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Characterization of Two Major Neutral Glyceroglucolipids of the Human Gastric Content†

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ABSTRACT: Two major neutral glyceroglucolipids (A and B) have been isolated from lipid extract of human gastric content by the procedure involving column fractionation on DEAE-Sephadex, silicic acid, and thin-layer chromatography. Both glycolipids contained glucose, glyceryl ethers, and fatty acids. The structures of these glycolipids were identified by mild alkaline methanolysis, oxidation with periodate and chromium trioxide, and permethylation studies. Based on the obtained data, we propose that glycolipid A is a monoalkylmonoacylglyceryl hexaglucoside and glycolipid B is a monoalkylmonoacylglyceryl octaglucoside. The diglyceride portion of these glycolipids consists mostly of 1-*O*-alkyl-2-*O*-acylglycerol.

Glycolipids of the gastric mucosa of the mammalian species consist of a group of compounds with internal variance in the carbohydrate chain and a common lipid core (McKibbin, 1976; Slomiany et al., 1976). The lipid cores of these glycolipids contain sphingosine and thus the compounds belong to the glycosphingolipids.

Recent studies on glycolipids of human gastric content indicate the presence of a new type of glycolipids, lipid core of which consists of diglyceride (Slomiany et al., 197 a,b). Glyceroglycolipids, originally found in the brain (Norton and Brotz, 1963) and more recently in testis and spermatozoa (Ishizuka et al., 1973; Kornblatt et al., 1972), so far are represented only by the neutral and sulfated monogalactosyl diglycerides. Our preliminary studies (Slomiany and Slomiany, 1977a) on the major neutral glyceroglucolipids of human gastric content indicated that these compounds are composed of monoalkylmonoacylglycerol and of variable number of glucose residues.

In this report, we describe the isolation of neutral glyceroglucolipids from the human gastric content and structures of two major components.

Experimental Procedure

Materials. Pentagastrin-stimulated human gastric secretion was obtained from several healthy individuals by gastric intubation. Methyl ethers of neutral sugars were donated by Drs. H. Choi and K. Meyer (Yeshiva University) and were also prepared by methylation of lactose, glucose 6-sulfate, glucitol, arabinitol, and xylitol (Hakomori, 1964). Alkyl 1-chlorides were obtained from the authentic glyceryl esters by BCl_3 treatment (Kates et al., 1965). Alkoxyacetaldehydes were prepared from glyceryl-1-*O*-alkyl standards by oxidation with periodate (Slomiany et al., 1977b). Monogalactosyl diglyceride (β-Gal-diglyceride) and digalactosyl diglyceride (β-Gal(1 → 6)-β-Gal-diglyceride) were from Supelco (Bellefonte, Pa.) and Analabs (north Haven, Conn.), respectively. Galactosylceramide (Gal→ceramide) and glucosylceramide (Glc→ceramide) were prepared from hog gastric mucosa.

Preparation of Glycolipids. Human gastric content (100 mL) was dialyzed against distilled water and lyophilized. The lyophilisate was extracted with chloroform–methanol and filtered through a sintered-glass funnel (Slomiany and Slomiany, 1977a). The lipids contained in the filtrates were concentrated, dissolved in a small volume of chloroform–methanol–water (30:60:8 v/v), and fractionated on a DEAE-Sephadex column (Yu and Ledeen, 1972). The neutral glycolipids were eluted from the column (1.2 × 35 cm) with 700 mL of the above solvent mixture and the acidic glycolipids with 1000 mL of 0.4 M sodium acetate in chloroform–methanol–water (30:60:8 v/v). The crude neutral glycolipid fraction, after removal of solvents, was dissolved in a small volume of chloroform with the aid of sonication and applied to silicic acid column (1.2 × 30 cm) equilibrated with chloroform. The column was developed first with 500 mL of chloroform followed by 700 mL of acetone, 700 mL of acetone–methanol (9:1 v/v), and finally with 900 mL of methanol. Each fraction was analyzed by thin-layer chromatography for glycolipids (Slomiany et al., 1977b). Further purification of glycolipids, contained in the acetone–methanol eluate, was accomplished by preparative thin-layer chromatography on silica gel HR plates developed in chloroform–methanol–water (65:30:8 v/v) and chloroform–acetone–methanol–water (50:40:20:5 v/v).

The homogeneity of the isolated glycolipids was determined

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on thin-layer plates developed in chloroform–methanol–acetic acid–water (60:20:20:1 v/v) and chloroform–methanol–water (65:35:8 v/v).

Glycolipids Composition. Methyl esters of fatty acids, glyceryl ethers, and methyl glycosides were obtained by methanolysis of glycolipids, as previously described (Slomiany and Slomiany, 1977a). The glyceryl ethers present in the hexane extracts of the methanolysates were separated from the methyl esters of fatty acids by thin-layer chromatography in hexane–diethyl ether–acetic acid (70:30:1 v/v). Aliquots of acid methanolysates prior to hexane extraction were dried and treated with BCl_3 (Kates et al., 1965). Following extraction of fatty acid methyl esters and alkyl chlorides with hexane, the methanolic phases were neutralized with silver carbonate and dried under reduced pressure at 4 °C, and the residues were analyzed for glycerol (Hughes and Clamp, 1972).

Alkaline Methanolysis. Mild alkaline methanolysis of the studied glycolipids was performed with 0.3 M NaOH in chloroform–methanol at room temperature for 1 h. After neutralization, the methyl esters of fatty acids were extracted with hexane and treated with BF_3 to assure complete esterification (Slomiany et al., 1977b). The glycolipids present in methanolic phase were chromatographed on thin-layer plates developed in chloroform–methanol–water (65:35:8 v/v) and subjected to acid methanolysis. Following extraction of glyceryl ethers with hexane, the hydrolysates were analyzed for methyl glycosides (Hughes and Clamp, 1972). The glyceryl ethers were dried and treated with BCl_3 (Kates et al., 1965). The hexane extracts of the reaction mixtures were analyzed for alkyl chlorides and the methanolic phases for glycerol.

Periodate Oxidation. Periodate oxidation of glyceryl ethers derived from the studied glycolipids was performed with 0.2 M sodium metaperiodate in aqueous chloroform–methanol (Slomiany and Slomiany, 1977a). The products of oxidation were recovered from the lower phase of chloroform–methanol–water partition system. Periodate oxidation of the glycolipids was performed according to the conditions described previously (Slomiany et al., 1974). The oxidation mixtures were treated with $NaBH_4$ to stop reaction and reduce the products, acidified, and freed of boric acid by codistillation with methanol. After acid methanolysis the products were analyzed for methyl glucoside, erythritol, and glycerol. The same procedure, with omission of methanolysis, was used for oxidation of partially methylated glucitol derived from the reduced hydrolyzates of the permethylated glycolipids.

Chromium Trioxide Oxidation. The purified glycolipids and monogalactosyl diglyceride, digalactosyl diglyceride, galactosylceramide and glucosylceramide standards were acetylated (in the presence of mannitol as internal standard) with pyridine–acetic anhydride (1:1 v/v) at 100 °C for 30 min and oxidized for 40 min at 45 °C in an ultrasonic bath with CrO_3 in acetic acid (Laine and Renkonen, 1975). The oxidation mixtures, diluted with H_2O, were extracted with chloroform–methanol (2:1 v/v) followed by extraction with chloroform. The combined organic extracts were washed repeatedly with water until the solutions became colorless (Itasaka et al., 1976). After removal of solvents, the oxidized samples were subjected to acid methanolysis and analyzed for methyl glycosides. The carbohydrate compositions of the original acetylation mixtures were analyzed by the same procedures, except for omission of CrO_3 oxidation.

Methylation Analysis. Methylation was performed with dimethyl sulfoxide, sodium hydride, and methyl iodide (Hakomori, 1964). The permethylated glycolipids and sugars were recovered from the methylation mixtures by extraction with chloroform, dried under stream of nitrogen at 4 °C, and purified by thin-layer chromatography (Kundu et al., 1975). Hydrolysis, reduction, and acetylation of the permethylated glycolipids and sugars were performed as described by Stellner et al. (1973). Permethylated penta-*O*-methylxylitol, penta-*O*-methylarabinitol, and hexa-*O*-methylglucitol were used for analysis with omission of hydrolysis and acetylation.

Gas–Liquid Chromatographic Analysis. Analyses of trimethylsilyl derivatives of glycerol, erythritol, and methyl glycosides were performed on the columns (180 × 0.2 cm) packed with 3% SE-30 on Chromosorb W, AW, DMCS (80–100 mesh) programmed at 2 °C/min from 100 to 210 °C. The same columns were used for the analysis of fatty acid methyl esters, trimethylsilyl derivatives of glyceryl ethers, alkyl chlorides, and alkoxyacetaldehydes. Temperature programs were 170–270 °C at 2 °C/min for fatty acids, 190–270 °C at 2 °C/min for glyceryl ethers, 130–240 °C at 3 °C/min for alkyl chlorides, and 150–260 °C at 2 °C/min for alkoxyacetaldehydes. Separation of partially methylated alditol acetates was achieved on ECNSS-M columns programmed at 1 °C/min from 150 to 170 °C. The same columns, operated isothermally at 90 °C, were used for analysis of penta-*O*-methylxylitol and penta-*O*-methylarabinitol. Hexa-*O*-methylglucitol was analyzed at 110 °C. The peaks were identified by comparing their retention times with that of standard compounds.

Other Procedures. For long-chain base analyses, glycolipids were hydrolyzed in 1.0 M HCl in aqueous methanol (Yang and Hakomori, 1971) and examined on thin-layer plates in the presence of authentic sphingosine standards (Slomiany and Slomiany, 1975). Glycolipids were also assayed for the presence of phosphorus (Bartlett, 1959), sulfate (Clarke and Denborough, 1971), and alkenyl ether group (Slomiany et al., 1972). Compounds were visualized on thin-layer plates with orcinol, ninhydrin, ammonium bisulfate, and iodine vapors (Slomiany et al., 1974).

Results

Thin-layer chromatography of the crude glycolipid fraction, eluted from DEAE-Sephadex with chloroform–methanol–water, revealed the presence of two major and several minor glycolipid components. The major glycolipids (A and B) were eluted from silicic acid column with acetone–methanol and purified to homogeneity by the preparative thin-layer chromatography in several different solvent systems. The purified glycolipids A and B were obtained in a yield of 5.2 and 4.7 mg, respectively, per 100 mL of gastric content or 630 mg of total lipid extract. The third glycolipid (spot 3, Figure 1), obtained in a yield of 1.2 mg, was not studied further. The isolated glycolipids were also homogeneous on thin-layer chromatography in chloroform–methanol–acetic acid–water (60:20:20:1 v/v), and chloroform–methanol–water (65:35:8 v/v). The R_f values in the acidic solvent system were: glycolipid A, 0.22, glycolipid B, 0.13 and 0.15 and 0.10, respectively, in the neutral solvent system.

Analyses of the methanolysis products of glycolipids A and B revealed the presence of glucose, alkyl ethers, and fatty acids. Methanolysates of the deacylated glycolipids contained glucose and alkyl ethers, but not the fatty acids. Sphingosine, phosphorus, sulfate, and alkenyl ethers were not detected. Analyses of trimethylsilyl derivatives of glycerol and methyl glucoside revealed that these components are present in a molar ratio of 1:6.3, in glycolipid A and 1:8.2, in glycolipid B. These results indicate that glycolipid A is hexaglucosyl diglyceride and glycolipid B is octaglucosyl diglyceride.

The carbohydrate linkages in glycolipids A and B were elucidated on the basis of the results of methylation analysis

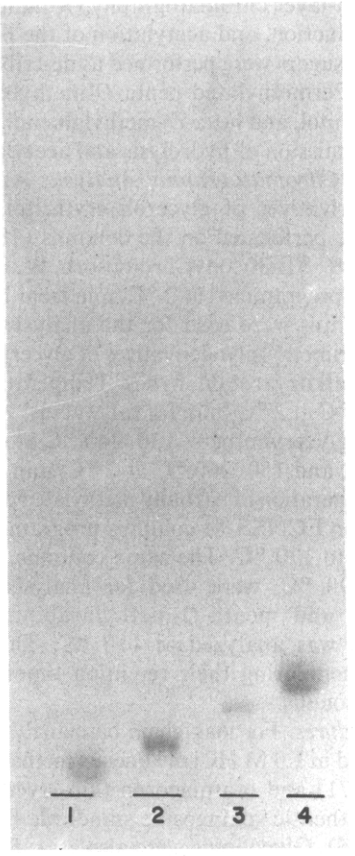

FIGURE 1: Thin-layer chromatogram of the glyceroglucolipids purified from human gastric content. (1) Glycolipid B; (2) glycolipid A; (3) minor neutral glycolipid (not studied); (4) major sulfated glycolipid (Slomiany et al., 1977b). Conditions: Silica gel HR 250 nm developed in chloroform–acetone–methanol–water (50:40:20:5 v/v). Visualization: orcinol reagent.

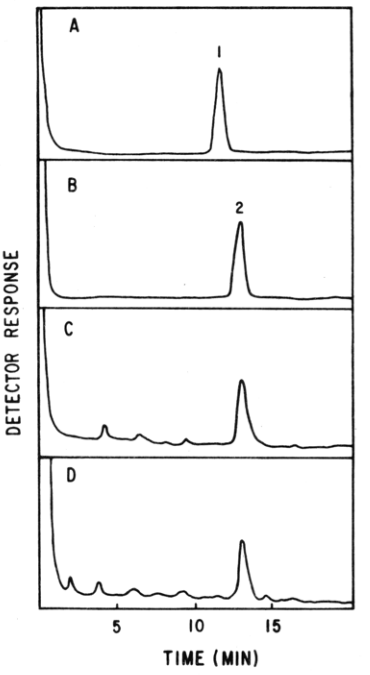

FIGURE 2: Gas–liquid chromatograms of penta-*O*-methylarabinitol (A-1), penta-*O*-methylxylitol (B-2), and products resulting from permethylated glycolipids A (C) and B (D), after hydrolysis, reduction, oxidation with periodate, reduction, and permethylation. Conditions: 1% ECNSS-M columns; temperature, 90 °C.

and oxidation with periodate. Oxidation with periodate resulted in a complete loss of glucose in both glycolipids. Gas–liquid chromatography of the reduced and methanolyzed oxidation fragments revealed only the presence of glycerol, in the molar quantities equal to that of the lost glucose. Erythritol was not detected among the reduced periodate oxidation products of either glycolipid. Thus, these results indicated the absence of C-3 and/or C-4 substitution on the glucose residues, in both glycolipids. Each of the permethylated glycolipids (A and B), after purification, hydrolysis, reduction, and acetylation, gave on gas–liquid chromatography, two peaks, which cochromatographed with 2,3,4,6-tetra-*O*-methylglucitol and 2,3,4-tri-*O*-methylglucitol standards. The ratio of 2,3,4,6-tetra-*O*-methylglucitol to 2,3,4-tri-*O*-methylglucitol was 1:4.8, in the hydrolysates of permethylated glycolipid A and 1:6.9 in the hydrolysates of permethyl glycolipid B. The above data strongly suggest the existence of (1→6) linkages between the glucose residues in the saccharide chains of both glycolipids. Also, it strongly argues against the existence of structures with a branched carbohydrate chain. The presence of (1→6) linkages between the glucose residues in glycolipids A and B is further supported by the results of periodate oxidation of the partially methylated glucitol, derived from the permethylated glycolipids A and B. Reduction and permethylation of the above products gave on gas–liquid chromatography (Figure 2) a peak with retention time corresponding to penta-*O*-methylxylitol standard. Penta-*O*-methylarabinitol, which would form in the above procedure from 3,4,6-tri-*O*-methylglucitol, was not detected in either glycolipid. Thus, the possible existence of (1→2) linkages between the glucose residues was excluded in both glycolipids.

TABLE I: Chromium Trioxide Oxidation of Glycolipids from Human Gastric Content.

Glycolipid	Recoveries (%) of monosaccharides after oxidation	
	Glc	Gal
Glycolipid A	98.7	
Glycolipid B	101.2	
Glucosylceramide	3.1	
Galactosylceramide		2.6
Monogalactosyl diglyceride		2.8
Digalactosyl diglyceride		3.3

The anomeric nature of the glycosidic linkages between the glucose residues in the studied glycolipids was elucidated with the aid of oxidation with chromium trioxide. The recoveries of monosaccharides from the oxidized acetylated glycolipids A and B, and several glycolipid standards, based on the amounts recovered from the glycolipids prior to oxidation, are shown in Table I. Glucose residues, in the glycolipids described here, were almost completely resistant to oxidation. At the same time, over 95% loss of carbohydrates was observed in the standard compounds carried through the chromium trioxide procedure (Table I). These results suggest that glucose residues in glycolipids A and B are linked α glycosidically.

The glyceryl ether and alkyl group composition of the isolated glycolipids is given in Table II. The major glyceryl ethers, identified from the purified glyceryl ether fractions of glycolipids A and B, were glyceryl monohexadecyl, glyceryl monooctadecyl, and glyceryl monoeicosyl. Treatment of the glyceryl ether fractions (from glycolipids A and B) with BCl_3 gave alkyl chlorides, which were identified as hexadecyl 1-chloride, octadecyl 1-chloride, and eicosanyl 1-chloride (Table

TABLE II: Glyceryl Ether and Alkyl Group Composition of the Isolated Glycolipids.

Short hand formula[a]	% total: Glyceryl ether[b] Glycolipid A	Glyceryl ether[b] Glycolipid B	Alkyl group[c] Glycolipid A	Alkyl group[c] Glycolipid B
16:0	36.8	38.7	35.7	36.2
18:0	21.1	20.2	20.9	20.9
20:0	22.0	25.6	23.4	26.1
Unidentified	20.1	15.5	20.0	16.8

[a] First number indicates the chain length; the second number indicates the number of double bonds. [b] Analyzed as trimethylsilyl derivative. [c] Analyzed as alkyl chloride.

TABLE III: Fatty Acid Composition of Glycolipids A and B from Human Gastric Content.

Shorthand formula	% total fatty acid: Glycolipid A	Glycolipid B
16:0	10.5	16.1
18:0	23.3	28.7
18:1	7.0	12.9
20:0	14.5	25.6
20:1	1.4	1.7
22:0	29.1	3.2
22:1	0.9	4.1
Unidentified	13.3	7.7

II). Both glycolipids also contained the glyceryl ethers of longer chains than C-20, which in absence of reference compounds are listed in Table II as unidentified. Treatment of the glyceryl ether fractions with periodate followed by analysis of alkoxyacetaldehydes, in both glycolipids, resulted in conversion of glyceryl monooctadecyl ether to octadecyloxyacetaldehyde, glyceryl monoeicosyl ether to eicosyloxyacetaldehyde and only partial (30%) oxidation of glyceryl monohexadecyl ether. These data indicate that the diglyceride portion of the studied glycolipids consists of a mixture of 1-*O*-alkyl and 2-*O*-alkyl ethers, with the former type being predominant.

Fatty acid composition of the isolated glycolipids is given in Table III. Docosanoate and octadecanoate were the major fatty acids of glycolipid A, whereas eicosanoate and octadecanoate were predominant in glycolipid B.

Discussion

The presence of glyceroglucolipids in human gastric content has been recently demonstrated in our laboratory (Slomiany et al., 1977a,b; Slomiany and Slomiany, 1977a). The major glycolipid from this source was found to contain glucose, glyceryl ethers, fatty acids, and sulfate. The structure of this glycolipid was characterized as monoalkylmonoacylglyceryl triglucoside sulfate (Slomiany et al., 1977b). In addition, several glycolipids devoid of sulfate ester group were also noted. These were found to contain diglyceride lipid core and a variable number of glucose residues (Slomiany and Slomiany, 1977a). The studies described here provide data as to the structure of two most abundant neutral glyceroglucolipid components of human gastric content. The carbohydrate portion of glycolipid A was found to be composed of six glucose residues, whereas eight glucose residues were present in the saccharide chain or glycolipid B. Diglyceride lipid core of both glycolipids was similar and resembled that of previously described monoalkylmonoacylglyceryl triglucoside sulfate (Slomiany et al., 1977b). The purified glycolipids were susceptible to deacylation under mild conditions, thus indicating the presence of ester-linked fatty acids. Furthermore, only glucose and glyceryl ethers were detected among the products of the deacylated glycolipids.

Results of periodate oxidation allowed conclusion as to the point of attachment of the alkyl and acyl groups to the glycerol. These data indicated that the diglyceride portion of the studied glycolipids consists mainly of 1-*o*-alkyl-2-*O*-acylglycerol, but 1-*O*-acyl-2-*O*-alkylglycerol, although in lesser quantities, was also present. Both glycolipids exhibited high content of alkyl ethers of longer chain than C-18, and in this respect differed from glyceroglycolipids of bovine brain (Norton and Brotz, 1963), rat testis (Kornblatt et al., 1972), and boar testis and spermatozoa (Ishizuka et al., 1973). The diglyceride portion of the above glycolipids consists mostly of glyceryl-1-hexadecyl.

Assignment of the (1 → 6) linkages between the glucose residues and the linear nature of the saccharide chains, in both glycolipids, was supported by: (1) complete susceptibility of glucose to periodate and identification of glycerol, but not the erythritol, among the reduced and hydrolyzed oxidation products; and (2) yield of 5 and 7 mol of 2,3,4-tri-*O*-methylglucitol against 1 mol of 2,3,4,6-tetra-*O*-methylglucitol from permethylated glycolipids A and B, respectively. Additional evidence for the presence of (1 → 6) linkages between the glucose residues was obtained from the results of periodate oxidation of partially methylated and reduced hydrolysis products of permethylated glycolipids. The reduced and permethylated products of oxidation showed the presence of penta-*O*-methylxylitol, but not penta-*O*-methylarabinitol.

Results of chromium trioxide oxidation indicated that all glucose residues in both glycolipids were linked α glycosidically. In the past, chromium trioxide procedure has been used successfully in structural studies of various glycolipids (Laine and Renkonen, 1974, 1975; Itasaka et al., 1976; Slomiany and Slomiany, 1977b).

Based on the data presented, we propose the following structures for glycolipids A and B.

Glycolipid A:

αGlc(1→6)αGlc(1→6)αGlc(1→6)-
αGlc(1→6)αGlc(1→6)αGlc(1→3)-diglyceride

Glycolipid B:

αGlc(1→6)αGlc(1→6)αGlc(1→6)αGlc(1→6)-
αGlc(1→6)αGlc(1→6)αGlc(1→6)αGlc(1→3)-diglyceride

Our preliminary studies indicate that compounds of similar chemical composition to those described here are also present in the secretions from the dog Heidenhain fundic pouch and rat stomach. It is therefore conceivable that glyceroglucolipids form an essential component of digestive secretions of mammals.

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Reaction of the Basic Trypsin Inhibitor from Bovine Pancreas with the Chelator-Activated 7S Nerve Growth Factor Esteropeptidase†

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ABSTRACT: The native 140 000 molecular weight nerve growth factor protein from the mouse submaxillary gland (7S NGF_n) is a multisubunit zinc metalloprotein which regulates the differentiation of sensory and sympathetic ganglia in vivo. The 7S NGF_n oligomer contains a masked trypsin-like proteolytic activity which is activated by the sequestering and removal of the 7S NGF_n-bound zinc ions by divalent metal-ion chelators. The proteolytic activity of the oligomer is associated with the γ subunit, while growth activity resides with the β subunit. In this study, the susceptibility of the proteolytic activity to inhibition by seven protein protease inhibitors, the basic trypsin inhibitor from bovine pancreas (PTI), soybean trypsin inhibitor, lima bean trypsin inhibitor, ovomucoid, human α_1-anti-trypsin, human antithrombin III, and human C-1 esterase inhibitor, has been investigated. Of these inhibitors, only PTI is an inhibitor for the proteolytic activity. By the use of sucrose density gradient sedimentation, isoelectric focusing gel electrophoresis, gel filtration, equilibrium sedimentation, and protease activity studies we have established that PTI does not react with 7S NGF_n; however, PTI undergoes rapid, stoichiometric reactions with both the EDTA-activated 7S NGF species (7S NGF_a) and with the isolated γ subunit. Reaction of PTI with 7S NGF_a results in the inhibition of the proteolytic activity and the dissociation of the 7S oligomer to a mixture of the α and β subunits and the γ subunit–PTI complex. In contrast to the reaction of NGF_a with PTI, the reaction of a low-molecular-weight substrate, α-*N*-benzoyl-L-argininamide, does not alter the state of aggregation of the 7S oligomer.

The 140 000 molecular weight nerve growth factor protein (7S NGF)[1] from the mouse submaxillary gland is a zinc metalloprotein (Pattison and Dunn, 1975, 1976a,b) composed of three types of subunits (designated α, β, and γ), each of molecular weight 20 000 to 30 000 (Varon et al., 1967a,b, 1968; Baker, 1975a,b). This protein is involved in the regulation of growth and differentiation for the sensory and sympathetic chains (Levi-Montalcini, 1965; Schenkein, 1972; Varon et al., 1967a,b). The 7S NGF subunits carry separate biological activities; neuronal outgrowth activity is associated with the β subunit, a potent trypsin-like protease activity is associated with the γ subunit, and the α subunit has been reported to enhance the survival of sensory neurons under conditions of mechanical stress (Varon et al., 1967a; Greene et al., 1968, 1969; Varon and Raiburn, 1972).

Previous work from this laboratory (Pattison and Dunn, 1975, 1976a,b) has shown that the reaction of divalent metal-ion chelators with native 7S NGF (hereafter designated

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[1] Abbreviations used are: NGF, nerve growth factor; PTI, pancreatic trypsin inhibitor; IAI, β-*trans*-indolacryloylimidazole; Tris-HCl, 2-amino-2-hydroxymethyl-1,3-propanediol; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, (ethylenedinitrilo)tetraacetic acid; BAPNA, α-*N*-benzoyl-D,L-arginine-*p*-nitroanilide; BAA, α-*N*-benzoyl-L-argininamide; BU, biological unit (Levi-Montalcini and Hamburger, 1953).