AN INVESTIGATION INTO

THE EFFECT OF PARTICULATE SOLIDS

ON CERTAIN ANTIMICROBIAL PRESERVATIVES

IN PHARMACEUTICAL AND COSMETIC SUSPENSIONS

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ABBREVIATIONS.

% m/v solution	-	grams of solute per 100 ml of solution
BP	-	British Pharmacopoeia
BPC	-	British Pharmaceutical Codex
BZC	-	Benzalkonium chloride
CMC	-	Critical micelle concentration
CPC	-	Cetylpyridinium chloride
CTAB	-	Cetyltrimethylammonium bromide
DHA	-	Dehydroacetic acid
IPA	-	International Pharmaceutical Abstracts
MDT	-	Mean Death Time
PMN	-	Phenylmercuric nitrate
QAC	-	Quaternary ammonium compound
Bism. carb.	-	Bismuth carbonate
Calc. carb. praecip.	-	Calcium carbonate, precipitated
Mag. carb. light	-	Magnesium carbonate, light
Mag. carb. heavy	-	Magnesium carbonate, heavy
Mag. ox. light	-	Magnesium oxide, light
Mag. trisil.	-	Magnesium trisilicate
Titan. diox.	-	Titanium dioxide

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INTRODUCTION:

Pharmaceutical and cosmetic preparations must be formulated so as to assure elegance of appearance, efficacy of ingredients and a satisfactory shelf life as the product. If the formulation is not self-preserving and if it contains material which could act as a substrate for growth of micro-organisms, the shelf life aspect involves, in addition to several other factors, the choice of a suitable antimicrobial preservative. Such preservatives, when present in the minimum effective concentration, are unfortunately prone to interact with many other materials. A number of papers on the inactivation of preservatives by containers, filters and formulation components have been published. The field has been adequately reviewed by de Navarre (1962), Wedderburn (1964) and, more recently, by Coates (1973). From these reviews and from a study of the literature it became apparent that relatively little work had been done on interactions between preservatives and solid particles in aqueous suspension.

Consequently, a range of preservatives not previously investigated in this respect was tested for loss of activity in the presence of fifteen powders commonly used in aqueous suspension in pharmaceutical and cosmetic formulations. In view of the information obtained in this preliminary study and after the development of more satisfactory analytical techniques, it was decided to study in greater depth the interaction between these powders and selected organomercurials and quaternary ammonium compounds.

CHAPTER 1 LITERATURE SURVEY.

1.1 REVIEW OF PRESERVATIVE INACTIVATION

The increased emphasis in recent years on the need for preservation of pharmaceutical formulations has been a reflection of the decline in the use of extemporaneous preparations and of the increase in the manufacture of ready-made products. Prior to 1950, preservatives were not widely used in pharmaceutical products. Normally such products were used soon after preparation and an extended shelf life was therefore not required. The few preservatives in general use had proved their worth in practice in the presence of a limited range of adjuvants.

With the proliferation of the manufacture of pharmaceutical products in the post-war period and the introduction of many new adjuvants which aided effective formulation, there was an increased demand for preservatives which would remain effective in the presence of these substances, which were often themselves susceptible to microbial attack. The introduction of plastic containers has further increased the demands made on preservative systems as many preservatives are inactivated by plastics. Thus, research into the incompatibilities of preservatives has taken place mostly in the last twenty five years.

For convenience, preservative interactions can be divided into the following categories:

1.1.1 Preservative/macromolecule interactions

This field has been adequately reviewed by Coates (1973b), who observed that:

(i) In certain cases higher molecular mass polymers

exhibit a greater tendency to complex preservatives.

(ii) The functional groups on both the preservative and the macromolecule are of importance in determining the strength of the bonds involved, although other groups may interfere with the bond.

(iii) The magnitude of the interaction may be temperature independant or it may increase or decrease with rise in temperature.

(iv) In some cases the extent of inactivation can be related to the hydrophile-lipophile balance of non-ionic macromolecules. As the non-ionic becomes more lipophylic, its power to inactivate preservatives tends to increase.

Since the publication of this review McCarthy and Myburgh (1974) have found that most commonly used preservatives do not bind readily onto tragacanth gel, thus confirming the observation of Taub et al. (1958) and Miyawaki et al. (1959) that the parabens should be satisfactory preservatives for formulations containing tragacanth.

Crooks and Brown (1974) have investigated the binding of various preservatives, alone and in pairs, to cetomacrogol and have discovered the existence of two distinct classes of binding sites in the cetomacrogol micelle. They concluded that preservative mixtures used in non-ionic surfactant systems may show an apparent synergism as a result of mutual competitive displacement from binding sites.

1.1.2 Preservative / container interactions

Due to the non-reactivity of glass, the reactivity of rubber and to the increased use of plastic packaging materials in recent years, researchers have understandably tended to concentrate their investigations in this area on preservative/rubber and preservative/plastic interactions. Both these fields have been reviewed by Coates(1973c), who concluded that such interactions are of widespread occurrence.

Due to the large number of different plastics, rubbers and preservatives available, no general conclusions are possible, although Armstrong (1972) has prepared a table summarising plastic/preservative systems that had been investigated to that date.

A problem peculiar to this field is that the precise composition of the plastic container or rubber closure is usually not known to the user. Testing of the final product in its intended container is therefore essential.

1.1.3 Preservative / filter interactions

The loss of benzalkonium chloride, chlorhexidine acetate, phenylethyl alcohol and phenylmercuric nitrate onto a variety of media used for filtration sterilisation has been investigated by Naidoo et al. and reported on in three separate publications (1970, 1971 and 1972). Their conclusion was that, in most cases, the loss was considerable with fibrous asbestos pads, significant with porcelain candles and sintered glass and slight with membrane filters.

Benzalkonium chloride, chlorhexidine gluconate and phenylmercuric borate have also been investigated for loss onto membrane filters from several different manufacturers by van Ooteghem and Herbots (1969).

The adsorption of hydroxyquinoline sulphate, phenol, orthochlorophenol and formaldehyde on membrane filters has been studied by Lambin et al. (1972) who observed that washing of the membranes with distilled water decreases the extent of adsorption.

1.1.4 Preservative / lipid interactions

The preservation of emulsions against microbial attack was the subject of an in depth study by Wedderburn (1964). Preservative/lipid interactions have been further reviewed by Coates (1973a).

It is clear that the activity of a preservative in the aqueous phase of an oil/water dispersion is influenced by the following factors.

(i) Overall concentration of preservative

(ii) Oil/water partition coefficient

(iii) Oil/water ratio

(iv) Tendency of the preservative to concentrate at the interface and the area of the interface.

Of particular interest is the following statement from Coates' review:

"Bean et al.[42] studying the bactericidal activity against Escherichia coli of phenol in oil:water dispersions found increased bactericidal activity associated with the formation of an interface and concluded that the interface was the cause or site of the increased activity. They found that bacteria were adsorbed at the interface, a phenomenon previously reported by Kamakaka[43]. From subsequent studies[35] it was deduced from interfacial tension measurements and the Gibbs adsorption equation that the concentration of phenol at the interface was greater than in the bulk of the aqueous phase."

The increase in antibacterial activity of an aqueous benzalkonium chloride solution in the presence of suspended powders as was observed in this study may be explained by a similar mechanism, i.e., bacteria concentrate at the solid/water interface where they come into contact with a

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(42. H.S.Bean, J.P.Richards and J. Thomas, Boll. chim-farm., 1962, 101, 339

43. T.R. Kamakaka, "Studies on the Bacterial Population of Two-phase Systems," PhD Thesis, London, 1956

35. H.S. Bean, S.M. Heman-Ackah and J. Thomas, J. Soc. Cosm. Chem. 1965, 16, 15.)

higher concentration of the preservative (For a full discussion of this matter see section 5.2).

Since Coates' review, Jacobs et al. (1975) have studied the influence of pH, emulsifier and accelerated aging upon preservative requirements of oil/water emulsions. Using twenty nine individual preservatives and sixteen combinations of two or more preservatives they discovered that less than 35% of the preservatives or systems tested were effective.

McCarthy (1973) has described a rapid, dialysis method of determining preservative release from creams and emulsions. Mitchell and Kazmi (1975) have attempted to quantify the distribution of preservative between oil, aqueous and micellar phases of an emulsified system, and Tufegdzic and Berberovic (1973) have investigated the release of sodium lauryl sulphate and cetrimide from commonly used ointment bases into agar gel.

1.1.5 Miscellaneous preservative interactions

The incompatibilities of commonly used preservatives, especially with other aqueous solutes, are well documented in standard reference works such as Martindale's Extra Pharmacopoeia (1977) and do not warrant review here. However, previously unknown interactions are discovered periodically, sometimes in well known formulations. For example: Richards and McBride (1973) have found that the activity of phenylmercuric nitrate, an official preservative in sodium sulphacetamide eye drops BPC 1968, is antagonised by another component of the eye drops, sodium metabisulphite.

Hart (1973) has established that phenylmercuric nitrate is lost from Zinc sulphate and adrenalin eye drops BPC 1968 when the preparation is subjected to autoclaving at 115-116^{o}C

and has therefore recommended that they be sterilised by a filtration method.

Recently, El-Nakeeb and Ali (1976) have investigated the binding of ten antibiotic preservative mixtures by sixteen different pharmaceutical materials using microbiological and spectrophotometric methods. Their results showed that benzalkonium chloride and, to a lesser extent, chlorocresol interfered variably with the interactions between the tested materials and certain antibiotics.

1.1.6 Preservative / powder interactions

Much of the research done in this field has centered around interactions between adsorbents known to be strong such as talc and kaolin, and preservatives known to be surface active, such as the quaternary ammonium and pyridinium compounds.

Batuyios and Brecht (1957), in investigating incompatibilities in compressed lozenges, studied the adsorption of cetylpyridinium chloride and benzalkonium chloride by talc and kaolin. The adsorption isotherms were determined at 25°C and plotted according to the Langmuir and Freundlich equations. Similar curves were obtained for talc and for kaolin for each compound, the powders differing only in adsorptive capacity. Cetylpyridinium chloride was adsorbed to a somewhat greater extent than benzalkonium chloride by both powders. Elution experiments revealed that, regardless of original concentration, approximately 5mg cetylpyridinium chloride per gram of talc is bound irreversibly and is therefore not available for diffusion back into the aqueous phase.

Clarke and Armstrong (1972), in studying the influence of pH on the adsorption of benzoic acid by kaolin, found that

the Langmuir adsorption isotherm was followed. This fact is interesting in view of the discovery of Batuyios and Brecht (1957) that cetylpyridinium chloride follows the Langmuir isotherm when adsorbed onto kaolin while benzalkonium chloride does not. This phenomenon could possibly be explained by the fact that, while benzoic acid and cetylpyridinium chloride are pure compounds, benzalkonium chloride is a mixture of homologues from which the higher homologues could tend to be adsorbed first. Yousef et al. (1971), working with benzalkonium chloride and magnesium trisilicate, also noticed that the adsorption isotherm did not follow the Langmuir equation. It seems that most, if not all, benzalkonium isotherms do not obey the Langmuir equation. A study of the other quaternary ammonium preservative which is a mixture of homologues, namely cetrimide, would be of great interest in this respect.

The paper of Clarke and Armstrong (1972) is of further interest because of their discovery that the degree of adsorption of benzoic acid onto kaolin can be controlled by adjusting the pH of the formulation. In only one other paper, that of Selleri et al. (1974), are specific attempts made to limit preservative loss onto powders by suitable manipulation of the formulation, although in other cases, the characteristics of preservative-powder systems have been investigated with the apparent objective of finding ways of limiting preservative inactivation. One such paper is a further investigation by Armstrong and Clarke (1973), in which the adsorption of benzoic acid and crystal violet on kaolin from solutions of varying salt and alcohol concentrations was determined in order to elucidate the influence of electrolyte content and system dielectric constant on these systems. A further paper by Armstrong and Clarke (1976) probes the effect of pretreatment of kaolin with cationic and anionic materials at various pH's on its electrophoretic mobility and adsorptive properties.

Bean and Dempsey (1967), in seeking to evaluate the influence of suspended solid particles and of the solid/liquid interface in a suspension on activity, chose a phenol/activated carbon system. They were able to establish that the bactericidal activity of phenol in aqueous suspensions of carbon is dependant on the residual aqueous phenol concentration after the adsorption equilibrium between phenol and carbon has been established, provided that allowance is made for the effect on activity of a leached extract from the carbon. It should be born in mind however that, although the solid chosen had a very high specific surface, the major part of it was internal and therefore not accessible to bacteria so this system was not characteristic of medicinal suspensions.

In subsequent research Bean and Dempsey (1971) made use of kaolin and procaine penicillin as more typical particulate solids used in pharmaceutical suspensions. They discovered, inter alia, that suspensions containing benzalkonium chloride possessed a greater activity than the corresponding supernatant solutions removed from contact with the kaolin owing to the bactericide adsorbed on the kaolin, becoming available to the bacteria. (See Section 5.2 for further discussion).

In contrast to this finding, Yousef et al.(1971) in their study of the adsorption of benzalkonium chloride by magnesium trisilicate discovered that almost complete adsorption of the benzalkonium chloride took place at benzalkonium chloride: magnesium trisilicate ratios of 1:20 and above and that the resultant suspension was completely devoid of antibacterial properties. In fact, magnesium trisilicate used in this ratio was found to have a greater neutralizing capacity for benzalkonium chloride than the commonly used lecithin-Tween mixture.

Blackman and Harrop (1968) studied the adsorption of cetyltrimethylammonium bromide (C.T.A.B.) onto Aerosil. Measurements of the effects of pH and concentration of CTAB

supported the concept of a cation-exchange mechanism. However, the infra red data indicated that the adsorption is associated with a charge transfer type reaction.

Also working with Aerosil (on amorphous colloidal silicic acid), Thoma et al.(1966)observed that "surface active quaternary ammonium and pyridinium compounds were very differently adsorbed depending on their concentration". This phenomenon has also been observed in the present study in which a number of different powders were used and will be further commented on in Section 5.1.2.

Ullman et al. (1968), continuing this study of cationic surfactants onto Aerosil, discovered that the rate of adsorption is dependant upon both the pH and the electrolyte concentration. They also proposed two mechanisms for the sorption of cationic drugs:

(i) drugs which cannot form micelles, such as alkaloids or acridine derivatives are bound exclusively by ion exchange mechanisms and

(ii) cationic surfactants, or phenothiazine derivatives, are bound as ions at low concentrations and, generally, as molecules at higher concentrations.

Other colloidal materials have also been investigated with regard to their interaction with preservatives. Harris (1961) assessed the compatibility of the suspending agent bentonite with various antiseptics both by bacteriological and chemical means. As was to be expected from a knowledge of the negatively charged nature of bentonite particles, cationic antibacterial substances were found to be inhibited or inactivated, while anionic and non-ionic types were not.

McGinty and Lach (1976), working with the closely related substance montmorillonite, came to similar conclusions (although they found that certain non-ionics were also tenaciously bound) and postulated a two step binding mechanism.

The appearance of mould growth in sulphadimidine mixture for infants BPC prompted Beveridge and Hope (1967) to investigate the inactivation of benzoic acid by sulphadimidine. They concluded that benzoic acid was removed from the mixture by the undissolved sulphadimidine, probably by physical adsorption.

Khalil and Nasipuri (1973) conducted a more detailed investigation into this system and showed that the adsorption isotherm for benzoic acid on sulphadimidine conformed to the Langmuir equation. Three hydrophilic polymers were found to suppress the adsorption of benzoic acid. Their order to effectiveness was, polyvinylpyrrolidone $>$ methylcellulose $>$ sodium carboxymethylcellulose. Continuing their study of this system Nasipuri and Khalil (1974a) found benzoic acid adsorption to be pH dependant and later (1974b) found the suppressive effect of polyvinylpyrrolidone on benzoic acid adsorption by sulphadimidine to be time dependant.

Goudah and Guth (1965) have investigated interaction between starches and a number of pharmaceutical compounds. They have established that starches form complexes in solution with benzoic acid, salicylic acid, the parabens, p-aminobenzoic acid and ethyl p-aminobenzoate.

In a broad ranging study, McCarthy (1969) studied the adsorption of eight commonly used preservatives onto fifteen powders used in pharmaceutical and cosmetic suspensions. This investigation was continued by Horn et al. (1970) who studied the adsorption of a further nine preservatives onto the same powders. The loss of three more preservatives onto a similar group of powders has recently been reported (McCarthy et al. 1977).

Many authors stress the importance of microbiological testing of the final formulation, as most attempts to predict the tendency of a preservative to be inactivated are based on

tests on simple systems which do not approach the complexity of most complete formulations.

1.2 INTERACTIONS BETWEEN POWDERS AND MATERIALS OTHER THAN PRESERVATIVES

This field of study has attracted much attention in recent years and the volume of published material available is already considerable. No attempt will be made therefore to review this field fully. Reference will be made only to those aspects thereof which are of relevance to this study.

Attention has tended to focus on interactions between solids and solutes which are likely to be used together in medicinal suspensions, especially intestinal adsorbents and antacids in combination with antibiotics and anticholinergics.

Aggag et al.(1977) in a study of neomycin bioactivity in antidiarrhoeal mixtures observed, inter alia, that the neomycin/kaolin adsorption isotherm conforms to the Langmuir-equation, that polyvinylpyrrolidone also protects neomycin from adsorption by kaolin and that the electrolytes sodium chloride, sodium citrate and magnesium chloride were able to suppress partially the adsorption of neomycin by kaolin, with magnesium chloride exerting the most powerful effect. Heating in an oven at 170°C for two hours and hydration in distilled water followed by autoclaving at 121°C for 20 min were found to have no significant effect on the affinity of kaolin for neomycin.

Kaolin was found by El-Nakeeb and Yousef (1968) to have a far greater affinity for basic antibiotics than for neutral or acidic antibiotics and they suggested that adsorption might be due to a base exchange mechanism. Armstrong and Clarke (1971) agreed with this theory and presented evidence that the adsorption of crystal violet by kaolin involves release of magnesium from the kaolin lattice and cation replacement

through electrostatic attraction. They also discovered that crystal violet adsorption by kaolin increases with increase in pH over the range of 2,5 to 9,5.

Referring to phenothiazine derivative adsorption by kaolin and talc, Sorby et al. (1966) concluded that the failure of sodium chloride to depress adsorption tends to discount simple ion exchange as a major mechanism of adsorption.

In two papers on the adsorption of atropine by kaolin, Ridout (1968) suggested that a discontinuity in the Langmuir isotherm was due to irregularities in the kaolin surface.

In a study of the electrophoretic mobility and adsorptive properties of kaolin Armstrong and Clarke (1976) have now been able to deduce the following: "The picture of kaolin that emerges is of a clay bearing a negative charge on the cleavage faces due to lattice defects, the edge charge being reflective of the exposed atoms. While the cleavage plane negative charge is unaffected by pH, the sign and magnitude of the charges on the edge faces are strongly dependent on pH. The base-exchange capacity of kaolin in acidic or neutral solution is due to isomorphous replacement within the lattice in a similar manner to that proposed for montmorillonite and other clays. In alkaline solutions, however, the edge faces of the kaolin become negatively charged and can participate in exchange reactions. Thus, the fact that kaolin has never been shown to exhibit a positive potential by decreasing the pH is due to the negative charge of the greater area of the cleavage surface outweighing the positive charge on the smaller edge face area."

It is clear that whenever kaolin is used investigations should be made to ensure that the kaolin is not interfering with the activity of some other substance. For example: Yu et al. (1976) observed that patients suffering from chronic diarrhoea and stabilised with codeine phosphate suffered relapse after kaolin mixture was added to their treatment regimen. Investigations revealed that a percentage of the

codeine phosphate was being adsorbed by the kaolin.

In a study of *in vitro* adsorption of some anticholinergic drugs by various antacids, Blaug *et al*. 1965 observed that magnesium trisilicate had the highest adsorptive capacity of the antacids studied. Later Khalil and Moustafa (1973) in a similar study of the adsorption of some tranquilizers, sedatives and anticholinergic compounds by six antacids came to a similar conclusion, namely that, in most cases, magnesium trisilicate and magnesium oxide showed the highest adsorptive capacity. They also showed that the degree of adsorption was correlated to the pH of the antacid suspensions, the chemical structure of the adsorbates and the duration of equilibration.

The high adsorptive capacity of magnesium trisilicate was noticed once again by Khalil *et al*. (1976) in a study of the adsorption of antibiotics onto the same six antacids. Calcium carbonate and aluminium hydroxide have intermediate adsorptive power and kaolin and bismuth oxycarbonate the least.

El Masry and Khalil (1974) have found the adsorption of atropine and hyoscine onto magnesium trisilicate to be appreciable, but observed that elution takes place in acid medium so that intestinal adsorption of these alkaloids is unlikely to be affected unless the antacid raises the pH of the gastric contents to a relatively high value. In the same study, adsorption rate was high, as equilibrium was attained within ten minutes.

Khalil (1974a and 1974b) has also investigated the adsorption of digoxin and digitoxin by some antacids and, once again, found magnesium trisilicate to be the most powerful adsorbant. Possible impairment of the bioavailability of these two glycosides was discussed.

In the case of chloroquine adsorption onto magnesium trisilicate, Khalil (1977) has determined that interaction "not only resulted in adsorption of the antimalarial drug (possibly altering its bioavailability) but also reduced the acid reactivity of magnesium trisilicate".

In an investigation into the interaction of isoniazid and magnesium oxide, Wu et al. (1970) established that isoniazid was both chemisorbed and physically adsorbed to the magnesium oxide surface.

Two approaches have been adopted to investigate ways of reducing undesired adsorption by magnesium silicates. Firstly, Tanada et al. (1974) have found that the manufacturing method of magnesium silicate can be altered to reduce the amount of methylene blue adsorbed without altering its antacid properties. Secondly, Daabis et al. (1976) have investigated various additives serving to reduce the adsorption of tetracycline hydrochloride on magnesium trisilicate and milk and found citric acid to be the most effective.

In following up reports by Fearnley and Ellwal (1972) and others on contamination of magnesium trisilicate mixtures with Gram-negative bacteria, Beveridge and Todd (1973) found that Escherichia coli "interacted strongly with magnesium trisilicate particles in buffered solution with aggregation of the particles and ready removal of bacteria from suspension, an equilibrium being attained between adhering and unattached cells." Such adsorption of bacteria to suspended powders is of great significance to the present study and will be discussed further under Section 5.2.

Moriguchi and Keneniwa (1969) also have investigated the protective effect of polyvinylpyrrolidone using cyanocobalamin and talc. They discovered that the decreased adsorption of cyanocobalamin on talc was caused by the

adsorption of the polyvinylpyrrolidone on the talc and not by any direct interaction between polyvinylpyrrolidone and cyanocobalamin. The mechanism of the protective effect is probably steric hindrance of the polyvinylpyrrolidone molecule interfering with the adsorption of the cyanocobalamin rather than simple competition for adsorption between the two species.

Finally, the adsorption properties of titanium dioxide surfaces have recently been discussed by Rupprecht (1976). These findings may be summarised as follows:

(i) Titanium dioxide is amphoteric in nature and is capable of acting as an anion exchanger below its isoelectric point of pH 6,5 and as a cation exchanger above that value.

(ii) Ionogenic surfactants are strongly bound by ion exchange mechanisms and by hydrophobic interactions.

(iii) Surfactant adsorption is increased by neutral salts if there is no competitive adsorption involving the salt ions.

(iv) Strongly adsorbed polyvalent ions can act as secondary adsorption sites for organic ions bearing the same electrical charge as the titanium dioxide surface under the given pH conditions.

1.3 ELEVATED TEMPERATURE INTERACTIONS

No reports could be found in the literature on the effect of temperature change on preservative/powder interactions. However the quaternary ammonium preservatives and the organomercurials, phenylmercuric nitrate and thiomersal, are known to be stable to autoclave temperatures in dilute solution in the absence of adjuvants. Walter and Errera (1967) have reported that autoclaving is not detrimental to quaternary ammonium compound solutions.

Tsuji et al. (1964) have shown that simple thiomersal solutions are very stable to heat but that thermal decomposition is

accelerated by the presence of Cu^{++}, Fe^{+++} or Zn^{++} ions but not by Ca^{++} or Mg^{++} ions.

Richards and Reary (1972) have determined that autoclaving of thiomersal and phenylmercuric nitrate in the presence of sodium thiosulphate or E.D.T.A. causes inhibition of their antibacterial activity while autoclaving them with sodium metabisulphite has no such effect.

As mentioned earlier, Hart (1973) has established that phenylmercuric nitrate is inactivated in Zinc Sulphate and Adrenalin Eye Drops BPC 1968 when subjected to autoclaving at 115°-116°C and the author has therefore recommended that these eye drops be sterilised by filtration.

Patel and Foss (1964) have observed that the binding of the parabens to polysorbate 80 decreases with increase in temperature, whereas with polyethylene glycol 4000, it increased with increase in temperature.

In the case of cyanocobalamin and methylparaben, Yazdany and Badii (1976) showed that it was the cyanocobalamin that deteriorated when these two substances were autoclaved together.

Jaminet et al. (1970) found that 0,02% chlorohexidine solutions are unaffected by a temperature of 100°C regardless of pH. Slight thermal decomposition took place after 30 minutes at 120°C however, and this effect tended to increase with increase in pH but was still insufficient to affect significantly the bactericidal activity of the solution.

Finally, Nair and Lach (1959) have shown that sterilisation of a 0,5% chlorobutanol solution at 121°C for 20 minutes causes hydrolysis which ranges from 3,3% at pH 2,3 to 94,4% at pH 6,6.

From what has been written in the preceding pages it is evident

that a host of factors can adversely affect the activity of antimicrobial preservatives. Wherever possible, these factors have been taken into account in studying to greater depth the interaction between certain powders and selected organomercurial and quaternary ammonium compounds respectively.

CHAPTER 2 MATERIALS AND METHODS.

2.1 MATERIALS USED

2.1.1 Powders

In acquiring powders for use in this study, care was taken to select grades that are commonly used in cosmetic and pharmaceutical formulation. For most powders, the BP (1973) or BPC (1973) quality was obtained from South African Druggists, a reputable local supplier. No attempt was made to acquire highly pure analytical reagent grades as it was felt that, because most of the preservatives tested were to be used in very low concentration, trace impurities in the powders could have a significant effect on preservative loss if they reacted with the preservative. Whilst it was realised that pharmacopoeial grades could show variation as regards level of trace impurities and particle size, it was nevertheless considered more realistic to use a grade which is commonly used in formulation. This meant that if an interaction took place between a preservative and an impurity in the powder, the extent of such interaction might vary considerably from batch to batch or brand to brand of the powder, but it would probably be observed. The use of an analytical reagent grade could result in a situation where an interaction, which would normally take place in practice, not being observed at all in these tests. It is worth noting that in the few cases in this work where tests done overlapped with those done by others, good agreement of results was observed despite the probability of having used different batches or makes of preservative and powder.

It should be borne in mind, therefore, that where the extent of an interaction is expressed quantitatively in this work it should not be considered as giving an absolute indication

of the amount or proportion of preservative which would be lost in practice but rather to give an indication of the extent to which the interaction varies within a group of related preservatives or within a group of related powders. The degree of inactivation of the preservative would probably be quite different in practice, even if the same batch of preservative and powder were to be used, due to the presence of adjuvants and active ingredients, or a different pH in the actual formulation.

In deciding what powders were to be tested, efforts were made to select as broad a range as possible of powders commonly used in pharmaceutical and cosmetic suspensions. Whilst liquid aqueous suspensions represent the main type of formulation which would be considered in this investigation, sight was not lost of the fact that the results of these tests would be relevant to any formulation or situation in which an aqueous liquid, an insoluble powder and an antimicrobial substance were mixed, i.e. this investigation could be of relevance to formulations as varied as antiseptic throat lozenges and semisolid aqueous creams. For this reason, certain powders such as zinc oxide, titanium dioxide and talc, which are not often used in fluid aqueous suspensions, have been included in the list of powders studied.

As centrifuging was to be the method of separating the suspended powders from the vehicle, the use of certain colloidal powders like bentonite was avoided, as their interactions are best studied by dialysis methods.

Brief reasons for the selection of each of the fifteen powders used are as follows:

Colloidal silicon dioxide (Degussa)
This material has found wide application as an adjuvant in recent years. It is an extremely fine, light powder of

particle size about 15 nm. It has the ability to absorb a large quantity of water without liquefying and, when excess water is added, the suspension so produced is viscous. It is therefore widely used as a suspending agent and thickener in suspensions, ointments and suppositories, as a stabilizer in emulsions, and as a filler in tablet coatings. Its water absorbing properties are used to prevent clogging of hygroscopic powders, a feature which enables it to improve the flow properties of powders and granules in tablet and capsule production.

Its pharmaceutical and cosmetic applications have been reviewed by Ferch (1970).

Aerosil brand colloidal silicon dioxide has been used in this study. For the sake of brevity, it will henceforth be referred to as Aerosil.

Bismuth carbonate (BPC)

Bismuth carbonate is the name used in the BPC for a basic bismuth carbonate of varying composition, approximating to the formula $(Bi\ O)_2\ CO_3$, $\frac{1}{2}H_2O$. It is also known as bismuth oxycarbonate and bismuth subcarbonate.

Although this compound is only a weak antacid and although there is little justification for its inclusion in antacid formulations, it is still widely used in proprietary antacid preparations, some of which are aqueous suspensions which may need preservation; hence, its inclusion here.

Although Martindale's Extra Pharmacopoeia makes no mention of any antibacterial action of this compound, it does mention the treponemicidal action of certain bismuth salts. One wonders, therefore, whether bismuth ions have an effect on bacteria too, and whether bacteria adsorbed to bismuth carbonate particles would be inhibited or even killed by

traces of bismuth ions passing into solution. Even if the inhibitory effect is only very slight, it may be sufficient to more than compensate for a degree of adsorption loss of preservative onto the powder. If this were the case, we would expect preservative solution/bismuth carbonate suspensions and supernatant liquids to retain antimicrobial activity despite chemical evidence of loss of preservative activity, although alteration of pH or concentration of preservative in an adsorbed layer on the surface of the powder could possibly also produce this effect.

Calcium carbonate (BP)

The names calcium carbonate and precipitated calcium carbonate distinguish this compound from the naturally occuring substance chalk. Calcium carbonate is an effective antacid, which tends to have a constipating effect. For this reason, it is also used to combat diarrhoea, often in combination with other antidiarrhoeals like kaolin. When used as an antacid, it is usually combined with magnesium carbonate or hydroxide to counteract the constipating effect. Assessment of preservative inactivation by this substance would perhaps be of greater relevance if the combined effect of calcium carbonate and kaolin or calcium carbonate and magnesium carbonate (or hydroxide) was investigated.

Calamine (BP)

Calamine BP is a basic zinc carbonate coloured pink with ferric oxide. It has a mild astringent action on the skin and is used in dusting powders, creams, lotions and ointments for a variety of skin conditions. It is often included in liquid formulations for its fleshlike colour, a feature which is widely utilised in cosmetic preparations. Interactions of calamine with preservatives could be due to either or both of its components.

Kaolin (BP)

Kaolin is a native hydrated aluminium silicate purified by elutriation. Both heavy and light kaolin are used for their adsorbent properties, light kaolin being used internally to adsorb toxic substances from the gut in the treatment of poisoning and various forms of diarrhoea and, externally, in dusting powders. The main use of heavy kaolin is as an ingredient of kaolin poultice.

Both grades of kaolin have been included in this study in order to assess whether variation in particle size, resulting in different total particle surface area, has a significant effect on the degree of inactivation of preservatives in the concentrations used.

Diatomite or Purified Kieselguhr (BPC 1949)

This material consists of an amorphous form of almost pure silicon dioxide. It consists chiefly of fragments of diatoms, suitably purified.

It is used in the preparation of dusting powders and, significantly, in disinfectant powders. Its use as a filter aid is of interest; for when used in this capacity, it could adsorb a significant amount of preservative and other solutes from solutions.

Magnesium carbonate (BP)

This substance is a basic hydrated magnesium carbonate. It is a weak antacid and a mild laxative, but as an antacid it is less effective than magnesium oxide and has the disadvantage of liberating carbon dioxide. It is available in two varieties, heavy and light. The light variety is a diffusible powder very suitable for the preparation of aqueous suspensions while heavy magnesium carbonate is used mainly for tablets and powders. They are equally effective as antacids.

As with kaolin, both grades are included in this study in order to assess whether the variation in particle size and related change in surface area has a significant effect on the degree of inactivation of preservatives in the concentrations used. The appearance of the two varieties is noticeably different. Light magnesium carbonate is a fluffy powder which diffuses into the air readily, while heavy magnesium carbonate is quite dense. The light variety is about six or seven times more bulky then the heavy.

Magnesium oxide (BP)

Magnesium oxide is a potent antacid and a mild laxative. It too is available in two varieties, but only the light form has been included in this study for the sake of comparison with light magnesium carbonate. Its main use is in the preparation of magnesium hydroxide mixture.

Magnesium trisilicate (BP)

This substance is a hydrated magnesium silicate corresponding to the formula $2MgO$, $3SiO_2$, with water of crystallisation. It has both antacid and adsorbent properties. Its adsorbing power is greater than that of light kaolin. Its antacid action is slow but prolonged, a valuable feature as long-acting antacids are required in the treatment of gastric and duodenal ulcers. Either alone or in combination with other antacids, it is a component of a number of proprietary and official antacid mixtures.

Starch (BP)

Starch BP may be obtained from maize, wheat, rice or potatoes. It contains the polysaccharides amylose and amylopectin. It has the ability to adsorb substantial amounts of water at relative humidities above 75 % and is, in fact, widely used in dusting powders for its absorbent properties. In boiling water starch forms a translucent viscous fluid or

jelly. In this study the behaviour of suspended maize starch has been investigated at low temperatures only since preservative interactions with jelled starch would best be studied by dialysis methods.

These investigations could be of relevance in the formulation of certain lotions in which ungelatinised starch is sometimes included and in the formulation of dusting powders containing antiseptic substances.

Purified Talc (BP)

This is a purified native magnesium silicate approximating to the formula $Mg_6(Si_2O_5)_4(OH)_4$. Talc is used mainly in dusting powders for its adsorptive and lubricating properties. Its use in clarifying liquids, especially those containing volatile oils, could enable it to interact with preservatives or other solutes present in low concentration. Furthermore, its use as a lubricant in the production of compressed tablets and lozenges would enable it to interact with antiseptic substances in these formulations.

Titanium dioxide (BPC)

Titanium dioxide is used for its soothing, protective effect on the skin in a number of semisolid preparations, which are used in the treatment of pruritis and certain exudative dermatoses. As it is an intensely white pigment, it has been included in suncream preparations which provide complete protection from the sun's rays. When used in such preparations and in cosmetics, a red pigment such as ferric oxide is often added to produce a flesh coloured product. It is also used to opacify hard gelatin capsules and tablet coatings when the active ingredients are light-sensitive.

Zinc oxide (BP)

This substance is also used in external preparations for its soothing, protective and mildly astringent effect on the skin.

A large number of official and proprietary creams, dusting powders, liniments, lotions and ointments contain zinc oxide. It is an ingredient of many cosmetic preparations too.

2.1.2 Preservatives

The basis for the selection of preservatives for use in this study was rather different from that used for the selection of powders. As trace impurities in the preservatives were unlikely to have a detectable influence on the tests and if the batch or brand of a preservative used differed in potency from other batches or brands of the same preservative these phenomena would not complicate the investigations provided that the same sample of preservative was used for all the tests. Nevertheless, preservatives of the highest available quality from well known manufacturers were used in all cases.

The preservatives selected for the preliminary study (Chapter 3) were chosen largely because they had not previously been subjected to this type of study in the concentrations used. Subsequent investigations into the interactions of organomercurials and of the quaternary ammonium compounds necessitated the inclusion of selected additional preservatives.

As the interactions of each preservative were studied at only one or two concentration levels, the concentrations selected were those which, as far as could be determined, are commonly used in practice.

Reasons for the selection of the concentration or concentrations of each preservative used were as follows:

2.1.2.1 Preliminary study

Benzoic acid - 0,1 %

Martindale's Extra Pharmacopoeia states that 0,1 % benzoic acid is an efficient preservative for pharmaceutical preparations, provided that the pH is not above 5, as its antimicrobial properties are due to the undissociated acid.

o - Chlorobenzoic acid - 0,1 %

This substance has been used as a preservative for cosmetic and toilet preparations. In the absence of specific information regarding concentrations used, a concentration of 0,1 % was selected.

Cetalkonium chloride - 0,1 %

A concentration of 0,1 % was chosen as the lowest concentration that could conveniently be assayed by a direct ultraviolet spectrophotometric method. A concentration of 0,01 % may have been more appropriate, however, as Rao et al. (1970) describes it as "a powerful bactericidal agent against certain gram negative and gram positive bacteria", and there is on the South African market, at least one product (a surface anaesthetic) which uses it in a concentration of 0,01 %.

Cetylpyridinium chloride - 0,1 %

This substance will, for the sake of convenience, be grouped with the QACs in this study. Cetylpyridinium chloride (CPC) is used in concentrations ranging from 0,01 to 1,0 %. The usual strength of the United States National Formulary CPC solution is 0,1 %. Thus, 0,1 % was chosen, as this concentration would also enable comparisons to be made with cetalkonium chloride solution of the same concentration.

Dehydroacetic acid - 0,05 %

Dehydroacetic acid (DHA) has been used mainly for the preservation of cosmetic products in the concentration range 0,02 to 0,2 %. An intermediate concentration, 0,05 %, was chosen for this study.

8 - Hydroxyquinoline sulphate - 0,1 %

This substance is used as the potassium salt in lotions and creams in concentrations ranging from 0,05 to 0,5 %. A concentration of 0,1 % was selected for use here.

Methyl hydroxybenzoate 0,1 %

This compound is usually used as a preservative in concentrations of 0,1 to 0,2 %. As 0,2 % is the limit of its solubility, 0,1 % was the concentration selected.

Phenol 0,5 %

The concentration of phenol, which is nearly always used as a preservative in pharmaceutical formulations, is 0,5 %, e.g. calamine lotion and certain multidose injections make use of this concentration, hence the use of 0,5 % phenol here.

Phenylmercuric nitrate 0,002 %

The BPC uses 0,002 % phenylmercuric nitrate (PMN) for preserving eye drops. This concentration is also used for sterilisation by heating with a bactericide. 0,001 % PMN is used for preserving injections in multidose vials but a concentration of 0,002 % was selected for this study because the colorimetric method of analysis used gave best results if a minimum concentration of 0,002 % was used.

2.1.2.2 Further investigations

Benzalkonium chloride 0,01 %

McCarthy (1969) used 0,1 % benzalkonium chloride (BZC) in his investigation of preservative/powder interactions because, at this concentration, it was easily assayed by ultraviolet spectroscopy. However, the BPC now uses 0,01 % BZC as an eye drop preservative, and this concentration would presumably be effective in preserving other aqueous formulations provided that incompatibilities do not exist. Because of the popularity of this preservative, it was felt that the

lower concentration also warranted investigation.

Cetrimide 0,01 %

This concentration of this preservative was investigated in order to allow comparison with 0,01 % BZC (See section 5.1.2)

Cetyltrimethylammonium bromide (CTAB) 0,01 % and 0,1 %

Similarly, these two concentrations of this preservative were investigated for the sake of comparison with the other quaternary ammonium compounds whose interactions had also been investigated at these concentrations.

Thiomersal 0,02 %

Thiomersal was investigated in order to establish whether other organomercurial preservatives exhibited similar interactions to those of PMN with powders. Some thought was given to whether it would be advisable to investigate it at the same concentration as was used for PMN, ie 0,002 %, but it was realised that the results of a test of this nature would be of purely theoretical interest because thiomersal is not used in such dilute solution in practice.

Thiomersal, in 0,01 to 0,02 % concentration, is used as a preservative in biological products, concentrations of 0,005 to 0,01 % have been used in eye drops and creams, and solutions containing 0,1 % have been applied to the skin. A concentration of 0,02 % was therefore selected for these tests.

2.2 BASIC TECHNIQUE USED IN INVESTIGATING PRESERVATIVE/POWDER INTERACTIONS

To make it possible to compare the extent of the various preservative/powder interactions observed, it was necessary to adopt a standardised approach for the testing of these systems. Complete standardisation, in the sence of testing the same concentration (or equimolar concentrations) of each of the preservatives, was not considered practicable as the

concentrations of preservatives used in practice vary considerably. Thus, a commonly used concentration of each preservative was selected.

A simple test for preservative/powder interaction would involve mixing the powder with the preservative solution, allowing the suspension so formed to stand with regular agitation for a period of time, then centrifuging, removal of the supernatant liquid and analysis thereof. In order to achieve standard conditions in performing a test of this nature, it was deemed necessary to:

(i) use a standard concentration of powder,

(ii) agitate the solution in a standardised manner at set intervals,

(iii) store the suspension in this manner for a set period of time,

(iv) store at a set temperature, preferably in an incubator set slightly above the prevailing room temperature so that cooling would not be necessary at any stage,

(v) use containers and closures that would not permit evaporation of the water or the preservative (if volatile) during the period of storage and which would not interact with the preservative in any way, and

(vi) repeat the test on a similar system containing distilled water in the place of the preservative solution so that allowance could be made for any positive assay results given by the water-soluble extractive from the powder.

The following factors were also considered but not implemented for the reasons given:

(i) A standardised pre-treatment of the powders, such as heating in an oven at a high temperature in order to

activate them so that adsorption of preservatives would be maximal. This was not done because: (a) such pre-treatment is not part of normal manufacturing procedure, (b) for reasons discussed previously, a certain variation could, in any case, be expected between different batches and brands of the same powder, the object of this study being mainly to establish the order of magnitude of preservative/powder interactions, and (c) in the case of neomycin adsorbed onto kaolin and veegum such pre-treatment has now been shown by Aggag et al. (1977) to have no significant effect on the extent of adsorption.

(ii) A standardised centrifuging procedure to separate the supernatant liquid from the powder. It was found that a number of powders separated rapidly under gravity leaving a clear supernatant and that centrifuging was only necessary to compact the sediment, while other powders only gave a clear supernatant after prolonged centrifuging at high speed. For example, some magnesium trisilicate suspensions required high speed centrifuging for approximately one hour before a clear supernatant was obtained. Standardisation was not even feasible for specific powders due to the effect of surface active preservatives on the properties of the suspensions. For example, with surface active preservatives magnesium trisilicate settled rapidly due to flocculation, so it would have served no purpose to centrifuge such systems for as long as the highly peptised magnesium trisilicate/water control tubes.

All test and control tubes were therefore centrifuged until a visually clear supernatant liquid was obtained.

2.2.1 Test procedure

The following standard test procedure was therefore adopted

for the preliminary tests described in chapter 3.

(i) 0,5 g of each powder was placed in rimless neutral glass test tubes of approximately 15 ml capacity. (In the case of Aerosil 0,1 g was used due to its bulk).

(ii) 10 ml of preservative solution was pipetted into each tube, which was then sealed with a latex rubber cap. The powder in each tube was brought fully into suspension by agitation with a swirling movement that ensured minimal contact of the solution with the rubber cap.

(iii) Similar tubes containing each powder, plus water, were prepared as controls. A further control tube contained 10 ml of the preservative solution only.

(iv) All tubes were placed in an incubator at 25 ^{o}C for at least two weeks with daily agitation using the same swirling technique.

(v) After this period, the tubes were quickly transferred to a Hettich Universal II centrifuge and centrifuged until a completely clear supernatant liquid was obtained in all cases. The time interval between removal of the tubes from the incubator and centrifuging was kept to a minimum in order to avoid change in the amount of preservative removed by the powder due to the slight drop in temperature.

(vi) An aliquot of each supernatant liquid from both test and control tubes was then removed with a pipette and analysed for preservative content. The liquid in the tube containing preservative solution only was analysed similarly.

(vii) After resuspension of the powder in each tube, the pH was measured with a Metrohm E520 pH meter.

The following procedural changes were introduced when the subsequent tests described in chapters 4 and 5 were done:

(i) The powder/preservative suspensions were stored in neutral glass ampoules of 10 ml nominal capacity sealed by fusion of glass. This provision facilitated agitation of the suspensions and also permitted them to be autoclaved for certain tests.

(ii) For tests requiring autoclaving of powder/preservative suspensions, the suspensions, prepared as before, were sealed in 10 ml ampoules, placed on their sides in an autoclave and subjected to steam sterilisation at 115 °C for 30 min. After allowing them to cool in the autoclave to 100 °C, the ampoules were removed, allowed to cool to room temperature and then stored for the same period and agitated in the same manner as for systems which had not been heated, as described in point (iii) below. Powder/water control suspensions in ampoules were subjected to the same treatment.

(iii) The period of storage at 25 °C was reduced to 24 hours with the ampoules containing the suspensions being shaken four times during this period. The ampoules were stored on their sides so that a maximum surface area of sediment was exposed to the supernatant liquid.

This change was introduced when it was realised from the following experiment and reports in the literature that equilibrium was attained in powder/preservative systems in a very short period of time:

(a) In an investigation into the effect of time on inactivation of 0,002 % phenylmercuric nitrate by certain powders it was found that, except in the case of starch, the percentage loss after two hours did not differ significantly from that observed after twelve weeks (see Section 4.3).

(b) El-Masry and Khalil (1974) in their study of the adsorption of atropine and hyoscine onto magnesium

trisilicate, observed that equilibrium was attained after 10 min.

(c) Armstrong and Clarke (1976) in a study of the rate of uptake of 0,05 % m/v gentian violet on kaolin at various pH's showed in each case that equilibrium is attained in about 10 min (Fig. 5).

(d) Bean and Dempsey (1971), referring to a benzalkonium chloride/kaolin system, stated that equilibrium was attained in six hours.

(e) Sorby (1968), in a study of adsorption of promazine onto attapulgite and charcoal, remarked that most of the interaction takes place in the first five minutes but that some further adsorption continues over a longer period of time.

2.2.2 Preparation of preservative solutions

It was not considered necessary to determine the percentage purity of each preservative used prior to preparing the solutions to be tested as the methods of analysis used had been shown to be reliable for determining the strength of preservative solutions relative to standard solutions. A slight variation in the concentration of a preservative solution used for different series of tests on a preservative would therefore be of no consequence, provided that, within a series of tests, the same solution was used for the tests and for the control tube containing preservative solution only, this latter tube being used as the standard with which the supernatant fluids were compared. Furthermore, all solutions of each preservative were prepared from the same sample of preservative throughout this project.

Solutions were prepared by careful weighing off of the required quantity of solid preservative on a sensitive balance, dissolving it in water and making up to volume in a volumetric flask. Only in the case of benzalkonium chloride was some

difficulty experienced in making up solutions, which did not vary significantly in concentration. This anomaly was due to the fact that this substance was obtained in the form of a 50 % m/v aqueous solution, which was very difficult to measure accurately due to its viscosity. Its specific gravity was therefore determined with a pycnometer as 0,984 and, thereafter, solutions were prepared by weighing off 0,984 x 2 x the quantity of benzalkonium chloride required and making up to volume in a volumetric flask.

2.3 ULTRAVIOLET SPECTROPHOTOMETRIC METHODS OF ANALYSIS

In deciding on the most suitable methods of analysis for the preservative solutions and supernatant liquids the following factors were borne in mind:

(i) Rapid techniques would be required as a large number of determinations would have to be done.

(ii) Extreme accuracy was not essential as the amount of preservative lost would be subject to variation with variation in pH, other ingredients of the formulation, the batch and particle size of the suspended powder used, the container and closure used, etc.

(iii) The volume of samples required for analysis should preferably not exceed 5 ml, as it would not be feasible to prepare, incubate and agitate large volumes of each preservative/powder system.

Ultraviolet spectroscopy was found to fit these requirements and to be a satisfactory technique provided that the preservative absorbed radiation strongly in the ultraviolet region and provided that a fairly large dilution of the preservative solution was required before reading the absorbance in a spectrophotometer. The reason for this latter proviso was twofold:

(i) If a supernatant liquid was very slightly cloudy or if a few particles of powder were inadvertently removed

with the supernatant, the absorbance reading could be affected, and

(ii) Water-soluble extractive from the powder could affect the absorbance reading if it absorbed light at the wavelength used for the analysis. In all ultraviolet spectroscopic determinations an attempt was made to negate the effect of water soluble extractive by analysing an aqueous control supernatant liquid in the same manner and by subtracting the absorbance reading so obtained from that obtained for the supernatant liquid under test. Unfortunately, in some cases, the quantity of water-soluble extractive in the supernatant liquid appeared to be affected by the presence of the preservative. This effect was most noticeable when surface active preservatives were tested. Thus, the water-soluble extractive content in such cases would be different in the test and the control samples. However, if the light absorbance of the preservative solution is very much higher than that of the water-soluble extractive, the effect of the latter would be slight and variation in water-soluble extractive content between the test and the control samples would have a negligible effect. If the preservative solution absorbs light very strongly at the required wavelength, a high dilution is required before analysis.

Thus in both the above cases dilution of the preservative solution must be considerable in order to negate the effect of potential errors arising from the analysis of suspension supernatant liquids. It follows, therefore, that ultraviolet spectrophotometry is most suitable for detection of preservative loss in the presence of powders when the preservative solution used absorbs light very strongly at the ultraviolet wavelength used for the analysis.

2.3.1 Preparation of standard curves

For each of the preservatives analysed in this manner:

(i) A suitable absorbtion peak in the ultraviolet region was located.

(ii) A stock solution of the preservative of the concentration decided upon for these tests was prepared as accurately as possible by use of a sensitive chemical balance to weigh off the preservative and making the solution up to volume in a volumetric flask.

(iii) Suitable dilutions of this solution were prepared by use of volumetric glassware.

(iv) The absorbance of these dilutions was measured at the required wavelength with a Beckman DB spectrophotometer and with distilled water in the reference cell.

(v) A standard curve of absorbance/concentration was plotted for each preservative.

The standard curves for the various preservatives and information relevant to the determination thereof as well as conclusions drawn were as follows:

Benzoic acid (Fig. 1)

Stock solution: 0,1 % m/v in water

Dilutions prepared: 1-50 (0,002 %), 1-25 (0,004 %), 3-50 (0,006 %) and 2-25 (0,008 %)

Wavelength used: 270 nm

Conclusion: Benzoic acid obeys Beer's Law at 270 nm over the above concentration range. A suitable dilution of the stock solution for analysis is 1-25, which corresponds to a concentration of 4×10^{-5} g/ml, and an absorbance value of approximately 0,26.

o-Chlorobenzoic acid (Fig. 2)

Stock solution: 0,1 % m/v in water

Dilutions prepared: 1-20 (0,005 %), 1-10 (0,01 %), 3-20 (0,015 %), 1-5 (0,02 %), 3-40 (0,0075 %) and 5-40 (0,0125 %)

Fig. 1 STANDARD CURVE FOR THE DETERMINATION OF BENZOIC ACID.

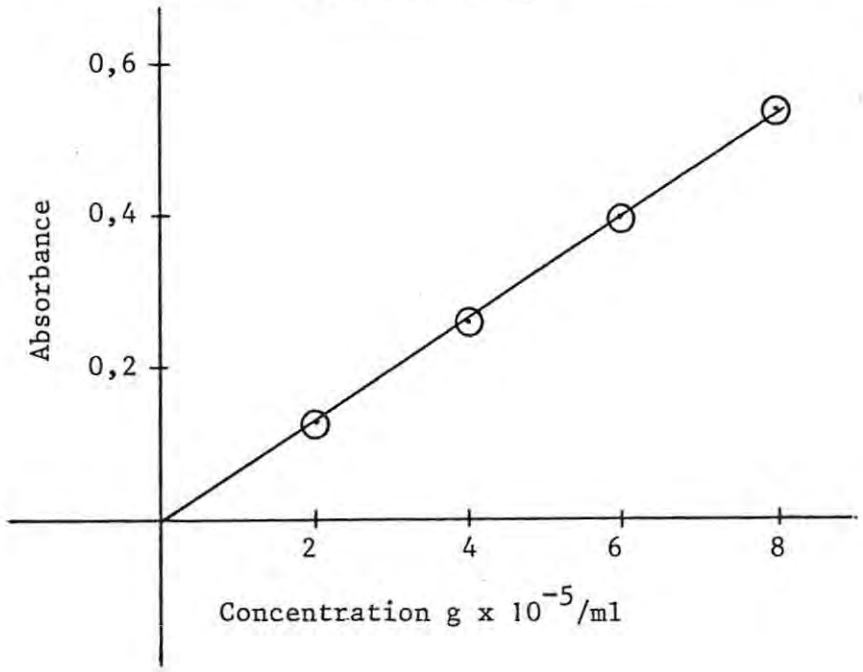


Fig. 2 STANDARD CURVE FOR THE DETERMINATION OF o-CHLOROBENZOIC ACID

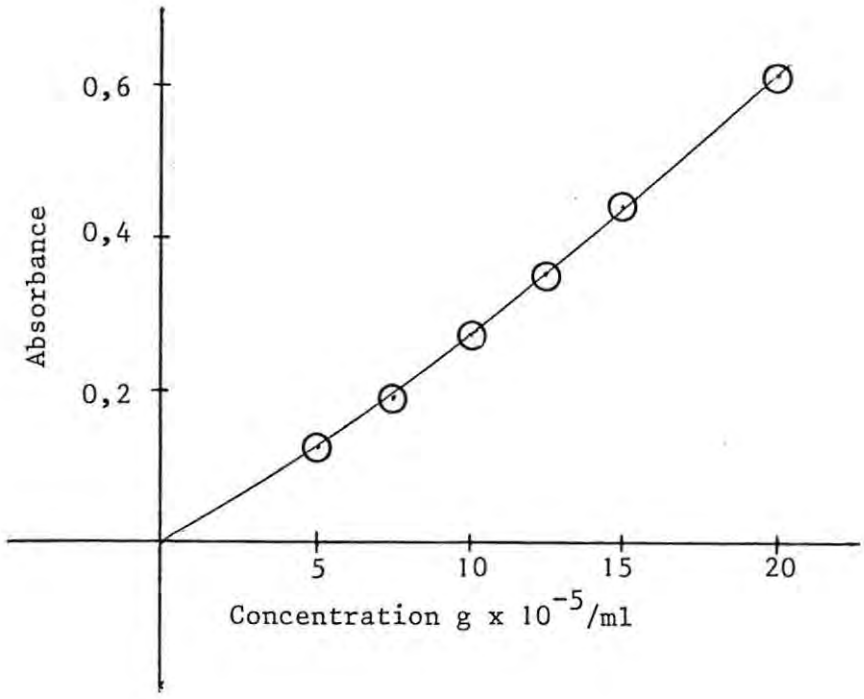


Fig. 3 STANDARD CURVE FOR THE DETERMINATION OF CETALKONIUM CHLORIDE

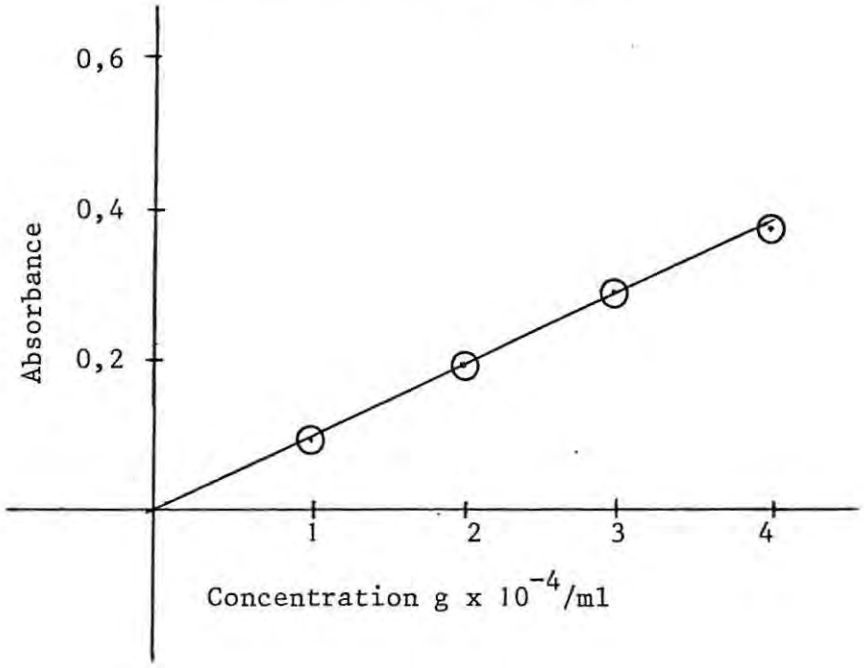
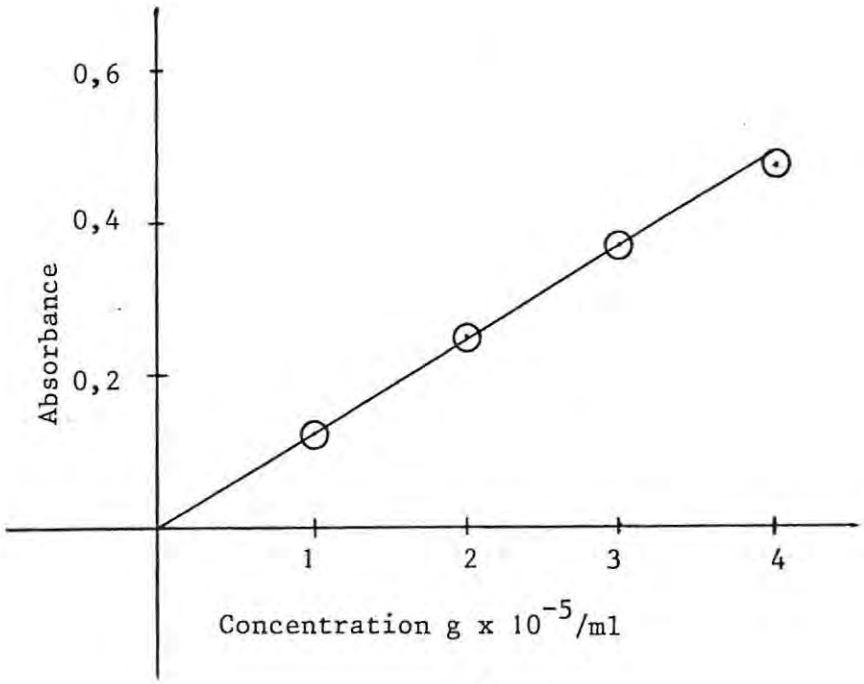


Fig. 4 STANDARD CURVE FOR THE DETERMINATION OF CETYLPYRIDINIUM CHLORIDE



Wavelength used: 272 nm

Conclusion: This substance does not obey Beer's Law at 272 nm as the graph is a slight but definite curve. The concentration of unknown solutions was therefore obtained from the graph. A suitable dilution of the stock solution for analysis is 1-10, which corresponds to a concentration of 1×10^{-4} g/ml, and an absorbance value of approximately 0,27.

Cetalkonium chloride (Fig. 3)

Stock solution: 0,1 % m/v in water

Dilutions prepared: 1-10 (0,01 %), 1-5 (0,02 %), 3-10 (0,03 %), 2-5 (0 04 %).

Wavelength used: 262 nm

Conclusion: Cetalkonium chloride obeys Beer's Law at 262 nm over the above concentration range. A suitable dilution of the stock solution for analysis is 1-4, which corresponds to a concentration of $2,5 \times 10^{-4}$ g/ml, and an absorbance value of approximately 0,24.

Cetylpyridinium chloride (Fig. 4)

Stock solution: 0,1 % m/v in water

Dilutions prepared: 1-100 (0,001 %), 1-50 (0,002 %), 1-25 (0,004 %)

Wavelength used: 259 nm

Conclusion: Cetylpyridinium chloride obeys Beer's Law at 259 nm in concentrations up to 0,003 %. It appears that the graph may not be linear above this concentration. Therefore all solutions were diluted so that absorbance readings of less than 0,37 were obtained. A suitable dilution of the stock solution for analysis is 1-50, which corresponds to a concentration of 2×10^{-5} g/ml, and an absorbance value of approximately 0,25.

Dehydroacetic acid (Fig. 5)

Stock solution: 0,05 % m/v in water

Dilutions prepared: 1-200 (0,00025 %), 1-100 (0,0005 %), 3-200 (0,00075 %), 1-50 (0,001 %)

Wavelength used: 302 nm

Conclusion: Dehydroacetic acid obeys Beer's Law at 302 nm over the above concentration range. A suitable dilution of the stock solution for analysis is 1-100, which corresponds to a concentration of 5×10^{-6} g/ml, and an absorbance value of approximately 0,27.

8-Hydroxyquinoline sulphate (Fig. 6)

Stock solution: 0,1 % m/v prepared in 0,1 M sodium hydroxide

Dilutions prepared: 1-100 (0,001 %), 1-50 (0,002 %), 3-100 (0,003 %) and 1-25 (0,004 %)

Wavelength used: 334 nm

Conclusion: This substance conforms to Beer's Law at 334 nm over the above concentration range. A suitable dilution of the stock solution for analysis is 1-50, which corresponds to a concentration of 2×10^{-5} g/ml and an absorbance value of approximately 0,29.

Methyl hydroxybenzoate (Fig. 7)

Stock solution: 0,1 % m/v in water

Dilutions prepared: 1-500 (0,0002 %), 1-250 (0,0004 %), 3-500 (0,0006 %), 2-250 (0,0008 %)

Wavelength used: 256 nm

Conclusion: Methyl hydroxybenzoate obeys Beer's Law at 256 nm over the above concentration range. A suitable dilution of the stock solution for analysis is 1-250, which corresponds to a concentration of 4×10^{-6} g/ml, and an absorbance value of approximately 0,40.

Fig. 5 STANDARD CURVE FOR THE DETERMINATION OF DEHYDROACETIC ACID

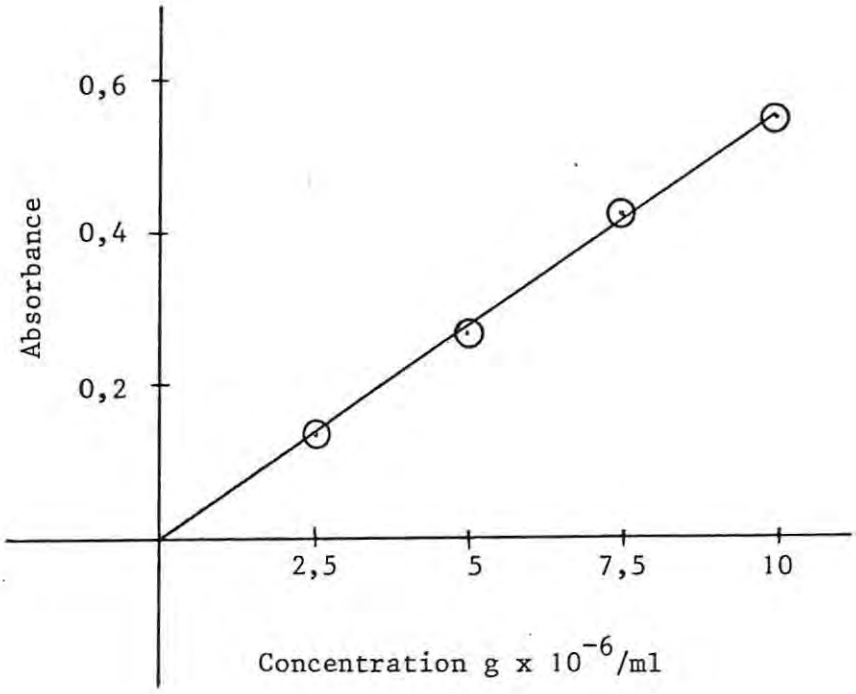


Fig. 6 STANDARD CURVE FOR THE DETERMINATION OF 8-HYDROXYQUINOLINE SULPHATE

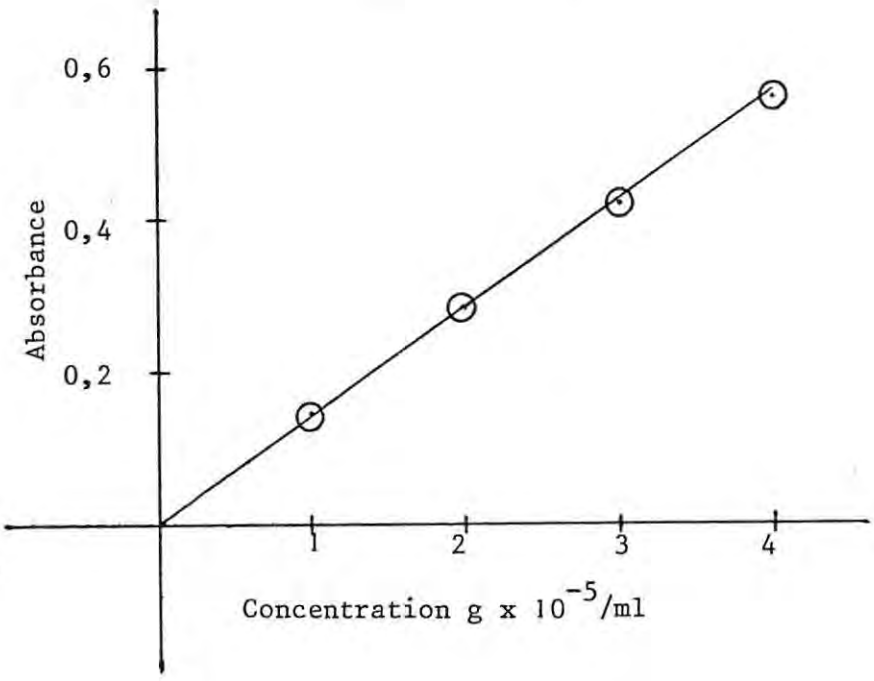


Fig. 7 STANDARD CURVE FOR THE DETERMINATION OF METHYL HYDROXYBENZOATE

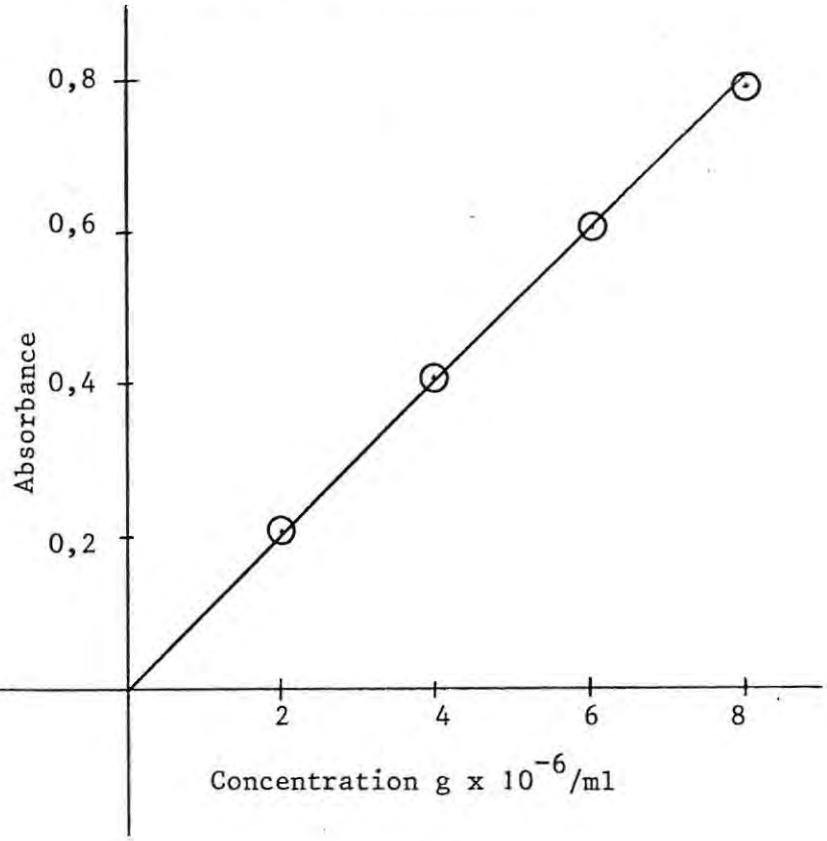
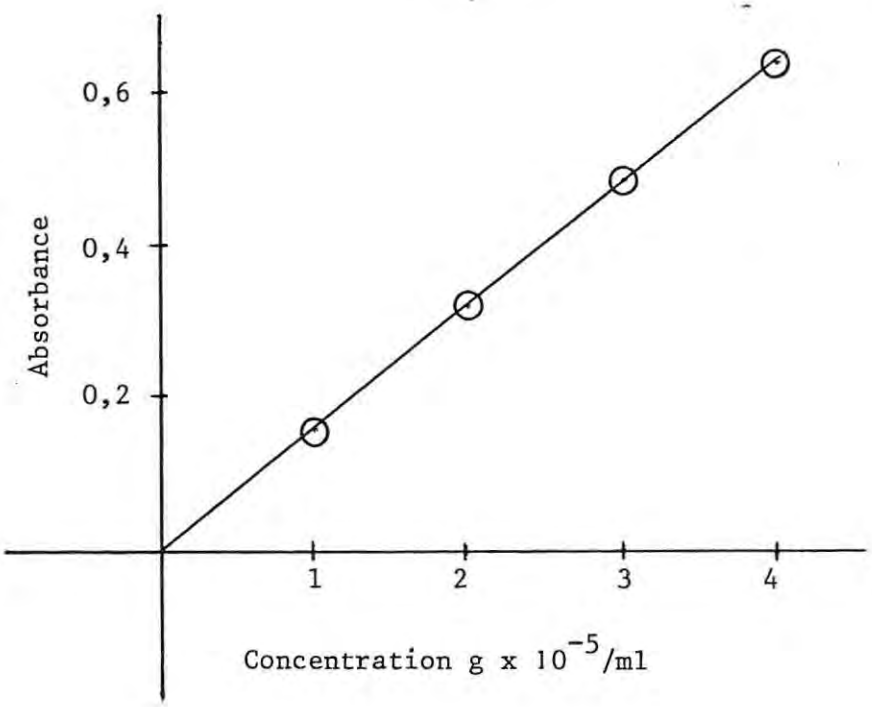


Fig. 8 STANDARD CURVE FOR THE DETERMINATION OF PHENOL



Phenol (Fig. 8)

Stock solution: 0,5 % m/v in water

Dilutions prepared: 1-500 (0,001 %), 1-250 (0,002 %), 3-500 (0,003 %), 2-250 (0,004 %)

Wavelength used: 269 nm

Conclusion: Phenol obeys Beer's Law at 269 nm over the above concentration range. A suitable dilution of the stock solution for analysis is 1-250, which corresponds to a concentration of 2×10^{-5} g/ml, and an absorbance value of approximately 0,32.

2.3.2 Analytical procedure

In order to determine the percentage loss of preservative in the supernatant liquid obtained from a powder/preservative solution suspension, the following procedure was adopted in each case:

(i) A sample of the same preservative stock solution which had been used for preparing the test suspensions and which had been treated in exactly the same way as the test suspensions was carefully diluted and its spectral absorbance measured at the selected wavelength.

(ii) A suitable dilution of the supernatant liquid was prepared and its absorbance measured in the same manner. The dilution used was not necessarily the same as that used for the preservative stock solution.

(iii) The absorbance of the supernatant liquid from the corresponding powder/water suspension was measured in the same manner, usually without dilution.

(iv) The loss of preservative due to interaction with the powder was calculated as in the following example in cases where the preservative used conformed to Beer's Law:

Absorbance of stock solution diluted 1-100 = 0,270

Absorbance of preservative/powder supernatant diluted 1-10 = 0,253

Therefore absorbance of preservative/powder supernatant diluted 1-100 = 0,0253

Absorbance of water/powder supernatant undiluted = 0,134

Therefore absorbance of water/powder supernatant diluted 1-100 = 0,0013

Percentage of preservative remaining in supernatant

$$= \frac{0,0253-0,0013}{0,270} \times \frac{100}{}$$

$$= \frac{0,024}{0,270} \times \frac{100}{}$$

= 8,8 % (rounded off to 9 %)

Therefore percentage loss of preservative = 100 - 9 %

= 91 %

(v) For preservatives like o-chlorobenzoic acid, which did not conform to Beer's Law the calculation, was modified as follows:

Absorbance of preservative/powder supernatant diluted 1-10 = 0,253

Absorbance of water/powder supernatant undiluted = 0,134

Therefore absorbance of water/powder supernatant diluted 1-10 = 0,013

Therefore absorbance of preservative in preservative/powder supernatant diluted 1-10 = 0,253 - 0,013

= 0,24

From graph concentration of preservative corresponding to this value = $9,1 \times 10^{-5}$ g/ml

At a 1-100 dilution this concentration would be $0,91 \times 10^{-5}$ g/ml

Absorbance of stock solution diluted 1-100 = 0,270

From graph, concentration of preservative corresponding to this value = $10,1 \times 10^{-5}$ g/ml

Therefore percentage of preservative remaining in supernatant $= \frac{0,91}{10,1} \times \frac{100}{}$

= 9 %

and percentage loss of preservative = 100-9

= 91 %

2.4 VISIBLE SPECTROPHOTOMETRIC METHODS OF ANALYSIS

For two of the preservative solutions included in this study, PMN 0,002 % and BZC 0,01 %, ultraviolet spectrophotometry was found to be insufficiently sensitive as a method of analysis for the low concentrations used. In both cases, very sensitive methods were found which involved conversion of the preservative to a colour-complex, extraction with an organic solvent and measuring the absorbance thereof at a wavelength in the visible region of the spectrum. In both cases, the methods were more time-consuming than simple untraviolet techniques, and certain problems were encountered which were eventually solved through suitable modification of the methods. In the case of the benzalkonium chloride method, the technique used was modified considerably over a period of time, and a far more reliable and accurate method was eventually developed.

Furthermore, in both cases, the modified methods were found to be suitable for the analysis of certain related compounds, provided that a standard curve was perpared for each such compound. Thus, the method used for PMN was subsequently used for thiomersal, while the method used for BZC was also used to analyse cetrimide, cetyltrimethylammonium bromide (CTAB) and cetalkonium chloride solutions.

In addition to their greater sensitivity, these colorimetric techniques possessed two further advantages over ultraviolet methods:

(i) The problem of water-soluble extractive absorbing light at the wavelength used for the analysis was overcome, as control tests revealed that in no case was water-soluble extractive converted to an organic solvent soluble complex which absorbed light at the wavelength used for the assay, and

(ii) If traces of powder were inadvertently drawn into the pipette with the sample of supernatant liquid, the analysis was not affected as the powder remained in the aqueous phase and did not cloud the organic extration solvent.

2.4.1 Analysis of PMN and thiomersal solutions by the "dithizone" method

This method is basically that recorded by Christensen and Dauv (1969). Their description of the method reads as follows: "3,00 ml of the (PMN) solution is diluted with 17 ml distilled water and 3,00 ml nitric acid is added. 10,00 ml dithizone 0,001 % dissolved in chloroform is then added, and after shaking for one minute in a separating funnel the chloroform phase is separated. The extinction of the phenylmercuric dithizone complex is measured at 481 nanometer in a spectro-photometer Beckman DU. A blank consisting of distilled water_____ is run in the same manner. The amount of phenylmercuric nitrate is found from a standard curve."

As the strength of the nitric acid used in this determination was uncertain, preliminary tests were carried out with various strengths of nitric acid. For convenience, the 17 ml of distilled water and the 3 ml of nitric acid were added as 20 ml of a more dilute nitric acid solution.

Initially, 0,05 % nitric acid was tried and was found to give a stable colour complex and a standard curve for phenylmercuric nitrate which obeyed Beer's Law. However it was feared that this concentration of acid might be too low. Therefore, on the assumption that the nitric acid used by Christensen and Dauv contained about 27 % of HNO_3, the concentration of dilute nitric acid used was increased to 4 % m/v of HNO_3. At this concentration of acid, the colour complex formed was extremely unstable and the colour in the chloroform layer faded

too rapidly for a spectrophotometric reading to be taken. It was realised that a much more dilute nitric acid solution would have to be used, so a 0,2 % m/v HNO_3 solution was tried next. This solution was found to produce a very stable colour complex and a standard curve for PMN identical to that obtained by use of 0,05 % HNO_3. It was therefore concluded that 0,05 % HNO_3 would suffice for this determination, but it was decided that 0,2 % HNO_3 should be used in case the lower strength did not give satisfactory results for thiomersal solutions as a standard technique was to be used for both PMN and thiomersal analyses, if possible.

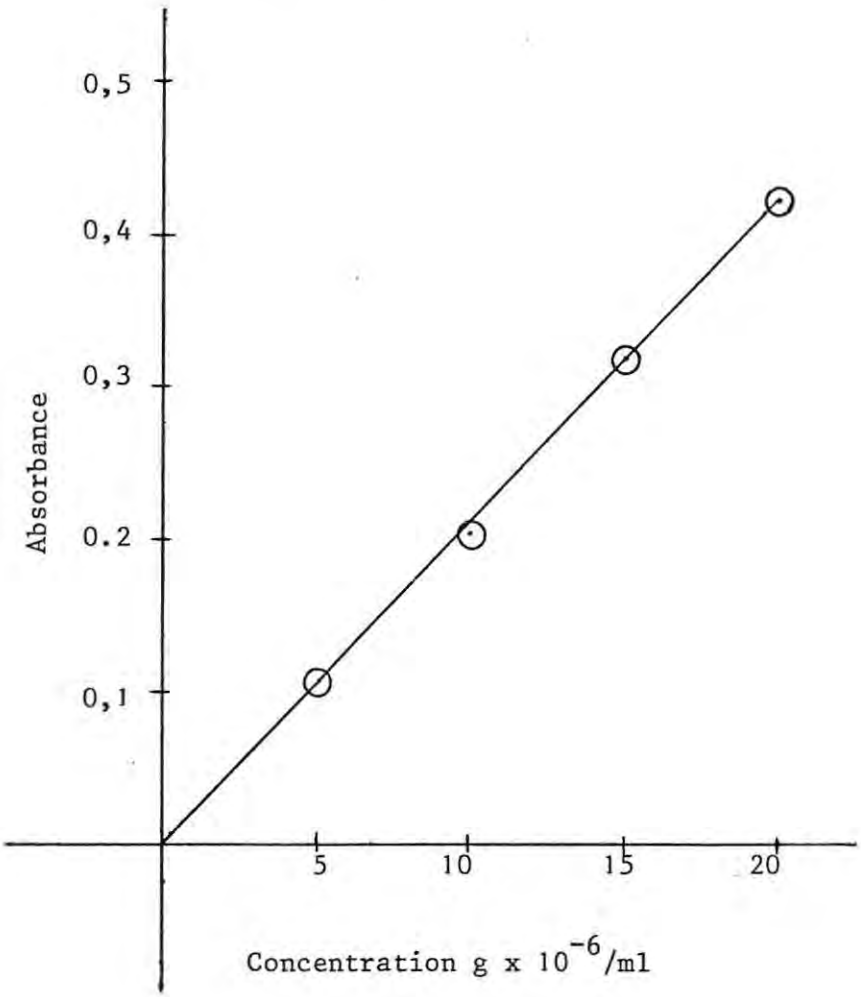
At a later stage, the following further observations were made:

(i) The dithizone used to make the 0,001 % solution in chloroform should be a reasonably fresh sample stored in a dark, well sealed container. The dithizone used initially was a sample that had been in stock for some years and had been exposed to light and air. Certain of the initial results obtained for preservative loss in PMN/powder systems could not be reproduced when fresh dithizone was used. Replicate determinations using the fresh dithizone produced consistent results however.

(ii) The 0,001 % dithizone solution in chloroform should be freshly prepared, a somewhat wasteful process as one litre was about the minimum quantity of so dilute a solution that could be prepared in one step. It was found that small quantities of this solution could conveniently be prepared by diluting 10 ml of a 0,01 % m/v chloroformic solution to 100 ml with chloroform in a volumetric flask. The 0,01 % solution could conveniently be prepared by dissolving 0,010 g of dithizone in sufficient chloroform to produce 100 ml. This solution could be stored in a refrigerator at 4 ^{o}C for at least one week without deterioration.

The modified method finally used for assaying PMN and thiomersal

Fig. 9 STANDARD CURVE FOR THE DETERMINATION OF PHENYLMERCURIC NITRATE



solutions was therefore as follows:

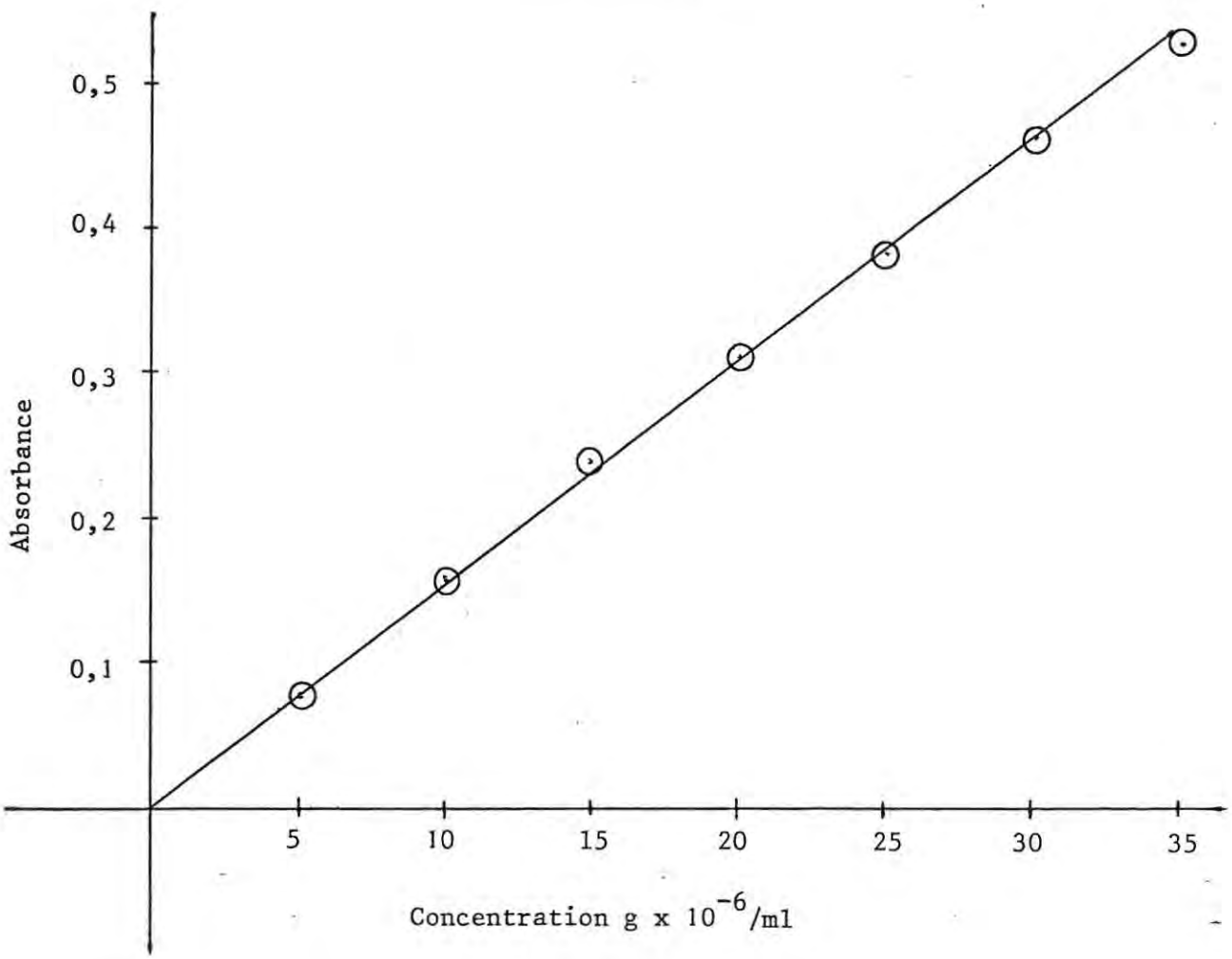
(i) To 20 ml of 0,2 % m/v HNO_3 in a separating funnel add 3 ml of organomercurial solution (diluted to about 0,002 % if necessary), plus 10 ml 0,001 % dithizone solution in chloroform.

(ii) Seal the funnel and shake it well for one minute.

(iii) Allow the layers to separate and read the absorbance of the chloroform layer at 481 nm against a blank solution prepared in the same way but using distilled water in the place of the organomercurial solution.

By use of this technique standard curves were prepared for PMN

Fig. 10 STANDARD CURVE FOR THE DETERMINATION OF THIOMERSAL



and thiomersal and for both substances obedience to Beer's Law was found.

Phenylmercuric nitrate (Fig. 9)

Stock solution: 0,002 % m/v in water

Dilutions prepared: 1-4 (0,0005 %), 1-2 (0,0010 %), 3-4 (0,0015 %) and undiluted (0,0020 %)

Thiomersal (Fig. 10)

Stock solution: 0,02 % m/v in water

Dilutions prepared: 1-40 (0,005 %), 1-20 (0,0010 %), 3-40 (0,0015 %), 1-10 (0,0020 %), 1-8 (0,0025 %), 3-20 (0,0030 %), 7-40 (0,0035 %).

2.4.2 Analysis of BZC, cetalkonium chloride, cetrimide and CTAB solutions by the "bromophenol blue" method

2.4.2.1 Modification of the method of Fokkens and Buurman (1969)

The following is a translation of their description of the method:

Glassware

All glassware must be cleaned with chromic acid. After rinsing with water and distilled water it should be dried. The use of soap and synthetic detergents is not recommended as minute residues thereof could upset the determination.

Reagents

Benzalkonium chloride solution 1 mg per ml (stock solution): Dissolve 100 mg benzalkonium chloride (USP) in water and dilute to 100 ml. Store this stock solution at 0-4 ^{o}C.

Benzalkonium chloride solution 80 µg per ml: Dilute 8 ml of stock solution to 100 ml with water. This solution should be freshly prepared.

Bromophenol blue solution 170 µg per ml: Dissolve 17 mg of bromophenol blue in 100 ml of 10 % $Na_2CO_3 10H_2O$. This solution may be kept for 48 hours at 0-4 ^{o}C.

Sodium phosphate solution 10 %: Dissolve 10 g anhydrous Na_2HPO_4 in water and dilute to 100 ml

Bromophenol blue reagent 34 µg per ml: Mix 10 ml bromophenol blue solution 170 µg per ml with 40 ml sodium phosphate solution 10 %. This reagent should be freshly prepared.

Dichloroethane (A.R. quality)

Method

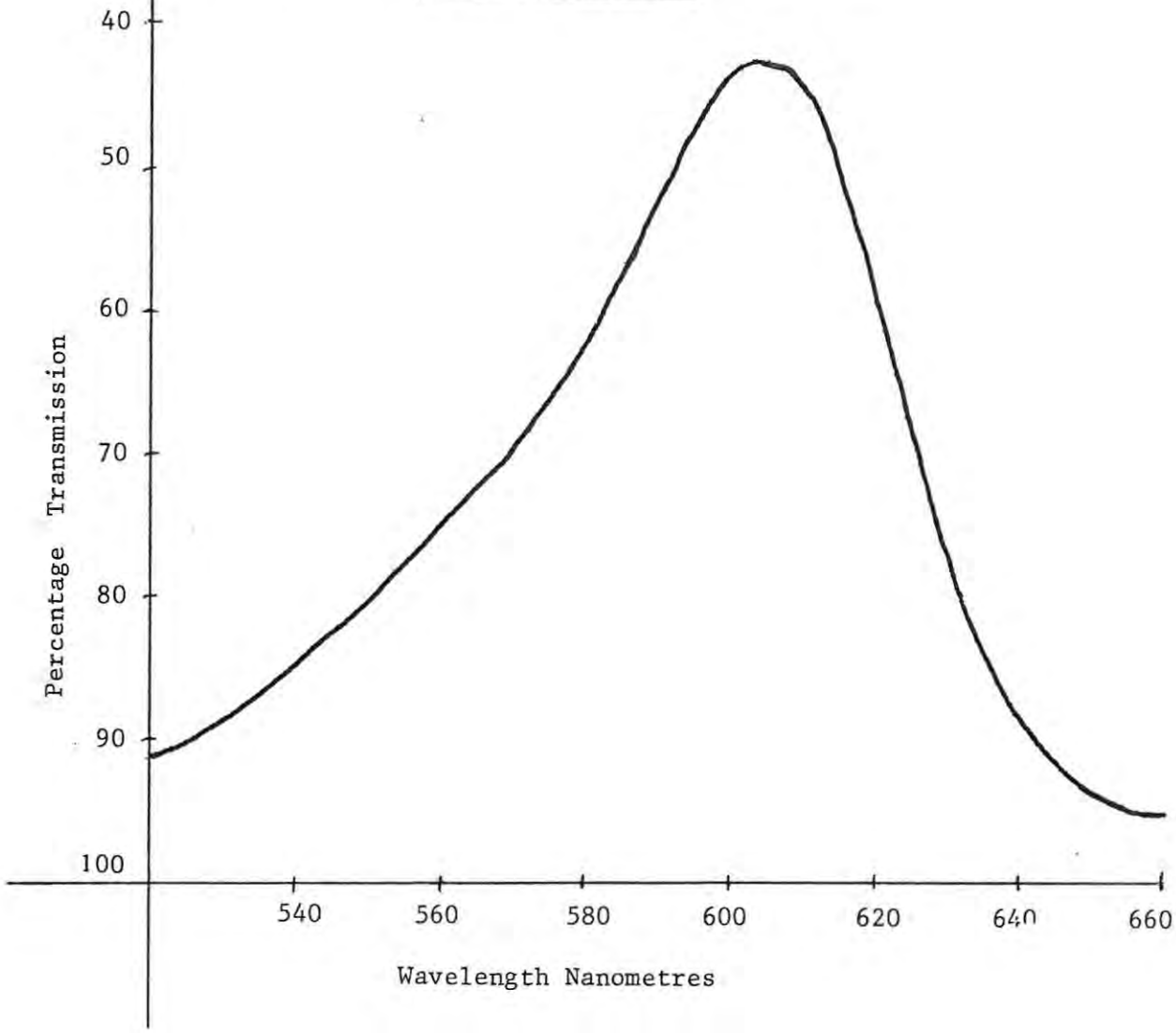
Dilute the test solution with water to an approximate benzalkonium chloride concentration of 8-16 µg per ml. Pipette 5 ml of this solution into a 100 ml separating funnel. Add 5 ml bromophenol blue reagent and mix. Add 10 ml dichloroethane and shake the mixture for 2 minutes. Allow all the liquid to run into a centrifuge tube and centrifuge for 10 minutes at 3000 r.p.m. Transfer the dichloroethane solution to a second centrifuge tube, seal it and centrifuge again for two minutes

at 3000 r.p.m. Measure the extinction of the clear, blue coloured solution in dichloroethane at 630 nm in a 1 cm cuvette against a dichloroethane blank. Determine the benzalkonium chloride content from the standard curve. The improvement of this method will be described in detail since considerable time was devoted to the development of this method of analysis. In attempting to use this method to prepare a standard curve certain difficulties soon became apparent.

Firstly, the wavelength of 630 nm mentioned in Fokkens and Buurman's description of the method was apparently a misprint as elsewhere in the same paper they state that the peak is at 603 nm. This was confirmed by scanning a dichloroethane solution of the colour complex in a Beckman D.B. spectrophotometer (See Fig. 11). A wavelength of 603 nm was therefore used for this determination.

Secondly, it was noticed that at concentrations below about 0,07 % m/v, BZC solutions rendered glassware repellant to water. This made accurate measurement of 5 ml volumes of these solutions difficult as water droplets tended to remain behind in the pipette adhering to the "waxy" surface. Therefore Fokkens and Buurman's suggestion that all glassware be absolutely clean and dry before use was implemented. It was found that if dilute BZC solutions were pipetted quickly with an initially clean and dry pipette the tendency for droplets of liquid to remain behind in the pipette was greatly reduced. In fact, a second volume of liquid could be pipetted fairly quickly with the same pipette and the number of droplets of liquid remaining behind in the pipette on the surface, now hydrophobic, would be minimal. It was felt at that stage that the slight error so introduced was tolerable. It was also found that pipettes and other glassware which had been rendered hydrophobic in this manner could be cleaned

Fig. 11 ABSORPTION SPECTRUM OF BROMOPHENOL BLUE/BENZALKONIUM CHLORIDE COLOUR COMPLEX IN DICHLOROETHANE



by rinsing them with methanol so as to elute the adsorbed BZC and then dried by sucking air through them.

Only pipettes cleaned in this manner and other glassware initially clean and dry, were employed for the preparation of an exactly 0,01 % m/v stock solution of BZC as described in Section 2.2.2, for accurate dilutions thereof and for their analysis by the above technique in order to construct a standard curve. Great difficulty was experienced in getting concordant results for duplicate determinations on the same solution. In fact, each solution was assayed many times and the absorbance readings obtained showed great variation. Even when duplicate determinations were done concurrently on the same BZC solution, the two absorbance readings so obtained differed by as much as 30 %. Then it was noticed that the first reading of a pair was always lower than the second and it was realised that the clean dry pipette used to measure the first volume of BZC dilution was probably adsorbing a large proportion of the BZC onto its surface. When the second volume was measured, the pipette was at least partially equilibrated with the solution so the loss of BZC would be less.

It was noticed that pipettes which had been rendered hydrophobic in this way could be rinsed repeatedly with distilled water without any visible alteration in the hydrophobic properties of the glass surface. It was therefore surmised that dilute BZC solutions deposited a monolayer of BZC molecules on glass surfaces and that it adhered tenaciously to the glass. The layer was hydrophobic, presumably because the polar heads of the molecules were attached to the glass with their hydrophobic hydrocarbon tails projecting into the solution. The fact that BZC solutions of concentration above about 0,07 % m/v wetted glass was taken as an indication that above this concentration further layers of BZC were adsorbed to the glass. It was apparent that such additional adsorbed layers were easily washed off the glass

because on rinsing with water the glass surface was rendered hydrophobic once more. Batuyios and Brecht (1957), in studying the adsorption of CPC onto talc noticed a similar irreversible binding phenomenon (see Section 1.1.6 page 7).

With the discovery that pipettes were adsorbing a proportion of the BZC in solutions being assayed it was then realised that the volumetric flasks in which the dilutions were prepared and any other glassware which came into contact with these solutions would adsorb BZC, thereby reducing their strength. The solution to this problem appeared to be the finding of a means of depositing a monolayer of BZC on the internal surfaces of glassware prior to use, but this raised a further problem, viz. the accurate measurement of volumes of dilute BZC solutions, as pipettes which were fully equilibrated with BZC solution retained appreciable volumes of solution in the form of droplets. Further investigation revealed that the volume of liquid retained in a pipette was related to the rate at which the meniscus moved over the glass surface while the pipette was draining. The faster the pipette drained, the greater was the volume of liquid retained in the form of droplets, and, because the meniscus moved rapidly in the narrow portions of the pipette and slowly in the bulb, almost all the retained droplets were deposited in the stem and neck of the pipette and hardly any in the bulb. It was found that provided that the downward travel of the meniscus was kept below a certain critical speed, no droplet would be left behind, provided further that the pipette had been scrupulously clean prior to equilibration with dilute BZC solution. Thus an accurate volume of dilute BZC solution could be delivered from an equilibrated pipette but the delivery time had to be greatly prolonged by restricting the rate of air inflow into the pipette. However, if a 5 ml graduated pipette was used instead of a bulb pipette the required volume of solution could be delivered somewhat more rapidly as the meniscus travelled more slowly over the glass

surface owing to the wider bore.

Volumetric flasks could be equilibrated by rinsing them with a 0,1 % m/v solution of BZC (a concentration which wetted the surface), allowing them to stand for five minutes and then rinsing them at least three times with distilled water to remove all adsorbed BZC except the firmly bound surface monolayer. Pre-treatment of pipettes involved thorough cleansing with chromic acid or suitable laboratory detergent followed by a number of rinses with distilled water. The pipette was then filled with the BZC solution to be pipetted, allowed to stand for at least five minutes, drained and rinsed twice more with the same solution prior to use. When a pipette prepared in this manner was used to measure a volume of dilute BZC solution, the interior of the pipette ended up quite dry and hydrophobic, provided that care had been taken to drain it slowly. Such pipettes could be used immediately to measure off a volume of BZC solution of different strength, provided that the small volume of liquid retained in the tip of the pipette was first removed by touching it against absorbant paper. In practice, pipettes were never used in this manner to measure off, successively, solutions which were appreciably different in concentration.

At this stage the possibility of adsorption of BZC by the other glassware used in the analysis was not seriously considered as all such glassware came into contact with the dichloroethane extraction solvent, which, it was assumed, would elute any adsorbed BZC.

In view of these problems and their probable causes, the procedure for the determination of the benzalkonium chloride standard curve and for the assay of solutions of unknown strength was modified to the following:

(i) The 0,01 % m/v benzalkonium chloride stock solution

was used to prepare the following dilutions in suitably equilibrated volumetric flasks: 2 ml - 100 ml (0,002 %), 3 ml - 100 ml (0,003 %), 4 ml 100 ml (0,004 %), 5 ml - 100 ml (0,005 %), 6 ml - 100 ml (0,006 %), 8 ml - 100 ml (0,008 %). The volumes of stock solution were measured into the volumetric flasks by slow delivery from an equilibrated burette to avoid droplet formation.

(ii) 5 ml volumes of freshly prepared bromophenol blue reagent were pipetted into clean dry 50 ml pear-shaped flasks fitted with ground glass joints.

(iii) 5 ml volumes of each dilution were added with an equilibrated 5 ml graduated pipette and a slow pipetting technique was used to avoid droplet formation.

(iv) 10 ml of dichloroethane was added to each flask, the flask stoppered and shaken for two minutes with a mechanical shaker.

(v) The entire contents of each flask was transferred to a 50 ml centrifuge tube and centrifuged for ten minutes at 3000 r.p.m.

(vi) A clean, dry 10 ml pipette was used to transfer approximately 8 ml aliquots of each dishloroethane layer to a 15 ml centrifuge tube. The dichloroethane solution was drawn up into the pipette slowly with slight suction to reduce the tendency for water droplets to form inside the pipette. These tubes were centrifuged for a further two minutes at 3000 r.p.m.

(vii) A clean dry 5 ml graduated pipette was then used to transfer approximately 3 ml volumes of these solutions to clean dry 10 mm cuvettes.

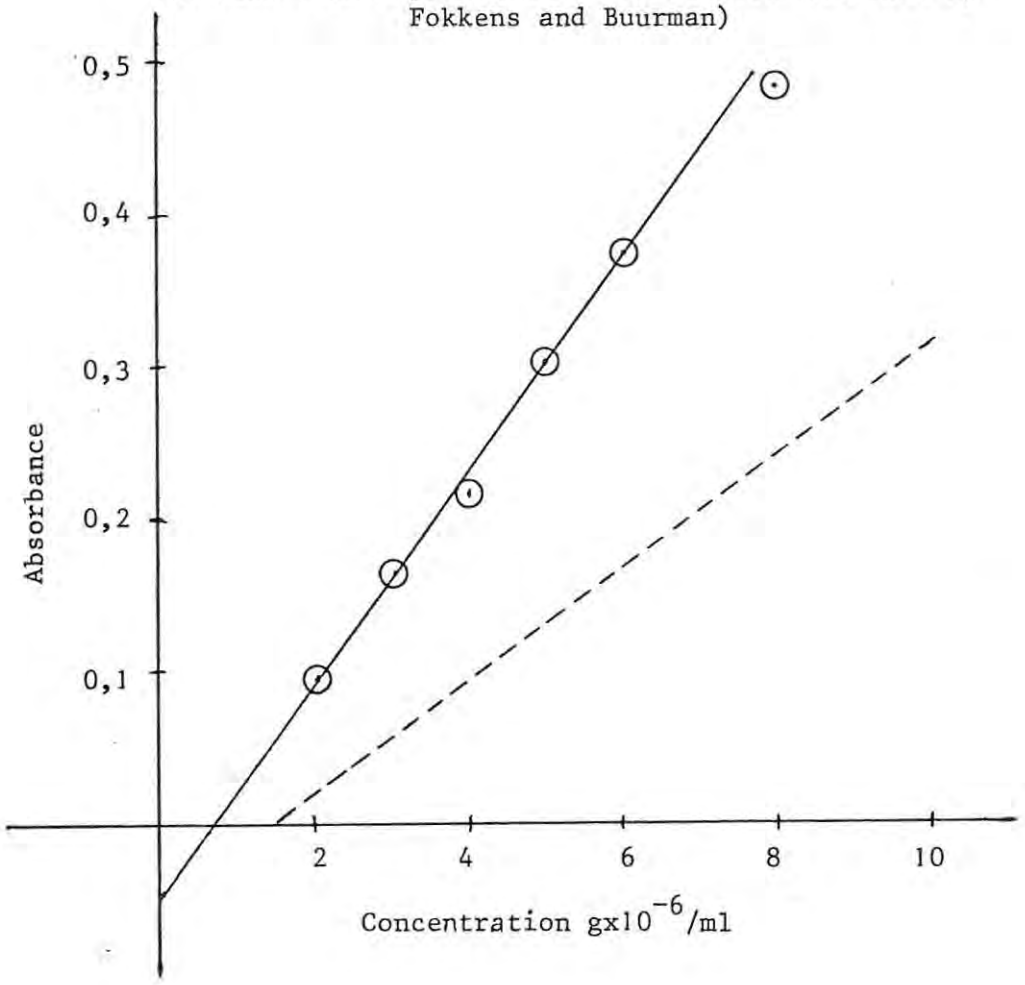
(viii) Absorbance readings were taken at 603 nm against a dichloroethane blank.

When the absorbance readings obtained for the various dilutions of BZC were plotted against concentration in order to prepare a standard curve, it was found that four of the six points lay

on a straight line with the remaining two points showing small deviations in position (see Fig. 12). The straight line did not cut the origin of the graph and therefore did not conform to Beer's Law. Analysis of the co-ordinates of these points by use of a polynomial regression programme in a Hewlett-Packard 9830A calculator revealed a correlation coefficient of 0,998126 for all six points and 0,999996 for the four points apparently on the straight line. The line drawn through these four points was therefore taken as the standard curve for benzalkonium chloride. In practice, the dilutions of BZC solutions used were such that absorbance values above 0,4 were not obtained as it was suspected that the standard curve might not be a straight line above this value. While calculating correlation coefficients with this calculator it was found that the same programme could be used to calculate the BZC content of unknown solutions from their absorbance values after supplying the co-ordinates for the standard curve. It was therefore not necessary to actually read the concentrations of unknown BZC solutions off the standard curve. Subsequently, whenever this method of analysis was used for solutions of quaternary ammonium compounds (QAC) the concentration of the QAC was calculated from the absorbance value in this manner.

Examination of Fig. 12 reveals that the standard curve obtained for BZC is very different from that obtained by Fokkens and Buurman. In general, much higher absorbance values were obtained for each concentration of BZC used. The explanation of this difference is probably that in Fokkens and Buurman's original method much of the BZC in their dilutions was adsorbed onto the glassware used to prepare them. There was, in fact, ample scope for such adsorption to take place as their dilutions were prepared in three stages with each solution being exposed to clean

Fig.12 STANDARD CURVE FOR THE DETERMINATION OF BENZALKONIUM CHLORIDE BY THE METHOD OF FOKKENS AND BUURMAN AS INITIALLY MODIFIED
(Broken line represents standard curve obtained by Fokkens and Buurman)



glass surfaces. It is a tribute to Fokkens and Buurman's consistency in technique that they obtained such consistent results, since the method that they used was subject to a constant loss of BZC onto glassware. For such a constant loss to be obtained all glassware would have to be scrupulously clean and an extremely consistent technique would have to be used. Furthermore, similar glassware of the same internal surface area and having the same adsorption characteristics would have to be used each time the determination was done, and similar volumetric flasks would have to be used for each dilution of BZC prepared, as a change in surface area to volume ratio could affect the concentration of the solution. Even the length of time that a BZC dilution is in contact with a glass container and the proportion of the surface of the container with which it comes into contact can have a noticeable effect on the absorbance reading obtained (see point (ii) below).

It is important to note that, while the losses of BZC onto glassware are small in terms of the mass of BZC adsorbed, they are not small in terms of the proportion of BZC lost from the dilute solutions used in this method of analysis. It was noticed, for example, that up to 30 % of the BZC in a solution containing 2×10^{-6} g/ml could be lost simply by drawing up a 5 ml volume into an unequilibrated pipette. Multiple transfers to unequilibrated glassware could obviously result in much greater losses even with more concentrated solutions. This statement is confirmed by examination of Fig. 12, which shows that, for any concentration of BZC, the absorbance value obtained by Fokkens and Buurman is less than half that obtained by the writer.

During the preparation of the BZC standard curve, further discoveries were made which had a bearing on the technique used:

(i) On shaking together a mixture of BZC dilution, bromophenol blue reagent and dichloroethane and then allowing it to stand, it was noticed that the dichloroethane layer which separated initially was colourless. Only when the separation of the two layers was virtually complete did the blue colour apparently spread downwards from the upper aqueous layer into the dichloroethane layer. Closer scrutiny revealed that, when the two layers first separated, the lower layer consisted of colourless dichloroethane with aqueous droplets dispersed in it, a phenomenon which gave it a whitish colour. These aqueous droplets moved upwards fairly rapidly and coalesced with the upper aqueous layer leaving the lower dichloroethane layer completely clear and colourless. The upper layer consisted of a blue aqueous solution (due to excess of reagent) with droplets of dichloroethane solution dispersed in it. These droplets moved gradually downwards and seemed to accumulate for a while at the interface between the two liquids. Eventually, with slight agitation, they would coalesce with the dichloroethane layer releasing a blue colour into it. The conclusion drawn from this observation was that, while the bromophenol blue/BZC colour complex partitions preferentially into the dichloroethane layer, it is still surface active and accumulates at dichloroethane/aqueous solution interfaces, preferentially at interfaces which are convex with respect to dichloroethane. It is clear therefore that centrifuging of these liquids must be very thorough so that no droplets of dichloroethane solution remain dispersed in the aqueous layer, as only a few such droplets are capable of adsorbing a large proportion of the colour complex.

(ii) At one stage, in an attempt to expedite the analyses,

5 ml volumes of BZC dilutions were pipetted into a series of eight 50 ml flasks. Following an interruption, the 5 ml volumes of bromophenol blue reagent were only added about half an hour later and the analyses then completed. The absorbance readings obtained for this series of dilutions were all unexpectedly low. The reason for this was probably that the flasks had adsorbed a monolayer of BZC on those areas of glass which had come into contact with the BZC solutions and that the bromophenol blue reagent had not reacted with the adsorbed molecules. The factors influencing this form of reduction in absorbance value would be, area of glass exposed to BZC dilution, time of contact and, possibly, concentration of BZC in the solution. It was therefore decided to pipette the bromophenol blue reagent into the flasks first, followed by the BZC dilutions pipetted directly into the reagent without contact with the glass. A repetition of this series of determinations with this modification of the procedure produced higher and more concordant absorbance values.

With respect to this source of error, it is interesting to note that, in Fokkens and Buurman's method, 5 ml of BZC dilution is pipetted into a 100 ml separating funnel, thus exposing the solution to a very large surface area of glass, followed by 5 ml of bromophenol blue reagent. The loss of BZC that must have taken place under these conditions further explains the differences between the two standard curves shown in Fig. 12.

(iii) In sucking up an aliquot of the dichloroethane layer into a pipette after centrifuging to effect initial separation of the two liquids, it was noticed that, if considerable suction was used, drops of aqueous

liquid tended to be deposited on the inner surface of the pipette by the dichloroethane solution. If the dichloroethane solution was drawn into the pipette slowly with gentle suction, no such separation of aqueous liquid occurred. Apparently excessive reduction in pressure caused water dissolved in the dichloroethane solution to separate out. This phenomenon was cause for some concern owing to the recent discovery that the colour complex tended to be adsorbed at dichloroethane/ aqueous liquid interfaces. Although the interface favoured was that of dichloroethane dispersed in aqueous liquid and not vice versa, it was felt that such separation of liquids should be avoided, if possible, as the extent to which the colour complex concentrated at such interfaces was unknown. Thus, the dichloroethane layer was henceforth drawn into the pipette with very gentle suction prior to the second centrifuging. This effectively prevented the formation of drops of aqueous liquid in the pipette.

(iv) The 10 % sodium phosphate solution used to prepare the bromophenol blue reagent is a solution saturated at about 20 ^{o}C. On a cold day, crystals of sodium phosphate tended to separate from this solution and from the bromophenol blue reagent as well if the ambient temperature was low enough. Therefore, both these solutions were stored in an incubator at 25 ^{o}C. The bromophenol blue reagent was never stored in this manner for more than two hours as it was required to be freshly prepared.

(v) Cold weather and slight evaporation could cause the temperature of the colour complex solution in dichloroethane to drop when it was transferred to a cuvette. This situation often caused a cloudiness to develop in the solution, once again due to

separation of aqueous droplets. Fortunately, such droplets occurred in a finely dispersed state and could be readily redissolved by applying the palms of the hands to the sides of the cuvette to warm the liquid slightly. Thereafter, care was taken to try and prevent the temperature of the liquids from falling, and the solutions in cuvettes were always carefully examined before taking an absorbance reading as any cloudiness could constitute a gross source of error.

The standard curve shown in Fig. 12, with four points in an almost perfectly straight line and two points off the straight line, is a fair indication of the reliability with which this determination could be done at this stage. Most of the time satisfactory results could be obtained but, at regular intervals and for apparently inexplicable reasons, a result would deviate appreciably from the expected value. The reasons for these inaccurate results were ultimately discovered and the method modified further, whereafter consistently accurate results could be obtained. These further modifications will be discussed in Section 2.4.2.3 but it was at the above level of reliability that the majority of the tests were done of QAC preservative/powder systems. Reliable results were obtained by multiple replication of determinations, where necessary, and rejection of readings which deviated excessively from the mean. It was also at this level of reliability, that the method was adapted for the determination of other QAC preservatives by using the same procedure and plotting standard curves for these preservatives.

Fokkens and Buurman claimed a precision of $\pm$ 5 % for their method. Whilst no attempt has been made to calculate the limits of error of this method at the above stage of

development, its precision is assumed superior to that of Fokkens and Buurman's original method due to the elimination of a number of potential sources of error and due to the fact that determinations were replicated and unreliable readings rejected. A variation of not more than 3 % between replicated results was required to establish concordancy.

2.4.2.2 Adaptation of Fokkens and Buurman's method (as modified) for the determination of other QAC preservatives

As will be explained in chapter 5, it became necessary to develop methods of analysis for dilute cetrimide and CTAB solutions so that comparisons could be made between the inactivation by powders of these compounds and of the QAC preservatives already studied. Fokkens and Buurman's method was used for cetrimide and CTAB as these compounds lacked suitable ultraviolet absorption peaks.

Cetalkonium chloride 0,1 % m/v solutions and supernatant fluids from powder/preservative systems were initially analysed by ultraviolet spectrophotometry, but, difficulty was experienced in getting reproducible results for losses in the presence of certain powders. This difficulty was probably due to variation in the content of ultraviolet-absorbing water-soluble extractive in the presence and absence of the QAC, which would have a profound effect on the absorbance reading obtained, owing to the low dilution of the preservative solution used for the analysis (only 1 in 4) (This potential source of error is more fully discussed in Section 2.3). Thus, for cetalkonium chloride a standard curve was prepared by use of the modified Fokkens and Buurman method and the powder/cetalkonium chloride systems, with which difficulty was being experienced, were retested by means of this method; whereupon satisfactory results were soon obtained.

For all three of these substances, 0,01 % m/v stock solutions

were carefully prepared as described in Section 2.2.2 by use of equilibrated volumetric flasks and the following dilutions thereof were made with equilibrated pipettes and volumetric flasks: 1-50 (0,002 %), 1-25 (0,0004 %), 3-50 (0,0006 %), 2-25 (0,0008 %), 5-50 (0,001 %) and 3-25 (0,0012 %). From these dilutions, absorbance readings were obtained and standard curves constructed in exactly the same manner as described for BZC. Comment on the standard curves obtained is as follows:

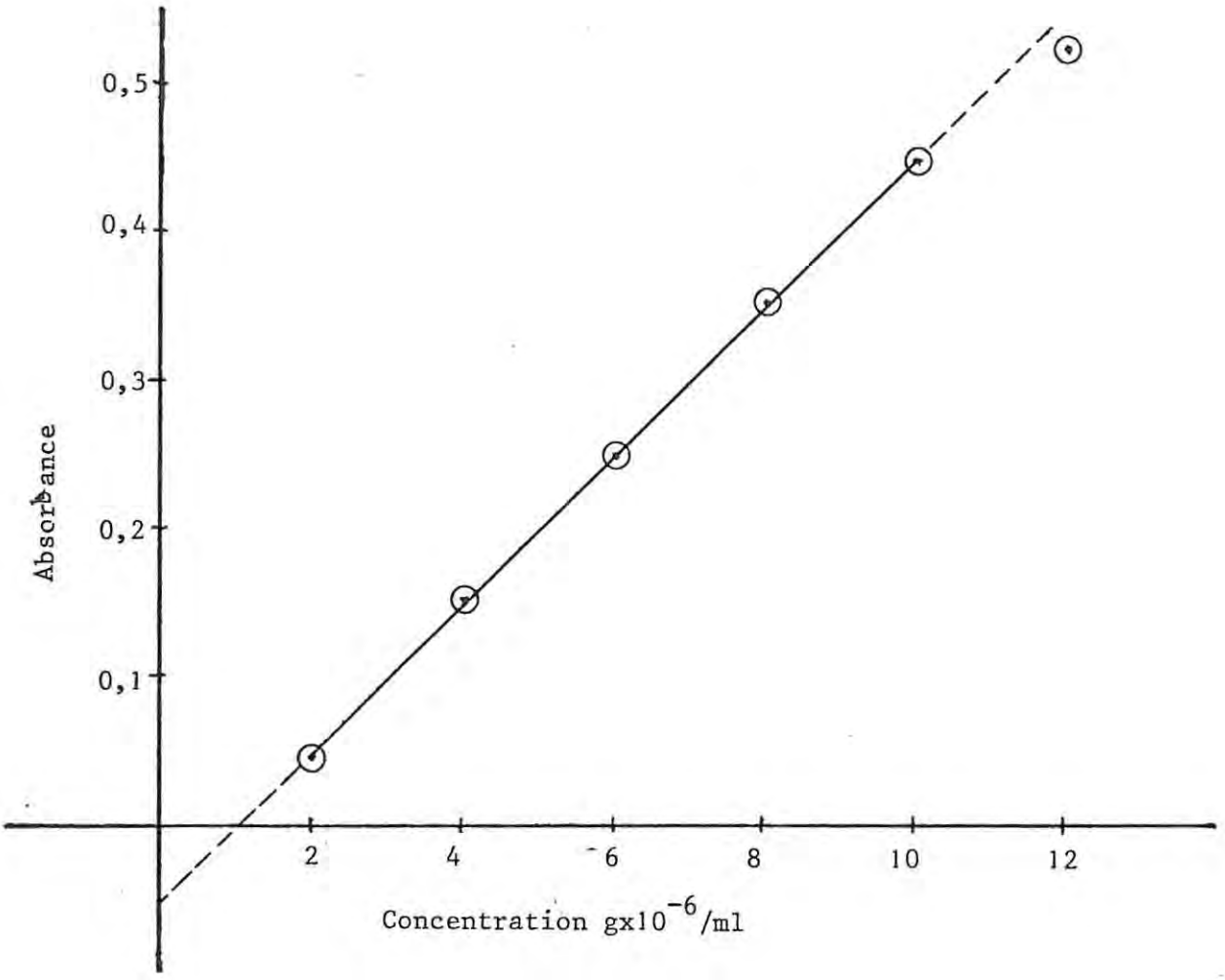
Cetalkonium chloride (Fig. 13)

Five of the six points plotted were found to lie in a straight line, but the highest concentration used, 12 x 10^{-6} g/ml, had an absorbance which was somewhat less than the expected value. It is interesting to note that, for the BZC standard curve, the highest value plotted, 8 x 10^{-6} g/ml, had an absorbance which was also somewhat less than the expected value and which was also in the region of 0,5. As these two substances are chemically closely related (cetalkonium chloride is one of the homologues contained in BZC, which is a mixture), it would appear that there is a tendency for the standard curves of BZC homologues to deviate from a straight line at absorbance values exceeding 0,4 to 0,5. From Fig. 13 it can be seen that the relationship between concentration of cetalkonium chloride and absorbance is linear between absorbance values of 0,05 and 0,45 when this method of analysis is used. Therefore, the dilutions of the cetalkonium chloride solutions analysed were always so chosen that absorbance values between 0,05 and 0,45 were obtained. The correlation coefficient for the five points on the straight line is 0,999939.

Cetrimide (Fig. 14)

Four of the six points were found to lie in a straight line, with the values for 2 and 12 g x 10^{-6}/ml deviating somewhat. The relationship between concentration and absorbance was

Fig. 13 STANDARD CURVE FOR THE DETERMINATION OF CETALKONIUM CHLORIDE



thus taken to be linear between absorbance values of 0,1 and 0,5, and the dilutions used for analysis by this method were so chosen that absorbance readings in this range were obtained. The correlation coefficient for the four points on the straight line is 0,999941.

Fig.14 STANDARD CURVE FOR THE DETERMINATION OF CETRIMIDE

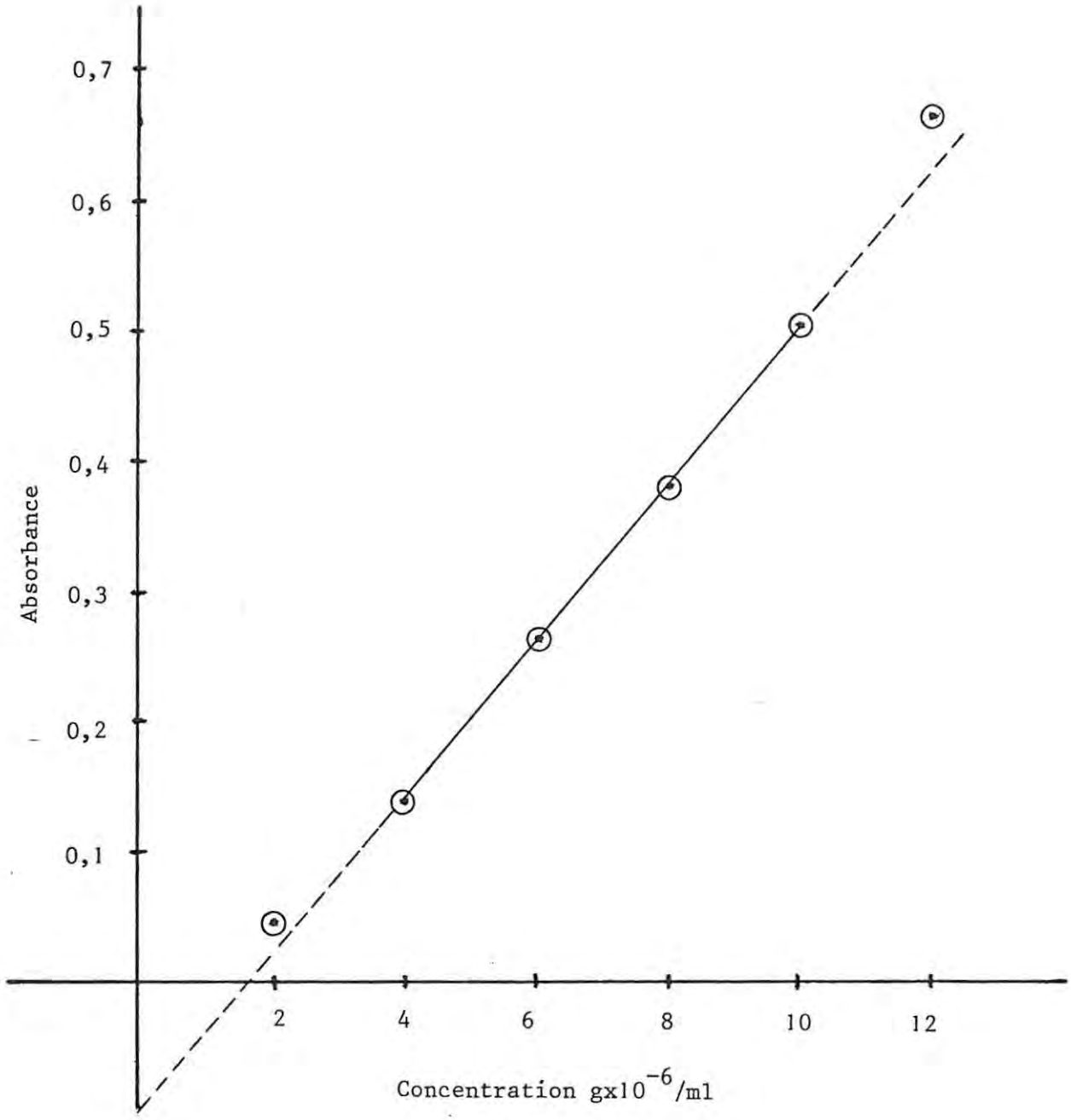
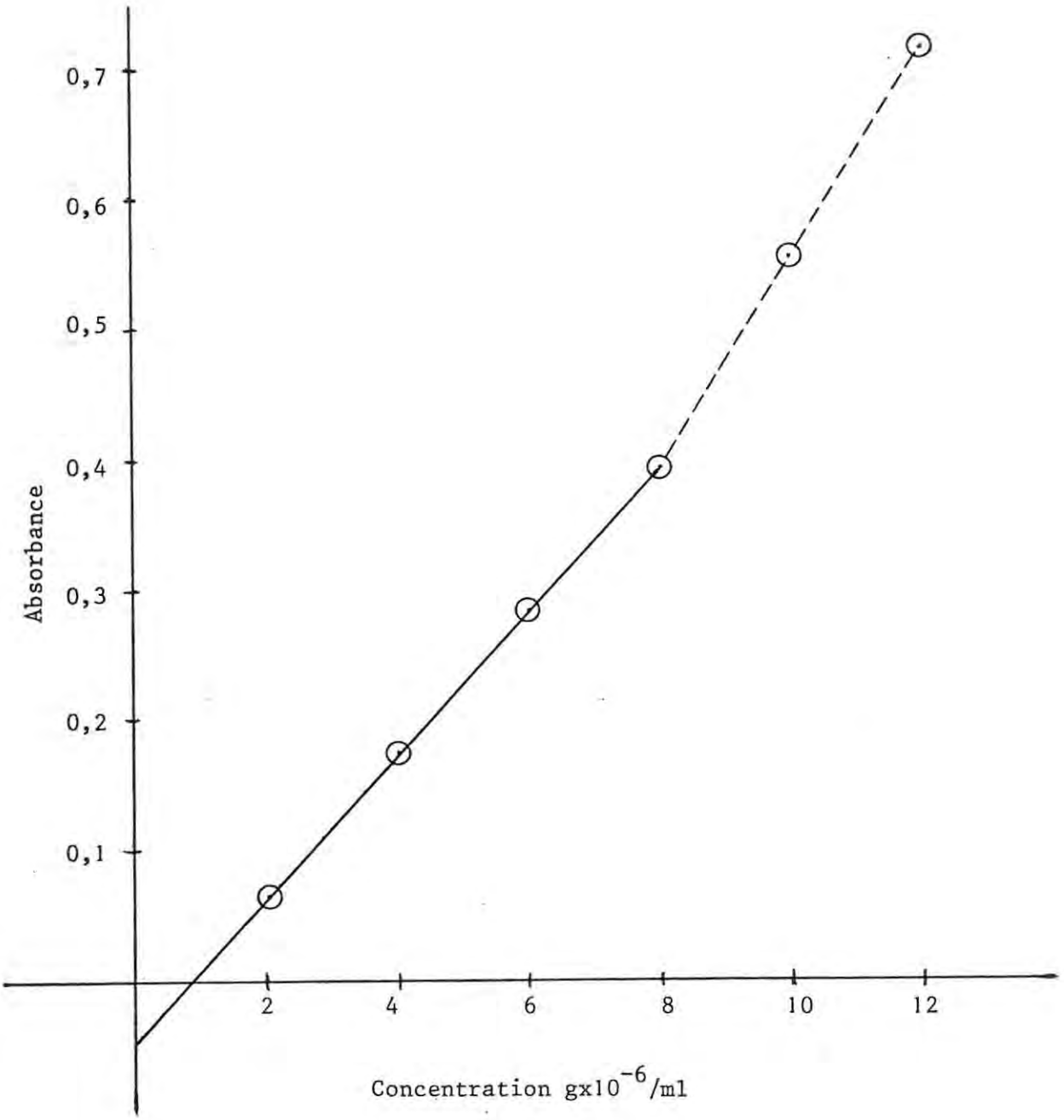


Fig. 15 STANDARD CURVE FOR THE DETERMINATION OF CETYLTRIMETHYLAMMONIUM BROMIDE



CTAB (Fig. 15)

Once again, four of the six points were found to lie in a straight line but, in this instance, it was the absorbance values for 10 and 12 g x10^{-6}/ml that showed deviations. The

points corresponding to the three higher concentrations used also formed a straight line, which was at a slight angle to the first one. Perhaps, if further points had been plotted for the cetrimide standard curve, at higher concentrations, a similar phenomenon would have been noticed, as CTAB is a homologue in the same series to which the mixture, cetrimide, belongs. It was decided to use only the lower straight line for analysis of solutions of CTAB and the relationship between concentration and absorbance was therefore taken to be linear between absorbance values of 0,06 and 0,40. Accordingly, dilutions were chosen so as to give absorbance readings within this range. The correlation coefficient for the four points on this straight line is 0,999997.

2.4.2.3 Further improvements made to Fokkens and Buurman's method for the determination of BZC solutions

As mentioned before, the method of Fokkens and Buurman, as modified, was used to analyse most of the supernatant fluids obtained from the QAC/powder systems tested. Reliable results were obtained by replication of analyses and rejection of unreliable values. These occasional deviant results, which were obtained in spite of careful attention to technique, were a source of constant concern.

Eventually, in the interests of speeding up the determinations, certain small modifications to technique were made which, it was felt, would not affect the results, i.e., the pipette used to transfer the aliquot of dichloroethane solution to the second centrifuge tube, the pipette used to fill the cuvette and the cuvette itself were used for up to four determinations without cleaning and drying. The pipettes were simply allowed to drain well between determinations and the mobile dichloroethane solution did drain away almost completely in this period of time. The cuvette was rinsed with solution prior to filling, but not for the first solution

of each series of four determinations as it was then clean and dry, having been rinsed with acetone after the previous series. Shortly after the adoption of these modifications it was noticed that an unreliable result was obtained approximately once every fourth determination. This let to the suspicion that a low absorbance reading could be due to adsorption of the colour complex onto glassware from solution in dichloroethane, a possibility not seriously considered previously as other organic solvents had proved to be effective in eluting BZC adsorbed to glass. The following simple tests were then performed to test this assumption:

(i) A volume of the colour complex in dichloroethane solution was transferred to a clean, dry cuvette and read in the spectrophotometer at 603 nm against dichloroethane. A reading of 0,312 was obtained. The cuvette was drained and then refilled with more of the same solution. This time a reading of 0,328 was obtained, indicating that some of the colour complex had been adsorbed onto the inner surface of the cuvette. The cuvette was drained and refilled with the same solution once more. The reading remained at 0,328.

(ii) The remaining solution was then poured into another clean dry test tube, transferred back to the same test tube, and a sample of the liquid poured into the above equilibrated cuvette. A reading of 0,308 was obtained indicating a further loss of colour complex from the solution.

(iii) After transferal of the same solution to two further clean, dry test tubes a reading of 0,281 was obtained.

(iv) The remaining solution was placed in a tube with a large air space, swirled to bring it into contact with the entire inner surface and the absorbance value of an aliquot read in the above cuvette. The liquid remaining in the tube was vigorously shaken, centrifuged

to remove the water droplets that appeared and a second absorbance reading taken. The two absorbance readings were the same, proving that it was not contact with the atmosphere that caused deterioration of the solution and, incidently, proving that the separation of drops of water from the solution did not affect the absorbance reading.

There is thus little doubt that adsorption of bromophenol blue/QAC colour complex in dichloroethane solution onto glassware does take place and does affect the results of this determination.

During the performance of the determination, as modified by the writer, the following glass surfaces could adsorb the colour complex:

(i) The surfaces of the 50 ml pear shaped flasks in which the organic and aqueous liquids are shaken together.

(ii) The surfaces of the 50 ml centrifuge tubes in which the two liquids are separated.

(iii) The surface of the 10 ml pipette used to remove the dichloroethane solution.

(iv) The surfaces of the 15 ml centrifuge tubes to which the solution is transferred.

(v) The surface of the 5 ml graduated pipette used to transfer solution to the cuvette.

(vi) The surface of the cuvette in which the reading is taken. (Presumably adsorption onto the side walls of the cuvette would reduce the observed absorbance - not adsorption onto the optical surfaces).

The extent of adsorption onto surfaces (i), (ii) and (iv) above should be fairly constant, provided that a standard cleaning procedure is used for glassware. Adsorption onto surfaces (i) and (ii) could not be prevented, since it would have been very difficult to equilibrate them in the presence

of two immiscible liquids. Adsorption losses onto these surfaces should have been fairly constant for each determination, as they were well rinsed with methanol to elute adsorbed QAC and allowed to dry before use.

Thus, (iii), (v) and (vi) were the main surfaces onto which adsorption losses could be controlled through equilibration, by rinsing with a small volume of the colour complex in dichloroethane solution, prior to allowing the bulk of the solution to come into contact with them.

In the light of the above observation it was decided to modify the procedure still further as follows:

(i) The pipette used to transfer an aliquot of the dichloroethane layer from the first centrifuge tube to the second, would first be rinsed with a few ml of the dichloroethane layer to equilibrate it with the colour complex. In subsequent determinations the same pipette would be used again without washing for similar transfers of liquid provided that it was well drained between transfers and similarly rinsed with a small quantity of the liquid to be transferred.

(ii) The 15 ml centrifuge tube would firstly be rinsed with the rinsings from the above pipette and then with a few ml more of the dichloroethane solution prior to the bulk of the liquid being run in.

(iii) After the second centrifuging, the dichloroethane solution would be poured into the cuvette to avoid the use of a pipette to transfer the liquid. This was made possible by the discovery that, if any aqueous droplets separated during the second centrifuging, they adhered to the sides of the centrifuge tube and did not get transferred to the cuvette if the liquid was poured.

(iv) The cuvette would be rinsed three times with small

portions of dichloroethane solution prior to filling it and taking an absorbance reading at 603 nm.

These modifications to the procedure produced absorbance readings which were so much higher than expected that it was decided to repeat the determination of the standard curve for BZC, incorporating these modifications. Thus a range of eight dilutions was prepared from a 0,01 % m/v stock solution of BZC, their absorbance readings determined by the newly modified technique and the standard curve shown in Fig. 16 constructed. Five of the eight points comprising this curve are virtually in a straight line with the three remaining points showing only minor deviations. The correlation coefficient for all eight points if 0,99857, whilst for the four best points it is 0,999935.

Using this standard curve and a technique incorporating all the modifications it has been found possible to obtain consistent results when replicate determinations are done on the same solution.

Also worthy of note is the fact that the slope of this curve is even steeper than that of the curve obtained previously and very much steeper than the slope of the curve obtained by Fokkens and Buurman. The new curve does not cut the origin, thus Beer's Law is still not obeyed but it does come closer to the origin than the previous plot. In fact, each time this method has been improved with equilibration of glassware the standard curve so obtained has come closer to cutting the origin. Perhaps, the fact that the curve still does not cut the origin can be taken as an indication that BZC, or its colour complex, is still being partly adsorbed onto glassware during the determination. The only unequilibrated surfaces remaining now are the interiors of the 50 ml pear-shaped reaction flasks and the large centrifuge tubes. If a means could be found of equilibrating these

vessels, the standard curve so obtained would probably conform to Beer's Law.

2.4.2.4 Description of Fokkens and Buurman's method for the determination of BZC, as fully modified.

As correct technique is of vital importance to the success of this determination, a full description of the method in its final form will be given:

Glassware

(i) All items of glassware should be cleaned initially with chromic acid. After rinsing thoroughly with water and distilled water, they should be rinsed with acetone or methanol and allowed to dry.

(ii) All volumetric flasks used to prepare dilutions of BZC should be equilibrated, after initial cleaning, by rinsing with a 0,1 % m/v aqueous BZC solution, allowing to stand for 5 min with a film of the BZC solution covering the entire inner surface and then rinsing at least three times with distilled water. Such volumetric flasks can be used again for the preparation of BZC dilutions after rinsing three times with water.

(iii) Pipettes used for measuring BZC solutions should be graduated pipettes and should be equilibrated, after thorough initial cleaning, by filling them with the BZC solution to be pipetted, allowing to stand for 5 min, draining and rinsing twice more with the same solution prior to use. Once pipettes have been equilibrated in this manner they can be used to pipette other BZC solutions, after rinsing with the solution to be pipetted. When BZC solutions of less than 0,07 % m/v concenteation are pipetted the liquid should be allowed to flow from the pipette at a sufficiently slow rate to prevent the formation of drops of liquid on the inner surface of the pipette.

Reagents

Bromophenol blue solution: Dissolve 0,017 g of bromophenol blue in 100 ml of 10 % $Na_2CO_3.10H_2O$. This solution may be kept for 48 hours at 0-4 ^{o}C.

Sodium phosphate solution 10 %: Dissolve 10 g anhydrous Na_2HPO_4 in water and dilute to 100 ml. Store at 25 ^{o}C to avoid crystal formation.

Bromophenol blue reagent: Mix 10 ml bromophenol blue solution with 40 ml sodium phosphate solution 10 %. This reagent must be freshly prepared.

Dichloroethane (AR grade)

Standard curve

(i) Use a benzalkonium chloride concentrate of accurately known concentration to prepare an exactly 0,01 % m/v stock solution in an equilibrated volumetric flask.

(ii) Use an equilibrated, graduated pipette (or burette) and equilibrated volumetric flasks to prepare a suitable range of dilutions of known BZC concentration.

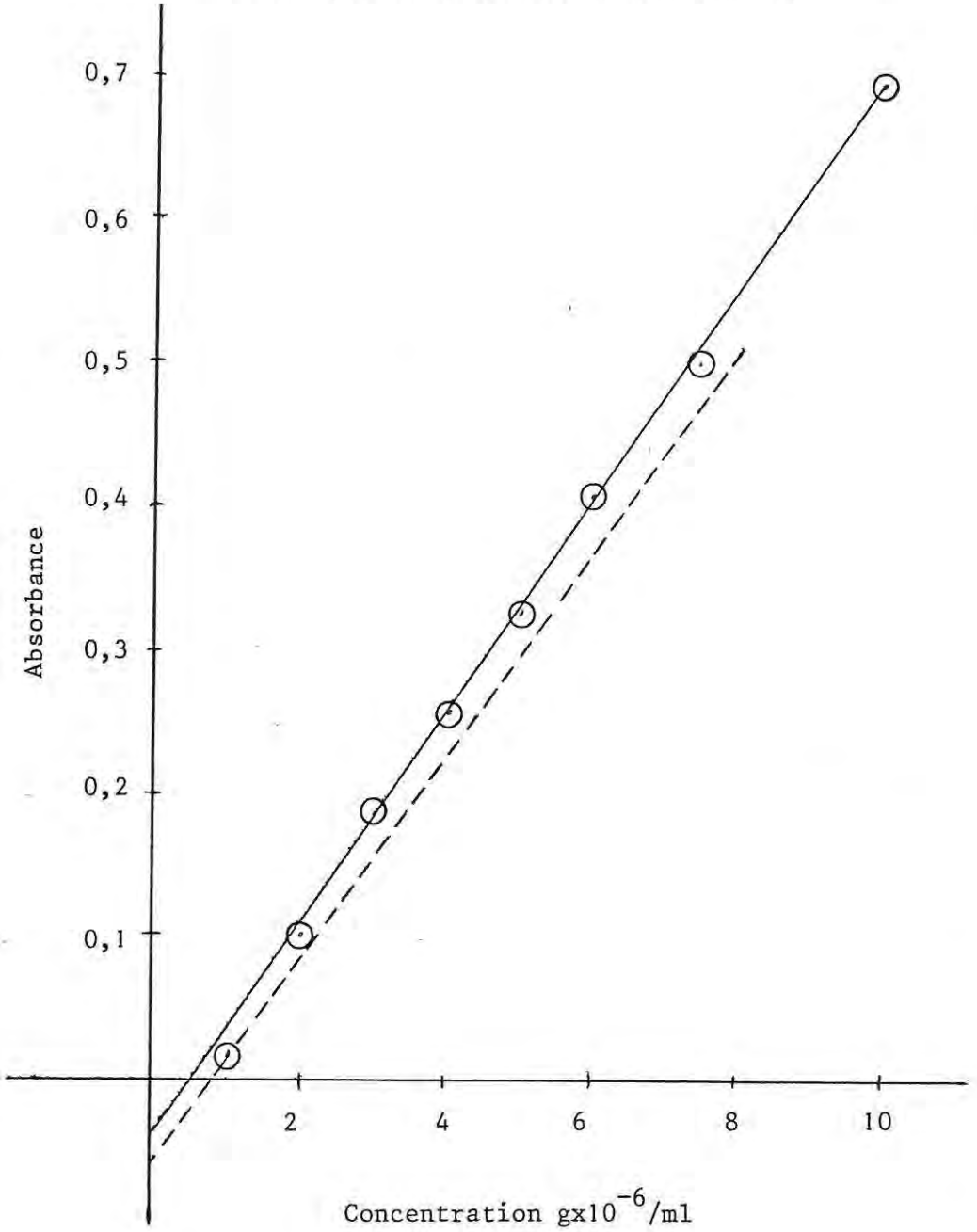
(iii) Determine absorbance values for these dilutions by the method which follows and use these values to construct a standard curve. This curve should correspond quite closely to that shown in Fig. 16, but the correspondence may not be exact due to the fact that the adsorption properties and surface areas of the 50 ml reaction flasks and 50 ml centrifuge tubes used may not be exactly the same as those used by the writer.

Method

(i) Pipette 5 ml of bromophenol blue reagent into a clean, dry 50 ml stoppered flask.

(ii) Pipette 5 ml of BZC dilution directly into the above bromophenol blue reagent using an equilibrated 5 ml graduated pipette and a slow delivery technique to avoid droplet formation inside the pipette. The BZC

Fig. 16 STANDARD CURVE FOR THE DETERMINATION OF BENZALKONIUM CHLORIDE BY THE METHOD OF FOKKENS AND BUURMAN AS FINALLY MODIFIED
(Broken line shows standard curve obtained by the initial modification of the method)



dilution should mix with the reagent without first touching the sides of the flask.

(iii) Add 10 ml of dichloroethane to the flask, seal it and shake the contents vigorously for 2 min on a mechanical shaker.

(iv) Transfer the entire contents of the flask to a 50 ml centrifuge tube, allowing the flask to drain well into the tube as a few dichloroethane droplets dispersed in aqueous liquid left behind in the flask can adsorb an appreciable quantity of the colour complex at the dichloroethane/water interface.

(v) Centrifuge at 3000 rpm for 10 min to effect complete separation of the liquids.

(vi) Draw up approximately 1 ml of the dichloroethane layer into a 10 ml bulb pipette and use it to rinse the pipette. When passing the tip of the pipette through the upper aqueous layer, care is taken to minimise ingress of the aqueous liquid by sealing the top of the pipette with the finger or pipette filler. After rinsing the pipette with a second approximately 1 ml volume of the dichloroethane layer, an aliquot of approximately 7 ml of the dichloroethane layer is drawn into the pipette with very gentle suction in order to avoid the separation of aqueous droplets inside the pipette. This pipette may be used again for the transfer of further aliquots of dichloroethane layers without cleaning and drying, provided that it is well drained between determinations, is rinsed as above and remains virtually free of droplets of aqueous liquid.

(vii) In turn, use the small quantities of dichloroethane layer which were used to rinse the 10 ml pipette to rinse a clean, dry 15 ml centrifuge tube. After a further approximately 1 ml rinse with the liquid in the pipette, run the remainder of the liquid into the centrifuge tube. Centrifuge for a further 2 min

at 3000 rpm.

(viii) Rinse a 10 mm cuvette three times with small quantities of the dichloroethane solution, then pour sufficient of the solution into the cuvette to fill it. (Do not use a pipette to transfer the liquid). Examine the solution in the cuvette critically for turbidity. Slight turbidity bay be dispersed by applying the finders or palms of the hands to the sides of the cuvette to warm the liquid slightly.

(ix) Measure the absorbance of the liquid in a spectrophotometer at 603 nm against a dichloroethane blank.

(x) The concentration of unknown solutions should be determined from the standard curve as it does not conform to Beer's Law.

This method can be used to assay solutions of other QACs as well, provided that standard curves are constructed for each such compound. To date, the method has been found satisfactory for the determination of solutions of cetalkonium chloride, cetrimide and CTAB, as well as BZC.

2.5 MICROBIOLOGICAL METHOD OF TESTING USED.

2.5.1 Development of test procedure

2.5.1.1 Selection of a method of testing for bactericidal efficacy

The main purpose of testing the preservative/powder systems by a microbiological method was to assess whether the supernatant fluids in which inactivation had been detected chemically were, in fact, reduced in bactericidal efficacy to the extent indicated by the chemical analysis. It was realised that factors such as alteration of pH, alteration of ionic content of the solution and selective adsorption of components of preservatives which are mixtures (eg. BZC and cetrimide) could produce in a supernatant fluid a bactericidal efficacy which was considerably more, or less, than what would be expected from a consideration of the

proportion of preservative lost from the fluid due to interaction with the powder.

Furthermore, as most of the powders tested remained in the formulations in which they were usually used, such formulations usually being shaken at regular intervals, it was desirable to test for any effect that the suspended powders might have on the bactericidal efficacy of the preservative in the system.

Thus it was decided to subject both the supernatant fluids and complete preservative/powder suspensions to microbiological tests.

A very large number of microbiological testing methods, which serve a variety of purposes, have been devised and reported in the literature. Such methods of preservative and disinfectant evaluation have been adequately reviewed by, for example, Hugo (1965), Kelsey (1968) and Cowen and Steiger (1976). No attempt will therefore be made to review the field here.

The method to be used in the present study, however, was required to:

(i) Provide a basis for the comparison of the bactericidal efficacy of different solutions of the same preservative.

(ii) Be suitable for the testing of suspensions as well as solutions.

(iii) Be reasonably rapid as fairly large numbers of systems were to be tested.

(iv) Preferably, make is possible to plot graphically, the relationship between bactericidal efficacy and concentration of preservative.

A study of the literature revealed that in two papers on preservative inactivation by powders, namely those of Batuyios and Brecht (1957) and Bean and Dempsey (1971), similar methods, involving addition of a standard inoculum of micro-organisms

to the test system and subculturing at intervals, had been used. In both these papers, the preservatives tested were quaternary ammonium compounds and the methods used satisfied the above criteria. The adoption of a similar testing method had the advantage of enabling comparisons to be drawn with the results obtained in these two papers. It was therefore decided to adopt a method based on that used by Bean and Dempsey owing to the greater degree of standardisation possible with their method.

It was also decided to test only the 0,01 % BZC/powder systems in this manner as it was not feasible to test all the preservative/powder systems in the time available.

2.5.1.1 Culture medium

Lubrol indicator broth as described by Bean and Dempsey (1971) was prepared according to the following formula:

Nutrient broth powder (Oxoid)		13 g
Lactose		10 g
Lubrol W (non-ionic surfactant)		1 g
Bromocresol purple		0,016 g
Distilled water	to	1 litre

The solids were dissolved with the aid of heat, the medium packed in 10 ml volumes in rimless test tubes and autoclaved at 115 ^{o}C for 30 min. 10 ml volumes of this medium were used, since, according to Bean and Dempsey, the minimum inhibitory concentration of BZC for Escherichia coli in this medium is 0,014 %. As 1 ml volumes of BZC solution with a maximum concentration of 0,015 % m/v were to be added to this medium the resultant concentration of BZC in the medium would be 0,00136 %, providing a tenfold safety margin.

This purple coloured medium changes to a yellow colour when organisms such as E. coli, which ferment lactose with the production of acid, grow in it.

2.5.1.3 Organism used

Bean and Dempsey used Escherichia coli NCTC 5933 for their

tests. Attempts to locate a culture of this strain in South Africa were unsuccessful, so Escherichia coli SATCC Esc 25, a strain used by the South African Bureau of Standards for testing QAC preservatives, was therefore used instead. This organism, obtained as a lyophilised specimen, was grown on nutrient agar slope cultures at 32 °C, which were then stored in a refrigerator at 4 °C for the duration of the tests.

2.5.1.4 Preparation of a standard inoculum of E. coli

In order to inoculate preservative solutions under test with a standard number of organisms on each occasion on which tests were done, the following procedure was adopted to establish the number of viable organisms in a bacterial suspension prepared in a standardised manner.

(i) A nutrient agar slope was inoculated with the above organism and incubated for 18 h at 32 °C

(ii) The cells were harvested with an inoculating loop and suspensed in 5 ml of sterile quarter-strength Ringer's solution.

(iii) Under aseptic technique, aliquots of this suspension were added to 50 ml of sterile quarter-strength Ringer's solution until a sample gave an absorbance of 0,20 when read in a spectrophotometer in a 10 mm cell at 360 nm against distilled water.

(iv) A viable count on this standard E. coli suspension, by use of a poured-plate technique, gave approximately 22 colonies/ml of 1 in 10^7 dilution. Therefore, the suspension contained approximately 220×10^6 viable organisms/ml and the volume required to produce a final viable population of 20×10^6 organisms/nil in 15 ml of preservative solution is given by the value for x in the equation:

$$\frac{x}{15+x} = \frac{20}{220}$$

$$x = 1,5 \text{ ml}$$

(v) Thus a standard E. coli suspension, providing 20×10^6 organisms/ml when 1,5 ml was inoculated into 15 ml of reaction mixture, could be prepared by suspending sufficient cells, harvested from an 18 h slope culture of E. coli, in quarter-strength Ringer's solution to give a spectrophotometric absorbance reading at 360 nm of 0,20 against distilled water, in a 10 mm cuvette.

2.5.2 Determination of Mean Death Times

In order to prepare a standard curve the following procedure was used to determine the mean death time of E. coli, (a) in simple solutions of known BZC concentration, in order to prepare a standard curve, (b) in the supernatant fluids and, (c) in the preservative/powder suspensions tested:

(i) For each solution or suspension to be tested, five 25 ml glass-stoppered tubes were equilibrated by rinsing with 0,1 % m/v BZC solution, allowed to stand for 5 min with a film of the BZC solution covering the surface, rinsed three times with distilled water and dried in an oven at about 100 °C. The glass stoppers were treated similarly.

(ii) 15 ml of the solution or suspension to be tested was pipetted into each of the five tubes, which were then stoppered and steamed at 100 °C for 20 min to kill any BZC-resistant lactose-fermenting organisms.

(iii) The tubes were allowed to cool, placed in a water bath at 25 °C in a laminar flow environment, and inoculated with 1,5 ml volumes of the standard E. coli suspension prepared as described in Section 2.5.1.3. A stopwatch was started as each tube was inoculated.

(iv) At time intervals approximately equal to one seventh of the expected mean death time, 1 ml samples of the above reaction mixtures were aseptically transferred,

in duplicate, to 10 ml volumes of Lubrol indicator broth. (Ranging tests were done as a preliminary to this experiment in order to determine approximate death times). Thus ten tubes of Lubrol indicator broth were inoculated at each time interval for each solution tested. For each time interval the tubes of broth were kept in the sequence in which they had been inoculated.

(v) The inoculated tubes were incubated for two days at 32 ^{o}C and a record made of the tubes which had turned yellow (indicating growth of the test organism).

(vi) Results were recorded and mean death times calculated as in the following example, which shows the actual results obtained for 0,015 % BZC in the preparation of the standard curve.

	0, 015 %									
	1	2	3	4	5	6	7	8	9	10
½ min	+	+	+	+	+	+	+	+	+	+
1 min	-	+	+	+	+	+	+	+	+	+
1½ min	+	+	-	+	+	-	-	-	-	-
2 min	-	-	-	+	-	-	+	-	-	-
2½ min	-	-	-	-	-	-	-	-	+	-
3 min	-	-	-	-	+	+	-	-	-	-

1+ 2+1½+2½+ 2+1½+1½+ 1½+ 1½+ 1½= 16½/10 = 1,65 min

The time corresponding to the first tube in each vertical column in which no growth was found was used, ignoring any subsequent "wild plusses", (which are a feature of tests of this nature when performed on surface active germicides). The mean of these times is the mean death time.

2.5.3 Preparation of a standard curve

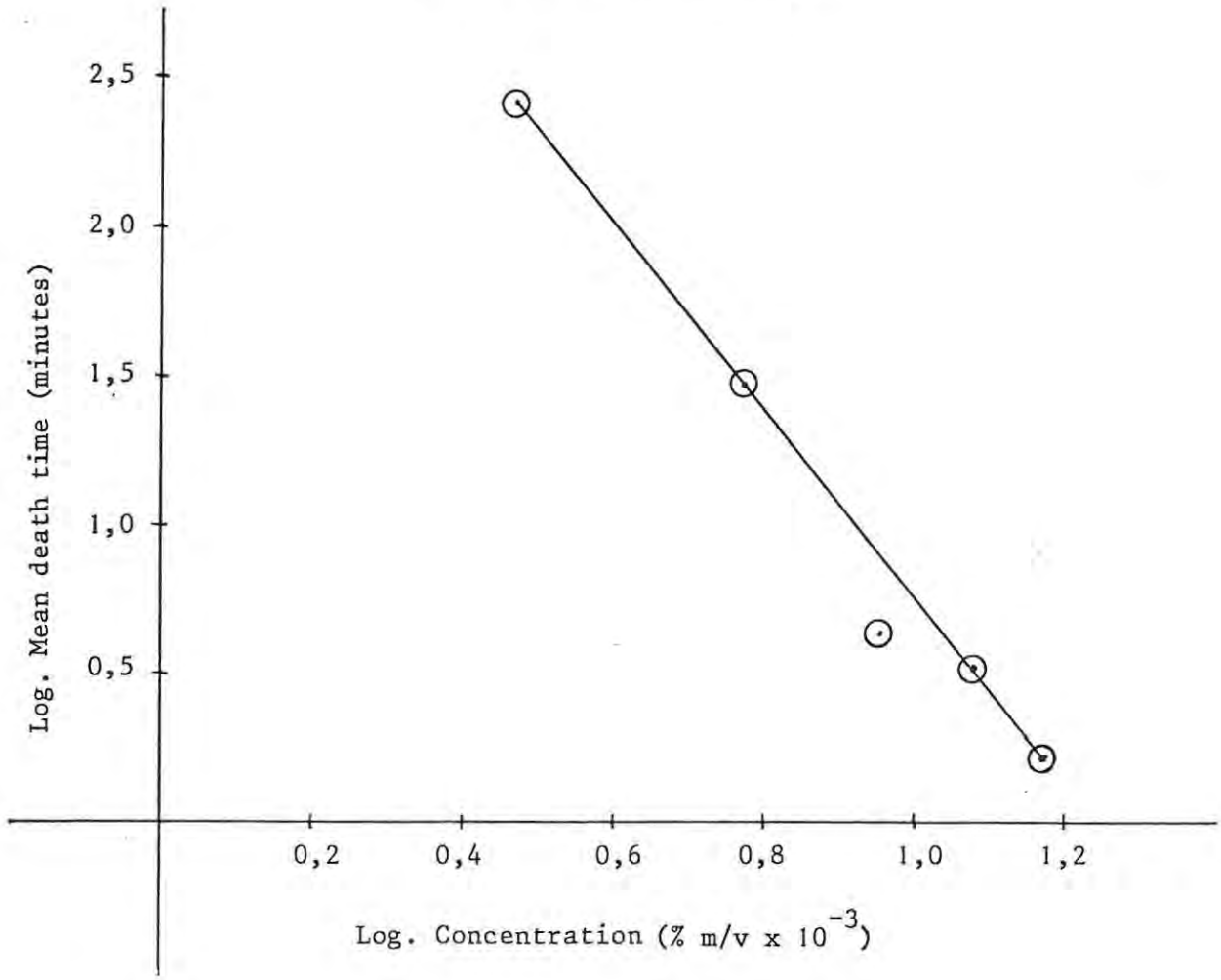
The above procedure was used to determine the mean death times of E. coli in five carefully prepared BZC solutions. Each solution was prepared $\frac{16,5}{15}$ times stronger than actually

required, in order to produce the required concentration when 1,5 ml of bacterial suspension was added to 15 ml of solution. Results obtained were as follows:

Final % m/v BZC	Mean death time
0,015	1,65
0,012	3,25
0,009	4,3
0,006	29,5
0,003	261

When the logarithms of the BZC concentrations were plotted against the logarithms of the mean death times, a standard curve was obtained in which four of the five points were in a straight line (see Fig. 17). The correlation coefficient for the five points was 0,9897.

Fig. 17 STANDARD CURVE FOR THE ACTIVITY OF AQUEOUS BENZALKONIUM CHLORIDE AGAINST ESCHERICHIA COLI (SATCC ESC 25) AT 25°C



CHAPTER 3 PRELIMINARY FINDINGS.

3.1 INTRODUCTION

The intention of this preliminary study was to identify those preservatives which interact with the selected powders to a significant extent so that interactions discovered could be further investigated. Conversely this investigation could also serve to identify those preservatives which were unlikely to interact with powders, to highlight any powder interactions that such preservatives might have and to enable the most suitable preservatives for use in the presence of each powder to be selected, not only from a knowledge of preservative/powder interactions, but also from a knowledge of the pH of the system and of the activity of the various preservatives at different pH values.

Therefore, a broad range of powders commonly used in pharmaceutical and cosmetic formulations, and a broad range of preservative solutions which had not previously been investigated in this manner, were selected. The effect of each powder on each preservative was then tested by the procedure described in Section 2.2.1. This procedure involved adding 5 % of the powder (1 % in the case of aerosil) to a commonly used concentration of the preservative in aqueous solution, allowing these suspensions to stand, analysing the supernatant liquid for content of preservative by one of the procedures in Section 2.3 and testing the pH of the test and control suspensions. Analytical results were expressed as percentage loss of preservative calculated as described in Section 2.3.2.

In the case of cetalkonium chloride 0,1 %, a few further tests were performed on somewhat more complex systems; that is, a third component was added to test and control suspensions,

similar to those used above, and the supernatant fluids analysed. The third components used were 1 % sodium chloride, 5 % propylene glycol and 0,02 M and 0,2 M phosphate-citrate buffers of pH 7,0.

3.2 RESULTS

The results of the tests on simple preservative/powder systems and on the cetalkonium chloride/powder/propylene glycol systems are shown in Table 1, while the pH values of test and control suspensions are recorded in Table 2.

The series of tests on cetalkonium chloride/powder systems containing 1 % sodium chloride was abandoned since, on standing at 25 °C, a large precipitate appeared in the control tube, containing 0,1 % cetalkonium chloride plus 1 % sodium chloride. Analysis of the supernatant fluid in this tube revealed an 81 % loss of cetalkonium chloride from the solution. It is clear therefore that an incompatibility exists between sodium chloride and cetalkonium chloride in aqueous solution, probably due to a common ion effect reducing the solubility of the latter compound. In an investigation into this incompatibility 0,5, 0,4, 0,3, 0,2 and 0,1 % respectively of sodium chloride was added to five samples of 0,1 % cetalkonium chloride. On allowing these solutions to stand overnight at 20 °C, a precipitate formed in all tubes except the one containing 0,1 % sodium chloride. However, a few days later, a precipitate was noticed in that tube also.

Tests on cetalkonium chloride/powder systems with phosphate-citrate buffers of pH 7,0 produced completely anomalous results as the apparent percentage losses obtained in most cases exceeded 100 %. This result must probably be ascribed to interaction between the preservative and buffer, or to a water-soluble extractive effect, as discussed previously. In any case, the tests were of little value as even the 0,2 M

TABLE 1. PERCENTAGE DECREASE IN PRESERVATIVE CONCENTRATION IN CONTACT WITH POWDERS

(5 g POWDER/100 ml PRESERVATIVE SOLUTION

	8-Hydroxyquinoline sulphate 0,1%	Phenylmercuric nitrate 0,002%	Methyl hydroxybenzoate 0,1%	Benzoic acid 0,1%	o-Chlorobenzoic acid 0,1%	Dehydroacetic acid 0,05%	Phenol 0,5%	Cetylpyridium chloride 0,1%	Cetalkonium chloride 0,1%	Cetalkonium chloride 0,1% in 5% propylene glycol
Aerosil (1 g/100 ml)	0	52	1	0	2	8	0	55	69	63
Bismuth carb.	67	6	26	28	36	41	2	30	83	62
Calamine	99	13	1	65	33	7	0	95	96	93
Calcium carb. ppt.	66	0	0	30	30	0	1	6	0	0
Kaolin (light)	56	45	0	12	16	19	1	100	100	100
Kaolin (heavy)	37	32	0	0	1	18	0	94	100	98
Kieselguhr	24	2	0	8	32	0	1	15	28	20
Magnes. carb. (light)	99	1	11	26	24	4	0	25	34	36
Magnes. carb. (heavy)	96	1	12	27	31	4	0	22	16	8
Magnes. oxide (light)	100	2	19	30	29	23	0	43	48	32
Magnes. trisilicate	100	53	12	29	32	0	0	100	100	100
Starch	10	88	10	11	7	8	4	74	78	61
Talc	99	12	4	27	27	4	1	29	27	28
Titan. dioxide	31	68	1	0	2	0	0	92	88	89
Zinc oxide	99	6	2	25	14	72	1	4	0	2

TABLE 2. OBSERVED pH DIFFERENCES IN POWDER/PRESERVATIVE SYSTEMS AND POWDER/WATER SYSTEMS

(5 g POWDER/100 ml WATER OR PRESERVATIVE SOLUTION)

	8-Hydroxyquinoline sulphate 0,1%		Phenylmercuric nitrate 0,002%		Methyl hydroxybenzoate 0,1%		Benzoic acid 0,1%		o-Chlorobenzoic acid 0,1%		Dehydroacetic acid 0,05%		Phenol 0,5%		Cetylpyridium chloride 0,1%		Cetalkonium chloride 0,1%	
	P	W	P	W	P	W	P	W	P	W	P	W	P	W	P	W	P	W
Aerosil(1 g/100 ml)	3,8	6,7	6,4	6,8	5,4	5,9	3,4	6,0	3,1	6,3	4,2	6,3	7,0	7,2	3,8	6,6	3,7	6,4
Bismuth carb.	3,8	7,3	7,6	7,6	5,8	7,0	3,8	7,1	3,3	6,5	5,1	7,4	6,9	7,4	6,9	7,8	8,4	8,1
Calamine	7,6	8,2	8,1	8,2	7,5	7,7	7,3	8,0	6,9	7,8	7,5	8,0	8,0	8,0	7,9	8,2	8,0	8,3
Calc. carb. ppt.	8,0	8,5	8,5	8,7	8,0	8,2	7,9	8,2	7,8	8,5	7,9	8,5	8,6	9,3	8,4	8,8	9,3	9,3
Kaolin (light)	3,8	6,9	7,2	7,1	6,7	7,0	4,2	7,1	3,4	7,2	5,3	6,9	7,3	7,0	6,6	7,2	7,3	8,1
Kaolin (heavy)	3,5	4,8	5,3	5,2	4,8	5,0	3,7	4,9	3,2	4,9	4,1	5,0	5,9	5,5	4,1	4,9	4,7	6,1
Kieselguhr	4,5	7,8	8,1	7,8	7,0	7,2	3,8	7,3	3,2	7,3	5,5	7,5	7,8	7,8	7,4	7,7	7,2	8,1
Mag. carb. (1.)	9,2	9,5	9,5	9,5	9,3	9,5	9,2	9,5	9,5	9,7	9,4	9,5	9,4	9,6	9,6	9,6	9,5	9,5
Mag. carb. (h.)	9,2	9,4	9,5	9,6	9,2	9,4	9,1	9,3	9,3	9,5	9,4	9,4	9,2	9,6	9,6	9,6	9,7	9,7
Mag. oxide (1.)	9,9	10,0	10,1	10,1	9,1	10,1	9,8	10,1	9,8	10,1	9,9	10,0	9,9	10,0	10,1	10,1	10,3	10,4
Mag. trisilicate	9,3	9,5	9,8	9,8	9,1	9,4	8,9	9,3	9,1	9,3	9,4	9,5	9,2	9,6	9,7	9.7	9,7	9,6
Starch	4,0	5,2	6,5	6,0	7,0	7,5	3,8	6,9	3,3	7,6	4,7	6,9	7,2	4,7	4,1	5,0	4,1	5,0
Talc	7,7	8,2	8,5	8,0	7,8	8,1	7,3	7,8	7,9	8,3	7,9	8,0	8,1	8,2	8,2	8,2	8,0	8,0
Titan. oxide	4,2	7,1	7,1	7,1	6,9	7,0	3,9	7,0	3,1	7,0	5,0	7,0	6,9	6,9	6,8	7,2	7,2	7,3
Zinc oxide	6,6	7,0	6,9	6,9	7,0	7,3	6,4	7,0	6,3	6,9	6,7	7,1	6,9	6,8	7,0	7,0	7,3	7,6

P = powder/preservative system W = powder/water system

buffer possessed insufficient buffer capacity to alter significantly the pH of suspensions of the more basic powders. In fact, some unexpected pH effects were observed; for example, calamine suspended in water has a pH of about 8,0 whereas, if it is suspended in 0,2 M phosphate-citrate buffer of pH 7,0, the pH rises to 9,8. In the case of zinc oxide, the rise in pH is even more dramatic, namely from pH 7,0 to 10,9.

3.3 COMMENT

3.3.1 On the effect of the various powders on each preservative.

Organic acids (benzoic, o-chlorobenzoic and dehydroacetic acids)

Benzoic and o-chlorobenzoic acids showed significant and fairly uniform (around 30 %) losses onto all the carbonate powders, talc and magnesium trisilicate, in spite of fairly large pH differences between suspensions of these powders. These apparent losses may be explained by the tendency of these preservatives to undergo peak shifts in alkaline solution. As benzoic acid dissociates above pH 5, ultra-violet absorbance readings taken above this pH value are suspect. This phenomenon would not explain inactivation by bismuth carbonate however as the pH in the systems containing the latter and these two preservatives was well below 5.

The apparent interaction of DHA with bismuth carbonate, magnesium oxide and zinc oxide is significant, although, in the latter two cases, peak shifts may again be at least part of the explanation.

Quaternary ammonium compounds

Losses with respect of CPC and cetalkonium chloride solutions were generally high and, in most cases exceeded the losses, observed by McCarthy (1969), from benzalkonium chloride solution of the same concentration.

High losses of these surface active compounds onto powders known to be strong adsorbants (eg. kaolin and magnesium trisilicate) were to be expected. However, the loss onto talc was unexpectedly low while that onto calamine was unexpectedly high in view of the fact that there was virtually no loss onto zinc oxide (calamine is largely zinc carbonate). The large difference in extent of inactivation of these two compounds by bismuth carbonate is also puzzling. In an attempt to ascertain which component of calamine is responsible for its ability to inactivate these compounds, similar tests were done on zinc carbonate and ferric oxide with cetalkonium chloride 0,1 %. The loss onto zinc carbonate was 95 % and onto ferric oxide 42 %.

8-Hydroxyquinoline sulphate

Since Martindale's Extra Pharmacopoeia states that this compound is incompatible with alkalies and many metal ions, certain losses were predictable. The high loss onto talc was not anticipated however. The apparent inactivation of this compound by calamine is of particular interest in view of the observation by Albert et al. (1953) that it forms two complexes with iron, one of which is bactericidal and the other not.

Methyl hydroxybenzoate

The only significant loss here is that onto bismuth carbonate. The somewhat smaller losses onto the magnesium carbonates, magnesium oxide and magnesium trisilicate are probably of little significance owing to reduction of the activity of this compound at high pH values, methyl hydroxybenzoate having a pH value of 8,3 and hence losing half its activity at an equal pH value.

Phenol

The very small losses observed here seem general for phenolic-type preservatives. McCarthy (1969) has recorded equally small losses for phenoxetol, chlorophenoxetol and

propylenephenoxetol and only slightly larger losses for chlorocresol. Phenol would thus, at first sight appear to be a good choice of preservative for most suspensions. However, it has been found that a degree of adsorption of preservative can actually enhance bactericidal efficacy in some cases - see Section 5.2

Phenylmercuric nitrate

In view of the low concentration used of this material, the losses onto kaolin, magnesium trisilicate and even titanium dioxide are to be expected but, bearing in mind that an approximately 1 % suspension of Aerosil was used, the losses onto this substance and onto starch are noteworthy. It became apparent that an investigation of the other commonly used organomercurial, thiomersal, would be of interest to assess whether it showed similar losses.

3.3.2 The effect of various preservatives on specific powders

Aerosil: When used as an approximately 1 % suspension, Aerosil seems to have the capacity to interact mainly with preservatives such as PMN, which are used in very low concentration, and with surface active preservatives such as the QAC's.

Bismuth carbonate: This substance interacts appreciably with all the preservatives under test, except with PMN and phenol.

Calamine: This is an interesting substance in view of the unexpected extent of its ability to inactivate the QAC s under test. Its interactions with benzoic acid and 8-hydroxyquinoline sulphate are predictable, although a microbiological test on its apparent interaction with the latter compound is warranted to establish whether the ferric compound, probably formed, is bactericidal.

Calcium and magnesium carbonates: For most preservatives, the extent of interaction with calcium carbonate is similar to that with the magnesium carbonates, although in the cases of methyl hydroxybenzoate, 8-hydroxyquinoline sulphate and the QAC's it is somewhat less for calcium carbonate than for the magnesium carbonates. Also worthy of note is the very small difference in ability between light and heavy magnesium carbonate to inactivate most preservatives, an unexpected observation in view of the very obvious difference in particle size between the two powders.

Magnesium oxide: The ability of this powder to inactivate preservatives is, in nearly all cases, somewhat greater than that of the magnesium carbonates.

Kaolin: As expected, kaolin inactivated most preservatives to a significant extent, so its minimal inactivation of methyl hydroxybenzoate and phenol is worthy of note. In most cases the loss onto light kaolin was appreciably greater than onto heavy kaolin in spite of a minimal difference in physical appearance between the two grades. This observation, although expected, contrasts with the observation made with respect to the ability of light and heavy magnesium carbonates to inactivate preservatives in solution.

Kieselguhr: In general, inactivation of preservatives by this substance was less than expected from a knowledge of its use as an adsorbent. Care would nevertheless have to be exercised if it were to be used as a filter acid for the filtration of small volumes of solutions containing 8-hydroxyquinoline sulphate, o-chlorobenzoic acid and the QAC s.

Magnesium trisilicate: Being a known strong adsorbent, this substance inactivated most preservatives to a

significant extent. In most cases losses onto magnesium trisilicate were higher than onto light kaolin, although DHA lost no activity in the presence of magnesium trisilicate but did show a small loss in the presence of kaolin. Like kaolin, magnesium trisilicate had no effect on the concentration of 0,5 % phenol.

Starch: All preservatives tested showed some loss in the presence of ungelatinised starch but no preservative was completely inactivated. It would be of interest to ascertain whether physical adsorption or a chemical interaction is the mode of inactivation for each preservative, for, if the latter, similar interactions may take place with other carbohydrates and the extent of interaction with starch may be time dependant due to a slow penetration of the starch grains by the preservative solutions.

Talc: As this powder is frequently used for its adsorbent properties the observed losses were somewhat less than expected, especially in the case of the QAC s. Its interaction with 8-hydroxyquinoline sulphate possibly represents a chemical reaction as this preservative is inactivated to a similar extent by other powders which are also magnesium compounds.

Titanium dioxide: Inactivation of the QAC s and PMN by this powder is probably explained on the basis of its large surface area and its ability to act as a cation exchanger above pH 6,5, and on the basis of the known surface activity of the QAC s and the low concentration of PMN used.

Zinc oxide: The fact that 8-hydroxyquinoline sulphate is 99 % inactivated by both the zinc-containing powders in this study is possibly an indication that a chemisorption process is involved. The interaction of zinc oxide with PMN is of interest because, initially, rather high and variable values

were obtained for percentage loss. Consistent and much lower figures were obtained when fresh dithizone was used for replicate determinations. However, these replicate determinations also corresponded to the use of a shorter storage time for the powder/preservative system (the original test systems were, unavoidably, incubated at 25 ^{o}C for six weeks before analysis). It was therefore not known whether it was the shorter time or the fresh dithizone, or both, which was responsible for the improved results.

3.3.3 pH Effects

Table 2 lists the pH values of the powder/preservative and powder/water systems. At first the pH variation between different Aerosil/water and calcium carbonate/water systems caused concern as to whether drift was occurring in the pH meter, but the stability of such systems as magnesium carbonate/water, magnesium oxide/water and titanium dioxide/water, which were examined concurrently, negated this possibility and highlighted the fact that the pH of a highly unbuffered system is difficult to determine accurately and that such systems are subject to pH variation due to the smallest influences. The most likely explanation of these pH differences is the slight differences in pH between different batches of distilled water used to prepare the suspensions.

It should be born in mind that the pH of a suspension can affect both the extent of inactivation of the preservative by the powder and the antimicrobial activity of the preservative. This study is therefore of a very superficial nature, and a clearer idea of the potential efficacy of a suspension preservative would be obtained if information regarding the extent of its inactivation by the various powders over a range of pH values were available. Formulators should therefore take into consideration the extent of

inactivation of the preservative, the actual activity of the preservative and the effect of adjuvants at the intended pH value when selecting a preservative for use in a suspension.

3.3.4 More complex systems

It is apparent from these results that the characteristics of complete formulations can be quite different from what would be expected, as shown by the addition of a pH 7,0 buffer to a system of pH 7,0 producing a final pH of 10,9, and the addition of as little as 0,1 % of sodium chloride to a 0,1 % cetalkonium chloride solution producing a precipitate. It is of interest, therefore, to note that the addition of 5 % propylene glycol to cetalkonium chloride 0,1 %/powder systems only produces a slight reduction in the loss of preservative in nearly all cases.

3.4 CONCLUSION

It is clear that, from the findings of this preliminary study, further investigations could have branched out in a number of different directions, for example:

(i) A study of the exact nature of the interactions of powders with 8-hydroxyquinoline sulphate, a case in which a chemisorption mechanism seems to be involved.

(ii) Investigation of possible methods of inhibiting preservative loss onto powders.

(iii) An investigation into the effects of adding common adjuvants to the preservative systems.

(iv) An investigation into the variation in the extent of inactivation of the different preservatives by powders with variation in pH.

(v) Confirmation of the results, already obtained, by microbiological methods.

The decision finally taken however was to investigate further various questions raised regarding the organomercurial and QAC interactions observed in this preliminary study and to

develop and use a suitable colorimetric method of analysis for the determination of QACs in view of certain difficulties experienced with ultraviolet spectrophotometric techniques when applied to preservative/powder systems. These further investigations are presented in the next two chapters.

CHAPTER 4

INACTIVATION OF ORGANOMERCURIALS BY POWDERS.

The main purpose of this further investigation into the interactions of organomercurials with the powders was to establish whether thiomersal, in a commonly used concentration, shows similar interactions with powders to those of PMN. There was also some evidence that a slow interaction of PMN with some powders may take place over a period of time, and that certain interactions may be hastened by, or possibly take place only at, elevated temperatures. Accordingly, the PMN and thiomersal solutions were subjected to the following three series of tests.

4.1 COMPARISON OF PMN/POWDER INTERACTIONS WITH THIOMERSAL/POWDER-INTERACTIONS

Thiomersal/powder interactions were tested according to the procedure described in Section 2.2.1, followed by analysis of the supernatant liquids as described in Section 2.4.1 and calculation of the percentage losses of preservative from solution. 0,002 % PMN and 0,02 % thiomersal in aqueous solutions were found to interact with powders at 25 ^{o}C to the extents shown in columns A and C in Table 3. It is apparent that there are big differences between these two preservatives in this respect, differences which cannot be explained entirely by the difference in concentration between the two preservatives.

The 52 % loss of PMN in the presence of Aerosil has already been remarked upon. Thiomersal interacts with Aerosil only very slightly; in fact the mass of thiomersal adsorbed is less than the mass of PMN adsorbed even though the thiomersal

TABLE 3. PERCENTAGE DECREASE IN CONCENTRATION OF ORGANOMERCURIAL PRESERVATIVES IN CONTACT WITH POWDERS, WITH AND WITHOUT AUTOCLAVING AT 115 °C FOR 30 min (5 g POWDER/100 ml PRESERVATIVE SOLUTION)

POWDER	PMN 0,002 %		Thiomersal 0,02 %	
	25°	115°	25°	115°
Aerosil (1 g/100 ml)	52	52	4	2
Bism. carb	6	9	53	41
Calcium carb. ppt.	0	1	2	1
Calamine	13	11	7	4
Kaolin, light	45	43	1	2
Kaolin, heavy	32	33	6	5
Kieselguhr	2	3	4	1
Magnes. carb. light	1	5	3	2
Magnes. carb. heavy	1	2	6	3
Magnes. oxide light	2	2	4	4
Magnes. trisilicate	53	55	5	3
Starch	88	-	13	-
Talc	12	12	3	3
Titan. dioxide	68	69	1	1
Zinc oxide	6	25	2	3
	A	B	C	D

solution is ten times more concentrated than the PMN solution. An even bigger difference in inactivating ability is seen in the case of bismuth carbonate, in whose presence the more concentrated solution, thiomersal 0,02 %, loses 53 % of its preservative content while the more dilute solution, PMN 0,002 %, loses only 6 %. This may be caused by a reaction between thiomersal and bismuth carbonate or be due to adsorption isotherms for both compounds on bismuth carbonate which are similar in shape to that in Section 5.1.3 involving

BZC on calamine. Note that the adsorption characteristics of BZC on bismuth carbonate were observed to be very similar to those of BZC on calamine. It is possible therefore that the above phenomenon could be due to similar behaviour in the case of organomercurials adsorbed on bismuth carbonate.

Losses of preservative in the presence of calcium carbonate and calamine were fairly minimal for both solutions, a somewhat unexpected result in the case of calamine in view of the known incompatibility between metallic iron and mercurials. In the presence of the kaolin powders adsorption is minimal from thiomersal solution but appreciable from PMN solution. As would be expected, light kaolin adsorbs PMN to a greater extent than does heavy kaolin, but the opposite is true for thiomersal. Perhaps this difference is due to a low pH favouring adsorbtion in the case of thiomersal (which is anionic) but not in the case of PMN (which is cationic) as the pH values for the light kaolin suspensions were 7,2 for PMN and 7,3 for thiomersal and for heavy kaolin, 5,3 for PMN and 5,8 for thiomersal.

The two preservatives were inactivated to a similar extent in the presence of kieselguhr. Kieselguhr is almost pure silicon dioxide, while Aerosil is pure silicon dioxide, yet the percentage loss of PMN in the presence of 1 % Aerosil is 52 % but in the presence of 5 % kieselguhr it is only 2 %. Assuming that the adsorbents have equal adsorptive capacity, it would seem that the surface area of unit mass of Aerosil is $\frac{52}{2} \times \frac{5}{1} = 130$ times greater than unit mass of kieselguhr. Whilst this ratio is quite possible, this observation is not confirmed by a comparison of the loss of thiomersal in the presence of these two powders, nor is it confirmed by comparing the loss of dilute QACs in the presence of these two powders. In fact, loss of QAC onto 5 % kieselguhr

invariably exceeds the loss onto 1 % Aerosil when 0,01 % QAC solutions are tested.

Percentage inactivation figures for both preservative solutions in the presence of the magnesium carbonates and magnesium oxide are minimal and do not warrant further comment.

In the case of magnesium trisilicate the adsorption loss is high for PMN and low for thiomersal. Starch, Aerosil and light kaolin behave similarly, so it would seem that PMN solutions are much more readily inactivated than thiomersal solutions by strong adsorbents. The minimal interaction of PMN with talc is therefore unexpected.

The large difference in the loss of the two preservatives onto titanium dioxide is almost certainly due to the fact that this powder acts as a cation exchanger above pH 6,5 and the fact that the pH of both systems was 7,1. PMN is a cationic compound, while thiomersal is anionic.

In conclusion, therefore, it appears that the fact that PMN and thiomersal are both organomercurials does not in any way predispose them to interact with the same powders.

4.2 THE EFFECT OF HEAT ON LOSS OF ORGANOMERCURIALS FROM SOLUTION IN THE PRESENCE OF POWDERS

In order to establish whether heat has any effect on the extent of inactivation of PMN and thiomersal by powders, preservative/powder systems containing these two organomercurials were subjected to one of the most severe heat treatments likely to be imposed on aqueous formulations, viz., moist heat sterilisation at 115 ^{o}C for 30 min. After a further 24 h. of storage at 25 ^{o}C the supernatant fluids were analysed and the percentage losses calculated. (see Sections 2.2.1, 2.3.3 and 2.4.1 for details of procedure, calculation and analysis, respectively). Powder/water

control samples and samples of the preservative solutions alone were treated similarly.

Examination of columns A and B of Table 3 shows that, in general, autoclaving has little effect on the percentage loss of PMN in the presence of powders. The only powder, in whose presence inactivation of PMN is enhanced, is zinc oxide. Hart (1973), working with zinc sulphate and adrenalin eye drops BPC 1968, has also noticed a loss of PMN from solution when this compound was heated in the presence of zinc ions. It would appear that the very low concentration of zinc ions provided by zinc oxide is sufficient to inactivate PMN partially on heating, but in the case of calamine the extent of inactivation is not increased by autoclaving, probably due to a lower concentration of zinc ions being yielded by the carbonate than by the oxide at 115 ^{o}C.

Examination of columns C and D of Table 3 reveals that heat treatment also has a minimal effect on percentage loss of thiomersal onto powders, an observation of some interest in view of the finding of Tsuji et al. (1964) that thermal decomposition of thiomersal is accelerated by Cu^{++}, Fe^{+++} or Zn^{++} ions but not by Ca^{++} or Mg^{++} ions. Presumably, the concentration of Fe^{+++} and Zn^{++} ions contributed by calamine and zinc oxide is too low to have a significant effect on 0,02 % thiomersal solution on autoclaving for 30 min. The small reduction in percentage loss of thiomersal in the presence of bismuth carbonate on autoclaving is also to be noted.

No loss of preservative in the control tubes, containing the preservative solution alone, was observed.

4.3 THE EFFECT OF TIME ON THE INACTIVATION OF PMN BY POWDERS

As mentioned previously, replicate determinations of the loss

of PMN in the presence of powders produced results which, for certain powders, were lower than those obtained originally. The replicate determinations corresponded to the use of fresh dithizone in the analytical procedure and to the use of a shorter storage time for the test systems. It was therefore not known whether the new figures, which were reproducible on further replication, were due to the use of fresh dithizone or to the use of a shorter storage period. The powders in question were Aerosil, light kaolin, starch, talc and zinc oxide. It was therefore decided to prepare a number of tubes containing each of these powders, plus preservative solution, to store them at 25 ^{o}C with regular agitation and to analyse the supernatant fluids at intervals. The results shown in Table 4 reveal that a progressive interaction over a long period of time takes place only in the case of starch. This further interaction is only slight, the percentage inactivation by starch increasing from 82 % after two hours to 89 % after twelve weeks. (A series of tubes was also analysed after one week but the results obtained were unreliable due to a faulty spectophotometer and are therefore not reported here.

TABLE 4. EFFECT OF TIME ON THE PERCENTAGE DECREASE IN CONCENTRATION OF 0,002 % PMN IN CONTACT WITH POWDERS (5 g POWDER/100 ml PMN SOLUTION)

POWDER	2h	1d	2d	21d	42d	84d
Aerosil (1 g/100 ml)	54	52	50	53	52	54
Kaolin, light	45	45	44	48	45	48
Starch	82	85	84	87	88	89
Talc	13	14	13	14	12	12
Zinc oxide	8	7	6	6	8	9

CHAPTER 5.

INACTIVATION OF QUATERNARY AMMONIUM COMPOUNDS BY POWDERS.

5.1 SPECTROPHOTOMETRIC TESTS

As discussed in Section 2.1.2.2, it was decided to determine the extent to which 0,001 % m/v solutions of BZC are inactivated by powders. This determination was carried out according to the procedure described in Section 2.2.1, the supernatant liquids assayed as described in Section 2.4.2.1, and the loss of BZC onto each powder calculated as described in Section 2.3.2.

5.1.1 Comparison of interactions of 0,01 % m/v BZC solution and those of other QAC solutions with powders

The percentage losses of 0,01 % m/v BZC solution onto powders are shown in Table 5, along with the figures already obtained for 0,1 % CPC and 0,1 % cetalkonium chloride solutions and similar figures obtained by McCarthy (1969) for 0,1 % BZC solution.

Comparison of columns A and B of Table 5 reveals that, as expected, the percentage loss of preservative in the presence of most powders is higher for the more dilute solution of BZC than for the more concentrated solution. However, the very much smaller percentage loss of BZC from 0,01 % solution than from 0,1 % in the presence of calamine is of interest and was considered worthy of further investigation (see Section 5.1.3). Aerosil, bismuth carbonate, calcium carbonate, heavy magnesium carbonate and light magnesium oxide also cause a smaller percentage reduction in preservative content in 0,01 % BZC than in 0,1 % BZC, but the difference between the two values in each of these cases is not nearly as pronounced as in the case of calamine.

TABLE 5. PERCENTAGE DECREASE IN CONCENTRATION OF FOUR QAC PRESERVATIVE SOLUTIONS IN CONTACT WITH POWDERS AT 25 °C (5 g POWDER/100 ml PRESERVATIVE SOLUTION)

POWDER	A [x]BZC 0,1 %	B BZC 0,01 %	C Cetalkonium chloride 0,1 %	D CPC 0,1 %
Aerosil (1 g/100 ml)	35	20	69	55
Bism. carb.	20	12	83	30
Calc. carb. praecip.	8	5	0	6
Calamine	33	6	96	95
Kaolin, light	55	100	100	100
Kaolin, heavy	40	100	100	94
Kieselguhr	20	48	28	15
Mag. carb. light	-	5	34	25
Mag. carb. heavy	11	7	16	22
Mag. ox. light	11	6	48	43
Mag. trisil.	93	100	100	100
Starch	25	100	78	74
Talc	29	81	27	29
Titan. diox.	20	58	88	92
Zinc oxide	8	10	0	4

[x]McCarthy (1969)

Comparison of columns A and C is also of value as they show the losses onto powders from solutions of two compounds which are structurally very similar, of similar molecular mass and present in the same concentration. Cetalkonium chloride is cetyldimethylbenzylammonium chloride, while BZC is a mixture of alkyldimethylbenzylammonium chloride homologues. It was expected, therefore, that similar adsorption losses onto powders would occur from these two solutions. However, Table 5 reveals that the percentage losses from 0,1 % cetalkonium chloride in the presence of Aerosil, bismuth carbonate, calamine, light and heavy kaolin, light magnesium oxide, starch and titanium dioxide are very much greater than similar losses from 0,1 % BZC. Paradoxically, the percentage losses from 0,1 % CPC in the presence of the same eight powders are, with the exception of bismuth carbonate, very similar to those obtained from 0,1 % cetalkonium chloride. This behaviour was considered quite remarkable in view of the dissimilar structures of cetalkonium chloride and CPC. However, cetalkonium chloride and CPC both have a cetyl group, while the equivalent group in most components of BZC have structures containing somewhat shorter chain lengths. Cetyl groups are aliphatic chains containing sixteen carbon atoms while the alkyl groups in BZC vary in chain length from eight to eighteen carbon atoms. The above facts, therefore, seem to indicate that increasing length of the hydrophobic group in a QAC, or related compound, favours increased adsorption onto a number of powders. In order to establish with greater certainty the correctness of this supposition it was decided to determine in a selection of certain other QAC solutions, the percentage losses of preservative in the presence of powders (see Section 5.1.2).

Whilst dealing with adsorption losses from BZC solutions, it is worth noting that the figures shown in Table 5 for talc are in good agreement with those obtained by Batuyios

and Brecht (1957). The lowest BZC concentration used by Batuyios and Brecht was 0,03 % m/v (30 mg/100 ml), and they used 6 g talc/100 ml of BZC solution. They found a loss of 74 %, which compares well with the 81 % loss for 5 g talc/100 ml of 0,01 % m/v BZC solution shown in Table 5. Furthermore, Batuyios and Brecht also tested a 0,1 % m/v BZC solution (100 mg/100 ml) and obtained a 34 % loss for 6 g talc/100 ml of solution which agrees well with McCarthy's report of 29 % loss for 5 g talc/100 ml of solution of the same strength.

5.1.2 Comparison of interactions of additional selected QAC solutions and those already discussed with powders

In order to investigate further the possibility that an increase in the length of the alphatic chain enhances the adsorption of a QAC by powders, the percentage losses, in the presence of powders, were determined for two additional preservatives, cetrimide and CTAB. Cetrimide bears a similar relationship to CTAB as does BZC to cetalkonium chloride. Cetrimide is a mixture of alkytrimethylammonium bromides in which the alkyl group ranges from twelve to sixteen carbon atoms in length, but contains predominantly the fourteen carbon homologue. CTAB is cetyltrimethylammonium bromide and, like cetalkonium chloride, is therefore a pure quaternary ammonium compound, containing a cetyl group. Cetrimide interactions were investigated with 0,01 % m/v solutions of the preservative only, but, in the case of CTAB, it was decided to determine the extent of its interactions with the selected powders in suspension containing both 0,01 % and 0,1 % m/v solutions of the preservative. As the other pure QACs containing cetyl groups had been investigated in 0,01 % concentration, this step would make possible comparisons between the interactions of BZC and cetrimide with the data obtained on CTAB at a 0,01 % concentration level as well.

Table 6 shows the extent of the interactions of the same four preservative solutions listed in Table 5 and of those of the three additional solutions, arranged in a sequence which facilitates comparison.

Comparison of columns B, C and D of the table shows, in the case of most powders, remarkable similarities in percentage of preservative adsorbed on each powder from 0,1 % m/v solution despite the considerable structural differences which exist between cetalkonium chloride, CPC and CTAB. The most noticeable common characteristic of these three compounds is the presence of a cetyl group in the structure of each compound.

Comparison of columns A and B of Table 6 reveals in studies with most powders, as mentioned previously, large differences in percentage loss from 0,1 % m/v solutions of BZC and cetalkonium chloride despite the structural similarity of these two substances.

The 0,01 % m/v solutions of QACs tested behave similarly. Comparison of columns E and F reveals similarities in percentage adsorption despite the relatively dissimilar structures of BZC and cetrimide and, once again, comparison of columns F and G reveals considerable differences in percentage adsorption onto most powders in spite of the structural similarity between CTAB and cetrimide.

Batuyios and Brecht (1957) made a similar observation with respect to BZC and CPC and commented that this phenomenon could be due to "difference in molecular orientation", while Thoma et al. (1966), having noticed that surface active quaternary ammonium and pyridinium compounds were very differently adsorbed on Aerosil, observed that little binding occurred at less than half the critical micelle concentration, but that strong adsorption was observed in

TABLE 6. PERCENTAGE DECREASE IN CONCENTRATION OF VARIOUS QAC PRESERVATIVE SOLUTIONS IN CONTACT WITH POWDERS AT 25 °C (5 g POWDER/100 ml PRESERVATIVE SOLUTION)

POWDER	A BZC 0,1 % m/v	B Cetalkon. Cl 0,1 %	C CPC 0,1 %	D CTAB 0,1 %	E BZC 0,01 %	F Cetrimide 0,01 %	G CTAB 0,01 %
Aerosil (1 g/100 ml)	35	69	55	55	20	13	22
Bism. carb.	20	83	30	76	12	7	53
Calc. carb. praecip.	8	0	6	1	5	1	17
Calamine	33	96	95	90	6	6	50
Kaolin, light	55	100	100	98	100	100	100
Kaolin, heavy	40	100	94	95	100	100	100
Kieselguhr	20	28	15	17	48	48	89
Mag. carb. light	-	34	25	27	5	5	40
Mag. carb. heavy	11	16	22	16	7	6	36
Mag. ox. light	11	48	43	44	6	9	54
Mag. trisil.	93	100	100	100	100	100	100
Starch	25	78	74	74	100	100	100
Talc	29	27	29	29	81	77	96
Titan diox.	20	88	92	90	58	45	94
Zinc oxide	8	0	4	3	10	11	5

the region of the CMC. This latter observation is interesting because length of the hydrophobic chain of a QAC does influence its CMC.

Whatever the explanation may be, there seems little doubt that increasing length of non-polar aliphatic groups on QACs causes increased adsorption onto most powders. Where there is a choice, therefore, it would seem wise, on the basis of this observation, for suspension formulators to select QAC preservatives of shorter chain length but bearing in mind that the most effective compounds have an alkyl chain length of about C_{12} to C_{14}. (However, in contrast to this statement, see Section 5.2.4).

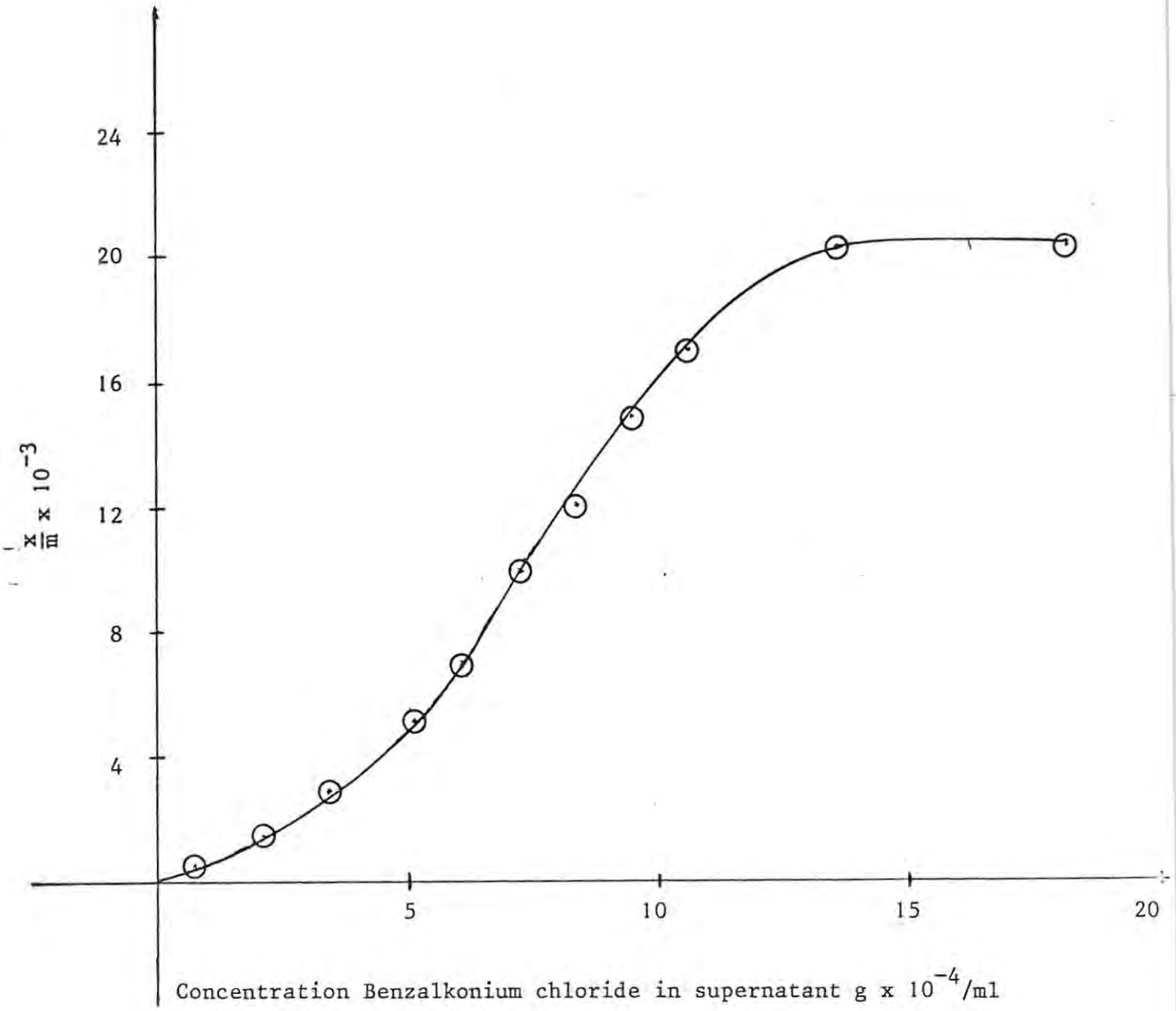
Also of interest in Table 6 is the fact that, for a number of powders, percentage losses from 0,1 % m/v cetalkonium chloride solution are somewhat higher than those from CTAB and CPC solutions of the same strength. This phenomenon may be due to the additional hydrophobic contribution of the benzyl group in the cetalkonium chloride molecule.

A final interesting observation is that the extent of interaction of QAC solutions with talc seems to depend more on the concentration of the QAC than on its structure. All 0,1 % m/v QAC solutions tested showed a 27 to 29 % loss in the presence of talc, while all 0,01 % solutions tested showed a 77 to 96 % loss.

5.1.3 Further investigation of the extent of adsorption of BZC by calamine with variation in concentration of BZC

It was mentioned in Section 5.1.1 that a 33 % loss of BZC onto calamine from a 0,1 % m/v solution, but only a 6 % loss from 0,01 % m/v solution seemed to be an anomaly. However, scrutiny of columns D and G of Table 6, shows that the percentage loss of CTAB onto calamine from 0,1 % solution (90 %) is also very much greater than that from

Fig. 18 ADSORPTION ISOTHERM FOR 0,5 g CALAMINE/10 ml AND BENZALKONIUM CHLORIDE AT 25 °C



0,01 % solution (50 %). The high losses of the other 0,1 % QAC solutions in Table 6 onto calamine and the low loss of cetrimide from 0,01 % solution, provide an extension of this observation to similar situations.

In order to elucidate further the QAC adsorption characteristics of calamine, the losses of BZC onto calamine from various known concentrations of BZC in aqueous solution were determined and the adsorption isotherm for 5 % m/v calamine and BZC plotted. (see Fig. 18). This curve is of the "S"

type according to the classification of Giles et al. (1960). According to Carter (1972), this shape of curve is characteristic of systems in which the adsorbed molecules have a single point of strong attachment to the adsorbent and adopt a vertical orientation due to intermolecular interaction within the adsorbed layer. This type of curve could also indicate strong competition by the solvent for the adsorbent sites, so all powders which adsorb a greater percentage of QAC from stronger, than from weaker, solutions would be expected to be very readily wetted by water. This is certainly the case with Aerosil, bismuth carbonate, calamine, heavy magnesium carbonate and light magnesium oxide, the five powders in respect of which this phenomenon was observed. Of course, the converse situation i.e., powders which do not exhibit this phenomenon, not being readily wetted by water, viz. talc, does behave in the opposite manner, for it adsorbs 81 % of the BZC from a 0,01 % m/v solution and 29 % from a 0,1 % m/v solution.

5.1.4 The effect of heat on loss of QACs from solution in the presence of powders

Since many formulations are heated to a greater or lesser extent during their preparation, it was thought desirable to test for possible QAC/powder interactions which either take place to a noticeable extent only at elevated temperatures, or which are enhanced by an elevated temperature. Such behaviour could possibly come about by slow chemisorption, and the process greatly accelerated by rise in temperature, or by the heat treatment which would displace previously adsorbed materials from adsorption sites on the powders, and thereby make it possible for adsorption of preservative to take place.

The method of testing was similar to that already described for similar tests on organomercurials in Section 4.2, i.e.,

preservative/powder suspensions packed in ampoules were exposed to moist heat sterilisation at 115 °C for 30 min, stored at 25 °C for 24 h, centrifuged and the supernatant fluids analysed for preservative content. (see Sections, 2.2.1, 2.3.3 and 2.4.2 for details of procedure, calculation and method of analysis respectively). Powder/water control samples and samples of the preservative solutions alone were treated similarly.

Percentage losses of selected QAC preservatives in two concentrations in the presence of powders, with and without autoclaving, are shown in Table 7. As was the case with similar tests on organomercurials, the differences in the percentage losses of the preservatives from heated and unheated suspensions is not significant. This holds true for nearly all systems. Only three observations are worth noting.

(i) For all four preservative solutions, significantly lower losses in the presence of Aerosil are recorded after the heat treatment than without it. This phenomenon may be due to the heat treatment changing the state of hydration of the particle surfaces and thereby changing their adsorption properties.

(ii) No satisfactory explanation has been found for the fact that heating increases the loss from the 0,1 % solution of cetalkonium chloride in the presence of heavy magnesium carbonate, but not from the 0,1 % solution of CPC in the presence of the same powder. If this phenomenon were due to additional adsorption sites becoming available due to the heat treatment, 0,1 % CPC would be expected to behave similarly. If it were due to the high pH of magnesium carbonate decomposing cetalkonium chloride at elevated temperatures, other high pH cetalkonium chloride suspensions would be expected to behave similarly.

TABLE 7. PERCENTAGE DECREASE IN CONCENTRATION OF SELECTED QAC PRESERVATIVE SOLUTIONS IN CONTACT WITH POWDERS, WITH AND WITHOUT AUTOCLAVING AT 115 °C FOR 30 min (5 g POWDER/100 ml SOLUTION)

	BZC 0,01 %		Cetrimide 0,01 %		Cetalk. Cl. 0,1 %		CPC 0,1 %	
POWDER	25°	115°	25°	115°	25°	115°	25°	115°
Aerosil (1 g/100 ml)	20	12	13	10	69	47	55	44
Bism. carb.	12	12	7	8	83	77	30	28
Calc. carb. praecip.	5	7	1	3	0	0	6	5
Calamine	6	3	6	6	96	95	95	93
Kaolin, light	100	100	100	100	100	100	100	100
Kaolin, heavy	100	100	100	100	100	100	94	94
Kieselguhr	48	70	48	63	28	27	15	18
Mag. carb. light	5	18	5	7	34	36	25	28
Mag. carb. heavy	7	5	6	6	16	37	22	25
Mag. ox. light	6	10	9	11	48	59	43	48
Mag. trisil	100	100	100	100	100	100	100	100
Talc	81	85	77	74	27	27	29	29
Titan. diox.	58	49	45	48	88	88	92	96
Zinc. oxide	10	10	11	12	0	0	4	1

(iii) The fact that autoclaving significantly increases the percentage losses from both 0,01 % BZC and 0,01 % cetrimide solutions in the presence of kieselguhr but not from 0,1 % cetalkonium chloride nor 0,1 % CPC solutions, is a phenomenon for which an explanation can be proposed.

If the heat treatment frees a certain number of previously blocked adsorption sites per unit mass of kieselguhr, the powder would be able to adsorb an additional quantity of QAC which would be of approximately equal mass for the four preservatives under test, due to similar molecular masses. This mass of preservative could be a significant proportion of the total mass of QAC originally in the solution in the case of 0,01 % solutions but it could be an insignificant proportion of the total mass in the case of 0,1 % solution. Thus, autoclaving could be expected to produce a noticeable increase in percentage loss onto kieselguhr from 0,01 % solution but not from 0,1 % solutions, and this is, in fact, what was observed.

5.1.5 An investigation into the extent of adsorption of BZC in eyedrops onto the surface of glass containers

Whilst developing the method used for the assay of QAC solutions, it was noticed that a single transfer of a 0,001 % BZC solution to a glass container could produce an appreciable reduction in BZC concentration due to adsorption onto the glass surface. This observation gave rise to some concern that multiple transfers of eyedrops, containing 0,01 % BZC, to various containers, during their preparation and packaging, could produce a significant reduction in BZC content and therefore a reduction in its ability to inactivate chance microbial contaminants of the eyedrops.

The following series of tests, designed to simulate the

packing of eyedrops in a bottle, the repacking of eyedrops in a second bottle, the preparation of 100 ml of eyedrops with and without filtration and the preparation of 20 ml of eyedrops with filtration, was therefore performed.

(i) A 0,01 % BZC solution was prepared and assayed by the fully modified method of Fokkens and Buurman described in Section 2.4.2.4.

(ii) A neutral glass eye drop bottle of approximately 15 ml total capacity was rinsed with distilled water, then acetone and allowed to dry. An equilibrated pipette was used to transfer 10 ml of the 0,01 % BZC solution to the bottle. The solution was swirled in the bottle, without a closure, for a few minutes in order to bring the solution into contact with the entire inner surface of the bottle for a sufficient length of time to allow adsorption of BZC to the glass surface to take place. The solution was then assayed.

(iii) Proceeded as for (ii) but the solution was transferred into a second clean, dry eyedrop bottle and swirled before analysis.

(iv) About 80 ml of the 0,01 % BZC solution was poured into a 250 ml beaker and the contents swirled. (This procedure corresponded to dissolving eyedrop solutes in the aqueous solvent). This solution was transferred to a 100 ml measuring cylinder and made up to the 100 ml mark with more of the 0,01 % BZC solution. The solution was transferred back to the 250 ml beaker and an all glass 10 ml syringe was used to pack 10 ml of the solution into a clean, dry eyedrop bottle. The contents of this bottle was assayed as before.

(v) Proceeded as described in point (iv) but the solution was passed through a 19 mm, no 3 porosity, sintered glass filter funnel before 10 ml was packed

in an eye drop bottle, as before, and its contents assayed.

(vi) Proceeded as described in point (v) but only 20 ml of simulated eye drops was prepared.

(vii) Proceeded as described in point (v) but all glassware used and the eye drop bottle were equilibrated three times with 0,01 % BZC solution before use.

Results:

	Treatment	Percentage loss of BZC
(ii)	Transfer to one bottle	0
(iii)	Transfer to two bottles	6
(iv)	100 ml simulated eye drop prep. without filtration	3
(v)	100 ml simulated eye drop prep. with filtration	3
(vi)	20 ml simulated eye drop prep. with filtration	6
(vii)	As for (v) but with equilibration	0

The results reveal that, although the loss of BZC from 0,01 % solution during preparation of eye drops is minimal, the proportion lost tends to increase if small volumes of eye drops are prepared, or if the eye drops are repacked or transferred to a number of different containers. It would therefore be wise to avoid repeated transfer of 0,01 % BZC solution to different containers and to avoid putting small volumes of such solutions in large containers of considerable surface area. If such practices are unavoidable, however, the use of equilibrated mixing and measuring vessels would prevent loss of BZC. In large scale production of solutions containing QACs, it should be remembered that passing a dilute QAC solution through a pipeline could result in the first volume of solution to emerge being devoid of QAC.

TABLE 8. MEAN DEATH TIMES AT 25 °C OF ESCHERICHIA (SATCC ESC25) FOR SUSPENSIONS CONTAINING 5 g POWDER/100 ml 0,01 % BZC SOLUTION AND ACTUAL AND EXPECTED MEAN DEATH TIMES FOR THEIR CORRESPONDING SUPERNATANT LIQUIDS

POWDER	% loss from 0,01 % BZC	% BZC in Supernatant Liquid x 10^{-3}	% BZC in Supernatant Liquid after dilution with E.coli Inoculum x 10^{-3}	Logarithm of BZC concentration x 10^{-3}	Logarithm of Expected Mean Death Time	Expected Mean Death Time (min)	Actual Mean Death Time for Supernatant Liquid (min)	Actual Mean Death Time for Suspension (min)	pH
Aerosil (1 g/100 ml)	20	8,0	7,3	0,862	1,152	14	16	3,3	6,3
Bism. carb.	12	8,8	8,0	0,903	1,021	10,5	5,4	1,9	8,8
Calc. carb. praecip.	5	9,5	8,6	0,936	0,916	8,2	3,6	2,2	9,8
Calamine	6	9,4	8,5	0,932	0,929	8,5	5,2	no growth	8,1
Kaolin, light	100	0	0			indefinite	>24h	>40h	7,1
Kaolin, heavy	100	0	0			indefinite	>24h	>40h	4,8
Kieselguhr	48	5,2	4,7	0,675	1,747	56	7,8	5,6	7,7
Mag. carb. light	5	9,5	8,6	0,936	0,916	8,2	7,2	1,4	9,8
Mag. carb. heavy	7	9,3	8,5	0,927	0,945	8,8	6,6	2,0	9,6
Mag. oxide light	6	9,4	8,5	0,932	0,929	8,5	10,5	1,8	10,1
Mag. trisilicate	100	0	0			indefinite	>24h	>40h	10,0
Starch	100	0	0			indefinite	>24h	>40h	6,1
Talc	81	1,9	1,7	0,237	3,141	1384	>24h	84	8,3
Titan. dioxide	58	4,2	3,8	0,582	2,043	110	18,5	4,2	7,0
Zinc oxide	10	9,0	8,2	0,913	0,989	9,7	10,2	no growth	7,1
0,01 % BZC	0	10,0	9,1	0,959	0,843	7,0	-	-	6,8

5.2 MICROBIOLOGICAL ASSESSMENT OF THE SIGNIFICANCE OF A SERIES OF PRESERVATIVE/POWDER INTERACTIONS AS DETECTED BY SPECTROPHOTOMETRIC METHODS

5.2.1 Tests on BZC/powder systems

The reasons for the testing of the preservative/powder systems by microbiological methods and the reasons for testing both supernatant liquids and complete suspensions have been discussed in Section 2.5.1.1.

After preparation of a standard curve showing variation in the logarithm of mean death time (MDT) of Escherichia coli (SATCC Esc 25), with variation in the logarithm of concentration of BZC, MDTs for 0,01 % BZC/powder supernatant liquids and whole suspensions were determined according to the procedure described in Section 2.5.2.

The results of these tests, together with the expected MDT, calculated from the co-ordinates of the standard curve, and the pH of each system are shown in Table 8.

5.2.2 Discussion of results

Table 8 reveals that in all systems where adsorption of BZC onto the powder was less than 100 % the MDT of the suspension was considerably less than that of the supernatant liquid, i.e., suspensions showed far greater activity against the test organism than their supernatant liquids. Possible general explanations of this phenomenon will be discussed in Section 5.2.3.

The MDTs of BZC/powder supernatant liquids should be expected to conform closely to the times calculated from the standard curve if it were not for the following factors which could influence the bactericidal efficacy of BZC:

(i) Change in pH. Increase in pH generally increases the activity of BZC, while decrease in pH decreases it.

(ii) According to Ridenour and Armbruster (1948), the

presence of cations in the solution can have an inactivating effect on BZC solutions.

(iii) Microbially toxic substances or ions extracted from the suspended powder could potentiate the antimicrobial activity of BZC.

Thus, the MDT of the Aerosil supernatant fluid is only slightly greater than the expected value, a phenomenon which is probably explained by the slight decrease in pH with respect to that of the 0,01 % BZC solution. Aerosil, being a pure form of silicon dioxide, is unlikely to increase the ionic content of the fluid significantly and the observed MDT is close to the expected value. Significantly though, the MDT of the suspension is very much less than that of the supernatant liquid.

The fact that the MDT of the bismuth carbonate supernatant liquid is about half of the expected value can be explained by the increase in pH with respect to that of 0,01 % BZC solution and to a lesser extent by the presence of a low concentration of bismuth ions, which could be slightly toxic to bacteria, since bismuth salts are known to have a treponemicidal effect and may, therefore, have a tendency to act against bacteria as well.

The MDT of 3,6 min rather than the expected 8,2 min for the calcium carbonate supernatant liquid could be explained by the appreciable rise in pH. It seems that calcium ions, albeit in low concentration, do not reduce the activity of BZC to a noticeable extent.

In the case of calamine the MDT of the supernatant liquid is somewhat lower than the expected value, possibly due to the rise in pH of the suspension but it is significant that, when 1 ml aliquots of the test suspension were inoculated into 10 ml volumes of Lubrol indicator broth, no growth was obtained in any of the tubes, even those corresponding to the shortest exposure time ($\frac{1}{2}$ min). Furthermore, when these

tubes of culture medium, plus calamine/BZC suspension, were inoculated directly with an E. coli culture, growth was still not observed after two days incubation at 32 °C. Apparently, a calamine/BZC suspension has the ability to inhibit the growth of E. coli even when diluted with ten times its volume of a culture medium containing more than sufficient Lubrol W to inactivate the BZC present. The fact that the zinc oxide suspension behaved in the same way seems to indicate that this property is in some way related to the presence of zinc in the suspended particles. In spite of this fact, the corresponding supernatant liquids are no more inhibitory to E. coli than would be expected from a consideration of their BZC concentrations and their pH values.

Both kaolin powders, magnesium trisilicate and starch, according to spectrophotometric determinations, removed 100 % of the BZC from 0,1 % solutions, and the microbiological tests confirm these observations since the relevant test suspensions and supernatant liquids were not able to inactivate E. coli inocula in 40 h and 25 h respectively.

In the case of kieselguhr, in which case the actual MDT is 7,8 min instead of the expected 56 min the only explanation of this behaviour that can be offered is to assume that a selective adsorption of the less active components of the BZC mixture takes place. This explanation seems unsatisfactory, however, as the MDT of the supernatant liquid is very close to that of 0,01 % BZC solution. There is a pH rise which would tend to reduce the MDT but not to the extent shown. The release of toxic material that could potentiate the activity of the remaining BZC also seems unlikely.

The fact that the supernatant liquids from the magnesium carbonate suspensions produce MDTs only slightly below the

expected values, in spite of significant increases in pH values, is probably due to the presence of a low concentration of magnesium ions, which would tend to reduce the antimicrobial activity of BZC and, therefore, increase the MDT. In the case of light magnesium oxide, the fact that the actual MDT slightly exceeds the expected value is probably also a result of the opposing effects of raised pH and magnesium ions on the antimicrobial activity of BZC.

The expected MDT for the supernatant fluid of the talc suspension was obtained by extrapolation of the standard curve into a region where the relationship is probably no longer linear because of the very low concentration of BZC in this solution. In fact, at this concentration BZC is probably no longer able to kill E. coli as is indicated by the lack of a result for the supernatant fluid MDT.

The titanium dioxide/BZC supernatant liquid had a MDT which was much less than the expected value. Possibly, this result could be due to the release of a slightly toxic substance or ion, contributed by the powder, potentiating the effect of the BZC in the solution, since the pH of the suspension is not significantly higher than that of the 0,01 % BZC solution.

The MDT of the zinc oxide/BZC supernatant fluid conforms closely to the expected value, a phenomenon which makes the strongly antibacterial activity of the suspension even more difficult to understand.

5.2.3 Possible mechanisms of the antibacterial activity of BZC/powder suspensions

Bean and Dempsey (1971) have compared the bactericidal activity of kaolin/BZC suspensions, kaolin/BZC supernatant liquids and aqueous BZC solutions in solutions of the same BZC concentration in the aqueous phase. They found that,

for very low BZC concentrations (0,00375 %), the bactericidal activity decreased as follows: aqueous solution > suspension > supernatant liquid. For somewhat more concentrated solutions the sequence was: suspension > aqueous solution > supernatant liquid.

They observed that, for a fixed BZC concentration in the aqueous phase, increase in amount of kaolin in the suspension produced a reduction in antimicrobial activity of the supernatant liquid but an increase in the antimicrobial activity of the suspension as a whole. They ascribed the reduction in activity of the supernatant liquid to the inactivating effect of inorganic cations displaced into the solution by more BZC being adsorbed onto the additional kaolin by a cation exchange mechanism. They stated further that the fact that suspensions had a greater activity than supernatant fluids, for a fixed aqueous phase BZC concentration, could be attributed to release of some of the adsorbed QAC from the suspended solid, but they went on to suggest that there may have been a direct contribution to activity by the adsorbed phase of the BZC, quite apart from any activity in the aqueous phase.

In Table 8, for any particular powder the figures in the columns headed "Expected Mean Death Time", "Actual Mean Death Time for Supernatant Liquid" and "Actual Mean Death Time for Suspension" give an indication of the activity of BZC, at a fixed aqueous phase concentration, in simple aqueous solution, in the supernatant liquid and in the suspension. It can be seen, therefore, that for most systems the order of decreasing bactericidal activity, is not, suspension > aqueous solution > supernatant liquid, as found by Bean and Dempsey for kaolin, but rather, suspension > supernatant liquid > aqueous solution. This phenomenon is not surprising in view of the high pH of most of the supernatant fluids relative to that of aqueous solutions

of BZC, a situation which would enhance their activity. Non identical pH values of the systems therefore, make direct comparison of the activities of supernatant liquids or suspensions with those of aqueous solutions impractical. However, a comparison of the activities of suspensions and supernatant liquids is quite possible, as the pH and ionic content of the aqueous phases must be the same in each case.

It is of considerable interest, therefore, to note that, in each case where it was possible to measure mean death times, the activity of the suspension was much greater than that of the supernatant liquid. While the explanation of Bean and Dempsey, that supernatant activity is reduced by the displacement of cations into the solution, is no doubt part of the explanation in this case, the great difference in activity between the suspensions and the aqueous BZC solutions of the same aqueous phase concentration, even in systems where the pH is low, seems to indicate that there are further reasons why BZC is so active in suspensions. Two complementory mechanisms will be proposed which are in fact attempts to explain in more detail Bean and Dempsey's inference that the adsorbed phase of BZC may make a direct contribution to the bactericidal activity of suspensions.

(i) In all cases where complete adsorption of BZC took place, the resultant suspension was devoid of bactericidal activity (see light and heavy kaolin, magnesium trisilicate and starch in Table 8). In all cases where adsorption of BZC was partial, the antibacterial activity of the suspension was far greater than that of the supernatant liquid.

Batuyios and Brecht (1957) have shown, in the case of BZC/talc system, that approximately 5 mg BZC/g of talc is irreversibly bound. Possibly, this corresponds to a tenaciously bound monolayer of BZC

the molecules of which would not be available for action against bacteria. It is probable that any subsequent layers of adsorbed BZC are not as tightly bound and are, in fact, in equilibrium with the BZC in the surrounding aqueous liquid. This would seem to be the case, as Batuyios and Brecht have also shown that such layers can be readily eluted by washing with water.

Thus, in systems where adsorption is complete, no more than a monolayer of BZC would exist on the surface of the powder and, being firmly bound, it would not be available to exert an antibacterial effect, even on co-adsorbed bacteria. In systems where adsorbtion of BZC from the solution is incomplete, there would be additional, loosely bound, layers of adsorbed BZC. Bacteria adsorbed on the powder would come into an area of relatively high concentration of BZC at the particle surface, which, being loosely bound, would be available to exert a rapid bactericidal effect on the bacteria.

(ii) It has been argued in Section 5.1.2 that the QACs with the highest molecular mass alkyl groups in the hydrophobic portion of the molecule are probably those which are the most strongly adsorbed by powders. In similar vein the BZC homologues with the highest molecular mass are presumed to be adsorbed to powders most readily. In those cases where adsorption is appreciable the adsorbed fraction of the BZC would tend to include preferentially such homologues in the mixture which are most active against bacteria, leaving the less active lower homologues in solution in the aqueous phase. In fact, a situation could arise in which the firmly bound monolayer consists largely of the highest homologues,

(say C 16 to C 18) which are not the most active, the loosely bound additional adsorbed layers consist largely of highly active intermediate homologues (say C 12 to C 16) and the fraction in simple solution in the aqueous phase consists largely of the lower, least active, homologues (say C 8 to C 12). Under these circumstances the suspension could be expected to have a very much greater antibacterial activity than the supernatant liquid, as it is likely that most bacteria are adsorbed onto the surfaces of most powders and therefore would enter the area of highest BZC activity.

Add to the above effects the diminution in antibacterial activity due to the release of cations into the solution, a phenomenon which takes place with a number of powders, and the large difference in antibacterial activity between whole suspensions and their isolated supernatant liquids becomes less enigmatic.

The observation that suspensions of powders in BZC solutions invariably have a much greater bactericidal efficacy than the supernatant liquid or the initial BZC solution, provided that adsorption of the BZC is not complete, could have a practical application.

It could be advantageous for formulators, wishing to preserve a suspension, to choose a QAC preservative which is appreciably adsorbed by the powder in suspension because of the enhanced activity of the preservative under such conditions.

SUMMARY.

Spectrophotometric methods for the determination of a number of preservative solutions, including a colorimetric method suitable for the determination of organomercurials, are presented.

The development of a modified colorimetric method for the determination of quaternary ammonium compounds in dilute solution and the development of techniques for the manipulation of dilute solutions of such compounds, without loss caused by adsorption onto containers, are described in detail.

The extent of interaction between aqueous suspensions of fifteen powders in common use in pharmaceutical and cosmetic formulations, and solutions of fourteen preservatives has been determined at 25 °C.

The effect of separately heating four quaternary ammonium compound and two organomercurial preservative solutions, at 115 °C for 30 min, in the presence of suspensions of the fifteen powders, has been assessed in a move to determine extent of inactivation.

The effect of time on the interactions between certain powders and 0,002 % m/v phenylmercuric nitrate solution has been determined at 25 °C. Starch is the only powder whose interaction with phenylmercuric nitrate solution takes place over a prolonged period of time.

The fact that an increase in the length of the aliphatic hydrophobic group on a quaternary ammonium compound facilitates adsorption by most powders is demonstrated.

In an attempt to elucidate an apparent anomaly in adsorption

of benzalkonium chloride by calamine, an adsorption isotherm is presented and certain deductions made.

The results of an investigation of the extent of adsorption of benzalkonium chloride onto glass containers and equipment used in the preparation of eye drops preserved with this substance are presented.

An attempt is made to confirm, by a microbiological method, the extent of the interactions between 0,01 % m/v benzalkonium chloride solution and fifteen selected powders. The observation that complete suspensions have a very much greater bactericidal activity than the supernatant liquids from the same suspensions and benzalkonium solutions of equivalent aqueous phase concentration to the supernatant leads to a discussion of possible mechanisms of this effect.

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