phate: the lesser quantities of phosphates give the basis for lesser respiratory response to pulses in the absence of sodium (see McIlwain, 1952), as observed in Fig. 5.

SUMMARY

1. Cerebral tissues incubated in oxygenated glucose-bicarbonate media rapidly gained sodium: about 40 μ equiv. of sodium/g. by exchange with potassium and a similar quantity by uptake of sodium chloride, mainly during the first minute's contact with the media.

2. Attempts were made to minimize these changes by alterations in the incubating medium. A number of added substances were without such effect; preincubation in media low in sodium did not lead to lower tissue sodium when, subsequently, slices were placed in media of normal sodium content.

3. Rapid preparation of tissue by cutting it *in situ* yielded samples of lower sodium after incubation; during incubation, a net extrusion of sodium was observed in such samples, at rates of 180–240 μ equiv./g. of tissue/hr.

4. Electrical stimulation increased the sodium content of the incubated tissue; after cessation of stimulation, the additional sodium was in part extruded, again at about 200 μ equiv./g. of tissue/hr.

5. Tissue incubated in media low in sodium content, between 0 and 75 mM, was low in potassium content; when stimulated electrically, the respiratory rate of such tissues changed by –50 to +60% rather than by the +100% shown in media of normal sodium content.

6. The intracellular to extracellular ratios of Na^+ and K^+ ions (based on chloride and inulin spaces) have been calculated for incubated tissues

prepared by different techniques and compared with the ratios (based on chloride space) in unincubated fresh tissue.

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Gangliosides, Phospholipids, Protein and Ribonucleic Acid in Subfractions of Cerebral Microsomal Material

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Potassium movements and excitability in cerebral tissues that have been kept in fluid at 0° are altered by added gangliosides; much of the tissues' native gangliosides is found in microsomal material (McIlwain, Woodman & Cummins, 1961; McIlwain, 1961; Wolfe, 1961). Excitability and

ion movements are likely to be membrane phenomena and the microsome fraction contains fragments of membranes, but it also contains other constituents, notably ribonucleic acid granules (Hanson & Toschi, 1959, 1960). Further knowledge of the subcellular localization of gangliosides can

therefore contribute to understanding their actions, and several processes that differentiate between microsomal constituents have now been applied to subcellular fractions from cerebral tissues; in the products, gangliosides, ribonucleic acid, protein and phospholipids have been measured.

EXPERIMENTAL

Tissue dispersion and primary fractions

The grey matter from the cerebral cortex of guinea pigs was obtained, dispersed at 0° in 0.32M-sucrose (pH 7; 10 ml./g. of cortex), and primary fractions were obtained by differential centrifuging as described by Deul & McIlwain (1961) and Wolfe (1961); centrifugal forces are quoted in Table 1.

In several experiments involving only the microsomal fraction, it was obtained as follows. The tissue dispersion was spun at 9460g for 15 min., the supernatant removed, the pellets were suspended in the 0.32M-sucrose (5 ml./g. of cortex), spun as before and this washing was repeated. The three supernatants were combined and spun as before, mitochondrial material being recovered; the supernatant now constituted a 'microsomal suspension'; it was in most cases spun at 105 000g for 95 min. to give 'microsomal pellets'.

Subfractionation and treatment of microsomal material

Differential centrifuging. Freshly prepared microsomal suspensions were spun at 0° as described in Table 2.

Density-gradient centrifuging. This was carried out in 5 ml. tubes in swing-out buckets of an ultracentrifuge rotor. Introduction of the sucrose gradient and sample zone, and sampling, were carried out as described by Britten & Roberts (1960), giving a continuous gradient between 0.8 and 1.5M-sucrose. Centrifuging was at 125 000g for 3 hr., and the resulting fractions were promptly diluted with water to make their sucrose 0.32M, giving suspensions that were spun at 100 000g for 2 hr. to collect their particles. These were drained from supernatant and suspended in water for analysis.

Density-gradient electrophoresis. Column-electrophoresis apparatus (LKB-Produkter Fabriksaktiebolag, Stockholm) was used as described by Svensson (1960). This involved the introduction of liquid density gradients as a supporting medium. Sodium phosphates, 0.01M, pH 7.4, constituted the light buffer and the heavy buffer supporting the gradient contained M-sucrose in addition. Sample and gradient were introduced with the system of series-coupled mixing chambers described by Svensson (1960), of which the upper reservoir contained buffered 0.8M-sucrose, the first mixing chamber (of 150 ml.) buffered 0.32M-sucrose, and the second mixing chamber (of 5 ml.) the microsomal sample in buffered 0.32M-sucrose. Descending electrophoresis was carried out at 0° and 500V for 15 hr. Although the sample formed a stable initial zone as part of the gradient, it was found difficult to operate the apparatus in such a way that part of the sample did not form droplets. In the experiments quoted, 40% of the sample was recovered as droplets; Fig. 2 gives the distribution of the material that remained suspended as particles. After the run, a series of about 40 samples (5 ml.) were collected,

their opacity was determined (extinction at 370 m μ) and, on the basis of the opacity readings, groups of samples were pooled for analysis.

Disruptive and solubilizing procedures. For the experiments of Table 4, freshly prepared microsomal pellets were suspended in either 0.32M-sucrose or the reagents quoted, each portion being derived from about 0.5 g. of cortex. After exposure, the suspensions were centrifuged at 105 000g for 95 min. and the resulting deposits and supernatants collected for analysis. Ultrasonic disruption was carried out in a vessel 5 cm. $\times$ 2.5 cm. diam., with a titanium probe 19 mm. diam. from an ultrasonic disintegrator (Measuring and Scientific Equipment Ltd.), which operated at 18–20 kcyc./sec.

Gangliosides of subcellular fractions

Gangliosides have been determined by the amount of *N*-acetylneuraminic acid yielded, in a method close to that of Wolfe (1961, Fig. 1), which uses the Bial reaction with *N*-acetylneuraminic acid as reference compound. The following notes give modifications or additional specifications to the numbered stages of that method. Stage 2: the subcellular fraction (0.1–1 g., containing 0.1–1 mg. of *N*-acetylneuraminic acid) was ground in a test-tube homogenizer in 19 vol. of the cold chloroform-methanol, filtered by gravity through a sintered-glass funnel, grade 2 porosity, and the apparatus and ground tissue were washed twice with 2.5 vol. of the solvent. Stage 3: when carried out, evaporation was from a bath at 60° and with a jet of air, after which the dry residue was re-extracted with dry solvent, filtered as before and made to its original volume. On other occasions this stage has been omitted. Stage 6: dialysis was carried out whenever sucrose was present, and three changes of water were used.

The modifications are based on the following observations. Stage 2: Wolfe (1961) separated tissue debris from the chloroform-methanol extract by centrifuging, but this has been found difficult. It was replaced by filtration by Balakrishnan & McIlwain (1961), who noted, however, that filter paper removed *N*-acetylneuraminic acid- and protein-containing substances from the chloroform-methanol solution. Filter paper has now been found to remove also a proportion of ganglioside *N*-acetylneuraminic acid (one ganglioside component, forming 40% of some specimens of gangliosides, was removed by 0.5 mg. of paper/ μ g. of specimen); however, filtration through the sintered-glass filter quoted has been found satisfactory. Evaporation and re-extraction at stage 3: at this stage 20–25% of the *N*-acetylneuraminic acid is not re-extracted, diminishing the content of extracted *N*-acetylneuraminic acid from 3.07 ± 0.2 (S.D.) μ moles/g. of cerebral cortex (11 samples) to 2.42 ± 0.39 (five samples). The material removed includes sialomucopolysaccharides or sialomucoproteins (Svennerholm, 1956), but an appreciable and variable loss of added gangliosides was also found at this stage ($17.2 \pm 14\%$; five samples). The process has therefore not usually been carried out.

Other determinations; materials

Protein. This was estimated according to Lowry, Rosebrough, Farr & Randall (1951); sucrose diminished the resulting colours, e.g. by 16% with 0.32M-sucrose, and samples were therefore diluted to relatively low sucrose contents and the same amount was included in the protein standards.

Phospholipid phosphorus. This was determined in samples of the lower phase at stage 4 of Wolfe (1961; Fig. 1). These were taken to dryness in a tube in a stream of air, digested with 10M-HClO₄ and inorganic phosphorus was determined by the method of Martin & Doty (1949).

Ribonucleic acid. The method of Scott, Fraccastoro & Taft (1956), as adapted to subcellular fractions by Littlefield & Keller (1956), was used, the extinction coefficient of ribonucleic acid at 260 m μ , after treatment, being taken to be 34.2 cm.²/mg.

Materials. *N*-Acetylneuraminic acid, synthesized according to Carrol & Cornforth (1960), and used as reference standard in the ganglioside determination, was kindly given by Mr D. A. Booth, as also was the ganglioside preparation, which contained 30.8% of *N*-acetylneuraminic acid (Booth, 1962). Lubrol (Lubrol W: a condensate of cetyl alcohol and polyoxyethylene) was from Imperial Chemical Industries Ltd. Other reagents were of analytical grade.

RESULTS

Ganglioside distribution in the primary fractions

The composition of primary fractions obtained from guinea-pig cerebral cortex by differential centrifuging is shown in Table 1. Over half the tissue ganglioside was obtained in the microsomal fraction, as found by Wolfe (1961); in addition, this fraction showed the greatest enrichment of ganglioside per unit protein. In much subsequent work

the microsomal fraction was prepared without separation of each of the earlier fractions; the composition of this material (microsomal pellets) is shown in Table 1 to be similar to that from the more lengthy fractionation.

An attempt was made to obtain a greater initial yield of ganglioside, or some purification, by removing the nuclear fraction and treating the remainder of the dispersion in an ultrasonic disintegrator, following an observation of Wolfe (1961). Although this process increased the amount of ganglioside in the fine microsomal material, this was accompanied by the transfer of still larger proportions of the protein and phospholipid to this fraction; ultrasonic treatment was not further employed.

Subfractionation of microsomes

Differential centrifuging. Table 2 shows experiments in which two distinct centrifuging sequences led to differential sedimentation of ribonucleic acid and gangliosides. In both cases the ribonucleic acid required a greater centrifugal force than did the ganglioside in order to deposit a given proportion of material. Deposition of protein and phospholipid followed a course intermediate between those of the ribonucleic acid and gangliosides. The findings suggested a partial separation of

Table 1. *Primary fractionation of guinea-pig cerebral cortex by differential centrifuging*

Fractions were prepared as described in the Experimental section.

Centrifugal force		Material	Protein (mg./g. of tissue) (a)	Extracted <i>N</i> -acetylneur- aminic acid (μ g./g. of tissue) (b)	(b)/(a)
g	min.				
—	—	Original dispersion	108.2	752	6.96
800	10	Nuclear	17.3	75	4.34
5 000	10	Mitochondrial	20	72	3.60
20 000	10	Intermediate	17.7	116	6.56
105 000	95	Microsomal	23.9	373	15.6
—	—	Supernatant	23.8	39	1.64
—	—	Recovery in sum of fractions	90%	95%	—
—	—	Microsomal pellets	21.1	378	17.8

Table 2. *Constituents of microsomal-pellet preparation and of subfractions derived by differential centrifuging*

Preparations and the procedure are described in the Experimental section. Expt. A employed a microsomal supernatant, and Expt. B the microsomal pellet, deposited at 105 000g in 95 min., and resuspended in 0.32M-sucrose. Values for Expts. A and B are given as percentages of the total.

Preparation (deposit at 105 000g unless stated otherwise). Microsomal pellet (seven samples)		Extracted <i>N</i> -acetylneur- aminic acid 378 $\pm$ 64 μ g./g.	Protein 21.2 $\pm$ 2.7 mg./g.	Phospholipid phosphorus 513 $\pm$ 37 μ g./g.	Ribonucleic acid 764 $\pm$ 70 μ g./g.
Expt. A.	30 min., 20 000g	61	26	51	25
	95 min.	34	21	46	55
	Supernatant	5	53	3	20
Expt. B.	30 min.	100	84.3	86.3	62.9
	60 min.	—	95.0	94.4	82.3
	90 min.	—	98.9	94.4	87.8
	120 min.	100	100	100	100

two categories of material with similar protein and phospholipid content but differing in the other constituents quoted.

Density-gradient centrifuging. This process, in 0.8–1.5 M-sucrose, resulted in separations shown in Fig. 1. The densest material formed a translucent pellet; above it was a clear fluid before a zone of whitish material at 1.3–1.4 M-sucrose, separated again by a clear portion before a similar zone at 1–1.2 M-sucrose, above which was a coppery opalescent zone that contained the bulk of the protein; a little material remained in the nearly clear fluid above.

Analysis again showed the behaviour of gangliosides to be differentiated from ribonucleic acid. The centrifuging was very effective in separating a ribonucleic acid-rich material, as found by Hanzon & Toschi (1960). No comparable enrichment of

gangliosides took place. Thus the ratio of ribonucleic acid to ganglioside, by weight, was about 1 in the lightest fraction but over 4 in the densest. The denser fractions were markedly poorer in phospholipids than were the lighter fractions.

Density-gradient electrophoresis. Density-gradient electrophoresis appears to have been little applied to fractionation of subcellular particles from animal tissues, but it seemed attractive to try to apply it to the present material, which from the foregoing results contained at least two components with characteristically different acidic groupings. In preliminary experiments material of the microsomal pellets was regularly seen to separate into two main bands during its movement towards the anode, while it was encountering buffered sucrose of progressively increasing density. In the experiment of Fig. 2 this was recorded by measuring the extinction of successive, small fractions run slowly out from the column. Groups of fractions, pooled as indicated in Fig. 2, gave the analytical data of Table 3. The protein content of the two fractions was similar, and phospholipid paralleled protein. However, in ribonucleic acid and in ganglioside content the two fractions diverged. Fraction I, which had migrated farther, contained most ganglioside, in total quantity and in relation to

Fig. 1. Density-gradient centrifuging of microsomal pellet preparation derived from 2.14 g. of guinea-pig cerebral cortex. The zones are described in the text, and their separation for analysis in the Experimental section. Lower curves: ●, ribonucleic acid; Δ, phospholipid phosphorus; ○, ganglioside *N*-acetylneuraminic acid.

Fig. 2. Density-gradient electrophoresis of microsomal pellet material from 2.1 g. of guinea-pig cerebral cortex. After electrophoresis as described in the Experimental section the column contents were run from the anode (densest sucrose) and collected in fractions (5 ml.), which were numbered consecutively. The Figure gives the extinctions on the basis of which the fractions were pooled in groups I and II as indicated; analysis of groups I and II is given in Table 4.

Table 3. *Composition of subfractions obtained by density-gradient electrophoresis*

The fractions were obtained, and pooled to form groups I and II, as indicated in Fig. 2.

Constituent	Group I		Group II	
	$\mu\text{g.}$	$\mu\text{g./mg. of protein}$	$\mu\text{g.}$	$\mu\text{g./mg. of protein}$
Protein	9 240	—	10 330	—
Ganglioside <i>N</i> -acetylneuraminic acid	251	27.2	188	18.2
Phospholipid phosphorus	316	34.2	341	33.0
Ribonucleic acid	350	37.9	469	45.3

Table 4. *Treatment of microsomal fraction with disruptive or solubilizing agents*

Samples of the microsomal-pellet fraction derived from about 0.5 g. of grey matter were exposed for 15 min. (unless otherwise indicated) in 5 ml. of the fluids. They were then centrifuged and the resulting pellets drained and suspended in water for analysis. Untreated samples of microsomal pellet were similarly centrifuged and analysed; these values are given $\pm$ S.D. when appropriate, with the number of observations in parentheses.

Treatment	Loss from pellet (%)			
	Ganglioside <i>N</i> -acetylneur- aminic acid	Protein	Phospholipid phosphorus	Ribonucleic acid
None	10 $\pm$ 6(4)	- 5 $\pm$ 3(6)	—	9(2)
Freezing and thawing three times in water	4	0	0	9
Ultrasonic; 0°; 0.32M-sucrose	51	50	60	23
0.1M-NaHCO ₃ , pH 8.1; 4°; 17 hr.	27	17	0	45
8M-Urea	3	46	19	—
Sodium deoxycholate (0.5% in 0.32M-sucrose)	55	60	71	—
Lubrol W (0.8% in 0.32M-sucrose)	88	85	85	—
Ethylenediaminetetra-acetic acid (2% in 0.32M-sucrose)	32	35	33	—

protein; in the less-mobile fraction II, ribonucleic acid occupied this position.

Disruptive or solubilizing procedures

A constituent was considered to be solubilized when it was not deposited at 105 000g in 95 min.; in control experiments, 90–100% of the constituents measured in the microsomal-pellet fraction was so deposited (Table 4). This was the case with the fraction suspended in sucrose or in water, and also when the aqueous suspension had been repeatedly frozen and thawed. A number of other treatments, however, led to much material remaining undeposited, and in most instances the different components measured were solubilized to different degrees.

Exposure to sodium hydrogen carbonate was based on its removal of ribonucleic acid granules from liver preparations (Hultin, 1957); Table 4 shows that, in the present preparation also, it took into solution a much greater proportion of ribonucleic acid than of other constituents. The conditions under which deoxycholate, Lubrol and ethylenediaminetetra-acetate were used were adopted from Hanzon & Toschi's (1959) study; it is significant that the first two are the most potent in removing gangliosides. Urea in concentrations in which it is a protein-denaturant (Haurowitz, 1950) caused loss of more protein than of the other com-

ponents noted. Ultrasonic treatment had markedly less effect on ribonucleic acid than on the other constituents measured.

DISCUSSION

Almost all the 10 separations or treatments applied to the microsomal fractions in the present studies gave products in which the relative amounts of four constituents measured had undergone appreciable change. The heterogeneity of the fraction thus cannot be doubted, and interest lies in the associations or divergencies that can be established among the changes observed in its constituents. Those measured in the present study are to be appraised together with the morphological constituents described by Hanzon & Toschi (1959, 1960), who found the main components to be ribonucleic acid granules and membrane structures. Of these, the ribonucleic acid granules proved denser and in sucrose gradients settled mainly below 1.2M-sucrose, whereas the membrane structures were distributed continuously throughout 0.9–1.5M-sucrose. This gradient in ribonucleic acid is reproduced in Fig. 2. with a much more even distribution of the gangliosides, which thus tends to associate them with the membrane structures.

Hanzon & Toschi (1959) found also that deoxycholate and Lubrol, under the conditions employed

in the present investigation, removed the greater part of the membrane structures. Table 4 shows that they were the most potent agents in removing gangliosides and phospholipids, of which 55–88% were solubilized. Ethylenediaminetetra-acetate, which left membrane structures largely intact but dispersed the granules, by contrast caused loss of much less ganglioside, as also did sodium hydrogen carbonate, which in other microsomal preparations removes ribonucleic acid (e.g. Hultin, 1957; Simkin & Work, 1957). Many observations therefore associate the gangliosides and phospholipids with the membrane structures and differentiate them from the ribonucleic acid granules.

Cell constituents judged by Hanzon & Toschi (1959) to contribute to the microsomal membrane structures included the endoplasmic reticulum and the plasma membrane; such origins make it understandable that the membrane structures exist in a range of sizes, formed by disrupting the larger but tenuous components of the cell during dispersion. Thus on differential centrifuging the larger of the membrane structures were deposited before the ribonucleic acid granules but smaller, otherwise similar, membrane structures were deposited with the granules. Gangliosides are seen to be deposited ahead of ribonucleic acid in the differential centrifuging of Table 2, and to be dispersed by the ultrasonic treatment of Table 4 into forms sedimented with greater difficulty, their dispersion occurring to a greater extent than that of ribonucleic acid. Again, these observations are consistent with the gangliosides being part of membrane structures, more fragile than ribonucleic acid granules. The density-gradient electrophoresis (Fig. 2 and Table 3) would suggest that the membrane structures have a greater density of negative charge, or a lesser specific gravity, than the ribonucleic acid granules.

The findings raise the possibility that, in neural tissues, gangliosides might be used as a chemical marker for a microsomal component enriched in the membrane structures, as ribonucleic acid is used for the granules. This would, however, require further specification of the ganglioside component, at least in terms of its two main constituents; and also consideration of the likely heterogeneity of the membrane fraction in terms of the cells from which it originates. Acetylcholinesterase has been proposed (Toschi, 1959) as an enzymic marker of the membrane structures, and indication from its substrate specificity is that the membrane structures come predominantly from neurones rather than from glial cells. The microsomal adenosine triphosphatase is also important here (Deul & McIlwain, 1961), and closer comparison of ganglioside components, of phospholipids and of the enzymes in microsomal subfractions would be relevant to the structure and functioning of neural membranes.

SUMMARY

1. In differential centrifuging of cerebral microsomal fractions, gangliosides required lesser centrifugal forces for depositing a given proportion of material than did ribonucleic acid; protein and phospholipids were intermediate in their properties.
2. On density-gradient centrifuging, ribonucleic acid was associated with denser material than the greater part of the gangliosides and phospholipids.
3. On density-gradient electrophoresis, a zone of particles enriched in gangliosides moved more rapidly towards the anode than a zone enriched in ribonucleic acid; phospholipid and protein were associated equally with the two zones.
4. Sodium deoxycholate and Lubrol caused greatest solubilization of gangliosides from microsomal material; sodium hydrogen carbonate (pH 8.1) solubilized most ribonucleic acid.
5. These and other properties of the fractions suggest that gangliosides may be a characteristic component of the membrane structures observed by Hanzon & Toschi (1959, 1960) in cerebral microsomal fractions; these are likely to contain also proteins and phospholipids.

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