[CONTRIBUTION FROM THE MICROBIOLOGICAL INSTITUTE OF THE NATIONAL INSTITUTES OF HEALTH, PUBLIC HEALTH SERVICE]

Synthesis of Protein-like Substances by Chymotrypsin[1a]

BY HENRY TAUBER[1b]

High molecular-weight, protein-like substances have been obtained at a physiological *p*H by the action of chymotrypsin on peptic digests of various proteins. It is not possible at present to state definitely whether in this reaction the small-size peptides are combined by peptide linkages. The synthesizing action of chymotrypsin is of biological importance since under optimum conditions the proteinase displays only one-third the hydrolyzing activity of crystalline trypsin. It is believed that this is the first time that protein-like substances of such high molecular weight (250,000–500,000) have been found to be produced by the action of an enzyme *in vitro*. The energy required for this reaction is apparently furnished by removal of the insoluble synthetic products.

Bergmann and Fruton[3] demonstrated that chymotrypsin can effect the synthesis of a single peptide linkage provided the end-product is insoluble. Two soluble simple amino acid derivatives were converted into an insoluble anilide.

Benzoyltyrosine + leucineanilide ⟶ Benzoyltyrosylleucineanilide

Tauber[4] has recently found that insoluble protein-like material is rapidly synthesized when a small amount of chymotrypsin is added to a concentrated solution of Witte peptone. Such protein-like substances have now been found to be produced readily by chymotrypsin from neutralized protein-free concentrates of peptic digests of egg albumin, bovine albumin, fibrin and zein. It will be shown in the present report that the molecular weights of these synthetic products are strikingly high and that they resemble natural proteins in many ways.

Experimental

Preparation of a Peptic Digest from Egg Albumin.—Into a 3-liter erlenmeyer flask were placed 100 g. of commercial egg albumin powder, 2 l. of distilled water containing 20 cc. of concentrated hydrochloric acid, 2 g. of "Difco" pepsin (1:10,000) and 10 cc. of toluene. The contents of the flask were well mixed and incubated at 37°. The *p*H of the digest was maintained at 1.5 to 1.8 by frequent additions of hydrochloric acid. A layer of toluene was kept over the digest. After 3 days of incubation another 2-g. portion of pepsin was added. After 7 days of digestion, a neutralized, filtered sample of the digest gave a faint turbidity with 0.3 *M* trichloroacetic acid. The flask was placed in a water-bath at 55° for 1 day. Twenty grams of Hyflo was mixed into the digest and was filtered by gravity. The clear filtrate was adjusted to *p*H 7.3 with 2 *N* sodium hydroxide.

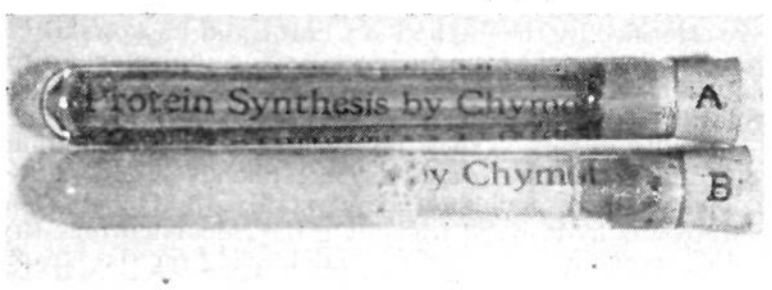


Fig. 1.

(1) (a) A preliminary report of this work was presented before the American Society of Biological Chemists at the meeting of the Federation of American Societies for Experimental Biology, at Atlantic City, N. J., April 16–21, 1950. (b) Venereal Disease Experimental Laboratory, U. S. Public Health Service, University of North Carolina, School of Public Health, Chapel Hill, North Carolina. Clinical Studies at the U. S. Marine Hospital, Staten Island, New York.

(2) H. Tauber, *Fed. Proc.*, **9**, 237 (1950).

(3) M. Bergmann and S. J. Fruton, *Ann. N. Y. Acad. Sci.*, **45**, 409 (1944).

(4) H. Tauber, THIS JOURNAL, **71**, 2952 (1949).

The filtrate was concentrated on the water-bath to 180 cc., 0.5 cc. of toluene was added, and the concentrate was kept at 6° for 1 day. The slight precipitate that formed was removed by centrifugation and was discarded. The solid content of the concentrate, on an ash-free basis, was 460 mg. per cc.

Synthesis of a Protein-like Material from a Peptic Digest of Egg Albumin.—To 175 cc. of the clear concentrate of *p*H 7.30, 30 mg. of crystalline chymotrypsin (Worthington Biochemical Laboratory), dissolved in 4 cc. of distilled water, was added. After 4 hours at 37° the solution became viscous and turbid. It turned into an almost solid gel in 6 hours and into a solid gel in 23 hours. The gel was suspended in 200 cc. of distilled water and the synthetic product was collected by centrifuging at 2500 r.p.m. The gel was washed 7 times with a total volume of 1400 cc. of distilled water; the last two supernatants were colorless.

The synthetic product was dissolved in 120 cc. of 0.1 *N* sodium hydroxide. A small amount of insoluble material was removed by centrifugation. The *p*H of the clear solution was 11.10. The solution was dialyzed for 2 days at 6° against 18 liters of distilled water which had been adjusted to *p*H 10.2 with sodium hydroxide, the outside fluid being replaced after 1 day with a freshly prepared sodium hydroxide solution of *p*H 10.2. Dialysis was resumed for an additional day against 18 l. of distilled water. The gelatinous material which precipitated was collected by centrifugation. The *p*H of the supernatant was 7.4. The precipitate was washed twice with 120 cc. of distilled water and was dried *in vacuo*. The yield was 2.7 g. of light tan-colored powder.

Rapidity of the Synthesis of the Protein-like Substance with Larger Quantities of Chymotrypsin.—When 2 mg. of chymotrypsin in 0.2 cc. of distilled water was added to 5 cc. of egg albumin digest concentrate, the solution turned viscous in 15 minutes and became a solid gel in 45 minutes (see Fig. 1, tube B. Tube A did not contain chymotrypsin). Similar results were obtained in a small-scale experiment with crystalline egg albumin.

Peptic digest-concentrates were prepared from bovine serum albumin (Fraction V, The Armour Laboratories), zein and blood fibrin, in a manner similar to the egg albumin peptic digest-concentrate. Synthesis, however, was not equally rapid with all protein digests. During synthesis sodium fluoride or toluene was used as the antiseptic.

General Properties of the Synthetic Protein-like Substances.—Synthesis of the protein-like substances was most rapid with egg albumin digest-concentrate as the substrate. Optimum concentration for synthesis was found to be at 40 to 45% solid concentration (on ash-free basis). Synthesis took place on both sides of the *p*H scale with a maximum close to 7.00. The synthetic products contain 13.81 to 15.09% nitrogen (see Table I). They are soluble in dilute acids and alkalies, and form gelatinous precipitates on the removal of most of these ions by dialysis or by neutralization. They are precipitated by protein precipitants such as trichloroacetic acid and phosphotungstic acid. The synthetic products are insoluble at *p*H 4.0 to 8.5, with the exception of the zein product which is soluble between 6.2 and 8.5, and in more alkaline and more acid solution. It is slightly

soluble in 70% ethyl alcohol whereas zein itself is quite soluble in this solvent. The other synthetic products are insoluble in 70% ethyl alcohol.

TABLE I

SOME PROPERTIES OF THE SYNTHETIC PRODUCTS

Source of product	Total N[a]	Amino N[a] 5-minute nitrous acid reaction	S_{20}
Egg albumin, dialyzed	14.36	..	15.2
Egg albumin, undialyzed	...	0.85	14.5
Bovine serum albumin, dialyzed	15.09	.92	Not detd.
Zein, dialyzed	13.81	.88	9.4
Blood fibrin, dialyzed	15.43	1.24	13.1

[a] These values are per cent., on ash-free and on dry weight, basis.

Amino Acid Content.—In Table II are shown quantitative results concerning some of the amino acids present in three of the synthetic protein-like substances. For comparison, published values for fibrin and egg albumin are also listed. The undialyzed egg albumin product listed in column 5 was prepared in a manner similar to the other products. It was extensively washed with distilled water and dried *in vacuo*.

TABLE II

AMINO ACID CONTENT OF A FEW SYNTHETIC PROTEIN-LIKE SUBSTANCES AS FOUND BY THE MICROBIOLOGICAL PROCEDURE[5]

	% Amino acid, on dry basis				
Amino acid	Synthetic dialyzed fibrin product	Fibrin[a]	Synthetic dialyzed egg albumin product	Synthetic undialyzed egg albumin product	Egg albumin[b]
Arginine	7.7	7.0	6.9	6.0	5.9
Glycine	3.2	5.4	1.6	1.7	1.9[a]
Histidine	2.2	2.1	2.4	2.3	2.3
Isoleucine	10.8	5.2	9.9	9.3	7.0
Leucine	10.7	16.2	10.1	9.2	9.2
Lysine	6.2	7.5	6.7	6.4	6.6
Methionine	2.6	2.4	4.2	4.0	4.1
Threonine	2.5	7.9	1.2	1.5	3.6
Valine	7.5	5.0	10.2	10.8	7.0
Tyrosine	5.7	5.5	3.9	3.5	3.2[c]
Phenylalanine	5.0	7.0	8.9	7.8	7.9
Tryptophan	4.7	3.5	1.5	1.4	1.4
Glutamic acid	7.4	13.8	5.2	5.1	16.3[a]

[a] Chemical assay averages from Block and Bolling.[6] [b] Microbiological assays from Stokes, *et al.*[7] [c] Microbiological assay from Gunness, *et al.*[8] For further data of amino acid composition of fibrin and egg albumin see reference 9.

In the synthetic products, the isoleucine and valine content is much higher and the threonine and the glutamic acid content is much lower than in the natural proteins. The synthetic fibrin product contains a much lower amount of glycine, leucine and lysine than the protein itself. There does not appear to be much difference between the dialyzed and undialyzed egg albumin products. The remainder of amino acid content does not differ to any great extent between fibrin and its synthetic product, and egg albumin and the synthetic product derived from it. From these data one could deduce that certain types of the peptides are selectively utilized in this synthesis by chymotrypsin. The amino acid assays were carried out by the microbiological procedure of Stokes and co-workers,[5,7,8] except for glycine which was determined by the method of Shankman, Camien and Dunn.[10] In the glycine test and for the other amino acid assays *L. mesteroides* P-60, as recommended by Shankman and associates, was employed as the test organism. In all of the assays however, the medium of Stokes and co-workers[7] was employed.

α-Amino Nitrogen.—The percentage of free α-amino nitrogen in these products, as determined by the Van Slyke procedure, was 0.85 to 1.2% (5 minute nitrous acid reaction), of the total nitrogen (see Table I).

Molecular Weights.—The average molecular weights of the synthetic products, as determined in an analytical ultracentrifuge, were strikingly high. The product obtained from egg albumin peptic digest-concentrate by the synthetic action of chymotrypsin at *p*H 7.3 had a sedimentation constant in Svedberg units of 15.2 (S_{20}). A preparation of synthetic undialyzed egg albumin product which was extensively washed but was not subjected to dialysis, showed an S_{20} value equal to 14.5. The product obtained from zein had a sedimentation constant of 9.4, and the product obtained from fibrin had a sedimentation constant of 13.1 (Table I). The centrifuge speed was 800 r.p.s. This corresponds to a centrifugal force of about 170,000 times gravity. For the molecular weight estimation the materials were dissolved in 1 cc. of 0.02 *N* NaOH per 20 mg. of synthetic products and were diluted with an equal volume of distilled water. The *p*H of the solutions was close to 9.00. Diffusion or viscosity measurements were not made. Assuming that the frictional ratios of my synthetic molecules are in the same range reported by other workers[11] for most corpuscular proteins, then the molecular weights are of the order of 250,000 to 500,000. The bulk of the material of each sample, however, was in a narrower range of molecular weights than the stated range. The molecular weight of the bovine serum albumin product was not determined. It is interesting to note in this connection that the molecular weights of the natural proteins from which the digest concentrates were prepared were much smaller than those of the synthetic products. Thus, the molecular weights as determined in the ultracentrifuge have been reported to be 40,000 for egg albumin, 40,000 for zein and 69,900 for fibrin.

Hydrolysis by Proteolytic Enzymes.—The synthetic products are readily hydrolyzed by crystalline pepsin at *p*H 1.5, fairly well by crystalline

(5) Described by J. L. Stokes in H. Tauber, "The Chemistry and Technology of Enzymes," John Wiley and Sons, Inc., New York, N. Y., 1949.

(6) R. J. Block and D. Bolling, "The Amino Acid Composition of Proteins and Foods," Charles C. Thomas, Springfield, Ill., 1945.

(7) J. L. Stokes, M. Gunness, I. M. Dwyer and M. C. Caswell, *J. Biol. Chem.*, **160**, 35 (1945).

(8) M. Gunness, I. M. Dwyer and J. L. Stokes, *ibid.*, **168**, 159 (1946).

(9) G. R. Tristram, "Advances in Protein Chemistry," Vol. 5 (1949).

(10) S. Shankman, M. N. Camien and M. S. Dunn, *J. Biol. Chem.*, **168**, 51 (1947).

(11) E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides," Reinhold Publ. Corp., New York, N. Y., 1943.

chymotrypsin at *p*H 7.6, and less readily by crystalline trypsin at *p*H 7.6. The pepsin digest contained 1 mg. of crystalline pepsin and 10 mg. of the respective products per 2 cc. of hydrochloric acid resulting in a *p*H of 1.6. The chymotrypsin and the trypsin digests contained 2 mg. of proteinase and 10 mg. of the respective synthetic products per cc. of the 0.1 *M* phosphate buffer of *p*H 7.6. The temperature was 37°. The incubation time was 20 minutes. For the measurement of proteolysis the Folin–Ciocalteu phenol reagent was employed using tyrosine as the standard as described by Northrop and associates.[12] These results are summarized in Table III. All the products, with the exception of zein, were used in the form of suspensions when digested by chymotrypsin and trypsin at *p*H 7.6. When 1 mg. of crystalline pepsin was added to 10 mg. of the synthetic products in a volume of 2 cc. the breakdown into small-size peptides was almost complete in 20 minutes at 37°. This did not take place with chymotrypsin and with trypsin when 2 mg. of each of the enzymes was used in the digests. There was only a small precipitate formed on the addition of 5% trichloroacetic acid to the digest. The trichloroacetic acid-soluble products gave a pink biuret test.

TABLE III

HYDROLYSIS OF THE SYNTHETIC (DIALYZED) PRODUCTS BY PROTEINASES

Source of product	Tyrosine liberated, mg. By pepsin	By chymotrypsin	By trypsin
Egg albumin	0.24	0.10	0.06
Bovine serum albumin	.17	.10	.08
Zein	.22	.18	.04
Blood fibrin	.28	.12	.09

(12) J. H. Northrop, M. Kunitz and R. M. Herriott, "Crystalline Enzymes," Columbia University Press, New York, N. Y., 1948.

Acknowledgments.—The author is grateful to Dr. S. J. Circle and Dr. M. MacKenzie of Columbia University, for the molecular weight estimations; to Dr. R. T. Major and Dr. J. J. Mayernik of Merck and Company, Inc., for the microbiological amino acid assays; to Dr. J. R. Weisiger of the Rockefeller Institute for Medical Research, for the free α-amino nitrogen determinations; and to Mr. P. P. Mazzella of this Laboratory for skillful technical assistance.

STATEN ISLAND, N. Y. RECEIVED JUNE 27, 1950

[CONTRIBUTION FROM THE RESEARCH LABORATORIES OF G. D. SEARLE AND COMPANY]

The Preparation of Some 2,3-Diaryl-2-pentenenitriles by the Knoevenagel Condensation

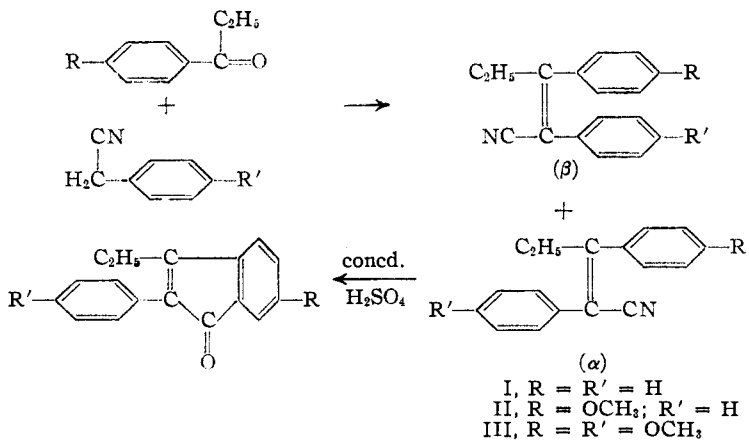
BY KURT RORIG

2,3-Diphenyl-2-pentenenitrile (I), 2-phenyl-3-(*p*-methoxyphenyl)-2-pentenenitrile (II), and 2,3-bis-(*p*-methoxyphenyl)-2-pentenenitrile (III) were prepared by the condensation of the appropriate arylacetonitrile and aryl ethyl ketone. Either sodamide in an aromatic solvent (toluene or xylene) or sodium methoxide in methanol effected these condensations. Both *cis*- and *trans*-2,3-diaryl-2-pentenenitriles were obtained from these reactions. The *trans* structure has been unequivocally assigned to the higher melting, less soluble 2,3-diphenyl-2-pentenenitrile (Iα) by virtue of its facile conversion to the known 3-ethyl-2-phenylindone (IV). Hydrogenation of I with Adams catalyst gave 2,3-diphenylvaleronitrile. With sodamide in boiling xylene, homoanisonitrile has been found to undergo autocondensation to give α,β-bis-(*p*-methoxyphenyl)-acrylonitrile.

Although condensations of aromatic aldehydes or diaryl ketones with arylacetonitriles are well known, aryl alkyl ketones have not been successfully used in such reactions. For example, the condensation of acetophenone with phenylacetonitrile in the presence of sodamide has been reported to fail.[1]

We have found that propiophenone and phenylacetonitrile do not react in the presence of acetic acid and ammonium acetate (the Cope conditions[2]). However, this condensation was effected by sodamide or sodium methoxide. In this manner 2,3-diphenyl-2-pentenenitrile (I) and its ring methoxylated analogs (II and III) were prepared.

Of these reactions, the sodamide-induced preparation of 2,3-diphenyl-2-pentenenitrile (I) was the one most thoroughly studied to achieve optimum conditions. This condensation was effected in maximum yield by heating equimolar amounts of the reactants in refluxing xylene. More than one equivalent of sodamide, or the lower reaction temperature obtained with benzene or ether as the solvent,[3] decreased the yield.



I, R = R′ = H
II, R = OCH_3; R′ = H
III, R = R′ = OCH_3

Two isomers (α and β) were obtained from these condensations. Trituration with petroleum ether

(1) F. Bodroux, *Bull. soc. chim.*, [4] **9**, 726, 758 (1911); *Compt. rend.*, **152**, 1594 (1911).

(2) A. C. Cope, C. M. Hofmann, C. Wyckoff and E. Hardenbergh, THIS JOURNAL, **63**, 3453 (1941).

(3) The sodamide used was a dry-packed commercial preparation. With freshly prepared sodamide, the optimum reaction temperature may well be lower.