Respiration and Phosphorylation in Preparations from Mammalian Brain

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Glucose constitutes the main substrate supporting the functional activity of the brain. The main end products from glucose in the brain *in vivo* are lactic acid and carbon dioxide, but the proportions in which these are formed vary greatly with the activity of the brain (for a recent assessment see McIlwain, 1950 *b*). These are also the main end products from the metabolism of glucose by slices of mammalian brain *in vitro*. We accordingly decided to study the action of substances which are known to alter the balance of carbohydrate metabolism in brain *in vivo* and *in vitro*. These include narcotics, convulsants, and certain compounds which increase aerobic lactic acid formation. As oxidative phosphorylation constitutes, in brain as elsewhere, a link between functional activity and the metabolism which supports such activity, we have studied the action of the compounds on this process.

The systems studied included that of Ochoa (1941), in which the phosphate esterified during oxidation is transferred to glucose and the phosphorylation followed by determining the loss of inorganic phosphate. In addition, a system has been devised in which labile phosphoric esters themselves can accumulate and be determined together with determination of loss of inorganic phosphate. This does not involve secondary reactions with glucose, and thus has some advantages in studying the actions of inhibitors on the phosphorylation itself.

EXPERIMENTAL

Reagents. Cytochrome *c* was prepared from horse heart according to Keilin & Hartree (1937). It was finally dialysed against water, standardized and preserved in a refrigerator as a solution without $CHCl_3$, as described by Potter (1945 *b*).

Potassium pyruvate was prepared in the light of the observations of Lipschitz, Potter & Elvehjem (1938) and Ochoa (1941) on the care necessary to avoid inhibitory substances. Pyruvic acid, purchased as a fine chemical, was distilled twice *in vacuo* and the fraction boiling at 55–60° (10 mm.) diluted to 5 M and kept in a refrigerator. For use it was diluted to 1 M, cooled in ice water, and brought to pH 7·4 with cold KOH. Purity of the pyruvate was checked by use of carboxylase. For determination of pyruvate in reaction mixtures, a method (Lardy, 1945), based on Case's (1932) dinitrophenylhydrazine procedure, was used.

Adenosinetriphosphate, purchased as Ba salt, was suspended in water and dissolved by adding the minimum quantity of M-HCl, keeping cold throughout. The theoretical quantity of K_2SO_4 was then added, the precipitated $BaSO_4$ centrifuged off, and the supernatant neutralized with KOH, still keeping cold. An 0·015 M solution was stable for a week or more when kept at 0°. With the specimen of triphosphate used, the final solution contained inorganic phosphate to the extent of about 8 % of its labile phosphate. Adenosine-5-phosphoric acid, purchased, was dissolved in KOH to give an 0·015 M solution.

Homogenates. Glass homogenizers of Potter & Elvehjem's type were used, made from stout tubes about 2·5 cm. internal diameter and driven directly by a $\frac{1}{16}$ h.p. motor (max. speed, 2000 rev./min.). The tube with 3 ml. of the suspending medium was weighed and cooled in ice water prior to use. A guinea pig was killed by decapitation, sometimes preceded by a blow on the neck, the cerebral hemispheres placed in the homogenizer tube and the whole weighed. Homogenizing was done by passing the pestle four times through the contents of the tube with maximum hand pressure, and occupied 4 sec. The tube was immediately returned to ice, and saline added to the contents to make a 1:5 suspension. The suspension was made uniform by using the pestle as a plunger without the motor running, and passed through a wisp of glass wool in a funnel to a test tube, also cooled in ice. This suspension was ready to pipette into experimental vessels 5 min. after the death of the animal, and its mean dry weight after subtracting the weight due to saline was 35 mg./ml. Few, if any, whole cells remained in the homogenate. The cerebral hemispheres weighed approx. 2 g., yielding approx. 9 ml. of filtered homogenate.

Dialysis of homogeuates. This must be rapid, as constituents of the phosphorylating system are labile; dialysis through cellophan tubing, obtainable commercially, has been found too slow. A suitable batch of collodion (for semi-permeable membranes: British Drug Houses Ltd.), already in solution, was added to an equal volume of a mixture of 11 vol. ethanol and 50 vol. diethyl ether, mixed, and the bottle left for some days to clear from bubbles. Tubes (12·7 × 1·6 cm.) were filled with the solution and emptied in about 30 sec. while rotating them to coat the walls uniformly; when only a few drops remained they were clamped upside down and left for 0·5 hr. at room temperature to drain and dry. A second coat was then applied in the same way. After the second drying (0·5 hr.) the membrane was moistened, removed from the tube and kept in water until needed. For dialysing homogenates, several litres of 0·05 M-phosphate buffer (from KH_2PO_4 and NaOH, pH 7·4) were kept overnight in a refrigerator. 2 l. were put into a 4 l. beaker, in a refrigerator, the homogenate put into a collodion tube and suspended in

the beaker, and the contents stirred with a motor, sufficiently powerfully to shake the sac well. Two sacs could be run together in 4 l. of phosphate.

Phosphorylation of glucose with dialysed homogenates. The reaction mixtures always contained glucose, fluoride, adenosinetriphosphate and potassium and magnesium salts, with sometimes additional phosphate and fumarate. Choice of the reactants and the concentrations used initially were based on the work of Banga, Ochoa & Peters (1939), and Ochoa (1941), who studied brain preparations, and also of Potter (1945 a), Green, Loomis & Auerback (1948) and Cross, Taggart, Covo & Green (1949) who investigated primarily kidney preparations. The experiments described in a later section were carried out to choose concentrations optimal for the present working conditions. These gave the following as the most generally adopted procedure.

For an experiment with some eight reaction mixtures, the following were mixed: M-glucose, 0·3 ml.; 0·015 M-potassium adenosinetriphosphate, 0·9 ml.; M-NaF, 0·3 ml.; 0·3 M-sodium fumarate, 0·1 ml.; 0·25 M-potassium pyruvate, 0·9 ml.; water or other addition, 0·2 ml. Warburg vessels (conical, with one side arm and a centre well; of about 15 ml.) were fixed in an ice tray, NaOH pipetted into their centre wells and 0·3 ml. 50 % (w/v) trichloroacetic acid pipetted into their side arms. Water, or a solution whose effect was being studied (0·1 ml.), was pipetted into their main vessels, followed by the above mixture (0·3 ml.), the homogenate (1 ml.) and 0·16 M-MgCl$_2$ (0·1 ml.). Papers were immediately put into the NaOH, and the vessels attached to the manometers and placed in the thermostat (37°). After 2·5 min. the joints were rubbed home and shaking commenced. After another 2·5 min. the manometers of experimental vessels were levelled and closed, while those of the controls were removed from the thermostat, their trichloroacetic acid mixed, the vessels detached and returned to the ice tray. Manometric readings for the remaining vessels were taken at intervals of 3 min. usually for 9 or 15 min., after which the reactions were stopped as described before. The series of vessels were put into and taken out of the bath at regular intervals of 15 or 30 sec. To all vessels, now in ice, 1·5 ml. water was added, the solutions each thoroughly mixed with a Pasteur pipette and transferred through coarse fluted papers to test tubes in ice. From these, portions were taken for analysis.

Phosphorylation with more dilute homogenates. Homogenates were prepared in some cases as described above, and in others by homogenizing for about 0·5–1 min. in a homogenizer with a looser pestle and less powerful motor; they were not dialysed. Except where specified otherwise, the cerebral hemispheres were added to 4 vol. of a solution containing 0·6 % potassium chloride and 0·5 % sodium fluoride. The mixture was used immediately after homogenizing, pipetting 0·6 ml. to the side arms of already prepared Warburg vessels; it was at pH 7 without any adjustment.

The vessels contained 2·4 ml. of reaction solution in their main compartments and KOH and paper in their centre wells.

The reaction mixture most generally adopted contained in the final reaction mixture of 3 ml.: 8·3 × 10⁻² M-glycylglycine buffer, pH 7·4; 1·7 × 10⁻³ M-phosphate buffer from KH$_2$PO$_4$ and NaOH, pH 7·4; 1·3 × 10⁻³ M-KCl (from homogenate); 2·1 × 10⁻² M-NaF (from homogenate); 1·7 × 10⁻² M-MgCl$_2$; 3·3 × 10⁻³ M-sodium fumarate; 3·3 × 10⁻² M-potassium pyruvate; 3·7 × 10⁻⁵ M-cytochrome *c*; 10⁻³ M-potassium adenosinetriphosphate; 10⁻³ M-potassium adenosine-5-phosphate. The constituents were prepared so that the majority could be mixed together for a group of six to ten vessels, and added as 2 ml. of solution. The homogenate and added substances whose effects on phosphorylation were being examined were added separately.

The course of the manometric experiment was similar to that described above, the reaction again being stopped by adding trichloroacetic acid, either from a second side arm or by pipette after detaching the vessel.

Determination of phosphates. Inorganic phosphate was determined on portions of trichloroacetic filtrates, according to Lowry & Lopez (1946). The molybdate reagent was kept in a waxed bottle at 2°, and the ascorbic acid used for reduction was prepared immediately before use. The molybdenum blue was read in a Beckman quartz spectrophotometer at 700 mμ. in a volume of 3 ml. Standards of about 1 μg. P/ml. were used for comparison. With this instrument, only occasional and small interference was observed with any of the coloured heterocyclic compounds whose action on phosphate distribution was being studied. Corrections were applied where necessary by taking blank readings after addition of the ascorbate but before addition of the molybdate.

The labile phosphates (possibly adenosinetriphosphate, diphosphate or pyrophosphate) were determined as follows. After trichloroacetic precipitation of either slices or homogenates, the cold suspensions were filtered into test tubes standing in ice. The filtrate was divided and inorganic phosphate determined on one portion. Another (1 ml.) was mixed with 1 ml. 2 N-HCl, the tubes placed in a boiling-water bath for 7 min. and then cooled in ice. NaOH (0·4 ml., 5 N) and sodium acetate (0·1 M, to 5 ml.) were added, and inorganic phosphate redetermined; the increase is referred to as labile (7 min.) phosphate. P/O ratios are calculated in atoms.

RESULTS

Dialysed homogenates

Respiration. Conditions were based initially on those of Banga *et al.* (1939) and Ochoa (1941). Homogenates were studied initially with the additions of fumarate, glucose, magnesium salts, adenosinetriphosphate and fluoride which are indicated above, and examined in the presence and absence of pyruvate. Respiration was high and only slightly affected by added pyruvate; dialysis was carried out in thin collodion sacs for 75 min. This gave a preparation whose respiration (see Table 1, Exps. 1–3) was dependent on added pyruvate, fumarate and magnesium and was almost linear during the short period required. Experiments with different concentrations of the reagents led to the choice of those indicated above, which are close to those of other authors.

Table 1. *Phosphorylation in dialysed homogenates*

(Reaction mixtures of 1·5 ml. were prepared as described in the experimental part. Final concentrations of reactants were: glucose, 0·022M; adenosinetriphosphate, 0·001M; NaF, 0·022M; sodium fumarate, 0·002M; potassium pyruvate, 0·017M; MgCl$_2$, 0·01 M and potassium and sodium phosphates (from the homogenate) approx. 0·02M.)

Exp. no.	Changes in reaction mixture	Oxygen uptake (μmol./vessel)	Loss of inorganic phosphate (μmol./vessel)	P/O ratio (atom/atom)
1	None	4·15	18·2	2·2
	Anaerobic	0	1·0	—
2	None	2·50	12·5	2·45
	No pyruvate or fumarate	0·65	0·8	0·61
	No Mg	1·9	1·7	0·4
3	None	4·35	15·5	1·8
	Fluoride to 0·03M	4·16	15·0	1·8
	Fluoride to 0·036M	3·5	13·1	1·87
4	No fluoride	5·0	0	—
5	None	2·54	11·0	2·25
	Ethyl red 2 × 10^{-7}M	2·15	6·3	1·46
	Ethyl red 6·7 × 10^{-7}M	1·95	4·25	1·12
	Ethyl red 2 × 10^{-6}M	1·70	3·0	0·91
	Ethyl red 6·7 × 10^{-5}M	1·06	1·0	0·47
6	None	3·58	18·5	2·6
	2:4-Dinitrophenol 5 × 10^{-5}M	3·70	10	1·3
	2:4-Dinitrophenol 1·66 × 10^{-4}M	2·64	0·4	0·1
7	None	3·38	14·7	2·17
	Phenosafranine 6·67 × 10^{-5}M	2·35	9·8	2·08
	Phenosafranine 10^{-4}M	1·55	1·0	0·3
	Phenosafranine 1·33 × 10^{-4}M	1·0	0·3	0·15
8	None	3·53, 3·30	19·2, 17·9	2·70, 2·7
	Chloral 1·5 mg./ml.	1·77, 1·79	15·6, 13·95	4·4, 3·9
9	None	2·74, 2·58	14·5, 14·5	2·66, 2·82
	Chloral 1·5 mg./ml.	1·20, 1·21	9·3, 9·3	3·88, 3·84
10	None	2·66	15·1	2·84
	Chloral 0·75 mg./ml.	1·65	10·9	3·3
11	None	3·67	6·7	0·91
	Chloral 0·75	3·36	7·8	1·16
	Chloral 1·5	2·43	5·9	1·21
	Chloral 2·25	1·68	4·2	1·25
12	Fumarate 1·5 × 10^{-4}M	1·53	4·8	1·6
	Fumarate 1·5 × 10^{-4}M; phenosafranine 4 × 10^{-5}M	1·43	2·8	0·98
	Fumarate 1·5 × 10^{-4}M; phenosafranine 6 × 10^{-5}M	1·3	0·7	0·27
	Fumarate 1·5 × 10^{-4}M; phenosafranine 8 × 10^{-5}M	1·3	0·1	0·04
	Fumarate 1·5 × 10^{-4}M; phenosafranine 10^{-4}M	1·06	1·2	0·5

The addition of fluorides during these experiments was necessary to prevent loss of the phosphates esterified, as active adenosinetriphosphatases exist in brain (Meyerhof, 1947; Utter, Reiner & Wood, 1945; Meyerhof & Wilson, 1948). Exps. 3 and 4 of Table 1 illustrate its action. Variations in fluoride concentrations between 0·02 and 0·03M had little effect on respiration, but 0·036M depressed it by about 25 %. At the concentration of 0·022M chosen finally, the respiratory quotient was determined during the oxidation of pyruvate, by the method of Warburg & Yabuso (1924). For the oxidation occurring during the first 9 min. an approximate value of 1·4 was found, and during the first 27 min., one of 1·22. Complete oxidation of pyruvate requires a value of 1·2.

The rate of respiration during different experiments with the full reaction mixture has been between 0·48 and 0·74 μmol./mg. dry wt./hr., or 81–127 μmol./g. corresponding fresh wt./hr. An example is given in Fig. 1. The 1 ml. of dialysed homogenate of a typical experiment contained material from 200 mg. fresh weight of brain, which was found to have 34–36 mg. dry wt. With the lower concentration of fumarate used in some experiments (Table 1, Exp. 12), respiration was at the rate of 0·18 μmol./mg. dry wt./hr., or 31 μmol./g. fresh wt./hr.

Phosphorylation. Rapid disappearance of inorganic phosphates was found aerobically, but not with the same reaction mixture in the absence of oxygen (Table 1, Exp. 1). In the absence of fluoride no inorganic phosphate was lost. During experi-

ments lasting 9 or 15 min. the ratio of inorganic phosphate lost to oxygen absorbed (ratio atoms P/atoms O) varied in different experiments from 1·6 to 3·0, with 2·4–2·8 as the most frequent range. The course of phosphorylation was not, however, found to be entirely uniform, and is illustrated in Fig. 1. The initial lag in phosphorylation is under-
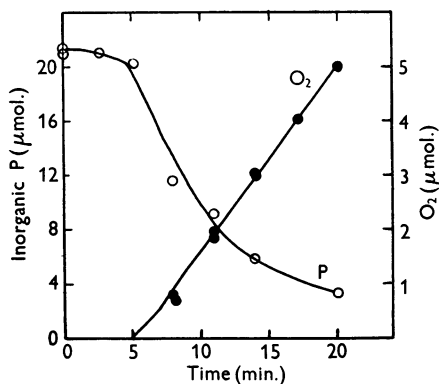

Fig. 1. Course of respiration and phosphorylation in dialysed homogenates. The reaction mixture was that described as normal in the experimental part, and was contained in eight vessels in which the reaction was stopped at the different times indicated. Dry weight of homogenate used/vessel, 34 mg. P/O ratio between 5 and 14 min.: 2·55.

standable, as zero time in the figure is the time at which the cold flasks are placed in the thermostat. Rapid phosphorylation is normally beginning at the time at which manometric observations of oxygen uptake are commenced, and phosphorylation is again slowing at the end of the 9 min. experimental period. During a brief period P/O ratios may be higher than average for the whole period, which is that quoted in Table 1.

Effect of added substances on respiration and phosphorylation. The effects of several substances on respiration, on loss of inorganic phosphate, and on the P/O ratio are quoted in Table 1. The substances include phenosafranine and ethyl red, which increase aerobic glycolysis in brain slices (Dickens, 1936; McIlwain & Grinyer, 1950), and 2:4-dinitrophenol (Loomis & Lipmann, 1948) which, as well as phenosafranine (Judah & Williams-Ashman, 1949), has been reported to inhibit the phosphorylation associated with oxidation in preparations from other tissues. Each of these substances lowered the P/O ratio in the present experiments. Chloral was examined also; this inhibited respiration and phosphorylation to about the same degree, so that the P/O ratio changed little or was slightly increased.

The results suggested that substances of the type of 2:4-dinitrophenol and phenosafranine were indeed inhibitors of aerobic phosphorylation. There were, however, certain sunatisfactory features

about the present findings, the main one being that the lowering of phosphorylation was usually associated with some inhibition of respiration. The different substances studied differed in this respect; the difficulty of discriminating between effects on respiration and phosphorylation was greater with phenosafranine than with 2:4-dinitrophenol. The inhibition of respiration was a phenomenon not seen in slices when these contained comparable concentrations of phenosafranine (McIlwain & Grinyer, 1950), and it varied in different reaction mixtures with homogenates. Thus Table 1 shows that the inhibition of respiration was less in homogenates which had a low concentration of fumarate; but here respiration was slow and the P/O ratio low.

It was also necessary in assessing experiments with inhibitors to consider the course of phosphorylation and respiration, and this is shown in Fig. 1. During the period of 5 min. equilibration before manometric readings were taken, phosphate changed relatively little; after this, rapid change took place until three-quarters of the inorganic phosphate was lost. Respiration was almost linear during the whole of the period of observation. In a normal experiment the period during which changes were observed was in the 9–12 min. after beginning respiration unless respiration was observed to be slow (e.g. with low fumarate concentrations), in which case experiments were continued for 15–20 min. During this time the changes in phosphate were approximately but not exactly linear. It is presumably such departure from linearity which permits the apparent increase in P/O ratio observed with chloral. It would have been desirable to observe the whole of the changes in both oxygen and phosphate by mixing the homogenate with the reaction fluid after the period of equilibration, but the phosphorylating systems in such dialysed homogenates are very labile to even a brief warming at 37° in the absence of substrates. These and other considerations led to the use of non-dialysed homogenates described below.

Non-dialysed, more dilute homogenates

A system more suited for examining the action of inhibitors on phosphorylation has been devised as follows: (i) the reactions involved in phosphorylation have been decreased in number by following the accumulation of labile phosphates themselves, rather than the result of the further transfer to glucose; (ii) the homogenate has not been dialysed, so that its phosphorylating systems remain more stable; (iii) the reaction conditions have been brought closer to those found by Elliott & Libet (1942), Elliott, Scott & Libet (1942) and Larner, Jandorf & Summerson (1949) to be optimal for respiration of brain homogenates. The addition of fluoride to minimize loss of adenosinetriphosphate remains necessary.

Respiration. Dilution of the dialysed homogenate led to a rapid fall in its respiration and phosphorylation, but with the present experimental arrangement, without dialysis, respiration remained high at concentrations of brain of some 6–8 mg. dry wt./ml. This enabled inorganic phosphate and adenosinetriphosphate to be used at concentrations approaching those normal to the tissue, and 1 to 2×10^{-3}M concentrations were chosen. Adenosine-5-phosphate was included as phosphate acceptor, also at 10^{-3}M. The low phosphate concentrations made necessary the use of an independent buffer and glycylglycine was chosen after finding veronal to be inhibitory.

Although labile phosphates are synthesized in the present reaction mixture, it was found necessary to add adenosinetriphosphate, as such, for optimal respiration and phosphorylation. This is shown in Fig. 2, together with the effect on respiration of omitting other constituents of the reaction mixture. Magnesium salts were seen to be necessary for optimal respiration and a similar experiment showed this to be the case also with cytochrome c. Respiration is, however, nearly independent of added inorganic phosphate and phosphate acceptors, some of which are already contained in the homogenate. Although cozymase and nicotinamide were found necessary by Larner *et al.* (1949) for optimal respiration in certain brain homogenates, we did not include them in the normal reaction mixture as we specifically wished to study the effects of such substances on phosphorylation. When added in the concentrations suggested by Larner *et al.* (1949) the compounds had little effect on respiration; our preparations presumably corresponded to the 'coarse homogenates' of these authors. The present preparations were found to retain some 25 % of the cozymase of the whole tissue, for at least 30 min. (Gore, Ibbott & McIlwain, 1950). Air was used in the gas space during most of the experiments. Oxygen appeared to have some very slight advantage when the manometric apparatus was stopped frequently for readings. It was never found detrimental to respiration during the present experiments, which usually lasted only 9 or 15 min.; Dickens's (1946) observations, on oxygen poisoning in brain suspensions (see Larner *et al.* 1949) concerned much longer experimental periods.

Respiration in the present system without added substrates was higher than with the dialysed homogenates (Fig. 2). The present system is thus less suited for following the fate of the added substrate. Without fumarate, respiration fell to about two-thirds of its rate and phosphorylation fell still further. Doubling or halving the normal concentration of pyruvate led to little change. We have not determined accurately the ratio of changes in pyruvate and oxygen, as the pyruvate was present in the normal mixture in large excess and a relatively small

change took place in it. On reducing its quantity to one-fifth, the rate of respiration fell, but some measure of the removal of pyruvate could be obtained. During 60 min. this was found to be about half the molar quantity of the additional oxygen absorbed. (Pyruvate lost: 1·4 of 10 μmol. Oxygen
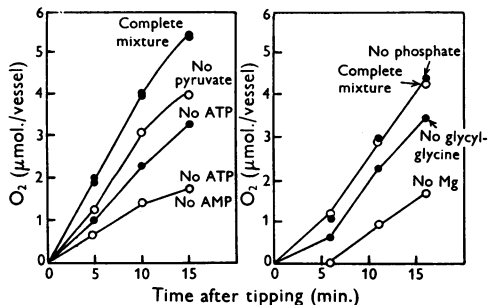

Fig. 2. Effect of different reagents on the respiration of dilute homogenates. Full medium: that described in experimental section, p. 2. ATP, adenosinetriphosphate; AMP, adenosinemonophosphate.

uptake in the presence of pyruvate, 4·73 μmol.; in absence, 1·95.) Although caution is needed in interpreting the change in oxygen uptake, there is some approximation to complete oxidation of pyruvate in the present reactions, and no considerable diversion of it to other reactions.

Phosphorylation. In the absence of fluoride, labile phosphate is lost from the reaction mixture and inorganic phosphate increases. Fig. 3 shows this, and also the actions of a range of fluoride concentrations on respiration and phosphorylation. Inhibition of respiration was noticeable even below 10^{-2}M (for comparable inhibitions in kidney homogenates see Potter, 1945a). In spite of this, the yield of labile phosphate as measured by the P/O ratio increased until about 2×10^{-2}M-fluoride was reached; it then fell. Concentrations between 1·6 and 2·7 $\times 10^{-2}$M were found optimal. On the basis of such results it was decided to incorporate in the phosphorylating system 2·1 $\times 10^{-2}$M-sodium fluoride. Ochoa (1943) employed 2·5 $\times 10^{-2}$M-fluoride with a muscle preparation and found comparable inhibition of respiration and of the breakdown of labile phosphates. In the present experiments, fluoride was found to have a greater effect in maintaining labile phosphates when it was present in the initial homogenizing fluid. Its action under these conditions is shown in Table 2. The reaction mixture here lacked only the pyruvate and fumarate of the normal system. Adenosinetriphosphate was added to one pair of vessels to give about 10^{-3}M (as in the reaction mixture), and to another pair 2·4 $\times 10^{-3}$M, which is somewhat greater than the usual final concentration after phosphorylation. Hydrolysis of labile phosphate is seen to be

fairly well controlled, though some loss occurs with the higher concentrations of adenosinetriphosphate. One result of the incomplete control of phosphatases was that certain potent inhibitors of phosphorylation could lead not only to lack of increase in labile phosphate, but to decrease in its quantity in certain reaction mixtures (Table 3).
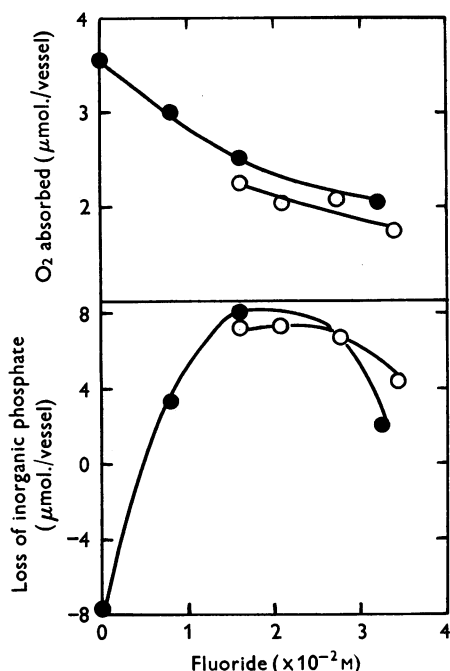

Fig. 3. Respiration and phosphorylation in dilute homogenates containing different concentrations of fluoride. Reaction period, 12 min.; 7 mg. dry weight homogenate/ ml. Two experiments, the results being indicated by ● and ○ respectively.

We have not determined the nature of the labile phosphoric ester which accumulates, beyond observing that the whole of the inorganic phosphate which disappears from the reaction mixture is recovered after 7 min. at 100° with N-HCl. It could therefore be the labile phosphate of the adenosine polyphosphates, or inorganic pyrophosphate (see Cross et al. 1949). Its quantity was decreased when adenosinemonophosphate was omitted from the reaction mixture. On the other hand, the inclusion of creatine (0·016M) was found to be without effect on the loss of inorganic phosphate, or on the formation of labile phosphate, in a variety of reaction mixtures.

The phosphorus/oxygen ratio. These findings gave the basis for the reaction mixture described in the experimental part. A series of results with it are quoted in Table 3. This shows that in the 15 min. of a typical experiment, more than half of the inorganic

phosphate became esterified and the concentration of labile phosphate was nearly doubled. Substances of the type under investigation could inhibit these changes or, in high concentrations, reverse them. Nevertheless, the inorganic phosphate lost was always accounted for completely, within experimental error, by the 7 min. phosphate formed. The

Table 2. *Control of adenosinetriphosphatase by homogenizing in fluoride*

(Vessels contained glycylglycine, pH 7·4, $8·3 \times 10^{-2}$M; KCl, $1·3 \times 10^{-2}$M; cytochrome c, $3·7 \times 10^{-5}$M; sodium fumarate, $3·3 \times 10^{-3}$M; MgCl$_2$, $1·7 \times 10^{-2}$M, and homogenate containing 18·6 mg. dry wt. brain. Initial inorganic P was found to be 84 μg. and labile P, 70 or 146 μg./ml. according to the quantity added.)

Adenosine-triphosphate added (10^{-3}M)	Fluoride (10^{-2}M)	Change (μg./ml.) during 15 min. at 37°	
		Inorganic P	Labile P
1·1	0	+61	−57
2·4	0	+109	−105
1·1	2·1	+2	+2
2·4	2·1	+19	−15

ratio between the changes in phosphate and in oxygen, quoted in the last column of Table 3, is comparable to ratios reported by many investigators. It is lower than that obtainable under conditions chosen as optimal for phosphate removal, such as those of the dialysed homogenate described above. The lower ratio in the present experiments appears likely to result from the increased difficulty of controlling adenosinetriphosphatases, caused by the greater concentration of adenosinetriphosphate in the present reaction mixture.

The course of changes in both phosphates and oxygen have been followed and found to be nearly linear during the 10 or 15 min. during which phosphorylation experiments were normally run; both processes had fallen to about three-quarters of their initial rates, at 15 min.

Actions of added substances on phosphorylation by diluted homogenates

2:4-*Dinitrophenol and azide.* The effect of the dinitrophenol in decreasing phosphorylation, noted above, was fully confirmed with the present homogenate. Low concentrations of it also consistently caused a slight increase in respiration associated with the first lowering of phosphorylation. Concentrations of about 10^{-4}M had a nearly maximum effect in lowering phosphorylation, and at this point respiration was little if at all affected (Fig. 4). It is especially notable that concentrations, even ten times those at which lowering of the P/O ratio was evident, did not lower respiration below the control value (Table 3), though still higher concentrations could do so. This latter effect may have been

Table 3. *Phosphorylation with more dilute homogenates: effects of added substances*

(Vessels contained the reaction mixture of Table 2 with K pyruvate, $3\cdot3 \times 10^{-2}$ M and NaF, $2\cdot1 \times 10^{-2}$ M, and were incubated for 15 min. Respiration was close to linear, with a small initial lag. Vessels were removed and their contents precipitated after 15 min. at 37°. Initial inorganic P, 216 μg./vessel or about 60 μmol./g. brain; labile P, 168 μg./vessel or about 145 μmol./g. brain.)

Exp. no.	Changes in reaction mixture (M concns. of added substances in parentheses)	Oxygen uptake (μmol./g. fresh wt.)	Change in phosphate (μmol./g. fresh wt.)	P/O ratio (atom/atom)
1	No pyruvate	23·4	40·4	0·86
	None	26·3	47·5	0·90
	2:4-Dinitrophenol (10^{-6})	25·0	43·0	0·86
	2:4-Dinitrophenol (10^{-5})	30·8	33·6	0·55
	2:4-Dinitrophenol (10^{-4})	24·3	− 15	− 0·31
	Sodium azide (10^{-3})	23·3	− 8·5	− 0·18
	Sodium azide (10^{-2})	17·6	− 4·5	− 0·13
2	None	16·3	22	0·68
	Synthalin B (10^{-5})	14·8	4·4	0·14
	Synthalin B ($3\cdot3 \times 10^{-5}$)	13·0	− 1·3	− 0·48
	Synthalin B (10^{-4})	9·5	− 3·5	− 1·32
3	None	12·1	12·2	0·50
	Nicotine (10^{-3})	12·1	10·4	0·43
	Nicotine ($3\cdot3 \times 10^{-3}$)	13·0	11·2	0·43
	Nicotine (10^{-2})	12·9	8·4	0·32
4	None	13·6	42	1·53
	Nicotine ($1\cdot7 \times 10^{-2}$)	12·1	23·2	0·96
	Nicotine ($3\cdot3 \times 10^{-2}$)	13·8	14·4	0·56
5	None	12·0	25·8	1·08
	Diazine green (10^{-6})	13·4	24·2	0·90
	Diazine green ($3\cdot3 \times 10^{-6}$)	13·1	22·4	0·86
	Diazine green (10^{-5})	12·6	15·4	0·61
6	None	12·1	12·2	0·50
	Diazine green (10^{-5})	10·8	1·0	0·05
	Diazine green ($3\cdot3 \times 10^{-5}$)	7·8	− 0·7	− 0·05
	Diazine green (10^{-4})	6·1	− 1·2	− 1·0
7	None	23·2	47·8	1·03
	Neutral red ($1\cdot7 \times 10^{-3}$)	21·5	20·2	0·47
	Neutral red (5×10^{-3})	12·8	7·2	0·28
	Pyocyanine ($3\cdot3 \times 10^{-4}$)	26·5	41·6	0·78
	Pyocyanine (10^{-3})	25·4	26·4	0·52
8	None	28·8	38·8	0·67
	Pyocyanine (10^{-3})	27·0	18·8	0·35
	Pyocyanine ($3\cdot3 \times 10^{-3}$)	18·5	− 3·6	− 0·10
	Pyocyanine (10^{-2})	7·4	− 11·8	− 0·80

secondary to the changes in phosphate, as it was always associated with a loss (and not merely a lack of increase) of labile phosphates in the reaction mixture, and absence of adenosinetriphosphate lowers respiration (Fig. 2). The effect of sodium azide was similar to that of the dinitrophenol (Table 3).

Phenazines, nicotine and synthalin. (For structures of some of these see McIlwain, 1950a.) These have certain characteristics in common with the dinitrophenol. All show effects on respiration and phosphorylation, which vary markedly with the concentration of added substance. Phenosafranine, diazine green and pyocyanine were observed to increase respiration at the lower concentrations of their active range (Table 3, Fig. 4). In most cases this was associated with lowered phosphorylation. Higher concentrations of these substances then led to inhibition of respiration with much greater inhibition of

phosphorylation. Lowered phosphorylation, accompanied by little if any change in respiration, was also observed to follow the addition of nicotine and neutral red. Lowering was also observed with synthalin B (undecane diamidine), but here it was associated with inhibition of respiration.

The results of several experiments on the action of phenosafranine are shown in Fig. 4 in comparison with those of the dinitrophenol. The similarity is considerable, but the figure illustrates also that the differentiation between inhibition of respiration and phosphorylation is not so great with phenosafranine as with the dinitrophenol. The difference in respiration and phosphorylation, between control and experimental vessels, has been selected in Fig. 4C as the best means of expressing the results of a number of experiments. This is because the actual level of phosphorylation itself varies with different

homogenates (Table 3). When so expressed, the results in Fig. 4C, which are from four different experiments at different times during the course of 6 months, are seen to be in approximate agreement.
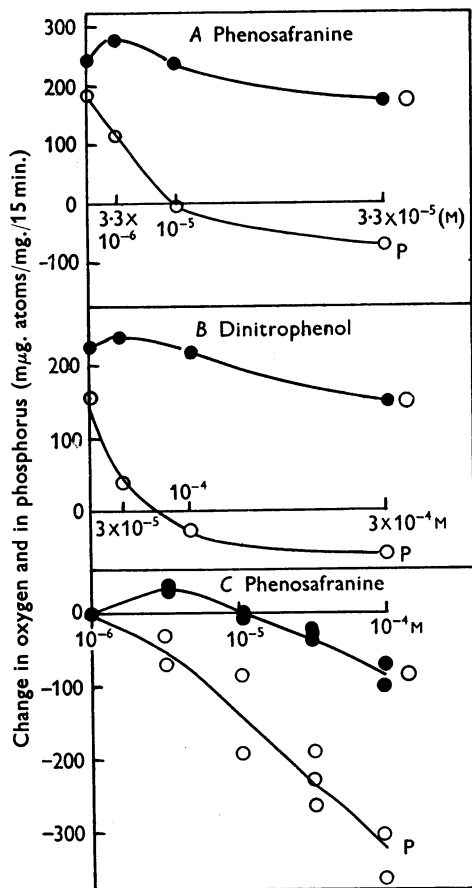

Fig. 4. Effect of different concentrations of phenosafranine and 2:4-dinitrophenol on respiration and phosphorylation in dilute homogenates. Ordinates: A and B, changes in individual vessels; C, differences of experimental from control vessels, in a series of experiments.

DISCUSSION

The balance between lactic acid and carbon dioxide as end products of carbohydrate breakdown in animal tissues has been the subject of many studies. It was early realized that the relation between the two end products was conditioned by the energy made available in their formation, and these relationships have been expressed more recently in terms of energy-rich phosphate derivatives (Johnson, 1941; Lipmann, 1942; Seits & Engel'gardt, 1949).

Brief accounts of the actions on aerobic phosphorylation of agents which affect the course of carbohydrate metabolism have appeared during the past 2 years. Loomis & Lipmann (1948, 1949) found a dinitrophenol to inhibit uptake of inorganic phosphate by a washed kidney suspension oxidizing glutamate; the range of concentration used was 5×10^{-6}–2×10^{-4}M, and respiration was little affected or slightly stimulated. Azide between $1 \cdot 7 \times 10^{-2}$ and $1 \cdot 7 \times 10^{-3}$M inhibited phosphorylation in a modified system. Judah & Williams-Ashman (1949), also using kidney preparations oxidizing glutamate, found 2:4-dinitrophenol, phenosafranine, and some related compounds to inhibit phosphorylation but not respiration. More recently, Tepley (1949) examined the action of 2:4-dinitrophenol in brain and liver preparations, and Eiler & McEwen (1949) found pentobarbital to inhibit both respiration and phosphorylation in a brain homogenate oxidizing pyruvate. In the present work the actions of several of these substances, and of some others, have been studied under two well defined sets of experimental conditions. This has enabled some comparative observations to be made on the types of effect given by different substances.

The first main differentiation in type of inhibition corresponds to one indicated by Eiler & McEwen (1949) and can be illustrated by the effects of chloral and of 2:4-dinitrophenol. In the concentrations in which it increases lactic acid formation in guinea pig brain slices (Buchel & McIlwain, 1950), chloral inhibits phosphorylation in homogenates of guinea pig brain. The effects both in slices and in homogenates are, however, always associated with inhibition of respiration. In homogenates, this occurs to a degree approximately equal to that of the inhibition of phosphorylation. The ratio of the two changes, or the P/O ratio, is therefore altered relatively little by chloral. These results parallel those reported briefly by Eiler & McEwen (1949) to be characteristic also of another narcotic, phenobarbitone. Hence one may conclude that a respiratory and not a phosphorylating step is primarily involved in the actions of these compounds. Michaelis & Quastel (1941) have already attempted to determine the respiratory step involved in the action of this class of compound. The effects of 2:4-dinitrophenol, when examined under the same conditions as chloral, are characteristically different in that respiration can be unaffected or even increased, while phosphorylation is greatly inhibited. The P/O ratio thus falls. These two types of inhibition of phosphorylation may correspond to the two types of action on aerobic glycolysis found by Warburg (1926) in different substances: an increase in glycolysis with decreased respiration, and an increase in glycolysis without decrease in respiration. Narcotics are cited as examples of the first action.

Differentiation of these two types of action assists in assessing the actions of the other substances examined. Of these, the phenazine derivatives

studied by Dickens (1936), McIlwain (1950a) and McIlwain & Grinyer (1950) form the largest group, and of them phenosafranine has been most fully examined. The actions of low concentrations of phenosafranine resemble those of the dinitrophenol, though inhibition of phosphorylation is not so well differentiated from inhibition of respiration. This is, however, a characteristic which varies with the system in which phenosafranine is examined. Differentiation is much better with the diluted than with the dialysed homogenates. The inhibition of respiration by phenosafranine in homogenates is a new phenomenon in the sense that it does not occur in slices which contain comparable concentrations of phenosafranine (McIlwain & Grinyer, 1950). It therefore seems that the action of lower concentrations of phenosafranine are most relevant to its action in slices. Here it is interesting to see that the homogenates retain a characteristic often shown by phenosafranine in slices, namely a stimulation of respiration at the lower concentrations at which its action on phosphorylation and glycolysis first became noticeable. The stimulation is seen also in the action of the dinitrophenol in homogenates.

An immediate conclusion from the findings with 2:4-dinitrophenol and low concentrations of phenosafranine might be that they uncoupled some normal link between phosphorylation and respiration, (Loomis & Lipmann, 1948). This conclusion appears to remain a valid one in spite of the observations of Tepley (1949) on the possible mechanism of the action of dinitrophenol. Before such a conclusion could be accepted with respect to the present system some subsidiary investigations were, however, necessary. The major difficulties in interpretation arose from the presence of phosphatases and the use of fluoride in controlling them. Thus, in the present studies we have considered the following points.

First, an acceleration of phosphatases would give a result equivalent, when examined by the present techniques, to decreased phosphorylation. It was therefore important to examine the effect of, for example, phenosafranine on the adenosinetriphosphatase of brain to see whether change was observed. This has been done (McIlwain, 1950a) and negligible effect observed at concentrations equal to, or greater than, those of the present experiments. Secondly, if 2:4-dinitrophenol or phenosafranine caused release of adenosinetriphosphatases from their inhibition by fluoride, the result would again be equivalent to inhibition of phosphorylation. It therefore appeared desirable to attempt an experiment in the absence of fluoride. With the dialysed homogenates this results only in a lack of any change in phosphate, but a graded effect can be seen in the experiments with diluted homogenates in reaction mixtures containing added adenosinetriphosphate. Fig. 5 gives the result of such an experiment. Here 2:4-dinitrophenol and low concentrations of

phenosafranine lead to a greater loss of labile phosphate than in their absence; the change in labile phosphate in their absence presumably represents the balance of its synthesis and breakdown. Phenosafranine and the dinitrophenol therefore show the same action in the absence as in the presence of fluoride.
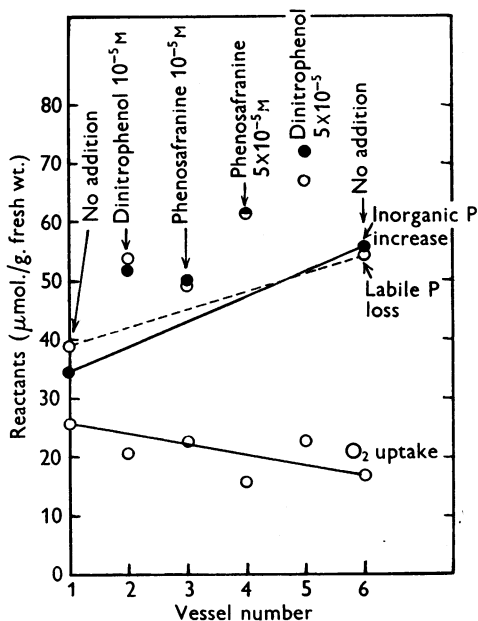

Fig. 5. Change in oxygen and phosphates in the absence of fluoride. After equilibration, brain homogenate was tipped from side arms of vessels to the main compartments, in the order of the vessel numbers quoted. Initial labile phosphate, 79 μmol./g. fresh wt.; duration of respiration, 9 min.

Thirdly, additional problems of interpretation are presented in systems in which the observed P/O ratio (of atoms P esterified/atoms O absorbed) is lower than the maximum. Using glucose or hexose monophosphate as phosphate acceptor, Ochoa (1941) found ratios of up to 2 with brain oxidizing pyruvate, and Cross et al. (1949) ratios approaching 2·5 with kidney oxidizing α-ketoglutarate. These values are reached during the present experiments with dialysed homogenates.

In diluted homogenates values are between 0·5 and 1·8, which presumably reflects the greater difficulty in controlling phosphatases during these experiments, when adenosinetriphosphate is present in considerable quantity. Ochoa (1943) noted such variation in phosphorylation in muscle preparations and gave the same explanation. In dialysed homogenates the value of 2·5 atoms/atom may even for a brief period be exceeded in the presence of concentrations of fumarate of the order of 0·02 M; this may

be due to fumarate itself acting as hydrogen acceptor. With lower fumarate both respiration and the P/O ratio are lower and therefore substances which prevent the participation of fumarate would yield a lowered P/O ratio. We have also observed an instance of a secondary lowering of P/O ratio through an action at pyruvate.

It therefore appears that the action of inhibitors in which lowering of P/O ratio is associated with any inhibition of respiration needs very careful assessing before it can be concluded that the primary action of an agent is on a phosphorylating rather than a respiratory step. In considering the actions of the phenazines and nicotine it is therefore especially noteworthy that phosphorylation can be inhibited in the diluted homogenates without any inhibition, or even with an increase, in respiration. To some extent, however, observation of increase in respiration brings its own problems, for it raises the question of whether all the oxygen absorbed is necessarily associated with phosphorylation at the same P/O ratio. If not, a change in overall P/O ratio could result from the action of an agent on some part of the respiration without acting differentially on the phosphorylation associated with it. For this reason we have considered it important to examine the actions of a variety of agents under the same experimental conditions, and observations with chloral form an important control in considering the action of 2:4-dinitrophenol and the phenazines.

It is concluded, therefore, that the lower concentrations of phenosafranine act like the dinitrophenol in inhibiting some step connecting phosphorylation with respiration. There are likely to be many such steps and differential inhibition of these could be of value in disentangling the processes involved. Differentiating characters in the action of dinitrophenol and the phenazines are therefore of interest. The inhibition of respiration by higher concentrations of phenosafranine and its much more potent action in inhibiting cozymase degradation (McIlwain, 1950a) may be examples of such characters.

Comparison of the present findings in brain homogenates with the reports available for the actions of similar compounds in preparations from mammalian kidney, show some similarities and some differences. Respiration in the present systems appears more susceptible to 2:4-dinitrophenol, but less so to azide. Thus Loomis & Lipmann (1949) found it necessary to use ferricyanide as hydrogen acceptor in order to dissociate the effect of azide on respiration of kidney homogenates, from its effect on phosphorylation. With brain differential inhibition was seen with oxygen as acceptor (Table 3). Our system contained added cytochrome c.

In the case of the phenazines and nicotine in our studies, it is possible to make some quantitative comparisons between their actions on phosphorylation in homogenates and their actions on aerobic lactic acid formation in slices. This is because the concentrations of the different substances in slices have been determined and correlated with their actions on glycolysis (McIlwain & Grinyer, 1950). Pyocyanine is excluded from the comparison because it is chemically changed in the slices, where it exists largely in reduced forms. The comparison is shown in Table 4. The actions on phosphorylation were derived from data such as that of Table 3, plotted as shown in Fig. 4. Two arbitrary degrees of action on phosphorylation were selected, which were reached with most of the compounds without an effect of more than 10 % on respiration. The concentrations of substances causing such action were then read from the graphs. The actions of these substances on aerobic lactic acid formation have already been compared by a similar method and the values of Table 4 are quoted from the previous study (McIlwain & Grinyer, 1950). The order of potency of the four compounds in bringing about the two changes is seen to be the same. The ratio of active concentrations of any two compounds in depressing phosphorylation is greater than the ratio of concentrations which increase lactic acid formation.

The correlation is thus suggestive only, and not demonstrative of connexion between phosphorylation and glycolysis. The lack of closer correspondence between the two sets of values does not count against the suggestion as there are many difficulties in correlating the actions of substances in slices and homogenates. Some of these have been discussed earlier (McIlwain & Grinyer, 1950). Also, homogenates and slices react differently towards adenosinetriphosphate and cozymase. The degrees of change in aerobic phosphorylation (100 and 200 μg.

Table 4. *Comparison of the concentrations of substances which affect aerobic phosphorylation and glycolysis*

(Derivation of the values is described in the text, this page.)

	Concn. ($\times 10^{-5}$M) decreasing aerobic phosphorylation by		Concn. ($\times 10^{-5}$M) raising rate of aerobic lactic acid formation to	
	100 μg. atom/g./hr.	200 μg. atom/g./hr.	15 μmol./g./hr.	30 μmol./g./hr.
Phenosafranine	0·9	1·6	6	16
Diazine green	1·3	5·6	7	24
Neutral red	130	280	140	550
Nicotine	2200	—	600	1100

atom/g./hr.) and lactic acid formation (15 and 30 μmol./g./hr.) chosen as bases for the comparison were selected for experimental convenience and no opinion is expressed regarding their actual values. Independent techniques are clearly required to show directly any connexion between actions on phosphorylation and glycolysis, and, for this reason, study of the levels of phosphates in tissue slices during their metabolism has been commenced (Buchel & McIlwain, 1950; McIlwain & Cheshire, 1950).

SUMMARY

1. Conditions were studied for obtaining maximum phosphorylation of glucose during respiration of dialysed brain homogenates. Ratios of atoms inorganic phosphorus esterified/atoms oxygen absorbed (P/O ratios) of about 2·5 were obtained.

2. In such homogenates, both phosphorylation and respiration were lowered by chloral, but the P/O ratio changed little, or rose. The P/O ratio was lowered greatly by 2:4-dinitrophenol with little lowering, and sometimes a stimulation, of respiration.

Ethyl red had an effect intermediate between those of the above compounds. Phenosafranine also lowered the P/O ratio, with a variable effect on respiration.

3. A system was also devised in which loss of inorganic phosphate and accumulation of labile phosphates catalysed by a brain homogenate could be studied. The homogenate in this case was not dialysed but was more dilute, and adenosine-5-phosphate was added as phosphate acceptor. P/O ratios of 0·6 to 1·8 were observed.

4. 2:4-Dinitrophenol again inhibited phosphorylation with little action on respiration, and azide had a similar effect. The actions of phenosafranine, diazine green, neutral red, and nicotine were also in general similar, though some differences were noted.

5. The findings are discussed in relation to the actions of the different substances on the course of carbohydrate metabolism.

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