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The Inhibition of Hexokinase by Disulphides

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A number of mammalian tissues reduce disulphides to the corresponding thiols. Human erythrocytes are able to reduce cystamine and a number of its derivatives when cell metabolism is maintained with an appropriate substrate such as glucose or adenosine. With glucose as substrate, high concentrations of these disulphides abolish the reducing ability of the cells (Eldjarn, Bremer & Börresen, 1962). There is indirect evidence that this 'disulphide poisoning' is due to an inhibition of glucose metabolism at the hexokinase level (Eldjarn & Bremer, 1962).

The present paper reports that similar 'disulphide poisoning' of glucose metabolism can also be produced in other mammalian tissues, such as diaphragm, brain, testicle and kidney of rat. The disulphide appears to interfere selectively with glucose phosphorylation. Experiments on crude calf-brain hexokinase show that this enzyme is inhibited by several disulphides and is partially reactivated by thiols. The inhibition can probably be ascribed to mixed disulphide formation of essential thiol groups of the enzyme.

MATERIALS AND METHODS

Albino rats of mixed breed were killed by a blow on the neck. The organs were rapidly removed and tissues prepared on ice as follows. Kidney and brain slices (0·5 mm. thick) were cut with a Stadie–Riggs microtome. To ensure constant ratios between white and grey matter, the hemispheres were separated; each hemisphere was sliced completely and all the tissue used. All slices obtained from one hemisphere were incubated in the same vessel in the presence of disulphide; the slices from the other hemisphere were used as controls. The whole diaphragms were cut out rapidly, one half of each diaphragm being incubated intact in the absence of disulphide as a control. Testicular tissue was prepared by teasing out bunches of tubules of approximately equal weight. The wet weight of the tissues was determined before incubation.

Crude calf-brain hexokinase was prepared according to Crane & Sols (1955); we have used the enzyme prepared by deoxycholate extraction that they called their 'CD preparation'. It contained about 5 units of enzyme activity/mg. of protein and the stock solution contained about 20 units/ml. (units are those of Crane & Sols, 1955).

Cysteamine and its *N*-dialkyl derivatives were obtained from Fluka A.-G., Buchs, Switzerland. The corresponding disulphides were prepared by oxidation with I_2 and purified by recrystallization four times from ethanol–HCl–ether. The following reagents were used: NADP and ATP (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.); glucose 6-phosphate dehydrogenase (C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany). All other chemicals were of analytical-reagent quality.

The incubations were carried out at 37°. The phosphate- and bicarbonate-buffered solutions were prepared according to Elliott (1955), with the exception of the glucose concentration (stated in the text). The pH of the incubating medium was 7·4 after the introduction of tissue. The bicarbonate-buffered media were equilibrated with CO_2+O_2 or CO_2+N_2 (5:95) for aerobic or anaerobic experiments respectively. The volume of the incubating medium was about 10 times the volume of tissue.

The photometric *o*-toluidine method of Hultman (1959), which is specific for aldoses, was used for glucose determination. The presence of cysteamine seriously impaired the colour development of the method. This is probably due to thiazolidine formation on the aldehyde groups, since the *N*-dialkyl-substituted cysteamines had no such effect. Tetramethylcystamine and tetraethylcystamine were therefore chosen as suitable disulphides for this study. Lactic acid was determined by Ström's (1949) modification of the method of Barker & Summerson (1941). The protein of the crude calf-brain hexokinase was estimated from its protein N. Total N and non-protein N (after protein precipitation with 8%, w/v, trichloroacetic acid) were determined by the micro-Kjeldahl method, giving the protein N of the enzyme preparation by difference.

Hexokinase activity was estimated by the formation of glucose 6-phosphate, which was determined spectrophotometrically by the reduction of NADP in the presence of glucose 6-phosphate dehydrogenase. The appearance of $NADPH_2$ was measured continuously at 340 mμ for 10 min. in a recording spectrophotometer (model RPQ 20A; Carl Zeiss, Jena, Germany). Silica cuvettes of 5 mm. were used. Unless otherwise stated each cuvette contained, in a total volume of 1·5 ml.: crude calf-brain hexokinase (0·1 unit, 21 μg. of protein), glucose 6-phosphate dehydrogenase in excess, glucose (7·5 μmoles), $MgCl_2$ (7·5 μmoles), ATP (5·0 μmoles), NADP (1·3 μmoles) and tris–HCl buffer (60 μmoles), the whole preparation being at pH 7·5.

RESULTS

When tissues are incubated in a medium containing 5·5 mM-glucose and 5 mM-tetramethylcystamine, glucose utilization gradually decreases in diaphragm, brain and testicle, whereas an initial increase in uptake of glucose appears to take place in kidney slices (Table 1). Maximum inhibition of

uptake of glucose occurs by about 30 min. The residual glucose uptake after incubation for 30 min. was therefore chosen as a measure of the inhibition due to a particular concentration of the disulphide (Table 2). Glucose utilization was inhibited by concentrations of the disulphide over 1 mM in all the tissues tested. Varying results were obtained when the concentrations of the disulphide were less than 1 mM, ranging from slight inhibition to apparent stimulation of uptake of glucose. Since the reduction of disulphides is known to take place in the tissues (Eldjarn *et al.* 1962), it should be stressed that the inhibitory effects presented in Tables 1 and 2 represent minimum values.

When tissues are incubated with tetramethylcystamine under anaerobic conditions, more lactic acid is produced than would correspond to the simultaneous glucose utilization (Fig. 1). Glucose utilization is almost completely abolished when cerebral slices or hemi-diaphragms are incubated anaerobically with 10 mM-tetramethylcystamine, whereas lactic acid is produced to an appreciable extent. Thus in brain slices the lactic acid produced in 2 hr. exceeds the glucose uptake by an amount corresponding to 5·7 μmoles of hexose/g. of tissue. This value is compatible with a complete conversion of cerebral glycogen into lactic acid (McIlwain, 1959*a*). In the absence of the disulphide, however, lactic acid production in brain slices roughly corresponds to the glucose uptake. In hemi-diaphragms, however, lactic acid production is considerably greater than the concomitant glucose uptake, even when they are incubated in the absence of the disulphide. The difference in the lactic acid production between the control and the inhibited hemi-diaphragm roughly corresponds to the glucose consumed by the former. These data suggest that the lactic acid production from endogenous substrates is largely unaffected by concentrations of the disulphide that completely inhibit glucose utilization.

Diaphragm, and to a less extent brain and kidney, contain glycogen, which may maintain lactic acid production for some time in the absence of exogenous substrates. This affords an opportunity for testing possible disulphide effects on the enzyme sequence between glycogen and lactic acid. Testicle is less suitable for this purpose owing to the low content of glycogen. Under anaerobic conditions tetramethylcystamine does not significantly alter the lactic acid production from endogenous substrates in any of the tissues tested

Table 1. *Inhibition of glucose utilization by tetramethylcystamine in rat tissues* in vitro

Tissue specimens were incubated aerobically in the absence or presence of tetramethylcystamine (5 mM) in a phosphate buffer solution containing glucose (5·5 mM). Results were calculated from the fall in glucose concentration in the medium.

Tissue	Concn. of tetramethylcystamine (mM)	Uptake of glucose (μmoles/g. of tissue) 30 min.	60 min.	120 min.
Hemi-diaphragm	0	3·1	5·6	10·8
	5·0	2·2	2·3	2·3
Brain	0	8·2	14·2	30·4
	5·0	5·3	6·7	8·6
Testicle	0	6·5	10·5	15·3
	5·0	4·4	5·6	5·6
Kidney	0	4·7	7·3	13·2
	5·0	7·0	9·7	11·0

Table 2. *Effect of concentration of tetramethylcystamine on glucose utilization in rat tissues* in vitro

Tissue specimens were incubated aerobically with various concentrations of tetramethylcystamine in a bicarbonate buffer solution that contained no glucose. After incubation for 30 min., glucose was added to 5·5 mM. Results were calculated from the fall in glucose concentration in the incubation medium for the subsequent 2 hr.

Concn. of tetramethylcystamine (mM)	Uptake of glucose (μmoles/g. of tissue during 2 hr.) Hemi-diaphragm	Brain	Testicle	Kidney
0	17·9	30·3	16·7	17·8
0·1	23·5	31·0	—	19·3
1·0	5·2	17·9	8·1	15·7
2·5	4·3	11·2	2·9	11·3
5·0	3·4	7·5	1·7	2·1

(Table 3). These findings suggest that (if glycogen is the major endogenous source of lactic acid) tetramethylcystamine inhibits glucose utilization by interference with a metabolic step preceding glucose 6-phosphate, i.e. either with the transport of glucose through the cell membrane or with glucose phosphorylation.

The inhibition of hexokinase by various potent –SH-blocking agents has been previously reported (Dixon & Needham, 1946; Griffiths, 1949; Sols & Crane, 1954; Villar-Palasi, Carballido, Sols & Arteta, 1957; Bhattacharya, 1959; Caramia, 1961). The present experiments have demonstrated that crude calf-brain hexokinase is inhibited by tetramethylcystamine (Table 4). Prolonged incubation of the enzyme with the disulphide is necessary to produce maximum inhibition. It is significant that under comparable conditions the same concentration of the disulphide produces the same degree of inhibition of glucose utilization in brain slices as of the activity of the isolated enzyme (Tables 2 and 4). A similar degree of inhibition of calf-brain hexo-

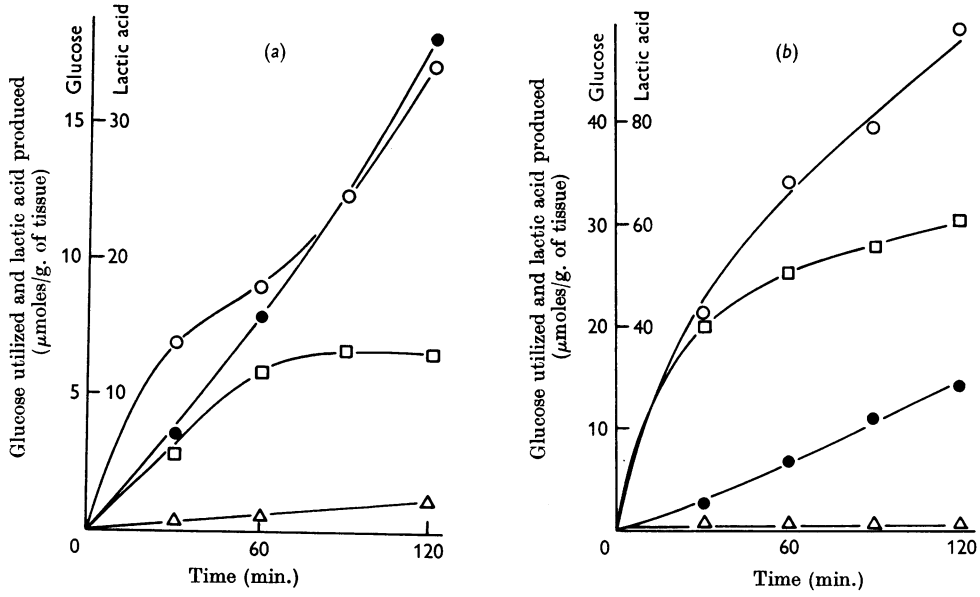

Fig. 1. Glucose utilization and lactic acid production in the presence of tetramethylcystamine in rat tissues *in vitro*. Tissues were incubated anaerobically in the absence or the presence of tetramethylcystamine (10 mM), in bicarbonate-buffered media containing glucose (5·5 mM). Results were calculated from determinations of the concentrations of glucose and lactic acid. ●, Glucose utilized in the absence of tetramethylcystamine; △, glucose utilized in the presence of tetramethylcystamine; ○, lactic acid produced in the absence of tetramethylcystamine; □, lactic acid produced in the presence of tetramethylcystamine. (*a*) Brain; (*b*) hemi-diaphragm.

Table 3. *Lactic acid production from endogenous substrates*

Tissue specimens were rapidly prepared and incubated anaerobically in the absence or presence of tetramethylcystamine (10 mM) in a bicarbonate buffer solution that contained no glucose.

Tissue	Concn. of tetramethylcystamine (mM)	Lactic acid produced (μmoles/g. of tissue) 30 min.	60 min.	120 min.
Hemi-diaphragm	0	56	81	106
	10	67	84	95
Brain	0	5·4	7·0	7·4
	10	5·3	7·2	7·3
Testicle	0	2·5	3·4	4·4
	10	2·3	2·8	3·5
Kidney	0	6·4	11·0	12·8
	10	7·1	8·6	14·8

Table 4. *Inhibition of crude calf-brain hexokinase by tetramethylcystamine*

Samples of crude calf-brain hexokinase (210 μg. of protein, 1 unit) were incubated in 2·5 ml. of tris buffer (40 mM, pH 7·5) containing various concentrations of tetramethylcystamine. Portions (0·25 ml.) were removed after incubation for 15, 30 and 60 min. and the residual hexokinase activity was measured. Results are given as percentages of the activity of an uninhibited control before incubation.

Concn. of tetramethylcystamine (mM)	Residual activity (%) after incubation		
	15 min.	30 min.	60 min.
0	89	86	80
0·5	74	57	28
1·0	60	43	20
2·5	46	23	7
5·0	27	7	0
10·0	15	3	0

Table 5. *Relative efficiency of cystamine, tetramethylcystamine and tetraethylcystamine as inhibitors of crude calf-brain hexokinase*

Samples of crude calf-brain hexokinase (210 μg. of protein, 1 unit) were incubated in a total volume of 0·5 ml. of tris buffer (40 mM, pH 7·5) containing various concentrations of cystamine, tetramethylcystamine and tetraethylcystamine. After incubation for 30 min. a portion (50 μl.) was removed from each sample and the residual hexokinase activity determined. Results are given as percentages of the activity of a control incubated in the absence of the disulphide.

Concn. of inhibitor (mM)	Residual enzyme activity (%) after incubation for 30 min.		
	Cystamine	Tetramethylcystamine	Tetraethylcystamine
0	100	100	100
0·1	95	90	77
0·5	78	73	50
1·0	53	50	20
5·0	20	9	0
10·0	15	3	0

kinase was produced by cystamine and tetraethylcystamine, the latter being slightly more potent (Table 5). The small differences in the efficiency of these inhibitors may be due to differences in their oxidation–reduction potentials. Oxidized glutathione, on the other hand, did not inhibit the enzyme.

Glucose utilization in testicle as well as the activity of crude calf-brain hexokinase can be partially restored by the addition of thiols (Fig. 2). Glucose utilization in the other tissues, however, could not be restored. In spite of the incomplete reactivation, it seems likely that the inhibitory mechanism is mixed disulphide formation of hexokinase –SH groups; bound cysteamine residues probably produce rapid denaturation with the irreversible loss of enzyme activity.

Glucose protects brain hexokinase against inhibition by disulphides (Table 6). The ATP–Mg^{2+} ion complex, the other substrate of hexokinase, also shows some protection, whereas neither Mg^{2+} ion nor ATP alone is effective. These findings indicate that the thiol groups of hexokinase either participate in the binding of substrate or become sterically protected, when the enzyme–substrate complex is formed. The protective ability of glucose does not seem to operate in slices (Table 1), as the inhibition corresponds fairly well to the inhibition obtained in the absence of glucose on crude calf-brain hexokinase (Table 4). This may be due to a low intracellular glucose concentration. Human erythrocytes, which are freely permeable to glucose, are more resistant to disulphide poisoning than other tissues, in spite of the lower activity of glutathione reductase (cf. Eldjarn & Bremer, 1962; Rall & Lehninger, 1952).

DISCUSSION

Eldjarn & Bremer (1962) reported an inhibitory effect of a number of disulphides on glucose metabolism in human erythrocytes, which they suggested to be due to a complete block at the hexokinase level. The present paper reports similar effects of tetramethylcystamine in various rat tissues, suggesting that 'disulphide poisoning' at the hexokinase level may be a general phenomenon in mammalian tissues. Furthermore crude calf-brain hexokinase was found to be inhibited by cystamine and its *NN'*-tetra-alkyl derivatives, and partially reactivated by thiols. It is suggested that the inhibition of this enzyme is caused by mixed disulphide formation of essential thiol groups. The same mechanism could operate in various rat tissues as well as in human erythrocytes (Eldjarn & Bremer, 1962).

Several enzymes in the glycolytic sequence (hexokinase, aldolase, phosphoglyceraldehyde dehydrogenase, pyruvate kinase and lactate dehydrogenase; Boyer, 1959), as well as in the pentose phosphate shunt (pentose phosphate epimerase, pentose phosphate isomerase; Dickens & Williamson, 1956), and glucose 6-phosphate dehydrogenase (Scheuch & Rapoport, 1962), are inhibited by thiol-blocking reagents. Our data show that hexokinase is probably the only enzyme in these pathways that is inhibited by disulphides of low oxidation–reduction potentials such as cystamine and its derivatives. A number of protein thiol groups that can be titrated with *p*-chloromercuribenzoate (and other thiol-blocking reagents) fail to interact with disulphides of low oxidation–reduction potentials (Pihl & Eldjarn, 1958). It has

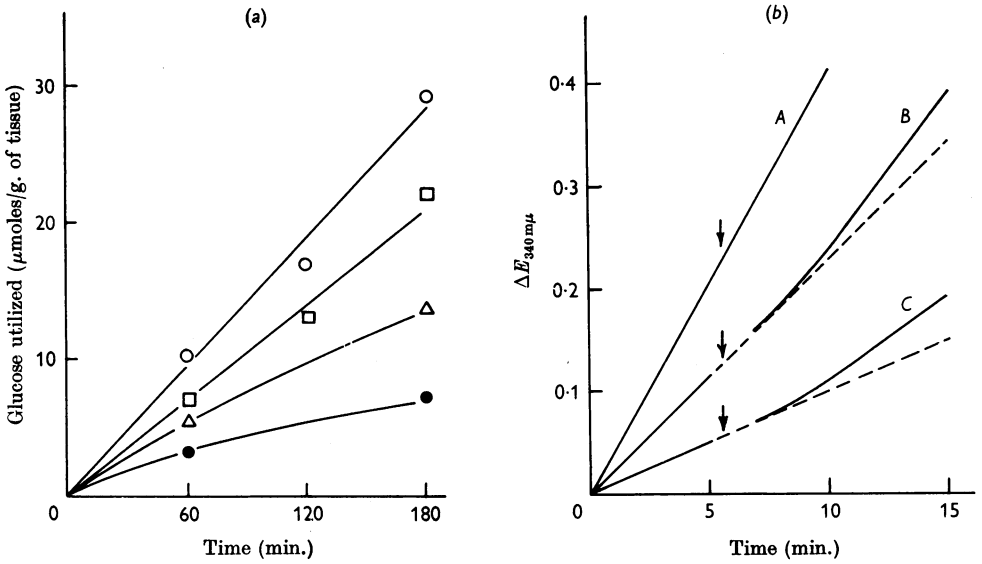

Fig. 2. Reactivating effect of thiols on glucose utilization in testicle tissue and on crude calf-brain hexokinase. (*a*) Glucose utilization was inhibited in three specimens of testicle tissue (0·5 g. wet wt.) by incubating with tetramethylcystamine (2·5 mM). A control specimen was incubated in the absence of the disulphide. After incubation for 30 min. the tissue specimens were removed from the incubation media and treated as follows: ●, inhibited control specimen: reincubated in buffer; △, inhibited, washed specimen: washed for 10 min. and reincubated in buffer; □, inhibited specimen, treated with thiol: washed for 10 min. in buffer containing 2-mercaptoethanol (25 mM) and reincubated in buffer; ○, uninhibited control specimen: incubated, washed and reincubated in buffer. The experiment was done aerobically in bicarbonate-buffered media containing glucose (5·5 mM) and the specific additions listed above. Volumes of buffer used: incubation, 5 ml.; washing, 20 ml.; reincubation, 5 ml. Upon reincubation the utilization of glucose was followed for 3 hr. (*b*) A portion (0·050 ml.) of crude calf-brain-hexokinase suspension (210 μg. of protein, 1 unit) was mixed with 0·450 ml. of ice-cold tris buffer (40 mM, pH 7·5) containing tetramethylcystamine (2·5 mM). Before incubation (*A*), as well as after incubation for 15 (*B*) and 30 (*C*) min. at 37°, portions (50 μl.) were removed and the hexokinase activities measured. After the formation of $NADPH_2$ had been recorded for 5 min., 50 μl. of freshly prepared, neutralized cysteine solution was added to the cuvettes (arrows), producing a concentration of 25 mM. To reveal the effect of the thiol, the formation of $NADPH_2$ was followed for another 10 min.

Table 6. *Substrate protection of crude calf-brain hexokinase against the inhibition by tetramethylcystamine*

Samples of crude calf-brain hexokinase (210 μg. of protein, 1 unit) were incubated in a total volume of 0·5 ml. tris buffer (40 mM, pH 7·5) containing tetramethylcystamine (5 mM). The buffered solutions contained in addition various concentrations of glucose, $MgCl_2$ or ATP. After incubation for 30 min. a portion (50 μl.) was removed from each sample and the hexokinase activity determined. Results are given as percentages of the activity of a control sample, which was incubated in the absence of the disulphide and in the presence of glucose (10 mM).

	Residual enzyme activity (%) after incubation with tetramethylcystamine (5 mM) for 30 min.						
Concn. of varying substrate (mM) ...	0	0·01	0·1	1·0	5·0	10·0	20·0
[Glucose] varying, [ATP] 0, [Mg^{2+}] 0	7	18	40	52	62	60	60
[ATP] varying, [Mg^{2+}] 10 mM, [Glucose] 0	4	3	6	6	10	12	11
[Mg^{2+}] varying, [ATP] 10 mM, [Glucose] 0	4	3	4	5	9	12	—

therefore been proposed that the reactivity of protein thiol groups could be estimated by testing their ability to interact with disulphides of different oxidation–reduction potentials (Pihl & Eldjarn, 1958; Pihl & Lange, 1962). The interaction of cystamine with a particular protein would thus indicate the presence of highly reactive thiol groups. Thiol groups that fail to interact with cystamine would still react with disulphides of higher oxidation–reduction potentials, such as tetrathionate, tetraethylthiuram disulphide or cystamine monosulphoxide. The last-named compounds would therefore be more potent inhibitors of thiol enzymes than cystamine. This assumption holds true for crude calf-brain hexokinase, since tetraethylthiuram disulphide (Strömme, 1963) and cystamine monosulphoxide (R. Nesbakken, unpublished work) inhibit the enzyme in lower concentrations than does cystamine. The present data therefore indicate that hexokinase possesses thiol groups with a greater reactivity towards cystamine than any other enzyme in the glycolytic sequence. A detailed study of the reactions and function of the thiol groups of hexokinase must await purification of the mammalian enzyme.

When tissues are incubated with disulphides the major reactions are mixed disulphide formation with particular protein thiol groups (reaction 1) (Eldjarn & Pihl, 1956) and with reduced glutathione (reactions 2, 3) (Eldjarn & Pihl, 1957).

$$\text{Protein SH} + \text{RSSR} \rightarrow \text{Protein SSR} + \text{RSH} \quad (1)$$

$$\text{GSH} + \text{RSSR} \rightarrow \text{GSSR} + \text{RSH} \quad (2)$$

$$\text{GSH} + \text{GSSR} \rightarrow \text{GSSG} + \text{RSH} \quad (3)$$

In all cells containing a glutathione-reductase system, disulphide reduction by reactions (2) and (3) will be maintained provided that cell metabolism can provide sufficient substrate:

$$\text{NADPH}_2 + \text{GSSG} \rightarrow 2\,\text{GSH} + \text{NADP}$$

The glutathione-reductase content of different tissues varies considerably. Rall & Lehninger (1952) found the proportions of this enzyme in muscle, brain and kidney to be 22, 60 and 400 respectively. The different degree of inhibition obtained in the various tissues with a particular concentration of the disulphide (Table 2) may depend on the disulphide-reducing capacity of the tissues rather than on differences in their hexokinases. The glutathione-reductase system obviously furnishes the cells with an efficient protective mechanism against the toxic effects of disulphides, and so the greater toxic effects would be expected in tissues having a low concentration of glutathione reductase. Other factors, however, such as differences in cell permeability for a particular disulphide and in intracellular glucose concentration, could also be of significance. Thus tetrathionate is known to possess a specific toxicity towards a certain area of the nephrons, despite the high activity of glutathione reductase in the kidney (Gilman, Philips, Koelle, Allen & St John, 1946). The thiol produced in reactions (1), (2) and (3) is spontaneously reoxidized to the corresponding disulphide by molecular oxygen or by tissue constituents such as cytochromes (Strömme, 1963) with the production of hydrogen peroxide. Furthermore, the thiol may be oxidized metabolically to the corresponding sulphenic acid or sulphinic acid (Eldjarn, 1954). Under anaerobic conditions the production of hydrogen peroxide as well as of the sulphenic acid or sulphinic acid would be considerably decreased. However, the inhibitory action of tetramethylcystamine on glycolysis was only slightly less under anaerobic conditions (Fig. 1). This could be due to a smaller effective concentration of the disulphide, since the production of the corresponding thiol is not diminished by reoxidation. It seems probable therefore that the inhibition of glycolysis can be entirely accounted for by the disulphide present.

Pure 'disulphide effects' can only be expected when the amount of disulphide present exceeds the disulphide-reducing capacity of the tissue. The possible metabolic effects of the corresponding thiol alone would be displayed at low disulphide concentrations. Thiols enhance glucose utilization in brain slices (McIlwain, 1959*b*), and in certain conditions of disturbed carbohydrate metabolism in human patients (Ciasca, 1959; Butterfield, 1960). The stimulation of glucose uptake seen in our experiments with low concentrations of disulphide is probably caused by similar effects of liberated thiol residues. The metabolic reaction which is stimulated by thiols is not known. Creatine phosphate and inorganic phosphate have been suggested as mediators in the stimulation in brain slices (McIlwain, 1959*b*) and interference with mitochondrial disulphide–thiol-interchange reactions was postulated by Ciasca (1959) and Butterfield (1960).

It has been suggested that variations in the intracellular disulphide–thiol balance might provide a physiological regulatory mechanism for enzyme activities (Barron, 1951). The lack of inhibition by oxidized glutathione makes it unlikely that mixed disulphides with this compound can participate in the regulation of glycolysis. It still remains possible, however, that enzymic reactions outside the glycolytic and pentose phosphate-shunt pathways may be regulated by reversible disulphide-interchange reactions with oxidized glutathione. The opposing effects of thiols and disulphides obviously can derange the normal regulation of glucose metabolism, and these effects must be taken into account when the toxicity as well as the pharmacological properties of the compounds are discussed.

SUMMARY

1. The effects of disulphides on glucose utilization in various rat tissues *in vitro* as well as on crude calf-brain hexokinase have been studied.

2. High concentrations of disulphides inhibit the utilization of glucose in diaphragm, brain, testicle and kidney, whereas the production of lactic acid from endogenous substrates is not inhibited.

3. Crude calf-brain hexokinase is inhibited by disulphides to the same extent as is the utilization of glucose in brain-tissue slices. Both inhibitions can be partially reversed by thiols.

4. The inhibition is tentatively ascribed to mixed disulphide formation on essential thiol groups of hexokinase.

5. Glucose and to a limited extent the adenosine triphosphate–Mg^{2+} ion complex protect crude calf-brain hexokinase against the inhibition by disulphides.

6. The apparent specificity of the enzyme inhibitory action of disulphides of low oxidation–reduction potentials, as well as the protective mechanism of the cells against this inhibition, are discussed.

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Periodic Activation of Lysosomal Enzymes during Regeneration of the Liver

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It has been suggested that an increase in the acid deoxyribonuclease content of a tissue is responsible for, or is directly associated with, an increase in the rate of cell division (Stern, 1960; Brody & Thorell, 1957). It is visualized that this would take place by a stimulation of DNA synthesis after the increase in concentration of precursors.

Acid deoxyribonuclease occurs in the lysosomes together with several other hydrolases, which also have an acid pH optimum (de Duve, 1959). These enzymes exhibit latency, i.e. when rat liver is gently homogenized in 0·25 M-sucrose the enzymes exhibit only a fraction of the activity shown in the presence of 0·1% Triton X-100. Total deoxyribo-