THE EFFECT OF NITROGEN MUSTARDS ON ENZYMES AND TISSUE METABOLISM*

II. The Effect on Tissue Metabolism

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It was shown in the preceding paper (1) that nitrogen mustards act as structural inhibitors for the enzymes where choline acts as a substrate, such as choline oxidase, acetylcholine esterase, and choline acetylase. Besides this type of inhibition, nitrogen mustards produced other enzyme inhibitions. *In vitro* studies with enzymes are an indication that such enzymatic reactions might be inhibited in experiments with tissues or in *in vivo* experiments. However, there is no certainty that they would actually be found, not only because the necessary concentrations might not be present, but also because the compound might have been transformed into others of different constitution or might have combined with other substances present in the extracellular milieu. We present in this paper studies on the effect of nitrogen mustards on tissue metabolism. Experiments with tissues permit the study of biochemical reactions which have not yet been accomplished in cell-free systems. These studies have shown that pyruvate metabolism in tissues is powerfully inhibited and that enzyme inhibitions in tissues increase in degree as the time of exposure of nitrogen mustards to tissue increases. Such experiments with tissue slices have finally been followed by a study in tissues of animals receiving nitrogen mustard of the same enzyme reactions that were inhibited in *in vitro* experiments.

Nitrogen Mustards and Tissue Respiration

A study of the effects of nitrogen mustards on the chemical activities of living cells is greatly complicated because of the rapid chemical transformations that these substances undergo when in solution, and by the rate of penetration through the cell membranes.

Toxicological tests have shown that the ethylenimonium derivatives are the most toxic of the transformation products of nitrogen mustards. Their concentration diminishes steadily so that at the end of 24 hours none is left in the case

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of TBA, about 9 per cent in that of MBA, and 47 per cent in that of ethyl-bis-(β-chloroethyl)amine HCl (EBA), as can be seen in Fig. 1 plotted from the data on hydrolysis at 25° given by Golumbic *et al.* (2, 3) and by Fruton and Bergmann (4). In all the experiments with tissue slices, the hydrochloride salts of nitrogen mustards dissolved in Ringer-phosphate buffer (phosphate, 0.02 M) were brought to pH 7.4 and were added to the tissue suspension 15 minutes after solution.

1. Tissue Respiration.— In the experiments with tissue slices and with leucocytes, tissues and cells remained in contact with the nitrogen mustards for

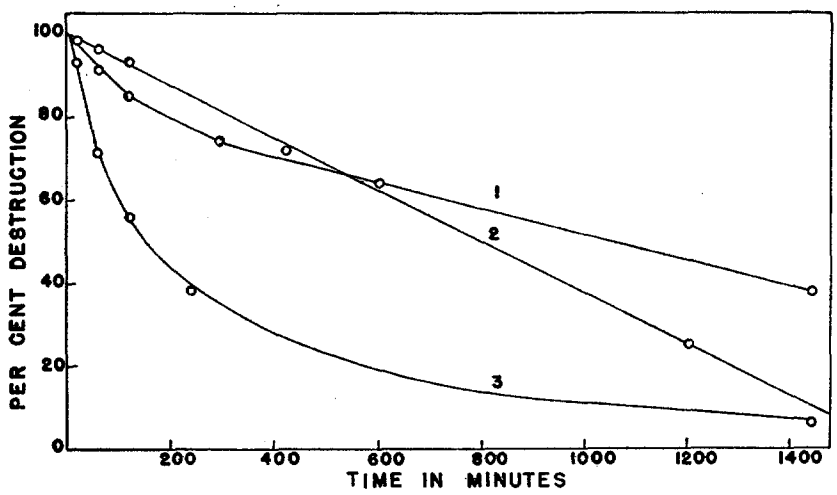

FIG. 1. The destruction of the ethylenimonium transformation product of nitrogen mustards in bicarbonate solution as determined by $Na_2S_2O_3$ consumed in 10 minutes per mM of nitrogen mustard. Temperature 25°. (1) Ethyl-bis(β-chloroethyl)amine; (2) methyl-bis-(β-chloroethyl)amine; (3) tris(β-chloroethyl)amine. (From data given by Golumbic *et al.* (2, 3).)

about 25 minutes before readings of the O_2 uptake were started. There was inhibition of respiration of all tissues studied, and in all cases the inhibition increased gradually so that inhibition in the 2nd hour was always greater than in the first. In some cases, as in that of leucocytes, there was no effect at all in the 1st hour, the inhibition becoming manifest in the 2nd hour (Table I).

The effect of four nitrogen mustards on the respiration of spleen (rat) is given in Table II. All four produced the same progressive inhibition. At the end of 2 hours, ethyl-bis(β-chloroethyl)amine, and isopropyl-bis(β-chloroethyl)amine had the most powerful inhibitory effect.

The respiration of lymphatic tissue (mesenteric and axillary lymph nodes) was inhibited by small concentrations of MBA. In fact, even 1×10^{-6} M (less than the LD_{50} for rabbits) produced definite inhibition (Fig. 2). Marked

TABLE I

Effect of Nitrogen Mustards on Tissue Respirations

Ringer-phosphate buffer. Inhibitor (0.001 M).

Tissue	Inhibition	
	TBA	MBA
	per cent	*per cent*
Leucocytes from myeloid leukemia		
1st hr.		None
2nd "		27.5
Leucocytes from lymphoid leukemia		
1st hr.	8	11
2nd "	13	45
Bone marrow, rat		
1st hr.		21
2nd "		52
Thymus, rabbit		
1st hr.		59
2nd "		88
Lymph nodes, rabbit		
1st hr.	12	15
2nd "	34	70
Liver, rat		
1st hr.	19	30
2nd "	23	72
Kidney, rat		
1st hr.	48	70
2nd "	77	92
Brain, rat		
1st hr.	20	14
2nd "	32	33

TABLE II

Effect of Nitrogen Mustards on the Respiration of Spleen Slices (Rat)

Glucose-Ringer-phosphate, pH, 7.4. Nitrogen mustard, 0.001 M.

Nitrogen mustard	O_2 uptake per mg. dry tissue	
	1st hr.	2nd hr.
	c.mm.	*c.mm.*
None	11.6	10.3
Methyl-bis(β-chloroethyl)amine	10.9	6.1
Tris(β-chloroethyl)amine	11.0	8.6
Isopropyl-bis(β-chloroethyl)amine	7.8	5.4
Ethyl-bis(β-chloroethyl)amine	7.2	4.4

histological alterations and the leucopenia are also indications of this marked sensitivity of lymphoid tissue to nitrogen mustards.

2. Tissue Glycolysis.—Nitrogen mustards inhibit somewhat the activity of hexokinase while they have no effect on the other enzymes which take part in the glycolytic process. In tissue slices, the anaerobic glycolysis of rat brain (slices) was not affected in experiments of 1 hour duration. In rabbit bone marrow there was slight inhibition in the 1st hour which became more marked in the 2nd hour. Anaerobic glycolysis of brain was unaffected.

3. Pyruvate Metabolism.—The metabolism of pyruvate by tissue slices was profoundly affected by nitrogen mustards. To differentiate the effect on

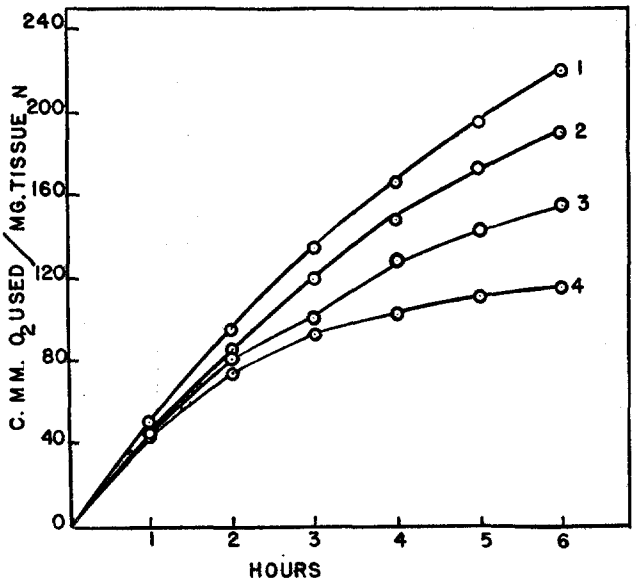

FIG. 2. Effect of various concentrations of MBA on the respiration of rabbit lymphatic tissue. (1) Control; (2) 1×10^{-5} M MBA; (3) 5×10^{-5} M MBA; (4) 1×10^{-4} M MBA.

pyruvate metabolism from the general inhibition of respiration, all the experiments were performed both in the presence and in the absence of pyruvate. The value Q_{O_2} pyruvate $- Q_{O_2}$ would represent the increase in O_2 uptake due to pyruvate oxidation. Furthermore, pyruvate analysis measured the amount of pyruvate utilized by the tissue. Four nitrogen mustards were tested: methyl-bis(β-chloroethyl)amine HCl (MBA), tris(β-chloroethyl)amine HCl (TBA), isopropyl-bis(β-chloroethyl)amine HCl (PBA), and ethyl-bis(β-chloroethyl)-amine HCl (EBA). All of them produced a striking inhibition of pyruvate metabolism at the concentration used (0.001 M), MBA being the most powerful inhibitor (Table III). The same increase of inhibition with time was observed. With 1×10^{-3} M MBA the inhibition started as soon as pyruvate was added to the tissues (30 minutes after addition of nitrogen mustard). With 1×10^{-4} M,

inhibition started only 30 minutes after pyruvate addition and increased with time so that at the end of the last half hour (experiments of 2 hours' duration) the inhibition was 61 per cent. With 5×10^{-5} M, the inhibition started 45 minutes after pyruvate addition. In the last 15 minutes, the inhibition had increased to 55 per cent. Finally with 1×10^{-5} M, the inhibition started only 90 minutes after measurement of the O_2 uptake and 105 minutes after addition of the nitrogen mustard (Fig. 3). The inhibitory effect of nitrogen mustard was also shown on measuring pyruvate utilization (Table IV). It must be mentioned, however, that at the end of 2 hours there is still a large proportion of ethylenimonium compound present in MBA (see Fig. 1). This inhibition of pyruvate metabolism seems to be confined to animal tissues, for the oxidation

TABLE III

Effect of Nitrogen Mustards on the Respiration of Kidney Slices (Rat) and on the Metabolism of Pyruvate

Ringer-phosphate, pH 7.4. Methyl-bis(β-chloroethyl)amine = MBA; tris(β-chloroethyl)-amine = TBA; isopropyl-bis(β-chloroethyl)amine = PBA; ethyl-bis(β-chloroethyl)amine = EBA. The figures give c.mm. per mg. dry weight per hour.

Measurements	Control	MBA	TBA	PBA	EBA
	c.mm.	*c.mm.*	*c.mm.*	*c.mm.*	*c.mm.*
O_2 uptake, 1st hr.	22.0	6.6	9.5	9.2	14.3
" " 2nd "	17.8	0.8	1.6	4.6	5.0
" " with pyruvate					
1st hr.	32.8	12.4	18.5	17.8	19.8
2nd "	30.5	1.1	6.4	5.9	8.6
Pyruvate utilization, 2 hrs.	35.1	6.4	17.3	11.4	12.0

of pyruvate by yeast was inhibited only to a small degree (Fig. 4). As the minimum lethal dose of MBA in subcutaneous injection in mice is 2.6 mg. per kilo, *i.e.* 1.35×10^{-5} M, inhibition of pyruvate metabolism can be obtained with amounts well within the minimum lethal dose.

4. Oxidation of Amino Acids by Kidney Slices.—The effect of nitrogen mustards on the oxidation of amino acids by kidney slices was studied by measuring the O_2 uptake of the tissues in the presence and in the absence of amino acids and by measuring the formation of NH_3. The following amino acids were used: glutamic acid, leucine, valine, and *d,l*-alanine. The oxidation of *l*-amino acids (measured by O_2 uptake as well as by NH_3 formation) was inhibited, that of glutamate showing the greatest effect. The oxidation of *d,l*-alanine (as measured by NH_3 formation) was increased in the presence of four nitrogen mustards (Table V). This increase was investigated further by measuring simultaneously the O_2 uptake, alanine utilization, and pyruvate and NH_3 formation. Both MBA and TBA produced an increase in the utilization of alanine

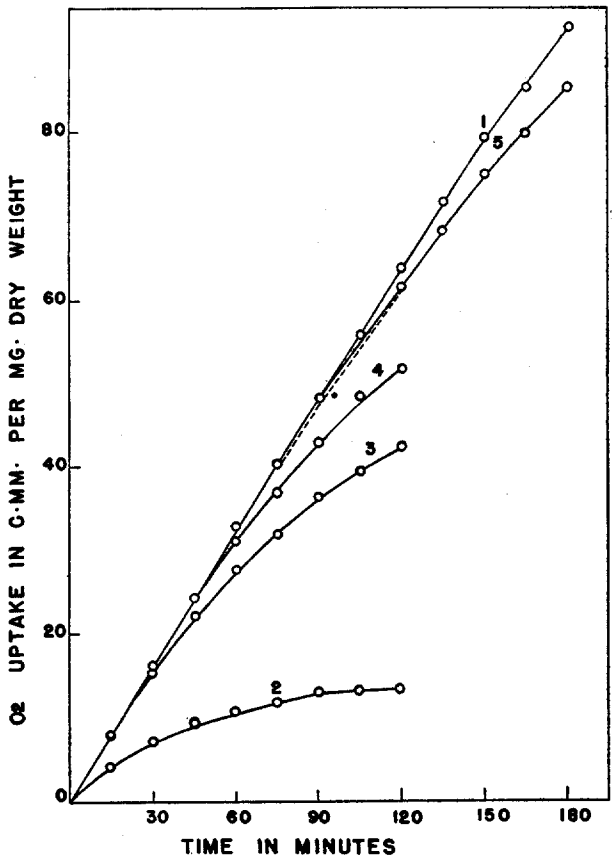

FIG. 3. Effect of methyl-bis(B-chloroethyl)amine (MBA) on the O_2 uptake of kidney slices in the presence of pyruvate. Pyruvate, 0.01 M. pH, 7.4. Temperature 38°. (1) Control; (2) MBA, 0.001 M; (3) MBA, 0.0001 M; (4) MBA, 0.00005 M; (5) MBA, 0.00001 M.

TABLE IV

Effect of Methyl-Bis(β-Chloroethyl)amine (MBA) at Different Concentrations on the Metabolism of Pyruvate by Kidney Slices (Rat)

Figures give pyruvate utilization at the end of 2 hours. Pyruvate utilization, control = 35.1 c.mm.

MBA concentration	Pyruvate utilization MBA	Inhibition
M	*c.mm.*	*per cent*
0.001	6.4	82
0.0001	19.8	43.5
0.00005	23.0	34.5
0.00001	30.4	12.4

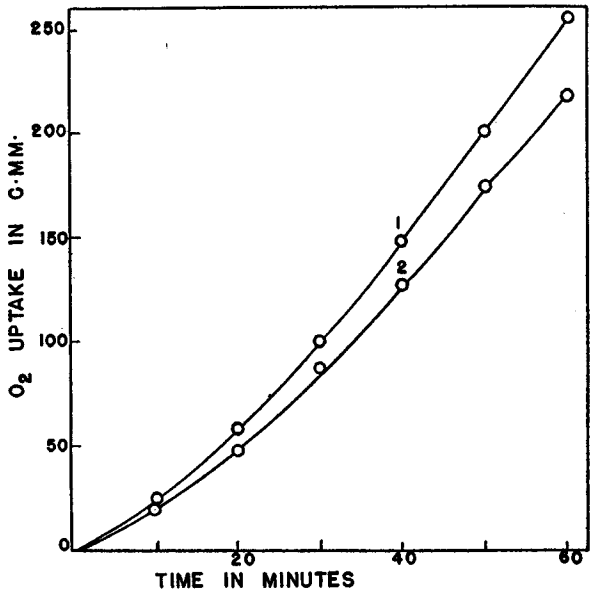

FIG. 4. Effect of methyl-bis(β-chloroethyl)amine HCl (MBA) on the oxidation of pyruvate by baker's yeast. Hippurate buffer, pH 3.8. MBA, 1×10^{-3} M; pyruvate, 1×10^{-2} M. Temperature 28°. (1) Control; (2) MBA, 1×10^{-3} M.

TABLE V

Effect of Nitrogen Mustards on the Oxidation of Amino Acids by Kidney Slices

Ringer-phosphate, pH 7.4; amino acid concentration, 0.01 M; nitrogen mustard, 0.001 M.

Substrate	Nitrogen mustard	O_2 uptake		NH_3 formation	
		Control	Inhibitor	Control	Inhibitor
		c.mm.	c.mm.	c.mm.	c.mm.
Glutamate	TBA	34.3	13.6	0.515	0.26
"	MBA	34.3	11.0	0.515	0.30
Leucine	TBA	22.9	12.6	1.0	0.845
Valine	"	17.9	13.9	0.995	0.885
"	MBA	17.9	11.8	0.995	1.053
Phenylalanine	TBA	12.8	10.0	0.966	0.834
"	MBA	12.8	10.0	0.966	1.011
d, l-Alanine	TBA	35.3	32.2	0.644	1.97
"	MBA	35.3	36.0		2.70
"	PBA	45.3	27.5	2.04	2.65
"	EBA	42.5	23.6	1.95	2.53

accompanied by an increased formation of pyruvate and NH_3. In the control experiments, 94 per cent of the pyruvate formed through the oxidation of

alanine was further utilized by the tissues while 83 per cent of the NH_3 formed was found in the solution. In the presence of MBA, 66 per cent of the pyru vate formed was found, while the accumulation of NH_3 was the same as in the control (Table VI).

TABLE VI

Effect of Nitrogen Mustards on the Oxidation of d,l-Alanine

Incubation time, 90 minutes. Ringer-phosphate, pH 7.4. Alanine concentration, 0.04 M. Tissue, rat kidney slices. Inhibitor, 1×10^{-3} M. Figures give micromoles per milligram of dry tissue.

	Control	TBA	Inhibition or increase	MBA	Inhibition or increase
	μM per mg.	*μM per mg.*	*per cent*	*μM per mg.*	*per cent*
O_2 uptake	1.66	1.34	−19	1.04	−31
Alanine utilization	1.11	1.62	+46	1.74	+57
Pyruvate formation	0.06	0.82	+1200	1.15	+1816
NH_3 "	0.92	1.25	+36	1.44	+46

TABLE VII

Effect of Nitrogen Mustards on Biochemical Reactions Leading to Synthesis

Synthesis	Tissue	TBA	Inhibition	MBA	Inhibition
		M	*per cent*	*M*	*per cent*
1. Carbohydrate, from pyruvate	Kidney	10^{-3}	71	10^{-3}	94
" " "	"	10^{-4}	61	10^{-4}	71
2. Urea, from glucose + NH_3	Liver	10^{-3}	68	10^{-3}	89
" " " " "	"	5×10^{-4}	62	5×10^{-4}	75
" " " " "	"	1×10^{-4}	2	1×10^{-4}	2
3. Creatine, from glycocyamine + methionine (3 hrs.)	"	1×10^{-3}	28	1×10^{-3}	26
Creatine, from glycocyamine + methionine (6 hrs.)	"	"	62	"	79
4. Amino acid, from NH_3 + ketoglutarate	Kidney	"	32	"	40

5. Effect of Nitrogen Mustards on Biochemical Reactions Leading to Synthesis.—On observing animals that have received toxic amounts of halogenated alkylamines, one is struck by the slow effect of these substances, which never produce rapid death; by the progressive disintegration of the animals. The partial inhibition of oxidation reactions accompanied by inhibition of those reactions leading to synthesis and to regeneration of tissue cells might be one of the mechanisms of the toxic action of halogenated alkylamines. The experiments reported in Table VII show that methyl-bis(β-chloroethyl)amine and tris(β-chloroethyl)amine inhibit those reactions leading to synthesis that have

been studied. The synthesis of carbohydrate from pyruvate by kidney slices was inhibited by TBA and by MBA. The synthesis of creatine by liver slices in the presence of glycocyamine and methionine was inhibited with both halogenated alkylamines, TBA and MBA. The synthesis of glutamic acid by the kidney in the presence of α-ketoglutarate plus NH_4Cl was inhibited by TBA and MBA. This marked effect of halogenated alkylamines on enzymatic processes leading to synthesis might explain the delay in the appearance of leucopenia. The compounds seem to act by slowing multiplication processes. This was beautifully shown by Friedenwald and Schultz (5), who found that amounts of alkylamine too small to inhibit the metabolism of the cornea produce inhibition of mitosis, and by the experiments of Auerbach *et al.* (6), who found that sublethal doses of MBA reduced the fertility of female *Drosophila* and produced considerable effects on mutation rate and chromosome arrangement as well as disturbances in the nuclear mechanism. Whether these effects are due to the halogen groups and are thus similar to the effects produced by mustard gas, or are due to structural inhibition of enzymatic reactions where choline is one of the substrates is not yet known. It is quite possible that some of these toxic symptoms may be due to disturbances of choline metabolism.

6. Bone Marrow.—It is known that nitrogen mustards produce marked alterations in the bone marrow even when used at concentrations far below the lethal dose. A series of experiments was performed with rabbit bone marrow slices with the purpose of finding an agent which would either prevent or reverse the injurious effects of nitrogen mustards when used for medical treatment. To insure a steady respiration, the slices were suspended in the following solution, which was shown by Warren (7) and by Goldinger *et al.* (8) to maintain respiration: 30 cc. of neutralized, sterile, unheated, beef serum ultrafiltrate, 30 cc. of Ringer solution (100 cc. of 0.154 M NaCl, 2 cc. 0.154 M KCl, 2 cc. 0.11 M $CaCl_2$), 40 cc. of 0.1 M phosphate, pH 7.4. Glucose was added to a concentration of 0.01 M. MBA at a concentration of 1×10^{-3} M inhibited the respiration 50 per cent in the 1st hour and 65 per cent in the 2nd hour. With low concentrations, the effect was similar to that produced in kidney slices. 5×10^{-5} M MBA, which had no effect on the respiration in the 1st hour, produced 30 per cent inhibition at the end of 5 hours. Even a concentration of 5×10^{-6} M gave a definite though small inhibition (Fig. 5). (This amount is about half the initial concentration in the blood of patients treated with MBA.)

With low concentrations of MBA most of it became fixed in the tissue and very little remained in the solution. This was demonstrated by keeping the slices in MBA (5×10^{-5} M) for 30 minutes at 38°. The slices were then washed and put into Warburg vessels containing no nitrogen mustard. Other slices were suspended in the fluid in which the previous slices had been kept, which originally contained 5×10^{-5} M MBA. At the end of 5 hours the respiration of the washed slices was inhibited 26 per cent, while that of the slices suspended in the fluid of the previous slices was inhibited only 14 per cent (Fig. 6).

Some preliminary experiments performed in 1943 indicated that choline may protect mice against lethal doses of MBA. The experiments in Fig. 7 show that choline added to bone marrow slices protected the tissue against the inhibition of respiration by MBA. Since choline in large concentrations inhibits tissue respiration, experiments were performed to find the maximum amount of choline which may be added to tissues without affecting respiration. Tissue slices were kept suspended for 45 minutes in the buffered solution containing

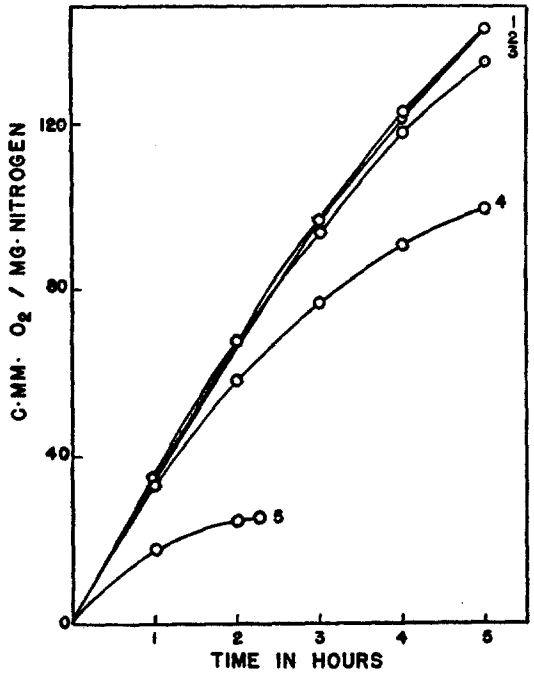

FIG. 5. Inhibition of bone marrow respiration by nitrogen mustards. (1) Control; (2) MBA, 1×10^{-6} M; (3) MBA, 5×10^{-6} M; (4) MBA, 5×10^{-5} M; (5) MBA, 1×10^{-3} M.

5×10^{-3} M choline before addition of nitrogen mustard (5×10^{-5} M). Tissues thus treated were scarcely affected by MBA, while in the absence of choline, MBA inhibited respiration by 43 per cent. A number of substances which enter into the cycle of biological choline interrelationships were tried as preventive agents: dimethylaminoethanol, betaine, methionine, and lecithin. Lecithin and betaine were without protective effect. 3×10^{-3} M dimethylaminoethanol plus 3×10^{-3} M methionine prevented almost completely (93 per cent) the inhibitory action of 5×10^{-5} M MBA;[1] dimethylaminoethanol alone

[1] Dimethylaminoethanol, at a ratio of 100:1 of nitrogen mustard, prevented the inhibition of choline oxidase by 53 per cent.

prevented it 81 per cent, while methionine alone had no effect at all (Fig. 8). Although the mechanism of this protective action has not yet been elucidated, it undoubtedly is an indication of the important rôle of choline in the metabolism of bone marrow. The methylation of dimethylaminoethanol to choline was demonstrated by du Vigneaud *et al.* (9). These experiments suggest the

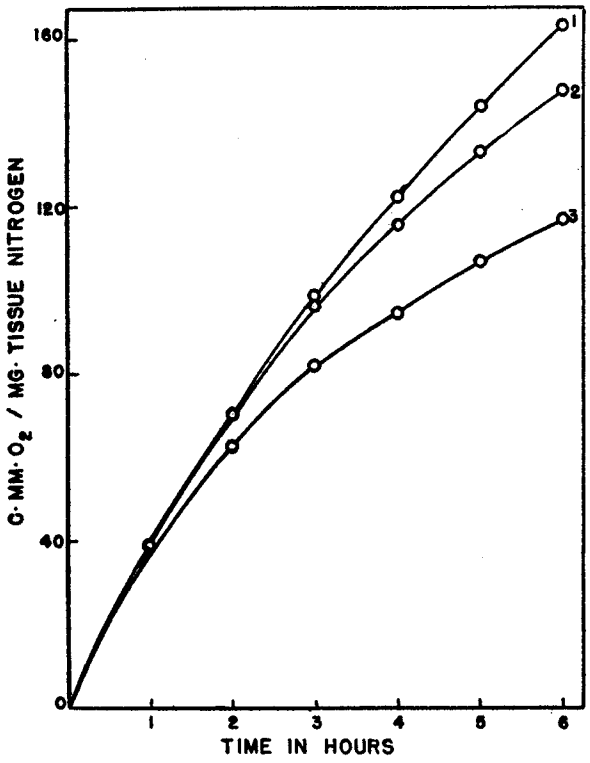

FIG. 6. Fixation of nitrogen mustards by bone marrow. (1) Control; (2) fresh bone marrow slices added to centrifuged supernatant fluid obtained from 30 minutes' incubation of other bone marrow slices (No. 3 below) with MBA, 5×10^{-5} M; (3) washed bone marrow slices from the above 30 minutes' incubation with MBA were resuspended in fluid free of MBA.

utilization of choline or choline precursors to avoid the injurious effects of nitrogen mustards on the bone marrow during nitrogen mustard therapy.

7. Toxicity of Methyl-Bis(β-Chloroethyl)amine to Growing Seeds.—Water solutions of nitrogen mustards retain toxicity for a longer time than nitrogen mustards in well buffered solutions. To study the toxicity of these water solutions to plants, tomato seedlings were grown in sand and distilled water in the presence and in the absence of 0.001 M methyl-bis(β-chloroethyl)amine. For 3 days the seedlings in both grew about equally. At the end of the 3rd day the

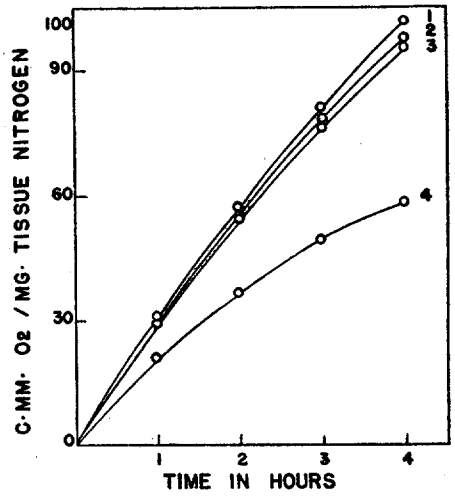

FIG. 7. Protection of nitrogen mustard inhibition of respiration by choline. (1) Control, bone marrow slices; (2) bone marrow + 5×10^{-3} M choline; (3) bone marrow kept for 45 minutes with 5×10^{-3} M choline; 5×10^{-5} M MBA, added afterwards; (4) bone marrow + 5×10^{-5} M MBA. O_2 uptake measurements are those obtained after the 2nd hour of incubation.

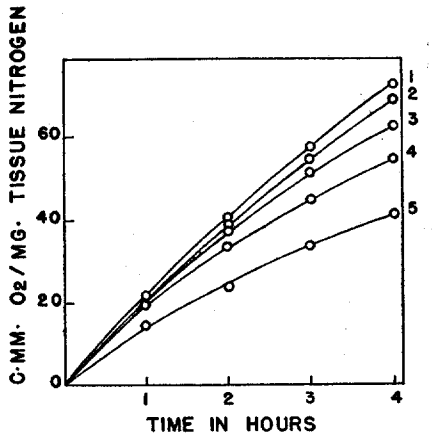

FIG. 8. Protection of nitrogen mustard inhibition of respiration by dimethylaminoethanol. (1) Bone marrow control; (2) dimethylaminoethanol, 3×10^{-3} M; (3) dimethylaminoethanol, 3×10^{-3} M + 3×10^{-3} M methionine + 5×10^{-5} M MBA; (4) dimethylaminoethanol, 3×10^{-3} M + MBA, 5×10^{-5} M; (5) MBA, 5×10^{-5} M. O_2 uptake measurements are those obtained after the 2nd hour of incubation.

seedlings containing MBA ceased to grow. At the end of 1 week the difference between the control seeds and the seeds containing MBA was remarkable.

Nitrogen mustards in water are thus toxic to growing seeds and the toxicity is manifested days after treatment.

Tissue Metabolism and the Activity of Some Enzyme Systems in Rats Treated with Methyl-Bis(β-Chloroethyl)amine (MBA)

In studies with isolated enzyme systems it has been shown that the most striking property of the halogenated alkylamines is the inhibition of choline oxidase, and next to it the inhibition of choline esterase, pyruvate oxidase, phosphocreatine phosphokinase, and inorganic pyrophosphatase. In studies with tissue slices it was found that a number of reactions leading to synthesis were inhibited as well as the total respiration. Whether these findings can be extended to the effect of these compounds on the living animal will depend on the distribution throughout the different tissues of this or that transformation product of the halogenated alkylamines. In searching for the mechanism of the toxic action of a compound, studies on enzymes must therefore be accompanied not only by observations of the enzymatic reactions in tissues but also by a determination of the enzyme activity of the tissues of animals treated with lethal amounts of alkylamine. Inhibitions found under these conditions may reasonably be considered among the factors responsible for toxicity. For this purpose rats were treated with amounts of MBA which allowed a survival up to 100 hours. Twelve rats together in each experiment received methyl-bis(β-chloroethyl)amine in a gas chamber and the rats were killed at different times for study of tissue metabolism.

1. Choline Oxidase by the Liver and Kidney of Rats Treated with MBA.—Kidney slices of rats treated with MBA and killed 2 and 5 hours after treatment showed a decreased Q_{O_2} value in the absence of choline (27 per cent decrease) when compared to the Q_{O_2} value of normal rats. On addition of choline there was no rise of the Q_{O_2} values 2 hours after treatment; *i.e.*, there was complete inhibition of choline oxidase. Complete inhibition was also found in rats killed 19 hours and 43 hours after treatment. The inhibition of choline oxidase was partial in the other rats and had disappeared at the end of 48 hours, an indication that in 48 hours there was new formation of the enzyme (Table VIII) (the inhibition produced by the alkylamines is irreversible). This resynthesis of the enzyme in such a short time is remarkable and is indicative that the continuous breakdown and synthesis of protein in the body apply also to the protein moiety of enzymes. The inhibition of choline oxidase in the kidney was confirmed by Cori (10).

In sharp contrast with this inhibition of choline oxidase in the kidney was the lack of effect in the liver. The choline oxidase activity of the treated rats, killed from 1 hour after treatment to 30 hours after, remained unimpaired.

2. Pyruvate Oxidation by the Kidney.—It has been shown that the oxidation of pyruvate by kidney slices is strongly inhibited by nitrogen mustards. The

TABLE VIII

Choline Oxidation by the Kidney of Rats Gassed with Methyl-Bis(β-Chloroethyl)Amine (MBA)

Q_{O_2} of normal kidney in the absence of choline = 18.3. In the presence of choline = 27.0: Q_{O_2} choline 8.7.

Amount of MBA	Time after gassing	Q_{O_2} without choline	Q_{O_2} with choline	Q_{O_2} choline	Inhibition of choline oxidation
mg. per liter per 10 min.	*hrs.*	*c.mm.*	*c.mm.*	*c.mm.*	*per cent*
0.71	2	13.0	13	0	Complete
0.71	5.2	13.5	18.2	4.2	51.7
0.71	12	18.5	21.6	3.1	64.5
0.80	19	16.0	14.3	0	Complete
0.80	24	10.0	16.8	6.8	21.8
0.80	31	18.5	19.3	0.8	90.8
0.80	43*	19.4	18.0	0	Complete
0.80	46.3	20.4	23.9	3.5	59.7
0.65	48	11.4	22.5	11.1	None

* Rat died 40 minutes before experiment started.

TABLE IX

Pyruvate Oxidation by the Kidney of Rats Gassed with Methyl-Bis(β-Chloroethyl)Amine (MBA)

Q_{O_2} of normal kidney in the absence of pyruvate = 18.3. In the presence of pyruvate = 27.5: Q_{O_2} pyruvate = 9.2.

Amount of MBA	Time after gassing	Q_{O_2} without pyruvate	Q_{O_2} with pyruvate	Inhibition of pyruvate oxidation
mg. per liter per 10 min.	*hrs.*	*c.mm.*	*c.mm.*	*per cent*
0.71	2	13.0	11.4	Complete
0.71	5.2	13.6	13.9	Complete
0.71	12	18.5	20.3	80.5
0.80	19	16.0	13.2	Complete
0.80	24	10.0	14.9	46.7
0.80	31	18.5	27.3	None
0.80	43	19.4	23.9	51.0
0.80	46.3	20.4	25.0	50.0
0.65	48	11.4	12.7	86.0
0.65	72	24.2	23.3	Complete
0.46	83	18.4	21.7	35.8

same inhibition was found in the kidney of rats treated with MBA. The inhibition was complete in rats killed 2, 5.2, 19, and 72 hours after treatment. In all the other rats, killed at different times up to 83 hours, there was partial inhibition (Table IX).

Whether the inhibition of pyruvate oxidation is found in all tissues or is confined to the kidney is not known. In one case, the oxidation of pyruvate by the

brain of a rat killed 2 hours after gassing was found to be the same as in normal rats. In another case, the utilization of pyruvate by the liver of a rat killed 24 hours after treatment was also found to be normal.

3. Urea Synthesis by the Liver.—It has been shown that the synthesis of urea by the liver is inhibited by alkylamines. In rats treated with MBA the synthesis of urea by the liver was followed, the animals being killed at different times after treatment, from 1 hour to 74 hours. In two rats which died half an hour before the experiments started there was 83 and 78 per cent inhibition of urea

TABLE X

The Activity of Urea Synthesis by the Liver in Rats Gassed with Methyl-Bis(β-Chloroethyl)Amine

The average value of Q urea of normal rats is: 4.87 (cubic millimeters of urea produced per milligram of tissue (liver) per 2 hours).

Amount of MBA	Time after gassing	Q urea	Inhibition
mg. per liter per 10 min.	*hrs.*	*c.mm.*	*per cent*
0.7	1	3.49	28.3
0.7	4.7	4.37	10.0
0.7	10.5	4.03	9.6
0.7	21	5.89	None
0.7	24	4.21	13.5
0.71	26	3.86	20.7
0.71	31	1.85	62.0
0.71	29	3.72	23.6
0.71	43*	0.81	83.2
0.71	74	3.72	23.6
0.71	46.5*	1.04	78.5
0.71	53	3.72	23.6
0.71	50	2.60	46.5

* Rat died ½ hour before the experiment started.

synthesis. In all the other cases, except one, there was partial inhibition (Table X).

4. Brain Choline Esterase.—It has been shown by Dixon, Thompson, and ourselves that brain choline esterase is inhibited by nitrogen mustards. However, the amount required to produce about 60 per cent inhibition was 1×10^{-4} M, which is 100 times higher than the lethal dose. As expected from these figures the choline esterase activity of the brain of rats killed from 1 hour to 55 hours after gassing remained remarkably within normal values.

5. Tissue Glycolysis and Coenzymes.—In *in vitro* experiments halogenated alkylamines produced some inhibition of glycolysis 1½ hours after the addition of alkylamine to the tissue. The anaerobic glycolysis of brain slices of rats treated with methyl-bis(β-chloroethyl)amine remained within normal values at different times after treatment.

The diphosphopyridine nucleotide and diphosphothiamine content of tissues was also normal, showing that MBA had no effect at all on these coenzymes.

Cori (10) injected methyl-bis(β-choroethyl)amine HCl (15 mg. per kilo) into rats. At the end of 3 hours, the following enzymes from the kidney were inhibited: choline oxidase, 100 per cent; hexokinase, 74 per cent; inorganic pyrophosphatase, 42 per cent. When the renal blood vessels were clamped for 20 minutes after the injection of nitrogen mustard choline oxidase inhibition dropped to 32 per cent. It seems that all the toxic substances capable of inactivating enzymes disappear from the circulating blood stream 20 minutes after injection.

DISCUSSION

In the experiments presented in this paper nitrogen mustards added to tissue slices produced a strong inhibition of pyruvate metabolism. They also inhibited the oxidation of *l*-amino acids and the utilization of NH_3. Furthermore there was inhibition of a number of synthesis reactions, such as the synthesis of carbohydrate from pyruvate, the synthesis of creatine from glycocyamine and methionine, the synthesis of glutamic acid, and the synthesis of urea. In plants, there was inhibition of the growth of seeds. Of these inhibitions, the inhibition of methylation reactions, such as the transfer of the methyl group from methionine to glycocyamine with the formation of creatine, perhaps belongs to the type of structural inhibition because labile methyl groups are found only in quaternary nitrogen compounds structurally similar to the active ethylenimonium derivative of nitrogen mustards. The inhibition of pyruvate metabolism and of *l*-amino acid oxidation might be due either to combination with the -SH groups of the protein moiety of these enzymes or to combination of other groups of the side chains of the protein with the highly reactive halogen groups of nitrogen mustards. The lack of inhibition of succinoxidase and phosphoglyceraldehyde dehydrogenase (which are sulfhydryl enzymes) is only evidence that nitrogen mustards do not inhibit all sulfhydryl enzymes. In fact, inhibition of mitosis by nitrogen mustards, sulfur mustard, and x-rays finds reasonable explanation in the assumption that the process is controlled by a sulfhydryl enzyme with very labile and reactive -SH groups, easily alkylated by the first two and oxidized by the last.

The great sensitivity of the bone marrow respiration explains the leucotoxic action of nitrogen mustards. The experiments on bone marrow slices in which the inhibitory action of nitrogen mustards was prevented on addition of choline or the choline precursors, dimethylaminoethanol and methionine, seem to demonstrate the rôle of choline in the metabolism of the bone marrow, and they point the way for the treatment of nitrogen mustard intoxication in the course of its medical use.

The mechanism of the toxic action of nitrogen mustards is thus complex and

must be due to the dual action of the compound which can act as a structural inhibitor and an inhibitor of enzyme reactions through combination with certain groups of the protein moiety essential for enzyme activity.

The inhibition of reactions leading to synthesis, the inhibition of pyruvate metabolism, of *l*-amino acid oxidation, of phosphokinases and of hexokinase together contribute to the striking symptoms of destruction and lack of formation of new cells leading to leucopenia and atrophy of the bone marrow. This inhibition of growth was clearly demonstrated in the inhibition of the growth of seedlings treated with nitrogen mustards. Furthermore, the large accumulation of NH_3, in the tissues where amino acids and nitrogen mustards were present, is indication of lack of utilization of NH_3 for the formation of nitrogenous compounds among which the proteins are obviously included.

SUMMARY

Nitrogen mustards at a concentration forty times the minimum lethal dose inhibited the respiration of all tissues studied but affected anaerobic glycolysis very little. The inhibiting effect increased with time. The respiration of lymphoid tissue was extremely sensitive to nitrogen mustard, as concentrations below the LD_{50} definitely inhibited the respiration of rabbit lymph nodes. In tissue slices nitrogen mustards inhibited the oxidation of pyruvate and of *l*-amino acids and the utilization of NH_3. A number of synthesis reactions were also inhibited, such as the synthesis of carbohydrate, of creatine, and of urea. When added to growing seeds, nitrogen mustards inhibited their growth. In rats given lethal doses of nitrogen mustards there were found complete inhibition of choline oxidation and strong inhibition of pyruvate oxidation by the kidney and partial inhibition of urea synthesis by the liver. Inhibition of bone marrow respiration by nitrogen mustards was prevented by the addition of choline, and of dimethylaminoethanol plus methionine. The possible mechanism of nitrogen mustard intoxication is discussed.

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