A Study of Respiration in Fluoroacetate-Poisoned Muscle Preparations

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The use of fluoroacetate as a specific indicator for the operation of the tricarboxylic acid cycle *in vivo* derives from the observations that: (i) substantial amounts of citric acid accumulate in most tissues of poisoned animals (Buffa & Peters, 1949); (ii) the toxicity of fluoroacetate is due to enzymic synthesis of fluorocitrate, which blocks the tricarboxylic acid cycle at the citrate stage (Peters, Wakelin & Buffa, 1953).

Although general agreement exists that the tricarboxylic acid cycle represents the principal pathway for respiration in mammalian tissues, the response of the various tissues to fluoroacetate poisoning has been observed to vary widely. Of the striated muscles of the rat, heart has been found to accumulate the largest amount of citrate (Buffa & Peters, 1949). However, the symptomatic response of rat heart is generally reported to be slight, compared with that of other species (Chenoweth, 1949), and the labile phosphate content of poisoned heart is practically unaltered (Dawson & Peters, 1955). On the other hand, rat skeletal muscle tends to accumulate much less citrate than the heart, although hypotonicity and decreased contractility of skeletal muscles is a common finding in fluoroacetate poisoning (Chenoweth, 1949). Recently Buffa, Carafoli & Muscatello (1960) observed inhibition of the respiration of slices of rat skeletal muscle but not of rat diaphragm, after administration of 20 mg. of fluoroacetate/kg. body wt. to the intact animals. However, accumulation of citrate and inhibition of pyruvate oxidation in isolated mitochondria were marked in both tissues.

Accumulation of citrate and inhibition of pyruvate oxidation in isolated mitochondria from heart, diaphragm and skeletal muscle indicate that the different behaviour of the three tissues with respect to inhibition of respiration does not reflect varying degrees of blocking of the tricarboxylic acid cycle. The question arises therefore whether alternative oxidative pathways are available in fluoroacetate-poisoned rat heart and diaphragm but not in rat skeletal muscle. The rate of glycolysis has been reported to increase in fluoroacetate-poisoned frog and mammalian muscles (Chenoweth, 1949). Interestingly enough, a continuously stimulated poisoned frog muscle accumulated less lactate in the bathing fluid than did a normal muscle (Clarke & Riker, 1950). Oxidation of glutamic acid also represents a possible metabolic pathway of the hydrogen supply in fluoroacetate-poisoned systems. Indeed, decreased concentrations of aspartate and glutamate have been found in heart and skeletal muscles of fluoroacetate-poisoned rats (Awapara, 1952).

In the present study the respiration of normal and fluoroacetate-poisoned muscle homogenates (oxidizing endogenous substrates, glycolytic substrates or glutamate) has been studied in the presence of various metabolic inhibitors. The present results indicate that rat heart and diaphragm muscles have a much higher capacity to oxidize glycolytic substrates than has gastrocnemius muscle, and that the metabolism of glutamate may account for a large part of the endogenous respiration in the poisoned muscle homogenates.

METHODS AND MATERIALS

Animals. The experimental animals were mostly young adult Wistar rats (200–250 g.) or pigmented rabbits (about 1 kg.). They were fed on a stock laboratory diet. Sodium fluoroacetate (FlAc, 20 mg./kg. body wt., rats; 1 mg./kg. body wt., rabbits) in about 0·2 ml. of 0·9% sodium chloride solution was administered by intraperitoneal injection 90 min. (rats), or about 60 min. (rabbits), before the animals were killed.

Preparation of tissue homogenates. The rats and rabbits were stunned by a blow on the head and bled from the carotid arteries. Heart, diaphragm and gastrocnemius muscles were quickly removed and placed in small beakers containing ice-cold 0·25 M-sucrose. The muscle tissues were blotted on filter paper, freed from connective tissue, weighed and finely minced with scissors without delay. Tissues were blandly homogenized in 0·25 M-sucrose or 0·25 M-sucrose–1 mM-EDTA, with the aid of an all-glass loosely fitting pestle homogenizer (four to six full excursions at 300 rev./min.). For measurements of cytochrome-oxidase activity, the muscles were homogenized thoroughly in water.

Manometric measurements. The ice-cold homogenates were pipetted into the main compartment of the Warburg vessels, which already contained all other constituents; 0·1 ml. of 20% KOH and a folded strip of filter paper were placed in the central well. The vessels were allowed to equilibrate in the bath for 5 min. (3 min. with stopcock open and 2 min. with stopcock closed) unless stated otherwise.

Portions of the tissue homogenates were incubated in a medium of the same basic composition as reported by Margreth, Muscatello & Andersson-Cedergren (1963), which, in a final volume of 3 ml. contained: 33·3–66·6 mM-tris buffer, pH 7·4; 6·6 mM-$MgCl_2$; 10 mM-nicotinamide; 0·53 mM-NAD; 1 mM-potassium phosphate buffer, pH 7·4; 1 mM-ATP. Incubation was at 30°, in air.

Cytochrome-oxidase activity of muscle homogenates was determined according to the method of Potter (1959), at 37°.

Analytical procedures

Determination of lactate and hexose 1,6-diphosphate. After incubation the reaction was terminated by adding 1 ml. of 33% trichloroacetic acid. Lactate and hexose 1,6-diphosphate were determined in the deproteinized acid extracts. Lactate was determined by the Barker & Summerson (1941) procedure under the conditions described by LePage (1959). Hexose 1,6-diphosphate was determined spectrophotometrically according to the adaptation by Slater (1953) of Racker's (1947) method.

Chromatographic determination of glutamate and aspartate. The reaction mixture was deproteinized by adding 1 ml. of 1·4 M-$HClO_4$ to 3 ml. of tissue suspension. Perchloric acid was neutralized with KOH in the cold and $KClO_4$ was removed by centrifugation. Portions (50 μl.) of the tissue extracts and standard solutions of glutamic acid and aspartic acid were applied to Whatman no. 1 (chromatographic grade) filter paper. The chromatograms were developed by descending chromatography in pyridine–acetic acid–water (10:7:3, by vol.; Decker & Riffart, 1950). The paper chromatograms were run for 10 hr., dried in a stream of air at room temperature and sprayed with ninhydrin solution (Meyer, 1957). The spots were detected by heating for 1–2 min. at 100°. In a limited number of experiments the spots were cut out, after light spraying with ninhydrin solution, and the colour formed on incubation of the pieces of paper with ninhydrin reagent was estimated at 578 mμ, essentially as described by Krebs & Bellamy (1960).

Determination of protein. Protein was determined on portions of the tissue homogenates by the biuret reaction (Gornall, Bardawill & David, 1949) or by a micro-Kjeldahl procedure (Markham, 1942). A factor of 6·25 was used to convert mg. of N into mg. of protein.

Chemicals. ATP, hexose 1,6-diphosphate, glucose 6-phosphate, glycerol 1-phosphate, sodium fluoroacetate and antimycin A were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. L-Cysteinesulphinic acid was purchased from California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A., Amytal from Eli Lilly, Indianapolis, U.S.A., and rotenone from S. B. Pennick and Co., New York. Oligomycin was kindly given by Dr H. A. Lardy. Other products were commercial-grade reagents.

RESULTS

Respiration of muscle homogenates from untreated rats. Differences in the oxygen uptake between different tissues depend on (i) the capacity of the various metabolic pathways to supply hydrogen to the respiratory chain, and (ii) the electron-transfer capacity of the terminal respiratory chain. The capacity of the terminal respiratory chain, as indicated by the activities of cytochrome oxidase in rat heart, diaphragm and gastrocnemius homogenates, was in the ratio 4:2:1 respectively (Table 1). Cytochrome-oxidase activity in rabbit heart had practically the same value as in rat heart.

The contribution of the various metabolic pathways to the endogenous respiration has been evaluated by studying the effect of known metabolic inhibitors. The endogenous respiration of heart, diaphragm and gastrocnemius homogenates was largely dependent on the continuous supply of oxaloacetate. Blocking of the formation of oxaloacetate from succinate by malonate, or removal of oxaloacetate by transamination to aspartate by addition of cysteinesulphinic acid (Singer & Kearney, 1956), resulted in 80% inhibition of the oxygen uptake in the three tissue homogenates. Inhibition of glycolysis at the triose phosphate-dehydrogenase stage by addition of iodoacetate resulted in a decrease of the endogenous respiration of 55%, 42% and 31% in heart, diaphragm and gastrocnemius homogenates respectively. The pathway of electron transport through the mitochondrial respiratory chain is indicated by the almost complete sensitivity of the oxygen uptake to the specific inhibitors, rotenone (Ernster, Dallner & Azzone, 1963) and antimycin A.

A useful means to measure the extent to which the oxygen uptake of tissue homogenates is limited by the availability of phosphate and phosphate acceptor, and thereby coupled to phosphorylation, is to test the effect of the energy-transfer inhibitor oligomycin (Lardy, Johnson & McMurray, 1958). It is seen in Table 2 that the endogenous respiration of heart, diaphragm and gastrocnemius homogenates was largely coupled to phosphorylation, as shown by the inhibition of about 70% on addition of oligomycin. Dinitrophenol did not produce a stimulation of the oxygen uptake. Endogenous respiration was actually inhibited by dinitrophenol, especially with heart and gastrocnemius homogenates, possibly because the utilization of endogenous fatty acids was blocked by lack of ATP.

Table 1. *Cytochrome-oxidase activity in heart, diaphragm and gastrocnemius homogenates*

Hearts (ventricles), diaphragm and gastrocnemius were homogenized in water. The homogenates were diluted to a final concentration of 2·5% (hearts) or 5% (diaphragm and gastrocnemius). Cytochrome oxidase was assayed at 37° on appropriate portions of the tissue homogenates, under the conditions described by Potter (1959). Values are the averages of two comparable experiments. Values for rat diaphragm and rat gastrocnemius were reported by Margreth, Muscatello & Andersson-Cedergren (1963).

	Uptake of O_2 (μl./hr./mg. of protein)		
Rabbit heart	Rat heart	Rat diaphragm	Rat gastrocnemius
657·6	698·5	380·0	166·5

Respiration of muscle homogenates from fluoroacetate-poisoned rats. The following results concern the effect of metabolic inhibitors on the oxygen uptake of fluoroacetate-poisoned muscle homogenates in the absence or in the presence of added substrates. Homogenates were prepared from heart, diaphragm and gastrocnemius muscles of rats which had received a lethal dose of fluoroacetate (20 mg./kg. body wt.). It is seen in Fig. 1(*a*) that the respiration of fluoroacetate-poisoned gastrocnemius homogenates was severely decreased; the inhibition was slightly lower for the diaphragm (Fig. 1*b*). In the heart (Fig. 1*c*) the initial rate of oxygen uptake was inhibited about 30 % by fluoroacetate, whereas the total oxygen uptake in a 40 min. incubation period was unchanged. The initial rate of respiration in the fluoroacetate-poisoned preparations was 50 % sensitive to the addition of oligomycin, both in the absence and in the presence of glycogen. The slight increase in the extent of oligomycin-insensitive respiration, as compared with the untreated rats (Table 2), was probably due to change in the nature of the substrate(s) being oxidized, rather than to mitochondrial damage. In fact the addition of dinitrophenol, at variance with the control homogenates, caused almost no inhibition of the oxygen uptake (Table 3).

Oxidation of glutamate. Due consideration has been given to the possibility that the respiration of fluoroacetate-poisoned muscle preparations might be supported by the oxidation of glutamate since (i) relatively large amounts of glutamate have been found in most animal tissues (Krebs, Eggleston & Hems, 1949) and especially in heart (Awapara, 1952), (ii) the oxidation of glutamate has been reported to be elevated in muscle homogenates (Krebs & Bellamy, 1960) and muscle mitochondria (Azzone & Carafoli, 1960; Borst, 1962). In Tables 4 and 5 it is shown that the addition of either malonate or arsenite strongly inhibited the oxygen uptake of heart, diaphragm and gastrocnemius homogenates from fluoroacetate-poisoned rats. The addition of malate, which did not itself markedly enhance the oxygen uptake, resulted in complete removal of the inhibition produced by malonate. This effect is consistent with present knowledge, according to which the malonate blocking of glutamate oxidation can be overcome by a continuous supply of oxaloacetate; it also indicates that the block of succinate oxidation by malonate only slightly affects the oxygen uptake of muscle homogenates, provided that oxaloacetate be supplied through the oxidation of malate. To determine whether the rate-limiting step for the respiration of muscle homogenates in the presence of malonate and malate was malate dehydrogenase or α-oxoglutarate dehydrogenase, the oxygen uptake was measured either in the presence of arsenite and malate or malonate and oxaloacetate. It is seen from Table 5 that the oxidation rate was very low in all three muscle homogenates in the arsenite and malate system, under which conditions malate was supposedly the main hydrogen source. On the other hand, oxaloacetate almost completely removed the inhibition of the oxygen uptake observed on addition of malonate, probably by allowing maximal rates of formation and oxidation of α-oxoglutarate (Fig. 2).

To test whether the decreased oxygen uptake of fluoroacetate-poisoned diaphragm and gastrocnemius preparations, as compared with the controls, might be due to a different availability of glutamate, the effect of the addition of glutamate on the respiration was studied. It is seen in Table 6 that the percentage stimulation of oxygen uptake, on addition of glutamate, was progressively lower in heart, diaphragm and gastrocnemius preparations of normal rats (61 %, 30 % and 4 % respectively), whereas it was almost equal (71 %, 64 % and 69 %) in the three poisoned preparations. The amounts of glutamate utilized and of aspartate formed were also estimated qualitatively by paper chromatography (Decker & Riffart, 1950) and were in good correlation with the Δoxygen values. In a limited number of experiments with rat heart, the chromatographic spots were eluted from the paper and the amounts of glutamate and aspartate quantitatively determined. The ratio Δglutamate:Δaspartate was about 1, and the ratio Δ(μg.atoms of oxygen):μmoles of aspartate formed was about 3.

Table 2. *Effect of metabolic inhibitors on the endogenous respiration of rat muscle homogenates*

The reaction medium contained, in a final volume of 3 ml.: 66·5 mM-tris buffer, pH 7·4; 6·6 mM-$MgCl_2$; 10 mM-nicotinamide; 0·53 mM-NAD; 1 mM-potassium phosphate; 1 mM-ATP; 1 ml. of tissue homogenate in 0·25 M-sucrose containing 0·5 mM-ethylene glycol-bis(aminoethyl)tetra-acetic acid and 2 mM-$MgCl_2$. Incubation was at 30°. Gas phase, air. Amount of protein: heart, 23·0 mg.; diaphragm, 41·4 mg.; gastrocnemius, 33·4 mg.

Additions	Uptake of O_2 (μl./20 min./mg. of protein)		
	Heart	Diaphragm	Gastrocnemius
None	9·54	3·58	3·80
Malonate (5 mM)	1·82	0·91	0·83
Cysteinesulphinic acid (2 mM)	1·43	0·54	0·46
Iodoacetic acid (1 mM)	4·33	2·08	2·65
Rotenone (0·01 mM)	0·73	0·18	0·16
Antimycin A (10 μg.)	1·71	0·35	0·25
Oligomycin (5 μg.)	2·67	1·32	1·31
Dinitrophenol (0·1 mM)	4·33	2·56	2·11

Utilization of carbohydrates. A low content of glycogen is found in striated muscles of rat, especially in the diaphragm (Lackey, Bunde, Gill & Harris, 1944; Beatty, Peterson & Bocek, 1960). The contribution of the glycolytic pathway to the respiration of fluoroacetate-poisoned homogenates was studied therefore both in the absence and in the presence of added glycolytic substrates, to make certain that the low concentration of substrate could not be a limiting factor in the system. The oxygen uptakes of the three untreated muscle homogenates were almost equally sensitive to iodoacetate (Table 7) whereas the inhibition of respiration, which followed on addition of fluoride, was

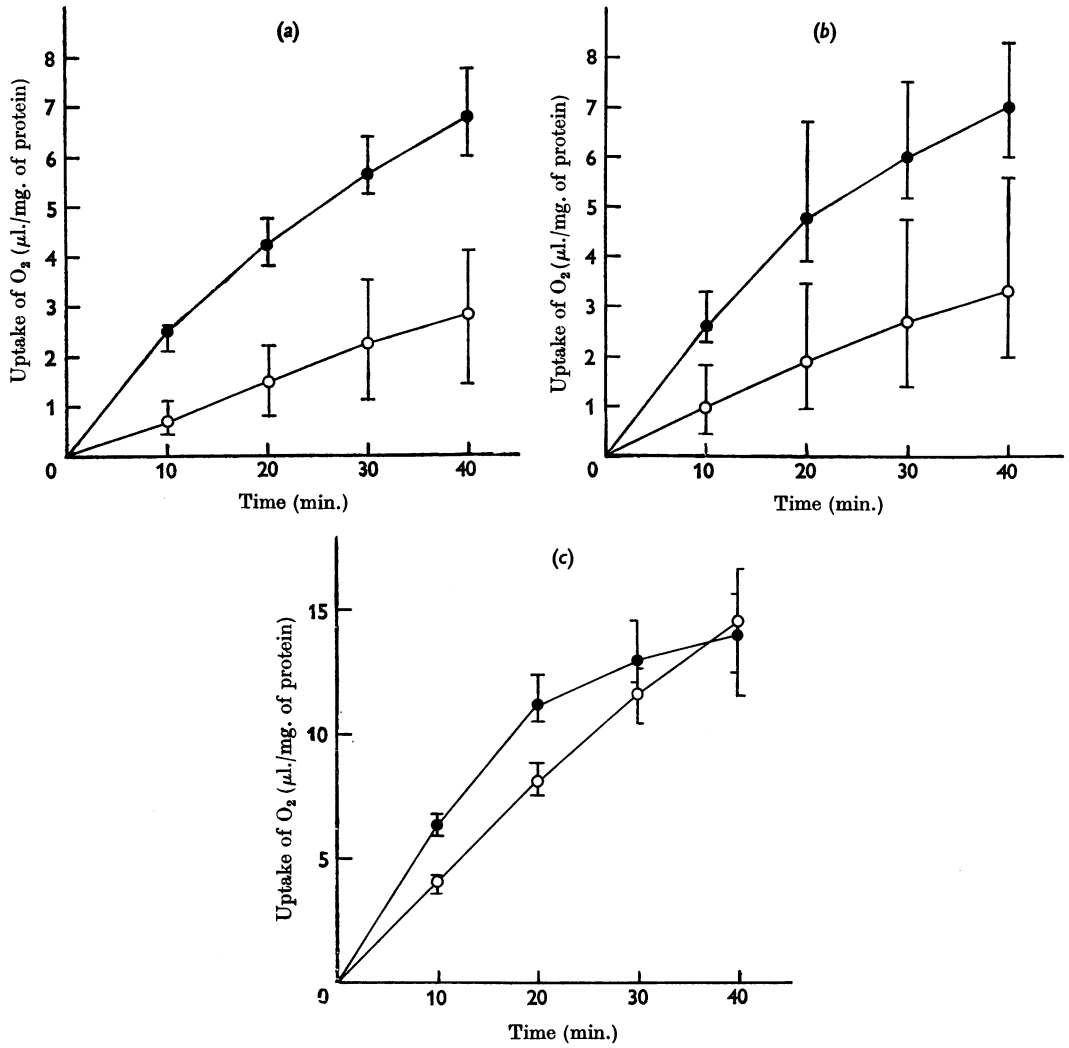

Fig. 1. Endogenous respiration in (*a*) gastrocnemius, (*b*) diaphragm and (*c*) heart homogenates from untreated and fluoroacetate-poisoned rats. One ml. of 10% homogenates (heart) or 20% homogenates (diaphragm and gastrocnemius) in 0·25M-sucrose was added to a reaction medium of the following composition, in a final volume 3 ml.: 66·6 mM-tris buffer, pH 7·4; 6·6 mM-$MgCl_2$; 10 mM-nicotinamide; 0·53 mM-NAD; 1 mM-potassium phosphate buffer, pH 7·4; 1 mM-ATP, potassium salt. Incubation was at 30°. Gas phase, air. Amount of protein (average value): heart, 16·4 and 17·2 mg. (untreated and fluoroacetate-poisoned rats respectively); diaphragm, 28·8 and 32·9 mg.; gastrocnemius, 30·2 and 35·8 mg. ●, Untreated rats, average values; ○, fluoroacetate-poisoned rats, average values; vertical bars show ranges of values in five experiments (heart), seven experiments (diaphragm), four experiments (untreated gastrocnemius), seven experiments (fluoroacetate-poisoned gastrocnemius).

progressively lower with gastrocnemius, diaphragm and heart in that order. The difference between the three muscles, with respect to sensitivity of the oxygen uptake to iodoacetate and fluoride, was greater in the poisoned preparations. Iodoacetate induced an inhibition of the oxygen uptake that was progressively stronger with gastrocnemius, diaphragm and heart, and conversely the inhibition by fluoride was progressively lower in the three poisoned muscles in the same order (Table 7).

Hexose 1,6-diphosphate was more effective, compared with glucose 6-phosphate and glycogen, in maintaining the respiration of poisoned heart homogenates, presumably because phosphorylase and phosphofructokinase activities were rate-limiting in this system (Table 8). The respiration supported by the hexose mono- and di-phosphate was not inhibited by fluoride.

A comparative study of the effect of glycolytic inhibitors on the oxygen uptake by diaphragm and gastrocnemius homogenates from fluoroacetate-poisoned rats, in the absence and in the presence of hexose diphosphate, is reported in Table 9. Malonate was also added to decrease the oxidation of substrates from other sources. It is seen that the

Table 3. *Degree of controlled respiration in muscle homogenates from fluoroacetate-poisoned rats*

Rats which had received 20 mg. of fluoroacetate/kg. body wt. intraperitoneally were killed after 90 min. Pooled heart, diaphragm and gastrocnemius muscles were homogenized in 0·25M-sucrose. The reaction medium had the same basic composition as given in Table 2; glycogen, 10 mg., oligomycin, 5 μg., or dinitrophenol, 0·1 mM, were added where indicated. Incubation was at 30° for 20 min. Amount of protein: heart, 15·4 mg.; diaphragm, 35 mg.; gastrocnemius, 38·1 mg.

Additions	Glycogen	Uptake of O_2 (μl./10 min./mg. of protein) Heart	Diaphragm	Gastrocnemius
None	–	5·04	1·60	0·53
Oligomycin	–	2·69	0·83	0·21
Oligomycin, 2,4-dinitrophenol	–	4·12	1·40	0·78
None	+	5·17	1·90	1·15
Oligomycin	+	2·41	0·99	0·41
Oligomycin, 2,4-dinitrophenol	+	4·49	1·82	1·25

Table 4. *Effect of malonate and malate on the endogenous respiration of muscle homogenates from fluoroacetate-poisoned rats*

The basic experimental conditions were the same as those described in Table 3. Concentration of malonate and malate was 5 mM. Incubation was at 30° for 40 min. Amount of protein: heart, 15·6 mg.; diaphragm, 29·2 mg.; gastrocnemius, 35·4 mg.

Tissue	Uptake of O_2 (μl./40 min./mg. of protein) Without malate	With malate
Heart	16·60	20·00
Heart with malonate	4·22	16·80
Diaphragm	5·59	5·86
Diaphragm with malonate	1·91	5·28
Gastrocnemius	4·10	3·76
Gastrocnemius with malonate	1·56	3·80

Table 5. *Effect of arsenite and malate on the endogenous respiration of muscle homogenates from fluoroacetate-poisoned rats*

Experimental conditions were the same as those described in Table 4. Concentration of sodium arsenite was 1 mM and that of malate was 5 mM. Amount of protein: heart, 14·6 mg.; diaphragm, 31·2 mg.; gastrocnemius, 34·4 mg.

Tissue	Uptake of O_2 (μl./40 min./mg. of protein) Without malate	With malate
Heart	14·45	17·40
Heart with arsenite	3·18	3·81
Diaphragm	3·46	4·37
Diaphragm with arsenite	1·22	1·56
Gastrocnemius	1·79	2·08
Gastrocnemius with arsenite	0·70	1·08

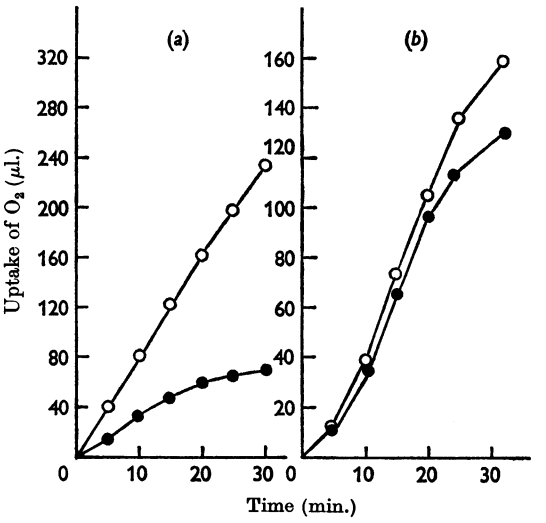

Fig. 2. Effect of malonate and oxaloacetate on the endogenous respiration of heart homogenates from fluoroacetate-poisoned rats. (*a*) Without oxaloacetate; (*b*) with oxaloacetate. Experimental conditions were the same as those described in Fig. 1, except that malonate (5 mM) and oxaloacetate (5 mM) were added as indicated. Incubation was at 30°. Gas phase, air. Amount of protein: 18·8 mg. ○, Malonate, absent; ●, malonate present.

oxygen uptake of diaphragm homogenates was more than doubled on addition of fructose 1,6-diphosphate. Iodoacetate prevented the stimulation of respiration by hexose diphosphate, and fluoride further increased it. Addition of hexose diphosphate resulted in a very low increase of respiration in gastrocnemius homogenates, even in the presence of fluoride. Glycerol 1-phosphate did not produce any significant stimulation of the oxygen uptake in either type of muscle (Expt. 2, Table 9).

The data presented in Table 10 show the balance sheet of utilization of hexose diphosphate, lactate formation and oxygen uptake, in both diaphragm

Table 6. *Effect of glutamate on the oxygen uptake of muscle homogenates from untreated and fluoroacetate-poisoned rats*

Hearts (ventricles), diaphragm and gastrocnemius from several rats were homogenized in 0·25M-sucrose. Homogenates were diluted with the sucrose solution to a final concentration of 10% (heart) or 20% (diaphragm and gastrocnemius). The incubation medium had the same basic composition as given in Table 2, except that 10 mM-glutamate, 10 mM-malate or both were added where indicated. Incubation was at 30° for 40 min. Gas phase, air. Amount of protein: about 17 mg. (heart) or about 32 mg. (diaphragm and gastrocnemius). Values are the average of two experiments.

	Uptake of O_2 (μl./40 min./mg. of protein)					
	Heart		Diaphragm		Gastrocnemius	
Additions	Untreated	Treated	Untreated	Treated	Untreated	Treated
None	14·83	12·97	6·55	3·32	5·85	2·88
Glutamate	23·87	22·21	8·52	5·45	6·11	4·86
Glutamate + malate	27·00	27·10	8·88	5·82	6·46	4·58

Table 7. *Effect of iodoacetate and fluoride on the respiration of muscle homogenates from untreated and fluoroacetate-poisoned rats*

Rats that had received 20 mg. of fluoroacetate/kg. body wt. intraperitoneally were killed after 90 min. The basic experimental conditions were the same as described in Table 2 except that 10 mg. of glycogen was added to the incubation medium. Incubation was at 30° for 20 min. Amount of protein: heart, 20·8 and 15·2 mg. (untreated and treated respectively); diaphragm, 41·4 and 35·3 mg.; gastrocnemius, 33·4 and 38·1 mg.

		Uptake of O_2 (μl./20 min./mg. of protein)	
Tissue	Additions	Untreated	Treated with fluoroacetate
Heart	None	9·61	10·20
	Iodoacetate (1 mM)	4·07	2·74
	NaF (10 mM)	6·26	8·80
Diaphragm	None	3·72	2·90
	Iodoacetate (1 mM)	1·76	1·97
	NaF (10 mM)	2·39	2·62
Gastrocnemius	None	4·50	2·08
	Iodoacetate (1 mM)	1·98	1·20
	NaF (10 mM)	1·80	1·68

Table 8. *Stimulation of oxygen uptake by glycolytic substrates in heart homogenates from fluoroacetate-poisoned rats*

Experimental conditions were the same as described in Table 7, except that 10 mg. of glycogen, or 10 mM-glucose 6-phosphate or hexose 1,6-diphosphate was added where indicated. Amount of protein: 17·7 mg.

Additions	Uptake of O_2 (μl./20 min./mg. of protein)			
Substrate added	None	Glycogen	Glucose 6-phosphate	Hexose 1,6-diphosphate
None	8·80	8·60	12·43	13·60
Malonate (5 mM)	2·22	4·44	8·25	12·10
Malonate + fluoride (10 mM)	1·53	3·05	—	12·50

Table 9. *Stimulation of oxygen uptake by hexose 1,6-diphosphate and glycerol 1-phosphate in diaphragm and gastrocnemius homogenates from fluoroacetate-poisoned rats*

Rats that had received 20 mg. of fluoroacetate/kg. body wt. intraperitoneally were killed after 90 min. The pooled diaphragm and gastrocnemius muscles from several rats were homogenized in 0·25 M-sucrose. The reaction medium had the same basic composition as given in Table 2. Concentrations of substrates and inhibitors added: hexose 1,6-diphosphate, 10 mM; glycerol 1-phosphate, 10 mM; malonate, 5 mM; iodoacetate, 1 mM; NaF, 10 mM; rotenone, 0·01 mM. Incubation was at 30° for 20 min. Amount of protein: 34 mg. in Expt. 1 and 11 mg. in Expt. 2.

Expt. no.	Additions	Uptake of O_2 (μl./20 min./mg. of protein) Diaphragm	Gastrocnemius
1	None	1·58	1·94
	Malonate	0·69	0·69
	Malonate, iodoacetate	0·59	1·03
	Malonate, NaF	0·77	1·48
	Hexose 1,6-diphosphate	4·09	2·36
	Hexose 1,6-diphosphate, malonate	2·78	1·79
	Hexose 1,6-diphosphate, malonate, iodoacetate	0·54	1·03
	Hexose 1,6-diphosphate, malonate, NaF	5·20	1·66
2	None	1·34	1·04
	Glycerol 1-phosphate	2·29	1·08
	Glycerol 1-phosphate, rotenone	1·38	0·63
	Hexose 1,6-diphosphate	4·57	1·61
	Hexose 1,6-diphosphate, NaF	7·90	1·25

and gastrocnemius homogenates prepared from untreated and fluoroacetate-poisoned rats. It is seen that the rates of aerobic glycolysis, as measured from the utilization of hexose diphosphate and formation of lactate, were equal in diaphragm and gastrocnemius homogenates of control rats. The addition of fluoride inhibited almost completely the formation of lactate, but affected the utilization of hexose diphosphate only 30–40 %. The utilization of fructose diphosphate was higher in fluoroacetate-poisoned diaphragm, as compared with the untreated preparation. The formation of lactic acid was conversely lower in the poisoned preparations than in the untreated controls. In agreement with the data shown in Table 9, the oxygen uptakes by both normal and fluoroacetate-poisoned diaphragm preparations were several times higher than the corresponding values of gastrocnemius homogenates. The difference in oxygen uptake between poisoned preparations of diaphragm and gastrocnemius was even greater in the presence of fluoride, which stimulated considerably the rate of respiration in diaphragm homogenates whereas it failed to stimulate respiration in gastrocnemius homogenates.

Utilization of glutamate and hexose diphosphate in rabbit heart. Table 11 shows that rabbit heart homogenates behaved similarly to rat heart homogenates, with respect both to the decrease of endogenous respiration after administration of fluoroacetate *in vivo* and to the stimulation of oxygen uptake on the addition of glutamate. The rates of disappearance of glutamate and formation of aspartate were also similar in both types of muscles, as revealed by chromatographic analysis. On the other hand, hexose diphosphate was found to be much less effective in maintaining high rates of

Table 10. *Effect of fluoride and fluoroacetate on glycolysis and respiration of rat diaphragm and gastrocnemius homogenates*

The pooled diaphragm muscles from several untreated and fluoroacetate-poisoned rats were homogenized in 0·25 M-sucrose. The reaction medium contained, in a final volume of 3 ml.: 33·3 mM-tris buffer, pH 7·4; 6·6 mM-$MgCl_2$; 10 mM-nicotinamide; 0·53 mM-NAD; 1 mM-potassium phosphate; 1 mM-ATP; 5 mM-hexose 1,6-diphosphate; 1 ml. of 5% tissue homogenate in 0·25 M-sucrose. Incubation was at 30° for 20 min. after equilibration for 6 min. Gas phase, air. Amount of protein: diaphragm, 7·8–8·4 mg.; gastrocnemius, 8·8–10·9 mg. n.d., Not determined.

	Rat treated with sodium fluoroacetate	Fluoride added	Hexose 1,6-diphosphate metabolized (μmoles/mg./hr.)	Lactic acid formed (μmoles/mg./hr.)	Oxygen consumed (μg.atoms/mg./hr.)
Expt. 1					
Diaphragm	−	−	3·66	4·35	2·78
Diaphragm	+	−	3·52	3·75	1·68
Gastrocnemius	−	−	n.d.	4·08	0·9
Gastrocnemius	+	−	3·14	3·50	0·55
Expt. 2					
Diaphragm	−	+	2·09	0·12	2·78
Diaphragm	+	+	2·96	0·44	2·84
Gastrocnemius	−	+	n.d.	0·41	0·87
Gastrocnemius	+	+	2·86	0·35	0·46

Table 11. *Uptake of oxygen in heart homogenates in the presence of glutamate and hexose* 1,6-*diphosphate*

Rats received 20 mg. of fluoroacetate/kg. body wt. intraperitoneally and were killed after 90 min. Rabbits were injected intraperitoneally with 1 mg. of fluoroacetate/kg. body wt. and were killed when evidently moribund (40 or 100 min. in two experiments). The hearts (ventricles) were homogenized in 0·25 M-sucrose. Final concentration of homogenates was 10%. The reaction medium had the same basic composition as given in Table 10. Incubation was at 30°. Amount of protein: about 15 mg.

	Uptake of O_2 (μl./40 min./mg. of protein)			
	Untreated		Poisoned	
Additions	Rat heart	Rabbit heart	Rat heart	Rabbit heart
None	16·9	17·9	14·5	13·5
Glutamate	23·8	23·2	22·6	24·3
Hexose 1,6-diphosphate	26·7	20·5	29·2	14·8

respiration in rabbit heart homogenates than it was in rat heart preparations, either in the normal or fluoroacetate-poisoned animals.

DISCUSSION

Evidence has been presented showing that in some types of striated muscles there occur other metabolic pathways, as well as the tricarboxylic acid cycle, which permit high rates of respiration. One such pathway is the glycerol 1-phosphate cycle (Bücher & Klingenberg, 1958; Estabrook & Sacktor, 1958), the operation of which probably accounts for a part of the respiration and energy supply in insect flight muscles. Another pathway concerns the oxidation of glutamate either via glutamate dehydrogenase or via transamination to aspartate (Krebs & Bellamy, 1960; Borst, 1962).

The use of metabolic inhibitors such as fluoroacetate to block the tricarboxylic acid cycle *in vivo* appears therefore of considerable interest to study the extent to which hydrogen may be supplied to the respiratory chain independently from the operation of the tricarboxylic cycle. The search for alternate oxidative pathways in different striated muscles is further encouraged by the observations, reported in the beginning of this paper, that the pharmacological and biochemical sensitivity to fluoroacetate poisoning varies considerably according to the type of muscle and the animal species.

In the present investigation a correlation has been sought between the actual insensitivity of some types of striated muscles to fluoroacetate poisoning, as far as physiological function is concerned, and the rates of fluoroacetate-insensitive respiration in slices or cell-free preparations of these muscles. The isolated heart (G. F. Azzone, E. Carafoli & U. Muscatello, unpublished work), diaphragm and skeletal muscle (Buffa *et al.* 1960) mitochondria from fluoroacetate-poisoned rats were about 80% inhibited with respect to the oxidation of pyruvate. The fluoroacetate-insensitive respiration in slices or cell-free systems of rat heart and diaphragm was therefore taken as evidence for a metabolic interplay between mitochondria and other cytoplasmic components of the cell. This interplay would permit, under conditions of fluoroacetate block, that tricarboxylic acid-cycle-dependent and -independent pathways be shifted in their relative importance to the economy of the cell.

As reported in the Results section the endogenous respiration was only slightly decreased in heart preparations from fluoroacetate-poisoned rats, with respect to the homogenates from the untreated controls, whereas a considerable inhibition was found in diaphragm and even more markedly in gastrocnemius homogenates. The effect of metabolic inhibitors, such as malonate and arsenite, indicated that a large part of the residual endogenous respiration in fluoroacetate-poisoned preparations was due to the utilization of glutamate. Since the data of Awapara (1952) show that the concentration of free glutamic acid in rat heart is about fivefold that in rat skeletal muscle the possibility was considered that the different extent of inhibition of the endogenous respiration, after administration of fluoroacetate *in vivo*, might reflect a difference between the two tissues in the capacity to form glutamate from protein sources. This possibility appears to be supported by the results shown in Table 6, where it can be seen that the addition of glutamate considerably stimulated the oxygen uptake of poisoned diaphragm and gastrocnemius homogenates. From the above observations it would appear therefore that a certain correlation exists between levels of glutamate oxidation and degrees of insensitivity of striated muscles to fluoroacetate poisoning.

Still another interesting correlation has been found in the present study, namely between insensitivity to fluoroacetate poisoning of certain types of striated muscles and capacity of hexose diphosphate to stimulate respiration. Indeed, stimulation of the respiration by hexose diphosphate was very small in rat skeletal muscle, and maximal in rat heart homogenates. Also the data on rabbit heart homogenates are in keeping with the hypothesis that carbohydrates play a major role in maintaining a fluoroacetate-insensitive respiration. The rabbit has been reported to be much more sensitive to fluoroacetate poisoning than the rat (LD_{50} 0·5 mg./kg. body wt. in pigmented rabbits, and 5 mg./kg. body wt. in albino rats; Chenoweth, 1949). In addition death generally occurs in poisoned

rabbits as a consequence of ventricular fibrillation, which is rarely seen in rats. Homogenates of rabbit heart showed almost no difference, as compared with homogenates of rat heart, with respect to the extent of inhibition of the endogenous respiration by fluoroacetate, the stimulation of respiration on addition of glutamate and the rate of conversion of glutamate into aspartate. On the other hand, insofar as the capacity of glycolytic substrates to stimulate respiration is concerned, rabbit heart homogenates behaved similarly to gastrocnemius homogenates. Fawaz, Tutunji & Fawaz (1958) observed that the performance of the dog heart–lung preparation was unaffected in the presence of inhibiting concentrations of malonate, provided that glycolysis proceeded at normal rates. Blocking of glycolysis by higher concentrations of malonate caused rapid heart failure.

The mechanism by which glycolytic substrates stimulate respiration in fluoroacetate-poisoned rat heart and diaphragm is still unknown. Fawaz & Fawaz (1962) have suggested that the oxidation of pyruvate in the isolated dog heart may not proceed solely via the tricarboxylic acid cycle. Our results appear to indicate that the formation of pyruvate is not an essential requirement for glycolysis to stimulate respiration in rat heart and diaphragm cell-free preparations. The observations that hexose diphosphate gives maximal rates of respiration, and that the respiration supported by the hexose diphosphate is inhibited by iodoacetate but not by fluoride, suggest that the main source of hydrogen in these systems is NADH generated at the triose phosphate-dehydrogenase level. Data relative to the mechanism by which glycolytically generated NADH is oxidized by the mitochondrial respiratory chain are presented by Margreth & Azzone (1964). It can only be postulated here that the transfer of hydrogen to the mitochondria does not occur through the operation of the glycerol 1-phosphate cycle (see Margreth & Azzone, 1964).

Metabolic patterns of striated muscles. Repeated attempts have been made to find a correlation between physiological function, ultrastructure and biochemical patterns of various types of muscles. It has been shown that generation of high-energy bonds is more dependent on aerobic metabolism in continuously active red muscles, and on anaerobic glycolysis in intermittently active white muscles (Lawrie, 1953). Another reported difference between red and white muscles has been found in *Locusta migratoria* (Vogell *et al.* 1959), where the 'jump' white muscles have a high lactate-dehydrogenase activity, at variance with 'flight' red muscles, which have a high glycerol 1-phosphate-dehydrogenase activity, both cytoplasmic and mitochondrial. The latter finding appears of special interest, because of the observation that the maximal rate of anaerobic and aerobic glycolysis is similar in homogenates of various striated muscles (LePage, 1950; Margreth *et al.* 1963; and this paper). It would therefore seem that the mechanism for the reoxidation of glycolytically generated NADH may represent an additional important difference between red and white muscles. In other words, intermittently active muscles reoxidize NADH mainly through pyruvate and lactate dehydrogenase, whereas continuously active muscles may reoxidize NADH also through the mitochondrial respiratory chain, either by way of a 'substrate cycle' or by other still unknown mechanisms. The data presented in this paper appear to support this difference in the mechanism of reoxidation of NADH in various types of muscles.

SUMMARY

1. The effect of the following inhibitors on the endogenous respiration of heart, diaphragm and gastrocnemius homogenates has been studied in normal and fluoroacetate-poisoned rats (20 mg. of fluoroacetate/kg. body wt.): arsenite, malonate, cysteinesulphinic acid, fluoride, iodoacetate, rotenone, antimycin A, oligomycin, dinitrophenol.

2. Fluoroacetate poisoning results in a strong decrease of the endogenous respiration in gastrocnemius homogenates. Respiration is less inhibited in rat diaphragm and only slightly inhibited in rat heart.

3. The endogenous respiration of heart, diaphragm and gastrocnemius homogenates from fluoroacetate-poisoned rats is largely coupled to phosphorylation, as indicated by the inhibition induced by oligomycin.

4. A large part of the endogenous respiration of heart, diaphragm and gastrocnemius homogenates from poisoned rats is dependent on the oxidation of glutamate, as indicated by the inhibition of the oxygen uptake by arsenite and malonate, and by the removal of malonate inhibition on addition of malate and oxaloacetate. Addition of glutamate stimulates the respiration in fluoroacetate-poisoned rat heart, diaphragm and gastrocnemius homogenates.

5. Glycogen, glucose 6-phosphate and, more markedly, hexose 1,6-diphosphate, stimulate the oxygen uptake of heart and diaphragm homogenates from fluoroacetate-poisoned rats. Almost insignificant stimulation is observed in gastrocnemius homogenates. The above stimulation is removed by iodoacetate, whereas it is unaffected by fluoride.

6. Oxygen uptake of fluoroacetate-poisoned rabbit heart homogenates is stimulated by the addition of glutamate, but only slightly by hexose 1,6-diphosphate.

7. The above differences between different muscle homogenates in utilizing glutamate and in oxidizing glycolytic substrates have been related to the different responsiveness of the intact muscles to fluoroacetate poisoning.

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Oxidation of Reduced Nicotinamide-Adenine Dinucleotide in Muscle Homogenates

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In the preceding paper (Margreth & Azzone, 1964) we suggested a correlation in some types of muscles between pharmacological insensitivity to fluoroacetate poisoning and the capacity of glycolytic substrates to support respiration. Hexose 1,6-diphosphate was found to stimulate considerably the oxygen uptake of rat heart and diaphragm homogenates, but not of rat gastrocnemius and rabbit heart homogenates. The hexose diphosphate-dependent respiration was inhibited by iodoacetate, and was insensitive to fluoride. These findings suggested that aerobic glycolysis stimulated the respiration through the generation of NADH at the triose phosphate-dehydrogenase level, and not by providing pyruvate. Oxygen uptake during aerobic glycolysis of hexose 1,6-diphosphate in the presence of fluoride has earlier been observed by Reif, Potter & LePage (1953) and was suggested to be due to the oxidation of NADH. The data in the present paper substantiate this conclusion. Evidence will also be given that glycolytically generated, external NADH is oxidized by the mito-