I. THE APPARENT ACID DISSOCIATION CONSTANTS OF PHENYLALANINE AND SOME OF ITS NUCLEARLY SUBSTITUTED DERIVATIVES

II. THE ISOLATION OF SPHINGOSINE FROM BOVINE SPINAL CORD

III. THE ULTRAVIOLET ABSORPTION OF SOME O-BENZOYL COMPOUNDS

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ABSTRACT

I. Apparent dissociation constants for the following compounds were determined by electrometric titration or ultraviolet spectroscopy: phenylalanine, ortho-, meta-, and para-chloro-phenylalanines, para-sulfamido-phenylalanine, benzene sulfonamide, toluene sulfonamide, and methane sulfonamide.

II. The basic hydrolysis of sphingolipids to obtain sphingosine and psychosine was investigated, using both liquid ammonia and anhydrous hydrazine. Dilituric acid was found to be an excellent reagent for the precipitation of the sphingosine bases. The presence of dihydrosphingosine in bovine spinal cord was confirmed by isolation of the tribenzoyl derivative. The ultraviolet light extinction curve of this derivative was determined in ethanol.

III. The ultraviolet light extinction curves of methyl benzoate, "n-Octadecyl" benzoate, d,l-dibenzoyl n-octadecandiol-1,2, and benzoic anhydride were determined in hexane and those of the first and third compounds in ethanol.

TABLE OF CONTENTS

I. THE APPARENT ACID DISSOCIATION CONSTANTS OF PHENYLALANINE AND SOME OF ITS NUCLEARLY SUBSTITUTED DERIVATIVES

PURPOSE.

In connection with the general study of protein structure underway at this Institute special attention is being directed to phenylalanine, its derivatives, and analogs* as possible substrates for studies in the enzymatic synthesis and degradation of peptides. Carboxypeptidase, chymotrypsin, and pepsin are particularly active on substrates containing phenylalanine moieties (5). It is hoped to obtain a range of properties in substrates incorporating such derivatives and analogs that would further characterize the enzyme systems. Two factors are at once evident which would influence the enzymatic reaction, namely: the shape of the substrate molecules and their acid strengths. The present investigation was undertaken to assemble data on the latter property of some amino acids.

It was originally planned to determine the three pK_a' values of p-sulfamido-phenylalanine by titration, assigning the different values to appropriate groups with the aid of a value for the dissociation of the sulfamido group independently established by ultraviolet spectroscopy. As a check on the technique employed, phenylalanine was then investigated--rather thoroughly when the constants found were significantly different from literature values; finally, the chloro derivative

*E.g., the pyridyl alanines (24), the fluoro-phenylalanines (4), the bromo- and iodo-phenylalanines (23), in addition to the chloro- and sulfamido-phenylalanines of this thesis.

was measured to obtain an idea of the magnitude of the purely inductive effects of substituents in the ring on the dissociation constants.

METHODS.

All the data reported were obtained by either a titration or an ultraviolet spectroscopic procedure . Both methods and the calculations used with each will be described and discussed first, before presenting the experimental results.

Titration Procedure : Twenty milliliter aliquots of 0.01-0.02 M amino acid in aqueous sodium chloride solutions of the desired ionic strength were titrated with standard 0.2 N acid or base. Adequate agitation during a run was secured by carrying out the titrations in a 50 ml. beaker mounted in a large cork which in turn was mounted on the shaft of an inverted air-stirrer. With the stirrer going the solution was carried past the stationary electrodes by the rotating beaker. Mixing is believed to have been sufficient, as after twenty seconds no fluctuations in pH were noticed.

The pH of the solution was measured after each addition of acid or base, using a Beckman pH Meter, Model G, equipped with external calomel (#1170) and glass (#1190E, for use in solutions containing sodium ion in the range pH 9 to 14) electrodes. The meter was standardized against Beckman buffers of pH 4.00, 7.00, or 10.00 before each series of runs and again at the end of the series; in no case did the instrument vary more than 0.02 pH-units from the standard after a series

of runs up to two hours long.

The temperature was measured at the end of each titration, with no attempt made to keep it constant during the run. All data was obtained in the range 22.3-27.5°C. The error thus introduced is probably small in pK'_{COOH} as the heat of ionization of the carboxyl group should be not more than ±500 cal.s/mole(9b). However, if the heat of ionization of the ammonium group is of the order of -10,000 cal.s/mole, as it is for most amino acids(9b), then a change of 2.5°C would produce a fluctuation in pK_a' of approximately ±0.06. This is about the limit of accuracy claimed for the values reported here, based on the observed deviations and the experimental technique.

Titration Calculations; Non-overlapping Ionizations (7a): If the dissociation constants of a polybasic acid or polyacidic base are sufficiently different in magnitude there is no interference in the ionization of one group by the second. In this case it is possible to treat the ionization of each group separately (i.e., as though each were a separate monofunctional acid or base) thus greatly simplifying the calculation of the ionization constants from titration data. The monoamino, mono-carboxylic acids belong in this category, since the acid dissociations of the ammonium and carboxyl groups differ by a factor of about a million (9a). The data for phenylalanine and the three chloro-phenylalanines were treated by this method.

The ionizations and constants formulated by the dipolar ion structure for an α-amino acid are:

$$H_3N^+ CHRCOOH \underset{+H^+}{\overset{-H^+}{\rightleftharpoons}} H_3N^+ CHRCOO^- \underset{+H^+}{\overset{-H^+}{\rightleftharpoons}} H_2NCHRCOO^- \qquad (1)$$

$$K_{COOH} = [H^+][H_3N^+CHRCOO^-] / [H_3N^+CHRCOOH] \qquad (2)$$

$$K_{NH_3^+} = [H^+][H_2NCHRCOO^-] / [H_3N^+CHRCOO^-] \qquad (3)$$

where the square brackets denote activities.

If we generalize the reaction involved in the titration of an amino acid solution with standard acid to:

$$B + H^+ \longrightarrow A \quad , \qquad (4)$$

then the dissociation constant, K_a, is given by:

$$K_a = [H^+][B] / [A] \quad . \qquad (5)$$

In this investigation the activity coefficients of A and B were assumed to be unity, and so we can replace activities by concentration in moles per liter of solution. Further, the titrations were carried out in solutions of finite ionic strength; therefore the dissociation constants found are 'apparent dissociation constants', K_d (9c).

If

$$pH = -\log_{10}(H^+)^* \quad , \qquad (6)$$

Eqn. 5 becomes:

$$K_a' = (H^+)(B)/(A) \quad , \qquad (5a)$$

where the brackets represent molar concentrations.

Since the total amount of amino acid present is fixed by the concentration of the solution used and the volume of the aliquot taken, if all the added hydrogen ion reacts with amino acid K_a' is given by Eqn. 7.

$$K_a' = (H^+)(C - y)/y \qquad (7)$$

where y = ml. of standard acid added

C = ml. of standard acid needed to react completely with all the amino acid present;

also $C = v_a m_a/n$,

if v_a = volume of amino acid solution aliquot

m_a = molarity " " "

n = normality of standard acid solution.

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*This is a working equation only and does not imply that the 'pH' measured is the quantity called by Bates (1) "p_cH". Note that it is 'pH' which is experimentally determined and that the concentration of hydrogen ion is calculated from it. Since the nature of the buffers used to standardize the pH meter is uncertain, the exact quantity measured and here denoted by 'pH' is uncertain; consequently it seems least objectional to call the hydrogen ion concentration calculated from Eqn. 6 the molar concentration.

However, all the hydrogen ion added does not react with amino acid. A correction for the amount not reacting can be found from the pH observed. Eqn. 7 then becomes:

$$K_a' = (H^+)(C - v_{hc})/v_{hc} \qquad (7a)$$

where v_{hc} = y - correction for H^+ not reacting with B.

Similar considerations lead to Eqn. 9 when titrating an amino acid solution with standard base according to the generalized equation:

$$A + OH^- \longrightarrow B + H_2O \quad ; \qquad (8)$$

$$K_a' = (H^+)\, v_{oh}/(C' - v_{oh}) \quad , \qquad (9)$$

where v_{oh} = corrected volume of base of normality n' added

$$C' = v_a m_a/n' \quad .$$

The correction for the amount of acid or base added which does not react with amino acid can be determined empirically by titrating a similar aliquot containing the same initial concentration of sodium chloride--the so-called 'water blank'--or one can assume that the pH meter corrects for the difference in activity coefficients of hydrochloric acid in sodium chloride solution over pure water and calculate the amount of hydrogen or hydroxyl ion necessary to cause the observed change in pH of the volume of solution involved. In the practical application of the empirical method the correction is made in two steps, one depending on the pH measured and the second

correcting for the difference in total volume between the amino acid solution (found by assuming that the volumes of aliquot and acid or base are additive) and the blank solution at the same pH.

In the pH range 9 - 11.5 the assumption involved in the second method was found to be valid within experimental error, and the corrections were made by either method. But in the range pH 1 to pH 3 the blank titration was higher than the calculated correction. Consequently all titrations to determine the carboxyl dissociations were corrected empirically.

The method used here to calculate K_a' is somewhat more involved than that usually employed in this field (21), which makes use of the relation:

$$pK_a' = \text{pH at half equivalence point.} \qquad (10)$$

A graph of pH versus the effective amount of reagent added is made and the pH of the point at which one half of the stoichiometric amount of reagent has been added is interpolated. A single value of K_a' is obtained from a titration, and this value is particularly sensitive to errors in the experimental points immediately adjacent to the half-equivalence point. The method of calculation used in this thesis, on the other hand, gives a value of K_a' for each addition of reagent; the average of these values is less sensitive to possible errors in a single point. Furthermore, having K_a' for each point makes it possible to evaluate the adequacy of the corrections for unre-

acted acid or base. Examination of the data (see Tables I and II for typical titrations) reveals that the values of K_a' are reasonably constant until the magnitude of the correction becomes comparable to, or exceeds, the effective volume of reagent added, when the dissociation constant calculated may deviate widely from the average value for the first part of the titration. One would expect errors to become more apparent toward the end of the titration, since: a) then the correction applied is a major fraction of the burette reading; b) at the higher concentrations of hydrogen or hydroxyl ions the assumptions made in deriving Eqns. 7a and 9 may be in serious error; c) the magnitude of any error made by the assumption of additivity of volumes is increased as the total volume increases; and d) possible impurities may begin to dissociate in the higher concentrations of hydrogen and hydroxyl ions.

This deviation of K_a' toward the end of the titration was noted early in the investigation; for this reason and because the magnitude of the correction made it difficult to estimate during the run the effective amount of reagent added, few points were taken past the half-equivalence point. The first part of the titration is further weighted in the average values of K_a' reported, since in the absence of a more satisfactory method of evaluation, all K_a' values were discarded which deviated from the arithmetic mean of the remaining values by more than three times the average deviation from the mean of

these remaining values*.

A number of K_a' values were also determined by the graphical method and in all instances the two values agreed within experimental error. For example, the data of Table II, which gives an average value of $pK'_{NH_3^+} = 9.10$, by the graphical method gave $pK'_{NH_3^+} = 9.11$.

A modification of the graphical method of determining the pK_a' values of amino acids from titration has been used by Hirsch (17), who calculates a function which when plotted against pH results in a curve similar to the normal titration curve. The pH of the mid-point of this curve is equal to pK_a'. There are no apparent advantages for Hirsch's more complicated method.

Titration Calculation; Overlapping Ionization: With dibasic acids whose dissociation constants differ by a factor of 1000 or less (2) it is no longer true that the dissociation of one group is complete before the second begins to ionize. Consequently Eqns. 7a and 9 cannot be used to calculate K_a'.

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*In error theory (10A), if we have a Gaussian distribution of values about a mean, M, whose standard deviation is k, then the probability of finding a value with a standard deviation from M of more than 3k is 0.002, i.e., 0.2%. The 'standard deviation' d_s, for which this probability is valid is somewhat larger than the 'average absolute deviation', d_a, used in this thesis, as the following definitions show:

$$d_a = (1/N_o)\sum|x_i - \bar{x}| \quad , \quad \text{and} \quad d_s = \sqrt{(1/N_o)\sum(x_i - \bar{x})^2},$$

where N_o = total number of values in the set
x_i = the i 'th value
$\bar{x}$ = arithmetic mean of all N_o values.

Instead we must consider the simultaneous ionization of both groups. The preceedure used in this thesis is that of Britton (7b), which makes it possible to calculate values of both K_1' and K_2' from each pair of experimental points. Two points are necessary, since there are five unknowns and only four equations relating them. Britton's equations are:

$$K_1' = (A'D - AD')/(BD' - B'D) \qquad (11)$$

$$K_2' = (AB' - A'B)/(AD' - A'D), \qquad (12)$$

where $A = (H^+)^2\left[a + (H^+) - (OH^-)\right]$

$B = (H^+)\left[a - c + (H^+) - (OH^-)\right]$

$D = 2c - a - (H^+) + (OH^-)$,

and a = total effective concentration of standard base added

c = total concentration of amino acid present;

primes denote values from a second experimental point.

The hydrogen ion concentration was found by Eqn. 6, the hydroxyl ion concentration from K_w taken equal to $10^{-14.00}$. Here also it is necessary to correct for the amount of added base which does not react with amino acid, as previously described.

Values of A, B, and D from a representative titration of p-sulfamido-phenylalanine are given in Table III and values of K_1' and K_2' calculated therefrom in Table IV. It is seen that there is a pH range in which it is possible to obtain valid values of both dissociation constants, indicating that in this range the two acid groups are dissociating simultaneously.

Deviation in the value of K_2' is noted toward the end of the titration.

Speakman (26) discusses a graphical method for determining the thermodynamic dissociation constants, K_1 and K_2, using the quantities A, B, and D of Britton's equations in terms of activities. One plots A/D versus (-B/D), obtaining a straight line whose slope is equal to K_1 and whose intercept on the A/D axis equals K_1K_2. The values of A/D and B/D vary over such a wide range that it is not convenient to plot all values obtained on a sufficient scale to determine the intercept accurately. Further, the accumulated errors fall on K_2. Despite the more laborious calculations it is felt that Britton's method yields more information. For the data of Table IV, Speakman's method (again assuming that all activity coefficients equal unity) gives $K_1' = 2.2 \times 10^{-9}$ and $K_2' = 6.0 \times 10^{-11}$, both within experimental error of the values obtained from Eqn. 11 and 12, namely: $K_1' = 2.3 \times 10^{-9}$ and $K_2' = 5.6 \times 10^{-11}$.

Bates (2) has recently discussed the determination of thermodynamic dissociation constants for polybasic acids of overlapping ionizations, using the acid salts in cells without liquid junctions. He gives references to the earlier literature.

Ultraviolet Spectroscopy. Procedure: In 1926 Stenström and Goldsmith (27) showed that it was possible to determine the dissociation constants of phenols from the change in their ultraviolet absorption spectra with hydrogen ion concentration. They

applied this method to phenol and tyrosine. It has since been used by others to determine the dissociation of phenolic hydroxyls in 2,4-dinitrophenol (16a), tyrosine (10), (12), 3,4-dihydroxy-phenylalanine (13), and 3,5-diiodo-tyrosine (10); for the aromatic amino groups in derivatives of p-amino-benzoic acid and sulfanilamide (18); for the carboxyl group of benzoic acid (16a); and for the addition of a proton to aniline, anthraquinone, and some carboxylic acids and acetophenones (16). In this investigation the method was used in an effort to determine independently the K_a' value of the sulfamido group in p-sulfamido-phenylalanine, after the procedure had been worked out for p-toluene sulfonamide and benzene sulfonamide.

Aqueous solutions approximately 0.001 M in the sulfonamide were prepared by dissolving weighed samples in enough sodium chloride solution of the desired pH to make 100.0 ml. The amounts of sodium chloride and hydrochloric acid or sodium hydroxide were adjusted to give a total ionic strength of about 0.12. The pH of the resulting solution was measured and its ultraviolet absorption determined, against the sodium chloride solution used as solvent, in a Beckman Model DU Quartz Spectrophotometer, using a hydrogen lamp as light source and matched 1 cm. quartz cells. Determinations were at room temperature, 25 ± 3°C. The spectra in 0.1 N hydrochloric acid and 0.1 N sodium hydroxide were measured at intervals of 2 mμ or less from the lower limit attainable with the spectro-

photometer out to 280 mμ, and then in 10 mμ steps to 320 mμ. These spectra were used to select the two or three wavelengths to be used for the determination of dissociation constants. Other solutions were then made up within 2 pH-units on either side of the expected pK_a' value and the extinction at the selected wavelengths measured. K_a' was calculated as outlined in the next section.

Theoretically any wavelength will serve to determine K_a', but in practice one picks a wavelength where the extinction in acid and base is sufficiently different to give good precision in the calculation and tries to use portions of the curve where the variation in extinction with wavelength is small.

Ultraviolet Spectroscopy. Calculation: The calculation of K_a' from the observed extinction is based on the following assumptions: a) in strong acid solution the absorbing species is the undissociated acid and in strong base it is the completely ionized salt; at intermediate pH values the observed extinctions are entirely due to varying amounts of the undissociated and dissociated molecules; as a corollary, b) there is no specific ion effect on the absorption of either form by the ions of the buffer system employed. In the case of the substituted phenylalanines one makes the further assumption that: c) the dissociations of the side-chain ammonium and carboxyl groups do not alter the absorption spectra of the molecule. There is some justification for this last assumption in that Stenström & Reinhard (28) found no difference in the ultra-

violet absorption spectrum of phenylalanine with changing pH*, and in the fact that with this assumption it has proven possible to calculate for a number of phenylalanine derivatives ionization constants in good agreement with values obtained by other methods (10), (18), (27).

The assumptions lead to the following equation for a given wavelength:

$$E_{obs} = E_d X + E_u(1 - X) \quad , \tag{13}$$

where E_{obs} = observed molar** extinction

E_d = molar extinction in strong base

E_u = molar extinction in strong acid

X = fraction of molecules ionized.

Hence $$X = (E_{obs} - E_u)/(E_d - E_u). \tag{13a}$$

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*See however the discussion of the work of the French school, below, and the results obtained with p-sulfamido-phenylalanine in this thesis.

** 'E' is defined according to the American convention (8):

$$e = Ecd = -\log_{10}(I/I_o),$$

where e = extinction read from spectrophotometer at a given wave length
E = molar extinction coefficient
c = concentration in moles per liter of solution
d = thickness of absorbing layer in centimeters
I = intensity of the light transmitted
I_o = intensity of the incident light.

In the British convention (6) this quantity is termed the "molecular" extinction coefficient.

Expressing Eqn. 5a in terms of X and substituting the above value, we get for K_a':

$$K_a' = (H^+)\ X/(1 - X)$$
$$= (H^+)(E_{obs} - E_u)/(E_d - E_{obs}) \quad . \qquad (14)$$

Other methods of calculation have been used by Stenström and Goldsmith (27) and by Vlès and Gex (30). The former authors plotted the observed extinction values against pH and obtained an ogee curve, similar to the usual titration curve. The pH corresponding to the mean value of $(E_d + E_u)$ was taken equal to the pK_a'. This is a convenient graphical method of averaging, but requires an excessively large number of determinations to achieve the desired accuracy.

Vlès and Gex use a considerably more complicated method. They plot the ratio of E_{obs} at two different wavelengths versus pH and by proper choice of the wavelengths obtain a curve with a number of "sinuosités". By a method of successive approximations a theoretical expression is obtained which fits the experimental points and which allows one to determine the K_a' values. The method has been used to calculate various constants in good agreement with values obtained by other means (12), (13), (30). The startling feature which throws doubt on the general validity of their method is that it yields all three K_a' values for tyrosine (12), contrary to the assumptions made by Stenström and Goldsmith (27) and by Crammer and Neuberger (10) that the side-chain carboxyl and ammonium groups did not contribute to the ultraviolet spectrum of tyrosine.

This fact in itself might mean that the French method is more versatile and powerful than Stenström and Goldsmith's. But Vlès and Gex by this method have obtained two dissociation constants for benzoic acid, four for nitric acid, and six for benzene, ranging from pK_1 of 1.73 to pK_6 of 11.5. This is certainly not in accord with the accepted structure and chemical properties of benzene, and requires explanation before any values obtained in this way can be accepted.

EXPERIMENTAL.

Materials: A student preparation of p-toluene sulfonamide was purified by solution in 5% sodium hydroxide and reprecipitation with dilute hydrochloric acid, followed by recrystallization from 50% aqueous ethanol; m.p. 135.7-137.0°C (corr.). The benzene sulfonamide was Eastman White Label, used without purification. The methane sulfonamide was prepared from the acid chloride in benzene by the addition of gaseous ammonia. It was recrystallized twice from benzene-ethanol; m. p. 91.0-91.6°C (corr.). Eastman White Label p-toluene sulfonic acid was used without purification.

Winthrop Chemical d,l-phenylalanine was recrystallized three times from water. The three isomeric chloro-phenylalanines were synthetic d,l-mixtures prepared by Dr. W. E. Shelberg and were used without further purification, except the o-chlorophenylalanine, which was found to contain a water-insoluble, colored impurity. Most of the data for this isomer were obtained on filtered solutions, but one solution

was prepared from material twice recrystallized from aqueous 90% methanol whose ultraviolet absorption showed that practically all the impurity had been removed; aliquots from this solution gave pK_a' values identical within experimental error with those from filtered solutions. The spectra of the ortho and para isomers shown in Fig. I are those of twice recrystallized material. The d,l-p-sulfamidophenylalanine was synthesized by Dr. Carl Niemann and was used without further purification.

Results: The data for the dissociation constants of phenylalanine are presented in Table V, followed by the K_a' values for the chloro-phenylalanines in Tables VI and VII.

The values of pK_a' reported in the tables are followed by the average absolute deviation from the mean of the data. These deviations indicate the scatter of the individual values, but are not intended as a measure of the accuracy of the constants. Measurements in solutions containing 0.050, 0.10, and 0.20 M sodium chloride were used to ascertain the effect of ionic strength on the ionization constants. It is seen from Tables V, VII, and VIII that the variations in the constants with ionic strength in this range are the same order of magnitude as the experimental error --$\pm$0.05 in pK_a' --for the three amino acids in question. Consequently the final average values of pK_a' given in Table XII include all values obtained, regardless of ionic strength.

The ultraviolet absorption spectrum of phenylalanine is given in Fig. V, those of the three chloro-phenylalanines in Fig. I. Because the volumetric equipment used was not specially calibrated it is not claimed that the molar extinction values reported are more accurate than ±5%. The wavelengths should be accurate to better than ±0.5 mμ (14).

The results of the ultraviolet method for the dissociation constants of the simple sulfonamides is given in Tables IX and X and in Figs. II to IV, while for comparison the spectrum of p-toluene sulfonic acid in the ultraviolet is also shown in Fig. III. In the case of the sulfonamides the dissociation which is assumed to occur is:

$$AR\text{-}SO_2NH_2 \longrightarrow AR\text{-}SO_2NH^- + H^+ \qquad (15)$$

The dissociation constants obtained from ultraviolet spectra for p-sulfamido-phenylalanine are given in Table XI and the results of titration in Table VIII. The ultraviolet light extinction curve for this amino acid in 0.1 N hydrochloric acid and in 0.1 N sodium hydroxide is shown in Fig. V.

TABLE I

K'_{COOH} FOR PHENYLALANINE FROM TITRATION

pH	Burette reading	pH corr'n	pH plus volume corr'n	Corr'd vol. HCl	$K' \times 10^3$
4.73	0.00	-	-	0.00	-
3.47	0.12	0.02	-	0.10	6
3.16	0.25	0.04	-	0.21	5.7
2.83	0.49	0.11	-	0.38	6.1
2.48	0.98	0.32	0.33	0.65	6.6
2.06	2.00	0.78	0.83	1.17	5.7
1.72	3.51	1.90	2.04	1.47	6.1
1.50	5.48	3.30	3.70	1.78	2.8
1.34	8.00	5.70	6.21	1.79	3.8

20.00 ml. 0.0231 M phenylalanine in 0.10 M sodium chloride titrated with 0.2089 N hydrochloric acid; final temperature was 26.0°C.

TABLE II

$K'_{NH_3^+}$ FOR PHENYLALANINE FROM TITRATION

pH	Burette reading	pH corr'n	pH plus volume corr'n	Corr'd volume NaOH	$K' \times 10^{10}$
4.76	0.00	-	-	0.00	-
7.74	0.10	0.01	-	0.09	8.
8.18	0.26	0.01	-	0.25	8.2
8.55	0.50	0.02	-	0.48	7.6
9.00	1.00	0.02	-	0.98	7.7
9.64	1.77	0.02	-	1.75	8.0
11.36	2.72	0.45	0.58	2.14	0.85

20.02 ml. of 0.02031 M phenylalanine in 0.10 M sodium chloride titrated with 0.1811 N NaOH; final temperature = 25.3°C.

TABLE III

TITRATION OF p-SULFAMIDO-PHENYLALANINE WITH STANDARD BASE

pH	v_{oh} (ml.)	Total vol. sol'n	a x 100	c x 100	A	B	D x 100
4.15	0.00	20.00	0.00	2.05	3.53×10^{-13}	-1.450×10^{-6}	4.10
7.42	0.13	20.15	0.117	2.03	1.68×10^{-18}	-7.25×10^{-10}	3.94
7.93	0.37	20.39	0.331	2.01	4.52×10^{-19}	-1.97×10^{-10}	3.69
8.49	0.94	20.96	0.81	1.958	8.4×10^{-20}	-3.7×10^{-11}	3.11
9.02	1.68	21.71	1.403	1.890	1.275×10^{-20}	-4.65×10^{-12}	2.38
9.60	2.46	22.49	1.981	1.822	2.242×10^{-21}	3.89×10^{-13}	1.667
10.16	3.28	23.31	2.55	1.760	1.210×10^{-22}	5.4×10^{-13}	0.98
10.78	4.07	24.21	3.05	1.694	8.19×10^{-24}	2.15×10^{-13}	0.40
11.37	4.46	24.99	3.24	1.640	5.45×10^{-25}	5.8×10^{-14}	0.27
11.75	4.76	26.00	3.32	1.579	8.70×10^{-26}	2.10×10^{-14}	0.40

20.00 ml. 0.02049 M p-sulfamido-phenylalanine in 0.050 M sodium chloride titrated with 0.1812 N sodium hydroxide; final temperature 27.5°C.

TABLE IV

K_1' AND K_2' FOR p-SULFAMIDO-PHENYLALANINE FROM TITRATION

pH_1-pH_2	$K_1' \times 10^9$	$K_2' \times 10^{11}$
4.15 - 7.42	(243)	-
7.42 - 7.93	2.3	-
7.93 - 8.49	2.3	negative
8.49 - 9.02	2.2	4.9
9.02 - 9.60	2.7	5.87
9.60 - 10.16	2.0	6.1
10.16 - 10.78	negative	5.34
10.78 - 11.37	-	(1.81)
7.42 - 8.49	2.3	-
7.42 - 9.02	2.3	-
7.93 - 9.02	2.5	-
9.02 - 10.16	-	6.1
9.02 - 10.78	-	5.5
9.60 - 10.78	-	5.5
Average of unbracketted val.	2.3	5.6

TABLE V

THE APPARENT DISSOCIATION CONSTANTS OF PHENYLALANINE

Sodium chloride (M/li.)	Amino acid (M/li.)	Final temp. (°C)	pK'_{COOH}	No. val.	Final temp. (°C)	$pK'_{NH_3^+}$	No. val.
0.050	0.02125	24.8	2.10 ± .02	9	24.3	9.14 ± .02	5
	"	25.0	2.10 .02	7	24.3	9.16 .04	6
	0.01907	23.0	2.22 .02	6	22.3	9.24 .01	4
	"	23.2	2.15 .04	8			
	Average all values		2.13 ± .05	30		9.17 ± .05	15
0.10	0.01928*	23.0	2.14 .03	7	24.5	9.13 .01	6
	0.02031	26.0	2.28 .04	7	25.3	9.10 .01	4
	"	26.0	2.22 .02	5			
	Average all values		2.21 ± .06	19		9.12 ± .02	10
0.20	0.02005	23.6	2.16 .05	5	24.0	9.12 .01	4
	"				24.0	9.11 .02	4
	Average all values		2.16 ± .05	5		9.12 ± .02	8

*Eastman White Label d,l-phenylalanine, not recrystallized.

TABLE VI

THE APPARENT DISSOCIATION CONSTANTS OF ORTHO- AND META-CHLORO-PHENYLALANINES IN 0.10 M SODIUM CHLORIDE

Amino acid	Conc'n M/li.	Final temp.	pK'_{COOH}	No. val.	Final temp.	$pK'_{NH_3^+}$	No. val.
o-Chloro-phenyl-alanine	0.01541	24.0	2.24±.03	6	22.6	8.94±.02	5
	"	24.0	2.25 .06	6	22.8	8.94 .01	5
	0.01768	22.5	2.21 .04	7	23.0	8.92 .02	6
	"	22.8	2.22 .06	7	23.3	8.93 .02	7
	0.01862*	25.7	2.22 .03	9	24.7	8.97 .02	8
	"	26.0	2.24 .05	11	25.0	8.97 .02	9
	Average all values		2.23±.04	45		8.94±.03	40
m-Chloro-phenyl-alanine	0.01519	25.0	2.19 .03	6	24.0	8.91 .02	6
	"	25.0	2.18 .01	5	24.3	8.91 .02	6
	0.01496	24.2	2.17 .04	6	24.6	8.90 .01	7
	"	24.0	2.17 .05	7	25.0	8.90 .01	6
	Average all values		2.17±.04	24		8.91±.01	25

*Twice recrystallized from 90% aqueous methanol

TABLE VII

THE APPARENT DISSOCIATION CONSTANTS OF p-CHLORO-PHENYLALANINE

NaCl (M/li)	Amino acid (M/li)	Final temp. (°C)	pK'_{COOH}	No. values	Final temp. (°C)	$pK'_{NH_3^+}$	No. values
0.050	0.00800	26.3	2.10±.04	3	23.7	8.99±.02	3
	"	26.3	2.19 .02	2	23.7	9.01 .01	2
	0.01502	25.2	2.13 .04	6	24.0	8.99 .02	3
	"	25.3	2.13 .02	4	24.0	9.00 .02	4
	Average all values		2.15±.04	15		9.00±.02	12
0.10	0.01114	23.7	2.13 .03	2	25.4	8.95 .02	4
	"	23.8	2.20 .05	4	25.7	8.93 .02	4
	Average all values		2.17±.05	6		8.94±.02	8
0.02	0.01543	24.7	2.00 .04	7	23.2	8.96 .02	5
	"	24.5	2.02 .04	8	23.3	8.95 .02	5
	0.01481	24.0	2.04 .05	7	24.3	8.92 .01	5
	"	24.0	2.05 .04	9	24.2	8.94 .03	5
	Average all values		2.03±.05	31		8.94±.02	20
Over-all average			2.08±.06			8.96±.03	

TABLE VIII

APPARENT DISSOCIATION CONSTANTS OF p-SULFAMIDO PHENYLALANINE FROM TITRATION

NaCl Conc'n (M/li)	Amino acid (M/li)	Final temp. (°C)	pK'_{COOH}	No.	Final temp. (°C)	$pK'_{NH_3^+}$	No.	$pK'_{SO_2NH_2}$	No.
0.050	0.02049	25.3	1.98±.01	7	27.5	8.64±.02	8	10.25±.03	10
0.10	0.02006	23.5	1.97 .03	11	25	8.64 .03	17	10.28 .04	17
	"	25.0	1.95 .02	3					
0.20	0.02004	25.3	2.01 .03	4	24.3	8.66 .04	6	10.21 .03	3
	"	25.5	2.01 .02	5	24.4	8.66 .04	6	10.23 .01	5
Over-all average			1.99±.03			8.64±.06		10.26±.03	

TABLE IX

THE APPARENT DISSOCIATION CONSTANT OF p-TOLUENE SULFONAMIDE FROM ULTRAVIOLET SPECTRA

pH	$E^{1}_{cm\ mol}$ at λ =		K' x 10^{11} at λ =		Buffer
	247 mμ	273 mμ	247 mμ	273 mμ	
1	305	315			**
13	1459	174			NaCl-NaOH
9.02*	377	-	6.4	-	NaH_2PO_4-NaOH
9.94*	713	-	6.28	-	
9.30*	442	304	6.8	(4)	NaCl-NaOH
9.86*	661	274	6.24	5.7	
10.23*	877	245	5.79	5.0	
10.44	1038	235	6.31	4.8	
11.18	1358	187	6.9	(6)	
11.40	1394	188	6.7	(4)	
Average			6.4±.2	5.2±.4	
pK_a'			10.19±.02	10.28±.04	
Over-all average pK_a'			10.21±0.05		

*Glass electrode #1190 used; observed value of pH corrected for sodium ion error according to Dole (11).

**Average of five determinations in 0.10 N H_3PO_4 (Two), 0.10 N HCl (2), and 0.10 N HCl plus 0.014 M NaCl. (One).

TABLE X

THE APPARENT DISSOCIATION CONSTANT OF BENZENE SULFONAMIDE FROM ULTRAVIOLET SPECTRA IN SODIUM CHLORIDE, SODIUM HYDROXIDE

pH	$E^{1\ cm}_{mol}$ at λ =			$K' \times 10^{10}$ at λ =		
	239 mμ	264.5 mμ	271 mμ	239 mμ	264.5 mμ	271 mμ
1	106	722	596			
13	2220	420	274			
9.33	495	675	531	1.052	0.86	1.18
9.64	924*	635	489	(1.447*)	0.93	1.14
10.29	1172*	524	376	(1.908*)	0.98	1.11
10.97	-	446	300	-	1.14	1.22
11.81	2129*	426	277	(0.34*)	(0.8)	(1.6)
Average unbracketted values				1.052	0.98	1.16
Average pK_a'				9.98	10.01	9.94
Over-all average pK_a'				9.96±0.04		

*The original solutions had to be diluted to obtain these points and the pH values of the diluted solutions were not determined.

TABLE XI

THE APPARENT DISSOCIATION CONSTANT OF THE SULFAMIDO GROUP IN p-SULFAMIDO-PHENYLALANINE FROM ULTRAVIOLET SPECTRA

pH	$E^{1\ cm}_{mol}$ at λ =		$K' \times 10^{10}$ at λ =		Buffer
	247. mμ	273.5 mμ	247 mμ	273.5 mμ	
1	272	543			**
13	2740	259			***
9.15*	1071	451	3.39	3.4	NaH_2PO_4-NaOH
10.48*	2035	310	0.825	1.51	
9.49*	1190	416	1.917	2.6	Glycine-NaCl-NaOH
9.88*	1561	413	1.441	1.11	
8.04	483	504	8.51	14.5	NaCl-NaOH
8.57	706	486	5.75	6.8	
9.53	1270	429	2.00	1.98	
11.36	2585	273	0.65	0.8	

*Glass electrode #1190 used; observed value of pH corrected for sodium ion error according to Dole (11).

**Average value of determinations in 0.10 N H_3PO_4 (Two) and 0.10 N HCl plus 0.03 M NaCl. (One)

***Average value of determinations in 0.10 N NaOH (Three) and 0.10 N NaOH plus 0.03 M NaCl (One).

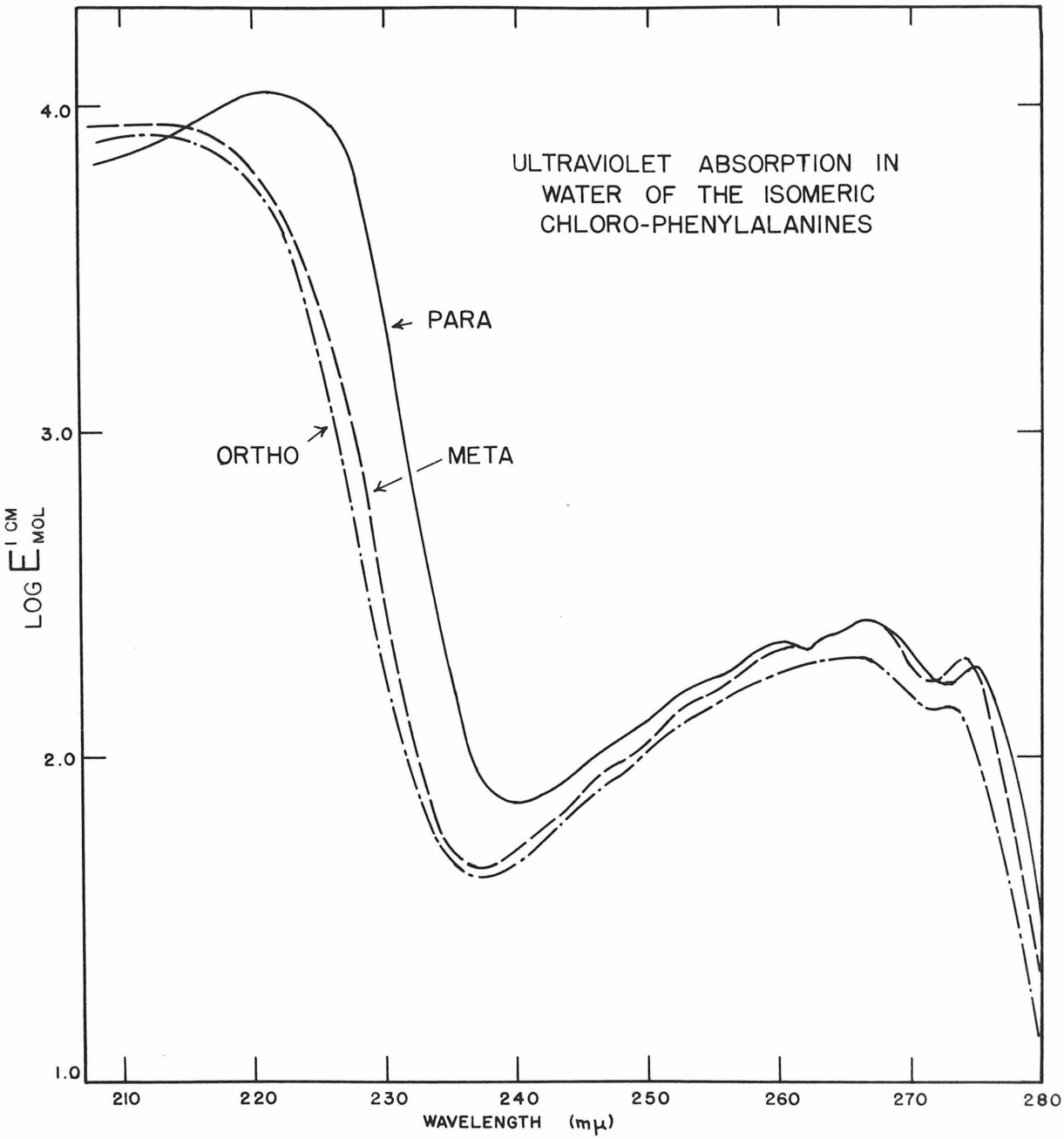

ULTRAVIOLET ABSORPTION IN
WATER OF THE ISOMERIC
CHLORO-PHENYLALANINES
PARA
ORTHO
META
LOG E 1 CM MOL
4.0
3.0
2.0
1.0
210
220
230
240
250
260
270
280
WAVELENGTH (mμ)

FIG. I

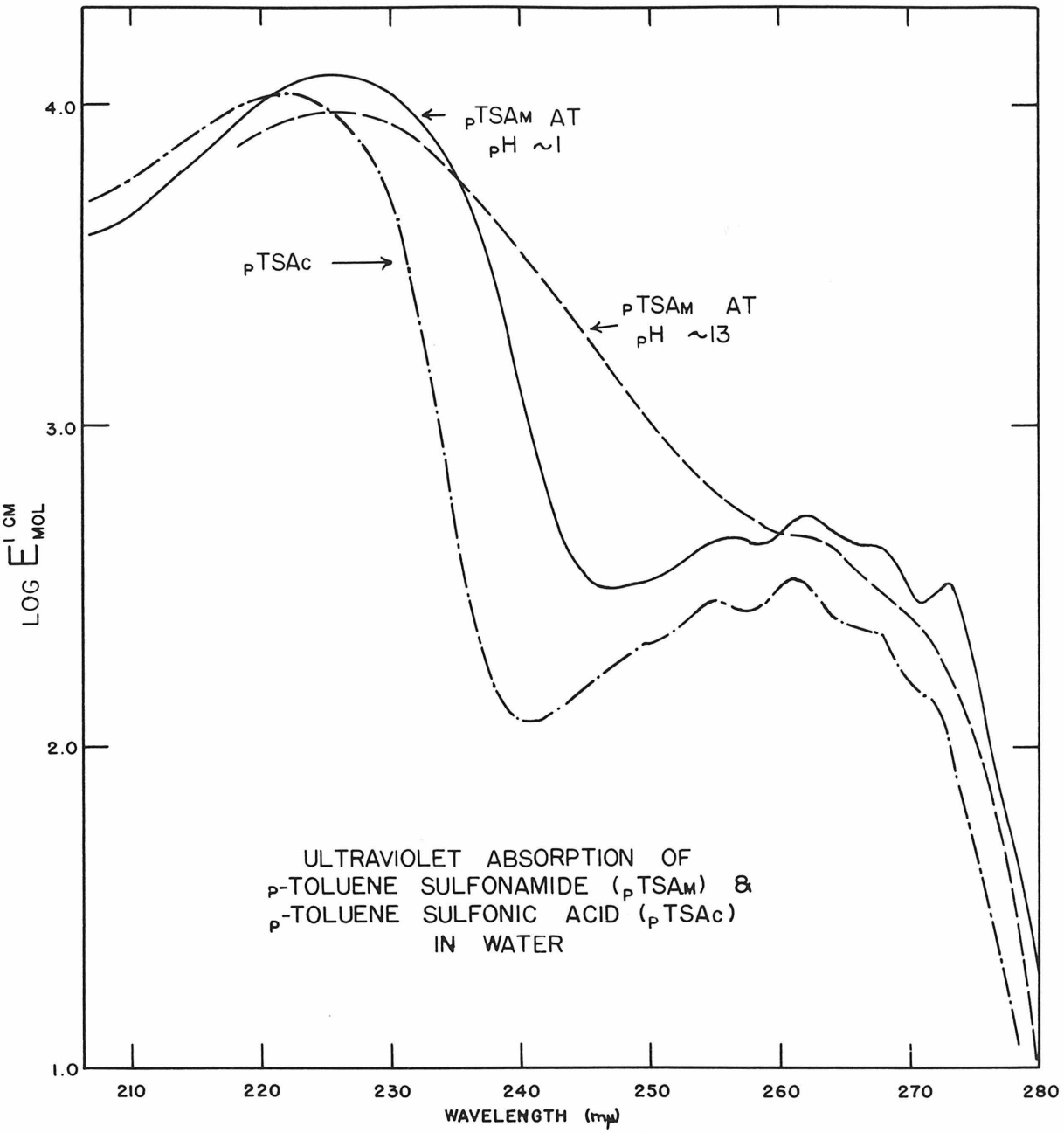

4.0
3.0
2.0
1.0
LOG $E^{1\,CM}_{MOL}$
$_pTSA_M$ AT $_pH$ ~1
$_pTSA_c$
$_pTSA_M$ AT $_pH$ ~13
ULTRAVIOLET ABSORPTION OF
$_p$-TOLUENE SULFONAMIDE ($_pTSA_M$) &
$_p$-TOLUENE SULFONIC ACID ($_pTSA_c$)
IN WATER
210
220
230
240
250
260
270
280
WAVELENGTH (mμ)

FIG. II

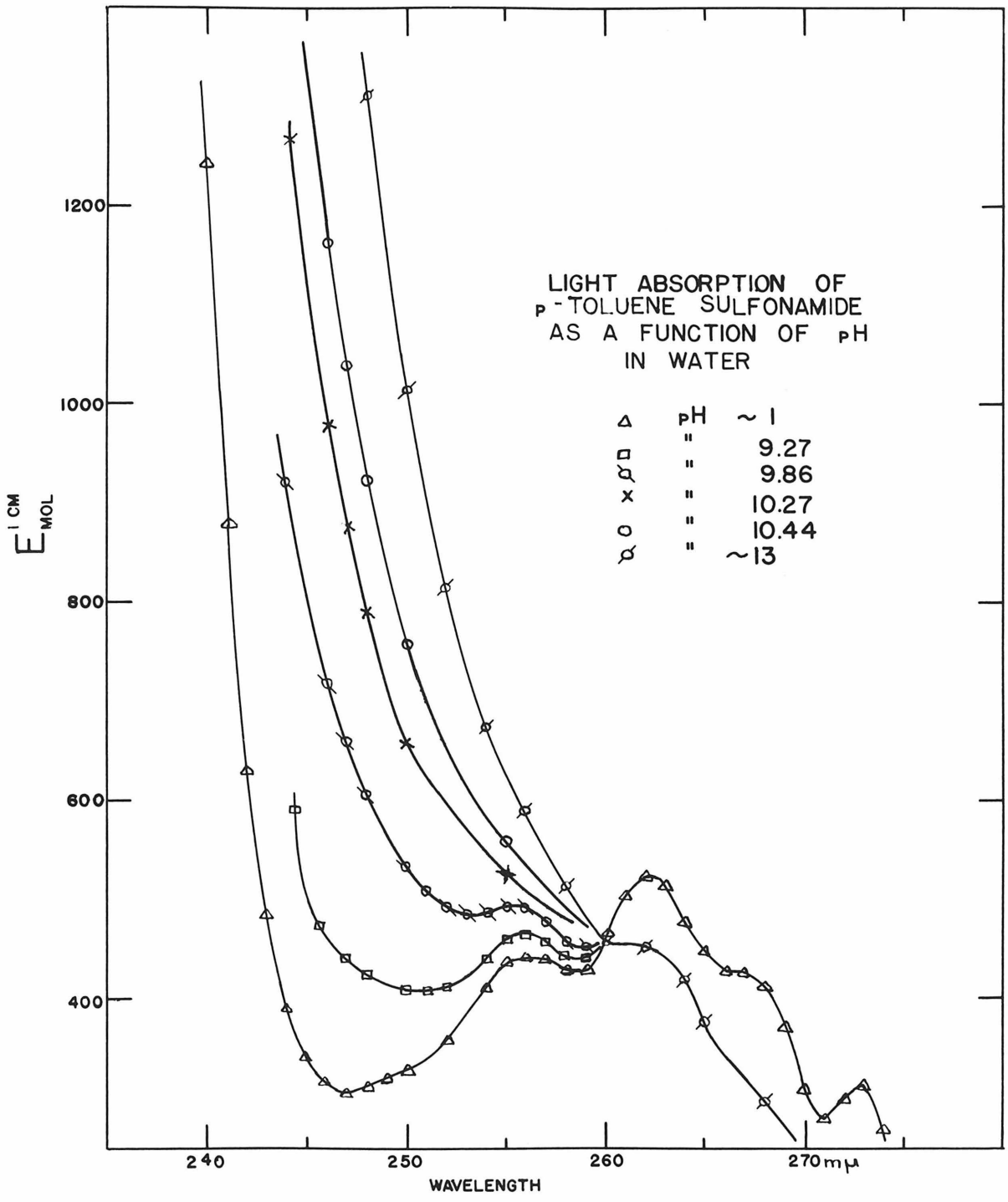

LIGHT ABSORPTION OF
p-TOLUENE SULFONAMIDE
AS A FUNCTION OF pH
IN WATER
pH ~ 1
" 9.27
" 9.86
" 10.27
" 10.44
" ~13
E 1 CM MOL
1200
1000
800
600
400
240
250
260
270 mμ
WAVELENGTH

FIG. III

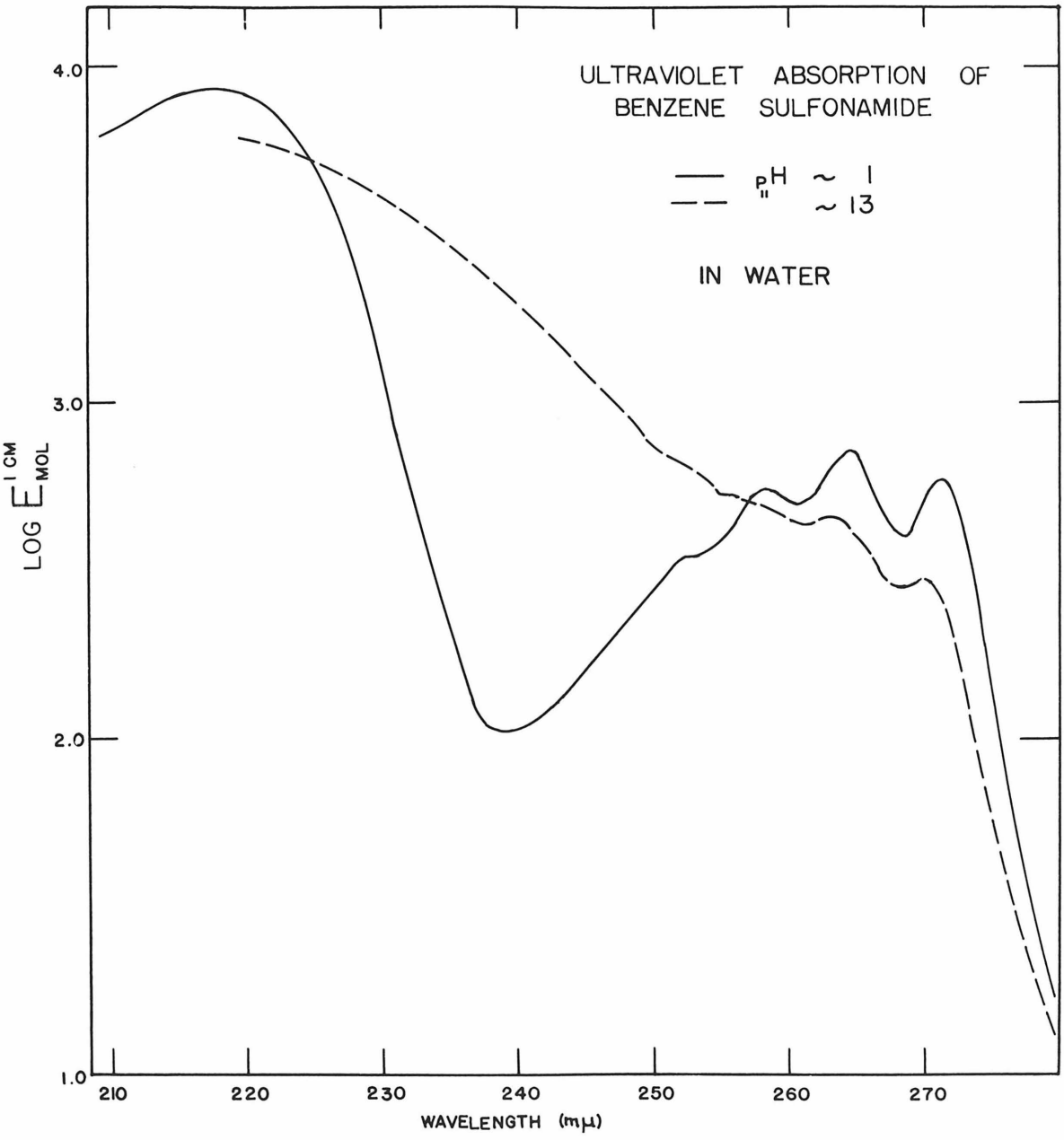

ULTRAVIOLET ABSORPTION OF
BENZENE SULFONAMIDE
pH ~ 1
" ~ 13
IN WATER
LOG E 1 CM / MOL
4.0
3.0
2.0
1.0
210
220
230
240
250
260
270
WAVELENGTH (mμ)

FIG. IV

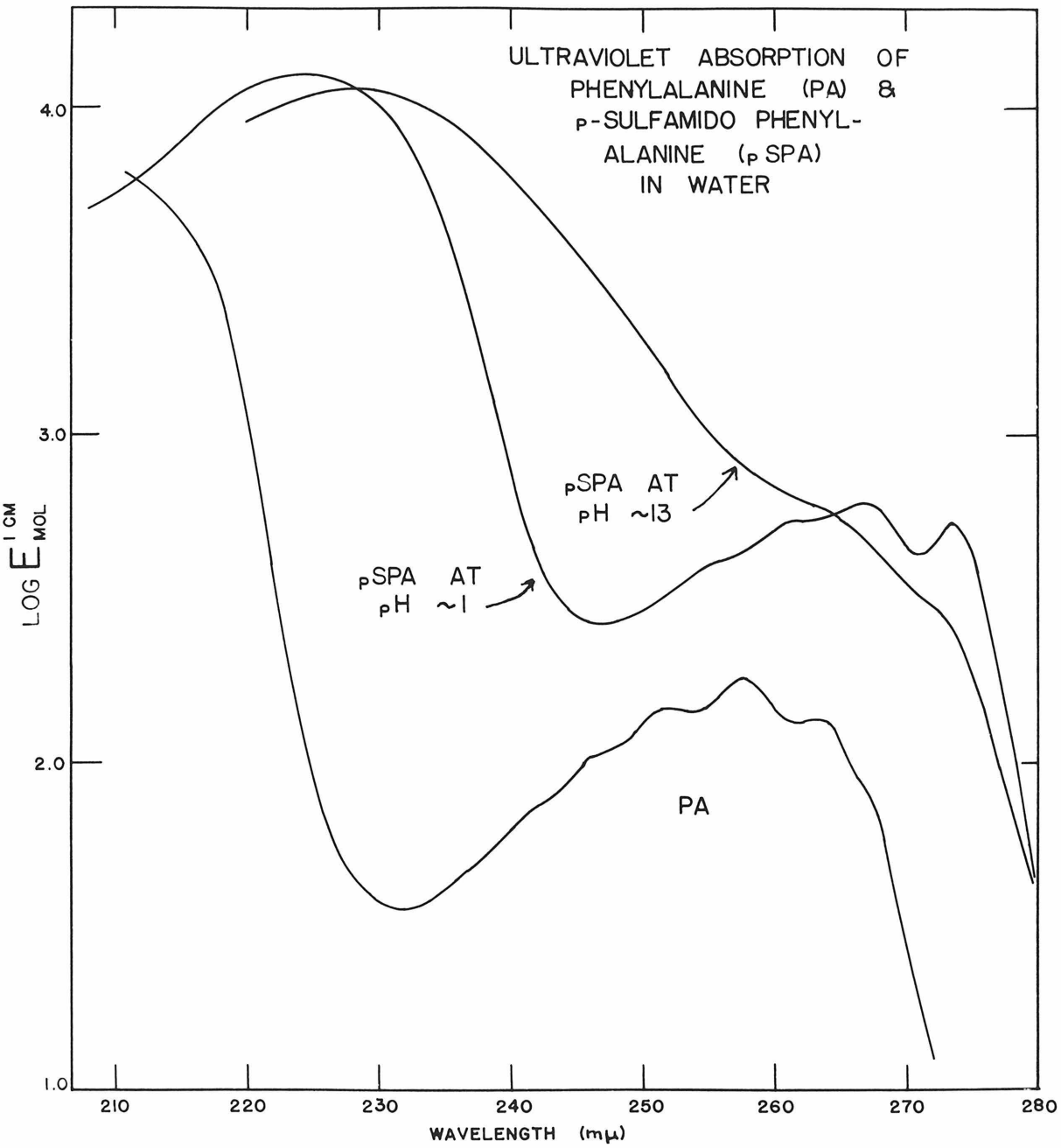

ULTRAVIOLET ABSORPTION OF
PHENYLALANINE (PA) &
p-SULFAMIDO PHENYL-
ALANINE (p SPA)
IN WATER
4.0
3.0
2.0
1.0
LOG E 1 CM MOL
pSPA AT
pH ~13
pSPA AT
pH ~1
PA
210
220
230
240
250
260
270
280
WAVELENGTH (mμ)

FIG. V

DISCUSSION.

Table XII summarizes the results of the amino acid investigation. Since the values are thought to be accurate to ±0.05 in pK ' it is seen that: a) substituting a chlorine for a hydrogen in the ring, regardless of its position, increases the acid strength of the ammonium group of phenylalanine by a factor of 1.5; b) there is no significant difference between the pK'_{COOH} of phenylalanine and the three isomeric chloro

TABLE XII

THE APPARENT DISSOCIATION CONSTANTS OF PHENYLALANINE AND DERIVATIVES

Compound	pK'_{COOH}	pK'_{NH3^+}	$pK'_{SO_2NH_2}$
Phenylalanine	2.16	9.15	
" (a)	1.83	9.13	
" (b)	2.58	9.24	
o-Chloro-phenylalanine	2.23	8.94	
m-Chloro-phenylalanine	2.17	8.91	
p-Chloro-phenylalanine	2.08	8.96	
p-Sulfamido-phenylalanine	1.99	8.64	10.26

(a) Hirsch (17). (b) Miyamoto and Schmidt (21).

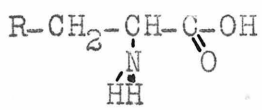
derivatives; c) the presence of the sulfamido group in the ring makes both the carboxyl and ammonium groups stronger--by factors of 1.5 and 3, respectively, over phenylalanine.

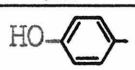
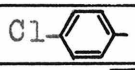
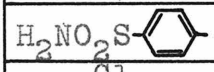
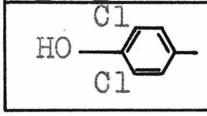
The value found for the ionization of the carboxyl group of phenylalanine is significantly different from those previously reported by Hirsch (17) and Miyamoto and Schmidt (21). The

value found in this investigation makes possible a consistent overall picture of the acid strengths of alanine, phenylalanine, tyrosine, the chloro-phenylalanines, and p-sulfamido-phenylalanine. From Table XIII we see that the effects on the dissociation

TABLE XIII

EFFECT OF SUBSTITUENTS ON pK'_{COOH} AND $pK'_{NH_3^+}$ OF ALANINE (9a):

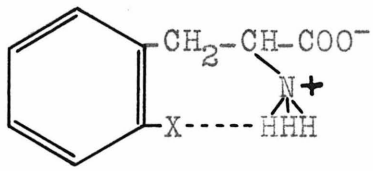


R	pK'_{COOH}	$pK'_{NH_3^+}$	pK'_{3}
H-	2.34	9.87	
	2.16	9.15	
	2.20	9.11	10.07
	2.08	8.96	
	1.99	8.64	10.26
	2.12	7.62	6.47

constants are directly proportional to the inductive effects of the various substituents predicted from modern theories of organic chemistry. The much larger effect on the ammonium group is presumably due to the closer proximity of the N-H bond over the O-H bond to the electronegative group attached to the β-carbon atom. The unexpectedly large magnitude of the effect on the ammonium group in the dihalogen tyrosines is qualitatively in the direction predicted.

One of the purposes in measuring the chloro-phenylalanines was for the light the results might throw on possible intra-

molecular hydrogen bond formation in the ortho derivative. Both o-chloro- and o-fluoro-phenylalanine (4) show anomalous physical properties; e.g., in contrast to the meta and para isomers, neither crystallizes well from water. It was thought this might be explained by intramolecular hydrogen bonding between the ammonium group and the halogen:



A structure of this type depending on the strength of the hydrogen bonding, might have a significantly lower $pK'_{NH_3^+}$ over the corresponding meta and para isomers, where analogous structures are less stable, and would perhaps show minor differences in ultraviolet absorption spectrum. In fact, with the chloro compound no significant difference in the dissociation constant from that of the other isomers was found. It is possible that the lower extinction and less prominent fine structure of the band centering at 266 mμ in the light extinction curve of the ortho isomer as compared to the meta and para compounds results from the hydrogen bond formation expected. The data on the fluoro compounds when available may be more enlightening, since fluorine is known to form strong hydrogen bonds and chlorine only very weak ones (25).

The spectrum of phenylalanine shown in Fig. V is in agreement with that reported previously by Smith (25A), but the band

at 257.5 mμ shows a fine structure not seen in Smith's curve. A comparison of Figs. I and V makes it evident that the chlorine has the expected batho- and hyperchromic effects when substituted in the aromatic ring of phenylalanine. The sulfamido group shows a moderate hyperchromic effect, but only an erratic bathochromic effect; e.g., in benzene sulfonamide and p-sulfamido-phenylalanine there is a shift of 9.5 mμ toward longer wavelengths of the band at 255-57 mμ in benzene and phenylalanine, but in p-toluene sulfonamide there is no change in wavelength as compared to toluene of the band at 262 mμ. Similarly, the sulfonic acid group in p-toluene sulfonic acid increases the extinction of the 262 mμ band of toluene, but shifts the maximum to 261 mμ-- a small hypsochromic effect. Ionization of a proton from the sulfamido group decreases the hyperchromic effect.

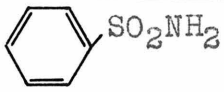
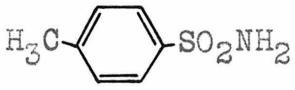
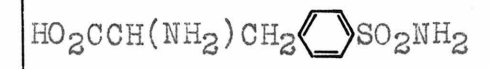
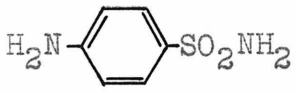
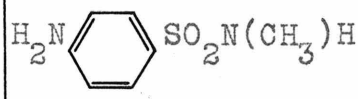
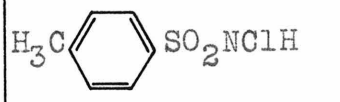
The peak at 212-221 mμ in the chloro-phenylalanines may either correspond to the high intensity E absorption of the benzene nucleus, shifted into the range of the spectrophotometer by the bathochromic effect, or to the K band produced by interaction of the benzene and chlorine groups (nomenclature of Braude (6), pp. 124-25). The same is true of the peak at 218-226 mμ observed for the sulfamido and sulfonic acid compounds. The former assignment would seem to be more logical, since the chloro group is not a chromophore and it is doubtful that the sulfamido group should be so considered.

While the attempted independent determination of the acid

dissociation constant for the sulfamido group in p-sulfamidophenylalanine was not successful (Table X) considerable confidence is felt in the assignment of the pK_a' values found by titration (Table VIII) because of their good agreement with representative values for other compounds with these functional groups (See Cohn and Edsall (9a) for typical pK'_{COOH} and $pK'_{NH_3^+}$ values and Table XIV for the available data on sulfonamides).

TABLE XIV

ACID DISSOCIATION CONSTANTS OF SOME SULFONAMIDES

Compound	$pK'_{SO_2NH_2}$	Method	Reference
	9.96	Ultraviolet	This thesis
	10.21	Ultraviolet	This thesis
	10.26	Titration	This thesis
	10.43	Titration	Bell and Roblin (3)
	10.77	Titration	Bell and Roblin
	4.55	Solubility	Morris, et.al. (22)
$CH_3SO_2NH_2$	10.62	Titration	This thesis*

*A single 20.00 ml. aliquot of 0.0995 M methane sulfonamide titrated with 0.4245 N NaOH; final temperature = 25.6°C.

The reason for the failure of the ultraviolet method in the

case of the sulfamido-phenylalanine cannot be decided with the evidence at hand. Two possibilities suggest themselves; specific ion effects, or changes in the absorption spectrum produced by the ionization of the ammonium group. Note that at 264.5 mμ the data for benzene sulfonamide (Table X) also shows a small trend in K'_a with pH*.

Dissociation constants for the sulfonamides investigated here have not previously been reported for aqueous solution, with the exception of mention by Morris, et. al. (22) that the K'_a for p-toluene sulfonamide might be as low as 10^{-10}. Kraus and Bray (19) determined a K_a of 1.39×10^{-4} for benzene sulfonamide in liquid ammonia by conductivity measurements. The ionization constants of a large number of N(1)-substituted sulfanilamides are given by Bell and Roblin (3). Graham and Macbeth reported the absorption spectra (in ethanol?) of p-toluene sulfonamide and of the sulfonamide plus sodium ethoxide, but it is presented in a form difficult to correlate with the data of this investigation.

The magnitude of the effect on K'_a produced by changes in the carbon chain attached to the sulfamido group is as would be expected for a group whose resonance forms involve only minor contributions from the benzene ring; such, for example, as the carboxyl group.

---

*The trend in this case is opposite to that for p-sulfamido-phenylalanine and can be explained by the presence of a small amount of benzene sulfonic acid in the sulfonamide, since for the acid E_{mol} is less than 340 at 264.5 mμ (13A), (22A), (29). Perhaps the fine structure in this region of the pH 13 curve (Fig. IV) may be attributed to the same impurity.

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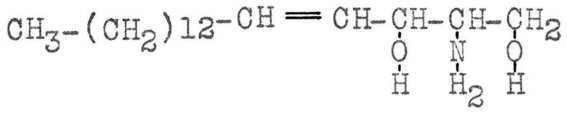
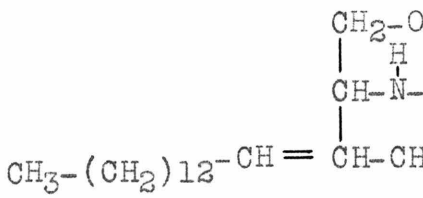
II. THE ISOLATION OF SPHINGOSINE FROM BOVINE SPINAL CORD

INTRODUCTION:

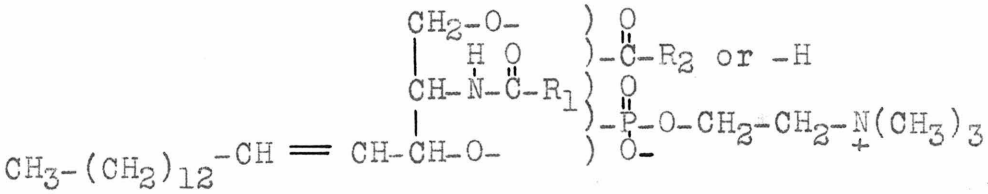
The amino glycol sphingosine, I, is the characteristic component of the group of compounds called by Carter (6) "sphingolipids." This group includes the cerebrosides, II, and sphingomyelins, (27) III, which are widely distributed in the tissues of higher animals, particularly in the brain and nervous tissues (28). The apparent absence of the sphingo-



I



II



III

lipids from plants and lower animals is probably less a real phenomenon that an indication of a lacuna in our knowledge.

Sphingosine was first isolated by Thudicum (29) in 1880,

and its structure established as a dihydroxy, monoamino n-octadecene-4 by the work of Thudicum, Thierfelder, Levene, Lapworth, and Klenk (this early structure work is summarized by Carter, et.al. (5)). Klenk and Diebold (13) believed the arrangement of hydroxyl and amino groups to be 1,2-dihydroxy-3-amino, based on their identification of the acid obtained from triacetyl sphingosine by ozonolysis and subsequent mild oxidation. However, Niemann and Nichols (20) showed that Klenk and Diebold's acid could not be an α-amino-, β,γ-dihydroxy-n-butyric acid as claimed; consequently Klenk and Diebold's assignment is untenable. Seydel (23), independently confirmed by Carter, et.al. (4), found that N-acylated sphingosine is not oxidized by periodic acid or lead tetraacetate. Since analogous compounds containing the other two arrangements possible in N-acylated contiguous monoamino-glycols are attacked by these reagents (19) only structure I remains for sphingosine.

The recent work of the Illinois group (4, 5, 8) is consistent with this assignment. From N-benzoyl-dihydrosphingosine they were able to form a cyclic benzilidene compound, which is possible only with 1,2- or 1,3-glycols. Under certain conditions of catalytic hydrogenation of triacetyl-sphingosine an acetoxy group is lost-- a result characteristic of a hydroxyl group in the allylic position to a double bond. Finally, a synthesis of dihydrosphingosine has been reported privately, but as yet not published (3).

Formula I, then, represents the presently accepted structure of sphingosine. The configuration at the double bond is not known, nor that around the two asymmetric carbon atoms. Niemann (17) has shown that it is possible to isomerize the sphingosine sulfate normally isolated to a less soluble compound which is more slowly hydrogenated, suggesting that the form isolated by the usual acid hydrolysis has the cis configuration around the double bond. Of interest is the natural occurrence of dihydrosphingosine, first demonstrated in the larvae of a tapeworm (14) and recently shown unequivocally to be present in bovine brain and spinal cord (7), although it was previously suggested by Mead (15) to explain the inertness of certain preparations of 'triacetyl-sphingosine' to olefin reagents. Its presence in bovine spinal cord was confirmed in this investigation. An additional high molecular weight base has been isolated by Niemann (18) from bovine brain and spinal cord, but is is as yet poorly characterized.

The investigation described in this thesis was originally undertaken to prepare sphingosine of known high purity as a preliminary to a study of the physical and chemical properties of the compound and some of its derivatives, which it was hoped would in turn lead to methods of characterization and criteria of purity for these compounds. Work had been initiated when the series of papers by Carter and co-workers (5, 6, 7b, 8) appeared, partially, at least, realizing our purpose. As a result, the emphasis in our investigation was shifted to the

preparation of sphingosine from sphingolipids by means other than aqueous or alcoholic acid hydrolysis.

DISCUSSION:

Isolation of C-S fraction. Previous work in this laboratory by Niemann and co-workers had shown that bovine spinal cord was the best starting material for large scale preparation of sphingosine. Carter, et.al. (6) and Mead (15) employed fresh spinal cord--which contains about 6% sphingolipids (6)--but because of its convenience this investigation used desiccated cord obtained from Wilson and Co., Chicago, Ill. The method for the isolation of the cerebroside-sphingomyelin fraction (abbreviated to 'C-S' fraction) developed by Mead (15) was applied with minor modifications. The material was extracted first with cold ethanol to remove lipids other than sphingolipids--i.e., fats, cholesterol, lecithin, etc.-- and then, exhaustively, with boiling ethanol to dissolve out the C-S fraction, which separated as a white solid on cooling the hot filtrates. A comparison in tabular form of the results with those of Carter and of Mead is given in Table XV.

Despite the apparent wide variations in yield of sphingolipid among the three proceedures, hydrolysis of any of the sphingolipid preparations gave approximately the same relatively low percentages of crude bases, namely: about 12-14% for Mead's C-S fraction, by acid hydrolysis; 11-23(?)% for the preparation obtained in this investigation, by acid hydrolysis

TABLE XV

YIELD OF SPHINGOLIPIDS FROM BOVINE SPINAL CORD

Investigator	Mead (15)	Nevenzel	Carter (6)
Method of desiccation	Distillation of water *in vacuo*	Extraction* with acetone, then benzene at room temp.	Extraction with acetone, then ether at room temp.
Material removed by cold EtOH extraction	25.2% of dried cord	8% of dried cord	No cold EtOH extraction used
C-S fraction	24%	20%	40% of dried cord
Residue from hot EtOH extraction	28%	60%	Not given
Weight of C-S fraction from 1 kilo fresh spinal cord	80 g.	25-30g.**	50 g.

*Carried out by Wilson and Co.

**Calculated assuming the residues from the hot ethanol extraction procedures of Mead and this investigation are comparable, and that Carter's acetone and ether extracted material is comparable to the acetone and benzene extracted material of this investigation.

and ammonolysis; and 18-20% for Carter's crude spinal cord sphingolipid preparations, by acid hydrolysis (7b). In all cases the crude sphingolipid fractions seem to be grossly contaminated, since on the basis of a ratio of cerebrosides to sphingomyelins of 3:1 (*cf.* (6)), and assuming saturated C_{20} acids exclusively, one can calculate that the theoretical yield of sphingosine should be 37%. An attempt to purify the C-S fraction by the use of liquid sulfur dioxide was only

partially successful, as only about 3.5% of the material was extracted after 24 hr. in a soxhlet. This extract, however, was principally non-sphingolipid in nature--cholesterol and fatty acids or glycerides, probably.

It is desired to eventually make in this laboratory a complete study of the components of spinal cord; consequently an effort was made to separate all filtrates and residues into a minimum number of solid fractions, employing the simplest and mildest proceedures possible and avoiding the use of reagents likely to introduce artifacts. The scheme adopted for the cold ethanol extracts is outlined in Fig.VI, that for the hot ethanol extracts in Fig.VII, in the Experimental section. The individual fractions were not further characterized except in case of A'-P_2, (A-2)-H_2O-P_1, and (C-G)-P_4, all of which consisted principally of potassium chloride. These fractions represented a total of about 60 g. of potassium chloride obtained from about 170 kg. of fresh cord, well within the amount possibly present as calculated from Weil's figures of 0.261% potassium and 0.130% chlorine in fresh bovine spinal cord (30).

Isolation of sphingosine. The method of Niemann (17) as modified by Mead (16) for the acid hydrolysis of the C-S fraction gives satisfactory results but requires the preparation of large amounts of concentrated methanolic hydrochloric acid solution. Furthermore most of the acid distills out during the first hours of refluxing. A study was therefore begun

TABLE XVI

METHANOLIC HCl HYDROLYSES OF C-S FRACTION

Batch No.	Normality of HCl	% MeOH	Yield of Sphingosine (as sulfate)*	Remarks
9	4	99	1.8 g.	
7	4	99	11.7	
3	4	99	10.8	HCl conc'n after reflux. for 36 hr. 0.7N
6	2	99	2.4	
4	2	99	13.8	HCl conc'n after 36 hr. refluxing 0.4N
1	2	99	9.0	
10	2	83	3.6	An addn'l 14.1 g. of diliturate obtained
5	1	99	2.5	HCl conc'n after 36 hr. refluxing 0.2N

*From batches 1-7 and 9 an additional 55.1 g.s of material was obtained as the diliturate.

of the effect on the hydrolysis (as measured by the yield of sphingsine salt obtainable) of the hydrogen ion and water concentrations. Using a standard proceedure the results shown in Table XVI were obtained. Unfortunately the advantages of the diliturate were only appreciated shortly before this phase of the investigation was dropped.

The cause of the erratic yields of sulfate has not been satisfactorily explained; a contributing factor is perhaps to be found in the observation that sphingosine can be extracted into petroleum ether from aqueous acid solution of high ionic strength. The precipitation of additional material with dilit-

uric acid is however not surprising. In a mixture of bases the dibasic sulfuric acid can be expected to form mixed salts, which may well be alcohol soluble; also, Carter (7b) has shown that during methanolic acid hydrolysis O-methyl ethers of sphingosine are formed, whose sulfates are much more soluble in alcohol than is sphingosine sulfate. The monobasic dilituric acid, however, cannot form mixed salts; further, its salts with a large number of organic bases are known to be but slightly soluble (21). Dilituric acid, then, is a more generally effective, although less specific, precipitating agent for sphingosine than is sulfuric acid.

It can be concluded from these incomplete data that 2 N and 4 N methanolic hydrochloric acid solutions are capable of equally good results. The use of still lower concentrations is not ruled out; the effect of water is not established; and no studies on the time necessary for complete hydrolysis were made. Carter, et.al. (7b) used 1.7 N sulfuric acid in 99% methanol and refluxed for 5-6 hr. They reported that in aqueous systems the yields of sphingosine were lower.

Basic hydrolysis of sphingolipids has not been generally applied, perhaps because a second step would be necessary to liberate sphingosine from the galactoside (psychosine) formed from cerebrosides by partial hydrolysis. High-pressure ammonolysis to obtain sphingosine, psychosine, and fatty acid amides seemed an attractive proceedure because of its convenience. Psychosine is of interest in itself and the amides

might be suitable for characterizing the fatty acids present.

The C-S fraction plus a large excess of liquid ammonia, with or without alcohol as solvent and with or without ammonium chloride as catalyst, were charged into a high pressure bomb and heated with continuous agitation for various periods of time in the range of 100-50°C. After cooling and venting the excess ammonia, the residue was recrystallized from alcohol, and the unreacted C-S fraction (65-80% of the starting material) filtered out. The bases could be precipitated from the filtrate with dilituric acid, leaving the amides in solution. The results are collected in Table XVII.

It is evident that reaction was not complete, but that increase in temperature and duration of heating increased the yield of dilituratates and decreased the amount of unreacted sphingolipid. No effect of added alcohol is apparent, although since the C-S fraction is insoluble in liquid ammonia at -33°C addition of a solvent seemed desirable. With regard to the lack of noticeable improvement in yield when ammonium chloride was added, note that ammonolysis of the sphingomyelins would form ammonium phosphate, which could provide ammonium ion to catalyze further reaction. Run XI was carried out having in mind the observation of Gordon, et.al. (11) that ammonolysis of simple esters in dioxane was speeded up by the presence of water. The results here were better, but not strikingly so, and on the basis of a single run it is not possible to draw valid conclusions.

TABLE XVII

AMMONOLYSES OF 25 g. OF C-S FRACTION

Run	Solvent (ml.)	Liquid NH_3 (ml.)	Insol. in cold alc. (g.)	Temp. (°C.)	Time (hr.)	Yield dilituratе (g.)
I	250 E[a]	75	20	85-115	7	-
II	250 M	100	10 (+9[b])	100-10	6.5	-
III	250 M	200	16	95-100	7)	
IV	none	300[c]	18.9 (+1[b])	85-100	8)	2.5
IVB[d]	none	250[c]	11.8 (+1[b])	99-102	7.5)	
V	100 M	300[c]	21.0	85-105	7.5	0.6
VI	100 M	300	16.3 (+2.3[b])	140-50	7.5	1.3
VII	100 M	300	19 (wet)	148-55	16	2.4
XI	95 M 5 W	300	16.9	130-35	10	3.0

[a] E = ethanol; M = methanol; W = water.

[b] Material insol. in hot alcohol.

[c] NH_4Cl added as catalyst: 10 g. to IV and V, 5 g. to IVB.

[d] Charge of 16.4 g. residue from Ammon. IV.

Two explanations of the limited reaction encountered are possible. First, that the rate of reaction is slow, due to inertness of the reactants, perhaps by reason of limited solubility in the system employed. Secondly, that the system reached an equilibrium state and that major improvement in yield was not achieved because the same ratios of ammonia, methanol, and C-S fraction were used in most runs. The available evidence perhaps favors the first theory, since the apparent direct dependence of yield of dilituratеs on duration of heating is in-

compatible with the idea that the system had already attained equilibrium with the shorter heating period. That the sphingolipids are relatively inert to basic hydrolysis is shown by the following facts: a) bayberry wax, which consists of myristyl and palmityl triglycerides (12), under the same conditions employed for the runs of TableXVII gave a yield of at least 75% of amides on heating to 130° for 8.5 hr.; and b) in the diethylene glycol method for the determination of the saponification equivalent (24) complete reaction was obtained in 1 hr. for the bayberry wax glycerides, in 3 hr. for the bayberry wax amides, but only in 6 or more hr. for the material (presumably C-S fraction) insoluble in cold alcohol after ammonolysis.

However, if the poor results were due to slow rate of reaction then: a) an increase in the concentration of catalyst should increase the rate; in fact, one can detect no difference on the addition of ammonium chloride, which in the ammonia system is a strong acid; and b) partial reaction might be expected to selectively remove material, leaving a more inert residue; but again, no difference was detected when in run IVB the residue from one ammonolysis was subjected to a second treatment. While these objections are not insuperable, further experiments are indicated in which the ratio of ammonia to C-S fraction should be varied and the duration of heating increased, before a reliable conclusion can be reached. A further increase in temperature, while probably desirable, is not recom-

mended without more knowledge of the behaviour of ammonia above its critical temperature (132.4°C.).

Several other systems for the splitting of sphingolipids were surveyed with the object of finding, ideally, a mild method for the isolation of sphingosine which would also give the fatty acids or sugars in a form convenient for characterization. The most promising procedure found was hydrazinolysis, i.e., the reaction of the C-S fraction with anhydrous hydrazine in a higher alcohol as solvent, leading to the formation of psychosine, sphingosine, and fatty acid hydrazides (cf. (26)). The unreacted sphingolipid separated on cooling the reaction mixture and was filtered off. The hydrazides were condensed with benzaldehyde by the procedure of Curtius and Dellschaft (9) and the remaining bases precipitated from the filtrate with dilituric acid. Reaction was at least as good as with ammonolysis--40% versus 20-35% for ammonolysis. However, this small increase hardly justifies the considerably more complicated procedure and more expensive reagents. Other systems, which showed little promise, were benzoic anhydride in benzoyl chloride at 100° and concentrated sulfuric acid at room temperature.

Attempted purification and characterization of sphingosine. As mentioned above, during this investigation dilituric acid was found to form a salt with sphingosine that was insoluble in ethanol in the presence of excess hydrogen ion and of basic impurities--conditions under which sulfuric acid formed soluble

mixed salts. This dilituratе, as is generally true with organic salts of dilituric acid (21), did not possess a characteristic melting point but decomposed above 205°. Repeated recrystallizations gave a cream powder whose analysis agreed better with dihydrosphingosine dilituratе than with the sphingosine salt, suggesting that one can separate the dihydrosphingosine in this way.

The most direct procedure for getting pure sphingosine would be chromatography of the crude bases from hydrolysis of the sphingolipids. It was hoped to extend Mead's observations (13) using different solvents and adsorbents. Both the free bases and the N-(2,4-dinitro-phenyl)-bases (cf. (22)) were chromatographed on silicic acid, alumina, and calcium hydroxide from petroleum ether and by a partition chromatographic method using commercial silicic acid and various solvents. Development of the chromatogram when using the free bases was followed by means of the fluorescence of some of them under ultraviolet illumination; the N-dinitrophenyl derivatives could be located visually by their yellow color. The results with the free bases were erratic, the findings not being reproducible, partly because it was hard to follow the zones with the ultraviolet lamp. Since there seemed no convenient way of regenerating the base from the N-di-nitrophenyl compounds this line of endeavour did not advance directly the main problem of the preparation of pure sphingosine, and was not pursued. Before the chromatographic work had made real progress it became apparent that a means

of characterizing the fractions was necessary; also the erratic results suggested that perhaps, in accord with Mead's conclusion (15), the crude free bases were slowly decomposing with liberation of ammonia, and that therefore one might expect to stabilize them by acylating the amino group. Interest then shifted to a study of the benzoyl derivatives.

The benzoylated sphingosines were chosen for investigation because the paper of Carter and Norris (7a) suggested that they were conveniently prepared and readily crystallized compounds. Our findings confirm those reported later by Carter, et.al. (7b). The N-benzoyl derivative was prepared by a Schotten-Baumann technique and was isolated as an amorphous powder melting at 117-19°, after recrystallization. On further benzolylation in pyridine it gave a crystalline compound that had the correct melting point and analysis for tribenzoyl dihydrosphingosine. The melting points of the crystalline residues after removal of the dihydro compound indicated the probable presence of tribenzoyl sphingosine admixed with other products, but it was not possible to isolate the pure compound. The observations of the Illinois group on the properties of tribenzoyl dihydrosphingosine were extended to include its ultraviolet absorption spectrum in ethanol solution (Fig. VIII).

Several other derivatives of sphingosine were cursorily surveyed as potential derivatives for characterization. None were purified or analyzed. The most promising of these was the tri-(phenyl-thiol-carbonyl)-derivative (10) of which a few

crystals were finally obtained. An effort to take advantage of the observation of Siegel and Moran (25) that cyclic acid anhydrides in the presence of bases react instantaneously with primary alcohol groups at room temperature, but not with secondary hydroxyls, led to an attempted preparation of the monosphingosine-amide of succinic acid. An amorphous solid was obtained of unsatisfactory melting behavior. The N-dinitrophenyl derivative previously mentioned was obtained only as an oil. The triacetyl compounds have been fully described and characterized by Carter, et.al. (7b); in our hands they were much less satisfactory than the corresponding benzoyl derivatives, which are far and away the best derivatives available for the characterization of sphingosine and derivatives.

EXPERIMENTAL:

Isolation of crude sphingolipids from spinal cord (cf. 15, 17). Finely ground desiccated bovine spinal cord* was covered with 95% ethanol using 3 liters of alcohol per kilogram of cord, and the suspension allowed to stand at room temperature, with thorough mechanical stirring two or three times, during 14 days. The brown-orange supernatant was then removed by use of an 11-inch basket centrifuge; this gave a solid cake still retaining about 1/7th of the original solvent. The extraction was repeated with the same volume of fresh ethanol for 14 days at room temperature, the resulting light brown solution being separ-

*A product of Wilson & Co., Chicago, Ill., who prepared it from fresh cord by extraction at room temperature with acetone and then benzene.

ated by centrifugation as above.

The centrifuge cake was crumbled manually and allowed to dry in air. The dried solid was extracted in 350 g. portions five times, in rapid succession, with boiling 92-95% ethanol, using 2.3 liters for the first and 1.6 liters for each succeeding extraction. The solid was introduced into the boiling alcohol and heating continued for 6 minutes; the suspension was then filtered through a large fluted filter into a receiver packed in ice. Further extraction of the residue with boiling absolute ethanol gave only a few hundred milligrams of additional C-S fraction.

The filtrates were placed in the cold room at 5° C for 5-10 days. The white solid which separated was filtered off and washed by covering with a minimum volume of ethanol in a flask kept overnight at 5°. After filtering and drying in vacuo over concentrated sulfuric acid, there were obtained from 350 g. of cold-ethanol-extracted cord 75-77 g. of an almost white, free-flowing powder which was not waxy to the touch. This was the cerebroside-sphingomyelin ('C-S') fraction. The exhausted residue from each 350 g. batch when air-dried weighed about 230 g.

Attempted purification of C-S fraction with liquid SO_2. Preliminary attempts to find a solvent in which the C-S fraction was reasonably soluble but which would give two phases with liquid sulfur dioxide were unsuccessful. Consequently a soxhlet-type extraction of solid C-S fraction by liquid sulfur

dioxide was carried out.

A 29.6 g. sample of air-dry C-S fraction prepared by Mead (15) was placed in a paper thimble in a vacuum jacketed soxhlet apparatus fitted with a dry ice condenser. Sulfur dioxide gas was passed in and allowed to condense until the appropriate volume of liquid had collected. The receiver was heated by a stream of tap water to secure a rapid relux rate. Six fractions of material were obtained in about 25 hr. total extraction time, amounting to more than 1 g. (3.5%) of the starting material. The initial fractions crystallized well from absolute ethanol; the recrystallized material melted 120-40° and gave a faint nitrogen test by CaO-Zn ignition (1), but a strong Liebermann-Burchard reaction (2). The final fractions did not form well developed crystals from absolute ethanol; the recrystallized materials melted 60-65° to cloudy liquids, and gave strong nitrogen tests and weak Liebermann-Burchard reactions.

Fractionation of the cold-ethanol extracts. The combined filtrates from the cold alcohol extractions were fractionated as outlined on the next page. All evaporations were carried out under maximum water-aspirator vacuum and at 50° or less. The use of a cyclic glass still of Dr. Niemann's design greatly speeded up the removal of solvents. A summary in tabular form of the weights and probable composition of the various fractions is given below (Table XVIII). These fractions were not investigated further except for a few

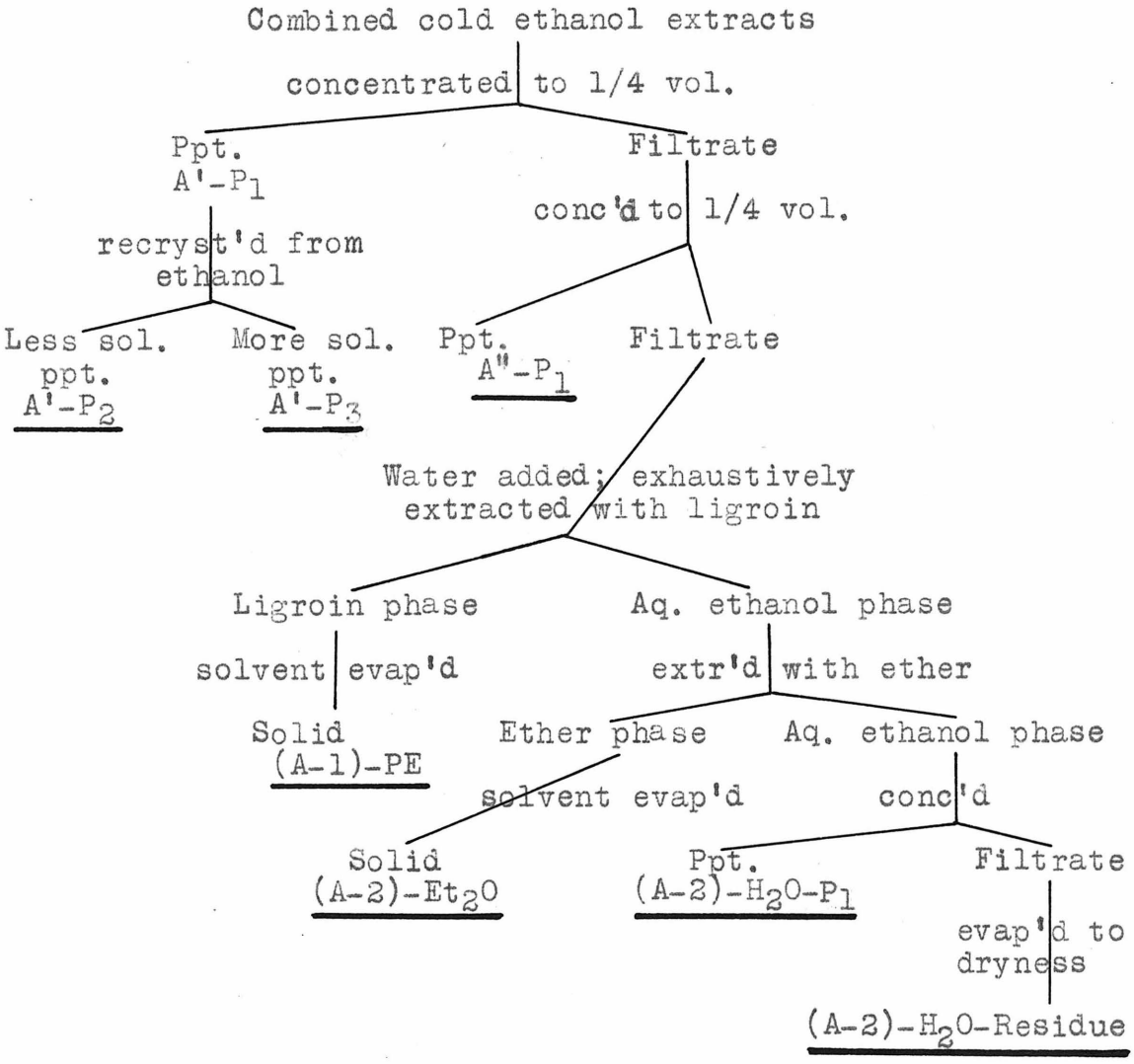

Combined cold ethanol extracts
concentrated to 1/4 vol.
Ppt. A'-P1
Filtrate
recryst'd from ethanol
conc'd to 1/4 vol.
Less sol. ppt. A'-P2
More sol. ppt. A'-P3
Ppt. A''-P1
Filtrate
Water added; exhaustively extracted with ligroin
Ligroin phase
Aq. ethanol phase
solvent evap'd
extr'd with ether
Solid (A-1)-PE
Ether phase
Aq. ethanol phase
solvent evap'd
conc'd
Solid (A-2)-Et2O
Ppt. (A-2)-H2O-P1
Filtrate
evap'd to dryness
(A-2)-H2O-Residue

Fig. VI

TABLE XVIII

YIELD OF FRACTIONS FROM COLD ETHANOL EXTRACTION

Fraction	Weight (g.)	Physical Properties	Probable Composition
A'-P_1)) A'-P_3)) A"-P_1)	568	White waxy powder	Phospholipids and KCl (contains N, P, Cl, and possibly S)
A'-P_2	10	White crystalline powder	KCl
(A-1)-PE	733	Brown plastic solid; strong blue-green fluorescence; characteristic odor	Free fatty acids and other lipids (forms insol. Ag and Pb salts)
(A-2)-Et_2O	193	Dark brown plastic solid; blue-green fluorescence; characteristic odor; insol. in ligroin	Lipids
(A-2)-H_2O-P_1	38	White crystalline powder	KCl
(A-2)-H_2O-Residue	316	Brown, very viscous liquid; blue-green fluorescence; burnt odor	Glycerol, inorganic ions and other water solubles.
Total	1858 g., or 8% of the dried bovine spinal cord		

qualitative tests. A'-P_2 was tentatively identified as KCl from the minor charring on ignition, the violet color through cobalt glass of a flame test, the solubility characteristics in aqueous ethanol, the formation of an insoluble dilituratе, and the chloride analysis of a crude sample--48.1% Cl found, theory for KCl is 47.6%.

Fractionation of the hot ethanol extracts. The combined filtrates after removal of the C-S fractions were fractionated as shown schematically in Fig. VII. The figures in parentheses

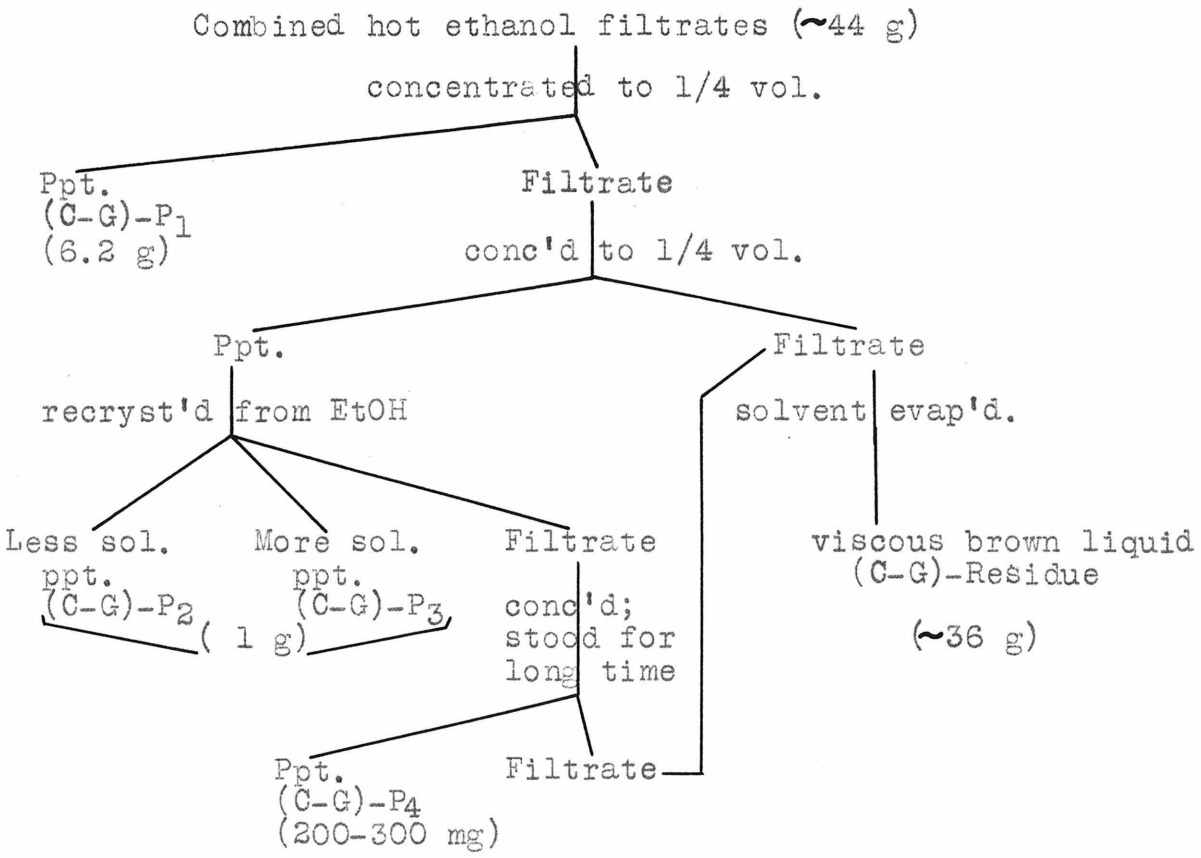


Fig. VII

are the weights of material obtained from each 350 g. batch of

cold alcohol extracted cord; they are tentative since only four batches have been extracted with hot ethanol. Again, only a few qualitative tests were made on the fractions obtained. From ignition, flame tests, and aqueous ethanol solubilities (C-G)P_4 is apparently also KCl.

Methanolic HCl hydrolysis of the C-S fraction. 100 g. of Mead's C-S fraction (15) were treated with one liter of methanolic HCl at the reflux temperature for 36 hours. The concentrations of HCl and of water used are listed in Table XVI (in the Discussion). After cooling, 100 ml. of water were added and the suspension kept at 5° for 24 hours. It was then filtered through a fluted funnel, the precipitate washed with a small volume of 90% methanol, and the filtrate plus washings extracted with 30-60° petroleum ether in a continous extractor for 24 hours. The aqueous methanol phase after concentration in vacuo to 300-400 ml. was made strongly basic with 6 N NaOH and extracted three times with 200 ml. portions of ethyl ether previously freed of peroxides by shaking with ferrous sulfate solution. The combined ether phases, after washing with sodium chloride solution, were dried over anhydrous calcium sulfate, filtered, and the ether removed in vacuo at 40° bath temperature. The residue was taken up in 100 ml. absolute ethanol, again filtered, and either made just acid to litmus with 8% H_2SO_4 in abs. ethanol, or made acid with excess saturated dilituric (5-nitrobarbituric) acid in 95% ethanol. After 12 hours at 5° the precipitated salt was filtered off, washed with a little 95%

ethanol and dried *in vacuo* over concentrated sulfuric acid or anhydrous calcium chloride. Yields are summarized in Table XVI.

Ammonolysis of C-S fraction. 25 g. C-S fraction and 100 ml. of methanol were placed in a monel liner and cooled in a dry ice-acetone bath. Then 300 ml. of liquid ammonia were added, the liner closed and placed in a precooled (dry ice-acetone) high pressure bomb, and the bomb sealed and mounted in a rocker fitted with a heating jacket. The bomb was kept at 85-155°C (see Table XVII) with continuous rocking for from 6 to 16 hours. After cooling, the excess ammonia was vented, the bomb dismantled, and the suspension removed from the liner with the aid of hot methanol. The suspension was taken up in the minimum amount of boiling alcohol, filtered, and allowed to cool. After standing overnight at 5° the precipitate was filtered off, washed with a little ice-cold alcohol, and dried *in vacuo* over sulfuric acid; yields are given in Table XVII. This material was identified as unreacted C-S fraction as detailed below.

The filtrate was concentrated at reduced pressure to remove additional ammonia and then treated with excess of a saturated solution of dilituric acid in 95% ethanol. After 12-24 hours at 5°, the dilituric acid were filtered off and dried; yields are given in Table XVII.

The filtrate after this last step could be freed from excess dilituric acid by treatment with ammonium hydroxide and filtration of the insoluble salt; removal of the ammonia and solvent at reduced pressure gave a crystalline fraction melt-

ing at 81-90°, which contained nitrogen and evolved a volatile base when boiled with methanolic KOH.

Properties of material insoluble in cold alcohol after ammonolysis. Melting behaviour: softens around 80°, darkens above 150°, melts 175-90°, gas evolution from the liquid begins around 195°. Elementary tests by CaO-Zn ignition method (1) show strong nitrogen, definite phosphorus and sulfur, and doubtful halogen tests. Solubility: soluble hot in methanol, ethanol, n-propanol, diethylene glycol, chlorofrom and chlorobenzene; insoluble cold in methanol, ethanol, ethyl ether, and acetone, partially soluble in cold chloroform and chlorobenzene; insoluble hot or cold in water, although emulsions form on shaking. Extraction of 17.8 g. of material in a soxhlet apparatus for 2 hours with ethyl ether removed 0.2 g.; two subsequent extractions with acetone each removed 0.6 g. The residue was used for Ammon. IVB; see Table XVII.

The saponification equivalent determined with 1 N KOH in diethylene glycol solution falls from about 4600 with 5 min. heating at 120-30°, to about 400 when heated for 6 hours at this temperature. The amount of volatile base liberated during the heating (calculated as ammonia) varied from 0.09% after 10 min. heating to 0.58% after 6 hours. Bayberry wax glycerides were completely saponified after 1 hour heating under these conditions, but the amides from this source required 3 hours for complete saponification and yielded one equivalent of volatile base.

5 g. of material was hydrolyzed with 4 N HCl in 83% methanol by the procedure used for the C-S fraction. The yield

of dilituratesw as 0.86 g., in good agreement with that obtained in the best hydrolyses of C-S fraction (cf. Table XVI.).

Hydrazinolysis of C-S fraction. 5.2 g. (0.23 M) of metallic sodium were dissolved in 100 ml. (0.92 M) of redistilled i-amyl alcohol contained in 500 ml. three necked flask fitted with a) a Friedrichs condenser and $CaCl_2$-Ascarite tube, b) a rubber tubing sealed mechanical stirrer, and c) a short rubber connection to a 50 ml. erlenmeyer flask containing 13.0 g. (0.125 M) solid hydrazine dihydrochloride. The sodium was dissolved with stirring and heating by means of a Glas-col mantle, and the hydrazine dihydrochloride in small portions was shaken into the solution over a period of 1 1/2 hours, while the heating and stirring continued; following the final addition of solid the heating rate was increased until moderate refluxing began. After 1/2 hour the flask was modified for distillation and alcohol and hydrazine were distilled into a 300 ml. balloon flask; after almost 100 ml. of distillate had collected, an additional 25 ml. of i-amyl alcohol were added to the boiler and distillation continued almost to dryness, the temperature of the vapor having risen to 126.5°.

5.0 g. of C-S fraction were added to this distillate and the solution refluxed with the exclusion of moisture for 17 hours. On cooling unreacted C-S fraction in the amount of 2.9 g. separated. The supernatant was concentrated at full water-aspirator vacuum and up to 100° bath temperature. The residue was taken up in 25 ml. of absolute ethanol, heated to boiling, and treated with 3 ml. (0.03 M) of redistilled benzaldehyde; refluxing was

continued for 1/2 hour. On cooling a solid formed; after filtration and drying there were obtained 0.65 g. of a buff colored powder melting at 63-85°. From the filtrate 0.3 g. of dilituratés were obtained by adding excess saturated dilituric acid solution in 95% ethanol.

Purification of crude dilituratés. 1 g. of the crude dilituratés from acid hydrolysis was recrystallized six times from a 1:1 mixture of methanol and ethanol. After drying 0.3 g. of a pale tan, microcrystalline powder was obtained, representing the least soluble portion. This material began to darken by 170° and decomposed at 205-20°C. It is photosensitive (as is dilituric acid) and on long exposure to bright light develops a rose-colored surface layer. Analysis. Calculated for $C_{22}H_{40}O_7N_4$: C, 55.92; H, 8.53; N, 11.86; for $C_{22}H_{42}O_7N_4$ (dihydrosphingosine dilituratе): C, 55.68; H, 8.92; N, 11.81. Found: C, 55.48; H, 8.88; N, 11.28.

Preparation of free bases. The free base was prepared from the sulfate by Seydel's method (23). However, since both sodium and potassium dilituratés are insoluble, this method lead to intractable emulsions and poor yields when applied to the dilituratе. Fortunately the benzyl-trimethyl-ammonium salt of dilituric acid was found to be water soluble. The solid dilituratе was triturated with a 50% aqueous solution of benzyl-trimethyl-ammonium hydroxide, diluted with aqueous methanol, and extracted three times with either petroleum ether (in the case of dilituratés resulting from acid hydrolyses) or chloroform (for dili-

turates from ammonolyses and hydrazinolyses, containing psychosine, which is insoluble in petroleum ether; since chloroform tended to form emulsions, it was generally less satisfactory than petroleum ether). The combined non-aqueous phases were washed twice with aqueous methanol, dried over calcium sulfate, filtered, and the solvent removed at reduced pressure. Yield, 90% of theory.

Psychosine. 2.3 g. of free bases obtained by ammonolysis were recrystallized from a minimum volume of ethanol. After standing at 5° overnight the amorphous precipitate was centrifuged off and washed with cold ethanol until the washings were colorless. After drying in vacuo over sulfuric acid there resulted 0.79 g. of a pale tan powder softening at 140° and melting 184-88°C. Additional material can be obtained from the supernatant by removal of solvent and extraction of sphingosine and dihydrosphingosine from the residue with petroleum ether, leaving crude psychosine.

N-Benzoyl dihydro-(?) sphingosine. 2.6 g. of recrystallized sulfates from acid hydrolysis were benzoylated by the acylation method of Carter and Norris (7a). The crude product was recrystallized from acetone-methanol three times to give an amorphous material which after drying in vacuo at 78° for 20 hours softened at 113° and melted at 117-19° (corr.d). Successive concentrations of the filtrates gave two additional crops. Total yield of crude material was 0.71 g. or about 25% of theory.

Tribenzoyl dihydrosphingosine. 0.22 g. of the above N-benzoylated bases were further benzoylated with 1 ml. of benzoyl

chloride in 1 ml. pyridine. The solution warmed up, developed a rose color, and deposited a white solid. It was allowed to stand at room temperature over night, and was then taken up in 5% aqueous sodium carbonate solution plus a petroleum ether-chloroform mixture. The non-aqueous layer was dried over drierite, the solvent evaporated off in vacuo, and the residue taken up in the minimum amount of warmacetone. On cooling, clusters of needles formed. After two recrystallizations from acetone these were snow-white, silky needles softening at 130°, melting 143-44°C (corr'd). Yield around 30%. Analysis. Calculated for $C_{39}H_{51}O_5N$: C, 76.31; H, 8.38; N, 2.28. Found: C, 76.23; H, 8.37; N, 2.56.

40 mg. of this material dissolved in 1 ml. warm carbon tetrachloride was treated with a 100% excess of 5% bromine in carbon tetrachloride solution. A little chloroform was added and the solution extracted with two portions of 5% sodium bicarbonate solution. The colorless organic phase was dried over calcium chloride, the solvent removed at reduced pressure, and the residue recrystallized from acetone to give white needles melting at 142-45°(corr'd). This material gave a positive elementary test for nitrogen, but a negative bromine test.*

*This work was completed in August, 1947, before Carter, et.al. (7b) published melting points for tribenzoyl sphingosine; consequently, at the time, it seemed necessary to ascertain if the 143-44° substance contained a double bond. The point was not pursued further, as the Illinois group's paper appeared in the next issue of the J.Biol.Chem. In agreement with these workers, it did not prove possible to obtain tribenzoyl sphingosine by benzoylation of the crude free bases (however, as indicated above the dihydrocompound was obtained in this manner, contrary to the finding of Carter, et.al.) The residues after removal of tribenzoyl dihydrosphingosine gave easily crystallized fractions melting from 95° to 130°. Repeated recrystallizations did not raise the melting point to 140°.

Properties of tribenzoyl dihydrosphingosine. This compound is very soluble in warm acetone and slightly soluble cold. It is soluble in hot 60-70° petroleum ether, but insoluble cold--not more than 1 mg. per 100 ml. in hexane, or about 10^{-4} M. It is very soluble in cold chloroform. Recrystallization from a small volume of acetone results in long interlacing needles, which form a mat on the funnel; from petroleum ether small spherical clusters of short needles are usually obtained, but a very dilute solution separates fan-shaped clusters of long, fine, flexible needles on cooling. The ultra-violet light extinction curve of an ethanol solution is given in Fig. VIII on the next page.

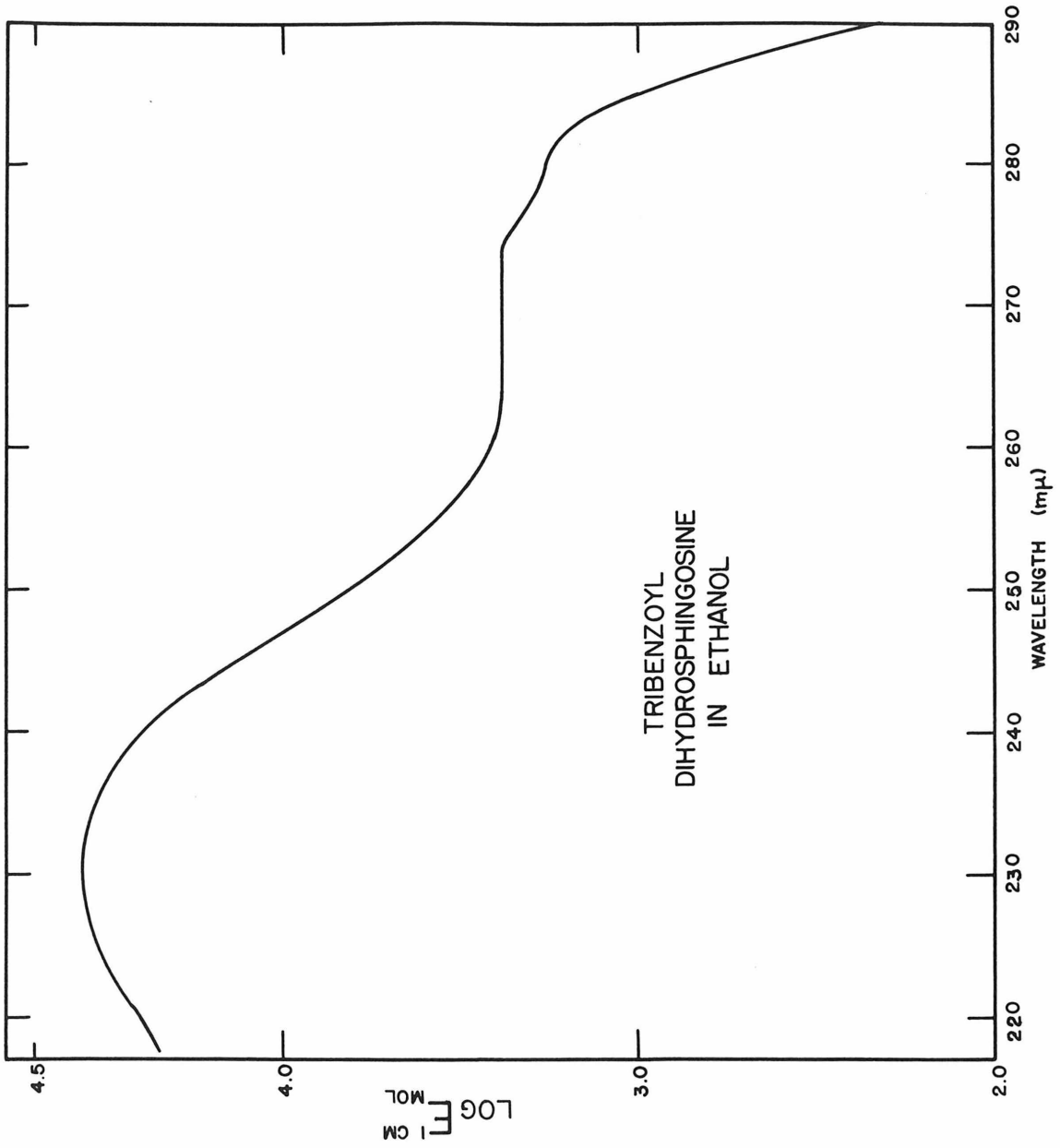

TRIBENZOYL
DIHYDROSPHINGOSINE
IN ETHANOL
LOG E 1 CM MOL
4.5
4.0
3.0
2.0
220
230
240
250
260
270
280
290
WAVELENGTH (mμ)

FIG. VIII

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III. THE ULTRAVIOLET ABSORPTION OF SOME O-BENZOYL COMPOUNDS

DISCUSSION:

In connection with the characterization of sphingosine and derivatives in Part II of this thesis the question rose as to the effect on the ultraviolet absorption spectrum which might be produced by changes in the structure of the carbon chain of the alcohol in O-benzoyl aliphatic alcohols (considering only changes not introducing additional absorbing groups). Recognizing that in such molecules the only chromophore would be the benzoyl group common to all, any effects were expected to be small. Lack of time permitted study of only four compounds: benzoic anhydride, methyl benzoate, 'octadecyl' benzoate, and d,l-dibenzoyl-n-octadecandiol-1,2. The following is not a theoretical treatment, but rather an empirical summary of the observed facts.

Even these limited data permit the conclusion that a change in the number of carbon atoms in the alcohol produces a negligible difference in the ultraviolet absorption. Two O-benzoyl groups in the molecule produce a small (0.5-1.0 mμ) shift in the maxima toward longer wavelengths and double the molar extinction coefficients. Three benzoyl groups in the molecule of tribenzoyl-dihydrosphingosine (Part II, Fig. VIII, this thesis) apparently cause a further slight shift toward the longer wavelength and triple the peak at 230 mμ. The effect in the region above 250 mμ is unexpected; in the absence of comparable data

for simple N-benzoyl compounds* one can speculate that it results from a displacement toward shorter wave lengths in N-benzoyl chromophores of the peaks at 266, 273, and 280 mμ for O-benzoyl compounds.

An additional feature of the light extinction curve of dibenzoyl-octadecandiol in hexane (Fig. XI) is the loss of the subsidiary peak at 266 ± 0.5 mμ in methyl and 'octadecyl' benzoates; its vestigial trace is perhaps the hump just visible at 266-68 mμ. In ethanol both methyl benzoate and dibenzoyl-octadecandiol show only small bumps in this region. The spectra in ethanol as compared to hexane show: (a) less fine structure, with the peaks broader and the valleys almost nonexistent in the region above 265 mμ; (b) a small shift (1.5-2.0 mμ) toward longer wavelength of the peak around 228 mμ; and (c) in the case of dibenzoyl-octadecandiol a significant increase in the height of this peak. Phenomena (a) and (b) conform to well established patterns (1), and agree well with Scheibe's findings for ethyl benzoate, where a peak at 227 mμ(log E around 4.2) in hexane was shifted to 229 mμ and raised to log E about 4.3 in methanol (8).

In Table XIX the data of this investigation are summarized and compared with values for some other compounds assembled from the literature. It can be said that, in the generalized

*A curve of benzamide (in alcohol?) is given by Ramart-Lucas and Grunfeld (6), but it does not extend below 235 mμ and shows only an inflection around 270 mμ, where log E is around 2.7.

benzoyl derivative

$$C_6H_5CO\text{-}Y\text{-}R,$$

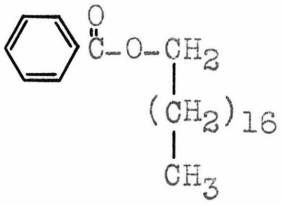
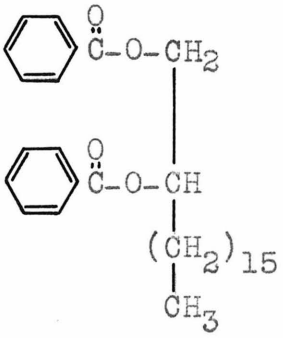
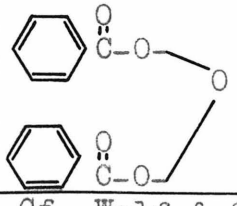
where -R, is either -H or a saturated alkyl radical, the nature of -Y- is of paramount importance in determining the secondary features of the ultraviolet light extinction curve, which, of course, is primarily that of the carbonyl chromophore coupled to the benzene chromophore. If -Y-R is a saturated group such as methyl, there is only a minor change from the prototype benzaldehyde spectrum (4). On the other hand if -Y- is an atom with one or more unshared pairs of electrons, such as oxygen or nitrogen, there is the possibility of additional resonance structures involving these electrons; consequently O-benzoyl derivatives show detectable differences from the benzaldehyde spectrum, as presumable do N-benzoyl derivatives also. However, again the size of -R has little or no effect, as it does not materially affect the availability of the unshared electron pairs on the oxygen or nitrogen atoms. In the case of benzoic anhydride -R is no longer a saturated group, but a second chromophore; since the unshared electron pairs of the bridge oxygen atom can interact with both benzoyl groups additional electronic states of the molecule are possible and there is a further shift from the spectrum of methyl benzoate.

EXPERIMENTAL:

Materials. The methyl benzoate was prepared by Dr. H.

TABLE XIX

THE ULTRAVIOLET ABSORPTION SPECTRA OF SOME BENZOYL DERIVATIVES

Solvent / Compound	Hexane		Ethanol	
	λ_{max} (mμ)	log E_{max}	λ_{max} (mμ)	log E_{max}
 (4)			243 279 325	4.2 3.25 1.25
 (4)			199 240 278 320	4.3 4.12 3.02 1.7
 (2)	231.0 273.5 276.0 283.0	3.70 2.85 2.93 2.93	227 267	4.15 3.25
 *	227.0 266.5 272.5 280.0	4.08 2.85 2.94 2.88	228.5 273.0 280.0	4.05 2.95 2.86
	227 266 272.5 279.5	4.11 2.85 2.95 2.89		
	228.0 273.0 280.5	4.31 3.25 3.18	230.0 273.5 280.0	4.39 3.26 3.17
	238 276	4.43 3.37		

* Cf. Wolf & Strasser (9).

Rinderknecht (7); b.p. 83° at 12 mm, 63-64° at 9 mm. The benzoic anhydride was Eastman Technical grade material twice recrystallized from benzene plus petroleum ether to colorless thin plates, softening at 40° and melting at 41.8-43.2°C (corr.) this material showed no absorption in the region 290-350 mμ.

The 'n-octadecyl'-benzoate was prepared from technical grade Eastman octadecyl alcohol which had been recrystallized once from carbon tetrachloride, twice from acetone, and finally from carbon tetrachloride again; melting behaviour: softened at 56°, melted 57.8-58.5°C (corr.). A sample of the recrystallized alcohol was benzoylated in pyridine with excess benzoyl chloride; the crude product was recrystallized three times from acetone, and once from methanol. The ultraviolet spectrum of this preparation was determined; after a second recrystallization from methanol the spectrum showed no change. It was a soft, white, apparently amorphous solid; m.p. 41.3-42.5°C (corr.). Analysis. Calculated for $C_{25}H_{42}O_2$: C, 80.17; H, 11.30. Found: C, 81.25; H, 11.53. The compound was recrystallized once more from ethanol. This gave needles and rosettes of needles, m.p. 41.2-42.4°C (corr.). Found: C, 81.24; H, 11.63. Assuming a mono benzoylated saturated aliphatic compound this corresponds to about a C_{23} alcohol; apparently the fractional crystallizations of the crude alcohol and benzoate concentrated the higher molecular weight alcohols. The spectra reported (Fig. XI) are those of material recrystallized three times from acetone and twice from methanol.

d,l-Dibenzoyl-n-octadecandiol-1,2 was prepared from 5.7 g. of d,l-n-octadecandiol-1-2 of m.p. 79.0° prepared by Dr. C. D. Wagner (5) by treatment with excess benzoyl chloride in pyridine. Yield of crude material was 93%. The product was recrystallized twice from acetone and twice from ethanol to give long silky needles softening at 32° and melting at 34.8-36.8°C (corr.). Analysis. Calculated for $C_{32}H_{46}O_4$: C, 77.68; H, 9.38. Found: C, 78.17, 77.85; H, 9.58, 9.21.

The hexane for optical measurements was purified by treatment with two successive portions of 30% fuming sulfuric acid overnight and then once with basic permanganate solution. After thorough washing with water and drying over drierite, the solvent was distilled through a short fractionating column. The optically pure ethanol was prepared by the sulfuric acid and silver oxide method of Leighton, Crary, and Schipp (3).

Methods. Weighed samples were made up to volume with optically clear hexane or ethanol. The volumetric flasks and pipettes used in dilutions were not specially calibrated. Measurement of the absorption were made with the Beckman Model DU Spectrophotometer, using matched 1 cm. quartz cells and a hydrogen lamp as light source. The values of extinction plotted in Fig.s IX-XI and tabulated in Table XIX are the molar extinction coefficients* at the wavelengths specified, for a 1 cm. thick layer of solution. The values of extinction are estimated to be accurate to better than 5%; those of the wavelengths to better than 0.5 mμ.

*American convention. See, in Part I of this thesis, "Ultraviolet spectroscopy. Calculation."

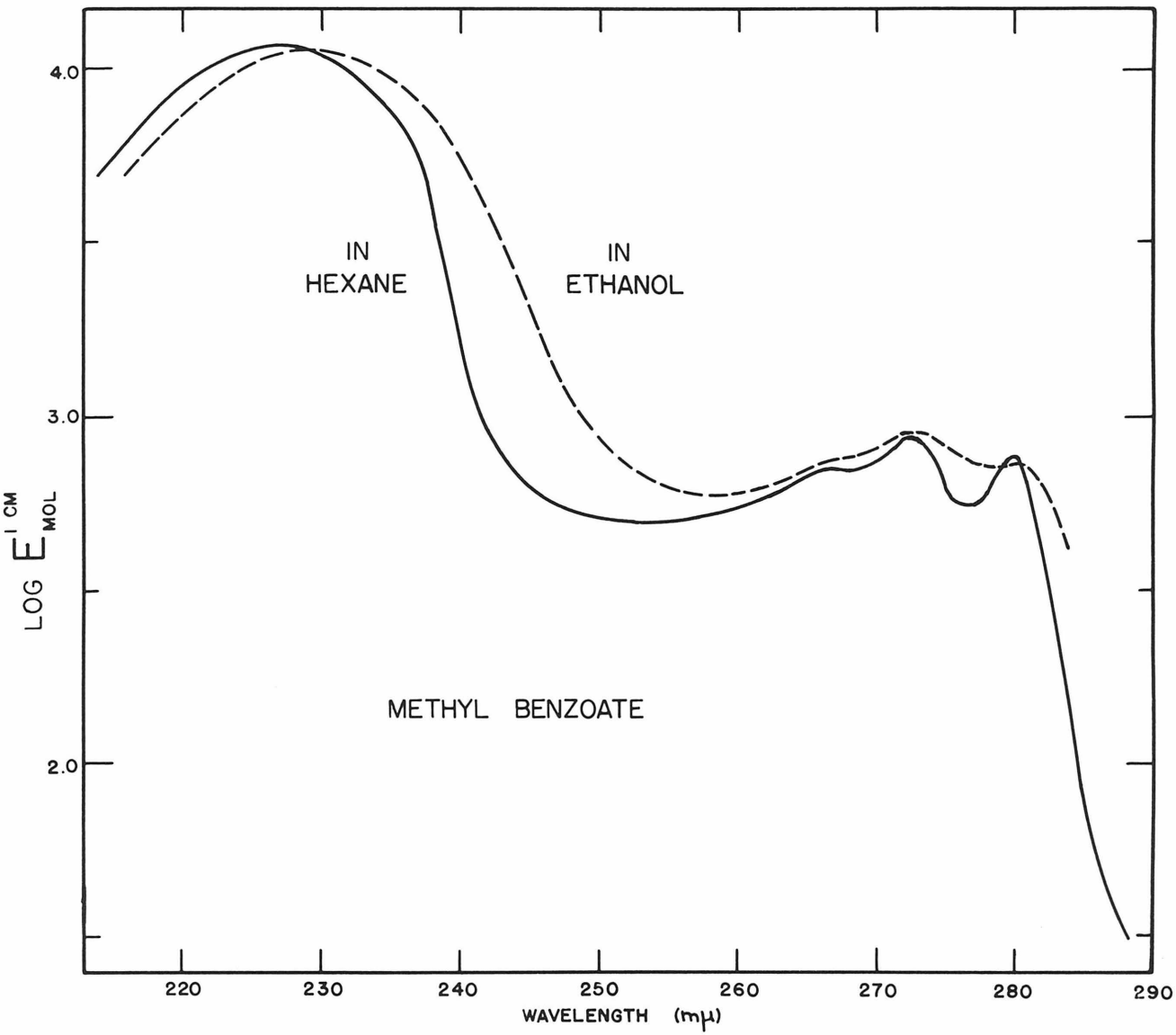

4.0
3.0
2.0
LOG $E^{1\,CM}_{MOL}$
IN
HEXANE
IN
ETHANOL
METHYL BENZOATE
220
230
240
250
260
270
280
290
WAVELENGTH (mμ)

FIG. IX

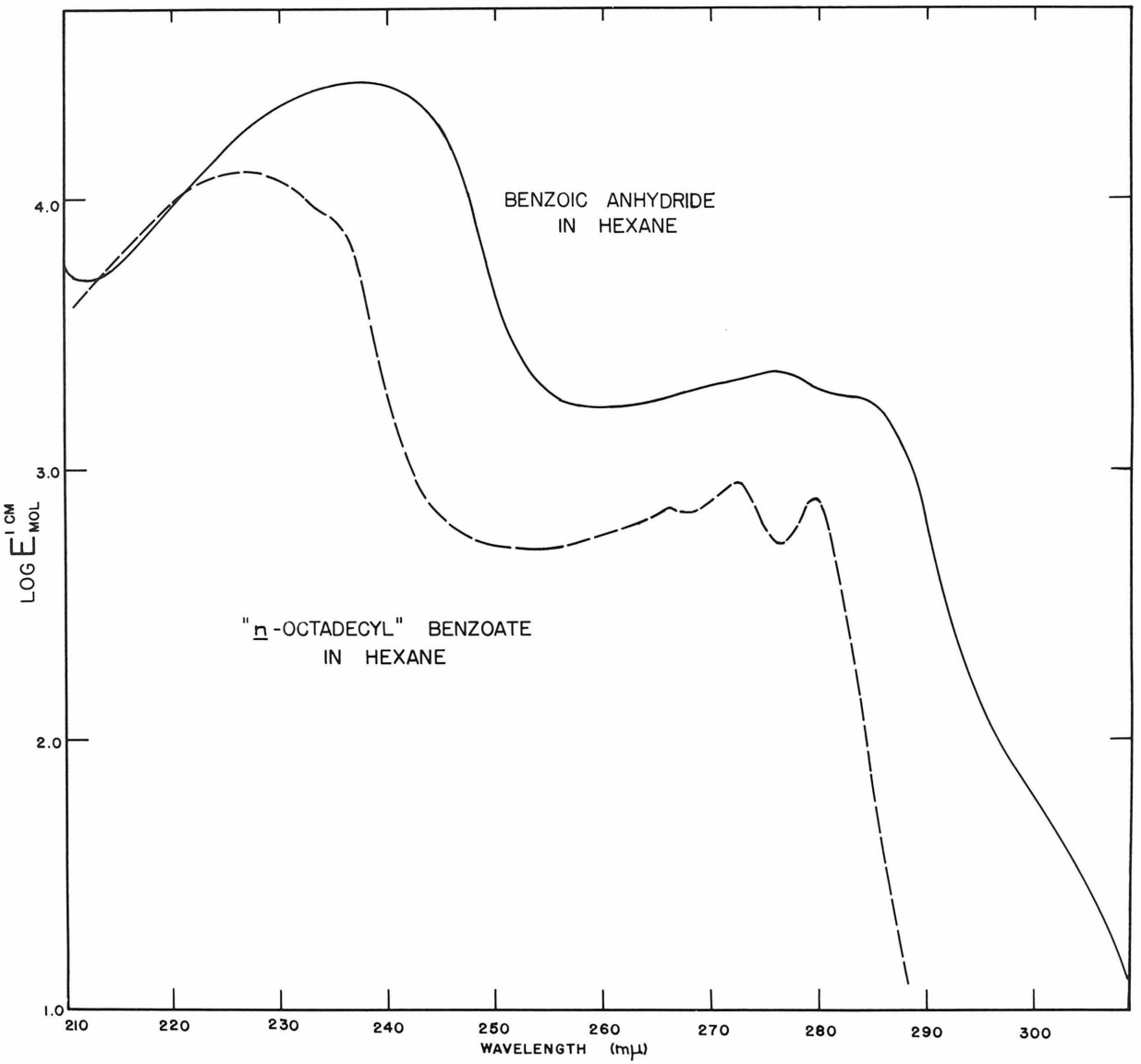

BENZOIC ANHYDRIDE
IN HEXANE
"n-OCTADECYL" BENZOATE
IN HEXANE
LOG E 1 CM MOL
4.0
3.0
2.0
1.0
210
220
230
240
250
260
270
280
290
300
WAVELENGTH (mμ)

FIG. X

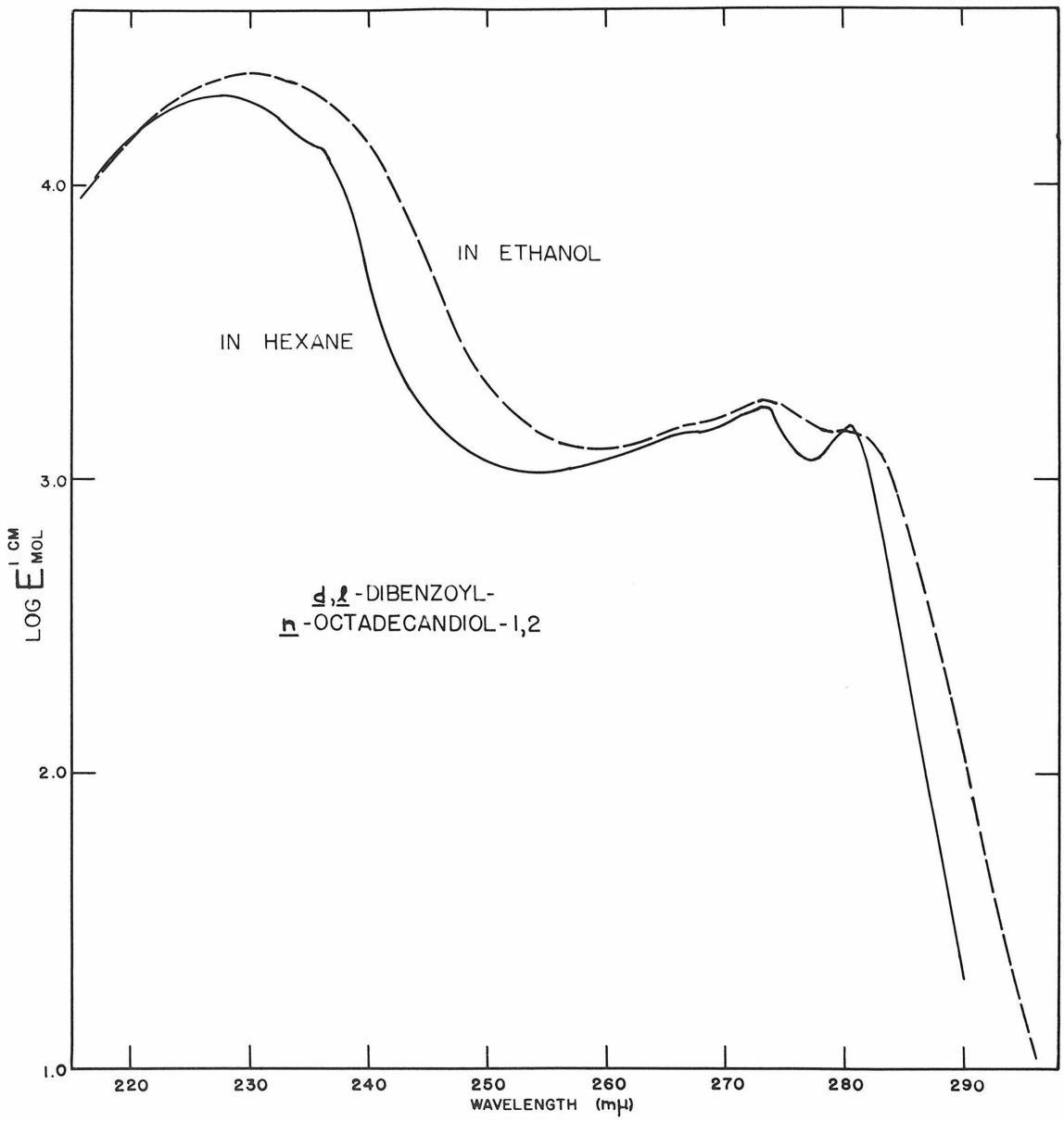

4.0
3.0
2.0
1.0
LOG E 1 CM MOL
IN ETHANOL
IN HEXANE
d,l-DIBENZOYL-
n-OCTADECANDIOL-1,2
220
230
240
250
260
270
280
290
WAVELENGTH (mμ)

FIG. XI

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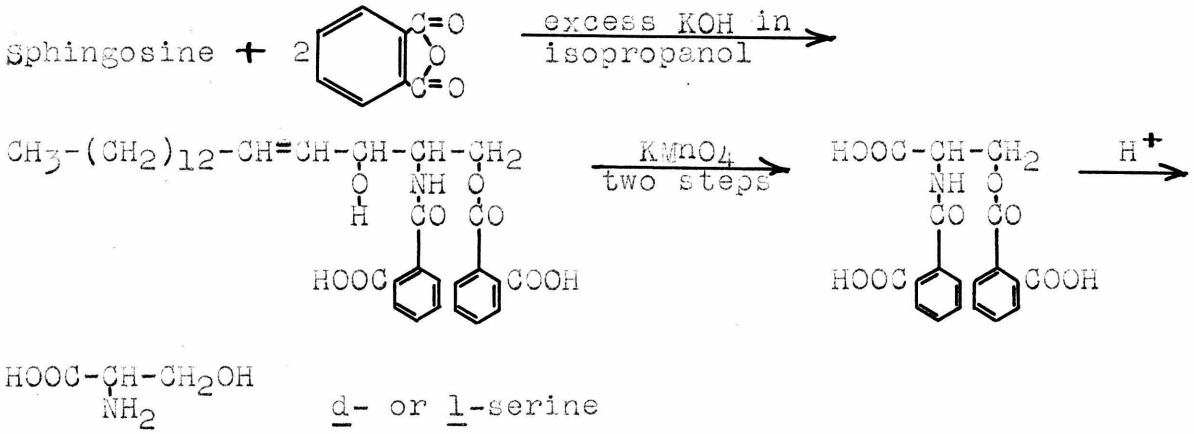
PROPOSITIONS

1. I propose that the pK_a' values of the dihalogen tyrosines can be adequately explained if the phenolic hydroxyl is considered to be present as the phenate ion during the ionization of the ammonium group and as the undissociated phenol during the ionization of the carboxyl group. From the Kirkwood-Westheimer theory* plus Traube's data** and some assumptions one can calculate a value for the separation of the O^- and N^+ of 6.3 Å, compared to about 7.8 Å measured on a model of the extended chain structure.

*J.Chem.Phys., 6, 513 (1938); **Ann., 290, 43 (1896).

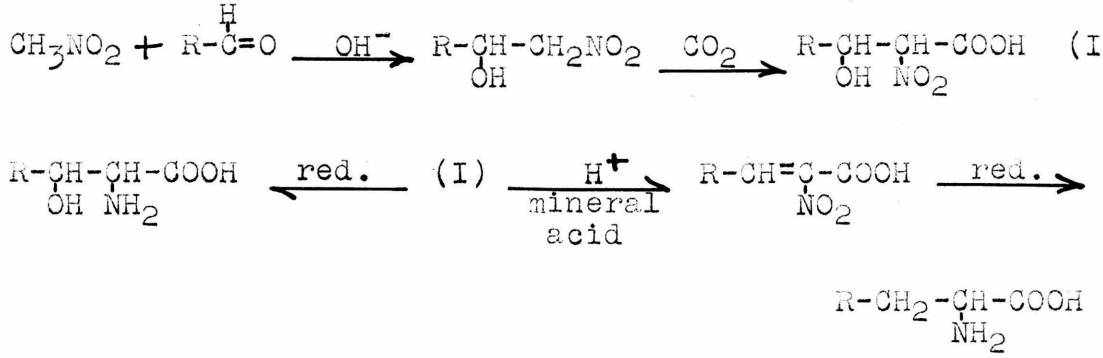
2. In order to establish the configuration about carbon atom two of sphingosine, I propose the following degradation to serine:



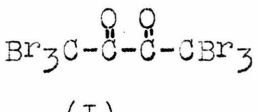
3. Siegel & Moran* interprete their results to mean that, "mono-esterification of dibasic acid anhydrides with primary... alcohols proceeds instantaneously and quantitatively at room temperature." I propose that in fact they observed only an instantaneous reaction between the anhydride and alcohol occurring during the addition of alcoholic KOH.

*J.Am.Chem.Soc., 69, 1457 (1947).

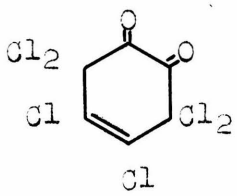
4. The following series of reactions constitute a general synthesis of amino acids, particularly the β-hydroxy-α-amino acids:



5. a) The normal reaction of periodate ion with carbonyl groups is postulated* to proceed through the hydrated form of the carbonyl group: $>C=O + H_2O \rightleftharpoons HO-\overset{|}{\underset{|}{C}}-OH$. On this basis (I) and (II) should react much more rapidly than the corresponding unsubstituted diketones, since stable hydrates are anticipated.



(I)

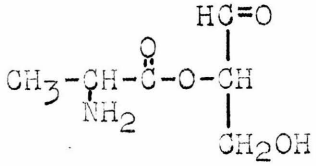


(II)

b) The formation of (I), above, from tetrabromo-o-benzoquinone can be explained by a simpler set of reactions than proposed by Jackson & Adams**.

*Org.React., 2, 341 (1944); **J.Am.Chem.Soc., 37, 2522 (1915).

6. Crystal structure determination of (I) would provide a direct relation of the configurations of the amino acids and carbohydrates.



(I) sec-O-(l-alanyl)-d-glyceraldehyde

7. Later work* does not entirely confirm the conclusions of Hantzsch** regarding the structure of benzamide. A repetition of Hantzsch's ultraviolet measurements with an instrument such as the Beckman, using solvents of varying polarities, should be instructive.

*Bull.soc.chim., 4, 478 & 944 (1937); **Ber., 64, 661 (1931).

8. The simultaneous study of the relation of iron to the formation of phycoerythrin or phycocyanin and to protein synthesis should be extremely rewarding in understanding the role of iron in plants.

9. a) A true measure of the permeability of cells to strong electrolytes can be obtained by the use of radioactive isotopes.

b) One could attack the problem of the mechanism of salt accumulation by plant cells by studying the material balance of the process.

10. The usefulness of ultraviolet absorption spectra for qualitative organic analysis could be greatly increased if the available data were collected and indexed according to the wavelengths of the principal maxima.