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A MANOMETRIC EVALUATION OF BACTERIOSTATIC ACTIVITY

by

EUNICE RUTH BONOW

A thesis submitted in partial fulfilment
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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UNIVERSITY OF WISCONSIN

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INTRODUCTION

In this study an attempt was made to develop a method for the determination of antibacterial properties of certain pharmaceuticals under conditions which simulate as nearly as possible the conditions under which they are used and secondly to apply this method to a bacterial culture in a synthetic medium to obtain information regarding factors which control the efficacy of the antibacterial agents.

It was the intention of the present investigation to determine whether any correlation could be developed between the thermodynamic activity of the antibacterial agent and its physiological activity. The thermodynamic activity of the antibacterial agent can be varied, for example, by the addition of alcohol or the alteration of the pH of the medium.

Because antibacterial properties are possessed by a diverse group of chemical compounds, the successful evaluation of antibacterial agents has been the object of many investigations. The method of the Food and Drug Administration (1) is now used almost exclusively in the United States for the evaluation of the bactericidal agents, however this method can not be employed for the evaluation of the many chemotherapeutic agents as they primarily exert a bacteriostatic action

and secondly, they are not related to phenol. A number of methods have been developed for the measurement of bacteriostatic activity as the agar-streak method (2,3), the agar cup method (1,4,5,6,7) and the turbidimetric method (8,9,10). The inhibition of growth and multiplication of the test organism in the presence of the antibacterial agent is measured.

It has been recognized for some time that a new method is needed for the evaluation of antibacterial agents in which the conditions of the test simulate as nearly as possible the conditions of the animal body when the agent is added. Bronfenbrenner, Hershey and Doubly (11, 12) introduced a method in which the effect of the germicide on the oxygen consumption of adult mouse liver cells and on a suspension of Escherichia coli was measured and compared. Bronfenbrenner and Hershey (13) had previously observed that when E. coli was grown under favorable environmental conditions "the oxygen uptake of a culture was directly proportional to the volume of cells determined nephelometrically, as well as to the amount of bacterial nitrogen at all stages of growth". Greig and Hoogerheide (14) extended the study to include several other organisms, namely Staphylococcus aureus, Proteus vulgaris, Pseudomonas fluorescens and one strain of yeast Willia anomala and noted "that the rate of oxygen consumption is

proportional to bacterial content determined nephelometrically".

For this study a rapid and reproducible method of measuring physiological activity was desired. An inhibition of metabolic activity as measured by an inhibition of oxygen uptake of the bacterial suspension has been suggested by Ely (15) as a method for the evaluation of antibacterial agents. He observed a correlation between the inhibition of oxygen uptake and the number of organisms killed. In this study the antibacterial activity was measured on the basis of the inhibition of total oxygen consumption of a suspension of E. coli in a glucose-ammonium salt medium.

A survey of the literature reveals that the effect of various types of antibacterial agents on the oxygen consumption by several different microorganisms has been studied in an attempt to elucidate the mode of action of these drugs on the bacterial cells and tissue cells and also to quantitatively evaluate them rather than the use of the F. D. A. phenol coefficient technique.

The Action of Drugs on Microorganisms

One class of compounds possessing antibacterial activity that has been studied rather extensively is the sulfonamide class. Sevag and Shelburn (16) studied the effect of sulfonamides on the respiration of Streptococcus pyogenes and pneumococcus Type I and observed that "under conditions favorable for growth the inhibition of respiration results in the immediate and proportional inhibition of growth". They suggested that this inhibition of respiration is due to the specific combination of compounds that are structurally similar to the whole or part of the coenzyme molecule with the protein portion of the respiratory enzyme. Sulfathiazole was found to inhibit the carboxylase activity of whole yeast and Staphylococcus aureus. Further study revealed that "sulfathiazole and sodium pyruvate compete for the active site of carboxylase. Presence or absence of inhibition depends on which of the two substances gets to the active site of the enzyme first". This inhibition could be reversed by the addition of para-aminobenzoic acid (17). The inhibitory effect of sulfanilamide on the aerobic and anaerobic respiration and growth of Escherichia coli in a synthetic medium containing glucose as the source of energy was observed by Clifton and Loewinger (18). The extent of

the inhibition was similar to that reported by Sevag and Shelburne(16). Clifton and Loewinger (18) suggest that sulfanilamide appears to inhibit respiration somewhere between the original dehydrogenase system and the final H-acceptor, possibly inhibiting a H-carrier system as well as the decarboxylase system. It is not evident whether the decreased rate of respiration is due to inhibition of growth or vice versa".

Fischer and Armstrong (19) studied the effect of sulfathiazole and propyl carbamate on the oxygen consumption and growth of E. coli and observed that concentrations "which are just sufficient to stop growth completely, lower the rate of oxygen consumption per unit of bacterial protoplasm to a value approximately 50 per cent of that seen in the absence of the inhibitor". This similarity indicates "in a general way, that the mechanism of action of these two inhibitors is similar". On further investigation Armstrong and Fischer (20) observed that when ammonium chloride is used as the sole source of nitrogen the peak of oxygen consumption is reached at the point of exhaustion of the ammonium chloride, the rate of oxygen consumption then fell to a level approximately 45 per cent of that existing immediately prior to the exhaustion of the ammonium chloride.

"It appears that fixation of ammonia, that is growth, requires approximately 55 per cent of the oxygen supplied by the growing cell". Clifton (21,22) noted that M/10 methyl urethan prevented the assimilation by Pseudomonas calcoaceticus during the oxidation of acetate. Similar results were observed when E. coli was used. He suggests that in certain concentrations cell poisons primarily inhibit synthesis and only secondarily respiration.

Not only have the effects of antibacterial agents on the total respiration of the bacterial cell been observed, but also the effects on isolated enzyme systems as the dehydrogenases which are concerned with the production of energy in the bacterial cell. Bach and Lambert (23) observed that a 5 per cent solution of ethyl urethane slightly inhibited the lactate, formate, glucose and butanol dehydrogenases and caused approximately a 50 per cent inhibition of the succinate, pyruvate and glutamate dehydrogenases, while phenyl-urethane had a greater inhibitory action on all the dehydrogenases. Cook, Haldane and Mapson (24) observed that phenylurethane did not inhibit the lactic and formic dehydrogenases of E. coli while Barron and Hastings (25) observed that urethane inhibited the dehydrogenases of the gonococcus.

Another class of compounds possessing antibacterial activity that has been studied to determine the effect on the respiration of the bacterial cell is the quaternary ammonium compounds. The effect of the quaternary ammonium compounds on the metabolism of Gram positive and Gram negative organisms has been studied by Miller, Baker and Harrison (26,27,28,29,30). In their search for a compound which would effectively limit the metabolism of microorganisms associated with human dental carries, they used washed suspensions of a lactobacillus, two strains of M. tetragenus, Staphylococcus albus, and an unidentified aerobic acid-producing Gram positive diplococcus and a Monilia buffered at the appropriate pH, and measured the effect of Zephiran (Alkyldimethylbenzylammonium chloride - Winthrop Stearns, Inc.) on the respiration and glycolysis in a Warburg respirometer. At a concentration of M/10,000 Zephiran completely and irreversibly inhibited one billion cells within five to ten minutes (30). They extended their studies to include additional quaternary ammonium compounds and additional micro-organisms and were able to show that the quaternary ammonium compounds studied effectively inhibited bacterial respiration at 1:3000 concentration, several were equally effective at 1:30,000 concentration. Maximum activity was found to occur in the alkaline pH

range. To determine whether this effect on respiration was limited to systems involved in the utilization of glucose, other substrates were substituted for glucose and it was found that "the oxidations by S. aureus of glucose, lactate, dl-alanine, succinate and glutamate were all inhibited to practically the same extent by 1:30,000 and 1:60,000 Zephiran" (26). Baker, Harrison and Miller (27) extended their study to germicidal properties of quaternary ammonium compounds and observed a fairly good correlation between germicidal action and inhibition of bacterial metabolism with the Gram positive organisms, however this correlation was less marked with the Gram negative organisms. "In general, the correlation of effectiveness against bacterial metabolism and the bactericidal properties... seems to be good enough to permit the assumption that the general relations found previously can be applied to the bactericidal effects with some degree of validity".

Ordal and Borg (31) included cetyl pyridinium chloride in their study of the effect of surface-active agents on the oxidation of lactate by molecular oxygen as compared with the anaerobic oxidation by methylene blue. They observed that cetyl pyridinium chloride inhibited the lactic dehydrogenase of both S. aureus and E. coli, however the lactic dehydrogenase of S. aureus was more

susceptible to its action. In order to compare the sensitivity of the aerobic and anaerobic oxidations of lactate by these organisms these authors determined the "critical" concentrations of cetyl pyridinium chloride, which they defined as the concentrations "inhibiting the reduction by methylene blue for sixty minutes in the tests for dehydrogenases, and the concentrations giving nearly complete inhibition after sixty minutes in tests for oxygen uptake". There was only a slight difference in the quantity of cetyl pyridinium chloride required to inhibit aerobic oxidation of lactate and the anaerobic oxidation by S. aureus. "Presumably, therefore, the cytochrome system normally concerned in the oxidation of lactate by oxygen in S. aureus possesses no special sensitivity to these surface active agents". The system concerned with the aerobic oxidation of lactate by E. coli is more sensitive to cetyl pyridinium chloride than is the anaerobic system. This suggests "that the cytochrome systems peculiar to E. coli are more sensitive to surface active agents than the other proteins concerned in the oxidations of the lactate, or are more available to the action of the agent". Roberts and Rahn (32) found that when E. coli was exposed to bactericidal concentrations of Zephiran or Ceepryn (Cetylpridinium chloride - Wm. S. Merrell Co.) practically all of the

dehydrogenase and oxidase is inactivated while catalase is only slightly effected.

Similar studies were conducted by Sevag and Ross (33) in the investigation of the mechanism of the inhibitory action of Zephiran on yeast cells. Zephiran inhibited both the aerobic and anaerobic oxidation of glucose by yeast, also the oxidation of para-phenylenediamine by the yeast cytochrome oxidase system. Addition of 1.8 per cent horse serum conteracted these inhibitions.

Knox, Auerbach, Zarundnaya and Sirtes (34) studied the effect of quaternary ammonium compounds on the intact cells of E. coli and several of its enzyme systems. As a measurement of the bactericidal amount of detergent they used the bactericidal ratio which was expressed as the micrograms of detergent per millegram of bacterial nitrogen or per millegram of protein nitrogen (for enzymes). These authors found a close correlation between the lethal action of the detergent with metabolic inhibition of E. coli by several quaternary ammonium compounds. "That half of the cells are dead and half of the glucose oxidation inhibited at the same amount of detergent has been found in all instances with many different batches of bacteria". The metabolic activity of the cell toward other substrates was not inhibited by a given amount of detergent to the same degree as glucose oxidation. "Several enzymes, the

reactions of which appeared to be inhibited in the cell by detergents, were therefore isolated to test their sensitivity to the detergents in the absence of cellular organization". The lactic acid oxidase, which appeared to be quite stable was selected for further study. It was prepared by grinding in a mill for three hours the stock suspension of E. coli, centrifuging this product and separating the supernatent liquid which contains the crude enzyme. This procedure indicates that although the bacterial cell is dead, the enzyme systems may continue to function. The lactic acid enzyme thus isolated differs from the lactic acid dehydrogenase which has been mentioned previously, in that it reacts directly with oxygen. The action of Zephiran on "glucose oxidation in the intact cell, viability and cell-free lactic acid oxidase" were parallel and dependent on the bactericidal ratio rather than the concentration of Zephiran. "This would tend to confirm the view that cationic detergents are bactericidal because the effect is on a specific enzyme system such as the lactic acid oxidase and not because they primarily alter the cell structure, since this enzyme still possesses, in the absence of cell structure, the intact cell's pattern of sensitivity to detergents".

The effect of antibiotics on bacterial respiration has also been studied in an effort to explain their mode of action. Chain, Duthie and Callow (35) determined the

effect of penicillin on the respiration of S. aureus and observed that penicillin "strongly inhibited and finally completely abolished" the respiration under favorable conditions for growth. They concluded that "penicillin appears to interfere with a metabolic function involved in the early stages of bacterial development".

Streptomycin has been shown to possess antibacterial action both in vitro and in vivo against the avian strain of Mycobacterium tuberculosis which is able to oxidize the higher fatty acids. Oginsky, Smith and Solotorovsky (36) showed that streptomycin "apparently inhibited the oxidation of the breakdown products of the fatty acids rather than the oxidation of the chain itself". They observed a similar inhibition of stearate oxidation by E. coli although "it is evident that the two organisms oxidized this substance somewhat differently".

Evaluation of Antibacterial Agents

It has been recognized for some time that a new method is needed for the evaluation of antibacterial agents in which the conditions of the test simulate as nearly as possible the conditions of the animal body when the agent is added. The determination of the inhibitory effect of the antibacterial agent on the metabolism of a microorganism is not new. Dreser (37) in 1917 and Pilcher and Sollmann (38) in 1929 studied the antiseptic activity of silver compounds by comparing their activity on the fermentation by yeasts with the action of a standard solution of silver nitrate. "The concentration of the silver compound which completely prevented carbon dioxide production was taken as the end point". This technique was applied by Peterson (39) to the study of mercury compounds, using mercuric chloride as the standard. He suggested that because of the ease of conducting this test, it should become widely accepted by technicians and clinicians. Several years later, Branhan (40) extended the work to a study of a number of antibacterial agents and found some difficulty in reproducing the previous work of Dreser, Pilcher and Sollmann and Peterson. She suggested several modifications, including the apparatus which would more accurately measure the carbon dioxide production by the yeasts. The end point of the reaction

was "expressed as the highest dilution which completely prevents gas formation by a mixture of 10 per cent yeast and 1 per cent cane sugar in one hour at 38°C." The results of this study indicate that "within the one hour period the degree of inhibition varied with the concentration of the antiseptic".

Bronfenbrenner, Hershey and Doubly (11,12)proposed a method in which the effect of the antibacterial agent on the oxygen consumption of adult mouse liver cells and E. coli suspensions was compared. It "consisted essentially in determining the concentration of disinfectant in the test fluid necessary to cause 50 per cent reduction in the rate of oxygen uptake, measured between the fifteenth and twentieth minutes after the beginning of the experiment". Using this method, these workers noted that "the per cent of inhibition of oxygen uptake observed at any time after the first few minutes is clearly a function of the concentration of the disinfectant but not of the duration of the exposure". In comparing the per cent of inhibition of oxygen uptake of bactericidal and bacteriostatic dilutions of mercuric chloride, merconyl, merthiolate, metaphen, hexyl resorcinol, and phenol on S. aureus and E. coli they noted that only a 10 per cent of oxygen uptake was effected by maximum bacteriostatic dilutions while effective bactericidal dilutions

effected a 80 per cent or more depression of oxygen uptake.

Ely (15) studied the relationship between the inhibition of oxygen consumption of a bacterial suspension by antibacterial substances and the subsequent ability of the bacterial suspension to initiate growth when subcultured. He observed that a concentration of merthiolate which produced an inhibition of respiration of 33 per cent killed only 7 per cent of the bacterial population where as a concentration which produced an inhibition of respiration of 61 per cent killed 43 per cent of the bacterial population. This suggests that merthiolate "affected the respiration of all of the organisms in the suspensions and killed first the weaker members of the population". They found that in order to achieve complete sterilization, 100 per cent inhibition of respiration was necessary. When using this method for the evaluation of the germicide in terms of the phenol coefficient, the effect of the germicide is compared with the effect of phenol on the oxygen consumption. "If comparable amounts of inhibition of respiration by different germicides indicate that comparable numbers of bacteria have been killed, then the phenol coefficients so determined should be accurate. However it is not certain at present that this is the case".

Greig and Hoogerheide (14) measured the inhibition of oxygen uptake by the germicidal solutions adjusted to pH 7.0 on Proteus vulgaris and S. aureus in a peptone-lactate broth at 30°C. They measured growth at the end of the fifth or sixth hour as turbidity by means of the photoelectric colorimeter. Their results indicated that the "oxygen uptake of growing cultures of bacteria was found to be directly proportional to bacterial content when the latter was determined nephelometrically". Since this relationship exists under favorable conditions, "the measurement of the rate of oxygen uptake constitutes a convenient method for the measurement of growth rate". In the presence of a germicide they observed that the "inhibition in the normal rate of increase of oxygen was found to be due to a corresponding inhibition of growth" (14).

PLAN OF STUDY

It has been observed that a correlation exists between the antibacterial activity and the physio-chemical properties of the antibacterial agent. Various types of chemical compounds have been investigated and two well known hypothesis have been proposed as a result of these studies. One is the theory of Overton (41) who related the drug activity to the lipoid/water distribution ratio and the other is that of Traube (42) who related the drug activity to the lowering of the surface tension of water.

In this study of the relation of antibacterial activity and the physical properties of the drug, the manometric technique as suggested by Greig and Hoogerheide (14,43) with some modifications has been used for measuring the bacteriostatic effect. A simple synthetic medium devoid of protein and containing glucose as the source of carbon was substituted for the peptone-lactate broth. Escherichia coli was selected as the test organism in preference to Proteus vulgaris and Staphylococcus aureus which they used. The temperature at which the determinations were made was increased to 38°C. The effect of the antibacterial agent has been expressed in terms of inhibition of oxygen consumption during the logarithmic phase of growth. The inhibition of oxygen consumption during this growth phase was

assumed to indicate a corresponding inhibition of growth as shown by Greig and Hoogerheide (14).

The data thus obtained was then related to the physical properties of the antibacterial agent to determine whether any correlation could be developed between the thermodynamic acitivity of the antibacterial agent and its physiological activity.

GENERAL PROCEDURE

The method used in the study of the bacteriostatic activity of ethyl alcohol, the parabens and chloramphenicol was a modification of the one described by Greig and Hoogerheide (14,43). It consisted essentially in the determination of the inhibition of oxygen consumption during the logarithmic phase of growth in the presence of the bacteriostatic agent.

Escherichia coli (American Type Culture Collection #8739) was used as the test organism. For these experiments the bacteria were grown on Difco Nutrient Agar in six ounce prescription bottles at 37°C. The bacterial suspension was prepared by washing off a twenty hour culture with sterile distilled water and adjusting the concentration of the suspension such that it would transmit 10 per cent incident light as measured in a Cenco colorimeter with a blue filter.

The oxygen consumption of the bacterial suspension during growth in a glucose-ammonium salt medium was measured in a Warburg respirometer. The synthetic glucose-ammonium salt medium was a modification of that used by Kohn and Harris (44) in their study of the effect of sulfonamides on the respiration of E. coli. It is prepared by dissolving 2 grams of glucose, 2 grams of monopotassium phosphate, 2 grams of diammonium hydrogen

phosphate, 4 grams of sodium chloride and 0.2 grams of magnesium sulfate in sufficient distilled water to make one liter. The pH of this medium, as measured with a Beckman Model "H" pH-meter, was adjusted to 7.2 with 10 per cent sodium hydroxide solution. This medium is then boiled for several minutes, filtered and tubed in 10 ml. quantities and sterilized at 15 pounds pressure for 20 minutes. The reaction vessels were thoroughly cleansed, rinsed with distilled water and dried in an oven. They were not sterilized as the duration of the experiment was relatively short. It was too short to permit foreign bacteria to become established and thus influence the manometric readings. Ely (15) observed that although absolute sterility was not maintained in the manometric evaluation of germicides, the number of foreign bacteria present was less than 1 per cent as determined by plate counts.

One-tenth ml. of the bacterial suspension and 2.9 ml. of a solution containing culture medium, buffer and antibacterial agent was placed on the floor of each reaction vessel, the central well of which contained 0.2 ml. of a 20 per cent solution of potassium hydroxide and a strip of filter paper for the absorption of carbon dioxide. Because of the fact that the ratio of culture medium, buffer and antibacterial agent was varied in the study of each antibacterial agent, the specific amounts

of each employed will be mentioned in the discussion of the individual antibacterial agents. A reaction vessel which contained only culture medium served as the thermobarometer. The reaction vessels were placed in a water bath at 38° C. and shaken for a period of two hours before the system was closed. Readings were taken at 15 minute intervals for a period of three to four hours.

ETHYL ALCOHOL

Introduction

A study of the effect of ethyl alcohol on the respiratory system of E. coli was included in this study as it was used as an agent to vary the physical properties of the parabens.

"Ethyl alcohol, either absolute or in 95 per cent strength is but an indifferent disinfectant, unless the material and organisms to be sterilized contain sufficient quantities of moisture to dilute the alcohol. The greatest disinfecting power is obtained in a strength of 70 per cent by weight" (45). Similar statements are found in other bacteriology text books, however no reference to the research supporting these conclusions is given. The original work suggesting the dilution of alcohol, within certain limits increased its germicidal properties was that of Epstein (46), who used several different organisms dried on silk threads and exposed them to various concentrations of ethyl alcohol. He observed that a 50 per cent solution was capable of killing bacteria in five to ten minutes. His study did not include the test organism of this study, E. coli. Minervini (47) extended the study to include E. coli and observed that the maximal germicidal effect was exhibited by 50 per cent and 70

per cent solutions. Utilizing the same technique, Harrington and Walker (48) extended the study to include the effects of alcohol on moist bacteria and observed "that dryness has a most important influence, for while the moist organisms are destroyed within five minutes by every strength above 50 per cent, the dried are affected by only 50 and 60 per cent and show a progressive resistance to the action of the stronger preparations". The bactericidal concentrations of ethyl alcohol have been extensively studied (49,50,51, 52,53).

Several investigators have studied the effect of highly diluted alcoholic solutions. Using the thread technique and recording the number of bacteria present, Stokvis (54) observed that a bacterial population of E. coli exposed to 6 per cent alcohol for 24 hours showed a decrease in number to about one-sixth of the original population. A bacterial population exposed to 4 per cent of alcohol showed a decrease after 24 hours, however an increase in numbers occurred after 48 and 72 hours exposure. Stokvis concluded that alcohol is a slow-acting poison. The influence of the incubation temperature on the effect of alcohol on E. coli was studied by Wirgin (55) who observed that E. coli in the presence of 8.5 per cent of alcohol-broth mixture, incubated at 37° C. for 24 hours showed no growth,

however lowering the temperature to 25°C. required 12 days to produce death, and at 18°C. 51 days.

Procedure

In this study the effect of dilutions of ethyl alcohol (1 to 5 per cent v/v) on the oxygen uptake of E. coli was studied using the manometric technique as described in the section on general procedure. The flask contents consisted of 1.9 ml. of a solution of equal parts of Mc Ilwaine buffer and the glucose-ammonium salt medium adjusted to a pH of 7.0 and 1.0 ml. of an ethyl alcohol solution of such a concentration that the percentage of alcohol per flask was as indicated.

Results and Discussion

A series of 15 determinations were made, the results are shown in Figures 1 and 2. The data indicates that an increase in the concentration of ethyl alcohol produces an increase in the inhibition of the oxygen uptake by the test organism, and an increase in the lag phase of growth. These results are in agreement with the observations of Dagley, Dawes and Morrison (56) who noted that 0.41 moles of ethyl alcohol inhibited the

growth of Aerobacter aerogenes by increasing the lag period. This effect could be removed if certain amino acids were supplied to the medium. On the basis of these observations they suggest that a possible mode of action of ethyl alcohol could be the retarding of the production of essential amino acids for growth and also a blocking of the conversion of one amino acid to another.

The data obtained in this study also indicates that the high dilutions of ethyl alcohol had very little inhibitory effect. Comstock (57) has suggested that "alcohol, just as it may serve in small quantities as an assimilable food for the human body, may serve to enrich the culture medium for microorganisms by yielding up readily oxygen for their catabolic requirements". Still (58) has shown that E. coli is able to oxidize ethyl alcohol to acetaldehyde. He has reported the preparation of a cell-free alcohol dehydrogenase from Bacterium coli commune which oxidizes ethyl alcohol through the cytochrome system to aldehyde.

THE EFFECT OF ETHYL ALCOHOL ON THE TOTAL OXYGEN CONSUMPTION OF *E. COLI*.

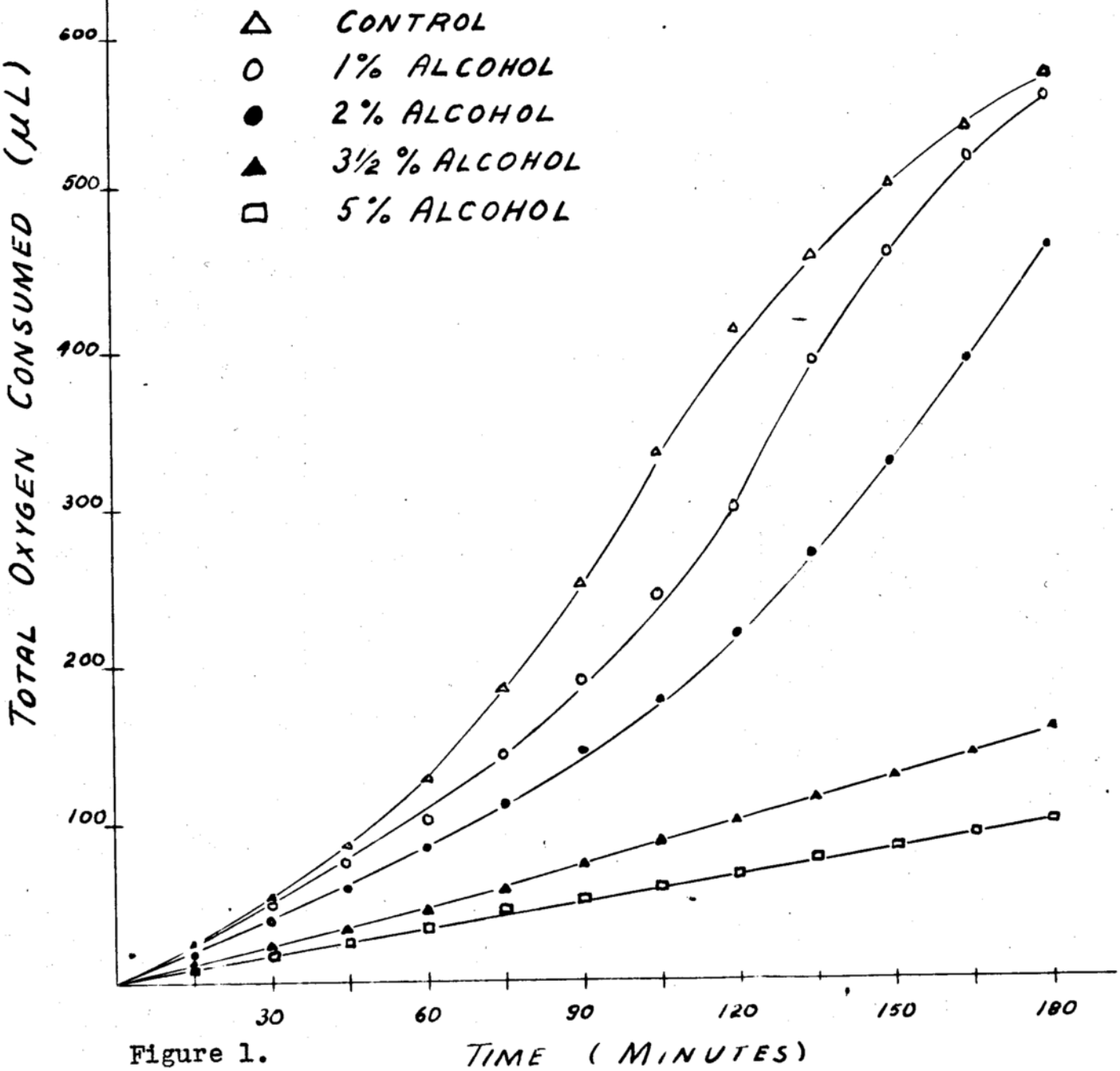
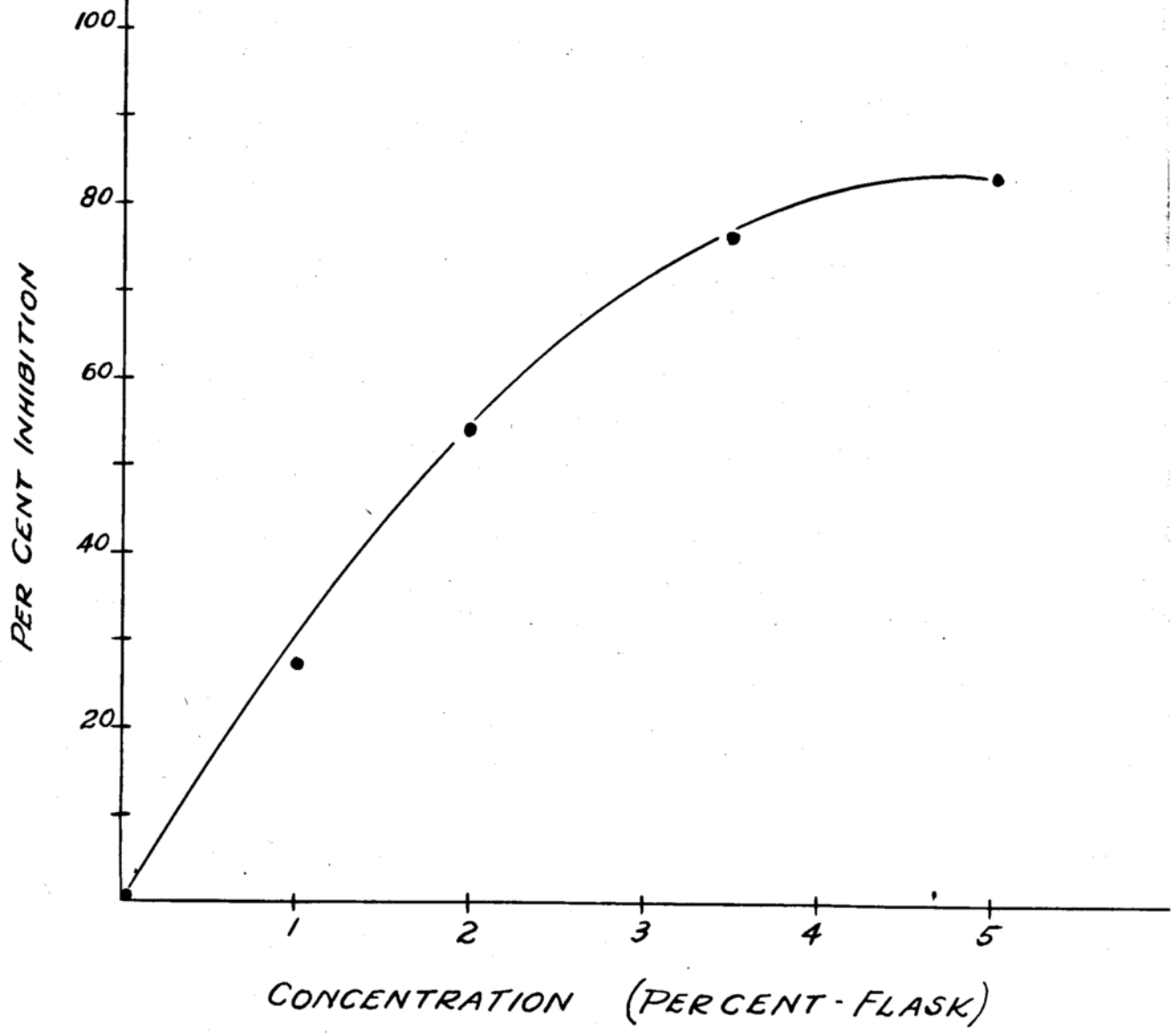


Figure 1.

Figure 2.

THE INHIBITION OF THE OXYGEN UPTAKE OF E. COLI AFTER 120 MINUTES EXPOSURE TO ALCOHOL.



THE PARABENS

Introduction

In 1929 Sabalitschka (59) reported a comparison of the antibacterial activity of the various esters of parahydroxybenzoic acid with that of phenol. The esters were found to be far more effective as antibacterial agents. The higher molecular weight of the alcohol used for the esterification, the greater was the antibacterial activity observed, thus suggesting that a correlation does exist between the physical properties of the drug and the antibacterial action.

Because of these previous investigations and because the physical and chemical character of these substances permitted ready and easy variation of their thermodynamic activities, they were chosen for this study. The thermodynamic activity of the parabens was varied by the addition of ethyl alcohol to the reaction system and the alteration of the pH of the system.

The Effect of the Parabens on the Total Oxygen Consumption of E. coli

Procedure

Because of the insolubility of the parabens in aqueous solution, the paraben solutions were prepared by dissolving the required amount of paraben in a solution containing equal quantities of glucose-ammonium salt medium and Mc Ilwaine phosphate buffer adjusted to pH of 7.0. The paraben series used in this study were Methyl Parasept, Ethyl Parasept, Propyl Parasept and Butyl Parasept supplied by the Heyden Chemical Co. of New York. Two and nine-tenths ml. of the paraben solution were placed on the floor of each reaction vessel. The phenol solution was prepared and used in a similar manner. The antibacterial effect of the parabens was determined by measuring the inhibition of growth, as indicated by the total oxygen uptake, in the presence of the paraben or phenol for the fifteen minute interval during which the peak of oxygen consumption was reached in the control.

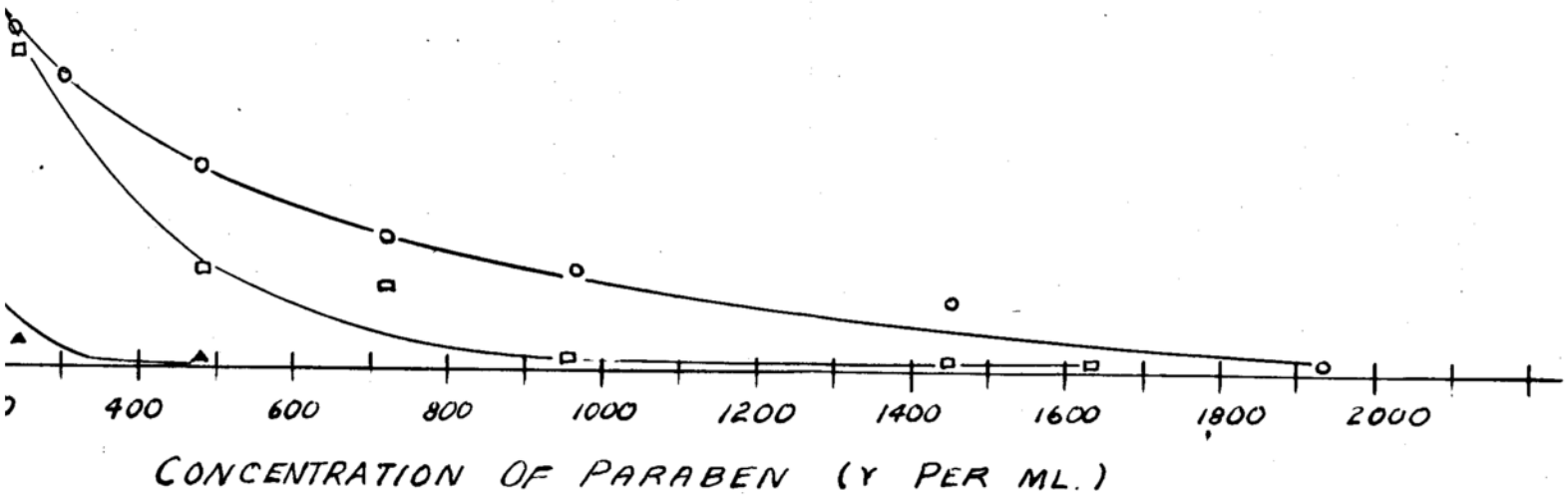
Results and Discussion

In this study it was observed that the antibacterial activity of the parabens increased with the length of the aliphatic chain. See Table I and Figure 3. This is in

agreement with the results found by other investigators when a homologous series was studied. Since the degree of insolubility is also directly proportional to the length of the side chain, it follows that the least soluble compound in the series is the most bacteriostatic. Tilley and Schaffer (60) observed that the bactericidal activity of a homologous series of primary, secondary and tertiary alcohols against Bact. typhosum was a function of the number of carbon atoms which they contain. Kamm (61) observed that the toxic action of the primary alcohols on the Paramecia exhibited a similar relationship.

The first theory to correlate the action of drugs on the cells to their solubility in fat was Overton (41) who found that the narcotic effect of chemically unreactive substances was related to their lipoid/water distribution ratio. In a study of the germicidal effect of benzoic acid, its hydroxy derivatives and their salts, Bayo (62) observed that the germicidal action was directly proportional to the lipoid/water distribution ratio. The determining factor appears to be the effect of the alkyl chain on the distribution coefficient. Thus the antibacterial activity is a function of the lipoid solubility and inversely proportional to the solubility in water.

THE EFFECT OF PARABENS
ON THE OXYGEN UPTAKE
OF E.COLI DURING THE
INTERVAL OF PEAK CONSUMPTION



The solubilities of the parabens in peanut oil and water are listed in Table I, in addition to the calculated distribution coefficients. The phenol coefficient was obtained by comparing the quantity of the paraben which produced 50 per cent inhibition of oxygen consumption during the 15 minute interval of peak oxygen consumption of the control culture of *E. coli* with the quantity of phenol which produced the same effect. The phenol coefficients and the distribution coefficients are plotted in Figure 4. There is a definite similarity in the two curves, both increasing rapidly with an increase in chain length.

Table I.

Comparison of the antibacterial activity of the parabens and their solubility

Compound	Solubility G./100 cc. water	Solubility G./100 cc. peanut oil	Distribution coefficient	Phenol coefficient
Methylparaben	0.25	0.5	2.0	1.4
Ethylparaben	0.17	1.0	5.9	2.1
Propylparaben	0.05	1.4	28.0	6.1
Butylparaben	0.02	5.0	250.0	26.0

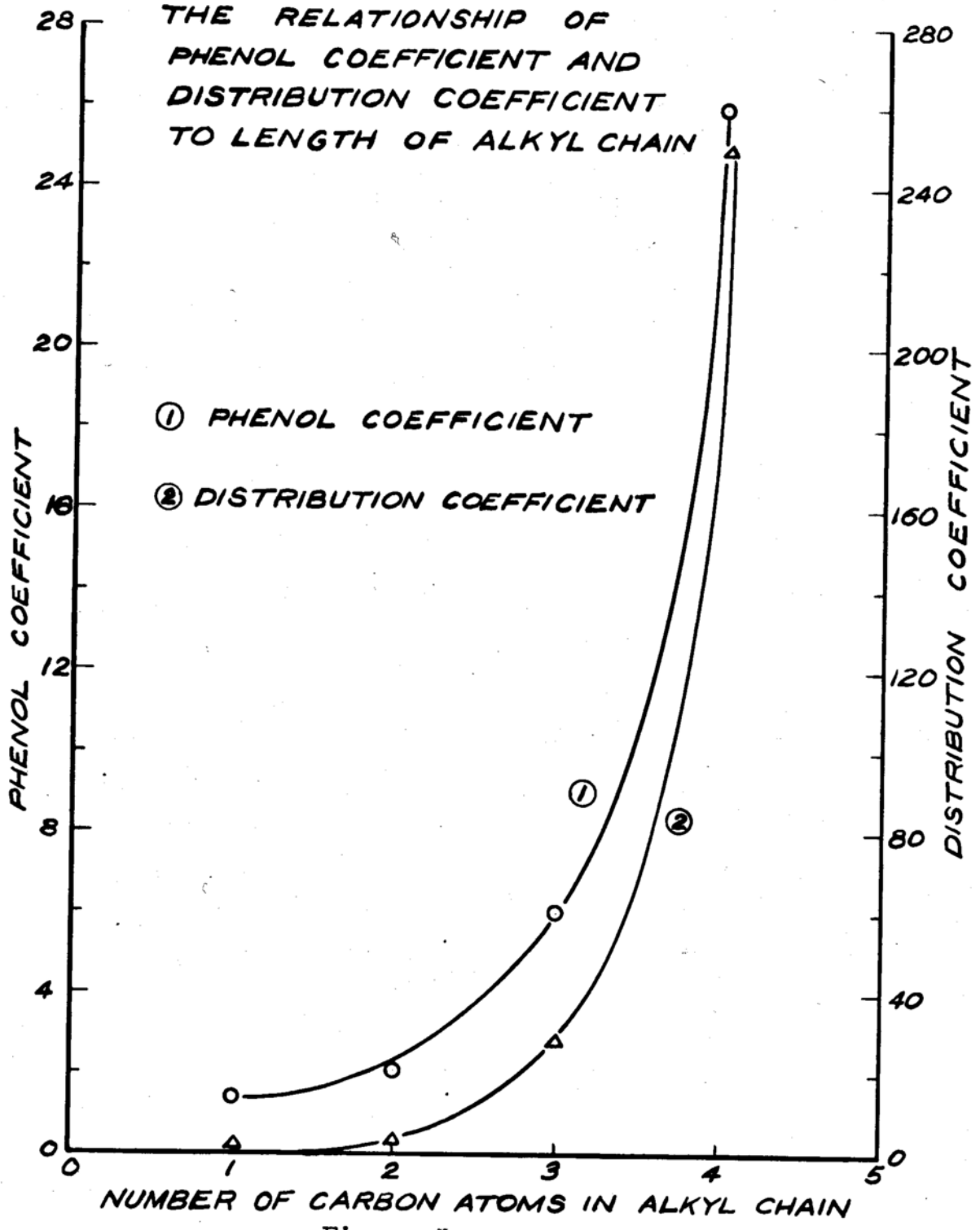

THE RELATIONSHIP OF
PHENOL COEFFICIENT AND
DISTRIBUTION COEFFICIENT
TO LENGTH OF ALKYL CHAIN
① PHENOL COEFFICIENT
② DISTRIBUTION COEFFICIENT
PHENOL COEFFICIENT
DISTRIBUTION COEFFICIENT
NUMBER OF CARBON ATOMS IN ALKYL CHAIN
0
4
8
12
16
20
24
28
40
80
120
160
200
240
280
1
2
3
4
5

Figure 4.

The Effect of Dilute Alcohol on the Antibacterial Activity of Methylparaben

Procedure

A stock alcoholic solution of Methylparaben was prepared and then diluted with sterile distilled water to the desired concentration of alcohol and the drug. One ml. of this methylparaben solution, 0.95 ml. of glucose-ammonium salt medium (pH 7.0) and 0.95 ml. of Mc Ilwaine buffer at pH 7.0 were used in each reaction vessel.

Results and Discussion

In the higher dilutions of methylparaben (165γ/ml. to 825γ/ml.) the presence of 1 to 5 per cent of ethyl alcohol increased the antibacterial action of the methylparaben, however the increase was not additative. The addition of a small quantity of alcohol increases the water solubility of the methylparaben and decreases the thermodynamic activity of the agent. The net result expected would be a decrease in the bacteriostatic activity attributed to the drug alone. The data (Tables II and III, and Figures 5,6, and 7) seem to bear this out qualitatively. In support of this observation, Wyss and Poe (63) observed "that alcohol-water mixtures presented no practical advantage over water as a

solvent for a comparative study of the germicidal value of the substituted benzoic acids".

Table II.

The Effect of Alcohol on the Antibacterial Activity of Methylparaben

Conc. of methyl-paraben (γ/ml.)	Conc. of alcohol %	Inhibition* Run No. 1	Run No. 2	Average
165	0	10	25	18
	1	49	56	53
	2	74	69	72
	3½	86	90	88
	5	87	89	88
330	0	59	60	59
	1	68	82	75
	2	78	87	82
	3½	83	96	95
	5	90	96	93
495	0	73	66	70
	1	89	83	86
	2	90	87	89
	3½	91	91	91
	5	95	96	95
660	0	88	92	90
	1	88	90	89
	2	89	98	93
	3½	89	92	91
	5	88	97	93
825	0	91	96	94
	1	90	97	94
	2	88	97	93
	3½	93	94	93
	5	97	95	96

Table II continued

Conc. of methyl-paraben (γ/ ml.)	Conc. of alcohol %	Inhibition* Run No. 1	Run No. 2	Average
990	0	94	90	92
	1	97	78	88
	2	100	86	93
	3½	98	90	94
	5	99	99	99
1165	0	96	98	97
	1	92	91	91
	2	90	95	93
	3½	95	96	96
	5	99	100	99

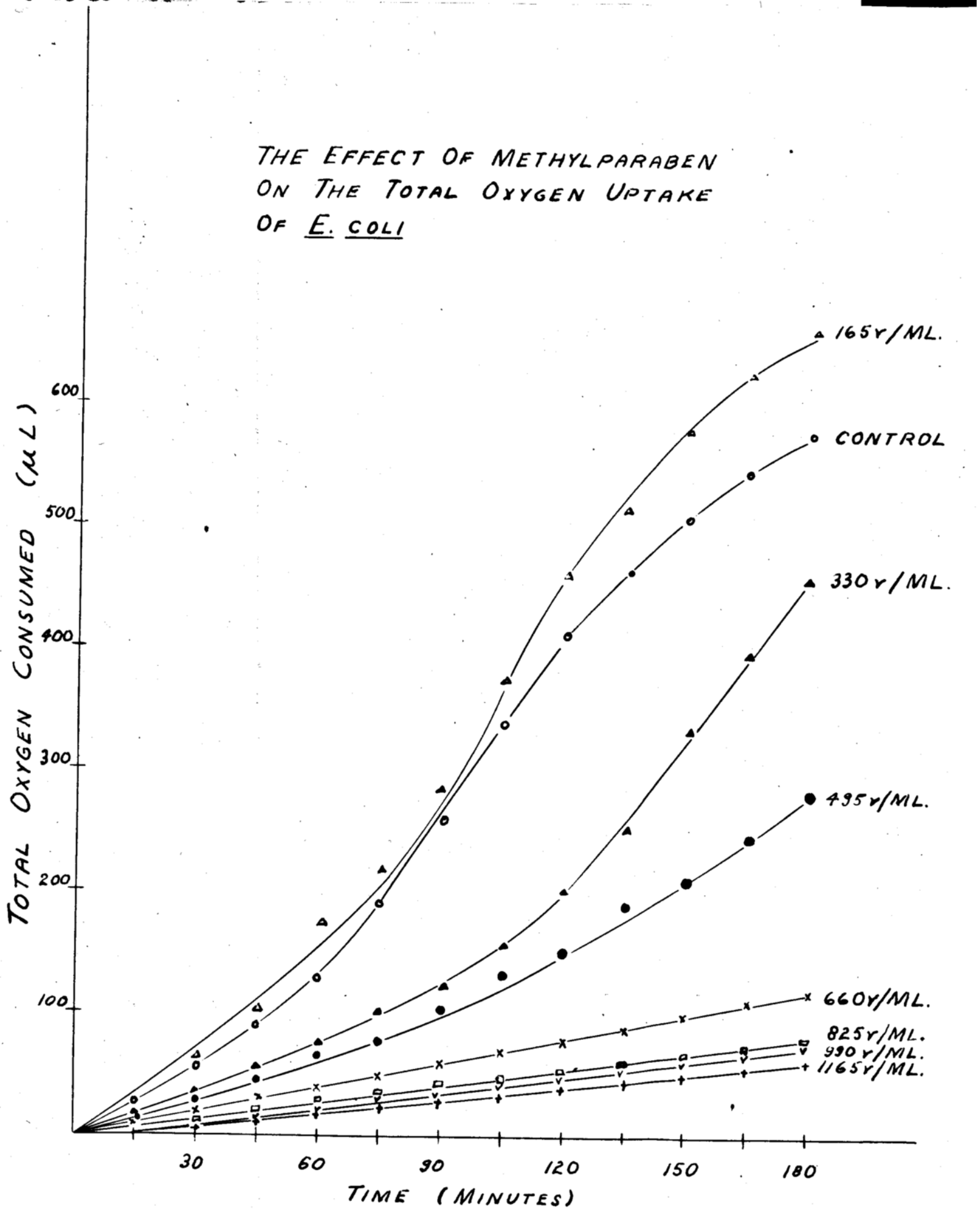
*Inhibition = the per cent of total oxygen consumed during the fifteen minute interval showing the peak of oxygen consumption in the control culture of E. coli in the absence of alcohol and methyl-paraben.

Table III.

The Effect of Ethyl Alcohol on the Total Oxygen Consumption of E. Coli

	Inhibition*			
	Concentration of ethyl alcohol			
Run No.	1%	2%	3½%	5%
1.	49	42	75	81
2.	30	51	71	81
3.	34	35	74	81
4.	21	59	77	89
5.	46	52	83	83
6.	30	53	86	86
7.	36	52	75	83
8.	28	46	71	83
9.	30	54	88	86
10.	38	56	75	84
11.	9	49	77	85
12.	45	56	82	88
13.	29	38	73	90
14.	0	10	63	80
15	18	48	66	78
ave.	29	47	76	84

* Expressed as per cent of total oxygen consumed during the fifteen minute interval showing the peak of oxygen consumption in the control.


THE EFFECT OF METHYLPARABEN
ON THE TOTAL OXYGEN UPTAKE
OF E. COLI
TOTAL OXYGEN CONSUMED (μL)
600
500
400
300
200
100
30
60
90
120
150
180
TIME (MINUTES)
165γ/ML.
CONTROL
330γ/ML.
495γ/ML.
660γ/ML.
825γ/ML.
990γ/ML.
1165γ/ML.

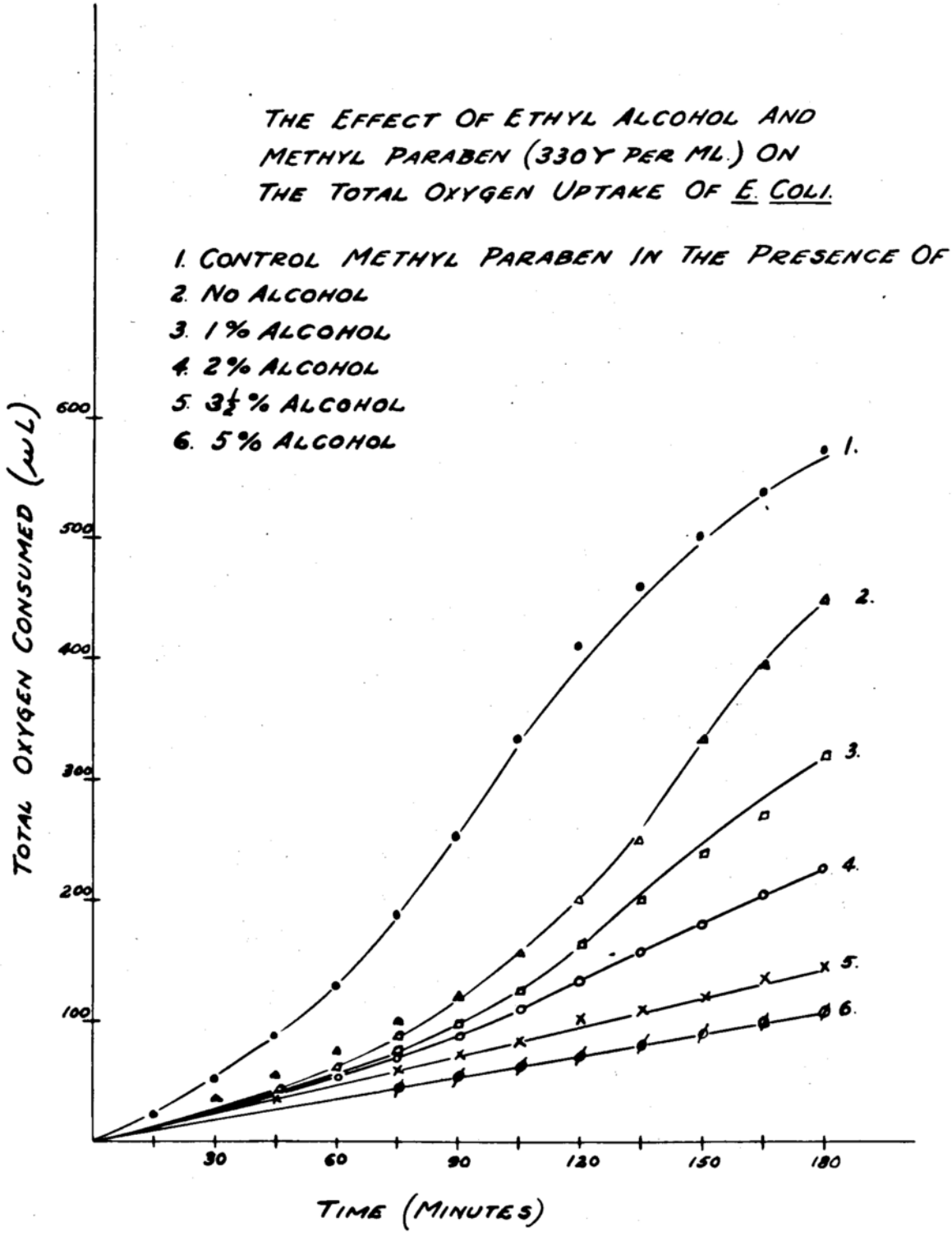

THE EFFECT OF ETHYL ALCOHOL AND METHYL PARABEN (330Y PER ML.) ON THE TOTAL OXYGEN UPTAKE OF E. COLI.
1. CONTROL METHYL PARABEN IN THE PRESENCE OF
2. NO ALCOHOL
3. 1% ALCOHOL
4. 2% ALCOHOL
5. 3½% ALCOHOL
6. 5% ALCOHOL
TOTAL OXYGEN CONSUMED (μL)
600
500
400
300
200
100
30
60
90
120
150
180
TIME (MINUTES)
1.
2.
3.
4.
5.
6.

Figure 6.

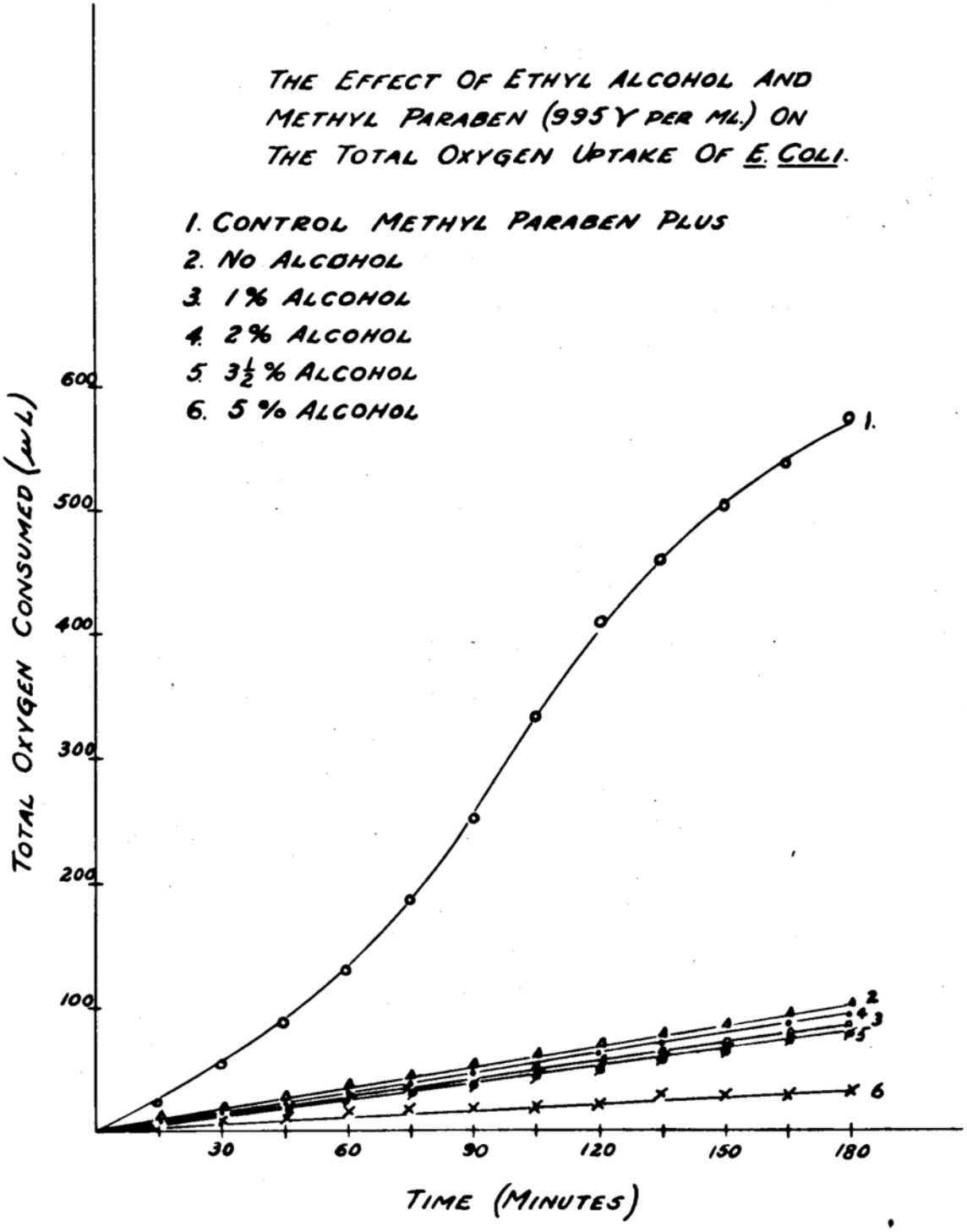

THE EFFECT OF ETHYL ALCOHOL AND METHYL PARABEN (995Y PER ML.) ON THE TOTAL OXYGEN UPTAKE OF E. COLI.
1. CONTROL METHYL PARABEN PLUS
2. NO ALCOHOL
3. 1% ALCOHOL
4. 2% ALCOHOL
5. 3½% ALCOHOL
6. 5% ALCOHOL
TOTAL OXYGEN CONSUMED (μL)
600
500
400
300
200
100
30
60
90
120
150
180
TIME (MINUTES)
1.
2
3
4
5
6

Figure 7.

The Effect of pH on the antibacterial activity of Methylparaben

Procedure

The two buffer systems used in this study were a phosphate buffer for the pH range of 7.0 to 8.25 and a carbonate buffer for the pH range of 8.5 to 9.0. The phosphate buffers were prepared from a 0.125M. solution of sodium pyrophosphate and a 0.1M. solution of phosphoric acid, while the carbonate buffers were prepared from a 0.1M. solution of sodium bicarbonate and a 0.1M. solution of sodium carbonate. Fifty ml. of the glucose-ammonium salt medium were mixed with 40 ml. of the buffer solutions adjusted so that the entire system was at the desired pH value. Because of the limited solubility of methylparaben in water, concentrated solutions of the paraben could not be added to the reaction vessel, thus the methylparaben was dissolved in the buffer-medium solution and 2.9 ml. of this solution used in the reaction vessel. The effect of pH and methylparaben was measured by the relative degree of inhibition of oxygen uptake produced, assuming the oxygen uptake of *E. coli* growing at pH 7 to represent 100 per cent growth.

In order to permit a limited correlation of the effect of pH on the bacteriostatic activity of methylparaben, the effect of pH alone was studied on the rate of total oxygen consumption of E. coli grown in a glucose-ammonium salt medium. Table IV and Figure 8 show this effect. Unfortunately, in this medium, the increase in pH caused a marked inhibition of oxygen uptake of E. coli and this inhibitory effect appeared to be less reproducible ab ve pH 8.0, the region primarily concerned in this study. These results are in agreement with the observations of Gale and Epps (64) who noted that E. coli would grow in a casein digest broth adjusted to pH values of 4.5 to 9.0.

The data recorded in Tables IV and V suggests that pH has an influence on the bacteriostatic activity of the methylparaben in the limited pH range studied. Initially this study was included in an attempt to correlate the antibacterial activity of methylparaben to the concentration of the unionized form, however in the pH range of 8.0 to 9.0 the growth of the organisms was markedly inhibited as indicated by the very slight volume of oxygen consumed. However, in the pH range studied, the

data reveals that as the pH of the medium approaches the pKa of the methylparaben, pKa = 8.47 (65) a sharp decrease in bacteriostatic activity is observed (Figure 9) thus suggesting that the unionized form is more active than the ionized form, however, these results are only qualitative and thus do not permit any mathematical analysis.

Table IV

The Bacteriostatic Effect of pH and Methylparaben on E. coli

pH	Growth* of E. coli in the presence of Methylparaben - Concentration (γ/ml.)					
	0	330	666	825	999	1330
7.0	100%			24%		
7.5	38%	28%	27%	26%	18%	12%
7.75	29%	44%	35%	21%	21%	18%
8.0	17%	25%	11%	20%	6%	4%
8.25	13%			16%		
8.5	6%	5%	6%		8%	3%

* Growth as measured by the per cent of total oxygen consumed during the logarithmic growth phase of the culture in relation to that consumed by the culture at pH 7 in the absence of methylparaben.

Table V

The Bacteriostatic Effect of Methylparaben on E. coli

pH	Growth* in the presence of 825 γ/ml. of methylparaben
7.0	18%
7.5	65%
7.75	83%
8.0	125%
8.25	116%
8.5	210%

*Growth in the presence of 825γ/ml. of methylparaben as compared to the growth in the absence of methylparaben at the indicated pH.

EFFECT OF THE pH OF THE MEDIUM ON THE TOTAL OXYGEN UPTAKE OF *E. COLI* AFTER 120 MINUTES INCUBATION.

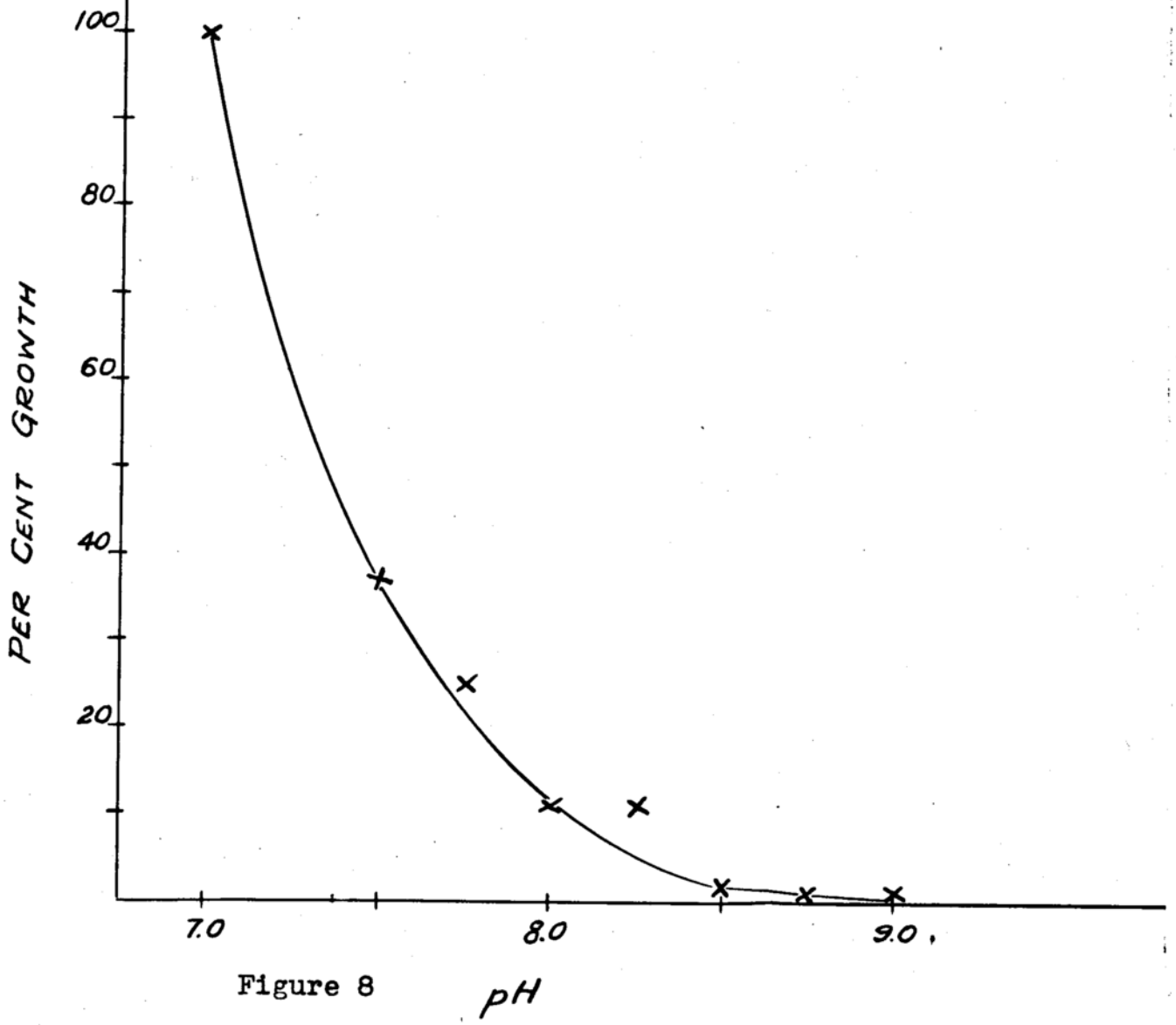


Figure 8

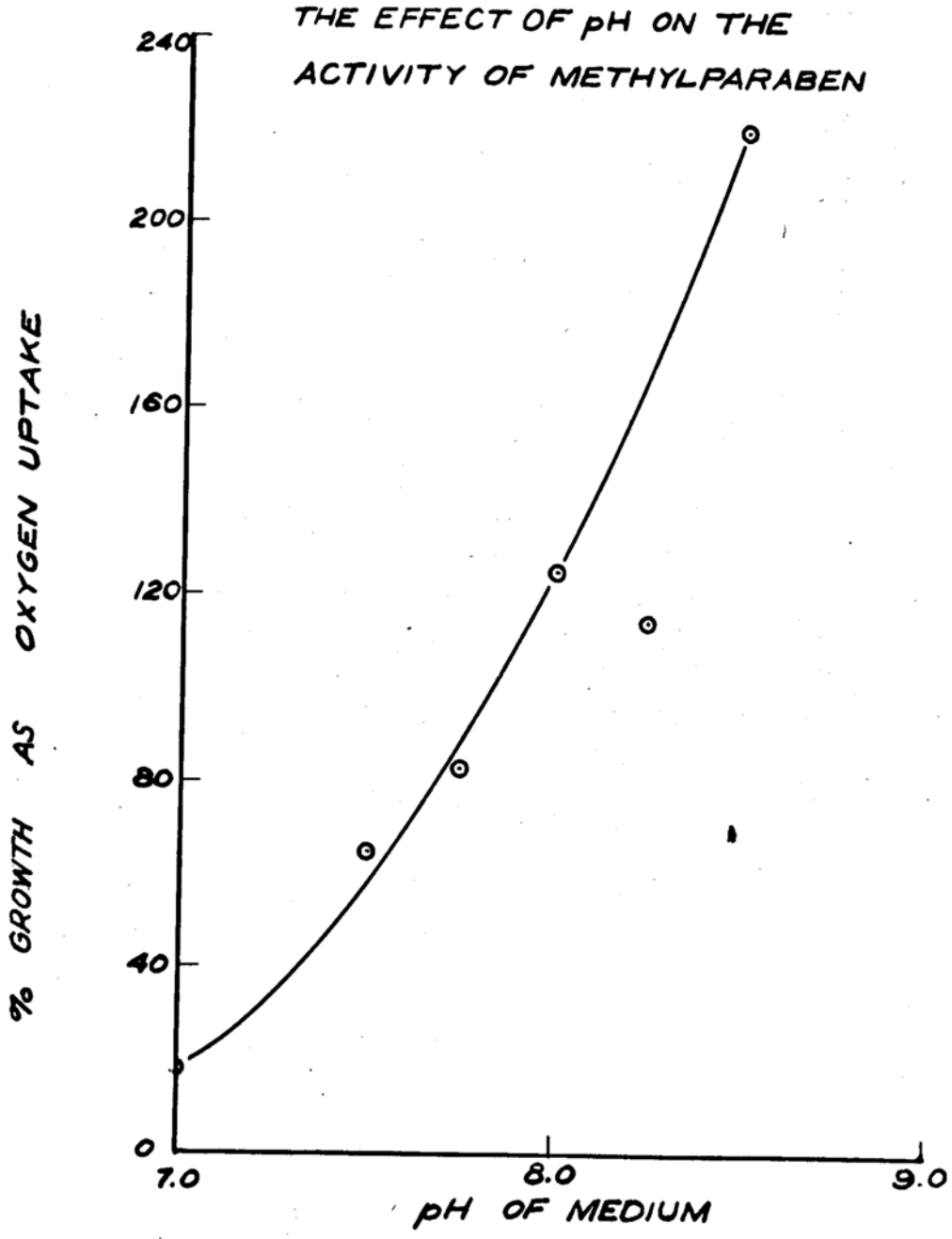

THE EFFECT OF pH ON THE
ACTIVITY OF METHYLPARABEN
% GROWTH AS OXYGEN UPTAKE
240
200
160
120
80
40
0
7.0
8.0
9.0
pH OF MEDIUM

Figure 9.

Introduction

Recently a new crystalline antibiotic substance, obtained from the filtrates of cultures of a species of *Streptomyces* which had been isolated from a soil sample collected near Caracus Venezuela (66) and also from a soil sample collected near Urbana Illinois, has been described (67,68). The name proposed for this substance was Chloromycetin (69) which has been adopted as the trade name by Parke, Davis and Company. This antibiotic substance has been identified chemically (70) as D(-)-threo-2-dichloro-acetamido-1-p-nitrophenyl-1,3-propanediol and titled Chloramphenicol in the United States Pharmacopoeia XIV. Eighteen genera of microorganisms have been observed to be susceptible to chloramphenicol and include *Aerobacter*, *Bacillus*, *Brucella*, *Corynebacterium*, *Diplococcus*, *Escherichia*, *Hemophilus*, *Klebsiella*, *Micrococcus*, *Neiseria*, *Pasteurella*, *Proteus*, *Pseudomonas*, *Salmonella*, *Sarcina*, *Shigella*, *Streptococcus*, *Vibrio* and *Rickettsiae* (71).

Chloramphenicol was included in this study because of the possible advantages in applying the manometric technique employed in the study of the parabens to the evaluation of the biological activity of an antibiotic

substance. At the present time, the antibacterial activity of chloramphenical is measured by three microbiological methods, namely the agar diffusion method (72,73,74), the overnight broth method (76), and the turbidimetric method (73,75,77). It was decided to apply the manometric technique to the study of the rate of degradation of chloramphenicol in solution.

Procedure

A standard solution of chloramphenicol containing 50 γ per ml. was prepared using sterile distilled water. This solution was stored in the refrigerator without any loss of potency as reported by Joslyn and Gailbraith (77). Dilutions were prepared using sterile distilled water so that one ml. of the dilution contained the desired amount of chloramphenicol. One ml. of this solution and 1.9 ml. of glucose-ammonium medium at pH 7.0 were placed in each reaction vessel. The bacteriostatic effect was determined as the per cent of inhibition of oxygen uptake for the fifteen minute interval at which the peak of oxygen consumption was observed in the control. Using this data a standard curve was constructed with the known dilutions and the potency of the unknown solutions read from this curve. The

rate of degradation of chloramphenicol was studied as a function of pH and temperature by dissolving a known quantity of chloramphenicol in the appropriate phosphate buffer system and incubating this solution at 92°C. for a period of 28 hours. At regular intervals, samples were withdrawn and assayed for chloramphenicol content as described above.

Results and Discussion

It was observed that the rate of growth of E. coli as measured by the per cent of oxygen consumed in the presence of chloramphenicol, decreases with the increase in concentration of chloramphenicol (Figure 10). Although this effect on E. coli can be reproduced with a standard known solution, when this method was applied as an assay procedure in the degradation studies, the results were not in accord with what was expected. The values noted when the manometric procedure was employed (Table VI and Figure 11) suggest that there maybe factors present in the solution of the degraded products that affect or contribute to the bacteriostatic activity of any unchanged chloramphenicol present. The increased bacteriostatic effect observed early in the degradation study might be due to the presence of an additional antibiotic compound formed during the

decomposition. Further study of this reaction by other workers is in progress.

Table VI

The Effect of pH and Heat (92°C.) on the Degradation of Chloramphenicol in Solution

Milligrams per milliliter in solution

pH	Length of Incubation (hours)															
	0	3	4	7	8	10	11	12	13	14	16	18	22	24	27	28
5.8	10											9.8	7.5			7.5
6.0	10											10.0	9.5			8.3
6.2	5		6.5		4.9			4.8			3.7			2.9		
6.4	5		5.0		7.0			4.8			3.7			2.8		
6.6	5	5.8		3.9		4.3				3.9				2.2	1.6	
6.8	5	5.6		4.5		4.3				3.7				2.7	1.9	
7.0	5	5.7		4.5		4.9				3.8				2.5	1.7	
7.2	5		5.6		5.7		5.3			3.7				1.7		2.5
7.6	5		7.0		4.3		4.5			3.8				2.5		1.8
7.8	5		5.8		4.1		3.0			2.7				2.0		1.3
8.0	5	4.8		4.3		3.9			2.7					1.7	1.3	

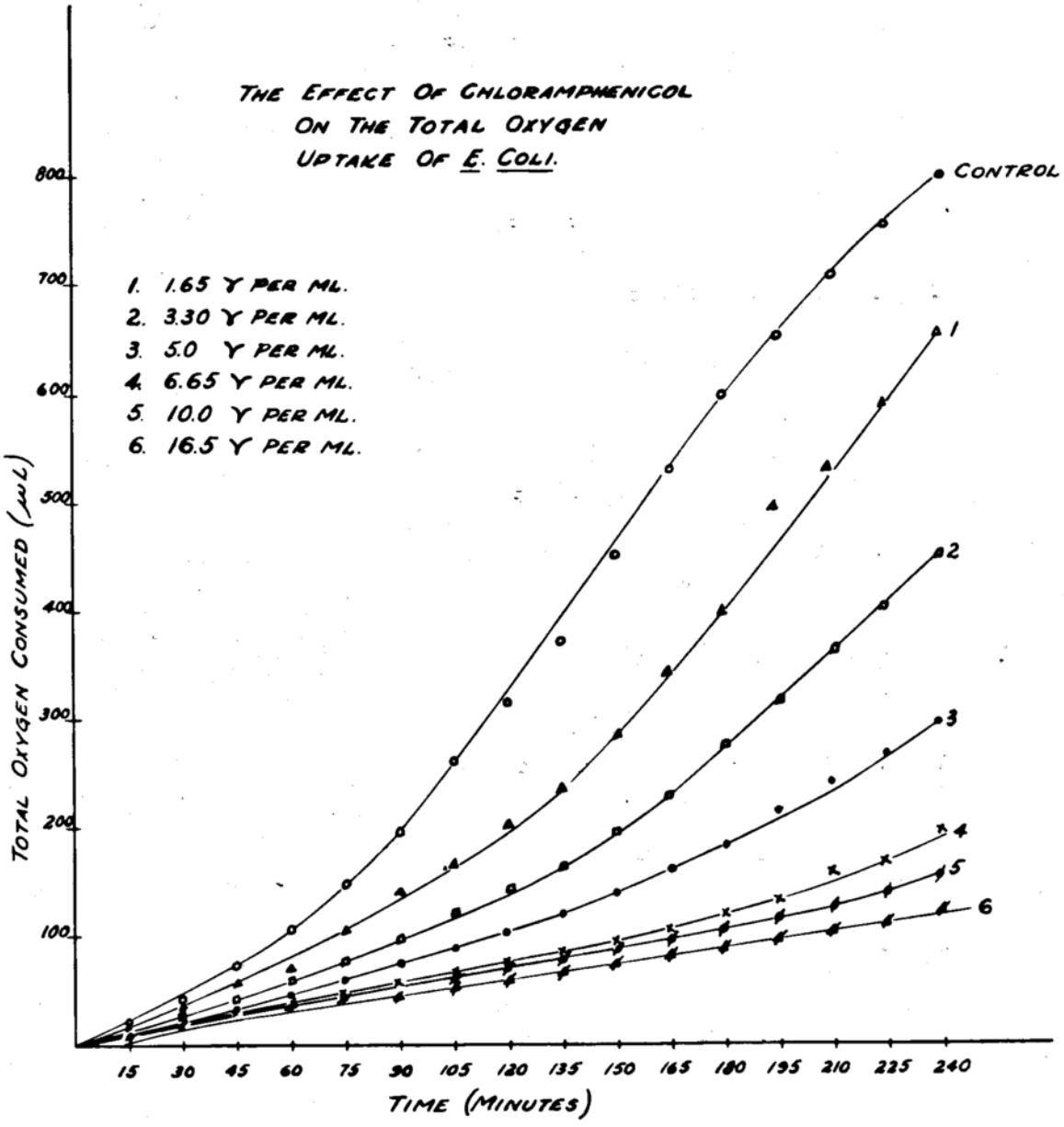
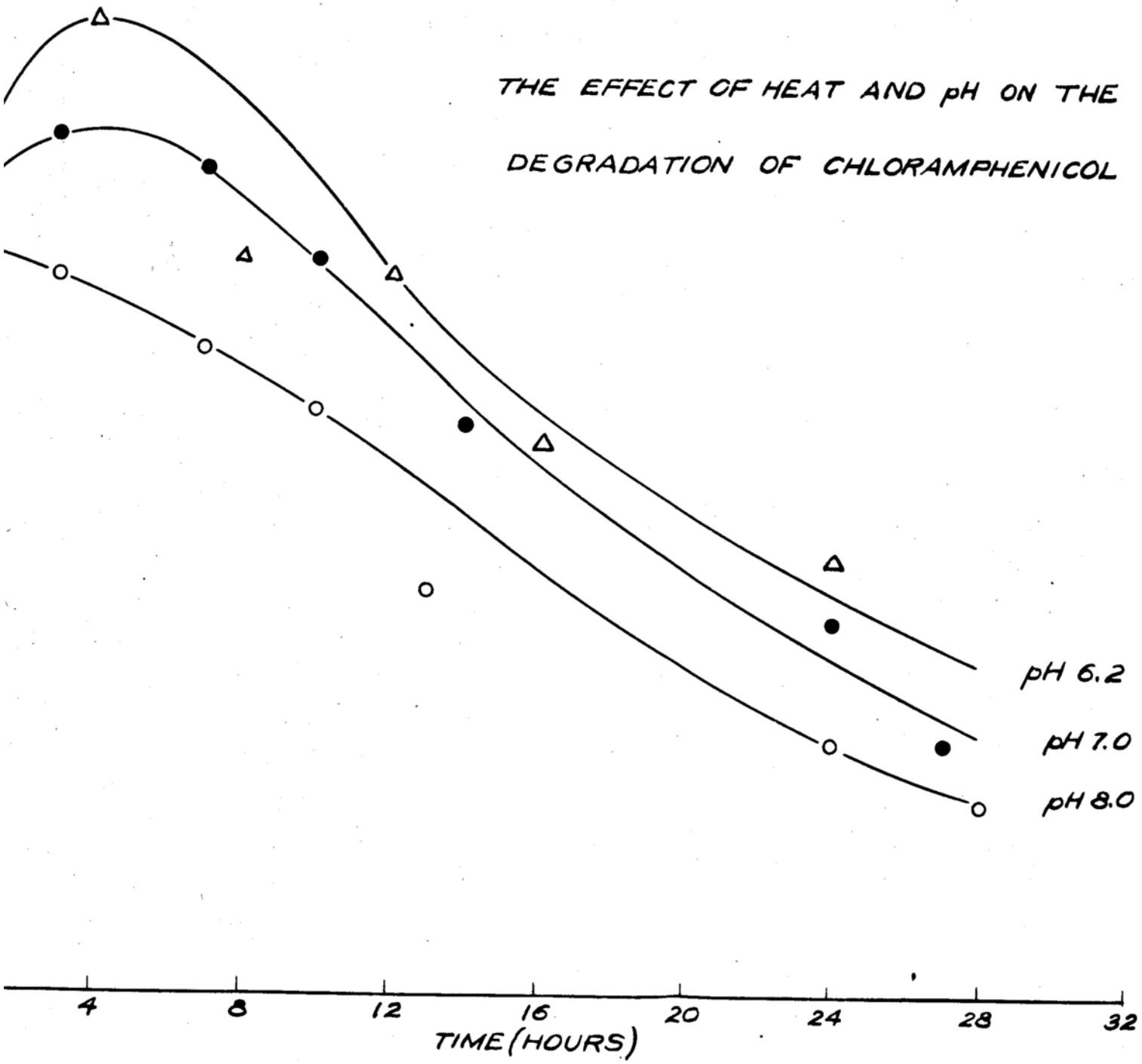

THE EFFECT OF CHLORAMPHENICOL
ON THE TOTAL OXYGEN
UPTAKE OF E. COLI.
1. 1.65 γ PER ML.
2. 3.30 γ PER ML.
3. 5.0 γ PER ML.
4. 6.65 γ PER ML.
5. 10.0 γ PER ML.
6. 16.5 γ PER ML.
CONTROL
1
2
3
4
5
6
TOTAL OXYGEN CONSUMED (μL)
100
200
300
400
500
600
700
800
15 30 45 60 75 90 105 120 135 150 165 180 195 210 225 240
TIME (MINUTES)

Figure 10.


THE EFFECT OF HEAT AND pH ON THE
DEGRADATION OF CHLORAMPHENICOL
pH 6.2
pH 7.0
pH 8.0
4
8
12
16
20
24
28
32
TIME (HOURS)

Page 54 is missing.

CONCLUSIONS

A manometric method for the measurement of bacteriostatic activity was developed and employed in a study of the thermodynamic relationship between the physico-chemical properties of the parabens and their bacteriostatic activity.

It was shown that the bacteriostatic activity of the parabens could be related to their lipoid/water distribution coefficient. The bacteriostatic activity is inversely proportional to their solubility in water. The addition of ethyl alcohol to the system increases the water solubility of the parabens and thus tends to decrease their activity, however the effect of this alteration on the physical property of the parabens was largely masked by the pronounced physiological effect of ethyl alcohol on the respiration of the microbial cells.

In the limited range of pH studied it was observed that as the pH of the medium approached the pKa of the paraben the bacteriostatic effect of the paraben was decreased thus suggesting that the concentration of the unionized form controls the bacteriostatic activity. These observations were largely masked by the marked effect of pH on the respiration of *E. coli* in the medium employed in this study.

This manometric procedure was employed as an assay procedure for the presence of chloramphenicol in solution, in the presence of degradation products of chloramphenicol. The results suggested the presence of additional factors to influence the antibiotic activity of chloramphenicol or the presence of an additional antibiotic substance which possesses higher activity than the original substance.

SUMMARY

The present study was originally undertaken in an attempt to learn the relationship, if any, between the magnitude of drug action and the thermodynamic activity of drugs. For such an investigation it was desirable to develop a reliable quantitative method of determining the magnitude of the physiological response evoked by a given concentration of an agent under study.

The approach that was adopted utilized the inhibitory property of antibacterial agents on the amount of oxygen absorbed over a given period. The logarithmic growth phase of Escherichia coli was chosen for the test and the respiration was followed by the use of Warburg manometers.

This procedure was applied to the study of the relationship between the thermodynamic activity of parahydroxybenzoate esters in solution and their bacteriostatic activity. Variations in the thermodynamic activity were achieved for fixed concentrations of the antibacterial agents by adding different amounts of ethyl alcohol to the system and by varying the pH of the system. Because of the greater solvent action of hydroalcoholic systems on the esters, additions of alcohol lead to decreases in the thermodynamic activity of the drug. Unfortunately,

because of concomittant action of alcohol alone on the biological system, the specific influence of alcohol on the physiological activity of the esters was largely masked. The results however were in qualitative agreement with the theory.

Because of the acidic nature of the parahydroxybenzoate esters, it is possible to vary the thermodynamic activity of the drug by varying the pH of the system employed. However, analagous to the preceding case, the effect of varying the pH alone on the respiration of the microorganisms was sufficient to render uncertain any quantitative attempt to correlate thermodynamic activity with biological activity. Nevertheless, the results were again in qualitative agreement with theoretical expectations.

The procedure which was developed for the study of the parahydroxybenzoate esters was also applied to a preliminary investigation of the rate of degradation of chloramphenicol in solution since the method appeared to have some value as an analytical tool. Results obtained suggest the possibility of the formation of a degradation product or products in solution which may possess higher antibiotic activity than the original drug.

As a part of the investigation a new chromatographic method of determining mixed parahydroxy-

benzoate esters was developed in cooperation with other workers.

APPENDIX

CHROMATOGRAPHIC SEPARATION AND DETERMINATION OF MIXTURES OF PARAHYDROXYBENZOATE ESTERS*

T. Higuchi, K. P. Patel, E. R. Bonow and J. Landsman

Although the parahydroxybenzoate esters (parabens) have been widely used as preservatives for pharmaceuticals, very little work appears to have been done on the determination of the esters in these products.

In certain cases, it may be well to analyse the preservative contents of the formulations. An aluminum hydroxide suspension supposedly preserved with a paraben, when analysed, showed zero concentration of the preservative. It was probably lost through hydrolysis. A method, therefore, is presented in this thesis suitable for the determination of the parabens and the mixtures of parabens such as may occur in common pharmaceutical products.

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* Journ. Am. Pharm. Assn., Scientific Edition, in press.

Theory

The method is based on preliminary extraction of parabens from the product, separation of the component esters by partition chromatography and subsequent determination of the individual constituents by ultraviolet spectrophotometry.

Parabens can be readily extracted from aqueous medicinal preparations with diethyl ether or chloroform since the partition coefficient is overwhelmingly in favor of the latter even with the methyl derivative (1/50). Double or triple extraction with small portions of ether is usually sufficient. With some additional manipulations, preservatives can be readily extracted quantitatively from even such items as suspensions, emulsions, etc.

Complex mixtures of parabens can be readily resolved by partition chromatography. This is illustrated in Figure 1, which is a partition chromatogram of a mixture of butyl, propyl, ethyl and methyl parabens. Although the chromatographic behaviour of higher molecular weight homologues were not investigated, it seems highly probable that the amyl, hexyl and even higher homologues can be

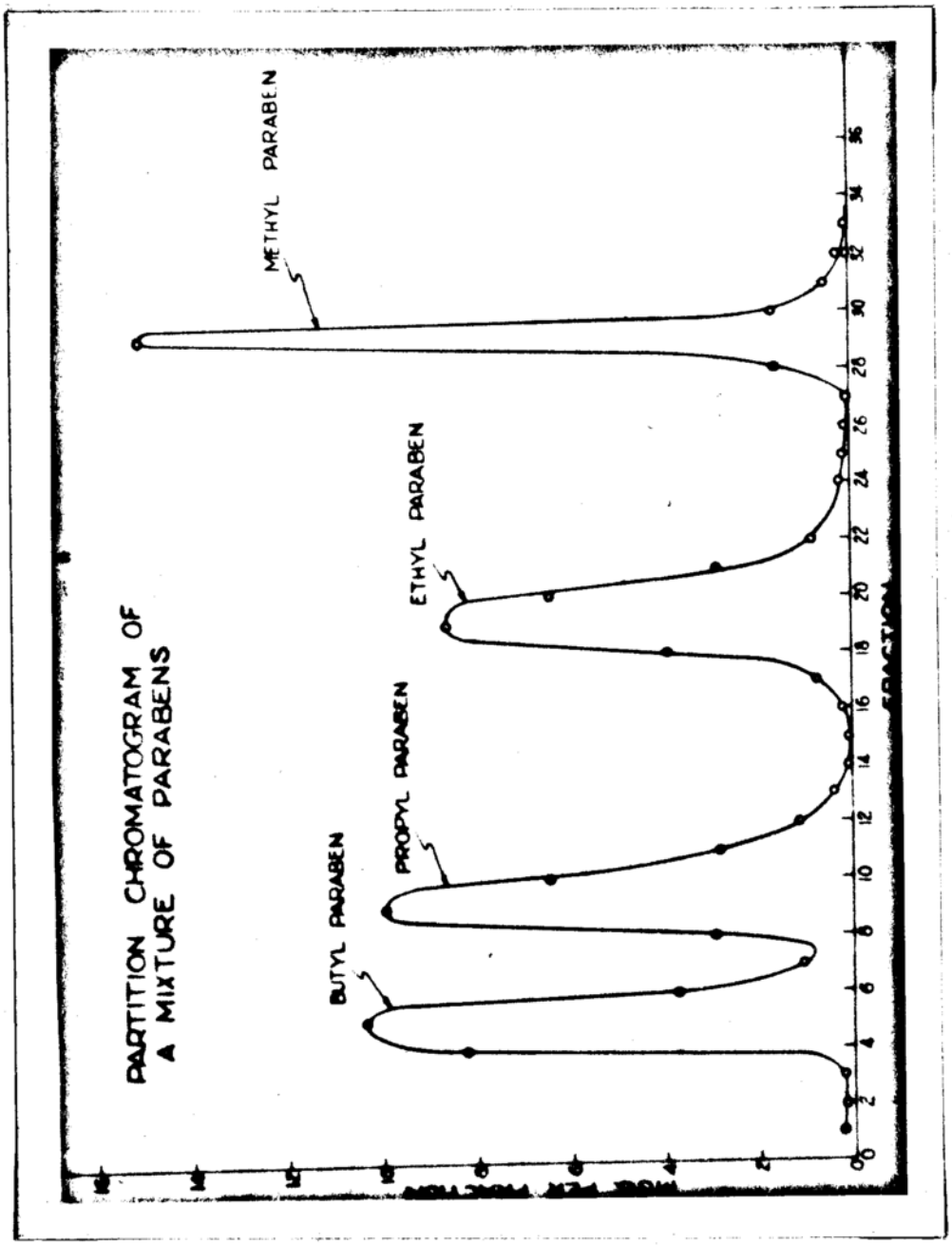
PARTITION CHROMATOGRAM OF
A MIXTURE OF PARABENS
METHYL PARABEN
ETHYL PARABEN
PROPYL PARABEN
BUTYL PARABEN
FRACTION
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36

Figure 1.

resolved by this method. In order to realize the full resolving power of a partition column, however, rather careful techniques and considerable amount of operational time are necessary. Fortunately, in practice it is usually sufficient to employ columns of limited resolving power, requiring less attention since only one or two parabens are present. For example, in separation and analysis of mixtures containing only methyl and propyl parabens, relatively few fractions need to be taken. The type of chromatogram found is shown in Figure 2. The propyl derivative was, in this instance, allowed to elute out of the column quickly by decreasing the methanol content of the stationary aqueous phase. Because of the wide separation achieved, it is possible for routine purposes in such cases to obtain the entire propyl component in a single large fraction. The eluant composition can then be changed and the entire methyl component obtained in another large fraction.

Ultraviolet Analysis of the Eluants

The paraben contents of the eluants can be determined either gravimetrically, as was done in obtaining the chromatograms shown above or spectrophotometrically. The gravimetric method is suitable where a relatively large sample is available; the spectrophotometric

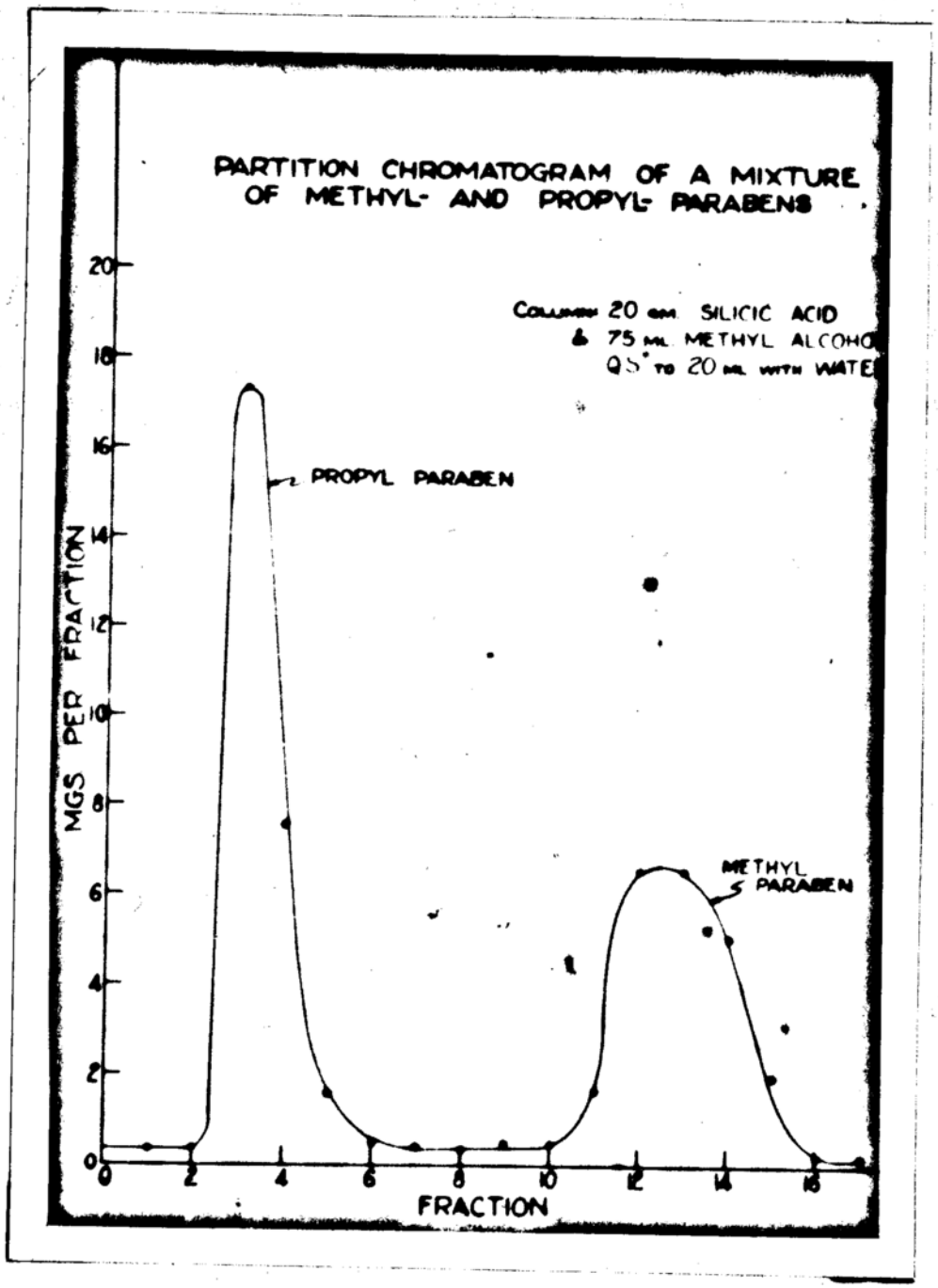
PARTITION CHROMATOGRAM OF A MIXTURE OF METHYL- AND PROPYL- PARABENS
Column 20 cm. SILICIC ACID
& 75 ml. METHYL ALCOHOL
QS to 20 ml. with WATER
PROPYL PARABEN
METHYL PARABEN
MGS PER FRACTION
FRACTION
0
2
4
6
8
10
12
14
16
18
20

Figure 2.

technique must be employed, however, if the sample contains less than 10 mgms. of the preservatives and is effective down to 10 micrograms.

The parahydroxybenzoate esters are relatively non-absorbing in the near ultraviolet region as is evident from Figure 3. Conversions of the parabens to their respective phenolates by making the solution mildly alkaline greatly enhances the extinction ceefficients and at the same time, shifts the first absorption peak to long wave length end as shown in in Figure 3 also. The shape of absorption spectra of the various esters are, however, very similar, the molecular extinction coefficients being approximately the same at all wave lengths. This similarity precludes any possibility of determining the different parabens by differential spectro-photometry. The concentration of the separated esters can, however, be readily determined on the alkaline solutions.

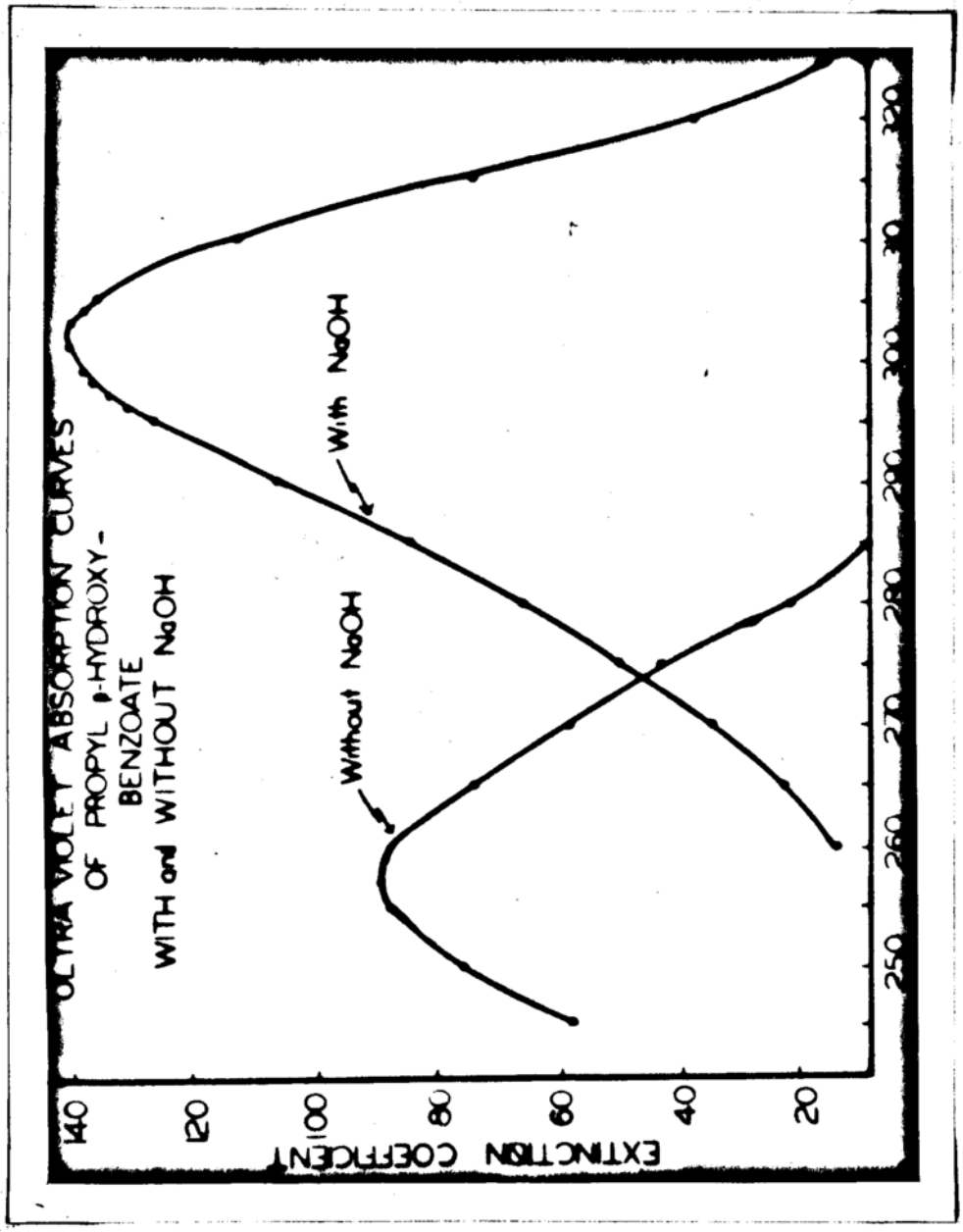
ULTRA VIOLET ABSORPTION CURVES
OF PROPYL p-HYDROXY-
BENZOATE
WITH and WITHOUT NaOH
With NaOH
Without NaOH
EXTINCTION COEFFICENT
140
120
100
80
60
40
20
250
260
270
280
290
300
310
320

Figure 3.

EXPERIMENTAL

To test the feasibility of the separate procedures outlined above, a method was developed for the determination of small concentrations of methyl and propyl parabens used as preservatives in a commercial preparation of histidine hydrochloride solution, put up in the form of 5 ml. ampuls. The label indicated that the solution was 0.18% with respect to the methyl derivative and 0.02% with respect to the propyl derivative.

Preparation of Sample Solution

Pipette 25 ml. of the solution to be analyzed into a 50 ml. separatory funnel. Acidify with a drop of concentrated sulfuric acid and extract with approximately 15, 10, 7 and 5 ml. portions of ether successively. Evaporate the combined ethereal extract in a 50 ml. volumetric flask by application of suction and gentle heat while swirling the flask. Dissolve the residue in 25 ml. of CCl_4, applying heat, if necessary. Make to mark with Skelly B. An aliquot of this solution is chromatographed.

Packing of the Column

A partition chromatographic column is prepared having an internal phase consisting of 20 gms. of silicic acid (Mallinchrodt, Chromatographic grade), 7½ ml. of methanol and 13 ml. of water containing one drop of concentrated sulfuric acid. An equal mixture of carbon tetrachloride and Skelly B is used as the external phase. More exact description of the apparatus used and the technique employed in packing of the column has been given for the analysis of APC combinations.

Chromatography of the Sample

A 5 ml. aliquot of the prepared sample solution is chromatographed on the prepared column. The entire propyl derivative in the sample is eluted off in a single fraction of 100 ml. with 50/50 mixture of carbon tetrachloride and Skelly B. The methyl component is then eluted out with 5/95 (V/V)butanol-chloroform mixture in another 100 ml. fraction.

Analysis of Eluate

Evaporate 25 ml. aliquot from each fraction to near dryness in 100 ml. vol. flasks by swirling the solution and heating gently under vacuum. Dissolve the residues by adding exactly 25 ml. of absolute alcohol and two drops of 0.2 N sodium hydroxide to each flask. Determine the absorbency of the solution immediately at 301 mμ. If the readings are higher than 0.9, dilute the solutions with absolute alcohol, which has been made slightly alkaline with sodium hydroxide, and repeat the absorbency readings at once.

Calculate the exact concentrations of the parabens on the basis of the absorbency readings, the total dilution factor and the specific extinction coefficients of the compounds as determined on the spectrophotometer used.

Results and Discussion

The above procedure was applied to two synthetic mixtures to test the feasibility of the method. The results obtained are shown in Table I. The recoveries with the first mixture was somewhat low; that for the second appears to be quite satisfactory.

In Table II, the results of the application to the previously mentioned histidine hydrochloride solution are given. Identical results were obtained for both runs which were consistent with the labelled quantities. The precision and the accuracy of the method seems to be satisfactory for most control purposes.

Table I

Recoveries from Known Samples

Mixt. No.	Components of the Mixture	Amounts Added	Amounts Recovered (average of 2)
1.	Propyl p-Hydroxybenzoate	0.0234%	0.0224%
	Methyl p-Hydroxybenzoate	0.1887%	0.1796%
2.	Propyl p-Hydroxybenzoate	0.0321%	0.0320%
	Methyl p-Hydroxybenzoate	0.1080%	0.1057%

Table II

Results of Analysis of Histidine HCl Ampuls

Run No.	Components of the Mixture	Labeled Amounts of each Present	Amounts Recovered (average of 2)
1.	Propyl p-hydroxybenzoate	0.02%	0.017%
	Methyl p-hydroxybenzoate	0.18%	0.16%
2.	Propyl p-hydroxybenzoate	0.02%	0.017%
	Methyl p-hydroxybenzoate	0.18%	0.16%

CONCLUSIONS

A method for separating and determining the mixtures of parahydroxy benzoate esters (parabens) such as may occur in pharmaceutical preparations has been developed. The method is based on three different steps:

(1) Preliminary extraction of parabens from the product by ether or chloroform.

(2) Partition chromatography of ether or chloroform extract.

In partition chromatography, silicic acid was used as adsorbent, a mixture of methanol and water was utilized as internal phase. Eluant was a mixture of carbon tetrachloride and Skelly B.

(3) Spectrophotometric determination of the eluates.

Data given supports the feasibility of the method as applied to the mixtures containing the methyl, ethyl, propyl and butyl derivatives, partition chromatography providing clean separation of each from others.

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