vestigated in a separate experiment. Five hundred nanograms nonradioactive norepinephrine and 50000 DPM norepinephrine-7-^{3}H were added, just prior to extraction in the homogenizer, to hearts obtained from rats dosed intraperitoneally 8 hr. previously with 3.1 mg./kg. reserpine. Six similar hearts, which received no added norepinephrine, were carried through the procedure and served as blanks for the spectrophotofluorometric determinations.

RESULTS AND DISCUSSION

Determination in the final 0.01 *N* hydrochloric acid extract of the two materials added to the reserpine-depleted hearts, and correction of nonradioactive norepinephrine values for percent extraction observed with norepinephrine-7-^{3}H, revealed that 102.4 ± 7.45% of the added norepinephrine was recovered.

Results of extraction efficiency studies are summarized in Table I. These findings indicate that norepinephrine extraction efficiency is increased 60 to 100%, while serotonin extraction efficiency is increased 25 to 55%. Serotonin extraction is nearly complete in all tissues, while norepinephrine extraction values are somewhat lower and appear to be partially dependent on the total weight of tissue extracted. That the extraction efficiency values are reproducible is indicated by the results obtained in two separate experiments with norepinephrine-7-^{3}H.

In this laboratory, screening is routinely carried out with these extraction efficiency values (yielding results similar to those illustrated in Figs. 1 and 2). For greater precision, or in cases where the weight of tissue employed may vary, it is advisable to add radioactive amine to some of the samples to serve as an internal standard.

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Consecutive First-Order Kinetic Consideration of Hydrocortisone Hemisuccinate

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Abstract □ The degradation of hydrocortisone hemisuccinate has been studied at 70° and at pH's of 6.9, 7.2, and 7.6. An expected first-order consecutive reaction was found to be operative and it is presumed that ester hydrolysis occurs by way of an intramolecular attack. The blue tetrazolium assay confirmed that the production of a species devoid of the 17-dihydroxyacetone side chain occurred subsequent to the formation of the steroid alcohol.

Keyphrases □ Hydrocortisone hemisuccinate—degradation kinetics □ Degradation, hydrocortisone hemisuccinate—first-order reaction □ Colorimetric analysis—spectrophotometer □ Blue tetrazolium—color reagent

The availability of a quantitative analytical method is essential to the formulator or scientist for the implementation of predictive kinetics. The application of various environmental parameters, such as temperature, permits the rapid approximation of relevant rates and produces data which aid in the mechanistic interpretation of chemical reactions. Thus, it is often found that dosage forms and their physical and chemical properties, such as solubility or buffer content, are predicated upon the nature of the active moieties and the rates at which decomposition occurs.

The hydrolytic pathway of the 21-hydrocortisone hemiester of succinic acid has been investigated under varied environmental conditions, such as pH and temperature (1, 2). The pH profile indicates that hydrolysis is due to a specific acid catalysis in the pH range of 1.0 to about 2.5 and specific hydroxyl catalysis from approximately 7.6 to 10.0. In the intermediate range, the compound may be subjected to an intramolecular attack of the anion on the ester carbonyl carbon or specific hydroxyl-ion catalysis of the undissociated hemiester.

Steroidal alcohols containing the 17-dihydroxyacetone side chain have been kinetically described under varying conditions of pH, temperature, buffer media, and oxygen deprivation. The rate of disappearance of the dihydroxyacetone function of prednisolone was investigated in solutions of varying hydroxyl-ion concentration, both in the presence and absence of air (3).

The oxygen-deprived system produced neutral and acidic steroidal components at a rate which exceeded that of the oxidative system. In addition, it was assumed that the oxidative degradations did not produce the neutral component. The effect of trace metal content in aqueous solutions of prednisolone has also been studied (4) and shown to significantly increase the production of the steroid devoid of the 17-dihydroxyacetone function.

Sequestering agents were demonstrated to reduce the degradation rate under similar conditions of pH and temperature. Hydrocortisone (cortisol) has also been shown to decompose to fractions devoid of the 17-dihy-

droxyacetone chain in a phosphate buffer at pH 7.4 and at temperatures of 16, 25, and 35° (5).

The complete decomposition of fluprednisolone acetate, from the ester to the free alcohol, and then to further degradation products was followed in an aqueous environment buffered at approximately neutrality (6). The data indicated the kinetic pathway to be consecutive first-order.

The objectives of this study were to kinetically study the degradation of hydrocortisone hemisuccinate at 70° and at pH values near neutrality.

EXPERIMENTAL

Determination of pH for Maximum Partitioning of the Steroid Alcohol—Aqueous solutions of the sodium salts of 21-hydrocortisone hemisuccinate of various hydrogen-ion concentrations were equilibrated with chloroform at room temperature. Subsequent separation of the layers and analysis of the aqueous phase indicated that essentially all of the hemiester remained in the aqueous portion at pH 10, which is consistent with the work of previous investigators (7).

Analytical Methodology, Colorimetric Analysis—Maximum color development, using the blue tetrazolium assay which is specific for the intact dihydroxyacetone chain, occurred at 90 min. and remained stable for at least 4 hr. Since hydrolysis of the ester must occur prior to color development (8), it was assumed that the steroid alcohol would have also reached its maximum color development during this time period. Hence, all experimental samples were allowed to develop in the dark for 90 min. ± 30 sec.

Beer-Lambert Relationship—Alcoholic solutions containing varying concentrations of sodium 21-hydrocortisone hemisuccinate were subjected to the blue tetrazolium assay and spectrophotometrically analyzed at 523 mμ. A plot of absorbance *versus* concentration of the steroid indicated a linear relationship over the required range.

Reagents—pH 10 Buffer: 71 g. of anhydrous sodium dibasic phosphate in 1 l. of water adjusted to pH 10 with 1.0 *M* sodium hydroxide; chloroform; glacial acetic acid, blue tetrazolium, lots 766100 and 764388, 50 mg. in 10 ml. of 95% ethanol; tetramethylammonium hydroxide, 10% aqueous solution diluted to 50 ml. with 95% alcohol. The latter two solutions were freshly prepared and used within 24 hr. as well as being refrigerated when not in use. Phosphate buffer solutions, monobasic and dibasic in concentrations of 0.02 *M*, were used to prepare noted pH levels. Where necessary, sodium chloride was used to maintain an ionic strength of 0.054. 21-Hydrocortisone hemisuccinate[1] was another reagent.

Equipment—A constant-temperature bath (Forma Temp model 2095), pH meter (Leeds Northrup), and spectrophotometer (Cary 16) were used.

Assay Procedure—A 2-ml. aliquot of 21-hydrocortisone hemisuccinate containing approximately 133 mg. of the ester was diluted with the appropriate buffer to 200 ml. Twenty-five milliliter portions of this solution were pipeted into 60-ml. screw-top bottles and immediately placed in an ice bath prior to commencing the kinetic run. These samples were then removed to a constant-temperature bath set at 70° and allowed to attain thermal equilibrium.

One sample designated as the "initial sample" was withheld from the 70° bath and immediately assayed. Ten milliliters of this sample was pipeted into a separator which contained 10 ml. of dilute HCl. Four successive extractions were performed with 20-ml. portions of chloroform. The chloroform phases were drained through a funnel containing a chloroform-wetted pledget of cotton into a 100-ml. flask and brought to volume with chloroform. Exactly 10 ml. of this solution was pipeted into a 50-ml. conical flask and the solvent extracted under reduced pressure at 30°. The residue was dissolved in 20 ml. of alcohol and 2 ml. of blue tetrazolium and 4 ml. of tetramethylammonium hydroxide solution added. Timing was started and the samples allowed to develop in the dark for 90 min. Subsequent to the development period, 1 ml. of glacial acetic acid was added to stabilize and quench the color reaction. The absorbance was then measured at 523 mμ against a blank containing everything except the steroid. All initial and subsequent samples were measured against a blank of this composition at 523 mμ. The pH of the initial sample was also measured.

At suitable time intervals, sample bottles were withdrawn from the temperature bath and immediately placed in an ice bath. After chilling, a 10-ml. aliquot was withdrawn and pipeted into a separator containing 5 ml. of pH 10 buffer and 5 ml. of distilled water.

Four extractions with 20-ml. portions of chloroform were performed and the chloroform phases drained through a funnel containing a chloroform-wetted pledget of cotton into a 100-ml. flask. The flask was then brought to final volume with chloroform.

Another 10-ml. portion was pipeted from the sample bottle and extraction procedures were performed as with the initial sample.

Ten-milliliter aliquots of these chloroform solutions from the acid and basic extractions were pipeted into separate 50-ml. conical flasks. These samples were subjected to drying and color development procedures identical to those of the initial sample.

Since the reaction is pH-dependent, samples from the bath were measured as the run progressed to ensure that the pH remained constant.

The initial and subsequent samples were calculated on the basis of the absorbance reading of the initial sample as 100% and the quantity of products devoid of the 17-dihydroxyacetone side chain found by difference. The data presented were the average results of at least three individual runs and periodic reassays at a given time interval.

RESULTS AND DISCUSSION

In the present study, the degradation behavior of the hemisuccinate ester of hydrocortisone was investigated over a narrow pH range. A temperature of 70° was chosen in order to accelerate the decomposition rate so that its measurement would be kinetically convenient. It has been assumed that the increased rate is mediated by thermal phenomena.

Since this reaction pathway is of the first-order consecutive type, typical concentration-time curves should be observed. In Fig. 1, the contents of the relevant species and their time course is shown for a pH value of 6.9. It can be seen that the concentration of the original ester moiety (A) decreases by a first-order rate. The intermediate alcohol product (B) rises to a maximum value after about 18 hr. and decreases in an approximately linear fashion to about 15% after 64 hr. The concentration of products devoid of the 17-dihydroxyacetone side chain (C), after an induction period of about 1–2 hr., rises rapidly and tails off after about 45 hr. due to reaching near com-

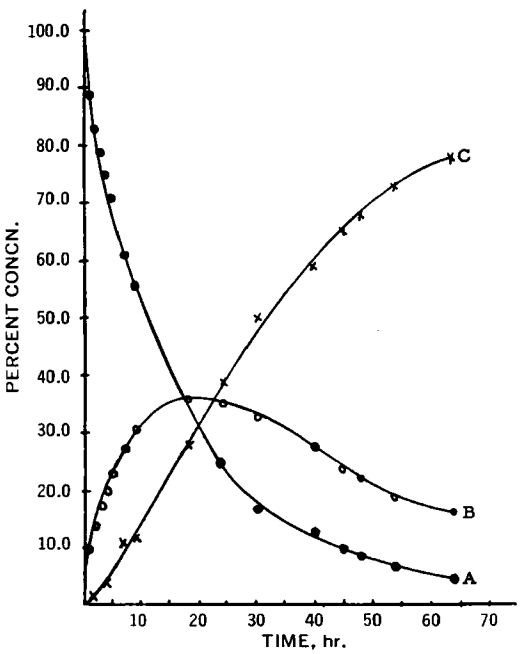

Figure 1—*Concentration-time curves for substances A (hydrocortisone hemiester), B (hydrocortisone alcohol), and C (degradation products) at 70° and a pH of 6.9.*

[1] Solu-Cortef, supplied through the courtesy of The Upjohn Company.

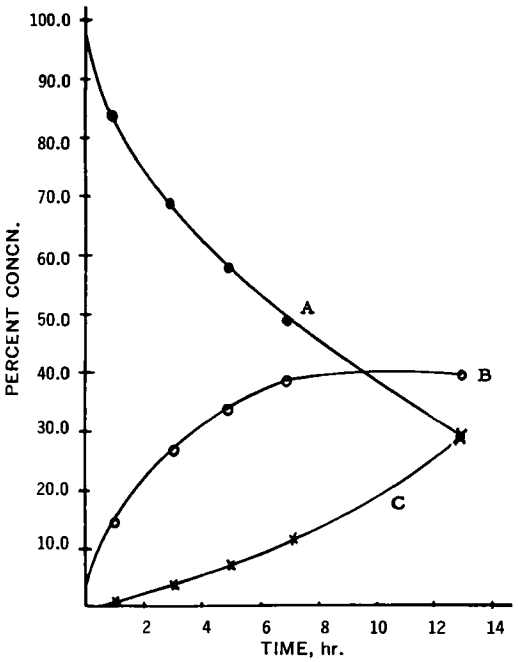

Figure 2—*Concentration-time curves for substances A (hydrocortisone hemiester), B (hydrocortisone alcohol), and C (degradation products) at 70° and a pH of 7.2.*

pletion of the irreversible reaction. The concentration of the final products, C, is seen to be about 80% after 64 hr.

It is also interesting to note that the initial content of the steroidal ester does not quite approach the theoretical concentration of 100%. This is possibly due to a small amount of steroidal alcohol initially present which could not be detected in significant quantities with the analytical procedure used.

In the above system and those subsequently studied at pH values 7.2 and 7.6, it should be pointed out that the hydrogen-ion concentration was maintained by the use of 0.02 *M* phosphate buffers at a constant ionic strength of 0.054.

Shown in Fig. 2 is a plot of the concentrations of the relevant species, A, B, and C, *versus* time at a pH value of 7.2.

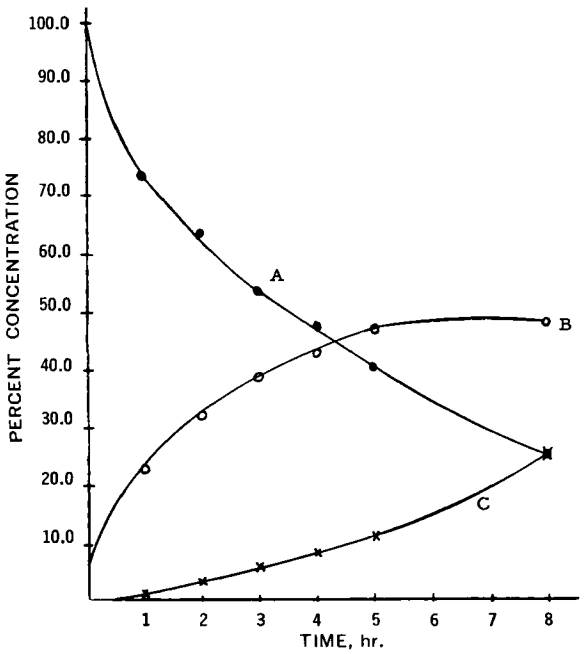

Figure 3—*Concentration-time curves for substances A (hydrocortisone hemiester), B (hydrocortisone alcohol), and C (degradation products) at 70° and a pH of 7.6.*

Table I—Percent Concentrations of the Steroid Ester (A), Steroid Alcohol (B), and Products Devoid of the 17-Dihydroxyacetone Side Chain (C) and Their Time Course at pH 6.9

Time, hr.	% A	% B	% C
1	89.5	10.1	0.4
2	83.2	14.5	2.3
3	79.4	17.9	2.7
4	75.7	20.1	4.2
5	71.5	23.5	5.0
7	61.1	27.9	11.1
9	56.1	31.1	12.7
18	35.4	36.0	28.5
24	25.4	35.2	39.4
30	17.0	33.4	49.6
40	13.1	28.4	58.6
45	10.4	24.1	65.5
48	9.2	22.8	68.0
54	7.4	19.5	73.1
64	5.2	16.7	78.1

Table II—Percent Concentrations of the Steroid Ester (A), Steroid Alcohol (B), and Products Devoid of the 17-Dihydroxyacetone Side Chain (C) and Their Time Course at pH 7.2

Time, hr.	% A	% B	% C
1	83.8	14.9	1.3
3	69.1	27.1	3.8
5	58.4	34.1	7.5
7	48.5	39.0	12.5
13	29.9	40.9	29.2

The concentration of A is seen to approach a final value asymtotically as the reaction proceeds toward completion. At all pH levels investigated, the decrease of A is by a pseudo first-order rate since the solvent (water) is present in large excess. The steroidal alcohol content, B, reaches a maximum after about 13 hr. and occurs at a substantially earlier time than with a pH of 6.9. The concentration of C reaches about 30% as B is maximized.

Figure 3 is a plot of the concentrations of A, B, and C *versus* time at a pH of 7.6. It will be noted that this figure parallels those previously shown although A decreases much more rapidly as the pH increases. Here, the content of B reaches a maximum after only 8 hr. It must be mentioned, however, that the second rate constant depends not only upon the time at which B is maximized, but also depends on the concentration at this point. Examination of the plot shows that B reaches its maximum at a concentration of about 48% compared to a maximum value of approximately 40% from Fig. 2. This indicates that less C has actually been formed at a pH of 7.6, although the maximum has occurred earlier. The concentration of products devoid of the 17-dihydroxyacetone side chain is about 25% at this point. Reference to Figs. 2 and 3 indicates that no more data were collected in time past the point where the concentration of B approximates a maximum. It was felt that the concentrations of B at these points were in fact approaching a finite value since the slopes are very close to zero. The data for the concentration of each species for all pH levels investigated are shown in Tables I–III.

The equations describing a first-order consecutive reaction were first integrated by Harcourt and Esson (10) and predict that the rate of hydrolysis of the steroid ester may be evaluated by a semilog plot

Table III—Percent Concentations of the Steroid Ester (A), Steroid Alcohol (B), and Products Devoid of the 17-Dihydroxyacetone Side Chain (C) and Their Time Course at pH 7.6

Time, hr.	% A	% B	% C
1	74.4	23.4	2.2
2	63.9	32.6	3.5
3	54.4	39.4	6.2
4	47.6	43.4	9.0
5	40.7	47.5	11.9
8	25.6	48.7	25.9

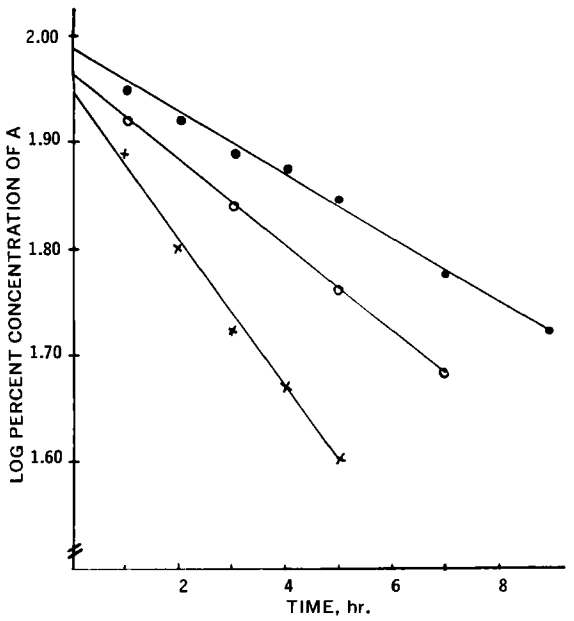

Figure 4—*Pseudo-first-order degradation of hydrocortisone sodium succinate at pH values of 6.9, 7.2, and 7.9 at 70°. Key: ●, pH 6.9; ○, pH 7.2; ×, pH 7.9.*

of the concentration of the ester *versus* time. Plots of this type for pH values of 6.9, 7.2, and 7.6 are shown in Fig. 4.

Examination of the figure indicates that the rates of hydrolysis of the steroidal ester increases with an increase in pH. This is consistent with the results of previous work (1, 2) done with 21-hydrocortisone hemisuccinate in this pH area. It can be seen that the hydrolysis rate at a value of pH 7.6 is approximately three times greater than at pH 6.9.

The calculated initial amount of steroidal ester is seen in Fig. 4 to deviate from the theoretical concentration of 100%. As mentioned previously, this is felt to be due to a small amount of steroid alcohol initially present.

Good linearity is observed at each pH level indicating that the disappearance of 21-hydrocortisone hemisuccinate is by a pseudo-first-order rate. The data for these plots were treated by the method of least squares in order to evaluate the slopes. The derived first-order rate constants are shown in Table IV.

Comparison of Tables IV and V indicates reasonable agreement between the experimental and theoretical values. The greatest deviation occurs at pH 7.6 which suggests that with decreasing hydrogen-ion concentration the hydrolysis may be attributed to a hydroxyl-ion catalysis. The validity of this assumption is substantiated by calculating a value from an equation derived to express the rate constant in terms of specific hydroxyl-ion catalysis (Eq. 6 of *Reference 1*). The rate constant calculated in this manner is approximately 0.1 per hour and agrees very well with the experimental value. Further, it should be noted that the expression used to calculate the values in Table V contains constants derived from the hydrolysis of hydrocortisone hemisuccinate in a media of 30% alcohol and 70% water, while the system under study was aqueous.

Table IV—First-Order Rate Constants for Hydrolysis of 21-Hydrocortisone Hemisuccinate in Aqueous Media[a]

pH	Rate Constant (k_1)/hr.
6.9	0.055
7.2	0.085
7.6	0.151

[a] Garrett (2) has derived an expression to calculate the first-order rate constants in the pH range where intramolecular hydrolysis is expected to occur. Theoretical values obtained from this equation are listed in Table V.

Table V—Derived First-Order Rate Constants for Hydrolysis of 21-Hydrocortisone Hemisuccinate

pH	Rate Constants (k_1)/hr.[a]
6.9	0.023
7.2	0.028
7.6	0.042

[a] Calculated from Eq. 15c of *Reference 2*.

Table VI—Summary of the β and $(1-\alpha)$ Values for Hydrocortisone Hemisuccinate at a pH of 6.9

Time, hr.	β	$(1-\alpha)$
1	0.101	0.105
2	0.145	0.148
3	0.179	0.206
4	0.201	0.243
5	0.235	0.285
7	0.279	0.389
9	0.311	0.439
18	0.360	0.646
24	0.352	0.746
30	0.334	0.830

There are several methods available (11–13) for the estimation of the second rate constant, which depend on the use of differential equations or dimensionless parameters derived from the rate expressions describing a consecutive first-order reaction.

Frost and Pearson (12) have described equations relating the maximum occurring with the intermediate component, B, and its relationship to k_2 by the use of various analytical expressions. Jensen and Lamb (6) have applied this method successfully to their data where $\beta_{max.}$ was obtained by plotting β *versus* $(1-\alpha)$ and evaluating the maximum from the graph.

The symbol α is defined as the fraction of ester (A_0) decomposed. The following equations illustrate this method.

$$\beta = B/A_0 \qquad \text{(Eq. 1)}$$

$$\alpha = A/A_0 \qquad \text{(Eq. 2)}$$

$$\kappa = k_2/k_1 \qquad \text{(Eq. 3)}$$

$$\beta_{max.} = \kappa^{\kappa/1-\kappa} \qquad \text{(Eq. 4)}$$

where β, α, and κ are dimensionless parameters and B is defined as the concentration of the intermediate species or the steroid alcohol, A and A_0 are, respectively, the concentration of ester at time t and initially. It will be noted from Eq. 4 that the ratio of k_2/k_1; *i.e.*, κ, is related to $\beta_{max.}$ and subsequent to the calculation of κ, k_2 may easily be evaluated since k_1 is already known. The data in Tables VI–VIII were evaluated in terms of the dimensionless parameters and plots of β *versus* $(1-\alpha)$ are shown in Figs. 5–7 for the three pH values used.

Table VII—Values of β and $(1-\alpha)$ at a pH of 7.2

Time, hr.	β	$(1-\alpha)$
1	0.149	0.162
3	0.271	0.309
5	0.341	0.416
7	0.390	0.515
13	0.409	0.701

Table VIII—Values of β and $(1-\alpha)$ at a pH of 7.6

Time, hr.	β	$(1-\alpha)$
1	0.234	0.256
2	0.326	0.361
3	0.394	0.456
4	0.434	0.524
5	0.475	0.593
8	0.487	0.744

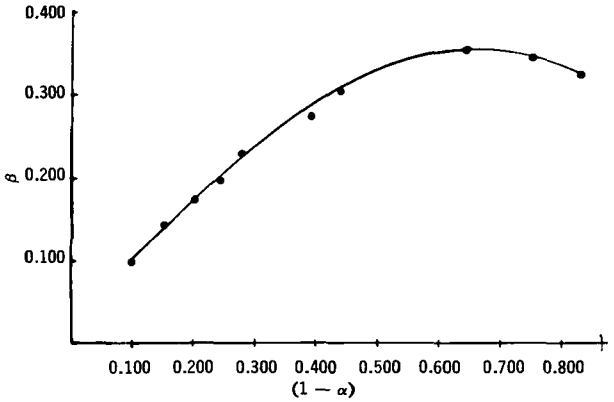

Figure 5—*Plot of* β versus $(1 - \alpha)$ *at a pH value of 6.9.*

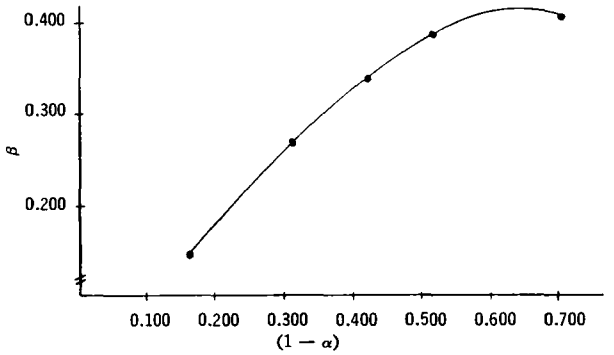

Figure 6—*Plot of* β versus $(1 - \alpha)$ *at a pH value of 7.2.*

Examination of the plots indicates that maximum is increasing in systems of increasing basicity. In Table IX the results of this method relative to the $\beta_{max.}$ and κ values are given. It can easily be seen that the values are decreasing with increasing $\beta_{max.}$ values. Hence, since k_2 is the product of k_1 and κ, k_2 depends not only on the magnitude of k_1, but also the point at which B is maximized.

Subsequent to the calculation of k_2/k_1, the second rate constants at the various pH levels may be derived. Table X summarizes the calculated k_2 values.

The magnitude of the second rate constants would seem to be substantiated by the data of Oesterling and Guttman (4) who

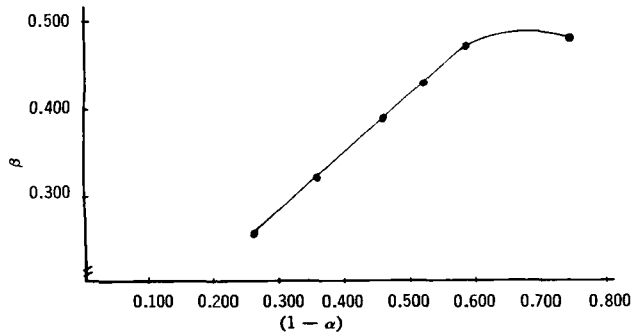

Figure 7—*Plot of* β versus $(1 - \alpha)$ *at a pH value of 7.6.*

Table IX—Values of κ and $\beta_{max.}$ at pH Values of 6.9, 7.2, and 7.6

pH	$\beta_{max.}$	$k_2/k_1 = \kappa$
6.9	0.363	0.907
7.2	0.415	0.790
7.6	0.490	0.530

Table X—Second Rate Constants for the Degradation of 21-Hydrocortisone Hemisuccinate

pH	Rate Constant (k_2)/hr.
6.9	0.050
7.2	0.067
7.6	0.080

graphically showed the degradation of prednisolone at 70° in an aqueous media to be approximately 0.06–0.07 per hour in the pH range of 7–7.5.

SUMMARY

1. The degradation of 21-hydrocortisone hemisuccinate was followed at 70° in aqueous solutions buffered at pH values of 6.9, 7.2, and 7.6. Resultant assay data indicated that the overall kinetic pathway at each pH level could be interpreted as consecutive first-order.

2. Ester hydrolysis is presumed to involve an intramolecular attack of the anion on the ester carbonyl carbon or specific hydroxyl-ion catalysis of the undissociated hemiester.

3. Rate constants corresponding to the degradation of the steroid ester to its free alcohol and products devoid of the 17-dihydroxyacetone side chain have been calculated.

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