SULFHYDRYL GROUPS IN RELATION TO THE METABOLISM AND MOTILITY OF HUMAN SPERMATOZOA

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During a wartime investigation of the effect of arsenicals on the motility of human spermatozoa (15, 11) it was apparent that the addition to suspensions of these cells of substances which inhibited —SH groups led to failure of their motile activity. It was shown further that such an inhibition could be reversed, in part at least, by subsequent addition to the cell-inhibitor suspension of dithiol compounds such as BAL, provided the inhibitor was not allowed to remain in contact with the cells for any appreciable length of time. The work to be reported is an extension and elaboration of these studies, with the use of compounds more "physiological" in their structure and with considerably less toxicity.

The wartime investigations which did so much to advance our knowledge of the functions of —SH groups have been reviewed in great detail (15, 12). It is now recognized universally that —SH groups are intimately involved in the normal functioning of a remarkably large number of enzymes (1, 5, 14). In the case of human spermatozoa, we already have demonstrated (8) that certain inhibitors which presumably affect —SH groups specifically, impair glycolysis in these cells and, simultaneously suppress their motile activity. Barron *et al.* (2) recently demonstrated the stimulatory effects in small concentrations of sulfhydryl reagents on sea urchin spermatozoa and the inhibitory effect of high concentrations. They did not relate these effects to the motile activity of these cells.

The studies to be presented here are concerned with the kinetics of Cu inhibition of the glycolysis and of the motility of human spermatozoa, and the protective effects of cysteine and glutathione against this inhibition. Cu was selected because it has an affinity for —SH groups and because it is a known inhibitor of such systems (6). Cu in low concentration produces a slow inhibition of the motility and the metabolism of human spermatozoa (11) and thus allows easier analysis of the kinetics of this inhibition and of the neutralizing effects of the protective substances. Because of their physiological nature, cysteine and glutathione were selected as the protective substances. In this connection it will be shown that cysteine and glutathione have widely different effects; and, indeed, that cysteine under certain conditions may act as an inhibitor of enzyme systems containing —SH groups.

Methods

The suspensions of spermatozoa were prepared by methods already described in detail (9). It should be emphasized that the spermatozoa were washed as free of seminal fluid as one washing with Ringer's solution and centrifugation would allow. The final cell suspensions were in Ringer's solution containing 100 mg. per cent glucose and bicarbonate. In all experiments, the suspensions contained 100 to 150 million cells/cc. of which approximately 50 to 60 per cent showed good motility just prior to the experimental runs. One cc. aliquots were added to Warburg vessels (7 cc. capacity) and equilibrated at 38°C. with gas mixtures containing 95 per cent N_2–5 per cent CO_2 or 95 per cent O_2–5 per cent CO_2 for anaerobic or aerobic experiments. When Cu inhibition alone was to be measured, the various concentrations were placed in the side-arms of the vessels and added to the cells in the main vessel at the end of the gas equilibration period and just prior to the zero time manometric reading. When the protective effects of cysteine and glutathione were studied, the Cu was added directly to the cells in the main vessels and the protective substances could be added to the cells at any selected time after the addition of the Cu. Because of the acidity of the cysteine (hydrochloride) and the glutathione, they were neutralized by bicarbonate so that upon their addition to the Ringer-bicarbonate in the manometers, there was only a minimal, initial displacement of CO_2. Aerobic and anaerobic glycolysis in the presence of these substances was measured as a rule for 3 hours at 38°C. Determinations of motility were made as routine at the end of each experiment in terms of quality (speed of forward progress) and percentage of active cells. Penicillin (10 units/vessel) was added to each vessel in all experiments to prevent bacterial growth.

EXPERIMENTS

The initial experiments were designed to determine the concentration of Cu necessary to produce complete inhibition of the motile activity of the spermatozoa at 38°C. over a period of approximately 2 hours. Hopkins *et al.* (7), using muscle tissue, obtained complete inhibition of succinic dehydrogenase with a Cu concentration of 300 gamma for each gram of muscle tissue. Since spermatozoa, considered as a tissue, are much more homogeneous than muscle and since, in terms of weight, the amount of spermatozoa used in these experiments is relatively small, it would be expected that if Cu would inhibit motility at all it would do so in low concentration. This proved to be the case. It was found that Cu in a concentration of 30 gamma/cc. of spermatozoa suspension would cause a progressive inhibition of the glycolysis and motility of the cells which was complete at about 2 hours. The progression is seen in Fig. 1. The inhibition of glycolysis is apparent in the first 30 minutes, becomes progressive during the next hour, and is virtually complete at 2 hours. Similarly, the failure of motile activity is obvious at 1 hour and is complete at 2 hours. Though in different experiments there were variations in these results, the data in Fig. 1 are representative of the general trend whether the measurements were made under aerobic or anaerobic conditions. The inhibition of the motility and of glycolysis in the spermatozoa by these concentrations of Cu is always found.

On the assumption that this inhibition involved enzyme systems the integrity of which depended on —SH groups, the suceeding experiments were designed to determine whether cysteine and glutathione would act as protective substances (1) when present in the system prior to addition of the Cu; and (2)

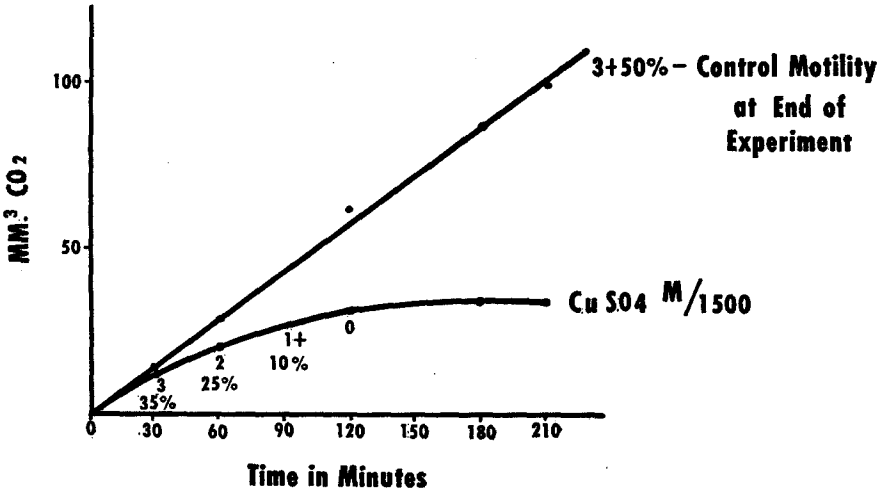

FIG. 1. Effect of Cu^{+} on glycolysis and motility of human spermatozoa. Per cent of active cells and quality of motility recorded at 30, 60, and 90 minutes on graph.

TABLE I

Protective Effects of Cysteine and Glutathione on Cu Inhibition of Motility of Human Spermatozoa

Time of addition of cysteine and glutathione after Cu	Cysteine N_2	Glutathione N_2	Cysteine O_2	Glutathione O_2
Prior to addition of Cu	Full protection	Full protection	None	Full protection
30 min.	Full protection	Full protection	None	Full protection
1 hr.	Partial to full protection	Partial to full protection	None	Partial to full protection
1½ hrs.	Only fair protection	Only fair protection	None	Only fair protection
2 hrs.	None	None	None	None

when added to the system at intervals after the addition of the Cu. The final concentrations of cysteine and glutathione used throughout were M/100. It was expected that if either cysteine or glutathione were in contact with the spermatozoa prior to the addition of the Cu, the Cu would be neutralized as an inhibiting agent by combination with the cysteine and glutathione in the external medium. The results are presented in Table I. Under anaerobic conditions the presence of cysteine and glutathione in the system prior to the addition of the

Cu fully protects the motility of the spermatozoa. The most reasonable explanation of this effect is that there is a rapid combination of the Cu with the sulfhydryl compounds in the external medium and that little free Cu is left to act upon the systems in the cell the inhibition of which produces loss of motile activity. Similarly, in all experiments done under anaerobic conditions in which cysteine or glutathione was added within 1 hour after the Cu, the motile activity of the cells was almost fully protected. It is apparent from Fig. 1 that at 1 hour after the addition of the Cu, a depressing effect on the motile activity already is obvious and therefore, that the Cu had already been attached to the systems responsible, in part at least, for maintenance of motility. A reasonable assumption, therefore, is that the addition of competing sulfhydryl groups to the

TABLE II

Effect of Cysteine on Motility of Spermatozoa under Aerobic Conditions*

Time after addition of cysteine	Motility in presence of cysteine		Control motility	
min.				
30	2^-	25 per cent	3^+	60 per cent
90	1^+	20 per cent	4	60 per cent
120	1	10 per cent	4^-	60 per cent
180		0	3^+	60 per cent

* The best quality of motility is designated arbitrarily as 4 and in terms of decreasing quality as 3, 2, 1, and 0. Any quality below 3 is considered subnormal as is any per cent of active cells below 50 per cent.

Cu-spermatozoa system within 1 hour not only protects the spermatozoa from any further loss of motile activity due to the Cu but restores the loss which already has taken place. However, if the sulfhydryl addition is made at $1\frac{1}{2}$ hours, the reversal of inhibition is not as striking though the remaining motility is protected. In this respect, glutathione exerts a better protective action than cysteine.

When these experiments are repeated under aerobic conditions there is a striking difference in the protective behavior of cysteine and glutathione. Glutathione exerts an equally protective effect in oxygen in spite of the fact that its oxidation under these experimental conditions is fairly rapid, as evidenced by the manometric readings. Cysteine, however, has no protective action whatsoever in oxygen, even though it is added to the system prior to the addition of the Cu. Further investigation (Table II) showed that within 30 minutes after addition of cysteine, a severe depression of motile activity ensues though complete failure of motile activity is not evidenced until 150 to 180 minutes. Concomitant with the failure of motility is a failure of glycolysis though in early stages this inhibition is from the manometric standpoint,

exaggerated due to the oxidation of the cysteine masking the CO_2 output of glycolysis. The toxic effect of cysteine remains even when its concentration is reduced tenfold. In this cellular system, therefore, and under these experimental conditions, cysteine even in low concentration is a toxic substance. It should be added that, like glutathione, the cysteine is oxidized, but not as rapidly as is glutathione.

The Effect of Tetrathionate and of Cysteine-Tetrathionate Combination

Gilman *et al.* (4) have shown that tetrathionate, the immediate oxidation product of thiosulfate, is, in moderate doses, toxic to the renal tubule. They have suggested that this action is due to the direct toxic action on catalytic

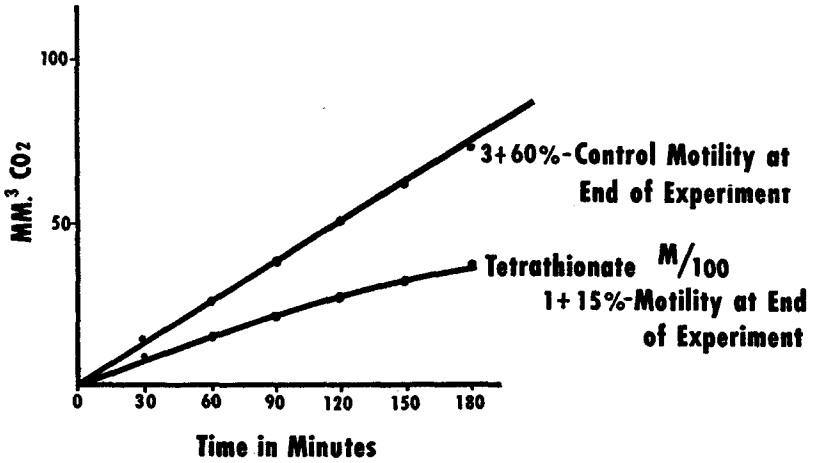

FIG. 2. Effect of Na tetrathionate on glycolysis and motility of human spermatozoa.

systems dependent on the presence of —SH, or by the removal of diffusible —SH compounds. In support of this postulate, they showed that cysteine, glutathione, and *l*-thiosorbitol, administered prophylactically, protect against the nephrotoxic action of tetrathionate. Philips *et al.* (13) have further demonstrated that *in vitro* tetrathionate is a powerful inhibitor of the succinic dehydrogenase.

In a test of additional sulfhydryl substances on the spermatozoa, tetrathionate was next selected. The final concentration of tetrathionate in contact with the spermatozoa, in most of the experiments to be described, was M/100 since it was found that this relatively high concentration had a depressing effect on motility and glycolysis slow enough to allow the addition of the protective substances up to 2 hours after addition of the tetrathionate. Fig. 2 shows a typical experiment in which, within 1 hour after the addition of the tetrathionate, the glycolysis of the treated spermatozoa was only about 50 per cent

of that in the control, no further inhibition taking place in the next 2 hours. These effects were the same under both aerobic and anaerobic conditions. The effect upon the motile activity of the spermatozoa of this concentration of tetrathionate is severe but not completely lethal. In all experiments, the motility of a large percentage of the cells was completely destroyed but, in every case, a residual percentage of cells (10 to 15 per cent) retained reasonably good motile activity. It can be stated, therefore, that tetrathionate, if given in high enough concentration, will inhibit the glycolytic activity of the spermatozoa and at the same time produce a severe but not complete depression of motile activity. In the latter respect, and as is true in inhibition of human sperm motility by any method, certain cells invariably are much more resistant to toxic substances.

Tetrathionate Plus Cysteine and Glutathione

Gilman *et al.* (4) have demonstrated that *in vivo* the prophylactic administration of cysteine and glutathione fully protects against the nephrotoxic action of tetrathionate, but that if the cysteine treatment is delayed for 15 minutes after the injection of tetrathionate no protection is afforded. We have repeated these experiments with the spermatozoa, with certain striking results regarding the different effects of cysteine and of glutathione. Since cysteine in itself had proved so toxic to the spermatozoa under aerobic conditions, its use as a prospective protective substance was limited to anaerobic experiments. It can be said immediately that the combination of cysteine-tetrathionate, irrespective of the order of addition of the substances, is lethal to the spermatozoa. As we have shown, cysteine alone is not toxic to the spermatozoa but when the tetrathionate is added, the combination of the two leads to pressure changes in the system which correspond to a rapid and large displacement of CO_2 from the bicarbonate present. It is obvious that this sudden burst of CO_2 liberation is not due to lactic acid production by the spermatozoa (chemical determinations confirm this), but rather is due to the interaction of the cysteine and tetrathionate. Gilman *et al.* (4) have measured this reaction *in vitro* in detail, and have shown clearly that cysteine reduces tetrathionate rapidly to thiosulfate; the cysteine is converted to cystine with the production of two hydrogen ions according to the equation:

$$2RSH + S_4O_6 \rightarrow 2S_2O_3 - RSSR + 2H^+.$$

The manometric changes we have observed, when equated with the amounts of cysteine and tetrathionate reacting in the system, are of the order of magnitude corresponding to the production of two mols of hydrogen ion for each mol of tetrathionate. The amount of H^+ ion produced in this system cannot be measured accurately by the manometric changes because (1) glycolysis is proceeding simultaneously, and (2) one cannot predict the changes in the cysteine occurring spontaneously either when it is in contact with cells prior to the addi-

tion of the tetrathionate or when it is in the side-arm of the vessel prior to addition to the tetrathionate–cell system. However, it seems certain that the nature of the reaction is as depicted in the above equation. What, then, is the nature of the lethal action of this combination? There are several possibilities, the most obvious being: (1) The formation of thiosulfate; (2) pH changes in the system; and (3) The formation of cystine.

These possibilities were tested and all but No. 3 eliminated. Thiosulfate is not toxic to the spermatozoa, nor is the combination of cysteine-thiosulfate. The pH change in the system is not enough to affect the motility of the spermatozoa. As further indication that pH is not a factor, the complete failure of the motility of the spermatozoa as a result of the cysteine-tetrathionate combination is not immediate but is delayed for 1 to 1½ hours after the reaction of the two substances which in turn leads rapidly to the pH change. In fact, the kinetics of the lethal effect of the combination are quite similar to the failure of motility in the presence of cysteine and oxygen. All the available evidence is in favor of postulate 3, namely that the formation of cystine in the presence of the spermatozoa leads to failure of motile activity.

Because of the relatively great insolubility of cystine as compared to cysteine it was not expected that, even in saturated solution, the addition of cystine to the spermatozoa would have any obvious effect; this proved to be the case. The motile activity of the cells is not affected by the amounts of cystine present in saturated solution.

In contrast to the toxicity of the products of cysteine-tetrathione interaction under anaerobic conditions, the substitution of glutathione for cysteine in the reaction produces the same rapid manifestation of pH change as depicted in the equation but has no inhibiting effect on the motility of the spermatozoa.

Effects in Oxygen of Other Sulfhydryl Substances

The effects of thiosorbitol, thioglycocollic acid, thioglycerol, and homocysteine have been investigated. In concentrations equivalent to the amount of cysteine used in these experiments, these substances have no striking effects on the motility of the spermatozoa under aerobic or anaerobic conditions. The combination of homocysteine-tetrathionate under anaerobic conditions has only the same effect on motility as tetrathionate alone. No further investigation of the effects of these substances was undertaken.

DISCUSSION

The evidence presented strongly suggests that —SH groups play an important, if not a fundamental, role in furnishing energy for the motility of human spermatozoa. Whatever the mechanism of the Cu inhibition the data show that once the Cu is attached to the cellular components which are involved in the simultaneous depression of metabolic and motile activity, the prompt addition

of competing —SH groups will detach the Cu and reverse the inhibition. We do not propose at this time to speculate on the particular enzyme systems affected in this process, except to point out that the failure of motile activity in the presence of Cu is closely related to the failure of glycolytic activity. Several of the enzymes essential for the breakdown of glucose to lactic acid are known to contain —SH groups and to depend for their adequate functioning upon the integrity of these groups (1). Hopkins *et al.* (7) have shown that the activity of the succinic dehydrogenase is inhibited by Cu, and that its activity can be completely restored by subsequent addition of glutathione to the system. But we have already shown (10) that, while human spermatozoa contain a very active succinic dehydrogenase, the motility of the cells is not dependent upon its integrity.

The toxic action of cysteine, under aerobic conditions is however, more conclusive proof that the inactivation of sulfhydryl groups in the spermatozoa leads to failure of motile activity. So far as we can determine, cysteine, in the concentrations used in these experiments, has not been thought of as toxic in biological systems. Hopkins and Morgan (6) in precise experiments showed that certain enzymes containing thiol groups could be inactivated by oxidized glutathione (GSSG), and that activity could be completely restored by subsequent exposure to reduced glutathione (GSH). These effects, however, were seen only in washed muscle extracts in which semipermeable membranes probably are disturbed and in which the GSSG would have ready access to the enzyme surface. There is good reason to believe, that the inhibiting effect of cysteine on sperm motility is a process similar to if not the same as that described by Hopkins and Morgan (6) in relation to oxidized glutathione. It should be emphasized (1) that cysteine (RSH) is toxic only in the presence of oxygen; (2) that manometric evidence of its slow oxidation under these conditions is definite; and (3) that cysteine is toxic under anaerobic conditions if a substance, *e.g.* tetrathionate, which can oxidize it, is present in the system. It is reasonable to suppose that cysteine can either penetrate the cell or be in the proximity of the enzyme surface in concentration, so that upon its oxidation to cystine (RSSR), oxidation of —SH groups on the enzyme to the inactive form (S-S) would take place. This would seem to be the most reasonable explanation of the toxicity of cysteine.

The failure of glutathione to affect motile activity under similar experimental conditions can be explained (1) by different rates of penetration of glutathione and cysteine or (2) by the two substances having dissimilar redox potentials. The latter are difficult to measure and are not known with any degree of accuracy (3). The first postulate is more tenable.

It is not hard to explain the non-toxicity of glutathione under these conditions. It is certain that it is oxidized in the aerobic experiments, and also anaerobically, in the presence of tetrathionate. In these respects, glutathione behaves in exactly the same fashion as cysteine, so far as its reactions with oxy-

gen and with tetrathionate are concerned, and yet the product of its reaction with these substances is not lethal to the motile activity of the spermatozoa.

There are two hypotheses which would explain the difference in the effects of cysteine and glutathione. If there were different rates of penetration of these substances, or different speeds of access to the enzyme surfaces affected, and if cysteine were the faster in either case, the cysteine would reach the inhibiting surfaces in higher concentration. If the cysteine were oxidized at this point (either by oxygen or by tetrathionate) cystine would be formed in concentration at the place where it would do the most harm, and where it could not reach by the addition of cystine alone (because of its relatively great insolubility) to the system.

The other hypothesis would assume that the redox potentials of cysteine and glutathione (or cystine and oxidized glutathione) are different. Unfortunately, the redox potentials of sulfhydryl systems are exceedingly hard to measure and are not known with any degree of accuracy (3).

In regard to the inhibitory effects of tetrathionate on metabolism and motility, no conclusive evidence has been presented that —SH groups alone are affected. The addition of glutathione to the cells within an hour after tetrathionate has been introduced does afford a good measure of protection to the motile activity; but this effect could be due, in part at least, to the reduction of the tetrathionate to thiosulfate, the latter being non-toxic. The fact that the cysteine-tetrathionate combination destroys motile activity does not necessarily contradict the observations of Gilman *et al.* (4) that prophylactic administration *in vivo* of cysteine protects the animal against the nephrotoxic action of tetrathionate. The two systems are not at all comparable.

SUMMARY

1. The motility and metabolism of human spermatozoa are inhibited by substances which have an affinity for sulfhydryl groups.

2. These inhibitions can be prevented, and in part, reversed, by the addition to the cell + inhibitor system of sulfhydryl compounds such as cysteine or glutathione.

3. Cysteine and glutathione, under aerobic conditions or in a system in which these substances can be oxidized, show widely different effects on the motility of the spermatozoa. Cysteine destroys the motility of the spermatozoa, whereas glutathione has no effect upon it.

4. Possible mechanisms of these effects are discussed.

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