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THE MECHANISM OF TOXICITY OF SYMMETRICALLY SUBSTITUTED DITHIOOXAMIDES

by
James Gregory Miller

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THE MECHANISM OF TOXICITY OF SYMMETRICALLY SUBSTITUTED DITHIOOXAMIDES

by James Gregory Miller

The objective of this research was to elucidate the mechanism of toxicity of substituted dithiooxamides in terms of altered biochemical activity.

The toxicity of dithiooxamide, N,N'-bis(2-hydroxyethyl)dithiooxamide, N,N'-di-secondary butyldithiooxamide and N,N'-diallyldithiooxamide was studied. N,N'-di-secondary butyldithiooxamide and N,N'-diallyldithiooxamide were shown to be the toxic members of the series. Their toxicity is characterized by a latent period at low dosages, ventricular fibrillation in dogs, cats, and rabbits, and central nervous system disturbances in all species. N,N'-di-secondary butyldithiooxamide exhibited an extreme cumulative toxicity in rabbits.

Homogenate and mitochondrial preparations of rat brain and liver were used to study the biochemical aspects of dithiooxamide toxicity. Purified preparations of liver alcohol dehydrogenase and glucose-6-phosphate dehydrogenase were also utilized. The study of these enzyme systems was also supplemented by the determination of coenzyme I levels in the liver of treated rats.

The toxic members of the series inhibited all coenzyme I-linked apoenzymes in both mitochondrial and purified preparations. The relatively nontoxic compounds did not exhibit any <u>in vitro</u> action.

The inhibition of coenzyme I-linked mitochondrial systems was competitive in nature and could be reversed by the addition of coenzyme I.

The coenzyme I content was markedly reduced in the liver of rats that had received an LD_{50} dose of N,N'-di-secondary butyldithiooxamide. The initial reduction consisted entirely of a decrease in the oxidized coenzyme I. This resulted in a reversal of the normal ratio of oxidized to reduced coenzyme I. Seventy-two hours after the administration of the compound, the content of reduced coenzyme I was decreased, restoring within normal limits the ratio of oxidized to reduced forms. The content of total coenzyme I was still very low.

The conclusion is drawn that coenzyme I is a pertinent characteristic of apoenzymes inhibited <u>in vitro</u> by the potent dithiooxamides. The altered levels of coenzyme I in treated animals indicate that this is probably an <u>in vivo</u> site of action of the compounds. The mechanism of toxicity of symmetrically substituted dithiooxamides is the occupation of coenzyme I sites of apoenzymes.

ACKNOWLEDGMENT

The author wishes to express his gratitude to Dr. Theodore M. Brody for his guidance and instruction throughout the biochemical phases of this problem.

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CHAPTER I

INTRODUCTION

The importance of enzymes in the activity of tissues is no longer a subject of controversy. Thousands of chemical reactions enable living tissue to carry out its varied and specialized functions. Many of these chemical reactions are not spontaneous because of the discrepancies in energy levels of the reactants and their products. Enzymes not only initiate, but often control, the rate of these reactions. At this period of almost universal acceptance of the vital role of enzymes in tissue functions, it is well to bear in mind that not all physiological events are of necessity enzymatic in nature. Certainly physicochemical phenomena also play a role.

The isolation, study, and integration of the function of enzymes is a rapidly expanding area of research and knowledge. In mammalian systems, the greatest advances in the elucidation of the integrated function of enzymes exists in the Krebs citric acid cycle and its related systems. Surely, there are vast areas of intermediary metabolism, undoubtedly vital to highly specialized cell functions, which are as yet undiscovered. To use an analogy, biochemical research has delineated a busy central area of chemical activity in the cell, vital to and responsible for many of its known functions. Far from discounting the possible existence of other important areas, our present knowledge makes their existence even more likely.

The relationship between drugs and enzymes has come into prominence as a means of explaining the action of such compounds. Certainly when a compound exerts a profound biological effect, the

interference with some vital enzyme function is a natural and attractive hypothesis. There are relatively few compounds whose in vivo action has been definitely linked with their relationship to an enzyme. The relationship of physostigmine to cholinesterase, cyanide to cytochrome oxidase, and fluorocitrate to aconitase rank as classic examples.

Pharmacological research cannot await the mapping of new areas of biochemical knowledge in which drug activity may lie, but must provide the tools, in the form of new potent drugs, the clues, in the form of some discernible altered function, with which new areas of enzyme research may be investigated.

One of the major problems in the application of the results of biochemical research on drug action, is to make sure that the data obtained by means of the available and increasingly ingenious methods, may be validly transposed into terms of cellular function. Welch and Bueding (1946) have outlined some basic criteria which aid in the transposition of in vitro biochemical data to the intact animal. These criteria have met with wide acceptance, assuming a role which is similar to that performed by Koch's postulates in the nineteenth century in the field of infectious disease research. Welch and Bueding suggest that before the in vitro action of a drug be considered as also the in vivo action the following criteria be met:

1. The concentrations of the drug or poison which produce the effect in vitro and which obtain at the anatomical site of action should be closely similar.

2. If a drug or poison exerts its effect primarily on a specific tissue in vivo, either the effect on this tissue in vitro should be more pronounced than the effects on other tissues, or the system inhibited must be shown to have more functional significance in this than in other tissues.

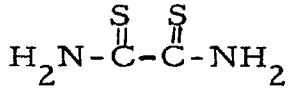
3. Among structurally related compounds there should be close parallelism between the pharmacological activities in vitro and

in vivo: all drugs of the same chemical series which are active in vivo must also be active in vitro, and those inactive in vivo must also be inactive in vitro, unless the discrepancy can be accounted for by poor absorption, by inadequate distribution to or penetration of the cells involved, by too rapid excretion, or by metabolic alteration.

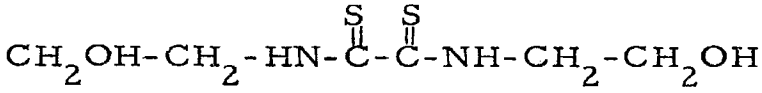
The application of these principles to this problem is discussed in Chapter V.

Four compounds form the subject of this investigation:

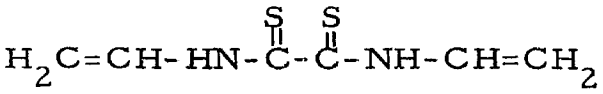
(a) Dithiooxamide



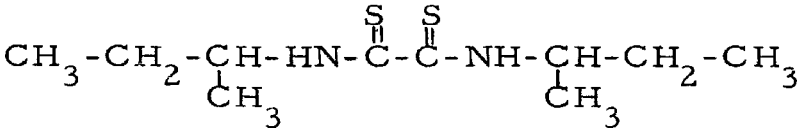
(b) N,N'-Bis(2-Hydroxyethyl)dithiooxamide



(c) N,N'-Diallyldithiooxamide



(d) N,N'-di-Secondary Butyldithiooxamide



N,N'-di-secondary butyldithiooxamide was used for most of the experiments; but, N,N'-diallyldithiooxamide was substituted in spectrophotometric experiments. N,N'-bis(2-hydroxethyl)dithiooxamide is a water-soluble compound. The other compounds are water-insoluble, but slightly soluble in ethanol.

Some of the compounds proved to be highly toxic in small dosages. Consequently, it was postulated that the mechanism of their toxicity might be referrable to an action on one or more enzyme systems. After delineating the nature of the toxicity, an attempt was made to explain their action by a biochemical approach.

CHAPTER II

TOXICITY

A. Ventricular Fibrillation

The toxic compounds characteristically produce death in dogs, cats, and rabbits by ventricular fibrillation. At the LD_{50} dose level, cardiac death is preceded by a latent period, frequently as long as six to nine hours; but with higher dosages the latency may be abolished. Ventricular fibrillation is sometimes heralded by various cardiac arrhythmias, such as extrasystoles or a multifocal ventricular tachycardia. A Sanborn Viso-Cardiette electrocardiograph was used to record the cardiac arrhythmias in dogs, cats, and rabbits. All records were made with a standard lead II. A control electrocardiogram was made following the preparation of the barbitalized animal for arterial blood pressure recordings. A continuous electrocardiogram accompanied the administration of the dithiooxamide, subsequent to which an intermittent record was made, as indicated by a continuous monitoring of the blood pressure and recording stylus. Some of these electrocardiograms are presented in Figures 1 to 5 to illustrate the types of cardiac arrhythmias encountered.

Rats, because of the small size of the heart, do not readily undergo ventricular fibrillation. However, cardiac irregularities, such as skipped beats and extrasystoles, are readily detected following the intraperitoneal administration of an LD_{50} dose of N,N'-di-secondary butyldithiooxamide. Most deaths occur within 24 hours of administration of the compound, and are frequently a result of cardiac arrest.

Figure 1. Dog. A. Control electrocardiogram lead II.[1] Subsequent to this 10 mgm./Kgm. of N,N'-di-secondary butyldithiooxamide were administered intravenously.

B. Thirty minutes after administration of the compound, absence of P wave, depression of ST segment.

C. Prolongation of QRS complex, inverted T wave, absence of P wave.

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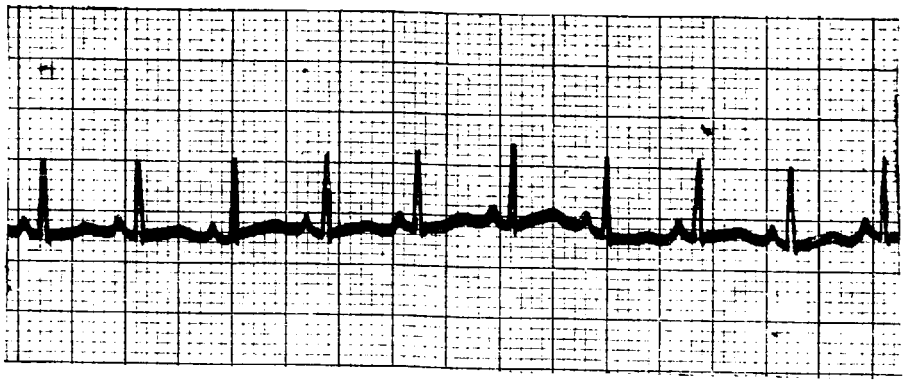
[1] In Figures 1 to 5, each heavy vertical line represents a time interval of 0.2 second.

A



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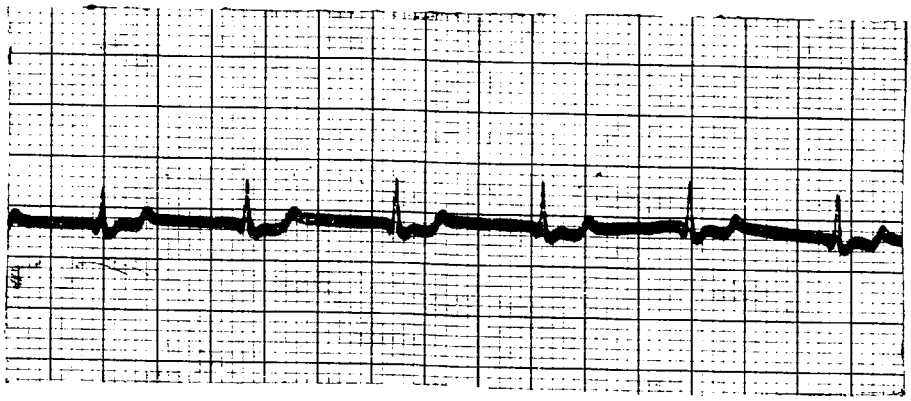

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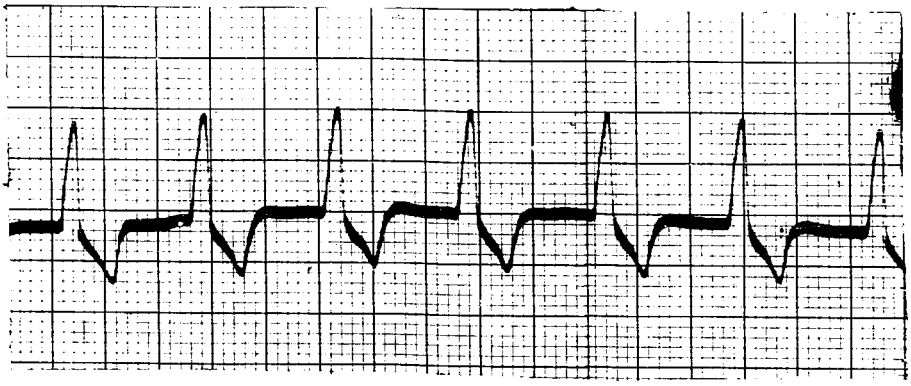


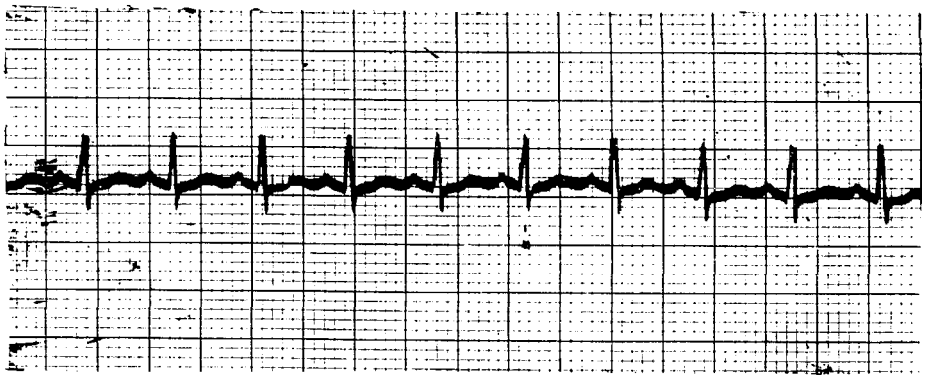
FIG. 1

Figure 2. Dog. A. Control electrocardiogram lead II. Subsequent to this 10 mgm./Kgm. of N,N'-di-secondary butyldithiooxamide were administered intravenously.

B. Fifty minutes after administration of the compound, this record shows a bigeminal rhythm.

C. Ventricular fibrillation.

A



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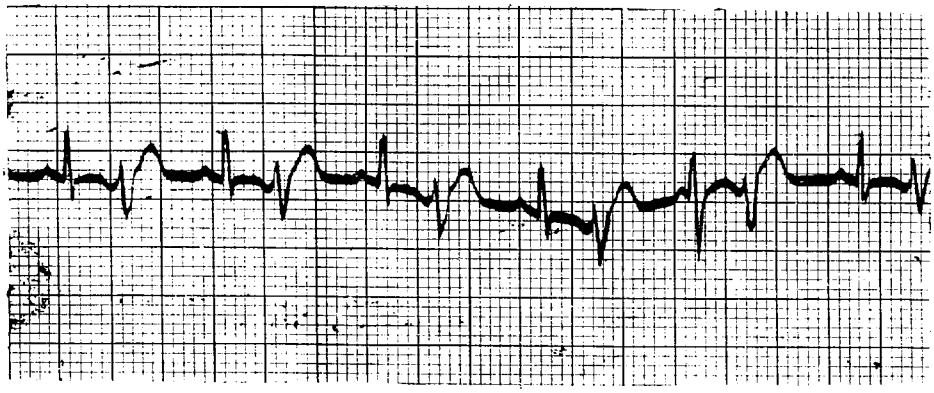

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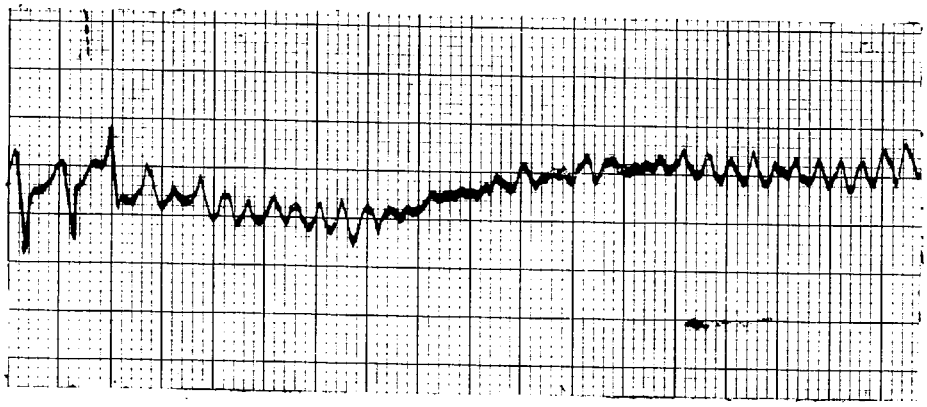
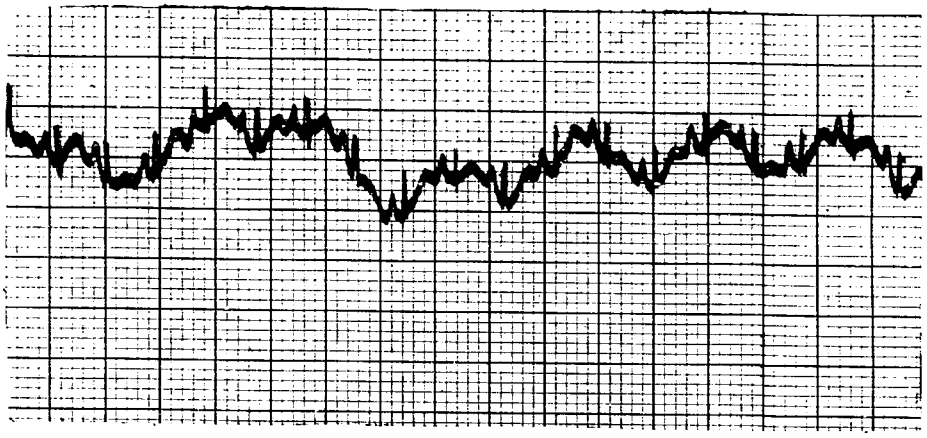


FIG. 2

Figure 3. Rabbit. A. Control electrocardiogram lead II, showing marked respiratory artifact. Subsequent to this 10 mgm./Kgm. of N,N'-di-secondary butyldithiooxamide were administered intravenously.

B. One hundred and twenty minutes after administration of the compound, this record shows a multifocal ventricular tachycardia.

A



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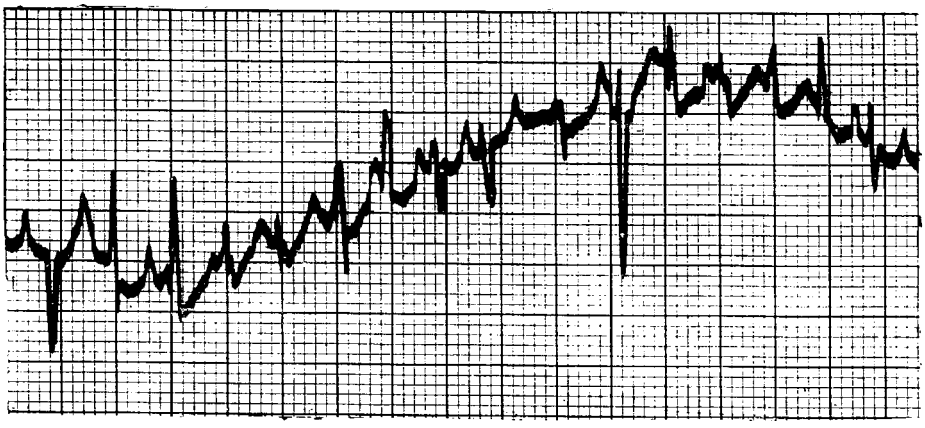
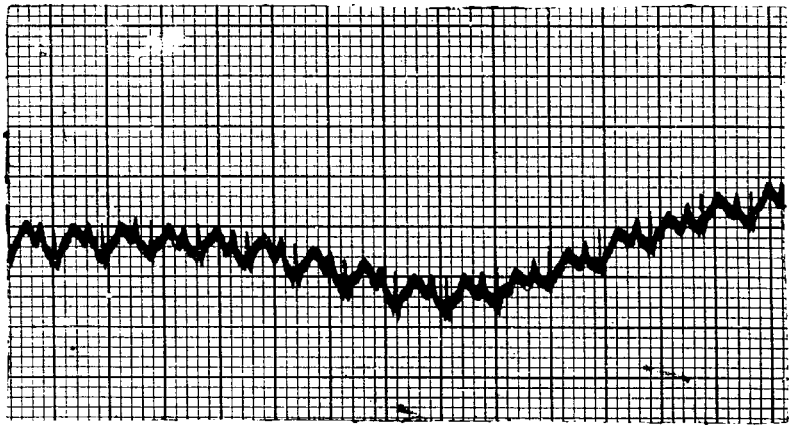


FIG. 3

Figure 4. Rabbit. A. Control electrocardiogram lead II. Subsequent to this 10 mgm./Kgm. of N,N'-di-secondary butyldithiooxamide were administered intravenously.

B. This record shows multifocal ventricular rhythm.

A



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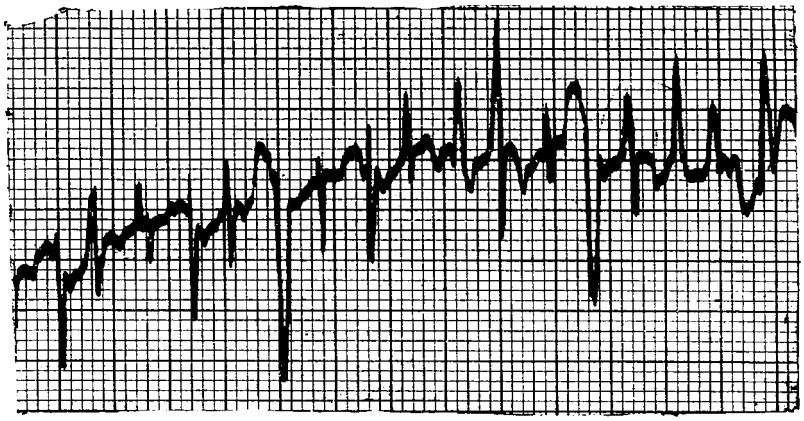
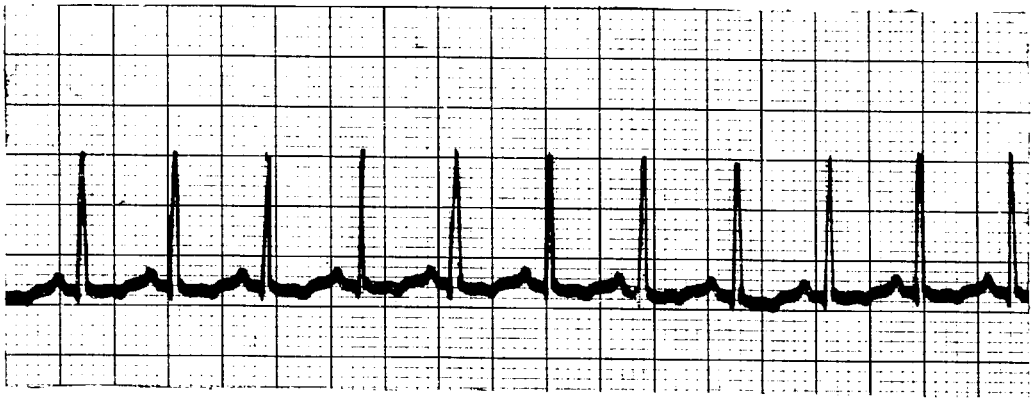


FIG. 4

Figure 5. Cat. A. Control electrocardiogram lead II. Subsequent to this 30 mgm./Kgm. of N,N'-di-secondary butyldithiooxamide were administered intravenously.

B. Ventricular fibrillation.

A



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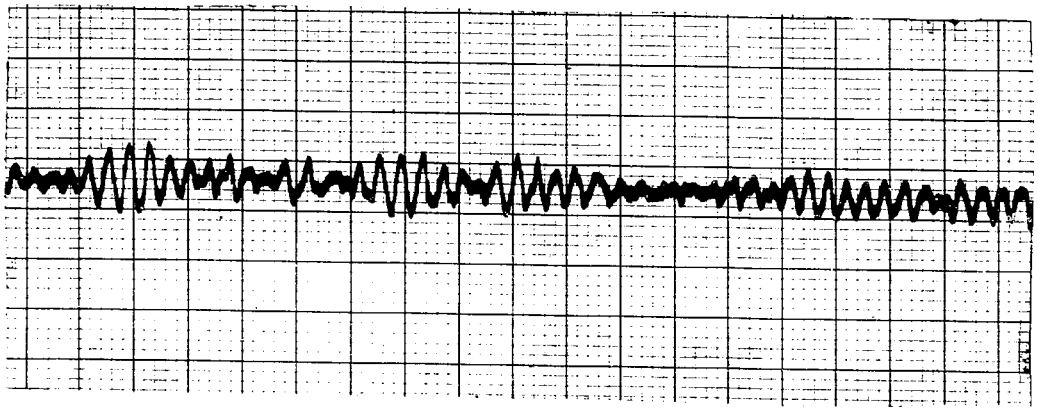

FIG. 5

B. Blood Pressure

The blood pressure response to these compounds is not striking; although there is an immediate fall in blood pressure when 10 mgm./Kgm. of N,N'-di-secondary butyldithiooxamide is injected intravenously. This response is attributed to Dowanol 50B®,[1] which is used as a solvent for the compound. Slow infusion of the Dowanol 50B® solution averts the fall in blood pressure. During the latent period following administration of the potent dithiooxamides, there is often a slow steady rise in blood pressure, not exceeding 25 mm. of mercury above the preinjection pressure. There is no observable change in pulse rate or in the electrocardiographic record prior to the onset of definite cardiac arrhythmias or ventricular fibrillation. The administration of 10 mgm./Kgm. intravenously of N,N'-di-secondary butyldithiooxamide to a barbitalized dog does not block or enhance the blood pressure response to acetylcholine or to epinephrine.

C. Central Nervous System

These compounds have definite effects on the central nervous system which are observable in all species. Convulsions of a clonic-tonic nature may be observed in nonanesthetized dogs and cats which have received N,N'-di-secondary butyldithiooxamide. These convulsions usually appear 20 to 24 hours after administration of the compound; and, therefore, have a considerably longer latent period than is seen in the event of cardiac death. Noise or excitement precipitate the clonic-tonic seizures. Death, apparently the result of central nervous

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[1] Dowanol 50B® is the registered trade mark for dipropylene glycol methyl ether which was obtained from the Dow Chemical Co.

system depression, usually occurs 24 hours after the start of the convulsions. There have been no observed recoveries following the onset of either cardiac or central nervous system toxicity.

Most rats exhibit some central nervous system derangement. All rats are profoundly depressed during the first 24 hours after administration of the compound, but in the next 24 hours the survivors undergo an almost complete behavioral change. They become hyperexcitable, aggressive, and sometimes even exhibit clonic-tonic convulsions. At this time the only observable pathological change is petechial hemorrhages in the walls of the lateral ventricles of the cerebrum. Rats which survive the first 48 hours undergo a severe weight loss over the next five days. This weight loss has on occasion amounted to 50 per cent of the initial weight of the animal. The lost weight is eventually regained over a period of 14 to 20 days.

D. Potentiation by Fluoroacetate

Because of the similarity of the gross toxicity between the dithiooxamides and fluoroacetate, experiments were designed to determine whether fluoroacetate potentiated these compounds. A dose of 2 mgm./Kgm. of sodium fluoroacetate combined with 3 mgm./Kgm. of N,N'-di-secondary butyldithiooxamide produces death in all rats. Either compound administered alone in these doses failed to produce any deaths.

E. Cumulative Toxicity

N,N'-di-secondary butyldithiooxamide exhibits a significant cumulative toxicity in rabbits. This is indicated by the results of

a series of experiments (see Table 1) in which 1 mgm./Kgm. was administered, intravenously, to adult rabbits. The dosage was repeated at intervals varying from thirty minutes to sixteen days. The total dose at death did not vary materially over the total time of administration, whether it was two hours or 48 days.

Table 1. The cumulative toxicity of N,N'-di-secondary butyldithiooxamide in adult rabbits.

1 mgm./Kgm. Administered IV every:	Total Dose at Death in Four Animals (mgm./Kgm.)
0.5 hour	4
1.0	4
2.0 hours	4
4.0	4
12.0	4
1 day	4
2 days	4
4	6
8	6
16	no deaths at 6 mgm./Kgm.

F. Determination of the Intravenous LD_{50} in Adult Albino Rabbits

The LD_{50} of N,N'-di-secondary butyldithiooxamide in rabbits weighing over two kilograms was determined by the method of Weil and Smyth (1953). Five rabbits were used at each dosage level. The dosage rates differed from one another by a factor of 1.26. A 1 per cent solution of N,N'-di-secondary butyldithiooxamide in

Dowanol 50B® was administered slowly via an ear vein. The rabbits were observed for a period of one week. A separate group of five rabbits received a dosage of Dowanol 50B® equivalent to that contained in the highest dosage of N,N'-di-secondary butyldithiooxamide. This group served as controls. The LD_{50} for adult albino rabbits was found to be 2.33 mgm./Kgm. All animals succumbed to a dose of 5.0 mgm./Kgm. (see Appendix 1).

G. Species Variation

A comparison of approximate LD_{50}'s in various species indicates no wide variation among mammals. Frogs survived 50 mgm./Kgm. of N,N'-di-secondary butyldithiooxamide administered into the ventral lymph sac; for one month after they exhibited clonic-tonic convulsions, which could be precipitated by noise or excitement.

Young rabbits weighing 500 grams exhibited definite resistance to the effect of N,N'-di-secondary butyldithiooxamide. They required 7.5 mgm./Kgm. to produce death. This resistance was not encountered in puppies or young rats.

Table 2. The approximate LD_{50} of N,N'-di-secondary butyldithiooxamide in various species.

Species	LD_{50} in mgm./Kgm.
Rabbit	2.3
Dog	5 - 10
Cat	5 - 10
Rat	5.0
Guinea pig	5 - 10
Frog	50.0

H. Routes of Administration

N,N'-di-secondary butyldithiooxamide produces death when administered by various routes. For instance, oral dosages to rats at 6 mgm./Kgm., application to the shaved abdomen of rabbits for 24 hours, or 10 minutes' contact in a rabbit's eye results in death. There is a marked tissue reaction, with phlebitis and necrosis frequently resulting from intravenous administration.

CHAPTER III

BIOCHEMICAL STUDIES

The nature of the acute toxicity of the dithiooxamides indicates that a severe interruption of biological energy production or utilization has taken place. The problem of first locating, then studying, the site of action of these compounds was approached through the use of biochemical techniques. These techniques included the utilization of homogenate and mitochondrial preparations of brain and liver, as well as purified enzyme systems.

A. Methods

1. Equipment

Animals. Sprague-Dawley rats weighing 125 to 200 grams were used throughout these experiments.

Homogenizer. The design of the homogenizer was similar to that of Potter and Elvehjem (1936). The pestle was made of lucite rather than glass, and the mortar consisted of a pyrex test tube, 20 × 140 mm, which was fitted to the pestle.

Motor. A Cenco (Central Scientific Co.) variable-speed stirrer, with a conical drive, was used to power the pestle.

Centrifuge. Centrifugations were carried out in Lusteroid tubes at 0° C. in the conical head No. 296 of the International Refrigerated centrifuge, Model PR-1.

Spectrophotometer. All spectrophotometric determinations were carried out in a Beckman Spectrophotometer, Model DU, using Corex quartz cells, with a light path of 1 cm.

2. Materials

Adenosine triphosphate. Dipotassium adenosine triphosphate (Pabst Laboratories and Sigma Chemical Co.) was used.

Cytochrome c. The cytochrome c was prepared from horse heart (Sigma Chemical Co.).

Diphosphopyridine nucleotide. A purified crystallized preparation (Pabst Laboratories) was utilized.

Triphosphopyridine nucleotide. The sodium salt of a 96.5 per cent pure preparation (Sigma Chemical Co.) containing $2H_2O$ per molecule was utilized.

Glycylglycine. Glycylglycine was obtained from the Sigma Chemical Co.

Glucose-6-phosphate. The sodium salt of glucose-6-phosphate was obtained from the Sigma Chemical Co.

dℓ-Isocitric acid. The trisodium salt was obtained from the H. M. Chemical Co.

α-Ketoglutaric acid. α-Ketoglutaric acid from the Sigma Chemical Co. was used.

ℓ-Malic acid (active). ℓ-Malic acid was obtained from the Nutritional Biochemical Corporation.

Citric acid. A chemically pure grade of citric acid from the Baker Chemical Co. was used.

ℓ-Glutamic acid. ℓ-Glutamic acid was obtained from the Nutritional Biochemical Corporation.

Sodium succinate. A reagent grade of sodium succinate was obtained from the Mallinckrodt Chemical Works.

Octanoate. Octanoate was made by redistillation of Eastman Kodak Co. octanoic acid and preparation of the sodium salt.

Ethanol. A 95 per cent preparation of ethanol was used.

Choline. Choline chloride (Eastman Kodak Co.) was used.

Tyramine. The hydrochloride salt was obtained from Eastman Kodak Co.

Enzymes:

Glucose-6-phosphate dehydrogenase. Glucose-6-phosphate dehydrogenase, type II, 0.82 units[1]/mgm. was obtained from the Sigma Chemical Co.

Hexokinase. A lyophilized preparation was obtained from Pabst Laboratories.

Alcohol dehydrogenase. A recrystallized preparation containing 22 mgm./ml. of alcohol dehydrogenase was obtained from the Sigma Chemical Co.

3. Methods

a. Preparation of homogenates

Liver and brain homogenates were prepared according to the method described by Potter (1936), using 0.25 M sucrose as the homogenization medium. Rats were decapitated and the tissues to be studied removed as quickly as possible and placed on crushed ice.

---

[1] A unit of enzyme is defined as that amount which causes an initial change in optical density of 1.000 per minute (room temperature 23° to 25° C.) under conditions described by Kornberg and Horecker (1955).

A sample of predetermined size was weighed. In experiments in which the brain was to be studied, only the cerebral hemispheres were used. The weighed tissue was then cut into small pieces with scissors, and approximately one gram at a time was placed in the homogenizer tube along with 10 ml. of chilled 0.25 M sucrose. After homogenization the final concentration of the homogenate was adjusted to 10 per cent in cold 0.25 M sucrose.

b. Preparation of mitochondria

Liver mitochondria were prepared according to the method outlined by Hogeboom, Schneider, and Palade (1948). A 10 per cent tissue homogenate, prepared as previously described, was centrifuged at 1,000 × "g"[1] for 10 minutes at 0° C. The supernatant was poured off into Lusteroid tubes and recentrifuged at 12,000 × "g" for 15 minutes at 0° C. The sediment from this centrifugation contained the mitochondria. The supernatant was discarded, the sediment resuspended in cold 0.25 M sucrose, and the preceding centrifugation repeated. The sediment obtained consisted of washed mitochondria which were homogenized to insure even distribution. The mitochondria were resuspended in a volume of cold 0.25 M sucrose equivalent to the original wet weight of the tissue used. Brain mitochondria were prepared by a modification of the above method as described by Brody and Bain (1952).

c. Manometric experiments

All experiments were conducted at 37° C. The following incubation medium was used in experiments in which the oxygen uptake of homogenates or mitochondria was measured:

0.3 ml. of glycylglycine buffer, 0.05 M, pH 7.4; + 0.1 M KCl + 0.08 M $MgCl_2$

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[1] "g" is the designation used for the relative centrifugal force acting on the center of the Lusteroid tube, where "g" = .00001118 × r × N^2. r = rotating radius in cm. N = rotating speed in r.p.m.

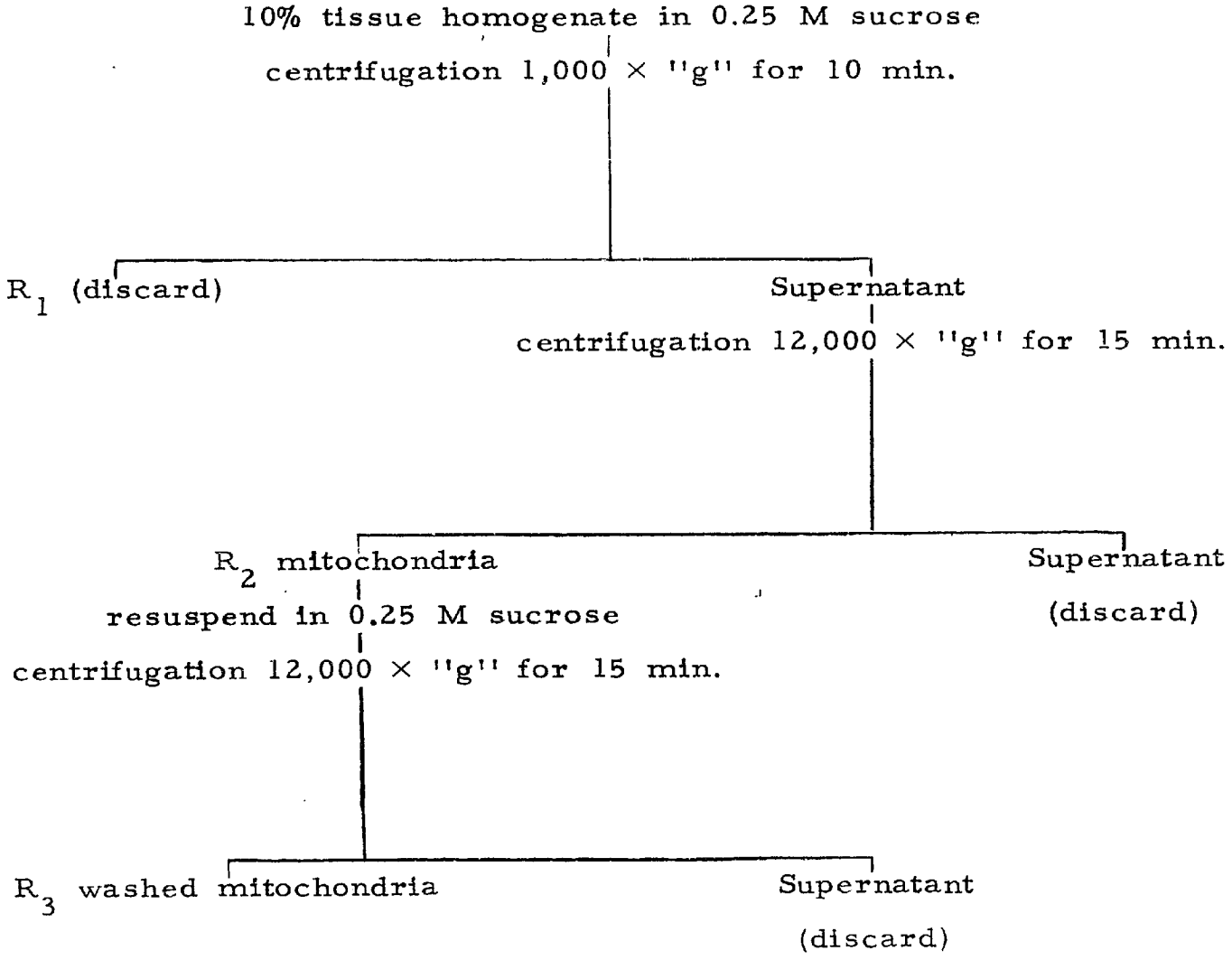


Figure 6. Preparation of liver mitochondria.

0.4 ml. phosphate buffer, 0.1 M, pH 7.4
0.1 ml. of cytochrome c, 0.0001 M
0.1 ml. of adenosine triphosphate, 0.05 M
substrate
a dithiooxamide, if needed
0.3 ml. of mitochondrial preparation
or 1.0 ml. of 10 per cent tissue homogenate preparation
and an additional volume of 0.25 M sucrose to bring the incubation medium up to a final volume of 2.0 ml.

In the center well, 0.2 ml. of 2N KOH was placed, with papers to absorb carbon dioxide.

(1) Procedure

The incubation medium was placed in the main compartment of Warburg flasks which were imbedded in crushed ice. A few minutes before the addition of the enzyme to the main compartment, 2N KOH was placed in the center well. The vessels were attached to the manometers and placed in a 37° C. bath, where they were allowed to shake for 10 minutes with the stopcocks open. This initial shaking period enabled the flask contents to equilibrate. The stopcocks were then closed, and the "zero" readings taken. Subsequent readings were taken every 10 minutes.

d. Spectrophotometric methods

(1) Reduction of coenzyme I by liver alcohol dehydrogenase

(a) Principle.--Ethanol, with coenzyme I as the electron acceptor, is oxidized by alcohol dehydrogenase to produce acetaldehyde. The formation of reduced coenzyme I may be followed spectrophoto-

metrically at 340 mμ (Bonnichsen and Brink, 1955). Oxidized coenzyme I has negligible absorption at this wave length.

$$\text{Alcohol} + \text{DPN} \underset{\text{ADH}}{\rightleftharpoons} \text{Acetaldehyde} + \text{DPNH} + \text{H}$$

(b) Reagents.--0.1 M glycine sodium hydroxide buffer, pH 9.6.

Coenzyme I stock solution containing 18 to 36 mgm./ml. of pure coénzyme I.

Ethanol 96 per cent.

Alcohol dehydrogenase, solutions containing 1.4 micrograms/ml. and 11.2 micrograms/ml.

Dowanol 50B®.

N,N'-diallyldithiooxamide 10^{-2} M etc., in Dowanol 50B®.

(c) Procedure.--Each cuvette contained:

2.3 ml. of glycine sodium hydroxide buffer, pH 9.6.

0.1 ml. of 96 per cent ethanol.

0.05 ml. of Dowanol 50B®.

0.03 ml. of stock solution of N,N'-diallyldithiooxamide, where applicable.

0.5 ml. of alcohol dehydrogenase solution.

The optical density was read at 340 mμ against a distilled water blank. Coenzyme I was then added, cell contents again mixed, and a "zero" reading taken. Subsequent readings of optical density were made at 1, 2, and 3 minute intervals.

(2) Reduction of coenzyme II by glucose-6-phosphate dehydrogenase

(a) Principle.--Glucose-6-phosphate is oxidized by glucose-6-phosphate dehydrogenase to form 6-phosphogluconate. Coenzyme

II plays the role of electron acceptor in the reaction (Kornberg and Horecker, 1955). The formation of reduced coenzyme II may be detected by the increase in optical density at 340 mμ.

Glucose-6-phosphate + TPN $\rightleftharpoons$ 6-Phosphogluconate + TPNH + H^+
(G-6-p Dehydrogenase)

(b) Reagents.--Glucose-6-phosphate, 0.02M.

Glycylglycine buffer, pH 7.4, 0.04 M.

$MgCl_2$, 0.1 M.

Coenzyme II, 1.3 mgm./ml.

Glucose-6-phosphate dehydrogenase, 0.8 units/ml.

Dowanol 50B®.

Stock solution of N,N'-diallyldithiooxamide in Dowanol 50B®.

(c) Procedure.--Each cuvette contained:

1.0 ml. of glycylglycine buffer.

1.4 ml. of glucose-6-phosphate, 0.1 M.

0.2 ml. of $MgCl_2$, 0.1 M.

0.05 ml. of Dowanol 50B®.

0.03 ml. of N,N'-diallyldithiooxamide stock solution.

0.02 ml. of glucose-6-phosphate dehydrogenase, 0.8 units/ml.

The optical density was read at 340 mμ against a distilled water blank; coenzyme II was then added, the cell contents mixed, and a "zero" reading taken. The optical density was again determined 3 minutes later.

B. Results

1. The Effect of N,N'-di-Secondary Butyldithiooxamide on Endogenous Oxygen Uptake of Brain and Liver Homogenates

Two controls were used in all experiments. These control determinations not only measured the oxygen uptake in the presence of the incubation medium and enzyme, but also determined the effect, if any, of the Dowanol 50B®. The addition of 0.05 ml. of stock solution of 4×10^{-2}M N,N'-di-secondary butyldithiooxamide to 1.95 ml. of incubation medium, gave a final concentration of 1×10^{-3}M of the compound. Similarly, 0.05 ml. of Dowanol 50B® was added to a like volume of incubation medium to constitute the "solvent control."

N,N'-di-secondary butyldithiooxamide, at a concentration of 1×10^{-3}M, markedly inhibited the endogenous oxygen uptake of brain and liver homogenates. Dowanol 50B® had only a minor effect, so that the inhibition was due to the compound and not to the solvent.

Table 3. The effect of 1×10^{-3}M N,N'-di-secondary butyldithiooxamide on tissue homogenate respiration.

Tissue	Respiration in Microliters O_2/hour		
	Control Endogenous Respiration	Dowanol 50B® Control	1×10^{-3}M N,N'-di-Secondary Butyldithiooxamide
Brain	109	100	22
Liver	238	206	61

2. The Effect of N,N'-di-Secondary Butyldithiooxamide on the Oxygen Uptake of Mitochondrial Enzymes

In view of the experiments indicating an inhibition of oxygen uptake in brain or liver homogenates, the mitochondrial enzyme systems were examined to determine the site and nature of this inhibition.

It was important to use a quantity of enzyme that was linearly related to oxygen uptake, in order that any alteration in enzyme function would be reflected in the oxygen consumption. To determine the relationship between amount of enzyme and oxygen consumption, an experiment was performed using varying amounts of liver mitochondrial enzymes. Glutamate was used as the substrate for this experiment.

Oxygen uptake was a linear function of mitochondrial concentration in the range of 120 to 900 mgm. equivalent wet weight of tissue. As a result of this experiment, 300 mgm. equivalent wet weight of tissue was used in most experiments with mitochondrial enzymes.

For the purpose of organization, the various enzyme systems were classified according to the type of electron transport with which they were associated.

a. Group I

An electron transport system consisting of:

→LTPP→Co A→Co I→Flavoproteins→Cytochrome System→Oxygen

Some enzyme systems for which this pathway has been described are:

(a) Octanoic acid oxidase.

(b) Pyruvic oxidase.

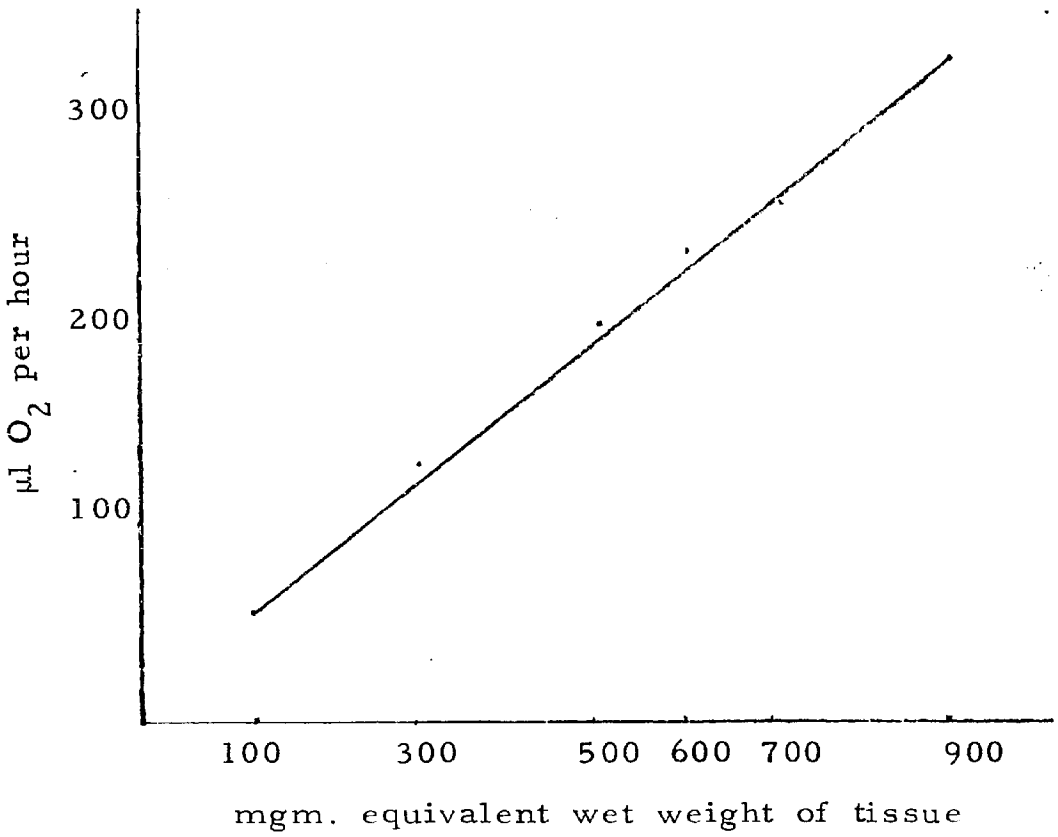


Figure 7. The relationship between oxygen uptake and liver mitochondria concentration.

(c) Alpha-ketoglutaric oxidase.

In the experiments with the above enzyme systems, alpha-ketoglutarate and pyruvate were used as substrates in a concentration of 1×10^{-2}M. Sodium octanoate was used as a substrate in a concentration of 3×10^{-3}M. Malate was added to both the pyruvic oxidase and octanoic oxidase systems in a concentration of 1×10^{-4}M, as a "sparker" to initiate oxidation (Kennedy and Lehninger, 1948).

The oxygen uptake of these enzyme systems was inhibited by N,N'-di-secondary butyldithiooxamide, at concentrations of 1×10^{-4}M to 5×10^{-4}M. Typical experiments showing the inhibitory effect on these systems are shown in Table 4.

Table 4. Effect of N,N'-di-secondary butyldithiooxamide on oxygen uptake of mitochondria utilizing octanoate, pyruvate, or alpha-ketoglutarate.

Tissue	Substrate	Microliters O_2/hour/300 mgm. Equivalent Wet Weight of Tissue				
		Control	Dowanol 50B® Control	Compound Conc./$\times 10^{-4}$M		
				1	5	10
Brain	Pyruvate	166	126	106	24	0
Liver	Pyruvate	224	224	187	72	0
Brain	Alpha-ketoglutarate	190	139	92	13	5
Liver	Octanoate	252	240	168	20	0

b. Group 2

An electron transport system consisting of:

→Co I→Flavoproteins→Cytochrome System→Oxygen

Some enzymes associated with this type of electron transport are:

(a) Malic dehydrogenase.

(b) Aconitase.

(c) Glutamic dehydrogenase.

(d) Choline oxidase.

The oxygen uptake of these mitochondrial enzyme systems was also inhibited by N,N'-di-secondary butyldithiooxamide. Typical experiments showing the effect of this compound on the above systems are shown in Table 5.

Table 5. Effect of N,N'-di-secondary butyldithiooxamide on oxygen uptake of mitochondria utilizing malate, citrate, glutamate, or choline.

Tissue	Substrate	Microliters O_2/hour/300 mgm. Equivalent Wet Weight of Tissue				
		Control	Dowanol 50B® Control	Compound Conc./× 10^{-4}M		
				1	5	10
Brain	Malate	95.7	61	54	0	0
Brain	Glutamate	148	114	73	22	14
Liver	Glutamate	262	260	261	131	9
Liver	Citrate	313	312	271	77	0
Liver	Choline	126	108	90	37	20

The inhibition of the various coenzyme I dependent systems in groups 1 and 2 by N,N'-di-secondary butyldithiooxamide follows a similar pattern. This is illustrated by the data presented in Figure 8, in which the inhibition of pyruvic oxidase and aconitase are compared.

c. Group 3

Isocitric dehydrogenase is an enzyme associated with an electron pathway consisting of:

→Co II→Flavoproteins→Cytochrome System→Oxygen

Isocitric dehydrogenase is adsorbed on mitochondria, and is largely removed from the washed preparation. Therefore, the activity of isocitric dehydrogenase was examined in a rat liver homogenate as well as in washed mitochondria, utilizing the equivalent of 600 mgm. of liver rather than 300 mgm. Although the electron transport system is represented as utilizing only coenzyme II, the isocitric dehydrogenase in rate liver is in reality a mixture of two enzymes, one utilizing coenzyme II, the other coenzyme I (Plaut and Sung, 1955). The coenzyme II-dependent isocitric dehydrogenase is present in far greater quantity than coenzyme I, so that rat liver could be used as a source of coenzyme II-linked isocitric dehydrogenase.

The oxidation of isocitrate was not significantly inhibited by N,N'-di-secondary butyldithiooxamide at 1×10^{-3} M. These data are shown in a typical experiment in Table 6.

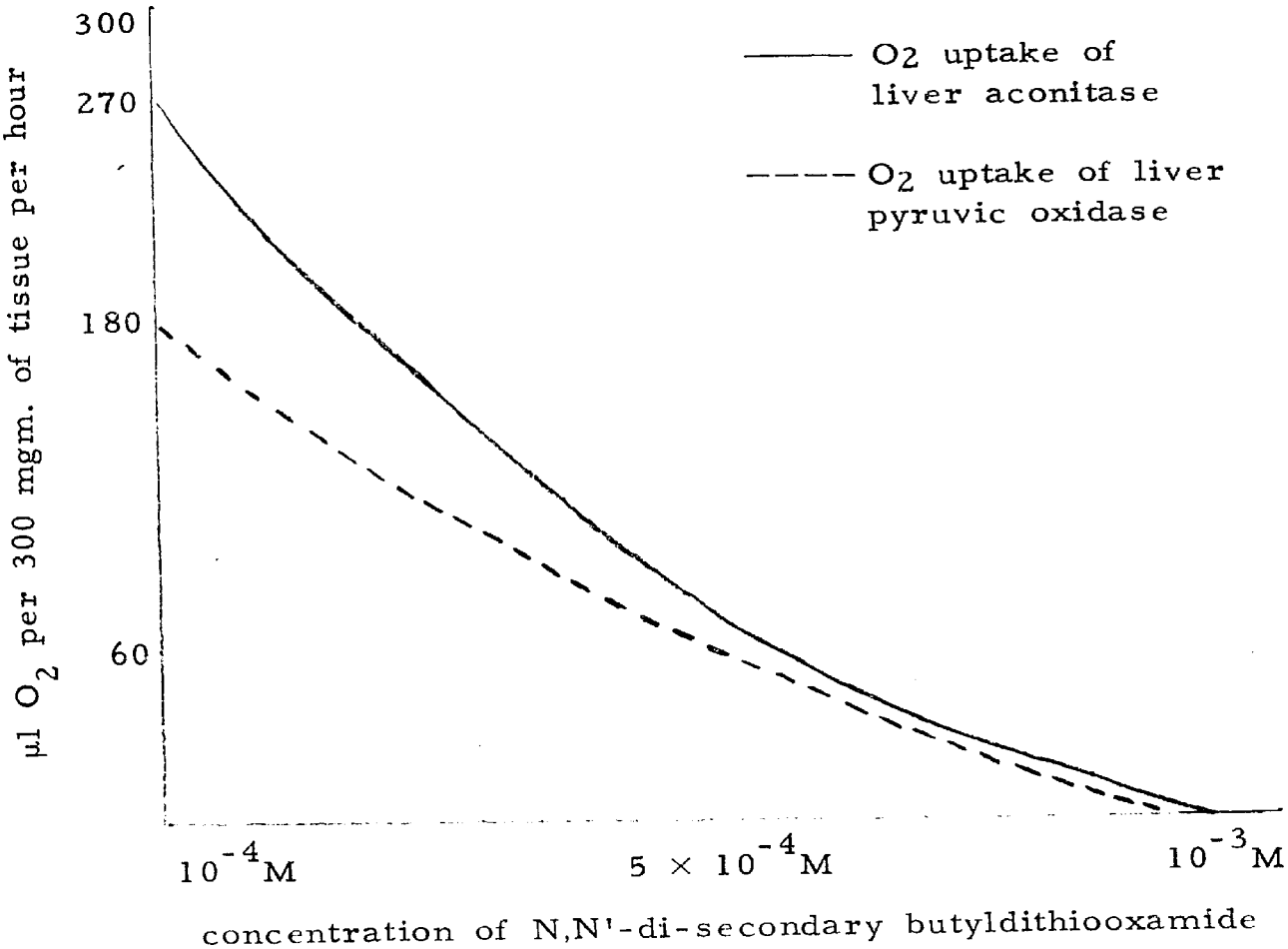


Figure 8. To illustrate the similarity of effect of N,N'-di-secondary butyldithiooxamide on two different enzymes.

Table 6. Effect of N,N'-di-secondary butyldithiooxamide on isocitric oxidation.

Tissue	Preparation	Microliters O_2/hour		
		Control	Dowanol 50B® Control	Compound 10^{-3}M
Liver	Homogenate	110	121	95
Liver	Mitochondria	64	51	49

d. Group 4

Enzymes associated with an electron transport system consisting of:

Substrate→Cytochrome b→Slater Factor[1]→Cytochrome c →Cytochrome a→Cytochrome a_3→Oxygen

Succinic dehydrogenase is the enzyme associated with this type of electron transport system.

In the first experiments, there was no apparent inhibition of oxygen uptake by N,N'-di-secondary butyldithiooxamide for the first twenty minutes, but a marked decrease in uptake appeared after this initial period. Since N,N'-di-secondary butyldithiooxamide would inhibit the oxidation of malic acid which is one step away in the Krebs citric acid cycle from succinic acid, it is reasonable to postulate that:

[1] This is a factor which acts between cytochrome b and cytochrome c (Slater, 1949). In this system electrons pass directly from succinate to cytochrome b, catalyzed by succinic dehydrogenase.

(a) The apparent inhibition in the succinic dehydrogenase system was due to the inhibition of malic oxidation.

(b) The latent period was a reflection of the time it takes malic acid to accumulate to the point where it would slow down the forward reaction from succinate.

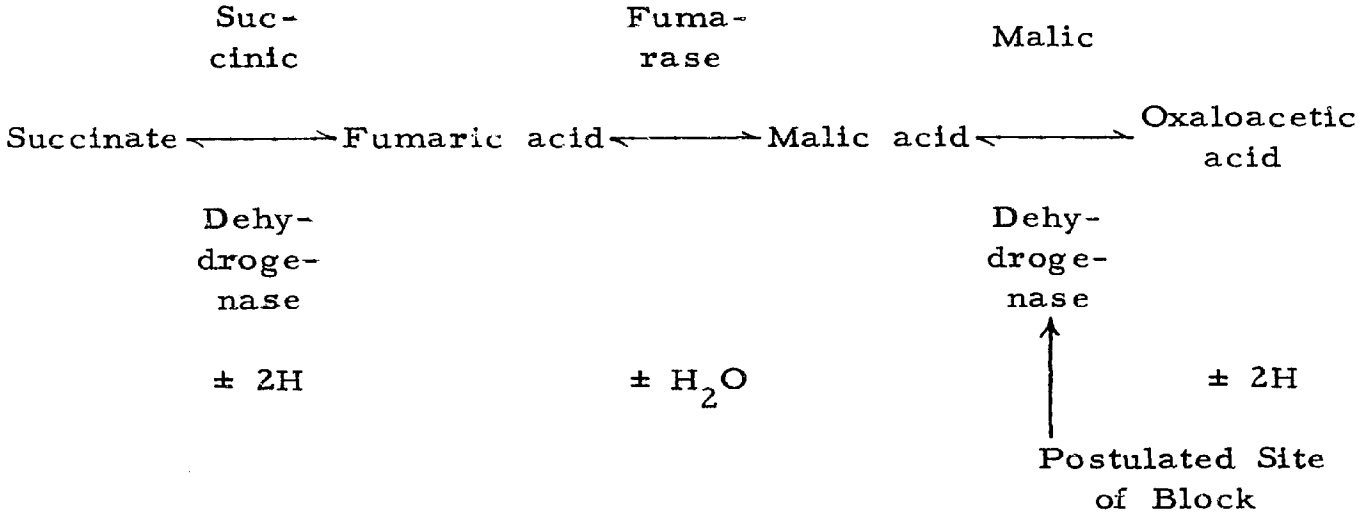


Figure 9. Illustration of the steps involved and possible site of inhibition in the oxidation of succinic acid.

The failure of N,N'-di-secondary butyldithiooxamide to inhibit the oxidation of succinate was demonstrated by the addition of potassium arsenite. Potassium arsenite inhibits malic oxidation; so that, oxygen uptake is then related only to the oxidation of succinate. In addition, the concentration of succinate was increased in order to reduce the influence of product accumulation on the rate of the forward reaction. Under these conditions, N,N'-di-secondary butyldithiooxamide did not inhibit the oxidation of succinate. Table 7 shows the

effect of N,N'-di-secondary butyldithiooxamide on succinate oxidation in the presence of potassium arsenite.

Table 7. Effect of N,N'-di-secondary butyldithiooxamide on the oxidation of succinic acid.

Potassium Arsenite (M. conc.)	Succinate (M. conc.)	µl O_2/hour/300 mgm. Equivalent Wet Weight of Tissue				
		Control	Dowanol Control	N,N'-di-secondary butyldithiooxamide		
				1×10^{-4}M	5×10^{-4}M	1×10^{-3}M
-	1×10^{-2}	222	219	191	158	119
3×10^{-2}	3×10^{-2}	230	220	233	203	206

e. Group 5

Oxygen uptake independent of an electron transport system. Monoamine oxidase is not dependent upon an electron transport system, but passes electrons directly to oxygen (Zeller, 1951). The effect of N,N'-di-secondary butyldithiooxamide on this enzyme was examined using tyramine as the substrate. Monoamine oxidase was not inhibited by N,N'-di-secondary butyldithiooxamide. The results of a typical experiment are shown in Table 8.

3. The Effect of N,N'-di-Secondary Butyldithiooxamide on Glycolysis

An attempt was made to study the effect of N,N'-di-secondary butyldithiooxamide on glycolysis in rat tissues. It was discovered

Table 8. Effect of N,N'-di-secondary butyldithiooxamide on monoamine oxidase.

Tissue	Preparation	μl O_2/hour/300 mgm. Equivalent Wet Weight of Tissue				
		Control	Dowanol Control	N,N'-di-secondary butyldithiooxamide		
				1×10^{-4}M	5×10^{-4}M	1×10^{-3}M
Liver	Mitochondria	85	78	74	76	70

that Dowanol 50B® interfered with the usual colorimetric method for lactic acid (Barker and Summerson, 1941) so that this method of studying glycolysis was impractical. An alternate approach was used. This consisted of the measurement of carbon dioxide evolved from the action of lactic acid on the sodium bicarbonate in the incubation medium. These experiments were conducted in an atmosphere of 95 per cent nitrogen and 5 per cent carbon dioxide.

There was no inhibition of glycolysis by N,N'-di-secondary butyldithiooxamide in the presence of coenzyme I which is usually added to the incubation medium. In the absence of coenzyme I, the reaction rate was considerably slower; however, there was still no inhibition by N,N'-di-secondary butyldithiooxamide. These data are shown in Table 9.

Table 9. The effect of N,N'-di-secondary butyldithiooxamide on glycolysis.

Tissue	Preparation	Coenzyme I	μl O_2 of CO_2 Evolution/ 30 min./50 mgm. Tissue		
			Control	Dowanol Control	N,N'-di-secondary butyldithiooxamide 1×10^{-3}M
Brain	Homogenate	-	56	51	59
Brain	Homogenate	2 μ M/ml.	110	86	106

4. The Comparative Activities of N,N'-di-Secondary Butyldithiooxamide, N,N'-Diallyldithiooxamide, N,N'-Bis(2-Hydroxyethyl)dithiooxamide, and Dithiooxamide

It was important to determine if the highly toxic members of the series were in the most potent in vitro, and also, if the relatively nontoxic compounds were active in vitro. This information was also required since it was desirable for reasons of solubility to substitute N,N'-diallyldithiooxamide for N,N'-di-secondary butyldithiooxamide in spectrophotometric determinations. For comparative purposes all four compounds were tested on the glutamic dehydrogenase system.

The two toxic compounds, N,N'-diallyldithiooxamide and N,N'-di-secondary butyldithiooxamide, both inhibited the glutamic dehydrogenase system. In contrast, the relatively nontoxic compounds, N,N'-bis(2-hydroxyethyl)dithiooxamide and dithiooxamide, failed to inhibit it. The results of these experiments are shown in Table 10.

Table 10. The effect of dithiooxamide and congeners on the oxygen uptake of glutamic dehydrogenase.

Tissue Preparation	Compound	µl O_2/hour/300 mgm. of Tissue			
		Dowanol Control	Compound Concentration		
			1×10^{-4}M	5×10^{-4}M	1×10^{-3}M
Liver mitochondria	N,N'-bis(2-hydroxyethyl) dithiooxamide	266	257	245	236
Liver mitochondria	Dithiooxamide	266	252	267	251
Liver mitochondria	N,N'-diallyl-dithiooxamide	212	195	0	0
Liver mitochondria	N,N'-di-secondary butyldithio-oxamide	262	261	131	9

5. The Action of N,N'-Diallyldithiooxamide on the Oxidation of Ethanol by Alcohol Dehydrogenase

This is a one-step system in which the oxidation of ethanol to acetaldehyde is dependent on the presence of coenzyme I as an electron acceptor. N,N'-diallyldithiooxamide was substituted for N,N'-di-secondary butyldithiooxamide because it was more soluble in the system used. Previous experiments had shown it to be as active as N,N'-di-secondary butyldithiooxamide.

N,N'-diallyldithiooxamide inhibited the reduction of coenzyme I and consequently the oxidation of ethanol by alcohol dehydrogenase. A typical experiment is shown in Table 11.

Table 11. The inhibition of liver alcohol dehydrogenase by N,N'-diallyldithiooxamide.

Compound M conc.	Enzyme conc. γ/ml.	Coenzyme I conc. γ/ml.	Δ Optical Density per Minute		Per Cent Inhibition
			Dowanol Control	Drug	
2.6×10^{-4}	1.86	20	.021	.010	50
1.6×10^{-5}	1.86	100	.070	.035	50

6. The Effect of N,N'-Diallyldithiooxamide on the Oxidation of Glucose-6-phosphate by Glucose-6-phosphate Dehydrogenase

This system is also a one-step reaction. In this instance coenzyme II is the acceptor of electrons from the oxidation of glucose-6-phosphate to 6-phosphogluconic acid. N,N'-diallyldithiooxamide did not inhibit the reduction of coenzyme II by glucose-6-phosphate dehydrogenase. The experimental data are shown in Table 12.

Table 12. Failure of N,N'-diallyldithiooxamide to inhibit glucose-6-phosphate dehydrogenase.

Compound M conc.	Enzyme conc. in units/ml.	Coenzyme II γ/ml.	Total Δ Optical Density for 3 Minutes	
			Dowanol Control	Drug
2.6×10^{-4}	.003	8.4	0.046	0.050
4×10^{-4}	.0026	8.4	0.025	0.023
4×10^{-4}	.0026	4.2	0.011	0.013

7. Experiments to Study Methods of Antagonizing the Inhibition of Oxygen Uptake by N,N'-di-Secondary Butyldithiooxamide

The following experiments were designed to attempt to antagonize the inhibitions with N,N'-di-secondary butyldithiooxamide described in section 2. The antagonists used were: ethylenediaminetetraacetic acid, magnesium chloride, methylene blue, and coenzyme I.

a. Attempted antagonism with ethylenediaminetetraacetic acid

Hunter and Ford (1955) have shown that the presence of small amounts of heavy metals and other miscellaneous compounds alter the permeability of mitochondrial membranes resulting in a loss of coenzyme I. Such systems as glutamic dehydrogenase, malic dehydrogenase, and other coenzyme I dependent systems were inhibited. By the addition of ethylenediaminetetraacetic acid to the incubation medium, they could antagonize the permeability effect of such compounds.

To determine whether the action of N,N'-di-secondary butyldithiooxamide was altering the membrane permeability in a manner analogous to the above compounds, ethylenediaminetetraacetic acid was added to a mitochondrial glutamic dehydrogenase system containing the dithiooxamide.

A final concentration of 1×10^{-3}M ethylenediaminetetraacetic acid in the incubation medium failed to antagonize the action of N,N'-di-secondary butyldithiooxamide (see Table 13). Liver mitochondria were also prepared in 0.25 M sucrose as described (see pages 23, 24) but containing 1×10^{-3}M ethylenediaminetetraacetic acid. The addition of such mitochondria to a similar system also failed to reverse the inhibition.

b. Attempted antagonism with magnesium chloride

Since magnesium plays a vital role in terminal oxidation and phosphorylation processes (Brody, Hurwitz, and Bain, 1954), it appeared necessary to determine whether dithiooxamides were binding this essential cation and producing an inhibition by this mechanism.

Magnesium chloride at a final concentration of 32 μM/ml. had no antagonistic effect on the inhibition of glutamic dehydrogenase by N,N'-di-secondary butyldithiooxamide, studied in liver mitochondria (see Table 13). These findings may be correlated with the in vivo observations that magnesium chloride failed to influence the toxicity of N,N'-di-secondary butyldithiooxamide in rabbits (Miller, unpublished data).

Table 13. A summary of the attempts to antagonize N,N'-di-secondary butyldithiooxamide and N,N'-diallyldithiooxamide in in vitro systems.

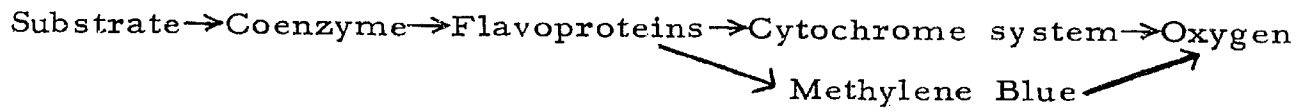
Inhibitor	Antagonist	Tissue Preparation	Substrate
N,N'-di-secondary butyldithiooxamide	EDTA 1×10^{-3}M	liver mitochondria	glutamate
N,N'-di-secondary butyldithiooxamide	$MgCl_2$ 32 μM/ml.	liver mitochondria	pyruvate
N,N'-di-secondary butyldithiooxamide	Methylene blue 1×10^{-5}M	liver mitochondria	glutamate
N,N'-di-secondary butyldithiooxamide	Coenzyme I 0.5 μM/ml.	liver mitochondria	glutamate
N,N'-di-secondary butyldithiooxamide	Coenzyme I 1.0 μM/ml.	liver mitochondria	glutamate
N,N'-di-secondary butyldithiooxamide	Coenzyme I 3.0 μM/ml.	liver mitochondria	glutamate
N,N'-diallyldithio-oxamide	Coenzyme I 0.5 μM/ml.	liver mitochondria	glutamate

Table 13--Continued

μl O_2 per Hour per 300 mgm. of Tissue					
Without Antagonist			With Antagonist		
Dowanol Control	Inhibitor		Dowanol Control	Inhibitor	
	5×10^{-4}M	1×10^{-3}M		5×10^{-4}M	1×10^{-3}M
246	0	0	230	0	0
149	39	-	155	44	-
179	-	0	210	-	0
185	-	0	221	-	95
260	-	9	352	-	126
277	-	31	452	-	140
203	-	0	252	-	113

c. Attempted antagonism with methylene blue

Methylene blue can antagonize those metabolic inhibitors whose site of action is in the cyctochrome portion of the electron transport system. This antagonism results from the ability of methylene blue to accept electrons from flavoproteins and pass them on to oxygen. The site of methylene blue action may be described as follows:



Methylene blue at a concentration of 1×10^{-5}M failed to antagonize the inhibition of glutamic dehydrogenase by N,N'-di-secondary butyldithiooxamide, in a liver mitochondrial preparation (see Table 13).

d. Coenzyme I antagonism of the inhibition of glutamic dehydrogenase

The studies above have demonstrated that all the coenzyme I-linked systems were inhibited by N,N'-di-secondary butyldithiooxamide. In view of the failure of the experiments already described to antagonize N,N'-di-secondary butyldithiooxamide, the addition of coenzyme I to the incubation medium was the next logical step.

Coenzyme I, in concentrations varying from 0.5 to 3 μM/ml., produced a marked increase in oxygen uptake in the presence of 1×10^{-3}M N,N'-di-secondary butyldithiooxamide. Although the increase in oxygen uptake equaled that of the control experiments without exogenous coenzyme I, it never reached the level of oxygen uptake attained by control experiments with corresponding amounts of coenzyme I (see Table 13).

8. A Study of the Relationship between Coenzyme I and N,N'-Diallyldithiooxamide

a. Mitochondrial systems

From the studies described in the previous section, it appears that in the mitochondrial system, coenzyme I can effectively antagonize the inhibition produced by N,N'-diallyldithiooxamide.

The relationship between coenzyme I and N,N'-diallyldithiooxamide in the mitochondrial glutamic dehydrogenase system was examined to determine if it was competitive or noncompetitive. The method outlined by Hunter and Downs (1945) was utilized. This approach plots $I\frac{\alpha}{1-\alpha}$ against [S] where:

I = molar concentration of inhibitor

α = fractional activity

[S] = substrate (antagonist) concentration

A competitive relationship between substrate and inhibitor results in a slope, whereas a noncompetitive relationship results in a horizontal line.

The concentration of coenzyme I was treated as the substrate concentration for this system. The above formula assumes that the substrate is the potential antagonist for the inhibitor. In the mitochondrial glutamic dehydrogenase system there are actually two compounds, glutamate and coenzyme I, which are functionally related to the apoenzyme. The concentration of glutamate was kept constant, so that it was nonlimiting. For any one series of experiments, the coenzyme concentration was the only factor varied. Therefore, it is valid, for the purposes of this analysis, to regard the coenzyme I as the substrate.

An analysis of the data presented in section 7 dealing with coenzyme I antagonism of N,N'-diallyldithiooxamide, indicates that

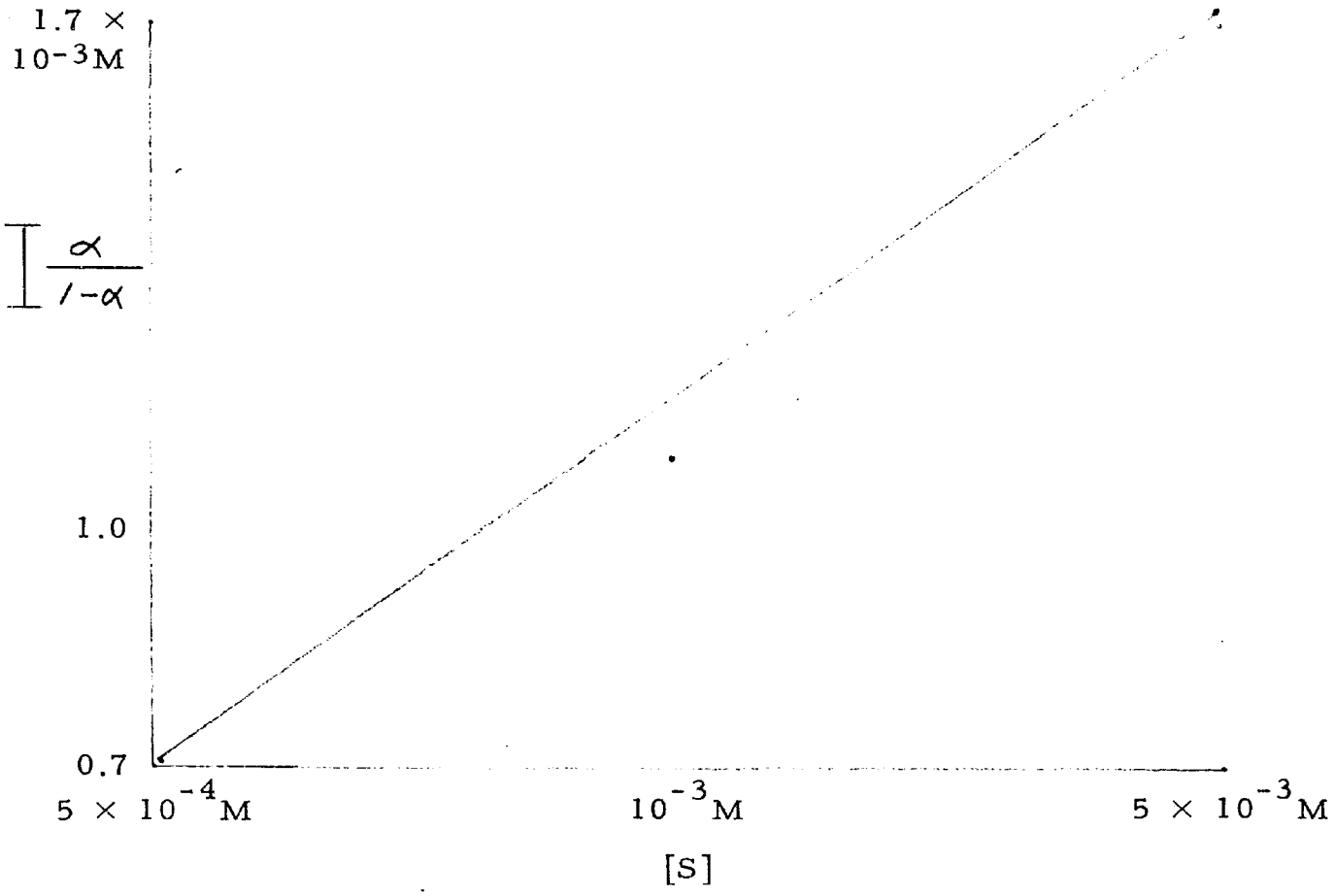


Figure 10. The competitive relationship of coenzyme I and N,N'-diallyldithiooxamide in a mitochondrial glutamic dehydrogenase system.

the relationship between these two agents is competitive (see Figure 10 and Appendix II).

b. Alcohol dehydrogenase system

In the alcohol dehydrogenase system the relationship between coenzyme I and N,N'-diallyldithiooxamide was examined according to the formula outlined by Hunter and Downs (1945). For the purpose of this approach coenzyme I was assumed to be the substrate for alcohol dehydrogenase, as ethanol is present in excess at all times (see section 8). The rate of utilization of coenzyme I was studied over a wide range of concentrations. The inhibitor concentration was also varied. Alcohol dehydrogenase was diluted to 1.4 micrograms/ml. so that the enzyme concentration was a limiting factor at all times. The data obtained were plotted as $I\frac{\alpha}{1-\alpha}$ (see section 8-a) on the ordinate; against substrate concentration on the abscissa. Plotting of the results meets all the requirements of a noncompetitive relationship between coenzyme I and N,N'-diallyldithiooxamide in this sytem (see Figure 11 and Appendix II).

c. Evidence against the interaction of N,N'-diallyldithiooxamide with coenzyme I

(a) N,N'-diallyldithiooxamide has a peak absorption at 300 mμ; however, there is still a significant absorption at 340 mμ. Oxidized coenzyme I has minimal absorption at 340 mμ and when added to a solution of N,N'-diallyldithiooxamide there is no change in absorption.

(b) In an alcohol dehydrogenase system containing 330 γ/ml. of enzyme, all the coenzyme I was reduced in the presence or absence of N,N'-diallyldithiooxamide. In both the control and drug-containing experiments the amount of coenzyme I reduced was equivalent to the calculated amount of coenzyme I added. This precludes

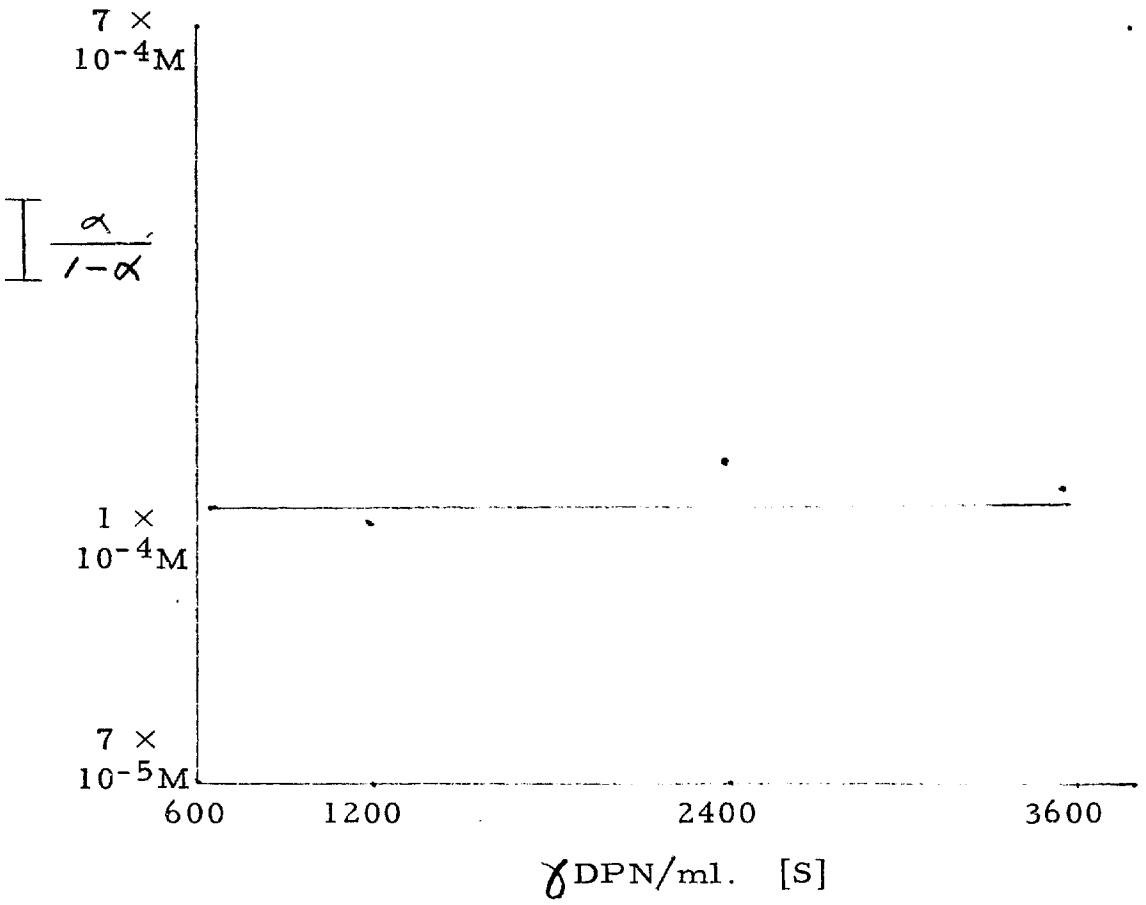


Figure 11. The noncompetitive relationship between coenzyme I and N,N'-diallyldithiooxamide in the alcohol dehydrogenase system.

the combination of N,N'-diallyldithiooxamide with coenzyme I to make it inaccessible for reduction. Alcohol dehydrogenase is present in such great excess that inhibition of a portion of the enzyme by N,N'-diallyldithiooxamide did not alter its ability to reduce the limiting amount of coenzyme I present.

CHAPTER IV

DIPHOSPHOPYRIDINE NUCLEOTIDE DETERMINATIONS IN RATS

The data presented in Chapter III indicate that diphosphopyridine is a common denominator of all N,N'-di-secondary butyldithiooxamide-inhibited enzymes. If the dithiooxamide interrupts the functional relationships of this coenzyme, then the in vivo action of the dithiooxamide may be reflected in altered tissue levels of diphosphopyridine nucleotide. This would provide additional data for the correlation of the in vivo and in vitro action of the compound. (For further elaboration see Chapter V.)

A. Methods

1. Extraction of Total Diphosphopyridine Nucleotide from Tissues

There are two basic difficulties in the determination of total diphosphopyridine nucleotide in tissues.

(1) Oxidized diphosphopyridine nucleotide (DPN) is stable at an acid pH. Reduced diphosphopyridine nucleotide (DPNH) is unstable at an acid pH and stable at a basic pH.

(2) Oxidized diphosphopyridine nucleotide is rapidly destroyed in homogenized liver and brain tissue, nicotinamide being hydrolyzed by diphosphopyridine nucleotidase.

An accurate method of determination had to be conducted at hydrogen ion concentrations that would conserve both forms of

diphosphopyridine nucleotide. Also, diphosphopyridine nucleotidase had to be inactivated without concomitant destruction of diphosphopyridine nucleotide.

A method, as described by Eichel and Spirtes (1954), was dependent upon an aqueous extraction at a pH of 7.4 with heat inactivation of the nucleotidase. In our hands this method did not yield results comparable to those published by the above authors. Low values were obtained for the oxidized form of diphosphopyridine nucleotide which was unstable at this pH under the conditions of this extraction.

The method eventually used was that described by Gloch and McLean (1955). It proved entirely satisfactory.

a. Procedure

(1) Two 500 mgm. tissue samples were minced. The tissue sample to be assayed for oxidized diphosphopyridine nucleotide was placed in a homogenizer tube containing 5 ml. of 0.1 N NaOH.

(2) The homogenizer tubes were placed in a boiling water bath within four and a half minutes after death of the animal. The tubes were left in the bath for thirty seconds, homogenized for fifty seconds, again placed in the bath for thirty seconds, then cooled in crushed ice. The total elapsed time cannot exceed two minutes.

(3) One ml. of 0.25 M glycylglycine, pH 8.7, was added to each homogenizer and the volume adjusted to 15.0 ml. with cold distilled water. Each sample was then neutralized with the aid of a pH meter, using 0.1 N HCl or 0.1 N NaOH, and brought up to a final volume of 20.0 ml.

(4) The neutralized homogenates were then centrifuged for twenty-five minutes at 25,000 × "g" at 0° C. An aliquot of the supernatant was assayed for diphosphopyridine nucleotide content.

2. Methods of Analysis of Oxidized and Reduced Diphosphopyridine Nucleotide

a. Analysis for oxidized diphosphopyridine nucleotide

(1) Principle

The method used is specific for diphosphopyridine nucleotide (Bonnichsen and Brink, 1955), and did not include triphosphopyridine nucleotide as is the case with nonenzymatic methods.

The oxidized diphosphopyridine nucleotide was reduced by alcohol dehydrogenase. Ethanol was utilized as the substrate. An excess of alcohol dehydrogenase and ethanol insured the reduction of all oxidized diphosphopyridine.

(2) Reagents

Ethanol, 96 per cent.

Alcohol dehydrogenase, 1 mgm./ml.

Glycine, NaOH, buffer, pH 9.6.

Aqueous tissue extract.

(3) Procedure

The following reagents were mixed in a cuvette:

2.2 ml. glycine sodium hydroxide, buffer, pH 9.6.

0.1 ml. of 96 per cent ethanol.

0.5 ml. of aqueous tissue extract.

This was duplicated in another cuvette. A third cuvette was set up, with all of the above contents except the aqueous tissue extract, for which a known amount of oxidized diphosphopyridine nucleotide was substituted. The latter cuvette was utilized as a control for alcohol

dehydrogenase activity. All experimental cuvettes were read against distilled water. Readings of optical density were made and 0.2 ml. of the alcohol dehydrogenase solution added to each cuvette, mixed, and a second determination made five minutes later. The reduction of 100 γ/ml. of diphosphopyridine nucleotide resulted in an increase of optical density of 0.835. From these data the concentration of diphosphopyridine nucleotide in the cuvette and in the original tissue sample may be calculated.

b. Analysis for reduced diphosphopyridine nucleotide

(1) Principle

The method used was specific for reduced diphosphopyridine nucleotide. The reduction of acetaldehyde to ethanol was carried out by alcohol dehydrogenase. Reduced diphosphopyridine nucleotide was the electron donor for the reaction. The method used is the backward reaction of the determination for oxidized diphosphopyridine nucleotide (see Chapter III, Methods). Spectrophotometric determinations were carried out at a pH of 7.1.

(2) Reagents

0.1 M K_2HPO_4 - KH_2PO_4, buffer, pH 7.1.

1 per cent redistilled acetaldehyde.

Alcohol dehydrogenase, 1 mgm./ml.

Aqueous tissue extract.

(3) Procedure

The following components were added to cuvettes:

0.3 ml. of 0.1 M K_2HPO_4 - KH_2PO_4 buffer, pH 7.1.

0.1 ml. of 10 per cent redistilled acetaldehyde.

1.0 ml. of aqueous tissue extract.

1.0 ml. of distilled water.

After mixing, duplicate determinations of optical density were made and 0.5 ml. of alcohol dehydrogenase solution added. Optical density was again determined five minutes later. Reduced diphosphopyridine nucleotide was calculated from the decrease in optical density resulting from the oxidation of reduced diphosphopyridine nucleotide (DPNH).

B. Results

The content of diphosphopyridine nucleotide was determined in the livers of rats treated with toxic doses of N,N'-di-secondary butyldithiooxamide. For this purpose a group of ten rats were given 5 mgm./Kgm. of the drug dissolved in Dowanol 50B® intraperitoneally. This was the approximate LD_{50} dose for rats. Rats were sacrificed by decapitation at various times after the compound was administered and the liver excised and assayed for pyridine nucleotides. The most severely affected survivors were utilized for these studies. The control group of rats were treated with a dose of Dowanol 50B® equivalent to that contained in the solution of N,N'-di-secondary butyldithiooxamide.

Rats treated with toxic doses of N,N'-di-secondary butyldithiooxamide exhibited a decrease in total diphosphopyridine content of liver. Initially this decrease consisted entirely of a decline in oxidized diphosphopyridine nucleotide, with an accompanying reversal in the ratio of the oxidized to reduced form. At seventy-two hours after administration of the compound, reduced diphosphopyridine had declined so that the ratio of oxidized to reduced diphosphopyridine was restored to the normal range. At this time the total

diphosphopyridine content of the liver was still approximately 50 per cent of the control level. Rats in the control group maintained normal diphosphopyridine levels and ratios throughout the experiments. There were no fatalities, prior to sacrificing, in the Dowanol 50B® control rats. The results of these experiments are shown in Table 14.

Table 14. A comparison of liver content of diphosphopyridine nucleotides in rats treated with N,N'-di-secondary butyldithiooxamide, and control rats.

Time After Drug Administration	γ/gram of Tissue, Wet Weight			Ratio DPN/ DPNH
	Oxidized DPN	Reduced DPN	Total DPN	
15 hours	198	307	505	0.64
Control	421	320	741	1.31
34 hours	188	301	489	0.62
Control	410	330	740	1.25
58 hours	160	198	358	0.89
Control	325	318	643	1.02
72 hours	226	140	366	1.6
Control	377	330	707	1.14

CHAPTER V

DISCUSSION

The aim of this research has been to determine the mechanism of toxicity of the potent dithiooxamides. This required the characterization of the toxicity and its subsequent biochemical investigation. The data will be discussed under the headings of:

(A) Verification of the biochemical data.

(B) Transposition of the biochemical data to the intact animal.

(C) The correlation of the biochemical studies with the characteristics of dithiooxamide toxicity.

A. Verification of the Biochemical Data

N,N'-di-secondary butyldithiooxamide inhibited the oxygen uptake of brain and liver homogenates. This indicates that the compound is active in an in vitro system, and that both brain and liver are susceptible to its action. The almost immediate and complete inhibition of homogenate respiration by N,N'-di-secondary butyldithiooxamide may mean that the compound is acting at many oxidative sites, or at a site common to most of the oxidative reactions.

The investigation of dithiooxamide activity was conducted at the mitochondrial level. Washed mitochondria not only contain most of the aerobic enzyme systems, but also allow a degree of selectivity as to the particular enzyme to be studied. The aerobic enzyme systems may be classified according to the type of electron pathway associated with substrate oxidation. The use of this type of

classification facilitates the correlation of dithiooxamide activity with a site of action in electron transport. If the action of the compound is specific for one particular enzyme system, then this will become apparent, regardless of the sequence of experiments. It is readily apparent that N,N'-di-secondary butyldithiooxamide inhibited seven different apoenzymes. However, these apoenzymes were associated with only two types of electron transport systems:

1.→LTPP→Co A→Co I→Flavoproteins→Cytochrome Systems→Oxygen

2.————————→Co I→Flavoproteins→Cytochrome Systems→Oxygen

Enzymes associated with the other types of electron transport systems or with direct passage to oxygen were not inhibited by N,N'-di-secondary butyldithiooxamide. These systems are composed of:

1.——→Coenzyme II→Flavoproteins→Cytochrome Systems→Oxygen

2.————————→"Slater Factor"→Cytochrome Systems→Oxygen

3.————————————————————————————→Oxygen

An inspection of these systems indicates that coenzyme I is the only common denominator that is not found in nonsusceptible pathways. The conclusion is that coenzyme I is intimately related to the in vitro mechanism of action of the potent dithiooxamides. This is supported by the following observations:

(1) The cytochrome system is functional in the presence of inhibiting concentrations of dithiooxamides. This is evidenced in both the coenzyme II and "Slater Factor" containing pathways. An additional point of evidence is the failure of methylene blue to antagonize the action of N,N'-di-secondary butyldithiooxamide. If the blockade of electron transport involved the cytochrome system, as is the case with cyanide, then methylene blue would have been an effective antagonist.

(2) The flavoproteins are not affected by the potent dithiooxamides. The same arguments used above are equally valid here.

The failure of N,N'-di-secondary butyldithiooxamide to inhibit the coenzyme II containing pathway is evidence for the functional status of the flavoproteins in the presence of effective concentrations of the compound.

This indicates that the site of dithiooxamide activity is at, or before, the coenzyme I stage of electron transport.

(3) If coenzyme I is a pertinent characteristic of susceptible apoenzymes, then the dithiooxamide inhibition should also be seen in other, nonmitochondrial, coenzyme I-linked enzymes. A purified preparation of horse liver alcohol dehydrogenase, coenzyme I-linked, was inhibited by N,N'-diallyldithiooxamide, in concentrations as low as 2×10^{-5}M. A purified preparation of glucose-6-phosphate dehydrogenase, a coenzyme II-linked enzyme, was not inhibited.

Additional evidence indicates that the relationship of coenzyme I to the dithiooxamide mechanism of action is not a nonspecific artifact.

(4) Hunter and Ford (1955) have shown that various substances, including heavy metals, exert a nonspecific action on in vitro mitochondrial preparations by increasing the loss of coenzyme I by diffusion. This nonspecific action, superficially similar to that of the dithiooxamides because of the susceptible systems, may be reversed by ethylenediaminetetraacetic acid. The inhibition of respiration by N,N'-di-secondary butyldithiooxamide cannot be reversed by ethylenediaminetetraacetic acid.

(5) Some dithiooxamides have chelating properties. Conceivably their biological activity could, in part, be due to the chelation of an essential enzyme-activating cation such as magnesium. The addition of magnesium failed to antagonize the action of N,N'-di-secondary butyldithiooxamide on the mitochondrial glutamic dehydrogenase system. The effect of other metals on this inhibition was not studied.

(6) The inhibition of a susceptible mitochondrial system by either N,N'-di-secondary butyldithiooxamide or N,N'-diallyldithioxamide was reversed by the addition of coenzyme I. This indicates that the relationship of coenzyme I to the inhibitor was competitive. Analysis of data from the attempted reversal in the alcohol dehydrogenase system indicates that a noncompetitive relationship was involved. The apparent conflict in results may be explained on the basis of apoenzyme affinity for the dithiooxamides and coenzyme I. If this affinity for the dithiooxamide were much greater in the alcohol dehydrogenase system than in the mitochondrial glutamic dehydrogenase system, then coenzyme I could not successfully compete for the alcohol dehydrogenase apoenzyme but may compete in the mitochondria. Alivisatos and Denstedt (1952) have shown differences in affinity between coenzyme II and glucose-6-phosphate dehydrogenase depending on whether manometric or spectrophotometric methods were used. With manometric methods the apoenzyme affinity for coenzyme II was 2,000 times greater than its affinity for nicotinamide and 17,000 times greater in the spectrophotometric system. The apparent differences in competitive and noncompetitive relationships of N,N'-diallyldithiooxamide and coenzyme I not only involve different methods and pH, but different apoenzymes. In all likelihood the competitive and noncompetitive relationships do not represent basic differences in dithiooxamide activity.

The evidence presented supports the original hypothesis that coenzyme I-linked apoenzymes are inhibited by the potent dithiooxamides. The competitive relationship in the mitochondrial system implies the action of the dithiooxamide is on sites of the apoenzyme having the specific configuration for coenzyme I. An interaction of dithiooxamide and coenzyme I is not likely on the basis of:

(1) The lack of significant change in absorption at 340 mμ when coenzyme I and N,N'-diallyldithiooxamide are mixed. Other reaction products with coenzyme I do exhibit changes in absorption spectra (Burton and Kaplan, 1954).

(2) The inability of N,N'-diallyldithiooxamide to bind coenzyme I available for reduction in the presence of excess alcohol dehydrogenase.

B. Transposition of the Biochemical Data to the Intact Animal

The hypothesis is advanced that the in vitro biochemical activity of the potent dithiooxamides is due to the occupation of the coenzyme I site on the apoenzyme. The transposition of this hypothesis to the intact animal requires that the criteria suggested by Welch and Bueding (1946) be met.

> The first criterion is, "The concentrations of the drug or poison which produce the effect in vitro and which obtain at the anatomical site of action should be closely similar."

The dose administered to rats was 5 mgm./Kgm. Assuming a random distribution of a water-soluble compound, this would reach a concentration of 2×10^{-5}M; on the basis of body water constituting 75 per cent of total body weight. The potent dithiooxamides are not water-soluble. In lieu of valid tissue distribution data a final concentration of 2×10^{-5}M will be utilized for the purposes of this discussion.

Susceptible mitochondrial systems are inhibited by a concentration of 2×10^{-4}M. This is of course no indication of the final concentration in the mitochondria. However, the difference between these extremes of in vivo and in vitro concentration is only tenfold,

which is not excessive. The alcohol dehydrogenase system is inhibited by N,N'-diallyldithiooxamide at a concentration of 2×10^{-5}M. This is directly comparable to the hypothetical in vivo concentration. It should be pointed out that there are no problems of membrane permeability in this system as there are in the mitochondrial system. The first criterion has been met.

> The second criterion is, "If a drug or poison exerts its effect on a specific tissue in vivo, either the effect on this tissue in vitro should be more pronounced than the effects on other tissues, or the system inhibited must be shown to have more functional significance in this than in other tissues."

Although the characteristic signs of potent dithiooxamide toxicity involve both cardiac and central nervous system tissue, the in vivo effect of these compounds is not necessarily directed specifically against these tissues. Both the brain and heart are immediately limiting as far as survival is concerned; other organs, such as the liver and kidneys, while vital, do not exhibit spectacular or immediate syndrome accompanying deranged function. The in vitro activity of the dithiooxamides does not show any specificity to brain. The postulated site of action is of pivotal importance to all active tissue.

In meeting this criterion it can be said that the in vitro site of activity would be of great functional significance in the tissues visibly affected.

> The third criterion is, "Among structurally related compounds there should be close parallelism between the pharmacological activities in vitro and in vivo: all drugs of the same chemical series which are active in vivo must also be active in vitro, and those inactive in vivo must be inactive in vitro, unless the discrepancy can be accounted for by poor absorption, by inadequate distribution to or penetration of the cells involved, by too rapid excretion, or by metabolic alteration."

This criterion is also fully satisfied. The two toxic compounds, N,N'-di-secondary butyldithiooxamide and N,N'-diallyldithiooxamide are active in the in vitro system, and the relatively nontoxic compounds, N,N'-bis(2-hydroxyethyl)dithiooxamide and dithiooxamide, are inactive at final concentrations of 1×10^{-3}M.

In addition to meeting the above criteria, the original hypothesis is further supported by the determinations of coenzyme I content in rats receiving N,N'-di-secondary butyldithiooxamide.

If the in vivo mechanism of action of the dithiooxamides involves the occupation of some of the coenzyme I apoenzyme sites, then this derangement of coenzyme I binding may be reflected in the tissue levels of the coenzyme. The displacement of coenzyme I may result in its diffusion into the cytoplasm. This would be aided by changes in mitochondrial permeability that accompany depression of cellular respiration. In certain tissues, such as liver and brain, there is an enzyme, diphosphopyridine nucleotidase, which destroys oxidized coenzyme I by the hydrolysis of nicotinamide (Mann and Quastel, 1941). The reduced form of coenzyme I is not susceptible to the action of this enzyme. Therefore, a postulated effect of the potent dithiooxamide would be a decrease in the oxidized form of coenzyme I. This decrease is seen in the livers of treated rats.

It should be emphasized that a reduction in the oxidized form of diphosphopyridine nucleotide is not a necessary condition for the transposition of the in vitro data to the intact animal. It is conceivable that the displaced coenzyme I could remain in the mitochondria, or as in the case of other tissues, not be destroyed upon entry into the cytoplasm. This would result in normal tissue levels of coenzyme I at a time when there was an acute functional deficiency of the coenzyme.

On the basis of the arguments presented, the inhibition of tissue respiration at the coenzyme I site of apoenzyme is an important *in vivo* mechanism of action of the dithiooxamides.

C. The Correlation of Biochemical Data with Toxicity

An agent which inhibits respiration of coenzyme I-linked apoenzymes could be expected to exhibit all the characteristic signs of toxicity described for the dithiooxamides. Such an inhibition of respiration would be reflected by cardiac muscle and nervous tissue in which the maintenance of normal function is dependent on the availability of large amounts of biological energy. The conduction system of the heart may be the first gross site of action in view of the arrhythmias observed.

The absence of significant species variation among mammals could be predicted for agents acting on the apoenzymes. The area of inhibition is so fundamental to the generation of energy in all mammals that there is no reason to predict wide species variations. Similarly, the potentiation of dithiooxamide toxicity by fluoroacetate can be rationally explained. The concomitant administration of fluoroacetate and a potent dithiooxamide would result in the partial inhibition of many oxidative reactions in addition to the aconitase inhibition by fluoroacetate. This would account for the potentiation observed. A similar potentiation may be predicted for any other Krebs cycle inhibitor.

The cumulative toxicity of N,N'-di-secondary butyldithiooxamide, while startling in its proportions, is readily explained by its mechanism of action. There are, in fact, two possible explanations.

(1) N,N'-di-secondary butyldithiooxamide may be retained on the coenzyme I-linked apoenzyme, either for prolonged periods, or long enough to inactivate the enzyme and require its regeneration. The repeated administration of 1 mgm./Kgm. of the dithiooxamide, even at widely separated intervals, then assumes the role of repeated insults to a vital system.

(2) A concurrent explanation could also embody the decrease of coenzyme I following the administration of N,N'-di-secondary butyldithiooxamide. Even if the apoenzyme-dithiooxamide bond were not of long duration, the repeated loss of coenzyme would eventually create a situation of acute deficiency. This, coupled with the short-lived apoenzyme bonding by the last dose of the compound, would precipitate death. The critical nature of cardiac and central nervous system function results in death before signs of chronic toxicity involving other tissues are apparent.

In conclusion, the mechanism of toxicity of N,N'-di-secondary butyldithiooxamide and N,N'-diallyldithiooxamide is the occupation of coenzyme I sites on apoenzymes. This is a new and unique mechanism of toxicity.

SUMMARY

The toxicity of dithiooxamide, N,N'-bis(2-hydroxyethyl)dithiooxamide, N,N'-diallyldithiooxamide, and N,N'-di-secondary butyldithiooxamide was studied. The diallyl, and secondary butyl derivatives proved to be the most toxic compounds, with LD_{50}'s in rabbits of approximately 15.0 and 2.3 mgm./Kgm., respectively. Dithiooxamide and the 2-hydroxyethyl derivative did not kill at 50 mgm./Kgm. The toxicity of the potent compounds is characterized by cardiac arrythmias and central nervous system disturbances. Death is most often the result of ventricular fibrillation. Other characteristics of the potent derivatives are a latent period, extreme cumulative toxicity, and a lack of significant species variations among mammals.

The in vitro biochemical effects of these compounds were examined. Only the toxic compounds proved to be active in vitro. Their in vitro activity was limited to coenzyme I-linked enzymes in mitochondrial and purified systems. The mitochondrial inhibition could be reversed by the addition of coenzyme I.

The livers of rats treated with N,N'-di-secondary butyldithiooxamide were assayed for their coenzyme I content. The oxidized form of coenzyme I was depressed to 50 per cent of the control levels; this resulted in a reversal of the oxidized coenzyme I to reduced coenzyme I ratio.

On the basis of the data presented, coenzyme I is a pertinent characteristic of apoenzymes inhibited in vitro by the dithiooxamides. The in vitro mode of action was transposed to the intact animal, using the criteria outlined by Welch and Bueding. The occupation of

coenzyme I sites of apoenzymes is advanced as an important *in vivo* action of the dithiooxamides; and their toxicity is discussed in terms of this hypothesis.

GLOSSARY

Co I = coenzyme I.

DPN = oxidized diphosphopyridine nucleotide, oxidized coenzyme I.

DPNH = reduced diphosphopyridine nucleotide, reduced coenzyme I.

TPN = oxidized triphosphopyridine nucleotide, oxidized coenzyme II.

TPNH = reduced triphosphopyridine nucleotide, reduced coenzyme II.

ADH = alcohol dehydrogenase.

EDTA = ethylenediaminetetraacetic acid.

LTPP = lipothiamide pyrophosphate.

Co A = coenzyme A.

APPENDIX I

DETERMINATION OF THE INTRAVENOUS LD_{50} OF N,N'-DI-SECONDARY BUTYLDITHIOOXAMIDE IN RABBITS

(The method of Weil and Smyth, 1953, was used.)

Data

Dose (mgm./Kgm.)	Number of Rabbits	Death on Days After Dosage
6.27	5	5
5.0	5	5
3.96	5	4
3.14	5	3
2.49	5	2
1.97	5	3
1.56	5	1
Dowanol 50B® Control	5	0

Calculations

$$\text{Log } LD_{50} = \text{Log Da} + d.(f+1)$$

where Da = lowest dose

d = geometrical progression between dosages

d.(f+1) = 0.175 (from tables of Weil and Smyth).

$$\text{Log } LD_{50} = 0.1931 + .175$$
$$= 0.368$$

Therefore, LD_{50} of N,N'-di-secondary butyldithiooxamide is 2.3 mgm./Kgm.

APPENDIX II

A. Competitive Antagonism of N,N'-Diallyldithiooxamide by Coenzyme I (Figure 10, page 48)

The competitive antagonism of N,N'-diallyldithiooxamide by coenzyme I in the mitochondrial glutamic dehydrogenase system is based on the plotting of $I\frac{\alpha}{1-\alpha}$ against [S].

The calculation of $I\frac{\alpha}{1-\alpha}$ is based on the following data.

$I = 1 \times 10^{-3}$ M N,N'-diallyldithiooxamide

$\alpha = V_1/V$

V = Control rate

V_1 = Inhibited rate

Experiment I

Coenzyme I, 0.5 μM/ml. [S]

$V = 282$

$V_1 = 114.5$

$I\frac{\alpha}{1-\alpha} = 0.68 \times 10^{-3}$ M

Experiment II

Coenzyme I, 1.0 μM/ml. [S]

$V = 320$

$V_1 = 168$

$I\frac{\alpha}{1-\alpha} = 1.08 \times 10^{-3}$ M

Experiment III

Coenzyme I, 1.5 μM/ml. [S]

$V = 321$

$V_1 = 204$

$I\frac{\alpha}{1-\alpha} = 1.74 \times 10^{-3}$ M

B. Noncompetitive Relationship of N,N'-Diallyldithiooxamide and Coenzyme I in the Alcohol Dehydrogenase System (Figure 11, page 50)

The plotting of $I\frac{\alpha}{1-\alpha}$ against [S] is based on the following data from experiments in which:

I = 4×10^{-5} M N,N'-diallyldithiooxamide

Enzyme = 0.5 cc of 1.4 micrograms/ml.

[S] = conc. of coenzyme I, varied

V = control rate

V_1 = inhibited rate

Experiment I

Coenzyme I, 600 micrograms/ml.

V = 0.60 Δ optical density/minute

V_1 = 0.49 Δ optical density/minute

$I\frac{\alpha}{1-\alpha} = 1.92 \times 10^{-4}$ M

Experiment II

Coenzyme I, 1200 micrograms/ml.

V = 0.71 Δ optical density/minute

V_1 = 0.53 Δ optical density/minute

$I\frac{\alpha}{1-\alpha} = 1.6 \times 10^{-4}$ M

Experiment III

Coenzyme I, 2400 micrograms/ml.

V = 0.71 Δ optical density/minute

V_1 = 0.54 Δ optical density/minute

$I\frac{\alpha}{1-\alpha} = 1.6 \times 10^{-4}$ M

Experiment IV

Coenzyme I, 3600 micrograms/ml.

V = 0.150 Δ optical density/minute

$$V_1 = 0.106 \ \Delta \text{optical density/minute}$$

$$I\frac{\alpha}{1-\alpha} = 1.08 \times 10^{-4} M$$

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