A NOVEL METHOD FOR THE DETERMINATION OF C-TERMINAL AMINO ACID IN POLYPEPTIDES BY SELECTIVE TRITIUM LABELLING

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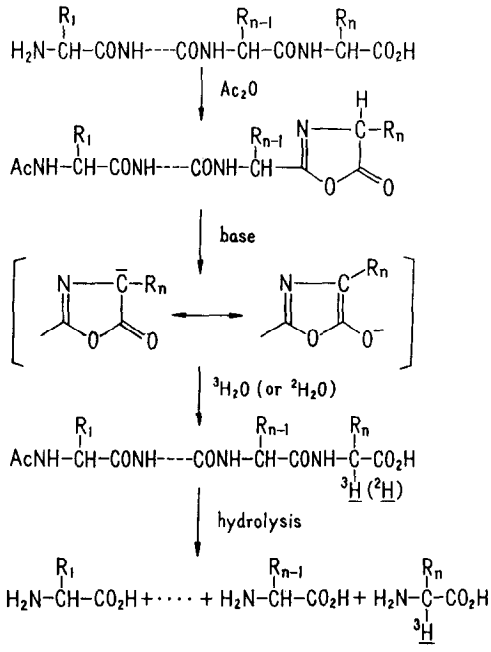
In our previous communication (Matsuo, 1965), we reported selective ^{2}H- and ^{3}H-labelling reaction at the C-terminal amino acid of several N-acetylpeptides through the C-terminal racemization mechanism. The selective deuteration or tritiation is based upon the C-terminal oxazolone formation in peptides by the action of acetic anhydride or dicyclohexylcarbodiimide, followed by the base-catalyzed hydrolytic ring-opening in deuterium oxide or tritium oxide as shown in the following scheme.

The above selective C-terminal tritiation has now been applied to the determination of the known C-terminal amino acids of angiotensin II (Skeggs, 1955) and beef-insulin (Sanger, 1955). The experimental results described in this communication will indicate that the C-terminal determination of polypeptides or proteins may be accomplished in a simple manipulation and in a micro-scale by this method.

A modification of the above procedure for identifying C-terminal aspartic acid and proline, which were two exceptions that resisted the oxazolone formation by the above method, will also be described.

C-Terminal Determination of Angiotensin II

A solution of angiotensin II (Hypertensine Ciba, Product of



Ciba Ltd.)*1(Asp-Arg-Val-Tyr-Val-His-Pro-Phe) (0.5 mg) in a mixture of acetic anhydride (2 ml) and anhydrous dioxane (2 ml) was brought to gentle boiling for 30 min. The mixture was allowed to cool to room temperature and evaporated in vacuo at 40° to dryness. The residue thus obtained was treated with one drop of pyridine and 1.0 ml of dimethylformamide containing 0.05 ml of $^{3}H_2O$ (ca. 50 mC) for 1 hr at room temperature. After evaporation in vacuo at 40°, addition of ordinary water, followed by evaporation, was repeated several times to remove completely the washable radio-isotope. The residue was hydrolyzed in 18% HCl at 110° for 8 hr and a small portion of the hydrolysate thus obtained was subjected to ascending paper-chromatography, using n-BuOH-AcOH-H_2O (4:2:1). Detection of radioactive spots on the paper-chromatogram was performed by the aid of Radio-chromatogram-scanner (Packard Model 7200). The radio-chromatogram

*1 The authors thank Professor H. Moriya, Tokyo College of Science, for this generous gift.

gave only a single radioactive spot (Rf 0.63), corresponding to the C-terminal phenylalanine, which was identified by direct comparison with a chromatogram colored by the Ninhydrin reagent (Fig. 1). For the purpose of further characterization, the radioactive spot was cut off, extracted with water, and re-chromatographed with two solvent-systems, n-BuOH-AcOH-H_2O (4:1:2) and phenol saturated with water. By direct comparison with paper-chromatograms of an authentic specimen of phenylalanine, the radioactive material was proved to consist of only phenylalanine.

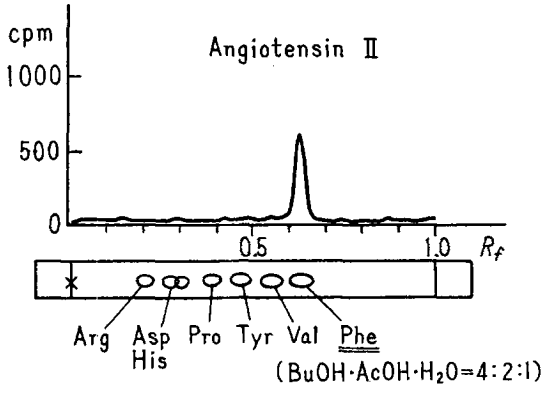


Fig. 1

Radio-chromatogram of the Hydrolysate of Angiotensin II scanned by Packard Radio-chromatogram-scanner (Model 7200)
Chart Speed : 1 cm/min

C-Terminal Determination of Beef-insulin

Since insulin is insoluble in non-polar solvent, the standard procedure described above, had to be modified in such a way that oxazolone formation and racemization took place in one step in aqueous media.

Crystalline beef-insulin*2 (2 mg) was dissolved in a mixture of 0.1 ml of ^3H_2O (ca. 100 mC) and 0.2 ml of pyridine with slight warming. After cooling to room temperature, 0.05 ml of acetic anhydride was added and the whole was kept at room temperature for 2-3 hr. After evaporation in vacuo at 40° and complete re-

*2 Crystalline beef-insulin used was donated from National Institute of Hygiene through the courtesy of Dr. S.Takenaka to whom our thanks are due.

moval of the washable isotope, the residue was subjected to performic acid-oxidation and then hydrolyzed in constant-boiling HCl for 24 hr. Paper-chromatography (phenol saturated with water) of a small portion of the hydrolysate gave two radioactive spots (Rf 0.20 and 0.60). By re-chromatographing extracts of the two radioactive spots along with authentic specimens in two solvent-systems, n-BuOH-AcOH-H_2O (2:1:1) and n-BuOH-pyridine-AcOH-H_2O (15:10:3:12), one of the two radioactive spots was identified as aspartic acid, a hydrolysis product of the C-terminal asparagine of the A-chain, while the other was identified as alanine, the C-terminal of the B-chain.

Determination of C-Terminal Aspartic Acid and Proline

In the C-terminal determination of insulin described above, it would be difficult to decide whether the radioactive aspartic acid had derived from aspartic acid or asparagine at the C-terminal end, if C-terminal aspartic acid as well as asparagine underwent the tritiation under the same conditions. In preliminary experiments, the deuterium exchange at the α-carbon of N-acetylaspartic acid and N-acetylasparagine was investigated by the aid of nuclear magnetic resonance technique (Matsuo, 1965). It was found that N-acetylaspartic acid, when subjected to the action of acetic anhydride, was converted to N-acetylaspartic anhydride (Barker, 1953), and not to the oxazolone derivative, unlike N-acetylasparagine. This anhydride resisted the deuteration at the α-carbon by the base-catalyzed procedure, but could incorporate deuterium by treatment with [carboxy-^{2}H]acetic acid. Therefore, it was anticipated that the C-terminal aspartic acid would not be tritiated by the procedure involving the base-catalyzed ring-opening of oxazolone, but would be labelled at the α-carbon if treated with a mixture of acetic anhydride and ^3H_2O.

A tripeptide, Pro-Lys-Asp[*3](2 mg), was refluxed for 45 min in a mixture of acetic anhydride (3 ml) and ^3H_2O (0.05 ml, ca. 50 mC) and, after removal of the washable isotope, the residue was hydrolyzed with 18% HCl and then worked up as described above. The result showed that radioactivity was located only at a spot corresponding to aspartic acid.

There is a similar problem in the determination of C-terminal proline, since C-terminal proline does not appear to form oxazolone by the action of acetic anhydride. In fact, peptides having C-terminal proline did not undergo tritiation under base-catalyzed conditions. However, the same procedure as in the case of C-terminal aspartic acid was successfully applied to a heptapeptide, Phe-Arg-Try-Gly-Ser-Pro-Pro[*3], by using a mixture of acetic anhydride and ^3H_2O, and proline was found to be the only radioactive amino acid obtained after hydrolysis. It is most likely that the racemization mechanism in the case of C-terminal aspartic acid or proline is different from the one involving the oxazolone formation in other cases.

In view of the data of the ^2H- and ^3H-labelling experiments described in the previous and present papers, one should be able to identify C-terminal Gly, Ala, Val, Leu, Ileu, Phe, Tyr, Try, Arg, Met, Ser, Thr, CySH, Glu[*4], Glu(NH_2) and Asp(NH_2) under the base-catalyzed conditions, while C-terminal Asp and Pro are determined by the modified method. Peptides having C-terminal Cys, Lys and His have not yet been examined.

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*3 The authors are indebted to Professor H.Yajima, the University of Kyoto, for generous gifts of these synthetic peptides.

*4 C-Terminal glutamic acid could be labelled under base-catalyzed conditions, making itself indistinguishable from C-terminal glutamine in this case, contrary to the case of aspartic acid.

The selective C-terminal labelling procedure presents a novel method for the C-terminal determination of polypeptides and particularly it will have a useful advantage that both the C-terminal determination and constituent amino acid analysis can be accomplished simultaneously in aqueous or non-aqueous media.

Work is continuing in this laboratory to study further application, scope and limitation of this method.

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