ON THE *IN VITRO* INCORPORATION OF ^{32}P-PHOSPHATE INTO PHOSPHOPROTEINS BY LACTATING MAMMARY GLAND*, **

by

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The mechanism of the introduction of the phosphorus-containing group into phosphoproteins is still unknown. It is possible either that phosphorylated hydroxyamino acids are precursors of these groups or that inorganic phosphate is directly transferred to the protein molecule. In the following, we shall report the results of experiments in which the influence of some added proteins on the incorporation of inorganic ^{32}P phosphate into the phosphorproteins by an enzyme preparation from lactating mammary gland was studied.

MATERIALS AND METHODS

Inorganic phosphate labelled with ^{32}P was obtained from the Atomic Energy Commission.

Casein "Hammarsten" was purchased from Eimer and Amend, Inc., crystallized bovine serum albumin from the Armour Laboratories, the barium salt of ATP was obtained from Sigma, Inc.

Enzyme preparation

A washed particulate fraction was prepared from lactating mammary glands of rabbits according to the procedure described for liver by LEHNINGER AND KENNEDY[1]. The first experiments were carried out with homogenates of fresh glands, but in the later experiments the glands were frozen in liquid nitrogen prior to the preparation of the homogenates, since fresh glands cannot be readily homogenized owing to their high content of connective tissue. The frozen material was powdered in a mortar, and 10 g of the powder were suspended in the desired medium and homogenized after thawing.

Procedure for the isolation of labelled phosphoprotein phosphorus

The procedure was based on the method of SCHMIDT AND THANNHAUSER[2] for the isolation of the phosphoprotein phosphorus from tissues. We modified the original procedure in two points in order to adapt it to the purposes of the present study. (1) Immediately before the deproteinization with trichloroacetic acid, 0.5 ml of a 12% solution of "carrier" casein were added to all samples which had not been incubated with added casein. This was done in order to facilitate the isolation of the very small amounts of phosphoprotein phosphorus in the samples which had not been incubated with added casein. Furthermore, the addition of unlabelled casein to the zero time samples permitted the detection of any adsorption of labelled phosphate on the phosphoprotein precipitates. (2) It was necessary to purify the protein fraction from acid-soluble contaminations with the added, labelled phosphate. Prior to the hydrolysis with potassium hydroxide, the lipid-free tissue powder was extracted overnight at 37° with dilute ammonia at pH 10.5 in a shaker.

* A preliminary report of some of the results reported in this paper was published in the Fifth Annual Report of the Cancer Research and Cancer Control Unit, Tufts College Medical School, (1953) page 17.

** This study was supported by research grants from the American Cancer Society, the Godfrey H. Hyams Trust Fund and the Bingham Associates Fund.

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The suspension was centrifuged and washed, and the combined supernatant solutions were precipitated with 7% trichloroacetic acid (final concentration). The precipitate was washed with a 7% solution of trichloroacetic acid, and dissolved by the dropwise addition of *N* ammonium hydroxide; the reprecipitation was repeated twice in a similar manner. The protein solution of the second reprecipitation was mixed with a solution of unlabelled sodium phosphate containing approximately 2 mg of inorganic phosphorus. The precipitate was washed until the washings were free of inorganic phosphoric acid. (3) The last precipitate was dissolved in 10 ml of 0.25 *N* sodium hydroxide and kept at 37° for 24 hours. The hydrolysate was neutralized with 0.2 ml of 5 *N* hydrochloric acid and precipitated with an equal volume of 15% trichloroacetic acid. The suspension was filtered, and the inorganic phosphorus was determined in a small aliquot according to the method of DELORY[3]. The main part of the filtrate was mixed with 1 ml of a 3% solution of unlabelled sodium phosphate; after addition of an excess of ammonia the phosphate was precipitated with magnesia mixture. The ammonium magnesium phosphate was recrystallized twice and dissolved in a few milliliters of dilute hydrochloric acid. The solution was brought to a volume of 5 ml, and the radioactivity was determined in 2 ml aliquots in a dip counter, Radiation Counter Laboratories, Inc., Chicago, Model 80S, of 12% efficiency. In control experiments in which casein was added to liver homogenates in a similar manner, the yield was 50% (± 10) of the added phosphoprotein phosphorus. Since the application of the procedure to pure casein solutions resulted reproducibly in recoveries of 80 (± 5)% of the casein, the considerable losses observed in the analyses of mixtures of casein with tissue homogenates were probably caused by the incompleteness of the extraction of the casein from the dried, defatted protein mixture with ammonia at pH 10. Because of the alkali lability of the phosphoproteins, no attempt was made to increase the yield by using solutions of higher alkalinity for the extraction of the phosphoproteins. Since the isolation of the phosphoprotein fraction was carried out as an isotope dilution method with the addition of equal amounts of casein to each sample, the incompleteness of the recoveries did not interfere with the quantitative comparison of the different samples with regard to their incorporation of labelled phosphate.

RESULTS

1. *Elimination of adsorbed inorganic ^{32}P from phospho-protein phosphorus*

The efficiency of the procedure described in the section on methods for the isolation of phosphoprotein can be evaluated from the data in Table I. The first two lines show that the incubation of pure casein with 0.1 *N* ammonia under the conditions described in the section on methods permitted the recovery of approximately 80% of the casein phosphorus, and that its characteristic lability toward alkali remained unchanged during the 24 hours extraction at pH 10.5. Line 4, 5, and 6 contain the results of an experiment, in which 10 ml of a 4% solution of casein had been mixed with 10 ml of a homogenate of rat liver containing 5 g of liver, and with 2 ml of a solution of ^{32}P ($2.33 \cdot 10^6$ counts per minute). This mixture was immediately fixed by

TABLE I

RECOVERY OF CASEIN PHOSPHORUS IN PROCEDURE USED FOR INCORPORATION EXPERIMENTS WITH ^{32}P

Conditions of materials		*Total P* (*mg in whole sample*)	*Alkali labile P* (*mg in whole sample*)	*Recovery* (*percent of initial amount*)	*Counts/min/mg P*
200 mg of casein*,	Initial	1.58	1.58	—	—
	Reprecipitated	1.27	1.25	79	—
400 mg of casein**,	Initial	3.16	—	—	—
mixed with liver	1st Reprec.	—	1.65	51.5	504 (± 8)
homogenate and ^{32}P-phosphate.	3rd Reprec.	—	1.39	43.5	16 (± 3)

* In 2% solution.

** In 4% solution.

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precipitation with trichloroacetic acid, and the phosphoprotein phosphorus was isolated according to the procedure described in the section on methods. It can be seen from the figures of the last column that the specific activity of the phosphoprotein phosphorus became negligible after the third reprecipitation of the phosphoprotein fraction. At this stage the yield amounted to 43.5% of the added casein phosphorus.

A comparison between the yields of the first and third reprecipitation shows that the main losses occurred before the first reprecipitation, probably owing to incomplete extraction from the dry mixture of casein and tissue proteins.

2. *Influence of some added proteins on the enzymic incorporation of ^{32}P into the phospho-protein fraction by lactating rabbit mammary gland*

Table II contains the results of a representative experiment in which the incorporation of added ^{32}P-phosphate into the phosphoprotein fraction by a washed particle fraction of lactating rabbit mammary gland was determined. After the interruption of the enzyme action in each sample by the addition of one tenth volume of an 80% solution of trichloroacetic acid, 0.5 ml of a 12% solution of serum albumin, 0.5 ml of a 12% solution of casein, and 0.5 ml of rabbit serum were added respectively to all those samples which did not contain these additions at the start of the experiment. This was done in order to carry out the isolation of the phosphoprotein phosphorus in all samples under closely similar conditions. Furthermore, the addition of a measured amount of carrier casein to each sample was necessary for the quantitative comparison of the rate of incorporation in the different samples since the amounts of phosphoproteins in the enzyme suspension were too small to permit their quantitative determination. The figures represent the total incorporation of ^{32}P under the conditions used for the incubation of each sample. It is obvious that the figures of column 2 do not permit conclusions concerning the specific activities of the tissue phosphoproteins.

The values of column 2 show that a measurable incorporation of ^{32}P into the

TABLE II

INFLUENCE OF ADDED PROTEINS ON THE ENZYMIC INCORPORATION OF ^{32}P INTO THE PHOSPHOPROTEIN FRACTION BY LACTATING MAMMERY GLAND

*Conditions of incubation**		*Counts/minute/mg carrier casein P*	*Recovery of carrier casein P (per cent of added casein P)*
1. Enzyme system + water	0 time	0	62
2. Enzyme system + water	Incubated	145	55
3. Enzyme system + bovine serum albumin	Incubated	120	41
4. Enzyme system + rabbit serum	Incubated	261	46
5. Enzyme system + casein	Incubated	1155	58
6. Enzyme without cofactors + casein	Incubated	768	64

* a. Washed particle fraction prepared according to LEHNINGER AND KENNEDY[1] from 10 g of gland after freezing in liquid nitrogen. Final sediment suspended in 7 ml of water, aliquots of 1 ml used in each sample.

b. Basic medium: 1.6 ml potassium chloride (0.5 *M*), 1.6 ml magnesium sulfate (0.05 *M*) 1.6 ml glycyl-glycine (0.1 *M*), 1.6 ml NaATP (0.0067 *M*), 1.6 ml malate (0.05 *M*), 1.6 ml sodium glutamate (0.05 *M*), 0.8 ml DPN (2%), 0.8 ml cytochrome *c* (2%), 0.8 ml sodium fructose diphosphate (0.05 *M*).

Incubation mixture: 1 ml of (a), 1.5 ml of (b), 0.2 ml of sodium ^{32}P-phosphate (110 uc per ml), 0.5 ml of 12% protein solution as indicated in the table. Incubation of 30′ at 37° in Dubnoff shaker in air.

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phosphoprotein fraction occurred during the incubation of the washed particles without addition of proteins. The incorporation was not influenced by the addition of bovine serum albumin, but it was significantly enhanced by the addition of serum of a lactating rabbit.

By far the largest incorporation was produced when the enzyme system was incubated in the presence of added casein. The maximal incorporation was observed when the washed particle fraction of mammary gland was incubated with casein in the presence of added substrates and coenzymes of cell respiration; even without such additions, however, the incorporation of ^{32}P into the phosphoprotein fraction in the presence of casein was approximately 3 times higher than that found after incubation of the enzyme with rabbit serum in presence of added intermediaries of the energy metabolism.

DISCUSSION

The enhancing effect of added casein on the *in vitro* incorporation of ^{32}P into phosphoproteins by lactating mammary gland is in analogy to the observations with liver homogenates recently described by BURNETT AND KENNEDY[4]. In view of this effect of casein, the increased ^{32}P-incorporation into phosphoproteins in the presence of serum of a lactating rabbit might have been caused by small amounts of circulating casein in lactating animals, although this assumption is conjectural, since no casein determinations in sera of lactating animals, have so far been carried out. LASKOWSKI[5] as well as ROEPKE AND HUGHES[6], however, demonstrated the presence of phosphoproteins in the plasma of laying hens already in 1935.

For this reason, the results of the present study do not justify any speculation regarding the possibility that the phosphorylation of a precursor protein rather than the utilization of phosphorylated amino acids would represent the mechanism of the biosynthesis of phosphoproteins. In agreement with observations of other authors[4,6], they suggest, however, an active metabolic role of the phosphoryl groups of casein beyond that of a source of phosphate for the growing organism.

SUMMARY

1. Suspensions of washed particles of lactating mammary gland were found to be capable of incorporating ^{32}P-phosphate into the phosphoprotein fraction of the tissue.

2. The addition of crystallized bovine serum albumin did not appreciably influence the rate of incorporation. A significant increase of this rate was found, however, when the incubation of the enzyme system was carried out in the presence of serum from a lactating rabbit. Much greater stimulations of the incorporation were observed when casein was added to the enzyme system.

RÉSUMÉ

1. Des suspensions de particules lavées de glande mammaire en lactation se sont montrées capables d'incorporer du phosphate ^{32}P dans la fraction phosphoprotéique du tissu.

2. L'addition de sérumalbumine bovine cristallisée n'influe pas de façon appréciable sur la vitesse d'incorporation. Une augmentation significative de cette vitesse s'observe toutefois lorsque l'incubation du système enzymatique est effectuée en présence de sérum d'une lapine en lactation. Des stimulations beaucoup plus importantes de l'incorporation s'observent lorsque de la caséine est a ajoutée au système enzymatique.

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ZUSAMMENFASSUNG

1. Es wurde festgestellt, dass Aufschlemmungen gewaschener Partikel aus der stillenden Milchdrüse imstande sind, ^{32}P-Phosphat in die Phosphoproteinfraktion des Gewebes einzuverleiben.

2. Die Hinzufügung von kristallisiertem Rinderserumalbumin hatte keinen wahrnehmbaren Einfluss auf die Einverleibungsgeschwindigkeit. Eine bedeutende Erhöhung dieser Geschwindigkeit wurde jedoch bei Inkubation des Enzymsystems in Gegenwart des Serums eines stillenden Kaninchens festgestellt. Es wurde eine viel grössere Steigerung der Einverleibung beobachtet falls Kasein dem Enzymsystem hinzugefügt wurde.

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Received May 20th, 1955