Biochemical Pharmacology, 1963, Vol. 12, pp. 705–715. Pergamon Press Ltd., Printed in Great Britain.

EFFECTS OF DIETHYLDITHIOCARBAMATE AND DISULFIRAM ON GLUCOSE METABOLISM AND GLUTATHIONE CONTENT OF HUMAN ERYTHROCYTES

JOHAN H. STRÖMME*

Institute of Clinical Biochemistry, Rikshospitalet, University of Oslo, Oslo, Norway

(*Received* 25 *February* 1963; *accepted* 2 *April* 1963)

Abstract—The effects of diethyldithiocarbamate on glucose metabolism of human erythrocytes have been investigated. At low concentrations of the compound a stimulation of the glucose consumption is observed, whereas at higher concentrations an inhibition of the metabolism develops. The mechanism underlying this inhihition involves the oxidation of diethyldithiocarbamate to disulfiram, which in turn inhibits the hexokinase. Disulfiram is found also to interfere with other steps of the carbohydrate metabolism, but to obtain this a higher concentration of the inhibitor is required. Methaemoglobin serves as the immediate electron acceptor in the conversion of diethyldithiocarbamate to disulfiram. This pigment is continuously formed by a diethyldithiocarbamate-catalyzed autoxidation of haemoglobin. Evidence indicates that the glutathione–glutathione reductase system offers an efficient protection against disulfiram poisoning of erythrocytes.

THE fundamental action of tetraethylthiuram disulphide (disulfiram) in the treatment of alcoholism appears to be a disulphide inhibition of enzymes involved in the metabolism of ethanol.[1, 2] This mode of action necessarily requires that the drug is present intracellularly in its disulphide form. As previously pointed out, however, a large body of indirect evidence indicates that disulfiram after administration to mammals will be reduced to the corresponding thiol (diethyldithiocarbamate).[3] The above theory requires therefore that an *in vivo* re-oxidation of diethyldithiocarbamate occurs and that this takes place at a rate exceeding that of the simultaneous reduction.

The present report deals with the oxidation of diethyldithiocarbamate and with the metabolic reduction of disulfiram in intact erythrocytes. Informations concerning these processes are obtained by applying the known effects of disulfiram on the hexokinase activity[3] and on the free glutathione.[4] The results indicate that diethyldithiocarbamate within erythrocytes is oxidized to disulfiram. This oxidation is caused by methaemoglobin which is formed continuously owing to the ability of diethyldithiocarbamate to promote a rapid oxidation of haemoglobin by atmospheric oxygen.[5] The accumulation of disulfiram thus formed is to a large extent prevented by a simultaneous metabolic reduction of the disulphide. Under conditions when the formation rate of disulfiram exceeds the reduction rate an inhibition of the glucose metabolism takes place.

EXPERIMENTAL

NADP, ATP, glutathione, sodium diethyldithiocarbamate, crystalline yeast hexokinase, and crystalline glucose-6-phosphate dehydrogenase were obtained as previously

* Fellow of The Norwegian Cancer Society.

reported.[3] Uniformly labelled ^{14}C-glucose (specific activity 3·77 mc/mmole) was obtained from The Radiochemical Centre, Amersham, Bucks., England. Human haemoglobin (2 × crystallized) was purchased from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. This haemoglobin was found to be 98 per cent in the form of methaemoglobin as determined spectrophotometrically.

Heparinized blood (freshly drawn) from the hospital blood bank was centrifuged, and the plasma and the buffy coat were removed. The erythrocytes were washed twice with 10 vol. phosphate-buffered saline, pH 7·4, containing (mequiv/l.): Na^+ ion, 160; K^+ ion, 2·2; Cl^- ion, 143; HPO_4^{2-} ion, 11. Erythrocytes containing methaemoglobin were prepared by treating the washed erythrocytes with amyl nitrite as previously described.[6] This treatment converted about 80 per cent of the total haemoprotein to methaemoglobin. The packed cells were finally suspended in a phosphate-buffered saline, pH 7·4 ,containing (mequiv/l.): Na^+ ion, 163; K^+ ion, 6·7; Cl^- ion, 110; HPO_4^{2-} ion, 33. Microhaematocrit values were taken to determine the final relative erythrocyte volumes.

Anaerobic conditions were obtained by equilibrating the blood against pure nitrogen in a rotating tonometer at room temperature for $\frac{3}{4}$ hr. The suspension was subsequently transferred under nitrogen to the incubation vessels.

The experiments were carried out by incubating the erythrocytes at 37° in the presence of diethyldithiocarbamate (for details, see legends to Figs.). Glucose was measurde by the method of Hultman,[7] and lactate by the method of Barker and Summerson[8] as modified by Ström.[9] The glutathione content of the erythrocytes was determined by the spectrophotometric method of Grunert and Phillips[10] as modified by Beutler.[11] The presence of diethyldithiocarbamate in the incubation medium did not disturb the glutathione measurements. Methaemoglobin was measured by the spectrophotometric method of Horecker and Brackett.[12] Before measurements, diluted samples were centrifuged at 20,000 × *g* for 10 min to obtain optical clear solutions. The total haemoprotein was determined spectrophotometrically as cyanmethaemoglobin by the method of Ceilous.[13]

The activity of the pentose phosphate shunt was evaluated by measuring the radioactivity of the $^{14}CO_2$ formed from ^{14}C-glucose by the erythrocytes, using a Tri Carb Liquid Scintillation spectrometer (Packard Instrument Co. Inc.). The method used is previously described.[6]

The experiments with crystalline yeast hexokinase and the determination of the hexokinase activity were carried out as previously described.[3]

RESULTS

Effects of diethyldithiocarbamate on the glucose consumption

The glucose consumption of "normal erythrocytes" (those not pretreated with amyl nitrite) proceeds linearly for more than 3 hr of incubation in the presence of diethyldithiocarbamate in concentrations below 10^{-2} M (Fig. 2), but at a higher rate than when no thiol is present (Figs. 1 and 2). At concentrations above about 10^{-2} M complete inhibition of the glucose uptake develops during the same period of incubation (Fig. 2). Also on the glucose consumption of "methaemoglobin erythrocytes" (those pretreated with amyl nitrite) diethyldithiocarbamate is found to stimulate at lower concentrations and to inhibit at higher concentrations. In these cells, however, the inhibition appears at far lower concentrations of the compound (Figs. 1 and 3).

A gradual increase in pH occurs during the incubation with diethyldithiocarbamate owing to the fact that a small fraction of the compound decomposes to carbon disulphide and the strong base diethylamine.[14] Such a pH change *per se* is known to increase the glucose metabolism in erythrocytes.[15] We found that in the range of pH 7·2 to 7·8 the rate of glucose uptake of washed erythrocytes suspended in phosphate

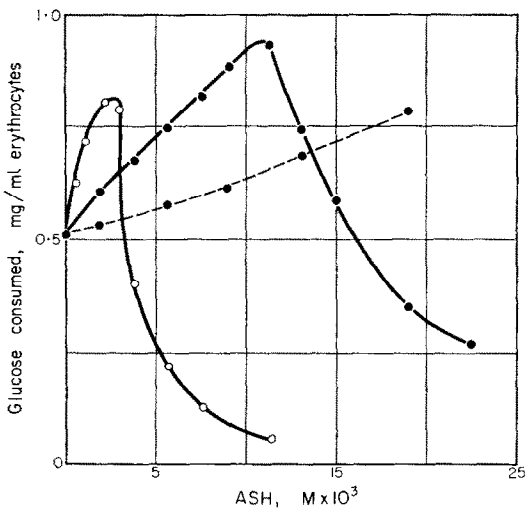


FIG. 1. Effect of increasing concentrations of diethyldithiocarbamate (ASH) on the total glucose consumed by erythrocytes in 1½ hr of incubation. Washed erythrocytes were preincubated for 20 min with glucose (initial conc.: 2·8 × 10^{-3} M). ASH was added at zero time. Final haematocrit value, approx. 33.

●, "normal erythrocytes" (those not pretreated with amyl nitrite).

○, "methaemoglobin erythrocytes" (those pretreated with amyl nitrite).

Broken curve: Increase in glucose consumption which can be ascribed to the mere pH shift during the incubation (for details, see the text).

buffers increased with about 18 per cent for each 0·1 pH unit as compared to the rate at pH 7·2. This finding, when correlated with the rise in pH when diethyldithiocarbamate is present, demonstrates that the mere pH shift can account for only a smaller part of the diethyldithiocarbamate-induced increase in the glucose consumption of erythrocytes (Fig. 1, broken curve).

Effect of diethyldithiocarbamate on the intracellular reduced glutathione

Figures 2 and 3 show that diethyldithiocarbamate causes a decrease in the content of glutathione in erythrocytes, and that this decrease precedes the inhibition of glycolysis. The concentration needed to bring about a complete or nearly complete disappearance of glutathione in "normal erythrocytes" (Fig. 2) as well as in "methaemoglobin erythrocytes" (Fig. 3) is the same as that required to obtain complete inhibition of the glucose consumption in the respective cells. At lower, non-inhibiting, concentrations of diethyldithiocarbamate, the glutathione content decreases only moderately and tends to return to normal levels in the course of incubation.

Role of methaemoglobin

The fact that the effects on glucose metabolism as well as on glutathione content appear at lower concentration of diethyldithiocarbamate in "methaemoglobin erythrocytes" than in "normal erythrocytes" indicates that methaemoglobin is involved in the mechanism of actions. In this connexion it should be recalled (see above)

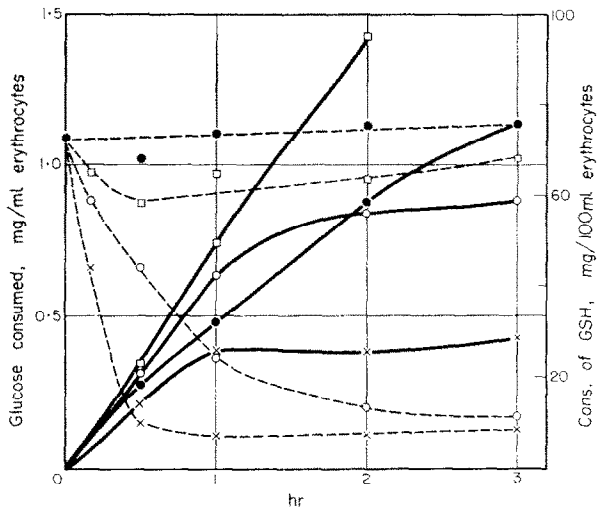


FIG. 2. Effect of diethyldithiocarbamate (ASH) on the glucose consumption (continuous curves) and on the glutathione content (broken curves) in "normal erythrocytes" during aerobic incubations. The erythrocytes were preincubated for 20 min with glucose (initial conc.: $4{\cdot}2 \times 10^{-3}$ M). ASH was added at zero time as follows: □, $7{\cdot}5 \times 10^{-3}$ M; ○, $1{\cdot}5 \times 10^{-2}$ M; ×, $2{\cdot}25 \times 10^{-2}$ M; ●, no ASH added. Final haematocrit value, approx. 33.

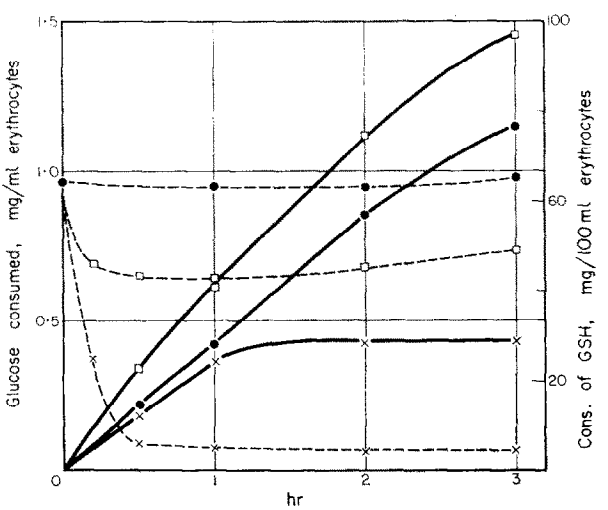


FIG. 3. Effect of diethyldithiocarbamate (ASH) on the glucose consumption (continuous curves) and on the glutathione content (broken curves) in "methaemoglobin erythrocytes" during aerobic incubations. The erythrocytes were preincubated for 20 min with glucose (initial conc.: $4{\cdot}2 \times 10^{-3}$ M). ASH was added at zero time as follows: □, $2{\cdot}5 \times 10^{-3}$ M; ×, $7{\cdot}5 \times 10^{-3}$ M; ●, no ASH added. Final haematocrit value, approx. 33.

that even the "normal cells" will contain methaemoglobin owing to the diethyldithiocarbamate-catalysed autoxidation of haemoglobin.[5] Since no methaemoglobin is formed under anaerobic incubations, the significance of this pigment is more clearly demonstrated under these conditions (Figs. 4 and 5). Thus, when depriving "normal cells" of oxygen, no significant changes occur in the presence of diethyldithiocarbamate either of the glucose metabolism or of the glutathione content (Fig. 4). In erythrocytes containing preformed methaemoglobin on the other hand, the same effects appear as when oxygen is present (Fig. 5).

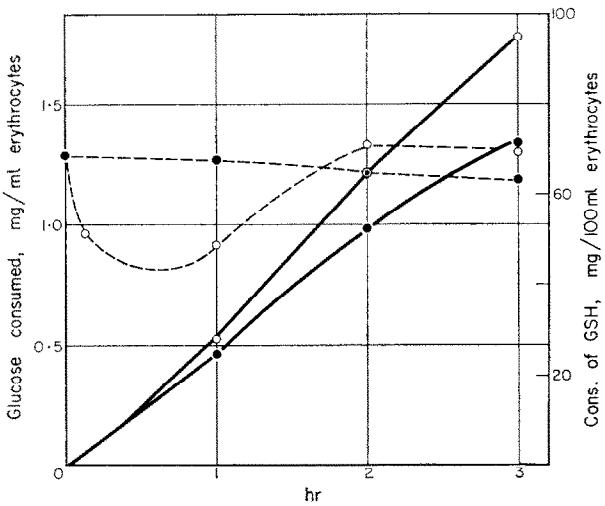


FIG. 4. Effect of diethyldithiocarbamate (ASH) on the glucose consumption (continuous curves) and on the glutathione content (broken curves) in "normal erythrocytes" during anaerobic incubations. The erythrocytes were preincubated for 20 min with glucose (initial conc.: $4{\cdot}2 \times 10^{-3}$ M). ASH was added at zero time as follows: ○, $2{\cdot}25 \times 10^{-3}$ M; ●, no ASH added. Final haematocrit value, approx. 33.

We have previously shown that oxidized cytochrome c stoichiometrically converts diethyldithiocarbamate to disulfiram.[3] Similar findings were obtained with crystalline methaemoglobin as oxidizing agent. Probably also within intact cells methaemoglobin brings about an oxidation of this thiol. The disulfiram thus formed most likely represent the immediate active agent in the effects observed both on the glucose metabolism and on the glutathione content. Support for this assumption is to be seen in experiments with crystalline yeast hexokinase. Thus Fig. 6 shows that diethyldithiocarbamate inhibits this enzyme only when in addition methaemoglobin is present in the mixture. Since hexokinase is known to be inhibited by disulfiram but not by diethyldithiocarbamate,[3] methaemoglobin obviously acts by converting the thiol to its disulphide. Methaemoglobin, in contrast to cytochrome c in similar experiments,[3] is catalytic in its function (Fig. 6). This is probably due to a continuous reformation of the methaemoglobin (see above).

Role of glutathione–glutathione reductase system

It is shown by the results of Figs. 3 and 5 that higher concentrations of diethyldithiocarbamate is needed to cause inhibition of the glucose consumption in ery-

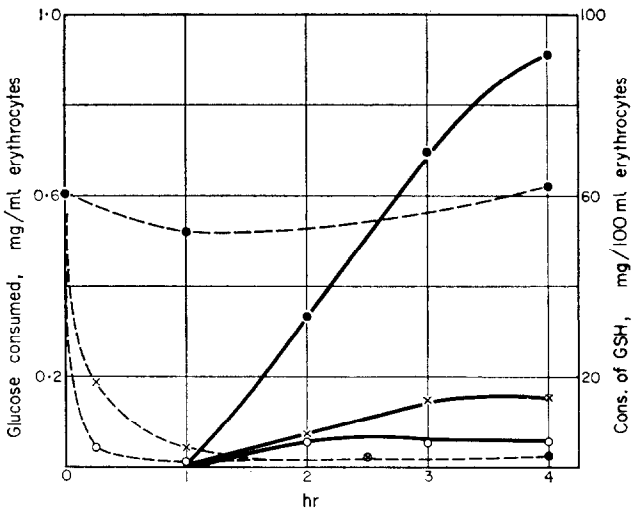


FIG. 5. Effect of diethyldithiocarbamate (ASH) on the glucose consumption (continuous curves) and on the glutathione content (broken curves) in "methaemoglobin erythrocytes" during anaerobic incubations. Glucose was added after 1 hr of incubation (initial conc.: 4·2 × 10^{-3} M). ASH was added at zero time as follows: × 10^{-3} M; ○, 3 × 10^{-3} M; ●, no ASH added. Final haematocrit value, approx. 33.

throcytes supplied with substrate than in those lacking substrate. This suggests that actively metabolizing cells possess a mechanism for protection against disulfiram poisoning. Disulfiram is known to react spontaneously with glutathione,[4] and the rapid disappearance of this thiol in erythrocytes deprived of glucose (Fig. 5) demonstrates that this interaction also occurs intracellularly. When substrate is available

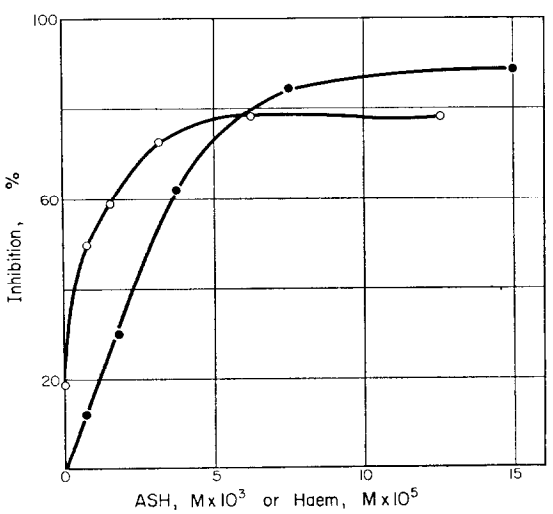


FIG. 6. Inhibition of crystalline yeast hexokinase by a mixture of diethyldithiocarbamate and crystalline methaemoglobin. The enzyme was preincubated for 1 hr at 37° as follows:

●, methaemoglobin (constant, 3·33 × 10^{-4} M, as Haem) plus diethyldithiocarbamate (ASH) in increasing concentrations.

○, diethyldithiocarbamate (constant, 7·5 × 10^{-3} M) plus methaemoglobin (Haem) in increasing concentrations.

the GSSG formed will be re-reduced by way of the glutathione–glutathione reductase system. One will expect that an intracellular accumulation of disulfiram, with subsequent inhibition of disulfiram-sensitive enzymes, will take place only after complete oxidation of the glutathione.

The metabolic reduction of GSSG in erythrocytes involves the glutathione reductase and the pentose phosphate shunt. A reduction of disulfiram via this system therefore should be reflected by a simultaneous increase in the production of CO_2 by the cells.[16] Table 1 shows that diethyldithiocarbamate may increase the CO_2 formation as much

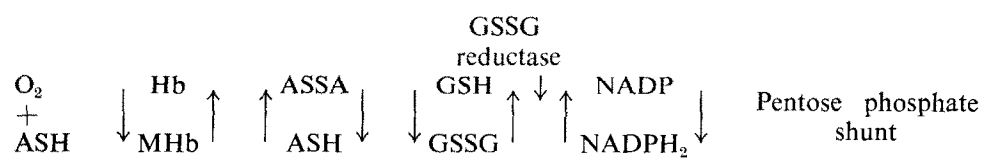
TABLE 1. EFFECT OF DIETHYLDITHIOCARBAMATE ON THE FORMATION OF CO_2 IN ERYTHROCYTES UNDER AEROBIC AND ANAEROBIC INCUBATIONS

Suspensions of erythrocytes not pretreated with amyl nitrite (Hb cells) and of erythrocytes pretreated with amyl nitrite (MHb cells) were preincubated with uniformly labelled ^{14}C-glucose (initial conc. 4×10^{-3} M) for 15 min. Diethyldithiocarbamate (ASH) was added at zero time and the suspensions were subsequently incubated for 2 hr. Final haematocrit, approx. 33. The cells pretreated with amyl nitrite contained 88 per cent of the total haemoprotein as methaemoglobin. Values (mean of duplicate experiments) for the CO_2 produced are expressed as μmole/ml packed erythrocytes.

Conc. of ASH (M)	Aerobic	Anaerobic	
	Hb cells	Hb cells	MHb cells
0	0·26	0·10	0·68
10^{-3}	0·40	0·10	1·00
2×10^{-3}	0·66	0·08	1·38
5×10^{-3}	2·20	0·12	2·02
10^{-2}	3·54	0·16	2·24

as 14 times under aerobic incubations. Under anaerobic incubations such a stimulation of the shunt is found only in "methaemoglobin erythrocytes", i.e. only in the cells in which an oxidation of diethyldithiocarbamate will take place. At the higher concentrations of the compound the metabolism of these cells is inhibited during the greater part of the incubation period. The results in Table 1 thus confirm the view that disulfiram is reduced via the glutathione–glutathione reductase system.

The likely sequence of reactions which take place when diethyldithiocarbamate (ASH) has been added to erythrocytes is illustrated by Scheme 1.



Scheme 1

Enzymes inhibited by disulfiram

The ability of disulfiram to inhibit isolated hexokinase[3] suggests that the observed metabolic disturbance of erythrocytes may be explained by an inhibition of this enzyme. In support of such a view are results recently obtained with intact cells by Eldjarn and Bremer[17] and Nesbakken and Eldjarn,[18] demonstrating a selective inhibition of

hexokinase by disulphides such as cystamine. In the case of disulfiram, however, a multi-enzyme inhibition would be expected since this disulphide has been shown to inhibit also other enzymes of the carbohydrate metabolism such as glucose-6-phosphate dehydrogenase[19] and glyceraldehyde-3-phosphate dehydrogenase.[20]

Information as to the localization was obtained by measuring the reduction of methaemoglobin and the formation of lactate under conditions when the hexokinase was by-passed by using inosine as substrate. Fig. 7 shows that when diethyldithiocarbamate is added to the erythrocytes simultaneously with the substrate no inhibition of either of these processes develops. At concentrations up to $7{\cdot}5 \times 10^{-3}$ M, which is

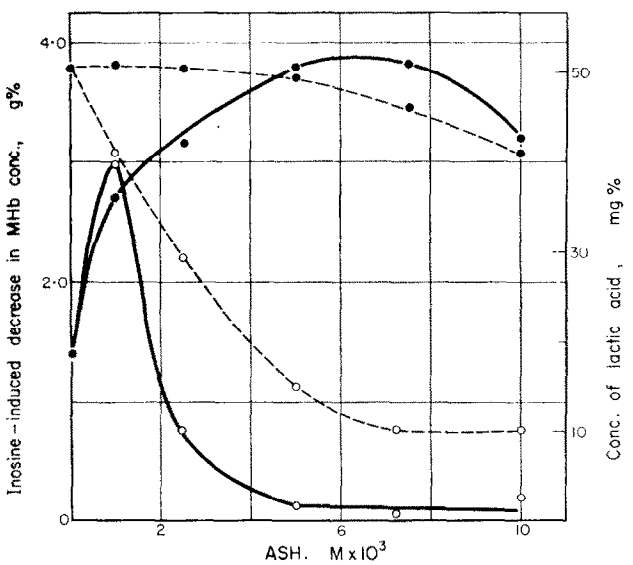


FIG. 7. Effect of increasing concentrations of diethyldithiocarbamate (ASH) on the metabolic reduction of methaemoglobin and on the formation of lactate when inosine is used as substrate. "Methaemoglobin erythrocytes" were preincubated for 1 hr with (○) and without (●) ASH. At zero time inosine (10^{-2} M) was added, and in addition ASH to the vessels not containing ASH during the preincubation period. The erythrocyte suspensions contained 7·95 g/100 ml haemoprotein, of which 81 per cent initially was methaemoglobin. The concentrations of methaemoglobin and of lactate were determined after 2 hr of incubation.
Continuous curves: Decrease in methaemoglobin (g/100 ml suspension). Corrections have been made for the changes in the concentrations of methaemoglobin which occur when inosine is omitted. The values given therefore represent the decrease which can be ascribed to the presence of this substrate. Broken curves: Concentration of lactate (mg/100 ml suspension).

about twice that needed to obtain inhibition of the glucose metabolism, an enhanced reduction of methaemoglobin is even observed. Evidently this is due to an increased reduction via the pentose phosphate shunt in accordance with the mechanism outlined in Scheme 1. Concomitantly a slight fall in the formation of lactate is observed. Since the hexokinase is the only enzyme by-passed when using inosine the results indicate that diethyldithiocarbamate, when added to actively metabolizing cells, may lead to a selective inhibition of hexokinase, thus leaving the other enzymes unaffected.

In contrast to the above findings, however, stand those obtained when erythrocytes had been preincubated with diethyldithiocarbamate in concentrations above $2{\cdot}5 \times 10^{-3}$ M but without substrate, i.e. under conditions when the protective effect of the glutathione–glutathione reductase system is abolished (Fig. 7). Subsequent addition of inosine in this case leads to an insignificant decrease in the methaemoglobin level as compared to that of the cells lacking substrate. An inhibition of the lactate formation is also observed, but the former effect is more pronounced, especially at concentrations of about $2{\cdot}5 \times 10^{-3}$ M. These results demonstrate that diethyldithiocarbamate, when added to erythrocytes deprived of substrate, may lead to inhibition also below the hexokinase level.

DISCUSSION

In the present report it has been shown that diethyldithiocarbamate may lead to an inhibition of the glucose metabolism of erythrocytes. The mechanism underlying this metabolic disturbance apparently involves an oxidation of diethyldithiocarbamate to disulfiram. Disulfiram in turn inhibits primarily the hexokinase, probably by blocking essential —SH groups through mixed disulphide formation as previously reported.[3] Evidence indicates that methaemoglobin serves as the immediate electron acceptor in the oxidation of diethyldithiocarbamate.

Diethyldithiocarbamate in non-inhibiting concentrations stimulates the glucose consumption of erythrocytes (Fig. 1). This is probably a consequence of the stimulation of the pentose phosphate shunt (Table 1). Support for such a view is to be seen in the fact that other shunt stimulatory agents such as methylene blue also causes a secondary increase in the glucose uptake of erythrocytes.[16, 21]

Eldjarn *et al.* have recently reported that erythrocytes reduce disulphides such as cystamine via the glutathione–glutathione reductase system.[22] The present data demonstrate that the same system is efficient in reducing also disulfiram (Scheme 1). In order to obtain an intracellular accumulation of this disulphide, therefore, the oxidation of diethyldithiocarbamate must proceed at a rate exceeding the disulfiram reducing capacity of the cells. Accordingly lower concentrations of diethyldithiocarbamate are needed to cause inhibition of the metabolism in "methaemoglobin erythrocytes" (i.e. relatively increased formation rate of disulfiram) and in erythrocytes lacking substrate (i.e. decreased reduction capacity) than of the metabolism in "normal erythrocytes" to which substrate is available.

According to the mechanism for the shunt stimulatory effect of diethyldithiocarbamate outlined in Scheme 1, the data of Table 1 permit calculations as to the disulfiram reducing capacity of erythrocytes. These calculations are based on the fact that the release of 1 mole CO_2 accompanies the formation of 2 mole $NADPH_2$, which via glutathione–glutathione reductase system are able to reduce 2 mole disulfiram. Table 1 shows that the presence of diethyldithiocarbamate (10^{-2} M) leads to an increase in the formation of CO_2 from 0·26 to 3·54 μmole/ml erythrocytes (2 hr of incubation), i.e. an increase of 1·64 μmole/hr per ml erythrocytes. If the whole increase is due to the reduction of disulfiram, about 3·3 μmole of this disulphide simultaneously is reduced per hr per ml erythrocytes. In other words, the total amount of erythrocytes in an adult will be able to reduce more than 50 g disulfiram in 24 hr. These data for the efficiency of the glutathione–glutathione reductase system in reducing this disulphide held together with the fact that the therapeutic dose of the drug is less than 0·5 g/day

strongly support the assumption that disulfiram will be subject to an immediate and quantitative reduction in patients receiving this drug.

Previously it has been shown that inosine increases the capacity of erythrocytes to reduce N,N'-diethylcystamine with a factor of about 2·5 as compared to the capacity when using glucose as substrate.[22] This suggests that care should be exercised in interpreting the results of Fig. 7 to indicate a special sensitivity of hexokinase toward disulfiram. Thus, inosine may increase the disulfiram reducing ability of "methaemoglobin erythrocytes" sufficiently to prevent an accumulation of the disulphide even in the presence of diethyldithiocarbamate concentrations as high as $7{\cdot}5 \times 10^{-3}$ M. Additional support, however, for the conclusion that a selective inhibition of the hexokinase may develop is to be seen in results obtained under conditions when no reduction takes place (no substrate available). In this case diethyldithiocarbamate in a concentration of 10^{-3} M within 1 hr of incubation brings about an inhibition of the glucose consumption (Fig. 5), whereas no significant interference with the metabolism of inosine could be shown (Fig. 7). It may therefore be concluded that disulfiram primarily inhibits hexokinase. The results thus support the view of Nesbakken and Eldjarn[18] that hexokinase possesses —SH groups with a higher ability for mixed disulphide formation than any other enzymes of the glycolytic sequence.

Disulfiram may interfere with the glucose metabolism at a level also below the hexokinase step (Fig. 7). This is in contrast to the selective inhibition of the hexokinase obtained with other disulphides such as cystamine.[17, 18] This disparity is probably explained by the higher oxidation potential of disulfiram. The increased ability to interact with protein —SH groups makes it a more potent inhibitor of the SH-enzymes. The results of Fig. 7 allows no definite conclusions as to the localization of the extended inhibition. Disulfiram is known to inhibit glyceraldehyde-3-phosphate dehydrogenase[20] and glucose-6-phosphate dehydrogenase.[19] Tetramethylthiuram disulphide has been reported to inhibit aldolase.[23] Furthermore, a number of enzymes of the glycolytic pathway and of the pentose phosphate pathway are inhibited by strong SH-blocking agents, such as *p*-chloromercuribenzoate (see ref. 18). The metabolic reduction of methaemoglobin, which in the presence of diethyldithiocarbamate for a greater part is linked to the pentose phosphate shunt (Scheme 1), seems more seriously affected by disulfiram than the formation of lactate (Fig. 7). These observations may suggest that the shunt (glucose-6-phosphate dehydrogenase?) is inhibited at somewhat lower concentrations of disulfiram than is the glycolytic pathway (glyceraldehyde-6-phosphate dehydrogenase?).

Chefurka has shown that purified glucose-6-phosphate dehydrogenase is markedly inhibited by dimethyldithiocarbamate as such in concentrations as low as 10^{-5} M. Diethyldithiocarbamate, which one would expect to possess similar enzyme-inhibitory properties as has dimethyldithiocarbamate, seems not to inhibit this enzyme in intact erythrocytes (Table 1).

One of the roles attributed to free glutathione of the cells has been to protect enzymes against oxidizing agents.[24] The present findings show that glutathione of erythrocytes to a large extent protects the hexokinase against oxidation by disulfiram. Functions in regulating the intermediar metabolism has also been ascribed to glutathione.[24] This regulation of enzyme activities should be demonstrated through a variation of the SH/SS balance of the cells. Considerable decrease, however, in the reduced glutathione level of erythrocytes is observed without concomitant decrease in

the rate of glucose consumption (Fig. 3). This is in agreement with results obtained in metabolic studies with brain tissues,[25] and throws some doubt on a regulatory function of glutathione.

Acknowledgement—The author is indebted to Professor L. Eldjarn for helpful discussion and suggestions.

REFERENCES

1. J. Hald, E. Jacobsen and V. Larsen, *Acta pharm. tox., Kbh.* **4**, 285 (1948).
2. F. E. Hunter and O. H. Lowry, *Pharm. Rev., Lond.* **8**, 89 (1956).
3. J. H. Strömme, *Biochem. Pharmacol.* **12**, 157 (1963).
4. C. D. Johnston, *Arch. Biochem. Biophys.* **44**, 249 (1953).
5. J. H. Strömme. *Biochem. Pharmacol.* In press.
6. J. H. Strömme and K. Eldjarn, *Biochem. J.* **84**, 406 (1962).
7. E. Hultman, *Nature, Lond.* **183**, 108 (1959).
8. S. B. Barker and W. H. Summerson, *J. biol. Chem.* **138**, 535 (1941).
9. G. Ström, *Acta physiol. scand.* **17**, 440 (1949).
10. R. R. Grunert and P. H. Phillips, *Arch. Biochem. Biophys.* **30**, 217 (1951).
11. E. Beutler, *J. Lab. clin. Med.* **49**, 84 (1957).
12. B. L. Horecker and F. S. Brackett, *J. biol. Chem.* **152**, 669 (1944).
13. E. Ceilous, *Ann. intern. Med.* **52**, 914 (1960).
14. M. Hallaway, *Biochim. Biophys. Acta*, **36**, 538 (1959).
15. J. R. Murphy, *J. Lab. clin. Med.* **55**, 286 (1960).
16. M. Brin and R. H. Yonemoto, *J. biol. Chem.* **230**, 307 (1958).
17. L. Eldjarn and J. Bremer, *Biochem. J.* **84**, 286 (1962).
18. R. Nesbakken and L. Eldjarn, *Biochem. J.* In press.
19. W. Chefurka, *Enzymologia*, **18**, 209 (1957).
20. A. P. Nygaard and J. B. Sumner, *Arch. Biochem. Biophys.* **39**, 119 (1952).
21. W. C. J. DeLoecker and T. A. J. Prankerd, *Clin. Chim. Acta*, **6**, 641 (1961).
22. L. Eldjarn, J. Bremer and H. C. Börresen, *Biochem. J.* **82**, 192 (1962).
23. H. D. Sisler and C. E. Cox, *Amer. J. Bot.* **42**, 351 (1955).
24. E. S. G. Barron, *Advanc. Enzymol.* **11**, 201 (1951).
25. H. McIlwain, *Biochem. J.* **71**, 281 (1959).