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Labelling of Phospholipid Phosphorus in Rat-Brain Dispersions

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In surviving slices of cat brain the *in vitro* incorporation of inorganic phosphate labelled with ^{32}P into phospholipid depends upon conditions optimum for oxidative phosphorylation (Strickland, 1954). Dinitrophenol, glutamate, glutamine, K^+ ion and NH_4^+ ion, in concentrations that do not decrease the oxygen consumption, greatly decrease phospholipid labelling (Strickland, 1954; Findlay, Magee & Rossiter, 1954). In addition, anaerobiosis, or the presence of respiratory inhibitors such as cyanide or azide, almost completely inhibits the labelling of lipid in brain, liver or kidney slices (Taurog, Chaikoff & Perlman, 1942; Schachner, Fries & Chaikoff, 1942; Strickland, 1954).

Schachner *et al.* (1942) and Strickland (1954) showed that if brain tissue was homogenized before incubation, the labelling of the phospholipid was reduced to very low levels. However, the respiratory activity of brain dispersions is low, unless suitable 'reinforcing' agents are added to the medium (Banga, Ochoa & Peters, 1939; Racker & Krinsky, 1945; Reiner, 1947).

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When inorganic ^{32}P was added to an actively respiring dispersion of rat brain 'reinforced' with glucose, hexose diphosphate, adenosine triphosphate, fumarate, diphosphopyridine nucleotide, nicotinamide, cytochrome *c* and Mg^{2+} ions, good labelling of phospholipid was obtained. An unexpected finding, however, was that the phospholipids were more strongly labelled when oxygen was excluded from the system. This was surprising, since Dawson (1953) demonstrated good phospholipid labelling in 'reinforced' dispersions of guinea-pig brain in the presence of oxygen, but a greatly decreased labelling under anaerobic conditions.

An investigation of the apparent discrepancy between our observations and those of Dawson (1953) revealed that in rat-brain dispersions there are two systems capable of supporting the labelling of phospholipids: (a) a glycolytic system, demonstrated in dispersions of brain prepared in water, and (b) an oxidative system, demonstrated in mitochondria isolated from isotonic dispersions. Best labelling is obtained under optimum conditions for glycolytic or oxidative phosphorylation respectively. In the present paper the properties of the glycolytic system are described. The

mitochondrial system is discussed subsequently (McMurray, Berry & Rossiter, 1957).

A preliminary account of this work has appeared (McMurray, Strickland & Rossiter, 1954).

METHODS

Rats were killed by decapitation. The cerebral hemispheres were rapidly removed and dispersions prepared in 4 parts of water at 0° in a loosely fitting homogenizer of the Potter-Elvehjem type. Portions of this hypotonic dispersion, representing 60 mg. wet wt. of brain, were pipetted into Warburg vessels containing an incubating medium similar to that described by Reiner (1947). The complete system contained: glucose, 0.028M; hexose diphosphate (HDP), 0.005M; adenosine triphosphate (ATP) or adenosine 5'-phosphate (AMP), 0.0007M; diphosphopyridine nucleotide (DPN), 0.001M; cytochrome *c*, 0.00006M; potassium fumarate, 0.0016M; nicotinamide, 0.04M; MgCl₂, 0.008M. The concentration of phosphate (pH 7.4) used by Reiner (1947) was decreased from 0.04 to 0.01M, and glycylglycine (0.083M), 2-amino-2-hydroxymethylpropane-1,3-diol (tris) buffer (0.05M) or, in some anaerobic experiments, KHCO₃ (0.048M) was added to increase the buffering capacity of the medium. With each buffer the final pH was 7.4. To each vessel was added 120 µC of neutralized carrier-free inorganic ³²P, supplied in the form H₂³²PO₄ by the Atomic Energy of Canada Ltd. The final volume was 3 ml.

In aerobic experiments 0.2 ml. of 10% (w/v) KOH was placed in the centre well for the absorption of CO₂. Vessels were gassed with O₂, and O₂ consumption was measured with the usual Warburg apparatus at 10 min. intervals for 1 hr. The incubation temperature was 37.5° and the vessels were shaken to ensure proper oxygenation. For anaerobic experiments N₂ (passed over hot copper to remove the last traces of O₂) replaced O₂. In some of the earlier experiments acid formation was determined by manometric measurements of the CO₂ evolved from the KHCO₃ buffer. In these experiments N₂ + CO₂ (95 + 5, v/v) replaced N₂.

At the end of the incubation period the reaction was stopped by the addition of 2 ml. of 20% (w/v) trichloroacetic acid (TCA). The suspension was centrifuged and a portion of the supernatant was taken for the determination of lactic acid (Barker & Summerson, 1941). Removal of contaminating acid-soluble P from the precipitate, and extraction of lipids, were carried out as described by Findlay *et al.* (1954). Last traces of inorganic ³²P were removed from the lipid extract by the purification procedure described by Kennedy (1953). The specific activity of the purified lipid P was determined as described previously (Findlay *et al.* 1954). When ATP was studied, it was separated from the supernatant remaining after the TCA precipitation by the chromatographic methods described by Krebs & Hems (1953).

Reagents. DPN (95% purity) and cytochrome *c* were obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio. Disodium ATP, dipotassium ATP, monosodium adenosine diphosphate (ADP), monosodium uridine triphosphate (UTP), disodium guanosine triphosphate (GTP) and disodium cytidine triphosphate (CTP) were obtained from Pabst Laboratories, Milwaukee, Wisconsin. Barium HDP and sodium AMP were products of Schwartz Laboratories Inc., Mount Vernon, New York. The barium salt of inosine triphosphate (ITP) was the product of Sigma

Chemical Co., St Louis, Missouri. Where necessary, the barium was removed by sulphate precipitation. The nucleotides were 'chromatographically pure', and were found to conform in purity to analytical values given by the manufacturer.

RESULTS

Aerobic experiments

Table 1 shows that a 'reinforced'-water dispersion of rat brain similar to that described by Reiner (1947) respire well and is capable of supporting good labelling of phospholipid. The oxygen consumption corresponded to a *Q*_{O₂} of the order of 17-24, measured over the whole 60 min. period. Similar *Q*_{O₂} values for mouse-brain dispersions were reported by Larner, Jandorf & Summerson (1949) for the first 20 min. of incubation.

From Table 1 it may be seen that omission of substrate (HDP and glucose), ATP, DPN and nicotinamide (Expt. 1), or Mg²⁺ ions (Expt. 3) caused a decrease in both the oxygen consumption and the labelling of phospholipid. Omission of ATP or Mg²⁺ ions had a greater effect on phospholipid labelling than on oxygen consumption. This is of some interest in view of the known requirement of various phosphokinase enzymes for Mg²⁺ ions. Recently, Bublitz & Kennedy (1954) isolated a Mg²⁺ ion-requiring enzyme, glycerokinase, from liver mitochondria. There is considerable evidence that this enzyme is involved in the incorporation of inorganic ³²P into phospholipids (Bublitz & Kennedy, 1954; Kennedy, 1956).

Expt. 2 shows that the omission of HDP was without significant effect on either the oxygen consumption or phospholipid labelling. The lack of effect of HDP on the oxygen consumption in the presence of glucose confirms the finding of Larner *et al.* (1949) for dispersions of mouse brain. The addition of DPN was necessary for optimum oxygen uptake and phospholipid labelling (Expt. 2), but the addition of nicotinamide was less important (Expt. 3). Larner *et al.* (1949) pointed out that the requirement of DPN and nicotinamide for optimum oxygen consumption depends upon the conditions of homogenization.

Table 1 also shows that, whereas the addition of cytochrome *c* and fumarate was necessary for optimum oxygen consumption, neither substance was necessary for optimum phospholipid labelling (Expt. 1). When the ATP of the system was replaced by AMP of the same concentration, phospholipid labelling was considerably increased, with a slight increase in oxygen consumption (Expt. 4).

The effect of replacing glucose with a number of other substrates is shown in Table 2. The addition of either glucose or HDP increased both oxygen consumption and labelling (Expts. 1 and 2). Mannose behaved similarly, but galactose was

Table 1. *Effect of omitting cofactors on the oxygen consumption and the specific activity of the lipid phosphorus of water dispersions of rat brain*

Dispersions were incubated for 70 min. at 37.5° in medium containing glycylglycine (0.083 M), pH 7.4, and [³²P]phosphate. Adenine nucleotide: ATP. Gas phase, O₂. Complete system is described in text.

Medium	O ₂ consumption		Specific activity	
	(μl. of O ₂ /60 mg. wet wt./hr.)	% of that with complete medium	(counts/min./μg. of P)	% of that with complete medium
Expt. 1				
Complete	230	100	34.1	100
No HDP or glucose	107	47	12.4	35
No ATP	97	42	5.6	16
No DPN or nicotinamide	46	20	7.2	21
No cytochrome c	117	51	38.8	114
No fumarate	195	85	35.3	104
Expt. 2				
Complete	210	100	46.7	100
No HDP	199	95	48.7	104
No DPN	54	26	11.1	24
Expt. 3				
Complete	263	100	47.0	100
No nicotinamide	250	95	40.7	87
No MgCl ₂	226	86	17.9	38
Expt. 4				
Complete	287	100	32.4	100
AMP for ATP	312	109	43.7	135

Table 2. *Effect of addition of substrates on the oxygen consumption and the specific activity of the lipid phosphorus of water dispersions of rat brain*

Dispersions were incubated for 70 min. at 37.5° in medium containing glycylglycine (0.083 M), pH 7.4, and [³²P]phosphate. Adenine nucleotide: ATP. Gas phase, O₂. Complete system is described in text.

Substrate	O ₂ consumption		Specific activity	
	(μl. of O ₂ /60 mg. wet wt./hr.)	(counts/min./μg. of P)	(μl. of O ₂ /60 mg. wet wt./hr.)	(counts/min./μg. of P)
	Expt. 1		Expt. 2	
None	80	16.5	127	30.9
Glucose (0.028 M)	221	40.1	255	54.2
HDP (0.005 M)	216	42.6	223	53.3
	Expt. 3		Expt. 4	
None	98	21.4	107	20.8
Mannose (0.028 M)	189	30.2	168	31.8
Galactose (0.028 M)	104	21.4	109	25.4
Citrate (0.028 M)	219	25.0	214	18.3
Malate (0.028 M)	207	19.5	211	24.2
Succinate (0.028 M)	207	19.7	222	21.5

almost without effect (Expts. 3 and 4). In like manner glucose and mannose were shown to support the incorporation of inorganic ³²P into the phospholipids of cat-brain slices, whereas galactose was found to be ineffective (Strickland, 1954). Tricarboxylic acid cycle intermediates citrate, malate and succinate all increased the oxygen consumption, but were ineffective in supporting lipid labelling.

The preceding experiments were all carried out under aerobic conditions. When the oxygen in the gas mixture was replaced with nitrogen, the labelling of the lipid was considerably increased (Table 3).

Table 3. *Effect of anaerobiosis on the specific activity of the lipid phosphorus of water dispersions of rat brain*

Dispersions were incubated for 70 min. at 37.5° in medium containing glycylglycine (0.083 M), pH 7.4, and [³²P]phosphate. Adenine nucleotide: ATP. Complete system is described in text.

Gas phase	Specific activity (counts/min./μg. of P)	
	Expt. 1	Expt. 2
O ₂	45.5	46.3
N ₂	58.8	55.7

This is in sharp contrast with previous findings for cat-brain slices (Strickland, 1954), and with the findings of Dawson (1953) for dispersions of guinea-pig brain.

The requirement for substrate, DPN, adenine nucleotide, fumarate, Mg^{2+} ions and cytochrome *c* for optimum respiration of brain dispersions is well known (see, for example, Banga *et al.* 1939; Racker & Krimsky, 1945; Reiner, 1947; Case & McIlwain, 1951). However, the conditions necessary for labelling of phospholipid have received less study. The most extensive investigation is that of Dawson (1953), who studied the labelling of phospholipid in a 'reinforced' dispersion of guinea-pig brain. The findings presented in Tables 1-3 differ from those reported by Dawson in a number of respects. In his system, Dawson found that (a) fumarate and cytochrome *c* were necessary for optimum labelling; (b) labelling was considerably reduced under anaerobic conditions; (c) tricarboxylic acid cycle intermediates increased both oxygen consumption and labelling. It is shown below that with dispersions of rat brain prepared in water the labelling was sustained largely by glycolytic phosphorylation, whereas in the experiments of Dawson (1953) with dispersions of guinea-pig brain prepared in KCl the phosphorylation was presumably mainly oxidative.

Anaerobic experiments

Table 4 shows the factors necessary for optimum glycolysis and labelling of phospholipid under anaerobic conditions. In these experiments the

complete system contained glucose, HDP, AMP, DPN, nicotinamide and Mg^{2+} ions. Fumarate and cytochrome *c* were unnecessary and were omitted from the system. Expt. 1 shows that, as in the aerobic experiments, omission of DPN and nicotinamide almost completely abolished lipid labelling. Glycolysis was also abolished. When only DPN was omitted from the system, both glycolysis and phospholipid labelling were still greatly reduced. However, when only nicotinamide was omitted, there was optimum glycolysis and the labelling of phospholipid was greater than that observed for the complete system containing nicotinamide. Nicotinamide prevents DPN breakdown by inhibiting DPN-ase (Mann & Quastel, 1941; Handler & Klein, 1942; McIlwain & Rodnight, 1949). However, nicotinamide has been shown also to depress the activity of certain DPN-requiring enzymes by competitive inhibition (Feigelson, Williams & Elvehjem, 1951). A similar mechanism may explain the inhibitory action of nicotinamide on the anaerobic ^{32}P incorporation, since this process exhibits a great requirement for DPN.

The omission of AMP or Mg^{2+} ions greatly decreased both glycolysis and the labelling of phospholipid (Expt. 2). As in the aerobic experiments, ATP could substitute for AMP. In the absence of substrate (glucose and HDP), there was almost no glycolysis or labelling of phospholipid (Expt. 3). Omission of glucose alone caused a great decrease in glycolysis, but no significant change in ^{32}P incorporation. On the other hand, omission of HDP alone caused a decrease in both glycolysis and

Table 4. *Effect of omitting cofactors on the anaerobic glycolysis and the specific activity of the lipid phosphorus of water dispersions of rat brain*

Dispersions were incubated for 70 min. at 37.5° in medium containing $KHCO_3$ (0.048M), pH 7.4, and [^{32}P]phosphate. Adenine nucleotide: AMP. Gas phase, $N_2 + CO_2$ (95+5). Complete system is described in text.

Medium	Anaerobic glycolysis		Specific activity	
	(μ moles of CO_2 /60 mg. wet wt./hr.)	% of that with complete medium	(counts/min./ μ g. of P)	% of that with complete medium
Expt. 1				
Complete	39.4	100	45.0	100
No DPN or nicotinamide	0.0	0	4.2	9
No DPN	11.4	29	12.1	27
No nicotinamide	38.0	97	62.7	139
Expt. 2				
Complete	36.6	100	42.8	100
No $MgCl_2$	20.4	56	12.8	30
No AMP	4.8	13	6.3	15
ATP for AMP	39.8	109	48.9	114
Expt. 3				
Complete	37.6	100	31.7	100
No HDP or glucose	0.3	0.8	1.4	4
No glucose	12.0	32	30.5	96
No HDP	0.7	2	2.4	8

Table 5. *Effect of hexose diphosphate on the anaerobic glycolysis and the specific activity of the lipid phosphorus of water dispersions of rat brain*

Dispersions were incubated for 70 min. at 37·5° in medium containing tris buffer (0·05M), pH 7·4, and [³²P]phosphate. Gas phase, N₂. Complete system is described in text. Hexose diphosphate was purified chromatographically.

	Adenine nucleotide	Medium	Anaerobic glycolysis		Specific activity	
			(μmoles of lactate/60 mg. wet wt./hr.)	% of that with complete medium	(counts/min./μg. of P)	% of that with complete medium
Expt. 1	AMP	Complete	58·9	100	33·9	100
		No HDP	3·3	6	3·5	10
Expt. 2	ATP	Complete	43·5	100	34·2	100
		No HDP	33·6	78	30·6	90

Table 6. *Effect of replacing adenosine triphosphate with other nucleotides on the anaerobic glycolysis, the specific activity of the lipid phosphorus and adenosine triphosphate, and the concentration of adenosine triphosphate in water dispersions of rat brain*

Dispersions were incubated for 70 min. at 37·5° in the medium described in Table 5, with ATP omitted. Gas phase, N₂. All nucleotides, 0·0007M.

Nucleotides added*	Anaerobic glycolysis (μ moles of lactate/60 mg. wet wt./hr.)	Specific activity of lipid P (counts/min./ μ g. of P)	ATP	
			$10^{-3} \times$ Specific activity (counts/min./ μ g. of P)	Concn. (μ g. of P/vessel)
Expt. 1				
ATP	38.6	32.4	17.6	139
ADP	16.5	10.9	5.6	74
UTP	8.5	3.9	5.9	49
UTP + ADP	32.4	23.3	13.4	107
ITP	6.5	3.4	4.1	53
ITP + ADP	11.5	6.8	6.2	54
Expt. 2				
ATP	49.2	62.2	12.6	118
ADP	52.3	48.1	9.4	139
GTP	14.4	13.7	8.5	42
GTP + ADP	43.5	61.3	13.7	119
CTP	7.7	17.0	5.4	47
CTP + ADP	8.8	15.8	3.9	45
Expt. 3				
ATP	44.2	63.5		
ADP	10.3	23.4		
UTP	4.5	3.4		
UTP + ADP	29.5	48.1		
GTP	1.1	4.5		
GTP + ADP	19.0	33.3		

* For abbreviations see Methods.

phospholipid labelling, almost to the level observed in the absence of substrate. The necessity for the presence of HDP when oxygen was excluded from the system contrasts sharply with the results obtained in the aerobic experiments. Under aerobic conditions, either HDP or glucose alone was capable of supporting both respiration and ³²P incorporation (Table 2).

The experiments reported in Table 5 go far to explain the requirement for HDP under anaerobic conditions. When AMP was the added adenine nucleotide, HDP was necessary for both glycolysis

and the labelling of phospholipid, since in the absence of HDP both were reduced to low levels (Expt. 1). When the AMP was replaced by ATP, good glycolysis and labelling of phospholipid were observed in the absence of HDP.

These experiments are consistent with the findings of Meyerhof (1947) and Meyerhof & Wilson (1947), who reported that either a labile-P donor (ATP) or a stable-P donor (HDP) was essential for initiating the utilization of glucose in brain homogenates under anaerobic conditions. For the utilization of glucose, ATP is necessary for the hexokinase

and phosphohexokinase reactions. The ATP may be provided as such, or it may be generated in the system from HDP and AMP by means of the triose phosphate dehydrogenase and subsequent phosphokinase reactions. In subsequent anaerobic experiments AMP was replaced by ATP, and HDP was omitted.

Replacement of ATP with other nucleotides

The effects of replacing ATP with the triphosphates of uridine, inosine, guanosine or cytidine (UTP, ITP, GTP or CTP) are shown in Table 6. When added to the system alone, none of the nucleotides tested supported either glycolysis or the incorporation of ^{32}P into lipid P to any significant extent. Generally, replacement of ATP by ADP also caused considerable decreases in glycolysis and labelling of phospholipid (with the exception of

Expt. 2, where the phospholipid labelling, but not the glycolysis, was decreased). However, the addition of either UTP or GTP, together with ADP, caused an increase in the anaerobic glycolysis and the labelling of the lipid P, which, in some instances, approached the levels observed in the presence of ATP. The addition of ITP or CTP, together with ADP, produced no such effect.

Table 6 also shows the specific activity and the concentration of ATP isolated from the TCA extract. In the presence of the four nucleoside triphosphates there was a decrease in the concentration and the specific activity of the ATP. When UTP or GTP was added with ADP, there was an increase in both the concentration and the specific activity of the ATP. This increase was not observed when ITP or CTP was added with ADP.

Table 7. *Effect of medium in which dispersion was prepared on the anaerobic glycolysis and the specific activity of the lipid phosphorus of dispersions of rat brain*

Dispersion was prepared in water, 0.25 M-sucrose or 0.15 M-KCl as indicated. Dispersions were incubated for 70 min. at 37.5° in the medium described in Table 5, with ATP as adenine nucleotide. Gas phase, N_2 .

	Anaerobic glycolysis		Specific activity	
	($\mu\text{moles of lactate/60 mg. wet wt./hr.}$)	% of that with water dispersion	(counts/min./ $\mu\text{g. of P}$)	% of that with water dispersion
Expt. 1				
Hypotonic (water)	66.8	100	43.8	100
Isotonic (sucrose)	13.2	20	9.1	21
Isotonic (KCl)	16.8	25	17.6	40
Expt. 2				
Hypotonic (water)	57.2	100	59.5	100
Isotonic (sucrose)	12.2	21	9.8	16
Expt. 3				
Hypotonic (water)	32.2	100	37.4	100
Isotonic (KCl)	7.7	24	14.8	40

Table 8. *Effects of 2:4-dinitrophenol, fluoride and iodoacetate on the oxygen consumption, glycolysis and specific activity of the lipid phosphorus of water dispersions of rat brain*

Dispersions were incubated for 70 min. at 37.5° in medium containing tris buffer (0.05 M), pH 7.4, and ^{32}P phosphate. Adenine nucleotide: ATP. Gas phase, O_2 or N_2 . Complete system is described in text. O_2 , $\mu\text{l. of O}_2/60\text{ mg. of fresh tissue/hr.}$ G , $\mu\text{moles of lactate/60 mg. of fresh tissue/hr.}$ $S.A.$, specific activity (counts/min./ $\mu\text{g. of P}$).

	Aerobic						Anaerobic			
	Expt. 1			Expt. 2			Expt. 1		Expt. 2	
	O_2	G	$S.A.$	O_2	G	$S.A.$	G	$S.A.$	G	$S.A.$
Control	218	22.5	19.8	280	37.4	26.9	46.1	33.1	53.1	43.2
DNP ($7.5 \times 10^{-5}\text{ M}$)	294	43.5	13.3	350	44.6	17.3	50.7	27.4	59.8	36.6
KF (10^{-2} M)	234	4.9	26.5	248	5.6	40.2	2.9	20.5	4.8	26.0
	Expt. 3			Expt. 4			Expt. 3		Expt. 4	
Control	151	47.7	43.9	155	30.1	21.1	40.4	50.0	40.5	33.8
Iodoacetate (10^{-6} M)	128	33.0	26.5	134	25.1	18.1	18.9	11.0	9.1	11.0
	% of control			% of control			% of control		% of control	
DNP	135	192	67	125	120	64	110	83	112	83
KF	107	22	134	92	15	150	6	62	9	60
Iodoacetate	85	69	61	87	83	86	47	22	22	33

Medium used for preparation of dispersion

At the outset, the brain dispersion was prepared in water, because such a preparation is known to respire well (Racker & Krinsky, 1945; Reiner, 1947; Lerner *et al.* 1949). For the anaerobic experiments the same preparation was used, for it is known to give good glycolysis (Racker & Krinsky, 1945; Utter, Reiner & Wood, 1945*a*; Utter, Wood & Reiner, 1945*b*; Utter, 1950). However, it can be seen from Table 7 that when the water of the medium was replaced with isotonic sucrose or isotonic KCl both the glycolysis and anaerobic ^{32}P incorporation were greatly decreased. Phospholipid labelling in the dispersion prepared in sucrose was somewhat less than that in the dispersion prepared in KCl.

Inhibitors

Table 8 shows that, at a concentration of $7.5 \times 10^{-5}\text{M}$, 2:4-dinitrophenol caused an increase in both the oxygen consumption and aerobic glycolysis, but a decrease (33–36%) in the aerobic incorporation of inorganic ^{32}P into phospholipid. When oxygen was excluded from the system there was a slight increase in glycolysis, but the inhibition of phospholipid labelling was less (17%) than that observed under aerobic conditions.

The addition of fluoride (10^{-2}M) caused little change in oxygen consumption, a great decrease in aerobic glycolysis and an increase in the aerobic labelling of phospholipid. In contrast, when oxygen was excluded from the system, the labelling of phospholipid was decreased. Anaerobic glycolysis, like aerobic glycolysis, was also greatly inhibited.

Table 8 also shows that under anaerobic conditions both glycolysis and the labelling of phospholipid were inhibited by iodoacetate (10^{-5}M), whereas in the presence of oxygen the degree of inhibition of both reactions was considerably less.

DISCUSSION

The experiments reported here confirm the earlier observations that suitably 'reinforced' hypotonic dispersions of brain tissue exhibit a high rate of respiration (Racker & Krinsky, 1945; Reiner, 1947; Lerner *et al.* 1949). Although the oxygen consumption was high (Q_{O_2} 17–24), the labelling of phospholipid under aerobic conditions was no better than that observed when oxygen was excluded from the system. On the other hand, Dawson (1953) demonstrated good aerobic labelling of phospholipid associated with quite low oxygen consumption (Q_{O_2} 3–5) in brain dispersions prepared in a potassium chloride solution. This would suggest that for good labelling of phospholipid the oxygen consumption need not be maximal.

In agreement with the reports of others (Utter *et al.* 1945*a*; Racker & Krinsky, 1945; Meyerhof & Wilson, 1947), it was found that the hypotonic dispersion of rat brain was also capable of a high rate of glycolysis. This glycolytic activity can support the labelling of phospholipid from inorganic ^{32}P . When glycolysis was inhibited by the addition of fluoride or iodoacetate, the labelling of phospholipid was similarly inhibited. The experiments also confirm the previous report of Utter *et al.* (1945*b*) that water dispersions of rat brain possess a more active anaerobic glycolysis than do dispersions prepared in isotonic media. It has now been shown that this increase in glycolysis may be associated with an increase in the labelling of phospholipid (Table 7).

The observation that UTP or GTP, when added with ADP, can substitute for ATP in maintaining anaerobic glycolysis and phospholipid labelling provides evidence for the existence in brain of nucleoside diphosphokinases catalysing reactions such as $\text{UTP} + \text{ADP} \rightleftharpoons \text{UDP} + \text{ATP}$. Presumably there is a similar reaction between ADP and GTP, but not between ADP and CTP or ITP. The ability of ADP to satisfy, in part, the ATP requirement may be related to the presence of adenylate kinase in brain (Barkulis & Lehninger, 1951).

It is probable that a large part of the phosphorylation that occurs under aerobic conditions in hypotonic dispersions of rat brain is of the glycolytic type. This is indicated by the experiments reported in Table 2, where it was shown that tri-carboxylic acid cycle intermediates, although they were capable of increasing the oxygen consumption, had little effect on the labelling of phospholipid. On the other hand, the addition of substrates that can undergo glycolysis, such as glucose, mannose or HDP, increased the labelling. The fact that the labelling of phospholipid was not greatly inhibited (30–40%) in the presence of $7.5 \times 10^{-5}\text{M}$ 2:4-dinitrophenol is further evidence that the labelling, even under aerobic conditions, was largely supported by glycolytic phosphorylation, for this concentration of dinitrophenol greatly inhibits the aerobic labelling of phospholipid in mitochondria that are capable of true oxidative phosphorylation (McMurray *et al.* 1957). It is known that the substrate-linked phosphorylation that occurs during glycolysis is not greatly sensitive to dinitrophenol.

The experiments provide evidence, however, that under aerobic conditions some oxidative phosphorylation takes place even in the hypotonic dispersions. With glucose as substrate and AMP as the added adenine nucleotide, glycolysis and labelling of phospholipid were found to proceed under aerobic, but not under anaerobic conditions. In the presence of oxygen sufficient ATP to initiate the

phosphorylation of glucose is formed by oxidative phosphorylation. In addition, other findings, e.g. the greater degree of inhibition of the phospholipid labelling observed in the presence of 7.5×10^{-5} M-2,4-dinitrophenol, and the stimulation of the labelling produced by the addition of fluoride, are consistent with the view that under aerobic conditions a small portion of the labelling of phospholipid is supported by oxidative phosphorylation.

The reasons for the good anaerobic labelling observed in the present experiments and for the poor anaerobic labelling reported by Dawson (1953) are now apparent. In the present experiments the dispersions were prepared in water, whereas the dispersions used by Dawson were prepared in 0.08 M-potassium chloride. Hypotonic dispersions are capable of a much more active anaerobic glycolysis and phospholipid labelling than dispersions prepared in potassium chloride (Table 7). In addition, Dawson (1953) supplied adenine nucleotide in the form of AMP. As shown in Table 5, with glucose as substrate either the AMP must be replaced by ATP or HDP must be added to initiate the glycolysis. With AMP in the absence of HDP, both anaerobic glycolysis and ^{32}P incorporation are reduced to low levels. In the system studied by Dawson (1953) the labelling of phospholipid was supported chiefly by energy derived from oxidative phosphorylation, whereas in the water dispersions used in the present study much of the energy was derived from glycolytic processes.

SUMMARY

1. Dispersions of rat brain, prepared in water and suitably 'reinforced', have been shown to incorporate inorganic ^{32}P into lipid phosphorus. Omission of cytochrome *c* or fumarate from the medium, or replacement of the oxygen of the gas phase with nitrogen, did not inhibit the incorporation.

2. Tricarboxylic acid cycle intermediates (citrate, malate or succinate) increased the respiration, but did not increase the aerobic ^{32}P labelling of lipid phosphorus.

3. Magnesium, diphosphopyridine nucleotide, adenosine 5-phosphate (AMP) or adenosine triphosphate (ATP), and substrate that can undergo glycolysis [glucose, mannose or hexose diphosphate (HDP), but not galactose] were necessary for the incorporation under both aerobic and anaerobic conditions.

4. Under anaerobic conditions a source of 'high-energy' phosphorus [ATP, HDP plus AMP, uridine triphosphate plus adenosine diphosphate (ADP), or guanosine triphosphate plus ADP] was essential for optimum glycolysis and labelling of lipid phosphorus.

5. Under anaerobic conditions, glycolysis and incorporation of inorganic ^{32}P into lipid phosphorus were best demonstrated in dispersions prepared in water. Dispersions prepared in isotonic media (potassium chloride or sucrose) were considerably less active.

6. Under aerobic conditions the addition of 2,4-dinitrophenol (7.5×10^{-5} M) caused increases in oxygen consumption and glycolysis, but a decrease in the labelling of phospholipid. Under anaerobic conditions there was little change in glycolysis and a much smaller decrease in phospholipid labelling.

7. The addition of fluoride (10^{-2} M) caused no significant change in oxygen consumption and large inhibitions of both aerobic and anaerobic glycolysis. The labelling of phospholipid was increased under aerobic conditions, but decreased when oxygen was excluded from the system.

8. The addition of iodoacetate (10^{-5} M) caused small inhibitions of oxygen consumption, aerobic glycolysis and aerobic labelling of phospholipid, but much greater decreases in glycolysis and phospholipid labelling under anaerobic conditions.

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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 0.25M-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 0.25M-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 supravitaly 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 0.028M to 0.01M. 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 0.25M-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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