THE INVERSION OF A DIPEPTIDE SEQUENCE DURING HYDROLYSIS IN DILUTE ACID

by

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In deducing the amino-acid sequence of a protein or polypeptide chain from the structure of the peptide fragments produced on partial hydrolysis, it is essential that the sequence present in the breakdown product is the same as was present in the original molecule. It has been pointed out[1] that an inversion of the order of amino-acids in a dipeptide could theoretically occur via the diketopiperazine. Thus if a dipeptide AB, which is less stable than the corresponding peptide BA, is exposed to conditions which catalyse ring formation, a conversion of AB to BA might be expected according to the equation;

$$AB \rightleftharpoons \begin{bmatrix} A \\ B \end{bmatrix} \rightleftharpoons BA$$

where $\begin{bmatrix} A \\ B \end{bmatrix}$ represents the diketopiperazine. Such inversion could occur without the accumulation of the intermediate diketopiperazine, since in general diketopiperazines are more labile than the corresponding dipeptides. In order to see if this reaction does in fact take place we have studied the effect of various conditions of "hydrolysis" on the dipeptide glycylvaline (Gly.Val), which is considerably less stable than the corresponding inverted peptide valylglycine (Val.Gly)[2,3]. ABDERHALDEN AND KOMM[4] showed that ring formation occurs at high temperatures in the presence of dilute acid, and the following experiment demonstrates that inversion can take place when hydrolysis is carried out in 0.1 *N* HCl at 100°.

Gly.Val (1 mg) was boiled under reflux with 0.1 *N* HCl (1 ml). Samples (0.1 ml) were withdrawn after 6, 11, 23 and 30 hours, evaporated to dryness on polythene and applied to a paper chromatogram together with control samples of Gly.Val and Val.Gly. The chromatogram was developed with collidine and better resolution could be obtained by attaching a folded half-sheet of filter paper to the bottom of the chromatogram so that the effective distance run by the solvent front was 1½ lengths. Fig. 1 is a diagram of the resulting chromatogram of the 24 hour sample and controls after spraying with ninhydrin. Besides spots corresponding in R_F value to glycine (R_F = 0.18), valine (R_F = 0.34) and unchanged Gly.Val (R_F = 0.40), a spot was present which had the same R_F value (0.45) as Val.Gly. Samples of the two faster-moving spots were collected from replicate chromatograms, using marker strips (sprayed with ninhydrin) from the same paper to locate the spots. The eluates of these spots were treated with 1:2:4-fluorodinitrobenzene as described by SANGER AND TUPPY[5]. The dinitrophenyl (DNP) peptides were extracted from the acidified reaction mixture into ether, and after removal of the solvent subjected to hydrolysis in boiling 5.7 *N* HCl for 3 hours. The hydrolysates were extracted with ether, and the DNP-amino-acids present in the extract identified using a buffered paper chromatogram developed with tertiary amyl alcohol[6]. The amino-acids remaining in the aqueous solution were identified on a phenol chromatogram. The results are given in Table I. They clearly show that the peptide with R_F = 0.45 was Val.Gly and that an inversion of the peptide Gly.Val had occurred during "hydrolysis" in boiling 0.1 *N* HCl.

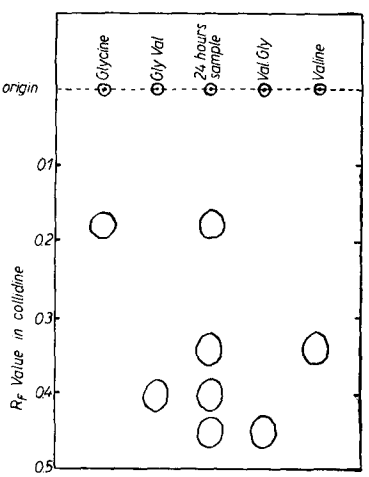

Fig. 1. Paper chromatogram of "partial hydrolysate" of Gly.Val.

These experiments indicate the need for caution in interpreting the results of partial hydrolysis experiments using dilute acid. During work on insulin[5,7] this type of hydrolysis has been used since it shows a rather different specificity from that shown by concentrated acids. However, no fundamental conclusions have been drawn from the structure of dipeptides identified in such a hydrolysate, and in fact no evidence of any inversion was obtained. Partial acid hydrolysis is normally carried out with 12 *N* HCl at 37° and under these conditions no inversion of Gly.Val could be demonstrated.

TABLE I

PROPERTIES OF HYDROLYSATES OF DNP-PEPTIDES

RF value of spot	*Products of hydrolysis of DPN peptide*	
	Ether extract	*Aqueous residue*
0.40	DNP-glycine	Glycine (?) valine (xx)
0.45	DNP-valine	Glycine (x) valine (?)

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6-HYDROXY NICOTINIC ACID AS AN INTERMEDIATE IN THE OXIDATION OF NICOTINIC ACID BY *PSEUDOMONAS FLUORESCENS*

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The ability of many strains of *Pseudomonas fluorescens* to oxidise nicotinic acid was first reported by ALLINSON[1]. Later studies[2,3,4] indicated that the oxidative system is adaptive and that CO_2 and ammonia are among the end products. There were however no indications of the intermediate steps of the oxidation. The following experiments show that 6-hydroxy nicotinic acid is an intermediate.

Five strains of *Pseudomonas fluorescens* isolated from soil were grown on a medium containing salts, 2% yeast extract and 0.1% nicotinic acid. The cells were washed and the oxidation of the nicotinic acid was followed manometrically in the Warburg apparatus. All strains oxidised both nicotinic acid and 5-fluoro nicotinic acid (cf. HUGHES[5]) at approximately the same rate (0.5–1.0 μmol nicotinic acid/mg dry wt./hr), and to the same extent (3.5–3.9 μmol O_2/μmol nicotinic acid), but none of the strains oxidised 6-fluoro nicotinic acid under the conditions tested. Variable results were obtained with 2-fluoro nicotinic acid but some batches of all the strains were able to oxidise the 2-fluoro-analogue with an uptake of 0.5 to 1.5 μmol O_2/μmol 2-fluoro nicotinic acid. These results suggested that the carbon atom six was important in the primary oxidative attack. Search for intermediates in the oxidation of the nicotinic acids in adapted cells was unsuccessful. Experiments were therefore carried out with cells not previously grown on nicotinic acid and the products of the oxidation examined as the cells became adapted. The non-adapted cells were grown on a medium containing 0.1% asparagine in the place of the nicotinic acid previously used. The cells were washed and placed in the Warburg apparatus suspended in 0.2 *M*-phosphate buffer, pH 6.5; 0.01 *M*-NH_4Cl and 0.025 *M*-nicotinic acid. No extra oxygen uptake over the endogenous respiration was found until 90–120 min had elapsed, after which the rate of oxygen uptake increased slowly until 0.5–0.8 μmol of extra O_2/μmol nicotinic acid had been taken up; then the rate of additional O_2 uptake increased until all the nicotinic acid was used. The final oxygen uptake varied between 3.8 and 4.0 μmol O_2/μmol nicotinic acid. During the initial period of increased oxygen uptake the presence of an intermediate was detected by paper chromatographic methods. This intermediate did not react with CNBr and *p*-aminobenzoic acid[6] but had an absorption curve between 215 and 300 mμ, typical of pyridine derivatives. This suggested that during the first slow extra oxygen uptake the pyridine ring was modified by substitution. From the results with the fluoro analogues it seemed likely that the substitution was in the six position and arguing by analogy to the oxidation of the benzene ring[7] it seemed possible that a hydroxylation occurred. Synthetic 6-hydroxy nicotinic acid was