PREPARATION OF PURE PROTEINS FROM HOG THYROID GLANDS BY COLUMN CHROMATOGRAPHY ON DIETHYLAMINOETHYL-CELLULOSE

By S. SHULMAN* and P. G. STANLEY*†

[*Manuscript received December* 19, 1960]

Summary

Saline extracts of hog thyroid glands were chromatographed on columns of diethylaminoethyl (DEAE)-cellulose using gradients of pH and salt concentration. Two major peaks of protein emerged from these columns. When rechromatographed under identical conditions, part of the first peak to emerge from the column redistributed itself to give two peaks which appeared in positions corresponding to those of the two peaks eluted from the first chromatographic run.

Examination of the ultracentrifugal components present in the eluted protein fractions and their comparison with the components present in the original saline extracts indicated that some resolution of the ultracentrifugal components had taken place during chromatography on DEAE-cellulose. After further purification by a run in the separation cell, about 96% of the sedimentary material was located in the peak with $S_{20,w} = 18$. Most such runs gave even higher purity.

Quantitative paper chromatography of the iodoamino acids of an enzymic hydrolysate of protein from the first peak and from three parts of a broad second peak eluted from a column of DEAE-cellulose indicated that the ratio of iodotyrosines to iodothyronines was 1·9, 3·7, 4·2, and 8·8, respectively.

I. INTRODUCTION

The first extensive study of methods for the preparation of thyroglobulin from thyroid glands of a number of species was described by Derrien, Michel, and Roche (1948). By fractional salting-out of the proteins present in saline extracts of sliced glands they obtained thyroglobulins which were homogeneous in the ultracentrifuge and during electrophoresis according to Tiselius (Derrien, Michel, and Roche 1948; Derrien *et al.* 1949). Crude thyroid extracts are known to contain three major and two minor ultracentrifugal components‡ (Shulman, Rose, and Witebsky 1955). Thyroglobulin prepared by the method of Derrien, Michel, and Roche (1948) exhibits three breaks in the salting-out curve which suggest that it comprises three molecular entities differing slightly in their solubility properties (Derrien, Michel, and Roche 1948). This concept is also supported by the finding of three precipitin bands by the method of Ouchterlony (1948) when human thyroglobulin and an antiserum prepared in a rabbit were allowed to diffuse together (Easty, Slater, and Stanley 1958).

* Department of Bacteriology and Immunology, University of Buffalo Medical School, Buffalo 14, New York.

† Present address: Unit of Clinical Investigation, The Royal North Shore Hospital of Sydney, Crow's Nest, N.S.W.

‡ In this paper the term "ultracentrifugal component" refers to material sedimenting in a particular peak during ultracentrifugal analysis. It is realized that such a "component" need not be homogeneous when subjected to other methods of fractionation.

A detailed description of salting-out methods for purification of the proteins of thyroid extract is being published from this Laboratory (Shulman and Witebsky 1961).

Sober *et al.* (1956) described the fractionation of serum proteins on columns of diethylaminoethyl (DEAE)-cellulose prepared according to Peterson and Sober (1956) and this procedure has now been applied to a study of the proteins present in saline extracts of hog thyroid glands in the hope of developing a simple and rapid method for the preparation of these proteins in a pure form. A preliminary report has been published (Shulman and Witebsky 1960).

II. Materials and Methods

Saline extracts were prepared as described by Shulman, Rose, and Witebsky (1955) from hog thyroid glands which had been stored in the frozen state. Protein concentrations were measured by the extinction at 280 mμ. Because of the variable composition of the buffers used for the elution of the proteins, the extinctions of the effluent fractions were read against a blank of distilled water; when samples of the fractions had to be diluted, 0·15M NaCl was used as diluent and the same solvent was used in the reference cell of the spectrophotometer. Additional readings of the optical densities of saline extracts and eluted fractions were taken at 407 mμ in order to record the intensity of the red-orange colour. Extracts were chromatographed immediately or were freeze-dried and stored at 2–4°C.

DEAE-cellulose, type 20, lot 1023, was obtained from the Brown Company, Berlin, New Hampshire, U.S.A. It was employed without pretreatment other than adjustment of the pH to the starting value. Used DEAE-cellulose was first regenerated by washing with a large excess of 1N NaOH followed by exhaustive washing with distilled water (Peterson and Sober 1956). DEAE-cellulose (29 g) was suspended in distilled water (1 l.) and the pH brought to 7·0–7·2 by gradual addition of 0·5M NaH_2PO_4 (about 35 ml) to the suspension with vigorous stirring. The column (2·5 cm internal diameter) was filled with 0·1M phosphate buffer, pH 6·96, and the suspension of DEAE-cellulose added gradually, permitting larger particles to settle on the fritted glass disk at the bottom of the column. The column was washed with 0·1M phosphate buffer (pH 6·96, 0·5–1·0 l.), then with an equal volume of starting buffer (0·005M phosphate buffer, pH 7·2). It was compressed with N_2 (10 lb/in^2, 20–60 sec). Saline extracts of hog thyroid glands (fresh or reconstituted from freeze-dried material) were dialysed with stirring against several changes of starting buffer. Initially the elution schedule was that used for human serum proteins by Sober *et al.* (1956, p. 759, fig. 3). Our subsequent modifications are described in the legends to Figures 2 and 4. Fractions of 6·1 ml were collected mechanically by a volumetric type of collector. The flow rates did not exceed 1 ml/min.

Protein solutions were concentrated by dialysis with stirring against concentrated solutions of polyvinylpyrrolidone. The polymer solutions had been exhaustively dialysed against 0·15M NaCl before use in order to free them of contaminants of low molecular weight. Alternatively, proteins were concentrated by freeze-drying and resolution.

Enzymic Hydrolysis.—Samples of proteins were subjected to enzymic hydrolysis in sealed tubes for 48 hr in the presence of toluene. A mixture of crystalline trypsin (14·9 mg), crude trypsin (4·9 mg), crystalline chymotrypsin (5·1 mg), and of a suspension of carboxypeptidase (0·5 ml) was used per 100 mg thyroidal protein in a 2-amino-2-hydroxymethylpropane-1,3-diol buffer, pH 8. The pH of the hydrolysates was adjusted to about 1·3 by gradual addition of 1N HCl and the iodoamino acids were extracted with three successive portions of *n*-butanol (25 ml), previously saturated with dilute HCl (pH 1·15), the pooled *n*-butanolic phases were made alkaline

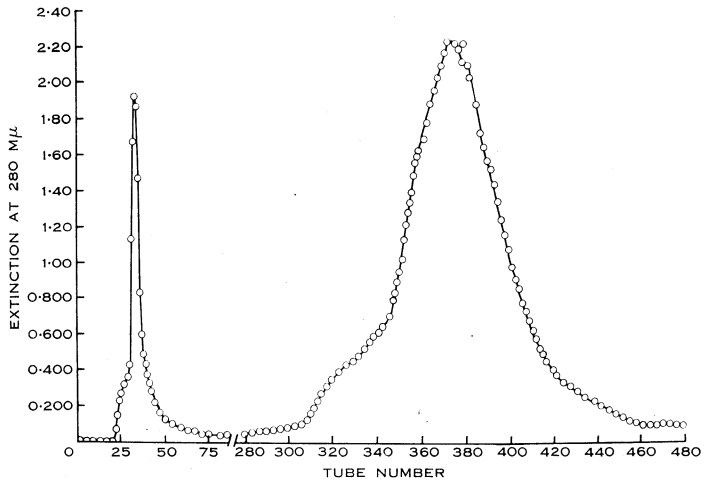

Fig. 1.—Elution pattern of thyroidal proteins (1·56 g, crude saline extract) applied to a column of DEAE-cellulose (column 3, 29 g, pH 7·1) and developed with the following buffers: 0·005M phosphate buffer (pH 7·2, 382 ml); 250 ml of the same buffer in mixing flask, gradient to 0·02M phosphate buffer (pH 6·0, 205 ml); gradient to 0·05M NaH_2PO_4 (pH 4·53, 480 ml); gradient to 0·05M NaH_2PO_4–0·02M NaCl (365 ml); gradient to 0·05M NaH_2PO_4–0·05M NaCl (pH 4·48, 750 ml); gradient to 0·05M NaH_2PO_4–0·1M NaCl (pH 4·43, 1150 ml). Volume of fractions: 6·1 ml.

by addition of 2N NH_4OH (15 ml) and the iodoamino acids stabilized by addition of thiouracil (1·33 mg in 1 ml water). The solutions were reduced to dryness and iodoamino acids determined by the method of Mandl and Block (1959).

Crude saline extracts of thyroid glands as well as protein fractions eluted from the columns of DEAE-cellulose were examined in the ultracentrifuge (Spinco, model E) either with or without prior adjustment of the solvent by dialysis against 0·15M NaCl. Concentrations of about 0·7% were found to be optimal for most of the fractions examined. Higher concentrations were used at times to detect impurities in isolated components.

III. Results

The extinctions at 280 mμ of the fractions collected from the column (column 3) are plotted against the number of the tube in Figure 1. This column was developed with the buffers described by Sober *et al.* (1956, p. 759, fig. 3). The protein emerged from the column in two major peaks, the recovery being 33% of the protein applied to the column.

The fractionation of thyroidal proteins on a new column (column 8) run with a modified system of buffers is illustrated in Figure 2; the buffers used are described in the legend. Again two major peaks of protein (*A* and *D*) were eluted from the column; 70% of the protein applied to this column was recovered in peaks *A* and *D*, an additional 2% in minor peaks. Part of the material from peak *A* of column 8 was rechromatographed on a fresh column (column 9) under identical conditions. The elution pattern is shown in Figure 2. Protein again appeared in two major peaks corresponding in position to peaks *A* and *D* of column 8. Of the protein applied to

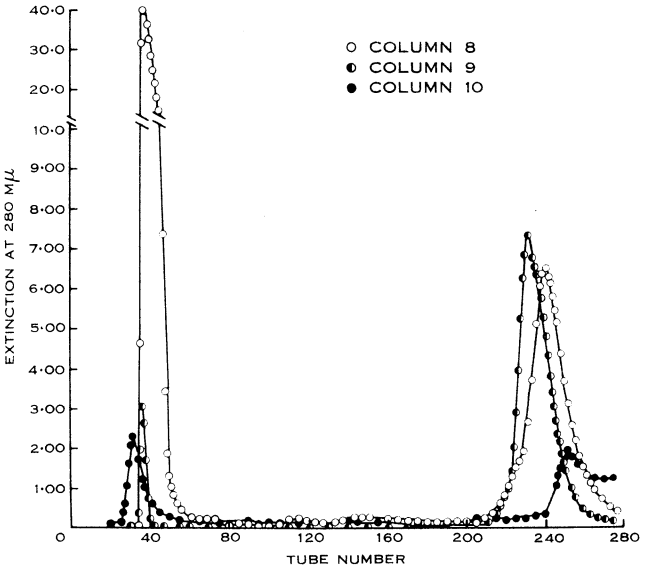

Fig. 2.—Elution patterns of thyroidal proteins applied to column 8 (3·96 g, crude saline extract), column 9 (1·21 g, peak *A* from column 8), and column 10 (67·5 mg, peak *A* from column 9) and developed with the following buffers: Columns 8 and 9: 0·005M phosphate buffer (pH 7·2, 350 ml); 250 ml of the same buffer in mixing flask, gradient to 0·05M NaH_2PO_4 (pH 4·53, 200 ml); gradient to 0·05M NaH_2PO_4–0·05M NaCl (pH 4·48, 300 ml); gradient to 0·05M NaH_2PO_4–0·1M NaCl (pH 4·43, 300 ml); gradient to 0·1M NaH_2PO_4–0·2M NaCl (pH 4·07, 950 ml). Column 10: Identical, except that the gradient to 0·05M NaH_2PO_4 was set up with 300 ml of 0·05M NaH_2PO_4, retarding the emergence of the second peak. Volume of fractions: 6·1 ml. Each column contained 29 g. of DEAE-cellulose (pH near 7). Extinctions have been multiplied by 20 for column 10.

column 9, 83% was recovered in peaks *A* and *D*. Part of the material from peak *A* of column 9 was re-run on a fresh column (column 10). The elution pattern is reproduced in Figure 2. The extinction values have been multiplied by 20. Again two peaks emerged from the column which corresponded in position to peaks *A* and *D* of column 8.

Examination of the proteins eluted in peak *A* of column 8 in the ultracentrifuge revealed that some fractionation of the ultracentrifugal components present in the original saline extract had taken place in the column, resulting in an almost complete absence of the slow component and the presence of only the 18-*S* and 29-*S*

components in this fraction. Four ultracentrifugal components were present in peak *D* of column 8. In a number of subsidiary experiments, material from peak *A* of column 8 was ultracentrifuged in a separation cell and an 18-*S* component of a high degree of purity isolated from the upper compartment. Although a small 11-*S* peak appeared in this particular fraction (run 2915 in Table 1), other samples obtained in exactly this way showed no detectable 11-*S* boundary, and thus appeared to contain close to 100% of the sedimenting material in the peak with $S_{20,w} = 18$. An example of such an ultracentrifugal appearance (run 2924) is shown in Figure 3 (*c*), where it

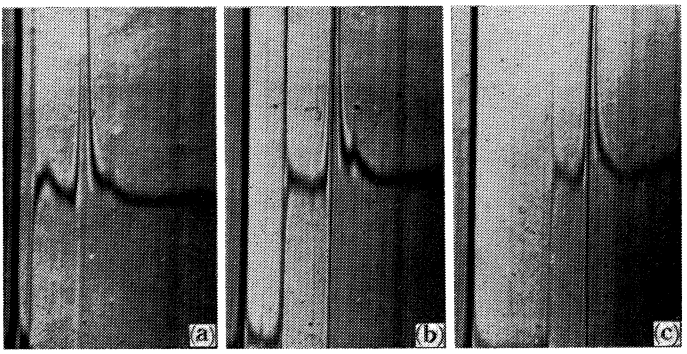

Fig. 3.—Ultracentrifugal patterns of (*a*) saline extract of hog thyroid tissue, run 2902; (*b*) peak *A* material from column 8, run 2909; (*c*) separation cell top component obtained from peak *A* of column 8, run 2924. Protein concentration 0·7% in each. Phase-plate angle 30°. Photographs were taken 8 min after rotor reached speed of 59,600 r.p.m.

can be compared with that of saline extract (run 2902) in Figure 3 (*a*), and peak *A* before use of the separation cell (run 2909) in Figure 3 (*b*). The $S_{20,w}$ values and percentage compositions of the proteins present in the original saline extracts and of some of the fractions are shown in Table 1.

In an additional column we modified not only the system of buffers but also the initial pH of the DEAE-cellulose and of the starting buffer. The elution pattern of this column (column 11) is shown in Figure 4; the buffers used are described in the legend. Two major peaks of protein emerged from this column in positions corresponding to peaks *A* and *D* of column 8, with an additional intermediate protein peak which coincided with a peak of the red-orange pigment (absorption maximum 407 mμ) of the thyroid extract. Although the final buffer used with column 11 was 0·25M NaH_2PO_4–2M NaCl, further elution of this column with 0·1N NaOH* led to the emergence of additional protein equivalent to about 11% of the protein applied.

Samples of material corresponding to peak *A* (column 8) and from three parts of a broad peak corresponding to peak *D* (column 8) were obtained from another chromatographic fractionation and were hydrolysed enzymically. The percentages of iodoamino acids in the four samples are listed in Table 2.

* We are indebted to Mr. J. Armenia for continuing the elution of column 11 with 0·1N NaOH.

IV. Discussion

The appearance of two major protein fractions during the development of the DEAE-cellulose columns suggested that the thyroid extracts contained two types of protein differing in their ionizing groups. The rapid appearance of the first protein

Table 1

$S_{20,w}$ values and approximate percentage compositions of the ultracentrifugal components present in the original saline extracts of hog thyroids and of fractions obtained from these by column chromatography on DEAE-cellulose

Run No.	Material	Concn. (%)	$S_{20,w}$	% of Total
2588	Saline extract used for column 3	0·50	28·4	10·0
			18·0	78·4
			4·3	11·5
2590	Column 3, pool B (peak *A*)	0·45	18·8	84·1
			*c.*4	15·9
2591	Column 3, pool D (peak *D*)	0·79	*	5·1
			17·7	74·8
			12·6	4·2
			0·68	15·9
2592	Column 3, pool C (peak *D*)	0·35	17·7	68·9
			12·2	3·9
			4·5	27·2
2593	Column 3, pool E (peak *D*)	0·59	*	20·1
			18·3	73·5
			12·2	6·4
2902	Saline extract used for column 8	0·70	25·7	11·3
			18·2	73·8
			4·7	14·9
2909	Column 8, peak *A*	0·70	29·8	10·6
			18·1	89·4
2915	Column 8, peak *A*, top of separation cell run	1·00	15·6	95·7
			11·1	4·3
2933	Column 8, peak *D*	0·76	29·1	6·0
			17·9	82·7
			11·4	3·0
			5·0	8·2
2934	Column 8, peak *E*	0·76	40·3	45·3
			18·5	39·0
			3·9	11·1
			*	4·5
2936	Column 8, peak *C*	0·68	8·5	38·9
			7·3	61·0
2952	Saline extract, not used for chromatography	0·80	27·9	7·8
			17·9	82·2
			4·0	10·0
2953	Column 9, tube 231	0·73	31·2	10·5
			17·3	85·3
			13·1	3·0
			*	1·2

TABLE 1 (*Continued*)

Run No.	Material	Concn. (%)	$S_{20,w}$	% of Total
2954	Column 9, tube 236	0·64	40·6	6·7
			19·0	89·2
			15·6	2·7
			11·7	1·4
2958	Column 9, peak *A*	0·73	25·1	10·7
			19·7	72·3
			13·9	2·6
			6·9	7·4
			2·6 }	7·0
			3·0 }	
2989	Column 10, peak *A*	0·56	6·5	37·0
			3·3	63·0
2992	Column 10, peak *D*	0·8	17·4	89·3
			11·5	10·7
2997	Column 10, peak *A*	0·56	6·8	41·2
			3·4	58·8
3012	Column 11, tube 34 (phosphate)	2·52	18·3	†
			12·6	†
			11·5	†
			3·0	†
3013	Column 11, tube 34	0·77	20·1	†
			14·4	†
			11·7	†
3015	Column 11, tube 34 (phosphate)	2·52	*	†
			11·6	†
			7·3	†
			2·2	†
3016	Column 11, tube 245 (phosphate)	1·23	19·1	†
			16·0	†
			12·5	†
			4·1	†

* Insufficient points to determine sedimentation rate.

† Concentration too high to determine percentages.

peak (peak *A*) after the start of development raised the possibility that this might be a "breakthrough" peak due to overloading of the column. The redistribution of material from peak *A* of column 8 when re-run under identical conditions on column 9 seemed to confirm this hypothesis, but the redistribution of protein observed with column 10 suggested that at least part of the protein invariably appeared in the position of peak *A*, since the possibility of overloading no longer existed in the running of columns 9 and 10. Column 11, run under such conditions of starting pH so as to increase the capacity of the DEAE-cellulose for proteins, nevertheless gave a major protein peak in the position of peak *A* of column 8. The exact nature of the transition which is responsible for the redistribution of protein during successive

chromatographic runs is not known. It is apparent that under certain conditions a resolution of the ultracentrifugal components constituting the thyroidal proteins may be possible. Examination of the data in Table 1 shows that there is reasonably good agreement between the rates and compositions of three saline extracts (runs 2588, 2902, and 2952). Two minor peaks (*E* and *C*) from column 8 (runs 2934 and 2936) contained high proportions, respectively, of a fast component with $S_{20,w} = 40 \cdot 3$ which may or may not be identical with component I of Shulman, Rose, and Witebsky (1955) and of a slow component ($S_{20,w} = 7 \cdot 3$) which is probably component IV of

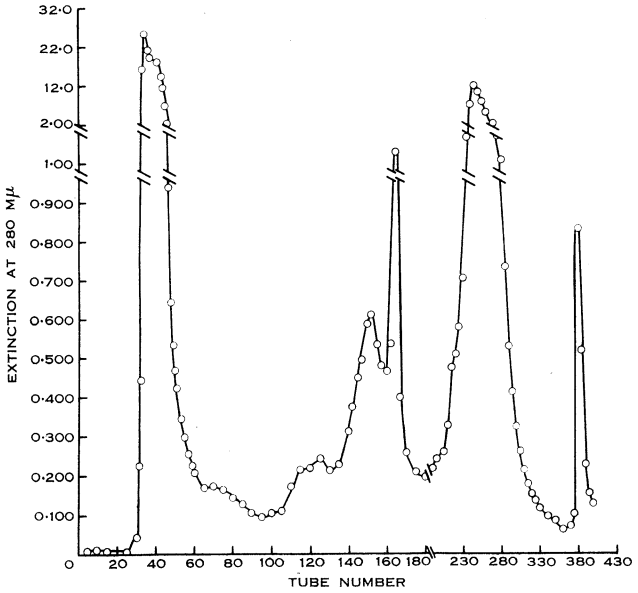

Fig. 4.—Elution pattern of thyroidal proteins (4·40 g, crude saline extract) applied to a column of DEAE-cellulose (column 11, 29 g, pH near 8) and developed with the following buffers: 0·005M phosphate buffer (pH 8·04, 400 ml); 250 ml of the same buffer in mixing flask, gradient to 0·05M NaH_2PO_4 (190 ml); gradient to 0·05M NaH_2PO_4–0·1M NaCl (333 ml); gradient to 0·1M NaH_2PO_4–0·2M NaCl (825 ml); gradient to 0·25M NaH_2PO_4–2·0M NaCl (500 ml). Volume of fractions: 6·1 ml.

Shulman, Rose, and Witebsky (1955). The additional component ($S_{20,w} = 8 \cdot 5$) of column 8, peak *C* (run 2936), may be a very minor component that does not occur in adequate concentration to be detected in the original saline extract, or it may even be non-thyroidal.

Examination of the iodoamino acids present in the peak *A* and in three positions of peak *D* suggested that there was some resolution of the iodoproteins depending on the nature of their constituent iodoamino acids. Proteins which emerged from the column in peak *A* had a higher ratio of iodothyronines to iodotyrosines than those emerging in the most distant (D_3) of three parts of peak *D*, and the ratios for the two intermediate fractions (D_1 and D_2) were intermediate between those of the proteins present in the extremes (*A* and D_3). The existence in thyroglobulin of proteins, similar in most respects, but differing markedly in their relative contents of iodo-

tyrosines and iodothyronines was already suggested by Ingbar, Askonas, and Work (1959), but these authors did not provide direct evidence for their existence. It should be pointed out, however, that our results are preliminary and require confirmation.

While this work was in progress, Ingbar, Askonas, and Work (1959) described the chromatography on DEAE-cellulose of ovine thyroglobulin prepared by the method of Derrien, Michel, and Roche (1948). They observed a peak corresponding to the peak *A* observed in our experiments, but it is possible that they failed to continue the development of the column long enough for a second peak to emerge. As these authors supply no data concerning the amount of protein applied to and

TABLE 2

DISTRIBUTION (% OF TOTAL) BETWEEN THE VARIOUS CONSTITUENTS OF THE IODINE PRESENT IN FOUR ENZYMIC DIGESTS OF IODOPROTEIN FRACTIONS ELUTED DURING THE CHROMATOGRAPHY OF CRUDE THYROID EXTRACT ON DEAE-CELLULOSE

Iodoprotein Fraction	Enzymic Digest			
	A	D_1	D_2	D_3
Origin (unknown)	10	15	12	16
3,5-Diiodotyrosine	41	36	42	38
3-Iodotyrosine	13	23	25	32
Iodide	9	10	4	6
Thyroxine	26	15	15	8
3,5,3′-Triiodothyronine	2	1	1	Trace
Total iodine in sample (μg)	258	179	330	463
Ratio* of $\frac{\text{iodotyrosines}}{\text{iodothyronines}}$	1·9	3·7	4·2	8·8

* For rat and human thyroglobulins the ratio usually found is 9 : 1 or 8 : 1 (R. J. Block, personal communication, 1960).

recovered from their columns, a strict comparison between their and our findings is impossible. It is also likely that they were using DEAE-cellulose of rather high pH, as it is virtually impossible to adjust the pH of freshly activated DEAE-cellulose to 6·8 by washing it with 0·01M phosphate buffer of that pH. Furthermore, the ultracentrifugal data reported by Ingbar, Askonas, and Work (1959) cannot be compared with ours, as there is no indication of the concentrations at which the solutions were run in the ultracentrifuge.

V. ACKNOWLEDGMENTS

This investigation was supported in part by a research grant, C-3737, from the National Cancer Institute of the National Institutes of Health, U.S. Public Health Service, Bethesda 14, Maryland. One of us (S.S.) gratefully acknowledges support through a Senior Research Fellowship (SF-118) from the U.S. Public Health Service.

We should like to express our appreciation to Professor E. Witebsky for his constant support and encouragement of this work. We are greatly indebted to Dr. R. J. Block, Boyce Thompson Institute for Plant Research, Yonkers, New York, for carrying out the quantitative analyses of iodoamino acids. We would also like to thank Mr. I. Kavai for doing the ultracentrifuge runs.

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