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The Inhibitory Effect at the Hexokinase Level of Disulphides on Glucose Metabolism in Human Erythrocytes

BY L. ELDJARN AND J. BREMER*
Institute of Clinical Biochemistry, Rikshospitalet, University of Oslo, Norway

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We have previously shown that, provided that a suitable substrate such as glucose or adenosine is present, intact human erythrocytes reduce a number of disulphides with the appearance of the corresponding thiol in the suspending medium. This reduction probably takes place via spontaneous exchange reactions with glutathione, leading to the formation of oxidized glutathione, which in turn is reduced by glutathione reductase. However, when higher concentrations of the disulphide were used, cystamine and several cystamine derivatives completely inhibited their own reduction. When glucose was oxidized in the presence of methylene blue, the oxygen consumption was also inhibited by higher concentrations of cystamine. However, various disulphides showed different effects in these respects. Thus some disulphides were reduced both at low and high concentrations whereas others neither became reduced nor demonstrated any toxic effect (Eldjarn, Bremer & Börresen, 1962).

Several explanations can be proposed for the 'disulphide poisoning' of erythrocytes. Higher concentrations of some disulphides are known to convert glutathione nearly quantitatively into the mixed disulphide form. Mixed disulphides with glutathione do not serve as substrates for glutathione reductase (Pihl, Eldjarn & Bremer, 1957). The disulphide-reducing system of the erythrocytes may thus be inhibited by the removal of its substrate (oxidized glutathione). However, preliminary findings did not support such a mechanism (Eldjarn *et al.* 1962).

In this paper we demonstrate that the 'disulphide poisoning' of erythrocytes can be ascribed to a reversible block of glucose utilization at the hexokinase level. This block is not due to a shortage of ATP or to an accumulation of glucose 6-phosphate. Despite the fact that many erythrocyte enzymes are usually classified as SH-enzymes, the experiments show that these other enzymes of the oxidative pentose shunt and of the Embden–Meyerhof glycolytic pathway are probably not inhibited in 'disulphide poisoning'. Also the glutathione-reductase system is most probably functionally intact. The inhibition of the disul-

* Fellow of The Norwegian Cancer Society.

phide-reducing capacity can be ascribed to $NADPH_2$ shortage due to the hexokinase inhibition. The nature of the inhibition at the hexokinase level remains obscure.

MATERIALS AND METHODS

The methods used for the collection and preparation of human erythrocytes were as described by Eldjarn *et al.* (1962). In most of the experiments 0.4×10^6 cells/mm.^{3} were incubated in a medium containing the following (m-equiv./l.): Na^+ ion, 142; K^+ ion, 6·7; Cl^- ion, 110; HPO_4^{2-} ion, 33·4; EDTA, 1. In all experiments incubation was at 37° with continuous shaking and with air as the gas phase. Lactate dehydrogenase, NAD and $NADH_2$ were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany; cysteamine and the cysteamine derivatives were obtained or prepared as described by Eldjarn *et al.* (1962). All other chemicals used were commercial products of highest purity.

The oxygen uptake in the presence of 0·05% methylene blue was measured in the conventional Warburg apparatus with 3 ml. of erythrocyte suspension in each vessel. Carbon dioxide was trapped with 0·2 ml. of 30% (w/v) potassium hydroxide in the centre well. Lactic acid was determined enzymically according to Ham & Bruns (1956). Checks for pyruvate formation were made spectrophotometrically at room temperature with lactate dehydrogenase and $NADH_2$.

AMP, ADP, ATP, IMP, hexose monophosphates and hexose diphosphates were isolated by column chromatography on Dowex 1 (formate form) according to Mills & Summer (1959). The amounts of nucleotides were calculated from the extinctions at 260 mμ of the column eluates. The following molar extinction coefficients were used: for AMP, ADP and ATP, 15×10^3; for IMP, 8×10^3. The amounts of hexose mono- and di-phosphates were determined in the column eluates according to the methods used by Bartlett (1959).

All results are given in μmoles/ml. of packed cells.

RESULTS

Fig. 1 demonstrates that cystamine may completely block the uptake of oxygen by erythrocytes in the presence of methylene blue when glucose is used as substrate, whereas the oxygen uptake is temporarily stimulated when adenosine is the substrate. Eldjarn *et al.* (1962) obtained essentially similar results with respect to disulphide reduction by erythrocytes, i.e. higher concentrations of cystamine blocked the reduction when glucose, but not when adenosine, was used as substrate. A moderate constant gas evolution was observed in poisoned cells when methylene blue was added (Fig. 1). Since the simultaneous formation of methaemoglobin could be demonstrated, this gas evolution was most probably due to the liberation of oxygen when oxyhaemoglobin is converted into methaemoglobin.

Erythrocytes appear to be virtually devoid of glycogen (Wagner, 1946) and cannot maintain their metabolism without exogenous substrates. Erythrocytes, when devoid of substrates, show only a small and temporary disulphide reduction. This limited activity must be ascribed to the pool of metabolic intermediates present. It would therefore be expected that preincubation of the cells with the disulphides alone should modify the development of the 'disulphide poisoning'. Figs. 2 and 3 demonstrate the inhibitory effects observed when increasing concentrations of cystamine are added simultaneously with, or 30 min. before, the glucose. In the preincubation experiment complete poisoning of the cells is obtained with a far lower concentration of the disulphide. The oxygen uptake is inhibited for several hours with a disulphide concentration as low as 1·5 mM. Fig. 2 also demonstrates that the degree of inhibition increases with the disulphide concentration. On the other hand, after preincubation with glucose (and methylene blue) for 30 min. with subsequent addition of cystamine (10 mM), more than an hour is needed for the inhibition to become complete. Thus, it seems that the development of 'disulphide poisoning' of erythrocytes is a relatively slow process, and that it depends on the metabolic condition of the cell.

Human erythrocytes possess the pentose monophosphate shunt, but this shunt is relatively dormant under normal conditions (Prankerd, 1961). Dische (1951), when studying the breakdown of adenosine in blood haemolysates, demonstrated that fructose 6-phosphate and fructose

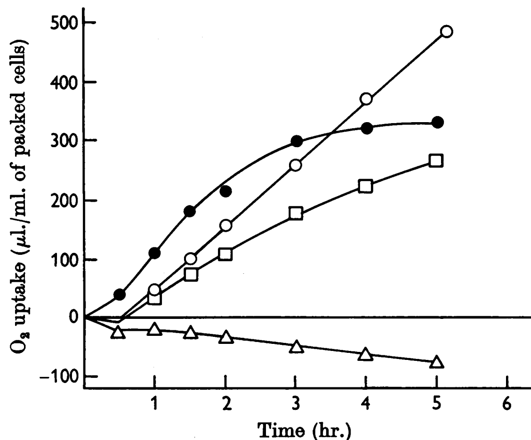

Fig. 1. Effect of cystamine on oxygen uptake by erythrocytes in the presence of methylene blue. Substrate [glucose (11 mM) or adenosine (5·5 mM)], methylene blue and cystamine (17 mM) were added at zero time. ●, Adenosine + cystamine; □, adenosine; ○, glucose; △, glucose + cystamine.

1,6-diphosphate accumulated when the glyceraldehyde 3-phosphate dehydrogenase was inactivated with bromoacetate. Thus the ribose of purine nucleosides can serve as substrate for erythrocytes. Brin & Yonemoto (1958) have shown that the oxygen uptake in erythrocytes in the presence of methylene blue can be ascribed mainly to glucose 6-phosphate oxidation via the pentose monophosphate shunt.

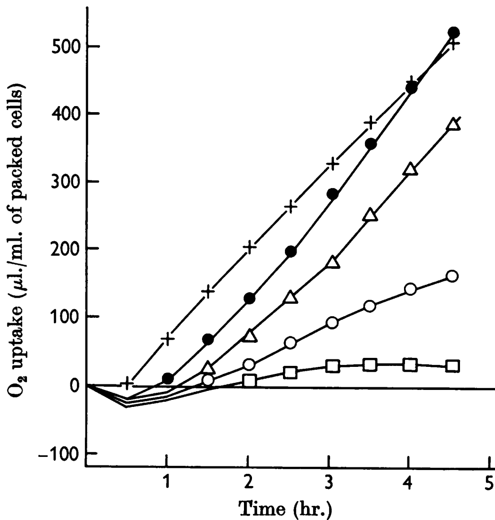

Fig. 2. Effect of increasing concentrations of cystamine on the oxygen uptake by erythrocytes in the presence of methylene blue. Glucose (11 mM), methylene blue and cystamine were added at zero time. Concentration of cystamine (mM): +, 0; ●, 1·5; △, 3; ○, 6; □, 10.

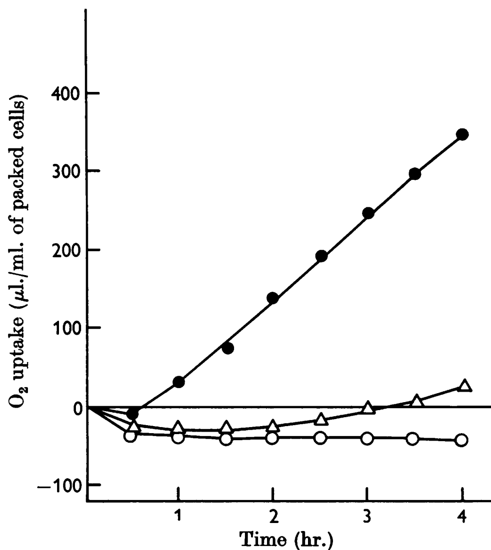

Fig. 3. Effect of preincubation with increasing concentrations of cystamine on the oxygen uptake by erythrocytes in the presence of methylene blue. The washed erythrocytes were preincubated for approx. 30 min. with cystamine. Glucose (11 mM) and methylene blue were subsequently added at zero time. Concentration of cystamine (mM): ●, 0; △, 1·5; ○, 3.

With this in mind our results strongly suggest that the 'disulphide poisoning' is due to a selective inhibition at the hexokinase level, whereas the pentose shunt as well as the other steps of the Embden–Meyerhof pathway are essentially unaffected. This interpretation is further supported by the effect of disulphides on lactate formation when glucose or adenosine is used as substrate (Table 1). Cystamine poisoning completely blocked lactate formation from glucose, whereas a significant but decreased formation of lactate took place when adenosine was used as substrate. This decrease in lactate formation was not due to pyruvate accumulation, as no pyruvate was detected by lactate–dehydrogenase–$NADH_2$ analysis of a trichloroacetic acid extract of the incubation mixture.

Table 1 also demonstrates that cysteamine, the thiol, does not inhibit lactate formation either from glucose or from adenosine. These observations are of particular significance in proving that the observed effects are due to a 'disulphide poisoning'. The effects of thiols on oxygen uptake in the presence of methylene blue could not be determined because of their rapid spontaneous oxidation.

One explanation for the blockage of glucose utilization by disulphides might be that the presence of the disulphide depletes the cells of some cofactor. The observed sluggishness of the process would support such a mechanism. The blockage at the hexokinase level might indicate that a drop in ATP concentration is responsible. Cooper (1959) has suggested that blocking of an inhibitory thiol group may activate adenosine triphosphatase.

Table 1. *Effect of preincubation for* 30 *min. with cystamine on lactate formation from glucose and adenosine in erythrocytes*

Substrate concentrations were 5 mM. Cystamine and cysteamine concentrations were 17 and 35 mM respectively. Experimental details are given in the text.

	Lactate found (µmoles/ml. of packed cells)		
Time (hr.)	0	1·5	3
Additions			
None	0·9	0·9	0·8
Glucose	—	3·2	5·8
Glucose + cystamine	—	0·8	1·2
Adenosine	—	6·5	9·4
Adenosine + cystamine	—	3·1	4·5
Glucose + cysteamine	—	2·8	6·7
Adenosine + cysteamine	—	5·6	10·1

Table 2. *Effect of preincubation with cystamine on the concentrations of adenosine mono-, di- and tri-phosphate and of inosine monophosphate in erythrocytes*

The concentrations of substrates and of cystamine are 5 mM and 17 mM respectively. Experimental details are given in the text.

Additions	Total incubation time (hr.)	Concn. (μmoles/ml. of packed cells) AMP*	ADP*	ATP*	IMP	Sum
None	0·5	0·030	0·30	0·87	0·035	1·230
Glucose (after 30 min.)	2·5	0·015	0·13	1·02	0·030	1·195
Adenosine (after 30 min.)	2·5	0·020	0·24	2·22	0·135	2·615
Cystamine (at zero time)	0·5	0·085	0·34	0·67	0·055	1·150
Cystamine (at zero time) + glucose (after 30 min.)	2·5	0·170	0·34	0·37	0·085	0·965
Cystamine (at zero time) + adenosine (after 30 min.)	2·5	0·045	0·28	1·00	0·465	1·790

* The normal concentrations of AMP, ADP and ATP are 0·01–0·02, 0·19–0·25 and 0·9–1·2 μmoles/ml. of packed cells respectively (Bartlett, 1959).

Table 3. *Effect of cystamine on the concentrations of adenosine mono-, di- and tri-phosphate, inosine monophosphate, and hexose mono- and di-phosphate in erythrocytes*

After preincubation without and with cystamine (17 mM) for 30 min., adenosine (5 mM) was added and the incubation continued for 2 hr. Experimental details are given in the text.

Additions	Concn. (μmoles/ml. of packed cells) AMP†	ADP†	ATP†	IMP	Hexose monophosphate*†	Hexose diphosphate*†
Adenosine (after 30 min.)	0·02	0·23	1·70	0·25	0·21	5·00
Cystamine (at zero time) + adenosine (after 30 min.)	0·03	0·29	0·72	0·63	0·04	0·86

* Estimations with the cysteine–carbazole method (Dische & Borenfreund, 1951) showed that the hexose phosphates present were mainly fructose phosphates.

† The normal concentrations of AMP, ADP, ATP, hexose monophosphate and hexose diphosphate were 0·01–0·02, 0·19–0·25, 0·9–1·2, 0·09–0·12 and 0·50–0·71 μmole/ml. of packed cells respectively (Bartlett, 1959).

However, the results presented in Table 2 make it extremely unlikely that such a mechanism can explain the inhibition observed. The ATP content of the cells preincubated with cystamine was only slightly lower than that of the control preincubated without cystamine, although the glucose utilization in the former cells is completely inhibited. A significant drop in the ATP concentration only became apparent after prolonged incubation of the cystamine-inhibited cells. Glucose evidently did not prevent this drop in ATP concentration of the poisoned cells whereas adenosine restored completely the ATP concentration.

The results further demonstrate that the sum of ADP, AMP or IMP is relatively constant in the cells incubated with cystamine or glucose or both. On the other hand, adenosine causes an increase in ATP.

Table 3 verifies the above conclusions concerning ATP and demonstrates that the well-known increase in hexose mono- and di-phosphates with adenosine as substrate becomes far less apparent when a high concentration of cystamine is present before and during the incubation. We have previously demonstrated that, with adenosine as substrate, toxic concentrations of cystamine increase the oxygen uptake in the presence of methylene blue, decrease the lactate as well as the ATP formation, and do not inhibit the disulphide-reducing system. Together these results indicate that, apart from a blockage at the hexokinase level, cystamine diverts glucose 6-phosphate from the Embden–Meyerhof glycolytic pathway to the oxidative pentose shunt. In accordance with this conclusion it was found that cystamine alone stimulated oxygen uptake in erythrocytes incubated with adenosine. This diversion of glucose 6-phosphate metabolism is most probably related to the relatively dormant pentose shunt of erythrocytes being brought into action by the oxidation of $NADPH_2$ by way of glutathione reductase. The resulting cysteamine finally consumes oxygen by spontaneous oxidation.

A likely explanation for the disulphide inhibition of glucose utilization at the hexokinase level would be an inhibition of hexokinase by mixed disulphide formation on essential protein thiol groups. If such

a view is correct, a revival of the poisoned erythrocytes should be possible either by prolonged incubation with adenosine or by washing with thiols.

Fig. 4 shows that such a reactivation of disulphide-poisoned cells can be effected by adenosine. The poisoned erythrocytes, from which the surplus cystamine had been removed by washing, did not take up oxygen with glucose as substrate, whereas the oxygen uptake with adenosine in low concentration tapered off after 3 hr., probably owing to exhaustion of substrate. When, in addition to the low concentration of adenosine, glucose was added, the oxygen uptake continued at a constant rate throughout the whole experiment (6 hr.), strongly indicating that glucose utilization was restored.

A partial reactivation of disulphide-poisoned cells with thiols could also be demonstrated (Fig. 5). Thus when the inhibition was brought about with tetramethylcystamine, a reactivation could be demonstrated (although with difficulty) after cysteamine treatment; however, 2-mercaptoethanol was much more effective in this respect. The difficulty in reactivating with cysteamine is most likely due to its rapid spontaneous oxidation. Oxidized 2-mercaptoethanol (bis-2-hydroxyethyl disulphide) inhibited neither its own reduction nor the oxygen consumption by erythrocytes in the presence of methylene blue.

The inhibition by tetramethylcystamine and the reactivation with 2-mercaptoethanol also show that the inhibition cannot be due to thiazolidine formation (Eldjarn, Nakken & Pihl, 1957).

No significant oxygen consumption could be demonstrated when galactose or mannose was used as substrate for human erythrocytes in the presence of methylene blue. Erythrocytes contain the enzymes necessary for the conversion of these compounds into glucose 6-phosphate and fructose 6-phosphate respectively, and galactose and mannose could sustain the disulphide reduction of erythrocytes at a slow rate (Eldjarn *et al.* 1962).

None of the pyrimidine nucleosides tested (cytidine, thymidine and uridine) were metabolized by human erythrocytes as judged from oxygen uptake in the presence of methylene blue. However, all the purine nucleosides were metabolized, with an oxygen consumption decreasing in the following order: inosine, adenosine, guanosine and xanthosine. As with adenosine, cystamine (17 mM) increased the oxygen uptake when inosine and guanosine were used as substrates. With xanthosine as substrate a pronounced inhibition in the oxygen uptake was caused by cystamine. The reason for this inhibition has not been studied in detail.

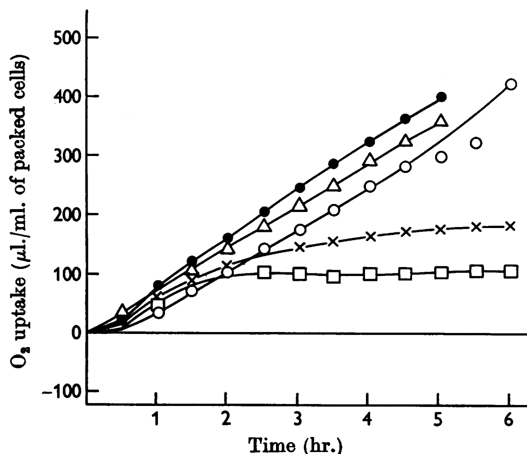

Fig. 4. Reactivating effect of adenosine on glucose utilization by cystamine-poisoned erythrocytes. Washed erythrocytes were poisoned by preincubation with cystamine (17 mM) for approx. 30 min. at room temperature. Most of the cystamine was subsequently removed from the cells by washing twice in 10 vol. of buffer. Incubations of the poisoned erythrocytes, as well as of untreated cells in the controls, were performed with methylene blue, glucose (11 mM), adenosine (2·5 mM) and cystamine (17 mM) as follows: ○, untreated cells with glucose; ●, untreated cells with glucose and adenosine; ×, poisoned cells with adenosine and cystamine; △, poisoned cells with glucose and adenosine; □, poisoned cells with adenosine. Glucose alone with poisoned cells did not give any oxygen uptake.

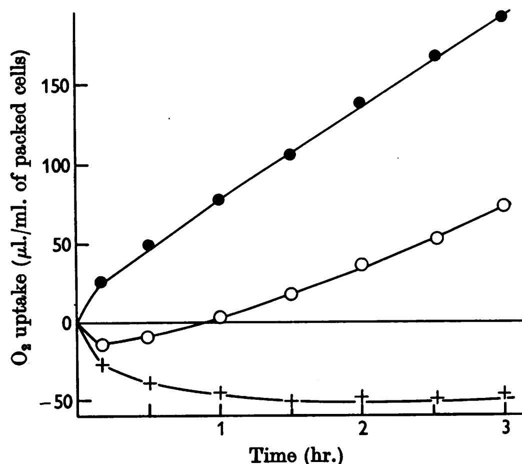

Fig. 5. Reactivating effect of 2-mercaptoethanol on glucose utilization in tetraethylcystamine-poisoned erythrocytes. Washed erythrocytes were poisoned by incubation with tetraethylcystamine (17 mM) for approx. 30 min. at room temperature. The tetraethylcystamine was subsequently removed from the erythrocytes by the procedures listed below and then incubated with glucose (11 mM) and methylene blue. ●, Untreated cells; +, poisoned cells washed twice in plain buffer and once with buffer containing EDTA (mM); ○, poisoned cells washed twice in plain buffer and once in buffer containing 2-mercaptoethanol (30 mM) + EDTA (mM).

DISCUSSION

In this paper we report that the 'disulphide poisoning' of human erythrocytes, which can be caused by high concentrations of a number of disulphides such as cystamine and its derivatives (Eldjarn *et al.* 1962), is due to a complete block in glucose utilization at the hexokinase level. Despite the fact that a number of the enzymes in the pentose phosphate shunt and in the Embden–Meyerhof pathway are classified as SH-enzymes, these other enzymes appear unaffected. Of the other metabolic reactions tested, only xanthosine metabolism appears to be affected by the 'disulphide poisoning'. However, the metabolic reactions of erythrocytes are limited in number.

The inhibition of hexokinase by disulphides could not be ascribed to a shortage of ATP, and it could be overcome by incubation with thiols. The intracellular glutathione reductase could furnish the reactivating thiol (GSH) provided that $NADPH_2$ could be synthesized. This was brought about by purine nucleosides which can provide pentose phosphates for the erythrocyte metabolism, thus bypassing hexokinase.

It has often been proposed that the significance of glutathione (GSH) and glutathione reductase is to maintain the intracellular SS/SH oxidation–reduction potential close to the reduced state in order to protect SH-enzymes. It has also been speculated that variations in this intracellular SS/SH balance might demonstrate a physiological regulatory mechanism for enzyme activities (Barron, 1951). However, the experimental evidence for such an intracellular function of GSH is meagre. Rapoport & Scheuch (1960) found that a decrease in the concentration of GSH in reticulocytes led to a concomitant drop in pyrophosphatase activity. Also the glyoxalase activity of erythrocytes depends on the GSH concentration (Jowett & Quastel, 1933). However, in the latter case GSH is known to function as a coenzyme in the reaction.

A possible mechanism by which a variation in the GSH/GSSG ratio could influence enzyme activity would be by mixed disulphide formation with enzyme thiol groups. Cystamine and cystamine derivatives form mixed disulphides with a number of thiols (Pihl & Eldjarn, 1958). However, these disulphides have not proved to be effective as enzyme inhibitors in experiments *in vitro*, probably owing to the fact that their oxidation–reduction potentials are too low (Eldjarn & Pihl, 1957). The inhibition of SH-enzymes in the purified state has so far only succeeded with disulphides of higher oxidation–reduction potentials, such as formamidine disulphide (Walker & Walker, 1960) and tetrathionate (Pihl & Lange, 1962).

The 'disulphide poisoning' of erythrocytes appears to be specifically limited to the hexokinase level, whereas the classical SH-enzymes such as glyceraldehyde 3-phosphate dehydrogenase seem to be unaffected. These findings cast doubt on the interpretation of the inhibition as being due to blockage of essential thiol groups by mixed disulphide formation.

SUMMARY

1. The effect of disulphides on glycolysis in human erythrocytes has been studied.

2. High concentrations of cystamine and cystamine derivatives inhibit the utilization of glucose, whereas utilization of adenosine or inosine is unaffected.

3. The inhibitory effect on glycolysis has been ascribed to a block at the hexokinase level.

4. This block cannot be explained by a depletion of the ATP of the cells.

5. The 'disulphide poisoning' can be reversed with adenosine or by treatment with thiols.

6. The possibility of a reversible mixed disulphide formation involving thiol groups of hexokinase is discussed.

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