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STUDIES ON THE BIOCHEMICAL BASIS OF SUSCEPTIBILITY AND RESISTANCE OF THE HOUSEFLY TO FLUOROACETATE

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SUMMARY

1. Administration of fluoroacetate to sensitive houseflies in amounts close to the $L.D._{50}$ range (0.25–0.3 μg/fly) brought about a prompt elevation of their citrate content. With about 10-fold higher doses of fluoroacetate a concurrent increase of both citrate and pyruvate levels took place in the fly tissues.

2. Incubation of sarcosomes of the sensitive housefly strain in the presence of oxidizable substrates and fluoroacetate resulted in accumulation of citrate, inhibition of respiration and uncoupling of oxidative phosphorylation. The magnitude of the effects varied considerably with the different substrates used, being particularly pronounced with pyruvate and malate and inappreciable with succinate and α-glycerophosphate.

3. The respiratory inhibition induced by a brief exposure in the cold of housefly sarcosomes to fluoroacetate, persisted after the sarcosomes had been washed free from fluoroacetate. The toxic effect of fluoroacetate on the respiratory chain could be prevented by an excess of simultaneously added acetate.

4. The susceptibility of the respiratory function of the sarcosomes to fluoroacetate inhibition was abolished by sonication. The unresponsiveness of the sonicated sarcosomes to fluoroacetate was attended by a loss of their respiratory chain phosphorylation activity.

5. Sarcosomes derived from a partially resistant housefly strain, when incubated in the presence of fluoroacetate, failed to accumulate citrate, but displayed the characteristic respiratory-inhibition response. Sarcosomes from a highly resistant strain showed no impairment of their functional capacity by fluoroacetate. However, all the different housefly strains tested proved to be equally sensitive to the deleterious effect of fluorocitrate on sarcosomal respiration.

6. The possible biochemical mechanisms underlying the toxicity of fluoroacetate in the housefly are considered with particular reference to the altered response of the target systems exhibited by the fluoroacetate-resistant strains.

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INTRODUCTION

It has been established by the fundamental investigations of PETERS[1] that the toxic action of fluoroacetate in mammals involves its prior enzymic conversion *in vivo* to fluorocitrate, a potent inhibitor of aconitase ((citrate (isocitrate) hydrolyase, EC 4.2.1.3)). The jamming of the tricarboxylic acid cycle due to aconitase inhibition was found to be reflected in a striking elevation of citrate levels in the organs of fluoroacetate-poisoned animals. Variations in the degree of sensitivity to fluoroacetate of different mammalian species were attributed to differences in their ability to catalyze the conversion of poison to the active antimetabolite, fluorocitrate[1].

The poisoning effect of fluoroacetate on cockroaches[2] and houseflies[3] has been also shown to manifest itself by elevated tissue levels of citrate. In addition, the occurrence in fluoroacetate-poisoned flies of another metabolic disturbance, unrelated to aconitase inhibition and expressed by pyruvate accumulation *in vivo*, has been reported in a preliminary communication[3]. In the present study the two inhibitory effects of fluoroacetate have been further characterized and shown to contribute jointly to its lethal action.

MATERIALS AND METHODS

Chemicals

The chemicals used were obtained from Calbiochem Inc. or from Sigma Chemical Co. Sodium fluoroacetate was acquired from Tull Chemical Co. (Oxford, Ala., U.S.A.). Barium fluorocitrate, synthesized by the method of RIVETT[4], was purchased from Sombert Chemical Co. (Brooklyn, N.Y.); the barium salt was dissolved in dilute HCl and converted to the sodium salt with an equivalent amount of sodium sulfate. Sodium fluoro[2-^{14}C]acetate was acquired from International Chemical and Nuclear Corporation (Yorktown, Calif.).

Strains

The following strains of housefly (*Musca domestica* L.) were used in the majority of the experiments: *S*, fluoroacetate sensitive (L.D.$_{50}$ of fluoroacetate, 0.3–0.4 μg), obtained through the courtesy of Dr. J. J. MENN, Stauffer Chemical Co. (Mountain View, Calif.); *Fm*, moderately resistant to fluoroacetate (L.D.$_{50}$, 3–4 μg), selected from the *S* strain for fluoroacetate resistance for up to 65 generations; *Fr*, highly resistant to fluoroacetate (L.D.$_{50}$, approx. 500 μg) derived from *Fm* by further selection for 16 generations.

Experimental procedure

Female flies starved for 24 h were immobilized with a stream of CO_2. Each fly was then fed 1 μl of an aqueous solution of fluoroacetate with the aid of an "Agla" microsyringe fitted with a 27-gauge needle, the feeding being observed under the low power of a dissecting microscope. For collective handling, the flies were immobilized by cooling in ice. The methods of rearing and selection of the flies and the assays of fluoroacetate toxicity have been previously described in detail[5].

Isolation of housefly sarcosomes and guinea-pig kidney mitochondria

All the operations were performed in the cold. The thoracic segments of 200–300 flies were dissected and homogenized by gentle grinding for 2 min in a chilled mortar with 6 ml of Medium A containing 0.25 M sucrose, 10^{-3} M EDTA (pH 7.4) and 1% (w/v) bovine serum albumin. The resulting homogenate was filtered through 8 layers of muslin, previously soaked with 0.25 M sucrose–10^{-3} M EDTA (pH 7.4) solution (Medium B) and then centrifuged in a refrigerated centrifuge at about 2° for 10 min at 4000 × *g*. Unless otherwise stated, the sedimented sarcosomes were washed once with 6 ml of Medium A, and a second time with the same volume of Medium B. The washed particles were dispersed in 6 ml of Medium B with the aid of a teflon pestle of low clearance to a final content of 5–7 mg protein per ml.

Guinea-pig kidney mitochondria were prepared according to the procedure described by TREBLE AND PETERS[6].

Disruption of sarcosomes by sonic oscillations

The sarcosome suspension in an ice-cooled container was sonicated for 2.5 min with the immersed probe of the "Sonifier" oscillator of Branson Instruments Incorp. (Stamford, Conn., U.S.A.) set at 2.5 A.

Measurement of respiration and oxidative phosphorylation of fly sarcosomes

The respiration of housefly sarcosomes was measured in a conventional Warburg apparatus under conditions similar to those described by VAN DEN BERGH AND SLATER[7]. The reaction mixture contained in a total volume of 2.5 ml the following components; 50 μmoles Tris–chloride buffer (pH 7.4), 40 μmoles potassium phosphate buffer (pH 7.4), 25 μmoles KCl, 2.5 μmoles EDTA, 20 μmoles $MgCl_2$, 5 μmoles ATP. The various substrates tested were added in the amounts specified in the tables. The amount of sarcosomes used was chosen according to Q_{O_2} values obtained with the different substrates tested and varied from 1 to 8 mg protein per flask. In experiments designed to measure oxidative phosphorylation, 50 μmoles glucose and about 10 international units of yeast hexokinase were added to the standard reaction mixture from the side-arm of the Warburg flask after 10 min allowed for temperature equilibration. The flasks were incubated at 25° in air. At the end of incubation the reaction was stopped by adding 0.5 ml of a 50% (w/v) trichloroacetic acid solution and the clear supernatant fluid obtained by centrifugation was used for chemical determinations.

Quantitative assays

About 30–40 female houseflies were ground with 4 ml of a 5% (w/v) trichloroacetic acid solution in a Potter–Elvehjem all-glass homogenizer immersed in an ice bath. Following centrifugation, appropriate samples of the resultant supernatant fluids were taken for determining pyruvate and citric acid and by the method of FRIEDEMANN AND HAUGEN[8] and a slightly modified procedure of NATELSON, PINCUS AND LUGOVOY[9], respectively. Alanine was estimated according to the paper-chromatographic procedure described by PRICE[10]. Protein was determined by the method of LOWRY *et al.*[11], using crystalline bovine serum albumin as reference. Inorganic phosphate was determined by the method of FISKE AND SUBBAROW[12]. The assays were carried out in duplicate and the results were averaged. Other pertinent details are indicated in the legends to the tables.

RESULTS

Effects of fluoroacetate and fluorocitrate on different housefly strains

A representative experiment recorded in Tables I and II shows that feeding of fluoroacetate to houseflies of the sensitive *S* strain in amounts close to the $L.D._{50}$ dose (0.25–0.3 μg/fly), resulted within 1–3 h in a 2- to 4-fold increase of their citrate content. With a dose of fluoroacetate about 10-fold higher, concurrent accumulation of both citrate and pyruvate took place in the housefly tissues. The elevated citrate level persisted in the surviving flies for at least 6 days, whereas the increased pyruvate content tended to return to normal values at the end of 3–4 h following fluoroacetate feeding. The rapid decline of the pyruvate excess was attended by a concurrent rise in the alanine level. Thus, the alanine content of the housefly tissues increased from the initial value of about 3 μmoles/g, up to 10 μmoles/g within 3 h following the administration of fluoroacetate. The occurrence of alanine accumulation in fluoroacetate-poisoned flies has been previously reported by RAY[13].

In the moderately resistant *Fm* strain the $L.D._{50}$ dose of fluoroacetate (3 μg/fly) produced no significant increase in the citrate level, but caused an accumulation of pyruvate similar in magnitude to that in the *S* strain. On the other hand, the highly resistant *Fr* flies showed no perceptible change of their pyruvate and citrate contents when dosed with 3 μg of fluoroacetate.

TABLE I

EFFECT OF FLUOROACETATE AND FLUOROCITRATE ON THE CITRATE CONTENT OF DIFFERENT HOUSEFLY STRAINS

Compound fed (μg/fly)	*Time after feeding (min)*	*Citrate content (μmoles/g housefly)*		
		S	*Fm*	*Fr*
None	—	1.9	1.7	1.7
Fluoroacetate (0.25)	60	3.6	1.5	—
Fluoroacetate (0.25)	180	6.0	1.9	—
Fluoroacetate (3)	60	5.3	1.4	1.7
Fluoroacetate (3)	180	6.9	1.6	—
Fluorocitrate (12)	60	5.0	—	4.1

TABLE II

CHANGES IN THE PYRUVATE CONTENT OF DIFFERENT HOUSEFLY STRAINS FOLLOWING FEEDING OF FLUOROACETATE AND FLUOROCITRATE

Compound fed (μg/fly)	*Time after feeding (min)*	*Pyruvate content (μmoles/g housefly)*		
		S	*Fm*	*Fr*
None	—	0.3	0.3	0.4
Fluoroacetate (0.25)	60	0.3	0.5	—
Fluoroacetate (0.25)	180	0.3	0.7	—
Fluoroacetate (3)	60	1.4	1.5	0.5
Fluoroacetate (3)	180	0.8	0.9	—
Fluorocitrate (12)	60	0.2	—	0.4

Fluorocitrate, in contrast, elicited an essentially equal response in the sensitive and resistant housefly strains, as manifested by the practically identical $L.D._{50}$ values (about 6 μg/fly), as well as the similar extent of citrate accumulation *in vivo*, with no detectable change in the pyruvate level.

Comparison of the effects of fluoroacetate and fluorocitrate on guinea-pig mitochondria and housefly sarcosomes

The action pattern of fluoroacetate on sarcosomes prepared from S flies differed essentially from that observed with a corresponding mammalian system, such as guinea-pig kidney mitochondria used in the studies of PETERS AND WAKELIN[14]. As

TABLE III

EFFECT OF FLUOROACETATE AND FLUOROCITRATE ON OXYGEN UPTAKE AND CITRATE ACCUMULATION BY HOUSEFLY SARCOSOMES AND GUINEA-PIG KIDNEY MITOCHONDRIA

Fumarate at a final concentration of $2 \cdot 10^{-2}$ M was added as substrate. The sarcosomes were prepared by the standard procedure (see MATERIALS AND METHODS), except that washing was omitted. Q_{O_2} denotes: μl O_2 per mg protein per h. For other experimental details see MATERIALS AND METHODS.

Additions to the standard reaction mixture (μmole/ml)		Q_{O_2}		*Citrate accumulated (mμmoles/mg protein per h)*	
		Housefly S sarcosomes	*Guinea-pig kidney mitochondria*	*Housefly S sarcosomes*	*Guinea-pig kidney mitochondria*
None		17.2	11.5	49	8
Fluoroacetate	(0.4)	5.9	10.2	92	118
Fluoroacetate	(1.0)	5.4	10.9	99	131
Fluorocitrate	(0.08)	13.4	7.9	76	163
Fluorocitrate	(0.2)	12.0	8.3	89	166

shown in Table III, housefly sarcosomes supplemented with fumarate as substrate and the other requisite ingredients of the reaction mixture, accumulated in the presence of fluoroacetate amounts of citrate which were about twice as high as in the control, whereas under the same conditions guinea-pig kidney mitochondria showed a nearly 15-fold increase of citrate above the normal control level. The two systems also displayed a similar discrepancy in the extent of citrate accumulation in response to fluorocitrate. Conversely, fluoroacetate caused a marked inhibition of oxygen uptake in the housefly sarcosomes, but showed no appreciable effect on the respiration of guinea-pig mitochondria. It should also be mentioned that the accumulation of citrate in the presence of fluoroacetate was readily demonstrable with unwashed housefly sarcosomes, but was considerably diminished or completely absent when washed particles were employed. The possible requirement for an extramitochondrial cell component, suggested by this observation, has not yet been further explored.

The magnitude of the inhibitory effects of fluoroacetate and fluorocitrate on the sarcosomal respiration varied with the different substrates tested according to a definite and characteristic pattern. Both compounds showed little if any effect on the rate of oxidation of succinate and α-glycerophosphate (Table IV).

TABLE IV

SUBSTRATE-DEPENDENT INHIBITIONS BY FLUOROACETATE AND FLUOROCITRATE OF THE RESPIRATION OF SARCOSOMES DERIVED FROM HOUSEFLY STRAIN *S*; RESISTANCE OF *Fr* STRAIN SARCOSOMES TO FLUOROACETATE

The substrates were added, as indicated, in the following amounts (μmoles/ml): pyruvate, 20; DL-malate, 40; citrate, 20; succinate, 20; DL-α-glycerophosphate, 80. NAD at a 0.4 mM final concentration was included in all the flasks except those containing succinate and α-glycerophosphate. Other details as in Table III.

Substrate	*Additions to the standard reaction mixture (μmoles/ml)*	*Strain S*		*Strain Fr*	
		Q_{O_2}	*Inhibition (%)*	Q_{O_2}	*Inhibition (%)*
Pyruvate	None	133	—	118	—
Pyruvate	Fluoroacetate (0.4)	46	66	118	0
Pyruvate	Fluoroacetate (1.6)	26	81	121	0
Pyruvate	Fluorocitrate (0.4)	114	14	95	20
Pyruvate	Fluorocitrate (1.6)	36	73	29	75
DL-Malate	None	77	—	67	—
DL-Malate	Fluoroacetate (0.4)	32	58	69	0
DL-Malate	Fluoroacetate (1.6)	31	60	68	0
DL-Malate	Fluorocitrate (0.4)	54	30	61	9
DL-Malate	Fluorocitrate (1.6)	28	64	37	45
Citrate	None	50	—	52	—
Citrate	Fluoroacetate (0.4)	42	16	54	0
Citrate	Fluorocitrate (1.6)	21	58	24	54
Succinate	None	80	—	86	—
Succinate	Fluoroacetate (0.4)	72	10	81	6
Succinate	Fluorocitrate (1.6)	72	10	71	17
DL-α-Glycerophosphate	None	290	—	298	—
DL-α-Glycerophosphate	Fluoroacetate (0.4)	298	0	300	0
DL-α-Glycerophosphate	Fluorocitrate (1.6)	290	0	286	4

Respiratory lesion induced by fluoroacetate or fluorocitrate not reversible by washing of the sarcosomes

Sarcosomes preincubated in the cold for 5–30 min in a substrate-less medium containing fluoroacetate or fluorocitrate and then washed and resuspended in fresh medium, exhibited on subsequent incubation under the standard test conditions a marked depression of their respiratory activity, as compared to parallel controls (Table V). The inhibition, which was more pronounced with pyruvate than with the other substrates tested, took place despite the fact that the washing procedure employed removed more than 99 % of the fluoroacetate added, as shown with the aid of ^{14}C-labeled fluoroacetate.

Protective effect of acetate against the inhibition of sarcosomal respiration by fluoroacetate

The inhibitory effect of fluoroacetate on the sarcosomal respiration was markedly relieved or even completely prevented by suitable amounts of acetate, provided that both compounds were added simultaneously to the incubation mixture. If, however, the addition of acetate was preceded by exposure to fluoroacetate for 30 min in the cold the resultant inhibition was no longer reversible (Table VI).

It should be pointed out that under our conditions acetate alone was not appreciably oxidized, nor did it significantly affect the rate of oxygen uptake when

added together with other Krebs cycle intermediates. The extent of protection afforded by acetate against inhibition of sarcosomal respiration by fluoroacetate seemed to be determined by the molar ratio of the two compounds rather than by their absolute concentrations, an about 25-fold excess of acetate being required for complete prevention of the inhibitory effect of fluoroacetate. Under otherwise similar conditions, acetate failed to obviate the injury of sarcosomal respiration caused by fluorocitrate (Table VI).

TABLE V

DECLINE OF RESPIRATORY ACTIVITY OF HOUSEFLY SARCOSOMES FOLLOWING TRANSIENT EXPOSURE IN THE COLD TO FLUOROACETATE OR FLUOROCITRATE

The sarcosomes suspended in Medium A were preincubated in an ice bath, as indicated. The preincubation was terminated by diluting the sarcosome suspension with 7 ml of ice-cooled Medium A. The sarcosomes were then sedimented by centrifugation, washed twice with Medium A and finally redispersed in the same medium and incubated in the standard reaction mixture as specified in the table. Other conditions were as in Table IV.

Additions to the preincubation medium (*μmoles/ml*)	*Time of preincubation* (*min*)	Q_{O_2}	
		Pyruvate	*Malate*
None	5	83	84
None	30	78	74
Fluoroacetate (1)	5	31	74
Fluoroacetate (1)	30	8	46
Fluorocitrate (4)	5	22	64

TABLE VI

PROTECTIVE EFFECT OF ACETATE AGAINST THE RESPIRATORY INHIBITION OF HOUSEFLY SARCOSOMES BY FLUOROACETATE

The Q_{O_2} values in the control experiment were: 130 for pyruvate and 76 for malate. The experimental conditions were as in Table IV.

Additions to the standard reaction mixture (*μmoles/ml*)	*Oxygen uptake* (*% of control*)	
	Pyruvate	*Malate*
Acetate (1)	104	109
Acetate (5)	103	125
Acetate (25)	105	129
Fluoroacetate (1)	38	37
Fluoroacetate (1)*	12	33
Fluoroacetate (1) + acetate (1)	43	38
Fluoroacetate (1) + acetate (5)	62	62
Fluoroacetate (1) + acetate (25)	104	113
Fluoroacetate (1) + acetate (25)**	25	43
Fluorocitrate (4)	30	—
Fluorocitrate (4) + acetate (25)	31	—

* Preincubated with fluoroacetate in an ice bath for 30 min.

** Acetate added after preincubation of the sarcosomes with fluoroacetate in an ice bath for 30 min.

Effect of NAD and other cofactors on fluoroacetate inhibition

In the course of this work it was observed that addition of NAD to the reaction mixture resulted in a considerable enhancement of the rate of oxidation of a number of substrates by the housefly sarcosomes. The effect of NAD was particularly prominent in stimulating the oxygen uptake in the presence of malate and α-oxoglutarate, but was inappreciable when pyruvate was used as substrate (Table VII). The relatively high demand for NAD was not attributable to an excessive activity of NAD glycohydrolase (EC 3.2.2.5) present in the sarcosomal preparation, since NAD was not spared by nicotinamide (40 mM), a known inhibitor of this enzyme[15]. Addition of NAD to the reaction mixture resulted in an increase of the inhibitory effect of fluoroacetate on the oxidation of the NAD-responsive substrates, relative to the enhanced respiration rate of the control (Table VI). A number of other known cofactors such as thiamine pyrophosphate (10^{-4} M), coenzyme A ($2 \cdot 10^{-4}$ M), carnitine ($8 \cdot 10^{-4}$ M) and reducing agents such as cysteine ($2 \cdot 10^{-3}$ M) or reduced glutathione ($2 \cdot 10^{-3}$ M) were without noticeable effect both on the respiration and the extent of its inhibition by fluoroacetate.

TABLE VII

STIMULATION OF SARCOSOMAL RESPIRATION AND ENHANCEMENT OF THE FLUOROACETATE EFFECT BY NAD

The concentrations of the respective substrates and NAD were as specified in Table IV.

Substrate	*Fluoroacetate (μmole/ml)*	*Without NAD added*		*With NAD added*	
		Q_{O_2}	*Inhibition (%)*	Q_{O_2}	*Inhibition (%)*
Pyruvate	0	117.0	—	128.0	—
Pyruvate	0.4	57.0	51	61.0	52
Fumarate	0	16.9	—	92.5	—
Fumarate	0.4	10.5	38	40.5	56
α-Oxoglutarate	0	33.0	—	51.5	—
α-Oxoglutarate	0.4	20.6	38	25.0	51
DL-Malate	0	17.3	—	68.0	—
DL-Malate	1	9.7	44	28.9	57

Lack of inhibition of NADH oxidase by fluoroacetate

While the preferential effect of fluoroacetate on the oxidation of NAD-linked substrates tended to implicate the NADH oxidase system as a possible site of its antimetabolic action, the disparity in the degree of inhibition obtained with the individual substrates seemed to militate against this assumption. The latter conclusion was corroborated by the finding that fluoroacetate at 1 mM concentration produced no perceptible inhibition of sarcosomal NADH oxidase when tested in the presence of NADH supplied as a hydrogen donor.

Uncoupling of oxidative phosphorylation

Exposure of *S* fly sarcosomes to fluoroacetate resulted in a decline of phosphorylation, which was considerably greater than the concomitant decrease in the rate of oxygen uptake. Unlike 2,4-dinitrophenol and related uncouplers (see ref. 16), however, fluoroacetate caused no detectable enhancement of the sarcosomal ATPase

activity above the normal range of hydrolysis of about 12–14 μmoles ATP per mg protein per h observed in the standard incubation system (except that P_i was omitted; see MATERIALS AND METHODS).

The linkage between the respiratory injury and the uncoupling of oxidative phosphorylation induced by fluoroacetate was reflected in the similar substrate specificity of the two effects, the protective action exerted by acetate against both phenomena and the failure of fluoroacetate to uncouple the oxidative phosphorylation in sarcosomes of the fluoroacetate-resistant *Fr* strain (Table VIII).

Correlation between the sarcosomal lesion and the lethal activity of fluoroacetate and fluorocitrate

The deleterious action of fluoroacetate on the sarcosomes appeared to be correlated with the degree of susceptibility of the different housefly strains to this poison.

TABLE VIII

UNCOUPLING EFFECT OF FLUOROACETATE ON OXIDATIVE PHOSPHORYLATION OF SENSITIVE HOUSEFLY SARCOSOMES; PROTECTIVE INFLUENCE OF ACETATE

Fluoroacetate and acetate were added, where indicated, in the amounts of 0.4 and 10 μmoles/ml reaction mixture, respectively. The amount of sarcosomes was 3 mg protein per reaction flask. The sarcosomes were prepared by the standard procedure, except that Medium A was used in the last washing and in the final suspension. Other details were as in Table IV.

Additions to the standard reaction mixture	*Strain S*		*Strain Fr*	
	Oxygen uptake (μatoms/h)	*P:O ratio*	*Oxygen uptake (μatoms/h)*	*P:O ratio*
Malate	18.9	2.5	24.4	2.6
DL-Malate + fluoroacetate	11.7	0.4	25.0	2.5
α-Oxoglutarate	11.2	2.1	14.2	2.0
α-Oxoglutarate + fluoroacetate	9.0	1.1	12.8	2.0
DL-α-Glycerophosphate	55.2	1.6		
DL-α-Glycerophosphate + fluoroacetate	59.1	1.5		
Pyruvate	44.1	3.0		
Pyruvate + fluoroacetate	6.9	1.0		
Pyruvate + acetate	39.9	2.9		
Pyruvate + acetate + fluoroacetate	38.7	2.9		

TABLE IX

DIFFERENTIAL EFFECTS OF FLUOROACETATE AND FLUOROCITRATE ON OXYGEN UPTAKE AND CITRATE ACCUMULATION BY SARCOSOMES OF FLUOROACETATE-SENSITIVE AND RESISTANT HOUSEFLY STRAINS

The experimental conditions were the same as in Table III.

Expt. No.	*Additions to the standard reaction mixture (μmoles/ml)*	*Oxygen uptake (% of control)*			*Citrate accumulation (% of control)*		
		S	*Fm*	*Fr*	*S*	*Fm*	*Fr*
1	Fluoroacetate (0.4)	34	62	—	188	109	—
	Fluorocitrate (0.2)	70	89	—	141	161	—
2	Fluoroacetate (1.0)	52	—	97	154	—	100
	Fluorocitrate (1.6)	26	—	55	142	—	151

Thus sarcosomes obtained from 3 different fluoroacetate-sensitive strains responded to fluoroacetate in a manner indistinguishable from that of the *S* strain. On the other hand, the respiratory inhibition induced by fluoroacetate in sarcosomes derived from the partially resistant *Fm* strain was less severe than in the *S* sarcosomes and was not attended by citrate accumulation (Table IX). Furthermore, the respiratory activity of sarcosomes from the highly resistant *Fr* strain was not appreciably affected by fluoroacetate (Tables IV, IX). In contrast, sarcosomes from both strains appeared to be equally susceptible to the toxic action of fluorocitrate.

The role of the sarcosomal lesion as a major site of the poisoning effect of fluoroacetate on the housefly was further ascertained by determining the respiration in sarcosomal preparations, isolated from *S* flies, 1–2 h following the administration of a lethal dose of fluoroacetate. As shown in Table X, sarcosomes from fluoroacetate-poisoned houseflies exhibited, by comparison to normal controls, a marked depression of respiratory rate and a decline of oxidative phosphorylation. Moreover, the substrate-dependent pattern of this alteration was similar to that observed *in vitro*, on adding fluoroacetate to sarcosomal suspensions (see Tables IV, VIII). Predictably, sarcosomes from similarly treated *Fr* flies showed no significant impairment of respiratory activity and coupled phosphorylation.

Reversal of the fluoroacetate inhibition by sonication of the sarcosomes

As may be seen from Table XI, exposure of the sarcosomes to sonic oscillations under controlled conditions abolished their susceptibility to fluoroacetate without affecting, however, their response to fluorocitrate. Moreover, the same treatment nullified the respiratory inhibition induced by pretreating the sarcosomes with fluoroacetate prior to their sonication. The acquired resistance of the sonicated sarcosomes

TABLE X

CORRELATION BETWEEN FLUOROACETATE SENSITIVITY AND INHIBITION OF THE RESPIRATORY ASSEMBLY IN SARCOSOMES FROM FLUOROACETATE-FED HOUSEFLIES

The sarcosomes were isolated from *S* houseflies 1–2 h following feeding of fluoroacetate (3 μg/fly). The sarcosomes were prepared as specified in Table VIII. Amount of sarcosomes: 5 mg protein per reaction flask. Other conditions were as in Table IV.

Housefly strain	*Substrate*	*Sarcosomes derived from*			
		Untreated flies		*Fluoroacetate-fed flies*	
		Oxygen uptake (μatoms/h)	*P:O ratio*	*Oxygen uptake (μatoms/h)*	*P:O ratio*
S	Pyruvate	45	2.9	7	2.1
	DL-Malate	38	2.5	19	1.8
	Citrate	22	0.6	10	0.2
	Succinate	33	0.4	28	0.4
	DL-α-Glycerophosphate	202	1.5	202	1.6
Fr	Pyruvate	26	1.8	29	2.1
	DL-Malate	68	2.4	62	2.3
	Citrate	47	0.4	48	0.4
	Succinate	61	0.3	61	0.3
	DL-α-Glycerophosphate	258	1.5	237	1.4

to fluoroacetate was attended by a virtually complete loss of their capacity to carry out oxidative phosphorylation. Addition of a mixture of cofactors (see legend to Table XI), which enhanced the oxidative activity of the sonicated sarcosomes (*cf.* ref. 7), did not restore their response to fluoroacetate.

TABLE XI

EFFECT OF SONICATION ON THE SUSCEPTIBILITY OF HOUSEFLY SARCOSOMES TO FLUOROACETATE AND FLUOROCITRATE

Sarcosome suspensions prepared from *S* flies as described in Table VIII were diluted prior to sonication with an equal volume of Medium B. The substrate used in the incubation system was DL-malate (40 mM). The mixture of cofactors consisted of: 0.1 mM coenzyme A, 0.4 mM thiamine pyrophosphate, 20 mM nicotinamide and 2 mM glutathione (reduced). Other experimental details were as in Table IV and as described under MATERIALS AND METHODS.

Additions to the standard reaction mixture (μmoles/ml)	Q_{O_2}			
	Intact sarcosomes		*Sonicated sarcosomes*	
	No cofactors added	*Cofactors added*	*No cofactors added*	*Cofactors added*
None	61	60	42	72
Fluoroacetate (0.4)	34	36	39	72
Fluorocitrate (1.6)	27	24	24	40

DISCUSSION

The data presented in this study appear to bear out the notion that two separate and essentially independent modes of action contribute to the overall toxicity of fluoroacetate in the housefly: (1) blocking of the tricarboxylic acid cycle, presumably at the stage of aconitase by a mechanism involving prior conversion of fluoroacetate to fluorocitrate[1]; (2) inhibition of the sarcosomal respiratory chain.

The significance of aconitase inhibition in governing the lethality of fluoroacetate at a dose as low as 0.25 μg/housefly is indicated by the accumulation of citrate, which occurred in the fluoroacetate-poisoned flies as well as in the isolated sarcosomes of the sensitive *S* strain, but not in the partially resistant *Fm* strain. This correlation, contrasting with the essentially equal susceptibility of both strains to fluorocitrate, is consistent with the interpretation that the partial resistance to fluoroacetate of the *Fm* flies stems from their inability to carry out the biosynthesis of fluorocitrate. A similar explanation has been offered for the differential response to fluoroacetate and fluorocitrate exhibited by the male rat liver[17,18] and pigeon brain mitochondria[14].

Thus, fluoroacetate at the relatively high dose of 3 μg/housefly appears to be active *per se* in eliciting in the *Fm* houseflies a toxic phenomenon, characterized by an elevation of their pyruvate content and an inhibition of the respiratory activity of the isolated sarcosomes. This conclusion is strengthened by the observation that the respiratory lesion could be induced by a brief exposure of the sarcosomes to fluoroacetate at about 0° and subsequent removal of the inhibitor by washing, conditions in which no substantial metabolic conversion of fluoroacetate to fluorocitrate is likely to take place.

The essential role of the sarcosomal injury in determining the lethal outcome

of fluoroacetate action in the housefly, is emphasized by the failure of this compound to produce a similar biochemical lesion in the highly resistant *Fr* strain. It may be pertinent to point out in this connection that fluoroacetate did not interfere with the sarcosomal oxidation of α-glycerophosphate which constitutes a major pathway for energy supply in insects[19]. Thus, the toxic action of fluoroacetate, being mediated primarily by the inhibition of the Krebs cycle, attests to the vital function of this system in the insect metabolism. It is interesting to note also that, although the "fluorocitrate mechanism" appears to be the main determinant of fluoroacetate toxicity in vertebrates[1], a strong depression of respiration has been recently reported to occur in homogenates[20] and mitochondria[21] of some organs from fluoroacetate-poisoned rats.

The available evidence seems to favour the conclusion that the deleterious effect of fluoroacetate on the respiratory assembly of the housefly sarcosomes bears primarily on some crucial step of the tightly coupled oxidative phosphorylation system. This interpretation gains further support from the observation that sonic treatment which destroyed the phosphorylative capacity of the sarcosomes, also abolished their susceptibility to fluoroacetate inhibition. The latter result suggests also that some labile structural component of the intact sarcosomal membrane may be the site of the biochemical lesion inherent in the fluoroacetate poisoning. However, the intimate nature of this derangement as well as the mechanism underlying the fluoroacetate resistance of the *Fr* strain remain as yet to be elucidated.

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