# THE STABILITY OF THIOGLYCOLLATE SOLUTIONS

## PART I. EFFECTS OF METHOD OF PREPARATION OF SOLUTIONS, pH AND TEMPERATURE UPON THE OXIDATION OF THIOGLYCOLLATE

BY A. M. COOK AND K. J. STEEL

*From the Department of Pharmaceutics, School of Pharmacy, University of London, Brunswick Square, London, W.C.*1

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The pH of, and amount of oxidation occurring in, thioglycollate solutions is influenced by the method of preparation of the solutions from thioglycollic acid. When heat is employed to effect solution, the oxidation of the resultant thioglycollate increases with increase in pH and temperature of storage. Solutions prepared without heat may exhibit a more alkaline reaction than expected. Storage of such solutions causes a fall in their pH, the extent of which is determined by the temperature and time of storage. The effect of storage at different temperatures upon the oxidation of thioglycollate is not as great with unheated solutions as in heated solutions. Oxidation of thioglycollate is increased by dilution. The dithiodiglycollate, produced on oxidation of thioglycollate, itself undergoes decomposition in alkaline conditions.

AMONGST the many diverse uses of thioglycollic acid is its use in bacteriology, both in the preparation of media for supporting the growth of anaerobic organisms[1] and as an inactivating agent for mercurial bacteriostats when testing preparations for sterility[2–5].

The use of sodium thioglycollate is common in the United States for these purposes, whilst it is more usual in this country to prepare the sodium salt *in situ* by neutralisation of the acid with sodium hydroxide. In his original paper, Brewer[1] considered sodium thioglycollate to be relatively stable and preferable to the acid which is a syrupy liquid and requires pH adjustment when incorporated into media.

Doubts on the stability of sodium thioglycollate have been expressed[3] and it has the disadvantages of being hygroscopic and tending to discolour on exposure to air, an aqueous solution of the discoloured material being yellowish.

During an investigation of the inactivation of mercurial bacteriostats by thioglycollate media, it was observed that peptone water containing 0·01 per cent of thioglycollic acid (neutraised to pH 7 with sodium hydroxide) after standing at room temperature for six hours, gave no red colouration with ammoniacal sodium nitroprusside whereas a freshly prepared sample produced an immediate red colour, indicating the presence of sulphydryl groups. Heating the older sample at 98–100° for up to 20 minutes and cooling before testing did not affect the result. Failure to detect sulphydryl in the stored medium implies that oxidation of the thioglycollate has occurred. As the value of thioglycollate media, both for the cultivation of anaerobes and the inactivation of mercurial

bacteriostats, is dependent upon the availability of sulphydryl groups, their oxidation will render the media useless for these purposes. It was decided therefore to investigate the oxidation of thioglycollic acid and factors associated with it.

## Experimental and Results

The thioglycollate medium has been prepared by the addition of a sterile solution of thioglycollic acid, neutralised to pH 7 with sodium hydroxide, to the peptone water immediately before use. The final medium had a pH of 7·4–7·5.

The peptone water and the thioglycollate solution were each tested for the presence of metallic ions which could catalyse the oxidation of thioglycollate. Peptone is known to contain traces of metals but these could not be detected in a 1 per cent solution. No positive reactions for cupric ions were obtained with α-benzoin-oxime ("cupron"), dithio-oxamide ("rubeanic acid") or sodium diethyldithiocarbamate; for ferric ions with ammonium thiocyanate or 7-iodo-8-hydroxyquinoline-5-sulphonic acid ("ferron"); or for ferrous ions with dimethylglyoxime or 2:2′-dipyridyl. As no gross contamination with metallic ions, which could catalyse the oxidation, was demonstrable, it was assumed that the loss of thioglycollate was a result of atmospheric oxidation, which might be expected to be accelerated at this low concentration and slightly alkaline reaction of the medium.

### *Effect of Heat on pH*

One per cent solutions of thioglycollic acid were prepared with the addition of 10·7, 10·85 and 11 ml. of N sodium hydroxide solution per 100 ml. of final solution. Theoretically, 1 g. of thioglycollic acid requires 10·85 ml. of N NaOH for neutralisation.

Determined at ten minute intervals up to three hours after preparation, the range of pH values (glass electrode) were 8·25 to 8·35, 8·45 to 8·6 and 8·95 to 9·15 respectively. Portions of the solutions autoclaved at 115–116° for 15 minutes and cooled had pH values of 5·4 ,7·2 and 8·8 respectively.

### *Effect of Storage on pH*

One per cent thioglycollic acid solutions containing varying amounts of sodium hydroxide solution were again prepared, half of each solution being autoclaved and the remainder unheated. The solutions were stored at 4, 20 and 37° in glass-stoppered bottles in the dark and their pH was determined initially and after 3, 8, 14, 24 and 40 days storage. As the dissociation constant Ka varies with temperature, samples were allowed to reach 20° before measuring their pH. The solutions prepared with heat maintained their reactions within the following limits, under the three storage conditions, "acid" solution, pH 5·35 to 5·45; "neutral" solution, pH 6·95 to 7·10; "alkaline" solution, pH 7·80 to 8·00.

The results for the solutions prepared without heat mostly showed a considerable fall, see Table I.

Storage of the autoclaved thioglycollate solutions produced no appreciable change in their pH. Solutions prepared without heat and stored at 37° showed a fall in pH, which after 14 days reached a value similar to that of the corresponding heated solutions. The unheated "acid" solution stored at 20° showed a gradual fall in pH until after 40 days storage its pH was only slightly above that of the heated "acid" solution.

TABLE I

EFFECT OF STORAGE UPON THE pH OF THIOGLYCOLLATE SOLUTIONS PREPARED WITHOUT HEAT

| Days of storage | 0 | 3 | 8 | 14 | 24 | 40 |
|---|---|---|---|---|---|---|
| "Acid" solution at 4° .. .. | 8·30 | 8·45 | 8·60 | 8·80 | 8·20 | 7·05 |
| 20° .. .. | 8·30 | 8·25 | 8·15 | 7·55 | 6·75 | 5·55 |
| 37° .. .. | 8·30 | 7·50 | 5·75 | 5·50 | 5·50 | 5·40 |
| "Neutral" solution at 4° .. | 8·40 | 8·55 | 8·80 | 8·90 | 8·50 | 8·10 |
| 20° .. | 8·40 | 8·45 | 8·45 | 8·30 | 7·75 | 6·95 |
| 37° .. | 8·40 | 8·10 | 7·20 | 7·00 | 7·00 | 7·00 |
| "Alkaline" solution at 4° .. | 8·50 | 8·65 | 8·90 | 9·00 | 8·80 | 8·60 |
| 20° .. | 8·50 | 8·55 | 8·70 | 8·90 | 8·60 | 8·20 |
| 37° .. | 8·50 | 8·30 | 8·00 | 7·60 | 7·60 | 7·60 |

The reactions of the remaining unheated solutions increased up to 8 to 14 days, after which their pH began to fall. All the unheated solutions stored at 4° were more alkaline after 40 days storage than the corresponding heated solutions, whilst the unheated solutions after 40 days storage at 20° had a pH similar to those of the heated solutions.

*Effect of Preparation upon the Oxidation of Thioglycollate*

One per cent thioglycollic acid solutions containing the theoretical quantity of sodium hydroxide solution for neutralisation were prepared. Half of the solution was autoclaved at 115–116° for 30 minutes whilst the remainder was sterilised by passing through a 5/3 sintered glass filters. After sterilisation, the solutions were stored in 100 ml. glass-stoppered bottles at 20° in the dark. Samples were removed for assay initially and after 24, 90 and 120 hours storage. Their thioglycollate content was determined by titration with potassium iodate solution in acid conditions[6], with the results shown in Table II.

*Effect of pH and Method of Preparation on the Oxidation of Thioglycollate during Storage*

On the results of the above preliminary experiments, the following investigation was designed. Solutions containing 1 per cent of thioglycollic acid and varying quantities of sodium hydroxide solution were prepared, and sterilised by heating in an autoclave or by filtration. The alkali content of each solution and its initial pH is shown in Table III. Portions of each solution were stored in glass-stoppered bottles in the dark at 4, 20 and 37°. After varying intervals, samples were withdrawn and their thioglycollate content determined by the potassium iodate method. As temperature is reported[7] to markedly affect the iodine consumption of thioglycollic acid, samples for assay were rapidly cooled to about

10° before being titrated. Some typical results are shown in Table IV which relates the percentage of the thioglycollate which has oxidised to the storage temperature and initial reaction of the solutions. Repetition of the experiments with 0·1 per cent thioglycollate solutions showed a much more rapid oxidation rate, up to 25 per cent of the thioglycollate being oxidised in 24 hours.

TABLE II

THIOGLYCOLLATE CONTENT (per cent w/v) OF NOMINAL 1 PER CENT SOLUTIONS STERILISED BY AUTOCLAVING OR FILTRATION, AFTER STORAGE AT 20°

| | Time of storage in hours | | | |
|---|---|---|---|---|
| | 0 | 24 | 90 | 120 |
| Autoclaved . . . . | 1·0 | 0·964 | 0·609 | 0·233 |
| Filtered . . . . | 0·907 | 0·871 | 0·677 | 0·534 |

TABLE III

ALKALI CONTENT AND INITIAL pH OF 1 PER CENT THIOGLYCOLLIC ACID SOLUTIONS, STERILISED BY AUTOCLAVING OR FILTRATION

| Solution | Amount of N NaOH per 100 ml. of solution | Initial pH | |
|---|---|---|---|
| | | Autoclaved | Filtered |
| "Acid" . . . . | 10·7 ml. | 5·4 | 8·3 |
| "Neutral" . . | 10·85 ml. | 7·4 | 8·4 |
| "Alkaline" . . | 11·0 ml. | 7·8 | 8·6 |

TABLE IV

PERCENTAGE OXIDATION OF THIOGLYCOLLATE OCCURRING IN AUTOCLAVED AND FILTERED 1 PER CENT THIOGLYCOLLATE SOLUTIONS, AFTER STORAGE AT DIFFERENT TEMPERATURES FOR 24 HOURS

| Storage temperature | Autoclaved solution, oxidation per cent | | | Filtered solution, oxidation per cent | | |
|---|---|---|---|---|---|---|
| | Acid | Neutral | Alkaline | "Acid" | "Neutral" | "Alkaline" |
| 4° | 2·8 | 5·4 | 8·0 | 5·0 | 6·2 | 6·5 |
| 20° | 4·3 | 7·1 | 9·7 | 5·3 | 8·1 | 8·5 |
| 37° | 8·0 | 9·9 | 11·8 | 5·3 | 6·3 | 6·3 |

*Presence of Dithiodiglycollate in Thioglycollate Solutions after Storage*

Thioglycollate solutions containing 1·0 or 0·1 per cent of the acid when first prepared and adjusted to an acid, neutral or alkaline reaction, were tested for the presence of sulphydryl groups, with ammoniacal sodium nitroprusside solution, after two months storage at 4, 20 or 37°. Samples in which a positive reaction was obtained were diluted with purified water until no red colouration was produced on addition of the reagent; dilution more than ten times was never required. The solutions were then tested for the presence of disulphide by the method of Walker[8], which reduces disulphides to sulphydryl compounds with potassium cyanide. Of the solutions tested, only those having an initial alkaline reaction and stored at 37° failed to give a positive reaction for the presence of disulphide.

## Discussion

Thioglycollic acid may be prepared by the action of sodium chloracetate on sodium hydrosulphide or by the reduction of dithiodiglycollic acid, prepared from sodium chloracetate and sodium disulphide. Besides thioglycollic acid, the reaction mixture before extraction and purification contains both dithiodiglycollic and thiodiglycollic acids with dithioglycollide and other thioglycollides[9]. After extraction, purification is by vacuum distillation.

Thioglycollic acid is commercially available in two forms, anhydrous and an aqueous preparation, containing about 97–98 and 75 per cent of thioglycollic acid respectively. Each contains a small quantity of dithiodiglycollic acid (about 0·5 per cent) and the remaining 2 per cent or so of unaccounted material, in the anhydrous product, probably consists of dithioglycollide.

By heating thioglycollic acid in a stream of nitrogen, esters of varying molecular weight have been isolated[10] which are considered to be straight chain polythioglycollic esters of structure

$$HSCH_2CO(SCH_2CO)_nSCH_2COOH$$

where $n$ can be any integer from 7 to 21.

$$(n+2)\ HSCH_2COOH \longrightarrow HSCH_2CO(SCH_2CO)_nSCH_2COOH + (n+1)H_2O$$

It is believed that on heating, these esters rearrange to form the cyclic dithioglycollide which can be separated by vacuum distillation. Dithioglycollide (2:5-dioxo-1:4-dithiane) may also be derived by the condensation of two molecules of thioglycollic acid with the elimination of the elements of two molecules of water[11], the reaction being analogous to the formation of lactide from lactic acid. Mulvaney[9] considers that such a dehydration occurs readily at the temperature required for distillation and expresses the reaction thus:

$$n\ HS{\cdot}CH_2{\cdot}COOH \longrightarrow (S{\cdot}CH_2CO)_n + nH_2O$$

It should be noted however that a monomeric cyclic thio-ester where $n = 1$ does not exist[11].

Dithioglycollide also forms at normal room temperatures, up to 4 per cent of the thioglycollic acid in a freshly distilled sample being dehydrated in a month[9]. The reaction resulting in its formation is reversible[9] but the rate of hydration is slow at ordinary temperatures; it may be accelerated by dilution, temperature rise or the use of mineral acid as catalyst.

Schöberl and Krumey[11] report that prolonged heating of dithioglycollide with water causes its hydrolysis, whilst alkalies rapidly hydrolyse it, the resultant thioglycollic acid being detectable both acidimetrically and iodimetrically. Their results showed that one of the CO–S bonds is rapidly broken by alkaline hydrolysis even at room temperatures forming *S*-thioglycollylthioglycollic acid. Other properties of dithioglycollide have been reported by Schöberl and Wiehler[10,12].

The heated thioglycollic acid solutions had the expected pH, whereas solutions prepared without heat remained alkaline (Table III). In the unheated solutions, it appears that all the thioglycollic acid has not reacted with the sodium hydroxide and it may be inferred that this unreacted acid is in some form other than thioglycollic acid. It could be present as dithiodiglycollic acid or as dithioglycollide. The first of these two compounds being a dicarboxylic acid would be expected to react with alkali in a manner similar to thioglycollic acid, and it is therefore more probable that dithioglycollide accounts for the unreactable material. The titration of thioglycollic acid with alkali does not show a drifting end point and presumably the dithioglycollide is stable towards alkali in the cold. In this respect it differs from the lactide of lactic acid, but this might be expected since sulphur compounds usually have a greater stability than the corresponding oxygen compounds.

In the heated thioglycollate solutions, more sodium hydroxide was utilised, causing a fall in pH, and this infers that hydrolysis of the dithioglycollide occurs on heating with alkali. This hydrolysis may be partial to produce *S*-thioglycollylthioglycollic acid, or complete to produce thioglycollic acid; in either case the production of carboxylic acids will decrease the amount of free alkali in the system.

Assay of samples of thioglycollic acid will not determine any dithioglycollide present, although some of it may be hydrolysed during the Pharmacopoeial assay process and its hydrolysis products will then be determined[6]. In a fresh sample of thioglycollic acid, the dithioglycollide content may be expected to be small (less than 2 per cent) but in older samples this value may be increased. If a sample contains an appreciable amount of dithioglycollide, the addition of sufficient alkali to neutralise the thioglycollic acid (based on the assay results) will produce a solution the pH of which will vary with the heat used in its preparation.

In bacteriological work, sterile thioglycollate solutions are usually required, and it may be questioned whether these solutions should be sterilised by heat or by filtration. Solutions sterilised by heat have the desired pH immediately; sterilised without heat, the use of sintered glass filters is essential to avoid contamination with metals. This method of preparation has the disadvantage that the final solution may have a more alkaline reaction than expected, especially if the thioglycollic acid had a high dithioglycollide content.

From the results in Table I of the effects of storage upon the pH of thioglycollate solutions, it is evident that some change is occurring in the unheated solutions which does not occur in heated solutions. The most probable explanation is that hydrolysis of dithioglycollide results from prolonged contact with alkali, since all the unheated solutions were initially alkaline. Schöberl and Krumey[11] have shown the hydrolysis of dithioglycollide by alkali to be accelerated by increase in temperature. This would explain the more rapid decrease in pH observed with those solutions stored at 37° compared with those stored at 20°, and the slight fall in pH of solutions stored at 4°.

*The Oxidation of Thioglycollic Acid*

The main loss of thioglycollic acid on storage is by oxidation to dithiodiglycollic acid. The oxidation is catalysed by copper[9,13–16], iron [9,13,14,17] and cobalt[18] but not by zinc[9]. The rate of oxidation varies with the pH[19], presence or absence of buffer[14] and the concentration of metallic catalyst. The disulphide formed on oxidation also acts as a catalyst in the auto-oxidation of thioglycollic acid[14,15,19,20]. Other reported oxidation products include hydrogen peroxide[16,21] and sulphuric acid[13].

Two main problems, apart from pH, appear to be associated with the question of sterilisation of thioglycollate solutions; first, does heat treatment accelerate the oxidation and, secondly, does the increased surface area of the solution during filtration cause increased oxidation? From the results in Table II it is seen that the unheated solution had a lower thioglycollate content than the heated solution when first prepared. As both solutions were made with the same quantity of thioglycollic acid, it is inferred that the different thioglycollate content is due to the presence of unreactable dithioglycollide in the unheated solution. On storage at 20°, the heated solution appeared to undergo oxidation more rapidly than the unheated solution. Fuller conclusions can be drawn from the later investigation, of which the results shown in Table IV are an example. From this more extensive investigation, the following conclusions were drawn.

Solutions which have been heated have a higher thioglycollate content initially than unheated solutions. The rate of oxidation of thioglycollate in heated solutions increases with increasing pH and storage temperature. The effects of the reaction of the solutions prepared without heat were not as obvious as those of the heated solutions. Storage of unheated solutions at 20° produced more oxidation than storage at 4° or 37°. It is believed that storage at 4° minimises oxidation, as in the case of heated solutions, whereas storage at 37° allows some hydrolysis of dithioglycollide which increases the sulphydryl content and reduces the alkalinity. "Acid" solutions appeared to undergo less oxidation than the "neutral" or "alkaline" solutions, but the difference between these two was much less well marked than in the heated solutions. Solutions containing 0·1 per cent of thioglycollate underwent much more rapid oxidation than the corresponding 1 per cent solutions.

The positive results obtained with the test for disulphide in old samples of thioglycollate solutions may be assumed to be caused by dithiodiglycollic acid, or its sodium salt, which is the oxidation product of thioglycollic acid. From the results reported it is seen that oxidation of thioglycollate occurred most rapidly in solutions adjusted to an alkaline reaction and stored at an elevated temperature; by the absence of disulphide in such solutions is it inferred that the disulphide has itself undergone further decomposition. The decomposition of sodium dithiodiglycollate has been reported[15,19,22,23], although the pH at which this decomposition occurs (above about 9 to 9·5) is higher than that in the solutions tested (initial pH about 8·5). Amongst the reported decomposition products are sodium glyoxalate[19] and sodium oxalate[22].

From this investigation, the following recommendations are proposed. For maximum stability, thioglycollate solutions should be adjusted to maintain an acid reaction; in the case of solutions which are to be mixed with bacteriological media, the buffering capacity of the medium should be normally sufficient to maintain the final mixture at the required pH. Similarly, thioglycollate solutions should be stored at as low a temperature as possible, preferably in a refrigerator. These solutions should be stored in well filled, well closed containers to reduce the volume of air in contact with the solution. Sterile solutions of thioglycollate may be prepared by autoclaving.

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