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

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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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serine and DNP-threonine were identified. An increase in the time of reaction to 24 hours did not increase the amount of DNP-asparagine set free.

WEYGAND AND JUNK[6] found that if the coupling was performed in buffer at pH 5.8 or pH 7, no DNP-aspartic acid was set free. When carboxypeptidase was coupled with FDNB in a 0.4 *M* phosphate buffer pH 7.1, DNP-asparagine (0.18 residues) still was liberated in an amount equivalent to the amount of DNP-serine found in the DNP-protein. Whereas "old yellow enzyme" was stable to aqueous sodium bicarbonate[6], carboxypeptidase was partially decomposed with the liberation of several amino acids or small peptides when shaken with a mixture of two parts ethanol and one part 10% aqueous sodium bicarbonate as during the reaction with FDNB. Thus, after coupling the material in the aqueous phase with FDNB, DNP-asparagine (0.2 residues) was isolated. This was the fragment obtained in highest yield and it was not possible to identify the other DNP-derivatives completely. After acid hydrolysis of these DNP-derivatives, however, DNP-aspartic acid, DNP-glutamic acid, DNP-serine, DNP-threonine, DNP-valine and DNP-leucine were identified on paper chromatograms[7]; this suggests a more extensive hydrolysis of peptide bonds than in the presence of FDNB. Similar results were obtained after incubation of the carboxypeptidase with di*iso*propyl fluorophosphate so that traces of chymotrypsin in the preparation were not producing this effect.

From these results it is evident that carboxypeptidase has a single polypeptide chain and that the N-terminal sequence is Asp(NH_2).Ser-. The next residue could be threonine, which would account for the free DNP-serine found in the mother liquors after FDNB treatment and the traces of DNP-threonine obtained in acid hydrolysates of the DNP-protein. A seryl-threonyl sequence is present in carboxypeptidase, since DNP-Ser.Thr was isolated from a chymotryptic digest of the protein subsequently coupled with FDNB. Experiments are in progress designed to confirm this sequence by an independent technique.

Both "old yellow enzyme" and carboxypeptidase, with N-terminal aspartic acid and asparagine residues, respectively, have shown liberation of DNP-aspartic acid and DNP-asparagine during coupling with FDNB in aqueous sodium bicarbonate solution. This is not a general property of all proteins which have N-terminal aspartic acid residues since human serum albumin which has a single N-terminal aspartic acid residue[8], was stable when treated with FDNB in the same way as was carboxypeptidase. One residue of DNP-aspartic acid was detected in acid hydrolysates in agreement with the previous work. Other proteins with N-terminal aspartic acid residues which are being studied in this laboratory[9] have also been shown to be stable during coupling with FDNB.

It is noteworthy that the mild treatment with bicarbonate solution is capable of splitting peptide bonds in carboxypeptidase and this possibly occurs with other proteins. BAILEY[10] has pointed out that the DNP procedure is one of the most sensitive techniques for the determination of protein purity but, if this criterion is to be used, it is essential that all the peptide bonds in the protein be stable under these conditions.

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