Biochem. J. (1971) **123**, 683–693
Printed in Great Britain

The Turnover of Myelin Phospholipids in the Adult and Developing Rat Brain

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(*Received* 15 *February* 1971)

1. Inorganic [^{32}P]phosphate, [U-^{14}C]glycerol and [2-^{14}C]ethanolamine were injected into the lateral ventricles in the brains of adult rats, and the labelling of individual phospholipids was followed over 2–4 months in both a microsomal and a highly purified myelin fraction. 2. All the phospholipids in myelin became appreciably labelled, although initially the specific radioactivities of the microsomal phospholipids were somewhat higher. Eventually the specific radioactivities in microsomal and myelin phospholipids fell rapidly at a rate corresponding to the decline of radioactivity in the acid-soluble pools. 3. Equivalent experiments carried out in developing rats with [^{32}P]phosphate administered at the start of myelination showed some persistence of phospholipid labelling in the myelin, but this could partly be attributed to the greater retention of ^{32}P in the acid-soluble phosphorus pool and recycling. 4. It is concluded that a substantial part of the phospholipid molecules in adult myelin membranes is readily exchangeable, although a small pool of slowly exchangeable material also exists. 5. A slow incorporation into or loss of labelled precursor from myelin phospholipids does not necessarily give a good indication of the rate of renewal of the molecules in the membrane. As presumably such labelled molecules originate by exchange with those in another membrane site (not necessarily where synthesis occurs) it is only possible to calculate the turnover rate in the myelin membrane if the behaviour of the specific radioactivity with time of the phospholipid molecules in the immediate precursor pool is known.

A good deal of uncertainty still exists as to the extent of the turnover of the phospholipid components of the myelin membrane subsequent to this being laid down in the developing brain. In the adult animal, myelin seems histologically to be rather an inert mass, built up of multilayers of membrane wound around the axon, and it has been argued that it is difficult to envisage extensive dynamic exchange processes occurring in such a structure (Davison, 1970). This view on the metabolic stability of myelin is supported by the lack of enzymes in the membrane (Adams, Davison & Gregson, 1963; Riekkinen & Clausen, 1969) and in particular those enzymes that are responsible for the synthesis of complex lipids (Aeberhard & Menkes, 1968; E. K. Miller & R. M. C. Dawson, unpublished work).

Myelin contains about half of the phospholipid present in the whole brain (Davison, 1970). August, Davison & Maurice-Williams (1961) showed that when [^{32}P]P_i was injected into neonatal rats a significantly higher proportion was retained in the phospholipids of a crude myelin fraction than the myelin from adult animals. Further, in long-term experiments with labelled precursors administered during myelination a remarkable persistence of phospholipid labelling was observed (Cuzner, Davison & Gregson, 1965). Using labelled acetate, the turnover of individual phospholipids in the myelin of adult rat brain was very slow when measured up to 51 days after administration (Cuzner, Davison & Gregson, 1966). However, Smith & Eng (1965) showed that label from [^{14}C]-acetate incorporated into the myelin of myelinating rats was slowly lost from the phospholipids although at different rates: serine, inositol and choline phosphoglycerides turned over faster than ethanolamine phosphoglycerides and sphingomyelin. Other workers have found a slow uptake of [^{32}P]P_i into phospholipids of crude myelin fraction obtained from adult (Mandel & Nussbaum, 1966) and developing rat brain (Abdel-Latif & Abood, 1965). Eichberg & Dawson (1965) found that the [^{32}P]P_i incorporated into guinea-pig brain after 3h was preferentially taken up by the phosphatidic acid and triphosphoinositide fractions of myelin. In adult rats, Smith (1968, 1969) observed both *in vivo* and *in vitro* an appreciable uptake of label from

[^{14}C]glucose into the myelin phospholipids, which was then slowly lost at different rates, depending on the phospholipid. Ansell & Spanner (1967) and Horrocks (1969) have reported a rapid incorporation of labelled ethanolamine into the ethanolamine phosphoglycerides of myelin after the base had been injected into the brains of rats and mice. Recently, radioautographic studies with labelled choline have led to the conclusion that a significant amount of myelin-related metabolic activity occurs in the mature myelin of rat peripheral nerve (Hendelman & Bunge, 1969).

To obtain further information about this problem we have carried out long-term experiments after intraventricular injection of various phospholipid precursors into both adult and myelinating rats. This route of injection leads to a higher incorporation of radioactivity into the brain phospholipid fractions. After the subarachnoid injection of [^{32}P]P_i, Lapetina, Rodríguez de Lores Arnaiz & De Robertis (1969) reported no difference between the rates of incorporation into the total phospholipids of myelin and the other subcellular fractions of brain. Chevallier & Gautheron (1969) found that the intraventricular route was the best for obtaining maximal synthesis of brain cholesterol from [^{14}C]-mevalonic lactone, and that the latter did not readily diffuse into the whole cerebrospinal fluid by this route of administration.

In our studies a comparison has been made of the long-term exchange of label in the individual phospholipid fractions of myelin and microsomal fractions, and attention has been paid to the label remaining in the water-soluble fraction of the brain tissue. The myelin was purified not only by differential and density-gradient centrifugation but also by osmotic shock treatment (Autilio, Norton & Terry, 1964), since it is known that the latter procedure removes a considerable amount of highly labelled phospholipid from crude myelin fractions (Eichberg & Dawson, 1965). It is likely that some membrane component in the microsomal fraction, probably endoplasmic reticulum, is responsible for the synthesis of the new phospholipid incorporated into myelin. Neither myelin nor brain mitochondria appear to be capable of synthesizing phospholipids (E. K. Miller & R. M. C. Dawson, unpublished work) and there is probably some type of transfer of intact phospholipid molecules from the synthetic site (Dawson, 1966). Evidence for such a transfer from the endoplasmic reticulum to other cellular membranes has been obtained for liver tissue (Wirtz & Zilversmit, 1968; McMurray & Dawson, 1969; Jungalwala & Dawson, 1970*a*).

EXPERIMENTAL

Injection of radioisotopes. Rats (200–225g, female albino) were anaesthetized with diethyl ether and clamped in a stereotaxic instrument designed by Dr B. A. Baldwin and constructed in the Institute's workshops. An incisor bar and screws in each ear orifice acted as reference points and an injection was made into the lateral ventricle of the brain. A longitudinal incision in the skin was made along the mid-line and the skull was drilled over the injection site with a dental drill using a 1mm-diameter burr (Ash, round 2) care being taken to limit damage to the pia-arachnoid membranes. The injection needle (no. 22 Record) was inserted into the third lateral ventricle by using the co-ordinates given by de Groot (1959) and König & Klippel (1963) (6.4mm anterior, 1.2mm lateral, 1.7mm above an horizontal plane which is 4.9mm above the intra-aural line). The label (20μCi of [U-^{14}C]glycerol, 5μCi of [2-^{14}C]ethanolamine, 0.5mCi of [^{32}P]P_i) in a maximum volume of 10μl of 0.9% NaCl was injected with an Agla micrometer syringe (Burroughs Wellcome Ltd., London N.W.1, U.K.) connected to the needle with thin nylon tubing. The injection was carried out over a period of 2min and the needle was not withdrawn until 1min later. The skull was closed with dental cement (De Treys zinc cement; Amalgamated Dental Trade Distributors Ltd., London W.1, U.K.), the repair being finished off level with the skull surface. An intraperitoneal injection of 10000i.u. of benzylpenicillin was given.

With young animals (10 days old, unsexed) where no co-ordinates were available, the radioisotope (10μl, 100μCi of [^{32}P]P_i) was injected intracerebrally at a site judged to be as near the ventricle as possible. The skull was pierced directly and the isotope injected 2–3mm from the surface. No penicillin was given. Survival in both groups was excellent, although when initially 0.5mCi of [^{32}P]P_i was given to young animals many died after several days, presumably owing to radiation damage.

Preparation of myelin and microsomal fractions. The rats were decapitated and the whole brain was removed, weighed, and homogenized for 1min in ice-cold 0.32M-sucrose (1g/10ml) in a motor-driven homogenizer with 0.008in clearance (Aldridge, Emery & Street, 1960). The isolation of myelin then followed a method based on those of Autilio *et al.* (1964) and Eichberg & Dawson (1965), all operations being carried out at low temperature (0–4°C). The homogenate was centrifuged at 755$g_{av.}$ for 11min in the SS34 rotor of a Servall refrigerated centrifuge. The supernatant was kept for isolation of the microsomal fraction. The pellet containing cell debris, nuclei and the heavy myelin fraction (approx. 70% of total myelin) was suspended in 5ml of 0.32M-sucrose and layered on 22ml of 0.8M-sucrose and then spun for 30min at 50000$g_{av.}$ in the SW25 rotor of the Spinco L2 centrifuge. The myelin-rich layer at the interface between the two layers was removed, diluted with 1.1vol. of water and again layered on 0.8M-sucrose and re-centrifuged as above. The re-isolated myelin band was diluted with 2.2 vol. of water and centrifuged at 755$g_{av.}$ for 11min. The pellet was treated with water amounting to ten times the volume of the supernatant from which the pellet had been collected. After 30min the osmotically shocked myelin suspension was centrifuged at 12100$g_{av.}$ for 10min. The pellet was suspended in 3ml of 0.32M-sucrose and layered on a continuous linear gradient (0.80–0.32M-sucrose, 23ml) which was centrifuged in the SW25 rotor for 1h at 50000g. The myelin formed two diffuse bands close together near the bottom of the tube. These were both

collected, combined, diluted with 2.2 vol. of water and, after centrifugation for 10 min in the Servall refrigerated centrifuge (SS34 rotor, 12100$g_{av.}$) the myelin pellet was collected. In very young rats (11–13 days old) so little myelin was present in the brain that the step involving centrifugation on the continuous gradient had to be omitted.

The microsomal fraction was isolated by centrifuging the original supernatant in the myelin preparation for 1 h at 12100g in a refrigerated Servall centrifuge (SS34 rotor). The supernatant from this centrifuging was re-centrifuged for 1 h at 290000$g_{av.}$ in an angle rotor in a M.S.E. 65 centrifuge. The microsomal pellet was collected.

Measurement of radioactivity in myelin and microsomal phospholipids. The myelin and microsomal pellets were suspended in 1.5 ml of 0.25 M-sucrose for extraction of lipids and marker-enzyme assays. A suitable sample (1.2 ml) was treated with 10 vol. of chloroform–methanol (1:1, v/v) and left overnight or longer at 4°C. The mixture was warmed for 15 min at 45°C, centrifuged and the extract filtered through a small pad of glass wool in a filter funnel. The pellet (almost non-existent with myelin) was re-extracted with 5 vol. of chloroform and the extract refiltered. The combined extracts were made up to 20 ml with chloroform–methanol (2:1, v/v) and washed with 0.2 vol. of 0.9% NaCl. The lower phase was washed six times with 0.2 vol. of a mixture containing chloroform–methanol–water (3:45:47, by vol.). In the first three of these washes, carrier was added depending on the radioisotope injected into the brain, e.g. 10 mM-KH_2PO_4, 1.5 mM-ethanolamine or 0.14 M-glycerol. The lower phase was made up to 20 ml with chloroform–methanol (2:1, v/v) and a 1 ml sample was taken for direct determination of phosphorus and radioactivity counting. The remainder was evaporated to dryness, and the phospholipids were successively degraded and the degradation products separated as described by Dawson, Hemington & Davenport (1962). After the initial alkaline ethanolysis of the myelin phospholipids, the separation of the water- and solvent-soluble components was greatly improved by inclusion of 0.25% cetyltrimethylammonium bromide in the aqueous phase. For assay of ^{32}P, the phosphorus-containing spots on the chromatogram, revealed (after spraying with acid molybdate) by u.v. irradiation, were digested with 72% (w/v) $HClO_4$ (Dawson *et al.* 1962). The digest was made up to 10 ml with water and the radioactivity was measured in a scintillation counter without addition of scintillator (Čerenkov radiation; von Haberer, 1965) followed by determination of P_i on the same sample (Bartlett, 1959). The counting was corrected for radioactive decay from the time of the injection but even after 78 days the amounts of radioactivity observed were satisfactory. For assay of ^{14}C, the spots on the paper were detected by spraying with an ethanolic 0.2% solution of quinine hydrochloride (Rorem, 1959) and examination in u.v. light. The fluorescing spots were cut out and arranged without overlapping around the circumference of a scintillation vial. The paper was then covered with scintillator solution (15 ml) containing 4 g of 2,5-diphenyloxazole and 0.1 g of 1,4-bis-(5-phenyloxazol-2-yl)benzene in 1 litre of toluene and the radioactivity counted in a Nuclear–Chicago automatic scintillation spectrometer. Tests showed that the use of quinine for locating the spots only resulted in about 10% quenching. After radioactivity counting, the paper was removed, washed three times with diethyl ether and, when dry, digested with perchloric acid for P_i assay as described above.

Marker-enzyme assays. 2′:3′-Cyclic nucleotide 3′-phosphohydrolase was determined by the method of Olafson, Drummond & Lee (1969), and NADPH–cytochrome *c* reductase and cytochrome *c* oxidase as described by McMurray & Dawson (1969). In these determinations myelin was dispersed by inclusion of 0.1% sodium deoxycholate in the incubation medium; addition of deoxycholate at this concentration to the microsomal preparation did not significantly affect the activities. Protein was measured by the method of Lowry, Rosebrough, Farr & Randall (1951) with crystalline bovine serum albumin as standard.

Materials. [2-^{14}C]Ethanolamine (27 mCi/mmol), [U-^{14}C]-glycerol (13.3 mCi/mmol) and 'carrier-free' [^{32}P]P_i were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

RESULTS

Purity of myelin fraction. When the isolated myelin fraction was examined under the electron microscope (kindly performed by Dr E. A. Munn) it appeared to have a typical myelinic membranous structure and was free of recognizable fragments of other cellular organelles or membranes. 2′:3′-Cyclic nucleotide 3′-phosphohydrolase, reported to be a marker enzyme for myelin (Kurihara & Tsukada, 1967, 1968; Kurihara, Nussbaum & Mandel, 1970), was present in the myelin fraction at an activity equivalent to 16μmol/min per mg of myelin protein, whereas the corresponding value for the microsomal fraction was 1.4μmol/min per mg of protein. Even if the enzyme occurs exclusively in myelin this indicates that the microsomal preparation is not contaminated with myelin fragments to any appreciable degree. A marker enzyme for endoplasmic reticulum, NADPH–cytochrome *c* reductase, was absent from myelin, and in control experiments added myelin did not inhibit the activity of this enzyme in the microsomal fraction. Cytochrome *c* oxidase could not usually be detected in the myelin fraction, although occasionally a very low activity was observed. However, the magnitude of this and also the absence of cardiolipin from the preparation suggested that contamination of the myelin with mitochondrial fragments was minimal.

Labelling of myelin and microsomal phospholipids in adult rats. The incorporation of various radioisotopic precursors into the individual phospholipids of the myelin and microsomal fractions after intracerebral injection is shown in Figs. 1–3. After administration of [^{32}P]P_i the specific radioactivity of the acid-soluble phosphorus pool, which was very high at short time-periods (1–2 days), fell steadily after 4 days (Fig. 1*a*), presumably representing the loss of ^{32}P from the brain and its equilibrium with the body phosphorus as well as its elimination from

the animal. Since the specific radioactivities of the individual components of the brain acid-soluble pool are known to come into rapid equilibrium after intracerebral injection of ^{32}P (Lindberg & Ernster, 1950) this pool presumably represented the ^{32}P specific radioactivity of the water-soluble precursors from which the individual phospholipids were synthesized.

The ^{32}P labelling of the individual phospholipids with time (Figs. 1*b*–1*h*) shows a consistent pattern. The specific radioactivity of each phospholipid in the microsomal fraction, apart from phosphatidic acid, increases more rapidly and reaches a higher peak than that in the myelin fraction. The height of the peak seems to vary somewhat with the type of phospholipid, being rather lower in both myelin and microsomal fraction with the 'so-called' myelinic phospholipids, ethanolamine, plasmalogen, phosphatidylserine and sphingomyelin. In this connexion Smith (1968) also noticed that in myelin fractions from adult rat brain the phosphatidylserine, sphingomyelin and ethanolamine phosphoglyceride (presumably mainly the plasmalogen) had a slower turnover after administration of [^{14}C]glucose. After 6–8 days the specific radioactivities declined rapidly in both subcellular fractions so that by 20 days the ^{32}P contents were less than one-tenth of those at the peak. This fall corresponded fairly closely to the loss of radioactivity from the acid-soluble phosphorus pool. The fall then continued at a much slower rate and in a second series of experiments with adult rats some radioactivity could still be detected in all the phospholipid fractions 78 days after injection, with no major differences between myelin and microsomal fractions. However, the acid-soluble pool was also labelled to more or less the same extent and some if not all of this persistent phospholipid activity presumably represents recycling of the radioisotope from the pool in the skeleton.

After injection of [^{14}C]glycerol there was a rapid loss of radioactivity from the acid-soluble fraction (Fig. 2*a*). For convenience the radioactivity is expressed in terms of the acid-soluble phosphorus present in the fraction but it cannot, of course, be assumed that this specific radioactivity will be quantitatively similar to the specific radioactivity of that component in the pool (presumably glycerophosphate) from which the ^{14}C-labelled phosphoglycerides are synthesized. However, as it is to be expected that radioactive equilibrium between the individual components of the acid-soluble pool would be achieved fairly rapidly, the glycerophosphate activity would be expected to fall in a similar manner. As with ^{32}P the major loss of ^{14}C radioactivity from the brain acid-soluble pool occurs within 20 days after injection of the [^{14}C]glycerol, followed by a slow loss, so that even at 100 or more days some radioactivity was present in this fraction. The specific radioactivity of the immediate [^{14}C]glycerol-containing precursor of the phosphoglycerides, phosphatidic acid (Fig. 1*b*), fell more rapidly than that of phosphatidylcholine, phosphatidylinositol, phosphatidylserine and phosphatidylethanolamine (Figs. 2*c*–2*f*). This is consistent with the specific radioactivity of a precursor being less than that of a product when the specific radioactivity of the latter is declining (Zilversmit, Entenman & Fishler, 1934). Phosphatidic acid itself showed no difference in the labelling pattern between the microsomal and myelin fractions. With the other phosphoglycerides there was initially, as with ^{32}P, a much slower incorporation of the ^{14}C label into the myelin fraction. The radioactivity of myelin fell with a similar general time-profile to that of the acid-soluble pool but slightly more slowly than the microsomal phospholipids, so that with time-periods above 20 days the specific radioactivity of the myelin fraction was in general above that in the microsomal fraction. Nevertheless 136 days after injection minimal radioactivity was usually present in the myelin phospholipids compared with that of the original incorporation, although the initial incorporation and subsequent fall was less marked with phosphatidylserine.

A similar pattern was observed after administration of [2-^{14}C]ethanolamine (Fig. 3). Although the individual points representing single animals unfortunately showed more scatter than with the other precursors there was clearly a slower incorporation into, and a slower loss, of the radioisotope from the ethanolamine-containing phospholipids of the myelin fraction compared with the microsomal fraction. Ansell & Spanner (1967) also reported that after the intraventricular injection of labelled ethanolamine the specific radioactivities of the two ethanolamine phosphoglycerides were initially higher in the brain microsomal fraction compared with the myelin fractions. At 30 days after injection the specific radioactivities of the phosphatidylethanolamine and ethanolamine plasmalogen became higher than the corresponding values in the microsomal fraction and remained so up to 128 days. Nevertheless, even after this period there was still considerable radioactivity in the microsomal phospholipids, presumably due to recycling. The specific radioactivities recorded in the two ethanolamine phosphoglycerides are very similar to those observed by Ansell & Spanner (1968) for the large myelin fraction of rat brain after the intracerebral administration of [^{14}C]-ethanolamine. The ethanolamine was not incorporated into any other phospholipids to a significant extent apart from phosphatidylcholine (also observed by Ansell & Spanner, 1967) and this is

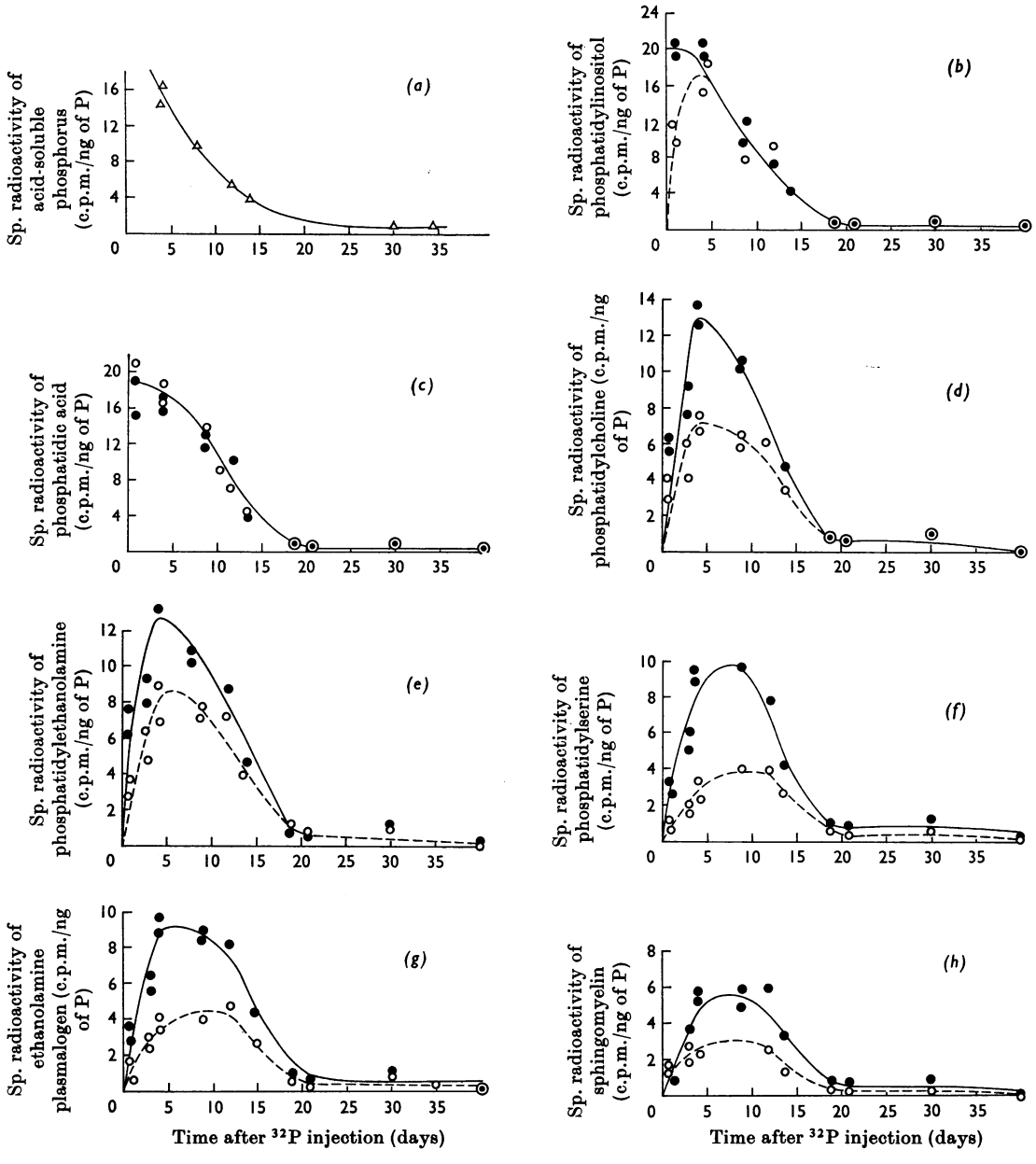

Fig. 1. Specific radioactivities of phospholipids in the microsomal and myelin fractions of brain after the intracerebral injection of [^{32}P]P_i into adult rats. ●, Microsomal fraction; ○, myelin. (*a*) acid-soluble phosphorus (△); (*b*) phosphatidylinositol; (*c*) phosphatidic acid; (*d*) phosphatidylcholine; (*e*) phosphatidylethanolamine; (*f*) phosphatidylserine; (*g*) ethanolamine plasmalogen; (*h*) sphingomyelin.

likely to represent a successive methylation of the labelled phosphatidylethanolamine either in the brain (Artom, 1965) or in the liver (Bremer & Greenberg, 1961), followed by recycling of the labelled choline to the brain.

Labelling of myelin and microsomal phospholipids in young rats undergoing myelination. [^{32}P]P_i was injected into the brains of young rats just at the commencement of myelination (10 days). Since no co-ordinates were available for locating the ventricle

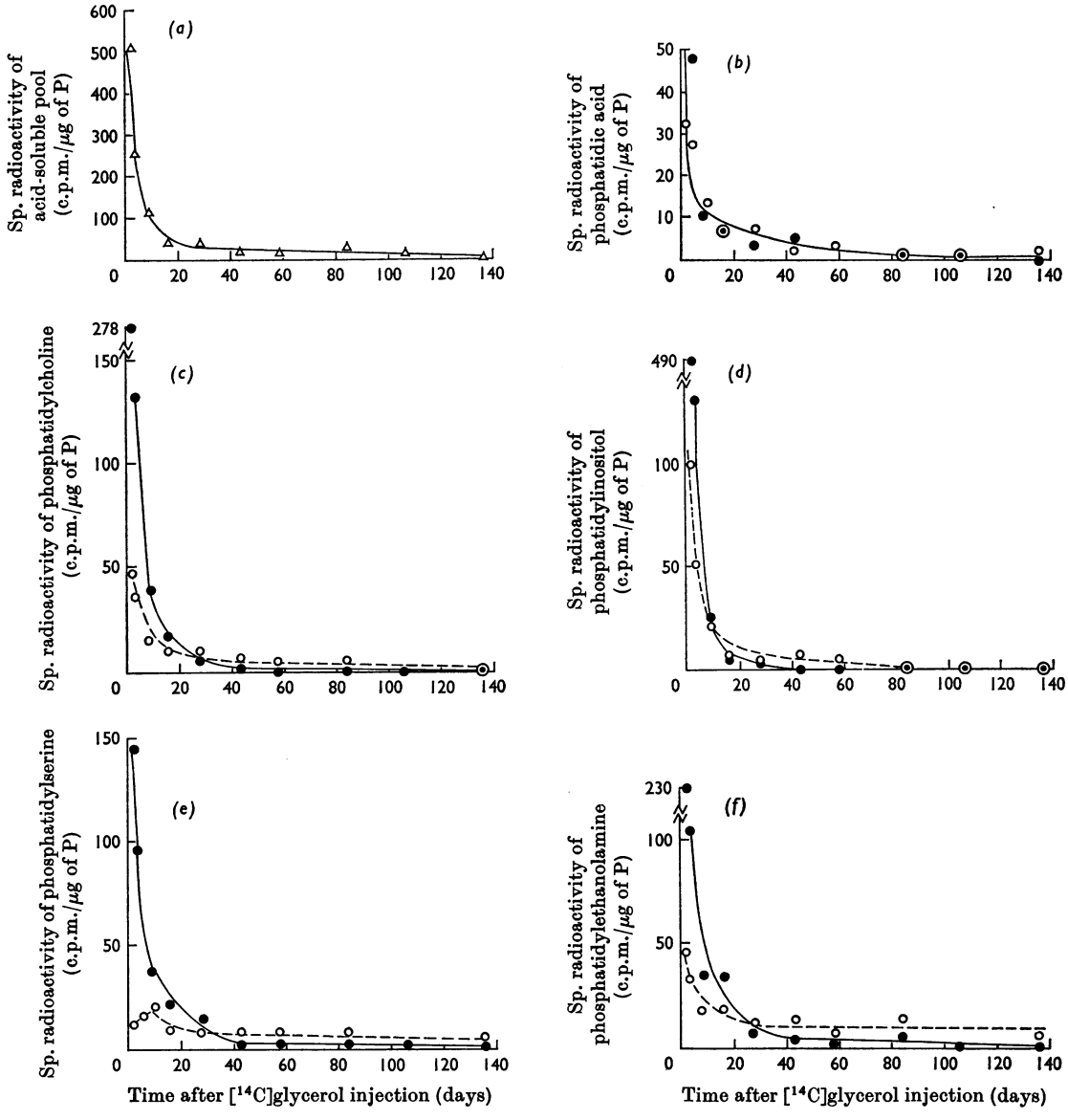

Fig. 2. Specific radioactivities of phospholipids in the microsomal and myelin fractions of brain after the intracerebral injection of [U-^{14}C]glycerol into adult rats. ●, Microsomal fraction; ○, myelin. (*a*) acid-soluble pool (△); (*b*) phosphatidic acid; (*c*) phosphatidylcholine; (*d*) phosphatidylinositol; (*e*) phosphatidylserine; (*f*) phosphatidylethanolamine.

in rats of this age, the sites of the injection were not as certain in this series of animals. Further, isolated myelin fractions from developing rat brain tend to be much more heterogeneous than those prepared from the adult brain (Banik & Davison, 1969). Those factors produced a much greater scatter of points compared with the ^{32}P-incorporation studies in adult rats. However, in general there was a similar pattern in the distribution of radioisotope with time to that seen in adult animals (Fig. 4). With phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, ethanolamine plasmalogen and sphingomyelin there was always a much higher specific radioactivity observed in the micro-

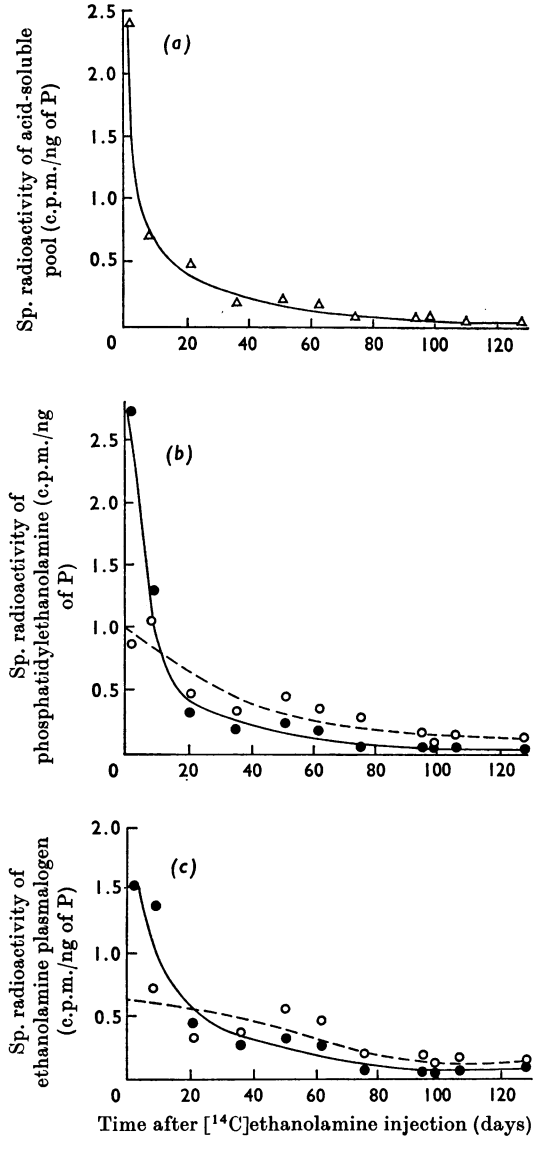

Fig. 3. Specific radioactivities of phospholipids in the microsomal and myelin fractions of brain after the intracerebral injection of [2-^{14}C]ethanolamine into adult rats. ●, Microsomal fraction; ○, myelin. (*a*) acid-soluble pool (△); (*b*) phosphatidylethanolamine; (*c*) ethanolamine plasmalogen.

somal fraction than the myelin initially, but as the specific radioactivity of the acid-soluble phosphorus pool fell this difference became less marked and by the time the animals were 50 days old or older, the specific radioactivity in the myelin fraction became consistently greater than that in the microsomal fraction (Figs. 4*a*–4*e*). The specific radioactivity of phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine in the microsomal fraction became very close to that of the acid-soluble phosphorus pool (Figs. 4*a*–4*c*). A greater proportion of the radioisotope incorporated into the phospholipids of myelin was retained at long time-periods compared with the adult animals.

DISCUSSION

The present results show that highly purified myelin obtained from adult rat brain shows an appreciable uptake into its phospholipids of radioisotopic precursors injected into the cerebral ventricles. From all the available observations on the myelin fraction it was not noticeably contaminated with fragments of other membranous structures. However, even though the myelin preparation had been treated by osmotic shock it cannot be assumed that it was entirely free of non-myelin elements. It is known that such contamination of crude myelin isolates and other so-called purified membrane fractions has in the past led to serious errors in the interpretation of radioisotopic-incorporation experiments (Eichberg & Dawson, 1965; Jungalwala & Dawson, 1970*b*). Adams & Fox (1969) have recently emphasized the difficulty of removing labelled microsomal material from a myelin preparation. Nevertheless, since myelin contains approx. 50% of the phospholipids of brain (Davison, 1970) and specific radioactivities rather than total radioactivities are being measured, any contaminating membrane component, unless present in major proportions, would have to have an enormous specific radioactivity to account for the radioactivity observed in the myelin. Thus contamination with the total microsomal fraction as judged from the marker-enzyme assays would be quite insufficient to account for the radioactivity found. Since the microsomal fraction shows the highest degree of phospholipid labelling from ^{32}P of the crude membrane fractions isolated from rat brain (Mandel & Nussbaum, 1966), it seems much more likely that the incorporation observed was produced by a genuine turnover of the phospholipid elements of the myelin membrane. This view is supported by the appreciable turnover observed of the ethanolamine plasmalogen of the isolated myelin, as this phospholipid is believed to be predominantly a myelin component (Eichberg, Whittaker & Dawson, 1964).

These results therefore support the findings of Ansell & Spanner (1967) and Horrocks (1969) of a substantial turnover of the ethanolamine phosphoglycerides in the myelin fractions (particularly the small myelin fraction) of rat brain after the intraventricular injection of [^{14}C]ethanolamine, and those of Lapetina *et al.* (1969), who found after the

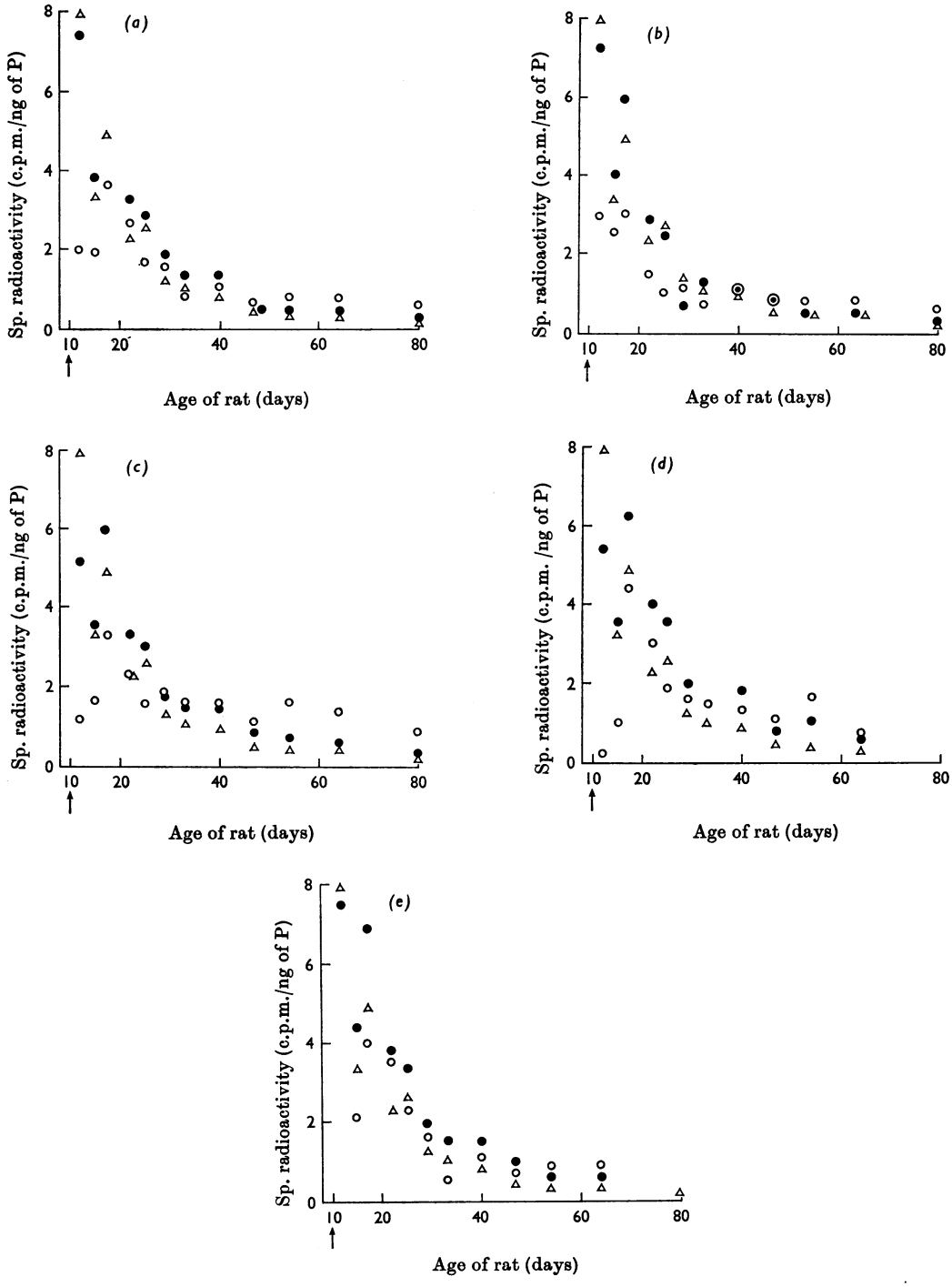

Fig. 4. Specific radioactivities of phospholipids in the microsomal and myelin fractions of brain after the intracerebral injection of [^{32}P]P_i in 10-day-old rats. ●, Microsomal fraction; ○, myelin. The specific radioactivities of the acid-soluble phosphorus pool (△) have been included on each plot for comparison. (*a*) phosphatidylcholine; (*b*) phosphatidylethanolamine; (*c*) phosphatidylserine; (*d*) sphingomyelin; (*e*) ethanolamine plasmalogen. Arrows denote ^{32}P injection.

subarachnoidal administration of [^{32}P]P_i a fairly even labelling of the phospholipids of all subcellular fractions including myelin. In addition, Wood & King (1971) have recently demonstrated a rapid turnover of experimental allergic encephalitogenic-basic protein in the brain of mature rats and this is known to be localized in the myelin sheath.

The intracerebral route is probably advantageous for demonstrating myelin turnover, since the tissue is subject to a sudden pulse of radioactivity rather than a slow build-up of a small percentage of the dose as is found after intraperitoneal or intravenous injection, owing to the impermeability of the blood–brain barrier. A partial exception to this is the use of [^{14}C]glucose as a precursor for the phospholipids (Smith, 1968). Since glucose is the sole energy-providing substrate used by the brain it can be assumed that the barrier is readily permeated by this carbohydrate. Again an appreciable turnover of the adult myelin phospholipids was demonstrated (Smith, 1968). With [^{32}P]P_i the intracerebral route permits a rapid equilibrium with the extracellular space and a ready access to all the membranous structures in the cerebral-cortical cells (Bakay & Lindberg, 1949; Lindberg & Ernster, 1950; Lajtha, 1962), whereas after intravenous or intraperitoneal injection the distribution can be comparatively uneven on a gross morphological level (Borell & Örström, 1947). The greater ^{32}P incorporation obtained allows observation over a longer period. In contrast with Lapetina *et al.* (1969) the actual initial ^{32}P-labelling of all the individual phospholipids was slower in the myelin fraction of adult brains than that in the microsomal fraction, but it cannot be assumed that the rate of renewal of phospholipid molecules was slower. If the labelled myelin phospholipids are not synthesized *in situ* but are obtained from a synthetic site (e.g. endoplasmic reticulum; Abdel-Latif & Abood, 1965), possibly through the intermediary of another membrane (e.g. plasma membrane; Davison & Gregson, 1966), then the initial specific radioactivity of the microsomal fraction will always be higher than that of the myelin irrespective of the rates at which synthesis *de novo* occurs in the former and exchange of whole phospholipid molecules in the latter. The rapidity of pool renewal in adult myelin can be seen in the decay curves after the initial few days. The specific radioactivities of the individual phospholipids in myelin fell at a rate that was very similar to the same phospholipid in microsomal fractions and of the precursor in the acid-soluble pool. The specific radioactivity of the latter presumably fell as the radioactivity was lost from the brain to the blood followed by its incorporation into the body pool and elimination by excretion. The fall in the specific radioactivities of sphingomyelin, phosphatidylserine and ethanolamine phosphoglycerides can be contrasted with the comparative stability of these phospholipids in myelin labelled by the intraperitoneal injection of [^{14}C]-glucose into rats (Smith, 1968). In the latter experiments, because of the blood–brain barrier and the site of injection, the labelling has come more quickly into equilibrium with the body-blood pool so that one is merely observing the slower loss equivalent to that observed by McMurray & Dawson (1969) for the decay of the specific radioactivity of the phospholipids in subcellular fractions of rat liver. One is therefore led to the conclusion that most of the myelin phospholipid molecules are in fairly rapid equilibrium with other components of the brain tissue. That this renewal does not represent a rapid exchange of parts of the phospholipid molecules, e.g. base or fatty acid exchange rather than whole-molecule turnover, is suggested by the three phospholipid precursors showing a similar pattern of incorporation and elimination from the myelin phospholipids.

The adult myelin phospholipids showed little evidence of persistence of radioactivity after ^{32}P incorporation. Although the radioactivity in these phospholipids fell very slowly after 20 days a similar phenomenon was observed with the microsomal fraction and the acid-soluble phosphorus pool and little consistent difference in the specific radioactivities could be observed from 20 to 78 days after the administration of label. It seems probable that this type of persistence is caused by recycling of the ^{32}P retained in the animal economy. With [^{14}C]glycerol and [^{14}C]ethanolamine there was some suggestion of a persistence of labelling in the nitrogen-containing myelin phosphoglycerides, which usually had higher specific radioactivities at the longer time-periods than those in the microsomal fraction. Studies by Norton, Poduslo & Suzuki (1969) have suggested that limited myelination may still be occurring even in adult rats, since the amount of myelin recovered from brain was a linear function of the logarithm of age, so the persistence of labelling previously observed with label administered to developing animals may still occur to a limited extent. This persistence of labelling was more clearly seen in the present series of experiments if [^{32}P]P_i was injected into the brain of animals just at the commencement of myelination, when at longer time-periods the specific radioactivities of the myelin phospholipids were appreciably higher than those in the microsomal fraction. However, appreciable radioactivity still remained in both the microsomal phospholipids and the acid-soluble phosphorus and part of the persistence in the myelin fraction can be explained by continued synthesis from ^{32}P retained within the brain. Thus at 30 days after injection into the adult brain the specific radioactivity of the acid-soluble phosphorus pool

had fallen to approx. 8% of that at 5 days, whereas the corresponding value for the developing brain was nearly 30%. This is probably explained not by a greater retention of the injected ^{32}P within the brain, but by the radioactivity coming into equilibrium with a much smaller body-and-skeleton pool.

It is difficult to be absolutely certain from the present results as to the stage of brain development when the turnover of the brain myelin phospholipids observed in the adult animal commences. The fall in the specific radioactivity in the myelin phospholipids after ^{32}P injection in 10-day-old animals seemed to correspond closely to that of the acid-soluble phosphorus pool, suggesting that rapid turnover was occurring. However, maximum phospholipid deposition in rat brain occurs between 10 and 25 days postnatally (Sheltawy & Dawson, 1969), a period corresponding to maximum myelination as judged by histological and behavioural patterns (Donaldson, 1924; Himwich, 1962). If, owing to the decline of activity in the acid-soluble phosphorus pool, this newly synthesized phospholipid had a lower specific radioactivity than that reached by the small myelin pool immediately after the injection it would tend by dilution to produce a fall in specific radioactivity just at the time that the maximum loss is observed.

The results in general therefore support the concept of Davison (1963) that two metabolic pools exist in myelin, one a rapidly exchanging pool and the other a more persistent and slowly turning-over part, which can only be significantly labelled in the developing animal. It has been suggested that the rapid exchange occurs at the surface of the myelin sheath and that during myelination the myelin layers become gradually enveloped in fresh myelin and so become unavailable to dynamic metabolic processes. Thus it has been calculated from studies with labelled sulphate ions that the fast-exchanging pool of myelin sulphatide amounts to only 0.2% of the total pool (Davison & Gregson, 1966). The present results showing the rather similar specific radioactivities of the individual phospholipids of microsomal fraction, myelin and acid-soluble phosphorus pool at times longer than 10 days after the injection into the brain and the similar rates of rapid decline would suggest that as far as phospholipids are concerned the buried pool in myelin must be small. A substantial part of the membrane phospholipids in myelin must be exchangeable rather than only those close to or on the surface of the sheath. Although approximately 30% of the myelin constituting the small myelin fraction was not examined in the present investigation the available evidence would suggest that the turnover of this may be even more rapid than that of the large myelin fraction (Ansell & Spanner, 1967, 1968). The results help to explain the observation of Rathbone (1965) that it is possible to change the fatty acid composition of myelin with diet. It is not difficult to comprehend how such an exchange could occur, since it merely requires an exchange of phospholipids between the successive unit membranes, which are assumed to constitute the sheath. The exchange of newly labelled phospholipid molecules brought from the synthetic site with the surface layer of myelin would be followed by a redistribution of the phospholipid molecules in the myelin lipid bilayers. The situation is somewhat analogous to the attack of phospholipases on large liquid crystals of phospholipid substrate. The water-soluble enzyme attacks the outermost bilayer of the substrate and then the lipoidal products of the reaction simply redistribute with fresh phospholipid molecules coming to the surface from the innermost bilayers (Bangham & Dawson, 1962). In a lipoprotein environment phospholipid molecules are believed to have much lower energy barriers against diffusion compared with those in a hydrated organized complex of pure phospholipid molecules (Dawson, 1968). Dobiašová & Radin (1968) have demonstrated an uptake *in vitro* of phosphatidylcholine from an aqueous suspension into purified myelin fractions especially in the presence of salts, but it is not clear whether this represents a true exchange or heterocoagulation of the two particles. As yet we have not been able to demonstrate an exchange of phospholipids between a labelled brain microsomal fraction and unlabelled myelin particles *in vitro* (E. K. Miller & R. M. C. Dawson, unpublished work).

F.B.J. gratefully acknowledges a Fellowship from the National Multiple Sclerosis Society of America. Miss N. Hemington is thanked for valuable technical assistance.

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