THE INTERACTION OF PENICILLINASE WITH PENICILLINS

by Clayton E. Rasmussen

(Under the supervision of Professor Takeru Higuchi)

These studies are concerned with investigations of the interactions of several penicillins with Bacillus cereus penicillinase (Neutrapen) based on the optical rotation or circular dichroism properties of the antibiotics. In the first part, the highly specific nature of the action of the penicillinase on the penicillins has been utilized to develop a highly sensitive and specific analytical procedure for these drugs. In the second part, the optical rotatory or circular dichroism changes occurring during these enzymatic reactions have been shown to be a convenient and rapid way of determining the kinetic parameters of such reactions.

Levy[1] has already shown, using a recording polarimeter of his own design,[2] that optical rotatory changes induced by penicillinase can be used for the analysis of penicillins which are sensitive to this enzymatic action. However, this method never became too popular since measurements were made in the visible region where the sensitivity was low and where there was a relatively large change in rotation due to mutarotationof the penicilloate ions. Also the end point Levy selected would be affected by the amount of enzyme used.

In the present study, an appropriate end point was selected based on theoretical analysis of the kinetics for penicillin-penicillinase reactions. This end point would be least affected by any variations in the system. By using a

more sensitive commercially available recording spectropolarimeter in the ultra violet region, an extremely precise assay procedure was developed. A precision of 0.3% was obtained from 197 assays on 26 solutions of three penicillinase-sensitive penicillins, benzylpenicillin, ampicillin and phenoxylmethyl-penicillin; and a precision of 0.5% from 34 assays on 5 solutions of a penicillinase-resistant penicillin, cloxacillin. These assay procedures are not affected by the presence of other optically active materials since the penicillin concentration is related to the changes in optical activity.

These optical rotatory and circular dichroism procedures were applied to assaying a mixture of a non-resistant and a resistant penicillin, ampicillin and cloxacillin, respectively.

Since these optical activity assay procedures continuously monitor the action of penicillinase on penicillin over a wide change in substrate concentrations, they were readily adapted to the determination of the Michaelis-Menten enzymatic kinetic parameters. It was found that a single recording of the reaction, and hence the integrated form of the Michaelis-Menten equation, could be used to obtain these kinetic parameters.

When applying these optical rotatory and circular dichroism methods to the study of the interaction of penicillinase with penicillin, several interesting points were observed. (1) The Michaelis constant for benzylpenicillin was found to be affected by the ionic strength while there was no effect on the maximum velocity. (2) This ionic strength effect was found to vary with the pH. At pH

5.7, there was no ionic strength effect, while the magnitude of the Michaelis constant increased with increasing ionic strength below pH 5.7 and decreased with increasing ionic strenght above pH 5.7. (3) For benzylpenicillin, at pH 5.00, sodium sulfate had a greater ionic strength effect on the Michaelis constant than sodium acetate, sodium chloride, or potassium chloride. (4) Boric acid was found to have a competitive inhibition effect on the penicillinase-benzylpenicillin system while the borate ions appeared to have no inhibition effect. (5) For the reaction between cloxacillin and penicillinase, there was a rapid initial change in rotation before the zero order portion of the reaction occurred.

1. G. B. Levy, Anal. Chem., 23, 1089-1092 (1951).

2. G. B. Levy, P. Schwed, and D. Fergus, Rev. Sci. Instruments, 21, 693-698 (1950).

APPROVED Louis G. Byrne

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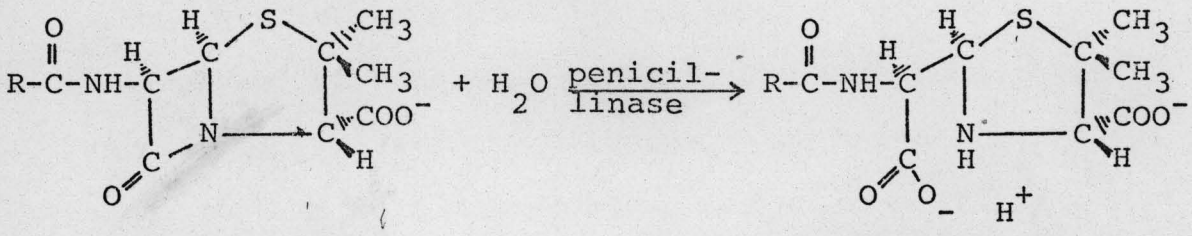
PART A.

Evaluation of Spectropolarimetric-Penicillinase or Circular Dichroism-Penicillinase Methods for Determining the Activity of Penicillin.

I. INTRODUCTION

An interesting polarimetric assay procedure specific for penicillins susceptible to penicillinase attack was proposed by Levy[1] in 1951. This method, in essence, relates the penicillin concentration to the changes in optical rotation of an aqueous solution of penicillin observed after hydrolysis of the β-lactam ring by penicillinase.



Penicillin D-α-Penicilloate ion

The change in rotation was determined on a custom built recording polarimeter[2] which used a General Electric mercury vapor lamp and two Corning filters to isolate the mercury line (5460 Å). The polarimetric assay procedure never became too popular since most of the measurements were made in the visible region where the sensitivity was low. In addition, the change in rotation due to the mutarotation of the penicilloate ion was relatively large. The selection of a proper end point was never determined thus reducing the precision of this assay procedure.

The specific aim of the present investigation was to develop a very precise, convenient and specific method for the analyses of penicillins based on the use of a more sensitive currently commercially available recording spectropolarimeter.

Any changes in optical activity resulting from the addition of penicillinase, a specific enzyme for the hydrolysis of the β-lactam ring, to an aqueous solution of penicillin is a direct result of the action of this enzyme on the penicillin. The presence of other optically active materials will have no effect on the assay since the assay relates the change in optical activity arising from the cleavage of the β-lactam ring by this specific enzyme to the penicillin concentration.

These studies have included application of the method to the analyses of both non-resistant and resistant classes of penicillin. The structural formulas of those studied and the names used in this dissertation are given in Table I.

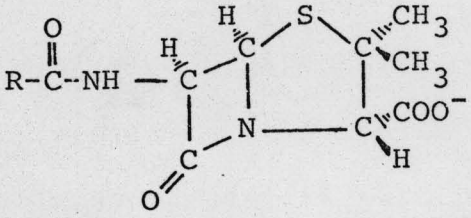
Present Methods of Assaying Penicillins.

Several chemical methods are available for the analysis of penicillin.[3] Among these, the iodometric and hydroxamic acid methods are the two most widely used methods. Results from these two chemical methods are in good agreement with microbiological data for a relatively pure substance, but poor agreement has been obtained with samples which contain relatively large amounts of degraded penicillin.

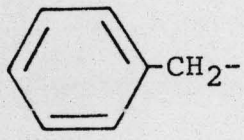
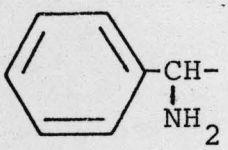
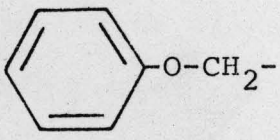
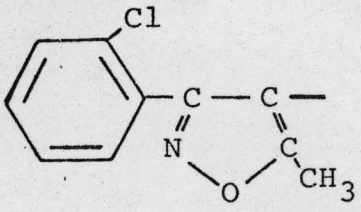
The principle of the iodometric method[3,4,5] is that the penicilloate ion takes up iodine while there is no consumption of iodine by the intact penicillin molecule. This method can also be made relatively specific by utilizing enzymatic cleavage of the β-lactam ring. This method is not, however, applicable to penicillins, such as cloxacillin,

TABLE I.

STRUCTURE OF THE PENICILLINS USED IN THIS STUDY.



general formula for penicillin

	Side Chain (R)	Penicillin
1.		Benzylpenicillin
2.		Ampicillin
3.		Phenoxymethylpenicillin
4.		Cloxacillin

which possess unsaturation in their side chain since the side chain will take up iodine regardless of whether the β-lactam ring is intact or not.[4] The iodine consumption appears to depend upon pH and iodine concentration even for systems where it is used.[3] The stochiometry of the reaction is not known exactly, but it appears that 8-9 atoms of iodine are taken up by each molecule of benzylpenicilloate.[4] Because of this variation, the iodine equivalence must be determined by analyzing samples of pure penicillin under the same assay conditions to be used in assaying the unknown samples. A blank estimate must also be made.

In the hydroxamic acid method, hydroxylamine reacts with the β-lactam ring of penicillin at pH 7 to form a hydroxamic acid.[3,4,5] Addition of ferric ions to the hydroxamic acid solution produces a color which is stable for about 5 minutes.[4] Different penicillins produce different color intensities.[4] Hydroxylamine will react only with the intact β-lactam ring and not penicilloate. On the other hand, hydroxylamine will react with other compounds, such as esters, which contain a carbonyl group.

II. EXPERIMENTAL

Apparatus.

Measurements of optical rotation and circular dichroism were made on a Cary 60 Recording Spectropolarimeter and with the Cary Model 6001 Circular Dichroism accessory. It was found that a 5 cm cell could be removed, shaken, and replaced without causing a significant variation in the spectropolorimetric reading. This was not true for the 1 cm cell. Therefore, the 1 cm cell was securely fastened to the cell holder with tape and the cell holder with the cell in it was shaken.

Penicillin samples were weighed on a Mettler microanalytical balance, model M5-5/A or a Cahn electrobalance, model G-2. A 10 and 50 microliter Hamilton syringe was used for the injection of penicillinase solution into the penicillin sample.

Chemicals and Reagents.

Bacillus cereus penicillinase solutions (Neutrapen injectable, 800,000 units/vial), a gift from Riker Laboratories, Northridge, Calif., were prepared by dissolving one vial in 10 ml of distilled water for the penicillinase-sensitive penicillin and one vial in 0.5 ml of distilled water for the penicillinase-resistant penicillin. Potassium benzylpenicillin (Wyeth's house standard, 1591 units/mg, Control No. W620248-S1), phenoxymethylpenicillin acid (Wyeth's house

standard, 1664 units/mg, Control No. E16-1), and ampicillin B (Wyeth's reference standard, 961 mcg/mg, Lot No. C-10351) were donated by Wyeth Laboratories, Philadelphia, Pa. Sodium cloxacillin (Ayerst reference standard, 905 mcg/mg as the free acid, Lot. No. BRL-1621) and samples of commercial grade ampicillin and sodium cloxacillin, whose microbiological assays were in good agreement with the chemical assays, were furnished, along with their assay data, by Ayerst Laboratories, Rouses Point, N.Y.

Potassium benzylpenicillin, purchased from Cal-Biochem, Los Angeles, Calif., and sodium cloxacillin, Ayerst commercial grade, were twice recrystallized by two different methods. In the first method, the penicillin salt was dissolved in a minimal amount of water and was precipitated by slowly adding approximately 20 volumes of 1-butanol.[6,7] The precipitate was then washed with ethylacetate. In the second method the penicillin salt was dissolved in a minimal amount of methanol and was precipitated by slowly adding approximately 5 volumes of ethylacetate.[6,7] The precipitate was then washed with ethylacetate.

Ampicillin, Ayerst's commercial grade, was once recrystallized by dissolving it in a minimal amount of 1 N hydrochloric acid at 70°C, precipitated by adding 2 N sodium hydroxide[8] at 70°C and the precipitate was washed with boiling ethylacetate.

Reagent grade potassium phosphate, monobasic and dibasic, sodium acetate, acetic acid, sodium carbonate, sodium hydroxide and hydrochloric acid were used to prepare the buffer solutions.

Procedure for Penicillinase-Sensitive Penicillin using ORD.

An aqueous solution of the penicillin to be studied was prepared by rapidly dissolving 0.10 to 0.15 grams, accurately weighed, of penicillin in 500 ml of 0.1 M phosphate buffer solution, pH 7.0. A 5 cm cell was filled with this aqueous penicillin solution and placed in the spectropolarimeter. The assay was carried out at a constant wavelength of 255 mμ (or 247 mμ) with a spectral bandwidth of 40 Å (slitwidth of 2.4 mm), a chart speed of 25 sec/division, and usually a full range scale of 0.4 degrees. The pen center was set at zero and the pen was adjusted with the manual zero and zero suppression to the 1.0 line on the chart paper.

With the filled cell in place, the rotation was recorded for 1-2 minutes. Then 50 μl of penicillinase solution (80,000 units/ml) was rapidly injected into the cell, which was shaken, then replaced, and the recording reinitiated.

Since spontaneous degradation of penicillin begins as soon as an aqueous solution is prepared, the time between the preparation of the solution and the assay was recorded. The timing was started as soon as the penicillin was dissolved and recorded when the cell was shaken. A kinetic curve of of the rapid drop in rotation versus time was recorded (Fig. 1). When the pen reached zero on the chart paper, the pen center was switched from zero to 1.0 so that the change in rotation covered twice the width of the chart paper. The pen continued to drop rapidly until all the penicillin was degraded by the penicillinase. This was followed by a slow reduction

in the rotation of the solution.

After each assay, the cells were filled with 3 N nitric acid and allowed to stand for a few minutes to inactivate the residual enzyme.

Procedure for Penicillinase-Sensitive Penicillin using CD.

For this situation, a wavelength of 231 mμ was used. This required that a smaller concentration of penicillin and a full range of 0.2 degrees be used. Approximately 3.5 mg, accurately weighed, of benzylpenicillin was dissolved in 50 ml of buffer solution. Except for these modifications, the procedure described for the penicillinase-sensitive system using ord was followed.

Procedure for Penicillinase-Resistant Penicillins using ORD.

For these samples, somewhat greater concentrations of both penicillin and penicillinase were used along with a reduction in the pathlength. Approximately 50 to 60 mg, accurately weighed, of the penicillin to be studied was dissolved in 100 ml of 0.1 M phosphate buffer solution, pH 7.0, and a 1 cm cell, which was affixed in a permanent position to the cell holder, was filled with this solution. Fifty μl of penicillinase solution (1,600,000 units/ml) was injected into the cell. Except for these modifications, the procedure described for the penicillinase-sensitive system using ord was followed.

Procedure for Assaying a Mixture of a Penicillinase-Sensitive and a Penicillinase-Resistant Penicillin.

Approximately 0.85 to 0.90 grams of the mixture, accurately weighed, was dissolved in 100 ml of 0.1 M phosphate buffer solution, pH 7.0. A 1 cm cell which was securely fastened to the cell holder was filled with this solution and a baseline for the rotation of the active mixture was run on the spectropolarimeter. Ten µl of penicillinase solution (80,000 units/ml) was rapidly injected into the solution in the cell and the cell and cell holder were shaken, replaced and the recording reinitiated. A kinetic curve of the rapid drop in rotation versus time was recorded. This curve represented the degradation of the penicillinase-sensitive penicillin by penicillinase.

For the determination of the amount of penicillinase-resistant penicillin, the special 1 cm cell was filled with a fresh sample of the above solution and a baseline of the active penicillins was run. Then 50 µl of penicillinase solution (1,600,000 units/ml) was injected, and the recording reinitiated. The observed curve represents the degradation of both the penicillinase-sensitive penicillin and the penicillinase-resistant penicillin.

III. RESULTS AND DISCUSSION

A Typical Polarimetric Assay Curve.

Fig. 1 shows a typical polarimetric recording obtained at 255 mμ as a function of time for a sample of benzylpenicillin following the addition of penicillinase. The segment A represents the optical rotatory activity of the penicillin prior to the addition of the enzyme. At point B, penicillinase solution was injected producing the observed rapid linear decrease in rotation (Segment C) corresponding to the enzymatic cleavage of the β-lactam ring to form D-α-benzylpenicilloate. This is followed by a very slow decrease in rotation seen in the terminal phase (segment D) due to the mutarotation of D-α-benzylpenicilloate.[9] This observed behavior is typical of all penicillinase-sensitive penicillins.

The ORD and UV Spectra for Benzylpenicillin and its Degraded Products.

An ord and uv spectra of benzylpenicillin and its degraded products were recorded in order to select an appropriate wavelength for the assay. The ord spectra for benzylpenicillin, D-α-benzylpenicilloate, and the final equilibrated mixture of the degraded product are shown in Fig. 2.

The optical activities of penicillins in the uv and visibile regions are largely due to the n - π* transition[10] of the carbonyl group in the β-lactam ring. It can be

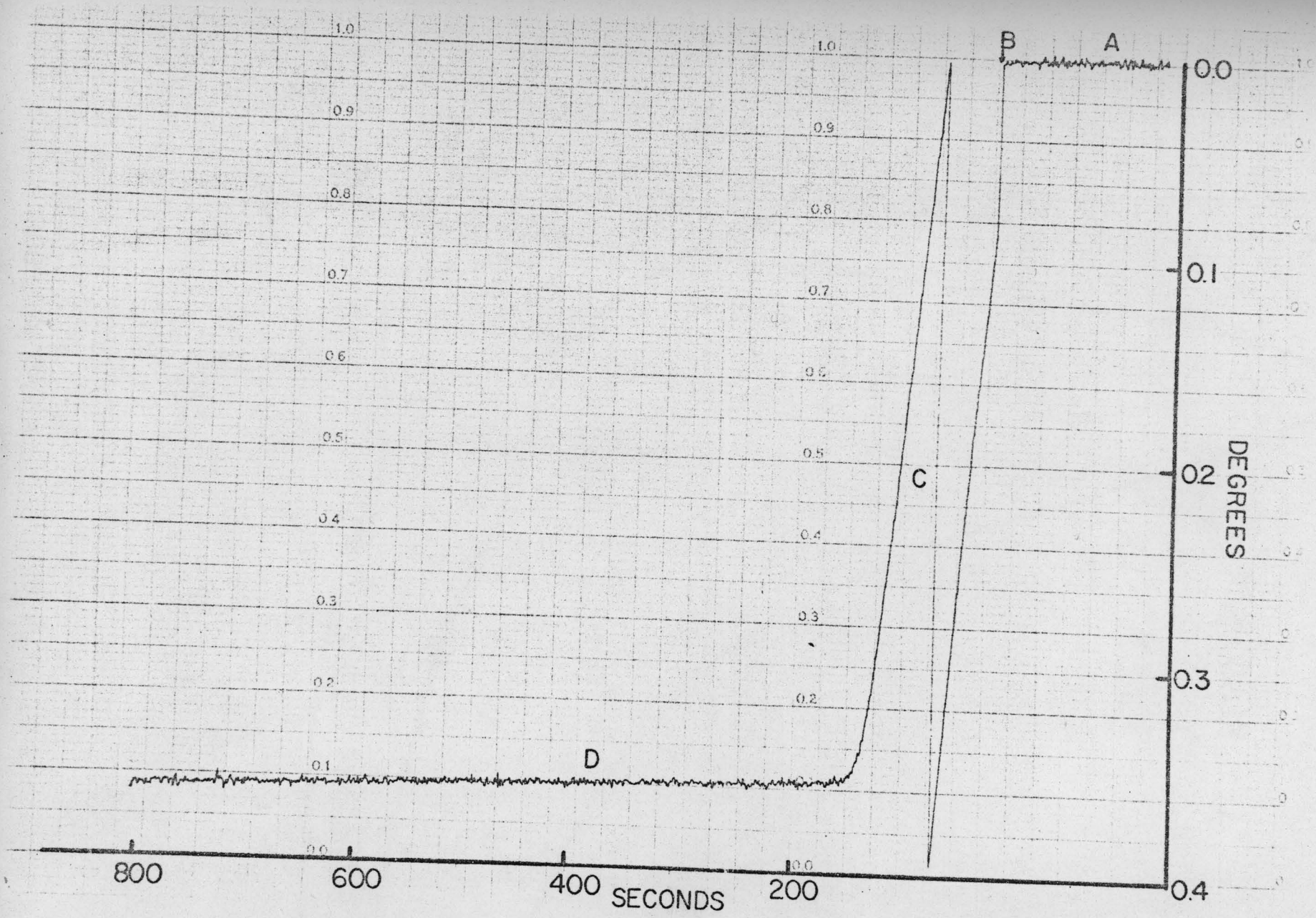


Fig. 1 - A Typical Polarimetric Assay Recording for Penicillinase-Sensitive Penicillin.

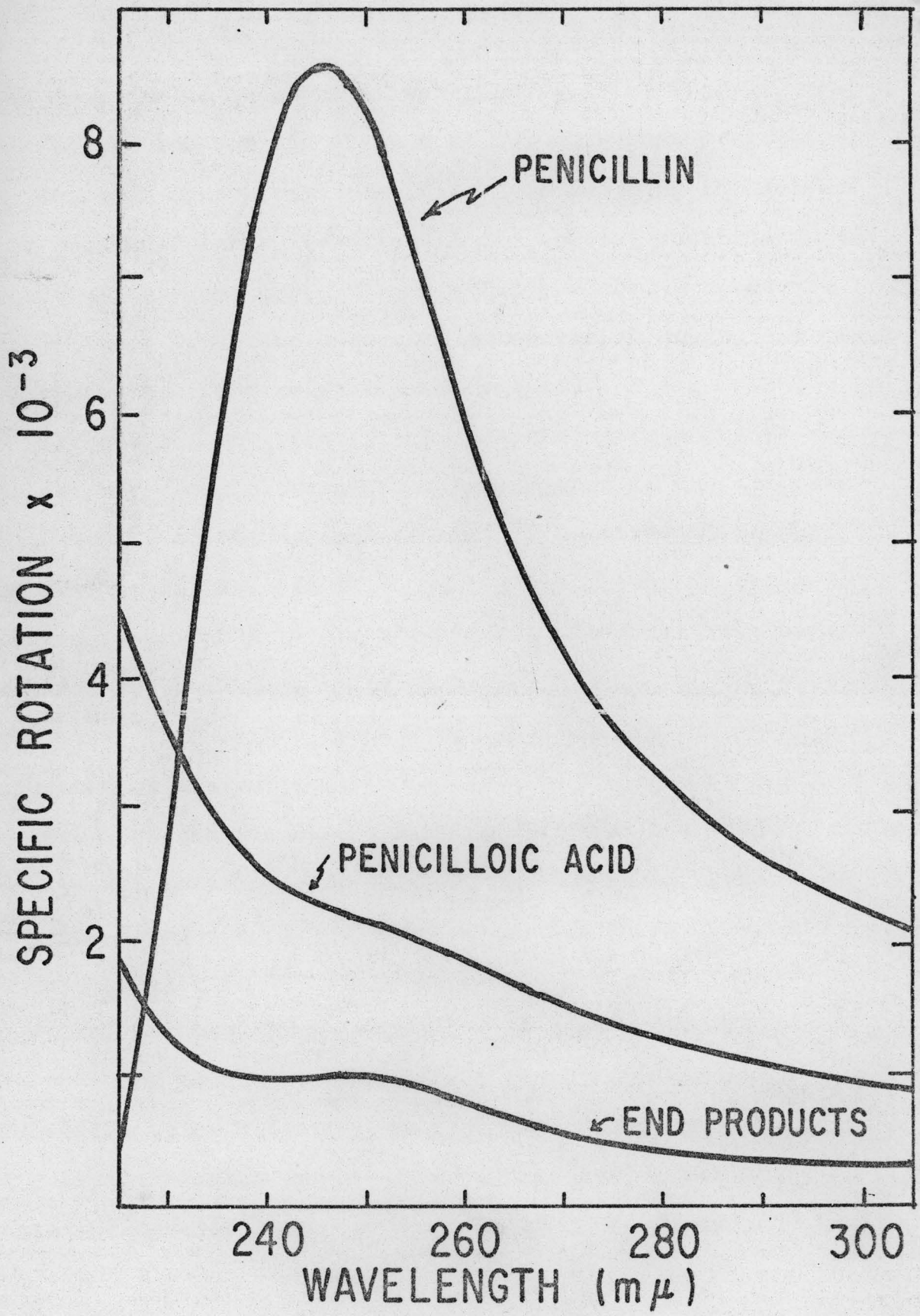


Fig. 2 - The ORD spectra of Benzylpenicillin, D-α-Benzylpenicilloate, and the Final Equilibrated Mixture in a 0.1 M Phosphate Buffer Solution, pH 7.00.

seen from the cd spectrum of benzylpenicillin, Fig. 12, that this transition has its maximum at 231 mμ. When penicillins are degraded by penicillinase, the β-lactam ring is cleaved thus causing a large reduction in the optical rotation in the uv and visible regions. The degraded product, D-α-benzylpenicilloate ion, then undergoes mutarotation giving the final ord spectrum of the equilibrated mixture.

It can be seen from Fig. 2 that the maximum change in optical rotation, arising from the cleavage of the β-lactam ring, occurs at the maximum in the ord spectrum of benzylpenicillin, 247 mμ. In this region, the change in optical rotation ascribable to the mutarotation is relatively small compared to the change in optical rotation due to the cleavage reaction. This is not true in the visible region, where the magnitude of the mutarotation is almost the same as the magnitude of the cleavage reaction. However the rate of change for the mutarotation step is much slower. This means that the relative magnitude of the terminal change in Fig. 1 would be much greater in the visible region.

Fig. 3 shows the uv spectrum for benzylpenicillin and its degraded products. There is a rapid increase in absorption below 255 mμ, somewhat limiting the concentration of penicillin which can be used in the assay. For this reason, the assays were carried out at both 247 and 255 mμ.

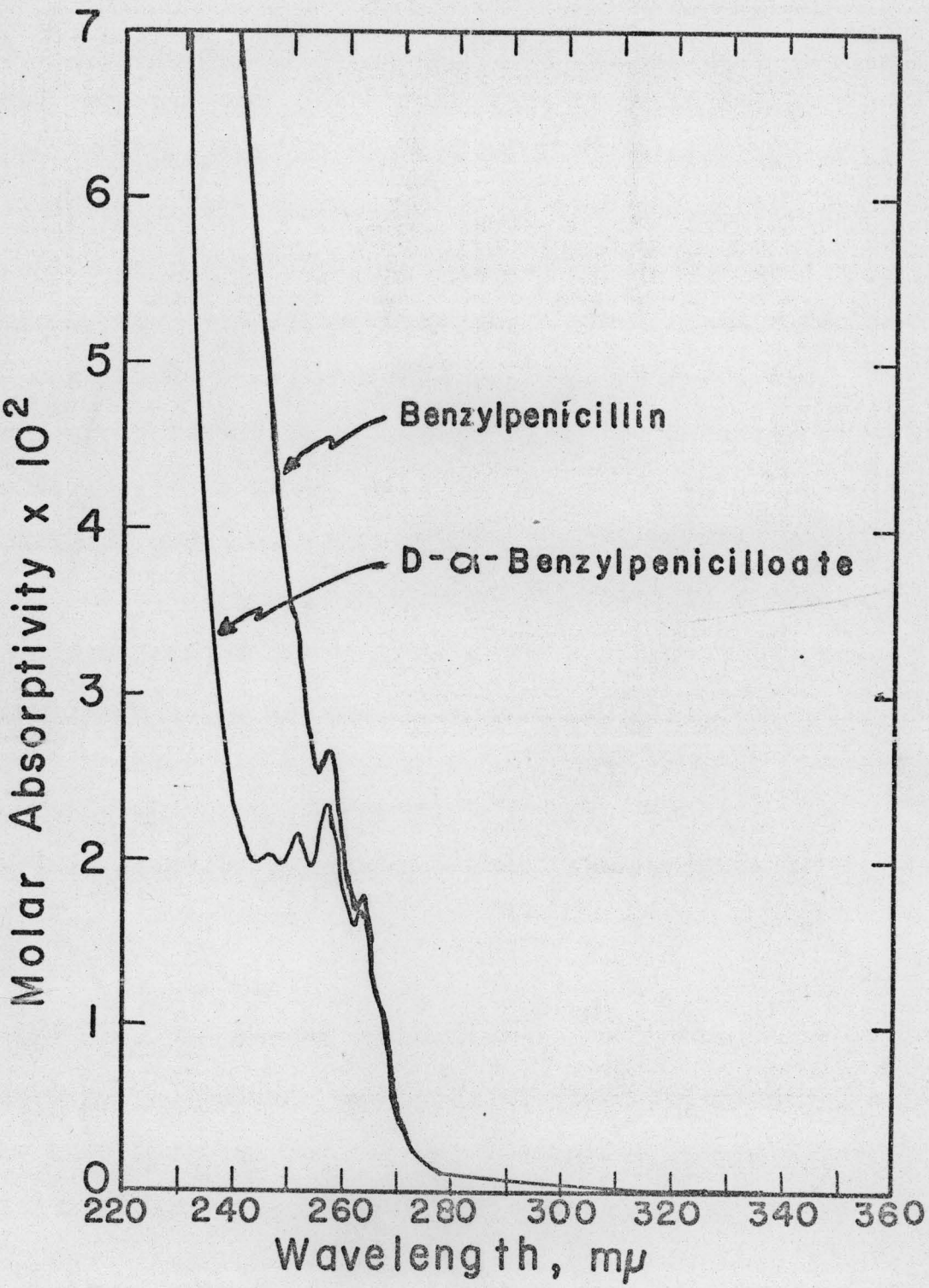


Fig. 3 - The UV Spectra of Benzylpenicillin and D-α-Benzylpenicilloate ion in 0.1 M Phosphate Buffer Solution, pH 7.00.

Effect of pH on the ORD Spectra of Benzylpenicillin and its Degraded Products and on the Change in Specific Rotation.

Fig. 4 shows the ord spectra of benzylpenicillin and its penicilloic acid derivative at pH 7.00 and 3.00. The ord spectra of the intact penicillin at these two pH's were essentially the same while there was a significant difference in the spectra of benzylpenicilloate. At pH 7.00, the carboxyl group formed by the cleavage of the β-lactam ring (pK_a = 4.7)[11,12] is essentially unprotonated, while at pH 3.00, this carboxyl group exists mainly in its protonated form. When this carboxyl group is protonated, there is a positive cotton effect around 243 mμ, while the unprotonated species has a positive plain ord curve in this region.

Fig. 5 shows the pH profiles for the change in specific rotation due to the hydrolysis of the β-lactam ring for benzylpenicillin at 247 mμ and 255 mμ and for ampicillin at 247 mμ.

Selection of a Proper End Point.

In order to convert the results from a polarimetric curve, such as that shown in Fig. 1, to an accurate quantitative measure of the amount of penicillin contained in the orginal sample, the penicillin concentration must be related directly to the decrease in the optical rotatory properties of the solution produced by the action of the enzyme. Levy[1] simply used the intersection of an extrapolated line from the initial rapid apparent zero order cleavage reaction and an extrapolated

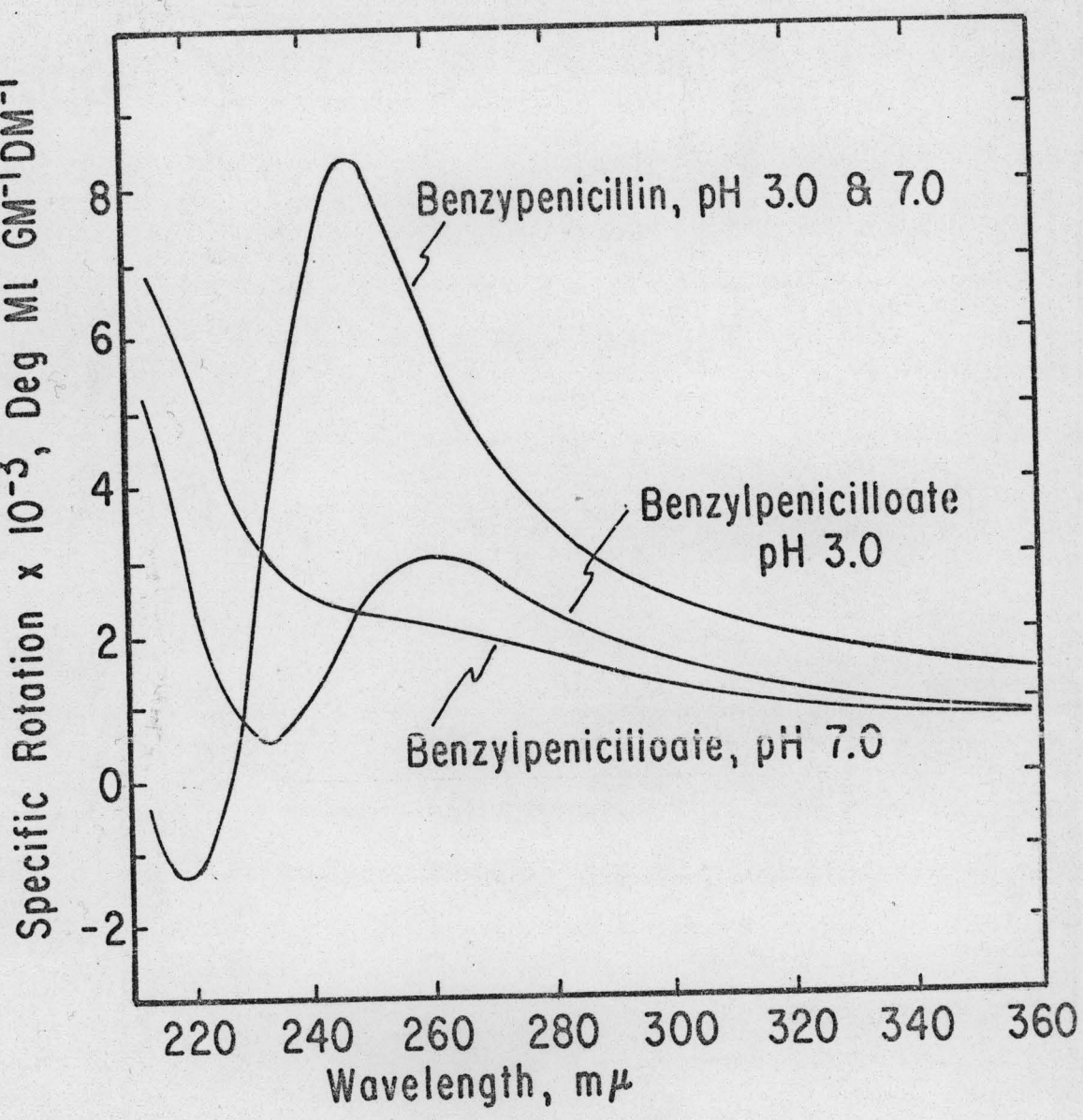


Fig. 4 - The ORD Spectra of Benzylpenicillin and its Penicilloic Acid Derivative at pH 3.00 and pH 7.00.

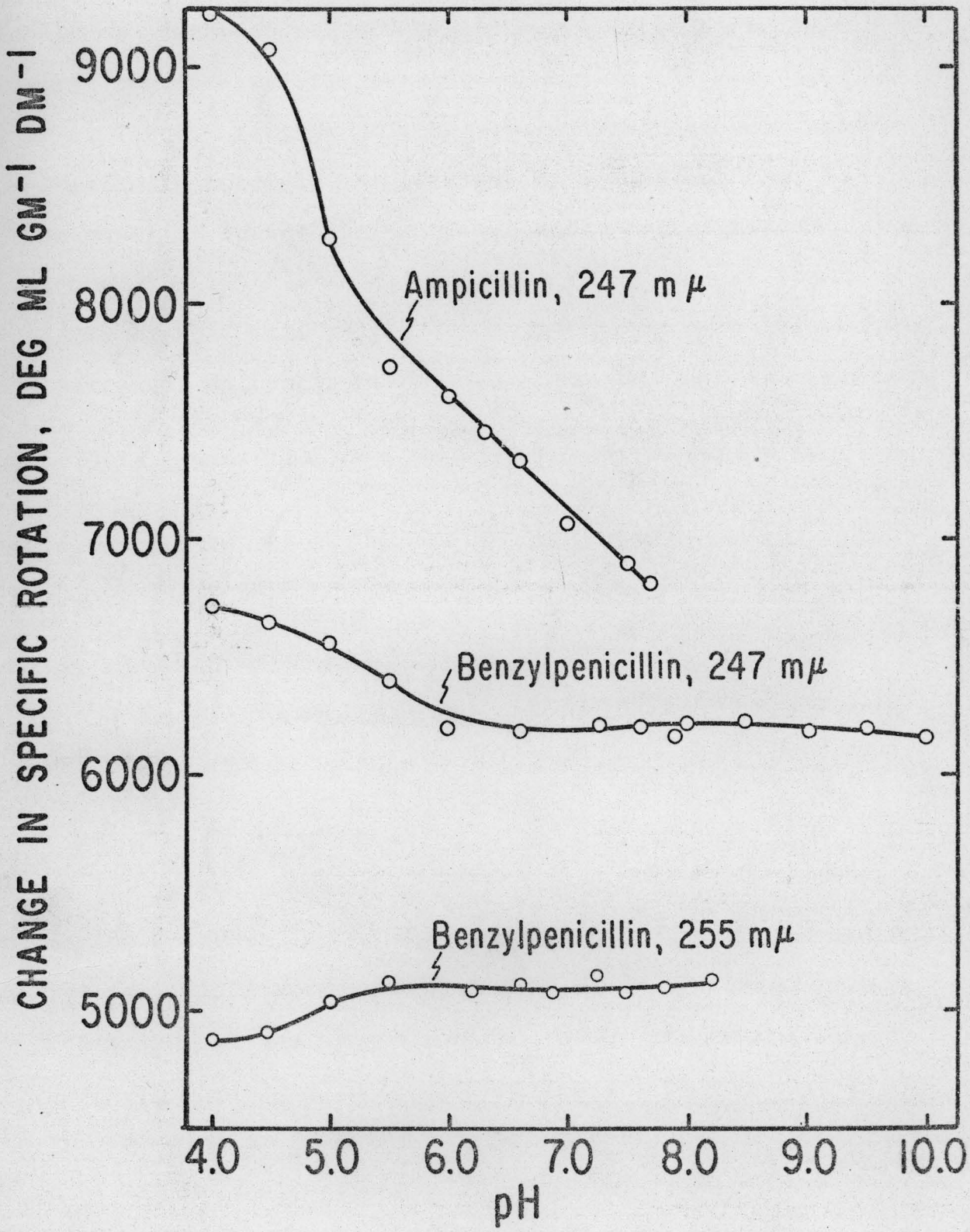


Fig. 5 - The Effect of pH on the Change in Specific Rotation for Benzylpenicillin at 247 mμ and 255 mμ and for Ampicillin at 247 mμ due to the Catalytic Hydrolysis of the β-Lactam Ring by Penicillinase.

line from the slower mutarotation. However, this point is dependent on the amount of enzyme used, particularly in the visible region where the relative change in the mutarotation step is large. In this section, the problem has been analyzed theoretically to yield the best way of estimating the theoretical ΔR equivalent to an instantaneous cleavage reaction.

The overall rate of the catalyzed cleavage reaction obeys the classical Michaelis-Menten mechanism for enzymatic systems:[13]

$$\underset{(S)}{\text{penicillin}} + \underset{(E)}{\text{penicillinase}} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \underset{(ES)}{\text{Enzyme-substrate complex}}$$

$$\underset{(ES)}{\text{Enzyme-substrate complex}} \xrightarrow{k_2} \underset{(P)}{\text{penicilloate}} + \underset{(E)}{\text{penicillinase}}$$

$$\underset{(P)}{\text{penicilloate}} \xrightarrow[\text{(mutarotation)}]{k_3} \underset{(P')}{\text{diasteroisomers}}$$

It can readily be shown that in these systems, the rate of degradation is given by:[14]

$$-\frac{d[S]}{dt} = \frac{k_2 E_T [S]}{K_m + [S]} \qquad (1)$$

where [S] is the concentration of the penicillin, K_m is the Michaelis constant, k_2 is the rate constant for the breakdown of the penicillin-enzyme complex, and E_T is the total concentration of the enzyme. Since K_m for the penicillinase-sensitive penicillins is always very small, it can be neglected in the denominator except at very low substrate concentrations. Thus at higher residual penicillin concentrations, a zero order kinetic equation is obtained.

$$-\frac{d[S]}{dt} = k_2 E_T$$

For penicillinase-sensitive penicillin - Schematic plots of penicillin and penicilloic acid concentrations versus time for a penicillin-penicillinase system having a low K_m value are shown in Fig. 6. In order to obtain a precise measurement for the change in rotation due only to the cleavage of the β-lactam ring by penicillinase, a point must be selected on the extrapolated mutarotation curve of the penicilloic acid which represents the concentration of penicilloic acid equivalent to the initial concentration of penicillin and which has not undergone any mutarotation. In the following derivation it will be shown that this time can be approximated at 1/2 t_α, where t_α is the hypothetical time required to complete the cleavage on the assumption that the reaction is strictly zero order.

In the strictly zero order range, the kinetic rate equation for the disappearance of [S] can be approximated by: $S_0 - [S] = k_2 E_T t$, the integration of eq. 1. When $t = t_\alpha$, $[S] = 0$, therefore:

$$t_\alpha = \frac{S_0}{k_2 E_T} \qquad (2)$$

Since the secondary change in rotation is presumably pseudo-first order, the differential rate equation describing [P] between $t = 0$ and $t = t_\alpha$ is:

$$\frac{d[P]}{dt} = k_2 E_T - k_3 [P]$$

Upon integrating and solving for [P]:

$$[P] = \frac{k_2 E_T}{k_3}(1 - e^{-k_3 t}) \qquad (3)$$

Expanding the exponential part in a power series and evaulating the expression at t_α, the following is obtained.

$$[P]_{t=t_\alpha} = S_0 - \frac{k_3 S_0}{2k_2 E_T} + \ldots\ldots \qquad (4)$$

For $t > t_\alpha$, after all the penicillin has been degraded to penicilloic acid, the secondary change in rotation is described by:

$$\frac{d[P]}{dt} = -k_3[P] \quad \text{or} \quad [P] = C\, e^{-k_3 t} \qquad (5)$$

The integration constant, C, can be evaluated at t_α. At t_α, just prior to the extrapolation, [P] is obtained by substituting t_α for t in eq. 3.

$$C\, e^{-k_3 t_\alpha} = \frac{k_2 E_T}{k_3}(1 - e^{-k_3 t_\alpha})$$

Solving for C, expanding the exponential in a power series, and substituting the expression for t_α (eq. 2), eq. 6 is obtained:

$$C = S_0 + \frac{k_3 S_0^2}{2k_2 E_T} + \ldots\ldots \qquad (6)$$

The integration constant, C, represents the hypothetical concentration of [P] that would be required at t = 0 for a hypothetical system in which there is only P going to P' (i.e. no S going to P) in order to give the same concentration changes for $t > t_\alpha$ in the above $S \rightarrow P \rightarrow P'$ system.

Now the problem is to select some time on the extrapolated P curve which represents the concentration of P equivalent to S_0. This point can be approximated by using eq. 4 with a power

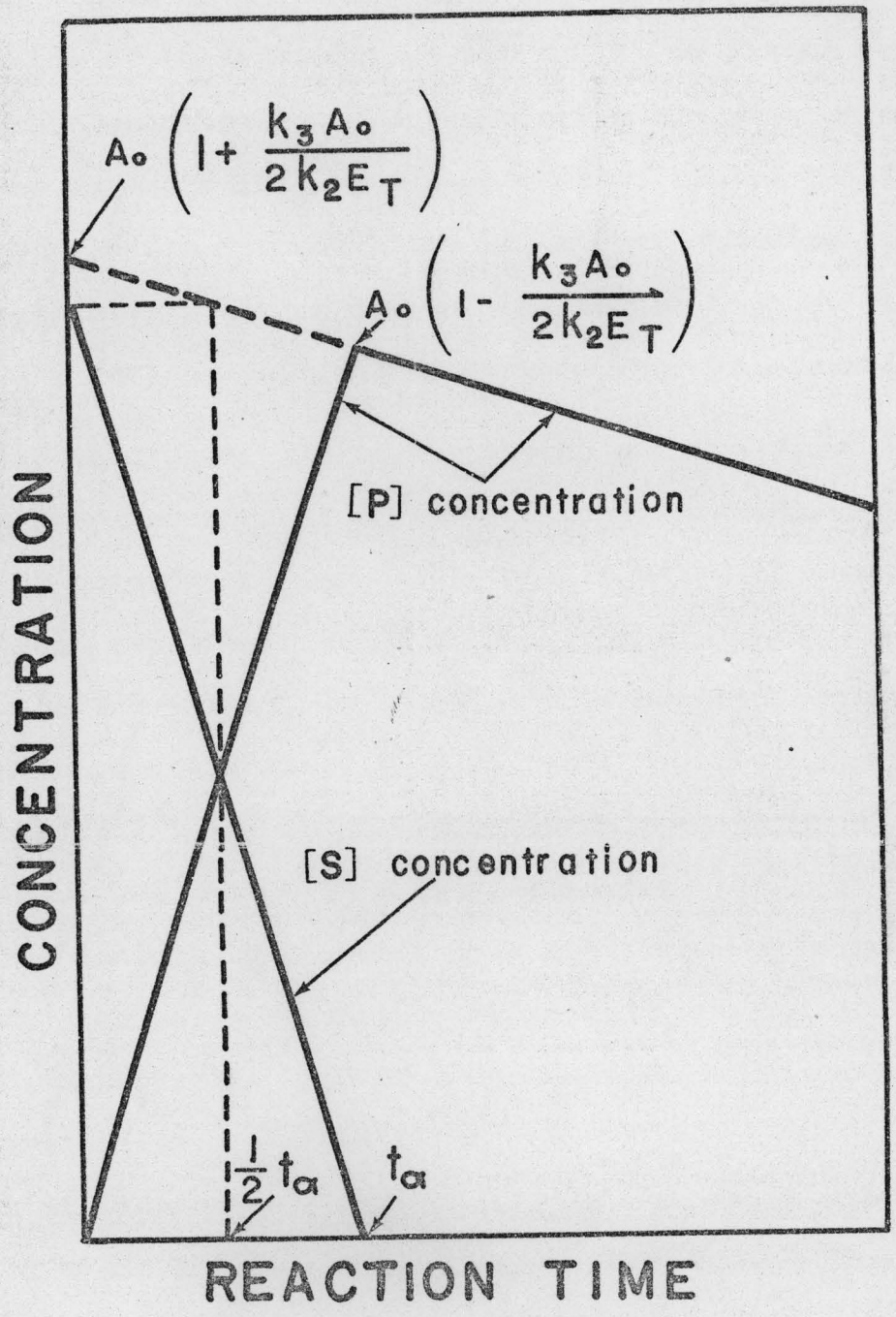


Fig. 6 - A Schematic Drawing of the Penicillin and its Penicilloic Acid Derivative Concentrations as a Function of Time for a Penicillinase-Sensitive Penicillin.

series expansion of the exponential and eq. 5, or it can be more easily seen graphically by extrapolating the right hand portion of the mutarotation curve back to zero time. This point does not yield a concentration value corresponding to the initial penicillin concentration, but is too high by the amount, $\frac{S_0^2 k_3}{2k_2 E_T}$, (eq. 6). On the other hand, the intersection of the initial zero order cleavage reaction and the slow mutarotation step occuring at t_α gives a concentration value too low by the same amount (eq. 3). On this basis we propose that the optical rotational value of penicilloate which can be correlated with the initial penicillin concentration occurs at 1/2 t_α on the optical activity-time plot, such as shown in Fig 7, for penicillins which cleave largely through zero order kinetics. The change in optical rotation which was related to the penicillin concentration was simply obtained by subtracting the value for the rotation at $\frac{1}{2}t_\alpha$ on the extrapolated mutarotation line from the initial rotation for the intact penicillin.

For penicillinase-resistant penicillin - Since these penicillins did not follow the conditions stated above, but instead exhibited pseudo-first order kinetics with respect to the penicillin for the cleavage reaction, a different approach must be used. In Fig. 8, a behavior of this type is evident in a polarimetric plot obtain during the cleavage of cloxacillin in the presence of Bacillus penicillinase.

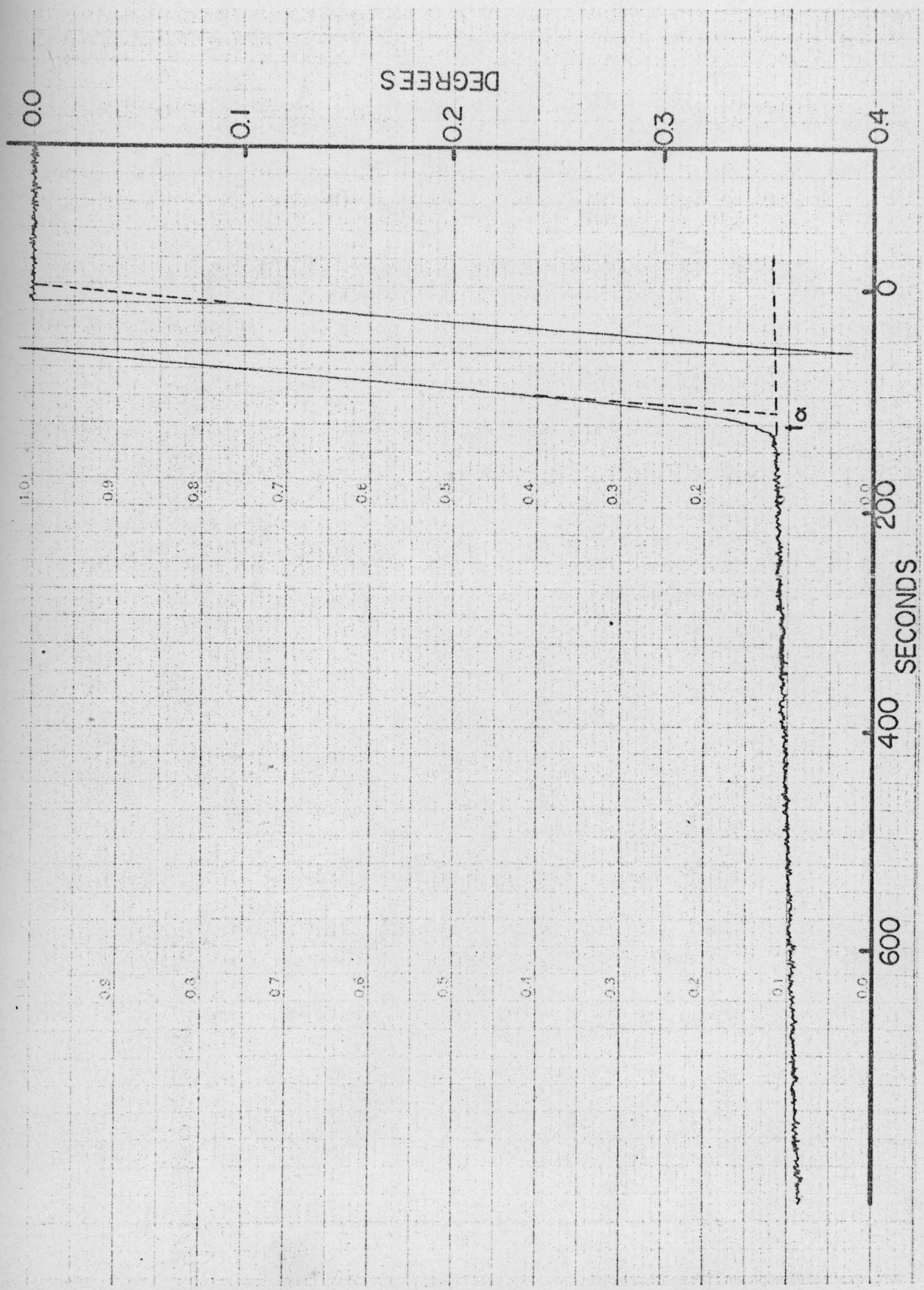


Fig. 7 - A Typical Polarimetric Assay Recording of Benzylpenicillin with the Extrapolated Lines Showing how the Proper End Point was Selected.

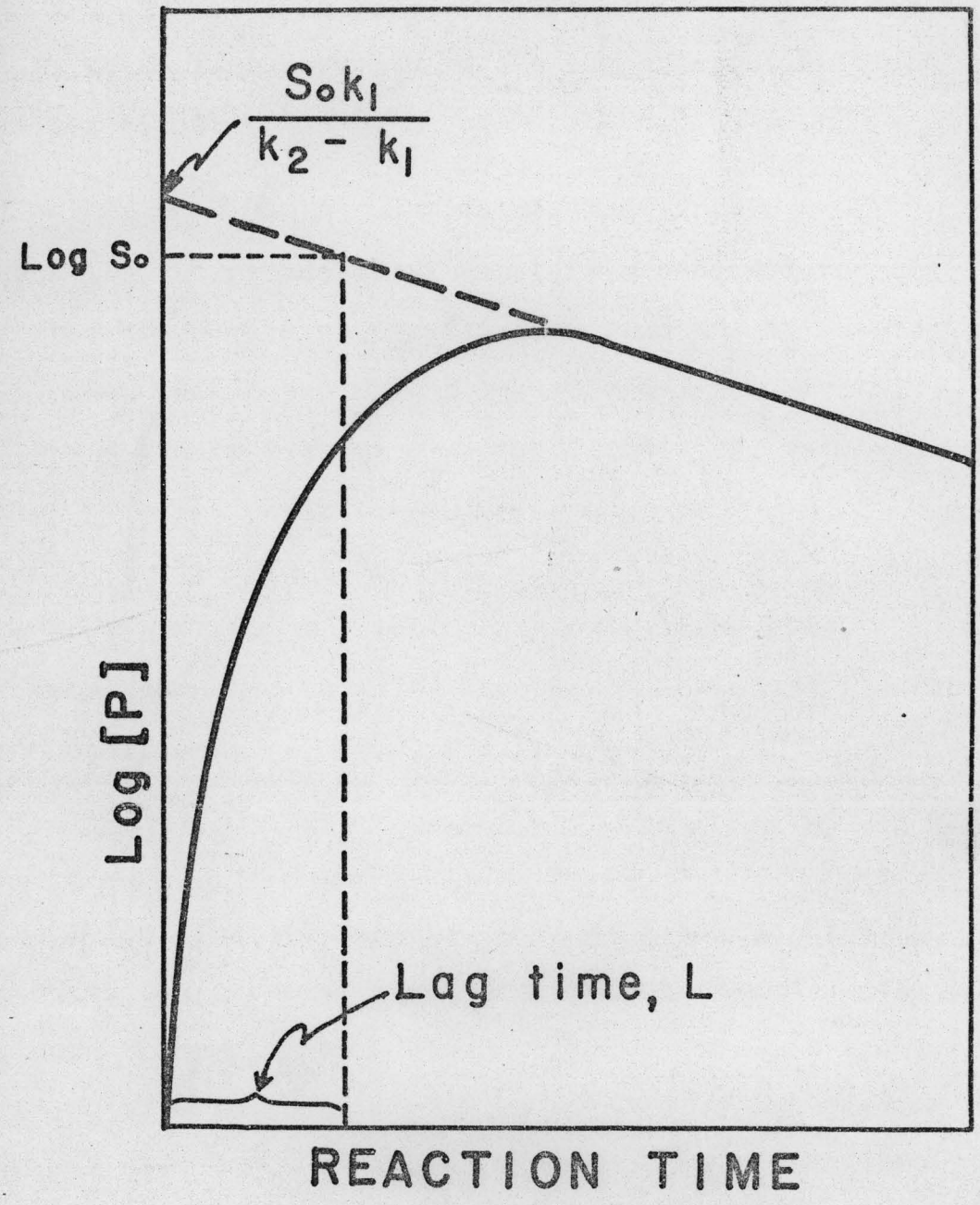


Fig. 8 - A Schematic Drawing of the Logarithm of the Penicilloate ion Concentration versus Reaction Time for a Penicillinase-Resistant Penicillin.

In these instances, there are two consecutive first order reactions:

$$S \xrightarrow{k_1} P \xrightarrow{k_2} P'$$

where k_1 is the pseudo-first order rate constant for the catalytic hydrolysis of the β-lactam ring and k_2 is the first order rate constant for the mutarotation.

The concentration of D-α-penicilloate, P, is given by:[15]

$$[P] = \frac{S_0 k_1}{k_2 - k_1}(e^{-k_1 t} - e^{-k_2 t})$$

Since $k_1 >>> k_2$, $e^{-k_1 t}$, at sufficiently large time, will be very small compared to $e^{-k_2 t}$ and can be neglected. Under these conditions:

$$[P] = \frac{S_0 k_1}{k_2 - k_1} e^{-k_2 t}$$

and a plot of log [P] versus t will be linear. If this linear portion of the curve is extrapolated back (see fig. 8), it will pass through a point where log[P] = log S_0. The time at which log[P] = log S_0, L, will be related to the rate constants by:

$$1 = \frac{k_1}{k_2 - k_1} e^{-k_2 L}$$

If this identity is expanded in a power series

$$k_1 - k_2 = k_1(1 - k_2 L + ...)$$

and all terms greater than the linear term are neglected, the following expression is obtained:

$$L = \frac{1}{k_1} = \frac{1}{\frac{0.693}{t_{1/2}}} = 1.44\ t_{1/2}$$

where $t_{1/2}$ is the first half-life of the reaction (S $\longrightarrow$ P).

Thus in Fig. 9 the value of rotational change which would be affected only by the initial penicillin concentration and independent of other variables in the system would occur only at the indicated point. Since the zero time is not known, the time between the first two half-lives was determined, multiplied by 0.44 and added to the first half-life time to obtain the proper end-point.

Correction for Spontaneous Degradation of Samples.

Another rather important factor which must be considered in the development of any really precise analytical method for these relatively unstable antibiotics is the significant spontaneous degradation which takes place as the sample stands. The loss is usually negligible for dry samples but becomes significant in aqueous solutions. Fig. 10 shows a typical plot for the enzyme induced change in apparent specific rotation at room temperature and pH 7.0 with standing time for a 0.1 M phosphate buffer solution of benzylpenicillin and phenoxymethylpenicillin. In this study, assays were carried out on benzylpenicillin, ampicillin, phenoxymethylpenicillin and cloxacillin. At room temperature and under the conditions generally employed, losses of approximately one percent were observed in 170 minutes for benzylpenicillin and cloxacillin and in 90 minutes for ampicillin and phenoxymethylpenicillin. The intercept represents the change in apparent specific rotation for the original dry sample before any spontaneous

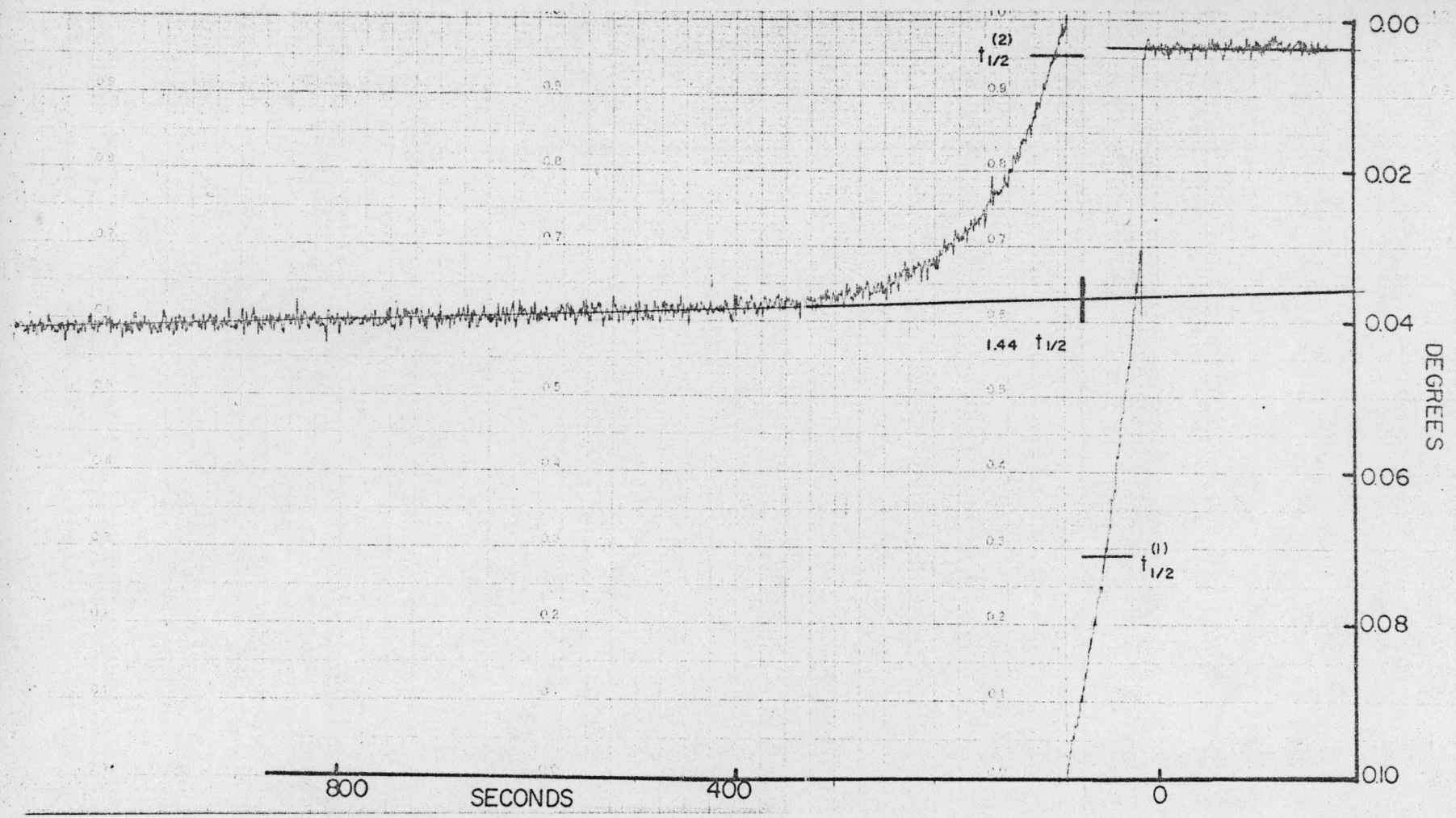


Fig. 9 - A Typical Polarimetric Assay Recording with the Extrapolated Mutrarotation Line for Cloxacillin in a 0.1 M Phosphate Buffer Solution, pH 7.00.

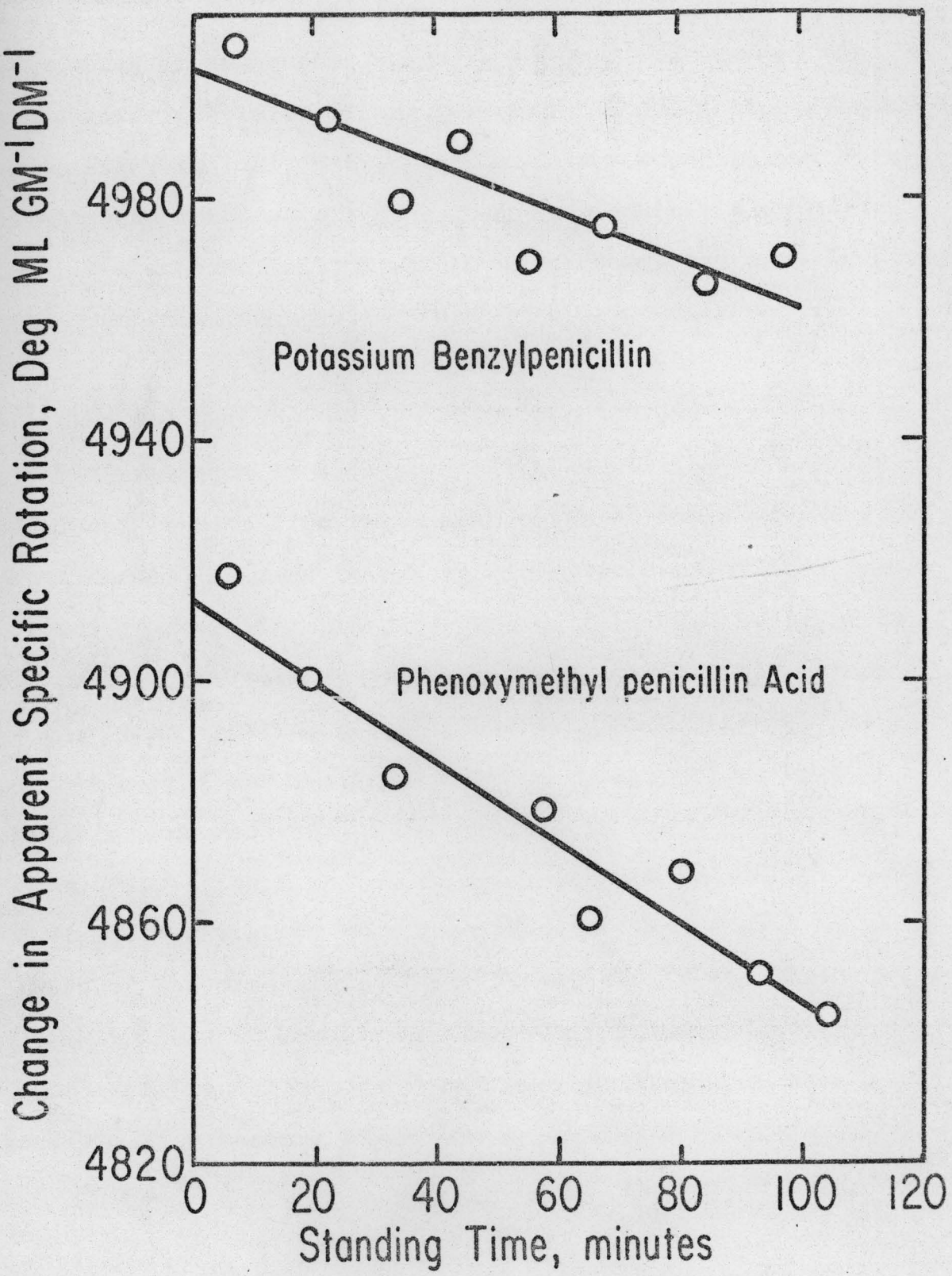


Fig. 10 - Shows the Decrease in the Assay Values for Benzylpenicillin and Phenoxymethylpenicillin as a Function of the Time the Solutions were Standing at Room Temperature prior to Assaying.

degradation of an aqueous solution occurs. Because of the much greater precision of the proposed method, this approach is necessary to take full advantage of its capability. With conventional methods, the losses in sample activities on standing are essentially ignored, even though the time involved may be very considerable.

Precision and Accuracy of the Assay Procedures.

The precision is a measure of the deviation of the individual results from their mean value.[16] Here, standard deviation will be used to express the precision of the assay. In order to determine the precision of this procedure, assays were carried out several times on the same solution. Since the change in apparent optical rotation decreased with the time the sample was standing prior to assaying, due to spontaneous degradation of the penicillin (Fig. 10), the standard deviation from a mean value can not be used. Instead the standard deviation from a regression line is used as a measure of the precision. The values given in the last column in Table II and III represent the standard deviation of the experimental points from the regression line and therefore is a measure of the precision for a single measurement.

TABLE II.

THE APPARENT CHANGE IN SPECIFIC ROTATION FOR SOME PENICILLINASE-SENSITIVE PENICILLIN DUE TO THE CATALYTIC HYDROLYSIS OF THE β-LACTAM RING BY PENICILLINASE.

Penicillin	conc. mcg./ml	No. of* assays	$[\Delta\alpha]^{25°C}_{255\ m\mu}$ †	Std. Dev. § from line
Potassium Benxyl-penicillin				
Commerical House Std.	320.14	8	5003.3 ± 6.0	8.3
1591 units/ml.	279.71	8	5029.2 ± 6.1	7.7
	197.40	8	5020.6 ± 6.3	17.6
	260.35	8	5038.2 ± 5.8	7.2
	313.00	8	5000.8 ± 6.0	7.4
	56.90	8	5058.0 ± 5.8	14.6
	316.99	9	4999.8 ± 5.5	6.9
Recrystallized from methanol	305.20	3	5035.5 ± 8.5	11.4
with ethylacetate	242.18	5	5061.4 ± 7.6	7.6
Recrystallized from water with	240.28	9	5061.7 ± 5.2	8.0
n-butanol	283.30	5	5101.5 ± 6.8	16.8
	276.60	10	5009.2 ± 5.2	9.7
Ampicillin B				
Commerical Ref. Std.	264.60	8	5688.0 ± 6.1	8.4
961 mcg./mg.	264.60	8	5665.7 ± 5.9	12.4
	255.13	3	5692.2 ± 9.9	13.9
	251.94	5	5670.0 ± 9.3	7.3
	222.00	14	5622.9 ± 4.3	20.8
Recrystallized from water	263.18	8	5533.1 ± 5.6	27.7
	263.18	8	5591.4 ± 5.3	27.3
(Phenoxylmethyl penicillin acid)				
Commerical House Std.	297.71	8	4904.9 ± 5.8	5.8
1664 units/mg.	297.56	9	4901.3 ± 5.7	6.2
	290.47	8	4911.7 ± 5.6	7.2
	308.01	5	4906.5 ±16.4	8.1
	303.31	8	4912.6 ± 5.7	6.8
	291.15	8	4919.5 ± 5.9	7.3
	301.78	8	4926.3 ± 7.1	12.1

*, †, § see footnotes at the end of table III.

TABLE III.

THE APPARENT CHANGE IN SPECIFIC ROTATION AT ZERO STANDING TIME FOR A PENICILLINASE-RESISTANT PENICILLIN DUE TO THE CATALYTIC HYDROLYSIS OF THE β-LACTAM RING BY PENICILLINASE

Penicillin	conc. mcg./ml.	No. of* assays	$[\Delta\alpha]^{25°C}_{255\ m\mu}$ †	Std. Dev. § from line
Sodium Cloxacillin				
Commerical grade	503.65	12	2681.8 ± 8.8	15.9
Commerical ref. std.	496.85	5	2799.0 ±13.2	5.0
905 mcg./mg. as free acid	503.70	5	2808.9 ±13.2	14.5
recrystallized from water with n-butanol	608.25	9	2877.1 ± 9.6	14.5
recrystallized from methanol with ethylacetate	678.45	3	2792.7 ±16.0	2.7

* The number of time the solution was assayed.

† The change in specific rotation at zero standing time was obtained from the intercept of a regression line through a plot of $[\Delta\alpha]^{25°C}_{255\ m\mu}$ versus the time the solution was standing before being assayed (see fig. 8). These values are not corrected for purity or dilution. The standard error in the intercept (ref. 13) was also recorded.

§ This value represent the deviation of the experimental points for the regression line (see fig. 8).

The joint estimate of standard deviation† of the change in apparent specific rotation for the experimental points from their regression lines for the penicillinase-sensitive penicillin was 13.3 deg ml gm^{-1} dm^{-1} and 14.2 deg ml gm^{-1} dm^{-1} for the penicillinase-resistant penicillin. This represents a precision of 0.27% and 0.50% respectively, which is a deviation of 0.05 inch in approximately a 18 inch measurement. Cary 60 "Instruction Manual"[17] claims a precision of 0.2% full range scale (10 inches) for a single reading when not limited by noise. Most of this error can be attributed to chart printing error. Since two readings and the switching of the pen center are required in this assay procedure, a precision much greater than 0.3% should not be expected if readings are taken off the chart.

Thus, the precision offered by this polarimetric method appears to be far superior to any of the presently available assay procedures. The hydroxylamine and iodometric methods do not give precisions greater than 1-2%, whereas the microbiological methods are at best semi-quantitative procedures. In this polarimetric assay procedure, a precision of at least 0.3% was obtained. It is felt that a far greater precision could be obtained by introducing some modifications

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† $\sigma = \sqrt{\frac{\Sigma_i (n_i-2)s_i^2}{\Sigma_i (n_i - 2)}}$ where n_i is the number of experimental points used to determine the regression line and s_i is the standard deviation of the points from their regression line. The summation is over all the regression lines to be combined.

into the method to improve the accuracy of the readout. Intinsically, the method appears to be capable of readily yielding precisions of the order of a few parts per 10,000 since polarimetric readings can discern differences of a few tenths of a millidegree.

These polarimetric assay procedures are based on relating the intercept value, obtained from a linear plot of the change in apparent specific rotation against standing time for the aqueous solution, to the corresponding values for an absolute pure compound. Thus the accuracy for these assay procedures depends on the determination of the absolute changes in specific rotation expected for the various penicillins. The intercept value of the regression lines along with the standard error[18] in determining the intercept are given in Tables II and III for non-resistant and resistant penicillins, respectively. The values accepted in this investigation for the absolute change in specific rotation for the pure penicillins used in this study are given in Table IV. They were obtained by correcting the intercept values (Table II and III) for the purity of the dry sample and averaging them. The value for benzylpenicillin appears to be very close to the true value. The value accepted here was the overall average for all the assays on the benzylpenicillin recrystallized both from water with 1-butanol and from methanol with ethylacetate. This value was slightly larger than the average value obtained from the commercial reference standard (Wyeth Laboratories, Philadelphia, Pa, Control No. W620248-S1). The values for

TABLE IV.

VALUED FOR CHANGE IN SPECIFIC ROTATION AT 255 mμ
ASSIGNED TO PURE PENICILLIN

Penicillin	$[\Delta\alpha]^{25°C}_{255\ m\mu}$ † (deg ml gm^{-1} dm^{-1})
Potassium Benzylpenicillin	5053.9
Ampicillin (anhydrous acid)	5897.8
Penicillin V acid	5006.3
Sodium Cloxacillin	2956.3

† These values given in the table are averages of the change in apparent specific rotation given in table I and II corrected for purity but not for the dilution caused by injecting 0.05 ml. of a penicillinase solution into 16 ml. of the penicillinase-sensitive penicillin solution or 0.05 ml. of a penicillinase solution injected into 3.2 ml. of the penicillinase-resistant penicillin solution.

ampicillin (Wyeth Laboratories, West Chester, Pa, Lot No. C-10351), phenoxymethylpenicillin acid (Wyeth Laboratories, Philadelphia, Pa., Control No. E 16-1), and sodium cloxacillin (Ayrest Laboratories, Rouses Point, N.Y., BRL-1621) were obtained from a single reference standard, so the accuracy of these values are dependent on the labeled value of their respective reference samples and the purity at the time of assay. These values are probably quite accurate but not as dependable as the value given to potassium benzylpenicillin.

Table V, shows a comparison of the spectropolarimetric-penicillinase assay procedure to microbiological and iodometric assay procedures. Ampicillin samples, whose microbiological assays were in good agreement with the chemical assays, were furnished by Ayerst Laboratories, Rouses Point, N.Y. along with the microbiological and chemical assay data. The values from the spectropolarimetric-penicillinase method were obtained approximately one month after the microbiological and chemical assays.

For any real precise assay method, there is another rather important source of error, the sampling error. Several lots of the penicillin with varying purity are mixed together, so the batch may not be completely homogeneous in purity. Since only very small samples (usually less than 150 mg to prepare a 500 ml solution) are used, the sampling error can become significant. This could account for some of the variation in Table V.

TABLE V.

A COMPARISON OF THE PENICILLINASE-SPECTROPOLARIMETRIC METHOD TO MICROBIOLOGICAL AND IODOMETRIC METHOD FOR ANALYZING THE ACTIVITY FOR SEVERAL SAMPLES OF AMPICILLIN.

Ampicillin Sample	MICROBIOLOGICAL ASSAY† Commerical Laboratory		IODOMETRIC METHOD† Commerical Laboratory			Penicillinase-Spectropolarmetric Method		
	No. 1	No. 2	No. 3	No. 1	No. 4			
1. Trihydrate	840	848	850	824		814	810	
2. Trihydrate	843	746	855	839		819	812	824
3. Trihydrate	860	848	863	835		824	818	
4. Trihydrate	841	848	860	840		818	813	
5. Trihydrate	846	840	858			830	827	
6. Trihydrate	821	826	847	836		823	823	
7. Anhydrous	956		976	950	961	957	957	

† Data furnished by Ayerst Laboratories, Rouses Point, N.Y.

Application of the Optical Rotatory Procedure for Assaying a Mixture of a Penicillinase-Sensitive Penicillin and a Penicillinase-Resistant Penicillin.

The results from assaying mixtures of ampicillin (a non-resistant penicillin) and cloxacillin (a resistant penicillin) by optical rotation at 255 mμ are give in Table VI. In this Table, a comparison was made of the concentration of the penicillins used to prepare the solutions and the activity of the penicillins determined by the assay. The activities of the penicillins used for preparing the mixtures were separately determined from the optical rotatory assay procedure for each respective penicillin.

The mixtures were prepared from known amounts of each penicillin. The ampicillin concentration was assayed by injecting 10 μl of penicillinase solution into the mixture. The selection of an end point was obtained by adjusting the point at zero time (i.e., the time at which the penicillinase solution was injected) on the extrapolated slower decreasing terminal phase. A small correction was made by extrapolating from the end point at zero time to 1/2 t_{α} on a line with a slope obtained by averaging the slopes for the mutarotation phase from the assays of ampicillin alone. This correction was only an increase of 0.3-0.4% over the assayed value for ampicillin obtained at zero time.

The concentration of cloxacillin in the mixture was determined by substracting the change in optical rotation determined above for ampicillin from the total change in optical rotation for both penicillins. The total change in

TABLE VI.

ASSAYS OF MIXTURES OF AMPICILLIN AND CLOXACILLIN

Penicillin†	Weighed conc. mcg./ml.	Activity* mcg./ml.	Change in Rotation, Degrees	Activity Measured mcg./ml.	Percent Activity Assayed
Cloxacillin	342.00	332.7	0.0983	332.5	99.9
Ampicillin	405.00	381.9	0.2204	373.6	97.8
Cloxacillin	386.25	375.8	0.1116	377.5	100.4
Ampicillin	541.45	510.6	0.2940	498.5	97.6
Cloxacillin	380.20	359.3	0.1062	359.2	100.0
Ampicillin	453.70	427.8	0.2474	419.5	98.0
Cloxacillin	234.10	221.2	0.0639	216.1	97.7
Ampicillin	503.55	474.8	0.2777	470.8	99.2
Cloxacillin	438.45	414.3	0.1320	446.5	107.8
Ampicillin	189.45	178.7	0.1043	176.8	99.0
Cloxacillin	275.25	260.1	0.0796	269.4	103.6
Ampicillin	386.85	364.8	0.2108	357.4	98.0
Cloxacillin	297.45	281.1	0.0681	230.3	82.0
Ampicillin	365.45	344.6	0.1990	337.4	97.9
Cloxacillin	301.65	285.1	0.0896	303.1	106.3
Ampicillin	593.85	560.0	0.3256	552.1	98.6

† The ampicillin sample used in all the above mixture was recrystallized from water at 70°C (see ref. 8). A purity of 94.3% was obtained by comparing the average change in specific rotation given in Table I to the change in specific rotation for a pure compound given in Table III. The first two mixtures contained sodium cloxacillin which was recrystallized from water with n-butanol and a purity of 97.3% was assigned by comparing the results in Table II to the value given in Table III. In the remaining six mixtures, the sodium cloxacillin was recrystallized from methanol with ethylacetate and a purity of 94.5% was assign by comparing the results in Table II to the absolute value given in Table III.

* Activity equals weighed concentration times the purity.

optical rotation was obtained by injecting 50 µl of penicillinase solution (1,600,00 units/ml) into the mixture. The selection of an end point for the total change in rotation was essentially the same as the procedure described previously for penicillinase-resistant penicillins.

From Table IV it can be seen that the change in specific rotation for ampicillin is about twice the change in specific rotation for cloxacillin. This means any error in determining the ampicillin concentration would have twice the effect on the determination of the cloxacillin concentration. For example, if the ampicillin concentration was underestimated by 0.5%, then the cloxacillin concentration would be overestimated by 1%.

General Comments on these Assay Procedures.

No effect on the change in apparent specific rotation due to the hydrolysis of the β-lactam ring by penicillinase was found when the ionic strength of the penicillin solution was changed from 0.005 M to 1.0 M. Fig. 11 shows the effect of temperature on the change in apparent specific rotation at 247 mμ due to the catalytic hydrolysis of the β-lactam ring in benzylpenicillin by penicillinase at pH 5.00 and pH 9.50.

The cell compartment in the Cary 60 has an operating temperature of 27°C, while the room temperature was kept at 25°C. This means that there may have been a small change in rotation due to temperature change. The time required for the cleavage reaction to go to completion was approximately 2 minutes. It was usually less than 5 minutes from the time the solution was placed in the cell compartment until the completion of the cleavage reaction. Sometimes there was a slight decrease in the baseline for the intact penicillin due to temperature change. Later when a temperature bath was used to bring the buffer solution to 27°C prior to the preparation of the penicillin solution, this slight decrease in the baseline was almost competely eliminated.

At a constant pH and temperature, it was found that the spectropolarimetric-penicillinase assay method for benzylpenicillin follows a Beer's type law from a concentration of 1×10^{-5} to 1.3×10^{-3} M. At lower concentrations, there was greater deviation on both sides of the line since a smaller full range (0.02 deg.) was used, thus increasing the

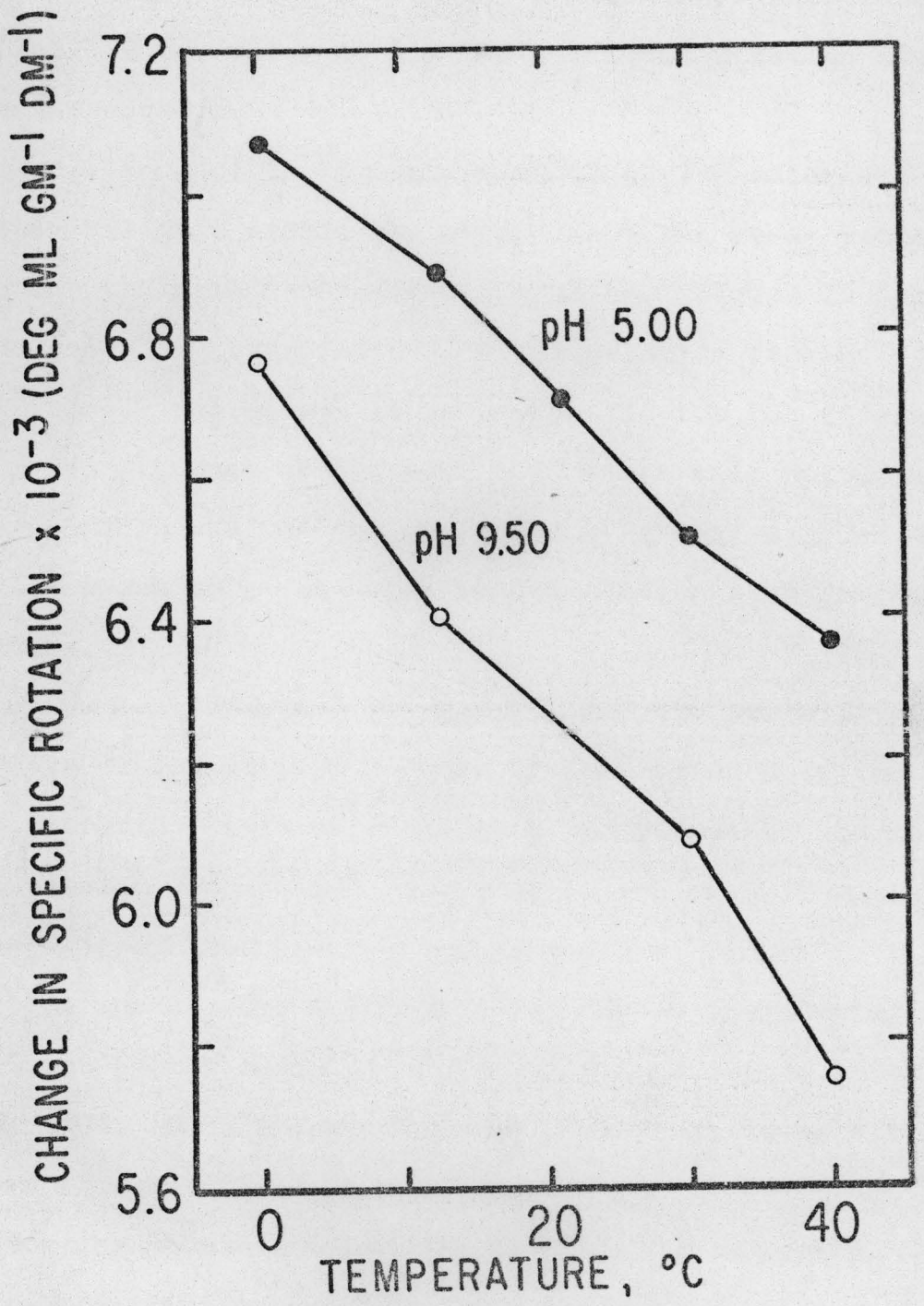

Fig. 11 - Shows the Effect of Temperature on the Change in Specific Rotation at 247 mμ due to the Catalytic Hydrolysis of the β-Lactam Ring in Benzylpenicillin by Penicillinase at pH 5.00 and pH 9.50.

noise. A concentration of 2×10^{-6} M for benzylpenicillin and ampicillin was close to the smallest concentration that could be detected.

The presence of any other optically active material, such as sugars, will not affect the assay since the assay procedure relates the penicillin concentration to the changes in optical rotation caused by the cleavage of the β-lactam ring by penicillinase. The enzyme is very specific for the β-lactam ring, so the presence of degraded penicillin will not affect the assay. This was checked by assaying several samples of ampicillin which were subjected to degradation study at room temperature.

Sodium benzylpenicillin tablets, 250,000 units or 400,000 units, were dissolved in one liter of the buffer solution. Without filtering, this solution with the suspended starch from the tablet was assayed at 255 mμ in a 5 cm cell with a full range of 0.2 and 0.4 degrees respectively. The degradation curves were a little noisy due to the scattering of the light by the suspended starch but an accurate assay could be made. When the solution was filtered, there was some loss of activity.

Since the change in specific rotation for ampicillin has a greater variation with pH than benzylpenicillin (see Fig. 5), it should be conceivable that a mixture of these two penicillins could be assayed by making measurements at two pH's, e.g. at pH 7.50 where the difference between the change in specific rotation for these two penicillins is

small and at pH 4.00 where this difference is significant. From these two readings, it should be possible to calculate the concentrations of both penicillins in the mixture.

Penicillin with uv absorption too great in the range of the present optical rotation measurement (247 or 255 mμ) can be assayed at a longer wavelength, but this increases the relative magnitude of the mutarotation and hence the selection of a proper end point becomes more critical.

Results from Assaying Benzylpenicillin by CD.

A circular dichroism phenomenonis associated with any medium which exhibits optical rotation. The cd spectra for benzylpenicillin and its penicilloic acid derivative at pH 7.00 and 3.00 are shown in Fig. 12. The cd spectra for the intact penicillin at these two pH's are essentially the same, showing a maximum around 231 mμ, which corresponds to the cotton effect for benzylpenicillin in Fig. 4. However, there is a significant difference in the spectra of benzylpenicilloate at these two pH's. At pH 3.00, where the carboxyl group formed by the cleavage of the β-lactam ring is mostly protonated, a positive circular dichroism spectrum is observed between 225 and 260 mμ with a maximum at 243 mμ corresponding to the positive cotton effect in the ord spectrum in Fig. 4. At pH 7.00 where this carboxyl group is mostly unprotonated, there is no cd spectrum for benzylpenicilloate in this region and hence no cotton effect is

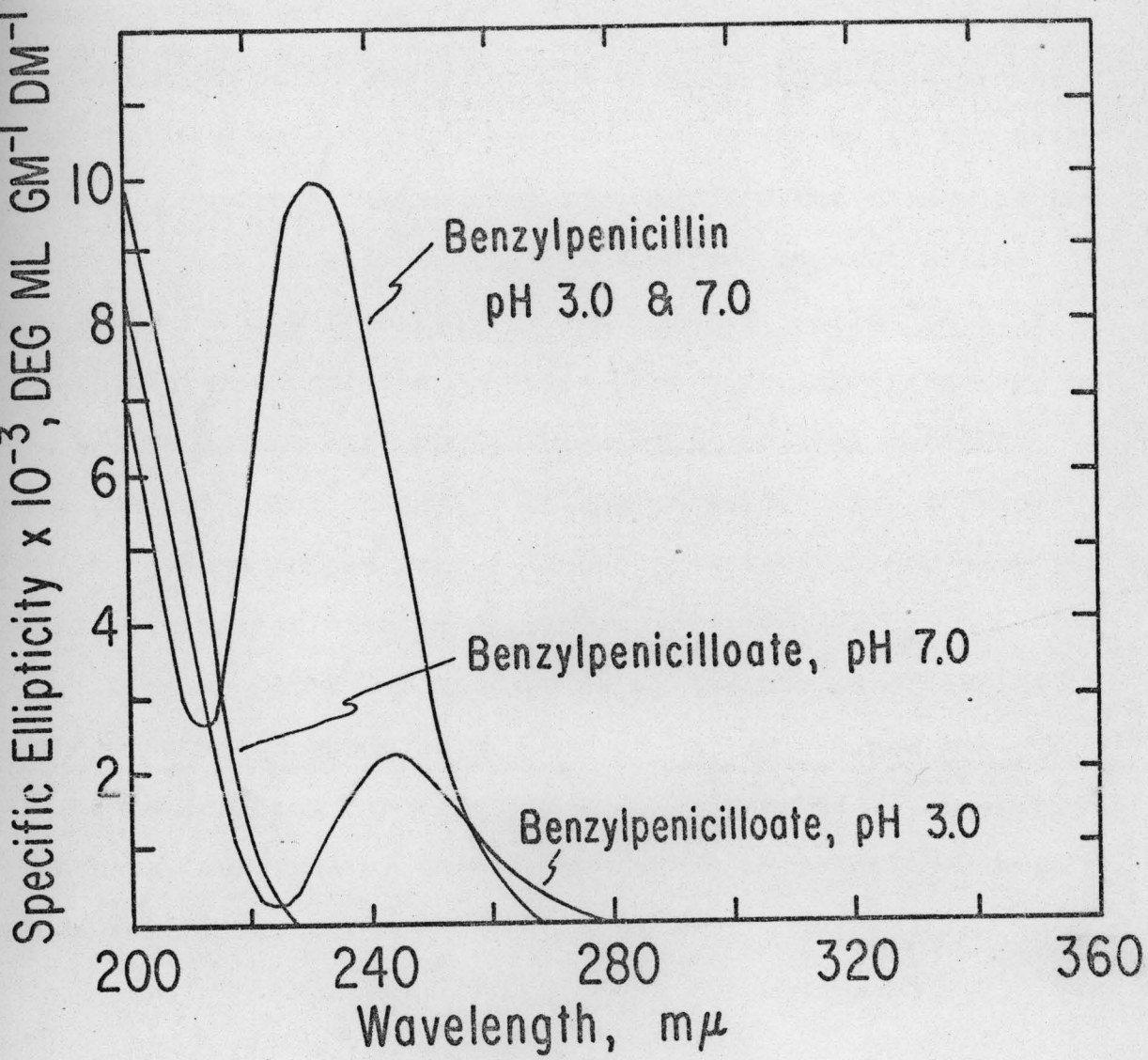

Fig. 12 - Circular Dichroism Spectra of Benzylpenicillin and its Penicilloic Acid Derivative at pH 3.00 and pH 7.00.

observedin the ord spectrum. Therefore for pH's above 5.50, the selection of an end point was greatly simplified by the fact that the mutarotation step was not observed in the assay curves. The final reading was constant and the change in the apparent specific ellipticity was obtained by subtracting the final reading from the initial reading. Below pH 5.50, the mutarotaion could be observed with circular dichroism, but not to the extent that was observed with optical rotation, thus the end point of 1/2 t_{α} should be used. Fig. 1 shows how the change in specific ellipticity at 231 mμ for benzylpenicillin varies with pH at 27°C.

Circular dichroism appears to be the method of choice when not limited by uv absorption at 231 mμ. Since most of the penicillins used today have much greater uv absorption than benzylpenicillin, this procedure is only applicable to a few penicillins.

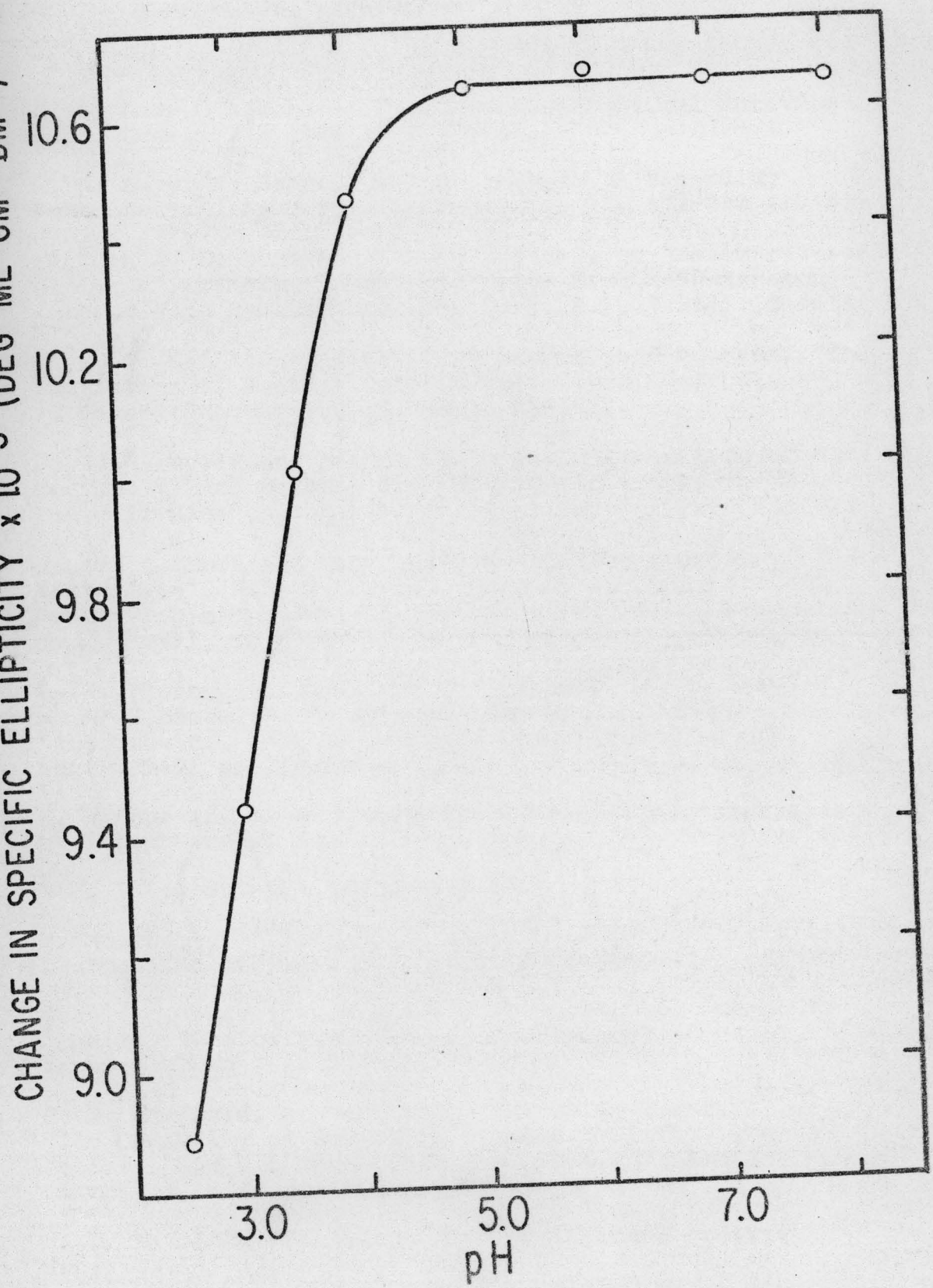

Fig. 13 - Shows the effect of pH on the Change in Specific Ellipicity at 231 mμ due to the Catalytic Hydrolysis of the β-Lactam Ring in Benzylpenicillin by Penicillinase.

IV. REFERENCES

1. G. B. Levy, "Automatic Measurement of Optical Rotation", Anal. Chem., 23, 1089-1092 (1951).

2. G. B. Levy, P. Schwed, and D. Fergus, "A Recording Polarimeter", Rev. Sci. Instruments, 21, 693-698 (1950).

3. Kenneth A. Connors and Takeru Higuchi, in "Pharmaceutical Analysis", Takeru Higuchi and Einar Brochmann-Hanssen, Interscience Publischers, New York, N.Y. (1961), Chapter 10.

4. J. M. T. Hamilton-Miller, J. T. Smith, and R. Knox, "The Estimation of Penicillins and Penicillin Destruction", J. Pharm. Pharmacol., 15, 81-91 (1963).

5. David A. Wolff and Morton Hamburger, "Evaluation of Some Currently Used Methods for Determining Staphylcoccal Penicillinase", J. Lab. Clin. Med., 59, 469-480 (1962).

6. Robert L. Peck and Karl Folkers, in "The Chemistry of Penicillin", Hans T. Clarke, John R. Johnson, and Sir Robert Robinson, Eds., Princeton Univ. Press, Princeton, N.J. (1949), pp 87-89.

7. H. W. Florey, E. Chain, N. G. Heathley, M. A. Jenning, M. A. Sanders, E. P. Abraham, and M. E. Florey, "Antibiotics, Vol. II", Oxford Univ. Press, London, Eng. (1949) pp 747-748.

8. K. W. Austin, A. C. Marshall, and H. Smith, "Crystalline Modifications of Ampicillin", Nature, 208, 999-1000 (1965).

9. Ref. 7, pp 803-804 and pp 840-846.

10. A. Moscowitz, "Theoretical Aspects of Optical Activity. I. Small Molecules", Adv. Chem. Phys., 4, 67- (1962).

11. Robert G. Benedict, William H. Schmidt, and Robert D. Coghill, "Penicillin. VII. Penicillinase", Arch. Biochem., 8, 377-383 (1945).

12. R. B. Woodward, A. Neuberger, and N. R. Trenner, in "The Chemistry of Penicillin", Hans T Clarke, John R. Johnson, and Sir Robert Robinson, Eds., Princeton Univ., Princeton, N.J. (1949), p 419.

13. J. E. Banfield, "Kinetics of Penicillinase Activity" Experientia, 13, 403-404 (1957).

14. Malcolm Dixon and Edwin C. Webb, "Enzymes", Academic Press, New York, N.Y. (1964), Chapter 4.

15. Keith J. Laider, "Chemical Kinetics, Second Edition", McGraw-Hill, Inc., New York, N.Y. (1964).

16. Howard A. Strobel, "Chemical Instrumentation", Addison-Wesley Publishing Co., Reading, Mass. (1960), pp 19-20.

17. "Cary Model 60 Spectropolarimeter Instruction Manual" Cary Instruments, 2724 South Peck Road, Monrovia, Calif.

PART B.

Optical Rotatory and Circular Dichroism Methods for Determining the Enzymatic Kinetic Parameters for the Action of Penicillinase on Penicillin.

I. INTRODUCTION

Since the optical activity methods described in the preceding section permit continuous monitoring of the action of penicillinase on penicillin over wide changes in the concentration of the substrate, it is evident that these methods may provide a simple, convenient means of evaluating Michaelis-Menten enzymatic kinetic parameters, K_m and V_{max}, for the action of this enzyme on these drugs. In this section, results of an investigation designed to test the feasibility of this approach are reported together with some new data on the nature of these enzymatic reactions.

Specifically the methods have been applied to the study of the catalytic effect of Bacillus cereus penicillinase (Neutrapen) on benzylpenicillin (non resistant), ampicillin (non resistant) and cloxacillin (resistant). Both optical rotation and circular dichroism were used interchangably and both gave essentially identical results. The methods have been applied to measurements of changes in the Michaelis constant caused by variation in ionic strength and pH. Competitive effects of the resistant and non resistant species acted on simultaneously are also discussed.

The optical activity methods are based on the fact that a linear relationship exists between the concentration of a penicillin solution and the changes in optical rotation or circular dichroism caused by the catalytic cleavage of the

β-lactam ring by penicillinase. Since the product produced during the reaction does not inhibit the reaction, a single continuous optical rotation (or circular dichroism)-time recording can be used to determine enzymatic kinetic parameters for the reaction between penicillinase and penicillin. The difference between the optical rotation (or circular dichroism) at any time, t, during the reaction and a final reading can be continuously related to the penicillin concentration at time, t. The rate of change of optical rotation (or circular dichroism) is thus directly related to the reaction rate. The optical rotation and circular dichroism methods have the advantage that the phenomena measured are a direct result of the reaction between the enzyme and substrate rather than an indirect result where the product must undergo a reaction, as in the manometric or iodometric methods. The only limitation of optical activity methods is due to the absorption of uv light, thus limiting the concentration of some penicillins and inhibitors with large uv absorption. A longer wavelength can be used with the optical rotation method to reduce the absorption, however, the sensitivity of this method would also be reduced.

Of the currently used methods for measuring penicillinase activity, the manometric and iodometric methods[1,2,3] are the most widely used for determining K_m and V_{max} values. The manometric method[4] depends on measurement of the amount of carbon dioxide released from a carbon dioxide-bicarbonate buffer on a Warburg respirometer. When penicillin is

catalytically hydrolyzed to penicilloate, a new carboxyl group (pK_a = 4.7)[5,6] and a weak secondary amine group are produced resulting in a net production of hydrogen ions which causes the release of carbon dioxide from carbon dioxide-bicarbonate buffer. This method is very time consuming since each measurement requires 20 minutes equilibration time for a system containing 5% carbon dioxide in nitrogen or oxygen. To determine K_m and V_{max} for a single system, the initial velocities at three to six substrate concentrations must be determined, duplicate or triplicate measurements being usually made at each concentration.

For the iodometric methods, the amount of iodine taken up, as the penicillin is hydrolyzed to penicilloic acid by penicillinase, is measured. In the iodometric titration method,[7,8] the cleavage reaction is carried out in an excess amount of iodine and back-titrated with a standard thiosulfate solution at arbitrary time intervals during the enzymatic reaction. Another method, the "timed iodometric method",[9] measures the time required to consume a known amount of iodine from a starch-iodine complex. The micro-iodometric method utilizes increased sensitivity of the iodometric method afforded by using a spectrophotometer. This method depends on the absorption by the I_3^- complex at 360 mμ[10] or 420 mμ[11] or on the absorption by the starch-iodine complex at 600 mμ[11] or 620 mμ.[12] This micro-iodometric method can measure initial rates for penicillin concentration from 2.5 to 800 μM[11] by using pathlengths from 4.00 to 0.25 cm and iodine concentrations

from 13 to 160 μM, respectively. Novick[11] found that the micro-method gave a 40% lower penicillinase activity measurement than the iodometric titration method.

A method used for the determination of the enzymatic kinetic parameters, K_m and V_{max}, must, as suggested by Cleland,[13] be accurate, rapid, and, preferably, a continuous recording method. If a timed assay method is used, the linearity of the reaction at all levels of substrate concentrations should be determined. In general, the timed assay procedures are less precise for velocity measurement than a continuous recording method.[13] Cleland[13] further suggested that the ideal concentration range is from 0.2 K_m to 5 K_m, giving velocities ranging from 1/6 V_{max} to 5/6 V_{max}. It is often not possible to use the entire ideal range of substrate concentrations becauses the method may not be sensitive at the lower concentrations while the upper limits are set by limited solubility or absorption of the substrate.

The optical activity methods do adhere very well to these requirements. For many of the penicillins, the optimal concentration range can be used. The upper part of the concentration range may be limited by uv absorption for a few penicillins. If a longer wavelength is used, the sensitivity will be reduced thus setting the lower limits, however, initial rate studies at a couple of wavelengths should allow the use of the entire concentration range for these penicillins. Continuous recordings are obtained from the optical rotatory and circular dichroism procedures used

here and the instruments respond very rapidly. The micro-iodometric method can be made a continuous recording method, however, a lag time of 3 to 4 minutes has been reported[11] before the decolorization rate becomes linear.

II. EXPERIMENTAL

Apparatus.

Measurements of optical rotation and circular dichroism were made on a Cary 60 recording spectropolarimeter and with the Cary model 6001 circular dichroism accessary. A 5 cm cell was used in the determination of K_m and V_{max} for benzylpenicillin and ampicillin and for the determination of inhibition constants for cloxacillin in benzylpenicillin-penicillinase systems. A 1 cm cell which was securely fastened to the cell holder with masking tape was used in the study of the effect of cloxacillin on penicillinase.

Penicillin samples were weighed on a Cahn Electrobalance, model G-2. A 10, 25, and 50 microliter Hamilton syringe were used for the injection of penicillinase solutions into the penicillin sample.

Chemicals and Reagents.

Bacillus cereus penicillinase solutions (Neutrapen injecable, 800,000 units†/vial, Lot. No. 66482, 77853, & 99139),

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† The units of penicillinase activity are those as defined by Levy,[14] that is, "A unit of penicillinase effects the inactivation of 10^{-7} moles of penicillin (35.6 mcg or 59.3 units of sodium benzylpenicillin) per hour at 25°C at pH 7.0. This action takes place in a phosphate buffer solution of a pure alkali salt of benzylpenicillin in sufficient concentration to maintain a zero-order reaction.

Another common unit of penicillinase activity is that of Pollock's,[15] where one unit of activity hydrolyzes 1 μmole of benzylpenicillin in 60 minutes at 30° and pH 7.0.

a gift from Riker Laboratories, Northridge, Calif., were prepared by dissolving one vial in 5 ml of distilled water for the determination of K_m, K_I, and V_{max} values and one vial in 1/2 ml of distilled water for the study of the effect of cloxacillin on penicillinase. The activity of penicillinase in a refrigerated solution was found to be fairly stable. The penicillinase solutions were prepared a day before their use. The enzyme solutions were stored in the refrigerator and kept in ice when not refrigerated. Solutions were usually kept for two weeks. The same Michaelis constant was obtained when repeated 3 months later with the same enzyme solution. However, there was some loss of activity.

Potassium benzylpenicillin, a gift from Wyeth Laboratories, Philadelphia, Pa., was twice recrystallized by dissolving in water and slowly precipatated by adding 20 volumes of 1-butanol,[16] the precipatated penicillin was then washed with ethylacetate. The ampicillin trihydrate, commerical grade, was a gift from Squibb and Co., New Brunswick, N.J., and sodium cloxacillin, commerical grade, was a gift from Ayerst Laboratories, Rouses Point, N.Y. Both ampicillin and cloxacillin were used without further purification.

Buffers - A hydrochloric acid-acetic acid buffer systems were used at pH 2.50 and 3.05. Sodium acetate buffer systems, pH 3.50 to 6.00, were prepared by adding glacial acetic acid to a sodium acetate solution. Sodium phosphate buffer systems, pH 6.00 to 8.00, were prepared by mixing solutions of monobasic and dibasic sodium phosphate solution of the same ionic strength.

Sodium carbonate buffers, pH 8.00 to 10.20, were prepared by mixing sodium bicarbonate and sodium carbonate solutions of the same ionic strength.

Optical Rotatory Procedure.

An aqueous solution of the penicillin to be studied was prepared by dissolving 10 to 12 mg of the penicillin in 50 ml of a buffer solution. A 5 cm cell was filled with this aqueous penicillin solution and placed in the spectropolarimeter. The optical rotatory measurements were carried out at a constant wavelength of 247 mμ (or 255 mμ for the determination of the inhibition constant for cloxacillin) with a slitwidth of 2.4 mm, a chart speed of 10 sec/division and a full range scale of 0.2 degrees. The pen center was set at zero and the pen was adjusted with the manual zero and zero suppression to the 1.0 line on the chart paper.

With the filled cell in place, the rotation was recorded for 1-2 minutes. Then 2-10 μl of penicillinase solution (160,000 units/ml) was injected into the cell, which was then shaken, replaced, and the recording reinitiated. A kinetic curve of the rapid drop in rotation versus time was recorded. When the pen reached zero on the chart paper, the pen center was switched from zero to 1.0 so the change in rotation covered twice the width of the chart paper. The pen continued to drop rapidly until all the penicillin was degraded by the penicillinase. This was followed by a slow reduction in the rotation of the solution.

After each run, the cells were filled with 3 N nitric acid and allowed to stand for a few minutes to inactivate the residual enzyme.

Circular Dichroism Procedure.

For this procedure, a wavelength of 231 mμ was used. Approximately 3.5 mg of benzylpenicillin was dissolved in 50 ml of buffer solution. Except for these modifications the procedure described for the optical rotatory procedure was followed.

Procedure for Studying the Effect of Cloxacillin on Penicillinase.

Aqueous solutions of cloxacillin were prepared by dissolving varying amounts of cloxacillin, 14-92 mg, in 50 ml of 0.50 M sodium acetate buffer solution, pH 5.00 or 5.50. The penicillin was degraded by injecting 20-100 μl of penicillinase solution (3,200,000 units/ml). Optical Rotatory measurements were made using the 1 cm cell which was securely fastened to the cell holder with tape. Measurements were made at 280 mμ with a chart speed of 10 and 100 sec/division. Except for these modifications, the optical rotatory procedure was followed.

III. RESULTS AND DISCUSSION

Application of the Optical Rotation (or Circular Dichroism)-Penicillinase Assay Methods for Determining Enzymatic Kinetic Parameters.

Fig. 1 shows a typical optical rotation recording as a function of time from which the enzymatic kinetic parameters were determined. Segment A represents the optical rotatory activity of benzylpenicillin prior to the addition of the enzyme. At point B, penicillinase solution was injected producing the observed rapid decrease in optical rotation (segment C) corresponding to the enzymatic cleavage of the β-lactam ring to form D-α-benzylpenicilloate. This initial decrease in optical rotation is mostly zero order converting to first order at the end of the reaction. There is a very slow decrease in rotation seen in the terminal phase (segment D) due to the mutarotation of D-α-benzylpenicilloate.

It is evident, from a plot such as Fig. 1, that the velocity of the cleavage reaction can readily be determined from the slope of the curve for several substrate levels, and hence the kinetic parameters of the reaction. Since a linear relationship exists between the optical rotation (or circular dichroism) and the penicillin concentration, the difference between the optical activity reading at time, t, and a value obtained from the extrapolated mutarotation line at the same time, t, is directly related to the penicillin concentration according to the relationship

$$c = \frac{\Delta\alpha}{[\Delta\alpha]^{T}_{\lambda} \cdot \ell} \qquad (1)$$

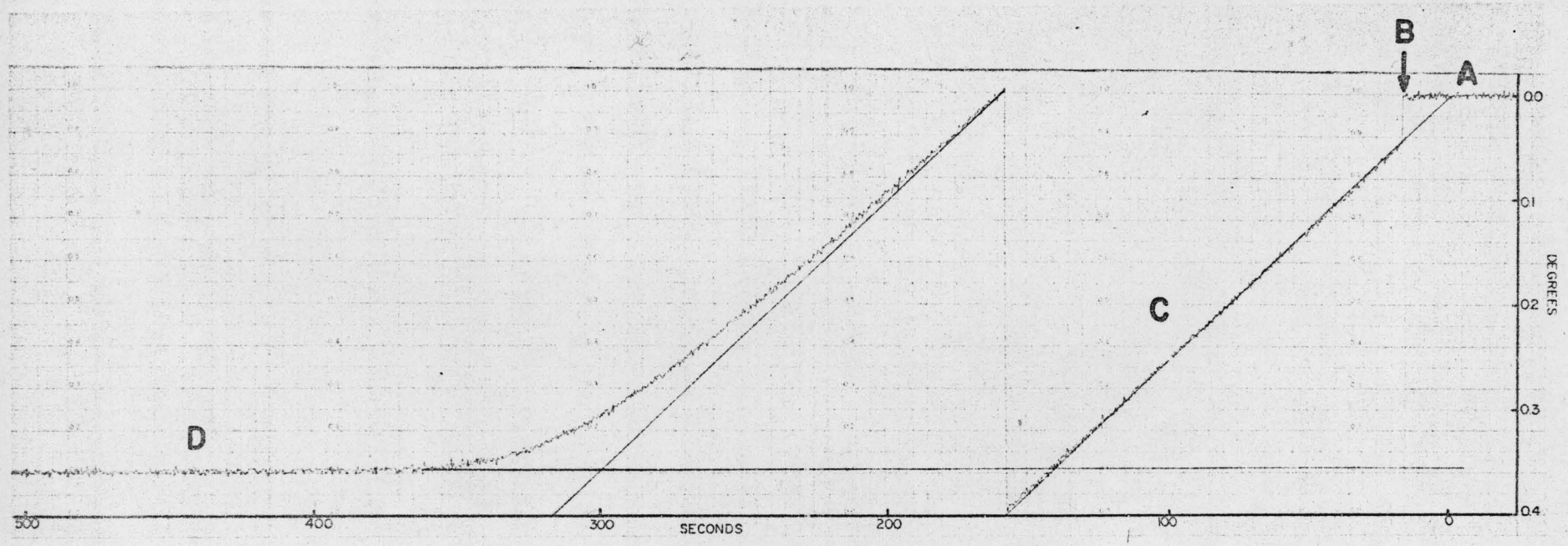


Fig. 1 - A Typical Polarimetric Recording Used for Determining the Enzymatic Kinetic Parameters, K_m and V_{max}.

where c is the concentration in gm/ml, ℓ is the pathlength in decimeters, $\Delta\alpha$ is the observe change in optical rotation, $[\Delta\alpha]_\lambda^T$ is the change in specific rotation, T and λ are the temperature and wavelength at which the reaction was carried out. The velocity of the reaction at time, t, can thus be determined from the slope of the curve at time, t, that is

$$\frac{dc}{dt} = \frac{1}{[\Delta\alpha]_\lambda^T \cdot \ell} \frac{d\alpha}{dt} \qquad (2)$$

where α is the optical rotation. Values for $[\Delta\alpha]_\lambda^T$ have already been reported in the first section.

Either (1) the full curve can be used to determine the Michaelis constants or (2) the initial segments of several curves for several initial substrate concentrations can be used (the initial velocity technique). For the analysis based on a single full curve, both the concentration and velocity are determined from one experimental run. For the initial velocity technique, the initial segment for the curve is determined at several substrate concentrations, the initial velocities being obtained by extrapolating the optical rotation curve back to zero time and calculating the slope of the curve at zero time.

The values obtained by these two methods are compared in Table I. For the initial velocity technique, six substrate concentrations were used with 8 to 12 initial velocity determinations at each substrate concentration. Cleland's computer program[13] for direct curve fitting of the Michaelis-Menten equation was employed to convert the rate measurements

TABLE I.

VALUES OF K_m § OBTAINED BY ANALYZING A SINGLE FULL CURVE AND BY AN INITIAL VELOCITY TECHNIQUE.

Method of Analyzing Data	Penicillinase Concentration	K_m §, μM
Initial Velocity Method	25 units/ml	41.2 ± 11.4
Analyzing a Single Curve	25 units/ml	39.3 ± 1.7
Analyzing a Single Curve	50 units/ml	38.2 ± 2.4

§ These values were determined by optical rotation at 255 mμ for benzylpenicillin at 27°C in a phosphate buffer system, pH 7.00 and an ionic strength of 0.18 M.

to the kinetic parameters. In this calculation, the average velocity at each substrate concentration was used and each data point was weighted inversely to the variance of the velocity.

The data obtained from the analyses of three full curves such as that shown in Fig. 1, using the same penicillinase concentration were found to lie on the same Lineweaver-Burk plot. Cleland's computer program[13] was used to analyze the combined data from the three curves; each point being given equal weight. It was concluded from this study (Table I) that the analyses of a single full recording gave essentially the same estimate for the Michaelis constant as the initial velocity method.

In the initial velocity method, the velocity measurements are dependent upon the amount of enzyme injected into the penicillin solution. Each velocity measurement requires a separate injection of enzyme which introduces an error in each velocity measurement due to variation in the amount of enzyme injected. Therefore duplicate or triplicate velocity measurements should be made.

By following a single continuous curve, the enzyme concentration is constant, thus increasing the accuracy for the determination of K_m. Since V_{max} is a function of the enzyme concentration, any error in the injection of the penicillinase solution will appear in V_{max}. However, the concentrations of the substrate are not known exactly as in the initial velocity method since they must be determined from the curve. But the change in rotation covered 15 to 19 inches of chart paper so the concentration can be determined fairly accurately. It was felt that the Michaelis constants could be determined more accurately from a single full curve rather than from an initial velocity method.

Since the entire curve can be used for determining the enzymatic kinetic parameters, it is evident that the following integrated form of the Michaelis-Menten equation can also be used to calculate these kinetic parameters,

$$S_0 - [S] = - K_m \ln(S_0/[S]) + V_{max}\, t \qquad (3)$$

where S_0 is the initial substrate concentration, [S] is the substrate concentration determined at time, t, and K_m and V_{max} are the enzymatic kinetic parameters to be determined.

It is also more convenient to collect data for the integrated form of the Michaelis-Menten equation since it requires only the determination of the concentration from the curve at a given time and not the velocity, that is the slope of the curve. For this reason, all values, unless otherwise stated, were obtained from a weighted least-square analysis of the integrated equation using the following weight expression,

$$\frac{1}{K_m^2(1/S_0^2 + 1/[S]^2) + 2} \quad . \qquad (4)$$

This weight expression was arrived at by giving equal weight to each substrate determination, but bivariate weighting the plotting variables according to the transformation that the concentration reading underwent. It was assumed that there was no correlation between the dependent and independent variables. Since this weight expression contains one of the regression coefficients which is to be determined from the weighted least-squares analysis, an iterative procedure is required using an initial guess for the K_m.

In order to use the integrated form of the Michaelis-Menten equation, the time should be known accurately. As can be seen in Fig. 1, there is a period of time between the injection of the enzyme into the penicillin solution and the time the recording can be reinitiated. Since the catalytic rate constant, k_2, for

$$S + E \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

is a first order rate constant, a point on the curve can

arbitrarily be selected for S_0. This will eliminate the extrapolation of the curve over the time the reaction was not recorded.

In order to achieve some idea of the reproducibility of these curves, several sets of Michaelis constants obtained from three or more similar systems are given in Table II. Most of the Michaelis constants were obtained on different days over a period of a year, using various vials of penicillinase from a single lot (Neutrapen, Lot. No. 77853). The joint estimate for the relative standard deviation of the Michaelis constant from Table II was 12%. The variation in the Michaelis constants appeared to be the same for those obtained on the same day as for those obtained on different days.

TABLE II.

MICHAELIS CONSTANTS WHICH WERE OBTAINED UNDER SIMILAR EXPERIMENTAL CONDITIONS.

pH	Ionic Strength	Michaelis Constants	Relative Standard Deviation
7.25	0.040	48.9, 50.0, 48.6	1.5 %
5.00	0.006	31.9, 21.1, 33.3	23.2 %
5.00	0.010	28.5, 19.2, 24.1	19.4 %
5.00	0.020	30.8, 29.1, 29.6	2.9 %
5.00	0.030	25.1, 32.8, 30.0, 33.2	12.3 %
5.00	0.060	32.4, 31.2, 39.0	12.3 %
5.00	0.200	67.7, 64.3, 64.4	3.0 %
5.00	0.60	119, 126, 138	7.4 %
5.00	1.00	156, 124, 145	11.4 %
4.00	0.040	25.9, 26.9, 30.3, 28,9	7.1 %

Advantages of the Optical Activity Methods.

The optical rotatory and circular dichroism methods presented here for the determination of Michaelis constants and maximum velocities for the reaction between penicillin and the enzyme, penicillinase, have many advantages over the currently used methods. These optical activity methods are extremely rapid and convenient. They are sensitive enough that the entire optimal concentration range (0.2 K_m to 5 K_m) can be used. There is a simple linear relationship between the change in optical rotation (or circular dichroism) and the penicillin concentration over this entire concentration range. The response of these instruments are very rapid, thus for all practical purposes, no lag time exists. This is not true for many of the other currently used methods. Because of the simple linear relationship, the rapid response of the instruments, and the fact that the product of the reaction does not inhibit the reaction, a continuous curve can be used to determine the kinetic parameters. The collection of data is greatly simplified by using the integrated form of the Michaelis-Menten equation. The optical rotation and circular methods use measurements of optical phenomena, therefore requiring no foreign materials as in the iodometric methods. The method developed here can be applied equally well over the entire pH range. The precision for determining these enzymatic kinetic parameters obtained in the method presented here is probable greater than the precision which can be obtained from any of the other currently used methods. Because

of all these advantages, the author believes that this method is far superior to any of the other methods currently in use.

Salt Effect on K_m for Benzylpenicillin.

It was found that the Michaelis constants were affected by ionic strength while the maximum velocity was not affected. This effect was checked at pH 5.00 by varying the concentration of sodium acetate buffer; or by using sodium acetate buffer (ionic strength = 0.006 M) and varying the sodium chloride or sodium sulfate concentration; or by using potassium acetate buffer (ionic strength = 0.010 M) and varying the potassium chloride concentration. For ionic strength less than 0.06 M, a linear plot was obtained when the pK_m† values, the negative logarithm of the Michaelis constants, were plotted against the square root of the ionic strength, $\sqrt{I}$. The statistical analysis of the regression lines are given in Table III. The sodium acetate, sodium acetate-sodium chloride, and potassium acetate-potassium chloride systems appeared to give the same results. The Michaelis constants for these three systems at a given ionic strength were averaged together and a semi-logarithm plot of the average $1/K_m$ values versus the square root of the ionic strength are shown in Fig. 2. Also shown in Fig. 2 is the ionic strength effect of a sodium acetate-

† Since the Michaelis constants is a dissociation constant, the pK_m (the logarithm of $1/K_m$) will be used in all plots and calculations. $1/K_m$ is an association constant and represents the binding between the enzyme and pencillin.

Fig. 2 - Shows the Ionic Strength Effect at pH 5.00.

Key: Values obtained from sodium acetate only, sodium acetate-sodium chloride, and potassium acetate-potassium chloride were averaged together and plotted as (●).

Values obtained from the sodium acetate-sodium sulfate systems were plotted as (○).

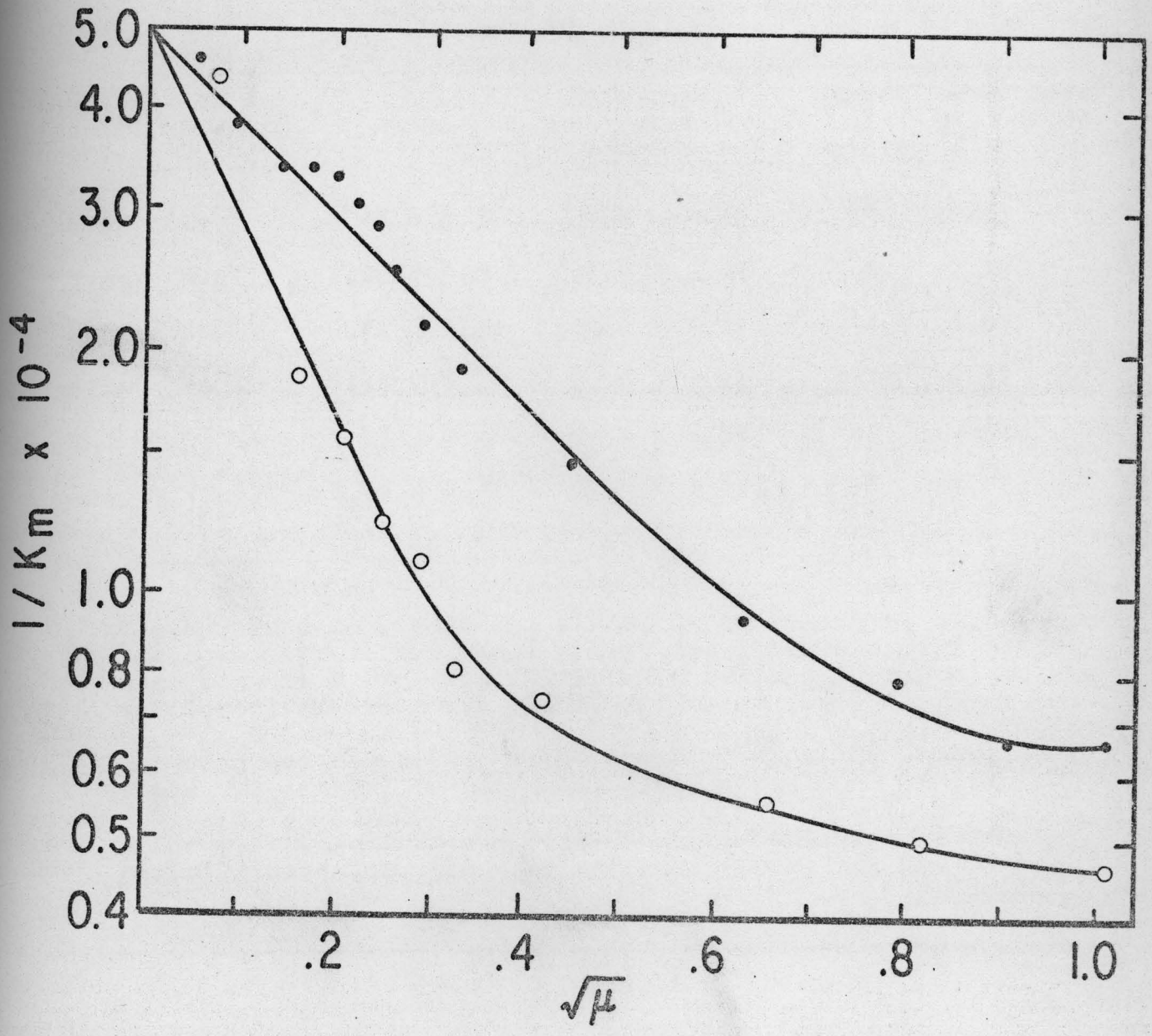


TABLE III.

REGRESSION COEFFICIENTS FROM LEAST-SQUARE ANALYSIS OF THE LINEAR PLOT, pK_m vs. $\sqrt{I}$, OBTAINED FROM SEPARATE EXPERIMENTS AT pH 5.00.

Salts used for Ionic Strength Effect	Slope	Intercept[§] Antilogarithm μM	Number of Points	Std. Dev.* (K_m units)
NaAc only	-0.44 ± 0.41	25.5 ± 3.84	9	7.36
NaAc - NaCl[†]	-1.35 ± 0.41	17.0 ± 3.68	5	2.26
NaAc - NaCl[†]	-0.69 ± 0.33	26.6 ± 4.27	4	3.57
NaAc - NaCl[†]	-0.65 ± 0.21	23.4 ± 2.02	7	2.51
KAc - KCl	-1.13 ± 0.41	19.3 ± 4.01	4	3.54
NaAc - Na_2SO_4	-2.14 ± 0.40	23.5 ± 4.89	10	7.66

§ These values represent the Michaelis constants at zero ionic strength. These intercept values and their standard errors were converted from the logarithmic scale (pK_m units) to the antilogarithm scale (K_m units, μM).

* The standard deviations were converted from the logarithmic scale (pK_m units) to the antilogarithm scale (K_m units, μM).

† These were seperated experiments using different vials of penicillinase form the same lot (Neutrapen, Lot No. 77853).

sodium sulfate system. It can be seen from Fig. 2 and Table III, that the sodium sulfate system has a greater ionic strength effect on the Michaelis constant than any of the other systems studied at pH 5.00.

The ionic strength of other buffer systems are compared in Table IV. From this Table, it was apparent that K_m was essentially the same for all buffer systems at the same pH and ionic strength. The tris buffer systems could not be used above pH 7.0, especially the tris-hydrochloride buffer system, since the penicillin was degraded by the tris buffer.

It was also found that boric acid acted as a competitive inhibitor (Table V), but that the borate ions appeared to have only an ionic strength effect, similar to that obtained for the phosphate ions.

From the above results, it was concluded that the three cations, Na^+, K^+, and $tris^+$, had the same ionic strength effect. Also the Ac^-, Cl^-, $H_2PO_4^-$, $HPO_4^=$, HCO_4^-, $CO_3^=$, and $H_2BO_4^=$ anions appeared to have the same ionic strength effect. However, the $SO_4^=$ anion appeared to have a specific interaction or salt effect.

Variation in Ionic Strength Effect with pH.

It was also found that the ionic strength effect varied with pH. This effect is shown in Fig. 3 and the results from the regression analysis of these lines are given in Table VI.

TABLE IV.

MICHAELIS CONSTANTS OBTAINED IN VARIOUS BUFFER SYSTEMS AT CROSS OVER pH'S.

pH	Ionic Strength	Buffer System	K_m, μM	Method	Wavelength, mμ
6.00	0.02	acetate	39.3	ord	247
6.00	0.02	acetate	42.9	cd	231
6.28	0.02	phosphate	44.7	ord	247
6.00	0.03	phosphate	39.5	cd	231
6.00	0.03	acetate	39.3	cd	231
6.00	0.03	acetate	39.4	cd	231
6.00	0.04	acetate	39.8	cd	231
6.10	0.04	acetate	40.9	ord	255
6.21	0.04	phosphate	44.0	ord	255
6.76	0.01	tris-acetate	62.8	ord	255
6.85	0.01	tris-HCl	73.8	ord	255
7.00	0.01	phosphate	75.8	cd	231
7.00	0.01	phosphate	68.0	cd	231
6.87	0.02	tris-acetate	45.5	ord	255
7.00	0.02	phosphate	60.7	cd	255
7.00	0.02	phosphate	56.4	ord	247
7.00	0.05	tris-acetate	47.0	ord	255
7.00	0.05	tris-HCl	50.6	ord	255
7.00	0.05	phosphate	47.4	cd	231
7.80	0.03	phosphate	65.4	cd	231
7.95	0.03	carbonate	63.1	cd	231
7.95	0.03	carbonate	62.4	ord	247

TABLE V.

INHIBITION CONSTANT FOR BORIC ACID

pH	K_{obs}, μM	K_m, μM	$f_{H_3BO_4}$ †	K_I, μM §
9.00	2160	96.8	0.5780	810
9.50	987	117.	0.3021	1070
10.00	1000	236.	0.1203	850

† $f_{H_3BO_4} = 1/(1 + K_1/[H^+] + K_1K_2/[H^+]^2)$, where $K_1 = 7.3 \times 10^{-10}$ $K_2 = 1.8 \times 10^{-13}$ are the first two dissociation constants of boric acid.[17]

§ $K_I = c \times f_{H_3BO_4}/(K_{obs}/K_m - 1)$, where c is the concentration of the boric acid buffer (0.03 M), K_{obs} is the Michaelis constant in the borate buffer system, and K_m is the Michaelis constant obtained from the sodium carbonate buffer system, ionic strength of 0.03 M.

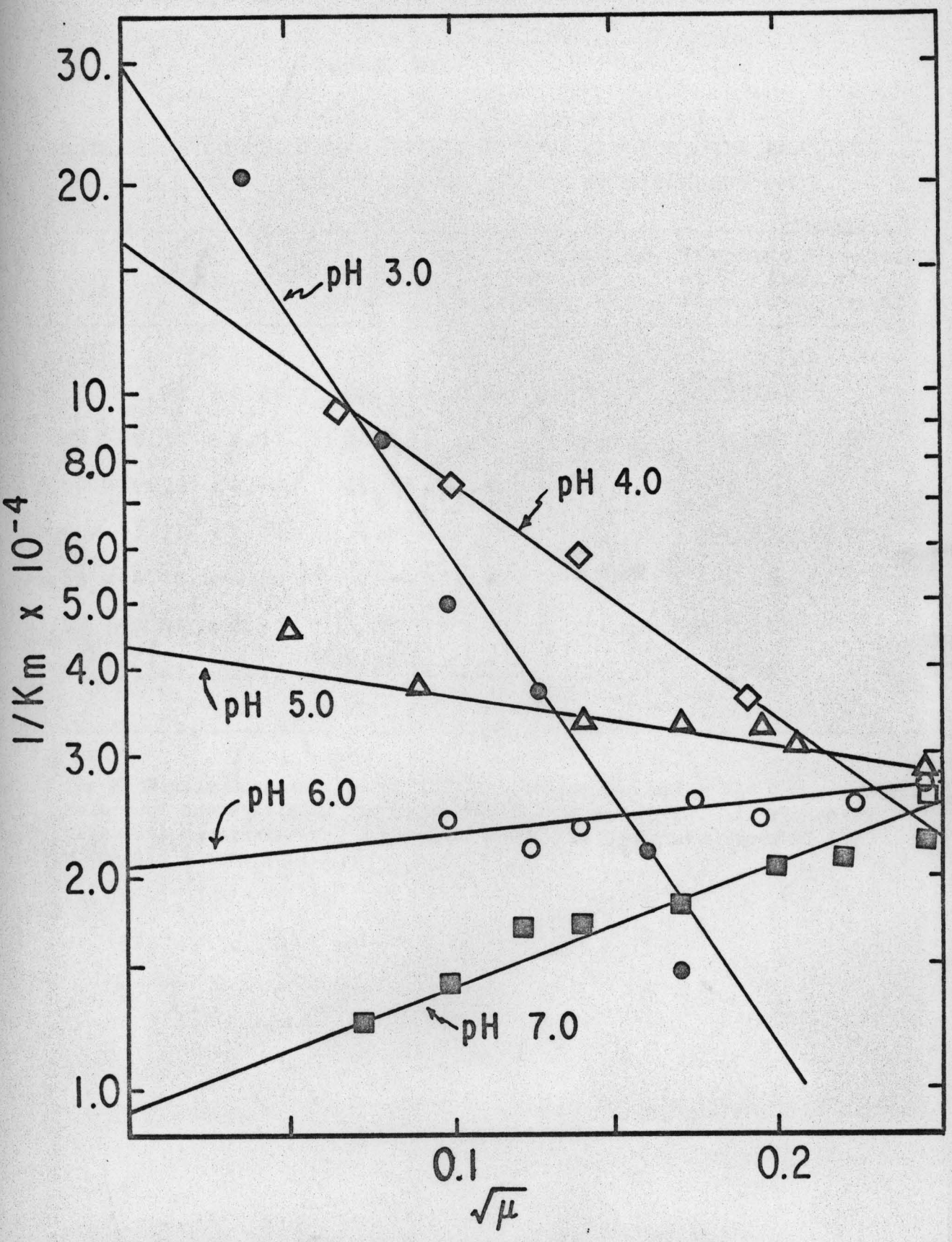


Fig. 3 - Shows How the Ionic Strength Effect on pK_m for Benzylpenicillin varies with pH.

TABLE VI.

REGRESSION COEFFICIENTS OBTAINED FORM LEAST SQUARE ANALYSIS OF THE LINEAR IONIC STRENGTH EFFECT AT DIFFERENT pH's.

pH	Slope	$K_m°$, $\mu\underline{M}$	Number of curves analysis	Standard Deviation of K_m from the line in K_m units
7.00	+1.74 ± 0.26	109.7 ± 9.4	13	7.36
6.00	+0.39 ± 0.17	48.1 ± 3.4	13	2.29
5.00	-0.73 ± 0.15	23.6 ± 1.5	32	3.77
4.00	-3.25 ± 0.26	6.06 ± 0.76	9	1.65
3.50	-4.45 ± 0.20	4.62 ± 0.48	5	1.42
3.00	-7.05 ± 0.32	3.31 ± 0.36	6	1.26
2.50†	-21.67 ± 2.22	1.13 ± 0.52	3	33.94

† The Michaelis constants at pH 2.5 were determined using the Gauss-Newton Method of curve fitting the Michaelis-Menten Equation. All the other Michaelis constants were determined using the Integrated form of the Michaelis-Menten Equation.

The effect of ionic strength on the Michaelis constant can be rationalized on the basis of eq. 5.[18]

$$pK_m = pK_m^\circ + 1.02\, Z_e Z_s \sqrt{I} \qquad (5)$$

where pK_m is the negative logarithm of the observed Michaelis constant at an ionic strength of I, Z_e is the charge on the enzyme and Z_s, the charge on the substrate. A plot of pK_m versus $\sqrt{I}$ gave a linear plot with a slope of $-1.02\, Z_e Z_s$ and an intercept of pK_m°, the pK_m at zero ionic strength. There is only one negative charge on the substrate, the carbonyl group with a pK_a of 2.9;[5,6] therefore the effect of pH on the slope obtained from the ionic strength plot could be due to a variation in the charges on the enzyme with the pH. This effect could be an overall effect on the enzyme rather than a specific effect at the active site.

pH Profiles of pK_m for Benzylpenicillin.

The pH profile of pK_m° and the pH profile of pK_m at a constant ionic strength of 0.03 M are shown in Fig. 4. These two profiles cross at pH 5.7. At this pH, there is no ionic strength effect, which from eq. 5 means that there is probably no net charge on the enzyme. This pH is probably the isoelectric point for this penicillinase. The isoelectric points for some penicillinases from several strains of Bacillus cereus have been reported to be slightly below pH 5.5.[19]

Since pK_m is a measure of the enzyme-substrate binding, a possible explanation for these pH profiles is as follows. The increase in the values of pK_m° below pH 5.7, and hence

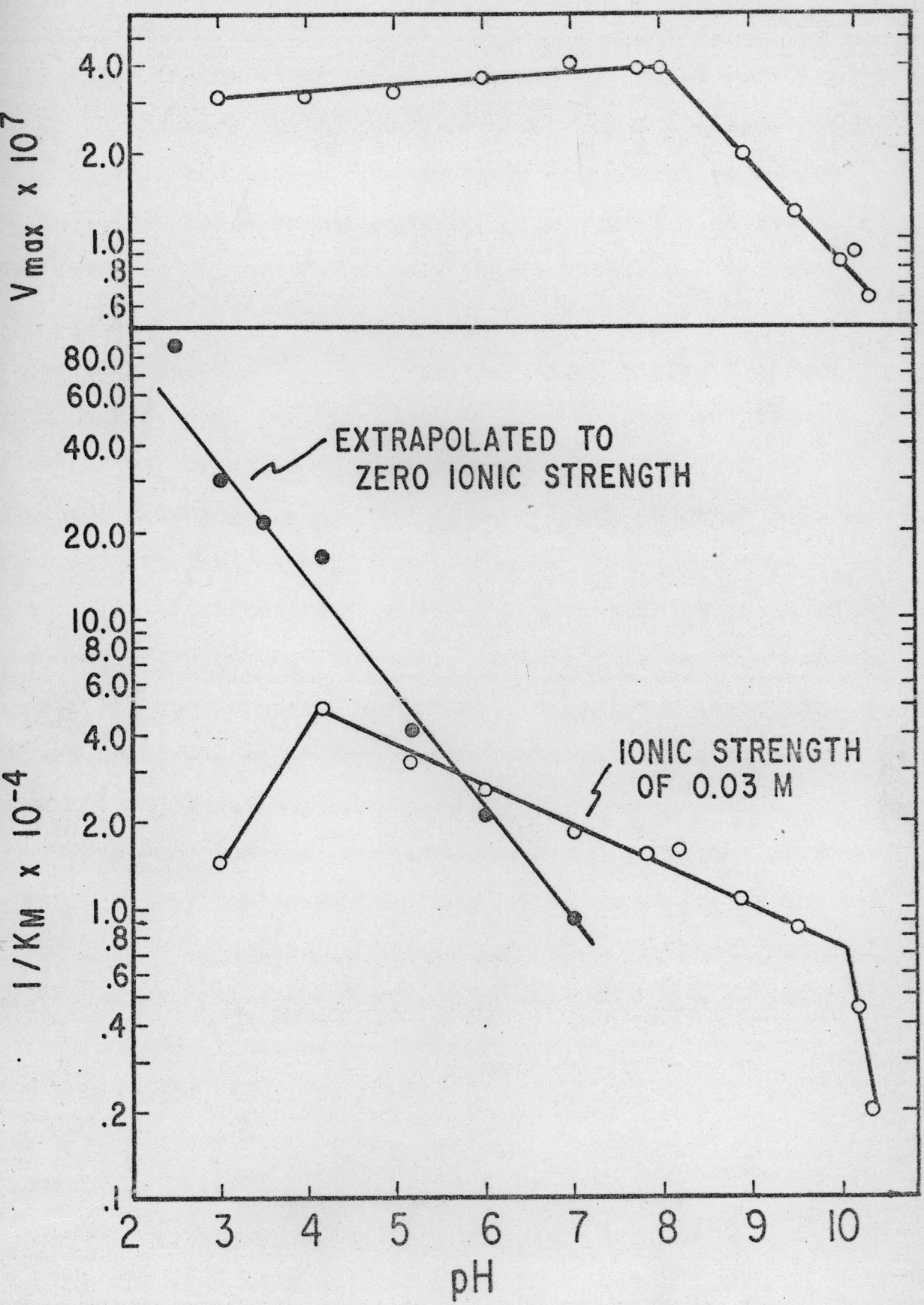


Fig. 4 - Shows (1) the pH Profile of pK_m°, the pK_m Extrapolated to Zero Ionic Strength, (2) the pH Profile of pK_m at an Ionic Strength of 0.03 M and (3) the pH Profile of V_{max} for Benzylpenicillin.

an increase in the enzyme-substrate binding, could possibly be due to an increase in the positive charge on the enzyme which could have an additional electrostatic attraction force for the negative charge on the penicillin in addition to the more specific enzyme-substrate attraction forces. Below pH 5.7, there is a negative ionic strength effect when pK_m is plotted against $\sqrt{I}$. This negative ionic strength effect would then account for the fact that for a given pH, the values of pK_m obtained at an ionic strength of 0.03 M are below the values for pK_m°. For values of pK_m° above pH 5.7, there is presumably a negative charge on the enzyme thus creating an electrostatic repulsion and decreasing the binding of the enzyme-substrate complex. According to eq. 5, there is a positive ionic strength effect and hence for a given pH, the values of pK_m at an ionic strength of 0.03 M will be above the pK_m° values.

These same results, however, can also be rationalized in terms of changes in the conformational structure of the enzyme. This is quite possible since there are no disulfide bridges to stabilize the tertiary structure of the enzyme.[20] Cross disulfide linkages in proteins are the most important, if not the only, covalent bonds which contributes to the stability and rigidity of the tertiary structure.[20] Protein molecules which do not contain a disulfide covalent linkage, such as penicillinase, have to rely on the weaker, more easily ruptured internal hydrogen bonds between carbonyl and the amide NH groups and the salt linkages between terminal carboxyl and terminal ammonium ions to preserve its folded state.[20]

Therefore penicillinase would be expected to have a greater degree of flexibility and to be more capable of unfolding and refolding than an enzyme which contains covalent disulfide linkages.

In order to explain the pH profile at zero ionic strength, a gradual change in the tertiary structure of the enzyme with pH would be required. The above salt effect can then be considered as a secondary salt effect, That is, the ionic strength is affecting the salt linkages and internal hydrogen bonds in the enzyme.

There is a break at pH 9.7 in the pH profile for pK_m determined at a constant ionic strength of 0.03 M. This break could possibly be the pK_a of a terminal amine group of lysine or the phenolic hydroxyl group of tyrosine.[21] There are two facts which favors the presence of tyrosine at the active site. First, boric acid was a competitive inhibitor for the reaction. Boric acid has been known to chelate with polyhydroxyl and phenolic compounds such as glycerine and epinephrine.[22] Secondly, Citri and Garber,[23] have suggested that the penicillinase enzyme unfolds in the presence of urea exposing a tyrosine residual which then reacts with iodine.

J. E. Banfield[24] has reported that the ionic strength effect on the rate of hydrolysis of benzylpenicillin by penicillinase from Bacillus subtilitis (a commercial preparation, Commonwealth Serum Laboratories, Australia) was negligble in the concentration range of 0.002 - 0.08 M. However, neither

the concentration nor the pH used for this determination was reported. If these results were obtained near the maximum velocity, their results would be consistant with the results obtained in the present study.

Salt Effect on V_{max}.

There was no ionic strength effect on the rate constant k_2. This can be explained either using the transition state theory or by conformational changes. According to the transition state theory,[18] the splitting of the complex ES (or EP) can be represented as follows

$$\text{ES (or EP)} \rightleftharpoons X^* \longrightarrow E + P$$

where X^* is the activated complex. The equation describing the effect of ionic strength on the rate constant is then given by:[18]

$$\log k_2 = \log k_2^\circ - 0.509\sqrt{I}(Z_{es} - Z_{X^*}) \qquad (6)$$

where Z_{es} is the charge on the enzyme-substrate complex and Z_{X^*} is the charge on the activated complex. It can be seen from this equation, that no ionic strength effect will be observed when the charges on ES (or EP) and the activated complex, X*, are the same.

If conformational changes occur at the active site when the enzyme combines with the substrate (Koshland "induced-fit" theory[25]), the activation process may occur in a region shielded from the medium. In this case, the application of eq. 6 would not be valid and the ionic strength would probably not have any effect on the rate constant, k_2.

Citri et. al.[15] have observed pronounced changes in the steric structures of penicillinase. They have shown that penicillinase exists in at least two distinct forms, α and γ forms, which may be in equilibrium. Both forms have penicillinase activity. The α form is less sensitive to free iodine and more thermostable than the γ form. The transition from the α form to the γ form can be brought about through adsorption on charged surfaces, such as glass; exposure to alkali (0.03 M) sodium hydroxide; high concentration of sodium chloride (3.3 M), urea (5 M), or guanidine hydrochloride (1.6 M).[15] The reversal was accomplished by elution from the glass surface, neutralization of the alkali, or dilution of sodium chloride, urea, or guanidine hydrochloride solutions.[15]

In their studies,[21] the enzyme appeared to retain full activity while completely converted to the iodine-sensitive state. They suggested that the modified penicillinase "reverts to the enzymatically active conformation through contact with the substrate". They found that the urea-treated enzyme was resistant to iodine in the presence of benzylpenicillin.

pH Profile of pK_m for Ampicillin at Constant Ionic Strength.

The pK_a of the carboxyl group of ampicillin is 2.65 and the pK_a of the amino group is 7.25.[26] The pI, that is the pH at the isoelectric point, is 4.95.[26] In the pH-pK_m profile for ampicillin at an ionic strength of 0.03 M, Fig. 5, there is a break in the curve around pH 5.3, which is closer to the pI of ampicillin than to either of the two pK_a values.

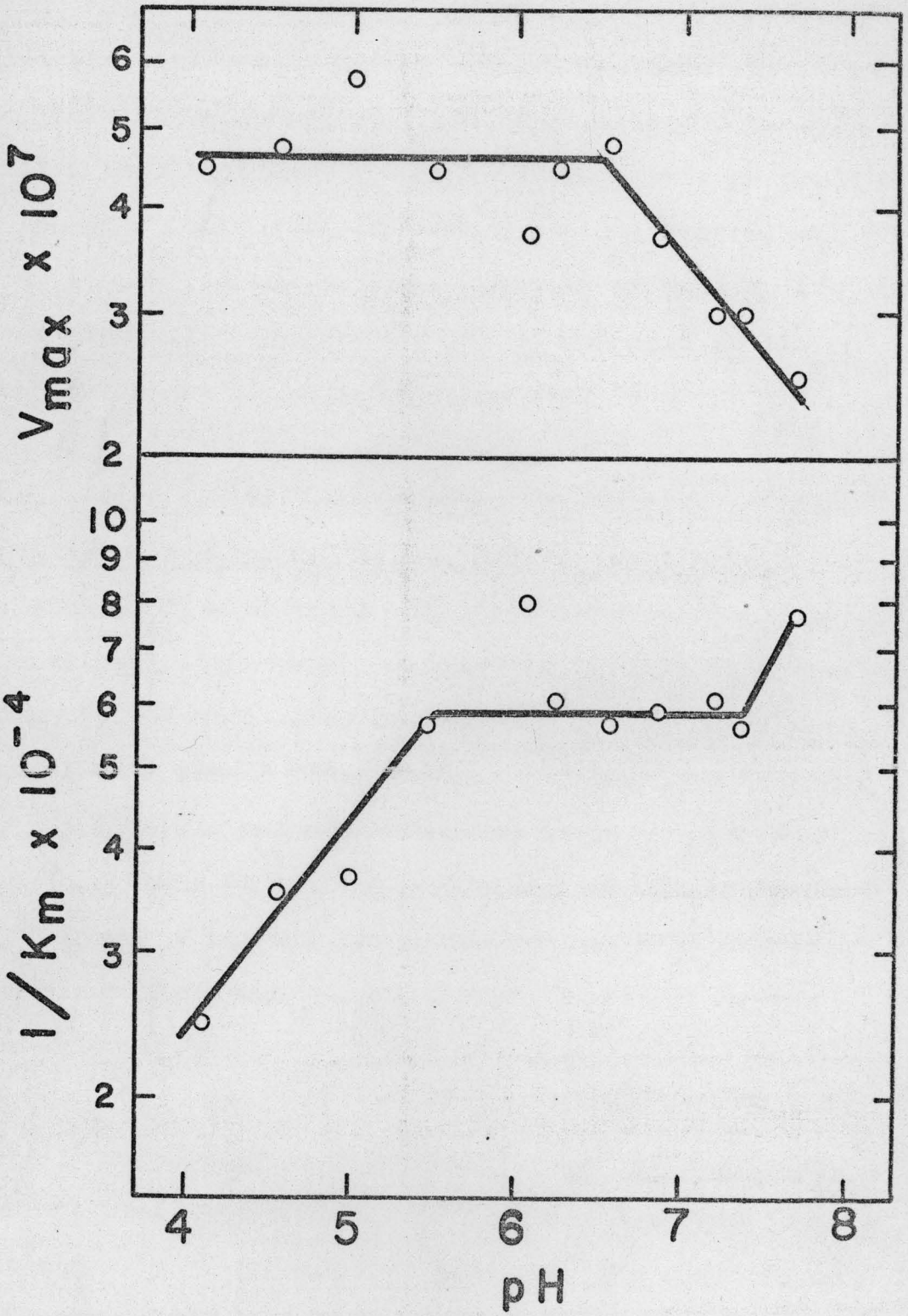


Fig. 5 - The pH Profile of $1/K_m$ and V_{max} for ampicillin at an Ionic Strength of 0.03 M.

The plot was obtained from single determinations and therefore did not have the accuracy that the pH profile for benzylpenicillin had. Not much can be said about this pH profile since the pH - ionic strength effect was not determined. However, at pH 6.74 and an ionic strength of 0.53 M, the observed Michaelis constant was 307 μM compared to 171 μM for the same pH at an ionic strength of 0.03 M.

The Competitive Inhibition Effect of Cloxacillin on the Reaction between Benzylpenicillin and Penicillinase.

The pH profile of the inhibition constants for cloxacillin in a benzylpenicillin-penicillinase system at a constant ionic strength of 0.04 M is shown in Fig. 6. These values were obtained from a single determination and the pH - ionic strength effect was not determined so again not too much can be said about this pH profile. However, it was found that cloxacillin was a competitive inhibitor over the pH range studied since there was no inhibition effect on the maximum velocity.

The Catalytic Hydrolysis of Cloxacillin by Penicillinase.

In attempting to obtain the Michaelis constant for cloxacillin at pH 5.00 and 5.50 from a single continuous curve, it was found that there was a more rapid decrease in rotation prior to the apparent zero order part. When the difference between the observed rapid decrease in rotation and an extrapolated line from the zero order portion of the curve was plotted, pseudo first-order rate constants (Table

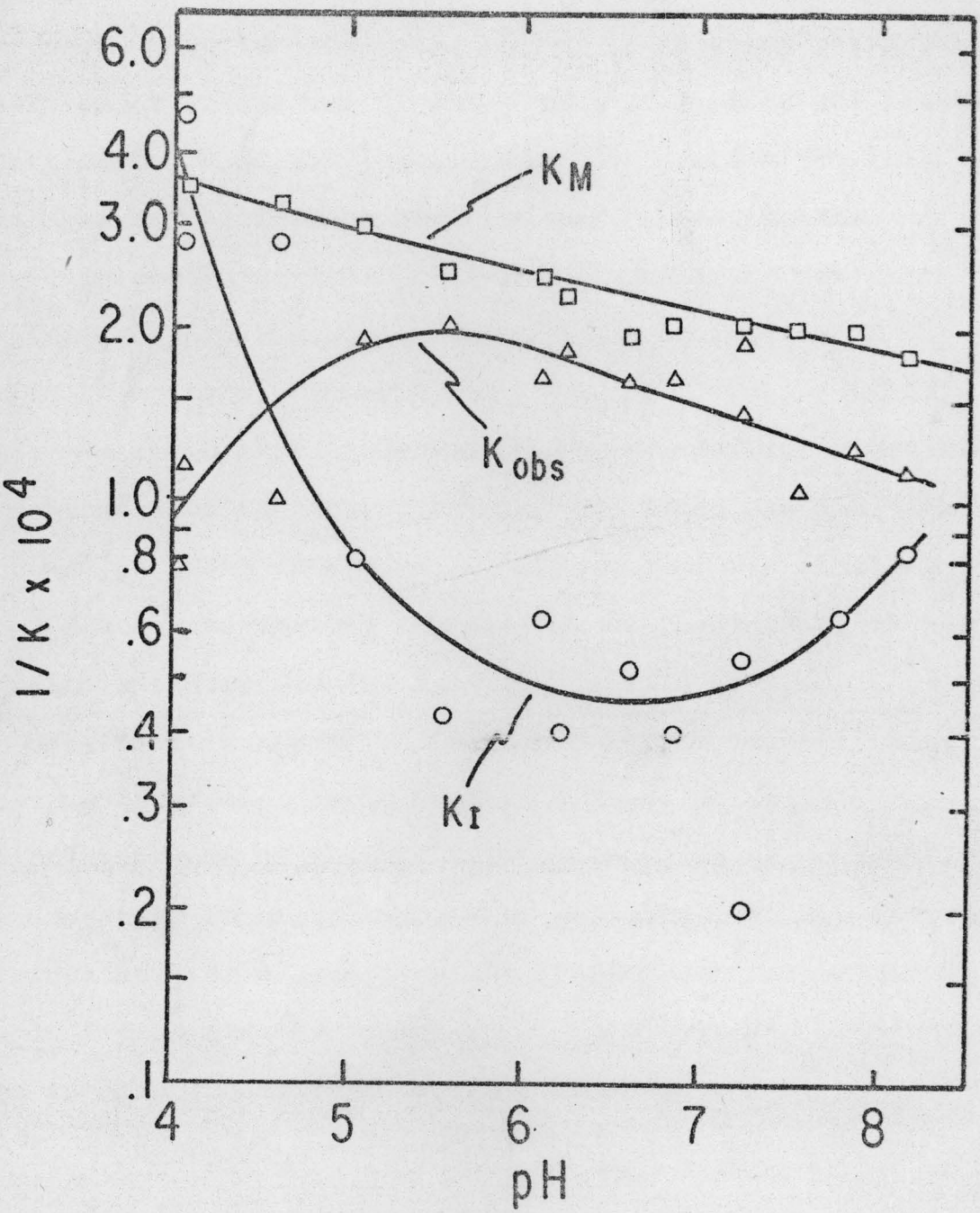


Fig. 6 - The pH Profile for the Competitive Inhibition Effect of Cloxacillin on the Benzylpenicillin-Penicillinase System at on Ionic Strength of 0.04 M.

Key: K_m is the Michaelis constant for benzylpenicillin;
K_{obs} is the apparent Michaelis constant for benzylpenicillin in the presence of the competitive inhibitor, cloxacillin; and
K_I is the competitive inhibition constant for cloxacillin on the benzylpenicillin-penicillin system.

VII) were obtained which were independent of the amount of enzyme used. However the total change in rotation prior to the zero order change was directly proportional to the enzyme concentration. When the total change in rotation for this initial pseudo first-order reaction was related to the cloxacillin concentration, the change in rotation per unit of penicillinase activity was found to be equivalent to 3.5×10^{-12} moles of cloxacillin.

It was found that these pseudo first-order rate constants were dependent on the concentration of cloxacillin and this dependence is shown in Fig. 7. A linear plot was obtained which did not pass through the origin, but instead there was a definite intercept value.

These results appear to substantiate the concept that the enzyme undergoes conformational changes during the reaction with some of the penicillinase-resistant penicillin.[27] Since substrate concentrations were so large that the enzyme was saturated, the observed pseudo first-order rate constant probably represents the change in maximum velocity with the change in enzyme conformation, that is:

$$V_{max} \xrightarrow{k_{obs}} V'_{max}$$

and from Fig. 6:

$$k_{obs} = k_1 + K_2[S] \qquad (7)$$

where k_{obs} is the observed pseudo first-order rate constant at a particular substrate concentration, k_1 is the intercept value, 5.89×10^{-3} sec^{-1}, corresponding to a pseudo first-order rate constant which is independent of the substrate

TABLE VII.

Pseudo First-Order Rate Constant for the Rapid Decrease in Rotation prior to the Apparent Zero-Order Portion of the Reaction.

pH	Ionic Strength M	Units of Penicillinase per ml	Concentration of Cloxacillin x 10^3	Pseudo First-Order Rate Constant x 10^3
5.00	0.05	25,000	3.98	8.08
5.00	0.50	40,000	4.22	7.19
5.00	0.50	25,000	4.09	8.87
5.00	0.05	40,000	4.09	8.84
5.50	0.05	20,000	5.43	11.28
5.50	0.05	40,000	7.81	15.35
5.50	0.05	50,000	7.81	13.54
5.50	0.50	50,000	10.69	18.16
5.50	0.50	80,000	10.69	14.10
5.50	0.50	50,000	17.98	24.10
5.50	0.50	200,000	17.98	24.74

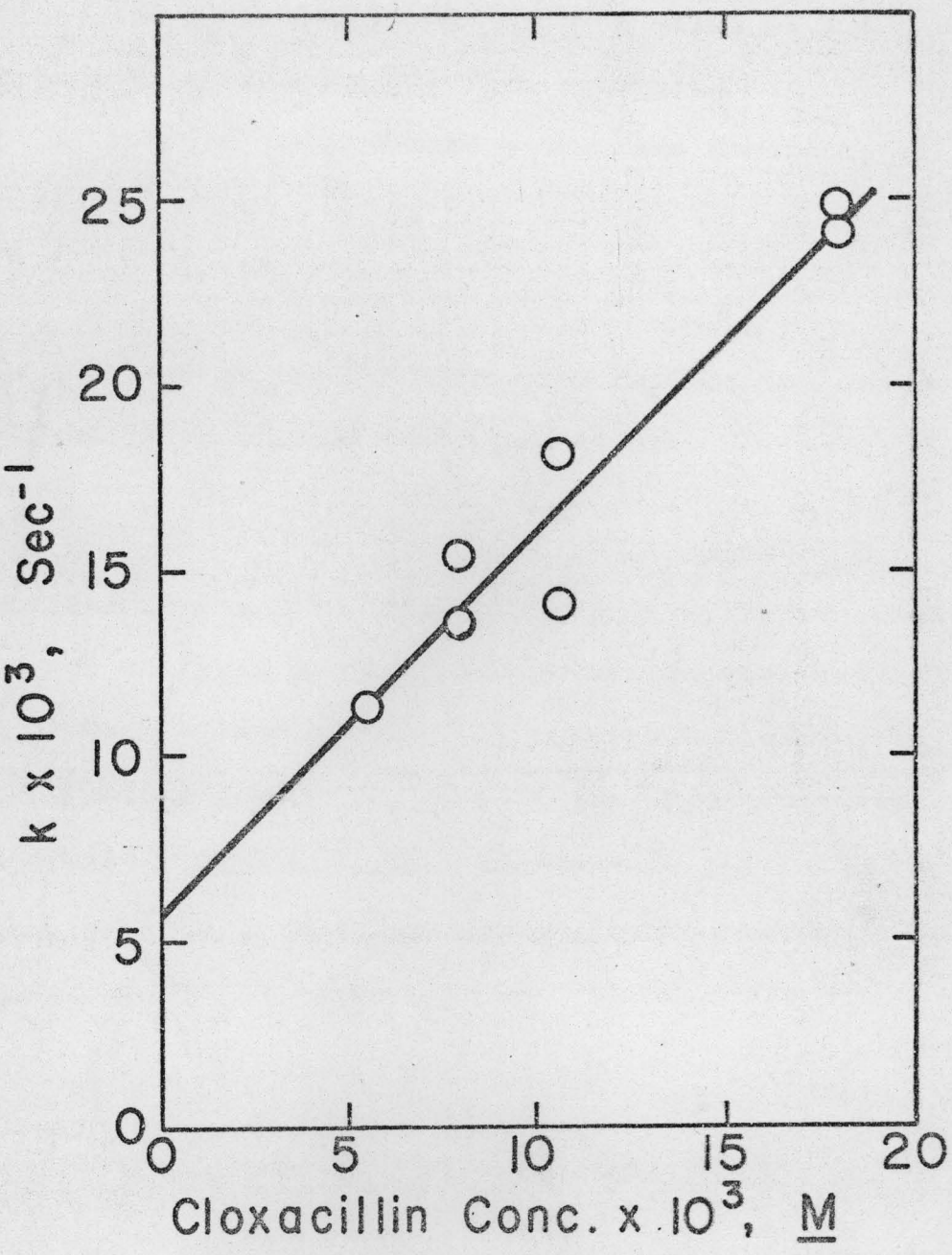


Fig. 7 - A Plot of the Pseudo First-Order Rate Constant against Cloxacillin Concentration.

concentration, and k_2 is the slope having a value of 1.02 and corresponds to a second order rate constant.

Since [S] >>> [ES], the second order rate constant could represent:

$$ES + S \xrightarrow{k_2} E'S + P$$

where E′ is a different conformation structure of the enzyme. While the first order portion could represent:

$$ES \xrightarrow{k_1} E' + P$$

The portion of the curve after this initial rapid change in rotation was analyzed using Michaelis-Menten kinetics to obtain the Michaelis constant for the reaction of cloxacillin catalyzed by penicillinase. A value of 238 μM was obtained for the Michaelis constant at pH 5.50 and 27°C.

IV. REFERENCES

1. R. H. Depue, A. G. Moat, and A. Bondi, "Relation between penicillin Structure and Penicillinase Activity", Arch. Biochem. Biophys., 107, 374-381 (1964).

2. J. M. T. Hamilton-Miller, J. T. Smith, and R. Knox, "The Estimation of Penicillins and Penicillin Destruction", J. Pharm. Pharmacol., 15, 81-91 (1963).

3. David A. Wolff, and Morton Hamburger, "Evaluation of Some Currently Used Methods for Determining Staphylococcal Penicillinase", J. Lab. Clin. Med., 59, 469-480 (1962).

4. Richard Henry and Riley D. Housewright, "Studies on Penicillinase. II. Manometric Method of Assaying Penicillinase and Penicillin Kinetics of Penicillin-Penicillinase Reaction, The Effect of Inhibitors on Penicillinase", J. Biol. Chem., 167, 559-571 (1947).

5. Robert G. Benedit, William H. Schmidt, and Robert D. Coghill, "Penicillin. VII. Penicillinase", Arch. Biochem., 8, 377-383 (1945).

6. R. B. Woodward, A. Neuberger, and N. R. Trenner in "The Chemistry of Penicillin", Hans T. Clarke, John R. Johnson, and Sir Robert Robinson, Eds., Princeton Univ. Press, Princeton, N.J. (1949) p 419.

7. R. G. Tucker, "A method for Determination of Penicillinase", Nature, 173, 85 (1954).

8. C. J. Perret, "Iodometric Assay of Penicillinase", Nature, 174, 1012-1013 (1954).

9. N. Citri, "Two Antigenically Different States of Active Penicillinase", Biochim. Biophys. Acta, 27, 277-281 (1958).

10. R. R. Goodwell and Roseda Davis, "Automatic Determination of Penicillin in Fermentation Both - A Improved Iodometric Assay", Analyst., 86, 326-335 (1961).

11. R. P. Novick, "Micro-Iodometric Assay for Penicillinase", Biochem. J., 83, 236-240 (1962).

12. Andres Ferrari, Frank M. Russo-Alesi, and Jacques M. Kelly, "A Completely Automated System for Chemical Determination of Streptomycin and Penicillin in Fermentation Media", Anal. Chem., 31, 1710-1717 (1959).

13. W. W. Cleland, "The Statistical Analysis of Enzyme Kinetic Data", Adv. Enzymol., 29, 1-32 (1967).

14. Gabor B. Levy, "A Unit of Penicillinase", Nature, 166, 740-741 (1950).

15. Nathan Citri and R. Pollock, "The Biochemistry and Function of β-Lactamase (Penicillinase)",' Adv. Enzymol., 28, 237-323 (1966).

16. H. W. Florey, E. Chain, N. G. Heathley, M. A. Jenning, M. A. Sanders, E. P. Abraham, and M. E. Florey, "Antibiotics, Vol. II", Oxford Univ. Press, London, Eng. (1949), pp 747-748.

17. Robert C. Weast, "Handbook of Chemistry and Physics, 50th Edition", The Chemical Rubber Co., Cleveland, Ohio, (1969), p D-119.

18. J. Leyden Webb, "Enzyme and Metabolic Inhibitors, Vol. I", Academic Press, New York, N. Y. (1963), pp 819-823.

19. Margot Kogut, M. R. Pollock, and E. J. Tridgell, "Purification of Penicillin-Induced Penicillinase of Bacillus cereus NRRL 569: A Comparison of Its Properties with those of Similarly Purified Penicillinase Produced Spontaneously by Constitutive Mutant Strain", Biochem. J., 62, 391-401 (1956).

20. M. R. Pollock and M. H. Richmond, "Low Cyst(e)ine Content of Bacterial Extracellular Proteins: Its Possible Physiological Significance", Nature, 197, 446-449 (1962).

21. Malcolm Dixon and Edwin C. Webb, "Enzymes", Academic Press, New York, N. Y. (1964), p 144.

22. Sidney Riegelman and Eliezer Z. Fischer, "Stabilization of Epinephrine against Sulfite Attack", J. Pharm. Sci. 51, 206-210 (1962).

23. Nathan Citri and Nachman Garber, "The Effect of Urea on the Activity of Penicillinase", Biochem. Biophys. Acta, 30, 664-665 (1958).

24. J. E. Banfield, "Kinetics of Penicillinase Activity", Experientia, 13, 403-404 (1957).

25. D. E. Koshland, Jr., "The Active Site and Enzyme Action", Adv. Enzymol., 22, 45-97 (1960).

26. Joseph P. Hou and John W. Poole, "The Amino Acid Nature of Ampicillin and Related Penicillins", J. Pharm. Sci., 58, 1510-1515 (1969).

27. Naomi Zyk and N. Citri, "The interaction of Penicillinase with Penicillins. V. Conformative Response Constants", Biochem. Biophys. Acta, 146, 219-226 (1967).

APPENDIX

The Statistical Analyses of Enzyme Kinetic Data Obtained from a Single Continuous Recording.

I. INTRODUCTION

The rate expression for an enzymatic reaction which follows Michaelis-Mentern Kinetics is represented by a rectangular hyperbola of the form:

$$v = \frac{V_{max}[S]}{K_m + [S]} \tag{1}$$

where v is the velocity, [S] is the substrate concentration, K_m is the Michaelis constant, and V_{max} is the maximum velocity. Since the relation between the dependent variable, v, and the independent variable, [S], is curvelinear, it is usually rearranged into one of the following three linear forms.

$$\frac{1}{v} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}} \frac{1}{[S]} \tag{2}$$

$$\frac{[S]}{v} = \frac{K_m}{V_{max}} + \frac{1}{V_{max}} [S] \tag{3}$$

$$v = V_{max} - K_m \frac{v}{[S]} \tag{4}$$

The statistical analysis of enzyme kinetic data has been well outlined by Wilkinson[1] and Johansen and Lumry.[2] Wilkinson's paper[1] covers the basic statistical principles and applied them to the regression analysis of the first two linear transformation, eqs. 2 & 3, and applied Gauss-Newton's method of curve fitting to eq. 1. He only considered the case when there was no error in the independent variable, [S].

Johanson and Lumry[2] considered the same three equations but also considered the case when both the independent and dependent variables were in error. Using the method of

Lagrangian multipliers as outlined by Deming[3] they arrived at the following weight expression for a linear line when both axes are in error.

$$W_i = \frac{\sigma^2}{b_1^2\,\sigma^2(Q_i) + \sigma^2(Y_i)} \qquad (5)$$

where σ^2 is an arbitrary scaling factor (independent of i) of the same dimension as Y; $\sigma^2(Q_i)$ is the variance in the dependent variable, $\sigma^2(Y_i)$ is the variance in the dependent variable and b_1 is the slope of the line. Note that the weight expression contains a regression coefficient, b_1, and, therefore, an iteration technique is required to determine the regression coefficients.

Hey and Hey[4] outlined a method for the normal fitting of a rectangular hyperbola of the form: $(x - a)(y - b) = c$. This can be readily adapted to normal fitting the Michaelis-Menten equation by putting eq. 1 in the following form.

$$(v - V_{max})([S] + K_m) = K_m V_{max} \qquad (6)$$

Cleland[5] has applied the statistical methods outline by Wilkinson,[1] and Johansen and Lumry,[2] and added a variety of ideas of his own. He also presented a very neat Fortran program. However, he only considers the case when all the error was in the velocity measurement.

Bliss and James[6] considered the case for fitting a rectangular hyperbola of the form

$$Y = a + b\left\{\frac{1}{x + d}\right\} \qquad Y = a + b\left\{\frac{x}{x + d}\right\}$$

When a = 0, the second form is the same as the eq. 1. They use what they called an auxiliary variable which is the second term in a Taylor series expansion and therefore is the same method for curve fitting used by Wilkinson,[1] Johansen and Lumry,[2] and Cleland.[5] They only considered the case for an error free independent variables. Hanson, Ling and Havir[7] wrote a Fortran program using the procedure described by Bliss and James.[6]

Dowd and Riggs[8] use the digital computer to generate 500 replicate computer experiments to estimate K_m and V_{max} using the three linear transformation, eqs. 2, 3, & 4. They generated normal random deviation in the initial velocities for 5 fixed substrate concentrations and plotted the histogram for the estimated K_m and V_{max} values from the three plots. They showed that the Lineweaver-Bulk method of plotting (eq. 2) gave the greatest variation in the calculation of the kinetic parameters.

In the Fortran program presented here, much of the notation and the matrix solution from Cleland's program[5] is used. This program is based on the analysis of a single continuous curve, where both the concentration and the velocity variables are in error. Three methods of analysis are used; the integrated equation, the Gauss-Newton method of curve fitting which has been outlined by Wilkinson,[1] and Johansen and Lumry,[2] and programed by Cleland;[5] and the method outlined by Hey and Hey[4] for normal fitting a retangular hyperbola.

II. GENERAL METHOD OF WEIGHTED LEAST-SQUARES.

Let eq. 7 be an estimate of the true regression function

$$Y = f(Q_1, Q_2, \ldots Q_n, b_1, b_2, \ldots b_n) \qquad (7)$$

where Y is the dependent variable or some tranformation of the dependent variable, $Q_1, Q_2, \ldots Q_n$ are the independent variables or some tranformation of the independent variables, and $b_1, b_2, \ldots b_n$ are the regression coefficients. The principle of weighted least square is to adjust the regression coefficients so that the weighted sum of squared residuals, eq. 8, is a minimum.

$$\sum_{i=1}^{NP} W_i res_i^2 = \sum_{i=1}^{NP} W_i (Y_i - f_i(Q_1, \ldots b_1, \ldots))^2 \qquad (8)$$

where W_i is the weight given to the i^{th} observation, Y_i and $Q_1, Q_2, \ldots Q_n$ are the dependent and independent variables or some transformation of them for the i^{th} observation, and $b_1, b_2, \ldots b_n$ are the regression coefficients which are to be minimized so that the weight for observed data variables, are a minimum. The summation is over all NP plotting points.

Weights - The relative weights for observed data variables, y and x, are inversely proportional to the variances, that is $w_y = 1/\sigma^2(y)$, and $w_x = 1/\sigma^2(x)$. If the Q variable is some function of the observed data elements, $x_1, x_2, \ldots x_m$, then according to the theory of propagation of error,[9] the variance in $Q(x_1, x_2, \ldots x_m)$ can be approximated by

$$\sigma_i^2(Q) = \sum_{j=1}^{m} \left(\frac{\partial Q}{\partial x_j}\right)_i^2 \sigma_i^2(x_j) \qquad (9)$$

where $\sigma_i^2(x_j)$ is the variance of the j^{th} observed data element for the i^{th} observation.

The overall weight expression used in eq. 8 denotes how the residual varies with the errors in all the observed data variables. Deming[10] using the method of Lagrangian multipliers arrived at the following expression, eq. 10, for the weight to be used for a single regression function that is linear in the regression coefficients.

$$\frac{\sigma^2}{W_i} = \left(\frac{\partial res}{\partial Q_1}\right)_i^2 \sigma_i^2(Q_1) + \left(\frac{\partial res}{\partial Q_2}\right)_i^2 \sigma_i^2(Q_2) + \ldots\ldots + \left(\frac{\partial res}{\partial Y}\right)_i^2 \sigma_i^2(Y) \qquad (10)$$

where σ^2 is a scaling factor, $\left(\frac{\partial res}{\partial Q_1}\right)_i^2$, $\left(\frac{\partial res}{\partial Q_2}\right)_i^2$ and $\left(\frac{\partial res}{\partial Y}\right)_i^2$ are evaluated for the i^{th} observation and $\sigma_i^2(Q_1)$, $\sigma_i^2(Q_2)$ and $\sigma_i^2(Y)$ are the variances for Q_1, Q_2, and Y, respectively. If the Q's or Y are transformations of the observed data elements, then eq. 9 is used to obtain their variances.

Adjusting the regression coefficient to minimize the weighted sum of square residuals - For the application of the least square technique, the expression for Y must be linear in the regression coefficient, b_1, b_2, ... b_n, that is:

$$Y = b_1Q_1 + b_2Q_2 + \ldots\ldots + b_nQ_n \qquad (11)$$

If b_1 is a regression coeficient for an intercept, then $Q_1 = 1$. A necessary condition for eq. 8 to be a minimum is that the partial derivatives of the weighted sum of squared residuals with respect to the regression coefficient, b_1, b_2, ... b_n,

should be zero. Substituting eq. 11 into eq. 8 and differentiating with respect to the regression coefficient yeilds n + 1 equations of the form:

$$\frac{\partial}{\partial b_j} \Sigma W (Y - b_1 Q_1 - b_2 Q_2 - .... - b_n Q_n)^2 = 0$$

or

$$- 2 \Sigma W (Y - b_1 Q_1 - b_2 Q_2 - .... - b_n Q_n) Q_j = 0$$

To simplify the notation, the i index is drop. When the index is dropped on a summation, it is to be taken over all observations.

The n + 1 regression coefficients can be obtained by solving these n + 1 simutaneous equations by a matrix method. Dividing through by 2 and redefining Y as Q_{n+1} the following argument matrix is obtained.

$$\left|\begin{array}{cccc|c} \Sigma W Q_1 Q_1 & \Sigma W Q_1 Q_2 & ..... & \Sigma W Q_1 Q_n & \Sigma W Q_1 Q_{n+1} \\ \Sigma W Q_2 Q_1 & \Sigma W Q_2 Q_2 & ..... & \Sigma W Q_2 Q_n & \Sigma W Q_2 Q_{n+1} \\ ... & ... & ..... & ... & ... \\ \Sigma W Q_n Q_1 & \Sigma W Q_n Q_2 & ..... & \Sigma W Q_n Q_n & \Sigma W Q_n Q_{n+1} \end{array}\right|$$

The method of inverting and solving this matrix is taken directly from Cleland's program.[5] The notation for the argumented matrix is simplified by defining:

$$S_{jk} = \Sigma W Q_j Q_k \qquad (12)$$

The argumented matrix can now be written as

$$\left|\begin{array}{cccc|c} S_{11} & S_{12} & ..... & S_{1n} & S_{1,n+1} \\ S_{21} & S_{22} & ..... & S_{2n} & S_{2,n+1} \\ .. & .. & ..... & .. & ... \\ S_{n1} & S_{n2} & ..... & S_{nn} & S_{n,n+1} \end{array}\right|$$

The matrix method from Cleland's program[5] is then used to solve the argument sums of squares and cross product matrix for the regression coefficients. During this process, the coefficient matrix was inverted.

Calculation of standard errors for the regression coefficients - When the matrix method is used to calculate the regression coefficient, the variance-covariance matrix is obtained by multiplying the inverse matrix by σ^2, that is

$$\begin{vmatrix} \text{var}(b_1) & \text{cov}(b_1,b_2) & \cdots & \text{cov}(b_1,b_n) \\ \text{cov}(b_2,b_1) & \text{var}(b_2) & \cdots & \text{cov}(b_2,b_n) \\ \cdots & \cdots & \cdots & \cdots \\ \text{cov}(b_n,b_1) & \text{cov}(b_n,b_2) & \cdots & \text{var}(b_n) \end{vmatrix} = \begin{pmatrix} \text{INVERSE} \\ \\ \text{MATRIX} \end{pmatrix} \times \sigma^2$$

where σ^2 is the weighted sum of the squared residuals divided by the degrees of freedom, the number of data points minus the number of regression coefficients to be determined.

$$\sigma^2 = \frac{\sum W \cdot (Y - f(Q_1, Q_2, \ldots Q_n \cdot b_1, b_2, \ldots b_n))^2}{NP - n}$$

For a linear plot, $y = b_1 + b_2 x$, σ^2 can also be obtained by:

$$\sigma^2 = S_{xy} - b_2^2 S_{xx} = S_{xy} - \frac{S_{xy}^2}{S_{xx}}$$

where $S_{xy} = \Sigma Wxy - (\Sigma Wx)(\Sigma Wy)/\Sigma W$

$S_{xx} = \Sigma Wx^2 - (\Sigma Wx)^2/\Sigma W$

$S_{yy} = \Sigma Wy^2 - (\Sigma Wy)^2/\Sigma W$

and the correlation coefficient, is given by:

$$R_{xy} = \frac{S_{xy}}{\sqrt{S_{xx}}\,\sqrt{S_{yy}}}$$

III. THE INTEGRATED MICHAELIS-MENTEN EQUATION.

The velocity is equal to the rate of disappearance of the substrate, so the Michaelis-Menten equation can by written as:

$$-\frac{d[S]}{dt} = \frac{V_{max}[S]}{K_m + [S]}$$

$$-V_{max}\int_0^t dt = K_m\int_{S_0}^{[S]}\frac{d[S]}{[S]} + \int_{S_0}^{[S]} d[S]$$

integrating and rearranging

$$S_0 - [S] = V_{max}t - K_m \ln\frac{S_0}{[S]} \qquad (13)$$

This equation is of the form: $Y = b_1Q_1 + b_2Q_2$

where $Y = S_0 - [S]$

$Q_1 = t$

$Q_2 = -\ln\frac{S_0}{[S]}$

$b_1 = V_{max}$

$b_2 = K_m$

Theoretically, the method of least-square should not be applied to the integrated equation since there is correlation between the Y and Q_2 coordinates, that is $Y = Y(S_0, [S])$ and $Q_2 = Q_2(S_0, [S])$. However, the method of least-squares does give good estimates for K_m and V_{max}. The effect of several possible weight expressions were investigated and the results form this investigation are given in Table I.

If it is assumed that there is no correlation between the coordinates, then the weight expression is given by eq. 10.

If the variance for all concentration determinations are assumed to be the same and that the variance for t is so small that it can be neglected, then,

$$\sigma^2(Q_1) = 0$$

$$\sigma^2(Y) = \sigma^2([S]) + \sigma^2(S_0) = 2\,\sigma^2([S])$$

$$\sigma^2(Q_2) = \sigma^2(\ln S_0) + \sigma^2(-\ln[S])$$

Applying eq. 9 to $\sigma^2(Q_2)$ yields

$$\sigma^2(Q_2) = \frac{\sigma^2(S_0)}{S_0^2} + \frac{\sigma^2([S])}{[S]^2} \qquad (14)$$

and eq. 10 becomes

$$\frac{\sigma^2}{W} = K_m^2 \left(\frac{1}{S_0^2} + \frac{1}{[S]^2} \right) \sigma^2([S]) + 2\,\sigma^2([S])$$

Solving for W

$$W = \frac{1}{K_m^2(1/S_0^2 + 1/[S]^2) + 2} \cdot \qquad (15)$$

Since σ^2 is a scaling factor, and $\sigma^2([S])$ is constant, $\sigma^2/\sigma^2([S])$ is constant and is just another scaling factor which cancels out in the calculation of the regression coefficients and their standard errors. For this reason, $\sigma^2/\sigma^2([S])$ was set equal to one.

Another possible weight expression was obtained by considering how the residual varied with error in [S] and S_0.

$$\frac{\sigma^2}{W} = \left(\frac{\partial res}{\partial [S]} \right)^2 \sigma^2([S]) + \left(\frac{\partial res}{\partial S_0} \right)^2 \sigma^2(S_0)$$

where $res = S_0 - [S] + K_m \ln S - K_m \ln[S] - V_{max} t$

By differentiating and setting $\sigma^2/\sigma^2([S])$ equal to one, the following weight expression is obtained for the integrated equation.

$$W = \frac{1}{K_m^2(1/S_0^2 + 1/[S]^2) + 2K_m(1/S_0 + 1/[S]) + 2} \qquad (16)$$

Since the observed variables, concentration and time are not coordinates of the plot, but some transformation of them, these weight expressions are used in the least-squares analysis to adjust for this transformation.

The catalytic rate constant in the Michaelis-Menten enzymatic kinetics is a first order rate constant for the break down of [ES]. Therefore it makes no difference what point on the curve is picked as the starting point. In this program, all possible combinations of [S] were made, that is for NP observation, there was $\binom{NP}{2} = \frac{NP(NP - 1)}{2}$ plotting points. There is a large correlation between these plotting points, so if $\frac{NP(NP - 1)}{2} - 2$ was used for the degrees of freedom, the standard errors would be underestimated. If only the first observed point were combined with the remaining observed points, then there would be NP - 3 degrees of freedom If making all possible combinations were considered as an averaging process, and that only NP - 2 degrees of freedom for calculation 2 regression coefficient from NP data points, then $\frac{(NP - 1)(NP - 2)}{2}$ would be the number of degrees of freedom which could be obtained by making all possible combinations.

After having obtained an expression for the weight and degrees of freedom, the weighted least-squares procedure outline in part 2 of this appendix was now applied. With $Q_1 = t$, $Q_2 = -\ln(S_0/[S])$ and $Q_3 = S_0 - [S]$, the S matrix was set up, solved, and inverted with $S_{11} = V_{max}$ and $S_{21} = K_m$ and standard error estimates of V_{max} and K_m are equal to $S_{12}^{-1} \times \sigma^2$ and $S_{23}^{-1} \times \sigma^2$ respectively.

The regression coefficients, K_m and V_{max}, just calculated were substituted back into the integrated equation, and the concentration was then calculated at each time, t. To accomplish this, the Michaelis-Menten equation, eq. 1, was integrated without limits.

$$[S] = - K_m \ln[S] + V_{max}t + C \qquad (17)$$

where C is the integration constant. The method of least-squares was used to evaluate this constant. The values for K_m, V_{max} and $[S]_i$ for i = 1, 2, ... NP are known. Therefore C is the only regression coefficient to be determined. By differentiating the unweighted sum of squared residuals and setting this expression equal to zero, the following expression for C was obtained.

$$C = \frac{\Sigma([S] + K_m \ln[S] - V_{max}t)}{NP} \qquad (18)$$

After having evaluated the integration constant, Newton's method for determining roots of an equation, eq. 23, was now applied to solve for [S] at each time, t.

$$[S]_{n+1} = [S]_n - \frac{f([S]_n)}{f'([S]_n)} \qquad (19)$$

where $[S]_n$ is the n^{th} guess for the concentration at time, t; $[S]_{n+1}$ would then be the next approximation for the concentration, $f([S]_n)$ is eq. 21 evaluated using $[S]_n$ as the value of [S]; and $f'([S]_n)$ is eq. 21 differentiated with respect to [S] and evaluated by substituting $[S]_n$ value for [S]. The observed value for [S] at t is used as the initial guess for [S]. Newton's method was then applied to obtain the next approximation, these approximation was then substituted back into Newton's method to obtain a second approximation for [S]. This iteration technique was continued until $\left|\frac{[S]_{n+1} - [S]_n}{[S]_{n+1}}\right| < \varepsilon$, where ε is the maximum relative error tolerated. The final iteration value for [S] obtained from Newton's method was then substituted into eq. 1 to obtain a value for v.

The standard deviation of concentration, $\sigma([S])$ and the standard deviation of the velocity $\sigma(v)$ were determined by the following formulas.

$$\sigma([S]) = \sqrt{\frac{([S]_{obs} - [S]_{calc})^2}{NP - 1}} \qquad (20)$$

$$\sigma(v) = \sqrt{\frac{(v_{obs} - v_{calc})^2}{NP - 1}} \qquad (21)$$

where $[S]_{obs}$ and v_{obs} are the observed data point and $[S]_{calc}$ and v_{calc} are the concentration predicted from Newton's method and the velocity calculated by subsituting $[S]_{calc}$ into eq. 1. The standard deviations were used to generate random points in part 6 of this appendix.

IV. GAUSS-NEWTON'S METHOD FOR DIRECT CURVE FITTING THE MICHAELIS-MENTEN EQUATION.

This method has been discussed in several papers[1,2,5,6] and computer programs have been given by two authors.[5,7] Cleland's program[5] has the advantage that it calculates the initial guess by using a weighted Lineweaver-Burk plot, while Hanson, Ling and Havir[7] requires an initial guess for K_m. Both programs deal with the situation in which the concentration is known exactly, but are readily converted to the bivariate situation by using the weighting factor given by Johansen and Lumry.[2]

$$W = \frac{\sigma^2}{\left(\frac{V_{max}^2 K_0^2}{(K_0 + [S])^4}\right)\sigma^2([S]) + \sigma^2(v)} \qquad (22)$$

where K_0 is an initial guess for K_m, σ^2 is a scaling factor, $\sigma^2([S])$ and $\sigma^2(v)$ are the variances of the concentration and velocity.

The method is essential as follows. Eq. 1 is linear in V_{max} but not in K_m. To make eq. 1 linear in K_m, expand eq. 1 in Taylors series about an initial guess for K_m which is called K_0.

$$v = \left(\frac{[S]}{K_0 + [S]}\right)V_{max} - \left(\frac{[S]}{(K_0 + [S])^2}\right)V_{max}(K_m - K_0) \qquad (23)$$

This equation is of the form: $Y = b_1Q_1 + b_2Q_2$

where $Q_1 = \frac{[S]}{K_0 + [S]}$, $Q_2 = \frac{[S]}{(K_0 + [S])^2}$, $b_1 = V_{max}$, and

$b_2 = V_{max}(K_0 - K_m)$.

The value for K_m obtained form the integrated equation is used as an initial guess, K_0, and the next successive guess is obtained by: $K_m = K_0 + b_2/b_1$

The iteration is continued until $\left|\frac{K_m - K_0}{K_m}\right| < \varepsilon$

After the iteration process has been completed, the second term becomes so small that it can be neglected leaving eq. 1.

V. HEY AND HEY'S METHOD OF NORMAL FITTING A RECTANGULAR HYPERBOLA.[4]

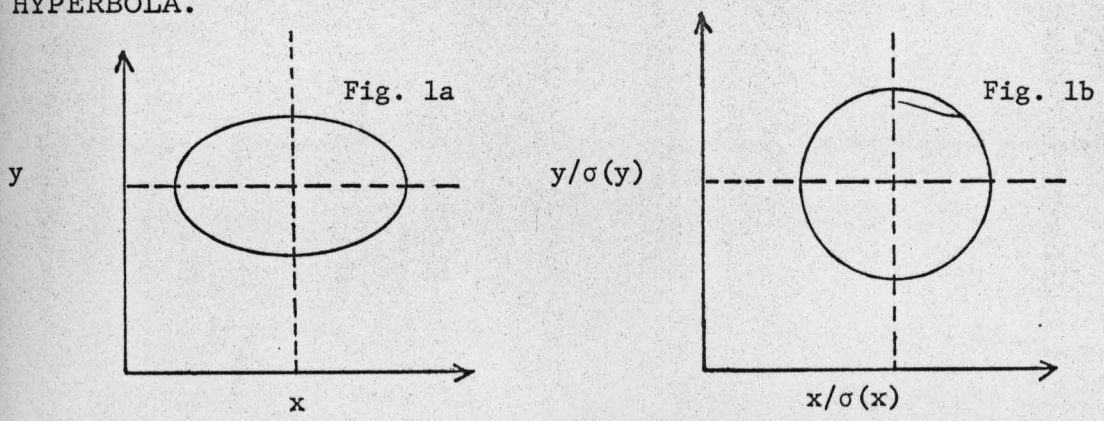


Fig. 1a shows a schematic representation of standard error contour for a system whose axes are not normalized in terms of their standard error. By normalizing the axes in terms of their standard error, this elliptical contour is converted into a circular contour (Fig. 1b).

Hey and Hey[4] used a geometric method to normal fit the rectangular hyperbola curve (Fig. 2). Consider the triangle CFP, if PT and PC are considered as the altitudes of this triangle with CF and PF as their respective bases, then the area of the triangle = 1/2 PT x CF = 1/2 PC x PF, therefore

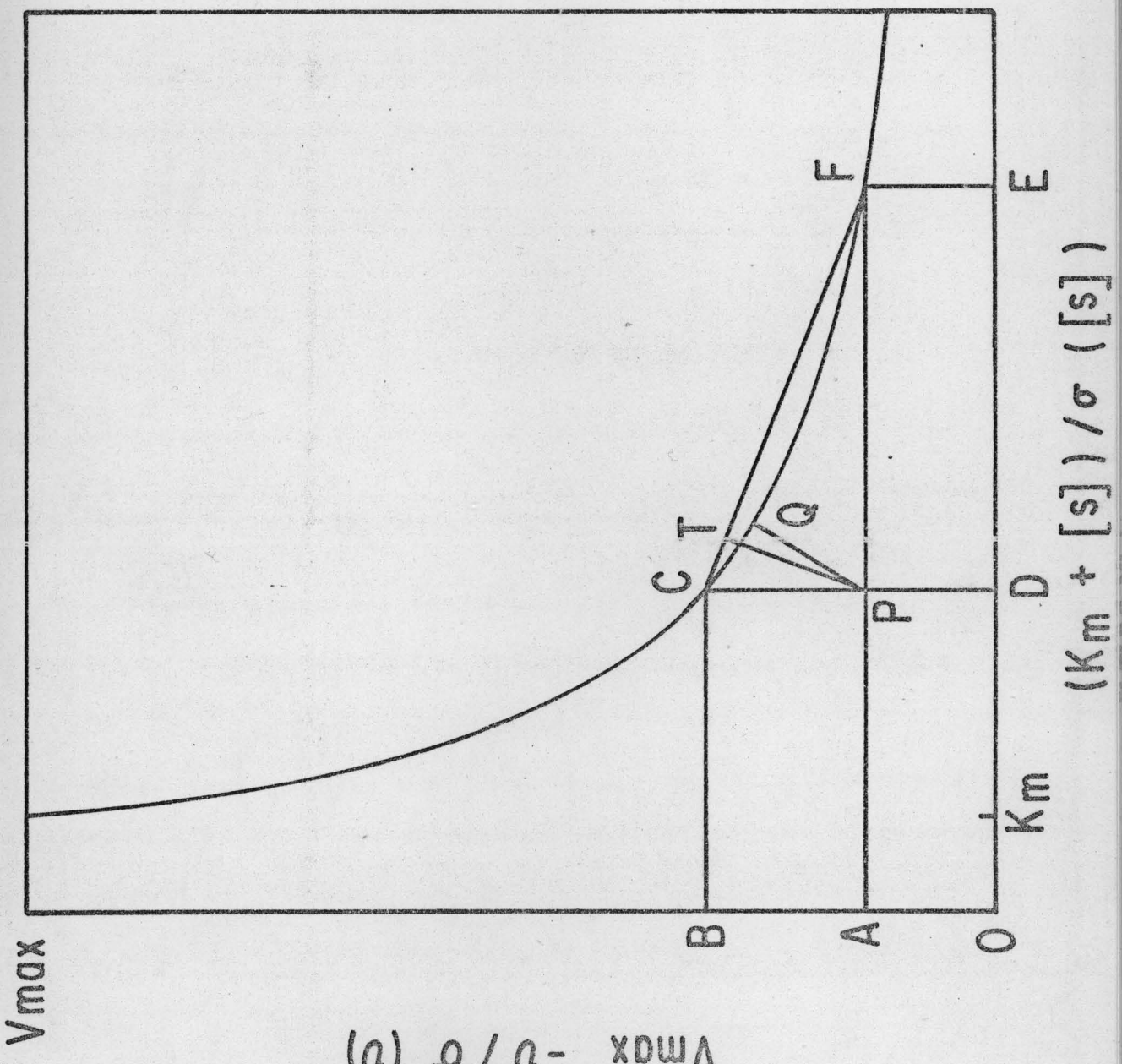
$$PT^2 = \frac{PC^2 \times PF^2}{CF^2}$$

Also $CF^2 = PC^2 + PF^2$, that is, the sum of the squares of the two sides of a right triangle equal the square of the hypotenuse. Now PT which is an approximation for PQ, the normal to the curve is related to PC and PF by:

$$PT^2 = \frac{PC^2 \times PF^2}{PC^2 + PF^2}$$

Now an expression for PC and PF are needed in terms of the residuals. From eq. 6, it can be seen that the area

Fig. 2 - A Schematic Diagram of Hey and Hey's Geometric Method for Normal Fitting of the Michaelis-Menten Equation.



for the retangulars form for all the points on the curve using y axis and V_{max} asymptotic line and lines parallel to these passing through the point on the curve are equal, that is,

$$K_m V_{max} = AF \times EF = BC \times DC = \text{etc.}$$

The normalized residual is equal to

$$res/\sigma(v)\cdot\sigma([S]) = AP \times DP - AF \times EF = - PF \times DP$$

$$= AP \times DP - AP \times PC = - AP \times PC$$

Since $DP = (V_{max} - v)/\sigma(v)$ and $AP = (K_m + [S])/\sigma([S])$

$$res^2 = PF\cdot(V_{max} - v)/\sigma^2(v) = PC\cdot(K_m + [S])/\sigma^2([S])$$

Now the expression for PT^2 which approximates PQ^2 becomes

$$PQ^2 \simeq PT^2 = \frac{res^2}{(K_m + [S])^2\cdot\sigma^2(v) + (V_{max} - v)^2\cdot\sigma^2([S])}$$

Define $F^2 = (K_m + [S])^2\cdot\sigma^2(v) + (V_{max} - v)^2\cdot\sigma^2([S])$

In order to normal fit this rectangular hyperbola, $\left(\frac{res}{F}\right)^2$ is to be minimized rather than res^2.

These same results can be obtained by using Johansen and Lumry's[2] expression for the weight factor for the Lineweaver-Burk method of plotting, eq. 2.

Let $Q_1 = 1$, $Q_2 = 1/[S]$, $Y = 1/v$, $b_1 = 1/V_{max}$, $b_2 = K_m/V_{max}$

and $$W = \frac{\sigma^2}{(K_m^2/V_{max}^2)\cdot\sigma^2([S])/[S]^2 + \sigma^2(v)/v^4}$$

The weighted sum of squared residuals is then,

$$\sum\left(\frac{\sigma^2\cdot v^2\cdot[S]^2\cdot V_{max}^2\cdot\left(\frac{1}{v} + \frac{K_m}{V_{max}}\frac{1}{[S]} - \frac{1}{V_{max}}\right)^2}{\left(K_m\frac{v}{[S]}\right)^2\cdot\sigma^2([S]) + \left(V_{max}\frac{[S]}{v}\right)^2\cdot\sigma^2(v)}\right)$$

From eq. 1, $\frac{[S]}{v} V_{max} = K_m + [S]$ and $\frac{v}{[S]} K_m = V_{max} - v$

Thus the weighted sum of squared residuals becomes:

$$\sum \left(\frac{\sigma^2 \cdot (v[S] + K_m v - V_{max}[S])^2}{(V_{max} - v)^2 \cdot \sigma^2([S]) + (K_m + [S])^2 \cdot \sigma^2(v)} \right)$$

This is Hey and Hey's expression[4] for the sum of squared normal residuals. Thus Hey and Hey's geometric method[4] of normal fitting the rectangular hyperbola, is the same as bivariate regression analysis of the Lineweaver-Burk plot, eq. 2, using eq. 5 as an expression for the weight.

VI. COMPUTER GENERATED EXPERIMENTS TO ESTIMATE THE STANDARD ERRORS OF THE REGRESSION COEFFICIENTS WHEN VARIOUS WEIGHTING FACTORS WERE USED.

In order to determine the best method for analyizing the experimental data, the computer was used to generate theortical computer experiments. Values of 30×10^{-6} M and 3×10^{-7} M-sec^{-1} were assigned as the true values for K_m and V_{max} respectively. For a particular time, t, Netwon's method for determining roots of an equation, eq. 23, was used to determine the true concentrations, [S]°. Fifteen points were determined. These points were equally spaced 60 seconds on the time scale starting at a concentration of 150×10^{-6} M (5 K_m). These true concentrations were then substituted into eq. 1 to determine the true velocities.

Hypothetical concentrations and velocities were then generated by adding a random error to the true concentrations and velocties.

$$[S] = [S]^\circ + \sigma([S]) \times RMS(I)$$

$$v = v^\circ + \sigma(v) \times RMS(I)$$

where [S]° and v° are the true concentration and velocity, $\sigma([S])$ and $\sigma(v)$ are the standard deviation of the concentration and velocity, eqs. 24 & 25, and RMS(I) is a computer function for generating random normal distributed numbers. The standard deviation of the concentration and velocity was estimated to be 3×10^{-7} M and 7.5×10^{-9} M sec^{-1} by averaging 41 experimental curves. One thousand computer experiments were carried out, 15 random concentrations and velocities were generated for each computer experiment. The computer

experiments were analyzed by the integrated equation, Gauss-Newton's method of curve fitting, and Hey and Hey's method of normal fitting. The Fortran program No. 1 in the next section was modified so that just the regression coefficients were calculated. These values were plotted in a histogram and the standard deviation of the Michaelis constants and maximum velocities were determined. The results for the analysis of the same 1000 computer experiments using various weighting factors are given in Table I. A weighting factor equal to one is equivalent to doing a unweighted least squares. The other weighting factors were used to account for the transformation that the observed data undergoes, but there was no weight given to individual data points since the standard errors are assume to be the same for all observation. For the integrated equation, three weighting factors were investigated. In the first weighting factor, all the error was considered to be in $\ln(S_0/[S])$; the second weighting factor, eq. 15, assume that there is no correlation between $(S_0 - [S])$ and $\ln(S_0/[S])$, and the third weighting factor, eq. 16, was obtained by considering how the residual varied with error in [S] and S_0.

The second weight expression, eq. 15, for the integrated equation gave the smallest standard error and range for both the Michaelis constants and maximum velocities, therefore, this weight expression was used in the least squares analysis of the integrated equation. When the weight expression, eq. 22, was used in the Gauss-Newton mentod, there was no significant

TABLE I.

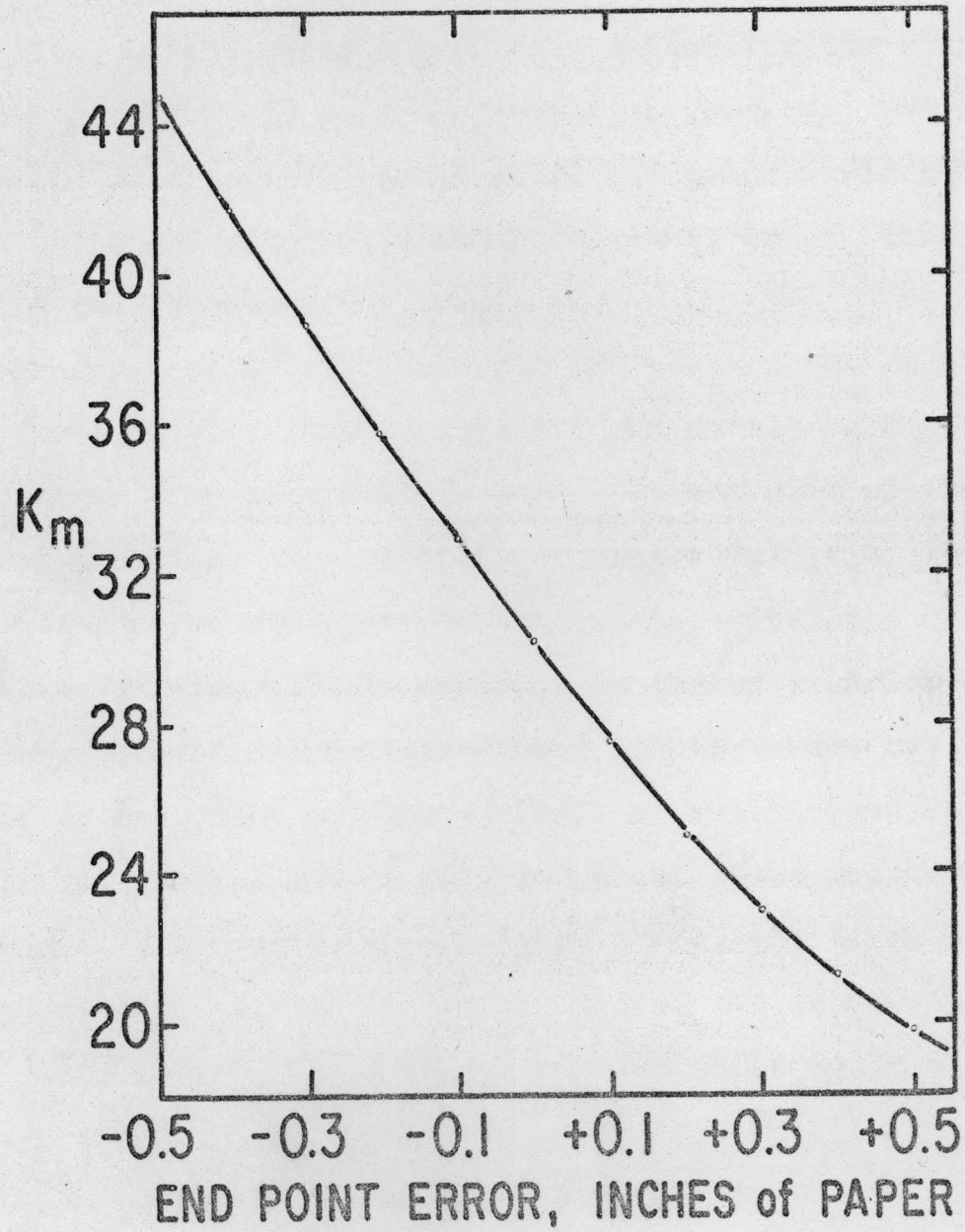
Statistical Summary of Results for Random Error in Each Concentration and Velocity Within a Single Computer Experiment. True $K_m = 30 \times 10^{-6}$ M and $V_{max} = 3 \times 10^{-7}$ M sec^{-1}

Weight Expression Used in the Regression Analysis	$K_m \times 10^{+6}$ M			$V_{max} \times 10^{+7}$ M sec^{-1}		
	Mean	Standard Error	Range	Mean	Standard Error	Range
Integrated Equation						
1	29.92	1.35	25.5 - 34.4	2.991	0.045	2.84 - 3.13
$\frac{1}{K_m^2(1/S_0^2 + 1/[S]^2)}$	29.92	0.92	26.9 - 32.9	2.997	0.029	2.91 - 3.09
$\frac{1}{K_m^2(1/S_0^2 + 1/[S]^2) + 2}$	29.90	0.71	27.3 - 32.6	2.996	0.027	2.90 - 3.09
$\frac{1}{K_m^2(\frac{1}{S_0^2} + \frac{1}{[S]^2}) + 2K_m(\frac{1}{S_0} + \frac{1}{[S]}) + 2}$	29.90	0.85	27.3 - 32.8	2.996	0.027	2.90 - 3.10
Gauss-Newton Method						
1	29.96	2.27	23.6 - 37.4	3.001	0.073	2.76 - 3.21
$\frac{1}{\frac{V_{max}^2 K_m^2}{(K_m + [S])^4}\sigma^2([S]) + \sigma^2(v)}$	29.93	2.26	23.1 - 36.8	3.000	0.073	2.77 - 3.22
Hey and Hey Method						
$\frac{1}{(V_{max}-v)^2\sigma^2([S]) + (K_m+[S])^2\sigma^2(v)}$	29.07	2.21	22.25 - 38.4	2.973	0.072	2.75 - 3.21

difference in the standard error, however, the mean value of the Michaelis constants and the range appeared to have shifted to lower values, and that the mean value of the unweighted Gauss-Newton method was closer to the true mean. In Table I, it can be seen that the mean values of the Michaelis constant obtained in all the methods of analyses are less than the true value (30×10^{-6} M). All the histrogram plots of the Michaelis constant form the integrated equation and the Gauss-Newton method with all the different weighting expressions appeared to have a normal distribution. However, this did not appear to be true for Hey and Hey's method.[4] This histogram plot appeared to be screwed toward the upper values. Hey and Hey's method[4] is equivalent to bivariate fitting of the Lineweaver-Burk plot, eq. 2. These results are similar to those observed by Dowd and Riggs[8] in which they found the the histogram plot obtained from the Lineweaver-Burk plot was screwed toward the upper values when only the velocity was considered to be in error.

Beside a random error in determining the optical rotation (and thus the concentration) from a single continuous recording, there is a possibility of a constant error due to an error in selecting the end point of the reaction. A plot of the Michaelis constants determined from the integrated equation versus an end point error in chart reading units is shown in Fig. 2. The theoretical data used above to generate the computer experiments were converted to chart reading units (0.2 degrees full range) and analyzed by Fortran program

Fig. 3 - Shows the Effect of an Error in Selecting the End Point on the Least-Squares Estimate of the Michaelis Constant from the Integrated Equation.



at the end of this appendix. The chart paper readings were round off to three significant figures, thus inducing a small round off error and the values obtained for K_m and V_{max} were 30.11×10^{-6} and 3.003×10^{-7}. A positive end point error is defined here as an end point reading above the true end point reading on the chart paper, thus decreasing the change in optical rotation for each concentration reading. For a negative end point error, the observed end point would be below the true end point reading on the chart paper, thus increasing the concentration readings.

The presence of a relative large end point error (a chart reading error of 0.10 inch or greater) can easily be detected in the Fortran program presented here. In the plot of the integrated equation, $(S_0 - [S])/t$ versus $1/t(\ln(S_0/[S]))$ the end point error will cancels out of $(S_0 - [S])/t$ term, so the $1/t(\ln(S_0/[S]))$ variable is the only variable affected by the end point error. When [S] is large, the effect of an end point on this variable is small, however, for small [S], the effect of this end point error becomes significant. For a positive end point error, too large of end point reading, causes the lower part of the decreasing plot to swing upward; while a negative end point would cause a downward swing.

Another effect of an end point error is on the concentration residual plot as shown in Fig. 4. When there is no end point error, the concentration residual distributes randomly about zero due to round off errors. However, when

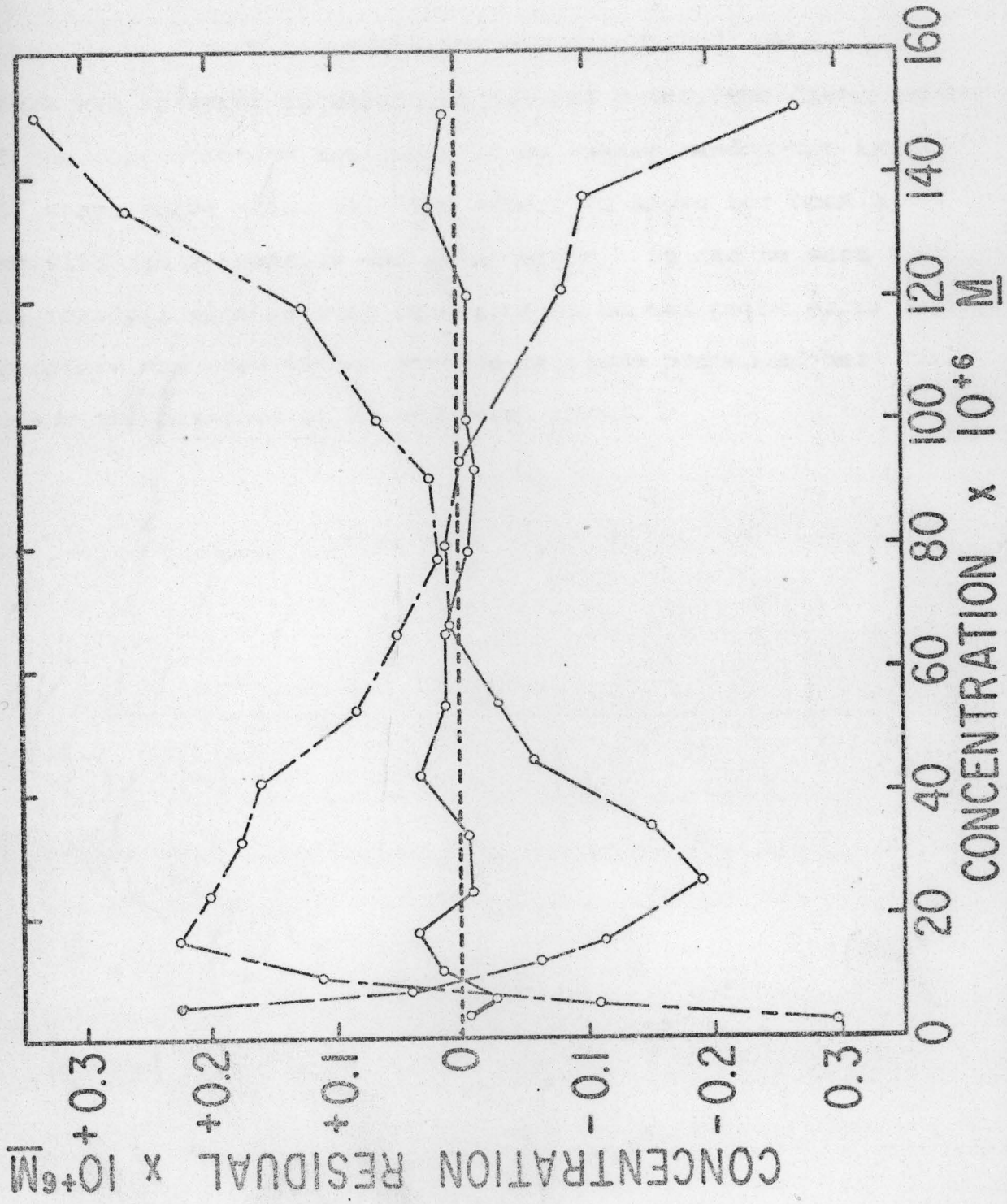


Fig. 4 - Shows the Effect of an Error in Selecting the End Point on the Concentration Residual Plot.

there was an error in selecting the end point, the distribution of the concentration residuals is no longer random but take a "S" shape curve (Fig. 3). The effect is shown for both a positive and a negative end point error. It can be seen that the residual plot is very sensitive to an end point error and therefore was used in the Fortran programs presented here to detect the presence of an end point error.

VII. THE FORTRAN PROGRAMS USED IN THIS STUDY FOR ANALYZING MICHAELIS-MENTEN ENZYME KINETIC DATA.

A. Description of Program No. 1.

The input data for the program are the readings taken from the continuous optical rotatory (or circular dichroism) curves obtained when penicillin is degraded by the enzyme, penicillinase. The input data (Fig. 4) are in chart reading units and converted to concentration and velocity readings. The converted data is then analyzed by the integrated equation, Gauss-Newton method and Hey and Hey's method.

In this Fortran program, both write and print statements are used for the output. Since a print statement was used first, all print statements will be printed out first, then the write statements. This allows a condensed summary of the output from all the curves analyzed to be printed out first (Fig. 5), followed by a more detailed output for each of the three different methods for analyzing the data from an individual curve (Fig 6-11).

Input - The input for this program consists of a title card in which the first two columns (column 1 & 2) are used to indicate the number of data points to be read in and any desired title is placed in the remaining columns (columns 3-80). The second card is for recording general information about the conditions of the reaction, and the information required to calculate a linear equation for the mutarotation baseline. In this and all data cards, no decimal points were typed in; they were aligned on the card (right justified) so that the computer would put the decimal point in the proper

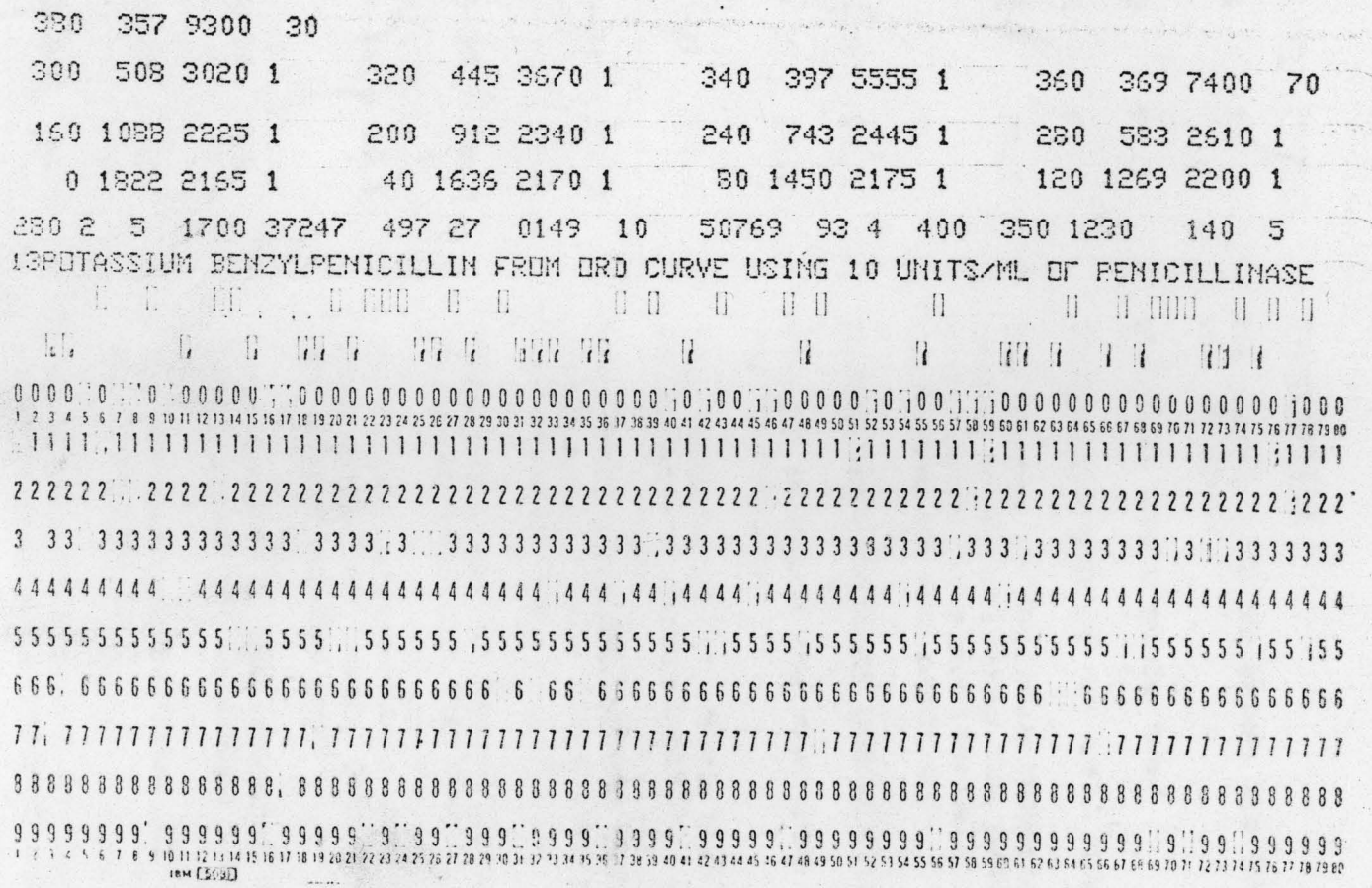


FIG. 4 - DATA DECK FOR PROGRAM NO. 1

place. The format for this general information card is given as follows.

Symbol	Column	Format	Discription
WAVLH	1-3	I3	The wavelength at which the curve was obtained
FRANGE	4-5	F2.1	The full range scale used
PATHLH	6-8	F3.1	The pathlength of the cell used
SPROT	9-14	F6.0	The change in specific rotation caused by hydrolyzing the β-lactam ring under the experimental condition of the reaction
MW	15-20	F6.2	Molecular weight of penicillin used
PH	21-25	F5.2	The pH at which the reaction was carried out
TEMP	26-29	F4.1	The temperature at which the reaction was carried out
IONIC	30-34	F5.4	The ionic strength of the penicillin solution in which the cleavage reaction was carried out
MICROL	35-38	F4.0	The microliter of penicillinase solution used for the hydrolysis
DATE	39-46	I4,I2,I2	The date the experiment was carried out; number of month, day, and last two digits of the year
NPAGE	47-50	I4	Page number in the notebook the information was recorded
NBOOK	51-52	I2	The notebook number the information was recorded in
BASEX	53-57	F5.0	The X axis of a point on the extrapolated mutarotation baseline
BASEY	58-62	F5.3	The corresponding Y axis for the same point on the mutarotaion baseline
BASEDX	63-68	F5.3	The delta x value for the slope of this baseline
SLOPE	69-73	F5.3	The corresponding delta Y value for the slope of the baseline

The remaining cards are the data cards. Four data points are put on each card. In columns 1-4, 21-24, 41-44, and 61-64 are placed the time readings in seconds. In columns 5-9, 25-29, 45-49, and 65-69 are placed the chart readings for the optical rotation (or circular dichroism). The Δx (time) coordinates for the slope of the curve are placed in columns 10-14, 30-34, 50-54, and 70-74 and the corresponding Δy (optical activity) coordinates are placed in columns 15-18, 35-38, 55-58, and 75-78. An example of the input deck is shown in Fig. 4

Arrays - Most of the symbols are the same as those used in Cleland's program[5] or are self explanatory except for the arrays. The "T" array is first used to store the time readings and represents time in the integrated equation calculation. In the Gauss-Newton method, the "T" array is then used (card 207) to store the predicted velocity for the velocity-concentration plot.

The "A" array is first used to store the chart readings (optical activity) taken from the curve. In card 29 of this program, these chart readings are converted to concentrations and are stored in the "A" array. The "A" array represents concentration throughout the remaining program.

The "W" array is first used for reading Δx portion of the slope, and to calculate the velocity in line 28. In line 31, the "W" array is used to store the logarithm of the concentration and represents the logarithm of concentration until line 103. From line 103 to 110, the "W" array is used to obtain the predicted value for the concentration from the integrated

equation using the estimated values of K_m and V_{max} obtained from the regression analyses of the integrated equation and Newton's method for approximating roots of an equation. The "W" array was again used in line 284 as Heys and Heys' F value.

The "V" array is used to read in the Δy portion of the slope and was converted to the velocity variable in line 28. The "V" array represented the velocity throughout the remaining program.

The "R" array is used to store the concentration residuals from the integrated equation (line 111) which is then used in the residual plot in line 334.

The "B" array is used to store K_m, V_{max}, and their standard errors which are printed out in the print statement.

The "X" and "Y" arrays are used for storing the dependent and independent variables of the integrated equation (line 70 and 71) which were obtained by making all possible combinations of the data points. These arrays are plotted in line 330. All combinations with the concentration at zero time are plotted as "+" while the remaining combinations are plotted as "X".

B. Description of Program No. 2.

This program uses only the integrated form of the Michaelis-Menten equation to obtain the reaction parameters, K_m and V_{max}. The input is essentially the same as program No. 1, except the Δx and Δy coordinates for the slope in the data card are not read in. These columns are just skip over when the data is read, hence they are usually just left blank. The notation is the same as that used in program No. 1.

C. Description of Program No. 3.

This program is an unweighted least-squares program for calculating the linear ionic strength effect. The relative error was considered to be the same for all Michaelis constants (or maximum velocities) rather than a standard error of equal magnitude in all the kinetic parameters. It can be shown that the weight required for the logarithmic transformation is cancelled by the weight for a constant relative error, thus reducing the weighted least-squares method to an unweighted least-squares method.

The input to this program consist of a title card in which the number of enzymatic kinetic parameters used for the least-squares analyses is put in columns 1 & 2 and any desired title in columns 3 through 80. This title card is followed by the data cards which were punched out either in program No. 1 or program No. 2. Only a single Michaelis constant and maximum velocity obtained from a single analyses of a curve is read into this program from one of these data cards.

PROGRAM NO. 1

```
C   ANALYSIS OF A SINGLE CONTINUOUS CURVE USING THE INTEGRATED EQUATION,
C   GAUSS-NEWTON'S METHOD, AND HEY  AND HEY'S METHOD

      DIMENSION  TITLE(13), T(30),A(30),W(30),V(30),R(30),Y(406),X(406),
     $WKM(3,20), VMAX(3,20), WVMAX(3,20), S(3,4), Q(3),SM(3),SS(3),B(12)
      REAL    IONIC(20),  MW, MICROL, KM(3,20), LOGK(30,3), KMZERO
      INTEGER   DATE(3), WAVELH
      EQUIVALENCE (R(1),LOGK(1,1)), (V(1),LOGK(1,2)), (A(1),LOGK(1,3))
      DATA  NSET, NGROUP, N, N1, N2 / 0, 0, 2, 3, 4 /
      CALL FLGEOF (5, NEOF)
    1 NUMBER = 0
      NGROUP = NGROUP + 1
      PRINT 7001
    2 NUMBER = NUMBER + 1
      IF (NUMBER .GT. 20)   NUMBER = 1
      NSET = NSET + 1
      READ (5,5002)  NP, (TITLE(K), K = 1, 13)
      IF (NEOF .EQ. 1)   STOP
      IF (NP .EQ. 1)   GO TO 1
      READ (5,5003)  WAVELH, FRANGE, PATHLH, SPROT, MW, PH, TEMP, IONIC(
     $NUMBER), MICROL, (DATE(K), K = 1, 3), NPAGE, NBOOK, BASEX, BASEY,
     $BASEDX, SLOPE, (T(K), A(K), W(K), V(K), K = 1, NP)
      WRITE (6,6004)   NSET, NGROUP, NUMBER, NP
      WRITE (6,6005)   (T(K), A(K), W(K), V(K), K = 1, NP)
      IF (NP  .GT.  30)    GO TO 2
      SLOPE = SLOPE/BASEDX
      BASEY = BASEY + SLOPE * BASEX
      IF (FRANGE .EQ. 0. .OR. PATHLH .EQ. 0. .OR. SPROT .EQ. 0.) GO TO 2
      FACTOR = 1000.*FRANGE/(PATHLH*SPROT*MW)
      DO 3 K = 1, NP
      V(K) = (V(K)/ W(K) - SLOPE) * FACTOR
      A(K)  = (A(K) - BASEY + SLOPE * T(K)) * FACTOR
      IF (A(K) .LT. 0.)    A(K) = 1.0 E-06
    3 W(K) = ALOG(A(K))
```

```
C
7001  FORMAT (//2X, 5HIONIC, 7X,19HINTEGRATED EQUATION, 2X, 16HFIT OF HY
     $PERBOLA, 2X, 19HMIN NORMAL RESIDUAL, 2X, 19HINTEGRATED EQUATION, 2
     $X, 16HFIT OF HYPERBOLA, 2X, 19HMIN NORMAL RESIDUAL / 1X, 8HSTRENGT
     $H, 2X, 3HRXY, 2X, 17H(KM+OR-S.E.)*E+06,2X, 17H(KM+OR-S.E.)*E+06, 2
     $X, 17H(KM+OR-S.E.)*E+06, 4X, 17H(VMAX+OR-SE)*E+07, 3X, 17H(VMAX+OR
     $-SE)*E+07, 2X, 17H(VMAX+OR-SE)*E+07 )
5002  FORMAT  (I2, 13A6)
5003  FORMAT  (I3, F2.1, F3.1, F6.0, F6.2, F5.2, F4.1, F5.4, F4.0, I4,
     $I2, I2, I4, I2, F5.0, F5.3, F6.1, F5.3  /    ( 4(F4.0, F5.3, F5.1,
     $ F4.2, 2X) ) )
6004  FORMAT (16H1DATA SET NUMBER, I3, 10X, 5HGROUP,I2, 3X, 6HNUMBER, I3
     $, 15X, I3, 12H DATA POINTS )
6005  FORMAT(/11H INPUT DATA / 4(6X, 4HTIME, 4X, 2HY2, 3X, 4HDELX, 3X, 4
     $HDELY  )  / (4(5X, F5.0, F7.3, F7.1, F6.2) ) )
C
C--------DETERMINING THE MICHAELIS CONSTANT USING THE INT  RATED EQUATIO
      ASSIGN 7 TO MM
      WRITE (6,6006)   (TITLE(K), K = 1, 13)
      WRITE (6,6007)  PH, FRANGE, TEMP, PATHLH, IONIC(NUMBER), SPROT,
     $WAVELH, MICROL, (DATE(K), K = 1, 3), NPAGE, NBOOK, BASEY, SLOPE
      KM(1,NUMBER) = 0.3E-04
      NT = 0
    4 NT = NT + 1
      XKM = KM(1,NUMBER)
      NCOUNT = 0
      SYY = 0.
      SUMW = 0.
      DO 5 J = 1, N2
      DO 5 K = 1, N1
    5 S(K,J) = 0.
      DO 6 L = 2, NP
      DO 6 J = L, NP
      NCOUNT = NCOUNT + 1
      WEIGHT = 1. / (KM(1,NUMBER)**2*(1./A(L-1)**2 + 1./A(J)**2) + 2.)
      Q(1) = T(J) - T(L-1)
```

```
      Q(2) = W(J) - W(L-1)                                                    68
      Q(3) = A(L-1) - A(J)                                                    69
      X(NCOUNT) = - Q(2)/Q(1)                                                 70
      Y(NCOUNT) = Q(3)/Q(1)                                                   71
      SUMW = SUMW + WEIGHT                                                    72
      SYY = SYY + Q(3)**2 * WEIGHT                                            73
      DO 6 M = 1, N1                                                          74
      DO 6 K = 1, N                                                           75
    6 S(K,M) = S(K,M) + Q(K)*Q(M) * WEIGHT                                    76
      SYY = SYY - S(1,3)**2 / S(1,1)                                          77
      SXX = S(2,2) - S(1,2)**2 / S(1,1)                                       78
      RXY = S(2,3) - S(1,2)*S(1,3)/S(1,1)                                     79
      S2 = (SYY - RXY**2/SXX) / FLOAT((NP-1)*(NP-2)/2)                        80
      S1 = SQRT(S2)                                                           81
      RXY = RXY / SQRT(SYY*SXX)                                               82
      GO TO 50                                                                83
    7 KM(1,NUMBER)  =  S(2,1)                                                 84
      VMAX(1,NUMBER)  =  S(1,1)                                               85
      AVRES = 0.                                                              86
      WRITE (6,6008)  NT, KM(1,NUMBER), NT, VMAX(1,NUMBER)                    87
      IF (NT .LT. 10  .AND.  ABS((XKM - S(2,1))/S(2,1)) .GT. 1.0 E-05)        88
     $GO TO 4                                                                 89
      DO 8  J = 2, N1                                                         90
      DO 8  K = 1, N                                                          91
    8 S(K,J) = S(K,J) * SM(K) * SM(J-1)                                       92
      SEKM  =  SQRT(S(2,3)) * S1                                              93
      WKM(1,NUMBER) = 1.0 / SEKM**2                                           94
      SEVMAX = SQRT(S(2,1)) * S1                                              95
      WVMAX(1,NUMBER)  =  1.0 / SEVMAX**2                                     96
      CONST = 0.                                                              97
      DO 9  K = 1, NP                                                         98
    9 CONST = CONST - A(K) -  KM(1,NUMBER)*W(K) -  VMAX(1,NUMBER)*T(K)        99
      CONST  =  CONST / FLOAT(NP)                                            100
      AVRES = 0.                                                             101
      DO 12 K = 1, NP                                                        102
      W(K) = A(K)                                                            103
```

```
      DO 10 J = 1, 10                                                          104
      PREDA = W(K)                                                             105
      W(K) = W(K) - (W(K) + KM(1,NUMBER)*ALOG(W(K)) + VMAX(1,NUMBER) *         106
     $T(K) + CONST) / (1.0 + KM(1,NUMBER)/W(K))                                107
   10 IF (ABS((PREDA - W(K)) / W(K) ) .LT. 0.00001)   GO TO 11                 108
      WRITE (6,6009)  K, A(K), W(K)                                            109
   11 R(K) = A(K) - W(K)                                                       110
   12 AVRES = AVRES + R(K)                                                     111
      AVRES = AVRES / FLOAT(NP) / FACTOR                                       112
      WRITE (6,6010)                                                           113
      AVR = 0.                                                                 114
      DO 13 K = 1, NP                                                          115
      CRESA = R(K) / FACTOR                                                    116
      S2A = S2A + R(K)**2                                                      117
      PREDV = VMAX(1,NUMBER) * W(K) / (KM(1,NUMBER) + W(K))                    118
      RESV = V(K) - PREDV                                                      119
      AVR = AVR + RESV                                                         120
      CRESV = RESV / FACTOR * B(K)                                             121
      S2V = S2V + RESV**2                                                      122
   13 WRITE (6,6011)   A(K), W(K), R(K), CRESA, V(K), PREDV, RESV, CRESV       123
      S2A = S2A / FLOAT(NP-N)                                                  124
      S2V = S2V / FLOAT(NP-N)                                                  125
      CRESA = AVRES * FACTOR                                                   126
      AVR = AVR / FLOAT(NP)                                                    127
      CRESV = AVR / FACTOR                                                     128
      S2 = S2 / SUMW * FLOAT((NP-1)*(NP-2)/2)                                  129
      S1 = SQRT(S2)                                                            130
      WRITE (6,6012)  CRESA, AVRES, AVR, CRESV                                 131
      WRITE (6,6013)  KM(1,NUMBER), SEKM, WKM(1,NUMBER),  VMAX(1,NUMBER)       132
     $, SEVMAX, WVMAX(1,NUMBER), S2, S1, RXY                                   133
      WRITE (6,6014) S2A, S2V                                                  134
      B(1) = KM(1,NUMBER) * 1.0 E+06                                           135
      B(2) = SEKM * 1.0 E+06                                                   136
      VMAX(1,NUMBER) = VMAX(1,NUMBER) / MICROL                                 137
      WVMAX(1,NUMBER) = WVMAX(1,NUMBER) * MICROL**2                            138
      B(7) = VMAX(1,NUMBER) * 1.0 E+07                                         139
```

```
      B(8) = SEVMAX / MICROL * 1.0 E+07                                  140
      PUNCH 9015, PH, IONIC(NUMBER), TEMP, KM(1,NUMBER), SEKM, VMAX(1,NU 141
     $MBER), SEVMAX, NUMBER                                              142
C                                                                        143
6006  FORMAT ( // 16X, 63HMICHEALIS CONSTANT DETERMINATION BY THE INTEGR 144
     $ATED EQUATION FOR / 16X, 13A6 )                                    145
6007  FORMAT (/6X,4HPH =, F6.2, 39X, 12HFULL RANGE =, F5.2, 8H DEGREES / 146
     $6X, 13HTEMPERATURE =, F5.1,19H DEGREES CENTIGRADE, 12X, 12HPATHLEN 147
     $GTH =, F4.1, 11H DECIMETERS  / 6X, 16HIONIC STRENGTH =, F7.4, 6H M 148
     $OLAR, 20X, 18HSPECFIC ROTATION =, F7.0, 36H DEGREES MILLILITER/(GR 149
     $AM DECIMETER)/6X,12HWAVELENGTH =,I4,12H MILLIMICRON,21X,49HMICROLI 150
     $TERS OF PENICILLINASE (160,000 UNITS/ML) =,F5.0 / 6X,4HDATE,I3,1H/ 151
     $, I2, 1H/, I2, 4X, 4HPAGE, I4, 15H IN BOOK NUMBER, I2, 7X, 14HBASE 152
     $LINE   Y =, F8.5, 2H -, F12.9, 18H * (REACTION TIME) // )           153
6008  FORMAT (10X, 3HKM(,I2,3H) =,2PE15.6,10X, 5HVMAX(,I2,3H) =,1PE15.7) 154
6009  FORMAT (64H NEWTON'S METHOD EXCEEDED 10 ITERATION,  ITERATION STAR 155
     $TED AT A(, I2, 3H) =, G15.8, 3X, 11HFINISHED AT, G15.8 )           156
6010  FORMAT (/5X,8HOBSERVED, 7X, 9HPREDICTED, 6X, 4HCONC,  9X, 12HRES I 157
     $N CHART, 5X, 8HOBSERVED, 7X, 9HPREDICTED, 6X, 8HVELOCITY, 5X, 12HR 158
     $ES IN CHART /  5X,  4HCONC,  11X, 4HCONC, 11X, 8HRESIDUAL,5X,13HRE 159
     $ADING UNITS,4X,8HVELOCITY, 7X, 8HVELOCITY, 7X, 8HRESIDUAL, 5X, 13H 160
     $READING UNITS )                                                    161
6011  FORMAT (1X, G14.6, 2G15.6, F15.6, 3G15.6, F15.8 )                  162
6012  FORMAT (/12X, 18HAVERAGE RESIDUAL =,G15.6,F15.6,30X,G15.6,F15.8 )  163
6013  FORMAT (//6X, 20HMICHAELIS CONSTANT =,2PE15.6,10X,16HSTANDARD ERRO 164
     $R =,0PE15.8,10X, 8HWEIGHT =, E15.8 //6X, 18HMAXIMUM VELOCITY =1PE1 165
     $7.7, 10X, 16HSTANDARD ERROR =,0PE15.8,10X, 8HWEIGHT =, E15.8 //6X, 166
     $ 10HVARIANCE =, G15.8, 10X, 20HSTANDARD DEVIATION =, G15.8, 10X,   167
     $25HCORRELATION COEFFICIENT =, G15.8 )                              168
6014  FORMAT(/6X,15HCONC VARIANCE =G15.8,5X,19HVELOCITY VARIANCE =G15.8) 169
9015  FORMAT (8HINT EQ. , F6.2, F7.4, F5.1, 2(2PE13.5), 2(1PE13.6), I2 ) 170
C                                                                        171
C---------LINEARIZATION CURVE FITTING OF THE HYPERBOLA  V = VMAX*A/(KM+A) 172
      ASSIGN 20 TO MM                                                    173
      NT = 0                                                             174
      WRITE (6,6004)   NSET, NGROUP, NUMBER, NP                          175
```

```
      WRITE (6,6016)  (TITLE(K), K = 1, 13)                                  176
      WRITE (6,6007)  PH, FRANGE, TEMP, PATHLH, IONIC(NUMBER), SPROT,        177
     $WAVELH, MICROL, (DATE(K), K = 1, 3), NPAGE, NBOOK, BASEY, SLOPE        178
      WRITE (6,6008)  NT,  KM(1,NUMBER), NT,  S(1,1)                         179
      KM(2,NUMBER) = KM(1,NUMBER)                                            180
      IF (KM(2,NUMBER) .LE. 0.)   KM(2,NUMBER) = 0.4 E-04                    181
   17 NT = NT + 1                                                            182
      SUMW = 0.                                                              183
      DO 18 J = 1, N2                                                        184
      DO 18 K = 1, N1                                                        185
   18 S(K,J) = 0.                                                            186
      DO 19 K = 1, NP                                                        187
      D = KM(2,NUMBER) + A(K)                                                188
      W(K) = S2V / ((VMAX(2,NUMBER)*KM(2,NUMBER)/D**2)**2 * S2A  + S2V)      189
      Q(1) = A(K)/D                                                          190
      Q(2) = Q(1)/D                                                          191
      Q(3) = V(K)                                                            192
      SUMW = SUMW + W(K)                                                     193
      DO 19 J = 1, N1                                                        194
      DO 19 L = 1, N                                                         195
   19 S(L,J) = S(L,J) + Q(L)*Q(J)*W(K)                                       196
      GO TO 50                                                               197
   20 KM(2,NUMBER) = KM(2,NUMBER) - S(2,1)/S(1,1)                            198
      VMAX(2,NUMBER) = S(1,1)                                                199
      WRITE (6,6008)  NT, KM(2,NUMBER), NT, S(1,1)                           200
      IF (ABS(S(2,1)/S(1,1)/KM(2,NUMBER)) .GT. 1.0E-05  .AND.  NT .LT.       201
     $ 10)   GO TO 17                                                        202
      S2 = 0.                                                                203
      AVRES = 0.                                                             204
      WRITE (6,6017)                                                         205
      DO 21 K = 1, NP                                                        206
      T(K) = S(1,1)*A(K) / (KM(2,NUMBER) + A(K))                             207
      RESV = V(K) - T(K)                                                     208
      AVRES = AVRES + RESV                                                   209
      AINV = 1./A(K)                                                         210
      VINV = 1./V(K)                                                         211
```

```
      WRITE (6,6018)  A(K),  V(K), RESV, AINV, VINV, W(K)
21    S2 = S2 + RESV**2 * W(K)
      S2 = S2/FLOAT(NP-N)
      S1 = SQRT(S2)
      AVRES = AVRES / FLOAT(NP)
      WRITE (6,6019)  AVRES
      SL = KM(2,NUMBER) / S(1,1)
      VINT = 1. / S(1,1)
      VK = 1. / SL
      DO 22 J = 2, N1
      DO 22 K = 1, N
22    S(K,J) = S(K,J) * SM(K) * SM(J-1)
      SEVMAX = S1 * SQRT(S(1,2))
      SEKM = S1 *  SQRT(S(2,3)) / S(1,1)
      SEVI = SEVMAX / S(1,1)**2
      S(1,3) = S1 *  SQRT(KM(2,NUMBER)**2 * S(1,2) + S(2,3) + 2. * KM(2,
     $NUMBER) * S(1,3))
      SESL = S(1,3)/S(1,1)**2
      SEVK = S(1,3)/KM(2,NUMBER)**2
      WKM(2,NUMBER) = 1./SEKM**2
      WVMAX(2,NUMBER) = 1./SEVMAX**2
      WSL = 1./SESL**2
      WVI = 1./SEVI**2
      WVK = 1./SEVK**2
      S2 = S2 / SUMW * FLOAT(NP)
      S1 = SQRT(S2)
      WRITE (6,6020)  KM(2,NUMBER), SEKM, WKM(2,NUMBER), S(1,1), SEVMAX,
     $WVMAX(2,NUMBER),SL,SESL,WSL,VINT,SEVI,WVI,VK,SEVK,WVK,S2,S1
      B(3) = KM(2,NUMBER) * 1.0 E+06
      B(4) = SEKM * 1.0 E+06
      VMAX(2,NUMBER)  =  S(1,1) / MICROL
      WVMAX(2,NUMBER) =  WVMAX(2,NUMBER) * MICROL**2
      B(9) = VMAX(2,NUMBER) * 1.0 E+07
      B(10) = SEVMAX / MICROL * 1.0 E+07
      PUNCH 9021, PH, IONIC(NUMBER), TEMP, KM(2,NUMBER), SEKM, VMAX(2,NU
     $MBER), SEVMAX, NUMBER
```

```
C                                                                       248
6016  FORMAT (/16X,56HDETERMINATION OF THE MICHAELIS CONSTANT BY LINEARI  249
     $ZATION  /16X, 57HCURVE FITTING OF THE HYPERBOLA   V = VMAX*A/(KM +  250
     $ A)  FOR  /16X, 13A6  // )                                        251
6017  FORMAT(//6X,13HCONCENTRATION,9X,8HVELOCITY,12X, 8HRESIDUAL, 17X,   252
     $ 6H1/CONC, 12X, 10H1/VELOCITY, 10X, 6HWEIGHT )                    253
6018  FORMAT (1X, G19.8, 5G20.8)                                        254
6019  FORMAT (/22X, 18HAVERAGE RESIDUAL =, G20.8)                       255
6020  FORMAT (//6X, 9H    KM  =, 2PE16.6, 4X, 15H    S.E.(KM)  =0PE16.8,  256
     $  4X, 3HW =, E16.8 /                                              257
     $/6X,9H   VMAX =1PE16.7,4X,15H   S.E.(VMAX) =,E16.8,4X,3HW =,E16.8/  258
     $/6X,9HKM/VMAX =,G16.8, 4X,15HS.E.(KM/VMAX) =,G16.8,4X,3HW =,G16.8/  259
     $/6X,9H 1/VMAX =,G16.8, 4X,15H S.E.(1/VMAX) =,G16.8,4X,3HW =,G16.8/  260
     $/6X,9HVMAX/KM =,G16.8, 4X,15HS.E.(VMAX/KM) =,G16.8,4X,3HW =,G16.8/  261
     $/ 6X, 10HVARIANCE =, G16.8, 4X, 7HSIGMA =, G16.8  )               262
9021  FORMAT (8HLIN FIT , F6.2, F7.4, F5.1, 2(2PE13.5), 2(1PE13.6), I2 )  263
C                                                                       264
C---------NORMAL FITTING THE RECTANGULAR HYPERBOLA BY HEY AND HEY'S METHOD  265
C         (V - VMAX)(A + KM) = KM*VMAX                                  266
      ASSIGN 26 TO MM                                                   267
      NT = 0                                                            268
      WRITE (6,6004)   NSET, NGROUP, NUMBER, NP                         269
      WRITE (6,6022)  (TITLE(K), K = 1, 13)                             270
      WRITE (6,6007)  PH, FRANGE, TEMP, PATHLH, IONIC(NUMBER), SPROT,   271
     $WAVELH, MICROL, (DATE(K), K = 1, 3), NPAGE, NBOOK, BASEY, SLOPE   272
      WRITE (6,6008)  NT,  KM(2,NUMBER), NT,  S(1,1)                    273
      KM(3,NUMBER) = KM(2,NUMBER)                                       274
      VMAX(3,NUMBER) = S(1,1)                                           275
      IF (KM(3,NUMBER) .LE. 0.)   KM(3,NUMBER) = 0.4 E-04               276
   23 NT = NT + 1                                                       277
      XKM = KM(3,NUMBER)                                                278
      SUMW = 0.                                                         279
      DO 24 J = 1, N2                                                   280
      DO 24 K = 1, N1                                                   281
   24 S(K,J) = 0.                                                       282
      DO 25 K = 1, NP                                                   283
```

```
      W(K)=(A(K) + KM(3,NUMBER))**2*S2  + (V(K) - VMAX(3,NUMBER))**2*S2A
      Q(1) =  A(K)
      Q(2) = - V(K)
      Q(3) = A(K)*V(K)
      SUMW = SUMW + W(K)
      DO 25 J = 1, N1
      DO 25 L = 1, N
   25 S(L,J) = S(L,J) + Q(L)*Q(J) / W(K)
      GO TO 50
   26 WRITE (6,6008)  NT, S(2,1), NT, S(1,1)
      KM(3,NUMBER) = S(2,1)
      VMAX(3,NUMBER) = S(1,1)
      IF (ABS((XKM - S(2,1))/S(2,1)) .GT. 1.0 E-05  .AND.   NT .LT. 10)
     $GO TO 23
      S2 = 0.
      AVRES = 0.
      WRITE (6,6023)
      DO 27 K = 1, NP
      RESV  =  V(K) - S(1,1)*A(K) / (S(2,1) + A(K))
      AVRES = AVRES + RESV
      WRITE (6,6018)  A(K),  V(K), RESV, W(K)
   27 S2 = S2 + ( A(K)*V(K) - S(1,1)*A(K) + S(2,1)*V(K) )**2 / W(K)
      S2 = S2/FLOAT(NP-N)
      S1 = SQRT(S2)
      AVRES = AVRES / FLOAT(NP)
      WRITE (6,6019)  AVRES
      DO 28 J = 2, N1
      DO 28 K = 1, N
   28 S(K,J) = S(K,J) * SM(K) * SM(J-1)
      SEVMAX = S1 * SQRT(S(1,2))
      SEKM = S1 * SQRT(S(2,3))
      WVMAX(3,NUMBER) = 1./SEVMAX**2
      WKM(3,NUMBER) = 1./SEKM**2
      WKM(3,NUMBER) = 1./SEKM**2
      S2 = S2 * SUMW * FLOAT(NP)
      S1 = SQRT(S2)
```

```
      WRITE (6,6024)  S(2,1), SEKM, WKM(3,NUMBER), S(1,1), SEVMAX, WVMAX      319
     $(3,NUMBER), S2, S1                                                      320
      B(5) = KM(3,NUMBER) * 1.0 E+06                                          321
      B(6) = SEKM * 1.0 E+06                                                  322
      VMAX(3,NUMBER)  =  VMAX(3,NUMBER) / MICROL                              323
      WVMAX(3,NUMBER) = WVMAX(3,NUMBER) * MICROL**2                           324
      B(11) = VMAX(3,NUMBER) * 1.0 E+07                                       325
      B(12) = SEVMAX / MICROL * 1.0 E+07                                      326
      PUNCH 9025, PH, IONIC(NUMBER), TEMP, KM(3,NUMBER), SEKM, VMAX(3,NU      327
     $MBER), SEVMAX, NUMBER                                                   328
      PRINT 7026, IONIC(NUMBER), RXY, (B(K), K = 1, 12)                       329
      CALL  PLT2 (2, X, Y, NCOUNT, X, Y, NP)                                  330
      WRITE (6,8027)    NSET, NCOUNT, IONIC(NUMBER), PH, TEMP                 331
      WRITE (6,8028)  NSET, NP, IONIC(NUMBER), PH, TEMP                       332
      CALL VPLT2 (A, R, NP, 1, 0, 1)                                          333
      CALL PLT2 (2, A, V, NP, A, T, NP)                                       334
      WRITE (6,8029)    NSET, NP, IONIC(NUMBER), PH, TEMP                     335
      IONIC(NUMBER) = SQRT(IONIC(NUMBER))                                     336
      GO TO 2                                                                 337
C                                                                             338
6022  FORMAT (/16X, 72HDETERMINATION OF THE MICHAELIS CONSTANT BY NORMAL      339
     $ FITTING TO RECTANGULAR /16X,79HHYPERBOLA WHEN THE COORDINATES ARE      340
     $ NORMALIZATED IN STANDARD DEVIATION UNITS FOR / 16X, 13A6 // )          341
6023  FORMAT(//6X,13HCONCENTRATION,9X,8HVELOCITY, 8X, 17HVELOCITY RESIDU      342
     $AL, 6X, 13HHEY'S F2 TERM )                                              343
6024  FORMAT (//6X, 20HMICHAELIS CONSTANT =,2PE15.6,10X,16HSTANDARD ERRO      344
     $R =,0PE15.8,10X, 8HWEIGHT =, E15.8 //6X, 18HMAXIMUM VELOCITY =,1PE      345
     $17.7,10X, 16HSTANDARD ERROR =,0PE15.8,10X, 8HWEIGHT =, E15.8 //6X,      346
     $ 10HVARIANCE =, G15.8, 16X, 20HSTANDARD DEVIATION =, G15.8 )            347
9025  FORMAT (8HNORM FIT, F6.2, F7.4, F5.1, 2(2PE13.5), 2(1PE13.6), I2 )      348
7026  FORMAT (1X, F6.4, F8.3, F9.3, F7.3, F12.3, F7.3, F12.3, F7.3, F14.      349
     $4, F7.4, F13.4, F7.4, F12.4, F7.4 )                                     350
8027  FORMAT (// 40X, 44HPLOT OF (A(0) - A)/T  VERSUS  1/T*LN(A(0)/A) //      351
     $ 16H DATA SET NUMBER, I3, 7X, I3, 11H DATA POINT, 10X, 16HIONIC ST      352
     $RENGTH =,F7.4, 6H MOLAR, 5X, 4HPH =, F6.2, 5X, 6HTEMP = F5.1, 6H D      353
     $EG C )                                                                  354
```

```
8028  FORMAT (16H1DATA SET NUMBER, I3, 7X, I3, 11H DATA POINT, 10X, 16HI      355
     $ONIC STRENGTH =, F7.4, 6H MOLAR, 5X, 4HPH =, F6.2, 5X, 6HTEMP =,      356
     $F5.1, 6H DEG C //35X, 51HPLOT OF CONCENTRATION RESIDUAL VERSUS CON    357
     $CENTRATION / )                                                        358
8029  FORMAT (// 20X,  78HPLOT OF OBSERVED VELOCITY, *, AND CALCULATED V    359
     $ELOCITY, X, VERSUS CONCENTRATION  // 16H DATA SET NUMBER, I3, 7X,     360
     $I3, 11H DATA POINT, 10X, 16HIONIC STRENGTH =, F7.4, 6H MOLAR, 5X,     361
     $4HPH =, F6.2, 5X, 6HTEMP =, F5.1, 6H DEG C )                         362
C                                                                           363
C---------SOLVING AND INVERTING THE MATRIX                                  364
  50  DO 51 K = 1, N                                                        365
  51  SM(K) = 1.0/SQRT(S(K,K))                                              366
      SM(N1) = 1.                                                           367
      DO 52 J = 1, N1                                                       368
      DO 52 K = 1, N                                                        369
  52  S(K,J) = S(K,J)*SM(K)*SM(J)                                           370
      SS(N1) = -1.                                                          371
      S(1,N2) = 1.                                                          372
      DO 54 L = 1, N                                                        373
      DO 53 K = 1, N                                                        374
  53  SS(K) = S(K,1)                                                        375
      DO 54 J = 1, N1                                                       376
      DO 54 K = 1, N                                                        377
  54  S(K,J) = S(K+1,J+1) - SS(K+1)*S(1,J+1)/SS(1)                          378
      DO 55 K = 1, N                                                        379
  55  S(K,1) = S(K,1)*SM(K)                                                 380
      GO TO MM,  (7, 20, 26)                                                381
      END                                                                   382
```

Fig. 5 - Summary of Computer Results from Print Statements.

SNUMB = N2172, ACTIVITY # = 02, REPORT CODE = 52, RECORD COUNT = 00206

pH 7.00

IONIC STRENGTH	RXY	INTEGRATED EQUATION (KM+OR-S.E.)*E+06		FIT OF HYPERBOLA (KM+OR-S.E.)*E+06		MIN NORMAL RESIDUAL (KM+OR-S.E.)*E+06		INTEGRATED EQUATION (VMAX+OR-SE)*E+08		FIT OF HYPERBOLA (VMAX+OR-SE)*E+08		MIN NORMAL RESIDUAL (VMAX+OR-SE)*E+08	
0.0053	0.999	72.117	0.215	75.092	4.077	73.237	4.076	33.744	0.059	34.151	0.859	33.776	0.860
0.0053	1.000	96.472	0.124	105.539	5.268	102.388	5.227	40.899	0.026	42.305	1.136	42.143	1.129
0.0103	1.000	75.769	0.104	82.083	2.870	81.134	2.856	35.078	0.031	36.930	0.598	36.740	0.595
0.0103	0.996	66.272	0.368	67.859	2.574	67.041	2.602	37.196	0.103	37.721	0.617	37.539	0.618
0.0153	0.997	56.609	0.450	61.014	3.382	59.583	3.416	35.961	0.142	36.790	0.824	36.467	0.826
0.0203	0.998	60.340	0.506	63.282	3.631	62.188	3.617	35.654	0.120	37.335	0.868	37.087	0.864
0.0303	0.537	35.976	6.949	53.870	3.623	46.338	4.041	34.267	1.907	37.814	0.999	36.130	0.914
0.0503	0.856	51.067	3.493	57.230	5.487	50.944	4.201	37.580	0.978	39.414	1.518	37.905	1.106
0.0603	0.923	51.588	2.653	53.953	4.208	50.805	3.598	39.092	0.657	38.917	1.219	38.113	0.973

pH 6.00

IONIC STRENGTH	RXY	INTEGRATED EQUATION (KM+OR-S.E.)*E+06		FIT OF HYPERBOLA (KM+OR-S.E.)*E+06		MIN NORMAL RESIDUAL (KM+OR-S.E.)*E+06		INTEGRATED EQUATION (VMAX+OR-SE)*E+08		FIT OF HYPERBOLA (VMAX+OR-SE)*E+08		MIN NORMAL RESIDUAL (VMAX+OR-SE)*E+08	
0.0105	1.000	41.330	0.133	48.027	1.240	47.644	1.263	30.489	0.046	31.979	0.305	31.938	0.293
0.0155	0.989	39.534	0.682	44.823	4.240	42.996	4.343	31.691	0.205	33.844	1.171	33.383	1.172
0.0205	0.983	42.546	0.824	85.595	46.531	13.959	15.335	33.016	0.274	40.780	10.302	23.137	4.490
0.0305	1.000	39.362	0.103	41.964	1.604	41.584	1.648	34.816	0.034	35.776	0.442	35.684	0.447
0.0405	0.998	36.964	0.257	39.428	1.754	39.539	1.689	30.145	0.083	31.853	0.480	31.074	0.438
0.0505	0.998	35.159	0.232	34.662	1.770	34.076	1.609	32.398	0.073	32.570	0.542	32.404	0.482
0.0605	1.000	36.525	0.090	37.850	1.615	37.549	1.651	36.557	0.022	37.287	0.557	37.194	0.565

pH 5.00

IONIC STRENGTH	RXY	INTEGRATED EQUATION (KM+OR-S.E.)*E+06		FIT OF HYPERBOLA (KM+OR-S.E.)*E+06		MIN NORMAL RESIDUAL (KM+OR-S.E.)*E+06		INTEGRATED EQUATION (VMAX+OR-SE)*E+08		FIT OF HYPERBOLA (VMAX+OR-SE)*E+08		MIN NORMAL RESIDUAL (VMAX+OR-SE)*E+08	
0.0050	0.997	24.842	0.217	27.499	1.379	27.046	1.291	0.015	0.000	0.015	0.000	0.015	0.000
0.0104	0.999	27.899	0.113	29.526	1.211	29.236	1.183	29.301	0.042	29.113	0.349	29.039	0.337
0.0154	0.998	28.389	0.170	30.031	1.216	30.093	1.046	26.788	0.057	26.769	0.324	26.980	0.265
0.0204	0.999	29.756	0.133	30.291	1.139	29.801	1.139	27.061	0.055	27.455	0.304	27.345	0.299
0.0304	0.997	25.942	0.225	26.528	1.163	26.097	1.185	28.463	0.085	28.482	0.348	28.375	0.346
0.0404	0.998	11.199	0.058	11.572	0.423	11.646	0.398	7.981	0.011	8.001	0.102	8.014	0.090
0.0504	0.997	33.285	0.289	34.165	1.285	33.565	1.235	29.910	0.103	30.235	0.360	30.086	0.340
0.0604	0.994	29.691	0.374	29.108	1.828	29.678	1.960	29.489	0.129	29.975	0.548	30.011	0.530

pH 4.00

IONIC STRENGTH	RXY	INTEGRATED EQUATION (KM+OR-S.E.)*E+06		FIT OF HYPERBOLA (KM+OR-S.E.)*E+06		MIN NORMAL RESIDUAL (KM+OR-S.E.)*E+06		INTEGRATED EQUATION (VMAX+OR-SE)*E+08		FIT OF HYPERBOLA (VMAX+OR-SE)*E+08		MIN NORMAL RESIDUAL (VMAX+OR-SE)*E+08	
0.0065	0.999	10.558	0.094	12.378	0.438	12.459	0.408	30.420	0.027	31.159	0.210	31.184	0.164
0.0105	0.999	13.644	0.109	14.648	0.675	14.592	0.708	30.757	0.027	31.139	0.365	31.112	0.368
0.0205	0.999	17.044	0.095	16.630	0.443	16.431	0.430	30.087	0.037	29.791	0.183	29.731	0.169
0.0405	0.999	25.851	0.112	29.837	0.913	28.790	0.893	29.723	0.047	30.749	0.300	30.697	0.290
0.0605	1.000	37.282	0.099	40.908	1.398	40.697	1.395	29.908	0.034	30.731	0.353	30.682	0.352

pH 3.50

IONIC STRENGTH	RXY	INTEGRATED EQUATION (KM+OR-S.E.)*E+06		FIT OF HYPERBOLA (KM+OR-S.E.)*E+06		MIN NORMAL RESIDUAL (KM+OR-S.E.)*E+06		INTEGRATED EQUATION (VMAX+OR-SE)*E+08		FIT OF HYPERBOLA (VMAX+OR-SE)*E+08		MIN NORMAL RESIDUAL (VMAX+OR-SE)*E+08	
0.0065	0.989	9.098	0.165	10.850	0.549	10.606	0.561	30.875	0.066	31.561	0.294	31.512	0.294
0.0105	0.994	12.459	0.141	12.719	0.531	12.464	0.533	28.423	0.064	29.584	0.227	29.508	0.212
0.0205	0.993	21.837	0.382	22.519	0.755	22.183	0.763	30.309	0.113	31.525	0.277	31.619	0.289

Fig. 6 - Analysis by Integrated Equation.

DATA SET NUMBER 3 GROUP 1 NUMBER 3 18 DATA POINTS

INPUT DATA

TIME	Y2	DELX	DELY	TIME	Y2	DELX	DELY	TIME	Y2	DELX	DELY	TIME	Y2	DELX	DELY
0.	1.822	216.5	1.00	40.	1.636	217.0	1.00	80.	1.450	217.5	1.00	120.	1.269	220.0	1.00
160.	1.083	222.5	1.00	200.	0.912	234.0	1.00	240.	0.743	244.5	1.00	280.	0.583	261.0	1.00
300.	0.508	302.0	1.00	320.	0.445	367.0	1.00	340.	0.397	555.5	1.00	360.	0.369	740.0	0.70
380.	0.357	930.0	0.30												

MICHEALIS CONSTANT DETERMINATION BY THE INTEGRATED EQUATION FOR
POTASSIUM BENZYLPENICILLIN FROM ORD CURVE USING 10 UNITS/ML OF PENICILLINASE

PH = 4.97
TEMPERATURE = 27.0 DEGREES CENTIGRADE
IONIC STRENGTH = 0.0149 MOLAR
WAVELENGTH = 280 MILLIMICRON
DATE 5/ 7/69 PAGE 93 IN BOOK NUMBER 4

FULL RANGE = 0.20 DEGREES
PATHLENGTH = 0.5 DECIMETERS
SPECFIC ROTATION = 1700. DEGREES MILLILITER/(GRAM DECIMETER)
MICROLITERS OF PENICILLINASE (160,000 UNITS/ML) = 10.
BASELINE Y = 0.39553 - 0.00011382 * (REACTION TIME)

KM(1) = 40.372619E-06 VMAX(1) = 2.9965287E-06
KM(2) = 40.170155E-06 VMAX(2) = 2.9947845E-06
KM(3) = 40.173111E-06 VMAX(3) = 2.9948099E-06
KM(4) = 40.173017E-06 VMAX(4) = 2.9948091E-06

OBSERVED CONC	PREDICTED CONC	CONC RESIDUAL	RES IN CHART READING UNITS	OBSERVED VELOCITY	PREDICTED VELOCITY	VELOCITY RESIDUAL	RES IN CHART READING UNITS
0.901121E-03	0.901567E-03	-0.446933E-06	-0.000707	0.284594E-05	0.286706E-05	-0.211153E-07	-0.02845119
0.786498E-03	0.787223E-03	-0.725457E-06	-0.001148	0.283922E-05	0.284940E-05	-0.101833E-07	-0.00023831
0.671875E-03	0.673038E-03	-0.151237E-05	-0.002869	0.283252E-05	0.282627E-05	0.625090E-08	0.01357130
0.560412E-03	0.561232E-03	-0.820837E-06	-0.001299	0.279952E-05	0.279476E-05	0.476004E-08	0.00145389
0.448948E-03	0.450283E-03	-0.134143E-05	-0.002122	0.276726E-05	0.274951E-05	0.177488E-07	0.03411652
0.340642E-03	0.341594E-03	-0.951326E-06	-0.001507	0.262773E-05	0.267967E-05	-0.519423E-07	-0.01491127
0.236759E-03	0.234656E-03	0.197033E-06	0.000312	0.251179E-05	0.256006E-05	-0.482681E-07	-0.00023642
0.138561E-03	0.138826E-03	0.254751E-06	0.000372	0.234845E-05	0.232080E-05	0.276566E-07	0.00000128
0.926204E-04	0.936646E-04	-0.134421E-05	-0.002126	0.201986E-05	0.209789E-05	-0.780283E-07	-0.00053194
0.542605E-04	0.553732E-04	-0.107766E-05	-0.001706	0.164939E-05	0.173516E-05	-0.857750E-07	-0.00006202
0.253764E-04	0.259165E-04	-0.540193E-06	-0.000855	0.106529E-05	0.117439E-05	-0.109099E-06	-0.00009145
0.912645E-05	0.891062E-05	0.215829E-06	0.000342	0.525664E-06	0.543676E-06	-0.180124E-07	-0.00001187
0.298394E-05	0.236153E-05	0.622411E-06	0.000985	0.131876E-06	0.166273E-06	-0.343967E-07	-0.00000665
AVERAGE RESIDUAL =		-0.599224E-06	-0.000949			-0.306003E-07	-0.00004876

MICHAELIS CONSTANT = 40.173117E-06 STANDARD ERROR = 0.27455543E-06 WEIGHT = 0.13265998E 14

MAXIMUM VELOCITY = 2.9948091E-06 STANDARD ERROR = 0.49717019E-08 WEIGHT = 0.40456643E 17

VARIANCE = 0.17200062E-11 STANDARD DEVIATION = 0.13114908E-05 CORRELATION COEFFICIENT = 0.99846219

CONC VARIANCE = 0.18673062E-09 VELOCITY VARIANCE = 0.48542219E-13

Fig. 7 - Analysis by the Gauss-Newton Method.

DATA SET NUMBER 3 GROUP 1 NUMBER 3 13 DATA POINTS

DETERMINATION OF THE MICHAELIS CONSTANT BY LINEARIZATION
CURVE FITTING OF THE HYPERBOLA V = VMAX*A/(KM + A) FOR
POTASSIUM BENZYLPENICILLIN FROM ORD CURVE USING 10 UNITS/ML OF PENICILLINASE

PH = 4.97
TEMPERATURE = 27.0 DEGREES CENTIGRADE
IONIC STRENGTH = 0.0149 MOLAR
WAVELENGTH = 280 MILLIMICRON
DATE 5/ 7/69 PAGE 93 IN BOOK NUMBER 4

FULL RANGE = 0.20 DEGREES
PATHLENGTH = 0.5 DECIMETERS
SPECFIC ROTATION = 1700. DEGREES MILLILITER/(GRAM DECIMETER)
MICROLITERS OF PENICILLINASE (160,000 UNITS/ML) = 10.
BASELINE Y = 0.39553 - 0.00011382 * (REACTION TIME)

KM(0) =	40.173017E-06	VMAX(0) =	2.9948091E-06
KM(1) =	44.596296E-06	VMAX(1) =	3.0115427E-06
KM(2) =	43.938107E-06	VMAX(2) =	3.0060713E-06
KM(3) =	43.948775E-06	VMAX(3) =	3.0061459E-06
KM(4) =	43.948661E-06	VMAX(4) =	3.0061491E-06

CONCENTRATION	VELOCITY	RESIDUAL	1/CONC	1/VELOCITY	WEIGHT
0.90112052E-03	0.28459403E-05	-0.20409573E-07	1109.7295	351377,71	0.99991583
0.78649801E-03	0.28392172E-05	-0.78374569E-08	1271.4590	352209,75	0.99985883
0.67187549E-03	0.28326250E-05	0.10944916E-07	1488.3710	353041,90	0.99974433
0.56041154E-03	0.27955201E-05	0.11979949E-07	1784.4030	357204,07	0.99949695
0.44894759E-03	0.27672569E-05	0.29152233E-07	2227.4315	361368,68	0.99886370
0.34064221E-03	0.26277256E-05	-0.34895692E-07	2935.6315	380557,23	0.99694027
0.23675851E-03	0.25117987E-05	-0.23700409E-07	4223.7076	398122,35	0.98930153
0.13856084E-03	0.23484538E-05	0.66194615E-07	7217.0466	425912,09	0.94294765
0.92620414E-04	0.21198624E-05	-0.18886784E-07	10796.756	495083,23	0.83821006
0.54260544E-04	0.16493863E-05	-0.11507197E-07	18429.596	606286,09	0.58079770
0.25376369E-04	0.10652947E-05	-0.35101849E-07	39406.741	938707,37	0.25599023
0.91264519E-05	0.52566400E-06	0.87469942E-08	109571,61	1902355,9	0.10569263
0.29839436E-05	0.15187608E-06	-0.59252530E-07	335126,96	7582876,3	0.67389694E-01

AVERAGE RESIDUAL = -0.65057371E-08

KM =	43.948661E-06	S.E.(KM) =	0.15993793E-05	W =	0.42199637E 12
VMAX =	3.0061491E-06	S.E.(VMAX) =	1.83796736E-08	W =	3.72725849E 15
KM/VMAX =	14.619614	S.E.(KM/VMAX) =	0.45363025	W =	4.8595493
1/VMAX =	332651.94	S.E.(1/VMAX) =	1816.5307	W =	3.0438922E-07
VMAX/KM =	6.8401258E-02	S.E.(VMAX/KM) =	2.1224144E-03	W =	221993,17

VARIANCE = 1.0188559E-15 SIGMA = 3.1919929E-08

Fig. 8 - Hey and Hey Method of Normal Fitting a Rectangular Hyperbola.

DATA SET NUMBER 3 GROUP 1 NUMBER 3 13 DATA POINTS

DETERMINATION OF THE MICHAELIS CONSTANT BY NORMAL FITTING TO RECTANGULAR HYPERBOLA WHEN THE COORDINATES ARE NORMALIZATED IN STANDARD DEVIATION UNITS FOR POTASSIUM BENZYLPENICILLIN FROM ORD CURVE USING 10 UNITS/ML OF PENICILLINASE

PH = 4.97
TEMPERATURE = 27.0 DEGREES CENTIGRADE
IONIC STRENGTH = 0.0149 MOLAR
WAVELENGTH = 280 MILLIMICRON
DATE 5/ 7/69 PAGE 93 IN BOOK NUMBER 4

FULL RANGE = 0.20 DEGREES
PATHLENGTH = 0.5 DECIMETERS
SPECFIC ROTATION = 1700, DEGREES MILLILITER/(GRAM DECIMETER)
MICROLITERS OF PENICILLINASE (160,000 UNITS/ML) = 10.
BASELINE Y = 0.39553 - 0.00011382 * (REACTION TIME)

KM(0) =	43.948681E-06	VMAX(0) =	3.0061451E-06
KM(1) =	42.736079E-06	VMAX(1) =	2.9980365E-06
KM(2) =	42.727455E-06	VMAX(2) =	2.9979705E-06
KM(3) =	42.727343E-06	VMAX(3) =	2.9979696E-06

CONCENTRATION	VELOCITY	VELOCITY RESIDUAL	HEY'S F2 TERM
0.90112052E-03	0.28429413E-05	-0.16315223E-07	0.91198948E-21
0.78649801E-03	0.28392172E-05	-0.42765365E-08	0.70530724E-21
0.67187549E-03	0.28325230E-05	0.13899205E-07	0.52541286E-21
0.56041154E-03	0.27995211E-05	0.13931615E-07	0.37800085E-21
0.44894759E-03	0.27672569E-05	0.29819753E-07	0.25624937E-21
0.34064221E-03	0.26277256E-05	-0.36113951E-07	0.17534529E-21
0.23675881E-03	0.25117917E-05	-0.27854669E-07	0.12372548E-21
0.13856034E-03	0.23484538E-05	0.57867897E-07	0.11226256E-21
0.92620414E-04	0.20198624E-05	-0.31691241E-07	0.19730936E-21
0.54260544E-04	0.16493835E-05	-0.27849456E-07	0.34918742E-21
0.25375369E-04	0.10652947E-05	-0.51739514E-07	0.70220861E-21
0.91264519E-05	0.52566401E-06	-0.19892888E-08	0.11440931E-20
0.29859436E-05	0.13187618E-06	-0.63925524E-07	0.15350272E-20

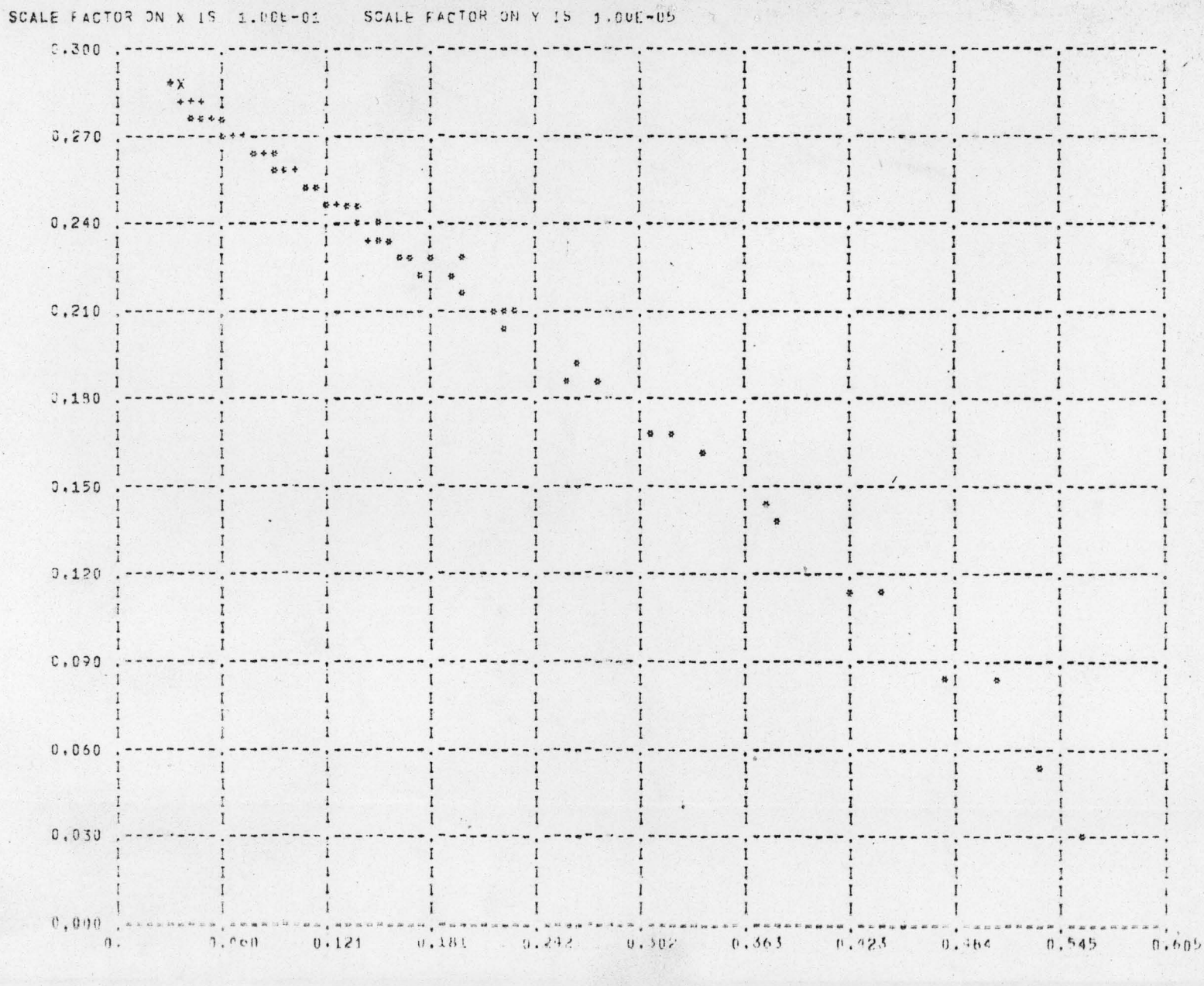
AVERAGE RESIDUAL = -0.11313588E-07

MICHAELIS CONSTANT = 42.727343E-06 STANDARD ERROR = 0.24125767E-05 WEIGHT = 0.17180577E 12

MAXIMUM VELOCITY = 2.9979696E-06 STANDARD ERROR = 0.16338324E-07 WEIGHT = 0.37461483E 16

VARIANCE = 0.35012695E-19 STANDARD DEVIATION = 0.18711145E-09

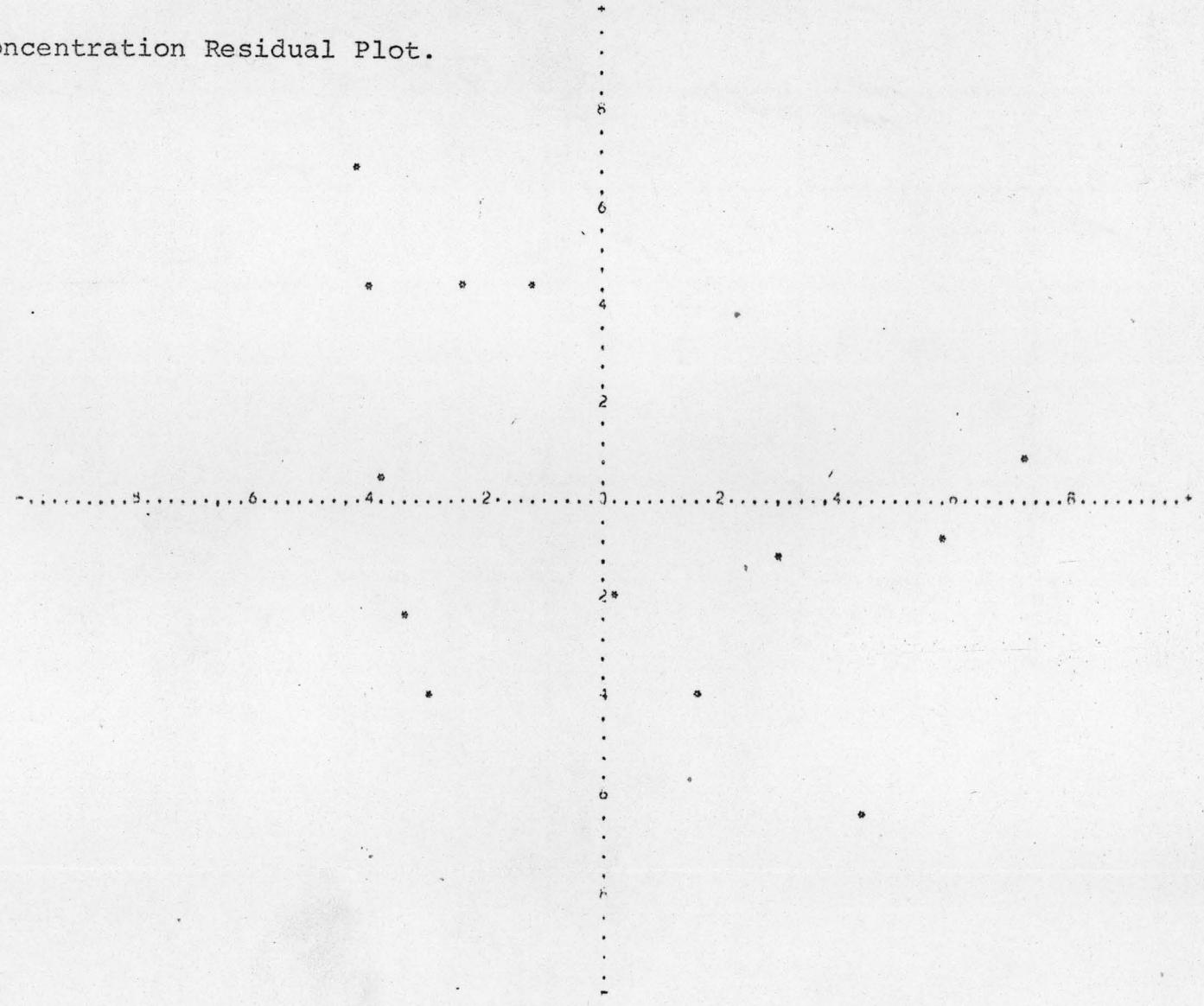
Fig. 9 - A Plot of the Integrated Equation.



DATA SET NUMBER 3 13 DATA POINT IONIC STRENGTH = 0.0149 MOLAR PH = 4.97 TEMP = 27.0 DEG C

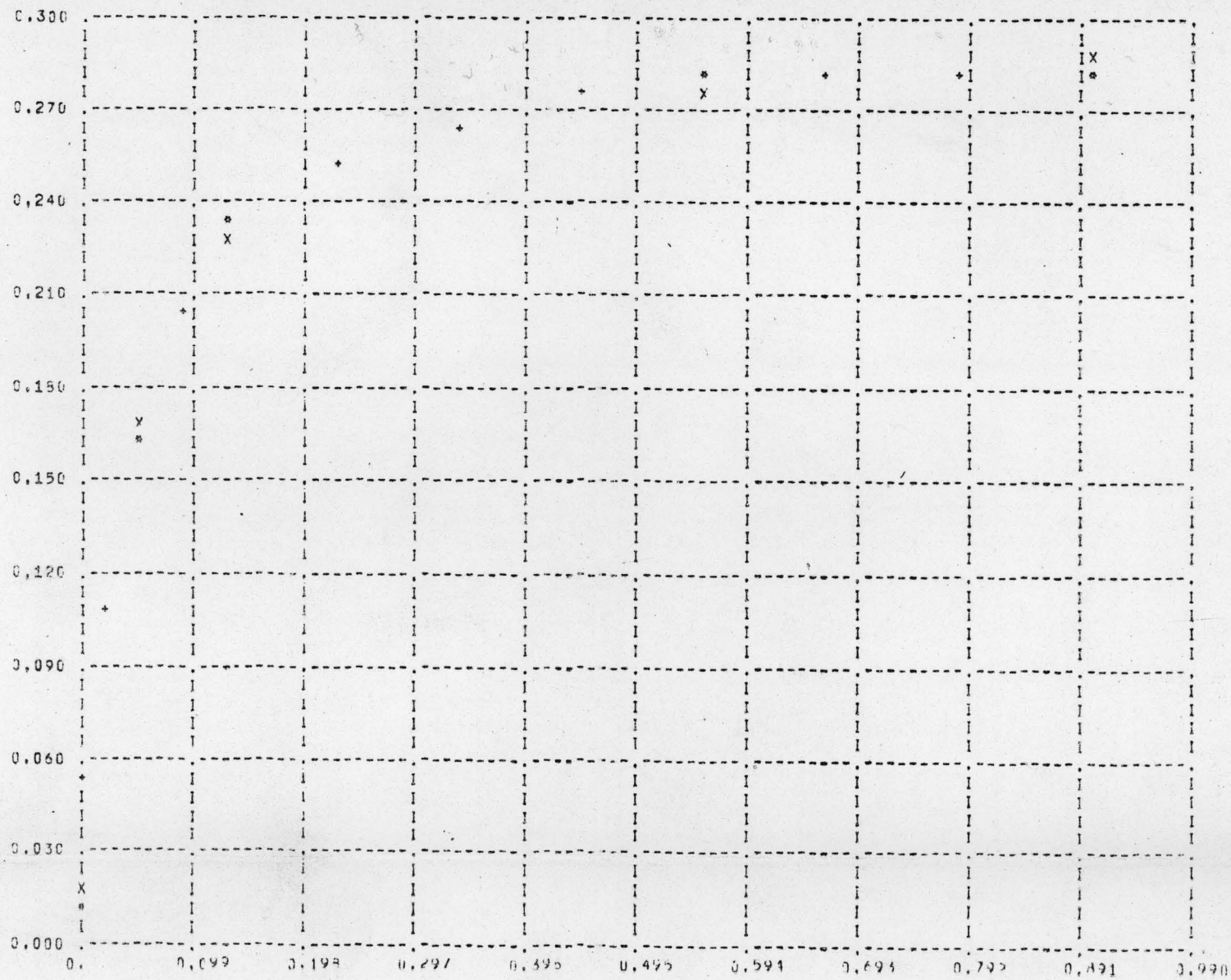
PLOT OF CONCENTRATION RESIDUAL VERSUS CONCENTRATION

Fig. 10 - Concentration Residual Plot.



* = 1 POINT, B = 2 POINTS, C = 3 POINTS, . . . , Z = 26 OR MORE POINTS.

Fig. 11 - A Plot of the Michaelis-Menten Equation, from the Analysis by the Gauss-Newton Method



PLOT OF OBSERVED VELOCITY, *, AND CALCULATED VELOCITY, X, VERSUS CONCENTRATION

```
C                              PROGRAM  NO. 2
C
C    ANALYSIS OF A SINGLE CONTINUOUS RECORDING FOR THE MICHAELIS CONSTANT
C    AND MAXIUM VELOCITY USING ONLY THE INTEGRATED EQUATION.
C
      DIMENSION  TITLE(13), T(30), A(30), W(30), R(30), Y(406), X(406),
     $WKM(20), VMAX(20), WVMAX(20), S(3,4), Q(3), SM(3), SS(3), C(30)
      REAL   IONIC(20),  MW, MICROL, KM(20), KMZERO
      INTEGER   DATE(3), WAVELH
      DATA  NSET, NGROUP, N, N1, N2 / 0, 0, 2, 3, 4 /
      CALL FLGEOF (5, NEOF)
    1 NUMBER = 0
      NGROUP = NGROUP + 1
      PRINT 7001
    2 NUMBER = NUMBER + 1
      IF (NUMBER .GT. 20)   NUMBER = 1
      NSET = NSET + 1
      READ (5,5002)  NP, (TITLE(K), K = 1, 13)
      IF (NEOF .EQ. 1)   STOP
      IF (NP .EQ. 1)   GO TO 1
      READ (5,5003)  WAVELH, FRANGE, PATHLH, SPROT, MW, PH, TEMP, IONIC(
     $NUMBER), MICROL, (DATE(K), K = 1, 3), NPAGE, NBOOK, BASEX, BASEY,
     $BASEDX, SLOPE, (T(K), A(K),  K = 1, NP)
      WRITE (6,6004)   NSET, NGROUP, NUMBER, NP
      WRITE (6,6005)   (T(K), A(K),  K = 1, NP)
      IF (NP  .GT.  30)    GO TO 2
      SLOPE = SLOPE/BASEDX
      BASEY = BASEY + SLOPE * BASEX
      IF (FRANGE .EQ. 0. .OR. PATHLH .EQ. 0. .OR. SPROT .EQ. 0.) GO TO 2
      FACTOR = 1000.*FRANGE/(PATHLH*SPROT*MW)
      DO 3 K = 1, NP
      A(K)  = (A(K) - BASEY + SLOPE * T(K)) * FACTOR
      IF (A(K) .LT. 0.)    A(K) = 1.0 E-06
    3 W(K) = ALOG(A(K))
C
7001  FORMAT (//7X, 2HPH, 5X, 4HTEMP, 4X, 5HIONIC, 10X, 3HRXY, 6X, 19H(K
```

```
     $M +OR- S.E.)*E+06, 5X, 19H(VMAX+OR-S.E.)*E+07 )                    32
5002  FORMAT  (I2, 13A6)                                                 33
5003  FORMAT  (I3, F2.1, F3.1, F6.0, F6.2, F5.2, F4.1, F5.4, F4.0, I4,   34
     $I2, I2, I4, I2, F5.0, F5.3, F6.1, F5.3 /  ( 4(F4.0, F5.3, 11X) ) )  35
6004  FORMAT (16H1DATA SET NUMBER, I3, 10X, 5HGROUP,I2, 3X, 6HNUMBER, I3  36
     $, 15X, I3, 12H DATA POINTS )                                       37
6005  FORMAT(/11H INPUT DATA / 4(6X, 4HTIME, 4X, 2HY2, 3X, 4HDELX, 3X, 4  38
     $HDELY  )   / (4(5X, F5.0, F7.3, 13X) ) )                           39
C                                                                        40
C--------DETERMINING THE MICHAELIS CONSTANT USING THE INTEGRATED EQUATION 41
      WRITE (6,6006)   (TITLE(K), K = 1, 13)                             42
      WRITE (6,6007)  PH, FRANGE, TEMP, PATHLH, IONIC(NUMBER), SPROT,    43
     $WAVELH, MICROL, (DATE(K), K = 1, 3), NPAGE, NBOOK, BASEY, SLOPE    44
      KM(NUMBER) = 0.3 E-04                                              45
      NT = 0                                                             46
    4 NT = NT + 1                                                        47
      XKM = KM(NUMBER)                                                   48
      NCOUNT = 0                                                         49
      SYY = 0.                                                           50
      SUMW = 0.                                                          51
      DO 5 J = 1, N2                                                     52
      DO 5 K = 1, N1                                                     53
    5 S(K,J) = 0.                                                        54
      DO 6 L = 2, NP                                                     55
      DO 6 J = L, NP                                                     56
      NCOUNT = NCOUNT + 1                                                57
      WEIGHT = 1. / (KM(NUMBER)**2 * (1./A(L-1)**2 + 1./A(J)**2) + 2.)   58
      Q(1) = T(J) - T(L-1)                                               59
      Q(2) = W(J) - W(L-1)                                               60
      Q(3) = A(L-1) - A(J)                                               61
      X(NCOUNT) = - Q(2)/Q(1)                                            62
      Y(NCOUNT) = Q(3)/Q(1)                                              63
      SUMW = SUMW + WEIGHT                                               64
      SYY = SYY + Q(3)**2 * WEIGHT                                       65
      DO 6 M = 1, N1                                                     66
      DO 6 K = 1, N                                                      67
```

```
    6  S(K,M) = S(K,M) + Q(K)*Q(M) * WEIGHT                                    68
       SYY = SYY - S(1,3)**2 / S(1,1)                                          69
       SXX = S(2,2) - S(1,2)**2 / S(1,1)                                       70
       RXY = S(2,3) - S(1,2)*S(1,3)/S(1,1)                                     71
       S2 = (SYY - RXY**2/SXX) / FLOAT((NP-1)*(NP-2)/2)                        72
       S1 = SQRT(S2)                                                           73
       RXY = RXY / SQRT(SYY*SXX)                                               74
       GO TO 50                                                                75
    7  KM(NUMBER)   = S(2,1)                                                   76
       VMAX(NUMBER) = S(1,1)                                                   77
       AVRES = 0.                                                              78
       WRITE (6,6008)  NT, KM(NUMBER), NT, VMAX(NUMBER)                        79
       IF (NT .LT. 10  .AND.  ABS((XKM - S(2,1))/S(2,1)) .GT. 1.0 E-05)        80
      $GO TO 4                                                                 81
       DO 8  J = 2, N1                                                         82
       DO 8  K = 1, N                                                          83
    8  S(K,J) = S(K,J) * SM(K) * SM(J-1)                                       84
       SEKM   =   SQRT(S(2,3)) * S1                                            85
       SEVMAX = SQRT(S(2,1)) * S1                                              86
       WKM(NUMBER)   =  1.0 / SEKM**2                                          87
       WVMAX(NUMBER)   = 1.0 / SEVMAX**2                                       88
       CONST = 0.                                                              89
       DO 9  K = 1, NP                                                         90
    9  CONST = CONST - A(K) -  KM(NUMBER)*W(K) - VMAX(NUMBER)*T(K)             91
       CONST  =  CONST / FLOAT(NP)                                             92
       AVRES = 0.                                                              93
       DO 12 K = 1, NP                                                         94
       C(K) = A(K)                                                             95
       DO 10 J = 1, 10                                                         96
       PREDA = C(K)                                                            97
       C(K) = C(K) - (C(K) + KM(NUMBER)*ALOG(C(K)) + VMAX(NUMBER)*T(K) +       98
      $CONST) / (1.0 + KM(NUMBER)/C(K))                                        99
   10  IF (ABS((PREDA - C(K)) / C(K) ) .LT. 0.00001)   GO TO 11               100
       WRITE (6,6009)  K, A(K), C(K)                                          101
   11  R(K) = A(K) - C(K)                                                     102
   12  AVRES = AVRES + R(K)                                                   103
```

```
      AVRES = AVRES / FLOAT(NP) / FACTOR                                    104
      WRITE (6,6010)                                                        105
      AVR = 0.                                                              106
      DO 13 K = 1, NP                                                       107
      CRESA = R(K) / FACTOR                                                 108
      S2A = S2A + R(K)**2                                                   109
   13 WRITE (6,6011)   A(K), C(K), R(K), CRESA, T(K)                        110
      S2A = S2A / FLOAT(NP-N)                                               111
      CRESA = AVRES * FACTOR                                                112
      S2 = S2 / SUMW * FLOAT((NP-1)*(NP-2)/2)                               113
      S1 = SQRT(S2)                                                         114
      WRITE (6,6012)  CRESA, AVRES                                          115
      WRITE (6,6013)   KM(NUMBER) , SEKM, WKM(NUMBER), VMAX(NUMBER),        116
     $  SEVMAX, WVMAX(NUMBER),    S2, S1, RXY                               117
      WRITE (6,6014) S2A                                                    118
      VMAX(NUMBER) = VMAX(NUMBER) / MICROL  * 0.5/PATHLH                    119
      SEVMAX = SEVMAX / MICROL  * 0.5/PATHLH                                120
      WVMAX(NUMBER) = WVMAX(NUMBER) * MICROL**2                             121
      PUNCH 9015, PH, IONIC(NUMBER), TEMP, KM(NUMBER), SEKM, VMAX(NUMBER    122
     $), SEVMAX, NUMBER                                                     123
      PRINT 7016,  PH, TEMP, IONIC(NUMBER), RXY, KM(NUMBER), SEKM, VMAX(    124
     $NUMBER), SEVMAX                                                       125
      CALL  PLT2 (2, X, Y, NCOUNT, X, Y, NP)                                126
      WRITE (6,8027)    NSET, NCOUNT, IONIC(NUMBER), PH, TEMP               127
      WRITE (6,8028)  NSET, NP, IONIC(NUMBER), PH, TEMP                     128
      CALL VPLT2 (A, R, NP, 1, 0, 1)                                        129
      IONIC(NUMBER) = SQRT(IONIC(NUMBER))                                   130
      GO TO 2                                                               131
C                                                                           132
 6006 FORMAT ( // 16X, 63HMICHEALIS CONSTANT DETERMINATION BY THE INTEGR    133
     $ATED EQUATION FOR / 16X, 13A6 )                                       134
 6007 FORMAT (/6X,4HPH =, F6.2, 39X, 12HFULL RANGE =, F5.2, 8H DEGREES /    135
     $6X, 13HTEMPERATURE =, F5.1,19H DEGREES CENTIGRADE, 12X, 12HPATHLEN    136
     $GTH =, F4.1, 11H DECIMETERS  / 6X, 16HIONIC STRENGTH =, F7.4, 6H M    137
     $OLAR, 20X, 18HSPECFIC ROTATION =, F7.0, 36H DEGREES MILLILITER/(GR    138
     $AM DECIMETER)/6X,12HWAVELENGTH =,I4,12H MILLIMICRON,21X,49HMICROLI    139
```

```
     $TERS OF PENICILLINASE  (80,000 UNITS/ML) =,F5.0 / 6X,4HDATE,I3,1H/      140
     $, I2, 1H/, I2, 4X, 4HPAGE, I4, 15H IN BOOK NUMBER, I2, 7X, 14HBASE      141
     $LINE   Y =, F8.5, 2H -, F12.9, 18H * (REACTION TIME) // )               142
6008  FORMAT (10X, 3HKM(,I2,3H) =,2PE15.6,10X, 5HVMAX(,I2,3H) =,1PE15.7)     143
6009  FORMAT (64H NEWTON'S METHOD EXCEEDED 10 ITERATION,  ITERATION STAR      144
     $TED AT A(, I2, 3H) =, G15.8, 3X, 11HFINISHED AT, G15.8 )                145
6010  FORMAT (/5X,8HOBSERVEL, 7X, 9HPREDICTED, 6X, 4HCONC,  9X, 12HRES I     146
     $N CHART     /  5X,  4HCONC,  11X, 4HCONC, 11X, 8HRESIDUAL,5X,13HRE      147
     $ADING UNITS , 4X, 4HTIME )                                              148
6011  FORMAT (1X, G14.6, 2G15.6, F15.6, F9.0 )                                 149
6012  FORMAT (/12X, 18HAVERAGE RESIDUAL =,G15.6,F15.6,30X,G15.6,F15.8 )        150
6013  FORMAT (//6X, 20HMICHAELIS CONSTANT =,2PE15.6,10X,16HSTANDARD ERRO       151
     $R =,0PE15.8,10X, 8HWEIGHT =, E15.8 //6X, 18HMAXIMUM VELOCITY =1PE1      152
     $7.7, 10X, 16HSTANDARD ERROR =, E15.8, 10X, 8HWEIGHT =,0PE15.8//6X,      153
     $ 10HVARIANCE =, G15.8, 10X, 20HSTANDARD DEVIATION =, G15.8, 10X,        154
     $25HCORRELATION COEFFICIENT =, G15.8 )                                   155
6014  FORMAT(/6X,15HCONC VARIANCE =G15.8)                                      156
9015  FORMAT (8HINT EQ. , F6.2, F7.4, F5.1, 4(1PE13.6), I2)                   157
7016  FORMAT (5X,F5.2,F8.1,F10.4,F13.4,6PF14.4,6PF9.4,7PF14.5,7PF10.5 )        158
8027  FORMAT (// 40X, 44HPLOT OF (A(0) - A)/T  VERSUS  1/T*LN(A(0)/A) //      159
     $ 16H DATA SET NUMBER, I3, 7X, I3, 11H DATA POINT, 10X, 16HIONIC ST      160
     $RENGTH =,F7.4, 6H MOLAR, 5X, 4HPH =, F6.2, 5X, 6HTEMP = F5.1, 6H D      161
     $EG C )                                                                  162
8028  FORMAT (16H1DATA SET NUMBER, I3, 7X, I3, 11H DATA POINT, 10X, 16HI       163
     $ONIC STRENGTH =, F7.4, 6H MOLAR, 5X, 4HPH =, F6.2, 5X, 6HTEMP =,        164
     $F5.1, 6H DEG C //35X, 51HPLOT OF CONCENTRATION RESIDUAL VERSUS CON      165
     $CENTRATION / )                                                          166
C                                                                             167
C--------SOLVING AND INVERTING THE MATRIX                                     168
  50  DO 51 K = 1, N                                                          169
  51  SM(K) = 1.0/SQRT(S(K,K))                                                170
      SM(N1) = 1.                                                             171
      DO 52 J = 1, N1                                                         172
      DO 52 K = 1, N                                                          173
  52  S(K,J) = S(K,J)*SM(K)*SM(J)                                             174
      SS(N1) = -1.                                                            175
```

```
      S(1,N2) = 1.                                                        176
      DO 54 L = 1, N                                                      177
      DO 53 K = 1, N                                                      178
53    SS(K) = S(K,1)                                                      179
      DO 54 J = 1, N1                                                     180
      DO 54 K = 1, N                                                      181
54    S(K,J) = S(K+1,J+1) - SS(K+1)*S(1,J+1)/SS(1)                        182
      DO 55 K = 1, N                                                      183
55    S(K,1) = S(K,1)*SM(K)                                               184
      GO TO 7                                                             185
      END                                                                 186
```

SNUMB = 1003[illegible], ACTIVITY = [illegible]2, REPORT CODE = 52, RECORD COUNT = 00084

Fig. 12 - The Print out for the Summary of the Results from Program No. 2.

PH	TEMP	IONIC	RXY	(KM +OR- S.E.)*E+06		(VMAX+OR-S.E.)*E+07	
4.08	27.0	0.0200	0.9760	45.9617	0.6173	0.82193	0.00413
4.55	27.0	0.0200	0.9997	29.0574	0.0836	1.28981	0.00034
4.55	27.0	0.0200	0.9928	26.4846	0.3650	0.59661	0.00059
5.03	27.0	0.0200	0.9999	30.7909	0.0367	1.27871	0.00016
5.50	27.0	0.0200	0.9999	32.9800	0.0513	1.36131	0.00022
6.01	27.0	0.0200	0.9999	39.3300	0.0327	1.54347	0.00016
6.28	27.0	0.0200	0.9999	44.7074	0.0491	1.53633	0.00020
6.59	27.0	0.0200	0.9999	55.4795	0.0740	1.68961	0.00027
6.92	27.0	0.0200	1.0000	56.4106	0.0521	1.69576	0.00019
7.24	27.0	0.0200	1.0000	49.6555	0.0478	1.60688	0.00019
7.54	27.0	0.0200	0.9999	55.4483	0.0764	1.53423	0.00029
7.85	27.0	0.0200	0.9999	61.6065	0.0764	1.56995	0.00026

PH	TEMP	IONIC	RXY	(KM +OR- S.E.)*E+06		(VMAX+OR-S.E.)*E+07	
3.00	29.5	0.0306	0.9943	69.0350	0.8395	1.53417	0.00417
4.00	29.5	0.0306	0.9994	20.3818	0.0662	3.09600	0.00286
5.00	29.5	0.0306	0.9996	32.8143	0.0844	3.27317	0.00381
5.00	29.5	0.0306	0.9982	29.9745	0.1804	3.12410	0.00729
5.95	29.5	0.0306	0.9999	39.5039	0.0564	3.56920	0.00215
5.98	29.5	0.0306	0.9999	39.2778	0.0623	3.62691	0.00219
7.00	29.5	0.0306	0.9993	25.0609	0.1622	3.95635	0.00603
7.80	29.5	0.0306	0.9999	65.3538	0.1174	3.88437	0.00272
7.95	29.5	0.0306	0.9998	63.0720	0.0871	3.87531	0.00341
7.95	29.5	0.0306	0.9994	62.4178	0.1985	3.86895	0.00289
8.80	29.5	0.0306	0.9979	92.6605	0.2129	2.45054	0.01050
9.50	29.5	0.0306	0.9992	117.0359	0.3542	1.23028	0.00131
9.85	29.5	0.0306	0.9985	248.2602	1.1752	0.84115	0.00283
9.98	29.5	0.0306	0.9998	222.8105	0.4078	0.84018	0.00094
10.10	29.5	0.0306	0.9988	819.5243	3.4465	0.90085	0.00222
10.10	29.5	0.0306	0.9992	347.1751	1.1864	0.62191	0.00078

PH	TEMP	IONIC	RXY	(KM +OR- S.E.)*E+06		(VMAX+OR-S.E.)*E+07	
4.06	27.0	0.0406	0.9993	26.9131	0.1703	1.41859	0.00090
4.06	27.0	0.0406	0.9999	30.2460	0.0594	0.66140	0.00042
4.10	27.0	0.0406	0.9967	28.9000	0.3195	1.01130	0.00347
4.57	27.0	0.0406	0.9972	30.6184	0.3094	0.95622	0.00276
5.05	27.0	0.0406	0.9997	34.1262	0.0941	1.10016	0.00110
5.54	27.0	0.0406	0.9989	39.9674	0.2341	1.17471	0.00264
6.10	27.0	0.0406	0.9910	40.9418	0.7459	1.37819	0.00699
6.21	27.0	0.0406	0.9996	44.0214	0.1756	1.35000	0.00193
6.57	27.0	0.0406	0.9999	51.8391	0.0728	1.39581	0.00073
6.85	27.0	0.0406	0.9974	49.9008	0.4469	1.31712	0.00473
7.22	27.0	0.0406	0.9997	48.9634	0.1442	1.42108	0.00178
7.25	27.0	0.0406	0.9991	49.9528	0.2359	2.01069	0.00171
7.25	27.0	0.0406	1.0000	48.6127	0.0520	1.03647	0.00061
7.53	27.0	0.0406	0.9987	50.0277	0.3155	1.36561	0.00326
7.81	27.0	0.0406	0.9993	50.1055	0.2325	1.36262	0.00233
8.17	27.0	0.0406	0.9965	55.8404	0.6307	1.35470	0.00639

PH	TEMP	IONIC	RXY	(KM +OR- S.E.)*E+06		(VMAX+OR-S.E.)*E+07	
4.06	27.0	0.0406	0.9995	126.5062	0.3534	1.39361	0.00236

PROGRAM NO. 3

```
C   ANALYSIS OF A SINGLE CONTINEOUS CURVE USING THE INTEGRATED EQUATION ONLY

      DIMENSION   TITLE(13), T(50), A(50), W(50), R(50), WKM(50), VMAX(5
     $0), WVMAX(50), S(3,4), Q(3), SS(3), SM(4), P(50), B(50), REL(50)
      REAL    IONIC(50), KM(50), KMZERO
      DATA  NGROUP, N, N1, N2  / 0, 2, 3, 4 /
      CALL  FLGEOF (5, NEOF)
   30 NGROUP = NGROUP + 1
      ASSIGN 36 TO MM
      ASSIGN 39 TO MMM
      READ (5,5001)  NUMBER, (TITLE(K), K = 1, 13)
      IF (NEOF .EQ. 1)   STOP
      PRINT 7030,      NGROUP, (TITLE(K), K = 1, 13)
      WRITE (6,6030)  NGROUP, (TITLE(K), K = 1, 13)
      WRITE (6,6001)
      DO 32 K = 1, NUMBER
      READ (5,5003)  IONIC(K), KM(K), SEKM, VMAX(K), SEVMAX
      WVMAX(K) = 1.0
      WKM(K) = 1.0
      WRITE (6,6002)  IONIC(K), KM(K), SEKM, WKM(K), VMAX(K), SEVMAX, WV
     $MAX(K)
      IONIC(K) = SQRT(IONIC(K))
      W(K) = WKM(K)
      A(K) =  KM(K)
      B(K) = 1.0/KM(K)
   32 P(K) = ALOG10(B(K))
   33 SYY = 0.
      DO 34 K =  1, N1
      DO 34 J = 1, N2
   34 S(K,J) = 0.
      DO 35 K = 1, NUMBER
      Q(1) = A(K)
      Q(2) = Q(1) * IONIC(K)
      Q(3) = Q(1) * P(K)
```

```
      SYY = SYY + Q(3)**2 * W(K)                                          33
      DO 35 J = 1, N1                                                     34
      DO 35 I = 1, N                                                      35
35    S(I,J) = S(I,J) + Q(I)*Q(J)*W(K)                                    36
      SYY = SYY - S(1,3)**2 / S(1,1)                                      37
      SXX = S(2,2) - S(1,2)**2 / S(1,1)                                   38
      RXY = S(2,3) - S(1,2)*S(1,3)/S(1,1)                                 39
      S2 = (SYY - RXY**2/SXX) / FLOAT(NUMBER-2)                           40
      S1 = SQRT(S2)                                                       41
      RXY = RXY / SQRT(SYY*SXX)                                           42
      GO TO 50                                                            43
36    DO 37 J = 2, N1                                                     44
      DO 37 K = 1, N                                                      45
37    S(K,J) = S(K,J) * SM(K) * SM(J-1)                                   46
      KMZERO  =  EXP(-2.30258509 * S(1,1))                                47
      SEA = 2.30258509 * KMZERO * SQRT(S(1,2)) * S1                       48
      WA = 1.0 / SEA**2                                                   49
      SEB = S1 * SQRT(S(2,3))                                             50
      WB = 1.0 / SEB**2                                                   51
      WRITE (6,6031)                                                      52
      GO TO MMM,  (39, 43)                                                53
39    WRITE (6,6034)  KMZERO, SEA, WA, S(2,1),  SEB, WB, S2, S1, RXY      54
      PRINT 6034, KMZERO, SEA, WA, S(2,1), SEB, WB, S2, S1, RXY           55
      WRITE (6,6035)                                                      56
      WRITE (6,6036)                                                      57
      S2 = 0.                                                             58
      SD = 0.0                                                            59
      DO 40 K = 1, NUMBER                                                 60
      T(K) = S(1,1) + S(2,1) * IONIC(K)                                   61
      R(K) = KM(K) - EXP(-2.30258509 * T(K))                              62
      REL(K) = R(K)/KM(K)                                                 63
      S2 = S2 + R(K)**2                                                   64
      SD = SD + REL(K)**2                                                 65
40    WRITE (6,6037)  IONIC(K), A(K), B(K), P(K), T(K), R(K), REL(K)      66
      S1 = SQRT(S2/FLOAT(NUMBER - 2))                                     67
      SD = SQRT(SD/FLOAT(NUMBER - 2))                                     68
```

```
      WRITE (6,6038)    S1, SD                                                    69
      CALL PLT2 (2, IONIC, P, NUMBER, IONIC, T, NUMBER)                           70
      WRITE (6,8038)  NGROUP                                                      71
      ASSIGN 43 TO  MMM                                                           72
      WRITE (6,6041)  NGROUP, (TITLE(K), K = 1, 13)                               73
      DO 42 K = 1, NUMBER                                                         74
      W(K) = WVMAX(K)                                                             75
      A(K) =  VMAX(K)                                                             76
   42 P(K) = ALOG10(A(K))                                                         77
      GO TO 33                                                                    78
   43 WRITE (6,6042)  KMZERO, SEA, WA, S(2,1),  SEB, WB, S2, S1, RXY              79
      WRITE (6,6035)                                                              80
      WRITE (6,6043)                                                              81
      S2 = 0.                                                                     82
      SD = 0.0                                                                    83
      DO 44 K = 1, NUMBER                                                         84
      T(K) = S(1,1) + S(2,1) * IONIC(K)                                           85
      R(K) = VMAX(K) - EXP(2.30258509* T(K))                                      86
      REL(K) = R(K)/VMAX(K)                                                       87
      S2 = S2 + R(K)**2                                                           88
      SD = SD + REL(K)**2                                                         89
   44 WRITE (6,6037)  IONIC(K), VMAX(K), P(K), T(K), R(K), REL(K)                 90
      S1 = SQRT(S2/FLOAT(NUMBER - 2))                                             91
      SD = SQRT(SD/FLOAT(NUMBER - 2))                                             92
      WRITE (6,6044)    S1, SD                                                    93
      CALL PLT2  (2, IONIC, P, NUMBER, IONIC, T, NUMBER)                          94
      WRITE (6,8044)  NGROUP                                                      95
      GO TO 30                                                                    96
C                                                                                 97
5001  FORMAT (I2, 13A6)                                                           98
5003  FORMAT (15X, F6.4, 5X, 4E13.6)                                              99
6001  FORMAT(//4X,5HIONIC/ 3X, 8HSTRENGTH, 5X, 9HKM * E+06, 2X, 13HS.E.(        100
     $KM)*E+06, 4X, 6HWEIGHT, 23X, 9HVMAX*E+07, 2X, 13HSE(VMAX)*E+07, 3X        101
     $, 6HWEIGHT  )                                                             102
6002  FORMAT (1X, F10.4, 6PF13.5, 6PF14.5, 1PE15.5, 15X, 7PF13.6, 7PF14.       103
     $5, 1PE15.5  )                                                             104
```

```
7030  FORMAT(/13H GROUP NUMBER, I3, 10X,      // 6X, 94HEQUATION FOR LINE
     $AR IONIC STRENGTH EFFECT   LOG(KM) = B*SQRT(IONIC STRENGTH) + LOG(
     $KM(0))  FOR / 6X, 13A6 )
6030  FORMAT (13H1GROUP NUMBER, I3, 10X,      // 6X, 94HEQUATION FOR LINE
     $AR IONIC STRENGTH EFFECT   LOG(KM) = B*SQRT(IONIC STRENGTH) + LOG(
     $KM(0))  FOR / 6X, 13A6 // )
6031  FORMAT (/3X,77HMICHAELIS CONSTANT AND MAXIMUM VELOCITY DETERMINED
     $BY THE INTEGRATED EQUATION / 3X, 46H1/T*LN(A(0)/A) = - 1/KM*(A(0)
     $- A)/T + VMAX/KM  )
6034  FORMAT ( 6X, 27HKM AT ZERO IONIC STRENGTH =,2PE15.6,6X, 16HSTANDAR
     $D ERROR =,1PE15.8,6X, 8HWEIGHT =, E15.8 / 6X, 7HSLOPE =, G15.8,
     $26X, 16HSTANDARD ERROR =, G15.8, 6X, 8HWEIGHT =, G15.8 / 6X,10HVAR
     $IANCE =, G15.8,  6X, 20HSTANDARD DEVIATION =, G15.8,  6X, 25HCORRE
     $LATION COEFFICIENT =, G15.8 )
6035  FORMAT (/1X,11HSQUARE ROOT / 1X, 8HOF IONIC, 12X, 19HINTEGRATED EQ
     $UATION )
6036  FORMAT (1X, 8HSTRENGTH,  9X,  2HKM, 14X, 4H1/KM, 18X, 3HPKM, 16X,
     $8HCALC PKM,  9X, 8HRESIDUAL,  6X, 24HRELATIVE RESIDUAL * E+02 )
6037  FORMAT (1X, F8.5, 2PE18.6, 1PE18.3, 3G19.8, 2PF19.8 )
6038  FORMAT (/10X, 56HSTANDARD DEVIATION OF MICHAELIS CONSTANT FROM THE
     $ LINE =, 2PE18.6 / 10X, 65HSTANDARD DEVIATION OF RELATIVE ERROR IN
     $ KM FORM THE LINE * E+02 =, 2PF11.6 )
8038  FORMAT (/15X,94HPLOT OF MICHAELIS CONSTANTS OBTAINED FROM THE INTE
     $GRATED EQUATION, *, AND THE VALUE CALCULATED / 15X, 96HFROM LOG(KM
     $) = B*SQRT(IONIC STRENGTH) + LOG(KM(0)), X, VERSUS THE SQUARE ROOT
     $ OF IONIC STRENGTH.   // 13H GROUP NUMBER, I3 )
6041  FORMAT(13H1GROUP NUMBER, I3      // 6X,104HEQUATION TO BE FITTED F
     $OR IONIC STRENGTH STUDY  LOG(VMAX) = B * SQRT(IONIC STRENGTH) + LO
     $G(VMAX(0))  FOR  / 6X, 13A6  )
6042  FORMAT  (6X, 29HVMAX AT ZERO IONIC STRENGTH =,1PE15.7,6X, 16HSTAND
     $ARD ERROR =0PE15.8,6X,8HWEIGHT =, G15.8 / 6X, 7HSLOPE =, G15.8,
     $28X, 16HSTANDARD ERROR =, G15.8, 6X, 8HWEIGHT =, G15.8 / 6X,10HVAR
     $IANCE =, G15.8,  6X, 20HSTANDARD DEVIATION =, G15.8,  6X, 25HCORRE
     $LATION COEFFICIENT =, G15.8 )
6043  FORMAT (1X, 8HSTRENGTH,   8X, 4HVMAX, 11X, 11HLOG OF VMAX, 8X,
     $13HCALC LOG VMAX,6X,8HRESIDUAL, 6X, 24HRELATIVE RESIDUAL * E+02 )
```

```
6044  FORMAT (/10X, 54HSTANDARD DEVIATION OF MAXIMUM VELOCITY FROM THE L   141
     $INE =, 1PE18.7/ 10X, 67HSTANDARD DEVIATION OF RELATIVE ERROR IN VM   142
     $AX FROM THE LINE * E+02 =, 2PF11.6 )                                143
8044  FORMAT (/15X,92HPLOT OF MAXIMUM VELOCITYS OBTAINED FROM THE INTEGR   144
     $ATED EQUATION, *, AND THE VALUE CALCULATED / 15X, 96HFROM LOG(VMAX   145
     $) = B*SQRT(IONIC STRENGTH) + LOG(VMAX(0)), X, VERSUS SQUARE ROOT O   146
     $F IONIC STRENGTH. // 13H GROUP NUMBER, I3 )                          147
C                                                                          148
C---------SOLVING AND INVERTING THE MATRIX                                 149
  50  DO 51 K = 1, N                                                       150
  51  SM(K) = 1.0/SQRT(S(K,K))                                             151
      SM(N1) = 1.                                                          152
      DO 52 J = 1, N1                                                      153
      DO 52 K = 1, N                                                       154
  52  S(K,J) = S(K,J)*SM(K)*SM(J)                                          155
      SS(N1) = -1.                                                         156
      S(1,N2) = 1.                                                         157
      DO 54 L = 1, N                                                       158
      DO 53 K = 1, N                                                       159
  53  SS(K) = S(K,1)                                                       160
      DO 54 J = 1, N1                                                      161
      DO 54 K = 1, N                                                       162
  54  S(K,J) = S(K+1,J+1) - SS(K+1)*S(1,J+1)/SS(1)                         163
      DO 55 K = 1, N                                                       164
  55  S(K,1) = S(K,1)*SM(K)                                                165
      GO TO 36                                                             166
      END                                                                  167
```

Fig. 13 - Analysis of the Linear Ionic Strength Effect for pK_m versus $\sqrt{I}$.

GROUP NUMBER 1

EQUATION FOR LINEAR IONIC STRENGTH EFFECT LOG(KM) = B*SQRT(IONIC STRENGTH) + LOG(KM(0)) FOR IONIC STRENGTH STUDY FOR BENZYLPENICILLIN AT PH 7.0

IONIC STRENGTH	KM * E+06	S.E.(KM)*E+06	WEIGHT	VMAX*E+07	SE(VMAX)*E+07	WEIGHT
0.0053	66.11070	0.38181	1.00000E 00	3.260900	0.02183	1.00000E 00
0.0053	96.47190	0.12446	1.00000E 00	4.089850	0.00524	1.00000E 00
0.0103	75.76880	0.10449	1.00000E 00	3.607760	0.00621	1.00000E 00
0.0103	68.00170	0.23271	1.00000E 00	3.745630	0.01297	1.00000E 00
0.0153	59.57180	0.44707	1.00000E 00	3.661170	0.02778	1.00000E 00
0.0203	60.70330	0.16587	1.00000E 00	3.673600	0.00782	1.00000E 00
0.0206	56.41062	0.05214	1.00000E 00	0.423941	0.00019	1.00000E 00
0.0306	55.06090	0.16216	1.00000E 00	3.966350	0.00603	1.00000E 00
0.0406	49.95263	0.23586	1.00000E 00	2.010689	0.00171	1.00000E 00
0.0406	48.96341	0.14422	1.00000E 00	1.421078	0.00178	1.00000E 00
0.0406	48.61273	0.05198	1.00000E 00	1.836468	0.00061	1.00000E 00
0.0503	47.36680	0.30570	1.00000E 00	3.694120	0.01785	1.00000E 00
0.0603	44.13080	0.34904	1.00000E 00	3.723820	0.01799	1.00000E 00

MICHAELIS CONSTANT AND MAXIMUM VELOCITY DETERMINED BY THE INTEGRATED EQUATION

1/T*LN(A(0)/A) = - 1/KM*(A(0) - A)/T + VMAX/KM

KM AT ZERO IONIC STRENGTH = 10.966478E-05 STANDARD ERROR = 9.41940277E-06 WEIGHT = 1.12707616E 10

SLOPE = 1.7366522 STANDARD ERROR = 0.26206082 WEIGHT = 14.561155

VARIANCE = 9.85475557E-12 STANDARD DEVIATION = 3.13922851E-06 CORRELATION COEFFICIENT = 0.89425585

SQUARE ROOT OF IONIC STRENGTH	INTEGRATED EQUATION KM	1/KM	PKM	CALC PKM	RESIDUAL	RELATIVE RESIDUAL * E+02
0.07280	66.110700E-06	1.51261445E 04	4.1797283	4.0863630	-0.15855906E-04	-23.98387268
0.07280	96.471900E-06	1.03657126E 04	4.0155992	4.0863630	0.14505295E-04	15.03577158
0.10149	75.768800E-06	1.31980446E 04	4.1205096	4.1361837	0.26858205E-05	3.54475789
0.10149	68.001700E-06	1.47055146E 04	4.1674802	4.1361837	-0.50812797E-05	-7.47228330
0.12369	59.571800E-06	1.67864661E 04	4.2249593	4.1747448	-0.73018705E-05	-12.25726008
0.14248	60.703300E-06	1.64735691E 04	4.2167877	4.2073677	-0.13310620E-05	-2.19273416
0.14353	56.410620E-06	1.77271584E 04	4.2486391	4.2091893	-0.53640838E-05	-9.50899646
0.17493	55.060900E-06	1.81617083E 04	4.2591567	4.2637228	0.57588003E-06	1.04589651
0.20149	49.952630E-06	2.00189661E 04	4.3014417	4.3098585	0.95878919E-06	1.91939680
0.20149	48.963410E-06	2.04234143E 04	4.3101283	4.3098585	-0.30430913E-07	-0.06215031
0.20149	48.612730E-06	2.05707434E 04	4.3132500	4.3098585	-0.38111070E-06	-0.78397304
0.22428	47.366800E-06	2.11118335E 04	4.3240260	4.3494233	0.26390864E-05	5.57159530
0.24556	44.130600E-06	2.26599111E 04	4.3552582	4.3863861	0.30523670E-05	6.91663641

STANDARD DEVIATION OF MICHAELIS CONSTANT FROM THE LINE = 73.632231E-07

STANDARD DEVIATION OF RELATIVE ERROR IN KM FORM THE LINE * E+02 = 10.442263

GROUP NUMBER 1

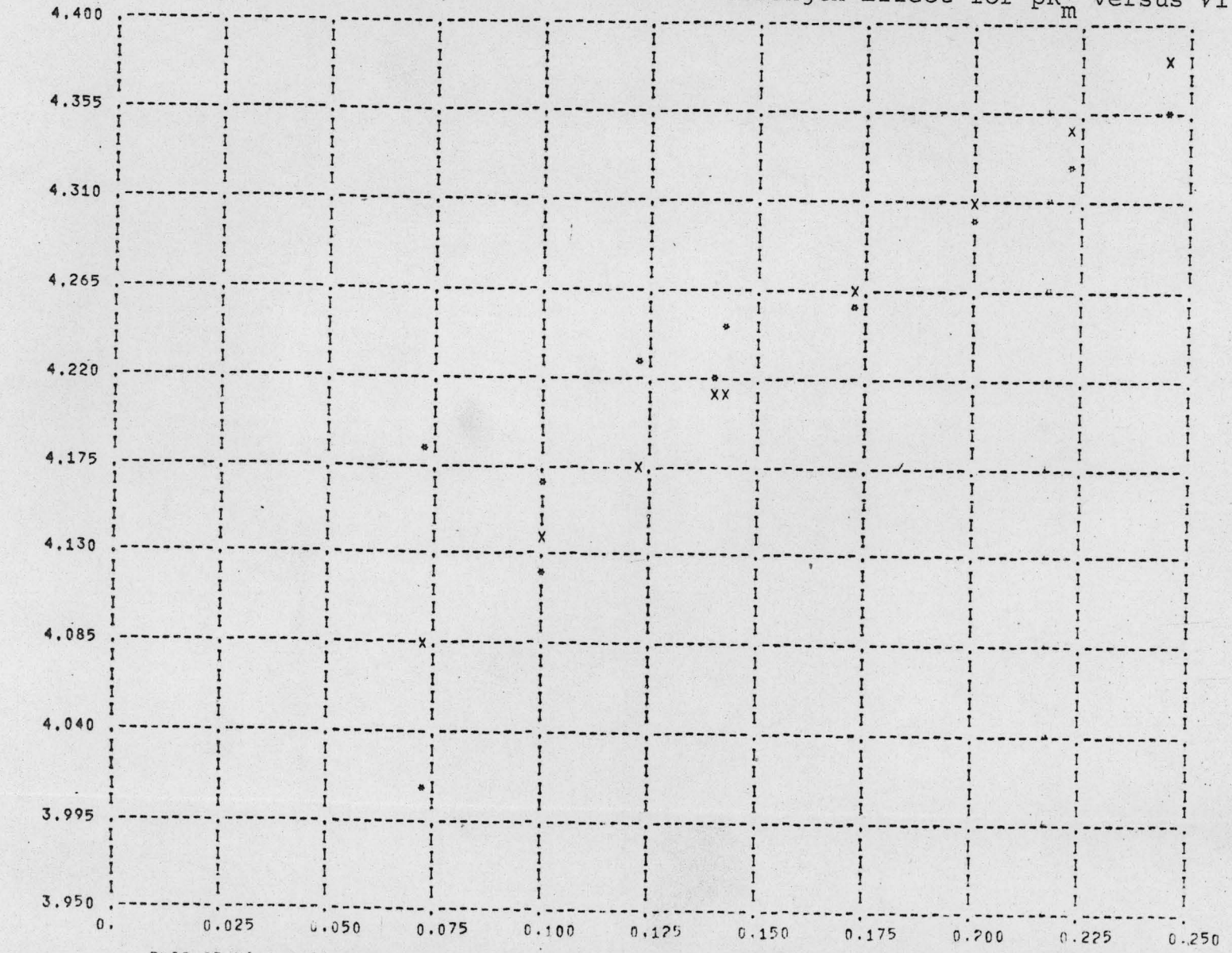


Fig. 14 - A Plot of the Linear Ionic Strength Effect for pK_m versus $\sqrt{I}$.

PLOT OF MICHAELIS CONSTANTS OBTAINED FROM THE INTEGRATED EQUATION, *, AND THE VALUE CALCULATED FROM LOG(KM) = B*SQRT(IONIC STRENGTH) + LOG(KM(0)), X, VERSUS THE SQUARE ROOT OF IONIC STRENGTH.

GROUP NUMBER 1

Fig. 15 - Analysis of the Linear Ionic Strength Effect for the Maximum Velocity.

GROUP NUMBER 1

EQUATION TO BE FITTED FOR IONIC STRENGTH STUDY LOG(VMAX) = B * SQRT(IONIC STRENGTH) + LOG(VMAX(0)) FOR IONIC STRENGTH STUDY FOR BENZYLPENICILLIN AT PH 7.0

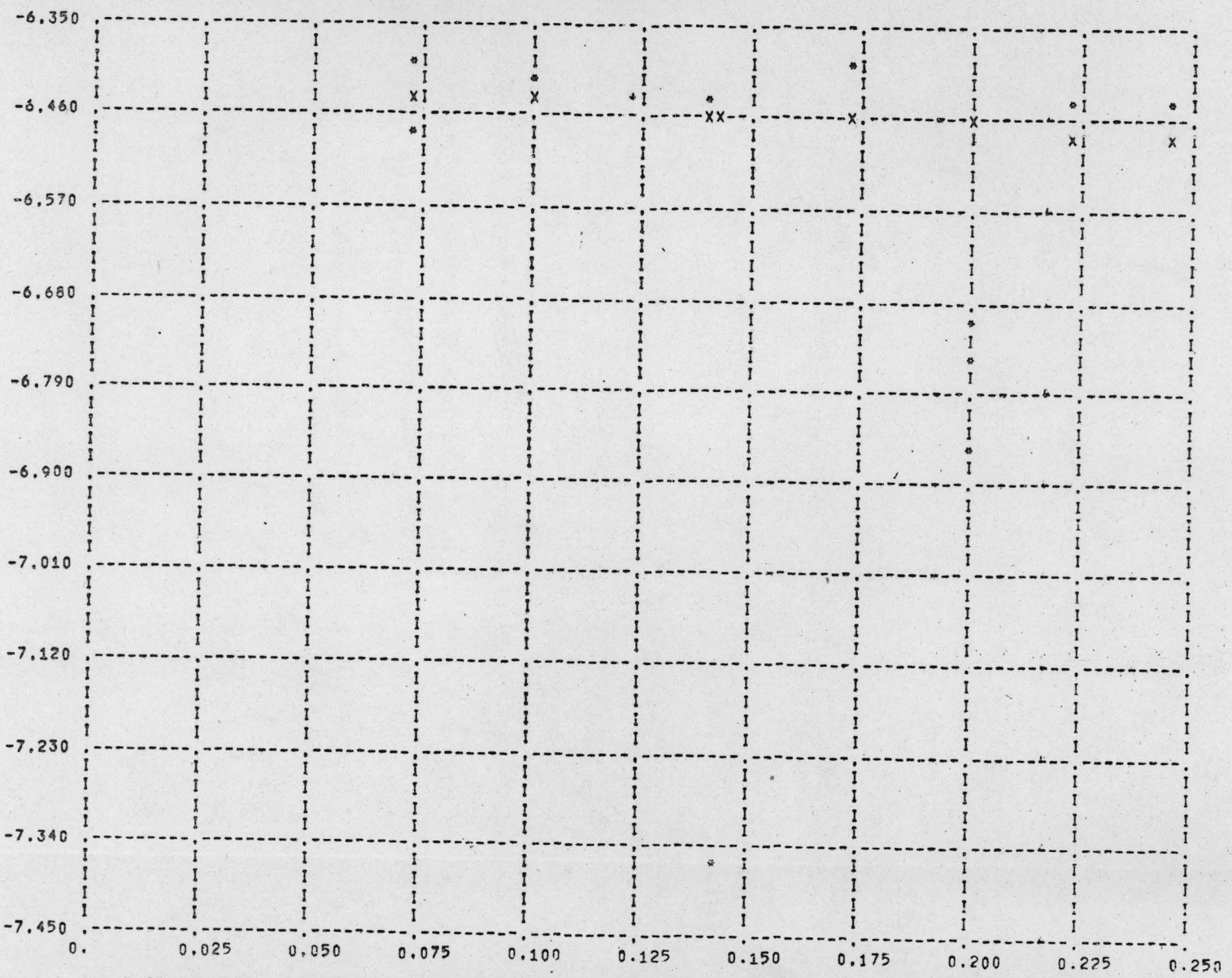
MICHAELIS CONSTANT AND MAXIMUM VELOCITY DETERMINED BY THE INTEGRATED EQUATION
1/T*LN(A(0)/A) = - 1/KM*(A(0) - A)/T + VMAX/KM

VMAX AT ZERO IONIC STRENGTH = 2.5341273E 06 STANDARD ERROR = 0.41812129E 06 WEIGHT = 0.57199923E-11
SLOPE = -0.32785231 STANDARD ERROR = 0.45736025 WEIGHT = 4.7806083
VARIANCE = 0.98354006E-15 STANDARD DEVIATION = 0.31361442E-07 CORRELATION COEFFICIENT = -0.21125606

SQUARE ROOT OF IONIC STRENGTH	INTEGRATED EQUATION VMAX	LOG OF VMAX	CALC LOG VMAX	RESIDUAL	RELATIVE RESIDUAL * E+02
0.07280	32.609000E-08	-6.48666251E 00	-6.4276965	-0.47421098E-07	-0.14542334
0.07280	40.898500E-08	-6.38829261E 00	-6.4276965	0.35473902E-07	0.86736437E-01
0.10149	36.077600E-08	-6.44276237E 00	-6.4371018	-0.47330228E-08	-0.13119007E-01
0.10149	37.456300E-08	-6.42647511E 00	-6.4371018	0.90539789E-08	0.24172112E-01
0.12369	36.611700E-08	-6.43638009E 00	-6.4443815	0.66835846E-08	0.18255325E-01
0.14248	36.736000E-08	-6.43490815E 00	-6.4505402	0.12987702E-07	0.35354153E-01
0.14353	42.394050E-09	-7.37269509E 00	-6.4508841	-0.31169773E-06	-7.3523933
0.17493	39.663500E-08	-6.40160900E 00	-6.4611792	0.50838346E-07	0.12817413
0.20149	20.106890E-08	-6.69665509E 00	-6.4698889	-0.13786194E-06	-0.68564527
0.20149	14.210780E-08	-6.84738207E 00	-6.4698889	-0.19682304E-06	-1.3850263
0.20149	18.364680E-08	-6.73601663E 00	-6.4698889	-0.15528404E-06	-0.84555810
0.22428	36.941200E-08	-6.43248898E 00	-6.4773580	0.36260402E-07	0.98157077E-01
0.24556	37.238200E-08	-6.42901134E 00	-6.4843360	0.44540466E-07	0.11960961

STANDARD DEVIATION OF MAXIMUM VELOCITY FROM THE LINE = 1.3098297E-07
STANDARD DEVIATION OF RELATIVE ERROR IN VMAX FROM THE LINE * E+02 = 228.099746

Fig. 16 - A Plot of the Linear Ionic Strength Effect on the Maximum Velocity.



PLOT OF MAXIMUM VELOCITYS OBTAINED FROM THE INTEGRATED EQUATION, *, AND THE VALUE CALCULATED FROM LOG(VMAX) = B*SQRT(IONIC STRENGTH) + LOG(VMAX(0)), X, VERSUS SQUARE ROOT OF IONIC STRENGTH.

GROUP NUMBER 1

VIII. REFERENCES.

1. G. N. Wilkinson, "Statistical Estimations in Enzyme Kinetics", Biochem. J., 80, 324-332 (1961).

2. Gordon Johansen and Rufus Lumry, "Statistical Analysis of Enzymic Steady-State Rate Data", Compt. Rend. Trav. Lab. Carlsberg, 32, 185-214 (1961).

3. W. Edward Deming, "Statistical Adjustment of Data", John Wiley and Sons, New York, N.Y. (1938), Dover, New York, N.Y. (1964), Chapter 4.

4. E. N. Hey and M. H. Hey, "The Statistical Estimation of a Rectangular Hyperbola", Biometrics, 16, 606-617 (1960).

5. W. W. Cleland, "The Statistical Analysis of Enzyme Kinetic Data", Advances in Enzymology, 29, 1-32 (1967).

6. C. I. Bliss and A. T. James, "Fitting the Rectangular Hyperbola", Biometrics, 22, 573-602 (1966).

7. K. R. Hanson, R. Ling, and E. A. Havir, "A Computer Program for Fitting Data to the Michaelis-Menten Equation", Biochem. Biophy. Res. Commun., 29, 194-197 (1967).

8. John E. Dowd and Douglas Riggs, "A comparison of Estimates of Michaelis-Menten Kinetic Constants from Various Linear Transformations", J. Biol. Chem., 240 863-869 (1965).

9. Ref. 3, Chapters 8 and 10.

10. Ref. 3, Chapter 3.

11. N. R. Drapper and H. Smith, "Applied Regression Analysis", John Wiley and Sons, New York, N.Y. (1966), Chapter 2.