The Inorganic Phosphate and Phosphocreatine of Brain Especially during Metabolism *in vitro*

By H. McILWAIN, L. BUCHEL (British Council Bursar) and J. D. CHESHIRE
Biochemical Laboratories, Institute of Psychiatry (British Post-Graduate Medical Federation, University of London), Maudsley Hospital, London, S.E. 5

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As phosphate derivatives form such important intermediates between the functioning of tissues and the metabolism which supports function, we decided to investigate the levels of certain phosphates in brain slices before and during metabolism *in vitro*. Investigation of this type appeared all the more necessary because there is now an extremely interesting group of observations on the levels of phosphorus derivatives in the brains of experimental animals, before and after they have been subjected to various treatments. (For references and an assessment see McIlwain, 1950; some results are quoted in Table 3 of the present paper.) The substances which have been determined following *in vivo* treatment of animals have included inorganic phosphate, creatine phosphate, and adenosine phosphates; and the treatments during which marked changes in these have been observed have included narcosis and induced convulsions. During these processes, the phosphate fractions in which greatest change has been observed have been inorganic and creatine phosphates, and we have accordingly commenced our studies by examining these compounds.

In contrast to the data available from experiments *in vivo*, relatively little information is available of the concentrations of phosphates during *in vitro* metabolism of, for example, brain slices, though Macfarlane & Weil-Malherbe (1941) studied some of these compounds, especially during anaerobic glycolysis, in investigating the course of glycolysis. This deficiency is probably due to the extremely rapid changes which have been reported to take place in these substances, following death or injury to the head. We have fully confirmed the rapidity and magnitude of such changes, but have also found that when brain slices are prepared and maintained under conditions normally regarded as satisfactory for metabolic studies *in vitro*, the changes in phosphates in many cases ceased and could be reversed. Relatively stable levels in inorganic and creatine phosphates were found, which were to some extent characteristic of metabolic conditions, and which could approach those normal to the tissue *in vivo*.

This study includes a subsidiary investigation into procedures for determining inorganic or creatine phosphates in small quantities of tissue. Two methods have been used. The first was an application of the well established calcium-ethanol separations, but determining, as well as phosphates, the creatine of creatine phosphate, precipitated at pH 8·2–8·5 by calcium salts in 80 % ethanol. The second depended on the determination of inorganic phosphate under both Fiske & Subbarow's (1925, 1929) conditions and under those of Lowry & Lopez (1946). The difference between the two values gave the phosphates with the lability of phosphocreatine.

Because of its convenience for metabolic studies we have largely employed guinea pig brain cortex during the present work. We have also determined the levels of phosphates *in vivo* in this species by the same methods as employed for the levels *in vitro*.

EXPERIMENTAL

Tissue for phosphates of brain after rapid fixing. (Guinea pigs weighing 250–350 g. were used, fed on a mixed diet.) Trichloroacetic acid was contained in a series of test tube homogenizers, and these were weighed, together with their pestles, and cooled in ice water. To obtain values for normal animals, the whole guinea pig, with minimum preliminary handling, was dropped head first into about 500 ml. liquid O_2 in a 1 quart wide-necked Thermos flask. After about 3 min. it was removed, held with a cloth, hair scraped from the head with a knife, and the skull opened with a $\frac{3}{8}$ in. (10 mm.) firmer chisel already cooled in liquid oxygen. Pieces were chipped from the brain with a cold $\frac{1}{8}$ in. or $\frac{3}{16}$ in. gouge and about 0·2 g. dropped into the trichloroacetic acid of each of the homogenizers. As far as possible, cortex was taken, but dissection was not accurate. Each specimen was homogenized immediately after its addition and the homogenizer returned to ice water. When all specimens had been taken the homogenizers were reweighed, their contents filtered and phosphates determined as described below.

To obtain values after decapitation, animals were guillotined, the head dropped into liquid air after the desired interval, and specimens taken as before.

Tissue for metabolic experiments. An animal was decapitated, the brain removed, and slices of about 0·35 mm. thick cut from the cerebral hemispheres, using a strip of safety-razor blading and a recessed microscope slide as guide. In some cases a Stadie & Riggs (1944) cutter was used. In a few experiments (indicated) the blade was used dry, the slices picked from it with a tared platinum carrier and weighed on a torsion balance before use. Normally, however,

the blade was moistened with the saline used in the later part of the experiment, and immediately after cutting each slice it was floated to a dish of the same saline through which the appropriate gas was passing. They were then picked from the dish with the carrier, adhering saline removed on moist, hardened filter paper or on a porcelain surface, the carrier and slice weighed, and the slice placed in saline in a Warburg vessel or analysed immediately. The times involved in these operations are quoted in the relevant tables. In some cases the slices were weighed at the end of the experiment, after draining on a glass or porcelain surface.

Salines and conditions of metabolism. Glycylglycine-saline contained: NaCl, 0·134 M; KCl, 0·0054 M; KH_2PO_4, $MgSO_4$ and $CaCl_2$, 0·00134 M, and glycylglycine brought to pH 7·4 with NaOH, 0·05 M. Glycine, treated similarly, replaced glycylglycine in a few experiments. Bicarbonate-saline contained: NaCl, 0·124 M; KCl, 0·005 M; KH_2PO_4, $MgSO_4$, and $CaCl_2$, 0·00124 M; $NaHCO_3$, 0·021 M and was gassed with 5% (v/v) CO_2 in O_2. Glucose was used as substrate at 0·012 M and D-glutamic acid, neutralized with NaOH, at 0·02 M.

In most cases the salines (3 ml.) were contained in conical Warburg vessels of approx. 20 ml., the slices added to them, and gas changes followed at 37° by the usual procedures for 15–150 min. The slices were then removed with a mounted, bent platinum wire, drained and sometimes reweighed, and dropped into extracting fluid. Typical experiments involved five or six slices, of which two were controls and others contained the substances under investigation, and in handling such a series each flask was kept in the bath, shaking, until the moment when the slice could be removed and homogenized (see below).

For larger scale experiments in which creatine phosphate was separated, some six or eight slices (total fresh weight about 0·35–0·5 g.) were added to 15 ml. of metabolic medium in a manometric vessel of about 100 ml. At the end of the experiment, any absorbing agent in the centre well was removed, and the slices separated by pouring into a small conical Büchner funnel. They were picked from this with a bent wire, being drained on the porcelain as they were removed. By means of the wire, they were transferred to homogenizer tubes containing deproteinizing agent, already weighed together with their pestles. The tissue was transferred and homogenized as rapidly as possible and after this the tube reweighed to give the weight of tissue.

Determination of inorganic phosphate and phosphocreatine by calcium-ethanol precipitations

These methods have been applied to brain in most detail by Kerr (1935) and Stone (1940, 1943). In the present studies we have adapted such methods to specimens as small as possible, as tissue slices are limited in size. Also, we have determined not only the phosphate distribution on precipitation, but also the creatine content of precipitates obtained under conditions which differentiate creatine phosphate from creatine present as such.

Extraction. Test tube homogenizers of Potter & Elvehjem's (1936) type, made from 150 × 15 mm. tubes, were used. The specimens (0·3–0·5 g.) were rapidly added to cold, 10% (w/v) trichloroacetic acid (1·5 ml.) in a tared homogenizer in ice water, promptly homogenized, and the apparatus reweighed. After leaving for at least 15 min. in ice, the pestles were removed, the tubes centrifuged and the largest convenient quantity (1 ml.) of supernatant removed and measured with a pipette. The tissue weight corresponding to the supernatant taken was calculated from the quantities added to the tube, supposing that the tissue contributed 80% of its weight of water. (The value of 80% was increased when the tissue was known to have absorbed a considerable amount of fluid: see below.) The creatine content of brain obtained by such calculation has been compared with that obtained by successive extractions, and close agreement found.

Inorganic phosphate. The measured portions of supernatants were transferred to further homogenizer tubes calibrated at 3·6 ml. These were already cooled in ice water and 1 drop of 0·1% phenolphthalein was added. With the homogenizer running, solid $Ca(OH)_2$ was added gradually until in slight excess. The pestle was washed with water, making the volume 3·6 ml. Ethanol (0·4 ml.) was then added and the tube left in ice for 15 min. The ethanol had been made just alkaline to phenolphthalein by NaOH. The tube was then centrifuged, the supernatant kept for the determination of creatine phosphate, and the tube drained to leave the precipitate. Inorganic phosphate was determined in the precipitate according to Fiske & Subbarow (see Hawk, Oser & Summerson, 1947).

Creatine of phosphocreatine. This was determined, after conversion to creatinine (see Bonsnes & Taussky, 1945), by the Jaffe reaction. 3·0 ml. of the supernatant after precipitation of inorganic phosphate (and other phosphates) was pipetted to a glass-stoppered tube holding about 20 ml. and calibrated at 10 ml., and 15 ml. of neutralized ethanol added. The supernatants were now adjusted if necessary to the same pH (faintly pink) by NaOH, and left overnight (16–18 hr.) in a refrigerator. In the morning their pH was, if necessary, readjusted with N-NaOH (about 0·01–0·02 ml.) and if adjustment had been made they were left for a further 1 hr. at 0°. The tubes were then centrifuged and the precipitate drained from the supernatant liquid. The precipitate was dissolved in 1·0 ml. 0·2 N-HCl and, if the phosphate of creatine phosphate was to be determined, a sample taken at this point. The portion chosen for creatine determination in the original tube was autoclaved at 15 lb. for 20 min. The tubes were cooled on removal, and 2·0 ml. 1% (w/v) picric acid, followed by 1·0 ml. 5% (w/v) NaOH, was added. The tubes were left 15 min. after mixing, and then made up to the 10 ml. mark with water. They were read against a reagent blank in an absorptiometer using Ilford 604 filters (green, maximum absorption at 520 mμ.).

Phosphate of phosphocreatine was determined in the sample taken, as described above, by Fiske & Subbarow's method.

Determination of inorganic phosphate and phosphates with the lability of creatine phosphate, without chemical separation (method of differential stability)

The method of Fiske & Subbarow (1925, 1929), when applied to biological materials, determines, as well as inorganic phosphate, certain phosphate derivatives which are easily converted to inorganic phosphate by the acid molybdate solution used as reagent. Phosphocreatine is the best known derivative of this type. Kerr (1935) characterized the phosphates of this lability present in trichloroacetic acid filtrates of brain, as creatine phosphate. This was done by the solubility of Ca and Cu salts and their analysis for creatine and P; by the rate of catalysed and non-catalysed hydrolysis; and by isolation of the Ca salt. Application of the method of

Fiske & Subbarow to trichloroacetic acid filtrates of brain preparations will thus determine inorganic plus creatine phosphates, and many of the observations of Tables 2 and 3 have been based on this. Determination of true inorganic phosphate has commonly been made after separation from creatine phosphate by precipitation with Ca or Ba and ethanol similar to that described above.

Lowry & Lopez (1946) have described conditions under which 'true' inorganic phosphate can be determined in the presence of creatine phosphate. They described application of the method to various tissue extracts, including trichloroacetic acid filtrates of brain, and concluded that substances such as acetyl phosphate were absent from the filtrates. Combination of this method with Fiske & Subbarow's appeared to us the simplest way of determining these two substances. We were glad to have also the opinion of Dr M. G. Macfarlane in this connexion. If this combination of methods were applicable to brain slices, it would avoid two Ca-ethanol precipitations and enable smaller quantities of tissue to be handled. We have, in general, found it to be applicable.

In applying Lowry & Lopez's (1946) method to brain extracts, optical density readings with each specimen were taken at 5 and 10 min. as recommended by the authors. The two readings were normally the same. Sometimes a small increase was observed. Usually when this happened, the same increase was found in the readings of the reagent control, so that the corrected readings remained steady; a fresh batch of reagents then commonly gave steady readings with the same brain extracts. In a few cases with extracts of fresh brain a slow increase in values, not paralleled by the control, was found. In these cases the procedure recommended by Lowry & Lopez (1946) was followed: readings were extrapolated to zero time, and the value so obtained regarded as true inorganic phosphate. This correction was necessary in only about 5% of the determinations made.

Application of method to mixture of inorganic and creatine phosphates. In applying their method to trichloroacetic acid filtrates of brain, Lowry & Lopez (1946) recommended dilution of the filtrates to some 150–250 times the volume of the original tissue. We accordingly attempted to apply the combination of methods to concentrations of inorganic and creatine phosphates which might be expected to be present in filtrates diluted to this extent. Creatine phosphate was prepared according to Ziele & Fawaz (1938) and purified by precipitation as Ca salt from ethanol (Fiske & Subbarow, 1929). It still, however, contained a little inorganic phosphate. The solutions indicated in Table 1 were prepared, and their phosphate determined by the two methods. In spite of the small quantities concerned, the recovery of added inorganic phosphate and the determination of creatine phosphate in its presence are seen to be accurate to within 6%.

Phosphates in tissue slices. A series of test tube homogenizers containing 3·3 ml. ice-cold 5% trichloroacetic acid was prepared. As soon as shaking in the manometric experiment stopped, a vessel was taken from the bath, its slice picked on a mounted, bent platinum wire, drained in the flask, dropped into a tube and homogenized. Shaking of the other vessels continued until their slices also were homogenized. Homogenizing occupied only some 10 sec.; the whole operations with six vessels occupied about 7 min. When all slices had been homogenized the mixtures were filtered through fluted papers, in a refrigerator, to test tubes standing in ice.

For inorganic phosphate, 1 ml. filtrate was pipetted into a glass-stoppered test tube in ice, 0·26 ml. N-NaOH (kept in waxed bottle) added to neutralize the trichloroacetic acid, and the volume made to 10 ml. with 0·1 N-sodium acetate of pH 4·0. Of this mixture, 3 ml. was mixed in a cell of a Beckman photoelectric absorptiometer (model DU) with 0·3 ml. of a fresh 1% ascorbic acid solution and 0·3 ml. 1% ammonium molybdate. Determination was completed according to Lowry & Lopez (1946), taking readings at 700 mμ. Corrections were applied for cell characteristics; reagent blanks were normally negligible. A standard was included with each set of determinations, and this was normally very close to a standard curve which had been established at the beginning of the investigation and which was checked at intervals.

For inorganic and creatine phosphate, 1 or 1·5 ml. of the trichloroacetic acid was taken, 1 ml. of the ammonium molybdate and 0·4 ml. of the aminonaphtholsulphonic acid reagents added (Hawk *et al.* 1947), the volume made to 10 ml. with 10% (w/v) trichloroacetic acid, and readings taken after 15 min.

Expression of tissue concentrations

The weights quoted for slices are, unless otherwise described, those after floating the slices in saline and then draining from excess saline. Such slices were known to increase in weight during metabolic experiments (Elliott, 1946; Stern, Eggleston, Hems & Krebs, 1949). The mean increase during typical instances in our experiments were: with glycylglycine buffer, glucose as substrate, aerobically for 75 min., 32%; with bicarbonate buffer and glutamate as substrate, aerobically for 75 min., 36%.

Table 1. *Determination of inorganic and creatine phosphates*

(Solutions of creatine phosphate, without or with the quantities of inorganic phosphate (as KH_2PO_4) indicated, were prepared in 5 ml. portions. 1 ml. was taken for determination according to Fiske & Subarrow and quantities between 0·2 and 2 ml. for determination according to Lowry & Lopez, 1946.)

	P (μg.) determined by		Phosphocreatine-P by difference (μg.)
	Lowry-Lopez	Fiske-Subbarow	
Creatine phosphate *A*	1·65	8·85	7·2
Creatine phosphate + 10 μg. P	12·0	19·5	7·5
Creatine phosphate + 25 μg. P	26·7	33·6	6·9
Creatine phosphate *B*	3·0	20·2	17·2
Creatine phosphate + 10 μg. P	13·9	30·8	16·9
Creatine phosphate + 25 μg. P	28·0	44·2	16·2

These are comparable to those found by Stern *et al.* (1949). We have not determined the weight of the slices at the end of each manometric experiment, because we have found that the slices became more fragile during manometric experiments and to drain them adequately was apt to involve some damage to them, and occupied a time of about 20 sec. during which the slices were not provided with substrate and were at an indefinite temperature. Knowing the lability of phosphates under such conditions, we preferred to drain the slices rapidly (about 2 sec.), but possibly less completely, against the walls of the vessels and not to weigh them.

The expression of concentrations of tissue constituents in terms of the original weight of tissue, which we have adopted, ensures that the quantities of substances are referred to a given weight of metabolizing tissue. We consider that this expression is more significant than one in which they would be referred to a final weight which would include additional material. It is not known how the additional material is associated with the tissue and it is difficult to say whether the swelling should be regarded as intra- or extra-cellular (for some related observations see Dodgson, 1948). If it is extracellular, then the concentrations of phosphate found in the present experiments are directly comparable with those of the fresh tissue *in vivo*; if intracellular, then the concentrations which we quote have been artificially increased by some 33%. But it still remains that they represent exactly the quantity of phosphates which a given original weight of tissue can cause to be kept separate from the salines in which it is metabolizing.

RESULTS

Determination after rapid freezing

Phosphates. Levels of phosphate fractions in brain of several animal species have already been studied. Kerr (1935) first developed adequate methods of fixing the tissue by using liquid air and the value of this method has been confirmed by other workers whose results are quoted in Table 2. Our values for the guinea pigs used in the present studies are seen to be comparable to those found for other small animals. In all determinations, the molar ratio of inorganic phosphate to creatine phosphate is of the order of 1 to 2.

Table 2. *Phosphates of normal brain*

Species (and material)	Reference	Inorganic P (μg./g.)	Phospho-creatine P (μg./g.)	Ratio inorganic P/ phospho-creatine P
Mouse (whole brain)	Stone (1940)	169	101	1·7
Rat (presumably whole brain)	Le Page (1946)	155	70	2·2
	Lindberg & Ernster (1950)	200	101	2·0
Guinea pig (largely cerebral cortex)	Present work: (*a*) eight results, by differential stability	146 (S.D. 26)	83 (S.D. 16)	1·8
Guinea pig (largely cerebral cortex)	(*b*) two results by Ca precipitation	118	102	1·2
Cat (cerebral hemisphere)	Klein & Olsen (1947)	145 (S.D. 54)	74 (S.D. 15)	2·0
	Olsen & Klein (1947*a*, *b*)	140 (S.D. 49)	76 (S.D. 14)	1·8
	Kerr (1935)	125	133	0·9
Dog	Gurdjian, Stone & Webster (1944)	77	97	0·8
Dog	Stone, Webster & Gurdjian (1945)	78	91	0·9
Dog	Kerr (1935)	90	80–120	—

We determined the change in phosphates on decapitation, as the animals from which tissue was taken for metabolic work were commonly killed in this way or by exsanguination. Very rapid changes in the phosphates were found. If the head was put into liquid oxygen about 2 sec. after cutting, creatine phosphate fell to half or a third of its normal value while inorganic phosphate was more than doubled (Table 3). The ratios of inorganic phosphate: creatine phosphate then became greater than 10. Kerr (1935) observed that 90% of the phosphocreatine of rat brain could be lost in 30 sec. and other findings are compared with ours in Table 3.

Creatine of creatine phosphate. Similar changes were found when creatine phosphate was followed by determining the creatine precipitable by calcium salts and ethanol. When intact animals were frozen some 100 μg./g. of phosphocreatine were found (Table 2); after decapitation, about 30 μg./g. The

Table 3. *Changes in the phosphates of brain*

(Percentage changes are calculated on the basis of the average of normal values quoted in Table 2.)

Species and worker		Treatment of animal	Inorganic phosphate		Creatine phosphate		Ratio inorganic P/ creatine P
			μg. P/g.	% change	μg. P/g.	% change	
Mouse	Stone (1940)	Dial	133	−21	136	+35	1·0
Mouse	Stone (1940)	Nembutal	125	−26	138	+37	0·9
Mouse	Stone (1940)	Decapitation	476	+181	27	−74	18
Rat	Le Page (1946)	Nembutal	155	0	93	+33	1·7
Guinea pig	Present work	Decapitation	400	+160	35	−58	11
Dog or cat	Kerr (1935)	Removal of brain in already prepared animal, occupying 3 sec.	—	—	39	−68	—
Dog	Stone *et al.* (1945)	Head injury	200	+160	40	−58	5
		30 min. after death	370	+380	About 4	−96	—

Table 4. *Inorganic and creatine phosphates in freshly cut slices of guinea pig brain cortex*

(Slices were cut from the cortex without additional saline ('dry') or with blade and cutter wet with saline. Tissue, cut or uncut, was left at room temperature until it was precipitated by homogenizing in aqueous trichloroacetic acid. Determinations were made by differential stability.)

Animal	Manner of cutting slice	Time between death and precipitation (min.)	Saline	Time of any sejour in saline (min.)	Inorganic phosphate P (μg./g.)	Creatine phosphate P (μg./g.)	Ratio inorganic P/ creatine P
1	Dry	8	—	—	307	—	—
1	Dry	16	—	—	480	—	—
1	Dry	22	—	—	363	68	5·4
2	Dry	8	—	—	343	—	—
2	Dry	10	—	—	372	60	6·2
2	Wet	17	Bicarbonate-glucose	4	229	37	6·2
2	Wet	22	Bicarbonate-glucose	9	192	10	19·2
3	Wet	21	Glycylglycine-glucose	10	195	40	4·9
3	Wet	21	Glycylglycine-glucose	10	166	21	8·0
4	Wet	23	Glycylglycine-glucose	10	195	24	8·1
4	Wet	23	Glycylglycine-glucose	10	152	33	4·6

values had fallen further by the time that slices had been prepared (Table 5).

Phosphates in freshly cut cortex slices

The inorganic phosphate P of slices cut dry, and fixed in trichloroacetic acid 8–20 min. after death of the guinea pig, was found to be 300–480 μg./g. or some three times that normal to the tissue (Table 4). When the slices were cut moist and left in saline solutions at room temperature for short periods, such as those incidental to metabolic studies, both these values and the values for phosphocreatine were lower. They were also very variable, especially in phosphocreatine (Table 4). Although both values fell, the ratio between inorganic and creatine phosphates remained erratic and several times that observed *in vivo*. During the preparation of slices for metabolic studies, therefore, creatine phosphate has been lost. Inorganic phosphate has been temporarily raised and then lowered to values which, however, remain greater than those normal to the tissue. The level of inorganic phosphate P which was left in the slices (150–230 μg./g.) remained markedly higher than that of the saline in which they had been placed (initially 38·5 or 41·5 μg./ml.; finally about 50 μg./ml.).

Resynthesis of creatine phosphate during metabolism in vitro

This has been demonstrated most clearly by our finding a large increase in creatine precipitable by calcium salts under conditions in which creatine phosphate, but not creatine itself, is precipitated. An equivalent increase in a phosphate with the lability of creatine phosphate was also demonstrated in the same precipitates. Confirmatory evidence was obtained by precipitation with copper salts, and by determination of phosphates by the method of differential stability. Results by the latter method are described in later sections.

Calcium-ethanol precipitation. The specificity of the method, when applied to trichloroacetic acid extracts of brain tissue following *in vitro* metabolism, was first examined. Table 5 shows that when small quantities of creatine, creatinine and creatine phosphate were added to such extracts, only creatine phosphate led to increase in creatine in the 80% ethanol precipitate. Creatine phosphate also led to an equivalent increase in the phosphate determinable by Fiske & Subbarow's method in the precipitates; recovery, judged by either determination, was good. satisfactorily precipitated when added to such extracts. However, we did not find conditions under which a proportion of added creatine was not also precipitated. When the method was applied to simple solutions of comparable quantities of creatine and creatine phosphate, both substances were precipitated. Less creatine was precipitated in the presence of glycylglycine or inorganic phosphate. The application to tissue extracts presumably depends on the presence of associated materials; we have not, therefore, reported results in detail.

Table 5. *Resynthesis of creatine phosphate in brain slices during metabolism* in vitro

(Determinations were by calcium-ethanol separation. The time (quoted) at which the specimen of brain was taken for the initial values, was that at which aeration of the brain slices at 37° was commenced.)

Exp. no.	Treatment	Content (μg. P/g.) of: Inorganic P	Creatine phosphate determined as: P	Creatine phosphate determined as: Creatinine
	Control experiments			
1*a*	Brain, 15 min. post mortem	—	9·9	11·5
b	As *a*, with 1·4 μmol./g. creatine phosphate	—	51·8	53·0
c	As *a*, with 1·6 μmol./g. creatine	—	9·0	12·4
2*a*	Brain, 5 min. post mortem	257	—	15·2
b	As *a*, with 1·6 μmol./g. creatinine	—	—	13·6
	Metabolic experiments			
3*a*	Brain, 15 min. post mortem	192·2	15·5	14·6
b	Slices from *a* after 40 min. respiration in glycylglycine saline	120·9	45·9	42·5
4*a*	Brain, 16 min. post mortem	290·2	—	9·3
b	Slices from *a* after 40 min. respiration in bicarbonate-glucose saline	122	—	41·2
c	As *b*, saline containing $1·5 \times 10^{-4}$ M-creatine	100	—	43·4
d	As *b*, saline containing $1·5 \times 10^{-3}$ M-creatine	102·6	—	45·3
5*a*	Brain, 20 min. post mortem	238·7	—	9·9
b	Slices from *a* after 20 min. respiration in glycylglycine-glucose saline	98·0	—	33·2
c	As *b*, after 70 min.	95·2	—	35·6
6*a*	Brain, 22 min. post mortem	293·6	—	11·8
b	Slices from *a* after 40 min. respiration in bicarbonate-glucose saline (O_2–5% CO_2)	90·8	—	44·9
c	As *b*, anaerobically (N_2–5% CO_2)	108·2	—	9·6

Table 5 also shows that creatine phosphate reappears in slices after *in vitro* respiration under a variety of conditions. The differences under the different conditions are discussed below. Creatine phosphate did not reappear anaerobically or (Buchel & McIlwain, 1950) aerobically in the presence of certain inhibitors.

Precipitation of material in the trichloroacetic acid filtrates as copper salts was also investigated. Fiske & Subbarow (1929) stated this to be especially suitable for differentiating between creatine and creatine phosphate in muscle extracts, and Kerr (1935) applied the findings to brain extracts. In our experiments, copper salts under Fiske & Subbarow's conditions were found to precipitate about the same quantity of creatine from extracts of brain as did calcium-ethanol. Creatine phosphate itself was

Phosphates in slices after metabolism in different solutions

Many determinations by the method of differential stability have shown that the inorganic phosphate and phosphocreatine of slices reach stable levels after *in vitro* metabolism. The data of Table 6 illustrate this. After 75 min. respiration with glucose as substrate in glycylglycine-buffered saline, 15 values are available from slices in independent experiments with different guinea pigs. These gave as mean values for inorganic phosphate P, 95 μg./g. (S.D. 9) and phosphocreatine P, 45·5 μg./g. (S.D. 8). The values at 15, 135 and 195 min. are all close to these averages. The rise in inorganic phosphate and fall in phosphocreatine which occurred shortly after death was thus stopped and even reversed by metabolism *in vitro*.

Further, the ratio of the concentration of inorganic to that of creatine phosphate became about 2·0, close to its value *in vivo*, though the levels of both compounds were lower than those *in vivo*.

The use of bicarbonate as buffer in place of glycylglycine (glucose still as substrate) made only a small change in the levels of phosphocreatine and inorganic phosphate in the slices. The level of inorganic phosphate in all these cases (approx. 100 μg./g.) remained markedly above the level initially present in the saline in which the slices were metabolizing (30–40 μg./ml.).

When glutamate replaced glucose as substrate, with glycylglycine as buffer, characteristic differences were found in phosphate distribution (Table 7). Inorganic phosphate levels became much higher. The values for phosphates with the lability of phosphocreatine became less constant, falling to about 10 μg./g. after 150 min. metabolism. The rate of respiration with glutamate as substrate was some 20–25% higher than its value with glucose. The effect was also examined of presenting both glucose and glutamate as substrates (glycylglycine as buffer). This gave, at 150 min., levels of both inorganic and phosphocreatine-like phosphates which were characteristic of glucose alone. The rate of respiration was markedly higher than that with either substrate alone (cf. Weil-Malherbe, 1936).

Balance of inorganic phosphate between slices and solution

Throughout the present experiments the level of inorganic phosphate in the metabolic mixture used has been initially at values between 30 and 35 μg. P/ml., or similar to those of blood plasma. The 3 ml. of metabolic mixture of typical experiments thus contained about 100 μg. P, and the slice which was added about 20 μg. P as inorganic phosphate and 1–7 μg. as creatine phosphate. The inorganic phosphate of the medium thus formed a reservoir of phosphate several times greater than that present as inorganic or creatine phosphates in the slice. Nevertheless as the quantity was small in comparison with the quantity of added substrate it was therefore important to follow any changes during the present experiments. We found in all cases an increase in the inorganic phosphate of the medium (Table 8). When expressed in terms of the tissue added this was between 90 and 140 μg. P/g. wet wt. Values for Exp. 3 of Table 5 show that in terms of the observed changes in inorganic and creatine phosphate, slices had lost about 45 μg. P/g. Other experiments showed losses of 60–80 μg./g. The medium thus gained some inorganic phosphate from compounds other than the inorganic and creatine phos-

Table 6. *Phosphates after respiration with glucose in various mixtures*

(All slices cut wet. Time between death and putting slices in appropriate saline, about 10 min.; between death and beginning of manometric experiments, about 35 min. Different values under one set of conditions refer to different experiments on different days. All salines contained glucose at 0·012M. Determinations: as Table 4.)

Saline	Period of metabolism (min.)	Rate of respiration (μmol./g. wet wt./hr.)	Phosphates (μg. P/g. wet wt.) Inorganic	Phosphates (μg. P/g. wet wt.) Creatine phosphate	Ratio inorganic P: creatine P
Glycylglycine	15	68, 69	78, 77	39, 51	1·7
Glycylglycine	75	68·5 (S.D. 4·8)*	95 (S.D. 9·0)*	45·5 (S.D. 8)*	2·1
Glycylglycine	135	65	83	37	2·2
Glycylglycine	195	69	109	50	2·0
Bicarbonate	75	—	115 (S.D. 15)†	44 (S.D. 10)†	2·6
Glycine	75	61, 59	89, 92	29, 30	3·0

* Mean and standard deviation of fifteen experiments. † Of eight experiments.

Table 7. *Phosphates after respiration with glutamate in various mixtures*

(Experimental conditions and recording of results, as in Table 6. D-Glutamate, final concentration 0·02M.)

Saline	Period of metabolism (min.)	Rate of respiration (μmol./g. wet) wt./hr.)	Phosphates (μg./g. wet wt.) Inorganic	Phosphates (μg./g. wet wt.) Creatine phosphate
Glycylglycine	75	74, 78, 75	200, 190, 171	30, 34, 39
Glycylglycine	150	73, 80	187, 190	0, 11
Glycylglycine-glucose	150	95	102	54
Bicarbonate	75	—	156	50

Table 8. *Phosphate of medium and slice after metabolism*

(Experimental conditions as in Table 6.)

Medium	Initial moist wt. of slices (mg./3 ml. medium)	Inorganic phosphate P (μg./g. or μg./ml.) Solution, initially	Solution, finally	Slice, finally
Glyclyglycine, glucose	152	33·5	38·6	111
Bicarbonate, glucose	58	31·2	33·0	105
Bicarbonate, glucose	104	30·5	33·4	—
Bicarbonate, glutamate	138	33·5	40·3	156

phates of the slices, but the concentration of inorganic phosphate in the medium was throughout much lower than that of the slice.

DISCUSSION

Resynthesis of creatine phosphate in muscle occurs after its separation from the animal (Meyerhof, 1930). Kerr (1935), however, emphasized that the lability of creatine phosphate in mammalian brain is much greater than in muscle, and the impression gained from the papers quoted in Table 3 is that resynthesis of creatine phosphate in brain *in vitro* would be most unlikely. As various forms of damage and disturbance to the central nervous system lead to lowered creatine phosphate, thin slices of brain might not seem promising material for observation of the resynthesis. Nevertheless, resynthesis has now been demonstrated in respiring slices, and this serves to emphasize the value of tissue-slice techniques and the considerable autonomy of mammalian tissues from a biochemical point of view. Krebs & Eggleston (1949) demonstrated comparable phenomena in brain slices with respect to their maintenance of potassium salts.

It appears likely that the rate of carbohydrate oxidation and glycolysis in tissues and the balance between these two processes are conditioned by the concentrations of labile phosphates and their breakdown products (Belitzer, 1939; Johnson, 1941; for an assessment in relation to brain, McIlwain, 1950). Such rates can be remarkably close in tissue slices to those normal to the tissue *in vitro* (Schmidt, Kety & Pennes, 1945). This is consistent with the restoration which we now observe in the levels of the phosphates investigated. Loss of phosphate from adenosinetriphosphate also occurs in brain after death (Kerr, 1941) and the resynthesis of creatine phosphate *in vitro* is probably preceded by resynthesis of adenosinetriphosphate. The equilibria concerned in brain have been considered by Klein & Olsen (1947) and Meyerhof & Wilson (1947).

The finding of a high rate of glycolysis for a short initial period during aerobic metabolism of brain slices (see, for example, McIlwain & Grinyer, 1950) may be related to their high concentrations of phosphate acceptors and inorganic phosphate at the beginning of such experiments. Because of the possible role of inorganic phosphate in regulating carbohydrate metabolism it is interesting to compare the concentrations which we find in brain slices with those known to affect intermediary metabolism in brain preparations. The concentrations which we find are about 5×10^{-3} M in normal brain, increasing to 12×10^{-3} M with damage. Banga, Ochoa & Peters (1939) found the respiration of brain dispersions to increase with phosphate concentrations especially over the range 1 to 20×10^{-3} M. Long (1945) found a reduction of methylene blue by fumarate to increase with phosphate over the range of about 1 to 5×10^{-3} M, and the formation of pyruvate from fumarate to increase between 1 and 20×10^{-3} M phosphate. It is evident, therefore, that the phosphate concentrations of the tissue are such that increase in them can be expected to bring increased carbohydrate metabolism. This is known also to be sensitive to the breakdown products of the labile phosphates, being increased in brain by creatine (see, for example, Meyerhof & Wilson, 1947) and adenylic acid (see, for example, Case & McIlwain, 1950). Phosphate also activates a glutaminase of brain (Errera & Greenstein, 1949).

The considerable changes in phosphates caused by glutamate appear likely to be related to the effects of this compound on brain respiration (Weil-Malherbe, 1936; Krebs, 1950) and on aerobic glycolysis (Weil-Malherbe, 1938). They are being investigated further. Elliott (1948) has postulated a glutamyl phosphate as intermediate in the synthesis of glutamine in brain, and the evidence of Elliott & Gale (1948) suggests that such a compound may take part in the assimilation of glutamic acid by microbial cells.

The general finding that creatine phosphate is maintained in brain tissue slices has enabled *in vitro* studies to be made of the mode of action of agents which affect the phosphates *in vivo*, as will be reported elsewhere.

SUMMARY

1. The inorganic phosphate and phosphocreatine in samples of about 0·5 g. of guinea pig brain have been determined after calcium-ethanol separation. Both phosphates and creatine have been estimated. Inorganic phosphate and phosphates with the lability

of phosphocreatine have been determined in specimens of about 0·1 g. brain without chemical separation.

2. After death, a very rapid fall in phosphocreatine and rise in inorganic phosphate takes place in the brain of guinea pig as of other species.

3. On slicing brain cortex and allowing the slices to respire with glucose under normal metabolic conditions, the inorganic phosphate falls and the phosphocreatine rises until the concentrations approach those normal to the tissue *in vivo*.

4. The concentrations reached vary with the metabolic mixture.

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The Reaction of Oxidizing Agents with Wool

3. THE INFLUENCE OF THE MORPHOLOGY ON THE RATE OF REACTION

By P. ALEXANDER, D. GOUGH and R. F. HUDSON
Department of Inorganic and Physical Chemistry, Royal College of Science, London, S.W. 7

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A detailed study of the kinetics of the reaction between wool and aqueous chlorine solutions at pH 2 by Alexander, Gough & Hudson (1949) has shown that the rate is controlled either by diffusion through a film of liquid surrounding the fibre or by diffusion within the fibre, depending on the conditions of treatment. The rate of the reaction increases with increasing agitation up to a limiting stirring rate, after which it becomes constant. At this point the temperature coefficient changes sharply, showing that a change in mechanism has occurred. The following observations show that the rapid rate at high stirring speeds with chlorine at pH 2 is not governed by the actual chemical reaction but by the diffusion of the chlorine to the site of reaction where it reacts instantaneously:

(1) The quantity of chlorine which has reacted increases with time according to a parabolic law which is to be expected for diffusion into the fibres under the experimental conditions employed.

(2) The observed activation energy (12·5 kg. cal./mol.) is the same as that found for a wide variety of