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Labelling of Phospholipid Phosphorus in Rat-Brain Mitochondria

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In the preceding paper (McMurray, Strickland, Berry & Rossiter, 1957) it was shown that, under anaerobic conditions, inorganic ^{32}P was incorporated into the lipid phosphorus of suitably 'reinforced' water dispersions of rat brain. In this system the phosphorylation was, for the most part, glycolytic. Under aerobic conditions the incorporation was no better than that observed when oxygen was excluded from the system; there was little evidence of phosphorylation coupled to the oxidation of added tricarboxylic acid cycle intermediates.

In order to investigate the incorporation of inorganic ^{32}P in a rat-brain preparation that was capable of carrying out true aerobic phosphorylation, the mitochondrial fraction was separated from a dispersion prepared in isotonic sucrose. In contrast with the findings for the dispersion prepared in water, the incorporation of inorganic ^{32}P by the mitochondrial preparation was found to require conditions optimum for oxidative phosphorylation. In the absence of oxygen the preparation was inactive. The effects of inhibitors, such as 2:4-dinitrophenol, cyanide, iodoacetate and fluoride, further emphasized the differences between the labelling of phosphorus compounds in hypotonic dispersions, associated with glycolytic phosphorylation, and the labelling in mitochondria, associated with oxidative phosphorylation.

METHODS

Preparation of mitochondria and other cell fractions from rat brain was carried out according to the method of Brody & Bain (1952). Freshly dissected rat cerebral hemispheres freed from corpus callosum were dispersed in 9 vol. of 0·25 M-sucrose in a Potter–Elvehjem hand homogenizer. This and all subsequent operations were carried out at 0°. The dispersion was centrifuged for 10 min. at 1500 *g* to yield a 'nuclear' fraction. The supernatant from the 'nuclear' fraction was then centrifuged at 8000 *g* for 20 min. The resulting sediment (mitochondrial fraction) was usually washed once with 0·25 M-sucrose and resedimented at 8000 *g* for 20 min. Supernatant and washings from the mitochondria were then spun for 30 min. at 25 000 *g*., yielding the microsomal fraction and a faintly opalescent final supernatant fraction.

In preliminary studies of the fractionation procedure, only the fraction sedimenting at 8000 *g* was found to stain supravitally with Janus green B. Examination with the phase-contrast microscope revealed that this fraction consisted chiefly of mitochondria. It contained approximately 50% of the lipid P, 20% of the ribonucleic acid P and negligible quantities of deoxyribonucleic acid P. The 'nuclear' fraction was found to consist chiefly of cell nuclei, but the fraction was contaminated with a few mitochondria, some whole nerve cells and some red blood cells.

For determination of P:O ratios, the medium described by Brody & Bain (1952) was used. The concentration of phosphate buffer was reduced from 0·028 M to 0·01 M. Hexokinase (200 units by the method of Berger, Slein, Colowick & Cori, 1946), together with 0·3 ml. of the cell fraction to be studied, was tipped into the reaction mixture from the side arm of a Warburg flask at zero time. Each cell fraction was either dissolved or suspended in 0·25 M-sucrose. After equilibration for 10 min. at 18°, control samples were removed and the proteins were precipitated with cold 20% (w/v) trichloroacetic acid (TCA). The O_2 consumption of the remaining samples was measured over the subsequent 30 min. period, after which the reaction was stopped by the addition of cold 20% TCA. The concentration of inorganic P in the TCA supernatant was determined by the method of Ernster, Zetterström & Lindberg (1950), the difference between that of control and incubated samples representing phosphate esterified in the 30 min. period during which O_2 consumption was measured.

In the experiments with ^{32}P, hexokinase and glucose were not added and the incubation temperature was 37·5°. To each vessel was added 120 μc of neutralized carrier-free inorganic ^{32}P. The specific activity of the phospholipid was determined as described in the previous paper (McMurray *et al.* 1957). Adenosine triphosphate (ATP), adenosine diphosphate (ADP) and adenosine monophosphate (AMP) were separated by the method of Krebs & Hems (1953).

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RESULTS

As may be seen from Table 1, the greater part of the phosphorylating ability of the whole dispersion was found in the mitochondrial fraction. The small amount of phosphorylation observed in the 'nuclear' fraction (Expt. 2) was probably the result of contamination with mitochondria. The values observed for the P:O ratio of just over 2 are similar to those reported by Abood & Gerard (1952), Brody & Bain (1952), Narayanaswami & McIlwain (1954) and Abood & Romanchek (1955) for mitochondria from brain, and to those reported by Ochoa (1941) and Case & McIlwain (1951) for brain dispersions. Dianzani & Scuro (1956) reported P:O ratios of 3·3–3·6 for rat-brain mitochondria. Such high values have never been observed in this Laboratory.

Table 2 shows the oxygen consumption and the radioactivity of the lipid P of the whole homogenate and of the various separated fractions. The mitochondria exhibited the greatest oxygen consumption and ^{32}P incorporation. In terms of specific activity (counts/min./μg. of P) the 'nuclear' fraction was as active as the mitochondria, but, because of the greater amount of lipid P in the mitochondrial fraction, the total incorporation (counts/min./100 mg. wet wt. of original tissue) in the 'nuclear' fraction was less. As reported above, the 'nuclei' had some respiratory activity that was probably due to contamination by mitochondria.

The mitochondrial fraction, known to play a role in the labelling of lipid P in liver tissue (Kennedy, 1953), was singled out for further study.

Cofactors. Table 3 shows that the omission of Mg^{2+} ions did not greatly affect the oxygen consumption, but that the incorporation of inorganic ^{32}P into phospholipid was greatly inhibited. When ATP was omitted, both respiration and incorporation were depressed. A subsequent experiment showed that AMP was more effective than ATP in this system. In later studies AMP was substituted for ATP.

The omission of KCl produced a slight increase in mitochondrial respiration (Table 3) and a considerable increase in the labelling of lipid P. Potassium chloride was added regularly to the incubation medium in the experiments of Brody & Bain (1952) with brain mitochondria, presumably to maintain

Table 1. *Oxidative phosphorylation in fractions obtained by differential centrifuging of dispersions of rat brain prepared in* 0·25 M-*sucrose*

Incubated at 18° for 40 min. Each vessel contained 0·008 M-$MgCl_2$; 0·05 M-KCl; 0·0025 M-sodium ATP; $8·3 \times 10^{-6}$ M-cytochrome *c*; 0·0005 M-DPN; 0·013 M-sodium pyruvate; 0·002 M-sodium malate; 0·012 M-NaF; 0·028 M-glucose; 0·01 M-sodium phosphate buffer, pH 7·4; 0·015 M-glycylglycine, pH 7·4, in the main compartment. The side arm contained the appropriate fraction (from 250 mg. of fresh brain) and 200 units of yeast hexokinase. Total volume, 3 ml. Gas phase, air.

Fraction	O_2 uptake (μg.atoms/100 mg. of original tissue)	P uptake (μg.atoms/100 mg. of original tissue)	P:O ratio (atom:atom)	O_2 uptake (μg.atoms/100 mg. of original tissue)	P uptake (μg.atoms/100 mg. of original tissue)	P:O ratio (atom:atom)
	Expt. 1			Expt. 2		
Homogenate	—	—	—	4·43	8·99	2·02
'Nuclei'	0·61	0·00	—	0·86	1·38	1·60
Mitochondria	1·91	4·13	2·16	2·31	4·63	2·00
Microsomes	0·45	0·54	—	0·01	0·02	—
Supernatant	0·94	0·00	—	1·04	0·38	—

Table 2. *Oxygen consumption and the specific activity of the lipid phosphorus of fractions obtained by differential centrifuging of sucrose homogenates of rat brain*

Incubated at 37·5° for 70 min. Medium was as described in Table 1, with [^{32}P]phosphate, but with no hexokinase or glucose. Gas phase, O_2.

Fraction	O_2 consumption (μl. of O_2/100 mg. wet wt. of original tissue/hr.)	Radioactivity (counts/min./μg. of P)	Radioactivity (counts/min./100 mg. wet wt. of original tissue)	O_2 consumption (μl. of O_2/100 mg. wet wt. of original tissue/hr.)	Radioactivity (counts/min./μg. of P)	Radioactivity (counts/min./100 mg. wet wt. of original tissue)
		Expt. 1			Expt. 2	
Homogenate	201	48·4	7890	159	40·2	6430
'Nuclei'	33	51·5	1720	27	51·8	1890
Mitochondria	73	55·8	2840	77	55·1	2790
Microsomes	7	15·1	150	6	28·1	390
Supernatant	18	11·3	105	10	4·7	51

the isotonicity, but no data were reported on its effect upon oxidative phosphorylation. The medium employed by Abood & Romanchek (1955) and Dianzani & Scuro (1956) also contained considerable quantities of K^+ ions.

When either cytochrome *c* or diphosphopyridine nucleotide (DPN) was omitted, both the oxygen consumption and the labelling of the phospholipid was decreased, but the decreases were less than those observed in the absence of either Mg^{2+} ions or ATP.

Substrates. Table 4 shows the extent to which glutamate and intermediates of the tricarboxylic acid cycle supported the respiration and labelling of phospholipid in brain mitochondria. Of the substrates tried, the best for both processes was pyruvate to which had been added a small amount of malate. All the other substrates, with the exception of citrate, increased both the respiration and the ^{32}P incorporation to levels above that observed in the absence of substrate. The addition of citrate caused a slight increase in the oxygen consumption, but the labelling of lipid P was below that observed when no substrate was present.

Inhibitors. The inhibitory effect of 2:4-dinitrophenol (DNP) on the labelling of phospholipid is demonstrated in Table 5. Respiration was stimulated maximally (50%) when the concentration of DNP was 5×10^{-5}M. At this concentration the incorporation of inorganic ^{32}P into lipid P was inhibited some 55%. When the concentration of DNP was increased to 10^{-4}M, the oxygen consumption remained slightly greater than that observed in the absence of DNP, but the labelling of phospholipid was greatly inhibited.

Preliminary experiments with rat-brain mitochondria respiring in such a medium, containing a relatively high concentration of adenine nucleotide, showed that a steady state exists with respect to the concentrations of ATP, ADP, AMP and inorganic P, but not with regard to the specific

Table 3. *Effect of omitting cofactors on the oxygen consumption and the specific activity of the lipid phosphorus of mitochondria from rat brain*

Incubated at 37·5° for 70 min. Medium was as described in Table 1, with [^{32}P]phosphate, but with no hexokinase or glucose. Gas phase, O_2.

	O_2 consumption (μl. of O_2/100 mg. wet wt. of original tissue/hr.)	O_2 consumption (% of that with complete system)	Specific activity (counts/min./μg. of P)	Specific activity (% of that with complete system)
Complete	46	100	36·2	100
No $MgCl_2$	32	70	4·8	13
No ATP	33	72	21·1	58
No KCl	53	115	53·8	149
No cytochrome *c*	42	91	28·9	80
No DPN	36	78	31·5	87

Table 4. *Effect of substrates on the oxygen consumption and the specific activity of the lipid phosphorus of mitochondria from rat brain*

Incubated at 37·5° for 70 min. Medium was as described in Table 1 with [^{32}P]phosphate, but with no hexokinase or glucose. AMP was substituted for ATP. Gas phase, O_2.

Substrate	O_2 consumption (μl. of O_2/100 mg. wet wt. of original tissue/hr.)	Specific activity (counts/min./μg. of P)	O_2 consumption (μl. of O_2/100 mg. wet wt. of original tissue/hr.)	Specific activity (counts/min./μg. of P)
	Expt. 1		Expt. 2	
None	13	27·1	11	12·5
Pyruvate (0·013M)	37	90·0	36	48·6
Pyruvate (0·013M) and malate (0·002M)	80	121	71	71·8
Citrate (0·013M)	28	13·4	21	6·0
α-Oxoglutarate (0·013M)	32	59·5	42	44·8
Glutamate (0·013M)	42	91·9	39	51·0
	Expt. 3		Expt. 4	
None	12	17·8	22	20·9
Pyruvate (0·013M) and malate (0·002M)	98	104	101	116
Succinate (0·013M)	72	64·1	99	85·8
Fumarate (0·013M)	38	44·8	47	53·8
Malate (0·013M)	57	55·0	48	61·4
Oxaloacetate (0·013M)	56	56·7	57	53·5

activities of these substances. However, as can be seen from Table 5, the addition of DNP caused changes in the specific activity of ATP and in the steady-state concentrations of ATP, ADP and AMP. The labelling of ATP was progressively decreased with increasing concentrations of DNP, whereas the concentrations of ATP and ADP were decreased and the concentration of AMP was increased.

The addition of sodium fluoride caused a decrease in the respiration. The specific activities of lipid P and ATP increased as the concentration of fluoride increased, up to 10^{-2}M. However, at a fluoride concentration of 5×10^{-2}M, the specific activity of ATP was not significantly different from that of the controls, but the specific activity of the lipid P was considerably greater. The steady-state concentrations of ATP and ADP also increased as the concentration of fluoride increased, up to 10^{-2}M. On the other hand, the concentration of AMP decreased as the fluoride was increased over the same concentration range. At a fluoride concentration of 5×10^{-2}M, the concentrations of ATP and ADP were smaller and the concentration of AMP was greater than the corresponding values observed in the presence of 10^{-2}M-fluoride.

Table 5 also shows that, whereas the addition of 10^{-5}M-iodoacetate had no significant effect on either respiration or the labelling of lipid P or ATP, the addition of 3×10^{-2}M-cyanide caused almost complete inhibition. The lack of effect of iodoacetate on the labelling of phospholipid is in contrast with the finding reported in the previous paper that this low concentration of iodoacetate caused a significant inhibition of the anaerobic labelling of phospholipid in water homogenates of rat brain (McMurray *et al.* 1957).

DISCUSSION

The labelling of phospholipid in cell-free preparations was demonstrated by Friedkin & Lehninger (1949) for washed particles from rat liver and by Abood (1954) for mitochondria from rat brain. The present experiments indicate that the cofactors required for the labelling of lipid P in the mitochondria are similar to those found by Brody & Bain (1952) to be necessary for oxidative phosphorylation. An unexpected finding was that the lipid labelling was greater when potassium chloride was removed from the medium. It is known that in brain-slice preparations a high concentration of K^+ ions causes an increase in the oxygen consumption, an increase in glycolysis, a decrease in the concentration of creatine phosphate and a decrease in the labelling of lipid P from inorganic ^{32}P, i.e. phosphorylation is 'uncoupled' from oxidation (see Findlay, Magee & Rossiter, 1954, for references).

The finding that substrates such as succinate and glutamate supported the labelling of lipid P in rat-brain mitochondria is of some interest. In slice preparations of cat brain these substances, although they are oxidized, are incapable of supporting the labelling of lipid P (Strickland, 1954). In general, the substrates that support the labelling of lipid P in brain mitochondria are those that give good P:O ratios (Brody & Bain, 1952; Abood & Gerard, 1952).

Citrate supported neither respiration nor the labelling of phospholipid. Of all the intermediates of the tricarboxylic acid cycle tested by Brody & Bain (1952) citrate gave the lowest P:O ratios. The failure of brain preparations to oxidize citrate was noted by Coxon, Liébecq & Peters (1949) for iso-

Table 5. *Effect of 2:4-dinitrophenol, fluoride, iodoacetate or cyanide on the oxygen consumption, specific activities of lipid phosphorus and ATP, and the concentrations of ATP, ADP and AMP of mitochondria from rat brain*

Incubated at 37·5° for 70 min. Medium was as described in Table 1 with [^{32}P]phosphate, with no hexokinase or glucose. AMP was substituted for ATP. Gas phase, O_2.

	O_2 uptake (μl. of O_2/100 mg. of original tissue/hr.)	Specific activity of lipid P (counts/min./μg. of P)	10^{-3} × Specific activity of ATP (counts/min./μg. of P)	Concentration (μg. of P/vessel)		
				ATP	ADP	AMP
Control	98	29·9	14·6	64·5	80·5	110
10^{-5}M-DNP	109	25·3	12·1	56·5	64·5	129
5×10^{-5}M-DNP	151	13·2	6·7	46·5	46·5	147
10^{-4}M-DNP	108	7·0	3·5	25·5	26·5	184
Control	94	25·5	9·0	40·0	40·5	118
5×10^{-3}M-NaF	74	54·5	12·5	122	133	93
10^{-2}M-NaF	69	68·0	13·2	157	149	74
5×10^{-2}M-NaF	50	40·2	8·4	65·5	38·5	127
Control	68	24·4	7·6	—	—	—
10^{-5}M-Iodoacetate	72	24·2	7·0	—	—	—
3×10^{-2}M-NaCN	10	0	0·9	—	—	—

tonic dispersions and by Christie, Judah & Rees (1953) for mitochondria. It is possible that brain mitochondria are impermeable to citrate (Gallagher, Judah & Rees, 1956). As noted previously (McMurray *et al.* 1957), when dispersions of brain are prepared in water, they are able to oxidize citrate as readily as succinate or malate. Plaut & Plaut (1952) reported that heart mitochondria are not able to oxidize citrate to any appreciable extent.

The labelling of phospholipid in the rat-brain mitochondria is more sensitive to DNP than is the phospholipid labelling in water dispersions, reported in the previous paper (McMurray *et al.* 1957). The addition of DNP also caused a decrease in the labelling of ATP. Although the specific activity of the phospholipid was much less than that of ATP, the relative changes in the specific activity of the phospholipid caused by the addition of DNP were of the same order as those found for the specific activity of ATP, suggesting that the latter were responsible for the former.

The effects produced by fluoride on the concentrations of the adenosine phosphates may be explained, in part, by the well-known effect of fluoride on ATP-ase. However, the observed increase in the specific activity of ATP is less readily explained. A comparison of the relative effects of fluoride on the specific activities of the lipid P and the ATP shows that there was no obvious relation between the two, as shown for DNP. For example, at a fluoride concentration of 5×10^{-2} M, the specific activity of the phospholipid was much greater than that of the control, but the specific activity of the ATP was not significantly different. In some respects, the specific activity of the lipid P appeared to be related to the concentration of ATP rather than to its specific activity. Such a finding probably indicates that ATP is required for the provision of energy for the labelling of phospholipid.

The results presented here, together with those reported in the previous paper (McMurray *et al.* 1957), indicate that there are two systems in brain capable of labelling ATP, and hence lipid P. These are the aerobic mitochondrial system described in this paper, and an anaerobic system, best demonstrated in dispersions of brain prepared in water. The activity of the dispersion was inhibited by fluoride or iodoacetate and was relatively insensitive to DNP. On the other hand, the activity of the mitochondria was inhibited by DNP or cyanide, was relatively insensitive to iodoacetate, and was stimulated by fluoride.

SUMMARY

1. Phospholipid was labelled from inorganic ^{32}P in mitochondria separated from dispersions of rat brain prepared in isotonic sucrose. The labelling was best observed under optimum conditions for oxidative phosphorylation.

2. The addition of adenine nucleotide, Mg^{2+} ions, diphosphopyridine nucleotide and cytochrome *c* was necessary for optimum ^{32}P incorporation.

3. The labelling was supported by the addition of glutamate and certain tricarboxylic acid cycle intermediates, but not by the addition of citrate.

4. The labelling of lipid phosphorus and adenosine triphosphate (ATP) was inhibited by the addition of 2:4-dinitrophenol or cyanide, was insensitive to iodoacetate and was stimulated by the addition of fluoride. The addition of dinitrophenol and fluoride also caused changes in the steady-state concentrations of ATP, adenosine diphosphate and adenosine monophosphate.

5. The results support the conclusion that there are two systems capable of supporting ^{32}P labelling in rat-brain dispersions: (*a*) an anaerobic glycolytic system best demonstrated in water dispersions, and (*b*) an aerobic system best demonstrated in mitochondria prepared from isotonic sucrose dispersions.

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