demonstrate carboxylase activity also excludes the carboxylation of acetaldehyde as a means of fixation. One route to which the data do seem to apply is that of a pentose diphosphate carboxylation. This mechanism has generally been associated only with photosynthetic cells. Santer & Vishniac (1955). Trudinger (1956), Aubert, Milhaud & Millet (1956) and Suzuki & Werkman (1958) described this type of fixation as occurring in chemoautotrophic bacteria of the genus *Thiobacillus* and found that both whole cells and cell extracts formed phosphoglyceric acid when supplied with carbon dioxide and ribulose diphosphate. According to the enediol mechanism suggested by Calvin (1954) 3-phosphoglyceric acid resulting from a carboxylation with $^{14}\text{CO}_2$ would contain ^{14}C only in the carboxyl carbon atom. A carboxyl-labelled 3-phosphoglyceric acid therefore, if metabolized via the Embden-Meyerhof route, should produce carboxyl-labelled lactic acid.

The presence of glucose 6-phosphate dehydrogenase and phosphoriboisomerase activity as well as the evidence for an Embden-Meyerhof system in this organism (Wirschafter & Jahn, 1956) lend support to the suggestion of the existence of such a system.

SUMMARY

1. The end product of the fixation of $^{14}\text{CO}_2$ by *Trichomonas vaginalis* was characterized by chromatography and by its infrared spectrum as lactic acid.
2. The ^{14}C occurred only in the carboxyl group of lactic acid.
3. Extracts of the organism contained glucose

6-phosphate dehydrogenase and phosphoriboisomerase, but no carboxylase.

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Phospholipid Metabolism in Nervous Tissue

2. METABOLIC STABILITY*

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Davison & Dobbing (1959) put forward a provisional hypothesis that the brain constituents, in common with the constituents of other tissues, may be divided into at least two main groups according to their biological half-lives. Evidence was presented for the possible existence of a phospholipid

compartment in the brain whose metabolic stability was much greater than could be accounted for by the known turnover rates of even its most slowly metabolized components. A possible explanation was that phospholipids of certain anatomical structures, for example the myelin sheaths, were metabolically inert, whereas in other parts of the brain these same phospholipids were metabolic-

* Part 1: Davison & Dobbing (1959).

ally active. This seemed to reconcile a current view, that brain phospholipids were rapidly metabolized at a rate comparable with that in other tissues, with our own findings with [$3\text{-}^{14}\text{C}$]serine and ^{32}P that labelled phospholipid molecules were still to be found in substantial quantity long after such activity had disappeared from other organs (Davison, Morgan, Wajda & Wright, 1959; Davison & Dobbing, 1959).

Much of the central nervous system, including the myelin sheath, undergoes no morphologically detectable change once maturity is reached, and there is virtually no possibility of new formation of neurons or myelin sheaths during adult life even when these structures are damaged by disease. Thus it is predictable on the basis of our hypothesis that it should be possible to demonstrate a non-structural, metabolically labile group of brain phospholipids in both young and adult animals; but that the metabolic inertness already shown when [$3\text{-}^{14}\text{C}$]serine is given to developing animals should not be demonstrable by the injection of isotopes into adults. Such a group of structural, stable constituents could, moreover, only be distinguished in long-term *in vivo* experiments in which they had been labelled during the course of myelinogenesis (Davison, Dobbing, Morgan & Wright, 1958, 1959; Davison, Morgan, Wajda & Wright, 1959).

The experiments presented here were designed to investigate this aspect of the problem and to determine to what extent metabolic inertness could be demonstrated in brain phospholipids by the use of ^{32}P .

METHODS

Two groups of white rats of different ages received an intraperitoneal injection of ^{32}P as phosphate in 0.9% sodium chloride soln. and were eventually killed by chloroform. In the first group, 19 rats (two litters) each received $25\text{ }\mu\text{C}$ of ^{32}P in 0.25 ml. of 0.9% sodium chloride soln. on the sixteenth postnatal day, when they weighed 30–35 g. They were reared in identical fashion and killed in pairs at intervals up to 116 days, and thereafter singly up to 189 days after injection. In the second group, seven adult rats of the same strain weighing 300–400 g. each received $1\text{ }\mu\text{C}$ of ^{32}P /g. body wt. as a solution containing $1\text{ }\mu\text{C}$ /ml. of 0.9% sodium chloride. The second group were killed singly at intervals up to 179 days after injection. All animals in both groups survived the rather high initial injection without any apparent ill effects. The developing series gained weight at a normal rate.

A third group of seven rats of the same litter each received $2\text{ }\mu\text{C}$ of [$1\text{-}^{14}\text{C}$]glycerol intraperitoneally on the thirteenth post-natal day, and a fourth group of adult rats of the same strain were each injected with $10\text{ }\mu\text{C}$ of [$1\text{-}^{14}\text{C}$]glycerol by the same route. These two groups were killed at intervals up to 110 days after injection.

Extraction procedures. Extraction of total lipid from brain, spinal cord and sciatic nerve by 20 times their volume of chloroform-methanol (2:1, v/v), together with

the trichloroacetic acid extraction of acid-soluble phosphorus from the blood, were by the conventional methods previously described (Davison & Dobbing, 1959).

Determination of ^{32}P radioactivity. Radioactivity of suitably diluted lipid extracts from the first two groups of animals was determined in a G.M. β -counter for liquids until 96 days after the injection of ^{32}P . After this time counting was continued in an Ekco type N612 liquid scintillation counter operated at room temperature, with an a.c. supply stabilized to $\pm 0.25\%$ by a Servomex a.c. stabilizer. A Dynatron N101 single-channel discriminator was employed which reduced the background of the counter to 14.3–18.2 counts/min. (see Table 1). The amplifier was used at a low-gain setting of $\times 50$.

The solvent used for counting was xylene (reagent grade, May and Baker Ltd.) with 2:5-diphenyloxazole as scintillator solute. Suitable portions of lipid extracts (up to about 30 ml. of brain extract) were evaporated to dryness and the residue was dissolved in xylene plus scintillator. Background counts were first determined on 20 ml. of xylene plus scintillator in a sample container, by repeated 5 min. counts, up to 15 times. The dried lipid extract was then dissolved in the same xylene plus scintillator and the radioactivity redetermined in the same sample container for an identical time. This procedure was necessitated by the finding that different sample containers gave different background counts (13.5–20.6 counts/min.; see Table 1). In addition, it was shown that the performance of the scintillation-counting equipment was stable throughout the lengthy period of the experiment, a background count being made on each occasion, with the same preparation of solvent and scintillator in a standard container (see 'Standard background', Table 1).

To correlate radioactivity determined in the liquid β -counter with that in the scintillation counter, several high-activity samples were counted by both methods. It was found that the scintillation technique gave a count which was constantly six times as high, and the results determined by this technique were therefore divided by six. The background counts for both methods were of a similar order.

Determination of ^{14}C radioactivity. Plasma was treated with 15 times its volume of acetone-ethanol (1:1, v/v). After being filtered, the extract was dried and the residue suspended in xylene and phosphor, and the radioactivity determined in the scintillation counter. The ^{14}C activities of whole-brain lipids and protein-free lipids were also determined by scintillation counting as previously described (Davison, Morgan, Wajda & Wright, 1959).

RESULTS

The whole-brain counts of phospholipid ^{32}P for 189 days after injection of inorganic ^{32}P into neonatal animals are shown in Fig. 1, together with the blood levels of trichloroacetic acid-soluble ^{32}P for the first 50 days of this period. No counts were made until the eleventh day after injection because events in the early stages had already been investigated (Davison & Dobbing, 1959). From 11 days onwards the pattern of the decline in brain-phospholipid ^{32}P has two main components: a steady rate of loss until about 100 days after in-

jection, after which time there is no demonstrable decline. The part of the curve drawn to represent lipid levels of radioactivity during the second half of the experiment is the calculated best straight line through the relevant points (96–189 days). The slope of the regression line (16 ± 12 counts/min./day) is consistent with the points falling on a horizontal line. Acid-soluble phosphorus had disappeared from the blood long before this second, stable component appeared in the results. A similar pattern is discernible in Fig. 2, which shows the persistence of phospholipid ^{32}P in the spinal cords and sciatic nerves of the same series of young animals. The small size of the sciatic-nerve sample gave rise to a greater degree of experimental error in the later samples of low activity, and for this reason it was found impracticable to investigate the radioactivity of peripheral nerve after 150 days.

The demonstration of the complete stability of a

Fig. 1. Radioactivity in phospholipids of whole brain (●) and acid-soluble phosphorus of blood (○). Rats were injected with ^{32}P when 16 days old. Radioactivity in blood is expressed as $10^{-3} \times$ counts/min./10 ml. of trichloroacetic acid extract. The continuous line is the best straight line calculated by the method of least squares for the points 96–189 days. See Table 1 for standard errors.

Fig. 2. Radioactivity in phospholipids of rat spinal cord (●) and sciatic nerve (○). Rats were injected with ^{32}P when 16 days old. Radioactivity is expressed as $10^{-3} \times$ counts/min./mg. of phosphorus. The relative standard errors increased from 1 to 28% for the spinal cord and from 3 to 50% for the sciatic nerve during the course of the experiment.

Fig. 3. Radioactivity in phospholipids of whole brain (●) and acid-soluble phosphorus of blood (○) of adult rats previously injected with ^{32}P . Radioactivity is expressed as $10^{-3} \times$ counts/min./whole brain. Radioactivity in blood is expressed as $10^{-3} \times$ counts/min./10 ml. of trichloroacetic acid extract. The relative standard errors increased from 1 to 18% during the course of the experiment.

Table 1. *Radioactivity of brain-lipid extracts*

A liquid β -counter was used up to 96 days after injection; thereafter radioactivity was determined by scintillation counting as described in the text.

Time after injection (days)	Time each sample counted (min.)	Counts/min.				Total corrected brain-lipid radioactivity (counts/min./whole brain)	Standard error (counts/min./whole brain)
		Standard background	Background for sample container	Sample count	Standard error		
96	40	—	13.8*	13.2	1.01	5390	412
96	40	—	11.8*	13.9	0.97	4780	334
116	20	14.3	15.6	30.4	1.75	2665	153
116	20	15.0	13.5	27.2	1.65	2840	172
130	60	18.0	15.5	24.7	0.97	4330	170
139	40	14.6	14.7	14.5	1.05	4070	294
154	40	15.4	15.4	9.8	1.01	2990	308
166	50	18.2	20.6	7.04	0.96	6450	882
175	50	15.3	19.5	4.18	0.93	4920	1100
183	50	14.8	17.7	4.04	0.89	6470	1430
189	75	18.1	20.2	1.69	0.75	4880	2160

* Background count in the G.M. β -counter tube.

proportion of the brain phospholipids depends greatly on the validity of the counting procedure during the last 80 days of the experiment. Table 1 expresses these particular results in more detail. The actual counts obtained on the brain-lipid extracts and their associated backgrounds are shown, together with the standard error of the samples (S.E. $\sqrt{[2B+S]/t}$, where B is total background count, S is total sample count, t is time; background and sample being counted for equal periods).

The changing levels of whole-brain-phospholipid ^{32}P in the adult animals are shown in Fig. 3, together with the values for trichloroacetic acid-soluble ^{32}P in whole blood. Unlike the results with the young animals, the adult-brain phospholipid has not achieved its maximum radioactivity 15 days after injection, but when it does so it subsequently declines at a rate very similar to that of the more labile phospholipids of the immature brain. The pattern of blood radioactivity in the older group, however, contrasts very markedly with that in the young. The level in the adult blood is at all times much higher. Fifty days after injection it is nearly eight times as high, owing to the more rapidly declining radioactivity in the young, and a quite unexpected persistence in the adult blood is demonstrable up to at least 116 days after injection. There is no evidence from these results of any complete metabolic inertness in the brain phospholipids of this series.

After injection of $[^{14}\text{C}]$ glycerol into young and old rats, the blood radioactivity fell within a week to negligible values in both cases. When expressed in terms of whole-brain activity, comparable and maximal amounts of ^{14}C were incorporated into brain lipids of the two groups on the first day

Fig. 4. Radioactivity in whole-brain lipids of young and adult rats injected with $[1-^{14}\text{C}]$ glycerol. Rats were injected with $[1-^{14}\text{C}]$ glycerol when 13 days old. Radioactivity of whole-brain-lipid extract (●) and protein-free lipid (○) are recorded. Results for adult rats are whole-brain-lipid extract (▲) and protein-free lipid (△) multiplied by two.

(Fig. 4). The rate of decline of activity in the old rats was, from the beginning of the experiment, however, consistently much greater than that in the young.

DISCUSSION

The most important conclusion to be drawn from these results is that a measurable proportion of the brain-phospholipid phosphorus, as is shown in Fig. 1, possesses a high degree of metabolic stability which may indeed be virtually complete. This apparently stable compartment could not have been revealed in an experiment lasting much less than 6 months: it did not even appear in our earlier, 70-day, experiment (Davison & Dobbing, 1959) because it is obscured for about 100 days under these conditions by the incorporation of ^{32}P into other, more labile, phospholipids. The extent to which this finding supports a hypothesis of metabolic inertness of the chemical constituents of the myelin sheath must now be discussed.

Complete proof for the hypothesis demands the demonstration of stability in all the differing constituent molecules that comprise the anatomical structure. So far this has been accomplished in similar experiments for the cholesterol molecule by the use of $[4-^{14}\text{C}]$ cholesterol (Davison *et al.* 1958; Davison, Dobbing, Morgan & Wright, 1959), and for those parts of certain phospholipids as well as cerebrosides and cholesterol into which the ^{14}C of $[3-^{14}\text{C}]$ serine was incorporated in developing animals. The present results suggest the same principle for phospholipid ^{32}P . The possibility must of course, be considered that a dynamic state, exists internally within the brain, or within the myelin sheath, in such a way as not to be revealed by this type of experiment. If this were so, however, the metabolic processes involved would have to take place by means of intermediates which did not themselves leak into the circulation, and the efficiency of re-incorporation into phospholipid would have to be complete. 'Internal turnover' in this sense remains a possibility, but seems most unlikely. ^{14}C and ^{32}P can both be incorporated in permanent fashion into brain phospholipid, although strictly, of course, this remains to be demonstrated simultaneously in the same molecules. Further support for the hypothesis may be derived from the evidence that the persisting $[4-^{14}\text{C}]$ cholesterol is mainly located in the white matter (Davison, Dobbing, Morgan & Wright, 1959) and that the same is true of the persisting phospholipid ^{32}P (Davison & Dobbing, 1960).

Our findings must also be discussed in the light of those of Thompson & Ballou (1954, 1956). These authors studied the biological half-lives of the components of a large number of tissues by means of long-term experiments with tritium as an indi-

cator, and, in their earlier paper, first demonstrated a general stability of brain phospholipids. Later they were able to show that biological half-lives, although presenting a wide range of values, may be divided grossly into two main groups. Thus more than half of the total brain constituents of rats exposed to tritium during their development have an average half-life as long as 150 days; and of the phospholipids in the entire body, one-third have an average half-life of about 220 days. Of the tissues used collagen was the only one that appeared to be virtually completely inert. Our approach differs essentially from theirs in being a more specific study of brain phospholipids. Presumably the main reason for the non-appearance in their figures of such stability of brain phospholipids as we have demonstrated is that they have inevitably included all the brain constituents labelled with tritium, many of which have very great metabolic activity, and by taking a single composite figure the stability of some of them has been obscured. Both their 'long-lived' tissue component and their 'shorter-lived' components would be included in our 'labile' group. The metabolically inert group of brain lipids is, in these terms, a third, 'permanent' component and is to be compared with collagen and possibly actomyosin whose inertness appeared likely to be 'not an intrinsic property of the molecules themselves, but an expression of the morphological stability of the fibrous structures of which these proteins form a part' (Henriques, Henriques & Neuberger, 1955).

A necessary corollary of the inertness of these anatomical structures within the brain is that it should be possible to incorporate injected material into them only during the course of their development, and not into the adult structure. Fig. 3 shows the results of an attempt to investigate the long-term fate of phospholipid ^{32}P after the injection of ^{32}P into adults. At first sight it has not been convincingly shown that there is no ultimate plateau in the curve, but several of its features

merit further discussion. Prominent amongst these is the very much greater persistence, both in quantity and duration, of the acid-soluble ^{32}P in the blood of the adults compared with the young. Measurable and significant quantities of precursor ^{32}P in the blood are being presented for synthesis into brain phospholipids as long as 116 days after injection, long after the acid-soluble ^{32}P in the blood of the immature series of animals has declined to negligible values. The old and the young can be more usefully compared for these purposes by taking these differing blood levels into account, as can be seen from Table 2 in which the results are expressed as the ratios of whole-brain phospholipid to blood concentration of acid-soluble ^{32}P in each series. In the young the ratio rapidly approaches infinity as the blood levels approach zero, whereas in the adults the values for the ratio rise more slowly and then begin to decline. This subsequent decline would be unlikely to forshadow an impending plateau in brain concentrations. The apparent similarity in the rate of decline of adult and young brain-phospholipid ^{32}P is therefore fortuitous and may be considered to be attributable to the greater persistence of precursor in the blood of the former. The same factor may equally well account for the delay in achieving maximum labelling of brain phospholipids in the adult series compared with the young. These results illustrate the importance of estimating blood levels of precursor in all experiments of this kind. With regard to ^{32}P , the differences of persistence of this in blood are probably largely due to the influence of the skeleton, which in both series takes up a high proportion of the injected dose. In the developing animals most of the phosphorus incorporated into the bones is retained, whereas in the adults a similar amount is slowly liberated into the circulation during the ensuing months after injection, maintaining a significant level of availability of precursor phosphorus for phospholipid metabolism (unpublished observations).

Table 2. Whole-brain phospholipid ^{32}P expressed in terms of blood acid-soluble ^{32}P concentration, after injection of ^{32}P into neonatal and adult rats

Neonatal rats		Adult rats	
Time after injection (days)	Brain-phospholipid ^{32}P / Blood-acid-soluble ^{32}P	Time after injection (days)	Brain-phospholipid ^{32}P / Blood-acid-soluble ^{32}P
11	11.5	15	17.5
33	52	25	25.5
50	175	49	55
75	∞	74	100
		102	97
		116	63.5

The experiments with [$1\text{-}^{14}\text{C}$]glycerol injected into both neonatal and adult rats were undertaken to test the influence of surviving blood levels of isotope on the labelling of brain lipids. Glycerol is predominantly incorporated by guinea-pig cortex *in vitro* into phosphoglycerides (Hokin & Hokin, 1958), and only to a small extent into non-phosphorus-containing lipids. In the present experiments the blood level of ^{14}C 1 week after injection was negligible in both series, and may therefore be presumed to have had a similar influence on the degree of incorporation into brain lipids in each group. Fig. 4 shows that, from the outset, the rate of decline of activity in the brains of the younger series is much slower than that in the old, and presents a picture more in keeping with the findings of Thompson & Ballou (1956). The experiment did not continue long enough, however, to demonstrate the presence or otherwise of stable components.

As can be seen from Fig. 2, the spinal cord and peripheral nerve show the same extended persistence of phospholipid ^{32}P when the isotope is administered to neonatal animals as has been shown in brain.

In conclusion, the suggestion that there exists in nervous tissue some anatomical structure or structures whose constituents are metabolically inert is considerably supported by these findings. Particularly when taken in conjunction with similar results from the use of [$4\text{-}^{14}\text{C}$]cholesterol and [$3\text{-}^{14}\text{C}$]serine, it is reasonable to suppose that the inertness is due less to the molecular structure and biochemical properties of the constituents than to their presence in a stable anatomical unit.

SUMMARY

1. The fate of injected ^{32}P has been followed in blood and in the phospholipids of brain, spinal cord and sciatic nerve of developing rats for a period of 189 days after injection.

2. The fate of injected ^{32}P has been similarly followed in mature rats up to 179 days after injection.

3. Brain-lipid metabolism in developing and mature rats was also studied by means of [$1\text{-}^{14}\text{C}$]glycerol.

4. A proportion of the phospholipids of rat nervous tissue appears to be metabolically inert.

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