The cornea also differs from lens in the severity of its radiation damage in that low doses of radiation do not produce any obvious long-term changes in the cornea. In lens, on the other hand, epithelial cells migrate to the posterior part of the lens and form abnormal fibres which are the precursors of the lens opacity.

SUMMARY

1. Whole lens and cornea of rabbits exposed to X-irradiation (1000 r.) were assayed 1, 4 and 14 days afterward for hexokinase and glutathione reductase activity. Corneal epithelium was assayed for hexokinase, glutathione reductase and isocitric dehydrogenase 4 and 14 days after X-irradiation. In spite of morphological changes under these conditions of irradiation, there was no alteration in enzyme content.

2. A sensitive method for the assay of glutathione reductase in tissue is presented. Its activity in tissues of the rat, rabbit and calf is compared.

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Cerebral Metabolism in the Sheep

1. NORMAL SHEEP

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The work reported in this paper was carried out to provide data for comparison with the results obtained with the brains from abnormal sheep, and to see if any difference could be found between the metabolism *in vitro* of sheep and other animals which could account for the greater resistance of the sheep to hypoglycaemia (Reid, 1951; McClymont & Setchell, 1956a).

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EXPERIMENTAL

Preparation of reagents

Sodium pyruvate was prepared by the method of Robertson (1942). Cytochrome *c* was prepared by the method of Keilin & Hartree (1945), and the concentration of the final solution determined spectroscopically by the method of Potter (1949).

The yeast-hexokinase preparation used was stage 3a of the method of Berger, Slein, Colowick & Cori (1946).

All other reagents were commercial products and, when available, of A.R. grade.

Buffers

pH measurements were made with a Pye Universal pH meter standardized at pH 4.0, 7.0 and 9.2 with the buffer tablets supplied.

Phosphate buffer. Na_2HPO_4 (104 or 400 mM) was adjusted to pH 7.4 with HCl.

Tris buffer. 2-Amino-2-hydroxymethylpropane-1:3-diol (500 mM; tris) was adjusted to pH 7.4 with HCl.

Glycylglycine buffer. Glycylglycine (250 mM) was adjusted to pH 7.4 with NaOH.

Media

The pH values of all media were checked before use and adjusted to 7.4 where necessary with HCl or NaOH.

Phosphate saline. This contained 134 mM-NaCl, 5.4 mM-KCl, 10.4 mM-phosphate buffer, 1.3 mM- KH_2PO_4 , 1.3 mM- MgSO_4 , 1.3 mM- CaCl_2 and, where present, 10 mM-glucose.

Potassium-rich medium for the study of pyruvate metabolism. This contained 76 mM-KCl, 1.3 mM- MgCl_2 , 1 mM-adenosine triphosphate (ATP), 40 mM-phosphate buffer and, where present, 3.3 mM-sodium pyruvate and 3.3 mM-sodium fumarate.

Sodium-rich medium. This contained 134 mM-NaCl, 5 mM-KCl, 1.3 mM- MgSO_4 , 10 mM-phosphate buffer, 1.3 mM- KH_2PO_4 , 1 mM-ATP and, where present, 3.3 mM-sodium pyruvate and 3.3 mM-sodium fumarate.

Tris saline and glycylglycine saline. These were the same as phosphate saline but with 50 mM-tris buffer or 25 mM-glycylglycine buffer respectively replacing the phosphate buffer.

Bicarbonate saline media. Bicarbonate saline (Krebs & Henseleit, 1932) was equilibrated with $\text{O}_2 + \text{CO}_2$ (95:5), and used without additions as 'no-substrate medium', with the addition of 20 mM-glucose as 'glucose medium' or with 10 mM-sodium glutamate and 20 mM-glucose as 'glucose + glutamate medium'.

Mitochondrial incubation medium. This contained 12 mM-sodium glutamate, 16 mM- Na_2HPO_4 , 1.2 mM-adenylic acid, 8 mM- MgCl_2 , 24 mM-KCl, 0.012 mM-cytochrome c, 24 mM-glucose and 40 mM-tris buffer.

Oxidative phosphorylation medium. This contained, in addition to the constituents of 'mitochondrial incubation medium', 10% of yeast-hexokinase preparation and 16 mM-potassium fluoride.

Analytical methods

Glucose. Glucose was determined by Nelson's (1944) method, the absorption being read at the maximum of 660 m μ , not at 520 m μ as was originally used.

Glycogen. Glycogen was estimated in brain slices by a micromodification of the method of Kerr (1936) similar to that used by LeBaron (1955). The brain slices (100–200 mg. fresh wt.) were digested in 0.5 ml. of aqueous 30% KOH soln. for 30 min. at 100° and then 1 ml. of ethanol was added to precipitate the glycogen. This was found to give more consistent recoveries than digestion with ethanolic KOH soln. The glycogen was sedimented by centrifuging for 30 min. at 3000 g after cooling. The precipitate was washed three times in the methanol- CHCl_3 mixture used by Kerr and the glycogen hydrolysed by treatment with $\text{N-H}_2\text{SO}_4$ (1 ml.) for 2 hr. in a boiling-water bath. The acid was neutralized with m-NaHCO_3 to pH 5–9, with wide-range indicator paper (British Drug Houses Ltd.) used

externally. This was more convenient than neutralization with NaOH. Solid NaHCO_3 added in slight excess gave erratic results. The reducing sugar in the resulting solution was estimated by the method of Nelson (1944).

Lactate. Lactate was determined by the method of Barker & Summerson (1941) as modified by Speck, Moulder & Evans (1946).

Pyruvate and α -oxoglutarate. Pyruvate and α -oxoglutarate were determined by the method of Friedmann & Haugen (1943), ethyl acetate being used to extract the 2:4-dinitrophenylhydrazones. The absorption was read at 390 and 520 m μ and the proportion of the two acids determined from the ratio of these two absorptions.

Citrate. Citrate was determined by the modification by McArdle (1955) of the method of Weil-Malherbe & Bone (1949).

Acetate. Acetate was determined in incubation media as the acid, which was steam-volatile in a Markham still from a mixture of saline and potassium oxalate-oxalic acid buffer, pH 4.0. This method is based on the method of Scarisbrick (1952) for volatile fatty acids in blood, without the paraffin and the *sec*-octyl alcohol. At this pH, lactate and pyruvate are not steam-volatile.

Phosphorus. Acid-soluble phosphorus was extracted from brain slices (about 100 mg. fresh wt.) by grinding the slices in 10% trichloroacetic acid (5 ml.). The mixture was centrifuged at 3000 g for 10 min. at 0–5°, sufficient under these conditions to yield a clear supernatant. Second extraction and centrifuging yielded a supernatant containing less than 5% of the phosphorus in the first extract. Centrifuging at 6000 and 20 000 g gave similar results. Heald (1956) found that centrifuging at 3000 g was insufficient to sediment all the phospholipids. Considerable amounts of phospholipids were also extracted by the second lot of trichloroacetic acid. Possibly under the conditions of the present work a coarser homogenate was produced, which was more readily sedimented, than that used by Heald.

The phosphorus in the supernatant was partitioned by the barium acetate-ethanol fractionation of LePage (1949). All manipulations were carried out either in the cold room or in tubes immersed in crushed ice. The final precipitates were dissolved in 0.1 N-HCl and phosphorus was estimated by the method of Berenblum & Chain (1938) modified by Long (1943), or latterly by the modification of Weil-Malherbe & Green (1951). The two modifications gave similar results.

Phosphorus in media, as in the oxidative phosphorylation studies, was determined by the vanadomolybdate method of Simonsen, Wertman, Westover & Mehl (1946).

Water. Water content of slices was calculated as the difference between the wet weight and the weight after drying for at least 24 hr. in a hot-air oven at 100°.

Sodium and potassium. Sodium and potassium were extracted from dried slices (10–20 mg. dry wt.) by incubation in stoppered glass tubes at room temperature for at least 48 hr. in 10 ml. of 0.1 N- HNO_3 (Whittam, 1955). The acid used was stored in polythene containers. To correct for any sodium or potassium extracted from the glass of the tubes, a blank of 10 ml. was incubated in a similar manner. Sodium and potassium were then determined, in a 1 in 10 dilution of the extract in ion-free water (also kept in a polythene container), with an EEL flame photometer. The intensity of the colour produced was compared with that

produced by a blank of ion-free water and by a standard containing 0.1 μ equiv. of both sodium and potassium. No interference was found, over the range of concentrations found in tissue slices, between sodium and potassium.

Chloride. Chloride was determined in a 1 ml. sample of the 0.1N-HNO₃ extract used for sodium and potassium determinations, by a potentiometric titration with silver and silver-amalgam electrodes (Cunningham, Kirk & Brooks, 1941; Seligson, McCormick & Sleeman, cited by Hawk, Oser & Summerson, 1954). The potential developed between the electrodes was measured after the addition of each 0.05 ml. of 0.001N-AgNO₃ soln. The end point of the titration was taken as the point of inflexion of the resultant sigmoid curve.

Preparation of tissues

The sheep used were mainly animals being slaughtered at an abattoir and had been fasted overnight. The heads were removed within a few minutes of death by stunning, cutting the throat and dislocating the neck. The top of the head was skinned and the top of the skull chopped away with a hatchet. With a little practice, this could be done in about a minute without tearing the dura. The brain was lifted out with the index and second fingers after breaking down its connexions with the medulla and cranial nerves.

Pieces of brain from which mitochondria were to be prepared were cut and immersed as quickly as possible in ice-cold 0.25M-sucrose soln., from which they were removed for weighing and homogenizing. The rest of the brain was cut into slices about 1 cm. thick and placed in small glass pots, which were then packed in crushed ice in a Thermos flask for transport to the laboratory. From death to final immersion of the pots in ice took no more than 10 min.

Guinea-pig and rabbit brains, where used for comparison, were similarly treated.

Chopped brain was prepared on a hand-operated modification (Setchell, 1957) of the chopper described by McIlwain & Buddle (1953).

Slices were cut by the method of Deutsch (1936) with a recessed microscope slide and a blade moistened with saline. When observations were made on ion movements, the slide and blade were used dry and the slices handled with fine forceps instead of being floated in a dish of saline. Slices cut in this way are referred to as dry-cut slices.

The homogenates used for the measurements of hexokinase activity were prepared with a mortar and pestle according to Long (1952).

Homogenates (20 and 10%, w/v) for the preparation of mitochondria and other purposes were made with an all-glass Potter-Elvehjem type of homogenizer, 0.25M-sucrose made with boiled ion-free water and neutralized with tris (250 mM) (Werkheiser & Bartley, 1957) being used.

Mitochondria were prepared from 10% (w/v) homogenates by the method of Schneider (1948) and resuspended in neutralized 0.25M-sucrose so that 0.5 ml. of the suspension was equivalent to approximately 500 mg. of fresh brain.

Incubation procedures

Oxygen uptake. Measurements were made with Warburg manometers and flasks, in oxygen as the atmosphere for slices and in air for chopped tissue, homogenates and mito-

chondria. The oxygen uptake was determined by calculating the line of best fit by the method of least squares (Mather, 1943).

Respiratory quotients. Determinations were made by the first method of Dickens & Šimer (1930), with slices or chopped tissue (about 50 mg. in 2 ml. of phosphate saline in the special flasks at 39°).

Metabolism of pyruvate. Studies were carried out with 200 mg. of chopped brain in 3 ml. of potassium-rich medium or sodium-rich medium in an atmosphere of air. Usually two flasks contained the complete system, two contained no fumarate and two neither pyruvate nor fumarate. Equilibration was for 5 min. and incubation for 30 min., during which time the oxygen uptake was measured. The reaction was stopped by adding 0.3 ml. of 80% trichloroacetic acid from the side arm and the contents of the flask were centrifuged. Pyruvate, α -oxoglutarate, citrate, lactate and acetate were estimated in the supernatant.

Phosphocreatine resynthesis. When the tissue was used for the study of phosphocreatine resynthesis, slices (100–150 mg. fresh wt.) were incubated for 20 min. at 39° in 3 ml. of glycylglycine saline or tris saline with 10 mM-glucose in an atmosphere of oxygen.

Glycogen resynthesis. For the study of glycogen resynthesis, slices (100–200 mg. fresh wt.) were incubated for 4 hr. in 3 ml. of phosphate saline at 39°, in an atmosphere of oxygen.

Ion and water movements. For studying the movements of inorganic ions and water, slices (100–150 mg. fresh wt.) were incubated for 1 hr. in 3 ml. of bicarbonate saline medium in O₂ + CO₂ (95:5). At the end of the incubation, slices were freed of adherent medium by blotting with hardened filter paper (Whatman no. 50) and transferred to tared tubes for weighing, drying and extraction as described above.

Hexokinase activity. Hexokinase activity was determined by the method of Long (1952).

Cytochrome-cytochrome oxidase (indophenol oxidase) activity. The activity of this system was determined by the method of Vernon (1911) with incubation for 20 min. at 28°. After the addition of ethanol and after centrifuging, the absorption at 550 m μ of the supernatant was measured and compared with that of a similar mixture of *p*-phenylenediamine and α -naphthol in which the formation of indophenol blue had been allowed to proceed to completion spontaneously.

Mitochondrial respiration. Mitochondrial suspension (0.5 ml.) in neutralized 0.25M-sucrose was added to 2.5 ml. of mitochondrial incubation medium. Incubation was at 39° in an atmosphere of air.

Oxidative phosphorylation. Mitochondrial suspension (0.5 ml.) or 1 ml. of 20% homogenate in neutralized 0.25M-sucrose was added to 2.5 ml. of oxidative phosphorylation medium. Incubation was at 39° in an atmosphere of air, usually for 15 min., when the reaction was stopped by the addition of 0.3 ml. of 80% trichloroacetic acid from the side arm. The contents of the flask were centrifuged and inorganic phosphate was estimated in the supernatant.

Electrical stimulation of slices

The effect of electrical stimulation on the respiration of slices was studied in flasks, described by McIlwain (1951) as type A, with tungsten wires sealed through the glass.

The slices were mounted in Perspex-silver wire grids, type H, described by Ayres & McIlwain (1953). Stimulation was with 50 cye./sec. a.c. from a transformer fed from the mains. Total current (r.m.s.) was observed continuously, and the current to the individual flasks usually at intervals of 5 min., with a rectifying moving-coil meter. One of the leads to each flask was fitted with a plug and socket which could be disconnected and plugged into the leads of the meter. Voltage was checked at the transformer at the beginning and end of each experiment.

RESULTS

Oxygen uptake and changes of the concentrations of substrate and products by sheep and other brains in the presence of various substrates

No substrate. As had been found earlier (Quastel & Wheatley, 1932), there is quite a considerable uptake of oxygen by sheep brain in the absence of substrate, but this gradually decreases with time. As a percentage of the initial rate, the decrease with time is much less than with guinea-pig brain. It is not certain whether this indicates the availability of more endogenous substrate in the sheep or whether it is simply a reflexion of the slower initial rate. The total oxygen uptake over the whole period is usually greater per gram of guinea-pig brain (Table 1).

The production of lactate by sheep brain is negligible in the absence of substrate.

Glucose. Sheep-brain slices took up glucose from saline *in vitro* and the oxygen uptake and lactate production were greater in the presence of glucose than with no substrate. The average oxygen uptake of slices of normal sheep brain in phosphate saline containing 10 mM-glucose was 46 μ moles/g. wet wt./hr. (range 36–61; 17 observations), and the production of lactate over a 90-min. period was 21 μ moles/g./hr. (range 15–26; six observations). Glucose disappeared from the medium at the rate of 26 μ moles/g./hr. (range 21–28) (six observations) measured at the end of a 90 min. period. Small amounts of keto acids, presumably pyruvate and α -oxoglutarate (1–2 μ moles/g./hr.), appeared in the

medium during incubation of brain slices in glucose-containing media.

The figure for oxygen uptake is 0.75 of that for guinea-pig brain under similar conditions (63 μ moles/g./hr.; range 58–70, 13 observations). A similar ratio for the two species was found by Krebs (1950) but the absolute rates he found for both were higher, possibly due to the inclusion in the medium he used of pyruvate, fumarate and glutamate as well as glucose.

Pyruvate. Most of the earlier work on pyruvate metabolism was done with dispersions of pigeon brain prepared in a mortar and pestle. However, the respiration of dispersions prepared in this way from sheep brain was very unreliable [Banga, Ochoa & Peters (1939) found that rat-brain dispersions were similarly unreliable], and as a method was wanted for application to individual abnormal brains, chopped brain was used in a potassium-rich medium (for details see Experimental). With this preparation, the oxygen uptake was greater in the presence of pyruvate than with no substrate, pyruvate was taken up from the medium and citrate and α -oxoglutarate appeared, all at reproducible rates. In this medium, the absence of fumarate, ATP or inorganic phosphate decreased the oxygen uptake and the pyruvate uptake; the absence of fumarate or ATP decreased the formation of α -oxoglutarate and citrate, but the absence of magnesium had no effect. In sodium-rich medium, the absence of fumarate had much less definite effects (Tables 2 and 3).

The respiration of chopped brain was greater in the potassium-rich medium than in the sodium-rich medium, although both were approximately isosmotic with plasma. This is contrary to the findings of Dickens & Greville (1935), who found an increase in respiration only if high concentrations of potassium salts were added to an already isosmotic medium. On the other hand, Dixon (1949) and Webb & Elliott (1951) found increases in respiration with high-potassium concentrations in isosmotic media.

Table 1. *Oxygen uptake of chopped brain in the absence of substrate*

	Guinea pig (five observations in three animals)	Sheep (seven observations in three animals)	P (Student's <i>t</i> test)
Total oxygen uptake in 5 hr. (μ moles/g. fresh wt.)	136 $\pm$ 5	104 $\pm$ 6	<0.01
Oxygen uptake during first hour (μ moles/g. fresh wt.)	56 $\pm$ 3	30 $\pm$ 2	<0.001
Oxygen uptake in succeeding hours as percentage of uptake in first hour			
Second hour	71% $\pm$ 5	82% $\pm$ 2	0.1 > P > 0.05
Third hour	40% $\pm$ 7	69% $\pm$ 3	<0.01
Fourth hour	25% $\pm$ 6	62% $\pm$ 3	<0.001
Fifth hour	13% $\pm$ 3	44% $\pm$ 3	<0.001

The preparation contained considerable quantities (3–4 μ moles/flask) of lactate, which were only slightly affected by incubation. Slight decreases were found in the flasks incubated without added pyruvate, and slight increase in those with pyruvate, but the changes were small compared with the initial concentrations.

Acetate was produced in measurable quantities by chopped brain when pyruvate and fumarate were present and the amount was increased by the absence of fumarate.

All these findings are in accord with those of Banga *et al.* (1939), Coxon, Liébecq & Peters (1949) and Coxon (1953), which were obtained with pigeon brain. The present findings, taken with those of Krebs, Eggleston, Kleinzeller & Smyth (1940), provide good evidence that the metabolism of pyruvate by the sheep brain proceeds via the tricarboxylic acid cycle.

Acetate. Acetate was not taken up from the

medium by sheep-brain slices and the oxygen uptake was unaffected by its presence. This is in accordance with the findings for the sheep and other species *in vitro* (Pennington, 1952; Elliott, Grieg & Benoy, 1937; Elliott, Scott & Libet, 1942; Kleinzeller, 1943; Mann, Tennenbaum & Quastel, 1938), and for sheep brain *in vivo* (McClymont & Setchell, 1956b).

Inositol. Inositol had no effect on the oxygen uptake of chopped sheep brain, in accordance with the finding of Young (1936) for rat brain and not with that of Das & Guha (1935).

Respiratory quotient

The R.Q. of sheep brain *in vitro* is not affected by the presence or absence of glucose in the medium. In this regard sheep brain is similar to the other species reported (Dickens & Greville, 1933) and the findings resemble those for rabbit brain in the present work (Table 4).

Table 2. *Pyruvate metabolism by chopped sheep brain*

All rates are given in μ moles/g. fresh wt./hr. Each figure is the average of two closely agreeing values. The complete system contained 10 μ moles of sodium pyruvate, 10 μ moles of sodium fumarate, 1 μ mole of ATP, 4 μ moles of $MgCl_2$, 230 μ moles of KCl and 120 μ moles of Na_2HPO_4 , pH 7.4, in 3 ml.

	Complete system	No fumarate	No ATP	No magnesium	Tris instead of phosphate	No pyruvate, fumarate or ATP
Pyruvate uptake	38	31	25	39	32	—
α -Oxoglutarate production	8	3	4	9	7	0
Citrate production	2.9	0	1.3	3.1	2.9	0
Acetate production	8	14	—	—	—	0
Lactate production	10	4	2	4	10	–8
Oxygen uptake	68	55	45	73	57	51

Table 3. *Pyruvate metabolism by chopped sheep brain in potassium-rich and sodium-rich media*

All rates are given in μ moles/g. fresh wt./hr. NS, No pyruvate, fumarate or ATP. The complete system contained 10 μ moles of sodium pyruvate, 10 μ moles of sodium fumarate and 3 μ moles of ATP. Potassium-rich medium also contained 4 μ moles of $MgCl_2$, 230 μ moles of KCl and 120 μ moles of Na_2HPO_4 , pH 7.4, in 3 ml. Sodium-rich medium also contained 400 μ moles of NaCl, 15 μ moles of KCl, 30 μ moles of Na_2HPO_4 , pH 7.4, 4 μ moles of KH_2PO_4 and 4 μ moles of $MgSO_4$ in 3 ml.

	Pyruvate uptake		α -Oxoglutarate production			Citrate production			Oxygen uptake		
	Complete system	Fumarate absent	Complete system	Fumarate absent	NS	Complete system	Fumarate absent	NS	Complete system	Fumarate absent	NS
Potassium-rich medium	46	26	12	3	0	2.6	0.3	0	61	43	39
Sodium-rich medium	23	27	3	1	0	0.3	0.2	0	38	36	32

Table 4. *Respiratory quotient of brain in vitro with and without glucose*

Species	Period of experiment (hr.)	Tissue preparation	Respiratory quotient		Oxygen uptake (μ moles/flask/hr.)	
			With glucose	Without glucose	With glucose	Without glucose
Sheep	2	Slices	0.94, 0.85	0.93, 0.93	5.8, 4.9	4.7, 3.7
	5	Chopped	0.97, 1.00	1.00, 1.00	2.9, 2.4	1.4, 1.4
Rabbit	1	Slices	0.85	0.95, 0.87	4.4	4.6, 3.6
	2	Slices	0.82	0.88, 0.90	6.8	6.2, 4.0

The lower values found in the shorter experiments may be due to too high a rate of oxygen uptake. Dickens (1936) pointed out a similar fault in the results of Elliott & Baker (1935), in which values even less than 0.90 were found for brain. The rate of oxygen uptake per flask was similar with or without glucose in the shorter experiments so presumably the lack of difference between the values with and without glucose is still valid.

Response to electrical stimulation

The oxygen uptake of sheep-brain slices increased when electrical stimulation (3.6 v a.c., 20–40 mA to each flask) was applied in the presence of glucose (Fig. 1). In this regard the sheep is similar to other species studied (guinea pig, rabbit, rat, human and monkey; McIlwain, 1951, 1953, 1954; McIlwain, Ayres & Forda, 1952).

However, unlike all these species, the oxygen uptake of sheep-brain slices showed a slight in-

Fig. 1. Effect of electrical stimulation on the oxygen uptake of sheep-brain slices (about 100 mg. fresh wt.) in 3 ml. of phosphate saline. Each point represents the oxygen uptake/g. fresh wt./30 min. period; three points linked by a line indicate successive periods for the same slice. ●, No stimulation; ○, electrical stimulation during the 30 min. period. Slices 1–12 were incubated in phosphate saline containing 10 mM-glucose, slices 13–19 in glucose-free phosphate saline. Lactate production and glucose uptake for some of the slices during the three 30 min. periods are given in Table 5.

crease when electrical stimulation was applied in the absence of substrate. When the increase in oxygen uptake of sheep-brain slices, judged as a percentage of the initial unstimulated rate, was compared with the effect in other species, the difference was highly significant ($P < 0.001$ by Student's t test). When the effect of electrical stimulation during the second of two periods of respiration was compared, on a percentage basis, with the respiration of sheep-brain slices in the absence of substrate during two successive unstimulated periods, the difference was highly significant ($P < 0.01$).

The reason for the increase in respiration rate in response to electrical stimulation in the absence of substrate is not known, but it could be taken as evidence for the more ready availability of some endogenous substrate.

The production of lactate by sheep-brain slices *in vitro* was also increased by electrical stimulation. The average unstimulated rate of production was $21 \mu\text{moles/g./hr.}$ or $32 \mu\text{moles/g./90 min.}$ period. In the flasks where electrical stimulation was applied for the second of the three 30 min. periods, the lactate production in the 90 min. period rose to about $60 \mu\text{moles/g.}$ The actual figures for individual slices are given in Table 5. If the rate of lactate formation is similar in the two unstimulated periods to that of slices unstimulated throughout, this would mean that glycolysis during the stimulated period proceeded at a rate of about $80 \mu\text{moles/g./hr.}$

As exactly comparable unstimulated slices were not available for all sheep, lactate production is given as the amount in $\mu\text{moles/g.}$ produced in 90 min., comprising two unstimulated 30 min. periods and one stimulated 30 min. period.

The uptake of glucose by brain slices from the medium was increased by electrical stimulation. The uptake during the 90 min. period was increased from an average of $39 \mu\text{moles/g./90 min.}$ when unstimulated throughout to about $60 \mu\text{moles/g./90 min.}$ when stimulated during the second

Table 5. Lactate production and glucose uptake by sheep-brain slices

See under Fig. 1.

Slice no.	Lactate production ($\mu\text{moles/g./}$ 90 min.)	Glucose uptake ($\mu\text{moles/g./}$ 90 min.)
6	48	59
7	51	96
8	84	70
9	62	67
10	61	53
11	54	56
Mean of six unstimulated slices	32 (range 22–39)	39 (range 31–42)

30 min. period. This is equivalent to a rate of 68 μ moles/g./hr. during stimulation if calculated in the same way as the lactate production. As comparable unstimulated slices were not always available, the uptakes are given in μ moles/g./90 min., including two unstimulated 30 min. periods and one stimulated 30 min. period (Table 5).

Resynthesis of creatine phosphate

Under aerobic conditions and in the presence of glucose, sheep-brain slices resynthesized creatine phosphate. The concentrations reached in sheep-brain slices *in vitro* were very similar to those found by other workers (McIlwain, Buchel & Cheshire, 1951; Heald, 1954), and in the present work, for guinea-pig slices. The changes in inorganic phosphate were opposite to those in creatine phosphate. The results for normal sheep and guinea pig from the present work are summarized in Table 6.

Resynthesis of glycogen

Glycogen resynthesis has been demonstrated in sheep- and guinea-pig-brain slices incubated aerobically in phosphate saline with glucose, but to a smaller extent than that observed in guinea-pig-brain slices by LeBaron (1955) and McIlwain & Tresize (1956). The last-named authors showed that the levels reached *in vitro* depended on the speed of preparation of the slices after death of the animal. A possible explanation of the lower levels found in the present work in sheep- and guinea-pig-brain slices was that all preparations were made single-handed and hence probably a good deal slower than in the experiments of McIlwain & Tresize

(1956) and LeBaron (1955). The concentrations found in sheep- and guinea-pig-brain slices in the present work are given in Table 7.

Glycogen concentrations in the brain in vivo

The brain-glycogen concentration, as μ moles of hexose/g. fresh wt., found in two sheep and a guinea pig killed in coma induced by the intra-peritoneal injection of 200 mg. of sodium iodoacetate/kg. body wt. were 3.4 (range 3.2–3.6; three samples) for the guinea pig and 4.0 (range 3.2–4.6; six samples) and 1.9 (range 1.7–2.2; five samples) for sheep nos. 1 and 2 respectively.

Before injection of the iodoacetate, the guinea pig and sheep no. 1 were normal. Sheep no. 2 was unable to stand, though quite conscious, as poor teeth had interfered with its feeding. The under-nutrition could be related to the lower concentration found in this sheep, but there is not sufficient evidence to generalize.

The concentrations found are of the same order as those found in cat, dog and rabbit brain by Kerr & Ghantus (1936); Chesler & Himwich (1943, 1944) and Ferris & Himwich (1946).

Sodium, potassium, chloride and water concentrations

When sheep-brain slices were placed in bicarbonate saline, up to 50 % of the potassium was lost from the slice into the medium within 5 min. at room temperature. On incubation at 39° in bicarbonate saline containing 10 mM-glutamate and 20 mM-glucose in an atmosphere of O₂ + CO₂ (95:5) (glucose + glutamate medium), potassium accumulated again against the concentration gradient

Table 6. *Concentration of creatine phosphate and inorganic phosphate in incubated and unincubated brain slices*

Species	Results are given as μ moles of P/g. fresh wt.			
	Concn. of creatine phosphate		Concn. of inorganic phosphate	
	Unincubated	Incubated	Unincubated	Incubated
Sheep	0.6, 0.6	1.2, 1.3	4.0, 5.9	2.5, 2.0
	0.6, 0.5	1.2, 1.2	5.0, 5.0	3.0, 3.0
Guinea pig	0.5	1.3, 2.0	4.8	3.0, 3.1
	0.6, 0.6	1.2, 1.4	5.4, 5.4	3.1, 3.5

Table 7. *Concentration of glycogen in incubated and unincubated brain slices*

Species	Time of incubation (hr.)	Results are given as μ moles of hexose/g. fresh wt.		
		Unincubated	Incubated	
			With glucose	Without glucose
Sheep	4	1.00, 0.50	1.16, 1.15, 1.00	0.47, 0.22
	4	0.69, 0.62	1.13, 1.11, 0.75	0.50, 0.44, 0.43
	4	0.40, 0.42	1.45, 0.81	0.34, 0.47, 0.32, 0.30
Guinea pig	4	0.64, 0.48	1.17, 1.13	

until, after incubation for 1 hr., the concentration in the slice/g. dry wt. was about the same as in dry-cut slices. In the absence of glucose and glutamate (no-substrate medium) further potassium was lost on incubation and with prolonged incubation in no-substrate medium under anaerobic conditions the potassium in the slices (per g. of tissue water) fell to a concentration only about twice that of the medium.

With glucose in the medium but no glutamate (glucose medium), some reaccumulation occurred but not as much as in glucose + glutamate medium.

Cyanide (mm) or 2:4-dinitrophenol (0.2 mm) in the medium prevented the reaccumulation of potassium by slices in glucose + glutamate medium.

When slices were incubated at 4° they contained less potassium, regardless of the medium, than slices incubated in no-substrate medium at 39° (Table 8).

When slices were placed in saline, as well as losing potassium they gained water, sodium and chloride. On subsequent incubation, however, these were not restored to the initial concentrations but increased further. Incubation in glucose medium was associated with a slightly smaller increase in water content and sodium and chloride concentrations than slices in the no-substrate medium. The water content of slices in the glucose + glutamate medium was usually intermediate between the other two but the differences were small.

There was less water in slices incubated at 4° than in those incubated at 39°, particularly in the no-substrate and the glucose + glutamate media. The sodium + potassium (total cation) concentration and the chloride concentration were also less after incubation at 4° than at 39° (Table 9).

The presence of inositol (1 mg./ml.) or glutathione (20 mm) had no effect on the water content of slices when added to glucose or glucose + glutamate media.

The potassium concentrations found after incubation were similar to those found by Turner, Eggleston & Krebs (1950) in guinea-pig brain. The results of Pappius & Elliott (1956*b*) with rat brain showed the same relative effects of glucose and glutamate but the concentrations of potassium in the incubated slices were not as high as in the dry-cut slices. One set of slices in the present work showed results similar to those of Pappius & Elliott (1956*b*), but these slices were cut much thicker than usual.

The water content of slices under the various conditions in the present work confirm the results of Elliott (1946), Stern, Eggleston, Hems & Krebs (1949), Aebi (1953) and Pappius & Elliott (1956*a*) that brain slices are not able to regulate their water content *in vitro* as efficiently as kidney and

Table 8. *Concentration of potassium in sheep-brain slices under various conditions of incubation*
Concentrations are given as m-moles/g. dry wt. Each figure is the average of two closely agreeing values.

	Cut-dry; unincubated	Incubated for 5 min. at room temp.	Incubated for 1 hr. at 39°					Incubated for 1 hr. at 4°		
			No substrate	Glucose	Glucose + glutamate	Glucose + glutamate + cyanide	Glucose + glutamate + 2:4-dinitro- phenol	No substrate	Glucose	Glucose + glutamate
1	0.54	—	0.38	0.47	0.56	—	—	—	—	—
2	0.53	—	0.29	0.45	0.52	—	—	—	—	—
3	0.53	0.30	0.28	0.37	0.44	0.21	0.30	0.19	0.20	0.21
4	0.49	0.33	0.29	0.38	0.53	0.22	0.33	—	—	0.16

Table 9. Potassium, sodium, chloride and water concentrations in sheep-brain slices after incubation for 1 hr. under various conditions

Ion concentrations are given as m-moles/g. dry wt. and water in g./g. dry wt. Figures are means of groups of slices, with standard deviations.

Conditions of incubation	No. of slices in group	Concentration of ions				Concn. of water
		K ⁺	Na ⁺	Na ⁺ + K ⁺	Cl ⁻	
Cut-dry; unincubated	15	0.52±0.03	0.32±0.09	0.84±0.10	0.26±0.07	4.3±0.3
No substrate; 39°	9	0.31±0.05	0.88±0.14	1.19±0.15	0.82±0.14	7.6±0.8
Glucose; 39°	8	0.39±0.08	0.77±0.17	1.16±0.21	0.70±0.13	6.9±0.7
Glucose + glutamate; 39°	8	0.53±0.05	0.69±0.12	1.22±0.16	0.65±0.12	7.5±0.5
Glucose + glutamate; 4°	10	0.18±0.03	0.80±0.06	0.98±0.05	0.63±0.07	6.1±0.6
Glucose + glutamate + inhibitors	8	0.27±0.05	0.86±0.09	1.13±0.06	0.74±0.09	7.2±0.5

Table 10. Uptake of oxygen and inorganic phosphate by sheep-brain mitochondria and homogenates

All uptakes are given as $\mu\text{g. atoms/flask/15 min.}$ Each flask contained mitochondria equivalent to about 500 mg. of fresh brain or homogenate equivalent to about 200 mg. of fresh brain.

	Homogenates			Mitochondria		
	Uptake of inorganic phosphate	Uptake of oxygen	P/O ratio	Uptake of inorganic phosphate	Uptake of oxygen	P/O ratio
Sheep 1	22	9.4	2.3	27	11.6	2.3
	19	11.6	1.6	29	11.8	2.5
Sheep 2	18	8.1	2.3	19	10.0	1.9
	17	8.6	2.0	22	10.0	2.2

liver slices under favourable conditions (Robinson, 1950, 1952), although slightly less swelling occurred in the presence of glucose than in its absence.

The effects of cold on the water content of incubated brain slices were similar but not as pronounced as in the results of Aebi (1953). The effect of cold is anomalous in terms of the effects on metabolism, especially as slices of other tissues swell more when incubated at lower temperatures (Robinson, 1950, 1952). Presumably, in brain slices there is a slower movement of water owing to reduced thermal diffusion at the lower temperatures.

Hexokinase activity

The hexokinase activity of 12 samples of sheep brain from four sheep was 150 $\mu\text{moles of glucose phosphorylated/g. fresh wt./hr. at } 30^{\circ}$ (range 90–224), and the hexokinase activity of nine samples of guinea-pig brain from three animals was 220 $\mu\text{moles of glucose phosphorylated/g./hr. (range 186–260)}$.

These values are of the same order as those reported in the literature for other species (Long, 1952; Crane & Sols, 1953; Utter, 1950; Weil-Malherbe & Bone, 1951).

Cytochrome-cytochrome oxidase activity

The cytochrome-cytochrome oxidase activity of sheep brain was 14 $\mu\text{moles of indophenol blue/g. fresh wt./hr. (range 13–16; six samples)}$ formed

Fig. 2. Oxygen uptake by sheep-brain mitochondria (equivalent to 500 mg. of fresh brain/flask). Details of incubation and media are given in the Experimental section.

from *p*-phenylenediamine and α -naphthol at 28°. Allowing for the temperature differences, this is of the same order as the activity of sheep brain determined by Vernon (1911-12).

Oxidative phosphorylation

Homogenates of sheep brain in 0.25 M-sucrose, and sheep-brain mitochondria suspended in 0.25 M-sucrose, took up oxygen and inorganic phosphate when incubated *in vitro* in the medium described above. The P/O ratio for both preparations was about 2, which was similar to the value obtained for other species (Brody & Bain, 1952; Abood, Gerard, Banks & Tschirgi, 1952; Christie, Judah & Rees, 1953) (Table 10).

The oxygen uptake of sheep-brain mitochondria, like that of rat-brain mitochondria, fell off with time to less than 10 % of the initial rate at the end of 90 min. (Fig. 2). Unfortunately the reagents were not available for testing whether the respiration of sheep-brain mitochondria could be prolonged by the addition of cofactors shown to be effective for rat-brain mitochondria by Christie *et al.* (1953), Gallagher, Judah & Rees (1956).

DISCUSSION

A close similarity is shown between those aspects of the metabolism of the sheep brain reported in the present paper and those of other species. No difference was apparent which could account for the resistance of the sheep to hypoglycaemia, except that the oxygen uptake of sheep-brain slices, unlike those of other species, increased slightly with electrical stimulation even without added substrate.

SUMMARY

1. Some aspects of the cerebral metabolism of normal sheep have been studied.

2. Oxygen and substrate uptake and the accumulation of metabolic products were studied with slices, with glucose and pyruvate as substrates and in the absence of added substrate. Respiratory quotients were measured and the effect of electrical stimulation on the metabolism of slices was examined with glucose and without substrate.

3. The resynthesis of creatine phosphate and glycogen *in vitro*, the concentration of glycogen *in vivo* and the movement of potassium, sodium and chloride ions and of water *in vitro* were studied in slices.

4. The oxidative phosphorylation and oxygen uptake of homogenates and mitochondria and the hexokinase and cytochrome-cytochrome oxidase (indophenol oxidase) activities were measured.

5. No differences between the sheep and other

species could be found which could account for the resistance of the sheep to hypoglycaemia, except that the oxygen uptake of sheep-brain slices, unlike those of other species, increased slightly with electrical stimulation even without added substrate.

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Cerebral Metabolism in the Sheep

2. IRREVERSIBLE HYPOGLYCAEMIC COMA

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Hypoglycaemia in the sheep is associated with symptoms similar to those in other animals, although hypoglycaemia may continue for up to 24 hr. in the sheep before symptoms begin. If symptoms are allowed to persist for long after a state of coma is reached, treatment by raising the blood-glucose concentration becomes ineffectual—so-called irreversible coma (McClymont & Setchell, 1956). A similar condition has been reported in man and in cats (Himwich, 1951) but is uncommon in rats (Himwich, 1951) and guinea pigs (Setchell, 1957).

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Apart from some observations on arteriovenous oxygen differences and cerebral metabolic rate *in vivo* (Himwich, Bowman & Fazekas, 1940; Fazekas, Alman & Parrish, 1951), little attention has been paid to the biochemical aspects of this condition and the present work was carried out in the hopes of finding a biochemical lesion which could explain the persistence of symptoms despite normal or raised blood-glucose concentrations.

EXPERIMENTAL

Methods were those described previously (Setchell, 1959). The sheep used for studying the effects of insulin hypoglycaemia were aged (i.e. more than 5 years old) Suffolk