dialyzed overnight in the cold against running tap water and lyophilized. The four diastereomers of α-hydroxy-β-methylvaleric acid were then subjected to the oxidative action of the supernatant and particulate fractions, so obtained.

Part of the enzymic data was obtained by Mrs. Betty Whitaker. The analyses were provided by Mr. Robert J. Koegel and his staff.

BETHESDA, MARYLAND

[CONTRIBUTION NO. 2053 FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY]

The Chromatographic Separation and Identification of Some Peptides in Partial Hydrolysates of Tussah Silk Fibroin

BY LOIS M. KAY, W. A. SCHROEDER, NANCY MUNGER AND NATALIE BURT

RECEIVED NOVEMBER 21, 1955

From a partial hydrolysate of Tussah silk fibroin, 27 peptides have been isolated and identified. About two-thirds of the protein molecule has been accounted for by these peptides and by free amino acids in the hydrolysate. The structure and quantity of the peptides lead to the conclusion that repetition of adjacent alanine residues is an important feature in Tussah silk fibroin but that there is little regularity in the arrangement of other amino acids. This structure is in contrast to that of *Bombyx* silk fibroin in which, for the most part, glycine residues occupy alternate positions along the polypeptide chain.

That the structures of *Bombyx mori* silk fibroin (BSF) and of Tussah silk fibroin (TSF) differ has long been known from the X-ray diffraction investigations of Trogus and Hess.[1] Although differences in the structures might arise simply from varied arrangements in the spatial configuration of polypeptide chains of identical sequence, complete amino acid analyses of these fibroins[2] have shown that the differences in structure more probably result from the very definite dissimilarities of amino acid composition; in both fibroins the quantities of glycine and alanine account for about 75% of the residues, but the major difference lies in the almost exact reversal in the relative proportions of these two amino acids in one fibroin as compared to the other. Marsh, Corey and Pauling[3,4] have interpreted the X-ray diffraction patterns of the two fibroins in terms of antiparallel chain pleated sheets. Their structure for BSF indicates that glycine residues alternate in position along the polypeptide chains for the most part; chemical data[5] on the sequence of amino acids confirm this view. In TSF, the alanine content is such that, for the most part, alanine residues could occupy alternate positions along the chains; the X-ray data, however, cannot distinguish between this possible regular arrangement or a more random one.

Many years ago, Abderhalden and co-workers[6,7] isolated alanine anhydride and what was thought to be ala-ala-ala-gly[8] from TSF. If these identifications are correct an alternate arrangement of alanine residues seems unlikely. The results of our investigation confirm this conclusion. In the present paper we shall describe the isolation and identification of 27 peptides in a partial hydrolysate of Tussah silk fibroin.

(1) C. Trogus and K. Hess, *Biochem. Z.*, **260**, 376 (1933). See also W. T. Astbury and F. O. Bell, *Tabulae Biologicae*, **17**, 90 (1939), for a résumé.

(2) W. A. Schroeder, L. M. Kay, B. Lewis and N. Munger, THIS JOURNAL, **77**, 3908 (1955).

(3) R. E. Marsh, R. B. Corey and L. Pauling, *Biochim. Biophys. Acta*, **16**, 1 (1955).

(4) R. E. Marsh, R. B. Corey and L. Pauling, *Acta Cryst.*, **8**, 710 (1955).

(5) L. M. Kay and W. A. Schroeder, THIS JOURNAL, **76**, 3564 (1954), and unpublished data.

(6) E. Abderhalden and A. Suwa, *Z. physiol. Chem.*, **66**, 13 (1910).

(7) E. Abderhalden and K. Heyns, *ibid.*, **202**, 37 (1931).

(8) Abbreviation of amino acid names and representation of sequences follow E. Brand (*Ann. N. Y. Acad. Sci.*, **47**, 187 (1946)) and F. Sanger (*Advances in Protein Chem.*, **7**, 1 (1952)).

Experimental

Source and Partial Hydrolysis of TSF.—The TSF which was used for hydrolysis was a portion of the preparation which had been completely analyzed in previous work.[2]

A 1.07-g. sample of TSF was placed in a 25-ml. volumetric flask and 10 ml. of J. T. Baker analyzed concd. hydrochloric acid was added. The mixture was maintained at 37° for 48 hr. with constant agitation which was supplied by attaching the stem of the flask parallel to a shaft which was inclined at 45° and rotated at 25 r.p.m. The greater portion of the fibroin dissolved rapidly to form a dark orange-brown solution which in the course of hydrolysis became darker and took on a greenish tinge. At the end of the period of hydrolysis, the mixture was cooled to room temperature, diluted to 25 ml. with water and a portion was used for the chromatogram. The solution contained suspended, undissolved material which was investigated as described below.

Ion-exchange Chromatography of the Partial Hydrolysate.—Chromatography was carried out on a column of Dowex 50-X4, 3.5 × 100 cm. in dimension. The resin (200–400 mesh, high porosity, Lot No. 3198-42) was purified exactly as Moore and Stein[9] describe for Dowex 50-X8 and was passed through a 120-mesh sieve.[10] The column was packed in the manner which Moore and Stein suggest. In the preparation of buffers, the detergent BRIJ 35, benzyl alcohol, thiodiglycol and disodium Versenate were omitted; *p*H 3.42 buffer was prepared as they describe and *p*H 5.5 (±0.05) buffer by minor modification of the quantities required for *p*H 5.0 buffer for basic amino acids.

The sample to be chromatographed was made up in the following way. A 13-ml. portion of diluted hydrolysate (preceding section) was pipetted into a mixture of 40 ml. of *p*H 6.7 buffer, 9 ml. of 6 *N* sodium hydroxide and 1 ml. of water. The *p*H of the solution was adjusted to 2.3 with 0.5 ml. of 6 *N* hydrochloric acid. After centrifuging to remove suspended material, 60 ml. of solution was placed on the column. On a moisture- and ash-free basis, the sample contained the soluble portion from 488 mg. of TSF.

Throughout the chromatogram, the temperature was maintained at 37°. Development was begun with *p*H 3.42 buffer and at the point shown in Fig. 1, change to *p*H 5.5

(9) S. Moore and W. H. Stein, *J. Biol. Chem.*, **192**, 663 (1951).

(10) More recently it has been determined that about one-third of the material in this sample of resin will pass a 200-mesh sieve. Consequently, the column as used contained a mixture of one-third through-200-mesh resin and two-thirds 120–200 mesh resin.

was made. The rate of solvent flow was 50 ml. per hr. and the fraction size was 10 ml. The progress of the chromatogram was followed by removing 0.5-ml. portions of alternate fractions and applying the ninhydrin method of Moore and Stein.[11]

Pooled fractions which contained a particular zone were neutralized to *p*H 6.5–7.5, evaporated to one-tenth their volume on a rotary evaporator,[12] finally evaporated to dryness over concd. sulfuric acid in a vacuum desiccator and stored at −10° until investigated.

Further Separation and Identification of the Peptides.—By the above chromatography on an ion-exchange resin, the complicated mixture of peptides is resolved into a number of simpler mixtures. Further separation is achieved by chromatographing on silicic acid–Celite after converting the peptides into dinitrophenyl (DNP) peptides. The DNP-peptide is then estimated and identified. The procedures have been described in detail.[13] Modification of the dinitrophenylation procedure sometimes was necessary; when the volume of water required to dissolve the material from an ion-exchange zone before dinitrophenylation was 5 ml. or more, the quantities of DNFB and sodium bicarbonate were doubled.

Because the amounts of the tri- and tetrapeptides were either small or the sequence was obvious from the composition (thus, ala-ala-ala), the actual sequence has been identified only in the case of gly-tyr-gly which was present in relatively large amount. The identification of the N-terminal amino acid is a part of the usual procedure and the C-terminal amino acid was identified by the hydrazinolytic method of Akabori, Ohno and collaborators in the modification described by Ohno.[14] The dry DNP-peptide was heated in a sealed tube for 3 hr. at 100° with 0.5 ml. of anhydrous hydrazine (Matheson). After removal of the hydrazine in a vacuum desiccator over sulfuric acid, the entire mixture was dinitrophenylated. The extractive separation of the now dinitrophenylated C-terminal amino acid was made essentially as described by Ohno.[14] Identification and quantitative estimation was done in the usual manner.[13] Trial experiments with known amounts of DNP-peptides with C-terminal glycine and alanine gave recoveries of the C-terminal amino acid which amounted to 50 to 70%. Kroner, Tabroff and McGarr[15] have also applied identical conditions of hydrazinolysis to DNP-peptides except that the temperature was 120°. Under these conditions, we failed to detect any C-terminal amino acid.

Investigation of Insoluble Portion of TSF.—The insoluble portion which was centrifuged down from the sample was washed repeatedly with water and recentrifuged. After the residue had been thoroughly dried, the weight corresponded to about 10% of the original fibroin.

In order to determine the amino acid composition of the insoluble portion, another sample of diluted hydrolysate equivalent to 40 mg. of fibroin was diluted with 4 ml. of water and centrifuged; the residue was washed with 5 × 3 ml. of *N* hydrochloric acid and then, without drying, transferred to a flask with 10 ml. of doubly distilled 6 *N* hydrochloric acid and hydrolyzed under reflux for 24 hr. After the hydrochloric acid had been removed with a rotary evaporator, the residue of amino acid was dinitrophenylated, and the components were identified and quantitatively estimated.

Results

Figure 1 shows the separations which resulted from the chromatography on Dowex-50. The chromatogram was carried no further because preliminary small-scale experiments had demonstrated that inappreciable amounts of material were eluted at other *p*Hs or even with 0.2 *N* sodium hydroxide. Contrary to previous experience,[5,13] some separations on the larger-scale chromatogram were less satisfactory than in the preliminary work. Thus, in

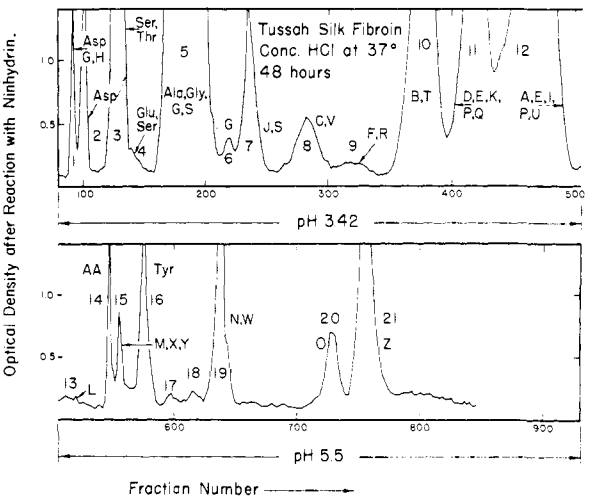

Fig. 1.—Separation of amino acids and peptides in a partial acidic hydrolysate of Tussah silk fibroin by chromatography on Dowex 50-X4. Column dimensions, 3.5 × 100 cm.; temperature of chromatogram, 37°; developers, buffers of *p*H shown; fraction size, 10 ml., sample, soluble portion from 488 mg. of TSF.

the preliminary chromatograms, zones 6 and 7 were resolved into three zones and zone 12 into two inadequately separated zones while, on the other hand, zones 1 and 2 were not resolved.

The 21 zones have been investigated and the qualitative and quantitative results are presented in Table I. One or more compounds have been identified in each zone except zones 17 and 18 which contained small amounts of several constituents. Minor components which were not investigated further were present in most of the zones.

Despite the fact that the individual zones of the ion-exchange chromatogram for the most part are not cleanly separated, there is relatively little mutual overlapping of compounds in adjacent zones (see Table I) and where it does occur one zone contains all but a small portion of the total. It is, therefore, the more surprising to isolate the same compound from non-adjacent zones, for example, asp-gly in zones 1, 5, and 6 and ser-asp in zones 5 and 7.[16] Further anomalous results were obtained when the DNP-peptides of zone 6 were separated on silicic acid–Celite prior to final identification. In addition to several insignificant zones of DNP-peptides, three main zones were obtained of which one contained about three times the sum of the other two. All three zones gave unequivocal identification as DNP-asp-gly. When a portion of the main zone was rechromatographed, three zones again appeared. It was definitely proved that the main zone contained DNP-aspartylglycine and not DNP-asparaginylglycine which might have produced a second zone, at least, by losing a portion of its amide group during the various operations. Although a definite cause for this unusual behavior cannot be adduced, it may be conjectured that the

(11) S. Moore and W. H. Stein, *J. Biol. Chem.*, **176**, 367 (1948).

(12) L. C. Craig, J. D. Gregory and W. Hausmann, *Anal. Chem.*, **22**, 1462 (1950).

(13) W. A. Schroeder, L. M. Kay, J. LeGette, L. Honnen and F. C. Green, THIS JOURNAL, **76**, 3556 (1954).

(14) K. Ohno, *J. Biochem.* (*Japan*), **40**, 621 (1953).

(15) T. D. Kroner, W. Tabroff and J. J. McGarr, THIS JOURNAL, **77**, 3356 (1955).

(16) A possible explanation was suggested by a referee of this paper. The high ionic strength of the sample (*ca.* 2 *N*) added to the column (0.2 *N* buffer) might be cause for some distortion. The observation is still foreign, however, to earlier studies on the use of such columns for the separation of peptides.

TABLE I

QUALITATIVE AND QUANTITATIVE DATA ON PEPTIDES FROM A PARTIAL ACID HYDROLYSATE OF TUSSAH SILK FIBROIN

Desig-nation	Compound	Isolated from zone no.	μ moles cpd. per g. TSF[a]	% [b] of the total amt. of amino acid in TSF accted. for by amt. of peptide isolated				
				Ala	Gly	Ser	Tyr	Asp	Others
A	Ala-ala	12	553.8	20.9					
B	Ala-ala-ala	10	149.7	8.5					
C	Ala-ala-ala-ala	8	34.4	2.6					
D	Ala-gly	11	157.5	3.0	5.0				
E	Ala-(gly,ala)	*11*,[c] 12	16.0	0.6	0.5				
F	Ala-(gly,ala$_2$)	9	2.0	0.1	0.1				
G	Asp-gly	1, 5, *6*	60.5		1.9			10.7	
H	Asp-gly-gly	1	3.1		0.2			0.6	
I	Gly-ala	12	35.3	0.7	1.1				
J	Gly-asp	7	11.9		0.4			2.1	
K	Gly-gly	11	85.5		5.4				
L	Gly-(ala,gly)	13	5.9	0.1	0.4				
M	Gly-(val,gly)	15	10.0		0.6				val = 13.5
N	Gly-tyr	19	74.3		2.3		12.7		
O	Gly-tyr-gly	20	37.6		2.4		6.4		
P	Ser-ala	11, *12*	106.7	2.0		7.6			
Q	Ser-ala-ala	11	46.2	1.8		3.3			
R	Ser-ala-ala-ala	9	6.8	0.4		0.5			
S	Ser-asp	5, 7	89.3			6.3		15.7	
T	Ser-gly	10	196.6		6.2	14.0			
U	Ser-(gly,ala)	12	18.8	0.4	0.6	1.3			
V	Ser-ser	8	8.4			1.2			
W	Ser-tyr	19	37.8			2.7	6.5		
X	Tyr-asp	15	1.4				0.2	0.3	
Y	Tyr-glu	15	1.5				0.3		glu = 1.6
Z	Tyr-gly	21	175.1		5.5		29.9		
AA	Val-gly	14	10.5		0.3				val = 14.2
	Total in peptides		1936.6	41.1	32.9	36.9	56.0	29.4	glu = 1.6 val = 27.7
	Ala	5	833	15.7					
	Asp	1, *2*	53					9.5	
	Glu	4	8						glu = 7.9
	Gly	5	1102		34.7				
	Ser	*3*, 4	268			19.0			
	Thr	3	4						thr = 23.2
	Tyr	16	82				13.9		
	Insoluble residue			25.0	1.7	5.3		2.6	
	Over-all total		4287	81.8	69.3	61.2	69.9	41.5	glu = 9.5 thr = 23.2 val = 27.7

[a] Recalculated to one g. of moisture- and ash-free TSF. [b] Based on the amino acid composition of TSF in reference 2. [c] The number of the zone which contained the major portion is italicized.

free carboxyl group of aspartic acid is responsible because the behavior was observed only with asp-gly and ser-asp; on the other hand, gly-asp did not behave unexpectedly. The quantities of asp-gly and ser-asp in Table I include all that was detected in the several zones.

Under the conditions which have been employed to dinitrophenylate compounds in the presence of buffer salts, the reaction of DNFB with the phenolic group of tyrosine may be incomplete. Thus, a mixture of N,O-di-DNP-tyrosine and N-mono-DNP-tyrosine was isolated from zone 16 after dinitrophenylation. Likewise, the reaction with gly-tyr-gly (zone 20) and tyr-gly (zone 21) was only partial. When N-mono-DNP-tyrosine is chromatographed on silicic acid–Celite under the conditions[17] which have been used in these investigations, its chromatographic behavior is almost indistinguishable from that of DNP-aspartic acid. Proof that mono-DNP-tyrosine is formed under some conditions is readily obtained by its further dinitrophenylation, whereupon a compound with the chromatographic properties of the di-DNP derivative results. It is apparent that this result was obtained but not recognized as such during the investigation of zone S-13 from a partial hydrolysate of *Bombyx* silk fibroin.[5]

About 90% of the fibroin which did not dissolve during the course of the partial hydrolysis was composed of alanine; smaller amounts of glycine, serine and aspartic acid also were present.

(17) F. C. Green and L. M. Kay, *Anal. Chem.*, **24**, 726 (1952).

Discussion

Quantitative Aspects of the Investigation.—As in previous investigations,[5,13] stress has been laid on

the acquisition of quantitative data. Some factors which influence the quantitative data have been discussed previously. In Table II, a comparison is made of the quantities which were isolated from each zone and the quantities which are calculated to have been present. The percentage isolated is generally better than 60% and where it is less the zone usually was rather insignificant. Caution must be exercised in the comparison of these data because of the assumption that the color yield of all compounds is equivalent to that of leucine. Zone 12 is a case in point; on the above basis, the recovery is only 63%, but if we take into consideration that ala-ala constitutes 80% of the peptides in zone 12 and that ala-ala is reported to have a color yield of 1.74,[18] the recovery is 100%.

TABLE II

COMPARISON OF AMOUNTS ISOLATED FROM ION-EXCHANGE ZONES WITH AMOUNTS CALCULATED FROM THE NINHYDRIN PROCEDURE

Zone	Isolated[a]	Calcd. from ninhydrin[a,b]	$\frac{\text{Isolated}}{\text{Calcd.}} \times 100$
1	30	38	79
2	52	63	83
3	269	287	94
4	10	16	63
5	1953	1991	98
6	32	25	128
7	86	115	75
8	43	74	58
9	9	18	50
10	346	470	74
11	309	399	77
12	710	1120	63
13	6	13	46
14	11	38	29
15	13	33	39
16	81	96	84
17	0	9	0
18	0	12	0
19	112	143	78
20	38	63	60
21	175	213	82

[a] Per g. of fibroin; values given to nearest μmole. [b] Assumption is made that color yield of all components is equivalent to that of leucine.

The data of Table I point out that about 67% of the TSF has been accounted for in one form or another. Of the 67%, about 35% is in the form of identified peptides, about 10% remained as undissolved fibroin and the remainder was present as free amino acids.

Conclusions about the Structure of TSF and a Comparison with the Structure of BSF.—Although the amino acid composition of TSF is such that, for the most part, the alanine residues could be arranged alternately along the polypeptide chains, the peptides which have been isolated suggest rather that considerable portions of the chains consist of a repetition of alanine residues. About 35% of the alanine in the fibroin was isolated in the form of ala-ala, ala-ala-ala, ala-ala-ala-ala, ser-ala-ala and ser-ala-ala-ala. Furthermore, about 25% of the alanine was present in the 10% of the fibroin which did not dissolve during the hydrolysis; actually about 90% of the amino acid residues in this insoluble portion were alanine and, on the average, therefore, about nine consecutive alanine residues must be present before the sequence is interrupted by a different amino acid. Apparently, then, some portions of the TSF are virtually polyalanine. This grouping of alanine residues of necessity decreases the probability that other amino acids will be associated with alanine and increases the probability of greater variety of combination of the other amino acids. The result is to be seen in such sequences as asp-gly, asp-gly-gly, gly-gly, ser-asp, ser-ser, ser-tyr, tyr-asp and tyr-glu although such anticipated sequences as ala-gly, gly-ala, etc., also occur.

There is great contrast both qualitatively and quantitatively in the peptides which may be isolated from TSF and BSF under identical conditions of hydrolysis (48 hr. at 37° in concd. hydrochloric acid). The six peptides which were isolated from both fibroins are compared quantitatively in Fig. 2. The dissimilarities are very evident. The large amount of ala-ala sequence (as well as ala-ala-ala and ala-ala-ala-ala) in TSF has reduced the probability of sequences such as ala-gly and gly-ala and accordingly they occur in greatly reduced amount. The sequence ser-gly is also less evident in TSF presumably because serine is also associated with alanine in ser-ala, ser-ala-ala and ser-ala-ala-ala. For the most part in both fibroins, tyrosine is linked at both the amino and carboxyl groups with glycine; although peptides of tyrosine and other amino acids have been isolated from TSF, peptides of tyrosine and glycine only have been found in BSF.

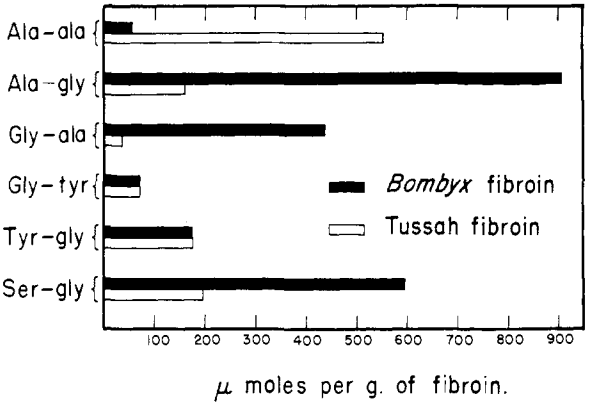

Fig. 2.—Quantitative comparison of certain peptides isolated both from BSF and TSF.

Qualitatively, the difference in the kind of peptides from the two fibroins is equally striking. With the exception of ala-ala which is quantitatively insignificant, all peptides from BSF contain glycine, a fact which is indicative of the alternation of glycine residues along the chains for the most part. Unpublished work on BSF has resulted in the isolation of ser-gly-ala-gly-ala-gly and all penta-, tetra-, tri- and dipeptides related to it in further substantiation of the alternation of glycine residues; indeed, quantitatively, this hexapeptide sequence appears to be very important in BSF. In TSF, on the other hand, there seems to be little regularity of sequence

(18) M. Ottesen and C. Villee, *Compt. rend. Lab. Carlsberg, Ser. chim.*, **27**, 421 (1951).

except for the repetition of adjacent alanine residues. This randomness makes itself evident in the greater array of peptides from TSF as compared to BSF. Thus, in BSF the sequence ser-gly seems to exist to the exclusion of other sequences such as ser-X where X is any amino acid; in TSF, a variety of sequences is to be found in ser-ala, ser-asp, ser-ser and ser-tyr in addition to ser-gly. Likewise, gly-gly has been isolated from the hydrolysate of TSF but no evidence of its presence in BSF hydrolysates has ever been found. Differences in the type of tyrosine-containing peptides have been noted above.

The chemical data on the sequence of amino acids in the two fibroins and the interpretation of the X-ray diffraction patterns are in accord. Thus, the X-ray pattern of BSF can best be explained[3] in terms of two spacings between adjacent pleated sheets and these spacings can be achieved only if glycine residues occupy alternate positions in the chains: the chemical data show this alternation. The X-ray pattern of TSF indicates a single spacing between pleated sheets[4] but no conclusions about regularity of sequence can be drawn; the chemical data show little evidence of regularity.

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[CONTRIBUTION FROM THE DEPARTMENT OF PHARMACOLOGY, SCHOOL OF MEDICINE, AND THE COBB CHEMICAL LABORATORY, UNIVERSITY OF VIRGINIA]

3-Iodo-, 3,3′-Diiodo- and 3,3′-Diiodo-5-bromothyronine[1]

BY CHALMERS L. GEMMILL, JAMES J. ANDERSON AND ALFRED BURGER

RECEIVED DECEMBER 1, 1955

Syntheses of DL-3-iodothyronine and D,L-3,3′-diiodo-5-bromothyronine have been performed, and these compounds as well as 3,3′-diiodothyronine have been described accurately. 3,3′-Diiodothyronine has weak or no thyromimetic activity while 3,3′-diiodo-5-bromothyronine is markedly active.

It is generally accepted[2] that in diphenyl ether derivatives structurally related to thyroxine, iodine substitution in positions 3 and 5 is necessary for minimal thyroxine-like activity, and that thyronine derivatives with halogens in 3′,5′ only are devoid of thyromimetic action. In view of the marked metabolic activity of (−)3,3′,5-triiodothyronine, introduction of iodine into position 3′ raises minimal activity to a high level. This fact reopens the question as to the significance of the 5-iodine atom. We began to synthesize 3,3′-diiodothyronine according to the general pattern set for 3,5-diiodothyronine by Harington and Barger,[3] starting with 3,4-diiodonitrobenzene and 3-iodo-4-methoxyphenol. While this work was in progress, a communication by Roche, Michel and Wolf[4] described the preparation of 3,3′-diiodothyronine by monodeiodination of 3,5-diiodothyronine, and subsequent monoreiodination of the resulting 3-iodothyronine. They reported[5] that DL-3,3′-diiodothyronine had about 82% of the antigoitrogenic activity of thyroxine in the rat; DL-3,3′,5′-triiodothyronine had little or no activity.

The last step of our synthetic approach to 3,3′-diiodothyronine was the reduction and hydrolysis of α-benzamido-3-iodo-4-(3′-iodo-4′-methoxyphenoxy)-cinnamic acid. Under a variety of conditions, using hydriodic acid, or combinations of hydriodic and hydrobromic acid with different amounts of phosphorus, one atom of iodine was lost, and a monoiodothyronine of melting point 235–237° was obtained in yields up to 64%. It was chromatographically homogeneous, and did not consist of a chance mixture of the 3- and 3′-iodo isomers. It differed in melting point from the 3-iodothyronine (m.p. 206°) and from 3′-iodothyronine (m.p. 207°) as reported by Roche, *et al.*[4] Confirmation of the structure of our monoiodo derivative as 3-iodothyronine was secured by unequivocal synthesis. Condensation of 3,4-diiodonitrobenzene with *p*-methoxyphenol to 2-iodo-4-nitro-4′-methoxydiphenyl ether was followed by reduction of the nitro group of this compound with iron and aqueous ethanol. A Sandmeyer reaction with the resulting amine gave 2-iodo-4-cyano-4′-methoxydiphenyl ether which was purified by chromatography and converted to the corresponding aldehyde by the Stephen method. The aldehyde was subjected to an azlactone synthesis to yield, in two steps, α-benzamido-3-iodo-4-(4′-methoxyphenoxy)-cinnamic acid which was reduced and cleaved to 3-iodothyronine. The product thus obtained was identical with the deiodination product of melting point 235–237° above, and vindicated our datum as contrasted with that in the literature.[4]

Since the direct synthesis of 3,3′-diiodothyronine had failed, we iodinated 3-iodothyronine and obtained a product which, as described,[4] melted at 198–199°. However, even after drying over phosphorus pentoxide at 115° (0.2 mm.) for eight hours it retained two molecules of water of crystallization. The iodine analysis reported[4] for the water-washed and dried product points to anhydrous material; this divergence cannot be explained at this time.

The only known thyroxine analog containing

(1) This investigation was supported in part by a research grant, A 649, from the National Institute of Arthritis and Metabolic Diseases of the National Institutes of Health, Public Health Service, and by a contract, No. AT-(40-1)-263, from the Atomic Energy Commission.

(2) E. Frieden and R. J. Winzler, *J. Biol. Chem.*, **176**, 155 (1948); C. Niemann, in "Fortschritte der Chemie der organischen Naturstoffe," VIII, L. Zechmeister, ed., Springer, Vienna, 1950, p. 167; H. A. Selenkow and S. P. Asper, Jr., *Physiol. Revs.*, **35**, 426 (1955), *cf.* p. 443.

(3) C. R. Harington and G. Barger, *Biochem. J.*, **21**, 169 (1927).

(4) J. Roche, R. Michel and W. Wolf, *Compt. rend.*, **239**, 597 (1954).

(5) J. Roche, R. Michel, W. Wolf and N. Etling, *Compt. rend. soc. biol.*, **148**, 1738 (1954).