us as urea (R_F , 0.25–0.31). We further observed that, on chromatographing with water-saturated isobutanol or phenol, urea separates well from PAS. The R_F values in isobutanol were: PAS, 0.40–0.45; urea, 0.20–0.25. In phenol values were: PAS, 0.57–0.60; urea, 0.65–0.70.

At plasma levels around 2 mg. total PAB/100 ml. the spot of PAB mostly failed to appear in the urine, the chromatogram indicating excretion predominantly of PAH. This seems to be an important fact concerning PAB clearance. The spot of PAB always appears at more elevated PAB serum levels, but the spot of PAH remains or even increases markedly in intensity. We eluted unsprayed PAH areas of our chromatograms and subjected the eluates to hydrolysis with mineral acid. The presence of glycine in the hydrolysate was then demonstrated on a second chromatogram sprayed with ninhydrin. Following mild hydrolysis of the urine with hydrochloric acid

the spot corresponding to PAB also becomes stronger and brighter. This may be due in part to hydrolysis of the *N*-acetyl derivative, but the phenomenon was not investigated in detail.

SUMMARY

1. A method using partition chromatography with filter paper permitted separation and determination of microgram quantities of *p*-aminobenzoic, *p*-aminohippuric, and *p*-aminosalicylic acids when all occurred together.

2. The method was used for investigating in what form *p*-aminobenzoic acid is excreted in humans. At all plasma levels investigated (up to 24 mg./100 ml.) *p*-aminohippuric acid was present in the urine.

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The Role of Glutamic Acid in the Transport of Potassium in Brain and Retina*

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In a previous paper from this laboratory (Stern, Eggleston, Hems & Krebs, 1949) it was shown that sliced brain tissue, when suspended in a saline medium containing glutamic acid, absorbs this amino-acid against a concentration gradient. Tissue slices thus afford a system in which problems of permeability and active transport can be studied under controlled conditions. The present paper is concerned with an *in vitro* study of the mechanism maintaining the high potassium gradient between nervous tissue and body fluids. The main result is

the demonstration that, apart from a source of energy such as glucose or lactate, L-glutamate is required for the maintenance of the high potassium concentration in brain and retina.

EXPERIMENTAL

Material. Guinea pig and rabbit brain were treated as described by Stern *et al.* (1949). Guinea pig kidney cortex and rat testis were handled in the same manner.

Ox eyes were removed at the abattoir immediately after the death of the animal, placed in ice water and carried to the laboratory. The eye-ball was opened by a circular cut

* Preliminary communication: Krebs & Eggleston (1949).

along the equator, the vitreous body poured off, the retina divided *in situ* by radial incisions into four to eight sectors of approximately equal size and then separated from the choroid by blunt dissection. Each piece was rapidly weighed on a torsion balance (weight between 50 and 150 mg.) and placed in the medium in the main compartment of a conical Warburg cup. The cups were kept in a tray of ice-cold water, until they were attached to their manometers and transferred to a water bath at 40°. At the end of the incubation period, usually 60 min., the tissues were removed from the cups, drained by sliding on a dry glass surface and again weighed on the torsion balance. They were then ground in a mortar with 2.5 ml. of a solution containing 330 mg. Na/100 ml. for K determinations by flame photometry or with 2.5 ml. of 0.4N-H₂SO₄ for other analyses. One portion of the tissue which was not incubated was used for the estimation of the initial composition of the tissue. Four different portions of one retina in a parallel experiment showed no difference in their K content initially and after incubation.

Media. Bicarbonate saline (Krebs & Henseleit, 1932), containing 5.93 m-equiv. K and 144 m-equiv. Na/l. and in equilibrium with a gas mixture containing 5% CO₂, was the standard medium. In most experiments 3.4 ml. of this medium were diluted to 4 ml. by the addition of solutions of substrates or of 0.9% NaCl. The amount of K in 4 ml. of the medium in the cups before the addition of the tissue portions was found by analysis (see below, p. 147) to be 0.796–0.804 mg., i.e. 5.0–5.2 m-equiv./l. In a few experiments phosphate saline without Ca (Krebs & Eggleston, 1940) was used.

All acidic substances were added as the neutral Na salts in solution. Glutathione and cysteine were neutralized immediately before use. Substances of low solubility were weighed directly into the Warburg flask.

Analytical methods

Estimation of potassium and sodium. A flame photometer was constructed by Mr G. Fletcher and Mr A. Renshaw in the departmental workshop according to information kindly supplied by Dr W. Klyne prior to publication of the paper by Domingo & Klyne (1949). The following minor modifications were found to improve its sensitivity for the determination of potassium. (1) A Cambridge Spot Galvanometer (sensitivity 160 mm./μa.) was used. The specified critical resistance of the instrument was 10,000 Ω; however, a resistance of 5000 Ω was found more effective in reducing erratic movement of the spot. (2) The photocell was a gas-filled caesium-silver oxide cell (Osram CMG 8). Since the cell was not sufficiently sensitive at its specified working voltage of 80 V., a potential of 108 V. was applied. This produced a galvanometer deflexion of about 2 cm. for every 0.5 mg. K/100 ml. on a linear galvanometer scale. Although the photocell was overloaded, it was possible, by avoiding excessive illumination, to prevent glow discharge. If, however, glow discharge occurred (after 6 months' continuous use), the cell recovered after a period of rest and could be used again.

Media were diluted in the way described by Domingo & Klyne (1949) for serum and plasma. For the estimation of K, 1 or 2 ml. samples of the ground tissues were diluted by the addition of 6–10 ml. of a solution containing 330 mg. Na/100 ml.; for the estimation of Na, samples were diluted ten times by the addition of distilled water. Each sample was determined at least twice and the flame photometer readings usually agreed within ±0.025 mg. K/100 ml. and ±0.02 mg. Na/100 ml. Since the content of K and Na of

the diluted tissue homogenates was of the order of 1–2 mg./100 ml., the error was less than ±2.5%.

In earlier experiments the cobaltinitrite precipitation method, as described by Eden (1943), was used for the estimation of K. Ashing of tissues and media was found to be necessary and this was done according to Van Slyke & Rieben (1944), except that vitreosil crucibles were used in place of platinum crucibles. Comparison of this method with flame photometry showed that the latter had not only the advantage of greater speed, but also gave more consistent results in duplicate determinations. In some experiments both methods were used at the same time and the cobaltinitrite methods gave slightly higher results (see Table 1). Ashing of the tissue was not necessary for flame photometry; analyses of ashed and unashed portions of the same sample agreed within the experimental error (see Stone & Shapiro, 1948).

Estimation of glutamic acid, glutamine and ammonia. The determination of the sum of glutamic acid and glutamine and the differential determination of the two substances were carried out by the decarboxylase method of Gale (1945) and Krebs (1948). In most experiments no differentiation was made between glutamic acid and glutamine. The analytical values under the heading of 'glutamate' therefore represent glutamic acid plus glutamine unless otherwise stated.

Calculation. Under certain conditions the weight of the tissues after incubation was found to be up to 20% higher than the initial wet weight. This increase was assumed to be due to adherent medium, and the results were corrected by subtracting the ionic content of an equivalent volume of the medium. In other cases the weight decreased and no further correction was applied. The resulting error was small in the estimation of K (see Stern *et al.* 1949, Table 1), but possibly large in the case of Na, which was present in the medium in high concentration. For this reason the technique employed cannot be regarded as very satisfactory for the study of the Na balance of the tissue.

Duplicate experiments. In many cases all stages of the experiment were duplicated. The K contents of different portions of the same tissue treated in a similar manner differed from the mean by less than ±5%.

RESULTS

Choice of material

The first experiments on the movements of ions were carried out on brain cortex (Krebs & Eggleston, 1949). Guinea pig kidney cortex and rat testis were found to be less suitable. In the case of testis duplicates did not agree satisfactorily; this was probably due to partial disintegration of the tissue during incubation, as shown by a loss of 20–40% of the initial wet weight. Kidney cortex contains much preformed substrate and therefore does not lend itself to studies of the effects of added substrates. Ox retina, on the other hand, was found to be a satisfactory material which has the advantage that slicing is not required so that mechanical injury is reduced to a minimum. Moreover, the store of respiratory substrates is low in retina. Sheep retina was also examined, but its isolation was more difficult owing to adherence to the vitreous body.

Experiments on brain-cortex slices

All experiments except one, in which rabbit brain was used, were carried out on guinea pig brain cortex. The results are shown in Table 1. It will be seen that under anaerobic conditions the potassium content of the slices fell to a low level approaching that of the medium (Exps. 1 and 2). Addition of

the joint addition of glucose and glutamate. In the presence of these substances the original concentration of potassium in the slice was maintained, or almost maintained. Aspartate had about the same effect as glutamate.

Results obtained by the cobaltinitrite precipitation method agreed essentially with those obtained by flame photometry.

Table 1. *Effect of various substrates on the loss of potassium from brain-cortex slices*

(Guinea pig brain in all experiments except Exp. 2 (rabbit brain) incubated in bicarbonate saline. Concentration of glucose 0.02 M, of L-glutamate 0.005 M unless otherwise stated, of DL-aspartate 0.01 M, of adenosinetriphosphate 0.001 M. Figures marked with an asterisk (*) represent results obtained by cobaltinitrite precipitation method, all others by flame photometry. Where two figures are given these represent results of duplicate experiments on different portions of the same brain; in Exp. 5 the ashed samples of each tissue portion were analysed by both flame photometry and cobaltinitrite method.)

Exp. no.	Substrates added	Experimental conditions	Incubation period (min.)	K found in tissue (m-equiv./kg.)		
				Initially	After incubation	Change
1	None	Anaerobic	60	90.4	9.3	- 81.1
	Glucose	Anaerobic	60	90.4	20.2	- 70.2
	Glucose; L-glutamate (0.01 M)	Anaerobic	60	90.4	12.3	- 78.1
	Glucose; L-glutamate (0.01 M) adenosinetriphosphate	Anaerobic	60	90.4	18.4	- 72.0
	Glucose; L-glutamate (0.01 M)	Aerobic	60	90.4	95.0	+ 4.6
2	None	Anaerobic	60	98.6	10.4	- 88.2
	Glucose	Anaerobic	60	98.6	38.3	- 60.3
	Glucose; L-glutamate (0.01 M)	Anaerobic	60	98.6	22.7	- 75.9
	None	Aerobic	60	98.6	28.6	- 70.0
	Glucose	Aerobic	60	98.6	38.8	- 59.8
3	Glucose; L-glutamate (0.01 M)	Aerobic	60	98.6	68.2	- 30.4
	None	Aerobic	15	118*	82.5*	- 35.5*
	None	Aerobic	30	118*	68.9*	- 49.1*
	None	Aerobic	60	118*	64.3*	- 53.7*
	Glucose; L-glutamate	Aerobic	60	118*	120.8*	+ 2.8
4	None	Aerobic	60	112*	48.0*	- 64.0*
	Glucose	Aerobic	60	112*	55.0*	- 57.0*
	Glucose; L-glutamate	Aerobic	60	112*	92.7*	- 19.3*
5	Glucose	Aerobic	60	110, 116*	71.3, 75.4*	- 38.7, - 40.6*
	Glucose	Aerobic	60	110, 116*	66.8, 74.5*	- 43.2, - 41.5*
	Glucose; L-glutamate	Aerobic	60	110, 116*	93.0, 97.5*	- 17.0, - 18.5*
	Glucose; L-glutamate	Aerobic	60	110, 116*	93.1, 108.8*	- 16.9, - 7.2*
	Glucose	Aerobic	40	90.5	51.7, 57.5	- 39.0, - 33.0
6	Glucose; L-glutamate	Aerobic	40	90.5	77.0, 77.5	- 13.5, - 13.0
	Glucose; L-glutamate	Aerobic	40	90.4	77.5	- 12.9
7	Glucose; L-glutamate	Aerobic	40	90.4	27.8, 29.0	- 62.6, - 61.4
	Glucose; L-glutamate	Anaerobic	40	90.4	27.8, 29.0	- 62.6, - 61.4
8	Glucose; L-glutamate	Aerobic	40	96.8	79.5, 82.5	- 17.3, - 14.3
	Glucose; DL-aspartate	Aerobic	40	96.8	80.5, 81.0	- 16.3, - 15.8

glucose or glutamate had no marked effect under anaerobic conditions. Aerobically about half of the potassium originally present in the slices was lost during incubation when no substrate was added to the medium. Most of the loss occurred within the first 30 min. (Exp. 3). After 30 min. the level appeared to become stabilized at about one-half the original potassium concentration. Glucose slightly reduced the loss in some experiments but the effect was not marked. Glutamate alone had about the same effect as glucose (Krebs & Eggleston, 1949). A very pronounced effect was produced by

Experiments on ox retina

Changes in sodium and potassium content of retina in the excised eye. Some eyes were carried from the abattoir to the laboratory in ice-cold water and the retinae removed 1-2 hr. after the death of the animal. These retinae showed a lower potassium, and a higher sodium, content than expected from analogy with other tissues, e.g. brain, the sodium level being higher than the potassium level (see Table 2). This was found to be due to changes occurring after excision of the eye. Retinae pre-

pared for analysis at the abattoir immediately after the death of the animal gave sodium and potassium values of the order expected with potassium predominating (Table 2). Thus potassium was lost during cold storage, presumably by diffusion into the vitreous body. At the same time the sodium

Table 2. *Potassium and sodium content of fresh, stored and incubated ox retina*

(A, retina removed from eye immediately after death of animal. B, retina removed from eye after 1-2 hr. storage in ice-cold container. (a) initial composition, (b) final composition after aerobic incubation with 0.02M-glucose and 0.005M-L-glutamate in bicarbonate saline for 60 min.)

	Retina no.	Cation concentration (m-equiv./kg. tissue)		
		Na	K	Na + K
A	1	52	70.5	122
	2	55	64.8	120
B	3a	107	29.1	136
	b	88	43.6	132
	4a	109	30.8	140
	b	83	55.0	138
	5a	97	31.1	128
	b	75	52.4	127
	6a	102	26.8	129
	b	65	68.0	133

content of the tissue increased by an approximately equivalent amount. When the retina was incubated at 40° in bicarbonate saline containing glucose and glutamate, these changes were reversed and the original distribution of ions was largely restored (Table 2).

In the subsequent sections the influence of the composition of the medium on ion transport was studied in detail, but as the procedure used is not suitable for accurate measurements of the sodium concentration of the tissue the investigation was confined to a study of potassium transport.

Effect of various substrates on the accumulation of potassium. When retina was incubated in bicarbonate saline without added substrate the potassium content of the tissue fell about 10% below the

Table 3. *Effect of various concentrations of glutamate on the accumulation of potassium in retina*

(Aerobic incubation in bicarbonate saline for 60 min.; 0.02M-glucose.)

	K found in tissue (m-equiv./kg.)		
	Initially	After incubation	Change
Glucose	33.7	38.6	+ 4.9
Glucose; L-glutamate (0.0002M)	33.7	38.0	+ 4.3
Glucose; L-glutamate (0.001M)	33.7	49.6	+ 15.9
Glucose; L-glutamate (0.002M)	33.7	47.6	+ 13.9
Glucose; L-glutamate (0.01M)	33.7	64.0	+ 30.3

starting (cold storage) value. In the presence of glucose, the potassium content was approximately maintained during incubation. As already stated, glucose and L-glutamate together caused a large increase in the potassium content of the tissue returning it to that of fresh retina (Tables 2 and 3).

Table 4. *Effect of various substrates on the accumulation of potassium in ox retina*

(Aerobic incubation in bicarbonate saline for 60 min.; 0.02M-glucose.)

Exp. no.	Substrates added	K found in tissue (m-equiv./kg.)		
		Initially	After incubation	Change
1	Glucose	31.8	38.2	+ 6.4
	Glucose; L-glutamine (0.01M)	31.8	42.0	+ 10.2
	Glucose; α -ketoglutarate (0.01M)	31.8	39.2	+ 7.4
	Glucose; L-glutamate (0.01M)	31.8	59.5	+ 27.7
2	Glucose	31.1	38.0	+ 6.9
	Glucose; L-aspartate (0.005M)	31.1	50.8	+ 19.7
	Glucose; L-glutamate (0.005M)	31.1	52.4	+ 21.3
3	Glucose	26.8	38.7	+ 11.9
	Glucose; L-aspartate (0.005M)	26.8	52.4	+ 25.6
	Glucose; L-glutamate (0.005M)	26.8	68.0	+ 41.2
4	None	32.8	29.9	- 2.9
	Glucose	32.8	35.1	+ 2.3
	Glucose; L-aspartate (0.01M)	32.8	43.8	+ 11.0
	Glucose; L-asparagine (0.01M)	32.8	32.7	- 0.1
	Glucose; L-glutamate (0.01M)	32.8	55.0	+ 22.2
	Glucose; L-glutamine (0.01M)	32.8	31.7	- 1.1
5	Glucose	35.6	37.6	+ 2.0
	Glucose; L-valine	35.6	35.9	+ 0.3
	Glucose; L-methionine (0.01M)	35.6	40.0	+ 4.4
	Glucose; glutathione (0.02M)	35.6	49.9	+ 14.3
	Glucose; L-glutamate (0.01M)	35.6	61.0	+ 25.4

Addition of DL-lactate or of pyruvate had the same effect as addition of glucose, but when these substances were present together with glucose the effects were not additive.

Glutamate alone did not cause accumulation of potassium, but like glucose maintained the initial potassium content of the tissue. When glucose was present, increasing concentrations of glutamate in the medium, within the range tested, caused increasing accumulation of potassium by the tissue (Table 3). The lowest concentration producing a measurable effect was 0.0005 M.

The following amino-acids when added to the medium in addition to 0.02 M-glucose had no effect on the potassium level of the tissue: glycine, DL-alanine, L-valine, L-leucine, DL-isoleucine, L-phenylalanine, L-tyrosine, DL-serine, DL-threonine, DL-cysteine, L-methionine, L-arginine, L-lysine, L-histi-

Conversion of aspartate to glutamate. The effect of aspartate on the accumulation of potassium may have been due to its conversion to glutamate. To test this, ox retina and slices of guinea pig brain cortex were incubated with glucose and aspartate (0.01 M) and the amounts of glutamate (plus glutamine) and potassium in tissues and medium were determined (Tables 5 and 6). On incubation with aspartate the glutamate content of the tissue was increased and glutamate was found in the medium in concentrations which, according to Table 3, influence the potassium concentration of the tissue. It is therefore possible that the effect of aspartate, at least in part, is due to its conversion into glutamate. The glutamate is presumably formed by transamination between aspartate and the α -ketoglutarate arising in the oxidative metabolism of the tissue.

Sodium, potassium and glutamic acid in intra-ocular

Table 5. *Formation of glutamate from aspartate in guinea pig brain-cortex slices*

(Aerobic incubation in bicarbonate saline for 40 min.; 0.02 M-glucose, 0.005 M-aspartate.)

Exp. no.	Substrates added in addition to glucose	Initial wt. of slice (mg.)	Glutamate found (μ l.)					Glutamate formed during incubation (μ l.)
			In slices			In 4 ml. medium	In tissue plus medium after incubation	
			Initially	Finally	Change			
1	None	83.5	27.8	25.4	- 2.4	32.0	57.4	+ 29.6
	Aspartate	75.0	24.8	36.3	+ 11.5	41.0	77.3	+ 52.5
2	None	76.0	25.2	25.0	- 0.2	14.6	39.6	+ 14.4
	Aspartate	95.5	31.6	39.3	+ 7.7	44.0	83.3	+ 51.7

Table 6. *Formation of glutamate and accumulation of potassium in the presence of aspartate in ox retina*

(Aerobic incubation in bicarbonate saline for 60 min.; 0.02 M-glucose, 0.01 M-L-aspartate.)

Exp. no.	Substrates added in addition to glucose	Initial wt. of tissue (mg.)	Glutamate found (μ l.)				Glutamate formed during incubation (μ l.)	K found in tissue (m-equiv./kg.)		
			In tissue			In 4 ml. medium		Initially		
			Initially	Finally	Change			Initially	Finally	Change
1	None	101	—	—	—	—	—	26.2	40.6	+ 14.4
	Aspartate	76.5	—	—	—	—	—	26.2	63.5	+ 37.3
	Aspartate	170.0	14.6	26.4	+ 11.8	41.6	+ 53.4	—	—	—
2	None	88	—	—	—	—	—	31.6	35.4	+ 3.8
	Aspartate	136	—	—	—	—	—	31.6	50.2	+ 18.6
	Aspartate	161	17.9	26.5	+ 8.6	24.9	+ 33.5	—	—	—

dine, L-proline, L-hydroxyproline and L-hydroxyaspartic acid. The final concentration of the L-amino-acids was 0.01 M, of the DL-acids 0.02 M. In contrast, L-aspartate caused an accumulation of potassium comparable to that produced by glutamate, whilst glutamine, asparagine, N-acetylglutamate and α -ketoglutarate were ineffective. Reduced glutathione had a small effect (see Table 4). Ammonium chloride (0.005 M), together with glucose, caused a fall in the potassium content of the tissue. DL-Glutamate (0.02 M) was almost as effective as L-glutamate (0.01 M).

fluid and in retina. In connexion with the preceding results, information on the sodium, potassium and glutamate concentration of the intra-ocular fluid of the ox eye is of interest. Enough fluid could be separated from the posterior half of the vitreous body for analysis. The potassium content varied from 5.33 to 6.44 m-equiv./l., average 5.8 m-equiv./l. (fourteen estimations), the sodium content from 131 to 142.5 m-equiv./l., average 137 m-equiv./l. (five estimations). In respect to these ions the composition of the intra-ocular fluid resembles that of serum (cf. Duke-Elder, 1942). The concentrations of sodium

and potassium are thus very different in intra-ocular fluid and in retina.

One sample of fluid separated from the vitreous body was analysed for glutamic acid plus glutamine; 1.34 $\mu\text{mol./ml.}$ was found. A pooled sample of two fresh retinae detached from the eyes at the abattoir immediately after the death of the animal, analysed as described by Krebs, Eggleston & Hems (1949), contained glutamic acid 5.66, glutamine 4.29 and ammonia < 0.9 $\mu\text{mol./g.}$ wet weight. The glutamine value is similar to that found in the brain of various

species, while the glutamic acid value is somewhat lower (cf. Krebs *et al.* 1949). After transport of the eye from the abattoir, involving cold storage for 1–2 hr., a lower value for glutamate plus glutamine was found in retina and this was raised about 4–7 times on incubation with glucose and glutamate (Table 7). Only a small amount of glutamate accumulated when glutamate alone was added. Sodium fluoride (0.02 M), 2,4-dinitrophenol (2×10^{-4} M) and malonate (0.02 M) inhibited the uptake of glutamate; iodoacetate (0.001 M) caused a fall of tissue glutamate

Table 7. *Effect of inhibitors on the accumulation of glutamate in retina*

(Aerobic incubation in bicarbonate saline for 60 min. Concentrations: glucose, fluoride and malonate, 0.02 M; DL-lactate, 0.01 M; iodoacetate, 0.001 M; 2,4-dinitrophenol, 2×10^{-4} M; glutamate, 0.005 M in Exps. 1 and 2, 0.01 M in Exp. 3.)

Exp. no.	Substrates and inhibitors added	Glutamate plus glutamine ($\mu\text{mol./g.}$)			
		In tissue			'Metabolized'
		Initially	After incubation	Change	
1	Glutamate	7.36	17.0	+ 9.6	- 26.0
	Glutamate; glucose; fluoride	7.36	15.6	+ 8.2	- 14.4
	Glutamate; glucose; malonate	7.36	29.2	+ 21.8	- 14.1
	Glutamate; glucose	7.36	37.7	+ 30.3	- 16.9
2	Glutamate	4.95	17.9	+ 12.9	- 21.4
	Glutamate; glucose; 2,4-dinitrophenol	4.95	18.5	+ 13.6	- 11.2
	Glutamate; glucose; iodoacetate	4.95	1.7	- 3.3	- 12.0
	Glutamate; glucose	4.95	34.8	+ 29.9	- 17.2
3	Glutamate; glucose; fluoride	6.34	18.0	+ 11.7	- 23.0
	Glutamate; glucose; malonate	6.34	26.0	+ 19.7	- 26.6
	Glutamate; glucose; 2,4-dinitrophenol	6.34	15.4	+ 9.0	- 22.8
	Glutamate; glucose; iodoacetate	6.34	7.1	+ 0.8	- 15.9
	Glutamate; DL-lactate; iodoacetate	6.34	14.7	+ 8.4	- 9.4
	Glutamate; glucose	6.34	28.7	+ 22.4	- 33.9

Table 8. *Effect of inhibitors on the accumulation of potassium in retina*

(Aerobic incubation in bicarbonate saline for 60 min.; 0.02 M-glucose, 0.01 M-glutamate.)

Substrates and inhibitors added	K found in tissue (m-equiv./kg.)		
	Initially	After incubation	Change
Glucose	34.3	42.4	+ 8.1
Glucose; glutamate; fluoride (0.02 M)	34.3	35.8	+ 1.5
Glucose; glutamate; malonate (0.02 M)	34.3	55.0	+ 20.7
Glucose; glutamate; iodoacetate (0.001 M)	34.3	8.6	- 25.7
Glucose; glutamate; 2,4-dinitrophenol (0.0002 M)	34.3	41.0	+ 6.7
Glucose; glutamate	34.3	68.3	+ 34.0

Table 9. *Accumulation of glutamate and of potassium in retina*

(Aerobic incubation in bicarbonate saline for 60 min. K and glutamate content was determined on different portions of same retina treated identically.)

Exp. no.	Substrates added	K found in tissue (m-equiv./kg.)			Glutamate found in tissue (mmol./kg.)			Glutamate metabolized (mmol./kg.)
		Initial	Final	Change	Initial	Final	Change	
1	Glutamate (0.001 M)	32.1	30.0	- 2.1	4.5	9.2	+ 4.7	- 16.9
	Glutamate (0.001 M); glucose (0.02 M)	32.1	43.4	+ 11.3	4.5	22.7	+ 18.2	- 11.2
2	Glutamate (0.002 M)	35.2	37.2	+ 2.0	5.8	10.8	+ 5.0	- 24.8
	Glutamate (0.002 M); glucose (0.02 M)	35.2	55.6	+ 20.4	5.8	31.5	+ 25.7	- 16.3

to the level of the medium. All four inhibitors reduced the amount of 'glutamate metabolized', i.e. the fraction of glutamate removed from the medium minus glutamate accumulated in the tissue (see Stern *et al.* 1949).

Glutamate accumulation in relation to accumulation of potassium. The question arises whether potassium is the cationic equivalent of the glutamate anion transported into the tissue. If this were so, the amount of glutamate transported should never be less than the amount of potassium transported, and further, substances which inhibit the transport of glutamate should also inhibit the transport of potassium. This is actually the case. Comparison of Tables 7 and 8 shows that approximately equivalent amounts of potassium and glutamate are transported. In the experiments recorded in Table 9, the changes in the potassium and glutamate concentrations were determined in the same tissue and somewhat more glutamate was transported than potassium. However, glutamate in these experiments represents the sum of glutamic acid and

Table 10. *Accumulation of glutamine, glutamic acid and potassium in retina*

(Aerobic incubation in bicarbonate saline for 60 min.; 0.02 M-glucose, 0.01 M-L-glutamate.)

Exp. no.		($\mu\text{mol./g. wet wt.}$)		
		Glutamine	Glutamic acid	Potassium
1	Initially	0.4	6.3	33.9
	After incubation	2.2	27.5	51.9
	Change	+1.8	+21.2	+18.0
2	Initially	1.7	9.1	36.0
	After incubation	8.1	20.3	50.1
	Change	+6.4	+11.2	+14.1

glutamine; differential determination of these substances showed that the increase of glutamine in the tissue during incubation was small and that approximately equivalent amounts of glutamic acid and potassium accumulated in the tissue (Table 10). The results are thus in accordance with the view that glutamate and potassium are transported together, but they do not prove it.

Experiments in phosphate saline. Phosphate saline without calcium (Krebs & Eggleston, 1940) was used in place of the bicarbonate saline and carbon dioxide was absorbed by alkali. As shown in Table 11, the substrate combination glucose plus glutamate again increased the potassium uptake by ox retina, but the amounts of potassium transported were somewhat smaller. There was no parallelism between potassium uptake and oxygen uptake.

Effect of drugs and hormone preparations. An attempt was made to study the effect of some drugs and hormones on the potassium content of retina incubated with glucose and glutamate, since it was thought that those substances might intervene in the mechanism involving the transport of potassium. Retina which had lost part of its physiological potassium content during cold storage was the test material. As already described, the potassium content of this tissue rose to its physiological level during incubation when no drug was added. Addition to the medium of picrotoxin (3×10^{-6} M), of phenobarbitone (2.5×10^{-4} M) and of these two substances together had no effect on the amount of potassium transported. No conclusive results were obtained in preliminary experiments with acetylcholine (10^{-5} M) plus eserine (5×10^{-5} M), with histamine (5×10^{-5} M), and with adrenal cortex extract, Eschatin (Parke, Davis and Co.).

Anaerobic experiments. When retina, which during cold storage had already lost about 50% of its original potassium content, was incubated anaerobically for 60 min. a further loss of potassium occurred in the absence of added substrates, resulting in an overall loss of more than 80% of the *in vivo* level. Glucose prevented the further loss of potassium on incubation, but failed to restore the potassium level to its *in vivo* value. In some experiments glutamate, when present in addition to glucose, caused a slight rise in the potassium level. Addition of adenosinetriphosphate had no effect (Table 12).

Effect of substrates on the disintegration of the tissue during incubation. Retina incubated in bicarbonate saline under aerobic conditions with glucose and glutamate increased in weight, usually by about 10–15%. Retina incubated without a substrate or

Table 11. *Accumulation of potassium in retina in phosphate saline without calcium*

(Incubation 60 min. at 40°; 0.02 M-glucose, 0.01 M-glutamate.)

Exp. no.	Substrates added	K found in tissue (m-equiv./kg.)			- Q_{O_2} ($\mu\text{l./mg. dry wt./hr.}$)
		Initially	After incubation	Change	
1	Glucose	30.1	34.5	+ 3.4	11.0
	Glutamate	30.1	34.4	+ 3.3	14.3
	Glucose; glutamate	30.1	40.2	+ 10.1	13.2
2	Glucose	31.7	41.6	+ 9.9	16.6
	Glutamate	31.7	33.9	+ 2.2	14.2
	Glucose; glutamate	31.7	50.6	+ 18.9	14.2

Table 12. *Accumulation of potassium in retina under anaerobic conditions*

(Incubation 60 min. at 40°; 0.02M-glucose, 0.01M-glutamate, 0.001M-adenosinetriphosphate (ATP).)

Exp. no.	Substrate added	K found in tissue (m-equiv./kg.)			$Q_{CO_2}^N$ (μ l./mg. dry wt./hr.)
		Initially	After incubation	Change	
1	None	34.9	12.0	- 22.9	2.4
	Glucose	34.9	28.8	- 6.1	24.9
	Glucose; glutamate	34.9	36.5	+ 1.6	22.3
2	None	32.9	11.2	- 21.7	3.1
	Glucose	32.9	29.7	- 3.2	22.7
	Glucose; glutamate	32.9	35.5	+ 2.6	22.9
3	Glucose	24.4	29.4	+ 5.0	23.6
	Glucose; glutamate	24.4	39.4	+ 15.0	27.6
	Glucose; glutamate; ATP	24.4	32.8	+ 8.4	28.8
4	Glucose	31.7	35.6	+ 3.9	26.8
	Glucose; glutamate	31.7	48.1	+ 16.4	28.1
	Glucose; glutamate	31.7	46.0	+ 14.3	29.1
	Glucose; glutamate; ATP	31.7	43.7	+ 12.0	29.6
	Glucose; glutamate; ATP	31.7	44.6	+ 12.9	32.8

with either glucose or glutamate alone, usually lost about 5–10% of its initial wet weight. Under anaerobic conditions the loss was about 15–30% in the absence of a substrate, and about 5–10% with glucose.

As already stated, the weight increase must have been due either to adherent medium or to swelling, whilst the loss of weight indicates disintegration of the tissue. It appears that glutamate reduces or prevents the disintegration of the tissue during incubation. Retina incubated without glutamate, even in the presence of glucose, appeared opaque and fragile. In the presence of glucose and glutamate the appearance of the tissue was normal.

Balance of ion exchanges between tissue and medium. Changes in the content of inorganic ions of the tissue are bound to cause corresponding changes in the medium. However, as the volume of the latter is much greater than that of the former the concentration changes are much smaller and measurements on the medium are therefore liable to greater experimental error. Further complications arise from the partial disintegration and swelling of the tissue which are more pronounced in brain slices (see Elliott, 1946) than in retina. Since the concentration of potassium in the tissue is much higher than in the medium, contamination of the medium by small amounts of disintegrated tissue causes a greater error than contamination of the tissue by the medium. This should be borne in mind in the interpretation of analytical data on the medium. Full experimental data on the balance of potassium changes in tissues and media are shown in Table 13.

Effect of temperature. Two experiments (Table 14) show that at 20° much smaller amounts of potassium are transported into the tissue in a given time than at 40°. This is presumably due to the lower rate of energy supply at the lower temperature.

DISCUSSION

Factors controlling potassium content of brain and retina. The main result of the experiments described in this paper is the demonstration that two types of substrate, namely, glucose (or lactate or pyruvate) and glutamate (or aspartate) must be present in the medium if the potassium content of brain-cortex slices is to be maintained *in vitro*. Furthermore, aerobic conditions are required. Ox retina behaves essentially in the same way as brain slices, but in the present experiments the state of the two tissues differed at the start of the incubation in one respect. Retina had an abnormally low potassium content owing to loss during transportation of the eye from the abattoir to the laboratory. Guinea pig brain slices, on the other hand, had a normal potassium level at the start of the experiment. The specific effect of aerobic incubation with glucose and glutamate in retina was to restore the physiological level.

Dixon (1949), working with rabbit-brain slices, reported that under anaerobic conditions 'the leakage of potassium was suppressed by glucose and was usually replaced by a small net absorption of potassium'. This may appear to be in disagreement with the results reported in the present paper, but the different findings may be accounted for by differences in technique. In Dixon's (1949) experiments the brain slices were washed in saline saturated with nitrogen containing 5% CO₂ and this probably reduced the potassium content of the tissue very appreciably. Therefore the starting level of potassium in the brain slices was probably low and comparable to that found in retina after cold storage. In retina, glucose had a small effect under anaerobic conditions similar to that reported by Dixon (1949) for brain. It should be pointed out that Dixon examined

Table 13. *Balance of exchanges of potassium between tissues and media*

(Exp. A, guinea pig brain-cortex slices. Exp. B, ox retina, incubated in bicarbonate saline at 40° for 60 min.; 0.02M-glucose, 0.001M-adenosinetriphosphate, all other substrates 0.01M. First portion of each tissue analysed for initial K content. When weight of tissue increased during incubation, the final K concentration was calculated from initial wet weight after correction for K in adherent medium. When weight of tissue decreased, the final K concentration was calculated from final weight, without further correction.)

Exp. tissue	Portion of tissue	Substrates added	Experi- mental conditions	Wt. of tissue (mg.)		K content of tissue (mg.)		K con- tent of ad- herent medium (mg.)	Final K con- tent corrected for ad- herent medium (mg.)	Concentration of K in tissue (mg./100 mg.)		K content of 4 ml. medium (mg.)		Sum of K contents of tissue and medium (mg.)		Re-covery (columns of 8 + 15) (11 + 16) (%)			
				Initial	Final	Initial	Final			Initial	Final	Initial	Final						
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)	(16)	(17)	(18)	(19)	
A																			
1		—	—	98.5	—	—	0.347	—	—	—	0.352	—	—	—	—	—	—	—	—
2	None		Anaerobic	89.0	163.5	+74.5	0.314	0.054	0.021	0.032	0.352	0.036	-0.316	0.804	1.120	1.118	1.152	103	
3	Glucose		Anaerobic	90.5	150.5	+60.0	0.319	0.089	0.017	0.072	0.352	0.079	-0.273	0.804	1.100	1.123	1.172	104	
4	Glucose; L-glutamate		Anaerobic	90.5	162.5	+72.0	0.319	0.063	0.019	0.044	0.352	0.048	-0.304	0.804	1.070	1.123	1.114	99	
5	Glucose; L-glutamate; ATP		Anaerobic	80.0	126.0	+46.0	0.282	0.070	0.012	0.058	0.352	0.072	-0.280	0.804	1.045	1.086	1.103	102	
6	Glucose; L-glutamate		Aerobic	66.0	132.5	+66.5	0.232	0.257	0.013	0.244	0.352	0.370	+0.018	0.804	0.778	1.036	1.022	99	
B																			
1		—	—	82.5	—	—	0.101	—	—	—	0.122	—	—	—	—	—	—	—	—
2	Glucose		Aerobic	63.0	55.0	- 8.0	0.077	0.077	—	0.077	0.122	0.140	+0.018	0.805	0.785	0.882	0.862	98	
3	Glucose; L-lysine		Aerobic	60.0	51.0	- 9.0	0.074	0.061	—	0.061	0.122	0.120	-0.002	0.805	0.814	0.879	0.875	99	
4	Glucose; L-tyrosine		Aerobic	59.5	39.0	-20.5	0.073	0.042	—	0.042	0.122	0.108	-0.014	0.805	0.836	0.878	0.878	100	
5	Glucose; L-leucine		Aerobic	77.5	78.0	+ 0.5	0.095	0.112	—	0.112	0.122	0.144	+0.022	0.805	0.770	0.900	0.882	98	
6	Glucose; L-methionine		Aerobic	70.5	69.0	- 1.5	0.086	0.109	—	0.109	0.122	0.158	+0.036	0.805	0.766	0.891	0.875	98	
7	Glucose; L-glutamate		Aerobic	78.0	87.5	+ 9.5	0.096	0.187	0.002	0.185	0.122	0.237	+0.115	0.805	0.693	0.901	0.878	98	

the changes in the medium only and did not measure the potassium content of the tissue before and after incubation.

There is a difference in the effects of glutamate (in the presence of glucose) in brain and retina under anaerobic conditions. In retina glutamate slightly increases the potassium content whilst in brain it lowers it. This difference is not unexpected as it is known that glutamate inhibits anaerobic glycolysis in brain but not in retina (Weil-Malherbe, 1938).

Table 14. *Effect of temperature on accumulation of potassium in retina*

(Aerobic incubation in bicarbonate saline for 60 min.; 0.02 M-glucose, 0.01 M-L-glutamate.)

Exp. no.	Temp.	K found in tissue (m-equiv./kg.)		
		Initially	After incubation	Change
1	40°	36.6	56.4	+19.8
	40°	36.6	57.7	+21.1
	20°	36.6	42.0	+ 5.4
	20°	36.6	37.2	+ 0.6
2	40°	34.8	61.0	+26.2
	40°	34.8	58.8	+24.0
	20°	34.8	40.3	+ 5.5
	20°	34.8	38.0	+ 3.2

Danowski (1941), Harris (1940, 1941) and Maizels (1948) have shown that red blood cells lose potassium when the energy-giving reactions are prevented by lack of glucose, low temperature or addition of inhibitors. This leakage of potassium from red cells in the absence of energy-giving reactions is very much slower than in brain and retina. It remains to be investigated whether in the case of red cells glutamic acid is required for the transport of potassium. In the experiments quoted the red cells were suspended in plasma, which contains about 5×10^{-4} M-glutamic acid (Krebs *et al.* 1949).

Specificity of glutamic acid. Among many substances tested, L-aspartate was the only one which had an effect comparable to that of L-glutamate. As aspartate can be readily converted into glutamate in brain and in retina, it is possible that aspartate has no effect as such.

The roles of glucose and glutamate in potassium transport. The two types of substances required for potassium transport probably have different functions, glucose being a source of energy and glutamate serving some other specific function. As the transport occurs against the concentration gradient, energy is required and it is therefore understandable that experimental conditions which reduce the supply of energy, e.g. the presence of respiratory inhibitors, low temperature and anaerobic conditions, also reduce the rate of potassium transport. If the energy requirement were the only

need which the substrates of the medium have to meet, the presence of carbohydrate in the medium should be sufficient. As glutamate is also required it may be argued that it is used for another purpose. It is remarkable that glutamate maintains the full rate of respiration in retina under the experimental conditions, and yet is not capable of maintaining the full rate of potassium transport if present as the only substrate. No attempt is made to explain this.

The fact that glutamine, in contrast to glutamate, does not promote potassium transport, suggests that the ionization of the γ -carboxyl group of glutamate is essential for potassium transport. Since the movements of glutamate and potassium are parallel and approximately equivalent, it may be assumed as a working hypothesis that potassium is the cationic equivalent of the glutamate anion. However, more knowledge of the behaviour of other ions is required before a detailed hypothesis can be considered.

Effect of glutamate and potassium on tissue metabolism. Ashford & Dixon (1935) found, and Dickens & Greville (1935) confirmed, that addition of 0.1 M-potassium chloride to an isotonic saline medium increased respiration and aerobic glycolysis of brain tissue. Effects of the same kind and magnitude are obtained on addition of L-glutamate (Weil-Malherbe, 1938). The effects produced by potassium and glutamate have presumably a common root: both substances raise the potassium concentration in the tissue slice.

SUMMARY

1. Slices of guinea pig and rabbit brain cortex when incubated in a saline medium lost potassium ions by diffusion into the medium. To prevent this loss two substrates, glucose and L-glutamate, had to be added together to the medium, and conditions had to be aerobic. Neither glucose nor glutamate, when present alone, prevented the loss.

2. The retinæ of ox eyes, stored for 1–2 hr. at 0°, lost about one-half of their potassium content and gained an equivalent amount of sodium. On incubation *in vitro* the *in vivo* levels of potassium and sodium were approximately restored if both glutamate and glucose were present in the medium. Glucose could be replaced by lactate or pyruvate, and L-glutamate by L-aspartate. Added alone, glucose and glutamate caused only small increases in the potassium content of the tissue.

3. L-Aspartate was converted into L-glutamate in brain and retina; it is probable that aspartate as such has no effect on potassium transport.

4. Glutamate and potassium were taken up by retina from the medium in approximately equivalent amounts. The possible significance of this equivalence is discussed.

5. Anaerobically glucose and glutamate had no major effects in brain slices. In retina, partly

depleted of its normal potassium content, small increases occurred on incubation with the two substrates.

6. It is suggested that glucose (or lactate or pyruvate) is required as a source of energy, whilst

L-glutamate serves another function in potassium transport.

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Studies on Suramin

8. THE ACTION OF THE DRUG ON ENZYMES AND SOME OTHER PROTEINS. GENERAL CONSIDERATIONS

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Suramin has a very powerful trypanocidal action *in vivo*, and furthermore, it has a prolonged prophylactic action; e.g. one injection of 1 or 2 g. may protect an individual against sleeping sickness for several weeks or even months. This long protection can almost certainly be accounted for by the long retention of the drug in the blood plasma and tissues, as a result of its firm combination with the body proteins (see Bournsnel & Wormall, 1939; Dewey & Wormall, 1946). The precise mode of action of suramin on trypanosomes or on the body defence mechanisms is, however, not known.

We have studied the action of suramin on proteins as a preliminary to an investigation of the direct action of the drug on trypanosomes and more particularly on the enzymes of the trypanosome. Some evidence is already available that suramin inhibits certain enzymes. Relatively high concentrations ($M/140$) inhibit trypsin (Beilinson, 1929), $M/6000$ suramin is toxic to fumarase but not to urease (Quastel, 1931), and it strongly inhibits hyaluronidase (Beiler & Martin, 1948). According to Stuber & Lang (1926) it retards blood glucolysis, but no evidence of this action was found by Teherniakofsky & Nattan-Larrier (1937). In spite of its

capacity to combine firmly with all the proteins we have tested, suramin is not a general enzyme poison.

This and the following paper describe investigations made with the following objects: (i) A study of the action of suramin on some enzymes taking part in carbohydrate metabolism. (ii) A qualitative and quantitative study of the combination of the drug with proteins and some enzymes, to determine as far as possible the nature of the protein groups involved and the number of molecules of the drug required to inactivate suramin-sensitive enzymes. (iii) Investigations on the relationship between concentrations required to inhibit some enzymes and the amounts of the drug present in the blood of injected animals. Preliminary accounts of a few of these experiments have been briefly recorded (Town, Wills & Wormall, 1949*a*, *b*; Town & Wormall, 1949; Wills & Wormall, 1949).

EXPERIMENTAL

Suramin determinations. These were made as described previously (Dangerfield, Gaunt & Wormall, 1938; Bournsnel, Dangerfield & Wormall, 1939). Where the amount of drug present was too small to permit the use of the Tintometer disks, e.g. in some of the enzyme studies, standards were made from acid-hydrolysed suramin suitably diluted with