The Biosynthesis of Sulphogalactosyldiacylglycerol of Rat Brain *in vitro*

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Triton X-100 extracts of rat brain microsomal fraction catalyse the formation of sulphogalactosyldiacylglycerol from galactosyldiacylglycerol and adenosine 3'-phosphate 5'-sulphatophosphate. Of the various subcellular fractions of brain assayed, the microsomal fraction contained most (79%) of the adenosine 3'-phosphate 5'-sulphatophosphate-galactosyldiacylglycerol sulphotransferase activity. The enzyme activity was stimulated by Triton X-100 and showed linearity with increasing time, concentrations of enzyme and added substrates. ATP and KF prolonged the linearity of the activity with time, but ATP had an overall inhibitory effect on the sulphotransferase. Both ATP and KF inhibit the degradation of adenosine 3'-phosphate 5'-sulphatophosphate, which probably causes the increased linearity of the sulphotransferase reaction with time. The enzyme preparation did not catalyse the transfer of sulphate from adenosine 3'-phosphate 5'-sulphatophosphate to either cholesterol or galabiosyldiacylglycerol (galactosylgalactosyldiacylglycerol). Significant differences between the formation of sulphogalactosyldiacylglycerol and cerebroside sulphate catalysed by the same enzyme preparation were noted. ATP and Mg^{2+} strongly inhibit the formation of sulphogalactosyldiacylglycerol but equally strongly stimulate the synthesis of cerebroside sulphate. The apparent K_m for galactosyldiacylglycerol is 200 μM , and that for cerebroside is 45 μM . Galactosyldiacylglycerol and cerebroside are mutually inhibitory toward the synthesis of sulphated derivatives of each. These data do not necessarily lead to the conclusion that two sulphotransferases are present, but they do indicate a possible means of controlling the synthesis of these two sulpholipids.

The galactosyldiacylglycerol (1,2-di-*O*-acyl-3-*O*- β -D-galactopyranosyl-*sn*-glycerol) of brain is closely associated with the process of myelination (Inoue *et al.*, 1971; Deshmukh *et al.*, 1971), and also occurs in brain as a sulphated derivative (Flynn *et al.*, 1975). Our initial studies on the biosynthesis of the sulphogalactosyldiacylglycerol *in vitro* indicated that galactosyldiacylglycerol functions as an acceptor of a sulphate moiety from adenosine 3'-phosphate 5'-sulphatophosphate in the presence of a microsomal preparation of rat brain (Flynn *et al.*, 1975). Investigations from other laboratories have shown that similar microsomal fractions of brain catalyse the transfer of sulphate from adenosine 3'-phosphate 5'-sulphatophosphate to a variety of water-soluble and lipid soluble acceptors (Cumar *et al.*, 1968; Farrel & McKhann, 1971; Nussbaum & Mandel, 1972; Farrel, 1974). Some of the data from these investigations suggest the possibility that more than one sulphotransferase may exist in this relatively crude source of enzyme. The results of our con-

tinued study on the biosynthesis of sulphogalactosyldiacylglycerol *in vitro* are reported here and show that this sulphotransferase reaction differs from other sulpholipid-biosynthetic reactions in several important parameters.

Experimental

Materials

Galactosyldiacylglycerol, galabiosyldiacylglycerol (galactosylgalactosyldiacylglycerol) and bovine cerebroside were bought from Analabs, New Haven, CT, U.S.A. Cholesterol was purchased from Eastman Organic Chemicals, Rochester, NY, U.S.A. The radioactive chemicals (adenosine 3'-phosphate 5'-[^{35}S]sulphatophosphate, NaB^3H_4 and carrier-free $H_2^{35}SO_4$) were bought from New England Nuclear Corp., Boston, MA, U.S.A. Amberlite XAD-4 was from Rohm and Haas, Philadelphia, PA, U.S.A. Adenosine 3',5'-bisphosphate and ATP were bought from P-L Biochemicals, Milwaukee, WI, U.S.A., and galactose oxidase was from Worthington Biochemical Corp., Freehold, NJ, U.S.A. All other chemicals used were of analytical grade.

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Methods

Preparation of ^3H -labelled galactosyldiacylglycerol. Galactosyldiacylglycerol was oxidized by galactose oxidase and subsequently reduced with NaB^3H_4 by the method described for lactosylceramide (Radin *et al.*, 1969). Galactosyldiacylglycerol (0.5 mg) was dissolved in 0.1 ml of tetrahydrofuran and incubated with 200 units of galactose oxidase (0.1 ml) in the presence of 0.1 ml of 5 mM-potassium phosphate buffer, pH 7.9, at 25°C for 4 h in a thermostatically controlled rotary shaker. The incubation was continued for 16 h after adding 100 units of galactose oxidase. At the end of the incubation, chloroform/methanol (2:1, v/v) and sufficient water to give a two-phase system were added. The oxidized galactosyldiacylglycerol was recovered from the chloroform phase. The oxidized galactosyldiacylglycerol was dissolved in 0.2 ml of tetrahydrofuran and reduced by adding 2 mCi of NaB^3H_4 dissolved in tetrahydrofuran. Complete reduction was achieved by addition of non-radioactive NaBH_4 (1.0 mg) solution in water. The reduced galactosyldiacylglycerol was purified by elution with acetone (100 ml) from a silicic acid column and by silica-gel t.l.c. developed with chloroform/methanol/water (65:25:4, by vol.).

Preparation of adenosine 3'-phosphate 5'-sulphatophosphate. Adenosine 3',5'-bisphosphate was sulphated chemically at the 5'-position as described by Baddiley & Sanderson (1963). Adenosine 3'-phosphate 5'-sulphatophosphate was purified by passing it through an Amberlite XAD-4 column (21 cm $\times$ 1 cm). Inorganic sulphate and other ions were removed by elution with 60 ml of triethylamine carbonate buffer, pH 7.6, and adenosine 3'-phosphate 5'-sulphatophosphate and adenosine 3',5'-bisphosphate were eluted with 60 ml of triethylamine carbonate buffer/methanol (1:1, v/v). Final purification of adenosine 3'-phosphate 5'-sulphatophosphate was carried out by paper chromatography with isobutyric acid/0.5 M- NH_3 (5:3, v/v) as the solvent system.

Preparation of enzyme and assay of sulphotransferase activity. Female Sprague-Dawley rats were killed at 21 days of age by cervical dislocation and the brains removed and homogenized in 0.32 M-sucrose. Preparation of enzyme and the assay of sulphotransferase activity were as described in the preceding paper (Pieringer *et al.*, 1977). The activities of galactosyldiacylglycerol sulphotransferase and cerebroside sulphotransferase were determined by measuring the radioactivity in the aqueous phase (for sulphogalactosyglycerolipid) and chloroform phase (for cerebroside sulphate) after deacylation.

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Radioactivity measurements were made in a Beckman LS-250 liquid scintillation spectrometer by

using the scintillation fluid containing 0.55% 2,5-diphenyloxazole and 0.0125% 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene in toluene/Triton X-100 (3:1, v/v).

Isolation of sulphogalactosyglycerolipid from brains. Sulphogalactosyglycerolipid was isolated from brains of 21-day-old rats injected with carrier-free $\text{H}_2^{35}\text{SO}_4$ as described by Flynn *et al.* (1975) and by Pieringer *et al.* (1977).

Analysis of adenosine 3'-phosphate 5'-[^{35}S]sulphatophosphate, adenosine 5'-[^{35}S]sulphatophosphate and $^{35}\text{SO}_4^{2-}$. The radioactive degradation products of adenosine 3'-phosphate 5'-[^{35}S]sulphatophosphate were analysed as follows. Portions of aqueous phase (derived from the sulphotransferase assay described above) were spotted on Whatman no. 1 paper and subjected to 2000 V, 75 mA, in a Camag electrophoresis apparatus in the presence of a pyridine/acetic acid/water (1:10:89, by vol.) buffer system for 30 min. The adenosine 3'-phosphate 5'-[^{35}S]sulphatophosphate, adenosine 5'-[^{35}S]sulphatophosphate and $^{35}\text{SO}_4^{2-}$ were detected with a strip scanner (Amersham-Searle), cut out and counted for radioactivity in a liquid-scintillation spectrometer.

Preparation of subcellular fractions of rat brain. The results of the adenosine 3'-phosphate 5'-sulphatophosphate-galactosyldiacylglycerol sulphotransferase activity in subcellular fractions recorded in the present paper were obtained from the same tissue fraction preparations used in a study reported in the preceding paper (Pieringer *et al.*, 1977).

Results

Subcellular localization of adenosine 3'-phosphate 5'-sulphatophosphate-galactosyldiacylglycerol sulphotransferase. The activity of the sulphotransferase was measured in different enriched fractions of rat brain. The microsomal fraction contained 79% of the total activity (Table 1). The remainder of the sulphotransferase activity was distributed in the small myelin and synaptic-vesicle (M_2) fractions. The activity in the latter fractions is probably caused by contamination with the microsomal preparation, since they are nearest-neighbour fractions to the microsomal fraction in the fractionation procedure, and they also possess the next highest activities (after the microsomal fraction) of the microsomal marker enzyme acetylcholinesterase (Pieringer *et al.*, 1977). The adenosine 3'-phosphate 5'-sulphatophosphate-galactosyldiacylglycerol sulphotransferase was not found in significant quantities in any other subfraction.

Optimal conditions for the assay of adenosine 3'-phosphate 5'-sulphatophosphate-galactosyldiacylglycerol sulphotransferase

The synthesis of sulphogalactosyldiacylglycerol increased with increasing amounts of enzyme up to

Table 1. Activity of adenosine 3'-phosphate 5'-sulphatophosphate-galactosyldiacylglycerol sulphotransferase in different subcellular fractions of rat brain

The activity of galactosyldiacylglycerol sulphotransferase was assayed by using 1% Triton X-100 extracts of different subcellular fractions as described in Pieringer *et al.* (1977). The incubation mixture consisted of 12.5 μ mol of imidazole/HCl buffer, pH 7.0, 1.0 μ mol of KF, 50 nmol of galactosyldiacylglycerol as a suspension in 1% Triton X-100 and 0.42 nmol of adenosine 3'-phosphate 5'-sulphatophosphate (250000 c.p.m.).

Fraction	Specific activity (pmol of sulphogalactosylglycerol formed/h per mg of protein)	Total activity (pmol of sulphogalactosylglycerol formed/h per g wet wt. of brain)
Crude nuclear		
Large myelin	0.51	1.21
Nuclei	0	0
Cell debris	0.09	0.53
Crude mitochondrial		
M ₁ -A, small myelin	3.74	24.9
M ₁ -B, synaptic membrane	0	0
M ₁ -C, mitochondria	0	0
M ₂ , synaptic vesicles and membrane	16.1	44.8
M ₃ , soluble	0	0
Microsomal fraction	23.3	270
Supernatant	0	0

Fig. 1. Effect of Triton X-100 on the activity of galactosyldiacylglycerol sulphotransferase

Freeze-dried microsomal powders were used as the enzyme source. Galactosyldiacylglycerol (50 nmol) was added as a solution in benzene and the solvent was evaporated. Triton X-100 (0.025 ml) of different concentrations was added and the activity of sulphotransferase was assayed by using 120–140 μ g of enzyme protein and 0.02 nmol of adenosine 3'-phosphate 5'-[³⁵S]sulphatophosphate (200000 c.p.m.). The assay mixture consisted of 12.5 μ mol of imidazole/HCl buffer, pH 7.9, 50 nmol of galactosyldiacylglycerol, 0.02 nmol of adenosine 3'-phosphate 5'-sulphatophosphate (200000 c.p.m.) and the enzyme (120–140 μ g of protein), with the specified concentration of Triton X-100, in a final volume of 0.15 ml. The incubation was carried out at 37°C for 10 min and the activity was measured from the radioactivity incorporated into deacylatable lipid sulphate. Average values from duplicate samples of a typical experiment were plotted. The concentrations of Triton X-100 in the final incubation volume are given in the Figure.

Fig. 2. Effect of increasing concentrations of galactosyldiacylglycerol and adenosine 3'-phosphate 5'-sulphatophosphate on the activity of sulphotransferase

The incubation mixture consisted of 12.5 μ mol of imidazole/HCl buffer, pH 7.0, 1.0 μ mol of KF, 0.025 ml of 1% Triton X-100, adenosine 3'-phosphate 5'-[³⁵S]sulphatophosphate and galactosyldiacylglycerol in a final volume of 0.15 ml. ○, Activity with increasing concentrations of adenosine 3'-phosphate 5'-sulphatophosphate (650000 c.p.m./nmol) and 50 nmol of galactosyldiacylglycerol; ●, activity with increasing concentrations of galactosyldiacylglycerol and 0.04 nmol (380000 c.p.m.) of adenosine 3'-phosphate 5'-sulphatophosphate. Other details are as described in Fig. 1.

160 μ g of protein and was stimulated by Triton X-100. The optimal final concentration of this non-ionic detergent was 0.4% (Fig. 1). The enzymic reaction was proportionally enhanced over a reasonable range by increasing amounts of the

substrates, galactosyldiacylglycerol and adenosine 3'-phosphate 5'-sulphatophosphate (Fig. 2). The apparent K_m values calculated from Lineweaver-Burk plots of these data were $208\ \mu\text{M}$ for galactosyldiacylglycerol and $77\ \mu\text{M}$ for adenosine 3'-phosphate 5'-sulphatophosphate. When cerebroside was substituted for galactosyldiacylglycerol the apparent K_m for this sphingoglycolipid was $45\ \mu\text{M}$ (results not shown). The latter value agrees well with the previously published K_m values for cerebroside of $33\text{--}85\ \mu\text{M}$ (Cumar *et al.*, 1968) or $80\ \mu\text{M}$ (Farrel & McKhann, 1971). The pH optimum of the sulphogalactosyldiacylglycerol-synthesizing enzyme was 7.0 and the reaction proceeded at a linear rate for up to 10 min (Fig. 3). The relative brevity of the linear portion of the time-curve suggested the possibility that competitive enzymes in the crude microsomal extract were acting on the substrates and/or product. KF and ATP were tested as potential deterrents of these competitive enzymes, and were found to have profound effects on the sulphotransferase reactions (Fig. 3). ATP significantly inhibited the reaction, but prolonged the linearity of the reaction for up to 1 h. KF had a very positive effect on prolonging the linearity of the reaction and brought about a maximal conversion of substrates into sulphogalactosyldiacylglycerol when the reaction was allowed to continue for 1 h.

Effect of ATP and KF on the degradation of adenosine 3'-phosphate 5'-sulphatophosphate

Adenosine 3'-phosphate 5'-[^{35}S]sulphatophosphate was rapidly expended the absence of ATP and

Fig. 3. Time-course of the activity of galactosyldiacylglycerol sulphotransferase

The assay mixture is the same as given in Fig. 1 with $127\ \mu\text{g}$ of enzyme protein and $0.068\ \text{nmol}$ of adenosine 3'-phosphate 5'-[^{35}S]sulphatophosphate ($340\,000\ \text{c.p.m.}$) and the total volume was $0.13\ \text{ml}$. ATP (neutralized with NaHCO_3) and KF were added as solutions in water. ●, No ATP or KF; ○, $1.0\ \mu\text{mol}$ of KF; ■, $1.0\ \mu\text{mol}$ of ATP. Averages from duplicates are plotted.

KF during the synthesis of sulphogalactosyldiacylglycerol (Fig. 4a). It is converted into a radioactive intermediate, believed to be adenosine 5'-sulphatophosphate, which in turn is further degraded to inorganic $^{35}\text{SO}_4^{2-}$ (Fig. 4a). In the presence of either KF or ATP the degradation of adenosine 3'-phosphate 5'-[^{35}S]sulphatophosphate is inhibited (Figs. 4b and 4c). ATP (Fig. 4c) preserves the concentration of adenosine 3'-phosphate 5'-sulphatophosphate better than KF (Fig. 4b). With ATP present, production of $^{35}\text{SO}_4^{2-}$ and adenosine 5'-[^{35}S]sulphatophosphate occurs to the same extent (Fig. 4c), but with KF the degradation product in highest concentration is $^{35}\text{SO}_4^{2-}$ (Fig. 4b). These data suggest that KF exerts its inhibition of adenosine 3'-phosphate 5'-sulphatophosphate degradation mainly by inhibiting phosphohydrolase activity, whereas ATP affects both phosphohydrolase and sulphohydrolase activities. The inhibition of adenosine 3'-phosphate 5'-sulphatophosphate breakdown by KF has been studied with

Fig. 4. Effect of ATP and KF on the degradation of adenosine 3'-phosphate 5'-sulphatophosphate

The radioactivity in adenosine 5'-[^{35}S]sulphatophosphate, adenosine 3'-phosphate, 5'-[^{35}S]sulphatophosphate and inorganic $^{35}\text{SO}_4^{2-}$ was measured as described under Methods in the aqueous phase of samples obtained in the time-course experiment (Fig. 3). The percentages of total radioactivity at various intervals of time are plotted. ○, Adenosine 3'-phosphate 5'-[^{35}S]sulphatophosphate; ●, adenosine 5'-[^{35}S]sulphatophosphate; ▲, inorganic $^{35}\text{SO}_4^{2-}$. (a) No ATP or KF; (b) $1.0\ \mu\text{mol}$ of KF; (c) $1.0\ \mu\text{mol}$ of ATP.

Table 2. *Effect of ATP, Mg²⁺ and KF on the activity of sulphotransferase*

The activity of sulphotransferase was assayed as described in Pieringer *et al.* (1977). The substrate (galactosyldiacylglycerol or bovine cerebroside) was dispersed in 0.025 ml of 1% Triton X-100. The enzyme preparation used in different experiments contained 120–135 μ g of protein and 0.04–0.06 nmol of adenosine 3'-phosphate 5'-[³⁵S]sulphatophosphate (200000–350000 c.p.m.). The final volume of incubation was 0.2 ml.

Expt.	Substrate	Compound added	Concentration (μ mol)	Activity (pmol of product formed/h per mg of protein)
I A	Galactosyldiacylglycerol	ATP	0	15.1
			1.0	6.3
			2.5	3.2
		Mg ²⁺	0	14.8
			2.5	8.3
			5.0	7.2
C		ATP+Mg ²⁺	0	8.0
			1.0+5.0	3.3
II A	Cerebroside	ATP+Mg ²⁺	0	15.3
			5.0+1.0	33.8
		ATP+Mg ²⁺	0	8.9
			1.0+5.0	15.6

a sheep brain preparation by Farooqui & Bala-subramanian (1969).

Opposite effects of ATP and Mg²⁺ on sulphogalactosyldiacylglycerol and cerebroside sulphate synthesis

In the cerebroside sulphate-synthesizing system, the presence of ATP was found by other investigators to have a positive overall effect on the reaction (Cumar *et al.*, 1968; Farrel & McKhann, 1971; Sarlieve *et al.*, 1976). As noted in Fig. 4, ATP has a strong inhibiting effect on the adenosine 3'-phosphate 5'-sulphatophosphate–galactosyldiacylglycerol sulphotransferase, and this inhibition is proportional to the concentration of ATP (Table 2). Mg²⁺ also has a stimulatory effect on cerebroside sulphate formation (Cumar *et al.*, 1968; Farrel & McKhann, 1971; Sarlieve *et al.*, 1976). However, the synthesis of sulphogalactosyldiacylglycerol is markedly inhibited. At 5 μ mol of Mg²⁺, Farrel & McKhann (1971) and Sarlieve *et al.* (1976) found that cerebroside sulphate synthesis was stimulated, whereas this concentration of Mg²⁺ inhibited the sulphogalactosyldiacylglycerol-synthesizing enzyme by more than 50% (Table 2). Our conditions of incubation are similar to those used by other laboratories, and we also observe a stimulation of cerebroside sulphate formation by ATP and Mg²⁺ (Table 2). Yet a simple substitution of galactosyldiacylglycerol for cerebroside brings about a completely opposite effect by ATP and Mg²⁺.

Competition between cerebroside and galactosyldiacylglycerol as substrates

The synthesis of cerebroside sulphate is inhibited by galactosyldiacylglycerol and the synthesis of

sulphogalactosyldiacylglycerol is inhibited by cerebroside (Figs. 5a and 5b). The two substrates were never observed to produce an additive effect.

Substrate specificity

The enzyme preparation used in these studies sulphated galactosyldiacylglycerol (15.5 pmol/h per mg above no-sulphate-acceptor control) and cerebroside (10.06 pmol/h per mg above control), but did not sulphate cholesterol (0.5 pmol/h per mg) or galabiosyldiacylglycerol (0.0 pmol/h per mg) to any significant extent.

Identification of sulphogalactosyldiacylglycerol synthesized in vitro

When [6-³H]galactosyldiacylglycerol was incubated with non-radioactive adenosine 3'-phosphate 5'-sulphatophosphate in the presence of the usual Triton X-100 extract of a brain microsomal preparation, a ³H-labelled lipid product was formed which chromatographed with [³⁵S]sulphogalactosyglycerolipid (generated *in vivo* by injecting H₂³⁵SO₄ intracerebrally) on thin layers of silica gel (developed with chloroform/methanol/water, 65:25:4, by vol.) (Fig. 6). The sulphogalactosyglycerolipid synthesized *in vivo* by rat brain and used as a standard here exists as an equimolar mixture of a diacyl (Flynn *et al.*, 1975; Pieringer *et al.*, 1977) and an alkylacyl (Levine *et al.*, 1975) derivative of sulphogalactosyglycerol. The incorporation of radioactivity from [³H]galactosyldiacylglycerol into a lipid product not only aids the characterization of the biosynthesized lipid as sulphogalactosyldiacylglycerol, but also establishes galactosyldiacylglycerol as a true lipid substrate in

Fig. 5. Competition between galactosyldiacylglycerol and cerebroside for the sulphotransferase enzyme

The assay mixture consisted of the substrates (of specified concentrations) dispersed in 0.025 ml of 1% Triton X-100, 12.5 μ mol of imidazole/HCl buffer, pH 7.0, 0.084 nmol of adenosine 3'-phosphate 5'-[35 S]sulphatophosphate (220 000 c.p.m.) and the enzyme (130 μ g of protein) in a total volume of 0.15 ml. The amounts of cerebroside sulphate and sulphogalactosyldiacylglycerol formed were calculated from the radioactivity incorporated into non-deacylatable and deacylatable lipid sulphates respectively. ○, Cerebroside sulphate; ●, sulphogalactosyldiacylglycerol. (a) Effect of increasing concentrations of galactosyldiacylglycerol in the presence of 50 nmol of bovine cerebroside. (b) Effect of increasing concentrations of cerebroside in the presence of 50 nmol of galactosyldiacylglycerol.

the reaction. The stimulation of the reaction by added exogenous galactosyldiacylglycerol is due to its accepting a sulphate group from adenosine 3'-phosphate 5'-sulphatophosphate rather than its functioning only as a detergent. The lipid product formed from adenosine 3'-phosphate 5'-[35 S]sulphatophosphate and galactosyldiacylglycerol was further characterized as sulphogalactosyldiacylglycerol by co-electrophoresis of the water-soluble deacylated 35 S product from the reaction *in vitro* and standard 3'-sulphogalactosylglycerol. Both standard and unknown ran 10 cm from the origin on Schleicher and Schuell no. 2040-B paper subjected to 2000 V for 30 min in pyridine/acetic acid/water (1:10:89, by vol.). These results indicate that little or no sulphogalactosylalkylacylglycerol was synthesized during the incubation.

Fig. 6. Co-chromatography of sulpho[3 H]galactosyldiacylglycerol synthesized *in vitro* with [35 S]sulphogalactosylglycerolipid isolated *in vivo*

Sulpho[3 H]galactosyldiacylglycerol synthesized *in vitro* as described under 'Methods' and [35 S]sulphogalactosylglycerolipid isolated *in vivo* were mixed and spotted on thin-layer plates of silica gel (0.25 mm thick). The chromatogram was run in chloroform/methanol/water (65:25:4, by vol.) and the radioactivity in 1 cm strips from the origin of the solvent front was determined and plotted. ○, 35 S; ●, 3 H.

Discussion

A number of properties of the enzymic synthesis of sulphogalactosyldiacylglycerol by brain preparations reported in this paper are similar to those found by others for the synthesis of cerebroside sulphate (Cumar *et al.*, 1968; Farrel & McKhann, 1971; Sarlieve *et al.*, 1976). Included among these are subcellular site of synthesis, extractability of enzyme from a microsomal preparation into aqueous detergent solution and pH optimum. However, we have also noted significant differences between the above two reactions. Both ATP and Mg^{2+} markedly stimulate the synthesis of cerebroside sulphate, but strongly inhibit the synthesis of sulphogalactosyldiacylglycerol. The apparent K_m of galactosyldiacylglycerol is 200 μ M and that for cerebroside is 45 μ M. These differences might indicate the presence of two enzymes. However, the addition of galactosyldiacylglycerol inhibits the synthesis of cerebroside sulphate, and cerebroside inhibits the formation of sulphogalactosyldiacylglycerol. Although this mutual inhibition does not rule out two enzymes, an additive effect by each substrate would have been more indicative of two different sulphotransferases. A more meaningful conclusion of this study is that these differences demonstrate possible mechanisms by which the synthesis of the two sulpholipids might be controlled *in vivo*.

The synthesis by a testicular enzyme of sulphogalactosylalkylacylglycerol from galactosylalkylacylglycerol and adenosine 3'-phosphate 5'-sulphatophosphate was also inhibited by ATP and also did not show an additive effect by galactosylalkylacylglycerol and cerebroside (Handa *et al.*, 1974).

Although ATP has an inhibitory effect on the overall synthesis of sulphogalactosyldiacylglycerol of brain, it did prolong the linearity of the synthesis with time. KF also prolonged the linearity of the reaction, but unlike ATP it did not inhibit the sulphotransferase. Therefore maximum sulphation of galactosyldiacylglycerol could be achieved by incubation for at least 1 h in the presence of KF. Both KF and ATP markedly inhibit the enzymic breakdown of adenosine 3'-phosphate 5'-sulphatophosphate. The preservation of adenosine 3'-phosphate 5'-sulphatophosphate undoubtedly is the means by which these two compounds prolong the linearity of the adenosine 3'-phosphate 5'-sulphatophosphate-galactosyldiacylglycerol sulphotransferase with time.

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