Influence of Nitrogen Mustards on Citric Acid Synthesis *in Vivo*.* (19830)

KENNETH P. DUBOIS, JERE DEROIN, AND KENNETH W. COCHRAN.

From the U. S. Air Force Radiation Laboratory and the Department of Pharmacology, University of Chicago.

During previous investigations on the influence of X-irradiation on intermediary carbohydrate metabolism we found(1) that citric acid synthesis is markedly depressed in hematopoietic tissues of irradiated rats. This effect was demonstrable through the use of the technic of sequential blocking of metabolic pathways developed by Potter(2) in which fluoroacetate is employed to inhibit the oxidation of citric acid *in vivo*(3-7). This procedure results in accumulation of citric acid in most tissues of normal rats in concentrations sufficient to permit quantitative measurements. The inability of certain tissues of irradiated rats to accumulate citrate following fluoroacetate treatment indicated that X-irradiation interferes with some reaction which normally leads to citrate formation. In contrast to an inhibition or no effect by X-ray on citrate formation in most rat tissues we observed that the livers of irradiated male rats accumulated large quantities of citric acid in the liver whereas normal male rats were unable to accumulate citric acid in the liver following fluoroacetate treatment(7-9). Recent studies in this laboratory(8) revealed that normal female rats and castrated or adrenalectomized male rats are able to accumulate citric acid in the liver following fluoroacetate treatment and thus resemble irradiated male rats. In addition, treatment of female rats with testosterone decreased citrate synthesis in the liver. These findings suggested that X-irradiation alters the metabolic pattern of the liver through interference with hormonal regulation of citrate synthesis.

The known similarities in the actions of nitrogen mustards and X-ray on mammalian tissues stimulated our interest in ascertaining whether the nitrogen mustards interfere with citrate synthesis in the same manner as X-ray. For this study various doses of the hydrochlorides of ethyl *bis*(2-chlorethyl)amine (HN1) and methyl *bis*(2-chlorethyl)amine (HN2) were administered to rats and the ability of various tissues to accumulate citric acid was measured following treatment of the animals with sodium fluoroacetate. These experiments demonstrated that the nitrogen mustards resemble X-irradiation in their effects on citrate formation in rat tissues *in vivo*.

Materials and Methods. Male Sprague-Dawley rats (175-225 g) were used for these experiments. Aqueous solutions of the hydrochlorides of HN1 and HN2 were administered intraperitoneally. In all the experiments rats were given 3.5 mg/kg of purified sodium fluoroacetate and sacrificed 3 hours later(2). Animals were sacrificed by decapitation and citric acid measurements were performed using the method of Natelson *et al.*(10) with the modifications recommended by Potter and Busch(7). The influence of HN2 on citrate formation and oxygen consumption *in vitro* was measured using the test system of Pardee and Potter(11) with various intermediates of the tricarboxylic acid cycle as substrates. Choline oxidase measurements were performed by the method of Williams *et al.*(12).

Results. Prior to studies on the influence of nitrogen mustards on citrate synthesis we measured the toxicity of HN1 and HN2 to rats under the conditions employed for this study. The approximate LD_{50} of the hydrochloride of HN1 was 0.75 mg/kg and the value for HN2 hydrochloride was 1.5 mg/kg when the compounds were given intraperitoneally to rats.

In previous studies we found(1) that X-irradiation exerts its most prominent inhibitory effect on citrate synthesis in spleen and thymus gland. Measurements of the influence of nitrogen mustards on citrate synthesis in spleen and thymus were, therefore, performed

* This study was supported by funds provided under contract with the USAF School of Aviation Medicine, Randolph Field, Texas.

TABLE I. Effect of Ethyl *bis*(chlorethyl)amine (HN1) Hydrochloride on Citrate Accumulation in Spleen and Thymus of Fluoroacetate-Treated Rats.

mg/kg HN1 hydrochloride	μg citric acid/g fresh tissue	
	Spleen	Thymus
Control	1425 (1245-1495)	1045 (965-1143)
1.5	892 (760-1150)	810 (745- 895)
2.0	895 (840-1160)	734 (610- 845)
2.5	744 (650- 830)	710 (545- 976)
5.0	420 (320- 688)	244 (130- 460)

in which both the dosage and time of sacrifice after HN1 and HN2 were varied. In all cases the animals were given 3.5 mg/kg of fluoroacetate 3 hours before they were sacrificed. The data in Table I show the effect of various doses of HN1 on the ability of spleen and thymus to accumulate citric acid following fluoroacetate treatment. The average values for 5 rats are presented together with the range of values for each group. The results of these measurements demonstrated that HN1 resembles X-irradiation in its inhibitory effect on citrate synthesis in spleen and thymus. However, in terms of the LD_{50} it was necessary to give higher doses of the nitrogen mustards than of X-ray to inhibit citrate synthesis in these tissues. Thus, 5 mg/kg of HN1 were required to produce an inhibitory effect comparable to that resulting from 800 r of X-ray. Whereas sublethal doses of X-ray (200 r) produced over 50% inhibition of citrate synthesis in hematopoietic tissues of rats only 38% and 22% inhibition of citrate synthesis occurred in spleen and thymus respectively in 48 hours after 1.5 mg/kg of HN1 which is twice the LD_{50} dose. Detailed studies on the influence of HN2 on citrate synthesis in spleen and thymus gland were also conducted in which several doses varying from 0.1 to 5 mg/kg of HN2 hydrochloride were given and groups of animals were sacrificed each day for 7 days following administration of the nitrogen mustard. After doses of 0.1 to 0.75 mg/kg of HN2 hydrochloride the amount of inhibition did not exceed 30% during the 7-day observation period. At 24 hours after 2 mg/kg of HN2 citrate accumulation was inhibited to the extent of 43% in spleen and 45% in thymus.

In the present investigation experiments were conducted to ascertain whether the nitrogen mustards resemble X-ray(1) in their effects on citrate accumulation in the livers of fluoroacetate-poisoned, male rats. The results of these experiments showed that the nitrogen mustards cause male rats to accumulate large quantities of citric acid in the liver following fluoroacetate treatment. In 24 hours after 1 mg/kg of HN1 hydrochloride the citrate content of the liver increased from an average for 6 rats of 64 μg/g to 354 μg/g of liver. With higher doses the effect was more pronounced as evidenced by an average of 729 μg of citric acid/g of liver in 24 hours after 2.5 mg/kg of the nitrogen mustard. More detailed studies were conducted with HN2 in which several sublethal doses were administered to male rats. Groups each containing 6 animals were treated with fluoroacetate and sacrificed at daily intervals for 7 days after each dose of HN2 and citric acid measurements were performed on the livers. An increase in citric acid accumulation in the liver was noted 24 hours after the administration of the doses employed in these experiments. The average value was generally highest about 4 days after administration of the nitrogen mustard. The data in Table II show the average value to-

TABLE II. Effect of Methyl *bis*(2-chlorethyl)amine Hydrochloride (HN2) on Citrate Accumulation in Livers of Fluoroacetate-Treated Rats.

mg/kg HN2 hydrochloride	μg citric acid/g fresh liver (4 days after HN2)
Control	64 (44- 71)
.1	269 (137- 430)
.25	338 (312- 364)
.5	449 (204- 812)
.75	642 (284-1025)
1	867 (331-1524)

TABLE III. Duration of Action of Methyl *bis*(2-chlorethyl)amine (HN2) Hydrochloride on Citrate Accumulation in Livers of Fluoroacetate-Treated Rats.

Days after 0.5 mg/kg HN2 hydrochloride	μg citric acid/g fresh liver
Control	64 (44- 71)
1	136 (101-182)
3	328 (255-453)
7	468 (198-872)
10	342 (230-434)
14	215 (123-418)
28	125 (61-226)

gether with the range for each group of animals at 4 days after poisoning. This experiment demonstrated that doses of HN2 far below the LD_{50} increase citric acid accumulation in the livers of fluoroacetate-treated. male rats.

The duration of the increase in citrate accumulation in the liver was measured by administering a single sublethal dose (0.5 mg/kg) of HN2 to rats and sacrificing groups containing 6 animals at intervals for a period of 4 weeks. The data in Table III show the persistence of the effect of HN2 on citrate accumulation in the livers of fluoroacetate-treated male rats. The average values together with the range of values for each group are presented in the table. These measurements demonstrated that the increase in citrate accumulation is evident at one day after administration of the nitrogen mustard and persists for several weeks. At 4 weeks after HN2 the values approached the normal range indicating that the change is eventually reversible following sublethal doses of the nitrogen mustards.

The experiments described above indicate that nitrogen mustards markedly alter carbohydrate metabolism in the liver. The nitrogen mustards are known to react with many biological compounds *in vitro*(13) but studies on the reactivity of these compounds with catalytic proteins have not yet provided an explanation for the toxic actions of low doses of the nitrogen mustards *in vivo*. In the present investigation the influence of HN2 on several oxidative reactions was measured using the test system of Pardee and Potter(11) with liver homogenates. Oxygen consumption and citrate formation was 50% inhibited by a final concentration of 2×10^{-3} M HN2 when pyruvate plus fumarate were used as substrates and the oxidation of acetate plus oxalacetate was 50% inhibited by 4×10^{-3} M HN2. The formation of acetoacetate from pyruvate in the presence of malonate(14) was 50% inhibited by 8×10^{-4} M HN2. The effect of HN2 on the oxidation of choline was also measured because choline oxidase has been reported(15) to be sensitive to the nitrogen mustards. The oxidation of choline by liver homogenates was inhibited 50% by 1×10^{-4} M HN2 when the test system of Williams *et al.*(12) was employed. These experiments demonstrated that relatively high doses of HN2 are required to affect these oxidative reactions *in vitro*.

To obtain a more direct evaluation of the action of HN2 on citrate synthesis and its effect on various oxidative reactions *in vivo*, experiments were performed on livers taken from rats which had been poisoned 2 days previously with 1 mg/kg of HN2. The ability of the livers of these animals to oxidize fumarate plus pyruvate and acetate plus oxalacetate was measured to obtain an indication of the effect of HN2 on enzymes involved in citrate synthesis. The formation of acetoacetate from pyruvate in the presence of malonate was measured to ascertain the influence of HN2 on pyruvate oxidation. Choline oxidase measurements were also performed on the livers of animals which had been treated with HN2. The results of these experiments are presented in Table IV in which the average values and the range of values for 4 animals are presented. These data show that 1 mg/kg of HN2 does not alter the ability of the liver to oxidize pyruvate plus fumarate or acetate plus oxalacetate. The oxidation of choline was likewise unaffected by this dose of the nitrogen mustard. The formation of acetoacetate from pyruvate was slightly increased. In contrast to the absence of effects by HN2 on enzymes involved in citrate formation by liver homogenates from HN2-poisoned animals the same dose of the nitrogen mustard caused a marked increase in the citrate content of the livers of fluoroacetate-treated rats. Thus, in two days after 1 mg/kg of HN2 the administration of fluoroacetate caused an increase in the citrate content of the liver of male rats to an average value of 889 μg/g as compared with 64 μg/g for normal male rats. The results of these experiments indicate that the increase in citrate formation in the livers of male rats after administration of HN2 occurs at dosages which have no direct effect on the enzymes involved in citrate synthesis.

Discussion. The results of these experiments have shown that nitrogen mustards produce alterations in citric acid accumula-

TABLE IV. Effect of Methyl *bis*(2-chlorethyl)amine Hydrochloride (HN2) *in vivo* on Ability of Liver to Oxidize Various Substrates. (Sacrificed 2 days after 1 mg/kg HN2).

Substrates	μM/50 mg liver/40 min.			
	Oxygen consumption		Citrate or acetoacetate formed	
	Control	HN2	Control	HN2
Pyruvate + fumarate	10 (9.3-10.8)	11.6 (7.7-14.5)	5.6 (4.8-6.4)	4.4 (3.9-5.4)
Acetate + oxalacetate	6 (5.5- 6.5)	7.7 (6 - 8.7)	2.2 (2.2-2.3)	2 (1.7-2.2)
Pyruvate + malonate	2.3 (1.9- 2.7)	3.3 (2.7- 4.2)	2.9 (2.3-3.5)	3.2 (3.1-3.4)
Choline	4.75 (4.2-5.3)	4.45 (4-5.1)		

tion in some tissues after treatment of rats with fluoroacetate according to the procedure developed by Potter(2). The nitrogen mustards thus resemble X-irradiation(1) qualitatively in their ability to alter citrate synthesis. In terms of LD_{50} values X-irradiation is considerably more effective than HN1 or HN2 in depressing citrate accumulation in spleen and thymus gland. Whereas doses below the LD_{50} of X-ray markedly inhibit citric acid formation in hematopoietic tissues it was necessary to administer lethal doses of the nitrogen mustards to obtain comparable effects. In contrast to the lesser effect of the nitrogen mustards than of X-ray on citrate synthesis in hematopoietic tissues the alteration in the metabolic pattern of the liver was more pronounced and consistent with the nitrogen mustards than with X-ray. However, in both cases male rats acquired the ability to accumulate large quantities of citrate following fluoroacetate treatment. That the increase in citrate accumulation following HN2 does not result from a direct action of the toxicant on reactions involved in the tricarboxylic acid cycle was suggested by the absence of significant changes in the ability of the livers from rats poisoned with HN2 to oxidize several intermediates of the citric acid cycle. The relatively high concentrations of the agents which were necessary to alter citrate formation and oxygen consumption *in vitro* also indicate that some indirect influence by the nitrogen mustards must be responsible for the metabolic changes observed by the use of the fluoroacetate technic(2). Our recent observations(8) that normal female rats and castrated or adrenalectomized male rats have the ability to accumulate citrate in the liver following fluoroacetate treatment suggest that a hormonal regulation of citrate synthesis is normally operative in the livers of male rats. The nitrogen mustards and X-ray may exert their influence on the liver through interference with the regulatory influence of hormones on citrate synthesis. The results of the present study have demonstrated a similarity in the action of X-ray and the nitrogen mustards on citrate synthesis in rat tissues. Elucidation of the exact mechanisms responsible for these biochemical changes requires further experimentation.

Summary. The influence of nitrogen mustards on citrate synthesis in rat tissues *in vivo* was studied using the fluoroacetate technic of Potter(2). The results of this study demonstrated that HN1 and HN2 produce alterations in citrate synthesis in certain tissues which are qualitatively similar to those produced by X-ray. Thus, a decrease in citrate synthesis was observed in spleen and thymus glands of nitrogen mustard-poisoned animals and a marked increase in citrate accumulation was observed in the livers of male rats. The increase in citrate accumulation in the liver was evident after doses far below the LD_{50} and persisted for several weeks following sublethal doses of the nitrogen mustards.

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Received August 25, 1952. P.S.E.B.M., 1952, v81.

Antidotal Efficacy of Vitamin B_{12a} (Hydroxo-Cobalamin) in Experimental Cyanide Poisoning. (19831)

CHARLES W. MUSHETT, KANE L. KELLEY, GEORGE E. BOXER, AND JAMES C. RICKARDS.
(Introduced by Hans Molitor.)

From the Merck Institute for Therapeutic Research and the Research Laboratories, Merck and Co., Inc., Rahway, N. J.

It has been shown that vit. B_{12} (cyanocobalamin) contains one cyano group bound coordinatively to the cobalt atom, whereas in vit. B_{12a} (hydroxo-cobalamin) a hydroxo group is present instead of the cyano group(1). The addition of cyanide ions to a solution of vit. B_{12a} results in the formation of vit. B_{12} (2). That the cyano group is tightly bound within the coordination complex of vit. B_{12} is indicated by the observation that dose levels of vit. B_{12} up to 1600 mg/kg both intraperitoneally and intravenously are nontoxic to mice(3). Calculation reveals that a dose of 1600 mg/kg of vit. B_{12} contains the equivalent of about 32 mg/kg of cyanide ion, or about 8 times the LD_{100} dose.

The ease with which vit. B_{12a} reacts with cyanide to yield vit B_{12} and the apparent irreversibility of this reaction in animals suggested that vit. B_{12a} might influence beneficially the course of cyanide poisoning in a manner analogous to that by which B.A.L. counteracts arsenic poisoning(4). The present communication demonstrates that vit. B_{12a} is capable both of preventing and of reversing the toxic effects of cyanide in mice.

Material and Methods. Male mice of the Harpaul and Barckmann stocks, weighing 20-28 g, were employed in these experiments. Freshly prepared aqueous solutions of potassium cyanide were injected intraperitoneally, preceded or followed by the intravenous administration of vit. B_{12a} or physiologic saline. The volume of intravenously injected solutions was kept constant at 0.2 ml/20 g body weight. Exp. 1, 2, and 3 (Table I) were controlled by using a saline-treated group of mice at the beginning and again at the end of the test. In all other experiments alternate (pair-weighed) mice were injected with vit. B_{12a} or saline. The vit. B_{12a} was prepared by irradiation and aeration from crystalline vit. B_{12}(5) and was recrystallized from acetone.

Results. Data in Table I show that the prophylactic injection of vit. B_{12a} intravenously, approximately 20 seconds before the intraperitoneal administration of potassium cyanide, was effective in reducing the mortality due to the latter substance. Respiratory distress and convulsions were prevented completely or were minimized. Doses of 50 or 250 mg/kg of vit. B_{12a} gave adequate protection against doses of 5.5-8.0 mg/kg of potassium cyanide, whereas doses of 25 mg/kg of vit. B_{12a} or less did not. No prophylactic effect against potassium cyanide toxicity (7-8 mg/kg, intraperitoneally) could be shown for vit. B_{12} (cyano-cobalamin) given intravenously in doses of 50-250 mg/kg. The encouraging results obtained by pretreatment with vit. B_{12a} prompted a study designed to determine whether this compound would effect recovery after the toxic manifestations of potassium cyanide were evident.

Table II shows that an intravenous dose of 250 mg/kg of vit. B_{12a} administered within