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Isolation and Partial Characterization of Anglerfish Proglucagon†

A. C. Trakatellis,* Kozue Tada, K. Yamaji, and P. Gardiki-Kouidou

ABSTRACT: Evidence is presented that proglucagon from anglerfish islets is a single chain polypeptide with 78 amino acid residues and that the glucagon portion of it is liberated after tryptic cleavage. The most striking characteristic in the conversion of the anglerfish proglucagon to glucagon is that the cleaved peptide bonds display enormous sensitivity toward trypsin. Thus, conversion of the prohormone to glucagon occurs very rapidly within 3–10 min with a 1:500–1:1000 molar ratio of enzyme to substrate. Further, tryptic cleavage of the anglerfish glucagon requires higher concentrations of trypsin (molar ratio 1:25 enzyme to substrate) and longer incubation time. The behavior of proglucagon and glucagon toward trypsin shows striking similarities with the tryptic conversion of anglerfish proinsulin to insulin.

Several published investigations have dealt with the problem of glucagon biosynthesis. These studies clearly indicated that glucagon is synthesized via a precursor (Rigopoulou et al., 1970; Noe and Bauer, 1971, 1973; Tung and Zerega, 1971; Hellestrom et al., 1972). In a previous brief communication we have described the isolation of anglerfish proglucagon (Trakatellis et al., 1973) and Tager and Steiner (1973) provided the primary structure of the amino terminal 37 residues of bovine/porcine proglucagon. We describe here the methods of isolation of a polypeptide consisting of 78 amino acid residues and which upon trypsin treatment yields glucagon.

Materials and Methods

Extraction of Anglerfish Islets. Anglerfish islets, frozen immediately after excision, were supplied by the New England Biological Associates, Narragansett, R.I. The islets were cleaned from the surrounding tissues and processed in batches of 100–200 islets. In a typical experiment, a suspension of 100 islets in 300 ml of ice-cold 10% trichloroacetic acid was homogenized (Sorvall Omni-Mixer set at maximum speed) for 2 min, and the mixture was centrifuged. The solid residue was suspended in 40 ml of 0.18 *N* HCl in 75% ethanol, allowed to stand for 1 hr, recentrifuged, and the clear supernatant fraction retained. The solid residue was reextracted twice more with 30-ml portions of acidified ethanol as described. All supernatant fractions were combined and concentrated in a rotary evporator to about 20 ml. The concentrate was adjusted to pH 4 with 1 *M* trisodium citrate and extracted with 100 ml of methylene chloride to remove lipids. The aqueous phase was made 10% in trichloroacetic acid and refrigerated overnight. The resulting precipitate was collected by centrifugation, washed successively three times with 40 ml of acetone–ether (1:1, v/v) and 40 ml of ether, and finally dried in vacuo.

† From the Departments of Biochemistry of Mount Sinai School of Medicine, New York, New York 10029, and University of Thessaloniki, Thessaloniki, Greece. *Received August 16, 1974.* This work was supported by U.S. Public Health Service Grant AM-12903 and by a grant from the National Service Research and Development of the Greek Government.

* Author to whom inquiries should be addressed: Department of Biochemistry, University of Thessaloniki, School of Medicine, Thessaloniki, Greece.

Incubation of Anglerfish Islets. The incubation procedure has been described before (Trakatellis and Schwartz, 1970). Briefly, the minced islets were incubated in 5 ml of incubation medium at pH 7.4 under 95% O_2/5% CO_2 in the presence of L-[^{3}H]tryptophan, in a Dubnoff metabolic shaker at 15°. The incubation medium was Hanks salt solution containing 350 mg/100 ml of glucose and 2 mg/100 ml of each of the naturally occurring amino acids in proteins, excluding tryptophan. After 6 hr of incubation Cl_3CCOOH was added to a final concentration of 10% and the islets were extracted as described above.

Sephadex G-50 Column. Sephadex G-50 (medium grade) was suspended in 5 *M* CH_3COOH containing 0.15 *M* NaCl and the suspension was poured into a column (4 × 50 cm). The Sephadex G-50 column (2 × 50 cm) equilibrated with 1 *M* acetic acid was prepared in a similar fashion.

Carboxymethylcellulose Column (0.9 × 25 cm). Preswollen, microgranular carboxymethylcellulose (Whatman CM 52/1) was employed. Acetate buffer was pumped through the column at 35–40 ml/hr until the conductivity of the effluent stabilized at 1.95 mmho. During the chromatography, the effluent was monitored by a Gilford recording spectrophotometer and by a conductivity meter (Radiometer, Copenhagen). Elution of various components chromatographed in this column was effected with an exponential salt gradient technique as previously described (Trakatellis and Schwartz, 1970; Katsoyannis et al., 1967).

Additional Techniques. Amino acid analyses of acid hydrolysates were performed in a Beckman-Spinco amino acid analyzer (Model 120 C) equipped with a digital read-out system (Model CRS-10AB, Infotronics Corp., Houston, Texas) according to the method of Spackman et al. (1958). Polyacrylamide disc gel electrophoreses were carried out essentially by the method of Davis (1964). The gels were prepared from 10% acrylamide and 0.6% *N,N*-methylenebisacrylamide. Electrophoreses were performed at pH 8.9 in Tris-glycine buffer, and the protein components were stained with Amido Schwarz or Coomassie Blue. Digestion with trypsin was carried out at 37° and pH 7.5 for various incubation times. TPCK-trypsin (L-1-tosylamido-2-phenylethyl chloromethyl ketone treated trypsin) was obtained from Worthington Laboratories. Formation of the picrate and hydrochlorides of various components were carried out as described by Randall (1964). Protein determinations were performed by the Lowry method (1951). The immunoassay for glucagon was carried out by the double-antibody technique (Morgan and Lazarow, 1963).

Results and Discussion

Preparation of Crude Proglucagon and Glucagon Fractions. Preparation of crude fractions of proglucagon and glucagon was achieved by gel filtration. The dried extract obtained from anglerfish islets was dissolved in 10–15 ml of 5 *M* CH_3COOH containing 0.15 *M* NaCl solution. The solution was centrifuged at 1000*g* for 20 min and the clear supernatant fraction was placed on a Sephadex G-50 column (4 × 50 cm) equilibrated with 5 *M* CH_3COOH containing 0.15 *M* NaCl. A typical chromatogram is presented in Figure 1A. Proglucagon is found in fraction II and glucagon and desamidoglucagon in fraction III. These fractions were concentrated in a rotary evaporator to about 15 ml and rechromatographed on the same Sephadex column under the same conditions. The discrete so obtained fractions II and III were concentrated in a rotary evaporator and converted to their picrate derivatives and then to the hydrochlorides as described by Randall (1964). The hydrochlorides of fractions II and III were processed by chromatography as described below.

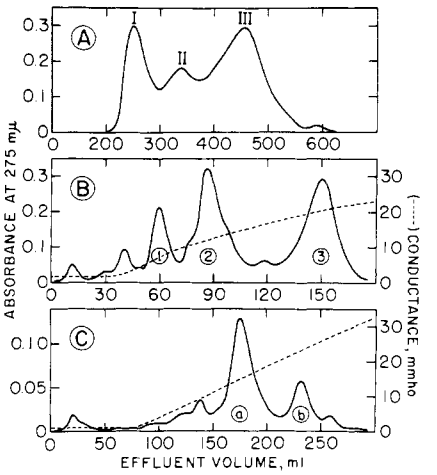

FIGURE 1: (A) Gel filtration chromatography of material extracted from anglerfish islets on a Sephadex G-50 column 4 × 50 cm, equilibrated and eluted with 5 *M* CH_3COOH containing 0.15 *M* NaCl. The effluent was monitored by a Gilford recording spectrophotometer. Fractions II and III were concentrated in a rotary evaporator to about 15 ml and rechromatographed on the same Sephadex column. The so obtained discrete fractions II and III were concentrated and converted to their picrate derivatives and then to the hydrochlorides. (B) Carboxymethylcellulose chromatography of the hydrochlorides of fraction III. Preswollen microgranular CM-cellulose (Whatman CM52/1) was employed. The CM-cellulose was cycled according to the directions of the manufacturer and poured into a column of 0.9 × 25 cm. The column was equilibrated with sodium acetate buffer (0.024 *M* brought to pH 3.3 with glacial acetic acid). During chromatography, the effluent was monitored by a Gilford recording spectrophotometer and by a conductivity meter. Elution of various components chromatographed in this column was effected with an *exponential salt* gradient technique as previously described. (C) Chromatography of fraction 2 (Figure 2B) on a DEAE column 0.9 × 25 cm. Preswollen microgranular Whatman DE-52 was employed. The resin was cycled according to the directions of the manufacturer, and then suspended in Tris–urea buffer (0.05 *M* Tris containing 2.5 *M* urea, brought to pH 9.0 with HCl) and poured into the column. The column was then equilibrated with Tris–urea buffer (0.01 *M* Tris containing 2.5 *M* urea, pH 8.0). The DEAE column was monitored exactly as the CM-cellulose column. Elution was effected with an exponential salt gradient technique.

Isolation of Glucagon and Desamidoglucagon from Fraction III. Chromatography of fraction III on CM-cellulose resulted in its separation into fractions 1, 2, and 3 (Figure 1B). Fraction 2 was found to contain the anglerfish glucagon and desamidoglucagon, and fraction 3 contained the anglerfish insulin, desamidoinsulin, and insulin intermediate which we have described in a previous publication (Yamaji et al., 1972). The separation of glucagon and desamidoglucagon was achieved on DEAE-cellulose (Figure 1C). Two major fractions a and b were observed. Fraction a contained a component which exhibited on disc electrophoresis (pH 8.9 gel 10%) the same mobility as bovine glucagon. This component which was eluted at the same position with bovine glucagon on gel filtration chromatography (Sephadex column G-50 2 × 50 cm) gave an amino acid analysis corresponding to the presence of 29 amino acid residues (Table I). The amino acid composition of this component therefore showed the same number of residues as bovine glucagon and several similarities as well as identical NH_2-terminal residues (Table I). Finally, this component reacted well with antibodies to bovine glucagon. On the basis of these results component a was identified as anglerfish glu-

Table I: Amino Acid Composition of Anglerfish Glucagon, Desamidoglucagon, and Proglucagon.

	Bovine Glucagon	Anglerfish Glucagon		Desamidoglucagon		Glucagon from Trypsin digested Proglucagon		Anglerfish Proglucagon	
Amino Acid	Theory	Found	Nearest Integer	Found	Nearest Integer	Found	Nearest Integer	Found	Nearest Integer
Lysine	1	1.9	2	1.9	2	1.8	2	2.9	3
Histidine	1	0.9	1	0.9	1	0.8	1	1.9	2
Arginine	2	2.2	2	2.1	2	1.7	2	3.8	4
Aspartic acid	4	3.4	3	3.3	3	3.2	3	8.7	9
Threonine	3	1.8	2	1.7	2	1.7	2	1.8	2
Serine	4	3.2	3	3.0	3	2.9	3	5.6	6
Glutamic acid	3	3.2	3	3.1	3	3.0	3	13.7	14
Proline	0	0.7	1	0.7	1	0.6	1	0.7	1
Glycine	1	1.9	2	2.0	2	1.8	2	5.8	6
Alanine	1	1.7	2	1.9	2	1.7	2	7.6	8
Cysteine	0		0				0		0
Valine	1	1.3	1	1.1	1	1.1	1	1.7	2
Methionine	1		0				0		0
Isoleucine	0		0				0		0
Leucine	2	2.1	2	2.0	2	2.3	2	15.8	16
Tyrosine	2	1.8	2	1.7	2	1.7	2	1.6	2
Phenylalanine	2	2.0	2	1.9	2	1.9	2	1.9	2
Tryptophan[a]	1		1		1		1	1	1
Total number of residues	29		29		29		29		78
NH_2-terminus[b]	Histidine	Histidine		Histidine		Histidine		Histidine	

[a] Tryptophan was determined by the method of Spies and Chambers (1949). [b] NH_2-terminal residues were identified by the dansyl method as described by Woods and Wang (1967).

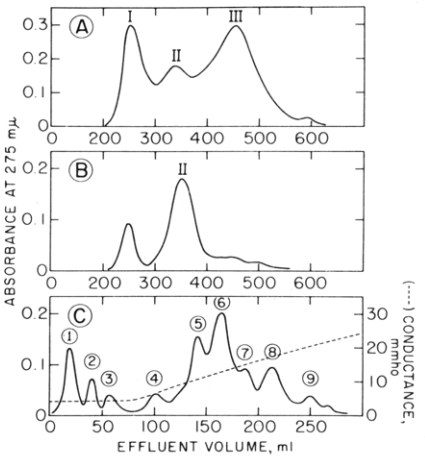

FIGURE 2: (A) Gel filtration chromatography of material extracted from anglerfish islets on a Sephadex G-50 column 4 × 50 cm equilibrated and eluted with 5 *M* CH_3COOH containing 0.15 *M* NaCl. The effluent was monitored by a Gilford recording spectrophotometer. (B) Rechromatography of fraction II on the same Sephadex G-50 column under the same conditions. (C) DEAE-cellulose chromatography of fraction II. Conditions of chromatography same as described in Figure 1C.

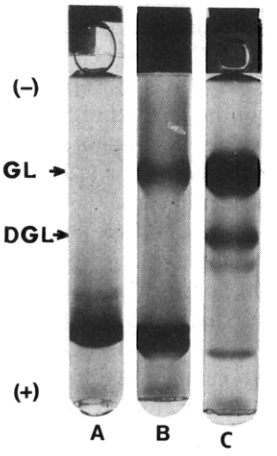

FIGURE 3: Polyacrylamide disc gel electrophoresis, pH 8.9, 60 min, 10% gel. Protein components were stained with Coomassie Blue. From left to right: (A) electrophoresis of fraction 8, obtained after DEAE-cellulose chromatography (Figure 2C); (B) electrophoresis of fraction number 8, after digestion with trypsin treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK) at pH 7.5, 37°, 5 min in a 1:500 enzyme to substrate molar ratio (note the appearance of glucagon); (C) electrophoresis of anglerfish glucagon obtained after DEAE-cellulose chromatography (Figure 1C). GL, glucagon; DGL, desamidoglucagon.

cagon. Treatment ofglucagon with carboxypeptidase B liberated lysine which is, therefore, the C-terminal residue of anglerfish glucagon. The amino acid composition, NH_2-terminal residue, and C-terminal residue of component b as well as its immunoreactivity toward bovine glucagon antiserum were comparable to that of anglerfish glucagon (Table I). This component, therefore, was identified as desamidoglucagon since its chromatographic and electrophoretic behavior showed that it was more negatively charged than glucagon.

Isolation of Proglucagon. Fraction II containing the prohormone (Figure 2A) was concentrated in a rotary evaporator to about 15 ml and rechromatographed on the same Sephadex column under the same conditions (Figure 2B). Separation of the proglucagon from the other components of fraction II was achieved by chromatography of this fraction on a DEAE-cellulose column (0.9 × 25 cm). This chromatographic procedure resulted in the separation of fraction II into several components (Figure 2C). From these components, component number 8, when digested with trypsin, gave rise to a substance which on disc electrophoresis migrated to the same position as anglerfish glucagon (Figure 3). Chromatography of this digest on a CM-cellu-

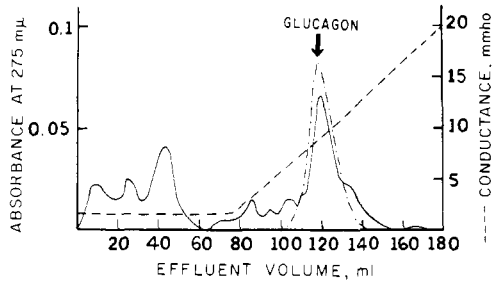

FIGURE 4: CM-cellulose chromatography of the digested proglucagon with TPCK-trypsin (1:500 enzyme to substrate molar ratio) for 10 min, at 37° and pH 7.5. Conditions of chromatography same as described in Figure 1B. The elution patterns of anglerfish glucagon are depicted by the broken line. Note that after proglucagon digestion a fraction is eluted at the same portion with anglerfish glucagon. This fraction was collected, concentrated to dryness, and hydrolyzed with 6 *N* HCl. The amino acid analysis of this hydrolysate gave a composition comparable to that of anglerfish glucagon (Table I).

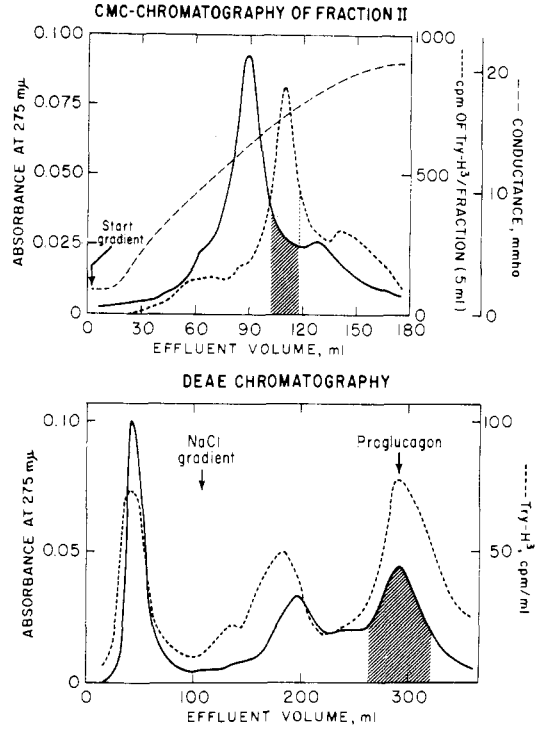

FIGURE 5: Column chromatography of material extracted from anglerfish islets incubated for 6 hr in the presence of L-[^{3}H]tryptophan. The minced islets were incubated in 5 ml of incubation medium at pH 7.4 under 95% O_2/5% CO_2 in the presence of L-[^{3}H]tryptophan, in a Dubnoff metabolic shaker at 15°. The incubation medium was Hanks salt solution containing 350 mg/100 ml of glucose and 2 mg/100 ml of the naturally occurring amino acids in proteins, excluding tryptophan. After 6 hr of incubation Cl_3CCOOH was added to a final concentration of 10% and the islets were extracted and subjected to gel filtration chromatography as described in Figure 1A. The obtained from gel chromatography fraction II was then subjected to CM-cellulose chromatography under the same conditions described in Figure 1B. Chromatography of the shaded fraction on DEAE-cellulose and elution with a salt gradient yielded ^{3}H-labeled proglucagon. Digestion of [^{3}H]proglucagon with trypsin yielded [^{3}H] glucagon.

lose column (Figure 4) gave rise to a component eluted at the same position as anglerfish glucagon. Amino acid analysis of this substance gave a composition comparable to that of anglerfish glucagon (Table I). On the basis of these results, component number 8 was identified as proglucagon.

Several of the other substances present in fraction II, together with proglucagon, can be removed prior to DEAE

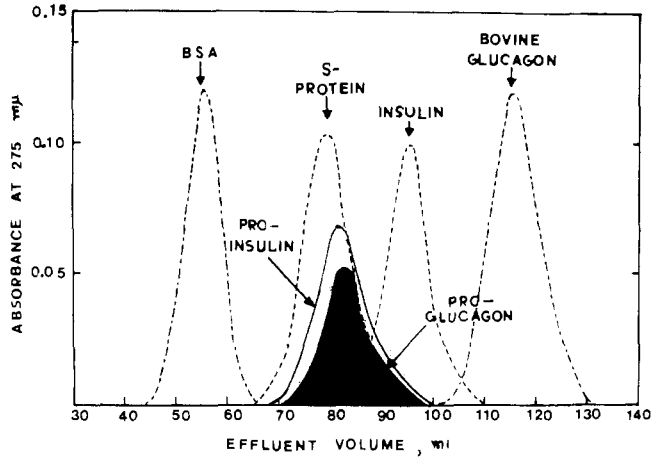

FIGURE 6: Gel filtration chromatography of anglerfish proglucagon and anglerfish proinsulin on a Sephadex G-50 column 2 × 50 cm. The column was equilibrated and eluted with 1 *M* CH_3COOH. Elution patterns of several protein markers are depicted by the broken lines. BSA, bovine serum albumin, S-protein; S-protein of ribonuclease. Anglerfish glucagon (not depicted) was eluted at the same position with bovine glucagon.

chromatography by chromatography on a CM-cellulose column. Thus, proglucagon synthesized by incubating anglerfish islets in the presence of [^{3}H]Trp was isolated in a highly purified state by this procedure (Figure 5). This procedure, although more tedious, is an improved method for isolating proglucagon, because several components present in fraction II are removed prior to DEAE chromatography (compare Figures 2C and 5).

The chromatography of proglucagon on a Sephadex G-50 column (2 × 50 cm) equilibrated with 1 *M* CH_3COOH was compared with that of bovine serum albumin, S-protein of ribonuclease, anglerfish insulin, and bovine glucagon, and the molecular weight was found to be in the range of 6000–10,000 (Figure 6). Amino acid analysis of proglucagon after acid hydrolysis showed the presence of 78 residues (Table I) corresponding to a molecular weight of about 9100. The NH_2-terminal residue was found to be histidine, the same with the NH_2-terminal of anglerfish proglucagon.

Our results, therefore, present strong evidence that anglerfish proglucagon is a single chain polypeptide with 78 amino acid residues. A strong possibility is that the NH_2-terminal 29 residues is the glucagon portion which is liberated after tryptic cleavage of the peptide bond located after the amino acid lysine which is at the 29th position of anglerfish glucagon.

The most striking characteristic in the conversion of the anglerfish proglucagon to glucagon is that the cleaved peptide bonds display enormous sensitivity toward trypsin. Thus, conversion of proglucagon to glucagon occurs very rapidly within 3–10 min with a 1:500–1:1000 molar ratio of enzyme to substrate. Further, tryptic cleavage of the anglerfish glucagon requires higher concentrations of trypsin (molar ratio 1:25 enzyme to substrate) and longer incubation time. The behavior of proglucagon and glucagon toward trypsin shows striking similarities with the tryptic conversion of anglerfish proinsulin to insulin.

Indeed, in our experiments in the in vitro conversion of anglerfish proinsulin to insulin with trypsin, we found that the cleaved peptide bonds display very good sensitivity to trypsin, whereas the tryptic cleavage of anglerfish insulin to yield the inactive deheptapeptide insulin required much higher concentrations of the enzyme (Yamaji et al., 1972).

In connection with the tryptic sensitivity our results agree with the many observations made in this field and especially those of Rigopoulou et al. (1970) and Noe and Bauer (1971). Tager and Steiner (1973) provided direct chemical evidence indicating the reason of the tryptic sensitivity of mammalian proglucagons.

From the various studies on the conversion of the prohormones proparathyroid (Habener et al., 1973), large gastrin (Gregory and Tracy, 1972), β-lipotrophin (Cretien and Li, 1967), proinsulin (Steiner et al., 1972), and now proglucagon to their respective hormones, a general pattern of conversion emerges in which tryptic-like enzymes play a fundamental role.

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Structures of Gangliosides from Bovine Adrenal Medulla†

Howard Price,‡ Samar Kundu,§ and Robert Ledeen*

ABSTRACT: Five gangliosides, accounting for over 95% of the total ganglioside fraction, were isolated from bovine adrenal medulla by preparative thin-layer chromatography and the carbohydrate structures determined by a combination of periodate oxidation and permethylation techniques. Partially methylated alditol acetates were generated from the neutral sugars of the fully methylated glycolipids and identified by gas–liquid chromatography. Substitution on *N*-acetylgalactosamine was determined by methanolysis of the permethylated ganglioside, acetylation of the products, and identification of the resulting substituted methyl glycosides by GLC. Periodate oxidation followed by borohydride reduction confirmed some of the linkages and demonstrated the absence of (2–8) linkages between sialic acid units. Mass spectrometry of the permethylated gangliosides gave information on sugar sequence at the nonreducing end.

Gangliosides were previously shown to occur at appreciable levels in bovine adrenal medulla (Ledeen et al., 1968a), the molar concentration amounting to approximately half that of bovine gray matter on a fresh weight basis. These gangliosides, although obtained from a component of the autonomic nervous system, appeared to have more in common with gangliosides of extraneural organs than of brain. Thus, over 90% of the mixture was hematoside, and the sialic acid was evenly divided between NANAc[1] and NANGly.[2] Lipophilic components were also of the extraneural

† From the Saul R. Korey Department of Neurology and Department of Biochemistry, Albert Einstein College of Medicine, Bronx, New York 10461. *Received October 7, 1974.* This work was supported by Grants NS-04834 and NS-03356 from the National Institutes of Health, U.S. Public Health Service.

‡ Recipient of NIMH Interdisciplinary Training Fellowship, MHO-6418. Present address: Department of Chemistry, Marshall University, Huntington, West Virginia 25701.

§ Recipient of NIHM Interdisciplinary Training Fellowship, MHO-6418.

[1] Abbreviations used are: NANAc, *N*-acetylneuraminic acid; NANGly, *N*-glycolylneuraminic acid; Gal, galactose; Glu, glucose; GalNAc, *N*-acetylgalactosamine; Cer, ceramide; NANAc-7, NANAc with two terminal carbons removed; NANGly-7, NANGly with two terminal carbons removed.

[2] Brain has little hematoside and very little NANGly (cf. Yu and Ledeen, 1970; Ledeen and Yu, 1973).