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A STUDY ON THE TOXICITY AND THE BIOCHEMICAL EFFECTS OF MONOFLUOROMALONATE

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Abstract—The toxicity of monofluoromalonate (FM) to guinea pigs, rats and mice has been examined. The symptoms of poisoning observed are similar to those obtained with fluoroacetate (FAc), but the LD_{50} of FM is found to be higher by an order of magnitude. The action of FM is accompanied by an accumulation of citrate in various tissues, the effect being most pronounced in the kidney.

It is shown that, under the experimental conditions employed, spontaneous decarboxylation of FM to FAc is negligible. However, evidence is presented that, with guinea pig kidney particles, the conversion of FM to FAc does take place. The experimental results substantiate the assumption that the metabolic effects of FM are due, at least partly, to FAc produced as an intermediary by enzymatic decarboxylation.

The inhibitory effect of FM on succinic dehydrogenase and oxaloacetic decarboxylase proves to be markedly smaller than that of malonate.

INTRODUCTION

IN THIS study a comparison is made between the metabolic pathway of malonate and that of monofluoromalonate (FM). The latter compound has been synthesized recently by Bergmann *et al.*[1] in the form of both its mono-potassium salt (KHFM), and its diethyl ester (DEFM).

The enzymatic decarboxylation of malonic acid was studied in *Pseudomonas fluorescens* by Hayaishi[2] and in rat tissues by Nakada *et al.*[3] Later work by Wakil[4] and by Formica and Brady[5], identified malonyl-CoA as an intermediate in fatty acid synthesis and established its formation by the enzymatic carboxylation of acetyl-CoA. According to these authors, the mechanism of malonate degradation may be summarized as follows:

$$\text{malonate} \longrightarrow \text{malonyl-CoA} \longrightarrow \text{acetyl-CoA} + CO_2$$

Assuming an analogous mechanism for the degradation of FM, the corresponding intermediate will be fluoroacetyl-CoA, i.e. the effect of FM is expected to be similar to that of fluoroacetate (FAc). The experiments carried out for testing this assumption are based on the investigations of Peters and his colleagues[6, 7] who have shown that, both *in vivo* and *in vitro*, FAc can be metabolized to fluorocitrate which causes accumulation of citrate.

Furthermore, since malonate is a well-known inhibitor of both succinic dehydrogenase and oxaloacetic decarboxylase, the effect of FM on these two systems was also investigated.

MATERIALS AND METHODS

KHFM and DEFM were supplied by Dr. E. D. Bergmann and Dr. S. Cohen.

For the *in vivo* experiments, KHFM was neutralized to pH 7·0 with KOH and diluted with saline to the appropriate concentration; DEFM was dissolved in propylene glycol to which saline was added in the ratio of 6 : 4. The injections were administered subcutaneously.

For the *in vitro* experiments, kidney (guinea pigs) and brain (pigeon) particles were prepared according to O'Brien and Peters[8]. Each flask contained: 1·0 ml enzyme preparation; 125 μmoles phosphate buffer pH 7·2; 1·2 μmoles disodium adenosine triphosphate (ATP); 4·0 μmoles $MgCl_2 \cdot 6H_2O$. Substrate for kidney: 22 μmoles fumarate. Substrate for brain: 32 μmoles sodium pyruvate plus 4·4 μmoles sodium fumarate. Various amounts of either KHFM (neutralized to pH 7·2) or FAc were added; in another set of experiments 1·2 ml of "kidney supernatant" was added instead. In all cases the volume was made up to 3·5 ml with 0·9 per cent KCl solution and incubation carried out at 38 °C. Gas phase: air.

Preparation of "kidney supernatant": Kidney particles were incubated in the usual way for 30 min with various amounts of either KHFM or FAc. After incubation the contents of the flask was heated in boiling water for 5 min, cooled, and centrifuged. The clear supernatant was used.

Succinic dehydrogenase was assayed either manometrically or colorimetrically using the tetrazolium method.[9] A 10 per cent (w/v) of rat kidney homogenate in 0·2 M phosphate buffer (pH 7·4) served as enzyme preparation. Oxaloacetic decarboxylase was assayed according to Ochoa[10]. Citrate was estimated by the method of Beutler and Yeh[11]. Glucose was determined according to the method of Nelson[12].

In all the manometric experiments the conventional Warburg techniques[13] were employed.

RESULTS

Spontaneous decarboxylation of FM

Since malonate forms acetate by spontaneous decarboxylation, it was of importance to ensure that the FM used did not contain any FAc. Hence, separate examinations were carried out with the following results:

(1) Chromatographic analysis[15] of KHFM showed that only traces of FAc were present (less than 0·1 per cent).

(2) Incubation of KHFM for 3 hr (in the usual medium without substrate and enzyme), at concentrations up to 1000 μmoles/flask did not cause any measurable liberation of CO_2.

(3) Heating of KHFM solution at 100 °C for 30 min did not change its toxicity to rats.

These three results prove that, in all the experiments performed, the amount of FAc formed by spontaneous decarboxylation is negligible.

Effects in vivo

KHFM and DEFM were injected subcutaneously to guinea pigs, mice, and rats in order to determine the LD_{50}. Guinea pigs were found to be far more sensitive than either mice or rats. Mice showed a higher sensitivity to DEFM than to KHFM[14] whereas in rats no difference between the two compounds was observed. The results

of the LD_{50} determinations are summarized in Table 1, which also includes the corresponding values for FAc.

Comparison of the first and third columns in Table 1 shows that the toxicity of KHFM—the only FM compound used in the following experiments—is 6–12 times lower than that of FAc.

TABLE 1. TOXICITY OF KHFM AND DEFM TO MICE, RATS, AND GUINEA PIGS

Animal*	LD_{50} (mg/kg)		
	KHFM	DEFM	NaFAc
Mouse	80	15	15
Rat	60	70	5
Guinea pig	2	—	0·25

* Six animals were used in each group.

The following symptoms of poisoning were observed in rats and mice injected with either DEFM or KHFM: after 15–20 min the animals became lethargic with their hair standing on end. About 2 hr later their rectal temperature decreased and their heart beats slowed down. The animals sprawled on their hind legs, stopped eating and drinking, and showed periodic convulsions.

No ketone bodies were detected in the urine of the injected animals whereas a rise in their blood glucose occurred, up to 2–2·5 times the normal value. Death was associated with a considerable accumulation of citrate in the different mouse tissues examined (kidney, heart, brain, liver and diaphragm), the increase being particularly marked in the kidney. However, when the injection of KHFM was immediately

TABLE 2. EFFECT OF KHFM AND MALONATE UPON CITRATE LEVELS IN DIFFERENT MOUSE TISSUES

Tissue	Citric acid (μg/g wet tissue)			
	Normal	KHFM	Malonate	KHFM + malonate
Kidney	16 ± 2	1150 ± 184	20 ± 3	363 ± 115
Heart	40 ± 4	302 ± 55	70 ± 5	186 ± 24
Brain	20 ± 2	207 ± 80	20 ± 2	175 ± 37
Liver	5 ± 1	65 ± 10	10 ± 2	40 ± 7
Diaphragm	70 ± 5	300 ± 40	225 ± 40	270 ± 106

Groups of five animals were used. Amount of KHFM injected: 60 mg/kg; of sodium malonate: 1·5 g/kg. Citrate tests carried out 1½ hr after injection. Figures are means ± standard deviations.

followed by an injection of malonate, the increase in the citrate level of heart and kidney was reduced, the kidney again being the most affected organ (Table 2). Similar results were obtained with rat tissues.

Effects in vitro

1. *Kidney particles.* Because of the higher sensitivity of guinea pigs to KHFM, the kidneys of these animals were chosen for the enzymic measurements.

KHFM, in the presence of fumarate, caused an accumulation of citrate and an inhibition of respiration (Fig. 1). However, the two effects differed with respect to their dependence upon the concentration of KHFM: While the inhibition of oxygen uptake increased monotonically with concentration in the whole range examined, the citrate content showed a rather flat maximum at a concentration of 4–5 μmoles/flask.

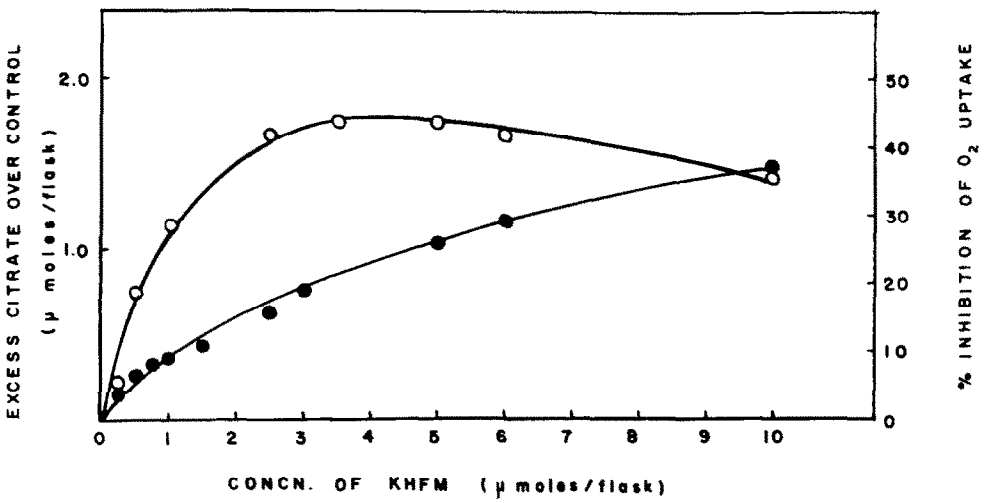

FIG. 1. Effect of KHFM on citrate accumulation (○) and respiration (●) of kidney particles.

The effect of KHFM was found to be counteracted by malonate, e.g. at a KHFM concentration of 4 μmoles/flask, addition of 2 μmoles malonate decreased the citrate content by 37 per cent and increased the oxygen uptake by 56 per cent.

It is of interest to mention in this connexion that addition of acetate instead of malonate produced variable results, e.g. 2 μmoles of acetate decreased citrate content by about 10 per cent while 5 or 10 μmoles acetate caused an increase of about the same percentage (concentration of KHFM, 4 μmoles/flask).

2. *Pigeon brain particles.* The results for the action of KHFM on pigeon brain particles with respect to respiration and citrate accumulation are summarized in Table 3(a). Values obtained with FAc have been included for comparison.

TABLE 3(a). EFFECTS OF KHFM AND FAc ON PIGEON BRAIN PARTICLES

Inhibitor	Concentration (μmoles/flask)	O_2 uptake (μmoles/30 min)	Citrate content (μmoles)
KHFM	0	3·1	0·33 ± 0·04
KHFM	1·0	3·0	0·41 ± 0·06
KHFM	3·0	2·9	0·46 ± 0·06
KHFM	5·0	2·7	0·51 ± 0·07
KHFM	10·0	—	0·50 ± 0·12
FAc	0·5	—	0·32 ± 0·04
FAc	1·0	—	0·37 ± 0·07
FAc	2·0	—	0·41 ± 0·07

Time of incubation 1 hr. Figures are means ± standard deviations.

It may be seen from the table that the inhibitory effect of KHFM on respiration was slight. The citrate content increased significantly (on the 5 per cent level) only at a concentration of 5 μmoles/flask; at lower and higher concentrations of KHFM the increase was not significant. Similarly, in agreement with the results of Peters and Wakelin[16], FAc did not cause any accumulation of citrate. On the other hand, these authors observed that fluorocitrate, when substituted for FAc, induced marked citrate accumulation in pigeon brain particles. Therefore, they attributed the toxic effects of FAc in guinea pig kidney particles to the fluorocitrate metabolically formed from FAc.

Assuming that FM incubated with kidney particles is degraded to FAc and then metabolized to fluorocitrate, the latter compound should be present in the "kidney supernatant" obtained with KHFM. The validity of this assumption was examined by incubating the "kidney supernatant" with pigeon brain particles. If any fluorocitrate is formed in the kidney supernatant, a notable accumulation of citrate[16] should occur. The results are presented in Table 3(b).

TABLE 3(b). EFFECTS OF "KIDNEY SUPERNATANT" ON PIGEON BRAIN PARTICLES

Inhibitor used for preparation of K.S.*	Conc. of inhibitor (μmoles/flask)	O_2 uptake (μmoles/30 min)	†Citrate content (μmoles)
	‡		
KHFM	0	5·1	0·54 ± 0·06
KHFM	0·5		0·58 ± 0·05
KHFM	1·0	4·9	0·92 ± 0·10
KHFM	2·0	4·5	1·01 ± 0·11
KHFM	3·0		1·06 ± 0·09
KHFM	5·0		1·35 ± 0·07
FAc	0·5	4·6	0·84
FAc	1·0	4·4	1·51
FAc	2·0	3·6	1·65

Time of incubation 1 hr.
* K.S. kidney supernatant.
† Original citrate content of added K.S. has been subtracted. Figures are means ± standard deviations.
‡ Compare first row of Table 3(a) for control without K.S.

In agreement with the assumption, the table shows a remarkable increase in citrate content caused by the action of "kidney supernatant". The increase rose monotonically with the concentration of KHFM used for the preparation of "kidney supernatant". Values obtained with FAc are included for comparison.

3. *Production of* CO_2 *from KHFM.* According to the assumed mechanism for the degradation of FM, CO_2 should be evolved concurrently. In order to determine whether CO_2 was in fact liberated and to measure its amount per hour, the following experiments were repeatedly carried out in the Warburg apparatus: kidney particles in the usual medium without substrate were incubated with KHFM at concentrations of 0–100 μmoles/flask, the gas phase being nitrogen. The results, duly corrected for the liberation of CO_2 from the control, are summarized in Table 4.

It may be seen from the table that, in the range of concentrations usually employed in this study, the amount of CO_2 liberated increased with concentration of KHFM.

Further increase in KHFM concentration, even up to 100 μmoles/flask, caused only a very slight further increase in the amount of CO_2 evolved.

These results substantiate the assumption that the effects of KHFM described in this study are associated with its degradation to FAc, CO_2 being evolved.

TABLE 4. LIBERATION OF CO_2 FROM KHFM ACTING ON KIDNEY PARTICLES

Conc. of KHFM (μmoles/flask)	CO_2 liberated* (μmoles/hr)
0	0·1 ± 0·1
2	0·3 ± 0·1
10	1·0 ± 0·1
50	1·2 ± 0·2
100	1·5 ± 0·3

Temp. of incubation 38 °C.
Period of incubation 1 hr.
The results are means ± standard deviations.
* Excess over control.

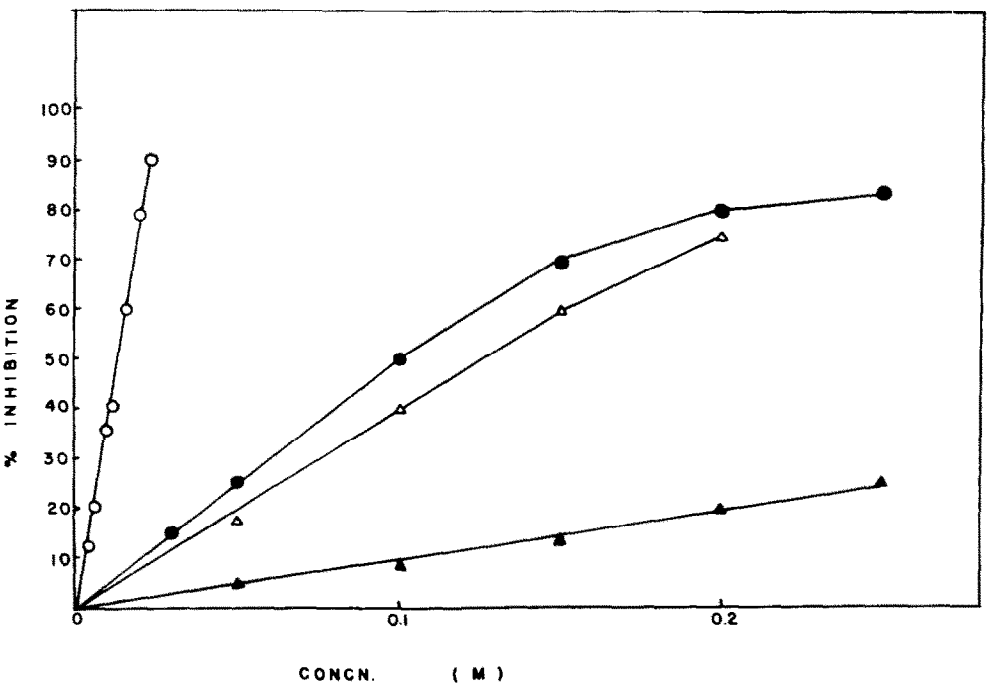

FIG. 2. Inhibitory effect of malonate (○), KHFM (●), methylmalonate (△) and dimethylmalonate (▲).

4. *Inhibitory effect of KHFM on succinic dehydrogenase and on oxaloacetic decarboxylase.* The results for the inhibitory effect on succinic dehydrogenase are summarized in Fig. 2. Parallel measurements for sodium malonate, sodium methylmalonate, and sodium dimethylmalonate are included for comparison. The values of

Fig. 2 were obtained by the colorimetric method.[9] The Warburg technique gave similar results. Fig. 2 shows that malonate was about ten times more effective than KHFM, the inhibiting action of methylmalonate and dimethylmalonate being even weaker than that of KHFM.

Experiments were carried out to compare the effect of malonate to that of KHFM on oxaloacetic decarboxylase at an identical concentration (10 μmoles/flask). Malonate proved to be almost twice as effective as KHFM (percentage inhibition by malonate, 35, by KHFM, 20).

DISCUSSION AND CONCLUSIONS

The experimental results conform with the assumption that the degradation of FM is analogous to that of malonate;[2–5] i.e. there appears to be no doubt that FAc (or fluoroacetyl-CoA) is an intermediate metabolite. However, it may be supposed that the conversion of FM to FAc is not complete and that possibly other intermediaries formed also play an important part. This supposition is based on the fact that, although the toxic action of FM (*in vivo* and *in vitro*) resembles that of FAc, the former requires higher concentrations than the latter for producing similar effects. Moreover, in the range of FM concentrations usually employed in this study (0–10 μmoles/flask), only about 10 per cent of the equivalent amount of CO_2 is liberated (Table 4).

It is to be pointed out, though, that no decisive proof could be given for the presence of fluoroacetyl-CoA: No fluoroacetyl hydroxamate could be detected by the method of Lipmann and Tuttle[17] when hydroxylamine trapping experiments[18] were carried out.

Malonate has proven to be a strong suppressor, *in vivo* and *in vitro*, of the effect of KHFM with respect to citrate accumulation in the kidney. It is not clear, to what degree the malonate influences the metabolic decarboxylation of FM to FAc. The action of "kidney supernatant", obtained by incubation of KHFM + malonate, on pigeon brain particles has not been examined. It is probable that the effect of the added malonate is due to the blocking of fluorocitrate formation[16] and, therefore, citrate accumulation in pigeon brain particles should also decrease significantly. Further investigation of these problems is indicated.

The comparison of the inhibitory influence on succinic dehydrogenase of malonate, KHFM, methylmalonate, and dimethylmalonate (Fig. 2) is of particular interest. Since the activity of KHFM lies between that of malonate and methylmalonate, one may assume that the size of the ligands of the central carbon atom is the determining factor: the larger their size, the lower the activity. It is an accepted fact that often the similarity in size between hydrogen and fluorine determines the biological activity of fluorinated compounds.

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