trations of lipid phosphorus in young rats and in the tumor were similar, and greater than in adult rats; 4) the concentration of an alkali-resistant phospholipid was greater in the tumor than in the carcass.

1. Mider, G. B., Sherman, C. D., and Morton, J. J., *Cancer Res.*, 1949, v9, 222.
2. Haven, F. L., Bloor, W. R., and Randall, C., *Cancer Res.*, 1949, v9, 511.
3. ———, *Cancer Res.*, 1951, v11, 619.
4. Mider, G. B., Fenninger, L. D., Haven, F. L., and Morton, J. J., *Cancer Res.*, 1951, v11, 731.
5. Sinclair, R. G., *J. Biol. Chem.*, 1931, v92, 245.
6. Ingle, D. J., *Endocrinology*, 1947, v41, 170.
7. Haven, F. L., and Levy, S. R., *J. Biol. Chem.*, 1941, v141, 417.
8. Hunter, F. E., *J. Biol. Chem.*, 1942, v144, 439.
9. Kuttner, T., and Lichtenstein, L., *J. Biol. Chem.*, 1930, v86, 671.
10. Haven, F. L., *J. Biol. Chem.*, 1937, v111, 111.

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Multiple Effects of Fluoroacetate on Pyruvate Metabolism *in vitro*. (19737)

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The injection of fluoroacetate into rats has been shown to result in the accumulation of large amounts of citrate in certain organs (1-5). No accumulation of citrate was found (3) in livers of fluoroacetate-treated rats; these rats were of the same sex and strain as those whose livers had previously been shown to produce large amounts of citrate *in vitro* (6).† A possible explanation for this discrepancy was furnished by studies(7,8) showing that an interruption in the Krebs cycle in the case of liver results in the diversion of C_2 fragments away from the Krebs cycle and into acetoacetate formation, and it might have been assumed that the results could be explained solely on this basis. However, when *in vitro* studies were carried out to test this simple hypothesis, new results were obtained, some of which appear to be unexplainable in terms of the inhibition of citrate removal alone.

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† The rats used by Potter and Busch(3) were normal males. Subsequent work in this laboratory and in that of Dr. K. P. DuBois makes it clear that hormonal and other physiological factors can alter the capacity of the liver to accumulate citrate following the injection of fluoroacetate. Fed female rats accumulated citrate in their livers following the injection of fluoroacetate, and male rats could be shown to do likewise under special conditions.

Experimental. Chemicals. Sodium monofluoroacetate was supplied by Dr. K. P. DuBois of the University of Chicago Toxicity Laboratory; adenosine triphosphate (ATP) was prepared by Dr. G. A. LePage and associates.

Homogenates. Male rats (weighing 200-300 g) obtained from the Holtzmann-Rolfsmeyer Rat Co. were used in this study. After the rats were decapitated, the kidneys were quickly removed and placed in ice-cold isotonic KCl solution; after weighing, they were placed in glass homogenizers(9) containing 9 volumes of ice-cold isotonic KCl solution and homogenized in the cold; the same procedure was followed with liver homogenates, but the homogenizers contained 5¼ volumes of KCl solution to give a concentration of 16% liver by weight.

Flask contents. a) *Reaction mixtures.* Each Warburg flask contained 1.0 ml of a basal medium composed of 0.01 M $MgCl_2$, 0.2 M KCl, and 0.01 M K phosphate buffer adjusted to pH 7.2(6) as well as 30 μM potassium pyruvate, 3 μM potassium ATP and sufficient 0.2 M or 0.02 M sodium monofluoroacetate‡ to produce the desired concentration. All substrates were added as potassium salts neutralized to pH 7.2-7.4. In all experiments other than those involving phosphate studies, an additional 0.1 ml of 0.1

M K phosphate was added. Center wells contained 0.2 ml of 2.5 M NaOH and a wick of filter paper. Cold homogenates were added to the cold reaction mixtures and the vessels were equilibrated 10 minutes before manometric readings were begun. The gas phase was air and the temperature was 38°C. b) *Homogenate systems.* Three homogenate systems were employed for the purpose of studying the effects of fluoroacetate on pyruvate oxidation. In addition to the basal medium, pyruvate and ATP noted above, the *liver-malonate* system contained 80 mg of liver tissue and 12 μM of potassium malonate; the *liver-fumarate* system contained 80 mg of liver tissue and 40 μM of potassium fumarate, while the *kidney-fumarate* system contained 40 mg of kidney tissue and 40 μM of potassium fumarate. Each of these reaction systems responds differently to fluoroacetate as will be shown below. The concentration of fumarate was selected following the demonstration that increasing the concentration of fumarate increased formation of citrate and decreased formation of acetoacetate by liver homogenates; at 40 μM of fumarate, both of these effects had reached plateau levels(8). Oxygen uptake in 60 minutes was proportional to tissue at concentrations up to 90 mg wet weight of liver per Warburg flask and up to 40 mg of kidney per flask; in general, kidney homogenates were much more labile than liver homogenates.

Determinations. Reactions were stopped by the addition of 0.3 ml of 50% perchloric acid to the main chamber. The protein-free supernatant fluid obtained on centrifugation of the contents of the flask was used for the analyses. Citrate(10), acetoacetate,§ α-ketoglutarate and pyruvate(11), and inorganic phosphate(12) were determined by standard methods.

Results. Comparative studies on kidney and liver homogenates. The data obtained with each of the 3 homogenate systems are presented in Fig. 1-4, and are plotted together for purposes of comparison. The following sections consider each of these systems separately.

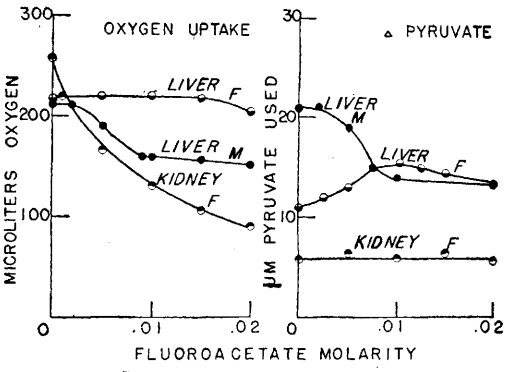

Fig. 1

Fig. 2

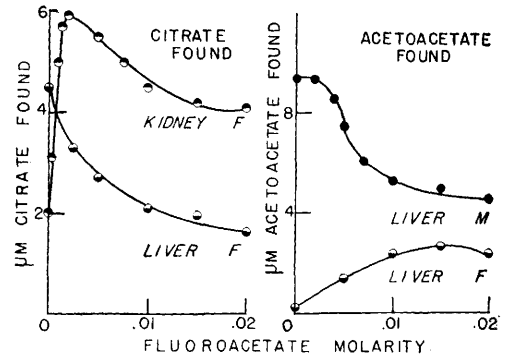

Fig. 3

Fig. 4

FIG. 1–4. Effects of fluoroacetate concentration on oxygen uptake, pyruvate utilization, citrate formation and acetoacetate formation by the liver-fumarate system (Liver F), liver-malonate system (Liver M) and kidney-fumarate system (Kidney F) described in the text. Time of incubation: 60 min.

Effect of fluoroacetate on the kidney-fumarate system. The sole pathway of pyruvate oxidation in this system is the citric acid cycle(13). At *low* concentrations of fluoroacetate a maximal accumulation of citrate was found (Fig. 3). However, as the concentration of fluoroacetate was increased, the block of oxidation was established earlier and/or more completely (Fig. 1) and this progressive depression of oxidation was paralleled by depression of citrate formation. While initial oxidation was depressed less in this system

‡ Trifluoroacetate inhibited neither the kidney-fumarate nor the liver-malonate system. Recrystallized difluoroacetate did not inhibit the liver-malonate system and the slight inhibition of the kidney-fumarate system at high concentrations may have been due to monofluoroacetate contaminating the product.

§Lehninger, A. L., Personal communication, see (8).

than oxidation at later time intervals, increasing concentration of fluoroacetate also depressed the initial oxidation.‖

The block of citrate oxidation is probably limited to a tricarboxylic acid step and at low levels of fluoroacetate it appears that the other reactions are not blocked, since a micromole of citrate is formed for each micromole of fumarate added to kidney homogenates inhibited *in vitro* (Fig. 5).¶

Effect of fluoroacetate on the liver-malonate system. The liver-malonate system also has one pathway for pyruvate oxidation, but in this case oxidative decarboxylation of pyruvate is followed by the condensation of the "C_2" fragments to acetoacetate. In this system, the depression of oxygen uptake (Fig. 1) was associated with a simultaneous diminution of pyruvate utilization (Fig. 2) and acetoacetate formation (Fig. 4) but these effects were not noted until *higher* concentrations of fluoroacetate were present as compared to the kidney-fumarate system. Moreover, the oxidation was increasingly depressed as the incubation progressed. The rate of oxidation of the blocked system here, however, was the same as that of the control until a time (30 minutes of incubation) when its activity suddenly decreased; oxidation by the control continued at a linear rate for at least 60 minutes. This delayed response accounts for the failure of high levels of fluoroacetate to produce more than a limited response in the measurements undertaken, and suggests that the action of the inhibitor must have been indirect just as in the kidney-fumarate system. It is unlikely, however, that the eventual decline in rate can be attributed to the formation of fluorocitrate since in this system essentially no oxidation takes place via the citric acid cycle, *i.e.*, no more than 0.25 μM of citrate were found in any of these flasks, and moreover there is no basis for explaining the results in terms of a block in the citric acid cycle, which was already blocked by malonate in this system. The time studies revealed that the inhibition of the liver-malonate system was not competitive; moreover, the inhibition was not prevented by addition of as much as 0.03 M $MgCl_2$(14).

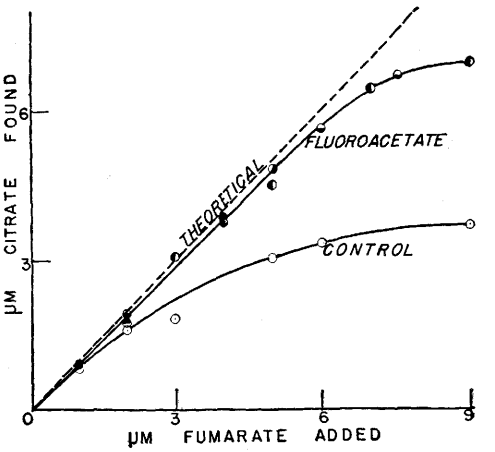

FIG. 5. Conversion of fumarate to citrate by kidney homogenates poisoned *in vitro* with fluoroacetate compared to controls. Additions as in kidney-fumarate system described in the text except for quantity of fumarate added. Flasks with fluoroacetate contained 6 μM.

Effect of fluoroacetate on the liver-fumarate system. The liver-fumarate system can oxidize pyruvate by both of the above mentioned pathways; these 2 pathways are in fact competitive, with citrate formation preferential when fumarate is available(8). Any differential effect of fluoroacetate would thus be revealed in this reaction as a shift away from

‖ Maintenance of bound phosphate was observed in the liver-fumarate system; bound phosphate of the kidney-fumarate system diminished as oxygen uptake decreased, in control flasks as well as in flasks containing fluoroacetate. Bound phosphate decreased rapidly even in control flasks of the liver-malonate system although oxidation proceeded at a linear rate, and at the end of 60 minutes of incubation, almost all the high energy phosphate had been released from ATP.

¶ In these experiments, oxygen uptake studies indicated that extra pyruvate disappearing was largely oxidized to completion and hence, fumarate must have cycled but was eventually trapped as citrate; for example, in a 60 minute incubation period, with 4 μM of fumarate and 30 μM of pyruvate as substrates, 6.7 μM of pyruvate disappeared, of which 3.8 μM entered into citrate formation; total oxygen uptake was 8.5 μM in the measured 50 minute period after the 10 minute equilibration period. The consumption of considerably more oxygen than the 1.9 μM necessary for conversion of pyruvate to citrate indicated that some fumarate proceeded around the cycle more than once but was eventually stopped only at the citrate level, suggesting that the other parts of the cycle were unaffected.

the normal ratio of citrate to acetoacetate. When the fluoroacetate concentration was increased, there was a diminution in citrate formation (Fig. 3) paralleled by a compensatory rise in acetoacetate formation such that pyruvate utilization actually increased (Fig. 2), while oxygen uptake continued unaffected (Fig. 1).

Discussion. The results indicate that fluoroacetate or its derivatives may inhibit 2 enzymatic steps in addition to aconitase, which has been studied in considerable detail by Peters and associates(15). The liver homogenate illustrates one of these inhibitions by decreasing the metabolism of pyruvate via the citric acid cycle and shifting to the alternative pathway of acetoacetate production. It seems likely that this result is due to a direct inhibition of an "acetokinase"(16) rather than to a shift resulting from the unavailability of C_4 acids, a mechanism studied by Lehninger(7), and by Recknagel and Potter(8), inasmuch as a considerable backlog of fumarate was present when fluoroacetate was studied. Differences in the K_I and K_M for these acetokinases and fluoroacetate or inhibitors formed from it must result in the metabolic shifts noted, and it appears that the acetokinase leading into the citric acid cycle is affected to a greater extent than the other acetokinases in liver. The decreased utilization of C_2 fragments for citric acid formation is reflected in an increased availability for acetoacetate formation *in vitro* and by the increased acetylation of foreign amines in intact rabbits treated with fluoroacetate(17). That the formation of acetoacetate can also be inhibited is shown by the data obtained with higher levels of fluoroacetate. The finding of Hagan *et al.*(18) of significantly less fluoroacetate in livers of poisoned rats than in other organs tested may result from utilization of "fluoroacetoacetate" formation as a means of detoxification of fluoroacetate by the liver.

The *in vitro* studies reported here suggest that the pharmacological effects of fluoroacetate may involve the production of more than one type of "biochemical lesion," while species differences may well reflect differing ratios of the several enzymes involved.

Summary. 1. Citrate formation is increased and oxidation is diminished by addition of fluoroacetate to kidney homogenates; under the same conditions, citrate formation is decreased and acetoacetate formation is increased while oxidation is unchanged in liver homogenates. 2. At higher concentrations of fluoroacetate, acetoacetate formation is also inhibited in liver homogenates.

1. Buffa, P., and Peters, R. A., *Nature,* 1949, v163, 914.
2. ———, *J. Physiol.,* 1950, v110, 488.
3. Potter, V. R., and Busch, H., *Cancer Res.,* 1950, v10, 353.
4. Potter, V. R., Busch, H., and Bothwell, J., PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 38.
5. Potter, V. R., PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 41.
6. Potter, V. R., Pardee, A. B., and Lyle, G. G., *J. Biol. Chem.,* 1948, v176, 1075.
7. Lehninger, A. L., *J. Biol. Chem.,* 1946, v164, 291.
8. Recknagel, R. O., and Potter, V. R., *J. Biol. Chem.,* 1951, v191, 263.
9. Potter, V. R., and Elvehjem, C. A., *J. Biol. Chem.,* 1936, v114, 495.
10. Natelson, S., Lugovoy, J. K., and Pincus, J. B., *J. Biol. Chem.,* 1947, v170, 597.
11. Friedemann, T. E., and Haugen, G. E., *J. Biol. Chem.,* 1943, v147, 415.
12. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.,* 1925, v66, 375.
13. Pardee, A. B., and Potter, V. R., *J. Biol. Chem.,* 1949, v181, 739.
14. Gillette, J. R., and Kalnitsky, G., *J. Biol. Chem.,* 1950, v187, 679.
15. Lotspeich, W. D., Peters, R. A., and Wilson, T. H., *Biochem. J.,* 1952, v51, 20.
16. Chou, T. C., and Lipmann, F., *J. Biol. Chem.,* 1952, v196, 89.
17. Chenoweth, M. B., *J. Pharm. and Exp. Therap.,* 1949, v97, 383.
18. Hagan, E. C., Ramsey, L. L., and Angel, R., *J. Pharm. and Exp. Therap.,* 1950, v98, 234.

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