sociated with binding of inhibitor, could alter the iodination rate of tyrosine residues elsewhere on the enzyme molecule and these are the ones isolated here.

We wish to thank Mrs. Lucy Emerson and Mr. Joseph Gurreri for technical assistance.

* Supported in part by the U.S. Public Health Service, grant AI 03962, from the National Institute of Allergy and Infectious Diseases.

† A unit of the New York State Department of Health.

[1] Simpson, R. T., J. F. Riordan, and B. L. Vallee, *Biochemistry*, **2**, 616 (1963).

[2] Vallee, B. L., J. F. Riordan, and J. E. Coleman, these PROCEEDINGS, **49**, 109 (1963).

[3] Pressman, D., and O. Roholt, these PROCEEDINGS, **47**, 1606 (1960).

[4] Roholt, O., G. Radzimski, and D. Pressman, *Science*, **141**, 726 (1963).

[5] In the case of the I^{125} label it is important to use some I^{131} along with the I^{125} as a composite label rather than pure I^{125} as the label. In subsequent steps of this procedure, the iodinated peptides derived from a mixture of the iodinated proteins are separated on paper chromatograms and their positions located by radioautography. Peptides containing I^{125} alone may be lost since the radioactivity of I^{125} exposes film relatively poorly. The I^{131} is added to the I^{125} so that the positions of the I^{125}-labeled peptides are well marked. The amount of iodinated peptide from the I^{131}-labeled enzyme present in any spot is calculated from the total I^{131} in the spot less the I^{131} on the I^{125}-labeled peptides in the spot. The I^{125}/I^{131} ratio is based on this corrected I^{131} value.

[6] The following abbreviations are used: Cpase, carboxypeptidase; H.V.P.E., high-voltage paper electrophoresis; MIT, monoiodotyrosine; DIT, diiodotyrosine; MIH, monoiodohistidine; DIH, diiodohistidine.

[7] Roholt, O., A. Shaw, and D. Pressman, *Nature*, **196**, 773 (1962).

[8] Bird, R., and H. E. A. Farran, *J. Clin. Endocrinol. Metab.*, **20**, 81 (1960).

[9] Dube, S. K., O. A. Roholt, and D. Pressman, *J. Biol. Chem.*, **239**, 1809 (1964).

---

*BIOSYNTHESIS OF THE TWO CHAINS OF INSULIN**

BY RENÉ E. HUMBEL†

THE ROCKEFELLER INSTITUTE

Communicated by George E. Palade, January 29, 1965

The insulin molecule, consisting as it does of two polypeptide chains interconnected by two disulfide bridges,[1] presents a special problem in the elucidation of biosynthetic mechanisms. Chymotrypsin, a protein consisting of three polypeptide chains, is known to be derived from a single-chain precursor, chymotrypsinogen. Insulin might be produced in the same way, but no single-chain insulin precursor has as yet been found. Alternatively, the two constituent chains of insulin might be separately synthesized and subsequently connected by two disulfide bridges. It can be assumed that the correct formation of the five intrachain disulfide bridges in chymotrypsinogen occurs spontaneously, like the intrachain disulfide bridge formation in reduced ribonuclease and lysozyme.[2, 3] For insulin, however, the situation seems to be different: it can be reconstituted *in vitro* from its reduced constituent chains with a yield of only 1–2 per cent.[4]

The purpose of the present work was to prove or rule out one of these two possibilities: (1) separate synthesis of the two constituent chains, with subsequent formation of the interchain disulfide bridges; (2) synthesis of a single-chain precur-

sor having three intrachain disulfide bridges, with subsequent peptide bond hydrolysis to yield the two chains.

It was first assumed that biosynthesis of insulin, or of its hypothetical precursor, starts at its N-terminal amino acid residue(s) and proceeds linearly toward the C-terminal end(s), a pattern which has been found for a number of proteins (α-amylase,[5] *E. coli* proteins,[6] globin chains,[7] egg white lysozyme,[8] and ribonuclease[9]). It was further assumed that a short time incubation of insulin-synthesizing tissue with a specific labeled amino acid will yield a gradient of specific radioactivities along the chains, the lowest specific activity being in the part where synthesis starts, since a number of unfinished chains of varied length are expected to exist at the start of the incubation.

Considering the low specific activities of insulin which probably would be obtained by pulse labeling, the use of mammalian pancreas with its small percentage of insulin-synthesizing tissue was abandoned in favor of the Brockmann body of anglerfish (goosefish, *Lophius piscatorius*), an organ consisting of practically pure islet tissue.[10] Previous work has established the proper conditions for *in vitro* studies of metabolic functions and of protein synthesis with isolated islet tissue of toadfish[11-13] and of anglerfish.[14, 15]

A simple and reproducible method for isolation of insulin was devised,[16] and the addition of a further purification step led to the isolation of pure insulin in good yield.[17] Since the structure of this particular fish insulin was not known, the determination of its amino acid composition and partial structure was undertaken, as reported elsewhere.[17] As a result, the positions of two of the three residues of proline in this molecule are known. These occur in the B chain, which corresponds to the phenylalanyl chain of beef insulin, at position B_2 and at B_{28}. The A chain, which corresponds to the beef glycyl chain, contains a single residue of proline,[18] probably at position A_9 or A_{10}, by analogy with bonito insulin II.[19]

Thus, proline, occupying positions near the NH_2-terminus and COOH-terminus of the B chain, and a position most probably near the center of the A chain, is the amino acid whose incorporation was studied in the present experiments.

Experimental Procedures.—Incubation of islet tissue: Brockmann bodies from freshly caught anglerfish (*Lophius piscatorius*) were isolated directly on the fishing trawler (New England Biological Assn., Point Judith, R.I.). They were kept and shipped in Krebs-Ringer solution on ice. Time delay due to shipping averaged 6–10 hr, after which experiments were immediately started. Fifteen to twenty-five minced Brockmann bodies (totaling 1 gm wet weight) were incubated in 3–6 ml Krebs-Ringer bicarbonate buffer, pH 7.4, under 95% O_2/5% CO_2 in the presence of 0.5 mM radioactive L-proline (either L-proline-3,4-H^3, 5 C/mM, or L-proline-C^{14}, 200 mC/mM, New England Nuclear Corp. in a Dubnoff metabolic shaker at 17°C. Puromycin (Lederle Laboratories, Pearl River, N.Y.), when added, was dissolved in Krebs-Ringer buffer before addition to the medium. In experiments in which incorporation of proline into insulin and bulk protein was compared, the term "bulk protein" refers to material insoluble in cold 10% TCA and in 75% ethanol/0.18 *N* HCl. For the experiments designed to yield a gradient of radioactivity along the insulin chains, one batch of islet tissue was incubated with proline-H^3 for 15–30 min, and another batch was incubated with proline-C^{14} for 5–8 hr. Incubation was stopped by adding TCA to 10% final concentration, whereupon the two batches were combined and processed together.

Isolation of insulin: Insulin extraction with acid ethanol from TCA precipitates and its purification on a column of Sephadex G-75 in 5 *M* acetic acid/0.15 *M* NaCl were performed as previously described.[16] The final purification was achieved by column chromatography on CM-cellulose with a formic acid gradient.[17]

Isolation of peptides: Isolated insulin was reduced and carboxymethylated[17] to yield the two chains which were subsequently treated with trypsin (Worthington) to split the only trypsin-susceptible bond Arg^{B22}-Gly^{B23}. The resulting three peptides (A chain, B_{1-22}, B_{23-29}), each containing one proline residue, were separated by paper electrophoresis in 0.2 *M* NH_4HCO_3, pH 8.1, at 20 v/cm for 3 hr and eluted in ascending fashion with 50% acetic acid.[17]

Isolation of proline: Each peptide was separately hydrolyzed in 6 *N* HCl in evacuated sealed tubes for 22 hr at 110°C. The hydrolysates were applied on the 50-cm column of an amino acid analyzer,[20] equipped with the accelerated system of Spackman.[21] As an additional monitoring device, the column effluent was run through a 2-ml anthracene-packed flow-cell in a scintillation counter (Nuclear-Chicago), and was then fed back to the analyzer for the automatic ninhydrin reaction. This design allowed unequivocal identification as well as estimation of the purity of the isolated peptide, and, by counting the column effluent, provided a convenient means for immediately checking the exact position of proline in the effluent. The short counting time of 2 min in the flow counter was, however, too low to be of value in the calculation of H^3/C^{14} ratios. The flow counter effluent containing the isolated proline (6 ml) was thus collected, desalted on a column (0.9 × 5 cm) of Dowex 1 × 8 (200–400 mesh, OH^- form) by eluting with 1 *M* acetic acid,[22] and counted in a hyamine-toluene system in a two-channel liquid scintillation counter (Packard Tricarb). The settings of the counter were so chosen as to yield highest possible channel ratios for both C^{14} and H^3. Efficiency for tritium counting in the first channel was 36%, for C^{14} in the second channel 57%. Calculations of C^{14} and H^3 counts followed the procedure of Okita *et al.*[23] using C^{14}- and H^3-toluene as internal standards.

Results.—The time course of incorporation of proline-C^{14} into bulk protein and insulin is shown in Figure 1. Linear incorporation with time occurs only after the first hour of incubation. When slices were preincubated without labeled substrate

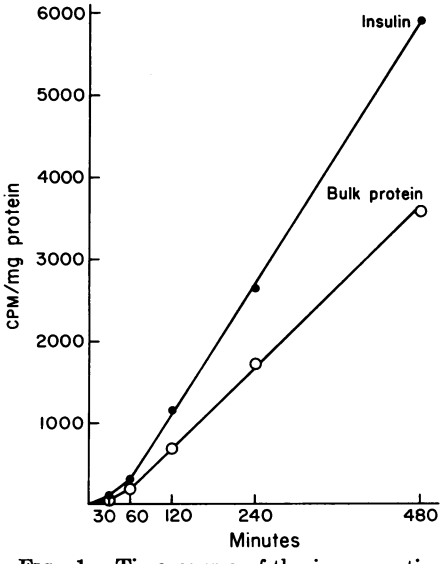

FIG. 1.—Time course of the incorporation reaction. Each flask contained about 250 mg wet weight of islet tissue minces in 1 ml of Krebs-Ringer bicarbonate buffer, pH 7.4, with L-proline-C^{14}, 200 μC/μM, 0.5 μM/ml. "Bulk protein" is the TCA- and 75% ethanol/0.18 *N* HCl-insoluble material. The purification of insulin was carried only to the Sephadex G-75 step, which leaves insulin contaminated with two peptides of small molecular weight and of low proline content.[17]

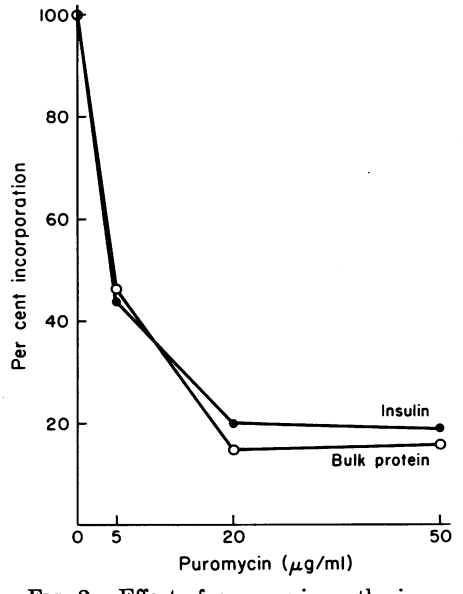

FIG. 2.—Effect of puromycin on the incorporation reaction. Results are expressed as per cent of control activity. Each flask contained about 300 mg wet weight of islet tissue minces in 1 ml of Krebs-Ringer bicarbonate buffer, pH 7.4, with L-proline-C^{14}, 200 μC/μM, 0.5 μM/ml. "Bulk protein" is the TCA- and 75% ethanol/0.18 *N* HCl-insoluble material. The purification of insulin included the final step of chromatography on CM-cellulose, which yields insulin devoid of any detectable impurities.[17]

for 1 hr at 17°C, essentially the same pattern was obtained. This lag period of 30–60 min is probably due to the time needed for the amino acid in the buffer to mix with the extracellular and intracellular amino acid pool of the minces.[24] After incubation for 1 hr or longer, proline incorporation into insulin is consistently higher than into bulk protein.

The effect of puromycin on amino acid incorporation is shown in Figure 2. Addition of 50 μg/ml (10^{-4} *M*) puromycin inhibited amino acid incorporation into bulk protein by 84 per cent and into insulin by 81 per cent.

In a first attempt to produce a gradient of radioactivity along the insulin chains, two batches of islet tissue minces were incubated for 30 min with proline-H^3 and for 5 hr with proline-C^{14}, as internal standard. When the three proline-containing peptides, rather than the isolated prolines, were counted, the amount of H^3-activity appeared to parallel the size of the counted peptide. Analysis of the proline-H^3 used in this experiment showed radiochemical impurities in the amount of 14 per cent, mostly in the form of aspartic acid. In the following experiments, therefore, the peptide-derived proline residues were isolated and counted, as outlined in the *Experimental Procedures* section.

The results of two experiments comparing the specific activities of the three isolated proline residues in insulin are shown in Table 1. In both experiments, the specific C^{14}-activities of the three prolines are not significantly different, whereas the specific H^3-activities are markedly different. Hence, incubation for 8 hr led to uniform labeling of all three proline residues in insulin, whereas incubation for 30 or 15 min led to uneven labeling. The difference in the labeling pattern due to differences in time of incubation is even more evident from the H^3/C^{14} ratios which are independent of the actual amounts of proline counted. In both experiments, the H^3/C^{14} ratios of the three proline residues are increasing in the order $B_2 < A < B_{28}$ (1:1.56:2.70 in experiment I, and 1:1.85:2.94 in experiment II).

Discussion.—The short length of the two insulin chains raises the possibility that these polypeptides might be assembled by mechanisms similar to the ones which serve for the synthesis of short bacterial polypeptides like tyrocidine.[25] Our experimental results show, however, that insulin is synthesized like protein, and unlike tyrocidine, for the following two reasons: (1) Puromycin, an inhibitor of protein synthesis, strongly inhibits amino acid incorporation into insulin as well as into bulk protein, while the synthesis of tyrocidine is not affected by inhibitors of pro-

TABLE 1

INCORPORATION OF H^3-PROLINE AND C^{14}-PROLINE INTO THE THREE PROLINE RESIDUES OF ANGLERFISH INSULIN

	Experiment 1: H^3-proline 30 min, C^{14}-proline 8 hr			Experiment 2: H^3-proline 15 min, C^{14}-proline 8 hr		
	dpm H^3/0.2 μM*	dpm C^{14}/0.2 μM*	H^3/C^{14}	dpm H^3/0.2 μM†	dpm C^{14}/0.2 μM†	H^3/C^{14}
Pro A	8,320	7,250	1.15	1,184	2,010	0.59
Pro B_2	6,430	7,920	0.81	740	2,290	0.32
Pro B_{28}	16,550	7,550	2.19	2,321	2,460	0.94

* Specific activities expressed as dpm/0.2 μM of original peptide as determined with the amino acid analyzer.
† Specific activities expressed as dpm/0.2 μM of isolated proline as determined by the ninhydrin reaction of an aliquot of the sample to be counted.
800 mg wet weight of islet tissue minces were incubated for 30 (Expt. 1) or 15 (Expt. 2) min in Krebs-Ringer bicarbonate buffer (pH 7.4) with H^3-proline, 5.0 mC/μM, 0.5 μM/ml. 200 mg wet weight of islet tissue minces were incubated for 8 hr in Krebs-Ringer bicarbonate buffer (pH 7.4) with C^{14}-proline, 200 μC/μM (Expt. 1) or 50 μC/μM (Expt. 2), 0.5 μM/ml. In both experiments, H^3- and C^{14}-labeled proteins were combined after incubation and processed together.

tein synthesis.[25] (2) Short time incubation with proline-H^3 (30 and 15 min at 17°C correspond to ~ 7.5 and 3 min, respectively, at 37°C) yielded nonuniformly labeled insulin. Since the H^3/C^{14} ratio of proline B_2, which is near the N-terminal end, is lower by a factor of 2.7 and 2.94, respectively, than the H^3/C^{14} ratio of proline B_{28} near the C-terminal end of the B-chain, it is concluded that biosynthesis of insulin B chain starts at its N-terminal end. This conclusion is independent of the final answer to the question whether insulin is synthesized as two separate chains or as a single-chain precursor, but the results are in keeping with recent results in the biosynthesis of other proteins,[5-9] and represent, therefore, further evidence that insulin is synthesized by mechanisms similar or identical to the ones operative in protein synthesis.

Kritzman *et al.*[26] have described uneven labeling of insulin by mere incubation of labeled phenylalanine with beef insulin. This type of incorporation, whatever its mechanism, could contribute to uneven labeling obtained with living tissue, but, in this case, the labeling should remain uneven even after long times of incubation. This was not the case in the present experiments. Hence, the type of amino acid replacement described by Kritzman *et al.* can be ruled out as a contributing factor to the results here recorded.

The fact that incubation with proline-C^{14} for 8 hr yielded uniformly labeled insulin is consistent with the assumption that islet tissue *in vitro* is able to synthesize new insulin chains, and not to complete only chains initiated *in vivo*. The fact that the

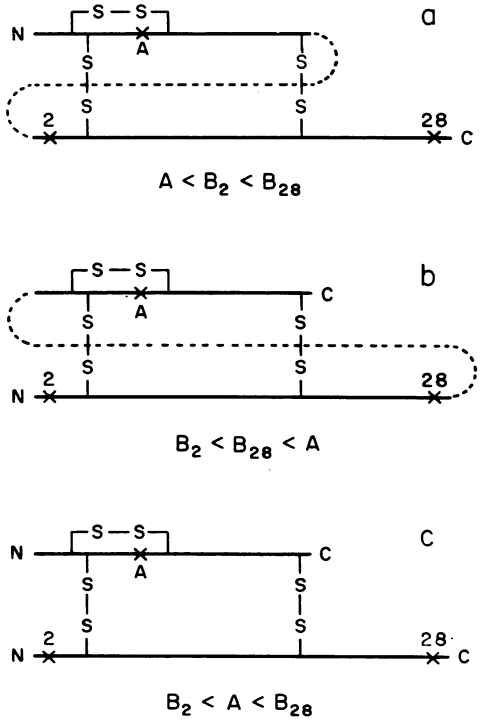

FIG. 3.—Expected labeling pattern of the three proline residues in Lophius insulin if (*a*) + (*b*) a single-chain precursor of insulin is assumed; (*c*) separate synthesis of insulin chains is assumed. See text for explanations.

time course of incorporation of radioactive proline into insulin remains linear over 8 hr further supports this assumption. Uniform labeling after prolonged incubation with labeled amino acids was also obtained in globin chains,[27] egg white lysozyme,[8] and ribonuclease.[9] An earlier report by Vaughan and Anfinsen,[28] however, describes uneven labeling of insulin and ribonuclease after incubation for 4 hr. No explanation has been offered for this discrepancy.[9]

As to the question put forward in the introduction, whether insulin is synthesized as two separate chains or as a single-chain precursor, three alternatives expressed in three different labeling patterns can be envisaged: (*a*) synthesis of a single-chain precursor starting at the N-terminal end of the A chain—in this case proline A is expected to have the lowest specific activity of all three prolines (Fig. 3*a*); (*b*) synthesis of a single-chain precursor starting at the N-terminal end of the B chain—in this case proline A is expected to have the highest specific activity (Fig. 3*b*); (*c*) separate synthesis of the two insulin chains—in this case proline A is expected to have a specific activity intermediate between the activities of prolines B_2 and B_{28}.

Patterns (*a*) and (*b*) (specific activity of proline A either lowest or highest) are required for the existence of a single-chain precursor, but do not prove it, for similar results can be produced by alternative (*c*), i.e., separate synthesis of the two chains, if the chains are synthesized at different rates, or the pools of finished A and B chains were of different sizes. Hence, experimental results in agreement with patterns (*a*) and (*b*) would be inconclusive.

While no labeling pattern could have excluded separate synthesis of the two chains, the observed data [pattern (*c*)] do eliminate the possibility of single-chain assembly. Hence, we can come to the conclusion that the two chains of insulin are synthesized as separate chains.

This still leaves open the question of how the two chains once finished combine *in vivo*, since, as mentioned earlier, reconstitution of active insulin from the two reduced chains takes place with very low yield *in vitro*.[4] It is of course possible that this low yield is a consequence of inadequate techniques. Du *et al.*[29] and Jiang *et al.*[30] claim to have obtained yields up to 20 per cent and 50 per cent, respectively. Since free chains have never been found in pancreas, chain-to-chain linkage *in vivo* must be rather efficient, suggesting the presence, in islet tissue, of an enzyme accelerating the rate of oxidation or of disulfide interchange in insulin. A similar enzyme for ribonuclease and lysozyme has been found in rat liver microsomes[31] and in pigeon and chicken pancreas.[32]

The finding of separate synthesis of the two insulin chains suggests that there may be cases of relative overproduction, with consecutive accumulation, of either chain, analogous to the accumulation of L-chains in Bence-Jones proteins.[33]

Summary.—Islet tissue minces from the fish *Lophius piscatorius* have been incubated with proline-C^{14} for 8 hr and with proline-H^3 for 15 or 30 min at 17°C. After incubation, the minces were combined, insulin was extracted, purified, and separated into three peptides, each containing one proline residue. The three proline residues were isolated and their H^3/C^{14} ratios determined. From these radioactivity values and from knowledge of the positions of proline in the chains, evidence was obtained for the conclusion that the two constituent chains of insulin are synthesized separately.

I thank Drs. G. E. Palade and P. Siekevitz for support and encouragement, Drs. S. Moore and W. H. Stein for providing their facilities, and Drs. A. M. Crestfield and T. Y. Liu for advice and assistance with the amino acid analyzer.

* This work was supported in part by USPHS grant AM-01635 (to Dr. P. Siekevitz).

† Fellow of the Helen Hay Whitney Foundation 1962–1965. Present address: Department of Biochemistry, University of Zürich, Switzerland.

[1] There is a third, intrachain disulfide bridge on the A chain.

[2] Anfinsen, C. B., and E. Haber, *J. Biol. Chem.*, **236,** 1361 (1961).

[3] Goldberger, R. F., and C. J. Epstein, *J. Biol. Chem.*, **238,** 2988 (1963).

[4] Dixon, G. H., and A. C. Wardlaw, *Nature*, **188,** 721 (1960).

[5] Yoshida, A., and T. Tobita, *Biochim. Biophys. Acta*, **37,** 513 (1960).

[6] Goldstein, A., and B. Brown, *Biochim. Biophys. Acta*, **53,** 438 (1961).

[7] Naughton, M. A., and H. M. Dintzis, these PROCEEDINGS, **48,** 1822 (1962).

[8] Canfield, R. E., and C. B. Anfinsen, *Biochemistry*, **2,** 1073 (1963).

[9] Luck, D. N., and J. M. Barry, *J. Mol. Biol.*, **9,** 186 (1964).

[10] Bargmann, W., in *Handbuch der Mikroskopischen Anatomie des Menschen*, ed. Möllendorff (Berlin, 1939), vol. 6, part II, p. 263.

[11] Lazarow, A., S. J. Cooperstein, D. K. Bloomfield, and C. T. Friz, *Biol. Bull.*, **113,** 414 (1947).

[12] Humbel, R. E., and A. E. Renold, *Biochim. Biophys. Acta*, **74,** 84 (1963).

[13] Humbel, R. E., *Biochim. Biophys. Acta*, **74,** 96 (1963).

[14] Bauer, G. E., and A. Lazarow, *Biol. Bull.*, **121,** 425 (1961).

[15] Humbel, R. E., unpublished results.

[16] Humbel, R. E., *Biochem. Biophys. Res. Commun.*, **12,** 333 (1963).

[17] Humbel, R. E., and A. M. Crestfield, *Biochemistry*, in press.

[18] Preliminary experiments have indicated that this proline is in a chymotryptic peptide containing the first 14 residues starting from the N-terminal end.

[19] Kotaki, A., *J. Biochem. (Tokyo)*, **53,** 61 (1963).

[20] Spackman, D. H., W. H. Stein, and S. Moore, *Anal. Chem.*, **30,** 1190 (1958).

[21] Spackman, D. H., *Federation Proc.*, **22,** 244 (1963).

[22] Dreze, A., S. Moore, and E. J. Bigwood, *Anal. Chim. Acta*, **11,** 554 (1954).

[23] Okita, G. T., J. J. Kabara, F. Richardson, and G. V. LeRoy, *Nucleonics*, **15,** 111 (1957).

[24] The inherent advantage of a cell-free system over minces was of course recognized, and attempts were made to devise a homogenate or microsome system capable of synthesizing proteins. However, amino acid incorporation into proteins in either whole homogenates or isolated microsome fractions of islet tissue was consistently lower than in minces by a factor of 10–50.[15] Further attempts to use a cell-free system were therefore abandoned.

[25] Mach, B., E. Reich, and E. L. Tatum, these PROCEEDINGS, **50,** 175 (1963).

[26] Kritzman, M. G., B. S. Sukhareva, and A. S. Konikova, *Nature*, **195,** 600 (1962).

[27] Dintzis, H. M., these PROCEEDINGS, **47,** 247 (1961).

[28] Vaughan, M., and C. B. Anfinsen, *J. Biol. Chem.*, **211,** 367 (1954).

[29] Du, Y. C., Y. S. Zhang, Z. X. Lu, and C. L. Tsou, *Sci. Sinica (Peking)*, **10,** 84 (1961).

[30] Jiang, R. Q., Y. C. Du, and C. L. Tsou, *Sci. Sinica (Peking)*, **12,** 452 (1963).

[31] Goldberger, R. F., C. J. Epstein, and C. B. Anfinsen, *J. Biol. Chem.*, **238,** 628 (1963); Givol, D., R. F. Goldberger, and C. B. Anfinsen, *J. Biol. Chem.*, **239,** PC3114 (1964).

[32] Venetianer, P., and F. B. Straub, *Biochim. Biophys. Acta*, **89,** 189 (1964).

[33] Edelman, G. M., and J. A. Gally, *J. Exptl. Med.*, **116,** 207 (1962).