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SULPHATE METABOLISM IN ACUTE EAE[1] RATS USING ISOLATED BRAIN PERFUSION TECHNIQUE

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Abstract—Metabolism of glycolipids and glycosaminoglycans were studied in rats in the acute stage of experimental allergic encephalomyelitis (EAE) using isolated brain perfusion technique. It was observed that there was a significant decrease in the concentration of cerebroside, sulphatide and GAG (hyaluronic acid and low sulphated GAG) when compared to normal and pairfed control rats. The radioactive sulphate incorporation into the cerebroside sulphate and sulphated GAG was significantly higher in the case of rats in the acute stage of EAE than the normal and pairfed control rats.

EXPERIMENTAL allergic encephalomyelitis (EAE) is a laboratory model of a demyelinating disease produced in animals and it has aroused considerable interest because of its close resemblance to certain demyelinating human diseases. This is an extremely suitable condition where the possible role of myelin constituents during the demyelination and remyelination of brain can best be followed. A number of studies have shown that EAE can be induced in animals by various types of neural preparations.

Biochemical studies have so far been focused on the [^{14}C]glucose incorporation into the various fractions of the total lipids in EAE using tissue slices of brain stem. SMITH (1965) showed high incorporation of [^{14}C]glucose into the phospholipids plus galactolipid fraction, while cholesterol synthesis was unchanged or slightly lower.

Studies by various investigators have emphasised the importance of sulphatide in the formation of myelin. It has been shown that there was an increase in the synthesis of sulphatide and a higher incorporation of [^{35}S]sulphate into the sulphatide during the peak period of myelination (DAVISON and GREGSON, 1962 and 1966; BALASUBRAMANIAN and BACHHAWAT 1965; MCKHANN, LEVY and HO, 1965). DAVISON and GREGSON (1962) have also shown that there is a very slow turnover of sulphatide in the adult brain.

So far no report has been made on the metabolism of glycosaminoglycans (GAGs) during the acute stage of EAE. Studies on GAG in the central nervous system indicated that there was an increased synthesis of sulphated GAG during the premyelination period and also the incorporation of [^{35}S]sulphate was high (GUHA, NORTHOVER and BACHHAWAT, 1960). Recently SINGH (1967) has shown that the sulphated-GAG reaches a peak prior to myelination and remains at a plateau during the peak period of myelination.

In view of this important role of sulphatide and sulphated GAG in myelin formation, a study was undertaken to investigate the metabolism of these compounds in the acute stages of EAE. The isolated brain preparation technique which has been developed in recent years (ANDJUS, SUHARA and SLOVITER, 1967; VASAN, J. ABRAHAM and BACHHAWAT, 1969) was adopted for this investigation.

Abbreviations used: EAE, experimental allergic encephalomyelitis; GAG, glycosaminoglycans; TLC, thin layer chromatography.

MATERIALS AND METHODS

Adsorbents for chromatography included Florisil, 60–100 mesh (Floridin Co., Florida, U.S.A.), Silica Gel G (E. Merck, A. G. Darmstadt, Germany), DEAE-cellulose (Carl Schleicher and Schuell and Co., New Hampshire, U.S.A.). DEAE-Sephadex A-25 was supplied by Pharmacia-Upsala, Sweden. Other chemicals were of analytical grade. Radioactive sulphate was obtained from the Atomic Energy Establishment, Trombay, Bombay, India. Freunds adjuvant (complete) purchased from Difco Laboratories, Detroit, Michigan, U.S.A.

The radioactivity was measured in a liquid scintillation counter (Beckman Model L.S. 100) using Toluene-PPO (2,5-diphenyloxazole)–POPOP (1,4-bis-2 (5-phenyloxazolyl)-benzene)–naphthaline mix (Toluene 100 ml; PPO, 0·05 g; POPOP, 1 g and naphthaline, 20 g). The aliquots of lipid samples were first evaporated in the counting vials and the scintillation mixture was added and they were then counted. The water soluble GAG samples were spotted on a 1·5 sq. cm filter paper dried and immersed in the scintillation mixture in the vials and counted.

Induction of EAE in rats. Adult male rats of about 250 g body wt. were injected intradermally with a 33% homogenate of guinea pig spinal cord, emulsified in an equal volume of Freunds adjuvant and containing a final phenol concentration of 0·25% (LEVINE and WENK, 1961). In earlier studies it was observed that when controls were injected with Freunds adjuvant alone, there was not any significant change in the glucose uptake, lactic acid production and [^{35}S]sulphate incorporation compared to that of normal rats. Therefore in the present study Freunds adjuvant-injected control rats were not employed.

The animals were judged to be in the acute stage of EAE when complete paralysis of hind limbs occurred, usually 12–16 days after injection. During the acute stage of EAE the common clinical symptoms—urinary incontinence, limping, loss of body weight, loss of appetite and weakness—were observed in addition to the paralysis of hind limbs. It was observed that the onset of EAE was accompanied by a decrease in food intake and loss of body weight of the rat. In order to study the effect of this decreased food intake and inanition on the metabolic studies, control rats ('Pair-fed'), which were given the same quantity of food as consumed by the EAE rats, were maintained and showed almost the same degree of weight loss. These 'Pair-fed' rats were used as controls throughout these studies.

Isolated brain preparation. The isolated brain preparation technique as described by ANDJUS *et al.* (1967) and standardised in this laboratory was adopted for this study. The rats were anaesthetised by hypothermia under combined hypoxia and hypercapnia as described elsewhere (VASAN *et al.*, 1969).

The perfusion fluid. The perfusion fluid or 'simplified blood' consisted of washed red cells of goat, bovine serum albumin (deionised) and Krebs–Ringers saline, to give a final haematocrit value of 20–25 per cent, a serum albumin concentration of 7–8% and pH 7·35. For [^{35}S]sulphate incorporation experiments the $MgSO_4$ was replaced with $MgCl_2$ in the original Krebs–Ringers saline. The electrophysiological and glucose uptake studies with this modified Krebs–Ringers saline showed no difference from the experiments where original Krebs–Ringer saline was used. Radioactive sulphate (2 mCi/25 ml) was added to the perfusion fluid. Glucose (2 mg/ml) was added initially to the perfusion fluid. Blood samples were collected every 30 min to estimate the glucose (SOMOGYI, 1945) and lactic acid (BARKER and SUMMERSON, 1947) levels.

Extraction of glycolipids. After 2 h of perfusion the brain was removed and homogenised in 19 vol. of chloroform–methanol (2:1, v/v) and filtered. The clear filtrate was shaken thoroughly with 0·2 vol. of 0·74% KCl. The lower phase was washed three times with theoretical upper phase containing 0·37% KCl. The washed lower phase was evaporated under vaccum at 40°C, dissolved in 3 ml of chloroform–methanol (2:1 v/v) and passed through a column of 4 g florisil. The cerebroside and sulphatide were eluted from the column using 100 ml of chloroform–methanol (7:3 v/v) containing 5% 2,2-dimethoxy propane (RADIN, LEVIN and BROWN, 1955). This fraction was concentrated and a portion was used for the quantitative estimation of galactolipid using anthrone reagent (RADIN, LEVIN and BROWN, 1963). Another portion was used for TLC and radioactive counting. Further fractionation of cerebroside and sulphatide was carried out on a DEAE-cellulose acetate column. The cerebroside fraction was eluted with chloroform–methanol (7:1 v/v) and sulphatide using chloroform–methanol (4:1, v/v) containing 1% ammonia (ROUSER, BAUMAN, KRITCHEWSKY, HELLER and O'BRIEN, 1961). The fraction was concentrated and quantitative assays of cerebroside and sulphatide were carried out. The homogeniety of the cerebroside and sulphatide was checked on a TLC plate. The TLC was done following the procedure as described by TAORI, MATHEW, BAKTHARIZIAM and BACHHAWAT (1969).

Isolation of GAG. The GAG was extracted from the lipid free brain residue as described by SINGH and BACHHAWAT (1968). The GAG was precipitated by cetylpyridium bromide and fractionated on a DEAE-Sephadex A-25 column. The different fractions of GAG were eluted using 0·5 M; 0·9 M and 1·25 M-NaCl in 0·01 M-HCl and designated fractions I, II and III respectively. It may be

mentioned here that because of the small amount of sample available, fractions I, II and III were not further characterised. However, earlier work from this laboratory has shown that fraction I, mainly consists of hyaluronic acid; fraction II, a mixture of low sulphated GAG and heparan sulphate and fraction III, higher sulphated GAG (SCHMIDT, 1962).

Constituent analysis. Uronic acid was determined by Dische's-Carbazole method (DISCHE, 1947) as modified by BITTER and MUIR (1962) using glucuronolactone as the standard.

RESULTS

Body weight curve. As shown in Fig. 1 there was a sudden drop of body weight of EAE rats from the day of the onset of clinical symptoms, to the stage of complete hind limb paralysis, which occurs 15–18 days after the injection. The decline in body weight coincided with the period of decreased food intake. Rats that are allowed to recover continue to lose weight up to 20–22 days and then gradually gain weight with a

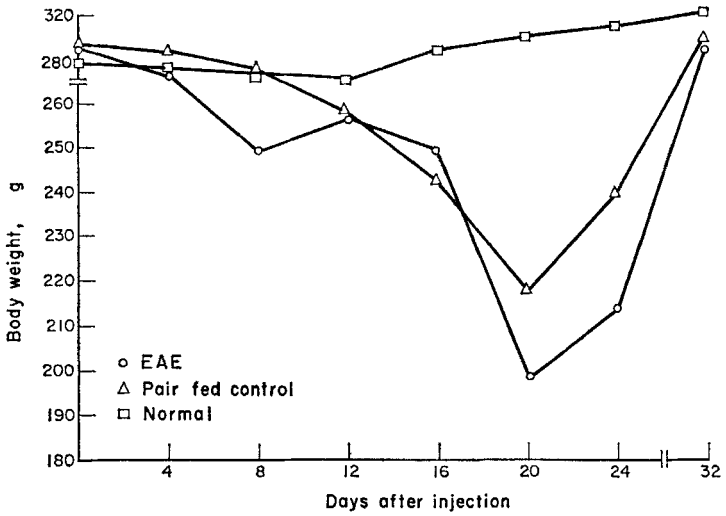

FIG. 1.—Body weight curve: Body weight were recorded from the day of induction of EAE to the stage of recovery. 'Pairfed controls' were maintained as described in the text.

gradual increase in food intake and the clinical symptoms disappear by 28 days. The pair-fed controls also exhibited a similar decrease in the body weight due to decrease in the food intake.

Glucose uptake studies. The glucose uptake studies are one of the routine parameters adopted by us to assess the viability of the isolated brain preparation. The glucose utilization by the isolated perfused brain preparations of normal, pair-fed control and EAE rats are depicted in Fig. 2. The glucose utilization was measured by the decrease in glucose concentration in the perfusion fluid. The isolated brain preparations were viable during the 2 h of perfusion, as shown by the fact that there had been glucose uptake. However, glucose uptake decreased in the EAE rat brain when compared with the control. After 2 h of perfusion the glucose uptakes by the normal, pair-fed and EAE brains were 3·00, 3·17 and 1·70 μmol respectively.

Lactic acid formation. There was an increase in lactic acid formation by the EAE rat brain compared to normal and pair-fed control as measured by an increase in concentration in the perfusion medium. The amounts of lactic acid formed during the

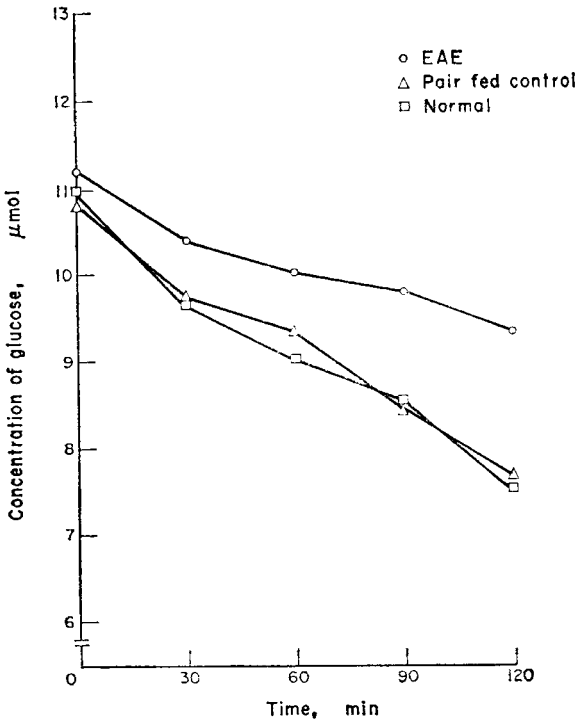

FIG. 2.—Glucose concentration: Blood samples were drawn at 30 min intervals and glucose analysis was done as described.

course of 2 hours of perfusion were 3·99, 3·55 and 4·57 μmol by the normal, pair-fed control and EAE rats respectively (Fig. 3).

Cerebroside and sulphatide metabolism in acute stage of EAE. The concentration of galactolipid in normal, pair-fed control and EAE rat brain is shown in Table 1. There was a striking decrease in the level of cerebroside and sulphatide in the case of rats in the acute stage of EAE and the decrease was about 60–65 per cent of the control value. There was no difference in the cerebroside and sulphatide levels between the normal and pairfed controls. The ratio of sulphatide:cerebroside was slightly decreased in the case of EAE compared to control rats, showing that the decrease in the sulphatide concentration was more marked than that of cerebroside. The TLC pattern of cerebroside and sulphatide isolated from EAE rat brain were similar to that of the control. The incorporation studies with isolated brain perfusion showed that the [^{35}S]sulphate incorporation into the glycolipid fraction in EAE rats was very high compared to that in control rats whereas there was no difference in the incorporation between the normal and pair-fed control (Table 1). The sulphated compound was identified as cerebroside sulphate by means of TLC and radioautography.

Metabolism og GAG in acute stage of EAE. As indicated in Table 2, compared to normal and pair-fed control, the GAG concentration decreased by 50–60 per cent in the acute stage of EAE.

The further fractionation on DEAE-Sephadex column shows that the fractions I and II were decreased to the same extent while fraction III was not much affected.

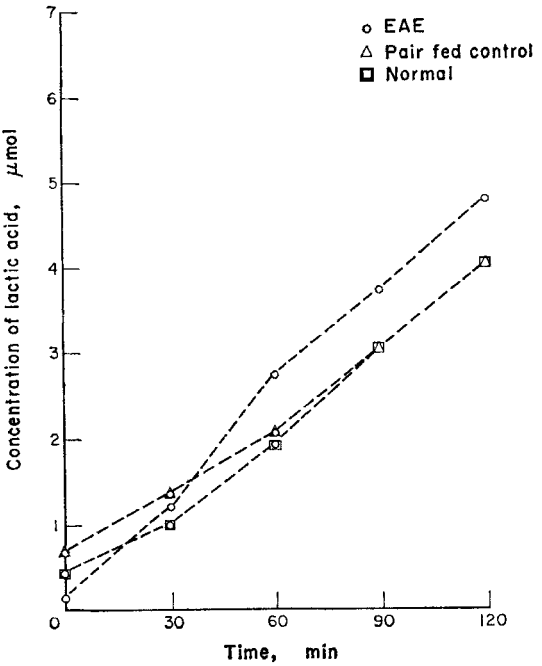

FIG. 3.—Lactic acid production: Increase in the concentration of lactic acid in the perfusion fluid at different intervals was estimated as given in Methods.

TABLE 1.—CONCENTRATION OF CEREBROSIDE, SULPHATIDE AND THE [^{35}S]SULPHATE INCORPORATION INTO SULPHATIDE IN THE WHOLE BRAIN

	Cerebroside (mg/g dry defatted tissue)	Sulphatide (mg/g dry defatted tissue)	Sulphatide:cerebroside ratio	c.p.m./mg sulphatide
Normal (6)	75·1 ± 7·7	23·8 ± 2·9	1:3·2	109 ± 8
Pairfed control (6)	75·1 ± 2·3	24·5 ± 2·1	1:3·1	124 ± 24
EAE (5)	34·3 ± 2·7	8·6 ± 1·9	1:4·1	3494 ± 220

Values are expressed as the mean ± S.E.M.
The number in brackets shows the number of experiments done.

The low sulphated GAG (Fraction II) was always slightly higher than hyaluronic acid (Fraction I) giving a similar ratio to that in normal adult rat brain (Table 3).

The radioactive sulphate incorporation into the total GAG is shown in Table 2. There was a very high incorporation in the case of EAE rats when compared to the control. This GAG when fractionated and eluted with 0·9 M-NaCl from the DEAE-Sephadex column contained the highest amount of radioactivity (Table 4).

DISCUSSION

The considerable interest in the metabolic studies of EAE has aroused in recent years the hope of better understanding the mechanism underlying such disturbances. The rat is an ideal subject for experimental studies of this nature because unlike the mouse and guinea pig it seldom dies from EAE and the genetic uniformity can be controlled.

The isolated brain perfusion technique adopted in the present study has advantages

TABLE 2.—CONCENTRATION OF TOTAL GLYCOSAMINOGLYCANS AND [^{35}S]SULPHATE INCORPORATION INTO GLYCOSAMINOGLYCANS IN THE WHOLE BRAIN

	Concentration μg/g dry defatted tissue	c.p.m./g dry defatted tissue $\times 10^{-3}$	c.p.m./μg uronic acid
Normal (4)	1380 ± 60	43·3 ± 0·1	31·4 ± 1·3
Pairfed control (6)	1283 ± 32	43·9 ± 3·7	34·2 ± 2·7
EAE (9)	638 ± 83	183·5 ± 44·5	269·2 ± 47·1

Values are expressed as the mean ± S.E.M.
The number in brackets shows the number of experiments done.

TABLE 3.—CONCENTRATION OF DIFFERENT GLYCOSAMINOGLYCANS FRACTIONS ISOLATED BY DEAE-SEPHADEX CHROMATOGRAPHY

	μg/g dry defatted tissue			Total
	Fraction I	Fraction II	Fraction III	
Normal (3)	563 ± 17	739 ± 51	78 ± 8	1380 ± 60
Pairfed control (3)	533 ± 34	667 ± 13	88 ± 9	1288 ± 37
EAE (3)	332 ± 20	356 ± 22	70 ± 4	758 ± 17

Values are expressed as the mean ± S.E.M.
The number in brackets shows the number of experiments done.
Glycosaminoglycan fractions were eluted from DEAE-Sephadex A-25 column by using 0·5 M; 0·9 M NaCl and 1·25 M NaCl in 0·01 M HCl and designated as Fraction I, II and III respectively.

TABLE 4.—INCORPORATION OF [^{35}S]SULPHATE INTO DIFFERENT FRACTIONS OF GLYCOSAMINOGLYCANS ISOLATED BY DEAE-SEPHADEX CHROMATOGRAPHY

	c.p.m./g dry defatted tissue $\times 10^{-3}$		
	Fraction I	Fraction II	Fraction III
Normal (3)	12·2 ± 0·4	23·9 ± 4·1	1·9 ± 0·1
Pairfed control (3)	9·9 ± 3·2	22·1 ± 8·4	3·5 ± 1·3
EAE (3)	4·9 ± 1·6	82·1 ± 4·9	3·3 ± 0·6

Values are expressed as the mean ± S.E.M.
The number in brackets shows the number of experiments done.
Fractions I, II and III were obtained as described in Table 3.

over the classical tissue slices and homogenate techniques in that it is more physiological because the brain is maintained viable. Moreover, the blood–brain barrier which is a physiological phenomenon is undisrupted. In addition, the influence of other organs does not come into play. The rats were anaesthetised by hypothermia, thereby eliminating the possible effects of chemical anaesthetics. The viability of the isolated brain preparation is monitored by the glucose uptake and lactic acid studies and the maintenance of electroencephalograms.

There was a decrease in the utilization of glucose and slightly higher lactic production in the acute stage of EAE. This is not unexpected because there may be mitochondrial damage in the acute state of EAE, as shown by the electronmicroscopic studies of ROIZIN (1964) and by the finding that the tricarboxylic cycle enzymes located in the mitochondria are also affected (SMITH 1966). SMITH (1965) using tissue slices study has reported that the EAE rat brain stem utilises as much glucose as the normal rat brain and its lactic acid formation was higher. The decreased uptake of glucose

by EAE brain observed by us, may be due to the difference in the technique employed and also to the fact that we have used whole brain instead of brain stem.

The destruction of myelin during the acute stage of EAE has been shown by BUBIS and LUSE (1964), ROIZIN (1959) and LAMPERT (1965) in the central nervous system. ROIZIN (1959) has reported that the process of demyelination is gradually followed by myelinolysis which in turn followed by lipolysis.

The decrease in the cerebroside and sulphatide level in EAE may be an effect of this myelinolipolysis. There is no difference in the concentration of cerebroside and sulphatide between the normal and pair-fed control indicating that the observed decrease in the case EAE rats is not due to inanition. This is in agreement with the findings of SMITH (1963) who reported that inanition did not affect the cerebral lipids in adult rats where lipids of central nervous system have largely a structural function and are not affected by starvation (FOLCH, 1947). BUNGE, BUNGE and RIS, (1961), BUBIS and LUSE (1964) and LAMPERT (1965) have reported that there is remyelination also taking place in central nervous system during acute stage of EAE. The high incorporation of [^{35}S]sulphate into the sulphatide during the acute stage of EAE (Table 1) suggest that the process of remyelination and perhaps tissue repair is taking place during this period. This is in agreement with the findings of SMITH (1965) that the maximum [^{14}C]glucose incorporation into the galactolipid occurs during the acute stage of EAE. The mechanism of remyelination was considered by BUNGE *et al.* (1961) to be similar to that of the initial myelination. In some of the above cases remyelination was concurrent with clinical improvement in the affected anaimals. Recently SMITH (1969) reported that there is an increased incorporation of [^{14}C]glucose into the lipids of brain homogenate from EAE rats as compared to normal and that this increased synthesis of this lipid might be occurring at a subcellular locus outside myelin sheath.

The studies on the effect of EAE on the GAG concentration, as well as [^{35}S]sulphate incorporation into the GAG, show certain interesting results. These results agree well with the report of BUNGE *et al.* (1961) that the mechanism of remyelination is similar to that of the initial myelination and also support the suggestion made by BRANTE (1958) as to the possible role of GAG in the process of myelination. The high rate of [^{35}S]sulphate incorporation in the fraction which may contain heparan sulphate and the low sulphated GAG (0·9 M-NaCl fraction) confirms the view reported earlier from this laboratory (GUHA *et al.*, 1960; SINGH and BACHHAWAT, 1968; BALASUBRAMANIAN and BACHHAWAT, 1964) GEORGE, SINGH and BACHHAWAT, 1970) that there exists a close relationship between the sulphated GAG and myelin formation. However, MARGOLIS (1967) has reported that there is no GAG in myelin.

It may be mentioned here that besides demyelination the EAE rat brain shows other changes such as mitochondrial damage etc. (ROIZIN *et al.*, 1964). In view of these facts it is too early to define the role of GAG in the myelination from the present data available.

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