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A KINETIC STUDY OF THE HYDROLYSIS OF CHLORAMPHENICOL AT ITS AMIDE LINKAGE

ACKNOWLEDGMENT

The author wishes to express sincere thanks and appreciation to Dr. Roger Gilmont for suggesting this investigation, guiding it to completion and for his unending patience in the most difficult moments.

Thanks are also due the Wisconsin Alumni Research Foundation for the financial support rendered during the course of this study.

By

ARNOLD DAVID MARCUS

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of
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1954

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This data signifies that time beyond which the concentration of the active ingredient or ingredients falls below the

INTRODUCTION

This investigation was undertaken to study the kinetics of chloramphenicol degradation in aqueous solution as caused by hydrolytic cleavage of the amide linkage in the drug. The hydrolytic reaction, which appears to be the only significant degradative pathway in neutral and acidic solutions, has been studied under a variety of catalyzed and uncatalyzed conditions.

Studies of a chemical kinetic nature with respect to the reactions responsible for spontaneous degradation of pharmaceuticals are conspicuous by their rarity. This is particularly unfortunate when it is realized that very many of the problems associated with product formulation arise through the necessity of maintaining stability. From the standpoint of economics alone, kinetic studies provide a means through which waste and loss may be minimized or avoided entirely.

Liquid preparations represent a class of pharmaceuticals which are particularly susceptible to loss of potency and these products are often characterized by short shelf lives even when kept under refrigeration. Many of the antibiotic preparations currently on the market, for example, must, by government regulation, be marked with an expiration date. This date signifies that time beyond which the concentration of the active ingredient or ingredients falls below the

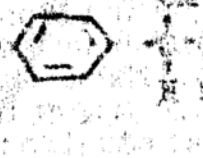
labelled amount. Vitamin preparations are another large class of pharmaceuticals very often marked with an expiration date. Subsequent to this date the product is assumed to be unfit for dispensing and is discarded. These instances represent substantial monetary losses to pharmaceutical industry as well as to retail pharmacists and their patrons.

Among the practices currently employed in attempts to lengthen the shelf lives of certain pharmaceuticals is the inclusion of an excess quantity of the active ingredient. The use of this excess will, it is assumed, allow for some deterioration to occur without reducing the concentration of the effective material below a certain minimum. Aside from the immediately obvious waste which may result from such a practice, more subtle factors such as uniform and adequate dosage place severe limitations upon any advantages derived. It is clear that any increases in effective shelf life are of necessity quite small. Where it is necessary to buffer a prod In view of these considerations, it is unfortunate that stability studies as most often conducted by those concerned with product formulation represent a purely empirical by a approach. Many man hours and large quantities of expensive l materials may be consumed during the course of such studies without yielding data which are of quantitative significance. The very shallowness of the data obtained may prevent proper conclusions from being drawn. Too often, pharmaceuticals quite similar in chemical constitution may be subjected to

totally separate studies even when the data gained from the study of one drug are directly applicable to the other. Factors such as the effect of neutral ions, buffer constituents, buffer capacity and even temperature effects may remain incompletely determined. The resultant costs represent inefficiently utilized time, manpower and material. In contrast to this, chemical kinetic studies provide quantitative data within the framework of an integrated picture. Each of the factors contributing to degradation may be evaluated separately but at no time is any single factor separate and distinct from the problem as a whole. If, for example, two important sources of degradative influence must be considered and the total correction of one only serves to accentuate the other, the quantitative character of a kinetic study permits a rational balance to be struck. Such a condition may be illustrated rather excellently in those instances where it is necessary to buffer a product so as to maintain rather narrow pH limits, and at the same time, certain buffer constituents serve to catalyze deterioration. In such an instance the data provided by a kinetic study will point toward that buffer system which will allow for maximum maintenance of pH limits with a minimum of deterioration resulting from the influence of buffer constituents. Only the quantitative and integrated character of kinetic studies permit these balances to be attained with optimum safeguards.

When regarded in relation to other drugs, and certainly with respect to other antibiotics, chloramphenicol exhibits unusual stability under a wide variety of conditions. Nevertheless, deterioration of chloramphenicol in aqueous solutions is a significant problem in the formulation of its preparations. When solutions or suspensions of the antibiotic must be sterilized prior to administration or are intended to retain their potency over long periods of storage the problem of deterioration may well be acute.

The various functional groups within the chloramphenicol molecule provide numerous foci for degradative attack. Those factors which contribute to the deterioration of a compound as stable as chloramphenicol may, moreover, assume overwhelming proportions in relation to similar but less stable compounds.



Therapeutic Implications

During the early clinical investigations into the therapeutic responses elicited by the antibiotic, it was recognized

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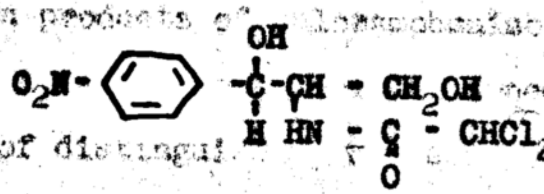
microorganisms and that its activity often coincided with that of penicillin and streptomycin. (11) More important was the early recognition of the fact that chloramphenicol offered great promise as an agent for the treatment of typhus, scrub typhus and typhoid fever. (12 - 16) At present, chlor-

PREVIOUS STUDIES CONCERNING CHLORAMPHENICOL

Isolation and Chemical Characterization:

Chloramphenicol, the antibiotic substance isolated from *Streptomyces venezualae* or prepared synthetically, was first described in 1947 and again, independently, in 1948. (1,2) From 1948 to 1949 extensive investigations were undertaken to determine the chemical nature of this broad spectrum antibiotic and, subsequently, to achieve total synthesis of the compound. (3 - 9).

In 1949 characterization and total synthesis were reported and chloramphenicol was shown to be D(-)threo-1-p-nitrophenyl-2-dichloracetamido-1,3-propanediol. (9)



Therapeutic Applications:

During the early clinical investigations into the therapeutic responses elicited by the antibiotic, it was recognized that chloramphenicol was effective against a wide number of micro-organisms and that its activity often coincided with that of penicillin and streptomycin. (11) More important was the early recognition of the fact that chloramphenicol offered great promise as an agent for the treatment of typhus, scrub typhus and typhoid fever. (12 - 16) At present, chlor-

amphenicol represents the agent of choice in the treatment of rickettsial infections.

Assay Procedures:

Coincident with the early chemical investigations into the structure and synthesis of chloramphenicol, a number of assay methods were devised. (1, 8 - 9, 17 - 22) Most of these were microbiological procedures making use of turbidimetric, serial dilution and disc plate techniques. A number of chemical procedures were also developed. These chemical procedures were essentially the same in that the nitro group was reduced, the resultant amine diazotized and coupled. The colored product obtained upon coupling was determined colorimetrically. Inasmuch as many of the degradation products of chloramphenicol would possess the nitro function intact, these chemical procedures were often incapable of distinguishing between chloramphenicol and various degradation products. (17,21,22)

Bodily Detoxication Routes and Products:

The effectiveness of oral administration of the antibiotic plus the additional information indicating that rectal administration was also effective (23), lends some insight into the metabolic stability of chloramphenicol. It may be safely assumed that gastric hydrochloric acid, in vivo, does not produce any significant degree of degradation. The data of Smith (24) as well as that of Gruhzit and his coworkers (25), reproduced below, offer additional insights into possible bodily detoxication processes.

TABLE I

SERUM AND URINE LEVELS OF CHLORAMPHENICOL AS ATTAINED THROUGH DIFFERENT ROUTES OF ADMINISTRATION. (25)

Route	Dose (mg./Kg.)	Serum (mcg./ml.)	Urine (mcg./ml.)
Oral	86 - 150	8	283
I.V.	19 - 50	7	644
I.M.	90 - 101	7	114

As may be seen from the above table, most of the chloramphenicol is unaccounted for. Since chloramphenicol was determined biologically, it may be assumed that most of the chloramphenicol is altered so as to provide a form or forms which does not possess any significant biological activity. Subsequent to this investigation, Glazko, Dill and Rebstock showed that less than 10% of an original dose of chloramphenicol appeared in the urine unchanged. (27) These workers also demonstrated that the major detoxication product of chloramphenicol metabolism is the glucuronide of the terminal hydroxyl group of the propanediol moiety. It was found, however, that degradation also proceeds through cleavage of the amide link. This does not, however, represent a significant metabolic pathway.

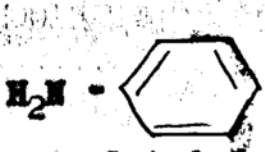
Stability and Degradative Studies:

A few of the early workers with the antibiotic made various attempts to study factors affecting the stability of

chloramphenicol but no intensive investigations in this direction were undertaken. These preliminary investigations indicated that chloramphenicol is stable in aqueous solution within the pH range 0.4 - 9.56 at room temperature. (4 - 7) It was shown that chloramphenicol was not hydrolyzed by papain, trypsin, chymotrypsin or pepsin. Hydrolysis was achieved, however, with bacterial enzymes from old cultures of P. vulgaris, B. subtillis and E. coli. (26)

Even though it was shown (27) that hydrolytic cleavage of the antibiotic at the amide linkage was not a significant route of detoxication, the very fact that the amine has been isolated as a degradation product points toward a possible degradative route. Hydrolysis at the amide linkage has also been demonstrated by Rebstock et al who showed that hydrolysis could be accomplished with strong acids or bases. (28) Periodic acid oxidations have shown the antibiotic to be converted to p-nitrobenzaldehyde (27) and although this oxidation was accomplished under rather vigorous conditions, oxidation, in general, could not be eliminated as a possible degradative route until the actual pathway was established.

The nitro group also presents a function subject to chemical change. Indeed, reduction of the nitro group results not only from the use of chemical agents (15 - 17) but has been accomplished, in vitro, with aqueous extracts of bacterial cultures (26) the amine



having been isolated.

Previous Kinetic Studies.

The only previously reported study of the kinetics of chloramphenicol degradation is that of Higuchi and Bias. (29) These investigators showed hydrolytic cleavage of the carbon - chlorine bonds to represent an important degradative pathway in aqueous solutions above pH 7. This study revealed that the rate of chloramphenicol degradation, as measured in terms of chloride ion production, could probably be regarded to represent two separate reactions. The first of these reactions, which was directly dependent upon the hydroxyl ion concentration of the system for the rate of chloride ion production, involved the hydroxyl ion catalyzed hydrolysis of the carbon - chlorine bonds in the antibiotic molecule. This initial reaction, it was found, was soon superceded by a second reaction through which the rate of chloride ion production became independent of the pH of the system.

This latter manifestation, it was postulated, resulted from hydrolytic cleavage of the amide linkage and formations of dichloracetate ion. This product was assumed to be the source of chloride ion production independent of the hydroxyl ion concentration. The work of Kunze (30) who showed chloride ion production from the hydrolysis of dichloracetate ion to be pH independent served to substantiate this hypothesis.

The very strong indications that hydrolytic cleavage of the amide function represents a major degradative pathway in alkaline solution, pointed toward a probable route of degradation in neutral and acid solutions as well and served as the starting point of the present investigation.

PREVIOUS WORK ON THE KINETICS OF AMIDE HYDROLYSIS

The evidence cited previously that amide hydrolysis represents a major pathway of chloramphenicol degradation made it advisable to review previous work on this subject. Although the literature pertaining to the kinetics of amide hydrolysis is by no means voluminous, it was felt that the results obtained by other workers might serve to illuminate the nature and possible dependencies of amide cleavage in the chloramphenicol molecule.

The first published investigation into the kinetics of amide hydrolysis is that of Remsen (31). This publication is rather sketchy and does little beyond showing that amide hydrolysis may be accomplished with aqueous solutions of strong acids or bases. Reid (32) reported the results of an extensive investigation of amide hydrolysis by both acids and bases. He found that the hydrolytic reaction was second order when catalyzed by either acids or bases in accordance with

$$\frac{d\,[\text{Amide}]}{dt} = -k\,[\text{Amide}]\,[OH^-]$$

$$\frac{d\,[\text{Amide}]}{dt} = -k\,[\text{Amide}]\,[H^+]$$

Crocker (33) investigating the kinetics of hydrolysis of various aliphatic amides in aqueous solutions of hydro-

chloric acid also found the hydrolytic reaction to be bimolecular. Crocker noted that the temperature dependency of the acid catalyzed reaction was quite large but at all temperatures studied the order of "relative reactivity" was maintained. He also made some attempt to correlate the "reactivity" of a particular amide with the nature of the acid employed as a catalyst but did not pursue the matter very far. He did, however, find the following relationship to hold:

$$\log k = \log k_o + B(1/T_1 - 1/T_2)$$

Crocker and Lowe (34) extended Crocker's investigations of amide hydrolysis and found the rate of hydrolysis to decrease with increasing molecular weight of the amide. These authors were the first to note that the temperature coefficient of hydroxyl ion catalyzed hydrolysis is much lower than for the hydrogen ion catalyzed reaction.

In 1907 Acree and Nerdlinger (35) investigated the kinetics of acetamide hydrolysis in an attempt to explain the hydrolytic reaction interms of Euler's theory of an ionic intermediate. (36-39) The velocity of the hydrochloric acid catalyzed reaction was measured at 65° C. and the results obtained seemed to substantiate Euler's postulation. In this respect it is interesting to note that both Bell (40) and Laidler (41) consider the hydrolysis of acetamide to represent the only documented evidence in support of Euler's theory.

Acree and Herdlinger found that during acid catalyzed hydrolysis of acetamide there was a gradual increase in the rate of the reaction. This increase was attributed to the effect of the ammonium chloride formed during the course of the reaction. These authors also found that sodium, potassium and aluminum chloride increased the velocity of the reaction whereas lithium chloride decreased the reaction rate. The postulation that these electrolytes contributed both positive and negative salt effects is in direct conflict, however, with the work of Taylor (42) who found the salt effect to be positive in all instances studied.

Euler and Olander (43) reported the magnitude of the catalytic constant for acid catalyzed hydrolysis of acetamide to decrease significantly as the concentration of acid was increased from 0.1 to 3.0 N. Any attempt to explain this decrease in terms of a negative salt effect runs contrary to the results obtained by Taylor as cited above. According to Laidler (44), the apparent decrease in the value of the catalytic constant may be explained from the fact that at different concentrations of acid the hydrolytic reaction is governed by separate relationships.

Reitz (45) showed the rate of acetamide hydrolysis in water to be quite different from the rate of the reaction in deuterium oxide. When the hydrogen ion concentration of the medium was 0.1 N., the rate of reaction in deuterium oxide was 50 percent greater than in water. As the concentration of hydrogen ion was increased to 2.3 N, however, the rates

became equal and in the presence of 3.0 N. acid the rate in water was 15 percent greater than in deuterium oxide. These data indicate that the hydrolytic cleavage of acetamide is subject to specific hydrogen ion catalysis, at least at lower concentrations of acid. Rabinovitch and Winkler (46) find the data of Reitz supporting specific hydrogen ion catalyzed hydrolysis to be somewhat in conflict with those of Krieble and Holst. (47) Rabinovitch and Winkler point out that the apparent relationship between the rate of acetamide hydrolysis and the acidity constant, H_o, in very high concentrations of acid as reported by Krieble and Holst indicate a deviation from specific hydrogen ion catalysis. Although this behavior may possibly be explained on the basis of general - acid base catalyzed hydrolysis of the amide, there are insufficient data to satisfactorily pursue this postulation.

Yatheraja and Sudborough (48) have reported that amides derived from unsaturated acids appear to be less susceptible to hydrolytic cleavage than their saturated analogues. The differences found by these investigators are summarized in Table II below.

TABLE II

FIRST ORDER RATE CONSTANTS FOR THE ACID AND BASE CATALYZED HYDROLYSIS OF CERTAIN AMIDES (48)

Compound	Base Catalyzed	Acid Catalyzed
Butyramide	0.111	0.0888
2,3-dehydro-butyramide	0.0697	0.0098
Dihydrocinnamide	0.172	0.0837
Cinnamide	0.0511	0.00748

As shown above, the rate of hydroxyl ion catalyzed hydrolysis of the unsaturated amides is one-third to one-half of the hydrolytic rate for the saturated compounds. The effect of unsaturation upon the rates of acid catalyzed hydrolysis is much more pronounced in which instance the presence of the double bond decreases the rate of the reaction by a factor of ten. Although the differences in polar characteristics of the molecule containing the double bond may be responsible for the lower rates of hydrolysis, the magnitude of the difference is not sufficiently great to permit valid conclusions to be drawn.

McKenzie and Smith (49) report that when 2-phenyl-2-hydroxyacetamide is hydrolyzed with concentrated potassium hydroxide the compound undergoes racemization. Although this amide is not entirely analagous to chloramphenicol in structure, the numerous assymetric centers within the chloramphenicol molecule present an ever present possibility that the antibiotic may undergo degradation through racemization to a form possessing less biological activity. As will be discussed in subsequent sections, this possibility was considered very seriously during the development of a procedure to determine residual concentrations of intact chloramphenicol.

In 1938 Krieble and Holst (47) reported the results of an extensive investigation into the hydrolysis of various aliphatic amides in solutions containing very high concentrations of mineral acids. These investigators showed the rate of hydrolysis to reach a maximum when sufficiently high

concentrations of either hydrochloric or sulfuric acid were employed to effect degradation. Such behavior had been reported previously for hydrochloric acid by Benrath (50) and Taylor (42) but Krieble and Holst are the first to report a similar maximum when sulfuric acid is used as the catalyst. Both Benrath and Taylor had used concentrations of sulfuric acid as high as 7 N. without encountering a maximum. It is, however, important to note that at such high concentrations, the term 'normality' loses much of its significance. Krieble and Holst have given all concentrations of acid used to effect hydrolysis in terms of molality, thus making comparison with other results somewhat uncertain.

It was determined that the catalytic activity of the two acids was quite different under different conditions. This is illustrated in Table III below.

TABLE III

CHANGE IN THE FIRST ORDER RATE CONSTANT WITH CHANGE IN ACID CONCENTRATION FOR THE HYDROLYSIS OF ACETAMIDE (47)

Molality HCl	k	Molality H_2SO_4	k
1.5	0.025	0.97	0.024
2.67	0.038	2.00	0.032
2.91	0.039	2.50	0.033
4.13	0.035	3.00	0.032
5.00	0.026	4.14	0.027

It is evident that the rate of the hydrolytic reaction is not any simple function of acid concentration. Furthermore, throughout the entire range of concentrations of the two acids, no simple relationship can be found between the rate of the reaction and the 'acidity' of the medium. The figures given above also show that prior to the maximum sulfuric acid is a better catalyst than hydrochloric but that this situation is reversed immediately after the maxima have been attained. It would therefore appear that 'acidity' is an aid to the reaction before the maximum is reached but hinders the hydrolysis afterwards. These authors have noticed, however, that with both acids the maxima are reached at approximately the same acidity. This characteristic of the reaction is illustrated in Table IV.

TABLE IV

VALUES OF H_0 FOR MAXIMA IN THE HYDROLYSIS OF FORMAMIDE AND ACETAMIDE BY BOTH HYDROCHLORIC AND SULFURIC ACIDS. (47)

Amide	Acid	H_0 at Maximum
Formamide	Hydrochloric	- 1.75
"	Sulfuric	- 1.70
Acetamide	Hydrochloric	- 1.02
"	Sulfuric	- 0.90

Rabinovitch and Winkler (46) have also investigated the rate of amide hydrolysis in solutions containing very high concentrations of mineral acids. This investigation was mainly concerned with detecting any changes in the Arrhenius constants

during the hydrolysis of acetamide as the concentration of the catalytic species changed. Such changes were found as was to be expected from the observations of von Kiss (51) who had shown large concentrations of electrolytes to cause a very definite alteration in the values of the Arrhenius constants. Similar changes in Arrhenius constants have been pointed out by Leninger and Kilpatrick for the hydrolysis of sucrose (52) and of ethylal. (53) This change in values of Arrhenius constants is ascribed by Rabinovitch and Winkler to the fact that very high concentrations of electrolytes may result in an alteration of certain fundamental properties of the medium, including the dielectric constant. This reasoning is analogous to that of Krieble and Holst who pointed out that very high concentrations of strong electrolytes may result in significant changes in the activity of water. (47)

Table V illustrates the decrease in the value of first order rate constants for the acid catalyzed hydrolysis of acetamide with increasing concentrations of acid. All the first order rate constants were calculated from the relationship

$$k = Ae^{-E/RT}$$

TABLE V

DECREASE IN THE FIRST ORDER RATE CONSTANT FOR ACETAMIDE HYDROLYSIS WITH INCREASING CONCENTRATIONS OF ACID. (46)

k = $Hours^{-1}$ x 100

Temperature °C.	Normality of Acid		
	1.00	4.00	8.47
40.6	13.2	16.9	4.02
45.7	20.6	29.4	-
51.9	-	50.6	-
52.0	-	-	12.8
59.6	71.4	107	26.9
70.7	183	-	80.0

Rabinovitch and Winkler have also shown that as the concentration of acid used to effect hydrolysis increases, there is an increase in the apparent heat of activation.

Meloche and Laidler (54) have recently shown that the nature of the substituent on the aromatic nucleus of benzamide may contribute polar effects of sufficient magnitude to markedly affect the rate of amide cleavage. This investigation was, however, carried out in a solvent system consisting of 60:40 ethanol - water and thus differs very markedly from the media employed in the degradation of chloramphenicol.

The kinetics of hydrolysis of the amide linkage in proteins and peptides was the subject of an investigation by Leach and Lindley. (55) These authors showed that the rates of

hydrolysis of L-asparginine to L-aspartic acid in hydrochloric acid (0.2 - 1.0 N.) and L-asparaginylglycine to L-aspartyl-a-glycine in 0.1 - 1.0 N. acid were first order with respect to both substrates although hydrolysis of the former is somewhat slower than that of the latter. Both rates are, however, comparable to those found for similar hydrolyses in proteins.

In a second publication (56) the same authors reported the results of a kinetic study of the hydrolysis of Glycyl- and L-leucyl-L-asparagine. The hydrolytic reactions were shown to be essentially pH independent in the presence of large amounts of mineral acids. The heats of activation for the four hydrolyses mentioned were calculated from the energies of activation according to

$$H_a = E_a - RT \quad (\text{at } 353^\circ \text{ K.})$$

and are summarized in Table VI below.

TABLE VI

HEATS OF ACTIVATION FOR THE AMIDE HYDROLYSIS OF CERTAIN PEPTIDES. (56)

Compound	Heat of Activation
L-asparagine	19.8 kcal./mole
L-asparaginylglycine	18.5
Glycyl-L-asparagine	24.0
L-leucyl-L-asparagine	22.2

Although a reasonably large number of investigations into the kinetics of amide hydrolysis have been made in the

past, these have, for the most part, not proven useful in providing insights into the course of amide cleavage with respect to chloramphenicol. Many of these previously reported investigations were concerned with determining the velocity of the hydrolytic reaction when catalyzed by hydroxyl ions. Moreover, the concentrations of hydroxyl ions used were such as to insure occurrence of hydrolysis. Inasmuch as it was desired to study the kinetics of chloramphenicol degradation without complication by hydroxyl ion catalyzed cleavage of the carbon - chlorine bonds, any data obtained by hydrolysis in alkaline media are not particularly suited to the purpose of this investigation.

Despite the results of previous researches into acid catalyzed hydrolysis of amides, the concentrations of acids which were employed far exceed the concentrations of hydrogen ion in the media used in the present investigation. Nowhere in the literature is there to be found any mention of amide hydrolysis in systems buffered so as to maintain the hydrogen ion concentration below that encountered at pH 2. Nevertheless, certain insights have been provided though these proved truly illuminating only after the present investigation was completed.

MECHANISMS OF AND FACTORS INFLUENCING AMIDE HYDROLYSIS

There can be little doubt that the mechanism by which hydrolysis of an amide takes place will be influenced by environmental conditions. It is possible, nevertheless, to postulate possible mechanisms for the hydrolytic cleavage of chloramphenicol at the amide function on the basis of previously proposed mechanisms for amide hydrolysis.

The report by Krieble and Holst (47) cited previously sums up much of the earlier thinking with respect to the mechanism of amide hydrolysis. These postulations were based upon the assumption that amides exist in a tautomeric equilibrium in aqueous solution according to

$$R - \overset{O}{\overset{\|}{C}} - NH - R' \rightleftharpoons R - \overset{OH}{\overset{|}{C}} = N - R'$$

Hantzsch and Geidel (57) reported the results of their studies on the absorption spectra of amides indicated the presence of an 'imido' form. Taylor (42), Calvet (58), and Bemrath (59) all proposed mechanisms for amide hydrolysis based upon the existence of the imido form in aqueous solution. This possibility derives credence from the fact that derivatives of both 'normal' and 'imido' amides have been prepared. Indeed, Wertheim (59) gives a mechanism for amide hydrolysis based upon this fact.

The following scheme, reproduced from the report of Krieble and Holst, sums up the early thinking on

mechanisms of amide hydrolysis:

$$R - \overset{O}{\overset{\|}{C}} - NH_2 \rightleftharpoons R - \overset{OH}{C} = NH$$

$$R - \underset{OH}{C} = NH + H^+ \longrightarrow R - \underset{OH}{C} = NH_2^+$$

$$R - \overset{OH}{C} = NH_2^+ + H_2O \longrightarrow R - \overset{O}{\overset{\|}{C}} - OH + NH_4^+$$

In attempting to explain the maximum observed, Benrath proposed that when the concentration of hydrogen ion was sufficiently high, all of the amide was converted into the cationic form. Taylor (42) lends support to this hypothesis by saying that increased concentrations of mineral acid result in the formation of an "unhydrolyzeable complex". These explanations for the maxima observed suffer, however, from a severe deficiency. If, as Krieble and Holst point out, these statements are true, the molality of the acid required to reach the maxima should vary with the concentration of amide used. Benrath used solutions 2.4 N with respect to the amide; Taylor employed solutions 0.05 N with respect to the amide and the solutions used by Krieble and Holst varied from 0.05 to 0.08 N with respect to amide.

As shown in Table VII below, the concentration of amide has almost no effect on the molality of the acid required for manifestation of the maximum.

TABLE VII

REALTION BETWEEN AMIDE CONCENTRATION AND MOLALITY OF HYDROCHLORIC ACID AT OBSERVED MAXIMA (47)

Amide Concentration	Molality HCl at Maximum
2.4 N.	3.18
0.05 N.	3.0
0.05 - 0.08 N.	3.25

It is apparent that the concentration of acid required for manifestation of the maximum is independent of the original concentration of amide. Therefore, the postulation that the maximum is manifested when all of the amide has been converted to the cationic form does not seem tenable. Similarly, Taylor's theory of an "unhydrolyzeable complex" also lacks tenability.

Krieble and Holst pursue explanation of the observed maxima from a different viewpoint. It has been noticed by these authors that in glacial acetic acid solution, amides will not react with nitrous acid until a significant quantity of mineral acid has been added to the solution. This lack of reactivity seems to indicate that mineral acids affect the amide - imide equilibrium in the direction of the amide form. Thus the maximum would be observed when the concentration of mineral acid is sufficient to convert all or most of the imide tautomer to the normal amide structure.

This postulation says, in effect, that the cationic

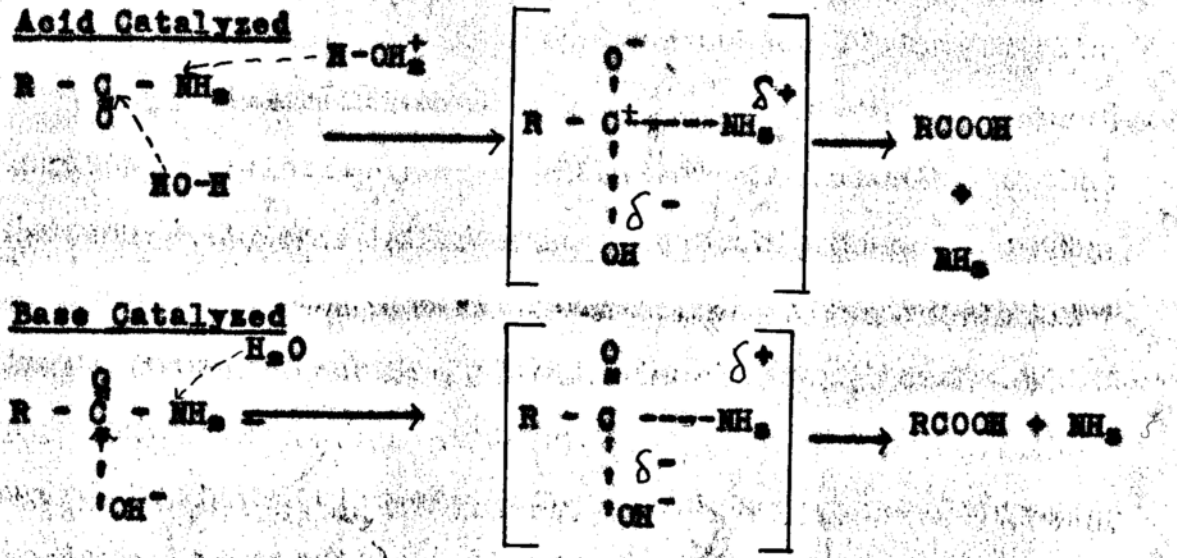
form which undergoes hydrolysis is gradually depleted as the imide is converted to amide. It would seem that this hypothesis also suffers from the fact that the maximum should then be affected by the original concentration of amide in solution.

It is also possible to object to this explanation on other grounds. It is well known that amides are distinctly weaker basic species than are amines. Therefore, the failure of amides to react with nitrous acid in glacial acetic acid solution may merely be a result of the weakly basic character of amides. Similarly, the lowrate of hydrolysis in glacial acetic acid solution may possibly be ascribed to the difficulty with which the weakly basic amides are transformed into the cationic intermediate.

Rabinovitch and Winkler (46) note that although acid catalyzed hydrolysis of amides has usually been interpreted on the basis of Euler's theory of interacting ions (60 - 61), there has been serious criticism of this theory by Bronsted (62) who has shown that in most cases, assumption of prior equilibrium between an ionic intermediate and the initial reactants is unnecessary to explain hydrolytic phenomenae. If, however, the equilibrium formation of the amide cation is considered preliminary to the formation of an activated complex, the maxima noted in the first order rate constants may result from maximum concentrations of amide cation. It would appear

that the objection stated before to this concept still retains validity and that the hypothesis is not an explanation of the maxima.

Rabinovitch and Winkler point out one possibility which may account for the unusual character of amide hydrolysis by very high concentrations of mineral acids. They indicate that although the magnitude of the 'frequency factor' in the Arrhenius expression is such as to place amide hydrolysis in the class of 'slow reactions, treatment of reaction velocity on the basis of ternary collisions results in values which are indicative of a 'normal reaction'. In this respect it is illuminating to examine the mechanism for amide hydrolysis proposed by Meloche and Laidler (54) as a result of studies on the hydrolysis of certain substituted benzamides. The proposed mechanism is given below



This mechanism involves:

1. Approach of water or hydroxyl ion to the carbonyl carbon.
2. Ionic fission of the carbon - nitrogen bond.
3. Approach of a water molecule or oxonium ion to the nitrogen atom.

The numerical designation does not represent the order of occurence but merely itemizes the steps required.

These authors found that substituents on the benzene ring markedly affected the heat of activation associated with the hydrolytic reaction. This change in activation energy is summarized below.

TABLE VIII

EFFECT OF RING SUBSTITUENTS ON THE HEATS OF ACTIVATION FOR THE HYDROLYSIS OF SUBSTITUTED BENZAMIDES (54)

Substituted Benzamide	Basic Hydrolysis	Acid Hydrolysis
p-nitro-	16.1 Kcal./M	24.6 Kcal./M
p-chloro-	17.6	23.7
no substituent	18.7	23.3
p-methyl-	18.9	22.0
o-methyl-	23.1	27.1

As is evident from the above data, electrophilic substituents cause a decrease in the heat of activation associated with base catalyzed hydrolysis whereas electrodotic substituents results in increased activation energies. The very reverse is true for acid catalyzed hydrolysis. In terms of the mechanism proposed by these authors, the effects of various substituents upon the

activation energy is explainable in the following manner:

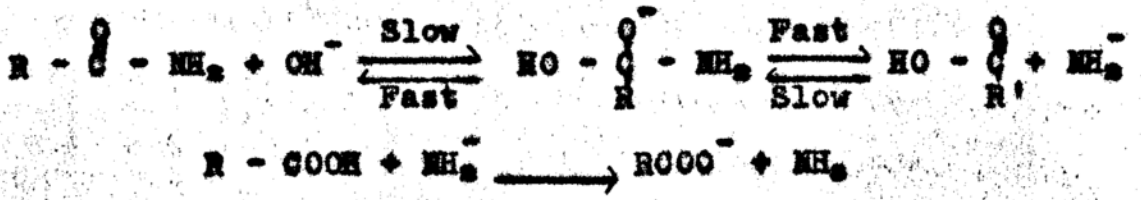
When R contains an electrophilic substituent, (1) is facilitated and 2 and 3 are hindered. The net effect depends, however, upon the relative weight of 1 vs. 2 + 3. In base catalyzed hydrolysis 1 must play a greater role than the other two effects according to the results shown in the table. This relative predominance of 1 in base catalyzed hydrolysis is explained by the fact that 1 involves a reaction between an ion, OH^-, and a substrate molecule; it being generally recognized that ion - molecule interactions are more important than molecule - molecule interactions.

With respect to acid catalyzed hydrolysis, 2 + 3 exert a greater effect than 1 for 3 now involves an ion - molecule interaction whereas 1 involves a molecule - molecule interaction. It is also found that the effect of substituents are more pronounced on the rate of base catalyzed hydrolysis than on acid catalyzed hydrolysis. This can be explained on the basis that the nitrogen atom, the focus of predominant attack during acid catalyzed hydrolysis, is much farther removed from the substituted aromatic nucleus than is the carbonyl carbon atom, the primary focus of attack during base catalyzed hydrolysis.

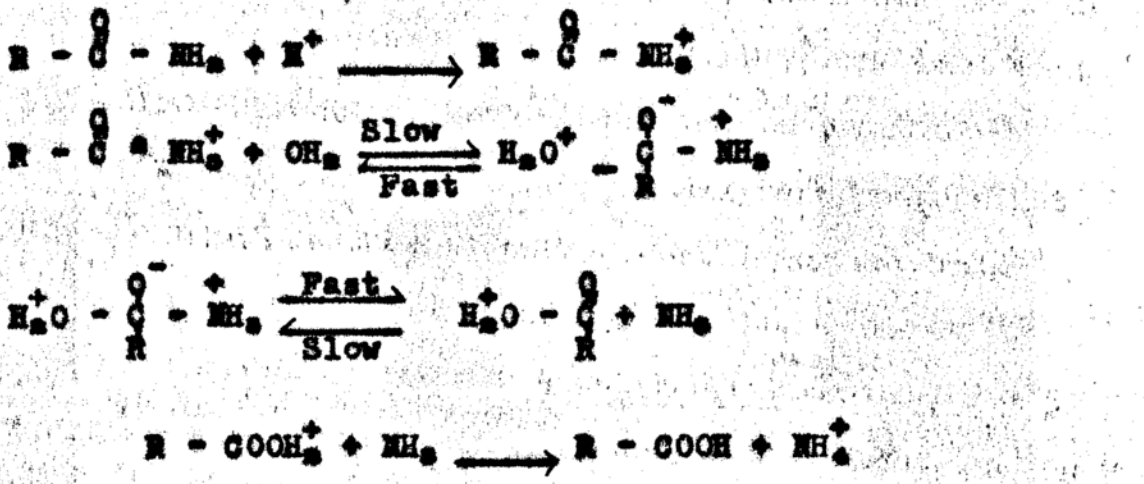
Ingold (63), referring to the results obtained by Reid for the acid and based catalyzed hydrolysis of amides, finds the second order character of the hydrolytic

reaction to indicate the following mechanisms:

Base Catalyzed



Acid Catalyzed



In attempting to apply the mechanisms proposed for amide hydrolysis to the possible hydrolytic cleavage of the amide function of chloramphenicol, a number of considerations must be observed. The peculiarities of the aromatic nucleus and attendant electronic effects may significantly alter the character of the hydrolytic reaction. The chloramphenicol molecule represents a complicated structure unlike most of the amides with which previous studies have dealt. Although many of the previous investigations into amide hydrolysis have dealt with derivatives of low molecular weight aliphatic acids, the

presence of the p-nitrophenylethyl substituent on the amide nitrogen may contribute electronic and steric effects which make the character of the hydrolytic reaction quite different when chloramphenicol is considered. The presence of two chlorine atoms of the beta carbon atom of the dichloracetate portion of the chloramphenicol molecule represent two strongly electrophilic functions which undoubtedly contribute greatly to the total electronic picture.

These studies are not without value, however, for any mechanism which may govern the rate of amide hydrolysis in the chloramphenicol molecule cannot be too far removed from the mechanisms which governthe hydrolytic rate for related aromatic and aliphatic amides.

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SCOPE OF THE INVESTIGATION

The rate of degradation of any chemical compound, whether the degradation proceeds through oxidation, reduction or hydrolysis is dependent upon a number of factors. These include temperature, pH of the media employed to effect degradation, presence of absence of neutral ions (primary or secondary salt effects), activity of acids and bases other than oxonium or hydroxyl ions and the mechanism through which the degradative reaction (s) proceed.

Inasmuch as it was the express purpose of this study to work within environmental conditions which would minimize hydrolytic cleavage of the carbon - chlorine bonds, this route of degradation could be very largely eliminated from consideration. All available evidence pointed toward the existence of a hydrolytic reaction which was assumed to be the major pathway of degradation in aqueous solution. The obvious necessity of having a suitable analytical method for the determination of residual concentrations of intact antibiotic therefore became the first requirement for successful pursuit of the investigation.

Subsequent to the development of a suitable analytical procedure, it was felt necessary to establish the order of the degradation over a wide range of conditions. Information gained from a knowledge of the order of a reaction very often provides valuable clues to the nature of the reaction. For example, demonstration

of first order character of the reaction over a wide range of experimental conditions would indicate the reaction to be of a simple ionic or molecular nature. If the results obtained could be duplicated with even reasonable precision, the possibility of redox reactions could be largely eliminated. In establishing both the order and rate of a reaction the factors cited above must be evaluated for their contributions. With this thinking in mind, the following factors were investigated with regard to their activity upon the rate of chloramphenicol degradation in aqueous solutions:

1. Order of the reaction.
2. pH dependency of the reaction.
3. Influence of neutral ions.
4. Catalytic activity of general acids and bases.
5. Catalytic contribution of hydrogen ions.
6. Magnitude of an uncatalyzed reaction.
7. Temperature dependency of the various reactions.
8. Evaluation of catalytic constants.
9. Possible mechanisms and route of degradation.

DEVELOPMENT OF THE ANALYTICAL METHOD

The experimental data concerning this section is given on pages 37 - 50 as a communication presented to the American Pharmaceutical Association, Scientific Section and are awaiting publication. These introductory pages will serve to amplify the thinking and considerations which preceded development of the analytical method.

Prior to undertaking a kinetic study of chloramphenicol degradation, it was essential to have available an analytical method which would permit differentiation between undegraded chloramphenicol and its degradation products. As noted previously, a number of assay procedures were already available, which made use of either microbiological or chemical techniques. It was felt however, that the former, in light of their inherent low degree of precision, cumbersomeness and time consuming nature would be unsatisfactory, for a kinetic study. The latter, based upon reduction of the nitro group, diazotization and subsequent coupling to form a colored product, have the immediately obvious disadvantage of being unable to distinguish between chloramphenicol and those of its degradation products which would possess an intact p-nitrophenyl moiety.

Determination of residual concentrations of intact chloramphenicol so as to provide useful information with respect to the order, and therefore the nature, of the

degradative reaction, with the requisite accuracy and precision necessary in a kinetic study appeared to be possible only through separation of the drug from its degradation products and subsequent determination.

Previous work in these laboratories (64) had shown that chloramphenicol lent itself very nicely to separation and determination through partition chromatography and subsequent spectrophotometric determination. Efforts were therefore directed toward evolving a partition chromatographic method for the selective elution of the antibiotic from partition systems containing it and its degradation products.

The aqueous nature of the media to be used to effect degradation of the drug presented an immediate problem in the analytical procedure. Ordinarily, samples to be analyzed are added to the partition systems in organic solvent solution. This was not possible in this investigation unless the water was removed and the residue redissolved in a suitable organic solvent. Such a procedure would however be very cumbersome and offer the possibility for additional quantities of the drug to undergo degradation. It was therefore felt that direct utilization of the aqueous samples would prove most fruitful.

This direct use of aqueous solutions was achieved by mixing an exact quantity of the aqueous solution with dry silicic acid and dispersing the mixture in an organic

solvent which would not elute the antibiotic from the aqueous phase. The resultant slurry could then be transferred to the partition column, tightly packed onto the body of the packing and the chloramphenicol eluted with a suitable solvent system.

From a standpoint of the structure of the antibiotic alone, it would seem that a strongly polar eluant would serve excellently to elute the drug. This thinking requires modification however, when it is considered that highly polar solvent systems would probably serve to elute degradation products as well. If hydrolysis, oxidation or reductions are assumed to be possible routes of degradation, the products resulting from such degradations would probably be more polar than the antibiotic. It therefore seemed more logical to attempt elution of the antibiotic with the least polar but satisfactory solvent system.

Unfortunately, such solvent systems would be required inrather large quantities for elution of the antibiotic which possesses a significant measure of polar character itself. If, instead of regrading the chloramphenicol molecule from a standpoint of its polar or non-polar characteristics alone, use is made of the electrophilic nature of the molecule, the apparent dilemma could be averted.

Chloramphenicol is very slightly soluble in water and even less soluble in chloroform. It is, however,

readily soluble in ethyl acetate and acetone. The solubility of the drug in these latter two solvents may be ascribed to their protophilic character. Of the two, ethyl acetate is available in very pure form and does not significantly absorb in the higher wavelengths of the ultra-violet spectrum. For these reasons it was assumed that effective partition chromatographic separation of chloramphenicol from its degradation products could be achieved with a solvent system composed of ethyl acetate in chloroform.

Although it seemed highly probable that chloramphenicol could be selectively eluted from a partition system containing it and its degradation products, the nature of the analytical procedure carried with it a possibly significant liability. Chloramphenicol is but one of a number of isomers, which differ only in the configurations of their assymetric centers. If, therefore, racemization at one or more of these centers represented a route of degradation, the proposed method of analysis would be incapable of distinguishing between the biologically active and inactive isomers, the character of the spectrophotometric method being such as to preclude this differentiation.

A microbiological procedure, by virtue of its selective response to biologically active materials would allow differentiation of any isomeric compounds. A microbiological assay method was therefore developed to serve as a check on the accuracy of the partition

chromatographic procedure. This microbiological method is described in detail in further pages.

The following pages report the experimental results obtained when a partition chromatographic method for the determination of chloramphenicol was compared with a microbiological procedure. These data show that the chromatographic method allows quantitative recovery and excellent precision. Most important, however, the very satisfactory agreement between the two procedures indicates that racemization is not a significant pathway of chloramphenicol degradation in aqueous solution.

COMPARISON OF A MICROBIOLOGICAL AND A CHROMATOGRAPHIC ASSAY METHOD FOR CHLORAMPHENICOL

Takeru Higuchi, Arnold D. Marcus and Charles D. Bias

ABSTRACT

It is shown in the determination of chloramphenicol that a microbiological method and a chromatographic method yield identical results when tested against partially degraded samples. The former is based on repression of growth as followed by Warburg manometry, and the latter on isolation of the antibiotic on a partition column and subsequent determination by ultraviolet spectrophotometry. Because of its greater precision and rapidity, the chromatographic method appears to be preferable for routine determinations.

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During the course of an investigation into the kinetics of the disappearance of chloramphenicol from aqueous solutions, it became expedient to follow the residual concentration of intact chloramphenicol molecules. Although an excellent microbiological method was already available, (1), it was thought desirable to develop a chemical assay for the antibiotic in the hope of achieving greater accuracy and precision. The present communication is concerned with such a chemical analytical method for the determination of chloramphenicol in the presence of its degradation products

as compared with a new microbiological method based upon respiratory inhibition of a bacterial culture.

THE MICROBIOLOGICAL METHOD

Biological methods are, in a sense, ideally suited for evalution of drug activity or concentration since the end use activity is determined directly. Such procedures are usually insensitive to the presence of similar but inactive contaminants or degradation products. They are, in most cases, very sensitive and are capable of detecting drugs present in extremely minute concentrations. In spite of the specificity and sensitivity characteristic of these procedures however, their adoption as analytical procedures is usually with reluctance and as a last resort because of their inherent low degree of precision.

In the present study the microbiological approach was mainly employed to validate the results obtained by the chemical method. Although the turbidimetric method proposed by Joslyn and Galbraith, (1), would have been equally suitable for this purpose, a manometric procedure was developed separately because of its somewhat greater rapidity. This procedure is based in principle upon the inhibition of oxygen uptake by Escherichia Coli in the presence of several solutions of chloramphenicol of known concentration, the inhibition produced by a sample of unknown concentration being compared against those shown by a series of standards.

The Experimental Method

Apparatus:

Warburg constant volume respirometer.

Preparation of the Media:

1. 23 g. of Difco nutrient agar are suspended in 100 ml. of freshly distilled water and the suspension is heated to boiling in order to effect solution. 25 ml. of the agar solution is then poured into ordinary prescription bottles which provide a fairly large surface for bacterial growth.
2. A synthetic glucose medium is prepared according to the method described by Kohn and Harris, (2).
3. Both solutions are autoclaved at a pressure of 15 lb./sq. in. for 20 minutes and stored for use when necessary.

Preparation of the Culture:

A culture of Escherichia Coli is transferred to a bottle of nutrient agar and incubated at 37° C. for 20 hours. The bacteria are then removed from the agar by washing with approximately 10 ml. of sterile distilled water and the resulting suspension is adjusted to an optical density of 1.0 (red filter) on a nephelometer. Five ml. of this suspension are poured into a bottle containing 100 ml. of the synthetic glucose medium, incubated for one and one-half hours and

cooled in a refrigerator for one hour before being used.

Preparation of Sample and Standard Solutions:

A standard solution of chloramphenicol containing 10 mcg./ml. is prepared by dissolving the drug in distilled water. Other samples of lower chloramphenicol content are prepared by proper dilution of this solution. The samples to be analyzed are weighed, dissolved and diluted with distilled water so that the concentration of the antibiotic will fall within the range 5 - 10 mcg./ml.

Preparation of the Warburg Flasks:

Exactly 0.2 ml. of a 20% solution of potassium hydroxide is pipetted into the well of each Warburg Flask and a 4 sq. cm. piece of fluted filter paper is inserted to absorb the alkali and to provide a large surface area for reaction with carbon dioxide. Two ml. of the cold glucose medium containing the suspended bacteria are pipetted into each flask, the suspension being shaken before each withdrawal to obtain uniform innoculation in each of the several flasks. One ml. of a standard chloramphenicol solution containing 5, 6, 7, 8, 9 or 10 mcg. of the antibiotic is added to each flask. In the same fashion, a one ml. aliquot of each sample solution is added to a separate flask. When higher accuracy is desired, duplicate or triplicate samples of the unknown solution are used.

The flasks are then attached to their corresponding manometers and placed in the water bath regulated at 37° C.

The manometers are allowed to equilibrate thermally for 10 minutes and are then closed to the atmosphere. Readings are taken every half hour. From these readings the corresponding volume of oxygen uptake for the contents of each flask is calculated according to the procedure described by Umbreit, (3).

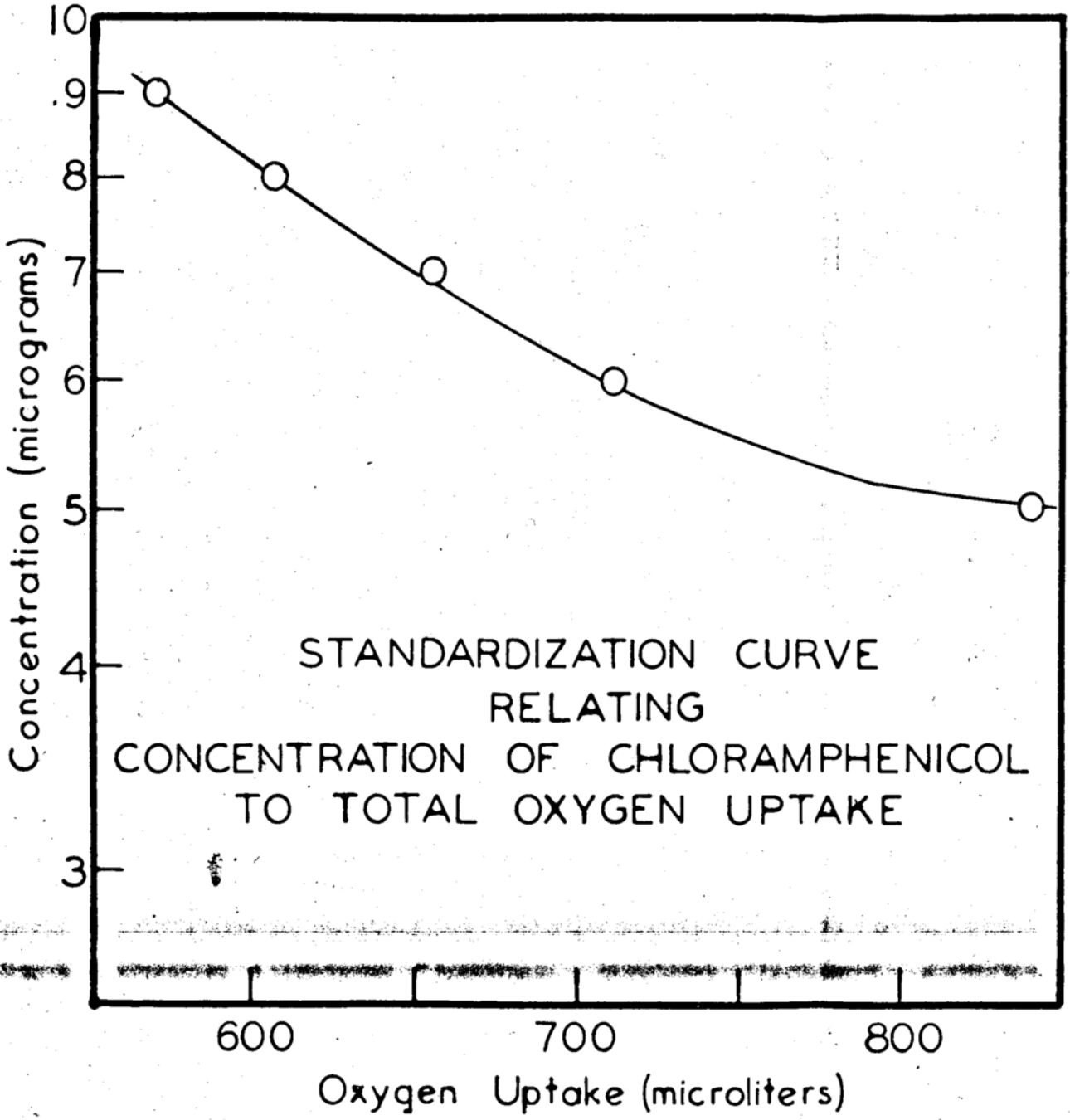
Each 'run' requires approximately 8 hours including about 4 hours in the Warburg bath.

Calculations:

The oxygen uptake in microliters is plotted against the time (in hours) as shown in Figure 1. A time is arbitrarily chosen during the logarithmic phase as a reference point. The oxygen uptake between this arbitrarily chosen time and zero time is then plotted against the logarithm of the chloramphenicol concentration of the standards as shown in Figure 2. By interpolation from this graph the chloramphenicol content of the unknown samples are obtained.

THE CHEMICAL METHOD BASED ON PARTITION CHROMATOGRAPHY

The chemical and instrumental methods of quantitative assay presently being employed are rather unspecific. Nearly all such procedures are essentially methods for determining the amounts of certain functional groupings present in the samples, very little differentiation being made, for example, between two alkaloidal nitrogens or between two carbonyl groups attached to different compounds found in the same sample. Thus in the analysis of chloramphenicol a number of


10
.9
8
7
6
5
4
3
Concentration (micrograms)
STANDARDIZATION CURVE
RELATING
CONCENTRATION OF CHLORAMPHENICOL
TO TOTAL OXYGEN UPTAKE
600
700
800
Oxygen Uptake (microliters)

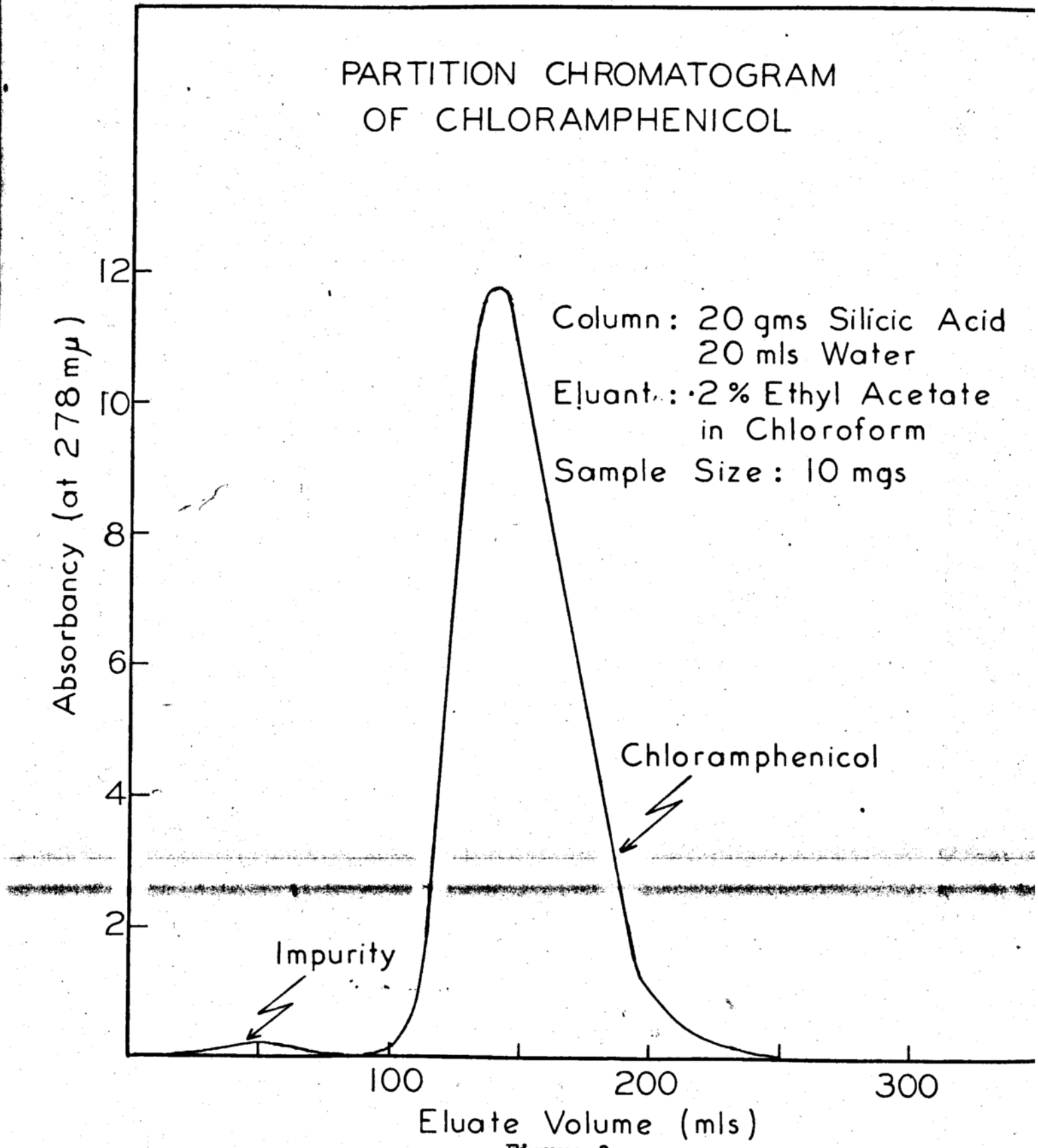

PARTITION CHROMATOGRAM
OF CHLORAMPHENICOL
Absorbancy (at 278 mμ)
12
10
8
6
4
2
Column: 20 gms Silicic Acid
20 mls Water
Eluant: 2% Ethyl Acetate
in Chloroform
Sample Size: 10 mgs
Chloramphenicol
Impurity
100
200
300
Eluate Volume (mls)

Figure 3.

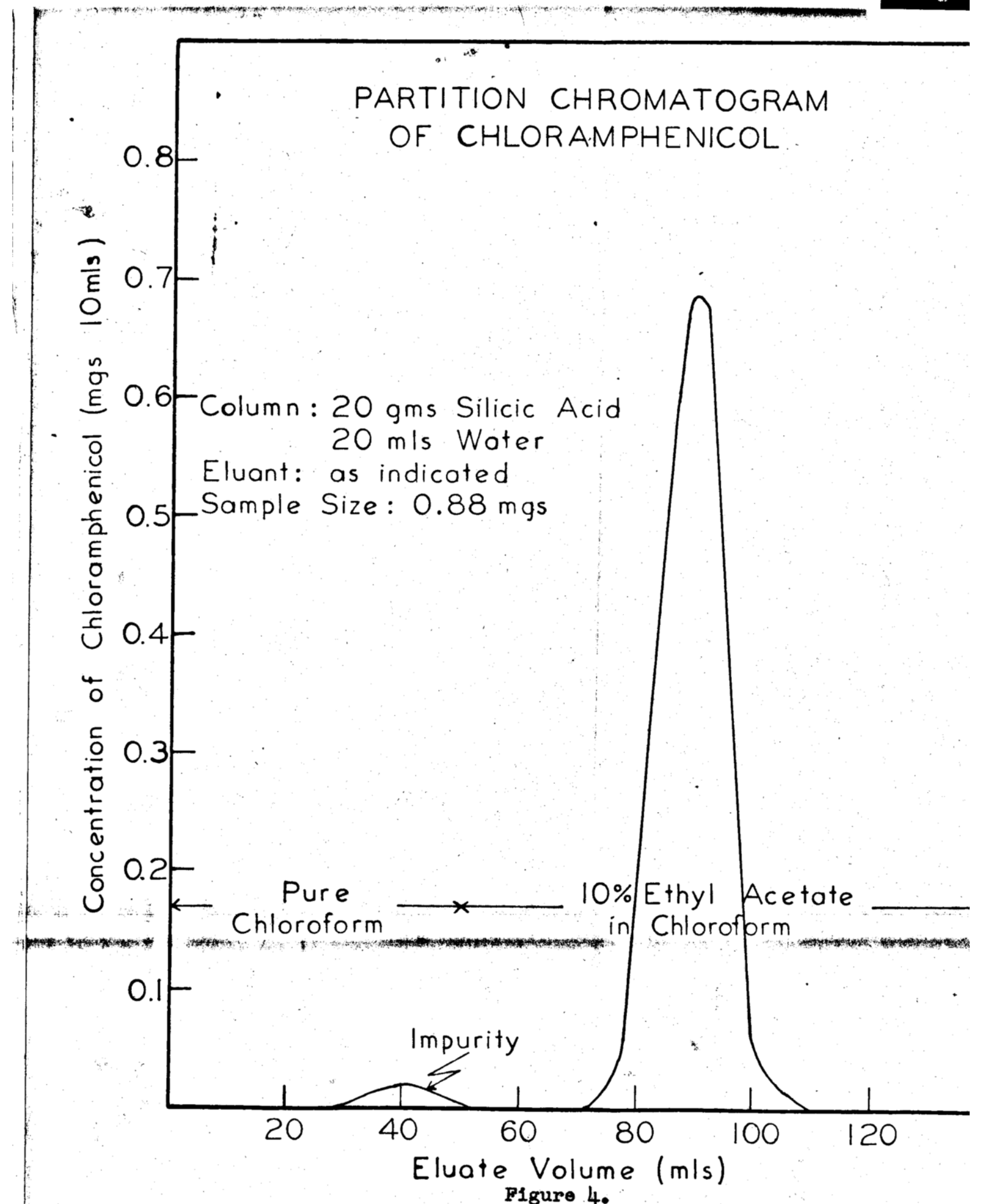

PARTITION CHROMATOGRAM
OF CHLORAMPHENICOL
Concentration of Chloramphenicol (mgs 10mls)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
Column : 20 gms Silicic Acid
20 mls Water
Eluant: as indicated
Sample Size : 0.88 mgs
Pure Chloroform
10% Ethyl Acetate in Chloroform
Impurity
20
40
60
80
100
120
Eluate Volume (mls)

Figure 4.

chloroform, the drug does not elute out even after 200 mls. of eluant had been consumed. To permit washing out of any impurities or components less polar than chloramphenicol from the column before the appearance of the drug in the eluate, it has been found convenient to use about 40 mls. of pure chloroform as the initial eluant. If this is followed by 60 mls. of 10% v/v ethyl acetate in chloroform, all the antibiotic is found in the corresponding eluate.

Since samples to be analyzed may be as aqueous solutions, solids, or dissolved in some organic solvent, a procedure has been developed for each case.

As is readily evident from Figure 5, the concentration of chloramphenicol in the eluate obtained after partition chromatography may be readily determined by spectrophotometric analysis over a relatively wide range of wavelengths. This method was therefore employed in analyzing the ethyl acetate - chloroform solutions for antibiotic content.

The Experimental Method

Reagents:

Ethyl acetate and chloroform, reagent grade. Silicic acid, chromatography grade.

Apparatus:

Pyrex glass chromatographic columns approximately 45 cm. long and 2 cm. in diameter. A tight fitting glass plunger to

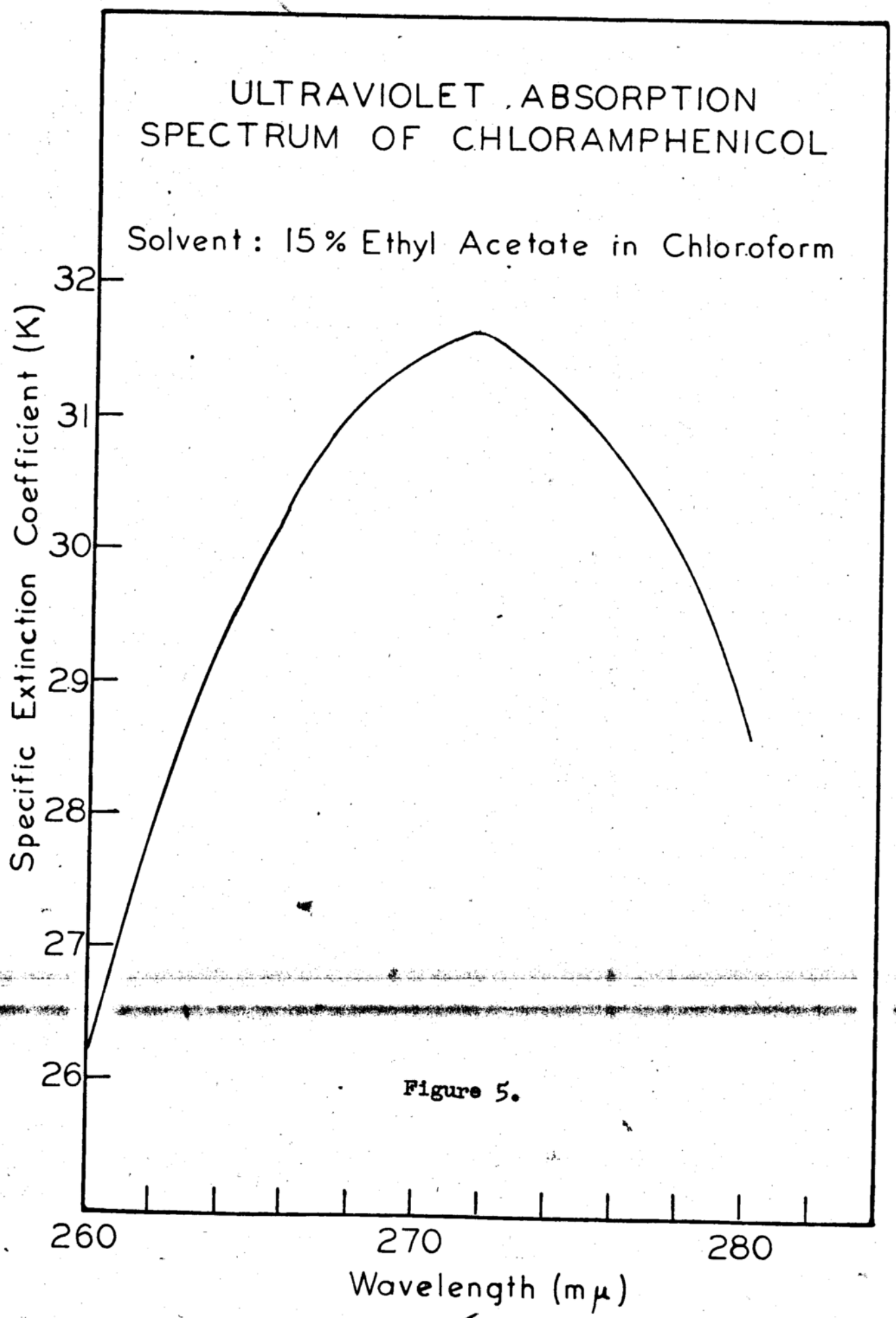

ULTRAVIOLET ABSORPTION SPECTRUM OF CHLORAMPHENICOL
Solvent : 15 % Ethyl Acetate in Chloroform
Specific Extinction Coefficient (K)
32
31
30
29
28
27
26
260
270
280
Wavelength (mμ)

Figure 5.

pack the columns. Beckmann Model DU Spectrophotometer.

Packing the Columns:

The columns are packed according to the procedure given by Higuchi and Patel, (7). Distilled water is used as the internal phase and pure chloroform is used as the external phase.

Procedure:

If the chloramphenicol is present in the solid state, it is dissolved in 10% (v/v) ethyl acetate in chloroform. If the chloramphenicol is present in an organic solvent solution, the solvent is removed by evaporation under reduced pressure (ca 25 mm.) and the drug is redissolved in 10% (v/v) ethyl acetate in chloroform.

The chloramphenicol is then separated as follows:

Exactly 5 ml. of the ethyl acetate - chloroform solution of the antibiotic is pipetted onto the surface of the column using care to avoid splashing the solution onto the sides of the column. A 100 ml. graduated cylinder is used to collect the eluate. After all but the last trace of supernatant liquid has entered the column, 5 ml. of pure chloroform are added to the column and allowed to drain into the mass. This is repeated with an additional 5 ml. At this point 25 ml. of chloroform are added to the column and a total of 40 ml. of the chloroformic eluate are collected in the cylinder. The eluant is then changed to 10% (v/v) ethyl acetate in chloroform and an additional 10 ml. of eluate are collected

in the cylinder. The cylinder is then removed, the contents discarded and the eluate collected in a 50 ml. volumetric flask. A total of 50 ml. of eluate is collected.

If the chloramphenicol is present in aqueous solution, the following procedure is employed;

Exactly 2 ml. of the aqueous solution are pipetted onto 2 g. of silicic acid in a 30 ml. beaker. The two are thoroughly mixed with a nichrome spatula and a slurry is made by adding 15 ml. of chloroform. The slurry is quantitatively transferred to the column, the beaker rinsed with 10 ml. of chloroform and the rinsings are added to the column. The slurry is then tightly packed onto the surface of the column. At this point the eluate is collected in a 100 ml. graduated cylinder. When removing the plunger great care should be exercised removing any adhering slurry. When almost all of the supernatant liquid has passed into the column, a second slurry prepared from 10 ml. chloroform, 1 g. silicic acid and 1 ml. distilled water is added to the column and tightly pressed onto the mass. After 40 ml. of chloroformic eluate have been collected in the cylinder the same procedure as above is followed.

The contents of the volumetric flasks are analyzed for chloramphenicol content by spectrophotometry.

CORRELATION BETWEEN THE TWO METHODS

Although the chemical method outlined in the previous section is considerably more convenient to employ than the microbiological procedure, the utility of the method depends upon the extent of the agreement between the results obtained by this chemical procedure with those obtained by the biological approach. The evidence presented in Table I and Figure 6 in which the analytical results obtained by the two methods for a series of samples containing chloramphenicol and its degradation products, indicates that the agreement is remarkably good. The results appear to be in complete agreement.

The relative precision of the two methods can be seen from the values shown in Tables I and II. In spite of best technique it is very difficult to obtain precision better than one part in 50 by any biological method because of the many variables involved. With the chromatographic procedure both the accuracy and precision obtainable are directly dependent on the manipulative care employed; good technique readily yielding precision of the order of two or three parts per thousand. This is apparent in Table II which shows recovery data on solutions of known composition.

In the procedures described above, smaller concentrations of the antibiotic can be determined by the microbiological method than by the chromatographic procedure. With certain

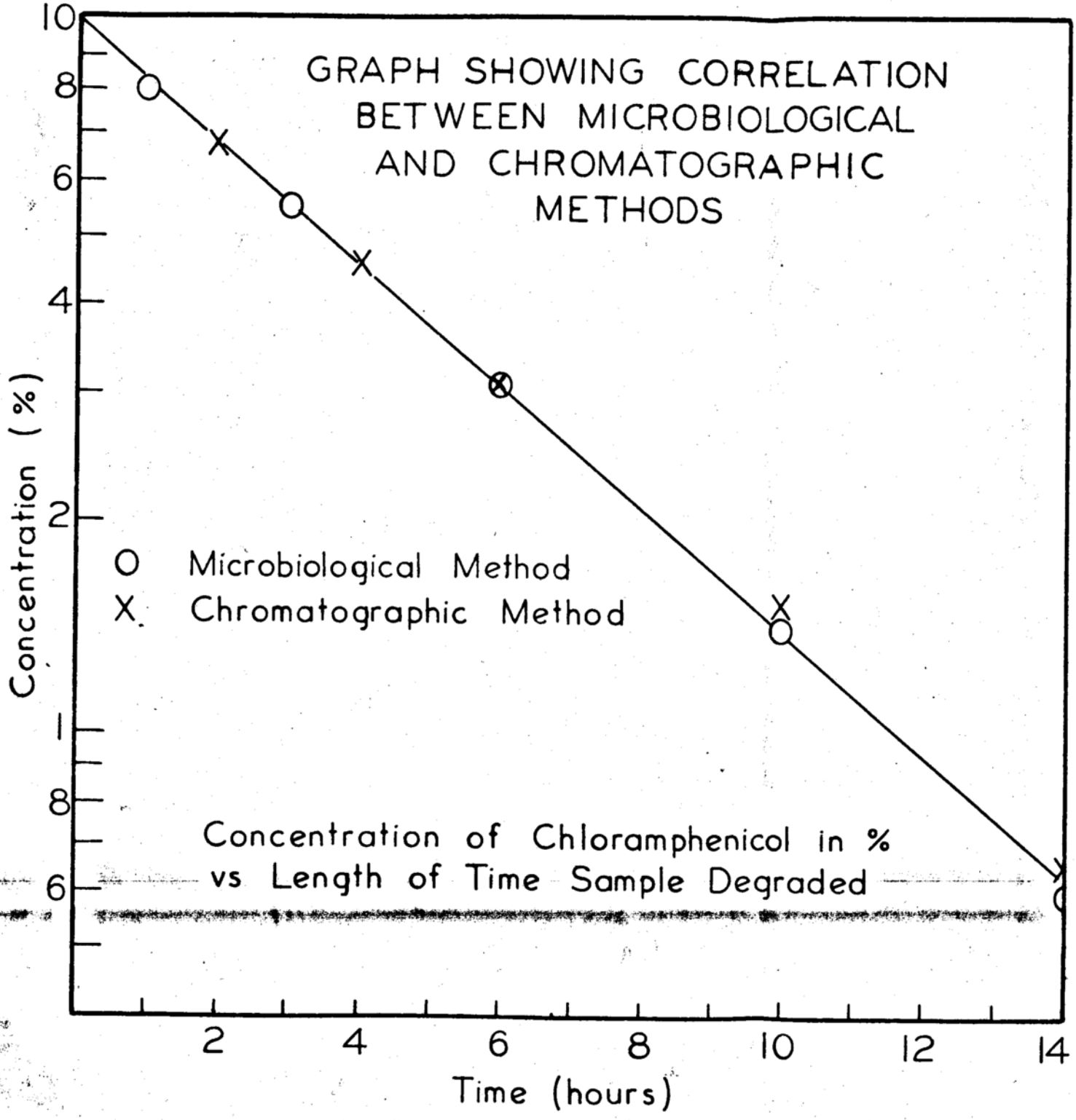

GRAPH SHOWING CORRELATION BETWEEN MICROBIOLOGICAL AND CHROMATOGRAPHIC METHODS
10
8
6
4
2
1
8
6
Concentration (%)
O Microbiological Method
X Chromatographic Method
Concentration of Chloramphenicol in % vs Length of Time Sample Degraded
2
4
6
8
10
12
14
Time (hours)

Figure 6.

obvious modifications, however, it is apparent that the latter method can be readily adapted so as to allow the detection and estimation of chloramphenicol concentrations of the order of a few micrograms.

The very nature of the chromatographic method prevents it from distinguishing between optical isomers. The close agreement obtained by the two methods therefore indicates that racemization is not a significant route of deterioration for chloramphenicol.

TABLE I

COMPARISON OF RESULTS OF MICROBIOLOGICAL AND CHROMATOGRAPHIC METHODS OF ANALYSIS

(Figures represent percent* of chloramphenicol remaining in degraded samples.)

Sample	Microbiological	Chromatographic
1	29 25.2 29 27	26.5
2	17.4 15 16.4 15.5	17.4
3	32.1 29.2	31
4	31 35 30.8	31
5	81 81.5	79
6	5.9 6.7 6.2	6.5

* The original concentration of chloramphenicol in the solution subjected to degradation was 0.25%.

TABLE II

RECOVERY OF CHLORAMPHENICOL CHROMATOGRAPHIC METHOD		
Theoretical (mg.)	Recovered (mg.)	Error (mg.)
4.99	4.99	0.00
4.43	4.39	-0.04
7.400	7.40	0.00
0.645	0.625	-0.02
0.344	0.343	-0.01

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KINETICS OF CHLORAMPHENICOL DEGRADATION IN SOLUTION

II. OVERALL DISAPPEARANCE FROM BUFFERED SOLUTIONS

The following pages describe the results obtained upon degradation of chloramphenicol in buffered aqueous solutions. All of the data were obtained at 97.30°C. In this study the effect of hydrogen ion concentration, neutral salts and buffer constituents upon the rate of disappearance of the antibiotic as well as the order of the reaction with respect to chloramphenicol were investigated through application of the partition chromatographic - spectrophotometric procedure described previously.

Since the results of this investigation have been accepted for publication in the Scientific Edition of the Journal of the American Pharmaceutical Association, no attempt has been made to reorganize the material.

THE KINETICS OF DEGRADATION OF CHLORAMPHENICOL IN SOLUTION

II. OVERALL DISAPPEARANCE RATE FROM BUFFERED SOLUTIONS

Takeru Higuchi, Arnold D. Marcus and Charles D. Bias

ABSTRACT

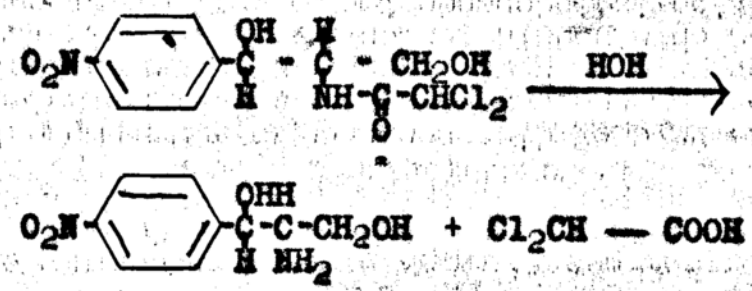
The rate of disappearance of chloramphenicol molecules, per se, from aqueous solution has been studied and shown to be first order over a wide range of hydrogen ion concentration. The data obtained indicates rather clearly that the rate of degradation is independent of the ionic strength of the medium employed for degradation. In addition, the rate of degradation appears to be substantially independent of the hydrogen ion concentration of the system within the pH range 2 - 7. Very strong evidence exists to indicate that chloramphenicol degradation is general acid - base catalyzed. For example, monohydrogenphosphate ions, undissociated acetic acid molecules and citrate - citric acid systems exert a definite catalytic effect upon the rate of antibiotic disappearance. The last cited example shows a rather complex relationship which might possibly be ascribed to the polybasic nature of citric acid and its ions, in which both electrophilic and nucleophilic functions are present within the same molecule.

****

In the preceding communication of this series (1), the mechanism and the influence of several variables upon the rates of the dehalogenation reactions responsible, in part, for the gradual loss of activity of chloramphenicol in aqueous solutions were treated. In the study the degradative rate was observed

experimentally by following the rate of chloride ion production in the aqueous solutions. In contrast, in the present investigation we were concerned primarily with the total rate of disappearance of chloramphenicol molecules, per se, from the reaction system, the analytical scheme employed being designed to determine the residual concentration of intact drug rather than any of its degradative products (2). As will be apparent from the experimental data presented, the dehalogenation reaction undergone by chloramphenicol appears to play but an insignificant part in the total degradative picture, at least in solutions below pH 7.

In the study referred to above, kinetic analysis indicated that one of the major causes of chloramphenicol breakdown can be attributed to the hydrolytic cleavage of the amide linkage in the drug according to the equation,



Subsequent observations on the overall disappearance rate appear to further substantiate this hypothesis, the rate being highly reproducible under any given experimental conditions and being strictly first order with respect to the drug. These are characteristics of molecular reactions of the hydrolytic type as contrasted to redox reactions which

are free radical mediated and rather difficult to reproduce.

Even if the degradative mechanism is assumed to be hydrolytic cleavage of the amide function, only a relatively small amount of help can be gained, for example, by reference to the literature for comparable reactions. Although a considerable amount of research effort has been devoted to the fundamentals of the kinetic of hydrolytic cleavage of esters, very little work seems to have been done on the similar cleavage of amides. This is probably due to the fact that amides, as a whole, are a great deal more stable than esters.

Certain theoretical assistance can be gained, however, by reference to the general theory of acid-base catalyzed reactions. In many instances the hydrolytic cleavage of ordinary esters and amides is catalyzed by hydrogen ions, hydroxyl ions or both. Hydrolysis of esters such as ethyl acetate, aspirin (3), and procaine (4) appears to be catalyzed by hydrogen ions or hydroxyl ions alone (i.e. specific hydrogen or hydroxyl ion catalysis). Hydrolysis of such simple amides as formamide and acetamide has been studied and shown to be catalyzed by hydrogen and hydroxyl ions. (5,6) In view of these instances of catalysis, it appears rather likely that the amide linkage in chloramphenicol may also be subject to hydrogen or hydroxyl ion catalyzed hydrolysis.

If we are to assume that hydrogen and hydroxyl ions are not unique as acidic or basic species but that other species may also be considered to function as acids and bases, a new

factor is introduced in considering the kinetics of hydrolytic reactions including degradation. It has been recognized in a number of instances that hydrolysis may be catalyzed by acids and bases other than hydrogen and hydroxyl ions, (i.e. general acid - base catalysis) (7). The hydrolysis of ethyl orthocarbonate and ethyl orthoacetate, for example, has been shown to be catalyzed by general acids as well as by hydrogen ions (8,9). More recently an investigation concerning the hydrolysis of diisopropyl fluophosphate revealed that hydrolysis of this compound is also general acid - base catalyzed (10). Among well known instances of non-hydrolytic general acid - base catalyzed reactions are the mutarotation of glucose and the decomposition of diazoacetate ion, (11,12).

Because of the several possible pathways by which amide cleavage may be effected, attempts have been made in the present investigation to study the kinetics of chloramphenicol breakdown as influenced by neutral ions, hydrogen ions, hydroxyl ions, general acids and general bases. Indeed, it was difficult to limit attention to hydrogen or hydroxyl ions alone inasmuch as maintenance of constant pH in the degradative systems necessitated the use of buffers which by their very nature contain significant amounts of general acids and general bases.

The results of the present study indicate that degradation of the antibiotic is general acid - base catalyzed but that within the pH range 2 - 7 the rate of degradation is substantially independent of hydrogen ion concentration.

EXPERIMENTAL

Reagents:

All reagents used in this investigation were of analytical grade. Silicic acid, chromatograph grade, was used to prepare the columns. The chloramphenicol was eluted from the columns with chloroform and a 15% (v/v) solution of ethyl acetate in chloroform. The buffers referred to below were prepared by dissolving reagent grade chemical in distilled water, adjusting and measuring the pH with a Beckmann Model G pH Meter.

Procedure:

250 mg. of chloroamphenicol were accurately weighed into 100 ml. volumetric flasks with the aid of a small glass funnel. Any of the compound adhering to the sides of the funnel was washed into the flask with approximately 90 ml. of the appropriate buffer solution. The flasks were then placed into a constant temperature bath at 97.30 / 0.05°C. and the contents allowed to attain the temperature of the bath. At that point the contents of the flasks were made up to the mark with the proper buffer solution and the flasks were securely stoppered. The first sample was withdrawn from each of the reaction vessels only after visible inspection showed that all of the chloramphenicol in each flask had dissolved and the timing of the reaction was started at this point. Samples, approximately 5 ml. in volume, were withdrawn with volumetric pipettes at appropriate intervals,

placed into glass vials (8 ml. capacity) and protected from the cap of the vial with aluminum foil.

The concentration of chloramphenicol in each sample was determined by partition chromatographic separation and subsequent spectrophotometric analysis according to a procedure reported elsewhere, (2).

DISCUSSION OF EXPERIMENTAL RESULTS

Sufficient experimental data have been collected in the present investigation to determine the order of the reaction with respect to chloramphenicol and to ascertain the relative catalytic activities of hydrogen ions, hydroxyl ions and certain general acids and bases within a pH range of 2-7.

Order of the Deterioration Reaction with Respect to Chloramphenicol.

As mentioned in the introduction, the rate of antibiotic disappearance from buffered aqueous solutions appears to be strictly first order with respect to antibiotic concentration. This relationship is apparently valid over widely differing hydrogen ion concentrations as is evident from Figure 1, 2 and 3. The linear logarithmic plots have been found to be extremely reproducible under all encountered experimental conditions and in no case have they deviated significantly from the overall first order relationship.

In much of the following discussion the rate of anti-

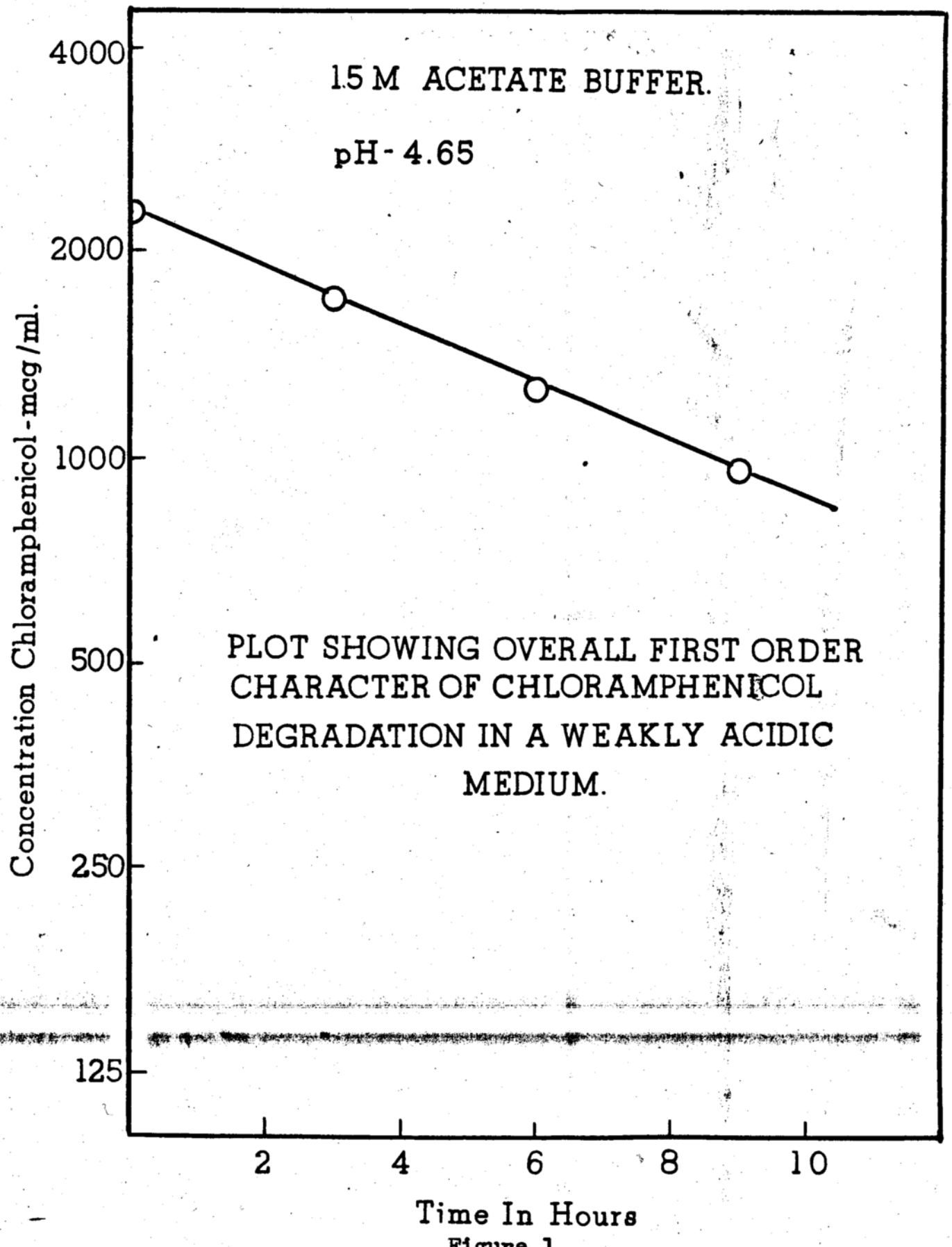

1.5 M ACETATE BUFFER.
pH - 4.65
PLOT SHOWING OVERALL FIRST ORDER CHARACTER OF CHLORAMPHENICOL DEGRADATION IN A WEAKLY ACIDIC MEDIUM.
Concentration Chloramphenicol - mcg/ml.
4000
2000
1000
500
250
125
2
4
6
8
10
Time In Hours

Figure 1.

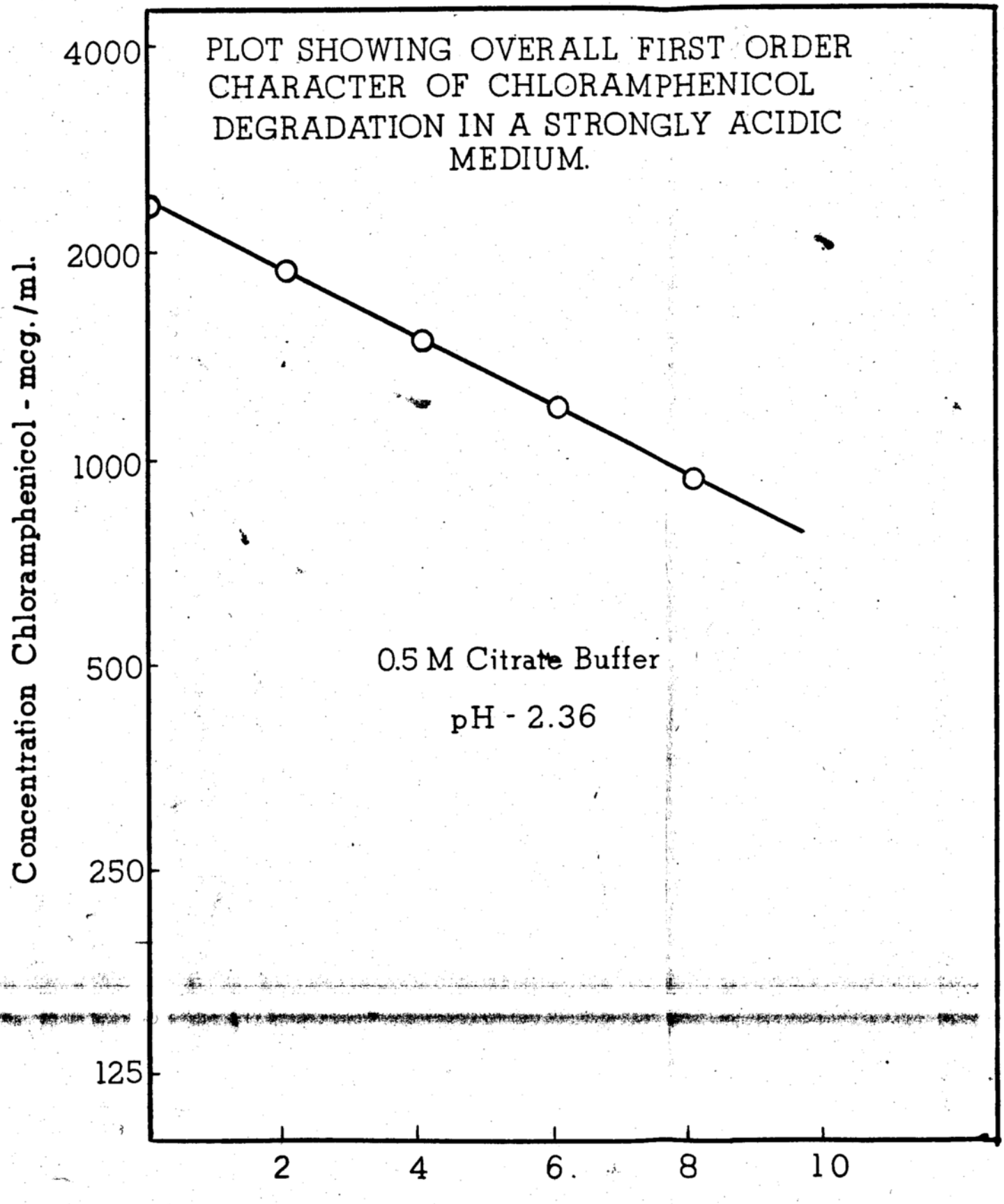

PLOT SHOWING OVERALL FIRST ORDER CHARACTER OF CHLORAMPHENICOL DEGRADATION IN A STRONGLY ACIDIC MEDIUM.
Concentration Chloramphenicol - mcg./ml.
4000
2000
1000
500
250
125
0.5 M Citrate Buffer
pH - 2.36
2
4
6
8
10
Time In Hours

Figure 2.

biotic disappearance is expressed in terms of half life of the drug because of the direct dependency of the deterioration rate upon the concentration of chloramphenicol. In certain instances, however, it was found convenient to make use of reciprocal half lives rather than the half lives themselves. This usage is particularly useful in determining the effect of general acid - base catalysis upon the rate of chloramphenicol disappearance. As may be seen from the relationship

$$k = 0.693/\text{half life}$$

where k is the first order rate constant, the value of the rate constant, in reciprocal time, is directly proportional to the reciprocal of the half life.

The strict adherence to first order dependency and the high degree of reproducibility suggests the likelihood that the rate determining step involves a simple ionic or molecular mechanism. Although free radical mediated reactions, such as are involved in all redox reactions, are not necessarily ruled out; they appear unlikely in view of the fact that such reactions often deviate greatly from first order dependency and are generally much less reproducible. This lends added weight to the suggestion made previously that the principle reaction responsible for chloramphenicol breakdown in neutral or acidic solutions is probably the hydrolytic cleavage of the amide function.

The absolute rate of chloramphenicol disappearance as determined from the plots shown in Figure 5, 6 and 7 indicates that the dehalogenation reactions contribute to but a very minor extent in the pH interval 6 - 7 and neglibly below pH 6.00, to the overall reaction. This is based on the fact that the maximum half life of chloramphenicol at 97.30° C. was 11 hours, whereas the minimum half life based on the dehalogenation reaction was over one hundred hours at pH 7 and much longer at lower hydroxyl ion concentrations (1).

Ionic Strength Independence of Degradation Rate.

The lack of a formal charge on the chloramphencol molecule would make it seem unlikely that the ionic strength of the buffers employed for degradation would be of significance in affecting the rate of degradation. However, in view of the partial ionic character of the nitro group and the fact that one of the resonance forms of an amide will show a separation of charge, it was thought that any dependency of the degradation rate upon ionic strength should be determined, so as to facilitate proper evaluation of data obtained in presence of buffers.

Degradation of chloramphenicol was therefore carried out at 97.30° C. in 0.062 M phosphate buffer adjusted to pH 7.00 and the half life thus obtained was compared with that obtained with the same buffer containing, in addition, 60 g. of sodium sulfate per liter. The difference in the ionic

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Degradation of chloramphenicol was therefore carried out at 97.30° C. in 0.06 M phosphate buffer adjusted to pH 7.00 and the half life thus obtained was compared with that obtained with the same buffer containing, in addition, 60 g. of sodium sulfate per liter. The difference in the ionic

strengths of the two systems was such that any dependency of the degradative rate upon the ionic strength of the medium would be indicated after analysis of the samples. As shown in Figure 4, practically identical results were obtained from both systems thus indicating that the rate of degradation is essentially independent of ionic strength. In both instances the half life of the antibiotic was 7.95 hours.

General Acid - Base Catalysis of Deterioration of Chloramphenicol.

In accordance with the thinking outlined previously, the possibility of general acid - base catalyzed deterioration was always present. Early in the course of the investigation it appeared that the rate of chloramphenicol disappearance was dependent upon the hydrogen ion concentration of the buffered systems in a rather complex manner. On the absence of any notable specific hydrogen or hydroxyl ion catalysis, the evidence pointed to the possibility of a general base or general acid present in the buffer acting as a catalyst.

An experimental verification of the part played by the components of a phosphate buffer is shown in Figure 5. In the plot, the reciprocal of the half lives of chloramphenicol in aqueous solutions containing varying amounts of phosphate buffer is shown as a function of the buffer concentration at 97.30° C. The pH of each solution was adjusted to 7.00 to minimize any effect of hydrogen or hydroxyl ions on the rate. It is evident from this figure that the rate of break-

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TABLE I

RATE OF CHLORAMPHENICOL DEGRADATION IN PHOSPHATE BUFFERS 0.2M WITH RESPECT TO MONOHYDROGEN PHOSPHATE ION AND CONTAINING VARYING AMOUNTS OF DIHYDROGENPHOSPHATE.

pH	Half Life - Hours
6.98	5.3 *
6.77	6.2
6.61	6.2
6.24	6.1
5.95	6.3

* The discrepancy introduced by this value, substantially below the others, may be ascribed almost entirely to hydroxyl ion catalysis of the dehalogenation reaction referred to in the introduction.

A similar investigation indicated that acetate buffers also act as catalysts for the deterioration of the antibiotic. In Figure 7, where experimentally determined reciprocal half lives have been plotted against total acetate concentration, i.e. the sum of the concentrations of equimolar sodium acetate and acetic acid, it is evident that the buffer exerts a small but definite catalytic effect on the overall rate. Unlike the phosphate systems, however, further studies have shown that the catalytic activity is associated almost entirely with the acidic component of the buffer and that the conjugate base possesses but slightly catalytic activity. Thus,

as is shown in Table II, systems containing constant amounts of acetic acid, variations in the acetate ion concentration have relatively little effect on the rate of degradation. These data suggest that the catalytic behavior is due essentially to general acid catalysis with but a small contribution resulting from general base catalysis.

TABLE II

RATE OF CHLORAMPHENICOL DEGRADATION IN ACETATE BUFFERS 0.05 M WITH RESPECT TO ACETIC ACID AND CONTAINING VARYING AMOUNTS OF SODIUM ACETATE.

pH	Sodium Acetate Concentration	Half Life - Hours
5.95	1.0 M	10.9 11.0 11.1
4.87	0.10 M	13.4 13.3
4.58	0.05 M	10.9 14.0 13.9

Although the catalytic properties of both phosphate and acetate systems can be ascribed to their acid-base characteristics, it is important to keep in mind that the effect may be a specific property of the catalytic species and only indirectly related to their acid-base character. This possibility is partially supported by a study of the degradative rate in presence of citrate buffers. Unlike the acetate or phosphate system, citrate systems present the possibility of

having more than one acidic or basic species in the buffer solution; thus there may be present

Citric acid	Monohydrogen citrate ion
Dihydrogencitrate ion	Citrate ion

all in equilibrium and possessing differing catalytic tendencies. This rather complex system can be expected to, and does, result in an involved dependency of the catalytic effect upon the hydrogen ion concentration although the degradative reaction itself is not specifically influenced, within this pH range, by hydrogen ions. (See below). The apparent dependency of the reaction rate, as expressed in half lives, on the hydrogen ion concentration is shown in Figure 8. It is evident that at higher pH values the half life is essentially unaffected by the buffer since the values are close to the value of 11 hours. In the pH region immediately below this, we find a rapid increase in the reaction rate which can be ascribed to the catalytic effect of the singly charged dihydrogen citrate and/or the doubly charged monohydrogencitrate. The minimum exhibited by the curve at pH approximately 3 and the very sharp reversal of the reaction rate appear difficult to rationalize. It is possible that either the singly or doubly charged ion or both possess significant catalytic activity but that the triply charged citrate ion and undissociated acid do not. These differences are difficult, however, to explain on the basis of general acid - base catalysis alone.

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A possible explanation of the unusual behavior of the

citrate systems may be found in the suggestion by Swain (13) that all reactions of this type, (i.e. general acid - base catalyzed) are termolecular in nature and involve both electrophilic and nucleophilic catalysts. The exceptional catalytic efficacy of the partially neutralized citrates being due, on this basis, to the dual nature of these acidic salts.

pH Independence of Rate of Chloramphenicol Degradation Over pH Range 2 - 7.

In the preceding two sections we tacitly assumed that the rate of the deterioration reaction was independent of hydrogen or hydroxyl ion concentration, i.e. not subject to specific hydrogen or hydroxyl ion catalysis. This assumption was based on the fact that the reaction rate in absence of any interfering buffer was found to exhibit pH independence.

Although it is impossible from a practical standpoint to directly determine the rate of the reaction at any given pH in absence of buffer because of the acidic nature of the reaction products, the corresponding rate can be obtained by extrapolation to zero buffer concentration of a series of results determined in presence of buffers of varying concentration. Thus in Figure 5, the intersection of the straight with the ordinate corresponds to the reciprocal half life at pH 7.00 and zero buffer concentration. In Figure 6, a similar plot is shown for a series of experimental determinations at pH 6.00. If the rate of chloramphenicol dis-

appearance due to the hydroxyl ion catalyzed dehalogenation reaction at pH 7.00 is subtracted from the overall rate of antibiotic disappearance at the same pH, the values at pH 6.00 and 7.00 are essentially the same, being equal in each case to 11 hours. The pH independence of the reaction rate is further demonstrated by noting that the extrapolated value for the reciprocal half life at pH 4.65 as shown in Figure 7 is identical with the value obtained similarly at pH 6.00. In addition, the data shown in Table I, when regarded in this light, strongly supports this view.

Further confirmation of the pH independence of the degradation rate within the pH range studied may be gained by reference to Figure 8. It may be seen that extrapolation of this plot to the pH extremes of the citrate systems employed once again give half life values of approximately 11 hours. Other data have shown that the deterioration reaction is not significantly affected by hydrogen ions above pH 2 nor by hydroxyl ions below pH 7.

PHARMACEUTICAL SIGNIFICANCE

Consideration of the experimental evidence presented can lead only to the conclusion that chloramphenicol possesses unusual stability in aqueous solutions, over a wide pH range. Despite the very great temperatures to which the antibiotic was exposed, the rate of degradation was relatively small. Inasmuch as the heat of activation for the dehalogenation has been shown to be 30,000 calories per mole, (1) and the indications, based on additional experimental determinations,

that the heat of activation for the postulated amide hydrolysis is of the order of 23,000 calories per mole, it is readily evident that within the pH range 2 - 7 the antibiotic shows almost no susceptibility to hydrolytic deterioration at ordinary temperatures.

At the same time, the very fact that a drug exhibiting the great stability of chloramphenicol is subject to degradation catalyzed by buffer constituents should point the way toward careful consideration of any buffers used in compounding or administering other pharmaceuticals which may be subject to hydrolytic degradation. Just as it would be prudent to dispense chloramphenicol in weakly buffered solutions, other pharmaceuticals possessing the amide linkage should be regarded very carefully with respect to stability in the presence of general acids and bases as well as hydrogen and hydroxyl ions. Thus the formulation of pharmaceuticals must consider not only manipulation of pH so as to maintain solubility or stability but also any deleterious effects upon the drugs so formulated by the very agents used to effect other pharmaceutically desirable properties.

In essence, an approach to the problems of drug deterioration from the standpoint of chemical kinetics provides very useful clues to proper formulation based upon actual knowledge of the character of the drug. In contrast, the purely empirical approach may overlook factors which are of substantial significance with respect to drug stability.

SUMMARY AND CONCLUSIONS

1. The overall degradation rate of chloramphenicol in aqueous solution has been shown to be first order with respect to the antibiotic over a wide range of hydrogen ion concentration.

2. The reproducibility of the experimental results along with the strict adherence to first order dependency indicate that in addition to hydrolytic cleavage at the carbon chloride bonds, similar cleavage occurs elsewhere in the molecule. This is assumed to be at the amide linkage.

3. The rate of chloramphenicol degradation is independent of the ionic strength of the medium employed for degradation and largely independent of hydrogen ion concentration within the pH range 2 - 7.

4. Definite evidence has been accumulated to show that chloramphenicol deterioration is catalyzed by species which are general acids and bases. Specifically, monohydrogenphosphate ion, undissociated acetic acid and both mono and dihydrogencitrate ions catalyze the rate of chloramphenicol degradation.

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ANALYTICAL PROCEDURE FOR SEPARATION AND DETERMINATION OF THE AMINE CORRESPONDING TO CHLORAMPHENICOL

The evidence noted previously that amide hydrolysis represents an important pathway for the degradation of chloramphenicol made it quite important to determine the magnitude of the hydrolytic reaction in the total degradative scheme. Although it appeared probable that hydrolysis was the only significant route of degradation in neutral and acidic solutions, this remained only an assumption until quantitative data could be obtained.

One of the products of hydrolytic cleavage of chloramphenicol at the amide linkage would be the amide, 1-(p-nitrophenyl)-2-amino-1,3-propanediol. Comparison of the rate of disappearance of chloramphenicol with the rate of appearance of the amide would serve to show whether or not reactions other than hydrolysis represented important routes of deterioration.

It was felt that the partition chromatographic method which had proven successful in separating chloramphenicol from its degradation products could be extended so as to allow selective elution of the amine. Certain minor changes were necessary for effective application of the chromatographic procedure but the general procedure remained unchanged. The utility of a chromatographic method was therefore made the subject of a separate investigation. The results obtained in this study are given in the following pages in the form of a note to be submitted for publication in Analytical Chemistry.

THE SEPARATION AND DETERMINATION OF CHLORAMPHENICOL AND 1-(p-nitrophenyl)-2-amino-1,3-propanediol [+]

Takeru Higuchi and Arnold D. Marcus

[+] A contribution from the School of Pharmacy, University of Wisconsin, Madison. This project was supported in part by the Research Committee of the Graduate School with funds supplied by the Wisconsin Alumni Research Foundation.

The present communication reports a partition chromatographic method for the simultaneous separation and determination of chloramphenicol and its main hydrolytic product, 1-(p-nitrophenyl)-2-amino-1,3-propanediol in aqueous solutions of degraded chloramphenicol. A paper partition chromatographic method for the qualitative identification of these products in similar systems has already been proposed by Glasko, Dill and Rebstock. (1) The filter paper method is, unfortunately, quite unsuited for the accurate quantitative determination of these components because of the small amounts of material available for final evaluation. The proposed procedure permits direct determination of much larger quantities and is an adaptation of the recently reported chromatographic method for quantitative determination of chloramphenicol alone. (2)

The partition systems employed in this investigation consisted of an aqueous internal phase held on silicic

acid and a mobile phase composed of either ethyl acetate or n-butyl alcohol in chloroform. Usually, samples to be analyzed are added to the partition columns in organic solvent solution. To obviate the necessity for removing water and attempting redissolution in a suitable organic solvent, it has been found more convenient to make direct use of aqueous solutions. The aqueous sample is mixed with dry silicic acid and added to the column in the form of a slurry dispersed in chloroform.

The elution of chloramphenicol from partition systems may be accomplished rather simply with 10% (v/v) ethyl acetate in chloroform. (2) The solubility characteristics of the amine require, however, a strongly polar solvent system to effect its elution. After a number of preliminary investigations with various solvent systems, it was found that 25% (v/v) n-butyl alcohol in chloroform was most satisfactory. It was also determined that the internal aqueous phase should be buffered so as to provide a weakly alkaline medium.

These findings were subjected to more critical evaluation by chromatographing an aqueous solution containing known amounts of chloramphenicol and a synthetic sample of 1-(p-nitrophenyl)-2-amino-1,3-propanediol. Ten per cent (v/v) ethyl acetate in chloroform was used to elute the antibiotic and 25% (v/v) n-butyl alcohol in chloroform was similarly employed to elute the amine. The eluate was

collected in 10 ml. fractions. The elution chromatogram obtained after analysis of the fractions is shown in Figure 1.

As was to be expected from the results of the investigation cited previously (2), the chloramphenicol peak is sharp and well defined. Figure 1 also shows that effective separation of the two components was achieved.

EXPERIMENTAL

Apparatus: Chromatographic columns of Pyrex glass, 50 cm. long and 2 cm. in diameter. Tight fitting plungers prepared from thick-walled capillary tubing were used to pack the columns. A Beckmann Model DU Spectrophotometer.

Reagents: Silicic acid, chromatography grade, sodium bicarbonate, sodium carbonate, ethyl acetate, n-butyl alcohol and chloroform.

All reagents were analytical grade.

The columns were prepared from 20 g. silicic acid, 20 ml. 0.6 M sodium bicarbonate solution and a sufficient quantity of chloroform in the manner described by Higuchi and Patel (3).

Procedure:

Pipet exactly 2 ml. of the aqueous sample into a 30 ml. beaker and add exactly 1 ml. of a saturated solution of sodium carbonate. Swirl to mix the contents and allow to stand for approximately 1 minute. Add 3 g. dry silicic acid and mix thoroughly with a nichrome spatula. Add 15 ml. chloroform and mix to form a homogenous slurry. Quantitatively transfer the slurry to the column, rinse the beaker with 10 ml. chloroform and transfer the rinsings

collected in 10 ml. fractions. The elution chromatogram obtained after analysis of the fractions is shown in Figure 1.

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Procedure:

Pipet exactly 2 ml. of the aqueous sample into a 30 ml. beaker and add exactly 1 ml. of a saturated solution of sodium carbonate. Swirl to mix the contents and allow to stand for approximately 1 minute. Add 3 g. dry silicic acid and mix thoroughly with a nichrome spatula. Add 15 ml. chloroform and mix to form a homogenous slurry. Quantitatively transfer the slurry to the column, rinse the beaker with 10 ml. chloroform and transfer the rinsings

to the column. Pack the slurry tightly onto the top of the column and collect the eluate in a 100 ml. graduate cylinder. Use caution when removing the plunger from the column to avoid concomitant removal of any adhering material.

When almost all of the supernatant liquid has entered the body of the column, use the plunger to push down any of the slurry which has adhered to the sides of the column. Then add to the column a slurry consisting of 1 g. silicic acid, 1 ml. 0.6 M sodium bicarbonate solution and 15 ml. chloroform. Pack this slurry very tightly onto the top of the column and remove the plunger with care.

After 40 ml. of the chloroformic eluate have been collected, change the eluant to 10% (v/v) ethyl acetate in chloroform and collect an additional 10 ml. of eluate in the cylinder. At this point, replace the cylinder with a 50 ml. volumetric flask and discard the previously collected eluate. When the eluate in the flask has reached the mark, replace with a suitable container and change the eluant to 25% (v/v) n-butyl alcohol in chloroform. (The 50 ml. of eluate collected contain all of the chloramphenicol.) Discard the next 20 ml. and replace the container with a 100 ml. volumetric flask. When the contents of this flask have reached the mark, collect an additional 25 ml. in a volumetric flask.

If the eluate in the 100 ml. flask is cloudy due to precipitation of water, add 10 ml. of 25% (v/v) n-butyl alcohol in chloroform at the first sign of cloudiness and swirl thoroughly. (The first 100 ml. of butanol - chloroform eluate contain the bulk of the amine. The additional 25 ml. contain that portion of the amine shown as the tail of the chromatogram in Figure 1.)

The chloramphenicol content of the 50 ml. flask is determined spectrophotometrically at 272 millimicrons. The amine contents of the other two flasks are determined similarly at 275 millimicrons.

The 125 ml. of butanol - chloroform eluate contain all of the amine originally present in the sample. In every instance the amine content of the 25 ml. flask was less than three per cent of the total. This amount is sufficiently significant, however, to prevent its being disregarded.

DISCUSSION

The applicability of this procedure was determined by analysis of several aqueous solutions containing known quantities of chloramphenicol and the amine. As shown in Table I, the recoveries of both components were quite satisfactory. The data of Table I show that the average error was in the neighborhood of 1%.

Inasmuch as the partition columns used to effect the separation were rather large and the size of the samples were correspondingly large, it is readily evident that much small quantities of chloramphenicol and 1-(p-nitrophenyl)-2-amino-1,3-propanediol can be determined by suitable reduction in the size of the column employed, the amount of sample added and the volume of eluate collected. There is no reason why the procedure cannot be adapted to the determination of microgram quantities of both components.

TABLE I

RECOVERY OF CHLORAMPHENICOL AND AMINE FROM AQUEOUS SOLUTIONS

Sample	Chloramphenicol Introduced	Chloramphenicol Recovered	Amine Introduced	Amine Recovered
1	1.19 mg.	1.18 mg.	0.94 mg.	0.93 mg.
2	1.19	1.20	0.94	0.95
3	1.19	1.20	0.94	0.93
4	-		2.89	2.80
5	-		2.89	2.86
6	-		2.89	2.88

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3. Higuchi, T. and Patel, K.P. Ibid 41, 171 (1952)

THE KINETICS OF DEGRADATION OF CHLORAMPHENICOL IN SOLUTION

III. THE NATURE, SPECIFIC HYDROGEN ION CATALYSIS AND TEMPERATURE DEPENDENCIES OF THE DEGRADATIVE REACTIONS.

Although the preceding studies strongly indicated that chloramphenicol degradation occurred primarily through hydrolytic cleavage of the amide function, the evidence available was purely circumstantial in nature. It was thought desireable, therefore, to establish conclusively whether this supposition was true or whether other degradative pathways contributed significantly to the overall rate of degradation. Such evidence could be obtained by comparing the rate of chloramphenicol disappearance with the rate of amine appearance, any difference in the two rates indicating the presence of other degradative reactions. These comparative studies were carried out through application of the analytical method developed for the separation and determination of chloramphenicol and its corresponding amine. The experimental data obtained in these studies are presented in the following section.

Despite the establishment of general acid - base catalyzed degradation of chloramphenicol, no specific hydrogen ion catalyzed degradation could be demonstrated within the pH interval 2 - 7. Since all previous studies concerning amide hydrolysis had shown that amide linkages are susceptible to catalytic hydrolysis by hydrogen ion, degradation of the antibiotic was investigated in media containing very high concentrations of the ion. The results of this study are also reported in the subsequent section.

In order to determine the temperature dependency of

the uncatalyzed (water) reaction discussed previously, degradation of chloramphenicol was carried out at two other temperatures. At the same time a similar study was initiated to determine the temperature dependency of the hydrogen ion catalyzed reaction. The data obtained in these studies permitted calculation of the heat of activation associated with the uncatalyzed and hydrogen ion catalyzed reactions. These data also made it possible to evaluate the constants for these reactions and to determine to a good approximation, the catalytic constant for undissociated acetic acid acting as a general acid.

Since these studies have been summarized in the form of a communication to be submitted for publication in the Journal of the American Pharmaceutical Association, Scientific Edition, no attempt will be made to duplicate the presentation.

THE KINETICS OF DEGRADATION OF CHLORAMPHENICOL IN SOLUTION *

III. THE NATURE, SPECIFIC HYDROGEN ION CATALYSIS AND TEMPERATURE DEPENDENCIES OF THE DEGRADATIVE REACTIONS.

Takeru Higuchi and Arnold D. Marcus

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* A contribution from the School of Pharmacy, University of Wisconsin, Madison. This project was supported in part by the Research Committee of the Graduate School with funds supplied by the Wisconsin Alumni Research Foundation.

ABSTRACT

The degradation of chloramphenicol in aqueous solutions below pH 7 has been shown to occur virtually entirely through hydrolytic cleavage of the amide function. It has been demonstrated that specific hydrogen ion catalyzed hydrolysis plays a major role in the degradation of the antibiotic in aqueous solutions below pH 2. The temperatures dependencies of the uncatalyzed (water) reaction and hydrogen ion catalyzed reaction have been determined and the catalytic constants for the various reactions contributing to the overall degradative rate have been evaluated. A possible mechanism to account for hydrogen ion catalyzed hydrolysis is also presented.

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In a previous communication (1) it was reported that chloramphenicol is subject to a type of general acid - base catalyzed degradation. These studies showed that an uncatalyzed reaction contributed significantly to the overall degradative rate. The results of a

further study establishing the chemical nature of the breakdown reaction, evaluating the heats of activation of the several reactions and demonstrating specific hydrogen ion catalysis during the degradative step are presented at this time.

The experimental procedures employed to effect degradation (1) of the antibiotic and the analytical methods (5,7) used have already been reported and will not be repeated here.

ESTABLISHMENT OF THE NATURE OF THE DEGRADATIVE STEP

The experimental evidences obtained previously (1,2) strongly indicated that amide cleavage represented the principal route of degradation. Furthermore, it had been shown that one of the products of chloramphenicol metabolism is the corresponding amine, 1-(p-nitrophenyl)-2-amino-1,3-propanediol (3) and it is known that this same compound is a product of the reaction between chloramphenicol and strong acids or bases. (3) These studies did not, however, entirely eliminate the possibility that other significant degradative pathways may exist. Indeed, it has been found that reduction of the nitro group takes place in the presence of certain bacterial extracts (4) and that the antibiotic may be oxidized to p-nitrobenzaldehyde. (3)

The extent of any secondary degradative reaction

can be readily established by analysing solutions of chloramphenicol at various stages of degradation for the residual drug and for the amine released by hydrolytic cleavage of the amide group. Any difference between the concentration of amine found by direct analysis and the amounts of amine calculated from the quantity of chloramphenicol which has undergone degradation can be attributed to some other degradative pathway. We have carried out this comparison for aqueous solutions of chloramphenicol at pH 6.00 and 4.65 utilizing an analytical procedure reported elsewhere. (5)

As shown in Figure 1 and 2, the rate of loss of the antibiotic from solution and the rate of appearance of the amine are essentially the same.[1] The points calculated from determination of chloramphenicol based upon the assumption that one amine molecule is formed for every molecule of the antibiotic lost are shown in both plots. It is evident that for all practical purposes the reaction involving amide cleavage was responsible for the entire observed loss of the antibiotic.

In view of the conclusions presented above and the known response of amides to high concentrations of hydrogen

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[1] The concentration of a product of any first order reaction is governed by the expression

$$-\ln (C - C_t) \quad kt + k'$$

where C is the concentration of the product at infinite time and C_t is the concentration at time 't'.

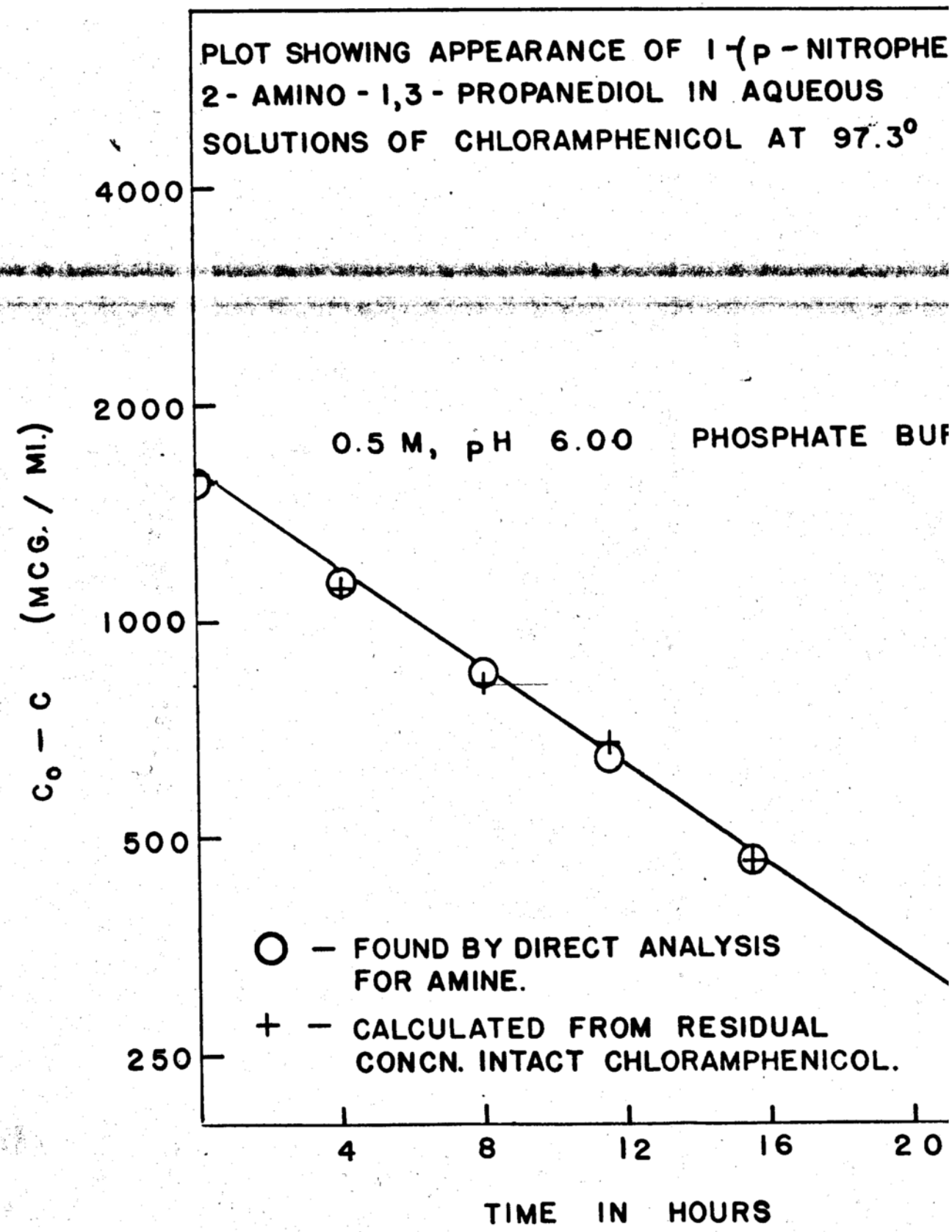
PLOT SHOWING APPEARANCE OF 1-(p-NITROPHE
2-AMINO-1,3-PROPANEDIOL IN AQUEOUS
SOLUTIONS OF CHLORAMPHENICOL AT 97.3°
0.5 M, pH 6.00 PHOSPHATE BUF
4000
2000
1000
500
250
$C_o - C$ (MCG./ML.)
O — FOUND BY DIRECT ANALYSIS FOR AMINE.
+ — CALCULATED FROM RESIDUAL CONCN. INTACT CHLORAMPHENICOL.
4
8
12
16
20
TIME IN HOURS

Figure 1.

ions, there can be very little doubt that in aqueous solutions below pH 7, hydrolytic cleavage of the amide group represents the only significant route of chloramphenicol degradation.

SPECIFIC HYDROGEN ION CATALYSIS

Although it was shown previously (1) that chloramphenicol degradation is independent of hydrogen ion concentration within the pH interval 2.3 - 6, there were reasons to believe that specific hydrogen ion catalysis would occur at very low pH values. Earlier investigations into amide hydrolysis showed that the hydrolytic reaction exhibited no significant range of pH independence. (6) Every previous investigation of the kinetics of amide hydrolysis had shown that hydrolytic cleavage was remarkably susceptible to catalysis by hydrogen ions (specific hydrogen ion catalysis). It has been shown, moreover, that when the antibiotic is exposed to solutions of strong acids, hydrolytic cleavage readily occurs with the formation of the corresponding amine. (3)

As part of the present investigation, experiments were carried out to determine the catalytic activity of hydrogen ions at relatively high concentrations. Perchloric acid solutions, ranging in concentration from 0.025 - 0.2 M., were employed as the source of the ion. This acid was chosen because of its very low volatility and because it is virtually totally dissociated in these concentrations.

The results of degradation effected in these media are summarized in Figures 3 - 5. The broken circles represent the magnitude of the uncatalyzed reaction these values being obtained by extrapolation to zero buffer concentration at pH 6.00 and 4.65 as shown in Figures 6 - 9. The plots in Figures 3 - 5 conclusively demonstrate specific hydrogen ion catalysis to play a major role in the degradation of chloramphenicol at sufficiently low pH values.

It is of particular interest to note that extrapolations of the straight line portions of Figures 3 - 5 pass through the origin. This can be interpreted only as resulting from a quenching action of hydrogen ion on the uncatalyzed reaction. This behavior can be rationalized on the basis that in the presence of sufficiently high concentrations of hydrogen ion there is a change in the nature of the amide substrate. The results of a study of the effect of hydrogen ion concentration upon the apparent solubility of the antibiotic supports this line of thinking. Figure 11 shows an increase in the apparent solubility of the drug at 30°C. with increasing concentrations of hydrogen ion. This increase is most logically attributable to the basic character of the amide linkage resulting in the formation of a protonated intermediate.[2]

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[2] Although the solubility study was carried out at 30°C. and the kinetic studies at much higher temperatures, the qualitative picture is very probably the same.

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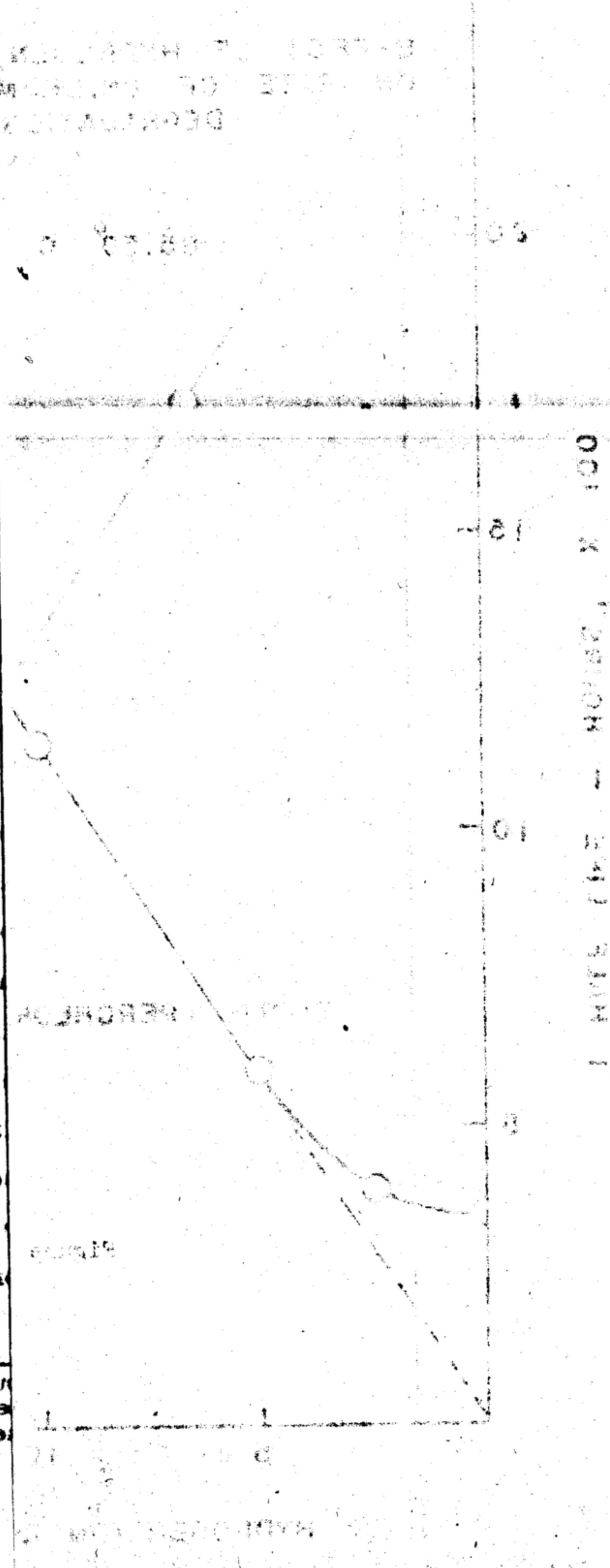

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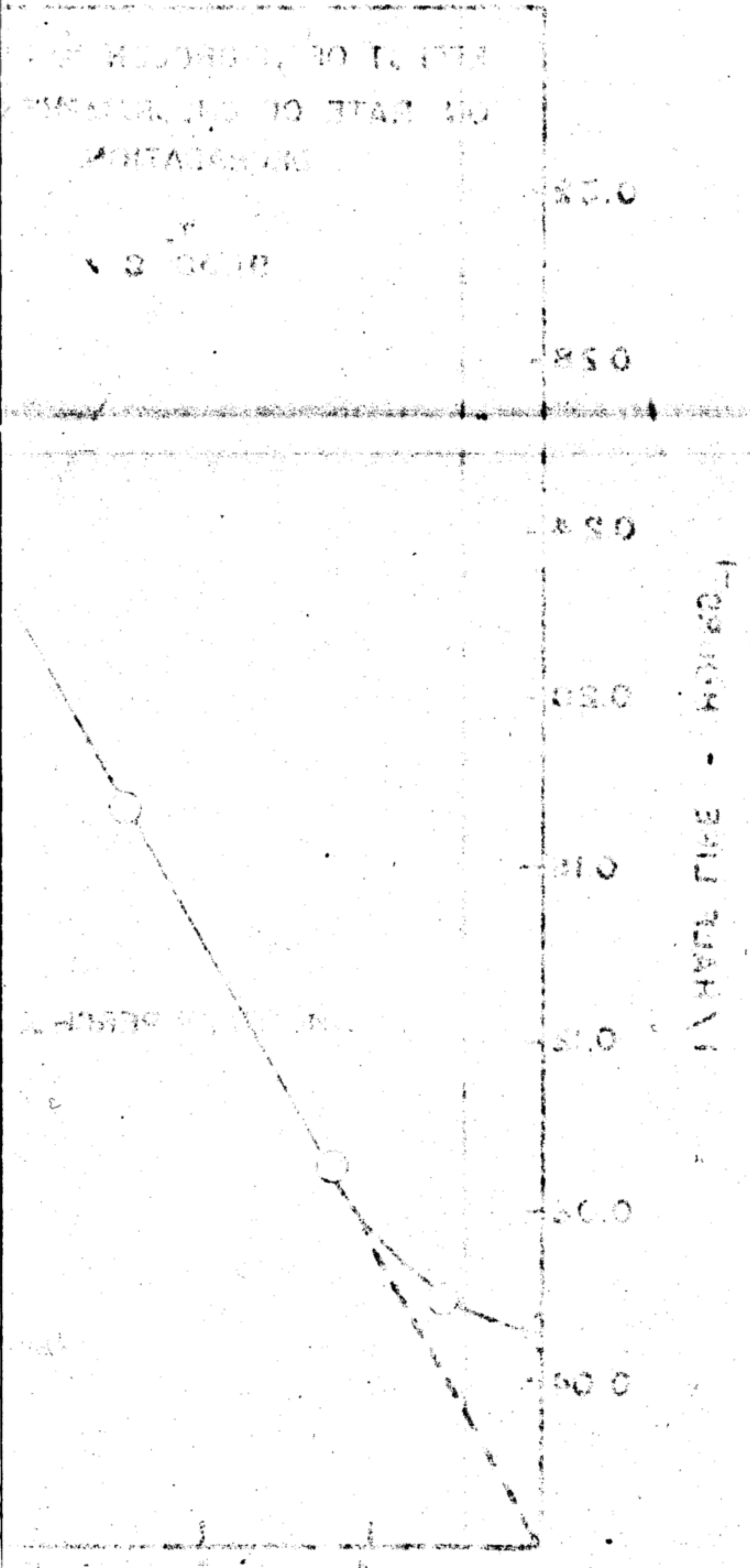


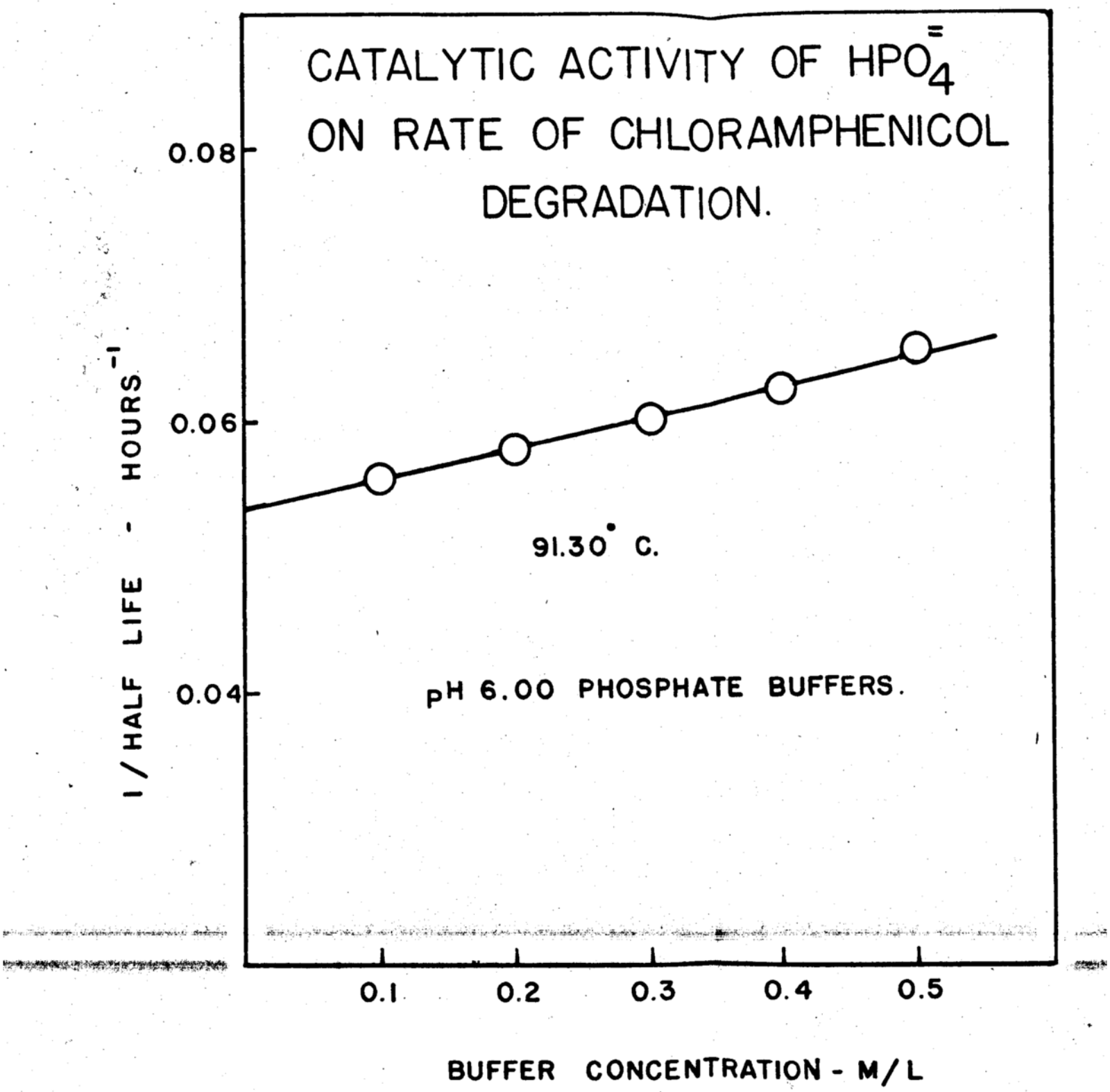
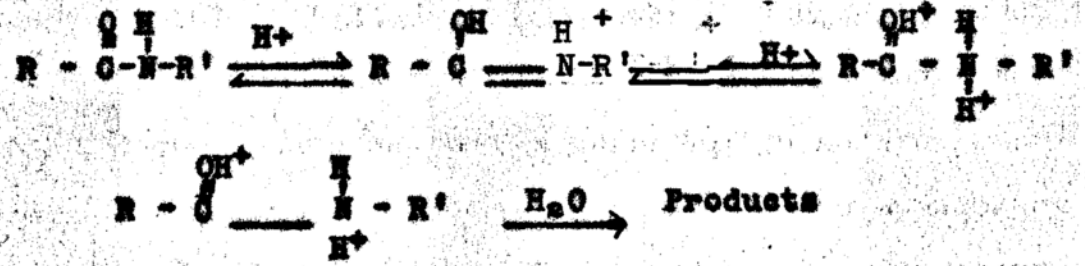

CATALYTIC ACTIVITY OF $HPO_4^{=}$
ON RATE OF CHLORAMPHENICOL
DEGRADATION.
0.08
0.06
0.04
1 / HALF LIFE - HOURS^{-1}
91.30° C.
pH 6.00 PHOSPHATE BUFFERS.
0.1
0.2
0.3
0.4
0.5
BUFFER CONCENTRATION - M/L

Figure 8.

Postulation of a mechanism to account for the hydrogen ion catalyzed hydrolysis of chloramphenicol is rather difficult. A scheme such as the following would appear, however, to represent the course of the reaction:



Despite the possible apparent theoretical difficulty involved in the formation of the doubly charged complex, this probably represents the mechanism of specific hydrogen ion catalyzed hydrolysis most accurately.

TEMPERATURE DEPENDENCY

The Uncatalyzed (Water) Reaction:

From previously reported data (1) and from the values of reciprocal half lives obtained by extrapolation to zero buffer concentration in Figures 6 - 9, it is possible to calculate the temperature dependency of the uncatalyzed hydrolytic cleavage reaction. Figure 12 shows that the reaction at both pH 6.00 and 4.65 yields a typical Arrhenius type plot. The heats of activation as determined from the slopes of these lines were 24.0 and 24.4 Kcal./mole. This slight variation is well within the limits of the experimental techniques.

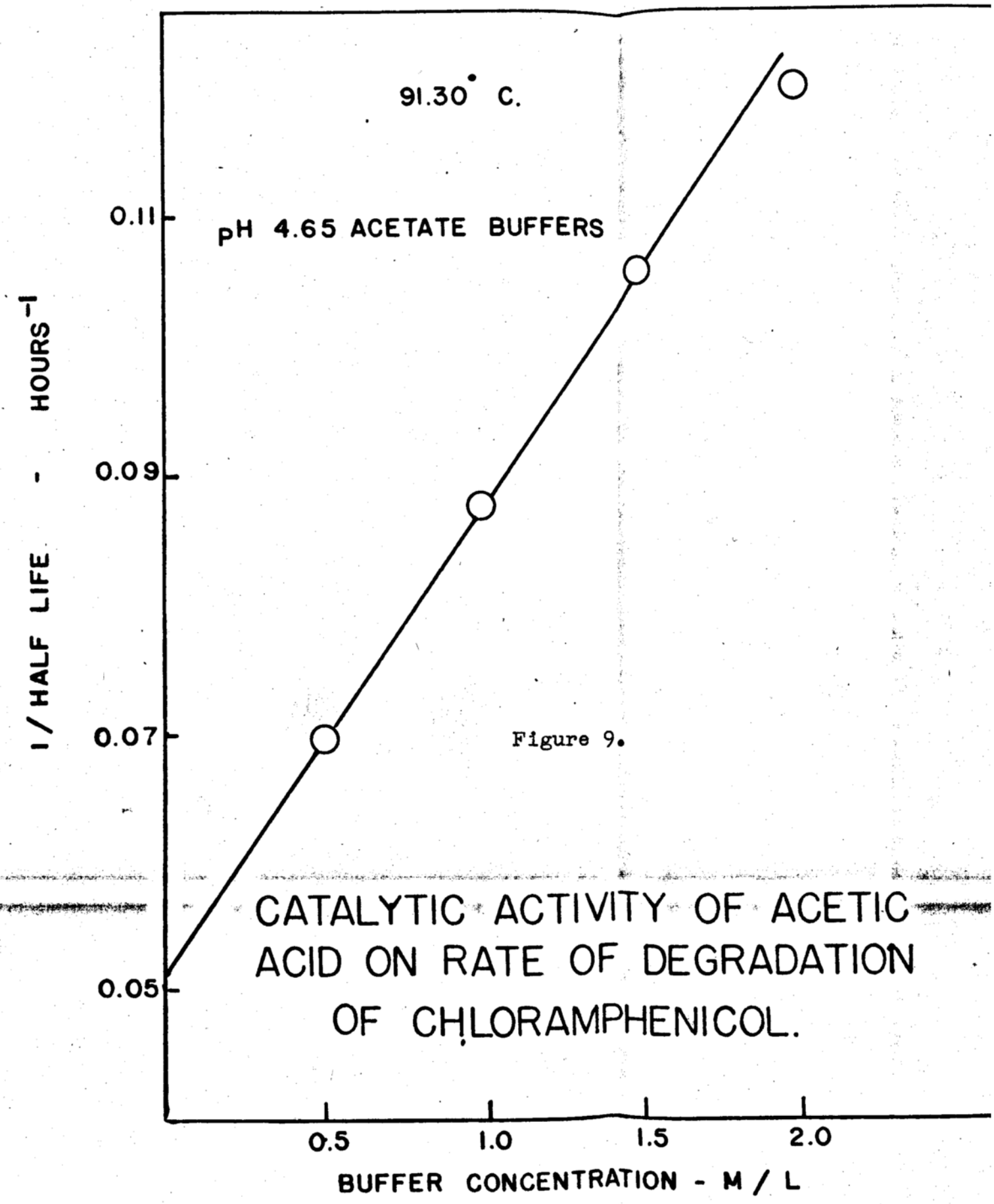


Figure 9.

CATALYTIC ACTIVITY OF ACETIC ACID ON RATE OF DEGRADATION OF CHLORAMPHENICOL.

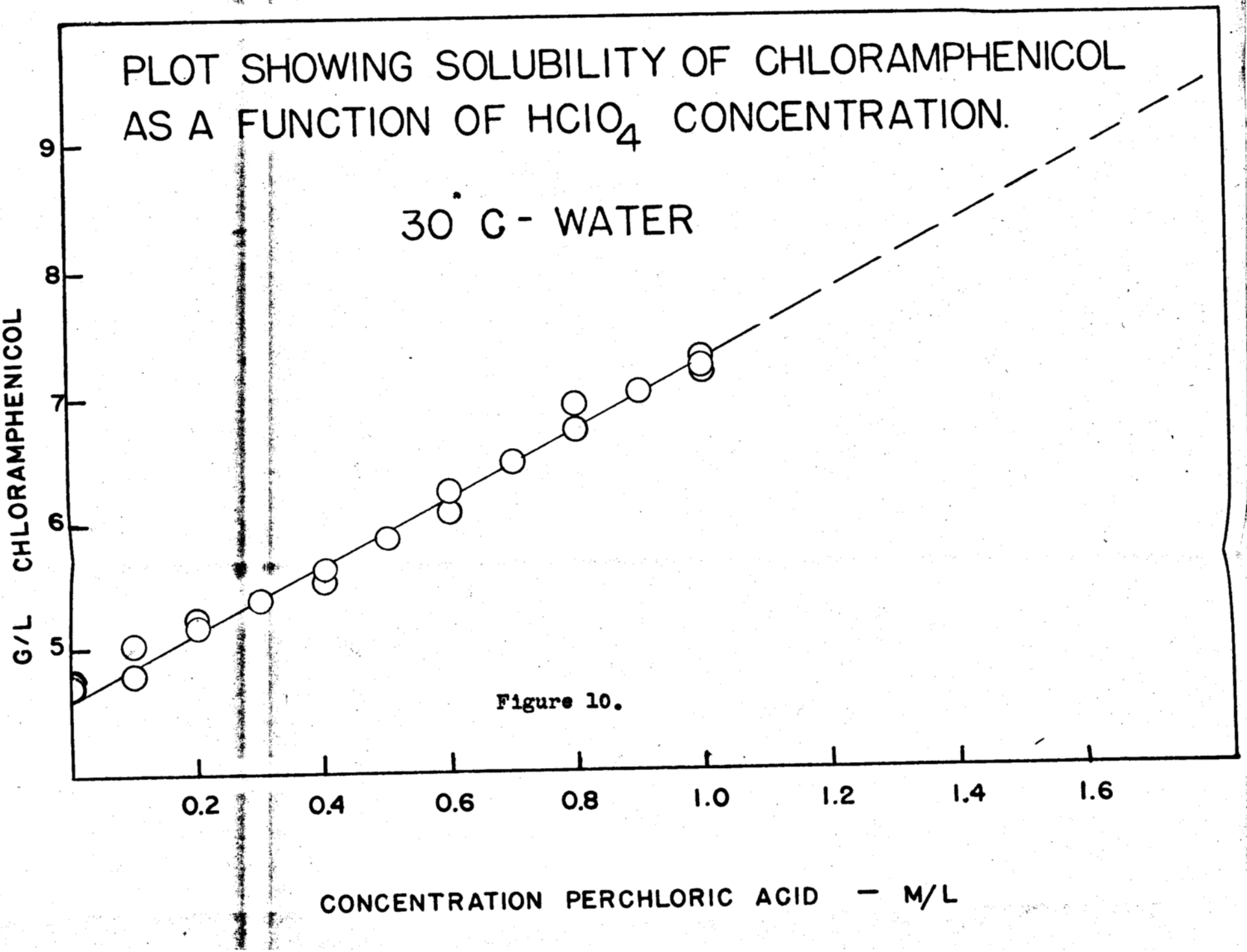

PLOT SHOWING SOLUBILITY OF CHLORAMPHENICOL
AS A FUNCTION OF $HClO_4$ CONCENTRATION.
30° C - WATER
G/L CHLORAMPHENICOL
9
8
7
6
5
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
CONCENTRATION PERCHLORIC ACID — M/L

Figure 10.

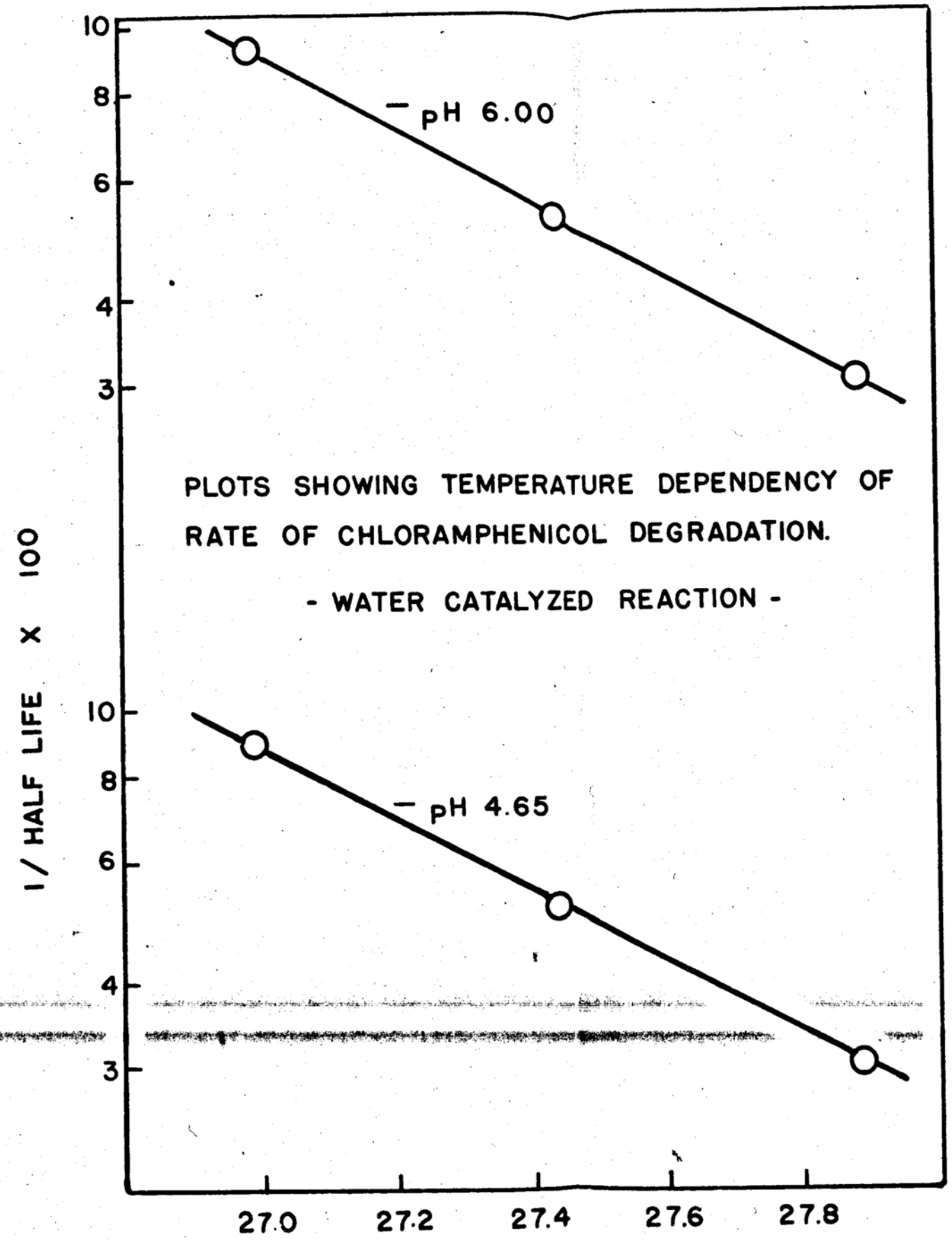

10
8
6
4
3
— pH 6.00
PLOTS SHOWING TEMPERATURE DEPENDENCY OF
RATE OF CHLORAMPHENICOL DEGRADATION.
- WATER CATALYZED REACTION -
1/ HALF LIFE X 100
10
8
6
4
3
— pH 4.65
27.0
27.2
27.4
27.6
27.8
1/T X 10^4

Figure 12.

The Hydrogen Ion Catalyzed Reaction:

The temperature dependency of the hydrogen ion catalyzed reaction, expressed as a second order reaction, is shown in Figure 13. The catalytic constants[3] expressed as reciprocal half lives, with the dimensions liters moles^{-1} hours^{-1}, were determined from the slopes of the straight line portions of Figures 3, 4 and 5. The heat of activation for this reaction as determined from the slope of the Arrhenius plot in Figure 13 was 19.5 Kcal./mole.

GENERAL RATE EQUATION

From present and previous considerations (1,2), it is evident that the degradation of chloramphenicol in aqueous solution is represented by no single reaction. Even in systems in which the rate of degradation is uncomplicated by hydrolytic cleavage of the carbon - chlorine bonds (2), the observed rate constant represents the sum of a number of catalytic constants. For aqueous solutions below pH 7, the observed rate of loss of activity, k_{obs}, may be given as

$$k_{obs} = k_{H_2O}\, H_2O + k_{H^+}\, H^+ + k_{OH^-}\, OH^- + k_{HB}\, HB + k_B\, B$$

where k_{H_2O}, k_{H^+}, k_{OH^-}, k_{HB}, and k_B are constants for the uncatalyzed, hydrogen ion, hydroxyl ion, general acid and general base catalyzed reactions respectively.

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[3] It is not entirely accurate to use the term "catalytic constant". The values used in calculating the slopes of the plots in Figures 3, 4 and 5 were actually reciprocal half lives. These half life values are, however, directly proportional to the values of the corresponding rate constants and the slopes of the lines remain unchanged.

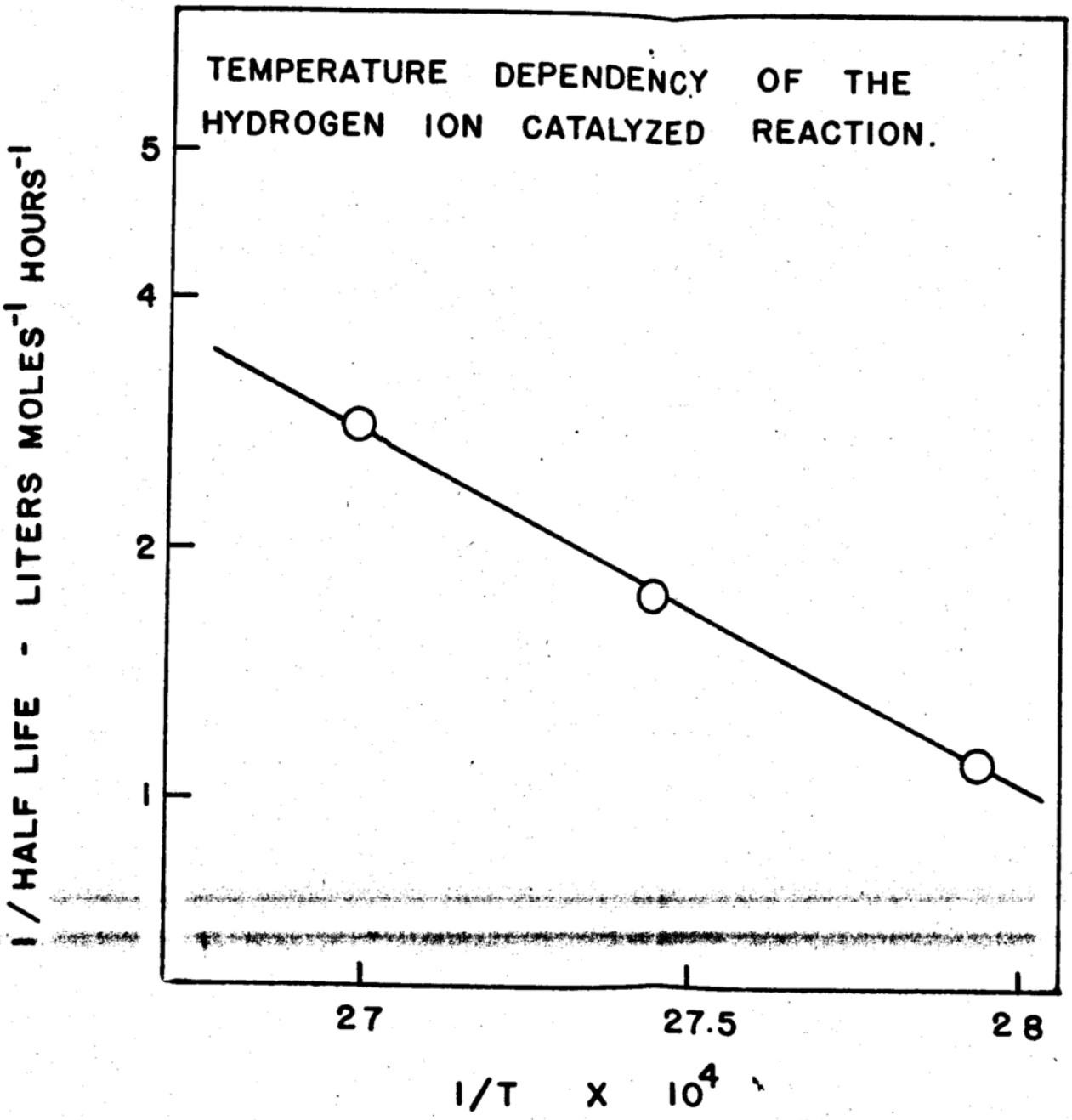
TEMPERATURE DEPENDENCY OF THE HYDROGEN ION CATALYZED REACTION.
1 / HALF LIFE - LITERS MOLES^{-1} HOURS^{-1}
5
4
2
1
27
27.5
28
1/T X 10^4

Figure 12.

Since the contribution of any catalytic species depends upon the pH of the system as well as the concentration and nature of the catalyst, the observed rate constant may often be represented more simply. In the case of those degradations carried out in the presence of moderate concentrations of a totally dissociated acid, for example, the above equation may be written

$$k_{obs} = k_{H^+} H^+$$

The rate constants for the uncatalyzed reaction and the catalytic constants for the hydrogen ion catalyzed reaction have been evaluated and are presented in Tables I and II. Table III gives the approximate values for the catalytic constant of undissociated acetic acid in the hydrolytic reaction. Although experimental data have been obtained for the catalytic activity of the general base $HOP_4^{=}$, the magnitude of its catalytic effect was too small to permit evaluation or reasonable approximation. The catalytic activities of any other general acids or bases must be determined separately by specific experimentation.

GENERAL DISCUSSION

As is apparent from these studies the degradation of chloramphenicol in aqueous solutions is obviously a complicated phenomenon representing a summation of many individual reactions. Separation and study of the several

degradative pathways have, however, been achieved. Our studies have so far been confined to degradative mechanisms operating in neutral and acidic systems. Other studies are presently in progress concerning degradation in alkaline media.

TABLE I

RATE CONSTANTS FOR THE UNCATALYZED (WATER) REACTION*

Temperature - ° C.	k - Hours^{-1}
97.30	0.0631
97.30	0.0651
91.30	0.0371
91.30	0.0371
85.36	0.0212
85.36	0.0212

* Two values for the rate constant are presented at each temperature because the rate constant of the uncatalyzed reaction was determined by extrapolation to zero buffer concentration at both pH 6.00 and 4.65.

TABLE II

CATALYTIC CONSTANTS FOR THE HYDROGEN ION CATALYZED REACTION

Temperature - ° C.	k_H - Liters Moles^{-1} Hours^{-1}
97.30	2.00
91.30	1.223
85.59	0.792

TABLE III

CATALYTIC CONSTANTS FOR ACETIC ACID CATALYZED REACTION*

Temperature - ° C.	k_{HOAc} - Liters Moles^{-1} Hours^{-1}
97.30	0.058
91.30	0.044
85.36	0.034

* Since these values were obtained from systems varying in buffer capacity but adjusted to the same pH values they represent only a good approximation.

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7. Higuchi, T., Marcus, A. and Bias, C., THIS JOURNAL, In Press

SUMMARY

Degradation of chloramphenicol in neutral, weakly acidic and strongly acidic aqueous solutions has been studied from a chemical kinetic standpoint. The rate, extent and dependencies of the degradative reactions were measured by partition chromatographic techniques for the separation of chloramphenicol and the corresponding amine, 1-(p-nitrophenyl)-2-amino-1,3-propanediol, and subsequent determination of these compounds by ultra-violet spectrophotometry. The analytical procedures employed were developed as part of the present investigation and show much promise for general application.

Degradation of the antibiotic has been shown to be the summation of a number of catalyzed and uncatalyzed reactions. An uncatalyzed (water) reaction, a specific hydrogen ion catalyzed reaction and general acid - base catalyzed reactions were determined to contribute to the overall breakdown of the drug. The data obtained upon comparison of the rate of chloramphenicol disappearance and amine appearance show rather conclusively that in neutral and acidic aqueous solutions hydrolytic cleavage of the amide function is the only significant degradative pathway.

It has been demonstrated that the hydrolytic reaction is independent of hydrogen ion concentration with the pH

interval 2 - 6. This represents the first report of any extensive range of pH independence for amide hydrolysis. This is also the first investigation to show that hydrolytic cleavage of an amide linkage may be subject to a type of general acid - base catalysis.

Despite the wide range within which the hydrolytic reaction is pH independent, specific hydrogen ion catalyzed hydrolysis has been demonstrated to occur at pH approximately 2 and below. The hydrogen ion catalyzed reaction apparently occurs through the formation of a protonated intermediate which appears to be the species which actually undergoes hydrolytic cleavage. This postulation is supported by a study which indicated the apparent solubility of chloramphenicol to increase linearly with hydrogen ion concentration.

The various catalyzed and uncatalyzed hydrolytic reactions obey the Arrhenius relationship without apparent deviation. The heats of activation of the uncatalyzed and the hydrogen ion catalyzed reactions have been determined to be 24.2 and 19.5 kcal./mole respectively. The constants for the uncatalyzed and hydrogen ion catalyzed reactions have been accurately evaluated. The value of the catalytic constant for undissociated acetic acid has been determined to a good approximation. The general base, monohydrogenphosphate ion, has been shown qualitatively to possess

significant catalytic activity, accurate quantitative evaluation being difficult because of the relatively low rate of this reaction as compared to the uncatalyzed reaction.