Reports

Calcium Phosphate Sequestering Phosphopeptide from Casein

By the use of ion-exchange columns, a number of crude phosphopeptide fractions have been separated from pancreatic casein hydrolyzates. One of these fractions exhibits, to a remarkable degree, the property of sequestering calcium phosphate in the *p*H range from 7 to 10.5. Although numerous investigations have been made on phosphopeptides derived from casein by various enzymatic hydrolyses (*1*), no mention of this calcium phosphate sequestering property has been found in the literature.

The finding of a casein fragment with the property of solubilizing calcium phosphate, or preventing its precipitation at relatively high *p*H's, has obvious implications in regard to an understanding of the role of casein in calcium and phosphate transport and assimilation. Apart from its interest in this connection, the phosphopeptide is of practical use in preventing the formation of calcium phosphate precipitates in culture media containing relatively high concentrations of phosphate and calcium ion.

Eighteen grams of the calcium phosphate sequestering phosphopeptide were obtained from 2 lb of Trypticase, a pancreatic casein hydrolyzate (*2*). The Trypticase was dissolved in 18 lit. of a buffer containing 0.1*M* sodium acetate and 0.1*M* acetic acid. This solution was passed through a 4- by 45-cm column of Dowex-1 (Cl form, 50 to 100 mesh). The column was washed with 16 lit. of the same buffer until the free amino acids, neutral peptides, and the inorganic phosphate had been removed. Since these operations require several days for completion, they were conducted in a cold room to avoid microbiological action. When negative tests for inorganic phosphate were obtained on the effluent solution, the column was removed to the laboratory and elution was begun with 0.05*N* hydrochloric acid. The eluant removed a large phosphopeptide fraction which was poor in calcium phosphate sequestering power. The peak of this fraction appeared in the 15th liter of eluant, and it dropped off rapidly thereafter. After 18 lit. of the dilute acid had been passed the concentration of hydrochloric acid was increased to 0.3*N*, and the active phosphopeptide was obtained in the next 2 lit. of eluant. It was isolated as its calcium salt by neutralization to *p*H 7 with sodium hydroxide, addition of 30 g of calcium chloride, and precipitation with three volumes of alcohol. The precipitate was collected by decantation and centrifugation, and it was washed successively with alcohol, acetone, and ether. After it had been dried in a vacuum desiccator at room temperature, the precipitate retained 12 percent of volatile solvent which could be removed by drying to constant weight at 100°C in high vacuum over P_2O_5.

Analyses on this preparation of calcium phosphopeptide were: N, 9.9; P, 6.6; Ca, 12.2. The optical rotation in water was $[\alpha]_D^{25} = -67.5°$. The preparation contained no inorganic phosphate.

The calcium phosphate sequestering power of the calcium phosphopeptide is illustrated by the following experiments. Four hundred and forty milligrams of the anhydrous calcium salt were dissolved in 10 ml of 0.1*M* phosphate buffer at *p*H 7. To this solution was added 1 ml of 1*M* calcium chloride solution. There was no turbidity or precipitation in the solution. By cautious addition of normal sodium hydroxide with stirring, the *p*H was increased to 10.5 without the appearance of turbidity. After the solution was adjusted to *p*H 9, it was boiled and allowed to stand in the refrigerator for 2 months; during this time no turbidity or precipitation occurred.

In a second experiment the ability of the peptide to dissolve precipitated calcium phosphate is illustrated. To a solution containing 100 μmole of phosphate in 0.1*M* trihydroxymethylaminomethane was added 150 μmole of calcium chloride. The final volume was 10 ml, and the *p*H was 8.5. Upon analysis after centrifuging it was found that 5 μmole of phosphate remained in the supernatant solution. Forty-four milligrams of the calcium phosphopeptide were stirred into the suspension of calcium phosphate, and centrifuging was repeated after 20 minutes. It was found that the solution contained 15.5 μmole of inorganic phosphate. The precipitate was again stirred and allowed to stand for 7 hours at room temperature, at which time 46.3 μmole of the phosphate were found to be in solution. Since inorganic phosphate of the peptide is not liberated by standing at room temperature at *p*H 8.5, the dissolved phosphate must have come from the calcium phosphate which had been precipitated in the first step of this experiment.

Under conditions favorable for calcium phosphate sequestering—that is, *p*H 7 to 9 and no great excess of either calcium or phosphate ion—the calcium phosphopeptide will sequester two-thirds of its weight of calcium phosphate (calculated as $CaHPO_4$) and hold it in solution through boiling or autoclaving. At room temperature and *p*H 9 it will hold in solution about one-half its weight of calcium phosphate in the presence of a 50-fold excess of either calcium or phosphate ion.

In other work (*3*) it has been learned that the calcium phosphate sequestering power is associated with material which migrates rapidly toward the anode upon electrophoresis on paper at *p*H 4.1 (phthalate buffer). The calcium phosphate sequestering property of the phosphopeptide is destroyed by acid or alkaline hydrolysis or by the action of phosphatases.

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References and Notes

1. G. Perlmann, *Phosphorous Metabolism* (Johns Hopkins Press, Baltimore, Md., 1952), vol. 2, p. 167; O. Mellander, *Upsala Läkarefören. Förh.* 52, 107 (1947); G. Agren and J. Glomset, *Acta Chem. Scand.* 7, 1071 (1953).
2. Trypticase is available from the Baltimore Biological Laboratory, Baltimore, Md.
3. A paper describing this work is in preparation.

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Radiocarbon Dating Up to 70,000 Years by Isotopic Enrichment

Isotopic enrichment of radiocarbon is an obvious method for dating samples which are too old and therefore have too small an activity. Enrichment by a factor of 2^n shifts the limit of counting by n half-lives. The amount of enriched material is fairly large, however, and this leads to considerable technical in-

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