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THE EFFECTS OF GANGLIOSIDE PREPARATIONS ON SMOOTH MUSCLE*

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Abstract—An impure preparation of gangliosides caused both contraction and relaxation of smooth muscles. The contractile effect was tachyphylactic. The relaxation induced by the impure ganglioside preparation resembled norepinephrine in being blocked by ephedrine and phentolamine. Norepinephrine was demonstrated in the impure ganglioside by a fluorometric method. With further purification, the ganglioside preparation lost all activity on smooth muscle.

EXTRACTS containing gangliosides, a class of water-soluble lipids containing N-acetylneuraminic acid, hexose, hesoxamine, sphingosine, and fatty acids, have been reported to exert pharmacological activity on various isolated tissues. Stimulation of the clam heart,[1] rabbit duodenum,[2] and guinea pig ileum[3] has been obtained, although the amounts of ganglioside required to elicit this activity were high. Since gangliosides are incompletely characterized, both with regard to purity of extracted samples and to composition,[4–7] it is possible that the biological activity attributed to gangliosides might be accounted for by active contaminants or other neuraminic acid-containing compounds.[8] To investigate this possibility, samples of gangliosides of increasing purity were prepared and assayed for pharmacological activity.

METHODS

The initial extract of gangliosides was prepared from brains of monkeys (*Macaca mulatta*) by the method of Trams and Lauter[6] with the following modifications. A mixed-bed ion-exchange resin consisting of equal quantities (w/w) of Amberlite IRA-410 and Amberlite CG-50 was used, rather than Amberlite MB-3; the barium salts of the gangliosides were dissolved in a solution of oxalic acid, rather than sulfuric acid, and barium was removed from the supernatant fraction by again passing it through the ion-exchange resin, rather than by dialysis, since dialysis tubing liberates nondialyzable sulfur-containing material.[9] The extracted material at this stage, designated as ganglioside A, was slightly yellow and of crystalline appearance; this material was further purified by chromatography on a silicic acid column, followed by gel filtration with Sephadex G-50.[10] The white crystalline material eluted from the

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silicic acid column with methanol was designated as ganglioside B. The effluent from the Sephadex column emerging with the neuraminic acid peak, which yielded white crystals upon evaporation, was termed ganglioside C (Fig. 1). Chemical analysis was carried out on these materials. Before being tested for biological activity the samples were dialyzed for 36 hr against distilled water to remove any residual barium. Tubing of the same size, containing only distilled water, was dialyzed and used as a control for the bioassay, since such tubing has been shown to liberate biologically active materials;[11] this control material had no action on the biological preparations used in these experiments.

N-acetyl neuraminic acid was determined by the method of Warren[12] and hexosamine according to Rondle and Morgan.[13] Catecholamines were extracted and measured by a modification[14] of the method of Shore and Olin.[15] Elemental analyses were performed by Schwartzkopf Microanalytical Laboratory, Woodside, N.Y.

Biological activity was examined on the rabbit duodenum and on the guinea pig ileum suspended in a 5-ml chamber containing aerated Tyrode's solution. Preparations from three rabbits and four guinea pigs were used. All samples were tested on each preparation in a multiple randomized manner, but when ganglioside A was seen to be tachyphylactic, on subsequent preparations gangliosides B and C were randomly tested before A. The responses were recorded with a model M5P Mini-Polygraph (Gilson Medical Electronic, Middletown, Wis.) and a force-displacement transducer F3-03 (Grass Instrument Co., Quincy, Mass.). With this apparatus the recording of the relaxation of the muscle could be eliminated when the contractile effect alone was under study. Unless stated otherwise, an interval of 3 to 4 min elapsed between applications of the materials.

RESULTS

Chemical analysis of the ganglioside preparations

The studies are summarized in Table 1. In the purification procedure, most of the phosphorus and all the sulfur were eliminated, while the relative content of N-

TABLE 1. ANALYSIS OF GANGLIOSIDE FRACTIONS

Fraction	Elemental analysis (%)					Hexosamine (μmoles/mg)	N-acetyl-neuraminic acid (μmoles/mg)	Contracting activity* (units/μmole neuraminic acid)	Relaxing activity† (units/μmole neuraminic acid)
	C	H	N	S	P				
A	49·69	7·75	3·37	0·29	0·56	0·43	0·56	10	150
B			3·07	0			1·15	0	80
C	50·12	7·88	2·71	0	0·24	0·60	0·95	0	0

* One unit is equivalent to 1 ng of acetylcholine as measured on the guinea pig ileum.

† One unit is equivalent to 1 ng of norepinephrine as measured on the rabbit duodenum.

acetylneuraminic acid was greatly increased as compared to that of preparation A. Hexosamine content, relative to that of neuraminic acid, decreased, probably because of loss of contaminating compounds; this also was reflected in the loss of nitrogen. Since the hexosamine content, as well as that of neuraminic acid, varies among the

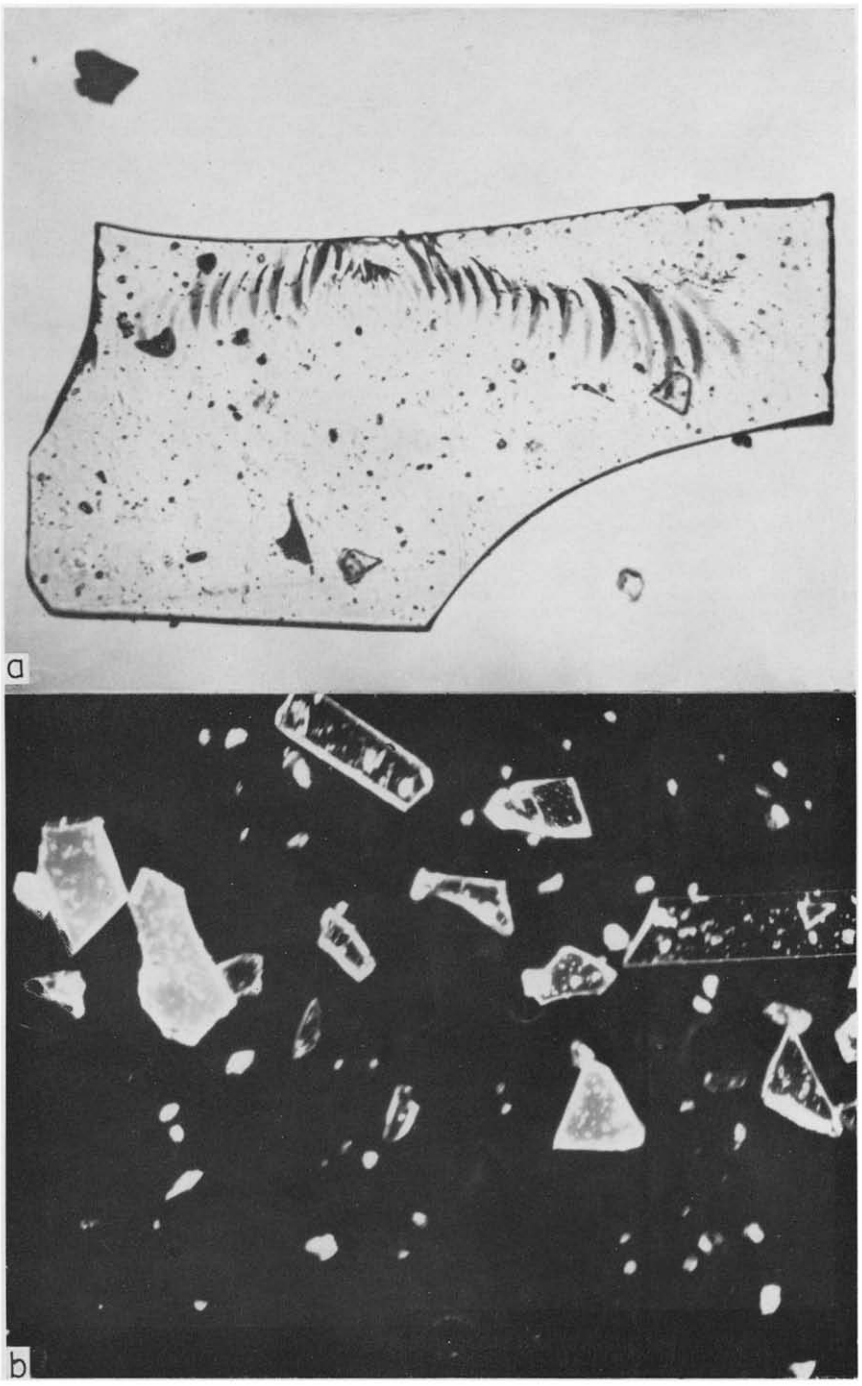

FIG. 1. Photomicrographs of ganglioside C taken in polarized light. Gangliosides crystallized in the evaporating flask as long filaments and were fragmented before photographing.

different gangliosides,[4–7] this result could be interpreted as a relative increase in a hexosamine-poor or neuraminic acid-rich ganglioside.

Action of the duodenum

On the rabbit duodenum (Fig. 2), ganglioside A had an almost immediate relaxing effect, and this was not characterized by tachyphylaxis; qualitatively, ganglioside B had the same action. Ganglioside C was not active even at levels containing twice the concentration of neuraminic acid involved in the tests of ganglioside A. The relaxing activity of gangliosides A and B resembled that of norepinephrine in being blocked by ephedrine[16] (Fig. 2). Ganglioside A was equivalent in relaxing activity to 150 ng of norepinephrine/μmole of neuraminic acid in the ganglioside; ganglioside B to 80 ng of norepinephrine (Table 1). It was noted also that dopamine (3-hydroxytyramine) was not active on the duodenum in concentrations up to 40 ng/ml, whereas norepinephrine induced an effect in concentrations as low as 2 ng/ml.

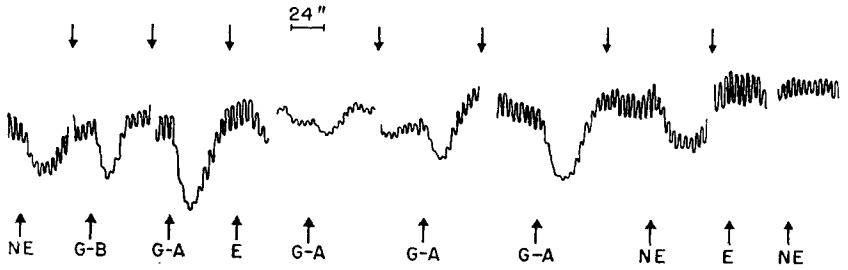

FIG. 2. The relaxing effects of ganglioside A, ganglioside B, and norepinephrine on the rabbit duodenum and their inhibition by ephedrine. Arrows above the tracing indicate change of the bathing fluid; note that after the addition of ephedrine (E), the fluid was not changed; after the addition of ephedrine, 5 min passed before the next addition. NE = 15 ng norepinephrine; G-B = ganglioside B (0·25 μmole neuraminic acid); G-A = ganglioside A (0·25 μmole neuraminic acid; E = 250 μg ephedrine.

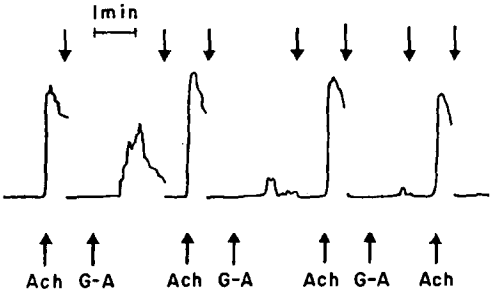

FIG. 3. The contracting effect of ganglioside A and acetylcholine on the guinea pig ileum. The ganglioside caused tachyphylaxis. The relaxation of the muscle was not recorded. Ach = 10 ng acetylcholine; G-A = ganglioside A (1 μmole neuraminic acid).

Action on the ileum

On the guinea pig ileum, ganglioside A showed both a contracting effect and a relaxing effect. Fig. 3 shows the contracting effect that was, in contrast with acetylcholine, typically slow-reacting in requiring 60 sec to become maximal. Ganglioside A

was equivalent in contracting activity to 10 ng of acetylcholine/μmole of neuraminic acid in the ganglioside (Table 1). But unlike acetylcholine, ganglioside A was strongly tachyphylactic: the second administration of the ganglioside caused a response equivalent to only 20% of that of the first, whereas a third administration was almost without effect. The muscle did not regain its sensitivity to the ganglioside even after a 30-min interval. The relaxing effect of ganglioside A on the guinea pig ileum, better seen after the development of tachyphylaxis to the contracting effect, was similar to the relaxing effect on the rabbit duodenum (Fig. 4); it was rapid in onset, not tachyphy-

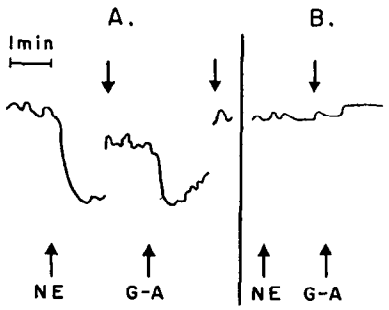

FIG. 4. The relaxing effects of ganglioside A and of norepinephrine on the guinea pig ileum before (A) and three minutes after (B) the addition of 150 mg phentolamine methanesulfonate (Regitine). NE = 200 ng norepinephrine; G-A = ganglioside A (1·5 μmoles neuraminic acid).

lactic and, like the action of norepinephrine, its action was blocked by phentolamine. On this preparation ganglioside A was equivalent in relaxing activity to that caused by 120 ng of norepinephrine or 12,000 ng of dopamine/μmole of neuraminic acid. Ganglioside B showed a slight but measurable relaxing effect but did not cause contraction. Ganglioside C was devoid of both of these smooth-muscle effects, even in twice the concentration used to test ganglioside A.

Chemical measurement of catecholamines in ganglioside A

Ganglioside A contained catecholamines equivalent to 7·5 ng of norepinephrine/μmole of neuraminic acid or 44 μmoles of norepinephrine/mole of neuraminic acid. Like norepinephrine, the ganglioside showed maximal wavelength for activation between 400 and 410 mμ and maximal emission between 500 and 510 mμ.

DISCUSSION

Although the possibility remains that the biological activity may result from a specific ganglioside that is lost during purification, the decline and final elimination of biological activity with sequential purification of the gangliosides (Table 1) is consistent with the hypothesis that such activity is attributable to impurities that accompany the ganglioside during the extraction procedure.

The contracting effect, which was slow in onset and tachyphylactic (Fig. 3), was seen only with the most impure extract (Table 1), ganglioside A. The identity of this slow-reacting material, which was lost in the first purification step, remains unknown.

The relaxing effect (Figs. 2 and 4) of the impure ganglioside, which persisted until the final purification step, was similar to that of norepinephrine in its action and in

being blocked by both ephedrine (Fig. 2) and phentolamine (Fig. 4). A fluorometric method showed that ganglioside A contains catecholamines. These observations and the fact that norepinephrine is the main catecholamine in the brain and the only known substance in brain that in small concentrations causes relaxation of smooth muscle suggest that norephinephrine is present in some impure preparations of gangliosides. On the basis of bioassay, ganglioside A contained 0·0084% norepinephrine; on the basis of chemical assay, 0·0004%. The 20-fold difference between the biological and chemical determination may be attributable to substances in the ganglioside preparation that potentiate the action of norepinephrine on the intestine.

The capacity of gangliosides to form complexes with amines has been emphasized before.[17, 18] It is not known whether a complex between the ganglioside and norepinephrine is present *in vivo* or whether the gangliosides sequester the amine during extraction. The amount of norepinephrine in ganglioside A was small; but since the extraction procedure included two passages through a mixed-bed ion-exchange column, solvent partition, and dialysis, the presence of even residual amounts of norepinephrine (or other catecholamine) testifies to the affinity of gangliosides for the amine.

The observation that preparations of gangliosides, a group of glycolipids, can contain pharmacologically active contaminants recalls previous work showing the presence of small amounts of acetylcholine (or a similar substance) in a highly purified lipid, cerebroside sulfate;[19] and the presence of a slow-reacting material(s) in highly purified glycopolymers.[20]

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REFERENCES

1. S. Bogoch and E. S. Bogoch, *Nature (Lond.)* **183**, 53 (1959).
2. W. Vogt, *Biochem. Pharmacol.* **12**, 415 (1963).
3. S. Bogoch, M. K. Paasonen and U. Trendelenburg, *Brit. J. Pharmacol.* **18**, 325 (1962).
4. R. Kuhn, H. Egge, R. Brossmer, A. Gauhe, P. Klesse, W. Lochinger, E. Röhm, H. Trischmann and D. Tschampel, *Angew. Chem., Int. Ed.* **72**, 805 (1960).
5. E. Klenk, W. Gielen and G. Padberg, in *Cerebral Lipidoses*, S. M. Aronson and B. W. Volk, Eds., p. 301. Academic Press, New York (1962).
6. E. G. Trams and C. J. Lauter, *Biochim. biophys. Acta* **60**, 350 (1962).
7. L. Swennerholm and A. Raal, *Biochim. biophys. Acta* **53**, 422 (1961).
8. W. G. Smith, *Life Sci.* **1**, 133 (1962).
9. J. D. Robinson, Jr. and J. P. Green, *Yale J. Biol. Med.* **35**, 248 (1962).
10. R. H. McCluer, E. H. Coram and H. S. Lee, *J. Lipid Res.* **3**, 269 (1962).
11. M. D. Lilley and J. Vallance-Owen, *Nature (Lond.)* **190**, 1196 (1961).
12. L. Warren, *J. biol. Chem.* **234**, 1971 (1959).
13. C. J. Rondle and W. T. J. Morgan, *Biochem. J.* **61**, 586 (1955).
14. I. A. R. Mead and K. F. Finger, *Biochem. Pharmacol.* **6**, 62 (1961).
15. P. A. Shore and J. S. Olin, *J. Pharmacol. exp. Ther.* **122**, 295 (1958).
16. B. Finkleman, *J. Physiol. (Lond.)* **70**, 145 (1930).
17. J. P. Green, *Advanc. Pharmacol.* **1**, 349 (1962).
18. R. W. Albers and G. J. Koval, *Biochim. biophys. Acta* **60**, 359 (1962).
19. J. P. Green, J. D. Robinson, Jr. and M. Day, *J. Pharmacol. exp. Ther.* **131**, 12 (1961).
20. J. P. Green, M. Day and M. Roberts, *J. Pharmacol. exp. Ther.* **132**, 58 (1961).