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Utilization of Phosphorus for Casein Biosynthesis in the Mammary Gland*

II. INCORPORATION OF P^{32} INTO FREE PHOSPHOPEPTIDES OF MILK AND OF MAMMARY GLAND

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Recent studies on casein biosynthesis have conclusively shown that the mammary gland utilizes the inorganic phosphorus of the blood for the formation of casein phosphorus and that the phosphorus incorporation occurs at a rapid rate (1–4). Very little is known, however, concerning the steps involved in this conversion. *o*-Phosphoserine as the basic phosphorus-containing unit of casein (4, 5) would appear to be the obvious choice as an intermediate in this reaction. The formation of *o*-phosphoserine by transamination of phosphohydroxypyruvate was first suggested by Le Bars *et al.* (6) and has subsequently received experimental support from recent studies on the biosynthesis of serine from carbohydrate in the liver (7).

In a study of the occurrence of P-serine[1] in mammary tissue, carried out in this laboratory, it was observed that the lactating mammary gland of the rat did not contain a detectable amount of this amino acid. These studies, on the other hand, brought to light the presence of traces of phosphorylated peptides in this tissue (8). The conditions of the experiments for the isolation of the mammary gland peptides (homogenization with trichloroacetic acid (8)) were such as to rule out the possibility of these arising as a result of the proteolytic action of the mammary gland. A possible role for these phosphopeptides as intermediates in the biosynthesis of casein is indicated from the observed similarity of their amino acid composition with that of phosphopeptones obtained by enzymatic degradation of casein (9) as also from the work of Thoai and Pin (10) who demonstrated the ability of goat udder homogenates to synthesize a phosphopeptide from a suitable complement of amino acids in the presence of adenosine triphosphate as energy donor. To throw more light on this aspect it was considered of interest to isolate the peptides, in a homogeneous state, from the mammary gland and milk of lactating rabbits to which P^{32}-labeled phosphate had been administered and to compare the extent of incorporation of P^{32} into these peptides with that observed in the phosphoprotein fraction of these sources. Evidence was also sought for the presence of free P-serine in the mammary tissue since the results of earlier nonisotopic experiments were not unambiguous enough to rule out the presence of traces of this amino acid which could have escaped detection by the chromatographic methods employed. The present paper describes the results of these experiments and discusses their possible significance in relation to the mechanism of phosphorus incorporation into casein.

EXPERIMENTAL

The materials employed in these investigations were as described in the previous paper (4).

Isolation of Phosphate Esters from Mammary Gland and Milk—P^{32}-labeled phosphate was administered intraperitoneally to the lactating rabbit (1 μc per g body weight), and the animal was killed at the end of 3 hours. The subsequent operations were carried out at 0–4°. The mammary glands were washed with 0.9% sodium chloride solution to remove adhering milk, and a water homogenate of the tissue obtained essentially according to the procedure described earlier (4). The homogenate was made alkaline (pH 9.7) with ammonia (0.1 N final concentration with respect to alkali) and after keeping overnight at 2° it was centrifuged in the cold. The supernatant solution was clarified by filtration through cotton-wool. In preliminary experiments, the extract was deproteinized by precipitating the bulk of the protein at pH 4.8 and removing the rest of it by the addition of 4 volumes of acetone. This procedure, however, resulted in poor recoveries of the phosphopeptides. In subsequent experiments the proteins were precipitated by adding trichloroacetic acid to the extract to 10% final concentration. The precipitated protein was centrifuged off and was washed once with 10% trichloroacetic acid. The washings were combined with the original supernatant, and trichloroacetic acid was removed from the solution by shaking with several portions of ether until the pH of the solution was about 4.0. The aqueous layer was neutralized with ammonia and the phosphorus-containing compounds in the solution were precipitated by adding sufficient (approximately 5 ml) of a 25% solution of $Ba(CH_3COO)_2$ followed by the addition of 4 volumes of acetone. The solution was stored in the cold overnight to complete the precipitation of the barium salts.

The precipitated barium salts were separated by centrifugation and were suspended in water. Barium was removed by adding the minimum amount of Dowex 50 (H^+ form, 200 to 400 mesh, 8X cross-linkage). The resin was washed once with 2 N

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[1] The abbreviation used is: P-serine, *o*-phosphoserine.

ammonia. This treatment did not displace barium from the resin. The washings and the original supernatant were combined and then made strongly alkaline with ammonia. Magnesia mixture (2 ml) was added to precipitate P_i. After storage in the cold overnight, the precipitated $MgNH_4PO_4$ was removed by filtration. The filtrate was neutralized with acetic acid and treated with 10 ml of a 25% solution of barium acetate. The suspension was kept at 0° for 6 hours and the precipitate, representing the water-insoluble fraction, was separated by centrifugation and washed once with water. The first supernatant solution and the wash water were combined and treated with 5 volumes of ethanol. The precipitate (the water-soluble fraction) was separated and was washed twice with 50% (volume for volume) ethanol. The two phosphorus-containing fractions were taken up separately in water, and after removal of barium with Dowex 50 (see above) the solutions were concentrated to dryness under reduced pressure over H_2SO_4.

The preparation of phosphorus-compounds from the trichloroacetic acid-soluble fraction of milk was carried out by a similar procedure.

Paper Electrophoretic Analysis—This was carried out in a horizontal open type electrophoresis apparatus (Arthur Thomas Company). Paper strips for electrophoresis (4 × 30 cm) were cut from acid-washed Whatman No. 1 sheets prepared according to Hanes and Isherwood (11). The runs were conducted at room temperature for 2 to 4 hours at 250 volts.

Location of Phosphopeptides—After completion of the electrophoretic run, the strips were dried at 100° for 10 minutes. They were cut lengthwise to obtain strips, 1 cm in width, to serve as guide strips. Peptides were revealed on the guide strips by spraying with the acidic ninhydrin reagent of Levy and Chung (12). Radioactivities were located on the same strips by a procedure described in the previous paper (4).

Determination of Specific Activity—The preparation of casein and of the mammary gland protein and analyses of these for phosphoprotein phosphorus were carried out as described in the previous paper (4).

For determining the specific activities of the phosphopeptides, these were hydrolyzed with constant boiling HCl (3 ml) in a sealed tube at 110° for 48 hours. Specific activity of the P_i was determined on an aliquot of the hydrolysate by the isobutanol method (4). All counting rates were corrected for background and decay.

Amino Acid Composition of Peptides—Hydrolyses of the peptides were effected as described above. Acid was removed by repeated evaporation of the hydrolysate over NaOH pellets under reduced pressure. The amino acids of the hydrolysate were separated by subjecting an aliquot of it to two-dimensional descending chromatography on Whatman No. 1 paper (46 × 57 cm). The solvent systems employed were butanol-acetic acid-water (4:1:5) for the first run and phenol-water (80:20) for the second. Estimation of amino acids on the chromatogram was carried out by the procedure of Giri *et al.* (13).

TABLE I

Distribution of radioactivity in ninhydrin-positive compounds of water-soluble phosphorus fraction from milk and mammary gland

Electrophoresis of the water-soluble phosphorus fraction was carried out in phthalate buffer (0.025 M, pH 5.6) at 250 volts for 3 hours. Movement of the peptides was towards the anode.

Source	Band No.	Distance from origin	Ninhydrin reaction*	Activity in band
		cm		*c.p.m.*
Mammary gland	1	−0.5–+1.0	+	105
	2	11.0–14.0	++++	6820
	3	14.0–14.5	+	800
	4	15.5–16.0	+	400
Milk	1	3.5–4.2	+	0
	2	4.2–5.2	+	20
	3	5.2–7.4	+	500
	4	7.4–10.4	++++	3235

* +, Faint color with ninhydrin; ++++, intense color with ninhydrin.

RESULTS

Isolation of Phosphopeptides—In earlier experiments the phosphopeptides were separated by paper chromatography employing butanol-acetic acid-water (4:1:5) solvent system (8). Since the peptides had low R_F values it was necessary to employ a prolonged run for a satisfactory separation of the peptides. Paper electrophoresis was found to be much more convenient for effecting a good resolution of the peptides. The use of Veronal buffer at pH 8.6 gave very good separation of the phosphopeptides from the nonphosphorus peptides as well as from other phosphorus compounds. Phthalate buffer at pH 5.6 was somewhat less satisfactory whereas tris(hydroxymethyl)aminomethane at pH 8.6 and borate buffer at pH 8.6 gave very poor resolutions. The best results were obtained by subjecting the phosphorus compounds to electrophoresis at pH 5.6 (phthalate buffer) to effect a partial separation of the peptides from other compounds and submitting the partially purified peptide to reelectrophoresis at pH 8.6 (Veronal buffer).

Electrophoresis at pH 5.6—The phosphorus esters, prepared as described earlier, were taken up in the minimum amount of water and subjected to electrophoresis employing 0.025 M phthalate buffer of pH 5.6. In the case of the water-insoluble fraction, two ninhydrin-positive bands were obtained, one of them at the origin, and the other with a low mobility, moving towards the anode. No activity was present in either of these bands and consequently this fraction was discarded. With the soluble fraction, a somewhat diffuse band giving an intense color with ninhydrin and containing the greater part of the activity of the fraction was obtained (Table I). In addition, several other ninhydrin-positive bands were present. These, however, did not contain appreciable activity. The active band was eluted from the strip by two successive extractions with 50% ethanol. The extracts were pooled, concentrated to a small volume by vacuum distillation, and evaporated to dryness under reduced pressure over H_2SO_4.

Re-electrophoresis at pH 8.6—The residue from the previous step was taken up in the minimum amount of water and was subjected to electrophoresis in 0.05 M Veronal buffer at pH 8.6. The phosphopeptide was well separated as a sharp band, which contained nearly the whole of the activity spotted on the paper (Figs. 1 and 2). In the case of mammary gland, a second band with a slightly lesser mobility and contiguous to the sharp band was also obtained. Two other ninhydrin-positive bands were also present. These had mobilities of 0.24 and 0.43 × 10^{-5} cm^2 per volt per sec, respectively, as against the value of about 2 × 10^{-5} cm^2 per volt per sec obtained for the active bands. These bands had no activity and possibly represented nonphosphoryl-

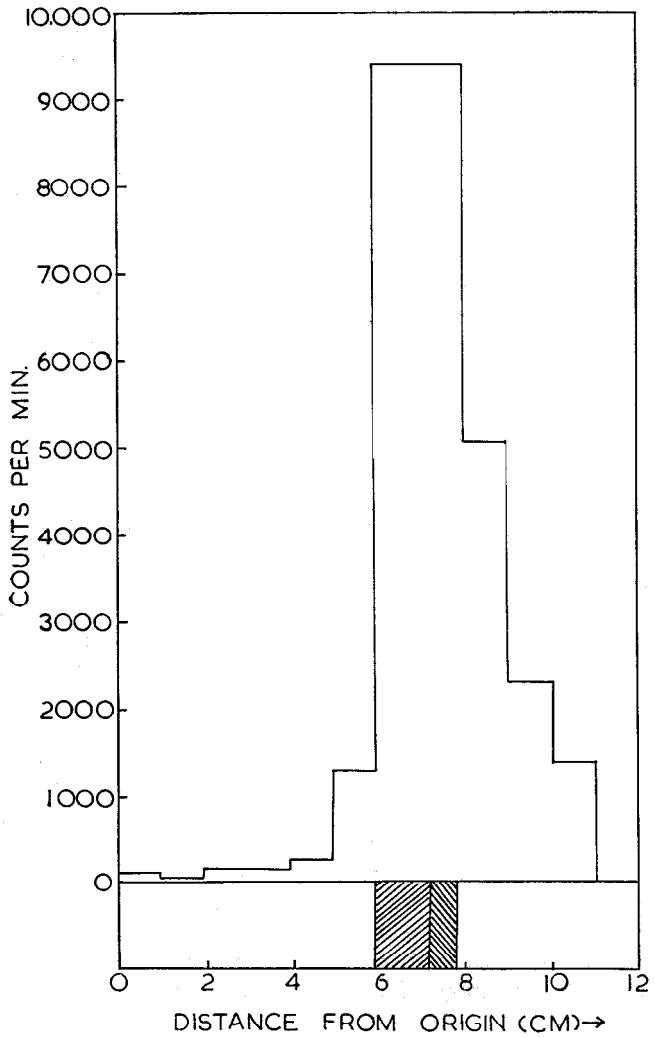

FIG. 1. Re-electrophoresis of mammary gland phosphopeptide. The partially purified peptide prepared by electrophoresis at pH 5.6 (see Table I) was subjected to re-electrophoresis in 0.05 M Veronal buffer at pH 8.6 at 250 volts for 2 hours. The shaded areas in the lower part of the figure represent the ninhydrin-positive bands. The upper part of the figure represents distribution of radioactivity in the strip. Movement of the peptides was towards the anode.

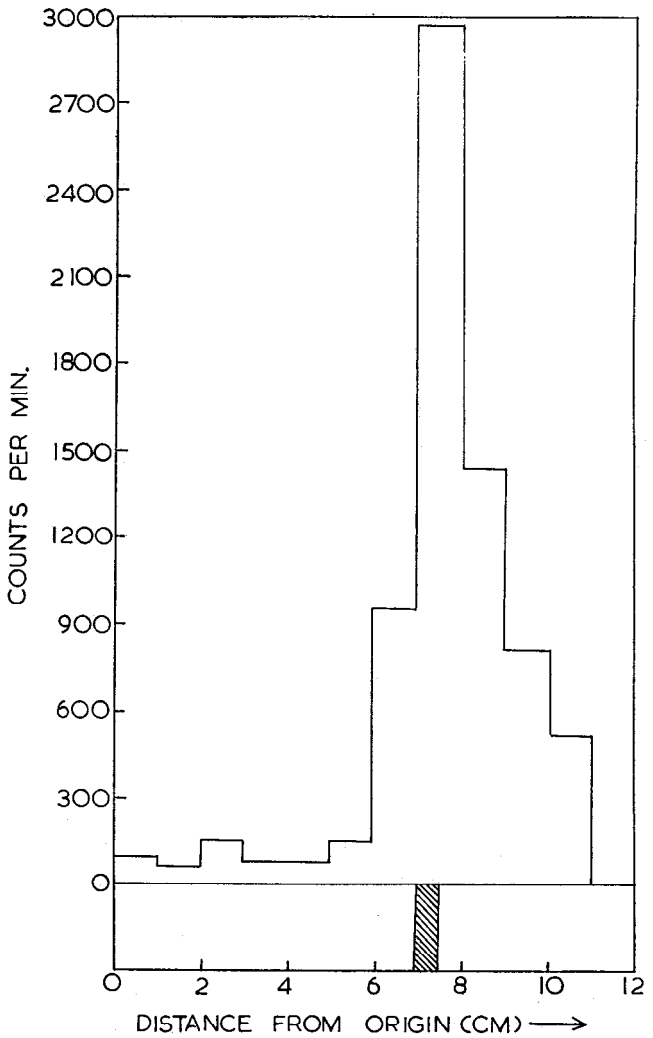

FIG. 2. Re-electrophoresis of milk phosphopeptide. The partially purified peptide prepared by electrophoresis at pH 5.6 (see Table I) was subjected to re-electrophoresis in 0.05 M Veronal buffer at pH 8.6 at 250 volts for 2 hours. The shaded area in the lower part of the figure represents the ninhydrin-positive band. The upper part of the figure represents the distribution of radioactivity in the strip. Movement of the peptide was towards the anode.

ated peptides similar to those found in the rat mammary gland in earlier studies (8). The active bands were eluted with 50% ethanol and concentrated to dryness as described earlier.

The peptides thus obtained appeared to be homogeneous since they gave a single band, reactive to ninhydrin and containing the whole of the radioactivity, on re-electrophoresis at pH 8.6. The peptides had somewhat lower mobilities as compared to P-serine at this pH (2×10^{-5} cm² per volt per sec as against 3.1×10^{-5} cm² per volt per sec for P-serine).

Specific Activity of Peptides—The specific activities of the phosphorus in the peptide preparations are shown in Table II. Also presented in the table are values for specific activities of phosphoproteins of milk and mammary gland of the same animal. It is evident that the specific activity of the phosphopeptide is in each case comparable to that of the phosphoprotein obtained from the same source.

Amino Acids of Peptides—The amino acid compositions of the phosphopeptides were determined by a semiquantitative paper

TABLE II

Specific activities of phosphorus in phosphopeptides and phosphoprotein fractions of mammary gland and milk

Peptide	Total phosphorus	Total activity in peptide	Specific activity	
			Peptide	Protein
	μg	c.p.m. × 10^5	c.p.m./mg phosphorus × 10^5	
Mammary gland*				
Peptide I	139	1.35	9.7	9.9†
Peptide II	126	1.31	10.4	
Milk peptide	49	0.25	5.0	4.3‡

* The numbering of mammary gland peptides is as given in Fig. 1.

† Value represents specific activity of phosphoprotein phosphorus in the acid-insoluble fraction of mammary gland.

‡ Value represents specific activity of casein phosphorus.

chromatographic method. The compositions of the peptides, as obtained in an experiment, were as follows:[2]

Mammary gland peptide I:[3] $Asp_4, Glu_{13}, Ser_5, Gly_{20}, Ala_9, Thr_6, Val_7, Leu_4$.
Mammary gland peptide II:[3] $Asp_4, Glu_{14}, Ser_4, Gly_{18}, Ala_6, Val_2, Leu_3$.
Milk peptide: $Asp_3, Glu_{15}, Ser_4, Gly_6, Ala_1, Leu_1$.

The amino acid compositions of the peptides were generally similar to that reported by Pin (14) for the phosphopeptides of goat milk. In contrast to his findings, basic amino acids were absent in our preparations. An unknown compound, giving an intense color with ninhydrin and with an R_F of 0.35 in butanol-acetic acid-water system, and 0.70 in phenol-water system, was also detected in the hydrolysates of all the peptides. The compound has not been characterized.

Identification of Phosphorus-containing Group in Peptide—Suitable aliquots of the peptides were hydrolyzed with 2 N HCl at 75° for 24 hours. Acid was removed by repeated evaporation under reduced pressure over NaOH pellets. The residues were taken up in water and analyzed by ascending paper chromatography in butanol-acetic acid-water solvent system. About 50% of the activity applied to the paper was recovered in a spot which was ninhydrin-positive and had the same R_F as an authentic sample of P-serine.

Absence of Free P-serine in Mammary Tissue—The phosphorus fractions, prepared from mammary gland as already described (see "Experimental") were tested for the presence of P-serine by electrophoretic analysis in Veronal buffer of pH 8.6. A synthetic sample of P-serine was used as marker. No radioactivity could be found in the test samples corresponding to the reference band of P-serine. To eliminate the possibility that P-serine was present in too low an amount to enable its precipitation from the acid-soluble extract of mammary gland, carrier P-serine (5 mg) was added to the extracts before precipitation of the phosphorus-compounds as the barium salts. P-serine which was separated by electrophoresis at pH 8.6 was still found to be devoid of radioactivity.

DISCUSSION

Any hypothesis concerning the mode of incorporation of phosphorus into casein must take into account the evidence, based on enzymatic and acid degradation studies carried out on the protein (5, 9, 15), that the phosphorus in the protein is present as P-serine and is linked to possibly the hydroxyl group of the amino acid residue. The occurrence of *N*-phosphoserine in casein does not appear likely in view of the failure to detect serine at the *N*-terminus of casein after enzymatic and alkali (barium hydroxide) dephosphorylation of the protein (16). The formation of casein phosphorus may be considered as occurring according to one or both of the following reactions. One possibility is that P-serine is involved as an intermediate. Alternately one may consider phosphorylation of the serine hydroxyl group as taking place at the peptide level. These possibilities have been kept in mind in the discussion which follows.

Failure to detect a phosphokinase for serine in the lactating mammary gland (17) raised some doubt as to the involvement of free P-serine in casein biosynthesis. The problem, had, however, to be considered more intensively in view of the observations of several workers (6, 7, 18) to the effect that P-serine might have its origin in a compound other than serine. The experiments of Ichihara and Greenberg (7) have conclusively shown that P-serine is formed from glucose by way of phosphohydroxypyruvate and that it is an obligatory intermediate in the biosynthesis of serine from carbohydrate. It is to be expected that P-serine, irrespective of its origin, must be metabolically active and turnover phosphorus at a significant rate, were it to be an intermediate in casein biosynthesis. From isotope distribution studies no evidence could be obtained for the presence of labeled P-serine in the mammary gland. It is thus unlikely that P-serine, in the free state, would serve as a precursor for casein.

The involvement of P-serine in casein biosynthesis is also open to doubt in view of the recent findings of Larson and Gillespie (19) that casein, lactalbumin, and lactoglobulin are synthesized from a common amino acid pool. Although the absence of P-serine in the last two proteins is explicable by assuming the operation of a selective mechanism for the incorporation of this amino acid into casein to the exclusion of the other two proteins, a more rational explanation would be that the serine residues are incorporated into casein mainly in the nonesterified state and that the presence of a suitable amino acid configuration around some of the serine hydroxyl groups in casein activates these groups to accept phosphorus from a suitable phosphate donor, a possibility already suggested by Burnett and Kennedy (20). Such a hypothesis would imply that phosphorylation occurs at the peptide or protein level rather than at the amino acid level. It may be of interest in this context to quote the analogous case of collagen biosynthesis where it has been established that the hydroxylysine residues are not incorporated into the protein as such and that hydroxylation of the lysine residues occur at the peptide level (21).

In the course of a diligent search for the presence of phosphoamino acids in the mammary tissue, the presence of trace amounts of phosphorylated peptides was discovered. The second possibility in regard to the mode of incorporation of phosphorus into casein, mentioned earlier, had to be considered in detail in view of this finding and also in view of the experiments of Thoai and Pin (10) on the synthesis *in vitro* of such phosphopeptides by homogenates of sheep udder. An observation of significance in this context is the close similarity in the amino acid pattern of these tissue phosphopeptides with that of casein phosphopeptone prepared by enzymatic degradation of casein. This naturally raises the question of their role in casein biosynthesis in the mammary gland. Studies *in vivo* with labeled phosphorus threw much light on this problem in that they demonstrated a high rate of turnover of phosphorus in the phosphoprotein fraction of mammary gland and milk (4) and of comparable rates of renewal in the free phosphopeptides derived from these sources (Table II). The high metabolic activity associated with the phosphopeptides is strongly suggestive of their utilization in casein synthesis. Of relevance in this context are recent reports on the occurrence of carboxyl-activated peptides in a variety of tissues and the suggestion that these may be on the path to protein synthesis (22–24).

With respect to the mode of synthesis of these phosphopeptides by mammary gland, it is of interest that serine, although of itself is incapable of accepting phosphorus, could be isolated in

[2] The amino acid compositions have been given in molar ratios. The values given for serine include phosphoserine and have been corrected for destruction during hydrolysis. Value for threonine carries similar correction.

[3] The numbering of mammary gland peptides is as given in Fig. 1.

the phosphorylated form from these peptides. It is quite likely that a particular amino acid sequence around serine activates an otherwise inert hydroxyl group of serine to get phosphorylated. The reactivity of the hydroxyl group of peptide-linked serine as contrasted with the inert nature of the hydroxyl group of the free amino acid has also been observed by several workers. Thus chymotrypsin is readily phosphorylated at a serine hydroxyl group of the protein by diisopropylphosphorofluoridate (25), although the free amino acid is phosphorylated by this reagent only to a very small extent (26). A detailed study of the mechanism of action of phosphoglucomutase has revealed that the enzyme is readily phosphorylated by its coenzyme glucose 1,6-diphosphate and that the site of phosphorylation is again a serine hydroxyl group in the protein (27). Further, these enzymes have a common amino acid sequence in the region of the reactive serine hydroxyl group (28–30), and it is of added significance that these amino acids, *viz.* aspartic acid, glutamic acid, glycine, alanine, serine, threonine, valine, and leucine, are also found in the phosphopeptides isolated in the present investigation. Another point of similarity between casein and the phosphoenzymes lies in the rapidity with which phosphorus incorporation into these proteins occurs (4). These observations thus add up to suggest strongly the involvement of certain basic mechanism in the addition of phosphorus to the protein, whether it be the phosphoprotein of the classical type like the phosphoproteins of milk or the phosphoenzymes of high metabolic activity. Whercas in the latter class of proteins addition of phosphorus occurs mainly at the protein level, it is possible that in the case of casein phosphorylation occurs at the protein or the peptide level. The possibility of peptide bond formation preceding phosphorylation, in casein synthesis, receives additional support from the demonstration of the existence of nonphosphorylated peptides in the mammary gland, which have the same amino acid pattern as the phosphorylated peptides of the gland (8). In addition the mammary gland possesses an enzyme system capable of phosphorylating polypeptides derived from casein (31). A detailed study of this enzyme for the nature of its physiological substrate and studies *in vitro* on the biosynthesis of the phosphopeptides and the extent of their incorporation into newly synthesized casein may help to throw more light on the role of these peptides in the biosynthesis of casein.

SUMMARY

The occurrence of phosphopeptides in the mammary gland and milk of lactating rabbits is reported. A method for their separation, based on paper electrophoresis of the acid-soluble phosphorus fraction of these sources, is presented. Isotopic experiments indicated that these peptides were metabolically active and that the rates of renewal of phosphorus in these were comparable to those observed in the phosphoprotein fractions. Further, these peptides had amino acid patterns similar to that in casein phosphopeptone obtained by enzymatic degradation of casein. The results have been discussed with reference to the possible utilization of these peptides by the mammary gland for the biosynthesis of casein.

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