CONDITIONS AFFECTING THE ACTION OF FLUOROACETATE ON THE METABOLISM OF NEMATODE PARASITES AND VERTEBRATE ANIMALS

By V. MASSEY*† and W. P. ROGERS†

[*Manuscript received April* 20, 1951]

Summary

The respiration of brei or mince prepared from pigeon breast muscle containing 0.0003M methylene blue was very little affected by 0.01M fluoroacetate. Under similar conditions, the respiration of preparations from *Ascaridia galli* and *Nematodirus* spp. was inhibited 35-50 per cent. This inhibition was lessened by adding intermediates of the tricarboxylic acid cycle. Succinate was most effective in lifting the inhibition; oxaloacetate was least effective. The formation of citrate in brei of *Nematodirus,* containing methylene blue and added acetate and oxaloacetate, was increased more than 100 per cent. when fluoroacetate, 0.01M, was present. The utilization of citrate in mince prepared from *Nematodirus* containing 0.0003M methylene blue was strongly inhibited by 0.01M fluoroacetate when a gas phase of air was used; under anaerobic conditions no inhibition was found. Similar preparations from pigeon liver and pigeon breast muscle were inhibited only to a small degree, about 20 per cent., under aerobic conditions, and not at all under anaerobic conditions. When methylene blue was not added, the inhibition of citrate utilization in pigeon breast muscle preparations in air rose to about 50 per cent.; under oxygen the inhibition rose to 100 per cent. When cytochrome *c,* 5×10^{-6}M, was added, the inhibition was 100 per cent., even in a gas phase of air.

The inhibition of citrate utilization in mince prepared from pigeon breast muscle was examined at different oxidation-reduction potentials maintained by electrolysis. At Eh levels below +50 mV., 0.01M fluoroacetate caused inhibitions of 0-25 per cent.; at levels above +120 mV. the inhibition ranged from 65 to 100 per cent.

The possibility that it was necessary for fluoroacetate to be metabolized for a period before inhibition occurred was also examined. Mince prepared from pigeon breast muscle, to which was added 0.025M oxaloacetate and fluoroacetate, was pre-incubated for 15 min. under oxygen, or under oxygen-free nitrogen. Thereafter, the inhibition of citrate utilization occurring under air, oxygen, or nitrogen during a period of 30 min. was determined. Inhibition in air after pre-incubation in nitrogen was 20-30 per cent.; after pre-incubation in oxygen, 80-90 per cent. After pre-incubation in oxygen, inhibition in nitrogen was nil, and in oxygen, 100 per cent.

It is suggested that these results support the hypothesis that fluoroacetate condenses with oxaloacetate to form the actual inhibitor. Both the formation of the inhibitor and the inhibiting reaction were increased in the presence of oxygen.

* Ian McMaster Scholar.

† Division of Animal Health and Production, C.S.I.R.O., McMaster Animal Health Laboratory, Sydney, N.S.W.

I. Introduction

It would appear that small species of nematode parasites inhabiting the small intestine of host animals are at least partially aerobic *in vivo* (Rogers 1949). In these organisms aerobic energy is probably obtained from the reactions of the tricarboxylic acid cycle (Massey and Rogers 1950*a*). Acetate is rapidly metabolized by the parasites, condensing, either as acetate or a closely related compound, with oxaloacetate to form citrate (Massey and Rogers, unpublished data). The early report that fluoroacetate could be regarded as a competitive inhibitor of biochemical reactions involving acetate (Bartlett and Barron 1947) led us to examine and compare its action on the metabolism of nematode parasites and host animals. The results of these experiments showed that it would be necessary to examine the effect of changes in oxidation-reduction potentials on the efficiency of fluoroacetate as an inhibitor and to examine the hypothesis advanced by Liebecq and Peters (1949) on the mode of action of the poison.

According to these authors, fluoroacetate itself is not an inhibitor, but it is activated and brought into the tricarboxylic acid cycle with the formation of fluoro derivatives of one or more of the normal components of the cycle. The fluoro compounds, it was suggested, cause a "jamming" of the cycle that leads to the accumulation of citrate. The results of an examination of some aspects of this hypothesis, together with a study of conditions affecting the efficiency of fluoroacetate, are reported in this paper.

The nematodes examined were *Nematodirus filicollis, N. spathiger,* and *Ascaridia galli.* For comparing vertebrate tissues with those of nematodes, pigeon breast muscle and pigeon liver were selected. These cannot be regarded as being truly representative of "host" tissues; they were selected as much-studied materials of which something of the metabolism is known.

II. Materials and Methods

Nematodirus filicollis and *N. spathiger,* which were not separated for use, were obtained from naturally infested sheep; *Ascaridia galli* was obtained from experimentally infected chickens. The parasites were separated from the hosts' intestinal contents and washed with saline, and then ground with quartz or minced in the mincer of the type described by Seevers and Shideman (1941). Pigeon breast muscle and pigeon liver were similarly treated. Unless stated otherwise, the tissue preparations were diluted with Krebs-Ringer phosphate buffer at pH 7.3 without calcium chloride. All materials added during experiments were brought to the appropriate pH.

Some of the fluoroacetic acid used in these experiments was obtained from Dr. B. C. Saunders, of the University of Cambridge; the remainder was prepared from commercial sodium fluoroacetate by acidifying and distilling at reduced pressure. Both fluoroacetate preparations had similar effects on biological systems.

Oxygen consumption was measured by the "direct" method of Warburg (1926). Citrate was determined by the Krebs and Eggleston (1944) modifica-

tion of the method of Pucher, Sherman, and Vickery (1936). In initial experiments the colorimetric method was used; later determinations were made by titration, which, though more time-consuming, was more accurate and consistent. Cytochrome *c* was prepared by the method of Keilin and Hartree (1937).

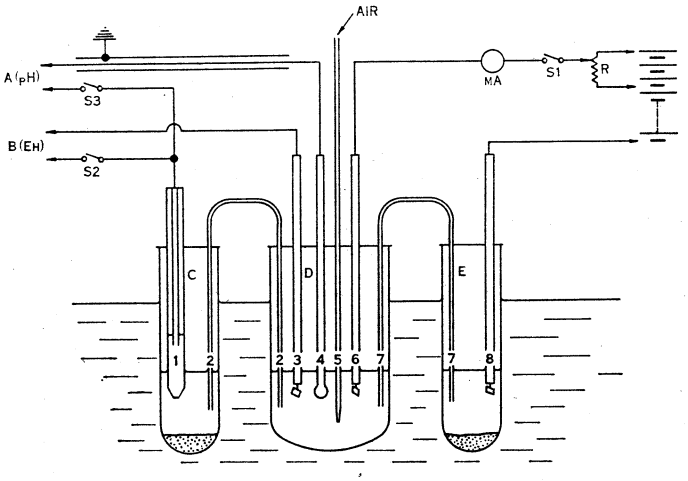

Fig. 1.—Apparatus used for controlling oxidation-reduction potentials by electrolysis. For explanation, see text.

Experiments involving the control of oxidation-reduction potentials were carried out in vessels shown in Figure 1. The potential was controlled by electrolysis as described by Hanke and Katz (1943). Vessel *C* contained saturated potassium chloride into which dipped the saturated calomel electrode, 1, and the potassium chloride-agar bridge, 2. The cell, *D*, was the reaction vessel in which the brei was incubated. It contained an arm of the bridge, 2; a bright platinum electrode, 3, for Eh determinations; a glass electrode, 4, for pH determinations; a glass tube, 5, from which air was bubbled through the brei; a platinum electrode, 6, and an arm of the sodium chloride- or sodium ammonium hydrogen phosphate-agar bridge to conduct the electrolysis current. Vessel *E* took the other arm of the bridge, 7, and a platinum electrode, 8, in contact with saturated sodium chloride, to complete the electrolysis current. Oxidation-reduction potentials and pH were read on separate potentiometers. When not in use, the glass electrode was removed from contact with the brei in cell *D*. The electrolysis current was provided by accumulators through the variable resistance, *R*, and the switch, S_1. The system of vessels and electrodes was duplicated and supported in a transformer oil-bath at 38°C.

The action of electrolysis in controlling the oxidation-reduction potential was tested by alternately oxidizing and reducing suitable indicators and observing the relation between colour changes and potential. Methylene blue (E_0 at pH 7.0, + 11 mV.), thionine (+ 63 mV.), toluyene blue (+ 115 mV.), and 2:6-dichlorophenolindophenol (+ 217 mV.) were examined in this manner. It was found that, starting with the dyes reduced with a little sodium

dithionite, oxidation and reduction could be controlled by electrolysis and the colours at different oxidation-reduction potentials agreed generally with those given by Hewitt (1950).

Before commencing an experiment, the electrodes for measuring potentials were tested by using quinhydrone in phthalate buffer and in 0.1N HCl. The error for the electrodes in both cells was never greater than 5 per cent. and was usually less than 2 per cent.

It should be noted that the potentials recorded on the platinum electrodes during experiments with biological preparations were not necessarily those of the "partial oxidation-reduction potentials" of the enzyme systems under examination. This error would not seriously affect the conclusions drawn from the results of the experiments.

The control of the oxidation-reduction potential of brei by electrolysis was found to be difficult. Only after a number of trials was it found possible to keep the Eh at within ± 10 per cent. of a required value. At times, though usually for periods of less than a minute, the potential of one or other of the two cells varied by as much as ± 20 per cent. from the required value. In the experiments described later in this paper the Eh was maintained at certain levels largely within ± 10 per cent. for periods of 30 min. by noting the E_1 every 3 min. and modifying the electrolysis current accordingly. Variations of more than ± 10 per cent. occurred only for very short periods.

The utilization of citrate in the biological preparations varied in different experiments, even when the Eh was kept constant. However, when aliquots from the one lot of mince were used and the Eh was kept at the same value in the two cells, the variation in the utilization of citrate was less than 8 per cent. for the three experiments carried out.

During the final experiments the mince in the two reaction cells was kept as near as possible to the selected Eh; one cell was used for fluoroacetate-poisoned tissue, and the other for the control. The mince in the cells was stirred by streams of air bubbles; the air for the fluoroacetate-poisoned preparation was passed through 0.1M phosphate buffer at pH 7.3 at 38°C. to which fluoroacetate was added to give the same concentration as that in the mince. Air from the control cell was passed through phosphate buffer only.

The separation of the components of the tricarboxylic acid cycle was accomplished by the paper partition chromatographic method of Lugg and Overell (1948). The phase mixtures found most suitable for separating the acids were amyl alcohol-water, with formic acid to a concentration of 5N in the water, and mesityl oxide-water, again with 5N formic acid.

The biological material was prepared for chromatography by precipitating the proteins with sodium tungstate and sulphuric acid, followed by continuous extraction of the protein-free filtrate with ether for 48 hr. The ether was then evaporated off and the residue dissolved in water. After adjusting the pH to 2.2 this solution was then applied to the filter paper for chromatographic separation.

Fluorine in the compounds which had been separated by paper chromatography was determined colorimetrically. Appropriate pieces were cut from the chromatogram and any organic fluoride present was converted to the inorganic form by fusing with metallic sodium at 400°C. *in vacuo* for one hour. Fluorine was freed from the digest by steam distillation after the addition of perchloric acid. The fluoride in the distillate was estimated by the zirconium-alizarin lake method of Scott (1941). Areas from the chromatogram that had been traversed by the developing fluid were used for blank determinations. By this method the fluorine in 20 μg. fluoroacetic acid placed on filter paper could be detected.

TABLE 1
EFFECT OF FLUOROACETATE ON OXYGEN UPTAKE

Biological Material	Inhibition Produced by Fluoroacetate	
	0.01M	0.02M
Nematodirus spp.	30-65%	
Mean	55% (10)	—
Ascaridia galli	20-45%	
Mean	35% (8)	—
Pigeon breast muscle	0%	0-10%
Mean	0% (8)	5% (6)

The figures within brackets refer to the number of experiments from which the mean results were calculated. The tissue was used as a brei suspended in Krebs-Ringer phosphate solution at pH 7.3, and contained methylene blue at a final concentration of 0.0003M.

III. PROCEDURE AND RESULTS

(*a*) *Effect of Fluoroacetate on Oxygen Consumption*

The action of fluoroacetate on the oxygen uptake of brei prepared from *Nematodirus* spp., *Ascaridia galli,* and pigeon breast muscle was examined at concentrations of 0.01M and 0.02M in Warburg manometers. All preparations contained methylene blue at a concentration of 0.0003M. The brei was in contact with the inhibitor during the preliminary 10 min. equilibration period before the taps were closed. The results given in Table 1 were calculated from the figures obtained during the first 15 min. of the experiment. Inhibition in pigeon breast muscle was small or absent even at the higher fluoroacetate concentration, whereas in the brei prepared from the parasites it was usually in the region of 34-45 per cent. at low fluoroacetate concentrations.

(*b*) *Effect of Intermediates of the Tricarboxylic Acid Cycle on Fluoroacetate Inhibition*

The effect of α-ketoglutarate, succinate, fumarate, malate, and oxaloacetate (0.01M) on the oxygen uptake of *Nematodirus* spp. and of *A. galli* brei in the absence of, and in the presence of, 0.01M fluoroacetate was determined in Warburg manometers. As before, the inhibitor, together with the substrate, was

present with the brei during the equilibration period, and the results (Table 2) were obtained from the first 15 min. of the experiments. Similar results were obtained when the experiment was repeated several times. All the substrates lessened the fluoroacetate inhibition; succinate was most effective and oxaloacetate the least effective in this regard.

TABLE 2
EFFECT OF SUBSTRATES ON FLUOROACETATE INHIBITION

	Nematodirus spp.		*Ascaridia galli*	
	Oxygen Uptake (μl.)	Inhibition (%)	Oxygen Uptake (μl.)	Inhibition (%)
No additions	— 160		— 115	
+ Fluoroacetate	— 70	56	— 70	39
+ α-Ketoglutarate	— 205		— 150	
+ α-Ketoglutarate + fluoroacetate	— 140	32	— 150	0
+ Succinate	— 290		— 205	
+ Succinate + fluoroacetate	— 280	3	— 200	2
+ Fumarate	— 220		— 165	
+ Fumarate + fluoroacetate	— 170	23	— 130	21
+ Malate	— 215		— 155	
+ Malate + fluoroacetate	— 180	16	— 130	16
+ Oxaloacetate	— 180		— 170	
+ Oxaloacetate + fluoroacetate	— 105	40	— 135	26

Each flask contained brei in Krebs-Ringer phosphate solution at pH 7.3 without calcium chloride and containing 0.0003M methylene blue. The substrates and the inhibitor were used at a final concentration of 0.01M. The results were obtained during the first 15 min. of the experiment.

(*c*) *Effect of Fluoroacetate on Citrate Formation*

Brei, prepared from *Nematodirus* spp. containing 0.0005M methylene blue, was shaken in Erlenmeyer flasks at 38°C. in air. Oxaloacetate, acetate, and fluoroacetate were added in different combinations to different flasks to give a concentration of 0.01M for each component. Samples for citrate determinations were taken after the flask contents had been shaken for a few minutes. One hour later samples were again taken for citrate determination. Typical results are shown in Table 3.

Though acetate and oxaloacetate did not stimulate citrate formation very much in the absence of fluoroacetate, the increase was very clear when the inhibitor was present. These experiments may be taken as showing that citrate formation in nematode parasites probably involves the condensation of oxaloacetate and acetate or a compound derived from acetate, and that fluoroacetate

either stimulates the formation of citrate by preventing the loss of one of the components involved in the condensation, or inhibits the utilization of the citrate formed.

(*d*) *Effect of Fluoroacetate on Citrate Utilization*

Mince prepared from *Nematodirus* spp., pigeon breast muscle, and pigeon liver, was suspended in Krebs-Ringer phosphate made without calcium chloride, and methylene blue was added to a concentration of 0.0005M. Aliquots were placed in Kreb's flasks and 2 mg. of neutralized sodium citrate was added to each. Control flasks and flasks containing 0.01M fluoroacetate were then incubated and shaken at 38°C., both with a gas phase of air and under anaerobic conditions. Anaerobiasis was obtained by gassing with nitrogen that had been passed over copper turnings at 400°C. and by absorbing any residual oxygen with freshly cut phosphorus. After two hours, samples for the determination of residual citrate were taken.

TABLE 3

EFFECT OF FLUOROACETATE ON THE FORMATION OF CITRATE IN *NEMATODIRUS* SPP.

System	Citrate Formation in 1 Hr. (mg.)
No addition	0.05
+ Oxaloacetate	0.05
+ Acetate	0.08
+ Oxaloacetate, acetate	**0.50**
+ Fluoroacetate	0.15
+ Oxaloacetate, acetate, fluoroacetate	1.12

Each flask contained 5 ml. of brei in Krebs-Ringer phosphate solution, without calcium chloride, at pH 7.3. Methylene blue (0.0005M) was present. The substrates and the inhibitor were used at a final concentration of 0.01M. The figures show the differences between initial and final citrate concentrations after shaking at 38°C. for 1 hr.

Typical results are shown in Table 4. Under aerobic conditions, citrate utilization by mince prepared from *Nematodirus* spp. was almost completely inhibited by fluoroacetate, whereas the inhibition of the utilization by pigeon breast muscle and pigeon liver was small. In the absence of oxygen, citrate utilization in the three preparations was still considerable but fluoroacetate did not inhibit.

The results of these experiments suggested that the efficiency of fluoroacetate inhibition might, in some way, be associated with the oxygen tension at which the experiment was conducted. This hypothesis was therefore examined.

(e) Effects of Oxygen Tension and Oxygen Carriers on Fluoroacetate Inhibition

Citrate was added to mince prepared from pigeon breast muscle in Krebs-Ringer phosphate without calcium chloride. Methylene blue, cytochrome *c*, and fluoroacetate were added to aliquots of the mince as shown in Table 5. Samples for citrate estimation were taken at the beginning of the experiment and after shaking in air for 2 hr. at 38°C. The results (Table 5) indicate that fluoroacetate was a much more effective inhibitor in the absence of methylene blue, and when cytochrome *c* was added. Similar results were obtained when these experiments were repeated, though in some cases the addition of cytochrome *c* caused an accumulation of citrate even in the absence of fluoroacetate. The accumulation was always increased in the presence of fluoroacetate, however.

TABLE 4
EFFECT OF FLUOROACETATE ON THE UTILIZATION OF CITRATE

System	Amount of Citrate Utilized (mg.)		
	Nematodirus spp.	Pigeon Breast Muscle	Pigeon Liver
No additions, aerobic	1.80	1.65	1.20
+ Fluoroacetate, 0.01M, aerobic	Nil	1.40	1.14
No additions, anaerobic	1.55	1.50	1.10
+ Fluoroacetate, 0.01M, anaerobic	1.46	1.47	1.10

Each flask contained 2 mg. citrate in 5 ml. of mince suspended in Krebs-Ringer phosphate solution at pH 7.3 without calcium chloride. Methylene blue (final conc. 0.0005M) was present. The figures show the utilization of citrate during shaking at 38°C. for 2 hr. Anaerobic conditions were established by gassing with oxygen-free nitrogen and absorbing any residual oxygen in the flasks with yellow phosphorus.

The fluoroacetate inhibition of citrate utilization in the absence of methylene blue was then examined at different oxygen tensions. After taking samples for the determination of the initial citrate concentrations, anaerobic conditions were established in some flasks, others were left open to the air, and others were gassed with oxygen for 5 min. before closing the taps. The flasks were shaken in a water bath for 40 or 60 min. at 38°C. after which samples were taken for the determination of the final citrate concentrations. The results of one set of experiments are shown in Table 6. It should be noted that the fluoroacetate concentration in the last group of experiments was 0.005M. The lower concentration was used because citrate was often found to accumulate when oxygen was used as the gas phase, even when fluoroacetate was not present. The inhibitor always increased the accumulation of citrate, however.

The results shown in Table 6 support the suggestion that the efficiency of fluoroacetate is associated with the oxygen tension at which the experiment is carried out. This hypothesis was then taken further by examining fluoroacetate inhibition of citrate utilization at different oxidation-reduction potentials.

TABLE 5

EFFECT OF METHYLENE BLUE AND CYTOCHROME *C* ON FLUOROACETATE INHIBITION OF THE UTILIZATION OF CITRATE IN PIGEON BREAST MUSCLE

System	Initial Citrate (mg.)	Final Citrate (mg.)	Inhibition (%)
No additions	1.20	0.65	
+ Fluoroacetate, 0.01M	1.20	0.95	55
+ Methylene blue	1.20	0.65	
+ Fluoroacetate, 0.01M, methylene blue	1.20	0.80	27
+ Cytochrome *c*	1.20	0.95	
+ Cytochrome *c*, fluoroacetate, 0.01M	1.20	1.20	100

Each flask contained 5 ml. of mince suspended in Krebs-Ringer phosphate solution without calcium chloride at pH 7.3. When methylene blue was added, the final concentration was 0.001M; the added cytochrome *c* gave a concentration of 5×10^{-6}M. The figures show the amounts of citrate present at the beginning of the experiment and after shaking the flasks at 38°C. for 2 hr.

(*f*) *Fluoroacetate Activity at Different Oxidation-Reduction Potentials*

Experiments were carried out with mince prepared from pigeon breast muscle in Krebs-Ringer phosphate solution at pH 7.3 without calcium chloride. The phosphate-buffer concentration was increased to give a final concentration of 0.05M. Methylene blue, 0.0004M, and citrate, 1 mg./ml., were also present. Fluoroacetate was used at a final concentration of 0.01M. A small amount of octyl alcohol was added to the mince from time to time to prevent frothing caused by the aeration. The utilization of citrate was not affected by small amounts of octyl alcohol.

In preliminary experiments the pH of the mince was adjusted to 7.6 when positive electrolysis was to be used, and to pH 7.0 for negative electrolysis. No great care was taken to keep the equilibration period constant or to prevent the loss of fluoroacetate from the mince caused by aeration. The experiments were carried out for an hour after the desired Eh had been obtained. During the experiment pH changes of the order of 0.6 took place when extreme oxidation-reduction potentials were used. Five experiments were carried out to determine the effect of fluoroacetate at Eh's below + 50 mV. Inhibition varied from nil to 25 per cent. In 12 experiments carried out at Eh's above + 150 mV. the inhibition varied between 65 and 100 per cent. One discordant result was obtained when no inhibition was found at + 250 mV.

In the final experiments, the technique was modified to maintain the equilibration period during which the required oxidation-reduction potential was attained, at 30 ± 5 min. At the end of the equilibration period fluoroacetate in buffer was added to the mince in one of the cells and buffer only to the other. Fluoroacetate was also added to the dilute buffer at 38°C. through which the air was passed before being bubbled through the mince. The experimental period was reduced to 30 min. Under these circumstances the removal of fluoroacetic acid from the poisoned mince by aeration was probably very slow, and the changes in pH caused by the electrolysis were much smaller, so that the divergence from pH 7.0, to which the mince was adjusted at the beginning of the equilibration period, was small. The pH was determined at the beginning and end of the experiment, the Eh was read at intervals of 3 min. and the electrolysis current modified accordingly. The results of these experiments are shown in Table 7.

TABLE 6

EFFECT OF OXYGEN TENSION ON FLUOROACETATE INHIBITION OF THE UTILIZATION OF CITRATE IN PIGEON BREAST MUSCLE

	Gas Phase	Duration of the Experiment (min.)	Initial Citrate (mg.)	Final Citrate (mg.)	Inhibition (%)
No additions	Nitrogen	60	1.30	0.66	Nil
+ Fluoroacetate, 0.01M			1.30	0.65	
No additions	Air	60	1.30	0.55	47
+ Fluoroacetate, 0.01M			1.30	0.90	
No additions	Oxygen	40	1.16	0.59	70
+ Fluoroacetate, 0.005M			1.16	1.00	
No additions	Air	40	1.32	0.57	32
+ Fluoroacetate, 0.005M			1.32	0.80	

Each flask contained 5 ml. of mince suspended in Krebs-Ringer phosphate solution at pH 7.3 without calcium chloride.

In a proportion of the experiments the utilization of citrate was small. The results of these experiments were discarded because inhibitions calculated from such results might have been inaccurate. In general, it was found that the utilization of citrate at high oxidation-reduction potentials was smaller than at low Eh's. At very high potentials fluoroacetate caused an accumulation of citrate.

(g) *Nature of Fluoroacetate Inhibition*

According to the hypothesis advanced by Liebecq and Peters (1949), there would be two phases in the action of fluoroacetate. First would come the formation of a fluoro compound, possibly fluorocitrate, followed by the inhibition arising from the action of this compound. Experiments were carried out to see if one or other of these phases was particularly associated with Eh conditions. Accordingly, attempts were made to determine whether the "fluorocitrate" was formed and to see if its formation was affected by the oxidation-reduction potential. The first part of the experiment was carried out using paper chromatography as a means of separating the "fluorocitrate." In the second part, the effect of the oxygen tension during a pre-incubation period on the subsequent inhibition of citrate utilization was examined.

TABLE 7

EFFECT OF OXIDATION-REDUCTION POTENTIAL ON FLUOROACETATE INHIBITION ON THE UTILIZATION OF CITRATE IN PIGEON BREAST MUSCLE

Eh (mV. at pH 7.2, 38°C.)		Inhibition	
Control	Fluoroacetate	(%)	Change in pH
44	49	4	7.0 — 7.3
82	73	Nil	7.0 — 7.2
89	90	Nil	7.0 — 7.2
128	132	100	7.0 — 7.1
170	158	70	7.0 — 7.1
170	178	100	7.0 — 7.1

The Eh figures give the average potential maintained over 30 min. during which the utilization of citrate was measured. The changes in pH occurring during the experiments are also shown.

Mince prepared from *Nematodirus* spp. and pigeon breast muscle to which oxaloacetate had been added was incubated, with and without fluoroacetate. By means of the paper chromatographic method of Lugg and Overell (1948), the tricarboxylic acid cycle components were separated. Citric, α-ketoglutaric, succinic, malic, fumaric, and oxaloacetic acids could be identified. Fluorine in the "citrate" spots obtained from mince which had been incubated with and without fluoroacetate was then determined. At times fluorine in the "citrate" spots from fluoroacetate-treated material was up to 5 μg. more than in the spot from the untreated mince. These results were not consistent, however, and no definite conclusions could be drawn from the experiments.

Since this work was carried out, Buffa *et al.* (1950) have reported that they have isolated, by chromatography from guinea pig kidney homogenates treated with fluoroacetate, a tricarboxylic fraction containing a small amount of fluorine that is active in preventing the utilization of added citrate. The failure to obtain a consistent separation of the fluoro compound in the present investigation was most probably due to the insensitivity of the methods used.

In the second part of the experiments on the nature of fluoroacetate inhibition, mince was prepared from pigeon breast muscle and citrate was added to a concentration of about 1 mg./ml. Oxaloacetate, 0.0025M, with and with-

out fluoroacetate, 0.0025M, was added and the mixture was incubated for 15 min. under oxygen or oxygen-free nitrogen. Thereafter, the inhibition of citrate utilization was determined over a period of 30 min., the gas phase being oxygen-free nitrogen, air, or oxygen. The results are shown in Table 8. This experiment was repeated several times and consistently showed that inhibition was much greater when oxygen was present, both during the pre-incubation and the experimental periods. The finding that the pre-treatment of the mince influenced the amount of inhibitor present supports the suggestion that fluoroacetate was changed in some way before the inhibition took place.

TABLE 8

EFFECT OF OXYGEN TENSION ON FLUOROACETATE INHIBITION OF THE UTILIZATION OF CITRATE IN PIGEON BREAST MUSCLE

Gas phase, pre-incubation period	N_2	O_2	O_2	O_2
Gas phase, experimental period	Air	Air	O_2	N_2
Inhibition of citrate utilization (%)	26	86	100	Nil

The mince in Krebs-Ringer phosphate without calcium chloride contained 0.0025M oxaloacetate, with and without 0.0025M fluoroacetate, and was incubated for 15 min. under the different gases before the experiment commenced. The inhibition of the utilization of citrate occurring under the different gases was determined over 30 min.

IV. DISCUSSION

One of the most characteristic actions of fluoroacetate is its inhibition of the utilization of citrate both *in vitro* (Kalnitsky 1948; Liebecq and Peters 1949; Massey and Rogers 1950*b*) and *in vivo* (Buffa and Peters 1949). The increased yields of citrate obtained from the condensation of acetate and oxaloacetate in biological systems poisoned with fluoroacetate (Kalnitsky 1949; Massey and Rogers 1950*b*) is an outcome of this action. Recent work (Buffa *et al.* 1950; Elliott and Kalnitsky 1950) has indicated that fluoroacetate, when incubated with the appropriate biological preparation, undergoes condensation with oxaloacetate to form a fluoro compound, probably fluorocitrate, which may be separated by chromatography or by precipitation with calcium. The inhibition leading to the accumulation of citrate may be attributed to this compound but it has no effect on aconitase, *iso*citric dehydrogenase, or oxalosuccinic decarboxylase in isolated systems (Buffa *et al.* 1950). The results of the present investigation, which supports and enlarges these findings, are discussed below.

(1) The action of fluoroacetate on the metabolism of preparations from certain nematode parasites was characterized by the inhibition of respiration and of citrate utilization, and by the increased accumulation of citrate formed by the condensation of acetate and oxaloacetate. The nematode parasites concerned are able to oxidize pyruvate via the tricarboxylic acid cycle (Massey and Rogers 1950*a*). It may be said, then, that fluoroacetate has a similar action on the microorganisms, the vertebrate tissues, and the nematode parasites that it poisons.

(2) The present investigation has shown that the efficiency of fluoroacetate as an inhibitor of the utilization of citrate is affected by the nature of the

oxygen carrier present, the oxygen tension, and the oxidation-reduction potential maintained by electrolysis. The action of the carriers, methylene blue (E_0, + 11 mV. at pH 7.0), which decreased the inhibition, and cytochrome *c* (E_0, + 270 mV. at pH 7.4) (Ball 1938), which increased inhibition, was paralleled by that of the oxidation-reduction potential controlled by electrolysis or by varying the gas phase, and it is probable that the potential changes were the fundamental factors affecting the activity of the drug. It seems unlikely that the results were obtained by direct toxic action of the agents used to influence the oxidation-reduction potential. Nor would it appear that enzyme denaturation, which might have occurred at high potentials, would have appreciably affected the results.

The action of the oxidation-reduction potential in controlling enzyme activity is well established. Thus the activity of urease (Sizer and Tytell 1941) and yeast invertase (Sizer 1941) has been shown to be a function of the oxidation-reduction potential; the relative hydrolytic and synthetic activities of α- and β-amylase are affected by the potential (Ito 1939); and the actions of autolytic proteases are strongly affected by the oxidation-reduction potential (Reiss 1938). It appears, then, that one explanation of the action of the oxidation-reduction potential on fluoroacetate inhibition might be that different enzyme systems are concerned with the metabolism of citrate at high and low potentials. The system functioning at low potentials would be the one unaffected by the poison.

It is notable that active utilization of citrate in mince prepared from pigeon breast muscle was obtained under anaerobic conditions. This might have occurred partly by way of the tricarboxylic acid cycle, with coenzyme-linked reactions leading to electron and proton transfer to substrates other than oxygen. In the formation of citrate under anaerobic conditions from oxaloacetate and acetoacetate, Hunter and Leloir (1945) found that oxaloacetate acted as an oxidant and that α-ketoglutarate was oxidized. However, if a similar type of reaction was concerned in the utilization of citrate under anaerobic conditions, fluoroacetate might be expected to retain its inhibiting action if the actual inhibitor acted in competition with the normal substrate, citrate, or some closely allied compound.

(3) The two reactions that are probably concerned in fluoroacetate inhibition, the formation of the inhibitory compound, and the actual inhibiting reaction, may be further defined by the effect of the oxygen tension upon the two reactions. The results in Table 8 show that the degree of inhibition obtained was not immediately dependent on the amount of fluoroacetate present and that the potential inhibiting activity increased when the preparations were incubated aerobically. One explanation of these results would be that fluoroacetate condensed with oxaloacetate during the pre-incubation period, thus forming the inhibitor proper. It is uncertain whether this reaction would be expected to proceed in the absence of oxygen. Under certain circumstances the formation of citrate from acetoacetate and oxaloacetate can take place anaerobically (Breusch and Keskin 1944; Hunter and Leloir 1945). Kalnitsky

(1949) found that citrate formation from oxaloacetate in rabbit-kidney homogenates was decreased by 52-55 per cent. when incubated in nitrogen instead of air. However, fluoroacetate was present in the reaction mixture and the results obtained must have been influenced by the action of the oxidation-reduction potential on the activity of the inhibitor.

The second phase of the action of fluoroacetate on the utilization of citrate, the inhibition proper, was also strongest at high oxygen tensions (see Table 8). Buffa *et al.* (1950) failed to obtain inhibition with the "active" fluoro compound obtained from fluoroacetate in isolated systems of aconitase, *iso*citric dehydrogenase, and oxalosuccinic decarboxylase. It seems that the study of isolated systems should receive further consideration in relation to oxidation-reduction potentials favourable for the action of the inhibitor.

V. Acknowledgments

The authors are indebted to Professor H. A. Krebs, University of Sheffield, for his advice on the metabolism of citrate. Thanks are also due to Dr. B. C. Saunders of the University of Cambridge, for several gifts of fluoroacetic acid.

The advice given by Dr. F. P. Dwyer, University of Sydney, on the method of controlling and measuring oxidation-reduction potentials is gratefully acknowledged.

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