THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 235, No. 3, March 1960
*Printed in U.S.A.*

# Utilization of Phosphorus for Casein Biosynthesis in the Mammary Gland*

## I. INCORPORATION *IN VIVO* OF $P^{32}$ INTO PHOSPHOPROTEIN OF MILK AND OF MAMMARY GLAND

T. A. SUNDARARAJAN,† K. S. V. SAMPATH KUMAR,‡ AND P. S. SARMA§

*From the University Biochemical Laboratory, Madras 25, India*

(Received for publication, July 21, 1959)

It is now well established that the synthesis of the major protein constituents of milk takes place exclusively in the mammary gland, utilizing as building materials the free amino acids of the blood (1). The research of Aten and Hevesy (2), confirmed subsequently by other workers (3, 4), leaves little room for doubt that the precursor of the casein phosphorus is the inorganic phosphorus of the blood. The rapid rate at which phosphorus incorporation into casein is effected has been noticed by several workers. The specific activity of casein phosphorus reaches a maximum 3 hours after intravenous injection of labeled phosphorus to the lactating animal (5) and is generally much higher than the activities in hexose monophosphate and phospholipid (2). These experiments do not, however, exclude the possibility that the casein owes its high activity to phosphoprotein enzymes possessing high rates of phosphorus turnover, which may be present as contaminants in the preparation. In view of recent findings on the phosphoprotein nature of various enzymes involved in phosphate transfer (6–9) a detailed characterization of casein with reference to the nature of its phosphorus appears desirable. A study of this aspect and a characterization of the phosphoprotein of mammary gland with a view to establishing its relationship with casein have been undertaken in the present investigation. It was also considered of interest to compare the specific activity of casein phosphorus with that in liver phosphoprotein since the latter has been shown to exhibit phosphorus turnover at a remarkably high rate (10–12).

### EXPERIMENTAL

#### *Materials*

The experimental animals employed in these studies were lactating rabbits, about 20 to 25 days after parturition.

$P^{32}$-labeled orthophosphate ($Na_2HPO_4$) was purchased from Radio Chemical Centre, Amersham, England, as a solution in 0.9% sodium chloride at pH 7.0.

P-serine[1] was a generous gift from California Foundation for Biochemical Research.

Pepsin (twice crystallized) and acid phosphatase (wheat germ) were obtained from Worthington Biochemical Corporation. The phosphatase preparation has no action on phosphodiester substrates (13). Yeast pyrophosphatase (twice crystallized) was a generous gift of Dr. M. Kunitz. "Phosphoprotein phosphatase" was prepared from rat spleen (14). This enzyme was free from diesterase activity as tested with diphenyl phosphate as substrate (15). Snake venom diesterase was prepared according to Sinsheimer and Koerner (16). The preparation was carried through the second acetone cycle. The enzyme preparation, 0.1 ml, caused complete hydrolysis of Ca[bis(p-nitrophenyl) phosphate]$_2$ under the assay conditions of Sinsheimer and Koerner.

#### *Methods*

*Fractionation of Phosphorus Compounds of Tissues*—The lactating rabbit was given an intraperitoneal injection of radioactive phosphorus (1 $\mu$c per g body weight) and was kept separated from the litters. The animal was killed after a time interval of 2 to 3 hours by a blow on the head and the liver and mammary glands (along with adhering milk) were dissected out and placed in beakers containing ice-cold 0.9% sodium chloride solution (100 ml). All subsequent operations were carried out at 0–4° unless otherwise specified. The mammary glands were cut into large bits to facilitate removal of milk. After a gentle swirling, the milk was transferred to a conical flask. The mammary tissue was washed twice with small portions (20 ml) of sodium chloride solution and the washings added to the milk. The tissue was washed thrice more with sodium chloride solution and the washings were discarded. The tissue was blotted and kept at −25° for an hour. The frozen tissue was ground well in a mortar which had previously been kept in the deep freeze. This operation repeated thrice at intervals of 30 minutes resulted in an easier homogenization of the tissue.

Weighed amounts (5 g) of the liver and mammary tissues were homogenized in 5 volumes of a 10% solution of trichloroacetic acid in a Potter-Elvehjem type of homogenizer for 2 minutes. The phosphorus-containing fractions of the homogenate were separated according to the scheme of Schmidt and Thannhauser (17) as modified by Friedkin and Lehninger (18).

*Preparation of Protein Fractions Insoluble at pH 4.6: From*

* The material in this paper was taken in part from the Ph.D. thesis submitted by K. S. V. Sampath Kumar to the University of Madras, in January 1958.

† Postdoctoral National Research Fellow, Ministry of Education, Government of India. Present address, Biology Division, California Institute of Technology, Pasadena, California.

‡ Present address, Biochemistry Department, University of Toronto, Toronto, Canada.

§ Present address, Biochemistry Department, Indian Institute of Science, Bangalore 12, India.

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

*Milk*—Milk sample obtained by washing the mammary glands with sodium chloride solution was centrifuged in the cold for 10 minutes and the supernatant filtered through cotton-wool to remove fat. Casein was precipitated from the filtrate by adjusting the pH to 4.6 with dilute acid. The precipitate was separated by centrifugation and was washed with water. To remove any adsorbed labeled $P_i$, the precipitate was dissolved with the minimum amount of ammonia and was dialyzed in cellophane sacs against several volumes of phosphate buffer of pH 7.0 (0.1 M), followed by dialysis against several changes of distilled water. The dialyzed solution was clarified by filtration through a layer of Celite (Fisher Scientific Company) on a Buchner funnel. The casein was precipitated as before and after thorough washing with water it was dried by several washings with cold (−20°) ethanol followed by a final washing with ether.

*From Mammary Gland*—Mammary tissue, prepared as described earlier, was homogenized in 4 volumes of distilled water, with the use of a Waring Blendor. The homogenate was made alkaline (pH 9.7) with ammonia (0.1 N final concentration with respect to alkali), and was kept overnight at 2° for extraction of the proteins. The suspension was centrifuged in the cold and the supernatant filtered through cotton-wool. The filtrate which was opalescent was brought to pH 4.6. The bulk of the proteins was precipitated at this pH. The protein was separated, washed, and was freed from contaminating $P^{32}$ as described for casein. The dialyzed solution contained considerable amount of lipid material. This was removed by shaking up the solution with an equal volume of *n*-butanol and separating the aqueous layer by centrifugation. The protein was precipitated from the aqueous layer by adjusting the pH to 4.6 and the precipitate washed and dried as previously described for casein.

*Analytical Methods*—Total nitrogen was determined by the method of Koch and McMeekin (19) and total phosphorus according to Fiske and SubbaRow (20). $P_i$ was determined by an isobutanol extraction method (21). In the case of solutions containing protein breakdown products, silicotungstate was also added, before molybdate, to eliminate interference in color development (22).

*Radioactivity Measurements*—Before measurement of activity the phosphorus in the samples was converted to $P_i$ by digestion with $H_2SO_4$ as in the case of acid-soluble and lipid phosphorus, or by incubation with 1 N KOH at 37° for 20 hours as with phosphoprotein phosphorus. Specific activities were calculated by determining the concentration and radioactivity of $P_i$ on the same sample according to Ernster *et al.* (23). The optical density of the blue color complex was read with a Klett photoelectric colorimeter after which the radioactivity due to $P^{32}$ in the sample was determined on the colored solution by the use of a Veall liquid counter (M6, 20th Century Electronics, Ltd.). The counting rate was corrected for background and decay.

For following activities in paper chromatograms and electrophoretic strips, the paper was cut into segments (1 $cm^2$ in area) and activities counted in stainless steel planchets with a mica window Geiger counter of conventional design.

*Paper Electrophoresis*—Electrophoretic analyses were conducted in a horizontal open type electrophoresis apparatus (Arthur Thomas Company). The run was conducted in acid-washed Whatman No. 1 filter paper strips (4 × 30 cm) at room temperature. Protein bands were revealed by staining with Amidoschwarz (Merck, Naphthalene black 12B200).

TABLE I

*Incorporation in vivo of $P^{32}$ into phosphoprotein fractions of liver and mammary gland*

$P^{32}$-labeled phosphorus was administered to a lactating rabbit which was killed after a time interval of 3 hours. Specific activities of phosphorus in the various fractions were determined as described in the text.

| Tissue | Fraction | Total phosphorus* | Total activity* | Specific activity |
|---|---|---|---|---|
| | | *mg* | *c.p.m.* × $10^5$ | *c.p.m./mg phosphorus* × $10^5$ |
| Liver | Acid-soluble | 1.34 | 5.24 | 3.91 |
| | Phosphoprotein | 0.02 | 0.07 | 3.50 |
| | Phospholipid | 1.25 | 0.18 | 0.14 |
| Mammary gland | Acid-soluble | 1.25 | 5.75 | 4.60 |
| | Phosphoprotein | 0.06 | 0.22 | 3.63 |
| | Phospholipid | 0.40 | 0.26 | 0.65 |
| | Casein | 3.40† | 7.34† | 2.16 |

* Values given per gram of fresh tissue.

† Values represent total yield and activity of casein phosphorus.

TABLE II

*Comparison of specific activities in casein and liver phosphoprotein*

| Rabbit No. | Specific activity | |
|---|---|---|
| | Liver phosphoprotein phosphorus | Casein phosphorus |
| | *c.p.m./mg phosphorus* × $10^5$ | |
| II | 2.15 | 3.47 |
| III | 0.92 | 0.41 |
| IV | 1.44 | 1.52 |
| V | 2.70 | 11.65 |
| VI | 3.45 | 2.16 |

## RESULTS

*Incorporation in vivo of $P^{32}$ into Phosphorus-containing Fractions of Mammary Gland and Liver*—Data on the incorporation of administered $P^{32}$ into some of the phosphorus compounds of the mammary gland and liver of lactating rabbits are presented in Table I. It may be seen that the acid-soluble fraction has the highest specific activity and is closely followed by the phosphoprotein fraction whereas the activity in the phospholipid is considerably less than that of the other fractions. The high rate of turnover of phosphoprotein phosphorus is in agreement with the finding of earlier workers (10–12). The present studies show further that the specific activity of casein phosphorus is comparable to and, at least in one instance, very much higher than that of liver phosphoprotein (Table II)—an indication of the remarkably rapid rate at which phosphorus incorporation into casein occurs.

The data correlating yield of casein in terms of protein phosphorus and the concentration of $P_i$ in the mammary gland are presented in Table III. The increase in casein synthesis is accompanied by an increase in the concentration of $P_i$. Since casein phosphorus accounts for a considerable proportion of the organic phosphate of milk, the results may be interpreted as indicating the dependence of mammary gland on $P_i$ fraction for

TABLE III

*Relationship between $P_i$ and amount of casein synthesized*

| Rabbit No. | $P_i$* | Casein phosphorus† | Specific activity | | | |
|---|---|---|---|---|---|---|
| | | | $P_i$ | Casein phosphorus | Mammary gland | |
| | | | | | Phosphoprotein phosphorus | Phospholipid phosphorus |
| | | | *c.p.m./mg phosphorus* × $10^5$ | | | |
| V | 0.36 | 1.17 | 7.42 | 11.65 | 7.51 | 0.98 |
| VII | 0.42 | 1.68 | 8.21 | 4.30 | 9.90 | 0.75 |
| II‡ | 0.48 | 1.91 | 10.33 | 3.50 | 3.39 | 0.50 |
| VI | 1.10 | 3.40 | 3.94 | 2.20 | 3.63 | 0.66 |

* Mg per gm of fresh tissue.
† Total yield of casein as mg protein phosphorus.
‡ Animals killed 2 hours after injection.

providing the phosphorus for casein synthesis. The inverse relationship between the amount of protein phosphorus synthesized and its specific activity is also evident from the results presented in Table III. This may be due to the following reasons: (*a*) the decrease in the specific activity of $P_i$, precursor of casein phosphorus, consequent on the increase in the total concentration of the $P_i$ of the gland, (*b*) dilution of the newly synthesized protein of high specific activity with the pre-existing unlabeled protein, and (*c*) a decrease in the rate of utilization of $P_i$ for casein synthesis owing to the large concentration of pre-existing casein. The specific activity of the mammary gland phosphoprotein exhibits likewise a dependence on the activity of $P_i$. Specific activity of the phospholipid fraction does not, however, show much variation. This may be taken to indicate that the rate of incorporation of phosphorus into phospholipid is much lower than the rate of its utilization for phosphoprotein synthesis.

*Characterization of Casein*—The casein preparation from rabbit milk contained 0.5% of phosphorus, all of it being labile to alkali. The specific activities of the total phosphorus and the alkali-labile phosphorus of the protein were consequently the same. Paper electrophoretic analysis of the protein in Veronal buffer (0.05 M) at pH 8.6 indicated the presence of a major protein band moving towards the anode and a minor one with a lower mobility. Radioactivity was present exclusively in the major protein component (Fig. 1).

The corresponding protein fraction from mammary gland exhibited considerable variation in its phosphorus content (Table IV). In contrast to casein only a small proportion of its phosphorus was labile to alkali. The amount of the labile phosphorus in the protein was also variable and appeared to be dependent on the yield of casein phosphorus. The specific activity of the fraction was of the same order as that of casein. These observations suggest that the fraction may be very similar to, if not identical with casein. The rest of the protein phosphorus possibly represents nucleic acid phosphorus. The specific activity of this fraction can be expected to be very much less than that of the phosphoprotein phosphorus.

Electrophoretic analysis of the mammary gland protein fraction at pH 8.6 indicated the presence of at least three protein components. The radioactivity in the electrophoretogram was too low, even when the maximum permissible amount of the protein was spotted, to draw any valid conclusion.

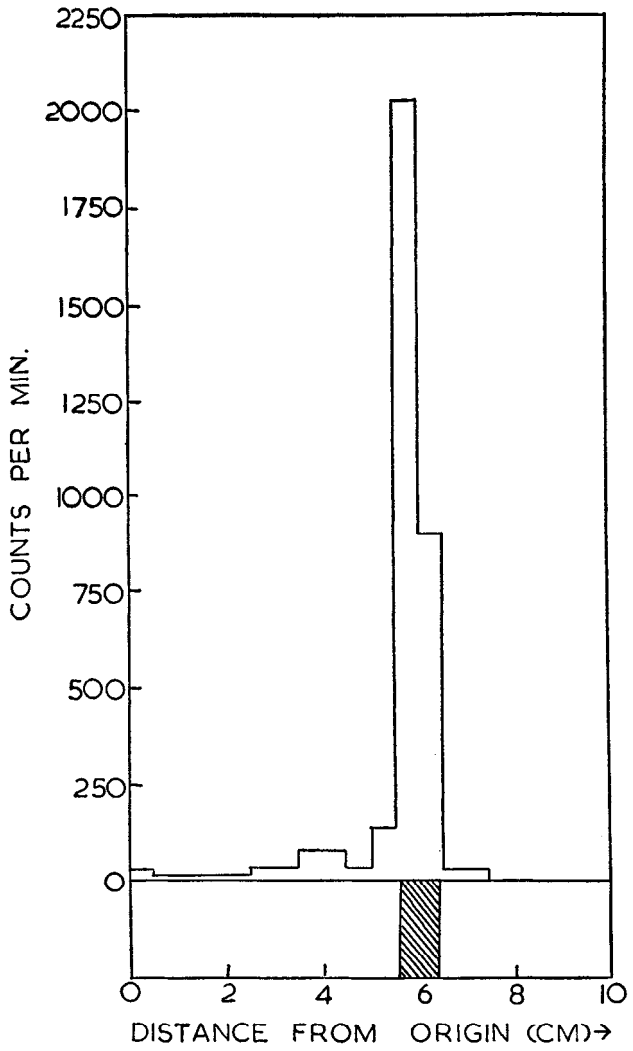


FIG. 1. Paper electrophoretic analysis of casein. Electrophoresis carried out in 0.05 M Veronal buffer, pH 8.6, at 280 volts for 3 hours. Shaded area in the lower part of the figure represents the major protein component in the preparation as revealed by staining with Amidoschwarz. Upper portion represents distribution of radioactivity in the strip.

TABLE IV

*Analysis of fraction from mammary gland insoluble at pH 4.8*

| Experiment No. | Mammary gland protein | | | | Casein | |
|---|---|---|---|---|---|---|
| | Total phosphorus* | Alkali-labile phosphorus* | Specific activity | | Yield*† | Specific activity |
| | | | Total phosphorus | Alkali-labile phosphorus | | |
| | % | % | *c.p.m./mg phosphorus* × $10^5$ | | *mg* | *c.p.m./mg phosphorus* × $10^5$ |
| I | 0.74 | 0.07 | 1.25 | 9.10 | 1.17 | 11.65 |
| II | 1.59 | 0.22 | 0.58 | 2.90 | 3.40 | 2.20 |

* The figures presented represent the maximum and minimum values obtained in these investigations.
† In terms of protein phosphorus.

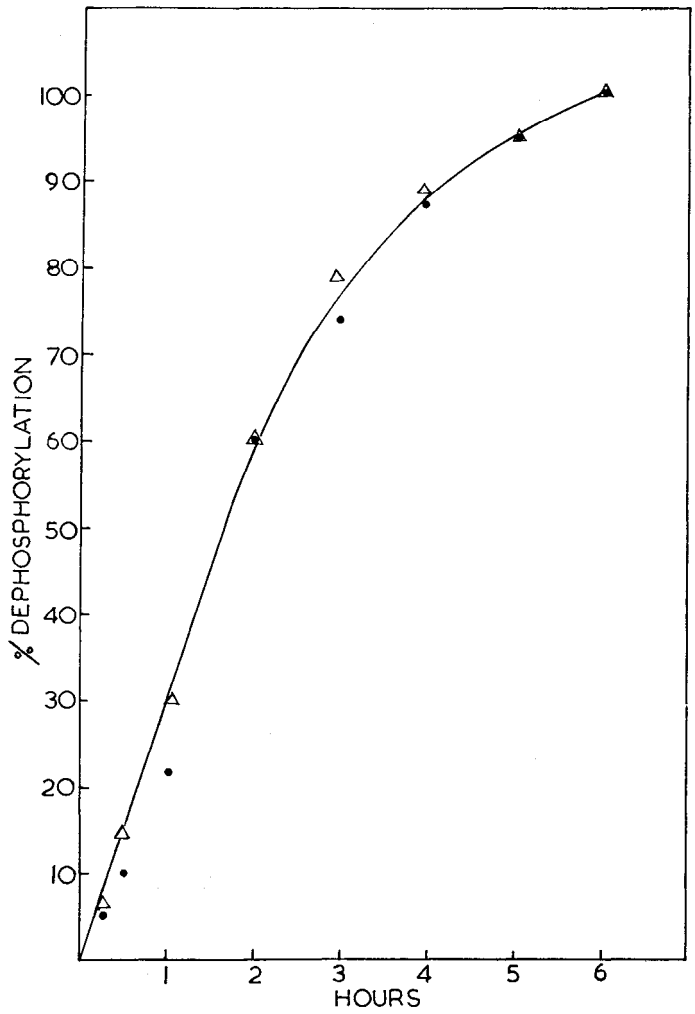


FIG. 2. Rate of dephosphorylation of casein by alkali. The protein (80 μg of phosphorus) was incubated with 2 ml of 0.5 N KOH at 37° for various time intervals. Reaction was stopped by the addition of 1 ml of 40% trichloroacetic acid. $P_i$ determined in the filtrate by colorimetric (●——●) and radioactivity (△——△) measurements.

*Dephosphorylation Studies*—The rate of liberation of $P_i$ from casein by alkali, as measured by colorimetric and radioactivity determination of the liberated $P_i$ is represented in Fig. 2. The equivalence in the rate, as measured by both the methods, suggest that all the phosphorus atoms of the casein have the same specific activity.

The results of studies on the enzymic dephosphorylation of casein are presented in Table V. The protein was readily dephosphorylated by phosphatase preparations from rat spleen and wheat germ and the specific activities of the $P_i$ released from the protein by the various reagents were nearly the same. Phosphodiesterase and inorganic pyrophosphatase had no dephosphorylating action on the protein, and incubation of the protein with these enzymes did not lead to the liberation of acid-soluble phosphorylated peptides.

*Degradation by Pepsin*—In an attempt to obtain more information on the distribution of the incorporated phosphorus into the casein, the protein was digested with pepsin. A 1% solution of the protein, adjusted to pH 1.8 with HCl, was mixed with crystalline pepsin (enzyme-substrate 1:50) and left at 37° for 20 hours. The small amount of precipitate which separated during the course of digestion, was separated by centrifugation, washed with water, and dissolved with the minimum amount of alkali. Radioactivity determination indicated that the activity in the precipitate accounted for only about 5% of the activity in the parent protein; the rest of the activity was recovered in the filtrate. Phosphopeptone was precipitated from the filtrate as the barium salt, according to the method of Damodaran and Ramachandran (24). The peptone was purified by dissolving it in water and reprecipitating with ethanol. After several washings with 50% ethanol, the precipitate was dissolved in water. A portion of it was analyzed for total nitrogen and phosphorus and for radioactivity. The phosphopeptone preparation had N:P atomic ratio ranging from 16 to 18 and had about the same specific activity as the parent protein (Table VI). When subjected to paper electrophoresis at pH 5.8 (0.025 M phthalate buffer) the peptone was resolved into two components. The radioactivity was present exclusively in these fractions (Table VII).

*Degradation by Acid*—Labeled phosphopeptides were also obtained on degradation of casein with concentrated HCl at 37° for a period of 48 hours. A phosphopeptide accounting for about 50% of the activity of the parent protein was isolated from the hydrolysate by descending paper chromatography in butanol-

TABLE V

*Dephosphorylation behavior of rabbit casein*

Incubation mixtures for enzyme experiments were made up as follows: buffer (0.05 M acetate buffer at pH 5.6 for spleen and wheat germ phosphatase, bicarbonate at pH 7.5 for diesterase and pyrophosphatase), activator (0.001 M thioglycollic acid for spleen phosphatase, 0.01 M $Mg^{++}$ for diesterase and pyrophosphatase), casein (63.5 μg of phosphorus), enzyme of sufficiently high concentration to ensure its excess, and water to a final volume of 4 ml. For alkali dephosphorylation the casein (63.5 μg of phosphorus) was dissolved in 2 ml of 1 N KOH. Incubations were at 37° for 20 hours. Reaction terminated by addition of 1 ml of 40% trichloroacetic acid. $P_i$ estimated in the filtrate by isobutanol procedure.

| Reagent | $P_i$ released | Activity in $P_i$ | Specific activity |
|---|---|---|---|
| | μg | *c.p.m.* | *c.p.m./mg phosphorus* × $10^4$ |
| Alkali | 63.5 | 2604 | 4.10 |
| Spleen phosphatase | 56.3 | 2230 | 3.96 |
| Wheat germ phosphatase | 57.8 | 2284 | 3.95 |
| Venom diesterase | 0.0 | 15* | |
| Yeast pyrophosphatase | 0.0 | 10† | |

* Total activity in acid-soluble fraction 130 c.p.m.

† Total activity in acid-soluble fraction 15 c.p.m.

TABLE VI

*Analysis of casein phosphopeptone*

| Preparation No. | Total phosphorus | Total activity | Specific activity | |
|---|---|---|---|---|
| | | | Peptone | Parent protein |
| | *mg* | *c.p.m.* × $10^5$ | *c.p.m./mg phosphorus* × $10^5$ | |
| 1 | 0.19 | 0.59 | 3.10 | 3.50 |
| 2 | 0.76 | 0.34 | 0.45 | 0.45 |
| 3 | 0.53 | 0.90 | 1.70 | 1.60 |

acetic acid-water (4:1:5) solvent system. The peptide had about twice the $R_F$ value of P-serine in this system and gave on complete hydrolysis the amino acids: aspartic acid, glutamic acid, serine, glycine, alanine, threonine, proline, valine, and leucine. These amino acids are also present in the phosphopeptones obtained by enzymic degradation of casein (25).

The site of binding of the phosphorus isotope in rabbit casein was investigated by subjecting the protein to partial acid hydrolysis according to the procedure of Lipmann (26). Acid was removed from the hydrolysate by repeated distillation under reduced pressure. The residue was taken up in water and any $P_i$ formed during hydrolysis was removed by treatment with magnesia mixture. After filtering off the precipitated $MgNH_4PO_4$, the filtrate was neutralized and the remaining phosphorus compounds were precipitated by the addition of $Ba(CH_3COO)_2$ (25% solution) and ethanol. The solution was kept in the cold overnight to complete the precipitation of the barium salts. The precipitate was separated by centrifuging, dissolved in water, and reprecipitated with ethanol. After removal of barium as $BaSO_4$, the free esters were analyzed by paper electrophoretic and chromatographic methods. P-serine appeared to be the only phosphorylated amino acid in the hydrolysate, as shown by the following experiments: (*a*) paper electrophoretic analysis at pH 8.6 (0.05 M Veronal buffer) indicated the presence of a component which gave an intense color with ninhydrin and had the same mobility as a synthetic sample of P-serine. Radioactivity was present exclusively in this component. (*b*) Chromatography in the butanol-acetic acid-water (4:1:5) system showed up a ninhydrin-positive spot with the same $R_F$ as P-serine and containing about 80% of the activity spotted on the paper. The spot was eluted and hydrolyzed with 6 N HCl at 110° for 48 hours. Serine was the only amino acid present in the hydrolysate as detected by paper chromatography. The results thus indicate a true incorporation of the phosphorus isotope into the protein, this being present as P-serine.

## DISCUSSION

The present studies confirm the findings of the earlier workers in demonstrating a rapid incorporation of phosphorus into casein in the mammary gland. This is evident from the observation that the specific activity of casein phosphorus is many times higher than that of phospholipid and possibly of nucleic acid phosphorus and that it is comparable to that of liver phosphoprotein. The latter fraction is known to have a high rate of phosphorus turnover (10–12). That the activity in casein is entirely due to incorporation of $P^{32}$ into this protein and does not arise from contaminating phosphoprotein enzymes, is quite evident from the following observations: (*a*) The radioactivity of the protein is fully recovered in a major protein component on electrophoresis of the preparation at pH 8.6. (*b*) All the phosphorus atoms of the protein possess the same specific activity, as shown by alkali dephosphorylation studies. (*c*) Phosphopeptone obtained by peptic digestion of the protein consists of two electrophoretically distinct components which are radioactive to more or less the same extent.

The observation that the specific activity of casein phosphorus exceeds that of hexose monophosphates (2) prompted Pin (5) to suggest that the phosphorus atoms of casein are not present exclusively as monoesters. According to his hypothesis, a part of the phosphorus atoms of the protein are incorporated at the same time as the formation of the initial peptide chains and that these phosphorus atoms are in the diesterified state serving to link together the peptide chains of the protein. Once the fundamental chain of the protein is formed, the rest of the phosphorus is assumed to be incorporated into the protein as monoesterified phosphate. These conclusions are not, however, borne out by our finding on the dephosphorylation behavior of casein. The presence of diesterified phosphate in rabbit casein can be definitely excluded in view of our observation that about 90% of the phosphorus of the protein is removed as $P_i$ by enzyme preparations completely lacking in diesterase activity (Table V). Failure to obtain phosphopeptides from casein after treatment of the protein with pyrophosphatase and phosphodiesterase indicates further that phosphate bridges may not be involved in cross-linking peptide chains in rabbit casein. The even labeling of the phosphorus atoms in casein, as revealed by dephosphorylation studies, also goes contrary to Pin's hypothesis. Taking into account the observation of Aten and Hevesy (2) that it takes about 1 hour for synthesis of casein to be effected by the mammary gland, one would expect appreciable variation in the specific activities of the phosphorus atoms of casein if these were to be incorporated into the protein at different rates.

TABLE VII

*Electrophoretic analysis of casein phosphopeptone*

Phosphopeptones prepared by peptic digestion of casein were analyzed by paper electrophoresis in 0.05 M phthalate buffer (pH 5.6) for 4 hours at 270 volts. Tracing of paper electrophoretic strips for radioactivity indicated that the activity was present mainly in the ninhydrin-positive components. Movement of the peptides was towards the anode.

| Peptide band | Distance of band from the origin | Activity in peptide | | |
|---|---|---|---|---|
| | | Experiment No. | | |
| | | 1 | 2 | 3 |
| | *cm* | *c.p.m.* | *c.p.m.* | *c.p.m.* |
| 1 | 6–6.8 | 512 | 570 | 1048 |
| 2 | 7–7.8 | 436 | 390 | 1252 |

Le Bars *et al.* (27) have investigated the protein constituents of the mammary gland of dog to establish their relationship to casein. The proteins were extracted from the tissue with 10% NaCl and were precipitated from the extract with trichloroacetic acid or by saturation with ammonium sulfate. The protein fraction thus obtained differed from casein in containing a higher amount of phosphorus. The specific activity of the protein phosphorus was very low and was in fact very much less than that of phospholipid phosphorus. They interpreted these findings as indicating a slow utilization of $P_i$ for casein synthesis. This conclusion is not supported by the result of the present studies which clearly establish the complex nature of the protein fraction prepared from the mammary gland. Only a small proportion of the phosphorus of the protein is labile to alkali (Table IV) whereas phospholipid and nucleic acid phosphorus constitute a large proportion of the protein phosphorus. Further, the specific activity of the alkali-labile phosphorus is very much higher than that in phospholipid and nucleic acid and is comparable to that of casein phosphorus. The low specific activity obtained for protein phosphorus by the French workers might also have been due to the fact that they killed the animal 5 days after injection of $P^{32}$-labeled phosphate—a period long enough to bring down the specific activity of $P_i$ of mammary

gland to a low value and in consequence that of protein phosphorus. It is possible that the alkali-labile phosphorus of mammary gland protein described in the present studies represents casein phosphorus, although conclusive proof for this is lacking.

SUMMARY

Intraperitoneal administration of $P^{32}$-labeled phosphate to lactating rabbit led to a rapid incorporation of the isotope into the phosphoprotein of milk and of mammary gland. Indication as to the rate of incorporation of phosphorus into casein was obtained from experiments which showed that the specific activity of casein phosphorus was comparable to that of liver phosphoprotein, which is known to have a high rate of phosphorus turnover.

$P^{32}$-labeled casein was prepared from the milk of rabbit given an injection of labeled phosphorus. The protein was characterized on the basis of its electrophoretic behavior, dephosphorylation by alkali and by enzymes, and through its degradation with pepsin and by acid. The results indicated that the high activity in the protein represented a true incorporation of $P^{32}$ into the protein as phosphoserine and that the phosphorus atoms in the protein were evenly labeled.

A protein fraction resembling casein in its solubility properties was prepared from mammary gland. The protein differed from casein in having a very low amount of alkali-labile phosphorus and appeared to be more complex electrophoretically. The specific activity of the labile phosphorus was of the same order as that of casein. The results are consistent with earlier findings on the rapid utilization of inorganic phosphorus for casein synthesis.

REFERENCES

1. Folley, S. J., *The physiology and biochemistry of lactation*, Oliver and Boyd, London, 1956, p. 130.
2. Aten, A. H. W. Jr., and Hevesy, G., *Nature*, **142**, 111 (1938).
3. Barry, J. M., *J. Biol. Chem.*, **195**, 795 (1952).
4. Kleiber, M., and Luick, J. R., *Ann. N. Y. Acad. Sci.*, **64**, 299, (1956).
5. Pin, P., *D.Sc. Thesis*, Paris University, 1955.
6. Wosilait, W. D., and Sutherland, E. W., *J. Biol. Chem.*, **218**, 469 (1956).
7. Agren, G., and Engstrom, L., *Acta Chem. Scand.*, **10**, 489 (1956).
8. Engstrom, L., and Agren, G., *Acta Chem. Scand.*, **10**, 877 (1956).
9. Kennedy, E. P., and Koshland, D. E., Jr., *J. Biol. Chem.*, **228**, 419 (1957).
10. Davidson, J. N., Frazer, S. C., and Hutchison, W. C., *Biochem. J.*, **49**, 311 (1951).
11. Norberg, B., *Acta Physiol. Scand.*, **23**, 205 (1951).
12. Agren, G., de Verdier, C. H., and Glomset, J., *Acta Chem. Scand.*, **8**, 503, 1570 (1954).
13. Kalan, E. B., and Telka, M., *Arch. Biochem. and Biophys.*, **79**, 275 (1959).
14. Sundararajan, T. A., and Sarma, P. S., *Enzymologia*, **18**, 234 (1957).
15. Sundararajan, T. A., and Sarma, P. S., *Biochem. J.*, **71**, 537 (1959).
16. Sinsheimer, R. L., and Koerner, J. F., *J. Biol. Chem.*, **198**, 293 (1952).
17. Schmidt, G., and Thannhauser, S. J., *J. Biol. Chem.*, **161**, 83 (1945).
18. Friedkin, M., and Lehninger, A. L., *J. Biol. Chem.*, **177**, 775 (1949).
19. Koch, F. C., and McMeekin, T. L., *J. Am. Chem. Soc.*, **46**, 2066 (1924).
20. Fiske, C. H., and SubbaRow, Y., *J. Biol. Chem.*, **66**, 375 (1925).
21. Koshland, D. E. Jr., and Clarke, E., *J. Biol. Chem.*, **205**, 917 (1953).
22. Martin, J. B., and Doty, D. M., *Anal. Chem.*, **21**, 965 (1949).
23. Ernster, L., Zetterstrom, R., and Lindberg, O., *Acta Chem. Scand.*, **4**, 942 (1950).
24. Damodaran, M., and Ramachandran, B. V., *Biochem. J.*, **35**, 122 (1941).
25. Mellander, O., and de Verdier, C. H., *Acta Soc. Med. Upsaliensis*, **57**, 218 (1952).
26. Lipmann, F., *Biochem. Z.*, **262**, 3 (1933).
27. Le Bars, H., Simonnet, H., and Sternberg, J., *Compt. rend.*, **232**, 1148 (1951).