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Effects of Experimental Galactosemia on the Measured Serotonin Receptor Activity of Rat Brain*

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ABSTRACT: Serotonin receptor activity was measured by a pharmacological assay which uses a rat stomach strip. Rats were made galactosemic by incorporating galactose as 40% of the total diet, from 19 to 80 days of age. Fractions isolated from the brains by chromatography were measured for serotonin receptor activity, and the galactosemic brains were found to contain less than 10% as much activity as controls. The activity was found in the galactolipid fraction. Chemical comparison of the fractionated galactolipids showed no significant differences in composition or quantity between the two groups of rats, except in the purified fractions which showed receptor activity. Purification of the receptor activity from normal brains yielded an active preparation from the ganglioside fraction which was not observable in the brains of galactosemic rats. Effects of galactosemia on the carbohydrate content of some of the minor galactolipids from the crude receptor fraction were also observed. It was concluded that galactosemia prevented the synthesis of normal serotonin receptor.

The possible relationship of this effect to the mental retardation of untreated human galactosemics was discussed.

The metabolic defect in galactosemia has been known for some time to be a genetic lack of the enzyme, galactose 1-phosphate uridyl transferase, such that galactose and galactose 1-phosphate accumulate in the tissues (Kalckar and Maxwell, 1958). The chemical explanation of the idiocy which results from the high levels of galactose and its derivatives in the tissues has not been found, aside from the demonstration that it is these excesses which provoke the mental failure. Several years ago, Woolley (1962) suggested that a deficiency of serotonin receptors might be the cause. It was later shown (Woolley and Gommi, 1964) that rats, made galactosemic by long-continued feeding of excessive galactose from 19 days to 11 weeks of age, had a specific deficiency of serotonin receptors in their stomachs. The specificity of the deficiency was indicated by the finding that the responsiveness to acetylcholine was normal. Responses to both acetylcholine and serotonin by uterus were unaffected by the galactosemia.

This work was followed by the development of a bioassay for serotonin receptor through the use of normal rat stomachs in which the receptor had been specifically damaged by neuraminidase and EDTA (Woolley and Gommi, 1966). Once it was possible to compare substances for receptor activity, it was shown that the most active materials were obtained from ganglioside preparations (Woolley and Gommi, 1965). Because gangliosides are galactolipids, they might be affected by derangements of galactose metabolism.

The present work was undertaken to learn whether the serotonin receptor of brain was demonstrably affected by galactosemia, and whether the amounts or composition of other galactolipids in brain were influenced by the disease. It was found that the major galactolipids were not significantly affected by the disease, but that a ganglioside fraction which contained receptor activity in the controls was affected. The lipid with the highest activity in the receptor assay was absent from galactosemic brains. Some other, minor lipids from this fraction also were found to differ in amount and composition between the two groups.

Experimental Section

Treatment of Rats. Female, 19-day-old albino rats from Carworth Farms were used. Groups of 15 rats were given either the highly purified diet of Sterling and Day (1951) (controls) or the same diet in which D-galactose replaced sufficient glucose to account for 40% of the total diet (galactosemics). Rats were individually caged on screen floors and fed the experimental diets *ad libitum* for at least 8 weeks. The con-

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trols then weighed about 200 g each and the galactosemics about 160 g each. Brains were the same size in the two groups, averaging 1.6 g. The galactose-fed animals exhibited the typical signs of galactosemia, such as cataracts of the eyes. Objective behavioral or intelligence tests were not applied to the animals. Rats were killed by being placed in a jar with a small amount of chloroform. Brains were quickly removed, weighed, and stored frozen until extracted.

Extraction Procedure. Total brain lipids were extracted by adding 20 ml of cold chloroform–methanol (2:1, v/v) per g of brain and homogenizing 4 min with a Lourdes Instrument Corp. multimixer. The cold extract was filtered into a graduated cylinder and the insoluble material extracted twice more with 10 ml of chloroform–methanol per g of brain for 3 min each.

The ganglioside fraction was prepared from the crude extract by the method of Folch *et al.* (1957). Water was used instead of the aqueous salt solution in order to get a more quantitative yield of the minor sphingolipids. The lower layer was washed twice with "pure solvents upper phase." The upper layers were combined, treated with one-tenth volume of 1-butanol to prevent frothing, and concentrated on a flash evaporator, below 40°, to approximately 10 ml. This solution was then dialyzed in Visking tubing against three changes of distilled water at 4°. The average dry weight of nondialyzable solids (crude ganglioside preparation) was about 5 mg/g of brain. The yield of the crude fraction was the same from galactosemic as from control brains.

Thin Layer Chromatography. Silica gel H (from Brinkmann Instruments, Inc., "according to Stahl") was spread on glass plates either at 0.25 or 0.50 mm thickness. The plates were heated overnight at 110° immediately before use. The sample of crude lipid was dissolved in a small volume of chloroform–methanol or in the chromatography solvent and applied to the starting line from a micropipet. Fractions from controls and from galactosemics were developed on separate plates in the same tank, simultaneously. The first solvent was a freshly prepared mixture of chloroform–methanol–water (60:35:8, v/v), which had equilibrated within the tank for 2–4 hr. Solvent was allowed to ascend 17 cm, the plates were removed, dried as suggested by Rouser *et al.* (1967), by keeping it in a stream of dry nitrogen for 1 hr, and replaced in the tank for a second excursion of the solvent. In the experiments using a second solvent system, propanol–water (7:3, v/v) was employed, with only one excursion. After drying in air, the bands were located by exposure to iodine vapor.

Elution from the thin layer plates was performed by the procedure of Goldrick and Hirsch (1963). Each band was eluted with a minimum of 20 ml of methanol in 5-ml portions. It was necessary to remove traces of iodine at this stage, in order to avoid interference in the bioassay and with the anthrone reaction. Iodine in untreated samples poisoned the stomach tissue which was used in the bioassay for serotonin receptor activity and caused high values of incorrect spectrum in the anthrone determination. To remove this iodine, solvent was evaporated from the eluates and the residue was treated, in neutral aqueous solution, with an excess of 0.1 M $Na_2S_2O_8$, followed by dialysis to remove salts. Controlled experiments with known gangliosides showed that these procedures effectively removed the interferences caused by iodine and resulted in no observable modification of the gangliosides.

It is useful to know the usual rate of migration, in thin layer systems, of substances studied routinely. For this reason, R_F values are mentioned, rounded off to the nearest multiple of 0.05. It has been observed that use of the nitrogen-drying procedure for 15 min just before each plate is put in its initial solvent and careful attention to routine procedure (Randerath, 1963) results in quite reproducible R_F values.

Figure 1 is a schematic representation of a typical thin layer chromatogram, which had the crude ganglioside fraction from brain applied to it, and was run once in the chloroform solvent and developed in iodine vapor. The bands were eluted in such a way that no silicic acid was left on the major part of the plate, as indicated on the figure.

Column Chromatography. Either the total lipid extract or the upper phase of the Folch partition was used on silicic acid columns. The method of Weiss (1956) for column chromatography and effluent analysis was followed. Columns were poured of Bio-Rad silicic acid (specially prepared for the chromatography of lipids, −325 mesh). When 1-cm diameter columns were used, the same gradient was applied as for 2-cm columns, but 2-ml fractions were taken.

Component Analysis. Hexose was determined by the anthrone method of Radin *et al.* (1955), with galactose as the standard. Glucose gave 150% as much color as galactose. Values were measured as galactose equivalents. Hexose was also determined, in the receptor fractions, by the gas chromatographic method of Sweeley and Walker (1964), procedure B. The liquid phase of the column was 2.5% SE-30, on Gas Chrom Q (Applied Sciences Laboratories), at 160°. A 6 ft × 3 mm column in an F & M Model 402 gas chromatograph was employed for all gas chromatography.

Sialic acid was determined by the resorcinol method of Svennerholm (1957) as modified by Miettinen and Takki-Luukkainen (1959). Hexosamine was determined by the method of Svennerholm (1956). Methyl esters of fatty acids were determined by gas chromatography on 10% EGSSX on Gas Chrom P at 150°. Bioassays for serotonin receptor activity were performed by the method of Woolley and Gommi (1966).

Results

Serotonin Receptor Activity Bioassays. Table I shows the results of serotonin receptor activity bioassays of comparable preparations from galactosemic and glucose control rats. Units of activity were calculated for the lowest dose of lipid that gave more than 1 cm activation. A fraction was designated inactive only after it gave no restoration of the tissue, even at

TABLE I: Serotonin Receptor Activity in Ganglioside Fractions of Control and Galactosemic Rat Brains.

Material Assayed	Units[a] of Activity/g of Fresh Brain: Controls	Units[a] of Activity/g of Fresh Brain: Galactosemics
Crude ganglioside fraction from whole brain	160	20
Receptor fraction from column effluents[b]	30	<3
Thin layer fractions		
R_F 0.40[c]	0	0
Receptor fraction (R_F 0.30)[c]	40	0
G1 (R_F 0.25)[c]	0	0
Disialogangliosides (R_F 0.15)[c]	(48)[d]	(40)[d]

[a] Units determined according to Woolley and Gommi (1966), and as described in text. [b] Fraction from a 1 × 40 cm column consisting of 20 ml of the column effluent between cerebrosides and G1. The G1 peak would be in the next 10–15 ml. [c] Purified by thin layer chromatography in chloroform–methanol–water (60:35:8, v/v). R_F given is value after a single excursion of the solvent. [d] Never gave full restoration of activity. Inhibition of the assay tissue seen at some dose levels. An activity is calculated only because a small activation was sometimes seen, but the dose–response relationship was quite different from that shown by the designated receptor fractions. This type of response was described by Woolley and Gommi (1965).

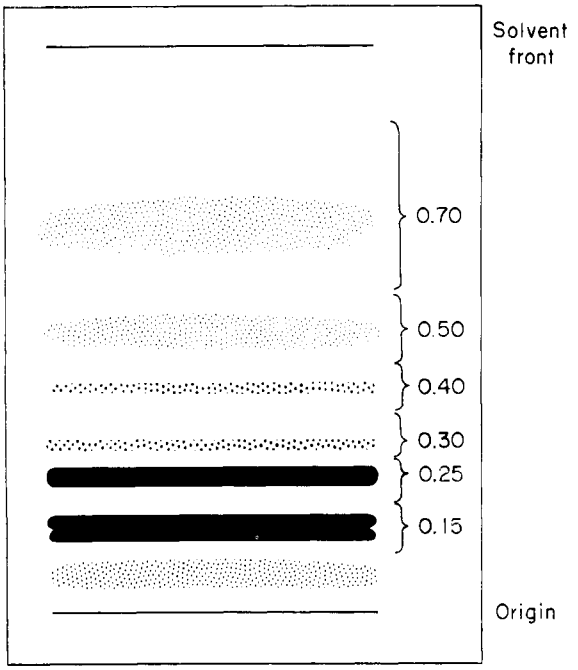

FIGURE 1: Schematic representation of thin layer chromatogram of crude ganglioside fraction of rat brain. Solvent was chloroform–methanol–water (60:35:8, v/v). Brackets indicate the extent of silicic acid removal for elution.

very high dosage, and subsequent application of an active fraction showed the tissue to be normally reponsive. In the assays shown in Table I, the units of activity were calculated from the responses obtained with fractions equivalent to 10–50 mg of fresh brain. The values shown in the table are averages from four or more determinations.

It can be seen readily from Table I that very different levels of receptor activity were present in the two kinds of rat brains. This difference was greatest in the R_F 0.30 band from thin layer plates and the column effluent fraction which moved just ahead of G1, the major monosialoganglioside of adult brain (Kuhn and Wiegandt, 1963). Both of these fractions contained material of very high specific activity from the controls, but the comparable fraction from galactosemics had less than 10% as much activity. Table II shows a typical tissue response to such an active fraction. There is a continuous increase of response of the tissue to a standard amount of serotonin as larger doses of lipid are applied. The preparation shown, on the basis of this test, would be said to have 36 units of activity, as defined above.

The receptor activity which was found in the disialogangliosides was anomalous, as was explained by Woolley and Gommi (1965). These lipids, although present in brain in relatively large amount, caused only a small activation of the tissue, and often caused inhibition of the tissue at the higher dose levels. A

TABLE II: Increase in Responsiveness to Serotonin Caused by Increasing Doses of a Purified, Active Ganglioside, When Applied to a Stomach Strip in the Standard Receptor Activity Bioassay.[a]

	Serotonin Response (cm)	Net Increase Owing to Active Lipid (cm)
Average response of stomach strip before lipid application	1.9	
Purified lipid fraction corresponding to:		
0.25 mg of brain	2.0	0.1
1.0 mg of brain	2.6	0.7
5.0 mg of brain	2.9	1.0
50.0 mg of brain	3.7	1.8

[a] Woolley and Gommi (1966).

TABLE III: Chemical Determinations of Ganglioside Fractions of Control and Galactosemic Rat Brains.

R_F[a] of Ganglioside Fraction	Controls			Galactosemics		
	Sialic Acid[b]	Hexose:[c] Sialic	Galactosamine:[c] Sialic	Sialic Acid[b]	Hexose:[c] Sialic	Galactosamine:[c] Sialic
0.40	0.03	>3	0	0.04	>3	0
0.30	0.02	4.5[d]	1.0	0.02	6.2[d]	0.9
0.25	0.25	3.7	0.8	0.27	4.0	0.8
0.15	1.35	1.8	0.6	1.36	2.0	0.6
Unfractionated gangliosides	1.62	2.2		1.68	2.3	

[a] Identification as in Table I. Receptor fraction is R_F 0.30. [b] Fresh brain (micromoles per gram). [c] Molar ratios. [d] Hexose determined by gas chromatography.

TABLE IV: Fractions Obtained by Thin Layer Chromatography[a] of the Receptor Fractions of Table I.

From Controls				From Galactosemics			
R_F[b]	Glucose[c]	Galactose:[d] Glucose	Stearic Acid:[d] Glucose	R_F[b]	Glucose[c]	Galactose:[d] Glucose	Stearic Acid:[d] Glucose
0.80	12	0.06	0.5	0.80	7	0.23	0.7
0.70	13	0.10	0.5	0.65	16	0.30	0.6
0.60	12	0.50	0.5	0.55	10	0.23	0.4
				0.50	12	1.0	0.5
0.45	16	0.23	0.2	0.40	20	0.23	0.2
0.35	6	0.26	0.3	0.35	9	0.30	0.2

[a] In propanol–water (7:3, v/v). [b] For a single excursion in the solvent. [c] Fresh brain (millimicromoles per gram). [d] Molar ratios, determined by gas chromatography.

continuous increase of response, with increasing dose of lipid, was not seen with these lipids. It will be noted that there was no significant difference in the serotonin receptor activity of this fraction in the two kinds of brains. In summation, no fraction from galactosemics was able to reactivate the assay tissue.

Chemical Determinations. Table III shows the results of chemical comparisons of sialic acid containing fractions from the two kinds of brains. The figures given are the average values obtained from four different experiments. It can readily be seen that the only significant difference between the two groups was in the hexose content of the R_F 0.30 fraction. This is the same fraction that, in the controls, had the major receptor activity. The fact that the galactosemic fraction had an increased hexose content and an unchanged mobility in thin layer chromatography necessitated a corollary increase in the lipophilic constituents of the fraction. Fatty acid analysis by gas chromatography of the methyl esters did, indeed, show a 24% increase of fatty acids in the galactosemic fractions, over controls.

The hexose content of the cerebroside fractions from the two groups of brains was also determined. The cerebrosides were isolated by column chromatography only. Kerasin peaks gave 1.9 mg of galactose equiv/g of brain from controls and 2.0 mg from galactosemics. Phrenosin peaks yielded 0.5 mg of galactose equiv/g of brain from controls and 0.49 mg from galactosemics. It was observed that the hot alcohol-extraction method of Payne and Platt (1961) gave only about one-half as much yield of cerebrosides and gangliosides as the chloroform–methanol extraction procedure.

In order to characterize further the differences in the receptor fractions from the two groups of brains, these bands (R_F 0.30 in Tables I and III) were eluted, treated with thiosulfate, dialyzed, and chromatographed in the second solvent.[1] Table IV shows the glucose content, galactose:glucose ratio, and stearic acid:glucose

[1] Propanol–water (7:3, v/v).

ratio of the resulting fractions. It will be noticed that the total hexose figures for the bands shown in Table IV agree quite well with the figures shown in Table III for the crude material. The total galactose in the fractions from galactosemic brains is increased 135% over controls, while glucose is increased only 25%. The fraction of R_F 0.60, from the controls, was different in composition from anything found in the galactosemics. This fraction was also significantly different in mobility on thin layer plates from any galactosemic fraction. It was clearly separable from all galactosemic fractions by thin layer chromatography, when the solvent was run a full 17 cm. Study of this fraction from normal brains has been hampered by the small quantities available. This material represents only 0.23% of the total brain ganglioside sialic acid. However, it has been shown to be a very active fraction from normal brains in the receptor bioassay. Both the dose–response activity curve and the total activation of the tissue are typical of the most active preparations studied.

In summation, the major chemical difference between brain galactolipids from galactosemics and controls was found in a chromatographic fraction just faster than G1. When this fraction from controls was examined by further chromatography, it yielded several bands, one of which exhibited strong serotonin receptor activity in the bioassay. This substance was not obtained from similar treatment of the corresponding fraction from galactosemics. Although this most active preparation is not yet well characterized, it contained glucose, galactose, sialic acid, and stearic acid in significant quantities. The conclusion that the synthesis of the serotonin receptor of brain is prevented by galactosemia seems reasonable.

Discussion

The demonstration that experimental galactosemia in rats causes a deficiency of brain serotonin receptor may be a significant step toward explaining the mental deficiency caused by untreated galactosemia in humans. Woolley and van der Hoeven (1965) showed that a lowered serotonin level in infant mice resulted in a learning defect which persisted even after the hormonal level was restored, later. This defect, caused by experimental phenylketonuria, could be reversed by 5-hydroxytryptophan or melatonin, administered in infancy. A deficiency of serotonin receptor in a developing brain, such as that demonstrated for experimental galactosemia, would be expected to result in a functional deficiency of the hormone, even in the presence of normal hormonal levels. This functional hormonal deficit could be the cause, by an unknown mechanism, of the mental defect which results from hereditary galactosemia, where the galactosemia persists from early infancy. Further work must be done, of course, to characterize the compound that has the receptor activity measured by the stomach assay, and to explain the mechanism of the inhibition of proper lipid synthesis resulting from high levels of galactose.

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