# THE EFFECT OF NITROGEN MUSTARDS ON ENZYMES AND TISSUE METABOLISM*

## I. The Effect on Enzymes

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The halogenated alkylamines, so called nitrogen mustards, investigated during the last war as a potential warfare agent, have become useful tools in biology and medicine. Possessing some of the properties of bis(β-chloroethyl) sulfide, and x-rays (such as injurious effects to the bone marrow and lymph glands which lead to leucopenia (1), inhibition of mitosis (2, 3), and production of mutants (3–5), they are being used with success in the treatment of blood discrasias (6–8). Some of the studies on the chemical properties of the nitrogen mustards have been published (9–14). We present in this paper experiments performed in this laboratory during the years 1942–43, and quite recently on the effect of nitrogen mustards on the activity of enzymes. These studies were undertaken to determine their mechanism of action. Nitrogen mustards were found to be powerful enzyme inhibitors belonging to the class of structural inhibitors, although different from most of the compounds of this group in the lack of easy reversibility and competition.

### *Nitrogen Mustards and Enzymes*

A number of investigators (9–15) have shown that tertiary halogenated alkylamines when in aqueous neutral solutions undergo a series of rapid transformations, the first of which is the formation of the ethylenimonium ring. In dilute solutions, according to Cohen (15), this first change is a rapid process while the rearrangement which ends with the formation of the dimer or the ethanolamine proceeds at a rate 100 times as slow as the rate of the first process. It is therefore reasonable to assume that in the first hour after the compounds have been brought into aqueous solutions, at pH values around neutrality,

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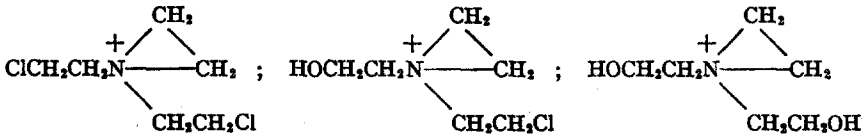
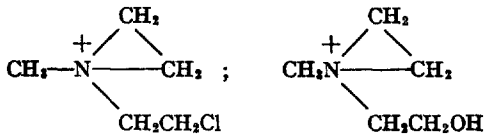
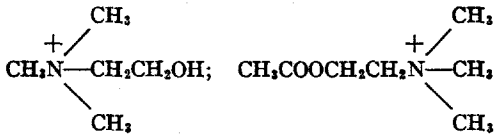
there are present at different concentrations three different ethylenimonium compounds:



in the case of tris(β-chloroethyl)amine; two compounds:



in the case of methyl-bis(β-chloroethyl)amine. The resemblance of these transformation products to choline and acetylcholine is striking:



Choline Acetylcholine

This similarity between the ethylenimonium derivatives of nitrogen mustards and choline and acetylcholine suggested the possibility that they may act as structural inhibitors of enzymes by combination with the protein moiety at the side chains where combination of choline and acetylcholine takes place. The validity of this assumption is demonstrated in the experiments with choline oxidase, acetylcholine esterase, and choline acetylase.

*1. Choline Oxidase.*—In agreement with the postulated assumption, choline oxidase was, of all the enzymes studied, the most sensitive to the action of nitrogen mustards. In these experiments choline oxidase was prepared from rat liver by a modification of the method of Bernheim (16); the enzyme suspension consumed no oxygen in the absence of choline. When methyl-bis-(β-chloroethyl)amine HCl (MBA) and tris(β-chloroethyl)amine HCl (TBA) were dissolved in phosphate buffer, 0.02 M pH 7.4, the degree of inhibition remained constant up to 2 hours after solution of the compound with TBA, while it decreased rapidly with MBA. When the compounds were dissolved in water, inhibition increased on standing (Table I). Isopropyl-bis(β-chloroethyl)amine HCl was also found to be a powerful inhibitor of choline oxidase (Table II). Since formation of the ethylenimonium derivative occurs more rapidly in phosphate buffer than in water (17), these experiments indicate that inhibition was due to the quaternary N derivative. Since inhibition disappeared when MBA was left standing in phosphate buffer for 2 hours, and TBA for 24 hours, it must be concluded that the end products of the series of reactions

TABLE I

*Inhibition of Choline Oxidase by Nitrogen Mustards. The Effect of Standing in Aqueous and Phosphate Solutions*

Methyl-bis(β-chloroethyl)amine HCl (MBA) and tris(β-chloroethyl)amine HCl (TBA) were dissolved and added to the enzyme suspension after remaining in solution for the time indicated in Table I.

| Time of standing | MBA | Inhibition | TBA | Inhibition |
|---|---|---|---|---|
| *min.* | *M* | *per cent* | *M* | *per cent* |
| *In phosphate buffer* | | | | |
| 0 | $1 \times 10^{-6}$ | 95 | $1 \times 10^{-6}$ | 74 |
| 15 | " | 72 | " | 79 |
| 30 | " | 38 | " | 84 |
| 60 | " | 13 | " | 74 |
| 120 | " | 0 | " | 75 |
| 1440 | " | 0 | " | 0 |
| *In water* | | | | |
| 0 | " | 42 | " | 34 |
| 30 | | | $2.5 \times 10^{-6}$ | 40 |
| 60 | $5 \times 10^{-7}$ | 32 | | |
| 90 | | | " | 57 |
| 120 | " | 42 | $1 \times 10^{-5}$ | 74 |
| 210 | | | $2.5 \times 10^{-6}$ | 62 |
| 480 | " | 76 | | |

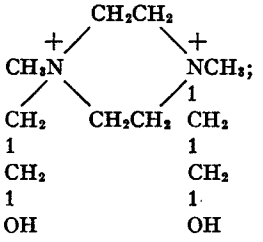
TABLE II

*Inhibition of Choline Oxidase by Isopropyl-Bis(β-Chloroethyl)amine HCl (IBA)*

Concentration of choline, 0.02 M. pH, 7.4. Temperature 38°.

| IBA | Inhibition |
|---|---|
| *M* | *per cent* |
| $7 \times 10^{-6}$ | Complete |
| $3 \times 10^{-6}$ | 97 |
| $1 \times 10^{-6}$ | 44 |
| $5 \times 10^{-7}$ | 22 |

which nitrogen mustards undergo in water solutions, namely the dihydroxy cyclic dimer (I) or tri-ethanolamine (II), have no inhibitory power.



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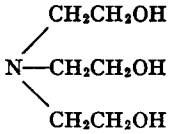


II

Further evidence that the inhibition is produced by the ethylenimonium transformation products is given by experiments in which the inhibitory power of nitrogen mustards was measured at different pH values. If the inhibition is produced by these quaternary N derivatives, it must increase as the pH values increase because the rates of transformation are so affected (15). In fact, $2 \times 10^{-6}$ M TBA, which produced 68 per cent inhibition at pH 6.4, inhibited completely at pH 7.4; $1 \times 10^{-7}$ M produced 15 per cent inhibition at pH 7.4, while it increased to 36 per cent at pH 8.4. Similar results were obtained with MBA (Table III).

It seems that inhibitors belonging to this group must be able to form the ethylenimonium derivative. β-chloroethylethylbenzylammonium chloride, β-chloroethylethylcyclohexylammonium chloride, and β-chloroethylethyl hexahydrobenzylammonium chloride[1] which form ethylenimonium derivatives

TABLE III

*Effect of pH on the Inhibition of Choline Oxidase by Alkylamines*

Phosphate buffer. Temperature 38°.

| pH | Methyl-bis(β-choroethyl)amine | | Tris(β-chloroethyl)amine | |
|---|---|---|---|---|
| | Concentration | Inhibition | Concentration | Inhibition |
| | M | per cent | M | per cent |
| 6.4 | $2 \times 10^{-6}$ | 68 | $2 \times 10^{-6}$ | None |
| 7.4 | $1 \times 10^{-7}$ | 15 | $2.5 \times 10^{-6}$ | 42 |
| | $2 \times 10^{-6}$ | Complete | $1 \times 10^{-6}$ | 27 |
| 8.4 | $1 \times 10^{-7}$ | 36 | " | 81 |

on solution in water were as powerful inhibitors as MBA. The quaternary N compound dimethyl-bis(β-chloroethyl)amine HCl which does not form ethylenimonium on solution had no inhibitory effect. Trimethylbenzylammonium chloride, dimethylethanolamine, and methylethanolamine had no effect at all (Table IV).

One important difference between nitrogen mustard inhibition and other substrate inhibitors (such as the classical malonate inhibition of succinoxidase discovered by Quastel and Wooldrige (18)) is that the former is not, like the latter, easily reversed, and as a consequence does not depend on the ratio of substrate to inhibitor. Once inhibition was produced with the nitrogen mustards it was difficult to reverse, and washing the enzyme with water brought no restoration of activity. This strong association of the enzyme with the nitrogen mustard in contrast with the reversible association with choline can be shown by measuring the half-saturation of the complex enzyme-choline and half-

[1] These compounds were kindly provided by Dr. Mark Nickerson, Department of Pharmacology, University of Utah, School of Medicine.

inhibition of enzyme activity. Half-saturation (Fig. 1) was reached with $2.5 \times 10^{-3}$ M choline while half-inhibition was reached with $1 \times 10^{-6}$ M TBA, $4 \times 10^{-6}$ M MBA, and $1.2 \times 10^{-6}$ M isopropyl-bis(β-chloroethyl)amine HCl.

TABLE IV

*Effect of Some Ethylenimonium-Forming Compounds on the Activity of Choline Oxidase*

Phosphate buffer, pH, 7.4. Choline, 0.01 M. Duration of experiments, 1 hour. Temperature 38°.

| Inhibitor | Concentration | $O_2$ uptake | Inhibition |
|---|---|---|---|
| | | *c.mm.* | *per cent* |
| None | — | 205 | — |
| MBA | $5 \times 10^{-6}$ | 41 | 80 |
| β-Chloroethylethylbenzylammonium chloride | " | 46 | 78 |
| β-Chloroethylethyl hexahydrobenzylammonium chloride | " | 10 | 95 |
| β-Chloroethylethylcyclohexylammonium chloride | " | 9 | 95 |

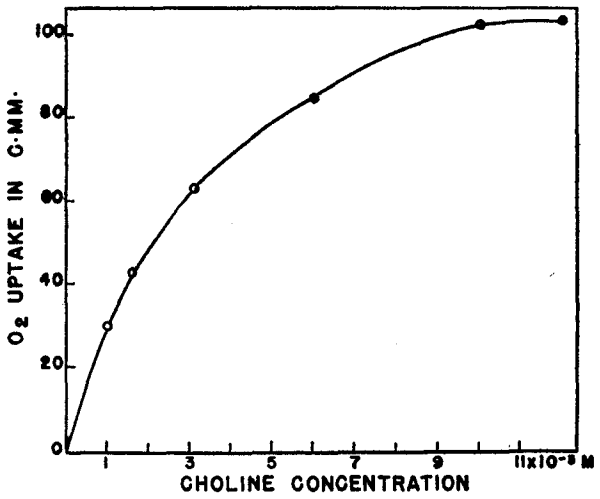



FIG. 1. Effect of choline concentration on the oxidation of choline by choline oxidase pH 6.7. Abscissa, choline concentration $\times 10^{-3}$ M. Ordinate $O_2$ uptake in 10 minutes. Temperature 38°.

Since the $LD_{50}$ values of nitrogen mustards lie around 2 to $5 \times 10^{-5}$ M, it can be seen that complete inhibition of choline oxidase occurred with amounts ten times lower.

Indication that the nitrogen mustards combine with the protein moiety of the enzyme (activating protein or dehydrogenase) at the place where combination with choline takes place, was given by experiments where choline was

added previous to or simultaneously with the nitrogen mustards, and by experiments where large amounts of choline were added after addition of nitrogen mustard to the enzyme. When choline was added to the enzyme previous to addition of MBA, or when choline and MBA were added simultaneously, there was no inhibition. When $2 \times 10^{-2}$ M choline was added to the enzyme after addition of MBA, the inhibition decreased from 80 per cent to 41 per cent; on increasing the concentration of choline to $1 \times 10^{-1}$ M the inhibition was further lowered to 35 per cent (Table V).

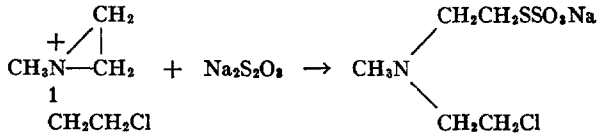
TABLE V

*Competition between Choline and MBA ($10^{-6}$ M) for the Activating Protein of Choline Oxidase*

Choline concentration, $1 \times 10^{-2}$ M. pH, 6.7. Temperature 38°.

| Experiment | $O_2$ uptake | | Inhibition |
|---|---|---|---|
| | Control | Inhibitor | |
| | *c.mm.* | *c.mm.* | *per cent* |
| MBA added to enzyme 15 min. before choline addition | 320 | 64 | 80 |
| MBA + enzyme mixed. Choline, $2 \times 10^{-2}$ M added later | 344 | 202 | 41 |
| Choline, $10^{-1}$ M | 309 | 210 | 35 |
| MBA + choline added simultaneously | 315 | 310 | None |
| Choline + enzyme mixed. MBA added later | 315 | 300 | 4 |

Thiosulfate reacts very rapidly with the ethylenimonium derivative of nitrogen mustards; *e.g.*, MBA:



Addition of thiosulfate ($1 \times 10^{-3}$ M) to choline oxidase previous to addition of MBA ($1 \times 10^{-5}$ M) prevented inhibition. When thiosulfate was added 5 minutes after contact of the enzyme with the nitrogen mustard, the inhibition was one-third of the original (Table VI). A number of substances known to combine rapidly with nitrogen mustards were tried to prevent enzyme inhibition. The inhibitor and the test substance were added to Ringer-phosphate, pH 7.4, and were allowed to react for 15 minutes, at the end of which they were added to the enzyme suspension. Of all the compounds tested, only thiosulfate had a significant preventive effect. A concentration of thiosulfate 1000 times greater than the concentration of nitrogen mustard brought about complete prevention of inhibition; when the ratio was 100:1 the prevention

dropped to 50 per cent; with a ratio of 10:1 there was no prevention at all. Reversal of inhibition with the preventive agents did not occur (Table VII). Tryptophane, tyrosine, inositol, cysteine, and histidine had no preventive effect.

*2. Acetylcholine Esterase.*—The inhibitory effect of methyl-bis(β-chloroethyl)-amine HCl on acetylcholine esterase was first observed by Thompson (19), who also described prevention of inhibition if acetylcholine had been added previously. Thompson's experiments were confirmed, as can be seen in Table VIII. If nitrogen mustards act as structural inhibitors, they must have no effect or very little on the hydrolysis of other esters. In fact, the hydrolysis of acetylcholine by brain choline esterase was inhibited 70 per cent by $1 \times 10^{-3}$ M MBA, while the hydrolysis of monobutyrin was not affected at all. With purified serum esterase, hydrolysis of acetylcholine was 40 per cent inhibited while hydrolysis of monobutyrin was inhibited 13 per cent (Table IX).

Thiosulfate prevented the inhibition. When it was added 5 minutes after addition of TBA, there was partial reversal (Table X).

TABLE VI

*Effect of Thiosulfate on the Inhibition of Choline Oxidase by Tris(β-Chloroethyl)amine HCl (TBA)*

| System | $O_2$ uptake | Inhibition |
|---|---|---|
| | *c.mm.* | *per cent* |
| Choline | 234 | — |
| " + $10^{-5}$ M TBA | 58 | 75 |
| " + $10^{-3}$ M $Na_2S_2O_3$ | 211 | — |
| " + $10^{-3}$ M $Na_2S_2O_3$ + $10^{-5}$ M TBA added 15 min. later | 188 | 11 |
| " + $10^{-4}$ M $Na_2S_2O_3$ + $10^{-5}$ M TBA | 80 | 58 |
| " + $10^{-5}$ M TBA + $10^{-3}$ M $Na_2S_2O_3$ added 5 min. later | 153 | 27 |

TABLE VII

*Inhibition of Choline Oxidase by Methyl-Bis(β-Chloroethyl)amine HCl (MBA) ($1 \times 10^{-6}$ M) and Its Prevention by Antidotes*

| Antidote | Concentration | Reactivation |
|---|---|---|
| | *M* | *per cent* |
| $Na_2S_2O_3$ | $10^{-3}$ | 90 |
| $Na_2S_2O_3$ | $10^{-4}$ | 52 |
| $Na_2S_2O_3$ | $10^{-5}$ | 0 |
| BAL | $10^{-3}$ | 30 |
| Urotropin | " | 21 |
| " | $10^{-4}$ | 0 |
| Glutathione | $10^{-3}$ | 17 |
| Thiourea | " | 17 |
| " | $10^{-4}$ | 0 |

*3. Choline Acetylase.*—Another reaction in which choline is one of the reactants is the synthesis of acetylcholine. Feldberg (20) reported that this reaction was inhibited by methyl-bis(β-chloroethyl)amine. This has been confirmed

TABLE VIII

*Effect of Nitrogen Mustards on the Hydrolysis of Acetylcholine by Serum and Brain Esterases*

In Ringer-bicarbonate ($N_2$:$CO_2$ as gas phase) pH, 7.4. Temperature 25°.

| System | TBA | Inhibition | MBA | Inhibition |
|---|---|---|---|---|
| | *M* | *per cent* | *M* | *per cent* |
| *Rat brain* | | | | |
| Inhibitor freshly prepared | $1 \times 10^{-4}$ | 81 | $1 \times 10^{-4}$ | 61 |
| 2 hrs. after | " | 32.5 | " | 69.5 |
| 6 " " | " | 22 | " | 65 |
| *Horse serum* | | | | |
| Purified enzyme Inhibitor, fresh. | $5 \times 10^{-4}$ | 73.4 | $1 \times 10^{-3}$ | 16.5 |
| 2 hrs. after | " | 20.7 | " | 17.0 |
| *Human serum* | | | | |
| Inhibitor, fresh | $1 \times 10^{-4}$ | 63.9 | $5 \times 10^{-4}$ | 46.6 |
| 2 hrs. after | " | 13.2 | " | 41.7 |
| | $1 \times 10^{-3}$ | Complete | $1 \times 10^{-3}$ | 88 |
| | $1 \times 10^{-4}$ | 54 | $1 \times 10^{-4}$ | 26 |
| *Chicken serum* | $1 \times 10^{-4}$ | 58.4 | " | 27 |
| | $5 \times 10^{-4}$ | Complete | $5 \times 10^{-4}$ | 50.8 |
| *Chicken brain* | $1 \times 10^{-4}$ | 57.5 | $1 \times 10^{-4}$ | 32 |
| Inhibitor in $H_2O$, fresh | $5 \times 10^{-4}$ | 80 | $5 \times 10^{-4}$ | 93 |
| " " " at 6 hrs. later. | " | 56 | " | 92 |

TABLE IX

*Effect of Methyl-Bis(β-Chloroethyl)amine HCl (MBA) (0.001 M) on the Activity of Esterases*

Buffer, Ringer-$NaHCO_3$ plus 3 cc. of 0.1 M $CaCl_2$ added to 100 cc. Saturated with $N_2$:$CO_2$. pH, 7.5. Substrate, 0.01 M. Duration of experiment, 20 minutes. Temperature, 38°.

| Experimental conditions | Serum esterase | | Brain esterase | |
|---|---|---|---|---|
| | $CO_2$ output | Inhibition | $CO_2$ output | Inhibition |
| | *c.mm.* | *per cent* | *c.mm.* | *per cent* |
| Acetylcholine | 146 | | 344.5 | |
| " + MBA | 87.4 | 40 | 102.9 | 70 |
| Monobutyrin | 124.8 | | 131.5 | |
| " + MBA | 108 | 13 | 130.8 | None |

in experiments in which acetylcholine synthesis was measured in acetone-dry brain extracts, anaerobically, and in the presence of citrate, adenosinetriphosphate, choline, and boiled yeast extract. Synthesis of acetylcholine was inhibited 70 per cent by $1 \times 10^{-3}$ M of MBA.

*4. Effect of Nitrogen Mustards on Other Enzymes.*—Nitrogen mustards in aqueous, weakly alkaline solutions are very reactive. They react rapidly with —SH groups like cysteine, glutathione, and the —SH groups of denatured albumin, removing half of the —SH groups in 5 minutes (21, 22). They react (pH 8) with a large number of organic compounds of biological importance ($NH_2$ groups and carboxyl groups of amino acids, peptides, and proteins, nicotinic acid, methionine, thiamine, adenylic acid, adenosinetriphosphate, pyridoxine, P organic compounds) (13, 14)), some of them essential for enzyme activity.

TABLE X

*Effect of Thiosulfate on the Inhibition of Choline Esterase by Tris(β-Chloroethyl)amine HCl*

Concentration, $2.5 \times 10^{-4}$ M; thiosulfate, $2.5 \times 10^{-2}$ M. Temperature 38°. Time of experiment, 60 minutes.

| System | $CO_2$ output | Inhibition |
|---|---|---|
| | *c.mm.* | *per cent* |
| Enzyme + AC* | 146.1 | |
| " + AC + $Na_2S_2O_3$ | 139.3 | 5 |
| " + TBA; AC from side arm | 18.7 | 87 |
| " + AC; TBA " " " | 126.5 | 9.6 |
| " + AC + TBA. $Na_2S_2O_3$ 5 min. later | 71.1 | 51 |
| " + AC + $Na_2S_2O_3$. TBA 5 " " | 132.7 | 9 |

* AC, acetylcholine.

TABLE XI

*Effect of Methyl-Bis(β-Chloroethyl)amine HCl on P Exchange Enzymes*

| Enzyme | MBA | Inhibition | Investigator |
|---|---|---|---|
| | *M* | *per cent* | |
| Phosphocreatine phosphokinase | $5 \times 10^{-3}$ | 75 | Cori *et al.* |
| Reduced state | $4 \times 10^{-3}$ | 84 | " " " |
| Oxidized " | " | 33 | " " " |
| Phosphopyruvate phosphokinase | " | 42 | " " " |
| Adenosinetriphosphatase (myosine) | $1 \times 10^{-3}$ | None | Barron *et al.* |
| Adenosinetriphosphatase | $4 \times 10^{-3}$ | 26 | Cori *et al.* |
| Myokinase | " | None | " " " |
| Inorganic pyrophosphatase | $1 \times 10^{-3}$ | 67 | " " " |
| Reduced state | $4 \times 10^{-3}$ | 80 | " " " |
| Oxidized " | " | 87 | " " " |
| Hexokinase | $2.5 \times 10^{-3}$ | 65 | Dixon |
| " | $4 \times 10^{-3}$ | 37 | Cori *et al.* |
| Deuterohexokinase | $1 \times 10^{-2}$ | 55 | " " " |
| Acid phosphatase | $1 \times 10^{-3}$ | 40 | Barron *et al.* |
| Alkaline " | " | 8 | " " " |

This high reactivity of the halogenated alkylamines would make them powerful inhibitors of a large number of enzymes. However, experiments performed by a number of investigators (Peters *et al.* and Dixon *et al.* in England, Cori and this laboratory in the United States) do not support this contention. In fact, if *in vitro* experiments with concentrations above 10 times the lethal

TABLE XII

*Effect of Nitrogen Mustards (0.001 M) on the Activity of Some Enzyme Systems. (Other than P Exchange Enzymes)*

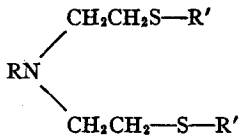
| Enzyme | Substrate | Determination | Inhibition | | Investigators |
|---|---|---|---|---|---|
| | | | TBA | MBA | |
| | | | *per cent* | *per cent* | |
| Choline oxidase | Choline | $O_2$ uptake | Complete | Complete | Barron *et al.* |
| Choline esterase | Acetylcholine | Acid formation, $CO_2$ | " | " | Thompson, Barron *et al.* |
| " " | " | " " " | " | " | Barron *et al.* |
| Betaine aldehyde oxidase | Betaine aldehyde | $O_2$ uptake | " | " | " " " |
| Pyruvate oxidase | Pyruvate | " " and pyruvate utilization | " | " | " " " |
| Adenosine deaminase | Adenosine | $NH_3$ formation | None | None | " " " |
| Uricase | Uric acid | $O_2$ uptake | | 27 | " " " |
| Phosphoglyceraldehyde dehydrogenase | Phosphoglyceraldehyde | DPN reduction | None | None | " " " |
| Polyphenol oxidase | Catechol | $O_2$ uptake | " | " | " " " |
| Carbonic anhydrase | $NaHCO_3$ | $CO_2$ output | " | " | " " " |
| Enolase | | | " | " | Cori *et al.* |
| Isomerase | | | " | " | " " " |
| Phosphoglucomutase | | | " | " | " " " |
| Lactic dehydrogenase | Lactate | DPN reduction | " | " | Barron *et al.* |
| Succinic oxidase | Succinate | $O_2$ uptake | " | " | " " " |
| Cytochrome " | Cytochrome C | Reduced cytochrome C oxidation | " | " | " " " |
| Diamine " | Histamine | $O_2$ uptake | " | " | " " " |
| Transaminase | Glutamate + pyruvate | Alanine | " | " | " " " |
| Carboxylase | Pyruvate | $CO_2$ output | " | " | " " " |
| Arginase | Arginine | $NH_3$ formation | " | " | " " " |
| Pepsin | Hemoglobin | Tyrosine | 6 | 6 | " " " |
| Trypsin | " | " | 11 | 11 | " " " |
| Papain | " | — | | None | Dixon |
| *d*-Amino acid oxidase | Alanine | Pyruvate | 26 | " | Barron *et al.* |

dose (about $1 \times 10^{-4}$ M) are discarded, the only enzyme systems inhibited were choline oxidase, acetylcholine esterase, and pyruvate oxidase.

Cori and his coworkers (24) have studied in detail the effect of methyl-bis-(β-chloroethyl)amine HCl upon the enzymes dealing with phosphorus exchange. They found that phosphocreatine phosphokinase, phosphopyruvate phosphokinase, and inorganic pyrophosphatase were considerably inhibited with $4 \times 10^{-3}$ M. We found that acid phosphatase was inhibited 40 per cent, while

adenosinetriphosphatase and alkaline phosphatase were not affected with $1 \times 10^{-3}$ M (Table XI). It could be concluded from these data that none of these enzymes is inhibited at concentrations around the lethal dose. However, Cori found that in certain tissues of animals injected with methyl-bis-(β-chloroethyl)amine HCl, or the ethylenimine transformation product there was partial inhibition of inorganic pyrophosphatase, phosphocreatine phosphokinase, and hexokinase. It may therefore be concluded that these three P exchange enzymes are inhibited by nitrogen mustards at concentrations around the lethal dose.

In Table XII are given data on the effect of TBA and MBA on other enzyme systems. It is surprising to find such highly reacting substances so ineffective on the activity of a large number of enzymes. For example, in spite of the high reactivity of the halogenated alklyamines with —SH groups with which they give compounds of the type succinoxidase, adenosinetriphosphatase,




papain, *d*-amino acid oxidase, phosphoglyceraldehyde dehydrogenase, carboxylase, transaminase—all requiring the presence of —SH groups for activity—were not affected at all by 0.001 M.

The findings that the $NH_2$ groups of thiamine, nicotinic acid amide, and pyridoxine combine with nitrogen mustards have not been confirmed in enzyme experiments where nitrogen mustard and enzyme were kept at pH 7. The activity of yeast carboxylase which requires diphosphothiamine, of lactate dehydrogenase which requires diphosphopyridine nucleotide, and of transaminase which requires pyridoxal (25) was not affected by nitrogen mustards.

## DISCUSSION

The experiments presented in this paper on inhibitions of enzyme reactions by nitrogen mustards show that these compounds act either as structural inhibitors or through combination with different reactive groups of the protein moiety of the enzyme. The halogenated alklyamines in aqueous neutral solutions are rapidly transformed into the quaternary ethylenimonium derivatives which are structurally similar to choline. Inhibition of the oxidation of choline by choline oxidase, of the hydrolysis of acetylcholine by the esterase, and of the synthesis of acetylcholine by choline acetylase, is due in every case to structural inhibition; *i.e.*, inhibition due to combination of the nitrogen mustard derivative with the protein moiety of the enzyme at the same side chains where combination of choline or acetylcholine and protein takes place. Indication that such a specific combination occurs was given by the lack of action of methyl-bis-

(β-chloroethyl)amine on the hydrolysis of monobutyrin, by prevention of inhibition on previous addition of choline or acetylcholine, and partial reversal on addition of choline after nitrogen mustard addition to the enzyme. There is, however, a difference between the other well known structural inhibitors and nitrogen mustards. In fact, while inhibition of succinoxidase by malonate depends on the ratio of malonate: succinate and not on the absolute amount of malonate (see reviews by Wooley (25–27), by Roblin (28), and by Welch (29)), nitrogen mustard inhibition occurs with very small concentrations and is not reversed completely. This must be attributed to strong association between the nitrogen mustard and the protein. This strong association was strikingly demonstrated when different substances known to react easily with nitrogen mustards were used to prevent inhibition. Thiosulfate, the most effective preventive agent, had to be added at a concentration 1000 times as great to prevent inhibition. The nitrogen mustards must, thus, be looked upon as a new subdivision of the general structural inhibitors, effective at low concentrations because of seemingly irreversible combination with the protein at the same specific side chain where combination with choline takes place.

The other enzyme inhibitions studied by Cori, as well as those found by Dixon and by us (inhibition of phosphokinases, pyruvate oxidase, etc.) have a different mechanism of action and require greater concentrations of inhibitor. They must be due to interaction of certain groups of the proteins ($NH_2$ groups, carboxyl groups, —SH groups) with the highly reactive halogen groups of nitrogen mustard.

## SUMMARY

Nitrogen mustards are powerful inhibitors for choline oxidase, acetylcholine esterase, and choline acetylase, half-inhibition of the first enzyme being produced with concentrations around $1 \times 10^{-6}$ M; *i.e.*, ten times less than the $LD_{50}$ values. Acetylcholine esterase and choline acetylase required higher concentrations. This inhibition seems to be due to the structural similarity of the ethylenimonium derivatives with choline and acetylcholine. A list of enzyme systems inhibited by nitrogen mustards is given.

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