The Deposition and Metabolism of Polyphosphoinositides in Rat and Guinea-Pig Brain during Development

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1. The deposition of triphosphoinositide and diphosphoinositide in rat and guinea-pig cerebral hemispheres during growth was measured. 2. The maximum increase in concentration of both of these phospholipids occurs during the period of myelination, but in the rat some di- and tri-phosphoinositide is present before significant myelination begins. 3. In guinea-pig cerebral hemispheres the polyphosphoinositides remaining after post-mortem breakdown are selectively enriched in dissected white matter compared with grey matter. 4. The polyphosphoinositides in the cerebral hemispheres of rats were labelled with injected ^{32}P very rapidly; the specific radioactivities were in the order triphosphoinositide > diphosphoinositide > monophosphoinositide > total lipid phosphorus. 5. The synthesis of triphosphoinositide in rat forebrain occurs at an appreciable rate before, and at the start of, myelination, but the amount formed per gram of tissue is four to five times greater in adult rat brains, thus maintaining a constant turnover time (about 1 hr.) for the whole triphosphoinositide fraction. This indicates that the rapid turnover of triphosphoinositide is independent of myelin deposition. 6. The specific radioactivity of the brain acid-soluble phosphorus pool referred to a constant dose of ^{32}P /g. body wt. falls rapidly with age, reaching a minimum at 13–14 days, and then rises again. The specific radioactivities of the polyphosphoinositides reflect this change. 7. Part of the polyphosphoinositides in rat and guinea-pig cerebral hemispheres is rapidly hydrolysed *post mortem* leaving a stable portion resistant to further breakdown. 8. The rate and extent of post-mortem hydrolysis of the polyphosphoinositides in both species decrease with age. 9. After ^{32}P labelling, the specific radioactivity of the triphosphoinositide remaining in the cerebral hemispheres of the rat after post-mortem breakdown is lower than the original triphosphoinositide fraction, suggesting two metabolically distinct pools.

Polyphosphoinositides are found mainly in the nervous system, where they are predominantly concentrated in myelin or in structures intimately associated with it (Eichberg & Dawson, 1965; Sheltawy & Dawson, 1966; Salway, Harwood, Kai, White & Hawthorne, 1968). Earlier experiments indicated that, in marked contrast with other phospholipids of myelin, polyphosphoinositides rapidly incorporate inorganic [^{32}P]phosphate (Eichberg & Dawson, 1965), and it is probable that most of the radioactivity resides in the mono-esterified phosphate groups (Brockerhoff & Ballou, 1962a,b).

Enzymes involved in the synthesis (Colodzin & Kennedy, 1965; Kai & Hawthorne, 1967; Kai, Salway & Hawthorne, 1968) and the hydrolysis

(Thompson & Dawson, 1964; Dawson & Thompson, 1964) of polyphosphoinositides have previously been studied in extracts, homogenates or subcellular organelles under conditions inevitably different from those of the intact tissue. More information was therefore needed on the synthesis and catabolism of these phospholipids in the intact brain, particularly in relation to the development of the nervous system.

In this paper we studied the metabolism of polyphosphoinositides in the developing and the adult brain of the rat and the guinea pig. The course of deposition of these phospholipids is described in both animals and the data are correlated with their patterns of myelination. The rate of incorporation of ^{32}P into the polyphosphoinositides and acid-soluble phosphorus of rat brain and its variation with development were also

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studied. In addition, some evidence bearing on the existence of brain triphosphoinositide in different pools was obtained by studying the variation of the course of post-mortem hydrolysis with age and the fate of incorporated ^{32}P during this hydrolysis.

To make our data as physiologically meaningful as possible we have limited our studies to the cerebral hemispheres. This is because myelination is known to begin at different times in different parts of the nervous system (Jacobson, 1963). Thus myelination proceeds from peripheral to central areas, from projection to association, and from sensory to motor centres. This pattern is repeated in the cerebral hemispheres themselves, although it is known from microscopical evidence that in the rat no significant myelination begins in these parts before the tenth day after birth. The guinea pig, on the other hand, is born with its cerebral hemispheres almost fully myelinated.

EXPERIMENTAL

Animals. Female rats or guinea pigs were weighed, and injected with 1 mc of [^{32}P]orthophosphate (The Radiochemical Centre, Amersham, Bucks.) in 0.9% NaCl containing 0.1 mg. of KH_2PO_4 phosphorus/ml. (when appropriate). In the experiment described in Fig. 3 the dose of ^{32}P was adjusted according to the body weight.

Collection of tissue. The animals, which were sometimes anaesthetized with 1.0–1.2 g. of urethane/kg. body wt., were killed by decapitation with a guillotine, and the heads were frozen as quickly as possible in liquid N_2 . Very young rats were killed by freezing them in liquid N_2 , and the heads were later separated from the frozen bodies.

In some experiments where the post-mortem hydrolysis of polyphosphoinositides was determined the decapitated heads were left for a specified time at room temperature, after which the cerebral hemispheres were dissected. They were immediately weighed and divided into two suitable portions for direct extraction with organic solvents and trichloroacetic acid respectively. In other experiments, the heads were frozen and the cerebral hemispheres were removed after the skull was chiselled away. The frozen brain tissue was carefully searched for bone fragments and then crushed either in a mortar or in a thick-walled centrifuge tube cooled with liquid N_2 . Care was taken not to allow the brain temperature to rise since accelerated post-mortem hydrolysis may occur on thawing (Hayashi, Yagihara, Nakamura & Yamazoe, 1966).

Extraction and analysis of lipids. Lipids were extracted by homogenizing the brain tissue once with 20 vol. of chloroform-methanol (1:1, v/v) and twice with 20 vol. of chloroform-methanol (2:1, v/v). To the combined extracts prepared by centrifuging, chloroform and 0.9% NaCl soln. were added to give final proportions of chloroform-methanol–0.9% NaCl soln. of 8:4:3 (by vol.). The lower phase that separated was then washed twice more with theoretical NaCl soln. upper phase by the method of Folch, Lees & Sloane-Stanley (1957). Portions were then assayed for radioactivity, as described below, and total lipid phosphorus (Fiske & Subbarow, 1925).

The bound polyphosphoinositides were extracted from the residue with acidified solvents by the method of Dawson & Eichberg (1966). As reported by Sheltawy & Dawson (1966), the addition of butylated hydroxytoluene (1 $\mu\text{g.}$ /60 $\mu\text{g.}$ of phospholipid phosphorus) to the extracting solvent improved both the recovery and the chromatographic behaviour of polyphosphoinositides. The extracts were washed and prepared for chromatography on formaldehyde-treated paper by the procedure of Sheltawy & Dawson (1966). The chromatograms were developed by ascending chromatography at 4° with the solvent system butan-1-ol–acetic acid–water (4:1:5, by vol.). The solvent was not stored for more than 6 weeks or else multiple bands of triphosphoinositide appeared. No significant difference was found between the specific radioactivities of polyphosphoinositides analysed by this technique and by acid hydrolysis (Dawson & Eichberg, 1965), thus excluding any possibility of contamination with tenaciously bound P_i sometimes encountered during the chromatography of whole phospholipids.

The phosphorus-containing spots were cut out and digested in HClO_4 (Dawson, 1960). Water (8.4 ml.) was added to the digest and when appropriate the radioactivity was determined with a liquid-counter tube (M6 tube; 20th Century Electronics Ltd., Croydon, Surrey). Counting was continued for a period long enough to yield a significance level better than 5%, and all counts were corrected back to the time of killing the animals. The phosphorus content of the samples was then determined by the method of Bartlett (1959).

Extraction and analysis of acid-soluble phosphorus. Cerebral hemispheres were ground with sand (acid- and organic solvent-washed), and then extracted twice with 10 vol. of ice-cold 10% (w/v) trichloroacetic acid. Crushed frozen tissue was similarly extracted. The combined extracts were washed twice with an equal volume of diethyl ether. (The ether washings contained no ^{32}P .) A suitable portion (containing 30–50 $\mu\text{g.}$ of phosphorus) of the aqueous phase was then digested and the radioactivity was determined as described above. Phosphorus in these digests was determined by the method of Fiske & Subbarow (1925) since the presence of cations in the original extract led to spurious colours in the Bartlett (1959) procedure. At least 30 min. was allowed for full colour development, since traces of trichloroacetic acid or its decomposition products inhibited colour development.

RESULTS

Concentration of polyphosphoinositides in rat and guinea-pig brain during growth. The accumulation of triphosphoinositide and diphosphoinositide in the forebrains of rats and guinea pigs during growth is shown in Figs. 1 and 2. Shortly after birth the rat forebrain contains small but significant concentrations of polyphosphoinositides (18% and 30% of adult values for tri- and di-phosphoinositide respectively). The concentration of triphosphoinositide increases until about 40 days of age, when the constant adult value is reached. However, the most marked increase occurs in the 12–25-days period. The accumulation of diphosphoinositide follows a similar course except that little change in

Fig. 1. Accumulation of polyphosphoinositides and total lipid phosphorus in rat forebrain during development: ●, triphosphoinositide; △, diphosphoinositide; ○, total lipid phosphorus.

Fig. 2. Concentration of triphosphoinositide (○) and diphosphoinositide (■) in guinea-pig forebrain during development.

Table 1. *Polyphosphoinositides in grey and white matter of guinea-pig cerebral hemispheres*

Guinea pigs weighed 600g., and an average of 1437 mg. of grey matter and 743 mg. of white matter was collected from each brain. Dissection took 10–15 min. to complete. Results from three animals are shown. Results are expressed as µg. of phosphorus/g.

Content (µg. of phosphorus/g.)			
Grey matter		White matter	
Triphospho- inositide	Diphospho- inositide	Triphospho- inositide	Diphospho- inositide
4.5	1.5	22.8	7.0
3.0	1.7	14.8	2.4
8.9	1.3	21.0	12.5

guinea-pig brain. The distribution of polyphosphoinositides between the grey and white matter of guinea-pig cerebral hemispheres was measured (Table 1). A comparison of the results with the values for the whole tissue (Fig. 2) clearly shows that appreciable post-mortem loss must have occurred during the time required for dissection, since an average of only 38% and 56% of the tri- and di-phosphoinositide respectively is recovered. Nevertheless, of the polyphosphoinositides remaining the major portion is localized in the white matter (67% and 71% of tri- and di-phosphoinositide respectively).

The incorporation of ³²P into the residual polyphosphoinositide of grey and white matter is given in Table 2. If it is assumed that the acid-soluble phosphorus is the precursor of triphosphoinositide, calculation shows that there is no major

concentration occurs in the first 10 days of life. The concentration of total lipid phosphorus in the cerebral hemispheres also increases most rapidly during the 12–25-days period (Fig. 1). In contrast, the guinea pig is born with a far higher content of triphosphoinositide in its brain, and its full complement of diphosphoinositide (Fig. 2). Moreover, the concentration of triphosphoinositide does not stabilize as in the rat brain but slowly increases during adult life.

Polyphosphoinositides in grey and white matter of

Table 2. Exchange of ³²P of polyphosphoinositides in grey and white matter of guinea-pig cerebral hemispheres

Guinea pigs weighed approx. 600g. and were injected intraperitoneally with 1 mc of inorganic [³²P]phosphate. Dissection took 10–15 min. to complete. —, Not determined.

Time of exchange (hr.) ...	Sp. radioactivities (counts/min./μmole)					
	White matter			Grey matter		
	3·5	5·5	8·5	3·5	5·5	8·5
Acid-soluble phosphorus	2270	2220	2040	1765	1960	4360
Triphosphoinositide	1030	—	1550	850	—	1570
Diphosphoinositide	870	1080	1100	570	880	1040

Fig. 3. Incorporation of [³²P]phosphate into the acid-soluble phosphorus, phosphoinositides and total lipid phosphorus of rat forebrain, after intraperitoneal injection. (a) Rats 150–170g., 50 day; 1·6 μC of ³²P/g. body wt.; (b) rats 17–21g., 12 day; 5 μC of ³²P/g. body wt.; (c) rats 9–11g., 6 day; 5 μC of ³²P/g. body wt. Δ, Acid-soluble phosphorus; ●, triphosphoinositide; ○, diphosphoinositide; ■, monophosphoinositide; □, total lipid phosphorus.

difference between the rate of synthesis of this phospholipid in grey and white matter, although it is probably slightly faster in white matter.

Turnover of ³²P of polyphosphoinositides in rat

forebrain. A constant dose of [³²P]phosphate was injected intraperitoneally into 150–170g. rats (young adults) and the time-course of the deposition of radioactivity into the forebrain phosphoinositides

Fig. 4. Variation of the incorporation of ^{32}P into the acid-soluble phosphorus, polyphosphoinositides and total lipid phosphorus of rat forebrain with development. The ^{32}P dose was $5\mu\text{C/g.}$ body wt., injected intraperitoneally. —, Exchange period 4-6 hr.; ----, exchange period 2-3 hr. ○, Acid-soluble phosphorus; ●, triphosphoinositide; △, diphosphoinositide; ■, total lipid phosphorus.

Fig. 5. Post-mortem depletion of polyphosphoinositides in the forebrain of rats at various stages of development (body wt.: ○, 300g.; ■, 200g.; ●, 100-110g.; △, 55g.). —, Triphosphoinositide; ----, diphosphoinositide.

was measured. Fig. 3(a) shows that the maximal specific radioactivity of the polyphosphoinositides is reached in about 3 hr. and that the incorporation into triphosphoinositide and diphosphoinositide and also monophosphoinositide is markedly greater than that into the whole phospholipid fraction. In fact, the phosphorus in the polyphosphoinositides is showing within 3 hr. practically the same specific radioactivity as the total acid-soluble phosphorus pool.

Similar time-course experiments with younger

animals (Figs. 3b and 3c) showed again a rapid incorporation of ^{32}P into the polyphosphoinositides. However, in these younger animals the specific radioactivity of the acid-soluble phosphorus fraction reaches a peak more rapidly.

The incorporation of ^{32}P into these phospholipids was also determined in a series of rats of various ages, after these had been injected with a constant dose of $[\text{}^{32}\text{P}]\text{phosphate}$ per g. body wt. (Fig. 4). Two exchange periods of 2.3 and 4.6 hr. were used in these experiments. The specific radioactivity-age curves of both the brain acid-soluble phosphorus and the phospholipids show a constant form, starting high in the younger animals, dipping to a minimum by 13-14 days for acid-soluble phosphorus, 17-19 days for diphosphoinositide and 20-27 days for triphosphoinositide. Thereafter the specific radioactivities of all fractions rise steadily (Fig. 4).

Post-mortem changes in the polyphosphoinositides of brain. The rapid partial depletion of the triphosphoinositide content of rat brain after death described by Eichberg & Dawson (1965) was confirmed. However, the rate and magnitude of the fall appear to depend on the stage of development, being appreciably faster and more extensive in the younger animal (Figs. 5 and 6). There is also an appreciable fall in the diphosphoinositide content of rat brain *post mortem* at all stages of development examined (Fig. 5). Unlike that with triphosphoinositide, the magnitude of the fall does not appear to depend on the body weight, but possibly any difference would be masked by the greater rate of diphosphoinositide formation from triphosphoinositide in the younger animal.

A similar depletion of both triphosphoinositide

Fig. 6. Post-mortem depletion of triphosphoinositide in forebrains of guinea pigs (○) and rats (●) at various stages of development.

Table 3. Post-mortem depletion of polyphosphoinositides in guinea-pig brain at various stages of development

Time post mortem (min.) ...	Triphosphoinositide (μg. of phosphorus/g.)		Diphosphoinositide (μg. of phosphorus/g.)	
	0	5	0	5
Body wt. (g.)				
100	27.5	12.6	6.7	3.2
300	33.8	23.5	7.5	2.1
400-500	38.0	29.4	6.0	2.5

and diphosphoinositide was observed in the guinea-pig brain (Table 3), and again the depletion of triphosphoinositide occurs more rapidly in the younger animal (Fig. 6).

These observations were made on animals anaesthetized with urethane, which had been decapitated and the heads immersed in liquid nitrogen at various times after death. However, no difference was detected in the post-mortem depletion of triphosphoinositide between two groups of rats decapitated with and without anaesthesia (Table 4).

Experiments were performed to investigate whether the post-mortem loss altered the specific radioactivity of triphosphoinositide previously labelled with ^{32}P (Table 5). The specific radioactivities of triphosphoinositide are expressed relative to that of the acid-soluble phosphorus to eliminate variations in absorption and injection of ^{32}P . Throughout the 20 min. post-mortem hydro-

Table 4. Post-mortem depletion of triphosphoinositide in control and anaesthetized rats

Time post mortem (min.) ...	Rats weighed 107-114 g. Triphosphoinositide (μg. of phosphorus/g. wet wt.)			
	0	2	10	20
	Control	27.5	23	20
Anaesthetized	39.5	27.5	—	21

Table 5. Effect of post-mortem changes on the relative specific radioactivity of triphosphoinositide in rat brain

Expt. no. ...	Sp. radioactivity of triphosphoinositide phosphorus (counts/min./μg.)	
	Sp. radioactivity of acid-soluble phosphorus (counts/min./μg.)	
Time post mortem (min.)	1	2
	4	12.3
Wt. of rats (g.) ...	50-65	150-200
0	1.12	1.040
	0.91	0.850
		1.060
2	0.78	
	0.94	
5		0.655
10		0.680
		0.435
15		0.750
		0.710
20	0.67	0.800
	0.68	
	0.64	

lysis no change occurs in the specific radioactivity of the acid-soluble phosphorus pool, this being 101% and 98.3% of the original values after 10 and 20 min. respectively. The relative specific radioactivity of triphosphoinositide falls significantly after periods of 5 min. or longer post mortem (Table 5), whereas that of monophosphoinositide or phosphatidic acid does not show a similar change.

DISCUSSION

The results presented agree in general with the conclusions reached from previous studies (Eichberg & Dawson, 1965; Sheltawy & Dawson, 1966; Salway *et al.* 1968) that the bulk of the polyphosphoinositides that occur in nervous tissue are actually part of, or intimately associated with, the myelin sheath. During growth the maximum accumulation of triphosphoinositide and diphosphoinositide in the cerebral hemispheres of the rat

occurs in the period (12–25 days) that is now generally considered to be that in which maximum myelination takes place, as judged by histological and behavioural patterns (Donaldson, 1924; Himwich, 1962). This also coincides with the period of maximum deposition of the so-called myelinic lipids such as cerebroside (Folch, 1955) and plasmalogen (Korey & Orchen, 1959), which are known from subcellular fractionation studies to be enriched in the myelin membrane (Eichberg, Whittaker & Dawson, 1964). This contrasts with the period of deposition of Nissl and ground substance (4–10 days after birth; Jordan, March & Messing, 1956) and the period of dendritic growth (6 days after birth; Donaldson, 1924).

However, our results, which agree with the concurrent studies of Rossiter & Gardiner (1966), Eichberg & Hauser (1967) and Wells & Dittmer (1967), clearly show that about 20% and 25% of adult concentrations of triphosphoinositide and diphosphoinositide respectively are already present in rat brain shortly after birth. This is before any significant myelination can have occurred. Two explanations of this are possible. First, polyphosphoinositides may be present in cellular membranes other than myelin that appear in the brain before significant myelination has occurred. Polyphosphoinositides do occur as minor components of non-neural tissues such as the kidney (Dawson & Eichberg, 1965), or even in non-innervated cells, e.g. erythrocytes (Hokin & Hokin, 1964) and Ehrlich ascites tumour (Palmer, 1965). Also, both Eichberg & Dawson (1965) and Kai & Hawthorne (1966) found small amounts of polyphosphoinositide in subcellular membrane fractions from brain other than myelin. The second explanation is that polyphosphoinositides may occur as components of the Schwann cells, which are present in the brain before myelination and which are, at a later stage, responsible for myelin formation. Certain lines of reasoning do in fact suggest that polyphosphoinositides, although intimately associated with myelin, are not components of the alternating layers of lipid and protein that constitute the myelin sheath (Dawson, 1966).

In contrast with the rat, the guinea pig is born with a far greater percentage of its adult complement of triphosphoinositide in the cerebral hemispheres, and the same diphosphoinositide concentration as is found in the adult (Fig. 2). This corresponds to the degree of neonatal neurogenesis in the two species. Thus the rat is born somatically and behaviourally immature, whereas the guinea pig can stand and run at birth. The rat brain at birth is not myelinated and its weight increases about sixfold post-natally, whereas the guinea-pig brain is partially myelinated at birth and its weight increases less than twofold during

post-natal life (Donaldson, 1924; Wender & Hierowski, 1960; Himwich, 1962).

The preponderance of both polyphosphoinositides in the dissected white matter of guinea-pig forebrain (Table 1) can be used as an additional argument for their being intimately associated with myelin structures. It is difficult to obtain a clean separation of the grey and white matter and this may account for much of the 33% and 29% of triphosphoinositide and diphosphoinositide respectively found in grey matter. However, the low recoveries obtained show that a good deal of post-mortem hydrolysis of these phospholipids had occurred. Previous workers also recovered only small concentrations of undifferentiated polyphosphoinositides from the grey matter (Hörhammer, Wagner & Holzl, 1960; Lébaron, McDonald & Sridhara Ramarao, 1963; P. Kemp & M. Kai, unpublished work). It is possible that in white matter enzymes destroying triphosphoinositide may be deficient, so that after post-mortem hydrolysis the depletion is selectively in grey matter. In this connexion, recent evidence suggests that triphosphoinositide phosphomonoesterase occurs only in low concentrations in isolated myelin membranes, and that it is predominantly a cytoplasmic enzyme (Salway, Kai & Hawthorne, 1967).

The results obtained on the incorporation of ^{32}P into the polyphosphoinositide fraction (Fig. 3) emphasize the remarkable metabolic activity of these phospholipids compared with the bulk of the phospholipids present in the tissue. That rapid metabolism of the brain phosphoinositide fraction occurs had been realized since the early work of Strickland (1952) on the undifferentiated fraction, and more recently it has become apparent that the monoesterified phosphate groups on the polyphosphoinositides are mainly responsible for the rapid turnover (Dawson, 1966).

There are many difficulties in calculating from the isotopic-labelling data the way in which the synthesis of polyphosphoinositides varies with the development of the animal. If one assumes that the precursor ATP used for synthesizing triphosphoinositide is in equilibrium with the forebrain acid-soluble phosphorus pool, then it is obvious from Fig. 3 that it is necessary to consider the whole curves of the time-course of labelling at each age group rather than the simpler specific-radioactivity ratios at a single time of isotope exchange. The manner in which the forebrain acid-soluble phosphorus specific radioactivity comes more rapidly to a peak in the younger animal is probably due to the greater permeability of the 'blood-brain barrier' to labelled phosphate in the circulation (Bakay, 1953; Himwich, 1962). However, calculations of the relative rates of synthesis of triphosphoinositide with age can be made, with allowance for

this differing form of the precursor time-curve and also for the variation in the size of the pool of this phospholipid (Fig. 1). These show that the forebrain before myelination (6 days) carries out an active synthesis of triphosphoinositide. The amount of triphosphoinositide phosphorus formed/g. of brain/hr. increases during myelination, and in the adult animal, with myelination complete, it is at a value that is approximately four to five times greater than in the 6-day-old animal. Thus the rapid ^{32}P turnover is maintained even when the full adult complement of triphosphoinositide has been laid down during the myelination (Fig. 1). Calculation of the turnover time for triphosphoinositide phosphorus (the time for synthesis of an amount equal to the tissue pool) shows this to remain rather constant during development (1 hr.), suggesting that the physiological role of the rapid triphosphoinositide phosphorus turnover can be divorced from the process of myelin deposition. The rates of synthesis observed *in vivo* are about half the activities of the triphosphoinositide-synthesizing enzyme found in homogenates (Salway *et al.* 1968), but they increase during development in a manner similar to that found by Salway *et al.* (1968). Such studies *in vitro* do, of course, measure only the potentiality of a tissue for synthesizing phospholipid, which is not necessarily the same as the actual synthesis that occurs in the intact animal.

The variation of the specific radioactivity of both brain triphosphoinositide and brain diphosphoinositide with development when referred to a constant dose per unit of body weight of the rat (Fig. 4) shows the same form as that obtained by Lebaron *et al.* (1963) for an undifferentiated phosphatidopeptide fraction, whose lipids probably consisted mainly of polyphosphoinositides. However, the present results show that this is probably partly due to the specific radioactivity of the acid-soluble phosphorus fraction from which these phospholipids are labelled (Fig. 4). In fact the total phospholipid extract shows the same trend, although this is in contrast with the results of Lebaron *et al.* (1963).

The rapid fall in specific radioactivities in early life (with a constant dose per unit body weight) initially observed is similar to the pattern shown by whole-brain phospholipids *in vivo* (Fries, Changus & Chaikoff, 1940) and by brain slices *in vitro* (Fries, Schachner & Chaikoff, 1942). This initial fall in the specific radioactivity can probably be partly accounted for by the greater permeability of the so-called 'blood-brain barrier' of newly born animals to the circulating ^{32}P (Bakay, 1953; Himwich, 1962). However, the observations of McMurray (1964) indicate that the same decline of the specific radioactivity of rat brain phospholipids with age occurs with brain homogenates, where such

permeability barriers are presumably circumvented. Probably an important factor is the increasing size of the pool of the phospholipids with age, which results in a greater dilution of the newly synthesized ^{32}P -labelled phospholipids.

Above the age of 20–27 days, the specific radioactivities of acid-soluble phosphorus, triphosphoinositide and diphosphoinositide in rat brain increase with age (Fig. 4). It is possible that as growth proceeds less of the injected ^{32}P is used to support the growth of other organs, especially the bones, so that the plasma radioactivity falls less rapidly after injection and more of the ^{32}P is available to be incorporated into the brain. Therefore with a constant dose of ^{32}P per unit of body weight the brain acid-soluble phosphorus pool becomes more radioactive in the older animal. Both diphosphoinositide and triphosphoinositide, which rapidly exchange phosphorus with the acid-soluble phosphorus pool, again reflect this change. It should be noted that, if the results are expressed as the percentage of the total dose injected that is incorporated into the phospholipids in 1 g. of brain, as in the investigation of Fries *et al.* (1940), the rise in the specific radioactivity of the total brain phospholipid is missed. However, these authors did note an abrupt change in the rate of decrease of phospholipid radioactivity when the rats achieved a body weight of 30–50 g. (i.e. age 15–30 days, Fig. 4).

The investigation of the post-mortem hydrolysis of brain triphosphoinositide has revealed that this is related to age, depletion being greater in the younger animal (Fig. 5). This agrees with the result of a preliminary experiment carried out concurrently by Eichberg & Hauser (1967). The earlier findings of Dawson & Eichberg (1965), which suggested that post-mortem breakdown was more extensive in the brain of the rat than in the guinea pig, can in fact be attributed to the different ages of the animals used.

The faster rate of breakdown in the younger animal cannot be ascribed to a greater concentration of triphosphoinositide phosphomonoesterase, since the activity of this enzyme increases with age and remains fairly constant once myelination is complete (Salway *et al.* 1968). It is doubtful also if the difference in the rate of post-mortem hydrolysis with age can be explained on the basis of incomplete myelination, with the myelin partially laid down in the younger animal being more accessible to enzymic decomposition; for in both species the fall in the rate of post-mortem hydrolysis with age still occurs over a period when myelination is generally considered to have finished. Moreover, the difference is independent of the initial amount of triphosphoinositide in the brain, since at all the different stages of development

examined in the rat the triphosphoinositide was at its constant adult value. Nevertheless, there is some evidence that in the adult rat two metabolically distinct pools of triphosphoinositide may exist. Thus in animals weighing over 100 g. the concentration of brain triphosphoinositide rapidly falls *post mortem* and then reaches a constant value. This stable part of the triphosphoinositide seems to increase with age (Fig. 5). In addition, when rat brain triphosphoinositide was labelled with ^{32}P before the rat was killed, its specific radioactivity was found to have decreased throughout the 20 min. allowed for post-mortem hydrolysis (Table 5) (cf. Eichberg & Hauser, 1967).

These results suggest the existence of more than one pool of triphosphoinositide in rat brain. Kai & Hawthorne (1966) reported that the mono- and di-phosphoinositide extracted from the subcellular fractions prepared from ^{32}P -labelled rat brain have different specific radioactivities, depending on their subcellular distribution and on whether acidified or neutral solvents were used for their extraction. It is possible that the decrease in relative specific radioactivity of triphosphoinositide may be explained on the basis that triphosphoinositide phosphomonoesterase, being localized in the cytoplasm (Salway *et al.* 1967), acts more readily on the substrate in peripheral layers of the myelin. If this accessible fraction of the triphosphoinositide is also the fraction most readily labelled from a cytoplasmic pool of ATP (see above), then a decrease in the specific radioactivity of the total pool of brain triphosphoinositide might be expected during post-mortem hydrolysis.

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