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INHIBITION OF HEXOKINASE BY DISULFIRAM AND DIETHYLDITHIOCARBAMATE

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Abstract—The inhibitory effect of disulfiram and of diethyldithiocarbamate on yeast hexokinase as well as on brain hexokinase has been studied. The results demonstrate that disulfiram is a potent inhibitor of hexokinase. Diethyldithiocarbamate inhibits the enzyme only when it is oxidized to disulfiram. Such an oxidation can be obtained by adding oxidized cytochrome *c* to the system. Reduced glutathione prevents the inhibition. Complete reactivation is obtained by cysteamine in small concentrations, indicating that the inhibition is due to a blocking of essential —SH groups on hexokinase. The results are discussed in relation to the therapeutic use of disulfiram.

WITHIN a short time after the introduction of tetraethylthiuram disulphide (disulfiram, Antabuse) as an adjuvant in the treatment of alcoholism[1] its mode of action seemed established. Based on enzyme studies[2–5] and supported by *in vivo* studies[6] it was assumed that the fundamental action of the drug was a disulphide inhibition of enzymes involved in the metabolism of ethanol. A secondary accumulation of acetaldehyde appeared to be responsible for the symptoms occurring after the administration of disulfiram and ethanol.[7] Subsequent studies, however, have thrown doubt on this simple picture of disulfiram action, and especially on the assumption that acetaldehyde alone can explain the symptoms.[8–10]

Since the basis for the theory outlined is an *in vivo* disulphide inhibition of one or several enzymes, the presence of disulfiram in the form of disulphide is required. Eldjarn[11] demonstrated, however, that an *in vivo* reduction of disulfiram takes place, and Linderholm and Berg[12] were not able to find non-reduced disulfiram at all in mammals receiving this drug. Subsequent studies have demonstrated that disulfiram readily is reduced by glutathione[13] and probably also by free —SH groups of proteins.[14] Recently, erythrocytes have been shown to possess a considerable disulphide reducing capacity, as have probably most cells containing the glutathione–glutathione reductase system.[15] The observation that diethyldithiocarbamate, when administered parenterally, has about the same clinical effect as has disulfiram[16] demonstrate that such initial *in vivo* reduction of the disulphide does not destroy the therapeutic effect of the drug. If the fundamental action of disulfiram, therefore, still should be based on a disulphide inhibition of enzymes involved in the metabolism of ethanol, a mechanism for re-oxidation of the thiol must exist in the cells.

Dithiocarbamates have attracted attention also because of their proven radioprotective properties.[17, 18] The optimal dose reduction factor of diethyldithiocarbamate in mice, with respect to the deleterious action of X-rays, is about 1·4, which classifies

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this thiol among the best chemical protectors known. The mechanism underlying chemical protection against ionizing radiation is still obscure. Although radiochemical reactions are supposed to play a major role in this protection, the possibility has been proposed that chemical protectors acts by altering the metabolism and the chemical composition of cells, thus bringing the tissues in a more radioresistent phase.[19] The biochemical effects of diethyldithiocarbamate, therefore, may be of interest also from this point of view.

In the present report the enzyme-inhibitory effect of disulfiram as well as of diethyldithiocarbamate in connexion with a possible physiological oxidizing agent has been studied. We have found both crystalline yeast hexokinase and crude particulate brain hexokinase to be suitable "marker" enzymes for these studies.

EXPERIMENTAL

Materials

Crude calf brain hexokinase, prepared by the method of Crane and Sols,[20] were kindly supplied by Dr. Ragnar Nesbakken of this Institute. The hexokinase preparation was dispersed in equal volumes of histidine–Tris–EDTA buffer (respective concentrations: 10^{-2} M, 10^{-2} M, 2×10^{-3} M) and glycerol. The specific activity of this dispersion was about 0·47 units/mg protein, defined as μmole substrate converted per mg protein per min at 37 °C.[21] Crystalline yeast hexokinase (specific activity given to be about 140 units[21]/mg protein) suspended in 3·0 M ammonium sulphate solution, as well as glucose-6-phosphate dehydrogenase (specific activity given to be about 70 units[21]/mg protein) suspended in 3·3 M ammonium sulphate solution, were obtained from Boehringer et Soehne, Mannheim, Western Germany. NADP, ATP, GSH and crystalline human albumin were purchased from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. Crystalline horse heart cytochrome *c* was obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. This cytochrome *c* was found to be 100 per cent in the oxidized form, as determined spectrophotometrically. Sodium diethyldithiocarbamate was obtained from E. Merck, AG, Darmstadt, Western Germany. Tetraethylthiuram disulphide (disulfiram) was prepared by oxidizing diethyldithiocarbamate by alcoholic iodine. The compound was washed with water and recrystallized three times from absolute alcohol to give white to faintly yellow crystalline needles with m.p. 70·5 °C.

Methods

The activity of hexokinase was evaluated by measuring the generation of $NADPH_2$ as the glucose-6-phosphate formed in the hexokinase reaction was enzymatically oxidized to 6-phosphogluconate. The increase in $NADPH_2$ was followed continuously in a Zeiss RPQ 20A recording spectrophotometer at 340 mμ and at 37 °C. The quartz cell contained: $3{\cdot}5 \times 10^{-1}$ M glucose, 10^{-2} M $MgCl_2$, $2{\cdot}8 \times 10^{-3}$ M ATP, 10^{-3} M NAOP and 2·5 μl (about 0·175 units[21]) glucose 6-phosphate dehydrogenase in 1·4 ml 4×10^{-2} M Tris(hydroxymethyl)aminomethan buffer pH 7·45. The blank cell contained the same except for the dehydrogenase and ATP. After temperature equilibration the reaction was started by adding to both cells about 2×10^{-2} units[21] hexokinase (20 μl of the hexokinase suspension to be tested). The reaction was followed for from 6 to 10 min. The glucose-6-phosphate dehydrogenase contained traces of hexokinase

(enough to give a change in $E_{340\ m\mu}$ of 0·005 per min in the system used) which was corrected for.

The enzyme-inhibitory effect of disulfiram and of diethyldithiocarbamate was tested by preincubating a hexokinase suspension (enzyme activity about 1 unit[21]/ml suspension) in the presence of these compounds. To obtain this enzyme concentration, the original enzyme suspensions were diluted with Tris buffer, 4×10^{-2} M, pH 7·6. The suspension of yeast hexokinase contained in addition glucose ($2{\cdot}8 \times 10^{-1}$ M) and human albumin ($1{\cdot}3 \times 10^{-3}$ M) in order to protect the enzyme from spontaneous inactivation during the incubation.

The hexokinase activity was measured immediately after the addition of the compounds to be tested and at suitable time intervals during the incubation. The activities are given as per cent of the initial activity, which never differed significantly from that of the controls without inhibitors. Due to the slight solubility of disulfiram in polar solvents, this compound was added to the preincubation mixture dissolved in equal volumes of Tris buffer and absolute ethanol. Thus, after the addition the enzyme suspension contained 2% ethanol which *per se* did not influence the activity of the hexokinase.

RESULTS

Inhibition of hexokinase by diethyldithiocarbamate

Slight inhibition of yeast hexokinase is observed with diethyldithiocarbamate in high concentrations. However, the addition of oxidized cytochrome *c* increases the inhibition several hundred times. Fig. 1 shows that in the presence of 5×10^{-4} M cyto-

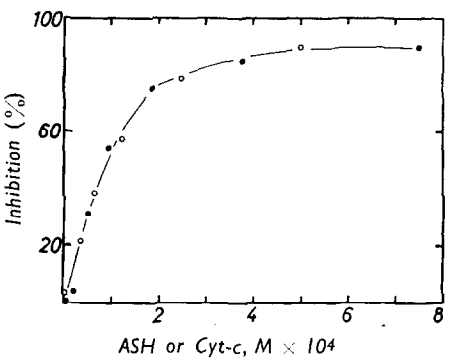

FIG. 1. Inhibition of yeast hexokinase by a mixture of diethyldithiocarbamate (ASH) and cytochrome *c*. The enzyme was preincubated for 30 min at 37 °C as follows:
●, cytochrome *c* (constant, 5×10^{-4} M) plus diethyldithiocarbamate in increasing concentrations;
○, diethyldithiocarbamate (constant, $7{\cdot}5 \times 10^{-4}$ M) plus cytochrome *c* in increasing concentrations.

chrome *c*, diethyldithiocarbamate in a concentration of 10^{-4} M causes a 50% inhibition of yeast hexokinase within 30 min of incubation. The inhibition develops gradually with time (Fig. 2).

Diethyldithiocarbamate alone is found to have a much stronger inhibitory effect on crude brain hexokinase than on yeast hexokinase. Thus, within 30 min of incubation a 50% inhibition is reached with diethyldithiocarbamate in a concentration of

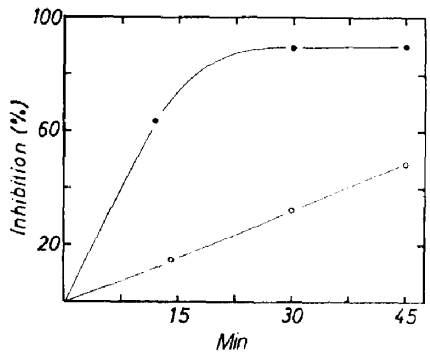

FIG. 2. Inhibition of yeast hexokinase by diethyldithiocarbamate as a function of time. The enzyme was preincubated at 37 °C as follows:
●, diethyldithiocarbamate (7.5×10^{-4} M) plus cytochrome *c* (5×10^{-4} M);
○, diethyldithiocarbamate (5×10^{-5} M) plus cytochrome *c* (10^{-3} M).

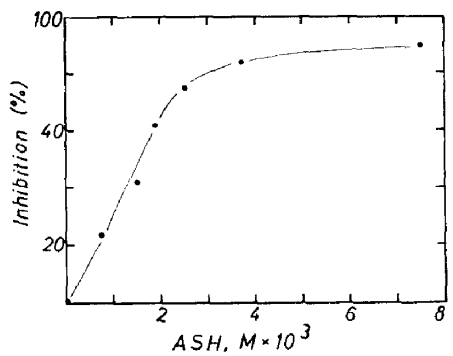

FIG. 3. Inhibition of crude calf brain hexokinase by increasing concentrations of diethyldithiocarbamate (ASH). The enzyme was preincubated at 37 °C for 30 min.

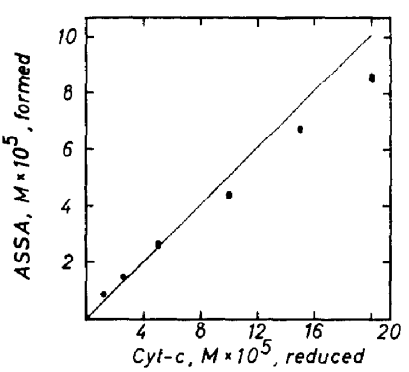

FIG. 4. Amounts of disulfiram (ASSA) formed when diethyldithiocarbamate (in excess) reacts with increasing amounts of cytochrome *c*. The expected amount of disulfiram formed if every 2 mole of cytochrome *c* reduced give rise to 1 mole of disulfiram is given by the continuous curve.

about $1{\cdot}6 \times 10^{-3}$ M (Fig. 3). Here also a significant increase in the inhibition is observed by the addition of cytochrome *c* (see below, Fig. 5).

Figure 1 also shows that essentially identical inhibition curves for yeast hexokinase are obtained whether the concentration of diethyldithiocarbamate is increased in the presence of an excess of cytochrome *c*, or vice versa. These findings may be explained

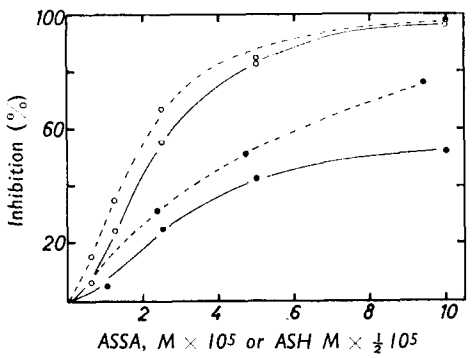

FIG. 5. Inhibition of yeast hexokinase and of brain hexokinase by increasing concentrations of disulfiram (ASSA, continuous curves) and by twice these concentrations of diethyldithiocarbamate (ASH) plus an excess of cytochrome *c* (5×10^{-4} M) (broken curves):
●, yeast hexokinase;
○, brain hexokinase.

by the assumption that the inhibitory agent is an oxidation product of diethyldithiocarbamate since it has been previously shown that this compound reduces cytochrome *c*.[22] The reaction product to be expected is disulfiram.

Oxidation of diethyldithiocarbamate to disulfiram by cytochrome c

For the identification and quantitative determination of the oxidation product of diethyldithiocarbamate the following procedure was used. Varying amounts of oxidized cytochrome *c* dissolved in Tris buffer (4×10^{-2} M, pH 7·6) was incubated at 37 °C for 15 min in the presence of excess diethyldithiocarbamate. After the incubation the cytochrome *c* was found spectrophotometrically to be completely reduced. The incubation solution was extracted with ½ vol. of carbon tetrachloride, which afterwards demonstrated the typical spectrophotometric absorbtion curve of disulfiram. As a blank the carbon tetrachloride extract from a solution of the same amount of diethyldithiocarbamate was used, but without cytochrome *c*. Fig. 4 shows that the amount of disulfiram formed in the diethyldithiocarbamate–cytochrome *c* solution correlates well with the amount of cytochrome *c* simultaneously reduced.

The oxidation of diethyldithiocarbamate (ASH) by cytochrome *c* probably proceeds according to the following equations:

(1) $$AS^- + \text{cyt-}c^{3+} \rightarrow AS + \text{cyt-}c^{2+}$$

(2) $$2\,AS \rightarrow ASSA$$

Thus either disulfiram (ASSA) or the free radical (AS·) may represent the oxidation product responsible for the enzyme-inhibitory effect of the diethyldithiocarbamate–cytochrome *c* system. The radical as inhibitor is made unlikely by the fact that the

active agent is stable for hours, as demonstrated by the following experiment. When preincubating a solution of diethyldithiocarbamate plus cytochrome *c* at 37 °C for more than 2 hr before the hexokinase is added, the same percentage inhibition is obtained as when the oxidation takes place in the presence of the enzyme. It should be noted that under the same conditions we found the oxidation of diethyldithiocarbamate to be complete within a few minutes. Apparently, the inhibition may be ascribed to disulfiram alone.

Inhibition of hexokinase by disulfiram

Disulfiram is found to be a potent inhibitor of hexokinase (Fig. 5). With the standard enzyme concentration used, a 50 per cent inhibition of brain hexokinase and of yeast hexokinase is reached within 30 min of incubation by disulfiram in concentrations of 2×10^{-5} M and 8×10^{-5} M, respectively. In the case of brain hexokinase an almost complete inhibition is obtained when the concentration of inhibitor is increased. Due to the slight solubility of disulfiram, a corresponding increase in the inhibition of yeast hexokinase could only be obtained when the time of incubation is increased (Fig. 6).

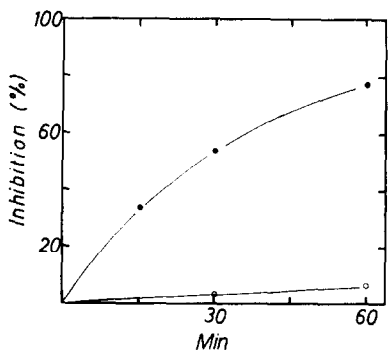

FIG. 6. Inhibition of yeast hexokinase by disulfiram as a function of the time. The enzyme was incubated at 37 °C as follows:
●, disulfiram (10^{-4} M);
○, nil (control).

The experiments presented in Fig. 5 confirm the assumption that disulfiram is the inhibitory agent in the diethyldithiocarbamate–cytochrome *c* system. Thus, almost identical inhibitions are obtained whether disulfiram is added as such, or is formed *in loco* by diethyldithiocarbamate plus cytochrome *c*. As is to be expected, the latter system is somewhat more potent in the saturation range of disulfiram.

Protection by reduced glutathione and reactivation by cysteamine

Fig. 7 shows that the inhibition of yeast hexokinase is completely abolished by the presence of GSH in a concentration of 5×10^{-4} M, i.e. equivalent with the amount of disulfiram formed in the inhibitory system. This is in agreement with the conclusion that disulfiram is the active agent, since the disulfiram formed at once will be reduced by GSH.[13]

The results of the reactivation tests are given in Table 1. GSH gives a slight but significant reactivation of the inhibited yeast hexokinase. Complete reactivation is obtained by cysteamine in concentrations as small as 3×10^{-3} M. Even with 10^{-3} M cysteamine the activity of the enzyme increased from 17 to 76 per cent during $\frac{1}{2}$ hr of incubation.

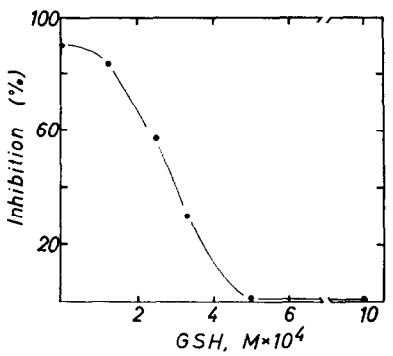

FIG. 7. Protective effect of GSH against inhibition of yeast hexokinase by diethyldithiocarbamate ($7{\cdot}5 \times 10^{-4}$ M) plus cytochrome *c* (5×10^{-4} M). Time of incubation at 37 °C was 30 min. Glutathione and the other compounds were added immediately prior to the incubations.

Brain hexokinase inhibited by diethyldithiocarbamate alone (Fig. 3) is also reactivated by cysteamine, and to the same degree as when the inhibition is caused by disulfiram (Table 1). Consequently, the two compounds probably inhibit the enzyme by the same mechanism. Possibly the inhibition by diethyldithiocarbamate is due to the presence of some oxidizing compounds (cytochromes, methaemoglobin) in the crude enzyme extract.

DISCUSSION

In the present paper we have reported that disulfiram is a potent inhibitor of hexokinase. Diethyldithiocarbamate inhibits the enzyme only when it is oxidized to disulfiram. Such an oxidation is obtained by adding oxidized cytochrome *c* to the system.

Disulphides such as cystamine have been shown to react with protein —SH groups by mixed disulphide formation.[14] Recently in this laboratory cystamine has been found to possess a similar inhibitory effect on hexokinase as has disulfiram.[23] It seems reasonable to believe, therefore, that disulfiram blocks essential —SH groups on the enzyme by mixed disulphide formation. The fact that complete reactivation is obtained by cysteamine in small concentrations support this conclusion. Previous studies with strong SH-blocking agents such as *p*-chloromercuribenzoate on yeast hexokinase[24] as well as on brain hexokinase[25] have indicated that free —SH groups are essential for the activity. The observation here reported that some disulphides, which are certainly more lenient and specific SH-blocking agents,[26] also inhibits hexokinase confirms this assumption.

For reasons given in the introduction we assume that soon after administration to mammals disulfiram will be reduced to the corresponding thiol, diethyldithiocarbamate. Here we have shown that this latter, relatively indifferent, compound is readily

TABLE 1. REACTIVATION OF INHIBITED HEXOKINASE BY REDUCED GLUTATHIONE AND CYSTEAMINE

Enzyme inhibition was obtained by 30 min of incubation (37° C) in the presence of the inhibitor. The reactivator was then added and the incubation continued for the time intervals indicated.

Hexokinase system	Inhibitors (molar conc.)		Reactivators (molar conc.)		Activity (%) at different time after addition of reactivator		
	ASH*	ASSA†	GSH	RSH‡	0 min	15 min	30 min
	7.5×10^{-4}	0	3×10^{-3}	0	10	15	19
Yeast hexokinase plus	7.5×10^{-4}	0	10^{-2}	0	9	15	21
cytochrome *c*	7.5×10^{-4}	0	0	10^{-3}	17	62	76
(5×10^{-4} M)	7.5×10^{-4}	0	0	3×10^{-3}	17	87	98
	7.5×10^{-4}	0	0	10^{-2}	16	100	
Yeast hexokinase without	0	10^{-4}	0	3×10^{-3}	41	83	98
cytochrome *c*	0	10^{-4}	0	10^{-2}	42	83	98
Crude brain hexokinase	3.7×10^{-3}	0	0	3×10^{-3}	27	72	76
without cytochrome *c*	3.7×10^{-3}	0	0	10^{-2}	35	74	78
	0	5×10^{-5}	0	3×10^{-3}	21	55	86

* Diethyldithiocarbamate.
† Disulfiram.
‡ Cysteamine.

converted to disulfiram by cytochrome *c in vitro*. Disulfiram has previously been shown to inhibit such enzymes as succinic dehydrogenase,[22] liver aldehyde oxidase,[2] Racker's liver aldehyde dehydrogenase,[3] xanthine oxidase[4] and glyceraldehyde-3-phosphate dehydrogenase.[5] This broad enzyme-inhibitory effect of disulfiram is probably due to its high redox potential.[27] Since blocking of essential —SH groups is probably the common mechanism for the inhibition of all these enzymes, it seems reasonable to believe that disulfiram is a general inhibitor of "SH-enzymes", affecting not only the enzymes mentioned, but more or less all "SH-enzymes". Possibly disulfiram also will interfere with the function of cofactors with essential —SH groups (CoA, thioctic acid).

On the basis of the present knowledge concerning disulfiram some possible modes of action of this drug will be considered.

(1) It has long been assumed that disulfiram is absorbed and exists as disulphides in mammals, and that the fundamental action of the drug is a disulphide inhibition of enzymes involved in the metabolism of ethanol (see introduction). The picture is more complicated, however, since a large body of evidence indicates that disulfiram exists mainly as the corresponding thiol in the tissues.

(2) Based on the fact that disulfiram is reduced to diethyldithiocarbamate upon administration, it may be proposed that the thiol, or some of its metabolic products, is responsible for the clinical disulfiram–ethanol reaction.[11, 28, 9] Little experimental evidence, however, has appeared in the literature in support of this theory.

(3) The possibility exists that an intracellular reoxidation of diethyldithiocarbamate is involved in the mode of action of this drug, and that the therapeutic action still may be explained by a disulphide inhibition of certain enzymes. In this oxidation perhaps cytochrome *c* and related compounds may serve as oxidizing agents. The disulfiram thus formed, however, has to be preserved from re-reduction by the glutathione–glutathione reductase system. In the present study we have shown that GSH in concentrations equivalent to that of disulfiram completely abolishes the inhibition of hexokinase, probably due to an immediate and stoichiometrical reduction of disulfiram. The intracellular GSH present, therefore, has to be in the form of GSSG before enzyme inhibition will occur. A general *in vivo* oxidation of GSH seems very unlikely. On the other hand one possibility would be that the oxidation of diethyldithiocarbamate takes place in certain intracellular compartments not containing glutathione and glutathione reductase (e.g. mitochondria[29]) but containing certain disulfiram sensitive enzymes.

(4) A possible mode of action of disulfiram previously not considered is that ethanol promotes the *in vivo* oxidation of diethyldithiocarbamate to disulfiram, thus perhaps inducing a general "disulphide poisoning". The immediate clinical reaction by ethanol in mammals receiving disulfiram has previously been ascribed to a simultaneous rise of acetaldehyde concentration. As mentioned in the introduction, doubt has been thrown on this interpretation. The theory here considered permits an explanation of the symptoms without assuming a selective interference of disulfiram with acetaldehyde metabolism. Furthermore, according to this theory disulfiram exists in its reduced non-toxic form provided that ethanol is not ingested. Thus, the paradox that patients may take a potent and general SH-blocking agent such as disulfiram for months with few untoward effects, is easily explained.

At present no conclusive evidence for either of these modes of action of disulfiram can be given. The two last mentioned theories seem to us most plausible and will form the basis for further work.

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