Some Effects of Synthetic Fluoro Compounds on the Metabolism of Acetate and Citrate

By E. M. GAL,* R. A. PETERS† AND R. W. WAKELIN

Department of Biochemistry, University of Oxford

(Received 1 November 1955)

The work described here was started to extend knowledge of the relative effects of fluoro compounds with special reference to fluoroacetate and fluorocitrate; it was hoped that it would have particular application to effects upon the liver and to the biochemical mechanism of convulsions induced by fluorocitrate.

Bartlett & Barron (1947) showed originally that acetate accumulates after fluoroacetate poisoning; but this effect is probably indirect, as Buffa & Peters (1949) found that the poisoning induced a citrate accumulation, and Peters, Wakelin, Buffa & Thomas (1953) demonstrated that this was due to the synthesis from the fluoroacetate of fluorocitrate, which blocked the tricarboxylic acid cycle at the aconitase stage. The specificity of this inhibition was clearly demonstrated on highly purified aconitase preparations by Morrison & Peters (1954). This work left questions still to be answered. For instance, the livers of male rats showed no increase in citric acid concentrations as a result of fluoroacetate poisoning, according to Buffa & Peters (1949), Potter & Busch (1950) and Fawaz & Fawaz (1953); but Ord & Stocken (1953) recently reported an apparent conversion of fluoroacetate into fluorocitrate by rat liver. There was also the problem why Hastings, Peters & Wakelin (1953) found no indication that convulsions induced by fluorocitrate were connected with any ionic imbalance.

[¹⁴C]Acetate was used *in vivo* to study inhibition of acetate metabolism as a whole. Previous work by Gould, Sinex, Rosenberg, Solomon & Hastings (1949) had shown that [*carboxy*-¹⁴C]acetate upon injection into rats was about 85% converted into labelled carbon dioxide in 4 hr., and Lifson & Swanson (1953) have indicated that in fluoroacetate-poisoned mice the rate of conversion of [¹³C]acetate into radioactive carbon dioxide was decreased by approximately 45%. These results were confirmed by us and extended to fluorocitrate and fluoropyruvate. The latter compound proved particularly

interesting because of its divergent action from fluoroacetate, both *in vivo* and *in vitro*.

A preliminary account of this work has appeared (Gal, Peters & Wakelin, 1954).

METHODS

Animals. Wistar-strain male rats (body weight, 70–300 g. except in rats used for experiments of Table 4) were used except for the isotopic studies when male rats of 45–55 g. were used exclusively. The animals, unless otherwise indicated, were fed upon a stock diet of rat cubes with water *ad lib*. In the experiments *in vivo*, the animals were killed by rapid immersion in liquid air. Organs were removed, weighed and worked up according to the requirements of the individual method. In the *in vitro* experiments pigeons and guinea pigs were also used. The rats for investigation of urinary-ketone substances were standardized over a period of 4 weeks before the actual experiment started; they received 20 g. of pellets daily at 4 p.m., and were weighed every 3 days. Every morning of the fifth week they were transferred to metabolic cages; the urine was collected between 9 a.m. and 4 p.m., and tested for ketone bodies by Rothera's test. In the sixth week they were divided into groups of three and injected with either fluoroacetate or fluorocitrate. Their collected urine was used for Rothera's test.

Citrate. This was determined by the method of Pucher, Sherman & Vickery (1936) as modified by Buffa & Peters (1949).

Pyruvate. This was estimated by the method of Friedemann & Haugen (1943), with 25 µg. of purified pyruvic acid as a standard. The blood samples (2 ml.) were withdrawn with heparinized needles from the hearts of unanaesthetized animals.

In the experiments with 2-¹⁴C-labelled sodium acetate (Radiochemical Centre, Amersham), the animals first received an intraperitoneal injection of the fluoro compounds and then 40 min. later 30 µg./kg. of acetate (about 2.5 mg./kg.), an amount which was within the range of that tested in the experiments of Gould *et al.* (1949). The animals were then placed in an all-glass metabolic apparatus for collection of CO₂; this was a version of the apparatus of Mackenzie *et al.* (1949), modified to permit the collection of urine. The flow meter was discarded, the rate being set at about 70 bubbles/min. and regulated with a fine high-vacuum stopcock. The solutions and traps were as used by Mackenzie *et al.* (1949). The collection and preparation of BaCO₃ was carried out as described by Popják (1950) with 2 cm.² polyethylene disks (Bettix Ltd., Arrow Works, Elm Grove, London). The radioactive measurement of the BaCO₃ was carried out in samples

* Postdoctoral Fellow of the National Foundation for Infantile Paralysis, U.S.A. Present address: Department of Pharmacology, UCLA Medical School, Los Angeles 24.

† Present address: Agricultural Research Council Institute of Animal Physiology (Biochemistry Department), Babraham Hall, Cambridge.

of 'infinite thickness', all samples being counted in duplicate. The brain, kidney, heart and liver were worked up separately, as recommended by Calvin, Heidelberger, Reid, Tolbert & Yankwich (1949). The rest of the organs were pooled with the carcass. The urine samples, whenever they could be collected, were also assayed, after neutralization with NaOH to about pH 7.2 and evaporation in a desiccator *in vacuo*. The residue was taken up in 0.5 ml. of water, plated and dried under a heating lamp.

Measurement of radioactivity. All measurements were carried out on a Dynatron scaler unit with a thin-end-window Geiger-Müller counter. The background varied between 12 and 18 counts/min., and was corrected for standard deviation. All samples of counts 20 times the background were counted for 30 min., recounted, and corrected for the background and total deviation. The background counts were measured for 30–45 min. at the beginning, the middle and the end of the course of the radioactive assays. The results are given $\pm$ s.d.

Chemicals. All reagents were AnalaR grade. The fluoro compounds were of analytical purity. Sodium fluoroacetate was purchased, and fluoroacetamide was given by Dr E. D. Bergmann through Dr S. Gitter. Sodium fluorocitrate was kindly given by Dr D. E. A. Rivett or synthesized according to his method (Rivett, 1953). Enzymic fluorocitrate was prepared in the laboratory by the method of Peters *et al.* (1953). Sodium fluoropyruvate was prepared from a solution of pure fluoropyruvic acid (kindly given by Mr R. Brown) by addition of an equivalent amount of 0.1 N-NaOH. Fluoropyruvic acid was also synthesized by us by a slight modification of the method of Mager & Blank (1954) and purified by microsublimation to m.p. 82°. Diethyl fluoro-oxaloacetate was prepared according to Rivett (1953). Dimethyl fluoromalate and fluoromalamide were synthesized by Taylor & Kent (1954) and were generously put at our disposal by them. Fluoroacetyl-hydroxamic acid was prepared by us through the reaction of methanolic hydroxylamine in ethyl fluoroacetate. The white solid, after its recrystallization from ethyl acetate, had m.p. 79°. It was also prepared by the Hoffmann (1889) method from the corresponding amide. Phosphate buffer consisted in all cases of KH_2PO_4 neutralized to the pH stated with NaOH.

RESULTS

Experiments with $[^{14}\text{C}]$ acetate

The average individual values calculated as percentage total activity of the recovered expiratory CO_2 are plotted in Fig. 1. About 50% of the activity in the acetate control animals appears in CO_2 in about 30 min. This is somewhat faster than was observed by Gould *et al.* (1949), but it agrees with the observations of Coniglio, Anderson & Robinson (1952) on the effect of $[^{14}\text{C}]$ acetate in fasted adult rats. All fluoro compounds injected were inhibitory; whereas in the control 75% of the acetate was excreted as CO_2 in 4 hr., with fluoroacetate it was 40, with fluorocitrate 30 and with fluoropyruvate 50%. It should be noted that since the acetate is injected 40 min. after the administration of the fluoro compound, considerable conversion of

fluoroacetate into fluorocitrate will have already taken place in tissues where it can occur.

At first glance the amount of sodium fluorocitrate injected seems to be unusually large, especially if it is assumed that each molecule of fluoroacetate is converted into fluorocitrate. The dose of fluoroacetate injected was 0.071 m-mole/kg. and that of fluorocitrate (synthetic) was 0.145 m-mole/kg. In enzymic tests the synthetic fluorocitrate has about half the activity of the enzymic owing to the presence of unnatural isomers, so that the effective doses of fluoroacetate and fluorocitrate in these experiments were approximately equal. This raises some points for discussion.

To get some idea of the distribution of the residual activity in some parts of the body, after injection of $[^{14}\text{C}]$ acetate, the percentage distribution was done (Table 1). The only observation of interest is that there is a trend towards increased residual activity in the carcasses of the poisoned animals. It is not possible to say whether there is some interference with acetate utilization, as suggested by Bartlett & Barron (1947), or whether in the presence of blocked aconitase there is increased conversion into other metabolites. To solve this, it would need an injection of acetate of at least 60 times the radioactivity to permit of isolation of the components for radioactive assays.

Fig. 1. Radioactivity of expiratory CO_2 from $[2-^{14}\text{C}]$ acetate from poisoned rats (male). Percentage $^{14}\text{CO}_2$ recovered. Fluoro compounds were injected 40 min. before the injection of 30 mg. of acetate/kg. The points are the averages of pooled collections from six animals for each curve, with the exception of the fluoroacetate curve where one animal died after 3 hr. and was discarded.

Table 1. *Distribution of radioactivity after injection of $^{14}\text{CH}_3\cdot\text{CO}_2\text{Na}$ (30 $\mu\text{C/kg.}$) into normal and poisoned rats*Average weight of rats 50 ± 5 g. Animals were killed at 4 hr. Doses of fluoro compounds as for Fig. 1.

	Acetate alone		Fluoroacetate and acetate		Fluoropyruvate and acetate		Fluorocitrate and acetate	
	(counts/min./g.)	(%)	(counts/min./g.)	(%)	(counts/min./g.)	(%)	(counts/min./g.)	(%)
Brain	382 ± 39	0.41	645 ± 132	0.70	675 ± 155	0.75	475 ± 187	0.53
Heart	555 ± 33	0.60	763 ± 164	0.82	768 ± 285	0.85	769 ± 196	0.85
Kidney	832 ± 146	0.90	906 ± 91	1.04	582 ± 34	0.64	$1\ 098 \pm 111$	1.22
Liver	958 ± 833	1.06	$2\ 080 \pm 735$	2.24	$1\ 087 \pm 182$	1.25	706 ± 276	0.78
Carcass	$17\ 000 \pm 2566$	18.5	$28\ 350 \pm 1960$	30.05	$22\ 290 \pm 1445$	24.50	$33\ 957 \pm 2380$	38.00
Urine	$1\ 840 \pm 378$	2.12	803 ± 143	0.93	776 ± 91	0.89	$7\ 828 \pm 892$	8.92
No. of determinations	(6)		(5)		(6)		(6)	

Table 2. *Effect of intraperitoneal injections of fluoroacetate and of fluorocitrate (synthetic) on the accumulation of citrate in male rats*Average weight of rats 50 ± 5 g. Results are expressed as $\mu\text{g.}$ of citrate/g. of wet tissue. The normal values for brain, kidney and liver are given in Table 4. Those for spleen and heart were found by Buffa & Peters (1949) to be 14 and 25 $\mu\text{g./g.}$ respectively.(a) *Upon accumulation of citrate in 2 hr.*

Tissue	Fluoroacetate 5 mg./kg.	Fluorocitrate	
		20 mg./kg.	80 mg./kg.
Brain	178 ± 7	151 ± 27	364 ± 34
Heart	798 ± 112	108 ± 14	365 ± 50
Kidney	1029 ± 40	1230 ± 130	3060 ± 567
Liver	33.5 ± 5	812 ± 89	1720 ± 205
Spleen	596 ± 71	510 ± 68	635 ± 84
No. of animals	(2)	(4)	(4)
Convulsion	+	-	+

(b) *Upon the rate of accumulation of citrate*

No. of animals in parentheses.

Tissue	Time (hr.)	Fluoroacetate (5 mg./kg.)	Fluorocitrate (40 mg./kg.)
Brain	1	172 ± 8 (2)	80.7 ± 7.3 (3)
	2	182.6 ± 83 (3)	221.5 ± 58.5 (4)
	3	200 ± 24.8 (4)	233 ± 25.6 (4)
	4	205 ± 25.3 (4)	248.5 ± 36.5 (4)
Kidney	1	597.6 ± 93 (3)	1508 ± 102 (4)
	2	1110 ± 83 (4)	2329 ± 153.9 (6)
	3	1499 ± 302 (4)	2725 ± 423 (4)
	4	1594 ± 751 (4)	2456 ± 251 (4)
Liver	1	30.6 ± 2.6 (3)	956 ± 44.6 (3)
	2	50.5 ± 17 (4)	1313 ± 179 (6)
	3	34.9 ± 1.9 (4)	1450 ± 126 (3)
	4	44.4 ± 5.7 (4)	1496 ± 236.5 (4)

Accumulation of citrate

Table 2a shows the average content of citric acid in the same strain of rats in various organs 2 hr. after poisoning by fluoroacetate and fluorocitrate. In fourteen out of fifteen cases, fluoroacetate induced no increase in the citrate concentration of the liver. It should be emphasized that these were male rats, as the citrate values are known to be different in female animals (Dubois, Cochran & Zerwic, 1951).

The picture for fluoroacetate at 1 hr. after injection confirms that of Buffa & Peters (1949); see also Potter & Busch (1950). The injection of fluorocitrate, however, even at 20 mg./kg., produced a surprising difference in the liver, where the citrate content increased to 24 times the normal. This was also shown by the triethyl ester of fluorocitric acid.

In Table 2b, the relative rates of citrate accumulation have been determined. It is to be noted

particularly that in both the liver and the kidney, but especially in the liver, there is an immediate rise of citric acid following fluorocitrate injection, suggesting that it produces its action rapidly. Taken together, the interpretation appears to be that fluorocitrate penetrates the liver cells directly and so blocks the tricarboxylic acid cycle, whereas fluoroacetate, even if it penetrates, is not synthesized to fluorocitrate. This latter behaviour would be consistent with the failure to find that fluorocitric acid is formed from fluoroacetate and oxaloacetate by pigeon-liver particles *in vitro* (Peters & Wakelin, 1952). In experiments *in vitro*, fluorocitrate caused large accumulations of citric acid in liver homogenates.

The results with brain were somewhat equivocal. There was a quicker rise in citric acid with fluoroacetate than with fluorocitrate; but this difference is not statistically significant. Fluoroacetate gave convulsions in 30 min., whereas with fluorocitrate the animals only convulsed with a dose of 80 mg./kg.; 40 mg./kg. did not give convulsions, nor could its action be potentiated by simultaneous administration of urea to increase penetration, or by injection of 0.8 mg. of fluoroacetate/kg.

In order to make it clear that in the rat also the intracerebral injection of fluorocitrate produces effects not given by fluoroacetate, some intracerebral injections were made in young rats with minimal doses. The previous comparison had been made on pigeons (Peters & Wakelin, 1953), and the experiments of Dawson & Peters (1955) made for another object had been done with amounts too large to determine this point. Intracerebral injections of 1.5 μ g. of fluorocitrate (enzymic) were convulsive in six animals of weight 48–55 g., whereas 10 μ g. of fluoroacetate did nothing, except in one animal out of six (the first tried, which convulsed after 55 min., but did not die). It may be reckoned that for these animals approximately 1.2 μ g. of fluorocitric acid was the limiting amount needed for producing convulsions. Table 3 gives the distribution of citric acid in brain, kidneys and the liver at death following such intracerebral injection; some of the injected fluorocitrate occasionally appears to get from the brain into the kidney, where it induces a rise of citric acid. A tendency for a similar phenomenon can be seen in the results of Dawson & Peters (1955), although their experiments were done with much larger amounts of fluorocitrate.

The experiments made *in vivo* give support to the *in vitro* experiments which have indicated that there is some difference in the pathway for the conversion of fluoroacetate and acetate into fluorocitric and citric acids respectively. This can be judged from the fact that, like fluorocitric acid, fluoroacetate cuts down the conversion of [14 C]acetate into CO_2 , and yet is clearly not activated by liver. In addition,

Table 3. *Effect of intracerebral injection of fluorocitrate (enzymic) on citrate accumulation*

Average weight of rats 50 ± 1.5 g.					
Fluorocitrate injected (μ g.)					
(as acid)	5.3	5.3	8		
First convulsion (min.)	36	33	37		
Killed (min.)	56	71	78		
	Citrate (μ g./g. of wet tissue)				
Brain	170	177	142		
Kidney	28.4	87.5	165		
Liver	11.3	5.6	8.5		

we have noticed that the urine of animals treated with fluorocitrate gave a positive Rothera reaction for acetoacetic acid. Quantitative assays are in progress by the method of Greenberg & Lester (1944). There may be a metabolic diversion of active acetyl fragments with fluorocitrate, presumably in the liver; this does not occur with fluoroacetate. One of us (Peters, 1955) has concluded that there must be two paths for acetate metabolism, only one of which is used in fluoroacetic acid activation. It seems certain now that fluorocitrate, as a component of the path for fluoroacetate, as this is implied by the experiments of Brady (1955) and of Elliott & Marcus (1955), who synthesized fluoroacetyl-CoA and showed that it gave fluorocitrate in the presence of oxaloacetate and condensing enzyme. Some difference was found, however, in the chemical nature, reactivity and resistance to hydrolysis between the acetyl- and the fluoroacetyl-CoA. To make certain that the inability to detect fluoroacetyl-CoA in enzymic tests was not due to failure of fluorocitrate to give the usual colour reaction with ferric chloride, we have synthesized fluoroacetylhydroxamic acid and found that it gives the iron reaction.

We have also tried several fluoro compounds along with fluorocitrate to find how far they interfere with the metabolism of pyruvate and acetate; the results of the intraperitoneal injections are given in Table 4. It will be noted that the fluorocitrate produced a significant increase of citric acid only in the kidney; yet, unexpectedly, it produced convulsions which appeared 90 min. after the administration of 8 mg./kg. Much larger doses of diethyl fluoro-oxaloacetate gave the same citric picture without convulsions. Fluoromalamide produced no effect except for a small rise in kidney citrate. In all these cases it appeared that there was little if any tendency to convert any active fluoroacetyl into fluorocitrate (as judged by citrate accumulation). The most remarkable result was found with fluoropyruvate, the preliminary results of which are reported below

Table 4. *Effect of the intraperitoneal injection of various fluoro compounds and of one iodo compound on the citrate accumulation*

Compound	Average wt. of animal (g.)	Dose (mg./kg.)	Time (hr.)	Citrate (μ g./g. of wet tissue)			No. of expts.
				Brain	Kidney	Liver	
Fluoroacetylhydroxamic acid	80	8.0	4	59 $\pm$ 17.5	251 $\pm$ 83	12.4 $\pm$ 3.8	6
Fluoroacetamide	100	10.0	2	120 $\pm$ 21	247 $\pm$ 61	20 $\pm$ 6.9	2
Dimethyl fluoromalate	85	150.0	2	43 $\pm$ 6	48.2 $\pm$ 2.5	16 $\pm$ 3.5	2
Fluoromalamide	100	300.0	2	46 $\pm$ 5	125 $\pm$ 33	62 $\pm$ 5.1	2
Diethyl fluoro-oxaloacetate	50	150.0	2	41 $\pm$ 3.8	119 $\pm$ 21	22.5 $\pm$ 2.5	2
	400	150.0	2	53 $\pm$ 7	344 $\pm$ 100	15.5 $\pm$ 0.5	2
Iodoacetic acid	612	1.80	1	—	74.2 $\pm$ 16	33 $\pm$ 9.2	2
Control	578	—	—	39 $\pm$ 6	45 $\pm$ 7	52 $\pm$ 12	2
	100	—	—	20 $\pm$ 6	29 $\pm$ 13	3.5 $\pm$ 0.4	2

at greater length, and show that this compound is metabolized differently from fluoroacetate or the even-numbered ω -fluoro fatty acids.

Fluoropyruvic acid

It has long been thought by those working in the field of fluoro compounds (see Chenoweth, 1949) that fluoropyruvic acid should be investigated. The compound recently became available through the work of Mager & Blank (1954), whose synthesis has been confirmed by Mr R. Brown and by one of us (E.M.G.) in England.

Experiments in vivo. When injected *in vivo* fluoropyruvate produced no change at all in the citric acid levels; yet some change in the animal must have occurred because of the convulsions it could produce in 4 hr. with a dose of 80 mg./kg. It is noteworthy that whereas the animals poisoned with fluoroacetate and fluorocitrate did not recover, the animals treated with fluoropyruvic and fluoroacetylhydroxamic acids survived at the dose levels tried. At first it was thought that fluoropyruvate might interfere with pyruvate metabolism, especially in view of the enzyme results given below (and of the report of Mager & Blank, 1954). Table 5 shows, however, that there was lower blood pyruvate than normal in the animals poisoned with fluoropyruvate. The following substances were tried with negative results in each of two animals, in the hope of reversing the poisoning: glucose, calcium gluconate, thiamine-HCl and pyruvic acid. This failure of fluoropyruvic acid to affect the tricarboxylic acid cycle is all the more surprising because it would be expected that, like pyruvic acid, it would yield an active fluoroacetyl fragment which in turn would form fluorocitrate, and consequently cause accumulation of citrate.

Experiments in vitro. The methods used have been described in previous papers; the particle preparations are similar to those of Buffa, Peters & Wakelin (1951) and of Liébecq & Peters (1949).

Table 5. *Effect of the intraperitoneal injection of fluoropyruvic acid (80 mg./kg.) on citrate and blood pyruvate accumulation in 4 hr.*

Rats: 100 $\pm$ 5 g. for citrate and 220 $\pm$ 15 g. for pyruvate.

	No. of determinations	Citrate (μ g./g. of wet tissue)
Brain	4	26.1 $\pm$ 9.2
Heart	4	34.1 $\pm$ 5
Kidney	4	39.8 $\pm$ 7
Liver	4	22.5 $\pm$ 3.1
		Blood pyruvic acid (mg./100 ml.)
Poisoned	4	0.578 $\pm$ 0.028
Control	2	1.317 $\pm$ 0.042

Table 6. *Effect of fluoropyruvic acid on carboxylase of yeast*

Each bottle contained 100 mg. of yeast in 1 ml. of 0.1 M phosphate buffer (pH 6.2), 0.1 ml. of $MgCl_2$ (4.0 μ moles of Mg^{2+}), 0.2 ml. of sodium pyruvate (54 μ moles) $\pm$ 0.1 ml. of neutralized fluoropyruvic acid (16.3 mM); the whole was made up to 3.2 ml. with phosphate buffer. The small residual CO_2 production was subtracted from the results. Pyruvate was added from the side tubes after equilibration. Temp. 28°.

	CO_2 evolved (μ l./30 min.)	Percentage of control
Pyruvate alone	732	—
Pyruvate and fluoropyruvate	272	37.1

(i) *Pigeon-brain particles.* In the experiments on pigeon-brain particles it was decided to test for oxidation and conversion into fluorocitrate by the use of the pigeon-brain pyruvate oxidase system. As has been shown in experiments in this Laboratory, when pigeon-brain particles are supplemented with pyruvate plus fumarate and reinforced with magnesium and adenosine triphosphate (ATP), the pyruvate is readily oxidized, and in the presence of fluorocitrate there is an accumulation of citric acid. If therefore fluoropyruvate is oxidized in a similar

Table 7. *Effect of fluoropyruvate upon (a) the synthesis of citric acid from fumarate, and (b) the metabolism of citric acid by kidney particles*

All conical flasks contained as a final addition 2 ml. of kidney particles (guinea pig) suspended in 1.6 ml. of 0.134 M-KCl and 0.4 ml. of 0.5 M phosphate buffer (pH 7.2); the flask also contained 0.1 ml. of Mg^{2+} (4.0 μ moles of $MgCl_2$) and 0.1 ml. of ATP (1.2 μ mole) in (a) 0.2 ml. of sodium fumarate (20 μ moles); (b) 0.4 ml. of sodium citrate (10 μ moles); 0.134 M-KCl was added to make the final volume of 3.0 ml. After shaking for 30 min. at 38.1°, the reaction was stopped with 1 ml. of 25% trichloroacetic acid, and after filtration the contents of duplicate flasks were made up to 16 ml., from which samples were taken for estimation of citric acid.

Blocking agent	Addition	Citric acid accumulated (μ moles)	Citric acid disappeared (μ moles)
Expt. 1 (a) None	Fumarate alone	0.16	—
	Fumarate + fluoropyruvate (6.29 mM)	0.13	—
(b) Malonate (0.034 M)	Citrate (10 μ moles)	—	9.51
	Citrate + fluoropyruvate (6.29 mM)	—	8.92
Expt. 2 (a) Fluorocitrate (20 μ g., synthetic) (0.095 μ mole)	Fumarate alone	7.52	—
	Fumarate + fluoropyruvate (3.14 mM)	0.76	—
	Fumarate + fluoropyruvate (6.29 mM)	0.58	—
(b) Malonate (0.034 M) (no fluorocitrate)	Citrate (10 μ moles)	—	9.83
	Citrate + fluoropyruvate (3.14 mM)	—	9.92
	Citrate + fluoropyruvate (6.29 mM)	—	9.93

way to pyruvate, it should lead to fluorocitrate formation, which should both block the respiration and cause an increased accumulation of citric acid. Fluoropyruvate causes a strong inhibition of pyruvate oxidation in the pigeon-brain system (Fig. 2). In the presence of 0.633 mM fluoropyruvate (0.2 mg./3 ml. in the flask) there is already an inhibition of approximately 35% in the oxygen uptake. This is not, however, associated with an accumulation of citric acid. In the legend to Fig 2, inhibitions of citric acid formation are recorded which have been found to accompany the decrease of oxygen uptake in these experiments. These are more variable and generally greater than the inhibition of oxygen uptake. It seems clear, therefore, that there must be interference with the pyruvate-dehydrogenase step in this oxidation and that there has been no synthesis of an inhibitor for the citric acid stage of the tricarboxylic acid cycle.

The inhibition of the early stage of metabolism of pyruvate has been shown again by studying the effect upon yeast decarboxylation of pyruvic acid. Table 6 shows the decrease caused by fluoropyruvate in this decarboxylation, one of three similar results with dried baker's yeast.

(ii) *Kidney particles (guinea pig)*. The brain-particle experiments indicated that there was no synthesis of fluorocitric acid by brain tissue; instead there was a definite interference with pyruvate oxidation which was reflected in the formation of citric acid. Since brain tissue, at any rate under these conditions, does not synthesize fluorocitric acid from fluoroacetate in the presence of pyruvate and fumarate, it was thought that the failure of fluorocitric acid synthesis should be confirmed with kidney tissue. In our ordinary experiments with

fumarate alone and kidney particles, sufficient active acetyl fragments are formed to make citric acid. It has been shown that in the presence of fluoroacetate and fumarate there is an extra accumulation of citric acid owing to the synthesis of fluorocitric acid (Liébecq & Peters, 1949; Peters *et al.* 1953). With fumarate there is a maximal accumulation of citrate when the preparation is blocked with fluorocitrate. On the other hand, when citrate itself is used as a substrate, fluorocitrate can be determined by its action in blocking aconitase and so preventing the disappearance of citrate. The latter has been the test consistently used in the isolation of fluorocitric acid. The experiments in Table 7 show that in kidney particles, when blocked with fluorocitrate, 3.14 mM fluoropyruvate blocked the formation of citrate by approximately 90%. On the other hand, this amount had no inhibiting effect upon citrate disappearance. In another experiment in the absence of a fluorocitrate block, fluoropyruvate induced no accumulation of citrate. Both these experiments, which have been confirmed, show that fluoropyruvate has an entirely different effect from the expectation if it had been activated to an active fluoroacetyl fragment. More work is required to decide the exact position of the block in the initial stages of pyruvate oxidation and to determine whether fluoropyruvic acid blocks other enzymes too.

In the preliminary note of these experiments it was stated that the effect was not reversible, and this failure to reverse has been found in a small number of experiments; but in further work there seemed to be some conditions under which some degree of reversibility can be observed. This matter, therefore, requires further exploration.

Fig. 2. Inhibition of pyruvate oxidation by fluoropyruvate in pigeon-brain particles; points at 0.633 mM with centrifuged particles, the rest with uncentrifuged particles. The total volume of 3.0 ml. contained in 1.0 ml. of fumarate (10 μ moles), pyruvate (27 μ moles), Mg^{2+} (4.0 μ moles $MgCl_2$), ATP (1.2 μ moles), KCl (0.134 M); and in the remaining 2.0 ml. of brain particles in KCl (0.134 M) and 0.1 M phosphate buffer (pH 7.2). Citric acid accumulations were also determined, and gave, as compared with the control (100%): at 0.157 mM 87%; 0.633 mM 31, 50, 53, 68, 79 %; at 1.572 mM 29, 34%; at 4.72 mM 29%.

DISCUSSION

It is clear that the difference in citric acid content of the liver after injection of fluorocitric acid forms a distinguishing clinical mark for identifying the presence of fluorocitric as distinct from fluoroacetic acid.

In our studies of the rate of carbon dioxide production from acetate there was striking agreement between our values and those of Lifson & Swanson (1953). These authors gave the rate 9.35 m-moles/hr./100 g. of mouse, while ours, as shown in Fig. 1, was 9.65 m-moles/hr./100 g. of rat. With fluoroacetate their rates were 5.59 m-moles/hr./100 g. for

10 mg. of fluoroacetate/kg. and 4.60 for 20 mg./kg.; ours was 4.09 m-moles/hr./100 g. for 5 mg./kg. in the rat. We found that the rate of CO_2 production was 5.30 m-moles/hr./100 g. for 40 mg. of (synthetic) fluorocitrate/kg., while the rate of CO_2 for fluoropyruvate (8 mg./kg.) was 8.15 m-moles/hr./100 g. In assessing the close correlation it is important to remember the wide variation in LD_{50} , even for different strains of rat (Chenoweth, 1949). Both fluoroacetate and fluorocitrate were given at levels causing convulsions, but fluorocitrate was much more inhibitory to the rate of production of respiratory CO_2 . Our results should not be interpreted as showing more than a trend.

The lack of citrate accumulation in the liver of male rats poisoned by fluoroacetate and its indication of failure to form fluorocitric acid suggested that there is a mechanism competing with the formation of fluoroacetyl-CoA.

SUMMARY

1. Injections into rats of some fluoro compounds followed by $[2-^{14}C]$ acetate led to reduction in the $^{14}CO_2$ expired as follows: fluoroacetate 40 %, fluorocitrate 30 %, and fluoropyruvate 50 %. The carcasses of the poisoned animals contained more radioactivity than the other tissues examined.

2. In male rats, whereas generally fluoroacetate induced no rise in citric acid content of the liver, fluorocitrate injections caused immediate rise in the citric acid level.

3. Fluorocitrate when injected intracerebrally has a high toxicity for the rat, as has been shown already in pigeons and rabbits.

4. The urine from animals injected with fluorocitrate gave a marked Rothera's test.

5. Fluoroacetylhydroxamic acid when injected gave convulsions in 90 min. with only slight increases in kidney citrate. Diethyl fluoro-oxaloacetate also increased citrate in kidney, but without convulsions. Fluoromalamic acid gave no effect.

6. Fluoropyruvic acid when injected gave convulsions in 4 hr., with no increase in citric acid. Several substances tried did not reverse the effect. *In vitro*, fluoropyruvic acid inhibited the metabolism of pyruvic acid.

One of us (E.M.G.) gratefully acknowledges the support of the National Foundation for Infantile Paralysis, Inc. (U.S.A.), throughout the entire course of these experiments. He also wishes to thank Professor Wendel H. Griffith of the Department of Physiological Chemistry, Medical Centre, University of California, Los Angeles, for the use of the departmental facilities for some of the experiments. The valuable help of Drs P. Buffa, P. W. Kent, L. A. Stocken, and particularly that of Mr N. F. Taylor, is greatly appreciated. Our thanks are also due to Mr H. Vincent for the skilled construction of the metabolism apparatus and other

glass equipment, and to Miss M. Southward, also to Miss R. Shawdon and Mr R. G. Hall of the Institute of Animal Physiology, Babraham.

REFERENCES

- Bartlett, G. R. & Barron, E. S. (1947). *J. biol. Chem.* **170**, 67.
 Brady, R. O. (1955). *Fed. Proc.* **14**, 186.
 Buffa, P. & Peters, R. A. (1949). *J. Physiol.* **110**, 488.
 Buffa, P., Peters, R. A. & Wakelin, R. W. (1951). *Biochem. J.* **48**, 467.
 Calvin, M., Heidelberger, C., Reid, J. C., Tolbert, B. M. & Yankwich, P. (1949). *Isotopic Carbon*. New York: John Wiley and Sons, Inc.
 Chenoweth, M. B. (1949). *Pharmacol. Rev.* **1**, 383.
 Coniglio, J. G., Anderson, C. E. & Robinson, C. S. (1952). *J. biol. Chem.* **198**, 525.
 Dawson, R. M. C. & Peters, R. A. (1955). *Biochim. biophys. Acta*, **16**, 254.
 Dubois, K. P., Cochran, K. W. & Zerwic, M. M. (1951). *Proc. Soc. exp. Biol., N.Y.*, **74**, 452.
 Elliott, W. B. & Marcus, A. (1955). *Fed. Proc.* **14**, 207.
 Fawaz, G. & Fawaz, E. N. (1953). *Proc. Soc. exp. Biol., N.Y.*, **84**, 680.
 Friedemann, T. E. & Haugen, G. E. (1943). *J. biol. Chem.* **147**, 415.
 Gal, E. M., Peters, R. A. & Wakelin, R. W. (1954). *Biochem. J.* **58**, xiii.
 Gould, R. G., Sinex, F. M., Rosenberg, I. N., Solomon, A. K. & Hastings, A. B. (1949). *J. biol. Chem.* **177**, 295.
 Greenberg, L. A. & Lester, D. (1944). *J. biol. Chem.* **154**, 177.
 Hastings, A. B., Peters, R. A. & Wakelin, R. W. (1953). *J. Physiol.* **120**, 50.
 Hoffmann, C. (1889). *Ber. dtsch. chem. Ges.* **22**, 2854.
 Liébecq, C. & Peters, R. A. (1949). *Biochim. biophys. Acta*, **3**, 215.
 Lifson, N. & Swanson, M. (1953). *J. Pharmacol.* **109**, 1.
 Mackenzie, C. G., Chandler, J. P., Keller, E. B., Rachele, J. R., Cross, N. & du Vigneaud, V. (1949). *J. biol. Chem.* **180**, 99.
 Mager, J. & Blank, I. (1954). *Nature, Lond.*, **173**, 126.
 Morrison, J. F. & Peters, R. A. (1954). *Biochem. J.* **58**, 473.
 Ord, M. G. & Stocken, L. A. (1953). *Proc. Soc. exp. Biol., N.Y.*, **83**, 695.
 Peters, R. A. (1955). *Johns Hopk. Hosp. Bull.* **97**, 1, 21.
 Peters, R. A. & Wakelin, R. W. (1952). Quoted by Peters, R. A. (1952). *Brit. med. J.* **2**, 1165.
 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.
 Popják, G. (1950). *Biochem. J.* **46**, 560.
 Potter, V. R. & Busch, H. (1950). *Cancer Res.* **10**, 353.
 Fucher, G. W., Sherman, C. C. & Vickery, H. B. (1936). *J. biol. Chem.* **113**, 235.
 Rivett, D. E. A. (1953). *J. chem. Soc.* p. 3710.
 Taylor, N. F. & Kent, P. W. (1954). *Nature, Lond.*, **174**, 401.

Indirect Analysis of Corticosteroids

3. THE DETERMINATION OF STEROIDAL DIHYDROXYACETONES*

By J. K. NORYMBERSKI AND R. D. STUBBS

Chemical Research Laboratory, Rheumatism Research Unit, Nether Edge Hospital, Sheffield

(Received 7 March 1956)

Earlier papers of this series (Appleby, Gibson, Norymberski & Stubbs, 1955; Appleby & Norymberski, 1955) introduced two analytical methods, each of which involved a sequence of specific reactions designed to transform an accurately defined group of corticosteroids into 17-oxosteroids and the measurement of the latter as chromogens in the Zimmermann reaction. The same general principle has now been employed to develop a further method of steroid analysis. It is based on the observation (Norymberski, 1956) that steroidal dihydroxyacetones (17 α :21-dihydroxy-20-oxopregnanes) undergo hydrogenolysis at the C₁₇-O bond when treated with zinc powder in boiling aqueous acetic acid; so, for instance, cortisone (I) was converted into 11-dehydrocorticosterone (II). Consequent upon the removal of the 17-hydroxyl group the dihydroxyacetones lose their 17-ketogenic property (convertibility into 17-oxosteroids on oxidation with bis-

muthate; see Brooks & Norymberski, 1953). On the other hand, it was expected that 17:20-diols and 17:20:21-triols, i.e. the two other structural types of the 17-ketogenic group, will resist the action of zinc and hence that it should be possible to measure them selectively as 'zinc-resistant 17-ketogenic steroids'. The proposed analytical procedure was briefly outlined in a preliminary communication (Norymberski & Stubbs, 1955).

The preparative experiments referred to above were performed with only a moderate excess of zinc (10:1, w/w). When cortisone was reduced under conditions considered more suitable for analytical purposes, wherein a much larger excess of zinc (10 000:1, w/w) was employed, it was found that after 1 hr. reduction with zinc, and following oxidation with bismuthate, the amount of Zimmermann chromogens was decreased to a constant level. The nature of the residual chromogenic contribution, whose magnitude corresponded to about 20 % of the quantity expected if the cortisone had not been

* Part 2: Appleby & Norymberski (1955).