PD and ALS isolates gave positive tests for 11 PD and five ALS isolates from different plants.

Previously noninfective (*9*) *H. circellata* placed for 2 days on symptomatic, suction-inoculated grapevines subsequently transmitted PD to healthy grapevine seedlings in five of five attempts. Transmission of the PD bacterium from cultures to grapevines via leafhopper vectors have been inconclusive. The mouthparts of 45 noninfective CO_2-anesthetized (*9*) *H. circellata* adults collected from natural populations were held in colonies of the PD bacterium until the insects regained mobility and were transferred to test plants. Only two leafhoppers treated in this manner transmitted the PD agent. In numerous other transmission attempts we injected suspensions of the PD bacterium into the body cavities of *H. circellata* as well as another leafhopper vector of PD, *Draeculacephala minerva* Ball, with fine glass needles or fed suspensions of the PD bacterium in sterile membrane sachets to both vector species (*9*). Neither of these methods resulted in PD transmission to grapevines.

The PD bacterium was also isolated from vector leafhoppers. Individual *H. circellata* were exposed to PD source plants for at least 2 days and subsequently transmitted PD to healthy grapevines. These leafhoppers were surface-sterilized, macerated, and then plated (*9*) on JD-2 medium. Of 21 isolation attempts, 11 yielded colonies resembling those of the PD bacterium. Seven of these isolates tested positively in slide agglutination tests (*13*) with antiserums to a cultured PD bacterium. No PD bacteria were isolated from *H. circellata* that failed to transmit PD to test plants.

We have cultured the "rickettsia-like" organism associated with PD by previous investigators (*2, 6, 10*), and we believe that this bacterium is the etiological agent of PD. The PD and ALS bacteria appear to be closely related if not identical.

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12. Six male rabbits weighing approximately 2 kg each were injected in pairs with one of three isolates. Two PD isolates, one each from Napa and Tulare counties, and one ALS isolate from Contra Costa County were grown in JD-2 medium without agar, pelleted by centrifugation, fixed with 2 percent glutaraldehyde, and washed in phosphate buffered saline. For intravenous injection, 10^9 bacteria per milliliter were suspended in physiological saline. Each rabbit was injected with increasing dosages of 0.5, 1.0, 2.0, 4.0, and 5.0 ml at 3-day intervals and bled 1 week after the final injection.
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Thyroid Gangliosides with High Affinity for Thyrotropin: Potential Role in Thyroid Regulation

Abstract. *Thyroid cell membranes contain a multiplicity of gangliosides, some of which inhibit thyrotropin binding to thyroid membranes. The most potent inhibitor is a ganglioside which is present in only trace amounts and appears to have a novel structure. Thyroid gangliosides may play a role in relaying the hormonal message to the thyroid cell.*

Gangliosides purified from brain inhibit the binding of thyrotropin (TSH) to thyroid membranes (*1, 2*). Since the thyroid gland itself contains a diversity of gangliosides, we postulated that gangliosides are involved in the interaction of TSH with thyroid membranes analogous to their role as receptors for cholera toxin (*3, 4*). In order to test this postulate, we examined the ability of gangliosides isolated from thyroid gland to inhibit the binding of TSH to thyroid membranes.

Fresh bovine thyroid glands (1.5 kg from about 75 adult animals) were homogenized in a stainless steel Waring blender with 10 liters of a mixture of chloroform and methanol (1 : 2, by volume). The homogenate was filtered, and the residue was extracted twice with 5 liters of the same solvent. A fourth extraction with 4 liters of buffered tetrahydrofuran (*5*) recovered little additional lipid-bound sialic acid (LBSA). The combined chloroform-methanol extracts were taken to dryness under reduced pressure and partitioned (*6*). The resulting lower phase was washed twice with theoretical upper phase; the combined upper phases were taken to dryness, dissolved in water, dialyzed, and lyophilized. The ganglioside mixture thus obtained contained 195 μmole of LBSA (*7*); analysis by thin-layer chromatography (*8*) revealed a complex pattern of gangliosides (Fig. 1A). Fractionation of the ganglioside mixture by column chromatography on diethylaminoethyl (DEAE)-cellulose (*9*) resulted in eight pooled fractions. When each of these pooled fractions was analyzed by thin-layer chromatography (Fig. 1B), at least 30 distinct resorcinol-positive bands could be visualized, that is, several minor ganglioside components present in the mixture become apparent only after enrichment by DEAE-cellulose chromatography. Each of these eight pooled fractions was further purified by silica gel column chromatography (*10*). This procedure resulted in 28 separate ganglioside fractions; some fractions contained a single pure ganglioside component when analyzed by thin-layer chromatography, others contained several ganglioside bands not further purified in this study. Several fractions discussed below are shown in the chromatogram in Fig. 2A.

Each of the 28 thyroid ganglioside fractions was tested for its ability to inhibit ^{125}I-labeled TSH binding to thyroid membranes. Binding was assayed by a filtration method with the use of cellulose acetate filters (Millipore, EHWP-02500) (*11*). ^{125}I-Labeled TSH and thyroid plasma membranes were bovine preparations (*11, 12*). In addition to the particular ganglioside fraction being tested, the incubation mixture contained (in 130 μl): 25 m*M* tris-acetate, *p*H 6.0, 0.5 percent bovine serum albumin, ^{125}I-labeled TSH, approximately 100,000 count/min (specific activity = 10.5 μc/μg, iodinated within 14 days of use, approximately 0.2 pmole), and 10 μg of membrane protein (*13*). Ganglioside fractions were first incubated with ^{125}I-labeled TSH for 30 minutes at 0°C prior to the addition of membranes. The percent inhibition of ^{125}I-labeled TSH binding was determined

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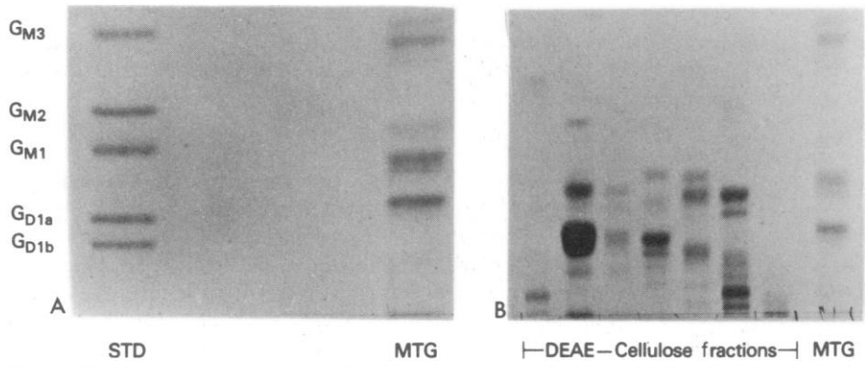

Fig. 1. Thin-layer chromatography of bovine thyroid gangliosides. (A) Ganglioside standards (*STD*) as indicated (*19*); mixed thyroid gangliosides (*MTG*) prior to fractionation on DEAE-cellulose. (B) Fractions collected from DEAE-cellulose column (seven of eight fractions shown). Each of these fractions was further purified by silica gel chromatography and resulted in 28 separate ganglioside fractions (see Fig. 2A). Gangliosides were visualized with resorcinol spray.

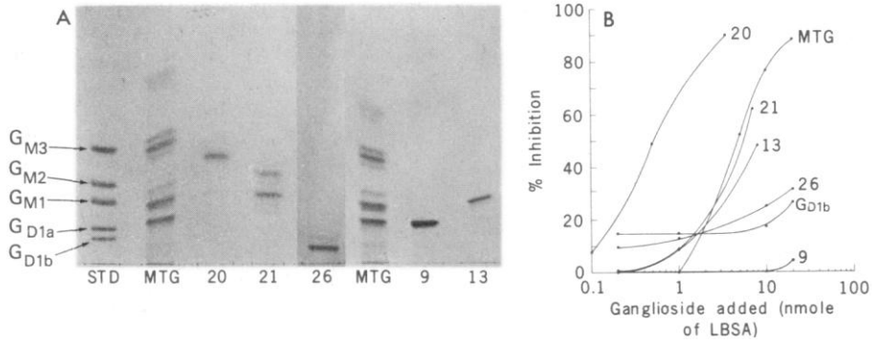

Fig. 2. Inhibition of TSH binding by thyroid gangliosides. (A) Thin-layer chromatography of ganglioside fractions plotted in (B). Fraction 9 is the predominant ganglioside of the bovine thyroid gland. (B) Binding of ^{125}I-labeled TSH (approximately $1.5 \times 10^{-9}M$) to thyroid membranes was measured in the presence of increasing concentrations of gangliosides. In the absence of ganglioside, 25,000 count/min were bound (± 10 percent). Each ganglioside fraction (1 to 28) was tested, but only those fractions clearly more potent than brain G_{D1b} are shown. Abbreviations: *STD*, ganglioside standards; *MTG*, mixed thyroid gangliosides; and *LBSA*, lipid-bound sialic acid.

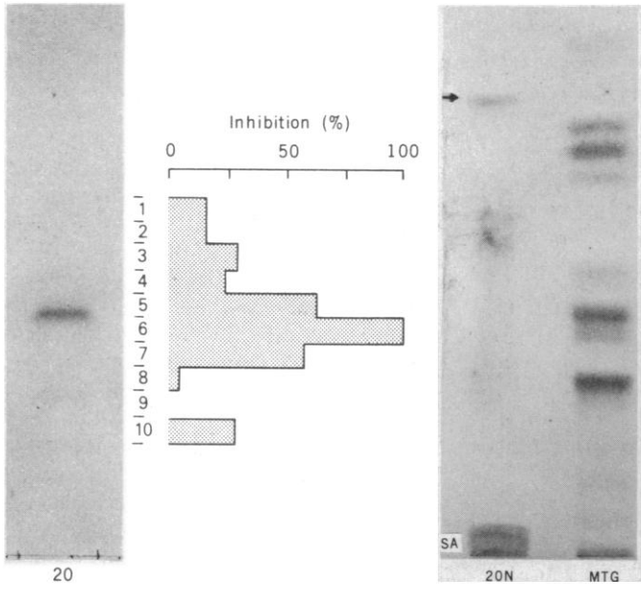

Fig. 3 (left). Localization of TSH inhibitory activity of fraction 20 to the major band. Fraction 20 (5 nmole of LBSA) was chromatographed on thin-layer silica gel (E. Merck). Consecutive strips (1 to 10) of the silica gel (1.5×0.5 cm) were scrapped, eluted with methanol, and tested in the ^{125}I-labeled TSH binding assay. In order to localize the major ganglioside band, the edge of each strip was retained on the plate and visualized with resorcinol. Material eluted from strip 6 caused total inhibition of ^{125}I-labeled TSH binding. Fig. 4 (right). Neuraminidase treatment of fraction 20 (3 nmole) was carried out in 20 μl of 0.05 *M* sodium acetate buffer, *p*H 5.5, containing 0.15*M* NaCl, 9 m*M* $CaCl_2$, and 10 units of Vibrio cholerae neuraminidase (Behringwerke AG) at 37°C for 18 hours. The arrow indicates new resorcinol-positive product. Abbreviations: *SA*, sialic acid; *20N*, fraction 20 after neuraminidase; and *MTG*, mixed thyroid gangliosides.

by comparison with control incubations in the absence of ganglioside or membranes, or both.

Several of the bovine thyroid ganglioside fractions were more potent than bovine brain G_{D1b}, the best inhibitor previously studied (*1*). Those ganglioside fractions that were clearly more effective than G_{D1b} are shown in a dose-response curve (Fig. 2B).

Fraction 20 is by far the most potent inhibitor of TSH binding; 500 pmole causes 50 percent inhibition of TSH binding. Thin-layer chromatography of fraction 20 (Fig. 2A) shows one major band (> 90 percent) migrating near the G_{M3} standard and two minor slower-moving bands. To be certain that inhibition was due to the major band, individual components were separated by preparative thin-layer chromatography and tested in the TSH binding assay. The inhibitory ability of fraction 20 corresponds to the major ganglioside band (Fig. 3). The yield of fraction 20 is approximately 20 nmole of LBSA per kilogram of fresh tissue (or 0.015 percent of the total LBSA). A detailed structural analysis of the active ganglioside in fraction 20 was not possible with the amount of material available. Treatment with neuraminidase, however, revealed that the major band of this fraction contains at least two sialic acid residues, one neuraminidase-sensitive and the other neuraminidase-resistant (Fig. 4). A ganglioside with such rapid mobility, that is, near G_{M3}, and containing two (or more) sialic acid residues appears to be novel.

TSH binding is inhibited to a lesser degree by fractions 21, 13, and 26. The inhibitory activity of fraction 21 is similar to that of the thyroid ganglioside mixture and about ten times less than fraction 20. Since this fraction eluted from the same silica gel column immediately after fraction 20, trace amounts of the active ganglioside of fraction 20 may be present (Fig. 2A). Preparative thin-layer chromatography and binding inhibition studies of this fraction indicate an inhibitory component corresponding to the active ganglioside component of fraction 20 (data not shown). The major component of fraction 26 is a ganglioside with a mobility similar to that of the bovine brain G_{D1b} (Fig. 2A). Also, its ability to inhibit TSH binding is similar to that of G_{D1b}.

Fraction 9, containing a single resorcinol-positive band, is the predominant ganglioside of the bovine thyroid gland (Fig. 2A). It comprises about 35 percent of the total LBSA, a value in agreement with the previous observation of Lagrou *et al.* (*14*). Treatment with neuraminidase resulted in sialic acid and a ganglio-

side with the mobility of G_{M1}, indicating a G_{D1a} structure. This ganglioside does not inhibit TSH binding in the concentrations tested, in agreement with the previous observation that G_{D1a} from brain is a poor inhibitor (*1*). Thus, the ganglioside present in the highest concentration in bovine thyroid shows little ability to inhibit TSH binding.

A multiplicity of gangliosides is present on thyroid plasma membranes (*15*). These gangliosides vary in their ability to inhibit TSH binding to thyroid membranes. The most potent inhibitor (fraction 20) contains at least two sialic acid residues and some structure still undescribed. It constitutes 0.015 percent of the total thyroid gangliosides, or approximately 10,000 molecules per thyroid cell (*16*), a figure similar to that postulated for the number of cholera toxin receptors on fat cells (*4*). The predominant ganglioside of the bovine thyroid (tentatively G_{D1a}) is a poor inhibitor of TSH binding. In our study, those thyroid gangliosides that were the best inhibitors are present in the lowest concentrations, while those gangliosides showing minimal ligand interaction were present in higher concentration.

An earlier study indicated that TSH can bind to a glycoprotein membrane component (*17*). Gangliosides with a high affinity for TSH may act alone or in concert with this glycoprotein in receiving and transmitting the hormonal message to the thyroid target cell. It has been shown (*18*) that the disialyl residue present in gangliosides with a high affinity for TSH (G_{D1b}, G_{T1}, and possibly fraction 20) also is present in some glycoproteins. Gangliosides and glycoproteins may both contribute to the formation of a functional TSH receptor on the thyroid cell.

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15. Lagrou *et al.* (*14*) showed that gangliosides are concentrated in the plasma membrane fraction of bovine thyroid tissue. Also, the thyroid membrane preparations used in our study contain five times more LBSA per milligram of protein than crude homogenates (*1*).
16. This figure assumes 2.8×10^9 follicular cells per bovine gland. It is calculated from published values for total DNA per gram of thyroid [D. J. Begg, E. M. McGirr, H. N. Munro, *Endocrinology* **76**, 171 (1965)] and DNA per bovine cell [R. Vendrely and C. Vendrely, *Int. Rev. Cytol.* **5**, 171 (1956)]. Half of the total thyroid cells are estimated to be follicular cells.
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20. We thank S. Wollman for discussion concerning the number of cells in thyroid tissue.

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Deficiencies of Glucosamine-6-Sulfate or Galactosamine-6-Sulfate Sulfatases Are Responsible for Different Mucopolysaccharidoses

Abstract. *[1-^{3}H]Galactitol-6-sulfate,* N-*[1-^{3}H]acetylgalactosaminitol-6-sulfate,* N-*[1-^{3}H]acetylglucosaminitol-6-sulfate,* N-*acetylglucosamine-6-sulfate, and 6-sulfated tetrasaccharides from chondroitin-6-sulfate have been used for the measurement of 6-sulfatase activity of extracts of normal skin fibroblasts and of fibroblasts cultured from patients with genetic mucopolysaccharidoses. With these substrates, extracts of fibroblasts derived from Morquio patients lack or have greatly reduced activities for galactitol-6-sulfate,* N-*acetylgalactosaminitol-6-sulfate, and 6-sulfated tetrasaccharides but have normal activity for* N-*acetylglucosamine-6-sulfate and its alditol; those derived from a patient with a newly discovered mucopolysaccharidosis have greatly reduced activity for* N-*acetylglucosamine-6-sulfate and its alditol but normal activity for galactitol-6-sulfate,* N-*acetylgalactosaminitol-6-sulfate, and the 6-sulfated tetrasaccharides. These findings demonstrate the existence of two different hexosamine-6-sulfate sulfatases, specific for the glucose or galactose configuration of their substrates. Their respective deficiencies, causing inability to degrade keratan sulfate and heparan sulfate in one case and keratan sulfate and chondroitin-6-sulfate in the other, are responsible for different clinical phenotypes.*

Morquio disease (mucopolysaccharidosis IV) (*1*) emerged as a separate entity from the various osteochondrodysplasias when it was demonstrated that the affected patients excrete in the urine excessive amounts of keratan sulfate and accumulate in cartilage keratan sulfate and chondroitin-6-sulfate (*2*).

In 1974 Matalon *et al.* (*3*) prepared polymeric $^{35}SO_4$-labeled chondroitin-4/6-sulfate in chick embryos; using this material, oligosaccharides derived from it, and purified 6-sulfated oligosaccharides as substrates, they demonstrated that a sulfatase present in extracts of normal skin fibroblasts was absent or greatly defective in extracts of fibroblasts obtained from Morquio patients (*4*).

In 1976 Singh *et al.* (*5*) assayed the enzyme *N*-acetylgalactosamine-6-sulfate sulfatase, measuring sulfate released from tetrasaccharides derived from purified chondroitin-6-sulfate of shark cartilage, and confirmed that the enzyme is

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