THE CHEMISTRY OF CHLORAMPHENICOL 3-MONOSUCCINATE

by Beverly J. Sandmann

(Under the supervision of Professor Takeru Higuchi)

Chloramphenicol 3-monosuccinate, a water soluble derivative of chloramphenicol, has been shown to undergo an isomerization in aqueous solution to form an equilibrium mixture which consists of itself and a rearranged compound. The present investigation has been concerned with the study of the general chemistry of chloramphenicol 3-monosuccinate in aqueous solutions, including 1) the pH profile for the rate of equilibrium formation, 2) the effect of buffer concentration and buffer type on the rate of equilibration, 3) the nature and chemical composition of the equilibrium mixture, and 4) isolation and characterization of the rearranged species.

The optical activity of solutions of chloramphenicol 3-monosuccinate was found to change at a rate directly relatable with that for the rate of equilibrium attainment as determined by column chromatography. Kinetic data for the approach to equilibrium as obtained by following the change in optical rotation of solutions of the monosuccinate ester at various pH values showed the reaction to be effectively first order with respect to substrate concentrations over the range of concentrations and pH employed in this study.

The rate of both the forward and reverse reaction was found to vary directly as hydroxyl ion concentration over a pH region in which the acid functional group is completely ionized. Rate data for the approach to equilibrium below pH 7 did not indicate a direct hydroxide ion dependency but seemed to be the result of an additional contribution to the rate from a very slow uncatalyzed rearrangement of the mono anionic species or hydroxide ion catalyzed attack on the uncharged form. The kinetic behavior best fits a simple $A \rightleftharpoons B$ model which for this system can be written:

$$(CMMSH)_l \underset{k_r}{\overset{k_f}{\rightleftharpoons}} (CMMSH)_r$$

where,

$(CMMSH)_l$ = chloramphenicol 3-monosuccinate

$(CMMSH)_r$ = the rearranged compound.

From the rate data the isomerization of chloramphenicol 3-monosuccinate was found to follow the rate law:

$$k_f = k_1(CMMSH)_l(OH^-) + k_2(CMMS^-)_l(OH^-)$$

where,

$(CMMS^-)_l$ = ionized chloramphenicol 3-monosuccinate.

By using the specific rate constants evaluated from experimental data and the dissociation constant for the ionization of the carboxylic acid group it was possible to

calculate a theoretical pH-rate profile which was in good agreement with the observed results.

Results of a study of the effect of various buffer systems and of ionic strength on the rate of approach to equilibrium revealed that neither buffer type nor ionic strength had any observable effect on the rate. Slight buffer catalysis by phosphate systems was observed above pH 7.

The influence of variables such as pH and concentration on the equilibrium as well as the composition of the equilibrium mixture was also studied. Over the pH range of 4.0 to 9.0 the equilibrium mixture was found to always consist of approximately 75% of chloramphenicol 3-monosuccinate and 25% of the rearranged compound. Spectral data of the rearranged component isolated from equilibrium mixtures obtained over the pH range studied indicated that only one isomeric species was being formed at all pH values.

Isolation and purification of the rearranged compound was achieved by using a reverse phase column chromatographic method and extraction technique. Repetitive attempts to recrystallize the semi-solid residue corresponding to the pure rearranged compound were unsuccessful although the residue was shown to be a single chemical entity by tlc and nmr. Immediate analysis of the isolated compound was required because of its tendency to degrade even under cold, dark and dry storage conditions.

Characterization of the rearranged compound was accomplished by the use of nmr, ir, and ord data. A comparison of the nmr spectra of chloramphenicol 3-monosuccinate and its equilibrium component showed several significant differences in the chemical shifts and splitting patterns of the propane skeleton protons, the succinate methylene protons and the hydroxylic hydrogens. From these data it was possible to propose that the structure for the rearranged compound was a cyclic hemi ortho ester. Further proof for this structure was obtained from ir and ord data. The difference in the carbonyl absorbance for these two compounds also supported the proposed structure. The shape of the ord curves of chloramphenicol 3-monosuccinate and the isolated rearranged compound was also significant. The ord curve for chloramphenicol 3-monosuccinate exhibited a positive Cotton-effect while that for the cyclic isomer showed a negative Cotton-effect.

The formation of the cyclic hemi ortho ester was proposed to take place by the attack of the ionized C-1 hydroxylic oxygen on the ester carbonyl carbon of the C-3 mono ester to give the cyclic meta-dioxane-like structure which is capable of collapsing to give back the more thermodynamically stable linear form of chloramphenicol 3-monosuccinate.

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by

BEVERLY J. SANDMANN

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

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TO MY PARENTS

For their constant encouragement to seek out the highest academic achievements, for their unselfish support of my educational desires, and for their love and understanding at all times, I am very grateful.

ACKNOWLEDGEMENTS

I wish to express my sincere appreciation to Professor Takeru Higuchi for suggesting this problem and for his helpful guidance and encouragement throughout my graduate studies at the University of Wisconsin.

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INTRODUCTION

Regulations governing sale and distribution of drugs in this country generally require that the chemical nature of at least the active ingredient or ingredients be well established. Superficially this requirement appears to be generally met and does not seem to offer any serious problem. What would appear to be a single chemical species, and is described as such, however, often exists actually in a number of different forms. Thus we could have mixtures of polymorphs, of solvated and unsolvated crystals, of keto-enol equilibrium species in solution, epimerization products in solution, etc. In most cases the existence of such a mixture does not significantly alter the therapeutic efficacy of the drug preparation. This may not always be true. The possibility of such subtle variations in the character of drug systems must always be kept in mind.

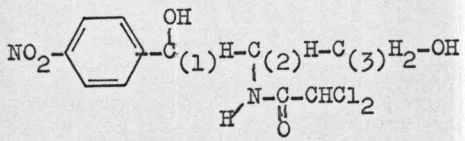
During the course of routine analytical research, Szulczewski and co-workers (1) found that sodium chloramphenicol-3-monosuccinate in aqueous solution at near neutral pH's was incapable of independent existence and formed an equilibrium mixture with a different molecular structure. Further work in our laboratory confirmed the equilibrium reaction and showed that this different molecular form was a cyclic hemi ortho ester. The present contribution is concerned with results of an

investigation designed to establish the pH profile of this interesting reaction; to determine the catalytic species involved; to isolate, if possible, the isomeric form; and to characterize the reaction product.

Being a derivative of the antibiotic chloramphenicol, consideration of the chemistry of the monosuccinate derivative must also be one of the parent compound. Hydrolysis of the amide linkage and the rate of loss of chloride ions have been carefully studied as well as the nature of the degradation products (2-4). The acyl migration of the dichloro acetyl group has been studied (5), and some of this work has been verified in the present contribution.

PAST WORK ON CHLORAMPHENICOL

Research on the chloramphenicol molecule has been both intense and varied since its discovery in 1947 (6). For a period of two years extensive investigations were undertaken to determine the chemical nature of this compound as well as its biological behavior and medical usefulness. This broad spectrum antibiotic was characterized in 1949. The total synthesis was reported and chloramphenicol was shown to be D(-)three-1-p-nitrophenyl-2-dichloroacetamido-1,3-propanediol, I, (7).



I

The complexity of the molecule as well as the great pharmaceutical interest in this antibiotic initiated several research projects to study its chemistry, stability and degradation.

Several kinetic studies were undertaken by various researchers to determine the route of degradation and the important hydrolytic pathways. In the first reported kinetic study on the degradation of chloramphenicol, Higuchi and Bias (2) showed the hydrolytic cleavage of the carbon to chlorine bonds, in aqueous solutions above pH 7.0, to represent an important degradative pathway. The results of this study showed that the overall rate of chloramphenicol degradation, as measured in terms of chloride ion production, could be considered as a summation of at least three first order reactions:

1. The uncatalyzed hydrolytic cleavage of chloride ions from intact chloramphenicol molecules.

2. The hydroxyl ion catalyzed cleavage of chloride from intact chloramphenicol molecules.

3. The hydrolysis of the amide linkage of chloramphenicol followed by subsequent displacement of the halogens of the dichloro-acetate anion hydrolysis product to yield chloride ions and other products.

Further kinetic studies were carried out by Higuchi, et al. (3,4) on the kinetics of chloramphenicol degradation in neutral and acidic aqueous solutions. Under these conditions the only significant pathway of degradation was via the hydrolytic cleavage of the amide linkage. They

reported that the rate of amide hydrolysis, followed by the disappearance of unreacted chloramphenicol, represented the summation of an uncatalyzed, hydrogen ion catalyzed and general acid-general base catalyzed reactions. All of these reactions were shown to conform to the first order rate law with respect to the disappearance of unreacted chloramphenicol.

Extrapolation of rate data over the pH interval 2.0 to 6.3 showed the hydrolytic cleavage of the amide group to be independent of the external hydrogen ion concentration. This was the first report of pH independence of amide hydrolysis over this range of hydrogen ion concentration and was postulated to be the result of an uncatalyzed water reaction (3).

Below pH 2 specific hydrogen ion catalysis has been found to play a major role in the degradation of chloramphenicol (4). This report postulated that the hydrogen ion catalyzed reaction proceeded by formation of a protonated intermediate which then underwent subsequent hydrolytic cleavage. A study of the solubility of chloramphenicol as a function of hydrogen ion concentration indicated that the apparent solubility of chloramphenicol increased linearily with the hydrogen ion concentration. Although this lent support to this proposed mechanism later studies by Marcus and Taraszka concerning the specific hydrogen ion catalyzed hydrolysis

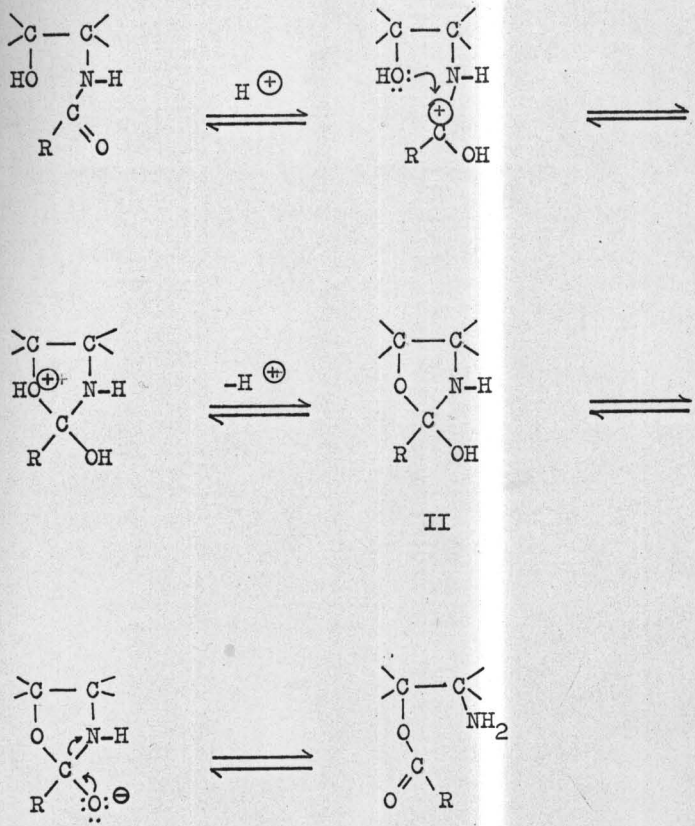
of chloramphenicol using solvents with varying dielectric constants (8) were not in agreement with the mechanism postulated by Higuchi and Marcus. Neither postulation is all inclusive and more recent work has failed to resolve this mechanism question.

Observations by Higuchi, et al. concerning the general acid-general base catalyzed hydrolysis of chloramphenicol have revealed several interesting cases of apparent bifunctional catalysis by various carboxylic acids (3,9). A study of chloramphenicol hydrolysis in the presence of citrate buffers showed that the partially neutralized salts of citric acid markedly enhanced the rate of degradation, while the totally dissociated and the totally undissociated forms of citric acid were essentially noncatalytic. This report alone suggests that the presence of both acidic and basic centers on the same molecule were necessary for increased rates of reaction. Further studies with dibasic acids as catalysts support the idea that the catalytic species is the monoanion of the dibasic acid. In general the results from this study indicated a rather nonspecific catalytic effect since significant alterations in the structure of the bifunctional compounds gave rise to relatively small changes in rates of reaction.

Of particular interest was the catalytic behavior of the singly ionized cis- and trans-cyclohexane dicarboxylic acids on the rate of chloramphenicol hydrolysis. The rate

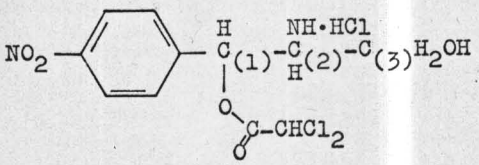
of degradation of chloramphenicol was found to be a linear function of the *trans* monoanion concentration whereas the *cis* isomer was shown to exhibit a limiting rate phenomenon suggestive of complex formation. Support for complex formation was gained by showing that the limiting rate behavior of the *cis* half-salt fit a Lineweaver-Burk plot. Phase solubility studies further substantiated complex formation but the nonlinear plots of chloramphenicol solubility as a function of *cis* cyclohexane dicarboxylic acid half-salt concentration suggested the occurrence of a rather involved interaction.

In addition to the hydrolytic and degradative studies on chloramphenicol some work has been done on the nitrogen-oxygen acyl migration of this complex molecule by mineral acids and organo-sulfonic acids. This acid catalyzed N $\longrightarrow$ O migration involves a transfer of the acyl group from the amide nitrogen to a neighboring hydroxyl group, as shown in Figure 1. The reverse migration occurring in mildly basic media to give back the N-acylated product. This reaction has been proposed to proceed via cyclic intermediate II which is capable of collapsing to give either the O- or N-acylated compound (10,11). Ueyanagi (6) reported the migration of the dichloro acetyl group using aqueous or alcoholic HCl in a suspension of the drug. The readily formed migration product was *d*-1-*p*-nitrophenyl-2-amino-1-dichloroacetoxy propane-3-ol-hydrochloride (III).



II

Figure 1



III

The possibility of formation of the O^3 migrated product was investigated but this isomer was not isolated from the reaction mixture. Treatment of a cold aqueous solution of III with sodium bicarbonate was reported to cause a reverse migration to give back optically active chloramphenicol (5,12).

The use of methane sulfonic acid to effect the migration resulted in 2 crystalline products, one being predominate over the other. The major product was proposed to be the O^1 isomer where the acyl group had migrated to the hydroxyl group on carbon 1 and the minor product to be the O^3 isomer. Reversal by alkali was reported to give back chloramphenicol.

In a later study of the O $\longrightarrow$ N acyl migration of chloramphenicol derivatives using dilute alkali, a difference in reaction for the dichloro acetyl as compared to the acetyl functional group was reported (12). The rearrangement was followed by potentiometric titration of

O^1 acyl chloramphenicol with 0.1 N NaOH to a stable voltage. Migration of the O^1 acetyl group proceeded quantitatively to the N-acetyl derivative as expected. However in the case of the O^1 dichloroacetyl group, it was reported that the ester linkage was cleaved by hydroxide ion during the titration and the cyclic intermediate necessary for migration was not formed.

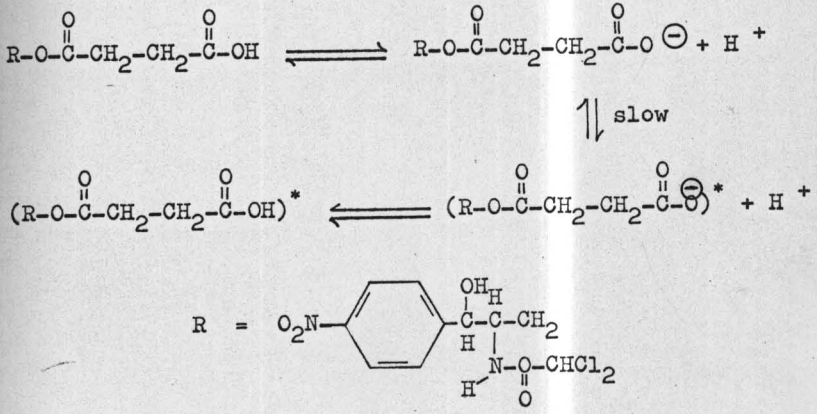
PAST WORK ON CHLORAMPHENICOL 3-MONOSUCCINATE

Although the low water solubility of chloramphenicol has hindered its use in a directly injectable dosage form, the salt form of its monosuccinate ester has provided a water soluble source of the antibiotic. The sodium salt of the mono ester has been widely used in parenteral preparations with no apparent *in vivo* effect on chloramphenicol activity. A study initiated by the need for an analytical method to distinguish between chloramphenicol and its hemisuccinate when present in a mixture led to the discovery of an unexpected form of the ester. Investigation of aqueous solutions of the pure monosuccinate revealed a change in the apparent chemical composition, with time, leading to further studies on this effect.

Szulczewski and co-workers (1) found that the anion of the monosuccinate in aqueous solutions at near neutral pH's formed an equilibrium mixture with a different molecular form. Repetitive chromatographic analysis of mixtures of pure acid monosuccinate and chloramphenicol gave quantitative recovery with the free chloramphenicol content being in agreement with that obtained microbiologically. When these mixtures were prepared using the sodium salt rather than acid succinate to supply the ester component or the sodium salt analyzed per se the indicated content of free chloramphenicol was grossly different from that added or obtained by microbiological assay. They stated that the

free chloramphenicol content was calculated on the basis of total absorbance of the material eluted in what was assumed to be the chloramphenicol peak (Figure 2).

Since this analysis was carried out under mild conditions no appreciable hydrolysis had taken place. The only explanation for the apparent increase in chloramphenicol content was the simultaneous elution of two different components, one being chloramphenicol, the other an unknown material present in the sodium salt but not in the acid succinate. By correcting the total absorbance at 278 mμ of the chloramphenicol peak for free chloramphenicol and, assuming the extra component to have the same molecular weight and absorptivity equal to that of the normal ester, all material in the solution was accounted for with the following model being suggested.



*denotes molecular form difference.

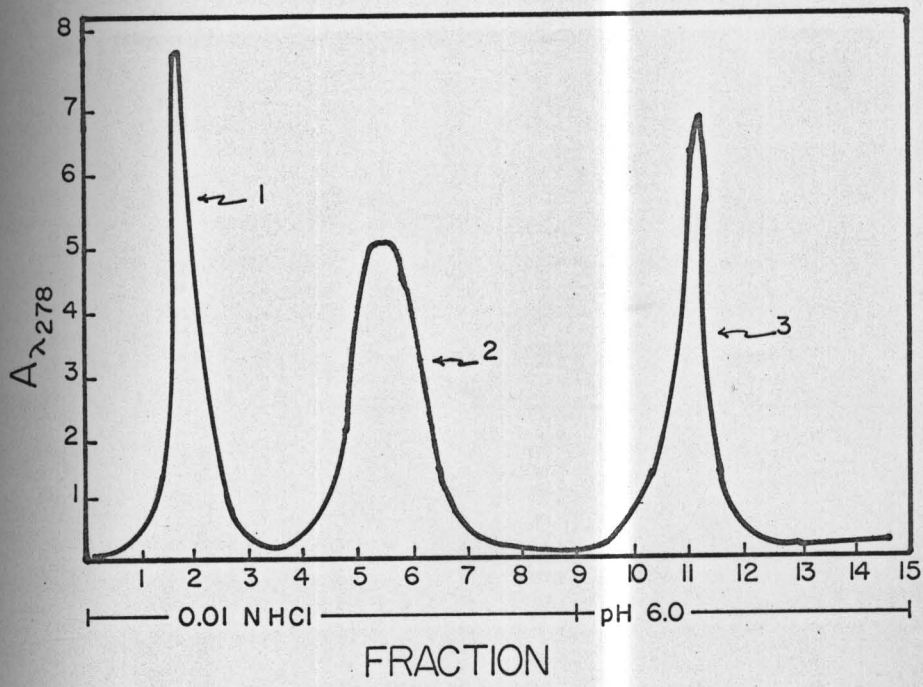


Figure 2. Chromatogram of a ternary mixture of D(-)threo-2-amino-1-(p-nitrophenyl)-1,3-propanediol (1), chloramphenicol (2) and chloramphenicol 3-succinate (3). Amyl acetate is the internal phase. Fraction volume is 20 ml.

Additional evidence for the existence of an extra component produced from chloramphenicol 3-monosuccinate in solution at pH's near neutrality or contained in the dried sodium salt was obtained from a phase solubility investigation. Based on the assumption that the preceeding model is correct it was assumed that the aqueous solutions of the sodium salt, when adjusted to pH 2, would produce essentially a biphasic system which would be expected to behave as at least a binary system with respect to absorbance at 278 mμ of the supernate. By knowing the free chloramphenicol content of the supernate, the data obtained in Figure 3 was found to be in agreement with that obtained chromatographically, where the per cent of the extra compound was 15%.

The effect of variables such as pH and concentration were also studied. Results of this portion of the work showed that at pH's sufficiently high to cause complete dissociation of the free carboxyl group, neither pH nor concentration influence the equilibrium composition. From these and other studies they found the equilibrium constant for the reaction,

in water at 25°C to be 0.293. The primary effect of pH in the region 7.0 or greater was seen to be on the rate of equilibrium attainment. Plotting the composition of such

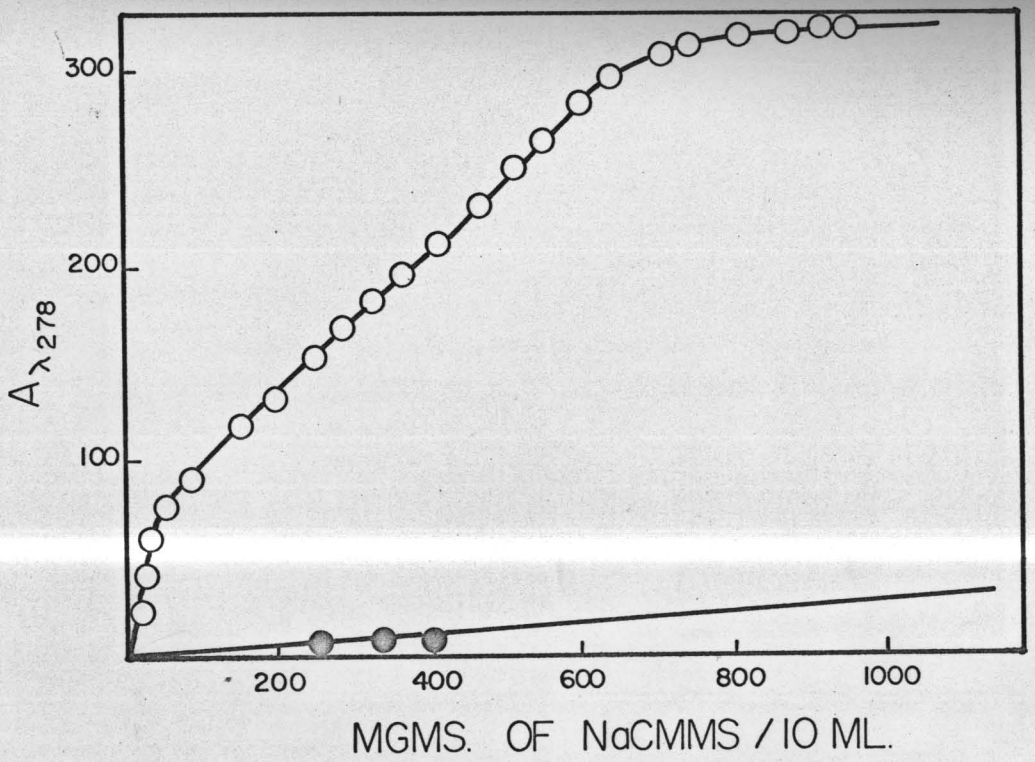
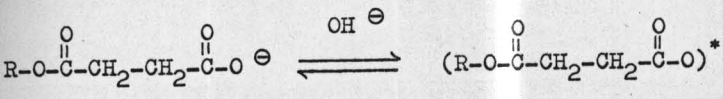


Figure 3. Solubility behavior of acidified solutions of chloramphenicol 3-monosuccinate sodium salt (NaCMMS). o = absorbance of supernate at 278 mu. ● = absorbance due to free chloramphenicol as determined microbiologically.

solutions as a first order approach to equilibrium yielded straight lines of changing slope. The rate of reaction was shown to vary directly as hydroxyl ion concentration over a pH region in which the fraction of total succinate present as anion is virtually 1.0. Catalysis of formation of the unknown compound by hydroxide ion was considered to be established and the following equation proposed:



Lowering pH caused a reduction in rate but not to the degree which would be anticipated if only the reaction occurring were the one above.

The current study was initiated by an intense interest in the structure of the extra peak component as well as a desire to investigate further the factors promoting formation of the equilibrium mixture. Data on the characterization of the unknown suggested a partial oxygen to oxygen acyl migration involving an unstable cyclic compound. The possibility for cyclization under the reaction conditions necessary for formation of the cyclic compound was substantiated by various reports on similar compounds capable of undergoing this reaction.

ACYL MIGRATIONS OF CHLORAMPHENICOL ESTERS

Several cases of acyl migration of chloramphenicol 1-esters have been observed by Fisher and Edgerton (13). They found that during alkaline hydrolytic procedures incorporated as part of the synthetic preparation of chloramphenicol 1-palmitate, an $O^1 \longrightarrow O^3$ acyl migration of the palmityl group had occurred and chloramphenicol 3-palmitate was recovered in good yield. However, when chloramphenicol 3-dichloroacetate 1-palmitate was hydrolyzed by weakly basic sodium acetate solution, no acyl migration occurred but an excellent yield of chloramphenicol 1-palmitate was obtained. An aqueous alcoholic solution of the 1-palmitate made basic with one drop of 1 N sodium hydroxide solution produced a good yield of chloramphenicol 3-palmitate, the shifted product. Control experiments under neutral and acidic conditions resulted in no migration.

On the theory that less bulky acyl moieties such as benzoyl and acetyl would have migration tendencies that would differ from the palmityl, work parallel to that described with the 1-palmitate was attempted with the 1-benzoate and 1-acetate. Hydrolysis of chloramphenicol 1-benzoate 3-dichloroacetate by alkali titration resulted in an $O^1 \longrightarrow O^3$ acyl migration to chloramphenicol 3-benzoate. The shift also occurred when chloramphenicol

1-benzoate was exposed to slightly alkaline conditions. Hydrolysis of chloramphenicol 1-benzoate 3-dichloroacetate by sodium acetate gave chloramphenicol 3-benzoate in good yield. Hydrolysis by sodium acetate, therefore, induced $O^1 \longrightarrow O^3$ migration of the benzoyl moiety much more readily than the palmityl. These authors also found that acetylation of chloramphenicol 3-dichloroacetate with acetic anhydride in pyridine unexpectedly gave a good yield of chloramphenicol 1-acetate. They proposed that 1-acetylation of the 3-dichloroacetate was followed by direct hydrolysis of dichloroacetyl group in the reaction mixture. However, chloramphenicol 1-acetate under basic migration conditions rearranged to the chloramphenicol 3-acetate.

The $O^1 \longrightarrow O^3$ acyl migration of chloramphenicol 1-esters under basic conditions was thought to be a general reaction of the series proceeding via a meta-dioxane-like intermediate similar to that proposed by several workers (14,15). While $N \longrightarrow O$ acyl migrations in the chloramphenicol series are well known (16) when the acyl group migrates to a free hydroxyl group, there has been little evidence that the shift to an acetylated hydroxyl group is possible. Acyl migration was found to take place readily either to the 1- or 3-oxygen depending largely on which O-acyl was the more labile. The order of lability of O-acyl groups under migration conditions was found to be roughly as follows:

acetyl > palmityl > dichloroacetyl > benzoyl

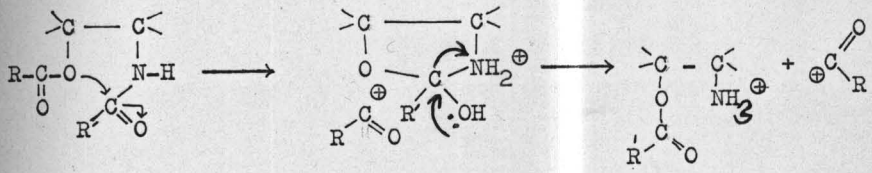
In no experiment of this series did the authors find that an O-benzoyl had been replaced directly by another acyl group by migration under acid conditions.

These workers also carried out a study of N ⟶ O acyl migrations of chloramphenicol diesters. The acyl migration of D-threo-1,3-O-diacetyl-1-(p-nitrophenyl)-2-palmitamido-1,3-propanediol is an example of replacing the 3-O-acetyl moiety by a palmityl group shifted from the amine nitrogen. The migration product was the diester amine hydrochloride which after dichloroacetylation was proven to be chloramphenicol 1-acetate 3-palmitate. Acyl migration of this diester gave D-threo-2-amino-1-O-dichloroacetyl-1-(p-nitrophenyl)-3-O-palmityl-1,3-propanediol hydrochloride which when treated with sodium carbonate solution gave chloramphenicol 3-palmitate.

The synthetic utility of the acyl migration of chloramphenicol diesters was pointed out by the preparation of chloramphenicol 1-benzoate. Chloramphenicol 3-palmitate was benzoylated and the resulting chloramphenicol 1-benzoate 3-palmitate was rearranged under acid conditions to give D-threo-2-amino-1-O-benzoyl-3-O-dichloroacetyl-1-(p-nitrophenyl)-1,3-propane hydrochloride which was neutralized to give chloramphenicol 1-benzoate in excellent yields with no trace of O ⟶ N migration of the 1-O-benzoate. No evidence of inversion of configuration

was noted in this work which is in agreement with the retention mechanism of N ⟶ O acyl migrations as proposed by several groups of investigators (16,10,11).

The retention mechanism for acyl shifts of unesterified acyl amino ethanols was thought to be applicable to the migration of esterified congeners with expulsion of an acyl carbonium ion rather than a proton during the shift. The carbonium ion then reacts with a mole of water to give the carboxylic acid as by-product.



The stability of the benzoyl and dichloroacetyl moieties in comparison with acetyl and palmityl was thought to confirm the mechanism in that the electron density on the hydroxyl oxygen would be much lower in the case of proximity of the electron withdrawing groups as phenyl and dichloromethyl.

Other mechanistic studies were carried out using N-ethylformyl derivatives. Since the ethyl formyl group would increase the electron density at the carbonyl carbon due to contribution of the neighboring ethoxy group, it was thought that N ⟶ O migration would not occur to any

significant degree and they recovered only a very small amount of the N → O shifted product. Similar work on other formates (17), carbamates (18,19), and sulfonyl migrations (20) resulted in unsuccessful N → O migration.

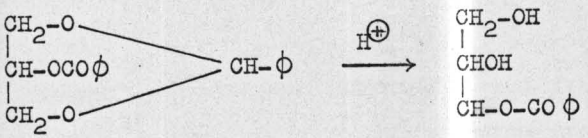
PAST WORK ON OTHER OXYGEN TO OXYGEN ACYL MIGRATIONS

Introduction

There have been numerous reports in the literature of neighboring hydroxyl group facilitation in ester hydrolysis (21-24) and amide hydrolysis (25-28) with a variety of mechanisms being proposed to account for these observations. Of particular interest in light of the current work is the migration of the acyl functional group from the ether oxygen of the ester linkage to the hydroxyl oxygen of the hydroxyl group. This reaction has been proposed to proceed through an intermediate cyclic derivative which subsequently undergoes ring scission to yield the migrated compound.

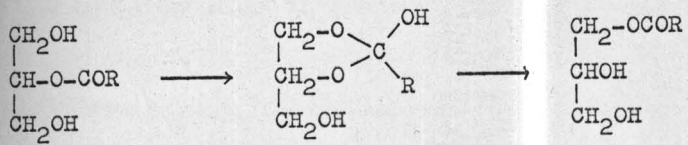
Evidence for the migration of an acyl functional group was first observed by Fischer (29) when the reaction between silver nitrate and some mono-acid and α,β-diacyl-glycerol-γ-iodohydrins in aqueous alcoholic solution did not lead to the expected α,β-diglyceride, but to the isomeric α,γ-diglyceride. Difficulty in the preparation of β-mono glycerides of fatty acids was also reported by Stimmel and King (30). Under the reaction conditions migration of the aliphatic acyl group from the beta to the alpha position was found to occur. In an alcoholic solution of N/20 hydrochloric acid at room temperature, β-mono palmitin underwent complete rearrangement to the

more stable alpha isomer in twenty-four hours, but in solutions more dilute than N/200 there was no significant rearrangement during the time interval. Somewhat higher concentrations of ammonium hydroxide gave similar results. Subsequent reports relating to both mono and diglycerides were always in agreement with the observation that acyl group always migrated from the β- to an α-position. Hibbert and Carter (31) reported that 1,3-benzylidene glycerol 2-benzoate, IV, on hydrolysis with dilute acids



IV V

yielded glycerol 3-benzoate (V) and not the β-benzoate as was expected. They were in agreement with the mechanism proposed by Fisher for such abnormal reactions whereby the assumption is made of the intermediate formation of a dioxolane derivative (VII), formed from the β-acyl derivative (VI) by the migration of a hydrogen atom of the hydroxyl group on the carbon atom adjacent to the carbonyl group:



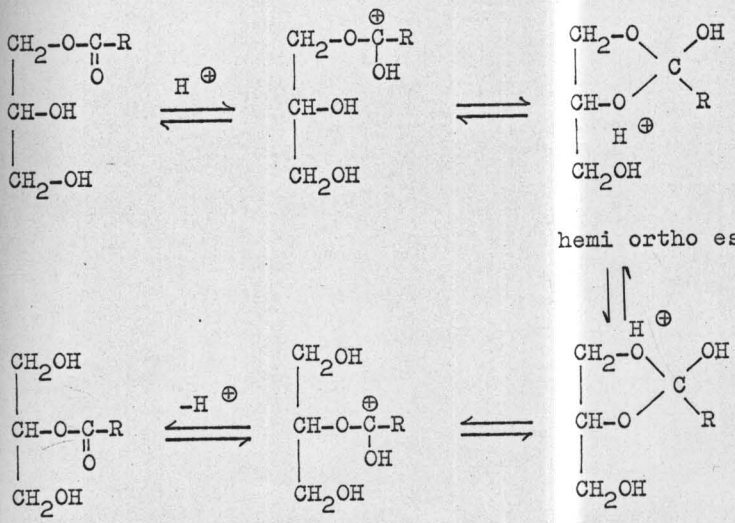
VI VII VIII

This dioxolano ring (VII) then undergoes ring scission with a second migration of the hydrogen atom taking place with formation of the acyl derivative (VIII).

Daubert and King (32) investigated the behavior of β-monopalmitoylglycerol and β-mono-p-bromobenzoyl glycerol towards alcoholic hydrogen chloride and "alcoholic" ammonium hydroxide of varying concentrations at room temperature and found a marked contrast in the ease of migration for aromatic as compared to aliphatic β-esters. In both dilute acid and alkali the shift for the aliphatic acyl group was always more facile.

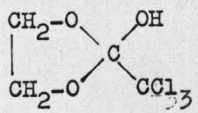
Verkade, et al. (33) challenged the completeness of this reaction by being the first to report the possibility of an equilibrium between the α- and β-monoglycerides. This was substantiated by Verkade and Van Lohugen in a later investigation (14) designed to prove that a mutual rearrangement of the two isomers is involved. Monoglycerides were again used as reactant with the catalyst and solvent being hydrogen chloride and ethanol-water mixtures, respectively. The reaction was followed by oxidation with periodic acid. It was of great interest

that the same position of the equilibrium was invaribly found: the equilibrium mixture contained about 88% of the α-monoglyceride and about 12% of β-monoglyceride. They also reported that under their reaction conditions no influence of the hydrogen chloride concentration on the position of the equilibrium was evident. Migration of the acyl group in the β position followed kinetics of the first order with the migration rate constant being directly proportional to the hydrogen chloride concentration. A mechanism proposed for the mutual rearrangement of the monoglycerides as shown below is closely related to that of alcoholysis or hydrolysis of the esters of carboxylic acids.



The carbon atom serves as the reaction center, and the approach of the adjacent hydroxyl group towards this reaction center was said to be the rate determining step.

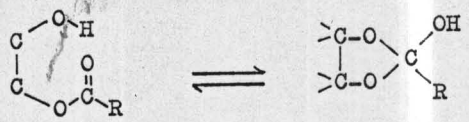
Isolation of a cyclic intermediate derivative by Hibbert and Greig (15) gave strong confirmatory support to the mechanism first proposed by Fisher and later by Hibbert and Carter (31) to account for the acyl migration reaction. They found that the mono-trichloroacetates of polyhydroxy compounds show a most remarkable tendency to pass over into a dioxolane or meta-dioxane ring, a behavior which appears to be a general property of the esters of trichloro acetic acid. All attempts to synthesize the mono-trichloro-acetate of ethylene glycol yielded only the corresponding dioxolane (IX).



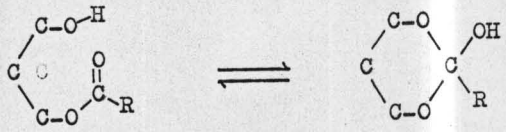
IX

On the basis of those and other studies, Hibbert and Grieg concluded that all organic monoesters of polyvalent alcohols, carbohydrates, polysaccharides, o-amino phenols, etc., containing a free hydroxyl group, the hydrogen atom of which is spatially in close proximity to the carbonyl group of the acyl radical, tend to pass over into a ring isomer which in all these cases would exist in an

equilibrium with the open-chain form.



dioxolane ring

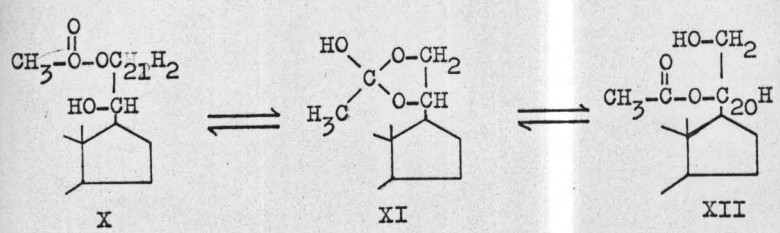


meta-dioxane ring

The tendency and ease of ring formation was postulated to be dependent on (a) the relatively labile character of the hydrogen attached to the hydroxyl group, (b) the negative polarity of the carbonyl group in the acyl radical, and (c) the spatial relationship of the migratory hydrogen atom with reference to the carbonyl group. Their experimental work with a series of glycol monoesters such as glycol monoacetate, glycol monochloroacetate, glycol mono-dichloroacetate, and glycol monotrichloroacetate has shown that the tendency towards cyclization did increase from the acetate to the trichloroacetate.

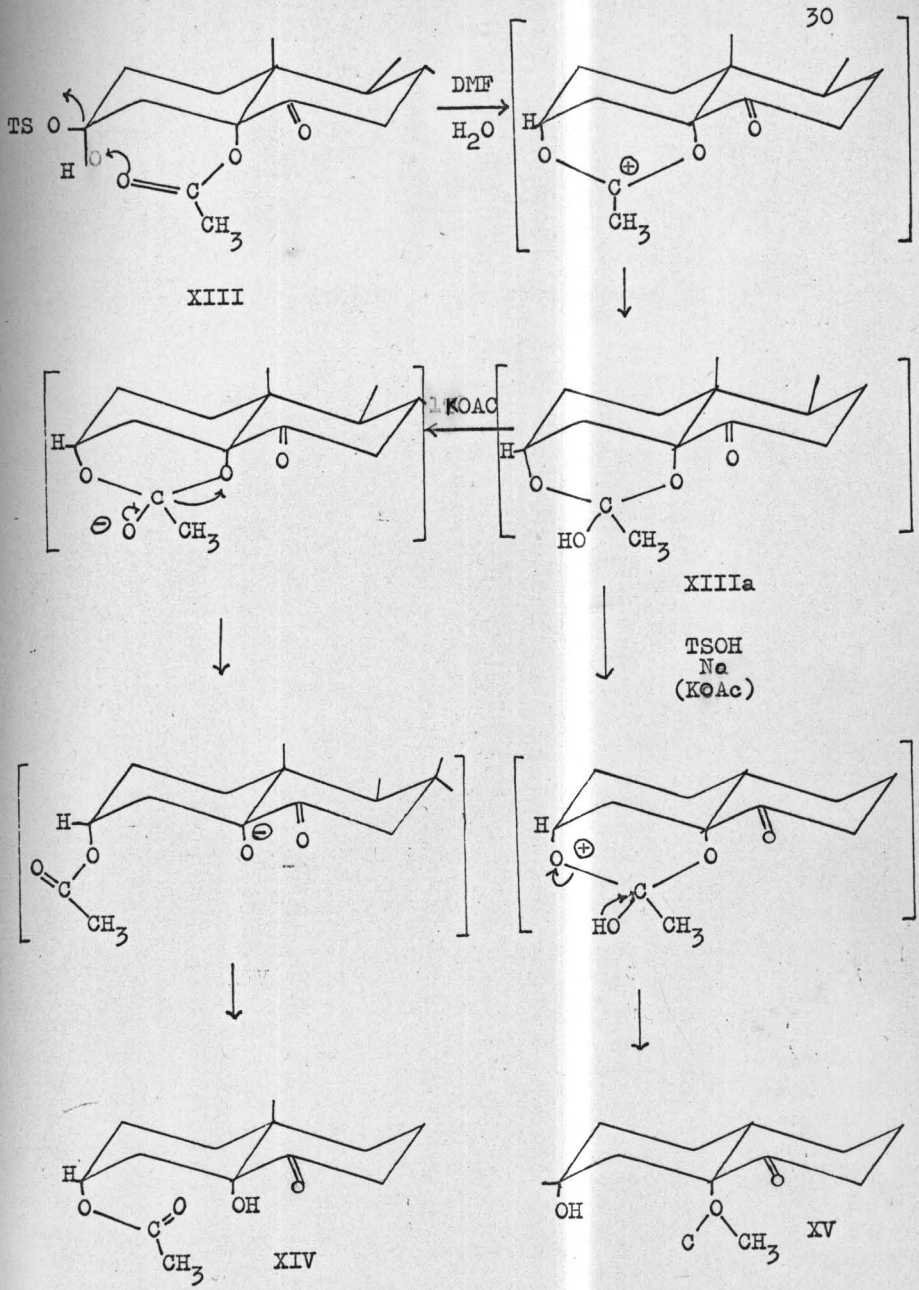
In a series of papers concerning the role of neighboring groups in replacement reactions, Winstein and

co-workers (34-36) reacted acetoxybromobutanes with silver acetate in acetic acid and proposed that a cyclic intermediate was probably involved. In the presence of water this cyclic acetoxonium ion intermediate was thought to react with a molecule of water to give a mono-ortho-acetate which then could collapse to the monoacetate or isomerize to give a migrated monoacetate. An Sn_1 mechanism with resulting steric requirements of predominant retention of configuration was thought to be involved. Displacement of sulfonate esters under solvolytic conditions with participation of neighboring acyl groups was reported to occur in refluxing collidine by Taub, et al. (37) when the C-20 β-acetate group of a cortisone derivative (X) rearranged to the C-21 position (XII). The rearrangement was depicted as proceeding through an ortho acetate intermediate (VI) which was probably in equilibrium with (X) and (XII), the amounts of each of the latter isolated in any given case being influenced by the reaction conditions. Further experiments using aqueous dimethyl formamide containing potassium bicarbonate revealed that mild base catalysis was strikingly effective in promoting acetyl transfer from C_{21} to C_{20} in this series.



X XI XII

The occurrence of acetyl migration in the steroid series has also been reported by Schultz (38). Solvolysis by p-toluene sulfonic acid of cholestan-3β,3α-diol-6-one-3-tosylate-5-acetate (XIII) with added potassium acetate gave a compound which unexpectedly proved to be cholestan-3α,5α-diol-6-one-3-acetate (XIV). In contrast, solvolysis of XIII in dimethyl formamide-water without added potassium acetate afforded cholestan-3α,5α-diol-6-one-5-acetate (XV). By reference to Plattner (39,40) who postulated a 3,5-bridged intermediate for a similar rearrangement, Schultz concluded that when potassium acetate is present, it may act as a base to remove the proton from a possible ortho ester cyclic intermediate (XIIIa) and the anion so formed could then collapse to an acetate and the alkoxide of greater stability. In this case this was the position alpha to the carbonyl group thereby forming cholestan-3α,5α-diol-6-one-5-acetate (XIV). In the absence of acetate buffer, the p-toluene sulfonic acid formed in the displacement is free to protonate one of the oxygens of the ortho ester. Since the oxygen attached to carbon-3 has greater electron density than the one attached to carbon-5 this would probably be the one protonated and this would account for the formation of cholestan-3α,5α-diol-6-one-5-acetate (XV).



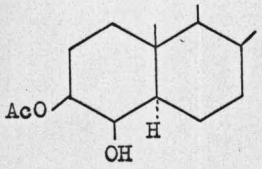
XIII

XIIIa

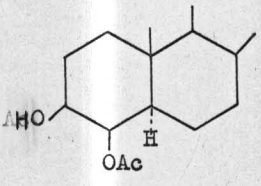
XIV

XV

In a series of papers on intermolecular catalysis, Kupchan and co-workers have reported several cases of facilitation of alkaline hydrolysis of certain steroid acetates by a neighboring hydroxyl group (23,24,41,42). The reactivity of esters at C_7 and C_{16} in germine derivatives was attributed to assistance by neighboring hydroxyl groups bearing cis-1,3-diaxial relationships to the ester groups (41). Cholestane-3β,4β-diol-3-acetate (XVI) upon treatment with basic alumina led to quantitative isomerization to cholestane-3β,4β-diol-4-monoacetate (XVII).



XVI



XVII

From subsequent kinetic studies it was found that the hydrolysis of the 3β-acetate was accelerated eight- or nine-fold when a 4β-hydroxy group was present in the molecule. Hydrolysis of the 3β-acetate was proposed to proceed via some hydrogen bonded intermediate; however, the base-catalyzed migration of the acetate from the 3- to the 4-position was suggested to involve a cyclic

ortho acetate intermediate. No further evidence for the cyclic orthoester was reported.

Further examples of acyl migrations can be obtained from work in the field of nucleoside chemistry (43-46). The migration of an acyl group attached to the ribose moiety of aminoacyl adenosine nucleoside isolated from amino acyl RNA has been postulated to account for the ambiguous results obtained during an attempt to ascertain which of the two terminal hydroxyl groups in aminoacyl RNA is involved in ester linkage with amino acids, since formation of a mixture of the 3'-adenylic acids was invariably obtained during the alkaline workup procedure (43).

The possibility of acyl migration was investigated, using closely related model compounds. Glycerol monoacetate was used in subsequent kinetic studies with the limited objective of providing an indication of the relative tendency toward base-catalyzed isomerization and hydrolysis of carboxylic esters bearing neighboring hydroxyl groups. Results of these studies showed that acyl equilibrium between α and β-positions was rapid, exceeding the rate of hydrolysis by a factor of more than 6000 in the neutral and alkaline range. The observed rate constants for isomerization and hydrolysis were plotted as a function of pH. The rates of both reactions were found to be strictly proportional to hydroxide-ion concentration, except at low pH values where there

appeared to be an additional contribution to the rate of isomerization from a very slow uncatalyzed or acid catalyzed reaction. It was concluded that amino acyl-RNA probably isomerizes virtually as soon as it is formed enzymatically, at least *in vitro*.

EXPERIMENTAL

Apparatus

Constant temperature water bath equipped with Sargent "Thermonitor" constant temperature control unit.

Jacketed reaction vessel with inlet and outlet flow-through connections.

Ten cm spectropolarimetric sample cell with connections for flow-through usage.

Pyrex glass chromatographic columns of assorted lengths and diameters.

Thin layer chromatography plates, developing tank and reagent sprayer.

Instrumentation

Cary 14 Recording Spectrometer.

Beckman IR5A and IR9 Infrared Spectrophotometer.

Cary 60 Spectropolarimeter.

Varian Associates A-60A Spectrometer - Nuclear Magnetic Resonance (NMR) Spectrometer with spin decoupler.

Radiometer pH meter and automatic titrator, type TTT1 with syringe burette recorder type SBR2.

Technicon automatic fraction collector.

Reagents and Chemicals

Chloramphenicol Monosuccinate, obtained from Parke-Davis and Co., mp 127-128°C.

Reagents and Chemicals - Cont.

Chloramphenicol, obtained from Parke-Davis and Co., recrystallized from aqueous alcohol, mp 150-151°C.

1-(p-Nitrophenyl)-2-amino-1,3-propanediol, obtained by hydrolysis of chloramphenicol and recrystallized from aqueous alcohol, mp 165-167°C.

All other chemicals were reagent grade or of the highest purity available.

Solvents for Nmr Spectroscopy

Silanar-A®

Deuterium oxide.

Procedure for Kinetic Studies

Various buffer solutions of chloramphenicol succinate were prepared such that the exact molar concentration of the succinate and the total buffer concentration were known. Although the present study, as well as a previous investigation (1), has shown the isomerization of chloramphenicol monosuccinate to be independent of salt concentration a constant ionic strength of 1.0 was maintained throughout all kinetic investigations. This was done to facilitate calculations of the various species involved.

For rate studies using the spectropolarimeter all solutions were prepared by dissolving chloramphenicol 3-monosuccinate in various buffer solutions so that the

total concentration was known and was between 5 and 25 mgm/ml. The solutions were filtered to insure clarity, then transferred to a strain-free sample cell in a compartment maintained at 25°. In most cases a few minutes was needed to equilibrate the solution to the compartment temperature. The change in optical rotation at 390 mμ associated with the cyclization of the substrate was recorded as a function of time using a time synchronized chart speed. All necessary precautions and considerations concerning the use and operation of the spectropolarimeter were taken into consideration during each kinetic study.

The isomerization was also followed using a reverse phase column chromatographic technique (1). The reaction solution was sampled at various time intervals, and chromatographed. The various fractions were analyzed for absorbance at 278 mμ and from this the rate of equilibration of the succinate species was determined.

Column Chromatographic Procedure

Solutions of the equilibrium mixture were acidified with dilute mineral acid and unreacted succinate was allowed to precipitate out and was removed by filtration. The filtrate was concentrated by flash evaporation to a few cc, then transferred to the column for separation.

The columns were prepared by treating celite 535 with dichlorodimethyl silane or Desicate ® to obtain a nonpolar hydrophobic support. A mixture of 10% mineral oil and

15% Hallcomid M-18® was intimately mixed with the silanized support and a slurry of this in an appropriate amount of water was subjected to a vacuum until all visible air bubbles were removed. The slurry was poured into a glass chromatographic column and allowed to settle with light pressure being used to compact the support. The packed column was then washed with 0.05 N HCl saturated with pre-washed Hallcomid M-18® and ethyl acetate until the absorbance of the elute at 278 mμ was less than 0.05 for all quantitative analyses. Elution with 0.05 N HCl, saturated with Hallcomid and ethyl acetate, was carried out until the cyclic compound was collected in the eluate. The unreacted succinate was then eluted with pH 7 phosphate buffer saturated ~~as~~ in the same manner. In most cases 10 ml fractions were collected using an automatic fraction collector and the elution monitored by ultraviolet (uv) analysis at 278 mμ. The columns were washed with eluting solvent and were successfully re-used for further separations.

Isolation of Cyclized Succinate

Fractions of the eluate containing the cyclic ortho ester were immediately combined and washed with chloroform. All the Hallcomid M-18® and any overlapping chloramphenicol were extracted from the aqueous layer. The acid solution was concentrated on a flash evaporator, and ethyl acetate was then used to extract out all the cyclic

material. The ethyl acetate portions were combined, washed with water to remove any hydrolysis products from ethyl acetate, and dried over anhydrous magnesium sulfate. Subsequent evaporation of the dried ethyl acetate solution gave an off-white, noncrystalline residue. Recrystallization from acetone-Skellysolve B, aqueous methanol, or acetone-diethyl ether failed to induce crystallization of the cyclic product.

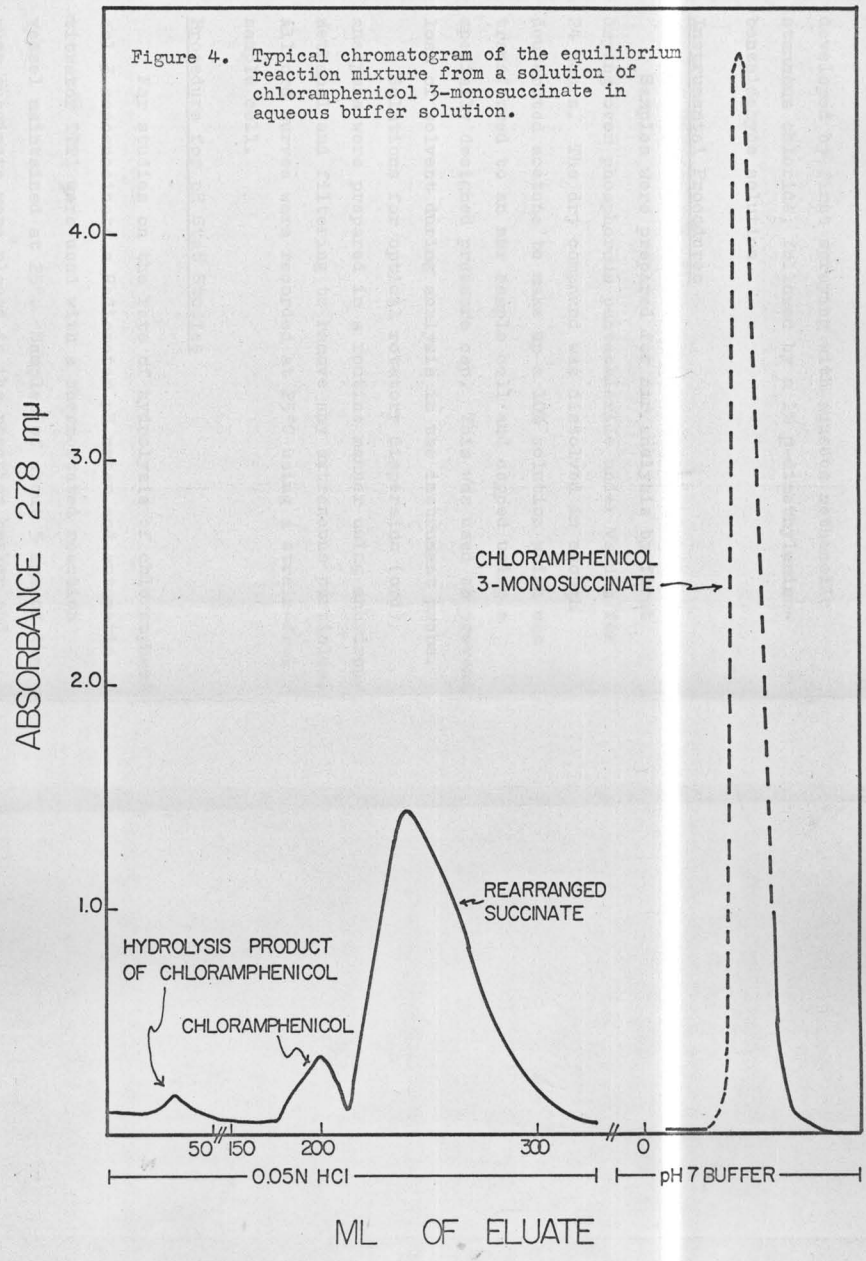
Analytical Chromatographic Procedure

Columns for quantitative analysis of the equilibrium mixture were prepared in the same manner described under the previous section on chromatographic procedures. Approximately a 1:1000 ratio of sample to column support was used to insure complete separation of each peak. Solutions for investigation were quenched with a 0.1 N HCl solution and diluted with 0.1 N HCl until a concentration of 5 mgm/ml was achieved. A 3 ml aliquot was then used for chromatography.

Thin Layer Chromatography

Thin layer chromatography (tlc) was employed to show the purity of the noncrystalline product as well as to verify its presence in various equilibrium mixtures. Complete separation of all compounds was achieved with a chloroform-methanol, glacial acetic acid (92:7:1) system using precoated silica gel G tlc plates. The spots were

Figure 4. Typical chromatogram of the equilibrium reaction mixture from a solution of chloramphenicol 3-monosuccinate in aqueous buffer solution.



developed by first spraying with aqueous methanolic stannous chloride, followed by a 1% p-dimethylamino-benzaldehyde solution.

Instrumental Procedures

Samples were prepared for nmr analysis by first drying over phosphorous pentachloride under vacuum for 24 hours. The dry compound was dissolved in enough deuterated acetone to make up a 10% solution which was transferred to an nmr sample cell and capped using a specially designed pressure cap. This was used to prevent loss of solvent during analysis in the instrument probe.

Solutions for optical rotatory dispersion (ord) analysis were prepared in a routine manner using anhydrous methanol and filtering to remove any extraneous particles. All ord curves were recorded at 25°C using a strain-free sample cell.

Procedure for pH Stat Studies

For studies on the rate of hydrolysis of chloramphenicol 3-monosuccinate a Radiometer pH meter and automatic titrator TTT1 were used with a thermostated reaction vessel maintained at 25°. Samples of about 5 mgm of the pure succinate were placed in the reaction beaker and 100 ml of dilute sodium hydroxide was added such that the solution was at a pH suitable for hydrolysis studies. The

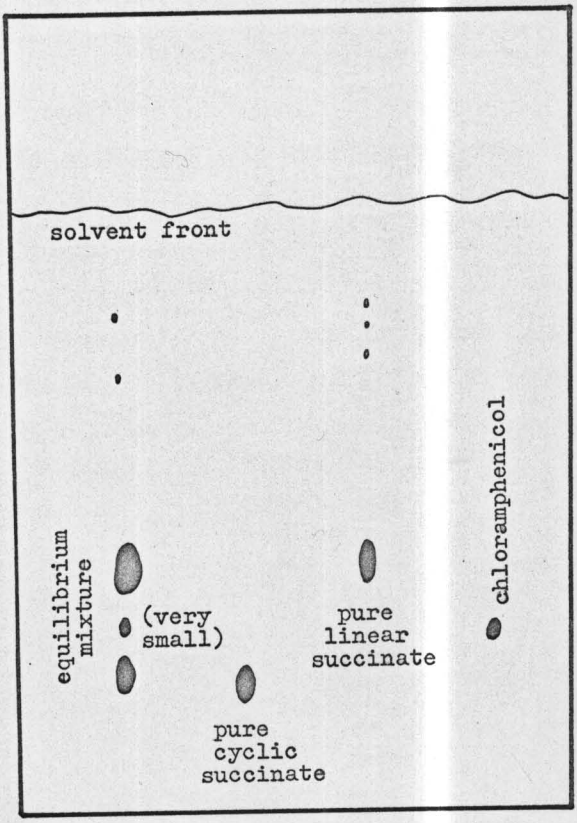


Figure 5. Representative tlc chromatogram showing the relative positions of pure chloramphenicol 3-monosuccinate, cyclic succinate and chloramphenicol.

titrant was 0.005 N sodium hydroxide and an ionic strength of 1 was maintained using potassium chloride.

The rate of change in optical rotation at 390 mμ of a 5 mgm/ml solution of chloramphenicol 3-succinate at zero buffer concentration and ionic strength of 1 was also followed using the automatic titrator to maintain the desired pH and using a flow through 10 cm strain-free cell. The reaction mixture was pumped into the sample cell from a thermostated reaction vessel, maintained at 25°. A constant pH for the entire equilibrium reaction was maintained using various sodium hydroxide titrants such that any change in total solution volume was negligible.

RESULTS AND DISCUSSION

Preliminary Remarks

The results of the present investigation substantiate the evidence obtained previously (1) that an equilibrium mixture composed of chloramphenicol 3-monosuccinate and another compound is formed when chloramphenicol 3-monosuccinate is dissolved in aqueous buffer solutions. In these studies the rate of equilibration was followed using both a spectropolarimetric method as well as by reverse phase column chromatography. The effect of buffer species, buffer concentration and ionic strength on the rate of reaction as well as on the equilibrium ratio of the compounds is reported. The structure of the new species has been established based on ir, nmr, and ord data.

The reaction is proposed to be one involving the intramolecular attack on the ester carbonyl by the anionic secondary hydroxylic oxygen to give a cyclic hemi ortho ester.

Change in Optical Rotation Associated with Equilibrium Formation

Solutions of chloramphenicol 3-monosuccinate in aqueous phosphate buffer at neutral pH were observed to undergo a change in optical rotation upon standing over a period of a few hours as illustrated in Figure 6. The

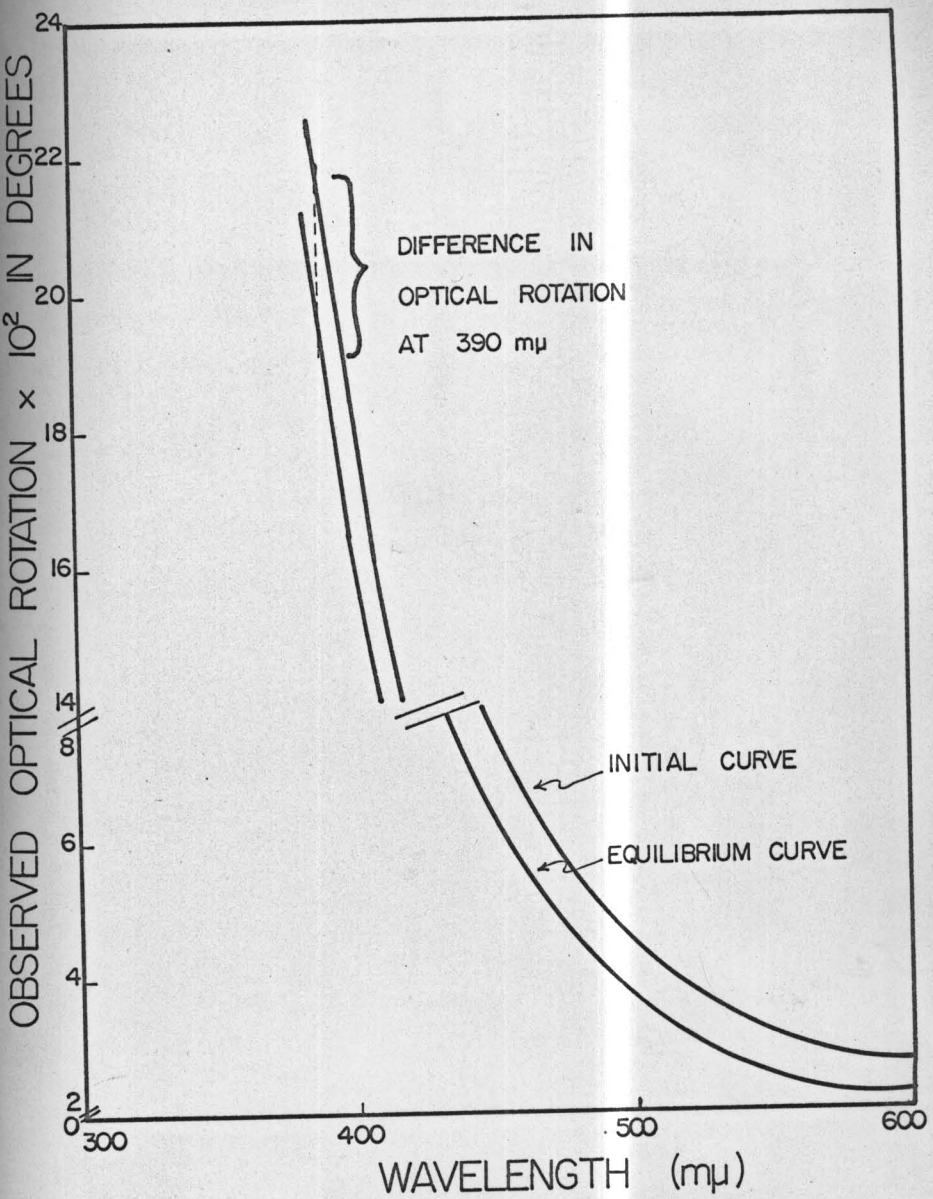


Figure 6. Change in the optical rotatory dispersion curve associated with equilibrium formation of chloramphenicol 3-monosuccinate in pH 7.5 phosphate buffer. T = 1.0; T = 25° C.

optical activity of a fresh solution followed at 390 mμ is shown in Figure 7, the plot exhibiting apparently first order decay to an equilibrium optical rotation value. Kinetic studies were carried out at this wavelength since the greatest change in rotation was observed under this condition with minimal interference from instrumental limitations due to absorbance of the compounds in solution.

The initial specific rotation of chloramphenicol 3-monosuccinate was found to be a function of the pH of the aqueous solutions; however, the difference between the initial optical rotation of a fresh solution and the equilibrium optical rotation was found to be a constant value over the range of pH studied (Table I).

TABLE I

pH	Initial observed optical rotation* α_i	Equilibrium observed rotation α_e	$\Delta\alpha=\alpha_e-\alpha_i$
5.50	0.221	0.196	0.025
6.10	0.223	0.198	0.025
7.23	0.225	0.199	0.026
7.70	0.226**	0.200	0.026

*Initial chloramphenicol 3-monosuccinate concentration was 250 mgm/25 ml.

**Value obtained by extrapolating to zero time.

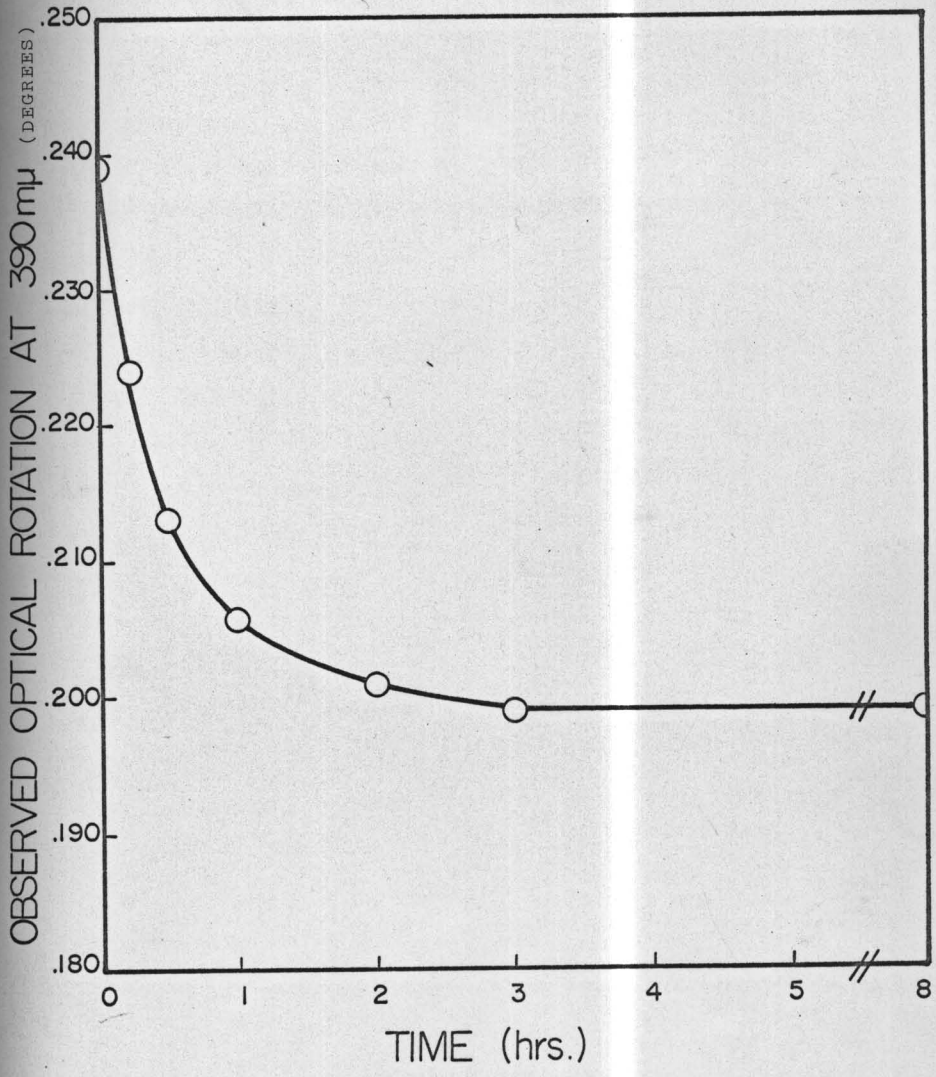


Figure 7. Typical plot of the approach to equilibrium of chloramphenicol 3-monosuccinate at 390 mμ in phosphate buffer at pH 7.7. I=1. T=25°

The change in the specific rotation of fresh solutions of chloramphenicol 3-monosuccinate with pH is thought to be a function of the dissociation of the free carboxylic acid. From pH 5 to 7 an increase in specific rotation of fresh solutions was observed; however, above pH 7 the specific rotation of chloramphenicol 3-monosuccinate, obtained by extrapolation to zero time, approached a constant value of +91.5 degrees. Complete investigation of this phenomena was impossible due to the low water solubility of the ester below pH 5.

Changes in the initial concentration of chloramphenicol 3-monosuccinate over the range 5 mgm/ml to 25 mgm/ml had no effect on the rate of attainment of the equilibrium as is shown in Figure 8 by a representative semi-logarithmic plot of the difference between the initial and equilibrium optical rotation at 380 mμ as a function of time. The independence of substrate concentration on the rate of equilibrium formation suggested that the equilibrium reaction was not an association of two or more monosuccinate molecules but rather an intramolecular reaction which appeared to be first order with respect to substrate over the range of substrate concentrations employed in this investigation.

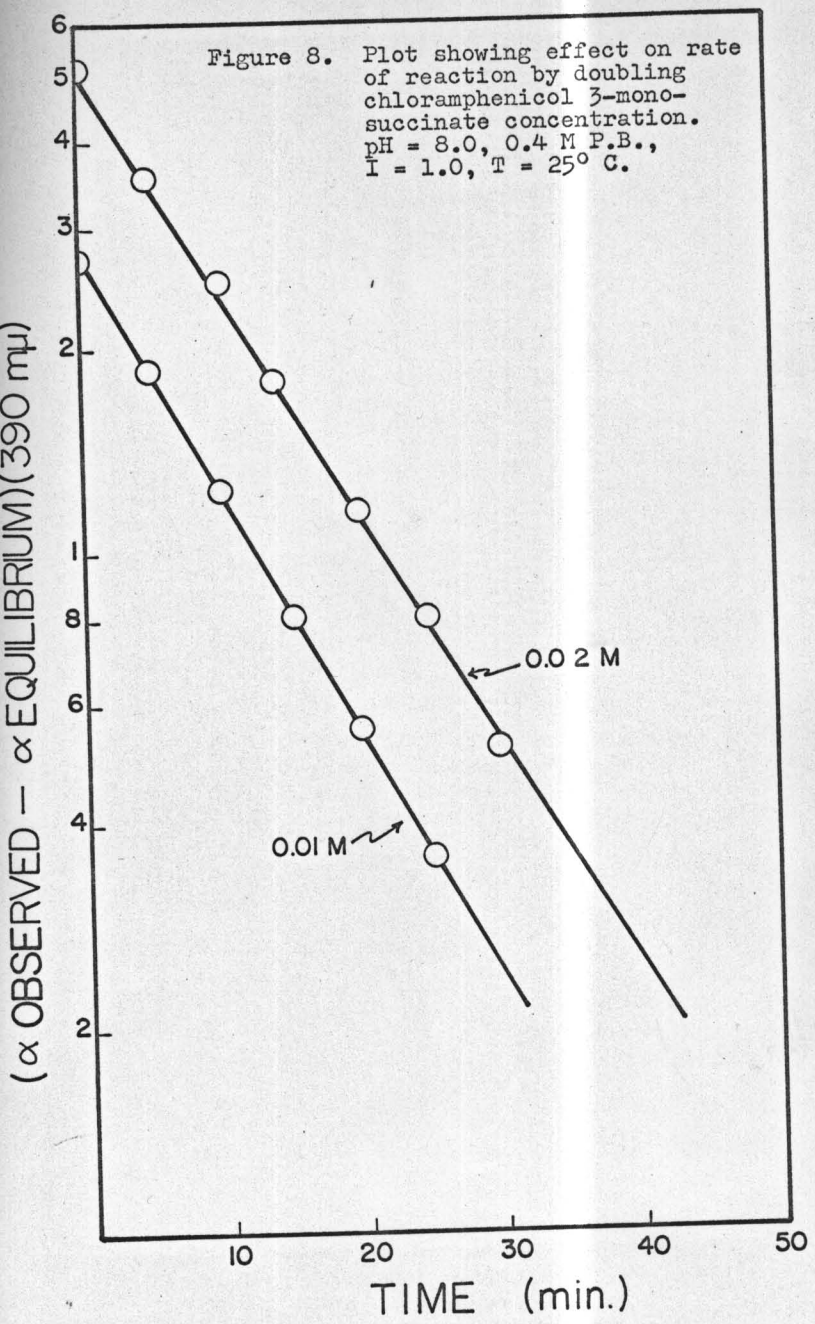


Figure 8. Plot showing effect on rate of reaction by doubling chloramphenicol 3-monosuccinate concentration. pH = 8.0, 0.4 M P.B., I = 1.0, T = 25° C.

pH Rate Profile for the Equilibration

Studies on the rate of equilibration of chloramphenicol 3-monosuccinate using various buffer systems indicated that changes in buffer type had no effect on the rate of formation of the equilibrium; therefore, phosphate buffer systems were used for most kinetic studies to maintain a constant pH. Typical rate data are shown in Figure 9. A plot of the observed rate constant obtained from optical rotation data as a function of phosphate buffer concentration is shown in Figure 10. Although no buffer concentration dependency was found below pH 7.5, slight buffer catalysis was observed at pH 8. Data for the rate of equilibrium formation in the absence of phosphate buffer at pH 8 and pH 7.5 were therefore obtained using a pH stat technique with no buffer present in the reaction system.

Results of an investigation of the rate of equilibration of chloramphenicol 3-monosuccinate over the pH range of 4.0 to 9.0 are shown in Figure 11. Calculation of the slope from the equation $\log k_{obs} = mpH + \text{Intercept}$ for the portion of the line from pH 7 to pH 9 gives an average value of $m = 1$, thus indicating a direct dependency of the rate on hydroxide-ion concentration. The portion of the line below pH 7 does not indicate a direct hydroxide-ion dependency but seems to be the result of an additional contribution to the rate of reaction from a very slow uncatalyzed rearrangement of the monoanionic species or

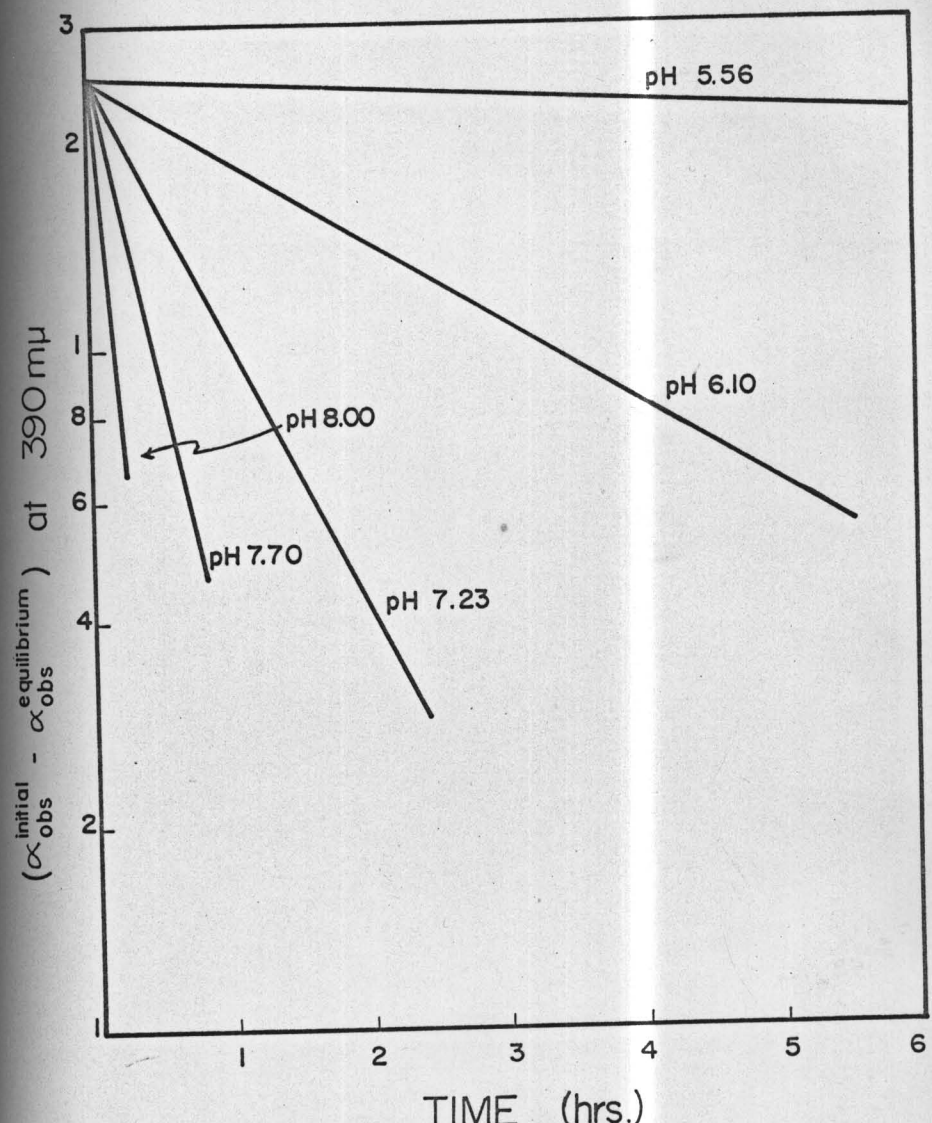


Figure 9. Plots showing apparent first order behavior of the rate of equilibrium formation of chloramphenicol 3-monosuccinate in 0.2 M phosphate buffer at 25° C for several pH values.

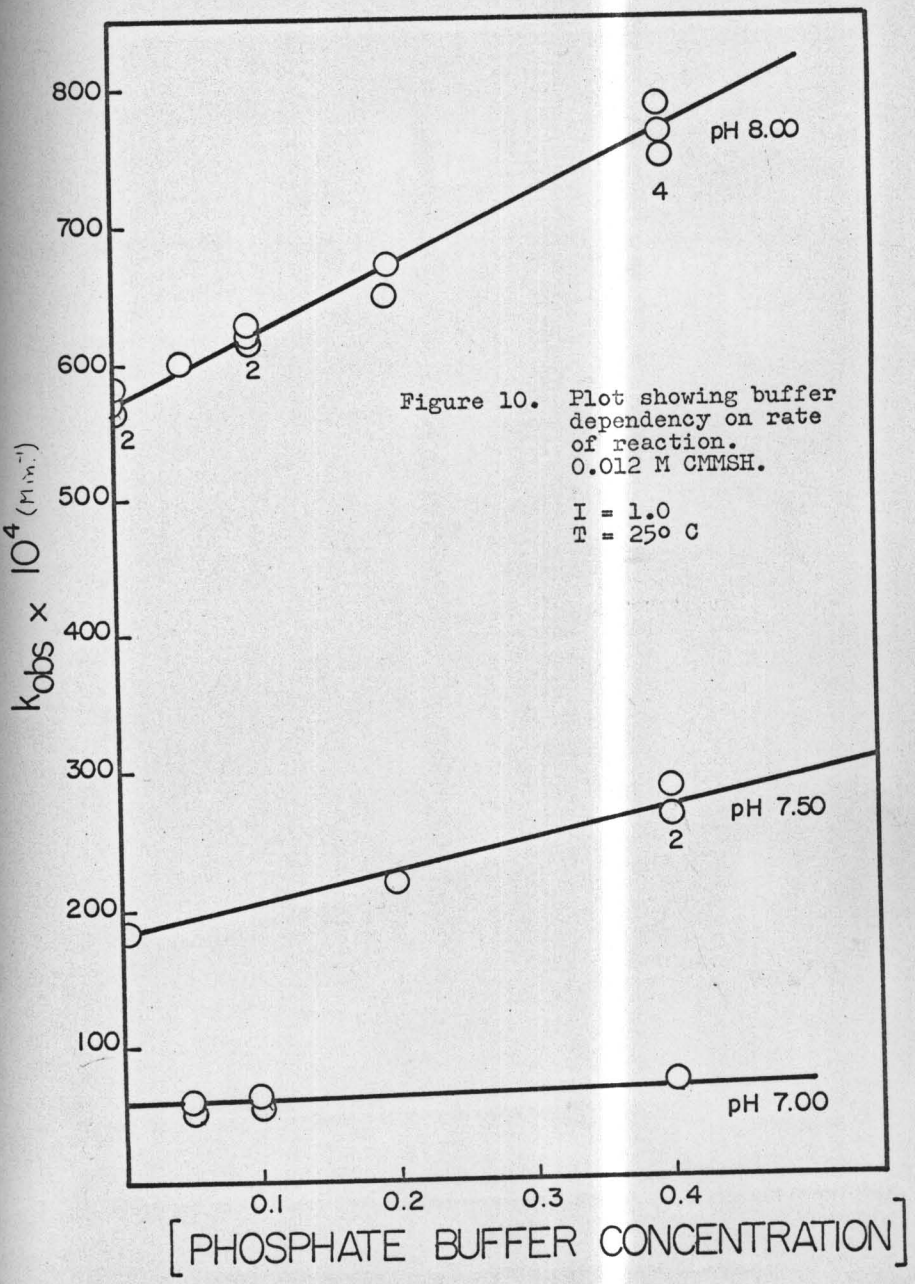


Figure 10. Plot showing buffer dependency on rate of reaction.
0.012 M CMMSH.

I = 1.0
T = 25° C

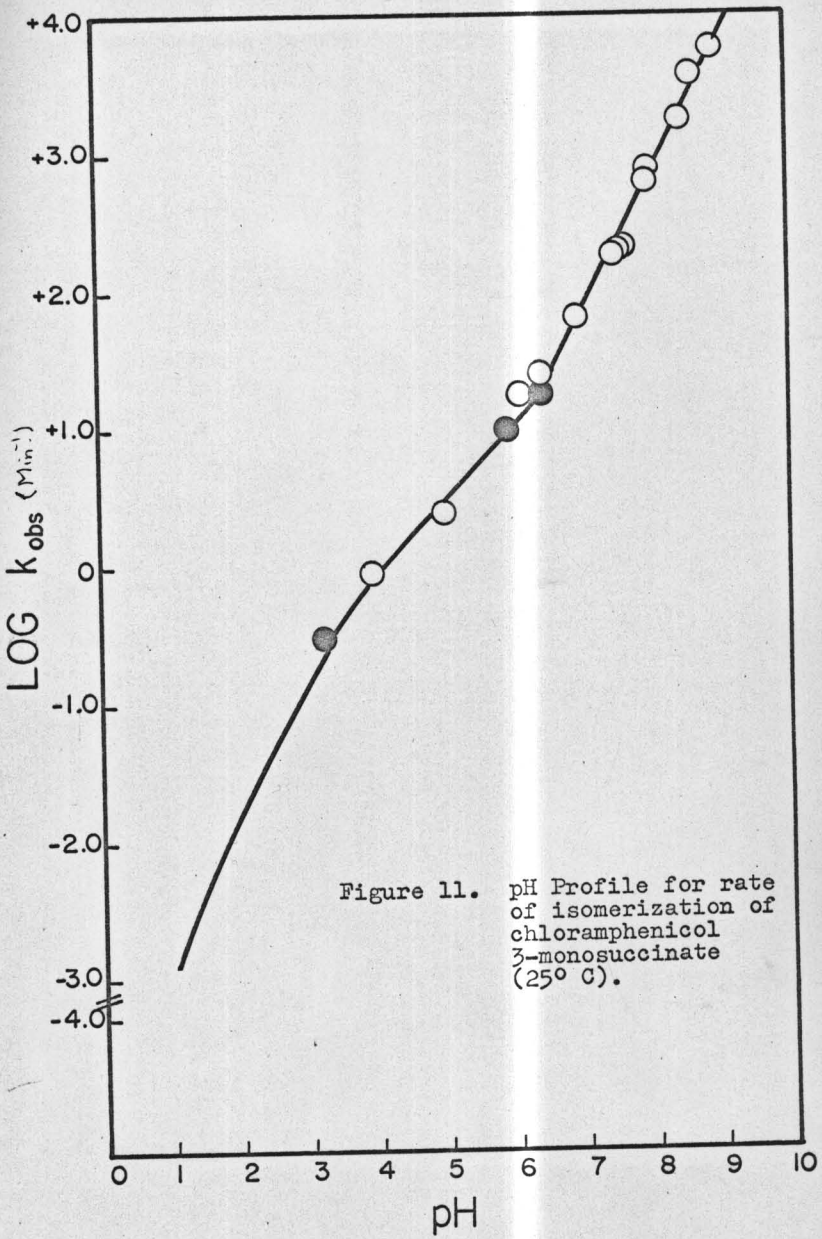


Figure 11. pH Profile for rate of isomerization of chloramphenicol 3-monosuccinate (25° C).

hydroxide-ion catalyzed attack on the uncharged form. From these data it can be shown that the isomerization of chloramphenicol follows the rate laws:

$$k_f = k_1(CMMSH)_{\ell}(OH) + k_2(CMMS^-)_{\ell}(OH)$$

where,

k_f = the rate constant for the approach to the equilibrium:

$$(CMMSH)_{l,total} \underset{k_r}{\overset{k_f}{\rightleftharpoons}} (CMMSH)_{r,\ total}$$

and where,

$(CMMSH)_l$ = concentration of the linear associated form of chloramphenicol 3-monosuccinate

$(CMMSH)_{l,total}$ = total drug concentration in linear form

$(CMMS^-)_l$ = concentration of linear ionized form

$(CMMSH)_{r,total}$ = total drug concentration in rearranged cyclic form

Evaluation of the rate constants at 25° gives,

$$k_1 = 2.56 \times 10^5 \text{ Moles}^{-1}\text{min}^{-1}$$

$$k_2 = 1.25 \times 10^4 \text{ Moles}^{-1}\text{min}^{-1}$$

Using the above values and the experimentally determined $K_{ionization} = 4.4 \times 10^{-5}$ and $K_{equilibrium} = 0.30$, theoretical k_{obs} values are obtained.

pH	$k_{obs} \times 10^4$ (min^{-1}) as calculated	$k_{obs} \times 10^4$ (min^{-1}) as determined spectro-polarimetrically
3.0	0.115	0.19
4.0	0.781	0.82
5.0	2.52	2.43
6.0	7.73	9.10
7.0	56.76	59.76
8.0	540	560
9.0	5400	5650

The theoretical pH profile is a fairly good representation of the experimentally determined values for the observed rate constant. The low water solubility of the unionized substrate and the very slow rate of rearrangement in the lower pH regions proved to be limiting factors in obtaining reliable data below pH 3. The reaction occurs at such a fast rate above pH 9 that the instrumental method used to follow the reaction was not suitable for following fast reactions whose half-life is less than 5 min.

It seemed of interest as part of this study to investigate the rate of hydrolysis of the nonisomerized ester in the pH region 7-9. Using the automatic titrator and pH stat technique no hydrolysis over the period required for equilibrium formation was observed. This gave further support to the migration reaction as being the

only major reaction being observed.

The effect of ionic strength on the rate of isomerization was also studied. Changing the ionic strength twenty-fold had no effect on the rate of the reaction. Since reaction can occur over a wide pH range with several possible catalytic species being involved a direct effect of ionic strength would not be expected.

Studies on the Equilibrium Mixture

An experiment to check the validity of the equilibrium was carried out using the pure cyclic succinate isolated by the reverse phase chromatographic procedure. A solution of the purified cyclic hemi-ortho-ester in pH 7 phosphate buffer was allowed to equilibrate, then chromatographed. Calculation of the concentrations of the components showed them to be in the same ratio as that found for the cyclization reaction, thus verifying that a true equilibrium is indeed involved.

A study of the reaction by reverse phase column partition chromatography was carried out to check the stability of the equilibrium as well as to identify all the compounds involved. One such set of data is shown below in Table II.

TABLE II

Time (hrs)	Other compounds	% Chloramphenicol	% Cyclic ester	% Chloramphenicol 3-monosuccinate
0	trace	0.023	0.254	99.5
3	trace	0.70	8.92	90.4
12.5	trace	0.88	20.5	78.5
23.5	trace	0.87	25.2	74.0
35.5	trace	1.54	25.3	73.4
121	trace	2.4	24.9	72.2
145	trace	3.1	22.8	74.5

A plot of the components of the reaction versus time in Figure 12 shows the formation of the equilibrium mixture and the stability of this mixture over a period of several days. Hydrolysis of the ester linkage to give chloramphenicol is seen to occur at a very slow rate. This is in agreement with several other studies on acyl migrations where the authors reported migration to occur at an extremely fast rate as compared to hydrolysis (43,15).

The equilibrium ratio can be calculated from extrapolation of the flat portion of the curve to zero time and reading the indicated per cent. This curve shows the ratio of linear chloramphenicol monosuccinate to cyclic succinate to be about 75:25; this ratio apparently varied, however, from 78:22 to 70:30 depending on the method used to measure it. Calculation from nmr integration data of

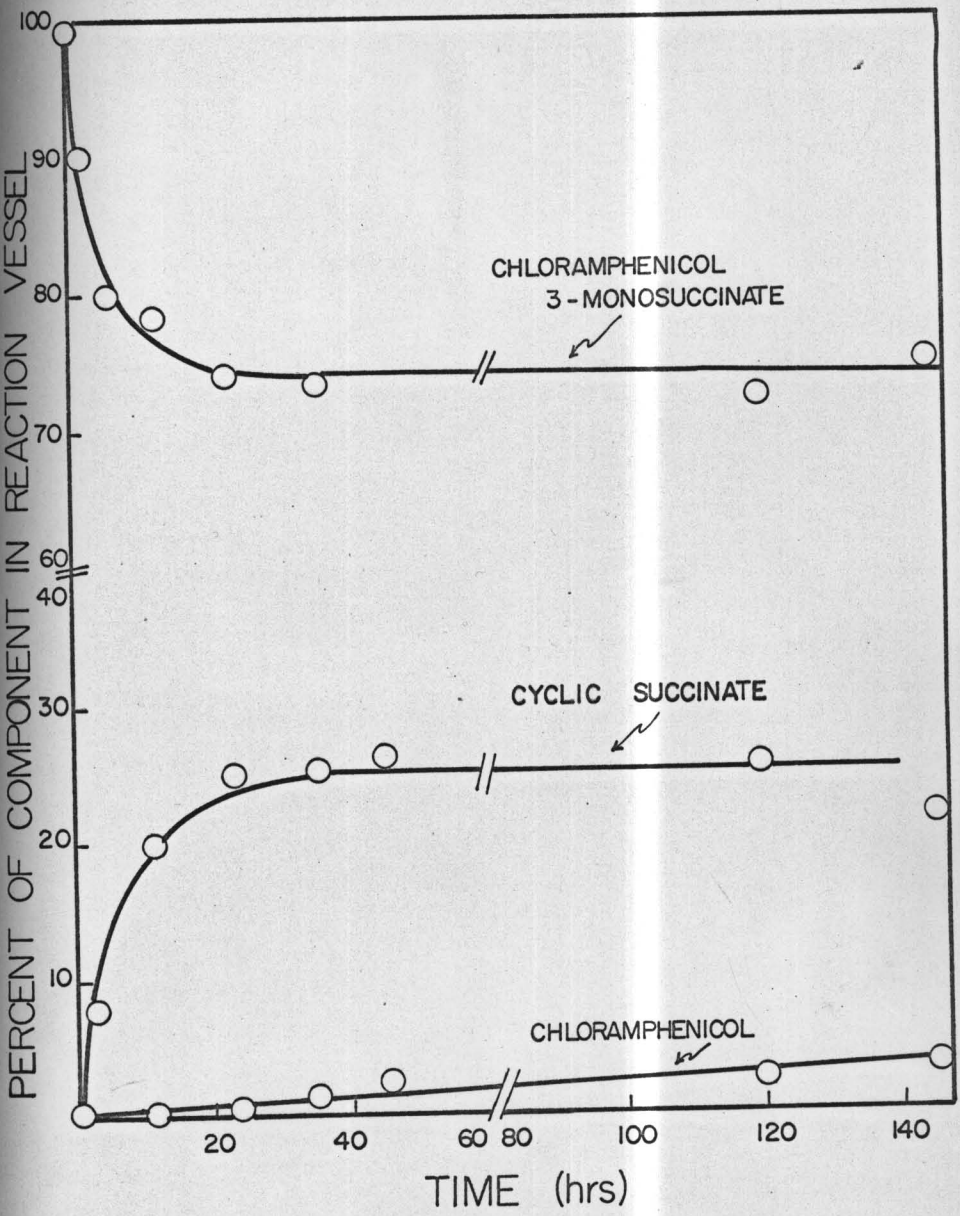


Figure 12. Plot of percent of each component in reaction vessel versus time, showing the approach to equilibrium and constancy of equilibrium composition. $T = 25^{\circ}$

the equilibrium ratios from aqueous buffer as well as mixed organic-aqueous systems gave values averaging 70:30 for the linear to cyclic form. A partial representative nmr spectra of the equilibrium mixture is shown in Figure 13 with peaks used for calculation of equilibrium ratio in solid black lines.

Nuclear Magnetic Resonance Data

Examination of the nmr spectra of chloramphenicol, Figure 15, and chloramphenicol 3-monosuccinate, Figure 16, shows the proton attached to the dichloroacetyl group and the one attached to C^1 of the propanediol portion to have the same chemical shift in both of these compounds. The line position of dichloroacetyl proton with chemical shift 6.35 ppm is the same for chloramphenicol, the linear and the cyclic rearranged succinate.

The nmr spectrum of chloramphenicol has been carefully studied by Jardetsky in a report on the conformation of chloramphenicol in solution (47). The spectrum of chloramphenicol in Figure 14 is one obtained by exchanging the hydroxyl and amide hydrogens for deuterium. It then consists of 5 sets of lines with a ratio of peak areas of approximately 4:1:1:1:2. Assignments for all the protons except the hydrogens on C(1) and C(2) and the exchangeable protons are possible by integral area and relative chemical shift measurements. Tentative placement of the C(1) proton at 5.28 ppm was verified by spin-spin decoupling. Collapse

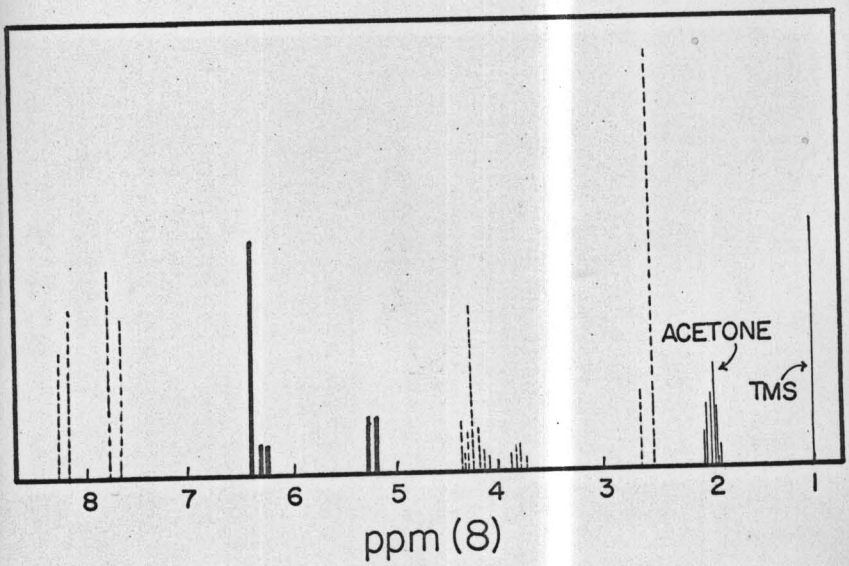


Figure 13. Nmr representations of spectra of equilibrium mixture of linear and cyclic chloramphenicol 3-monosuccinate in pH 7.0 phosphate buffer. Solvent is acetone d6.

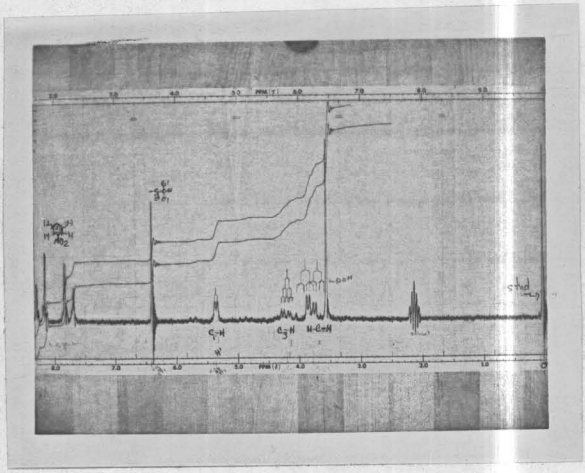


Figure 14. 60 Megacycle nmr spectrum of chloramphenicol in deuterated acetone with deuterium oxide used to replace the exchangeable hydrogens with deuterium and tetramethylsilane as internal standard.

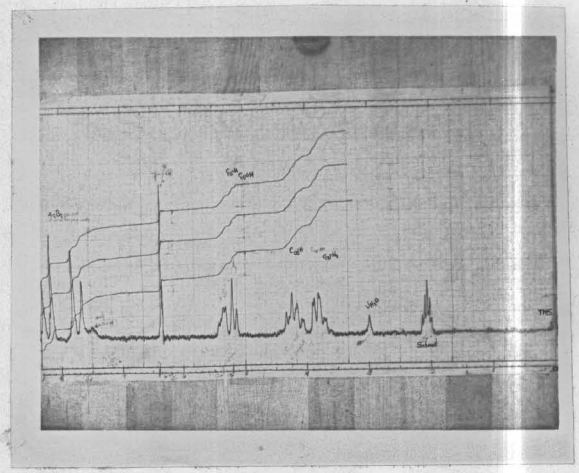


Figure 15. 60 Megacycle nmr spectrum of chloramphenicol in deuterated acetone with tetramethyl silane as internal standard.

of the doublet (J = 2.4 cps) at 5.28 ppm when decoupled with the set of peaks at 4.29 ppm, proved these two protons to be on adjacent carbon atoms. Further decoupling of the two protons on C(3) with the multiplet at 4.29 ppm fixed the positions of the two protons on C(1) and C(2). The C(3) hydrogens appear as a multiplet at 3.86 ppm. A more detailed analysis of the splitting patterns is presented by Jardetsky (47). He also assigned the chemical shifts for the exchangeable protons which for the N-H, C(1)-OH and C(3)-OH in deuterated acetone are 7.46, 3.31, and 4.20 ppm respectively.

The spectrum of chloramphenicol 3-monosuccinate in deuterated acetone (Figure 16) is somewhat similar to that of chloramphenicol (Figure 15). The aromatic ring protons form the same A_2B_2 system centered at 7.95 ppm. The singlet at 6.35 ppm is the C-H-Cl_2 proton. The other singlet at 2.63 ppm integrates for 4 protons and is assigned to the succinate methylene protons. The four carbon linked hydrogens can be partially assigned on the basis of spin decoupling experiments. The C_1(H) proton is assigned to the doublet (J = 2 cps) at 5.26 ppm. The C(2)H and C(3)-H_2 hydrogens are a complex multiplet centered at 4.35 ppm. The single secondary hydroxyl proton C(1)-OH occurs as a broad peak at about 6 ppm and the amide proton is present at 7.46 ppm; however, the acid proton was undistinguishable at probe temperature.

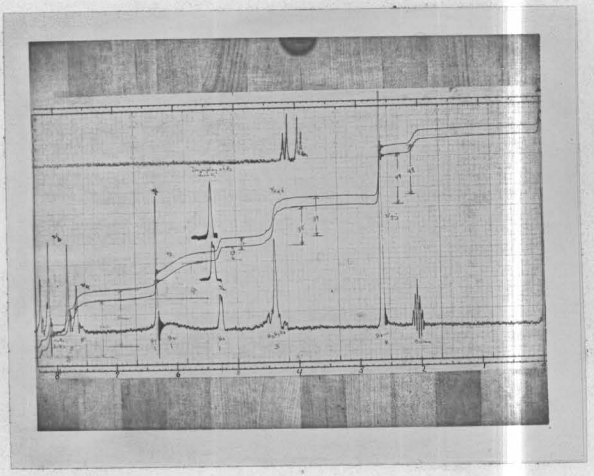


Figure 16. 60 Megacycle nmr spectrum of chloramphenicol 3-monosuccinate in deuterated acetone with tetramethylsilane as internal standard.

Line positions: ν_b = center of the ring A_2B_2 quartet; ν_c = $-CHCl_2$; ν_a = $C_{(1)}H$; ν_d = $C_{(2)}H$; ν_e and ν_f = $C_{(3)}H$'s; ν_{g-j} = succinate methylenes; ν_k = NH; ν_l = $C_{(1)}OH$.

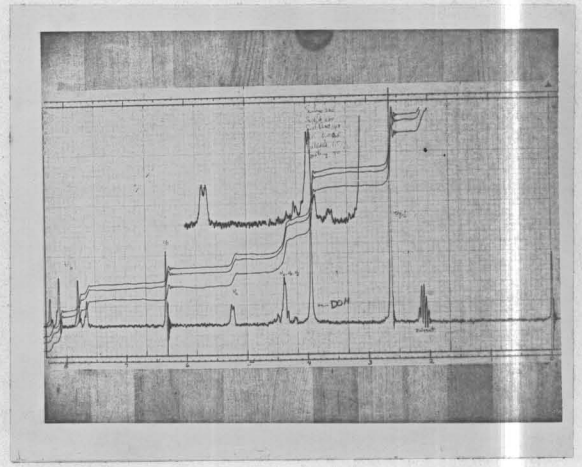


Figure 17. 60 Megacycle nmr spectrum of chloramphenicol 3-monosuccinate in deuterated acetone with deuterium oxide used to replace the exchangeable hydrogens with deuterium and tetramethylsilane as internal standard.

The cyclic succinate has certain structural differences which are distinctly shown in its nmr spectrum taken in deuterated acetone, Figure 18.

A comparison of the peaks for the amide nitrogen, the aromatic ring protons and the $CHCl_2$ hydrogen between the linear and cyclic form shows essentially no change in the frequency of absorbance for these protons. The $C_1(H)$ hydrogen for the cyclic compound has undergone a paramagnetic shift of 57 cps and appears as a doublet at 6.23 ppm. The deshielding effect produced on esterifying a hydroxyl group on the proton or the protons on the carbon holding the hydroxyl group is well documented (48). Narayanan and Sanna reported a downfield shift of about 1 ppm for a proton on a secondary hydroxylic carbon when the hydroxyl is acetylated (49). Acetylation of the free hydroxyl in chloramphenicol monosuccinate results in a similar shift as shown in Figure 20. The C(1)-H hydrogen for the acetylated derivative has a chemical shift of 6.2 ppm which is the result of a downfield shift of about 1 ppm upon acetylation.

Also for the cyclic ester, the splitting of the C(2)-H hydrogen by the C(1)-H and the two C(3)-H protons give a quartet centered at 4.44 ppm. The two hydrogens on C(3) have shifted upfield to 3.7 ppm and appear as a pair of overlapping doublets. Table III shows the chemical shifts of the propyl hydrogens for the compounds of interest.

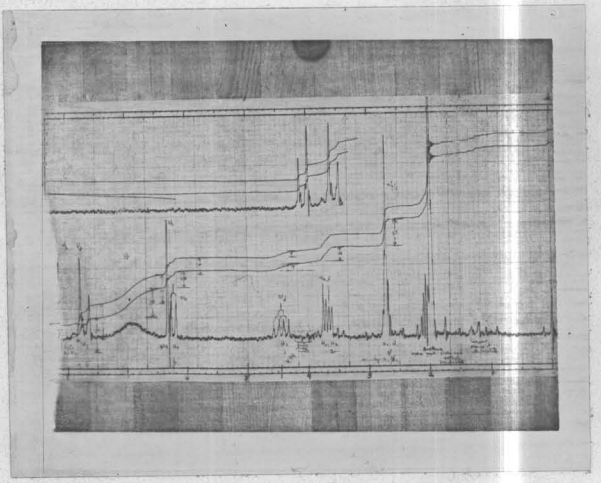


Figure 18. 60 Megacycle nmr spectrum of chloramphenicol cyclic hemi ortho succinate in deuterated acetone with tetramethylsilane as internal standard.

Line positions: v_b = center of the ring A_2B_2 quartet; v_c = $-CHCl_2$; v_a = $C_{(1)}H$; v_d = $C_{(2)}H$; v_e and v_f = $C_{(3)}H_2$; v_{g-j} = succinate methylene triplet overlapping pair; v_k = NH; v_l = other replaceable hydrogens.

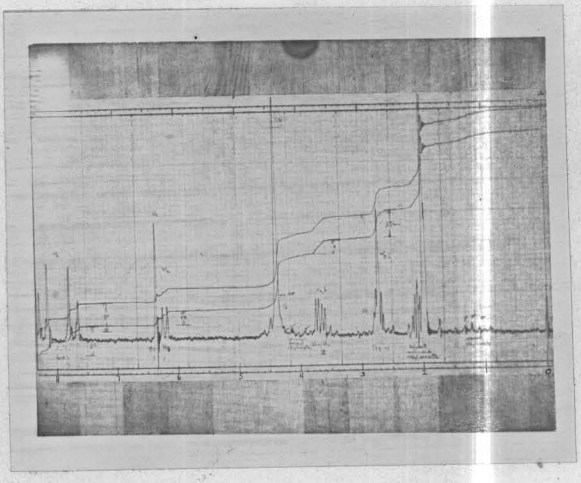


Figure 19. 60 Megacycle nmr spectrum of chloramphenicol cyclic hemi ortho succinate in deuterated acetone with deuterium oxide used to replace the exchangeable hydrogens with deuterium and tetramethylsilane as internal standard.

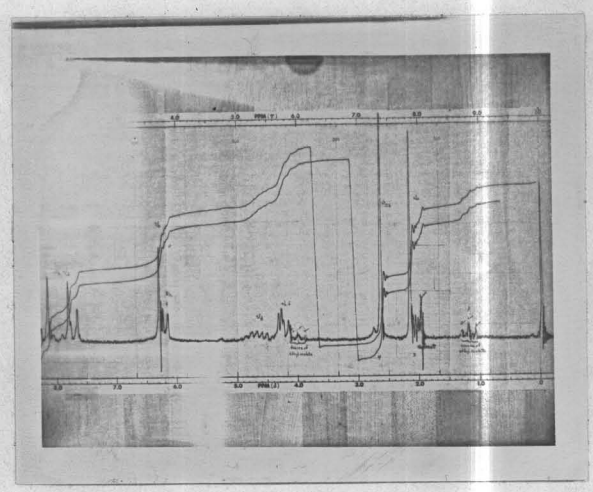


Figure 20. 60 Megacycle nmr spectrum of O^1-acetyl chloramphenicol 3-monosuccinate in deuterated acetone with tetramethylsilane as internal standard.

TABLE III

Chemical shift values for propanediol hydrogens of chloramphenicol and its derivatives

	Hydrolysis product of chloramphenicol	Chloramphenicol	Chloramphenicol 3-monosuccinate	Cyclic succinate
C_1H	4.83	5.30±0.02	5.26	6.23
C_2H	3.1	4.2 ±0.08	4.4	4.44
C_5 H H	3.7	3.8 ±0.06	4.4	3.7

The succinate methylene protons in the cyclic ortho ester do not give rise to a singlet as in the case in the linear molecule. The signal for these hydrogens in the cyclic compound is an A_2B_2 system which arises from a pair of overlapping triplets. The nmr spectra of other monoesters of succinic acid all show the methylene hydrogens to appear as a singlet at between 2.5 and 3.0 ppm. The A_2B_2 system for the cyclic ortho ester is centered at 2.7 ppm.

One explanation for the unique splitting pattern of the methylene hydrogens may be the difference between an sp^2 and sp^3 hybridized carbon adjacent to the protons. In a monoester of succinic acid both carbons are sp^2 hybridized and interact through resonance with the oxygens of the carbonyl or carboxyl functional group. The carbon center of the cyclic ortho ester is sp^3 hybridized and is not part

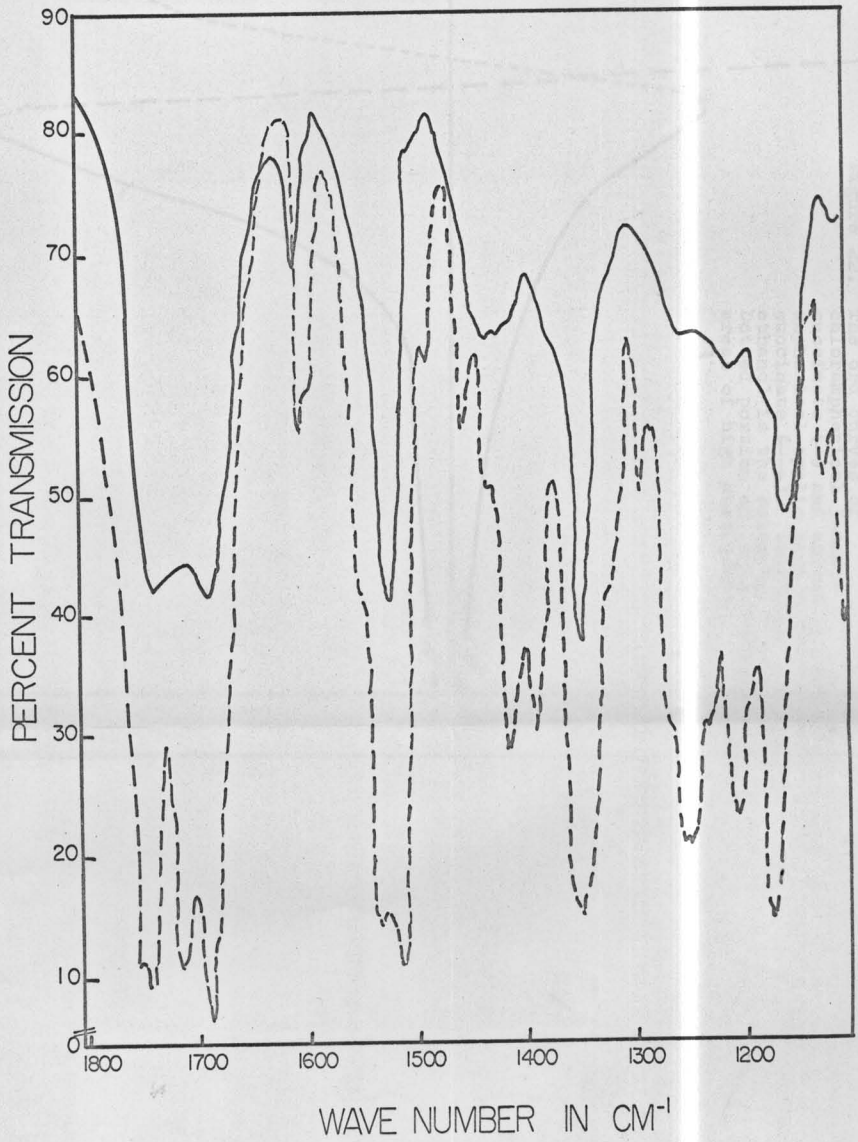
of a resonance cloud thereby having a different electronic effect on the neighboring protons.

Other Spectral Data

Additional structural evidence was obtained from ir and ord data. The ir spectra of both the cyclic and linear compounds is shown in Figure 21. The amide, ester, and acid carbonyl of the linear ester absorb at 1690 cm^{-1}, 1718 cm^{-1} and 1745 cm^{-1} respectively. Assignments of these bands was based on published values for similar compounds (50,51). A comparison of the carbonyl region and C-O-C stretch portion of both spectra can be made. The poorly defined carbonyl region for the cyclic ester suggests the loss of ester carbonyl absorption as does the less detailed C-O-C stretch region.

Since the proposed structure for the rearranged succinate is a cyclic hemi ortho ester, an attempt was made to derivatize the free hydroxyl by reaction with diazomethane to convert the compound to an ortho ester to be used for ir analysis. Reaction of the isolated reaction product from chloramphenicol 3-monosuccinate with diazomethane proved to be very complex. Tlc analysis of the reaction mixture showed five products and none of the succinate ester of chloramphenicol. Apparently, the ring had opened and hydrolysis of the ester linkage had taken place as the succinate methylenes were not observed in the nmr spectrum of the isolated reaction mixture.

Figure 21. Representation of the ir spectra of chloramphenicol 3-monosuccinate (- -) and chloramphenicol cyclic hemi ortho succinate (——), ~~cyclic ester (——)~~ using a KBr pellet, ~~CHCl3~~ (- - -).



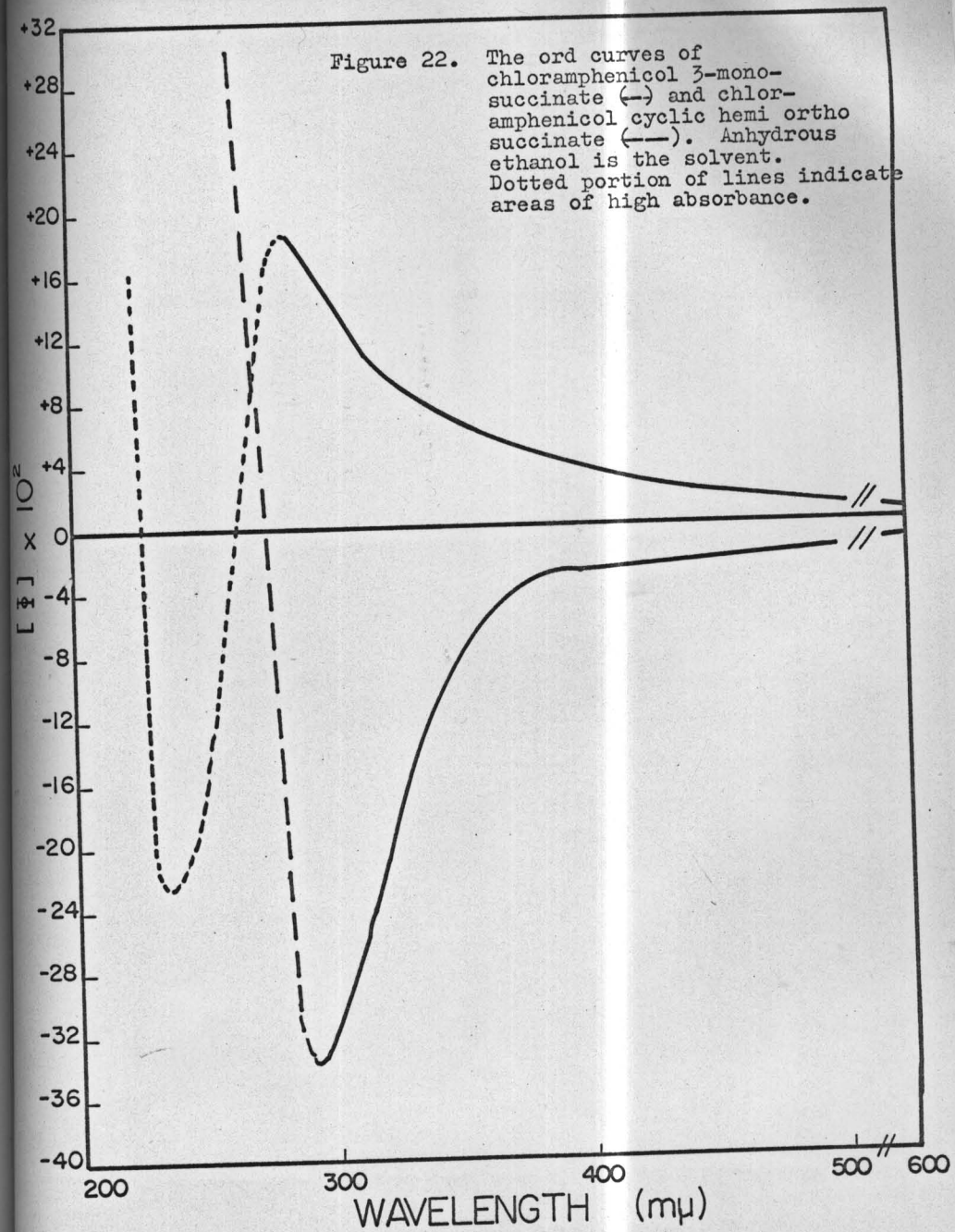
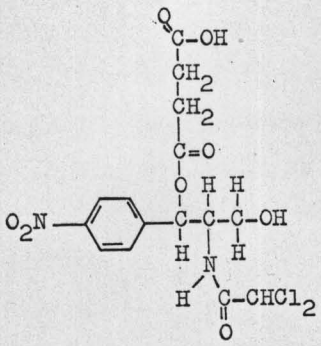


Figure 22. The ord curves of chloramphenicol 3-monosuccinate (—) and chloramphenicol cyclic hemi ortho succinate (– – –). Anhydrous ethanol is the solvent. Dotted portion of lines indicate areas of high absorbance.

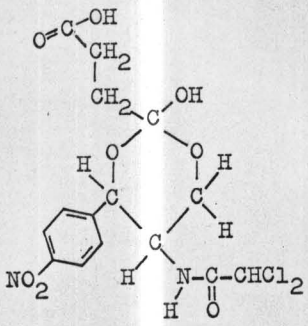
The ord curve for the cyclic and linear ester is shown in Figure 22. Due to high absorption of both compounds below a wavelength of 280 mμ this region is an approximation based on repeated determinations and several dilutions. Chloramphenicol 3-monosuccinate shows a positive Cotton-effect whereas the rearranged ester shows a negative Cotton-effect. The ord curve of O^1-acetylated chloramphenicol 3-monosuccinate and O^1-dichloroacetylchloramphenicol 3-monosuccinate also show a negative Cotton-effect. Esterification of the secondary hydroxyl group seems to be associated with a change in optical rotation as observed for these compounds, resulting in a negative ord curve for the O^1 ester compound whose ord curve prior to esterification was positive. Also characteristic of esterification of the O^1-hydroxyl group is a paramagnetic shift of the $C_{(1)}H$ as observed in the nmr spectra. Since both of these phenomena occur in the rearrangement of chloramphenicol 3-monosuccinate, involvement of the O^1-hydroxyl is definitely established.

Structural Proposal and Mechanism for Formation of the Cyclic Hemi Ortho Ester

It is possible to propose two structures for the rearranged succinate whose spectral data would be very similar. These are shown below as compound A and B.



Compound A



Compound B

However, certain characteristics of the nmr and ir spectra of the isolated rearranged species are unique and can only be explained in terms of structure B. The signal for the methylene protons for a compound such as A would be expected to be a singlet based on data for similar chloramphenicol derivatives and related hemi-succinate esters and on the similarity of the electronic environment. A splitting pattern would be expected for the methylene protons of B due to the difference in electronic environment caused by the difference between sp^2 and sp^3 hybridization of the adjacent carbon atoms. Since a distinct A_2B_2 splitting pattern is observed in the spectrum for the rearranged succinate this observation is in support of structure B.

If compound A were the correct structure a peak for the primary hydroxylic proton would be expected at about 4.2 ppm. Based on the observation by Jardetzsky (47) that the chemical shift of the C_3-(OH) proton for chloramphenicol are constant over a wide range of concentrations it would be expected that a peak for this proton would be observed near this frequency for a structure such as A. There was no evidence of any exchangeable proton in this region of the nmr spectrum as seen in Figure 18. The exchangeable protons for the rearranged succinate have chemical shifts of 7.8 and 7 ppm. The peak at 7.8 ppm is the amide nitrogen. The broad peak at 7 ppm integrates for more than one hydrogen and probably indicates the presence of a hydrate.

The 6-membered meta-dioxane ring of compound B would be expected to assume a fairly rigid chair conformation as proposed for other meta-dioxane derivatives (52). The well defined splitting patterns of the three propanediol protons observed in Figure 18 would be expected for a structure with predominately one conformation.

As previously stated the ir spectrum of the isolated rearranged succinate is not particularly conclusive but the carbonyl region does indicate the possible loss of ester carbonyl as would be expected for the hemi ortho ester.

Acetylation of the rearranged succinate with acetic anhydride was attempted in an effort to obtain a derivative

which might be made to crystallize. However, a complex reaction mixture was obtained and nmr data on this reaction indicated that cleavage of the ring and hydrolysis of the succinate group had occurred. A similar reaction with chloramphenicol 3-monosuccinate gave the O^1-acetylated derivative whose nmr is shown in Figure 20 and ord curve in Figure 23.

All attempts to synthesize compound A were without success (1) so it is not surprising that the data indicate that compound A is incapable of existence in equilibrium with chloramphenicol 3-monosuccinate to the extent of 25% as reported in this study. Therefore, the structure for the rearranged compound is proposed to be the cyclic hemi ortho ester referred to as compound B.

The pH profile shows a definite hydroxide catalyzed reaction above pH 7. The following mechanism is proposed for this region of the profile.(see page 78).

The hydroxide presumably acts as a specific base, removing the hydroxyl proton from the alcoholic oxygen which then is free to attack the ester carbonyl carbon which is a reactive nucleophilic center. The p-NO_2 phenyl moiety lowers the pK_a of this alcoholic group so that its proton is more easily removed than would be the case for a normal benzyl alcohol. Formation of the six-membered meta-dioxane ring is obviously a function of the pK_a of the hydroxylic proton. Below pH 7 several factors may be responsible for the increase in rate of reaction of the

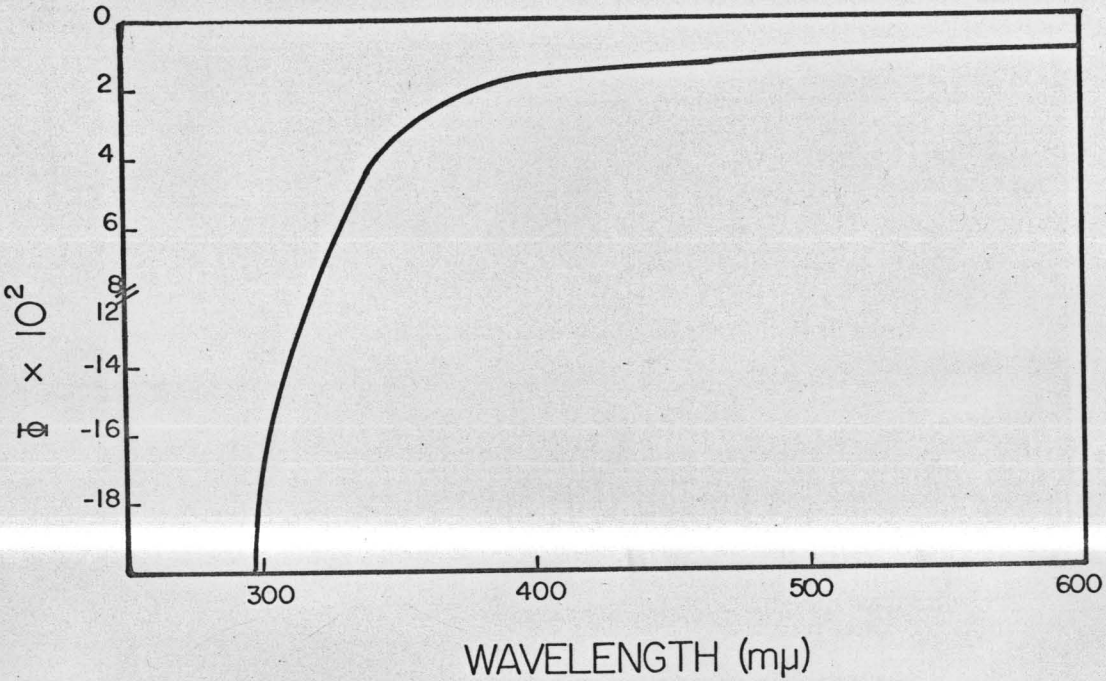
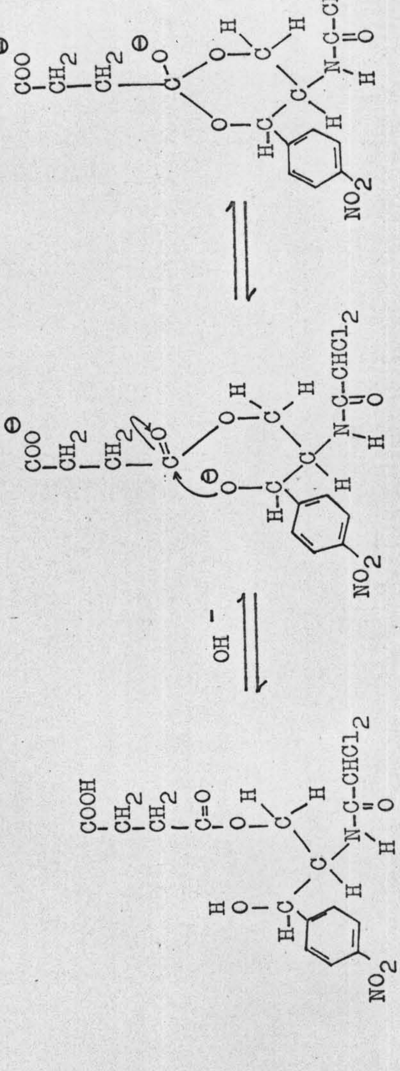


Figure 23. Ord curve of O'-acetyl chloramphenicol 3-monosuccinate in anhydrous methanol.



linear succinate. The dissociation of the free carboxylic acid would be expected to change the pK_a of the secondary hydroxyl, thus the rate of the reaction. Participation by other isoprotonic mechanisms does not seem too probable. Removal of the hydroxylic proton of the cyclic hemi ortho ester by hydroxide ion results in collapse of the ring structure to give back the more favored equilibrium component, the linear form of chloramphenicol 3-monosuccinate.

APPENDIX

STUDIES ON THE N → O MIGRATION OF CHLORAMPHENICOL

This investigation was also concerned with the N → O acyl migration of chloramphenicol. The acid catalyzed migration of the dichloroacetyl group from the amide nitrogen to the secondary hydroxyl oxygen has been reported to occur with several chloramphenicol derivatives (16). It was of interest in connection with the present study to determine if the dichloroacetyl group was capable of migration under the same conditions necessary for the isomerization of chloramphenicol 3-monosuccinate.

In this study solutions of hydrogen chloride gas in anhydrous methanol were prepared by first saturating the alcohol with the gas and then diluting to the appropriate concentration of hydrogen chloride to cause N → O migration of the dichloroacetyl group in chloramphenicol. The reaction was followed using a spectropolarimetric technique and also by paper chromatography. A definite change in the ord curve of the reaction mixture of chloramphenicol was associated with the N → O acyl migration. By following the change in optical rotation at 430 mμ as a function of time the half-life for the migration was found to be 20 minutes at 25°, where the hydrogen chloride concentration was 25%.

Solutions of chloramphenicol in aqueous ammonium hydroxide were also studied using a spectropolarimetric

procedure. These basic solutions were particularly susceptible to decomposition by the action of base on the nitro group of the benzene portion of the molecule as observed by a change in ord curve of the solution as well as by a change in color of the reduction mixture. However, aqueous buffer solutions of chloramphenicol at near neutral pH showed no change in optical rotation over a period several times that necessary for the isomerization of chloramphenicol 3-monosuccinate.

Therefore, by assuming that the results for this study of the N $\rightarrow$ O acyl migration of chloramphenicol are also true for chloramphenicol 3-monosuccinate, it is possible to rule out N $\rightarrow$ O acyl migration as being a significant reaction in the case of the isomerization of chloramphenicol 3-monosuccinate.

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SUMMARY

Chloramphenicol 3-monosuccinate, a water soluble derivative of chloramphenicol, has been shown to undergo an isomerization in aqueous solution to form an equilibrium mixture which consists of itself and a rearranged compound. The present investigation has been concerned with the study of the general chemistry of chloramphenicol 3-monosuccinate in aqueous solutions, including 1) the pH profile for the rate of equilibrium formation, 2) the effect of buffer concentration and buffer type on the rate of equilibration, 3) the nature and chemical composition of the equilibrium mixture and 4) isolation and characterization of the rearranged species.

The optical activity of solutions of chloramphenicol 3-monosuccinate was found to change at a rate directly relatable with that for the rate of equilibrium attainment as determined by column chromatography. Kinetic data for the approach to equilibrium as obtained by following the change in optical rotation of solutions of the monosuccinate ester at various pH values showed the reaction to be first order with respect to substrate concentrations over the range of concentrations and pH employed in this study.

The rate of both the forward and reverse reaction was found to vary directly as hydroxyl ion concentration over a pH region in which the acid functional group is completely ionized. Rate data for the approach to equilibrium below

pH 7 did not indicate a direct hydroxide ion dependency but seemed to be the result of an additional contribution to the rate from a very slow uncatalyzed rearrangement of the mono anionic species or hydroxide ion catalyzed attack on the uncharged form. The kinetic behavior best fits a simple A $\rightleftharpoons$ B model which for this system can be written:

$$(CMMSH)_l \underset{k_r}{\overset{k_f}{\rightleftharpoons}} (CMMSH)_r$$

where,

$(CMMSH)_l$ = chloramphenicol 3-monosuccinate

$(CMMSH)_r$ = the rearranged compound

From the rate data the isomerization of chloramphenicol 3-monosuccinate was found to follow the rate law:

$$k_f = k_1(CMMSH)_l(OH) + k_2(CMSS^-)_l(OH)$$

where,

$(CMMS^-)_l$ = ionized chloramphenicol 3-monosuccinate.

By using the specific rate constants evaluated from the experimental data and the dissociation constant for the ionization of the carboxylic acid group it was possible to calculate a theoretical pH-rate profile which was in good agreement with the observed results.

Results of a study of the effect of various buffer systems and of ionic strength on the rate of approach to equilibrium revealed that neither buffer type nor ionic strength had any observable effect on the rate. Slight buffer catalysis by phosphate systems was observed above pH 7.

The influence of variables such as pH and concentration on the equilibrium as well as the composition of the equilibrium mixture was also studied. Over the pH range of 4.0 to 9.0 the equilibrium mixture was found to always consist of approximately 75% of chloramphenicol 3-monosuccinate and 25% of the rearranged compound. Spectral data of the rearranged component isolated from equilibrium mixtures obtained over the pH range studied indicated that only one isomeric species was being formed at all pH values.

Isolation and purification of the rearranged compound was achieved by using a reverse phase column chromatographic method and extraction technique. Repetitive attempts to recrystallize the semi-solid residue corresponding to the pure rearranged compound were unsuccessful, although the residue was shown to be a single chemical entity by tlc and nmr. Immediate analysis of the isolated compound was required because of its tendency to degrade even under cold, dark and dry storage conditions.

Characterization of the rearranged compound was accomplished by the use of nmr, ir, and ord data. A comparison of the nmr spectra of chloramphenicol 3-mono-

succinate and its equilibrium component showed several significant differences in the chemical shifts and splitting patterns of the propane skeleton protons, the succinate methylene protons and the hydroxylic hydrogens. From these data it was possible to propose that the structure for the rearranged compound was a cyclic hemi ortho ester. Further proof for this structure was obtained from ir and ord data. The difference in the carbonyl absorbance for these two compounds also supported the proposed structure. The shape of the ord curves of chloramphenicol 3-monosuccinate and the isolated rearranged compound was also significant. The ord curve for chloramphenicol 3-monosuccinate exhibited a positive Cotton-effect while that for the cyclic isomer showed a negative Cotton-effect.

The formation of the cyclic hemi ortho ester was proposed to take place by the attack of the ionized C-1 hydroxylic oxygen on the ester carbonyl carbon of the C-3 mono ester to give the cyclic meta-dioxane-like structure which is capable of collapsing to give back the more thermodynamically stable linear form of chloramphenicol 3-monosuccinate.