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EFFECTS OF OUABAIN, GLUTAMATE AND CATIONS ON PHOSPHATE INCORPORATION IN BRAIN SLICES

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Abstract—Guinea-pig brain cortex slices were incubated in media in which the concentrations of sodium, potassium, calcium, glutamate and ouabain were varied, and the absolute levels and rate of phosphate incorporation into ATP and 5 acid-insoluble phosphate fractions were measured. The level of inorganic phosphate and of the acid-insoluble phosphate fractions, except for phosphoprotein, did not vary significantly under different conditions. ATP levels were lowered by ouabain, glutamate or omission of sodium from the medium.

The rate of phosphate incorporation into all acid-insoluble phosphate fractions was lowered by 75% when sodium was replaced by choline and by 60% by glutamate. Incorporation was stimulated by 35% by the omission of calcium from the medium and by up to 20% on raising the potassium concentration from 2·5 to 7·5 mM, except for phospholipid, incorporation into which was reduced by 35% by raised potassium.

Ouabain at 10 μM caused a 35% inhibition of incorporation in the sodium medium in both rat and guinea-pig cortex slices. Lower concentrations of ouabain gave smaller inhibitions. The rise in incorporation caused by omission of calcium and the fall caused by glutamate, were abolished by ouabain. The fall in incorporation into phospholipid, and the rise in all other acid-insoluble fractions, caused by raised potassium, were both reversed by ouabain. All effects of glutamate, calcium and ouabain were abolished when sodium was replaced by choline.

THE phosphate metabolism of brain cortex slices has frequently been studied, and changes in the rates of incorporation of inorganic ^{32}P into nucleotides and various of the acid-insoluble phosphates present in the tissue have been demonstrated under differing conditions of incubation.[1–22] Amongst the factors studied have been oxygen,[3] electrical pulses,[9, 14–16] a variety of drugs[4–13] and the ionic composition of the medium.[17–22, 25] In particular, the effects of the cardiac glycoside ouabain on phosphate incorporation into some fractions has been examined and the results used to suggest hypotheses of ouabain action.[9–12] Such studies, though, have tended to focus attention on changes in incorporation in only one of several different phosphate fractions, notably either phospholipid[7, 8, 10, 11, 13, 25] or phosphoprotein.[9, 12, 23, 24] Hence it is not established whether some of the effects that have been reported for cations or for ouabain are in fact confined to certain specific phosphate fractions, or represent more generalised enhancement or depression of phosphate incorporation.

In order to test this possibility, experiments have been made in which brain cortex slices were incubated with ^{32}P under a variety of different conditions, and the rate of phosphate incorporation into ATP and into 5 different acid-insoluble phosphate

fractions, together accounting for all the acid-insoluble tissue phosphate, was measured. In this paper, the effects of the ionic composition of the incubation medium, of glutamate and of ouabain on phosphate incorporation are reported.

METHODS

Salines. The composition of the various salines used for incubation is shown in Table 1. The standard medium used throughout was the sodium saline of the Table. In addition, experiments were performed in which ouabain at various concentrations was added to the salines either at the start of the experiment, or just prior to addition of ^{32}P. To increase the rate of uptake of ^{32}P into the slice, media were phosphate-free throughout. Preliminary experiments showed that omission of phosphate from standard Krebs–Ringer saline was without effect on maintenance of ATP levels in the slice.

TABLE 1. COMPOSITION OF SALINES

All salines used for incubation contained, as well as the components listed in the Tables, $MgCl_2$ (2 mM), Tris-HCl, pH 7·5, (10 mM), glucose (10 mM). All figures in the Table refer to concentrations in mM in the final incubation medium.

Saline	Component				
	NaCl	KCl	Tris glutamate (pH 7·5)	$CaCl_2$	choline chloride
Sodium	150	2·5	—	2·5	—
Na^+, high K^+	150	7·5	—	2·5	—
Na; Ca^{2+} free	150	2·5	—	—	—
Na^+, glutamate	150	2·5	10	2·5	—
Na^+, high K^+, glutamate	150	7·5	10	2·5	—
Choline	—	2·5	—	2·5	150
Choline; Ca^{2+} -free	—	2·5	—	—	150
Choline, glutamate	—	2·5	10	2·5	150

Incubation procedure. Guinea-pigs were stunned by a blow on the back of the neck, the brains exposed, and rapidly removed onto a Petri dish packed in ice. Slices of cerebral cortex were cut with a razor and guide lubricated with sodium saline, and each slice, as made, was placed in a dish containing ice-cold sodium saline until all had been prepared. Usually, three slices, each of about 100 mg wet weight, were cut from each hemisphere. A single experiment with guinea-pig cortex normally required 5 brains, and the time between the killing of the first animal and the completion of slicing was up to 20 min. The pooled slices were then divided into approximately 1 g lots and transferred to 50 ml conical flasks each containing 10 ml of an appropriate incubation medium. The flasks were gassed with oxygen, stoppered, and placed in a shaking bath at 37°. After a preliminary incubation of 15 min to allow steady-state levels of ATP and phosphoprotein to be reached by the slices, 100 μC of ^{32}P, as carrier-free orthophosphate buffered in 0·1 M tris, pH 7·5, was added to each of the flasks, and incubation continued for a further period, usually 30 or 60 min. At the end of this time, the slices were removed from each of the flasks in turn, blotted with filter paper, and the slices from each flask divided into three portions, of approximately 300 mg each. Each portion was rapidly weighed on a torsion balance, transferred to a

glass homogenising tube, and dispersed with 2 ml of ice-cold 10% (w/v) trichloroacetic acid (TCA) by hand-grinding with a glass pestle. Time from the removal of slices from their incubation medium to fixing with TCA varied from 30 sec to 2 min.

Treatment of TCA-fixed tissue. In an individual experiment, four different incubation conditions were investigated. As the slices from each incubation medium were divided into 3 lots to provide triplicates for analysis, this procedure yielded 12 samples in all. The homogenates were transferred into 15 ml stoppered tubes and centrifuged at 2,000 rev/min on an anglehead bench centrifuge for 7 min, the supernatants removed with a fine-drawn Pasteur pipette, and stored at − 16° until analysis. The homogenising tubes were rinsed with 5 ml portions of 10% TCA, and the rinsing fluid added to the TCA-insoluble residues, which were centrifuged and the supernatants discarded. The residues were similarly washed twice more to remove the last traces of adherent inorganic ^{32}P. They were then extracted by the procedure outlined in Fig. 1, which was, with minor modifications, essentially that of Heald.[16]

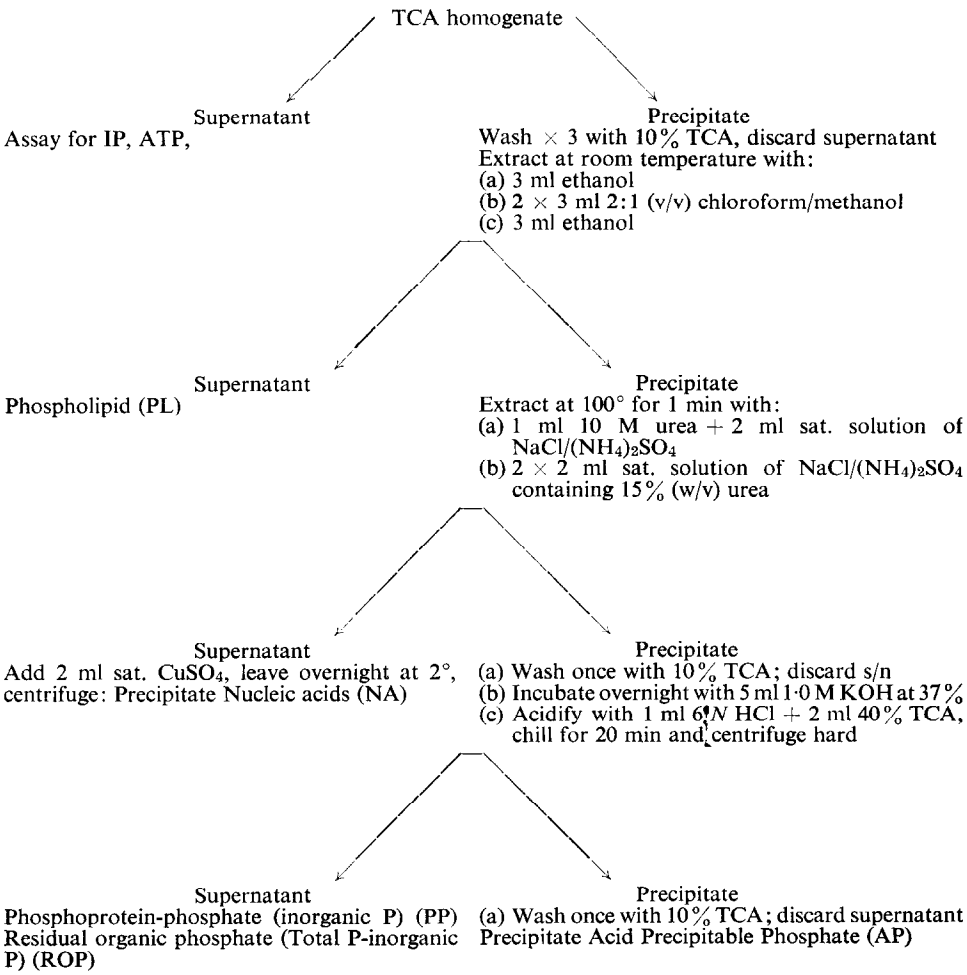

FIG. 1. Extraction of tissue slices. Slices were incubated as described in Methods, blotted, weighed and dispersed with 2 ml ice-cold TCA.

This method yields 5 major groups of phosphate compounds, although no fraction is homogeneous,[27-31] phospholipids (PL), nucleic acids (NA) phosphoproteins (PP), residual organic phosphate (ROP) (mainly phosphatidopeptides), and acid-precipitable phosphate (AP) (a heterogenous fraction consisting in part of nucleic acid not extracted by the procedure of Fig. 1). Total phosphate was determined in aliquots of the separated fractions by ashing with 70% perchloric acid (0·1–0·4 ml), diluting with water and estimating inorganic phosphate by the method of Martin and Doty.[32, 33] Phosphoprotein phosphate was determined as inorganic phosphate released on alkaline hydrolysis (Fig. 1), and for this fraction, saturated sodium silicotungstate solution was added to precipitate interfering TCA-soluble material prior to analysis.[32] Residual organic phosphate was calculated as the difference between total and inorganic phosphate in the alkaline hydrolysate.

ATP and inorganic phosphate (IP) were determined in the initial TCA supernatant by ascending chromatography in the solvent of Gerlach, Weber & Döring.[34] The chromatograms were sprayed with Hanes–Isherwood reagent,[35] and the blue ATP and inorganic phosphate spots cut out, ashed with perchloric acid, and phosphate determined.

Radioactivity. Radioactivity was measured in the blue *iso*-butanol-benzene extract, following phosphate determination, by counting in an M6H liquid counter (20th Century Electronics Ltd.).

Reagents. Ouabain was obtained as Strophanthin-G, British Drug Houses Ltd., Poole. Solutions of ouabain were prepared freshly on the same day they were to be used. ^{32}P was carrier-free orthophosphate, Radiochemical Centre, Amersham, and was diluted with tris-HCl, pH 7·5,(0·1 M) prior to use.

Expression of results. Absolute levels of various tissue fractions are expressed as μmoles P/g wet wt., this tissue weight refers to that subsequent to incubation. Preliminary experiments showed that tissue slices increased in weight by, on average, 10 ± 2% over the fresh-cut material during preparation and floating in ice-cold sodium saline. During incubation for 60 min, slices swelled a further 17 ± 1% over the weight after floating for 15 min in cold saline. No significant differences in swelling occurred during incubation in different media, except where the sodium of the saline was replaced by choline; slices incubated in a choline medium increased in weight by only 10 ± 1% during 60 min. Levels of tissue components after incubation in choline media were thus some 7% higher than in sodium due to differences in water content of the slices, irrespective of changes in the absolute amount of any individual fraction.

Radioactivity is expressed either as specific activity (counts/min per μmole P) for each fraction, or as relative specific activity (r.s.a.), (counts/min per μmole P × 100 divided by counts/min per μmole inorganic phosphate in the slice). Specific activity of ATP is calculated on the assumption that the β- and γ-phosphates of the molecule were labile and had identical activities, whilst the α-phosphate was stable and unlabelled (e.g. ref. 42). In calculations of the specific activity of intracellular inorganic phosphate, a figure for extracellular water of 51 μl/100 mg fresh weight of tissue[36] was adopted.

In the tables, the r.s.a. found for each fraction after incubation in the various media is calculated as a percentage of that found for the same fraction in the standard "sodium" medium.

RESULTS

Maintenance of tissue phosphates

Absolute levels of the seven different phosphate fractions studied after incubation in various media are set out in Table 2. The results for incubation in the sodium medium were in good agreement with those in the literature,[2, 16] after allowance for tissue swelling, with the exception of the phosphoprotein fraction, which was 50% lower than the figure of 1·0 μmoles P/g fresh wt. found by Heald.[16] This is due to rapid changes in phosphoprotein levels which occur during cold storage and the first 15 min of incubation; after this period the phosphoprotein levels stabilise at the figure shown in Table 2 (Rose, unpublished results).

Of the phosphate fractions examined, inorganic phosphate, phospholipid, nucleic acid and acid-precipitable phosphate were essentially unaltered in level by variations in the incubation medium. The residual organic phosphate fraction was higher by 30% in the standard sodium medium than under any other condition (except for the medium (sodium; calcium-free)). In all other conditions there were approximately 2·5 μmoles ROP-P/g tissue of; this was also the figure found by Heald.[16] Phosphoprotein levels were lowered by up to 25% in the presence of ouabain or choline, but maintained or slightly raised by glutamate even when ouabain or choline were also present.

In sodium media, ATP levels were maintained at 3·25 μmoles ATP-P/g independently of calcium or potassium concentrations. Addition of glutamate reduced the level by 45%, and of ouabain (10 μM) by 23%. Glutamate and ouabain together lowered ATP levels by 37%. Essentially similar results were obtained in high or low potassium media. Replacement of sodium by choline lowered ATP levels by about one half, and this reduction occurred irrespective of other variations in the composition of the incubation medium.

Incorporation of ^{32}P into tissue phosphates

Uptake of ^{32}P into the slice was slow, and even after incubation for 1 hr, the intracellular phosphate of the slice, after allowance had been made for adhering extracellular water, had reached only 20% of the specific activity of the medium. This value was not significantly altered by changes in the ionic composition of the medium, or by the presence of glutamate or ouabain, and it was therefore possible to calculate a relative specific activity, in all conditions examined, by referring the specific activity of the phosphate fractions to that of total slice inorganic phosphate as described under Methods.

A. Sodium

In the standard sodium medium, incorporation of phosphate into ATP was rapid. At the end of 60 min, as also at shorter time intervals measured but not reported here, the specific activity of the two terminal phosphates of ATP was 52% of that of the total inorganic phosphate of the slice, or 85% of the specific activity of the intracellular inorganic phosphate after allowance had been made for extracellular water. Incorporation into other tissue phosphates was much slower. After incubation for 1 hr, the specific activity of the phosphoprotein fraction was 46%, of the residual organic 23%, of the acid-precipitable 2·3%, of the nucleic acid 1·9% and of the

TABLE 2. LEVELS OF TISSUE PHOSPHATES AFTER INCUBATION IN VARIOUS MEDIA

Cortex slices were incubated at 37° for 75 min in the media of Table 1, weighed, dispersed with TCA and fractionated as described under Methods. Levels of phosphate fractions are expressed as μmoles P/g wet wt., and are quoted ± s.e.m. where more than 5 determinations were made; other figures represent means only. Levels found after incubation in the sodium medium are taken as standard; values differing significantly from these ($p < 0.01$) are set in bold type. All ouabain concentrations 10 μM.

Incubation medium	Ouabain (+) 10 μM	Phosphate levels; μmoles/g swollen wet wt.											
		IP		ATP		PP		ROP		AP		PL		NA	
Sodium	−	3·34	± 0·17	3·25	0·13	0·388	± 0·022	3·24	± 0·09	1·02	± 0·06	38·9	0·75	1·91	± 0·10
Sodium	+	**2·67**	**± 0·17**	**2·54**	**0·30**	**0·297**	**± 0·036**	**2·47**	**± 0·24**	0·92	± 0·11	38·0	2·0	2·05	± 0·13
Sodium; Ca^{2+} free	−	2·95	± 0·40	3·16	± 0·15	0·380	± 0·012	3·25	± 0·40	1·25	± 0·11	41·0	1·6	1·87	± 0·24
Sodium; Ca^{2+}-free	+	3·2		**1·73**		0·355		3·12		0·8		41·5		2·2	
Sodium, high K^+	−	2·80	± 0·12	3·44	± 0·28	0·380	± 0·008	**2·35**	**± 0·18**	0·97	± 0·06	36·5	± 0·8	**2·31**	**± 0·14**
Sodium, high K^+	+	2·92	± 0·35	2·95	± 0·15	**0·366**	**± 0·012**	**2·25**	**± 0·09**	0·95	± 0·02	**33·0**	**± 1·7**	1·82	± 0·08
Sodium, glutamate	−	3·27	± 0·27	**1·76**	**± 0·05**	0·405	± 0·023	**2·70**	**± 0·16**	0·86	± 0·03	38·5	± 3·0	1·85	± 0·16
Sodium, glutamate	+	3·04	± 0·40	**2·05**	**± 0·12**	**0·305**	**± 0·023**	**2·77**	**± 0·16**	**0·78**	**± 0·09**	42·0	± 2·0	2·24	± 0·14
Sodium, high K^+, glutamate	−	3·1		**1·78**				**2·37**		0·875				1·7	
Sodium, high K^+, glutamate	+			**2·68**		0·33		**2·62**		**1·08**				1·75	
Choline	−	3·3	± 0·07	**1·56**	**± 0·13**	**0·295**	**± 0·04**	**2·27**	**± 0·04**	0·96	± 0·03	40·5	± 1·5	2·0	± 0·12
Choline	+	3·0	± 0·30	**2·10**	**± 0·15**	**0·330**	**± 0·04**	**2·72**		1·04		42·2		2·06	
Choline, Ca^{2+} free	−	2·96	± 0·14	**1·23**		**0·360**	**± 0·014**	**2·30**	**± 0·25**	1·01	± 0·16	37·0	± 1·7	**1·4**	
Choline, glutamate	−	3·25	± 0·24	**1·85**		0·410	± 0·030	**2·51**	**± 0·19**	0·88	± 0·05	**49·0**	**± 1·5**	1·85	± 0·14
Choline, glutamate	+	3·1		**2·42**		0·34		**2·20**		0·89		42·5		2·27	

phospholipid 1·0% of that of the β- and γ-phosphates of ATP. Preliminary experiments also showed the increase in specific activity of the acid-insoluble phosphates, with the exception of residual organic phosphate, to be practically linear over periods of incubation ranging from 15 min to one hour. In subsequent experiments, therefore, incubation periods of one hour were adopted.

B. Changed ionic concentrations

1. *Choline.* Replacement of sodium in the incubation medium by an equivalent amount of choline had a marked effect on phosphate incorporation (Table 3). Relative specific activity of all fractions, including ATP was *reduced* to 25–30% of that in the presence of sodium. No significant differences of effect between the fractions were observed.

TABLE 3. EFFECT OF SODIUM AND CHOLINE ON PHOSPHATE INCORPORATION

Guinea-pig brain cortex slices were incubated for 60 min in the presence of ^{32}P, in the following media; (a) sodium; (b) choline, as defined in Table 1. The tissue was then dispersed in TCA and fractionated as described under Methods. Abbreviations, and definition of r.s.a., as in Methods. Results are means of six determinations. Specific activity of inorganic P of slice: 9×10^5 counts/min per μmole P.

Fraction	r.s.a.		$\left(\frac{\text{r.s.a. + choline}}{\text{r.s.a. + sodium}}\right) \times 100$
	Na^+	Choline	
IP	100	100	—
ATP	56 ± 3·0	12·5 ± 3·0	23
PP	24·5 ± 2·0	6·5 ± 0·8	26
ROP	8·8 ± 2·0	1·9 ± 0·4	22
AP	0·95 ± 0·15	0·23 ± 0·04	24
NA	1·15 ± 0·15	0·27 ± 0·04	25
PL	0·63 ± 0·06	0·15 ± 0·02	24

2. *Calcium.* Omission of calcium from the incubation medium, which was without effect on the absolute levels of any of the phosphate fractions, resulted in an *increase* of 30–40% in the specific activity of all acid-insoluble fractions. (Table 4.) No significant differences of behaviour between the fractions were observed. When the sodium of the medium was replaced by choline, the effects of omission of calcium on phosphate incorporation were abolished. (Table 5.)

3. *Potassium.* Raising the potassium level of the medium from 2·5 to 7·5 mM K^+ resulted in a slight (up to 20%) *stimulation* of incorporation into phosphoprotein, residual organic phosphate, acid precipitable phosphate and nucleic acid. By contrast, incorporation into phospholipid was *reduced* by some 35%. (Table 4.) This reduction could be observed after as little as 30 min incubation. In preliminary experiments, the effects of further increasing the potassium concentration of the medium to 50 mM has been measured, and found to result in a decrease in the labelling of all fractions of up to 60%. This latter observation is similar to the finding of Findlay, Magee and Rossiter[17] for cat brain. (See also refs. 20, 25.)

C. Effects of glutamate

Addition of glutamate to a sodium medium depressed ATP levels (Table 2). Table 4 shows the effect of glutamate on ^{32}P uptake into the various fractions in a sodium medium. Incorporation into phosphoprotein, residual organic, acid-precipitable and

TABLE 4. EFFECT OF VARIOUS INCUBATION MEDIA ON PHOSPHATE INCORPORATION IN BRAIN SLICES

Guinea-pig brain cortex slices were incubated for 60 min, in the presence of ^{32}P, in the following media: (a) Sodium; (b) Sodium; calcium-free; (c) Sodium; potassium; (d) Sodium; glutamate; (e) Sodium; ouabain; (f) Sodium, calcium-free; ouabain; (g) Sodium; potassium, ouabain; (h) Sodium; glutamate; ouabain, as defined in Table 1. All ouabain concentrations 10 μM. The tissue was then dispersed in TCA and fractionated as described under Methods. Abbreviations and definitions of r.s.a. as in Methods. Results are expressed ± s.e.m. when more than 3 determinations were performed. Specific activity of inorganic P of slice 3–9 × 10^5 counts/min per μmole P.

Fraction	r.s.a., sodium	$\left(\frac{\text{r.s.a.}}{\text{r.s.a. in } Na^+}\right) \times 100$ for each fraction					
		$Na^+ - Ca^{2+}$	Na^+, K^+	Na^+, glut	Na^+ + ouabain	$Na^+ - Ca^{2+}$ + ouabain	Na^+, K^+ + ouabain	Na^+, glut + ouabain
IP	100	100	100	100	100	100	100	100
ATP	52·0 ± 3 (20)	76 ± 5 (8)	106 ± 3 (5)	63 ± 3 (6)	74 ± 3 (8)	71 (3)	84 ± 5 (5)	66 ± 5 (8)
PP	24·0 ± 1·3 (21)	135 ± 5 (7)	118 ± 3 (5)	46 ± 4 (6)	73 ± 3 (8)	66 (3)	82 ± 7 (5)	68 ± 5 (9)
ROP	12·0 ± 1·4 (21)	140 ± 5 (8)	105 ± 4 (5)	39 ± 5 (6)	61 ± 6 (9)	76 (3)	82 ± 4 (5)	58 ± 4 (9)
AP	1·20 ± 0·17 (18)	149 ± 11 (7)	105 ± 5 (5)	43 ± 8 (5)	65 ± 5 (8)	81 (3)	**65** **6*** (5)	60 ± 7 (9)
NA	0·98 ± 0·12 (18)	140 ± 11 (8)	122 ± 7 (5)	43 ± 3 (6)	60 ± 4 (8)	75 (3)	81 ± 5 (5)	62 ± 3 (9)
PL	0·52 ± 0·05 (21)	130 ± 9 (9)	**67** **3*(5)**	**60** **3*(4)**	**82** **4*(6)**	90 (3)	**100** **3*(5)**	**84** **2*(9)**

* Change in phosphate incorporation significantly different than that for other acid-insoluble phosphate fractions ($P < 0.01$) set in bold type.

nucleic acid phosphate was reduced by 60% and into phospholipid by 40%. Similar depressions occurred at 7·5 mM potassium. These figures are comparable to those found by Findlay, *et al.*[17] If the sodium of the medium was replaced by choline (Table 5), the glutamate-induced depression was abolished, the turnover of all fractions being lowered to 20–30% of that in the standard medium.

TABLE 5. EFFECT OF CHOLINE ON CALCIUM, GLUTAMATE AND OUABAIN-INDUCED CHANGES IN PHOSPHATE INCORPORATION

Guinea-pig brain cortex slices were incubated for 60 min in the presence of ^{32}P in the following media: (a) choline; (b) choline, calcium-free; (c) choline, glutamate; (d) choline, 10 μM ouabain; and (e) choline, glutamate, 10 μM ouabain; media were as defined in Table 1. The tissue was then dispersed in TCA and fractionated as described under Methods. Abbreviations and definition of r.s.a. as in Methods. Results are the means of at least 3 determinations. Specific activity of inorganic phosphate of slice: 4×10^5 counts/min per μmole P.

Fraction	r.s.a.				
	Choline	Choline, Ca^{2+} free	Choline glutamate	Choline, ouabain	Choline glutamate ouabain
IP	100	100	100	100	100
ATP	8·0	8·2	7·6	7·9	8·3
PP	6·5	5·0	4·2	6·0	7·1
ROP	1·60	1·58	1·45	1·60	1·75
AP	0·40	0·34	0·40	0·31	0·35
NA	0·32	0·28	0·26	0·23	0·24
PL	0·16	0·14	0·18	0·16	0·17

TABLE 6. INHIBITION OF PHOSPHATE INCORPORATION IN SODIUM MEDIUM AT DIFFERENT CONCENTRATIONS OF OUABAIN

Guinea-pig brain cortex slices were incubated for 30 min in the presence of ^{32}P in the following media: (a) sodium; (b) sodium to which ouabain at various concentrations had been added at the same time as ^{32}P, after a 15 min preincubation. Media were as defined in Table 1. The tissue was then dispersed in TCA and fractionated as under Methods. Abbreviations and definition of r.s.a. as in Methods. Results are means of at least 3 determinations in each case. Specific activity of inorganic phosphate of slice: 7×10^5 counts/min per μmole P.

Ouabain conc. μM	$\left(\frac{\text{r.s.a.} + \text{ouabain}}{\text{r.s.a.} - \text{ouabain}}\right) \times 100$ for each fraction					
	ATP	PP	ROP	AP	NA	PL
0	100	100	100	100	100	100
0·1	97·5	87	89	72·5	84	65
0·5	91	95	98	83	88	96
1·0	87	96	99	75	80	63
2·0	62	71	66	62·5	66	53
10·0	75	67	43·5	59	53	57

D. *Effects of ouabain*

1. *Sodium medium.* Incorporation of ^{32}P into all the acid-insoluble fractions was *lowered* by up to 40% by ouabain at 10 μM (Table 4). Lower concentrations of ouabain, down to 0·1 μM, resulted in correspondingly smaller inhibitions of incorporation, without, however, altering in any way the uniformity of the effect on the various individual fractions (Table 6). On the other hand replacing the sodium of the medium

by choline abolished the ouabain-induced lowering of incorporation; specific activities in the choline medium were 20–30% of those in sodium, independently of the presence or absence of ouabain (Table 5).

This uniform depression was unexpected, in that it had been reported[26] that ouabain at 10 μM is without effect on the respiration of rat brain cortex slices, although at that concentration significant effects on transport of glutamate and of potassium could be observed. Accordingly, in one experiment, slices of rat cerebral cortex were used in place of guinea-pig. Table 7 shows that whilst after 30 min incubation there was little effect of the ouabain on incorporation, after 60 min a uniform inhibition of incorporation of up to 50% was observed in all fractions.

TABLE 7. EFFECT OF OUABAIN ON PHOSPHATE INCORPORATION IN RAT BRAIN

Rat brain cortex slices were incubated for 30 min and 60 min in the presence of ^{32}P in the following media: (a) sodium; (b) sodium to which ouabain at 10 μM was added at the same time as ^{32}P after a 15 min preincubation. Media were as defined in Table 1. The tissue was dispersed in TCA and fractionated as under Methods. Abbreviations and definition of r.s.a. as in Methods. Specific activity of inorganic phosphate of slice after 60 min; 10 × 10^5 counts/min per μmole P. Results are means of 3 determinations in each case:

Fraction	30 min r.s.a.			60 min r.s.a.		
	Na^+	Na^+ + ouabain	$\left(\frac{Na^+ + \text{ouabain}}{Na}\right) \times 100$	Na^+	Na^+ + ouabain	$\left(\frac{Na^+ + \text{ouabain}}{Na}\right) \times 100$
IP	100	100	—	100	100	—
ATP	31	34·5	110	78	33	42
PP	11·5	10·5	91	20	12·5	63
ROP	4·2	4·4	103	10·3	5·15	50
AP	0·72	0·63	88	1·24	0·64	51
NA	1·18	1·28	109	2·86	1·50	53
PL	0·60	0·48	80	1·17	0·57	48

2. *Ouabain at high potassium concentrations.* The effect of ouabain (10 μM) on slices incubated in 7·5 mM K^+ is shown in Table 4. Incorporation into ATP, nucleic acid, acid-precipitable phosphate, phosphoprotein and residual organic phosphate was *depressed* by up to 35% compared with that in 7·5 mM K^+ without ouabain.

However incorporation into phospholipid was *stimulated* by 50% by ouabain. As increasing the medium potassium level from 2·5 to 7·5 mM K^+ resulted in a depression of incorporation into phospholipid, the net effect of the ouabain-induced stimulation was to restore the incorporation to that found at 2·5 mM K^+. In no case was incorporation increased above this level. Preliminary experiments suggest that when labelling in all fractions is *depressed* by increasing the potassium concentration in the medium to 50 mM, addition of ouabain then results in a stimulation of incorporation into all fractions to a level less than or equal to that found in the standard medium.

3. *Ouabain in calcium-free media.* Lack of calcium stimulated phosphate incorporation into all fractions. Addition of 10 μM ouabain to the calcium-free medium abolished this stimulation, and reduced incorporation to a level similar to that found for the standard medium in the presence of ouabain (Table 4).

4. *Ouabain in glutamate-containing media.* In the presence of glutamate, in the sodium medium, addition of 10 μM ouabain resulted in an increased incorporation of phosphate into all fraction of up to 35% above that in the absence of ouabain. This

stimulation occurred at both high and low potassium concentrations, but was abolished, as shown in Table 5, when sodium was replaced by choline in the medium. Closer inspection of the ouabain-induced stimulation of incorporation, however, revealed that the apparent stimulation represented a partial reversal of the glutamate-inhibition of incorporation.

Thus, in Table 4, the effect of ouabain in a sodium medium was to reduce incorporation, and in a (sodium; glutamate) medium to stimulate incorporation, to a resultant level intermediate between that found with sodium alone, and that with (sodium; glutamate) alone.

DISCUSSION

Evidence based on the effects of drugs or of altered ionic conditions on phosphate incorporation into phospholipid or phosphoprotein has hitherto been used in an attempt either to deduce a specific locus for the site of action of the drug (e.g. ouabain, (9–12) acetylcholine[7, 8, 25]), or a particular role for the phosphate fraction under examination.[7–10] The experiments described here have surveyed the effects of a variety of changed metabolic conditions on the rate of incorporation of inorganic phosphate into ATP and the acid-insoluble phosphates of respiring brain slices. They have shown that, in fact, ouabain, glutamate and altered ionic milieu affect the incorporation of inorganic phosphate into all of the phosphate fractions examined. These fractions are admittedly heterogeneous,[27–31] and these studies cannot rule out either the presence in any one of them of a small subfraction whose rate of labelling is affected in a specific and different manner to the bulk of the tissue phosphates, or the occurrence of small, rapid changes in the rate of incoporation over the first 15 min of incubation. Neither of these possibilities would be detectable in the type of experiment reported here (e.g. ref. 37). Nonetheless, it remains that deductions as to the locus of ouabain action based on observations on the effect of ouabain on phosphate incorporation into one specific phosphate fraction become difficult to sustain. It would clearly be desirable to extend such examination also to the effects of other substances such as acetylcholine or chlorpromazine.

Effects of ouabain. The effects of ouabain on respiration in cerebral slices have been widely studied, but agreement as to the interpretation of results has been lacking. Thus Wollenberger[38] found for guinea-pig brain 10 μM ouabain first stimulated and then depressed respiration. Langemann, Brody and Bain[39] noted, in a magnesium-free medium only depression. 10 μM ouabain was found to have no effect on respiration of rat cortex slices by Gonda and Quastel[26], whilst effects on cation and glutamate transport were marked. For rabbit, Whittam[40] found only depression of respiration in the absence of calcium. For guinea-pig, Schwartz[12] found only stimulation in the presence or absence of calcium. Effects of ouabain on phosphate turnover in brain have been partly studied. Yoshida *et al.*[11] found that ouabain stimulated incorporation into phospholipids; Nicholls *et al.*[10] found only a transient increase, followed by a fall, with 10 μM ouabain; with glutamate also present only the stimulation was observed. Schwartz,[12] found no effect on phosphoprotein turnover. Ahmed, Judah and Wallgren[9] observed that whilst ouabain was without effect on phosphoprotein turnover in unstimulated slices, it abolished the increase in turnover associated with electrical stimulation.

In our experiments, it has been shown that ouabain, at concentrations between 0·1 and 10 μM, reduced incorporation into ATP and all the acid-insoluble fractions. The extent to which incorporation was reduced depended on the concentration of ouabain used, reaching a maximum of 45% at 10 μM ouabain, and was independent of the duration of incubation, and of the time of addition of ouabain, either with or without preincubation (Tables 4, 6).

That this is more complex than a simple uncoupling of oxidation from phosphorylation is shown by the fact that ouabain also abolished the increased incorporation associated with diminished calcium or raised potassium in the medium. In this context, the effects of ouabain on incorporation into phospholipid are of particular interest. In contrast to its effects on other acid-insoluble fractions, raised potassium depressed incorporation into phospholipid, and here, in agreement with the findings of Yoshida *et al.*[11] and Nicholls *et al.*[10] the addition of ouabain stimulated incorporation, though never to a level greater than that found in the standard medium. Thus in many respects, the addition of ouabain appears to have similar effects, both on respiration,[40] and on phosphate incorporation (Table 4), to raising the Ca^{2+}, or diminishing the K^+ concentration, of the incubation medium. It is well known that levels of respiration in brain slices depend on the ratio of K^+ to Ca^{2+} in the medium,[41] and it is possible that the effect of ouabain on phosphorylation demonstrated here is in some way mediated through an alteration of the K^+/Ca^{2+} balance within the cell by the glycoside.

In a glutamate medium, the effect of ouabain was (Table 4) partially to reverse the glutamate-induced suppression of phosphate incorporation, resulting in an apparent stimulation. In this respect ouabain acts in a manner predicted by Gonda and Quastel[26] who assumed that its site of action was at the membrane. They argued that entry of glutamate into the cell and its subsequent utilisation involved consumption of $\sim$ P, and hence a reduced availability of ATP for other metabolic pathways. Hence the blocking of glutamate entry into the cell results in an increased availability of ATP for incorporation. This prediction has been confirmed by the present work.

It is difficult to reconcile these various effects of ouabain with any hypothesis demanding a single site of glycoside action. Its effects would appear diverse, and could depend more upon some general ability of the glycoside molecule to complex with, and disorientate, cellular lipoprotein structures, than on any more specific enzyme-inhibitory action.

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