The Incorporation of Labelled Phosphate into the Lipids of a Brain Dispersion

By R. M. C. DAWSON*
Department of Biochemistry, University of Oxford

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It is now known from isotopic investigations with labelled phosphate that the phospholipid phosphorus present in adult brain tissue is in a state of dynamic equilibrium with the inorganic phosphate in the cerebral cells. On the basis of results obtained by the intravenous injection of labelled phosphate, it was originally thought that this phosphorus exchange of the brain phospholipids was extremely slow. However, when a correction is made for the very slow rate at which phosphate ions present in the circulation pass into the brain (Dawson, 1952) the rate of phospholipid phosphorus exchange becomes appreciable and comparable with that of other tissues.

In spite of the obvious importance of this process in the nervous system, it has been little investigated. Fries, Schachner & Chaikoff (1942) demonstrated that rat-brain slices could incorporate labelled phosphate into their lipids which suggested that part at least of the radioactive brain phospholipids found after *in vivo* isotopic experiments was actually synthesized in cerebral tissue. A continuation of these studies (Schachner, Fries & Chaikoff, 1942) showed that the uptake was accelerated by hexoses and largely abolished by anaerobic conditions, suggesting that the incorporation was associated with the respiration of the tissue. However, on disruption of the cellular organization by 'homogenization' the rate of uptake was greatly reduced and was no longer changed on the addition of glucose to the incubation medium.

The advantage of using tissue dispersions for investigating the process of phospholipid phosphorus exchange is that factors of cellular permeability do not complicate the interpretation of the results. It has been found in the present investigation that freshly prepared and correctly fortified cell-free dispersions of guinea-pig brain readily incorporate labelled phosphate into their lipids, at a rate which is easily measured by conventional counting methods. A technique has been developed by which the lipid fraction can be easily extracted and freed from contaminating inorganic phosphate and acid-soluble phosphate esters of high specific activity which are formed during the incubation. The purification is therefore better than methods which depend upon dilution of the contaminating radioactivity with inorganic phosphate. Hahn & Tyrén (1946) have shown that these dilution methods are unsatisfactory for removing contaminating radioactivity from lipid extracts of liver.

The results of experiments with brain dispersions show beyond reasonable doubt that the replacement of lipid phosphorus is a process which is dependent upon an active oxidative phosphorylation in the tissue, which makes it extremely unlikely that the incorporation of radioactive phosphorus (^{32}P) is due to the reversibility of the enzymic processes by which phospholipids are hydrolysed into inorganic phosphate and other simpler components. These hydrolytic enzymes are appreciably active in brain tissue (Sloane-Stanley, 1952) and consequently no net increase in total phospholipid can be detected.

It was observed by Friedkin & Lehninger (1949*a*) that the incorporation of labelled phosphate into the lipids of a liver particulate preparation was accelerated when the preparation was actively respiring with malate as substrate so that the phosphate was rapidly esterified. In a recent note Kennedy (1952) has reported that the uptake of phosphate into the lipids of isolated liver mitochondria is inhibited by the presence of 2:4-dinitrophenol.

A preliminary communication about this work has already been presented to the Biochemical Society (Dawson, 1953).

METHODS

Preparation of dispersions and incubation. A young adult male guinea pig was stunned, decapitated, and its brain rapidly dissected out and weighed. A dispersion of the whole brain was prepared by grinding in an ice-cold mortar according to the description of Banga, Ochoa & Peters (1939). After thoroughly grinding the brain into a uniform paste, a cold solution of 0·08M-KCl and 0·12M-NaF was very gradually worked into the paste until the dispersion was of the requisite dilution. It was then filtered through muslin or glass wool and stored until required at 0°, its ability to respire and to incorporate phosphate into its lipids being reasonably stable under these conditions. Histological examination of these dispersions by Mr E. H. Leach showed them to be virtually cell-free, although they

* Betty Brookes Research Fellow.

contained intact nuclei and mitochondria, together with fibrils and innumerable small particles. After incubation for 1 hr. in the presence of a substrate the mitochondria became less numerous and the number staining with Janus green decreased. Brain dispersions prepared in a Waring blendor showed a low capacity for incorporating phosphate into their lipids.

Samples of the dispersion were pipetted into Warburg vessels containing the incubation medium and labelled phosphate (approx. 6 μc) and 0·2 ml. 10% KOH in the centre cup. The vessels were immediately transferred to a Warburg bath and shaken at 37°. Respiration readings were commenced at 6 min. by which time the contents had attained temperature equilibrium with the bath. In experiments in which the carrier-free phosphate was tipped from the side arm the results were generally inconsistent. This is probably due to variable adsorption of the labelled phosphate on the glass (Harrison & Raymond, 1951; Lamerton & Hariss, 1951). At the end of the incubation 5–10 ml. of 20% (w/v) trichloroacetic acid were added to the contents of each flask. This destroys some of the plasmalogens which are unstable to acid even at room temperature (Feulgen & Bersin, 1939; Hack, 1947), but it is an important factor in ensuring that the final lipid extract is free from acid-soluble phosphate esters of high specific activity.

Filtration, extraction and washing of the lipids. The filtration of the precipitated lipids and proteins, and the subsequent solvent extraction of the lipids, were both carried out in the same Soxhlet extraction vessel. A series of these vessels were mounted as illustrated in Fig. 1. Each extractor of about 15 ml. capacity was tightly packed with glass wool to a depth of 2·5 cm., followed by a loose layer of glass wool. The mixture was transferred to the extractor with the aid of a funnel, when the trichloroacetic acid extract percolated through the glass wool, siphoning over into the bulb, where it collected as a clear filtrate. A sample of this filtrate was removed when required for examination of the acid-soluble phosphorus. Filtration was rapid provided that no more than 0·14 g. brain tissue which had been precipitated with trichloroacetic acid was filtered through 1 sq.cm. of the filter. The precipitate was then washed with about 60 ml. of 5% (w/v) trichloroacetic acid solution followed by 60 ml. of water; the final washings had a pH of 3–4. The end of a bent glass rod covered with soft rubber tubing was then inserted into the vapour orifice of the extractor, and air sucked through the precipitate until it was free from surplus supernatant. Subsequently the precipitate was extracted for 5 hr. with a mixture of equal volumes of $CHCl_3$ and methanol. Siphoning of the solvent was normal provided the bore of the siphon tube was not too narrow. At first troublesome and violent bumping of the solvent was experienced, and this was not eliminated by the usual remedies. It was then found that if the extraction flasks were washed after each run by refluxing $CHCl_3$: methanol in them a layer of insoluble matter gradually collected on the glass, which completely cured any tendency to bump. After extraction the solution of lipids was cooled, and poured into a separating funnel containing 60 ml. of N-HCl. The flask was washed with two 5 ml. portions of $CHCl_3$ which were added to the separating funnel. This was shaken mechanically for 5 min. and the lower $CHCl_3$ layer which separated rapidly was filtered through a Whatman no. 2 paper. The aqueous phase contained the contaminating acid-soluble phosphorus and experiments showed that further washing of the $CHCl_3$ layer with N-HCl did not remove any more radioactivity. Suspended in the aqueous phase was a curdy, white precipitate which was initially soluble in a $CHCl_3$: methanol mixture, but on standing in HCl became insoluble. Initially this precipitate contained a little phosphorus which had a specific activity equal to that of the lipid fraction, but on standing and then washing with ether the phosphorus content became negligible (<0·1%). When the precipitate was hydrolysed in 6 N-HCl and a two-dimensional chromatogram of the hydrolysate prepared, about fourteen amino acids could be detected by ninhydrin spraying, including large amounts of alanine and leucine isomers. It is therefore highly probable that the precipitate contains proteolipids whose presence in brain has recently been described by Folch & Lees (1951).

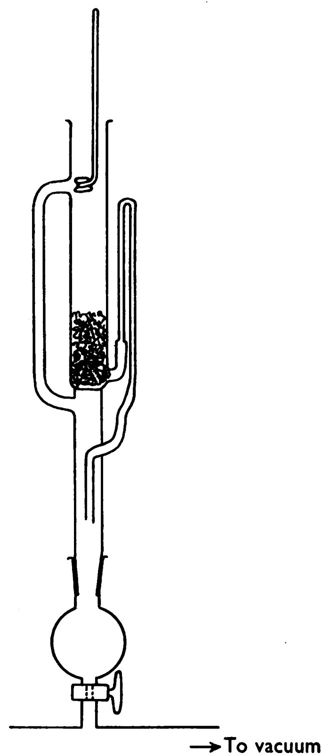

Fig. 1. Filtration and extraction unit.

After the $CHCl_3$ layer had been evaporated to dryness the lipids were oxidized and assayed for radioactivity by methods previously described (Dawson & Richter, 1950). Phosphorus estimations on duplicate samples were carried out by the method of Fiske & Subbarow (1929), the colour being read in a Beckman spectrophotometer at 660 mμ.

Materials

Glycylglycine. The commercial product (10 g.; British Drug Houses Ltd.) was dissolved in 48 ml. water, filtered under pressure, and 240 ml. ethanol slowly added to the filtrate. The precipitate was filtered, washed with ethanol and dried at room temperature. This reduced the inorganic phosphorus content to below 0·01%. The peptide was then dissolved in water and titrated to pH 7·2 with KOH.

Sodium pyruvate. This was prepared by a method based on the observations of Peters (1936) and Lipschitz, Potter & Elvehjem (1938). Pyruvic acid doubly distilled *in vacuo* was diluted with an equal volume of water and neutralized to pH 5 with NaOH solution, keeping the temperature below 30°. The salt was then crystallized by adding acetone until precipitation just commenced, followed by cooling to 0°. The sodium pyruvate was recrystallized at frequent intervals by the method of Robertson (1942).

Cytochrome *c* was prepared by the method of Keilin & Hartree (1937), but in later experiments a commercial preparation (Light & Co., Slough) was used.

The adenosine triphosphate (ATP) and diphosphopyridine nucleotide (DPN) used were samples prepared in this laboratory by Dr L. A. Stocken.

Radioactive phosphate. It was found that even traces of microflora growing in the stock carrier-free labelled phosphate solution greatly increased the zero-time 'incorporation' values. It could be stored satisfactorily for several weeks by diluting to a convenient volume with sterile water and storing in a closed container at 0°. Before pipetting the labelled phosphate solution into the Warburg vessels the pipette was rinsed six times with the solution to minimize adsorption effects.

Expression of results. The incorporations observed have been expressed in terms of the specific activities of the isolated lipid fraction, as this eliminates any error in the quantitative isolation of the lipids. In no experiment did the actual concentration of the lipid phosphorus alter sufficiently to change appreciably the specific activity of the fraction. It was calculated that the specific activity of the lipid phosphorus in an extract prepared from a brain dispersion respiring for 1 hr. in a complete medium could be measured with an accuracy of $\pm 4\%$.

RESULTS

The efficiency of the preparation of lipid

The method used in the present investigation to prepare lipid phosphorus for radioactive assay was designed to enable the lipid to be easily obtained from brain tissue in good yield and free from acid-soluble phosphorus. Experiments showed that only a further 1 % more lipid phosphorus was obtained on prolonging the solvent extraction to 15 hr. The precipitation with trichloroacetic acid resulted in the loss of about 5–15 % of the total phospholipid phosphorus present in the brain tissue. This loss was caused by the decomposition of a phosphorus-containing lipid which is estimated as sphingomyelin in the conventional method of estimation, and also by the release of some acid-soluble phosphorus from plasmologens. Measurements showed that less than 5 % of the lipid phosphorus present in an extract was lost during the purification procedure.

Zero-time 'incorporation'. The extreme difficulty of completely freeing lipid extracts from contaminating acid-soluble phosphorus has been noted by Fries *et al.* (1942) and McKibbin & Taylor (1949). With the present method of purification about 99 % of the radioactivity was removed from a lipid extract which had been deliberately contaminated with carrier-free labelled inorganic phosphate. In further experiments a solution of labelled acid-soluble phosphorus compounds was prepared from a brain dispersion which had been incubated with labelled phosphate. This solution was added to another dispersion which was then precipitated with trichloroacetic acid. A lipid extract of this precipitate after washing with hydrochloric acid possessed negligible radioactivity, showing that the labelled phosphorus compounds had been adequately separated from the lipids.

The specific activity of the lipid phosphorus isolated from a dispersion which had been immediately treated with trichloroacetic acid after adding labelled phosphate amounted to less than a few per cent of that isolated after incubation for 1 hr. in a complete medium. This residual, or zero-time, radioactivity appeared to be tenaciously 'bound' to the lipids and no easy method of removing it was discovered. It did not appear to be bound by enzymic action as it occurred with boiled dispersions and was increased somewhat by adding inorganic phosphate (0·005 M) to the medium. When labelled phosphate was added to a dispersion immediately after incubation for 1 hr. the zero-time 'incorporation' was appreciably less than that of the fresh dispersion, even after correcting for the differing concentrations of inorganic phosphate in the media. For this reason the values obtained in the present investigation have not been corrected for zero-time 'incorporation', as there is no evidence that such a correction would be valid.

Factors which affect the enzymic incorporation of labelled phosphate into the lipids

Initial experiments using dialysed dispersions showed a comparatively low rate of incorporation of labelled phosphate into the lipid fraction, and studies were therefore confined to the undialysed dispersions at various dilutions. It was found that the incorporation of phosphate occurred at a maximum rate during the first hour of an incubation at 37° the subsequent uptake being relatively slow (Fig. 2). The associated respiration also declined but it fell off much less rapidly than the incorporation. It is surprising that there is no apparent time lag at the beginning of the incorporation during which labelled precursors of the phospholipids are synthesized, and this must mean that such precursors attain a maximal specific activity very rapidly. In this connexion recent experiments have shown that labelled phosphate added to a kidney-cortex mitochondrial preparation reaches radioactive equilibrium with external ATP within a few minutes (Bartley & Davis, 1952). A similar rapid rate of exchange of the labile groups of ATP is found when

a liver dispersion is incubated with labelled phosphate (Ennor, 1952).

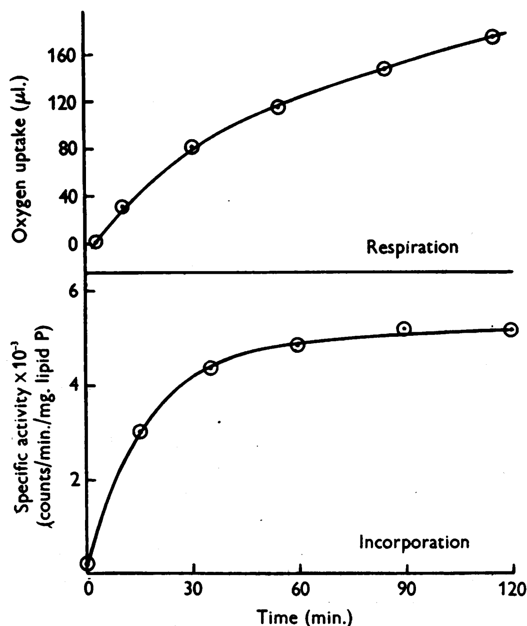

Fig. 2. The incorporation of labelled phosphate into the lipids of a guinea-pig brain dispersion. Medium: glycylglycine buffer, pH 7·2, 0·1 M; adenylate, 0·001 M; Mg^{2+}, 0·017 M; pyruvate, 0·03 M; fumarate, 0·0033 M; F^-, 0·017 M; cytochrome *c*, $3\cdot2\times10^{-5}$ M; ^{32}P, 10^6 counts/min. 240 mg. fresh brain in 3 ml.; 37°.

The composition of the medium used in the present investigation was based on the experiments of Case & McIlwain (1951) who investigated the conditions necessary for optimal phosphorylation and respiration in brain dispersions. However, glucose, ATP and some of the phosphate were usually omitted, because in general these were found to reduce the incorporation of labelled phosphate into the lipids. The incubations were carried out in the presence of fluoride which markedly increased the ^{32}P uptake. The rate of incorporation did not change appreciably when veronal or bicarbonate:carbon dioxide buffers replaced the glycylglycine buffer usually employed.

Additions to the incubation medium which were found necessary to obtain a maximum incorporation of labelled phosphate into the lipids are shown in Table 1 which presents the results of two typical experiments. The effects shown were all confirmed on two or three independent brain dispersions. For maximum incorporation the system requires the presence of an oxidizable substrate, magnesium ions, cytochrome *c*, adenylic acid and oxygen. It seems likely that these factors are necessary to ensure a high rate of oxidative phosphorylation and consequent esterification of the ^{32}P present in the incubation medium. It was shown by Friedkin & Lehninger (1949*b*) that both magnesium ions and cytochrome *c* are required in the system which esterifies inorganic phosphate during the passage of electrons from reduced diphosphopyridine nucleotide to oxygen. In the present experiments it was found that when magnesium ions were left out of the medium there was usually a slight stimulation of the respiration which was not observed when a phosphate buffer was also present in the medium. The presence of adenylic acid is probably necessary to ensure a supply of suitable phosphate acceptor (adenosine diphosphate, ADP) through the myokinase system (Ochoa, 1944; Hunter, 1949). On the addition of a limited amount of ATP to a dilute dispersion (40 mg. brain/ml.) a slight stimulation of the respiration was observed and this was usually

Table 1. *The incorporation of labelled phosphate into the lipids of a guinea-pig brain dispersion*

(Medium: glycylglycine buffer, pH 7·2, 0·1 M; adenylate, 0·001 M; Mg^{2+}, 0·017 M; pyruvate, 0·03 M; fumarate, 0·0033 M; F^-, 0·019 M; cytochrome *c*, $3\cdot2\times10^{-5}$ M; ^{32}P, 10^6 counts/min. 120 mg. fresh brain in 3 ml. total vol., incubated at 37° for 1 hr. in air.)

Experiment	Medium	Respiration (µl.)	Respiration % of that with complete medium	Specific activity lipid P (counts/min./mg. P)	Specific activity (% of that with complete medium)
1	Complete	77·5	100	12 600	100
	No Mg	87	112	5 680	45
	No adenylate	79	102	10 650	84
	No cytochrome *c*	86	111	7 580	60
	No pyruvate or fumarate	51·5	66	2 760	22
	Unfortified	67	86	2 260	18
	Complete + ATP, $2\cdot5\times10^{-4}$ M	80	103	13 700	109
2	Complete	77	100	12 800	100
	No fumarate	66	86	4 400	34
	No cytochrome *c*	75	97	9 900	77
	No incubation	—	—	79	0·6
	Complete + glucose, 0·031 M	79	102	3 820	30
	Complete + glutamate, 0·011 M	92	120	14 800	116

Table 2. *Effect of adenosine triphosphate on the incorporation of labelled phosphate into the lipids of a guinea-pig brain dispersion*

(Medium: glycylglycine buffer, pH 7·2, 0·05 M; adenylate, 0·001 M; Mg^{2+}, 0·017 M; pyruvate, 0·03 M; fumarate, 0·0033 M; cytochrome *c*, $2·2 \times 10^{-5}$ M; phosphate buffer, pH 7·2, 0·003 M; F^-, 0·022 M; ^{32}P, 10^6 counts/min. 240 mg. brain in 3 ml. total vol., incubated at 37° in air.)

ATP added* (M × 10^{-4})	Time of incubation (hr.)	Final concn. inorganic P (M × 10^{-3})	Respiration (μl.)	Specific activity lipid P (counts/min./mg. P)
0	0	3·75	—	256
0	1	2·49	180	6850
2·5	1	2·61	205	5720
5·1	1	2·59	207	5450
7·7	1	2·93	218	4320
12·2	1	3·00	196	4010

* ATP was a pure sample with a low inorganic pyrophosphate content; labile phosphate was determined by 10 min. hydrolysis in N-HCl.

associated with an increase in the rate of incorporation of ^{32}P into the lipids. At higher concentrations of ATP or on adding ATP to less dilute dispersions (80 mg./ml.) there was a definite inhibition of ^{32}P uptake (Table 2). It would seem, therefore, that because of the rapid post-mortem depletion of ATP (Maleci, 1950) it is necessary to add a limited amount to dilute dispersions to obtain a satisfactory concentration of ADP for phosphate acceptance. Above this concentration it inhibits the uptake into the lipids either by a direct isotopic dilution effect or by breakdown into inorganic phosphate which lowers the specific activity of the labelled phosphate from which the labelled phospholipid precursors are synthesized.

The addition of a coenzyme A concentrate (20 units) to a respiring dispersion (80 mg. brain/ml.) produced no appreciable change in the incorporation of ^{32}P into the lipids. Two DPN samples assaying at 40 and 85% pure both produced a slight inhibition of the incorporation when added at a concentration of 0·001 M.

Influence of substrates. The necessity of added oxidizable substrate for the incorporation of ^{32}P into the lipids is clearly seen in Table 1. It is surprising that the incorporation is inhibited far more than the respiration by the absence of added substrate. This could be due to utilization of residual substrates in the brain homogenates whose oxidation is not so favourably coupled with the esterification of ^{32}P as the oxidation of pyruvate plus fumarate. Glucose at low concentrations had a pronounced inhibitory action on the incorporation so that when present as the sole added substrate the incorporation was less than 10% of that with pyruvate plus fumarate as substrate (Table 3). This inhibitory effect of glucose was also observed in the presence of pyruvate plus fumarate (Table 1), and it is therefore likely that, as hexokinase is present in the dispersion, the glucose is competing with phospholipid precursors for the labelled phosphate bonds of ATP. A similar but smaller inhibition of the incorporation was observed on adding 2×10^{-2} M-creatine to the incubation medium, and it is probable that here the creatine is competing with phospholipid precursors for the labelled phosphate bonds of ATP through the Lohmann reaction.

Table 3. *Effect of different substrates on the incorporation of labelled phosphate into the lipids of a guinea-pig brain dispersion*

(Medium: glycylglycine buffer, pH 7·2, 0·1 M; phosphate buffer, pH 7·2, 0·001 M; adenylate, 0·001 M; Mg^{2+}, 0·017 M; F^-, 0·019 M; cytochrome *c*, $3·2 \times 10^{-5}$ M; $^{32}P = 10^6$ counts/min. 120 mg. fresh brain in 3 ml. total vol., incubated at 37° for 1 hr. in air.)

Substrate	Respiration (μl.)	Specific activity lipid P (counts/min./mg. P)
Pyruvate, 0·03 M + fumarate, 0·0033 M	86	6210
Fumarate, 0·02 M	76	5290
α-Ketoglutarate, 0·02 M	103	3880
Glutamate, 0·02 M	97	5700
Malate, 0·02 M	96	7680
Succinate, 0·02 M	240	2950
Glucose, 0·02 M	50	540

The use of glutamate as an oxidizable substrate resulted in a rate of incorporation similar to that found with pyruvic plus fumarate as substrate (Table 3) which agrees with the satisfactory utilization of glutamate as a substrate for oxidative phosphorylation by cerebral mitochondria (Abood & Gerard, 1952; Brody & Bain, 1952). On adding glutamate to a dispersion respiring with pyruvate plus fumarate as substrate there was a slight stimulation of both the respiration and the incorporation. The possibility that this increased incorporation was due to an inhibition of phosphatase action by glutamic acid (Bodansky, 1948) was considered, but experiments showed that the addition of histidine had no stimulatory action on the incorporation. The utilization of succinate as oxidizable substrate

resulted in a high rate of respiration, but the incorporation was only about half that obtained with pyruvate plus fumarate as substrate (Table 3). This is in keeping with the results of Ochoa (1941) and Brody & Bain (1952), who found that cerebral particulate preparations had a high rate of respiration and a low P/O ratio when succinate was the substrate. The succinic-oxidase system is known to be very stable on incubation at 37°, but at the same time the oxygen uptake becomes uncoupled from phosphorylation.

Anaerobic conditions. In anaerobic experiments the Warburg vessels were flushed with nitrogen and shaken for 5 min. with yellow phosphorus in the centre cup. The labelled phosphate was then tipped into the main compartment from the side arm. The results of anaerobic experiments in which the dispersions used were prepared with and without fluoride are presented in Table 4. Anaerobiosis resulted in a considerable reduction in the rate of incorporation of labelled phosphate into the lipids but the lowered rate of incorporation was increased somewhat by the addition of glucose. The stimulation produced by glucose was greatest in the absence of fluoride. This may be due to the higher rate of glycolysis in the dispersion producing a lower residual glucose. Although the quantitative interpretation of the results is complicated by the inhibitory action of glucose on the incorporation, there seems little doubt that anaerobic glycolysis can to a limited extent support the uptake of ^{32}P into the lipids. The use of yellow phosphorus resulted in a high degree of anaerobiosis, but it was shown that the phosphorus pentoxide fumes often resulted in a transfer of phosphorus into the medium. Presumably the metaphosphate formed would be quickly converted into orthophosphate but it was calculated that the amount transferred in the present experiments would not significantly affect the results obtained.

Table 4. *Anaerobic glycolysis and the incorporation of labelled phosphate into the lipids of a guinea-pig brain dispersion*

(Medium: glycylglycine buffer, pH 7·2, 0·1 M; adenylate, 0·001 M; Mg^{2+}, 0·017 M; fumarate, 0·0033 M; cytochrome *c*, $3{\cdot}2\times10^{-5}$ M; ^{32}P, 10^6 counts/min. 240 mg. fresh brain in 3 ml. total vol., incubated at 37° for 1 hr.)

	Specific activity lipid P (counts/min./mg. P)	
Conditions	No fluoride	+0·022 M Fluoride
Glucose (0·0154 M), aerobic	1060	1830
Glucose (0·0154 M), anaerobic	470	520
No glucose, anaerobic	200	450

Addition of fluoride. When a dispersion was incubated with pyruvate plus fumarate as substrate, the addition of fluoride resulted in a marked increase in the incorporation of labelled phosphate into the lipids and a decrease in the respiration. The curve relating fluoride concentration and incorporation (Fig. 3) reaches a maximum at 0·02 M, and its general shape is very similar to that obtained by Case & McIlwain (1951) when they determined the relationship of fluoride concentration and phosphorylation in brain dispersions. It is therefore likely that fluoride inhibits the active adenosine triphosphatase present in brain tissue and thus prevents the breakdown of labelled ATP enabling it to be used for phosphorylating precursors of the phospholipids.

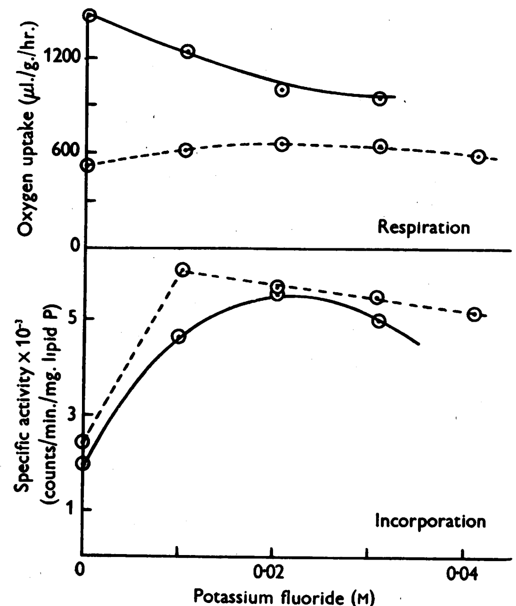

Fig. 3. The effect of fluoride on the incorporation of labelled phosphate into the lipids of a guinea-pig brain dispersion. Medium: glycylglycine buffer, pH 7·2, 0·1 M; with and without phosphate buffer, pH 7·2, 0·003 M; with and without adenylate, 0·001 M; Mg^{2+}, 0·017 M; pyruvate 0·03 M; fumarate 0·0033 M; ^{32}P, 10^6 counts/min. 240 mg. fresh brain in 3 ml., 1 hr., 37°. ⊙--- ⊙, Adenylate and phosphate buffer omitted; ⊙—⊙, adenylate and phosphate buffer added.

When adenylic acid and phosphate were omitted from the incubation medium it was observed that although there was markedly increased incorporation on adding fluoride, the oxygen uptake was not inhibited, but actually slightly increased (Fig. 3). Further experiments indicated that the decreased respiration on adding fluoride was only observed if adenylic acid was present in the medium and that the inhibition was independent of the presence of a phosphate buffer.

Table 5. *Effect of inhibitors on the incorporation of labelled phosphate into the lipids of a guinea-pig brain dispersion*

(Medium: glycylglycine buffer, pH 7·2, 0·1 M; adenylate, 0·001 M; Mg^{2+}, 0·017 M; phosphate buffer, pH 7·2, 0·001 M; pyruvate, 0·03 M; fumarate, 0·0033 M; F^-, 0·022 M; cytochrome *c*, $2 \cdot 1 \times 10^{-5}$ M; ^{32}P, 10^6 counts/min. 240 mg. fresh brain in 3 ml. total vol., incubated at 37° for 1 hr. in air.)

Inhibitor	Concentration (M)	Increase (+) or decrease (−) in respiration (%)	Incorporation of ^{32}P into lipids. Inhibition (%)
Arsenate	$4 \cdot 4 \times 10^{-3}$	+5	32
	$8 \cdot 8 \times 10^{-3}$	+6	42
Azide	10^{-3}	+4	78
Calcium chloride	1×10^{-3}	−16	77
	$3 \cdot 5 \times 10^{-3}$†	−45	89
2:4-Dinitrophenol	10^{-4}	+81	47
Gramicidin	5×10^{-6}‡	+28	66
Iodoacetate	3×10^{-3}	−16	21
Malonate	$4 \cdot 3 \times 10^{-3}$	−3	16
	$4 \cdot 3 \times 10^{-2}$	−26	45
Methylene blue	$2 \cdot 5 \times 10^{-4}$	+2	77
	10^{-3}	−24	81
2-Octanol	$3 \cdot 6 \times 10^{-2}$ added	−86	92
Phlorrhizin	$4 \cdot 5 \times 10^{-3}$	−57	72
Potassium cyanide*	10^{-3}	−48	76
Potassium sulphate	$3 \cdot 3 \times 10^{-2}$	+20	23

Respiration of control dispersions = 126–148 µl.
Specific activity of lipids of control dispersions = 5710–7225 counts/min./mg. P.

* KCN/KOH in centre Warburg cup.
† Phosphate buffer 0·003 M.
‡ 0·015 ml. 10^{-3} M in 66% ethanol.

When adenylic acid is present, the myokinase in the dispersion will ensure that the respiration will not be inhibited by the lack of phosphate acceptor (ADP), but on the addition of fluoride the inhibition of myokinase produced (Barkulis & Lehninger, 1951) will limit ADP formation and so inhibit respiration. In the absence of adenylic acid little or no formation of ADP by myokinase action will occur, and the respiration will be limited by the lack of a phosphate acceptor. Under these conditions the addition of small quantities of fluoride is not likely to have an appreciable inhibitory effect on the respiration. The fact that there is a slight stimulation of the oxygen uptake could possibly mean that the removal of the limited supplies of ADP by myokinase is inhibited more than its formation by the action of adenosine triphosphatase (Ochoa, 1944; Hunter, 1949; Barkulis & Lehninger, 1951).

Action of inhibitors. The effects of various inhibitors on the respiration and incorporation of labelled phosphate into the lipids of brain dispersions are shown in Table 5. These experiments were all conducted in the presence of fluoride and with pyruvate plus fumarate as substrate. Inhibitors of respiration, namely cyanide, malonate, iodoacetate, octanol and phlorrhizin all caused a reduction of the incorporation of ^{32}P which was always greater than the corresponding inhibition of the respiration. Agents which can cause an uncoupling of oxidative phosphorylation, e.g. arsenate, azide, 2:4-dinitrophenol, gramicidin, methylene blue and sulphate ions, all produced at a suitable concentration a stimulation of the respiration, and a reduction in the incorporation. In these experiments with inhibitors measurements were made of the inorganic phosphate plus labile phosphate present in the final incubation mixture, and in no instance did the concentration differ appreciably from that of a corresponding control.

The effect obtained on adding calcium ions to a dispersion is also shown in Table 5, and it is seen that even at a normal serum concentration of ionizable calcium (10^{-3} M) there was a profound inhibition of the incorporation with a slight reduction in the rate of oxygen uptake. Keltch & Clowes (1951) have shown that a low concentration of calcium inhibits the aerobic phosphorylation of glucose in particulate brain preparations, with little or no effect on the respiration.

Tonicity of incubation medium. When potassium or sodium chloride was added to an incubation medium, which was already isotonic, there was a marked inhibition of the incorporation of labelled phosphate into the lipids (Fig. 4). The effect on the respiration was far less marked and an inhibition only became apparent when the tonicity had increased by about 50%. Increasing the tonicity by the addition of sucrose also produced a decreased incorporation, but the inhibition was much less than that found with the electrolytes. Lehninger

(1949) has observed that the coupled phosphorylation of liver mitochondria is dependent upon the tonicity of the incubation medium, and Brody & Bain (1952) have noticed that cerebral mitochondria prepared in hypertonic sucrose (0·88 M) had very low P/O ratios. On the other hand, Slater & Cleland (1953) have recently reported that the P/O ratio of the α-ketoglutaric-oxidase system in heart-muscle preparations is only slightly reduced when the tonicity is increased by the addition of saline.

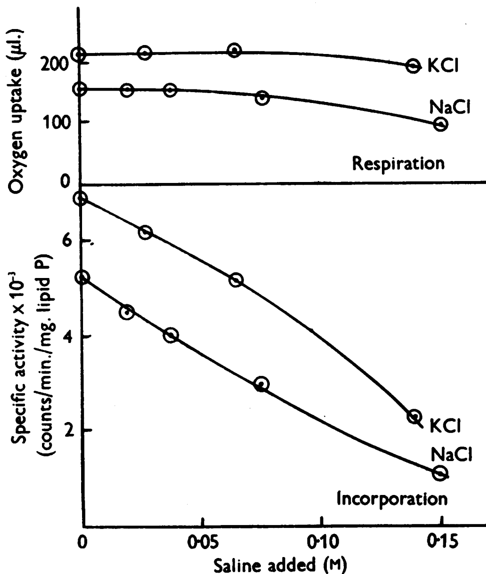

Fig. 4. Effect of hypertonic saline on the incorporation of labelled phosphate into the lipids of guinea-pig brain dispersion. Medium: glycylglycine buffer, pH 7·2, 0·05 M; phosphate buffer, pH 7·2, 0·003 M; adenylate, 0·001 M; Mg^{2+}, 0·017 M; pyruvate, 0·03 M; fumarate, 0·0033 M; F^-, 0·022 M; cytochrome *c*, $2 \cdot 1 \times 10^{-5}$ M. ^{32}P 10^6 counts/min. 240 mg. fresh brain in 3 ml., 1 hr., 37°.

Optimum pH. The optimum pH for the incorporation was measured using low molarity phosphate buffers (Na_2HPO_4/KH_2PO_4) in which the phosphate concentration was kept constant. Each medium was approximately adjusted to the required pH before the introduction of the buffer. It is seen from Fig. 5 that the pH optimum is near 6·5 and different from that of the respiration.

Phosphate concentration. Addition of inorganic phosphate to the incubation media reduced the incorporation of ^{32}P into the lipids. At the same time there was a stimulation of the oxygen uptake, and this probably accounts for the deviation of the curve plotting incorporation against phosphate concentration (Fig. 6) from that predicted by only considering the changing specific activity of the labelled phosphate precursor. It was consistently observed that the zero-time 'incorporation' of

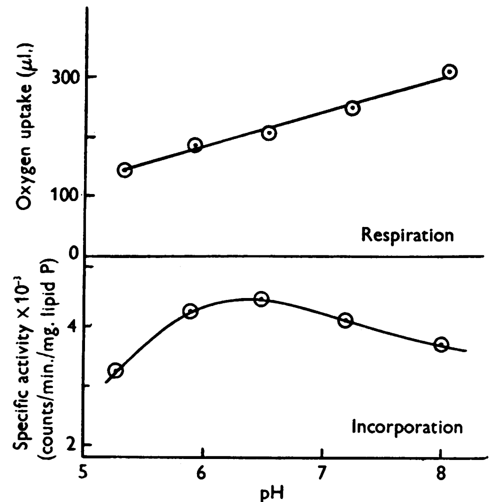

Fig. 5. Effect of pH on the incorporation of labelled phosphate into the lipids of a guinea-pig brain dispersion. Medium: phosphate buffer, 0·003 M; adenylate, 0·001 M; Mg^{2+}, 0·017 M; pyruvate, 0·03 M; fumarate, 0·0033 M; F^-, 0·022 M; ^{32}P, 10^6 counts/min. 240 mg. fresh brain in 3 ml., 1 hr., 37°.

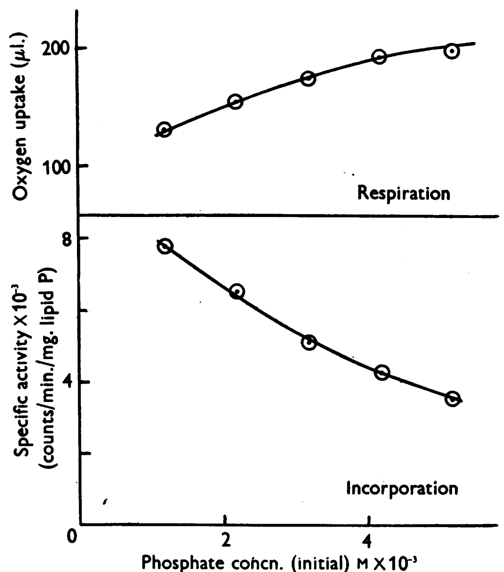

Fig. 6. Effect of initial phosphate concentration on the incorporation of labelled phosphate into the lipids of a guinea-pig brain dispersion. Medium: glycylglycine buffer, pH 7·2, 0·1 M; adenylate, 0·001 M; Mg^{2+}, 0·017 M; malate, 0·011 M; cytochrome *c*, $2 \cdot 1 \times 10^{-5}$ M; F^-, 0·022 M; ^{32}P, 10^6 counts/min. 240 mg. fresh brain, 1 hr., 37°.

labelled phosphate became higher when the inorganic phosphate concentration in the medium was increased. During the present investigation the concentration of phosphate which was added to the incubation medium in some experiments never exceeded the *in vivo* concentration of inorganic phosphate in the brain.

Addition of possible phospholipid precursors. Experiments in which various possible precursors of the phospholipids were added to the incubation medium are reported in Table 6. None of these additions produced an appreciable increase in the rate of ^{32}P incorporation into the lipid fraction, and it is therefore concluded that if any of these substances are required for the synthesis of labelled phospholipids they are present in sufficient concentration in the dispersion or they are unable to penetrate to the active centre of synthesis. The effect of oleate at a low concentration (2×10^{-3} M) is interesting, as it inhibits the respiration and even more markedly the incorporation. Peters & Wakelin (1938) have reported a similar inhibition of the respiration of a pigeon-brain brei on adding sodium oleate adjusted to pH 7·4. Moreover, the inhibitory action of fatty acids on the fatty-acid oxidase system is thought to be due to their toxic effect on some phase of oxidative phosphorylation (Lehninger, 1951). It is possible, therefore, that the soluble soap has a powerful destructive effect on the biochemical organization of the particulate matter in cell-free brain preparations. It is surprising, however, that under the present conditions of incubation, stearate had no similar action on the respiration or incorporation.

Table 6. *Effect of adding possible precursors on the incorporation of labelled phosphate into the lipids of a guinea-pig brain dispersion*

(Medium: glycylglycine buffer, pH 7·2, 0·05 M; adenylate, 0·001 M; Mg^{2+}, 0·017 M; malate, 0·02 M; F^-, 0·022 M; cytochrome *c*, $2·1 \times 10^{-5}$ M; phosphate buffer, pH 7·2, 0·004 M; ^{32}P, 10^6 counts/min. 240 g. fresh brain in 3 ml. total vol., incubated at 37° for 1 hr. in air.)

Substance	Concentration (10^{-3} M)	Respiration (% of control)	Specific activity of lipid P (% of control)
Control	—	100	100
Choline	8	94	102
Betaine	8	95	100
Ethanolamine	8	96	108
L-Serine	8	99	106
Inositol	8	97	102
Glycerol	8	100	102
Stearate	3	119	110
Oleate	2	59	17

Respiraton of controls = 199–264 μl.
Specific activity of lipid P of controls = 3355–3610 counts/min./mg. P.

On the addition of either α- or β-glycerophosphate at a concentration of 0·0025 M there was no appreciable change in the rate of incorporation of ^{32}P from a labelled 0·005 M-phosphate buffer. Although these phosphate esters both caused a slight stimulation of the respiration (see Peters & Sinclair, 1933) there is no evidence that they are penetrating to the active centres of phospholipid synthesis. From the results of experiments on the *in vivo* turnover of phospholipids in rat liver Popják & Muir (1950) have concluded that glycerophosphates are precursors of liver lecithin and kephalin, but they were unable to obtain confirmation of this from *in vitro* experiments. In a recent note Kennedy (1952) has reported that unlabelled glycerophosphate inhibited the incorporation of ^{32}P into the phospholipids of isolated liver mitochondria.

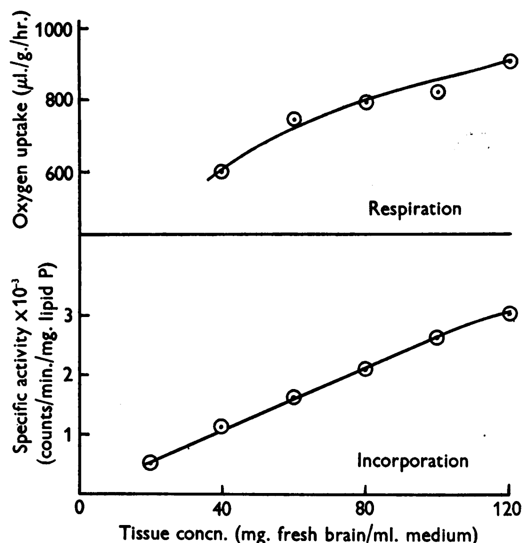

Fig. 7. Effect of tissue concentration on the incorporation of labelled phosphate into the lipids of a guinea-pig brain dispersion. Medium: glycylglycine buffer, pH 7·2, 0·1 M; ATP, 0·00025 M; Mg^{2+}, 0·017 M; pyruvate, 0·015 M; fumarate, 0·0033 M; F^-, 0·011 M; ^{32}P, 10^6 counts/min./g. fresh brain tissue. Incubated 1 hr. at 37°.

Tissue concentration. It is seen from Fig. 7 that both the respiration and the incorporation of ^{32}P into the lipids per unit weight of tissue increased with increasing tissue concentration. No stimulation of the uptake was observed on adding to the incubation medium an aqueous extract prepared from a boiled brain dispersion.

DISCUSSION

The present investigation has indicated that the incorporation of labelled phosphate into the brain lipids is associated with oxidative phosphorylation.

The technique of separation used ensures that the lipid fraction is adequately separated from contaminating acid-soluble phosphorus of high specific activity. There seems little doubt therefore that the active lipids isolated represent a genuine conversion of labelled phosphate into substances soluble in organic solvents. It is highly likely, but not proven, that this represents a synthesis of complete phospholipid molecules or their immediate precursors (e.g. phosphatidic acids). No net synthesis of lipid phosphorus could be detected, presumably because of the presence in the dispersion of catabolic enzymes which attack the phospholipids (Sloane-Stanley, 1952). However, the initial rate of incorporation observed in a dispersion respiring in a medium containing a physiological concentration of phosphate represents a minimal formation of 44 mg. phospholipids/100 g. tissue/hr. It is interesting that the corresponding *in vivo* figure for mouse brain calculated using a number of assumptions and approximations was 65 mg. phospholipids/100 g. tissue/hr. (Dawson & Richter, 1950).

It is a significant fact that many of the diverse agents which produce degenerative lesions of the nervous system are of a type which are known to interfere with energy-giving reactions in nervous tissue. They include, for example, anoxia (Morrison, 1946), cyanide, azide, carbon monoxide (Hurst, 1940, 1942), malononitrile (Hicks, 1950) and a deficiency of vitamins of the B group (Gildea, Kattwinkel & Castle, 1930). It is therefore possible that the interference with energy-giving reactions found in these conditions is inhibiting the normal dynamic replacement of such structural elements as phospholipids and proteins (see Peterson & Greenberg, 1952), and this may in turn initiate the production of degenerative lesions. The generation of such lesions in the central nervous system by feeding unnatural fats may also depend on such a blocking of the normal replacement of lipid structural elements (Schaltenbrand & Schorn, 1948).

SUMMARY

1. A simple technique has been devised for preparing a lipid extract of brain tissue containing a minimum of contaminating acid-soluble phosphorus.

2. The conditions have been determined for the maximum incorporation of labelled phosphate into the lipids of a guinea-pig brain dispersion.

3. For optimum incorporation the process requires oxidizable substrate, oxygen, phosphate acceptor, magnesium ions and cytochrome *c*, and is greatly accelerated by the presence of fluoride ions.

4. Hypertonic media and low concentrations of calcium ions and glucose strongly inhibit the incorporation.

5. The use of inhibitors has shown that those which are known to uncouple oxidative phosphorylation also uncouple the respiration of a dispersion from the incorporation of labelled phosphate into its lipids.

6. It is concluded that the initial step in the incorporation is an esterification of the ^{32}P into high-energy phosphate compounds, which then phosphorylate phospholipid precursors.

The author wishes to thank Prof. Sir Rudolph A. Peters, F.R.S., for his continual advice and interest in this investigation. He is greatly indebted to Mr E. H. Leach for his histological investigation of suspensions and also to Mr V. Butlin and Mr R. Lincoln for valuable technical assistance.

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The Amino Acid Composition of Salmine

BY M. C. CORFIELD AND A. ROBSON
Wool Industries Research Association, Torridon, Headingley, Leeds

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During recent years several authors have investigated the amino acid composition of salmine, employing a variety of classical and modern methods of analysis. Reference may be made to Tristram (1947), Hamer & Woodhouse (1949), Block, Bolling, Gershon & Sober (1949), Velick & Udenfriend (1951) and Mills (1952). The analyses of these authors clearly show that salmine contains only the seven amino acids: glycine, valine, alanine, isoleucine, proline, serine and arginine; Velick & Udenfriend (1951) identified a small amount of glutamic acid in their salmine hydrolysates but regarded it as an impurity. A careful examination of all these analytical data shows that, whereas there is good agreement for the glycine and arginine contents of salmine, there are fairly wide differences in the estimated amounts of the other five amino acids.

Blackburn & Robson (1953) found that the ratio of isoleucine to valine in salmine hydrolysates was almost exactly 1:3; a finding which agrees fairly well with that of Block *et al.* (1949), but not with the other analyses. We have, therefore, carried out a full analysis of our salmine sulphate sample to see if its amino acid composition is significantly different from that of samples hitherto analysed.

EXPERIMENTAL

Chromatographic techniques

The amino acid separations were carried out by chromatography on starch columns 30 cm. in length and 0·9 cm. in diameter by the methods of Stein & Moore (1948). Two samples of starch were used, one being manufactured by Gordon Slater Ltd., Manchester, and the other (labelled Jalan A48) supplied by James Laing & Co. Ltd., Manchester. Both samples gave satisfactory resolution of the amino acids but the Jalan A48 starch was the finer of the two, a 30 cm. column having a flow rate of 1·3 ml./hr. under 15 cm. Hg pressure, whilst the Gordon Slater starch gave a flow rate of 1·5 ml./hr. under a pressure of 6 cm. Hg. The Jalan A48 starch, however, had a much higher column blank than the Gordon Slater starch, whose blank was equal to that of the developing solvent.

During early work with starch chromatograms it became apparent that the optimum load of 3 mg. of amino acids quoted by Stein & Moore (1948) was not sufficient to afford accurate analyses of isoleucine and alanine in salmine as they each only constitute about 1% of the protamine. It was found, in common with Stein & Moore, that loads greater than 3 mg. of amino acids caused flattening and broadening of the peaks, and prevented separation of proline from alanine and of serine from glycine. By modifying the method of applying the amino acid mixture to the column, however, we found that we could increase the load up to 8 mg. of amino acids without impairing the resolution of isoleucine, valine, proline and alanine. A suitable portion of amino acid mixture or protein hydrolysate was delivered from a microburette on to a circle of Whatman no. 3 filter paper (0·9 cm. diam.) and dried in a stream of warm air. After the surface of the starch column had been carefully dried and tamped, the paper circle was dropped on to it and pressed gently into place with a stainless-steel rod. 0·5 ml. of developing solvent was run over the end of the rod, allowed to drain on to the column surface, and driven in under pressure. A further two 0·5 ml. amounts of solvent were similarly added. The column was then prepared for development and transferred to the fraction cutter, which was similar in design to that used by Stein & Moore (1948).