TABLE IV

REACTION OF 1 MOLE OF 1-OCTENE AND 3 MOLES OF DIBUTYL PHOSPHITE

5 mole per cent. (on the phosphite) di-*t*-butyl peroxide; 120°

Time, hr.	$n^{20}D$	Amount of reaction 1-Octene, mmoles/g.	Dibutyl phosphite, mmoles/g.	Ratio olefin: phosphite reacted	Chain length
0	1.4201	0	0	..	
0.75	1.4275	1.23	0.96	1.3	221
1	1.4297	1.58	1.27	1.3	221
1.5	1.4297	1.82	1.27	1.5	
2	1.4300	1.73	1.26		
2.5	1.4298	1.73	1.27		
3	1.4300	1.81	1.30		
3.5	1.4300	1.70	1.32		
4	1.4302	1.79[a]	1.31[b]		

[a] 100% conversion of 1-octene. [b] 32% conversion of dibutyl phosphite.

ber Co.; the 2-butene and diisobutylene were obtained from Shell Oil Co. and the cyclohexene was Eastman Kodak Co. white label. All olefins were refractionated before use.

The experimental procedure was comparatively simple. The mixed reactants were heated at the desired temperatures either in sealed glass bombs or, if sufficiently high boiling, in open vessels. The product mixtures were then fractionated. In some cases the course of the reaction was followed by periodic sampling and analyses.

Analytical. The analysis of mixtures of dibutyl phosphite and olefin consists of (1) determining the neutral equivalent, which is the direct measure of the phosphite content and (2) determining the bromine number. The difference between the two values is then the measure of the olefin content.

(1) **Neutral Equivalent of Olefin-Dibutyl Phosphite Mixtures.**—A weighed sample (200–300 mg.) was dissolved in 25 ml. of 50% aqueous alcohol and allowed to react with excess 0.1 *N* alkali for 10 minutes and then titrated to the phenolphthalein end-point with 0.1 *N* acid.

(2) **Bromine Number of Olefin-Dibutyl Phosphite Mixtures.**—A weighed sample (200–400 mg.) was allowed to react for 45 min. with an excess of 0.1 *M* bromine in carbon tetrachloride in an atmosphere of dry nitrogen. At the end of this period the excess bromine was determined as previously described.

In Table V are contained the 1-octene and phosphite contents of several mixtures (all containing peroxide) used in rate studies. It will be noticed that as the phosphite concentration is increased, the reliability of the analysis for octene is decreased. This may be due to the large amount of HBr liberated when the phosphite is present in excess.

TABLE V

ANALYSIS OF STOCK SOLUTIONS

Ratio octene: phosphite	1-Octene, mmoles/g. Calcd.	Found	Dibutyl phosphite, mmoles/g. Calcd.	Found
3:1	5.70	5.67	1.86	1.84
1:1	3.19	3.10	3.19	3.19
1:3	1.41	1.64	4.19	4.14

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[CONTRIBUTION FROM THE EASTERN REGIONAL RESEARCH LABORATORY[1]]

The Phosphopeptones Obtained from α-, β- and Whole Casein by Partial Hydrolysis with Pepsin[2]

BY M. L. GROVES, N. J. HIPP AND T. L. MCMEEKIN

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Methods are described for fractionating the gel phosphopeptones obtained from partial peptic hydrolysates of α-, β- and whole casein. The phosphopeptone fraction prepared from β-casein, insoluble at *p*H 3.5, contained two electrophoretic components and a large proportion of the phosphorus of β-casein. These two components were separated by fractionation with ammonium sulfate. They were also prepared in good yield from whole casein by the same method.

Many investigators have studied the insoluble phosphopeptone (para- or pseudo-nuclein) formed during the early stages of the hydrolysis of casein by pepsin.[3–6] Holter, Linderstrøm-Lang and Funder[4] studied the rate of formation of the gelatinous insoluble phosphopeptone by means of viscosity and splitting of peptide bonds. The insoluble phosphopeptone was formed when the increase of amino nitrogen amounted to only 1.5% of the total nitrogen. They also found that the insoluble phosphopeptone had a nearly constant nitrogen-to-phosphorus ratio which was independent of the casein fraction used and the time of digestion. Mellander[6] has extensively reviewed the literature, has investigated the action of pepsin on cow's casein and human casein, and has found, in agreement with the literature, that the insoluble phosphopeptone is formed only from cow's casein.

The present investigation was undertaken in order to prepare large homogeneous phosphopeptones and to obtain information concerning the phosphorus linkage in casein. The isolation of the insoluble phosphopeptones from α-, β- and whole casein is described.

Materials and Methods

Samples of α-, β- and whole casein were prepared as previously described.[7] Crystalline pepsin was used. Nitrogen was determined by Nesslerization[8] and phosphorus by Sumner's[9] modification of the Fiske and Subbarow method.[10] All nitrogen and phosphorus values were corrected for 10% moisture. Zone electrophoretic determinations were made on Whatman No. 1 paper strips at 3° in a Durrum type cell using veronal buffer, 0.05/μ, *p*H 8.6. About 7.5 mg. of

(1) A laboratory of the Eastern Utilization Research and Development Division, Agricultural Research Service, U. S. Dept. of Agriculture. Article not copyrighted.

(2) Presented at the 129th Meeting of the American Chemical Society, Atlantic City, N. J., September, 1956.

(3) R. H. Chittenden and H. M. Painter, *Trans. Conn. Acad. Arts Sci.*, **7**, 362 (1885–1888).

(4) H. Holter, K. Linderstrøm-Lang and J. B. Funder, *Compt. rend. trav. lab. Carlsberg*, **19**, No. 10, 1 (1933).

(5) K. Linderstrøm-Lang, *Ergebnisse Physiol.*, **35**, 415 (1933).

(6) O. Mellander, *Upsala Läkarefören. Förh.*, **52**, 107 (1947).

(7) N. J. Hipp, M. L. Groves, J. H. Custer and T. L. McMeekin, *J. Diary Sci.*, **35**, 272 (1952).

(8) F. C. Koch and T. L. McMeekin, THIS JOURNAL, **46**, 2066 (1924).

(9) J. B. Sumner, *Science*, **100**, 413 (1944).

(10) C. H. Fiske and Y. Subbarow, *J. Biol. Chem.*, **66**, 375 (1925).

material was dissolved in 0.25 ml. of veronal buffer, and sodium hydroxide was carefully added when necessary since the phosphopeptones were quite acidic. A 0.01-ml. sample of the solution was placed on the paper which had been previously wet with the buffer, and 75 volts were applied for approximately 16 hours. The papers were then dried, sprayed with a ninhydrin reagent,[11] and scanned photoelectrically the next day. All the phosphopeptones moved toward the positive electrode.

Experimental Results

Solutions of α-, β- and whole casein (2%) were made to *p*H 2.0 with 0.1 *N* hydrochloric acid, with or without lactic acid.[12] On digesting with 0.01% crystalline pepsin at 30° for about 1.5 to 2.5 hours, a gel formed which was immediately separated by centrifugation. After centrifugation and washing with water, the insoluble gel was suspended in water, dissolved and adjusted to *p*H 7.0 by adding a saturated solution of barium hydroxide. The gel supernatant and washings were also made to *p*H 7.0 with barium hydroxide. Under these conditions the pepsin was inactivated. On the addition of an equal volume of alcohol and cooling to 4°, most of the phosphorus-containing material was precipitated as barium salts. The percentage distribution of nitrogen and phosphorus in these precipitates and also in the combined filtrates from each casein are shown in Table I. Nearly all the phosphorus from β-casein is found in the gel fraction, while only a fourth of the phosphorus from α-casein is found in this fraction. Almost one-half of the phosphopeptones from whole casein is in the gel fraction, which is consistent with the fact that whole casein is a mixture of three parts of α-casein and one part of β-casein.

TABLE I

DISTRIBUTION OF NITROGEN AND PHOSPHORUS IN PHOSPHOPEPTONE FRACTIONS FROM PARTIAL PEPTIC HYDROLYSATES OF α-, β- AND WHOLE CASEIN

Fraction	α-Casein % of total P	α-Casein % of total N	Whole casein % of total P	Whole casein % of total N	β-Casein % of total P	β-Casein % of total N
Gel phosphopeptone	28–74[a]	11–22[a]	44–46	15–14	92–97[a]	21–24[a]
Non-gel phosphopeptone	48–	16–	40–38	13–12	3–	1–
Filtrates (by diff.)	24–26	73–78	16–16	72–74	5–3	78–76

[a] In these experiments total phosphorus and nitrogen values were done only on the combined gel and non-gel fractions.

The alcohol precipitates of the barium salt of the gel fractions obtained by the action of pepsin on 15 g. of α-, β- and whole casein were each dissolved in water, precipitated at *p*H 3.5, and analyzed, along with the barium precipitates from the supernatants (non-gel phosphopeptones), for nitrogen and phosphorus; Table II. The relatively large amount of barium present in the barium phosphopeptone precipitate obtained from the supernatant sharply decreases the nitrogen and phosphorus content of these fractions. The atomic nitrogen-to-phosphorus ratios, however, indicate that the two fractions are comparable and the values of 12 to 15 reflect the large increase in phosphorus in the phosphopeptones, since this ratio for α- and β-casein is 34 and 56.

TABLE II

COMPOSITION OF PHOSPHOPEPTONE FRACTIONS FROM α-, β- AND WHOLE CASEIN

	α-Casein Gel *p*H 3.5	α-Casein Non-gel Ba. ppt.	Whole casein Gel *p*H 3.5	Whole casein Non-gel Ba. ppt.	β-Casein Gel *p*H 3.5	β-Casein Non-gel Ba. ppt.
Yield, %	7.4	25.1	12.0	16.9	19.5	2.7
N, %	15.1	10.3	14.3	9.9	14.2	8.6
P, %	2.2	1.8	2.2	1.7	2.3	1.6
Atomic ratio N/P	15.2	12.7	14.4	12.9	13.7	11.9

(11) A. L. Levy and D. Chung, *Anal. Chem.*, **25**, 396 (1953).

(12) β-Casein does not dissolve readily in hydrochloric acid at room temperature, but does dissolve when warmed to 60° and then rapidly cooled. In some experiments the β-casein was first dissolved in lactic acid and the solutions were then adjusted to *p*H 2.0 with hydrochloric acid.

The phosphopeptones from the gel fraction precipitated at *p*H 3.5 (Table II) were further fractionated by dissolving in dilute barium hydroxide and precipitated at varying *p*H values with hydrochloric acid. The major portion of the phosphopeptones was precipitated at *p*H 4.7 and 3.5. Three-fourths of the gel fraction from α-casein was insoluble at *p*H 4.7, while only a small amount (4%) was precipitated at *p*H 3.5. Only one-third of the gel fraction of β-casein was insoluble at *p*H 4.7, while two-thirds was precipitated at *p*H 3.5. About one-half of the gel fraction of whole casein was insoluble at *p*H 4.7 and two-fifths was insoluble at *p*H 3.5. Paper electrophoretic determinations showed that the gel fractions from each of the caseins contained three similar components in different amounts. The electrophoretic patterns on the fractions from α-casein insoluble at *p*H 4.7 and 3.5 show that both of these fractions are essentially one component, with a small amount of slow moving material. The fractions insoluble at *p*H 4.7 and 3.5 contained three components in somewhat different proportions from the original materials.

Ammonium sulfate was used to separate the two components from the gel fraction of β-casein precipitated at *p*H 3.5 as follows: A 2% solution of the phosphopeptones, Fig. 1 (I), was made to *p*H 6.6 with ammonium hydroxide; saturated ammonium sulfate solution, previously adjusted to *p*H 6.6, was added dropwise and with stirring until the phosphopeptone solution was 74% saturated. The precipitate formed, which contained the slower-moving peak (B), was removed by centrifugation. The supernatant was then made to 93% saturation by adding solid ammonium sulfate and then adjusting to *p*H 6.6. The resulting precipitate was removed by centrifugation and filtration. This fraction contained both electrophoretic components. The filtrate, which contained mostly the faster-moving peak (A), was then precipitated by adjusting the *p*H to 3.5. On repeating this procedure on the material insoluble in 74% ammonium sulfate and the material insoluble in 93% ammonium sulfate at *p*H 3.5, the two peaks were essentially resolved as shown in Fig. 1 (II, III). These fractions were shown to have small amounts of a minor component by

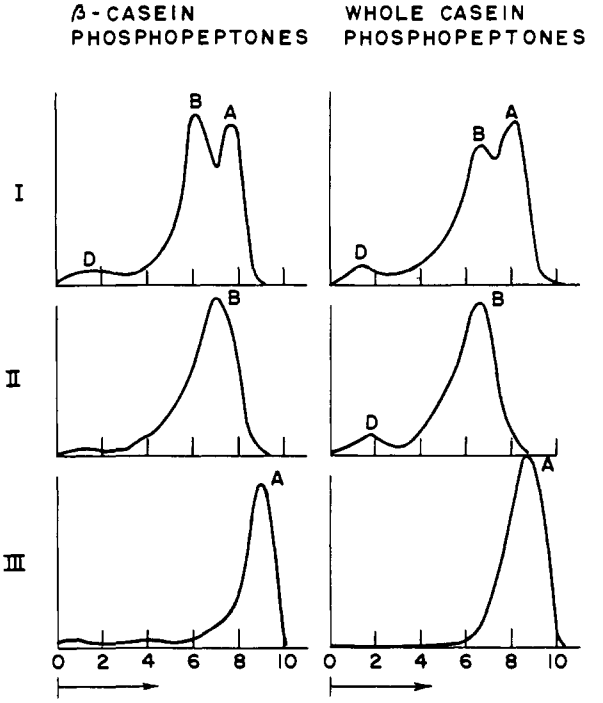

Fig. 1.—Paper electrophoretic patterns of phosphopeptones obtained from the gel fractions of β- and whole casein by fractionation with ammonium sulfate; veronal buffer, *p*H 8.6, 0.05 ionic strength, 16 hours, 2.5 v./cm.: I, phosphopeptones from β- and whole casein; II, fraction least soluble in ammonium sulfate; III, fraction most soluble in ammonium sulfate.

means of the Tiselius method. The principal component of fraction A had a mobility of $u = -7.7$ and of fraction B, $u = -6.4$ in veronal buffer at pH 8.5 and 0.1 ionic strength.

In order to determine whether the two β-casein phosphopeptones can be obtained in good yield from whole casein by this method, 60 g. of whole casein was dissolved in hydrochloric acid and made to 3 liters. The solution was adjusted to pH 2.0, and then digested with pepsin as previously described. The barium precipitate from the gel fraction, amounting to 11.75 g., had 11.4% nitrogen and 2.06% phosphorus (N/P 12.2). From the supernatant (non-gel fraction) 9.71 g. of barium precipitate was obtained which contained 12.2% nitrogen and 2.07% phosphorus (N/P ratio 13.0). The electrophoretic composition of these two fractions is shown in Fig. 2.

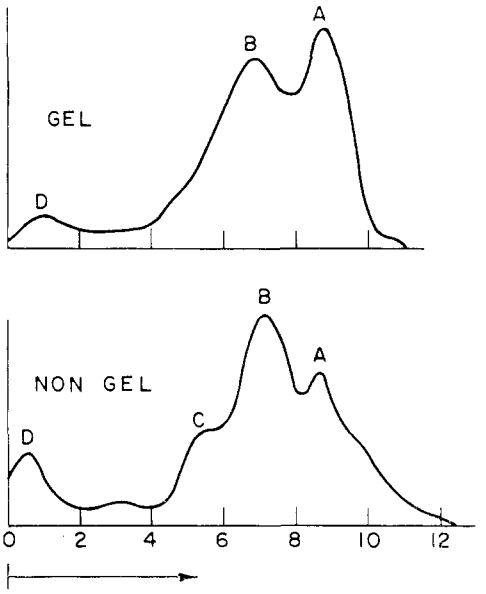

Fig. 2.—Paper electrophoretic patterns of phosphopeptones from whole casein by the action of pepsin; veronal buffer, pH 8.6, 0.05 ionic strength, 16 hours, 2.5 v./cm.

A 3% aqueous solution of the barium salt from the gel fraction was precipitated at pH 5.0, 4.5 and 3.5 by the addition of 0.1 N hydrochloric acid dropwise and with stirring. The fraction insoluble at pH 3.5, which amounted to 3.41 g., was then fractionated by ammonium sulfate as previously described for the β-casein phosphopeptones. The fraction insoluble at 74% saturated ammonium sulfate, representing the slower-moving component, amounted to 0.78 g. as compared to 1.31 g. of the faster-moving component, insoluble in 93% saturated ammonium sulfate, pH 3.5. The electrophoretic patterns of these two fractions compared to the unfractionated material are shown in Fig. 1. Essentially the same patterns were obtained for the phosphopeptones of whole casein as for those of β-casein. Analytical data on three purified fractions, one obtained from β- and two from whole casein are shown in Table III. The nitrogen and phosphorus contents of these phosphopeptones are in good agreement and furnish additional evidence to the electrophoretic patterns that the β-casein phosphopeptones can be prepared from whole casein.

Discussion.—Peptic hydrolysis of α-casein, at the time of formation of the insoluble gel, is more extensive than that of β-casein. Van Slyke amino nitrogen determinations showed that 7% of the total peptide bonds of α-casein were split at the time of separation of the gel while only 2.5% of the bonds of β-casein were broken. The finding that α-casein gives only a small amount of gel phosphopeptone when digested with pepsin is also consistent with the idea that it is more extensively hydrolyzed by the pepsin and produces a more complex mixture of phosphopeptones than does β-casein. It is of interest to note that β-casein is also more resistant to hydrolysis by acid. This may indicate that the phosphorus groups in β-casein are closer together than in α-casein.

TABLE III

COMPOSITION OF PHOSPHOPEPTONES OBTAINED BY AMMONIUM SULFATE FRACTIONATION

	Component B			Component A		
	P, %	N, %	N/P	P, %	N, %	N/P
β-Casein	2.2	14.9	15.0	2.8	14.4	11.4
Whole casein (1)	2.3	15.1	14.5	2.9	15.1	11.5
Whole casein (2)	2.3	15.8	15.2	2.8	15.2	12.0

The mobilities of the purified phosphopeptones from β-casein are proportional to their phosphorus content, that is, the faster-moving phosphopeptone (A) has the larger phosphorus content. The mobility is determined, however, by the total number of charged groups rather than the total amount of phosphorus. Perlmann[13] has concluded that the phosphorus of β-casein is in the form of a diester of orthophosphoric acid, while the phosphorus of α-casein is divided into monoesters of orthophosphoric acid, pyrophosphates and nitrogen diesters of orthophosphoric acid.

The phosphopeptones from the gel fraction do not dialyze through cellophane. A preliminary determination of the sedimentation constant of a slightly impure phosphopeptone with a mobility of component B gave a value of $S_{20} = 1.3$ at room temperature. Both of these findings are consistent with a fairly large molecular size. The phosphorus contents of 2.3 and 2.8% for the pure phosphopeptones from β-casein indicate minimum molecular weights of one-fourth to one-fifth of that of β-casein, which has a molecular weight of about 24,000 below 17°,[14] and a phosphorus content of 0.6%.

The nitrogen and phosphorus values for the gel phosphopeptone given by Holter, *et al.*,[4] gave a value of about 17 for the atomic nitrogen-to-phosphorus ratio for this fraction. This compares with the value of 14 herein reported for the fraction from whole casein, 12 for the purified A component, and 15 for the B component.

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(14) R. A. Sullivan, M. M. Fitzpatrick, E. K. Stanton, R. Annino, G. Kissel and F. Palermiti, *Arch. Biochem. and Biophys.*, **55**, 455 (1955).