.)	Fluoroacetate (0.5 mg./kg.)
Kidney	0.21	0.59	0.84
Liver	0.09	0.12	0.14
Lung	0.42	0.51	0.20
Heart	0.23	0.22	0.78
Spleen	0.19	0.17	0.34
Muscle	0.19	0.21	0.40
Brain	0.16	0.14	0.51
Blood	0.09	0.10	0.12

of fluoroacetate on urinary citrate. The increase in urinary citrate after administration of potassium hydrogen carbonate was not altered by the simultaneous injection of fluoroacetate (Table 2), even though the higher dose of fluoroacetate doubled the concentration of citrate in renal tissue. It can therefore be concluded that fluoroacetate does not poison a mechanism for citrate excretion and that potassium hydrogen carbonate does not release mitochondrial citrate. These results strongly suggest that the mitochondrial citrate cannot diffuse into the lumen of the kidney tubule and that the effect of potassium hydrogen carbonate is not related to mitochondrial accumulation. Furthermore, if mitochondrial citrate does not move into the lumen it would seem unlikely that a citrate precursor would cause the kidney to secrete synthesized citrate directly into the urine.

In the dog, Herrin & Lardinois (1958) have shown that citrate is normally absorbed from the tubules. None of the excretion rates reported here exceed the glomerular filtration rate (Friedman, 1947) in the rat (0.7 ml./100 g. body wt./min.). As the mitochondrial citrate does not move into the lumen, absorption, if accomplished by the same cells that accumulate citrate in the presence of fluoroacetate, signifies that citrate moves across the cell membrane in one direction only.

The small increase in renal-tissue citrate, without any significant increase in urinary concentration after treatment with free acids, is probably caused by mitochondrial accumulation or synthesis (Table 5). The absence of an increase in urinary citrate further supports the proposal that renal mitochondrial citrate does not escape in the direction of the lumen.

Orten & Smith (1939) showed that administration of sodium malate in the rat produced a large increase in urinary citrate and citrate of renal

tissue but not blood citrate. This was taken as evidence that the kidney synthesizes and secretes citrate. Experiments with potassium and sodium hydrogen carbonates yield similar results (Crawford *et al.* 1959; Table 5). Previously the effect of hydrogen bicarbonate has been associated with pH change and that of malate with net synthesis.

The evidence for citrate excretion being dependent on intracellular pH (Milne, Scribner & Crawford, 1958) is:

(1) That citrate clearance can be increased in alkalosis and decreased in acidosis either of respiratory or metabolic origin (Clarke *et al.* 1955; Crawford *et al.* 1959).

(2) That the carbonic-anhydrase inhibitor, acetazolamide, which increases urinary pH but reduces systemic pH, suppresses citrate excretion (Harrison & Harrison, 1955; Gordon & Sheps, 1957; Evans, MacIntyre, Macpherson & Milne, 1957).

(3) That citrate excretion in potassium deficiency follows intracellular pH rather than urinary pH (Cooke *et al.* 1954; Clarke *et al.* 1955).

The similarity between the results of the sodium malate, malonate and succinate experiments with those of sodium carbonate (Tables 4 and 5), taken together with the inability of the free acids to cause any significant rise in citrate excretion, suggests a common denominator, possibly the effect of alkalosis brought about by administration of large amounts of Na⁺ ion in the presence of a weak acid. The alteration in citrate clearance that occurs indicates renal involvement in both instances. It is probable that the increase in urinary citrate after carbonate and malate is due to changes in renal function with respect to citrate rather than metabolism and secretion.

The above conclusion would also explain the apparent discrepancy between the fluoroacetate and malonate experiments. Both inhibitors cause an accumulation of substrates; malonate causes an increased excretion of citrate whereas fluoroacetate does not. The effect of fluoroacetate on tissue accumulation is observed at less than one-thousandth of the malonate dose, and as a salt it should not affect acid-base balance. On the other hand, malonate is effective only at doses at which sodium carbonate produces similar increases in citrate excretion.

The inhibition of succinic dehydrogenase by malonate *in vitro* is considered to be due to competition for receptor sites, arising from the chemical similarity between succinic acid and malonic acid (Quastel & Wooldridge, 1928; Leloir & Dixon, 1937; Thorn, 1953). If an organic acid-transport system (or systems, e.g. for di- and tri-carboxylic acids) exists it would be expected that infusion of one member of a group would result in an increased

excretion of other members of the same group. A parallel situation occurs with the amino acids, where administration of lysine results in increased excretion of arginine, cystine and ornithine because of saturation of a common absorption process (Beyer, Wright, Skeggs, Russo & Shamer, 1947; Kamin & Handler, 1951). A similar situation might be occurring in the kidney with the organic acids.

The effect of fluoroacetate is uncomplicated by the above issues. The effect of sodium malonate could involve a pH effect on citrate excretion or an effect due to competition for organic acid transport or both. Intracellular accumulation could occur but need not be related to urinary citrate.

The free acids caused an increase in tissue citrate and this would seem to be entirely intracellular. The greater increase brought about by the salts could either be due to a greater intracellular concentration or to a combination of intracellular and extracellular citrate. The suppression of citrate absorption that occurs must increase the citrate content of the lumen very considerably and this would be analysed with whole tissue. This raises the question of whether or not the sodium carbonate also increases intracellular citrate through an influence on metabolism. Clarke *et al.* (1955) drew attention to the remarkable pH-dependence of the condensing enzyme (Stern, Ochoa & Lynen, 1952) and proposed that alkalosis increased the rate of condensation and consequently increased synthesis and secretion of citrate.

It would appear from the present paper that citrate secretion does not take place but increased intracellular synthesis is still possible as a contributory factor to the high amounts of tissue citrate. In an attempt to see if other tissues possessing the condensing enzyme reacted in a similar manner analyses were made after treatment with potassium hydrogen carbonate. The kidney was the only tissue to respond (Table 7); this finding is in agreement with results from a smaller survey by Orten & Smith (1939). Either the pH-dependent condensing enzyme is not the mediator or else the pH change in the kidney is considerably greater than in other tissues. The mechanism whereby alkalosis suppressed citrate absorption still remains to be elucidated.

Evidence has been accumulating that the kidney is the principal site of citrate utilization (Martensson, 1940; Freeman & Chang, 1950; Herndon & Freeman, 1958); in particular it has been shown that nephrectomy gives rise to an increase in plasma citrate whereas arterial ligation does not (Freeman & Chang, 1950). Citrate absorbed from the lumen may therefore be metabolized by the renal tubular mitochondria, which are densely packed in the cells of proximal and

distal convoluted tubules (Rhodin, 1958). It is probable that malate or carbonate partially suppress this utilization, giving rise to the increased excretion rates.

SUMMARY

1. Fluoroacetate does not increase urinary citrate despite citrate accumulation in renal tissue.

2. Hydrogen carbonate increases citrate excretion and renal-tissue citrate. This urinary increase is not influenced by fluoroacetate, despite doubly increased kidney citrate.

3. Fluoroacetate does not interfere with the excretion of α -oxoglutarate after either hydrogen carbonate or malate.

4. Sodium hydrogen malonate, sodium hydrogen malate and sodium hydrogen succinate increase citrate excretion in a manner quantitatively similar to the increase seen with equimolar sodium hydrogen carbonate.

5. The free acids succinic acid and L-malic acid did not increase citrate excretion and it is suggested that the acid-base balance is an over-riding factor.

6. The fact that malonate increased citrate excretion whereas fluoroacetate did not is discussed in terms of acid-base changes.

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The Distribution of Succinate Dehydrogenase and Malate Dehydrogenase among Components of Tobacco-Leaf Extracts

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In a study of the localization of malate dehydrogenase and succinate dehydrogenase [L-malate-NAD oxidoreductase, EC 1.1.1.37, and succinate-(acceptor) oxidoreductase, EC 1.3.99.1] in etiolated pea stems, Price & Thimann (1954) concluded that the succinate dehydrogenase was almost exclusively in the mitochondrial fraction, whereas the malate dehydrogenase was distributed between the mitochondrial fraction and the supernatant fluid. The 'plastid' fraction appeared to contain neither enzyme. Davies (1961) confirmed the presence of malate dehydrogenase in at least two fractions of pea-epicotyl extracts, and found small differences between the two. Similarly, Hiatt (1961) confirmed the presence of succinate dehydrogenase in mitochondrial preparations of roots and leaves.

However, there is evidence that malate dehydrogenase occurs in mature and immature chloroplasts. Sorokin & Thimann (1960) demonstrated it histologically in the prolamellar bodies of prochloroplasts of *Avena* coleoptiles, and the enzyme was detected in concentrated and well washed preparations of chloroplasts by Leech & Ellis (1961) and Zelitch & Barber (1960). There is similar evidence for the occurrence of succinate dehydrogenase in chloroplasts (Sorokin & Thimann, 1960; Sissakian, 1958), although in this case there are also contradictory claims (Zelitch & Barber, 1960).

There seemed therefore good cause to extend the work of Price & Thimann (1954) to extracts of mature leaves, by using methods of leaf disintegra-

tion and fractionation by gradient centrifuging that are known to separate chloroplasts, smaller chlorophyll-bearing fragments and mitochondria (Pierpoint, 1962). It seemed desirable to use a method of estimating succinate dehydrogenase in which phenazine methosulphate was the electron acceptor. This, unlike the method used by Price & Thimann, would be expected to measure the enzyme in both free and bound form, and not only the amount attached to electron-transfer factors probably specific to the mitochondria (Singer, Kearney & Massey, 1957).

EXPERIMENTAL

Leaf preparations. Chloroplasts and mitochondrial preparations were made from tobacco leaves by the methods and materials previously described (Pierpoint, 1962). In experiments where the washing procedure was altered, details are given. The preparations were suspended in either a small volume of the washing medium, or, when they were to be centrifuged in a sucrose gradient, in 15 ml. of a solution containing sucrose (0.15 M), tris (5 mM, adjusted to pH 7.4 with HCl) and EDTA (1 mM).

Centrifuging in sucrose gradients. Sucrose gradients were made by layering buffered sucrose solutions of decreasing densities in 100 ml. glass centrifuge tubes, and allowing the interfaces of the layers to become smooth by standing for 5 hr. in the cold (0–5°). Details of constructing the gradients, centrifuging and fractionating the tube contents were described by Pierpoint (1962). The densities (g./ml.) of fractions 1–9 obtained in a typical experiment were 1.017, 1.021, 1.036, 1.045, 1.074, 1.127, 1.141, 1.162 and 1.199 (Pierpoint, 1962).