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.

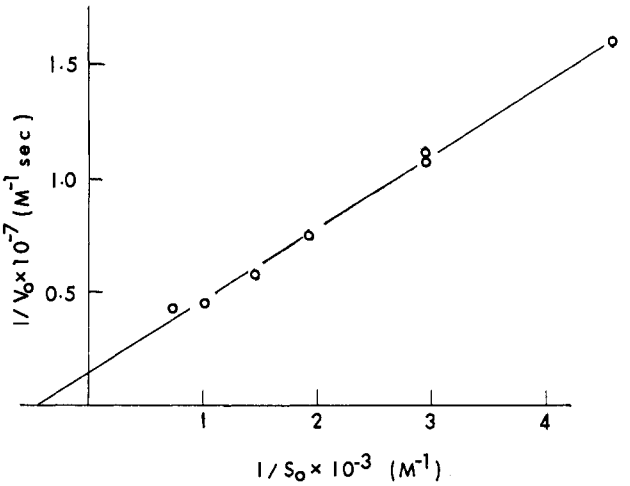

Figure 2. Lineweaver–Burk plot of the pepsin-catalyzed hydrolysis of N-acetyl-L-phenylalanyl-L-tyrosine at 35°, pH 2.0, 3.4% methanol; [E] = 1.41×10^{-5} *M*.

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.

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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

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(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).

which lead to the formation of stable micelles are complex and depend on the concentrations of α_s- and κ-caseins, the ratio of these proteins, the concentrations of mono- and divalent cations, and on the specific way in which interactants are mixed.[7-13] The binding site(s) and role of the divalent cation are therefore among those factors of fundamental interest.

Considerable controversy has centered around the nature of the organic phosphorus atoms in the caseins. The work of Perlmann[14] and Van Thoai, *et al.*,[15] indicates the presence of organic phosphomonoester, phosphodiester, and pyrophosphate. Sundararajan and Sarma,[16] Hofman,[17] Kolan and Telka,[18] and Belec and Jenness[19] present evidence indicating exclusively the monoester form. Lipmann[20] some time ago in fact found O-phosphoserine in the caseins.

This communication concerns the use of infrared spectroscopy as a technique for identifying divalent cation binding sites.

Experimental

For infrared studies a Perkin-Elmer Model 421 or 521 spectrophotometer, both for thin dry films and aqueous samples, was used. Dry films were prepared by spreading a drop of sample on a silver chloride disk (Harshaw Chemical Co.) and drying in a silica gel desiccator or under a heat lamp. Aqueous samples were confined in a type F-05BFT cell (Limit Research Co.) having BaF_2 windows and a 0.025-mm. path length. The reference sample was distilled water. A few preliminary samples were prepared as KBr pellets and examined by a Baird Model B spectrophotometer.

All chemicals including chloride salts of the divalent cations were analytical reagent grade and were used without further purification. O-Phosphoserine was purchased from Nutritional Biochemicals Corp. (lot No. 1389). The general supply of laboratory distilled water was passed through a Barnstead mixed-bed resin cartridge and then through an HA Millipore filter. The pH of the distilled water was between 6.0 and 7.0.

Purified bovine α_s-casein was prepared according to the method of Waugh and co-workers[2] and κ-casein essentially by the method of R. G. Wake.[5] The molecular weight of α_s-casein is 27,300[2,21] and that of κ-casein is assumed to be 30,000.[5,22] The lyophilized proteins were dissolved in 0.01 *M* potassium citrate and dialyzed at 4°, initially against 0.01 *M* potassium citrate for about 8–12 hr., then against 0.01 *M* KCl for another 8–12 hr. to remove citrate, and finally against "boiled" distilled water for 20–30 hr., with at least three changes of distilled water and until the dialysate was chloride free. Those samples of α_s- and κ-casein used in aqueous infrared studies were then concentrated by dialyzing against 20% polyethylene glycol, mol. wt. ~20,000 (Fisher Scientific Co.). Protein concentrations were determined from optical density using $\epsilon^{1\text{ cm}}_{1\%,\,280} = 10$ for α_s-casein[2] and κ-casein.[23]

Results and Discussion

The absorption characteristics of inorganic and organic phosphate groups, our primary interest, have been examined by Corbridge,[24] Bellamy,[25] and Herzberg.[26] Epp, *et al.*,[27] have examined the effects of interaction of ATP, ADP, and AMP with Mg^{+2}, Co^{+2}, and Mn^{+2}. We find that O-phosphoserine alone or after interaction with divalent ions gives results similar to those obtained by these investigators. Results

Table I. Effects of Divalent Cations on Ionic Phosphate Groups in *o*-Phosphoserine and Bovine Caseins at pH 7

Material	KCl, *M*	Divalent cation Nature	Concn.,[a] *M*	Ionic phosphate, cm.$^{-1}$ Region II[b]	Region III[b]
O-Phosphoserine	0.01		0	1090	978
O-Phosphoserine	0.01	Mg^{+2}	0.0076	1112	1007
O-Phosphoserine	0.01	Ca^{+2}	0.0076	1110	999
O-Phosphoserine	0.01	Sr^{+2}	0.0076	1097	985
O-Phosphoserine	0.01	Ba^{+2}	0.0076	1093	980
O-Phosphoserine	0.01	Ni^{+2}	0.0076	1087	992
O-Phosphoserine	0.01	Co^{+2}	0.0076	1082	993
α_s-Casein	0.05		0	1084	974
κ-Casein	0.05		0	1079	975
α_s-κ-Caseins	0.05		0	1085	973
α_s-κ-Caseins	0.10		0	1085	973
α_s-Casein	0.05	Ca^{+2}	0.0015	1096	978
α_s-Casein	0.05	Ca^{+2}	0.0050	1100	994
κ-Casein	0.05	Ca^{+2}	0.0020	1107	994
κ-Casein	0.05	Ca^{+2}	0.0500	1101	999
α_s-κ-Caseins	0.05	Ca^{+2}	0.0050	1103	994
α_s-κ-Caseinate (precipitate)	0.05	Ca^{+2}	0.0100	..	992
α_s-κ-Casein micelles	0.05	Ca^{+2}	0.0200	..	996
α_s-κ-Casein micelles	0.05	Ca^{+2}	0.0500	1100	999
α_s-κ-Casein micelles	0.10	Ca^{+2}	0.0200	1098	998
α_s-κ-Casein micelles	0.10	Ca^{+2}	0.0500	1103	998
α_s-κ-Caseins[c]	0.10		0	1084	977
α_s-κ-Casein micelle[c]	0.10	Ca^{+2}	0.0300	1092	993
α_s-κ-Casein micelle[c]	0.10	Ca^{+2}	0.0600	1092	992

[a] The concentration given in this table refers to the solution concentration before film drying. [b] The notations of regions II and III correspond to those given in Figure 1. [c] The spectrum was taken in aqueous medium.

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(27) A. Epp, T. Ramasarma, and L. R. Wetter, *J. Am. Chem. Soc.* **80**, 724 (1958).

are summarized in Table I. The concentrations of O-phosphoserine and divalent ion, in 0.01 *M* KCl at pH 7, were each 7.6×10^{-3} *M*. Characteristic ionic phosphate absorption peaks at 1090 and 978 cm.$^{-1}$ are shifted on interaction with alkaline earth or transition metal divalent cations to frequencies between 1112–1082 and 1007–980 cm.$^{-1}$, respectively, depending on ion type. Attention should be directed to the existence of a peak at 1420 cm.$^{-1}$ which is not altered appreciably on ion interaction. Absorption in this region has been assigned to the carboxylate ion.[25,28] It should be pointed out also that water is relatively transparent over the range 1500–900 cm.$^{-1}$.[29,30]

Films of α_s-casein, κ-casein, and α_s-κ-casein mixtures have been prepared from solutions, at pH 7, which contained 2% α_s-casein, 2% κ-casein, or a 2.5% α_s-κ-casein mixture of weight ratio 4:1. The ratio of 4 is that present in milk.[7,31] These solutions also contained various amounts of KCl from 0.05 to 0.1 *M* and $CaCl_2$ from 0 to 0.05 *M*.

Infrared spectra for dry protein films free of divalent ion are similar over the range 1500–900 cm.$^{-1}$ and all change in the same way on adding divalent cation. Figure 1 refers to an α_s-κ-casein mixture. The solid line gives the absorption spectrum in 0.1 *M* KCl before the addition of $CaCl_2$. The dotted line gives the spectrum after the addition of 0.05 *M* $CaCl_2$. For convenience the spectrum is divided into five regions: *Region I* has a broad absorption peak between 930 and 920 cm.$^{-1}$. This broad peak disappears when the concentration of $CaCl_2$ is greater than 0.005 *M*. Although an assignment of this peak is not made here, this region has been suggested as due to pyrophosphate absorption.[24,25,32] *Regions II and III* have absorption peaks at 1084 and 974 cm.$^{-1}$, due as stated above to ionic phosphate. Upon the addition of $CaCl_2$, these peaks are shifted toward 1100 and 999 cm.$^{-1}$, respectively. *Region IV* has small bands between 1180 and 1150 cm.$^{-1}$. These undergo ill-defined alterations on the addition of Ca^{+2}. *Region V* has absorption peaks near 1445 and 1395 cm.$^{-1}$. The peak at 1395 cm.$^{-1}$ gradually disappears as the ratio of Ca^{+2} to casein-P atoms exceeds 1.4. The peak at 1445 cm.$^{-1}$ does not seem to be affected. Changes in absorption in the region 1440–1390 cm.$^{-1}$ could be due to effects on carboxylate or ammonium ions or on phenolic or unsubstituted amide groups.[25,28]

Table I summarizes the effects of several divalent ions under a variety of conditions. Only changes in regions II and III are given. The addition of Ca^{+2} produces changes expected from ionic phosphate groups. We estimate that the accuracy for measuring the frequency shift is about 5 cm.$^{-1}$ for the peak at 1085 cm.$^{-1}$ and about 2 cm.$^{-1}$ for the peak at 974 cm.$^{-1}$. Evidence suggests that Sr^{+2}, Ba^{+2}, Ni^{+2}, and Co^{+2} also produce upward shifts in the ionic phosphate frequencies of the caseins, but some shifts are not as marked as those produced by Ca^{+2}.

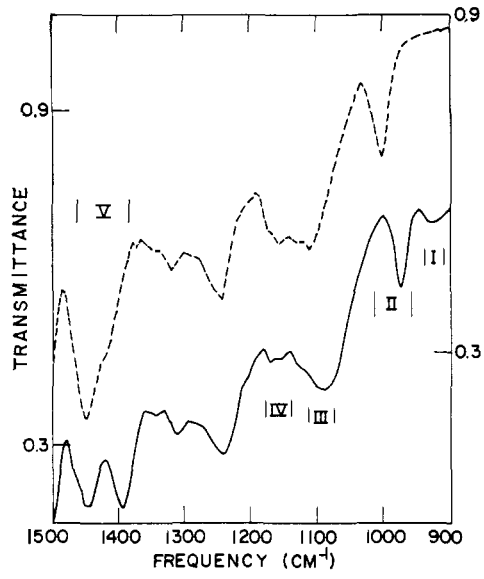

Figure 1. The spectra were obtained from thin films dried on silver chloride disks. The α_s-κ-casein solution contained 2.5% protein, at a weight ratio 4:1, in 0.1 *M* KCl at pH 7. The solid line is for the divalent ion free protein and refers to the transmittance on the left. The dotted line is for the same protein after the addition of 0.05 *M* $CaCl_2$ and refers to the transmittance on the right.

Infrared absorption studies have been carried out on α_s-κ-casein mixtures in aqueous media at pH 7 in the absence or presence of calcium ion. The total protein concentration was 7.5% and the α_s- to κ-casein weight ratio was 4. The Ca^{+2} concentration was varied from 0 to 0.06 *M*. Effects similar to those with dry films were obtained in regions II and III (see Table I). In region V, although the peak at 1395 cm.$^{-1}$ decreased, the decrease relative to the unaltered peak of 1445 cm.$^{-1}$ was partial and, even at a Ca^{+2}/P ratio of 2.8, much less than that observed in dry films at a ratio of 2.0. As expected for aqueous media, however, even at 7.5% protein the relative absorption was much less than that of the dry films.

The infrared spectra suggest that in bovine caseins the primary sites for Ca^{+2} binding are organic phosphate groups. At a Ca^{+2}/P ratio of 0.2 partial shifts in regions II and III are observed while changes in other regions of the spectrum are not observed. Shifts in regions II and III are essentially complete at a Ca^{+2}/P ratio of 2.0. Near this same ratio (Ca^{+2}/P $\sim$1.4) is initiated the second most noticeable change, a decrease in the relative size of the peak at 1395 cm.$^{-1}$ in region V. A disappearance of this peak has been obtained with dry films but only a decrement in aqueous media. Data for micelle formation[9,10] allow comparison. At a Ca^{+2}/P ratio of 1.2 solutions containing 2% α_s-casein are clear. At ratios between 2.5 and 4 complete micelle formation takes place in systems having at least up to 2% protein and α_s-κ-casein weight ratios below 10. Complexity must also be noted, however, for at a Ca^{+2}/P ratio of about 1.4 appreciable precipitation of α_s-casein from 2.2% α_s-κ-casein mixtures takes place, even at α_s-κ-casein weight ratios of about 4.

According to spectroscopy the interaction of calcium ions with casein phosphate groups is clearly well advanced just prior to the point where Ca α_s-caseinate

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precipitation takes place from solutions either of α_s-casein alone or α_s-κ-casein mixtures. Over the Ca^{+2}/P range where micelles are present in the absence of any precipitate, interaction of calcium ion with phosphate groups is approaching completion. Over this latter Ca^{+2}/P range, but not before, are also observed alterations in the absorption band of region V. Possibly interactions of calcium ions with group(s) responsible for absorption in this region are involved in micelle formation. Of course, protein–protein interactions or conformational changes not involving calcium ions or organic phosphate groups could be responsible.

2-Pyridone, 2-Pyridthione, and 2-Pyridselenone. Hydrogen-Bonding Ability as Determined by Dipole Moment and Molecular Weight Determinations[1]

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The tendency of 2-pyridone, 2-pyridthione, and 2-pyridselenone, simple models of nucleic acid antagonists, to form hydrogen-bonded dimers was investigated by means of dielectric constant and molecular weight measurements in benzene and in dioxane. The propensity of 2-pyridone to dimerize exceeded that of its sulfur and selenium analogs; however, all these compounds must be considered as being powerful hydrogen bonders. The dipole moments of 2-pyridone, 2-pyridthione, 2-pyridselenone and their N-methyl derivatives were investigated. Polarization increased progressively as oxygen of 2-pyridone was replaced by sulfur and selenium.

Numerous heterocyclic sulfur compounds (*e.g.*, 6-mercaptopurine, 2-thiouracil derivatives, 2-thiobarbiturates, penicillin) have found widespread application as medicinal agents. Because the often considerable differences in the biological activities of oxygen-, sulfur-, and selenium-substituted heterocyclic compounds are likely to be related to differences in their strengths of binding to receptor sites, rather than to differences in their abilities to fit receptor sites, a systematic investigation of the hydrogen-bonding and hydrophobic-bonding capacities of oxygen, sulfur, and selenium isologs was undertaken.

Interest in such a study was raised by the report[3] that the antineoplastic activity of 6-thioguanine may be related to the ability of this compound to be incorporated into deoxyribonucleic acid (DNA). This observation led to the postulate[4] that the antitumor action of 6-thioguanine[5] and 6-selenoguanine[4,6] might be due to the formation of unusual hydrogen bonds between the sulfur or selenium of the guanine analogs and the amino group of cytosine, which would be facing these atoms within the double helix of DNA. A similar interaction has been proposed as taking place between the thiocarbonyl group of thionicotinamide and the amino group of adenine in the sulfur analog of dihydrophosphopyridine nucleotide.[7]

Since the complexity and insolubility in organic solvents complicate the study of the bonding tendencies of DNA bases and of their analogs, a series of simple heterocyclic compounds, 2-pyridone, 2-pyridthione, and 2-pyridselenone, was chosen for detailed investigation.

It was shown recently by comparison of the ultraviolet spectra and ionization constants of these compounds with those of their N-methyl, O-methyl, S-methyl, and Se-methyl derivatives that for 2-pyridone,[8] 2-pyridthione,[9] and 2-pyridselenone[10] equilibrium favors predominantly amidic rather than imidic tautomers. Molecular weight determinations carried out at moderately high concentrations showed 2-pyridselenone[10] to be dimeric in benzene solution. Similarly, 2-pyridone and 2-pyridthione form hydrogen-bonded dimers in benzene[10] and in chloroform.[11] To obtain information about the relative abilities of the above compounds to form hydrogen bonds, as well as about the relative degree of polarization of heterocyclic carbamyl, thiocarbamyl, and selenocarbamyl groups, a series of dipole moment and molecular weight measurements in benzene and in dioxane was undertaken.

Experimental

Materials. Reagent grade 2-pyridone was purified by two distillations followed by recrystallization from benzene. A commercial sample of N-methyl-2-pyridone was distilled twice *in vacuo* (b.p. 53.5–54.5 (0.2 mm.)). 2-Pyridthione,[12] N-methyl-2-pyridthione,[13] 2-

(1) This work was supported, in part, by grants from the National Science Foundation (G-19329) and the National Cancer Institute of the Public Health Service (CA-03937).

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