THE DETERMINATION OF THE TOTAL AND SPECIFIC ACTIVITY OF CALCIFIED TISSUE IN TRACER EXPERIMENTS USING ^{32}P. ADVANTAGES OF THE EXTRACTION OF THE INORGANIC PHOSPHATE WITH THE HELP OF A 10% TRICHLOROACETIC ACID SOLUTION

by

V. CLAASSEN AND B. S. J. WÖSTMANN

Laboratory of Physiological Chemistry of the Municipal University of Amsterdam and Netherlands Institute of Nutrition, Amsterdam (Netherlands)

In the course of our work on the phosphorus metabolism of the skeleton of the young rat it was necessary to study the total activity and specific activity of the inorganic fraction of the bone phosphate after intravenous injection of ^{32}P. To achieve this, two methods are generally used. In the first the dried and defatted bone is ashed and the ash dissolved in mineral acid. Aliquots of this solution are analyzed for ^{31}P and ^{32}P. As a result, all the phosphorus present in the defatted bone (still containing about 40% organic material) contributes to the values found for the specific activity. In the second method the bone is boiled for an hour or more in an ethyleneglycol-KOH mixture. Thus the organic part is hydrolyzed and extracted, leaving the inorganic part for ^{31}P and ^{32}P analysis. We found, however, that after boiling for one hour up to 17% of the total phosphorus might be present in the ethyleneglycol-solution. This makes it probable that, besides the organic material, at least some of the inorganic material must have been dissolved. As a result the phosphate analysis in the remaining inorganic part will turn out too low. From the extracted organic material now in solution, on the other hand, inorganic phosphate will have been liberated. In short time metabolism experiments with ^{32}P the specific activity of this originally organic fraction must be much lower than the specific activity of the inorganic phosphate at the surface of the bone crystals, as these surfaces are known to accumulate ^{32}P by exchange-adsorption from the inorganic phosphate of the plasma at a very rapid rate[1]. As a result losses of ^{32}P from the solid inorganic material will occur due to the fact that the exchange between phosphate groups in the solution and on the bone surface will desorb part of the ^{32}P originally accumulated in the bone.

To avoid these difficulties we extracted the fresh bone with trichloroacetic acid (T.C.A.), a possibility mentioned recently by Zetterström[2]. In this way only a very small amount of phosphorus containing esters is extracted besides the inorganic phosphate. This will have no practical influence on the inorganic phosphate content determined according to Fiske and Subarow. The rest of the organic material (mainly collagen, osseomucoid and fatty substances) is not decomposed and just left behind. During the extraction no exchange of any practical importance takes place between the phosphorus present in the residue and ^{32}P dissolved in the T.C.A.-solution.

After the bone has been dissected free of adhering flesh it is frozen in dry ice and pulverized. The powder is brought in a 50 ml beaker and extracted with 10 ml of a 10% T.C.A. solution at room temperature while stirring constantly. After two hours the T.C.A. solution is renewed and the extraction continued for another two hours. The extracts are combined, filtered and made up to 25 ml. Aliquots are analyzed directly for ^{31}P and ^{32}P.

We analyzed two series of six 200-mg-samples of fat-free bonepowder by both direct ashing (600° C) and by T.C.A. extraction. Direct ashing gave 14.0 ± 0.18 (stand. error) mg of P for a 200 mg sample. T.C.A. extraction gave a value of 14.5 ± 0.23 mg P while the residue left after extraction (digested with HNO_3–H_2SO_4) still contained 572 ± 32 microgram of P or 3.9% of the total P content. Thus the total P content amounted to 15.1 mg/200 mg bone and proved to be significantly higher than the value obtained by direct ashing (P value according to Wilcoxon < 0.01). This might be explained by losses due to volatilization of P during the dry ashing of the bone. The data prove that the T.C.A. extraction is in any case as good as other methods for determining the amount of inorganic phosphate present.

In another series of experiments we determined the extractable phosphate contents in the femora of six normal and eight rachitic rats and compared these with the amounts of P present in the residue after extraction. In normal femora of rats weighing around 100 g the extractable inorganic phosphate amounted to 10.2 ± 0.4 mg for one femur while 465 ± 58 micrograms of P were left behind, being 4.3 ± 0.54% of the total. In rachitic femora, with an average extractable inorganic phosphate of 5.0 ± 0.5 mg, 370 ± 31 micrograms or 6.9 ± 0.58% were left behind. Statistical treatment showed that there was no significant difference between the amounts of "organic" P found in rachitic and normal femora. In per cent of the total amount of phosphorus present, however, the average amount left behind after T.C.A.-extraction was significantly higher in the case of ricketts (P value < 0.01).

These data give an impression of the error made by ashing the whole, defatted bone when determining the specific activity of the bone inorganic phosphate. They also give an idea of the amount of "organic" phosphorus of comparatively low specific activity which, after being extracted by the ethyleneglycol-KOH solution, may be converted into inorganic phosphate and thus give rise to losses due to exchange with phosphate groups at the highly active crystal surfaces. In extracting the bone with T.C.A. these complications are avoided.

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THE N-TERMINAL SEQUENCE OF CARBOXYPEPTIDASE*

by

E. O. P. THOMPSON

Laboratory for the Study of Hereditary and Metabolic Disorders, University of Utah College of Medicine, Salt Lake City, Utah (U.S.A.)

When carboxypeptidase (five times recrystallized) was examined for N-terminal residues by the fluorodinitrobenzene (FDNB) method[1], the only dinitrophenylamino acids (DNP-amino acids) detected in acid hydrolysates[2] were DNP-aspartic acid, DNP-serine, DNP-threonine and ε-N-DNP-lysine. Quantitative separations on "Celite 545" columns[3] yielded approximately 0.7 residues of DNP-aspartic acid, 0.3 residues of DNP-serine, 17–18 residues of ε-N-DNP-lysine and only trace amounts of DNP-threonine per molecule of carboxypeptidase (M.W. 34,000)[4]. The yield of DNP-aspartic acid and DNP-serine from different preparations of DNP-protein varied somewhat but the sum of the yields of these two derivatives was always close to one residue.

Since the carboxypeptidase preparations were homogeneous by several criteria, the presence of an impurity with N-terminal serine and threonine residues seemed a less likely possibility than the occurrence of partial splitting of bonds involving the amino groups of these two amino acids; it is known[5] that such bonds are more labile than most peptide bonds. Attempts to obtain DNP-peptides by partial acid hydrolysis of DNP-carboxypeptidase were unsuccessful, only the above DNP-amino acids being obtained. This result suggested the presence of a labile bond adjacent to the N-terminal aspartic acid residue.

As DNP-aspartic acid is one of the slowest moving DNP-amino acids on buffered silica columns, attempts were made to isolate DNP-aspartyl (or DNP-asparaginyl) peptides from peptic digests of carboxypeptidase, subsequently coupled with FDNB. Two such peptides were identified, DNP-Asp(NH_2).Ser and DNP-Asp(Glu,Thr) or DNP-Asp(NH_2)(Glu,Thr). Since DNP-asparagine moves more rapidly than DNP-aspartic acid, from the rate of travel it seemed probable that the DNP-Asp(NH_2)Ser was in the amide form; the higher yield of this peptide also suggested that if either of these two sequences were the N-terminal sequence, it was probably this one.

The sequence Asp(NH_2).Ser- not only accounts for the failure to detect DNP-aspartyl peptides after partial acid hydrolysis but also explains the presence of DNP-serine in hydrolysates of the DNP-protein, since WEYGAND AND JUNK[6] have previously drawn attention to the lability of the N-terminal aspartic acid residues of "old yellow enzyme" during coupling with FDNB. When carboxypeptidase was coupled with FDNB under the conditions recommended by PORTER[2] (3 h shaking) and the mother liquors and washings (3 times with water, ethanol, ether) were examined for DNP-amino acids, DNP-asparagine was recovered in an amount almost exactly equivalent to the DNP-serine isolated from hydrolysates of the DNP-protein. In addition, trace amounts of DNP-

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