accelerated by increasing the temperature to 37° and was not inhibited by 1 mM-iodoacetate.

3. The binding of inorganic phosphate by thymus nuclei was investigated. Linkage to nucleoprotein is probable but the exact nature of the complex is not yet certain.

4. Entry of inorganic phosphate to the nucleus did not require prior uptake into adenosine triphosphate. Entry into the cytoplasm was possibly linked with glucose utilization; its dependence on high-energy phosphate compounds is discussed.

5. Arginine also could be extracted from the nuclei with 0.5–1.0N-perchloric acid at 0°. The molar ratio of total inorganic and labile phosphates: arginine was approximately 2:1.

6. The arginine in the nuclei exchanged rapidly with [¹⁴C]arginine at 0°.

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Phospholipid Metabolism in Nervous Tissue

4. INCORPORATION OF ³²P INTO THE LIPIDS OF SUBCELLULAR FRACTIONS OF THE BRAIN*

By C. AUGUST, A. N. DAVISON AND FAITH MAURICE-WILLIAMS

Departments of Biochemistry and Pathology, Guy's Hospital Medical School, London, S.E. 1

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In earlier investigations (Davison & Dobbing, 1960a, b; Davison, Morgan, Wajda & Payling Wright, 1959) it was shown, by introduction of radioactive precursors into developing animals,

that part of the brain lipids remained metabolically stable during growth and subsequently in the adult animal. It was suggested that this metabolic stability was associated with the myelin sheath and possibly other anatomical structures in the brain. This concept was supported by the demonstration

* Part 3: Davison & Dobbing (1960b).

that stable radioactive lipid was found more in white matter than in grey (Davison & Dobbing, 1960*b*) and that radioactivity was retained only by lipids known to be present in myelin. This paper describes attempts to extend these observations by determining radioactivity incorporated into the lipids of subcellular fractions and myelin-enriched fractions from the brain of rats at intervals after injection of suitable precursors.

METHODS

In the first experiment five 16-day-old white rats from one litter were given 0.25 ml. of 0.9% sodium chloride solution containing $10\mu\text{C}$ of ^{32}P as phosphate by intraperitoneal injection. Five rats from the same litter were injected with 0.2 ml. of 0.9% sodium chloride solution containing $8\mu\text{C}$ of ^{35}S as sulphate. The rats were killed at various intervals after injection and the brain of each was removed, washed free of blood, dried with filter paper and weighed. The brain was then suspended (10%, w/v) in ice-cold 0.25M-sucrose by homogenization in a MSE homogenizer for 5 min. at a predetermined speed. Two samples (0.5 ml.) of the suspension were extracted by refluxing with 10 ml. of ethanol-acetone (1:1, v/v). The mixture was filtered and the residue was re-extracted by the same procedure, and the combined filtrates were evaporated to dryness. The dry residue was extracted with 10 ml. of chloroform-methanol (2:1, v/v) and filtered. The extract was washed with 0.2 vol. of water (Folch, Lees & Sloane-Stanley, 1957) and repeatedly with synthetic upper phase (Folch *et al.* 1957) until free from radioactivity. Radioactivity in the lower phase was determined either for ^{32}P in the β -counter for liquids or for ^{35}S by scintillation counting (Davison & Dobbing, 1960*a*). Phosphorus was determined as described previously (Davison & Dobbing, 1959).

Differential centrifuging. The remaining brain suspension was fractionated by differential centrifuging as described by Brody & Bain (1952), except that only one mitochondrial fraction was prepared (i.e. $R_2 + R_3$). Lipid was extracted from particulate fractions as described above. The supernatant was mixed with 15 vol. of ethanol-acetone (1:1, v/v) and, after refluxing for 10 min., the mixture was filtered and

the filtrate taken to dryness. The dried residue was then extracted with chloroform-methanol (2:1, v/v) and washed as described above.

Fractionation in sucrose density gradients. In the second experiment four young adult rats (body weight 219–241 g.) and one older animal (329 g.) were given intraperitoneally $50\mu\text{C}$ of ^{32}P as phosphate in 0.5 ml. of 0.9% sodium chloride solution. Two neonatal rats were injected with 0.25 ml. of 0.9% sodium chloride solution containing $10\mu\text{C}$ of ^{32}P as phosphate. Animals were killed at stated times after injection. A myelin-rich fraction of brain was then prepared by a modification of the techniques of Korey (1957) and Whittaker (1959). Brains were suspended in 0.25M-sucrose as described before. A portion (10 ml.) of the suspension was layered over a discontinuous density gradient consisting of 10 ml. of 0.5M- and 10 ml. of 1.75M-sucrose. The tubes were centrifuged at $15\,000g$ at 10° for 30 min. Three layers separated: a topmost translucent layer, a middle fluffy layer and a red layer at the interface of the 0.5M- and 1.75M-sucrose layer. The middle fluffy layer (in approx. 5 ml. of 0.5M-sucrose) was removed, diluted with 10 ml. of water and centrifuged for 20 min. at $12\,000g$. The supernatant from the centrifuge tube was decanted and the remaining pellet suspended in 10 ml. of 0.8M-sucrose and centrifuged again at $25\,000g$ for 2 hr. at 10° . The white material floating at the top of the centrifuge tube was separated from the supernatant and precipitate (particulate fraction) and diluted to 60 ml. with water. A pellet was then collected on centrifuging at $12\,000g$ for 10 min. at 10° . Radioactivity in the original suspension and final pellet was determined as described above.

Extraction with salt solution. In a third experiment two adult rats (390 g.) were injected intraperitoneally with 0.2 ml. of 0.9% sodium chloride solution containing $60\mu\text{C}$ of ^{32}P phosphate. The animals were killed after 2 days and the brains removed. Each brain was suspended in 20% (w/v) sodium chloride solution at 0° , 10 ml./g. of fresh tissue (LeBaron & Folch, 1959). The suspension was then centrifuged at $30\,000g$ for 40 min. at 4° . The supernatant was removed and radioactivity and lipid phosphorus were determined on extracts as described above. The brain precipitate was then separated exactly as described above in the second experiment. Lipid sulphate was estimated by the method of Long & Staples (1961).

Table 1. *Intracellular distribution of radioactive lipid in the brain of rats previously injected with ^{32}P phosphate or ^{35}S sulphate*

Rats (15 days old) were injected either with 0.2 ml. of salt solution containing $8\mu\text{C}$ of ^{35}S sulphate or with 0.25 ml. of salt solution containing $10\mu\text{C}$ of ^{32}P phosphate. The rats were killed at intervals after injection; the brain was removed and treated as described in the text. Figures given represent results for one rat and radioactivities are corrected for the equivalent of a dose of $1\mu\text{C}$ of ^{32}P /g. body wt.

Time after injection ...	^{32}P (counts/min.)		^{35}S (μC)		
	4 hr.	13 days	4 hr.	2 days	26 days
Fraction					
Whole-brain lipid extract (total radioactivity)	260	11 900	5 010	34 700	42 500
	Percentage of total brain-lipid radioactivity				
Debris and nuclei	18	33.2	6.4	11.5	12.5
Mitochondria	26.8	47.5	65.4	50	80.7
Microsomes	37.6	13.4	0.6	15.2	7.1
Supernatant	17.6	13	27	8.7	4.4

Histology. Different brain fractions were smeared on to glass slides and fixed in formalin vapour. Haematoxylin and eosin and Sudan black were used for staining. Samples for electron microscopy were fixed by the method of Chou & Meek (1958).

RESULTS

The results shown in Table 1 indicate distribution of radioactive lipid in all the cell fractions of the brains of neonatal rats 4 hr. after injection of [32 P]phosphate; 13 days after injection nearly half the total radioactivity was present in lipids of the mitochondrial fraction. This concentration of radioactivity in the lipids of the mitochondrial fraction was more marked when [35 S]sulphate was used as a precursor even 4 hr. after injection.

Histological examination showed that each brain fraction was of mixed composition and contained sudanophilic material as well as subcellular particles and debris (see also Aldridge, 1957; Petersen & Schou, 1955). Most of the lipid-staining material, probably myelin, was, however, associated with the mitochondrial fraction. The large amount of labelled sulpholipid found in the mitochondrial fraction also suggests the presence of a high proportion of myelin, for there is evidence to suggest that sulphatide is one of the most characteristic lipids of the myelin sheath (Davison, 1961). It was therefore necessary to separate the brain suspension by an alternative method in order to investigate the relationship between metabolic stability and the myelin sheath.

The method of preparation of a myelin-rich brain fraction makes it unlikely that it would contain any particulate matter; furthermore, microscopic examination showed that cells and nuclei were absent whereas characteristic staining for myelin could be demonstrated. The absence of cells and nuclei and the concentration of myelin was confirmed by electron microscopy. At low power ($\times 7800$) in the electron microscope typical myelin forms were seen and no other particulate matter was detected; at higher magnification ($\times 76\ 250$) lamellar complexes of similar periodicity to that of the intact myelin sheath were observed. (These observations were kindly undertaken by Dr C. P. Wendell-Smith.) It therefore seems that myelin-sheath fragments are concentrated in this fraction, although the extent of any contamination by lipid from other sources is not known. In the second series of experiments [32 P]phosphate was injected into neonatal and adult rats. The animals were killed and each brain was suspended in sucrose and separated into a myelin-enriched and a myelin-depleted fraction. Lipid was extracted from each fraction and radioactivity and lipid phosphorus were determined. Comparison of the specific activities of both fractions shows the extent of uptake

Table 2. *Distribution of radioactivity in lipids of myelin-rich fractions from the brain of rats injected with [32 P]phosphate*

Age when injected	Time after injection (days)	Number of animals	Initial body wt. (g.)	Brain wt. (g.)	Myelin concentrate			Remainder of brain lipid			Ratio: specific activity of myelin concentrate specific activity of remainder
					Phospholipid (mg. of phosphorus)	Radioactivity (counts/min.)	$10^{-3} \times$ Specific activity	Phospholipid (mg. of phosphorus)	Radioactivity (counts/min.)	$10^{-3} \times$ Specific activity	
13 days	2	2	25	1.28	0.131	500	3.83	1.869	6 550	3.52	1.09
14 days	36	1	25	1.65	0.38	2250	5.92	1.445	6 050	4.19	1.42
Adult	2	1	329	1.96	0.59	4420	7.5	1.54	17 580	11.4	0.66
Adult	2	4	230	1.87	0.315	1388	4.4	2.115	17 542	8.35	0.53

Radioactivity and phosphorus estimations were as described earlier (Davison & Dobbing, 1959). The procedure for isolation of myelin concentrate is given in the text. Animals were given different doses of 32 P as phosphate by intraperitoneal injection. To facilitate comparison results are corrected to the equivalent of a dose of $1\ \mu\text{Ci/g. body wt.}$ Where more than one animal was used mean figures are recorded. Specific activity = (counts/min.)/mg. of lipid phosphorus.

Table 3. *Distribution of radioactivity in the lipids of different brain fractions*

An adult rat (387 g.) was injected with 50 μC of [^{32}P]phosphate in 0.2 ml. of 0.9% sodium chloride solution. The animal was killed 2 days after injection and the brain suspended in 20% (w/v) sodium chloride solution and fractionated as described in the text. Radioactivity has been corrected to the equivalent of a dose of 1 μC /g. body wt. Specific activity = (counts/min.)/mg. of lipid phosphorus.

Brain fraction	Total phospho- lipid (mg. of phosphorus)	Total radioactivity (counts/min.)	$10^{-3} \times$ Specific activity	Lipid sulphate Lipid phosphorus
Whole brain	3.24	24 400	7.15	—
Supernatant (20% NaCl)	0.174	3 240	18.6	—
'Myelin' fraction	0.238	1 350	5.67	0.59
Final particulate fraction	0.567	10 400	18.3	0.17

of [^{32}P]phosphate into each. The specific activities of myelin-enriched and myelin-depleted fractions were similar 2 days after injection of radioactive phosphate into a 13-day-old rat, but 36 days after injection there was relatively more radioactivity in the myelin concentrate from the brain (Table 2). Different results were obtained 2 days after injection of adult rats. In this case much less radioactivity was found in the lipid of the myelin concentrate than in the rest of the brain.

In the third experiment adult rats were again injected with [^{32}P]phosphate and after 2 days the brain was removed. The brain suspension was, however, first extracted with salt solution of high ionic strength to remove brain lipoprotein, then myelin concentrate and a particulate fraction were obtained as described above. During the course of the fractionation procedure much of the brain suspension is rejected (e.g. upper and lower layers after sucrose density separation), and the results shown in Table 3 therefore represent only part of the total brain myelin or particulate fraction. The ratio of lipid sulphate to lipid phosphorus was shown to be higher in the myelin-enriched fraction than in the brain particulate fraction, as would be expected if sulphatide is a characteristic myelin lipid (Davison, 1961). A typical result is shown in Table 3. In this example the specific activity of the myelin-enriched lipid fraction is about a third that in the particulate fraction and in the supernatant lipid.

DISCUSSION

In earlier papers it has been suggested that brain phospholipids, in common with other constituents, can be divided into metabolically stable and metabolically active compartments (Davison & Dobbing, 1960*a, b*). There is evidence to suggest from experiments on developing animals that this metabolic stability is associated with the myelin sheath and structural cellular constituents. This paper describes experiments designed to confirm the hypothesis by the use of alternative methods for fractionation of the brain. In the first investigations [^{32}P]phosphate was injected into developing rats

and brain suspensions were separated by differential centrifuging into subcellular fractions. The results indicate that, 13 days after injection, the highest proportion of radioactivity was found in the mitochondrial fraction. Since the number of cerebral mitochondria increases threefold during the first 21 days after birth in the rat (Samson, Balfour & Jacobs, 1960) it could be that radioactivity was largely incorporated into developing mitochondria. But subsequent histological examination and similar experiments with [^{35}S]sulphate as precursor indicated that this fraction was of mixed composition and probably contained much of the myelin from the brain. Furthermore it seems likely that myelin fragments to a variable extent contaminated all subcellular fractions from the brain and that this contamination would vary both with the age of the animal and with the efficiency of the initial homogenization. For these reasons another type of separation was undertaken and a comparison was made between the radioactivity incorporated into myelin-enriched and myelin-depleted brain fractions.

If it be true that lipids of the myelin sheath are metabolically inert then it should be possible to incorporate ^{32}P or other suitable precursors into the lipids of this structure only during development and not subsequently. The preliminary experiments reported here are entirely consistent with the hypothesis that the metabolic activity of the myelin lipids from adult rat brain is less than that of other brain lipid. Thus a month after injection of neonatal rats, more radioactivity was found in the myelin-enriched brain fraction than in the rest of the brain phospholipid, whereas significantly less radioactivity was found in the myelin-enriched fraction from the brain of adult rats, previously injected with ^{32}P , compared with the radioactivity in the remainder of the brain (Table 2). An increased difference can be demonstrated if lipoprotein is first removed from the brain suspension: in adult rats three times more radioactivity is present in lipoprotein lipid and the brain particulate fraction than in the myelin-enriched phospholipid fraction. Nevertheless, it is clear from Tables 2

and 3 that a significant amount of radioactive phosphorus is incorporated into the myelin-enriched fraction obtained from the brain of adult rats. However, the method of preparation of the myelin-enriched brain fraction described above is necessarily crude and the criteria for homogeneity, both chemical and microscopic, are only qualitative. To this extent therefore these experiments have failed to give a clear-cut answer and further investigations with more refined methods will be necessary to determine whether or not myelin lipids and other brain structural constituents are completely inert in the adult animal.

SUMMARY

1. Radioactive phosphate and sulphate have been injected into neonatal rats.

2. Most of the persistent radioactivity is found in the lipids of the brain mitochondria, but this fraction also probably contains myelin.

3. In other experiments radioactive phosphorus as phosphate was injected into neonatal and adult rats and a myelin-enriched fraction isolated from the brain.

4. Relatively more radioactive lipid was found in the myelin-enriched fraction from the brains of neonatal rats than in the rest of the brain lipid.

5. In contrast less radioactive phospholipid was obtained from the myelin-enriched fraction from adult rats than from the remaining brain lipid.

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An Electrophoretic Investigation of Interactions between Bovine Plasma Albumin and Charged Dextran Derivatives

BY T. E. THOMPSON* AND W. M. McKERNAN†

Department of Experimental Pathology, University of Birmingham and Medical Research Council Industrial Injuries and Burns Unit, Birmingham Accident Hospital

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The formation of soluble complexes through the interactions of macromolecules bearing net charges of the same sign is a phenomenon of considerable interest. Particular examples of this type of complex may be found in the antigen-antibody system (e.g. Singer & Campbell, 1955a, b, c; Singer, Eggman & Campbell, 1955), the polymerization of acid insulin (Oncley, Ellenbogen, Gitlin & Gurd, 1952; Doty & Myers, 1953), the polymerization of

α -chymotrypsin (Kegeles & Rao, 1958), the interaction between nucleic acids and proteins (Goldwasser & Putnam, 1950) and the interactions of plasma proteins with charged polysaccharides (Walton, 1952; Noguchi, 1956; Bernfeld, Donahue & Berkowitz, 1957; Sasaki & Noguchi, 1959).

The work reported in this paper is an investigation, with free-boundary electrophoresis, of the effect of variations of pH, ionic strength and molecular size on the formation of soluble complexes between bovine plasma albumin and synthetic derivatives of dextran. Two types of dextran derivatives have been employed: a sulphate ester and an ether-linked diethylaminoethyl derivative. Interactions between the bovine plasma albumin

* Present address: Department of Physiological Chemistry, John Hopkins School of Medicine, Baltimore 5, Maryland.

† Present address: Biochemistry Department, British Glues and Chemicals Ltd., 123 Grange Road, London, S.E. 1.