ted N-benzoyltyrosine. Complications arise from the fact that aniline, which is produced by the competing hydrolysis of phenyl isocyanate, also reduces the Folin reagent. Correction for this hydrolysis was made in a blank run containing no N-benzoyltyrosine and curve II (Fig. 1b) was obtained. Examination of the curve indicates that the phenylcarbamoyl derivative initially formed undergoes hydrolysis at the pH of the reaction (8.50). The aniline formed in the latter hydrolysis causes an apparent increase in the concentration of N-benzoyltyrosine which could not be corrected for. This probably accounts for the rise of the curve. In any case the experiment indicates that by using the present method for partial substitution of proteins, substitution at tyrosine residues would be unlikely in the presence of unreacted amino groups. Since phenyl isocyanate is known to react much faster with amines than with alcohols the same would appear to hold true for the hydroxyl groups of serine and threonine residues.

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Received October 6, 1954.

Phenylcarbamoyl Derivatives of Insulin

WILLIAM ANDERSEN

*Carlsberg Laboratory, Chemical Department,
Copenhagen, Denmark*

Much research has in the past been devoted to the chemical modification of insulin, mainly because of its relative stability and unique physiological action. The advent of refined separation techniques has made it possible to investigate further the complexity of such protein derivatives.

This was done for the first time by Harfenist and Craig¹ who were able to separate DNP-substituted insulin derivatives by countercurrent distribution. In the present communication is reported the synthesis and separation of phenylcarbamoyl derivatives of insulin.

An aqueous solution of pork insulin (60 micromoles, based on the molecular weight 5777 for a molecule consisting of one A and one B chain) was reacted at pH 8.50 and 40° with phenyl isocyanate (42 μ moles) liberated in the solution by Lossen rearrangement of potassium dibenzohydroxamate as previously described². The partially substituted insulin was subjected to partition chromatography on a Hyflo Super-Cel column, using the solvent system 2-butanol—0.01 *N* trichloroacetic acid³. The elution chromatogram consisted of 3 peaks (Fig. 1) of which the first appeared in the same fractions as insulin itself and obviously was the unreacted protein. According to Sanger and coworkers⁴ the two-chain model of insulin contains three primary amino groups, the N-terminal α -amino groups of glycine and phenylalanine and the ϵ -amino group of a lysine residue. The two α -amino groups should react more rapidly under the above conditions (pH 8.5) than the latter owing to the difference in their pK's (7.5 and 11.9, respectively). Consequently, by reasoning along the same lines as Craig¹, one would expect D_1 and D_2 to be mono- and di-substituted derivatives, respectively, substitution having occurred only on the α -amino groups of the insulin molecule.

The isolated components were hydrolysed in strong acid (5 *N* hydrochloric acid at 100° for 18 hours) which is known to cause cyclisation of phenylcarbamoyl derivatives of α -amino acids and peptides, thus affording the corresponding hydantoins, (p. 1724).

The hydantoins were extracted from the hydrolysis mixture with ether and determined spectrophotometrically in alkaline solution (0.1 *N* sodium hydroxide) under which conditions they were shown to develop strong absorption peaks at 239 $m\mu$, with a molecular extinction of 1.60 and $1.65 \cdot 10^4$ for the glycine (Gly.hyd.) and phenylalanine derivative (Phe.hyd.), respectively.

The hydantoins were identified by paper chromatography in two solvent systems (xylene-acetic acid-water, 3:2:1, and *n*-pentanol saturated with water). The spots were located on the paper by spraying with *N* sodium hydroxide followed by photo-

graphy in ultraviolet light. An estimate of the ratio of the two hydantoins was obtained by cutting out the spots, eluting with 0.1 *N* alkali and determining the optical density of the eluates at 239 m μ . The method is not very exact ($\pm 10\%$) owing to a rather high and varying absorption of the paper blanks. The results obtained are summarised in Table 1.

18 hours. The corresponding figure for Phe.hyd. is 90 %. This difference in stability of the two hydantoins indicates that the actual molecular ratio of $D_{1,\text{Gly}}$ to $D_{1,\text{Phe}}$ is even greater than 10. It will be noticed that the yield of Gly.hyd. from D_1 , calculated on the basis of a minimum molecular weight for insulin of 12 000 would be $>100\%$. The present experi-

Table 1.

Peak	Gly.hyd. + Phe.hyd. moles per mole of derivative		Molecular ratio Gly.hyd. : Phe.hyd.	
	theoret.	found	theoret.	found
<i>I</i>	0	0	—	—
D_1	1	0.7	—	10 : 1
D_2	2	0.85	1	1 : 3

D_1 is obviously a mixture of the two monosubstituted derivatives, consisting mainly ($>90\%$) of the mono-derivative substituted on the N-terminal glycine ($D_{1,\text{Gly}}$). The yield obtained of Gly.hyd. (about 63 %) is consistent with the fact that Gly.hyd. is unstable under the strongly hydrolytic conditions of the cyclisation, 57 % of Gly.hyd. being recovered after

ments thus confirm the molecular weight of 6 000 found by Craig¹ and recently by Kupke and Linderström-Lang⁵, provided that D_1 is the monosubstituted derivative.

The molecular ratio of the two hydantoins obtained from D_2 corresponds fairly well to their respective stability during the cyclisation. The total yield of the hydantoins, however, is somewhat low compared

Fig. 1. Elution chromatogram of partially substituted insulin. Absc.: Number of fractions (23 ml). Ord.: Optical density at 277 m μ .

with that obtained from D_1 . The possibility that D_2 is a mixture of di-derivatives must not be overlooked. It seems reasonable to suppose, however, that the derivative substituted on both N-terminal amino-groups is the major component.

The course of the substitution may be presented as follows:

where k_1 (k'_1) and k_2 (k'_2) represent the (second order) rate constants for substitution at the N-terminal glycine and phenylalanine residue, respectively. Examination of Fig. 1 shows that the amount of each component is approximately $I = D_1 = 3 \cdot D_2$ and from Table 1 is obtained $D_{1,\text{Gly}} \gg D_{1,\text{Phe}}$. These facts cannot be reconciled unless it is assumed that $k'_2 > k_2$, that is: the amino group of the N-terminal phenylalanine must react faster in the mono-derivative $D_{1,\text{Gly}}$ than in insulin itself. The supposition that this «unmasking» is an intermolecular effect caused by a higher degree of dissociation of the mono-derivative into subunits as compared with insulin, is not substantiated by ultracentrifuge experiments. The possibility remains that the effect is of intramolecular origin. This would seem to require the distance between the groups involved to be rather short, or in different terms: The A and B chains of the insulin molecule would appear to be parallel rather than antiparallel. This last conclusion is in agreement with the allocation of the cystine bridges recently published by Sanger⁶. Further details will be published in *Compt. rend. trav. lab. Carlsberg, Sér. chim.*

The author is greatly indebted to Professor K. Linderstrøm-Lang for encouragement and helpful discussions during the course of this work and to Nordisk Insulin Laboratorium for financial support and for a gift of crystalline pork insulin.

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Received October 6, 1954.

Additional Note on α -Aminopimelic Acid in Green Plants

ANN-MARIE BERG and ARTTURI L. VIRTANEN

Laboratory of the Foundation for Chemical Research, Biochemical Institute, Helsinki, Finland

In an earlier paper¹ we reported the detection of α -aminopimelic acid in *Asplenium septentrionale* by the paper chromatographic method. We have now been able to isolate α -aminopimelic acid from the same plant by cutting out the spot in question from some paper chromatograms before the treatment with ninhydrin (thick Munktell 20 paper was used). The pieces of paper were extracted with water, the extract evaporated to dryness, and the solid rest extracted with a mixture of abs. alcohol and ether in order to remove browncoloured impurities. The white microcrystalline substance (2 mg from 280 g fresh *Asplenium*) which on the paper chromatogram gave only one spot identical with the spot of α -aminopimelic acid, had a melting point of 204° C. Synthetic α -aminopimelic acid had a melting point of 203° C and the mixture of both 204° C. 1.4 mg of the isolated substance used 0.76 ml 0.01 N NaOH (0.304 mg NaOH), calculated for α -aminopimelic acid 0.80 ml (0.320 mg NaOH).

The isolated α -aminodicarboxylic acid is thus α -aminopimelic acid. Its R_F -value in phenol + NH_3 is 0.40 using Whatman 4 paper; in butanolacetic acid the R_F -value corresponds to that of γ -aminobutyric acid.

We have found free α -aminopimelic acid by the paper chromatographic method also in the seeds of a legume tree, *Ceratonia siliqua*.

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Received October 6, 1954.