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

The Separation and Amino Acid Composition of a Pure Phosphopeptone Prepared from β-Casein by the Action of Trypsin[2]

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The preparation of a relatively large electrophoretically homogeneous phosphopeptone by the action of trypsin on β-casein is described. From the amino acid composition data the empirical residue formula is calculated to be HOOC-Arg_1, Asp_1, Glu_7, $(NH_2)_3$, Gly_1, Val_2, Leu_3, $Ileu_2$, Ser_4, Thr_1, $(PO_4)_5$, Pro_1, Arg-NH_2. The minimum molecular weight of this phosphopeptone is calculated to be 3111 which is of the same order as the determined molecular weight of 4100. The results indicate that arginine is both N-terminal and C-terminal in the phosphopeptone.

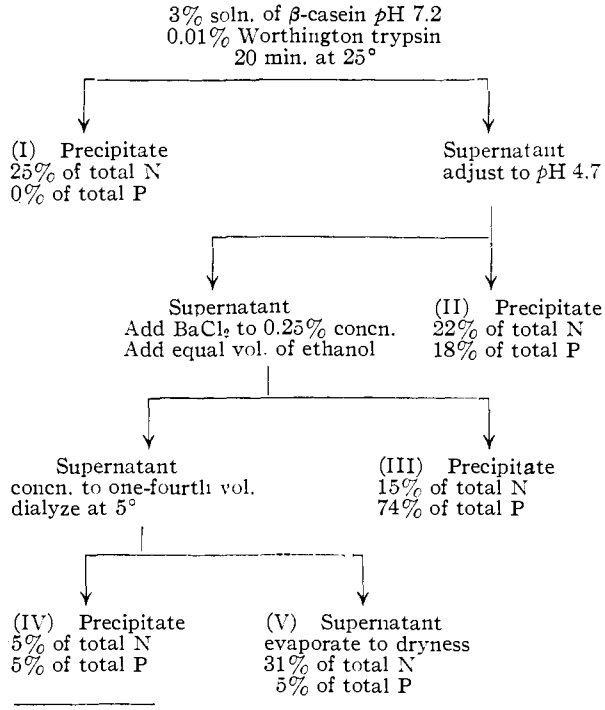
Numerous phosphopeptones have been prepared by the action of trypsin or trypsin plus pepsin on unfractionated casein.[3-6] Since casein is a mixture of proteins,[7] the action of proteolytic enzymes on its purified components should lead to less complicated mixtures of phosphopeptones; consequently, the phosphopeptones produced from β-casein by the action of trypsin have been investigated. In the study of the structure of proteins the desirability of producing large peptide fragments has been indicated by Sanger.[8] Christensen[9] observed that a large increase in viscosity of β-casein solutions was produced after short periods of tryptic hydrolysis and that the viscosity decreased rapidly after longer periods of digestion. Preliminary experiments showed that a number of large peptides could be separated easily after trypsin acts on β-casein for short periods. This communication describes the separation and composition of a large phosphopeptone produced by the action of trypsin on β-casein for about 20 minutes.

Materials and Methods

β-Casein was produced by the method of Hipp, *et al.*[10] Crystalline trypsin containing 50% magnesium sulfate obtained from the Worthington Chemical Co.[11] was used. The extent of hydrolysis was measured by means of Van Slyke amino nitrogen determinations using a separate reaction chamber.[12] Nitrogen was determined by the micro-Kjeldahl method. Phosphorus was determined by the A. O. A. C. method,[13] after a preliminary decomposition in the Carius bomb. Amide ammonia was measured by the procedure of Mellon, *et al.*[14]

Preparation of Phosphopeptone.—A 3% solution of β-casein at *p*H 7.0–7.5 was prepared by dissolving β-casein in dilute sodium hydroxide. Trypsin, dissolved in water, was added to the β-casein solution to give a 0.01% solution. The solution became turbid in five minutes and coagulated in about 20 minutes. Amino nitrogen determinations by the Van Slyke method increased from an initial value of 0.75% to a value of 1.14% at the time of coagulation as shown in Fig. 1. Since β-casein has a minimum molecular weight of 24,100[15] the increase in amino nitrogen of 0.39% due to tryptic hydrolysis indicates that seven peptide bonds were broken at the time of coagulation. The trypsin was then destroyed by heating the beaker in boiling water for 8 minutes. After heating, the insoluble material (fraction I), which is essentially free from phosphorus, was removed by centrifugation. The supernatant was adjusted to *p*H 4.7 with 0.1 *N* hydrochloric acid and the insoluble material removed by centrifugation (fraction II). The supernatant was then made to 0.25% barium chloride by addition of a 10% barium chloride solution. Then an equal volume of absolute ethanol was added which precipitated the barium phosphopeptone (fraction III). The precipitate was removed by centrifugation and then dried with acetone and ether. The supernatant was concentrated on the Craig evaporator to one-fourth its volume and then dialyzed at 5° against several changes of water. A small precipitate (fraction IV) was removed by centrifuging. The solution was then evaporated further on the Craig evaporator to a glassy material (fraction V).

The method of separation and distribution of nitrogen and phosphorus of the fractions of β-casein are as indicated



(1) A laboratory of the Eastern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

(2) Presented, in part, at the 126th Meeting of the American Chemical Society, New York City, September 1954.

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(4) O. Mellander, *Upsala Lakareforen Forh.*, **52**, 107 (1947).

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

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(14) E. F. Mellon, S. J. Viola and S. R. Hoover, *J. Am. Leather Chem. Assoc.*, **49**, 710 (1954).

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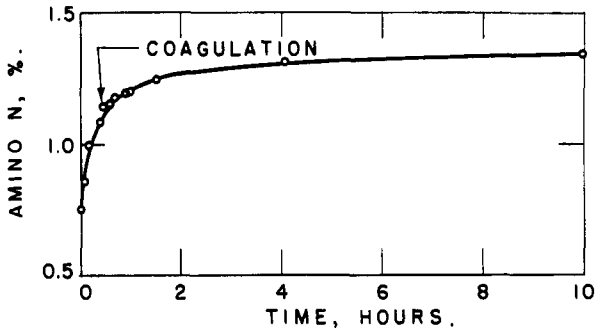

Fig. 1.—Increase in amino nitrogen with time, as determined by the Van Slyke method, produced by the action of 0.01% trypsin acting on a 3% β-casein solution at *p*H 7, 25°.

The electrophoretic composition of each of the five fractions thus obtained was determined by the Tiselius method in glycine–sodium hydroxide buffer of *p*H 9.0 and 0.1 ionic strength. The results are shown in Fig. 2 and the mobilities are given in Table I.

TABLE I

MOBILITIES OF HYDROLYTIC PRODUCTS OBTAINED FROM β-CASEIN AT *p*H 9.0 IN GLYCINE BUFFER 0.1 IONIC STRENGTH

Fraction	Major components u^a	Minor components u
I	−2.7	−6.6
II	−2.4	−3.0
		−5.1
		−9.4
III	−8.8	−9.3
IV	−2.6	−1.8
		−4.0
		−9.8
V	−4.3	−2.8
β-casein	−3.5	

[a] u = cm.^{2} volt^{-1} sec.$^{-1}$ × 10^{-5}—calculated from the descending patterns.

Fraction III, containing about 2.4% phosphorus, had a component with a mobility of −9.3 and a small amount of a second component moving slightly faster. The electrophoretic patterns in Fig. 2 also show that fractions II and IV contain small amounts of components with similar mobilities to fraction III. By reworking fraction II a further small yield of fraction III was obtained.

It was of interest to compare the phosphopeptone fraction (fraction III) obtained from α-casein with that of β-casein using the same short digestion time and method of preparation. It was found that the α-casein phosphopeptone fraction contained at least three electrophoretic components whereas the β-casein phosphopeptone contained only one major component. Unfractionated caseins gave a phosphopeptone fraction with four electrophoretic components, indicating that the phosphopeptones of α- and β-casein are different.

Purification of the Phosphopeptone (Fraction III).—The crude barium phosphopeptone, 6.7 g., containing about 2.4% phosphorus, was dissolved in water. A small amount of material was insoluble and was removed. Further purification was attained by adjusting the *p*H of the solution to 3.5 with hydrochloric acid and adding an equal volume of acetone to the resulting solution. The dried phosphopeptone amounted to 3.6 g. Electrophoretic analysis of this material dissolved in glycine buffer at *p*H 9.0 showed essentially one component with a diffuse falling boundary; it had the composition

Ash, %	14.3
Phosphorus, %	4.5 (ash-free basis)
Nitrogen, %	14.2 (ash-free basis)
N/P ratio (atomic)	7.0

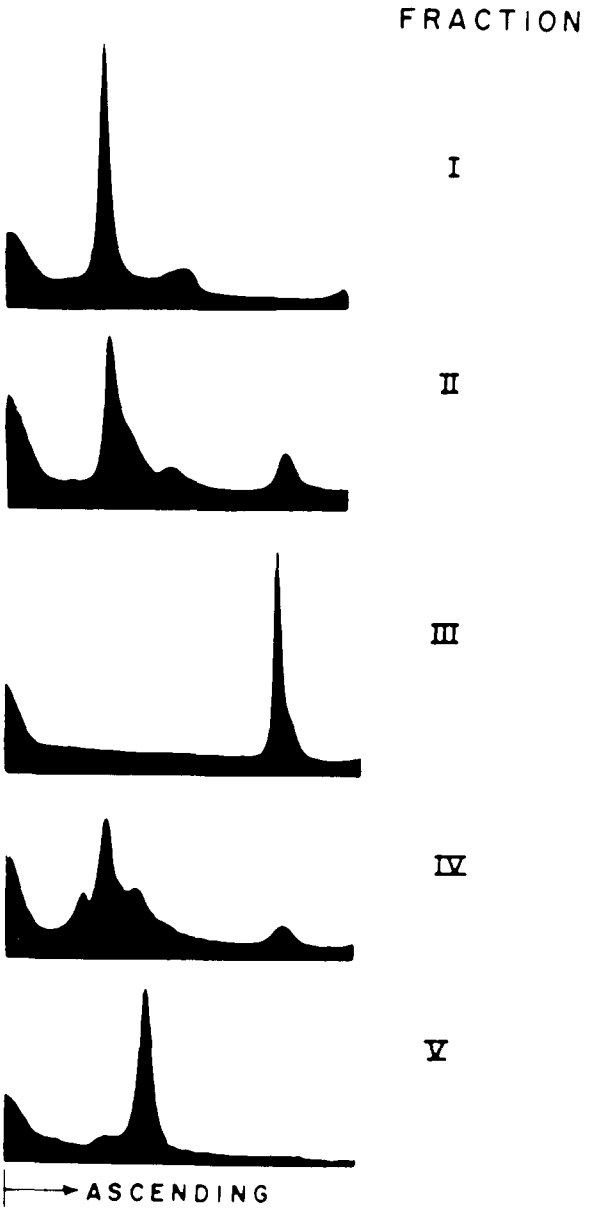

Fig. 2.—Ascending electrophoretic patterns of fractions obtained by the action of trypsin on β-casein: (I) insoluble at *p*H 7.0; (II) insoluble at *p*H 4.7; (III) barium phosphopeptone insoluble in 50% alcohol; (IV) insoluble on dialysis at *p*H 4.7; (V) soluble material after dialysis. The patterns were made by the Tiselius method using a glycine–sodium hydroxide buffer at *p*H 9.0 and 0.1 ionic strength with a field strength of 5 volts per cm., after 10,600 seconds.

The barium phosphopeptone was further purified by either (1) column chromatography on ion-exchange resin, or (2) by chemical fractionation at *p*H 2.0.

(1) **Column Chromatography.**—Barium was removed from the phosphopeptone by passing an aqueous solution through a Dowex 50 × 8 resin in the acid state. Electrophoretic analysis by means of the Perkin–Elmer apparatus using the 2-ml. micro cell revealed one main component with a small amount of faster component. The phosphopeptone was found to be firmly held on a Dowex 50 × 2 column but it could be eluted by a 0.1 ionic strength ammonium formate buffer at *p*H 3.4. In order to secure good resolution 100-cm. columns of the resin were eluted with a buffer with an increasing *p*H obtained by continuous mixing

of the *p*H 2.9 buffer with *p*H 4.5 buffer.[16] By this means the phosphopeptone was separated into one main and two minor components. The ammonium formate buffer was removed by sublimation[17] and the ammonium phosphopeptone was freed of ammonia by passing through a Dowex 50 × 8 column containing the acid form of the resin. The solution was evaporated at 25° to dryness. A yield of 0.53 g. of electrophoretically homogeneous material (Fig. 3b) was obtained from 1.0 g. of the purified barium phosphopeptone. The phosphopeptone was found to have the following composition

Ash, %	1.40
Phosphorus, %	4.5 (ash-free basis)
Nitrogen, %	13.9 (ash-free basis)
N/P ratio (atomic)	6.9

(2) **Chemical Fractionation at *p*H 2.0.**—The previously described barium phosphopeptone precipitated at *p*H 3.5 was dissolved in water to make a 2.5% solution and made to *p*H 2.0 with hydrochloric acid. The precipitate obtained was removed by centrifugation and the remaining phosphopeptone was precipitated from the supernatant at *p*H 3.5 by the addition of an equal volume of acetone. The phosphopeptone prepared from the supernatant was electrophoretically homogeneous (Fig. 3a) at *p*H 7.0 and contained

Ash, %	15.6
Phosphorus, %	4.0 (ash-free basis)
Nitrogen, %	14.3 (ash-free basis)
N/P ratio (atomic)	7.95

The amino acid composition of the purified phosphopeptones prepared by these two methods was determined by the procedure of Stein and Moore.[18] The sample, 10.00 mg., was hydrolyzed in a sealed evacuated glass tube with 100 times its weight of 5.7 *N* redistilled hydrochloric acid at 120° for 20 to 40 hours. An aliquot of 3.20 mg. was applied to the 100-cm. columns. The amino acid composition of the phosphopeptones is shown in Table II. The orginal data used in Table II, expressed as micromoles of amino acid in the sample, were converted to molar ratios by taking aspartic acid as 1.00. These ratios are recorded in Table III. All of the amino acids, with the exception of arginine and alanine, are close to whole numbers. The large amount of glutamic acid interfered with the determination of proline since the

TABLE II

AMINO ACID COMPOSITION OF PURIFIED PHOSPHOPEPTONES

	Amino acid residue (g./100 g. of dry phosphopeptone, ash free)					
	Free acid from column chromatography				From chemical fracn. Ba salt purif. at *p*H 2	β-Casein for comparison[a]
	Prep. 1		Prep. 2			
Amino acid	Hydrol. 20 hr.	Hydrol. 20 hr.	Hydrol. 41 hr.	Hydrol. 40 hr.	Hydrol. 40 hr.	
Asp	3.36	3.74	3.65	3.23	3.95	4.23
Glu	26.00	...	27.36	23.68	27.55	20.35
Gly	1.85	...	1.93	1.62	1.89	1.18
Ala	0.48	...	0.48	0.64	0.50	1.58
Val	5.62	6.06	6.59	5.66	6.69	8.63
Leu	10.65	11.38	11.24	9.39	10.83	10.01
Ileu	6.35	6.91	7.21	6.20	7.16	4.74
Ser[b]	11.30	11.45	11.30	11.11	14.66	5.63
Thr[b]	3.04	3.27	3.04	3.03	3.58	4.33
Pro	2.34	3.21	4.16	4.44	2.53	12.74
Arg	7.18	8.09	8.15	...	7.82	3.05
Amide–NH_3	1.64	...	1.64	...	1.30	1.60
PO_3H[c]	11.62	11.62	11.62	...	10.22	1.57
Total	91.43	...	98.40	...	98.68	...

[a] Only those amino acids are given which are in phosphospeptone.[19] [b] Extrapolated to zero time of hydrolysis. The extrapolation was based on the 1st and 3rd column figures for prep. 1. At 20 hours, 16.8% serine and 8.6% threonine were destroyed; at 41 hours, 38.0 and 18.8%, respectively. [c] P content calculated as the monoester.

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(17) C. H. W. Hirs, S. Moore and W. H. Stein, *ibid.*, **195**, 669 (1952).

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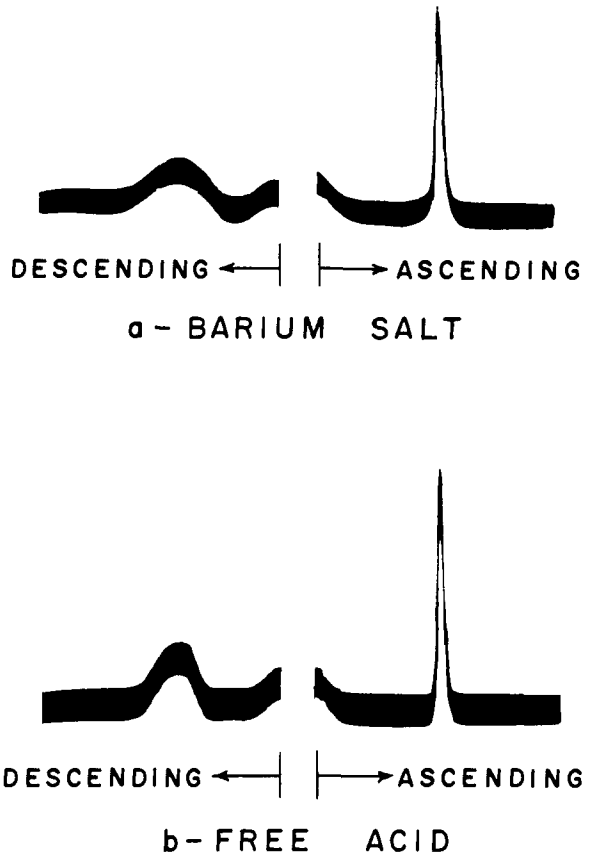

Fig. 3.—Electrophoretic patterns of the purified phosphopeptone in 0.2 ionic strength 0.02 phosphate, 0.18 NaCl buffer *p*H 7.0 after 3,840 seconds at 10 volts per centimeter.

TABLE III

AMINO ACID COMPOSITION OF PHOSPHOPEPTONES EXPRESSED AS MOLAR RATIOS

	Molar ratios of amino acids in phosphopeptone (asp. = 1.00)			
	Free acid from column chromatography		From chemical fractionation Ba salt purif. at *p*H 2	β-Casein for comparison[a]
Amino acid	Av. for prep. 1	Prep. 2 Hydrol. 40 hr.	Hydrol. 40 hr.	
Asp	1.00	1.00	1.00	9
Glu	6.79	6.50	6.21	40
Gly	1.09	1.01	0.96	5
Ala	0.25	0.32	0.26	3
Val	1.96	2.02	1.97	22
Leu	3.13	2.95	2.74	22
Ileu	1.92	1.94	1.85	11
Ser[b]	4.18	4.55	4.55	16
Thr[b]	0.99	1.06	1.04	11
Pro	1.01[c]	1.62	0.75	35
Arg	1.60	..	1.65	5
Amide–NH_3	3.0	..	3.0	29
PO_3H	4.67	..	3.76	5

[a] Residues per 25,000 mol. wt. Only amino acids given which are in phosphopeptone, to nearest whole number.[19] [b] Extrapolated to zero time of hydrolysis. [c] This figure obtained by Chinard's reagent and is considered to be more accurate.

peaks were not resolved. Consequently, Chinard's reagent, which is specific for proline, was used in analyzing that region of the chromatograph for the second 20-hour hydrolysate.[20]

The presence of the alanine as a persistent constituent in all the preparations could indicate that the molecular weight of the phosphopeptone was 4 or 5 times that calculated from aspartic acid = 1.00. However, ultracentrifuge data indicate that the lowest molecular weight is the correct value; therefore, alanine is probably an impurity.

Molecular Weight of Phosphopeptone.—The molecular weight of the phosphopeptone, freed from barium, was determined in the Spinco model E ultracentrifuge, using the artificial boundary cell.[21] In 0.2 ionic strength buffer, 0.02 phosphate, 0.18 NaCl, *p*H 7.0 at 25° the substance moved as a single peak with a sedimentation constant S_{20} of 0.69×10^{-13}. A calculation of the diffusion constant from this run gave a value of 13.5×10^{-7}. The amino acid analysis was used to calculate[22] a value of 0.697 for the partial specific volume. Using these values, a molecular weight of 4,100 was calculated for a 1% solution. A consistent value of 4,400 was calculated for its minimum molecular weight from the amino nitrogen content of 0.32% as determined. A minimum molecular weight of 3,111 can be inferred from the amino acid determinations for aspartic acid, glycine or proline.

The electrophoretic mobilities of the two phosphopeptone preparations of slightly different phosphorus contents were determined in the Perkin–Elmer apparatus. The values obtained at *p*H 4.5 and 7.0 are shown in Table IV. The mobilities of preparation 2 were somewhat lower both at *p*H 4.5 and 7.0 than those of preparation 1, which is consistent with its lower phosphorus content. The value of −0.2 for the mobility at *p*H 2.0 indicates that the phosphopeptone is close to the *p*H value of zero net charge.

TABLE IV

MOBILITIES OF PURIFIED PHOSPHOPEPTONES

The free acid at *p*H 2.0 had a mobility of −0.21 in oxalate–NaCl buffer. The *p*H 4.5 buffer was acetate 0.02, NaCl 0.18; *p*H 7.0 buffer was phosphate 0.02, NaCl 0.18.

	Preparation	P, %[a]	Mobility × 10^5[b] *p*H 4.5	*p*H 7.0
(1)	Barium salt ppt., *p*H 3.5	4.5	−6.4	−11.3
	Free acid (from chromatography of 1)	4.5	−6.7	−11.05
(2)	Barium salt ppt., *p*H 2	4.0	−5.1	−10.02

[a] *P* calculated on ash-free basis. [b] All mobilities calculated from ascending boundaries in 0.2 ionic strength buffers. *u* is in cm.^{2} volt^{-1} × 10^{-5}.

The N-Terminal Amino Acid of the Phosphopeptone.—The phosphopeptone was treated with fluorodinitrobenzene (FDNB) in alcohol-sodium bicarbonate solution by the procedure described by Fraenkel-Conrat.[23] After removing the excess FDNB with ether, the DNP-phosphopeptone was hydrolyzed for 16 hours at 105° with a 100-fold excess of 5.7 *N* hydrochloric acid. The solution was diluted to 1 *N* HCl concentration and again extracted with ether. No color or derivative went into the ether. A paper chromatogram of the aqueous solution, developed with 4-1-5 butanol–acetic acid–water showed only one spot and it ran at the same rate as DNP-arginine; consequently, arginine is the only N-terminal amino acid present.

Further information on the linkage broken in β-casein to produce the phosphopeptide was obtained by the action of trypsin on β-casein previously treated with FDNB. As shown by Anfinsen[24] only arginine linkages can be broken by this means in a DNP protein derivative. A yellow barium phosphopeptone, insoluble in 50% ethanol, was obtained by the separation procedure. This contained 2.2% phosphorus which compares with 2.4% for fraction III without purification and was close in mobility at *p*H 8.6 by paper electrophoresis to the phosphopeptones prepared without DNP. This evidence places the second arginine shown by the amino acid analysis at the carboxyl end of the phosphopeptone. Therefore, both chain ends are arginine. The N-terminal arginine could be one of the end groups detected by Mellon, *et al.*,[25] in β-casein.

Discussion

The phosphopeptone produced by the action of crystalline trypsin on β-casein for 20 minutes contains 24 amino acid residues of 10 different amino acids, as shown in Table III. This phosphopeptone differs markedly from those previously described,[3-6] which were produced by the action of crude trypsin or pancreatic extracts acting on casein from 2 to 40 days and which contained only from 2 to 10 amino acid residues of 2 to 4 different amino acids. This new phosphopeptone is similar to the previously described phosphopeptones in that it contains large amounts of glutamic acid, serine, leucine and isoleucine. The use of crystalline trypsin, which is considered to break only the peptide bonds involving the carboxyl groups contributed by lysine or arginine, results in a much larger and probably a more homogeneous phosphopeptone than could be obtained by the use of crude pancreatic extracts.

The minimum molecular weight of 3,111 as obtained from the sum of the amino acid residues in Table III is about one-eighth of the molecular weight of β-casein which has been found to be 24,100,[15] and the phosphopeptone may contain the same number of phosphorus atoms as β-casein. A comparison of the amino acid composition of the phosphopeptone with that of β-casein (Table II) shows that the phosphopeptone contains: (1) a greater amount of serine, glutamic acid, arginine and isoleucine; (2) about the same quantity of glycine, threonine, leucine and aspartic acid; (3) less alanine, valine and proline; and (4) no methionine, phenylalanine, lysine, histidine, tyrosine and tryptophan. The absence of tyrosine and tryptophan in the phosphopeptone were further confirmed by the measurement of its ultraviolet absorption spectrum.

The phosphopeptone prepared from β-casein differs from previous phosphopeptones in containing arginine. The evidence indicates that the two arginine groups are terminal. That arginine in the phosphopeptone is N-terminal is based on the isolation of DNP-arginine from hydrolyzed DNP-phosphopeptone. The isolation of apparently the same DNP-phosphopeptone by the action of trypsin on DNP-β-casein also indicates that arginine is at the carboxyl end of the phosphopeptide since trypsin hydrolyzes only peptide linkages in DNP proteins where the carboxyl is derived from arginine.[24]

The amino acid content of three phosphopeptone preparations as recorded in Table II are in agreement with each other within the experimental variations. The preparations purified finally by column chromatography contained 4.5% phos-

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(22) T. L. McMeekin, M. L. Groves and N. J. Hipp, THIS JOURNAL, **71**, 3298 (1949).

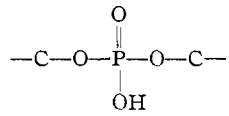
(23) H. Fraenkel-Conrat, J. I. Harris and A. L. Levy, "Methods of Biochemical Analysis," Vol. 2, Interscience Publishers, New York, N. Y., 1955, p. 360.

(24) R. R. Redfield and C. B. Anfinsen, *J. Biol. Chem.*, **221**, 385 (1956).

(25) E. F. Mellon, A. H. Korn and S. R. Hoover, THIS JOURNAL, **75**, 1675 (1953).

phorus and 13.9% nitrogen, while the preparation which was purified entirely by chemical fractionation contained 4.0% phosphorus and 14.3% nitrogen. These differences in composition appear to be real since the mobilities given in Table IV are greater for the preparation containing the larger amount of phosphorus. Since the preparations were equally homogeneous by electrophoresis it is concluded that they vary slightly in composition. It may be that these peptides vary only in their phosphorus content. A phosphopeptone of the amino acid composition $Asp_1Glu_7Gly_1Val_2Leu_3$-$Ileu_2Ser_4Thr_1Pro_1Arg_2(NH_2)_3(PO_3H)_5$ (as calculated from the results in Table III on the preparations prepared by chromatography using the closest whole number and omitting alanine) would have a phosphorus content of 5.0% and a nitrogen content of 14.9%. This is considered to be in reasonable agreement with the values found of 4.5% phosphorus and 13.9% nitrogen, particularly in view of the possible uncertainties in correcting for the ash content.

While there is no direct evidence for the position of phosphorus or its type of linkage in the phosphopeptone, the composition of the phosphopeptide as given in Table III does not appear to support the conclusion of Perlmann[26] that the phosphorus of β-casein is in the form of a diester. The diester form of phosphorus as postulated by Perlmann involves two hydroxy amino acids for each phosphorus as



The data in Table III show that there are four moles of serine and one mole of threonine and five moles of phosphoric acid in the phosphopeptone; consequently, there are not enough hydroxy-amino acids present in the phosphopeptone for the formation of diester bonds with phosphoric acid. It is possible that trypsin breaks diester bonds in the formation of the phosphopeptone; however, the evidence indicates that trypsin does not break phosphoric acid diesters. Sinsheimer and Koerner,[27] who purified snake venom diesterase, found that it hydrolyzed bis-(*p*-nitrophenyl)-phosphoric acid. The trypsin used in this work was tested for possible action on secondary phosphate bonds by incubating the calcium salt of bis-(*p*-nitrophenyl)-phosphoric acid with a 0.1% solution of Worthington trypsin for 3 hours at 37°. No hydrolysis of the diester occurred. Under similar conditions, the diesterase from calf intestinal mucosa[28] hydrolyzed this diester.

Acknowledgments.—We are indebted to Dr. Clyde Ogg and Mrs. Ruth Kelly who made the phosphorus, ash and nitrogen determinations. Mrs. Betty Harrington made the Van Slyke amino nitrogen determinations.

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PHILADELPHIA 18, PA.

[CONTRIBUTION FROM THE DEPARTMENT OF BIOLOGICAL CHEMISTRY, UNIVERSITY OF MICHIGAN]

Three Schiff Base Types Formed by Amino Acids, Peptides and Proteins with Pyridoxal and Pyridoxal-5-phosphate[1]

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Amines react with pyridoxal-5-phosphate to form yellow Schiff bases which appear to be hydrogen-chelated, as shown earlier for pyridoxal. An accompanying product with maximal absorption at 278 to 285 mμ probably has the same structure lacking the double bond in conjugation with the ring, possibly because of hydration to the carbinolamine. In addition the yellow Schiff base passes gradually over to a variable extent to a third type of Schiff base in which the H-bonding is believed to be lost so that the 3-hydroxypyridine structure is in the zwitterionic form. In this product the bathochromic effect of the aldimine double bond is small or absent, perhaps also because of conversion to the carbinolamine. Upon protonation spectrophotometric evidence for reversion to the hydrogen-bonded form is obtained. The conversion to the non-hydrogen bonded form is attributed to the electron-attracting action of vicinal groups, especially the carbonyl group of amino acid esters and peptides. Proteins show analogous reactions, bovine serum albumin binding pyridoxal phosphate very tightly mostly as the non-hydrogen-bonded Schiff base.

The yellow Schiff base formed when pyridoxal (pl) reacts with amino acids has its H^+-dissociations oddly displaced from their positions in pyridoxal.[2] A pK' of 10.5 is ascribed by Metzler to the *phenolic* group (4.2 in pyridoxal), H-bonding to the imine-N (formula IIC, Fig. 1) serving to explain both the firmness with which this H^+ is held and the presence of an absorption band at about 415 mμ.[2] An additional consequence is the dissociation of the *pyridinium*-H^+ at about *p*H 5.9 rather than at 8.6 as in pyridoxal. A similar situation now has been found to apply to the Schiff base of pyridoxal-5-phosphate with valine. With pK' values of 5.9 and 10.5 for the pyridine N and the phenolic group of pl in that order very little of the Schiff base would ordinarily be expected to exist as the zwitterion.

A second much less abundant product of the reaction which absorbs maximally at 330 mμ was also noted by Metzler.[2] He proposed an aminoacetal structure (the hemiacetal corresponding to the carbinolamine form of the Schiff base, structure VI Fig. 1), taking into account the reactivity

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(2) D. E. Metzler, THIS JOURNAL, **79**, 485 (1957).