XX. THE FORMATION OF CITRIC AND α-KETOGLUTARIC ACIDS IN THE MAMMALIAN BODY

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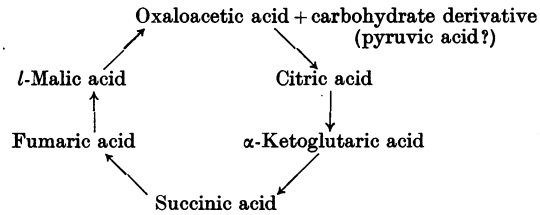
FROM experiments reported in a previous paper [Krebs & Johnson, 1937] we concluded that carbohydrate is oxidized in animal tissues through the following series of reactions:



The evidence in support of this scheme, the "citric acid cycle", was derived from work on isolated tissues. In the present paper experiments will be reported which supplement the previous evidence by experiments on the intact organism. It will be shown that considerable amounts of citric and α-ketoglutaric acids appear in the urine of rabbits and rats after intravenous injection of succinic, fumaric, *l*(−)-malic or oxaloacetic acids. Furthermore α-ketoglutaric and succinic acids are excreted if citric acid is injected. It is thus possible to demonstrate the occurrence of the "citric acid cycle" in the living organism.

Experiments of this kind were suggested to us by a paper of Orten & Smith [1937] who injected 21 different substances intravenously into dogs and studied the excretion of citric acid. They found that only succinic, fumaric, *l*(−)-malic and malonic acids increased the citric acid excretion. Our own experiments confirm and extend the findings of Orten & Smith and the results are in good agreement with the work on isolated tissues. The effects of succinic, fumaric, malic and oxaloacetic acids on the one hand and the effect of malonic acid on the other are due to two different mechanisms. The first group forms citric and α-ketoglutaric acids through the "citric acid cycle", whereas malonic acid acts as an enzyme poison.

The yields of citric and α-ketoglutaric acids are necessarily not quantitative and the evidence is therefore only of a qualitative nature. The question to what extent oxidations are brought about through the "citric acid cycle" can as yet not be decided by work on the intact normal organism, but only by experiments on isolated tissues.

Experimental procedure

Rabbits weighing about 2 kg. were used for most experiments. The solution was infused into an ear vein with the "slow perfusion apparatus" of Burn & Dale [1925]. The animal was kept in a metabolism cage and the urine was collected

in flasks containing a few ml. 20% H_2SO_4 as antiseptic. As a rule, 100 ml. 0·2 *M* solutions of the substrate were infused. Two-thirds of the acid equivalents were neutralized with NaOH, unless otherwise stated. The acid solutions prevent development of the alkalosis arising from the oxidation of the infused sodium salts of combustible acids. The rate of infusion was 1–2 ml. per min. No serious ill-effects were observed during or after the infusion except in the case of citric acid. Citrate and citric acid, as is well known, are highly toxic if injected intravenously [Salant & Wise, 1916]. We observed dyspnoea and convulsions after injection of only 10 ml. 0·2 *M* sodium citrate. The toxic effect was abolished by calcium and we have therefore used a mixture of disodium citrate and acid calcium citrate for the infusion.

A few experiments were performed on rats to which the substances were administered subcutaneously.

All determinations were carried out on 24 hr. specimens of urine. Citric acid was determined according to Pucher *et al.* [1936]. α-Ketoglutaric acid was estimated by the method of Krebs [1938]. Special care was taken to destroy interfering substances such as oxaloacetic acid before the determination of citric acid [see Krebs & Johnson, 1937]. Succinic acid was determined in the following way: the urine was deproteinized with tungstic acid and α-ketoglutaric acid was removed from the solution as dinitrophenylhydrazone, as described in the preceding paper. The aqueous phase was then freed from ether and treated exhaustively with permanganate. The volume of the solution was measured, the MnO_2 formed was filtered off and succinic acid was extracted from an aliquot of the filtrate and determined manometrically [Krebs, 1937]. The "succinic" acid found thus includes the succinic acid formed by oxidation with permanganate. The total quantity of "succinic acid" found in normal urine is too small to allow one to distinguish between preformed succinic acid and succinic acid formed by oxidation.

Results

Each type of experiment was carried out two or three times with uniform results. Typical results are recorded in Tables I and II and Figs. 1 and 2.

It will be seen that citric and α-ketoglutaric acids appear in the urine in considerable quantities after the injections. Assuming that the acids arise from the injected C_4-dicarboxylic acids through the citric acid cycle we calculate the following yields:

		Citric and α-ketoglutaric acids found	
Acid injected	Micromols.	Micromols.	Yield %
Fumaric	20,000	694	3·5
Oxaloacetic	20,000	886	4·4
l(−)-Malic	20,000	484	2·4
Succinic	20,000	154	0·8

If citric acid is injected, only a small fraction appears unchanged in the urine [see Östberg, 1931], for instance 13% in the experiment recorded in Table I. Thus only a small fraction of citric acid present in the body escapes oxidation and is passed into the urine; it is therefore justifiable to assume that very much more citric and α-ketoglutaric acids are actually formed in the body after the injection than are found in our experiments.

Malonic acid is known to be excreted unchanged; it is not metabolized. The work on isolated enzyme systems shows that the acid is an enzyme poison [Quastel & Wooldridge, 1929]. Malonate inhibits in the first place succinic dehydrogenase and we find accordingly a large output of succinic acid after

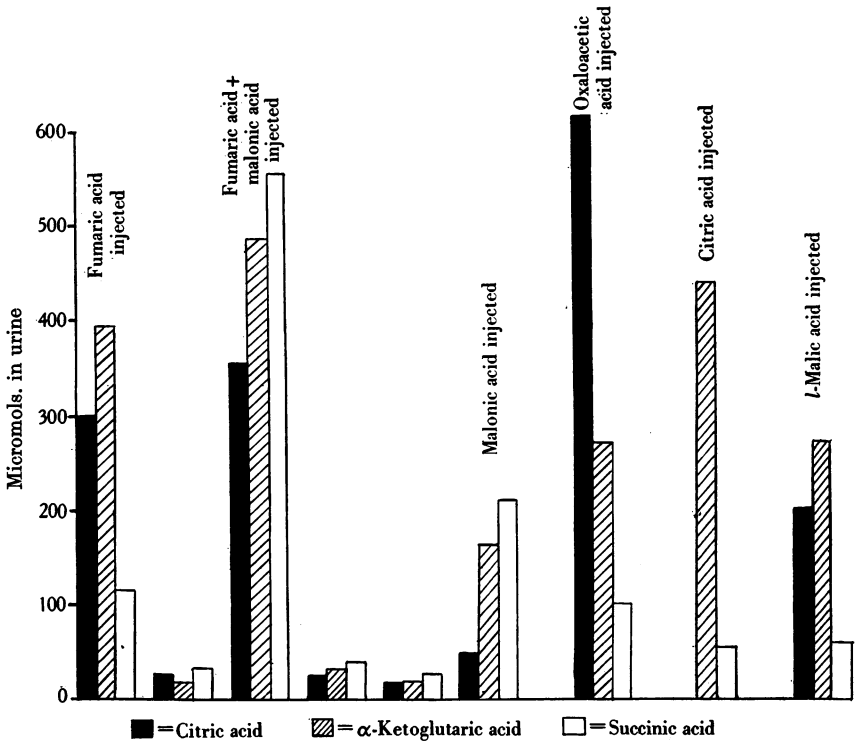


Fig. 1. Citric, α-ketoglutaric and succinic acids in the urine of rabbits after intravenous injection of fumaric, malonic, oxaloacetic, citric and *l*(−)-malic acids.

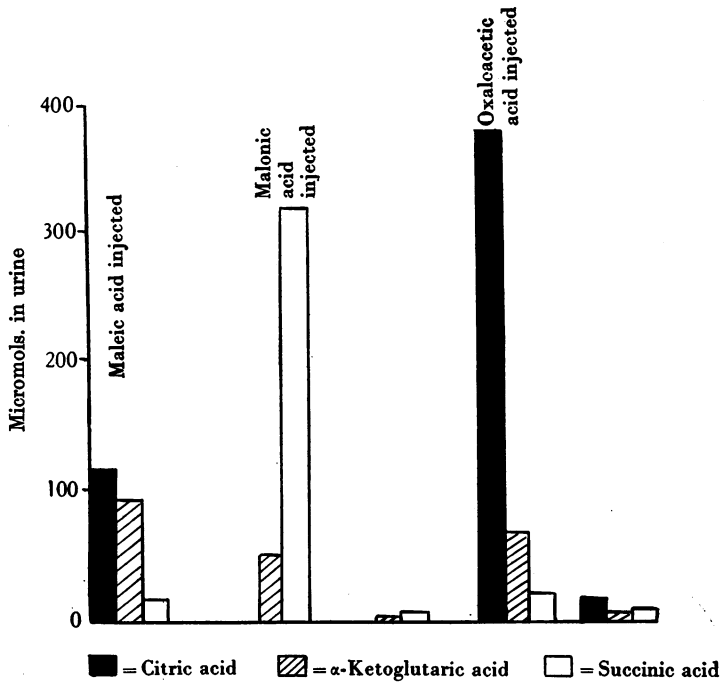


Fig. 2. Citric, α-ketoglutaric and succinic acids in the urine of rats after subcutaneous injection of maleic, malonic and oxaloacetic acids.

Table I. *Experiments on rabbits*

Wt. 1900–2000 g.; the acids injected were partly neutralized (see text) except malonic acid which was injected as neutral Na salt; "citric acid" was a solution of 3·8 g. anhydrous citric acid, 1 g. $CaCO_3$, 0·8 g. NaOH in 100 ml.

Rabbit no.	Day	Solution injected	Excreted (micromols.) in 24 hr. Citric acid	α-Keto-glutaric acid	Succinic acid
I	1	96 ml. *M*/5 fumaric acid	300	394	116
	2	Nil	26	18	32
	3	100 ml. *M*/5 fumaric acid *M*/10 malonate	355	487	554
	4	Nil	25	31	40
	5	Nil	17	18	25
	6	100 ml. *M*/5 neutral malonate	49	161	210
	11	100 ml. oxaloacetic acid	615	271	99
	12	Nil	208	72·2	59
II	1	100 ml. citric acid	(2310)	440	52
	2	Nil	25·5	12	2
	5	100 ml. *M*/5 succinic acid	84	70	(6300)
III	1	100 ml. *M*/5 *l*(−)-malic acid	201	273	59
	2	Nil	77	25	39

Table II. *Experiments on rats*

Four rats used for each experiment. Wt. in Exp. I: 218, 260, 225 and 207 g.; in Exp. II: 178, 184, 221 and 228 g.

Exp. no.	Day	Solution injected (per rat)	Micromols. excreted in 24 hr. Citric acid	α-Keto-glutaric acid	Succinic acid
I	1	Nil	—	1·3	5·4
	2	2 ml. *M* Na-malonate	—	51·4	324
	3	Nil	—	2·2	8·8
	4	Nil	9·7	5·3	12·4
	5	2 ml. *M* Na oxaloacetate	382	60·0	30·0
	6	Nil	17	4·9	8·0
II	1	0·5 *M* Na maleate	120	96·5	20·5
	2	Nil	—	12·8	8·7

injection of malonate. It increases too, to some extent, the excretion of citric and α-ketoglutaric acids. The magnitude of this side effect seems to vary in different species.

Greville [1936] and Weil-Malherbe [1937] have recently suggested that succinic dehydrogenase may be protected from malonate in the structurally intact tissue. Our experiments show, however, an inhibition of succinic dehydrogenase in the intact body.

Maleic acid causes an increased citric acid excretion in Orten & Smith's and our experiments (Table II). It is probable that maleic acid, too, is an enzyme poison, but we cannot exclude the possibility that it acts as a precursor like fumaric acid. Thunberg [1920] and Laki [1935] reported that maleic acid is metabolized in animal tissues.

Summary

1. Citric and α-ketoglutaric acids appear in the urine of rabbits and rats after injection of succinic, fumaric, *l*(−)-malic or oxaloacetic acids. This is considered to be evidence in support of the "citric acid cycle".

2. Malonate, injected into rabbits or rats, causes an excretion of succinic acid and, to a lesser extent, of citric and α-ketoglutaric acids. Since malonate is not metabolized in animal tissues its effect must be due to the inhibition of the enzymes concerned with the breakdown of succinic, citric and α-ketoglutaric acids.

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Note added 4 January, 1938. Breusch has recently questioned the existence of the "citric acid cycle" in muscle tissue [*Hoppe-Seyl. Z.* **250**, 262]. We are, however, unable to accept his arguments and shall discuss them in full in a subsequent paper.