

## Commentary by

Claude Liébecq

*University of Liège, Liège (Belgium)*

on 'The toxicity of fluoroacetate and  
the tricarboxylic acid cycle'

by C. Liébecq and R.A. Peters

*Biochim. Biophys. Acta* 3 (1949) 215–230

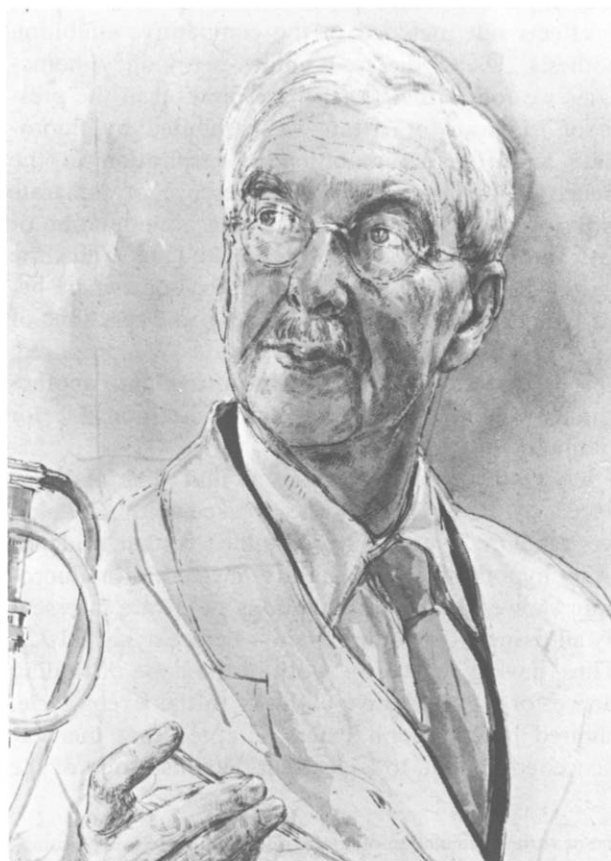
The reader of this essay will find it difficult to believe that the junior author (C.L.) had never heard of the tricarboxylic acid cycle when he joined Rudolph Peters' laboratory in 1947, just after having completed his medical studies at the University of Liège.

The paper of Krebs and Johnson [1] describing the role of citric acid in the intermediary metabolism of

animal tissues had been published in 1937, but the citric acid cycle hypothesis had probably been considered too new and perhaps too daring to be included in a course of general biochemistry given to young medical students in the academic year 1940–1941. The better-known paper of Krebs and Eggleston [2] remained unknown to teachers of biochemistry in the occupied territories of



Claude Liébecq



Part of a drawing of Sir Rudolph Peters by H.A. Freeth (1954)

the European continent until the end of the war.

Not all biochemists had accepted the general significance of the so-called Krebs cycle, however. Peters was one of them; his main reason was that the increased oxygen uptake his group had observed with pigeon-brain dispersions on addition of fumarate as well as pyruvate could not be observed with citrate, which he could not therefore consider an intermediate in the metabolism of pyruvate (see page 320 in Ref. 3).

Peters proposed that I use fluoroacetate as a tool for the study of the oxidative pathway of pyruvate metabolism, in the hope of finding an alternative to the citric acid cycle.

Fluoroacetate, extremely toxic to animals, had been shown by Bartlett and Barron [4], not to react with thiol-containing compounds, not to inhibit thiol-containing enzymes, nor to affect the oxidative steps of the tricarboxylic acid cycle oxidizing isocitrate,  $\alpha$ -ketoglutarate (2-oxoglutarate), succinate or malate. Still, it inhibited acetate oxidation and induced accumulation of acetate during in vitro experiments in which pyruvate was the oxidizable substrate. These authors therefore postulated that fluoroacetate acted as a substrate-competitive inhibitor of acetate utilization. This was taken as evidence for the existence of an oxidative pathway of pyruvate metabolism via acetate, alternative to the Krebs citric acid cycle.

Our experiments showed that fluoroacetate had further effects not included in the competitive-inhibition hypothesis. Using dialysed guinea-pig kidney homogenates we found that (a) their respiration in the presence of fumarate or citrate was inhibited by fluoroacetate and (b) the inhibition of respiration in the presence of fumarate or in the presence of fumarate plus pyruvate was accompanied by an accumulation of citrate without accumulation of acetate [5,6]. Thus, the tricarboxylic acid cycle was interrupted somewhere beyond the citrate level, whereas none of the reactions of the cycle is inhibited by fluoroacetate. We proposed, therefore, that fluoroacetate is transformed into another substance which is the actual inhibitor responsible for the jamming of the cycle \*.

I left Oxford for Cambridge at that time and was replaced by Paolo Buffa, who worked with Rudolph Peters for 2 or 3 years. They obtained further evidence for this hypothesis in vivo. Rats injected with fluoroacetate showed large accumulations of citrate in essentially all tissues – including brain – before dying [11,12].

Thus, having started the work in the hope of finding evidence for an alternative pathway to the Krebs cycle, I admired how Rudolph Peters accepted that the new evidence led, in fact, to a clear-cut demonstration of the

unique role of the Krebs cycle (which he occasionally called the Krebs tri-cycle) in the intermediary metabolism of all animal tissues.

This could have been the end of a story, the closing chapter of a disputed theory now accepted with enthusiasm by one of its leading opponents. This would ignore Peters' genius for new approaches to cell biology and medical problems, and his insistence at using biochemistry to interpret pharmacology and toxicology \*\*.

Peters had previously introduced the concept of 'biochemical lesion' [13]. He then introduced the concept of 'lethal synthesis' to describe the synthesis of toxic fluorocitrate from non-toxic fluoroacetate [14]. His group had indeed found that the inhibitor was fluorocitrate [15], as correctly guessed by Martius [10], and that the jamming of the cycle was due to the inhibition of aconitase by the accumulating fluorocitrate [16,17].

Lethal synthesis offers new possibilities in the development of drugs; advantage has been taken of it in the development of cytostatic agents in cancer therapy [18]. Thus, non-toxic but diffusible 5-fluorouracil is transformed in vivo into 5-fluoro-2'-deoxyuridylate, a suicide inhibitor (inactivator) of thymidylate synthase (Refs. 19, 20; see also p. 615 in Ref. 21), thus preventing DNA synthesis and cell division by lack of dTMP.

I remember having lectured along these lines at the Universidad Nacional Mayor de San Marcos de Lima in 1965. I had started the fluoroacetate story with the work of Grant R. Bartlett and E.S. Guzman Barron, of course. I did not fail to tell the audience that the American biochemist Guzman Barron was of Peruvian origin. This pleased them, but I had not anticipated that the newspapers, next day, would announce (headlines on first page) that a Peruvian biochemist had discovered a cure for cancer. Yes indeed, the impact of our BBA paper has been considerable.

## References

- 1 Krebs, H.A. and Johnson, W.A. (1937) *Enzymologia* 4, 148–156.
- 2 Krebs, H.A. and Eggleston, L.V. (1940) *Biochem. J.* 34, 442–459.
- 3 Coxon, R.V., Liébecq, C. and Peters, R.A. (1949) *Biochem. J.* 45, 320–325.
- 4 Bartlett, G.R. and Barron, E.S.G. (1947) *J. Biol. Chem.* 170, 67–82.
- 5 Liébecq, C. and Peters, R.A. (1948) *J. Physiol. (Lond.)* 108, 11P.
- 6 Liébecq, C. and Peters, R.A. (1949) *Biochim. Biophys. Acta* 3, 215–230.
- 7 Kalnitsky, G. (1948) *Arch. Biochem.* 17, 403–411.
- 8 Kalnitsky, G. and Barron, E.S.G. (1948) *Arch. Biochem.* 19, 75–87.
- 9 Kalnitsky, G. (1949) *J. Biol. Chem.* 179, 1015–1025.
- 10 Martius, C. (1949) *Liebigs Ann. Chem.* 561, 227–232.
- 11 Buffa, P. and Peters, R.A. (1949) *Nature (Lond.)* 163, 914.
- 12 Buffa, P. and Peters, R.A. (1949) *J. Physiol. (Lond.)* 110, 488–500.
- 13 Gavrilescu, N. and Peters, R.A. (1931) *Biochem. J.* 25, 2150–2161.

\* The in vitro accumulation of citrate due to fluoroacetate poisoning was also reported by Kalnitsky [7–9] and by Martius [10]. The latter even proposed that the actual inhibitor was fluorocitrate.

\*\* Peters was one of the founders of 'Biochemical Pharmacology' published by Pergamon Press.

- 14 Peters, R.A. (1952) Proc. R. Soc. Lond. B Biol. Sci. 139, 143–170.
- 15 Peters, R.A., Wakelin, R.W., Buffa, P. and Thomas, L.C. (1953) Proc. R. Soc. Lond. B Biol. Sci. 140, 497–507.
- 16 Lotspeich, W.D., Peters, R.A. and Wilson, T.H. (1951) Biochem. J. 51, 20–25.
- 17 Morrison, J.F. and Peters, R.A. (1954) Biochem. J. 58, 473–479.
- 18 Heidelberger, C., Chaudhuri, N.K., Danneberg, P., Mooren, D., Griesbach, L., Duchinsky, R., Schnitzler, R.J., Plevin, E. and Scheiner, J. (1957) Nature (Lond.) 179, 663–666.
- 19 Cohen, S.S., Flaks, J.G., Barner, H.D., Loeb, M.R. and Lichtenstein, J. (1958) Proc. Natl. Acad. Sci. USA 44, 1004–1012.
- 20 Hartmann, K.U. and Heidelberger, C. (1961) J. Biol. Chem. 236, 3006–3013.
- 21 Stryer, L. (1988) Biochemistry, 3rd Edn., Freeman, New York.

Correspondence: C. Liébecq, L'Université de Liège, Quai Marcellis 14/011, B-4020 Liège, Belgium.

*Erratum* to the original article as reproduced here. On p. 225 (p. [264] of this Volume), last line of paragraph 1: 'to acetate itself' should read 'to fluoroacetate itself'.

THE TOXICITY OF FLUOROACETATE AND THE TRICARBOXYLIC  
ACID CYCLE\*

by

CLAUDE LIÉBECQ AND RUDOLPH ALBERT PETERS

*Department of Biochemistry, Oxford (England)*

The toxic action of the rat poison sodium fluoroacetate presents many features of interest, especially because the molecule is so small and because the C-F bond is so stable (SWARTS<sup>1</sup>) that it cannot combine with -SH groups. BARTLETT AND BARRON<sup>2</sup> have advanced the hypothesis that fluoroacetate is a competitive inhibitor for biochemical reactions in which acetate takes part. Neither they nor others have found any case in which a single enzyme is inhibited by fluoroacetate; but with slices of tissue during the oxidation of pyruvate and other substances, they found that acetate accumulated. In connection with other work upon the paths of oxidation of pyruvate, we have reinvestigated the action of fluoroacetate, in kidney tissue from the guinea pig, and have concluded that the poison has further effects not included in the competitive inhibition hypothesis.

## EXPERIMENTAL

## PREPARATIONS

*Chemicals*

- Pure sodium fluoroacetate (NaFlAc) was kindly provided by Dr B. C. SAUNDERS (*Cambridge*).
- Sodium acetate "Analar".
- Sodium pyruvate, prepared from pure crystalline pyruvic acid.
- Sodium fumarate "B.D.H."
- Sodium citrate "Analar".
- Sodium malonate prepared from recrystallized malonic acid "KAHLBAUM".
- Cozymase was a crude preparation kindly supplied by Dr L. A. STOCKEN, containing about 40% pure cozymase.
- Adenosine triphosphate (ATP) "Boots" was used. Its purity, determined by BAILEY'S<sup>3</sup> method was about 90%.
- Oxaloacetic acid was prepared by R. W. WAKELIN from diethylxaloacetate. Hydrolysis with 4 vol. conc. HCl at -10° C for 2 days. Crystals were filtered and recrystallized from acetone-chloroform mixture.
- Oxalosuccinic acid was prepared by R. W. WAKELIN according to OCHOA<sup>4</sup>.

*Enzyme preparations*

As animals, unfasted adult pigeons and guinea pigs fed on ordinary laboratory diet were used. Pigeons were killed by decapitation, guinea pigs by a blow on the head. The *homogenate of pigeon brain* was prepared according to BANGA, OCHOA, AND PETERS<sup>5</sup> and PETERS AND WAKELIN<sup>6</sup>. The *water extract of the acetone powder of pigeon liver* was prepared according to EVANS, VENNESLAND, AND SLOTIN<sup>7</sup>. Ringer phosphate pH 6.5 was used instead of acetate buffer pH 5 (MOULDER, VENNESLAND AND EVANS<sup>8</sup>) in order to avoid any possible influence of acetate on the effects to be observed with

\* Some preliminary statements about this work were presented in the *Dixon Memorial lecture* (June 1948) and in *Proc. Physiol. Soc.*, 26 June 1948.

*References p. 229/230.*

sodium fluoroacetate.  $\text{MnSO}_4$  (1 mM) and cozymase ( $6 \cdot 10^{-2}$  mM) were added. Liquid phase amounted to 3 ml.

The *kidney brei* (guinea pig) was prepared by removing the kidney quickly and mincing the cortex first with scissors and then with a sharp spatula. From 50 to 200 mg of tissue were used per respirometer bottle. Medium was Ringer phosphate: total volume of fluid was 3 ml; gas phase was  $\text{O}_2$  (80%).

The *kidney homogenate* (guinea pig) was prepared as follows; the kidneys were quickly removed, cut in two, and cooled in ice according to POTTER<sup>9</sup>. A two minutes' stay in the ice seems to be a maximum and may be even too long as our last experiments seem to indicate. They were trimmed, the medulla discarded, quickly weighed and ground in an ice-cold mortar; 0.9% KCl (ice-cold) was then added gradually and squeezed through muslin. Grinding in a mortar was deliberately preferred to various homogenizers.

The homogenate containing approx. 150 mg of tissue p. ml was then centrifuged for 30 min at 5000 to 5500 R.P.M. (3500–4700.g) in the cold room and the precipitate suspended in ice-cold 0.9% KCl buffered with M/10 phosphate pH 7.35.

2 ml containing an amount of tissue corresponding to 150 mg of the kidney cortex were pipetted into respirometer bottles surrounded by ice and already containing  $\text{Mg Cl}_2$  (1.33 mM), ATP (equivalent to 1 mg of the Ba salt p. bottle) 0.2 ml of M/2 phosphate buffer pH 7.35, the inhibitors and sufficient 0.9% KCl to bring the volume to 3 ml after addition of both enzyme preparation and substrates. The latter were added two minutes after the enzyme preparation.

## METHODS

### *Manometric measurements*

Unless especially specified, measurements were carried out at 38° C, in Barcroft's differential manometers. Rate of shaking was between 110 and 120 p. min.

### *Acetate estimation*

LONG'S<sup>10</sup> modification of WEIL-MALHERBE'S<sup>11, 12</sup> method, based on steam distillation at low pressure and pH about 2, was used. However, phenol red was used as indicator instead of bromothymol blue; the end-point was determined by comparison with a sample of Ringer phosphate pH 7.35 containing a suitable amount of phenol red. 50 ml were collected instead of 55 ml. Recovery, checked each time, was about 80%.

Deproteinization was carried out with metaphosphoric acid (final conc. 5%) instead of sulphuric acid.

*Fluoride estimation* was based on the method used by ARMSTRONG<sup>13</sup>. The preparation of the sample in the case of biological material was done by removing the proteins by trichloroacetic acid (final conc. 4%), neutralizing the filtrate and precipitating the undesirable anions (oxalate, citrate and phosphate) by suitable amounts of  $\text{AgNO}_3$  while maintaining the pH at about 7.0 with NaOH: the solution was centrifuged and the fluoride measured in the supernatant fluid.

### *Citrate estimation*

PUCHER'S *et al.*<sup>14</sup> colorimetric reaction was used after the recommendations of LARDY<sup>15</sup>.

Proteins were precipitated by metaphosphoric acid (final conc. 5%) according to KREBS AND EGGLESTON<sup>16</sup>. Samples from 0.5 to 3 ml were added to 0.5 ml  $\text{H}_2\text{SO}_4$  50 vol %, made up to 5 ml and reduced to 1.5 to 2.0 ml by boiling. This treatment was omitted when no acetate was used as substrate. Treatment with bromine water was always omitted since no reducing material of vegetable origin was expected. Controls showed that these omissions were justifiable in the cases examined. The final extraction was done with 2 ml  $\text{Na}_2\text{S}$ -dioxane- $\text{H}_2\text{O}$  instead of 10 ml. Readings with the Hilger Spekker photometer (1 cm deep micro cells and Ilford filter 601) gave values of  $d = 0.30$ – $0.35$  for 100  $\mu\text{g}$  citric acid. Standard curves were run simultaneously. Recovery was within the limits of error of the method ( $\pm 0.02$  drum reading).

## CHEMICAL PURITY OF SAMPLES OF SODIUM FLUOROACETATE

We were able to confirm the high degree of purity of the sample of sodium fluoroacetate provided by Dr B. C. SAUNDERS; the fluoride content was 0.05–0.10%.

As SWARTS<sup>1</sup> already showed, the stability of the C-F bond is such that it is not broken by boiling with conc.  $\text{H}_2\text{SO}_4$  nor by nascent hydrogen.

Sodium fluoroacetate does not react with the -SH group of cysteine (BARTLETT AND BARRON<sup>2</sup>) and does not inhibit the oxidation of pyruvate by the pigeon brain (PETERS AND WAKELIN, 1943, unpublished experiments; this paper), a most sensitive biological test for some -SH poisons. From a chemical point of view, the fluoride atom in a C-F bond seems to be unreactive.

*References p. 229/230.*

Sodium fluoroacetate gives the lanthanum test of KRÜGER AND TSCHIRCH<sup>17</sup> for acetate. Using the amounts and concentrations of reagents recommended by LONG<sup>10</sup>, we however found it approximately 3 times less sensitive to fluoroacetate than to acetate.

In the estimation of acetate by our method when fluoroacetate is present, account must be taken of the "acetic acid" values given by fluoroacetate; 9% of the added amount of fluoroacetate is recovered and titrated as acetic acid. Whether this is due to actual steam distillation of fluoroacetate or to an untimely partial splitting to hydrofluoric and acetic acids has not been determined. It is to be noted that by FRIEDEMANN'S<sup>18</sup> method less than 5% fluoroacetate is recovered. Fluoroacetate is not split by the tissues into fluoride and acetate. Such a phenomenon would have explained both inhibition of the oxygen consumption and the accumulation of acetate. A further proof that the action of fluoroacetate is not due to liberated fluoride is the fact that the residual respiration of a kidney brei was 57.5% inhibited by sodium fluoroacetate (16.6 mM) and only 28% by sodium fluoride (16.6 mM): moreover no traces of freed fluoride could be detected after 2 hours' incubation with the brei: also fluoride inhibits the respiration of brain tissue with pyruvate as substrate, whereas fluoroacetate does not.

## RESULTS

### GENERAL FEATURES OF THE OXIDATION OF FUMARATE AND/OR PYRUVATE BY HOMOGENATES OF KIDNEY CORTEX

It was our aim to study the action of fluoroacetate in a system free from residual respiration and this was achieved, because the homogenates of kidney cortex prepared as indicated are virtually free from oxidizable substrates. They readily oxidize fumarate; pyruvate alone gives only a small oxygen uptake; the addition of pyruvate to fumarate produces an increase of the oxygen uptake over fumarate alone (Fig 1), but this is not comparable with that observed with dialysed brain dispersions (BANGA, OCHOA, AND PETERS<sup>19</sup>). Practically no oxidation occurred in the absence of adenine nucleotides. By using small amounts of fumarate alone, it can be observed that the oxygen taken up accounts for more than the oxidation of fumarate to oxaloacetate (Table I).

As will be seen below, this system is able to synthesize and to oxidize citrate. From experiments with isotopes (BUCHANAN, SAKAMI, GURIN, AND WILSON<sup>20</sup>; WEINHOUSE, MEDES, AND FLOYD<sup>21</sup>; FLOYD, MEDES, AND WEINHOUSE<sup>22</sup>) it is clear that the principal activity of kidney cortex homogenates is to oxidize acetate, acetoacetate and oxaloacetate via the "tricarboxylic" cycle (KREBS AND JOHNSON<sup>23</sup>; KREBS<sup>24</sup>).

In the preparation of this homogenate a somewhat similar system is obtained to that called by GREEN, LOOMIS, AND AUERBACH<sup>25</sup> "cyclophorase"; that is to say it is an enzymatic system oxidizing members of the tricarboxylic cycle via this cycle. For instance it oxidizes citrate well. For these reasons it may be reasonably assumed that the following observations made by us are observations upon the tricarboxylic cycle.

References p. 229/230.

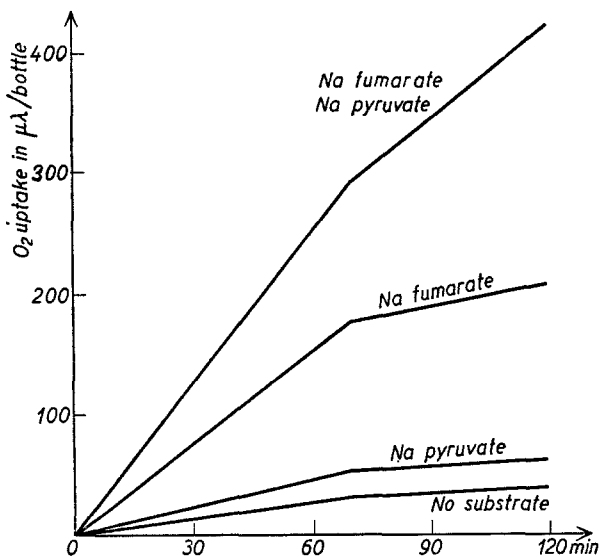


Fig. 1. O<sub>2</sub> uptake by guinea pig kidney homogenate (completed with ATP and Mg<sup>++</sup>). Sodium fumarate: 3.3 mM; Sodium pyruvate: 18.2 mM

TABLE I  
EFFECT OF FUMARATE

Oxygen uptake of centrifuged kidney homogenates with varying amounts of sodium fumarate as substrate (in  $\mu$  moles  $O_2$ /bottle; 2 hours' incubation at  $38^\circ C$ ). All samples contained 1.33 mM  $Mg^{++}$ , 0.1 M phosphate, 0.4 mM ATP\*

		Net	Required for oxidation to oxaloacetate
No fumarate . . . . .	0.98	—	—
2 $\mu$ moles fumarate/bottle . . . . .	2.06	1.08	1.0
4 $\mu$ moles fumarate/bottle . . . . .	4.60	3.62	2.0
6 $\mu$ moles fumarate/bottle . . . . .	5.09	4.11	3.0

\* approx. value

SODIUM FLUOROACETATE AS INHIBITOR OF THE OXIDATION OF FUMARATE BY  
HOMOGENATES OF KIDNEY CORTEX

If the only action of sodium fluoroacetate is that of inhibiting utilization of acetate, inhibition of the oxidation of fumarate should be accompanied by acetate accumulation. Our experiments showed that in the presence of fluoroacetate, the oxidation of fumarate, measured by the  $O_2$  consumption, is inhibited, but that this inhibition is not accompanied by an accumulation of acetate (Table II). Hence there must be some other effect of the poison. In the attempt to locate this, other reactions were studied.

TABLE II  
SODIUM FLUOROACETATE AND FUMARATE

Inhibition by sodium fluoroacetate of the oxidation of sodium fumarate (6.6 mM) by centrifuged kidney homogenates, and the effect upon formation of citric and acetic acids. All samples contained 1.33 mM  $Mg^{++}$ , 0.1 M phosphate and 0.4 mM ATP.  $O_2$  uptake and acids formed during incubation in air at  $38^\circ C$  for 2 hours

Experiment	NaFlAc (mM)	$O_2$ uptake ( $\mu$ l/bottle)	Inhibition	Acetic acid (mg/bottle)	Citric acid (mg/bottle)
2669	0.0	512	—	None	0.32
	7.5	258	50 %	None	0.83
2685	0.0	462	—	0.10	0.18
	3.3	275	40 %	0.10	0.37

ATTEMPTS TO STUDY SINGLE REACTIONS OR GROUPS OF REACTIONS INVOLVED IN THE  
TRICARBOXYLIC CYCLE

BARTLETT AND BARRON<sup>2</sup> were not able to find any single enzymatic reaction which shows a sensitivity to fluoroacetate. Particularly interesting is the fact that most of the enzymes involved in the tricarboxylic cycle and subsequent hydrogen transfer were among the enzyme studied: cytochrome oxidase, isocitrate dehydrogenase,  $\alpha$ -keto-glutarate oxidase, succinoxidase, malate dehydrogenase and yeast carboxylase.

Animal pyruvate dehydrogenase can be added to this list.

References p. 229/230.

Table III shows that the oxidation of pyruvate by brain homogenates of the pigeon, is not inhibited by fluoroacetate, whereas it is inhibited by sodium fluoride, a result already obtained by PETERS AND WAKELIN (1943, unpublished experiments).

TABLE III  
PIGEON BRAIN HOMOGENATE  
The effect of sodium fluoroacetate and sodium fluoride upon the oxygen uptake (in  $\mu\text{l}/\text{bottle}$ ) of sodium pyruvate (9.1 mM) in the presence of sodium fumarate (3.3 mM)

Exp.	O <sub>2</sub> uptake (30 min)		
2631 <sup>1, 2</sup>	No inhibitor	426	38% inhibition No inhibition
	NaF (24 mM)	262	
	NaFlAc (16.6 mM)	439	
2643 <sup>3, 4</sup>	No inhibitor	99	No inhibition
	NaFlAc (7.5 mM)	96.5	

<sup>1</sup> Residual O<sub>2</sub> uptake not subtracted.

<sup>2</sup> Dialysed preparation (4 1/2 h), completed with 0.19 mM adenylic acid and 1.33 mM Mg<sup>++</sup>: 220 mg tissue.

<sup>3</sup> Residual O<sub>2</sub> uptake subtracted.

<sup>4</sup> Undialysed preparation: 167 mg tissue.

Since one of the components of the pyruvate oxidase system is pyruvate dehydrogenase, these experiments show that the oxidative decarboxylation of pyruvate is not impaired by fluoroacetate.

In order to obtain some information upon other enzymes of the cycle, *i.e.*, aconitase, oxalosuccinate decarboxylase, fumarase and oxaloacetate decarboxylase, which were not included among the enzymes studied by BARTLETT AND BARRON, the acetone powder of the pigeon liver was used.

This preparation was showed by EVANS *et al.*<sup>7</sup> and MOULDER *et al.*<sup>8</sup> to carry out the following reactions: added fumarate is converted to malate by the fumarase present in this preparation; malate is dehydrogenated to oxaloacetate, this oxidation being coupled with the reduction of pyruvate to lactate; oxaloacetate itself is decarboxylated to pyruvate and carbon dioxide, both spontaneously and enzymatically. The net result is the disappearance of a certain amount of fumarate and its recovery as lactate. Similar reactions occur in the case of added citrate, aconitase, isocitrate dehydrogenase, oxalosuccinate decarboxylase and lactate dehydrogenase being the enzymes involved. Illustration of these processes is summarized in the following scheme: as Fig. 2.

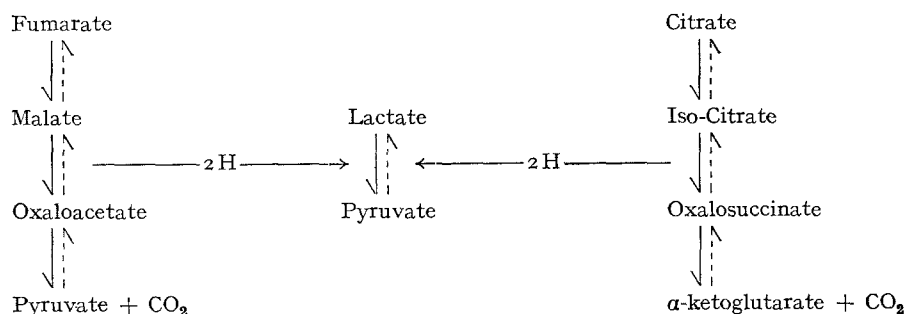


Fig. 2

(All these reactions are reversible, the continuous arrow indicating the predominant direction under the conditions used).

References p. 229/230.



TABLE IV

DECARBOXYLATION OF OXALOACETATE AND OXALOSUCCINATE BY AN ACETONE POWDER OF PIGEON LIVER  
Substrates provided as sodium fumarate (3.3 mM) and sodium citrate (3.3 mM) respectively. Pyruvate conc.: 20 mM; NaFlAc conc.: 10 mM. Results in  $\mu\text{l CO}_2/\text{bottle}$ . Incubated in air for 1 h at 38° C

Experiment	Fumarate as substrate*		Citrate as substrate*	
	Without NaFlAc	With NaFlAc	Without NaFlAc	With NaFlAc
2645	176	175	159	149
2646	168	168	159	150
2647	173	150	158	151

\* Residual subtracted

TABLE V

DECARBOXYLATION OF OXALOACETATE (3.3 mM) AND OXALOSUCCINATE (3.3 mM) BY AN ACETONE POWDER OF PIGEON LIVER

Substrates tipped into the main compartment from KEILIN's tubes at  $t_0$ . Temp. 28° C. NaFlAc 10 mM; Na malonate 25 mM; no pyruvate, no cozymase ( $\mu\text{l CO}_2/\text{bottle}$ )

Experiment	Substrate	Spontaneous (boiled enzyme)	Control	NaFlAc	Malonate
2706	oxaloacetate	115*	189*	194*	122*
2707	oxalosuccinate	110**	159**	183**	167**

\* 30 min incubation      \*\* 10 min incubation

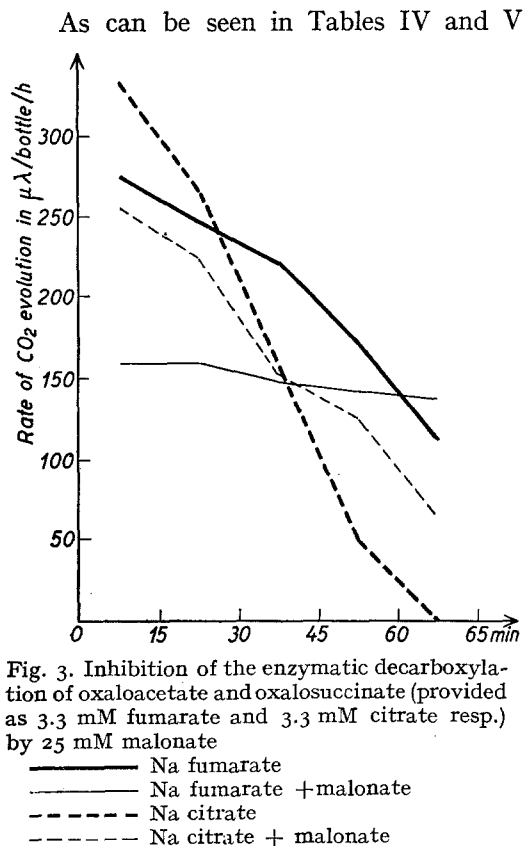


Fig. 3. Inhibition of the enzymatic decarboxylation of oxaloacetate and oxalosuccinate (provided as 3.3 mM fumarate and 3.3 mM citrate resp.) by 25 mM malonate

— Na fumarate  
 - - - Na fumarate + malonate  
 . . . Na citrate  
 - . - . Na citrate + malonate

References p. 229/230.

As can be seen in Tables IV and V, none of these reactions is inhibited by fluoroacetate. Fig. 3 shows the effect of sodium malonate (25 mM) upon these reactions. In the presence of malonate, the speed of the group of reactions is decreased but the total volume of carbon dioxide evolved at the end of the experiment is almost identical and nearly amounts to the theoretical value for complete decarboxylation. This could be explained if malonate inhibits the enzymatic but not the spontaneous decarboxylation.

EVANS *et al.*<sup>7</sup> showed it was so in the case of oxaloacetate decarboxylase. Table V confirms this opinion. However we were unable to observe an inhibition of the oxalosuccinate decarboxylation by malonate.

#### ACCUMULATION OF CITRATE ACCOMPANYING THE INHIBITION BY FLUOROACETATE

The only part of the cycle which had not yet been investigated was the condensation step supposed to occur between a

"C<sub>2</sub> active fragment" derived from pyruvate by oxidative decarboxylation, and oxaloacetate, to give *cis*-aconitate.

TABLE VI  
FUMARATE AND CITRATE OXIDATION

Oxidation of sodium fumarate (6.6 mM) and sodium citrate (6.6 mM) by centrifuged kidney homogenate and inhibition by sodium fluoroacetate (3.3 mM); 2 h incubation at 38° C. All samples contained 1.33 mM Mg<sup>++</sup>, 0.1 M phosphate, 0.4 mM ATP.

Exp.	Fumarate as substrate			Citrate as substrate		
	O <sub>2</sub> uptake (μl/bottle)		Inhibition	O <sub>2</sub> uptake (μl/bottle)		Inhibition
	Without NaFlAc	With NaFlAc		Without NaFlAc	With NaFlAc	
2696	407	267	34 %	503	333	32 %
2700	357	260	27 %	428	282	34 %

Since aconitase was shown to be insensitive to fluoroacetate, it should be possible to study the condensation reaction by means of citrate estimations. As can be seen in Table II, instead of a decreased citrate formation, a strong accumulation was observed, whereas citrate is well oxidised by a normal preparation (Table VI).

With various concentrations of fluoroacetate ranging from 0.66 mM to 33.3 mM (from 0.2 to 10.0 mg NaFlAc/bottle) the inhibition of O<sub>2</sub> uptake increases but the accumulation does not increase to the same extent (Table VII and Fig. 4).

Both inhibition (8–16%) and accumulation of citrate can be observed with concentrations of fluoroacetate as minute as 0.05 mM (15 μg NaFlAc/bottle) that is to say the concentration required to kill a rat in 12–36 hours

(5 mg/kg). Fig. 5 shows that an accumulation of citrate increasing in parallel with the inhibition of the O<sub>2</sub> uptake, is observed between 0.05 and 0.5 mM.

Assuming that extrapolation from an acetone powder of pigeon liver to a homogenate of guinea pig kidney cortex is permitted, the problems seems to come to an apparent contradiction; apparently none of the reactions of the cycle is inhibited by fluoroacetate, whereas the cycle itself is interrupted somewhere below the citrate level.

It seems that the only hypothesis to explain this apparent deadlock is that, in the kidney preparation, fluoroacetate is not the inhibitor but that it is transformed into another substance which is inhibitory. This transformation does not occur in the liver acetone powder.

References p. 229/230.

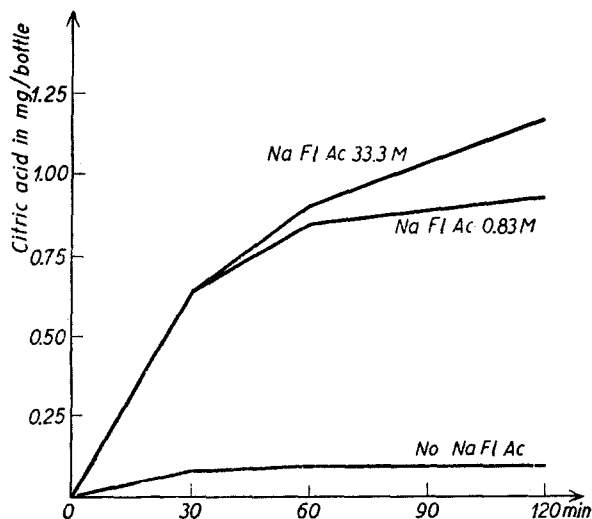


Fig. 4. Accumulation of citrate in the presence of fluoroacetate with sodium fumarate (6.6 mM) as substrate

A possibility which will be examined in the discussion is that fluoroacetate activated to a "fluoro C<sub>2</sub> active fragment" enters the cycle and blocks it somewhere.

TABLE VII

COMPARISON BETWEEN THE INHIBITION BY DIFFERENT CONCENTRATIONS OF FLUOROACETATE OF THE OXYGEN UPTAKE AND THE CITRATE ACCUMULATION IN A CENTRIFUGED KIDNEY HOMOGENATE

Sodium fumarate (6.6 mM) as substrate; 2 hours' incubation at 38%. All samples contained 1.33 mM Mg<sup>++</sup>, 0.1 M phosphate, 0.4 mM ATP.

Experiment	NaFlAc (mM)	Inhibition of the O <sub>2</sub> uptake	Citric acid (mg/bottle)
2672	0.00	—	0.27
	1.66	32 %	0.32
	16.6	64 %	0.47
2674	0.00	—	0.31
	1.66	37 %	1.30
	16.6	51 %	1.26
2677	0.00	—	0.09
	0.83	34 %	0.94
	33.3	50 %	1.18
2678	0.00	—	0.13
	0.83	—	0.92
	33.3	—	0.91

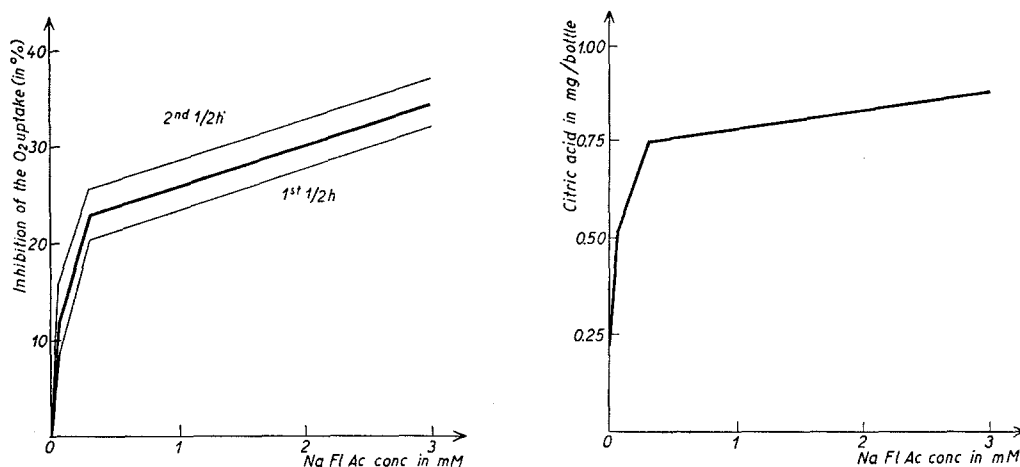


Fig. 5. a) Relation between inhibition and concentration of fluoroacetate

b) Relation between accumulation of citrate and concentration of fluoroacetate

(In this experiment, the homogenate was centrifuged 45 min; boiling of the samples with H<sub>2</sub>SO<sub>4</sub> was performed before citric acid was estimated; 1 h incubation).

The following experiments are consistent with this hypothesis.

The fact that very small amounts of fluoroacetate produce nearly the same accumulation of citrate as larger amounts, together with the fact (*vide infra*) that the factor bringing the acetate into the cycle is not very active in these preparations, suggest

References p. 229/230.

that a very small amount of the added fluoroacetate only is activated and that we may deal with far less actual inhibitor than we think.

Whatever the interpretation of these results may be, the striking fact is that citrate accumulates and that acetate does not, a fact which cannot be explained by BARTLETT AND BARRON'S theory.

This accumulation of citrate is not inconsistent with the inhibition of the citrate synthesis observed by KALNITZKY AND BARRON<sup>26</sup> who used baker's yeast as enzyme preparation and acetate as substrate.

#### INHIBITION BY FLUOROACETATE WITH FUMARATE AND PYRUVATE AS SUBSTRATES

With sodium fumarate (6.6 mM) and sodium pyruvate (10 mM) as substrate, the O<sub>2</sub> uptake is 20 to 50% higher than with fumarate alone and there is an increased production of citrate even in the absence of fluoroacetate. The inhibition by fluoroacetate is lower and larger amounts of citrate accumulate than with fumarate alone. No significant amount of acetate accumulates under these conditions (Table VIII).

TABLE VIII  
CENTRIFUGED KIDNEY HOMOGENATE

Inhibition of the oxygen uptake and accumulation of citrate in the presence of sodium fluoroacetate (3.3 mM), with sodium fumarate (6.6 mM) and sodium pyruvate (10 mM) as substrates. 2 hours' incubation at 38° C. All samples contained 1.33 mM Mg<sup>++</sup>, 0.1 M phosphate, 0.4 mM ATP.

Exp.	Substrate	O <sub>2</sub> uptake (μl/bottle)		Inhibition by NaFlAc	Acetic acid (mg/bottle)		Citric acid (mg/bottle)	
		Without NaFlAc	With NaFlAc		Without NaFlAc	With NaFlAc	Without NaFlAc	With NaFlAc
2685	F	462	275	40%	0.10 (± 0.05)	0.10 (± 0.05)	0.18	0.36
	F+P	545	461	15%	0.05 (± 0.05)	0.15 (± 0.05)	0.60	1.00
2699	F	436	246	43%	0.11 (± 0.03)	0.13 (± 0.03)	0.18**	0.75**
	F+P	561	406	27%	0.07 (± 0.03)	0.13 (± 0.03)	0.43**	2.02**
2700	F	375	260	27%	0.28 (± 0.03)	0.23 (± 0.03)	0.25	0.75
	F+P	532	389	27%	0.23 (± 0.03)	0.21 (± 0.03)	0.59	1.60
2672	F	272	184*	32%	—	—	0.26	0.32
	F+P	346	311*	10%	—	—	0.42	1.20

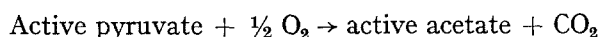
Figures between brackets mean the possible error.

\* NaFlAc 1.66 mM

\*\* Samples were boiled with H<sub>2</sub>SO<sub>4</sub> before estimation of citric acid.

F = fumarate; P = pyruvate.

This lifting of the inhibition by pyruvate may be only apparent. It is possible that "C<sub>2</sub> active fragment" formed from pyruvate competes with "fluoro C<sub>2</sub> active fragment" formed from fluoroacetate. A more likely alternative is that the increase of the O<sub>2</sub> uptake observed by addition of pyruvate is not due to a general increase of all the reactions of the cycle but to the increase of the step:



as the increased formation of citrate is evidence of it. Thus a larger proportion of the O<sub>2</sub> uptake is due to a reaction not inhibited by fluoroacetate.

References p. 229/230.

## INHIBITION BY FLUOROACETATE WITH FUMARATE AND ACETATE AS SUBSTRATES

With sodium fumarate (6.6 mM) and sodium acetate (10 mM) as substrates, the  $O_2$  uptake is slightly higher (4-11%) than with fumarate alone and there is more citrate present at the end of the incubation period (Table IX). This is interpreted as an indication that the factor responsible for bringing the acetate into the cycle is not very active in this kidney preparation. Usually the inhibition by fluoroacetate is very slightly lower as well as the accumulation of citrate, which is consistent with a competition at this stage between acetate and fluoroacetate, as envisaged in the theory of BARTLETT AND BARRON<sup>2</sup>. In one experiment (2696) in which the increase of citrate formation without fluoroacetate was slight, there was no change in the inhibition and no decrease in the accumulation of citrate when fluoroacetate was added. Not much stress can be laid on these experiments with acetate owing to the small changes, but they are consistent with BARRON's theory.

TABLE IX

## CENTRIFUGED KIDNEY HOMOGENATE

Inhibition of the oxygen uptake and accumulation of citrate in the presence of sodium fluoroacetate (3.3 mM), with sodium fumarate (6.6 mM) and sodium acetate (10 mM) as substrates. 2 hours' incubation at 38° C. All samples contained 1.33 mM  $Mg^{++}$ , 0.1 M phosphate, 0.4 mM ATP.

Experiment	Substrate	$O_2$ uptake ( $\mu$ l/bottle)		Inhibition by NaFlAc	Citric acid (mg/bottle)	
		Without NaFlAc	With NaFlAc		Without NaFlAc	With NaFlAc
2685	F	462	275	40 %	0.18	0.36
	F + A	510	309	40 %	0.32	0.26
2688	F	474	307	35 %	0.24	0.85
	F + A	528	374	29 %	0.33	0.70
2696	F	407	267	34 %	0.22	0.53
	F + A	378	254	35 %	0.29	0.56
2699	F	436	246	43 %	0.18	0.75
	F + A	454	296	34 %	0.23	0.53

F = fumarate; A = acetate

## DISCUSSION

*Inhibition of citrate oxidation.* The observations in this paper (excluding those in the appendix) have been made upon a finely ground homogenate, as distinct from organized tissue slice preparations; it was also freed from residual substrates. This renders interpretation somewhat easier. It has been shown on the basis of experiments by BARTLETT AND BARRON<sup>2</sup> by Webb (see DIXON AND NEEDHAM<sup>27</sup>), by ourselves (PETERS AND WAKELIN, unpublished) and this report, that none of the reactions of the tricarboxylic cycle yet studied separately can be considered responsible for the inhibition of the respiration of a homogenate of guinea pig kidney cortex, when fumarate is used as substrate. In order to explain the accumulation of citrate under these conditions, it has been proposed as a hypothesis that fluoroacetate, as well as acetate (BUCHANAN *et al.*<sup>20</sup>) can be activated and brought into the cycle. All the experiments published in *References p. 229/230.*

this paper support this view though they do not prove it. It must be added that this conception is in perfect agreement with the fact known since fluoroacetate was prepared (SWARTS<sup>1</sup>) that the C-F bond is very stable and unreactive. The size of the fluorine atom is not much greater than the size of the hydrogen atom, and it can be expected that fluoroacetate might be metabolized to some extent as acetate, until a compound is reached, like fluoromalate for instance (COOK-CH<sub>2</sub>-CFOH-COOK) which cannot be dehydrogenated. Our hypothesis is in agreement with the fact shown by BARTLETT AND BARRON<sup>2</sup> that tissues or preparations unable to oxidize acetate are not inhibited by fluoroacetate, a fact which is confirmed in the case of the pigeon brain. It is also in accordance with the very interesting observations by SAUNDERS and collaborators (SAUNDERS<sup>28</sup>) who showed that among the substituted fluorofatty acids, only the even numbered were inhibitory, thus drawing the attention to the CH<sub>2</sub>F.CO. grouping, that is to say, to 'active fluoroacetate' rather than to acetate itself.

*Acetate.* In a pigeon brain homogenate, the active C<sub>2</sub> fragment is stabilized as acetate in absence of added fumarate, (COXON, LIÉBECQ, AND PETERS<sup>29</sup>). The absence of accumulation of acetate in the kidney homogenate can be explained by the large excess of fumarate present, so that the C<sub>2</sub> fragments formed from oxaloacetate via pyruvate are immediately used in synthesis. In one experiment with a homogenate, in which 30 μmoles of pyruvate and 20 μmoles fumarate were used as substrates, 15.65 μmoles of citrate were formed after 3 hours of incubation (NaFlAc 0.33 mM). Since the inhibition by fluoroacetate is not immediately maximal, it may be assumed that a part of the citrate formed was oxidized; another part should be present as *cis*-aconitate and isocitrate which are not estimated by the method used. Thus the transformation of 20 μmoles fumarate to 15.65 μmoles citrate represents a nearly complete one. As far as we know these are the best conditions ever realized to prove the reality of the synthesis of citrate itself in animal tissues, which has been a disputed phase of the tricarboxylic cycle (BREUSCH<sup>30</sup>). This high citrate figure makes it clear that fluoroacetate or 'active fluoroacetate' does not interfere with the synthesis of citrate. Any possible competition between the 'C<sub>2</sub> active fragment' and the 'fluoro C<sub>2</sub> active fragment' cannot be inhibitory.

*Pyruvate metabolism.* In regard to the relation of our observations to the degradation of pyruvate, the work upon the avitaminous brain supports the idea of the C<sub>2</sub> intermediates. Our results are one more argument in favour of the formation of such a C<sub>2</sub> active fragment from pyruvate followed by a condensation with oxaloacetate rather than the formation of a C<sub>7</sub> compound pyruvate and oxaloacetate, which has had little experimental support.

*Relation to the biochemical lesion.* We have confirmed the observations in the literature that doses of 5 mg/kilo are toxic to rats (CHENOWETH AND GILMAN<sup>31</sup>); rats injected with this dose do not show signs for at least 30 min and usually die within 12-36 hours. The LD<sub>50</sub> dose for the guinea pig is given as 0.35 mg/kg. In most cases we have used larger concentrations than this in our experiments *in vitro*, but it was found that a concentration of 0.05 mM (*i.e.*, 5 mg/kg) produced an inhibition of 16% in the second half hour of the experiment and a strong accumulation of citrate. These amounts are sufficiently close to the doses *in vivo* to suggest that the toxicity is really due to interference with the tricarboxylic cycle. It must be remembered that the proportion of fluoroacetate brought into the cycle according to our hypothesis is not known; from the fact that large amounts of fluoroacetate induced the same accumulation of citrate as

*References p. 229/230.*

small amounts, it can be deduced that a limiting factor is involved in the activation of fluoroacetate. It is consistent with other evidence from arsenic poisoning that an attack upon the tricarboxylic cycle should induce a biochemical lesion, and this shows the outstanding importance of this cycle (KREBS AND JOHNSON<sup>23</sup>; KREBS<sup>24</sup>) in the intermediate metabolism. Since nervous signs are induced, the presence of the tricarboxylic cycle in brain can be inferred, for which direct evidence is given in another paper. It is interesting to note that BACQ<sup>32</sup> classed fluoroacetate among the "substances vésicantes" as it gave the LUNDGAARD effect in muscle.

*Specificity of malonate.* That malonate may not be a specific inhibitor of succinodihydrogenase is a view which has already been raised by WEIL-MALHERBE<sup>11</sup>. STARE AND BAUMANN<sup>33</sup> found that malonate 1 mM inhibited the oxidation of citrate in muscle tissue by 42% and that of succinate by only 16%. EVANS *et al.*<sup>7</sup> showed that the enzymatic decarboxylation of oxaloacetate was completely inhibited by 10 mM malonate. As has been shown in the experimental part of this paper, we were able to confirm this observation (using 25 mM malonate). Whether this is of importance when various substrates forming oxaloacetate are oxidized by the enzyme system is doubtful, because it would be expected that this inhibition would be lifted by addition of pyruvate. This was not always the case as can be seen in the experiment shown in Table X; the spontaneous decarboxylation seemed to be nearly sufficient in this system.

TABLE X  
INHIBITION OF THE OXYGEN UPTAKE BY SODIUM MALONATE (25 mM) IN A CENTRIFUGED KIDNEY HOMOGENATE  
Sodium fumarate (6.6 mM) and pyruvate (10 mM) as substrates. 2 hours' incubation at 38° C. All samples contained 1.33 mM Mg<sup>++</sup>, 0.1 M phosphate, 0.4 mM ATP.

Experiment	Substrate	O <sub>2</sub> uptake (μl/bottle)		Inhibition by malonate
		Without Malonate	With Malonate	
2666	F	499	304	39%
	F + P	594	370	38%
2672	F	272	90	67%
	F + P	346	153	56%

F = fumarate; P = pyruvate

We failed to show the same inhibitory effect of malonate on the rather similar decarboxylation of oxalosuccinate. This is taken as one more evidence for the non-identity of the enzymes concerned. On the other hand, Fig. 3 shows that there is a slowing of the transformation of citrate to  $\alpha$ -ketoglutarate when malonate was added. This confirms the point of STARE AND BAUMANN that malonate also acts somewhere between citrate and succinate and excludes the attack on the oxalosuccinate decarboxylation, which is mainly a spontaneous reaction under the conditions used, even more so than the decarboxylation of oxaloacetate.

(During the completion of this manuscript, we received the interesting paper by G. KALNITSKY, *Arch. Biochem.*, 17 (1948) 403, in which he has found using kidney tissue that fluoroacetate can induce an accumulation of citrate).

References p. 229/230.

## APPENDIX

*Experiments with kidney brei* (guinea pig). During the course of this research some experiments with a kidney brei have been made, which merit a brief note. The residual respiration of a kidney brei (unwashed) was inhibited about 50% by fluoroacetate; increases in acetate formed during this inhibition are slight and hardly significant, even not constant. In one experiment where very small amounts of fluoroacetate were used, as little as 0.05 mM sodium fluoroacetate produced a very marked accumulation of citrate (Table XI). The small changes in acetate accumulation and the poisoning are in agreement again with our view that the 'cycle' is attacked.

TABLE XI  
INHIBITION OF THE RESIDUAL RESPIRATION OF A KIDNEY BREI BY SODIUM FLUOROACETATE  
2 hours' Incubation at 38° C

Experiment	NaFlAc (mM)	O <sub>2</sub> uptake (μl/g tissue)	Inhibition by NaFlAc	Acetic acid (mg/g tissue)	Citric acid (mg/g tissue)
2613	0	2920	—	3.2	—
	15	1320	55%	4.3*	—
2617	0	1840	—	0.2	—
	15	825	55%	0.0	—
2618	0	2510	—	0.2	—
	15	1180	53%	0.9	—
2697	0.00	2192	—	—	0.23
	0.05	2100	4%	—	0.62
	0.15	1905	13%	—	0.85
	0.50	1695	22%	—	1.26
	3.00	1495	31%	—	1.61
	15.0	1365	37%	—	1.44

\* This accumulation of 1.1 mg acetic represents a variation of 0.05 mg acetic acid between the values actually measured, *i.e.*, within the limits of error.

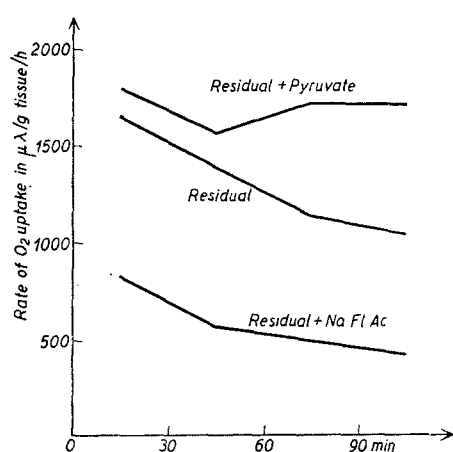
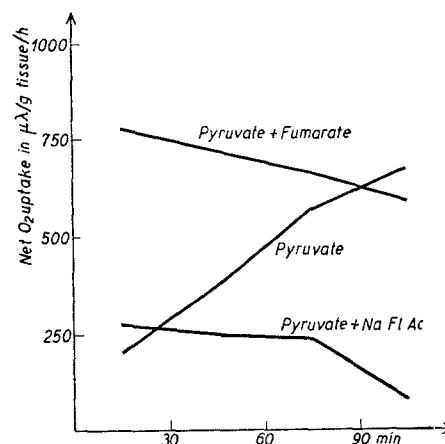


Fig. 6. a) Increase of the oxygen uptake due to the addition of sodium pyruvate (18 mM) to a brei of guinea pig kidney; inhibition of the residual respiration by sodium fluoroacetate (15 mM)



b) Net oxygen uptake due to the addition of sodium pyruvate (18 mM) and its inhibition by sodium fluoroacetate (15 mM), or due to the addition of sodium pyruvate (18 mM) and sodium fumarate (3.3 mM)

Referen ces p. 229/230.



When pyruvate was added, a curious phenomenon was seen; the extra oxygen uptake due to the addition of pyruvate increased regularly. Fig. 6a shows that this was not a stabilization of the rate of oxygen uptake by the brei; the increase of the net  $O_2$  uptake due to pyruvate is inhibited by fluoroacetate (Fig. 6b). If fumarate was also present, the net oxygen uptake due to pyruvate plus fumarate was immediately maximal (Fig. 6b). These facts strongly suggest that the increase was due to the formation of fumarate from pyruvate, that the synthesis of this was inhibited by fluoroacetate. Unlike brain brei (BANGA *et al.*<sup>5</sup>), the kidney brei must be permeable to fumarate. These facts are consistent with the idea that the fumarate concerned is formed via the tri-carboxylic cycle. It is more difficult to draw sharp conclusions from the brei as the residual respiration represents about 75% of the total respiration.

We are indebted to Dr B. C. SAUNDERS for a gift of sodium fluoroacetate; to the British Council for a Scholarship to one of us (C.L.), and to R. W. WAKELIN for skilful technical assistance.

#### SUMMARY

1. The hypothesis that fluoroacetate is a competitive inhibitor for acetate in tissue metabolism (BARTLETT AND BARRON) has been examined and found incapable of explaining some of the enzymatic effects of the poison.

2. Using a homogenate from guinea pig kidney, free from residual oxidizable substrates, and reinforced with  $Mg^{++}$  and adenine nucleotides, it was found that this readily oxidizes fumarate and citrate. In presence of fluoroacetate, citrate accumulates during the oxidation of fumarate, without an accompanying accumulation of acetate.

3. With the same homogenate, fumarate and pyruvate together give 20–50% increased oxygen uptake as compared with fumarate alone, and there is even formation of citrate in absence of poison. Fluoroacetate produces inhibition without accumulation of acetate, but this is less than with fumarate alone.

4. Fluoroacetate has no action upon the pyruvate dehydrogenase component of the brain pyruvate oxidase system. It also has no effect upon the activity of the enzymes aconitase, oxalosuccinate decarboxylase, fumarase and oxaloacetate decarboxylase, as tested by a pigeon liver preparation.

5. The fact that no single enzymatic reaction has been found to be inhibited by fluoroacetate and yet that the reactions of the tricarboxylic cycle are stopped with accumulation of citrate requires a hypothesis in addition to that proposed by BARRON AND BARTLETT.

6. It is suggested that, in the kidney preparation, fluoroacetate is not the inhibitor but that it is transformed into another substance which is inhibitory.

7. The accumulation of citrate can be observed with concentrations of 0.05 mM (15  $\mu g$  Nafluoroacetate per bottle) which approximates to the amounts causing toxic effects *in vivo*.

8. It was confirmed that malonate inhibits the enzymatic decarboxylation of oxaloacetate.

9. Some experiments upon kidney brei are described.

#### RÉSUMÉ

1. Nous avons examiné l'hypothèse proposée par BARTLETT ET BARRON, selon laquelle le fluoroacétate inhibe le métabolisme tissulaire par compétition avec l'acétate, et avons constaté qu'elle était incapable d'expliquer certains effets du poison sur les réactions enzymatiques du cycle des acides tricarboxyliques.

2. Un "homogénat" de rein de Cobaye, dépourvu de substrats oxydables, oxyde parfaitement le fumarate et le citrate, pour autant qu'on y ajoute des nucléotides d'adénine et des ions  $Mg^{++}$ . En présence de fluoroacétate, du citrate s'accumule au cours de l'oxydation du fumarate, sans accumulation simultanée d'acétate.

3. Au contact de fumarate et pyruvate au lieu de fumarate seul, la même préparation enzymatique consomme 20 à 50% d'oxygène supplémentaire et la formation de citrate est nettement accrue, même en l'absence de tout inhibiteur. Le fluoroacétate produit une inhibition de la consommation d'oxygène (sans accumulation d'acétate), moindre cependant qu'au contact de fumarate seul.

References p. 229/230.

4. Le fluoroacétate n'a aucune action sur la déshydrogénase pyruvique, une des pièces du système oxydase pyruvique du cerveau. Il n'a pas plus d'action sur l'aconitase, la décarboxylase de l'oxalosuccinate, la fumarase et la décarboxylase de l'oxaloacétate, enzymes étudiées à l'aide d'une poudre de foie de Pigeon.

5. Le fait qu'aucune réaction enzymatique isolée ne s'est montrée sensible à l'action du fluoroacétate, mais que d'autre part le cycle des acides tricarboxyliques est bloqué avec accumulation de citrate, nécessite une hypothèse supplémentaire à celle de BARTLETT ET BARRON.

6. Nous suggérons que, dans les préparations de rein utilisées, le fluoroacétate lui-même n'est pas l'inhibiteur, mais qu'il est transformé en une autre substance, responsable de l'inhibition.

7. On peut observer l'accumulation de citrate avec des concentrations de fluoroacétate de 0.05 mM (15  $\mu$ g de fluoroacétate sodique par auge), c'est-à-dire une concentration provoquant l'intoxication *in vivo*.

8. Nous confirmons l'inhibition de la décarboxylation enzymatique de l'oxaloacétate par le malonate sodique.

9. Nous décrivons quelques expériences faites avec une pulpe de cortex rénal.

### ZUSAMMENFASSUNG

1. Die Hypothese, dass Fluoracetat ein Hemmstoff für Acetat im Gewebestoffwechsel sei (BARTLETT UND BARRON), wurde geprüft. Dabei wurde festgestellt, dass die Hypothese nicht imstande ist, einige enzymatische Wirkungen des Giftes zu erklären.

2. Bei Benutzung eines "Homogenats" von Meerschweincheniere, das frei von oxydierbaren Restsubstraten war und mit  $Mg^{++}$  and Adeninnukleotiden verstärkt war, wurden Fumarat und Citrat schnell oxydiert. Bei Anwesenheit von Fluoracetat wird während der Fumaratoxydation Citrat angehäuft, und zwar ohne dass diese Anhäufung von einer Anhäufung von Acetat begleitet wäre.

3. Bei demselben Enzympräparat ergeben Fumarat und Pyruvat zusammen eine Erhöhung der Sauerstoffaufnahme von 20–50 % im Vergleich mit Fumarat allein, und bei Abwesenheit des Giftes tritt sogar Citratbildung auf. Fluoracetat verursacht eine Hemmung ohne Anhäufung von Acetat, diese Hemmung ist jedoch geringer als bei Fumarat allein.

4. Fluoracetat hat keinen Effekt auf die Pyruvatdehydrogenasekomponente des Pyruvatoxydationssystems des Gehirns. Es hat auch keine Wirkung auf die Aktivität der Enzyme Aconitase, Oxalsuccinatdecarboxylase, Fumarase und Oxalacetatdecarboxylase, wie mit einem Taubenleberpräparat geprüft wurde.

5. Die Tatsache, dass, wie festgestellt, keine einzelne enzymatische Reaktion durch Fluoracetat gehemmt wurde, und dass doch die Reaktionen des Tricarbonsäurecyklus unter Anhäufung von Citrat gehemmt wurden, erfordert noch eine Hypothese neben der von BARTLETT UND BARRON:

6. Es wird angenommen, dass bei dem Nierenpräparat nicht Fluoracetat der Hemmstoff sei, sondern dass dieses in eine andere Substanz umgesetzt wird, die eine hemmende Wirkung hat.

7. Die Citratanhäufung kann bei Konzentrationen von 0.05 mM (15  $\mu$ g NaFluoracetat pro Gefäß) wahrgenommen werden, was in der Nähe der Mengen, die die toxischen Wirkungen *in vivo* ausüben, liegt.

8. Es wurde bestätigt, dass Malonat die enzymatische Decarboxylierung von Oxalacetat hemmt.

9. Einige Versuche mit Nierenbrei werden beschrieben.

### REFERENCES

- 1 F. SWARTS, *Bull. Acad. roy. Belg.*, IIIe s., XXXI (1896) 675.
- 2 G. R. BARTLETT AND E. S. G. BARRON, *J. Biol. Chem.*, 170 (1947) 67.
- 3 K. BAILEY, *Biochem. J.*, 36 (1942) 121.
- 4 S. OCHOA, *J. Biol. Chem.*, 174 (1948) 115 and personal communication.
- 5 I. BANGA, S. OCHOA, AND R. A. PETERS, *Biochem. J.*, 33 (1939) 1109.
- 6 R. A. PETERS AND R. W. WAKELIN, *Biochem. J.*, 40 (1946) 513.
- 7 E. A. EVANS JR., B. VENNESLAND, AND L. SLOTIN, *J. Biol. Chem.*, 147 (1943) 771.
- 8 J. W. MOULDER, B. VENNESLAND, AND E. A. EVANS JR., *J. Biol. Chem.*, 160 (1945) 305.
- 9 V. R. POTTER, *Arch. Biochem.*, 6 (1945) 439.
- 10 C. LONG, *Biochem. J.*, 32 (1938) 1711.
- 11 H. WEIL-MALHERBE, *Biochem. J.*, 31 (1937) 299.
- 12 H. WEIL-MALHERBE, *Biochem. J.*, 31 (1937) 2202.
- 13 W. D. ARMSTRONG, *Ind. Eng. Chem., Anal. Ed.*, 8 (1936) 384.
- 14 G. W. PUCHER, C. C. SHERMAN, AND H. B. VICKERY, *J. Biol. Chem.*, 113 (1936) 235.

- <sup>15</sup> H. A. LARDY; in W. W. UMBREIT, R. H. BURRIS, AND J. F. STAUFFER, *Manometric techniques and related methods for the study of tissue metabolism*, Minneapolis (Minn.), Burgess Publ. Co., (1945) 106.
- <sup>16</sup> H. A. KREBS AND L. V. EGGLESTON, *Biochem. J.*, 38 (1944) 426.
- <sup>17</sup> D. KRÜGER AND E. TSCHIRCH, *Ber.*, 62 (1929) 2776.
- <sup>18</sup> T. E. FRIEDEMANN, *J. Biol. Chem.*, 123 (1938) 161.
- <sup>19</sup> I. BANGA, S. OCHOA, AND R. A. PETERS, *Biochem. J.*, 33 (1939) 1980.
- <sup>20</sup> J. M. BUCHANAN, W. SAKAMI, S. GURIN, AND D. W. WILSON, *J. Biol. Chem.*, 159 (1945) 695.
- <sup>21</sup> S. WEINHOUSE, G. MEDES, AND N. F. FLOYD, *J. Biol. Chem.*, 166 (1946) 691.
- <sup>22</sup> N. F. FLOYD, G. MEDES, AND S. WEINHOUSE, *J. Biol. Chem.*, 171 (1947) 633.
- <sup>23</sup> H. A. KREBS AND W. A. JOHNSON, *Enzymologia*, 4 (1937) 148.
- <sup>24</sup> H. A. KREBS, *Biochem. J.*, 34 (1940) 460.
- <sup>25</sup> D. E. GREEN, W. F. LOOMIS, AND V. H. AUERBACH, *J. Biol. Chem.*, 172 (1948) 389.
- <sup>26</sup> G. KALNITSKY AND E. S. G. BARRON, *J. Biol. Chem.*, 170 (1947) 83.
- <sup>27</sup> M. DIXON AND D. M. NEEDHAM, *Nature*, 158 (1946) 432.
- <sup>28</sup> B. C. SAUNDERS, *Nature*, 160 (1947) 179.
- <sup>29</sup> R. V. COXON, C. LIÉBECQ, AND R. A. PETERS (1948) in preparation for the Press.
- <sup>30</sup> F. L. BREUSCH, *Z. physiol. Chem.*, 250 (1937) 262.
- <sup>31</sup> M. B. CHENOWETH AND A. GILMAN, *J. Pharm. Exptl. Therap.*, 87 (1946) 90.
- <sup>32</sup> Z. M. BACQ, *Bull. acad. roy. méd. Belg.*, VIe s., VII (1942) 108.
- <sup>33</sup> F. J. STARE AND C. A. BAUMANN, *Cold Spring Harbor Symposia Quant. Biol.*, 7 (1939) 227.

Received August 26th, 1948

#### NOTE ADDED AT CORRECTION

In regard to Fig. 2, while our paper was in the press, MEHLER, KORNBERG, GRISOLIA, AND OCHOA, *J. Biol. Chem.*, 174 (1949) 961, showed that some reactions with the pigeon liver preparation take a different course and include TPN; this does not seem to upset our arguments. There also appeared a further report by KALNITSKY AND BARRON, *Arch. Biochem.*, 19 (1948) 75; working with undialysed rabbit kidney preparation, they have observed an increased synthesis of citrate in presence of fluoroacetate; in our work, the reduction of the residual respiration makes interpretation less difficult.