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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hydrogenase, it should be possible to study the effect of malonate *in vivo* by measuring its effect upon the citric acid accumulation in individual organs in an animal treated with fluoroacetate, which has recently been shown to block the oxidation of citric acid *in vivo* (6-8).

The present study was undertaken as a model for what might result from the combined action, *in vivo*, of two inhibitors, each of which acts on the same metabolic sequence but upon *different* enzymes within a limited portion of the sequence. The combined use of two or more inhibitors in this manner will be referred to as *sequential blocking*.

In attempting chemotherapy by preventing the formation of an "essential metabolite" two inhibitors may be better than one from the following considerations. Both in the phenomenon of competitive inhibition by reversible inhibitors and in substrate "interference" in the case of irreversible inhibitors (9) an enzyme is less liable to combination with the inhibitor if its specific substrate is present in excess. When one inhibitor is used, the inactivation of part of the enzyme results in an accumulation of its substrate, which tends to protect the remaining enzyme from attack. However, the reaction might be slowed sufficiently so that if the next enzyme in the sequence is attacked by a second inhibitor the formation of its substrate by the prior enzyme may be so limited that the protection afforded to the second enzyme by its substrate becomes negligible. In this case the inactivation of the second enzyme should proceed farther toward completion, and the formation of product from the sequentially blocked system should be less than could have been accomplished with either inhibitor alone.

The study of sequential blocking *in vivo* requires a fairly complete knowledge of the individual steps and converging pathways in a metabolic sequence, the availability of effective inhibitors for at least two of the steps, and satisfactory analytical methods for the accumulating metabolites or the inhibited enzymes. There are indeed relatively few instances in which these requirements are fulfilled. In the case of the Krebs cycle, the ease with which citrate may be determined, the availability of malonate and fluoroacetate as inhibitors, and the immense amount of literature on the main metabolic pathway and its side reactions made the experimental manipulations in this study fairly simple. Nevertheless, it was found that the results obtained could not have been fully predicted on the basis of existing *in vitro* data.

The nature of the Krebs cycle is such that the product of citrate oxidation is ultimately converted to oxalacetate, which combines with acetate(C_2) to form more citrate, and, in the absence of ancillary reactions that produce the acids of the Krebs cycle, the amount of citrate that could accumulate would be no larger than the summation of these intermediates(10). The amounts of citrate that are actually found(6-8) are so large in some tissues that it might be assumed that proteins or other sources were, in fact, being mobilized as precursors of the Krebs intermediates during the development of the fluoroacetate block. On the basis of the known side reactions, these precursors might enter the Krebs cycle either between citrate and succinate or between fumarate and oxalacetate.

If sodium malonate acts *in vivo* according to what might be expected from *in vitro* studies, its primary effect should be to interrupt the Krebs cycle at the point of the conversion of succinate to fumarate. Thus the proper dosage of sodium malonate might be expected to alter citrate production in the presence of a fluoroacetate block in a manner that would depend upon the nature and extent of the side reactions. The present study shows that malonate affects citrate production differently in various organs and thus leads to the inference that the contribution of the side reactions differs widely from one

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organ to another.

Experimental. Young male rats weighing from 180 to 240 g were obtained from the Holtzman Rat Co. Neutral 1 M solutions of sodium malonate were injected subcutaneously in several sites on the back, following the indications from previous studies(1-5). The concentration was not varied but the dose was varied and increased beyond what had been used hitherto. Preliminary trials with *potassium* malonate intraperitoneally resulted in almost immediate deaths, and no experiments of this type are reported. Freshly prepared solutions of sodium monofluoroacetate (Monsanto) were injected intraperitoneally at a dosage of 3.5 mg per kg in all cases, either into rats that received no other treatment, or into rats that had received sodium malonate. This level of fluoroacetate was shown to result in the maximum rate of citrate production during the first 3 hours(6). In general, one animal was injected with fluoroacetate alone and tested in parallel with 2 or 3 animals that had received additional treatment. The animals were decapitated at 1 to 3 hours after the fluoroacetate injection, and the organs were quickly removed and chilled as before(6). In this study, only the heart, brain, kidney, thymus and spleen were removed for analysis. Protein-free filtrates were analyzed as before, using the modified pentabromoacetone method(8,11). Results are expressed in terms of micrograms of citric acid per gram of fresh tissue.

Results. The results of the experiments with combinations of malonate and fluoroacetate are given in Fig. 1.† The first point to be emphasized is that the 5 different tissues responded individually just as in the case of fluoroacetate alone, again suggesting that the results depend essentially on the metabolism of the individual tissues. Thus in the malonate treated animals, the data have to be discussed for each tissue. The fact that strikingly different results were obtained suggests that no individual result can be ascribed to a general systemic effect.

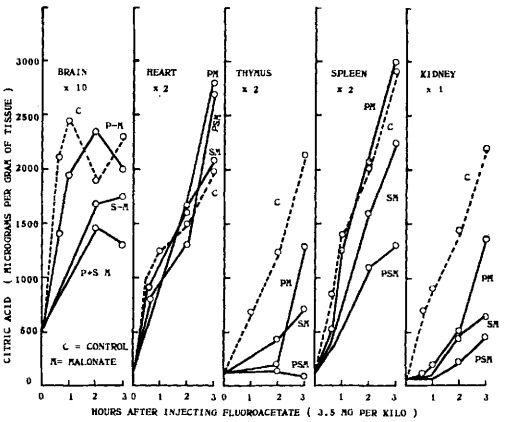

FIG. 1.

Citric acid accumulation in rat tissues following *sequential blocking* of the Krebs citric acid cycle by various combinations of malonate and fluoroacetate. Animals receiving fluoroacetate only are called controls, and the data are given in dashed lines labeled C. Malonate was given in various combinations in addition to fluoroacetate. Curves labeled PM (prior malonate) represent 1.2 cc of 1 M malonate per 100 g 60 min. prior to the fluoroacetate. Curves labeled SM (simultaneous malonate) represent the same dose of malonate, but the malonate was given at the same time as the fluoroacetate. Curves labeled PSM (prior and simultaneous malonate) represent a dosage of 1.2 cc of 1 M malonate per 100 g followed in 60 min. by *one-half* this amount of malonate and the usual dose of fluoroacetate. The data have been multiplied by the numbers indicated for each tissue in order to use the same scale for the ordinates. Each point represents the average of 2 to 14 animals (usually 3) and the control, PM, SM, and PSM curves represent 17, 5, 9 and 5 animals respectively, except in the case of thymus in which 12, 5, 4, and 5 analyses were obtained, owing to insufficient samples in the remaining animals.

The most decisive malonate block of citrate formation was obtained in the case of *thymus* when 1.2 ml of 1 M malonate was followed

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† Fig. 1 represents only the data obtained with the more effective doses of malonate and fluoroacetate. A number of tests were carried out with lower doses of both inhibitors. Two animals received 0.8 cc of 1.0 M malonate per 100 g followed immediately by the standard 3.5 mg dose of fluoroacetate. Three hours later the average citric acid values were: brain 215, kidney 1000, thymus 720, spleen 1315, and heart 1200. The kidney and thymus values are significantly lower than the controls and all of the values support the findings in Fig. 1. Tests with lower doses of malonate in combination with 1 mg of fluoroacetate were inconclusive. When 1.2 cc of malonate per 100 g was injected and no fluoroacetate was given, the citrate values were not elevated (27-49 μg per gram).

in one hour by one-half this dose with the fluoroacetate given at the time of the second malonate injection (curves labeled PSM, prior and simultaneous malonate, in Fig. 1).

When only one dose of malonate was given, the results depended on the time of administration: the *prior* dose of malonate (PM) suppressed citrate formation for two hours, but then wore off, indicating that the effective malonate concentration was maintained for a total of only 3 hours following a single subcutaneous injection. This conclusion is in harmony with the recent report that malonate can be metabolized in mice but that the CO_2 arising therefrom appears in the first 2 to 3 hours(12). Similar experiments on rats, including tests on urine would be of interest. When the *simultaneous* dose of malonate (SM) was given there was evidently some citrate synthesis before the malonate block became effective. Only in the case of the PSM dosage was the block fully effective.

Of the other tissues studied, only the *kidney* yielded data resembling that obtained from thymus. In the case of kidney the same phenomena were observed but the block was quantitatively less complete. In the case of *spleen* the prior malonate (PM) dose was without effect although the PSM dose showed a significant decrease in citrate accumulation. *Brain* showed a response that was very much like that of spleen except that the total amounts of citrate were much smaller at all combinations.

Heart showed a striking deviation from the other tissues. When prior malonate was given, the citrate formed during the third hour after fluoroacetate was markedly *increased* above that found in the animals receiving fluoroacetate alone. This finding suggests that in heart, oxalacetate may be formed from a source other than succinate under these conditions.‡ Had the PM or PSM dosages resulted in citrate accumulations no greater than in the controls it might have been possible to suggest that the amount of succinate formed in the malonate system was great enough to break through the competitive inhibition which is characteristic of malonate *in vitro*. However, the finding of a significantly *greater* citrate than in the case of fluoroacetate alone is not satisfactorily explained in these terms.‡ A clue to the alternative source of oxalacetate stems from experiments on fasting animals. In fasting rats, the injection of fluoroacetate results in a greater citrate accumulation in heart than is observed when fed animals are injected(13). The heart utilizes fatty acids(14), and moreover hearts from fasting(15) and diabetic(16,17) animals have higher than normal glycogen levels, while injected ketone bodies lead to increased heart glycogen values(18). Since malonate has been reported to induce ketonemia(1), the question arises as to whether, *in the heart,* ketone bodies may be convertible to both glycogen and to citrate by a pathway involving oxalacetate but not succinate.

The heart findings are of interest from another standpoint, since regardless of the explanation of how the oxalacetate became

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‡ Among the well-known sources of oxalacetate that are independent of succinate oxidation are (1) the transamination between aspartic acid and α-ketoglutaric acid to yield oxalacetic and glutamic acids, and (2) the Wood-Werkman reaction, or its modifications, by which pyruvate and CO_2 lead through a condensation reaction to oxalacetate. Since it was difficult to see how these reactions would be accelerated in the presence of malonate our attention was led to a consideration of ketone body formation, a reaction that is *increased* in the presence of malonate in liver *in vitro* (*cf.* Recknagel and Potter, to be published) and on this basis ketone bodies were discussed in the above text. Isotope experiments are in progress which may help to decide which, if any, of the 4 possible sources of oxalacetate predominates in the hearts of the malonate-poisoned animals.

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available for citrate synthesis in the heart, it apparently was not available to kidney or thymus via the bloodstream.

Discussion. The results of these experiments are complicated by the fact that fluoroacetate *per se* does not inhibit citrate oxidation but has to be converted to some compound (possibly fluorocitrate or a derivative of it)(8,19,20) that is the true inhibitor. However, in the malonate experiments for example, where the data indicate a failure to form citrate, a failure to form fluorocitrate would yield the same result, the difference being that in the one case the citrate oxidation would be impaired while in the other it would not. This point can be tested experimentally by studying the oxidation *in vitro* using tissues from the poisoned animals. Busch and Potter(10) found a marked decrease in the ability of kidney homogenates from fluoroacetate-treated animals to oxidize citrate, without a concomitant decrease in the rate of oxidation with pyruvate plus fumarate. Further tests, including thymus, remain to be made.

In these experiments it has been tentatively assumed that malonate blocked succinate oxidation in all tissues, but the results in certain tissues might lead to alternative hypotheses that in these tissues malonate did not enter the cells, or was rapidly destroyed, or was overwhelmed by accumulating succinate. While the determination of succinate and of malonate is not as feasible as that of citrate, it is hoped to determine the amounts of these substances chromatographically. Earlier *in vivo* studies do not provide an answer to the tissue differences, but do indicate that succinic dehydrogenase was blocked, because increased amounts of succinate appeared in the urine(3,4).

It should be emphasized that the failure to form citrate after some pretreatment that is followed by fluoroacetate does not imply that the pretreatment, *e.g.* malonate, necessarily inhibited some enzyme in the metabolic sequence immediately prior to citrate. It was understood at the outset that the experimental basis for sequential blocking would have to be fairly well established by independent studies before a sequential block could be assumed to have occurred. The fluoroacetate block might result in no citrate formation in a tissue in which the cells have received lethal or near lethal treatment of any kind, even though the animal as a whole is destined to die much later. Through the cooperation of Dr. K. P. DuBois(21), unpublished experiments may be cited in which rats that received lethal doses of 800 r of X-ray irradiation, were given the standard fluoroacetate block (3.5 mg per kg and killed 3 hours later) at various times after irradiation. Non-injected animals died 4 to 5 days after irradiation. The injected animals were found to have a marked decrease in citrate production in spleen and thymus, a definite decrease in the case of kidney, and no effect in heart or brain. These changes began within 0 to 3 hours after irradiation and continued until death. It seems likely that in this and numerous other instances a destruction of some cell component will have repercussions that may involve the Krebs cycle and be revealed by the technic herein described before pathological changes will be evident microscopically. It would not be appropriate to consider such phenomena as sequential blocking in the absence of further information as to which specific enzyme, if any, is involved. However, the data illustrate another example of the usefulness of the fluoroacetate block in revealing differences in the metabolism of individual organs in animals following some treatment administered to the whole animal.§ Further studies to be reported later have been carried out in this laboratory using the fluoroacetate block in fasting animals and following the administration of aminopterin, alloxan, cortisone, and insulin. In all cases, the individual organs do not give parallel responses. It appears that the technic may have wide applicability in the study of tissue metabolism and in combination with iso-

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topically labeled precursors should permit the determination of the rate of entry of a given metabolite into the Krebs citric acid cycle in individual organs.

From the therapeutic standpoint, combinations of inhibitors or inhibitors and hormones that act sequentially on metabolic pathways such as protein and nucleic acid synthesis should be sought. Not only should simultaneous injections (*cf.* Skipper)(21) be attempted, but the individual drugs should also be given separately and in different sequences of administration.

Summary. The action of two or more inhibitors, each of which acts on the same metabolic sequence but upon different enzymes within a limited portion of the sequence, was defined as *sequential blocking*. As a model of such a system, the combined action of malonate and fluoroacetate was studied *in vivo*. It was shown that malonate decreased citrate formation to varying degrees in thymus, kidney, spleen and brain, but increased citrate formation in heart following the injection of fluoroacetate into rats.

§ Note added Jan. 30, 1951: Livers from irradiated male rats were found to accumulate large amounts of citrate following the fluoroacetate treatment(21) in contrast to livers from normal male rats, which do not accumulate citrate. It was also found that normal *female* rats showed a liver response to fluoroacetate and the latter finding has been confirmed in this laboratory, in which preliminary tests with *males* given dessicated thyroid have given liver responses in 4 out of 9 animals, suggesting that several hormone relationships may be involved.

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Effect of Yolk Removal in Day-old Chicks on Early Nutritional Requirements.* (18384)

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(Introduced by H. R. Bird.)
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Several investigators have found a positive relationship between early chick growth and the diet of the dam. Rubin and Bird(1) demonstrated that an unidentified growth factor present in cow manure, later shown to be identical with vit. B_{12}, was transmitted through the egg and thence to the chick. Subsequently, Rubin and Bird(2) reported the chick growth factor to be chiefly concentrated in the yolk of the egg. Bethke *et al.* (3), McGinnis and Carver(4), Wiese *et al.* (5), Robblee *et al.*(6), and Csonka and Olsen (7) also found that the diet of the breeders had a definite influence on the growth of the progeny. It is evident, therefore, that nutritional studies involving the chick are limited by the extent to which the chick can be depleted of the nutrients under consideration. Studies concerning vit. B_{12} and unidentified chick growth factors conducted by Menge *et al.*(8) in this laboratory have involved the use of chicks from dams fed rations contain-

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