in fact, a special substrate and can only react under conditions when the enzyme is active. In support of this conclusion, trypsinogen was found not to be labelled by $DF^{32}P$.

The rapidity of separation of labelled trypsin and free DFP (and DIP) on the Dowex-50 column (5–7 minutes) enabled a search to be made for an E_1DIP complex as postulated above, but none could be detected either during the reaction with trypsin in the presence of 8 *M* urea nor when "cold" DIP-trypsin (3 times crystallized) was treated with $DF^{32}P$. In the first case, both sites E_1 and E_2 would be available, since no substitution had occurred; while in the case of DIP-trypsin, since any shift must have been completed during the lengthy preparation and crystallization of the protein, the E_1 site would be unoccupied and therefore available for further phosphorylation. Under conditions, therefore, where the imidazole side chains of trypsin are available but where the usual course of inhibition finally resulting in DIP stably bound to serine is prevented, no labelling of the protein is observed even though the analytical technique would ensure the detection of very labile intermediates. Moreover, even if the intermediate existed only on the surface of the enzyme and were rapidly decomposed by water to form free DIP and enzyme, a DFP-ase effect of trypsin would be manifest by an increased formation of DIP. Comparison with a control in which the same amount of DFP was incubated in 8*M* urea under the same conditions of temperature and pH showed, however, no difference in the amount of DIP produced.

In summary, therefore, it may be said that no evidence has been found to support the hypothesis that the phosphorylation of imidazole constitutes the initial stage of the combination of DFP with trypsin.

Our thanks are due to Miss DOROTHY KAUFFMAN for technical assistance. This work has been carried out in part, under Contract No. Nonr-477-04 between the University of Washington and the Office of Naval Research, Department of the Navy.

GORDON H. DIXON
HANS NEURATH

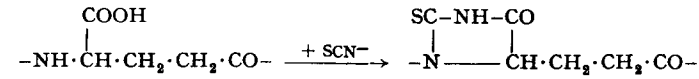
Department of Biochemistry, University of Washington, Seattle, Wash. (U.S.A.)

[1] B. J. JANDORF, H. O. MICHEL, N. K. SCHAFFER, R. EGAN AND W. H. SUMMERSON, *Discussions Faraday Soc.*, (in the press.).
[2] I. B. WILSON, *Discussions Faraday Soc.*, (in the press).
[3] N. K. SCHAFFER, S. HARSHMAN, R. R. ENGLE AND R. W. DRISKO, *Federation Proc.*, 14 (1955) 275.
[4] R. A. OOSTERBAAN, P. KUNST AND J. A. COHEN, *Biochim. Biophys. Acta*, 16 (1955) 299.
[5] G. H. DIXON, S. GO AND H. NEURATH, *Biochim. Biophys. Acta*, 19 (1956) 193.
[6] A. K. BALLS AND E. F. JANSEN, *Adv. Enzymol.*, 13 (1952) 321.
[7] T. WAGNER-JAUREGG AND B. E. HACKLEY, *J. Am. Chem. Soc.*, 75 (1953) 2125.
[8] B. S. HARTLEY AND B. A. KILBY, *Biochem. J.*, 50 (1952) 672.
[9] J. I. HARRIS AND K. LINDERSTRØM-LANG, private communication.
[10] T. VISWANATHA AND I. E. LIENER, *Fed. Proc.*, 14 (1955) 298; *J. Biol. Chem.*, 215 (1955) 777.
[11] T. VISWANATHA AND I. E. LIENER, *Nature*, 176 (1955) 1120.

Received April 3rd, 1956

Condensation of γ-glutamyl peptides with thiocyanate*

Ammonium thiocyanate in the presence of acetic anhydride has been used as a reagent for the determination of C-terminal amino acids in proteins or peptides[1–5]. Using this reaction we found that proteins combine with 10–20 $S^{14}CN$-residues per 10^5 g of protein[6–8]. The radioactive $S^{14}CN$-residues were not exchanged with nonradioactive thiocyanate; this suggested binding by covalence, and seemed to indicate the combination of SCN-residues with the free α-carboxyl groups of γ-glutamyl or β-aspartyl residues in the peptide chain; this reaction would result in the formation of thiohydantoin rings in the chain:



Our results did not exclude, however, the possibility that α-glutamyl residues are converted into γ-glutamyl residues under the drastic conditions of the treatment with thiocyanate. Rearrangements of this type have been described[9–11]. We examined, therefore, the reaction of α- and γ-polyglutamic acid with thiocyanate.

Radioactive ammonium thiocyanate was prepared from $Na^{14}CN$ and sulfur and recrystallized from ethanol with added nonradioactive ammonium thiocyanate as carrier. Polyglutamic acid preparations were mixed with the radioactive thiocyanate, acetic anhydride and acetic acid as

described by JOHNSON AND GUEST[12] and kept for 30 minutes in a boiling water bath. The cooled mixtures were poured into water and after decomposition of the anhydride repeatedly washed with nonradioactive thiocyanate, then extracted with hot methanol and benzene, and finally dried. Approximately 60 mg of the dry preparations were plated on planchets and the activities determined in a flow-gas counter.

TABLE I

Preparation	*Weight (mg)*	*Counts/min*	*Mol. wt.*	*SCN-residues per mole* [a]
Poly-γ-glutamic acid[b,c]	62.8	9408	12,000	5.24
Poly-α-glutamic acid[b,d]	63.5	513	6,500	0.15
5-*iso*Butyl-2-thiohydantoin[e]	60.3	2475	172	(1.0)

[a] after correction for self-absorption.
[b] sent to us by Dr. E. KATCHALSKI (The Weizmann Institute of Science, Rehovoth, Israel) to whom we are greatly indebted for these preparations.
[c] from *Bac. subtilis*[13].
[d] from poly-γ-benzyl-L-glutamic acid by HBr in glacial acetic acid[14].
[e] prepared by condensing leucine with a mixture of 1 part of radioactive thiocyanate and 51.1 parts of nonradioactive thiocyanate.

Our results assembled in Table I demonstrate that only γ-polyglutamic acid condensed with thiocyanate. The low value observed may, partly, be due to the rearrangement of γ- into α-glutamyl residues. However, even if only γ-glutamyl residues were present, quantitative reaction cannot be expected because of steric hindrance.

From the nonreactivity of α-polyglutamic acid we conclude that no rearrangement of α- into γ-glutamyl bonds takes place under the conditions of the condensation. It is reasonable to assume, therefore, that this is also valid for proteins and to conclude that the condensation of proteins with a considerable number of thiocyanate residues is due to the presence of γ-glutamyl residues in their peptide chains. The presence of such residues is also indicated by the formation of succinyl-peptides when partial hydrolysates of proteins are oxidized with sodium hypobromite[15] and by the formation of γ-amino-δ-hydroxyvaleric acid when proteins are hydrolyzed after reduction by $LiAlH_4$[16]. Whereas γ-glutamyl bonds occur in several natural products such as glutathione or folic acid, the occurrence of β-aspartyl residues has not yet been observed. The occurrence of β-aspartyl residues in proteins is, therefore, less probable.

FELIX HAUROWITZ
Department of Chemistry, Indiana University, Bloomington, Ind. (U.S.A.) JACK HOROWITZ

[1] P. SCHLACK AND W. KUMPF, *Z. physiol. Chem.*, 154 (1926) 125.
[2] V. H. BAPTIST AND H. B. BULL, *J. Am. Chem. Soc.*, 75 (1953) 1727.
[3] S. G. WALEY AND J. WATSON, *J. Chem. Soc.*, (1951) 2394.
[4] J. TIBBS, *Nature*, 168 (1951) 910.
[5] S. W. FOX, T. L. HURST, J. F. GRIFFITH AND O. UNDERWOOD, *J. Am. Chem. Soc.*, 77 (1955) 3119.
[6] F. HAUROWITZ, R. L. HARDIN AND M. ZIMMERMAN, *Federation Proc.*, 13 (1954) 225.
[7] R. L. HARDIN, *Ph. D. Thesis*, Indiana University, 1952.
[8] W. ZIMMERMAN, *Ph. D. Thesis*, Indiana University, 1955.
[9] W. D. JOHN AND G. T. YOUNG, *J. Chem. Soc.*, (1954) 2870.
[10] D. W. CLAYTON AND G. W. KENNER, *Chemistry & Industry*, (1953) 1205.
[11] J. KOVACS, K. MEDZIHRADSZKY AND V. BRUCKNER, *Naturwiss.*, 19 (1954) 450.
[12] T. B. JOHNSON AND B. H. NICOLET, *J. Am. Chem. Soc.*, 33 (1911) 1973.
[13] M. BOVARNICK, F. EISENBERG JR., D. O'CONNELL, J. VICTOR AND I. OWADES, *J. Biol. Chem.*, 207 (1954) 593.
[14] W. E. HANBY, S. G. WALEY AND J. WATSON, *J. Chem. Soc.*, (1950) 3239.
[15] F. HAUROWITZ AND F. BURSA, *Biochem. J.*, 44 (1949) 509.
[16] C. FROMAGEOT AND M. JUTISZ, in G. E. W. WOLSTENHOLME AND MARGARET P. CAMERON, *Chemical Structure of Proteins*, J. A. Churchill, Ltd., London, 1953, p. 82.

Received March 26th, 1956

* Support of this work by contracts of Indiana University with the Office of Naval Research and the U.S. Atomic Energy Commission, and by research grants from the National Science Foundation and the U.S. Public Health Service, is gratefully acknowledged.