XXXI. CARNOSINE, CONSTITUTION AND SYNTHESIS.

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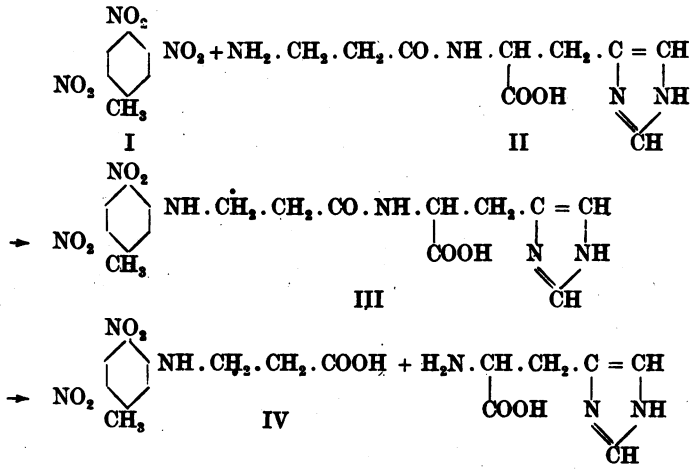
While engaged on work relating to the physiological behaviour of the trinitrotoluenes we observed that the β- and γ-isomerides condense with amino-acids on boiling in dilute alcoholic solution; the amino-acid becomes attached to the benzene ring by its amino-group, in replacement of a reactive nitro-group, which is eliminated. This reaction does not occur with imino-groups, whether present in a glyoxaline ring or in peptides, but does take place with the free amino-groups of peptides. The resulting compounds which are therefore *N*-dinitrotolylamino-acids resist boiling with concentrated hydrochloric acid and hence this reaction provides a suitable means of determining the constitution of peptides, for it is only necessary to combine the peptide with β- or γ-trinitrotoluene and hydrolyse the reaction product, when only those amino-acid radicles which originally had free amino-groups remain attached to the benzene ring, and these can therefore be identified. We applied this reaction to the dipeptide carnosine. Gulewitsch [1911] has shown that this base, one of the most important of those occurring in muscle extracts, is an anhydride of histidine and β-alanine, but whether β-alanylhistidine or histidyl-β-alanine, remained undecided.

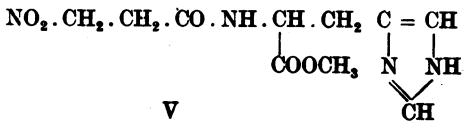
This question had formerly occupied one of us in conjunction with Dr A. J. Ewins, and some experiments on the hydrolysis of deaminocarnosine, which the latter carried out in 1914, and has kindly allowed us to quote below, indicated that carnosine is β-alanylhistidine. It was, however, impossible to isolate the deaminocarnosine in a pure condition or to obtain more positive evidence of the deamination by isolating β-hydroxypropionic or acrylic acid after hydrolysis. The condensation product of γ-trinitrotoluene with carnosine on hydrolysis furnished dinitrotolyl-β-alanine in a

pure condition, without any trace of the corresponding histidine compound, thus proving carnosine to be β-alanylhistidine and this result was communicated to the Biochemical Society at a meeting at the Lister Institute in December, 1917.

The reactions involved may be formulated as follows:



γ-Trinitrotoluene is 2 : 4 : 5-trinitrotoluene, in which the 5-nitro-group is labile, as shown by the production of 2 : 4-dinitrotoluene by the action of ammonia, diazotising and boiling with alcohol. Its condensation product (III) with carnosine (II) is a carboxylic acid and (owing to the presence of the glyoxaline ring) also a base; it is therefore insoluble in ether. It is hydrolysed by boiling acids to IV, a carboxylic acid without basic properties, and soluble in ether, which was found to be identical with the substance obtained by condensing β-alanine with γ-trinitrotoluene. This result encouraged us to attempt the synthesis of carnosine. The action of ammonia on β-chloropropionylhistidine (prepared like the α-bromo-isocaproyl derivative of Fischer and Cone [1908]) did not yield the desired result. We therefore condensed histidine methyl ester with β-nitropropionyl chloride, and reduced the resulting product (V). After hydrolysis of the



ester grouping this yielded carnosine, which we eventually isolated with considerable loss as the copper salt and identified. Our single experiment on a small scale yielded only 19 mg. of the copper salt, and being mainly occupied

with questions of a more practical kind, we did not repeat the experiment on a larger scale. Subsequently our attention was called to a paper by Baumann and Ingvaldsen [1918] who had also determined the constitution of carnosine and obtained the synthetic base in much larger quantity and in the free state. As our methods both of establishing the constitution, and of synthesis, differ from those given by Baumann and Ingvaldsen, we consider it advisable to publish our rather incomplete experiments.

A. Constitution.

Experiments by Dr A. J. Ewins (1914). 0·1 g. of carnosine was kept for two days at room temperature with one molecular proportion of silver nitrite and of hydrochloric acid. After filtration from silver chloride, a small amount of silver was removed from the filtrate by hydrogen sulphide. The solution was then concentrated to a small bulk and alcohol was added, when a gum separated. This was dissolved in concentrated hydrochloric acid and boiled for 6 hours, then evaporated to dryness. After redissolving in a little water and filtering from an amorphous impurity, the filtrate on standing deposited large colourless crystals of histidine dihydrochloride, M.P. 260°. The salt gave a picrate, M.P. 90–93°. In another experiment with 1 g. of carnosine and 1½ molecular proportions of silver nitrite and hydrochloric acid numerous attempts were made to crystallise the deaminocarnosine, without success; nor did it yield a crystalline phosphotungstate. The yield of histidine dihydrochloride was from the main bulk 0·55 g. or 55 per cent. of the theory.

Action of γ-trinitrotoluene on carnosine. 0·5 g. of γ-trinitrotoluene and 1·0 g. of carnosine (two mols.) were dissolved in slightly diluted alcohol and boiled under a reflux condenser for one hour. As in all similar cases the liquid soon became deep yellow in colour. After evaporation on the water-bath, absolute alcohol was added and the evaporation was repeated to remove the small amount of water present. A bright yellow amorphous solid separated, very little soluble in absolute alcohol, even on boiling, but readily soluble in water. When damp with alcohol it was very deliquescent, but no longer so after washing with dry ether. This substance may be regarded as the carnosine salt of the condensation product of carnosine with γ-trinitrotoluene (III). It was dissolved in water and acidified with acetic acid when the condensation product itself was thrown down as a bright yellow precipitate, resembling the corresponding product from histidine and γ-trinitrotoluene. It was quite insoluble in ether, very little soluble in absolute alcohol and boiling water,

much more readily in boiling 50 per cent., alcohol. On concentrating its solution in the latter solvent it separated in yellow granules but could not be obtained distinctly crystalline.

Hydrolysis of the condensation product; 0·3 g. was boiled for 5 hours with 30 per cent. aqueous sulphuric acid under a reflux condenser, when all but a trace of dark coloured resinous material had dissolved. The liquid was extracted with ether which removed a yellow substance. The ethereal solution was extracted with a small quantity of dilute ammonium carbonate and the resulting deep yellow alkaline extract acidified with sulphuric acid when a yellow solid separated. The latter crystallised from hot water or dilute alcohol in tufts of small, bright yellow needles, melting at 166°. The substance was evidently not the condensation product of γ-trinitrotoluene with histidine, which is insoluble in ether and decomposes violently at 265°, but rather the condensation product of γ-trinitrotoluene with β-alanine. This was synthesised and isolated in a similar manner, and found to be identical with the substance from carnosine. A mixture of the two melted at 166°. The synthetic compound was analysed.

0·1291 g.; 0·2102 CO_2; 0·0500 H_2O. C = 44·4, H = 4·3.
Calc. for $C_{10}H_{11}O_6N_3$. C = 44·6, H = 4·1.

B. Synthesis.

We first attempted the synthesis in the way used by Fischer and Cone [1908] for the synthesis of leucylhistidine, and accordingly acted on histidine methyl ether with β-chloropropionylchloride. The crude condensation product, which could not be crystallised, was treated with ammonia at various temperatures; this did not result in the formation of any appreciable quantity of carnosine, but apparently of an unsaturated compound. With copper carbonate we obtained indications of carnosine copper salt, but the use of β-chloropropionyl chloride had to be abandoned. Baumann and Ingvaldsen found the same difficulty but were successful when they used the chloride of β-iodopropionic acid. We met this difficulty by using the chloride of β-nitropropionic acid, and reducing the compound formed. From glycerol we prepared successively glyceric acid, β-iodopropionic acid and β-nitropropionic acid, the last named according to Lewkowitsch [1879]. β-Nitropropionyl chloride had not been prepared hitherto; we heated the acid with excess of thionyl chloride on the water-bath for two hours, removed the excess of thionyl chloride by gently warming in a vacuum, and then distilled the residue in small quantities at a time by plunging the flask evacuated to

10 mm. into a metal bath previously heated to 160°. The object of this precaution was to limit the duration of heating, as otherwise the chloride is apt to decompose. We thus obtained an almost colourless liquid, B.P. 123°/10 mm.

0·1425 g.; 0·1511 AgCl. Cl = 26·2

Calc. for $C_3H_4O_3NCl$. Cl = 25·8.

The β-nitropropionyl chloride was next condensed with histidine methyl ester. 2 g. of the acid chloride in 20 cc. of dry chloroform was added gradually, with vigorous shaking, to a chloroform solution of rather less than two equivalents of the ester, cooled in a freezing mixture. As soon as the mixture ceased to give an alkaline reaction, the addition was discontinued and the chloroform solution of the condensation product was decanted from the histidine methyl ester monohydrochloride, which had separated. It was found impossible to isolate the condensation product itself since it decomposed on gently warming the solution, and quite appreciably during a few hours at ordinary temperatures. It was therefore at once reduced, by adding excess of stannous chloride in dilute hydrochloric acid to the above mentioned chloroform solution; the chloroform was removed by very gentle warming under reduced pressure. The temperature was then gradually raised and finally maintained at 100° for one hour. After cooling, the tin was removed as sulphide, and the filtrate was made slightly alkaline and kept over night to hydrolyse the ester. On evaporation to dryness the residue, consisting largely of sodium chloride, was extracted with methyl alcohol, which was distilled off, leaving a viscid residue of crude carnosine. As it could not be crystallised, we precipitated with phosphotungstic acid and obtained on regeneration 0·5 g. of a base, which on digestion with copper carbonate gave a deep blue solution. The latter was concentrated and deposited first an amorphous copper salt which was removed from time to time. Finally, on keeping the concentrated mother liquor for some days, ill-formed six-sided crystals separated, which were recrystallised and then yielded deep blue hexagonal plates, characteristic of the copper salt of carnosine. We finally only obtained 19 mg. of the pure salt. The recrystallisation is very troublesome and attended with great loss, as was pointed out by Gulewitsch and Amiradzibi [1900]. Perhaps we would have done better to attempt the crystallisation of the nitrate of the base regenerated from the phosphotungstate. The copper salt was dried *in vacuo* over sulphuric acid for some months, and was then analysed by Pregl's micro-Kjeldahl method [see Abderhalden, 1912].

I. 2·85 mg.; 2·65 cc. *N*/70 HCl. N = 18·6,

II. 5·7 mg.; 5·0 cc. *N*/70 HCl. N = 17·5.

For the sake of comparison natural carnosine copper salt was also analysed.

3·25 mg.; 3·0 cc. *N*/70 HCl. N = 18·5.

Calc. for $C_9H_{14}O_3N_4 . CuO$. N = 18·3.

We also determined the rotation. It was considered inadvisable to attempt the removal of the copper from so small a quantity. Hence we merely dissolved the salt in excess of dilute acid.

9 mg. Cu salt in 200 mg. 13 per cent. sulphuric acid gave a pale blue solution containing 6·6 mg. carnosine.

$c = 3·3$ per cent.; $l = 0·5$ d.m.; $a_D = + 0·15°$; $[\alpha]_D = + 9°$.

Gulewitsch [1913] gives for carnosine nitrate dissolved in excess of nitric acid, $[\alpha]_D = + 12°$ to 13°.

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