IN VITRO INHIBITION OF LIVER ALDEHYDE DEHYDROGENASE BY TETRAETHYLTHIURAM DISULPHIDE

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Several investigators[1,2,3,4,5] have reported that a characteristic syndrome develops in man or lower mammals upon the ingestion of alcohol after prior dosage with tetraethylthiuram disulphide. It has been suggested[3,4,5] and experimental evidence has been offered in substantiation[6], that these symptoms, at least in large part, are due to the accumulation of acetaldehyde in the body. This suggests that this substance interferes with the normal course of alcohol metabolism by slowing the action of the aldehyde oxidase system.

Edwards[7] investigated its effect on the respiration of tissue homogenates and reported that the drug, at what probably was a relatively high concentration, inhibited endogenous respiration by about 85 per cent. He suggested that it broke down to yield cyanide or cyanate which brought about the inhibition. Further experiments by Nowinski *et al.*[8], illustrated the inhibition by the drug of alcohol oxidation by rat liver homogenate. This inhibition was overcome by the addition of large amounts of ascorbic acid. It was postulated that tetraethylthiuram disulphide acted as a competitive hydrogen acceptor and that ascorbic acid overcame the inhibition by acting as an additional hydrogen donor in the system. The investigation, the results of which are presented here, was undertaken with the purpose of further elucidating the mechanism of action of tetraethylthiuram disulphide.

Part I. Homogenate Studies

Experimental Method and Results. In the present study, normal rat liver was homogenised in physiological saline solution with the Potter and Elvehjem apparatus[9] and was diluted to 5 per cent.. w/v concentration. Using the modified Thunberg technique of Friedemann and Hollander[10], the effects of incubating this homogenate with tetraethylthiuram disulphide prior to the addition of various substrates were tested. By this means it was shown (see Table I) that incubating for 40 minutes at 38°C. with 1·0 mg. of the drug per ml. suspended in the homogenate, inhibited the subsequent breakdown of endogenous metabolites and of added citrate, succinate, and ethanol.

Table II shows that when succinate was used as substrate, increasing the duration of contact between homogenate and tetraethylthiuram disulphide prior to substrate addition, increased the degree of inhibition produced. It will be observed that inhibition was more marked where the

homogenate was more dilute; a fact which may be explained in part on the basis that the reaction rate was slower and therefore duration of contact between enzymes and the drug was greater. Inhibition was observed to increase with increasing amounts of added drug.

TABLE I

THE EFFECT OF TETRAETHYLTHIURAM DISULPHIDE ON THE ACTIVITY OF RAT LIVER HOMOGENATE

Substrate	Inhibition (per cent.)
Endogenous	50
0·4M citrate	40
0·4M succinate	63
0·1M ethanol	77

Table I.—5 per cent. w/v of rat liver homogenate was incubated for 40 minutes at 38°C. prior to use. The control contained no additions while the test homogenate contained 1·0 mg. of tetraethylthiuram disulphide per ml. as a suspension. The digest consisted of 1·0 ml. of homogenate, 0·5 ml. of substrate, 0·5 ml. of 0·002 M methylene blue, 0·5 ml. of distilled water, and 2·5 ml. of 2 per cent. w/v agar buffered to *p*H 7·4 with phosphate. Decolorisation of the methylene blue was followed visually for 120 minutes: The results are expressed as percentage inhibition from the formula $\left\{1 - \frac{\text{control reduction time}}{\text{test reduction time}}\right\} \times 100$

TABLE II

EFFECT OF INCREASED CONTACT TIME OF VARIOUS CONCENTRATIONS OF TETRAETHYLTHIURAM DISULPHIDE ON THE DEHYDROGENATION OF SUCCINATE BY RAT LIVER HOMOGENATE

Homogenate per cent. (w/v)	Tetraethylthiuram disulphide mg./ml. of homogenate	Incubation time in minutes				
		0	15	30	45	60
		Inhibition (per cent.)				
2·5...	0·0	0	3	0	6	3
2·5...	0·25	10	22	39	61	87
2·5...	0·5	18	33	60	>90	>90
2·5...	1·0	20	51	89	>90	>90
2·5...	2·0	20	54	>90	>90	>90
5·0...	0	0	0	15	15	8
5·0...	0·25	0	15	21	31	52
5·0...	0·5	0	21	21	45	71
5·0...	1·0	0	21	31	54	77
5·0...	2·0	0	27	52	82	90

Table II.—The system consisted of 1·0 ml. of 5 per cent. w/v rat liver homogenate, 0·5 ml. of 0·4 M succinate, 0·5 ml. of 0·002 M methylene blue, 0·5 ml. of distilled water, and 2·5 ml. of 2 per cent. w/v agar solution buffered to *p*H 7·4 with phosphate. Tetraethylthiuram disulphide was added to the test samples of homogenate as a dry powder, suspended by shaking and incubated at 38°C. for the times indicated prior to addition to the system. Reduction of the dye was followed for 120 minutes.

Similar results were obtained when the tetraethylthiuram disulphide was added to the homogenate in propylene glycol solution. As might be expected, the drug when added by this means was a more potent inhibitor

than when added in solid form. Significant inhibition of acetaldehyde breakdown was produced by a 1×10^{-5} M concentration when the inhibitor was added at zero time. These data are shown in Figure I. It will be noted that reduced glutathione can reverse, in large degree, the inhibition by tetraethylthiuram disulphide of acetaldehyde oxidation in rat liver homogenate. With other substrates, similar reversal of inhibition by reduced glutathione was observed.

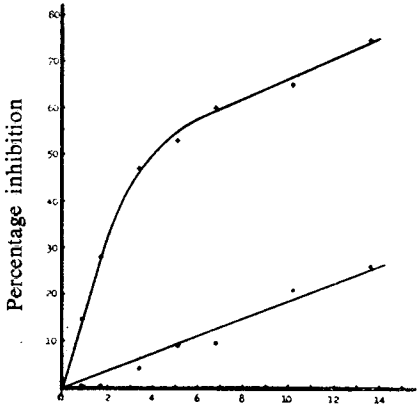

FIG. 1.—1·0 ml. of aged 5 per cent. rat liver homogenate, 0·3 ml. of 0·5 M acetaldehyde, 0·2 ml. of 2 mg./ml. crude diphosphopyridine nucleotide solution, 0·1 ml. of 2 per cent. nicotinamide, 0·1 ml. of propylene glycol containing tetraethylthiuram disulphide, 0·3 ml. of distilled water, 0·5 ml. of 0·002 M methylene blue, and 2·5 ml. of 2 per cent. agar buffered to *p*H 8 with phosphate. Reduced glutathione 0·1 ml. of 20 mg./ml. was added (lower graph) at the expense of water (total volume 5·0 ml.).

These studies served to indicate that tetraethylthiuram disulphide could inhibit the *in vitro* oxidation of a considerable range of substrates by rat liver homogenate. However, the results were rather variable and the inhibition seemed to be rather non-specific. The degree of inhibition produced in fresh homogenate was generally less than that produced in 1 to 2 day old preparations and, in the case of ethanol or acetaldehyde oxidation, inhibition by a given concentration of tetraethylthiuram disulphide was less when diphosphopyridine nucleotide* was added to the reaction. These inhibitions were largely reversed by the addition of reduced glutathione suggesting that tetraethylthiuram disulphide at these concentrations was a non-specific inhibitor acting as an oxidiser of essential -SH groups.

PART II. PARTIALLY PURIFIED ENZYME STUDIES

Experimental Methods and Results. Attention was turned to the theory that acetaldehyde metabolism was disrupted by tetraethylthiuram disulphide. Kjeldgaard[12] has investigated the mechanism of action of the drug and has presented evidence that, at 1 in 1×10^7 dilution, it will inhibit the activity of a liver aldehyde dehydrogenase preparation by some 50 per cent. In the work with homogenates described above very considerably higher concentrations were required. At the time Kjeldgaard's paper appeared, similar work was in progress in this laboratory and results have been obtained which corroborate Kjeldgaard's findings in some degree. Further, the mechanism of the *in vitro* inhibi-

* Schwarz diphosphopyridine nucleotide was used throughout. Analysis according to the method of Green and Dewan[11] indicated the presence of 49 per cent. of diphosphopyridine nucleotide.

tion of liver aldehyde dehydrogenase by tetraethylthiuram disulphide has been elucidated.

The liver aldehyde dehydrogenase of Gordon *et al.*[13] used by Kjeldgaard[12] was believed not to require a coenzyme for its activity. As prepared by this method, the enzyme was found, in this laboratory, to require added diphosphopyridine nucleotide for maximum activity as measured by Thunberg or Warburg techniques. The preparation did, however, have a very significant activity in the absence of added diphosphopyridine nucleotide. When the enzyme was tested for its sensitivity to tetraethylthiuram disulphide, inhibition at the levels indicated by Kjeldgaard's work could not be demonstrated, particularly in the presence of added disphosphopyridine nucleotide.

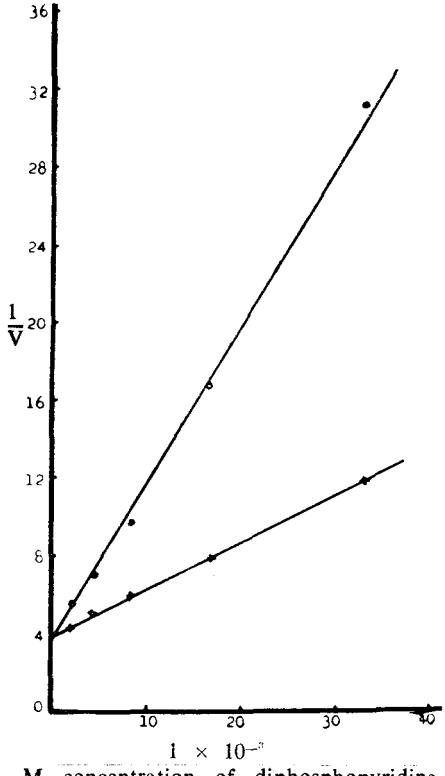

FIG. 2.—1·0 ml. of enzyme solution, 0·1 ml. of 0·05 M acetaldehyde, 3·0 ml. of 0·1 M pyrophosphate buffer *p*H 9·3, varying amounts of diphosphopyridine nucleotide solution and water to make 5·0 ml. Tetraethylthiuram disulphide was added (upper graph) at the expense of water to give a final concentration of $3{\cdot}4 \times 10^{-6}$ M. Readings of optical density at 340mμ were made 9 minutes after addition of the enzyme.

The Racker aldehyde dehydrogenase of liver[14] has been shown to require diphosphopyridine nucleotide. Using this enzyme it was possible to demonstrate a variable degree of inhibition by tetraethylthiuram disulphide depending entirely upon the concentration of added diphosphopyridine nucleotide. These results are shown in Table III.

An inhibition of 50 per cent. at a 1 in 2×10^7 dilution of tetraethylthiuram disulphide has been found when the diphosphopyridine nucleotide concentration was $1{\cdot}5 \times 10^{-5}$ M. From Figure 2, it may be seen that the former apparently is a competitive inhibitor, competing with the latter for the active centres of the enzyme. It would appear that the aldehyde dehydrogenase preparation of Gordon *et al.*[13] contains variable amounts of diphosphopyridine nucleotide. In Kjeldgaard's preparation[12], the concentration must have been lower than in the similar preparation made here.

The data in Table III have been used to calculate the Michaelis and Menten constants[15], Km and Ki: the latter value has been derived also

through use of the formulæ of Lineweaver and Burk[16]. The values obtained are listed in Table IV. From other data, Figure 3, the Km for the enzyme-acetaldehyde complex has been calculated and also is reported in Table IV. The inhibition in this case is non-competitive. It may be

TABLE III

EFFECT OF DIPHOSPHOPYRIDINE NUCLEOTIDE CONCENTRATION ON THE DEGREE OF INHIBITION OF LIVER ACETALDEHYDE DEHYDROGENASE PRODUCED BY TETRAETHYLTHIURAM DISULPHIDE

Tetraethylthiuram disulphide addition final concentration M × 10^6	Diphosphopyridine nucleotide final concentration M × 10^6				
	30	60	120	240	480
0	84*	129	170	200	237
3·4	32 (62)†	62 (52)	104 (39)	144 (28)	185 (22)
0·68	42 (50)	87 (33)	118 (30)	164 (18)	232 (2)

* Optical density × 1000. † Per cent. inhibition.

Table III.—Digest contained 1·0 ml. of enzyme solution in 0·1 M pyrophosphate buffer *p*H 9·3, 3·0 ml. of buffer, 0·1 ml. of 0·05 M acetaldehyde, varying amounts of diphosphopyridine nucleotide solution and distilled water to make 5·0 ml. Tetraethylthiuram disulphide in fresh aqueous solution was added at the expense of water. (The solutions were prepared by initially dissolving the drug in propylene glycol, 1 mg. per ml., and diluting this preparation, with rapid mixing, with water. Homogeneous solutions containing 0·01 mg./ml. could be thus prepared.) Readings were taken at 1·5 minute intervals for 15 minutes after the addition of the enzyme in the Beckman DU spectrophotometer at 340 μm against a blank lacking only acetaldehyde. Optical density changes usually followed a linear path for 10 to 12 minutes and the readings used throughout this work were those made 9 minutes after the addition of the enzyme.

TABLE IV

KINETIC DATA ON ACETALDEHYDE DEHYDROGENASE OF LIVER

Km (acetaldehyde)	$5·0 \times 10^{-4}$M
Km (diphosphopyridine nucleotide)	$6·4 \times 10^{-5}$M
Ki Michaelis-Menten	$1·7 \times 10^{-6}$M
Lineweaver-Burk	$1·4 \times 10^{-6}$M

seen that the affinity of the enzyme protein for its coenzyme is about 10 times its affinity for acetaldehyde.

The affinity of the enzyme for tetraethylthiuram disulphide is much greater than that for diphosphopyridine nucleotide (46 times) or for acetaldehyde (357 times). This high degree of affinity of the enzyme for its inhibitor is rather a rare occurrence. The measured tetraethylthiuram disulphide/diphosphopyridine nucleotide ratio of 1/46 compares closely with the malonate/succinate ratio observed by Potter and Du Bois[17]. Competition between coenzyme and inhibitor rather than between substrate and inhibitor has been reported previously by Hellerman *et al.*[18] and by Euler[19]. It would appear that these results might explain some of the variability observed in the response of homogenates to tetraethylthiuram disulphide where diphosphopyridine nucleotide content will depend on the type of tissue, the age of the homogenate and the fineness of grind[20,21]. It is interesting to note that Bergstermann[22]

found that a similar situation obtained in the case of triose phosphate dehydrogenase where the addition of diphosphopyridine nucleotide greatly increases the resistance of this enzyme to selenite poisoning.

When acetaldehyde and diphosphopyridine nucleotide concentrations were held constant and tetraethylthiuram disulphide concentration was varied, a curve illustrated by Figure 4 was obtained.

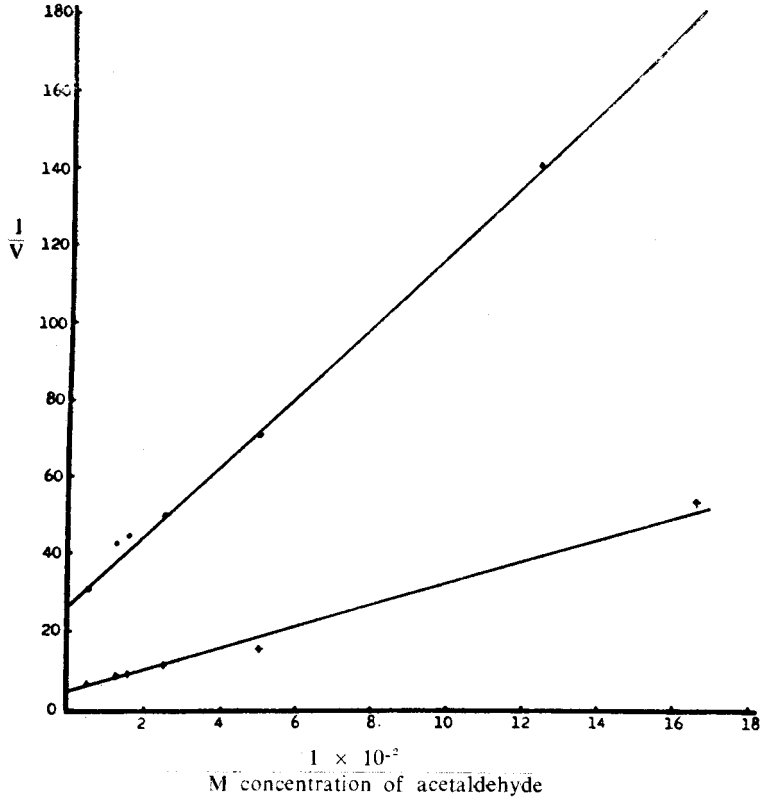

FIG.—1·0 ml. of enzyme solution, 0·2 ml. of 4 mg. per ml. crude diphosphopyridine nucleotide solution, 3·0 ml. of 0·1 M pyrophosphate buffer *p*H 9·3, varying amounts of acetaldehyde solution and water to make 5·0 ml. Tetraethylthiuram disulphide was added (upper graph) at the expense of water to give a final concentration of $4{\cdot}1 \times 10^{-6}$ M. Readings of optical density at 340mμ were made 9 minutes after addition of the enzyme.

These data indicate that at suboptimal concentrations of diphosphopyridine nucleotide the inhibited system operated in Zone B of Straus and Goldstein[23]. However, the addition of optimal amounts returned the system toward operation in Zone A. Determination of the rate of reaction at different enzyme concentrations with and without inhibitor in the manner described by Ackermann and Potter[24] indicated that tetraethylthiuram disulphide inhibition is of the pseudo-irreversible type at suboptimal concentrations of diphosphopyridine nucleotide. Conse-

quently the Ki reported in Table IV may be erroneous. From calculations based on the methods of Straus and Goldstein values of Ki of about 1/10th this magnitude were obtained. Allowing the enzyme to remain in contact with various concentrations of tetraethylthiuram disulphide for one half-hour prior to the addition of substrate had no effect on the degree of inhibition produced.

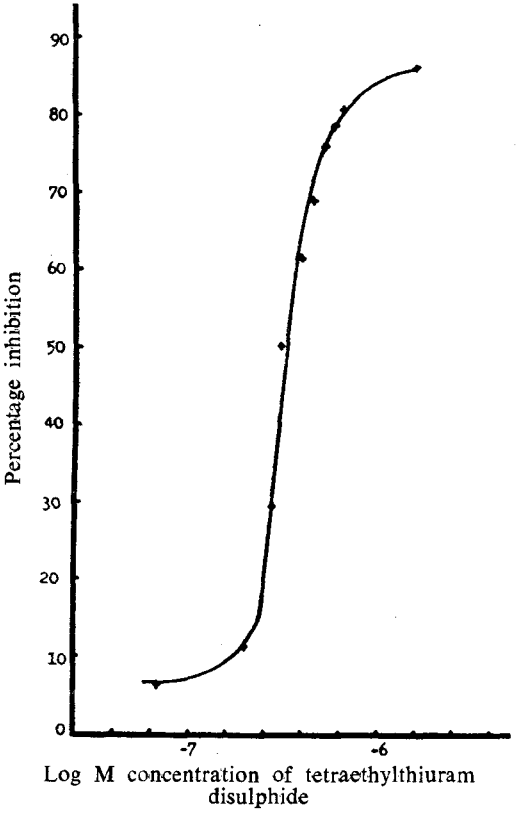

FIG. 4.—1·0 ml. of enzyme solution, 0·2 ml. of 4 mg. per ml. crude diphosphopyridine nucleotide solution, 3·0 ml. of 0·1 M pyrophosphate buffer *p*H 9·3, 0·2 ml. of 0·1 M. acetaldehyde, varying amounts of tetraethylthiuram disulphide solution and water to make 5·0 ml. Readings of optical density at 340mμ were made 9 minutes after addition of the enzyme.

It was previously shown that reduced glutathione would reverse the inhibitory effect of tetraethylthiuram disulphide on the dehydrogenation of various substrates by rat liver homogenate. With the purified aldehyde dehydrogenase system of Gordon *et al.*[13] Kjeldgaard[12] reported that reduced glutathione had no effect on the inhibition produced by tetraethylthiuram disulphide. This finding is at variance with the results obtained in this laboratory. Reduced glutathione at a very low concentration was able to reverse the effect of tetraethylthiuram disulphide on the Racker aldehyde dehydrogenase of liver as is illustrated in Figure 5. To restore the enzyme to 50 per cent. activity a molar ratio of reduced glutathione to tetraethylthiuram disulphide of only 3 to 1 was required under the conditions employed.

Ascorbic acid has been found to be effective in alleviating tetraethylthiuram disulphide-alcohol toxicity[2] and in offsetting the inhibitory effect on tissue metabolism[8]. The latter effect was confirmed in the present study and further investigated on the semi-purified Racker enzyme. The results illustrated in Figure 6 show that, while ascorbic acid tended to relieve the inhibition, it was required in rather high concentration. These results indicate that ascorbic acid is much less specific in its reversal of

tetraethylthiuram disulphide inhibition than is reduced glutathione and, to restore the enzyme to 50 per cent. activity, a molar ratio of ascorbic acid to tetraethylthiuram disulphide of 1700 to 1 was required.

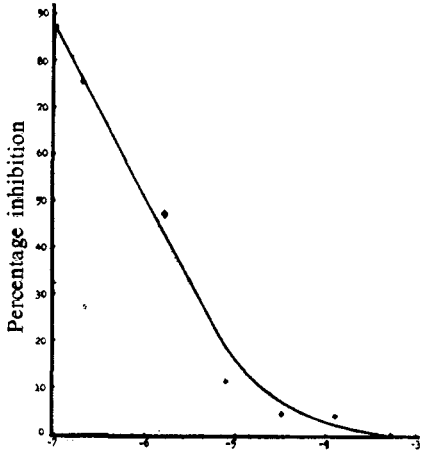

FIG. 5.—1·0 ml. of enzyme solution, 0·2 ml. of 4 mg./ml. crude diphosphopyridine nucleotide solution, 0·2 ml. of 0·1 M acetaldehyde, 3·0 ml. of 0·1 M pyrophosphate buffer *p*H 9·3, tetraethylthiuram disulphide solution to give a final concentration of $6·8 \times 10^{-7}$ M and water to make 5·0 ml. Reduced glutathione solution was added at the expense of water. Readings of optical density at 340mμ were made 9 minutes after addition of the enzyme.

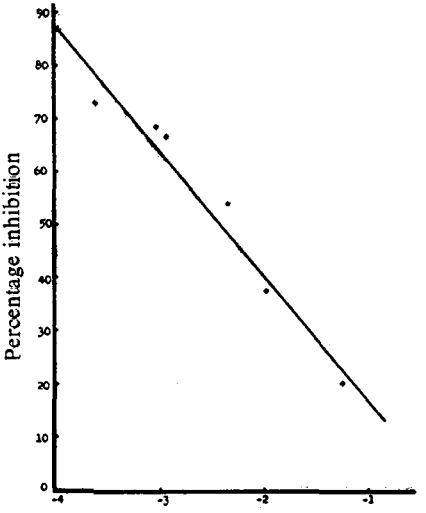

FIG. 6.—1·0 ml. of enzyme solution, 0·2 ml. of 4 mg./ml. crude diphosphopyridine nucleotide solution, 0·2 ml. of 0·1 M acetaldehyde, 3·0 ml. of 0·1 M pyrophosphate buffer *p*H 9·3, tetraethylthiuram disulphide solution to give a final concentration of $6·8 \times 10^{-7}$ M and water to make 5·0 ml. Ascorbic acid solution, freshly prepared and neutralised, was added at the expense of water. Readings of optical density at 340mμ were made 9 minutes after addition of the enzyme.

SUMMARY

1. The dehydrogenation of various substrates by normal rat liver homogenate was inhibited by tetraethylthiuram disulphide. This effect was largely reversed by reduced glutathione.

2. Acetaldehyde dehydrogenase of liver was found to be very strongly inhibited by low concentrations of tetraethylthiuram disulphide. It was found that this substance apparently acted as a competitive inhibitor, competing with diphosphopyridine nucleotide for the active centres of the enzyme. The affinity of the enzyme protein for tetraethylthiuram disulphide was approximately 50 times its affinity for reduced glutathione and 350 times its affinity for acetaldehyde.

3. The inhibitory effect of tetraethylthiuram disulphide on acetaldehyde dehydrogenase of liver was reversed by low concentrations of reduced glutathione and less completely by relatively high concentrations of ascorbic acid.

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