AN INVESTIGATION OF CHLORBUTOL IN OPHTHALMIC AND PARENTERAL SOLUTIONS.

A thesis presented in partial fulfilment of

the requirements for the degree of

MASTER OF SCIENCE

at

RHODES UNIVERSITY

GRAHAMSTOWN

by

ROBERT S. SUMMERS

B.Sc.(PHARM.), B.Sc.(HONS.), M.P.S.

DECEMBER, 1966.

ACKNOWLEDGEMENTS.

Acknowledgements and thanks are extended to:

a. Professor C.H. Price - the director of this project, whose assistance, at all times, was greatly appreciated.

b. R.E. Coss, Esq. - for assistance in proof-reading.

c. Addressograph-Multigraph (Pty.) Ltd. - this thesis was printed on a Multilith Offset 1250.

Chapter I. Introduction. 5

Chapter II. Investigation of the efficiency of chlorbutol as a bactericide in the process "Heating with a Bactericide". . 10

2.1. Introduction. 10

2.2. The selection of suitable bacterial strains for the investigation. 10

2.3. The selection of the experimental conditions. 12

2.3. 1. Containers. 12

2.3. 2. Chlorbutol concentration. 13

2.3. 3. Hydrogen ion concentration of solutions. 14

2.4. Processing of test solutions. 14

2.4. 1. Design of apparatus. 14

2.4. 2. Preparation of test solutions. 15

2.4. 3. Processing of solutions. 16

2.5. Bacteriological testing of solutions. 16

2.5. 1. Introduction. 16

2.5. 2. Experimental procedure. 17

2.5. 3. Results. 18

2.6. Discussion of bacteriological results. 20

2.6. 1. Tabulated results. 20

2.6. 2. Relative resistance of bacteria. 20

2.7. Abstract. 21

Chapter III. Investigation of the degradation of chlorbutol in pharmaceutical processes. 22

3.1. Introduction. 22

3.2. The selection and adaptation of a suitable assay procedure for residual chlorbutol in solution. 22

3.2. 1. Selection of a suitable assay procedure. 22

3.2. 2. Preliminary testing of the selected procedure. 23

3.2. 3. Assay of 'test' quantities of chlorbutol in solution using the adapted procedure. 26

3.2. 4. Interferences with the procedure. 28

3.3. The degradation of chlorbutol in solution during the pharmaceutical processes of heat sterilisation. 29
3.3. 1. Introduction. 29
3.3. 2. The degradation of chlorbutol during the "steaming" of its solutions. 30
3.3. 3. The degradation of chlorbutol during the autoclaving of its solutions. 32
3.3. 4. Summary of results of degradation determinations. 36
3.4. The effects of the degradation of chlorbutol during heat processing on its preservative properties in ophthalmic and parenteral solutions. 36
3.4. 1. Introduction. 36
3.4. 2. Bacteriological testing of processed solutions of chlorbutol for self-sterilising properties. 37
3.5. Discussion. 39
3.5. 1. Spectrophotometric assay results. 39
3.5. 2. Results of bacteriological tests. 40
3.6. Abstract. 40

Chapter IV. Investigation of the effect of the silicone coating of containers on the degradation of chlorbutol.....41
4.1. Introduction and theoretical considerations. 41
4.1. 1. The relationship between the degradation of chlorbutol and the glass of the container. 41
4.1. 2. The structure of glass. 41
4.1. 3. The structure and properties of silicone fluids. 42
4.2. Silicone coating the containers. 43
4.2. 1. Introduction. 43
4.2. 2. Procedure. 44
4.3. The degradation of chlorbutol in solutions in silicone-coated containers. 44
4.3. 1. Introduction. 44
4.3. 2. Procedure.....45
4.3. 3. Results of the assay procedure. 45
4.3. 4. Discussion of results. 45

4.4. The bacteriological testing of chlorbutol solutions, in silicone-coated containers for self sterilising properties after processing....47
4.4.1. Introduction....47
4.4.2. Procedure....47
4.4.3. Results of bacteriological tests....47
4.4.4. Discussion of results....48
4.5. Discussion of the effects of the silicone-coating of containers on chlorbutol solutions....48
4.6. Abstract....49

Chapter V. Formulation of chlorbutol solutions for maximum antibacterial potency....50
5.1. Introduction....50
5.2. The investigation of the use of dilute alcohols....51
5.2.1. Introduction....51
5.2.2. Solubility tests....52
5.2.3. Discussion of results of solubility tests....52
5.3. Bacteriological tests with diluted stock solutions....53
5.3.1. Introduction....53
5.3.2. Bacteriological tests after heat processing....53
5.3.3. Bacteriological tests after filtration sterilisation....56
5.4. Investigation of possible interference in the spectrophotometric assay procedure by 4% ethanol....57
5.4.1. Introduction....57
5.4.2. Procedure....57
5.4.3. Discussion....57
5.5. Investigation of the possible loss of chlorbutol, from its solutions, by sorption onto membrane filters during filtration sterilisation....57
5.5.1. Introduction....57
5.5.2. Procedure....58
5.5.3. Results....58
5.5.4. Discussion of results....58
5.6. Discussion....59
5.7. Abstract....59

Chapter VI. Investigation of the nature of the degradation products of chlorbutol in ophthalmic and parenteral solutions.....61.
6.1. Introduction and theoretical considerations.....61
6.2. Selection of degradation products for investigation.....62
6.3. Investigation of the selected degradation products.....63
6.3. 1. Introduction.....63
6.3. 2. Determination of degradation products by thin-layer chromatographic methods.....64
6.3. 2. a. Standard procedure.....65
6.3. 2. b. Determination of α-hydroxyisobutyric acid and isopropanol.....65
6.3. 2. c. Determination of acetone.....70
6.3. 2. d. Determination of formic and acetic acids.....73
6.3. 2. e. Determination of chlorides.....78
6.3. 3. Determination of degradation products by specific tests..81
6.3. 3. a. Introduction.....81
6.3. 3. b. Determination of chloroform.....81
6.3. 3. c. Determination of hydrogen ions.....84
6.4. Discussion.....85
6.5. Abstract.....86

Chapter VII. Discussion.....88

APPENDIX 1.....90
Presentation of experimental data.....90
1. Tables.....90
2. Graphs.....125
3. Diagrams.....152

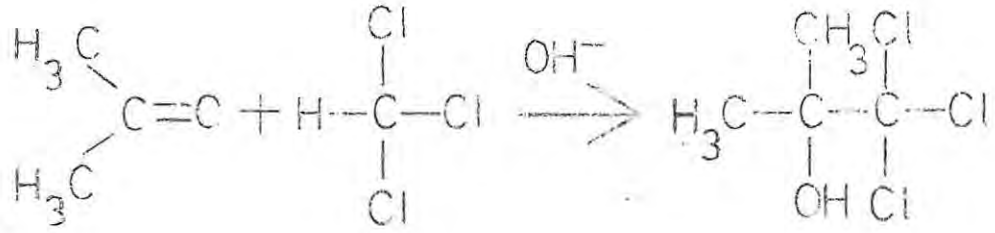
APPENDIX 2.....164
Bibliography.....164

CHAPTER I.

Introduction.

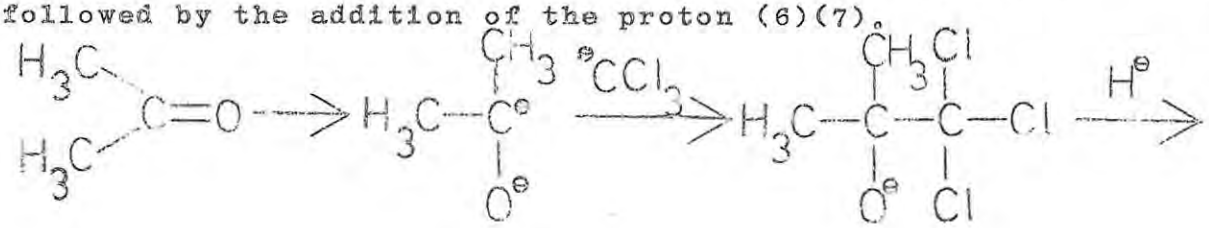
Chlorbutol, which is tri-chlor-tertiary-butanol, was first prepared by Willgerodt in 1886 (1). The reaction he used for its preparation is still used today, though slightly modified (2)(3)(4), and is suggested by its original name "acetone-chloroform". The substance was prepared by adding solid potassium hydroxide to a cold mixture of acetone and chloroform (5).

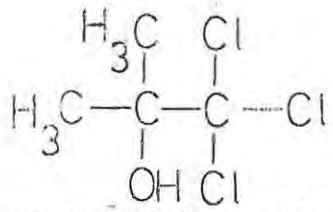
Chlorbutol is a derivative of the trichlorinated derivative of methane, and its formation may best be described by the use of structural formulae.



Carbonyl compounds form condensation products with hydrogen-containing compounds. One electron pair of the carbonyl bond tends to become detached from the carbon atom so as to form a lone pair on the oxygen atom.

The carbonyl group becomes polarised and it can be surmised that the primary reaction in the formation of chlorbutol is the nucleophilic attack of the OH^- on the polarised carbonyl group. This is followed by the addition of the proton (6)(7).





The use of such a substance as a preservative for ophthalmic and parenteral solutions is based upon the antibacterial effect of the chlorine in the molecule (8).

Parenteral solutions were first used over one hundred years ago, and by 1853 a simple syringe with a hollow needle had been invented (9). Serious complications often followed medication by this route. Substances such as chloral hydrate and glycerin were added to the solutions as antiseptics to reduce these adverse effects. Hypodermic tablets, to be dissolved immediately before injection , were also used in an attempt to reduce febrile reactions (9).

In 1910, intra-muscular and intra-venous injections were first described in a pharmacopoeia (10), but their current widespread use had to await the discovery of a suitable means of preservation from bacterial and fungal contamination. Many substances, including phenols, cresols, mercurials, p-hydroxybenzoic acid esters, and chlorbutol, were suggested and tested for this purpose.

Today, a large number of agents are available. Their variety suggests that there is no single substance satisfactory for use in all pharmaceutical preparations and such is indeed the case.

The use of ophthalmic solutions has a much shorter documented history. Eye-drops and eye-lotions were widely used by 1932 (11), but were not included in the British Pharmacopoeia of that year (12).

By 1938 a more realistic attitude to ophthalmic solutions was apparent, and in Great Britian the sterility of such solutions was considered necessary. Sterilisation was achieved by the adaptation of the methods used in the production of parenterals (13).

No such criteria had been introduced in the United States before 1951 (14). Soon after, however, suitable regulations, under which all ophthalmic solutions prepared by pharmaceutical manufacturers had to be sterile, were promulgated (15). Hospitals and retail pharmacies were exempt from the regulations. The use of chemical anti-

bacterial agents in such solutions is essential if serious ophthalmic infections are to be avoided. As in the case of parenterals, the selection of a suitable agent is difficult and no single substance, covering all contingencies, has yet been discovered.

The properties which a suitable preservative for ophthalmic and parenteral solutions should possess are ennumerated below. It should be borne in mind that many and varied requirements of physical properties, toxicity and compatibility may be encountered in practice.

Desirable properties which a preservative should possess are:

1. High antibacterial activity at low concentration.
2. Possession of few physical and chemical incompatibilities.
3. Retention of efficiency over a wide pH range.
4. Efficiency against a wide range of micro-organisms.
5. Ready solubility at effective concentration.
6. Stability under heat sterilisation and storage conditions.
7. Absence of odour and colour.
8. Absence of toxic and irritant effects on the patient at effective antibacterial concentrations. (16)(17)(18)(19).

The efficiency of a suitable agent can be affected by factors which may arise under manufacturing conditions, viz:

1. The amount of nutrient present in the medicament and preparation.
2. The pH of the product.
3. The presence of interfering substances which may decrease the antibacterial activity of the agent e.g. nonionic surfactants (20).
4. The presence of other inherently antibacterial compounds.
5. The micro-organisms involved. (17)

Hence, the efficiency of any compound as an antibacterial agent in solution depends upon it possessing certain desirable properties, and on its ability to remain unaffected by the system in which it is incorporated.

In this investigation, the chlorinated alcohol, chlorbutol, was examined for its ability to comply with the criteria of the theore-

tically ideal preservative, with special reference to parenteral and ophthalmic solutions.

The preservative properties of chlorbutol were first described in 1911 (21). It was tested as a preservative in 1919 and found "effective" (22). The first instance of its use in a parenteral preparation was in 1920, when a saturated solution was used as a vehicle for bacterial vaccines (23).

Official recognition of its antibacterial properties had to wait till 1923 (24). By 1935 its inclusion in parenteral solutions had been more precisely evaluated (25).

According to Hamilton (26), chlorbutol had a phenol coefficient of 1·2, and was completely bacteristatic at a concentration of 0·8% (i.e. at saturation). Briggs and Callow (27) found it effective against non-sporing organisms at a concentration of 0·5%.

Since 1950 chlorbutol has been widely used as a preservative for both parenteral and ophthalmic solutions, and is described as such in most official monographs from that date until the present time (28)(29)(30)(31)(32)(4)(33). The concentration has not always been specified, but a 0·5% solution has generally been used.

Chlorbutol has a great advantage over many other preservatives in that it exerts a local analgesic and anaesthetic effect at the site of administration (34)(21)(23)(35)(36)(28)(29)(4). A parenteral or ophthalmic solution incorporating chlorbutol would be highly acceptable to the patient as it would reduce local pain.

Incompatibility between agent and medicament is an aspect of some import, but the studies of McEwan and McMorran (37) and of Goldstein and Ryan (38) have shown that chlorbutol has a high level of compatibility. This is yet another reason for its current widespread use.

In one aspect, however, chlorbutol does not appear to conform to the requirements for the "ideal" preservative. This is in regard to its stability under conditions of heat sterilisation.

In 1937, Rosenthaler (39) reported that when chlorbutol was heated with alkali, it decomposed into its components, acetone, and chloroform, and yielded their characteristic reactions. More important was the investigation of Taub and Luckey (35), in which

the decomposition was more closely examined, and appeared to be considerable under certain conditions of temperature and pH (i.e. above 65° and at a pH higher than 6).

Subsequently, other investigations have been performed with varying results, (36)(40)(41), but no complete evaluation of the extent of the degradation of chlorbutol under pharmaceutical conditions had as yet been carried out. Doubt also existed as to the nature of the decomposition products. It had been proposed that these were acetone, carbon monoxide, α-hydroxyisobutyric acid (trace quantity) and free chloride ions (42), but confirmation had not been obtained.

This investigation therefore, was mainly concerned with the evaluation of the extent of the degradation of chlorbutol when its solutions were subjected to pharmaceutical processes, and with the elucidation of the nature of the products of the degradation. Also investigated were methods of reducing the degradation of the compound, and of increasing its antibacterial potency.

CHAPTER II.

The investigation of the efficiency of chlorbutol as a bactericide in the process "Heating with a Bactericide".

2.1. Introduction.

Provision is made for the sterilisation of thermolabile substances by heat processing in the B.P. process "Heating with a Bactericide". (33). In this process, the solution, which must contain an antiseptic at least as effective as 0.5% phenol, is subjected to steaming at 98°-100° for thirty minutes. The combination of antiseptic and temperature, which is not nearly as high as for the autoclaving of solutions, renders the product sterile.

Chlorocresol and phenylmercuric nitrate have been tested for their efficiency in this process (43), but although they are specified in the B.P. (33), they are not effective against all bacteria (44)(45)(46).

Tricresol has also been investigated (47), and 0.5% chlorbutol solutions have been observed to possess 'bactericidal' properties (48).

This investigation was therefore commenced with a full evaluation of the efficiency of chlorbutol as a bactericide in the process "Heating with a Bactericide" (33).

2.2. The selection of suitable bacterial strains for the investigation.

In an investigation of the antibacterial properties of any substance, it is essential that the substance be tested against as wide a range of micro-organisms as possible. The test range must be selected so that micro-organisms exhibiting extremes of pathogenicity and of resistance are included. An evaluation, as complete as possible under the experimental conditions, is therefore obtained. However, it must be borne in mind that the conditions prevailing in a research laboratory

are not always reproducible in a manufacturing establishment. Under the comparatively ideal conditions of the research laboratory, the substance might well prove more effective than if tested in a pharmacy or manufacturing laboratory.

A representative cross-section of common pathogenic bacteria, which may contaminate an ophthalmic or parenteral solution at source, or during its compounding, is required.

The following bacterial strains were selected as the test organisms for the investigation:-

2.2. 1. Staphylococcus aureus.

This organism is amongst the most resistant of all pathogenic non-sporing bacteria (8). It is frequently found in dust, in which it may remain viable for months. Its normal habitat, in the human being, is just inside the nostril, an ideal situation for droplet dispersion (46). S. aureus is a Gram-positive aerobe and facultative anaerobe, and is pyogenic, causing boils, carbuncles and conjunctivitis. These facts indicate that it is an important possible contaminant of ophthalmic and parenteral solutions (36)(49)(50)(51).

2.2. 2. Bacillus subtilis.

There are a number of varieties in this group, ranging from highly susceptible to highly resistant organisms (e.g. B.globigii (52)(8)). B. subtilis was selected for inclusion to simulate dust and air-borne contamination, due to its ubiquitous nature (36)(46).

2.2. 3. Bacillus cereus.

This is a member of the B. subtilis group, and is highly resistant, due to the common spore-forming properties of the group. Although believed to be non-pathogenic it is included because of its extremely high resistance to both chemical and heating methods of sterilisation (52).

2.2. 4. Pseudomonas fluorescens.

This Gram-negative flagellate is included as a substitute

for Ps.aeruginosa, one of the most highly infective ocular contaminants (53)(55), which was not available. Kohn, Gershenfeld and Barr (53) emphasised the incidence and severity of eye infections from this source and their conclusions have been amply confirmed (54)(55)(56). Any investigation of a bactericide for ophthalmic solutions must include a member of the genus (18)(51)(57). Ps. fluorescens is only slightly less pathogenic than Ps. aeruginosa (52).

2.2. 5. Proteus vulgaris.

This organism frequently occurs as a secondary invader in wound infections and is widely distributed (8). It is a Gram-negative, non-sporing facultative anaerobe (52), and has been identified as a common contaminant of ophthalmic solutions (18).

2.2. 6. Escherichia coli.

This organism is a Gram-negative, non-sporing facultative anaerobe (52), and is highly infective (8).

Gram-positive and Gram-negative aerobes and anaerobes were included. Their presence ensured that, in this investigation, the bactericidal effect of chlorbutol in solution was adequately tested against a wide range of micro-organisms.

2.3. The selection of the experimental conditions.

2.3. 1. Containers.

As the bactericidal properties of chlorbutol solutions during heat processing were being investigated under pharmaceutical conditions, a selection of containers for parenteral and ophthalmic solutions must be used. It was possible that the bactericidal properties of the compound would be affected by the nature of the container.

2.3. 1.a. For parenteral solutions.

1. 1 ml. amber glass ampoules.
2. 2 ml. white glass ampoules.

3. 5 ml. white glass ampoules.

4. 10 ml. white glass ampoules.

5. 15 ml. Clinbritic bottles (amber glass).

2.3. 1.b. For ophthalmic solutions.

1. 15 ml. amber glass eye drop bottles.

2.3. 1.c. Closures for the containers.

1. Ampoules were sealed by fusion of glass.

2. Clinbritic bottles were sealed with rubber caps, retained by aluminium sealing rings. The absorption of bactericides by such closures has been well documented (58)(51)(41). The B.P. requirements (33) for their use were strictly adhered to, except that a double strength solution of the concentration of chlorbutol employed in the determination could not be used due to solubility factors. Hence, a saturated solution of chlorbutol (approximately 0.8%) was used for the equilibration process (33).

3. Eye-Drop Bottles. Due to the low resistance of the rubber teats on the droppers to temperature and pressure changes, these closures were discarded as a method of sealing the containers. Instead, plastic screw caps were used. These were unaffected by the physical changes and any deleterious effect on the solutions was avoided by the use of aluminium foil liners.

2.3. 1.d. Test for limits of alkalinity of glass.

All containers used complied with the requirements of the B.P. test for limits of alkalinity of glass (33), although the 5% glacial acetic acid washings were necessary for the amber glass containers to conform to the standards. The test was performed on the relevant containers immediately before use.

2.3. 2. Chlorbutol concentration.

The use of a 0.5% w/v chlorbutol solution as a preservative has been recommended by many authors, (27)(36)(38)(18)(49)(59), and this is the concentration in which it is most generally used.

This concentration was therefore selected for investigation for bactericidal properties. The establishment of its efficiency as a bactericide in the process "Heating with a Bactericide" (33), at this concentration, facilitated the subsequent investigation of its preservative and self-sterilising properties.

2.3. 3. Hydrogen ion concentration of solutions.

The hydrogen ion concentrations of ophthalmic and parenteral solutions vary considerably (33). The pH values of official injections sterilised by "Heating with a Bactericide" vary from approximately 3 (Emetine Injection. B.P.) to approximately 8 (Mersalyl Injection. B.P.).

The pH values selected for the investigation must therefore cover this range. The following pH values were selected for the test solutions.

a. pH 3.5

b. pH 5.6

c. pH 7.4

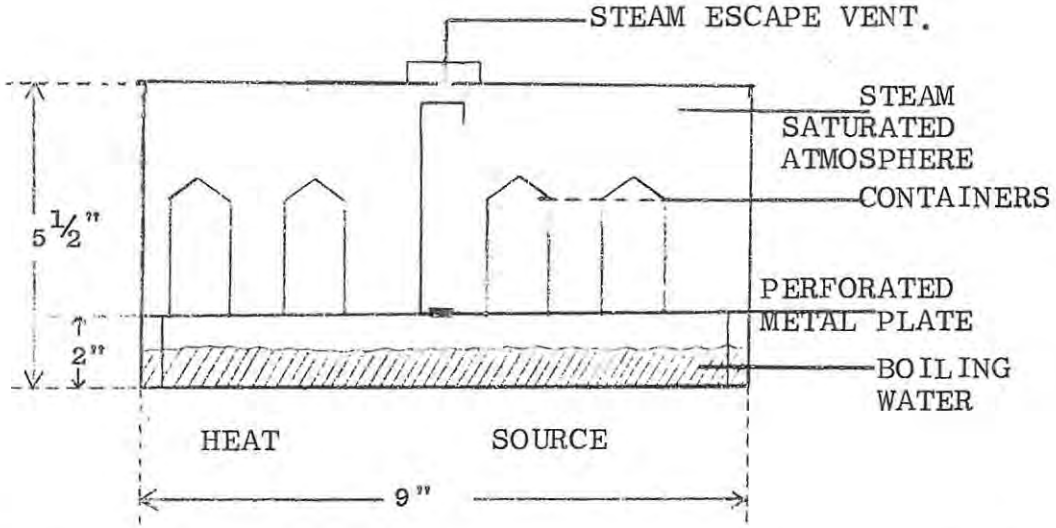
Such solutions are well tolerated when used ophthalmologically (60).

In certain instances, the B.P. specifies that ophthalmic and parenteral solutions must be buffered to a specific pH value, so, for a complete evaluation, the bactericidal effect of chlorbutol in both unbuffered and buffered solutions was determined. Reasons for buffering are discussed by Carter and Gunn (46).

2.4. Processing of test solutions.

2.4. 1. Design of apparatus.

The time-temperature requirements of the process "Heating with a Bactericide" are thirty minutes at 98°-100° (33). This may be effected by placing the containers of test solutions in boiling water, but a more satisfactory method is to use a steamer. A steamer incorporating the principles outlined by Carter and Gunn (46) was designed and made.



By using this apparatus, in conjunction with a constant level device, the test solutions were maintained at 98°-100° for as long a period as required.

2.4. 2. Preparation of test solutions.

Sufficient 0.5% w/v chlorbutol solutions with buffered and unbuffered pH values of 3.5, 5.6 and 7.4 respectively were prepared by shaking.

The buffered solutions of pH 3.5 and pH 5.6 were prepared by the use of phthalate buffer (61). The buffered pH 7.4 solution was prepared with phosphate buffer (61).

The unbuffered solutions were standardised against standard buffer solutions using the Radiometer pH meter, which was also used to check the pH values of the buffered solutions immediately before transfer to the containers.

The respective solutions were transferred to the specified containers (2.3.1.) and inoculated with one loopful of the mixed bacterial suspension (see 2.5.). The containers were sealed in the prescribed manner (2.3.1.).

Control solutions, with the same pH values, buffered and unbuffered, but without chlorbutol, were prepared, and transferred to containers. The solutions were inoculated and the containers were sealed.

2.4. 3. Processing of solutions.

Sufficient inoculated containers of each type, containing 0.5% w/v chlorbutol solutions at the respective pH values were placed in the steamer. Five minutes were allowed to elapse before the commencement of timing to enable the test solutions to reach the required temperature.

As the determination was performed in quadruplicate, four containers of each type, and pH value of solution, were removed from the steamer at fifteen minute intervals, commencing at zero time plus fifteen minutes. The process was continued until zero time plus ninety minutes to give a complete evaluation over an extended period. By this method, the bactericidal activity of 0.5% w/v chlorbutol solutions could be related to time during heat processing.

The contents of the containers were then subjected to bacteriological tests for viable bacteria (see 2.5.).

NOTE: All solutions used in the determination were continuously refrigerated at 4°, except when being processed or tested. This was done for two reasons.

a. To maintain the concentration of the 0.5% w/v chlorbutol solutions.

b. To maintain the viable bacteria at the status quo between processing and bacteriological testing. The bactericidal properties of chlorbutol and other antibacterial agents are markedly decreased when the temperature is reduced. Bacterial growth and proliferation are also reduced (46).

2.5. Bacteriological testing of solutions.

2.5. 1. Introduction.

Due to uncertainty and difficulties in the inactivation of antibacterial agents in conventional sterility tests (49) (46)(53), the method adopted in this investigation was that of bacterial filtration.

In 1946, Davies and Fishburn indicated the drawbacks of

the official method of sterility testing, and pointed out the advantages of bacterial filtration methods (112). The widespread use of the method had to await the production of the membrane filter in 1959 (62), which enabled high flow rates to be obtained.

Minor advantages of the method are numerous (46). The main advantage is that a total estimate of all viable bacteria in the test solution can be obtained. As all the test solution is passed through the filter, errors due to dilution factors are avoided.

The use of the method does, however, require a high standard of asepsis and of technique (46).

2.5. 2. Experimental procedure.

2.5. 2.a. Growth of micro-organisms and inoculation of test solutions.

The test organisms (2.2) were stored under refrigeration at 4° in 1/4 strength Ringer's solution (84) and were subcultured onto nutrient agar slopes twenty-four hours before inoculation.

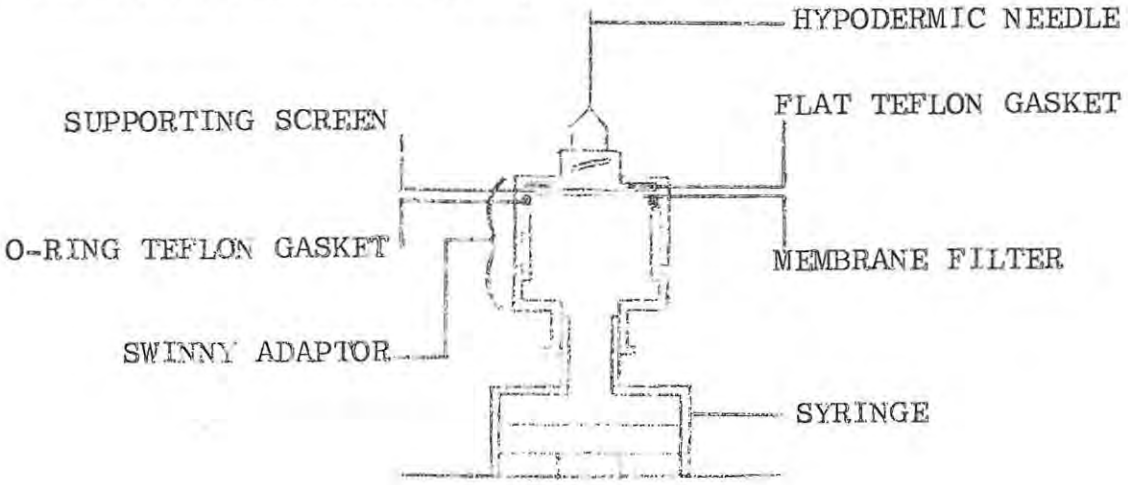
The mixed bacterial suspension was prepared by adding 2 ml. of sterile 1/4 strength Ringer's Solution to the slope, rotating to effect suspension of the micro-organisms, and transferring the suspension to a sterile bulk container. The test solutions were inoculated before closure of the container, by transferring a loopful of the mixed bacterial suspension to the solution.

NOTE: This process, and all others in this investigation which required asepsis as a condition, were performed under a sealed screen of the type developed by Royce and Sykes (63).

The test solutions were processed as described under 2.4.3.

2.5. 2.b. Testing for sterility of solutions.

The Millipore membrane filter type HA (pore size 0.45μ), with Swinny hypodermic adaptor, was used in this investigation. The unit, with the membrane in position, may be sterilised by autoclaving at 115° for thirty minutes without any adverse effect on the filter (64).



In each test, the solution was withdrawn from the container into a sterile syringe, onto which was then placed the sterile filtration unit. The solution was forced through the filter and the unit removed from the syringe. The membrane was transferred from the unit to a sterile agar plate and was incubated at 30°-32° for seven days (33)(46).

At the end of the incubation period, the plates were examined for bacterial growth.

2.5. 3. Results.

No quantitative estimation of the viable bacteria was attempted. What was required was a knowledge of whether the processed solution was sterile or not. However, a qualitative determination was performed, from which an evaluation of the relative resistance of the test organisms to the antibacterial agent and to the process was obtained.

2.5. 3.a. The following symbols were used in all tabulated bacteriological tests performed in this investigation.

1. The minus sign indicated that no bacterial growth was observed on the plate, signifying that the test solution was sterile.

2. The plus sign indicated that bacterial growth was observed on the plate, signifying that the test solution was not sterile.

3. +M. All the test organisms were identified on the plate by the taxonomic growth characteristics of the colonies.

a. S. aureus.
b. B. subtilis.
c. B. cereus.
d. Ps. fluorescens.
e. P. vulgaris.
f. E. coli.

4. +S. The following four organisms only were identified on the plate.

a. S. aureus.
b. B. subtilis.
c. B. cereus.
d. P. vulgaris.

5. +B. The following organism only was identified on the plate.

a. B. cereus.

The identity of this organism was subsequently confirmed by the South African Institute for Medical Research.

2.5. 3.b. Results for unbuffered solutions.

1. At pH 3·5. TABLE 1.
2. At pH 5·6. TABLE 2.
3. At pH 7·4. TABLE 3.

2.5. 3.c. Results for buffered solutions.

1. At pH 3·5. TABLE 4.
2. At pH 5·6. TABLE 5.
3. At pH 7·4. TABLE 6.

2.6. Discussion of bacteriological results.

2.6. 1. Tabulated results.

The efficiency of 0.5% w/v chlorbutol as a bactericide, in the process under investigation, was dependent on the pH of the vehicle to some extent, but was not much affected by the buffering of solutions. As the pH value of the test solutions rose from 3·5 to 7·4, so the antibacterial potency of chlorbutol decreased.

As well as being due to the antibacterial effect of the pH itself, this could also have been due to the decomposition of chlorbutol in alkaline medium (39)(35)(36)(65)(40)(41). Confirmatory evidence was supplied by the fact that the buffered solution at pH 7·4 was less effective than the unbuffered solution. In the unbuffered solution, a decrease in pH resulting from the decomposition of chlorbutol was thought to excercise a self-limiting effect on further degradation (42). As the pH of the buffered solution would not be reduced to such an extent, such self-limiting action was not exerted, and the decomposition was thought to be greater, which would result in decreased efficiency.

The results of Erne and Söderlundh (47) and of Murphy et al (48) were similar to these results. However, it must be emphasised that heating for at least forty-five minutes at 98°-100° was required for a 0.5% w/v solution of chlorbutol, at pH 7·4, to exhibit adequate bactericidal effect. At pH 5·6, thirty minutes heating was necessary, whilst at pH 3·5, fifteen minutes heating appeared to kill the test organisms.

2.6. 2. Relative resistance of bacteria.

B. cereus was the most highly resistant organism to chlorbutol under the conditions of the determination, probably due to

its spore-forming properties. Slightly less resistant were S. aureus, B. subtilis and P. vulgaris, though not necessarily in that order. Ps. fluorescens and E. coli appeared to be more susceptible to the compound and to a lowering of pH than were the other test organisms.

It was concluded that a 0.5% w/v chlorbutol solution was not sufficiently bactericidal, in alkaline medium, to be used as a bactericide in the process "Heating with a Bactericide".

2.7. Abstract.

A 0.5% chlorbutol solution was tested for its efficiency as a bactericide, in various containers, in the process "Heating with a Bactericide" against S. aureus, B. subtilis, B. cereus, Ps. fluorescens, P. vulgaris and E. coli. The effect of buffered and unbuffered vehicles of pH 3·5, 5·6 and 7·4 respectively on the efficiency of the compound was investigated by the application of a method of sterility testing incorporating bacterial filtration using membrane filters.

CHAPTER III.

The investigation of the degradation of chlorbutol in pharmaceutical processes.

3.1. Introduction.

The degradation of chlorbutol by an hydrolysis process is well documented (39)(35)(36)(40)(41). Taub and Luckey (35) measured the extent of degradation after processing and storage by determining the pH decrease of the solution, and the change in chloride ion concentration.

Gershenfeld (36) also measured pH changes before and after processing but the effect of environmental conditions (e.g. nature of containers and alkalinity of glassware) on these pH values was not discussed.

Other investigations have been carried out (40)(66)(67), but have either been mainly concerned with the degradation on storage or with the mechanism of the degradation. No investigation of the degradation under strictly pharmaceutical conditions, relating chemical and bacteriological results, had yet been performed.

The degradation of chlorbutol in pharmaceutical processes was therefore investigated.

3.2. The selection and adaptation of a suitable assay procedure for residual chlorbutol in solution.

3.2. 1. Selection of a suitable assay procedure.

The primary requirement of the investigation of the degradation of chlorbutol was the selection of an assay procedure applicable to the sensitive determination of residual chlorbutol in ophthalmic and parenteral solutions which had been subjected to specific pharmaceutical processes.

The official method (33)(68) involves further degradation of the compound so was not considered.

The pH decrease which occurs on the degradation of chlorbutol has been used as a measure of that degradation (35)(36),

as have the liberated chlorides (35)(69)(70), but these methods were not further considered because of factors of sensitivity and specificity.

Amperometric (71) and polarographic (72) methods have also been applied to the determination of small quantities of chlorbutol in solution, but a spectrophotometric method, utilising a colour reaction specific for chlorbutol, was thought to most nearly meet the requirements of this investigation.

Two colorimetric assays for chlorbutol, based on the following reactions, have been described.

a. Fujiwara-alkali-pyridine reaction, resulting in a pink complex (73).

b. Ferric ion reaction with the hydroxamic derivative of chlorbutol, resulting in a reddish-brown complex (74).

The latter method, originally described by Rehm and Mader (74), was utilised by Lachman et al (75) and by Patwa and Huyck (67) in their respective investigations.

This method was selected for testing for its suitability in this project.

NOTE: A further potentiometric determination of chlorbutol was described after this investigation was commenced (76).

3.2. 2. Preliminary testing of the selected procedure.

The experimental procedure of Rehm and Mader (74) was found to be unsuitable for the conditions prevailing in this determination and was modified accordingly.

3.2. 2.a. Absorbance spectrum of the complex.

3.2. 2.a. 1. Preparation of reagent solutions.

Reagent grade chemicals were used throughout the investigation. The following reagent solutions were prepared.

a. Standard chlorbutol solution containing 1 mg./ml. in deionised water. This solution was refrigerated at 4°

whilst not in use to prevent degradation of the chlorbutol and was freshly prepared daily.

b. Hydroxylamine hydrochloride reagent solution containing molar hydroxylamine hydrochloride in deionised water (69·5G./litre). This solution was refrigerated at 4° when not in use to prevent its deterioration.

c. Sodium hydroxide reagent solution containing 3·5 molar sodium hydroxide in deionised water (140G./litre).

d. Hydrochloric acid reagent solution containing 3·5 molar hydrochloric acid in deionised water (345 ml. of reagent grade hydrochloric acid/litre).

e. Ferric chloride reagent solution containing 0·3 molar ferric chloride in 0.1 molar hydrochloric acid (48·6 G. of ferric chloride plus 29 ml. of 3.5 molar hydrochloric acid reagent solution per litre).

3.2. 2.a. 2. Preparation of the coloured complex.

a. 5 ml. of the standard chlorbutol solution was diluted to 10 ml. (so as to contain 0.5 mg./ml.) and transferred to a 25 ml. A Grade volumetric flask.

b. 2 ml. of hydroxylamine hydrochloride reagent solution was added, followed by 2 ml. of sodium hydroxide reagent solution. The reaction mixture was left to stand at room temperature for twenty minutes.

c. 2 ml. of hydrochloric acid reagent solution was added, followed by 2 ml. of ferric chloride reagent solution.

d. After five minutes the reaction mixture was shaken and made up to 25 ml.

NOTE: All reagents were added from 50 ml. A Grade burettes as experience showed that any error in the addition of reagents led to variations in the absorbance of the solution.

3.2. 2.a. 3. Absorbance spectrum.

The absorbance spectrum of the complex was recorded on the recorder of the Beckman DB Spectrophotometer against a reagent blank, in 1 cm. cells. (GRAPH 1.).

3.2. 2.a. 4. Discussion of the absorbance spectrum.

The ferric-hydroxamic complex of chlorbutol exhibited a wide absorbance peak in the region 500-540 millimicrons ($m\mu$). In this wavelength range, the absorbance of the reagent blank was low, being least at 500 $m\mu$.

This wavelength was therefore selected for the absorbance/concentration calibration of the ferric-hydroxamic complex of chlorbutol.

3.2. 2.b. Conformation with Beer's Law.

Before the ferric-hydroxamic derivative of chlorbutol could be used in the quantitative determination its ability to conform to Beer's Law (113) was investigated.

3.2. 2.b. 1. Procedure.

a. 6, 5, 4, 3, 2 and 1 ml. quantities of standard chlorbutol solution were diluted to 10 ml. (to give solutions containing 0·6, 0·5, 0·4, 0·3, 0·2 and 0·1 mg./ml. respectively) and transferred to 25 ml. A Grade volumetric flasks.

b. The procedure described under "Preparation of the coloured complex" (3.2.2.a.2.) was performed on these solutions and their absorbance at 500$m\mu$ was determined against a reagent blank, in 1 cm. cells, at a fixed time interval of twenty minutes after the addition of the ferric chloride reagent solution.

c. The results were tabulated (TABLE 7) and graphically represented (GRAPH 2).

3.2. 2.b. 2. Discussion of results.

The complex conformed to Beer's Law, in that its absorbance increased linearly with concentration. This only occurred in the (initial) concentration range 0·2-0·5 mg./ml. 0·1 and 0·6 mg./ml. concentrations respectively were therefore discarded as standard concentrations for the calibration curve.

3.2. 2.c. Fading of the complex with time.

As the complex had been visually observed to fade with the passage of time after reaction, a more detailed examination of this phenomenon was performed.

3.2. 2.c. 1. Procedure.

a. 5 ml. of the standard chlorbutol solution was diluted to 10 ml. (to give a concentration of 0.5 mg./ml.) and transferred to a 25 ml. A Grade volumetric flask.

b. The procedure described under "Preparation of the coloured complex" (3.2.2.a.2.) was performed on this solution.

c. The absorbance of the solution at 500 mμ was determined, in 1 cm. cells against a reagent blank, at five minute intervals from five to sixty minutes after the addition of ferric chloride reagent solution.

d. The results were tabulated (TABLE 8) and graphically represented (GRAPH 3).

3.2. 2.c. 2. Discussion of results.

The complex faded rapidly for the first fifteen minutes after reaction, after which the rate of fading decreased.

Twenty minutes after reaction time was selected for the determination of the absorbance of the chlorbutol solutions because:

a. At times less than this period, a very slight variation would have resulted in relatively large errors.

b. Practical considerations precluded any possibility of the determination being made within seventeen minutes of reaction time.

The selected time interval of twenty minutes was subsequently experimentally shown to be suitable.

3.2. 3. Assay of "test" quantities of chlorbutol in solution using the adapted procedure.

The usual concentration in which chlorbutol is used (0·5% w/v) has been shown to possess a degree of bactericidal

activity when used in conjunction with heat processing. (Chapter II).

Such a solution contains 0·5 G. chlorbutol per 100 ml., which is equivalent to 5 mg. per ml. As this concentration was ten times that found to be suitable for the assay procedure, it was reduced to the same concentration range by the application of a dilution factor of ten. To test the accuracy of the method when applied in this way to bacteriologically active solutions of chlorbutol the following procedure was carried out.

3.2. 3.a. Assay procedure on bacteriologically active solutions of chlorbutol.

1. Two test solutions containing 0·25% and 0·35% chlorbutol were prepared as Test Solutions X and Y respectively.

2. 1 ml. of each of the test solutions was diluted to 10 ml. and transferred to 25 ml. A Grade volumetric flasks, as were 5, 4, 3 and 2 ml. quantities of standard chlorbutol solution, to obtain the calibration curve.

3. The procedure "Preparation of the coloured complex" (3.2.2.a.2.) was carried out on both the "unknown" and standard solutions.

4. The absorbances of the solutions at 500 mμ were determined against a reagent blank, in 1 cm. cells, twenty minutes after the addition of the ferric chloride reagent solution.

5. The results were tabulated (TABLE 9) and graphically represented (GRAPH 4).

3.2. 3.b. Discussion of results.

The procedure provided an accurate method for the determination of small quantities of chlorbutol in aqueous solution.

Comparing Graph 4 with Graph 2, the calibration curve in Graph 2 was observed to be steeper than that in Graph 4. It was therefore concluded that, although the absorbance of the complex increased linearly with concentration, the magnitude

of the absorbance in any one determination, which was affected by the experimental conditions prevailing for that determination, was not related to the magnitude of absorbance exhibited in any other determination.

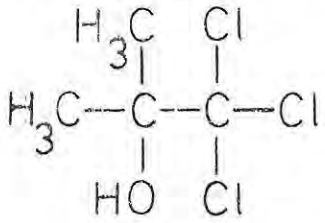
Therefore, in each and every determination, a calibration curve was included as the basis for correct quantitative results. The calculation method of Rehm and Mader (74) did not give sufficiently accurate results for this investigation.

3.2. 4. Interferences with the procedure.

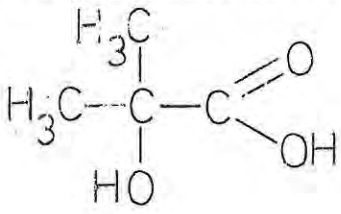
The majority of substances which might have been expected to interfere in this assay have been discussed by Rehm and Mader (74), but as most of them were not likely to be found in ophthalmic or parenteral solutions, they were not considered to be relevant to this investigation.

One substance which might have formed a coloured complex similar to that of chlorbutol was α-hydroxyisobutyric acid, which has been suggested as a degradation product of chlorbutol (42).

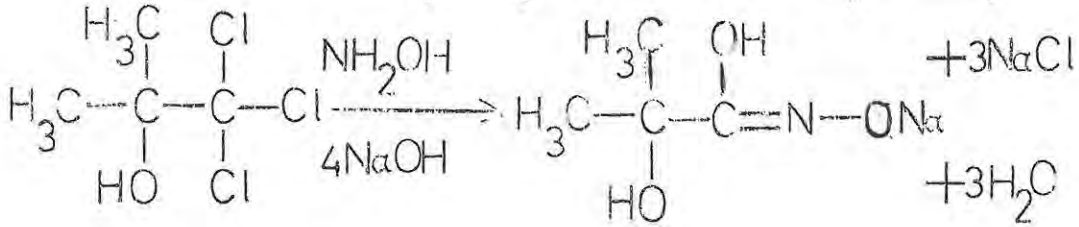
CHLORBUTOL



α-HYDROXYISOBUTYRIC ACID.



It appeared possible that a reaction analagous to that between chlorbutol and hydroxylamine (hydrochloride) in alkaline solution might occur with this substance. The reaction between chlorbutol and hydroxylamine can be expressed:



However, investigation of the absorbance spectrum of this compound, both alone, and in combination with chlorbutol indicated that no interference occurred in the vicinity of 500 mμ, even in as high a concentration as 5 mg./ml. (i.e. ten times as high as that of the chlorbutol) (GRAPH 5).

The absorbances of the α-hydroxyisobutyric acid containing solutions were identical to those given by the standard chlorbutol solution and the reagent blank at 500 mμ.

Hence, the only substance which appeared likely to interfere in the determination of chlorbutol, by the spectrophotometric assay used in this investigation, was shown to have no effect on the absorbance of the chlorbutol complex at 500 mμ.

This assay method was therefore adopted for the quantitative determination of residual chlorbutol in solution.

3.3. The degradation of chlorbutol in solution during the pharmaceutical processes of heat sterilisation.

3.3. 1. Introduction.

When chlorbutol is incorporated in ophthalmic and parenteral solutions subjected to heat sterilisation methods, the compound is exposed to a number of variable factors.

a. pH and buffering effects. This has been discussed (2.3.3).

b. Temperature effects. In the sterilisation of ophthalmic and parenteral solutions by heat processes, the applied temperature may vary from 100° (in "Heating with a Bactericide") to 122° (in Autoclaving)(46).

c. Effects due to the nature of the container. This has been discussed (2.3.1).

These factors may be expected to affect both the rate and the magnitude of the degradation, which was followed, in this investigation, by the application of a spectrophotometric assay method for residual chlorbutol in solution after processing.

3.3. 2. The degradation of chlorbutol during the steaming of its solutions.

3.3. 2.a. Selection of containers.

In the process "Heating with a Bactericide", steaming is used in conjunction with a bactericidal compound to effect the sterility of the solution. The process is therefore applied to both single-dose and multi-dose containers of thermolabile solutions. The containers and closures used have been described (2.3.1.).

3.3. 2.b. Preparation of the test solutions.

The test solutions were prepared as described (2.4.2.). As a chemical evaluation was being performed, inoculation with the mixed bacterial suspension was NOT carried out. The containers were sealed in the prescribed manner (2.3.1.c.).

3.3. 2.c. Processing of test solutions.

The procedure described (2.4.3.) was carried out on the test solutions.

3.3. 2.d. Results of the spectrophotometric assay for residual chlorbutol.

1. Introduction.

The assay procedure described (3.2.3.a.1-4) was carried out on the test solutions. The respective concentrations of chlorbutol in the test solutions were obtained by the graphical method, incorporating a calibration curve, as illustrated by the example . (TABLE 10. GRAPH 6).

By the incorporation of an unprocessed standard solution of 0.5% chlorbutol, the percentage degradation of the compound in the respective solutions was calculated. The results were tabulated and graphically represented.

2. Results for unbuffered solutions.

a. At pH 3·5 TABLE 11. GRAPH 7.

b. At pH 5·6 TABLE 12. GRAPH 8.

c. At pH 7·4 TABLE 13. GRAPH 9.

3. Results for buffered solutions.

a. At pH 3·5 TABLE 14. GRAPH 10.

b. At pH 5·6 TABLE 15. GRAPH 11.

c. At pH 7·4 TABLE 16. GRAPH 12.

3.3. 2.e. Discussion of results, with special reference to the bacteriological results of the investigation of chlorbutol in the process "Heating with a Bactericide".

The results of the determination of the degradation of chlorbutol when its solutions were subjected to "steaming" at 98°-100° were summarised (i.e. data from TABLES 11-16 and GRAPHS 7-12).

1. The rate of degradation increased with increasing pH of the solution. The degradation was highest at pH 7·4, when after ninety minutes at 98°-100°, it was of the order of 12·5% of the original concentration of chlorbutol. At this pH the antibacterial potency of chlorbutol was lowest (TABLES 3 and 6).

2. The rate of degradation was dependent on the type of container, and was higher in amber than in white glass containers. This effect was probably due mainly to the nature of the glass itself as well as to the effective volume of the container.

NOTE: It has been demonstrated that the total extracted matter and the pH of test solutions varied in different containers when distilled water was sterilised and stored in ampoules (77). As alkali was the first component extracted by such conditions (78) (79) and as Anderson (66) has shown that the degradation of chlorbutol is an hydroxyl ion catalysed reaction (42), the differences in the degradation rates in different containers were most probably due to differences in the alkalinity of the glass used in the production of the containers. That such differences do occur was confirmed previously. (2.3.1.d.).

3. The effect of the buffering of solutions on the rates of degradation of chlorbutol in unbuffered vehicles appeared to be dependent on the pH of the solution.

a. At pH 3·5, the buffered solutions showed slightly decreased degradation rates, and differences due to containers were slightly reduced.

b. At pH 5·6, the buffered solutions showed similar rates of degradation to those in unbuffered solutions. The differences due to containers were slightly reduced.

c. At pH 7·4, the buffered solutions showed increased rates of degradation compared to the unbuffered solutions. That this was due to the self-limiting effect on the degradation by unbuffered solutions, in which the pH could fall and thus retard the degradation of chlorbutol, seemed certain. There appeared to be only a very slight effect on the differences due to the containers.

That the results of the bacteriological tests described in Chapter II (TABLES 1-6) were intimately related to the physical breakdown of chlorbutol in ophthalmic and parenteral solutions was self-evident, as was the actual relationship. The hypotheses submitted as explanations for the varying antibacterial potency of chlorbutol in solution in the process "Heating with a Bactericide" were confirmed by the spectrophotometric assay results.

3.3. 3. The degradation of chlorbutol during the autoclaving of its solutions.

3.3. 3.a. Introduction.

When a small volume of solution is autoclaved at 115° for thirty minutes, or at 122° for twenty minutes, that solution is sterile (46). The process is applied to thermostable compounds, and the incorporation of chlorbutol in multidose containers of solutions of such compounds rests entirely on its preservative properties.

In the same way that the bactericidal properties of chlorbutol were adversely affected by its physical degradation, it had been suggested that its preservative properties were also affected (36)(17)(49).

Considerations similar to those which applied to the investigation of the relationship between the bactericidal potency of chlorbutol and its physical degradation applied to the investigation of its preservative properties. (2·1). No complete evaluation of the preservative properties of the compound, as related to its physical breakdown, had yet been performed.

As preservative properties are only required in multi-dose containers, in which the possibility of contamination of the solution during or between successive administrations is high, the investigation of the degradation of chlorbutol during autoclaving was confined to one multi-dose container for parenteral solutions, and one for ophthalmic solutions.

These were

1. 15 ml. Clinbritic bottle.
2. 15 ml. Eye Drop bottle.

Two temperatures, 115° and 122°, are commonly used for the sterilisation of ophthalmic and parenteral solutions by autoclaving (46), and the degradation of chlorbutol at both these temperatures was investigated.

3.3. 3.b. The degradation of chlorbutol during autoclaving of its solutions at 115°.

1. Preparation of test solutions.

Sufficient 0·5% w/v chlorbutol solutions with pH values of 3·5, 5·6 and 7.4 respectively were prepared. They were packed in the prescribed containers (3.3.3.a. 1 and 2) which were sealed in the prescribed manner. (2.3.1.).

2. Processing of test solutions.

To obtain a complete evaluation of the degradation process, sufficient of the test solutions was autoclaved at 115° for periods of fifteen, thirty, forty-five and sixty minutes respectively. The assay procedure (3.2.3.a. 1-4) was performed on the test solutions. The respective concentrations of residual chlorbutol in the test solutions were obtained using a calibration curve (as in 3.3.2.d.1.) and the percentage

degradation for specific autoclaving times was calculated.

3. Results of the spectrophotometric assay.

The results were tabulated and graphically represented.

a. Results for unbuffered solutions.

1. At pH 3·5	TABLE 17.	GRAPH 13.
2. At pH 5·6	TABLE 17.	GRAPH 14.
3. At pH 7·4	TABLE 17.	GRAPH 15.

b. Results for buffered solutions.

1. At pH 3·5	TABLE 17.	GRAPH 16.
2. At pH 5·6	TABLE 17.	GRAPH 17.
3. At pH 7·4	TABLE 17.	GRAPH 18.

4. Discussion of results.

Results similar to those obtained when the test solutions were subjected to steaming were demonstrated by the test solutions autoclaved at 115°. These results were discussed previously. (3.3.2.e.). The degradation rates for autoclaved solutions were considerably higher, almost certainly due to the increased temperature, which was the only altered factor. Maximum degradation was again shown in buffered solutions of pH 7·4. Extrapolation of the plot indicated as much as 20% degradation, of the original chlorbutol concentration, after a theoretical ninety minutes autoclaving at 115°. This was a large increase over the corresponding degradation in solutions steamed at 98°-100°, which was approximately 12·5%.

3.3. 3.c. The degradation of chlorbutol during the autoclaving of its solutions at 122°.

1. Introduction.

The test solutions were prepared, packed, processed and assayed in identical manner to those used in the determination at 115° (3.3.3.b.), except that the autoclaving temperature was increased to 122°.

2. Results of the spectrophotometric assay.

The results were tabulated and graphically represented.

a. Results for unbuffered solutions.

1. At pH 3.5 TABLE 18. GRAPH 19.
2. At pH 5.6 TABLE 18. GRAPH 20.
3. At pH 7.4 TABLE 18. GRAPH 21.

b. Results for buffered solutions.

1. At pH 3.5 TABLE 18. GRAPH 22.
2. At pH 5.6 TABLE 18. GRAPH 23.
3. At pH 7.4 TABLE 18. GRAPH 24.

3. Discussion of results.

The effects of pH and of buffering observed in this determination were similar to those observed in the two previous determinations.

The highest percentage degradation was again found in buffered solutions of pH 7·4. Extrapolation of the plot indicated a probable 29% degradation of the chlorbutol in solution after autoclaving at 122° for a theoretical ninety minutes.

These results confirmed the direct relationship between the degradation of chlorbutol and temperature, which had been intimated by the results of the two previous determinations. However, in this determination, a greater increase in degradation resulted from a much smaller rise in temperature. Degradation rose by approximately 10% over a 7° rise in temperature. In the determination at 115°, the corresponding increase in degradation was approximately 7·5% for a temperature rise of 15°.

This suggested that, at temperatures higher than approximately 120°, some breakdown step resulted in a marked increase in the rate of degradation of chlorbutol.

The results for the individual containers indicated that, in every case, the degradation rates in solutions in 15 ml. Clinbritic bottles were higher than those in solutions in 15 ml. eyedrop bottles. Hence, the glass of the 15 ml. Clinbritic bottles appeared to posses a higher alkaline content than the glass of the 15 ml. eyedrop bottles. These differences were reduced by the buffering of the solutions, which resulted in very similar

rates in both containers.

3.3. 4. Summary of results of degradation determinations.

The results of these determinations, which were previously discussed (3.3.2.e.; 3.3.3.b.4; 3.3.3.c.3.) were summarised.

a. The rate of degradation of chlorbutol increased with increasing pH of the solution.

b. The rate of degradation of chlorbutol varied with the nature of the container.

c. The rate of degradation of chlorbutol was affected by the buffering of its solutions. At low pH the effects were slight, but at higher pH (7-8) the degradation rate was greatly increased in buffered solutions.

d. The rate of degradation of chlorbutol increased in a geometric rather than arithmetical ratio with increasing temperature.

These four factors directly affected the rate of degradation of the compound in ophthalmic and parenteral solutions sterilised by heating methods.

3.4. The effects of the degradation of chlorbutol during heat processing on its preservative properties in ophthalmic and parenteral solutions.

3.4. 1. Introduction.

Although chlorbutol has been widely used as a preservative for ophthalmic and parenteral solutions since 1950, (Chapter I), no comprehensive bacteriological examination of the compound with results which would justify such widespread use appeared in the literature.

In pharmaceutics, "preservation" of solutions has come to mean the maintenance of a bacterial status quo, with no increase or decrease in the number of micro-organisms introduced to the solutions, at one time or another. Due to the fact that "preservatives" in solutions are normally diluted out of effective concentration on administration, and because the counter-attack by the body's defences is not always adequate,

such an effect is not enough. What is required is that such solutions should be self-sterilising due to their probable high level of casual contamination.

Both Gershenfeld (36) and Gladhart et al (17) have shown that the antibacterial potency of chlorbutol decreased after heat processing. The determination of the extent of the degradation of chlorbutol carried out in this investigation (3.1; 3.2; 3.3) enabled a quantitative determination of the effect of the degradation on the self-sterilising properties of the compound to be made.

That the products of the degradation might, in themselves, possess antibacterial properties has apparently not been considered, but a quantitative determination as described, could take such effects into account.

3.4. 2. Bacteriological testing of processed solutions of chlorbutol for self-sterilising properties.

3.4. 2.a. Procedure.

The bacteriological testing of the efficiency of chlorbutol as a bactericide has been described. (Chapter II). The same principles and methods formed the basis of this evaluation, in which an attempt was made to ascertain the time interval necessary for the inoculated test solutions to regain their original sterility.

The methods used in the preparation, packing and processing of the test solutions of chlorbutol have been described. (3.1.).

After processing, the test solutions were inoculated with the bacterial suspension (2.5.2.a.) and, at fixed time intervals after inoculation, the contents of the respective containers were drawn up into a sterile syringe and forced through a sterile membrane filter bacterial filtration unit. The membrane filters were transferred to sterile agar plates, which were incubated at 30°-32° for seven days (33).

After the incubation period the agar plates were examined for bacterial growth.

3.4. 2.b. Results of the bacteriological testing of the processed solutions for self-sterilising properties.

1. Results for steamed solutions.

a. For unbuffered solutions.

1. After 15 minutes steaming. TABLE 19.
2. After 30 minutes steaming. TABLE 20.
3. After 45 minutes steaming. TABLE 21.

b. For buffered solutions.

1. After 15 minutes steaming. TABLE 22.
2. After 30 minutes steaming. TABLE 23.
3. After 45 minutes steaming. TABLE 24.

c. Discussion of results for steamed solutions.

As in the determination of the bactericidal efficiency of chlorbutol, the efficiency of the compound as a self-sterilising agent for solutions decreased with increasing pH and time of exposure to steaming.

Unbuffered solutions did not show marked differences between processed and unprocessed samples, except at pH 7·4, when the self-sterilising properties of the test solutions decreased quite rapidly with increased steaming times.

At the two lower pH values, the buffering of the test solutions did not have any adverse effect, but at pH 7·4 the results for processed solutions were once more unfavourable, compared to both unprocessed and unbuffered processed solutions.

Comparison of the results of the spectrophotometric assays with those of the bacteriological tests indicated that a decrease in the self-sterilising properties of processed solutions of chlorbutol occurred when the degradation reached approximately 3% of the original concentration. That such a relatively low percentage degradation should be responsible for the decrease of these properties indicated that the original concentration of chlorbutol used for the self-sterilisation of ophthalmic and parenteral solutions was itself critical.

The results of the spectrophotometric assays corresponded so closely with the bacteriological results that it

appeared that the degradation products, per se, did not exert any effect on the antibacterial properties of the test solutions.

The results also indicated that the widely used and supposedly effective concentration of 0·5% chlorbutol was not entirely satisfactory as a self-sterilising agent for ophthalmic and parenteral solutions. Forty-eight hours after inoculation the test solutions had not regained their original sterility. Some justification for this adverse result might lie in the fact that the offending organism is known to be highly resistant.*

NOTE: Determinations of the effect of the degradation of chlorbutol in autoclaved solutions on their self-sterilising properties were not performed as the percentage degradation observed to occur under conditions of steaming had adequately demonstrated the relationship between degradation and the reduction in antibacterial potency.

3.5. Discussion.

The results of these determinations did not, in some instances, agree with those obtained by other workers.

3.5. 1. Spectrophotometric assay results.

Previous workers, using less accurate and less specific assay methods (e.g. pH determinations) have obtained results both higher (35)(76) and lower (40) than these results, but the same tendencies, due to pH and buffering, were observed in all investigations.

Differences due to methods of calculation were also observed, e.g. Patwa and Huyck (67), although using the same complex formation, did not use a calibration curve for the quantitative examination of their results. In this investigation, a calibration curve was found to be essential for any degree of accuracy to be obtained.

* B. cereus. (2.2.3.). This organism is ubiquitous in nature (80) and could therefore be a common contaminant of ophthalmic and parenteral solutions.

The determination of the percentage degradation of chlorbutol, at stages throughout the processing of solutions under different conditions, which was carried out in this investigation, enabled a complete quantitative evaluation of the degradative processes to be obtained.

3.5. 2. Bacteriological results.

Comparison of these results with those obtained by other workers (36)(17)(18)(81)(53) indicated that chlorbutol was slowly bactericidal against certain bacterial species. This investigation showed that 0·5% chlorbutol was not entirely satisfactory as a self-sterilising agent for ophthalmic and parenteral solutions, before or after heat processing, particularly against the highly resistant organisms of the B.subtilis group.

3.6. Abstract.

A spectrophotometric assay process was investigated, adapted and utilised in the quantitative evaluation of the degradation of chlorbutol when subjected to heat processing under various conditions, in ophthalmic and parenteral solutions. Parallel bacteriological tests enabled a relationship to be established between the degradation of the compound and the reduction in antibacterial potency.

CHAPTER IV.

The investigation of the effect of the silicone coating of containers on the degradation of chlorbutol.

4.1. Introduction and theoretical considerations.

4.1.1. The relationship between the degradation of chlorbutol and the glass of the container.

In this investigation, chlorbutol has been observed to be reasonably effective as a self-sterilising agent against all except the most resistant micro-organisms. That the effective concentration is critical has also been observed.

It was considered that if some method of stabilisation of the compound could be developed, the antibacterial properties might be improved, or at least would not decrease to the extent observed. A method of eliminating or reducing the degradation was therefore required.

As the nature of the glass was considered to be partly responsible, this was the first factor investigated with a view to decreasing the degradation, which had been shown to be higher in amber than in white glass containers. Theoretically, on these results, amber glass should have a higher alkali content than white glass.

4.1. 2. The structure of glass.

Glass is formed by fusing silica, limestone, and soda-ash at 1300° (82). The proportions of the ingredients naturally influence the final constitution of the glass. If a high concentration of soda-ash is used the glass would have a higher alkali content and would be more susceptible to atmospheric and chemical attack. On the other hand, glass with a high silica content would be considerably less alkaline in nature and would possess a high degree of resistance to degradation.

The reasons for these effects lie in the lattice struc-

ture of glass, which mainly consists, in stable silica glasses, of silicon and oxygen atoms covalently bonded.

The incorporation of alkali metal and other metal ions results in an increase in electrovalent bonding, and this bonding, being weaker than the former type, is responsible for the low resistance to attack of glass with a high alkali content (82). The resultant breakdown of the glass liberates metal ions into the solution, where the formation of hydroxides would result in the breakdown of alkali-sensitive compounds. This could occur in the case of chlorbutol.

In alkaline solutions this effect is additive, as decomposition of glass is more rapid in such solutions. The resultant increase in alkalinity of the solution then leads to further breakdown of the glass (82).

The presence of a far higher percentage of alkali and other metal ions in amber glass, than in white (82), confirmed the theoretical explanation of its effect.

Hence, chlorbutol in solution is subjected to two main degradative factors:

a. The pH of the solution.

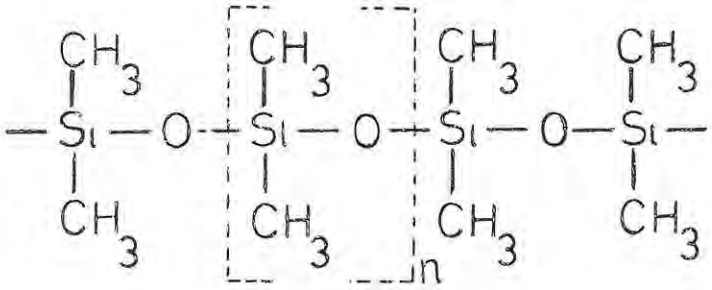
b. The alkalinity of the glass of the container.

As the pH of the solution varies considerably with the nature of the medicament and other factors, methods of reducing the effect of the alkalinity of the glass were investigated.

Glass exerts its influence on compounds in solution because its components enter the solution. Therefore, some means of preventing solution and container from coming into contact would eliminate these effects. The use of a water-repellent silicone film on the glass surface was investigated.

4.1. 3. <u>The structure and properties of silicone fluids</u>.

The polymethyl siloxanes, which are used in the formation of water-repellent films, can be represented by the structural formula:



(83)(84)(46)(85).

Water-repellent coatings on glass are formed by a rapid reaction between the silicone and glass because of their similarities of structure and bonding (86)(46). It is thought that the organic groups project out from the surface forming a very thin film which alters the contact angle of the surface for water, and hence renders it water-repellent (86).

The properties of silicones are by now well established (83)(84)(32)(4). Those of value in this investigation were its lack of toxicity by any route, its solubility in benzene and the lighter hydro-carbons, and its insolubility in both natural and synthetic rubbers, causing them neither to swell nor to soften (86).

4.2. Silicone coating the containers.

4.2. 1. Introduction.

The methods described for the silicone coating of glass containers are all based on either the solvent or volatilisation methods. In the solvent method a solution of the silicone, varying in concentration from 2% to 4%, in one of the volatile lighter hydro-carbons, is used as a dip-solution for the containers, which are then drained and air-dried. Subsequent baking at fairly high temperatures stabilises the film on the surface (87)(88)(84)(4)(46). Maximum resistance to wetting has been obtained by baking at 300° for two hours (83).

The method used in this investigation was basically that

described in "The Extra Pharmacopoeia" (84). "I.C.I. Silicone Fluid F111/100" was used in place of the recommended "Midlands Silicone 200", which was not available.

4.2. 2. Procedure.

4.2. 2.a. Preparation of silicone solutions.

Of the volatile solvents in which silicone fluids are soluble, carbon tetrachloride was used (84). A 3% solution of "I.C.I. Silicone Fluid F111/100" in this solvent was prepared.

4.2. 2.b. Treatment of the containers.

The containers to be coated were thoroughly washed and dried. Each container was dipped into the 3% silicone solution for 30 seconds. The dipped containers were allowed to drain and air-dry.

4.2. 2.c. 'Baking' of the containers.

The dried containers were oven-baked at 300° for two hours to fix the silicone film to the glass surface (83). After cooling, the coated containers were stored in a cool, dry, dustfree atmosphere.

4.3. The degradation of chlorbutol in solutions in silicone-coated containers.

4.3. 1. Introduction.

As the highest rate of degradation of chlorbutol had been observed when test solutions of pH 7·4 were autoclaved at 122°, it was decided that a comparison of the rates in uncoated and coated containers, under these conditions, would best enable an evaluation of the effect of the silicone coating of containers on the degradation of chlorbutol in solution to be made.

If the degradation rate was substantially decreased under these adverse conditions, it could be expected to be proportionately further decreased under the milder conditions which

produced lower rates of degradation (i.e. in solutions of pH 5·6 and 3·5, during autoclaving at 115° and steaming at 100°).

4.3. 2. Procedure.

Buffered and unbuffered 0·5% w/v chlorbutol solutions of pH 7·4 were prepared and packed in the prescribed manner (2.3.1.c.2 and 3.), in silicone-coated containers, all of which complied with the requirements of the B.P. test for limits of alkalinity after coating.

Sufficient containers of each type were autoclaved at 122° for periods of fifteen, thirty, forty-five and sixty minutes. The processed solutions were assayed (as in 3.3.2.d.1.). The percentage degradations for specific autoclaving times were calculated.

4.3. 3. Results of the assay procedure.

The results were tabulated and graphically represented against the degradation plots obtained from test solutions in uncoated containers, subjected to the identical conditions, to enable a direct visual comparison of the rates to be made.

4.3. 3.a. Results for unbuffered solutions.

TABLE 25. GRAPH 25.

4.3. 3.b. Results for buffered solutions.

TABLE 25. GRAPH 26.

4.3. 4. Discussion of results.

In both buffered and unbuffered solutions the effect of using silicone-coated containers was to considerably reduce the rates of degradation of chlorbutol. Extrapolation of the rate plots to a theoretical ninety minutes autoclaving at 122°, for unbuffered solutions, showed a decrease in degradation from approximately 19% in uncoated containers to approximately 8% in silicone-coated containers. The corresponding reduction in degradation for buffered solutions was from

approximately 29% to approximately 9%. Hence, the reduction in the magnitude of the degradation brought about by the use of silicone-coated containers was much greater in buffered than in unbuffered solutions.

It was suggested that this was so because a buffered solution of chlorbutol, of pH 7·4, is not affected to the same extent as an unbuffered solution by the pH-reducing effect when chlorbutol decomposes (35)(36)(18). Hence, it would be more corrosive against the glass surface for a longer period, the result of which would be to increase the alkalinity of the solution, due to the breakdown of the glass. (4.1.2.).

As the glass was silicone-coated, this breakdown did not occur. The reduction in the degradation of chlorbutol effected by the silicone-coating of the glass surface was therefore greater in buffered than in unbuffered solutions.

Another effect of the silicone coating of the containers for chlorbutol solutions was to practically eliminate the differences in degradation rates exhibited by the two containers. This provided further evidence in support of the hypothesis that, under pharmaceutical conditions, a high proportion of the degradation of chlorbutol was caused by the nature of the container. Therefore, the degradation rates in silicone-coated containers corresponded most closely to the actual degradation rate of chlorbutol with temperature and time, when its solutions are isolated from extraneous factors.

The plots A'-A'' and B'-B'' in Graphs 25 and 26 therefore gave a truer picture of the actual degradation rate of chlorbutol in ophthalmic and parenteral solutions than has been given by any determination to date. As the compound was subjected to the most adverse conditions of temperature and pH in this determination, its actual stability in pharmaceuticals was seen to be greater than might have been expected from the results of other workers (49)(40)(50)(66).

It was considered that the marked decrease in the degradation rates of chlorbutol observed in this determination would apply at least equally to its degradation under milder

processing conditions, even so far as to almost entirely eliminate the degradation when conditions were favourable. (e.g. Buffered solution of pH 3·5-4·0, steamed at 98°-100°).

4.4. The bacteriological testing of chlorbutol solutions, in silicone-coated containers, for self-sterilising properties after processing.

4.4. 1. Introduction.

As the silicone coating of containers had been observed to exert such favourable effect on the degradation of chlorbutol when solutions of pH 7·4 were autoclaved at 122° (4.3), its effect on the self-sterilising properties of chlorbutol solutions, after processing under identical conditions, was investigated.

4.4. 2. Procedure.

The identical procedure to that previously described (4.3.2.) was carried out, but the processed solutions were bacteriologically tested for self-sterilising properties (as in 3.3.2.a.). The procedure was also carried out using uncoated containers for the test solutions, so that a direct comparison could be made.

4.4.3. Results of the bacteriological tests.

4.4. 3.a. Results after autoclaving at 122° for fifteen minutes.

1. For uncoated containers. TABLE 26.
2. For coated containers. TABLE 27.

4.4. 3.b. Results after autoclaving at 122° for thirty minutes.

1. For uncoated containers. TABLE 28.
2. For coated containers. TABLE 29.

4.4. 3.c. Results after autoclaving at 122° for forty-five minutes.

1. For uncoated containers. TABLE 30.
2. For coated containers. TABLE 31.

4.4. 3.d. Results after autoclaving at 122° for sixty minutes.

1. For uncoated containers. TABLE 32.
2. For coated containers. TABLE 33.

4.4. 4. Discussion of results.

A marked improvement in the antibacterial properties of processed chlorbutol solutions was effected by the use of silicone-coated containers. Test solutions autoclaved at 122°, for periods of fifteen and thirty minutes respectively, showed virtually no loss in self-sterilising properties, in coated containers, compared to unprocessed chlorbutol solutions. A marked decrease in antibacterial potency was observed for test solutions in uncoated containers.

For longer autoclaving periods (i.e. forty-five and sixty minutes) improvements of this nature were not observed.

These results were related to the degradation of chlorbutol during processing. GRAPHS 25 and 26.

For the two longer autoclaving periods, the magnitude of the degradation of chlorbutol was such that even the marked reduction in degradation, brought about by the silicone-coating of the containers, had virtually no effect on the antibacterial properties of the test solutions.

For shorter autoclaving periods, however, the utilisation of silicone-coated containers reduced the percentage degradation to a level below that which had been shown to be critical for a reduction in antibacterial properties, compared with unprocessed solutions.

Hence, chlorbutol solutions, in silicone-coated containers, autoclaved at 122° for periods of fifteen and thirty minutes respectively, exhibited self-sterilising properties similar to those of unprocessed solutions.

4.5. Discussion of the effects of the silicone-coating of containers on chlorbutol solutions.

This determination showed that a large part of the degradation of chlorbutol in ophthalmic and parenteral solutions

was due to the alkalinity of the glass of the container. By eliminating this factor, a truer picture of the degradation of the compound was obtained, and its stability was found to be greater than previously thought (49)(40)(66).

The main effect of the silicone-coating of the containers for chlorbutol solutions was to considerably reduce its degradation in solutions, and hence to maintain their self-sterilising properties.

The quantitative relationship between degradation and the reduction in self-sterilising properties previously discussed (3.4.2.b.1.c.) was confirmed. i.e. Degradation higher than 3% of the original 0·5% chlorbutol led to a decrease in the antibacterial potency of the solution.

The silicone-coating of the containers was therefore observed to have a markedly retarding effect upon the degradation of chlorbutol in ophthalmic and parenteral solutions.

The reported flaking and removal of silicone films upon repeated autoclaving of coated glass containers (82)(89) was considered to be of no relevance in this determination, as containers for ophthalmic and parenteral solutions are normally discarded immediately after their contents have been administered, and are not re-used.

4.6. Abstract.

The silicone coating of glass containers for ophthalmic and parenteral solutions containing chlorbutol was investigated, and was found, by chemical and bacteriological determinations, to exert a highly beneficial effect on the self-sterilising properties of the solutions, by retarding the rate of degradation of the compound. The previously established quantitative relationship between degradation and reduction in antibacterial potency was confirmed.

CHAPTER V.

The formulation of chlorbutol solutions for maximum antibacterial potency.

5.1. Introduction.

As previously observed in this investigation, even unprocessed 0·5% chlorbutol solutions were not completely self-sterilising, particularly against certain bacillus species. (3.4.2.c.TABLES 19-24). For this reason, the formulation of chlorbutol solutions possessing maximum antibacterial potency was investigated.

The raising of the effective concentration appeared to be the first step in this direction, as a number of investigators have stated that a saturated solution of chlorbutol (0·75-0·8%, dependent on temperature) was self-sterilising (36) (90). This investigation had also indicated that the antibacterial potency of the compound increased with its concentration.

At first sight this appeared a simple problem, but chlorbutol is so slowly soluble that at least twelve hours continuous shaking is necessary to produce a saturated solution (91). This process would be tedious and time-consuming and is entirely impracticable from the point of view of the practising pharmacist.

The use of non-ionic surfactants and of alcohols to aid the solution of chlorbutol was therefore considered.

Non-ionics have been used for this purpose in the past (48), but more recent investigations have shown that, in some cases, their use has led to a reduction of the antibacterial properties of chlorbutol solutions (20)(49)(50). One compound, which was used to solubilise chlorbutol in 1955 (48) (Tween 20) was subsequently found to be the most effective inactivating agent of a series of surfactants, of chlorbutol in solution (53).

It was suggested that this was due to the formation of a complex, by a binding or association reaction between the two

compounds. This resulted in a reduction in the amount of chlorbutol biologically available (19). An alternative, or perhaps additional, explanation was that the surfactant produced some effect on the cell wall which prevented effective inter-action of the antibacterial agent and the micro-organism. (92)(93).

These inconclusive findings led to the rejection of the use of nonionic surfactants as a method for the solubilisation of chlorbutol.

Similar investigations to those carried out with nonionic surfactants have been performed on solutions of chlorbutol in various dilute alcohols. Ethanol (91), benzyl alcohol (20)(50) and phenylethyl alcohol (20)(50) have been investigated for this purpose, and in each case the tendency appeared to be towards a slight increase in antibacterial properties. These favourable results led to the further investigation of the use of dilute alcohols as a means of increasing the rate, and possible the degree, of solution of chlorbutol.

5.2. The investigation of the use of dilute alcohols.

5.2. 1. Introduction.

The use of a substance, which itself possesses some antibacterial potency, as a means of accelerating the solution of chlorbutol, should increase the self-sterilising efficiency of its solutions. This applied to the use of alcohols, which, theoretically, could be used in the preparation of concentrated stock solutions of chlorbutol, to be diluted to the required concentration during compounding. The use of concentrated stock solutions is time-saving and economical, but great accuracy must be exercised in their preparation and dilution (94).

Chlorbutol is highly soluble in ethanol (91)(33), which was selected for investigation as a suitable solvent for concentrated chlorbutol solutions.

The limiting factor in the use of ethanol in this way was its adverse physiological effect. Too high a concentra-

tion would lead to local irritation of mucous membranes in ophthalmic solutions, and to haemolysis and tissue damage in parenterals (46). It therefore became necessary to ensure that the final concentration of ethanol, after dilution of the stock solution, was not higher than 10%, a concentration which is used in some injections (33)(46).

A high concentration of chlorbutol might also produce adverse physiological effects. It has been observed, however, that concentrations of up to 1% in dilute alcohols exerted no effect on the conjuctiva and cornea of rabbits (20)(95).

5.2. 2. Solubility tests.

Although a saturated aqueous solution of chlorbutol contains 0·75-0·80% of the compound, it was considered that a more stable solution would result if a solution of slightly lower concentration was used. (e.g. 0·7%). A concentrated stock solution containing ten times this concentration would therefore lead to ease of calculation and dilution. The alcohol content of this concentrated solution should be such that after a ten-fold dilution it would not exceed 10%. A reduction in this figure would be favourable to the decrease of adverse physiological effects. The lowest concentration of alcohol in which 7% chlorbutol was easily soluble was therefore determined.

In a short test, 7% chlorbutol was found to be readily soluble in 50% ethanol, so alcohol concentrations below this were investigated.

The method used was to shake 7·0G. of chlorbutol in 100 ml. of 30%, 35%, 40% and 45% ethanol respectively. The shake flasks were examined for undissolved chlorbutol at ten-minute intervals. The results of this determination were tabulated. TABLE 34.

5.2. 3. Discussion of results of solubility tests.

From the tabulated results, solution (C) was selected as a suitable concentrated stock solution of chlorbutol. This

solution contained 7% chlorbutol in 40% ethanol, and combined the advantage of a short period of shaking to effect solution with a low final concentration of ethanol.(4%).

Dilution tests showed that no precipitation of chlorbutol occurred when the concentrated stock solution (C) was slowly added, with shaking, to the bulk of the diluent, with subsequent further addition of diluent to volume.

5.3. Bacteriological tests with diluted stock solutions.

5.3. 1. Introduction.

As has previously been observed, the degradation of chlorbutol resulted in a decrease in the self-sterilising properties of its processed solutions. This can be expected to apply to higher concentrations than that tested.

Solutions are autoclaved to render them sterile, hence if an alternative method of preparing sterile solutions, without the use of heat, could be applied, it might be possible for sterilised chlorbutol solutions to exhibit self-sterilising properties identical to those of unprocessed solutions.

The use of filtration methods of sterilisation for chlorbutol solutions was therefore investigated, in addition to the heating methods previously described.

5.3. 2. Bacteriological tests after heat processing.

5.3. 2.a. Procedure.

Buffered 0·5%, 0·6% and 0·7% chlorbutol solutions of pH 7·4 were prepared from concentrated stock solutions.(5.2.3). The solutions were packed in sufficient silicone-coated containers in the prescribed manner (2.3.1) and were autoclaved at 122° for periods of fifteen, thirty and forty-five minutes respectively.(3.3.3.c.1.).

The bacteriological tests for self-sterilising properties (3.4.2.a.) were performed on the processed test solutions and on unprocessed solutions.

5.3. 2.b. Results of bacteriological tests.

The results of the bacterological tests for the self-sterilising properties of processed solutions containing 0·5%, 0·6% and 0·7% chlorbutol were tabulated.

1. After autoclaving at 122° for 15 minutes.
   TABLE 35.
2. After autoclaving at 122° for 30 minutes.
   TABLE 36.
3. After autoclaving at 122° for 45 minutes.
   TABLE 37.

5.3. 2.c. Discussion of results.

The results of this determination indicated that certain general trends occurred with regard to the antibacterial properties of chlorbutol.

1. The antibacterial potency of chlorbutol increased with increasing concentration in solution.

2. The antibacterial potency of chlorbutol decreased with increasing times of autoclaving, regardless of the concentration.

3. The 4% ethanol present in the final solution exerted no observable additive effect on that of the chlorbutol itself. TABLES 27, 29, 31 and 35, 36, 37.

4. Even a 0·7% solution of chlorbutol was not completely self-sterilising, although unprocessed solutions of this concentration exhibited results very close to ideality.

The previously observed fact that the compound exhibited higher antibacterial potency and greater stability in solutions of lower pH values, taken in conjunction with the results of this determination, indicated that the antibacterial properties of 0·7% chlorbutol solutions of pH 5·6 and 3·5 should be investigated.

5.3. 2.d. Bacteriological tests for the self-sterilising properties of 0·7% chlorbutol solutions of pH 5·6 and 3·5 after heat processing.

1. Procedure.

As the degradation rates of chlorbutol in buffered and unbuffered solutions with these pH values had been observed to be similar (3.3.2.e.3.) the self-sterilising properties of buffered solutions only, after autoclaving at 122° for periods of fifteen, thirty and forty-five minutes respectively, were investigated.

0·7% chlorbutol solutions with buffered pH values of 5·6 and 3·5 respectively were processed in the manner previously described (5.3.2.a.), and were bacteriologically tested for self-sterilising properties (3.4.2.a.).

2. Results of bacteriological tests.

The results of the bacteriological tests were tabulated.

a. After autoclaving at 122° for 15 minutes.
TABLE 38.

b. After autoclaving at 122° for 30 minutes.
TABLE 39.

c. After autoclaving at 122° for 45 minutes.
TABLE 40.

3. Discussion of results.

At the lower pH values used in this determination, marked improvements in the self-sterilising properties of the 0·7% chlorbutol solutions were observed. The results for processed and unprocessed solutions of pH 3·5 were very similar even after forty-five minutes autoclaving at 122°. Processed solutions of pH 5·6 were, however, slightly less effective than unprocessed solutions.

The results for unprocessed solutions indicated that a low pH was essential if chlorbutol was to exert its maximum antibacterial effect as a self-sterilising agent for ophthalmic and parenteral solutions.

A buffered pH value of approximately 4·0 was suggested for the stabilisation of processed 0·7% chlorbutol as a most effective self-sterilising solution, in silicone-coated containers.

5.3. 3. Bacteriological tests after filtration sterilisation.

5.3. 3.a. Introduction.

As a result of the degradation of chlorbutol during heat sterilisation procedures, the need for some other form of sterilisation of its solutions arose.(5.3.1.).

The adaptability and efficiency of the membrane filtration methods utilised in this investigation recommended their application to the production of sterile chlorbutol solutions. Preliminary investigations showed that the utilisation of membrane filtration methods resulted in the production of sterile solutions.

5.3. 3.b. Procedure.

Sufficient 0·7% chlorbutol solutions with buffered pH values of 7·4, 5·6 and 3·5 respectively were prepared from concentrated stock solutions (5.2.3.), and sterilised by passage through a sterile membrane filter unit, attached to a syringe, into sterile sealed containers. The test solutions were inoculated with the test organisms.

NOTE: As inoculation into sealed containers had to be carried out, a syringe was used for this purpose. One drop of bacterial suspension (2.5.2.a.) was introduced to each container as the inoculum.

The bacteriological tests for self-sterilising properties (3.4.2.a.) were performed on the test solutions and on unfiltered chlorbutol solutions. The results of these tests were tabulated, TABLE 41.

5.3. 3.c. Discussion of results.

No differences in the antibacterial properties of filtered and unfiltered chlorbutol solutions were observed, although the slight differences in potency due to pH effects were still present.

The method was therefore strongly recommended for the production of sterile ophthalmic and parenteral solutions with

high self-sterilising potency.

It had been observed throughout the bacteriological tests that the micro-organism most resistant to chlorbutol was B. cereus. This is an extremely common organism, and has been found to be non-pathogenic (80). Its survival indicated that the final test solutions, although highly suitable as self-sterilising ophthalmic and parenteral vehicles due to their efficiency against many common bacteria, were not bactericidal to all micro-organisms.

5.4. Investigation of the possible interference in the spectrophotometric assay procedure by 4% ethanol.

5.4. 1. Introduction.

Possible interference by the 4% ethanol in the final solution, in the spectrophotometric determination of chlorbutol was investigated before the procedure could be applied to the investigation of the possible loss of chlorbutol from its solutions, by sorptive processes onto membrane filters.

5.4. 2. Procedure.

The absorbance spectra of the coloured complex of chlorbutol and the reagent blank, with and without 4% ethanol, were recorded, on the Beckman D.B. Spectrophotometer, using 1 cm. cells. GRAPH 27. The coloured complexes were prepared in the usual way. (3.3.2.d.1.).

5.4. 3. Discussion.

No interference, by 4% ethanol, in the absorbance spectrum of the complex was observed in the region of 500 mμ, the wavelength used in the spectrophotometric assay.

5.5. Investigation of the possible loss of chlorbutol, from its solutions, by sorption onto membrane filters during filtration sterilisation.

5.5. 1. Introduction.

Although the bacteriological tests on filtered and un-

filtered 0·7% chlorbutol solutions exhibited no differences in their respective self-sterilising properties, the possibility of the loss of a small proportion of the compound, from the solution by sorptive processes onto the membrane filter, was investigated. As the membranes are paper-thin and the pores occupy approximately 80% of the filter volume (46), such loss could be expected to be low.

5.5. 2. Procedure.

0·7% chlorbutol solutions with buffered pH values of 3·5, 5·6 and 7·4 respectively were prepared from concentrated stock solutions. (5.2.3.).

Sample solutions were filtered through sterile membrane filter units into silicone-coated containers (in triplicate). The filtered solutions and unfiltered standard solutions were assayed for residual chlorbutol by the spectrophotometric method. (3.3.2.d.1.). The concentrations of the test solutions were exactly halved, by dilution, before complexation, to ensure that the concentration range fell on the calibration plot between 0·2 and 0.5 mg. chlorbutol per ml., the concentration limits for accurate results (3.2.2.b.2.).

5.5. 3. Results of assay procedure for residual chlorbutol.

TABLE 48.

5.5. 4. Discussion of results.

A very slight decrease in the concentration of residual chlorbutol in solution was observed to be the result of passage through the membrane filter. The reduction was of the order of 0·004%, which was equivalent to 0.5715% of the original concentration of chlorbutol in solution.

The observed similarity of the self-sterilising properties of filtered and unfiltered solutions (5.3.3.c.) was the result of this very slight reduction in concentration, which was in agreement with that predicted (5.5.1.), due to the low sorptive capacity of the membrane filters used in the process.

5.6. Discussion.

This determination showed that the antibacterial potency of chlorbutol was proportional to its concentration, the increase of which to 0·7% resulted in almost completely self-sterilising ophthalmic and parenteral solutions.

However, the increase of the chlorbutol to 0·7% resulted in the compound being present in solution in a concentration very close to saturation, the result of which was to considerably retard its solution processes. The problem was overcome by the formulation of concentrated stock solutions in various concentrations of ethanol.

The self-sterilising properties of these ethanolic solutions of chlorbutol, which had been diluted to final concentrations of 0·7% chlorbutol and 4% ethanol, were still affected by heat processing. As in previous determinations, low pH values decreased the extent of degradation in solution, and it was observed that if chlorbutol was to exert the maximum possible antibacterial effect a low pH was essential. It was suggested that this was due both to the stabilisation of the chlorbutol at low pH, and to the antibacterial effect of the low pH itself.

Bacterial filtration using membrane filters, into sterile silicone-coated containers, was observed to be a highly successful alternative method for the sterilisation of chlorbutol solutions. By eliminating the need for degradative sterilisation procedures, this process resulted in the maintenance of the self-sterilising properties of sterilised solutions of chlorbutol. This was observed to be due to the extremely low sorption of the compound from its solutions onto the membrane filter discs.

5.7. Abstract.

The formulation of chlorbutol solutions for high antibacterial potency was investigated and the use of dilute alcohols was adopted. Solutions prepared from concentrated stock solutions of chlorbutol in various concentrations of ethanol were evaluated and bacteriologically tested for self-

sterilising properties. These properties were found to decrease with increasing times of heat processing, but were increased in solutions of low pH. The possible interference by ethanol in the spectrophotometric assay of chlorbutol was investigated. Filtration methods of sterilisation of chlorbutol solutions were bacteriologically evaluated and were found to be far more suitable than heating procedures. The loss of chlorbutol from its solutions by sorptive processes onto membrane filter discs was investigated.

CHAPTER VI.

The investigation of the nature of the degradation products of chlorbutol in ophthalmic and parenteral solutions.

6.1. Introduction and theoretical considerations.

The degradation of chlorbutol has been observed by many workers (70)(36)(71)(40), but, whilst the nature of the reaction has been examined (49)(40)(50)(66), the products resulting from it have not received much attention.

This investigation has shown that the degradation products did not, in themselves, exert any marked antibacterial properties, with the exceptions of the chlorine, from which the compound derives its antibacterial effect, and the reduction in pH.

The degradation products characterised by Nair et al (71)(42) had not been confirmed, as their investigation appeared to be the only one to date. It resulted in the identification of acetone, carbon monoxide, hydrogen and chloride ions, plus a trace of α-hydroxyisobutyric acid.

When chlorbutol is used as a self-sterilising agent for ophthalmic and parenteral solutions, it is exposed to different environments (e.g. H^+ or OH^-) and to various temperatures, both of which have been shown to affect the degradation processes. Hence, the formation of varied degradation products may result.

Therefore, the degradation products of chlorbutol, when its solutions were subjected to various conditions of pH and temperature, in uncoated and in silicone-coated containers, were investigated.

The various possible degradative reactions of chlorbutol, and the possible degradation products were illustrated. (DIAGRAM 1.). These reactions would, in many cases, only occur under the most extreme conditions, which would allow each individual reaction to reach equilibrium. Any or all of the reactions might occur simultaneously. Hence, the actual

degradation of chlorbutol was expected to proceed by a very complex route, which included both side- and reversible reactions.

The resultant effect of all these reactions, under pharmaceutical conditions, was the degradation of only a relatively small proportion of the chlorbutol present in solution. It was most probable, even under the most adverse pharmaceutical conditions of pH, temperature and time, that the processes did not go to completion.

The overall picture of the theoretical degradative processes of chlorbutol nevertheless facilitated the determination of the degradation products of the compound in ophthalmic and parenteral solutions.

6.2. The selection of degradation products for investigation.

Examination of DIAGRAM 1 indicated that the possible degradation products of chlorbutol were many and varied. The determination of each one would be time-consuming and unnecessary, hence certain probable products were selected for investigation.

The products for investigation were selected on the basis of two criteria.

1. The probability of their occurrence, due to one or more common mechanisms for their formation.

2. Their likely contribution to the determination of the degradation route(s) followed by chlorbutol in ophthalmic and parenteral solutions.

From the primary reactions the most probable degradation products were considered to be:

1. α-hydroxyisobutyric acid.
2. Isopropanol.
3. Chloroform.
4. Acetone.
5. Chloride ions.
6. Hydrogen ions.

Secondary reactions were considered likely to result in the formation of:

1. Formic acid (or formate).

2. Acetic acid (or acetate).

It was considered that the detection of these probable degradation products would facilitate the determination of the actual pathway(s) of degradation of chlorbutol.

Their presence, as degradation products of chlorbutol in ophthalmic and parenteral solutions, was therefore investigated.

6.3. The investigation of the selected degradation products.

6.3. 1. Introduction.

In this investigation, it was observed that approximately 30% of the original concentration of chlorbutol was degraded when its solutions were subjected to the most adverse pharmaceutical conditions of pH, temperature and time. As the original concentration of the compound was 0·7% (the result of bacteriological tests for antibacterial potency), the total concentration of the degradation products would be approximately 0·21%.

The large number of possible products indicated that the concentration of each would be extremely low, so that the utilisation of highly sensitive detection methods was essential if significant results were to be obtained. Furthermore, the volatility of some of the possible products necessitated the adoption of detection methods which would not lead to volatilisation losses and/or to the further degradation of the compound or its products.

Due to its applicability to the detection and separation of extremely low concentrations of compounds in solution (97) (98), thin-layer chromatography was adopted as the main determinative procedure for the selected degradation products. In some instances, the procedure could not be applied and other specific tests had to be used for the identification of the compounds.

6.3. 2. The determination of degradation products by thin-layer chromatographic methods.

6.3. 2.a. Standard procedure.

In thin-layer chromatography it is essential that a standard procedure be applied if accuracy and reproducibility of results are to be obtained (98). The following standard conditions were used throughout this investigation.

1. Saturation of chamber.

The atmosphere in the rectangular trough tank, with ground-glass cover, was saturated with the developing solvent by lining the walls with filter paper soaked in the solvent. Immediately before a determination, the tank was tilted so that the filter paper was again soaked with the solvent. This procedure resulted in "chamber saturation". (98).

The chamber was stored and used in a cool, dry atmosphere at constant temperature to prevent effects resulting in oblique solvent front movement (97)(98).

2. Preparation of the layer.

A layer, 250 mμ in thickness, was formed, on glass plates 20 cm. square, by triturating 25G. of Silica Gel G (Merck and Co.) with 35 ml. deionised water to form a smooth paste. A further 15 ml. of deionised water was incorporated, and the suspension was transferred to the Desaga spreader, and spread onto the clean, dry plates.

The layers were subjected to successive drying procedures.

a. Air-drying for ten minutes.

b. Drying with hot-air blower for ten minutes.

c. Oven-drying, in the vertical position, at 110° for thirty minutes.

This procedure resulted in activated layers. The dried, activated layers were immediately transferred to a dessicator, in which they were stored over blue silica gel for protection from laboratory fumes and for the maintenance of the activity of the layer.

Immediately before use, the layers were divided into strips 1 cm. in width by drawing boundaries in the layer. This procedure had been experimentally observed to reduce edge effects in a previous investigation. (Summers, 1965. Unpublished work).

The starting points were situated 15 mm. from the lower edge of the plate, and the standard length of run was 100 mm. The layer, after spotting with the standard and test solutions, was inserted in the solvent to a depth of 5 mm.

3. Spotting of solutions onto the layer.

The Agla micro-syringe* was used for the accurate application of uniform quantities of the solutions to the layer as semi-quantitative determinations were to be carried out.

4. Spraying of reagents.

The reagents, for the detection of the spots after migration, were applied to the layer in a fine spray by the "Aerolak Propellant Power Pak". This unit produced a fine mist which greatly facilitated the application of uniform quantities of the reagent(s) to the whole layer.

6.3. 2.b. The determination of α-hydroxyisobutyric acid and isopropanol.

6.3. 2.b.1. Experimental selection of a suitable procedure.

As the direct determination of volatile compounds by thin-layer chromatography was not feasible (98), an indirect method incorporating the utilisation of derivatives was investigated.

Steam-volatile hydroxy-group containing substances have been determined by the separation of their 3,5-dinitrobenzoates (99)(100)(101). Due to the presence of the hydroxy group on their respective molecules, both α-hydroxyisobutyric acid and isopropanol might be detected in solution in this manner. The method might also be applicable to the detec-

*Burroughs, Wellcome and Co. London.

tion of chlorbutol, by thin-layer chromatography, due to it containing an hydroxy group.

In practise, the presence of a substance in the test solution was detected by running the suitably treated test solution against the standard solution of the compound or its relevant derivative.

6.3. 2.b.1. a. Identification of standard compounds.

The identities of the standard compounds were confirmed by the formation of suitable derivatives and melting-point determinations (102)(33).

6.3. 2.b.1. b. Preparation of alcohol-free solvents.

Alcohol-free ether and benzene were prepared by refluxing commercial grades with an excess of 3,5-dinitrobenzoyl chloride for sixty minutes over a water-bath, and collecting the distillate (98).

6.3. 2.b.1. c. Preparation of standard derivatives.

The following standard solutions were prepared:

1. 0·7% chlorbutol in 4% ethanol.
2. 4% ethanol.
3. 0·05% α-hydroxyisobutyric acid in 4% ethanol.
4. 0·05% isopropanol in 4% ethanol.

Each standard solution was subjected to the following procedure, 10 ml. being selected as the original volume of solution because it is a common volume for ophthalmic and parenteral solution packings.

1. 10 ml. of the standard solution was extracted three times with 5 ml. portions of alcohol-free ether, which were combined and dried over dessicated sodium sulphate.

2. 2·0G. of 3,5-dinitrobenzoyl chloride was added and the mixture was refluxed over a water-bath for thirty minutes.

3. 10 ml. of deionised water was added to remove the unused acid chloride (98), and the mixture was made alkaline (to pH 9-10) by the dropwise addition of 10% sodium hydroxide solution.

4. The ether layer was separated off, and the aqueous layer extracted three times with 5 ml. portions of alcohol-free benzene.

5. The ether and benzene extracts were combined and dried over dessicated sodium sulphate.

6. The benzene-ether mixture was distilled off, and the respective derivative(s) crystallised out in the flask.

7. The derivative(s) were dissolved by the addition of two successive 0·5 ml. portions of alcohol-free benzene, which were transferred, in turn, to a sample tube, which therefore contained the standard derivative solution.

6.3. 2.b.1. d. Selection of solvent system.

The standard derivative solutions (prepared as in 6.3.2.b.1.c.) were chromatographed under the standard conditions (6.3.2.a.), using the solvent system: cyclohexane-carbon tetrachloride - ethyl acetate (10 + 75 + 15). The resulting chromatograph did not exhibit sufficient differentiation between the derivatives.

Under the standard experimental conditions, gradual increases in the polarity of the solvent system resulted in greater differentiation (98). The solvent system: ethyl acetate - acetone - methanol (45 + 45 + 10) was observed to give the maximum separation of the derivatives. DIAGRAM 2.

"Tailing" of the spots occurred and was reduced by the addition of two drops of N/50 hydrochloric acid per 10 ml. of solvent. DIAGRAM 3. These results were reproducible.

Both Rhodamine B, and 2', 7'-dichlorofluorescein spray reagents were used (98). The sprayed chromatographs were viewed under ultra-violet light, and better results were observed with Rhodamine B spray reagent.

The last solvent system mentioned, and the procedure described previously (6.3.2.b.1.c.) were therefore applied to the determination of α-hydroxyisobutyric acid and isopropanol as degradation products of chlorbutol in ophthalmic and parenteral solutions.

6.3. 2.b.2. Application of the selected procedure to the determination of α-hydroxyisobutyric acid and isopropanol.

6.3. 2.b.2. a. Introduction.

As the procedure was being applied to the determination of α-hydroxyisobutyric acid and isopropanol as degradation products of chlorbutol in ophthalmic and parenteral solutions, the same factors regarding pH and containers, which have been discussed previously, applied (2.3.3. and 2.3.1.).

Therefore, the presence of these compounds, in processed and unprocessed solutions of various pH values packed in silicone-coated and in uncoated containers was investigated.

6.3. 2.b.2. b. Containers and processing.

The degradation of chlorbutol in Clinbritic bottles (for parenterals) and in eye-drop bottles had been shown to be similar, hence, in order to eliminate time-consuming repetition the degradation products in only one type of container were investigated. Due to ease of closure the amber glass eye-drop bottle was used.

In this determination, solutions for processing were subjected to autoclaving for forty-five minutes at 122°, so that the maximum degradation of the compound possible under pharmaceutical conditions would occur.

6.3. 2.b.2. c. Selection of pH values.

Although the degradation of chlorbutol in solutions of pH 3·5, 5.6 and 7·4 had been determined, solutions of pH 3·5 were not investigated for their degradation products. The degradation at this pH was so low that such a determination was unrealistic.

The remaining pH values of 5·6 and 7·4 were highly suitable, as the degradation products in both acidic and basic media could be determined. Buffered solutions were used, as the degradation of the compound had been observed to be higher in such solutions at the respective pH values.

6.3. 2.b.2. d. Details of test solutions.

The following test solutions, containing 0·7% chlorbutol in 4% ethanol, were prepared, packed, and processed where necessary.

Solution.	Container.	pH.
1. Unprocessed.	Uncoated.	7·4.
2. Unprocessed.	Uncoated.	5·6.
3. Processed.	Uncoated.	7·4.
4. Processed.	Uncoated.	5·6.
5. Unprocessed.	Coated.	7·4.
6. Unprocessed.	Coated.	5·6.
7. Processed.	Coated.	7·4.
8. Processed.	Coated.	5·6.

6.3. 2.b.2. e. Treatment of test solutions.

The test solutions were treated in the manner described previously. (Preparation of derivatives. 6.3.2.b.1.c. 1-7). The derivative solutions were transferred to sample tubes.

6.3. 2.b.2. f. Chromatography of test derivative solutions.

The test derivative solutions were chromatographed against the standard derivative solutions (6.3.2.6.1.c.) under the standard conditions (6.3.2.a.), using the solvent system: ethyl acetate - acetone - methanol (45 + 45 + 10) plus two drops of N/50 hydrochloric acid per 10 ml. of solvent. The chromatograph was sprayed with Rhodamine B spray reagent, and viewed under ultra-violet light. The results obtained were reproducible. DIAGRAM 4.

6.3. 2.b.3. Discussion of chromatogram.

a. Determination of α-hydroxyisobutyric acid.

In no instance was the 3,5-dinitrobenzoic acid ester of this compound detected. Therefore, it was either not formed during the degradation of chlorbutol, or, alternatively, it was formed in quantities not detectable by this method. As concentrations at least as low as 0·05% were readily determined however, if present the concentration of the compound

was extremely low.

b. Determination of isopropanol.

The 3,5-dinitrobenzoic acid ester of this compound was detected in Test Derivative Solutions 1,3,4,7 and 8. This indicated that isopropanol was the main hydroxy-group-containing compound formed during the degradation of chlorbutol in pharmaceutical processes. It was observed to be present in processed solutions of pH 7·4, and a slight amount was detected in the unprocessed solution of pH 7·4 in uncoated containers. Trace quantities of the derivative of isopropanol were detected in processed solutions of pH 5·6.

The results of this determination were:

a. α-hydroxyisobutyric acid. NEGATIVE.

b. Isopropanol. POSITIVE.

6.3. 2.c. The determination of acetone.

6.3. 2.c. 1. Experimental selection of a suitable procedure.

6.3. 2.c. 1.a. Introduction.

As this compound is volatile, an indirect method of determination by thin-layer chromatography, using derivative formation was investigated. 2,4-dinitrophenylhydrazones have been utilised in the separation of ketones by thin-layer chromatography (99)(100)(101), and this method was investigated for its applicability to the determination of acetone.

6.3. 2.c. 1.b. Identification of standard compound.

Acetone was distilled from a commercial sample. The fraction distilling over at the boiling-point range 55·5°-56·5° was collected, and its identity was confirmed by the preparation of the 2,4-dinitrophenylhydrazone (m. pt.126°) and the semicarbazone (m.pt. 187°).

6.3. 2.c.1. c. Preparation of standard derivative.

6.3. 2.c. 1.c. 1. Preparation of reagent solution.

The reagent solution was prepared by dissolving

0·25G. of 2,4-dinitrophenylhydrazine in a mixture of 20 ml. of concentrated hydrochloric acid and 25 ml. of deionised water, by gently heating over a water-bath. After cooling, and the addition of a further 30 ml. of deionised water, the reagent solution was filtered (104).

6.3. 2.c. 1.c. 2. Preparation of derivative.

The following standard solutions were prepared.

a. 0·7% chlorbutol in 4% ethanol.

b. 0·05% acetone in 4% ethanol.

Each standard solution was subjected to the following procedure.

a. 10 ml. of the standard solution was transferred to a 50 ml. round-bottom Quick-fit flask. 10 ml. of the 2,4-dinitrophenylhydrazine reagent solution (6.3.2.c.1.c.1.) was immediately added.

b. The mixture was left to stand for fifteen minutes, after which period the inside of the flask was scratched with a glass rod. The mixture was left to stand a further ten minutes.

c. The contents of the flask were filtered and the filter was washed three times with 3 ml. portions of deionised water, to remove excess reagent.

d. After drying the filter was transferred to a sample tube, to which was added 1·0 ml. of alcohol-free benzene to effect solution of the derivative on the filter. (i.e. the standard derivative solution).

6.3. 2.c. 1.d. Selection of solvent system.

The standard derivative solutions (6.3.2.c.1.c.2.) were chromatographed under standard conditions (6.3.2.a.), using the solvent system: benzene - petroleum ether (75 + 25) (100). The resulting chromatograph did not exhibit sufficient migration of the 2,4-dinitrophenylhydrazine derivative of acetone, after spraying with Rhodamine B spray reagent and viewing under ultra-violet light.

Under identical experimental conditions other

solvent systems were tested. Sufficient migration of the derivative was observed to occur with the solvent system : benzene-ethyl acetate (95 + 5). The results obtained under these conditions and with this solvent system were reproducible. DIAGRAM 5.

6.3. 2.c. 1.e. Discussion.

As a well-defined spot was produced by the 2,4-dinitrophenylhydrazine derivative of acetone, this method was adopted for the determination of low concentrations of acetone in aqueous solution. For this purpose, it provided a sensitive (at least as low a concentration of acetone as 0.05% was detected), simple and elegant procedure.

6.3. 2.c. 2. Application of the selected procedure to the determination of acetone.

6.3. 2.c. 2.a. Introduction.

The factors and conditions discussed with regard to the determination of α-hydroxyisobutyric acid and isopropanol as degradation products of chlorbutol in ophthalmic and parenteral solutions (6.3.2.b.2.a-c.) applied equally to the determination of acetone.

6.3. 2.c. 2.b. Test solutions.

Test solutions, identical to those previously described (6.3.2.b.2.d.), were prepared, packed, and processed where necessary.

6.3. 2.c. 2.c. Treatment of test solutions.

The test solutions were treated in the manner previously described. (Preparation of derivative. 6.3.2.c.1.c.2. a-d).

6.3. 2.c. 2.d. Chromatography of test derivative solutions.

The test derivative solutions were chromatographed against the standard derivative solution (6.3.2.c.1.c.2.d.) under standard conditions (6.3.2.a.) using the solvent

system: benzene-ethyl acetate (95 + 5). The chromatograph was sprayed with Rhodamine B spray reagent and viewed under ultra-violet light. The results obtained were reproducible. DIAGRAM 6.

6.3. 2.c. 3. Discussion of chromatogram.

No spots other than those produced by the standard derivative solution were observed. The spots produced by the standard derivative solution were so intense that concentrations of acetone much lower than that of the standard solution (0·05%) would have been detected.

Hence, acetone was not isolated as a degradation product of chlorbutol in either processed or unprocessed solutions.

This result did not, however, rule out its possible formation and immediate further degradation under the test conditions.

The result of this determination for acetone was NEGATIVE.

6.3. 2.d. The determination of formic and acetic acids.

6.3. 2.d. 1. Experimental selection of a suitable procedure.

6.3. 2.d. 1.a. Introduction.

As acetic and formic acids are liquids (105), an indirect method of determination, utilising derivative formation and thin-layer chromatography, was investigated. Of the relatively large number of derivatives and salts of these acids (104), the benzylthiuronium salts were selected for the investigation of their application in this determination.

6.3. 2.d. 1.b. Identification of standard compounds.

The identity of reagent grade samples of the acids was confirmed by the preparation of derivatives.

1. Formic acid.

a. p-bromophenacyl ester. m.pt. 99°.

b. Benzyl-thiuronium salt. m.pt. 152°.

2. Acetic acid.

a. p-bromophenacyl ester. m.pt. 85°.

b. Benzylthiuronium salt. m.pt. 134°. (104)

6.3. 2.d. 1.c. Preparation of standard salts.

6.3. 2.d. 1.c. 1. Preparation of reagent solution.

A 10% solution of benzylthiuronium chloride in distilled water was prepared.

6.3. 2.d. 1.c. 2. Preparation of salts.

The following standard solutions were prepared.

a. 0·5% formic acid.

b. 0·5% acetic acid.

The standard solutions were subjected to the following procedure, modified from that of Mann and Saunders (104).

a. 10 ml. of the standard solution was neutralised to phenolphthalein (first trace of pink)by the dropwise addition of 5% sodium hydroxide.

b. N/50 hydrochloric acid was added dropwise until the solution was practically colourless.

c. The solution was transferred to a beaker containing 2 ml. of the reagent solution.(6.3.2.d.1.c.1.).

d. The mixture was stirred and cooled. After five minutes the inside surface of the beaker was scratched with a glass rod, and a crystalline precipitate formed.

e. After standing for fifteen minutes, the precipitate was filtered off, recrystallised from 90% ethanol, and dried.

f. The identity of the benzylthiuronium salts of the two acids was confirmed by melting point determinations. (6.3.2.d.1.b.1.b. and 2.b.).

Due to the probably extremely low concentration of the two acids in the test solutions, which would have resulted in a minute quantity of precipitate, leading to filtra-

tion difficulties, the application of an extraction procedure was investigated.

Solubility tests on the benzylthiuronium salts of the acids indicated that they were highly soluble in chloroform and almost insoluble in deionised water.

6.3. 2.d. 1.c. 3. Extraction procedure for the preparation of salts.

Standard solutions containing 0·05% formic and acetic acid respectively were prepared and subjected to the following procedure.

a. 10 ml. of the standard solution was neutralised to phenolphthalein by the dropwise addition of 5% sodium hydroxide.

b. The procedure described under 6.3.2.d.1.c.2. b-d. was carried out.

c. The suspension of the salt was extracted three times with 2 ml. portions of chloroform, which were combined, washed with 2 ml. of deionised water, and dried over dessicated sodium sulphate.

d. The solution was filtered free from sodium sulphate and evaporated down to 1 ml.

e. The 'salt' solution was transferred to a sample tube.

6.3. 2.d. 1.c. 4. Identification of the 'salts' produced by the extraction procedure.

The identification of the salts produced by the extraction procedure was carried out by chromatographing the solutions, under standard conditions (6.3.2.a.) against solutions of the standard salts (6.3.2.d.1.c.2.), using the solvent system: chloroform-ethyl acetate-acetone (40 + 40 + 20) plus one drop of N/10 hydrochloric acid per 10 ml. of solvent.*

The chromatograph was sprayed with 2',7'-dichlorofluorescein spray reagent and viewed under ultra-violet light. The results obtained were reproducible. DIAGRAM 7.

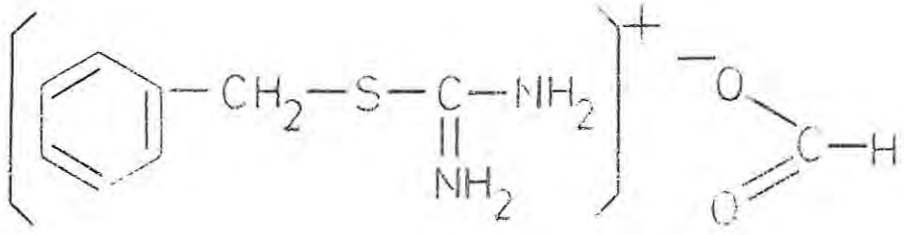
The salt solutions produced by the extraction

procedure were chromatographically identical to the standard salt solutions.(6.3.2.d.1.c.2.).

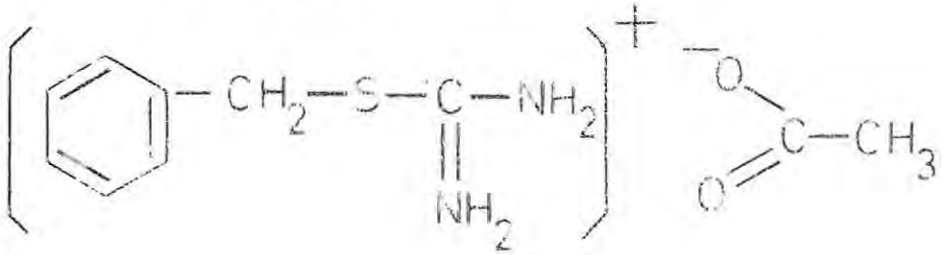
*Note: Selection of solvent system.

The structures of the benzylthiuronium salts of formic and acetic acids can be represented as:

a. Salt of formic acid.



b. Salt of acetic acid.



(104)

Due to the presence of the $-NH_2$ and carboxyl groupings, and their resultant polarity, these salts would be strongly adsorbed on the layer, hence would require a polar solvent system on a non-active adsorption layer (98).

The solvent system: chloroform-ethyl acetate-acetone (40 + 40 + 20) plus one drop of N/10 hydrochloric acid per 10 ml. of solvent, was experimentally found to give optimum separation of the two salts.

Thin layers which had not been activated were used in this determination.

Throughout the experimental determination of a suitable

solvent system, the salts produced by the extraction procedure exhibited chromatographic characteristics identical to those of the standard salts.(6.3.2.d.1.c.2.).

6.3. 2.d. 1.c. 5. Discussion.

By the application of the extraction procedure, concentrations of 0•05% of formic and acetic acids were detected. The method was therefore adopted for the determination of low concentrations of these acids in aqueous solution.

6.3. 2.d. 2. Application of the selected procedure to the determination of formic and acetic acids.

6.3. 2.d. 2.a. Introduction.

The factors and conditions discussed previously (6.3.2.b.2. a-c) applied equally to the determination of formic and acetic acids.

6.3. 2.d. 2.b. Test solutions.

Test solutions, identical to those described previously (6.3.2.b.2.d.) were prepared, packed and processed where necessary.

6.3. 2.d. 2.c. Treatment of test solutions.

The test solutions were subjected to the procedure previously described.(Extraction procedure for the preparation of salts. 6.3.2.d.1.c.3.). The solutions were transferred to sample tubes.

6.3. 2.d. 2.d. Chromatography of test solutions.

The test salt solutions were chromatographed against the standard salt solutions (6.3.2.d.1.c.3.), under standard conditions (6.3.2.a.), using the solvent system: chloroform - ethyl acetate - acetone (40 + 40 + 20) plus one drop of N/10 hydrochloric acid per 10 ml. of solvent.

The chromatograph was sprayed with 2',7'-dichlorofluorescein spray reagent and viewed under ultra-violet light

The results obtained were reproducible. DIAGRAM 8.

6.3. 2.d. 3. Discussion of chromatogram.

The benzylthiuronium salt of neither formic nor acetic acid was observed to be present in processed or unprocessed solutions of chlorbutol, i.e. formic and acetic acid were not isolated as degradation products of chlorbutol, although concentrations at least as low as 0·05% were readily detected by this method.

The results of this determination were:

a. Formic acid. NEGATIVE.

b. Acetic acid. NEGATIVE.

6.3. 2.e. The determination of chlorides.

6.3. 2.e. 1. Experimental selection of a suitable procedure.

6.3. 2.e. 1.a. Introduction.

Chlorides produced by the degradation of chlorbutol during pharmaceutical processes would be present in the solution as free chlorine or as the anion.

The potassium and sodium salts of the halides have been fractionated by thin-layer chromatography (98), and the application of the procedure to this determination was investigated.

The alkali halide could be determined by chromatography against a standard halide, after the addition of sodium hydroxide to the solution. The determination should be performed immediately after the addition of the base, so that further degradation of the residual chlorbutol, due to the hydroxyl ion, would not occur.

6.3. 2.e. 1.b. Preparation of standard solution.

A 0·1% sodium chloride solution in deionised water was prepared.

6.3. 2.d. 1.c. Preparation of neutralised chloride solution.

1 ml. of N/50 hydrochloric acid was neutralised

with N/2 sodium hydroxide.

6.3. 2.e. 1.d. Selection of solvent system.

The standard solution (6.3.2.e.1.b.) and the neutralised chloride solution (6.3.2.e.1.c.) were chromatographed under standard conditions (6.3.2.a.), using the solvent system: acetone - n-butanol - concentrated ammonia solution - deionised water (65 + 20 + 10 + 5) (98).

The chromatograph was sprayed with 0·1% bromocresol purple spray reagent (98). The results obtained were reproducible. DIAGRAM 9.

6.3. 2.e. 1.e. Discussion.

The R_f values obtained with the standard sodium chloride solution, and with the neutralised chloride solution were identical (0·15). As the spots obtained were very well defined, the method was suitable for the determination of low concentrations of chlorides in aqueous solutions.

6.3. 2.e. 2. Application of the selected procedure to the determination of chlorides.

6.3. 2.e. 2.a. Introduction.

The factors and conditions previously discussed (6.3.2.b. a-c.) applied equally to the determination of chlorides.

6.3. 2.e. 2.b. Test solutions.

Test solutions identical to those previously described (6.3.2.b.2.d.) were prepared, packed, and processed where necessary.

6.3. 2.e. 2.c. Treatment of test solutions.

The test solutions were subjected to the following procedure.

1. 1 ml. of the test solution was transferred to a sample tube, and neutralised (to pH indicator paper) with N/2 sodium hydroxide.

2. The tube was shaken once, and the solution was immediately spotted onto the thin-layer.

The procedure on one test solution was completed before that on the next was commenced.

6.3. 2.e. 2.d. Chromatography of test solutions.

The treated test solutions were chromatographed against the standard chloride solution (6.3.2.e.1.b.) and the neutralised chloride solution (6.3.2.e.1.c.), under standard conditions (6.3.2.a.), using the solvent system: acetone - n-butanol - concentrated ammonia solution - deionised water (65 + 20 + 10 + 5).

The chromatograph was sprayed with 0·1% bromocresol purple spray reagent. The results obtained were reproducible. DIAGRAM 10.

6.3. 2.e. 3. Discussion of chromatogram.

Chlorides were isolated from every test solution, and certain conclusions were drawn from a detailed examination of the chromatograph.

a. Solutions of pH 7·4 exhibited lower chloride concentrations than solutions of pH 5·6.

b. Processed solutions exhibited lower chloride concentrations than unprocessed solutions.

c. Solutions in uncoated containers exhibited lower chloride concentrations than solutions in coated containers.

Therefore, low chloride concentrations appeared to depend upon two factors.

a. The alkalinity of the solutions and of the containers.

b. The extent of degradation.

The low chloride levels determined in processed alkaline solutions of chlorbutol were responsible for the decreased antibacterial potency of these solutions, and it was evident that chlorides were lost from the solution during the degradation of the compound.

A high concentration of chlorides was dependent upon a high concentration of chlorbutol.

It was therefore concluded that the antibacterial properties of solutions of the compound were dependent upon both the concentration of undegraded chlorbutol and the concentration of free chlorides, of which free chlorine was considered to play the major part, due to its intense reactivity (8).

6.3. 3. The determination of degradation products by specific tests.

6.3. 3.a. Introduction.

The determination of possible degradation products of chlorbutol, in ophthalmic and parenteral solutions, by thin-layer chromatography, was not applicable to the detection of two of the selected products.

1. Chloroform.
2. Hydrogen ions.

Sensitive specific tests for these compounds were therefore investigated.

6.3. 3.b. The determination of chloroform.

6.3. 3.b. 1. Experimental selection of a suitable procedure.

6.3. 3.b. 1.a. Introduction.

Although a search of the literature had indicated that microdeterminations of chloroform in aqueous solutions were carried out by gas chromatography (e.g. (106)), the method was not applied in this determination because a qualitative test only was required.

The high sensitivity of the phenyl-isocyanide test, which has been used for the identification of both chloroform and chlorbutol (30)(6)(104)(33), recommended its further investigation.

6.3. 3.b. 1.b. Identification of the standard compounds.

The identities of the chloroform and chlorbutol standards used in this determination were confirmed by the official tests (33).

6.3. 3.b. 1.c. Testing of the method.

The method described by Mann and Saunders (104) was adapted to the requirements of this investigation.

1. Preparation of reagent solution.

0·5 ml. of aniline was dissolved in 10 ml. of N/10 alcoholic sodium hydroxide.

2. Testing of the procedure.

Preliminary tests indicated that the method was sufficiently sensitive for the detection of a concentration of 0·025% chloroform in aqueous solution.

The following standard solutions were prepared.

a. 0·025% chloroform in 4% ethanol.

b. 0·7% chlorbutol in 4% ethanol.

1 ml. of the reagent solution (6.3.3.b.1.c.1.) was added to 10 ml. of the respective standard solutions and the reaction mixtures were immediately gently warmed.

The characteristic odour of phenyl isocyanide was produced by solution a. (0·025% chloroform) within thirty seconds, but positive results were not obtained with solution b. (0·7% chlorbutol), even after gentle warming for three minutes.

6.3. 3.b. 1.d. Discussion.

This test is official for the identification of both chlorbutol and chloroform (33). Its application in the former case rests upon the preliminary degradation of the compound in alkaline medium. Under the experimentally determined conditions of this evaluation, the chlorbutol in solution was not subjected to sufficient degradation for a positive result to be obtained i.e. the experimental conditions were so mild as not to cause the degradation of the compound. Positive re-

sults were given when chloroform was included in the test solution.

The modified procedure was therefore applied to the detection of chloroform in solution in the presence of residual chlorbutol.

6.3. 3.b. 2. Application of the selected procedure to the determination of chloroform.

6.3. 3.b. 2.a. Introduction.

The factors and conditions previously discussed (6.3.2.b.2. a-c) applied equally to the determination of chloroform.

6.3. 3.b. 2.b. Test solutions.

Test solutions identical to those previously described (6.3.2.b.2.d.) were prepared, packed, and processed where necessary.

6.3. 3.b. 2.c. Treatment of test solutions.

1 ml. of the reagent solution (6.3.3.b.1.c.1.) was added to 10 ml. of the respective test solution, and the reaction mixture was gently warmed.

The detection of the characteristic odour of phenylisocyanide within thirty seconds was evaluated as a positive result.

Standard solutions of chloroform and chlorbutol (6.3.3.b.1.c.2.) in 4% ethanol were used for comparative purposes.

6.3. 3.b. 2.d. Results.

The results of this determination were tabulated.

TABLE 43.

6.3. 3.b. 3. Discussion of results.

Chloroform was detected in all processed test solutions, but was not detected in any unprocessed test solution.

Differences due to the pH values of the solutions

were not observed, as the method was not quantitative.

These results indicated that chloroform was a degradation product of chlorbutol in ophthalmic and parenteral solutions subjected to heat processing.

The result of this determination for chloroform was POSITIVE.

6.3. 3.c. The determination of hydrogen ions.

6.3. 3.c. 1. Introduction.

As the formation of hydrogen ions during the degradation of chlorbutol in solution has been firmly established (35)(17)(40)(76), this determination was mainly directed towards the ratification of previous findings.

The pH values of test solutions before and after processing were therefore obtained with the Radiometer pH meter.

6.3. 3.c. 2. Experimental procedure.

6.3. 3.c. 2.a. Test solutions.

Buffered and unbuffered test solutions identical to those previously described (6.3.2.b.2.d.) were prepared, packed, and processed where necessary.

6.3. 3.c. 2.b. Measurement of pH values of test solutions.

The Radiometer pH meter was calibrated against standard buffer solutions of pH 7·4 and 5·0 respectively and was used to determine the pH values of the test solutions.

6.3. 3.c. 2.c. Results.

The results of the determination were tabulated. TABLE 44.

6.3. 3.c. 3. Discussion of results.

The results indicated that hydrogen ions were produced during the degradation of chlorbutol in pharmaceutical processes.

A reduction in pH was observed to occur with all the test solutions after processing, and was much greater in unbuffered than in buffered solutions.

Solutions in silicone-coated containers were observed to possess lower pH values than solutions in uncoated containers. This fact provided further evidence in support of the hypothesis previously submitted in explanation of the observed greater degradation of chlorbutol in solutions in uncoated containers. (Chapter 4.).

The results were in agreement with those of Kerckhoffs and Huizinga (76), except that no general reduction of the pH to approximately 2·5, upon autoclaving unbuffered chlorbutol solutions, was observed to occur.

The result of the determination of <u>hydrogen ions</u> was POSITIVE.

6.4. <u>Discussion</u>.

The degradation products of chlorbutol which were detected in this investigation were:

1. Isopropanol.
2. Chlorides.
3. Chloroform.
4. Hydrogen ions.

They were outlined in DIAGRAM 1, and the results indicated that the degradation of chlorbutol was likely to occur by reaction paths 1 and/or 2.

The fact that α-hydroxy-isobutyric acid was not isolated led to the rejection of reaction path 3 as a possibility, although Bressanin and Segré (107), and Nair and Lach (42) hypothesised that the formation of this compound by a competitive reaction was possible. Reactions of the type envisaged are highly favoured in alcoholic medium (108), and the reaction would therefore have been aided by the prevailing experimental conditions.(i.e. 4% ethanolic solutions). The fact that the compound was not detected, under these favourable conditions,

eliminated the possibility of its formation during the degradation of chlorbutol in pharmaceutical processes.

Another probable degradation product not isolated was acetone. It was considered that the negative result was due to its formation and immediate further degradation, as the thermal decomposition of the substance has been shown to proceed by a free radical mechanism. Such mechanisms result in extremely rapid reactions (6).

The formation of the compounds which were isolated was feasible.

It seemed likely, however, that chloroform was a terminal, and not an intermediate, degradation product. Its rate of degradation in aqueous solution is extremely low compared to that of chlorbutol, and for complete degradation it must be refluxed with sodium hydroxide for several hours (109)(110).

Chloroform has been observed to be (directly or catalytically) active against bacteriophage, by the production of toxic components in the solution (111). It was suggested that its antibacterial properties were similarly based, and were due to the gradual release of free chlorine into the solution.

This hypothesis was equally applicable to an explanation of the mode of action of chlorbutol itself, due to the formation of chloroform.

In conclusion, it was considered that, whilst the detection or isolation of a particular degradation product was not positive proof that degradation occurred through or to that product, the results obtained were evidence that degradation along the indicated reaction path was probable.

This investigation indicated that the reaction paths most probably followed during the degradation of chlorbutol, in ophthalmic and parenteral solutions, were those designated as 1 and 2 in DIAGRAM 1.

6.5. Abstract.

Certain possible degradation products of chlorbutol in

ophthalmic and parenteral solutions were selected for investigation on the basis of their probability of formation and likely contribution to the elucidation of the degradation path(s) of the compound.

Sensitive methods of detection by thin-layer chromatography were devised and applied. Where such techniques were unsuitable, other specific tests were utilised.

Isopropanol, chlorides, chloroform and hydrogen ions were detected, and their contribution to the elucidation of the degradative processes of chlorbutol was discussed. An hypothesis regarding the mode of antibacterial action of chlorbutol was proposed.

CHAPTER VII.

Discussion.

This investigation demonstrated the advantages and limitations of chlorbutol as a self-sterilising agent for ophthalmic and parenteral solutions, and enabled positive steps to be taken to reduce or to eliminate the limitations.

Chlorbutol was adversely affected by high pH values and high temperatures. These two factors greatly increased its rate of degradation, which was similarly affected by the alkalinity of the glass of the containers in which its solutions were packed.

The results of the spectrophotometric assay procedure indicated that the observed reduction of antibacterial properties of processed solutions of the compound was the direct result of its physical breakdown.

Bacteriological tests against a representative cross-section of common micro-organisms indicated that solutions with a chlorbutol concentration of 0·5% possessed inadequate self-sterilising properties, although solutions of this concentration were bactericidal when used in conjunction with heating processes for the purpose of sterilising solutions.

Increasing the concentration to 0·7%, by the utilisation of ethanolic solutions, resulted in a marked increase in the self-sterilising properties, although the antibacterial potency of these solutions was also adversely affected by heat and high pH values.

Filtration sterilisation procedures, with membrane filters, resulted in the maintenance of the original concentration, and the self-sterilising properties, of the compound. 0·7% chlorbutol solutions sterilised in this manner virtually complied with all the requirements of the "ideal" self-sterilising and preservative agent. (Chapter 1.).

The investigation of the degradation products of the compound indicated that the degradation path(s) were complex, and included both side and reversible reactions.

The proposed hypothesis that the antibacterial properties of chlorbutol resulted from the formation of chloroform, with the subsequent slow release of chlorine, most nearly complied with the experimental results.

The results of this investigation necessitated that a recommendation be made regarding the use of chlorbutol, for its antibacterial properties in solution.

Recommendation.

For the production of almost completely self-sterilising solutions, 0·7% chlorbutol, in 4% ethanol, should be incorporated as the antibacterial agent, by the dilution of concentrated stock solutions. For the optimum stability of the compound and of its antibacterial properties, sterilisation should be carried out by bacterial filtration procedures using membrane filters. Where heat sterilisation methods are unavoidable, solutions should be buffered to pH 3·5-4·0. Silicone-coated containers should always be used for both ophthalmic and parenteral solutions.

APPENDIX 1.

Presentation of experimental data.

A.1. TABLES.

A.1.1. Results of bacteriological tests for the bactericidal properties of 0·5% chlorbutol solutions used in conjunction with heat processing.

TABLE 1.

Unbuffered solution. pH 3·5.

Container		Sterile Control	Control No chlorbutol	0	Steaming times in minutes. 15	30	45	60	75	90
1 ml. amber ampoules	A	-	+S	+S	-	-	-	-	-	-
	B	-	+S	+S	-	-	-	-	-	-
	C	-	+S	+S	-	-	-	-	-	-
	D	-	+S	+S	+B	-	-	-	-	-
2 ml. white ampoules	A	-	+S	+S	-	-	-	-	-	-
	B	-	+S	+S	-	-	-	-	-	-
	C	-	+S	+S	-	-	-	-	-	-
	D	-	+S	+S	-	-	-	-	-	-
5 ml. white ampoules	A	-	+S	+S	-	-	-	-	-	-
	B	-	+S	+S	-	-	-	-	-	-
	C	-	+S	+S	-	-	-	-	-	-
	D	-	+S	+S	-	-	-	-	-	-
10 ml. white ampoules	A	-	+S	+S	-	-	-	-	-	-
	B	-	+S	+S	-	-	-	-	-	-
	C	-	+S	+S	-	-	-	-	-	-
	D	-	+S	+S	-	-	-	-	-	-
15 ml. Clinbritic bottles	A	-	+S	+S	-	-	-	-	-	-
	B	-	+S	+S	-	-	-	-	-	-
	C	-	+S	+S	-	-	-	-	-	-
	D	-	+S	+S	-	-	-	-	-	-
15 ml. eye-drop bottles	A	-	+S	+S	-	-	-	-	-	-
	B	-	+S	+S	-	-	-	-	-	-
	C	-	+S	+S	-	-	-	-	-	-
	D	-	+S	+S	-	-	-	-	-	-

TABLE 2.

Unbuffered solution. pH 5·6.

Container		Sterile Control	Control No chlorbutol	Steaming times in minutes. 0	15	30	45	60	75	90
1 ml	A	-	+M	+S	-	-	-	-	-	-
amber	B	-	+M	+S	+B	-	-	-	-	-
ampoules	C	-	+M	+S	-	-	-	-	-	-
	D	-	+M	+S	-	-	-	-	-	-
2 ml.	A	-	+M	+S	-	-	-	-	-	-
white	B	-	+M	+S	+B	-	-	-	-	-
ampoules	C	-	+M	+S	-	-	-	-	-	-
	D	-	+M	+S	-	-	-	-	-	-
5 ml.	A	-	+M	+S	-	-	-	-	-	-
white	B	-	+M	+S	-	-	-	-	-	-
ampoules	C	-	+M	+S	+B	-	-	-	-	-
	D	-	+M	+S	-	-	-	-	-	-
10 ml.	A	-	+M	+S	-	-	-	-	-	-
white	B	-	+M	+S	-	-	-	-	-	-
ampoules	C	-	+M	+S	-	-	-	-	-	-
	D	-	+M	+S	-	-	-	-	-	-
15 ml.	A	-	+M	+S	+B	-	-	-	-	-
Clinbritic	B	-	+M	+S	+B	-	-	-	-	-
bottles	C	-	+M	+S	+B	+B	-	-	-	-
	D	-	+M	+S	-	-	-	-	-	-
15 ml.	A	-	+M	+S	-	-	-	-	-	-
eye-drop	B	-	+M	+S	-	-	-	-	-	-
bottles	C	-	+M	+S	-	-	-	-	-	-
	D	-	+M	+S	-	-	-	-	-	-

TABLE 3.

Unbuffered solution pH 7·4.

Container		Sterile Control	Control No chlorbutol	Steaming times in minutes. 0	15	30	45	60	75	90
1 ml. amber ampoules	A	-	+M	+S	+S	-	-	-	-	-
	B	-	+M	+S	+S	-	-	-	-	-
	C	-	+M	+S	-	+B	-	-	-	-
	D	-	+M	+S	+S	+B	-	-	-	-
2 ml. white ampoules	A	-	+M	+S	+S	+B	-	-	-	-
	B	-	+M	+S	+S	-	-	-	-	-
	C	-	+M	+S	+S	+B	-	-	-	-
	D	-	+M	+S	+S	-	-	-	-	-
5 ml. white ampoules	A	-	+M	+S	+S	-	-	-	-	-
	B	-	+M	+S	+S	-	-	-	-	-
	C	-	+M	+S	+S	+B	-	-	-	-
	D	-	+M	+S	+S	-	-	-	-	-
10 ml. white ampoules	A	-	+M	+S	+S	-	-	-	-	-
	B	-	+M	+S	+S	-	-	-	-	-
	C	-	+M	+S	+S	+B	-	-	-	-
	D	-	+M	+S	-	-	-	-	-	-
15 ml. Clinbritic bottles	A	-	+M	+S	-	-	-	-	-	-
	B	-	+M	+S	-	-	-	-	-	-
	C	-	+M	+S	+S	-	-	-	-	-
	D	-	+M	+S	+S	+B	-	-	-	-
15 ml. eye-drop bottles	A	-	+M	+S	+S	-	-	-	-	-
	B	-	+M	+S	+S	+B	-	-	-	-
	C	-	+M	+S	+S	+B	-	-	-	-
	D	-	+M	+S	+S	-	-	-	-	-

TABLE 4.

Buffered solution pH 3.5.

Container		Sterile Control	Control No chlorbutol	Steaming times in minutes. 0	15	30	45	60	75	90
1 ml. amber ampoules	A	-	+S	+S	-	-	-	-	-	-
	B	-	+S	+S	+B	-	-	-	-	-
	C	-	+S	+B	-	-	-	-	-	-
	D	-	+S	+S	-	-	-	-	-	-
2 ml. white ampoules	A	-	+S	+S	-	-	-	-	-	-
	B	-	+S	+B	-	-	-	-	-	-
	C	-	+S	+B	+B	-	-	-	-	-
	D	-	+S	-	-	-	-	-	-	-
5 ml. white ampoules	A	-	+S	+S	-	-	-	-	-	-
	B	-	+S	+S	-	-	-	-	-	-
	C	-	+S	+S	-	-	-	-	-	-
	D	-	+S	+S	-	-	-	-	-	-
10 ml. white ampoules	A	-	+S	+S	-	-	-	-	-	-
	B	-	+S	+S	-	-	-	-	-	-
	C	-	+S	+S	-	-	-	-	-	-
	D	-	+S	+S	-	-	-	-	-	-
15 ml. Clinbritic bottles	A	-	+S	+S	+B	-	-	-	-	-
	B	-	+S	+B	-	-	-	-	-	-
	C	-	+S	+S	-	-	-	-	-	-
	D	-	+S	+S	-	-	-	-	-	-
15 ml. eye-drop bottles	A	-	+S	+S	-	-	-	-	-	-
	B	-	+S	+S	-	-	-	-	-	-
	C	-	+S	+S	-	-	-	-	-	-
	D	-	+S	+S	+B	-	-	-	-	-

TABLE 5.

Buffered solution. pH 5·6.

Container		Sterile Control	Control No chlorbutol	Steaming times in minutes, 0	15	30	45	60	75	90
1 ml. amber ampoules	A	-	+M	+S	-	-	-	-	-	-
	B	-	+M	+S	-	-	-	-	-	-
	C	-	+M	+S	+B	-	-	-	-	-
	D	-	+M	+B	-	-	-	-	-	-
2 ml. white ampoules	A	-	+M	+S	-	-	-	-	-	-
	B	-	+M	+S	-	-	-	-	-	-
	C	-	+M	+S	-	-	-	-	-	-
	D	-	+M	+S	-	-	-	-	-	-
5 ml. white ampoules	A	-	+M	+S	-	-	-	-	-	-
	B	-	+M	+S	-	-	-	-	-	-
	C	-	+M	+S	-	-	-	-	-	-
	D	-	+M	+S	+B	-	-	-	-	-
10 ml. white ampoules	A	-	+M	+S	-	-	-	-	-	-
	B	-	+M	+S	-	-	-	-	-	-
	C	-	+M	+S	-	-	-	-	-	-
	D	-	+M	+S	-	-	-	-	-	-
15 ml. Clinbritic bottles	A	-	+S	+S	-	-	-	-	-	-
	B	-	+M	+S	-	-	-	-	-	-
	C	-	+M	+S	+B	+B	-	-	-	-
	D	-	+M	+S	+B	-	-	-	-	-
15 ml. eye-drop bottles	A	-	+M	+B	-	-	-	-	-	-
	B	-	+M	+S	-	-	-	-	-	-
	C	-	+S	+S	-	-	-	-	-	-
	D	-	+M	+S	-	-	-	-	-	-

TABLE 6.

Buffered solution, pH 7·4.

Container		Sterile Control	Control No chlorbutol	Steaming times in minutes. 0	15	30	45	60	75	90
1 ml. amber ampoules	A	-	+M	+S	+S	+B	-	-	-	-
	B	-	+M	+M	+S	+B	-	-	-	-
	C	-	+M	+S	+S	+B	-	-	-	-
	D	-	+M	+M	+S	-	-	-	-	-
2 ml. white ampoules	A	-	+M	+S	+S	+B	-	-	-	-
	B	-	+M	+S	+S	+B	-	-	-	-
	C	-	+M	+S	+S	-	-	-	-	-
	D	-	+M	+S	+S	+B	-	-	-	-
5 ml. white ampoules	A	+	+M	+M	+S	-	-	-	-	-
	B	-	+M	+S	+S	-	-	-	-	-
	C	-	+M	+S	+S	+B	-	-	-	-
	D	-	+M	+S	+S	+B	-	-	-	-
10 ml. white ampoules	A	-	+M	+S	+S	-	-	-	-	-
	B	+	+M	+M	+S	+B	-	-	-	-
	C	-	+M	+S	+S	-	-	-	-	-
	D	-	+M	+S	+S	-	-	-	-	-
15 ml. Clinbritic bottles	A	-	+M	+M	+S	+B	-	-	-	-
	B	-	+M	+S	+S	+B	-	-	-	-
	C	-	+M	+S	+S	+B	-	-	-	-
	D	-	+M	+M	+S	+B	-	-	-	-
15 ml. eye-drop bottles	A	-	+M	+S	+S	+B	-	-	-	-
	B	-	+M	+S	+S	+B	-	-	-	-
	C	-	+M	+M	+S	+B	-	-	-	-
	D	-	+M	+S	+S	+B	-	-	-	-

A.1.2. Investigation of spectrophotometric assay procedure.

TABLE 7.

Conformation of the coloured complex with Beers' Law.

Experimental data for GRAPH 2.

Initial concentration of chlorbutol (mg./ml.)	0·1	0·2	0·3	0·4	0·5	0·6
Absorbance of solution.	0·051	0·116	0·241	0·359	0·499	0·587

TABLE 8.

Fading of the coloured complex with time.

Experimental data for GRAPH 3.

Time after reaction in minutes.	5	10	15	20	25	30	35
Absorbance of solution.	0·405	0·359	0·332	0·326	0·316	0.304	0·298
Time after reaction in minutes.	40	45	50	55	60		
Absorbance of solution.	0·293	0·291	0·289	0·286	0·283		

TABLE 9.

Assay of 'test' quantities of chlorbutol in solution.

Experimental data for GRAPH 4.

a. Calibration curve data.

Initial concentration of chlorbutol (mg./ml.)	0·2	0·3	0·4	0·5
Absorbance of solution.	0·201	0·262	0·318	0·380

b. Experimental data.

'Unknown' solution	X	Y
Absorbance	0·228	0·293

c. Calculation.

1. Concentration of solution X from graph = 0·247 mg./ml.
   Original concentration of solution X = 0·25 mg./ml.
   ∴ Error expressed as a percentage of initial concentration = 1·20%

2. Concentration of solution Y from graph = 0·350 mg./ml.
   Original concentration of solution Y = 0·350 mg./ml.
   ∴ Error expressed as a percentage of initial concentration = 0·0%

TABLE 10.

Example of method used to obtain percentage degradation of 0·5% chlorbutol in solution.

Experimental data for GRAPH 6.

a. Calibration curve data.

Initial concentration of chlorbutol (mg./ml.)	0·2	0·3	0·4	0·5
Absorbance of solution.	0·177	0·256	0·328	0·429

b. Experimental data.

Heating time in minutes.	0	15	30	45	60	75	90
Absorbance of solution.	0·417	0·414	0·405	0·397	0·393	0·386	0·382
Concentration of solution from graph.	0·499	0·495	0·483	0·475	0·469	0·461	0·454
Calculated percentage degradation.	0·2	1·0	3·4	5·0	6·2	7·8	9·2

A.1.3. Results of spectrophotometric assays for residual chlorbutol after steaming (i.e. percentage degradation values).

TABLE 11.

Unbuffered solution. pH 3.5.

Experimental data for GRAPH 7.

Time of steaming in minutes.	0	15	30	45	60	75	90
A. 1 ml. amber ampoules.	0.2	1.6	2.8	3.8	5.2	6.8	7.6
B. 2 ml. white ampoules	0.0	0.8	1.6	2.6	3.4	4.0	4.8
C. 5 ml. white ampoules	0.0	0.6	1.6	1.8	2.8	3.2	4.0
D. 10 ml. white ampoules.	0.2	0.4	0.8	0.8	1.2	1.4	1.8
E. 15 ml. Clinbritic bottles.	0.2	1.8	3.2	5.2	6.4	8.0	10.0
F. 15 ml. eye-drop bottles.	0.2	1.0	3.4	5.0	6.2	7.8	9.2

TABLE 12.

Unbuffered solution, pH 5.6.

Experimental data for GRAPH 8.

Time of steaming in minutes.	0	15	30	45	60	75	90
A. 1 ml. amber ampoules	0.0	1.6	3.4	4.8	6.8	8.2	9.8
B. 2 ml. white ampoules	0.0	1.6	3.2	4.4	6.0	7.2	9.0
C. 5 ml. white ampoules	0.0	1.4	2.6	4.0	5.4	6.6	7.8
D. 10 ml. white ampoules.	0.0	1.0	2.4	3.4	4.8	5.6	7.0
E. 15 ml. Clinbritic bottles.	0.2	2.0	3.8	6.0	7.6	9.8	11.4
F. 15 ml. eye-drop bottles.	0.0	1.8	3.6	5.2	7.2	9.0	10.4

TABLE 13.

Unbuffered solution, pH 7.4.

Experimental data for GRAPH 9.

Time of steaming in minutes.	0	15	30	45	60	75	90
A. 1 ml. amber ampoules	0.2	2.2	4.0	6.4	8.0	10.2	12.2
B. 2 ml. white ampoules	0.0	2.0	3.6	5.4	7.4	9.4	10.8
C. 5 ml. white ampoules	0.0	1.8	3.6	5.2	7.2	8.8	10.6
D. 10 ml. white ampoules	0.0	1.4	3.4	5.2	6.6	8.4	10.0
E. 15 ml. Clinbritic bottles.	0.4	2.6	5.0	7.2	9.2	11.4	13.8
F. 15 ml. eye-drop bottles.	0.2	2.4	4.4	6.4	8.8	10.8	12.8

TABLE 14.

Buffered solution, pH 3·5

Experimental data for GRAPH 10.

Time of steaming in minutes.	0	15	30	45	60	75	90
A. 1 ml. amber ampoules	0·0	1·0	1·6	2·8	3·8	4·4	5·2
B. 2 ml. white ampoules	0·0	0.8	1·2	2·0	2·4	2·8	3·6
C. 5 ml. white ampoules	0·0	0·4	1·2	1·4	1·6	2·4	2·6
D. 10 ml. white ampoules	0·0	0·4	0·6	0·6	1·2	1·2	1·4
E. 15 ml. Clin-britic bottles	0·2	1·4	2·4	3·6	5·0	6·2	7·0
F. 15 ml. eye-drop bottles.	0·2	1·2	2·4	3·4	4·4	5·6	6·6

TABLE 15.

Buffered solution, pH 5·6.

Experimental data for GRAPH 11.

Time of steaming in minutes	0	15	30	45	60	75	90
A. 1 ml. amber ampoules.	0·0	1·6	3·2	5·0	6·4	8·0	9·8
B. 2 ml. white ampoules	0·0	1·2	3·0	4·4	5·8	7·6	8·8
C. 5 ml. white ampoules	0·0	1·6	2·6	4·4	5·6	6·8	8·4
D. 10 ml. white ampoules	0·0	1·4	2·8	3·6	5·2	6·4	7.6
E. 15 ml. Clin-britic bottles	0·2	2·2	4·0	5·6	7·4	9·4	11·0
F. 15 ml. eye-drop bottles	0·2	2·0	3·6	5·4	7·0	8·8	10·4

TABLE 16.

Buffered solution, pH 7.4.

Experimental data for GRAPH 12.

Time of steaming in minutes.	0	15	30	45	60	75	90
A. 1 ml. amber ampoules.	0.2	2.6	5.0	7.6	10.2	12.6	15.0
B. 2 ml. white ampoules	0.0	2.4	4.8	6.8	9.4	11.6	13.8
C. 5 ml. white ampoules	0.0	2.2	4.6	6.6	9.2	11.4	13.4
D. 10 ml. white ampoules	0.0	2.2	4.6	6.4	8.6	11.0	13.0
E. 15 ml. Clinbritic bottles.	0.4	3.2	6.0	8.4	11.4	13.8	16.6
F. 15 ml. eye-drop bottles.	0.4	3.0	5.6	7.8	10.4	13.2	15.4

A.1.4. Results of spectrophotometric assays for residual chlorbutol after autoclaving.

TABLE 17.

Percentage degradation values of chlorbutol solutions in uncoated containers autoclaved at 115° for varying periods. Experimental data for GRAPHS 13-18.

pH	State of solution	Container	Times of autoclaving in minutes 0m.	15m.	30m.	45m.	60m.	Graph Number.
3·5	Unbuffered	CBB	0·4	2·2	4·2	5·6	7·8	13
		EDB	0·2	1·8	3·0	4·2	5·8	
5·6	Unbuffered	CBB	0·4	2·4	4·0	6·2	7·6	14
		EDB	0·2	1·8	3·4	5·2	6·4	
7·4	Unbuffered	CBB	0·2	3·0	5·6	8·8	11·4	15
		EDB	0·0	2·2	4·8	7·0	9·4	
3·5	Buffered	CBB	0·2	2·0	3·2	5·2	6·6	16
		EDB	0·2	1·4	2·8	4·4	5·8	
5.6	Buffered	CBB	0·2	2·4	4·8	7·0	9·2	17
		EDB	0·0	1·8	3·6	5·2	7·4	
7·4	Buffered	CBB	0·0	3·4	6·6	9·8	13·4	18
		EDB	0·2	3.0	6·2	9·0	12·2	

CBB = 15 ml. Clinbritic bottle.
EDB = 15 ml. eye-drop bottle.

TABLE 18.

Percentage degradation values of chlorbutol solutions in uncoated containers autoclaved at 122° for varying periods. Experimental data for GRAPHS 19-24.

pH	State of solution	Container	Times of autoclaving in minutes 0m.	15m.	30m.	45m.	60m.	Graph Number
3·5	Unbuffered	CBB	0·0	2·4	4·4	6·8	8·6	19
		EDB	0·0	1·8	3·6	5·6	7·2	
5.6	Unbuffered	CBB	0·4	2·8	5·2	7·4	10·0	20
		EDB	0·0	2.2	4·2	6·6	8·4	
7·4	Unbuffered	CBB	0·2	3·6	6·6	10·4	13·4	21
		EDB	0·2	3·0	6.2	8·6	11·8	
3·5	Buffered	CBB	0·0	2·2	4·2	6·2	8·6	22
		EDB	0·0	1·8	4·0	5·8	8·0	
5·6	Buffered	CBB	0·0	2·6	5·8	8·4	11·2	23
		EDB	0·2	3·0	5·2	8·0	10·4	
7·4	Buffered	CBB	0·0	4·6	9·6	14·2	19·2	24
		EDB	0·2	4·8	9·2	13·6	18·8	

A.1.5. Results of bacteriological tests for the self-sterilising properties of 0·5% chlorbutol solutions after steaming.

TABLE 19. Unbuffered solutions. After steaming for fifteen minutes.

a. At pH 3·5

Containers		Control Technique	Control No chlorbutol	Test solutions Time after inoculation 30m.	60m.	90m.	24hr.	48hr.	0·5% chlorbutol. No process. Time after inoculation 90m.	24hr.	48hr.
15 ml.	A	-	+S	+S	+S	+S	+B	+B	+S	+B	+B
Clinbritic	B	-	+S	+S	+S	+S	+B	+B	+S	+B	-
bottle	C	-	+S	+S	+S	+S	+B	+B	+S	+B	+B
15 ml.	A	-	+S	+S	+S	+S	+B	+B	+S	+B	+B
Eye Drop	B	-	+S	+S	+S	+S	+B	+B	+S	+B	+B
bottle	C	-	+S	+S	+S	+S	+B	+B	+S	+B	+B.
b. At pH 5·6											
15 ml.	A	-	+M	+S	+S	+S	+B	+B	+S	+B	+B
Clinbritic	B	-	+M	+S	+S	+S	+B	+B	+S	+B	+B
Bottle	C	-	+M	+S	+S	+S	+B	+B	+S	+B	+B
15 ml.	A	-	+M	+S	+S	+S	+B	+B	+S	+B	-
Eye Drop	B	-	+M	+S	+S	+S	+B	+B	+S	+B	+B
Bottle	C	-	+M	+S	+S	+S	+B	+B	+S	+B	+B
c. At pH 7·4											
15 ml.	A	-	+M	+S	+S	+S	+B	+B	+S	+B	+B
Clinbritic	B	-	+M	+S	+S	+S	+B	+B	+S	+B	+B
Bottle	C	-	+M	+S	+S	+S	+B	+B	+S	+B	+B
15 ml.	A	-	+M	+S	+S	+S	+B	+B	+S	+B	+B
Eye Drop	B	-	+M	+S	+S	+S	+B	+B	+S	+B	+B
Bottle	C	-	+M	+S	+S	+S	+B	+B	+S	+B	+B

TABLE 20. Unbuffered solutions. After steaming for thirty minutes.

a. At pH 3·5

Containers	Technique	Control	Control No chlorbutol	Test solutions Time after inoculation 30m.	60m.	90m.	24hr.	48hr.	0·5% chlorbutol. No process. Time after inoculation. 90m.	24hr.	48hr.
15 ml. Clinbritic Bottle	A	-	+S	+S	+S	+S	+S	+B	+S	+B	+B
	B	-	+S	+S	+S	+S	+B	-	+S	+B	+B
	C	-	+S	+S	+S	+S	+S	+B	+S	+B	-
15 ml. Eye Drop Bottle	A	-	+S	+S	+S	+S	+B	+B	+S	+B	+B
	B	-	+S	+S	+S	+S	+B	+B	+S	+B	+B
	C	-	+S	+S	+S	+S	+B	+B	+S	+B	+B

b. At pH 5·6

15 ml. Clinbritic Bottle	A	-	+M	+M	+S	+S	+S	+S	+S	+B	+B
	B	-	+M	+S	+S	+S	+S	+S	+S	+B	+B
	C	-	+M	+S	+S	+S	+S	+S	+S	+B	+B
15 ml. Eye Drop Bottle	A	-	+M	+S	+S	+S	+S	+S	+S	+B	+B
	B	-	+M	+M	+S	+S	+S	+S	+S	+B	+B
	C	-	+M	+S	+S	+S	+S	+S	+S	+B	+B

c. At pH 7·4

15 ml. Clinbritic Bottle	A	-	+M	+M	+S	+S	+S	+S	+S	+B	+B
	B	-	+M	+M	+S	+S	+S	+S	+S	+B	+B
	C	-	+M	+M	+S	+S	+S	+S	+S	+B	+B
15 ml. Eye Drop Bottle	A	-	+M	+M	+S	+S	+S	+S	+S	+B	+B
	B	-	+M	+M	+S	+S	+S	+S	+S	+B	+B
	C	-	+M	+M	+S	+S	+S	+S	+S	+B	+B

TABLE 21. Unbuffered solutions. After steaming for forty-five minutes.

a. At pH. 3·5

Containers		Control Tech-nique	Control No chlorbutol	Test solutions Times after inoculation 30m.	60m.	90m.	24hr.	48hr.	0·5% chlorbutol. No heating. Times after inoculation. 90m.	24hr.	48hr.
15 ml.	A	-	+S	+S	+S	+S	+B	+B	+S	+B	+B
Clinbritic	B	-	+S	+S	+S	+S	+B	+B	+S	+B	+B
Bottle	C	-	+S	+S	+S	+S	+B	+B	+S	-	-
15 ml.	A	-	+S	+S	+S	+S	+B	+B	+S	+B	+B
Eye drop	B	-	+S	+S	+S	+S	+B	+B	+S	-	-
Bottle	C	-	+S	+S	+S	+S	+B	+B	+S	+B	+B

b. At pH 5·6

15 ml.	A	-	+M	+M	+M	+M	+S	+S	+S	+B	+B
Clinbritic	B	-	+M	+M	+M	+S	+S	+S	+S	+B	+B
Bottle	C	-	+M	+M	+M	+S	+S	+S	+S	+B	+B
15 ml.	A	-	+M	+M	+M	+S	+S	+S	+S	+B	+B
Eye Drop	B	-	+M	+M	+M	+S	+S	+S	+S	+B	+B
Bottle	C	-	+M	+M	+M	+S	+S	+S	+S	+B	+B

c. At pH 7·4

15 ml.	A	-	+M	+M	+M	+S	+S	+S	+S	+B	+B
Clinbritic	B	-	+M	+M	+M	+S	+S	+S	+S	+B	+B
Bottle	C	-	+M	+M	+M	+S	+S	+S	+S	+B	+B
15 ml.	A	-	+M	+M	+M	+S	+S	+S	+S	+B	+B
Eye Drop	B	-	+M	+M	+M	+S	+S	+S	+S	+B	+B
Bottle	C	-	+M	+M	+M	+S	+S	+S	+S	+B	+B

TABLE 22.

Buffered solutions. After steaming for fifteen minutes.

a. At pH 3·5.

Containers		Control Technique	Control No chlorbutol	Test solutions Times after inoculation 30m.	60m.	90m.	24hr.	48hr.	0·5% chlorbutol. No heating. Times after inoculation. 90m.	24hr.	48hr.
15ml.	A	-	+S	+S	+S	+S	+B	+B	+S	+B	+B
Clinbritic	B	-	+S	+S	+S	+S	+B	+B	+S	+B	+B
Bottle	C	-	+S	+S	+S	+S	+B	+B	+S	+B	+B
15 ml.	A	-	+S	+S	+S	+S	+B	+B	+S	+B	-
Eye Drop	B	-	+S	+S	+S	+S	+B	+B	+S	+B	+B
Bottle	C	-	+S	+S	+S	+S	+B	+B	+S	+B	+B
b. At pH 5·6											
15 ml.	A	-	+M	+S	+S	+S	+B	+B	+S	+B	+B
Clinbritic	B	-	+M	+S	+S	+S	+B	+B	+S	+B	+B
Bottle	C	-	+M	+S	+S	+S	+B	+B	+S	+B	+B
15 ml.	A	-	+M	+S	+S	+S	+B	+B	+S	+B	+B
Eye Drop	B	-	+M	+S	+S	+S	+B	+B	+S	+B	+B
Bottle	C	-	+M	+S	+S	+S	+B	+B	+S	+B	+B
c. At pH 7·4											
15 ml.	A	-	+M	+M	+S	+S	+S	+B	+S	+B	+B
Clinbritic	B	-	+M	+M	+M	+S	+S	+S	+S	+B	+B
Bottle	C	-	+M	+S	+S	+S	+S	+S	+S	+B	+B
15 ml.	A	-	+M	+S	+S	+S	+S	+S	+S	+B	+B
Eye Drop	B	-	+M	+M	+S	+S	+S	+B	+S	+B	+B
Bottle	C	-	+M	+S	+S	+S	+S	+S	+S	+B	+B

TABLE 23 Buffered solutions. After steaming for thirty minutes.

a. At pH 3·5

Containers		Control Tech-nique	Control No chlorbutol	Test solutions Times after inoculation 30m.	60m.	90m.	24hr.	48hr.	0·5% chlorbutol. No heating. Times after inoculation. 90m.	24hr.	48hr.
15 ml.	A	-	+S	+S	+S	+S	+S	+B	+S	+B	+B
Clinbritic	B	-	+S	+S	+S	+S	+B	-	+S	+B	+B
Bottle	C	-	+S	+S	+S	+B	+B	+B	+S	-	-
15 ml.	A	-	+S	+S	+S	+S	+B	+B	+S	-	-
Eye Drop	B	-	+S	+S	+S	+S	+B	+B	+S	+B	+B
Bottle	C	-	+S	+S	+S	+S	+B	+B	+S	+B	-

b. At pH 5·6

15 ml.	A	-	+M	+M	+S	+S	+S	+S	+S	+B	+B
Clinbritic	B	-	+M	+M	+S	+S	+S	+S	+S	+B	+B
Bottle	C	-	+M	+M	+S	+S	+S	+S	+S	+B	+B
15 ml.	A	-	+M	+M	+S	+S	+S	+S	+S	+B	+B
Eye Drop	B	-	+M	+M	+S	+S	+S	+S	+S	+B	+B
Bottle	C	-	+M	+M	+S	+S	+S	+S	+S	+B	+B

c. At pH 7·4

15 ml.	A	-	+M	+M	+M	+S	+S	+S	+S	+B	+B
Clinbritic	B	-	+M	+M	+M	+S	+S	+S	+S	+B	+B
Bottle	C	-	+M	+M	+M	+S	+S	+S	+S	+B	+B
15 ml.	A	-	+M	+M	+M	+S	+S	+S	+S	+B	+B
Eye Drop	B	-	+M	+M	+M	+S	+S	+S	+S	+B	+B
Bottle	C	-	+M	+M	+M	+S	+S	+S	+S	+B	+B

TABLE 24. Buffered solutions. After steaming for forty-five minutes.

a. At pH 3·5

Containers		Control Technique	Control No chlorbutol	Test solutions Times after inoculation 30m.	60m.	90m.	24hr.	48hr.	0·5% chlorbutol. No heating. Times after inoculation. 90m.	24hr.	48hr.
15 ml.	A	-	+S	+S	+S	+S	+B	+B	+S	+B	+B
Clinbritic	B	-	+S	+S	+S	+S	+B	+B	+S	+B	+B
Bottle	C	-	+S	+S	+S	+S	+B	+B	+S	+B	+B
15 ml.	A	-	+S	+S	+S	+S	+B	+B	+S	-	-
Eye Drop	B	-	+S	+S	+S	+S	+B	+B	+S	+B	+B
Bottle	C	-	+S	+S	+S	+S	+B	+B	+S	+B	+B

b. At pH 5·6

15 ml.	A	-	+M	+M	+M	+S	+S	+S	+S	+B	+B
Clinbritic	B	-	+M	+M	+M	+S	+S	+S	+S	+B	+B
Bottle	C	-	+M	+M	+M	+S	+S	+S	+S	+B	+B
15 ml.	A	-	+M	+M	+M	+S	+S	+S	+S	-	-
Eye Drop	B	-	+M	+M	+M	+S	+S	+S	+S	+B	+B
Bottle	C	-	+M	+M	+M	+S	+S	+S	+S	+B	+B

c. At pH 7·4

15 ml.	A	-	+M	+M	+M	+M	+S	+S	+S	+B	+B
Clinbritic	B	-	+M	+M	+M	+M	+M	+S	+S	+B	+B
Bottle	C	-	+M	+M	+M	+M	+S	+S	+S	+B	+B
15 ml	A	-	+M	+M	+M	+S	+S	+S	+S	+B	+B
Eye Drop	B	-	+M	+M	+M	+M	+S	+S	+S	+B	+B
Bottle	C	-	+M	+M	+M	+M	+S	+S	+S	+B	+B

A.1.5. Results of spectrophotometric assays for residual chlorbutol after autoclaving 0·5% solutions in SILICONE-COATED CONTAINERS at 122° for varying periods.

TABLE 25.

Percentage degradation values of chlorbutol solutions of pH 7·4.

Experimental data for GRAPHS 25 and 26.

State of Solution	Container	Times of autoclaving. 0m.	15m.	30m.	45m.	60m.	Graph Number.
Unbuffered	CBB	0·0	1·2	3·0	4·2	5·6	25
	EDB	0·2	1·6	2·6	4·0	5·2	
Buffered	CBB	0·2	1·8	3·2	4·8	6·2	26
	EDB	0·2	1·6	3·2	4·6	6·2	

A.1.6. Results of bacteriological tests for self-sterilising properties of 0·5% chlorbutol solutions, of pH 7·4, in coated and uncoated containers, after autoclaving at 122° for varying periods.

TABLE 26. After autoclaving at 122° for fifteen minutes.

a. Uncoated containers. Unbuffered solutions.

Containers	Control Technique.	Control No chlorbutol	Test solutions Time after inoculation 30m.	60m.	90m.	24hr.	48hr.	0·5% chlorbutol. No process. Time after inoculation. 90m.	24hr.	48hr.
15 ml. Clinbritic Bottle	A	-	+M	+S	+S	+S	+B	+S	+B	-
	B	-	+M	+S	+S	+S	+B	+S	+B	+B
	C	-	+M	+M	+S	+S	+B	+S	+B	+B
15 ml. Eye Drop Bottle	A	-	+M	+S	+S	+S	+B	+S	+B	+B
	B	-	+M	+S	+S	+S	+B	+S	+B	-
	C	-	+M	+S	+S	+S	+B	+S	+B	+B

b. Uncoated containers. Buffered solutions.

15 ml. Clinbritic Bottle	A	-	+M	+M	+M	+S	+S	+S	+B	+B
	B	-	+M	+M	+S	+S	+S	+S	+B	+B
	C	-	+M	+M	+M	+S	+S	+B	+B	-
15 ml. Eye Drop Bottle	A	-	+M	+M	+M	+S	+S	+S	+B	+B
	B	-	+M	+M	+M	+S	+S	+S	+B	+B
	C	-	+M	+M	+M	+S	+S	+S	+B	+B

TABLE 27. a. Coated containers. Unbuffered solutions.

15 ml. Clinbritic Bottle	A	-	+M	+S	+S	+S	+B	+B	+S	+B	+B
	B	-	+M	+S	+S	+S	+B	+B	+S	+B	+B
	C	-	+M	+S	+S	+S	+B	+B	+S	+B	+B
15 ml. Eye Drop Bottle	A	-	+M	+S	+S	+S	+B	+B	+S	+B	+B
	B	-	+M	+S	+S	+S	+B	+B	+S	+B	+B
	C	-	+M	+S	+S	+S	+B	+B	+S	+B	+B

b. Coated containers. Buffered solutions.

15 ml Clinbritic Bottle	A	-	+M	+S	+S	+S	+B	+B	+S	+B	+B
	B	-	+M	+S	+S	+S	+B	+B	+S	+B	+B
	C	-	+M	+S	+S	+S	+B	+B	+S	+B	+B
15 ml. Eye Drop Bottle	A	-	+M	+S	+S	+S	+B	+B	+S	+B	-
	B	-	+M	+S	+S	+S	+B	+B	+S	+B	+B
	C	-	+M	+S	+S	+S	+B	+B	+S	+B	+B

TABLE 28. After autoclaving at 122° for thirty minutes.

a. Uncoated containers. Unbuffered solutions.

Containers	Control Technique	Control No chlorbutol	Test solutions. Time after inoculation 30m.	60m.	90m.	24hr.	48hr.	0·5% chlorbutol. No process. Time after inoculation. 90m.	24hr.	48hr.
15 ml.	A	-	+M	+M	+M	+M	+S	+S	+S	+B	+B
Clinbritic	B	-	+M	+M	+S	+S	+S	+S	+S	+B	+B
Bottle	C	-	+M	+M	+M	+M	+S	+S	+S	+B	+B
15 ml.	A	-	+M	+M	+M	+M	+S	+S	+S	+B	+B
Eye Drop	B	-	+M	+M	+M	+M	+S	+S	+S	+B	+B
Bottle	C	-	+M	+M	+M	+S	+S	+S	+S	+B	+B

B. Uncoated containers. Buffered solutions.

15 ml.	A	-	+M	+M	+M	+M	+S	+S	+S	+B	+B
Clinbritic	B	-	+M	+M	+M	+M	+S	+S	+S	+B	+B
Bottle	C	-	+M	+M	+M	+M	+S	+S	+S	+B	+B
15 ml.	A	-	+M	+M	+M	+M	+S	+S	+S	+B	+B
Eye Drop	B	-	+M	+M	+M	+M	+S	+S	+S	+B	-
Bottle	C	-	+M	+M	+M	+M	+S	+S	+S	+B	+B

TABLE 29. a. Coated containers. Unbuffered solutions.

15 ml.	A	-	+M	+M	+S	+S	+B	+B	+S	+B	+B
Clinbritic	B	-	+M	+M	+S	+S	+B	+B	+S	+B	+B
Bottle	C	-	+M	+M	+S	+S	+B	+B	+S	+B	+B
15 ml.	A	-	+M	+M	+S	+S	+B	+B	+S	+B	+B
Eye Drop	B	-	+M	+M	+S	+S	+B	+B	+S	+B	+B
Bottle	C	-	+M	+M	+S	+S	+B	+B	+S	+B	+B

b. Coated containers. Buffered solutions.

15 ml.	A	-	+M	+M	+S	+S	+B	+B	+S	+B	+B
Clinbritic	B	-	+M	+M	+S	+S	+B	+B	+S	+B	+B
Bottle	C	-	+M	+M	+S	+S	+B	+B	+S	+B	+B
15 ml.	A	-	+M	+M	+S	+S	+B	+B	+S	+B	-
Eye Drop	B	-	+M	+M	+M	+S	+B	+B	+S	+B	+B
Bottle	C	-	+M	+M	+S	+S	+B	+B	+S	+B	+B

TABLE 30. After autoclaving at 122° for forty-five minutes.

a. Uncoated containers. Unbuffered solutions.

Containers	Control Technique	Control No chlorbutol	Test solutions. Time after inoculation 30m.	60m.	90m.	24hr.	48hr.	0·5% chlorbutol. No process. Time after inoculation. 90m.	24hr.	48hr.
15 ml.	A	-	+M	+M	+M	+M	+S	+S	+S	+B	+B
Clinbritic	B	-	+M	+M	+M	+M	+S	+S	+S	+B	+B
Bottle	C	-	+M	+M	+M	+M	+S	+S	+S	+B	+B
15 ml.	A	-	+M	+M	+M	+M	+S	+S	+S	+B	+B
Eye Drop	B	-	+M	+M	+M	+S	+S	+S	+S	+B	+B
Bottle	C	-	+M	+M	+M	+M	+S	+S	+S	+B	+B

b. Uncoated containers. Buffered solutions.

15 ml.	A	-	+M	+M	+M	+M	+S	+S	+S	+B	+B
Clinbritic	B	-	+M	+M	+M	+M	+S	+S	+S	+B	+B
Bottle	C	-	+M	+M	+M	+M	+S	+S	+S	+B	+B
15 ml.	A	-	+M	+M	+M	+M	+M	+S	+S	+B	+B
Eye Drop	B	-	+M	+M	+M	+M	+S	+S	+S	+B	-
Bottle	C	-	+M	+M	+M	+M	+S	+S	+S	+B	+B

TABLE 31. a. Coated containers. Unbuffered solutions.

15 ml.	A	-	+M	+M	+M	+S	+S	+S	+S	+B	+B
Clinbritic	B	-	+M	+M	+M	+S	+S	+S	+S	+B	+B
Bottle	C	-	+M	+M	+M	+S	+S	+S	+S	+B	-
15 ml.	A	-	+M	+M	+M	+S	+S	+S	+S	+B	+B
Eye Drop	B	-	+M	+M	+M	+S	+S	+S	+S	+B	+B
Bottle	C	-	+M	+M	+M	+S	+S	+S	+S	+B	+B

b. Coated containers. Buffered solutions.

15 ml.	A	-	+M	+M	+M	+S	+S	+S	+S	+B	+B
Clinbritic	B	-	+M	+M	+M	+M	+S	+S	+S	+B	+B
Bottle	C	-	+M	+M	+M	+S	+S	+S	+S	+B	+B
15 ml.	A	-	+M	+M	+M	+M	+S	+S	+S	+B	+B
Eye Drop	B	-	+M	+M	+M	+S	+S	+S	+S	+B	+B
Bottle	C	-	+M	+M	+M	+S	+S	+S	+S	+B	+B

TABLE 32. After autoclaving at 122° for sixty minutes.

a. Uncoated containers. Unbuffered solutions.

Containers	Control Technique	Control No chlorbutol	Test solutions. Time after inoculation 30m.	60m.	90m.	24hr.	48hr.	0·5% chlorbutol. No process. Time after inoculation 90m.	24hr.	48hr.
15 ml.	A	-	+M	+M	+M	+M	+S	+S	+S	+B	+B
Clinbrtic	B	-	+M	+M	+M	+M	+S	+S	+S	+B	+B
Bottle	C	-	+M	+M	+M	+M	+S	+S	+S	+B	+B
15 ml.	A	-	+M	+M	+M	+M	+S	+S	+S	+B	+B
Eye Drop	B	-	+M	+M	+M	+S	+S	+S	+S	+B	+B
Bottle	C	-	+M	+M	+M	+M	+S	+S	+S	+B	+B

b. Uncoated containers. Buffered solutions.

15 ml.	A	-	+M	+M	+M	+M	+S	+S	+S	+B	+B
Clinbritic	B	-	+M	+M	+M	+M	+S	+S	+S	+B	+B
Bottle	C	-	+M	+M	+M	+M	+S	+S	+S	+B	+B
15 ml.	A	-	+M	+M	+M	+M	+S	+S	+S	+B	+B
Eye Drop	B	-	+M	+M	+M	+M	+S	+S	+S	+B	+B
Bottle	C	-	+M	+M	+M	+M	+S	+S	+S	+B	+B

TABLE 33. a. Coated containers. Unbuffered solutions.

15 ml.	A	-	+M	+M	+M	+M	+S	+S	+S	+B	+B
Clinbritic	B	-	+M	+M	+M	+S	+S	+S	+S	+B	+B
Bottles	C	-	+M	+M	+M	+M	+S	+S	+S	+B	+B
15 ml.	A	-	+M	+M	+M	+S	+S	+S	+S	+B	-
Eye Drop	B	-	+M	+M	+M	+M	+S	+S	+S	+B	+B
Bottle	C	-	+M	+M	+M	+M	+S	+S	+S	+B	+B

b. Coated containers. Buffered solutions.

15 ml.	A	-	+M	+M	+M	+S	+S	+S	+S	+B	+B
Clinbritic	B	-	+M	+M	+M	+S	+S	+S	+S	+B	+B
Bottle	C	-	+M	+M	+M	+M	+S	+S	+S	+B	+B
15 ml.	A	-	+M	+M	+M	+M	+S	+S	+B	+B	-
Eye Drop	B	-	+M	+M	+M	+M	+S	+S	+S	+B	+B
Bottle	C	-	+M	+M	+M	+S	+S	+S	+S	+B	+B

A.1.7. Results of solubility determinations.

TABLE 34. 7% chlorbutol in ethanolic solutions.

Percentage concentration of ethanol	Times of shaking in minutes.					
	10	20	30	40	50	60
A. 30%	+	+	+	+	+	-
B. 35%	+	+	+	+	-	-
C. 40%	+	-	-			
D. 45%	+	-	-			

\+ = undissolved chlorbutol present in the flask.

\- = no chlorbutol visible in the flask i.e. the chlorbutol entered solution.

A.1.8. Results of bacteriological tests for self-sterilising properties of 0·5%, 0·6% and 0·7% chlorbutol solutions, of pH 7·4, in 4% ethanol, in coated containers, after autoclaving at 122° for varying periods.

TABLE 35. After autoclaving at 122° for fifteen minutes.

a. 0·5% chlorbutol in 4% ethanol.

Containers		Control Technique	Control No chlorbutol	Test solutions Time after inoculation. 30m.	60m.	90m.	24hr.	48hr.	0·5% chlorbutol. No process. Time after inoculation. 90m.	24hr.	48hr.
15 ml. CBB	A	-	+M	+S	+S	+S	+S	+B	+S	+B	+B
	B	-	+M	+M	+S	+S	+B	+B	+S	+B	+B
	C	-	+M	+S	+S	+S	+B	+B	+S	+B	+B
15 ml. EDB	A	-	+M	+M	+S	+S	+B	+B	+S	+B	+B
	B	-	+M	+M	+S	+S	+B	+B	+S	+B	+B
	C	-	+M	+S	+S	+S	+B	+B	+S	+B	+B

b. 0·6% chlorbutol in 4% ethanol — Control 0·6% chlorbutol

15 ml. CBB	A	-	+M	+M	+S	+S	+B	+B	+S	+B	+B
	B	-	+M	+S	+S	+S	+B	+B	+S	+B	+B
	C	-	+M	+S	+S	+S	+B	+B	+S	+B	+B
15 ml. EDB	A	-	+M	+S	+S	+S	+B	+B	+S	+B	+B
	B	-	+M	+S	+S	+S	+B	+B	+S	+B	+B
	C	-	+M	+S	+S	+S	+B	+B	+S	+B	+B

c. 0·7% chlorbutol in 4% ethanol — Control 0·7% chlorbutol

15 ml. CBB	A	-	+M	+S	+S	+B	+B	+B	+B	+B	-
	B	-	+M	+S	+B	+B	+B	+B	+B	+B	-
	C	-	+M	+S	+S	+S	+B	-	+B	+B	+B
15 ml. EDB	A	-	+M	+S	+B	+B	+B	-	+B	+B	-
	B	-	+M	+S	+S	+B	+B	-	+B	-	-
	C	-	+M	+S	+S	+B	+B	+B	+B	-	-

TABLE 36. After autoclaving at 122° for thirty minutes.

a. 0·5% chlorbutol in 4% ethanol

Containers	Control Technique	Control No chlorbutol	Test solutions Time after inoculation. 30m.	60m.	90m.	24hr.	48hr.	0·5% chlorbutol. No process. Time after inoculation. 90m.	24hr.	48hr.
15 ml. CBB	A	-	+M	+M	+S	+S	+B	+B	+S	+B	+B
	B	-	+M	+M	+M	+S	+B	+B	+S	+B	-
	C	-	+M	+M	+S	+S	+S	+B	+S	+B	+B
15 ml. EDB	A	-	+M	+M	+S	+S	+B	+B	+S	+B	+B
	B	-	+M	+M	+S	+S	+B	+B	+S	+B	+B
	C	-	+M	+M	+S	+S	+B	+B	+S	+B	+B

b. 0·6% chlorbutol in 4% ethanol — Control 0·6% chlorbutol

15 ml. CBB	A	-	+M	+M	+S	+S	+B	+B	+S	+B	+B
	B	-	+M	+M	+S	+S	+B	+B	+S	+B	+B
	C	-	+M	+M	+S	+S	+S	+B	+S	+B	+B
15 ml. EDB	A	-	+M	+M	+S	+S	+B	+B	+S	+B	+B
	B	-	+M	+M	+S	+S	+S	+B	+S	+B	+B
	C	-	+M	+M	+S	+S	+B	+B	+S	+B	+B

c. 0·7% chlorbutol in 4% ethanol — Control 0·7% chlorbutol

15 ml. CBB	A	-	+M	+M	+S	+B	+B	+B	+B	+B	+B
	B	-	+M	+S	+B	+B	+B	+B	+B	-	-
	C	-	+M	+M	+S	+B	+B	+B	+B	+B	-
15 ml. EDB	A	-	+M	+S	+B	+B	+B	+B	+B	-	-
	B	-	+M	+S	+B	+B	+B	+B	+B	+B	+B
	C	-	+M	+M	+S	+B	+B	+B	+B	-	-

TABLE 37. After autoclaving at 122° for forty-five minutes.

a. 0·5% chlorbutol in 4% ethanol.

Containers		Control Technique	Control No chlorbutol	Test solutions Time after inoculation. 30m.	60m.	90m.	24hr.	48hr.	0·5% chlorbutol. No process. Time after inoculation. 90m.	24hr.	48hr.
15 ml.	A	-	+M	+M	+M	+S	+S	+S	+S	+B	+B
CBB	B	-	+M	+M	+M	+M	+S	+S	+S	+B	+B
	C	-	+M	+M	+M	+S	+S	+S	+S	+B	+B
15 ml.	A	-	+M	+M	+M	+M	+S	+S	+S	+B	+B
EDB	B	-	+M	+M	+M	+S	+S	+S	+S	+B	+B
	C	-	+M	+M	+M	+S	+S	+S	+S	+B	+B

b. 0·6% chlorbutol in 4% ethanol — Control 0·6% chlorbutol

15 ml.	A	-	+M	+M	+S	+S	+S	+S	+S	+B	+B
CBB	B	-	+M	+M	+S	+S	+S	+S	+S	+B	+B
	C	-	+M	+M	+S	+S	+S	+S	+S	+B	+B
15 ml.	A	-	+M	+M	+S	+S	+S	+S	+S	+B	+B
EDB	B	-	+M	+M	+S	+S	+S	+S	+S	+B	-
	C	-	+M	+M	+S	+S	+S	+S	+S	+B	+B

c. 0·7% chlorbutol in 4% ethanol — Control 0·7% chlorbutol

15 ml.	A	-	+M	+M	+S	+B	+B	+B	+B	+B	+B
CBB	B	-	+M	+M	+S	+B	+B	+B	+B	+B	-
	C	-	+M	+M	+S	+B	+B	+B	+B	+B	-
15 ml.	A	-	+M	+M	+S	+B	+B	+B	+B	-	-
EDB	B	-	+M	+M	+S	+B	+B	+B	+B	+B	-
	C	-	+M	+M	+S	+B	+B	+B	+B	+B	

A.1.9. Results of bacteriological tests for self-sterilising properties of 0·7% chlorbutol solutions, of pH 5·6 and 3·5 respectively, in 4% ethanol, in coated containers, after autoclaving at 122° for varying periods.

TABLE 38. After autoclaving at 122° for fifteen minutes.

a. At pH 5·6

Containers		Control Technique	Control No chlorbutol	Test solutions Time after inoculation. 30m.	60m.	90m.	24hr.	48hr.	0·7% chlorbutol. No process. Time after inoculation. 90m.	24hr.	48hr.
15 ml.	A	-	+M	+S	+B	+B	+B	-	+B	+B	-
CBB	B	-	+M	+S	+B	-	-	-	+B	+B	-
	C	-	+M	+S	+B	+B	-	-	+B	-	-
15 ml.	A	-	+M	+B	+B	+B	-	-	+B	+B	-
EDB	B	-	+M	+B	+B	+B	-	-	+B	-	-
	C	-	+M	+S	+B	-	+B	-	+B	+B	-
b. At pH 3·5											
15 ml.	A	-	+S	+B	+B	+B	-	-	+B	+B	-
CBB	B	-	+S	+B	+B	-	-	-	+S	-	-
	C	-	+M	+B	+B	-	-	-	+B	+B	-
15 ml.	A	-	+S	+B	+B	-	-	-	+B	-	-
EDB	B	-	+S	+B	+B	+B	-	-	+B	-	-
	C	-	+M	+B	-	-	-	-	+B	-	-

TABLE 39. a. At pH 5·6. After autoclaving at 122° for thirty minutes.

15 ml.	A	-	+M	+S	+B	+B	+B	+B	+B	+B	-
CBB	B	-	+M	+S	+S	+B	+B	-	+B	+B	-
	C	-	+M	+B	+B	+B	+B	+B	+B	-	-
15 ml.	A	-	+M	+S	+B	+B	+B	+B	+B	+B	-
EDB	B	-	+M	+S	+B	+B	-	-	+B	-	-
	C	-	+M	+S	+S	+B	+B	-	+B	+B	-
b. At pH 3·5											
15 ml.	A	-	+S	+B	+B	+B	-	-	+B	+B	-
CBB	B	-	+S	+B	+B	+B	-	-	+B	-	-
	C	-	+M	+B	+B	+B	-	-	+B	-	-
15 ml.	A	-	+M	+B	+B	+B	-	-	+B	-	-
EDB	B	-	+S	+B	+B	+B	-	-	+B	-	-
	C	-	+S	+B	+B	+B	-	-	+B	-	-

TABLE 40 After autoclaving at 122° for forty-five minutes.

a. At pH 5·6

Containers	Control Technique	Control No chlorbutol	Test solutions Time after inoculation 30m.	60m.	90m.	24hr.	48hr.	0·7% chlorbutol. No process. Time after inoculation. 90m.	24hr.	48hr.
15 ml.	A	-	+M	+M	+S	+B	+B	+B	+B	+B	-
CBB	B	-	+M	+M	+S	+B	+B	+B	+B	-	-
	C	-	+M	+S	+B	+B	+B	+B	+B	+B	-
15 ml.	A	-	+M	+S	+S	+B	+B	+B	+B	+B	-
EDB	B	-	+M	+S	+B	+B	+B	+B	+B	-	-
	C	-	+M	+M	+S	+B	+B	-	+B	-	-
b. At pH 3·5											
15 ml.	A	-	+S	+B	+B	+B	+B	-	+B	+B	-
CBB	B	-	+S	+B	+B	+B	-	-	+B	-	-
	C	-	+M	+B	+B	+B	-	-	+B	-	-
15 ml.	A	-	+S	+B	+B	+B	+B	-	+B	-	-
EDB	B	-	+M	+B	+B	+B	-	-	+B	-	-
	C	-	+S	+B	+B	+B	-	-	+B	-	-

A.1.10 Results of bacteriological tests for self-sterilising properties of 0·7% chlorbutol solutions, of pH 7·4, 5·6 and 3·5 respectively, in 4% ethanol, after filtration sterilisation.

TABLE 41.

a. At pH 7·4

Container	Control Technique	Control No chlorbutol	Test solutions Time after inoculation 30m.	60m.	90m.	24hr.	48hr.	0·7% chlorbutol. No process. Time after inoculation. 90m.	24hr.	48hr.
15 ml. CBB	A	-	+M	+S	+B	+B	+B	-	+B	+B	+B
	B	-	+M	+S	+B	+B	+B	-	+B	+B	-
	C	-	+M	+S	+B	+B	+B	-	+B	+B	-
15 ml. EDB	A	-	+M	+S	+B	+B	-	-	+B	+B	-
	B	-	+M	+S	+B	+B	+B	-	+B	+B	-
	C	-	+M	+S	+B	+B	+B	-	+B	-	-

b. At pH 5·6

15 ml. CBB	A	-	+M	+S	+B	+B	+B	-	+B	+B	-
	B	-	+M	+S	+B	+B	+B	-	+B	+B	-
	C	-	+M	+B	+B	+B	-	-	+B	-	-
15 ml. EDB	A	-	+M	+B	+B	+B	-	-	+B	-	-
	B	-	+M	+B	+B	+B	+B	-	+B	+B	-
	C	-	+M	+S	+B	+B	-	-	+B	-	-

c. At pH 3·5

15 ml. CBB	A	-	+S	+B	+B	+B	-	-	+B	-	-
	B	-	+S	+B	+B	+B	-	-	+B	-	-
	C	-	+M	+B	+B	+B	-	-	+B	-	-
15 ml. EDB	A	-	+S	+B	+B	+B	-	-	+B	-	-
	B	-	+M	+B	+B	+B	-	-	+B	-	-
	C	-	+S	+B	+B	+B	-	-	+B	-	-

A.1.11. Results of spectrophotometric assays for residual chlorbutol after filtration sterilisation of 0·7% chlorbutol solutions of pH 7·4, 5·6 and 3·5 respectively.

TABLE 42.

a. At pH 7·4

Containers		Residual chlorbutol (unfiltered)		Residual chlorbutol (filtered)	
15 ml. CBB	A	0·698		0·694	
	B	0·698	Mean = 0·698	0·692	Mean = 0.694
	C	0·698		0·696	
15 ml. EDB	A	0·698		0·696	
	B	0·698	Mean = 0·698	0·694	Mean = 0·694
	C	0·696		0·692	
b. At pH 5·6					
15 ml. CBB	A	0·698		0·694	
	B	0·698	Mean = 0·698	0·698	Mean = 0·696
	C	0·698		0·696	
15 ml. EDB	A	0·700		0·696	
	B	0·702	Mean = 0·700	0·698	Mean = 0·697
	C	0·698		0·698	
c. At pH 3·5					
15 ml. CBB	A	0·700		0·698	
	B	0·702	Mean = 0.701	0·696	Mean = 0·697
	C	0·700		0·698	
15 ml. EDB	A	0·700		0·700	
	B	0·702	Mean = 0·700	0·696	Mean = 0·699
	C	0·698		0·700	

d. Calculation.

Original concentration of chlorbutol = 0·700%

Maximum reduction after filtration = 0·004%

Therefore percentage reduction due to filtration

$$= \frac{0{\cdot}004}{0{\cdot}700} \times 100$$

$$= 0{\cdot}5715\%$$

TABLE 43. Results of phenyl isocyanide test for the determination of chloroform.

Standard Solutions = SS

a. 0·025% chloroform in 4% ethanol

b. 0·7% chlorobutanol in 4% ethanol

Test Solutions = TS i - viii as specified in Diagram 4.

TDS i - viii.

Solutions	A	B	C
SS a	+ve	+ve	+ve
SS b	-ve	-ve	-ve
TS i	-ve	-ve	-ve
TS ii	-ve	-ve	-ve
TS iii	+ve	+ve	+ve
TS iv	+ve	+ve	+ve
TS v	-ve	-ve	-ve
TS vi	-ve	-ve	-ve
TS vii	+ve	+ve	+ve
TS viii	+ve	+ve	+ve

TABLE 44, pH values of 0·7% chlorobutol in 4% ethanol solutions before and after autoclaving at 122° for 45 minutes.

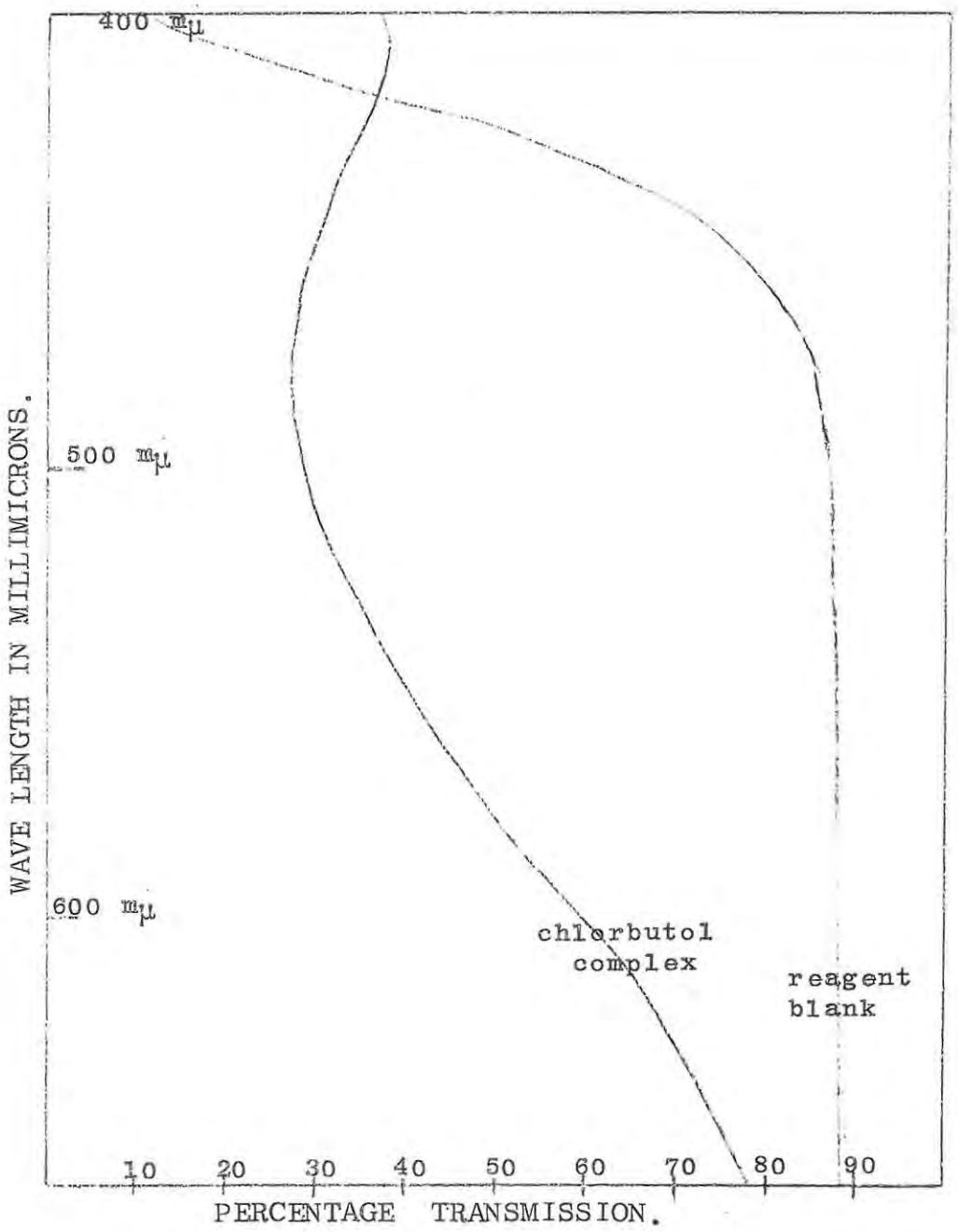
Test Solutions = TS i - viii as specified in Diagram 4.
TDS i - viii.

Solution	Buffered		Unbuffered.	
	pH before	pH after	pH before	pH after.
TS i	7·43	UP	7·45	UP
TS ii	5·62	UP	5·65	UP
TS iii	7·44	6·73	7·46	3·12
TS iv	5·63	5·15	5·64	3·03
TS v	7·40	UP	7·41	UP
TS vi	5·61	UP	5·60	UP
TS vii	7·40	6·38	7·40	2·81
TS viii	5·60	4·95	5·60	2·52

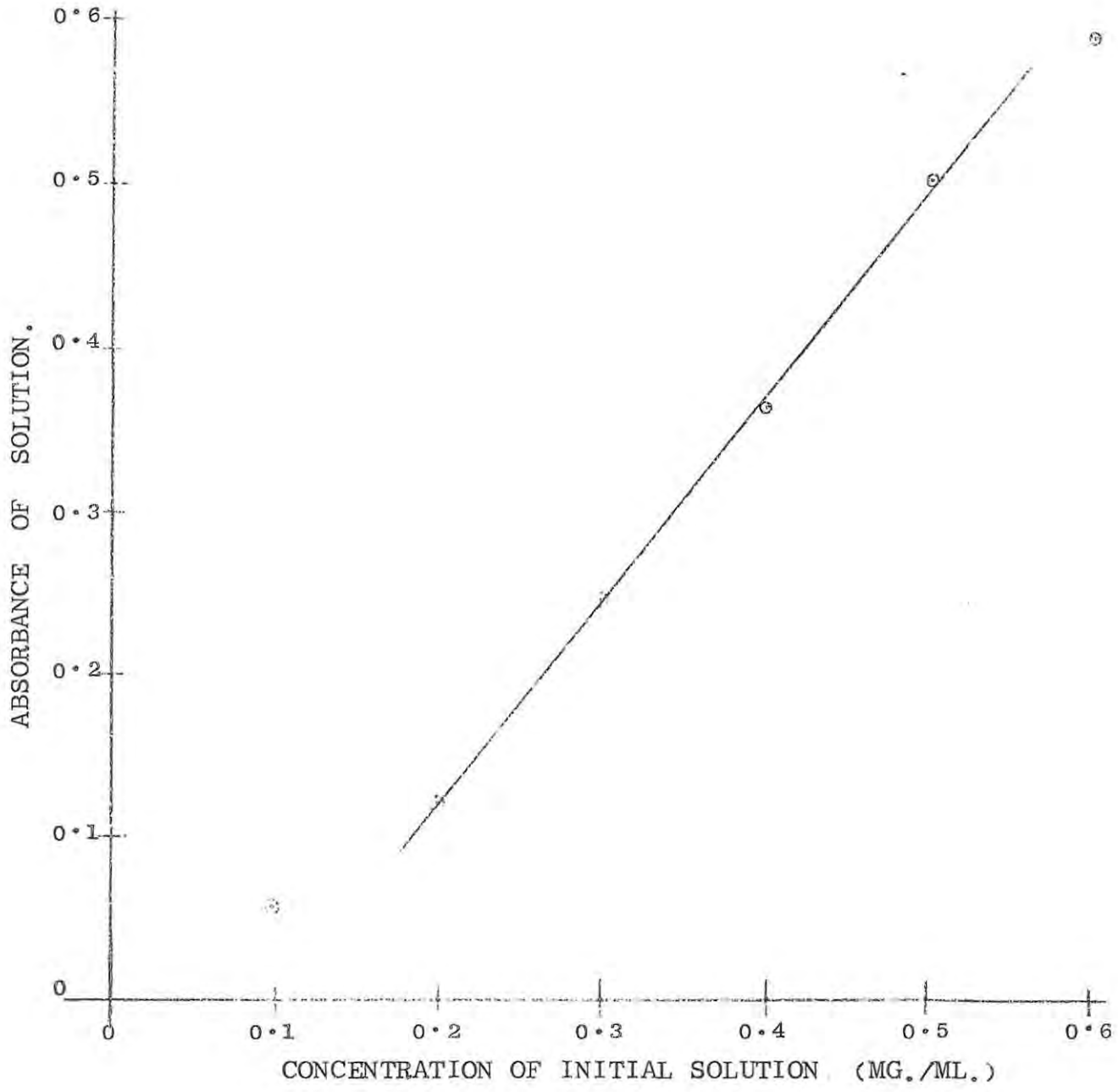
UP = unprocessed solutions.
The pH values given in the table are the mean of three determinations.

A. 2. GRAPHS.

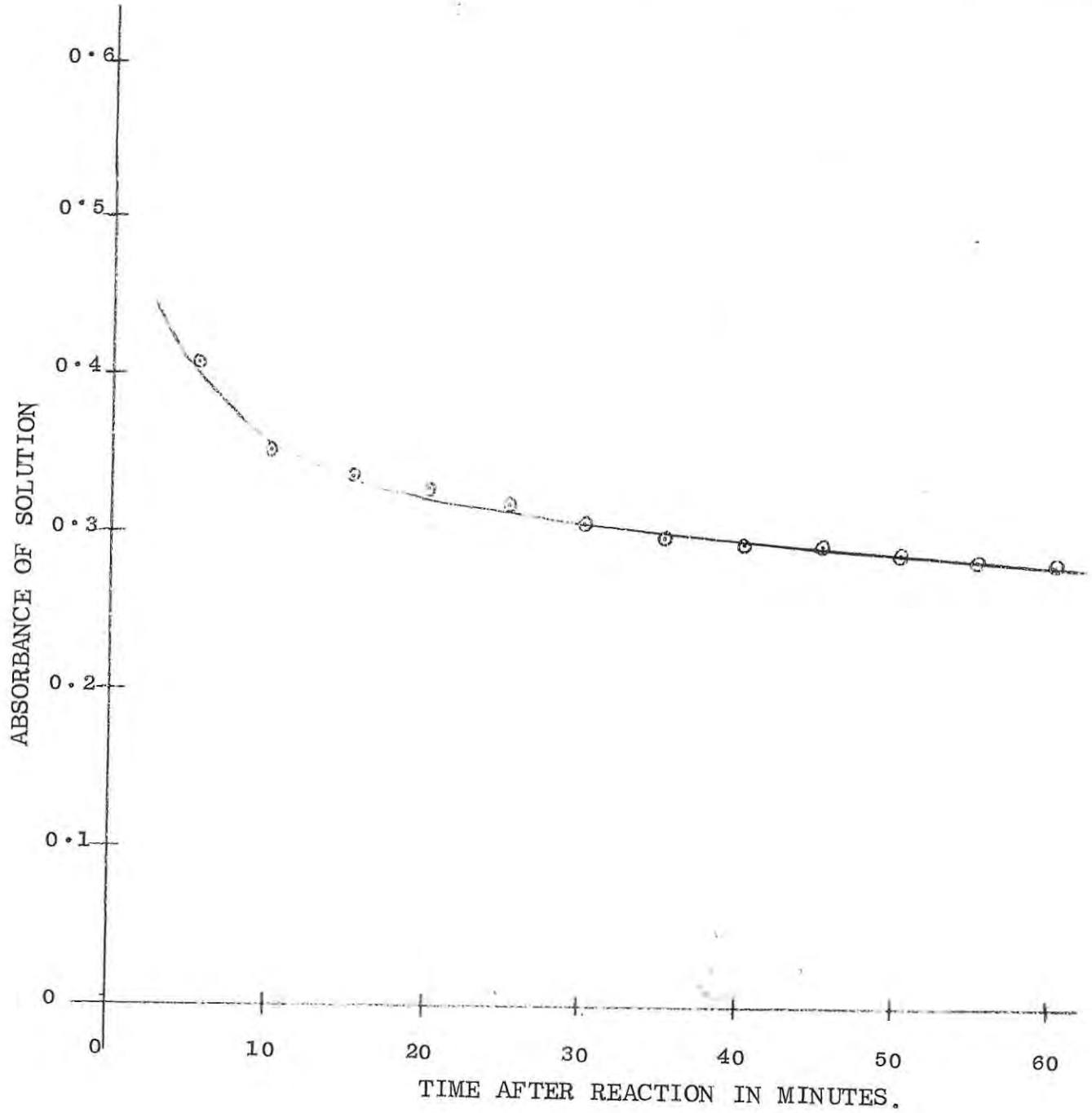
GRAPH 1. Absorbance spectrum of the ferric-hydroxamic derivative of chlorbutol.



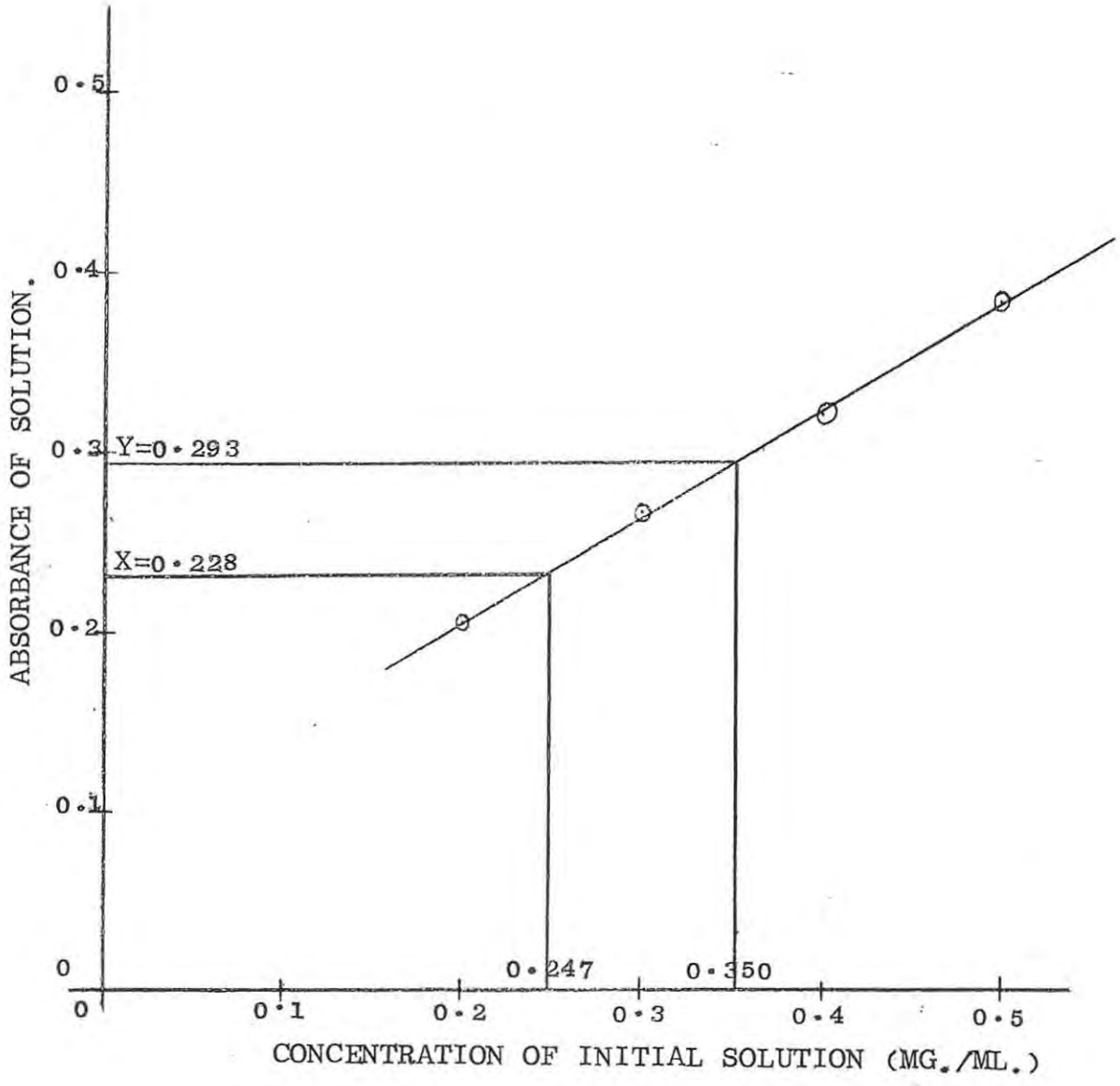
GRAPH 2. Absorbance/concentration plot of the ferric-hydroxamic derivative of chlorbutol.



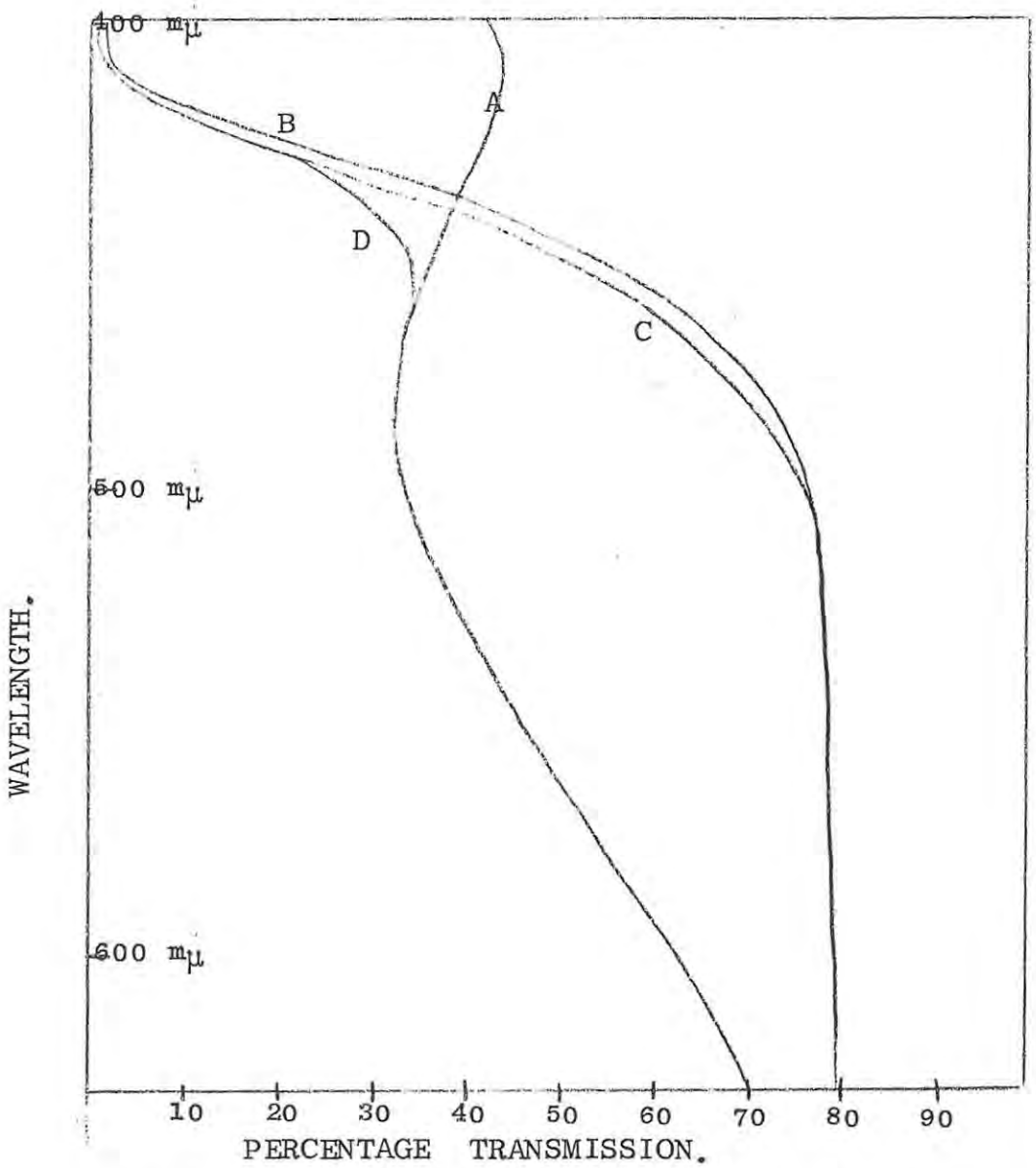
GRAPH 3. Absorbance/time plot of the ferric-hydroxamic derivative of chlorbutol.



GRAPH 4. Trial assay of test solutions of chlorbutol.



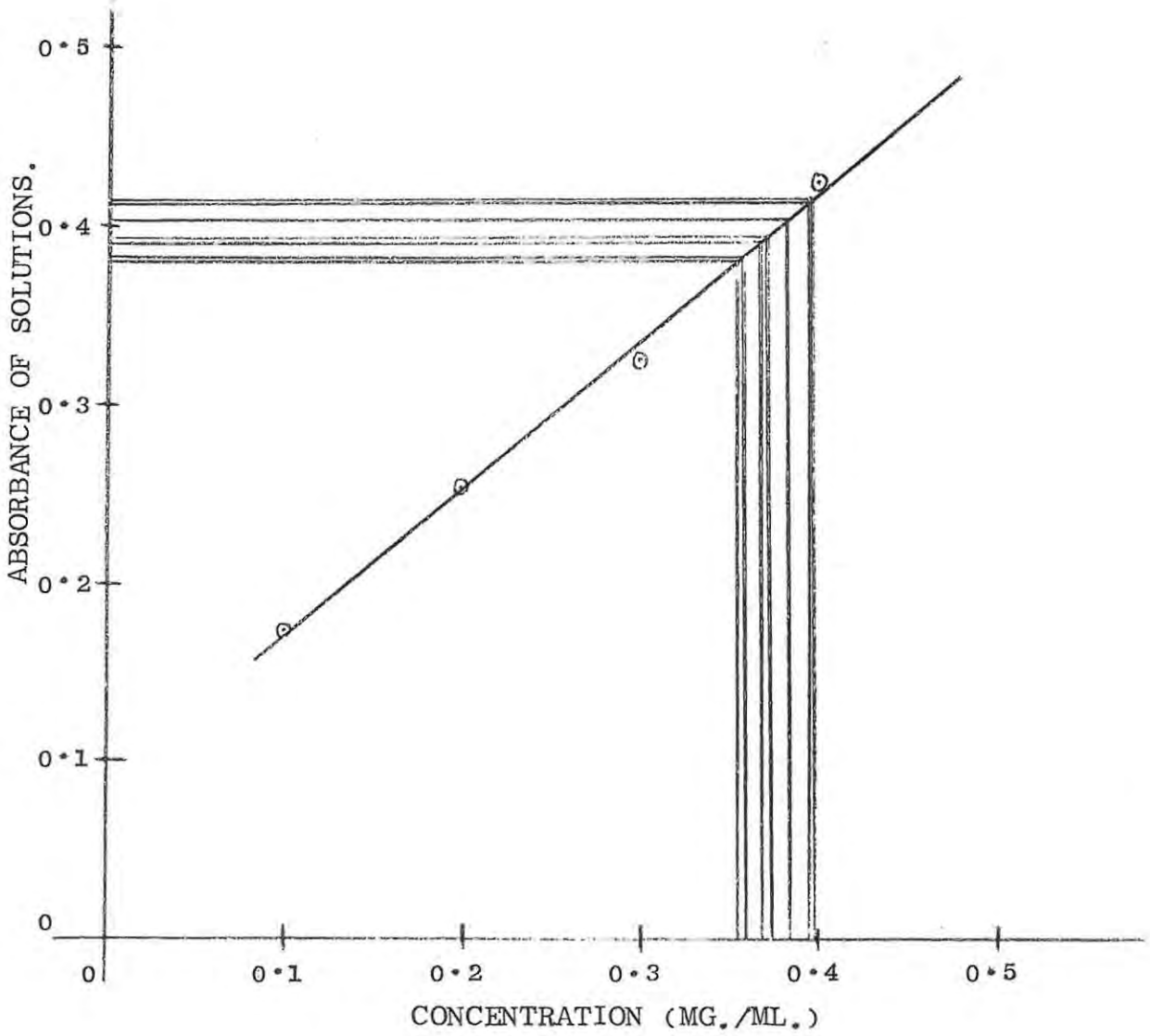
GRAPH 5. Investigation of possible interference by α-hydroxy-isobutyric acid in absorbance spectrum of ferric-hydroxamic complex of chlorbutol.



Absorbance spectra of:

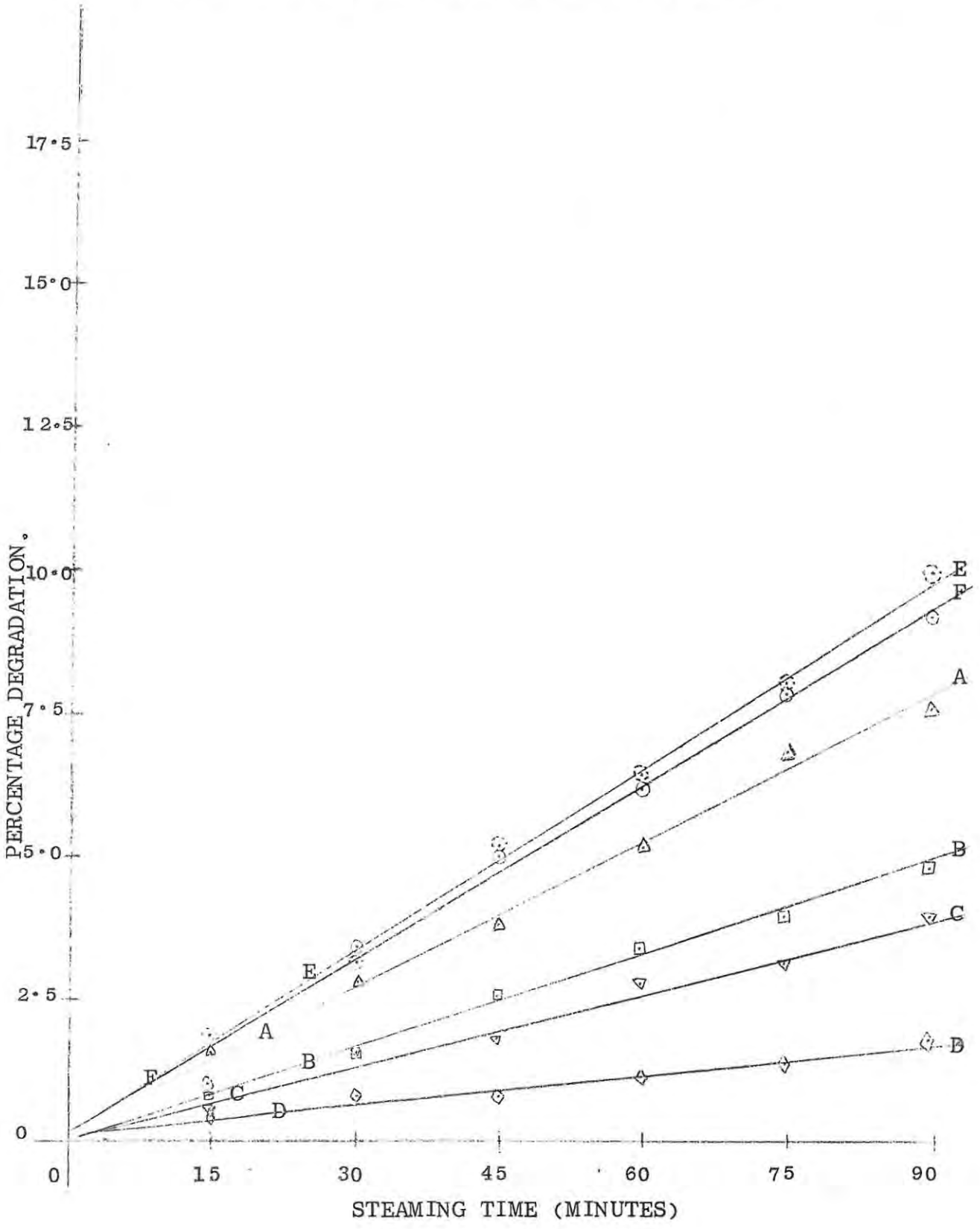
A. Standard chlorbutol solution.
B. Reagent blank.
C. Reagent blank + α-hydroxyisobutyric acid 5 mg./ml.
D. Standard chlorbutol solution + α-hydroxyiso-butyric acid. 5 mg./ml.

GRAPH 6. Graphical method for the determination of residual chlorbutol in solution by utilising a calibration curve (See TABLE 10). After steaming 0·5% solutions of chlorbutol, of pH 3·5, in 15 ml. eye-drop bottles for varying periods.



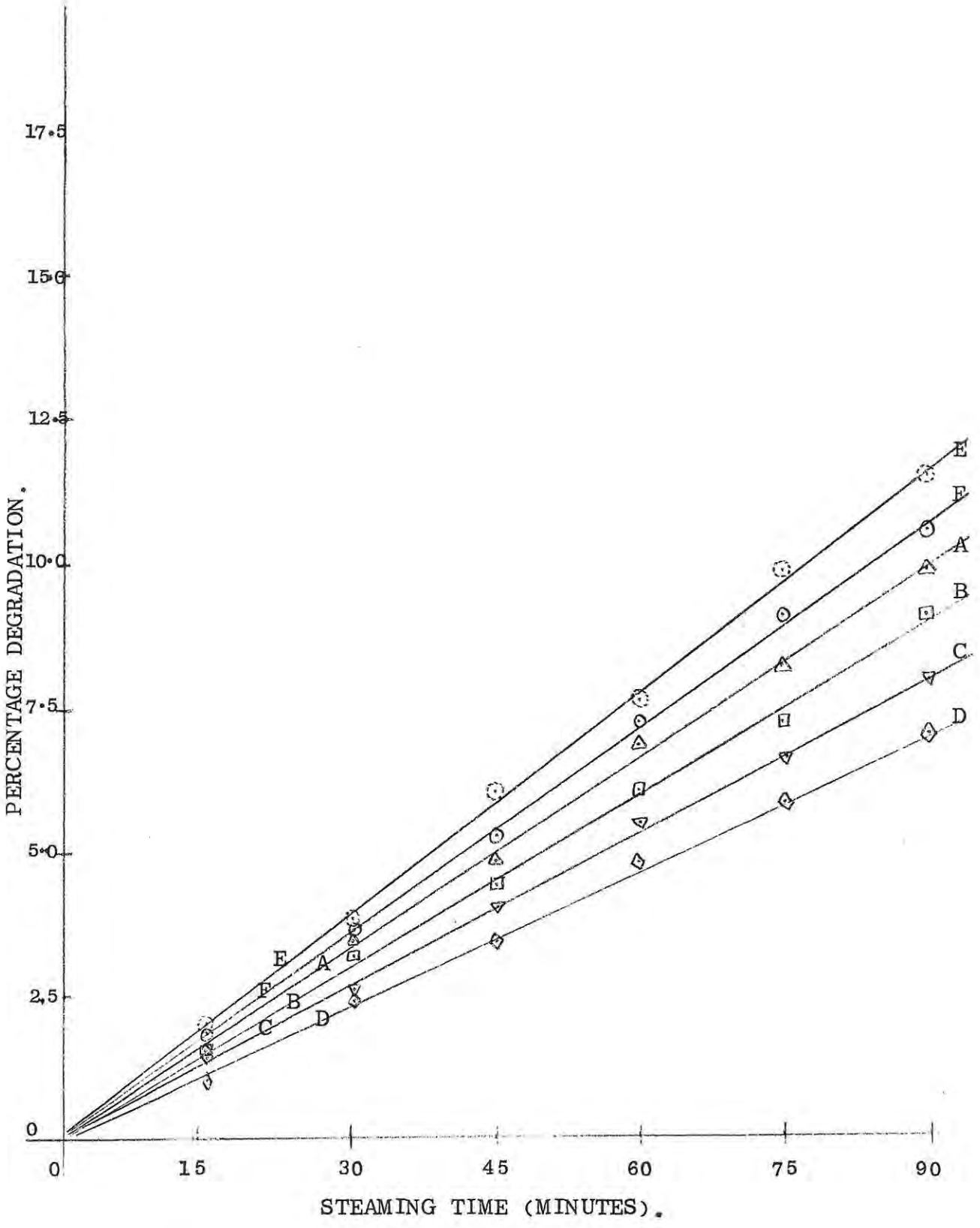
GRAPH 7. Unbuffered solution pH 3·5

Percentage degradation/time of steaming plot



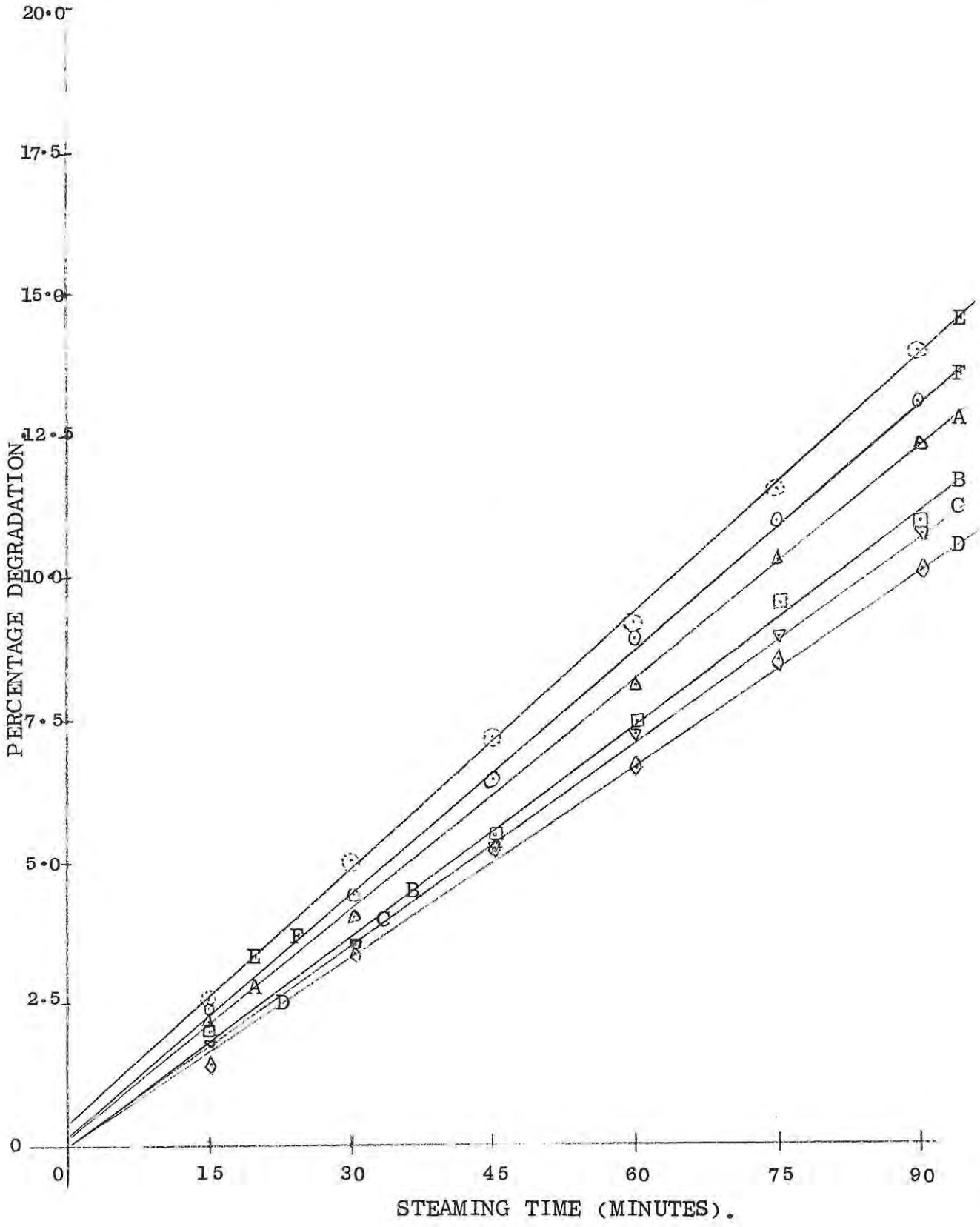
GRAPH 8. Unbuffered solutions pH 5·6

Percentage degradation/time of steaming plot.



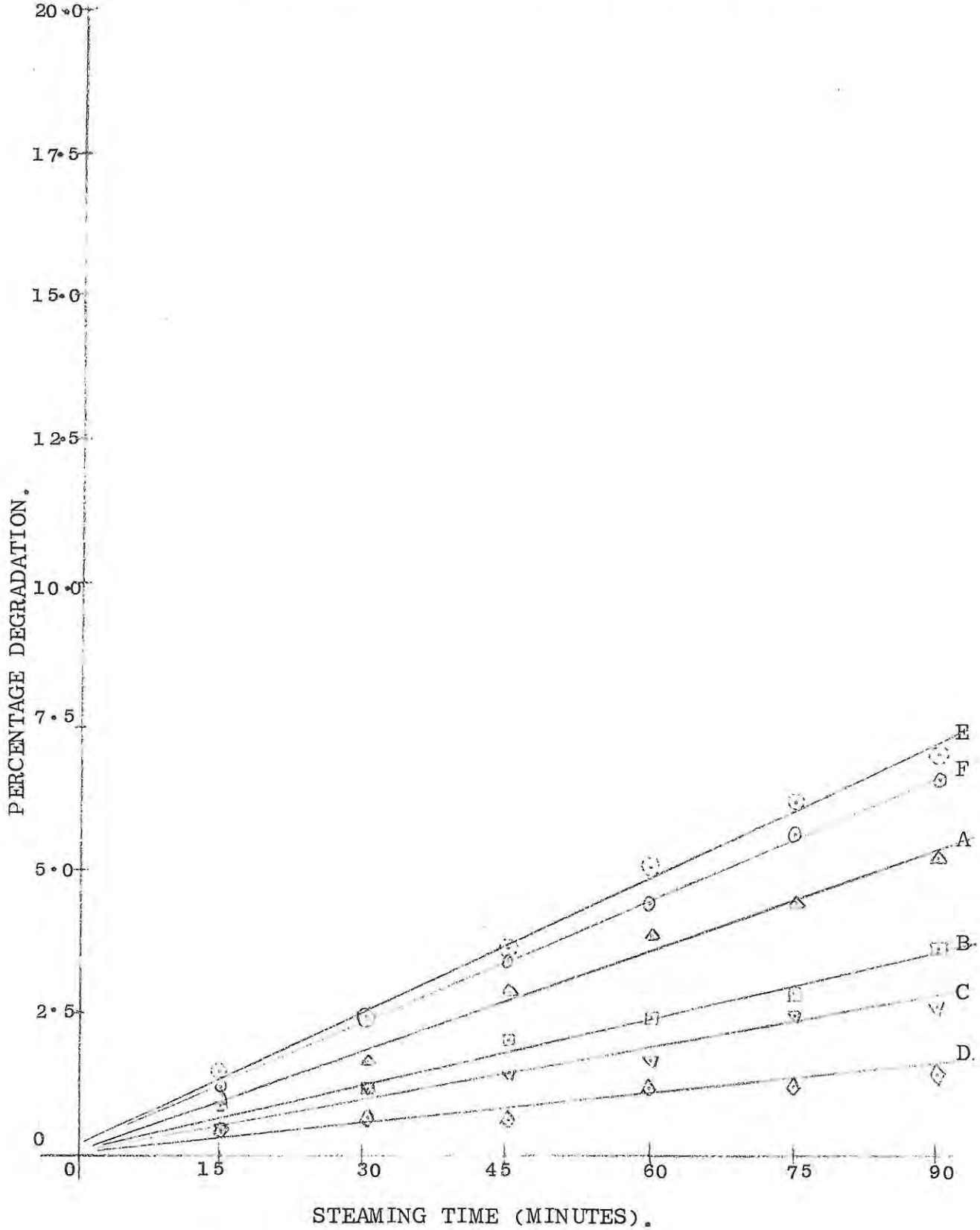
GRAPH 9. Unbuffered solution pH 7·4

Percentage degradation/time of steaming plot.



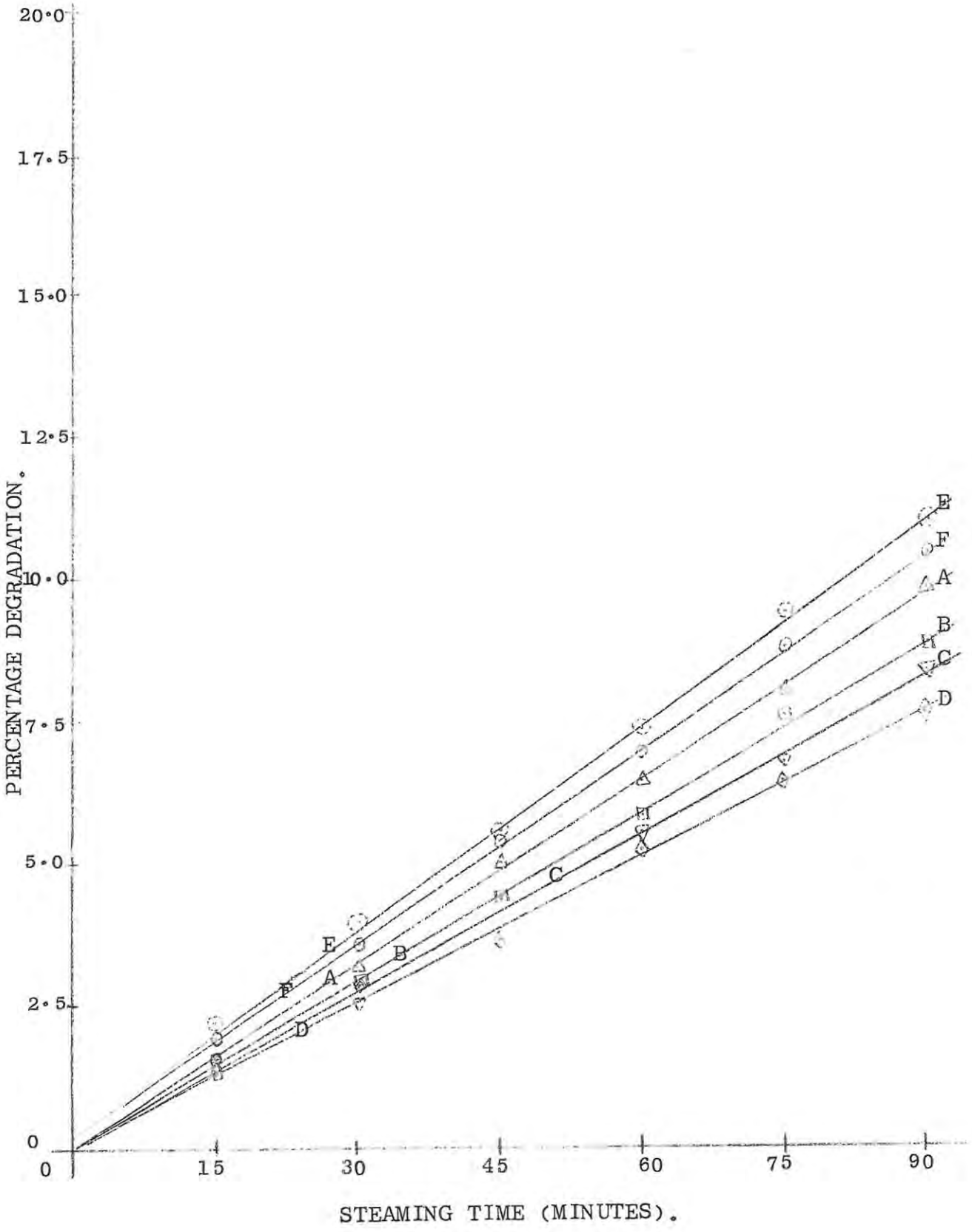
GRAPH 10. Buffered Solution pH 3·5.

Percentage degradation/time of steaming plot.



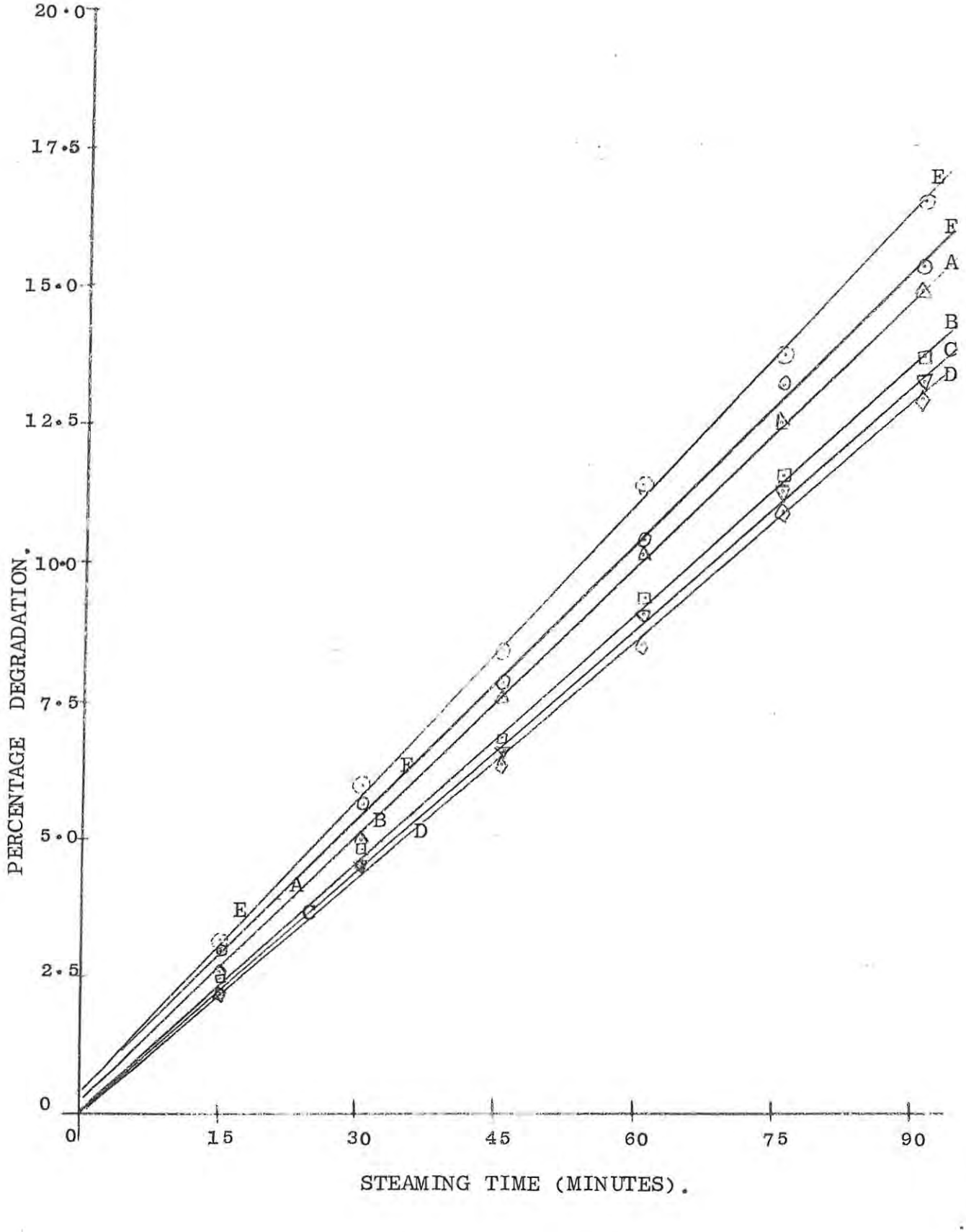
GRAPH 11. Buffered solution. pH 5·6

Percentage degradation/time of steaming plot.



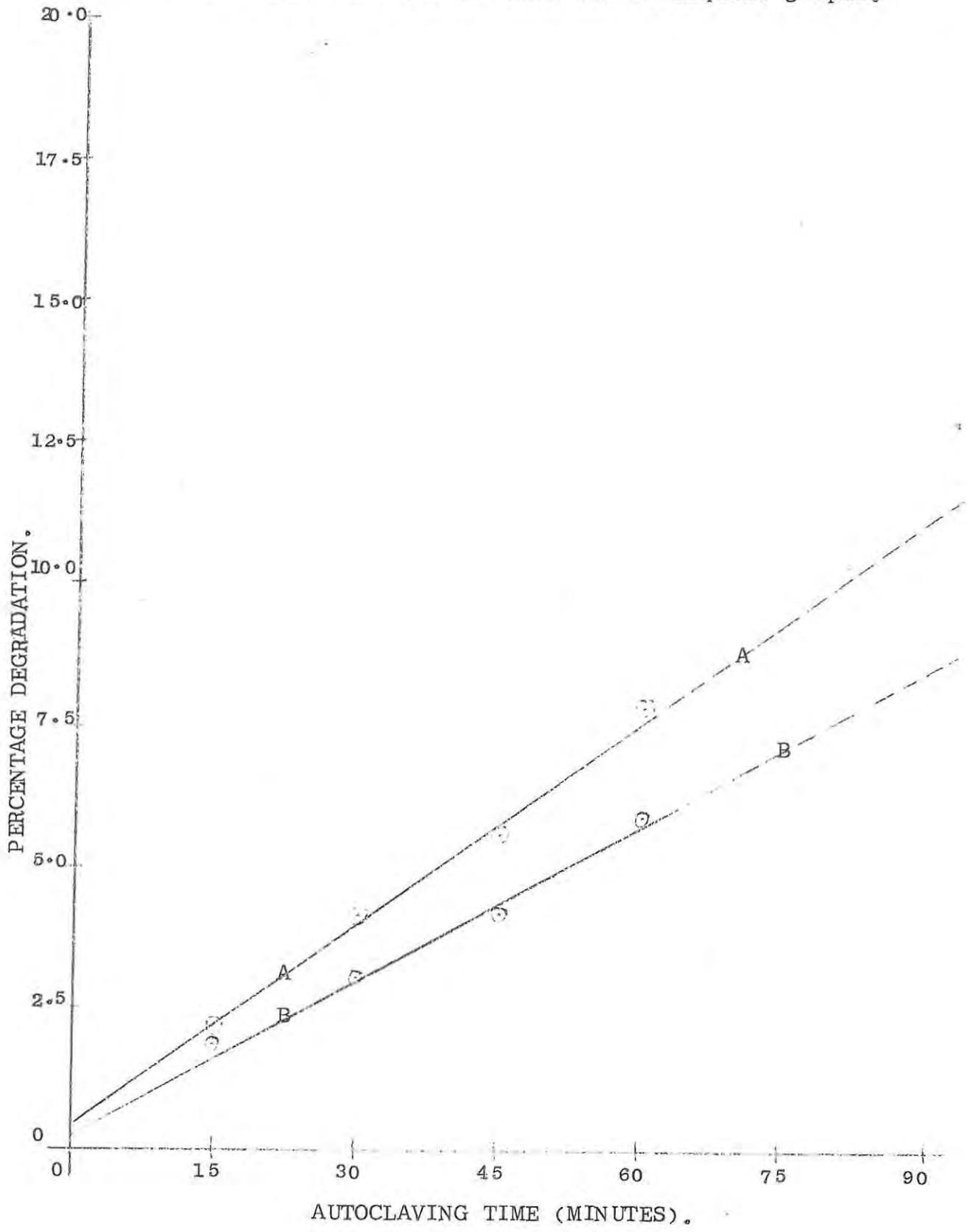
GRAPH 12. Buffered solution. pH 7·4

Percentage degradation/time of steaming plot.



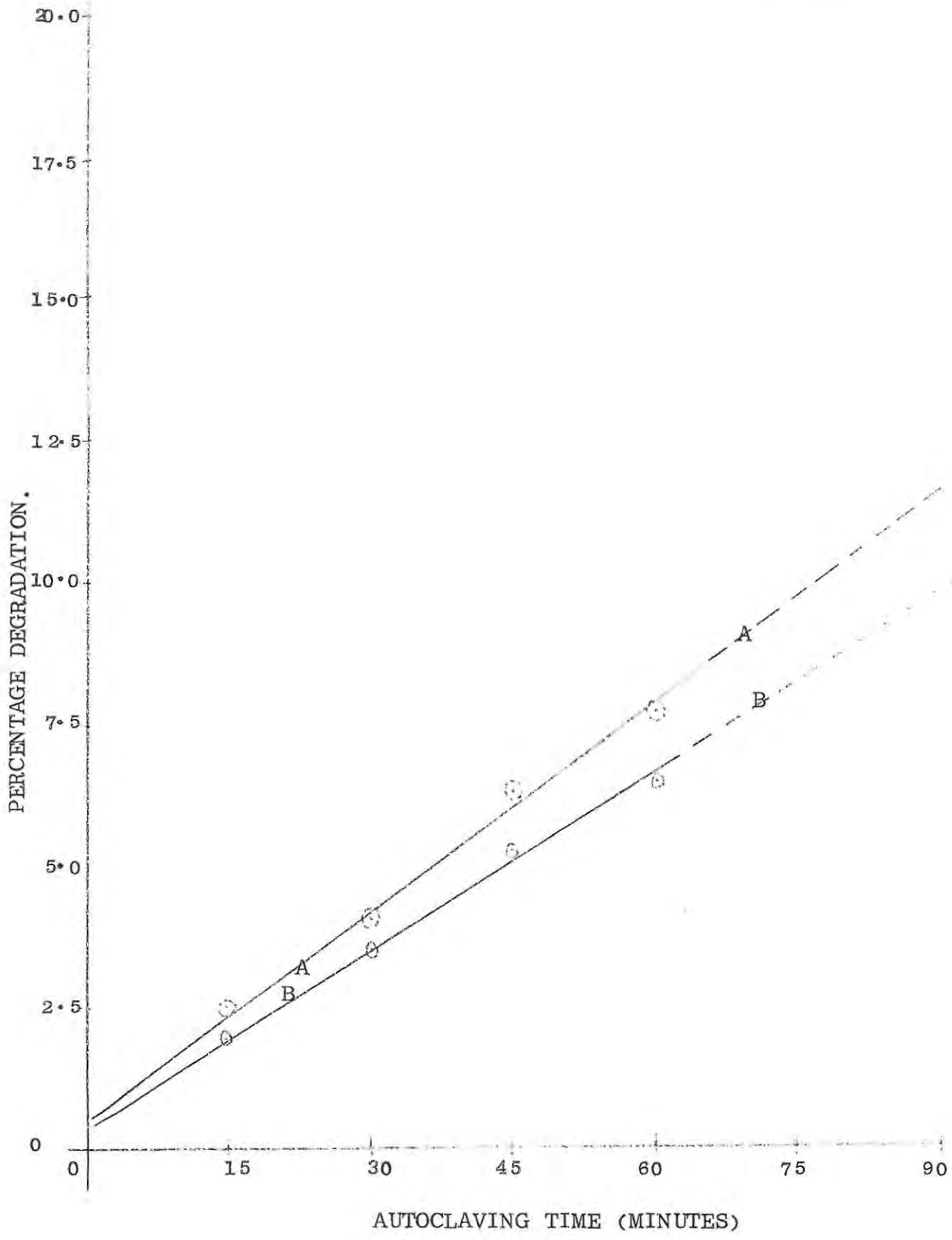
GRAPH 13. Unbuffered solution. pH 3·5

Percentage degradation/time of autoclaving at 115°.
A = CBB. B = EDB in this and subsequent graphs.



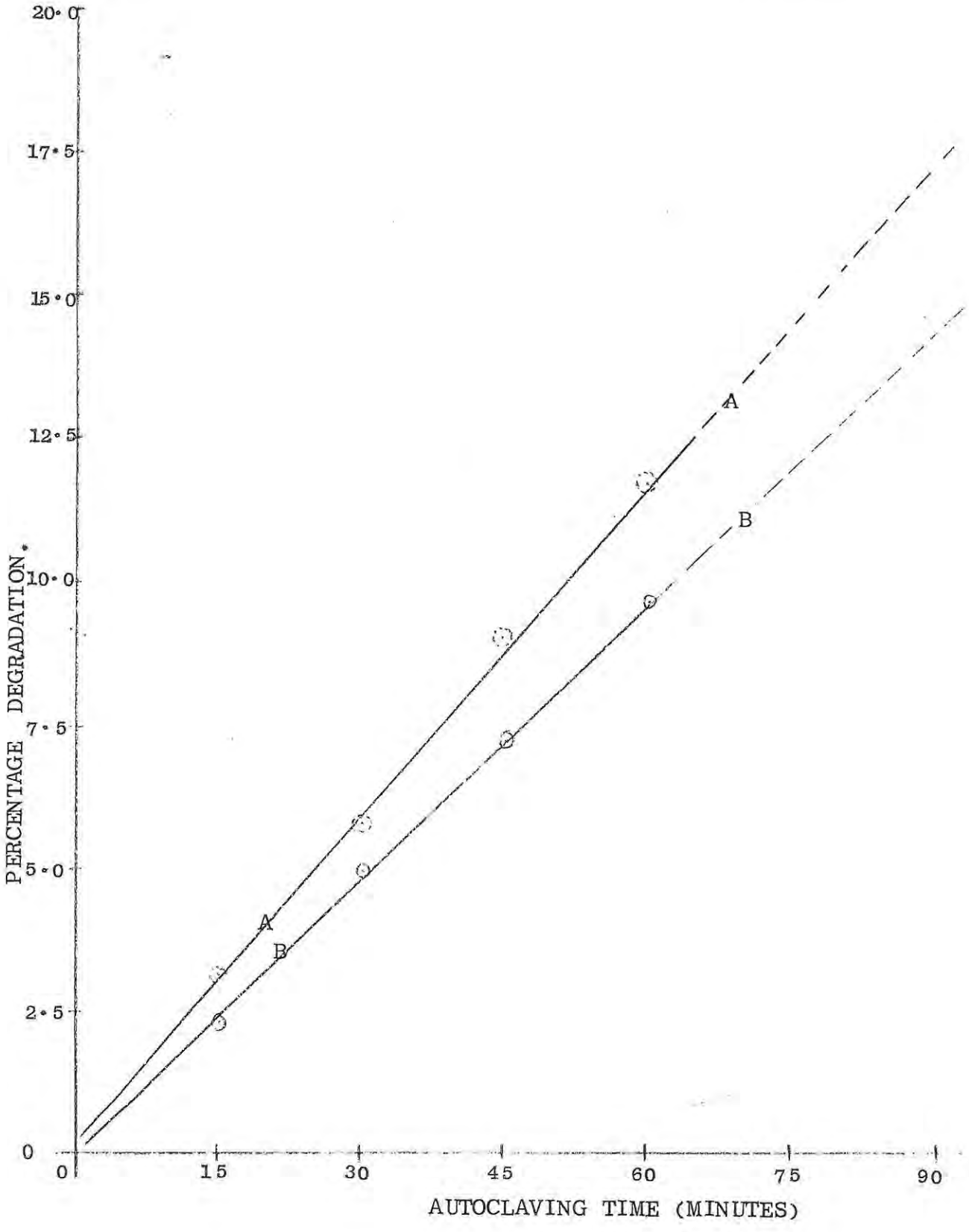
GRAPH 14. Unbuffered solution. pH 5·6.

Percentage degradation/time of autoclaving at 115°.



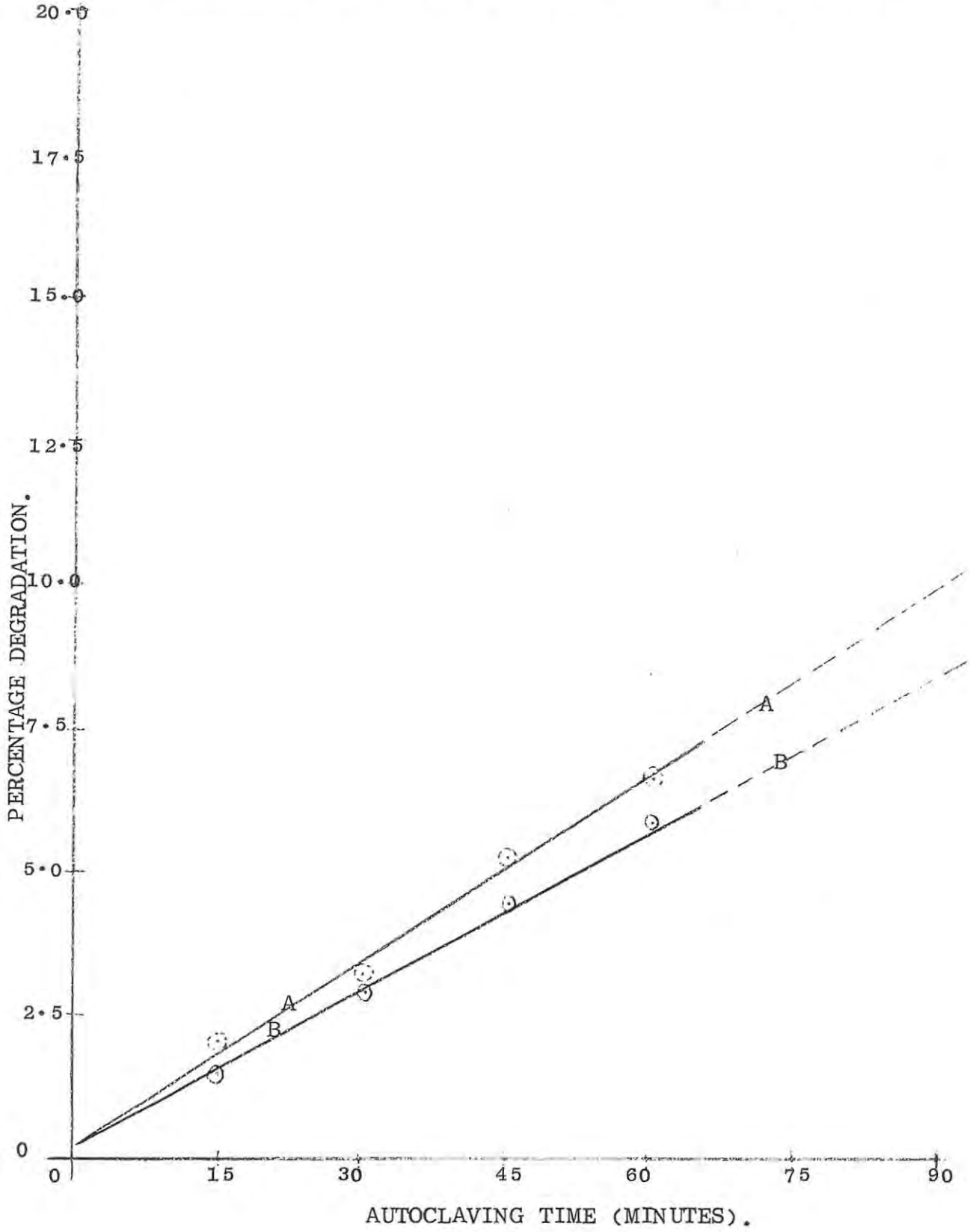
GRAPH 15. Unbuffered solution. pH 7·4

Percentage degradation/time of autoclaving at 115°.



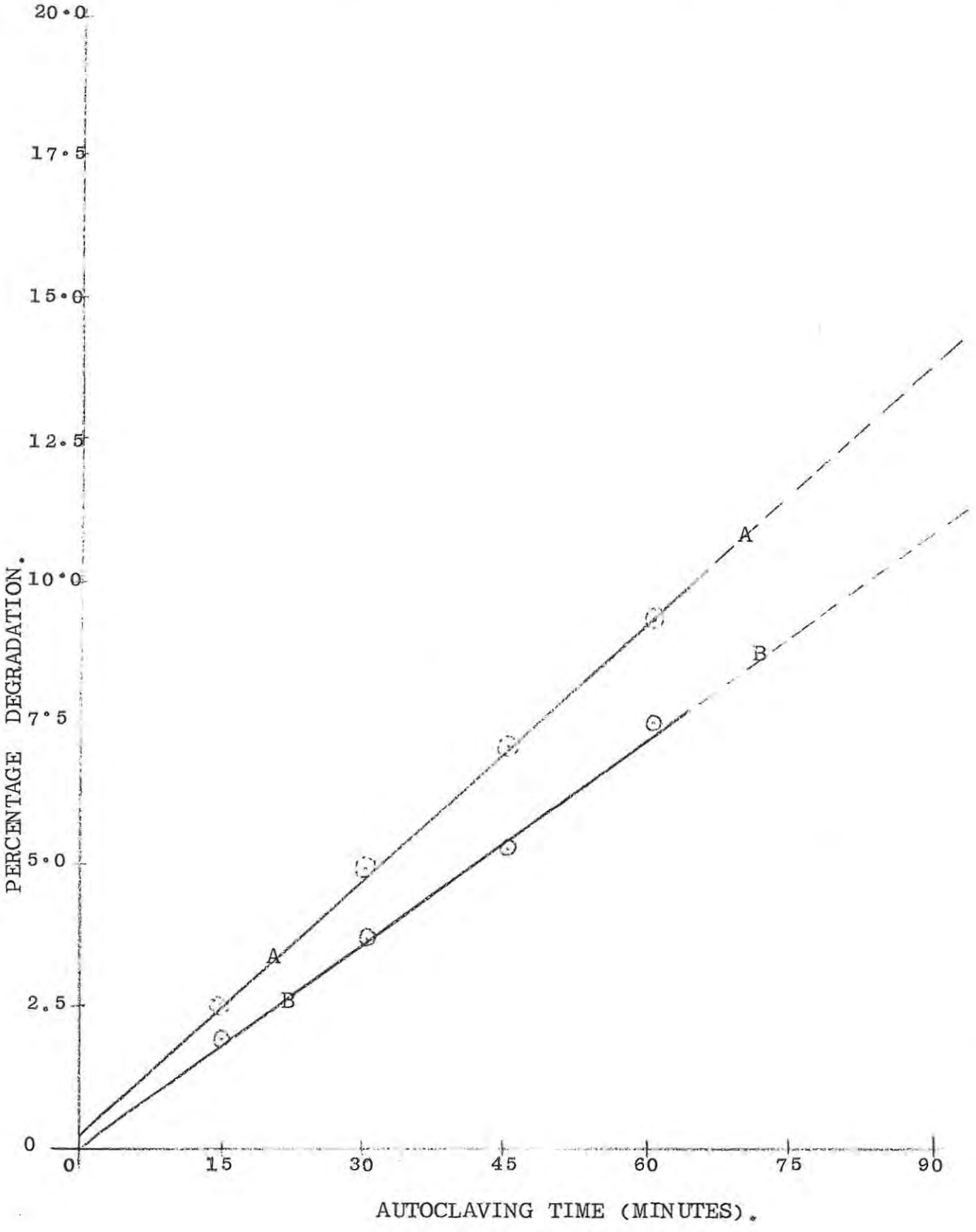
GRAPH 16. Buffered solution. pH 3·5.

Percentage degradation/time of autoclaving at 115°.

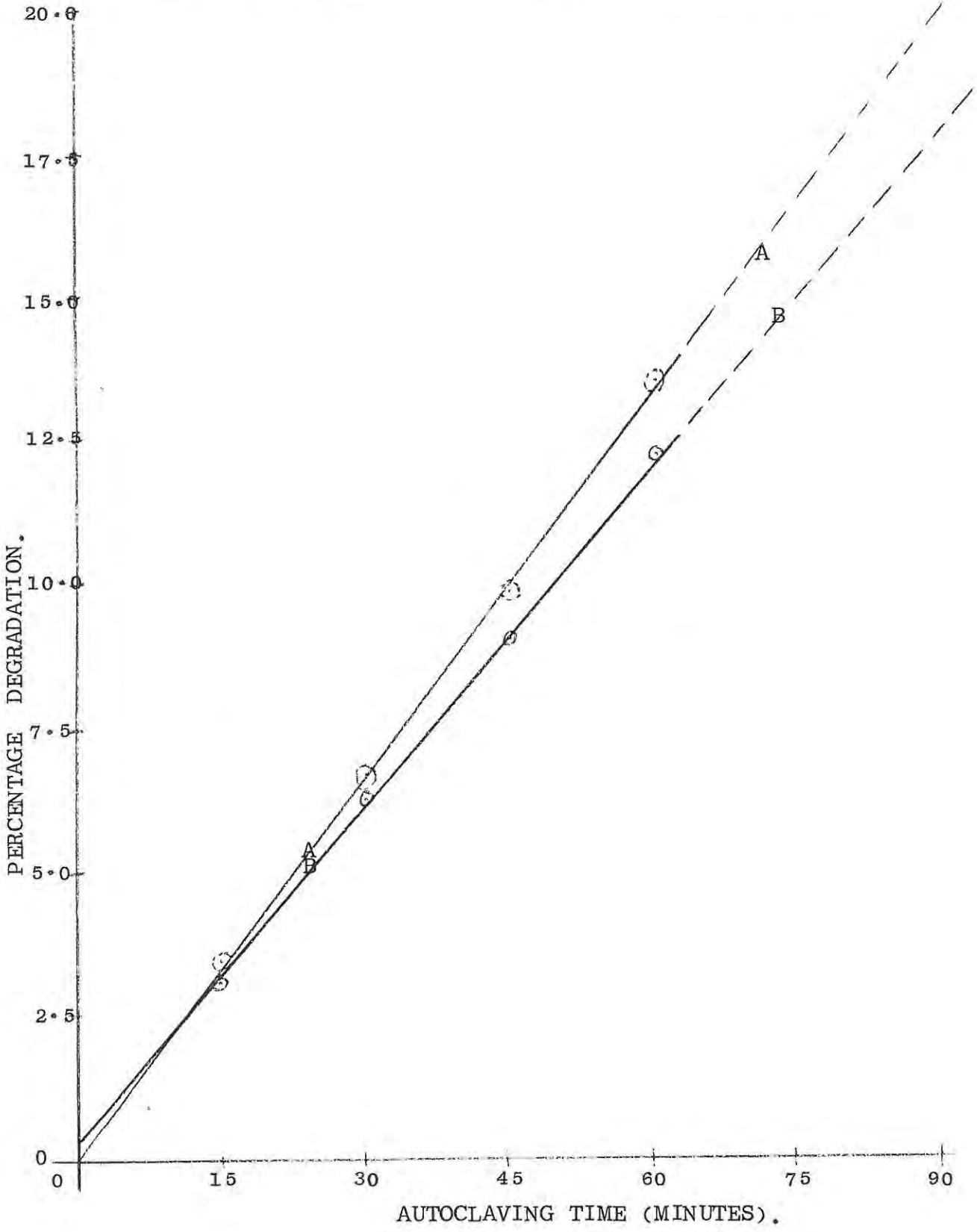


GRAPH 17. Buffered solution, pH 5·6

Percentage degradation/time of autoclaving at 115°.

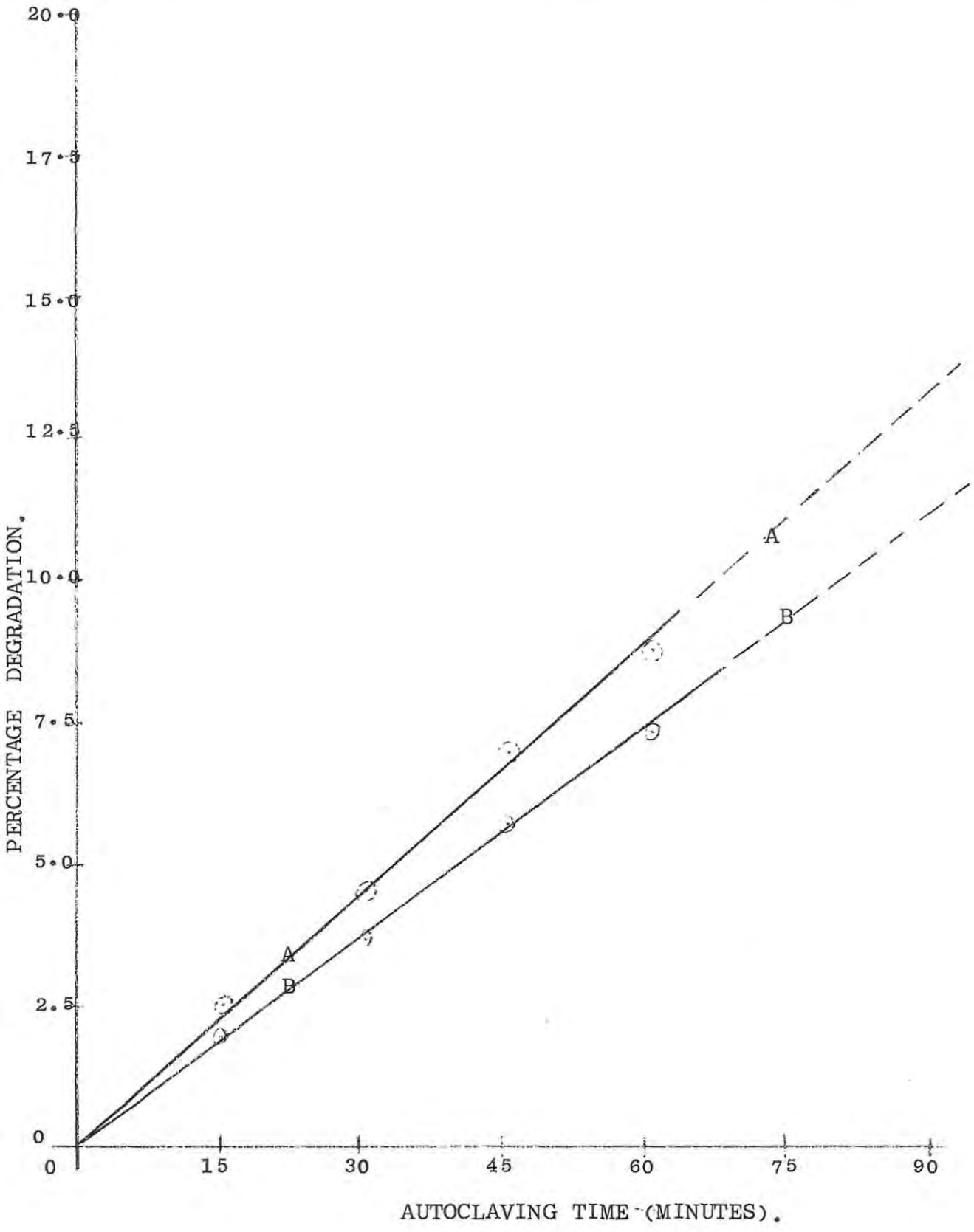


GRAPH 18. Buffered solution. pH 7·4.
Percentage degradation/time of autoclaving at 115°.



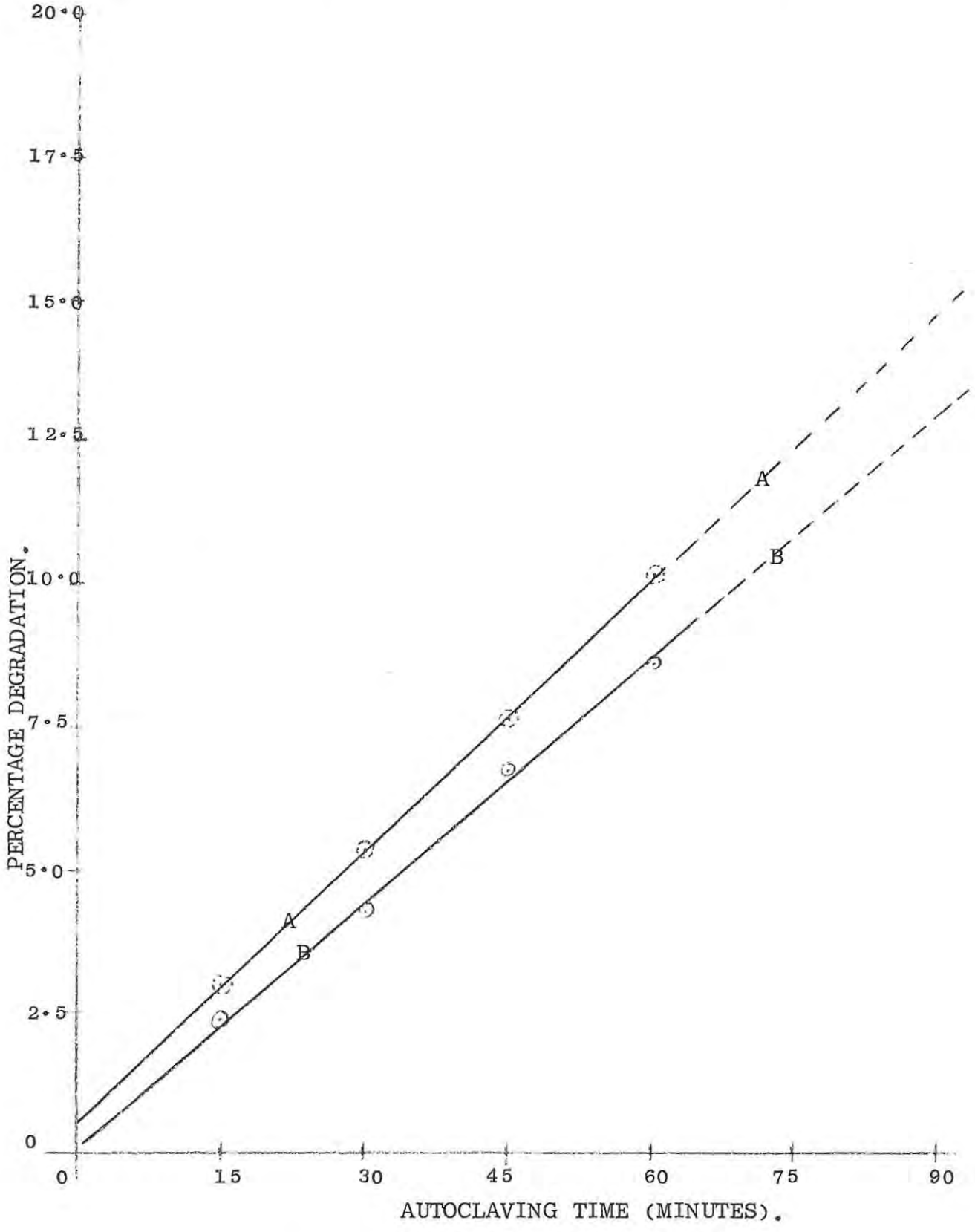
GRAPH 19. Unbuffered solution. pH 3·5

Percentage degradation/time of autoclaving at 122°.



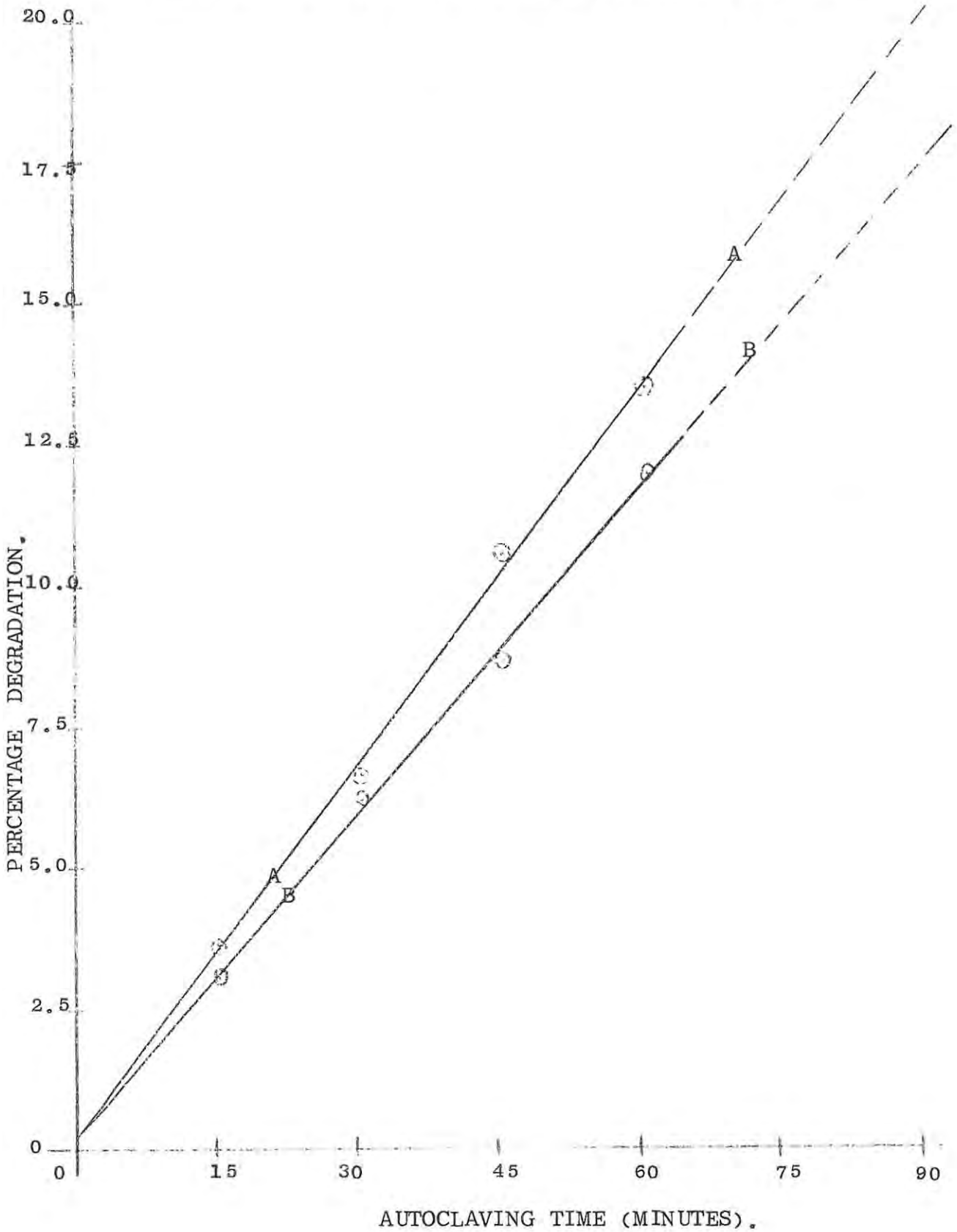
GRAPH 20. Unbuffered solution. pH 5·6

Percentage degradation/time of autoclaving at 122°.



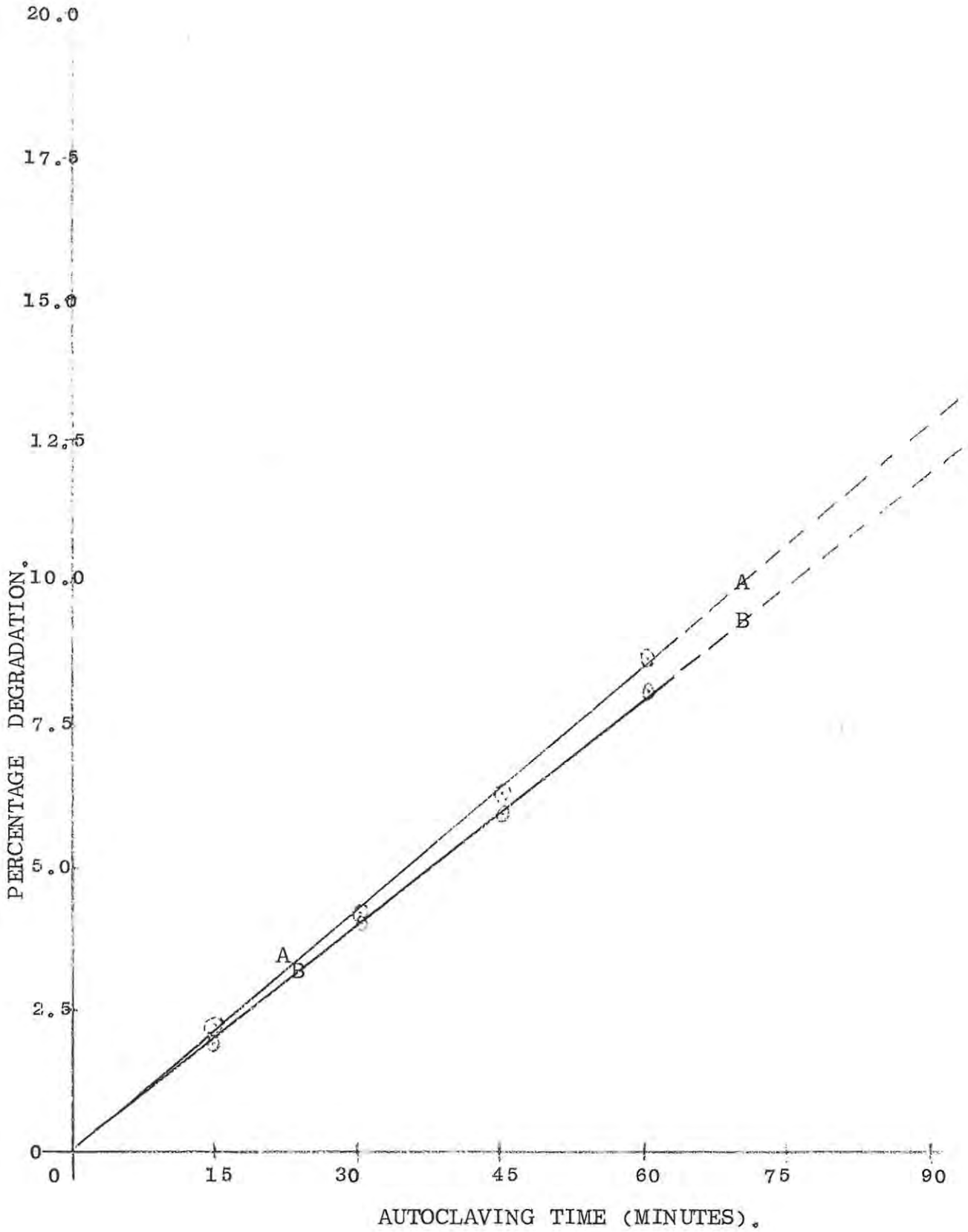
GRAPH 21. Unbuffered solution. pH 7·4

Percentage degradation/time of autoclaving at 122°.

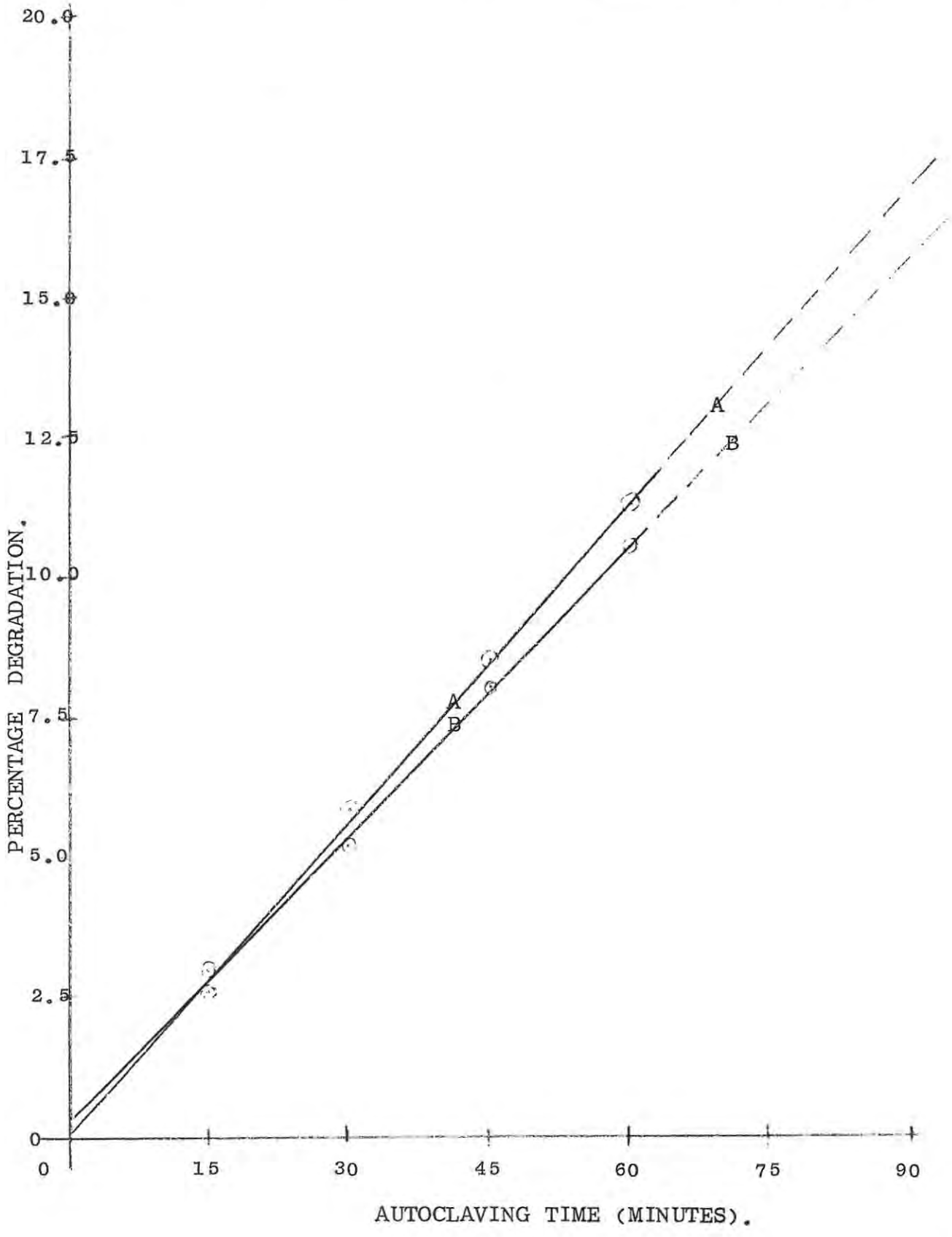


GRAPH 22. Buffered solution. pH 3·5

Percentage degradation/time of autoclaving at 122°.

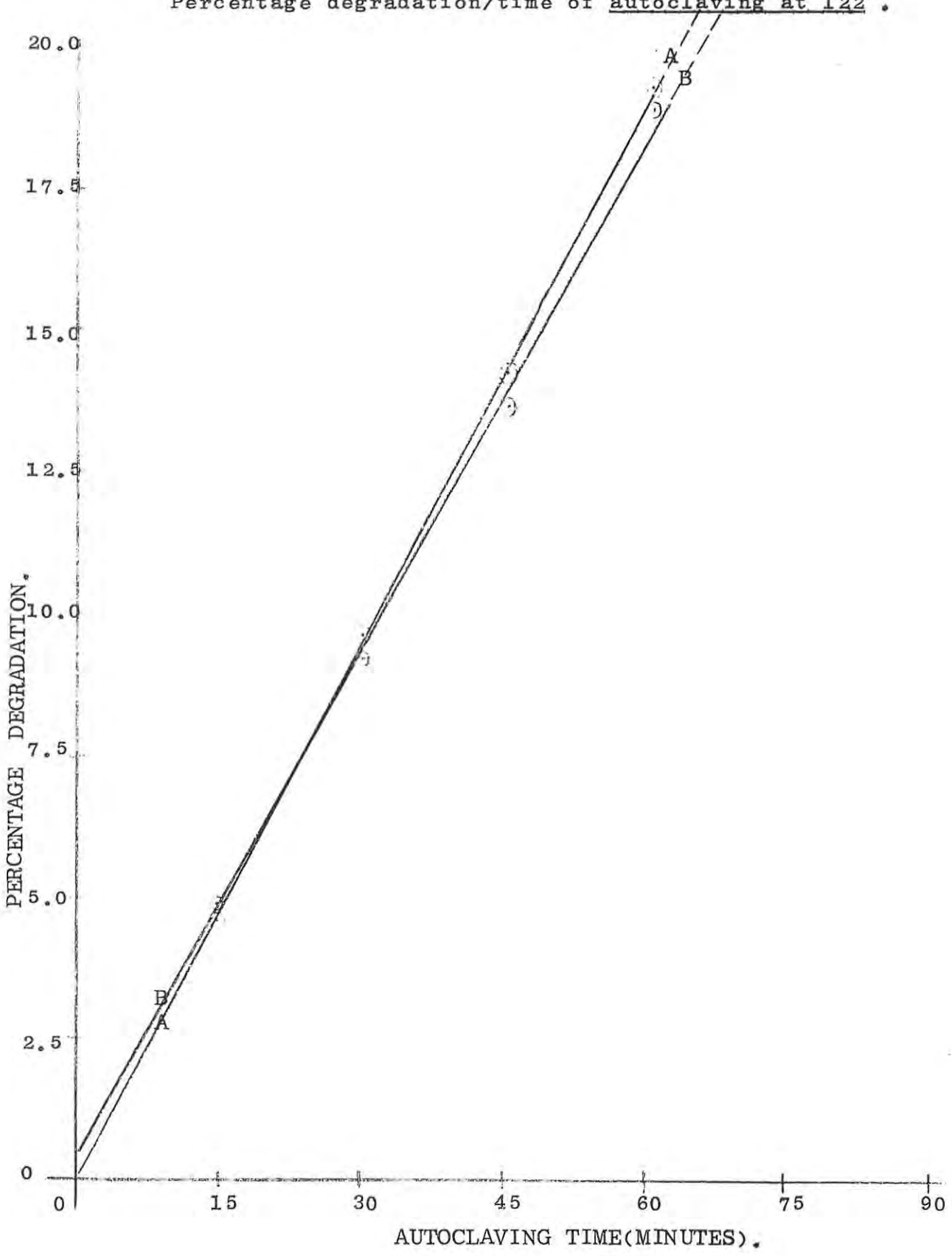


GRAPH 23. Buffered solution. pH 5·6.
Percentage degradation/time of autoclaving at 122°.

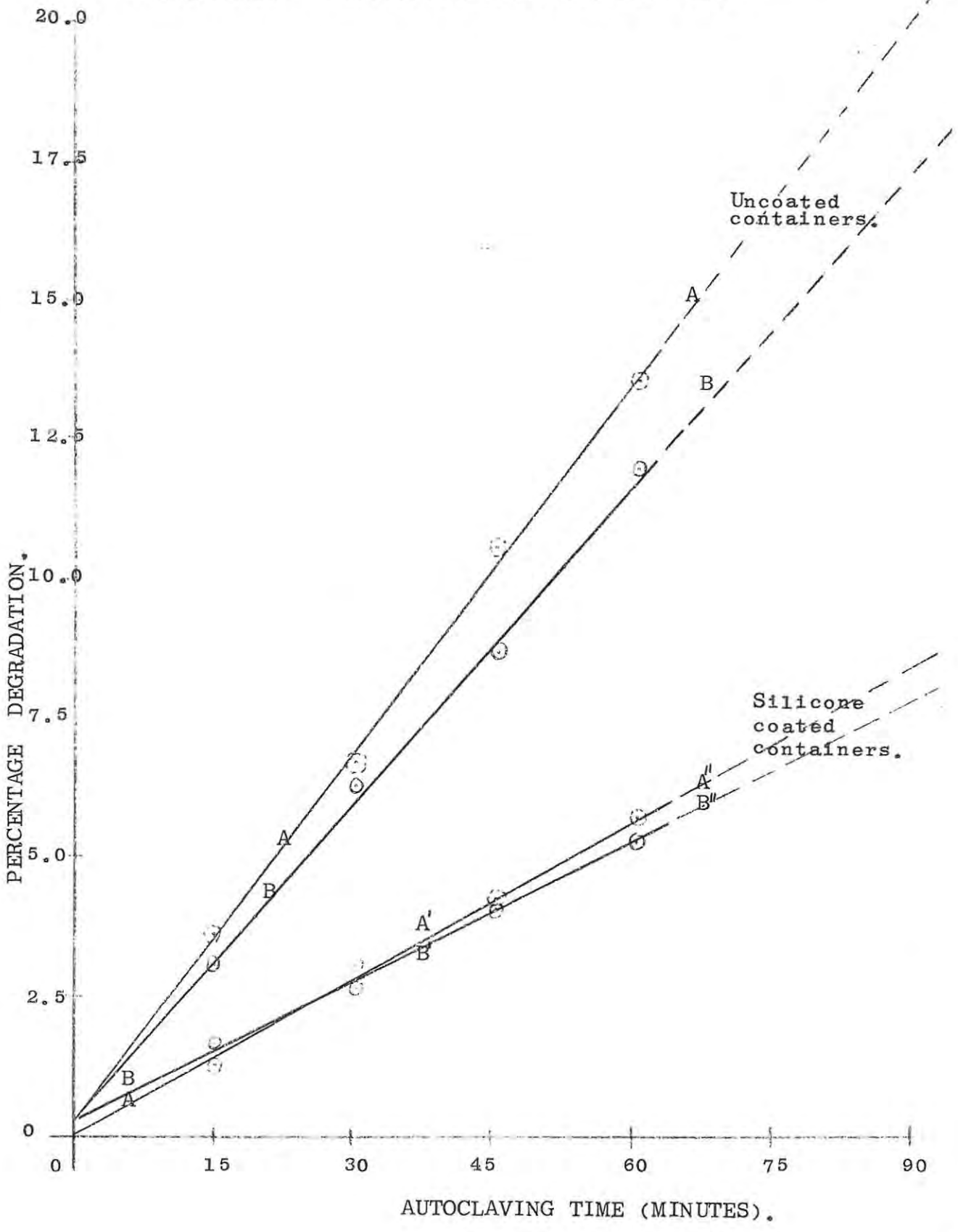


GRAPH 24. Buffered solution. pH 7·4

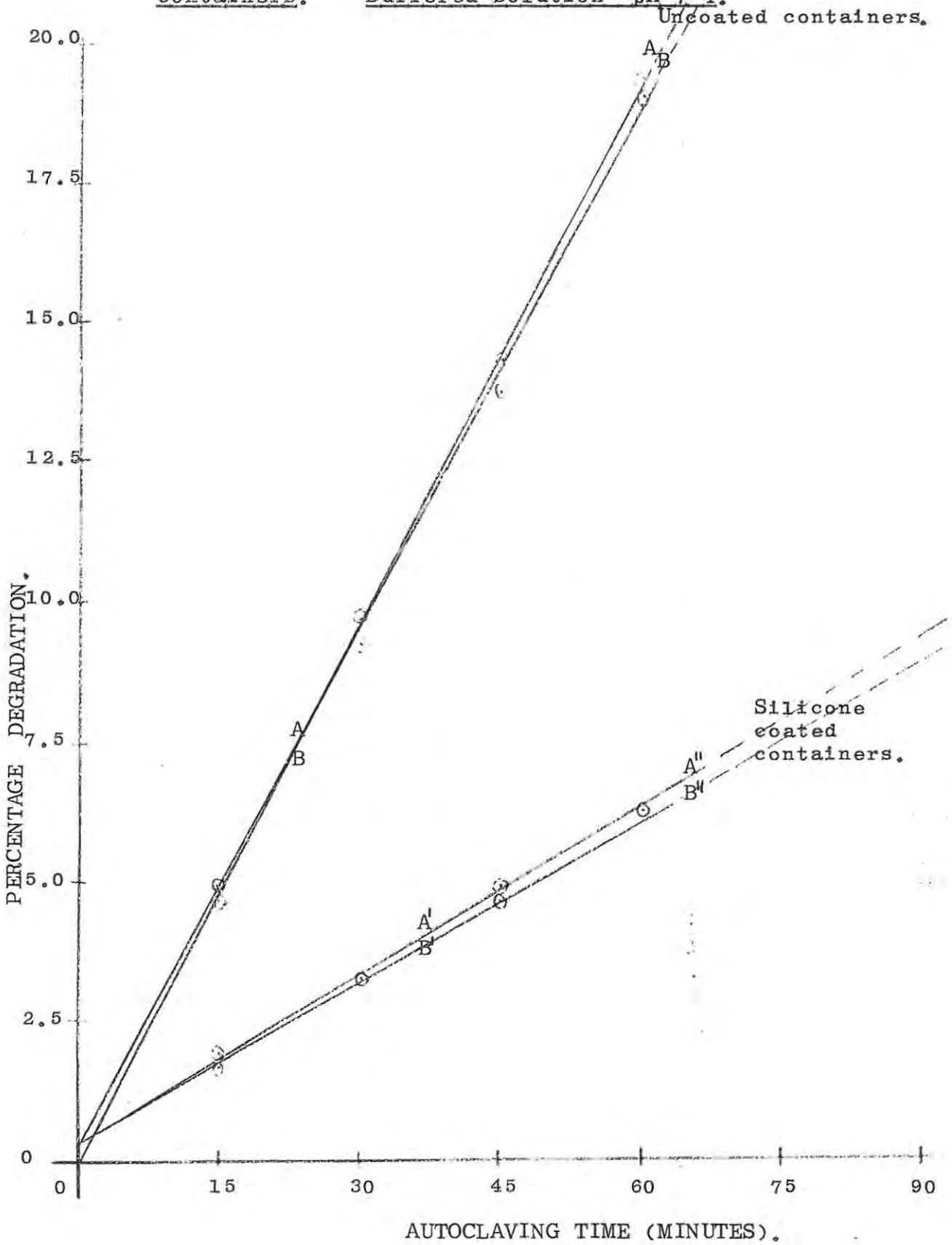
Percentage degradation/time of autoclaving at 122°.



GRAPH 25. Degradation plots in silicone-coated and uncoated containers. Unbuffered solution. pH 7·4.



GRAPH 26. Degradation plots in silicone-coated and uncoated containers. Buffered solution pH 7·4.



GRAPH 27. Investigation of possible interference by 4% ethanol in the absorbance spectrum of the ferric-hydroxamic derivative of chlorbutol.

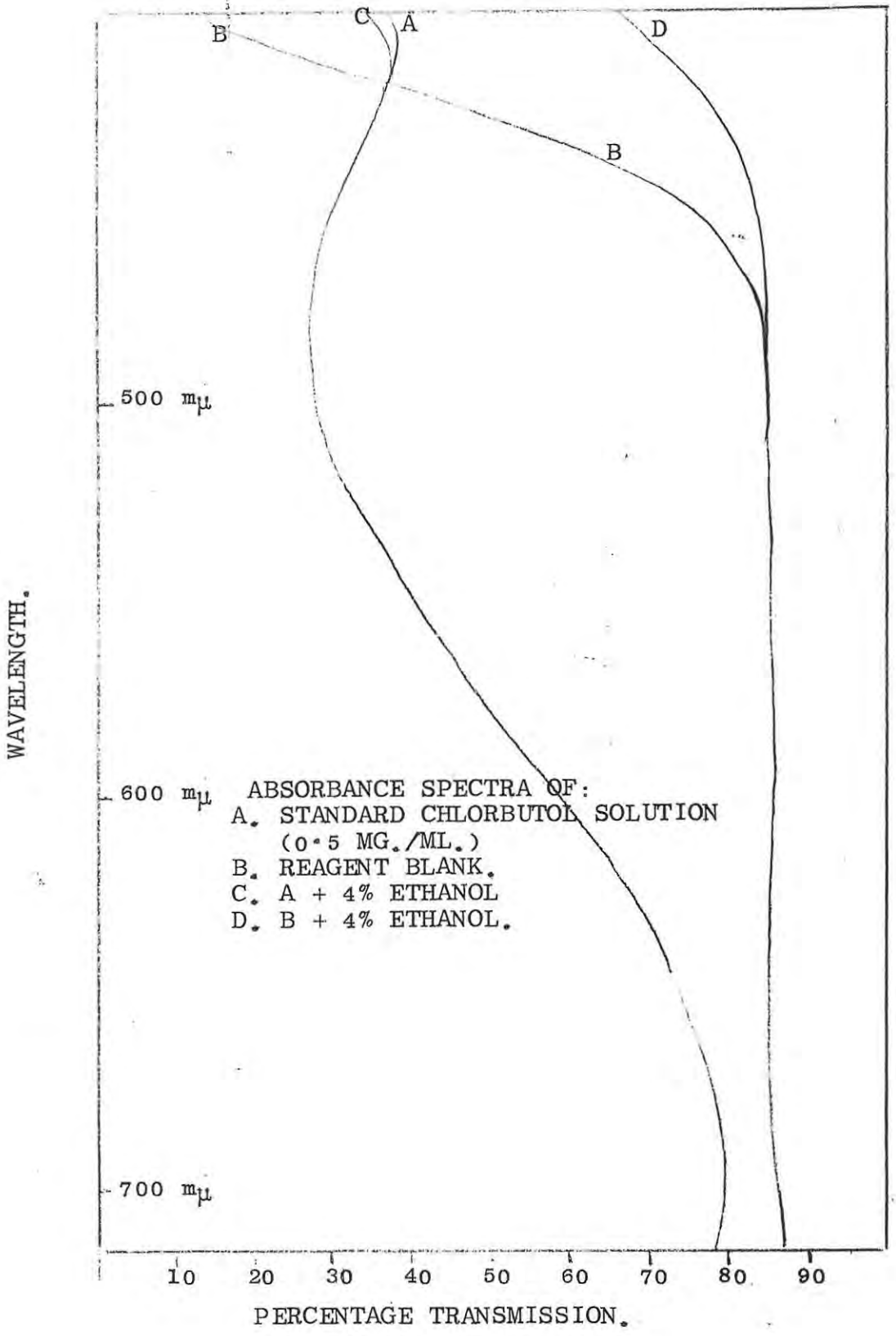


DIAGRAM 2. Thin Layer Chromatogram of 3,5-dinitrobenzoic acid esters of α-hydroxy isobutyric acid, isopropanol and chlorbutol, with ethanol, using the solvent system: ethyl acetate-acetone-methanol (45 + 45 + 10).

		0·7% chlorbutol in 4% ethanol.
		4% ethanol.
		0·05% α-hydroxyisobutyric acid in ethanol.
		0·05% isopropanol in 4% ethanol.
		mixt. of the standard derivative solutions.

R_f values obtained for the respective derivatives were:

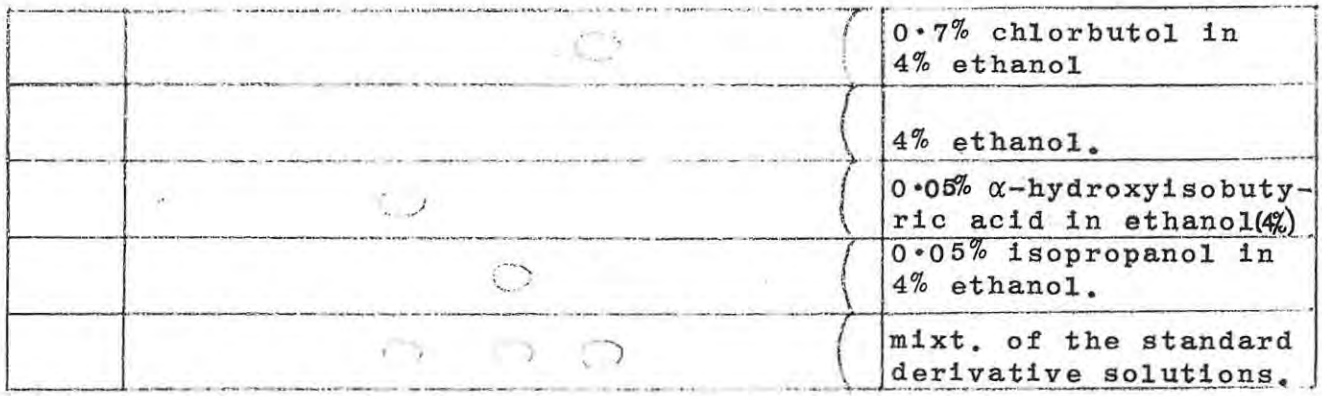
a. 0·7% chlorbutol. R_f = 0·52.

b. 4% ethanol. R_f = 1·0.

c. 0·05% α-hydroxyisobutyric acid. R_f = 0·35.

d. 0·05% isopropanol. R_f = 0·44.

DIAGRAM 3. Thin Layer Chromatogram of the 3,5-dinitrobenzoic acid esters of α-hydroxyisobutyric acid, isopropanol, and chlorbutol, with ethanol, using the solvent system: ethyl acetate-acetone-methanol. (45 + 45 + 10) + 2 drops of N/50 hydrochloric acid per 10 ml. of solvent.



R_f values obtained for the respective derivatives were:

a. 0·7% chlorbutol $R_f = 0·63$.

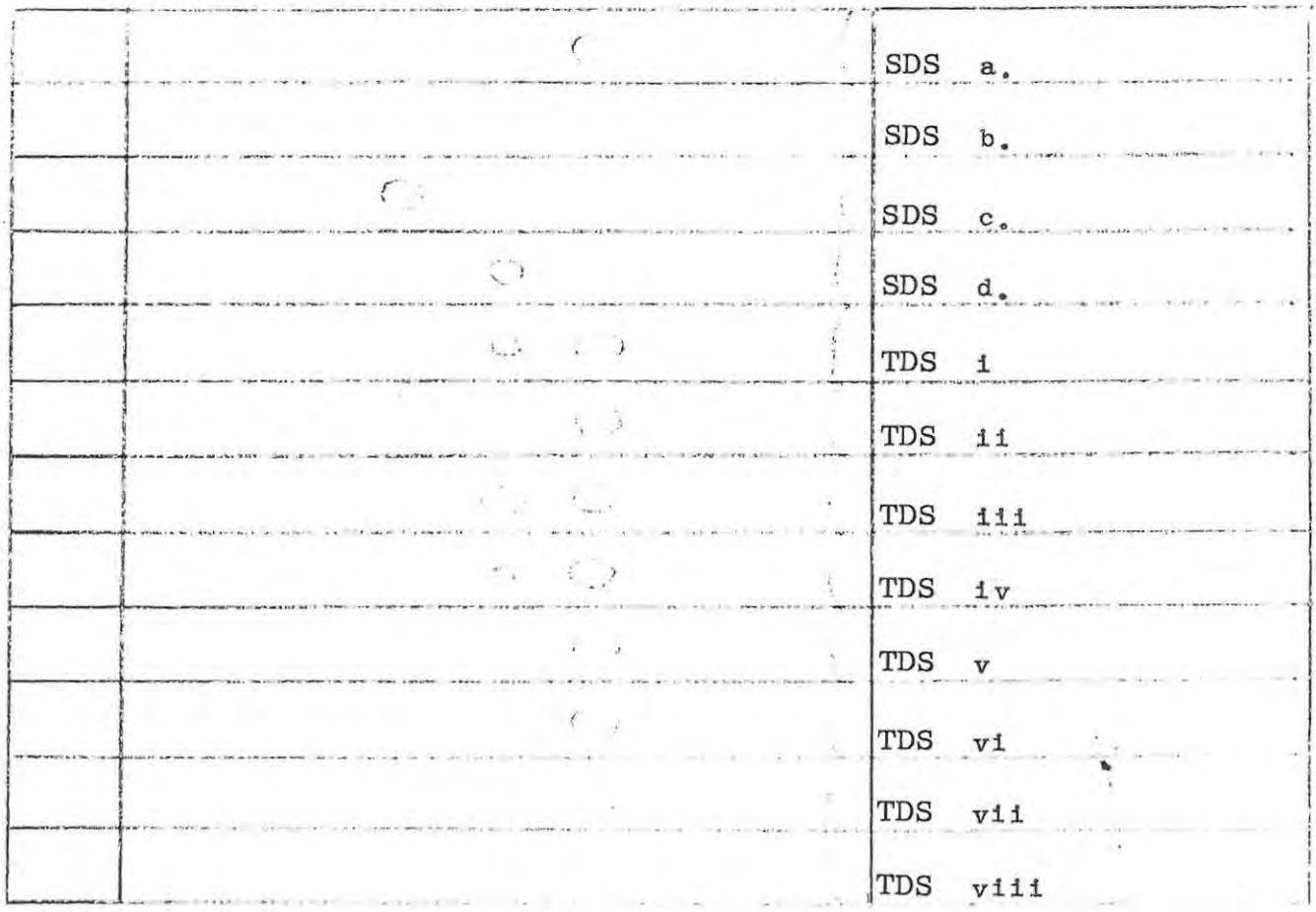
b. 4% ethanol $R_f = 1·0$

c. 0·05% α-hydroxyisobutyric acid $R_f = 0·37$

d. 0·05% isopropanol $R_f = 0·51$

Effective separation of the derivatives was obtained with this solvent system.

DIAGRAM 4. Thin Layer Chromatogram of test derivative solutions, for the determination of α-hydroxyisobutyric acid and isopropanol, using the solvent system: ethyl acetate-acetone-methanol. (45 + 45 + 10) plus 2 drops of N/50 hydrochloric acid per 10 ml. of solvent.



Standard Derivative Solution = SDS.

a. 0·7% chlorbutol R_f = 0·63
b. 4% ethanol R_f = 1·0
c. 0·05% α-hydroxyisobutyric acid R_f = 0·37
d. 0·05% isopropanol R_f = 0·51

Test Derivative Solutions = TDS.

i.	Unprocessed.	Uncoated.	pH 7·4.
ii.	Unprocessed.	Uncoated.	pH 5·6.
iii.	Processed.	Uncoated.	pH 7·4.
iv.	Processed.	Uncoated.	pH 5·6.
v.	Unprocessed	Coated.	pH 7·4.
vi.	Unprocessed.	Coated.	pH 5·6.
vii.	Processed.	Coated.	pH 7·4.
viii.	Processed.	Coated.	pH 5·6

DIAGRAM 5. Thin Layer Chromatogram of 2, 4-dinitrophenyl hydrazine derivative of acetone, against a chlorbutol solution, with ethanol, using the solvent system: benzene-ethyl acetate. (95 + 5).

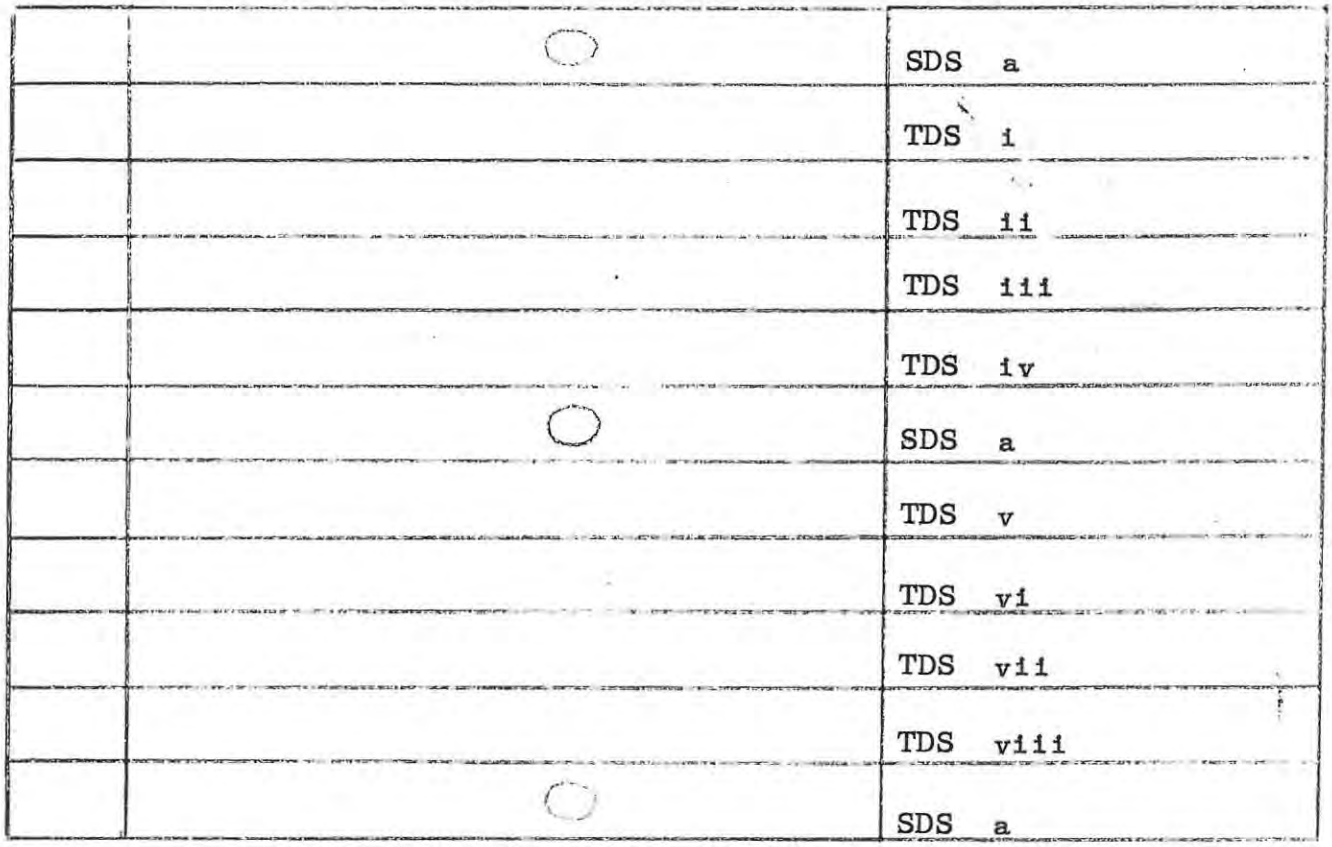
R_f value obtained for the derivative was.

a. 0·7% chlorbutol in 4% ethanol. = No spot.

b. 0·05% acetone in 4% ethanol. R_f = 0·59.

No interference in the determination was shown by either 0·7% chlorbutol or 4% ethanol.

DIAGRAM 6. Thin-Layer Chromatogram of test derivative solutions for the determination of acetone, using the solvent system: benzene-ethyl acetate (95 + 5).



Standard Derivative Solution = SDS.

a. 0·05% Acetone R_f = 0·59.

Test Derivative Solutions = TDS.

i.	Unprocessed.	Uncoated.	pH 7·4.
ii.	Unprocessed.	Uncoated.	pH 5·6.
iii.	Processed.	Uncoated.	pH 7·4.
iv.	Processed.	Uncoated.	pH 5·6.
v.	Unprocessed.	Coated.	pH 7·4.
vi.	Unprocessed.	Coated.	pH 5·6.
vii.	Processed.	Coated.	pH 7·4.
viii.	Processed.	Coated.	pH 5·6.

DIAGRAM 7. Thin Layer Chromatogram of benzyl-thiuronium salts of formic and acetic acids produced by the extraction procedure, against standard derivatives of these acids, using the solvent system: chloroform-ethyl acetate- acetone (40 + 40 + 20) plus 1 drop of N/10 hydrochloric acid per 10 ml. of solvent.

R_f values obtained were:

i. Standard derivative solutions.

a. 0·5% formic acid. $R_f = 0·44$.

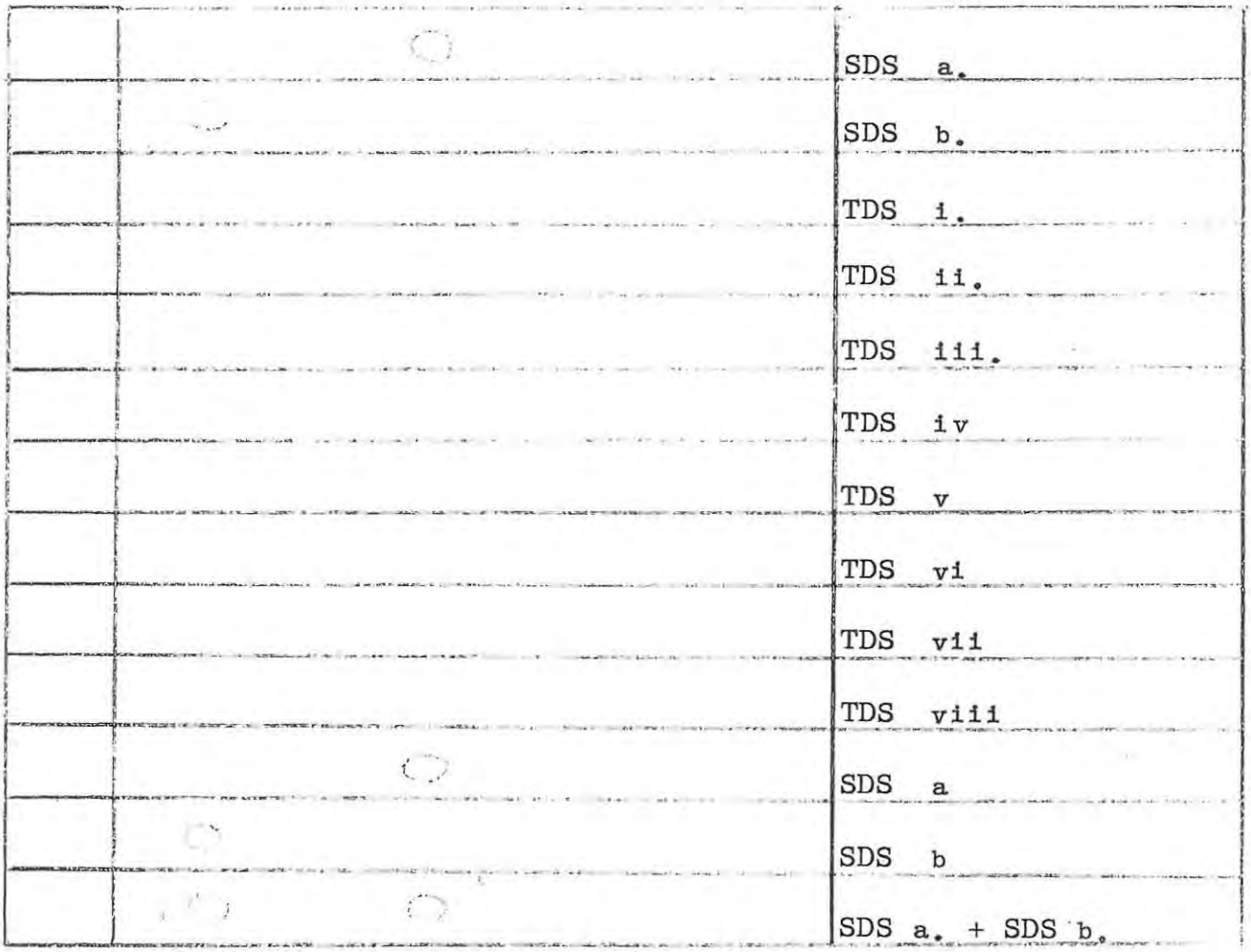
b. 0·5% acetic acid. $R_f = 0·13$.

ii. Derivative solutions produced by extraction procedure.

a. 0·05% formic acid. $R_f = 0·44$.

b. 0·05% acetic acid. $R_f = 0·13$.

DIAGRAM 8. Thin Layer Chromatogram of test solutions for the determination of formic and acetic acids, using the solvent system: chloroform-ethyl acetate-acetone (40 + 40 + 20) plus one drop of N/10 hydrochloric acid per 10ml. of solvent.



Standard derivative solutions = SDS.

a. 0·05% formic acid. $R_f = 0·44$

b. 0·05% acetic acid. $R_f = 0·13$

Test derivative solutions = TDS.

i.	Unprocessed.	Uncoated.	pH 7·4.
ii.	Unprocessed.	Uncoated.	pH 5·6.
iii.	Processed.	Uncoated.	pH 7·4.
iv.	Processed.	Uncoated.	pH 5·6.
v.	Unprocessed.	Coated.	pH 7·4.
vi.	Unprocessed.	Coated.	pH 5·6.
vii.	Processed.	Coated.	pH 7·4.
viii.	Processed.	Coated.	pH 5·6.

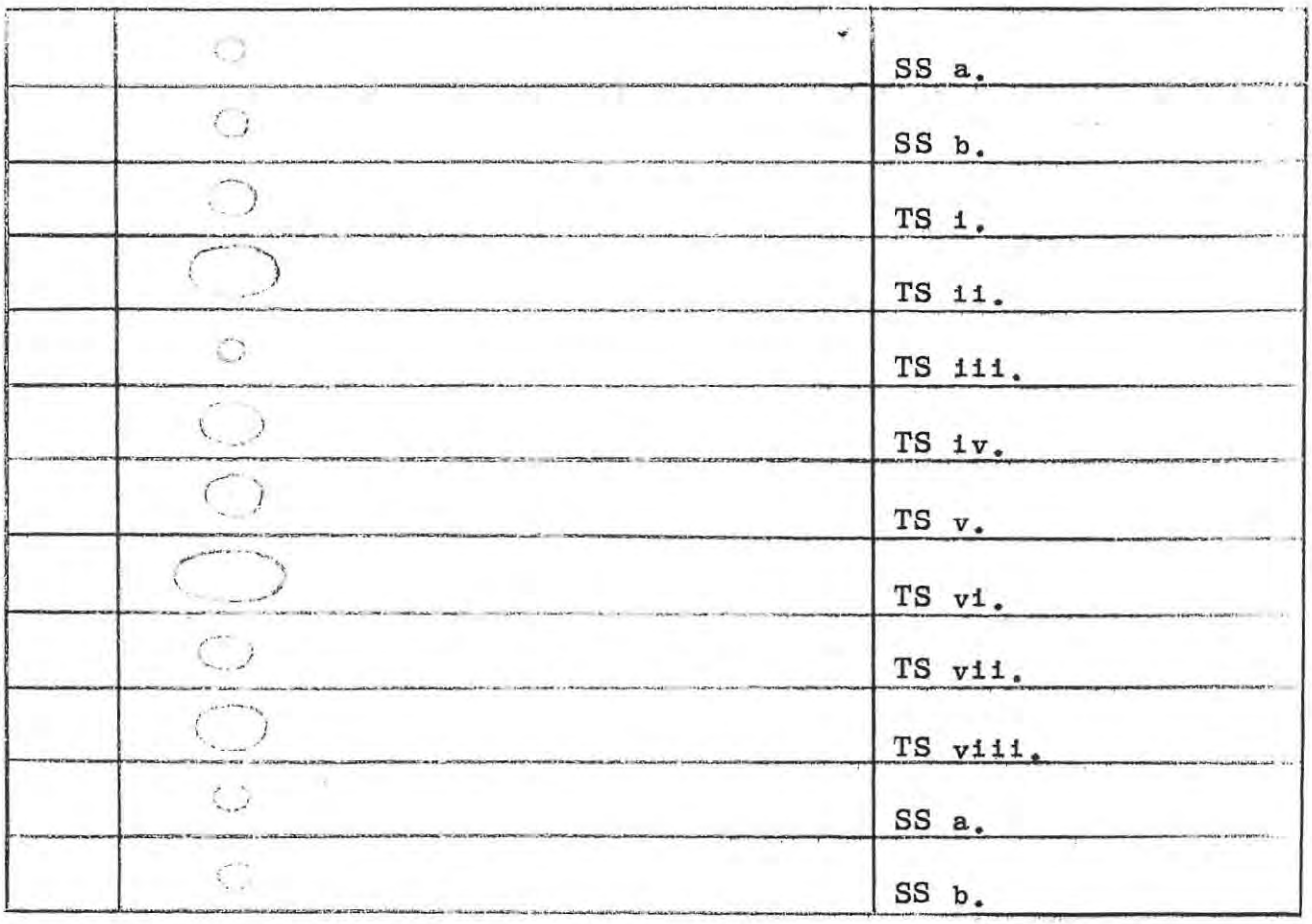
DIAGRAM 9. Thin Layer Chromatogram of alkali halides using the solvent system: acetone- n-butanol-concentrated ammonia solution-distilled water. (65 + 20 + 10 + 5).

R_f values obtained were:

a. 0·1% sodium chloride. R_f = 0·15.

b. neutralised chloride solution R_f = 0·15.

DIAGRAM 10. Thin Layer Chromatogram of test solutions for the determination of chlorides, using the solvent system: acetone-n-butanol-concentrated ammonia solution-distilled water (65 + 20 + 10 + 5).



Standard solutions = SS.

a. 0·1% sodium chloride. R_f = 0·15.

b. Neutralised chloride. R_f = 0.15.

Test Solutions = TS.

i.	Unprocessed.	Uncoated.	pH 7·4.
ii.	Unprocessed.	Uncoated.	pH 5·6.
iii.	Processed.	Uncoated.	pH 7·4.
iv.	Processed.	Uncoated.	pH 5·6.
v.	Unprocessed.	Coated.	pH 7·4.
vi.	Unprocessed.	Coated.	pH 5·5.
vii.	Processed.	Coated.	pH 7.4.
viii.	Processed.	Coated.	pH 5·6.

APPENDIX 2.

Bibliography.

1. "The Extra Pharmacopoeia". MARTINDALE AND WESTCOTT. (1932).
2. FISHBURN AND WATSON. J. Am. Pharm. Assoc. 28. 491. (1939).
3. HARRINGTON. U.S. Patent. (February, 1949). (Abstract).
4. British Pharmaceutical Codex 1963.
5. "Watt's Dictionary of Chemistry". Vol.1. MOR-LEY AND MUIR. (1888).
6. "Organic Chemistry". Vol. 1. 3rd Edition. FINAR. (1959).
7. "Textbook of Pharmaceutical Chemistry". BENTLEY AND DRIVER (1960).
8. "Disinfection and Sterilisation". SYKES (1958).
9. PFENDER. 'Historical Synopsis of the Development of Hypodermic Medication'. Reprint from Washington Medical Annals. 10. 348-58. (Abstract).
10. Pharmacopoeia of the London Hospitals. 8th Edition. (1910).
11. "Materia Medica and Therapeutics." 14th Edition. BRUCE AND DILLING (1933).
12. British Pharmacopoeia. 1932.
13. Pharmaceutical Pocket Book. 1938.
14. THEODORE AND MINSKY. J. Am. Med. Assoc. 147. 1381 (1951).
15. Food and Drug Administration. Federal Register. 18. 351. (1953). (Abstract).
16. GERSHENFELD AND PERLSTEIN. Am. J. Pharm. 111. 277. (1939).
17. GLADHART et al. Bulletin Am. Soc. Hosp. Pharm. 12. 534-9. (1955).
18. LAWRENCE. J. Am. Pharm. Assoc. (Sci. Ed'n) 44. 457-64 (1955).
19. BRIGGS AND COOK. Proc. Congr. Int. Fedn. Soc. Cos. Chem. (2nd) London, 1962. 227-39.
20. DEEB AND BOENIGK. J. Am. Pharm. Assoc. 47. 807-9 (1958).
21. The Modern Materia Medica. (1911).
22. FARWELL. J. Am. Pharm. Assoc. (Sci. Ed'n) 8. 1053. (1919).
23. OWEN, MARTIUS AND BROSIUS. J. Lab. Clin. Med. 6. 47 (1920).
24. British Pharmacopoeia. 1923.
25. DAVIS. Quart. J. Pharm. and Pharmacol. 8. 362. (1935).
26. U.S. Dispensatory. HAMILTON. 22nd Ed'n. (1937).

27. BRIGGS AND CALLOW. Quart. J. Pharm. and Pharmacol. 14. 127. (1941).
28. British Veterinary Codex. 1953.
29. British Pharmaceutical Codex. 1954.
30. Pharmacopoeia of the United States. Fifteenth Revision (1955).
31. British Pharmacopoeia. 1958.
32. British Pharmaceutical Codex. 1959.
33. British Pharmacopoeia. 1963.
34. "The Extra Pharmacopoeia". MARTINDALE AND WESTCOTT.(1901).
35. TAUB AND LUCKEY. J. Am. Pharm. Assoc. 32.28-31 (1943).
36. GERSHENFELD. Am. J. Pharm. 124. 363-6 (1952).
37. McEWAN AND McMORRAN. Pharm. J. 158. 260-2 (1947)
38. GOLDSTEIN AND RYAN. Drug.Standards. 20. 133-7. (1952).
39. ROSENTHALER. Scientia Pharm. 8. 9-10. (1937).
40. ANDERSON AND STOCK. Aust. J. Pharm. 41. 1018-21 (1960).
41. LACHMAN et al. J. Pharm. Sci. 52. 241-43. (1963).
42. NAIR AND LACH. J. Am. Pharm. Assoc. 48. 390-95. (1959).
43. COULTHARD. Pharm. J. 142. 79-81. (1939).
44. BERRY, JENSEN AND SILLER. Quart. J. Pharm. 11. 729-35. (1938).
45. DAVIES AND DAVISON. Quart. J. Pharm. 20. 212-8. (1947).
46. "Dispensing for Pharmaceutical Students". 11th Edition. Edited by CARTER AND GUNN. (1965).
47. ERNE AND SÖDERLUNDH. Svensk. Farm. Tid. 50. 21-30. (1946).
48. MURPHY et al. Am. Med. Assoc. Arch. of Ophthalm. 53. 63.(1955).
49. ANDERSON AND STOCK. Aust. J. Pharm. 39. 1110-12. (1958).
50. STOCK. ibid. 43. 1143-46. (1962).
51. SYKES. J. Pharm. and Pharmacol. 10S. 40T-46T. (1958).
52. "An Introduction to Microbiology". HUGO.
53. KOHN et al. J. Pharm. Sci. 52.967-74. (1963).
54. THEODORE AND FEINSTEIN. Am. J. Ophthalm. 35. 656. (1952).
55. VAUGHAN. ibid. 38. 55. (1955).
56. ALLEN. ibid. 35. 99. (1952).
57. FOSTER. Manuf. Chemist. 36. 46, 47 and 50. (1965).
58. ROYCE AND SYKES. J. Pharm. and Pharmacol. 9. 814-23 (1957).
59. SCHRODIE AND MILLER. J. Am. Pharm. Assoc. (Pract. Pharm. Ed'n.) 20. 197. (1959).

60. REIGELMAN AND VAUGHAN. ibid. 19. 474-7. (1958).
61. "The Extra Pharmacopoeia". MARTINDALE. VOL. 2. 23rd Edition. (1955).
62. SYKES AND HOOPER. J. Pharm. and Pharmacol. 11. 235T - 239T. (1959).
63. ROYCE AND SYKES. ibid. 7. 1046-52 (1955).
64. Millipore Filter Corporation. (1963).
65. GOLDSTEIN. J. Am. Pharm. Assoc. (Pract. Pharm. Ed'n.). 14. 498-500 and 524. (1953).
66. ANDERSON. Aust. J. Pharm. 43. 309-10. (1962).
67. PATWA AND HUYCK. J. Am. Pharm. Assoc. NS6. No. 7. 372-3. (1966).
68. "Practical Pharmaceutical Chemistry". APPLEYARD AND LYONS. (1957).
69. JENSEN AND JANNKE. J. Am. Pharm. Assoc. 37. 37. (1948).
70. RAY AND BASU. Indian J. Pharm. 12. 6-7. (1950).
71. NAIR, LACH AND BLAUG. J. Am. Pharm. Assoc. 47. 46-48. (1958).
72. BIRNER. J. Anal. Chem. 33. 1955. (1961).
73. CHAFETZ AND MAHONEY. J. Pharm. Sci. 54. 1805-6. (1965).
74. REHM AND MADER. J. Am. Pharm. Assoc. 46. 621-3. (1957).
75. LACHMAN, WEINSTEIN et al. J. Pharm. Sci. 51. 224-32. (1962).
76. KERCKHOFFS AND HUIZINGA. Pharm. Weekblad. 101. 701-12. (1966).
77. HINSON, SMITH AND GREENE. J. Am. Ceram. Soc. 30. (7). 211. (1947).
78. REXER. Keram. Rund. 38. 387. (1930)(Abstract).
79. TURNER et al. J. Soc. Glass Tech. 19. T171. (1935).
80. "Medical Microbiology." 16th Edition. CRUIKSHANK. (1965).
81. REIGELMAN, VAUGHAN AND OKUMOTO. J. Pharm. Sci. 45. 93. (1956).
82. DIMBLEBY. J. Pharm. and Pharmacol. 5. 969-89. (1953).
83. TOD. J. Soc. Glass Tech. 34. 58-65. (1950).
84. "The Extra Pharmacopoeia". Vol.1. 24th Edition. MARTINDALE. (1958).
85. "Textbook of Organic Chemistry". 2nd Edition. FERGUSON. (1965).
86. "Chemistry of the Silicones". ROCHOW. (1947).
87. THOMAS. Alchemist. 18. 24. (1954).
88. BERGSTEIN. Am. Prof. Pharm. 22. 522. (1956).
89. BERRY. J. Pharm. and Pharmacol. 5. 1008-17. (1953).
90. KLEIN et al. ibid. 6. 725-34. (1954).