Fig. 2. Two-dimensional paper chromatogram of the reduced hydrazones. Full-lined spots = with ninhydrin produced distinct spots of 1 = gly, 2 = ala, 8 = ser, 17 = glu, X = γ -hydroxy- α -aminopimelic acid (brownish), Z = lactone of γ -hydroxy- α -aminopimelic acid (yellowish), T = unknown. Dot-lined spots = with ninhydrin produced faint spots. 71 = α -aminopimelic acid; unnumbered ninhydrin spots are unidentified.

Among the keto acids which we have found *hydroxypyruvic acid*, *α -ketopimelic acid* and *γ -hydroxy- α -ketopimelic acid* especially attract attention as they have not earlier been found in any plant as far as we know. In addition there are some ninhydrin positive unidentified spots on the chromatogram, among which spot T is particularly intense. The characterization of γ -hydroxy- α -ketopimelic acid has been possible only because the corresponding amino acid was earlier isolated from *Asplenium*¹.

In germinating pea seeds formation of *α -ketoadipic acid* could be shown. Even this acid has not earlier been found in any organism.

Our investigations suggest that *α -amino-adipic acid*, *α -aminopimelic acid* and *γ -hydroxy- α -aminopimelic acid* are formed *via* transamination from the corresponding keto acids. We are going to investigate these transamination reactions more closely. The formation of serine from hydroxy-

pyruvic acid, and the formation of glycine from glyoxylic acid seems also probable.

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The Synthesis of Phenylcarbamoyl Derivatives by Lossen Rearrangement of Dibenzohydroxamic Acid

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Reports on the modification of proteins by reaction with organic isocyanates (notably phenyl isocyanate) have been published by numerous authors (for review, see Herriott¹). One common feature of these experiments has been the use of an excess of the reagent. The consequence of this has probably been the production of a very complex mixture of highly substituted protein derivatives and in most cases, when the reactions were performed in aqueous solution, hydrolysis of an uncontrolled amount of the reagent has occurred.

A method by which proteins and peptides in aqueous solution could be partially substituted by reaction with phenyl isocyanate under strictly controlled conditions would be highly desirable. It appeared to us that the well known Lossen rearrangement of dibenzohydroxamic acid might be useful in this respect.

This reaction, in which phenyl isocyanate is produced by rearrangement of the dibenzohydroxamate anion in alkaline solution, has been thoroughly investigated by Hauser and coworkers^{2,3} who found that the reaction in 0.1 *N* ammonia solution followed first order kinetics with respect to the concentration of the dibenzohydroxamate anion. We have confirmed this for potassium dibenzohydroxamate under slightly different conditions. The phenyl isocyanate produced during the rearrangement was brought into reaction with an excess of β -alanine at constant pH (8.50) according to the scheme:

The reaction was followed on a recording autotitrator⁴. Dibenzohydroxamic acid is a rather weak acid ($\text{pK} = 7.4$ in 50 % ethanol at 40° C). Accordingly the pH must be kept at (or above) 8.5 in order that the reagent be present mainly in the anionic form which undergoes rearrangement. The kinetic data were corrected for dissociation of the ammonium group of β -alanine ($\text{pK} = 10.2$) at pH 8.5. The first order rate constants were determined at 35.0° and 40.0°. Mean values of several determinations were as follows:

$$k_{35}^\circ = 2.88 \times 10^{-3} \text{ min}^{-1} (t_{\frac{1}{2}} = 241 \text{ min})$$

$$k_{40}^\circ = 5.83 \times 10^{-3} \text{ min}^{-1} (t_{\frac{1}{2}} = 119 \text{ min})$$

By using these rate constants it is possible, under the conditions specified above, to generate in aqueous solution almost any amount of phenyl isocyanate down to about 1 micromole. The reaction is conveniently quenched after the predetermined time by addition of acid to pH 5 which causes precipitation of the insoluble dibenzohydroxamic acid.

It is well known¹ that phenyl isocyanate reacts readily with amino groups of proteins at or above pH 8. However, when working with dilute solutions of these compounds, hydrolysis of the reagent might conceivably become a significant side reaction. In order to investigate this problem the model peptide, triglycine (GGG), was reacted at pH 8.5 and 40° with phenyl isocyanate liberated from an excess of potassium dibenzohydroxamate. Under these conditions 85 % of the GGG is present as the free amine. The disappearance of the peptide was followed on aliquots by the ninhydrin method. The experiment (Fig. 1a) shows that the reaction

between phenyl isocyanate and the amino group is practically quantitative down to a concentration of the latter of about 15 microequivalents per 10 ml (1.5×10^{-3} equivalents per l). Below that concentration competing hydrolysis of the reagent becomes detectable. However, more than 90 % of the GGG has reacted at the time when the equimolar amount of phenyl isocyanate has been liberated. Experiments at 35° gave similar results.

Craig and his coworkers⁵ have observed that substitution at the hydroxyl group of tyrosine residues of peptides occurred with dinitrofluorobenzene. Since the reports

Fig. 1a — Curve I: Calculated liberation of phenyl isocyanate at pH 8.50 and 40.0° from potassium dibenzohydroxamate (121.7 micromoles) added at zero time to 10 ml of water containing 31.8 micromoles of GGG. Curve II: Disappearance of GGG.

Fig. 1b — Curve I: Calculated liberation of phenyl isocyanate at pH 8.50 and 35.0° from potassium dibenzohydroxamate (132.7 micromoles) added at zero time to 10 ml of water containing 30.4 micromoles of *N*-benzoyltyrosine. Curve II: Disappearance of *N*-benzoyltyrosine.

on the reaction of phenyl isocyanate with tyrosine residues have been ambiguous¹, we decided to study the reaction with *N*-benzoyltyrosine under conditions similar to those given above for GGG, using the Folin method for determination of unreacted

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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Phenylcarbamoyl Derivatives of Insulin

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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-