Two crystallizations from methanol brought the m.p. to 111–112°, not raised by further recrystallization, $[\alpha]_D$ +24.0°. *Anal.* Calcd. for $C_{25}H_{40}O_3$ (388.6): C, 77.27; H, 10.38. Found: C, 77.1; H, 10.2. This was probably identical with the product of Barnett and Reichstein[17] prepared similarly but with pyridine as solvent and assigned the Δ^3-structure with no comment. Their material had m.p. 110–111° out of methanol; no rotation reported. (b) By pyrolysis of methyl desoxycholate 3-cathylate,[25] a product in 88% yield, m.p. 106.5–109° after recrystallization from methanol, was obtained.

(25) L. F. Fieser and S. Rajagopalan, THIS JOURNAL, **72**, 5530 (1950).

Ethyl 12α-Hydroxy-3-cholenate (54).—(a) By dehydrotosylation of ethyl 3α-tosyloxy-12α-hydroxycholanate (26) a 98% yield of 92–95° melting material was produced, which, when recrystallized from methanol, melted at 92–97°. When pyridine was used instead of lutidine, the yield of product dropped to 32%. (b) By ester interchange: After 13 days at room temperature in ethanolic HCl methyl 12α-hydroxy-3-cholenate (53) gave a 60% yield of product melting 96–99° out of ethanol–H_2O (2:1). After chromatography, the m.p. was 97.5–99.2°, $[\alpha]_D$ +27.0°*.

Anal. Calcd. for $C_{26}H_{42}O_3$ (402.60): C, 77.56; H, 10.52. Found: C, 77.53; H, 10.26.

MEMPHIS 3, TENN.

[CONTRIBUTION FROM THE DEPT. OF PATHOLOGY, UNIVERSITY OF PITTSBURGH SCHOOL OF MEDICINE]

Potentiometric Study of Gelatin and its Methyl Ester[1a-c]

BY SAM KATZ AND PAUL H. MAURER

RECEIVED NOVEMBER 9, 1956

The proton dissociation isotherm for gelatin was established by both titration and equilibration techniques at 37.0 ± 0.02°, 0.15 ionic strength. It was demonstrated that even though the isotherms obtained by titration were reproducible and reversible they were approximately 10% lower than those obtained by the equilibration technique. Similar curves evaluated from equilibration data were analyzed in terms of the number and dissociation constants of the constituent prototropic groups. The resulting values are in good accord with those established by amino acid analyses. A method is proposed for the quantitative determination of the degree of esterification of the methyl ester of gelatin. This is based on the selective alkaline hydrolysis of the ester linkages. The analytical values are considered accurate to 4%.

Introduction

In the course of the investigation of gelatin and some of its derivatives as possible plasma expanders, it was observed that the solubility and gelation properties of the methyl ester of gelatin were markedly different from the parent material.[2] This observation suggested that if this effect were real, *i.e.*, not due to the hydrolysis of gelatin during the esterification procedure, some information relevant to gelation might be derived.

The information derived from potentiometric titrations is important for the interpretation of the results obtained from the other physical techniques such as viscosity, osmometry, etc.[3] From these data a provisional estimate of the electrostatic charge of the gelatin as a function of *p*H may be made. Furthermore, the analysis of the dissociation isotherm will reveal not only the number and the dissociation constants of the prototropic groups but also their relative distribution as a function of *p*H. By comparison of the titration curves of the original and modified gelatins, a quantitative measure of the extent of reaction can be made. If extensive hydrolysis of the protein were to occur, this would be reflected by an increase either in the number of the carboxyl and/or amino groups.

Experimental

Materials.—The beef bone gelatin, lot #148-1B, was kindly supplied by Dr. D. Tourtellote of the Knox Gelatine Company. Analytical grade reagents were utilized without further purification. The standard solutions of NaOH (0.2 *N*) and HCl (0.2 *N*) were prepared by diluting Stansol solutions (Standard Solution Company, Menasha, Wisconsin) to the desired concentrations and restandardized before use. The NaOH was protected from the atmosphere by a tube packed with activated Alumina and Caroxite, a CO_2 absorbent. Nitrogen, water pumped, stated as being 99.6% pure was employed whenever necessary.

Prior to titration, the gelatin solutions were dialyzed at 37° in the presence of toluene against 6 changes of solvent (0.15 *M* KCl) for at least 3 days; the ratio of the gelatin (inner solution) to the solvent being 1:4. The dialysis sac was rotated during this period.

Protein concentrations were determined by dried weight at 115 ± 5°, correcting for the salt content. The validity of this correction was established by demonstrating that the dry weight of the outer solution differed from that of the 0.15 *M* KCl by less than 0.1%.

The methyl ester of gelatin (Metgel) was prepared by the procedure of Fraenkel-Conrat and Olcott.[4] The preparation was washed once with methanol and then repeatedly with acetone. The solvent was removed *in vacuo* at room temperature. The product, which was obtained in 80% yield, was identical in gross appearance to the parent substance.

Equipment.—The *p*H was determined with a Beckman Model G *p*H meter with a type E-2 all-*p*H glass electrode in conjunction with a saturated calomel electrode. The instrument was calibrated at 37° against 0.05 *M* potassium hydrogen phthalate, *p*H 4.03,[5] and at 6.98 and 9.88 using 2 standard buffers.

The iso-ionic *p*H for gelatin was established by electrodialysis employing both parchment paper membrane and Amberplex ion resin membranes (donated by Rohm and Haas).

Titration.—The standard acid or alkali was introduced with a Koch micro-buret, 5-ml. capacity, into 15.00 ml. of 2% gelatin solution contained in a 50-ml. water-jacketed beaker. The temperature was maintained at 37.0 ± 0.2°. Control experiments in which the protein was eliminated were performed in order to evaluate the activity coefficient of the reactants. Prior to the initiation of any experiment, the electrodes were standardized against two of the buffers, and upon completion of the run they were rechecked. In any case, where the drift in reading exceeded 0.03 *p*H unit the experiment was discarded. In the alkaline range of ti-

(1) (a) This investigation was supported by a grant from the Office of the Surgeon General, Department of the Army, Contract No. DA-49-007-MD-248, upon the recommendation of the Subcommittee of Shock of the National Research Council. (b) Presented in part at the 130th Meeting of the American Chemical Society, Atlantic City, New Jersey, Sept., 1956. (c) Publication No. 95 of the Department of Pathology, University of Pittsburgh School of Medicine.

(2) P. H. Maurer, *Arch. Biochem. et Biophys.*, **58**, 205 (1955).

(3) S. Katz and P. H. Maurer in preparation.

(4) H. A. Fraenkel-Conrat and H. S. Olcott, *J. Biol. Chem.*, **161**, 259 (1945).

(5) National Bureau of Standards, Letter Circular LC 933 (1948).

tration, absorption of atmospheric CO_2 was minimized by continuously bubbling nitrogen gas through the system. The reversibility of the reaction was demonstrated by back-titration and agreed to within 4% of theoretical values. The resulting isotherms were calculated in the conventional manner making the customary assumptions with respect to the additivity of volumes, junction potentials and activity coefficients.[6]

Equilibration.—The equilibration method for determining the association isotherm for gelatin was performed by adding measured quantities of reagents to 50-ml. weighing bottles so that the total volume was 20.0 ml. and the resultant gelatin concentration was about 2%. The system was equilibrated at 37 ± 0.02° for about 24 hr. before *p*H measurements. The electrodes were standardized at frequent intervals. The deviation of any individual reading about the average as a rule did not exceed 0.01 *p*H unit at *p*H values lower than 5. When alkali was added a nitrogen atmosphere was maintained as previously described. The reversibility of the process was established by addition of the appropriate reagent to the equilibrated system, after which another equilibration period was allowed before *p*H measurement. The results agreed within 3% on the acid and 5% on the alkaline[7] sections of the curve.

Alkaline Hydrolysis.—The alkaline hydrolytic method for assaying the methoxyl content of Metgel was performed as follows. A measured excess of NaOH was added to 50.00-ml. aliquots previously adjusted to *p*H 6.2. The solution was then transferred to a 100-ml. volumetric flask, sealed with a greased stopper and allowed to equilibrate for 24 hr. at 37°. The residual alkali was determined by back-titration of 25.00-ml. aliquots.

Formol Titrations.—The procedure was essentially similar to that reported in the review by French and Edsall.[8] In order to determine the α- and ε-ammonium residues and minimize the imidazolium contribution, the solution of gelatin was adjusted to *p*H 7.8. To aliquots containing approximately 2% gelatin, measured volumes of 36% reagent grade formaldehyde, *p*H 7.0, were added so that the final formaldehyde concentration was 8 ± 1%. The end-point was determined by plotting Δ *p*H/Δ ml., the incremental change of *p*H with respect to alkali, *versus* ml. of NaOH added.[9]

Results and Discussion

The iso-ionic point[10] for beef bone gelatin was found to be 4.96 ± 0.02 at 37° by electrodialysis. Equilibration required 24 hr. when parchment-paper membranes were used; however, with ion-exchange resins, Amberplex C-1 and A-1, the time was decreased to 5 hr. When a 2% salt-free gelatin solution was made 0.15 *M* with respect to KCl the resultant iso-ionic *p*H of 4.94 indicated little or no binding of KCl by gelatin, in agreement with Carr and Topol.[11]

Potentiometric Titrations.—The proton dissociation curves for gelatin are given in Fig. 1. The titration isotherm represented by the lower curve was reproducible to within 3% and was found to be reversible. However, when compared with the data obtained by equilibration these proved to be 10% too low. Subsequent investigation revealed that the 30-min. period allowed for reaction during titration was not sufficient to permit equilibration. In contrast with these findings, Kenchington and Ward[12] titrated another type of gelatin successfully; however, the time allowed for equilibration was not indicated. It is difficult to attribute this deviation to heat effects, change of ionic strength (the change was less than 4%), or the 20% dilution of the protein. Assuming the absence of systematic errors, the most plausible explanation is that equilibration of the gelatin with the acid requires an appreciable time. It should be noted that a variation of 0.10 *p*H unit at *p*H 2.2 for the system studied would be equivalent to a change of 0.08 milliequivalent protons bound per g. gelatin. On this basis, no attempt was made to analyze the gelatin isotherms derived from titration.

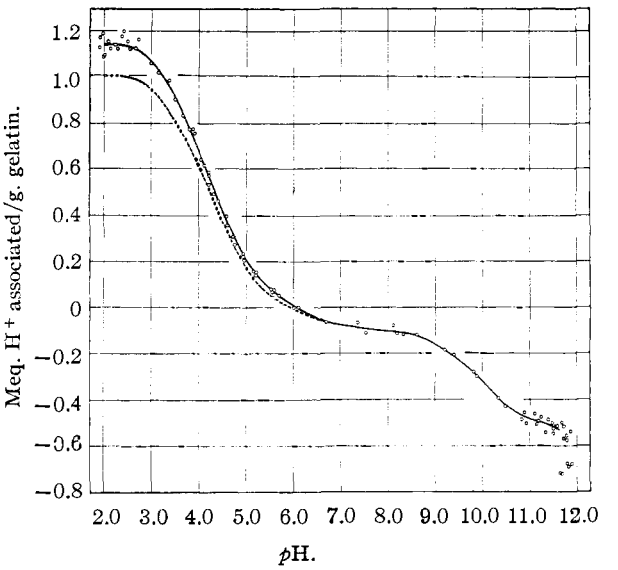

Fig. 1.—The proton association isotherm for beef bone gelatin as determined at 37°; μ = 0.15. The titration data are represented by the broken line; the equilibration data by the curve through the open circles.

The equilibration isotherm, upper curve, is typical for this process,[12,13a-c] except for the region more alkaline than *p*H 11.5. That the downward trend of the data is due to the effect of the guanidinium residues and not due to the experimental error was demonstrated by reversibility experiments.

The theory of the analysis of similar isotherms has been the subject of many reviews[14a-c]; therefore, this aspect of the discussion will be limited to some of the essential equations.

The relationship between h_i, the number of protons dissociated from the ith set of n proton donor groups and the apparent dissociation constant, K_i, for any statistically equivalent unit in this set is given by equation 1 where (H^+) is the proton concentration.

(6) E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides," Reinhold Publ. Corp., New York, N. Y., 1943, pp. 444–502.

(7) No correction was made for the slight change in ionic strength.

(8) M. Levy, *J. Biol. Chem.*, **105**, 157 (1934); R. A. Kekwick and R. K. Cannan, *Biochem. J.*, **30**, 235 (1936); D. French and J. T. Edsall in M. L. Anson and J. T. Edsall, "Advances in Protein Chemistry," Vol. 2, Academic Press, Inc., New York, N. Y., 1945, pp. 302–317.

(9) M. S. Dunn and A. Loshakoff, *J. Biol. Chem.*, **113**, 359 (1936).

(10) S. P. L. Sørensen, K. U. Linderstrøm-Lang and E. Lund, *J. Gen. Physiol.*, **8**, 543 (1927); G. Scatchard and E. S. Black, *J. Phys. Chem.*, **53**, 88 (1949).

(11) C. W. Carr and L. Topol, *ibid.*, **54**, 176 (1950).

(12) A. W. Kenchington and A. G. Ward, *Biochem. J.*, **58**, 202 (1954).

(13) (a) J. H. Bowes and R. H. Kenten, *ibid.*, **43**, 358 (1948); (b) W. M. Ames, *J. Sci. Food Agr.*, **12**, 579 (1952); (c) J. Salvinien and S. Combet, *Compt. rend.*, **242**, 114 (1956).

(14) (a) R. K. Cannan, *Chem. Revs.*, **30**, 395 (1942); (b) E. J. Cohn and J. T. Edsall, *ibid.*, **30**, 395 (1942); (c) J. Steinhardt and E. M. Zaiser in M. L. Anson, K. Bailey and J. T. Edsall, "Advances in Protein Chemistry," Academic Press, Inc., New York, N. Y., Vol. 10, 1955, pp. 152–226.

$$h_i = \frac{n_i K_i}{(H^+) + K_i} \qquad (1)$$

Since $\bar{h}$, the mean number of protons dissociated, is equivalent to the summation of the individual h_i it is readily shown that the fraction contributed by any of the given sets, *e.g.*, the lth set, may be calculated from the equation

$$h_1/\bar{h} = 1/\left(1 + \sum_{i=2}^{i=n} \frac{h_i}{h_1}\right) \qquad (2)$$

where all the terms have their usual significance.

The relationship based on Linderstrøm-Lang's[15] hypothesis and initially tested by Cannan, Palmer and Kibrick[16] is given as

$$p\mathrm{H} = pK_{\mathrm{int}} + \frac{\log \alpha}{1 - \alpha} - \frac{2\bar{Z}\omega}{2.303} \qquad (3)$$

where pK_{int} is the intrinsic dissociation constant, α is the fraction of any given set of prototropic groups dissociated at the given pH, $\bar{Z}$ is the mean electric charge of the protein, and ω is a function of electrostatic potential of the protein species involved.[14a]

In order to evaluate the contribution of the imidazolium groups a tentative assignment of the α-ammonium groups was made on the premise that the gelatin studied was essentially a linear polymer with a number average molecular weight of about 50,000.[13a,17,18] The number and the pK's of the imidazolium and the α-ammonium groups were obtained by solving simultaneous equations[19] for values of $\bar{h}$ at pH 6.5 and 7.5.

The number of ϵ-ammonium groups was determined from the titration values between pH 8.0 and 11.25 as corrected for the residual contribution of the α-ammonium, imidazolium and guanidinium groups. The greatest single source of error is the difficulty in ascertaining the guanidinium contribution since its pK is not well established even at 25°, while the extrapolation to 37° (van't Hoff equation) introduces another element of error. If the pK were taken as being 13.0 then its contribution would be 0.009 meq./g., if it were 12.5 the corresponding value would be 0.027 meq./g., and similarly for pK 12 it would be 0.078 meq./g. By inspection of the curve it appeared that the value of about 12.5 would be justified for this calculation.

The pK_{int} of the carboxylate groups (uncorrected for α-carboxyl) was determined by plotting $p\mathrm{H} - \log \alpha/(1 - \alpha)$ as ordinate *versus* $\bar{Z}^*$, the electronic charge of the protein calculated on a gram basis. The term $\bar{Z}^*$ was calculated from the titration curve, implicitly omitting any salt correction.[11] From Fig. 2, the value for pK_{int} of 4.35 ± 0.04 at 37° was determined in good agreement with that of 4.28, at 25° reported by Salvinien and Combet.[13c] The plot is linear over 80% of the range. The reversal of the curve is too large to be attributed to the α-carboxyl contribution and must be ascribed to higher order effects which have been neglected in the simple model used. A similar plot for the ϵ-ammonium groups is given in Fig. 3. The apparently anomalous behavior may be explained by considering that the ϵ-ammonium groups are contributed by both the lysine and hydroxylysine residues whose pK's are 10.28 and 9.50, respectively.[20] Therefore it is apparent that equation 4 is not applicable.

A comparison of the number of dissociable groups deduced from titration data with that determined by Eastoe[21] from amino acid analysis is offered in Table I. The titration value for the free carboxyl groups (inclusive of the α-carboxyl) of 1.13 ± 0.03 meq./g. gelatin, is in excellent accord with Eastoe's. The figure for the imidazolium residues obtained by titration, though corrected for the α-ammonium contribution, is somewhat high but within the experimental error. If the titration value for the ϵ-ammonium groups is adjusted for the tyrosyl hydroxyls the resulting value of 0.37 meq./g. is again in excellent accord with the English work. This is further corroborated by the value of 0.36 meq./g. obtained by formol titrations. The number of guanidinium residues, 0.46 meq./g., was calculated from the difference between the maximum acid binding quantity, 0.923 meq./g., and the anionic basic groups. This value is lower than that established by amino acid analysis. Furthermore it must be recognized that the initial quantity is more suspect due to incorporation of accumulative errors.

TABLE I

IONIZABLE GROUPS—BEEF BONE GELATIN

Group	Meq. per gram: Titration[a]	Meq. per gram: Analysis[b]	pK: This study	pK: Lit.[c]
Carboxyl (free)	1.13[d]	1.12[e]	4.35 ± 0.04[f]	3.0–4.7
Imidazolium	0.075	0.045	6.7	5.4–6.8
α-Ammonium	.019	...	8.2	7.3–8.1
ϵ-Ammonium	.38[g]	.346	...	9.1–10.3
Phenolic hydroxyl	...	.013	...	.....
Guanidinium	.46	.52	...	.....

[a] Determined at 37.0 ± 0.02°, μ = 0.15. [b] J. E. Eastoe, *Biochem. J.*, 61, 589 (1955). [c] These values, corrected to 37°, are from E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides," Reinhold Publ. Corp., New York, N. Y., 1943, p. 445. [d] This includes the contribution of the α-carboxyl groups. [e] This value is corrected for the amide contribution. [f] This corresponds to pK_{int}. [g] This includes the phenolic hydroxyl contribution.

(15) K. U. Linderstrøm-Lang, *Compt. rend. trav. lab. Carlsberg*, **15**, No. 7 (1925).

(16) R. K. Cannan, A. H. Palmer and A. C. Kibrick, *J. Biol. Chem.*, **142**, 803 (1942).

(17) A. Courts, *Biochem. J.*, **59**, 382 (1955); J. W. Williams, W. M. Saunders and J. S. Cicirelli, *J. Phys. Chem.*, **58**, 774 (1954); J. Pouradier and A. M. Venet, *J. Chim. Phys.*, **47**, 11 (1950); G. Scatchard, J. L. Oncley, J. W. Williams and A. Brown, THIS JOURNAL, **66**, 1980 (1944).

(18) If the gelatin contains an appreciable fraction with a molecular weight much less than 30,000 the validity of this assumption is seriously compromised. However, the trend to the other extreme would not introduce as serious an error.

(19) The equations used were

at pH 6.5

$$2 = \frac{K_1}{3.16 \times 10^{-7} + K_1} + \frac{5K_2}{3.16 \times 10^{-7} + K_2}$$

at pH 7.5 =

$$4.5 = \frac{K_1}{3.16 \times 10^{-8} \times K_1} + \frac{5K_2}{3.16 \times 10^{-8} + K_2}$$

The values for h were corrected for carboxyl contribution. Even though these equations are based on an estimated molecular weight of 50,000, the results are independent of this assumption.

(20) D. D. Van Slyke, A. Hiller, J. A. Macfayden, A. B. Hastings and F. W. Klemperer, *J. Biol. Chem.*, **133**, 287 (1940).

(21) J. E. Eastoe, *Biochem. J.*, **61**, 589 (1955).

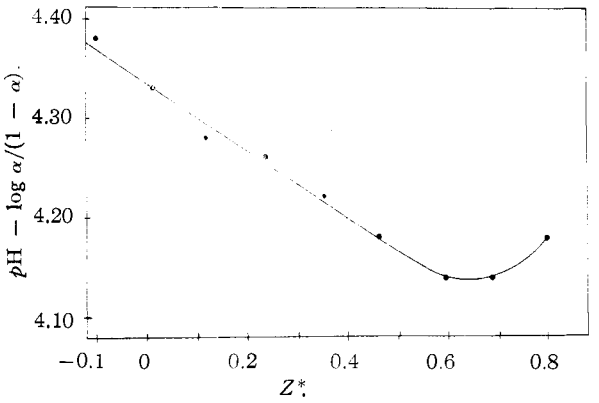

Fig. 2.—The plot of pH − log $\alpha/(1-\alpha)$ *versus* Z^*, the mean electric charge on gelatin calculated on a gram basis. This curve is for the free carboxyl group of beef bone gelatin at 37°, $\pi = 0.15$.

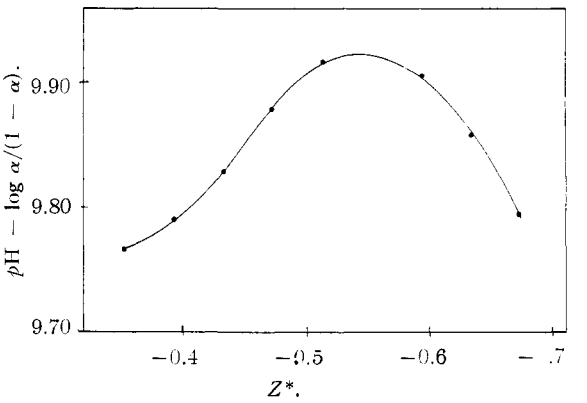

Fig. 3.—A plot, similar to Fig. 2, for the ϵ-ammonium groups.

Another method for interpreting this isotherm is the approach of Cannan, *et al.*,[14a,16] whereby certain arbitrary sections of the titration curve are attributed to given acidic groups. This analysis gave the following results: 1.145 meq./g. for the carboxyl groups (pH 2.0–6.125), 0.10 meq./g. for the imidazolium and α-ammonium groups (pH 6.125–8.0), 0.395 meq./g. for the ϵ-ammonium and phenolic hydroxyl groups (pH 8.0–11.25) and the calculated value for guanidinium was 0.44 meq./g. Thus it may be concluded that this approach is a successful first approximation.

Metgel.—From the difference between the carboxyl content of the original and modified proteins as determined potentiometrically, the extent of esterification of these polar groups may be evaluated. However, when the equilibration method was attempted for Metgel, it was observed that the readings were apparently stable at pH values lower than 5 but that there was a negative drift at higher pH values, *e.g.*, at pH 6.9 the rate of decrease was about 0.01 pH unit/min. When Metgel, with an initial pH of 4.4, was electrodialyzed the pH reached a maximum of about 9; however, upon prolonged electrodialysis the pH dropped asymptotically to 5. These observations are in general agreement with the findings of Saroff, *et al.*,[22b] for the methylated ester of bovine serum albumin which were interpreted as an indication of the lability of the methyl ester linkage in alkaline medium.

On this basis, the titration technique was employed to determine the acid section of the isotherm since it could be completed before extensive hydrolysis would occur. Even though the resulting data did not deviate more than 5% about the mean, no attempt was made to evaluate the reliability of these results due to the variables involved. Yet, if reference is made to Table III, where the degree of methylation was determined using these somewhat doubtful results, the conclusions appear to be quite satisfactory. This apparent anomaly may be rationalized by recognition of the fact that when a small value with a relatively large uncertainty is subtracted from a large number the error introduced by the initial value is comparatively small.

Alkaline Hydrolysis.—In order to determine the degree of esterification of Metgel by a less ambiguous method, the following procedure was developed which is based on the lability of the methyl ester bond in an excess of base.

The stoichiometry of the alkaline hydrolysis of the methyl ester bond in Metgel is indicated by the equation

$$\mathrm{G{-}\overset{\overset{O}{\|}}{C}{-}OR + OH^- \longrightarrow G{-}\overset{\overset{O}{\|}}{C}{-}O^- + ROH} \quad (4)$$

For quantitative results, an excess of alkali must be employed in order to drive the reaction to the right. The possibility exists that this determination might be complicated by the competitive hydrolysis of either amide and/or peptide bonds. However, Bowes and Kenten[23] have shown that the peptide bond is resistant to alkaline hydrolysis under conditions more drastic than those used here, but that the amide group is somewhat more labile. If peptide hydrolysis were to occur it is evident that the back-titration to pH 6.2 would compensate for this effect since the liberated amino groups would be titrated. Evidence indicating that the ester hydrolysis was quantitative was the observation that the value for the free carboxyl groups as determined by continuing the titration to pH 2.1[24] agreed with that for the original gelatin (±2%). The possibility exists that this agreement may have been due to a fortuitous combination of incomplete ester hydrolysis coupled with amide and/or peptide cleavage. However, the fact that the latter effect was negligible was demonstrated by the results of formol titrations on regenerated Metgel, *i.e.*, Metgel neutralized after exposure to alkali, which were about 0.015 meq./g. higher than that of gelatin. Further corroboration was offered by the observation that a 24 hr. exposure of gelatin at pH 13, 37°, resulted in a consumption of ± 0.015 meq. alkali/g.

The effect of different concentrations of hydroxide ion on the ester hydrolysis is summarized in Table II. From these data it appears that providing the final hydroxide ion concentration is in

(22a) S. Blackburn, E. G. H. Carter and H. Phillips, *Biochem. J.*, **35**, 627 (1941); (b) H. A. Saroff, N. R. Rosenthal, E. R. Adamik, N. Hages and H. A. Scheraga, *J. Biol. Chem.*, **205**, 255 (1953).

(23) J. H. Bowes and R. H. Kenten, *Biochem. J.*, **43**, 365 (1948).

(24) Sufficient time should be allowed in order to ensure that equilibrium is reached.

the *p*H range indicated, a reasonable accuracy is obtainable. The increase of the reaction period to 48 hr. caused an additional uptake of 0.01 meq. hydroxide ion/g. indicating that the reaction is essentially completed in 24 hr.

TABLE II

THE EFFECT OF HYDROXIDE ION CONCENTRATION ON THE METHYL ESTER DETERMINATION OF METGEL

Expt. no.	Equilibrium[a] *p*H	Meq. OH^- consumed / g. Metgel
1	7.75	0.90
2	8.00	.88
3	8.4	.90
4	8.5	.90
5	8.5	.93
6	9.0	.94
		.91

[a] The *p*H of the system after 24 hr. incubation.

A check of the validity of this technique is offered by a comparison of these results with those derived by other methods. The methyl ester content of Metgel may be calculated from the difference in the titration curves of the initial and final materials. The validity of these results has been discussed in a previous section. Still another method for methoxyl analysis is the micro-Zeisel determination.[25] A compilation of these analyses for two different preparations of Metgel is given in Table III.

TABLE III

ANALYTICAL VALUES FOR THE METHOXYL CONTENT OF METGEL (MEQ./G. METGEL)

Method	Lot 2	Lot 3
Alkaline hydrolysis	0.71	0.91
Titration (difference)	.71	.94
Micro-Zeisel[a,b]	..	.87[c]

[a] Analyses performed by Elek Microanalytical Labs., L. A., Calif. [b] The value for gelatin was 0.10 meq./g. as compared to the calculated value of 0.04 meq./g. (computed for the methionine content). [c] Not corrected for the gelatin blank.

From these data it may be concluded that the alkaline hydrolysis method for methoxyl analysis is relatively simple and reliable to ± 4%.

Acknowledgment.—The authors wish to acknowledge the able assistance of Mr. Trayon Onett in the latter phase of this problem.

(25) J. B. Niederl and V. Niederl, "Micromethods of Quantitative Organic Analysis," John Wiley and Sons, Inc., New York, N. Y., 1942, pp. 239–244.

PITTSBURGH, PA.

[CONTRIBUTION FROM THE DEPARTMENT OF PHYSIOLOGICAL CHEMISTRY, UNIVERSITY OF MINNESOTA]

Sulfhydryl Groups in Relation to Aldolase Structure and Catalytic Activity[1]

BY A. D. SWENSON AND P. D. BOYER

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Spectrophotometric measurements readily reveal a graded reactivity of *p*-mercuribenzoate with the –SH groups of rabbit muscle aldolase; some react rapidly at *p*H 7 and 25°, some react much more slowly, and others react only in the presence of urea. With 4.8 *M* or higher urea concentrations, 28 *p*-mercuribenzoate molecules react rapidly per aldolase molecule. The catalytic activity is progressively decreased to 0 as urea concentration is increased up to 3.6 *M*; inactivation by up to 4 *M* urea is completely reversible upon dilution. The rate and extent of reaction with *p*-mercuribenzoate is not influenced by the presence of an excess of substrates. Up to 10 –SH groups per mole of aldolase can react with *p*-mercuribenzoate without decrease in the catalytic activity. Additional reaction with *p*-mercuribenzoate causes a reversible loss of catalytic activity which appears to be associated with structural changes in the aldolase.

Introduction

Considerable information has been accumulated about the status of sulfhydryl groups in enzymes and their catalytic activity.[2] For example, the pioneering studies of Hellerman, *et al.*,[3] with urease showed that the most reactive –SH groups were not essential for catalytic activity but that reaction of additional –SH groups resulted in marked activity loss. Quantitative observations on the relationships between enzyme structure, number of –SH groups reacted with a particular reagent, and the catalytic activity have been limited. Particularly lacking are results which give a clear indication of whether the essential –SH groups have a primary role in the catalysis or whether reaction of the –SH groups results in secondary changes leading to loss of activity. The latter possibility has been suggested by others, including Desnuelle and Rovery[4] and Nygaard.[5]

The rate and extent of the reaction of –SH groups of proteins with the mercaptide forming agent, *p*-mercuribenzoate, at neutral *p*H and under mild conditions, may be measured conveniently by a spectrophotometric technique.[6] This paper gives results of application of the spectrophotometric procedure to measurements of the –SH groups of rabbit muscle aldolase together with catalytic activity measurements in the presence and absence of various urea concentrations. Rabbit muscle aldolase was chosen for study because it is readily obtainable in pure form,[7] conveniently assayed, and has been reported to be inhibited by reaction with

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