SUMMARY

1. A colorimetric method is described for the quantitative estimation of thymine which is suitable for quantities between 50 and 200 $\mu\text{g.}$ in 2 ml. of solution.

2. The application of this method to the estimation of thymine in 50 mg. samples of deoxyribose-nucleic acid is reported.

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Anaerobic Leakage of Potassium from Brain

By K. C. DIXON, *Department of Pathology, University of Cambridge*

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Interruption of blood supply to tissues and organs is one of the main causes of organic disease. Characteristic histological changes follow such vascular occlusion, and it seems likely that these have their origin in chemical and metabolic disturbances produced by deprivation of oxygen and food substances from the tissue cells. The present communication describes a study of some of the chemical events taking place when the supply of oxygen and glucose to the cells of the brain is interrupted. These two deficiencies are probably the immediate determinants in the dislocation of cerebral function following vascular accidents, and thus may be regarded as the primary factors in ischaemic damage.

The intracellular concentration of potassium is many times greater than that outside the cells. Danowski (1941) showed that this intracellular potassium diffuses out of red blood cells when the blood sugar is used up, or when glycolysis is inhibited by fluoride. Harris (1941) reported similar results. Dickens & Greville (1933) found that brief anaerobic incubation of slices of cerebral cortex, without substrate, causes inhibition of glycolysis, and Macfarlane & Weil-Malherbe (1941) ascertained that pyrophosphate is destroyed at the same time. Colfer & Essex (1947) observed a loss of potassium from brain during convulsions. Potassium ions are known to cause intense aerobic stimulation and anaerobic inhibition of the glycolytic metabolism of cerebral cortex (Ashford & Dixon, 1935; Dickens & Greville, 1935). For these reasons the present work was designed to find out if leakage of potassium also

occurs from brain when deprived of oxygen and glucose. In this tissue a rise in the external concentration of potassium might produce profound metabolic changes in neighbouring areas outside the initial region of ischaemia as well as affecting radically the internal milieu of the cells primarily affected.

METHODS

Slices of rabbit cerebral cortex were suspended in bicarbonate-Ringer solution without glucose made up according to Dickens & Greville (1935) and previously saturated with N_2 containing 5% CO_2 . After washing in this solution, the slices were transferred to Warburg manometer cups containing 1.5 ml. of fluid. In general, half the slices were incubated in bicarbonate-Ringer without substrate, and the remaining slices in the same volume of the same salt solution containing in addition 0.2% glucose. In some experiments half the slices were incubated in this glucose-Ringer, and the remainder in the same glucose-Ringer containing 0.05% NaF. In a few experiments three samples of the slices were used, one without substrate, one with glucose and one with glucose and fluoride. The manometers were filled at room temperature with N_2 containing 5% CO_2 the gas being passed through for 3–3.5 min.

Potassium estimations were made as follows. Practically the whole of the 1.5 ml. of fluid remaining in the manometer cups after incubation at 38° was pipetted away from the slices into centrifuge tubes using Pasteur pipettes. This fluid was then centrifuged at high speed for 15 min. and 1 ml. of the clear supernatant fluid was pipetted off into Pyrex crucibles and evaporated to dryness in an oven at 100–120°. Conc. HNO_3 (0.5 ml.) was then added to each, and again the crucibles were heated to dryness. The crucibles were then incinerated for 3 hr. in an electric muffle furnace at 400–450°, to destroy ammonium salts.

The colourless residues were extracted twice with 0.5 ml. portions of 0.1N-HCl and twice with 0.5 ml. portions of distilled water. During extraction the crucibles were briefly heated, each time on a hot plate, till steaming commenced. The four washings from each crucible were transferred, using Pasteur pipettes, to numbered graduated centrifuge tubes and each made up to 2 ml. (a little fluid is lost by evaporation during the heating). Sodium cobaltinitrite solution (1 ml.; see King, 1946) was added to each tube, and after standing for 45 min. the precipitated potassium cobaltinitrite was separated by centrifugation. After draining and washing twice with 70% ethanol, the cobalt in the precipitate was estimated by the method of Jacobs & Hoffman (1931) using the colour reaction with choline chloride and $\text{Na}_4\text{Fe}(\text{CN})_6$. The volume at the final stage was made up to 9 ml., and the colours were compared with similarly treated standard K and standard Co solutions in an Evans photoelectric colorimeter, as designed by King (1946), using an Ilford tricolor red filter as suggested by this author. A sample of the Ringer solution used in the experiment was included in each batch of estimations as a check on recovery following incineration. The recovery of K from this bicarbonate saline was always at least 90 and often 100% (see Table 1). Equivalent amounts of KCl alone cannot be estimated in this way as recovery is extremely poor, presumably owing to unshielded K salts attacking the Pyrex crucibles above 400°. In the ashed bicarbonate-Ringer, on the other hand, only a minute fraction of the contained K salts probably comes into sufficiently intimate contact with the Pyrex to cause appreciable loss. It will be evident from the figures given that the recovery of K contained in the incinerated bicarbonate saline is practically complete using the method described above.

Anaerobic glycolysis (Q_L^{N}) was measured manometrically. Readings were taken every 10 min. and the values of Q_L^{N} were calculated over the first 0.5 hr.

Considerable uniformity in the values of anaerobic glycolysis was shown with different specimens of rabbit-brain cortex. Furthermore, glycolysis was fairly constant throughout incubation, only falling by 14% (average of 17 experiments) over 40 min. This constancy contrasts with observations of previous workers using rat brain. For the sake of brevity protocols have had to be omitted.

RESULTS

Table 1 summarizes the results of estimations of potassium in Ringer solution before and after incubation with brain slices for 1 hr. The mean dry weights of tissue used are also shown. Considerable increase in the potassium concentration of the fluid surrounding the tissue was observed, when the medium was devoid of glucose, or contained fluoride as well as glucose. With glucose alone, on the other hand, this increase was eliminated and was generally replaced by slight diminution in the potassium concentration.

A clearer picture of these results is given in Table 2 by evaluating the amounts of potassium liberated or absorbed per unit dry weight of tissue. Mean values for anaerobic glycolysis are also shown. The amount of potassium liberated or absorbed is expressed in $\mu\text{l.}$, on the basis of 1 $\mu\text{equiv. K} = 11.2 \mu\text{l.}$ The number of $\mu\text{l.}$ of potassium liberated or absorbed/mg. dry wt. of tissue/hr. gives values of convenient order. A positive value signifies liberation of potassium and a negative value a corresponding absorption. Owing to stringency of space individual values have had to be omitted. In every case except one the results obtained refer to parallel experiments under at least two of the conditions studied with the tissue of each of the rabbits used.

From Table 2 we see that, in the absence of glucose, potassium was in every case liberated into the fluid surrounding the brain tissue, at an average rate of 2.2 $\mu\text{l.}/\text{mg.}/\text{hr.}$ The leakage of potassium was suppressed by glucose and was usually replaced by a small net absorption of potassium (0.4 $\mu\text{l.}/\text{mg.}/\text{hr.}$). In the presence of glucose the anaerobic glycolysis was high ($Q_L^{\text{N}} = 28 \pm 3$), but in the absence of glucose very small ($Q_L^{\text{N}} = 2 \pm 1$). It appears that when glycolysis can take place the amount of

Table 1. *Potassium leakage from brain tissue*

(Showing K concentration in Ringer before and after anaerobic incubation for 1 hr. at 38° with slices of rabbit-brain cortex. The slices were suspended in 1.5 ml. of fluid. Observations on the tissues of 22 rabbits are included.)

		Initial Ringer solution	Initial Ringer after incineration	Ringer after incubation with brain slices in the presence of		
				Glucose 0.2%	No substrate	Glucose 0.2% NaF 0.05%
K concentration m.equiv./l.	Mean	2.45	2.37	2.08	3.86	3.62
	Range	2.3-2.6	2.1-2.6	1.7-2.6	3.1-4.9	3.1-3.9
	S.D.	0.11	0.14	0.26	0.48	0.23
	S.E. of mean	0.03	0.03	0.06	0.12	0.07
Dry weight of tissue used (mg.)	Mean	—	—	13.48	11.43	11.22
	Range	—	—	8.1-20.2	7.7-17.3	8.1-16.3
	S.D.	—	—	3.51	2.99	3.15
	—	13	16	—	—	—
No. of determinations on initial solutions	—	13	16	—	—	—
No. of rabbits from which tissue was used	—	—	—	21	16	10

Table 2. Potassium leakage from brain in terms of dry weight

(K liberation into the fluid bathing the brain slices during incubation for 1 hr. at 38° is expressed as $\mu\text{l. of K/mg. dry wt. of tissue}$. Positive values indicate liberation of K, negative values indicate absorption. $1\mu\text{equiv. of K}=11.2\mu\text{l.}$ The anaerobic glycolysis is also shown. The figures are from the same series of experiments on the tissues of 22 rabbits referred to in Table 1.)

	$\mu\text{l. K/mg. dry wt./hr.}$			Q_L^N		
	Glucose 0.2%	No substrate	Glucose 0.2%, NaF 0.05%	Glucose 0.2%	No substrate	Glucose 0.2%, NaF 0.05%
Mean	-0.41	+2.24	+1.89	27.7	1.6	1.9
Range	-1.0 to +0.4	+1.7 to +3.2	+1.2 to +2.5	23-32	0-4	1-3
S.D.	0.36	0.38	0.42	2.6	1.0	0.9
S.E. of mean	0.08	0.10	0.13	0.6	0.3	0.3
No. of rabbits	21	16	10	22	15	10

potassium leaving the cells was more than compensated by increased entry, so that there was usually no net loss of potassium from the cells into the surrounding fluid, and often there was an uptake from the fluid by the cells. In the absence of glycolyzable substrate, however, there was always a highly significant loss of potassium from the cells into the surrounding fluid.

Table 2 also shows that glycolysis was practically eliminated by fluoride ($Q_L^N=2\pm 1$). Danowski (1941) found that the addition of fluoride produced leakage of potassium from human red cells even when blood sugar was present. The results of Table 2 amply confirm this for rabbit brain. When glycolysis was suppressed by fluoride, potassium leaked out into the surrounding fluid just as in the absence of glucose at the rate of 1.89 $\mu\text{l./mg. dry wt./hr.}$ In each parallel experiment using glucose alone this leakage was prevented and was often replaced by the actual removal of potassium from the fluid. The suppression of potassium leakage was not due to the mere static presence of glucose, but to its dynamic utilization in supplying energy to the glycolyzing cell, since when glycolysis was inhibited leakage again became evident.

In the absence of glycolyzable substrate appreciable potassium leakage occurred even in half an hour. This is apparent from Table 3 which records observations with brain tissue of two rabbits incubated for only half an hour; in these two experiments about 1.0 $\mu\text{l. K/mg. dry weight}$ was liberated in half an hour. Approximately twice this amount (average 2.2 $\mu\text{l.}$) leaked out in 1 hr. (Table 2). In Table 3 the effect of glycolyzable substrate in curbing leakage and promoting uptake is also evident.

Table 3. Potassium accumulation/mg. dry weight in fluid incubated with brain slices for 0.5 hr.

Rabbit no.	$\mu\text{l. K liberated/mg. dry wt./0.5 hr.}$		
	Glucose	No substrate	Glucose-fluoride
10	-1.0	+1.0	+1.1
11	-1.0	+1.0	—

Some potassium leakage may even occur while setting up the experiments at room temperature. Thus in three cases Ringer solution without substrate or with glucose-fluoride was treated with brain slices, and equilibrated with nitrogen/carbon dioxide under the same conditions as in the actual experiments up to the point of the start of incubation. Then, instead of being placed in the bath at 38°, the fluid was removed from the tissue slices for analysis without incubation. In two cases without substrate the potassium concentration of the fluid had risen so that 0.9 and 0.6 $\mu\text{l. of potassium}$ were liberated per mg. dry weight. In the third case with glucose-fluoride, however, no increase was detected. After incubation for 1 hr., tissue from these three rabbits liberated 2.0-2.2 $\mu\text{l. of K.}$

When both glucose and oxygen were supplied to the cells (just as with glucose alone) no net leakage of potassium occurred, and the uptake of potassium from the fluid by the tissue is apparently even more definite than with glucose alone. Thus with tissue from two of the rabbits parallel experiments were set up with glucose and oxygen/carbon dioxide as well as the anaerobic experiments recorded in Table 2. In one case with glucose alone the rate of disappearance of potassium was -0.6 $\mu\text{l./mg. dry wt./hr.}$, but with glucose and oxygen this was -1.0 $\mu\text{l./mg. dry wt./hr.}$, while in the absence of both glucose and oxygen, there was a net accumulation of potassium at the rate of +1.7 $\mu\text{l./mg. dry wt./hr.}$ Similarly, in the other case, with glucose alone, the disappearance of potassium was -0.6 $\mu\text{l./mg. dry wt./hr.}$, but with glucose and oxygen this was -1.2 $\mu\text{l./mg. dry wt./hr.}$, whereas the anaerobic accumulation with glucose and fluoride was +2.1 $\mu\text{l./mg. dry wt./hr.}$ It thus appears that glucose and oxygen checked the leakage and promoted uptake of potassium even more effectively than glucose alone.

How far potassium leakage is accompanied by tissue disintegration is uncertain. Lutwak-Mann (1947) found tissue disintegration of gastric mucosa to be promoted by anaerobic substrate deprivation. In the present experiments with brain also more

debris was usually formed in the absence of substrate than when glucose was present. In one experiment an exceptionally large amount of debris was formed without substrate; this was much more marked than with glucose and even more marked than with glucose-fluoride. However, the leakage of potassium was no greater without substrate than with glucose-fluoride. This indicates that potassium leakage is probably not mainly occasioned by mere tissue disintegration, but the two phenomena may well progress together. In the later experiments of this series the spun debris was included in the dry weight determinations.

DISCUSSION

We may conclude from the results recorded in Tables 1-3 that when both the supply of glucose and oxygen to the brain cells is cut off there is considerable leakage of potassium into the extracellular fluid. This loss of potassium may be one of the basic factors responsible for ischaemic injury to cells, as potassium is doubtless an essential intracellular constituent on whose presence in high concentration many enzymic processes may depend. Furthermore, in the case of brain, it is possible that the loss of potassium from damaged cells may have other consequences since in brain potassium ions have such remarkable effects on metabolism. The irritation and paralysis associated with vascular

accidents may thus depend in part on these metabolic effects produced by potassium ions which have emerged from the primary focus of ischaemic damage out into adjacent regions, as well as on the escape of essential intracellular constituents (such as potassium) out of the initially injured tissue.

SUMMARY

1. During anaerobic metabolism in the presence of glucose there was no increase in the concentration of potassium in the fluid bathing slices of cerebral cortex. In some cases there was a decrease.

2. Under the same conditions, but in the absence of glucose, there was always a significant rise in the potassium concentration of the fluid surrounding the brain slices.

3. The action of glucose in preventing this loss of potassium is dynamic: it depends on the active utilization of glucose by the glycolyzing cell. Where glycolysis was inhibited by fluoride potassium leaked into the environmental fluid just as in the absence of glucose. Brain thus resembles red blood cells for which Danowski (1941) has described a similar effect.

4. The significance of these findings on the nature of ischaemic damage is briefly considered.

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The Activation of Phosphoglucomutase by Metal Ions

By L. H. STICKLAND

Department of Experimental Pathology and Cancer Research, University of Leeds

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The existence of glucose-1-phosphate and its conversion to glucose-6-phosphate in muscle were first described by Cori & Cori (1936). Kendal & Stickland (1938) claimed that Mg^{++} and fructofuranose-1:6-diphosphate were both essential for this conversion, but Cori, Colowick & Cori (1938) stated that 'the contention of Kendal & Stickland that hexosediphosphate acts as a coenzyme and is essential for the reaction is not substantiated by our findings'. They found, on the other hand, that

Mn^{++} and Co^{++} are more efficient activators than Mg^{++} , and that the enzyme (named by them phosphoglucomutase) shows a large residual activity in the absence of any added metal. This has led to the view, expressed typically by Sumner & Somers (1947), that 'phosphoglucomutase requires no coenzyme'.

The present paper deals with some of the effects of metallic ions on the activity of phosphoglucomutase. In a later publication the author hopes to