Glycosphingolipids in the spleen of developing rats

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ABSTRACT Splenic cholesterol, and glucosyl, lactosyl, trihexosyl, and sialyl lactosyl ceramides were studied in developing normal rats from birth to 96 days of age.

Total lipid, extracted from pooled organs for each age group in the study, were subjected to mild alkaline hydrolysis and separated into purified glycolipid fractions in high yield, by a series of silicic acid column and thin-layer chromatographic procedures. Enzymatic and colorimetric methods were applied to quantitative analysis of each splenic glycosphingolipid fraction, and the fatty acids were analyzed by gas-liquid chromatography.

Glycosphingolipid content in the total spleen increased over the period of the study. The most rapid increase occurred during the third and fourth weeks. There was also a 2.5-fold change in the concentration (per gram of tissue) of glucosyl and sialyl lactosyl ceramide, the principal glycosphingolipids of rat spleen. In contrast, cholesterol concentration increased only slightly.

A precisely equimolar ratio of glucosyl and sialyl lactosyl ceramide was observed throughout all stages of splenic development. Fatty acid compositions of these two lipid fractions were always significantly different.

SUPPLEMENTARY KEY WORDS cholesterol • glucosyl ceramide • lactosyl ceramide • trihexosyl ceramide • sialyl lactosyl ceramide • total spleen lipids • fatty acid composition

THERE IS A lack of basic information concerning essential progressive changes in splenic glycosphingolipids relative to normal mammalian organ development. This study describes changes which occur in splenic glycosphingolipids of developing rats as the animals progress from birth to maturity. Rate of growth of the developing animals, spleen growth, disparate increases in splenic content of specific glycosphingolipids, and a progressive selective change in fatty acid composition of certain of the latter compounds are described.

MATERIALS AND METHODS

Experimental animals were obtained from Wistar strain Sprague-Dawley rats maintained on Purina laboratory chow. A total of 36 adult females were mated with males of the same species. Date of birth for each individual litter was denoted the first day of life. Groups of pups were killed at selected intervals by decapitation, with no attempt to separate the animals according to sex; the spleens were removed, weighed, and frozen. The adult females were then remated until sufficient offspring were obtained for at least 4 g of tissue for each of the specimen ages chosen. A total of 995 rats were sacrificed for the experiment over a period of two years.

Glycolipid Standards

Verified standards of glucosyl ceramide and lactosyl ceramide (cytolipin H) were used, the former obtained as described previously (1), and the latter a generous gift from Dr. Maurice Rapport. Further amounts of these compounds, and preparations of pure triglycosyl ceramide and sialyl lactosyl ceramide (hematoside) standards, were obtained from a spleen from a 26-year-old woman with confirmed Gaucher's disease. The spleen was removed at surgery and immediately frozen until use. 165 g of the tissue was homogenized and extracted in a Waring Blender as described by Folch, Lees, and Sloane Stanley (2), and the homogenate was filtered. The filtrate was dialyzed in the cold for 3 days against...
running the interface and the chloroform phase were mixed with 225 g of 60–100 mesh Florisil. The mixture was divided into two portions and added as a slurry to two 70 x 2 cm (i.d.) glass columns. The columns were washed exhaustively with chloroform. Glucosyl ceramide was eluted with chloroform–methanol 92:8 (v/v). Remaining glycolipids on the column were eluted with chloroform–methanol–water 1:3:0.4 (v/v/v).

The glucosyl ceramide was purified further by chromatography on Florisil. The Florisil column was washed with chloroform–methanol 98:2 (v/v). Glucosyl ceramide was eluted with chloroform–methanol 95:5 (v/v). It was chromatographically pure on TLC, migrating with known glucosyl ceramide, and free of chloroform–methanol-insoluble material. Dry samples were weighed for use as glucosyl ceramide standard.

The lipid residue from the chloroform–methanol–water 1:3:0.4-eluate was saponified (3). The glycolipids were separated by preparative TLC in chloroform–methanol–water 2:1:0.1 (v/v/v). The lactosyl ceramide band was scraped off and the lipid was eluted with chloroform–methanol–water 1:3:0.4 (v/v/v). The preparation was further purified by preparative TLC in chloroform–methanol–water 3:1:0.1 (v/v/v). The quantity of lipid was determined, after hydrolysis in 3 N HCl (1), by measurement of glucose, using the glucose oxidase method (4). Verified samples of cytolympin were used as reference standards. Trihexosyl ceramide and sialyl lactosyl ceramide bands were similarly eluted and purified by preparative TLC in chloroform–methanol–water 1.5:1:0.1 (v/v/v). The sialyl lactosyl ceramide, being more polar, was distinguished from the trihexosyl ceramide by a slower mobility, and it was identified by a positive resorcinol reaction (5) and release of lactosyl ceramide upon incubation with sialidase from Clostridium perfringens (6) (Worthington Biochemical Corp., Freehold, N.J.). The quantity of lipid was determined by measurement of sialic acid according to the method of Suzuki (7). Trihexosyl ceramide was hydrolyzed in 3 N HCl (1), and the sugars were quantitatively analyzed with glucose oxidase (4) and galactose oxidase (Galacto-stat, Worthington Biochemical Corp.), using lactosyl ceramide as a reference. The quantitative ratio of galactose to glucose was 2:16:1.

Extraction of Lipids

Rat spleens were thawed, homogenized, filtered, and dialyzed as described above for the Gaucher spleen. The aqueous phase was collected and evaporated in a Buchler Flash Evaporator at 37°C. The chloroform phase and interface were added to the dried residue and similarly evaporated. The total residue was redissolved in dry chloroform–methanol 2:1 (v/v), transferred to tared tubes, dried under N₂, and then dried to a constant weight in a vacuum desiccator. Weighed samples were then redissolved in chloroform–methanol, and aliquots were taken for cholesterol determination (8), and for fatty acid determination and glycolipid quantitation, as described later.

TLC Separation of Glycolipids

All TLC separations were performed on precoated 20 x 20 cm silica gel (F-254) plates (E. Merck A.G.) obtained from Brinkmann Instruments Inc., Westbury, N.Y. The plates were divided into two groups. One group was used for the unknown and the other for the reference compounds. The plates were developed in paper-lined tanks in the solvent mixtures noted in the text. The reference plates with known pure standards were developed in the same tank with each of the sample plates. The standards were visualized in an iodine vapor bath and the sample plates were scored for elution of the lipids on the basis of the Rᵢ values of standards. The sample plates were sprayed with deionized water to visualize the bands. They were scraped with a spatula, while still wet, into a beaker containing a small amount of eluting solvent, and then transferred to a column to complete elution.

A greater degree of purification is necessary for the precise identification of the fatty acids of the glycosphingolipid fractions than for the quantitative analysis of these fractions, since the latter depends upon relatively specific reactions for the glycosyl components. A special purification scheme was therefore developed to remove the very small amounts of contaminating lipid material, as follows.

Fatty Acid Determination

Aliquots of total rat spleen lipid for each age group were dried under N₂, saponified by the method of Suomi and Agranoff (3), and the glycolipids were separated by TLC in chloroform–methanol–water 2:1:0.1 (v/v/v). They were eluted from the silica gel with 200 ml of chloroform–methanol–water 1:3:0.4 (v/v/v). Quantities of lactosyl ceramide and trihexosyl ceramide were insufficient for fatty acid determination. Glucosyl ceramide fractions of these samples were spotted on TLC plates, developed with chloroform–methanol–water 5:1:0.5 (v/v/v), and eluted as above, three successive times. Hematoside fractions were developed twice with chloroform–methanol–water 1.5:1:0.1 (v/v/v), and similarly eluted in order to remove contaminating globoside. Glucosyl and sialyl lactosyl ceramide fractions gave single, anthrone-positive spots in each TLC system. Samples were divided into aliquots, dried, and methanolized in 16% BF₃ in methanol (w/w) by a method previously published (9). The hexane extract was dried...
and analyzed in a Research Specialties model 600 flame ionization gas chromatograph at 173°C; column packing was 15% Hi-Env-2BP on Chromosorb W(AW) 80–100 mesh (Applied Science Laboratories Inc., State College, Pa.). Methyl esters of fatty acids were identified by comparison with known standards and quantitated by triangulation.

Quantitation of Glycolipids
Aliquots of rat spleen lipids equivalent to 2 g of wet tissue were added to the freshly prepared silicic acid columns in chloroform, and eluted according to the method of Vance and Sweeney ([10]), except that the recommended volume of acetone–methanol was doubled. TLC plates, 10 x 20 cm, were predeveloped in chloroform–methanol–water 1.5:1:0.1 (v/v/v) to remove an unidentified substance which reacted with anthrone. After they were dry they were spotted with the eluate from the columns. The plates were developed in chloroform–methanol–water 2:1:0:1 (v/v/v), and the individual glycolipid bands were eluted with 100 ml of chloroform–methanol–water 2:1:0:2 (v/v/v). The eluate was reduced to dryness on a flash evaporator and redissolved in dry chloroform–methanol 2:1 (v/v) for final anthrone ([11, 12]) or resorcinol ([7]) determination. Blank TLC plates, eluted in the same manner, served as controls.

Anthrone Determination
Serial dilutions of glucosyl ceramide, lactosyl ceramide, and trihexosyl ceramide standards were prepared. Aliquots were pipetted and dried under nitrogen. They were then hydrolyzed for 90 min in a boiling water bath with 0.5 ml of 3 N HCl in screw-cap tubes flushed with nitrogen and sealed with Teflon tape. The tubes were cooled, and free fatty acids were extracted with 0.5 ml of hexane or chloroform and discarded. The tubes were placed in an ice bath and 2.5 ml of anthrone reagent was added (11, 12). The tubes were capped, mixed, and heated in boiling water for 15 min; they were then cooled while warm and shaken on a Vortex mixer in a well-ventilated hood to allow the escape of gaseous HCl and hexane or chloroform vapors. The samples were then cooled for 20 min. Tubes that contained hexane were centrifuged if they were not completely clear. Tubes with chloroform were shaken for a short time in a boiling water bath in order to clear them. Samples were then read at 520 nm on a Bausch and Lomb recording spectrophotometer. The absorbances followed Beer's law, and the results were reproducible in a given experiment to an accuracy of ±5%. Multiple determinations of standards over a period of one year were reproducible to an accuracy of ±6.5%. Samples of rat glycolipids were treated in the same manner as the reference substances; the eluates from blank TLC plates were used for the "blanks" in the anthrone determination.

Results
Physiological Data
The number of animals used in each age group, their weights, and lipid content of their spleens are shown in Table 1. A larger sampling of younger animals was necessary in order to obtain sufficient tissue for analysis. As the animals grew older, fewer were needed to obtain sufficient tissue.

The initial slow rate of growth in the first eight days was followed by a rapid increase until 61 days of age. The spleen weight, however, increased at a more constant rate from birth until 61 days. Pups were weaned between the 16th and 32nd day.

Fatty Acid Composition
The fatty acid composition of glucosyl ceramide from 1- to 61-day-old rats is shown in Table 2. Similar data for hematoside are in Table 3. The compositions of the two lipid fractions are dissimilar. A higher proportion of odd-numbered fatty acids is noted in glucosyl ceramide than in hematoside, and the content of oleic acid in hematoside, though it falls from the very high levels of the first few days, remains greater than that in glucosyl ceramide throughout.

Comparing percentage composition of fatty acids of chain length C<sub>18</sub> and longer with those below C<sub>20</sub> there is an increase in the percentage of longer chain fatty acids in glucosyl ceramide with age, but no comparable change in hematoside.

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>WEIGHT AND LIPID CONTENT OF Spleen in Growing Rats</th>
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</thead>
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<td>Age</td>
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</tr>
<tr>
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<td></td>
</tr>
<tr>
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<td>288</td>
</tr>
<tr>
<td>2</td>
<td>255</td>
</tr>
<tr>
<td>4</td>
<td>210</td>
</tr>
<tr>
<td>8</td>
<td>127</td>
</tr>
<tr>
<td>16</td>
<td>79</td>
</tr>
<tr>
<td>32</td>
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<td>12</td>
</tr>
<tr>
<td>96</td>
<td>4</td>
</tr>
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</table>

* Standard deviations were calculated for pooled litters.
TABLE 2  FATTY ACID COMPOSITION OF SPLENIC GLUCOSYL CERAMIDE

<table>
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<tr>
<th>Age</th>
<th>14:0*</th>
<th>15:0</th>
<th>15:1</th>
<th>16:0</th>
<th>16:1</th>
<th>17:0</th>
<th>18:0</th>
<th>18:1</th>
<th>18:2</th>
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<th>24:0</th>
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<th>25:0</th>
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<td></td>
<td></td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>5.3</td>
<td>1.3</td>
<td>7.2</td>
<td>23.1</td>
<td>T†</td>
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<td>15.9</td>
<td>T†</td>
<td>15.4</td>
<td>T†</td>
<td>—</td>
<td>4.6</td>
<td>3.9</td>
<td>13.1</td>
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</tr>
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<td>15.8</td>
<td>2.4</td>
<td>2.8</td>
<td>10.1</td>
<td>6.2</td>
<td>9.1</td>
<td>3.0</td>
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<td>2.6</td>
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<td>2.6</td>
<td>15.2</td>
<td>6.9</td>
<td>—</td>
<td>3.7</td>
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<td>1.5</td>
<td>2.8</td>
<td>21.7</td>
<td>T†</td>
<td>2.4</td>
<td>13.4</td>
<td>T†</td>
<td>9.5</td>
<td>1.2</td>
<td>3.0</td>
<td>T†</td>
<td>8.2</td>
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<td>8.7</td>
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<td>18.9</td>
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<td>—</td>
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<td>5.8</td>
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<td>7.6</td>
<td>—</td>
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<td>1.3</td>
<td>—</td>
<td>1.2</td>
<td>11.1</td>
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<td>2.0</td>
<td>10.4</td>
<td>4.0</td>
<td>11.0</td>
<td>2.4</td>
<td>—</td>
<td>5.7</td>
<td>8.4</td>
<td>4.8</td>
<td>28.7</td>
<td>6.9</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>61</td>
<td>2.8</td>
<td>—</td>
<td>8.8</td>
<td>16.5</td>
<td>1.4</td>
<td>1.4</td>
<td>10.8</td>
<td>1.7</td>
<td>10.0</td>
<td>0.6</td>
<td>—</td>
<td>0.4</td>
<td>10.8</td>
<td>6.6</td>
<td>20.2</td>
<td>7.0</td>
<td>1.7</td>
<td>—</td>
</tr>
</tbody>
</table>

* Number of carbon atoms: number of double bonds.
† Trace.

The overall fatty acid compositions of the two glycolipids are dissimilar for all age groups.

Recovery of Glycolipids

To determine the amount of recovery of glycolipids by the method described for their quantitative analysis, the lipids of 16 g of normal rat spleens were extracted and divided into eight aliquots. Four of these were analyzed as described, and to the other four were added known quantities of each glycolipid to be measured. These samples were then analyzed in the same manner. The results, together with the average TLC for these glycolipids, are summarized in Table 4. Over-all recovery was similar to TLC recovery alone, suggesting a complete recovery from the column. Recorded data from the experiment are corrected to the theoretical yields on this basis.

Lipids of Rat Spleens

The glycolipids are reported, in micrograms, as the standard compounds against which they were measured. The sialyl lipid, measured as sialic acid, is reported as hematoside. This is done on the assumption that the principal ganglioside of rat spleen is the same as that which Svennerholm has identified in human spleen (13).

A substantial increase in quantity of glycolipid per gram of tissue was seen. As shown in Table 5, glucosyl ceramide and hematoside increased in concentration in rat spleen approximately 2.5-fold over the period of the study, and the dihexoside also increased considerably. These glycolipids also comprised an increasing proportion of the total lipid of the spleen. The presence of excess quantities of spurious chromogens in the 8- and 16-day samples of dihexoside and in the samples of trihexoside after the eighth day prevented accurate measurement, so these figures are not presented. A small
TABLE 5 QUANTITY OF LIPIDS PER GRAM OF SPLEEN (Wet Weight)

<table>
<thead>
<tr>
<th>Age</th>
<th>Total Lipid mg</th>
<th>Total Cholesterol mg</th>
<th>Glucosyl Ceramide µg</th>
<th>Lactosyl Ceramide µg</th>
<th>Trihexoside µg</th>
<th>Hematoside µg</th>
</tr>
</thead>
<tbody>
<tr>
<td>days</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>21.0</td>
<td>2.64</td>
<td>66.8</td>
<td>13.1</td>
<td>11.2</td>
<td>92</td>
</tr>
<tr>
<td>2</td>
<td>21.0</td>
<td>2.59</td>
<td>65.1</td>
<td>25.6</td>
<td>11.7</td>
<td>100</td>
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<tr>
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<td>131</td>
</tr>
<tr>
<td>8</td>
<td>22.3</td>
<td>2.55</td>
<td>87.6</td>
<td>—</td>
<td>—</td>
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<td>222</td>
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<tr>
<td>96</td>
<td>27.2</td>
<td>—</td>
<td>184.6</td>
<td>52.8</td>
<td>—</td>
<td>245</td>
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</table>

increase in trihexoside concentration was noted in the first four days, however. The quantity of glycosphingolipids in the total organ increased slowly for the first 16 days and rapidly thereafter until the 61st day.

Only a slight increase in the quantity of total lipid and cholesterol per gram of tissue occurred after the eighth day of life. The cholesterol fraction of total lipid remained relatively constant at all times. Thus, the amount of total lipid and cholesterol in the whole organ increased proportionally with organ growth.

The increase in glucosyl ceramide was paralleled very closely by that of hematoside. These compounds had a ratio by weight of approximately 1:1.4 in all the animals. The average ratio of glucosyl ceramide to hematoside at all stages of splenic development was equimolar (1.04:1; range 0.92-1.10). As the micrograms of rat glucosyl ceramide were calculated on the basis of micrograms of standard, the molecular weight used in calculation (782) was that of the standard glucosyl ceramide. This was calculated on the basis of average fatty acid molecular weight of 338, as determined by GLC analysis.

DISCUSSION

The very small quantity of tissue available for analysis, and the need to perform at least duplicate analyses on each sample, led to modifications of standard procedures in order to decrease loss of sample and increase the sensitivity of the measurements. Though the sensitivity of the GLC procedure was sufficient to measure the fatty acid composition of glucosyl ceramides and hematosides because of the greater concentration of these compounds, it was insufficient for the di- and trihexosyl ceramides.

Critical to the sensitive measurement of glycolipids was improvement of TLC yields. Use of commercial precoated plates improved these recoveries, and the additional step of spraying the bands with water and their removal with a spatula while still wet resulted in complete separation of the silica gel from the glass without residual material remaining, and further prevented loss from dust formation. This step improved yields by 10%.

There was a twofold purpose in hydrolyzing the glycolipids in 3 n HCl before measuring their hexose content by the anthrone reaction. First, the anthrone determination produced a 50% greater color development on hydrolyzed samples than on nonhydrolyzed samples, thus increasing the sensitivity of the procedure. Second, chromogens present as trace contaminants produced an interfering color in anthrone determinations made directly. Hydrolysis and extraction with hexane or chloroform partially removed spurious chromogens in the solvent extract, thus resulting in better color development. Of the two solvents, chloroform was more efficient than hexane in removing the chromogens. This was not discovered until after the dihexoside and trihexoside measurements. Had chloroform been used on these, the missing results could have been rescued.

Direct colorimetric measurement by anthrone or resorcinol immediately after TLC separation, rather than through preparation of trimethylsilyl derivatives and measurement by GLC, as in the method of Vance and Sweeley (10), had the advantage of saving a number of steps in preparation of derivatives and calculation of results. There was, however, the obvious disadvantage that the hexose composition was not available as a check of the validity of the separation. With reproduc-
able TLC systems for separation, this disadvantage was minimized.

The problem of contamination of hematoside with globoside in the TLC separation was obviated by measuring the sialic acid rather than the hexose in the hematoside.

The differing fatty acid composition of glucosyl ceramide and hematoside in all age groups would suggest that the latter compound is not the sole precursor of the former unless a catabolic selection of compounds with specific fatty acids occurred. Though the fatty acid composition of globoside was not studied, the dissimilarity of composition of glucosyl ceramide and hematoside in this study is consistent with Radin's suggestion that red cell globoside (14) may be a precursor for splenic glucosyl ceramide. The increase in the percentage composition of long-chain fatty acids with age observed in glucosyl ceramide may stem from maturation of enzyme systems for elongating fatty acids.

Our finding that accumulation of glycolipids occurs in normal spleens suggests either that degradation decreases with age or that these compounds may be of importance in the subcellular structure of the spleen in the developing animal. The quite constant (unit) molar ratio of glucosyl ceramide to hematoside in the spleens throughout development may relate to such a structural configuration.

The work of Coles, Hay, and Gray (15) showing variation in glycosphingolipid content in mouse kidney according to the sex of the animal, and that of Kwiterovich, Sloan, and Fredrickson (16) suggesting that there is variation in liver glycosphingolipid content in humans according to sex, had not yet appeared by the time the present study had been completed. Separation of spleens from male and female rats was not made for our study. Hildebrand and Hauser (17) have previously reported that glucosyl ceramide is the major glycosphingolipid component in spleens of rats weighing 100–170 g. Presumably, those animals correspond to an age group falling between 32 and 61 days in the present study. We show here that glucosyl ceramide is constantly a major splenic glycolipid component in rats from birth to 96 days of age, and our finding that there is always an equimolar splenic content of glucosyl ceramide and sialyl lactosyl ceramide indicates that both these compounds may be considered the major glycosphingolipid components of rat spleen.

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