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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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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vestments: the large counter in operation in Groningen requires 8 liters of CO_2. A sample enriched 16 times contains as much C^{14} as 128 liters of the original material. Since the depletion of the reservoir should be at most about 30 percent in order to avoid an appreciable decrease in the transport of C^{14}, about 350 liters of sample are required at the least.

The enrichments are performed at the Laboratory for Mass Spectrography in Amsterdam. The CO_2 obtained from the original material (*1*) is reduced to CO by leading it over zinc at a temperature of 380° ± 5°C. The CO is enriched in five thermal diffusion columns connected in parallel, each of them having a length of 430 cm. The enrichment is derived from the O^{18} abundance in the CO (*2*). For the activity measurements, the enriched CO is oxidized again by leading it over CuO. Enrichment by a factor of 16 takes at present about 2 months; this enrichment shifts the limit of counting to about 70,000 years.

Up to now three samples have been measured. The first sample consisted of recent carbon dioxide diluted with a known amount of inactive CO_2. The enrichment predicted by the abundance of O^{18} was 8.70 ± 0.5; the enrichment measured was 8.06 ± 0.05.

The second sample consisted of dead CO_2 (from anthracite). If all steps are completely free from contamination by recent carbon, the enriched sample should still be inactive. The activity was actually 0.04 ± 0.02, corresponding to an apparent age of 73,000 years. The activity is not really significant, however. This experiment will be repeated in due course.

The third sample consisted of wood from a well-exposed profile at Amersfoort (Netherlands). The situation has been described in more detail by H. de Vries (*3*); it is indicated as Amersfoort XII. The present sample dates the end of the Last Interglacial or an early Würm interstadial separated from the Last Interglacial by a not very cold period. The age found by enrichment was 64,000 ± 1100 years (sample Gro-1397). The limits of error include the statistical error only. Errors introduced by the enrichment and measurement of the enrichment amount to about 800 years. The most important source of error, however, for these old samples is infiltration of recent material (see also *3*). The wood had been cleaned thoroughly by chemical treatment. Humus extracted from it has an age of 42,300 ± 900 years only. So even this wood, protected by a shield of compressed peat and 9 meters of sand, had acquired some recent hume (the extracted humus is a mixture of original material and infiltrated material). Because of possible infiltration, all dates are more or less minimum dates, but our opinion is that the present date is fairly reliable. It fits fairly well with the chronology developed by de Vries (*3*, *4*).

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4. We thank J. van Wel for the construction of the columns. The analyses of O^{18} were carried out by the mass spectrometric group of the Laboratory for Mass Spectrography in Amsterdam. The isotopic enrichments were carried out as part of the research program of the Stichting voor Fundamenteel Onderzoek der Materie with the support of the Nederlandse Organisatie voor Zuiver Wetenschappelijk Onderzoek.

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Extending the Range of Dose-Effect Curves for Irradiated Mice

A recurring problem in experiments yielding results to be expressed in the form of dose-effect curves is the need to use responses measured in different units at different parts of the dosage scale. Any attempt to combine two such responses rests on the assumption that they reflect the same phenomena. A number of methods for extending the dosage scale by combining percentage survival and survival time into a single response have been proposed (*1*). We report here a simple method for combining information on survival which we have recently found useful in our studies on the effect of colchicine and its derivatives in irradiated mice (*2*).

When given 24 hours before exposure to radiation which would kill all controls in an average of 9 days or longer, trimethylcolchicinic acid methyl ether d-tartrate increased the percentage surviving. When given to animals which radiation would kill in 8 days or less, no effect on survival was obtained. After the results were plotted in the usual way (upper half of Fig. 1*A*), the ordinate at zero survival was equated with the mean survival time of the animals which had received the lowest radiation dose required for approximately zero survival (in these experiments, 1000 r and 10.5 ± 0.6 days). At higher radiation doses, survival time gradually decreases to a plateau value of approximately 4 days (*3*). It was noted that this plateau was reached at a radiation dose which is the same distance above the approximate LD_{100} as the dose which would kill almost no animals is below the LD_{100} (1300 r, 1000 r, and 700 r). The ordinate was extended downward to 4 days, and the distance between 4 and 10.5 days was made to equal the distance between 0 and 100 percent survival, with the LD_{100} becoming, on the combined scale, the mid-dose, which for a single response would be the ED_{50} (lower half, Fig. 1*A*). The data, when plotted in this way, yield a smooth radiation dose-effect curve, permitting an expression of percentage mortality and mean survival time

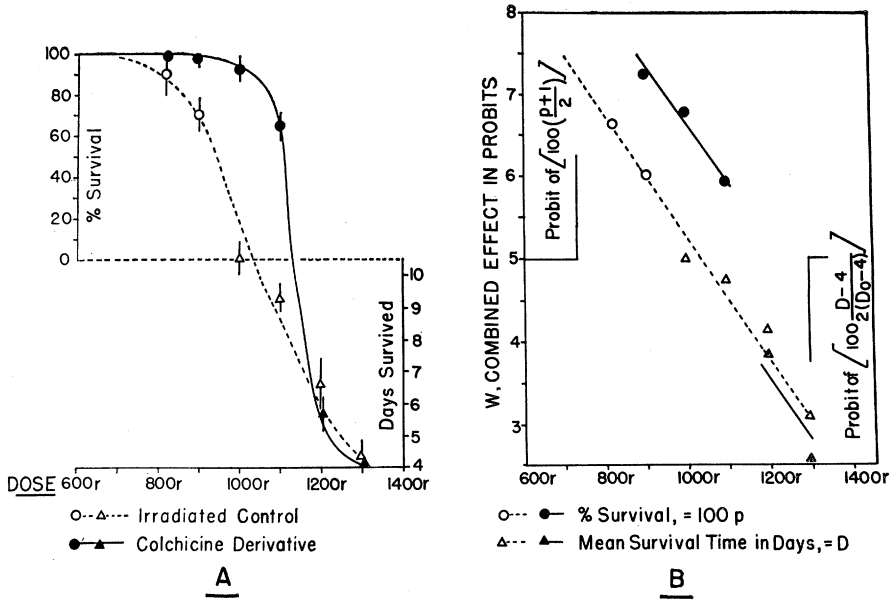

Fig. 1. (*A*) Plot of percentage survival and mean survival time by radiation dose for control mice and those treated with a colchicine derivative (1 mg of trimethylcolchicinic acid methyl ether *d*-tartrate, administered intraperitoneally 24 hours before irradiation). (*B*) Same data plotted in probits.