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ENZYMIC STUDIES ON SULPHATIDE METABOLISM IN DIFFERENT STAGES OF EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS

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Abstract—The activities of three enzymes—cerebroside sulphotransferase, 3′-phosphoadenosine 5′-phosphosulphate synthesizing enzyme and arylsulphatases A and B have been studied in various developmental and recovery stages of experimental allergic encephalomyelitis. The concentrations of cerebroside and sulphatide were also analysed during these stages. It was observed that the sulphatide concentration decreased during the development of the disease, with a concurrent increase in the activity of arylsulphatase and vice versa during the recovery stages. 3′-Phosphoadenosine 5′-phosphosulphate synthesis as well as sulphotransferase activity increased during the pre-acute stage of the disease, reached a maximum at the acute stage and decreased during recovery stages.

IN OUR earlier studies with isolated rat brain preparations we have shown that there is a marked decrease in the concentration of glycolipids as well as a higher incorporation of radioactive sulphate into sulphatide fractions during the acute stage of experimental allergic encephalomyelitis (EAE) (VASAN, ABRAHAM and BACHHAWAT, 1971). Since it has been well established that sulphatide is an important constituent of the myelin sheath (CUZNER, DAVISON and GREGSON, 1965; EVANS and FINEAN, 1965) it was of interest to study the enzymes involved in the metabolism of this myelin lipid in this experimental model of demyelination disease.

Enzymic synthesis of sulphatide by the transfer of sulphate from 3′-phosphoadenosine 5′-phosphosulphate (PAPS) to an endogenous protein-bound cerebroside in brain has been reported by BALASUBRAMANIAN and BACHHAWAT (1962) and to galactocerebroside by MCKHANN, LEVY and HO (1965) and CUMAR, BARRA, MACCIONI and CAPUTTO (1968). BALASUBRAMANIAN and BACHHAWAT (1961) have also shown the synthesis of PAPS in rat brain. Deficiency of arylsuphatase A in metachromatic leucodystrophy led to the suggestion that this enzyme may have a regulatory role in the concentration of sulphatide (AUSTIN, ARMSTRONG and SHEARER, 1965). Although purified brain arylsulphatase A (BALASUBRAMANIAN and BACHHAWAT, 1963) did not degrade sulphatide, MEHL and JATZKEWITZ (1968) conclusively demonstrated that sulphatide is the physiological substrate for arylsulphatase A in the presence of a heat-stable cofactor. In the present paper the activities of these enzymes which play a regulatory role in the concentration of sulphatide in myelin has been studied in different stages of EAE.

MATERIALS AND METHODS

Absorbents used for chromatography included Florisil 60–100 mesh (Floridin Co., Florida, U.S.A.) and DEAE-cellulose (Carl Schleicher and Schuell and Co., New Hampshire, U.S.A.). Radioactive sulphate was obtained from the Bhabha Atomic Research Centre, Trombay, Bombay, India.

Abbreviations used: EAE, experimental allergic encephalomyelitis; PAPS, 3′-phosphoadenosine 5′-phosphosulphate.

Freunds adjuvant (complete) was purchased from Difco Laboratories, Detroit, Michigan, U.S.A. and the dipotassium salt of nitrocatechol sulphate was obtained from Sigma Chemical Co., U.S.A. Triton X-100 was from Rohn & Haaz, Philadelphia, Pa., U.S.A. All other chemicals used were of analytical grade.

Induction of EAE in rats. The procedure has been described previously (VASAN *et al.*, 1971). The different stages in the development of EAE are arbitrary and the degree of paralytic involvement of the hind limbs was taken as the guide line. This was done by drawing on the experience and constant observation of the development of the disease for well over 2 years. The different stages of the development of the disease are classified in the following way:

Stage 1: Onset of the disease: The rats show an involvement of the hind limbs and drag the limbs. They take less food. This usually occurs between 12 and 13 days after induction of EAE.

Stage 2: One day after stage 1 but a day before the acute stage, the rats show definite symptoms of paralysis and manage to move a little by dragging the limbs. They show a loss of body weight. This is followed by an acute attack.

Stage 3 (acute stage): There is complete hind limb paralysis, inability to move about, with food refusal and a marked decrease in body weight.

Stage 4: On the 3rd day after the acute attack (stage of recovery), the rats show a little improvement and move about with dragging hind limbs. Food intake is considerably improved.

Stage 5 (complete recovery): This takes 8–12 days from the acute attack and by then the rats move about and take food normally; the body weight increases.

Extraction of glycolipids. The brain was homogenized in 19 vol. of chloroform:methanol, 2:1 (v/v) and the filtrate was shaken thoroughly with 0·2 vol. of 0·74% (w/v) KCl (RADIN, LEVINE and BROWN, 1955). The lower phase was washed three times using the theoretical upper phase and the crude lipid subjected to further fractionation as follows: (1) For Florisil column chromatography, 4 g of regenerated Florisil was used and the fraction containing cerebroside and sulphatide was eluted from the column using 100 ml of chloroform:methanol, 7:3 (v/v) (RADIN *et al.*, 1955). (2) DEAE-cellulose acetate chromatography: Further fractionation of cerebroside and sulphatide was done on a DEAE-cellulose acetate column. The cerebroside was eluted with chloroform:methanol, 7:1 (v/v) and sulphatide with chloroform:methanol, 4:1 (v/v) containing 1% ammonia (ROUSER, BAUMAN, KRITCHEWSKY, HELLER and O'BRIEN, 1961).

Analysis of glycolipids. The different fractions were concentrated and quantitative assays of cerebroside and sulphatide were done using anthrone reagent (RADIN *et al.*, 1955).

Preparation of [^{35}S]PAPS. Active sulphate synthesizing enzyme was prepared according to the method of PANIKKAR and BACHHAWAT (1968) using rat liver instead of sheep liver. After ammonium sulphate fractionation the enzyme was passed through Sephadex G-75 for desalting. [^{35}S]PAPS was prepared from ATP and inorganic ^{35}Ssulphate in the presence of active sulphate synthesizing enzyme as reported by MUKHERJI and BACHHAWAT (1966). This carrier-free [^{35}S]PAPS was used in all the experiments described here. The radioactivity was measured in Panax solid B scintillation counter. Protein concentrations were determined by the method of LOWRY, ROSEBROUGH, FARR and RANDALL (1951) using crystalline bovine serum albumin as standard.

Assay of cerebroside sulphotransferase enzyme activity. All the enzyme preparations were carried out at 0–4°C unless otherwise stated. The brains and kidneys were taken out after decapitation, chilled immediately in ice and homogenized in 2 vol. of 0·05 M-tris–HCl buffer, pH 7·4. The crude supernatant fraction obtained after centrifugation at 1000 *g* for 20 min at 0°C was used as the enzyme source. The assay system was essentially the same as described by BALASUBRAMANIAN and BACHHAWAT (1965), with a slight modification (BHANDARI, 1970). The complete system comprised 50 μmol of potassium phosphate buffer, pH 7·4; 2 μmol of EDTA, pH 7·4; 5 μmol of reduced glutathione; [^{35}S]PAPS equivalent to 500,000 c.p.m. and the enzyme in a total vol. of 0·5 ml. After incubation for 60 min at 37°C, 2·5 ml of chloroform:methanol, 2:1 (v/v) were added and the mixture centrifuged. The lower phase was washed twice with theoretical upper phase and counted.

Assay of PAPS synthesizing enzyme activity. The brain was homogenized in 5 vol. of 0·05 M-tris–HCl buffer, pH 7·4. The homogenate was centrifuged at 18,500 *g* for 30 min at 0°C and the supernatant fluid was used for the assay of the enzyme. The incubation system was largely as described by MUKHERJI and BACHHAWAT (1966) except that pyrophosphatase was omitted. After 60 min of incubation at 37°C the reaction was stopped by immersion in a boiling water bath for 30 s. A sample of the protein-free supernatant fluid was chromatographed using a solvent system comprising ethanol: M-ammonium acetate (7·5:3, v/v), pH 7·2 (ADAMS, 1963). The portion of the paper containing PAPS was cut into 1 cm pieces and scanned for radioactivity.

Assay of arylsulphatases. The enzyme was prepared in the same way as described for cerebroside sulphotransferase activity. The supernatant fluid was dialysed for 8 h in 0·001 M-tris–acetate buffer, pH 6·9, with three changes of buffer. Arylsulphatases A and B were assayed by the method of BAUM, DODGSON and SPENCER (1959) using nitrocatechol sulphate as standard with some modification (FAROOQUI and BACHHAWAT, 1971).

RESULTS

Cerebroside and sulphatide concentrations. The concentrations of galactolipids in normal and different stages of EAE are shown in Fig. 1. The concentrations of both cerebroside and sulphatide decreased gradually from the day of onset of the disease to the acute stage of EAE. The recovery as estimated symptomatically was associated with an increase in the concentration of these two myelin lipids. The cerebroside content in the completely recovered rats was almost the same as that of the normal, but sulphatide was still somewhat lower than the normal level.

Cerebroside sulphotransferase activity. The enzyme activity in different stages of EAE is shown in Fig. 2. It is seen that the activity of this enzyme was similar to that of a normal rat during the first two stages of development of EAE. During the acute stage of the disease, there was a marked increase in the enzyme activity which then decreased during the recovery stage. In contrast to the brain, sulphotransferase activity in kidney was found to be decreased in the acute stage of EAE compared to normal, thus indicating that the kidney is also affected in this condition. The product of the sulphotransferase activity from EAE brain was confirmed as cerebroside

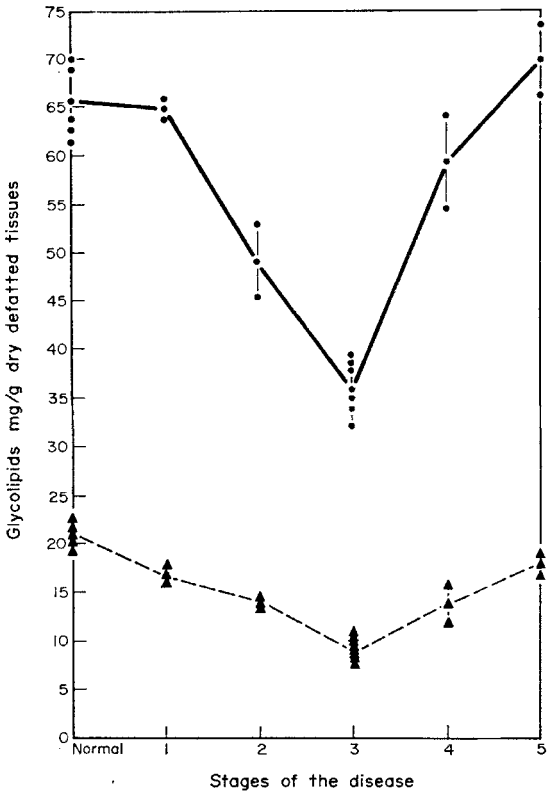

FIG. 1.—Concentrations of cerebroside and sulphatide in different stages of EAE. The concentrations of these glycolipids were estimated by the method of RADIN *et al.* (1955) as described in Methods. The results are expressed as mg/g dry defatted tissue. The different stages of EAE are described in the text.
● Cerebroside. ▲ Sulphatide.

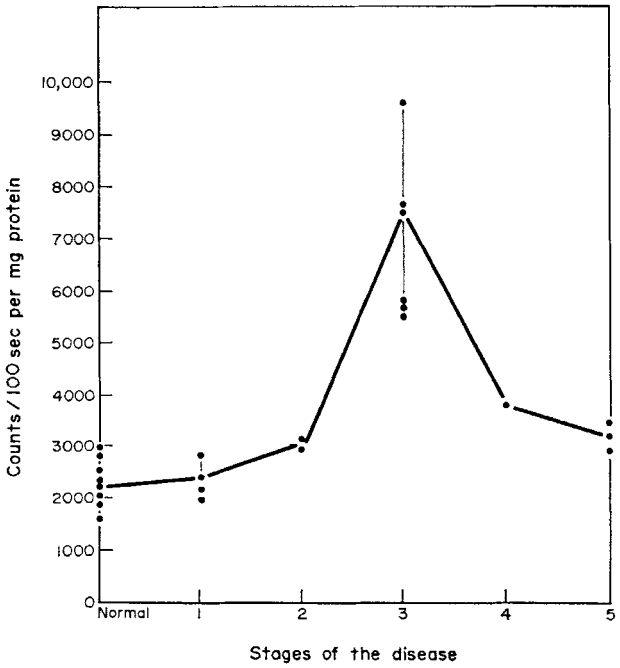

FIG. 2.—Cerebroside sulphotransferase activity. The enzyme activity was assayed in different stages of EAE following the procedure of BHANDARI (1970). The details of the procedure are described in the text.

sulphate by TLC and radioautography. The compound formed was found to have the same mobility as that of authentic sulphatide.

PAPS synthesizing enzyme activity. It can be seen from Fig. 3 that the enzyme activity was higher than normal during the onset of the disease and reached a peak at the acute stage. The recovery stage was characterized by a decrease in PAPS synthesis when compared to the acute stage. However, the activity was higher than normal even after complete recovery.

Arylsulphatase activity. Both arylsulphatases A and B were increased during the onset of the disease and reached a peak activity during the second stage. This was followed by a decline to normal during the acute stage and remained the same throughout the recovery period (Fig. 4).

DISCUSSION

The destruction and degeneration of myelin during the acute stage of EAE in the CNS has been described by BUBIS and LUSE (1964), ROIZIN (1959) and LAMPERT (1965). The concentrations of both cerebroside and sulphatide decreased gradually from the day of onset of the symptoms to the acute stage of EAE and showed an increase during the recovery stages. This is in parallel with the remyelination which is also concurrent with the clinical improvement in the affected animals. SMITH (1965) studied lipid biosynthesis at different stages of EAE and measured the [^{14}C]glucose incorporation into phospholipids. She came to the conclusion that recovery from the disease was due to tissue repair and perhaps that remyelination was taking place.

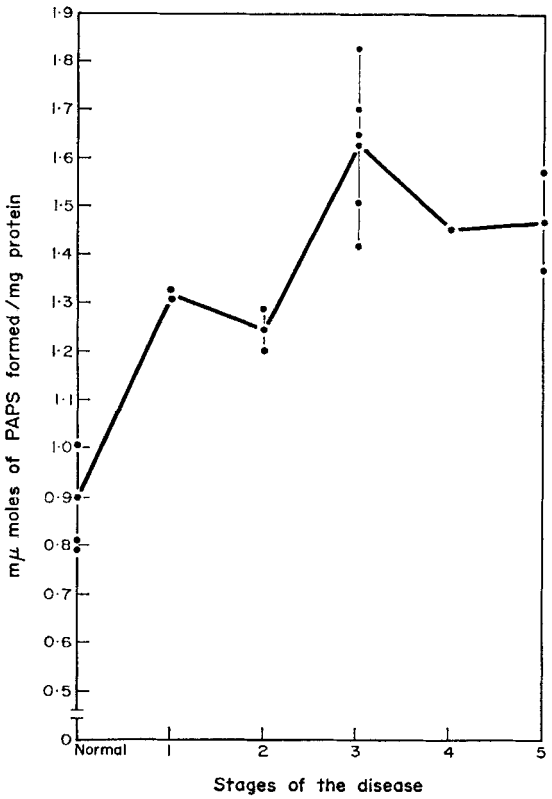

FIG. 3.—PAPS synthesizing enzyme activity. The active sulphate synthesizing enzyme activity in different stages of EAE was done as described in the Methods.

The cerebroside sulphotransferase activity was maximal during the acute stage and decreased during recovery. This is similar to the pattern seen in the developing rat brain (BALASUBRAMANIAN and BACHHAWAT, 1965) at least during the period of active myelination. Moreover, we have previously reported (VASAN *et al.*, 1971) that there was a high [^{35}S]sulphate incorporation into sulphatide during the acute stage of EAE when compared to normal. Biosynthesis of PAPS which is the sulphate donor for the synthesis of sulphatide was also at a maximum during the acute stage of EAE. However, during the recovery stage when the sulphotransferase activity has returned to normal, the synthesis of PAPS was still at a higher level. Since PAPS is known to be the sulphate donor for many sulphation reactions in the brain other than sulphatide synthesis, it is reasonable to assume that the synthesis of other sulphate compounds such as sulphated glycosaminoglycans is still taking place during the recovery stage. The presence of a glycosaminoglycan sulphotransferase activity in the rat brain has already been reported from our laboratory (BALASUBRAMANIAN and BACHHAWAT, 1964; GEORGE, SINGH and BACHHAWAT, 1970). Moreover, glycosaminoglycan metabolism is also known to be disturbed in EAE (VASAN *et al.*, 1971).

It has been shown by BALASUBRAMANIAN and BACHHAWAT (1961) that during the development of rat brain there are two peaks of PAPS synthesis—one at birth and the other during myelination. The present observation is consistent with the finding

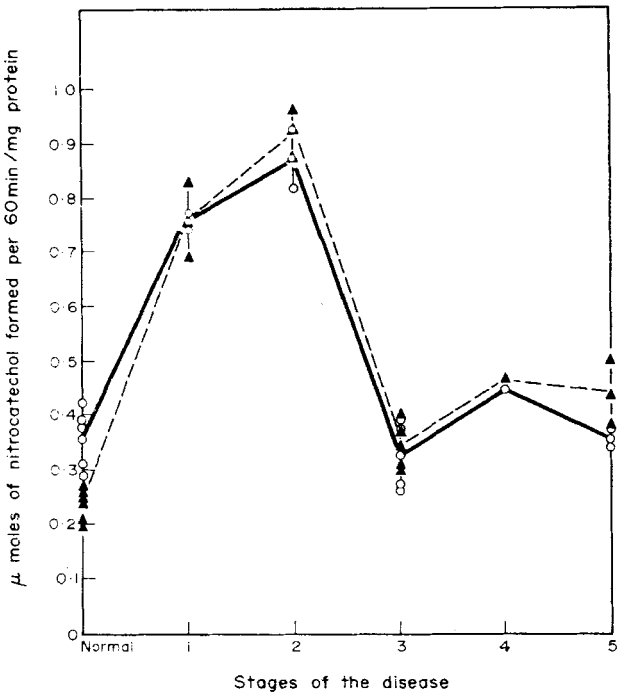

FIG. 4.—Activities of arylsulphatases A and B. Differential assay procedures for A and B were done according to the method of BAUM *et al.* (1959), with some modifications. Assay mixture for arylsulphatase A: 0·1 ml of reagent A (0·01 M-nitrocatechol sulphate in 0·05 M-sodium acetate–acetic acid buffer containing 0·5 mM-sodium pyrophosphate and 10% (w/v) NaCl, pH 5·0); 0·1 ml of 1% Triton X-100 and enzyme in a total vol. of 0·3 ml.

The mixture was incubated for 60 min at 37°C, the reaction stopped by the addition of 2·7 ml of 0·11 M-NaOH and the colour developed was read at 500 nm.

Arylsulphatase B: 0·1 ml of reagent B (0·05 M-nitrocatechol sulphate in 0·05 M-sodium acetate buffer containing 10 mM-barium acetate, pH 6·0); 0·1 ml of 1% Triton X-100 and enzyme in a total vol. of 0·3 ml.

Tubes were incubated at 37°C for 30 min and 90 min and the reaction was stopped as indicated for arylsulphatase A.

● Arylsulphatase A.
▲ Arylsulphatase B.

that PAPS synthesis is important in the myelination process. The concentration of glycolipids as well as the activities of the enzymes studied did not reach the normal level even after an apparent clinical recovery, indicating that the remyelination is still taking place.

The arylsulphatases A and B showed a maximal activity during the second stage of the disease, i.e. after the onset and a day before the acute stage, and a return to the normal level during the acute and subsequent recovery stages. Arylsulphatase A deficiency was found in metachromatic leucodystrophy (AUSTIN, BALASUBRAMANIAN, PATTABIRAMAN, SARASWATHI, BASU and BACHHAWAT, 1963; MOSER, MOSER and MCKHANN, 1967; TAORI, MATHEW, BHAKTAVIZIAM and BACHHAWAT, 1969). It was found to be the primary enzyme defect responsible for the accumulation of sulphatides in these diseases (MEHL and JATZKEWITZ, 1965). The marked decrease of aryl-

sulphatase A activity at the acute and in the recovery stage may be related to the increased synthesis of sulphatide during the recovery stage. At present the role of arylsulphatase B is not known, even though it has been implicated in the regulation of sulphated glycosaminoglycan. Moreover, in our earlier studies we found that the glycosaminoglycan concentration was decreased at the acute stage of EAE.

While our earlier work has shown a rapid incorporation of sulphate into sulphatide during the acute stage of EAE suggesting a high turnover of this compound, the present results indicate that a net synthesis of sulphatide is also taking place. Thus, the metabolic changes observed during different stages of the disease studied bear close resemblance to the initial myelination. It may also be mentioned here that the other tissues such as kidney are also affected in EAE and we have observed low kidney sulphotransferase activity during the acute stage of this disease. WAJDA, LEE and NEIDLE (1969) have reported that the liver is also affected, from their studies on transglutaminase levels during the early stages of EAE.

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