SUCCINATE ACCUMULATION IN VIVO FOLLOWING INJECTION OF MALONATE

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Among the factors involved in the accumulation of citrate by a tissue following the injection of fluoroacetate in vivo is the ability of the tissue to convert precursors into 4-carbon acids (1). An indirect measure of the importance of this conversion was reported in a recent study (2) in which the Krebs cycle was blocked both by fluoroacetate and malonate, but in this study concurrent analyses for succinate were not attempted. Recently developed chromatographic techniques (3) have made it possible to determine not only the concentration of succinate, the metabolite accumulated, but also the concentration of malonate, the inhibitor, in tissue extracts following the injection of malonate. Previous studies on the effects of malonate in vivo have dealt with the metabolism of C4-malonate (4–6) and the effects of malonate on components of blood and urine (7–10). In the present study, the optimal conditions for the accumulation of succinate in tissues in vivo were defined, and the rate of excretion of succinate, citrate, and malonate in the urine was determined.

EXPERIMENTAL

Tissue Studies—Female rats, bearing 10 day-old Flexner-Jobling tumors maintained on a normal stock diet, and weighing 140 to 180 gm., were used in these studies. The aggregate weight of the four tumor masses per rat averaged 2.5 gm. At the indicated time intervals after the injection of malonate (Figs. 1 and 2), the rats were decapitated and blood was obtained from the neck vessels. The liver, kidneys, and tumors were excised, chilled, and then homogenized in a volume of 0.6 N perchloric acid sufficient to produce a final concentration of 0.33 N. The precipitated protein was removed by centrifugation and the supernatant solutions were neutralized with KOH, causing precipitation of the bulk of the perchlorate ion as potassium perchlorate. The neutralized supernatant solutions were passed into Dowex 1 resin columns (formate form) which were subjected to a slowly increasing concentration of formic acid, as previously de-

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scribed for the isolation and quantitative determination of succinate and malonate (3). In comparison to the melting point of an authentic sample of succinic acid, 187–189°, the melting points of the succinic acid from liver and kidney samples ranged from 165–178° and the melting points of the succinic acid of tumor samples from 178–183°. The purity of the succinic acid of urine samples was higher, as manifested by a melting point of 188°. The quantity of acid present in each fraction was determined by titration after evaporation of the eluent (3), and the values presented in the graphs represent the total titration in the succinate and malonate peaks.

Time and Dose Studies—One group of animals received one injection of 1.2 ml. of 1 M sodium malonate per 100 gm. of body weight, injected subcutaneously into the inguinal region or the back, and the experiment was terminated at ³⁄₄, 1, or 2 hours after the injection. Another group of animals received two injections, the above dose being supplemented at the end of the 1st hour with either a repetition of the initial dose or with one-half the initial dose; the initial dose established a level of malonate on which the second dose was superimposed. The animals receiving two injections were all killed 2 hours after the initial injection. The highest dose employed resulted in marked dyspnea in most animals, convulsions in a large percentage, and death in a few cases by the end of the 2nd hour; hence higher dosage levels or longer periods of treatment are not feasible. The results of these studies are presented in Figs. 1 and 2, in which each point is the average of two column separations with samples that represent pooled tissues from two rats. Twenty-four rats were used to obtain the data reported, but no more than two rats were injected on any given day. The average deviation from the values noted on the graphs was 0.77 μeq. for succinate, and 1.4 μeq. for malonate except for the malonate of the kidney, for which the deviation was 8.5 μeq. In addition, the curves reinforce the individual values. The concentration of malonate in the kidney greatly exceeded that of other tissues and, like that of the blood, reached a maximum ½ hour after a single injection (Fig. 1). Presumably the blood transports the malonate from the subcutaneous depot to the kidney where it is concentrated and excreted. Maximal concentration of malonate in the liver and tumor was reached 1 hour after the injection of a single dose. With increasing dosages, the concentration of malonate rose sharply and concomitantly in the blood and in the tumor, but little malonate was found in the liver until the largest dose was given. It is possible that the liver destroys or fails to retain the malonate in quantities supplied at the lower levels.

With a single injection of malonate, the maximal succinate accumulation in the tissues was found at 1 hour after the injection and correlates
with the maximal malonate content in liver and tumor (Fig. 2). The discrepancy in the case of kidney may indicate that not all the malonate present is effectively blocking the available succinic dehydrogenase at ½ hour and may mean that a significant amount of the malonate is not located at the particulate sites of succinic dehydrogenase activity. By the end of the 2nd hour, the concentration of succinate in the tissues had fallen to the level noted at ½ hour. With increasing dosage, a steady rise in the concentration of succinate was found both in the kidney and in the tumor. Although the highest concentrations of succinate were found in the kidney, the difference in concentration between the kidney and other tissues for this substance was not as marked as in the case of malonate (Fig. 1). The concentration of succinate in the liver paralleled the concentration of malonate and also did not increase until the highest doses were administered. Little succinate was found in the blood at any time or dose, although significant amounts were found in the presence of the highest levels of malonate (Fig. 3). These data indicate that equilibration of the succinate of the tissues and that of the blood was incomplete; a similar incomplete equilibration of citrate was found in fluoroacetate poisoning (1, 11, 12).

The ratios of succinate to malonate were approximately 1.0 for liver, 0.4 for kidney, and 0.1 for blood throughout the range studied (Fig. 3).
SUCCINATE ACCUMULATION IN TISSUES

Such a simple relationship did not hold for the Flexner-Jobling carcinoma:
at low levels of malonate, the ratio of succinate to malonate was as great
as 3, while at higher levels, the ratio diminished to 0.5. After the in-
fection of the curve (Fig. 3), the slope approaches the slope of the line for
blood. This limitation of succinate accumulation in the tumor may mean
that the supply of 4-carbon acids and their precursors is rapidly depleted,
with the result that the tumor becomes dependent on a limited blood-borne
supply (see the discussion). Recent studies which indicate a low rate of
protein breakdown in this tumor under fasting conditions (13) support
this idea. The slope of the lines in Fig. 3 represents molar ratios of the
substrate of the inhibited enzyme to the concentration of the inhibitor
present; these values are seldom assessable in pharmacological studies.
In this case, they could include malonate which is present in the tissue
but not capable of causing accumulation of succinate. Hence the ratios
are resultants of the permeability of the mitochondria and the cell to
malonate, the total amount of enzyme within the cell, and other vectors
as yet unknown.

Excretion Studies—The rats used in this study were similar to those
employed in the above studies with the exception of the fact that they
did not bear tumors. Following the injection of 1.2 ml. of 1 M sodium
malonate per 100 gm. of body weight, the rats were placed in a metabolism cage and the urine was collected into flasks containing 2 ml. of 6 N perchloric acid. Determinations of succinate and malonate content were made on the urine samples excreted in four periods: the 1st hour, 2nd and 3rd hours, 4th to 6th hours, and the 7th to 24th hours. Malonic acid was identified by its position on the chromatogram (3), the melting point (133°, which was the same as the reagent injected), and the neutral equivalent (53 as compared with the known value of 52). The succinic acid was identified by its position on the chromatogram (3) and its melting point of 188°. Citrate was determined on aliquots of the perchloric acid filtrates of the urine by the method of Natelson, Lugovoy, and Pincus (14). The rate of urinary excretion of these acids is shown in Fig. 4. At 1 hour, 30 per cent of the injected malonate had been excreted, 55 per cent at 3 hours, and almost 70 per cent by 24 hours. The remaining malonate, unaccounted for by excretion, may have been oxidized, since Lifson and Stolen (5) found that in the 1st hour after the injection of malonate carboxyl-C\textsuperscript{13} into mice 10 per cent of the label was found in the breath, 20 per cent after 3 hours, and 26 per cent after 24 hours. Moreover, Lee and Lifson (6) found that the rat also oxidizes malonate. Although the rate of succinate excretion was similar to that of malonate, citrate was eliminated more slowly and most of it appeared in the 21 hours after the bulk of the malonate had been excreted.

**DISCUSSION**

A number of studies have demonstrated succinate in the urine following the injection of malonate in vivo (4, 6, 9). These findings have constituted

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In a series of experiments designed to test the effects of malonate on the growth of Flexner-Jobling tumors, malonate was injected into rats every 3 hours for 4 days in a dosage of 1.2 ml. of 1 M malonate per 100 gm. of body weight. In this case, a milk supplement was added to the regular grain diet. In one series of experiments some diminution of the growth of the tumor was noted, and in another, in which the tumors were smaller initially, an increased tendency of the tumor to invade the deeper musculature was noted. In addition to these effects, a scirrhous edema of the liver was noted. Although two injections of this dose of malonate 1 hour apart were lethal for some animals, in this case most animals received thirty-two injections and all survived, indicating that the 3 hour interval enabled the animals to excrete or detoxify the injected malonate. The total dose given in the 4 day period was approximately 75 mm per rat. No change was found in succinoxidase concentration of liver, tumor, or kidney.

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There is evidence that citrate excretion in the urine can result from alkalosis (15) which could result from oxidation of malonate (5, 6). In quantities equivalent to those of the malonate used in this study, sodium oxalacetate, which has a greater tendency to produce alkalosis on a molar basis, resulted in marked excretion of citrate, but had little effect on excretion of succinate (9).
evidence for the operation of the Krebs cycle in vivo. Quantitative data presented here corroborate and extend the earlier data. Moreover, evidence for succinate accumulation in the kidney, liver, and tumor has been obtained, and time and dose studies on these tissues provided a basis for determination of optimal conditions for succinate accumulation. It is evident that a number of factors can influence the succinate content of a given tissue following the injection of malonate and include activity of the Krebs cycle, availability of amino acid precursors, the concentration of malonate in the tissue and the extent to which it is effective in blocking succinic dehydrogenase, the rate at which succinate formed by tissues diffuses into the blood, and the rate at which succinate from other tissues enters any given tissue via the blood. The last two factors in particular call attention to the possibility that the dose of malonate of greatest utility in the study of the metabolism of a given tissue in vivo may be less than the dose giving maximal succinate formation.

It is apparent that the amount of succinate accumulated greatly exceeds the amount of Krebs' cycle intermediates available for conversion to succinate and that precursors from outside the cycle must be utilized (cf. (2)). The data may be more valuable in assaying the ability of a tissue to convert glutamate, for example, to succinate, than in deciding the rôle of the Krebs cycle in the individual tissue. However, supplementary information on the latter point can be gained by using isotopically labeled precursors such as pyruvate and acetate, as experiments in progress have shown.

This report and those preceding it (1–3, 11) constitute an experimental approach to the metabolism of individual organs in the whole animal, which can conveniently be referred to as "in vivo metabolic blocking techniques." While the data so obtained are obviously the resultant of many vectors, it is already established that marked individual differences between organs can be demonstrated. Moreover, the data show the effect of changes in the physiology of the animal ((16, 17) and unpublished studies from this laboratory). It would seem that the method should be applicable to the study of other enzyme inhibitors and other metabolic pathways.

The use of specific enzyme inhibitors to produce biochemical lesions in the whole animal by blocking a metabolic pathway is comparable to the blocking that results when there is a deficiency of a specific factor, such as insulin or a vitamin. However, for the study of metabolism, the use of a specific inhibitor has the advantage that the block may be initiated at a more definite point on the time scale.

4 These studies in vivo also constitute an experimental approach to the mechanism of action of enzyme inhibitors as chemotherapeutic agents.
SUMMARY

1. The injection of 1.2 ml. of 1 M sodium malonate per 100 gm. of body weight into rats bearing Flexner-Jobling carcinomas, followed in 1 hour by a repetition of this dose, resulted in the accumulation of increasing amounts of succinate in the liver, kidney, and tumors up to 2 hours. The time studies indicated that the maximal concentrations of succinate were reached 1 hour after the injection of a single dose of malonate and the second dose resulted in the superimposition of the second maximum on the base level created by the first dose.

2. A linear relationship was found between succinate and malonate in the liver, kidney, and blood, but the relationship for the Flexner-Jobling tumor was more complex.

3. Within 6 hours, 55 per cent of the malonate injected was recoverable in the urine. The excretion of succinate paralleled that of malonate, but the excretion of citrate did not.

4. The utility of this type of metabolic block for the study of the metabolism of individual organs in vivo was discussed.

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