A CHEMICAL ANALYSIS OF BREWER'S MEDIUM FOR THE AEROBIC CULTURE OF ANAEROBES

By V. B. D. SKERMAN*

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Summary

Polarographic methods for the estimation of thioglycollic acid, cysteine, and glutathione in culture media are described.

A study of the rate of oxidation of thioglycollic acid and cysteine in a peptone-yeast-extract medium in the presence and absence of glucose and agar has shown that neither glucose nor agar (0.1 per cent.) influences the rate of oxidation. The oxidation of 0.1 per cent. thioglycollic acid in a medium 7 cm. deep exposed to air occurs in 80 hr.; 0.1 per cent. cysteine HCl approximately 120 hr.

The disulphides formed on oxidation are partially hydrolysed on heating, the extent depending on the time and temperature. The continued usefulness of media used for the "aerobic" culture of anaerobes appears to be dependent on this property.

I. INTRODUCTION

Brewer's medium or one of its modifications has been used extensively for the cultivation of anaerobes in sterility tests on various medicinal substances. In his discussion on the function of his medium Brewer (1940) made the following statement, which largely stimulated the investigation:

"Some effort has been made to find an explanation for the seemingly reversible reaction taking place in the medium, but no definite conclusion has been reached. It has been found that 80 cc. of sterile air per minute can be bubbled through a tube of the medium for thirty minutes, and it will still support growth of *Clostridium novyi,* one of the most strict anaerobes."

The constituents of Brewer's medium which might be expected to achieve and maintain an anaerobic state are glutathione in the pork infusion and the thioglycollic acid, aided perhaps by glucose. Both of these substances contain the autoxidizable sulphydryl group. In the glutathione it is present as the amino acid cysteine. The oxidation of the sulphydryl groups to the disulphides would result in the removal of oxygen from the medium and the reduction of the oxidation reduction potentials to a more negative level. It seems logical to assume that the complete oxidation of the sulphydryl groups would result in the destruction of the anaerobic state and hence the usefulness of the medium. The investigations reported in this paper were primarily conducted to determine the rate of oxidation of these components.

* Department of Bacteriology, University of Queensland.

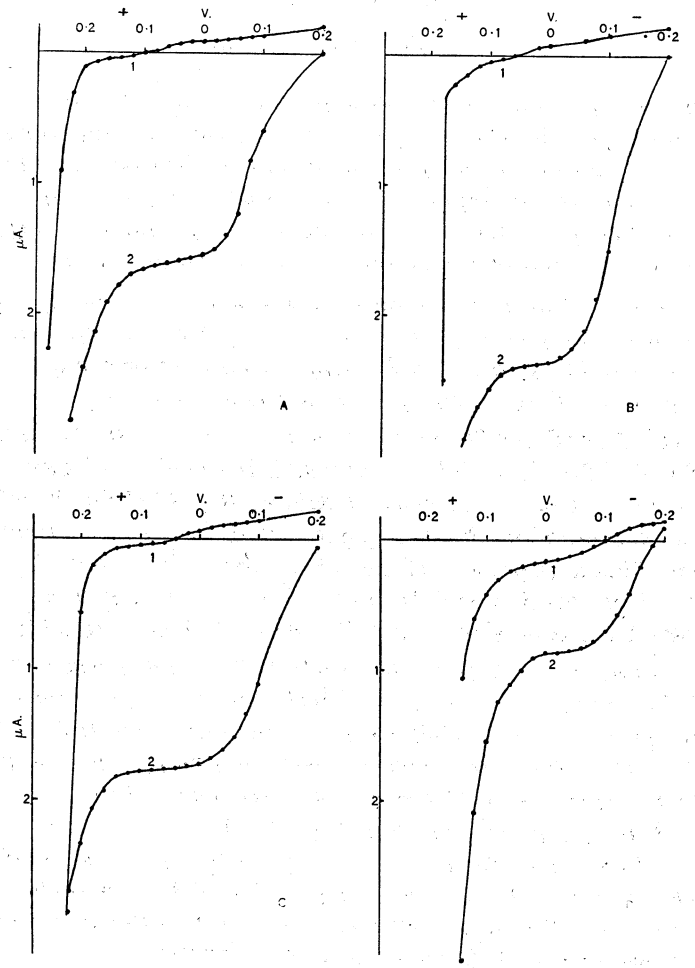

Fig. 1.—*A.* Current-voltage curves for deoxygenated (1) 0.01N $HClO_4$, and (2) 4.3×10^{-4}M thioglycollic acid in 0.01N $HClO_4$, using a saturated calomel reference electrode.

B. Current-voltage curves for deoxygenated (1) 0.01N $HClO_4$ containing 10 per cent. YEPW, and (2) 6.3×10^{-4}M thioglycollic acid in 0.01N $HClO_4$ containing 10 per cent. YEPW, using a saturated calomel reference electrode.

C. Current-voltage curves for deoxygenated (1) 0.001N HCl, and (2) 4.5×10^{-4}M thioglycollic acid in 0.001N HCl, using a saturated calomel reference electrode.

D. Current-voltage curves for deoxygenated (1) YEPW at pH 3, and (2) 1.8×10^{-4}M thioglycollic acid in YEPW at pH 3, using a saturated calomel reference electrode.

II. Methods of Analysis

(*a*) *Thioglycollic Acid*

Kolthoff and Barnum (1940) described a method for the estimation of cysteine in 0.1N perchloric acid. The latter provides a supporting electrolyte in which the equilibrium potential of the dropping electrode is sufficiently positive to permit analysis of other substances with a limiting current potential more positive than the reference electrode and it maintains a pH sufficiently low to prevent oxidation of the sulphydryl group during analysis.

The same procedure was examined by the author for the determination of thioglycollic acid. Perchloric acid (0.01N) was substituted for 0.1N perchloric acid to reduce risk of explosion, and a concentration of 36.5 p.p.m. (0.00043M) of the concentrated thioglycollic acid was found to yield, in the absence of oxygen, a well-defined anodic wave with the limiting current measurable at 0.06 V. *v.* S.C.E. (Fig. 1*A*). This represented the analysis of pure thioglycollic acid in perchloric acid. In practice, however, the thioglycollic acid was employed at a concentration of 1000 p.p.m. (0.012M) in a medium consisting of 1 per cent. "Bacto-peptone," 0.5 per cent. "Bacto-Yeast extract," and 0.5 per cent. NaCl. This medium will be referred to in the paper as YEPW. For some purposes 1 per cent. glucose and 0.1 per cent. agar were added.

It was necessary to determine what effect these substances had on the production of the sulphydryl wave. Kolthoff and Barnum (1940) stated that hydrochloric acid was unsatisfactory as a supporting electrolyte for cysteine determinations since the anodic chloride wave interfered with the measurement of the cysteine wave. The YEPW medium contains chloride in *c.* 0.1M concentration. With an initial concentration of 1000 p.p.m. thioglycollic acid a dilution of 1/10 in perchloric acid was necessary to reduce the sulphydryl wave to measurable limits. This dilution contained *c.* 0.01M chloride—sufficient to give an anodic wave of *c.* 80 μA. It was suspected that this might interfere with the direct measurement of thioglycollic acid in YEPW.

The formation of the wave was examined in the following supporting electrolytes:

(i) 0.01N $HClO_4$,
(ii) 0.01N $HClO_4$ containing:
  (1) 10 per cent. YEPW,
  (2) 10 per cent. YEPW containing 0.1 per cent. agar,
  (3) 0.05 per cent. NaCl,
  (4) 0.1 per cent. "Bacto-peptone."

The polarograms for ii (1) are shown in Figure 1*B*. The presence of the YEPW caused a small change in the residual current and produced a steep anodic wave at *c.* 0.17 V. *v.* S.C.E. Comparison with the residual current curves (not illustrated) for ii (3) and ii (4) showed that this wave was due to the chloride. The peptone caused a movement in the wave to a more positive value than that obtained with perchloric acid alone.

The agar had no effect on either the residual current or the sulphydryl wave.

Since the chloride had no apparent effect on the determination of thioglycollic acid in perchloric acid an attempt was made to determine it in hydrochloric acid alone. Figure 1*C* shows the residual current (1) and thioglycollic acid wave (2) obtained in deoxygenated 0.001N HCl. The lower chloride ion concentration is responsible for the more positive potential at which the chloride wave appears as compared with that found in Figure 1*B*. The residual current curve and thioglycollic acid wave were practically identical with those obtained in 0.01N $HClO_4$. There was obviously no chloride interference similar to that reported by Kolthoff and Barnum for cysteine.

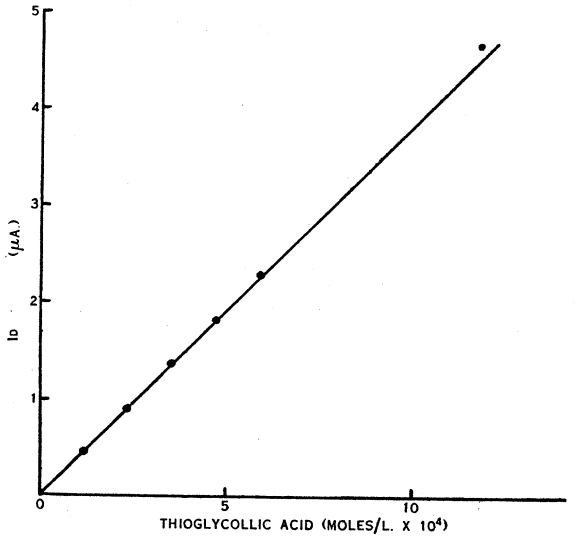

Fig. 2.—Diffusion current-thioglycollic acid calibration curve.

This finding suggested that direct analysis of small concentrations of thioglycollic acid, e.g. 0-50 p.p.m., might be determined directly in YEPW by acidification with hydrochloric acid to pH 3. Figure 1*D* shows the residual current curve (1) and sulphydryl wave (2) obtained in this manner. The curves, though similar in other respects to the previous waves, were displaced to a more negative potential with the limiting current measurable at —0.02 V. *v.* S.C.E.

The presence of 0.1 per cent. agar had no effect on these curves.

Relationship between the diffusion current and thioglycollic acid concentration.—For the purpose of the investigation the maximum amount of thioglycollic acid present in a 1/10 dilution of YEPW was 100 p.p.m. (0.0012M). The diffusion current was found to be proportional to the concentration of thioglycollic acid to within 0.5 per cent. over the range of 0-100 p.p.m. (see Fig. 2). The ratio was found to be the same in all the supporting electrolytes tested both in the presence and absence of agar.

Oxygen had to be excluded from all tests since it interferes with the measurement of the sulphydryl wave. Its removal was effected by bubbling commercial nitrogen through the sample till a zero oxygen reading was obtained at — 0.6 V. *v.* S.C.E. (Skerman and Millis 1949).

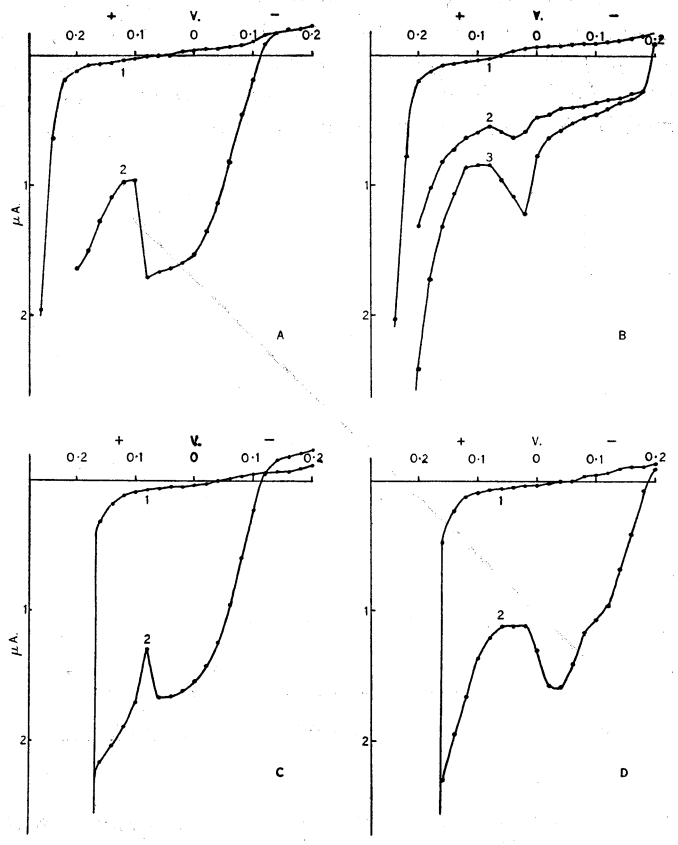

Fig. 3.—*A.* Current-voltage curves for deoxygenated (1) 0.01N $HClO_4$, and (2) 6.4×10^{-4}M cysteine HCl in 0.01N $HClO_4$, using a saturated calomel reference electrode.

B. Current-voltage curves for deoxygenated (1) 0.001N HCl, (2) 6.4×10^{-4}M cysteine HCl in 0.001N HCl, and (3) 12.8×10^{-4}M cysteine HCl in 0.001N HCl, using a saturated calomel reference electrode.

C. Current-voltage curves for deoxygenated (1) 0.01N $HClO_4$ containing 10 per cent. YEPW, and (2) 6.4×10^{-4}M cysteine HCl in 0.01N $HClO_4$ containing 10 per cent. YEPW, using a saturated calomel reference electrode.

D. Current-voltage curves for deoxygenated (1) 0.001N HCl containing 10 per cent. YEPW, and (2) 6.4×10^{-4}M cysteine HCl in 0.001N HCl containing 10 per cent. YEPW, using a saturated calomel reference electrode.

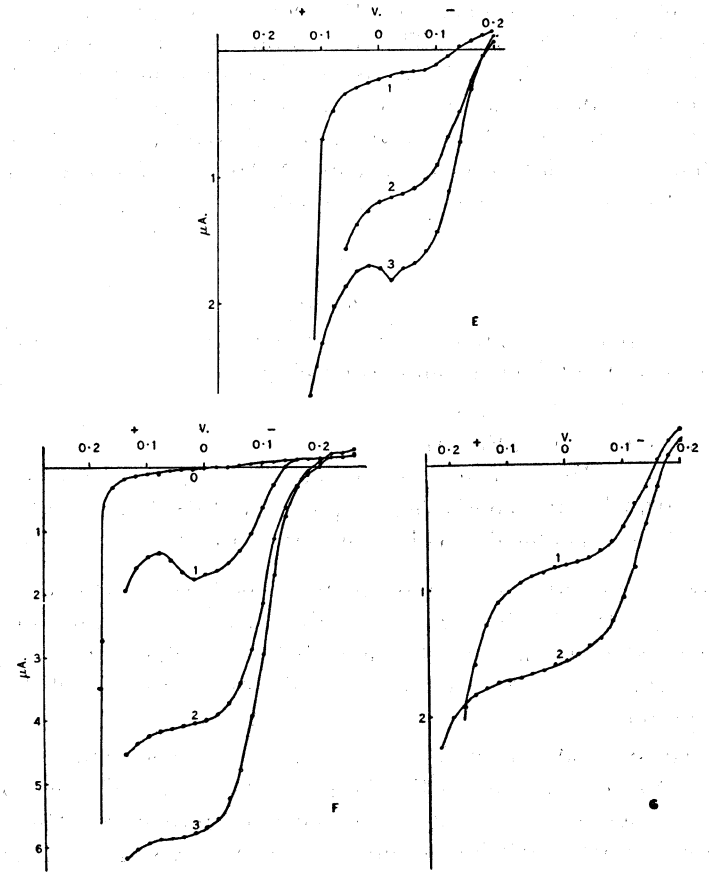

Fig. 3.—*E.* Current-voltage curves for deoxygenated (1) YEPW at pH 3, (2) (3) after autoclaving, and (3) 6.4×10^{-4}M cysteine HCl in YEPW at pH 3, using a saturated calomel reference electrode.

F. Current-voltage curves for deoxygenated (0) 0.01N $HClO_4$ containing 10 per cent. YEPW, (1) 6.4×10^{-4}M cysteine HCl in 0.01N $HClO_4$ containing 10 per cent. YEPW, (2) 10.5×10^{-4}M thioglycollic acid in 0.01N $HClO_4$ containing 10 per cent. YEPW, and (3) 6.4×10^{-4}M cysteine HCl and 10.5×10^{-4}M thioglycollic acid in 0.01N $HClO_4$ containing 10 per cent. YEPW, using a saturated calomel reference electrode.

G. Current-voltage curves for deoxygenated (1) 3.2×10^{-4}M glutathione in 0.001N HCl, and (2) 6.4×10^{-4}M glutathione in 0.001N HCl, using a saturated calomel reference electrode.

Plotting the rate of oxidation of thioglycollic acid in YEPW.—During the course of experiments reported in a later section of this paper the concentration of thioglycollic acid varied from 1000 to 0 p.p.m. During the initial stages measurements were made by diluting the medium 1/10 in 0.01N $HClO_4$, thereby reducing the pH to *c.* 3 and preventing further oxidation. The 10 ml. sample

was deoxygenated with nitrogen and the polarogram plotted over the range +0.1 to —0.1 V. (*v.* S.C.E.). This precaution was always taken to ensure that the wave had not altered its position. The concentration of thioglycollic acid could then be determined from the reading at +0.02 V. *v.* S.C.E.

When the reading for the 1/10 dilution was reduced to 2-5 mm. samples of the medium were acidified to pH 3 with 1N HCl, deoxygenated, and polarograms plotted in the same manner, final readings being taken at —0.02 V. *v.* S.C.E.

(*b*) *Cysteine*

Following upon observations made on thioglycollic acid the cysteine waves were examined in the following supporting electrolytes:

(i) 0.01N $HClO_4$ (Fig. 3*A*),
(ii) 0.001N HCl (Fig. 3*B*),
(iii) 0.01N $HClO_4$ containing 10 per cent. YEPW (Fig. 3*C*),
(iv) 0.001N HCl containing 10 per cent. YEPW (Fig. 3*D*),
(v) YEPW acidified to pH 3 with HCl (Fig. 3*E*).

Although a proportional increase in the anodic wave at 0.02 V. *v.* S.C.E. was obtained in 0.001N HCl (Fig. 3*B*) the waves were most unsatisfactory. Waves illustrated in Figures 3*A*, 3*C*, and 3*D* are similar to those reported by Kolthoff and Barnum (1940) who have discussed the significance of the 'maximum' produced.

The waves illustrated in Figure 3*E* (3) and (2), were obtained before and after heating the cysteine in YEPW respectively. The heating caused some unidentified change which prevented the maximum formation. Dilutions of the heated medium in 0.01N $HClO_4$ resulted in smooth, sigmoid waves without the maximum shown in Figure 3*C*.

The maximum was also suppressed by mixing thioglycollic acid with cysteine in the unheated medium (Fig. 3*F* (3)).

Relationship between cysteine concentration and diffusion current.—The ratio of diffusion current to cysteine concentration was found to be constant over the range 0-200 p.p.m. (0.0013M) of cysteine HCl (Fig. 4). This ratio was the same in all electrolytes tested and was not affected by the presence of agar.

In studying the oxidation of cysteine in YEPW initial determinations were made on 1/10 dilutions in 0.01N $HClO_4$ which were deoxygenated and the current measured at 0.06 V. *v.* S.C.E. (Fig. 3*C*) after plotting the polarogram between 0.1 and —0.1 V. *v.* S.C.E. When readings were reduced to 2-5 mm. further determinations were made directly on YEPW acidified to pH 3 and deoxygenated. Polarograms were plotted over the same range and readings taken at —0.02 V. *v.* S.C.E. (Fig. 3*E*).

It must be emphasized that the actual position of a wave depends largely on the composition of the medium. Slight changes have been the cause of a slight shift in the potential of the limiting current. Plotting of the wave ensured that measurements were always taken at the correct potential.

(c) *Combined Measurement of Thioglycollic Acid and Cysteine*

Figure 3*F* shows the waves obtained with cysteine (1) and thioglycollic acid (2) alone and in a mixture (3). The waves of these two substances coincide and are additive. In addition the maximum of the cysteine wave is not produced in the mixture.

The measurement of this single wave does not permit the expression of results in terms of cysteine and thioglycollic acid independently but does permit the rate of the oxidation of the combined mixture to be determined.

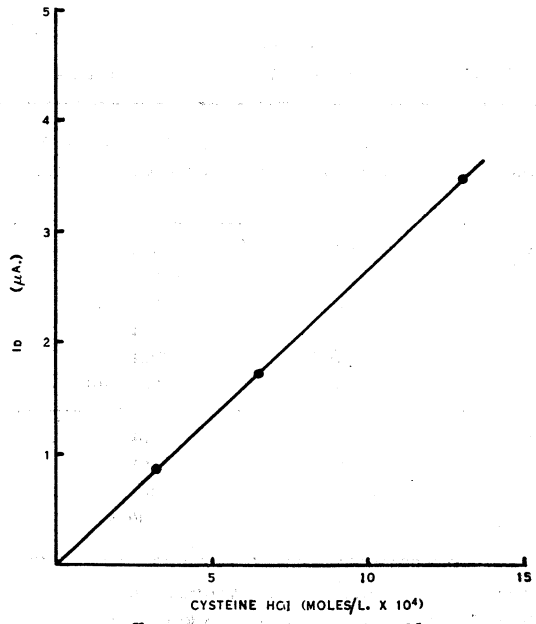

Fig. 4.—Diffusion current-cysteine HCl concentration calibration curve.

(d) *Glutathione*

The formation of an anodic depolarization wave by glutathione was examined in 0.001N HCl, in which it yields a clearly defined wave with the diffusion current proportional to the concentration over the range of 0-3 $\times$ 10^{-4}M. Two waves are shown in Figure 3*G*. Similar waves are obtained in YEPW with the diffusion current measurable at 0.0 or —0.02 V. *v.* S.C.E. (cf. cysteine).

(e) *Effect of By-products on the Calibration of the Polarograph for Measuring Cysteine and Thioglycollic Acid*

It seemed possible that the accumulation of cystine and dithioglycollic acid in the medium during the course of oxidation might affect the residual current measurements. Dithioglycollic acid was found to have no effect whatsoever. Cystine caused a change in portion of the residual current curve at potentials more negative than those employed in measuring cysteine but did not alter the residual current at the critical potentials.

(f) Calibration of the Diffusion Currents in Terms of Sulphydryl Concentration

It was intended that the nitroprusside test should be used in the titration of the sulphydryl with *p*-chlormercuribenzoate for the calibration of the polarograph. Parallel tests were conducted with the nitroprusside reagent against measurements made in the polarograph. Equal quantities of the various sulphydryl dilutions and 33 per cent. ammonia were mixed and 5 drops of a freshly prepared solution of sodium nitroprusside added. The colour change was observed up to 1 min. The results of these observations are tabulated in Table 1.

TABLE 1

COMPARISON OF NITROPRUSSIDE TESTS AND POLAROGRAPH MEASUREMENTS

—SH	Concentration (p.p.m.)	Galvanometer Deflection (mm.)	Initial Colour with Nitroprusside Reagent	Rate of Decolourization
Thioglycollic acid	1000 (0·012M)	1000	Purple	—
	100	100	Red	—
	50	50	Pink-red	—
	40	40	Pink	—
	20	20	Pink	+
	10	10	Pale pink	++
Cysteine HCl	400 (0·026M)	133	Pink	—
	200	67	Pink	+
	100	33	Pink	+++
	50	17	Yellow	

— = Not decolourized in 1 min.; + = decolourized in 1 min. or slightly less; ++ = decolourized in less than 30 sec.; +++ = transient colour only.

This table shows that the polarograph is capable of making quantitative estimations to a higher degree of sensitivity than that attainable with the nitroprusside test. With thioglycollic acid the sensitivity is only slightly greater but with cysteine hydrochloride a negative nitroprusside test is obtainable, particularly in YEPW, with concentrations up 150 p.p.m. For this reason an alternative method was sought for estimation of the sulphydryl by means of an amperometric titration using the polarograph itself. It was found that thioglycollic could be estimated by titration with mercuric chloride in 0.001N HCl provided the mercuric chloride was added to excess thioglycollic acid and not vice versa. Quantities of thioglycollic acid yielding diffusion currents of *c.* 3 μA. (66 mm. deflection on the galvanometer) were added to 0.001N HCl and the actual diffusion current measured at + 0.06 V. (see Fig. 1*C*). When quantities of 0.025 per cent. mercuric chloride were added to the thioglycollic acid the decrease in diffusion current was found to be strictly proportional to the amount of mercuric chloride added. Values given in Figure 2 were estimated in this way.

Reaction between the thioglycollic acid and mercuric chloride was instantaneous and there was no evidence of the formation of the highly insoluble mercurous salt.

When thioglycollic acid is added to excess mercuric chloride a white precipitate of mercurous salt is initially formed. It was not possible to use this method in the polarograph.

A similar attempt to estimate cysteine by the same procedure as that employed for thioglycollic acid was not successful. The titration could not be performed in HCl for reasons already indicated (see Fig. 3*B*). When an attempt was made to titrate in perchloric acid the changes in diffusion current with successive aliquots of mercuric chloride were not equal and a series of complicated anodic waves was encountered. The concentrations given for cysteine in Figure 4 were obtained gravimetrically. This should introduce very little error since the dry acid salt is quite stable. Fresh solutions in perchloric acid showed no evidence of the cathodic wave obtainable for cystine at more negative potentials.

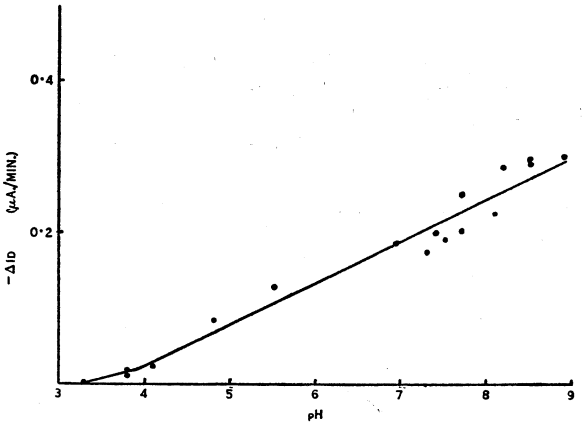

Fig. 5.—Effect of pH on the rate of reduction of oxygen concentration in YEPW by 0.1 per cent. thioglycollic acid expressed as rate of decrease in the oxygen diffusion current in μA. per min.

III. Observations on the Oxidation of Thioglycollic Acid and Cysteine in YEPW

(*a*) *Effect of pH on the Rate of Oxygen Reduction by* 0.1 *per cent. Thioglycollic Acid and* 0.1 *per cent. Cysteine HCl in YEPW*

In both cases 2 per cent. solutions of the reagents were prepared in glass-distilled water in sealed bottles. The quantity of 1N/NaOH required to neutralize 1 ml. of each was determined with a glass electrode and the concentration of the NaOH adjusted so that this amount was obtained in 1 ml.

The pH of the YEPW was adjusted to the desired value and 18 ml. placed in the cell, 1 ml. of the alkali added followed by 1 ml. of the reducing agent.

The rate of oxygen reduction (— ΔId) was plotted at 1-min. intervals for 10 min. and then the final pH was determined. The rate was not constant but diminished with time. The curves obtained were therefore not linear.

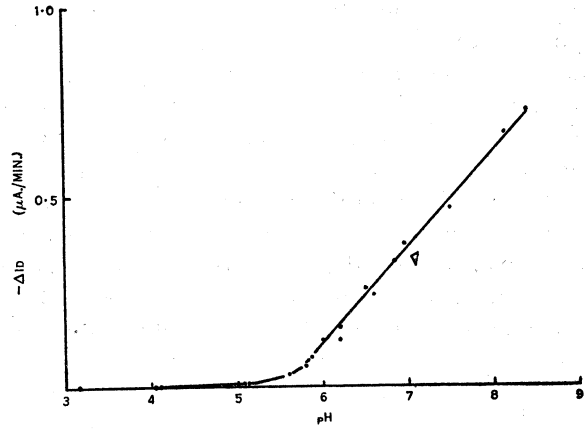

Fig. 6.—Effect of pH on the rate of reduction of oxygen concentration in YEPW by 0.1 per cent. cysteine HCl expressed as rate of decrease of the oxygen diffusion current in μA. per min.

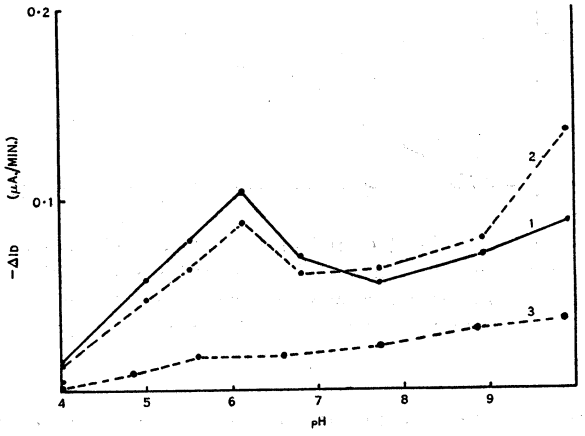

Fig. 7.—Curves (1) and (2) show the rate of reduction of oxygen (1) and sulphydryl (2) concentrations at varying pH values when 0.01 per cent. thioglycollic acid is added to YEPW saturated with oxygen at 37°C. Curve (3) shows the rate of reduction of sulphydryl concentration in the deoxygenated medium. All rates are expressed as rates of decrease in diffusion currents in μA. per min.

Figures 5 and 6, in which the tangents to the curves 2 min. after the addition of the reducing agent are plotted against pH, represent results obtained

with thioglycollic acid and cysteine respectively. The 2-min. interval was chosen to allow turbulence caused by mixing of the reagents adequate time to subside.

No attempt was made to trace the rate of oxidation of the sulphydryls in these experiments. The complete removal of oxygen from the YEPW after the latter had been equilibrated with air at 30°C. caused too small a change in sulphydryl concentrations at the concentrations employed to permit any accurate analysis.

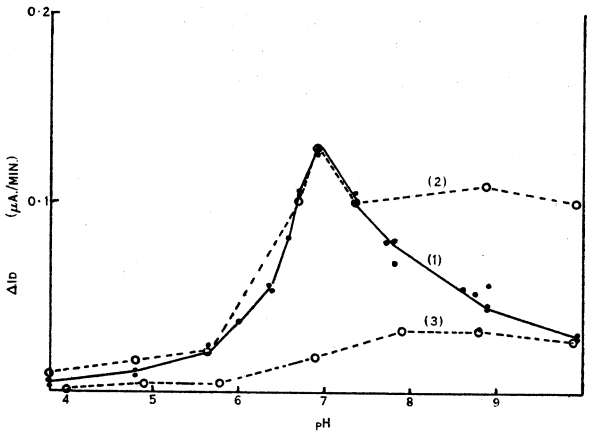

Fig. 8.—Curves (1) and (2) show the rate of reduction of oxygen (1) and sulphydryl (2) concentrations at varying pH values when 0.01 per cent. cysteine HCl is added to YEPW saturated with air at 37°C. Curve (3) shows the rate of reduction of sulphydryl concentration in the deoxygenated medium. All rates are expressed as rates of decrease in diffusion currents in μA. per min.

(*b*) *Effect of pH on the Rate of Reduction of Oxygen and Rate of Oxidation of* 0.01 *per cent. Cysteine and* 0.01 *per cent. Thioglycollic Acid in YEPW*

Previous unpublished experiments have shown that *Clostridium perfringens, Cl. tetani,* and *Cl. sporogenes* are all capable of growth in YEPW when inoculated after the concentration of reducing agents had fallen to 0.01 per cent. The behaviour of the reducing agent at these levels is therefore of considerable interest.

The small change in pH associated with the addition of such small quantities of acid obviated the necessity to take any special precaution to neutralize them on addition to the medium. Solutions (0.1 per cent.) were prepared in 0.001N HCl. Aliquots of 5 ml. of each were added to 45 ml. of YEPW and the rate of oxygen reduction (— ΔId) plotted at 1-min. intervals for 4 min. At the 5-min. interval a 10-ml. sample was removed and the pH immediately adjusted to pH 3 by the addition of 0.25 ml. 1N HCl. The sample was deoxygenated and analysed for residual sulphydryl.

The rate of oxygen reduction was plotted at 1-min. intervals for a further 10 min., when a second sample was removed for residual sulphydryl estimation. The final pH was determined on the remaining broth.

In this manner the amount of sulphydryl oxidized to oxygen reduced could be estimated. The results are shown in Figures 7 and 8. In both figures the rate of reduction of oxygen (1) and oxidation of sulphydryl (2) are plotted as rate of change in their respective diffusion currents in μA. per min.

Provided the sulphydryl reduces only the oxygen in the medium or reduces oxygen and some other component at a uniform rate over the time interval employed the curves should be parallel. Any sharp increase in the rate of sulphydryl oxidation over oxygen reduction indicates a preferential reduction of some component other than oxygen in the medium.

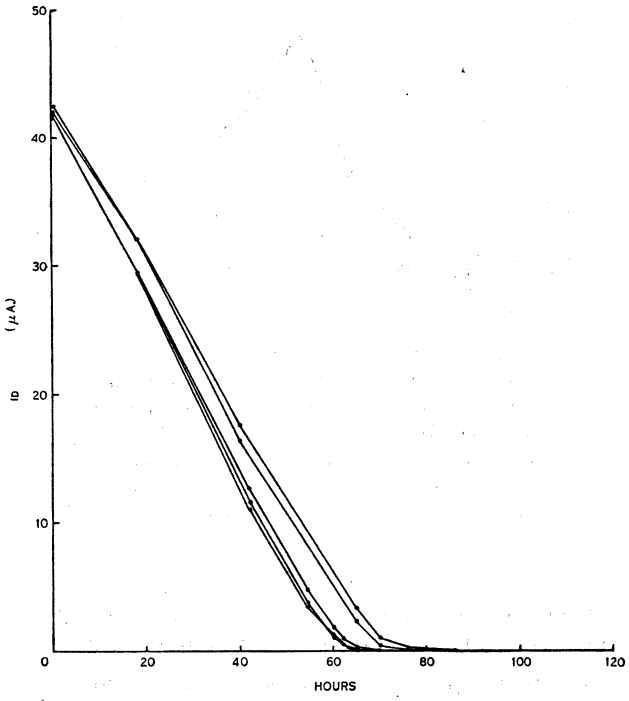

Fig. 9.—Rate of oxidation of 0.1 per cent. thioglycollic acid in YEPW (pH 7.0) exposed to air at 37°C. in a depth of 7 cm. 1 μA. $\equiv 2.6 \times 10^{-4}$ moles/l.

For cysteine (Fig. 8) the curves coincided over the range pH 5-7.3. The curves should have been parallel. Their coincidence is entirely fortuitous. Calculations show that the cysteine oxidized is entirely accounted for by the oxygen reduced. At higher pH values the cysteine appears to be preferentially oxidized by some other component of the medium. The rate of reaction with oxygen reached a maximum at the isoelectric point (pH 6.86).

Figure 8 (3) shows the rate of cysteine oxidation in the deoxygenated medium.

With thioglycollic acid the curves 1 and 2 remain parallel over the range pH 5-9. In the range pH 5-7.5 the amount of oxygen reduced exceeded the calculated by approximately 30 per cent. It approached the theoretical value between pH 7.5 and 9. Beyond this point the amount of sulphydryl oxidized greatly exceeded that calculated for the amount of oxygen reduced. No satisfactory explanation can be given for these findings. Similar results were obtained in nine replications of the experiment. It could not be explained by a change in the ratio of diffusion current to concentration of reactants at varying pH values.

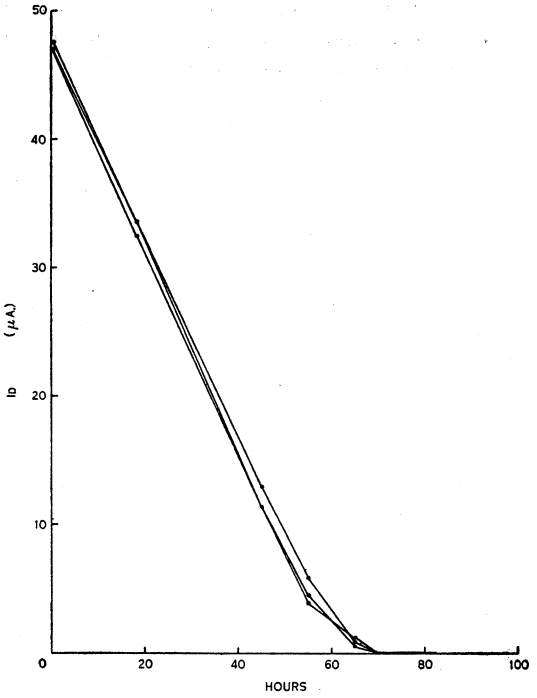

Fig. 10.—Rate of oxidation of 0.1 per cent. thioglycollic acid in YEPW + 0.1 per cent. agar (pH 7.0) exposed to air at 37°C. in a depth of 7 cm. 1 μA. ≡ 2.6×10^{-4} moles/l.

(*c*) *Oxidation of Thioglycollic Acid and Cysteine in YEPW and other Media*

Brewer (1940) claimed that pork infusion containing 0.1 per cent. agar is a suitable medium for the cultivation of anaerobes. His final medium contained in addition thioglycollic acid and glucose although, from the tables supplied, the addition of these substances does not appear to have improved the medium. Since pork infusion *per se* did not indicate the presence of any specific reducing substance the initial step in the investigation was to study the oxidation of the thioglycollic acid in various media.

Thioglycollic acid (to 0.1 per cent.) was added to the medium and the pH adjusted to pH 7.0. The medium was dispensed in 330-350 ml. quantities in tall 800 ml. beakers to give a depth of 7 cm. The beakers were loosely plugged with cotton wool. A length of ½-in. glass tubing through which samples could be withdrawn was placed through the centre of the plug and covered with an aluminium cap. Care was taken in the preparation of the plugs to see that replicates were as uniform as possible since some earlier work has shown that the tightness of the plug was an important factor in the rate of oxidation through its ability to regulate the freedom of air movement. The flasks were autoclaved at 120°C. for 20 min., cooled, and placed in a 37°C. water bath with the water level ¼ in. above the level of the liquid in the beaker to minimize convectional movements. Analyses of the contents for residual — SH were conducted at intervals determined by the trend of the oxidation itself.

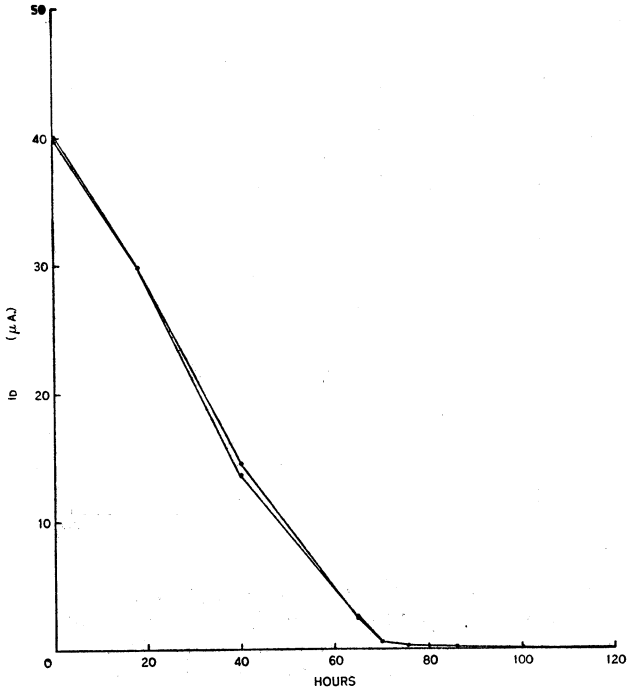

Fig. 11.—Rate of oxidation of 0.1 per cent. thioglycollic acid in YEPW + 1.0 per cent. glucose (pH 7.0) exposed to air at 37°C. in a depth of 7 cm. 1 μA. ≡ 2.6×10^{-4} moles/l.

The rate of oxidation of thioglycollic acid was studied in the following media:

(i) YEPW alone (Fig. 9),
(ii) YEPW + 0.1 per cent. agar (Fig. 10),
(iii) YEPW + 1.0 per cent. glucose (Fig. 11),
(iv) YEPW + 1.0 per cent. glucose + 0.1 per cent. agar (Fig. 12).

Reference to these figures will show that 0.1 per cent. thioglycollic acid is completely oxidized in 70-80 hr. in YEPW and that the addition of agar and glucose had no detectable influence on this rate.

Comparative experiments in which *Clostridium perfringens, Cl. tetani,* and *Cl. sporogenes* were tested for their ability to grow in YEPW + 0.1 per cent. thioglycollic acid showed that growth of *Cl. perfringens* would occur in the medium up to and slightly beyond the point where approximately 0.005M thioglycollic acid (Id = 2 μA.) was left. *Cl. sporogenes* ceased growth approximately at this point and *Cl. tetani* slightly before it.

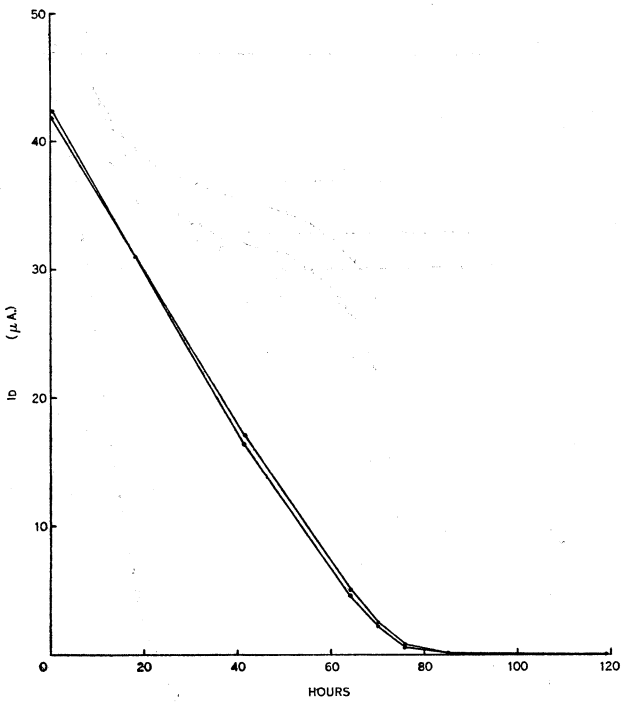

Fig. 12.—Rate of oxidation of 0.1 per cent. thioglycollic acid in YEPW + 1.0 per cent. glucose + 0.1 per cent. agar (pH 7.0) exposed to air at 37°C. in a depth of 7 cm. 1 μA. $\equiv 2.6 \times 10^{-4}$ moles/l.

Other experiments conducted with *Cl. perfringens* in YEPW, thioglycollic acid, and agar showed that agar prolonged the period of growth for 24-48 hr. beyond the complete oxidation of the thioglycollic acid.

None of these observations suggested why the Brewer medium should have remained effective for the period claimed. An examination was made therefore of pork infusion for the presence of an anodic wave suggestive of a sulphydryl compound. Minced pork in the proportion of 500 g./l. was steamed for 2 hr. in 0.5 per cent. NaCl. The hot infusion was filtered and a sample rapidly cooled, acidified to pH 3 with 1N HCl, deoxygenated with nitrogen, and a polarogram plotted. An anodic wave similar to that obtained with cysteine

and thioglycollic acid in YEPW was obtained and the presence of a sulphydryl group was confirmed with the nitroprusside reagent.

Curves were plotted on similar extracts from ox liver, ox muscle, and veal. These curves are illustrated in Figure 13. It seems likely that the substance produced was cysteine, probably as glutathione (Hopkins 1929; Kendall, McKenzie, and Mason 1929). The possibility that this may have contributed to the prolonged effectiveness of Brewer's medium led to the investigation on the determination of cysteine and glutathione reported in an earlier section of this paper.

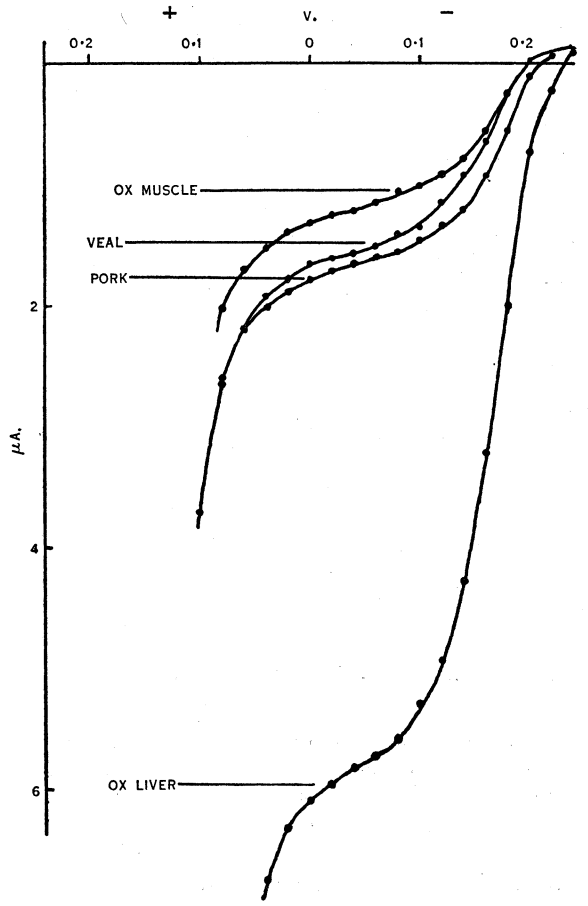

Fig. 13.—Sulphydryl waves obtained with tissue extracts prepared by steaming 50 per cent. w/v suspension for 2 hr., acidifying to pH 3 with HCl, and deoxygenating with nitrogen.

Owing to the high cost of glutathione only a single experiment was conducted with it. A 0.1 per cent. solution in YEPW at pH 7.0 equivalent in sulphydryl content to 0.05 per cent. cysteine HCl, was completely oxidized in 26 hr. at 37°C.

The oxidation of 0.1 per cent. cysteine HCl was studied in the following media: (i) YEPW alone (Fig. 14); (ii) YEPW + 0.1 per cent. agar (Fig. 15); (iii) YEPW + 1.0 per cent. glucose + 0.1 per cent. agar (Fig. 16).

Cysteine is initially rapidly oxidized until approximately 60 p.p.m. are left. The remainder is very slowly oxidized. Growth of *Cl. perfringens* ceases at the point where rapid oxidation ceases (20 hr.) in the absence of agar and 24-48 hr. later in the presence of agar.

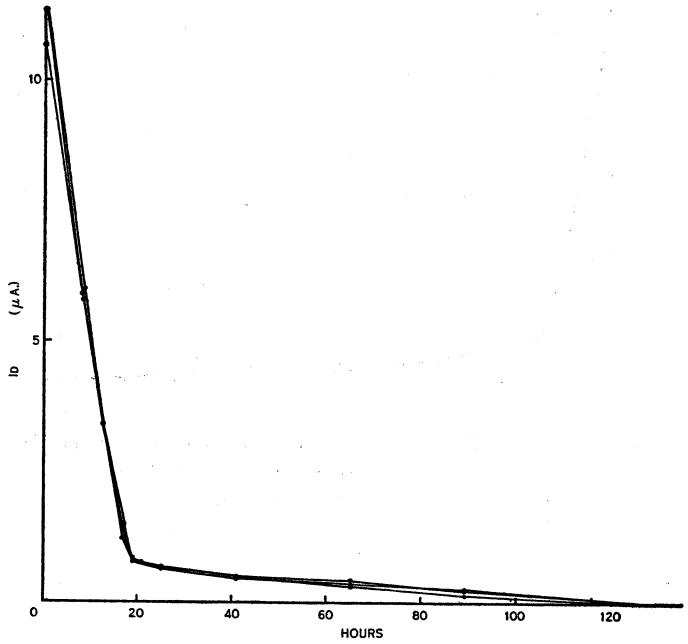

Fig. 14.—Rate of oxidation of 0.1 per cent. cysteine HCl in YEPW (pH 7.0) exposed to air at 37°C. in a depth of 7 cm. 1 μA. ≡ 3.6×10^{-4} moles/l.

The results of duplicate experiments in which 0.1 per cent. thioglycollic acid, 0.1 per cent. cysteine HCl, 1.0 per cent. glucose, and 0.1 per cent. agar were added to YEPW are shown in Figure 17. They are somewhat remarkable. It was expected that the time taken for oxidation of the combined sulphydryls would at least equal the sum of both. Instead the time barely exceeded that for thioglycollic acid alone and the slow process of oxidation of cysteine alone was completely absent.

Examination of pork infusion alone yielded results similar to those obtained with YEPW + 0.1 per cent. cysteine HCl.

(*d*) *Oxidation of Sulphydryl Groups in "Difco" Brewer's Medium and "Difco" Liquid Thioglycollate Medium*

In view of the results reported in the foregoing sections samples of the dehydrated "Difco" media used for sterility testing were obtained, prepared

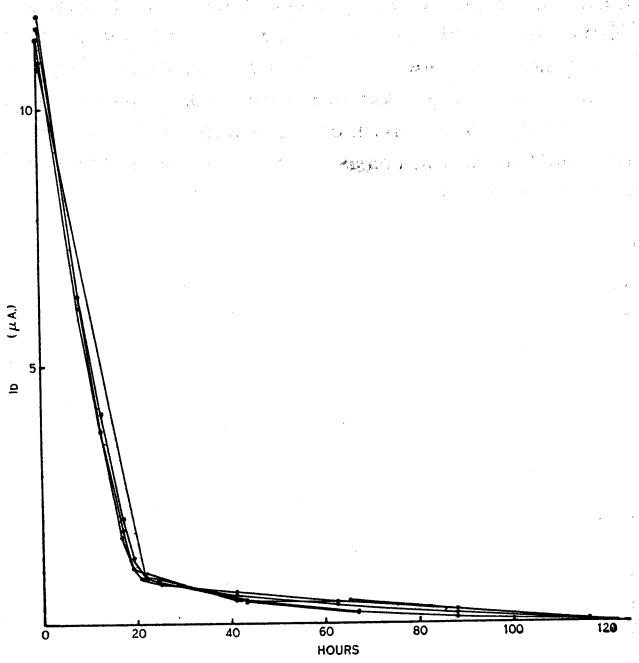

Fig. 15.—Rate of oxidation of 0.1 per cent. cysteine HCl in YEPW + 0.1 per cent. agar (pH 7.0) exposed to air at 37°C. in a depth of 7 cm. 1 μA. ≡ 3.6×10^{-4} moles/l.

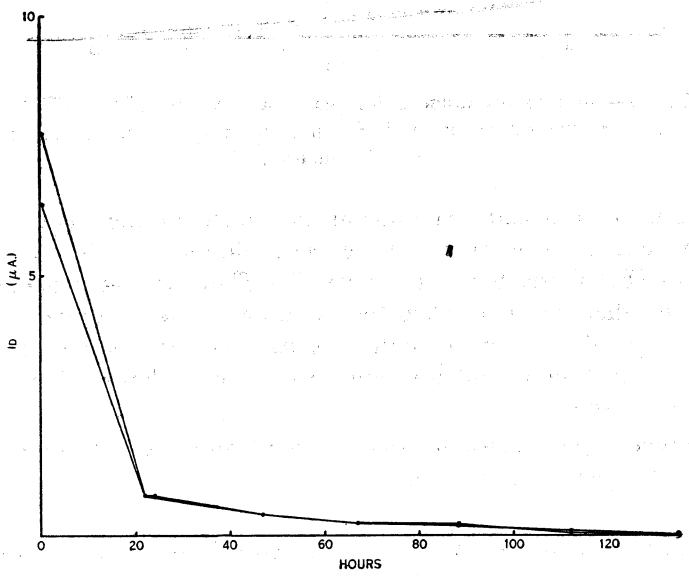

Fig. 16.—Rate of oxidation of 0.1 per cent. cysteine HCl in YEPW + 1.0 per cent. glucose + 0.1 per cent. agar (pH 7.0) exposed to air at 37°C. in a depth of 7 cm. 1 μA. ≡ 3.6×10^{-4} moles/l.

according to directions, and subjected to a similar course of study. The results are shown in Figures 18 and 19.

It was impossible with these media to determine the residual current for the — SH-free base medium. The anodic waves in the freshly autoclaved media occurred in the same position as that obtained for thioglycollic acid and cysteine in YEPW. The change in diffusion current was plotted to at least 100 hr. beyond the time when the media failed to support the growth of *Cl. perfringens.*

Neither of these media showed any better performance than those already reported. The idea that the prolonged action of these media was due to some mechanism that preserved a relatively high sulphydryl concentration had to be abandoned and the answer sought in some other direction. Two possibilities existed:

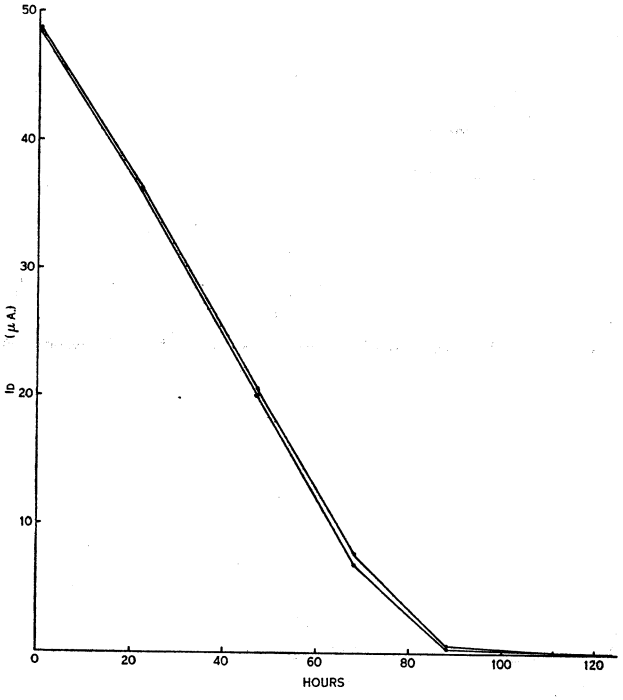

Fig. 17.—Rate of oxidation of a mixture of 0.1 per cent. thioglycollic acid and 0.1 per cent. cysteine HCl in YEPW + 1.0 per cent. glucose + 0.1 per cent. agar (pH 7.0) exposed to air at 37°C. in a depth of 7 cm.

(i) That the prolonged action was due to the method of dispensing, or

(ii) That the commonly advised procedure of boiling the medium prior to inoculation to "exclude dissolved oxygen" caused a regeneration of the sulphydryl.

That the method of dispensing is an important factor cannot be denied. "Difco" Liquid Thioglycollate medium dispensed in cotton-wool-plugged tubes in 7 cm. depth turns pink in less than 24 hr. and will support growth of *Cl. perfringens* for little more than 100 hr. if it is not reheated. Dispensed in screw-

capped bottles it retains its yellow colour and effectiveness for months provided the seals are good.

Instructions are given to users of the medium to the effect that, once the medium has become oxidized to a point where it will no longer support anaerobic organisms, it can be reheated to remove dissolved oxygen and re-used.

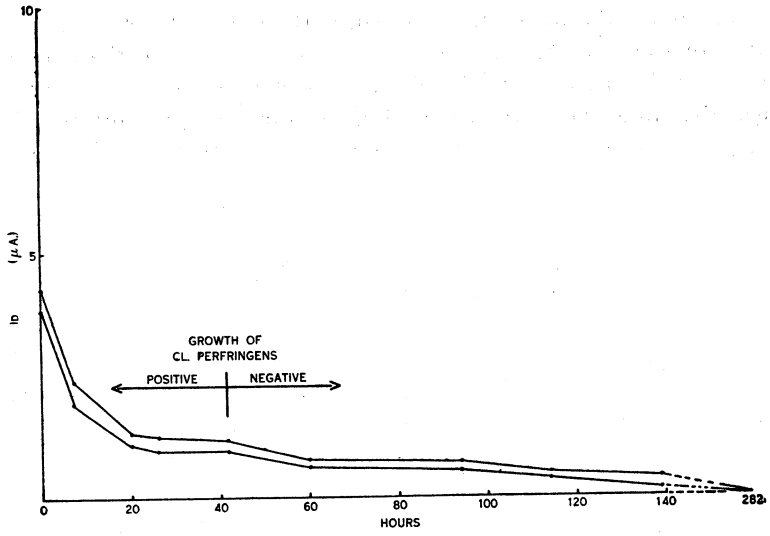

Fig. 18.—Rate of oxidation of sulphydryls in "Difco" Brewer's Medium.

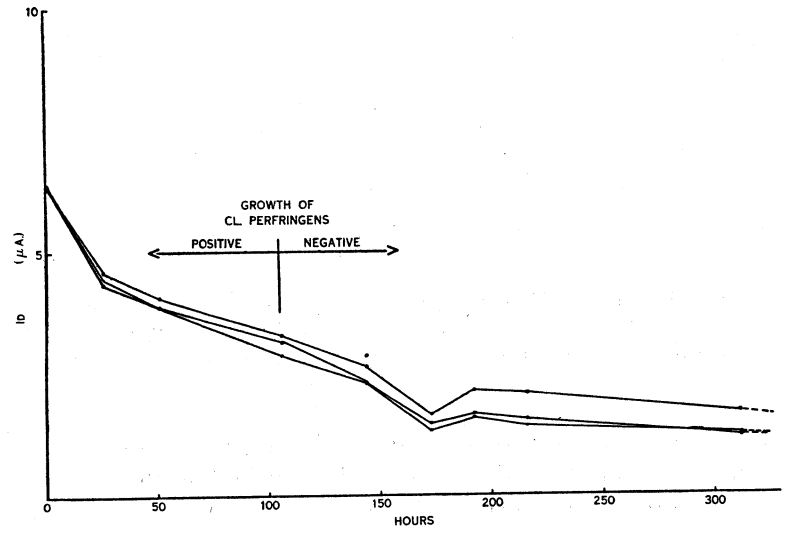

Fig. 19.—Rate of oxidation of sulphydryls in "Difco" Liquid Thioglycollate Medium.

It has been found that when YEPW is deoxygenated with nitrogen and exposed freely to air at a depth of 7 cm. at 37°C. without agitation, reoxygena-

tion occurs to saturation level in a period of 8-10 hr. Media which have been reheated remain suitable for anaerobic culture for longer periods than this. It seemed logical to assume that heating regenerated the sulphydryl group. This supposition was put to test and found to be true.

Regeneration of — SH groups from disulphides in oxidized culture media.— Heating samples of YEPW containing the oxidation products of cysteine, thioglycollic acid, and glutathione at 121°C. for 20 min. at pH 7.0 resulted in regeneration of sulphydryl equivalent, on an average, to 20 per cent. of the initial sulphydryl content. According to Shinohara and Kilpatrick (1934) the process is one of hydrolysis. They demonstrated that the degree of hydrolysis increases with a rise in pH and is dependent on time. These observations have been substantiated by the author. The process is accelerated by constituents of the medium.

When cystine is autoclaved in the presence of free thioglycollic acid, as in "Difco" Liquid Thioglycollate Medium, the cathodic reduction wave (Kolthoff and Barnum 1941) for this substance disappears. It is only partially reduced in the absence of the sulphydryl compound under similar conditions. It seems possible therefore that the process may not be one of simple hydrolysis.

IV. Discussion

From the data presented it is abundantly clear that the suitability of Brewer's and similar media for the cultivation of anaerobic bacteria is dependent on the presence of free sulphydryl groups. Once these have been oxidized the medium will no longer support growth. Subsequently, heating of the medium will result in a regeneration of portion of the sulphydryl content as well as removal of the oxygen.

Glucose does not aid the establishment of anaerobic conditions. Its principal function is probably as a ready source of fermentable carbohydrate which would aid in the rapid establishment of a bacterial population.

Agar is claimed by Hitchens (1921) to support the growth of several types of organisms, both aerobic and anaerobic. In sulphydryl culture media it does not affect the rate of sulphydryl oxidation, and direct experiments (unpublished data) have shown that it has no effect on the rate of diffusion of oxygen in a concentration of 0.1 per cent. This supports the findings of Rahn and Richardson (1941). The action of agar is most noticeable during and after the final stage of oxidation of the sulphydryl. Where duplicate tubes have been inoculated and only one has shown growth it can be demonstrated, as pointed out by Hitchens, that growth occurs initially on small particles of separated agar. It has been the author's experience that such separation only occurs in tubes where growth occurs and would appear to be initiated by the cells themselves. Attempts to demonstrate that the separated agar had adsorbed and concentrated small quantities of sulphydryl have so far been abortive.

The author cannot support Brewer's claim that his medium is suitable for the support of anaerobes after exposure to air for one month. It seems likely that the medium has been reheated prior to inoculation.

In examining the large volumes of fluid for residual sulphydryl, samples were removed from the base of the flask as this was considered to be the region most significant in relation to growth of organisms in open tubes. This was based on the assumption that an oxygen gradient would exist from the surface to the base of the solution, the fluid at the base remaining deoxygenated for the longest period of time. Since the samples had to be acidified immediately on removal, it was not possible in these experiments to determine the oxygen concentration in the sample at the same time. When it was demonstrated that growth of selected anaerobes continued after the concentration of residual thioglycollic acid had reached 0.001M, a method was devised whereby it was possible to determine simultaneously both the concentration of sulphydryl and the oxygen in the medium and, at the same time, to determine the distribution of oxygen throughout the solution. The method and observations resulting from its application will be published in a subsequent paper. Pertinent to the present discussion are the findings that:

(i) The medium remains completely deoxygenated from a distance of 2 mm. and probably less from the surface from shortly after the addition of 0.01M thioglycollic acid until the concentration of the latter is reduced to approximately 0.00025M;

(ii) Resolution of the oxygen commences in this region and that its concentration rises uniformly throughout the solution until saturation is reached for the prevailing temperature. No concentration gradient was detectable in the solution either of oxygen or residual sulphydryl.

The studies were made on a 7 cm. depth of fluid held perfectly still in a temperature-controlled bath with the water-level of the bath above that of the containing vessel. Convectional movement of the solution, if any, was not detectable by the electrodes, which are particularly sensitive to such movements.

From these observations and those of the nitroprusside test (Table 1) it would appear that a medium yielding a pink to pale pink or yellow colour with the nitroprusside reagent may contain oxygen and requires reheating prior to inoculation. No purpose would be served in heating media yielding a deep pink to red colour.

The use of the dye resazurin is not recommended. Its oxidation to pink resorufin which occurs very quickly in open culture is not an index of complete sulphydryl oxidation or a return of oxygen to the medium and its presence complicates the application of the nitroprusside test.

V. Acknowledgments

My thanks are due to Mr. Noel Henry of the Brisbane General Hospital for the supply of "Difco" Brewer's medium used in this investigation, and to Miss Shirley Hean for considerable technical assistance.

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