The Metabolism of Sulphatides in Cerebral Tissues

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The sulphatides of brain consist essentially of galactocerebrosides, the galactose moiety of which is singly esterified on C-6 by sulphuric acid (Thannhauser, Fellig & Schmidt, 1955). The various preparations described by Blix (1933), Lees, Folch, Sloane-Stanley & Carr (1959), Thannhauser *et al.* (1955) and Jakewitz (1960*b*), if they differ, would appear to do so only in the nature of the fatty acid part of the parent cerebrosides (Lees *et al.* 1959; Jakewitz, 1960*b*).

The metabolism of brain sulphatides has been little studied. In a classical investigation it was shown that the sulpholipids of rat brain increased in quantity throughout life (Koch & Koch, 1913), a study which has not since been repeated. More recently, marked increases in the quantities of sulpholipids in human brain have been demonstrated in certain demyelinating diseases (Austin, 1959; Jakewitz, 1960*a*, *b*).

Experiments with radioactive sulphate *in vivo*, however, led to the conclusion that in normal brain the sulphatides are relatively metabolically inert. Thus, after the injection of radioactive sulphate into rats or rabbits, the radioactivity accumulating in the brain sulpholipids was extremely low and declined very slowly over periods of up to several weeks (Bostrom & Odeblad, 1953; Dziewiatkowski, 1953; Radin, Martin & Brown, 1957; Green & Robinson, 1960). Such experiments, however, parallel in their results earlier experiments of a similar nature conducted with radioactive phosphate [see Heald (1960) for review], in which the low radioactivity of the phospholipids was wrongly attributed to a generally slow metabolism rather than to a slow rate of penetration of radioactive phosphate into the brain. It was therefore considered desirable to re-examine the incorporation of sulphate into the sulpholipids of brain under conditions *in vitro* where more precise information might be obtained. Since it has been shown (Bostrom & Odeblad, 1953) that the cortical surfaces of rat brain take up sulphate more rapidly than other areas, the work was performed on slices of guinea-pig cerebral cortex.

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MATERIALS AND METHODS

Tissues and salines. Slices of adult-guinea-pig cerebral cortex were used. Animals were killed by a blow on the neck and rapidly exsanguinated. Three slices, 0·30–0·35 mm. thick, were cut from each hemisphere by using a recessed plate as a guide and a strip of razor blade (McIlwain, 1951). Each slice was floated into the appropriate saline and trimmed rapidly with a scalpel. Excess of saline was removed by draining against a glass plate. The slices were weighed on a torsion balance, and adjusted to a weight of 110–120 mg. by trimming with scissors if necessary to standardize as far as possible the tissue–fluid ratios in each vessel. They were again floated into saline and transferred to manometric vessels. Since two slices of cortex were required for a single estimation of sulphatide, experiments were arranged so that, with the six slices obtained from a single brain, two different treatments were compared with a control either containing glucose or without any added substrate. Each vessel contained 3·5 ml. of saline composed of: NaCl, 127 mM; KCl, 6·3 mM; $MgCl_2$, 1·28 mM; tris, 0·05 M (adjusted to pH 7·4 with HCl). Slices were incubated at 37·5° under O_2. Substrates in the concentrations stated in the appropriate Tables were included in the salines in which the slices were cut and trimmed. 2:4-Dinitrophenol was added from the side arms of the manometric vessels.

Determination of sulphatides

These were determined as sulphate released on hydrolysis (Folch & Lees, 1959) essentially by the method of Long & Staples (1961) (cf. Jones & Leytham, 1956), modified to permit the determination of radioactive sulphate in extracts of tissue slices.

Preparation of extracts. After incubation each pair of slices was tipped out into a basin and the slices were rapidly drained and transferred to 5 ml. of chloroform–methanol (2:1, v/v). The incubation media were retained for the determination of radioactivity in appropriate samples. The slices were dispersed in the chloroform–methanol by grinding in a small centrifuge tube with a fitting pestle for 3–4 min. and centrifuged at 12 000*g* for 10 min. at −5°. The supernatants were removed, the residues again extracted with a further 5 ml. of chloroform–methanol, and the combined supernatants from each pair of slices were brought to 10 ml. with chloroform–methanol. To each extract 0·05 M-NaCl (0·2 vol.) was added, and after vigorous shaking the mixtures were centrifuged and the upper layers removed and discarded. The lower layers were again extracted three times with 2 ml. of chloroform–methanol–0·05 M-NaCl (5·2:50:47·4, by vol.),

and the upper layers discarded. This procedure, designed for the removal of non-lipid substances from lipid extracts (Folch, Lees & Sloane-Stanley, 1957), completely removed all traces of radioactive sulphate added to the extracts.

Determination of sulphatide sulphur. The washed lipid extracts were evaporated to small volume and made up precisely to 1·0 ml. with ethanol or chloroform–methanol. A portion (0·8 ml.) of the extract was transferred to conical tubes (3 ml. capacity) and evaporated to dryness in a stream of N_2. To the residues HCl (5·5 N; 0·3 ml.) was added, and the tubes were capped and heated at 100° for 1 hr. with frequent mixing of the contents. They were then cooled and 1·5 ml. of chloroform–methanol (2:1, v/v) was added and mixed thoroughly with a capillary pipette. The lower layer was removed and the upper layer washed twice with 1 ml. of chloroform–methanol (17:3, v/v), the lower layer being discarded each time. The aqueous upper layer was evaporated to dryness in a stream of N_2 and the residue taken up in precisely 0·15 ml. of water. 4-Amino-4′-chlorobiphenyl reagent (4-amino-4′-chlorobiphenyl hydrochloride, 0·224 g.; cetyltrimethylammonium bromide, 0·10 g. in 100 ml. of 0·1 N-HCl) (0·5 ml.) was added. The tubes were capped, mixed and kept in the refrigerator overnight. They were centrifuged, 0·1 ml. of the supernatant was diluted to 10 ml. with water, and the extinction read at 254 mμ. Duplicate sulphate standards and blanks were carried through the procedure from the point of hydrolysis onwards. Sulphate was calculated from the difference in extinction between the blank and the samples (ΔE).

Sulphate activation

Adenosylsulphatophosphatase was estimated by the method of Wilson & Bandurski (1958). This method depends on the reaction:

$$ATP + SO_4^{2-} \rightleftarrows AMP\text{-}SO_4 + \text{pyrophosphate}$$

moving rapidly to the right in the presence of pyrophosphatase, and is enhanced by the presence of molybdate ions, which form a less stable adenosyl derivative than does sulphate. Inorganic phosphate formed by the pyrophosphatase thus provides a measure of the overall reaction. Brain contains a highly active pyrophosphatase (Gordon, 1950) and addition of yeast pyrophosphatase (cf. Wilson & Bandurski, 1958) was not considered necessary.

Incubation mixtures contained: ATP (0·01 M) and either sodium molybdate (0·01 M) or Na_2SO_4 (0·01 M); $MgCl_2$ (2 mM); tris–HCl buffer (pH 7·4; 0·1 M); sodium ethylenediaminetetra-acetate (0·6 mM); enzyme suspension (0·2 ml.) Total vol., 0·5 ml. Sodium molybdate and sulphate were replaced by chloride for the measurement of adenosine triphosphatase. Incubation was at 37·5° for 30 min. The reaction was stopped by boiling for 2 min. The enzyme suspension consisted of guinea-pig cerebral cortex dispersed in 10–20 vol. of water, sucrose (0·25 M) or NaCl (0·15 M).

Determination of radioactivity

In sulphatides. The precipitated 4-amino-4′-chlorobiphenyl sulphate was taken up in not more than 0·1 ml. of water and plated on polythene disks (1·75 cm.²). After evaporation of the solution to dryness the radioactivity was counted under an end-window counter to an accuracy of 5%. No correction for self-absorption was made, since the quantities of sulphate determined were usually less than 0·5 μmole and over the range 0·3–0·75 μmole of sulphate the recoveries of radioactivity were 99·1% [S.E.M. ± 2·65 (9)] without any correction being applied.

In incubation media. A portion (0·1 ml.) of the medium was plated as described above and counted in a similar fashion. Usually the counts were of the order of 2×10^5 counts/min./ml. of medium.

Units. Specific activity = counts/min./μmole of sulphate calculated to a standard count of 10^5 counts/min. in each ml. of the incubation medium.

Reagents

^{35}S was obtained as carrier-free sulphate from The Radiochemical Centre, Amersham, Bucks. 4-Amino-4′-chlorobiphenyl was obtained from L. Light and Co. Ltd., Colnbrook, Bucks. All other reagents were standard laboratory chemicals of AnalaR or P.B.C. quality.

RESULTS

Determination of sulphatide sulphate

At the start of this work no information was available on the effectiveness of the method of Long & Staples (1961) for the quantitative determination of radioactive sulphate, and it was necessary to examine the method from this point of view. As described by Jones & Leytham (1956) and Long & Staples (1961), sulphate is precipitated by the addition of a volume of the reagent equal to that of the sulphate solution. Although this method yielded an apparently straight-line relationship between the concentration of sulphate and ΔE (Fig. 1*A*), when radioactive sulphate was included a similar proportionality between quantity and radioactivity was not obtained (Fig. 1*B*). Presumably the relation between sulphate concentration and ΔE is also a curve under these conditions but this is not easily detectable, unless sulphate is measured by a more sensitive method such as the determination of radioactivity. A straight-line relationship was obtained, however, if the quantity of reagent was doubled (Fig. 1*B*). Under these conditions also, a straight-line relationship existed between the concentration of sulphate and the ΔE, the latter values being higher than those obtained when equal volumes of reagent and sample were used. Recoveries of radioactivity over the range 0·3–0·75 μmole of sulphate were 99·1 ± 2·65% (9 determinations). To achieve this, samples were kept for 16–18 hr. at 2°. Shorter periods, 2–3 hr. (Jones & Leytham, 1956), yielded recoveries of only 76·0 (S.D. of the mean ± 3·2)% (6 determinations). Owing to the extremely low solubility of the sulphate (Belcher, Nutter & Stephen, 1953) the lines relating sulphate concentration to ΔE pass through the origin. Quantities above 0·8 μmole of sulphate were not tested, being greater than any encountered with slices of cerebral tissue.

Under the conditions examined orthophosphate up to 4·0 μmoles or β-glycerophosphate up to 10 μmoles did not interfere with the determination of sulphate. Addition of radioactive phosphate showed that contamination by orthophosphate, present in a concentration of 6·6 mM, did not exceed 3%. Heating β-glycerophosphate in the presence of sulphate, under the conditions used for the hydrolysis of sulphatides, did not affect the recovery of sulphate. It was concluded that the major phosphorylated products likely to be released during acid hydrolysis of the lipid extract would not interfere with the determination of sulphate.

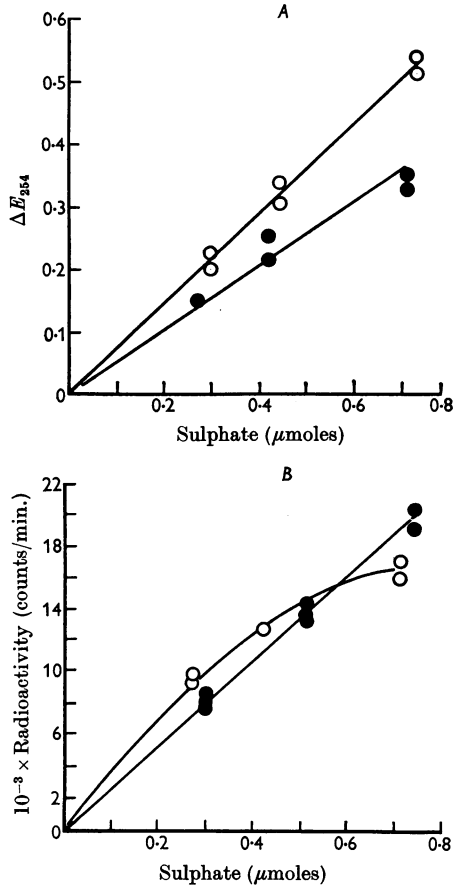

Fig. 1. The precipitation of radioactive sulphate from 0·15 ml. of aqueous solution by different quantities of 4-amino-4-chlorobiphenyl reagent. For experimental details and method of estimating ΔE see the Materials and Methods section. *A*, The relationship between ΔE and quantities of sulphate: ○, with 0·30 ml. of reagent; ●, with 0·15 ml. of reagent. *B*, The relation between radioactivity of the precipitated sulphate and the quantity originally present in solution: ●, 0·30 ml. of reagent added; ○, 0·15 ml. of reagent added.

With the quantities of radioactive sulphate employed (40 μc/vessel) it was estimated that 0·1 ml. of solution, or 15 000–20 000 counts/min., would be carried over with the slices into the chloroform–methanol. Radioactive carrier-free sulphate added to the tissue extracts was quantitatively removed by the washing procedure of Folch *et al.* (1957). More than 95% of the radioactivity was removed in the first two washings and the remainder by the third and fourth washings. Since this method has been shown to leave virtually all the tissue lipids in the chloroform-rich lower phase (Folch *et al.* 1957), it was concluded that the sulphatides could be adequately separated from radioactive sulphate carried over from the incubation medium. Radioactive sulphate added to the lipid extracts immediately before hydrolysis and carried through the remaining procedure was quantitatively recovered. Thus in four experiments with lipids extracted from 400 mg. of tissue the recoveries were 96·5 ± 4·5% (S.E.M.).

Metabolic experiments

Initial experiments carried out in salines containing 1·28 mM-sulphate showed that the radioactivity incorporated was too small to be measured by the methods used. A measurable amount was incorporated if the medium was free from added sulphate. Under these conditions the degree of incorporation into the sulphur-containing lipids was not altered by the buffer used. Thus, in saline media containing glucose, replacement of the buffer tris–HCl by sodium phosphate (pH 7·4; 0·05M) or glycylglycine–HCl (pH 7·4, 0·05M) produced little variation in the radioactivity incorporated into the sulpholipids. Radioactive sulphate was also incorporated into the lipid-free residue, which presumably contains chondroitin sulphate (Brante, 1957). Attempts to determine the ester sulphate in this fraction were unsuccessful and further examination was not attempted.

Table 1. *Effects of anaerobiosis and lack of glucose on the incorporation of* ^{35}S *into sulphatides of guinea-pig cerebral slices*

Slices were cut, weighed and incubated under conditions described in Materials and Methods. Glucose was included where stated. All vessels contained 40 μc of ^{35}S from the start of the experiment. Incubation was for 2 hr. at 37·5°. Specific activity = counts/min./μmole of sulphate calculated to a standard count of 10^5 counts/min./ml. of medium.

Conditions	Gas phase	Specific activity Expt. 1	Specific activity Expt. 2
Glucose (10 mM)	O_2	1160	1930
Glucose (10 mM)	N_2	9·6	231
No glucose	O_2	Not estimated	314

Table 1 shows that both substrate and oxygen were necessary for maximal incorporation of ^{35}S into sulphatides. Incorporation was markedly reduced by the presence of 2:4-dinitrophenol (Table 2). This occurred both at concentrations (6×10^{-4} M) sufficient to decrease the concentrations of adenosine triphosphate and phosphocreatine in the tissues (Kratzing & Narayanaswami, 1953) and at lower concentrations where levels of phosphocreatine only are decreased.

The effect of various substrates in supporting sulphate incorporation into the sulphatides is shown in Table 3. In these experiments the various substrates were compared for their effectiveness with slices incubated both in the absence of additional substrate and in the presence of glucose. Of the metabolites examined only pyruvate was able both to maintain a respiration and an incorporation of sulphate equivalent to glucose. Mannose and fructose, though maintaining respiration and supporting sulphate incorporation, did so to a less degree than did glucose. Succinate even at concentrations maintaining a high respiration was almost without effect upon sulphate incorporation, as were galactose, glutamate, citrate and fumarate.

Table 2. *Effect of 2:4-dinitrophenol on the incorporation of ^{35}S into sulphatides of guinea-pig cerebral cortex*

Slices were incubated as described in Materials and Methods. Glucose was included in the saline. Dinitrophenol was added from the side arm after equilibration of the vessels for 10 min. at 37·5°.

Concn. of 2:4-dinitrophenol	Oxygen uptake (μmoles/g. wet wt.) 0–60 min.	60–120 min.	Specific radioactivity
0	58	57	966
0·6 mM	74	48	595
0	62	62	5250
6 μM	122	126	1580
0	60	60	2320
6 μM	80	80	1180
60 μM	108	108	730

Table 3. *Incorporation of ^{35}S into sulphatides of guinea-pig-brain slices when metabolizing in the presence of different substrates*

Experimental conditions and determination of sulphatides were as described in Materials and Methods. Specific activity = counts/min./μmole of sulphate calculated to a standard count of 10^5 counts/min./ml. of medium.

Expt. no.	Substrate	Concn. (mM)	Oxygen uptake (μmoles/g. wet wt.) 0–60 min.	60–120 min.	Specific activity of sulphate
1	Glucose	10	72	72	916
	Mannose	10	65	65	485
	Fructose	10	61	61	610
2	No substrate	—	33	15	364
	Mannose	10	65	65	1870
	Fructose	10	64	64	2590
3	Glucose	10	66	66	560
	Galactose	10	49	21	172
	Succinate	10	75	40	295
4	No substrate	—	42	17	264
	Galactose	10	57	25	210
	Succinate	10	61	39	374
5	Glucose	10	63	63	2400
	Pyruvate	20	55	55	2120
	Glutamate	10	72	58	550
6	No substrate	—	33	16	184
	Glutamate	10	60	37	121
7	Glucose	10	61	61	2600
	Citrate	20	36	14	475
8	No substrate	—	40	20	575
	Pyruvate	20	66	66	1255
	Citrate	20	42	21	666
9	Glucose	10	61	61	1300
	Fumarate	20	45	29	300
10	No substrate	—	50	32	845
	Fumarate	20	50	35	740
11	Glucose	10	67	67	1030
	Succinate	10	56	45	373
	Succinate	50	108	48	243

Table 4. *Sulphate activation by dispersions of guinea-pig cerebral cortex*

For experimental details see the Materials and Methods section. Values are μmoles of inorganic phosphate released and are corrected for adenosine-triphosphatase activity.

	Tissue equivalent (mg.)	Phosphate liberated (μmoles)		Phosphate liberated (μmoles/g. wet wt./hr.)	
		With MoO_4^{2-}	With SO_4^{2-}	With MoO_4^{2-}	With SO_4^{2-}
Water dispersion	2·5	0·39	—	78·0	—
Water dispersion	12·5	—	0·73	—	29·2
Water dispersion	12·5	1·83	0·82	73·2	32·8
Dispersion in 0·25 M-sucrose	12·5	3·53	—	157·0	—
Dispersion in 0·15 M-NaCl	8·0	0·42	0·15	105·0	38·0

Rate of metabolism of sulphate by brain tissue. The radioactivity of sulphatide sulphate obtained from 200 mg. of slices incubated for 2 hr. with glucose was generally between 400 and 600 counts/min. These values were equivalent to 0·08–0·12 μc of ^{35}S (carrier-free) counted under the same conditions and in the same apparatus. Since each slice was incubated with 40 μc of ^{35}S and the sulphatides from two slices were pooled for analysis, these quantities of 0·08–0·12 μc represented no more than 0·1–0·15 % of the sulphate added. Although this level of incorporation was similar to that described for gastric mucins (Pasternak, Kent & Davies, 1958), it seemed possible that the low incorporation was due to a low permeability of the tissue slices to sulphate rather than to a low rate of metabolism.

In an attempt to decide the potential rate at which sulphate could be metabolized by brain dispersions, the rate of sulphate activation by guinea-pig cerebral cortex was measured. The results are presented in Table 4 and show the speed of the adenosine triphosphate-sulphurylase reaction. Since the product of the reaction is highly unstable when molybdate replaces sulphate, the rate of the reaction in the presence of this anion is measurably greater (Wilson & Bandurski, 1958). Even so, the speed of reaction in the presence of sulphate (30–38·0 μmoles of phosphate released/g. wet wt. of tissue/hr.) was considerable. Since the inorganic phosphate determined is liberated by pyrophosphatase from pyrophosphate produced during sulphate activation, the values represent speeds of reaction of 39–78·0 μmoles/g. wet wt./hr. in the presence of molybdate and 15–19 μmoles/g. wet wt./hr. in the presence of sulphate.

Incubation of sulphatide A from ox brain in this system in the presence of radioactive sulphate did not result in any measurable incorporation of radioactivity into the sulpholipid fraction finally obtained. This was so whether the incubation was with an aqueous dispersion or with a dispersion made in iso-osmotic sucrose. This result could not be attributed to breakdown of sulphatide by sulphatases in the dispersion since incubation of 1·3 μmoles of sulphatide A with 50 mg. of tissue dispersion either unbuffered in water or buffered at pH 5·9 with sodium acetate–acetic acid (0·125 M) did not release any measurable quantities of sulphate into the solution. Sulphate added to this system was quantitatively recovered. It was concluded that under these conditions added sulphatide is not metabolized.

DISCUSSION

It has been shown that slices of cerebral cortex from the adult guinea pig, when respiring in a medium containing glucose, incorporate inorganic sulphate added to the medium into sulpholipids. The sulpholipids involved have not been isolated, but it seems probable that the results reported here concern a single major substance. Thus although the sulphatides isolated by Blix (1933) and Thannhauser *et al.* (1955) represented only a small sample of the total sulpholipid sulphur, this has been shown to be due to the methods employed. Using a simple technique Lees *et al.* (1959) have convincingly demonstrated that a sulphatide, 'sulphatide A', could be readily obtained in a high yield and that an associated sulphatide (sulphatide B) accounted for the remaining sulpholipid sulphur. Chemically, the two were not distinguishable. More recently Green & Robinson (1960) showed that only one radioactive sulphatide could be isolated from extracts of rat brain after injection of radioactive sulphate *in vivo*. In human brain, however, two sulphatides can be distinguished, differing in the fatty acid components of the molecule (Jakewitz, 1960*a*). Similar fractions from the brain of other mammals have not been reported.

Incorporation of sulphate into the sulphatides was inhibited by 2:4-dinitrophenol, an uncoupler of oxidative phosphorylation (Table 2). Further, substrates that were unable to support sulphate incorporation are similar to those unable to support adequate levels of energy-rich phosphates such as phosphocreatine in cerebral slices (Heald, 1960), and suggest that a common factor may be involved.

Of several, the simplest would appear to be adenosine triphosphate. Since sulphate activation, generally, involves adenosylsulphatophosphate formation (Gregory & Robbins, 1960) the inability of certain substrates to maintain adequate levels of energy-rich phosphates in brain slices would appear to present a plausible explanation of their failure to support sulphate incorporation. However, it seems likely that other factors are also involved. Thus the ability of pyruvate to support the incorporation of sulphate (Table 3) is not paralleled by its ability to support the incorporation of phosphate into phospholipids, whereas fructose, supporting sulphate incorporation to an extent greater than mannose (Table 3), was much less effective in supporting phosphate incorporation into phospholipids (Findlay, Rossiter & Strickland, 1953; Findlay, Magee & Rossiter, 1954). Since phosphate incorporation into phospholipids also involves adenosine triphosphate these differing effects of substrates may reflect the ability or otherwise of the substrate to synthesize sulpholipid precursors.

Although dispersions of cerebral tissue contain adenosine triphosphate sulphurylase acting at a rate of up to 19 μmoles of sulphate/g. wet wt./hr., no sulphate was incorporated into added sulpholipid under similar conditions. Although the formation of adenosine 3′-phosphate 5′-sulphatophosphate was not measured there is little reason to doubt that tissues containing adenosine triphosphate sulphurylase are capable of forming it (Gregory & Robbins, 1960). Failure to incorporate sulphate into sulphatide A may therefore imply either a loss of or absence of a sulphokinase or that the sulphatide was not a suitable acceptor. Sulphatide A itself was not degraded by dispersions of whole brain, to yield any measurable quantities of sulphate. This latter result is in agreement with those of Green & Robinson (1960), who were similarly unable to demonstrate any release of sulphate from added sulphatide 'A' on incubation with brain dispersions for 3 hr. In the intact tissue different conditions may obtain, for the concentrations of sulphatide sulphur in slices incubated for 2 hr. with glucose were ($\pm$S.E.M.) $0{\cdot}84 \pm 0{\cdot}05$ (28 determinations) μmole of sulphate/g. wet wt. of tissue, and without added substrate $0{\cdot}61 \pm 0{\cdot}11$ (6) μmole of sulphate/g. wet wt.

The results appear to show that the sulphatides of brain tissue, once formed, are capable of little further metabolic transformations. This applies *in vivo* and extends to the galactose fragment of the sulphatide. Thus, after the injection of the [^{14}C]-galactose or $^{35}SO_4^{2-}$ into both young and adult rats, the radioactivity of the sulpholipids isolated from the brain reached a peak in 3 days and thereafter remained constant for at least 16 days (Radin *et al.* 1957). Green & Robinson (1960) and Ringertz (1956) found that the decrease in radioactivity of the brain sulpholipids, labelled by intraperitoneal injection, persisted for up to several weeks. In the absence of any demonstrable sulphatidase activity in brain dispersions it is considered that incorporation of sulphate into sulphatides in brain represents the slow synthesis of new sulpholipid and is not simply an exchange of existing sulphate.

The extremely slow metabolism of the sulphatides parallels that described by Davison & Dobbing (1960) for certain cerebral phospholipids and also for cholesterol (Davison, Dobbing, Morgan & Payling-Wright, 1959), both of which substances are considered to be components of the myelin sheath. The precise localization of the sulpholipids in brain is not clear, a distribution between both the 'mitochondrial' and 'nuclear' fractions having been found by Green & Robinson (1960). The early work of Koch & Koch (1913) in which the maximum increase of sulpholipids coincided with the period of maximum myelination has also been interpreted to indicate a localization in the myelin sheath and much of the metachromatic staining properties of the sheath have been attributed to the presence of sulphatides (Diezel, 1957). Also, in the Scholtz leucodistrophy degeneration of the myelin is accompanied by a marked increase in the sulphatide (Jakewitz, 1960*a*, *b*). In so far as the properties of such a structure require a degree of metabolic stability of its components, the apparent metabolic inertness of the sulphatides would not be inconsistent with a location in the myelin sheath.

SUMMARY

1. The incorporation of radioactive sulphate into the sulphatides of guinea-pig cerebral slices is dependent upon oxidative phosphorylation. Glucose or pyruvate was essential for maximum incorporation; mannose and fructose were less effective than glucose, while succinate, citrate, fumarate and glutamate did not support incorporation.

2. Dispersions of whole brain activated sulphate via the adenosylsulphatophosphate system at rates up to 20 μmoles/g. wet wt. of tissue/hr. Sulphatides added to this system containing $^{35}SO_4^{2-}$ did not exchange sulphate, nor was any sulphate liberated from added sulphatide by incubation for up to 24 hr. In the intact slice the incorporation of radioactive sulphate in 2 hr. amounted to only 0·1–0·15% of the sulphate in the medium.

3. It is concluded that the sulphatides of brain are metabolically inert and may be localized in the myelin sheath. The incorporation of sulphate into the sulphatides of the brain slices is considered to be the result of a total synthesis of new sulphatide.

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Stimulation of Ascorbic Acid Synthesis and Excretion by Carcinogenic and other Foreign Compounds

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Increases in the hepatic ascorbic acid concentrations of mice occur after a single injection of a variety of carcinogenic hydrocarbons (Kennaway, Kennaway & Warren, 1944; Daff, Hoch-Ligeti, Kennaway & Tipler, 1948) although experiments with smaller doses of some carcinogenic compounds had not shown such increases (Boyland & Mawson, 1938).

Longenecker, Fricke & King (1940) demonstrated that narcotics such as chloretone raise the excretion of ascorbic acid in rats and considered that this was associated with an increase in detoxication processes. Chloretone or barbital treatment stimulates the biosynthesis of glucuronic acid and ascorbic acid (Burns & Evans, 1956; Burns, Evans & Trousof, 1957; Evans, Conney, Trousof & Burns, 1960). This increase is abolished by hypophysectomy but not by adrenalectomy (Conney, Gastel & Burns, 1959), nor is it an effect of the drug on the mechanism for the renal tubular reabsorption of ascorbic acid since it can also be produced in nephrectomized rats (Burns *et al.* 1960). Increase in ascorbic acid excretion also occurs after the injection of aminopyrine, antipyrine, diphenhydramine, chlorcyclizine, orphenadrine, meprobamate and phenylbutazone (Burns *et al.* 1960). These authors suggest that the raised biosynthesis of ascorbic acid that takes place after an injection of a wide variety of chemical compounds represents an adaptive response to foreign compounds although borneol and aniline, which are conjugated with glucuronic acid, do not affect ascorbic acid excretion. An increase in ascorbic acid excretion after treatment with carcinogenic hydrocarbons has