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The Prevention of Thiol Autoxidation in Biological Systems by Means of Thiolated Sephadex

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The autoxidation of biologically important thiols has been prevented for periods up to 2 days in neutral and slightly alkaline solution. This is accomplished by the addition of a small quantity of the insoluble electron exchange material "thiolated Sephadex" (SH-Sephadex).

The level of cysteamine, cysteine, reduced glutathione, AET and diethylcysteamine added to biological materials can be maintained constant for more than 20 h at pH 7.5 and 37° in the presence of SH-Sephadex. When cupric and ferric ions are present the system is more unstable.

The stabilizing effect of SH-Sephadex involves (*a*) prevention of the disulfide catalysed thiol autoxidation and partial removal of heavy metals, resulting in a 30 fold decreased rate of oxidation and (*b*) a continuous reduction of the disulfide molecules formed.

Most thiols of low molecular weight are relatively stable in acid medium, but highly unstable in neutral and alkaline solution. Oxidation to the corresponding disulfides rapidly takes place with the simultaneous consumption of oxygen. Autoxidation is in general catalysed by heavy metals, particularly cupric and ferric ions.[1–5] The metallic ions of Ag, Hg, Be, Co, Cr, Mn, Ni, Pb, U, W, and Zn are less efficient catalysts.[5] The autoxidation of a thiol is furthermore accelerated by the oxidized, *i.e*, disulfide form, either of itself or of some other sulfhydryl compounds.[6,7] The mechanism of the thiol autoxidation, which is not yet fully understood, is apparently rather complex [7–9] and does not fit in with any simple and general theory. In fact, there is evidence which indicates that the reaction mechanism particularly in presence of metal catalysts or chelating agents, are quite different for several thiols.[4,8–12] Different results may consequently be obtained from thiol to thiol under identical conditions, *e.g.* the spontaneous oxidation of cysteine is accelerated by small amounts of ferric ions, whereas iron has a stabilizing effect on cysteamine.[11] It has been demonstrated that the rate of autoxidation, apart from the nature of the substrate, the pH, the amount and kind of heavy metal

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as well as disulfide present, also to a large extent depends upon the nature and concentration of the buffer and the presence or absence of chelating agents, and when present, also on the concentration of the latter.[10]

We may distinguish between two essentially different biochemical applications of thiols. Firstly, in a great number of experiments concerning SH-enzymes or other SH-proteins, it is customary to add a thiol to the system in order to "protect" the protein sulfhydryl groups, *i.e.* to maintain them in the reduced state and to avoid blockage by heavy metals. In this type of study a large excess of the thiol is usually employed, and only a small fraction will be oxidized during the experiment. The spontaneous oxidation ot the thiol therefore offers only a minor problem in such cases.

In the second type of experiments involving a thiol, *viz.* the *in vitro* studies on the biochemistry of the thiol itself, its metabolism, toxicity, X-ray protective effect, or other modes of action, the autoxidation has represented an important and difficult obstacle for many years. Small concentrations of the thiols are usually employed in these studies and autoxidation may consequently convert a substantial fraction into the corresponding disulfide in the course of a few minutes. It is thus doubtful if the true biochemical effect of a thiol like cysteine or cysteamine has ever been observed completely free from interference by their own oxidation product.

No satisfactory method for preventing the autoxidation of thiols of low molecular weight in neutral and alkaline solution has been described. The most widely used reagent for attempting to stabilize a thiol is probably EDTA which chelates with the traces of heavy metals always present.[8] In many cases the autoxidation process may indeed be slowed up to a certain degree. EDTA, however, must be used with great caution, since it is known to exhibit a strong pro-oxidative effect.[10–13] Thus EDTA may, particularly in presence of biological materials which contain traces of ascorbic acid [13] accelerate the oxidation of the thiol instead of preventing it. (See also Refs.[10,12]) In other cases EDTA has no effect, as in the autoxidation of glutathione in presence of blood serum.[14]

In the present paper it is reported that the concentration of low molecular weight thiols can be kept completely constant while the disulfide level is kept at zero, for periods up to 2 days under conditions which normally would have caused oxidation of the thiol in the course of an hour. This remarkable stabilization can be accomplished through the addition of a small amount of thiolated Sephadex (SH-Sephadex), which is a new insoluble electron exchange material developed in our laboratory.[15]

EXPERIMENTAL

Materials. Thiolated Sephadex, which is used for preparation of the chromatographic material "organomercurial polysaccharide"[15] was synthesized from Sephadex G-25. (Obtained from Pharmacia, Uppsala, Sweden). The latter was aminated with 2-aminoethyl hydrogensulphate and finally thiolated with N-acetylhomocysteine thiolactone to yield SH-Sephadex as described in a previous publication.[15]

Cysteamine, cysteine, dimethylcysteamine and N-acetyl DL-homocysteine thiolactone were obtained from Fluka AG., Chemische Fabrik Buchs, Switzerland and [^{35}S]cystamine and [^{35}S]tetrathionate from the Radiochemical Centre, Amersham, England. Tetraethylcystamine and N-piperidylcysteamine were generously supplied by Deutsche Gold und Silber

Scheideanstalt, Frankfurt am Main, Germany. 2-aminoethyl isothiouronium bromide (AET) and glutathione were purchased from Nutritional Biochemical Corporation, Cleveland, Ohio. The corresponding disulfides were prepared by oxidation with iodine and purified by recrystallization from ethanol-HCl-diethylether. All other chemicals used were commercial products of highest purity.

Chemical properties of thiolated Sephadex. The SH-Sephadex which was used throughout the present investigation contained approximately 0.5 % SH, *i.e.* one sulfhydryl group per 35 glucose units of the cross-linked dextran. Vacuum dried SH-Sephadex is a white insoluble powder which swells in water forming gel-grains with the same appearance and gel-filtration properties as the original Sephadex G-25. The SH-groups of the thiolated material are remarkably stable towards oxidation by air; thus no oxygen uptake was demonstrated in 24 h, at 37° and pH 7.4 (Fig. 4). The nitroprusside test for SH-groups is strongly positive. The material possesses a very high affinity for certain heavy metals such as Ag and Hg, and as a typical polythiol, it shows pronounced reducing properties. Thus all disulfides tested so far, *i.e.* cystine, cystamine, tetramethylcystamine, tetraethylcystamine, oxidized AET, oxidized glutathione, piperidylcystamine and tetrathionate are immediately reduced by the SH-Sephadex in a neutral medium. The reduction is accompanied by an oxidation of the sulfhydryl groups of the SH-Sephadex.

When thiolated Sephadex is oxidized by disulfides there exists a possibility for mixed disulfide formation between the SH-groups of the Sephadex and the added disulfide. Fortunately this reaction occurs only to a small extent, *viz.* less than 6 %. This could be demonstrated by means of ^{35}S-labelled cystamine and tetrathionate. Only 2 – 6 % of the added radioactivity became fixed to the insoluble SH-Sephadex while 94 – 98 % appeared in the supernatant in the reduced, *i.e.* thiol form. It may therefore be assumed that when SH-Sephadex acts as a reducing agent, the reduction will be accompanied by the production of intra- or intermolecular disulfide groups in the cross-linked dextran, rather than a mixed disulfide formation.

Methods. The experiments were performed in a Warburg bath with constant shaking at 37°. The basic system usually consisted of a thiol, the SH-Sephadex and a buffer (0.1 M phosphate-,veronal-, or carbonate-bicarbonate buffer). The additional components, *viz.* rat liver and rat kidney homogenates, EDTA, and various heavy metals were applied in different combinations. The shaking was interrupted at intervals, the SH-Sephadex allowed to settle, and aliquots of the supernatant fluid were withdrawn and immediately acidified to pH 2 with hydrochloric acid. The thiol content was subsequently determined by amperometric silver titration at the rotating platinum electrode.[16,17]

RESULTS

Most of the experimental data have been obtained on cysteamine which was chosen as a typical and representative thiol for the present studies. A detailed study on the autoxidation of cysteamine in presence or absence of various heavy metals with or without EDTA was performed prior to the experiments with SH-Sephadex. Phosphate buffer, pH 8.0 was employed and the concentration of the heavy metals and EDTA were 2×10^{-5} M and 10^{-2} M, respectively. The results are shown in Fig. 1. It is evident that the cupric ion is a particularly efficient catalyst, whereas the ferric ion has a certain stabilizing effect, as previously shown by Hagen and Koch.[11] Silver and mercuric ions stimulate the reaction only to a small extent. Addition of EDTA has a pronounced stabilizing effect on cysteamine when no heavy metals have been added. In presence of mercury and especially iron, EDTA exhibits a pro-oxidative effect, whereas the copper and silver catalysed oxidation of the thiol is slowed up to a certain degree by the chelating agent. Particular attention should be paid to the shape of the curves in Fig. 1, which indicate that the autoxidation of cysteamine is catalysed by its own oxidation product.

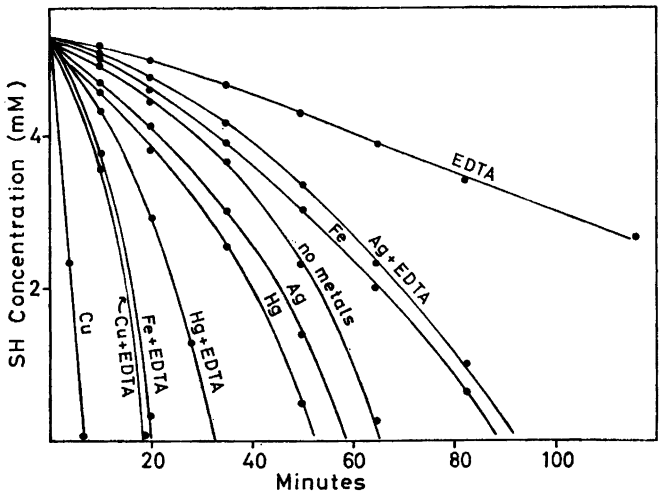

Fig. 1. Effect of heavy metals and EDTA on the autoxidation of cysteamine (RSH). The thiol (final concentration 5 mM), dissolved in phosphate buffer (5 ml 0.1 M) was incubated in presence of Cu, Fe, Ag and Hg (2×10^{-5} M) with and without EDTA (0.01 M) at pH 8.0 and 37°. Aliquots were withdrawn at intervals and the SH concentrations were determined by amperometric silver titration.

The effect of SH-Sephadex on the spontaneous oxidation of cysteamine was studied under similar experimental conditions as described above. The results are shown in Figs. 2 and 3. In Fig. 2 the stability of cysteamine in phosphate buffer at 37° and pH 7.5 has been compared with the stability in the same buffer containing EDTA, or SH-Sephadex. The figure shows that in absence of stabilizing agents the thiol was fully oxidized in one hour, and in the course of 5 h when EDTA had been added. In presence of SH-Sephadex, hower, the concentration of the thiol was kept completely constant for 48 h. During this period the disulfide level was consequently maintained at zero. After 48 h there was a slight decline in the SH-titer, partly due to bacterial growth, but even after 5 days with constant shaking at 37° and pH 7.5 there were detectable amounts of cysteamine remaining.

The stability of cysteamine in the presence of various heavy metals (concentration 2×10^{-5} M) and SH-Sephadex is shown in Fig. 3. As seen neither silver nor mercury interfered with the stabilization of the thiol. Even in the presence of copper and iron it was possible to maintain a constant thiol level for 45 min. This period could be extended by adding more SH-Sephadex to the system. The copper catalysed autoxidation in absence of thiolated Sephadex is included in Fig. 3 for comparison, and in the latter case the cysteamine was fully oxidized in less than 5 min.

Whether the stabilizing effect of SH-Sephadex is merely due to its reducing properties or if the actual rate of spontaneous oxidation is also changed, was settled by studying the oxygen consumption during an experiment similar to that described in Figs. 2 and 3. The results are shown in Fig. 4.

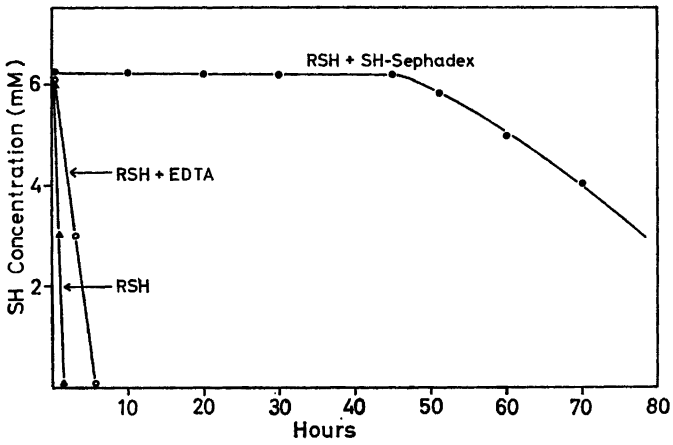

Fig. 2. The stabilizing effect of SH-Sephadex on cysteamine (RSH) The incubations were performed at 37° and the medium consisted of SH-Sephadex (0.15 g), the thiol (final concentration 6 mM) and phosphate buffer of pH 7.5 (10 ml 0.1 M). The control experiments contained cysteamine and phosphate buffer only and cysteamine, phosphate buffer and EDTA (0.01 M). The SH concentrations were determined as before.

The autoxidation of SH-Sephadex itself is negligible in absence of soluble thiols and heavy metals, and even in the presence of the most efficient catalyst, *i.e.* cupric ions, the oxygen uptake was very small (1.6 μl O_2/h). In the system consisting of SH-Sephadex, cysteamine and the phosphate buffer, a constant

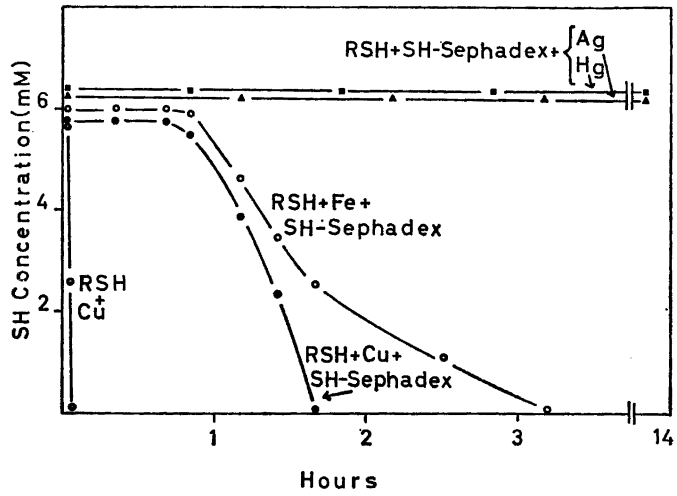

Fig. 3. The stabilizing effect of SH-Sephadex on cysteamine (RSH) in presence of heavy metals. The experimental conditions were as described in Fig. 2, except the additional heavy metals (final concentration 2×10^{-5} M).

rate of oxygen uptake was observed during the whole experimental period. This rate of oxygen consumption however, was very small (11 μl O_2/h) in comparison with the oxygen uptake of cysteamine alone (340 μl O_2/h). It is thus evident that apart from acting as an S—S reducing agent capable of maintaining a constant thiol level, addition of SH-Sephadex also decreases the oxygen consumption by a factor of 30. This also holds true in presence of 2×10^{-5} M silver and mercuric ions. A reduction of the rate of autoxidation was observed also in the presence of cupric ions. In a system consisting of copper (2×10^{-5} M), cysteamine and buffer the oxygen uptake was 3500 μl O_2/h. When SH-Sephadex was added, the consumption was 125 μl O_2/h, *i.e.* a decrease by a factor of 28. If the medium in addition contained EDTA (10^{-2} M) the oxygen uptake was further diminished (uptake 60 μl O_2/h.)

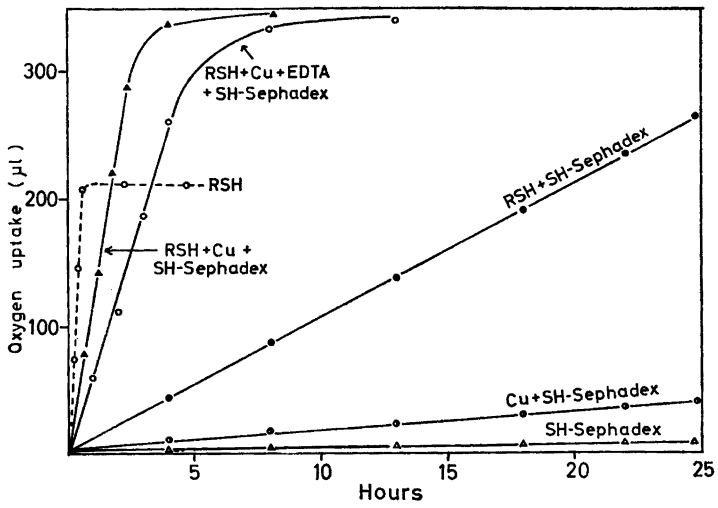

Fig. 4. The effect of SH-Sephadex on the oxygen consumption by cysteamine (RSH) in absence or presence of Cu^{++} and EDTA. The Warburg flasks contained SH-Sephadex (90 mg), cysteamine (final concentration 6 mM), phosphate buffer (3 ml 0.1 M), $CuSO_4$ (2×10^{-5} M) and EDTA (0.01 M) in different combinations. The oxygen uptake of cysteamine in presence of cupric ions and buffer only, was very large (3500 μl O_2/h) and is therefore not plotted in the above figure.

The autoxidation of thiols is in general faster at higher pH values when the dissociation of the sulfhydryl group is more complete. It was therefore of interest to examine how the stabilizing effect of SH-Sephadex was influenced by the pH. The results obtained showed that the SH-Sephadex was able to maintain a constant thiol level for at least 20 h when the pH was below 8.5. At higher pH the system gradually became more unstable, but even at pH 10.7 the thiol level could be kept constant for 3 h.

The amount of SH-Sephadex employed was found to be of importance. At pH 7.5 and in absence of cupric or ferric ions, 0.1—0.2 g dry SH-Sephadex

Table 1. Stabilization of thiols by SH-Sephadex in biological systems. The incubations were performed at 37° and pH 7.5. The medium contained the thiols (final concentration 4 mM), SH-Sephadex (0.1 g), rat liver or rat kidney homogenate equivalent to 200 mg tissue, and phosphate buffer (5 ml 0.1 M). RSH = cysteamine, CySH = cysteine, Et_2RSH = diethylcysteamine, AET = aminoethylisothiouronium bromide.

Time (hours)	SH-conc. (mM)					
	RSH + Liver homogenate	RSH + Kidney homogenate	CySH + Liver homogenate	Et_2RSH + Liver homogenate	GSH + Liver homogenate	AET + Liver homogenate
0	4.1	3.9	4.0	4.0	4.1	4.0
10	4.0	4.0	4.0	4.0	4.0	4.0
20	4.1	4.0	4.2	4.1	4.0	4.0

per 10 ml solution was sufficient for maintaining a constant SH-level for more than 24 h. The same amount kept the thiol level constant only for 30—40 min in presence of 2×10^{-5} M copper or iron.

Stabilization of thiols in systems containing biological materials. Several experiments were performed in order to study the stabilizing effect of SH-Sephadex on various thiols of low molecular weight in the presence of different biological materials. The results are summarized in Table 1. As seen from the table, all the tested thiols were completely stabilized for at least 20 h by the SH-Sephadex in media containing rat liver and rat kidney homogenate.

DISCUSSION

The remarkable stabilizing effect of the insoluble SH-Sephadex may be ascribed to at least three different factors, *viz.* (*a*) prevention of the disulfide catalysed oxidation of the thiol, (*b*) removal of heavy metals, and (*c*) a continuous re-reduction effect.

The autoxidation of a thiol is catalysed by its own oxidation product as well as by other disulfides (Ref.[6,7] and Fig. 1). Since SH-Sephadex behaves as a reducing agent which readily converts a disulfide into its thiol form, the level of the disulfide will always be maintained at zero as long as SH-Sephadex is present in excess. Thus the autocatalysed oxidation of the thiol is completely avoided.

Thiolated Sephadex which is an insoluble polythiol, may to some extent extract from its surrounding solution certain heavy metals with affinity for the sulfhydryl group. When cysteamine or other thiols are added, however, an equilibrium will be attained between the heavy metals bound by the SH-Sephadex and complexed to the thiols as mercaptides. A partial removal

of metals from the solution may nevertheless be accomplished, and the metal catalysed autoxidation of the thiols thereby diminished.

A combination of the two effects described probably explains the greatly diminished oxygen uptake (Fig. 4) in presence of SH-Sephadex. The figure clearly shows, however, that the oxygen consumption is not zero, although the level of cysteamine remains unchanged. This can be explained by the re-reduction effect which continuously converts the disulfide formed back to the thiol.

As a result of the continuous electron exchange between SH-Sephadex and the disulfide formed, the level of the thiol can be expected to remain constant only as long as the Sephadex contains available SH-groups. Thus, when the level of the soluble SH begins to decline, it indicates that the "electron supply" has been exhausted. In absence of copper and iron, the SH-Sephadex lasts for about 48 h at pH 7.5 and 37° (Fig. 2), but in presence of these metals the electron supply is exhausted in 45 min (Fig. 3). The faster drainage of electrons when Cu and Fe have been added, is obviously due to the stimulating effect of the latter metals on the rate of autoxidation. In biological experiments the stability of any thiol in presence of SH-Sephadex therefore very much depends upon the absence or presence of significant amounts of cupric and ferric ions. Fortunately such interfering quantities are probably not present in most biological materials (Table 1).

It should be emphasized that unless one deals with a relatively thin liquid layer, (*e.g.* 1 cm) the mixture should be shaken since the stabilization mechanism involves continuous electron exchange between a solution and a completely insoluble material. If allowed to stand the SH-Sephadex may settle, and autoxidation of the thiol in the supernatant may take place.

Table 1 has demonstrated that it is possible to maintain a constant thiol level for at least 20 h in presence of several biological materials. This period should give ample time to carry out almost any studies on the mode of action of the thiol, and the results obtained in this way should be devoid of interference from the corresponding disulfide. Such experiments are in progress.

The insoluble SH-Sephadex particles are very large in comparison with cells, mitochondria and other cellular particles, and are unable to penetrate any biological membrane. The thiolated material is furthermore non-adsorbent and inactive towards proteins and nucleic acids, and does not interfere with mitochondrial respiration, as demonstrated by S. Skrede* in our laboratory. Apparently the only effect of SH-Sephadex is to keep the disulfide level at zero and the thiol level constant in the surrounding medium.

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* Personal communication.

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