THE STABILITY OF THIOGLYCOLLATE SOLUTIONS

PART II. MISCELLANEOUS FACTORS ASSOCIATED WITH THE OXIDATION AND STABILITY

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 rate of oxidation of thioglycollate in aqueous solution increases with increasing pH, but when dextrose is present, neutral solutions are oxidised more rapidly than alkaline solutions. The effect of sodium metabisulphite on the oxidation was indecisive. Boiling thioglycollate solutions evolved hydrogen sulphide. Heating old solutions of thioglycollate caused regeneration of the oxidised material but the extent of this regeneration was less than might have been expected. A yellow colour in some thioglycollate solutions is believed to be due to the action of alkali on the thioglycollic acid. The tetracarboxy acid occurring in samples of the acid was isolated. It is recommended that thioglycollate media be stored in air-tight containers at as low a temperature as possible.

IN Part I of this paper[1] the oxidation of thioglycollate in aqueous solution was shown to be dependent upon whether heat was employed or not in preparation, the pH of the solution and the temperature at which the solution was stored. In this second part, the effects of added dextrose and of sodium metabisulphite are reported. The effect of heat on thioglycollate solutions has been studied together with other miscellaneous factors associated with thioglycollic acid and its solutions. An attempt has been made to correlate the results in parts I and II with their application to thioglycollate media for bacteriological use.

EXPERIMENTAL AND RESULTS

Effect of Dextrose on the Oxidation of Thioglycollate

Solutions containing 1 per cent of dextrose and 0·1 per cent of thioglycollic acid with 10·7, 10·85 or 11·0 ml. of 0·1N sodium hydroxide per 100 ml. of solution were prepared with the aid of heat. These solutions are referred to as acid, neutral or alkaline, respectively, with pH values of 5·6, 7·2 and 7·8, respectively. Similar solutions without dextrose were prepared at the same time.

Samples of each solution were stored in glass-stoppered bottles in the dark at 4, 20 and 37°. At varying intervals, portions were withdrawn and their thioglycollate content determined by titration with potassium iodate[2]. Dextrose was found not to interfere with the assay.

In all cases, the rate of oxidation of the thioglycollate was reduced when dextrose was present in the solution. Figure 1 shows the oxidation of the acid solutions on storage at the different temperatures; essentially similar graphs are obtained with the neutral and alkaline solutions. Solutions containing dextrose prepared to have an approximately neutral

reaction were oxidised more rapidly than alkaline solutions which, in turn, were oxidised more rapidly than acid solutions. This is shown in Figure 2. The phenomenon was noted at all three storage temperatures.

Effect of Sodium Metabisulphite on the Oxidation of Thioglycollate

Solutions containing 0·1 per cent of thioglycollic acid, 0·05 per cent of sodium metabisulphite and varying quantities of 0·1N sodium hydroxide as before, were prepared and autoclaved. This concentration of sodium metabisulphite was chosen as it had previously been shown to be below the bacteriostatic concentration for all of the nine different bacterial species used by Cook, Steel and Wills[3]. The solutions were stored as before.

As sodium metabisulphite reacts with potassium iodate, it was not possible to estimate the thioglycollate content of the stored solutions in the usual manner. Attempts were made, however, to follow the oxidation qualitatively by the colorimetric reaction with ammoniacal sodium nitroprusside. This reaction was found to have a sensitivity of 20 μg. of thioglycollate and concentration limits of 1 in 5×10^5. Akiba and Ishii[4] reported the sensitivity to be 1 μg. of sodium thioglycollate per ml. Sodium metabisulphite and its decomposition products interfere with this colorimetric reaction, producing an amber to orange colouration with the reactants.

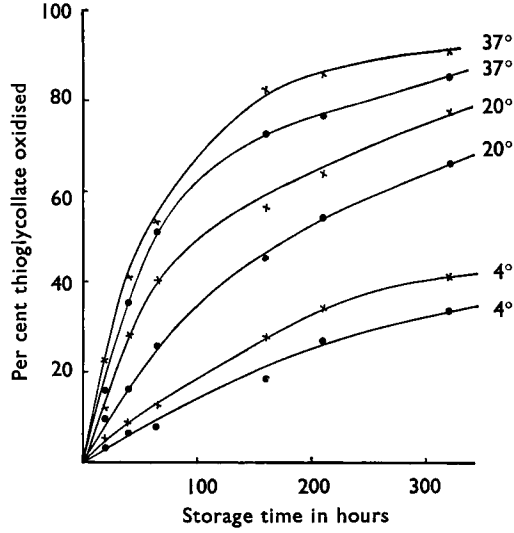


FIG. 1. The oxidation of thioglycollate in a 0·1 per cent. solution, with and without 1 per cent of dextrose, on storage at 4°, 20° and 37°.

×—× Aqueous solution.
●—● Solution with dextrose.

After up to 3 months storage, all the solutions containing the antoxidant still gave a positive reaction for thioglycollate. Only those adjusted to an acid pH and stored at 37° failed to give a strong positive reaction. The reactions obtained were, in all cases, stronger than those produced by simple aqueous thioglycollate solutions with or without dextrose after storage under comparable conditions.

Effect of Heat on Thioglycollate Solutions

Aqueous solutions and peptone water mixtures containing varying concentrations of thioglycollic acid or its sodium salt were boiled and the vapours tested for the presence of hydrogen sulphide by means of

lead acetate paper. The odour of hydrogen sulphide was appreciable from preparations containing 0·5 per cent or more of thioglycollate and the gas was detected, although its odour was not discernible, from solutions containing 0·1 per cent of thioglycollate but not less. There was no difference in the behaviour of the aqueous solutions or the broth mixtures towards boiling.

Solutions containing 1·0 or 0·1 per cent of thioglycollic acid, adjusted to an acid, neutral or alkaline reaction and with or without 1·0 per cent of dextrose were assayed for their thioglycollate content after storage for between 17 and 50 days. Samples of the solutions were then heated at 98–100° for 30 minutes and, after cooling, were re-assayed.

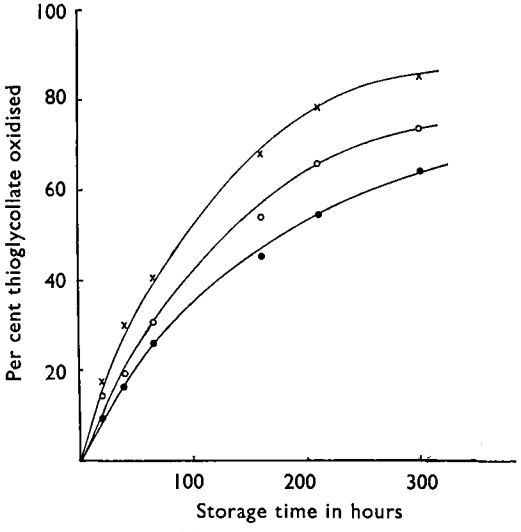


FIG. 2. The oxidation of thioglycollate in a 0·1 per cent solution containing 1 per cent of dextrose, on storage at 20°.

●—● Acid solution.
×—× Neutral solution.
○—○ Alkaline.

A comparison of the thioglycollate contents of the solutions before and after heat treatment by means of a paired *t* test suggested that a hypothesis that there was no difference between the two sets of results was in reasonable accord with the experimental data. Closer inspection of the results showed that the original unheated solutions could be roughly classified into two groups; the first consisting of solutions which still contained about 5–10 per cent or more of their original thioglycollate content, and the second of solutions which had undergone further oxidation so that their thioglycollate content had been reduced to about 5 per cent or less of the original value. Heating solutions of the first group caused no change or further loss in their thioglycollate content, whilst solutions of the second group showed an increase in their thioglycollate content after heating.

The Yellow Colour Appearing in Thioglycollate Solutions

During the work, some 1 per cent thioglycollate solutions were observed to develop a distinctly yellow colour on storage. This colouration was seen in heated solutions having an alkaline reaction; unheated solutions developed the colouration on storage at 4 or 20°, but at 37° only those containing an excess of alkali showed this yellow colour. A freshly

prepared 2 per cent solution of a commercial sample of sodium thioglycollate was also yellowish in colour.

The Storage of Thioglycollic Acid

Two samples of thioglycollic acid (nominally 97–98 per cent w/w $HS{\cdot}CH_2{\cdot}COOH$) were examined. One had been stored at room temperature for 17 months and was hazy in appearance with some sedimentation of white material at the base of the container, whilst the other had been stored at 4° for 29 months and was perfectly clear. Both bottles had been opened and some of their contents used, thus permitting contact with air and possibly water vapour. Assay of the two samples showed thioglycollic acid contents of 83·9 and 86·2 per cent w/w respectively.

To the first sample, containing the sediment, the addition of water or dilute hydrochloric acid produced no change in appearance whereas the addition of N sodium hydroxide solution caused an almost immediate solution of the precipitated material resulting in a clear solution. The mean results for the thioglycollic acid content of aliquots of the first sample assayed by the potassium iodate method immediately or after 10 minutes alkali treatment were 83·87 and 83·85 per cent w/w respectively. Thus alkali does not produce any –SH when added to the precipitate.

The sample containing the white precipitate was centrifuged to collect the sediment which was re-suspended in purified water and washed by re-centrifuging. This washing and centrifugation was repeated until the supernatant was free from sulphydryl. The white material obtained was insoluble in water but readily soluble in sodium hydroxide solution and in sodium bicarbonate solution with effervescence. It was soluble in concentrated sulphuric acid producing an amber coloured solution which on standing became yellowish. When treated with a mixture of concentrated sulphuric acid and perchloric acid (60 per cent $HClO_4$) an orange-red colour was produced which passed to yellow on standing and on the addition of water became colourless.

Discussion

Dextrose in a concentration of 1 per cent is included in Brewer's medium[5] both as a fermentable carbohydrate and as a reducing agent to keep the thioglycollate in the reduced state and to maintain the oxidation-reduction potential of the medium at a low level. Skerman[6], however, reported that dextrose does not aid the establishment of anaerobic conditions. From our results it appears that the presence of dextrose in thioglycollate solutions does reduce the rate of oxidation compared with plain aqueous solutions. The rate of oxidation of thioglycollate in aqueous solution has been shown to increase with increasing pH[1] but when dextrose is present in the solutions, neutral solutions were found to be oxidised more rapidly than alkaline solutions which, in turn, became oxidised more rapidly than acid solutions.

Dilute acids have little or no effect upon dextrose (α-glucose), and in the solutions adjusted to an acid or neutral reaction the mutarotation

occurring is expected to produce an equilibrium between the α- and β-forms involving no overall change in the cyclic pyran structure[7]. The action of dilute alkali upon dextrose is to cause re-arrangement without scission of the carbon chain, tending to result in the formation of an equilibrium mixture of glucose, fructose and mannose. The fructose in such a mixture will itself undergo mutarotation which, in this case, involves a furanose to pyranose shift and vice versa[7]. In such a mixture of the three monosaccharides the existence of the acyclic hydrated aldehyde (aldehydrol) and the enolic (1 : 2-enediol) forms of glucose are postulated[8]. It is possible that this phenomenon may occur in the thioglycollate solution adjusted to an alkaline pH, the overall effect being to enhance the reducing activity of the dextrose. This would explain the decreased oxidation occurring in the alkaline solution. The possible effect of dextrose upon the oxidation-reduction potential of the systems has not been investigated.

The reactions obtained for the presence of thioglycollate in originally dilute solutions containing 0·05 per cent of sodium metabisulphite point to the efficacy of this material as an antoxidant for thioglycollate. However, only quantitative determinations will prove whether sodium metabisulphite is of value for this purpose, and it is proposed to continue this investigation using a quantitative colorimetric reaction in which sodium metabisulphite does not interfere. No explanation of the apparent value of sodium metabisulphite as an antoxidant for thioglycollate is as yet advanced.

The evolution of hydrogen sulphide when thioglycollate solutions and broth mixtures are boiled has been confirmed in a personal communication to the authors by Sykes, who believes that this decomposition is normal and not caused catalytically by constituents of the broth. He states that the sterilisation of media containing 0·4 per cent of sodium thioglycollate is accompanied by evolution of hydrogen sulphide and this is responsible for the dark colour which sometimes appears in thioglycollate media after sterilisation.

Brewer's medium, before use, is usually heated for half an hour in a boiling water bath to remove dissolved air, the removal being accompanied by a discharge of the green colour of the oxidation : reduction potential indicator (methylene blue) added to the medium. Whilst this shows that dissolved air has been removed and that the oxidation : reduction potential has been reduced, it does not show whether the thioglycollate is in the reduced or oxidised state, although it is believed that removal of dissolved air is accompanied by reduction of any disulphide present to the sulphydryl form. Practically, it is well known that a sample of thiocollate medium which has become oxidised and gives no reaction for sulphydryl with ammoniacal sodium nitroprusside will, after heating, give a positive reaction for sulphydryl, support the growth of anaerobic organisms and antagonise the effects of mercurial bacteriostats.

Thus it appears that the effect of heating thioglycollate solutions which have deteriorated is to cause some regeneration of their sulphydryl content. The greatest reversal was obtained with those solutions which had

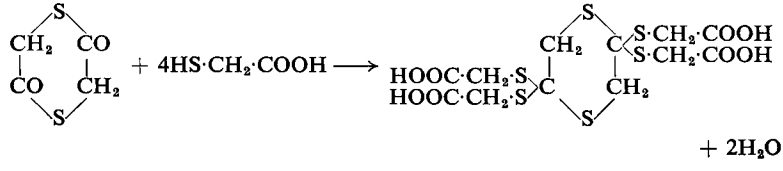
become oxidised to the greatest extent. These results lend support to the work of Skerman[6], who demonstrated that the disulphide formed on oxidation was hydrolysed to the extent of about 20 per cent to the sulphydryl compound on heating, the actual amount of this hydrolysis depending upon the temperature and the time of heating. Skerman believes this regeneration is accelerated by the other constituents of thioglycollate media.

The production of a yellow colour in thioglycollate solutions, apart from the solution made with the sodium salt, was not observed until they had been stored for at least 14 days, and from our present and previous results[1] seems to be associated with the action of sodium hydroxide upon thioglycollic acid.

The effect of storage conditions upon thioglycollic acid are well shown by the appearance and $HS{\cdot}CH_2{\cdot}COOH$ content of the two samples examined. The older sample stored at a lower temperature had undergone less decomposition than a fresher sample kept at room temperature. These results are in agreement with the conclusions reached in Part I of this paper[1] where the oxidation of thioglycollic acid was found to increase with dilution and temperature rise. A World Health Organisation report[9] recommends that thioglycollic acid for the preparation of sterility test media be periodically assayed and rejected if its content falls below 75 per cent. It further advises storage of thioglycollic acid in tightly stoppered bottles which prevent the access of moisture, protected from light and in a cool dry place. Leussing and Kolthoff[10] reported that the oxidation of the acid could be reduced to a very low level if the container was flushed with nitrogen during and after removal of samples.

Since no significant difference existed between the $HS{\cdot}CH_2{\cdot}COOH$ content of the sample of thioglycollic acid containing a white sediment before or after alkali treatment it appears that the sodium hydroxide merely reacts to form a sodium salt with the precipitated material, which is water-soluble, but produces no other change. The amount of alkali required to clarify the sample of thioglycollic acid was less than 5 per cent of that required for complete neutralisation of the acid, and it seems likely that the precipitated material is a stronger acid.

Under certain conditions, dithioglycollide will react with more thioglycollic acid to form a tetracarboxylic acid[11]:



This compound (2:2:5:5-tetracarboxymethylmercapto-1:4-dithiane) has been shown to be identical with the white crystalline material which separates from thioglycollic acid on prolonged storage. Some properties and reactions of this compound have been reported[12], of which the colour reaction with perchloric and sulphuric acids is one.

Whilst insufficient of the white material occurring in the old sample of thioglycollic acid was obtained for melting point determination, elemental analysis and other tests, it is believed that it was the tetracarboxy acid which Schöberl and Wiehler[12] have isolated.

Application of the Results to Thioglycollate Media

Brewer[5], in his original paper, stated that his medium remained anaerobic for a month and did not require heating before use. He also reported that bubbling air through the medium had no adverse effects upon the growth of strict anaerobes afterwards inoculated into it. The deterioration of thioglycollate media has been noted by several workers[13–15], who have pointed out that this may be accompanied by an increased toxicity. Whilst suggested modifications of the formula for thioglycollate media have been studied[16], comparatively little attention has been paid to the oxidation of thioglycollate in such media.

Using a polarographic method for the determination of thioglycollate, Skerman[6] showed that 0·1 per cent of thioglycollate in a medium 7 cm. deep exposed to air at 37° was completely oxidised in 80 hours. He showed the rate of oxidation to be unaffected by the presence of agar in the medium. Sykes, in a personal communication, reports that by the use of the colorimetric method of Schöberl and Ludwig[17] he has shown that of 0·1 per cent thioglycollate in a meat medium, approximately half is decomposed on autoclaving whilst the remainder (in plugged tubes) has decomposed after one week. Sykes, Royce and Hugo[18] consider there is always some slight loss of thioglycollate on autoclaving and this loss usually represents about 0·05 per cent in thioglycollate concentration. Hence in a medium containing 0·1 per cent of thioglycollate there may be a serious deficiency. Apart from this initial loss during sterilisation, they report that media containing 0·4 per cent of sodium thioglycollate stored in screw-capped bottles shows negligible loss by oxidation up to one month. To overcome the deterioration of thioglycollate media on storage, Australian workers[19] prepared their media daily by the addition of sufficient of a sterile 10 per cent thioglycollic acid solution to the otherwise complete medium; this addition caused a reduction in the pH of the medium from 8·1 to 7·1. Sufficient of a 10 per cent thioglycollic acid solution was prepared for one to three weeks use and it was stored in small sterile air-tight containers.

Brewer[5] recommended that his medium be stored at room temperature as low temperature storage increased the amount of air dissolving in it. Skerman[6] suggests the use of screw-capped containers in place of cotton wool-plugged tubes. Sykes (personal communication) concludes that plugged tubes are unsuitable for thioglycollate media unless they are to be used immediately or have a paraffin seal, and reports bottled media is only suitable for about a week unless a rubber liner is incorporated to make an air-tight seal.

Conclusions

From a consideration of the results obtained in this investigation, the following conclusions are made. Whilst the value of dextrose as a

reducing agent in thioglycollate solutions has been shown, it is possible that the addition of an antoxidant may minimise the rate of oxidation of the thioglycollate. Thioglycollate media should be adjusted to as low a pH value as is compatible with bacterial growth. Provided thioglycollate media is contained in air-tight containers it should be stored at as low a temperature as possible. Storage at 37° to check on the sterility of the media is obviously detrimental. The preparation of thioglycollate media in small batches will provide for more rapid turnover of the media. During sterilisation, the caps of the containers should be loosened to permit the escape of hydrogen sulphide, which otherwise results in a darkening of the media, but must be screwed down tight immediately on removal from the autoclave.

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