The Synthesis of Fluorocitric Acid and its Inhibition in Acetate

BY R. A. PETERS AND R. W. WAKELIN

Department of Biochemistry, Oxford, and A.R.C. Institute of Animal Physiology
(Biochemistry Department), Babraham, Cambridgeshire

(Received 25 February 1957)

The theory that the citric acid formation induced by the poison fluoroacetate *in vitro* (Liébecq & Peters, 1949; Martius, 1949) and *in vivo* (Buffa & Peters, 1949) is due to the synthesis of a fluorotricarboxylic acid has been proved by the isolation of a crystalline fluorocitric acid (Peters, Wakelin, Buffa & Thomas, 1953) and by the observation that this substance inhibits the enzyme aconitase *in vitro* (Lotspeich, Peters & Wilson, 1952; Morrison & Peters, 1954). Two forms of aconitase are known, one present in mitochondria, the other obtainable in solution. Working with the soluble aconitase, Peters & Wilson (1952) and Morrison & Peters (1954) found that there is a competition between the inhibitor and the tricarboxylic acid substrates when the inhibition is induced by enzymically made fluorocitric acid. With synthetic fluorocitric acid (Rivett, 1953) the relations are more complex.

More information on the mechanism of synthesis of the fluorocitric acid from the fluorinated C_2 fragment was needed to place this upon a firm biochemical basis, partly for theoretical reasons and partly for the practical objective of understanding protective therapy. Bartlett & Barron (1947) advanced the hypothesis that fluoroacetate interfered with the metabolism of acetate, a hypothesis which was shown by Liébecq & Peters (1949) not to apply to the accumulation of citric acid, because they found conditions in which this accumulated in the presence of fluoroacetate without a change in acetic acid content. In connexion with the protective action of C_2 substances, it was early found (Hutchens, Wagner, Podolsky & McMahon, 1949) that acetate with or without ethanol protected against fluoroacetate poisoning in some animals. Farah, West & Angel (1950) also found that acetate prevented the pharmacological toxicity of fluoroacetate to preparations of rabbit small intestine, and there have been other instances since of such protective effects (see Peters, 1957). The failure of acetate in some animals might well be due to failure of the initial activation stage; it was later established (Chenoweth, Kandel, Johnson & Bennett, 1951) that monoacetin was highly protective, presumably owing to its liberation of active acetyl groups *in situ*. On similar lines Gitter, Blank & Bergman (1953) (see also Gitter, 1956) found that

acetamide was protective; this might be expected to penetrate mitochondria.

None of these researches established whether acetate itself interfered at some active stage with the synthesis of fluorocitric acid. Peters (1952) reported the results of some preliminary experiments by R. W. Wakelin and himself on the effect of acetate on the synthesis of fluorocitric acid *in vitro*, and on the conditions leading to the best synthesis of fluorocitric acid using kidney preparations from the guinea pig. The experiments suggested that in fact it is acetate which interferes with the synthesis of fluorocitric acid from fluoroacetate. Subsequently these experiments were extended and more carefully controlled, partly because in this earlier work the apparent effects of acetate stopped short of the maximum. Difficulties were found on account of the small amounts of fluorocitric acid synthesized in experiments of this type; this makes it necessary to estimate the fluorocitric acid by the inhibition produced upon citrate metabolism. Estimations were done with kidney particles in the first instance, but later the results were confirmed with brain tissue.

METHODS

Reagents. Unless otherwise stated these were of A.R. quality. Sodium pyruvate was prepared by R. W. Wakelin in the laboratory. Synthetic fluorocitrate was a specimen prepared by Dr D. E. A. Rivett. Enzymic fluorocitrate was prepared by ourselves. Adenosine triphosphate (ATP) was from a commercial specimen of a barium salt, not more than 70% pure; tests, however, showed that it fulfilled the function required. Sodium fluoroacetate was from the Monsanto Chemical Co.; sodium malate was mostly from L-malic acid obtained commercially from U.S. sources, and not further purified. A test by Mr R. J. Hall showed that it was chromatographically homogeneous.

Estimations. Citric acid estimations were made by the method of Pucher, Sherman & Vickery (1936) as modified by Buffa & Peters (1949).

Preparation and use of kidney particles. These were prepared from guinea-pig kidneys by our usual methods. To get full activity the details of these preparations are important. The tissue was first squashed thoroughly in a small ice-cold mortar with the pestle, and ice-cold KCl (1%) to about 10.0 ml. was added drop by drop at first, with constant stirring. If the initial additions were too large and not stirred in properly, the tissue appeared to coagulate and the

preparations were less active. The suspension was squeezed through muslin, and the homogenate centrifuged at 3500–4700 *g* for 30 min., after which the pellet of particles was separated and stirred into 1% KCl. Phosphate buffer (made from KH_2PO_4 neutralized with NaOH to pH 7.2) was added to 0.1 M immediately before pipetting into the flasks.

This particle suspension was reinforced with Mg^{2+} and ATP and exposed to various substrates with or without sodium fluoroacetate, being shaken in the water bath at 38° in 50 ml. Erlenmeyer flasks. At the completion of the experiments, the contents of the flasks were transferred quickly to thin test tubes and heated for 5 min. in a boiling-water bath, after which they were cooled and centrifuged. This procedure does not appear to alter the fluorocitric acid, and permits subsequent estimations of fluorocitrate. The supernatants from duplicate flasks were usually combined; this ensures sufficient solution for analysis where the citric acid content is low. In enzyme experiments of this nature it is preferable to shake each flask under exactly the same conditions rather than to make up a larger bulk. The effect of any possible changes in the enzymes during pipetting can be reduced by adding them to alternate flasks in the series. Suitable amounts of the respective supernatants from the first stage of the experiment were then taken for estimation of fluorocitric acid with a fresh set of kidney particles (second stage of the experiment), by using 10 μmoles of citric acid and 0.03 M-malonate to block re-entry of citrate breakdown products into the tricarboxylic acid cycle.

RESULTS

In our earlier series of experiments it was considered sufficient to set up controls with fluoroacetate but no substrate other than citrate; these showed no synthesis of fluorocitric acid when tested with the malonate-blocked particles as judged by interference with the metabolism of the citric acid in this second stage. Later experiments, however, demonstrated that even in the malonate-blocked kidney system of the second stage some synthesis of fluorocitrate from residual fluoroacetate could still take place from any residual amount of malate or similar substrate which might be still present in the supernatant from the first stage, and which escaped

the malonate block. Table 1 illustrates this with the details of an experiment in which it is shown clearly that by itself fluoroacetate has no effect in the presence of malonate, i.e. the citric acid metabolism is blocked presumably at the succinate stage so that there is no synthesis of fluorocitric acid. However, the addition of small amounts of malate or fumarate are enough to give a marked synthesis of fluorocitrate. Hence there must be a control for traces of substrate as well as of fluoroacetate left from the first stage of the experiment. This control proved to be elaborate and difficult, especially where the amounts of fluorocitric acid formed were small. Experience proved that it was best to reduce the magnitude of the interference in the second stage by using not more than 1/15 of the 3.0 ml. from the first stage, and to make experiments with dummy mixtures of malate, fluoroacetate, acetate, ATP and Mg^{2+} ions so that the citrate formed in these could be also used in correction. With these corrections, it was no longer found that the apparent effect of acetate stopped short of the maximum; and this fact gives confidence that the controls were adequate. The two-stage experiment summarized in Table 2 is typical of several satisfactorily controlled experiments, which place the preliminary observations upon a solid basis.

In the first stage of the experiment, which is summarized in Table 2 (a), with kidney particles, L-malate and fluoroacetate, there was a 12–25% reduction of citrate accumulation in the presence of acetate. This effect is variable. In the second stage of the experiment with malonate [Table 2 (c)] it is seen that the acetate present in the first stage, even at 1.25 μmoles , has made a big difference in formation of fluorocitric acid as judged by the effects on citrate disappearance in the second stage, after correction as described in Table 2 (b). From Table 2 it is clear that the method is sensitive because even the small amounts of fluorocitrate formed in the presence of 2.5 μmoles of acetate (in

Table 1. *Synthesis from fluoroacetate of the inhibitor for citric acid metabolism in the presence and absence of fumarate and malate, in kidney particles blocked with malonate*

Twelve flasks were set up containing in each 1.9 ml. of enzyme particles from the kidneys of one guinea pig made up to contain 0.1 M- K_2HPO_4 neutralized to pH 7.2 with NaOH and malonate (0.0334 M), 0.1 ml. of MgCl_2 (4.0 μmoles), 0.1 ml. of ATP (1.2 μmoles), 0.4 ml. of sodium citrate (10 μmoles), additions of sodium fluoroacetate (0.2 ml., 8.0 μmoles), sodium fumarate or malate (0.1 or 0.2 ml.), with KCl (1%; 0.1–0.5 ml.) to make 3.0 ml. total volume. The flasks (in duplicate) were shaken for 30 min. at 38°, and the contents treated with trichloroacetic acid as usual.

Additions	Citrate estimated at end ($\mu\text{moles/flask}$)	Diff. for substrate (μmoles)
None	0.94	—
Fluoroacetate	0.92	—
Fluoroacetate + fumarate (3.12 μmoles)	3.63	2.71
Fluoroacetate + fumarate (1.56 μmoles)	2.22	1.3
Fluoroacetate + malate (7.46 μmoles)	5.28	4.36
Fluoroacetate + malate (3.73 μmoles)	3.00	2.08

Table 2. *Summary of the two stages of an experiment with kidney particles (guinea pig)*

The first stage is designed for synthesis of fluorocitric acid, and the second for estimating the amounts synthesized. For further experimental details of second stage, see (b) and (c).

(a) *First stage*: kidney particles from one guinea pig were made up to 26 ml. in 1% KCl containing 0.1M-phosphate buffer, pH 7.2. Portions were shaken in a volume of 3.0 ml. for 30 min. at 38°. 3.0 ml. contained 1.9 ml. of enzyme, 0.1 ml. of $MgCl_2$ (4.0 μ moles), 0.1 ml. of ATP (1.2 μ moles), 0.1 ml. of sodium L-malate (19 μ moles), 0.2 ml. of sodium fluoroacetate (20 μ moles), with additions of 0.1–0.2 ml. of sodium acetate, and KCl (1%; 0.4–0.6 ml.) to total volume. At the end of the experiment the flasks were quickly emptied into thin test tubes, which were kept cold on ice. Each test tube was then immersed in the boiling-water bath for 5 min. and centrifuged after cooling. The supernatant was available for the second stage, in which the fluorocitric acid formed was estimated. 1/15 of flask contents from the first stage was used for the second stage; for details see (b) and (c).

		Second stage (with citrate and malonate; no malate)		
	First stage (malate as substrate)	Citric acid* not metabolized (μ moles)		Estimated fluorocitrate per flask of first stage (μ mole)
	Citrate formed (μ moles)	Per flask of 2nd stage	Per flask of 1st stage	
A. Fluoroacetate only†	8.12	2.20	33.0	0.171
B. Fluoroacetate + acetate (0.31 μ mole)‡	7.14	1.95	29.3	0.152
C. Fluoroacetate + acetate (0.625 μ mole)‡	6.95	1.62	24.3	0.126
D. Fluoroacetate + acetate (1.25 μ moles)†	6.57	0.67	10.0	0.052
E. Fluoroacetate + acetate (2.5 μ moles)†	6.16	0.015	0.2	0.001
No fluoroacetate or acetate	0.59	—	—	—
Fluorocitrate, 0.0143 μ mole (3.0 μ g.)	—	2.75	—	—
Fluorocitrate, 0.0286 μ mole (6.0 μ g.)	—	3.64	—	—
	* As corrected.	† Duplicate.	‡ Triplicate.	

* As corrected.

† Duplicate.

‡ Triplicate.

(b) *Second stage (control).*

Three mixtures were made up with the following composition:

	(i)	(ii)	(ii)
L-Malate (mg.)	2.5	2.5	2.5
Fluoroacetate (mg.)	2.0	2.0	2.0
Acetate	—	0.2 ml. ($\equiv$ 0.042 mg.)	0.2 ml. ($\equiv$ 0.34 mg.)
ATP (ml.)	0.1	0.1	0.1
Mg (ml.)	0.1	0.1	0.1
KCl-phosphate to a total volume (ml.) of	3.0	3.0	3.0

These were added to the control flasks of the same general composition as in the heading for the second stage below, and shaken under similar conditions:

Additions (ml.)	Citrate found after 30 min. (μ moles)	Diff. (μ moles)
Nil	0.938	—
0.2 (i)	1.213	+ 0.275
0.2 (ii)	1.344	+ 0.406
0.2 (iii)	1.025	+ 0.087
0.2 (i) + fluorocitrate (3 μ g.)	3.96	+ 3.022 - (i) = 2.747
0.2 (i) + fluorocitrate (6 μ g.)	4.84	+ 3.902 - (i) = 3.627

(c) *Second stage (estimation of fluorocitrate formed in first stage with kidney particles blocked with malonate).*

Kidney particles from two guinea pigs were made up in the usual KCl-phosphate mixture to which was added enough malonate to make this 0.0334M. The flasks were shaken for 30 min. at 38°. Each flask contained, in a total volume of 3.0 ml., 1.9 ml. of enzyme solution, 0.1 ml. of $MgCl_2$ (4.0 μ moles), 0.1 ml. of ATP (1.2 μ moles), 0.4 ml. of sodium citrate (10 μ moles) and additions of 0.2 ml. with KCl (1%, 0.1–0.5 ml.) to bring to total volume.

Additions (0.2 ml.)*	Citrate found after 30 min. (μ moles)†	Diff. (μ moles)	After final correction‡
—	0.938	—	—
A	3.415	2.477	2.202
B	3.298	2.360	1.954
C	2.959	2.021	1.615
D	1.697	0.759	0.672
E	1.042	0.104	0.017

* 1/15 of contents of flasks from first stage.

† Corrected for preformed citrate added in A–E.

‡ Corrected for the citrate formed from the small additions of malate and fluoroacetate as determined in the second stage (control).

3.0 ml. of the mixture) are enough to indicate a slight change of citrate catabolism in the mitochondrial preparation.

Errors. In this experiment, which is reported in full and which was the last to be done, the results show the inhibitory effect of acetate on the formation of fluorocitrate, the values for the latter giving a smooth curve when estimated by its effect on the metabolism of citric acid. With 2.5 μ moles of acetate there was almost complete suppression of fluorocitric acid formation, and even with a quarter of this amount the action was marked. These facts give confidence that the methods employed are sufficiently accurate, and this is reinforced by a large number of earlier experiments, all giving substantially the same answer. At the same time it is difficult to state the exact errors of such an experiment. The duplicate citric acid analyses are the average of figures differing by less than 0.02 μ g. This will be magnified by multiplication and be a greater percentage error where less is estimated; but in a series of six duplicates the mean differed from the duplicate estimation by ± 5 , 3, 5, 0.5, 0.25 and 3 %. From this it may be inferred that a single estimation may be 5 % out, and the mean ± 3.0 %. Hence a difference between two experiments will be of the order of ± 6.0 %. Therefore, when allowance is made for inhomogeneity of enzyme preparation, the final figures might not be more accurate than ± 10 %. Hence the drop in the citric acid not metabolized (Table 2) from 33 to 29.3 μ moles, taken by itself, is within experimental error. It becomes significant only when taken in conjunction with earlier experiments, all of which show effects at these relatively low concentrations of acetate.

It was interesting to know the amounts of fluorocitric acid corresponding to these inhibitions of citric acid metabolism. In Table 2 the inhibitory response was not linear with malonated kidney

particles between 3 and 6 μ g. of fluorocitric acid (enzymic) and that it was lower than the responses in kidney untreated with malonate, which might be expected to be 5–10 μ moles (Peters *et al.* 1953). This point was investigated in another experiment (Table 3) in which a direct comparison was made. The value with fluorocitric acid (0.0142 μ mole) without malonate was 4.51, rather lower than the 5.0 μ moles which might be expected for the enzymic compound. With malonate the values were 2.75 and 4.27, 61 and 64 % of the values obtained in the absence of malonate. The value 2.75 μ moles for 0.0142 μ mole was confirmed in another experiment, but the value found for 0.0285 μ mole was lower. In another case, however, the relation was approximately linear up to 3.0 μ g. of fluorocitric acid (0.0142 μ mole). From the value 2.75, assuming a linear relation, provisional values of fluorocitric acid present were calculated which are shown in Table 2. It is clear that malonate reduces the accumulation of citrate in presence of fluorocitrate, perhaps due to inhibition of re-cycling of any metabolite of citrate which escapes the aconitase block. As the sodium malonate increases the tonicity of the media, in a control experiment the tonicity was increased by addition of NaCl, with little effect on citrate metabolism; hence an effect of change of tonicity can be excluded.

Brain 'particles'. In view of the complicated nature of the controls required for estimating fluorocitric acid with kidney particles, it appeared advisable to get some different proof of the same point, which was done as follows. In earlier papers (Liébecq & Peters, 1949; Peters & Wakelin, 1953) it had been proved that the pyruvate oxidase system present in brain particles (from the pigeon) under our conditions does not synthesize fluorocitric acid from fluoroacetate, and that fluorocitric acid blocks the metabolism of pyruvate with

Table 3. *Effect of fluorocitric acid (neutralized) on citric acid metabolism of kidney particles (from the guinea pig) in the presence and absence of malonate*

Kidney particles prepared from one guinea pig were suspended in 26 ml. of 1 % KCl containing $\frac{1}{2}$ vol. of 0.5M-phosphate buffer (KH_2PO_4 neutralized with NaOH to pH 7.2); to half sodium malonate was added so that each 3.0 ml. contained 15 mg. Twelve flasks were set up, each containing a total volume of 3.0 ml. made up of 1.9 ml. of kidney suspension, 0.1 ml. of MgCl_2 (4.0 μ moles), 0.1 ml. of ATP (1.2 μ moles), 0.4 ml. of citric acid neutralized (10 μ moles), KCl (1 %; 0.3–0.5 ml.), with additions 0–0.2 ml. of fluorocitrate (0.1 ml. $\equiv$ 3 μ g. of fluorocitric acid). Immediately after the final addition of enzyme the flasks were shaken at 38° for 30 min.; then 1 ml. of trichloroacetic acid (25 %) was added, and citrate estimated.

Additions	Citrate present (μ moles)	Diff. (μ moles)
Malonate present		
Citrate only	0.726	—
Citrate + fluorocitrate (0.0142 μ mole)	3.48	2.75
Citrate + fluorocitrate (0.0285 μ mole)	5.00	4.27
No malonate		
Citrate only	0.413	—
Citrate + fluorocitrate (0.0142 μ mole)	4.92	4.51
Citrate + fluorocitrate (0.0285 μ mole)	7.02	6.61

accumulation of citric acid and a diminution of oxygen uptake. Though less accurate this gives a good way to estimate fluorocitric acid in the presence of traces of fluoroacetate and malate or fumarate, because the latter must be present in any case in the pyruvate experiment where an addition of fumarate to the pyruvate is necessary for full activity. Accordingly, the first stage of the experiment with kidney particles, in which the system was arranged to synthesize fluorocitrate, was performed as before. The supernatant from this first stage was added in suitable quantities to a particle preparation from pigeon's brain, made by grinding according to our usual methods, in order to see how inhibitory it was to the citrate metabolism and oxygen uptake. The experiments in Table 4 demonstrate quite clearly that by this method there is no detectable synthesis of fluorocitric acid from fluoroacetate in the presence or absence of pyruvate; hence the method is valid in detecting fluorocitric acid in the presence of fluoroacetate. A point has been raised as to whether the addition of CoA would

improve the synthesis. Reinforcement with CoA is essential in the choline acetylase system from pigeon brain (Balfour & Hebb, 1952; Gardiner & Whittaker, 1954). Such brains have been treated with acetone, which evidently alters permeability relations. Expt. 2 in Table 4 shows quite clearly that our particle preparations are not influenced by additions of CoA.

In the experiment given in Table 5, it is shown by the use of brain dispersions that the synthesis of fluorocitrate by kidney particles is inhibited by the presence of acetate.

DISCUSSION

Both types of experiment agree in demonstrating that 0.416 mM-acetate markedly inhibits the formation of fluorocitric acid from fluoroacetate (6.67 mM), and that even the effect of 0.2 mM-acetate is detectable. The advantage of the estimations with brain particles is that with these the formation of more fluorocitric acid during the estimation is

Table 4. *Failure of washed brain particles (pigeon) to synthesize fluorocitrate from fluoroacetate*

Experiment 1

In the first stage, pigeon-brain particles were exposed to fluoroacetate + pyruvate. In the second stage, extracts from this suspension were tested with fresh brain particles for the effect on citrate accumulation.

First stage. Brain-particle dispersions, from one pigeon, in KCl (1%) were centrifuged and made up in 14 ml. of KCl-phosphate mixture, pH 7.2, containing 0.05 M-phosphate. Six Erlenmeyer flasks were shaken in the bath at 38° for 40 min.; each contained 1.9 ml. of enzyme preparation, 0.1 ml. of $MgCl_2$ (4.0 μ moles), 0.1 ml. of ATP (1.2 μ moles), 0.1 ml. of sodium fumarate (10 μ moles), 0.1 ml. of sodium fluoroacetate (10 μ moles), and 1% KCl to total volume of 3.0 ml. Three flasks had an addition of 0.1 ml. of sodium pyruvate (30 μ moles).

	Citrate formed (μ mole/flask)
A. No pyruvate	0.18
B. + pyruvate	0.81

The contents of flasks A and B were heated in thin-walled tubes in the boiling-water baths for 10 min. The supernatant fluid after cooling and centrifuging was used for the second experiment.

Second stage. The brain particle dispersion was made up in 18 ml. of KCl-phosphate mixture. Other details were the same, except that all the flasks contained fumarate and pyruvate, and that 8 flasks were used (4 sets of duplicates).

	Citrate formed (μ moles/flask)
No addition	0.54
+ A (1/10 of flask contents)	0.59*
+ B (1/10 of flask contents)	0.50*
+ fluorocitrate (synthetic) 5 γ	1.31

Experiment 2. Failure of addition of CoA to influence the synthesis

In a similar set of two experiments carried out with the addition of CoA, there was also no appreciable citrate formation, even though the preparation was more active.

	Citrate formed (μ moles/flask)
<i>First stage:</i> A. No pyruvate + CoA (36 units)	0.18
B. + pyruvate + CoA (36 units)	0.64
<i>Second stage:</i> No addition	0.99
+ A	0.99*
+ B	0.87*
+ fluorocitrate	2.46

* Citrate present in 1/10 of A and B has been subtracted.

excluded. There can be no doubt therefore that in the kidney-tissue homogenate acetate stops fluorocitric acid formation. It is curious that on the whole there seems little correlation between O_2 uptake and the citric acid metabolism, but this has been also a feature of our earlier work on the fluorine-containing compounds.

Our biochemical results *in vitro* provide the biochemical proof in cell-free preparations that acetate is a powerful protective agent, in the sense that it stops the formation of fluorocitric acid. *In vivo*, as mentioned above, the effective behaviour of acetic acid itself has proved variable; except in mice (Tourtellotte & Coon, 1949; 1951) it works poorly. In the intact animal it appears therefore that acetic acid does not always reach the active centres concerned.

Though acetate has been here proved by two independent methods of estimation to inhibit fluorocitrate synthesis from fluoroacetate, the centre concerned with fluoroacetate activation is not on the ordinary pathway for acetate, because it has been a general experience that the known isolated acetate-activating systems do not activate fluoroacetate. For instance, some time ago we tried the acetone-dried pigeon-liver preparation (Stern & Ochoa, 1949). In two experiments with liver enzyme which easily formed citrate from oxaloacetate, in the presence of ATP, Mg^{2+} ions and cysteine, there was no detectable formation of fluorocitrate from oxaloacetate and fluoroacetate.

Similar negative results have been obtained by us with acetate-activating enzymes from bacteria, kindly given to us by Dr W. H. Elliott. Others have had like experiences, for instance P. Hele (personal communication), Brady (1955), Dominguez, Shide-man, Mahler & Hift (1954). Since it seems unlikely that acetate is merely blocking a centre through which it is not metabolized at all, it can be concluded that some stage of the pathway for the activation of fluoroacetate is independent of the usual main path for acetate activation; the final stage, however, appears to be fluoroacetyl-CoA as shown by Brady (1955) and Marcus & Elliott (1956). Hence the acetate inhibition of fluorocitrate synthesis appears to occur by some subsidiary pathway for acetate (Peters, 1955). It has been found by others (Hendershot & Chenoweth, 1954) that butyrate does not protect against fluoroacetate, but protects against fluorobutyrate. In a few experiments we have found that butyrate does not stop fluorocitric acid formation from fluoroacetate with the kidney particles, even at a concentration of $20 \mu\text{moles}/3.0 \text{ ml}$. Hence, though the final step again should be fluoroacetyl-CoA, the inhibition by fluoro derivatives of higher fatty acids cannot occur on the same initial enzyme centre as that which activates the fluoroacetate; the position has been represented diagrammatically by Peters (1957). One further point may be mentioned: the experiment given in Table 1 confirms that the substances taking part in fluorocitrate synthesis belong to the

Table 5. *Effect of acetate on the synthesis of fluorocitric acid with kidney particles as tested by experiments with brain particles*

First stage. Kidney particles from guinea pig, prepared in KCl-phosphate mixture (see Table 3). Each flask contained 1.9 ml. of enzyme, 0.1 ml. of $MgCl_2$ ($4.0 \mu\text{moles}$), 0.1 ml. of ATP ($1.2 \mu\text{moles}$), additions as stated (0.2 ml. of malate ($20 \mu\text{moles}$), 0.1 ml. of fluoroacetate ($20 \mu\text{moles}$), 0.05–0.2 ml. of sodium acetate and KCl to bring to volume of 3.0 ml.). The flasks were shaken for 50 min. at 38° , cooled and immersed separately in thin test tubes in the boiling-water bath for 5 min. The supernatant was used for tests in the second experiment after centrifuging; it was stored in the cold.

Additions	Citrate accumulated ($\mu\text{moles}/\text{flask}$)
A. Malate alone	0.53
B. Malate + fluoroacetate	5.80
C. Malate + fluoroacetate + sodium acetate ($0.625 \mu\text{mole}$)	5.10
D. Malate + fluoroacetate + sodium acetate ($1.25 \mu\text{moles}$)	5.13
E. Malate + fluoroacetate + sodium acetate ($2.50 \mu\text{moles}$)	6.40

Second stage. Brain-particle homogenate from two pigeons, prepared in KCl-phosphate mixture (not centrifuged) made up to 26.0 ml. Each respirometer flask contained, in a total volume of 3.0 ml., 1.9 ml. of enzyme, 0.1 ml. of $MgCl_2$ ($4.0 \mu\text{moles}$), 0.1 ml. of ATP ($1.2 \mu\text{moles}$), 0.2 ml. of pyruvate ($30 \mu\text{moles}$), 0.1 ml. of fumarate ($10 \mu\text{moles}$), 0.4 ml. of KCl-phosphate and additions (0.2 ml.) (i.e. 1/15 flask from 1st experiment). The flasks were shaken for 40 min. at 38° , and subsequently deproteinized with trichloroacetic acid for citric acid estimation. The effect is very marked with D ($1.25 \mu\text{moles}$ of acetate).

Additions	O_2 uptake ($\mu\text{l.}$)	Citrate accumulation (μmoles)
A	346	0.83
B	229	3.08
C	267	1.46
D	301	0.90
E	311	0.60

part of the tricarboxylic acid cycle not blocked by malonate, i.e. coming after succinate.

From the information available in this paper, in explaining fluoroacetate toxicity it is still unnecessary to go outside the hypothesis that fluorocitric acid is the toxic agent. On this hypothesis, the effect of the protective agents, monoacetin or acetamide when given early enough, is to stop the formation of fluorocitric acid *in vivo*, and so stop the poisoning.

In regard to the problem of acetate and fluoroacetate activation in brain tissue, there are some facts which are not yet reconciled: (1) No increase of oxygen uptake on adding acetate to brain-particle or -brei preparations has ever been found, i.e. these preparations do not activate acetate. (2) Acetate used with acetone-dried preparations of brain tissue can take part in the synthesis of acetylcholine via acetyl-CoA (Balfour & Hebb, 1952; Gardiner & Whittaker, 1954). (3) Fluoroacetate does not cause citrate accumulation *in vitro* in pigeon-brain tissue, which therefore lacks some of the enzymes needed for synthesis of fluorocitric acid. (4) Fluoroacetate when injected into the brain cortex of the dog *in vivo* does cause citric acid accumulation (Hendershot & Chenoweth, 1955). (5) Fluoroacetate when injected intraperitoneally or systemically into several species of animal causes convulsions with rise of citrate in brain tissue; this means that somehow fluorocitrate does get into the brain. (6) Fluorocitrate when injected into the subarachnoid space of pigeons, rats and rabbits induces convulsions at a much smaller concentration than fluoroacetate; in fact it is doubtful whether the latter produces convulsions directly at all (Hastings, Peters & Wakelin, 1955).

From (5) and (6) it seems certain that fluorocitrate is the actual initial stimulus to the convulsions; (4) has not actually been tried for dog's brain tissue *in vitro*. Points (3) to (6) would be reconciled if the tissue constituting the blood-brain barrier (whether blood vessel or otherwise) could synthesize fluorocitrate from fluoroacetate; the discrepancy between (1) and (2) may be merely a matter of relative concentration or of spatial separation.

SUMMARY

1. The effect of acetate *in vitro* on the synthesis of fluorocitric acid from fluoroacetate by particulate preparations from the guinea-pig kidney has been examined. Estimation of fluorocitric acid formed was made by two independent enzymic methods, with kidney and brain 'particles' respectively.

2. Both methods are in agreement in establishing that the presence of acetate in much lower concentrations than that of the fluoroacetate inhibits the synthesis of fluorocitric acid.

3. Since there is evidence that the usual acetate-activating systems do not activate fluoroacetate, the activation concerned here must take place on some independent centre.

4. In animals that are not protected by acetate, this substance must fail to penetrate to the active centres *in vivo*.

REFERENCES

- Balfour, W. E. & Hebb, C. (1952). *J. Physiol.* **118**, 94.
 Bartlett, G. R. & Barron, E. S. G. (1947). *J. biol. Chem.* **170**, 67.
 Brady, R. O. (1955). *J. biol. Chem.* **217**, 213.
 Buffa, P. & Peters, R. A. (1949). *J. Physiol.* **110**, 488.
 Chenoweth, M. B., Kandel, A., Johnson, L. B. & Bennett, D. R. (1951). *J. Pharmacol.* **102**, 31.
 Dominguez, A. M., Shideman, F. E., Mahler, H. R. & Hift, H. (1954). *Fed. Proc.* **13**, 1149.
 Farah, A., West, T. C. & Angel, R. (1950). *J. Pharmacol.* **98**, 234.
 Gardiner, J. F. & Whittaker, V. P. (1954). *Biochem. J.* **58**, 24.
 Gitter, S. (1956). *Biochem. J.* **63**, 182.
 Gitter, S., Blank, I. & Bergman, E. D. (1953). *Proc. kon. ned. Akad. Wet.* **56**, 4C, 423, 427.
 Hastings, A. B., Peters, R. A. & Wakelin, R. W. (1955). *Quart. J. exp. Physiol.* **40**, 258.
 Hendershot, L. C. & Chenoweth, M. B. (1954). *J. Pharmacol.* **110**, 344.
 Hendershot, L. C. & Chenoweth, M. B. (1955). *J. Pharmacol.* **113**, 160.
 Hutchens, J. O., Wagner, H., Podolsky, B. & McMahon, T. M. (1949). *J. Pharmacol.* **95**, 62.
 Liébecq, C. & Peters, R. A. (1949). *Biochim. biophys. Acta*, **3**, 215.
 Lotspeich, W. D., Peters, R. A. & Wilson, T. H. (1952). *Biochem. J.* **51**, 20.
 Marcus, A. & Elliott, W. B. (1956). *J. biol. Chem.* **218**, 823.
 Martius, C. (1949). *Liebigs Ann.* **561**, 227.
 Morrison, J. F. & Peters, R. A. (1954). *Biochem. J.* **58**, 473.
 Peters, R. A. (1952). *Brit. med. J.* **2**, 1165.
 Peters, R. A. (1955). *Johns Hopk. Hosp. Bull.* **97**, 1.
 Peters, R. A. (1957). *Advanc. Enzymol.* (in the Press).
 Peters, R. A. & Wakelin, R. W. (1953). *J. Physiol.* **119**, 421.
 Peters, R. A., Wakelin, R. W., Buffa, P. & Thomas, L. C. (1953). *Proc. Roy. Soc. B*, **140**, 497.
 Peters, R. A. & Wilson, T. H. (1952). *Biochim. biophys. Acta*, **9**, 310.
 Pucher, G. W., Sherman, C. C. & Vickery, H. B. (1936). *J. biol. Chem.* **113**, 235.
 Rivett, D. E. A. (1953). *J. chem. Soc.* p. 3710.
 Stern, J. R. & Ochoa, S. (1949). *J. biol. Chem.* **179**, 491.
 Tourtellotte, W. W. & Coon, J. M. (1949). *Fed. Proc.* **8**, 339.
 Tourtellotte, W. W. & Coon, J. M. (1951). *J. Pharmacol.* **101**, 82.