MONOFLUOROACETIC ACID (COMPOUND 1080), ITS PHARMACOLOGY AND TOXICOLOGY

ERNEST KUN, Department of Pharmacology, The University of California, San Francisco, San Francisco, California 94143

ABSTRACT: The molecular mechanism of toxic action of fluoroacetate is analyzed in the perspective of scientific developments of the past 30 years. Stereospecific enzymatic conversion of fluoroacetate via fluoroacetyl-CoA + oxalacetate to (-)-erythrofluorocitrate in mitochondria is the metabolic pathway that converts the nontoxic fluoroacetate to the toxic intracellular effector molecule. The mode of toxic effect of (-)-erythrofluorocitrate cannot be equated with its reversible inhibitory effect on a mitochondrial enzyme (aconitase) as had been originally thought by Peters (1963) and is still propagated in textbooks. Instead, the chemical modification of inner mitochondrial membrane proteins by (-)-erythrofluorocitrate, comprising a novel, as yet incompletely understood biochemical mechanism, is the molecular basis of toxicity. Research in this new area may eventually explain selective (species-dependent) toxicity, the development of resistance to this poison and can lead to the scientific basis of the development of antidotes.

INTRODUCTION

The discovery of detailed mechanism of action of highly toxic agents inevitably reveals new areas of cellular physiology, as first pointed out by Claude Bernard (1). It is evident that if an agent in minute quantities is capable of terminating life processes, the site of action of the toxic substance has to represent a vitally important biological system. Despite the plausibility of this axiom, only relatively few poisons have been studied in sufficient depth to allow the precise identification of their critical target systems, and their proposed mechanisms of action are frequently borrowed from concepts of a branch of biochemistry that happens to be fashionable at that time. As in all areas of science, especially in biology, the scientific explanations of cellular physiology or, more correctly stated, the scientific hypotheses that should predict (2) the operation of complex cellular processes, reflect the developmental history of science. This is especially true of pharmacology that critically depends for mechanistic interpretations on the developmental stage of both biochemistry and molecular biology. At the time Sir Rudolph Peters (1963) in the early 1950s recognized that fluoroacetate requires metabolic conversion to the fluorotricarboxylic acid, fluorocitrate (cf. 3) biochemistry was preoccupied with "metabolic pathways." This area should be defined as enzymatic interconversion of small molecules. Explanation of cellular physiology by the great complexity of metabolic pathways was the promise of "metabolic biochemistry", which despite important advances in enzymology and intermediary metabolism could not live up to expectations. It is now clear that without considering the metabolism of macromolecules and of heterogenous, notably membrane systems, no understanding of cellular physiology is possible, and metabolic pathways merely represent a first approximation similar to a wiring diagram, which by itself can be misleading. In the following section I wish to examine the scientific evidence that leads to the still popular metabolic explanation of fluoroacetate poisoning and then proceed to new developments.

METABOLIC ACTIVATION

The metabolic hypothesis of fluoroacetate poisoning.

The main event that survived the test of time is the metabolic activation of fluoroacetate as proposed by Peters (cf. 3). However, details of this process were unknown and required further chemical and enzymological studies. The conversion of fluoroacetate (1080) to fluoroacetyl-CoA is presumed to take place in much the same way as acetate is converted to AcCoA, except the rate of the fluoro analog formation is probably much slower than acetate conversion to AcCoA, similar to results observed in all enzymatic reactions of monofluoro-carboxylic acids (4, 5). The exact rate of F-acetyl-CoA formation is still unknown. The next step, the enzymatic condensation of F-acetyl-CoA with oxalacetate has been extensively studied in our laboratory (6, 7) and results are shown in Table 1.

Table 1. Summary of kinetic properties of citrate synthase from pig heart (cf. 4).

Constant	Substrate or inhibitor	Kinetic constant
K_m	Acetyl-CoA	25 μM
K_m	Fluoroacetyl-CoA	23 μM
K_i	Fluoroacetyl-CoA	2.2 μM
V_{max}	Acetyl-CoA	2.77 (μmoles DPNH/mg/min)
V_{max}	Fluoroacetyl-CoA	0.00845 (μmoles DPNH/mg/min)

It is evident that the affinities of F-AcCoA and AcCoA toward the condensing enzyme are the same, but the V_{max} of the condensation reaction is only 1/350th with F-AcCoA as compared to AcCoA. This means

1982. Proceedings Tenth Vertebrate Pest Conference (R.E. Marsh, Ed.), Univ. of California, Davis, Calif.

that fluorocitrate formation proceeds at 1/350th the rate of citrate synthesis under comparable conditions and effectively F-acetyl-CoA is a potent competitive inhibitor of citrate synthesis in an enzymatic system. It can be predicted that large doses of acetate would be moderately effective in counteracting the formation of the toxic fluorocitrate, by competing for F-AcCoA formation and therefore acetate may diminish F-acetate toxicity. This was shown to be the case using glycerol-monoacetate that was somewhat protective in monkeys against F-acetate poisoning (8). However, only large doses are effective as would be anticipated from Table 1 and this agent only slows down fluorocitrate formation but does not alter fluorocitrate toxicity.

The consecutive and most important product is formed from F-acetyl-CoA and oxalacetate and this substance was identified in our laboratory by enzymatic (6, 7) and chemical (9) synthesis and its precise sterochemistry established by NMR (6, 7) and X-ray diffraction (10, 11) to be (-)-erythro-fluorocitric acid or expressed in more precise stereochemical nomenclature as 1R-2R-1-fluoro-2-hydroxy-1, 2, 3-propanetricarboxylic acid. (Fig. 1) or 2R, 3R (-)-erythrofluorocitric acid, as shown in Fig. 1.

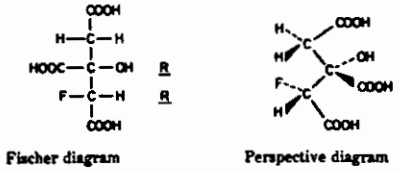

Fig. 1. This is the isomer described by Dummel and Kun (1969, J. Biol. Chem. 244, 2966) and defined as (-)-erythro-fluorocitrate by them. We report here structural studies of the mirror image (enantiomer) of this isomer. Crystals of the complex of the diethyl ester of (+)-erythro-fluorocitrate with (-)-methylbenzylamine were studied by X-ray diffraction techniques. Since the absolute configuration of (-)-methylbenzylamine has already been determined experimentally, the absolute configuration of the (+)-erythro isomer of fluorocitrate is thereby established. This isomer is shown to be noninhibitory with the enzyme aconitase, while its racemate is a powerful inhibitor. Thus it is proved that the absolute configuration of the isomer of fluorocitrate that is formed from fluoroacetyl-CoA by the enzyme citrate synthase, and that inhibits aconitase, is the 2R, 3R, isomer (cf. 11).

The metabolic conversion of F-acetate to the toxic factor (-)-erythrofluorocitrate is clearly a slow process that can be modified by the preexistence of competing physiological metabolites and this explains the slow onset of toxic symptoms after ingestion of F-acetate. The nature of the molecular toxicology of (-)-erythrofluorocitrate has been proposed by the metabolic hypothesis to be an inhibitory effect on the mitochondrial enzyme aconitase that maintains an equilibrium between citrate and isocitrate. My concern about the correctness of this hypothesis is based on results of <u>enzyme kinetics</u>.

Enzyme kinetics shows that with highly purified aconitase enzymes the inhibition is competitive and reversible (Fig. 2).

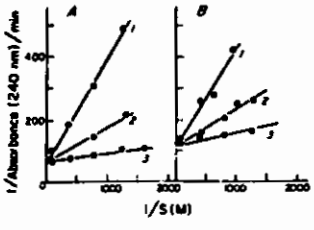

Fig. 2. Competitive inhibition of cytoplasmic (A) and mitochondrial (B) aconitase of pig liver by (-)-erythrofluorocitrate. Rates of cis-aconitate formation from citrate were measured at 240 nm (10) in 0.15 M Tris-HCl, pH 7.5, at 25°. In A, 1.5µg (protein) of cytoplasmic aconitase, and in B, 32 µg of mitochondrial aconitase, were used per test system (5-cm light path; 3-ml volume) at varied concentrations of citrate (abscissa). Curve 1, 500 µM fluoro-citrate; 2, 100 µM fluorocitrate; 3, no fluorocitrate (cf. 12).

This information predicts that if citrate accumulates, as is the case in F-acetate poisoning, the inhibition by fluorocitrate should disappear; therefore, the metabolic hypothesis of F-acetate poisoning, based on <u>reversible</u> kinetics, cannot be correct because despite large citrate accumulation in cells, F-acetate poisoning is not alleviated.

The metabolic theory has been tested in our laboratory with a number of other F-substituted carboxylic acid analogs of the citric acid cycle (cf. 5) which proved to be selective reversible competitive inhibitors of mitochondrial as well as cytoplasmic enzymes. The inhibitory effect of some F-carboxylic acids (e.g. monofluoro-oxalacetate on malate dehydrogenase) is much larger than the inhibitory effect of (-)-erythrofluorocitric acid on aconitase, yet F-carboxylic acids that cannot be effectively converted to (-)-erythrofluorocitric acid are relatively nontoxic. Properties of F-carboxylic acids are summarized in Table 2.

Table 2. Substrate and inhibitory properties of F-carboxylic acids (cf. 5).

No.	Enzyme	F-carboxylic acid	K_m	K_i	$\frac{Ma(S)}{Ma(F-S)}$
1	Kidney MDH(mito)	β-F-oxalacetic	0.5 μM	0.5 μM	101
2	Kidney MDH(mito)	ββ'-F_2-oxalacetic	4.0 mM	--	1.0
3	Liver GOT(mito)	ββ'-F_2-oxalacetic	--	45. μM	--
4	Liver GOT(mito)	βF-oxalacetic	transaminative defluorination		
5	Liver GDH	βF-glutaric	--	330 μM	--
6	Liver GDH	α-F-glutamate($NADP^+$)	1800 μM	710 μM	13.0
7	Liver GDH	α-F-glutamate(NAD^+)	640 μM	330 μM	15.0
8	Liver malic enzyme (decarboxylating)	ββ-difluoromalate	--	300 μM	--
9	Kidney MDH	(-)-erythrofluoromalate	--	13 μM	--
10	Muscle LDH	L(+)β-fluorolactate	--	300 μM	--

LEGEND: MDH = malate dehydrogenase; GOT = glutamate oxalacetate aminotransferase; GDH = glutamate dehydrogenase; LDH = lactate dehydrogenase; Ma = molecular activity; (S) = with physiological substrate; (F-S) = with fluoro-analogue.

If the enzyme inhibitory mechanism of F-acetate poisoning based on reversible competitive inhibition were correct, then any of the F-carboxylic acid analogs of the citric acid cycle that competitively inhibited a citric acid cycle enzyme should be a highly potent poison, which was not the case.

These results therefore oppose the metabolic hypothesis of F-acetate toxicity that is based on a reversible competitive enzyme kinetics.

The metabolic inhibitory hypothesis of F-citrate toxicity is also incompatible with results shown in Table 3. Depending on the cellular origin of mitochondria, addition of F-citrate not only does not inhibit mitochondrial O_2 uptake, but with glutamate and liver mitochondria, respiration is augmented by fluorocitrate (13). Concentrations of F-citrate employed were sufficient to inhibit aconitase, yet a significant increase in O_2 uptake and obviously ATP synthesis was maintained by glutamate despite aconitase inhibition. It follows that in vivo availability of ubiquitous glutamate as mitochondrial substrate results in a bypass of the inhibition of aconitase-dependent mitochondrial energy production, as tested by conventional metabolic assays (Table 3).

Table 3. The effect of synthetic Monofluorocitrate (0.6 mM) on respiration and citrate accumulation of mitochondria prepared from various tissues[a] (cf. 13).

Mito-chon-dria	Experimental conditions	Glutamate			Oxalacetate			Pyuvate			Malate			Malate + Pyruvate		
		O_2'	O_2"	C	O_2'	O_2"	C	O_2'	O_2"	C	O_2'	O_2"	C	O_2'	O_2"	C
Liver	Endogenous	120														
	+substrate	480	252	0	0[b]	117	0	60	132	130	0	185	126	480	266	86
	+substrate + F-citrate	270	371	79	0	20	139	60	186	130	0	185	170	300	283	200
	change (%)	-43	+32	Large	0	-83	Large	0	+40	0	0	0	+32	-30	0	+130
Kidney	Endogenous	270														
	+substrate	350	620	5	565	280	40	0	60	0	790	560	50	1740	910	200
	+substrate + F-citrate	75	365	50	0	0	200	0	10	0	365	370	250	1740	775	410
	change (%)	-80	-41	1000	-100	-100	+500	0	-80	0	-53	-34	+500	0	-15	+100
Brain	Endogenous	80														
	+substrate	120	450	0	80	116	9	130	162	0	160	150	10	500	540	110
	+substrate + F-citrate	35	240	26	0	69	62	50	99	0	60	150	80	500	400	325
	change (%)	-71	-46	Large	-100	-30	+700	-60	-40	0	-62	0	+800	0	-26	+200
Heart	Endogenous	700														
	+substrate	1300	1033	0	1300	122	0	100	81	0	1100	558	0	2300	1413	40
	+substrate + F-citrate	1300	1082	0	1300	180	75	100	34	0	600	285	60	2300	963	160
	change (%)	0	0	0	0	+49	Large	0	-58	0	-54	-50	Large	0	-30	+400

[a]All rates are expressed as μmoles/g protein/30 min; all contents as moles/g protein. O_2', polarographic O_2 uptake calculated from initial rates; O_2", Warburg respirometer O_2 uptake measured over 30-minutes duration; C, citrate formed in Warburg vessels. Substrates were present at a final concentration of 5 mM, and ADP at 2.3 mM. Fluorocitrate (0.6 mM) was added where indicated.

[b]No increase over endogenous.

The main lesson deduced from these data is that reversible competitive inhibition of aconitase or any other citric acid cycle enzyme by a F-carboxylic acid does not inhibit cellular respiration and energy production as, for example, is the case if one inhibits mitochondrial cytochrome oxidase by cyanide.

Reactions of (-)-erythrofluorocitric acid with mitochondrial proteins.

We have known ever since 1963 (7) that (-)-erythrofluorocitric acid exerts an inhibitory effect on aconitase activity of crude mitochondrial extracts that is not explained by a linearly competitive reversible inhibition, as illustrated in Fig. 2. Much smaller concentrations of (-)-erythrofluorocitric acid than would be predictable from K_i values derived from initial velocity analysis cause a complete inactivation of this enzyme, if sufficient time is allowed for this process of inactivation to proceed as illustrated in Fig. 3. The effect under these conditions is irreversible and is reminiscent of kinetics of enzyme inactivation by active site-oriented irreversible inhibitors.

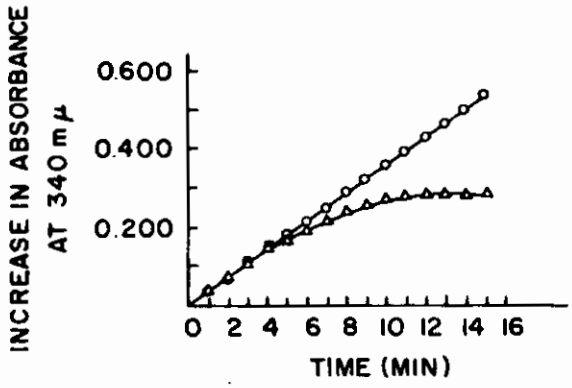

Fig. 3. Lower curve: rate in the presence of 1 μM F-ci (cf. 4).

One possibility appeared to be an enzymatic conversion of (-)-erythrofluorocitrate to a reactive species, such as F-aconitate, that could alkylate thiol groups of the enzyme. However, it was shown (14) that purified aconitase catalyzes the defluorination of (-)-erythrofluorocitric acid in the presence of cysteine and Fe 2^+ and converts it to hydroxy-citrate, which is only a reversible inhibitor of aconitase and does not bind to aconitase protein. Subsequent work in our laboratory with [^{14}C] labeled (-)-erythrofluorocitrate of high specific activity opened up a new territory of investigation (16). It was shown that in crude extracts of mitochondria (-)-erythrofluorocitric acid was bound to macromolecules (Fig. 4).

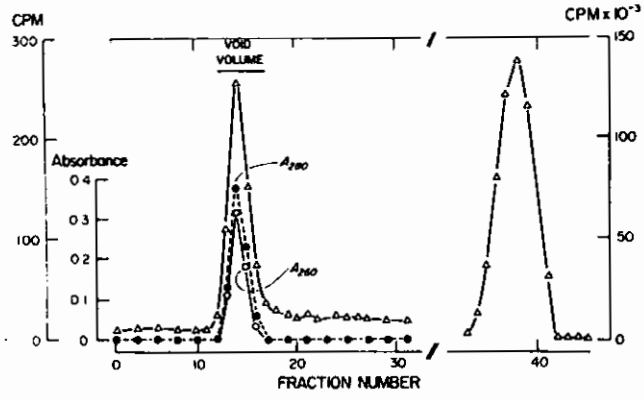

Fig. 4. Inner mitochondrial membrane vesicles were incubated with ^{14}C-(-)-erythrofluorocitrate and after dissolution in guanidine HCl passed through a Sephadex G-200 molecular filter column. Unreacted F-citrate separates (peak at right) from protein bound F-citrate (cf. 15).

Furthermore, evidence was obtained that at least three molecular species of proteins served as ligands for (-)-erythrofluorocitrate, as illustrated in Fig. 5.

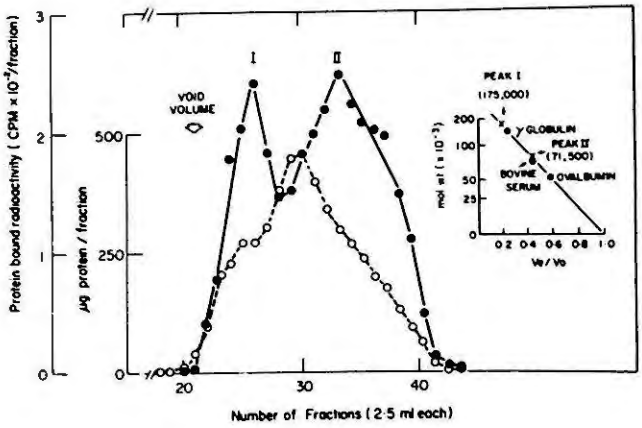

Fig. 5. G-200 Sephadex chromatography, I, II and inflection on II indicate 3 molecular species of F-ci binding proteins (cf. 15).

The chemical bond between (-)-erythrofluorocitric acid and protein resisted acid treatment but was hydroxylamine sensitive, yielding a fluorocitrate derivative tentatively identified as a hydroxamate of (-)-erythrofluorocitric acid (Fig. 6).

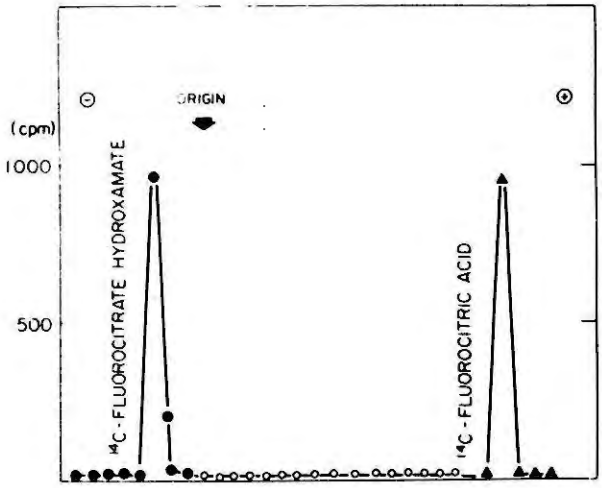

Fig. 6. Separation of (-)-erythrofluorocitrate from its hydroxamate derivative by paper electrophoresis (cf. 15).

Synthesis of authentic 2-hydroxamate of (-)-erythrofluorocitric acid was only recently successful (16) and the identification of the chemical bond between (-)-erythrofluorocitric acid and proteins is presently pursued.

The process of protein fluorocitrylation is illustrated in Fig. 7.

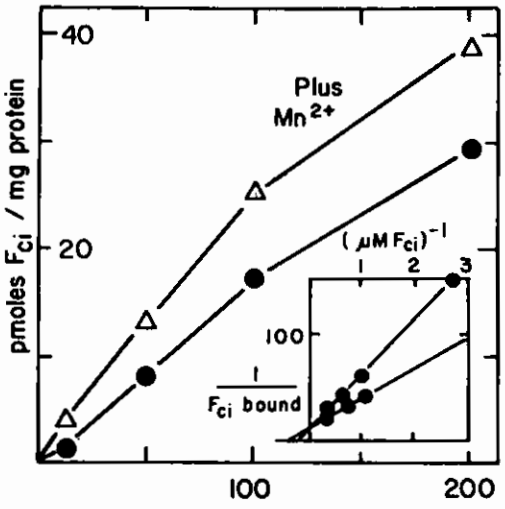

Fig. 7. Process of protein fluorocitrylation. Abscissa = μM F-citrate, Ordinate = rate of protein binding (cf. 15).

The reaction is clearly a catalytic process, but we are still uncertain about the nature of its detailed mechanism that is, I believe, the central problem of fluorocitrate toxicity. One experimentally well-documented consequence of macromolecular association of (-)-erythrofluorocitric acid with mitochondrial proteins is the irreversible inactivation of metabolism-connected citrate in and outflux in isolated mitochondria (15). There is controversy about the relevance of another type of citrate flux in mitochondria, which is termed as exchange diffusion and is measured in the absence of metabolism, with inhibitors of metabolic functions present. I believe that the metabolism-connected much slower rate of citrate transport that we are studying is the one that is inactivated by nmolar concentrations of (-)-erythrofluorocitric acid and this process is the physiologically significant transport of citrate. It can be postulated that in certain animal species, where the biosynthesis of the brain transmitter substance acetylcholine is entirely dependent on the export of intramitochondrially generated citric acid, the membrane transport site of mitochondria for citrate may be a target site that could explain the neurotoxic effect of (-)-erythrofluorocitric acid. I believe this to be an interesting but, perhaps not crucial, mechanism and our research work tends to indicate an even more complex inner mitochondrial system that, according to my present state of information, is the real target site of F-acetate poisoning on a molecular level. Briefly, we isolated a new mitochondrial protein, which is likely to be the 180 kd protein shown in Fig. 5. Characteristics of this protein are shown in Fig. 8, which is a gel electrophoresis pattern at various stages of purity.

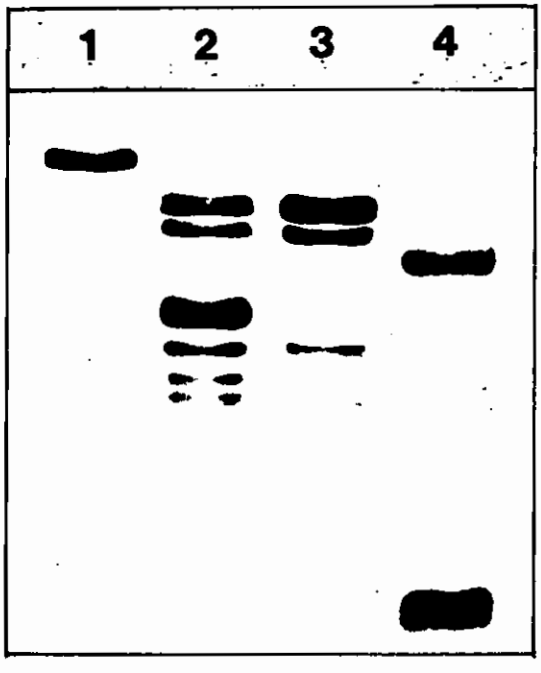

Fig. 8. Gel electrophoresis (SDS) characteristics of the new mitochondrial protein at various stages of purity. 1 = Purified aconitase, 2 = Fe-S protein after isoelectric purification, 3 = Fe-S protein after aminohexyl sepharose chromatography. Main protein = 40 kd., 4 = Standards: BSA and cytochrome C.

In the gel the subunit structure is illustrated, corresponding to a major 40 kd subunit of the tetrameric protein (160 kd), plus apparently associated further subunits that make up the sum of 180 kd mass. This protein is an iron sulfur protein which contains also covalently bound glutathione. Its physiological function is unknown except that it binds (-)-erythrofluorocitrate; and, according to present hypothesis, transfluorocitrylation to other iron sulfur proteins, like aconitase, takes place in successive enzymatic steps. Since specific iron-sulfur proteins in the mitochondrial electron transport system exist at critical stages of energy transfer (sites of oxidative phosphorylation), the possibility exists that the ultimate toxic mechanism of (-)-erythrofluorocitrate, and, of course, fluoroacetate, is related to a chemical blocking of mitochondrial energy transduction. This is our present state of ignorance related to this complex problem. It is anticipated that clarification of these questions will reveal a hitherto unsuspected macromolecular membrane apparatus, but very much needed research in these basic areas can only promise slow progress.

Probable correlations of biochemical mechanisms to animal toxicology.

The macroscopic toxicology of fluoroacetate is not very revealing, except for the well-documented latency which can vary -- as predictable from the known pathway of intramitochondrial (-)-erythro-fluorocitric acid biosynthesis. In susceptible species the effect appears to be neurotoxicity, with repeated onset of convulsions and eventual central paralysis. As in all types of convulsive seizures, loss of consciousness is a general accompaniment, thus contrary to laymen's impressions, death by fluoroacetate poisoning is not unusually troublesome. It is not known what the precise reasons for neurotoxicity are, except for the experimental fact (17) that submicrogram amounts of (-)-erythrofluoro-citric acid if injected into the third cerebral ventricle causes relatively rapid neurotoxic death. If this effect is due to inhibition of cholinergic centers in the brain, then the citrate export-dependent acetyl choline biosynthesis-related mechanism (15) may play an important role. However, very little is known about the function of cholinergic centers in the central nervous system. It has to be also assumed that in species that respond with neurotoxicity to fluoroacetate extramitochondrial, sources of AcCoA must be negligible in the target cells. In some species death is presumably due to cardiac arrest, but it is unclear whether or not this effect is due to central or peripheral mechanisms.

Questions have been asked whether or not secondary intoxication, i.e. toxicity by ingestion of F-acetate poisoned animals, is due to F-acetate or (-)-erythrofluorocitric acid. Based on calculated metabolic rates of F-carboxylic acids, as discussed earlier, the most probable substance that may cause secondary poisoning is remaining unmetabolized F-acetate itself.

An unsolved problem of F-acetate toxicology is the marked variation in susceptibility and development of tolerance depending on animal species, a matter that is not explainable by the "metabolic hypothesis" of the toxicology of F-acetate. Significant contributions to this subject were made by Australian colleagues (18-21) who showed that apparent genetic determinants can modify the development of susceptibility or resistance to F-acetate in various species and that this phenomenon is unrelated to metabolic defluorination of F-acetate (22). Again, this entire phenomenology is unexplained by the blocking of an ubiquitous metabolic pathway (3), and I predict that a better understanding of the inner mitochondrial membrane-related novel system is likely to yield clues.

In conclusion, I believe that the most rational application of F-acetate in agriculture and animal ecology should be done on the basis of selective protection of desired species by the feeding of an appropriate antidote to species that are to be preserved. This, of course, depends on much research on a basic level, which has yielded possible approaches, but under present circumstances research in this field appears to be not favorable because of apprent lack of appreciation of this problem by funding agencies.

REFERENCES

1. BERNARD, C. 1857. Leçons sur les effects des substances toxiques. Paris (Revue des Deux Mondes Sept. 1864).
2. POPPER, K. 1961. The logic of scientific discovery. Science Editions Inc. New York.
3. PETERS, R.A. 1963. Biochemical lesions and lethal synthesis. Macmillan Publ. Co. Inc., New York.
4. KUN, E. 1969. Mechanisms of action of fluoro analogues of citric acid cycle compounds: an assay in biochemical tissue specificity. In: Citric Acid Cycle Ed. J.M. Lowenstein, Marcel Dekker Publ. New York. Chapter 6, p. 297-337.
5. KUN, E. 1976. Fluorocarboxylic acids as enzymatic and metabolic probes. ACS Symposium Series 28, p. 1-22. Biochemistry involving carbon-fluorine bonds. (Ed. R. Filler)
6. FANSHIER, D.W., L.K. GOTTWALD and E. KUN. 1962. J. Biol. Chem. 237, 3588-3596.
7. FANSHIER, D.W., L.K. GOTTWALD and E. KUN. 1964. J. Biol. Chem. 239, 425-434.
8. CHENOWETH, M.B., A. KANDEL, L.B. JOHNSON, and D.R. BENNETT. 1951. J. Pharmacol. Exptl. Ther. 102, 31-49.
9. DUMMEL, R.J. and E. KUN. 1969. J. Biol. Chem. 244, 2966-2969.
10. CARRELL, H.L. and J.P. GLUSKER. 1973. Acta Crystalogr. 29, 674-682.
11. STALLINGS, W.C., C.T. MONTI, J.F. BELVEDERE, R.K. PRESTON and J.P. GLUSKER. 1980. Arch. Biochem. Biophys. 203, 64-72.
12. EANES, R.Z. and E. KUN. 1974. Mol. Pharmacol. 10, 130-139.
13. KUN, E. and P. VOLFIN. 1966. Biochem. Biophys. Res. Commun. 23, 696-701.
14. VILLAFRANCA, J.J. and E. PLATUS. 1973. Biochem. Biophys. Res. Commun. 55, 1197-1207.
15. KIRSTEN E., M.L. SHARMA and E. KUN. 1978. Molecular Pharmacol. 14, 172-184.
16. MCLICK, J., E. KIRSTEN and E. KUN. 1982. Paper in preparation.
17. MORSELLI, P.L., S. GARATTINI, F. MARCUCCI, E. MUSSINI, W. REWERSKY, L. VAZELLI and R.A. PETERS. 1968. Biochem. Pharmacol. 17, 195-202.

18. WHEELER, S.H. and D.S. HART. 1979. Austr. Wildl. Res. 6, 57-62.
19. OLIVER, A.J., D.R. KING and R.J. MEAD. 1979. Austr. J. Zool. 27, 363-372.
20. KING, D.R., A.J. OLIVER and R.J. MEAD. 1978. Austr. J. Zool. 26, 699-712.
21. OLIVER, A.J., D.R. KING and R.J. MEAD. 1977. Search, Vol. 8, No. 4, 130-132.
22. MEAD, R.J., A.J. OLIVER and D.R. KING. 1979. Austr. J. Biol. Sci. 32, 15-26.