## A Novel Method of a Peptide Bond Formation

The conventional methods of synthesizing peptide bonds require rather laborious chemical protection and activation of the functional groups in amino acids and peptides concerned. Although automating devices have been introduced recently, more simplified methods are desirable as daily laboratory practice to synthesize simple peptides. Such a case may be illustrated by the synthesis of a dipeptide, carnosine or β-alanyl-1-histidine[1].

In the present work, ion exchange resins have been used to synthesize dipeptides. The intention of using the resin is quite different from the recently developed solid phase peptide syntheses[2]; in our case the ion exchange resins are used as the catalyst of the peptide bond formation.

Positively charged amino group, $-NH_3^+$, in an amino acid is adsorbed on a cation exchange resin and it is 'eluted' with the acid group of another amino acid. When the first amino acid is eluted, the naked or unprotected active amino ion reacts with the carboxyl group of the second amino acid. In such a manner, probably, an ammonium salt of the amino acid is formed primarily, and this is then converted to the amide or peptide linkage on heating. When the first amino acid is adsorbed on a resin, it is thought to be activated by the resin and the heat of adsorption of the second amino acid or heat of desorption of the first amino acid on or from the resin, or both, may contribute to the formation of the ammonium salt which eventually is converted to the peptide bond.

The main purpose of this study was to synthesize carnosine for practical interests, and a more detailed study was performed for carnosine. Then the method was extended to other dipeptides: glycylglycine, glycylphenylalanine, glycylserine, leucyltyrosine and some amino-blocked dipeptides.

As stated above, the reactions may be proceeded in the following general manner. However, detailed kinetic studies were not yet made.

Theoretically, both the cation and anion exchange resins may be used to activate amino and carboxy groups respectively, but the preliminary experiments revealed that the cation exchange resin is more suitable. The resin used was mainly Dowex 50 × 2, $H^+$ form, which was activated by the usual method. Amberlite IRA-400 was used as the anion exchange resin.

In the present investigation, the minimum protection of a functional group on the first amino acid adsorbed on a resin was necessary, and thus the carboxy group was transformed to its ester, in the case of histidine, but it was found later that more simple amino acids do not require such protection. The resin used in this study was a cation exchange resin, and an amino group blocked amino acid did not naturally form the peptide bond since it was not adsorbed on the resin. The possibility of peptide formation using an anion exchange resin with amino group blocked amino acids is now under investigation.

Results performed for carnosine methyl and ethyl esters are listed in Table I. The method using a cation exchange resin was extended to other simple dipeptides, containing relatively neutral amino acids (isoelectric point range, 5.48–5.98), Table II.

In preliminary experiments, the formation of the peptide was followed by paper chromatography. The first amino acid was adsorbed on a resin, and the resin was washed with distilled water after heating the mixture of the resin and amino acid, so that no free amino acid molecule was detected. The amino acid adsorbed resin was then suspended in 95% ethanol and a slightly acidic ethanolic solution of the second amino acid was added to the suspension. The peptide formation was checked at intervals taking the aliquot, and peptide bond formation was detected 10 min after initiating the reaction. Spots on the paper strip corresponding to the peptides were eluted with the developing solvent of paper chromatography, acetic acid–*n*-butanol-water (1:4:5, upper layer), and the yield was determined spectrophotometrically by colouring the eluent with ninhydrin at appropriate wave-lengths for individual peptides against the standard peptides.

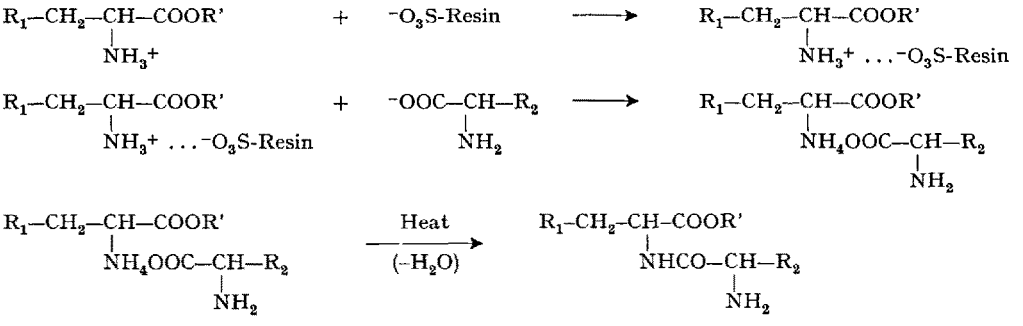


Table I. Studies of peptide bond formation on carnosine

| Mode of reaction | Yield (%) | |
|---|---|---|
| | Batch | Column |
| Histidine (free) on resin (C)[a] + β-alanine | 0 | 0 |
| Histidine HCl on resin (C) + β-alanine | 0 | 0 |
| Histidine Et ester on resin (C) + β-alanine | 5 | 30 |
| Histidine Me ester on resin (C) + β-alanine | 10 | 50 |
| Histidine Me ester + β-alanine on resin (A)[b] | 0 | 0 |
| Histidine Me ester + β-alanine | 0 | 0 |

[a] Cation exchange resin; [b] anion exchange resin. The yields were calculated based on the first amino acid adsorbed on the resin.

[1] R. H. Sifferd and V. du Vigneaud, J. biol. Chem. *108*, 753 (1935).

[2] R. B. Merrifield, J. Am. chem. Soc. *89*, 2149 (1963).

In a preparative experiment [mg scale] reactions were carried out in the following manner. The first amino acid was dissolved in 0.01 *N* hydrochloric acid, and the solution was added to the activated ion exchange resin. The mixture was heated for 10 min. The excess amino acid was removed by filtration after cooling the mixture and the resin was washed with cold water until ninhydrin positive material was removed. The resin was then put into a Meyer flask or packed in a glass column with a heating jacket. The second amino acid was dissolved in slightly acidic (hydrogen chloride) ethanol or methanol, and mixed with the resin in the Meyer flask or passed through the heated column slowly. The temperature of reaction, both by the column and the batch method, was about 60° (± 5°). Heating in the flask was made for about 15 h. Yields of the product were better in the column method than in the batch method.

*Zusammenfassung.* Eine neue Methode der Peptidsynthese, z.B. von Carnosin, GlySer, GlyGly, LeuTyr und GlyPhe mit Ionenaustauschharzen als Katalysatoren wird beschrieben.

S. YAMASHITA and N. ISHIKAWA

*Biochemistry Division, Hoshi College of Pharmacy, Shinagawa-Ku, Tokyo (Japan), 27 May 1968.*

Table II. Comparison of Rf values and colour spots (by ninhydrin) of the reactants and the products on paper chromatograms and yield of the products determined spectrophotometrically

| Reactant and product | Rf[a] | Colour of spot[b] | Yield of the product |
|---|---|---|---|
| β-Alanine | 0.05 | Indigo | |
| Histidine HCl | 0.10 | Violet | |
| Histidine Me ester | 0.18 | Violet | |
| Carnosine Et ester | 0.23 | Sky blue | [c] |
| Carnosine | 0.35 | Sky blue | |
| Carnosine Me ester | 0.37 | Sky blue | [c] |
| Glycine | 0.17 | Violet | |
| Serine | 0.21 | Violet | |
| Leucine | 0.60 | Violet | |
| Tyrosine | 0.44 | Violet | |
| Phenylalanine | 0.55 | Violet | |
| Glycylglycine | 0.23 | Violet | 70%[d] |
| Glycylserine | 0.20 | Violet | 25% |
| Leucyltyrosine | 0.29 | Violet | 30% |
| Glycylphenylalanine | 0.54 | Violet | 50% |

[a] AcOH:*n* BuOH:$H_2O$ = 1:4:5, upper layer. [b] By ninhydrin. [c] Yield was obtained spectrophotometrically at 467 mμ and listed in Table I. [d] Yield was obtained spectrophometrically at 570 mμ in the column method.

## A Simple Method for Estimating Melanophore Responses to Drugs in Fishes

In a study of the reactions of fish melanophores to autonomic drugs, the need arose for a speedy, simple method for estimating the melanophore responses without handling or disturbing the fish unnecessarily. In the majority of teleost fish studied to date, the movements of pigment within the skin melanophores are coordinated by the sympathetic nervous system. In addition blood-borne pituitary hormones affect the state of melanophore pigment in similar, but slower fashion. These changes are conventionally referred to as 'physiological' changes, to distinguish them from the much slower 'morphological' changes in numbers of melanophores in the skin which follow prolonged exposure to one shade of background[1,2].

Recordings of the amount of melanophore response have been made in the past by observing the melanophores microscopically[3], or by observing the overall shade of the fish[4]. However, in the fish under study, *Phoxinus phoxinus* (L), handling the fish for microscopical examination brings about fluctuations of melanophore state[5]. The pharmacological study was carried out using the Derived Ostwald Index (DOI) of HEALEY[6] to discover whether standard grey papers could be used to estimate melanophore responses accurately. It was, however, necessary to discover the effects of morphological changes and of temperature on the observed shade of the fish.

Minnows were collected from Hertfordshire and kept in white or black stock tanks in the laboratory as described by HEALEY and Ross[6]. Fish from a stock tank were placed in beakers of tap water on trays painted the same shade as the stock tank and illuminated from above. The shades of the fish were estimated using the shade standards, and the beakers transferred to the opposite (black or white) background. Estimates of the shade of the fish were then made at intervals during which time the tap water was slowly changed to prevent fouling. Figures 1–4 represent the shade changes of the fish subjected to background reversal at different temperatures and after different histories on a particular background. Increase in DOI value indicates darkening, and vice versa, following the numerical convention adopted by HOGBEN[3] for his Melanophore Index (MI) based on microscopical observation of melanophores. It is notable that prolonged exposure to 1 background or lowering the temperature slows background adaptation. The former effect is probably due to changes in overall pigmentation of the minnow which masks the physiological changes whilst changes in environmental temperature of poikilothermic animals are likely to affect metabolism in the effector cells[7]. Too brief an exposure to 1 background (Figures 2 and 4), whilst allowing nervously coordinated responses to occur, fail to allow the circulating pituitary hormone titres to adapt to the new environment[5]. Accord-

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