Method for the Study of Tissue Metabolism *in Vivo* Using Fluoroacetate.* (18382)

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Sodium monofluoroacetate was shown by Buffa and Peters(1,2) to produce marked accumulations of citrate in various organs of rats following injections of lethal doses of the compound. Potter and Busch(3) confirmed their findings for normal rat tissues and, in addition, showed that in several kinds of malignant tissue from rats, no citrate accumulated following the injection of fluoroacetate.

When fluoroacetate is administered, an enzyme necessary for citrate oxidation becomes inactivated, while the enzymes necessary for citrate production appear to be relatively unaffected(4-6). The continued production of citrate without adequate means for its further metabolism leads to the accumulation of citrate in some tissues to the extent of 30 to 50 fold over the normal operating level. Although the level of citrate that develops in any given tissue is the resultant of at least 5 separate factors(3) that have yet to be individually evaluated, it appears that *the rise in citrate is primarily an expression of the metabolism of the individual organ.* This conclusion is supported by the fact that the individual organs in the fluoroacetate-treated animal exhibit citrate values that differ widely from each other, and from the blood level (*cf.* Buffa and Peters(2), p. 494). Moreover, as will be shown(7), in combination with other inhibitors, the various tissues produce or fail to produce citrate in a manner that suggests lack of effective equilibration via the blood.

The present study was undertaken to extend the area covered by the previous reports in terms of both the dosage and the rate at which citrate accumulates. The object of the experiments was to develop an experimental approach to the problem of studying the operation of the Krebs citric acid cycle and ancillary reactions in individual organs *in situ*. The accompanying report(7) shows results obtained with animals under altered physiological conditions.

Experimental. Young male rats weighing from 180-240 g were obtained from the Holtzman Rat Co. Freshly prepared solutions of sodium monofluoroacetate (Monsanto) were injected intraperitoneally and the rats were allowed access to food and water either for a fixed period of time or until death was imminent. Parallel tests were made in some cases with purified fluoroacetate† but no significant difference was detected. It is doubtful that the biological tests would reveal the difference between 90% and 100% purity unless a highly toxic impurity were present. The dosages and times were varied in each experiment in order that each point would involve the use of different batches of animals. The animals were killed by decapitation and the organs were quickly removed and analyzed for citrate as previously described(3). The dosage of fluoroacetate was varied from 0.3 to 50 mg per kg and the time was varied from 45 minutes to 48 hours.

Results. The data for brain, heart, thymus,

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spleen, and kidney are shown in Fig. 1. Data for the 0.3 mg per kg dose are omitted. The citrate accumulation was not followed beyond 2 hours in the case of the 5 mg dose, or beyond 6 hours in the case of the 2.0 and 3.5 mg doses for the reason that death occurred in the majority of animals that were observed longer than these times. A few animals at the 3.5 mg dose die before 3 hours, and an appreciable number die between 3 and 6 hours, so that the 6 hour animals are not average specimens. The LD_{50} on the basis of 24 hour survival lies between 1.0 and 2.0 mg per kg. With the former dose almost no deaths occurred before 24 hours and with the 2.0 mg dose there were no survivals after 12 hours.

The citrate accumulation was greatest at the 3.5 mg dosage, in all organs studied. The animals that received 5.0 mg per kg accumulated citrate as fast or faster than the animals receiving 3.5 mg but death prevented them from attaining the higher levels shown at the lower dosage.‡ Not shown in Fig. 1 are the

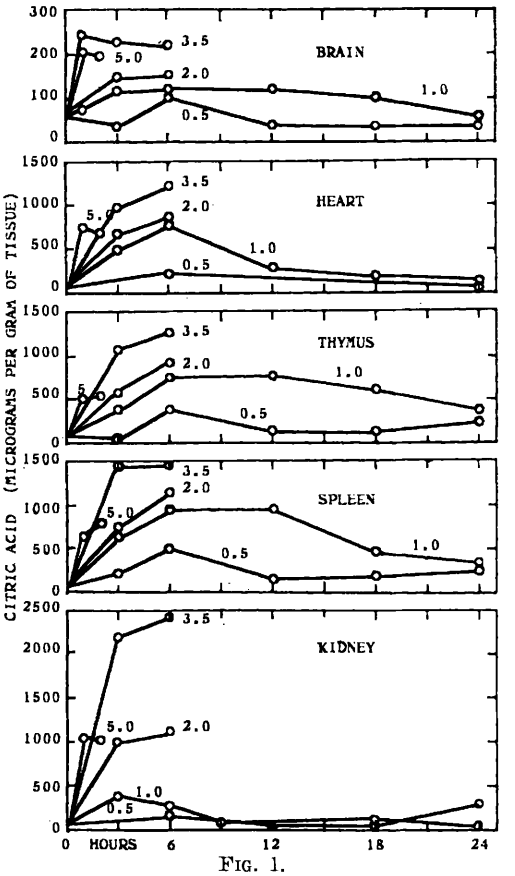

FIG. 1.

The citrate content of brain, heart, thymus, spleen, and kidney at various time intervals after the injection of doses of fluoroacetate ranging from 0.5 to 5.0 mg/kg. Each point is the average of 2 to 14 rats, usually 3 or 4 rats, with the major emphasis on the peak values. The curves for the various doses from 0.5 to 5.0 respectively represent the average values from 25, 32, 8, 18, and 11 rats respectively. The data on controls and on the 5 mg/kg dose are taken from a previous study(3).

results of a series in which 50.0 mg per kg was given to six animals. These animals survived for 22-70 minutes.‡ The average citric acid content of their tissues (μg per gram) was as follows: blood 186, brain 204, thymus 555, heart 877, lung 880, liver 46, kidney 1540, testes 108, spleen 291, pancreas 939 and muscle 171. These values show some marked deviations from the values obtained with 5.0 mg per kg (*cf.* Fig. 1 and previous reports) (2,3). At the 5 mg dosage the blood citrate was but slightly elevated (78, normals 58 μg per ml). However, at the 50 mg dosage the blood citrate was elevated to 186 μg per ml.

‡ Fig. 1 does not show any data on either liver or tumor tissue. Earlier studies(3) showed that neither of these tissues accumulated citrate following a lethal dose of 5 mg of fluoroacetate per kg. In order to supplement the earlier study, a number of experiments were carried out to determine whether a higher or lower dose of fluoroacetate would lead to citrate accumulation in these tissues. The 3.5 mg dosage was given to 2 rats bearing the Flexner-Jobling carcinoma and to 3 rats bearing the Jensen sarcoma, and the animals were killed at 6 hours. The average citrate values were 150 and 85 μg/g respectively in comparison with the 121 and 85 μg/g found in uninjected controls(3). When a still lower dosage was given, 0.5 mg/kg, to 3 rats bearing Flexner-Jobling tumors the citrate content at 6 hours was 118 μg/g. The high dosage of 50 mg per kg was given to 6 tumor-bearing animals and the normal tissues as well as the tumors were analyzed when death was imminent, as in the case of 6 non-tumorous rats described in the text. The results were about the same or slightly lower than in the non-tumorous animals (see text) except in the case of kidney in which the value was 644 μg/g in comparison with a value of 1540 μg/g in the non-tumorous animals, accentuating the trend seen earlier in tumor-bearing animals at the lower dose(3). The tumors contained less citrate than the tumors from the non-injected animals.

This finding suggests that at this dosage, possibly some organs were damaged sufficiently to become more permeable to citrate. This explanation may find support in the fact that the citrate content of the spleen was much lower than it was in the case of the lower dosage. The lung and pancreas, however, accumulated more citrate at the higher dosage.

Of considerable interest is a comparison of the relation between the dose of fluoroacetate injected and the amount of citrate accumulated in the different organs. If the data in Fig. 1 are visualized in a graph (not shown) in which the citrate per gram of tissue at 3 or 6 hours is plotted against the dose in mg per kg, it can be seen that for each tissue the amount of citrate accumulated rises with increasing dose and reaches its maximum level at a fluoroacetate dose of about 3.5 mg per kg. At higher doses, the plateau level is not attained, owing to the early death of the animals at doses of 5 or 50 mg per kg.

It is assumed that the maximum level represented by the 3.5 mg dose is a rough measure of the maximum capacity of the respective tissues to produce citrate, and since the level is about the same at 3 and 6 hours (Fig. 1) the best comparison of the ability of the various tissues to produce citrate may be obtained by analyzing the tissues 3 hours after the administration of 3.5 mg per kg. Further support for the use of this time period is obtained from studies(7) showing that the rate is almost linear on the basis of time periods of 1, 2, and 3 hours, and this combination of time and dosage constitutes the method herein proposed for further studies, with supplementary studies on animals killed at 1 and 2 hours. Since the exact values are not apparent from Fig. 1, they are tabulated in Table I, which includes data from a concomitant investigation(7).

If the relation between citrate accumulation and dose of fluoroacetate is examined further, it may be seen that the shape of the resulting curves is not uniform from tissue to tissue. Kidney, which on the basis of results obtained at 3.5 mg per kg has the greatest citrate-forming potential, accumulates much less citrate at the 0.5 and 1.0 mg dosages than do the heart, spleen, and thymus. This result would not be unexpected if the latter tissues contained less of the citrate-oxidizing enzyme component that is inhibited by fluoroacetate and if the lower doses of fluoroacetate "titrated" a fixed amount of this enzyme from each tissue (*cf.* Ackermann and Potter)(8), since such a titration would leave a larger amount of effective citrate-oxidizing enzyme in the kidney tissue to prevent citrate accumulation at the lower levels of fluoroacetate. The fact that at the high dosages kidney accumulates more citrate than the other tissues suggests that it may actually have more of the enzymes involved in citrate formation and removal than do the other tissues.

This marked difference between the response of kidney and other tissues may actually form the basis of a crude assay for fluoroacetate§ since for example, the ratio of kidney

TABLE I. Citrate Content of Various Rat Organs Following a Dose of 3.5 mg of Fluoroacetate per kg.

Organ	Time after inj. of fluoroacetate			
	0	1	2	3
Kidney	56(5)	887(3)	1440(4)	2211(7)
Heart	49(5)	636(3)	749(4)	988(7)
Thymus	55(5)	338(2)	620(3)	1070(6)
Spleen	59(5)	717(3)	1012(4)	1462(7)
Brain	57(5)	238(3)	189(4)	230(7)

Figures in parentheses indicate number of animals, data given in μg of citric acid per g of tissue, wet weight.

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§ The question has been raised as to whether the liver and tumor tissue had been penetrated by the fluoroacetate, since they failed to accumulate citrate. Potter and Busch(6) showed evidence that the liver might shift the balance of citrate production: acetoacetate production in favor of the latter, thereby shunting fluoroacetate into acetoacetate rather than into citrate with a consequent return of the fluorine derivative to the blood-stream. By direct analysis, Hagan *et al.*(10) have shown that the liver contains somewhat less fluoroacetate than certain other tissues, following injections of fluoroacetate but no data were available on tumor nor was the method available. A bioassay was therefore attempted (*cf.* Frick and Boebel)(11) using the carcasses of the 6

citrate to spleen citrate is roughly 0.4, 1.0 and 1.67 at the 1, 2, and 3.5 mg doses of fluoroacetate, respectively.

The data in Fig. 1 show that the citrate content of the tissues falls with time, after the maximum has been reached, in the case of the non-lethal doses. This suggests that in the case of the higher doses, although no decline is seen, owing to the death of the animals, the apparent rise is merely the resultant of the rate of production and the rate of removal, so that the amount actually accumulated is not a full measure of the citrate-producing potential of the tissue. In independent studies elsewhere (personal communication from Dr. J. Schubert(9), Argonne National Laboratory) these 2 processes have been examined in greater detail, but the results of these studies have not been applied here.

Summary. Sodium monofluoroacetate was administered to Holtzman white rats in doses ranging from 0.3 to 50 mg per kg, and the citrate content of kidney, thymus, brain, spleen, heart, liver and tumors was determined at times ranging from 20 minutes to 48 hours. As in previous studies liver and tumors did not accumulate citrate, but the remaining tissues did, with the maximum occurring in all cases with a dose of 3.5 mg per kg at 3 to 6 hours after the injection. As a standard procedure this dose was recommended for the study of certain phases of the metabolism of individual tissues *in vivo*.

rats that received the 50 mg/kg dose as a source of tissues. Twelve-gram portions of pooled tumor tissue, pooled livers, and combined hearts, spleens, lungs, and kidneys were fed, respectively, to 3 groups of 2 rats each, in the absence of other food. Eighteen hours later, the tissues had been completely eaten, and one of the tumor-fed animals was dead, having shown the typical signs of fluoroacetate poisoning. The remaining rats were killed, and the spleens and kidneys from the six rats (one post-mortem), *cf* Buffa and Peters(2) were analyzed for citrate. The citrate content in μg per gram in the spleens was as follows: tumor-fed, 1404 and 790 (dead specimen); liver-fed, 1440 and 1130, pooled organ fed, 970 and 1310. The citrate contents in the respective kidneys were 457 and 242, 192 and 200, and 238 and 614. On the basis of the fact that the spleens contained more than the kidneys, in every case, it would appear that the fluoroacetate given was equivalent to an injected dose of less than 2.0 and more than 1.0 mg per kg, and that the tumors contained approximately as much as the other tissues.

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Sequential Blocking of Metabolic Pathways *in vivo*.* (18383)

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Malonate is well known as an inhibitor of succinic dehydrogenase in *in vitro* systems. Relatively few studies have been made of its action *in vivo*, and these have been limited to the metabolism of the organism in terms of blood(1,2) or urine(3,4) changes or to the phosphate balance in a single organ(5). Since malonate presumably should interrupt the citric acid cycle by blocking succinic de-

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