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Cerebral Metabolism in the Sheep

2. IRREVERSIBLE HYPOGLYCAEMIC COMA

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(Received 18 August 1958)

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

ewes which were given 10 units of insulin, ordinary or Ultralente (Burroughs Wellcome and Co.)/kg. subcutaneously. The sheep were fasted for 24 hr. before being given the insulin. They were killed in coma when, on the basis of previous observation (McClymont & Setchell, 1956), it was considered extremely unlikely that they would recover if the blood-glucose concentration were raised. Two sheep (11/12 and 8/1) were given glycerol *per os* (120 ml.) to increase the blood-glucose concentration (Setchell & McClymont, 1955*b*) and when no change in their condition occurred within 3 hr., they were killed by cutting the throats and dislocating the necks. The brains were removed and treated as described previously (Setchell, 1959). The seven sheep studied are designated 18/10, 12/11, 11/12, 8/1, 18/1, 11/5 and 15/5.

RESULTS

Metabolism of brain slices in glucose saline and the effects of electrical stimulation. The unstimulated oxygen uptake of brain slices from the seven sheep in irreversible coma was not markedly different from that of slices from normal brains. Some were

rather lower, particularly some from sheep 8/1, but most fell within the normal range (Fig. 1).

The respiration of brain slices from all insulin-treated sheep (except 18/1) showed a reduction, or absence of the increase, in response to electrical stimulation. (3.6 v a.c., 20–40 mA to each flask.) Although there was a general reduction in or an absence of the response to electrical stimulation, the degree of reduction of the response was variable in different slices from the one sheep. Some slices, in fact, showed a decreased respiration in response to electrical stimulation, and in three slices from three different sheep the rate of respiration was greater in each successive period. No explanation can be offered for these abnormal responses.

The variability of response is understandable in view of the variability of the development of the symptoms seen during hypoglycaemia and the persistence of some symptoms and not others after treatment in some cases. There seemed to be no correlation between the area from which the slices were taken and the degree of reduction in the response to electrical stimulation.

Despite the variability between slices, the effect of electrical stimulation on the oxygen uptake of slices was significantly less ($P < 0.001$ by Student's *t*-test) for each of the insulin-treated sheep (except 18/1) than for the normal slices described in the previous paper, when the oxygen uptake during the period with stimulation was expressed as a percentage of the average oxygen uptake during the two periods without stimulation.

The rate of production of lactate by brain slices from sheep in irreversible coma was similar to that by brain slices from normal sheep and generally was not affected by electrical stimulation in those sheep from which some slices were unstimulated. In those sheep in which all slices were stimulated, the total lactate production over the two unstimulated periods and one stimulated period was no higher than in brain slices from other insulin-treated sheep or from normal sheep, unstimulated throughout. The lactate production for the 90 min. of the experiment is shown in Table 1.

The uptake of glucose by unstimulated brain slices from sheep in irreversible coma was somewhat higher than by slices from normal sheep. Consequently the effect of electrical stimulation could be decided only when unstimulated slices from the same sheep had been incubated. No effect of electrical stimulation could be shown in brain slices from the two insulin-treated sheep thus studied (Table 1).

Sheep 18/1 showed a normal response in lactate production and glucose uptake to electrical stimulation in two of the three slices studied. These were not the two slices which showed the normal response of respiration to electrical stimulation.

Fig. 1. Effect of electrical stimulation on the oxygen uptake of brain slices (about 100 mg. fresh wt.) from sheep in irreversible hypoglycaemic coma, incubated in 3 ml. of phosphate saline with 10 mM-glucose. 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. Lactate production and glucose uptake for some of the slices during the three 30 min. periods are given in Table 1.

Table 1. *Lactate production and glucose uptake by sheep-brain slices*

See under Fig. 1.

Sheep no.	Slice no.	Electrical stimulation during second period	Lactate production (μ moles/g. fresh wt./90 min.)	Glucose uptake
12/11	1	+	29	58
	2	+	35	54
	3	+	30	57
	4	-	31	56
	5	-	25	46
11/12	1	+	39	55
	2	+	35	65
	3	+	46	59
	4	+	40	43
	5	+	43	45
	6	+	69	49
8/1	1	+	34	39
	2	+	26	43
	3	+	25	52
	4	+	22	41
	5	+	36	37
	6	-	31	41
18/1	1	+	32	21
	2	+	60	81
	3	+	119	83
	4	-	40	57
	5	-	43	56
	6	-	49	52
11/5	1	+	26	—
	2	+	37	—
	3	+	27	—
	4	+	39	—
	5	+	38	—
15/5	1	+	32	—
	2	+	26	—
	3	+	27	—
	4	+	25	—
	5	+	36	—
	6	+	29	—
Mean of 6 normal unstimulated slices		-	32 (Range 22-39)	39 (Range 31-42)

Metabolism of pyruvate. The uptake of pyruvate and oxygen and the production of α -oxoglutarate were studied with chopped brain from the seven sheep in irreversible coma and, in some, the production of citrate, lactate and acetate was also studied. There were no differences from normal in the pyruvate or oxygen uptake with or without fumarate nor in the production of α -oxoglutarate, citrate, lactate or acetate with or without fumarate (Table 2). The oxygen uptake in the absence of substrate was slightly lower in chopped brain from insulin-treated sheep, especially when considered as a percentage of the rate in the complete medium. This is in accord with the findings of Holmes (1930), Ashford & Holmes (1931), Wortis (1935) and Elliott, Scott & Libet (1942) of a lowered rate of respiration by brain from an insulin-treated animal in the absence of substrate but not when substrate was present.

Resynthesis of creatine phosphate. The resynthesis of creatine phosphate was studied in brain slices from six of the seven animals in irreversible coma. In five of those there was no resynthesis of creatine phosphate when the slices were incubated under aerobic conditions in glucose saline (Table 3). In fact, the concentration of creatine phosphate in the incubated slices was slightly lower than in the unincubated slices, but as the concentrations involved were near the limit of sensitivity of the method this fall is not certain.

In sheep 18/1 there was resynthesis of creatine phosphate in two of the three slices studied, from an initial concentration of 0.3 μ mole/g. to 0.9 μ mole/g. The third incubated slice contained 0.5 μ mole of creatine phosphate/g. Thus the slices from the brain of sheep 18/1 showed a normal response of respiration to electrical stimulation and a normal resynthesis of creatine phosphate.

Resynthesis of glycogen by brain slices in vitro. In brain slices from the one insulin-treated sheep studied, there was resynthesis of glycogen of the same order as that seen in normal slices (Table 4). However, as there is only a small amount of glycogen resynthesized by slices of normal brain under the present conditions, it would be possible to be certain of the significance of only extreme changes in this resynthesis.

Concentrations of potassium, sodium, chloride and water. The concentration of potassium in the slices cut without moistening with saline (dry-cut slices) was significantly lower in slices from sheep in irreversible coma. This confirms the findings of Setchell & McClymont (1955a).

Whereas after incubation for an hour in glucose + glutamate medium the concentration of potassium in brain slices from normal sheep was about the same as the concentration in dry-cut slices, the concentration of potassium in brain slices from the insulin-treated sheep after incubation was considerably less than in dry-cut slices from the same brain.

The concentration of potassium in brain slices from insulin-treated sheep incubated in glucose medium and in no-substrate medium was less than in brain slices from normal sheep incubated under the same conditions. There was less difference between the slices incubated in glucose medium and slices in glucose + glutamate medium when the slices were taken from an insulin-treated sheep (Table 5).

Sheep 18/1 was less affected than the other insulin-treated sheep. The potassium concentration in slices incubated in glucose + glutamate medium, although not the same as in dry-cut slices, was less reduced than in brain slices from the other insulin-treated sheep.

The water, sodium and chloride concentrations in the incubated slices were not markedly abnormal in slices from insulin-treated animals. There was less difference between the water content of slices in glucose medium and those in no-substrate medium in some insulin-treated sheep, but the large changes in water content of slices of normal brain make changes in this already small difference difficult to demonstrate.

Hexokinase activity. There was no consistent difference in the hexokinase activity between homogenates made from the brains of insulin-treated and normal sheep. There was a lowered activity in sheep 8/1, possibly associated with the lower rate of oxygen uptake seen in this sheep. The mean activity of three values for each sheep are given in Table 6. The greatest difference between the three tubes from any one sheep was 30 μ moles/g./hr.

Cytochrome-cytochrome oxidase activity. There was no difference in the cytochrome-cytochrome

Table 2. *Pyruvate metabolism by chopped brain from normal sheep and from sheep in irreversible hypoglycaemic coma*

Each figure is the average of two closely agreeing values. All rates are in μ moles/g. fresh wt./hr. (a) Complete system; (b) no fumarate; (c) no fumarate, pyruvate or adenosine triphosphate (ATP). Complete systems 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.

Sheep no.	Pyruvate uptake			α -Oxoglutarate production			Citrate production			Lactate production			Acetate production			Oxygen uptake			Slices in sodium-rich medium with glucose
	(a)	(b)	(c)	(a)	(b)	(c)	(a)	(b)	(c)	(a)	(b)	(c)	(a)	(b)	(c)	(a)	(b)	(c)	
Normal	43	33	6	2	0	0	2.8	1.1	0	14	8	—	—	—	—	65	57	51	48
	38	31	8	3	0	0	3.3	0	0	10	4	—8	—	—	—	68	55	51	38
	46	26	10	3	0	0	2.6	0.3	0	—	—	—	8	14	61	43	39	64	35
Insulin-treated	42	34	10	3	0	0	2.5	0.4	0	11	3	—6	—	—	—	76	60	41	39
	34	20	6	1	0	0	2.1	0.5	0	—	—	—	—	—	—	60	39	30	39
	38	24	7	1	0	0	1.7	0.2	0	—	—	—	8	21	56	26	24	43	45
	37	28	8	2	0	0	2.6	0.5	0	—	—	—	—	—	—	59	42	31	30
	45	32	10	3	0	0	5.1	0.9	0	—	—	—	—	—	—	69	50	41	51
18/1	46	34	9	5	0	—	—	—	—	—	—	—	6	23	54	35	27	50	48
11/5	48	41	10	4	0	—	—	—	—	—	—	—	8	22	56	41	35	62	50

oxidase activity between the brains of sheep in irreversible coma and normal sheep. The activity determined for five of the insulin-treated sheep is given in Table 7.

Oxidative phosphorylation. The respiration and oxidative phosphorylation of brain homogenates

made from two sheep in irreversible coma was no different from that of normal sheep.

However, the mitochondria prepared in the usual way from these homogenates showed much reduced respiration and little or no associated phosphorylation. The actual extent of the phosphorylation was difficult to determine owing to the small changes involved (Table 8).

A similar state of normal oxygen and phosphate uptake by homogenate, but much reduced uptake of both oxygen and phosphate by mitochondrial preparations derived from the homogenate, could be induced by ageing a homogenate of normal brain for 2 hr. at 2° before separation of the mitochondria.

Table 3. *Creatine phosphate concentrations in brain slices from normal sheep and sheep in irreversible hypoglycaemic coma*

Concentrations are given in μ moles of P/g. fresh wt. Each figure is the average of three closely agreeing values, except for incubated slices from sheep 18/1, where the individual concentrations for each slice are given.

Sheep no.	Unincubated slices	Incubated slices
Normal		
1	0.6	1.3
2	0.6	1.2
Insulin-treated		
12/11	0.5	0.3
11/12	0.3	0.2
8/1	0.3	0.3
18/1	0.3	0.9, 0.9, 0.5
11/5	0.6	0.2
15/5	0.5	0.3

DISCUSSION

The metabolic lesion found in the brains of sheep in irreversible hypoglycaemic coma does not seem to involve the conversion of the various substrates of metabolism into their end products but rather the utilization of the energy produced from these processes, of the fundamental nature of which little is known. Further elucidation of the nature of the lesion must await advances in the knowledge of the normal mechanisms.

Table 4. *Glycogen concentration in brain slices from normal sheep and from one sheep in irreversible hypoglycaemic coma*

Concentrations are given in μ moles of hexose/g. fresh wt. Values given are averages with range and number of observations.

Sheep	Unincubated slices	Incubated slices	
		With glucose	Without glucose
Normal	0.60 (0.42–1.00; 6)	1.07 (0.75–1.45; 8)	0.39 (0.22–0.50; 9)
Insulin-treated 18/10	0.60, 0.60	0.80 (0.60–1.00; 6)	—

Table 5. *Potassium concentration in brain slices from normal sheep, and from sheep in irreversible hypoglycaemic coma, under various conditions of incubation*

Concentrations are given in m-moles/g. dry wt. Each figure is the average of at least two closely agreeing values.

Sheep no.	Slices cut dry and unincubated	Incubated slices		
		Glucose + glutamate	Glucose	No substrate
Normal				
1	0.54	0.56	0.47	0.38
2	0.53	0.52	0.45	0.29
3	0.52	0.44	0.37	0.28
4	0.49	0.53	0.38	0.29
Insulin-treated				
12/11	0.47	0.38	0.33	0.20
11/12	0.48	0.28	0.27	0.18
8/1	0.43	0.30	0.28	0.24
18/1	0.48	0.43	0.34	0.24
11/5	0.49	0.32	0.35	0.28
15/5	0.46	0.34	0.34	0.20

To relate the present findings to the effects of hypoglycaemia on the arteriovenous oxygen difference and cerebral metabolic rate *in vivo*, it must be decided whether the metabolism *in vivo* is better correlated with the unstimulated or the stimulated metabolism *in vitro*. The overall metabolism *in vivo* is probably somewhere intermediate between the stimulated and unstimulated meta-

bolism *in vitro*, but the rate of oxygen uptake *in vivo* corresponds more closely with the stimulated rate *in vitro* (McIlwain, 1955).

If this is so, then a reduced rate of respiration with electrical stimulation should be reflected in a lower oxygen uptake *in vivo*. In other species, this is certainly the case (Himwich, 1951), but in the one sheep for which data are available (McClymont & Setchell, quoted by Setchell, 1957) no marked changes in arteriovenous oxygen difference were demonstrated during hypoglycaemia. As no estimate of cerebral blood flow was made, this finding is not unequivocal evidence and further work is necessary.

The finding of a failure in the resynthesis of creatine phosphate *in vitro* is possibly related to the findings of a lowered creatine phosphate concentration in the brains of hypoglycaemic animals *in vivo* (Olsen & Klein, 1946), although the concentration of creatine phosphate was normal in the brains of hypoglycaemic animals which had been anaesthetized (Kerr & Ghantus, 1936). Unfortunately, neither report gives the concentration of creatine phosphate in brains of animals in irreversible coma after treatment with glucose, so the changes found may have simply been a temporary reflexion of the lowered production of energy from the metabolism of glucose rather than the unavailability of the energy produced.

The lowered reaccumulation *in vitro* of potassium by brain slices from sheep in irreversible coma may be related to the lowered concentration of potassium found in the brains of sheep and cats in irreversible hypoglycaemic coma (Yannet, 1939; Setchell & McClymont, 1955*a* and the present work). In the brains of insulin-treated sheep, the concentration of potassium *in vivo*, although less than the normal concentration *in vivo*, was greater than the concentration in slices from insulin-treated sheep incubated *in vitro* in 'glucose + glutamate medium'. This is unlike normal brain, in which a concentration is maintained *in vitro* in

Table 6. *Hexokinase activity of brain from normal sheep and from sheep in irreversible hypoglycaemic coma*

Activity is given in μ moles of glucose phosphorylated/g. fresh wt./hr.

Sheep no.	Activity
Normal	156 $\pm$ 11
18/10	150
12/11	125
11/12	174
8/1	70
18/1	178
11/5	138
15/5	146

Table 7. *Cytochrome-cytochrome oxidase activity of chopped brain from normal sheep and sheep in irreversible hypoglycaemic coma*

Activity is given in μ moles of indophenol blue formed/g. fresh wt./hr. Each figure is the average of two closely agreeing figures.

Sheep no.	Activity at 28°
Normal	
1	16
2	13
3	13
Insulin-treated	
18/10	15
12/11	12
11/12	12
8/1	> 11*
18/1	15

* Slightly cloudy; the colour was compared visually with standard.

Table 8. *Oxygen and inorganic phosphate uptake by brain homogenates and mitochondria from sheep in irreversible hypoglycaemic coma*

All uptakes are given in μ 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.

Sheep no.	Homogenates			Mitochondria		
	Inorganic phosphate uptake	Oxygen uptake	P/O ratio	Inorganic phosphate uptake	Oxygen uptake	P/O ratio
Normal	18	8.4	2.2	21	10.0	2.1
Normal (homogenate aged for 2 hr. at 2°)	17	9.0	1.8	0	0	—
11/5	20	8.8	2.3	0	1.1	—
	15	8.0	1.9	0	0.8	—
15/5	18	8.8	2.1	2	3.0	0.7
	17	8.2	2.1	0.5	2.4	0.2

'glucose + glutamate medium' of the same order (on a dry-weight basis) as the concentration *in vivo*. Presumably this difference is due to the essentially unphysiological conditions of incubation emphasizing the difference from normal brains.

It should be emphasized that the findings in the present work, although reasonably consistent in themselves, are only of a preliminary nature. This is a result of the small numbers of sheep used and of the fact that all these animals were studied in the same stage, the early stages of irreversible coma. Nothing is known of the metabolism of brains from sheep after protracted periods of coma or those showing early symptoms. By analogy, perhaps unwise, from the brains of other animals in early coma (Holmes, 1930; Ashford & Holmes, 1931; Elliott *et al.* 1942; Wortis, 1935; Setchell, 1957), it would seem likely that the metabolism of the brains of sheep in early coma would be normal if glucose were present.

SUMMARY

1. The metabolism has been studied of the brains of sheep in irreversible hypoglycaemic coma after the injection of large doses of insulin.

2. Abnormalities were found in the response of oxygen and glucose uptakes and lactate production to electrical stimulation, in the resynthesis of creatine phosphate, in the reaccumulation of potassium ions *in vitro* and in the oxygen uptake and oxidative phosphorylation by mitochondria.

3. The metabolism of glucose and pyruvate in the absence of electrical stimulation, the hexokinase and cytochrome-cytochrome oxidase activities and the oxygen uptake and oxidative phosphorylation of homogenates were normal.

4. The implications of this 'metabolic lesion' are discussed.

I would like to thank the Walter and Eliza Hall Trust for the Walter and Eliza Hall Veterinary Science Research Fellowship of the University of Sydney, during the tenure of which this work was carried out, and the N.S.W. Department of Agriculture for leave to enable me to carry out the work.

It is a pleasure to thank Professor W. I. B. Beveridge and the members of his Department for the hospitality accorded to me by the Department during my stay in Cambridge. More especially, I would like to thank my supervisor, Mr A. A. Wilson, for his invaluable help and encouragement.

I would also like to thank Mr R. Kimberley for invaluable help with the sheep and for general assistance and to Miss Jill Mizen for part-time assistance in the early part of the work.

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Studies on Phospholipids

5. THE SEPARATION OF DINITROPHENYLATED AND METHYLATED PHOSPHOLIPIDS BY COUNTERCURRENT DISTRIBUTION*

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(Received 19 September 1958)

Collins & Wheeldon (1957) have described the preparation of *N*-2:4-dinitrophenylated and methylated phospholipids and, by the use of these derivatives, have demonstrated the presence in

rat liver, ox brain and egg yolk of unknown forms of aminophospholipids in addition to phosphatidylethanolamine and phosphatidylserine and the corresponding plasmalogens. Further study of these hitherto unrecognized forms of phospholipids depended on the development of efficient means of separation and of adequate criteria of purity.

* Part 4: Collins & Wheeldon (1958).

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