FLUORINE CHEMISTRY

Edited by

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VOLUME III

Biological Effects of Organic Fluorides

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1963

ACADEMIC PRESS · NEW YORK and LONDON

BIOLOGICAL EFFECTS
OF
ORGANIC FLUORIDES

THE TOXICITIES OF ELEMENTAL FLUORINE and the inorganic fluoride ion have long been known and studied in detail; Roholm, for example, listed almost 3500 references in his classical review published in 1935. Systematic toxicological investigations of fluorine-containing *organic* compounds, however, are almost nonexistent. This situation is due in large part to the newness of these materials as a class; relatively little work was published prior to World War II in the field of organic fluorine chemistry. With few exceptions, most of the toxicity data so far published have been obtained in the course of studies in which fluorine analogs were included only incidentally as members of a series of compounds investigated.

The toxic effects of the organic fluorine compounds are unlike those of inorganic fluorides; in general, the effects are not consequent on the liberation of the fluoride ion. With two or three notable exceptions, the mechanisms of action of these compounds are unknown. Very few data are available describing the metabolic fate of these materials.

The most important organic fluorine compounds from a physiological viewpoint are the fluoroacetates, the fluorophosphates, the freons and other fluorocarbons, and the fluorine-containing steroids.

Fluoroacetate

ACUTE HUMAN POISONING

The toxicity of fluoroacetate constitutes a hazard to any community in which this rodenticide is used. In March of 1951, fluoroacetate made the headlines—"Tiny Poison Cups Peril Community." The newspaper described a "purple, jellylike substance" distributed in little paper cups like those used in restaurants for mayonnaise. Some of the cups were lost and a search for them was set off by the death of two dogs, a cat, and a number of birds. Parents were warned that "poison could kill a child who touches it and then puts his finger in his mouth" (*Boston Post*, Monday, March 12, 1951). Providentially, no children were killed. To avoid undue hazard to the public, fluoroacetate must be used only by qualified rodent control experts.

In Table I (on p. 55) are listed the first records of human deaths. Nonfatal poisonings are indicated in Table II (p. 56).

As shown in these tables, there have been at least twenty-three reported instances of poisoning by sodium fluoroacetate since this compound was

introduced as a rodenticide in 1945.[1] Of these, sixteen or two-thirds terminated fatally.

Fluoroacetate (FAc) received widespread attention as one of the "supertoxics" (50 μg/kg is fatal for dogs) and later as a research tool to explore one of the basic metabolic pathways furnishing cellular energy. FAc kills either by central nervous action (convulsions in some species, depression in others) or by cardiac failure (ventricular fibrillation in some species). Characteristically there is a "long and essentially irreducible latent period" (Chenoweth, 1949) because FAc itself is relatively inactive biologically. The story of how the body turns FAc into a highly lethal substance is an exciting chapter in recent biochemistry.

Polish chemists (Gryszkiewicz-Trowkiemowski *et al.*, 1947) brought to England during World War II the news of the toxicity of fluoroacetate. To our knowledge, fluoroacetate was never used as a chemical warfare agent; however, work begun during the war by Saunders and colleagues in England (McCombie and Saunders, 1946a) and by Chenoweth and Gilman (1944) at the Army Chemical Center in the United States ultimately led to the introduction of sodium fluoroacetate as a rodenticide and pesticide by Treichler and Ward of the U.S. Fish and Wildlife Service in 1945.

Species Variations

The canny rat, always difficult to poison, apparently cannot taste or smell FAc and will accept food containing lethal amounts. FAc, however, has important drawbacks as a rodenticide; the most striking is the dangerous toxicity for other species (Table III, p. 56). The dog, for example, is killed by 1/100 of the lethal dose for the rat. In carefully staged tests as a rodenticide, the toll of death among other species has been impressive; e.g., in one test an undisclosed number of rats were killed and at the same time a calf, three dogs, four cats, six chickens, and twenty-five wild birds were found dead from the poison. All species are not equally susceptible; the South African clawed toad requires a gram and a half per kilogram to be lethal. Man is fairly resistant. Lord Adrian found in himself that the lethal dose for dogs could be tolerated; a lethal range of 2–5 mg/kg is cited by Chenoweth. The extraordinary range in toxicities (Tables IV* and V*) presumably is related to the metabolic differences between species and is at present by no means accounted for in detail. Additional references are as follows: Nichols *et al.* (1949), Sayama and Brunetti (1952), Schnautz (1949), and Tourtellotte and Coon (1950).

[1] Mr. J. C. Ward, U.S. Department of Agriculture, has assisted in compiling these tabulations; his cooperation is acknowledged with thanks.

* Table IV is on p. 57; Table V, on p. 58.

Occurrence in Nature

One economic aspect of FAc toxicity has been identified only a decade ago. Large areas of the Transvaal must be fenced off to prevent cattle and sheep from grazing on a native plant, *Dichapetalum cymosum*, known locally as Gifblaar which especially in the spring and autumn has been the cause of death of many head of stock. The histories of such deaths indicate that some time (hours) after eating even as little as a double handful of the leaves of the plant animals convulse or become ill, and die. Marais found the active principle to be FAc (Marais, 1944).

A closely related species, *Dichapetalum toxicarium*, or ratsbane, has been notoriously associated with the practices of certain witch doctors who can produce "broke-back" in their victims. When the leaves and fruit of the plant were ingested, a pattern of poisoning followed: first vomiting and diarrhea, followed by trembling and then by a paralysis, beginning with the lower extremities, that sometimes progressed fatally. Peters (1955) *et al.* found, surprisingly, that FAc is not the toxic principle although FAc could be recovered after treating the fruit with pancreatin. Apparently some fluorofatty acid is the main toxic component, perhaps a C_{16} fluoroacid (Peters *et al.*, 1959). Fluorofatty acids produce toxic changes that resemble in many ways those of FAc but differ characteristically (for example, there are contrasts in the relative citrate concentrations in the organs of poisoned animals).

Chemical Factors

Chemically FAc is surprisingly stable, even withstanding hot concentrated sulfuric acid. First synthesized by Swarts in Ghent in 1896, the potentialities were recognized only in World War II rodenticide research. The strong binding of F to C in this molecule is also indicated by the toxicities of fluoroacetate congeners (see Table VI*). Only compounds in which FAc is present or can easily be produced by simple ionization or hydrolysis exhibit the excessive toxicity of FAc. Another indication of the tight binding is found in the toxicities of fatty acids having an odd number of carbon atoms. Acids containing an even number of carbon atoms, assumed to be broken ultimately to FAc, show toxicities as great or greater than FAc; odd-numbered acids were not toxic or only slightly toxic. Structure-toxicity relationships have been discussed at length by Saunders (Saunders, 1957), Pattison *et al.* (1956 a, b, c, d, e, f, g, 1957 a, b; Pattison, 1953, 1954; Pattison and Fraser, 1955; Pattison and Howell, 1956 a, b; Pattison and Norman, 1957; Pattison and Saunders, 1949; Pattison and Woolford, 1957 a, b), and by Parker and Walker (1957).

* Table VI is on p. 58.

The increase of toxicity with chain length is an important point and will be mentioned later.

FAc is absorbed with nearly equal effect by any route of administration except dermal. The skin is a fairly efficient barrier but not to the extent that careless handling can be countenanced. Distribution is reasonably uniform in the tissues examined (Hagan *et al.*, 1950); the liver is notably low in FAc content (*vide infra*); however, recoveries of 40–70% were reported 24 hours after administration. FAc evidently is metabolized (as is discussed below in detail). Tolerance of a treatment can be demonstrated in some but not all species.

Cause of Death

For reasons not now clear, FAc poisoning presents a puzzling variability from species to species in the symptom complex. Profound convulsions or ventricular fibrillation, or in some species both, are the striking actions. "Death may result from (*a*) respiratory arrest following severe convulsions, (*b*) gradual cardiac failure or ventricular fibrillation, or (*c*) progressive depression of the central nervous system with either respiratory or cardiac failure as the terminal event" (Chenoweth, 1949).

1. *Central Nervous System: Prototype Species—Dog*

After a lag period that may be hours long, agitation, hyperactivity, and tonic and clonic convulsions appear. Respiratory paralysis follows repeated and protracted convulsions; death is never primarily cardiac in origin.

2. *Cardiac: Prototype Species—Rabbit*

After a lag period of minutes, a seizure sometimes precedes relaxation, gasping, and death. By electrocardiography, convulsive bouts can always be shown to follow cardiac syncope (Chenoweth and Gilman, 1946 a).

3. *Mixed Reponse: Man: Prototype Species—Rhesus Monkey*

The lag period is noted. "The first indication of poisoning in man is the onset of epileptiform convulsions after an initial period of nausea and mental apprehension" (Foss, 1948). ". . . in man . . . repeated and severe fits indistinguishable from status epilepticus are observed" (Foss, 1948). Monkeys are apparently unconscious during the period of convulsions but gradually regain their feet in about 30 minutes. "Generally, the animal becomes weaker over the period of the next few hours (see cardiac status), but is often standing or otherwise exerting himself when suddenly stricken by ventricular fibrillation and death" (Chenoweth, 1949).

4. *Depression: Prototype Species—Rat*

Tonic convulsions are usually observed after a 1- or 2-hour lag period. Death, however, comes in a gradually instituted respiratory depression occurring much later (5–24 hours in some strains). Survivors exhibit an intention tremor and bradycardia.

Pharmacological Properties

Chenoweth's review presents a detailed survey of the literature. Only a few high points will be mentioned here.

The electrical activity of the brain develops a spike and dome pattern such as is often seen in clinical *petit mal* epilepsy. The intimate mechanism of the origin of the convulsions is not known; calcium depletion by citrate complexing is not the sole factor (Peters, 1955). Intracerebral injection of synthetic fluorocitrate precipitated convulsions after a short delay (Peters, 1955) in a pigeon even by doses as small as 11 μg. FAc in much greater amounts did nothing. The bulk of evidence supports the hypothesis that citric acid accumulation is not the direct cause of convulsions or of death (Kandel and Chenoweth, 1952 a; Kandel *et al.*, 1951; Potter *et al.*, 1951; Hendershot and Chenoweth, 1954; Awapara, 1952 b; Peters, 1952; Fawaz and Fawaz, 1953).

Arrhythmias of the heart indicate a disorganization of the excitation and conduction mechanisms. Pulsus alternans has been reported in at least one instance of fatal human poisoning. "Ventricular fibrillation . . . appears to be initiated by mechanisms similar to those . . . for electrically induced ventricular fibrillation" (Chenoweth, 1949). Loss of contractile power precedes failure.

Additional references are as follows: Allsopp and Fell (1950), Aronson (1952), Bennett and Chenoweth (1951), Busch *et al.* (1958), Chenoweth and Pengstritong (1950), and Pardo (1951).

Pathological changes are minimal and presumably secondary to cardiac failure.

Biochemical changes have been reported in the blood and tissue levels of various constituents, e.g., in serum inorganic phosphate, in plasma potassium, in calcium, in blood glucose, in lactic acid, and in pyruvic acid; these do not account for the toxic effects. Phosphate esters in the brains of poisoned rats are not significantly altered (Dawson and Peters, 1955). The critical biochemical lesions will be discussed in detail below.

Antidotes available are not very powerful. Acetate in general does not influence favorably the course of poisoning *in vivo*. The control of the convulsions by barbiturates does not prevent the eventual lethal outcome. Monoacetin is a "practical antagonist" (Chenoweth *et al.*, 1951).

A tentative dose schedule recommended for man is "—small doses, 0.1–.05 ml/kg (6–30 ml for a 60-kg man) given at least hourly for several hours." Contraindicated are (*a*) injections of calcium, potassium, sodium chloride, bicarbonate, acetate, (*b*) digitalis. Antifibrillatory drugs are of no use. "It does not now appear warranted to use other measures, beyond suitable nursing care and gastric lavage."

Oral administration of 4 ml/kg of an equal mixture of 50% ethyl alcohol (whiskey) and 5% acetic acid (vinegar), or either one alone if both are not available, to be repeated in 3–4 hours has been suggested if monoacetin cannot be obtained (Hutchens *et al.*, 1949).

The symptoms and treatment of fluoroacetate poisoning have recently been reviewed (Gleason *et al.*, 1957). A list of symptoms is given for human poisoning; these include vomiting, apprehension, facial twitching, central nervous excitation, epileptiform convulsions, and a dangerous and fatal ventricular fibrillation. Treatment procedures are suggested stressing (*a*) the removal of any unabsorbed material, (*b*) the administration of monoacetin in an effort to antagonize the fluoroacetate ion, (*c*) the control of convulsions by barbiturates, and (*d*) supportive therapy.

Clinical experience in the treatment of one nonfatal and two fatal cases of sodium fluoroacetate is also described by Gajdusek and Luther (1950), by Harrisson *et al.* (1952 b), and by Brockman *et al.* (1955). Harrisson *et al.* (1952 b) determined the fluoride content of a number of organs removed from their case at autopsy. The milligrams of compound per organ in liver, brain, and kidney were 100, 109, and 22 (both kidneys), respectively. A total of 465 mg of compound was recovered in the organs, urine, and stomach contents analyzed. It is estimated by Harrisson *et al.* that the victim must have ingested at least 6 mg of compound per kg of body weight. Brockmann *et al.* (1955) were able to show (qualitatively) the presence of fluoride only in the bile.

Additional references are as follows: Bacq *et al.* (1957, 1958), Fairhall (1952), Johnson and Chenoweth (1950), and Saunders (1953).

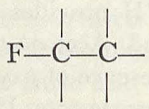
Essential Biochemical Lesions

Fluoroacetate poisoning has provoked an exciting series of biochemical researches leading to the discovery of (*a*) a "lethal synthesis," (*b*) the nature of the real poison, and (*c*) the metabolic site of the poisonous effect. The story is inherently so interesting and the unfolding has so much suspense that a more or less chronological plan will be followed in presenting it (cf. also Peters, 1957). Several facts emerged early:

(1) The fluorine-to-carbon bond in fluoroacetate is a stable one, chemically. Presumably this bond is not broken in the body.

(2) This delayed convulsant is a highly toxic one, in some species a "supertoxic."

(3) The toxicity resided in the



group. Characteristic toxic effects were observed in animals following the administration of molecules yielding fluoroacetate by hydrolysis or oxidation in the body.

(4) Many isolated enzyme systems including those investigated in the early research were not inhibited by fluoroacetate. The hypothesis that FAc competes directly with acetate in some metabolic process was so clearly visualized by the early investigators that special attention was directed from the beginning to the question of interference with acetate metabolism.

The first evidence of metabolic interference came from Bartlett and Barron's study (1947) of *in vitro* systems utilizing pyruvate and acetate. Typical data are presented in Table VII.* When kidney slices were allowed to respire in the presence of oxygen in a medium containing 0.02 M pyruvate with or without 0.01 M fluoroacetate, *pyruvate utilization* was reduced by fluoroacetate to 52% of the control value. Oxygen utilization was reduced correspondingly by 60%. In the second experiment (column 3) in the presence of 0.014 M acetate, kidney slices respiring in the presence of oxygen showed a 62% inhibition of *acetate oxidation* following the addition of 0.005 M fluoroacetate. [Compare the reduction in metabolism of C^{14}-2-acetate in a rat given FAc; exhaled $C^{14}O_2$ serves as the criterion (Gal *et al.*, 1954).] The reduction in oxygen uptake and the equivalent reduction in the oxidation of acetate fits the idea that fluoroacetate is competing directly by blocking acetate utilization. The marked reduction in pyruvate utilization was explained on the grounds that if pyruvate is necessarily transformed to acetate, a block in acetate oxidation would be reflected as a block in pyruvate utilization. Bartlett and Barron concluded that "fluoroacetate probably acts by inhibiting the formation of 'active' acetate (the so-called C_2 compound) which may be an acetyl derivative or an acetate radical." The important contribution of this first paper was the clear indication of the existence of a toxic metabolic lesion. The singling out of the transacetylases (as they are now called) was, as will be shown, a blind alley.

* Table VII is on p. 67.

Bartlett and Barron's experiments with other enzymes led them to reject the possibility that the tricarboxylic acid cycle was involved because they could find no effect of fluoroacetate on oxidative steps involving isocitric, alpha-ketoglutaric, succinic, and malic acids. Nevertheless, their data as illustrated in Table VII provides evidence that the tricarboxylic acid cycle was indeed involved. Acetoacetate formation from acetate by liver slices respiring in the presence of oxygen (column 4) was more than doubled in the presence of fluoroacetate. If fluoroacetate acted by inhibiting the formation of active acetate, why should there have been an increase in acetoacetate formation? Some later step must also have been inhibited. Coupled with a decrease in citrate formation from added acetate, the inhibition of acetate oxidation by respiring yeast and bacterial suspensions was held by Kalnitsky and Barron (1947) to be additional evidence of a fluoroacetate blockade of the acetate activating enzyme. Kalnitsky and Barron presented what we believe to be the first instance *in vitro* of a delay in the development of the fluoroacetate effect. Yeast cells oxidized alcohol at a normal rate in the presence of fluoroacetate (Fig. 1) for over 30 minutes before a retardation in oxidation became apparent.

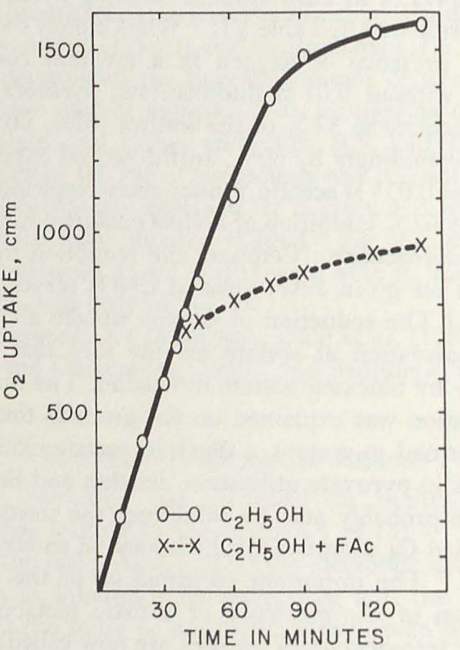


FIG. 1. Effect of FAc (0.01 *M*) on the oxidation of ethyl alcohol by bakers' yeast (Kalnitsky and Barron, 1947).

Citrate Concentration Increase

Within a few months attention was directed to citrate metabolism; the first indication that fluoroacetate increases the formation of citrate was furnished by Kalnitsky in 1948 (Table VIII*). He attributed the increase in citrate concentration to an increased formation from oxalacetate, although he recognized that some inhibition of citrate removal was involved. The marked increase in citric acid formation from oxalacetate in the kidney homogenate was confirmed by Kalnitsky and Barron (1948) under conditions in which there was a complete suppression of acetate utilization. Fluoroacetate inhibited the oxidation of citric acid but only after a delay of about an hour during which approximately 10% of the citric acid had been oxidized. The significance of this delay soon became apparent.

Independent of and nearly simultaneous with the publication of Kalnitsky and Barron, Liebecq and Peters announced in 1948, and a year later described in full, their work establishing the marked increase in citrate concentration (Liebecq and Peters, 1949). These authors also reported the decrease in oxygen utilization previously described by Bartlett and Barron but showed that no change in acetate concentration necessarily occurred, thus pointing to some action of fluoroacetate other than the direct competition with the acetate activating system. The simplest hypothesis explaining an increase in citrate concentration would involve a block in citrate metabolism, i.e., in the tricarboxylic acid cycle. In the words of Liebecq and Peters, "the cycle itself is interrupted somewhere below the citrate level."

Nature of the Inhibitor

The remarkable potency of FAc when quantitative comparisons with other enzyme poisons were made gave a clue as to the nature of the inhibition. For example, Liebecq and Peters (Fig. 2) found that as small a concentration as 0.8 m*M* FAc would produce nearly as great an increase in citrate content as a manyfold larger concentration (viz., 33 m*M*). The true inhibitor must be active in very low concentrations. If this is a metabolic antagonism, it possesses a high degree of specificity.

Impelled by a baffling contradiction, Liebecq and Peters were driven to a new assumption, one that proved to be a golden guess. The contradiction involved (*a*) on the one hand the absence of enzyme blockade by FAc *in vitro*, and (*b*) on the other hand the increase in citrate concentration that must mean a tricarboxylic acid cycle blockade. The force of this contradiction is brought home by reviewing the list (below) of more or less purified enzymes found *not* to be sensitive to FAc *in vitro*.

* Table VIII is on p. 68.

Enzyme	Investigators
Isocitrate dehydrogenase	Bartlett and Barron
Oxalosuccinic decarboxylase	Liebecq and Peters
alpha-Ketoglutarase	Bartlett and Barron
Succinoxidase	Bartlett and Barron
Fumarase	Liebecq and Peters
Malic dehydrogenase	Bartlett and Barron
Oxalacetate decarboxylase	Liebecq and Peters
Aconitase	Liebecq and Peters
Cytochrome oxidase	Bartlett and Barron
Yeast carboxylase	Bartlett and Barron
Amine acetylases	Brady
Thiol acetylases	Brady

If these enzymes were not affected by FAc, how could a blockade of the tricarboxylic acid cycle be explained? Liebecq and Peters found a satisfactory hypothesis: the real inhibitor is not fluoroacetate but something synthesized by metabolic processes from fluoroacetate and active in very small concentrations.

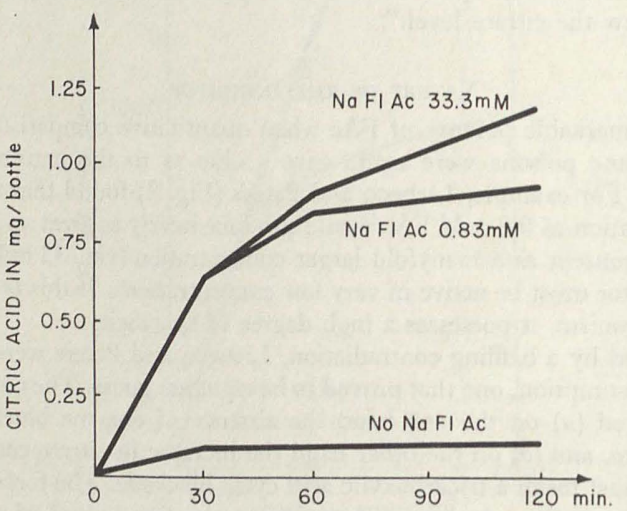


FIG. 2. Accumulation of citrate in the presence of FAc with sodium fumarate (6.6 *mM*) as substrate (Liebecq and Peters, 1949).

As so frequently happens, another investigator, Martius, independently at this time first established the fact that citric acid oxidation was inhibited by fluoroacetate (submitted for publication in 1948) (Martius, 1949). In a preparation of heart muscle brei initially containing 25 mg of citric acid, Martius found about 26 mg of citric acid after a 2-hour incubation period in the presence of 0.04 *M* fluoroacetate; a control preparation contained only about 1 mg of citric acid (Table IX*). In companion studies using a series of dicarboxylic acids, viz., oxalacetate, fumarate, succinate, and malate, Martius discovered that the *synthesis* of citric acid was relatively unimpaired by fluoroacetate. Martius, a pioneer in the studies of the enzyme aconitase, was fortunately in a position to grasp the significance of this pair of findings. He offered a reasonable explanation based on the assumption that a citrate metabolizing enzyme was blocked. This would not interfere with the synthesis of citrate but would effectively prevent its utilization. The inhibitor he correctly deduced was not FAc but the transformation product, fluorocitrate.

". . . dass in den Versuchen mit Gewebe die als solche wahrscheinlich gänzlich ungiftige Fluoressigsäure erst in das eigentliche Enzymgift umgewandelt wird. . . .

". . . glaube ich mit Bestimmtheit sagen zu können, dass es sich bei diesem um eine *Fluorcitronensäure* (oder Isocitronensäure) handelt. . . .

". . . einer Konkurrenz beider Säuren in dem Sinne, dass sie beide in gleicher Weise in den Stoffwechsel einbezogen werden, d.h. über die vermutliche Zwischenstufe einer Acet—bzw. Fluoracet—essigsäure in Citronensäure bzw. Fluorcitronsäure übergehen. Letztere bewirkt dann eine kompetitive Hemmung an der Isocitricodehydrase, was bei der Festigkeit der Fermentssubstrat-Bindung gerade dieses Enzymes offenbar schon bei sehr niedrigen Giftkonzentrationen möglich est" (Martius, 1949).

It is ironical that Martius proposed the enzyme isocitric dehydrogenase as the site of the inhibitory effect, a proposal ultimately shown to be in error.

The *in vitro* finding that FAc increased citrate concentrations was immediately extended to *in vivo* systems. Rats given 5 to 10 mg of fluoroacetate per kilogram body weight exhibited prompt and marked increases in citrate concentrations of most of the body tissues (Buffa and Peters, 1949; Potter and Busch, 1950 a). The magnitude of the citrate increase can be seen in the data of Potter and Busch (Table X†). Control tissues contained from 31 to 75 μg of citrate per gram. In contrast, 1 hour after a

* Table IX is on p. 68.

† Table X is on p. 68.

dose of 5 mg of fluoroacetate per kilogram several organs had concentrations of 200 to 1000 μg of citrate per gram. Two tissues in which the citrate concentration did not rise deserve mention: tumor and liver. For tumor, this was not unexpected; tumors maintain ATP levels by glycolysis but do not oxidize oxalacetate through the Krebs cycle. For liver, the picture is complicated. Immature female rats showed no increase in liver citrate after fluoroacetate (DuBois *et al.*, 1951); however, with age there is an increasing ability to develop high concentrations of citrate. In male rats, castration or exposure to X-irradiation significantly increased the citrate concentration after fluoroacetate (Ord and Stocken, 1953). Starved rats showed less increase in liver citrate than did fed rats. These facts point to the existence in liver of a number of alternate pathways for the metabolism of the active two carbon fragments whereas other tissues depend to a much greater extent on the tricarboxylic acid cycle. As a commentary on this point, Ottey and Daniel (1955) have recently reported that vitamin B_{12} reversed fluoroacetate inhibition in *Escherichia coli* perhaps by providing an alternate pathway through which acetate oxidation bypasses the tricarboxylic acid cycle. There is some evidence that the tricarboxylic acid cycle in liver can be blocked by fluorocitrate. Liver does synthesize fluorocitrate *in vivo* (Gal *et al.*, 1954); however, when rats were given fluorocitrate intraperitoneally the liver citrate levels were markedly elevated. When the fluoroacetate story began to unfold, the tricarboxylic cycle was little more than an appealing hypothesis and was unsupported by direct experimental test. The elevation of the citrate concentration in living tissues following the administration of fluoroacetate was strong support for the concept that the tricarboxylic acid cycle is indeed a reality and an important part of the metabolic machinery.

Evidence for Fluorocitrate

Martius stated the hypothesis (1) that fluoroacetate was converted to fluorocitrate, and (2) that this molecule was the inhibitor. Evidence has established the soundness of this hypothesis almost beyond reasonable doubt (as will be indicated, a question or two remain unanswered). The data came from two types of studies:

(*a*) Metabolic Studies:

1. Liebecq and Peters suggested early in their work that FAc, like acetate, is activated, brought into the cycle, and perhaps metabolized in part as is acetate.
2. The inhibitor competes with citrate in citrate oxidation (Elliot and Kalnitsky, 1950 c); a Lineweaver-Burk plot (Fig. 3) shows a clear-cut

instance of competitive inhibition. The inhibition could be reversed by increasing the concentration of citrate, viz., when the added citrate was increased from 10 to 50 μmoles the oxygen uptake increased from 14 to 63 μl corresponding to a decreased inhibition (from 83 to 25%). The inhibitor also competitively inhibits isocitrate metabolism (Peters and Wilson, 1952 a, b) as shown both by kinetic analyses and by direct demonstration of reversal.

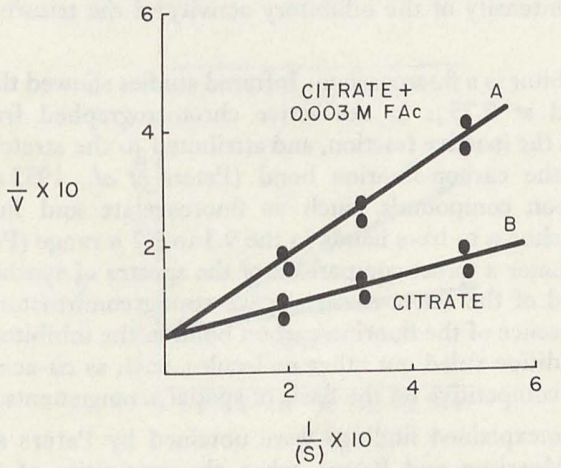


FIG. 3. Competitive inhibition of citrate oxidation by FAc (Elliott and Kalnitsky, 1950 c).

(*b*) Chemical Studies:

1. The inhibitor contains fluorine. Elliot and Kalnitsky (1950 c) recovered 32 μmoles of citrate following the incubation of oxalacetate and FAc (200 μM) with a homogenate of rabbit kidney cortex. The citrate, isolated in the form of pentabromacetone, contained identifiable quantities of fluoride. Control experiments in which the FAc was added after deproteinization gave recoverable amounts of pentabromacetone containing no fluoride.
2. The inhibitor is a tricarboxylic acid, traveling on the paper chromatograph with the tricarboxylic acid fraction (Buffa *et al.*, 1951). Using kidney homogenates from various species, the inhibitor was concentrated by an elaborate system of separation. Ultimately the active fraction was chromatographed in propanol-ammonia-water. Traces of

dicarboxylic acids were found but were inactive when tested in a kidney homogenate system. All of the activity was located in the tricarboxylic acid fraction.

3. The fluorine-containing molecule in the tricarboxylic acid fraction is not FAc. The addition of FAc to the active fraction prior to chromatography (*a*) gave rise to a spot containing all of the added FAc and located much in advance of either the di- or the tricarboxylic acid spots, and (*b*) did not induce activity in the dicarboxylic fraction nor alter the intensity of the inhibitory activity of the tricarboxylic acid fraction.

4. The inhibitor is a fluorocarbon. Infrared studies showed the presence of a band at 9.75 μ in the active chromatographed fraction, not present in the inactive fraction, and attributed to the stretching vibrations of the carbon-fluorine bond (Peters *et al.*, 1953 a). Several fluorocarbon compounds, such as fluoroacetate and fluorolactate, had been shown to have bands in the 9.3 to 9.7 μ range (Peters *et al.*, 1953 b). Later a direct comparison of the spectra of synthetic fluorocitrate and of the active material gave strong confirmatory evidence of the presence of the fluorine-carbon bond in the inhibitor molecule, and in addition ruled out other molecules, such as *cis*-aconitate, that might be competitive on the basis of spatial arrangements.

5. As yet unexplained findings were obtained by Peters and Wilson and by Morrison and Peters, when the properties of the natural inhibitor and the synthetic fluorocitrate were compared. Peters and Wilson (1952 a, b), in tests with a crude aconitase in two forms (a homogenate of pigeon breast muscle or a precipitate from ammonium sulfate), clearly showed in a quantitative fashion that the natural inhibitor, i.e., the inhibitor in the fraction separated from these mixtures after incubation, competitively inhibited (*a*) the transformation of citrate to *cis*-aconitate, and (*b*) the transformation of isocitrate to *cis*-aconitate. Morrison and Peters (1954 a) found that approximately twenty times more natural inhibitor was needed to inhibit a soluble aconitase than the crude aconitase in particles from kidney homogenate. Synthetic fluorocitrate, half as potent as the natural inhibitor on crude aconitase, was more active than the natural inhibitor on soluble aconitase (Fig. 4); furthermore, soluble aconitase was partially irreversibly inhibited by the synthetic fluorocitrate. These confusing facts are at present unresolved into a coherent description. *Fluorocitrate, the real inhibitor, has been produced from FAc by a "lethal synthesis."* The facts marshall themselves in an understandable array around this simple concept.

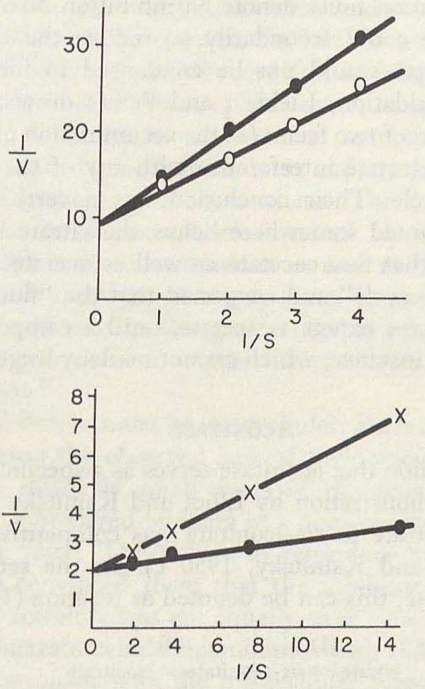


FIG. 4. Competitive inhibition of aconitase. Upper graph, inhibition by natural fluorocitrate. O, control; ●, fluorocitrate, 3.2×10^{-5} *M*. Lower graph, inhibition by synthetic fluorocitrate. ●, control; X, fluorocitrate, 2.4×10^{-5} *M* (Morrison and Peters, 1954a).

THE SITES OF INHIBITION

It seems reasonable that fluoroacetate should somehow interfere with the utilization of acetate. A direct fluoride effect can be ruled out. Because FAc is not broken in the body to release fluoride ions, the enzymes inhibited by fluoride, such as enolase, are not affected (Bartlett and Barron, 1947). Acetate oxidation, however, is reduced proportionately with oxygen consumption; this fact led Bartlett and Barron to locate the blockade at the initial activation of acetate (by an enzyme now described as a transacetylase). While transacetylase blockade may explain some of the various observed derangements in fluoroacetate poisoning, other more important sites of inhibition by fluoroacetate are known.

Acetate is oxidized principally via the tricarboxylic cycle. An early step is the coupling of the two-carbon active fragment (acetyl CoA) with oxalacetate by the condensing enzyme. Elliot and Kalnitsky observed that oxalacetate could reverse in part the inhibition of acetate oxidation and

concluded that the increase in concentration of citrate despite the redu tion in oxygen utilization must denote an inhibition below citrate in t cycle. This blockade could secondarily so reduce the concentration oxalacetate that acetate would not be condensed to form citrate, thu depressing acetate oxidation. Liebecq and Peters drew attention to th essential contradiction of two facts: (*a*) the accumulation of citrate; (*b*) lack of evidence of fluoroacetate interference with any of the enzymes of the tricarboxylic acid cycle. Their conclusion was nevertheless clear: "the cycle itself is interrupted somewhere below the citrate level." They set up the hypothesis "that fluoroacetate as well as acetate can be activated and brought into the cycle" and suggested that the "fluoroacetate might be metabolized to some extent as acetate, until a compound is reached, like fluoromalate for instance, which cannot be dehydrogenated."

Aconitase

The first recognition that aconitase serves as a specific target of inhibition came in the demonstration by Elliot and Kalnitsky in 1950 that the transformation of citrate to *cis*-aconitate was competitively inhibited by fluoroacetate (Elliot and Kalnitsky, 1950 c). In the series of equilibria catalyzed by aconitase, this can be denoted as reaction (1).

$$\text{citrate} \underset{(2)}{\overset{(1)}{\rightleftharpoons}} \textit{cis}\text{-aconitate} \underset{(4)}{\overset{(3)}{\rightleftharpoons}} \text{isocitrate}$$

The normal tendency for most of the material represented by these three molecular species to be held in the citrate form (80% more or less) should be recalled. Busch and Potter (1952 a) confirmed the observation that reaction (1) is inhibited and also showed that reaction (2), the transformation of *cis*-aconitase to citrate, was little affected. The over-all reaction to the right, citrate to isocitrate (reactions (1) plus (3)) by a partially purified aconitase, was inhibited by FAc (Lotspeich *et al.*, 1952), thus confirming the demonstration that, in the crude aconitase preparation of Elliot and Kalnitsky, citrate oxidation could be nearly completely blocked without necessarily interfering with the transformation of *cis*-aconitate to isocitrate (reaction (3)). When the inhibitor was incubated with the enzyme prior to adding substrate, the inhibition of the isocitrate to *cis*-aconitate transformation (reaction (4)) was progressively decreased. Evidently the enzyme-inhibitor complex readily dissociated, thus satisfying this criterion of a competitive inhibitor (Peters and Wilson, 1952 a). The kinetics of the citrate to isocitrate transformation permitted the deduction that the inhibitor competitively inhibited citrate in reactions (1) plus (3) (Peters and Wilson, 1952 a). The isocitrate to citrate transformation also can be

inhibited; thus, in the words of Lotspeich *et al.* (1951), "both the forward and the reverse action of aconitase are inhibited strongly."

The final word for the moment on the inhibition of aconitase has come from the work of Morrison and Peters (1954 a) using the highly purified aconitase preparation of Morrison. The action of synthetic fluorocitrate removes any real doubt as to the chemical nature of the inhibitor. As shown in Fig. 4 both the natural inhibitor and synthetic fluorocitrate are competitive inhibitors of purified aconitase. The natural inhibitor is a reversible inhibitor at all concentrations tested. Synthetic fluorocitrate is a reversible inhibitor at concentrations as low as 0.6×10^{-5} *M* but is partially irreversible at higher concentrations. In the words of Morrison and Peters, "it would seem as though the isomers present in the synthetic material, but not present in the natural fluorocitrate, are responsible for the different effects."

The site of inhibition must be intracellular. Buffa *et al.* (1951) reached this conclusion from the observed lack of toxic response when what are now regarded as relatively small doses of the active fraction, i.e., the natural inhibitor as prepared by them in a homogenate, were injected into two rats and two mice. The failure to reproduce symptoms of fluoroacetate poisoning convinced them that the inhibitor, however potent it might be *in vitro*, certainly was not equally so *in vivo*. The inhibitor, they thought, must penetrate cellular membranes with difficulty. At any rate, this concept is consonant with the intracellular location of the enzymes of the tricarboxylic acid cycle in the mitochondria.

Other enzymes than aconitase must be inhibited to some degree in fluoroacetate poisoning. At present, of the two groups of enzymes having to do with acetate metabolism, transacetylases and acetokinases, only the latter have been implicated. The relative importance of these blocked functions in the clinical picture of FAc poisoning has not yet been assessed. To return to the hypothesis of Bartlett and Barron, considerable detail can now be added. Surprisingly enough, the original hypothesis has not yet been critically tested. Studies of transacetylases are available and whether these enzymes will or will not "activate" FAc has been determined, but the inhibition of acetate activation has not been investigated.

An undeniable implication of some enzyme earlier in the cycle than aconitase was furnished by the effects of FAc on a kidney homogenate in the presence of fumarate. In the kidney, pyruvate is oxidized only through the tricarboxylic acid cycle; pyruvate was not utilized at all when FAc was added. Oxygen utilization was reduced and citrate concentrations increased with smaller concentrations of FAc but less citrate was synthesized when the FAc content increased (Busch and Potter, 1952 b). Obviously some earlier step was being blocked.

The discussion of the reactions involving or influenced by FAc can be more easily presented with the aid of a diagram (Fig. 5). Here acetyl CoA or F-acetyl CoA are placed centrally with donors, i.e., transacetylases, on the left, and acceptors, i.e., acetokinases, on the right.

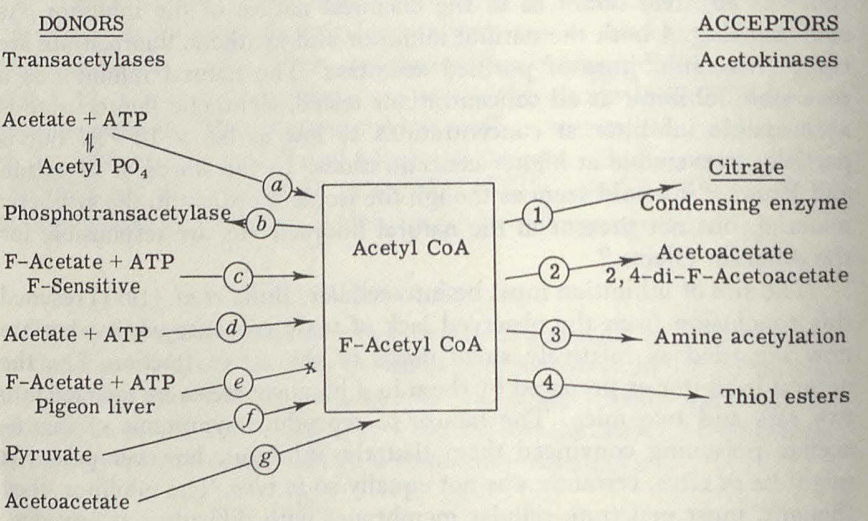


FIG. 5. Reactions involving or influenced by fluoroacetate.

CONDENSING ENZYME

That the condensing enzyme (1, Fig. 5) might discriminate between F-acetyl CoA and acetyl CoA was first hypothesized by Peters to explain the decrease in fluorocitrate formation in the presence of large concentrations of acetate. In his experiments, however, the effect could have been either on the activating system (transacetylases) or on the condensing enzyme.

Indirect evidence obtained by Busch and Potter had pointed to an effect of fluoroacetate on the condensing enzyme system; a direct demonstration of this inhibition, however, was furnished by Brady (1955). Using a purified condensing enzyme preparation and chemically prepared fluoroacetyl CoA, Brady found that the transformation of acetyl CoA to citrate was competitively inhibited by fluoroacetyl CoA (Fig. 6). The condensation product proved to be fluorocitrate; the oxidation of citrate was inhibited by the product of the condensation reaction (technique of Buffa, Peters, and Wakelin). Using a synthetic fluorocitrate, the condensation

reaction of fluoroacetyl CoA with oxalacetate was shown to be only slightly reversible. In partial confirmation, Marcus and Elliot (1956) observed that fluoroacetyl CoA and acetyl CoA were synthesized equally to "citrate" although the product was not further identified.

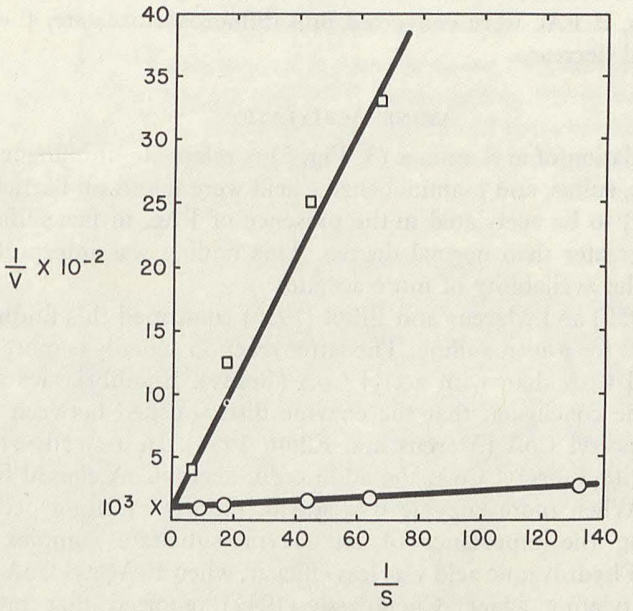


FIG. 6. Competitive inhibition of condensing enzyme by F-acetyl S CoA; □: acetyl S CoA in presence of F-acetyl CoA (52% purity), 0.038 μM; ○: acetyl S CoA (77% purity), 0.0075–0.085 μM (Brady, 1955).

ACETOACETATE FORMATION

The complexity of the events involving FAc began to appear when the liver malonate system was examined (Busch and Potter, 1952 b). Here pyruvate is decarboxylated and condensed to form acetoacetate (2, Fig. 5). FAc reduced both pyruvate and oxygen utilization and, when the concentration of FAc was high, acetoacetate formation decreased. In these systems essentially no citrate was formed; it can hardly be doubted that some acetokinase other than the condensing enzyme must be inhibited.

Under Brady's conditions, both acetyl CoA and F-acetyl CoA formed an acetoacetate-type molecule. Initially the velocity of the reaction with acetyl CoA was faster but as time went on the rates became comparable and the final concentrations were roughly the same whether acetyl CoA

or F-acetyl CoA was the starting material. In the latter case the product was identified as 2,4-difluoroacetoacetate.

This reaction offers a plausible explanation for the finding that liver has less FAc than other tissues in a poisoned animal (Busch and Potter, 1952 b; Hagan *et al.*, 1950). The analytical method of Hagan *et al.* (1950) was specific for FAc and would not reflect fluoride bound as difluoroacetoacetate. Thus, if FAc were converted into difluoroacetoacetate, the FAc content would decrease.

Amine Acetylation

The acetylation of aryl amines (3, Fig. 5) is relatively uninfluenced by FAc. Choline, sulfas, and *p*-aminobenzoic acid were found by Bartlett and Barron (1947) to be acetylated in the presence of FAc, in fact sulfas and PABA to a greater than normal degree. This finding was interpreted as evidence of the availability of more acetate.

Brady (1955) and Marcus and Elliot (1956) confirmed this finding for sulfas and also for *p*-nitroaniline. The latter reaction actually is more rapid with F-acetyl CoA than with acetyl CoA (Brady). Equilibria are set up permitting the conclusion that the enzyme distinguished between acetyl CoA and F-acetyl CoA (Marcus and Elliot, 1956). In a steady state of acetylation with F-acetyl CoA, the addition of acetyl CoA caused further acetylation. When more enzyme was added, more acetylation occurred, demonstrating the importance of an enzyme-substrate complex. The acetylation of hydroxamic acid was less efficient when F-Acetyl CoA (FAc) was the acetylating agent. Chenoweth (1949) reported that rats and rabbits *in vivo* exhibited increased acetylation of sulfanilamide and PABA.

Thiol Esters

Ester formation was equally rapid when 2-mercaptoethanol was acetylated (4, Fig. 5) with acetyl CoA or with F-acetyl CoA (Brady, 1955).

In summary, of the acetokinases only the condensing enzyme clearly is inhibited by F-acetyl CoA. This inhibition probably contributes little to the clinical picture of FAc poisoning. High concentrations of FAc inhibit acetoacetate production but in lower concentrations the synthesis of 2,4-difluoroacetoacetate was accomplished about as easily as that of acetoacetate. Amine and thiol acetylation were not blocked.

Transacetylases

Bartlett and Barron assumed that FAc effects could be explained by a direct inhibition of the formation of "active acetate," now known to be acetyl CoA. The enzymes that catalyze these reactions, the transacetylases,

are numerous; some detail will be presented describing some of the reactions. Three generalities can be stated as follows:

(1) None of the transacetylases are known to be inhibited by FAc;
(2) Some transacetylases "activate" FAc just as efficiently as acetate;
(3) One transacetylase does not "activate" FAc.

Phosphotransacetylase

Under proper conditions, it is possible to separate acetyl PO_4 from the medium in which acetate and ATP have reacted. Phosphotransacetylase converts acetyl PO_4 to acetyl CoA (a, Fig. 5) and can convert F-acetyl PO_4 just as efficiently to F-acetyl CoA. The reverse reaction (b, Fig. 5) was studied by Brady.

F-Sensitive System

"Dialyzed extracts of an acetone powder of rabbit kidney homogenate contain an active fluoroacetate-activating system which appears to be somewhat different from an acetate-activating system also present in crude extracts" (Brady, 1955). Both systems showed an absolute dependence on ATP but the FAc-activating system was markedly inhibited by KF (c, Fig. 5) whereas the acetate-activating system was not (d, Fig. 5) and the FAc-activating system was more dependent on supplemental CoA than was the acetate-activating system.

Pigeon Liver Preparation

The acetate-activating system in pigeon liver (e, Fig. 5) that converts acetate and ATP to acetyl CoA will not activate FAc (Brady). Peters reported that the pigeon liver preparation could not form fluorocitrate from FAc and oxalacetate; presumably the fault lay in this system.

Other Sources

Pyruvate (f, Fig. 5) and acetoacetate (g, Fig. 5) are acetate donors that have been utilized to form acetyl CoA in various studies of the mechanism of FAc actions. In neither reaction does FAc interfere. As a comment on the fluorocarbons, the effects of fluoropyruvate may be mentioned briefly: (*a*) $C^{14}O_2$ production in the exhaled breath of the rat given C^{14}-2-acetate is reduced; (*b*) citrate accumulates *in vivo* (Blank *et al.*, 1955); (*c*) convulsions may be seen; and (*d*) pyruvate metabolism is depressed. *In vitro*, fluoropyruvate is a noncompetitive inhibitor of lactic dehydrogenase (Busch, 1956). Whether reaction (f) is involved is not known.

A direct inhibition of acetate utilization has been postulated from the findings that less citrate is formed from fumarate and acetate (Terner, 1955; Liebecq and Peters, 1949) or from oxalacetate and acetate (Watland *et al.*, 1957 c) in the presence of FAc.

Ethyl difluoroacetoacetate, ethyl fluoroacetate, and diethyl fluorooxalacetate inhibited citrate formation from oxalacetate by rabbit kidney cortex mitochondria (Watland *et al.*, 1957 c). Mitochondria appear to lack esterase activity.

Metabolic Mechanisms: Summary

To the nonbiochemist, the detailed story of FAc poisoning may obscure the well-established essentials. The picture may be summarized as follows:

1. *Basic Background*

The basic oxidation mechanism for supplying cellular energy is the tricarboxylic acid cycle, a machine for burning two-carbon fragments. A four-carbon acid, oxalacetate, is condensed with the activated two-carbon fragments (acetyl CoA) to form a six-carbon acid, citrate. By successive steps using oxygen, citrate is degraded to five- and then to four-carbon acids, ultimately regenerating oxalacetate. A schematic outline follows. High-energy phosphates are produced to serve as the energy source for cellular metabolism. A blockade of this cycle causes metabolic death.

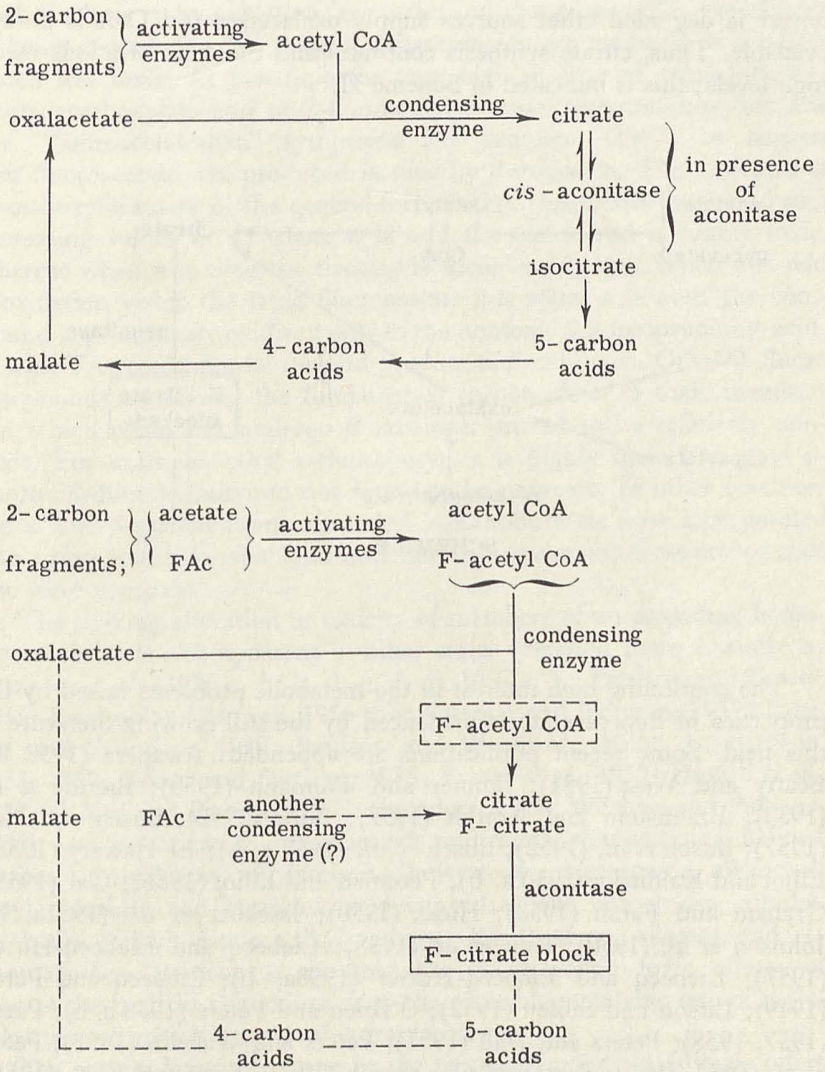
Two enzymes of the cycle, aconitase and the condensing enzyme, are blocked in FAc poisoning. Aconitase interconverts citrate, *cis*-aconitate, and isocitrate, three contiguous members of the cycle; a blockade of aconitase is followed by an increase in the concentration of citrate. A blockade of the condensing enzyme prevents citrate formation and is followed by increasing concentrations of other acetylated molecules (e.g., acetoacetate).

2. *Lethal Synthesis*

FAc enters the pool of two-carbon fragments and is "activated" to F-acetyl CoA and subsequently condensed with oxalacetate to fluorocitrate. Malate may also serve as the four-carbon fragment probably condensed with FAc by a different condensing enzyme (Peters, 1955). These changes are shown in the accompanying Scheme I.

Fluorocitrate blocks aconitase; the tricarboxylic acid cycle cannot function; metabolic death ensues. This is the "lethal synthesis;" fluorocitrate is the real poison. Minute concentrations of fluorocitrate exert a

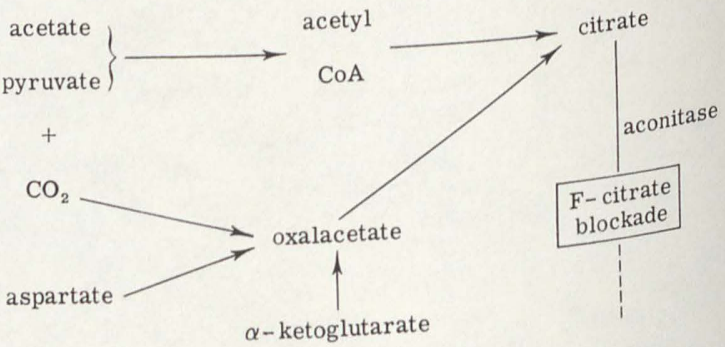
profound blocking action; for example, 1 mg of fluorocitrate will block the disappearance of 300 mg of citrate in 30 minutes. F-acetyl CoA competitively inhibits the condensing enzyme but the significance of this inhibition has not been evaluated.



SCHEME I

The characteristic delay in the action of this "delayed convulsant" may now be ascribed to the time required for FAc (*a*) to diffuse into the cell, (*b*) to reach the mitochondria, and (*c*) to be condensed into fluorocitrate in sufficient quantities to inhibit aconitase.

The high concentrations of citrate in most tissues, characteristic of FAc poisoning, arise because when the cycle is blocked and citrate no longer is degraded other sources supply oxalacetate and CoA is always available. Thus, citrate synthesis continues and the concentrations reach high levels; this is indicated in Scheme II.



SCHEME II

The continuing high interest in the metabolic problems raised by the properties of fluoroacetate is evidenced by the still growing literature in this field. Some recent publications are appended: Awapara (1952 b); Beatty and West (1951); Bonner and Thimann (1950); Braude *et al.* (1952); Braunstein and Azarkh (1957); Buffa (1952); Busch and Nair (1957); Busch *et al.* (1952); Busch *et al.* (1957 a, b); El Hawary (1955); Elliot and Kalnitsky (1950 a, b); Freeman and Elliot (1956); Gal (1958); Graham and Farah (1952); Hicks (1950); Issekutz *et al.* (1951a, b); Johnson *et al.* (1950); Kun *et al.* (1958); Liebecq and Liebecq-Hutter (1957); Liebecq and Liebecq-Hutter (1958a, b); Liebecq and Peters (1949); Lifson and Stolen (1952); O'Brien and Peters (1958 a, b); Peters (1957, 1958); Peters and Hall (1957); Peters and Wakelin (1957); Peters *et al.* (1952, 1959); Scott and Chenoweth (1950); Serif and Wick (1958); Terner (1958); Tietz and Ochoa (1958); Van de Berg (1957, 1958 a, b); Ward and Peters (1957); and Wollemann and Feuer (1957).

Structure-Toxicity Relations

Although the toxicities of compounds containing a F—C group are in general unpredictable, certain compounds were early recognized as highly toxic and the toxicity was related to the fluoroacetate structure. By 1943 Saunders *et al.* had concluded that compounds yielding FCH_2CO *in vivo*, by hydrolysis or by oxidation, are toxic, and that compounds not capable of being degraded *in vivo* to the fluoroacetate ion are nontoxic, or certainly much less toxic. At that time the very high toxicity of ethyl-5-fluoropentanecarboxylate and of 2-fluoroethyl-5-fluoropentanecarboxylate and the "fluoroacetate-like" symptoms led Saunders (1957) to suggest that fluoroacetate was produced *in vivo* by β-oxidation. The toxicities of ω-carboxylic esters of the general formula $F(CH_2)_nCO_2R$ fluctuated with increasing values of n; where n is odd the compound is highly toxic, whereas when n is even the toxicity is considerably less. When n is odd β-oxidation yields the toxic fluoroacetate but when n is even the compound is presumably oxidized only to the nontoxic β-fluoropropionic acid; at least FAc could not be the end product of β-oxidation. Other ω-fluoro compounds containing the fundamental carbon chain of toxic members but which could not undergo β-oxidation proved to be relatively nontoxic. For example, ethyl γ-fluorobutyrate is highly toxic but ethyl γ-fluoro-$\beta\beta$-dimethylbutyrate was found to be nontoxic. In other instances the α- and β-carbon atoms of methyl-γ-fluorobutyrate were incorporated into a ring system incapable of undergoing β-oxidation; these compounds also were nontoxic.

The striking alteration in toxicity of members of an ascending homologous series is also apparent in other series prepared more recently by Pattison *et al.* (1956 a, b, c, d, e, f, g; 1957 a, b; Pattison and Fraser, 1955; Pattison and Howell, 1956 a, b; Pattison and Norman, 1957; Pattison and Saunders, 1949; Pattison and Woolford, 1957 a, b; Pattison, 1953, 1957; Fraser and Pattison, 1955; Fraser *et al.*, 1957; Howell *et al.*, 1957; O'Neil and Pattison, 1957; Saunders, 1947; Wilshire and Pattison, 1956) who investigated ω-fluoro acids and esters, alcohols, alkyl halides, nitrites, nitroalkanes, alkylamines, thiocyanates, mercaptans, alkanesulfonyl chlorides and fluorides, acid chlorides, acid anhydrides, amides, aldehydes, alkyl esters, alkyl ethers, β-ketoesters, alkynes, and 1-fluoroalkanes. Pattison *et al.* used the characteristic toxic effects of FAc as the basis of deductions as to the probable metabolic fate of molecules that ultimately furnish this fragment.

The accumulation of citrate in the tissues of poisoned animals offers convincing proof that the toxic ω-fluoro compounds indeed act as fluoroacetate sources *in vivo*. Parker and Walker (1957) compared representative

ω-fluoro compounds containing odd- or even-numbered carbon chains. High toxicity paralleled high citrate concentrations in the kidneys of mice. (See Table XI, p. 69.)

Comparing compounds having the same functional group, those having an even-numbered carbon chain are appreciably more toxic than those having an odd number of carbons. The even compounds cause a pronounced accumulation of citric acid whereas the odd do not. In fact, the citric acid levels after administration of odd compounds fall in the normal range.

Differences in toxicity in members of a series, and between series, have been pointed out by Pattison *et al.* With few exceptions toxicities of the ω-fluoroalcohols were found to be comparable to those of the corresponding acids, whereas the esters usually were less toxic. The high specificity of the ω-fluorine atom for pharmacological activity is illustrated by the difference in toxicity between 18-fluorostearic acid (LD_{50}, 5.7 mg/kg, mice) and 9(10)-fluorostearic acid (LD_{50}, > 400 mg/kg, mice). The presence of unsaturated linkages appears not to affect the toxicity.

Saunders (1957, pp. 133 *et seq.*) classifies compounds containing the F—C bond on the basis of their toxicities.

Hughes and Saunders (1954 b) described an enzymatic breaking of the F—C bond fluoroaniline as the first known instance of such an enzymatic reaction.

The branched ω-fluorocarboxylic acids present several interesting features. In compounds of the general formula $F(CH_2)_nCHMe(CH_2)_mCO_2H$ where m is odd, the introduction of one or more methyl groups in the β-position sharply alters the toxicological pattern; the branched chain acids were appreciably different in toxicity than the corresponding straight chain acids. β-Oxidation of the main chain must not occur in the usual manner. In contrast, where m is even the toxicities of the branched chain acids approximate those of the unbranched compounds; introduction of the methyl group does not alter the general toxicological pattern. The point is well illustrated with the two acids $F(CH_2)_8CHMeCH_2CO_2H$ and $F(CH_2)_7CHMeCH_2CO_2H$. The LD_{50} (mice) of the branched chain (m = odd) compound is 2.42 mg/kg, some twenty times more toxic than the corresponding unbranched acid (LD_{50} = 57.7 mg/kg). For $F(CH_2)_7CHMeCH_2CH_2CO_2H$ in which m is even, the LD_{50} is 52 mg/kg, approximately equal to that of the unbranched acid (57.5 mg/kg). The substitution of a β-methyl group in 8-fluorooctanoic acid was found to reduce the toxicity of the latter compound a hundredfold from an LD_{50} of 0.64 mg/kg to 118 mg/kg; the substitution of an additional β-methyl group gave a negligible change: LD_{50}, 121 mg/kg.

The toxicity of ω-fluoroesters and ω-fluoroalcohols in which n is

odd increases with increasing chain length (Table VI). It would be expected, however, that the toxicity should decrease because of the increasing chain length which must be oxidized before fluoroacetate is produced. In 1949 Buckle *et al.* (1949 b) suggested that the observed higher toxicities might arise from a higher lipid solubility of the longer chained members and, therefore, ultimately from higher intracellular concentrations of fluoroacetate. However, long-chain esters of fluoroacetic acid and of 2-fluoroethanol did not show the expected higher toxicities. Apparently hydrolysis of the ester occurs before the increased fat solubility effectively increases intracellular concentrations.

The 1-fluoroalkanes have proven to be among the most toxic ω-fluoro compounds. This fact, coupled with their volatility, suggests caution in handling. The toxicities of the 1-fluoroalkanes, especially those where n is even, are similar to the corresponding acids. The relatively few ω-fluoroalkanes investigated proved to be toxic regardless of the number of carbons in the chain. The fluoroalkynes all caused citric acid accumulation, regardless of whether n is odd or even; this phenomenon was also noted with the fluoroalkyl methyl ketones. The ω-fluorophenyl ketones investigated were relatively nontoxic, possibly because the phenyl groups inhibited oxidative mechanisms.

Toxicities of the alkyl halides demonstrates that the halogen atom of higher members is more labile than that of the 2-fluoroethyl halides. The chlorides and iodides are more toxic than the bromides. The sulfonyl fluorides investigated apparently are toxic more by virtue of their anticholinesterase activity than by ultimate degradation to fluoroacetate.

The ω-fluoro amines have proven to be nearly as toxic when placed on the skin as when injected.

The 2-fluoroethyl compound in any series has proven to be considerably less toxic than the higher even members in the same series. Possibly the influence of the 2-fluorine atom on other functional groups and their metabolic behavior outweighs solubility and steric effects.

Additional references are as follows: Pattison (1957) and Walker and Parker (1958).

Phosphofluoridates[1]

The biological effects of certain fluorophosphates recorded by Lange and Kreuger in 1932 were exploited in the search for new chemical warfare agents during World War II (McCombie and Saunders, 1946 b;

[1] We are grateful to Drs. McGehee Harvey and David Grob of the Johns Hopkins Medical School, and to Colonel Millard Bayliss and colleagues, Army Chemical Center, for comments and suggestions which have been incorporated into this section.

Comroe *et al.*, 1946 b; Harvey *et al.* 1946). The best known member of this group, diisopropyl phosphofluoridate, DFP, proved to be an inhibitor of cholinesterase of extraordinary potency. The development of the nerve gases, and of the phosphate insecticides, has made the general subject of cholinesterase inhibitors of major importance. In these new series of compounds, however, fluorine is not an essential or even a common substituent. Methods for the detection and estimation of nerve gases have been described by Megregian (1954), Goldenson (1957), and Gehauf and Goldenson (1957). At present, interest in DFP is almost lacking except historically and because of some clinical use in the treatment of eye diseases. Two aspects of DFP justify detailed consideration: (*a*) the variations in properties of its derivatives, and (*b*) DFP metabolism, especially the properties of the enzymes classed as the DFPases.

"The problems of civil defense against chemical attack . . . can be reduced to the consideration of a single group of chemical agents, the nerve gases, until such time as an enemy may be able to establish a base at or within our borders." So wrote General John R. Wood in October, 1950, in discussing the medical problems in chemical warfare (Wood, 1950).

Nerve gases are organic phosphorous compounds with oxygen or sulfur, alkyl or aryl substituents, and may have a halide, cyanide, amide, or other group. Examples of nerve gases are diisopropyl phosphofluoridate (DFP), isopropyl methylphosphonofluoridate (Sarin, GB), dimethylamidoethoxy-phosphorylcyanid (Tabun), and pinacolyl-methylphosphonofluoridate (Soman). Comparative toxicities of certain of these compounds are indicated in Table XII.* Diisopropyl phosphofluoridate (DFP) is a colorless, odorless liquid, toxic by any route (swallowed, splashed on the skin or in the eyes, or inhaled as a vapor). Some medical uses have been found for this material: benefit to patients suffering from myesthenia gravis has been reported in certain cases. The prolonged duration of therapeutic effect makes DFP one of the drugs of choice in the treatment of glaucoma (an eye disease characterized by abnormally high intraocular pressure) and certain other eye diseases.

The nerve gases inactivate cholinesterase. To understand the symptoms that accompany nerve gas poisoning, a word should be said about the role of acetylcholine in the function of nerves. When a nerve impulse courses down certain nerve fibers and reaches the terminus, the electrical impulse elicits the release of a tiny but essential amount of acetylcholine. This chemical mediator, in some manner at present unknown, triggers the response, i.e., the contraction of a muscle, the outpouring of a secretion

* Table XII is on p. 69.

from a gland, or the transmission of the nerve impulse to a second nerve fiber. In the presence of acetylcholine, the contractile or secretory or transmitting process continues to operate. Some mechanism for removing this stimulus must be provided to make rapid repeated responses possible. This mechanism in fact is an enzyme, cholinesterase, of exceptional capacity, whose specific function is the hydrolysis of acetylcholine; the products, acetate and choline, have very little of the activity of the parent compound. The inactivation of cholinesterase leads to the accumulation of acetylcholine in the nervous system. The effects of some of the most powerful nerve poisons thus develop, e.g., of nicotine and of muscarine poisoning and, in severe poisoning, effects like those of curare. The recommended antidote at the moment is atropine. In fact, solutions for parenteral injection are available commercially,[1] packaged, and marked "for nerve gas poisoning."

Under normal conditions acetylcholine reacts with the cholinesterases in such a way that the carbonyl group of the acetylcholine apparently forms a covalent bond with the esteratic site of the enzyme, producing an acetyl enzyme. Normally this complex reacts with water in a few microseconds to release acetate and regenerate the enzyme surface. When an organic phosphate with anticholinesterase activity reacts, the phosphorus atom forms a covalent bond with the same group on the enzyme surface; the result is a phosphorylated enzyme. If this complex does not react with water the access of acetylcholine to the esteratic site is blocked.

Following reports that oximes and hydroxamic acids react with cholinesterase, and also reactivate cholinesterase inhibited by anticholinesterase compounds (Hackley *et al.*, 1955; Wilson *et al.*, 1950, 1955; Childs *et al.*, 1955), a number of investigators have sought compounds in which the hydroxylamine was placed at a proper atomic distance from a nitrogen group. This nitrogen grouping could be attracted to the negative anionic site in the enzyme surface which is located at precisely the distance for rapid hydrolysis of acetylcholine. Studies by Wilson and Ginsberg (1955), Kewitz (1957), Kewitz and Nachmansohn (1957), Kewitz *et al.* (1956), Kewitz and Wilson (1956), and Davies and Green (1956) have shown pyridine-2-aldoxime (PAM) to be a highly potent antidote. Using a rat diaphragm preparation, Kewitz (1957) has shown that PAM regenerates the cholinesterase functional group. Kewitz and Nachmansohn

[1] There are practical reasons why information about the dangers, the effects, and the treatments of this class of compounds should be disseminated. Some of the newer and most useful insecticides, such as parathion, TEPP (tetraethylpyrophosphate), and EPN (ethylphenylparanitrophenylthionophosphate), are "powerful though less dangerous anticholinesterases." These are not fluorine-containing compounds but are mentioned because of their similarity to DFP in their mechanism of action.

(1957) have also demonstrated the recovery of cholinesterase activity in the brain. With critically chosen doses of anticholinesterases, PAM is a powerful antidote in mice against doses of DFP up to five times the LD_{50}. This range of doses, however, is too narrow to make it generally useful (Loomis, 1956). Askew (1956) has tested twenty-three oximes and nine hydroxamic acids as possible antidotes. Dultz *et al.* (1957) also have screened a series of nineteen oximes. Childs *et al.* (1955) have screened forty-one similar compounds. Of these ninety-two compounds, 2-oximino-3-butanone (DAM) has proven the most effective. DAM protects mice against 3 LD_{50}'s of Sarin and rats against 20 LD_{50}'s.

Additional studies on the treatment of poisoning have been presented in the following references: Askew (1957), Askew *et al.* (1957), Brown *et al.* (1957), Davies and Green (1959), Davies *et al.* (1959), DeCandole and McPhail (1957), Edery and Schatzberg-Porath (1958), Fleisher *et al.* (1958 a, b), Green (1958), Green and Smith (1958a , b) Grob and Johns (1958), Hackley *et al.* (1959), Hobbiger (1956, 1957 a, b, c), Jager and Stagg (1958), Larsson (1957, 1958 a, b) Rajapurkar and Koelle (1958), Rutland (1957, 1958), Steyn (1958), Wilson (1958), Wilson and Ginsburg (1955, 1959), and Wilson *et al.* (1958).

The mechanism of action of anticholinesterases has been described by Cohen and Posthumus (1955). The kinetics of reactivation of inhibited cholinesterase by oximes has been investigated in detail by Davies and Green (1956).

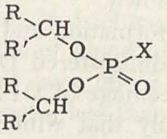
The treatment of acute poisonings by DFP follows the procedures recommended for treatment of poisonings by the organic phosphate insecticides. The toxic effects, symptoms, and recommended treatment in detail have recently been summarized (Gleason *et al.*, 1957). Symptoms progress from nausea, vomiting, and abdominal cramps to the full galaxy of the effects of overwhelming stimulation of the parasympathetic nervous system, e.g., difficulty in breathing, mental confusion, and death primarily as a result of respiratory failure. Treatment procedures are aimed (*a*) at the removal of unabsorbed poison, (*b*) at controlling the peripheral symptoms by the administration of atropine (which also alleviates in part the central nervous system effects), (*c*) at the maintenance of respiratory exchange, and (*d*) at supporting the patient.

Two industrial cases of poisoning due to DFP have been described by Moore (1956). Visual disturbances, nausea, and vomiting were experienced in both cases, and plasma cholinesterase levels were reduced approximately 66%.

Additional references are as follows: Bergner and Durlacher (1951), Bidstrup and Bonnell (1953), Bidstrup *et al.* (1953), Fredriksson (1958 b), Rubin and Goldberg (1957), and Rubin *et al.* (1957).

Toxicity of DFP and Derivatives

The toxicity of DFP in several species is given in Table XIII*, of related compounds in Table XIV*; in compounds of the type shown in the structure below, the myotic effect and toxicity depend upon the



nature of the various substituents. Where X is fluorine, high toxicity and myotic properties are present. These effects are increased where R = R′ = —CH_3; R′ = CH_3, R′ = —C_2H_5; RR′ = cyclohexyl; R = —CH_3, R′ = —$CH_2CH(CH_3)_2$. For greater toxicity R and R′ should be unsubstituted hydrocarbons, of which one should be a methyl group. Where R′ is H and also where R is unsubstituted, greater toxicity is achieved. Diphenyl substituents also result in low toxicity.

If X is replaced by —H, —C_2H_5, —OCH_2CH_2Cl, —OCH_2CH_2F, —Cl, —NH_2, —$NHCH_3$, —NHC_6H_5, —CH_2F, —CH_2CH_2F, —CN, —SCN, or morpholino, myotic effects are absent, and toxicity is low. Loss of activity also was noted when the single-bonded oxygen atoms were replaced by sulfur atoms. Structure-activity relationships of the organophosphorus compounds have been discussed in detail by Saunders (1957), Kodama *et al.* (1955), Holmstedt (1951), and DuBois and Coon (1952).

DFP Metabolism

The dynamic aspects of DFP metabolism in the body can be aptly described by the term "protein interaction." This interaction is responsible for the toxicity of DFP as well as for its enzymatic destruction.

In aqueous solution, spontaneous DFP hydrolysis is relatively slow, about 1% in 1 hour at 25°C. The alkyl ester linkage is extremely stable and is not affected even by boiling 10% NaOH. The phosphorus-fluorine bond, however, is easily broken by protein interaction (Mazur, 1946 b). Upon rupture of this bond, fluorine is liberated as the F ion and the phosphorus-containing molecule either becomes attached to an enzyme esteratic site, causing inhibition, or is released as diisopropyl phosphoric

* Table XIII is on p. 70; Table XIV, on p. 71.

acid (DIP). DIP is nontoxic, exerts no antienzyme action, and is rapidly excreted via the kidney.

Interaction of DFP with hydrolyzing enzymes is therefore a detoxication mechanism. Enzymes accomplishing this hydrolysis will be termed DFPases in this discussion. They are not specific for DFP, but also hydrolyze other fluorophosphates and organic phosphate esters. Occurrence of DFPases is widespread in animal tissues and microorganisms. Their natural substrate is unknown.

Because of the rapid DIP formation and urinary excretion following DFP hydrolysis, most of an administered DFP dose is swiftly removed from the body. The small percentage of DFP which leaves the organism at a very slow rate is probably that which has combined tightly and irreversibly with various tissue proteins. Held in this form, DFP would leave the body only when the protein molecule binding it was degraded.

In addition to the cholinesterases and DFPases, DFP interacts with such proteins as liver esterase, milk lipase, kidney phosphatase, chymotrypsin, and trypsin (Jansen *et al.*, 1949 a, b, Webb, 1948 a). Interactions with these enzymes result in their inhibition.

Additional references on DFP metabolism are as follows: Dixon *et al.* (1958 a, b), Fleisher *et al.* (1950), Jansen and Balls (1952), Klevens (1953), Matsubara and Nishimura (1958), Michel (1952), Mounter and Shipley (1958), Mounter *et al.* 1957 a, b), Neurath *et al.* (1952), Oosterbaan *et al.* (1958 a, b), Rapp and Sliwinski (1956), and Wilson *et al.* (1950).

Studies of DFP^{32} Metabolism

Because of the high toxicity of DFP and the lack of sufficiently sensitive chemical methods, it has been necessary to use radioactively labeled DFP to follow its fate in the animal organism. Studies have been carried out in the rabbit and the human. These studies have emphasized two important features, the importance of protein binding and the detoxication to DIP.

Jandorf and McNamara (1950), after administering toxic doses of DFP^{32} to rabbits, found a rapid disappearance from body tissues. In sacrifice experiments, 65, 12, and 11% of an intravenously injected dose (0.5–2.0 mg/kg) could be accounted for at 15 minutes, 5 hours, and 19 hours in the soft tissues. Kidney, liver, and lung retained the most activity. The expected metabolite DIP^{32} was also injected (1.1 mg/kg) with less than 1% recovery in the tissues in 18 hours, demonstrating its rapid excretion. The high retention of DFP^{32} by kidney, lung, and liver was due to strong protein binding. After 4 hours, essentially *all* of the P^{32} activity of these organs was bound firmly to protein and could not be

extracted from tissue homogenates by trichloroacetic acid. As will be mentioned later, these organs are rich in DFPases.

Plasma radioactivity after injection fell rapidly to 10% in 6 hours, nil in 6 days; plasma cholinesterase inhibition continued for several days and regained normal levels in 6 days. Erythrocytes took up radioactivity relatively slowly but retained it for a longer period than plasma. Unfortunately these authors did not quantitate the high urine radioactivity.

Cohen and Warringa (1954) administered nontoxic doses of DFP^{32} intramuscularly to humans (0.24–1.99 mg/human) and reported irreversible DFP binding to proteins of blood plasma and red cells. Within 24 hours, 25–35%, and within 9 days, 60–65% of the radioactivity had appeared in the urine; at 2–3 weeks, less than 4% had appeared in the feces. Paper chromatography of urine revealed only one radioactive metabolite, DIP, and presumptive evidence was obtained that none of the injected DFP was excreted unchanged.

Pretreatment of a patient with octamethyl pyrophosphoramide (OMPA) was shown to alter greatly the blood distribution of radioactivity. By combining with the same sites of the protein as DFP, OMPA prevented the binding of DFP and therefore free DFP was more rapidly removed from the blood than in normal patients.

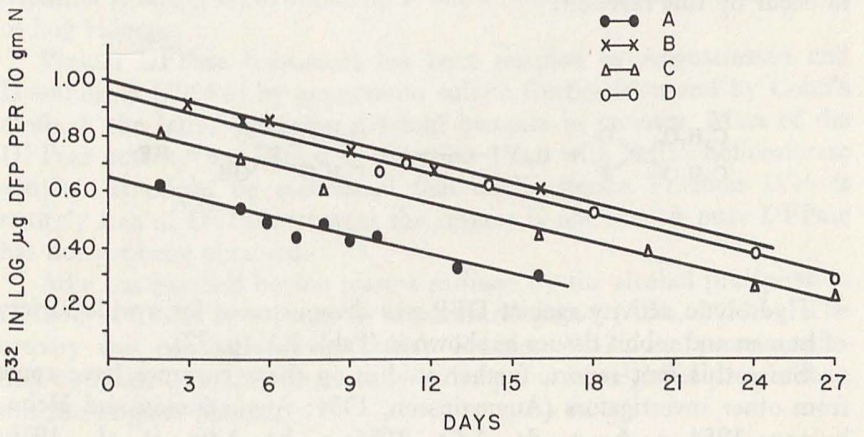


FIG. 7. Plasma bound radioactivity after injection of DFP^{32} into subjects A, B, C, and D. From Cohen and Warringa (1954).

DFP^{32} binding by plasma and red blood cells was so tight that calculations of protein turnover could be made. The protein was presumed to be a pseudocholinesterase. From curves such as those shown in Fig. 7,

Cohen and Warringa obtained half-lives for plasma protein of 12, 13, 13, and 14 days, and for red cell protein half-lives of 58 and 64 days. These figures compare favorably with accepted values obtained for serum albumin, serum globulin, and red blood cells. Leeksma and Cohen (1956), using similar methods, have shown the life span of blood platelets in man to be 8–9 days.

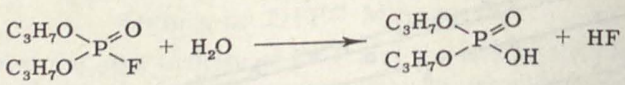
It is evident from the DFP^{32} studies that DFP is both bound to tissue proteins and degraded by them. This enzymatic degradation is accomplished by the DFPases.

Additional references are as follows: Cohen and Warringa (1957 b), Gladner *et al.* (1958), and Schaffer *et al* (1958).

Enzymes Hydrolyzing DFP (DFPases)

1. *Background*

Mazur (1946 b) reported the existence of enzymes capable of hydrolyzing the alkyl fluorophosphates at the P—F bond. Although DFP was primarily studied, the dimethyl, diethyl, and ethylmethyl phosphofluoridates were also found to be hydrolyzed at rates equal to or greater than DFP. Using Warburg manometric techniques, DFP hydrolysis was found to occur by this reaction:



Hydrolytic activity against DFP was demonstrated for a wide variety of human and rabbit tissues as shown in Table XV (p. 72).

Since this first report, further studies on these enzymes have come from other investigators (Augustinsson, 1954; Augustinsson and Heimburger, 1954 a, b, c, d; Adie, 1956 a, b; Adie *et al.*, 1956; Hoskin, 1956 a; Mounter and Chanutin, 1953; Mounter and Dien, 1954, 1956; Mounter, 1954; 1955; Mounter *et al.*, 1953, 1955 a, b). It seems apparent at this time that these groups are studying the same reactions. However the nature of the DFPases is complex. It has not been obtained as a pure enzyme. DFPase in this discussion simply denotes the ability of a preparation to hydrolyze DFP at the P—F bond.

2. *Occurence*

DFPase activity can be found in the tissues of practically all species. Initially described in rabbit and human, it has subsequently been demonstrated in rat, cat, guinea pig, pigeon, and turtle (Mounter *et al.*, 1955 b), cow and pig (Augustinsson and Heimburger, 1954 a), and many microorganisms (Mounter *et al.*, 1955 a). Organs containing high DFPase activity are the liver (greatest activity by virtue of its large mass), kidney (highest activity per milligram protein nitrogen), lung, intestinal mucosa, and adrenals (rat, rabbit). Cholinesterase activity is not directly related, but varies rather in inverse proportion to DFPase activity.

Plasma contains essentially all of the blood DFPase. The following species order is found (Augustinsson, 1954; Augustinsson and Heimburger, 1954 a): rabbit > dog > horse > human > rat, cat > cow > guinea pig.

3. *Purification*

Rabbit kidney extract had been subjected to ethanolic precipitation by Mazur (1946 b) resulting in a 13-fold purification of DFPase activity. Further improvement of the ethanolic precipitation technique enabled Mounter *et al.* (1953) to obtain up to one hundred times the initial activity of hog kidney.

Plasma DFPase (tabunase) has been purified by Augustinsson and Heimburger (1954 a) by ammonium sulfate fractionation and by Cohn's method, the latter achieving a 4-fold increase in potency. Most of the DFPase activity was found in Fraction IV–1 with little cholinesterase activity. It might be mentioned that cholinesterase Fraction IV-6 is entirely free of DFPase whereas the reverse is not true. A pure DFPase has not yet been obtained.

Adie has purified bovine plasma sarinase by the alcohol precipitation method of Cohn *et al.*, and by ultracentrifugation (Adie, 1956 a). The activity was concentrated in Cohn's Fraction VI-2; the protein solution obtained was 1250 times more active per unit weight of protein than was the original plasma.

4. *Specificity and Mechanism*

Enzymes hydrolyzing DFP can hydrolyze many other compounds containing the P—O group (Mazur, 1946 b; Mounter and Dien, 1954; Augustinsson and Heimburger, 1954 d). Augustinsson and Heimburger (1954 a) suggested the general name, phosphoryl phosphatase; for all such

enzymes but used "tabunase" to denote activity against dimethyl amidoethoxy-phosphoryl cyanide.

Comparative studies performed on tabun and DFP indicated the identity of tabunase from cow and pig kidney and adrenals with the DFPase of Mazur (1946 b) and Mounter *et al.* (Mounter *et al.*, 1953, 1955 a, b; Mounter and Chanutin, 1953; Mounter and Dien, 1954, 1954 b; Mounter, 1954, 1955, 1956).

One enzyme in rabbit serum was believed (Mounter, 1954) to hydrolyze DFP, TEPP, Paraoxon, and *p*-nitrophenyl acetate. Evidence for a single enzyme was obtained by inhibition studies (heat, chemicals), electrophoretic fractions, and experiments with mixed substrates. It was simultaneously shown (Augustinsson and Heimburger, 1954 a) that several different enzymes must have been involved. Although tabunase and DFPase are identical, TEPP was hydrolyzed by at least one other of the Cohn serum fractions prepared by Augustinsson. Purified hog kidney DFPase (Mounter and Dien, 1954) hydrolyzed dibutyl, diethyl, and diisopropyl phosphofluoridate giving 1 mole of F^- per mole of phosphate ester. This enzyme also hydrolyzed diisobutyl, di-*n*-propyl, and di-*n*-amyl phosphofluoridate, and TEPP, as well as other esters and hexose phosphate (Mounter and Dien, 1956).

The mechanism of the enzymatic hydrolysis of DFP (as well as that of the other organic phosphate esters) has been suggested to be similar to that of cholinesterase inhibition (Augustinsson and Heimburger, 1954 a, b). In both reactions the same chemical bond is broken, *i.e.*, between phosphorus and the acid group—halogen, cyanide, organic radical. The difference then lies in the fate of the phosphorus compound. In inhibition, this portion becomes irreversibly bound to the esteratic site; otherwise the liberated compound is nontoxic and is eliminated by the organism. Hoskin (1956 a) showed that isopropyl methylphosphoric acid was the only phosphorus-containing product of the enzymatic hydrolysis of sarin. The hydrolysis is apparently a single first-order reaction (Adie *et al.*, 1956).

5. *The Complex Nature of DFPases*

Activation, inhibition, and specificity studies of DFPases from various sources indicate differences in behavior. These studies point to a number of enzymes possessing the ability to hydrolyze DFP.

As one example, rat kidney and liver DFPase is activated by Mn^{++} to a greater extent than by Co^{++}, which is the reverse of that found for cat and human liver (Mounter *et al.*, 1955 b). Furthermore, rat and hog liver appear to contain two different DFPases: one soluble, activated by Mn^{++} and Co^{++} and hydrolyzing DFP and di-*n*-butyl phosphofluoridate (DBFP) at equal rates; the other insoluble, activated by Ca^{++}, inhibited by Mn^{++},

Co^{++}, and Mg^{++}, and hydrolyzing dibutyl phosphofluoridate much more readily than DFP. Kidney of rat and hog contains only a soluble enzyme activated by Mn^{++} (Mounter and Chanutin, 1953; Mounter, 1955). The status of DFPases remains complex.

A high DFPase activity is presumably the reason that *Proteus vulgaris* and *Pseudomonas aeruginosa* can grow in 10 m*M* DFP. Numerous other microorganisms can also hydrolyze DFP (Mounter *et al.*, 1955 a); metal activation and chemical inhibition studies indicate a number of different DFPases.

6. *Significance of DFPases*

The DFPases must play an important role as a primary detoxication mechanism against DFP. While interaction with other tissue proteins certainly removes some circulating DFP, the presence of a tissue DFPase is probably the major factor in lowering the blood level of free DFP.

Consideration of the species difference in blood disappearance of DFP^{32} will point out this factor (Cohen and Warringa, 1954; Mazur, 1946 b; and Jandorf and McNamara, 1950). Thus in the rabbit, a species having high plasma DFPase, cholinesterase has a rapid fall of P^{32} activity, while in the human with less DFPase, cholinesterase P^{32} activity falls at a slower rate.

Information about the enzymatic hydrolysis of DFP has given some appreciation of the role of DFPase in studies of DFP action. Use of untreated human serum greatly affected experiments of DFP cholinesterase inhibition *in vitro* (Augustinsson and Heimburger, 1954 c), because two simultaneous DFP interactions occurred, first, the DFP inhibition of cholinesterase and second, the hydrolysis of DFP by DFPase.

Additional references dealing with the enzymatic hydrolysis of phosphofluoridates are: Adie (1958), Adie and Tuba (1958), Augustinsson and Heimburger (1954 a, b, c, d), Bell and Mounter (1958), Bergmann *et al.* (1957), Cohen and Warringa (1957), and Mounter *et al.* (1958).

Acute Effects of Organofluorine Compounds in Experimental Animals

Burger (1951) characterizes the fluorinated compounds as much less active physiologically than the corresponding derivatives containing the other halogens and even less active than the nonfluorinated compounds. The stability of fluorine derivatives he attributes to the relatively shorter distance between carbon and fluorine atoms and to the strong resonance. Some years ago, the inertness of certain saturated fluorocarbons led to the

hypothesis that marked physiologic potency would be limited to unsaturated fluorinated compounds, but this generality has not been established. At present the listing of compounds and their effects may at least call attention to possibilities of modifying the physiological properties of molecules by fluorination.

Plan of Review

So many organic fluoride compounds (and so many types of these compounds) have been administered to experimental animals and so many kinds of effects have been discovered that no manner of consistent unifying presentation has been found. Some guidance will be helpful in examining the compilation of information that follows. The following plan has been evolved:

(1) The acute effects are described and a table listing acute doses of a large number of fluorine-containing organic compounds is presented. In this table are compounds that have been discussed previously, such as the fluoroacetates and phosphofluoridates, but there are also many other fluorine-containing compounds not previously referred to. (*A*) The approximate lethal atmospheric concentrations of a number of halogenated compounds (*a*) as the undecomposed vapor and (*b*) as thermally decomposed vapor are next presented.

(2) A lengthy table listing the effects of many fluorine-containing compounds has been prepared. Some of the effects have been the object of considerable study. These are discussed in the following order: (*A*) Central nervous system effects, (*a*) general anesthesia, (*b*) local anesthesia, (*c*) depressants of the spinal cord; (*B*) Sympathetic nervous system effects; (*C*) Metabolic effects, (*a*) tyrosine analogs, (*b*) steroids, (*c*) enzymes, (*d*) antitumor compounds; (*D*) Effects on microorganisms, (*a*) antimalarials, (*b*) fungi, (*c*) viruses; (*E*) Miscellaneous pharmacological properties; (*F*) Rodent repellency; (*G*) Insecticidal activity.

(3) Chronic effects. This classification evolved from the varied topics that have claimed the interest of research workers and by no means represents a balanced approach to the properties of the fluorinated organic compounds. For many of these topics the studies are fragmentary; the reviews and tabulations are of value only in giving an entry to the recorded data.

In Table XVI (p. 72) are listed the acute doses for a number of organofluorine compounds as determined in several species by various investigators.

Marked differences are observed in the acute toxicities of the organofluorine compounds; the range is greater than for the soluble inorganic,

fluorides. Of the compounds investigated to date, fluoroacetamide, ethyl-5-fluorohexoate, 2-fluoroethyl-5-fluorohexoate, ethyl 9-fluorodecoate, sodium and methyl fluoroacetate, and diisopropylfluorophosphate are among the more toxic. Less than 1 mg/kg of each of these compounds will kill the average animal (LD_{50}) of at least one species (Table VI).

Inhalation Toxicity

In Table XVII* are given, for a number of halogenated compounds, the approximate lethal concentrations in milligrams per liter and in ppm (*a*) of the undecomposed vapor in an exposure chamber and (*b*) of the thermally decomposed vapor (at 800°C) cooled to room temperature before exposing the rats. The approximate lethal concentration (ALC) was obtained by exposing one rat in each test to a series of increasing concentrations with a 50% increase in concentration from test to test. The lowest concentration producing death in a 15-minute exposure was called the approximate lethal concentration. It is obvious that some of these compounds, notably CF_3Cl and CF_4, have remarkably low acute toxicities. All of these compounds produced irritation to the respiratory system at various levels. Most of the deaths resulted from pulmonary edema and in some cases from pulmonary hemorrhage. A few rats died apparently from the anesthetic action. Both the *undecomposed* and the pyrolized vapors produced pulmonary irritation when administered in lethal and near lethal concentrations. The pathological changes in the other organs were notably insignificant. In Table XVIII† are listed the toxic effects by inhalation of a number of fluorocarbons.

The principal interest has been directed toward the polymers of tetrafluoroethylene and especially to the thermal decomposition products (Anonymous, 1955; Challen *et al.*, 1955; Sherwood, 1955; Treon *et al.*, 1955 b). Other fluorohydrocarbon decomposition products are discussed by Sendroy *et al.* (1952) and by Treon *et al.* (1955 b).

Effects of Acute Doses of Fluorine-Containing Organic Compounds

In contrast to the inorganic fluorides which, in general, give a consonant pattern of toxic effects depending mostly on the ability to furnish fluoride ions, a variety of biological effects has been noted in experimental animals following the acute administration of organofluorine compounds.

* Table XVII is on p. 89.
† Table XVIII is on p. 89.

A number of these effects are recorded in Table XIX.* Miscellaneous biological properties of organofluorine compounds are also to be found in: Behrman and Stainer (1957), Berger *et al.* (1957), Buu-Hoi *et al.* (1949), DeGarilhe *et al* (1951), Eden and Rennerfelt (1946), Fabre (1947), Fisher and Joullie (1958), Helin and Vanderwerf (1952), Hermann and Muhlemann (1958), Knight and Young (1958), Lands *et al.* (1958), and Miller *et al.* (1957).

Anesthetic Effects

The low toxicity of many of the fluorinated organic compounds, their high degree of stability, and their relative nonflammability have encouraged the search among these compounds for new anesthetic agents. Krantz and his group have been particularly active (Krantz *et al.*, 1953). Among the fluorine-containing ethers the trifluoroethylvinyl ether (fluoromar) has received most attention (Dundee and Dripps, 1957; Musser *et al.*, 1957; Park *et al.*, 1957; Sadove *et al.*, 1956, 1957). Recently the new compound, 1,1,1-trifluoro-2,2-bromochlorethane (Fluothane, Halothane), has been used under carefully observed conditions. A report to the Medical Research Council by a committee headed by J. H. Burn (Anonymous "Fluothane"—A Report, 1957) summarizes the pharmacological actions. Clinical investigations are summarized by a committee headed by Dr. Burns (Anonymous "Fluothane"—A Report, p. 483, 1957). Other references are: Brindle *et al.* (1957); Chang *et al.* (1957); Gain and Paletz (1957); Given *et al.* (1957); Hudon *et al.* (1957); Junkin *et al.* (1957); Pittinger *et al.* (1957); Raventos (1956); Bryce-Smith and O'Brine (1956).

Literature on the properties and uses of Fluothane has continued to increase, as indicated by the following references: Beaton (1959), Boivin *et al.* (1958), Brown (1959), Brown and Woods (1958), Burns *et al.* (1957), Burton (1958), Delaney (1958), Devine *et al.* (1958), Dobkin (1959 a, b), Epstein (1957), Feldman and Morris (1958), Griffith *et al.* (1958), Gusterson (1959), Hall and Norris (1958), Hudon *et al.* (1958), Johnstone (1958), Krantz *et al.* (1958 a), Long *et al.* (1958), MacKay (1957), MacKay and Kalow (1958), Mapleson (1957), Marrett (1959), McGregor *et al.* (1958), Ngai *et al.* (1958), Payne *et al.* (1959), Pope (1957), Raventos and Dee (1959), Robson and Sheridan (1957), Robson and Welt (1957 a, b), Seiflow (1957), Severinghaus and Cullen (1958), Sniper (1958), Stephen *et al.* (1957, 1958 a, b), Suckling (1957), Virtue *et al.* (1958), Watland *et al.* (1957 b, c), and Wyant *et al.* (1958).

It is interesting to note that hexafluorodiethyl ether has convulsant, not anesthetic, effects.

* Table XIX is on p. 92.

The low water solubility and relatively high lipid solubility of the saturated fluorocarbons (up to C_6) suggested to Struck and Plattner (1940) the possibility that these compounds might show valuable anesthetic properties (Table XX*). Decafluorobutane, decafluorocyclopentane, and dodecafluorocyclohexane were investigated; ansthesisa was not produced in the mouse and the compounds proved to be toxic. Pathologic examination of the animals indicated that death was due to an "irritating gas." The effects of the higher boiling C_5F_{10} compound were more severe than for the C_4F_{10} compound. With the increased interest in fluorocarbons and their use as refrigerants and aerosol propellents, Lester and Greenberg (1950) investigated the toxicity of a number of halogenated derivatives of methane and ethane. Of the nine compounds tested, vinyl and vinylidene fluoride, 1,1,1,2-tetrachloro-2,2-difluoroethane and 1,1,1,2-tetrachloro-1,2-difluoroethane, did not show anesthetic properties. The latter compound, as well as 1,1-difluoroethane, 1-chloro-1,1-difluoroethane, and 1,1-difluoro-1,2-dibromoethane, were pulmonary irritants. None of the compounds appeared to damage the liver.

Local Anesthetics

Campaigne *et al.* (1941) investigated the local anesthetic effects of various alkamine esters of fluorobenzoic acid; all but diethylaminoethyl *p*-fluorobenzoate proved superior to procaine and produced considerable local irritation. Diethylaminoethyl *p*-fluorobenzoate produced little irritation and appeared similar to procaine in both the goldfish and dermal wheal tests. Young and Zbarsky (1944) isolated *p*-fluorophenylmercapturic acid in the urine of rats injected subdermally or subcutaneously with fluorobenzene; these authors thus conclusively demonstrated that all monohalogenated benzenes are similarly converted *in vivo*.

A number of additional references to the anesthetic properties of fluorine-containing organic compounds follow: Esquibel *et al.* (1957, 1958), Jones *et al.* (1958), Krantz *et al.* (1957 a, b, Ling *et al.* (1959), Lu *et al.* (1953), and Truitt *et al.* (1958).

Mephenesin Analogs

Lindenstruth *et al.* (1950) have prepared and investigated a series of fluorine-containing α- and γ-substituted glycerol ethers. In these compounds the methyl group of 3-(2-methylphenoxy)propane-1,2-diol, or mephenesin, is replaced by a trifluoromethyl group or the fluorine atom. The compounds were comparable in depressant effect to the corresponding bromine and chlorine analogs but were slightly less effective than the

* Table XX is on p. 114.

parent compound. The α,γ-disubstituted ethers showed only insignificant effects, while the trifluoromethyl analogs, particularly when this group was in the *meta* position, were extremely effective. The *o*-fluoro analog was the most powerful compound of the fluoro-substituted series.

Sympathomimetic Amines

Fluorinated sympathomimetic amines have been investigated by Suter and Weston (1941), by Marsh (1948), and by Burger (1951). In general, the compounds showed pressor activity in dogs and guinea pigs, and depressor activity in rabbits. Replacement of the *para*-hydrogen by fluorine in phenethylamine and phenethylmethylamine resulted in compounds more active as sympathomimetic amines; the fluorinated analogs were not as effective as *p*-hydroxyphenethylamine (tyramine).

Metabolic Effects of Tyrosine Analogs

Several fluorine analogs of tyrosine and phenylalanine have been investigated as possible therapeutic agents in the treatment of thyroid disorders. While clinical trials have been reported in Germany (May, R., 1950; May, W., 1942) these agents have received less favorable attention in this country. In fact, Boyer *et al.* (1941), following experiments with these agents on the basal metabolic rate of the rat, suggest that, under the conditions of their experiments, the use of these compounds is contraindicated because of their high toxicity. The basal metabolic rate was not affected by 3-fluorotyrosine and 3-fluorophenylalanine at dosages up to one half the minimal lethal dose; a slight drop was obtained with 3,5-difluorotyrosine at approximately three fourths of the minimal lethal dose. The latter compound apparently is the least toxic member of the series. Very good agreement is noted among the acute toxic doses as determined by Litzka (1936 b), Boyer *et al.* (1941), and Niemann and Rapport (1946). Niemann (personal communication) states that 3-fluorophenylalanine has about the same toxicity as 3-fluorotyrosine, a finding in agreement with that reported by Boyer (1941). According to Niemann, 2-fluorophenylalanine, 2-fluorotyrosine, and 4-fluorophenylalanine are relatively non-toxic to mice; toxicities are sometimes 500-fold less than those for the *meta* compounds. Casterra (1947) has reported the treatment of a series of 500 cases of hyperthyroidism in man with 3-fluoro-4-hydroxyphenylacetic acid; the lethal dose of this material for white mice is reported to be 3500 mg/kg with no effect on the bones and teeth. Euler *et al.* (1949) report the same lethal dose.

The thyroxinelike activities of a number of fluorine-containing compounds, investigated by Cortell (1949), Martin *et al.* (1950), and

Barker *et al.* (1951) (cf. Table XIX), have been reviewed by Selenkow and Asper (1955). Apparently the fluorine analogs of thyronine are the least effective and require the presence of iodine in the 3,5 positions. As far as is known, halogenation in the 3,5 positions of thyronine is essential for thyroxinelike activity; the order of effectiveness is I > Br > Cl > F. The same order of relative activity has been found for halogenation of the 3′,5′ positions in compounds already 3,5-halogenated.

9α-Fluorocorticosteroids

In 1953 Fried and Sabo reported marked glucocorticoid activity for a new group of derivatives of cortisone and hydrocortisone, in which the 9α-hydrogen atom is replaced by halogen. The activity was found to be inversely proportional to the size of the halogen atom; for the chloro derivatives, the activity was four times that of the parent hormones. Following this lead, the fluoro derivatives were also prepared (Fried and Sabo, 1954; Fried *et al.*, 1955 a) and in several instances found to be much more active than the parent compounds. Table XXI* shows the relative effectiveness of a number of these fluorinated corticosteroids with respect to sodium retention and glucocorticoid activity. No significant differences in activity were found between 11β-hydroxy and 11β-keto derivatives. The 9α-fluoro-11β-hydroxy and 11β-keto derivatives of progesterone approximately equaled cortisone acetate in glucocorticoid activity, though both are lacking the 17- and 21-hydroxy groups. 9α-Fluorocorticosterone acetate and 9α-fluorodehydrocorticosterone acetate were the most potent mineralocorticoids, approximating the activity of aldosterone.

Swingle *et al.* (1955) found that adrenalectomized dogs could be maintained in a symptom-free state by the daily oral administration of 6.5–13.75 mg of 9α-fluorocorticosterone acetate, or 27.5 mg of 9α-fluorohydrocorticosterone acetate. The former compound was approximately twenty times as effective as deoxycorticosterone (DOC) (and approximately equal to aldosterone) in maintaining the test animal, whereas the latter, though more potent than DOC, has only one fourth to one fifth the activity of aldosterone. Large oral doses of 9α-fluorohydrocorticosterone acetate gave rise to polydipsia and polyuria with edema of the hind legs and scrotum.

Liddle *et al.* (1954) reported that 25–100 mg of 9α-fluorohydrocortisone acetate per dog was 2.4–9.2 times as effective as DOC in sodium retention in the adrenalectomized animal. These dosages were without effect on circulating eosinophils, or on the glomerular filtration rate. At levels of 200–800 mg/dog, eosinopenia and the glomerular filtration rate both

* Table XXI is on p. 120.

increased, and there were losses of Na and K in the urine; the latter element was lost at all doses investigated. The paradox with regard to sodium retention and loss is explained by the assumption that, though tubular resorption of sodium increases at low dosages, at higher levels the increased filtration rate presents an excessive level of sodium to the tubules.

9α-Fluorohydrocortisone acetate was less effective than deoxycorticosterone acetate in promoting growth and survival of adrenalectomized rats, though somewhat more effective than the reference compound in effecting sodium retention. Thus, there is not always a direct correlation between these two properties (Borman *et al.*, 1954). This compound also was reported to be more effective than cortisone acetate in maintaining the body temperature of adrenalectomized mice given *Brucella* somatic antigen (Halberg and Spink, 1955).

Singer and Borman (1955) investigated the anti-inflammatory properties of several fluorocorticosteroids; 9α-fluorohydrocortisone acetate was 13 times as effective as hydrocortisone acetate, 9α-fluorocorticosterone acetate 4.1 times as effective, 9α-fluoro-11β,17α-dihydroxyprogesterone 0.4 times as effective, and 9α-fluoro-11β-hydroxyprogesterone was without effect.

Boland and Hendley (1954) and Ward *et al.* (1954) have reported on the use of these compounds in rheumatoid arthritis. The latter investigators found the rheumatic symptoms to be reduced in patients treated with 4–8 mg/day for 12–28 days; increased sodium, chloride, and water retention was noted, together with a loss of potassium. Liddle *et al.* (1954) found that 0.5 mg of 9α-fluorohydrocortisone acetate given orally to two patients corrected within 4 hours the symptoms of Addisonian crisis. Goldfien *et al.* (1955) also have investigated the effects of these compounds in patients with adrenal insufficiency.

No explanations are yet available as to how the fluorocorticosteroids bring about their effects. The possibility that they are more slowly metabolized in the liver, which is a primary site of steroid metabolism, has been disposed of by Todd and Hechter (1955) who showed that 9α-fluorohydrocortisone is destroyed only slightly more slowly than is hydrocortisone.

Structure-activity relations in this group of compounds have been comprehensively reviewed by Fried (1957). Recent information relating to the pharmacological and therapeutic aspects of these materials is included in a monograph edited by Miner and Jailer (1955).

Additional references dealing with the fluorocorticosteroids are: Adeymo *et al.* (1957), Arth *et al.* (1958 a, b), Barger *et al.* (1958), Bernstein *et al.* (1957), Bischoff *et al.* (1953), Boland (1956), Bowers and Ringold

(1958), Brown and Anason (1958), Busch and Mahesh (1958 a, b), Camerino *et al.* (1956), Cope and Harrison (1955), Dulin *et al.* (1958), Engel and Noble (1957), Feinberg *et al.* (1958), Fitzpatrick *et al.* (1955), Fried (1957, 1955), Fried *et al.* (1955, 1958 a, b), Garrod (1955), Greene (1958), Gurling *et al.* (1958 a, b), Halberg (1955), Hershberger and Calhoun (1957), Hertz and Tullner (1956), Hogg *et al.* (1955 a, b, c, 1957), Jenkins and Schemmel (1958), Jenkins and Spence (1957), Kissman *et al.* (1959), Kleeman *et al.* (1958), Kupperman *et al.* (1958), Leith and Beck (1957), Liddle (1958), Liddle *et al.* (1956), Lipschutz *et al.* (1956, 1957), Maffii and Bianchi (1958 a, b), Maffii and Virga (1956), McAleer *et al.* (1958), McGavack and Seegers (1958), Mills *et al.* (1959), Munro (1958), Nayler (1957), Oliveto *et al.* (1958 a, b, c), Poutsiaka *et al.* (1957), Ringold *et al.* (1958), Rosemberg and Dorfman (1958), Sala and Baldratti (1957), Schreier *et al.* (1958), Schriefers and Korus (1958), Segaloff *et al.* (1958), Selye and Bois (1956), Shewell (1957), Silber and Morgan (1956), Singer and Borman (1956), Spero *et al.* (1956, 1957), Sturtevant *et al.* (1957), Sulzberger (1955), Swingle (1955), Swingle *et al.* (1957, 1958), Tanz and Whitehead (1957), Tanz *et al.* (1956, 1957), Taubenhaus *et al.* (1956), Thorn *et al.* (1955 a, b), Velardo (1956), Villa *et al.* (1956), Wakai and Prickman (1954), Ward and Hench (1955), West (1958), and West and Cervoni (1955).

Effects of Organic Fluorine Compounds on Enzyme Systems

The effects of fluorine-containing organic compounds on certain enzyme systems are indicated in Table XXII*. The effects of these inhibitors cannot be attributed to the fluoride ion, since the *in vivo* results produced by them are dissimilar to the *in vivo* effects of the F ion. The range of concentrations required for 50% inhibition indicates that diisopropylfluorophosphate and its derivatives are effective against cholinesterase in extreme dilutions.

This inhibition has been used as a basis for differentiating between true and pseudo cholinesterases; the latter enzyme is inhibited at lower concentrations of DFP than is the former (Aldridge, 1953). Aldridge *et al.*, (1947) have presented evidence suggesting that organophosphorus compounds are substrates for cholinesterase and are hydrolyzed, but that the substituted phosphoric acid resulting remains attached to the enzyme The more stable the compound is toward hydrolysis, the less effective it is as an inhibitor. The nature of the alkyl groups attached to phosphorus also determine the effectiveness by affecting the "fit" of the substrate on the enzyme surface. According to Mackworth and Webb

* Table XXII is on p. 121.

(1948), the most potent esters are those with short, branched-chain alkyl groups. The reaction of cholinesterase with P^{32}-labeled DFP has been investigated by Michel and Krop (1951) and by Jansen *et al.* (1952). The former investigators found that completely inhibited cholinesterase had combined with 2.1×10^{-10} mole of DFP per unit of enzyme activity; the latter workers showed that the phosphorus of the inhibitor was incorporated into the inactivated enzyme to the extent of 0.0023%. Boursnell and Webb (1949) showed that 1gm of DFP combined with 96,000 gm of liver esterase, with complete inactivation of the enzyme. These results agree well with those of Jansen *et al.* (1949 a, 1950) who showed that complete inhibition of α-chymotrypsin by DFP resulted in the incorporation of 1.1 moles of phosphorus per mole of enzyme. Two isopropyl groups are introduced per mole of enzyme, but no fluorine was found in the inert protein. Hydrofluoric acid was formed during the reaction, however, to the extent of about 1 mole per mole of α-chymotrypsin. These data are interpreted to mean that DFP reacts with α-chymotrypsin by a condensation reaction with the evolution of fluorine as hydrogen fluoride. Koelle and Gilman (1949) have calculated that following the administration of a lethal dose of DFP in the monkey (0.2 mg/kg), the concentration of DFP in the total body water will be approximately 1.7×10^{-6} *M*. At death the cholinesterase activity of the brain is virtually zero. The marked specificity of DFP for cholinesterase is clearly evident. The *in vivo* action of DFP in the rabbit has been investigated by Nachmansohn and Feld (1947); it was concluded that the cause of death in poisoning by this agent is the direct result of the complete inhibition of cholinesterase, with a consequent breakdown in the transmission of nerve impulses.

Koelle *et al.* (1950) have utilized the anticholinesterase activity of DFP as the basis of a histochemical method for differentiation of types of cholinesterases and their localizations in tissues. Specific cholinesterase was found in certain regions of the central nervous system, in skeletal muscle, and in smooth muscle in certain regions. Nonspecific cholinesterase was localized chiefly in the sensory ganglia, the adrenal pericapsular ganglia, the hepatic cells, the carotid body, and smooth muscle from other sites. Both types of enzyme were found in most autonomic ganglia and effector cells. Myers and Kemp (1954) have suggested that dimethylcarbamoyl fluoride and alkanesulfonyl fluorides also inhibit cholinesterases by attaching to the enzyme and leaving an hydrolysis product bound to the previously active center.

Hydrolysis of esters of fluoroacetic acid by cholinesterase and dog liver esterase was investigated by Bergmann and Shimoni (1953) who found that the maximal rate of hydrolysis of *n*-alkyl fluoroacetates by cholinesterase increases with increasing chain length. Branched alkyl chains resulted in

much lower hydrolysis rates by both enzymes, the effect on cholinesterase being greater than that on liver esterase.

Additional references in this field are: Cohen and Posthumus (1955), Comba and Costa (1954), DeCandole *et al.* (1953), Gladner and Laki (1956), Jansen *et al.* (1947, 1948, 1949, b,) Miller and Van Vunakis (1956), Myers (1951, 1952, 1956 a, b,), Myers and Kemp (1954), Myers *et al.* (1957), Oosterbaan *et al.* (1955, 1956).

Anti-Tumor Action

Among the compounds (Table XXIII*) that have been tested for possible usefulness for the chemotherapy of cancer are: fluorinated pyrimidines (Bollag, 1957; Heidelberger *et al.*, 1957 b; Wong and Benson, 1957); pyruvate (Busch *et al.*, 1957 b); fluoroacetate (also fluorocitrate) (Dietrich and Shapiro, 1956; Potter and LePage, 1949); fluorocholine (Luhrs and Bothe, 1956); fluorinated dimethylazobenzene (Miller *et al.*, 1953; Miller *et al.*, 1949; Price *et al.*, 1950, 1952); and miscellaneous compounds (Sellei *et al.*, 1953).

Additional references are: Ansfield and Curreri (1959), Bosch *et al.* (1958), Chaudhuri *et al.* (1958), Cohen *et al.* (1958), Cranston (1959), Curreri *et al.* (1958), Danneberg *et al.* (1958), Davern and Bonner (1958), Duschinsky *et al.* (1957), Eidenoff *et al.* (1957), Elion *et al.* (1958), Gordon and Staehelin (1958), Heidelberger *et al.* (1958), Kennedy (1957), Melnick *et al.* (1958), Montgomery and Hewson (1957), Nemeth *et al.* (1957), Reichard *et al.* (1959), Rich *et al.* (1958), Sirota and Wishnow (1958), Skold (1958), Stone and Potter (1956, 1957 a, b), Theret (1957).

Effects of Organofluorine Compounds on Microorganisms

The effects of organofluorine compounds on a number of microorganisms are listed in Table XXIV (p. 131); the actions range from "ineffective" to "bactericidal."

Kalnitsky and Barron (1947) found the oxidation of yeast to be inhibited by the addition of small amounts of sodium fluoroacetate. In contrast, seven more complex molecules investigated by Eichler *et al.* (1949) were found to be without effect on yeast fermentation processes. Suter and Weston (1939) found the bactericidal effects of certain alkylphenols to be enchanced somewhat by the replacement of a ring hydrogen by fluorine; more pronounced effects were obtained, however, upon substitution of chlorine or bromine. Bacteriostatic properties of a number of aromatic acids were compared by Hager and Starkey (1943); in general,

* Table XXIII is on p. 128.

the introduction of a nuclear fluorine atom increased the bacteriostatic effect over that of the parent compound; again bromine was more effective.

The biosynthesis and characterizaiton of several fluorobenzyl penicillins are described by Clarke *et al.* (*The Chemistry of Penicillin*, Princeton University Press, 1949) and by Behrens *et al.* (1948 a, b); Behrens and Kingkade (1948). In general, these compounds are not as effective against microorganisms as is benzyl penicillin.

Several other compounds have been tested against other organisms. Abbot (1945) found *Physarella oblonga* Morgan to be inhibited by sodium fluoroacetate at a concentration of 0.75 mg/ml. Mitchell and Niemann (1947) found 3-fluoro-DL-phenylalanine in concentrations of 0.04 mg/ml to reduce the growth of *Neurospora crassa* 50%; D, L, and DL isomers of 3-fluorotyrosine inhibited growth of this organism by 50% at concentrations of 0.41, 0.15, and 0.23 mg/ml, respectively.

Additional references are: Agarwala *et al.* (1954), Aldous and Rozee (1958), Baker *et al.* (1958), Dagley and Walker (1956), Dunn and Smith (1957), Elsden and Ormerod (1956), Fowler and Werkman (1952), Horowitz *et al.* (1958), Manly *et al.* (1959), and Thoma *et al.* (1957).

Antimalarials

In Table XXV* data are given for the fluorine-containing compounds screened in the survey of antimalarial drugs done in 1941–1945 under the auspices of the Office of Scientific Research and Development (Wiselogle, editor, 1946). In Table XXVI* are listed similar data obtained by various authors for additional compounds. None of these compounds give much promise as candidate antimalarial agents.

Fungistats

During the period 1950–1954 a series of fluorine-containing aromatic organic compounds synthesized by chemists of the Illinois State Geological Survey (Finger *et al.*, 1952, 1955) were tested for fungistatic properties against four common fungi (see Table XXVII, on p. 148). An arbitrary rating was given to indicate the constitution-potency relations:

Very high potency (< 10 ppm)—Dinitrofluorobenzenes

High potency (10–200 ppm)—Fluoronitrophenols, fluorobiphenols, and fluorobiphenol sulfide

Fair potency (200–400 ppm)—Fluorophenols, mononitrofluorobenzenes, and fluoronitroanilines

Low potency (400–1000 ppm)—Fluorobenzoic acids, fluoronitrotoluenes, and fluorobenzoquinones

Very low potency (< 1000 ppm)—Fluoroanisoles, fluorohydroquinones, and diacetate derivatives

* Table XXV is on p. 140; Table XXVI, on p. 147.

The most effective compound tested was 1-fluoro-3-bromo-4,6-dinitrobenzene, which at a concentration of only 0.8 ppm completely inhibited growth of *A. niger*. Several of the compounds were tested for their effectiveness in maintaining the breaking strength of cotton thread exposed to *Mycothecium verrucaria*. The compounds showing most promise as protective agents are indicated in Table XXVIII.* The incorporation of 0.25% of 1-fluoro-3-bromo-4,6-dinitrobenzene in polyvinyl chloride conferred complete protection against *A. niger*, *A. flavus*, *Trichoderma* sp., and *Penicillium luteum*.

The Prevention of Deterioration Center of the U.S. Department of Agriculture also has tested a number of compounds for fungistatic behavior toward *A. niger*. Of these, α-(*p*-chlorophenoxy)α-α^3,α^3,α^3-trifluoro-*m*-acetotoluidide was the most effective; 43% inhibition was produced at the test concentration of 250 ppm.

Additional references dealing with fungicidal properties of organofluorine compounds are: Discher *et al.* (1958), Hamm and Speziale (1957), Jeney and Zsolnai (1957), and Johnston *et al.* (1957).

Viruses

The effects of sodium fluoroacetate on growth and infectivity of several viruses have been investigated. The growth of Eastern equine encephalomyelitis virus was decreased in mice previously treated with fluoroacetate, and the survival time of the host was prolonged; the course of the infection was not otherwise affected. Prior treatment of the virus with fluoroacetate did not affect either its infectivity or its effects in mice (Watanabe *et al.*, 1952). The early growth phases of poliomyelitis virus, Lansing strain, in mice treated previously with fluoroacetate were depressed, and the appearance of the first signs of illness were delayed (Ainslee, 1952). Again, prior treatment of the virus with the compound was without effect on its infectivity. Multiplication of influenza viruses A and B are reported to be delayed by fluoroacetate (Mogabgab and Horsfall, 1952), while growth of mumps virus and pneumonia virus of mice is only slightly depressed. Growth of the pneumonia virus of mice was not affected by prior treatment with fluoroacetate.

Some studies of the use of fluorohydrocarbons in removing anticomplement in the purification of various virus preparations have been reported by Epstein (1958), Gessler *et al.* (1956 a, b), Holt and Epstein (1958), Hummeler and Hamparian (1957), Pearson *et al.* (1952), Porter (1956), and Manson *et al.* (1957). Fluorinated halogen hydrocarbons also have been discussed by Flick (1954).

* Table XXVIII is on p. 150.

Additional references on the topic of microorganisms are: Aldous and Rozee (1956), Berry and Mitchell (1953), Elsden and Ormerod (1953), Ingram and Salton (1957), Mager *et al.* (1955), Tanenbaum (1957), Windfuhr (1952), and Scheiner *et al.* (1957).

Miscellaneous Pharmacological Effects of Organic Fluorides

From time to time reports appear of the biological actions of certain fluorinated compounds. A diversity of subject matter has been included, as the following list testifies:

(1) A blood pressure effect (Lands, 1952);

(2) A membrane transfer of tetraethylammonium derivatives (Farah and Frazer, 1957; Farah *et al.*, 1950, 1953, 1955);

(3) A depression of adrenal ascorbic acid content (Little *et al.*, 1954);

(4) Sympathetic nervous system blockade (Ulloyt and Kerwin, 1956);

(5) Fluorine derivatives of curare derivatives (Adank *et al.*, 1953; Asmis *et al.*, 1954; Bickel *et al.*, 1954; Kebrle *et al.*, 1953; Waser, 1954);

(6) A loss of hypnotic effect in barbiturates (Bruce and Huber, 1953).

Rodent Repellency

Rodent-repellent properties of a number of fluorine-containing compounds have been investigated by the Fish and Wildlife Service of the U.S. Department of the Interior, according to techniques described by Bellack and DeWitt (1949, 1950). Results obtained are listed in Table XXIX, on p. 151.

Additional references are: Curculio (1951), Horsfall (1957), Hüter (1952), Robinson (1948, 1953), Ward (1946 a, b), and Welch (1951).

Organic Fluorine Compounds Useful as Insecticides

In Table XXX* are listed the results of numerous investigations of the activity of 2,2-bis(*p*-fluorophenyl)-1,1,1-trichloroethane (DFDT) against various insects. Similar investigations of the activity of miscellaneous organofluorine compounds are included in Table XXXI, on p. 154.

Summerford (1950) has prepared an excellent review of the chemistry, insecticidal activity, and toxicity of organofluorine compounds. The contact activity of DDT-type compounds apparently is associated with the presence of one or two *p*-substituted phenyl rings on one carbon of an ethane chain, with a di- or tri-halogen group on the second alkyl carbon atom. The lipid-soluble —CX_3 grouping has been suggested as being necessary for penetration of the molecule. Kirkwood and Dacey (1946)

* Table XXX is on p. 152.

investigated the insecticidal activities of three fluorine analogs of DDT and found that those compounds containing the —CF_3 grouping were practically ineffective against *Drosophila melanogaster*, whereas 1,1-bis-(*p*-fluorophenyl)-2,2,2-trichloroethane was somewhat effective (cf. Table XXX). According to these investigators, these results were to be expected, since CHF_3 has been shown to be physiologically inert. A number of compounds containing the 1,1-(*p*-chlorophenyl)ethane bridge, but with residues corresponding to inhalation anesthetics other than chloroform, were investigated and found to show activities roughly proportional to the partition coefficient of the parent anesthetic. Although Kirkwood and Phillips (1946) showed DFDT to be ten times more soluble than DDT in fat, Summerford points out that both compounds are approximately equal in effect as insecticides and suggests that the distribution between fat and water may be more important than solubility in fat. Busvine (1946) also tested a series of DDT analogs and showed that solubility in oil was not essential to activity. Picard and Kearns (1949) feel that the lipid-soluble —C—C—Cl_3 grouping and the electronegative character of the *p-p'*-substituents are not solely responsible for insecticidal activity, but that the entire steric properties of the molecule must be considered. Browning *et al.* (1948 b) also consider steric factors to be of importance.

Metcalf (1948 a) believes the bis(*p*-fluorophenyl)methylene linkage to be more toxic than the bis(*p*-chlorophenyl)methylene structure; however, because of the greater volatility of the fluoro compounds an apparently lower toxicity may result, due to the lesser amount of residue.

Martin and Wain (1944) have advanced the theory that the insecticidal activity of DDT and certain related compounds is related to the ease with which hydrochloric acid is released, which then presumably affects vital centers in the insect. DDT and DFDT, however, show very different rates of dehydrohalogenation, though their insecticidal activities are comparable. Browning *et al.* (1948 b) could see no relation between toxicity and ease of dehydrohalogenation in the series of compounds which they compared.

Browning *et al.* feel that the attribution of specific properties to specific portions of the molecule is at present unjustified. Picard and Kearns (1949) suggest that the potency of related compounds should be determined in more than one species before relationships between structure and function are postulated.

Relations between structure and activity of large numbers of insecticides, including fluorine compounds, have been reviewed by Riemschneider (Riemschneider, 1947 a, b, c, d, e, f, 1951, 1954, 1955; Riemschneider and Kuhl, 1947; Riemschneider and Rohrmann, 1950), and by Frear and Seiferle (1947).

Bis[2-(2-fluoroethoxy)ethoxy]methane (Questel and Bradley, 1951), bis(2-fluoroethoxy)methane (Martin, 1949), and sodium fluoroacetate (David, 1950) are unusually interesting compounds in that they are absorbed by plants in concentrations sufficient to kill feeding insects. Heretofore, only esters of certain polyphosphoric acids and some selenium compounds have shown this property.

Additional references dealing with insecticidal and pesticidal properties of fluorine-containing organic compounds are: Applegate and Howell (1958), Applegate *et al.* (1957), Ascher (1957, 1958 a, b), Bornstein *et al.* (1957), Cohen and Tahori (1957), David and Gardiner (1951, 1953), David *et al.* (1958), Manefield and McDougall (1951), Menon *et al.* (1951), Smallman and Fisher (1958), Tahori *et al.* (1958), and Winteringham *et al.* (1957).

Compounds acceptable as insecticides must not excessively damage the plant nor retard its growth when applied in concentrations that show adequate activity against various pests. The effects of a number of organofluorine compounds tested as plant growth regulators are indicated in Table XXXII, on p. 167.

Additional references in this field are: McLane *et al.* (1953), McIntosh (1955), Minarik *et al.* (1951), Oláh (1950), Oláh and Pavlath (1954 a, b, 1955), and Ready and Minarik (1954).

Chronic Experimental Intoxication

The essential facts concerning several experiments on chronic toxicity from organofluorine compounds are listed in Table XXXIII (p. 170.) In Table XXXIV (p. 178), the organ systems involved, together with the changes encountered, are given.

According to Boyer *et al.* (1941), levels of 3-fluorotyrosine as low as 0.0005% inhibit the growth of young rats; levels of 0.004–0.005% were lethal in 1–22 days. In reasonable agreement with this level is the dosage of 2–20 mg/kg six times per week used by Euler and Eichler (1942); their rats survived 1–156 days. Boyer *et al.* indicate that a dietary level of 0.0025% produced growth retardation equivalent to 0.1% sodium fluoride. While Euler and Eichler report tooth and bone changes in their rats similar to those caused by inorganic fluorides and greater changes per unit of organic fluoride, Boyer *et al.* found skeletal fluoride concentrations lower in treated rats than in control rats from which they conclude that the effects of 3-fluorotyrosine are not the result of the fluorine per se.

May and Litzka (1939) found the mouse to be more resistant to fluorotyrosine; moreover, the formation and growth of transplanted tumor in treated mice was inhibited. Niedner (1941) could show no effect on the tumor viability by daily intraperitoneal injections of fluorotyrosine.

Euler *et al.* (1949) investigated a series of organofluorine compounds and found the adrenals, thyroid, and parathyroid to be slightly affected, while the kidney, liver, and teeth were not significantly injured. These authors used a higher dosage of 3-fluoro-5-iodotyrosine (15–20 mg/kg/day) than Boyer *et al.*

Kempf and Nelson (1936) found *p,p'*-difluorobiphenyl, *p*-fluorobenzoic acid and fluorobenzene to be ineffective toward the rat's teeth, as was 3-trifluoromethyl-4-chlorophenol (unpublished data, Sterling-Winthrop Research Institute, Dr. C. M. Suter).

Of the three species in which the chronic toxicity of diisopropylfluorophosphate was studied, the rat appears to be the most resistant (Koelle and Gilman, 1946 b). The dog showed cardiospasm, hind-leg paralysis, and urinary incontinence. In both the dog and monkey the formed elements of the blood, blood sugar, protein and nonprotein nitrogen, and hepatic function were not directly affected. The monkey was predisposed to bronchopneumonia.

Greenberg and Lester have investigated the inhalation toxicity of some difluoroethanes for the rat (Greenberg and Lester, 1950; Lester and Greenberg, 1950). The symmetrical tetrachlorodifluoroethane proved to be a pulmonary irritant; neither isomer was effective upon ingestion. 1,1-difluoroethane and 1-chloro-1,1-difluoroethane, at atmospheric concentrations of 10 and 1% respectively, produced only a mild diffuse round cell infiltration of the lungs upon 2 months' exposure. The latter compound, however, at 10% in the atmosphere produced an extensive hepatization and consolidation of the lungs, and was lethal to all animals within 9 days.

Several additional chronic studies are described in: Jacobson *et al.* (1959), Oberst *et al.* (1956), Punte *et al.* (1958), and Treon *et al.* (1954).

Azouz *et al.* (1952) have investigated the metabolism of benzene and of fluoro-, chloro-, bromo-, and iodobenzenes in the rabbit. Relatively large amounts of benzene and of fluorobenzene were excreted unchanged in the exhaled air, whereas lesser amounts of the other halogenated benzenes were so excreted. About 30% of fluorobenzene was oxidized *in vivo*; of this about one third was excreted as glucuronides and about two thirds as ethereal sulfate. Only 1–2% appeared in the urine in the form of mercapturic acid. About 25% of the other halogenated benzenes appeared in this form. The authors concluded that the metabolic fate of fluorobenzene more closely resembles that of benzene than it does that of the other halogenated benzenes.

Bray *et al.* (1958) investigated the fate of *o*-, *m*-, and *p*-fluoro-, bromo- and iodonitrobenzenes in the rabbit. All three fluoro compounds were shown to undergo hydroxyldehalogenation to some extent; in the other

halogen nitrobenzenes the halogen group was not sufficiently labile to be replaced by hydroxyl. Over 30% of the dose of the *ortho* and *para* fluoro compounds was excreted as mercapturic acids; the *meta* compound did not form this metabolite. About 30–60% of the dose of the *ortho* and *meta* compounds was excreted as phenols conjugated with glucuronic and sulfuric acids; less than 10% of the *para* compound was so excreted. The main phenolic metabolite of *p*-fluoronitrobenzene is *p*-aminophenol. Less than 10% of the dose of all three fluoro isomers is excreted as the corresponding anilines.

Miscellaneous

Occasionally fluorine-containing organic compounds have been utilized as aids in unraveling metabolic pathways. Examples of this approach have been described earlier in the work of Pattison *et al.* with ω-fluoro-labeled compounds. Another instance is to be found in the calcification studies of Solomons *et al.*

Solomons and Irving (1958) have recently used 1-fluoro-2,4-dinitrobenzene as a reagent in establishing differences between the reactivity of certain amino groups of soft tissue collagens that do not calcify and hard tissue collagens that do calcify. The key amino acids are lysine and hydroxylysine. The epsilon lysyl and hydroxylysyl amino groups in intact soft tissue collagen react with fluorodinitrobenzene to the extent of about two thirds of those groups present, whereas, in the hard tissue collagens, as decalcification proceeds the reactive epsilon amino acid groups satisfactorily account for all those unmasked. In decalcified dentine collagen small amounts of *N*-terminal aspartic and glutamic acids were present. Use of this technic introduced by Sanger (1945) has led Solomons and Irving to postulate that the epsilon lysyl and hydroxylysyl amino groups may be a part of the template on which hydroxylapatite crystals deposit in the bone or other hard tissues when calcification begins.

Appropriate references are Sanger (1945) and Solomons and Irving (1955, 1956 a, b).

TABLE I

ACUTE FATAL INTOXICATION CAUSED BY SODIUM FLUOROACETATE[a]

Year	Cause	Age	Sex	Total
1946	Accident	8 mo	—	1
1947	Suicide	Middle age	M	3
	Suicide	—	M	—
	Accident	15 yr	M	—
1948	Accident	11 mo	F	2
	Accident	1 yr	F	—
1949	Accident	14 yr	M	7
	Suicide	—	M	—
	Suicide	—	F	—
	Accident	18 mo	F	—
	Accident	Young adult	M	—
	Accident	2 yr	F	—
	Suicide[b]	49 yr	M	—
1951	Suicide[c]	Young adult	M	2
	Accident	19 mo	F	—
1955	Suicide[d]	17 yr	M	1

[a] Unpublished data from J. C. Ward, U.S. Department of Agriculture, and from R. Moore and W. W. Dykstra, U.S. Fish and Wildlife Service, unless otherwise indicated.

[b] Harrison *et al.* (1952 b).

[c] Unconfirmed estimate of dose, somewhat less than 14 gm.

[d] Brockmann *et al.* (1955).