upon the addition of water indicating that the anhydride ring was intact.

Polylysyl-bovine Plasma Albumin.—Two grams of crystalline bovine plasma albumin (Pentex, Inc., Kankakee, Ill., Lot A 1201) dissolved in 100 ml. of cold saturated sodium bicarbonate solution, was added to the freshly prepared N-carboxy-L-lysine anhydride hydrobomide, which dissolved in a few minutes. In other preparations the anhydride was divided into several portions and the protein solution added successively to each batch. The reaction mixture was allowed to stand 4 to 5 hours in the cold, dialyzed exhaustively against distilled water, followed by electrodialysis. The protein solution was then lyophilized and stored in the cold.

N-Carboxy-L-glutamic Acid Anhydride.—Ten grams of γ-benzyl-L-glutamic acid[22] was converted to γ-benzyl N-carboxy-L-glutamic acid anhydride in a 99% yield by the method of Farthing.[20] The anhydride (9.9 g.) was dissolved in 100 ml. of dioxane and the γ-benzyl group removed by hydrogenation at room temperature in the presence of 2 g. of 10% palladized charcoal. After 24 hours, when hydrogen uptake was complete and theoretical amount of hydrogen was consumed, the catalyst was filtered, washed with dioxane and the filtrate containing the anhydride was concentrated *in vacuo* to a colorless viscous oil.

Polyglutamyl-bovine Plasma Albumin.—Two grams of crystalline bovine plasma albumin dissolved in 100 ml. of cold saturated sodium bicarbonate solution was added to the freshly prepared N-carboxy-L-glutamic acid anhydride. After standing overnight in the cold, the reaction mixture was dialyzed exhaustively against distilled water and lyophilized.

Poly-L-leucyl- and Poly-DL-phenylalanyl-proteins.—Four hundred mg. each of finely powdered N-carboxy-L-leucine and N-carboxy-DL-phenylalanine anhydrides was suspended in 100 ml. of cold $^1/_{15}$ *M* sodium bicarbonate buffer containing 2.0 g. of crystalline bovine plasma albumin and magnetically stirred for 48 hours. The reaction mixtures were centrifuged at 18,000 r.p.m. using No. 30 head of the Spinco model L centrifuge. The supernatant was dialyzed exhaustively against cold distilled water and lyophilized.

Preparation of Protein Solutions for Electrophoresis.—An approximately 1% (w./v.) solution of the lyophilized protein was dissolved in 0.1 ionic strength buffer and dialyzed with agitation against two changes of the same buffer. Samples of lyophilized protein dried over phosphorus pentoxide at 100° and 0.03 mm. pressure to constant weight showed an average loss of 6% moisture. The electrophoretic analyses were carried out on 0.94% (w./v.) solutions to correct for this 6% moisture.

Electrophoretic Analysis.—A Spinco model H electrophoresis-diffusion apparatus was employed for the electrophoresis measurements. All experiments were conducted in a 1.8-ml. capacity quartz micro Tiselius cell equipped with Alberty electrodes at a bath temperature of 2.07°. Conductivity measurements were made at 0° in a No. 038-0081 electrolytic conductivity cell (Perkin–Elmer Corp., Norwalk, Conn.) using a L. and N. portable conductivity bridge.

MADISON, WISCONSIN

(22) W. E. Hanby, S. G. Waley and J. Watson, *J. Chem. Soc.*, 3239 (1950).

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, UNIVERSITY OF OREGON]

The Ionization of Cysteine[1]

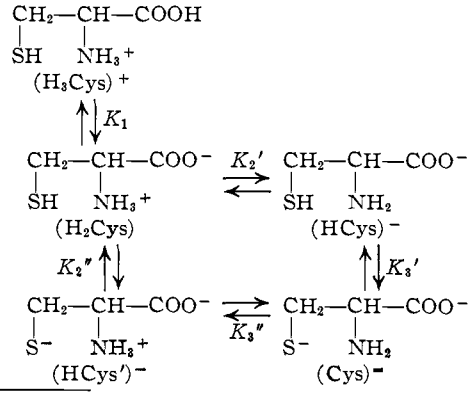
BY GEORGE GORIN

RECEIVED AUGUST 23, 1955

The ultraviolet absorption spectra of thioglycolic acid and its anion $^-SCH_2COO^-$ have been examined; a large increase in absorption toward longer wave lengths accompanies the ionization of the -SH group. Cysteine hydrochloride develops a similar absorption during the titration of its second proton (pK 8.27 at 25° in 0.10 *M* potassium chloride) and it is concluded that the ion $^-SCH_2CH(NH_3^+)COO^-$ is produced in this process. This ion may be in equilibrium with its tautomer, $HSCH_2CH(NH_2)COO^-$, but the presence of the latter can be estimated only on the basis of a rather uncertain assumption.

The mode of ionization of the amino acid cysteine has been the subject of considerable discussion, and the main point of uncertainty is of determining importance for defining the ionization of this substance in biological systems. Since cysteine, in its free and combined forms, plays an important role in the biochemistry of living organisms,[2] a clarification of the problem is very desirable.

The status of present knowledge may be summarized in the scheme



There is general agreement that the carboxyl hydrogen in $(H_3Cys)^+$ is the first to ionize, but there is a difference of opinion concerning the subsequent steps: (a) Cohn and Edsall,[3] in their classical work, indicate that the second proton comes from the ammonium group and the third from the thiol, giving, successively, $(HCys)^-$ and $(Cys)^=$; (b) Calvin[4] suggests the opposite view, that the ionization proceeds from (H_2Cys) through $(HCys')^-$ to $(Cys)^=$; and (c) Ryklan and Schmidt[5] propose that $(HCys)^-$ and $(HCys')^-$ are formed contemporaneously and in nearly equal quantities.

As was pointed out by Edsall,[6] the ionization constants which are determined experimentally from measurements of the hydrogen ion activity are related to those of the scheme above by the relations, $K_2 = K_2' + K_2''$; $1/K_3 = 1/K_3' + 1/K_3''$, $K_2K_3 = K_2'K_3' = K_2''K_3''$; the respective views are equivalent to making the assumptions, for (a), that $K_2' >> K_2''$, for (b), that $K_2'' >> K_2'$, and, for (c), that $K_2' \sim K_2''$. Recently, Grafius and

(1) Presented at the 128th National Meeting, American Chemical Society, Minneapolis, Minn., September, 1955.

(2) E. S. G. Barron, *Advances in Enzymology*, **11**, 201 (1951).

(3) E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides," Reinhold Publ. Corp., New York, N. Y., 1943, p. 85.

(4) M. Calvin, "Mercaptans and Disulfides," in "Glutathione," Academic Press, Inc., New York, N. Y., 1954, pp. 8–11.

(5) R. L. Ryklan and C. L. A. Schmidt, *Arch. Biochem.*, **5**, 89 (1944).

(6) J. T. Edsall, quoted in ref. 5.

Neilands[7] have produced evidence in favor of scheme (c).

The arguments for this scheme are based upon comparison of the ionization constants of cysteine with those of related compounds. Ryklan and Schmidt based their calculations on the values $pK_2 = 8.33$ and $pK_3 = 10.78$ for cysteine,[8] and assumed $pK_2' = 8.60$, the value which had been found for S-ethylcysteine[9]; Grafius and Neilands used the values $pK_2 = 8.30$ and $pK_3 = 10.40$ for cysteine, $pK_2 = 8.75$ for S-methylcysteine, and $pK_2 = 8.65$ for cysteine betaine. All the values in the latter investigation were determined by the authors in 0.15 M sodium chloride, and, for the purposes of comparison, it is best to have such a set of data, determined in a consistent way and based on a uniform convention. Despite the differences in the values of the constants which were assumed, the results were not too different with respect to the ratio $(HCys')^-/(HCys)^-$ calculated in each case; the calculation of Grafius and Neilands gives 1.3.

Results and Discussion

Measurements of the hydrogen ion activity do not, *per se*, distinguish the source of the hydrogen ions, and it is therefore clear that the more intimate investigation of the species involved in the ionization of cysteine must be based on a property characteristic for these species. Noda, Kuby and Lardy[10] showed, in the course of an investigation upon the hydrolysis of thiol acetates, that *n*-butyl mercaptide ion absorbs ultraviolet light much more strongly than the mercaptan from which it arises, and it seemed likely that this property might permit the detection and determination of species $(HCys')^-$ and $(Cys)^=$, which contain $-S^-$.

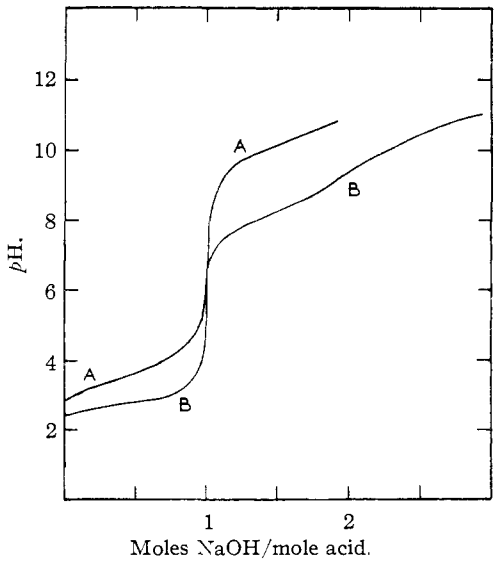


Fig. 1.—Titration curves: (A) thioglycolic acid; (B) cysteine hydrochloride; 0.01 M solutions in 0.10 M potassium chloride.

(7) M. A. Grafius and J. B. Neilands, THIS JOURNAL, **77**, 3389 (1955).
(8) H. Borsook, E. L. Ellis and H. M. Huffman, *J. Biol. Chem.*, **117**, 281 (1937).
(9) S. Ratner and H. T. Clarke, THIS JOURNAL, **59**, 200 (1937).
(10) L. H. Noda, S. A. Kuby and H. A. Lardy, *ibid.*, **75**, 913 (1953).

In order to test this expectation, a study was first made of thioglycolic acid. Its titration curve, shown in Fig. 1, indicates that this acid is dibasic; the buffer regions are widely separated, and, therefore, in the first one we deal only with (H_2Tgl) and $(HTgl)^-$, and in the second only with $(HTgl)^-$ and $(Tgl)^=$.

CH_2—COOH	CH_2—COO^-	CH_2COO^-
SH	SH	S^-
(H_2Tgl)	$(HTgl)^-$	$(Tgl)^=$

Figure 2 shows the absorption spectra of some solutions of interest. Curve A is the spectrum of a 0.01 M solution of acid in 0.10 M potassium chloride, and the spectrum of a solution to which one half of a mole of base has been added is almost identical; therefore, it appears that the absorption of $(HTgl)^-$ is very similar to that of (H_2Tgl). Curve B is the spectrum of 0.01 M thioglycolic acid in initially 0.10 M sodium hydroxide, where the acid must have been converted to $(Tgl)^=$, and it is seen that this solution absorbs more strongly and at longer wave lengths. At these concentrations, it is not possible to locate any maxima; the shift of comparable points on the two curves is about 37 Å.

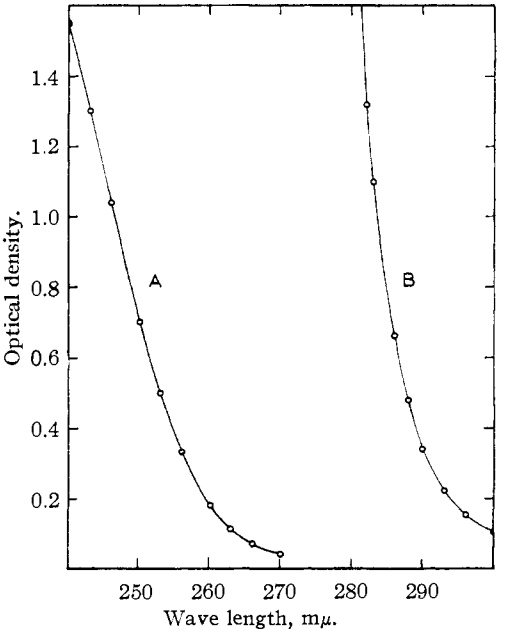


Fig. 2.—Spectra of thioglycolic acid: (A) 0.01 M in 0.10 M potassium chloride; (B) 0.01 M in initially 0.10 M sodium hydroxide.

The spectra of the solutions, particularly the alkaline ones, change considerably with time, and are not reproducible with great precision; apparently, some side reactions take place, which result in a permanent alteration of the molecule—reaction with oxygen, which was carefully minimized but not completely avoided, as well as other processes, are, apparently, responsible. Two experiments, however, show that the spectral changes observed are due in the main to the process of ionization, and not to adventitious effects. In the first place, an alkaline solution, with spectrum (B), again gives

spectrum (A) when restored to the *p*H of a 0.01 *M* thioglycolic acid solution; deviations amounted to less than 5% throughout the spectral range observed. Secondly, performance of a spectrophotometric titration of 0.01 *M* thioglycolic acid at an appropriate wave length, *e.g.*, 283 mμ, indicates that the absorption is proportional to the amount of base added after the first mole. A typical experiment is shown in Fig. 3, and the result of the spectrophotometric titration is in good agreement with the alkalimetric one.

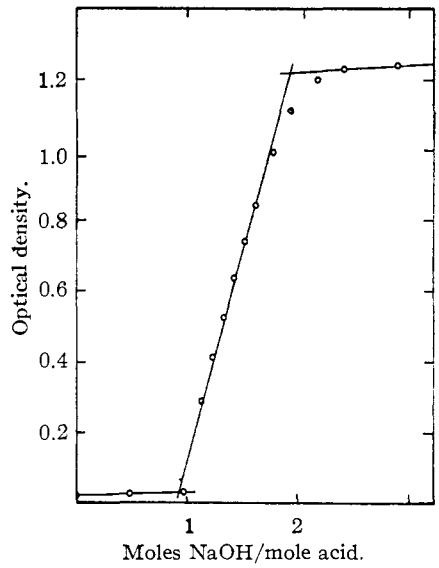


Fig. 3.—Spectrophotometric titration of thioglycolic acid at 283 mμ; end-point 1.96 moles of NaOH/mole of acid.

The behavior of cysteine hydrochloride in comparable conditions can be seen in Figs. 1 and 4. The former shows the titration curve of 0.01 *M* cysteine hydrochloride in 0.10 *M* potassium chloride; the second and third ionization steps are the ones which concern us here. The "practical ionization constants" (see Experimental section for definition of these quantities) are calculated to be 8.27 and 10.42; taking the values of 0.82 and 0.47 for the activity coefficients of $(HCys)^-$ and $(Cys)^=$,[11] respectively, gives 8.36 and 10.66 for the thermodynamic constants, in fair agreement with the results of Borsook, *et al.*[8]

Figure 4 shows the spectra of interest. Curve A is that of 0.01 *M* cysteine hydrochloride, and the curve of a solution to which one half of a mole of base has been added does not differ appreciably from this; hence, the spectra of $(H_3Cys)^+$ and of (H_2Cys) must be nearly the same in the region investigated. Curve B is that of a solution to which 1.5 moles of base has been added; since the concentration of the species formed in this step is, at this point, approximately 0.005 *M*, the optical densities observed are doubled in order to make this curve comparable to the other two. Curve C is the spectrum of 0.01 *M* cysteine hydrochloride in initially 0.10 *M* sodium hydroxide. It is seen that the titration of the second hydrogen is accompanied by a large increase and shift in the ultraviolet absorption, wholly similar to that observed when the thiol hydrogen is removed in thioglycolic acid; in this case, the shift of comparable points on the curves is about 38 Å. The removal of the third hydrogen causes a further, but much smaller, change.

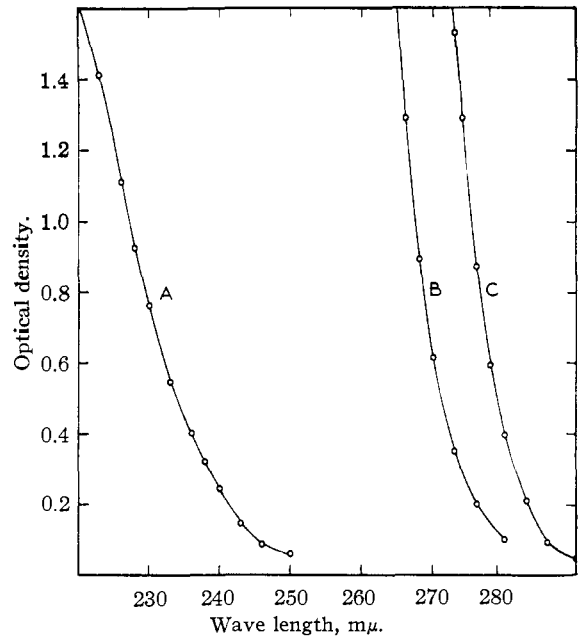


Fig. 4.—Spectra of cysteine: (A) 0.01 *M* $(H_3Cys)^+Cl^-$ in 0.10 *M* KCl; (B) 0.01 *M* $(H_3Cys)^+Cl^-$ + 1.5 moles NaOH, optical densities × 2; (C) 0.01 *M* $(H_3Cys)^+Cl^-$ in 0.10 *M* NaOH.

The spectra of cystine in 0.10 *M* hydrochloric acid and 0.10 *M* sodium hydroxide are very little different, as reported by Beaven and Holiday,[12] and confirmed in this work. We must, accordingly, conclude that the large shift and increase in absorption in going from cysteine hydrochloride to the middle of the second buffer region (*i.e.*, from curve A to B in Fig. 4) must be due to the formation of ion $(HCys')^-$.

Figure 5 shows the results of a typical spectrophotometric titration, at 274 mμ; the behavior, *e.g.*, at 278 mμ, is entirely similar. The plot of optical densities against the amount of base added is seen to consist of three segments. The first, corresponding to the formation of (H_2Cys) from $(H_3Cys)^+$, has a negligible absorption, as we should expect from curve A of Fig. 4. The second segment has a slope of 0.330, and this corresponds to an extinction coefficient of 33 for the species being formed, either $(HCys')^-$ or a mixture of $(HCys)^-$ and $(HCys')^-$; the segment deviates slightly from linearity in its upper portion, because the absorption of the species formed in the third titration step is comparatively high and the second and third steps are not widely separated. A line representing the best linear extrapolation is drawn through the points. The third segment deviates downward in its upper portion, because the correspondence between $(Cys)^=$ and the amount of base added is no longer stoichiometric above *p*H 10.4;

(11) J. Kielland, THIS JOURNAL, **59**, 1675 (1937).

(12) G. H. Beaven and E. R. Holiday, *Advances in Protein Chem.*, **7** 319 (1952); see Fig. 4, p. 328.

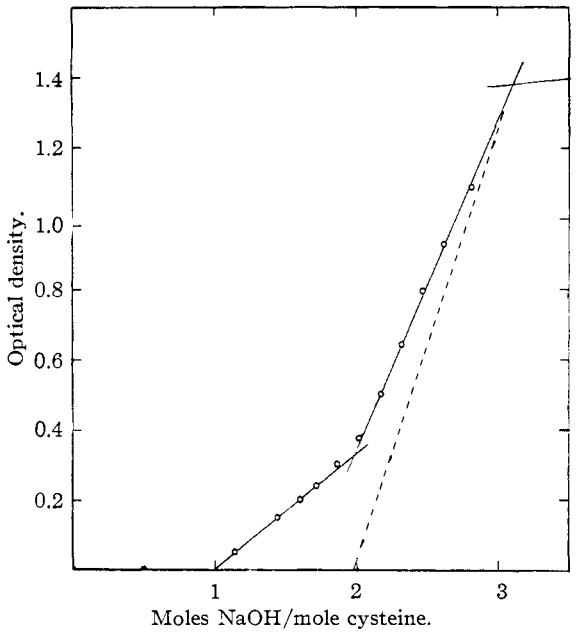


Fig. 5.—Spectrophotometric titration of cysteine hydrochloride; end-points: 0.98, 2.00 and 3.10 moles of NaOH/mole of cysteine.

again, the solid line represents the best linear extrapolation. The points of intersection of the extrapolated segments are in good agreement with stoichiometric expectations. Since the formation of $(Cys)^{=}$ is accompanied by the disappearance of $(HCys')^{-}$, the absorption of which is not negligible, a correction must be made for this, and the dashed line represents the segment which results. Its slope is 1.3, and the extinction coefficient is 130, in satisfactory agreement with that found for $(Cys)^{=}$ at this wave length in 0.10 *M* sodium hydroxide solution.

If we had an independent measure of the extinction coefficients of $(HCys)^{-}$ and $(HCys')^{-}$, the slope of the second segment would permit us to calculate, as we would surely like to do, if, and how much of, the former species might be present. Unfortunately, we lack this information. For the purpose of making a rough estimate, we can consider the pair cystine–cystinate ion, which is a fairly close analog of $(HCys')^{-}$ and $(Cys)^{=}$; the difference between the two members of each pair is an H^{+} in the $-NH_3^{+}$ group. The ratio of the optical densities of cystine and cystinate ion at a comparable wave length is approximately 1 to 1.4, and, on the same basis, the extinction coefficient of $(HCys')^{-}$ at 274 mμ would be 93. The absorption of $(HCys)^{-}$ at this wave length should be negligible. Therefore, the observed optical density of 0.330 would correspond to a $(HCys')^{-}/(HCys)^{-}$ ratio of 0.54. *This result is based on a very uncertain assumption.* It is possible, indeed likely, that the difference in optical density between $(Cys)^{=}$ and $(HCys')^{-}$ would be greater than for cystinate–cystine, and this would have the effect of increasing the estimated proportion of $(HCys')^{-}$. Accordingly, the ratio 0.54 should be regarded as a lower limit; $(HCys')^{-}$ might, possibly, be the only species present.

Other experiments, designed to resolve this uncertainty and based on yet a different assumption, are now in progress.

Acknowledgments.—It is a pleasure to acknowledge the financial support of the Research Corporation of New York and of the Graduate School of the University of Oregon. Dr. Wendell M. Graven afforded the author encouraging and stimulating discussion.

Experimental

Materials.—Thioglycolic acid of practical grade was distilled twice, rather rapidly, under a low pressure of nitrogen, only the center one-third of the distillate being retained each time; $n^{27}D$ 1.5115, neut. equiv. 45.6 (calcd. 46.1), -SH (spectrophotometric titration) 98%. One sample was partially frozen; the frozen portion (one third) and residue (two thirds) gave the same spectrum. Cysteine hydrochloride is not easily purified. Two samples from different lots were purchased from the California Foundation for Biochemical Research: N, 7.8–8.05% (calcd. 7.98%); α_D (6 *N* HCl) +5.09–6.03°; HCl (alkalimetric titration) 100%; -SH (Lavine's method[13]) 96–99%; the average results are given. Sodium hydroxide was prepared carbonate-free from a filtered saturated solution. All other reagents were of Reagent Grade. All solutions were made up in distilled water which had been boiled, cooled under a stream of nitrogen, and stored out of contact with atmospheric oxygen.

pH Measurements.—A Beckman Model G *p*H meter was used for all measurements, with extension calomel and Type "E" (high *p*H) glass electrodes. The instrument was calibrated with National Bureau of Standards buffer at *p*H 4.00, and did not read true with a NBS buffer at *p*H 10.00; a correction (+0.06) was applied. The temperature was 25 ± 0.1°.

Interpretation of Titration Curves.—The thermodynamic ionization constant K° of an acid HA is defined in terms of activities

$$K^{\circ}_{HA} = \frac{a_H a_A}{a_{HA}}$$

In this work, only the concentrations of HA and A^{-} are known, and the activity of H^{+} as measured by the *p*H meter. A "practical ionization constant" is defined in terms of these quantities

$$K_{HA} = \frac{a_H C_A}{C_{HA}}$$

In the 0.10 *M* potassium chloride solution employed as a medium, the activity coefficients, γ, may be regarded as constant, and

$$K^{\circ}_{HA} = K_{HA}\frac{\gamma_A}{\gamma_{HA}}$$

K_{HA} was estimated by graphical location of the *p*H at the point where $C_{HA} = C_A$.

Absorption Spectrum Measurements.—A Beckman Model DU Spectrophotometer was used in all measurements, with glass-stoppered silica cells of 1.00-cm. light-path length. Solutions were prepared by weight in air-free distilled water, were stored under nitrogen, and contact with the air was minimized during transfers; however, some exposure could not be avoided. In general, the absorption of a sample stored in a stoppered cell first increased slowly with time, then levelled off, and finally started to decrease; it would appear that some oxidation occurs during transfer of the solution into the cell, and that the oxidation intermediates which are first formed absorb very strongly. However, no great improvement was obtained, *e.g.*, by adding 1% sodium sulfite to the solutions, and this suggests that other processes are also at work. The effects were minimized by measuring the spectra quickly after the solutions had been mixed—usually, within 15 minutes; in this interval, the change in spectrum was small, but appreciable.

EUGENE, OREGON

(13) T. F. Lavine, *J. Biol. Chem.*, **109**, 141 (1933).