pentane-4,5-dione-1,3-dicarboxylate (**6**) (1.21 g., 0.005 mole) in glacial acetic acid (15 ml.) was saturated with dry hydrogen chloride gas. After a reaction time of 9 hr. at room temperature, the mixture was poured into water (100 ml.), and the yellow precipitate (1.5 g.) was collected on a filter and washed with water. Recrystallization from ethanol afforded yellow needles, m.p. 203–205°.

Anal. Calcd. for $C_{17}H_{16}O_7$: C, 61.44; H, 4.85. Found: 61.21; H, 5.17.

5,7-Dimethoxycyclopentenon[3,2-c]coumarin (**8**). The ketoester **7** (400 mg.) was dissolved in a mixture of 5% hydrochloric acid (15 ml.) and dioxane (20 ml.), and the resulting solution was heated at 105° for 3 hr. The dark yellow solution was then diluted with water (500 ml.) and the mixture was extracted five times with chloroform (20 ml. each). All extracts were combined and washed with 5% aqueous sodium bicarbonate and water; the solution was dried with magnesium sulfate. After concentration to 5 ml. the material was poured onto a column of Merck acid-washed alumina (70 g.) and the material eluted with chloroform. The early eluates on evaporation produced yellow crystals (164 mg.). One recrystallization from ethanol–chloroform furnished yellow needles: m.p. 178–179°; λ_{max}^{EtOH} 245, 268, and 356 mμ (ε 13,200, 8700, and 9000); $\nu_{max}^{CHCl_3}$ 1726 (broad), 1614, and 1565 cm.$^{-1}$.

Anal. Calcd. for $C_{14}H_{12}O_5$: C, 64.61; H, 4.65. Found: C, 64.55; H, 4.85.

5,7-Dimethoxy-4-(2′-methoxycarbonylethyl)coumarin (**10**). A solution of phloroglucinol dimethyl ether (**5**) (4.6 g., 0.03 mole) and the ketoester **9**[22] (6.2 g., 0.03 mole) in glacial acetic acid (80 ml.) was saturated with dry hydrogen chloride gas. After 8 hr. at room temperature, the product (8.5 g.) was isolated in the manner described above. Recrystallization from a mixture of chloroform and cyclohexane gave silky needles: m.p. 124–124.5°; $\nu_{max}^{CHCl_3}$ 1725, 1620, 1608, and 1560 cm.$^{-1}$.

Anal. Calcd. for $C_{15}H_{16}O_6$: C, 61.64; H, 5.52. Found: C, 61.64; H, 5.67.

5,7-Dimethoxycyclopentenon[2,3-c]coumarin (**11**). The ester **10** (8.5 g.) was heated with polyphosphoric acid (250 g.) at 105–110° with occasional stirring. After 2 hr. the brown reaction mixture was poured into ice–water (2 l.) and the crystalline precipitate (7.9 g.) collected by filtration. Chromatography over Merck acid-washed alumina (150 g.) using chloroform as eluent gave starting ester **10** (400 mg.) and colorless crystals (7.0 g.). Recrystallization from chloroform–methanol yielded pure tricyclic ketone **11**: m.p. 248–249°; λ_{max}^{EtOH} 215, 237 (shoulder), 257, 345 (shoulder), and 355 mμ (ε 22,200, 14,600, 9650, 25,800, and 26,800); $\nu_{max}^{CHCl_3}$ 1759 (very intense), 1685 (weak), 1614, 1594, and 1550 cm.$^{-1}$.

Anal. Calcd. for $C_{14}H_{12}O_5$: C, 64.61; H, 4.65. Found: C, 64.46; H, 4.83.

Acknowledgment. Financial support of this investigation by the National Science Foundation (G-22647) and the National Cancer Institute is gratefully acknowledged.[23]

(22) D. K. Banjeree and K. M. Sivanandaian, *J. Org. Chem.*, **26**, 1634 (1961).

(23) NOTE ADDED IN PROOF. Professor A. F. Peerdeman and Mr. B. van Soest (Utrecht) have confirmed the structure of aflatoxin B_2 using X-ray analysis (private communication from Dr. D. A. van Dorp).

Spectrophotometric Determination of the Kinetics of the Pepsin-Catalyzed Hydrolysis of Certain Dipeptide Substrates

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Contribution from the Department of Chemistry, Amherst College, Amherst, Massachusetts. *Received August 14, 1964*

The kinetics of the pepsin-catalyzed hydrolysis of N-carbobenzoxy-L-phenylalanyl-L-tyrosine and N-acetyl-L-phenylalanyl-L-tyrosine at pH 2 in 3.4% methanol at 35° have been determined by a spectrophotometric technique. The kinetic parameters for the former compound are $K_0 = 2.1 \pm 0.3 \times 10^{-4}$ M, $k_0 = 1.24 \pm 0.08 \times 10^{-2}$ sec.$^{-1}$, and for the latter, $K_0 = 1.95 \pm 0.18 \times 10^{-3}$ M, $k_0 = 4.66 \pm 0.44 \times 10^{-2}$ sec.$^{-1}$. The results with the acetyl compound are in agreement with earlier data obtained by a different procedure.

Only two quantitative experiments have been performed on the kinetics of the hydrolysis of simple synthetic substrates by pepsin.[2] Casey and Laidler[3] utilized a potentiometric formol titration to investigate the hydrolysis of N-carbobenzoxy-L-glutamyl-L-tyrosine and N-carbobenzoxy-L-glutamyl-L-tyrosine ethyl ester. Baker[4] employed primarily a ninhydrin analysis in her studies on the hydrolysis of N-acetyl-L-phenylalanyl-L-tyrosine (Ac-PheTyr) and N-acetyl-L-tyrosyl-L-tyrosine. In this paper we report a spectrophotometric technique which promises to be useful in pursuing detailed kinetic investigations on pepsin and in quantitatively evaluating pepsin activity. The pro-

(1) (a) This investigation was supported in part by Grant AM 08005-01 of the U. S. Public Health Service; (b) taken in part from the B. A. Thesis of J. J. S.

(2) (a) F. A. Bovey and S. S. Yanari in "The Enzymes," Vol. 4, P. D. Boyer, H. Lardy, and K. Myrback, Ed., 2nd Ed., Academic Press Inc., New York, N. Y., 1960, Chapter 4; (b) R. M. Herriott, *J. Gen. Physiol.*, **45**, part 2, 57 (1962).

(3) E. J. Casey and K. J. Laidler, *J. Am. Chem. Soc.*, 72, 2159 (1950).

(4) L. E. Baker, *J. Biol. Chem.*, **211**, 701 (1954).

cedure calls for observation at 237 mμ of the enzymatic hydrolysis of N-acetyl-L-phenylalanyl-L-tyrosine or the corresponding N-carbobenzoxy compound (Z-PheTyr), and is somewhat analogous to the Schwert and Takenaka[5] scheme for determining the α-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tyrosine ethyl ester at pH 7 by measuring the decrease in optical density at 237 mμ (1). Dipeptides with C-terminal tyrosine are expected to provide a larger change in optical density than those with N-terminal tyrosine upon undergoing hydrolysis in the pH range (~2) of maximum pepsin efficiency (eq. 2 *vs.* 3),[6] and Ac-PheTyr is one of the most reactive synthetic substrates for pepsin currently known.[7]

$$\text{Ac-TyrCOOEt} \xrightarrow{\text{pH 7}} \text{Ac-TyrCO}_2^- \quad (1)$$

$$\text{X-CONH-TyrCOOH} \xrightarrow{\text{pH 2}} {}^+\text{NH}_3\text{-TyrCO}_2^- + {}^+\text{NH}_3\text{-TyrCOOH} \quad (2)$$

$$\text{X-TyrCONHR} \xrightarrow{\text{pH 2}} \text{X-TyrCOOH} \quad (3)$$

Experimental

Pepsin was Lots PM 691 and 699 of the Worthington Biochemical Corporation, Freehold, N. J.

N-Carbobenzoxy-L-phenylalanyl-L-tyrosine was obtained from the Cyclo Chemical Corporation, Los Angeles, Calif., Lot M-1156, and had m.p. 182–187.5° (lit.[8] m.p. 183–184°) after one recrystallization from methanol–water and $[\alpha]^{29}D$ −7.4° (*c* 3, 5% ammonia). Cyclo reports the original material homogeneous on paper chromatography. Enzymatic hydrolysis established that the compound was of high optical purity. The recrystallized sample apparently contained 1 mole of water.

Anal. Calcd. for $C_{26}H_{26}O_6 \cdot H_2O$: C, 64.99; H, 5.87; N, 5.83. Found: C, 64.70, 64.85; H, 6.06, 5.94; N, 6.02.

N-Acetyl-L-phenylalanyl-L-tyrosine was Cyclo Lot S-1020-B and had m.p. 220–222° (lit.[6] m.p. 230°). Cyclo reports $[\alpha]^{22}D$ +14.2° and Baker[7] gives $[\alpha]^{27}D$ +14.5° (*c* 2, pyridine). Enzymatic hydrolysis established that the material was of high optical purity. Cyclo reports the compound homogeneous in two systems in paper chromatography.

Anal. Calcd. for $C_{20}H_{22}N_2O_5$: C, 64.85; H, 5.99; N, 7.56. Found: C, 64.47; H, 6.14; N, 7.50.

Other Materials. A typical batch of solvent, μ ~0.2, was prepared by diluting 44 g. of reagent grade 85% phosphoric acid and 54.4 g. of potassium dihydrogen phosphate to 2 l. with water. Spectrograde methanol was used throughout. Distilled water was redistilled through an all-glass apparatus. N-Carbobenzoxy-DL-phenylalanine, Cyclo Lot K-4201, m.p. 101–104° (lit.[9] m.p. 103°), N-acetyl-L-phenylalanine (Ac-PheCOOH), Cyclo Lot M-1113, m.p. 169–170° (lit.[10] m.p. 172°), and L-tyrosine, Cyclo Lot H-1073, were used as received. All glassware was carefully cleaned and steamed out.

pH Measurements were made at room temperature with a Radiometer PHM 4c meter, standardized against an 0.01 *M* potassium tetroxalate solution,[11] pH 2.15. The different batches of buffer used for the kinetics read in the range pH 1.93–2.02 on the meter. The addition of 100 μl. of methanol per 3 ml. of buffer increased the apparent pH by 0.04 units; the presence of reactants or products had no effect on the pH.

Kinetic Measurements. All rates were followed at 237 mμ in a Cary 14 recording spectrophotometer equipped with thermostated cell holder and cell compartment. The temperature of the reaction mixtures was 35.1 ± 0.2°.

In a typical series of experiments, about 170 mg. of pepsin was dissolved in 10.0 ml. of water. The solution was centrifuged for 30 min. at 10,000 r.p.m. and used as the enzyme stock solution. Enzyme solutions were freshly prepared every day.

A solution of 3.0 ml. of buffer plus 100 μl. of enzyme stock solution in an open cuvette was equilibrated in the cell holder. Reaction was initiated by the addition of 100 μl. of methanolic substrate solution, the cell was loosely stoppered, and recording of the optical density *vs.* time curve at 237 mμ commenced. Reactions were monitored for 20–40 min.

Enzyme concentrations were calculated from the optical density at 278 mμ of a solution of 100 μl. of enzyme stock in 3.0 ml. of buffer, assuming a molar extinction coefficient (ε) for pepsin of 51,500.[12] The spectrophotometrically determined concentrations ran about 89% of the value calculated on the basis of weight, assuming a molecular weight for pepsin of 35,000.[13]

Absorption Measurements. The molar extinction coefficients of the various species involved were determined at 237 mμ, 35°, in the presence of enzyme and are as follows: Ac-PheTyr 1620; AcPheCOOH 110; tyrosine 1040; Z-PheTyr 1725; and Z-PheCOOH 134. The change in ε for the reaction of Ac-PheTyr is calculated to be 470, and that for Z-PheTyr 550. The values for the Δε's were checked by determining the the optical densities of reaction mixtures which had reached equilibrium (about eight half-lives, with no further change in optical density for 0.5 hr.).

pK_a Measurements. Crude spectrophotometric determinations of the pK_a of tyrosine and Ac-PheTyr were performed at 236.25 mμ. Spectra in 0.5 *M* hydrochloric acid and in pH 5.5 buffer were taken as representing the completely protonated and dissociated carboxyl group, respectively, and two intermediate points were chosen for determining the pK_a. The average values were tyrosine 2.17, and Ac-PheTyr 3.40 (*cf.* footnote 6).

Results and Discussion

Proof that it is feasible to follow the pepsin-catalyzed hydrolysis of Ac-PheTyr and Z-PheTyr spectrophotometrically is most clearly seen in the agreement of the

(5) G. W. Schwert and Y. Takenaka, *Biochim. Biophys. Acta*, **16**, 570 (1955).

(6) The pK_a of the carboxyl group of AcPheTyr is ~3.45, of tyrosine ~2.20, in water at 25°. At pH 2, the former will be completely protonated and the latter considerably dissociated. The data are estimated from J. T. Edsall and J. Wyman, "Biophysical Chemistry," Academic Press Inc., New York, N. Y., 1958, Chapter 8.

(7) L. E. Baker, *J. Biol. Chem.*, **193**, 809 (1951).

(8) H. Neumann, Y. Levin, A. Berger, and E. Katchalski, *Biochem. J.*, **73**, 33 (1959).

(9) M. Bergmann and L. Zervas, *Ber.*, **65**, 1192 (1932).

(10) V. du Vigneaud and O. J. Irish, *J. Biol. Chem.*, 122, 349 (1938).

(11) R. G. Bates, G. D. Pinching, and E. R. Smith, *J. Res. Natl. Bur. Std.*, **45**, 418 (1950).

(12) G. E. Perlmann, *Proc. Natl. Acad. Sci. U. S.*, **45**, 915 (1959).

(13) G. R. Tristram and R. H. Smith in "The Proteins," Vol. I, H. Neurath, Ed., 2nd Ed., Academic Press Inc., New York, N. Y., 1963, p. 47.

Table I. Kinetics of the Pepsin-Catalyzed Hydrolysis of Two Dipeptide Substrates[a] at 35° in 3.4% Methanol, pH 2

Compd.	$[S]_0$[b] × 10^4, M	$[E]_0$[b] × 10^6, M	K_0[c] × 10^3, M	k_0[c] × 10^2, sec.$^{-1}$	n[d]
Z-PheTyr[e]	0.73–3.29	1.20–1.38	0.214 ± 0.034	1.24 ± 0.08	7–12
			0.163 ± 0.080	1.07 ± 0.23	
Ac-PheTyr[e]	1.16–13.6	1.34–1.58	1.95 ± 0.18	4.66 ± 0.44	7–10
			1.68 ± 0.35	4.08 ± 0.51	
	30–125[f]	Varied	2.4	14	

[a] Details provided in text. [b] Total range of $[S]_0$ utilized in these studies. [c] Each value is the average determined from six independent Lineweaver–Burk plots. The uncertainties are standard deviations. [d] Number of points in the Lineweaver–Burk plots. [e] Upper set of values for K_0 and k_0 from integrated rate equation; lower, from initial slopes. [f] Estimated from ref. 4, assuming for pepsin mol. wt. 35,000 and nitrogen content of 14.6%. Refers to pure water at 37°, pH 2.

$\Delta\epsilon$'s observed for reactions which had reached equilibrium with the $\Delta\epsilon$'s calculated from the molar extinction coefficients of the appropriate species. Four hydrolyses of Ac-PheTyr gave $\Delta\epsilon = 490 \pm 15$, and four hydrolyses of Z-PheTyr gave $\Delta\epsilon = 539 \pm 13$. The data establish the optical purity of the substrates and suggest that large amounts of transpepditation products[2a] do not accumulate under the present experimental conditions.

General Treatment of the Kinetic Data. The hydrolysis of neither Z-PheTyr nor Ac-PheTyr exhibited zero-order kinetics (Figure 1). Each individual run with both compounds yielded a good apparent first-order plot, in agreement with the observations of Baker[4] on the hydrolysis of Ac-PheTyr. Figure 1 illustrates a typical first-order plot.

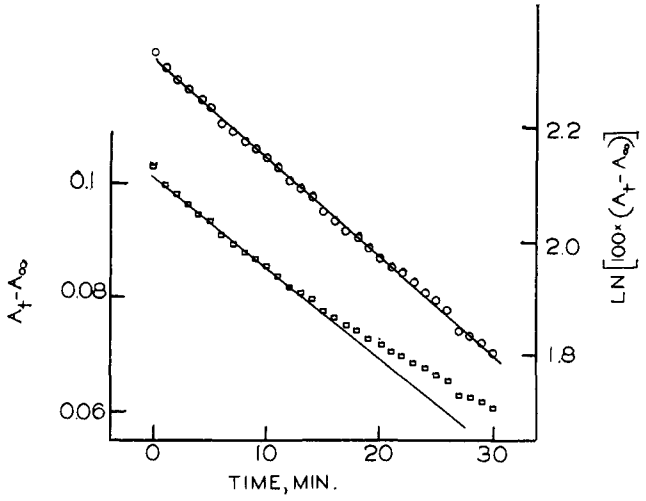

Figure 1. Apparent first-order kinetics in the pepsin-catalyzed hydrolysis of N-acetyl-L-phenylalanyl-L-tyrosine at 35°, pH 2.0, 3.4% methanol: [E] = 1.41×10^{-5} M, $[S]_0 = 2.19 \times 10^{-4}$ M, O, first-order plot of ln (Δ_{abs}) *vs.* time; □, zero-order plot of Δ_{abs} *vs.* time.

Green[14] has suggested one possible explanation for the apparent first-order kinetics. Equation 4 is the usual expression for the rate of an enzymatic reaction, and (5) is the rate expression for reaction in the presence of a simple competitive inhibitor. Equation 6 follows[15] as the rate of a product-inhibited reaction, if one assumes that $K_I = K_0$ and recognizes that [I] = $[S]_0 - [S]$. An enzymatic hydrolysis obeying (6) will exhibit apparent first-order kinetics for every $[S]_0$, with k given by (7). Baker[16] reported in 1956 that Ac-PheCOOH is an inhibitor of the hydrolysis of Ac-PheTyr by pepsin, and we confirm this observation. Quantitative evaluation of this inhibition, independent of the observation that the hydrolysis of Ac-PheTyr and Z-PheTyr obey (6), has not yet been completed.[17]

$$v = k_0[E][S]/(K_0 + [S]) \quad (4)$$

$$v = k_0[E][S]/([S] + K_0(1 + [I]/K_I)) \quad (5)$$

$$v = k_0[E][S]/([S]_0 + K_0) \quad (6)$$

$$k = k_0[E]/([S]_0 + K_0) \quad (7)$$

Two procedures have been used to evaluate the initial velocity, v_0, of the kinetic runs. The more indirect but more accurate method, which took full advantage of the relatively large change in optical density in the course of 40–50% reaction, employed the integrated rate equation. The slope of a conventional first-order plot of ln (O.D.$_t$ − O.D.$_\infty$) *vs.* time yielded the rate constant k, and v_0 was calculated as $k[S]_0$. Alternatively, v_0 was determined directly from the initial (less than 12% reaction) rate of decrease of optical density. This procedure was especially inaccurate at low $[S]_0$ where small changes in optical density were involved. Plots of $1/v_0$ *vs.* $1/[S]_0$ determined[18] the values for k_0 and K_0 listed in Table I. The method of least squares was used throughout the calculations.

The individual kinetic runs appeared to bear out the interpretation put on k by eq. 7 (see Figure 2). In a series of nine runs with Ac-PheTyr, as $[S]_0$ varied from 2.76 to 12.3 × 10^{-4} M, the average difference between k calculated from (7) (using the values of k_0 and K_0 of Table I) and k observed was 2%.

Carbobenzoxy-L-phenylalanyl-L-tyrosine. The ready availability of this compound made it our choice for kinetic studies. Unfortunately, the low solubility of Z-PheTyr restricted its utility, 3.5×10^{-4} M representing a nearly saturated solution under the experimental conditions. The Lineweaver–Burk plots for Z-PheTyr consequently rely heavily on reactions of low initial substrate concentrations, and it is not surprising that the plots derived from the initial slopes procedure showed much greater scatter than those ob-

(14) N. M. Green, *Nature*, **178**, 145 (1956).
(15) The treatment is essentially that of K. J. Laidler, "The Chemical Kinetics of Enzyme Action," Oxford University Press, London, 1958, Chapter IV.
(16) L. E. Baker, *Nature*, **178**, 145 (1956).
(17) A further consequence of the Green hypothesis, in the light of the more favorable K_0 for Z-PheTyr *vs.* Ac-PheTyr, is that Z-PheCOOH must be a considerably better inhibitor than Ac-PheCOOH.
(18) H. Lineweaver and D. Burk, *J. Am. Chem. Soc.*, **56**, 658 (1934).

tained from the integrated rate equation. Table I lists the average values for k_0 and K_0 and demonstrates that the two methods for determining v_0 were in satisfactory agreement.

Acetyl-L-phenylalanyl-L-tyrosine. The upper concentration limit for studies with this substrate was dictated by the difficulty in working with the highly concentrated methanolic stock solutions and by the high optical density of the substrate solutions at 237 mμ. The latter problem can be circumvented by going to higher wave lengths, where the molar extinction coefficients of enzyme and substrate are smaller, and $\Delta\epsilon$ for hydrolysis is also smaller. A run at $[S]_0 = 2 \times 10^{-3}$ *M* was observed at 240° mμ in one series of experiments, and $1/v_0$ for this point fell along the Lineweaver–Burk plot determined by the 237 mμ kinetics. The values of k_0 and K_0 calculated from the initial slopes and from the integrated rate expression are in close correspondence for Ac-PheTyr. The ratio k_0/K_0 for Ac-PheTyr is 24 M^{-1} sec.$^{-1}$, somewhat smaller than the 58 M^{-1} sec.$^{-1}$ for Z-PheTyr, but the more favorable K_0 of the latter is almost offset by the more favorable k_0 of the former.

Conclusions

This study entirely substantiates the feasibility of spectrophotometric determination of the kinetics of the pepsin-catalyzed hydrolysis of blocked phenylalanyl-tyrosine dipeptides at pH 2. The data on Ac-PheTyr from our experiments and those[4] from Baker's cover a range of 100-fold in substrate concentration (Table I). The satisfactory agreement between the results of the two investigations, carried out under somewhat different experimental conditions and utilizing entirely different methods of analysis, constitutes additional proof for the validity of the spectrophotometric method.

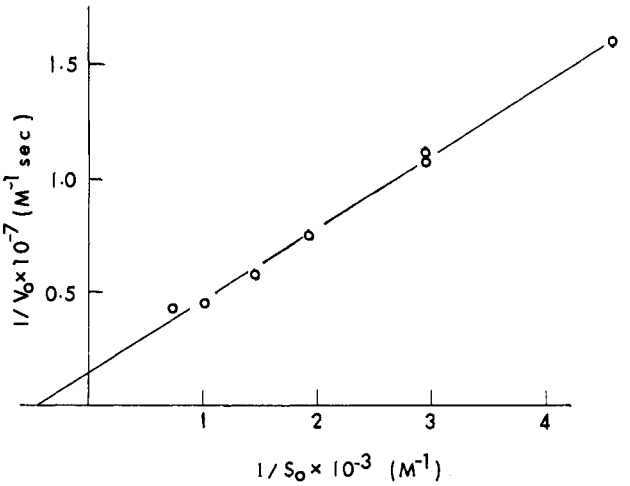

Figure 2. Lineweaver–Burk plot of the pepsin-catalyzed hydrolysis of N-acetyl-L-phenylalanyl-L-tyrosine at 35°, pH 2.0, 3.4% methanol; [E] = 1.41×10^{-6} *M*.

Experiments in progress in this laboratory indicate that the procedure is applicable over a considerable pH range, so that careful determination of the pH profiles of K_0 and k_0 is possible. The technique should be extendible to N-acetyl-L-phenylalanyl-L-diiodotyrosine[6] and has been applied to N-carbobenzoxy-L-phenylalanyl-L-tryptophan. The latter is too insoluble for detailed studies, and the N-acetyl analog will be investigated.

Interactions of Bovine Caseins with Divalent Cations[1]

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Infrared spectroscopy has been used to investigate the divalent cation binding sites of bovine α_s-casein, κ-casein, and mixtures of the two. Thin films on silver chloride disks, samples in KBr pellets, and aqueous solutions confined in cells with BaF_2 windows were used to obtain spectra. Even at a ratio of calcium ions to casein phosphorus atoms of 0.2 spectral shifts at 1084 and 974 cm.$^{-1}$, indicative of divalent ion interaction with organic phosphate groups, are observed in the absence of any other changes. At this Ca^{+2}/P ratio α_s-casein itself does not precipitate. At ratios of 2.5–4, where micelle formation takes place in the absence of any precipitate formation, alterations associated with organic phosphate groups approach completion and changes in other regions of the spectrum, e.g., 1395 cm.$^{-1}$, are observed.

(1) This work was supported by Grant No. GM 05410 from the Division of Research Grants, National Institutes of Health.

Introduction

Bovine α_s-, β-, and κ-caseins, having 1.0,[2] 0.5–0.6,[3,4] and 0.2–0.35%[5] phosphorus, respectively, are all normally involved in the formation of casein micelles. The minimum requirements are, however, α_s-casein, κ-casein, and a divalent cation.[6–8] The set of interactions

(2) D. F. Waugh, M. L. Ludwig, J. M. Gillespie, B. Melton, M. Foley, and E. S. Kleiner, *J. Am. Chem. Soc.*, **84**, 4929 (1962).

(3) M. P. Thompson and L. Pepper, *J. Dairy Sci.*, **47**, 633 (1964); J. Garnier, B. Ribadeau-Dumas, and G. Mocquot, *J. Dairy Res.*, **31**, 131 (1964).

(4) G. W. Dresdner and D. F. Waugh, *Federation Proc.*, **23**, 474 (1964).

(5) κ-Casein prepared by the method of R. G. Wake (*Australian J. Biol. Sci.*, **12**, 479, 1959) using fraction-S[8] as the starting material has a phosphorus content of about 0.35%. Values near 0.2% phosphorus have been obtained for the pellets centrifuged from fraction-S[7]. Both have been found to be equally effective in micelle formation.[8,9]

(6) D. F. Waugh and P. H. von Hippel, *J. Am. Chem. Soc.*, **78**, 4576 (1956).