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FLUORIDE
Quarterly Reports
Issued by
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The International Society for Fluoride Research will hold its Sixth Conference in historic Williamsburg, Virginia, November 7 to 9, 1974. This town is easily accessible by air to foreign and U.S. participants. It offers an unusual opportunity to observe, at first hand, the way of life experienced during the early years of our nation. Reservations should be made through Miss Ernestine Stowell, 42 Mountain View Street, South Hadley, Massachusetts 01075.

The program committee is now soliciting abstracts up to 300 words of papers dealing with any phase of fluoride research. They should be submitted in triplicate to the Society's office, P.O. Box 692, Warren, Michigan 48090. The deadline for papers is June 15, 1974.

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MANUSCRIPTS for publication should be submitted in English, double-spaced with generous margins. References should be arranged according to the order in which they are cited in the text, and written as follows: Author, title, journal, volume, pages and year. Each paper must contain a summary of not more than 20 lines.

Contributors will receive 10 copies of the issue of FLUORIDE containing their paper, free of charge.

FLUORIDE is listed in
Current Contents Agricultural
Food and Veterinary Sciences
EDITORIAL

FLUOROACETATE TOXICITY

The biological effect of a certain element is largely dependent upon the other elements with which it is combined, especially upon whether the compound is inorganic or organic. Recent advances in our understanding of the toxicity of such elements as lead and mercury have brought into focus the fact that the great differences in their biological action are due to the make-up of the compound whether it is in an organic or an inorganic form.

With respect to organic fluorides, some are relatively inert whereas others are extremely toxic. Among the latter, fluoroacetate and its salts have received much attention in the literature mainly because of their toxicity to domestic and wild animals. They occur in the biosphere as a rodenticide known as compound 1080. They are also found naturally in poisonous plants such as Dichapetalum cymosum (Gifblaar). In both instances they constitute a serious threat to livestock.


Sir Rudolf Peters of the Department of Biochemistry, the University of Cambridge, was first to identify fluoroacetate as the toxic agent in gifblaar. He and his collaborators have carried out most extensive research on the subject. His contribution to the symposium summarizes his wide experience with this material. Dr. M. N. Egyed of the Kirmon Veterinary Institute, Bet Dagan, Israel, has studied extensively the clinical phase of the toxicity of fluoroacetate and fluoroacetamide in sheep. Dr. Peter Ward of the Department of Biochemistry, Agricultural Research Council, of the Institute of Animal Physiology, Cambridge, reported on his studies concerned with the biochemical aspect of organofluorides, especially their conversion to fluoroacetate in plants. Prof. P. Buffa of the Institute of General Pathology, University of Modena, Italy, has made significant contributions to the metabolic action of the compound in animals which he outlines in detail in this issue.

One of the vital questions on the subject of organofluorides is the possibility that minute amounts might be present in the human organism either through conversion from inorganic fluoride or through ingestion of food containing the toxic agent.

In 1968, Cheng, Ming-Ho, Miller and Wilkie (1) demonstrated, in forage plants, the presence of fluoride taken up from the air or from the soil and its conversion into fluorocitrate. Ward and Huskisson (2) showed that fluorocitrate is present in lettuce in microgram amounts. These observations were supplemented by Peters and Shorthouse who detected trace amounts of fluorocitric acid in fluoroosed bones of cattle. On page 187 of this issue Miller, Yu and Psenak have presented data strongly suggestive of the presence...
of fluoroorganic compounds in the heart and kidneys of cattle and horses with skeletal fluorosis.

Whether or not under certain conditions such minute amounts of organic fluorides are present in the human organism, whether the human body is able to convert inorganic fluorides to organic form, and whether or not the presence of micro amounts of these toxic agents has any significant effects on human health, constitutes one of the most challenging aspects of fluoride research.

Bibliography


EDITORIAL

"OPTIMAL" FLUORIDE IN DRINKING WATER

A vast array of publications emphasizes that a level of 1 part of fluoride in one million parts of water (ppm) is the optimal concentration for reduction in the incidence of tooth decay. A somewhat lower level (0.6 to 0.7 ppm) is officially designated optimal in hot climates.

A recent survey, by Yoshitsugu Imai written in Japanese and abstracted in detail in this issue appears to upset this widely held concept. This research was carried out on a large scale: Well water at 485 locations and tap water, in 491 cities and towns, serving 63,710,760 people was analyzed for its fluoride content. The number of school children in grades 1 to 6 afflicted with dental caries was related to the fluoride levels of their drinking water. Dr. Imai's method differs from that used in the customary official statistical studies which compare the number of decayed, missing and filled (DMF) teeth at various ages in a fluoridated town to the number of DMF teeth of the same age group without fluoridation.

The survey demonstrates that the most effective level for prevention of tooth decay lies between 0.2 and 0.4 ppm. Should these observations be confirmed, it would constitute a significant contribution to our understanding of the action of fluoride on teeth.
KEYNOTE AND HISTORICAL PERSPECTIVE
OF ORGANIC FLUORIDES IN PLANTS

by

R. A. Peters
Cambridge, United Kingdom

Not long ago the sole interest in fluorine in the biological field was the part which it plays as inorganic fluoride (1). Attention was concentrated on the toxic effects of fluoride in excessive doses. Now, this is no longer true, mainly because we are aware that fluoride can take part in the metabolism of plants and animals. Prominent among such metabolites is fluoroacetate (FCH₂·COO⁻), with its CF bond, which is stable even in boiling sulphuric acid. First synthesized in 1896 by Swarts in Belgium, its toxic properties were subsequently explored by Schrader, and in 1939/40 were examined in connection with the war. The really startling discovery was made by Marais in 1943/44 (2) that fluoroacetate was the toxic principle of the S. African plant, Gifhlaar, (Dichapetalum cymosum), a dangerous cattle poison in the Transvaal. Other species of the plant in Africa are known to be toxic; in one, in Nigeria, fluorooleic acid in the seeds is its main toxic component. Since 1961, many other toxic plants which may contain large amounts of fluoroacetate, have been found in Australia, in the North Western Territory, and in Western Australia, and also in S. America. They constitute a serious financial risk to the farmer. As Compound 1080, fluoroacetate is used to kill rabbits and vermin, and may endanger human life. The toxicity of plants varies, and is most pronounced in the early shoots (3). The extensive analyses by Hall (4) show a wide variation in the distribution of fluoride and of organically bound fluorine in different parts of the toxic plants.

It is well-known that in some chemical respects F⁻ behaves differently from other halogen compounds: The stability of the CF bond in fluoroacetate is an example. In other CF compounds, this bond may be less stable. For instance, if CO is inserted into fluoroacetate to make fluoropyruvate (FCH₂·CO·COO⁻), the CF bond combines with thiol (-SH) (5) compounds as does iodoacetate. Biologically also there are differences. For instance Epstein (6) found that, with barley roots, bromide inhibits chloride absorption competitively but that fluoride does not.

In our experiments (7) - working both with homogenates of seedlings and with single cell cultures of the Australian Acacia georgeanae - we found a conversion of inorganic F⁻ to CF compounds, although this change did not occur in all seedlings; some required extra doses of fluoride. At the same time there was an apparent loss of added fluoride. In experiments with homoge-n
nates of ordinary flowers (8) like buttercups and daisies (but not grass), we found losses of fluoride over and above the experimental error (Table 1).

**TABLE 1**

<table>
<thead>
<tr>
<th>Plant</th>
<th>$F^-$ added</th>
<th>$% F^-$ disappearing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acacia georgineae</td>
<td>40</td>
<td>33.8</td>
</tr>
<tr>
<td>Pea Seedlings</td>
<td>90.5</td>
<td>28.0</td>
</tr>
<tr>
<td>Seeds</td>
<td>11.6</td>
<td>26.0</td>
</tr>
<tr>
<td>Daisy</td>
<td>15.9</td>
<td>41.5</td>
</tr>
<tr>
<td>Asclepias</td>
<td>55.3</td>
<td>31.0</td>
</tr>
<tr>
<td>Curassavica (leaves)</td>
<td>26.2</td>
<td>52.5</td>
</tr>
</tbody>
</table>

Note. Losses also occurred with homogenates of Doronicum flowers, Thea chinensis and Acacia armata (New Leaves and fine roots), Aquilegia canadensis, varying from 33 - 44%.

Data from Peters and Shorthouse, Nature (London) 216, 80 (1967)

We have now traced some of these to the formation of fluoroacetone ($FCH_2CO.CH_3$); but clearly much more work needs to be done. Nevertheless, the experiments make clear that a metabolism of fluoride occurs in plants, about which you will hear more later.

We have tried to find the pathway by which the CF bond is synthesized in *A. georgineae*. We think that it does not go via a chloro intermediate (7), or by addition to a double bond in fumarate. Nor is there evidence for the hypothesis that it is formed via fluorophosphate (9), even in single cell cultures, although we have seen the formation of this on a paper chromatograph from a homogenate. It has been suggested that "fluoride is covalently bound to a C3 entity linked to pyridoxal phosphate, and then is converted to fluoroacetate by decarboxylation and transamination". Experiments on this question have not as yet been reported (10). The most promising hypothesis, we think, is that it combines with the known plant metabolite ethylene to form vinyl fluoride or fluoroethane (11). Here again much work is needed.

In animal tissues there is no evidence that the CF bond is synthesized. As a step in search for an antidote, it was discovered in 1948 independently, that treatment of particulate fractions from guinea pig kidney (12) and from ox heart (13) with fluoroacetate in vitro produced large increases in citrate. This occurred in vivo in rats and in other animals (14).
The hypothesis that a "Lethal Synthesis" occurred in the mitochondria via the condensing enzyme, synthase, and fluoroacetyl CoA, to form fluorocitrate was established by the isolation of fluorocitrate from poisoned kidneys (15). The Lethal Synthesis occurs because fluorocitrate, in small amounts, blocks the enzyme aconitase (Aconitate hydratase), thereby interrupting the citric acid cycle and producing large accumulations of citrate. The block is ultimately irreversible and, so far, no antidote to it has been found to reverse this block.

This Lethal Synthesis is presumed to be the cause of the tonic convulsions and/or the bradycardia and hypothermia occurring in various animals. The toxic doses vary widely for different animals, ranging from 0.05 mg/k in the dog, 0.4 mg/k in the sheep or rabbit, 5 mg/k in the rat to approximately 400 mg/k in the toad. The 4 isomers of fluorocitric acid have been isolated in brilliant work by Kun et al. (16); only one, the L-erythro-compound, is biologically active and capable of blocking the reactions of aconitase in very low amounts. When fluorocitrate is injected into the sub-arachnoid space of rats (under ether), the equivalent of 0.05 µg killed every 200g rat. In spite of extreme toxicity inside the brain or in mitochondria, when given by the intraperitoneal or intestinal route its toxicity is perhaps 1/200 less, and we have evidence that the animal can split off the F⁻ group to leave citrate and inorganic fluoride (17).

In vitro, the brain tissue of rats and pigeons does not convert fluoroacetate to fluorocitrate, in our experiments. It was stated recently that this was due to our use of ATP instead of ADP in our media; but we have found that no synthesis takes place even when ADP is used (Peters and Shorthouse, unpublished). The dog's brain may be an exception (18). An organically combined form of fluoride has been reported to be present in human plasma (19). We have found one fluorosed bone (cattle) (20) to contain detectable fluorocitrate by gas chromatography. On the other hand, it was not found in bone tissue from cattle fed upon fluoride, which was kindly sent to us by Dr. Suttie. We now think that the presence of fluorocitrate in bone depends upon the ingestion of fluoroacetaetate in the diet. In two rats, fed upon sublethal doses of fluoroacetate, we have detected small amounts of fluorocitrate in the bones, viz. 16 and 60 µg/g. This may possibly explain the results of experiments by Miller and Phillips (21), in which they detected an increased amount of fluoride in the bones of rats fed upon fluoroacetate.

The origin of the convulsions due to fluoroacetate has not, as yet, been satisfactorily explained. König (22) suggests that they are due to an immobilization of Mg²⁺ by the increased content of citrate. On the other hand, in view of the formation of fluoroacetyl CoA, the fluoroacetyl moiety could theoretically take part in several reactions in which acetyl CoA itself acts.

It is disappointing that so far no antidote to the poisoning has been found except monoacetin. The latter prevents entry into the citric acid cycle (23) and is, therefore, only useful if administered very early.

FLUORIDE
The fact that we found (17) relatively large amounts of fluoride (15 to 20 ppm) in the ordinary rat pellets in use in Great Britain for feeding rats prepared us for the finding that inorganic fluoride in small amounts was unlikely to be harmful. Now, of course, there is evidence in rodents from two sources that fluoride may be an essential element in the diet (24, 25).

Acknowledgements

I am grateful to Professor Sir Frank Young, F. R. S. for facilities provided; to the Wellcome Trust for grants for assistance and expenses, and to the Royal Society for provision of a gas chromatograph.

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22. Koenig, H., Personal communication.


FLUORIDE


METABOLISM OF FLUOROACETATE BY LETTUCE

by

P. F. V. Ward
Cambridge, Great Britain

The inhibition of aconitase by fluorocitrate formed from fluoroacetate in vivo was discovered over 20 years ago (1). The fatal effect of this reaction on living organisms and the ready in vivo conversion of a number of organofluorides to fluoroacetyl CoA has given the impression that many organofluorides always behave toxically. Recent work on fertility in mice (2, 3) suggests that fluorine is an essential trace element. It is also known to concentrate in bones and teeth where it is believed to be beneficial, provided the levels do not rise too high. No information seems to be available on the mechanism involved in the transport of fluorine to the appropriate organs. Although inorganic fluorine is found in bones and teeth, there is no reason why organofluorides may not be involved in this transport mechanism. The discovery by Taves (4) of a non-diffusible fluorocompound in normal human blood serum and a similar compound in the urine of workers engaged in the manufacture of hydrofluoric acid by Cernik, Cooke and Hall (5) supports this hypothesis. The levels of organic fluorine involved, should such a mechanism exist, would be so low as to be non-toxic even to the dog which is killed by as little as 50 μg/kg of fluoroacetate, and there would be no possibility of blocking the tricarboxylic acid cycle in any living organism. It was decided therefore to study the metabolism of fluoroacetate at sub-toxic levels. In order to isolate sufficient metabolites for structural studies, a plant system was chosen since plants are able to withstand much higher levels of fluoroacetate than most animals. Lettuce was used because of its convenient size and ready availability throughout the year.

From the Department of Biochemistry, Agricultural Research Council, Institute of Animal Physiology, Babraham, Cambridge CB2 4AT.

* * * * * * * * * *


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A cabbage lettuce, weighing 50-200 g, was dug from the ground, washed free from soil and incubated in the apparatus shown in Fig. 1 with metabolites. The metabolites were injected, dissolved in 0.1 ml of water, at the base of the stem with a Hamilton syringe. Three different incubations were used with the following metabolites: (a) for 43 hours with sodium $^{14}$C acetate (1 mg/kg wet wt.), (b) for 24 hours with sodium fluoroacetate (50 mg/kg wet wt.), followed by 43 hours with sodium $^{14}$C acetate (1 mg/kg wet wt.), (c) for 43 hours with sodium $^{14}$C or $^{2}$C fluoroacetate. Radioactive fluoroacetate was prepared from $^{14}$C chloroacetate and purified by the method of Ward and Huskisson (6).

**Fig. 1**

Apparatus for the Incubation of Lettuces With Either $^{14}$C Acetate, or Fluoroacetate and $^{14}$C Acetate, or $^{14}$C Fluoroacetate

The lettuce, after injection of metabolites as detailed in the text, was placed in the 20 cm, diam, desiccator with its roots immersed in 300 ml of water. Air from a cylinder was bubbled through the water. The effluent gas was first dried in a CaCl$_2$ tube, then bubbled via a sintered-glass dispersion disc through a tube containing 21 ml of 2-methoxyethanol-ethanolamine (2:1, v/v) followed by another tube containing 6 ml of the same mixture. The gas escaped through a soap-bubble flow-meter used to set the air flow to approximately 50 ml/min. The apparatus was illuminated by four white fluorescent tubes (61 cm, 20W) placed 30 cm above it.
In the $^{14}$C acetate treated plants, 15-20% of the radioactivity was expired as CO$_2$ and this was unaffected by preincubation with fluoroacetate, but with $^{14}$C$^+$ fluoroacetate treated plants there was only a very small amount (approximately 0.5%) of $^{14}$CO$_2$ expired. The evolution of $^{14}$CO$_2$ from plants incubated with $^{14}$C acetate started within a few minutes of injection and the rate of production and % conversion to $^{14}$CO$_2$ was similar even when the level of $^{14}$C acetate was increased to 50 mg/kg or when preincubation with sodium fluoroacetate had taken place. It was concluded that at the end of the incubations nearly all the acetate that had been directly converted to acetyl CoA and had entered the tricarboxylic acid cycle had been converted to CO$_2$ and that fluoroacetate at 50 mg/kg did not affect the ability of the cycle to deal with the quantities of acetate given. Radioactivity was found in many classes of compounds in $^{14}$C acetate treated plants. Table 1 gives the citrate levels in a number of plants after incubation with acetate or fluoroacetate. Although there is a considerable variation de-

<table>
<thead>
<tr>
<th>Substance injected</th>
<th>Acetate mg/kg</th>
<th>Fluoroacetate 50 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrate content of plant</td>
<td>18.2</td>
<td>39.8</td>
</tr>
<tr>
<td>$\mu$mol/g dry wt.</td>
<td>13.4</td>
<td>27.2</td>
</tr>
<tr>
<td>10.2</td>
<td>29.0</td>
<td></td>
</tr>
<tr>
<td>14.1</td>
<td>23.7</td>
<td></td>
</tr>
<tr>
<td>13.4</td>
<td>31.3</td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>13.9</td>
<td>30.6</td>
</tr>
</tbody>
</table>

pending on the age and size of the plant, citrate is invariably higher after incubation with fluoroacetate and, on average, is about twice as high.

Ethanol water extraction by the method of Canvin and Beavers (7) removed all the radioactivity from $^{14}$C fluoroacetate treated plants and preliminary experiments with ion-exchange resins showed that the radioactivity was entirely associated with acidic material. When the dried extract was acidified with $\text{H}_2\text{SO}_4$ and extracted continuously with ether some radioactivity appeared in the ether. The amount depended very much on the size and age of the plant. For young plants with a high leaf to stem ratio very little was extracted but for some older plants it increased to as much as 50%. Gas chromatography of the ether extract after methyla-
tion with diazomethane showed that the only radioactive components were unchanged fluoroacetate and a little fluorocitrate, about 2% of the initial radioactivity injected into the plant.

The appearance of fluorocitrate and the increase in citrate content of plants incubated with fluoroacetate at 50 mg/kg indicated that some interference with the tricarboxylic acid cycle had taken place. This interference is so small, however, as to have no noticeable effect on the efficiency of the cycle in oxidizing acetate to CO₂.

Examination of the distribution of fluorine after incubating lettuces with fluoroacetate showed that it was entirely extracted into ether under acidic conditions. There was a close correlation between the measured organically bound fluorine and that calculated for the unchanged fluoroacetate and fluorocitrate found in the ether extract. The remainder which ranged between 50 and over 90% of the fluorine added as fluoroacetate at the start of the incubation was inorganic. The endogenous fluoride in the lettuces was only about 3% of the fluorine added as fluoroacetate. Preliminary experiments showed that fluoride was quantitatively extracted into ether from water under acidic conditions. These results showed that all the metabolized fluoroacetate with the exception of the small amount of fluorocitrate had undergone scission of the C–F bond and the elimination of fluorine as fluoride.

Examination of the behaviour of the radioactive substances in ¹⁴C fluoroacetate treated lettuces on an Amberlite IR-120 (H) cationic-exchange column showed that unchanged fluoroacetate and fluorocitrate together with much pigmented material was rapidly eluted by water but the metabolized radioactive substances were eluted much more slowly and could be partially purified by this method. The partially purified metabolites were freed of high molecular weight impurities on a Sephadex G-25 column eluted with water. No radioactivity emerged with the high molecular weight materials. The metabolites could now be resolved into four radioactive components by elution from an Amberlite IR-120 (H) column eluted with water as shown in Fig. 2. After component R-4 was mostly eluted, the eluting solvent was changed to ammonia solution, and shortly afterwards a further small radioactive peak emerged. This peak proved to be residual R-4 remaining on the column after the water elution. Component R-1 which represented about 5% of the eluted radioactivity was a breakdown product of components R-2 and R-3 on the column, for whenever R-2 and R-3 were rechromatographed more of R-1 was formed. R-4 could be rechromatographed without breakdown. The components R-1 to R-4 were collected separately and purified to constant specific radioactivity by paper chromatography. Elementary analysis of the components showed that they were all devoid of fluorine, but R-2, R-3 and R-4 contained both nitrogen and sulphur. Qualitative tests showed that in all cases the sulphur was bivalently linked, but not as a disulphide or a thiol. Fig. 3 shows the infrared spectrum of R-3 which has many peaks characteristic of amino acids and a peak at 1530 cm⁻¹ typical of a

**FLUORIDE**
secondary amide group as found in peptides. The spectrum of R-4 was similar but lacked the peak at 1530 cm\(^{-1}\) which was replaced by one at 1490 cm\(^{-1}\) suggesting the absence of a peptide bond. All three components gave a purple color with ninhydrin. Fig. 4 shows the analysis of R-2, R-3 and R-4 and their hydrolysis products on a Moore and Stein Amino Acid Analyser fitted with a radioactivity detector (8). R-2 produced an unresolved double peak which ran earlier than R-3, but the hydrolysis products of both the compo-

**Fig. 2**

**Chromatography of Radioactive Metabolites of \(^{14}C\) Fluoroacetate**

Lettuce on a Column (25 cm x 1 cm diam.; VO 15 ml) of Amberlite IR-120 (H) Ion-Exchange Resin Eluted With Water for 1200 ml, Then With aq. NH\(_3\) Solution (sp. gr. 0.88) - Water (1:9, v/v)
The disconnected peaks are polystyrene wavenumber markers.

components appeared identical. These consisted of component R-4 and equimolar amounts of glutamate and glycine. More vigorous hydrolysis in both cases produced extra glycine which was radioactive and ninhydrin negative acidic radioactive peaks. R-4 itself was not hydrolysed but under vigorous hydrolytic conditions it too broke down to give ninhydrin negative acidic radioactive peaks. The N-trifluoroacetyl methyl ester of R-4 could readily be prepared by the method of Darbre and Islam (9) and this was sufficiently volatile to give a good mass spectrum. From this it could be inferred that R-4 was S-carboxymethylcysteine \([\text{HO}_2\text{C-CH(NH}_2\text{-CH}_2\text{-S-CH}_2\text{-CO}_2\text{H}}]\) (8). The same derivative of a commercial sample of S-carboxymethylcysteine gave an identical mass spectrum to the R-4 derivative. Since R-4 is S-carboxymethylcysteine, it may be deduced that either R-2 or R-3 is S-carboxymethylglutathione but this could not be confirmed by mass spectroscopy. The difficulty appeared to be in the preparation of a volatile derivative, both components gave a mixture of compounds when attempts were made to prepare a number of different derivatives, and the original components could not be recovered when the derivative groups were hydrolyzed off.

S-carboxymethylglutathione was synthesized by mixing solutions of equimolar amounts of neutralized reduced glutathione and neutralized 1-\(^{14}\)C iodoacetic acid and titrating the HI as it was formed against NaOH to maintain neutrality. The reaction was complete in approximately 2 h and the radioactive products were identical to the radioactive products of 1-\(^{14}\)C fluoroacetate treated lettuce. R-3 was the most abundant synthetic product
Fig. 4

Chromatography of Components R-2, R-3 and R-4 and Their Hydrolysis Products on a Moore and Stein Amino Acid Analyser Fitted With a Radioactivity Detector

<table>
<thead>
<tr>
<th>Component</th>
<th>Hyp</th>
<th>Glu</th>
<th>Gly</th>
</tr>
</thead>
<tbody>
<tr>
<td>R-2 + Hyp</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R-2 mildly hydrolysed</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R-2 strongly hydrolysed</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R-3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R-3 mildly hydrolysed</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R-3 strongly hydrolysed</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R-4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R-4 mildly hydrolysed</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R-4 strongly hydrolysed</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Time (h) 0 2 4 6 8 10 14 16

Each chromatogram shows peaks of the intensity of ninhydrin color (increasing upwards) and radioactivity (increasing downwards). The position of hydroxyproline is shown as a marker. The buffer gradient was pH 2.5 - 3.25 and the column temperature was 30°C.
and, as with the lettuce compounds, R-1 was formed when R-2 or R-3 were rechromatographed on an Amberlite IR120 column eluted with water.

The chemistry of S-carboxymethylglutathione appears to be complex and needs further investigation but the major product R-3 of both the synthesis from iodoacetate and the biosynthesis from fluoroacetate is very likely to be S-carboxymethylglutathione itself. It is tentatively suggested that R-2 is the two possible lactam ring structures formed from S-carboxymethylglutathione by the elimination of water between the free amino group of the glutamate and either the carboxyl group of the carboxymethyl group or the carboxyl group of the glycine. As can be seen with atomic models, these two ring structures are very compact and their formation would account for the very low ninhydrin color yield from component R-2 and, because of their similar and low pK values, for the rapid elution and composite nature of the component on an amino acid analyzer.

Since S-carboxymethylglutathione is readily formed from iodoacetate and glutathione, it was decided to examine the rates of reaction of the other monohaloacetates with glutathione at 25°C and pH 7. The rate was maximum for iodoacetate, less for bromoacetate, still less for chloroacetate and zero for fluoroacetate. This shows that unlike the reaction between fluoropyruvate and thiols, the reaction between fluoroacetate and glutathione is not spontaneous and that in vivo an enzyme is likely to be involved.

The synthesis of S-carboxymethylglutathione from iodoacetate produced the structurally very closely related R-2 component and S-carboxymethylcysteine as by-products and the isolation of these same compounds from fluoroacetate-treated lettuces makes it unclear whether all these compounds are formed within the lettuce or whether S-carboxymethylglutathione is the sole metabolite and the others are artefacts of the isolation procedure. An extract of 2-14C fluoroacetate-treated lettuce partially purified without using ion-exchange columns when chromatographed on the amino acid analyzer column showed radioactive peaks in the position of R-2 and S-carboxymethylcysteine as well as S-carboxymethylglutathione but a purified synthetic sample of 14C carboxymethylglutathione also gave similar peaks on the same column. The answer to this problem must await a greater understanding of the chemistry of S-carboxymethylglutathione.

The reaction between fluoroacetate and glutathione strongly resembles the metabolism of many aliphatic and aromatic halogen compounds by mammalian liver in which the halogen is split off and the conjugate formed with glutathione is excreted (10). It is possible that this detoxifying reaction occurs in the liver of fluoroacetate-poisoned animals since the level of citrate found in this organ is always less than in the other organs tested (11, 12). The relatively simple metabolism of fluoroacetate by lettuce reported here does not resemble that found for Acacia georgiana, peanut, castor bean or kidney bean by Preuss et al. (13). This is not unexpected but it is surprising for the other plants. Attempts were made to repeat the work of Preuss et al. with germinating peanut seedlings but only S-carboxymethyl-

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glutathione and its associated compounds could be identified as metabolites of fluoroacetate.

Bibliography


PRESENCE OF FLUOROORGANIC COMPOUNDS
IN HIGHER PLANTS

by

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SUMMARY: A number of exotic plants are known to contain
fluoroorganic compounds. The toxic component of more
than a dozen plant species is known to be fluoroacetate. Ind-
direct evidence using extraction with non-polar solvents com-
bined with paper chromatography, inhibition of aconitase and
infra-red spectrometry indicated the presence of fluoroace-
tate and fluorocitrate in soybean and crested wheat grass ex-
posed to atmospheric fluoride.

Gas chromatographic analyses of organic acids isolated from
crested wheat grass collected from areas of high atmospher-
ic fluoride pollution showed the presence of two peaks that
corresponded with the methyl esters of authentic fluoroace-
tate and fluorocitrate. The study also showed that the or-
ganic acid pattern in fluoride-exposed plants was signifi-
cantly changed compared to control tissue.

Introduction

The exact mechanism by which fluoride causes damage to plants is
poorly understood. Certain physiological responses, however, are known to be
associated with fluoride injury.

It has been established that treatment of plant tissue with fluoride
alters the normal respiratory rate of the tissues (1). Either a stimulation
or inhibition of the respiratory rate may occur depending on a number of fac-
tors such as the species and age of plant, the fluoride concentration used,
and the length of treatment.

The reasons for respiratory inhibition are fairly obvious since flu-

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oride is known to inhibit respiratory enzymes in plants when associated with mitochondria including malic, succinic and NADH dehydrogenases (2). An increase in total organic acids (especially malate and citrate) has been found in HF-fumigated plants (3). This suggests an in vivo effect of fluoride on TCA cycle reactions and specifically malic dehydrogenase and aconitase. Aconitase, which metabolizes citrate, has been shown in vitro to be insensitive to inorganic fluoride (4) but is very sensitive to an organic fluoride compound, fluorocitrate.

A number of plants that are toxic to animals are known to contain fluoroorganic compounds. The toxic component of Dicapetalum cymosum (Hook) Engl. (5), Acacia georginae, F. M. Bailey (6) and Palicourea marcgravii, St. Hill (7) has been demonstrated to be fluorooacetate. More than two dozen plant species are known to make fluorooacetate. Most of these are in the genera Gastrolobium R. Br. and Oxylbium Andr. (8). The toxicity of these plants to livestock and man led to the discovery of their toxic constituents and to the theory of "lethal synthesis" by Peters (9).

Indirect evidence suggests the presence of fluoroorganic acids in soybeans fumigated with HF (4), and in crested wheat grass collected in the field (10). Lettuce was found by Ward and Huskisson (11) to form fluorocitrate when 14C-fluoroacetate was added to a plant extract.

Peters and Shorthouse have shown recently that monofluorocarbon acids can be formed from inorganic fluoride by single cell cultures of A. georginae (12), tea (12) and Glycine max, Merr. (13). Fluorocitrate was also shown to be present in small amounts in commercial specimens of tea and oatmeal (12).

In gas chromatographic experiments, Weinstein et al. (14) were unable to detect fluoroorganic compounds in crop plants exposed to atmospheric fluoride in the field, HF fumigation in the laboratory or aqueous solutions of NaF.

In this manuscript, experiments from my laboratory are reviewed suggesting that the change in organic acids and the presence of fluoroorganic acids are directly related to fluoride treatment of crop plants.

Material and Methods

Some of the tissues used in the present study were obtained from soybean plants fumigated with approximately 60 ppb of atmospheric HF for 4 days. Leaves from soybean plants grown in a chamber fumigated with ambient air were used as controls. Kidneys and hearts from cows and horses suffering severe fluorosis were used in further studies.

Tissue used for organic acid extraction was extracted in 95% ethanol for 4 days using a Soxhlet-type apparatus. The extract was condensed,
water added and extracted several times with petroleum ether to remove some pigment and lipid materials. The aqueous extract was passed through successive columns of Amberlite IR 120 (H+) and IR 4B (OH-). Organic acids were eluted from the IR 4B column using 2N \( \text{H}_2\text{O} \). The \( \text{NH}_4\text{OH} \) eluate was condensed, diluted with \( \text{H}_2\text{O} \) and acidified. The mixture was filtered and the filtrate then extracted with sulfuric-ether using a separatory funnel. The ether extracts were combined, dried over anhydrous sodium sulfate and decolorized with a small amount of activated carbon. After filtration the extract was methylated using diazomethane (15) or boron trifluoride-methanol reagent (16).

The aqueous layer was neutralized using \( \text{NaOH} \) and the extract was passed through a Dowex 50-X8 (H+ form) column and extracted with distilled water. The eluates were evaporated to dryness and the residue was taken up in methanol and methylated using diazomethane.

An F & M model 402 gas chromatograph (Hewlett Packard) was used with a flame ionization detector. Analyses were carried out on dual columns using helium carrier gas. Columns were packed with either 10% Reoplex 400 or 10% polyethylene glycol 6000 on Chromosorb W AW/DMSC (60-80 mesh) (17).

Young soybean plants exposed to fluoride were analyzed indirectly for fluoroorganic acids. Separation and identification of fluoroacetate and fluorocitrate was accomplished by paper chromatography using strip chromatography (18) and micro analysis of fluoride (19).

Results

The methyl esters of citric, trans-aconitic, succinic, fumaric, malic, oxalic and malonic acids were identified gas chromatographically (Fig. 1). Table 1 shows the comparison of organic acid content in both the HF treated plants and the controls. The increases were: citric acid 130%, succinic acid 204%, fumaric acid 107%, malic acid 298%, malonic acid 164% and oxalic acid 218%. Trans-aconitic acid showed a decrease of approximately 30%.

The increase in citrate may be a reflection of an inhibition of the aconitase enzyme. This could be the result of a block in the metabolism of citrate by a form of fluoride.

Plants were analyzed for fluoroacetate and fluorocitrate. The chromatographed organic acid extracts from HF fumigated plants contained fluoride in paper segments 1 through 7, but little or none in segments 8, 9 and 10. Segments 1, 2 and 7 contained the highest concentrations (Fig. 2). The chromatographed organic acid extracts from plants treated with fluoroacetate contained various amounts of fluoride from segments 1 to 8 but segments 2 and 7 again contained the highest concentrations, although the relative concentrations in segments 2 and 7 were reversed (Fig. 3). When commercial fluoroacetate was chromatographed, fluoride was located in segments 6 and 7 of the paper strip (Fig. 4).
Fig. 1

Gas Chromatographic Separation of Methyl Esters of Organic Acids From Normal Soybean Leaf Tissue (----) and Fluoride-Treated Tissue (-----).

Numbers refer to peaks of following organic acids: 1, oxalate; 2, malonate; 3, fumarate; 4, succinate; 5, oxalacetate (?); 6, malate; 7, trans-aconitate; 8, citrate.
Fluoride in Chromatographic Strips

Profile Analyses of

FIG. 2

TABLE

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<th>Acids</th>
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Changes in Organic Acid Content of HF-Fumigated Soybean Leaves

Organic Fluorides in Plants

Notes: Concentration of org. flu in mmoles/8 days tissue.
Fig. 1
Profile Analysis of Fluoride in Chromatographic Stips

Fig. 3
Profile Analysis of Fluoride in Chromatographic Stips

Organic acids extracted from plants treated with fluoracetate. Aliquots of the extract used in chromatography corresponded to 1 gram of fresh leaves.
The infrared spectra of commercial citric acid, fluorocitric acid, and suspected fluorocitric acid isolated from the organic acid fraction of NaF-treated plant leaves, are shown in Figure 5, A, B, and C, respectively. A marked difference in the spectra is seen between commercial citric and fluorocitric acids. The latter showed strong absorption bands between 9 to 10 microns whereas the former did not. The spectrum obtained with the sample in question was closely similar to that of the commercial fluorocitric acid, as shown in Figure 5, B and C.

Plant samples collected from areas of high atmospheric fluoride (10) were shown to have fluoride in paper segments corresponding to fluoroacetate and fluorocitrate using short strip chromatography (18) and micro fluoride analysis (19). Aliquots of these same samples were used for organic acid separation using gas chromatography (4). Organic acids were also determined from the kidneys of horses that had ingested hay containing high amounts of fluoride.

**Fig. 5**

Infrared Spectra of Citrate Compounds

Wavenumber, cm

A. Commercial Citric Acid; B. Commercial Fluorocitric Acid  
C. Extract From Field-Grown Plants.
Several columns packed with different liquid phases (including 5% Versamid, 5% diethylene glycol adipate, 10% Tween 80, and 10% polyethylene glycol 20,000) were tested, but only those packed with 10% polyethylene glycol 6000 or with 10% Reoplex 400 on Chromosorb W gave satisfactory results for the organic acid separation. Table 2 shows the retention data for methylacetate, methylfluoroacetate, trimethylcitrate, and trimethylfluorocitrate separated on these columns. The columns had to be preconditioned and aged for maximum resolution and separation. Columns prepared with less liquid phase would have shortened aging time.

### TABLE 2

<table>
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<th>Reoplex 400b</th>
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<td>202</td>
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<tr>
<td>Fluorocitrate</td>
<td>55</td>
<td>213</td>
</tr>
</tbody>
</table>

a Temperature programmed from 65-215° C. at 3°/min.

b Temperature programmed from 60-200° C. at 3°/min.

Data from Yu and Miller, Envir. Sci. and Techn., 4:492-495, 1970.

The chromatograms obtained with extracts from plant and animal tissues are shown in Figures 6 through 9. Peaks 1 and 2 were tentatively identified as methylfluoroacetate and trimethylfluorocitrate, respectively, based on retention data and cochromatography.

From the chromatograms of the methyl esters of organic acids extracted from pods of *Acacia georginae* (Fig. 6) it is evident that there is a peak (peak 1) in a position corresponding to one produced by an authentic sample of methylfluoroacetate. This was confirmed by cochromatography. The chromatogram lacked a peak which would correspond with one produced by trimethylfluorocitrate.

On the chromatogram of an extract from crested wheatgrass (*Agropyron cristatum*) collected near a phosphate plant there are two peaks of methyl esters (peaks 1 and 2, Fig. 7) that correspond in retention data to those produced by methylfluoroacetate and trimethylfluorocitrate. The positions coincided when authentic esters were cochromatographed (Fig. 7). Good resolution of the peaks identified as the methyl esters of fluoroace-
Fig. 6
Chromatographic Separation of Methyl Esters of Organic Acids from Pods of Acacia Georganae

Fig. 7
Chromatographic Separation of Methyl Esters of Organic Acids from Crested Wheatgrass (Agropyron Cristatum) Harvested Near a Phosphate Plant

FLUORIDE
Fig. 8

Chromatographic Separation of Methyl Esters of Organic Acids From Greenhouse-Grown Agropyron Cristatum in the Absence of Significant Fluoride

10% polyethylene glycol 6000 on Chromosorb W (60-80 mesh)

Fig. 9

Chromatographic Separation of Methyl Esters of Organic Acids From Kidney of Horse That Had Grazed Fluoride-Containing Hay

10% polyethylene glycol 6000 on Chromosorb W (60-80 mesh)
tate and fluorocitrate was obtained with 10% Reoplex 400 on Chromosorb W. Not all grass samples collected showed the presence of these components. The presence of the components may be related to the specific sampling area in relation to the fluoride source. Further experiments are necessary to establish any correlation between presence of components and sampling area.

Chromatograms of methyl esters of organic acids from greenhouse-grown crested wheatgrass did not have peaks which corresponded to the two fluoroorganic acids (Fig. 8). The dotted lines illustrate cochromatographic positioning of the methyl esters of fluoroacetate and fluorocitrate.

Horses and cows suffering severe fluorosis were sacrificed and organic acid extracts prepared from hearts and kidneys. Chromatograms of the extract from the kidney of a horse gave two peaks which corresponded in retention data with the methyl esters of the fluoroorganic compounds (Fig. 9). Cochromatography with authentic samples coincided with the suspected compounds. No positive results were obtained with other samples.

As noted previously, malic, succinic and NADH dehydrogenases are inhibited in vitro by fluoride when the enzymes are assayed in intact mitochondria (2). Increases in organic acids such as malic acid are probably only indirectly related to inorganic fluoride since purified isoenzymes (malic dehydrogenase) are not inhibited by fluoride (20). The effect of fluoride on membrane integrity is a probable direct relationship inducing secondary changes such as increases in malic acid.

The results obtained indicate the presence of both fluorocitrate and fluoroacetate in some of the fluoro-contaminated tissue studied. These together with observations of Peters and Shorthouse (12, 13) suggest that inorganic fluoride may be incorporated into organofluoride compounds by crop plants. Although it has not been established that the biosynthesis of fluoroacetate and fluorocitrate is a general property of plants, experimental data appear to favor this view. Peters and Shorthouse (12) showed the presence of fluorocitrate in oatmeal and tea. Single cell cultures of tea or soybean were able to synthesize fluoroacetate and fluorocitrate (12, 13). Ward and Huskisson (11) have reported the conversion of fluoroacetate to fluorocitrate by lettuce.

The evidence indicates the presence of both fluorocitrate and fluoroacetate in cultivated crops. The compounds were present in microgram quantities far below the yields obtained with Acacia georginae or other exotic plants.

**Bibliography**


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CLINICAL, PATHOLOGICAL, DIAGNOSTIC AND THERAPEUTIC ASPECTS OF FLUOROACETATE RESEARCH IN ANIMALS

by

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In the following review, mainly the practical aspects of fluoroacetate research with emphasis on domestic animals will be summarized.

In the fifties and at the beginning of the sixties, most cases of large-scale poisoning of livestock in Israel, especially sheep, were due to thallium which was used as a rodenticide. This rodenticide was withdrawn

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from the market at the request of various authorities and was replaced by another supposedly less harmful one called fluoroacetamide. In an attempt to solve one problem, another one was created as bad or worse than one we were trying to correct. However, at that time, we did not know the details concerning the two incidents of organofluoride poisoning in England which occurred in 1963. In one case, an entire herd of cattle had to be slaughtered in Kent; in a second case, meat from a poisoned pony sold for animal consumption resulted in illness or death to about 100 dogs and cows which had eaten it. Only after a long investigation based on circumstantial, clinical, pathological and laboratory evidence was it established that the first case of poisoning was due to an organically bound fluoride compound, obviously fluoroacetamide, which was manufactured in a nearby factory, the effluents of which had contaminated the pasture (1, 2). As a result of these incidents, the use of fluoroacetamide (FAA) and sodium fluoroacetate (FAC) is permitted in England only in ships and sewers. In Israel the situation is somewhat different. Despite the fact that only licensed pest control operators are permitted to use FAA, since its introduction many incidents of poisoning have been diagnosed in our laboratory, mainly in sheep, but also in cattle, horses, dogs, cats and even in seals. Under these circumstances as much information as possible was gathered on the symptomatology, pathology, laboratory diagnosis and therapy of FAA and to some extent of FAC poisoning.

Source of Poisoning

Approximately 36 species of plants which belong to various botanical families are known to contain fluoroacetate (3). Professor Peters reviewed this aspect during this morning's session. I have no information about the occurrence of these plants in Israel. However a bait consisting of wheat grains mixed with 0.2% FAA and colored with a green dye is widely used as a rodenticide (4). FAC to a lesser extent is used as a rodenticide. It should be mentioned that both FAC and FAA are water-soluble. They are relatively stable compounds and have no specific odor or taste. Since the toxic principle in FAC or FAA poisoning is also very stable, the consumption of poisoned carcasses may cause secondary poisoning in non-target animals, mainly in dogs and cats.

Symptomatology

One of the most interesting features of the toxicology of fluoroacetates is the extraordinarily wide variation in response in various species of animals with regard to sensitivity and clinical manifestations. Toxic doses range from 20 - 60 µg/kg body weight in dogs, to 4-14 mg/kg in monkeys. Limited data indicate that toxicity in man is intermediate. Frogs require about 300 mg/kg to induce toxic symptoms (5). Two types of clinical manifestations are characteristic. Ventricular fibrillation can be observed in the rabbit, goat, sheep, horse and spider monkey; convulsions are seen in the dog, and guinea pig. Both of these symptoms may be present in cats, pigs and
Fluoroacetamide Poisoning

Rhesus monkeys. In rats and hamsters, however, convulsive seizures may occur in the animals dying of respiratory paralysis. There is no explanation of these species differences with regard to the clinical symptoms. However, a working hypothesis can be established if we consider that the myocardium and nerve cells are the first to show energy depletion.

It has been stated that FAA is considerably less toxic than FAC (6), since the former is not hydrolyzed rapidly in vivo to fluoroacetic acid; therefore, in FAA poisoning lower concentrations of fluorocitric acid should be expected (7). The ratio of the values of LD₅₀ for FAC and FAA is about 1:5 (8). The LD₅₀ of fluoroacetic acid, methylfluoroacetic acid and sodium fluoroacetic acid is identical for rabbits if injected i. v. (9). Practically any compound which can form FAC in vivo by some biochemical process is toxic. Examples of such compounds are esters, salts, amides, acid halides and anhydride of fluoroacetic acid (10).

A relatively long latent period between the intake or administration of the poison and the onset of symptoms is characteristic. In FAA poisoning this latency is somewhat longer than in FAC poisoning, since FAA is hydrolyzed first to FAC before fluorocitric acid is formed.

As far as the lethal dose is concerned, 20 mg/kg FAA given orally to sheep proved to be lethal in every instance (11). One or two hours after the administration of a fatal dose of FAA, increased respiratory rates with superficial and labored breathing occurred. After another one to two hours, grinding of teeth, drooping and leaning of the head against a wall was observed. Subsequently the animals became reluctant to move. They stood with abnormal forward extension of the forelimbs, with the hind limbs pointing backward. At this stage frequent urination was a characteristic feature. In a more advanced stage the sheep lay down with eyelids partially closed. In the terminal stage, a short period of excitement characterized by running-like movements of the limbs, preceded the sudden collapse. This was followed by extremely rapid labored breathing, accompanied by abnormal moaning. Frothing at the mouth and nostrils were signs of approaching death. The terminal symptoms in general lasted for 20 to 60 minutes, and the sheep died about 6 to 8 hours after the administration of the lethal dose.

As mentioned previously in sheep (and in some other herbivores) FAC (or FAA) has a distinct cardiac effect. The heart rate increased from 90 to 125 per minute to 150 to 250, 4 to 5 hours after administration of a lethal dose. In the terminal stage, the heart rate was well above 400 per minute, or was so rapid, that it was impossible to make an accurate count by the use of the stethoscope. In one instance, we succeeded in taking a suitable ECG recording approximately 1/2 hour before death. The electrocardiogram indicated ventricular fibrillation in all the 6 leads.

Irrespective of the route of administration, 0.4 mg/kg FAC body weight proved fatal within 4 to 6 hours. Increased hypersensitivity was caused by 0.2 mg/kg and similar doses, given at intervals of 2 and 11 days.
proved rapidly fatal (12).

The symptoms of acute FAC poisoning in cattle are similar to those in sheep. Steyn gave an excellent description of the clinical picture of poisoning due to the ingestion of the leaves of Dichapetalum cymosum in South Africa (13) and to the ingestion of Acacia georgiana in Australia (14). Withholding water delays the onset of symptoms. Recent investigations revealed that the LD50 and 95% confidence limits for sodium fluoroacetate were 0.393 (0.247 to 0.625) mg/kg for Hereford cows and 0.221 (0.149 to 0.327) mg/kg for Hereford steers and heifer calves. The lowest dose causing death was 0.156 mg/kg in one steer calf and the highest dosage from which one cow and one steer calf survived, was 0.312 mg/kg. The most characteristic feature was the absence of detectable gross symptoms of poisoning until just prior to death. Terminal symptoms, such as frequent urination, staggering, falling down, slight spasms, lasted for 3 to 20 minutes (15). This clinical picture closely resembles field cases of FAA poisoning, in which investigations subsequently revealed FAA poisoning in cattle.

In dogs, the species most susceptible to FAC poisoning, the clinical symptoms are manifested by distress, frothing around the mouth and nostrils, labored breathing, tetanic convulsions (resembling strychnine poisoning) and motions of limbs similar to swimming and running. A relatively long latent period elapsed between administration of poison or poisoned meat (11) and the appearance of symptoms. After convulsive seizures, the dog appears to be normal. Later, convulsions may start again and large amounts of saliva appear around the mouth and face and often bloody fecal material is excreted. Stimuli such as loud noises do not intensify the convulsions. The longer latent period between the intake of the poison and onset of symptoms is a valuable aid in the differential diagnosis between FAC and strychnine poisoning.

Pathology

Despite the marked clinical symptoms, the gross and microscopic pathology are of little value towards detecting the cause of death. Congestion and hemorrhages in the lungs, rumen, intestines, liver, kidneys, heart and adrenals in experimental FAA poisoning in sheep are the principal findings. Subcutaneous petechiae and frothy pink mucus in the trachea, bronchi and bronchioli are also observed. The liver has rounded edges and is congested, enlarged and purple in color. The lungs contain areas of dark red and purple discoloration and pits on pressure. Pink, frothy mucus oozes from their surfaces. Petechial and ecchymotic areas were also noted on the diaphragm in one sheep which died about 20 hours after administration of the poison. Similar changes were found on the epicardial and myocardial surfaces and on the mucosae of the rumen and intestines. Congestion throughout the cortex and medulla of the kidneys and focal hyperemic areas in the cortex of the adrenal glands can be seen. Histologically, pulmonary congestion with acute intra-alveolar hemorrhages and edema of the lungs are found. Congestion and hemorrhage and slight superficial coagulative necrosis of gastrointestinal mucosa are observed. Congestion with equivocal to slight degenera-
tion of tubular epithelium are found. The cortex of the kidneys exhibits hyperemia and vascular engorgement in experimental FAA poisoning in sheep (11).

The pathological changes in cattle and sheep in Gifblaar poisoning are practically identical. The heart is always in a flabby dilated state (16). In Acacia georginae poisoning, significant myocardial lesions were found in cattle: irregular areas of pallor appeared in the underlying muscles. On section, the myocardium itself was occasionally mottled. It was concluded that the myocardium is the most susceptible target organ as indicated by the pathological lesions (17); this is in accordance with the clinical manifestations. In dogs, in secondary FAA poisoning (by feeding meat from FAA poisoned sheep) the post-mortem changes were similar and even more striking than in the sheep themselves. Hemorrhagic gastroenteritis constitutes the most marked pathological change (11). Some of the changes found are associated with terminal convulsive seizures prior to death.

It is noteworthy that low daily doses of FAA (50 mg/kg food) fed to rats for 30 to 90 days cause testicular degeneration (18). This finding has been confirmed in guinea pigs and in a young ram (19).

Diagnosis

The laboratory diagnosis of FAC and FAA poisoning is fraught with difficulties. The estimation of organic fluoride, and the difference between total and inorganic fluoride, is too complicated a procedure for routine diagnosis (2). The most accurate and specific method is the isolation and identification of FAC or its metabolite, fluorocitric acid in biological material by gas chromatography (20). However because this procedure is time-consuming and requires sophisticated equipment it is utilized mainly for research purposes. The presence of fluorocitric acid in soft tissues or bones is not necessarily a consequence of FAC or FAA poisoning, since these compounds may be present in the tissues of fluorotic animals (20, 21). A rapid method which gives satisfactory results is the spectrochemical detection of fluorine from FAA treated wheat grains (22). The use of an ion-selective fluoride electrode for screening of FAC in bait or biological material meets in many respects the requirements of routine diagnostic purposes (23). Since the last two methods mentioned cannot differentiate between inorganic and organic bound fluorides, the results must be interpreted with special care. Circumstantial, clinical and pathological evidence should be considered before drawing final conclusions. Feeding of dogs with poisoned bait or meat induces secondary poisoning with characteristic clinical symptoms and specific biochemical lesions, but the method cannot be recommended for general use due to its cruel nature (11).

Among the various biochemical changes, a few can be utilized to support the tentative diagnosis. Accumulation of citric acid (24) in various organs, especially in the heart and kidneys, is a relatively easy to detect procedure. However this method gives satisfactory results only with fresh carcasses because the elevated citric acid disappears from the tissues as soon
as one hour after death (25). To be sure the disappearance of citric acid can be delayed to some extent by refrigerating or, even better, by freezing the tissues (2, 4). It should be borne in mind that citric acid accumulation occurs not only in FAC or FAA poisoning. Even acetamide and monoacetin, which are recommended as antidotes for the treatment of FAC or FAA poisoning, may cause an increase of citric acid in the tissues (8).

In view of the many difficulties, attempts were made to develop a method for diagnostic purposes by utilizing the effect of these compounds on citrate metabolism and by inducing secondary poisoning in guinea pigs, thus eliminating the dog as a host. This method was found suitable for supporting the diagnosis of chronic FAA poisoning in cattle (2), in experimental FAA poisoning in sheep (26) and in establishing diagnosis in field cases of FAC and FAA poisoning in cattle, sheep, and dogs (27). The method is based on intraperitoneal injections followed by analysis of tissue extracts and urine from FAA poisoned sheep. Levels of citric acid in the tissues of these guinea pigs are 2, 4 to 8, 5 times the levels in the control animals. The degree of citric acid elevation was dependent on the type of the injected tissue and the conditions of storage of the tissues (26). These investigations indicate that the method can be successfully used in diagnosis of poisoning even as late as 72 hours after death. This demonstrates that a metabolite of FAC, obviously fluorocitric acid, is very stable, even in decomposed carcasses. Injection of tissue extracts and urine from normal sheep did not induce accumulation of citric acid in the kidneys of guinea pigs.

Some other biochemical changes in FAC or FAA poisoning include ketonemia (28), hyperglycemia (29, 30), elevated blood lactic acid levels (2, 4) elevated serum phosphorus as well as increase in the concentration of various blood enzymes such as ICDH, LDH, SDH, SGOT and SGPT (31). These findings show the multiple effects of FAC and FAA on the metabolism of the poisoned organism. The measurement of the degree of inhibition of the enzyme aconitase by an extract obtained from the kidneys and heart muscle of FAA poisoned sheep is a simple, rapid and direct method. This method has the advantage that the enzyme inhibitor is present in the kidneys as long as 40 hours after death (32).

**Therapy**

FAC bears a pronounced structural resemblance to acetic acid and the two can compete with each other with regard to incorporation in the tricarboxylic acid cycle. Therefore one might assume that any acetate donor (such as sodium acetate, monoacetin and acetamide) would afford protection against poisoning by competing with FAC. In ruminants, acetate is a major end product of fermentation in the rumen, and the concentration of acetate in the blood of these animals is usually 3 to 10 times higher than in that of non-ruminants (12). Certain feeds (like lucerne and gluten) were actually found to have some protective effect in induced FAC poisoning in sheep, due probably to increased production of volatile fatty acids (including acetates) by these feeds in the rumen (33). Yet in experimental sodium FAC
poisoning in sheep, acetate itself given intraruminally had very limited prophylactic value (12). Monoacetin prevented or reversed the neurotoxic and cardiotoxic effects of FAC in monkeys as long as 30 minutes after the ingestion of the poison, when convulsions had already started to develop (34).

Since acetamide (an acetate donor) is capable of penetrating into the mitochondria, it was assumed that it would be able to interfere with fluoro-citrate formation (35). Acetamide protected white rats against FAC poisoning when given simultaneously or up to 24 hours before the dose of FAC. It had no protective effect when given 30 minutes or more after the administration of FAC. The maximum permissible interval between FAC and acetamide administration to prevent poisoning was found to be 8 minutes (8). In experimental FAA poisoning in sheep, a dose of acetamide (200 mg/kg) given orally one hour after the administration of 20 mg/kg of FAA, had no protective effect. Another sheep given the two substances simultaneously also succumbed. Similarly repeated doses of 0.4 ml/kg of monoacetin failed to preserve the life of a sheep which had received a lethal dose of FAA. Neither acetamide nor monoacetine accomplished anything toward reversing the occurrence of hyperglycemia (36). The failure in our trials of monoacetin and acetamide to protect the poisoned sheep is probably due, at least in part, to the fact that the sheep is one of the most susceptible species as far as the LD50 of FAC is concerned. Attempts to develop an antidote to reverse the effect of fluoro-citric acid, the actual toxic agent in FAC poisoning, have proved futile. Of over 400 substances examined, only malonates had such an effect in vitro. The results of in vivo experiments, however, were much less promising (37). In view of these findings, in FAC or FAA poisoning no ameliorating treatment is known. However, attempts should be made to remove any unabsorbed poison from the gastrointestinal tract and to control the specific symptoms. This measure is the only one which might prove beneficial, when sub-lethal amounts of poison have been ingested.

Bibliography


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METABOLIC EFFECTS OF FLUOROOACETATE POISONING IN ANIMALS

by

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Modena, Italy

SUMMARY: Fluorooacetate poisoning causes a variety of metabolic effects in animals: block in the tricarboxylic acid cycle at the citrate stage; accumulation of citrate and lowering of adenosine triphosphate in tissues; increased production of ketone bodies; rapid hydrolysis of glycogen in liver, skeletal muscle and heart tissues and a parallel increase in blood glucose and lactate.

Injected fluorooacetate is rapidly distributed in all the tissues of the rat and 3% of it is transformed into fluorocitrate. The synthesis of fluorocitrate occurs in the mitochondria, in the complex formed by the inner membrane and the matrix. In isolated

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rat liver mitochondria fluorocitrate is formed from pyruvate plus fumarate or from acetate plus fumarate but not from fatty acids plus fumarate. Endogenous fluorocitrate inhibits specifically the mitochondrial-bound aconitase hydratase and not the enzyme present in the soluble cytoplasm. The primary biochemical lesion is soon followed by secondary inhibitions at various sites in the tricarboxylic acid cycle and ultrastructural changes in the mitochondria.

The increased production of ketone bodies is likely to be induced by inhibition of citrated synthase caused by fluoroacetyl-SCoA and the accumulated citrate.

Glycogen depletion in all probability is due to adrenalin release or intense sympathetic stimulation as a result of the biochemical changes caused by fluoroacetate. The parallel hyperglycemia and increased lactate formation can be explained by the rapid glycogenolysis.

The metabolic changes caused by fluoroacetate poisoning are discussed in the light of the present knowledge on cell biochemistry.

***

Here in Oxford, Sir Rudolph Peters and his collaborators (1, 2) first demonstrated that the toxic compound FAC (\(\text{CH}_2\text{F-COO}^-\)) in the animal body is transformed into FCIT and that this substance inhibits a basic biochemical mechanism of the aerobic cells, the tricarboxylic acid cycle (Fig. 1). Subsequent work from various laboratories provided further information on the changes leading to the biosynthesis of FCIT and proved that this compound in vivo inhibits the first of the two reactions catalyzed by

*Abbreviations used in this paper are: ADP, adenosine 5'-pyrophosphate; AMP, adenosine 5'-phosphate; ATP, adenosine 5'-triphosphate; CoA, coenzyme A; cyclic AMP, adenosine-3'5'-phosphate; EDTA, ethylenediaminetetraacetate; FAC, sodium fluorocitrate; FCIT, sodium fluorocitrate; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; LD, lethal dose; MFA, methylfluorocitrate; 100n mole of oxygen consumed by 1 mg of mitochondrial protein in one hour at 25°C.
the enzyme aconitate hydratase, i.e., the dehydration of citrate to cisaconitate (Fig. 1) (3-5).

The knowledge of the metabolic changes induced by FAC poisoning in the organism is far from complete; however, it seems that FCIT plays a central role in FAC toxicity. In fact, the biochemical modifications so far discovered in the animal organism in FAC poisoning seem to depend either on the impairment of the pathway of reactions involved in the biosynthesis of FCIT or on the inhibition of the mitochondrial aconitate hydratase.

In this report the main metabolic effects of FAC poisoning in animals will be reviewed and some recent results from our laboratory will be presented.

The Distribution of Injected Fluoroacetate in the Rat Organism

Gal and co-workers (6) showed that after intraperitoneal injection of sodium fluoroacetate-2,14C in the rat the label rapidly distributed in all the tissues of the animal (Table 1). Four hours after the administrat-

---

**Fig. 1**

Accepted View of the Biosynthesis of Fluorocitrate from Fluoroacetate in the Animal Cell and the Block of the Tricarboxylic Acid Cycle at the Citrate Stage Caused by Fluorocitrate

![Diagram of metabolic pathways involving aconitate hydratase and citrate metabolism.](https://via.placeholder.com/150)
TABLE 1*

Distribution of Radioactivity in Rat Tissues After Administration of Fluoroacetate-2-\(^{14}\)C

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Fluoroacetate Concentration ((\mu g/g) wet weight)</th>
<th>Radioactivity recovered (percent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>26.9</td>
<td>2.26</td>
</tr>
<tr>
<td>Liver</td>
<td>22.3</td>
<td>11.95</td>
</tr>
<tr>
<td>Heart</td>
<td>21.4</td>
<td>1.26</td>
</tr>
<tr>
<td>Kidneys</td>
<td>20.1</td>
<td>2.49</td>
</tr>
<tr>
<td>Intestine and Stomach</td>
<td>17.0</td>
<td>10.33</td>
</tr>
<tr>
<td>Lungs</td>
<td>16.8</td>
<td>4.05</td>
</tr>
<tr>
<td>Spleen</td>
<td>16.2</td>
<td>2.00</td>
</tr>
<tr>
<td>Testes</td>
<td>14.4</td>
<td>2.28</td>
</tr>
<tr>
<td>Carcass</td>
<td>8.2</td>
<td>59.70</td>
</tr>
</tbody>
</table>

**Treatment:** FAC-2-\(^{14}\)C 10.53 mg/kg body weight/4 h, by intraperitoneal injection.

*From Gal et al. (6)

The distribution of the poison the highest concentrations of the label were found in the brain, liver, heart and kidney tissues. Approximately 2% of the radioactive carbon of FAC appeared in the respiratory CO\(_2\) and this finding confirmed that the animal organism has a limited ability to split the C-F bond of the FAC molecule. Nearly 32% of the label was found in the urine excreted in 2-4 days subsequent to the administration of the poison and about 3% of the radioactivity appeared as fluorocitrate. Further, some label was incorporated into fatty acids and in a variety of compounds existing in the urine.

Thus it appears that the injected FAC in the rat organism concentrates in metabolically active tissues; however, a comparison between the concentration of the label and the accumulation of citrate in the tissues (see Table 4) shows that there is no direct relationship between the two variables. This is particularly evident in the liver tissue of the starved rat in which the rise in citrate concentration is very small in spite of the high hepatic level of FAC.

The results of Gal et al. (6) showed also that FCIT seems to be the main derivative of FAC in the animal body and this fact lends support to the idea that FCIT plays a primary role in FAC toxicity.
The Transformation of Fluoroacetate into Fluorocitrate in the Cell

In the organism, citrate originates from a condensation reaction between oxaloacetate and acetyl-SCoA catalyzed by the enzyme citrate synthase. Oxaloacetate is the terminal four-carbon acid of the tricarboxylic acid cycle and acetyl-SCoA may derive either from pyruvate or from fatty acids or from acetate. It is generally assumed that FCIT is synthesized in the cells along a similar metabolic pathway. FAC would be transformed into fluoroacetyl-SCoA and this compound would condense with oxaloacetate to form FCIT. This condensation reaction has been demonstrated to take place in vitro with synthetic fluoroacetyl-SCoA plus oxaloacetate and purified enzyme preparations (7-9). It is likely that the same reaction occurs in vivo and is catalyzed by the citrate synthase.

The formation of fluoroacetyl-SCoA from FAC in the living cell has not as yet been clearly demonstrated. FAC has been shown to be enzymatically activated in vitro by an acetate activating system (8); however, the results so far reported with acetate activating systems in vitro have not yielded unequivocal results (4,7,8) and more work is needed to elucidate the activation of FAC.

The synthesis of FCIT in the animal cells takes place in the mitochondria. First shown by Gal and Smith (10) in isolated rat liver mitochondria, we have confirmed and extended this finding. In our system, the formation of FCIT was inferred by the accumulation of citrate following addition of FAC.

In Table 2 the results of a typical experiment are reported. It can be noted that pyruvate was more active than acetate in promoting citrate accumulation and that the total oxygen taken up in one hour was not decreased in the presence of FAC. However, an analysis of the kinetics of the oxygen uptake showed that at first FAC caused an increase in respiration followed by a diminished respiratory rate compared with the control. The two changes compensated each other and the total oxygen taken up in one hour apparently was not modified significantly. These aspects of FAC action are being investigated in detail.

FAC was converted to FCIT by rat liver isolated respiring mitochondria when either pyruvate plus fumarate (or L-malate) or acetate plus fumarate were added as substrates. However, when medium or long chain fatty acids, like octanoate or oleate (plus fumarate) were used as the substrates, oxidation occurred but very little citrate accumulated thus indicating that rather small amounts of FAC had been converted into FCIT. These results are in agreement with the early observation that in the starved rat poisoned with FAC very little citrate accumulates in the liver tissue (11-13). In fact, liver respiration in the starved rat is mainly supported by fatty acid oxidation (17).
### TABLE 2

Citrate Accumulation Caused by Fluoroacetate in Intact Isolated Rat Liver Mitochondria in Vitro

<table>
<thead>
<tr>
<th>Additions</th>
<th>Oxygen Uptake (µl O₂/mg protein/h)</th>
<th>Citric Acid (µg/mg protein/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.6 mM Pyruvate, plus 2.4 mM Fumarate</td>
<td>19.8</td>
<td>8.5</td>
</tr>
<tr>
<td>9.6 mM Pyruvate, plus 2.4 mM Fumarate</td>
<td>19.3</td>
<td>36.9</td>
</tr>
<tr>
<td>10.0 mM Fluoroacetate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.6 mM Acetate, plus 2.4 mM Fumarate</td>
<td>16.8</td>
<td>7.6</td>
</tr>
<tr>
<td>9.6 mM Acetate, plus 2.4 mM Fumarate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.0 mM Fluoroacetate</td>
<td>16.5</td>
<td>26.6</td>
</tr>
</tbody>
</table>

The experiments were carried out in polarographic vessels. The system contained, 220 mM mannitol, 70 mM sucrose, 2.5 mM sodium phosphates pH 7.4, 2.0 mM HEPES pH 7.4, 10 mM sodium EDTA, 1.8 mg bovine albumine, 2.5 mM sodium ATP, 6 mg mitochondrial protein. Final volume 3.6 mL. The mixture was stirred continuously with a magnetic bar and the temperature was 25°C. After one hour the reaction was stopped with trichloroacetic acid, 8% final concentration. The precipitate was discarded by centrifugation and citric acid estimated on the supernatant by the method described by Buffa and Peters (11).

Thus it seems that when either pyruvate or acetate are transformed into acetyl-SCoA, FAC is activated and transformed to FCIT. So far we have only circumstantial evidence that FAC is first converted into fluoroacetyl-SCoA.

With regard to the localization of the FAC activating system in the mitochondrion, it was found not to be associated with the outer mitochondrial membrane. In fact the mitochondria deprived of the outer membrane showed oxidative accumulation of citrate from pyruvate plus fumarate and FAC as the intact mitochondria. Similar results were obtained with isolated intact kidney mitochondria.

These results on isolated mitochondria together with the obser-
vations on the accumulation of citrate in the tissues of the FAC poisoned animals would suggest that in vivo FAC is converted into FCIT in all the cells whose respiration is mainly supported by pyruvate. However, this suggestion should be considered merely a working hypothesis since the biochemistry of the fluoroorganic compounds often has proven to be elusive.

The Consequence of the Intramitochondrial Formation of Fluorocitrate

Aconitate hydratase, the enzyme inhibited by FCIT, catalyzes the reversible interconversion of citrate, cisaconitate and isocitrate. In the cell, this enzyme has a dual distribution (14): part of it is located in the inner compartment of mitochondria in all probability bound to the mitochondrial matrix, and part of it is extramitochondrial and dispersed in the soluble cytoplasm. The available evidence indicates that the two aconitate hydratases have different properties (14, 15); in fact they have dissimilar affinities for FCIT. In the liver of the FAC poisoned rat only the mitochondrial-bound enzyme is inhibited and solely the first reaction, the dehydration of citrate to cisaconitate, is affected by the inhibitor (Table 3). These findings have been confirmed by experiments in vitro on isolated mitochondria and on the cytoplasmic enzyme.

This selective biochemical lesion confined in the mitochondria involves relevant effects for the functioning of the cell and the changes that follow the primary biochemical damage. The inhibition of the mitochondrial-bound aconitate hydratase causes accumulation of citrate, which continues to be formed at a reduced rate by the condensing enzyme. This compound accumulates in all organs and tissues of the FAC-poisoned animal (Table 4) (11,

<table>
<thead>
<tr>
<th>Mitochondrial-Bound Enzyme (*) Inhibition of Oxidation of</th>
<th>Soluble Cytoplasmic Enzyme (**) Inhibition of Conversion to Cisaconitate of</th>
</tr>
</thead>
<tbody>
<tr>
<td>CITRATE</td>
<td>CISACONITATE</td>
</tr>
<tr>
<td>53%</td>
<td>14%</td>
</tr>
</tbody>
</table>

Treatment: FAC 10 mg/kg body weight/1h, by intraperitoneal injection.

* Polarographic determination of oxygen uptake with a glass electrode on intact mitochondria isolated from poisoned livers. Inhibition refers to State 3 respiration.

** Spectrophotometric determination at 240 nm according to Anfinsen (16), using the cell sap rapidly separated from homogenates of poisoned livers.

TABLE 3

Inhibition of Rat Liver Aconitate Hydratases Caused by in vivo Poisoning with Fluoroacetate

Volume 6  Number 4
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### TABLE 4*

**Accumulation of Citrate in the Rat Poisoned with Fluoroacetate**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Controls</th>
<th>Poisoned</th>
<th>Controls</th>
<th>Poisoned</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>3</td>
<td>50</td>
<td>54</td>
<td>74</td>
</tr>
<tr>
<td>Brain</td>
<td>21</td>
<td>166</td>
<td>57</td>
<td>212</td>
</tr>
<tr>
<td>Heart</td>
<td>25</td>
<td>677</td>
<td>49</td>
<td>632</td>
</tr>
<tr>
<td>Kidney</td>
<td>14</td>
<td>1036</td>
<td>56</td>
<td>1029</td>
</tr>
<tr>
<td>Spleen</td>
<td>0</td>
<td>413</td>
<td>59</td>
<td>652</td>
</tr>
<tr>
<td>Liver (fasted animals)</td>
<td>0, 8</td>
<td>31</td>
<td>47</td>
<td>50</td>
</tr>
<tr>
<td>Lung</td>
<td>9</td>
<td>257</td>
<td>75</td>
<td>285</td>
</tr>
<tr>
<td>Stomach</td>
<td>37</td>
<td>386</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Small intestine</td>
<td>36</td>
<td>368</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Large intestine</td>
<td>21</td>
<td>248</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uterus (Virgin Animals)</td>
<td>217</td>
<td>207</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thymus</td>
<td></td>
<td></td>
<td>55</td>
<td>525</td>
</tr>
<tr>
<td>Testis</td>
<td></td>
<td></td>
<td>73</td>
<td>114</td>
</tr>
<tr>
<td>Muscle</td>
<td>5</td>
<td>41</td>
<td>31</td>
<td>54</td>
</tr>
<tr>
<td>Diaphragm</td>
<td>0</td>
<td>400</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pancreas</td>
<td></td>
<td></td>
<td>53</td>
<td>276</td>
</tr>
</tbody>
</table>

*From Buffa and Peters (11) *From Potter and Busch (12)

Treatment: in both cases the rats were intraperitoneally injected with FAC 5 mg/kg body weight/1h.

12) and the degree of the rise in citrate concentration appears to parallel the respiratory activity of the tissues: the metabolically active tissues like the heart and kidney, show the largest citrate accumulations. In liver, the rise in citrate concentration depends upon the nutritional state of the animal (13); in the normally fed rats, substantial accumulations can be found whereas, in the fasted animals, the levels of liver citrate increase little above the normal values. In the fed rat, the liver glycogen concentration is high and the respiration is supported mainly by pyruvate; in the fasted animal, the liver...
glycogen is greatly reduced and the respiratory substrates are mainly fatty acids. Under this latter condition, FAC is scarcely if at all transformed into FCIT (6, 17) and the liver aconitate hydratase is not inhibited.

The citrate accumulated in the liver cells can be found in large proportion in the mitochondrial fraction after differential centrifugation of the homogenized tissue (18). After FAC poisoning, over 43 percent of the liver endogenous citrate was found in the mitochondrial fraction (17). This finding provides further evidence that the mitochondrial-bound aconitate hydratase is the inhibited enzyme.

Apart from the conspicuous intracellular citrate accumulations consequent to the inhibition of the tricarboxylic acid cycle, FAC poisoning causes also other metabolic changes which are disclosed by abnormal quantitative variations of metabolites in tissues and blood and by a variety of modifications in the respiratory biochemistry of mitochondria isolated from the tissues of poisoned animals.

### TABLE 5*

<table>
<thead>
<tr>
<th>Animal and Condition</th>
<th>Treatment</th>
<th>Material Examined</th>
<th>Ketone Bodies (mg per 100 ml)</th>
<th>Data of</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasted Rat, give Butyrate</td>
<td>FAC 2.5/24 h</td>
<td>Urine</td>
<td>Control: 7.4</td>
<td>Poisoned: 11.4</td>
</tr>
<tr>
<td>Fasted Rat</td>
<td>FAC 8/24h</td>
<td>Blood</td>
<td>Control: 4.7</td>
<td>Poisoned: 20.9</td>
</tr>
<tr>
<td>Fed Rat</td>
<td>FAC 6/24h</td>
<td>Blood</td>
<td>Control: 3.0</td>
<td>Poisoned: 16.0</td>
</tr>
<tr>
<td>Fed Rat</td>
<td>FAC --</td>
<td>Urine</td>
<td>Control: Negative</td>
<td>Rothera Reaction</td>
</tr>
<tr>
<td>Fed Rat</td>
<td>FCIT --</td>
<td>Urine</td>
<td>Control: Positive</td>
<td>Rothera Reaction</td>
</tr>
<tr>
<td>Fasted Sheep</td>
<td>FAC 0.4/5h</td>
<td>Blood</td>
<td>Control: 4.5</td>
<td>Poisoned: 5.0</td>
</tr>
</tbody>
</table>
Increased Formation of Ketone Bodies

The concentrations of these compounds in blood and urine of FAC and FCIT poisoned animals tend to increase (Table 5). FAC caused marked ketonemia in both fasted (19) and fed (20) rats (Fig. 2) and the intravenous infusion of sodium octanoate increased the concentration of blood ketone bodies in FAC injected animals (21). A relatively small increase in blood ketone bodies concentration was observed also in the FAC poisoned sheep (22). With regard to ketonuria, FCIT caused a positive Rothera reaction in fed rats, whereas FAC poisoning was accompanied by a negative Rothera reaction (23). On the other hand, FAC poisoning caused a significant increase in urine ketone bodies in fasted rats given butyrate by oral administration (24).

Similar effects are produced by FAC in vitro on perfused organs and liver homogenate. Peters et al. (25) found a tenfold increase in acetoacetate formation by the isolated and perfused udder of the cow after addition of FAC to the perfusion liquid, and Busch and Potter (26) observed increased acetoacetate production from pyruvate and fumarate by rat liver homogenates poisoned in vitro with FAC.

Increase of Lactate

Early in the study on the metabolic effects of FAC poisoning, in-
increases in blood lactate were observed in the rabbit (27, 28). Subsequently Annison et al. (22) found, in a fasted sheep injected with 0.4 mg FAC per kg body weight for 6.5 hours, an increase in blood lactate concentration from 12.9 mg to 126 mg per 100 ml of blood, and recently we observed accumulation of lactate in the liver of the FAC poisoned rat (Fig. 3).

**Fig. 3**

*Effect of Fluoroacetate Poisoning on Rat Liver Lactate*

![Graph showing lactate levels](image)

The liver tissue was prepared as described by Buffa et al. (17) and lactate was measured by enzymatic analysis by means of lactate dehydrogenase.

The available evidence, although not extensive, suggests that the increase in lactate concentration in blood and tissues, like the increase in tissue citrate levels, is a common metabolic change caused by FAC poisoning in animals.

**Effects on Carbohydrate Metabolism**

FAC poisoning induces dramatic changes on carbohydrate metabolism which manifest themselves as a rapid fall in glycogen concentration in the liver, skeletal muscle and heart tissues and by an increase in the level of
blood glucose.

Depletion of liver glycogen was first observed by Mazur et al., in the rabbit (27). Later Annison et al. (22) recorded a change in glycogen level in the liver from 35 mg to 0.7 mg per g wet tissue in a sheep given a single lethal dose of FAC (0.4 mg/kg) whereas no change was observed in animals given repeated sublethal doses of FAC (0.1 mg/kg FAC at 3 day intervals). In normal fed rats injected with 10 mg FAC/kg body weight per 2 hours, Buffa et al. (17) recorded a decrease in liver glycogen from 44 to 3 mg per g wet tissue. In the abdominal muscle of a sheep injected with 0.4 mg FAC/kg, Annison et al. (22) observed a change in glycogen contents from 4.5 mg to 1.1 mg per g moist tissue and in one experiment, on three rats intraperitoneally injected with 10 mg FAC per kg body weight for 2 hours, we found that the heart glycogen had decreased from 1.47 mg to 0.63 mg per g wet tissue.

Consistent increases in blood glucose levels have been recorded in various animals after FAC poisoning (Table 6 and Fig. 4). Hyperglycemia was observed in both fed (20) and fasted rats (19, 30) and Annison et al. (22) observed parallel rises in concentration of glucose and lactate in the blood of sheep injected either with a single lethal dose of FAC (0.4 mg/kg body weight) or following successive administration of a non-lethal dose of FAC (0.1 mg/kg).

### Table 6

**Effect of Fluoroacetate Poisoning on Blood Glucose**

<table>
<thead>
<tr>
<th>Animal and Conditions</th>
<th>Treatment (mg/kg/h)</th>
<th>Blood Glucose (mg/100 ml)</th>
<th><em>Data of</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit</td>
<td>LD of MFA</td>
<td>-</td>
<td>Increased</td>
</tr>
<tr>
<td>Rabbit</td>
<td>-</td>
<td>-</td>
<td>up to 400</td>
</tr>
<tr>
<td>Fed Rat</td>
<td>FAC 1, 5/48 h</td>
<td>110</td>
<td>248</td>
</tr>
<tr>
<td>Fasted Rat</td>
<td>FAC 8/24 h</td>
<td>88</td>
<td>220</td>
</tr>
<tr>
<td>Fasted Rat</td>
<td>FAC 6/6 h</td>
<td>82</td>
<td>188</td>
</tr>
<tr>
<td>Fed Rat</td>
<td>FAC 6/16 h</td>
<td>90</td>
<td>216</td>
</tr>
<tr>
<td>Fasted Sheep</td>
<td>FAC 0.4/1.5 h</td>
<td>40</td>
<td>140</td>
</tr>
<tr>
<td>Fasted Sheep</td>
<td>FAC 0.4/6.5 h</td>
<td>30</td>
<td>130</td>
</tr>
<tr>
<td>Fed Sheep</td>
<td>FAC 0.1/6 h</td>
<td>45</td>
<td>250</td>
</tr>
</tbody>
</table>
**Fig. 4**

Effect of Fluoroacetate Poisoning on Rat Blood Glucose

![Graph showing the effect of Fluoroacetate on blood glucose levels.](image)

From data of Cole et al. (20).

**The Effect of Fluoroacetate on Rat Liver Glycogen in vivo and in vitro**

The rapid breakdown of tissue glycogen provoked by FAC poisoning could be a direct effect of the compound on some cellular mechanism capable of activating the hydrolysis of glycogen, or an indirect effect of FAC mediated by adrenalin secretion or sympathetic stimulation.

In order to find out whether FAC induces glycogen hydrolysis by a direct effect on the liver cell, slices were prepared from the liver of normally fed rat and incubated with FAC under conditions favoring active respiration and the glycogen was determined at the start and at the end of the incubation. The results are summarized in Fig. 5 and compared with the effect of FAC on liver glycogen in vivo. The glycogen which disappeared after 60 min, incubation at 38 C was 26 percent in the control slices and only approximately 10 percent in the slices treated with FAC. In vivo, under comparable condi-
Effect of Fluoroacetate on Liver Glycogen in vivo and in vitro

The experiments were carried out and the glycogen was determined as described by Buffa et al. (17).

These results are interpreted as indicating that the hydrolysis of glycogen provoked by FAC poisoning in the animal tissues is due to some factor active only in the whole organism on the glycogen-containing cells. In view of the fact that FAC poisoning causes a marked depression of energy production, which in the rat is revealed also by a fall of the body temperature (32) it is likely that the breakdown of glycogen is promoted by increased secretion of adrenalin or/and intense sympathetic stimulation.

Effects on Adenosine Triphosphate

Early work on the behavior of inorganic phosphate in blood and tissues of FAC-poisoned animals pointed to a decrease of high energy phosphates (27, 33, 34), and later studies demonstrated marked lowering of ATP in the liver, skeletal muscle, and heart tissues after administration of the
poison (17, 35, 36). The observed decreases in ATP concentration in the liver (17) and the heart (36) of the rat were of the order of 50 percent after 1-2 hours of the administration of a lethal dose of FAC. In the liver tissue, ATP diminution is accompanied by a parallel rise in ADP, AMP and inorganic phosphate concentrations (Guarriero-Bobyleva and Buffa, unpublished experiments).

It is relevant to note that the lowering of ATP is not caused by uncoupling of oxidative phosphorylation. In fact, the mitochondria isolated from the tissues of FAC-poisoned rats showed a normal phosphorylative ability when tested in vitro (14, 17, 35, 37, 38). This was demonstrated by P:O or ADP:O ratios within the normal ranges when the experiments were carried out by manometric methods or by polarographic analysis, respectively. Further, FCCP added in vitro to intact normal rat liver mitochondria caused inhibition of citrate oxidation but did not affect the coupling mechanism of the organelles (14, 17).

Effects on Mitochondria

In addition to the metabolic changes revealed by abnormal concentrations of intermediates in blood and tissues of FAC-poisoned animals, a variety of biochemical abnormalities have been shown in mitochondria isolated from various tissues of the FAC-injected rat. These abnormalities consisted in modifications in their ability to oxidize added substrates in vitro. The results obtained by various workers are summarized in Table 7.

It is apparent that the mitochondria from different tissues were variously affected by FAC poisoning. Moreover, in spite of the fact that in all the tissues used for the preparation of mitochondria FAC intoxication caused accumulation of citrate (Table 4), only liver and kidney mitochondria showed inhibition of citrate oxidation. It is also interesting to note that brain mitochondria appeared not to be affected at all whereas kidney mitochondria showed a reduced ability to oxidize the five substrates tested, which involve enzymes variously located in the mitochondrial structure.

Apart from the functional changes just mentioned, FAC poisoning also causes structural damage in the mitochondria of various tissues, as revealed by the electron microscope (17, 40, 41, 42, 43). Within a few minutes of FAC administration, changes occur in the mitochondrial matrix which become transparent to the electrons, the matrix space expands and the mitochondria become swollen. The changes in volume are accompanied by dislocation and disintegration of the cristae and stretching and ruptures of the limiting membranes. The ultrastructural changes are confined to the mitochondria and only minor modifications can be seen in other cell components except for the reduction of glycogen in the liver of the fed rat acutely poisoned with FAC.

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October, 1973
TABLE 7

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Glutamate</th>
<th>Pyruvate</th>
<th>Isolated from Mitochondria</th>
<th>Mitochondria of Respiratory Activity of Mitochondria Isolated from Rats Acutely Poisoned with Lethal Doses of Fluorocetate</th>
<th>Changes in Respiratory Activity of Mitochondria Isolated from Rats Acutely Poisoned with Lethal Doses of Fluorocetate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Kidney</td>
<td>42</td>
<td>40</td>
<td>78</td>
<td>40</td>
<td>42</td>
</tr>
<tr>
<td>Heart</td>
<td>32</td>
<td>1.1</td>
<td>63</td>
<td>1.1</td>
<td>32</td>
</tr>
<tr>
<td>Skeletal Muscle</td>
<td>25</td>
<td>100</td>
<td>100</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>Liver</td>
<td>100</td>
<td>100</td>
<td>1.1</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>124</td>
<td>47</td>
<td>1.1</td>
<td>124</td>
<td>124</td>
</tr>
</tbody>
</table>

Chance and Williams (39). The liver mitochondria were isolated of normal mitochondria in respiratory state 3, as defined by 2°C.

The figure 100 indicates a conventional value attributed to the Ox.
Some Concluding Remarks

The transformation in the organism of FAC into FCIT, by means of physiological biochemical mechanism present in normal cells, and the consequent inhibition of the mitochondrial-bound aconitate hydratase appear to be the basic events in the toxic effects of FAC.

The inhibition by FCIT of the mitochondrial-bound aconitate hydratase, an enzyme of the tricarboxylic acid cycle, results in relevant changes in the physiology of the cells. The following main events of FAC poisoning at the cellular level take place: 1) the block in the tricarboxylic acid cycle at the stage of the transformation of citrate into cisaconitate (Fig. 1); 2) the lowering of energy production and, consequently, the decrease in concentration of cellular ATP; 3) the accumulation of citric acid in the mitochondria and the cells, especially in metabolically active tissues; 4) substructural and functional changes in the mitochondria.

The accumulation of citrate takes place because, in the FAC poisoned mitochondria, the enzyme citrate synthase continues to form citrate which only in part is transformed into cisaconitate because of the inhibition of aconitate hydratase induced by FCIT. The enzyme inhibited by FCIT is located in the inner mitochondrial compartment and, for this reason, citrate accumulates primarily in the mitochondria and subsequently in the cytosol. The exchange of citrate between the mitochondria and the soluble cytoplasm takes place in part by simple diffusion and in part is mediated by a specific carrier localized in the inner mitochondrial membrane (44).

Citrate in the cell under normal conditions has different metabolic alternatives. However, when it is present in concentrations abnormally high, as in the case of FAC poisoning, it is likely that the normal pattern of reactions of this compound is altered. Further, the accumulation of citrate involves changes in cation concentration, and the movements of the compound between the mitochondria and the cytosol is accompanied by movements of counterions. Finally, citric acid is a modifier of some enzyme activities. In fact, citrate has been reported to stimulate isocitrate dehydrogenase (45) and acetyl CoA carboxylase (46) and to inhibit phosphofructokinase (47, 48) and pyruvate dehydrogenase (49). Of course it remains to be seen if, in the complex environment of the cell where most of the enzymes are organized in defined structures and where other modifiers are also present, citrate is able to act on these enzymes. Indeed it appears that in the liver of the FAC poisoned rat, phosphofructokinase is not inhibited by the accumulated citrate (Guarriero-Bobyleva and Buffa, unpublished experiments).

It is likely that the accumulation of citrate and the resulting ionic and osmotic modifications together with the decline in ATP concentration are sufficient to induce the functional cardiac and nervous changes seen in FAC poisoned animals. The dramatic lowering of the body temperature induced by FAC in the rat suggests a diminution of the production of metabolic

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energy in all the tissues; however, a direct effect of the biochemical lesion on the cells of the nervous centers concerned with temperature regulation is also likely to occur.

If it is assumed that in the cells FAC is first transformed into fluoroacetyl-SCoA and that this compound condenses with oxaloacetate in a reaction catalyzed by the enzyme citrate synthase, as depicted in Fig. 1; then two concurrent mechanisms may be postulated for the accumulation of ketone bodies. The activity of citrate synthase could be inhibited by fluoroacetyl-SCoA, as demonstrated by Brady (7) in an in vitro system, and by the accumulated citrate, the end product of the reaction of citrate synthase. As a consequence of this inhibition, acetyl-SCoA would tend to accumulate and give rise to ketone bodies. The formation of ketone bodies would be further stimulated by the increased oxidation of fatty acids in the glycogen depleted tissues, especially in the liver.

The rapid drop in glycogen concentration in liver, skeletal muscle and myocardial tissue that occurs in FAC poisoning in all probability is caused by adrenalin release or/and intense sympathetic stimulation and the consequent activation of the phosphorylase system mediated by cyclic AMP. The lack of effect of FAC on glycogen in isolated liver slices in vitro is consistent with this view.

Cole et al. (20) and Engel et al. (21) suggested that the increase in blood glucose concentration in FAC poisoning is due to a reduced insulin secretion by the pancreas A-cells damaged by the toxic compound and named the phenomenon "FAC diabetes". Karam and Grodsky (31) showed that the pancreatic insulin content is increased in FAC poisoned hyperglycemic rats; thus the production of the hormone does not seem to be impaired in FAC poisoning. In fact the increased insulin production is the physiological response of the pancreatic islets to high concentrations of blood glucose (50).

The hyperglycemia observed in FAC poisoning can be accounted for by the rapid hydrolysis of the large glycogen deposit in the liver followed by hydrolysis of glucose-6-phosphate catalyzed by glucose-6-phosphatase. In addition, high blood glucose would be favored by a reduction of hexokinase activity which is likely to take place as a result of the lowered ATP concentrations in the tissues of the intoxicated animal.

The intense hydrolysis of glycogen could also account for the rise in concentrations of lactic acid found in blood and tissues. Increased formation of lactic acid would take place especially in muscular tissues in which the enzyme glucose-6-phosphatase is lacking. Consequently in these tissues glycogen is metabolized down the entire glycolytic pathway and lactic acid is produced in excess, due also to the concomitant impairment of citrate synthase activity caused by FAC poisoning.
The fact that the main biochemical lesion provoked by FAC involves the tricarboxylic acid cycle, and the fact that the enzyme inhibited by FCIT, aconitate hydratase, is organized in the mitochondrial structure, of the numerous attempts made, to date, to find an antidote to FAC primarily depends on the localization of aconitate hydratase in the mitochondrial structure as well as the high affinity of FCIT for this enzyme (14).

In spite of the considerable insight gained into the biochemistry of FAC intoxication, the actual mechanism by which the poison provokes the death of the animals has yet to be discovered. To our knowledge, no animal species is known to be resistant to FAC. However, FAC is not lethal to animal cells cultured in vitro in concentrations which kill the embryo or the adult animal from which the cells are obtained and cultured (51-53). It has even been possible to cultivate, in vitro, chick embryo heart myoblasts for several months in the presence of 5 mM FAC (Buffa, unpublished experiments). These observations, together with the information gained on the biochemistry and pharmacology of FAC poisoning, point to an effect of the toxic compound on an integrated function essential for the life of the animal. It is likely that the failure of such a function may depend on a relatively simple biochemical change such as an ion imbalance or a lowering of the available free energy in a specialized group of cells.

The experimental work reported in this paper was supported by grants from the Consiglio Nazionale delle Ricerche of Italy.

We wish to thank Mrs. Maddalena Gualtieri for her expert technical assistance.

Bibliography


FLUORIDE

**Discussion**

Dr. Schepers: I should like to show you some interesting results that I obtained through poisoning animals with extremely minute doses of organic lead. Although lead poisoning is not directly related to organic fluoride intoxication it is nevertheless of interest in connection with poisoning from any organic compound.

Using tetraethyl lead, we determined the lowest toxic dose, divided it by a hundred times and poisoned the animals with this 1/100th dose daily over a period of one hundred days following which the animal was sacrificed. The various speakers have shown the modern methods of using infrared spectrographs and gas chromatography. I employed an older method known to all of you, namely, the emission spectrograph. Although it has not been mentioned today, it is still useful for biological research on metallic elements.

My first slide shows what happens to the brain when an animal has been poisoned by organic lead i.e., tetraethyl lead or tetramethyl lead. If the different chemical elements in the brain tissue of the poisoned rat are analyzed - assuming the normals (for a rat) for these different elements to be the baseline of the bar graph - you will find first that the brain contains no lead in spite of the fact that organic lead poisoning is capable of producing toxic symptoms referable to the brain. Most animals convulse and eventually die in a state of encephalopathy. Microscopically too the damaged brain cells contain no lead at all. If these brain cells are not poisoned by lead, they may be poisoned by elements such as calcium, silicon, zinc, potassium which accumulate excessively in the brain or the poisoning may result from elution from the brain of other elements such as copper, sodium, strontium, etc. This interesting line of research, I believe, should be followed in connection with the organofluorine compounds. Unfortunately I am not in a position to pursue this research at the present time. I hope someone else will.
The following is another interesting result. Male and female rats do not react in the same manner when they are poisoned by lead alkyls. In this instance, as shown on the slide, for some strange reason calcium, silicon and nickel accumulated excessively in the male rat brain but did not accumulate in the female rat brain.

The last speaker discussed glycogen metabolism and very elegantly showed how it is achieved and modified by fluoroacetate poisoning. The red stain in the slide I am now projecting shows the glycogen in the liver cells of a normal rat. The next slide shows what happens after poisoning with organo-lead compounds. Note the lack of glycogen in the liver cells. After poisoning a rat for one hundred days with infinitesimally small quantities of organic lead, no glycogen can be demonstrated in the liver cell. I am sure similar results can be achieved and demonstrated with reference to fluoride compounds. Data which I shall show you this afternoon lead me to arrive at this conclusion.

Finally, one of the gentlemen spoke about the elevation of blood sugar (hyperglycemia) during some phase of fluoroacetate poisoning. This may be due to the effect on the pancreas. In the slide I now am projecting, the blue stain shows fibrous tissues formed inside the island of Langerhans of the pancreas of a rat which has been poisoned with organic lead. One way to render rats diabetic is to poison them with minute quantities of organic lead. Such rats would then develop hyperglycemia as did the animals poisoned with fluoroacetate.

Dr. Grunder: Is there any difference in toxicity in sheep and in cattle? Furthermore what is the minimum lethal dose and the maximum tolerable dose of fluoroacetate?

Dr. Egyed: I mentioned the LD50 related to cattle according to research carried out in Denver, Colorado and published in 1971. As far as I know, no publications have appeared with respect to the minimal lethal dose of fluoroacetate in sheep. However, according to experiments carried out by Anderson and his co-workers including Prof. Peters, 0.4 mg/kg is the established LD100 in sheep. No information about the LD50 is available with respect to sheep. To carry out such experiments on sheep and cattle would be very expensive. Only a wild guess can be made concerning the minimum lethal dose or maximum tolerable dose by extrapolating the data from the LD50's.

Sir Rudolf Peters: I do not know whether I should say anything in relation to this question. I hope I can make it intelligible but I haven't looked at my paper for some little time. I think we gave 0.4 mg/kg body weight at definite intervals of a week. The first two doses might not kill, but finally about 3 hours after the third dose was administered the animal would die in what looked like a heart attack. I could not understand this at all because I could not see the fluoroace-
tate accumulating. I felt that the explanation was found when I visited Australia where it had been noted that the sheep developed cloudy swelling from Acacia Georgina poisoning. It seemed to me - and this ought to be studied further - that each dose caused a certain amount of heart damage. After the passage of perhaps one or two days the heart recovered but with cell damage. With additional doses and then a final dose, the heart was unable to cope with fluoracetate. This is only an hypothesis but it does fit some of the facts.

Dr. Cooke: Prof. Buffa showed that fasted rats did not suffer in the same way and could recover from a dose of fluoracetate because of the fatty acid that they were feeding on. Should an animal be poisoned by fluoracetate, what would be the effect of giving it, even a well fed animal large doses of fatty acid?

Prof. Buffa: I did not explain this point clearly enough. If a fasted rat is injected with fluoracetate we have shown that in the liver alone citrate does not accumulate to any extent; in other organs citrate accumulates extensively. Therefore the intoxication affects the whole animal. Although the liver does not accumulate citrate, it is nevertheless damaged.

As to your question: What would happen if an animal were fed large amounts of fatty acid? With regard to antidotes I think the problem of acetate is interesting. Recently we found in experiments with isolated mitochondria that acetate, if given as a substrate and fumarate to produce an oxylacetate, under this condition fluoracetate is activated and fluorocitrate is formed. Therefore I can now see why the administration of acetate did not produce any positive results because, in practice when acetate is incorporated into citrate, fluoracetate is also activated.

Dr. Ingram: Is there anything known about long-term effect of very small doses rather like the type of thing Dr. Schepers has just mentioned in regard to organic lead compounds? Sir Rudolf says he does not think that fluorocitrate accumulates. I was wondering if any of the other speakers have any evidence on this point, i.e., the ingestion of minute concentrations over a very long time.

Dr. Egyed: That is something to which I had not paid much attention in chronic poisoning with fluoracetates. I can say only that such studies are extremely difficult to carry out because the range between the tolerable and lethal dose and level which produces chronic poisoning is very narrow and one can kill the animal very rapidly. However I recall that some years ago in America some experiments were carried out by using minute doses. The citrate concentrations in organs were recorded. I cannot remember the exact results. I can only remember the curve with a citrate concentration in various tissues.

FLUORIDE
RELATIONSHIP BETWEEN FLUORIDE CONCENTRATION IN DRINKING WATER AND DENTAL CARIES IN JAPAN

by

Yoshitsugu Imai
Tokyo, Japan

(Abstracted from the Koku Eisei Gakkai Zasshi, 22:144-96, 1972)

The chloride, fluoride, and hydrogen ion concentrations for well water (485 locations) and tap water (491 cities and towns) serving 63,710,760 people are tabulated. In the areas examined, 69.5% of the people were using water with a fluoride ion concentration of less than 0.1 ppm. Only 0.2% of the population or about 150,000 people were using water with a fluoride ion concentration greater than 1 ppm.

Table A (author’s table 13) indicates the fluoride concentration used by 20,272 grade school students from 48 different schools. The examination for tooth decay of the students in grades 1 through 6, yielded the data which appears in table B (author’s table 14 which is presented in condensed form)

<table>
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<tr>
<th>Number of Children Examined</th>
<th>Fluoride Content of Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>12,357</td>
<td>0.013-0.08</td>
</tr>
<tr>
<td>3,404</td>
<td>0.1-0.19</td>
</tr>
<tr>
<td>3,068</td>
<td>0.2-0.29</td>
</tr>
<tr>
<td>2,686</td>
<td>0.3-0.39</td>
</tr>
<tr>
<td>1,020</td>
<td>0.4-0.49</td>
</tr>
<tr>
<td>1,838</td>
<td>0.5</td>
</tr>
<tr>
<td>Total 20,272</td>
<td></td>
</tr>
</tbody>
</table>

Graph C (author’s graph 18) relates the percentage of students with caries to the various levels of fluoride ion concentrations.

It should be noted that the statistic used in this paper represented the percentage of students with caries. It differs from the DMF statistic.

From the Department of Preventive Dentistry, School of Dentistry, Tokyo Medical and Dental University, Tokyo, Japan

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<table>
<thead>
<tr>
<th>Numbers in brackets ( ) indicate children examined.</th>
</tr>
</thead>
<tbody>
<tr>
<td>% 56.6% 79.2% 74.9% 55.3% 37.3% 16.3% 0.5 &gt;</td>
</tr>
<tr>
<td>1.0 2.4 1.3 2.7 1.2 3.3 2.9</td>
</tr>
<tr>
<td>308 312 343 343 343 343 295</td>
</tr>
<tr>
<td>67.3% 78.5% 77.8% 78.3% 51.0% 24.4% 0.4 0.4</td>
</tr>
<tr>
<td>1.0 2.0 1.0 1.4 1.0 1.0 0.0</td>
</tr>
<tr>
<td>186 123 127 127 153 153 49</td>
</tr>
<tr>
<td>45.4% 73.2% 75.3% 65.0% 43.3% 43.2% 3.0 0.3</td>
</tr>
<tr>
<td>1.0 2.0 1.0 1.0 1.0 1.0 1.0</td>
</tr>
<tr>
<td>286 278 286 246 286 286 286</td>
</tr>
<tr>
<td>9.8% 51.1% 51.7% 51.7% 51.7% 51.7% 51.7% 0.2 0.2</td>
</tr>
<tr>
<td>1.0 1.0 1.0 1.0 1.0 1.0 1.0</td>
</tr>
<tr>
<td>94 94 94 94 94 94 94 94</td>
</tr>
<tr>
<td>62.9% 81.2% 80.2% 66.2% 66.2% 66.2% 66.2% 0.1 0.1</td>
</tr>
<tr>
<td>1.0 2.0 2.0 1.0 1.0 1.0 1.0</td>
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<tr>
<td>94 94 94 94 94 94 94</td>
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<td>1.0 1.0 1.0 1.0 1.0 1.0 1.0</td>
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<td>Drinking fluoride</td>
<td>Fluoride</td>
<td>Fluoride with tooth decay</td>
<td>Fluoride with tooth decay</td>
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<tr>
<td>Average</td>
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<td></td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Number of children with tooth decay</th>
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</table>

**TABLE B**
Fluoride in Chromatographic Strips

Profile analysis of

**FIG. 2**

TABLE 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>Control Expt.</th>
<th>Exp. 1</th>
<th>Exp. 2</th>
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</thead>
<tbody>
<tr>
<td>Malonic acid</td>
<td>30.2</td>
<td>4.9</td>
<td></td>
</tr>
<tr>
<td>Oxalic acid</td>
<td>62.4</td>
<td>30.3</td>
<td></td>
</tr>
<tr>
<td>Malic acid</td>
<td>6.0</td>
<td>4.9</td>
<td></td>
</tr>
<tr>
<td>Fumaric acid</td>
<td>15.2</td>
<td>3.2</td>
<td></td>
</tr>
<tr>
<td>Succinic acid</td>
<td>1.5</td>
<td>2.7</td>
<td></td>
</tr>
<tr>
<td>Isocitric acid</td>
<td>1.9</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>Trans-acrylic acid</td>
<td>0.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyclic acid</td>
<td>6.9</td>
<td>7.6</td>
<td></td>
</tr>
<tr>
<td>Other acids</td>
<td>4.9</td>
<td>1.5</td>
<td></td>
</tr>
</tbody>
</table>

Changes in Organic Acid Content of HF-Fumigated Soybean Leaves

*Notes*: Experiments 1 and 2 were conducted to compare the effects of HF-fumigation on the organic acid content of soybean leaves.
10% Reoplex 400 on Chromosorb W (60-80 mesh)

Retention time, minutes

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Chromatographic Separation of Methyl Esters of Organic Acids from Pods of Acacia Georgeana

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Chromatographic Separation of Methyl Esters of Organic Acids from Crested Wheatgrass (Agropyron Cristatum) Harvested Near a Phosphate Plant

---

FLUORIDE
The figure 100 indicates a conventional value attributed to the Ox 2.9°C.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Ox 1.4%</th>
<th>42°C</th>
<th>63°C</th>
<th>78°C</th>
<th>91°C</th>
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</thead>
<tbody>
<tr>
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<td>100</td>
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<td>100</td>
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<td>100</td>
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<tr>
<td>Kidney</td>
<td>67</td>
<td>0.4</td>
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<td>0.4</td>
<td>0.4</td>
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<tr>
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</tr>
<tr>
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<td>100</td>
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<td>32</td>
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<td>Liver</td>
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<td>1.4</td>
<td>1.4</td>
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<td>1.4</td>
</tr>
</tbody>
</table>

**TABLE 74**

Rats Acutely Poisoned with Lethal Doses of Fluorocacetate

Changes in Respiratory Activity of Mitochondria isolated from FAC Metabolism in Animals

---

**BLUE**

---

**FAC Metabolism in Animals**

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**239**
in that, as the group grows older the percent of students with caries will approach 100. This, therefore, implies that there will be an age at which this statistic will be most sensitive. From the above graph it can be seen that this point occurs at grade 4.

When the percentage of students with caries is plotted against the fluoride concentration for 4th grade students, as in graph D (author's graph 19), a sharp reduction in caries takes place at fluoride levels between 0.2 and 0.4 ppm. Thus the optimum level for the prevention of caries is between 0.2 and 0.4 ppm fluoride.

* * * * * * * * * * * * * *

IN VITRO BIOSYNTHESIS OF BONE MATRIX IN BONES OF RABBITS INTOXICATED WITH FLUORIDE

by

G. Sri Ranga Reddy and B. S. Narasinga Rao
Hyderabad, India


The authors induced skeletal fluorosis in rabbits and determined collagen and hexosamine synthesis in bones of these animals in vitro using labelled precursors.

Method

Four groups of animals were placed on a standard diet adequate in all nutrients. Three animals served as controls, five received the controlled diet plus fluoride, two a low-calcium diet and four a low-calcium diet plus fluoride. The fluoride in the form of NaF, 175 μg per gram of diet, was mixed in it during the first 13 weeks. In the next two weeks it was raised to 250 μg per gram of diet, to 300 μg per gram in the following 13 weeks, and to 500 μg per gram during the final 16 weeks. After 44 weeks the animals were killed; the right femur was processed according to Deiss et al, the aliquots of the minced bone substance were transferred to Erlenmeyer flasks containing

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either $^{14}C$-(U) proline or $^{14}C$-Glucose-(1) in 3 ml of Krebs Ringer bicarbonate buffer solution at pH 7.4. The flasks were incubated under 95% O$_2$ - 5% CO$_2$ at 37° in a metabolic shaker at 100 oscillations per minute for 5 hours. After incubation the bone fragments were processed and hexosamine, radioactivity in glucosamine and hydroxyproline were determined. Total protein, ash and fluoride content of the bone were also determined.

Results

The control rabbits, which had received adequate diets, gained more weight compared to animals of all the other three groups. The latter showed no significant differences in weight. All five animals of group IV (low calcium plus fluoride) exhibited exostosis on the radius. In all but one animal, the exostoses were unilateral. Exostoses were also found on the humeri and the tibiae. In contrast, only two out of five animals of group II which received an adequate diet plus fluoride, exhibited the exostoses, one on the left radius, the other at the lower end of the left humerus.

The mean specific activity of hydroxyproline was not different in the animals on the control diet from that in the animals which received fluoride added to the control diet. Hexosamine activity was higher in the fluoride animals than in the controls. The mean specific activities of hydroxyproline and those of hexosamine were higher in the animals on the low calcium diet with or without fluoride than in animals on the adequate calcium diet. Collagen protein and total protein content did not differ in all four groups.

On the adequate calcium diet, fluoride supplementation induced only an insignificant increase in the ash content. Fluoride feeding produced a definite increase in the fluoride level in the femur both in animals on the adequate diet and in those on the low calcium diets. However the rabbits which received a low calcium diet plus fluoride, retained significantly more fluoride in the femora than animals whose diets contained adequate calcium.

These studies indicated that the rate of synthesis of bone matrix collagen and mucopolysaccharides is unaltered in animals fed fluoride. The authors concluded that the development of fluorotic skeletal changes may be due to the reduction in osteolytic activity rather than to an increased bone formation. These experiments also confirm previous observations that low calcium diets produce higher retention of fluoride and greater incidence of exostosis in bones of rabbits than diets containing adequate calcium.

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EFFECT OF CALCIUM AND VITAMIN D ON FLUORIDE METABOLISM IN THE RAT

by

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(Abstracted from Nutrition and Metabolism, 14:257-61, 1972)

Male rats of 30-40 g weight and of a local strain derived from Wistar rats, were divided into 6 groups of 10 animals each. Groups 1-3 received a high-calcium diet and groups 4-6 a low-calcium diet. The diets contained 1.02 mg fluoride and 14.32 or 1.20 g calcium/kg respectively. The 1st and 4th groups received additional vitamin D₂, 100 IU/100 g body weight, 3 times a week by stomach tube. Ten mg fluoride (as sodium fluoride) were added to the rations of groups 2 and 5. The diets of groups 3 and 6 were supplemented with both vitamin D₂ and fluoride.

After an adaptation period of 1 week, urine and feces were collected daily for 30 days. At the end of this period, the animals were weighed and decapitated.

When fluoride was added to the high-calcium diet, a larger proportion of it was excreted in the feces, showing that less fluoride was absorbed than when it supplemented a low-calcium diet. Moreover, when fluoride was added to the low-calcium diet a larger proportion of the dose was retained.

Plasma, liver and kidney fluoride levels in rats placed on supplemented diets, showed a higher concentration of fluoride. However, the fluoride levels were significantly higher in groups 5 and 6 which received the low-calcium diet. Vitamin D caused a decrease in plasma fluoride level in both high- and low-calcium diets.

Dietary supplementation with fluoride increased the fluoride concentration of long bone ash. Vitamin D, added to the high-calcium diet, significantly increased the fluoride content of bone ash (groups 2 vs. 3), but had only a slight effect on the low-calcium diet (groups 5 vs. 6).

The enzymatic changes in those rats which received the high fluoride diet were an inhibition of fatty acid and carbohydrate metabolism. In animals with low calcium and fluoride, liver glucose-6-phosphate dehydrogenase level decreased significantly.

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GALLIUM FLUORIDE POISONING: A PROBABLE CASE WITH SKIN EFFECTS AND NEUROLOGICAL SEQUELAE

by

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(Absimted from the Journal of Occupational Medicine, 14:925-26, 1972)

A 43 year-old white female chemist was exposed to fumes from a vial of gallium fluoride crystals into which she had poured distilled water. She developed petechial hemorrhages on the right wrist and forearm. The following day she complained of pain on the right side of her neck, in the right shoulder and along the radial surface of the upper arm and forearm. This condition was associated with abdominal cramps and was followed by clumsiness of the right hand. About a month after exposure she was unable to pick up books or a bottle of milk with her right hand. The pain in the right arm had been decreasing gradually but weakness persisted for six weeks. The diagnosis was mild radial palsy of the muscles supplied by the deep branch of the radial nerve in the forearm and weakness of the extensors of the wrist. The condition gradually subsided.

The element gallium is not considered particularly toxic and no reports of it as an occupational hazard are available. The author postulated that hydrogen fluoride had formed. Complexed with gallium it had affected the nerve tissue of the arm.

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