Mechanism of Action of Mercurial Diuretics II.* (21275)

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In a previous paper(1) it was reported that at the height of diuresis produced by a therapeutic dose of mersalyl, kidney slices showed no change in oxygen consumption. The succinic oxidase activity of kidney homogenates as studied by the method of Schneider and Potter(2) was likewise unaffected. In this paper we are reporting on the effect of mercurial diuretics on an –SH containing coenzyme, coenzyme A, utilizing kidney slices and the method of Wieland and Rosenthal(3). All these methods involving the use of homogenates and slices are open to the criticism that the mercury might be diluted by the medium, that is, it might not remain bound to the cell constituents. We have therefore sought to study the effect of mercurials on the Krebs cycle *in vivo* by using the "sequential blocking" method introduced by Potter(4). This author utilized fluoroacetate-poisoned rats and studied the change in citrate accumulation using malonate as a second block, which acted on a different link in the Krebs cycle. If the mercurials were to inhibit one of the steps of the citric acid cycle one would expect a decrease in accumulation of citrate in the kidney of fluoroacetate-poisoned animals, as was observed with malonate. In the previous paper(1) we have also stated that even if a therapeutic dose of the mercurial were to inhibit an enzyme system, it does not necessarily follow that that particular enzyme system is responsible for the reabsorption of sodium or chloride in the renal tubules. More direct evidence would be to produce diuresis by a specific inhibitor of the enzyme system in question. We have thus studied the effect of sodium malonate, an inhibitor of succinic dehydrogenase, on intact hydrated rats and compared it with that of an equiosmotic dose of an "osmotic" diuretic like potassium nitrate, and an equimolar dose of diethylmalonate. The latter compound is interesting because it inhibits the succinic oxidase system not only in kidney slices but also in the intact kidney as shown by the method of "sequential blocking."

Finally, we are reporting in detail on the method of measuring mercurial diuresis in the rat which was briefly mentioned earlier(1), stressing in particular the narrow therapeutic range of mercurial diuretics in the rat. Recent statements(5,6) that inhibition of succinic oxidase is responsible for mercurial diuresis may be questioned on the following grounds: The amounts of mercury used to demonstrate enzymatic inhibition had not been tested to determine whether they were the minimum doses which produced maximum diuresis. Furthermore, no evidence was presented that the time at which the animals were killed corresponded to the period of maximum diuresis. It is known that succinic dehydrogenase is one of several enzyme systems containing sensitive —SH groups and that it can be inhibited by mercury compounds. If, therefore, sufficient mercury were added to such a system *in vitro,* or injected *in vivo* one would naturally expect an inhibition of the succinic oxidase activity.

Methods. Local male and female albino rats were used(7). For the mercurial assays 7-month-old male rats were used which were obtained from Tierzüchterei Brünger, Halle i.W., Germany. All rats intended for diuretic studies were kept on a constant diet for at least one week prior to the day of the experiment. Food but not water was withheld after 5 P.M. on the previous day. Three rats were put in one cage, and if possible, the 3 were selected so as to have the total weight in each cage the same. The assay was performed on several consecutive days, each day including the total dosage range of the diuretic. Salyrganic acid, 125 mg, was dissolved in 2.5 cc N/10 NaOH as a stock solution. The mercurial was injected *intravenously* into the tail vein

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in a volume of 0.2 cc per 100 g body wt, using a 27 gauge needle. The intravenous injection was followed immediately by the intraperitoneal administration of isotonic solution of sodium chloride, 5 cc/100 g body wt. The urine output was measured at hourly intervals for the next 12 hours and again at the end of 24 hours. Unless otherwise stated, the above procedure was also utilized in the metabolic experiments involving diuresis, in particular with regard to the method of hydration. Fluoroacetate, 3 mg/kg, was administered intraperitoneally 30 minutes after the mercurial in one set of experiments and after 3 hours in another set. In both cases the animals were killed 2 hours after treatment with fluoroacetate. The organs were analysed for citrate by Natelson's(8) modification of the pentabromacetone method as previously described(7). Dry weights of tissues were measured in experiments involving diuresis. In the set of experiments in which fluoroacetate was injected 3 hours after the mercurial we used the acetic anhydride-pyridine method of Saffran and Denstedt(9). Here, 2 blanks were run, one on a pure 5% trichloroacetic acid solution and another on a trichloroacetic acid tissue extract as intended for the determination of aconitic acid. This method compares favorably with the pentabromacetone method, but as the authors themselves state(9), it cannot be used in the presence of large amounts of organic acids. For experiments involving the use of added or injected metabolites we have used the pentabromacetone method. Although the amounts of citrate are expressed in terms of mg per gram wet tissue, an appropriate correction was made for the change in percentage dry weight of kidneys of animals treated with mersalyl. *Coenzyme A* activity was measured by using the procedure of Wieland and Rosenthal(3), who added oxalacetate and acetoacetate to kidney brei and isolated citrate. We used slices instead of brei, and $MgCl_2$ instead of $BaCl_2$. Slices of 100 mg were suspended in 2 cc solution and the mixture incubated with shaking at 38°C for an hour. It is important to use a small quantity of slices of uniform thickness since the oxalacetate cannot be added in unlimited amounts. Trichloracetic acid was then added to make a final concentration of 10% and the filtrate used for citrate determination. The medium was always prepared without oxalacetate, the latter being added just before the experiment. This is important as some oxalacetate changes spontaneously into citrate in a solution containing $MgCl_2$(10). In addition, blank experiments containing all constituents except tissue slices, were run simultaneously and the nonenzymatic citrate production subtracted from the corresponding values obtained with slices. Each rat served as its own control. The left kidney, removed under ether anesthesia, was analysed after saline injection, and the right kidney, removed a week later, was analyzed 2½ hours after the injection of mersalyl; control experiments having shown that there was no difference in the activity of either kidney removed at such different intervals. *Malonate* experiments were performed on intact rats, and kidney and heart slices of the rat. When intact animals were used, sodium malonate, diethylmalonate, and malonic acid diamide were injected subcutaneously in a dose of 1.15 millimoles per 100 g body wt. Thirty minutes later, fluoroacetate (3 mg/kg) was injected intraperitoneally and the animal killed after 2 hours for the measurement of citrate in tissue. When the diuretic action of the malonate derivatives was studied, 5 cc isotonic solution of sodium chloride per 100 g body wt were injected simultaneously with the malonate. The effect of malonate on tissue slices was studied by the conventional Warburg method. The

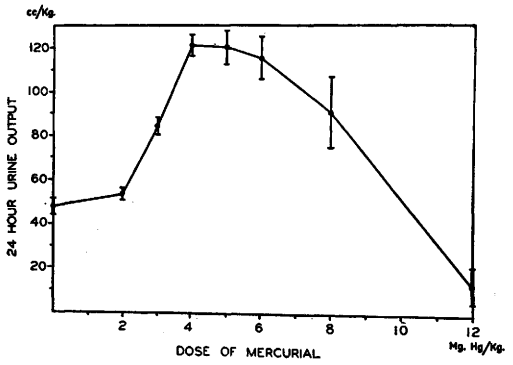

FIG. 1. Effect of increasing doses of mersalyl on 24-hr urine output of hydrated rats. Bars indicate standard error.

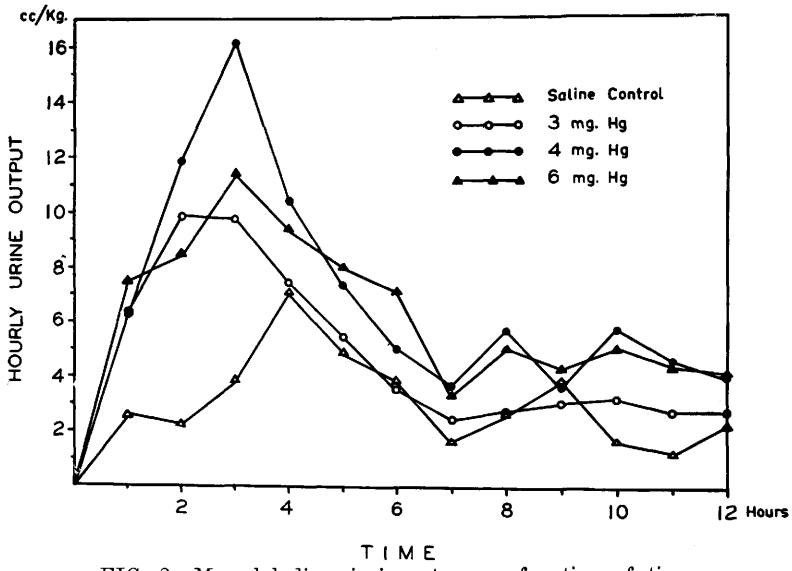

FIG. 2. Mersalyl diuresis in rats as a function of time.

medium used was Krebs-Ringer phosphate according to Webb *et al.*(11) with or without 0.02 M succinate. The inhibitor was added from the side-arm after the respiration had attained a steady rate (20 min.) in an atmosphere of oxygen. Respiration was measured for another 30 minutes.

Results. Effect of varying doses of Mersalyl on Diuresis and Tubular Histology. Fig. 1 illustrates the dose-response relationship for mersalyl. Each point on the curve is an average of 5 experiments and included 15 rats. The bars indicate the standard error. It will be seen, in agreement with many other unrecorded experiments, that with 4 mg of mercury per kilo, maximum diuresis is reached. It is unnecessary to use higher dosage. Amounts of 8 mg Hg/kg produce less diuresis, and 12 mg produces almost complete anuria. All rats receiving 12 mg of mercury and 50% of those receiving 8 mg mercury/kg died within a week after the injection. Kidneys removed 24 hours after the injection showed damaged tubules with all the doses of mercury used, the extent of the injury being proportional to the dose. With the highest dose used —12 mg Hg/kg—not only the proximal but also part of the distal convoluted tubules were involved. In the animals that survived, complete tubular regeneration took place within two weeks. Since in our experiments pure mersalyl was injected intravenously, it is quite possible that tubular damage may be more extensive than if the mercurial were injected subcutaneously and with theophylline. Furthermore, different strains of rats may react differently. Fig. 2 represents diuresis as a function of time. It is seen that maximum

TABLE I. Effect of Mersalyl (4 mg Hg/kg) on Accumulation of Citrate in Heart and Kidney of Fluoroacetate-Poisoned Rats.

No. of animals	Sex	Age, mo	Time between mersalyl and fluoroacetate inj. (hr)	Method	Citrate in heart (μg/g wet tissue), mean $\pm$ S.E.	Citrate in kidney (μg/g wet tissue), mean $\pm$ S.E.
9	♀	18	3	Acetic anhydride	1453 $\pm$ 68	865 $\pm$ 40
8	♀	18	S*	"	1402 $\pm$ 54	894 $\pm$ 70
9	♂	3.5	3	"	1227 $\pm$ 42	1024 $\pm$ 80
9	♂	3.5	S	"	1244 $\pm$ 47	977 $\pm$ 80
10	♂	4	½	Pentabromacetone	1310 $\pm$ 44	1250 $\pm$ 95
10	♂	4	S	"	1393 $\pm$ 29	1396 $\pm$ 78

* S = Saline controls.

TABLE II. Effect of Sodium Malonate, Ethyl Malonate and Potassium Nitrate on Diuresis in Rats. (All rats hydrated by injecting 50 cc saline/kg intraperitoneally.)

Substance	No. of rats	Mean urine flow in cc/kg body wt/hr					24 hr urine flow in cc/kg
		1st hr	2nd hr	3rd hr	4th hr	5th hr	
Saline controls	9	2.2	2.2	4.7	4.6	6.1	83
Sodium malonate, 1.15 mM/100 g	33	23	15.5	9.1	5.6	4.7	89
KNO_3, 1.72 mM/100 g	12	17.6	13.3	12.5	8.1	7.4	84
Ethyl malonate, 1.15 mM/100 g	12	2.7	5.9	3.2	4.7	4.1	69

diuresis is attained 2 to 3 hours after the administration of mersalyl.

Effect of mersalyl on citrate accumulation in fluoroacetate poisoned animals. Table I summarizes the results obtained by 2 different citrate methods and by the use of several batches of rats at different times. The amount of citrate in tissues varies, among other things, with the nutritional state and age of the animals, hence controls should be taken from the same batch. Since these tests were performed on starved animals, an increase in the amount of citrate in the heart is noted in agreement with the finding of Potter(4) and also a decrease in the amount in the kidney, as compared with values previously reported from this laboratory(7). The table shows that there was no significant decrease in the accumulated kidney citrate as would have happened had the mercurial, like malonate, blocked one of the steps of the citric acid cycle. The coenzyme activity of kidney slices as measured by Wieland's reaction: oxalacetate + acetoacetate → citrate, also does not appear to be diminished in rats at the height of mercurial diuresis. In 8 control rats treated with saline, 5 cc per 100 g body wt, the left kidney synthesized 8.4±0.38 mg citrate per gram wet tissue per hour, and the right kidney, removed a week later, synthesized 8.9±0.41 mg. In the next group of 8 rats, the left kidney synthesized 8.8±0.37 mg citrate per g per hour after intraperitoneal saline injection whereas the right kidney, removed a week later, synthesized 9.4±0.56 mg after the intraperitoneal injection of salt solution and intravenous mersalyl.

Malonate experiments. Sodium malonate is strongly diuretic when injected subcutaneously into hydrated rats in a dose that substantially inhibits the kidney succinic dehydrogenase, that is, 1.15 mM per 100 g body wt(4). This effect appears to be a purely osmotic one, since the injection of an equiosmotic dose of KNO_3, 1.72 mM, also produces strong diuresis. However, this comparison is at best semi-quantitative as diuresis depends on the amount of osmotic diuretic excreted rather than the amount injected. Convincing proof that the diuretic action of sodium malonate is osmotic and not enzymatic is furnished by the fact that an equimolar dose of diethylmalonate which inhibits the kidney succinic dehydrogenase to the same extent as sodium malonate (Table III) had no diuretic action. This is shown in Table II.

Table III illustrates the action of malonate in inhibiting the succinic oxidase activity of the kidney *in vivo*, confirming the findings of Potter *et al.*(4). Here it is seen that half the regular dose of sodium malonate is less effective than the full dose. Ethylmalonate has the same inhibitory action on the kidney as an equimolar dose of sodium malonate.

Ethylmalonate, but not malonic acid diamide, also inhibits succinate oxidation by renal tissue but has less effect on slices of heart.

TABLE III. Effect of Malonate Derivatives on Accumulation of Citrate in Heart and Kidney of Fluoroacetate-Poisoned Rats. 4 rats in each series.

Substance	Citrate in heart (μg/g wet tissue), mean ± S.E.	Citrate in kidney (μg/g wet tissue), mean ± S.E.
Na malonate 1.15 mM/100 g	1170 ± 71	443 ± 40
Na malonate 0.57 mM/100 g	998 ± 9	863 ± 32
Diethyl malonate 1.15 mM/100 g	897 ± 28	468 ± 63
Malonic diamide 1.15 mM/100 g	1097 ± 46	1970 ± 97

TABLE IV. Effect of Sodium Malonate and Ethyl Malonate on Oxygen Consumption of Rat Heart and Kidney Slices with and without Succinate (0.02 M).

Tissue	Substrate	No inhibitor	Sodium malonate, .02 M	Ethyl malonate, .02 M
Heart	Succinate	$Q_{O_2}^{O_2} = 36$	$Q_{O_2}^{O_2} = 6.1$	$Q_{O_2}^{O_2} = 15.5$
Kidney	—	14.3	4.5	2.5
"	Succinate	58.5	15.8	6.2

This is shown in a typical experiment illustrated in Table IV. In both tissues the malonic ester appears to undergo some hydrolysis, for the pH at the end of the experiment was lower than at the beginning: 6.3 in the heart and 5.6 in the kidney, in the presence of succinate. Webb *et al.*(11) have shown that a pH of 6.3 does not affect oxidation in heart slices. However, the greater inhibition of succinate oxidation in kidney slices by ethylmalonate as compared to sodium malonate may well be due, in part at least, to the difference in pH.

Discussion. If Potter's concept of "sequential blocking"(4) be valid, and if the observed decrease of accumulated citrate in fluoroacetate-poisoned rats after malonate be due to the (*in vivo*) inhibitory action of malonate on the renal succinic oxidase system, then the diuresis produced in rats after therapeutic doses of mersalyl cannot be due to an inhibition of one of the Krebs cycle enzymes. Otherwise, mersalyl would have acted like malonate by decreasing the accumulation of citrate in fluoroacetate-poisoned rats.

Ethylmalonate is as effective as sodium malonate in decreasing the amount of accumulated citrate in fluoroacetate poisoned rats. However, ethylmalonate, unlike sodium malonate, does not produce diuresis in rats. This is evidence that sodium malonate is diuretic by virtue of its osmotic effect rather than its inhibition of activity of succinic oxidase.

The results reported in this and the previous paper(1) do not exclude the possibility that mercurial diuresis is due to interaction of mercury with –SH groups or –SH enzymes in the kidney. We feel, however, that such –SH groups ought to be sought elsewhere than among the Krebs cycle enzymes or coenzymes.

Summary. 1) A method for measuring mercurial diuresis in the rat is described. The effect of varying intravenous doses of mersalyl on hydrated rats has been studied. Maximum diuresis is obtained with a dose of 4 mg Hg/kg and the height of diuresis is reached in 2 to 3 hours. The extent of tubular damage is proportional to the dose of the mercurial. No rats survived a dose of 12 mg Hg/kg and only 50% survived a dose of 8 mg. In the animals that survived, complete tubular regeneration took place within two weeks. 2) The results reported in this paper do not support the view that mersalyl, in therapeutic doses, has any effect on the oxidative enzymes of the Krebs cycle. Mercurial diuresis may be due to interaction of mercury with –SH groups or –SH enzymes in the kidneys. It is felt, however, that such –SH groups be sought elsewhere than among the Krebs cycle enzymes and coenzymes.

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1. Fawaz, G., and Fawaz, E. N., PROC. SOC. EXP. BIOL. AND MED., 1951, v77, 239.

2. Schneider, W. C., and Potter, V. R., *J. Biol. Chem.*, 1943, v149, 217.

3. Wieland, H., and Rosenthal, Ch., *Liebig's Ann.*, 1943, v554, 241.

4. Potter, V. R., PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 41.

5. Handley, C. A., and Lavik, P. S., *J. Pharmacol.*, 1950, v100, 115.

6. Rennels, E. G., and Ruskin, A., PROC. SOC. EXP. BIOL. AND MED., 1954, v85, 309.

7. Fawaz, G., and Fawaz, E. N., *ibid.*, 1953, v84, 680.

8. Natelson, S., Pincus, J. B., and Lugovoy, J. K., *J. Biol. Chem.*, 1948, v175, 745.

9. Saffran, M., and Denstedt, O. F., *ibid.*, 1948, v175, 849.

10. Breusch, F. L., *Hoppe Seyler's Z. f. Physiol. Chem.*, 1937, v250, 262.

11. Webb, J. L., Saunders, P. R., and Thienes, C. H., *Arch. Biochem.*, 1949, v22, 444.

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