Biochemistry of Sheep Tissues

ENZYME SYSTEMS OF LIVER, BRAIN AND KIDNEY

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Little information is available on the enzyme activities of sheep tissues *in vitro*. Kleiber (1941, 1947) measured the oxygen consumption of liver slices of rats, rabbits and sheep, and attempted to correlate the Q_{O_2} of this tissue with the metabolic rate of the living animal and to establish a direct relationship to body size. However, Krebs (1950), using more refined and standardized techniques, has shown that, although the Q_{O_2} values of the tissues of larger animals are lower than the corresponding values for smaller species, there is no direct relationship to the basal heat production of the intact animal. The Q_{O_2} value for most tissues changes much less with body weight than does the rate of heat production. Krebs attributed the differences in basal heat production in animals of different size mainly to variation in the Q_{O_2} of muscle, and suggests that the metabolic rate of other tissues is governed by the specific energy requirements of the particular tissue and not by the heat requirements of the whole body. Comparative Q_{O_2} values from various tissues and species, including sheep, are published by Krebs (1950) and Albritton (1954). These figures are of general interest but cannot supply information on the activities of isolated enzyme systems. More specific data are available on the metabolism of short-chain fatty acids by sheep-rumen epithelium and slices of kidney and liver (Pennington, 1952, 1954; Annison & Pennington, 1954; Pennington & Sutherland, 1954, 1955, 1956*a*), ketone-body production from certain substrates by rumen epithelium (Pennington & Sutherland, 1956*b*) and the metabolism of acetate and glucose by sheep mammary-gland slices, particularly in relation to lipogenesis (Folley & French, 1950; Balmain, Folley & Glascock, 1952, 1954; Duncombe & Glascock, 1953, 1956).

The aims of the present study were: to measure the activity of enzyme systems of sheep liver, brain and kidney; to observe where and how the metabolic pattern differs from that of other species; to note variations which might be attributable to the age, sex or nutritional history; and to establish normal reference data for studies in chemical pathology.

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METHODS

Enzyme preparations

Sheep were bled to death by severing the cervical vessels. Large samples, at least 10 g., were selected from the same area of liver parenchyma, cerebral cortex and kidney cortex in each animal. Tissues were removed rapidly and immersed in ice-cold 0·25 M-sucrose solution. Homogenates were prepared in 0·25 M-sucrose with glass homogenizers and fractionated by differential centrifuging in the cold (Schneider, 1948). Mitochondria were re-suspended in 0·25 M-sucrose unless otherwise indicated.

Hexokinase was prepared from yeast by the method of Berger, Slein, Colowick & Cori (1946) as modified by Cross, Taggart, Covo & Green (1949). The fraction precipitating between 25 and 40% (v/v) of ethanol was dissolved in 1% glucose solution and stored frozen. The optimum addition for oxidative phosphorylation and glycolysis was determined by assay with rat enzymes.

Reagents

Adenosine triphosphate (ATP), adenosine 5′-monophosphate (AMP), coenzyme I (Co I), coenzyme II (Co II), coenzyme A (CoA), nicotinamide, reduced glutathione (GSH), glucose, glucose 1-phosphate, glucose 6-phosphate, fructose 1:6-diphosphate (HDP), pyruvate, citrate, α-oxoglutarate, L-glutamate, succinate, fumarate, L-malate, oxaloacetate, acetate, propionate, butyrate, DL-β-hydroxybutyrate, octanoate, palmitate, L-ascorbic acid and inorganic reagents were obtained commercially. Cytochrome *c* was prepared by the method of Keilin & Hartree (1937) but dialysed against distilled water; it was also obtained commercially. New reagents were checked for inhibitory impurities in the reactions for which they were to be used, rat-tissue enzyme preparations for which the reaction rates are well established being employed. Inhibitory reagents were identified by elimination and substitution and were purified as follows: AMP was recrystallized twice from hot water. Cytochrome *c* (the commercial product) was dialysed at 0–1° for 24 hr. against three changes of distilled water and standardized by the method of Potter (1957). Pyruvic acid was redistilled *in vacuo* and crystallized as described by Lardy (1957). Succinic acid was recrystallized three times from hot water, then dissolved, neutralized and precipitated as the sodium salt with ethanol, and the salt was crystallized from 80% ethanol. Octanoic acid was

Table 1. *Anaerobic glycolysis by liver and brain homogenates*

System: ATP mM; AMP mM; KCl 25 mM; $MgSO_4$ 6·7 mM; sodium potassium phosphate buffer, pH 7·4, 13·3 mM; sodium pyruvate 6·7 mM; $KHCO_3$ 0·02 M; NaF 0·01 M; Co I 0·5 mM; nicotinamide 0·04 M; GSH 0·67 mM; enzyme equivalent to 80–100 mg. of fresh tissue in 0·25 ml. of 0·25 M-sucrose; water to 1·5 ml. final vol.; gas phase $N_2 + CO_2$ (95:5); temp. 38°; equilibration period 10 min. S.D., Standard deviation.

	$Q^{N_2}_{CO_2}$ (μl. of CO_2 evolved/mg. fat-free dry wt. of enzyme/hr.)							
	Liver				Brain			
Additions	No. of animals	Range	Mean	S.D.	No. of animals	Range	Mean	S.D.
None	15	7–20	14	±4	18	4–14	9	±3
HDP (0·01 M)	15	23–51	36	±9	18	37–76	54	±12
HDP (mM)	15	12–26	19	±4	18	14–31	22	±5
Glucose (0·01 M)	15	6–17	12	±3	—	—	—	—
HDP (mM) + glucose (0·01 M)	15	10–22	17	±4	18	47–107	76	±17
None	1	17	—	—	—	—	—	—
HDP (mM)	1	21	—	—	—	—	—	—
HDP (mM) + glucose 1-phosphate (0·01 M)	1	75	—	—	—	—	—	—
HDP (mM) + glucose 6-phosphate (0·01 M)	1	74	—	—	—	—	—	—
HDP (mM) + fructose (0·01 M)	1	24	—	—	—	—	—	—

purified by distillation *in vacuo*. Sodium pyruvate, α-oxoglutaric acid and L-ascorbic acid were dissolved and neutralized with dilute NaOH just before use. Other substrates were neutralized and stored frozen as sodium salts for 3–4 weeks. Sodium potassium phosphate buffer, pH 7·4, 0·1 M, was prepared by the method of Umbreit (1957).

Analytical methods

Oxygen uptake and CO_2 evolution were measured by Warburg manometers. Unless specified the standard system used for respiratory experiments was: AMP mM; $MgSO_4$ 6·7 mM; KCl 25 mM; sodium potassium phosphate buffer, pH 7·4, 13·3 mM; cytochrome *c* 10 μM; substrate 0·01 M; enzyme in 0·5 ml. of 0·25 M-sucrose; water to 3 ml. final volume; 0·1 ml. of 20% KOH was placed in the centre well to absorb CO_2; gas phase, air; temperature, 38°; equilibration period, 10 min.

Respiratory and glycolytic activities are expressed without correction for endogenous rates in the absence of added substrate unless otherwise indicated.

Nitrogen was determined by the micro-Kjeldahl method of McKenzie & Wallace (1954), inorganic phosphate by the method of Fiske & Subbarow (1925) and glucose by the method of Somogyi (1952).

Dry weights were determined after extracting the sample three times with boiling ethanol–diethyl ether (3:1, v/v) to remove lipids, drying in an oven at 100° for 24 hr. and cooling in a desiccator.

RESULTS

The data reported below are the pooled results of experiments on all sheep studied, except where otherwise indicated.

Anaerobic glycolysis

Table 1 shows that brain and liver homogenates glycolyse HDP well but that although brain catabolizes glucose very actively, liver preparations apparently do not use this substrate. It was found that liver can glycolyse the phosphorylated sugars, glucose 1-phosphate and glucose 6-phosphate, but not fructose (Table 1).

Table 2. *Effect of hexokinase on anaerobic glycolysis by liver*

System: as in Table 1; hexokinase in 1% glucose was added at 0·02 ml./flask. Supernatant enzyme was prepared from homogenates in 0·25 M-sucrose by centrifuging at 9000 *g* for 20 min. Results are the means of three experiments.

	$Q^{N_2}_{CO_2}$ (μl. of CO_2 evolved/mg. fat-free dry wt. of enzyme/hr.)	
Additions	Homogenate	Supernatant
None	—	15
Hexokinase	—	25
HDP (mM)	20	31
HDP (mM) + hexokinase	30	46
Glucose (0·01 M)	—	11
Glucose (0·01 M) + hexokinase	—	26
HDP (mM) + glucose (0·01 M)	19	18
HDP (mM) + glucose (0·01 M) + hexokinase	41	58

These results suggest that the inability to use glucose is associated with lack of phosphorylation by glucokinase. This is confirmed in Tables 2 and 3, where the addition of yeast hexokinase enables liver enzymes to metabolize glucose.

It will be noticed in Table 2 that hexokinase also stimulates to a lesser extent the $Q^{N_2}_{CO_2}$ of mM-HDP. This is explained by the fact that glucose is added, 1·2 μmoles with the hexokinase and about 1·2 μmoles with the enzyme preparation, to give a final concentration of at least 1·5 mM, as a variable amount

of glucose would presumably also be produced during incubation from glycogen in the enzyme preparation.

Anaerobic glycolysis by liver homogenates of two new-born lambs was studied. Neither preparation used glucose at a measurable rate. Thus the deficiency in glucose utilization by sheep-liver homogenates is present from birth.

Oxidative metabolism

Glucose oxidation by liver. Liver mitochondria oxidize glucose, as shown in Table 3, but only upon the addition of hexokinase. The stimulatory effect of Co II suggests that part at least of the oxidation occurs via glucose 6-phosphate dehydrogenase and the phosphogluconate shunt.

Oxidation of tricarboxylic acid cycle and related intermediates. The rates of oxidation of a variety of metabolic substrates by homogenate and mitochondrial preparations are shown in Tables 4 and 5. Controls without substrates were carried out in about half of the mitochondrial experiments reported in Table 5. However, the rate of endogenous oxidation was so low that these controls were discontinued. Endogenous oxidations were higher for brain than liver mitochondria but still negligible. The studies of Christie, Judah & Rees (1953) have shown that brain mitochondria do not metabolize well *in vitro* unless supplemented by respiratory cofactors. Consequently the reaction mixtures used in the present study to assay the brain mitochondrial activities have been supplemented with diphosphopyridine nucleotide (DPN), nicotinamide, GSH and ATP, as indicated in Table 5. In general, the Q_{O_2} values range from one-third to one-half of the values reported for rat-liver and -brain enzymes (Christie & Judah, 1953; Christie *et al.* 1953; Gallagher, Judah & Rees, 1956*a*, *b*).

Oxidation of fatty acids. Addition of fatty acids to homogenates of sheep liver, brain and kidney does not cause an increase in oxygen uptake over the rate resulting from the oxidation of endogenous substrates and 0·67 mM-L-malate, added as a 'primer'. However, compared with mitochondria, tissue homogenates have a high rate of endogenous oxidation which may mask the oxidation of an exogenous substrate. It was observed, too, that the addition of 0·67 mM-L-malate increased greatly the oxygen uptake of liver and kidney homogenates. This suggested that L-malate was 'sparking' the oxidation of some components of the homogenates, probably in large part fatty acids. It is thus possible that the enzymes of the fatty acid oxidation chain are saturated or nearly so with endogenous substrate in sheep-liver and -kidney homogenates and so the addition of more fatty acid either does not affect the rate of oxygen consumption or depresses it by raising the fatty acid concentration to inhibitory levels. In these studies exogenous fatty acids did in fact lower endogenous oxygen uptake.

Table 3. *Oxidation of glucose by liver mitochondria*

System: ATP mM; AMP mM; $MgSO_4$ 6·7 mM; KCl 25 mM; sodium potassium phosphate buffer, pH 7·4, 13·3 mM; cytochrome *c* 10 μM; Co I 0·5 mM; nicotinamide 0·04 M; GSH 0·67 mM; mitochondria equivalent to 150 mg. of fresh tissue in 0·25 ml. of 0·25 M-sucrose; water to 1·5 ml. final vol.; gas phase, air; temp. 38°; equilibration period, 10 min. Q_{O_2} determination is based upon oxygen consumption during 35 min. after equilibration. Results are the means of two experiments.

Additions	$Q_{O_2}^{(N)}$ (μl. of O_2 taken up/mg. of enzyme N/hr.)
None	46
Hexokinase (in 0·02 ml. of 1% glucose)	69
Hexokinase + Co II (0·25 mM)	76
Glucose (0·01 M)	45
Glucose (0·01 M) + hexokinase	86
Glucose (0·01 M) + hexokinase + Co II	103

On the other hand, mitochondria of both liver and kidney oxidize fatty acids actively (Tables 6 and 7). As with other species, brain mitochondria apparently do not catalyse fatty acid oxidation. However, by analogy with the rat (Vignais, Gallagher & Zabin, 1958), it is probable that sheep brain does oxidize fatty acids, but to a degree detectable only by isotope techniques.

The system used for fatty acid oxidation differs from that for other oxidations in one important respect. The concentration of KCl necessary for optimum oxidation of fatty acids is 0·05 M, twice as much as for other substrates. Where mitochondria are added in 0·15 M-KCl, the necessary adjustment is made to give a final concentration of 0·05 M-KCl. Octanoate and shorter-chain fatty acids are oxidized at the same rate whether the mitochondria are re-suspended in 0·25 M-sucrose or 0·15 M-KCl, but palmitate oxidation is facilitated by suspension in 0·15 M-KCl.

Palmitate, octanoate, butyrate and propionate are oxidized well by liver mitochondria but β-hydroxybutyrate and acetate are oxidized poorly. The feeble oxidation of the ketone body, β-hydroxybutyrate, is in contrast with the brisk oxidation of this substrate by enzyme preparations of rat liver (Christie & Judah, 1954). Sheep-kidney mitochondria, on the other hand (Table 7), oxidize acetate and octanoate well but have a rather poor rate of oxidation for propionate.

Oxidative phosphorylation. The efficiency of oxidative phosphorylation by sheep-mitochondrial preparations (Table 8) is of the order found with mitochondria of other species in the same reaction mixture.

Table 4. *Oxidative metabolism of tissue homogenates*

System: standard; enzyme equivalent to 50 mg. of fresh liver and 100 mg. of fresh brain was added in 0·5 ml. of 0·25 M-sucrose, except for succinate oxidation in which the equivalents of 25 mg. and 50 mg. were added respectively. Q_{O_2} calculations are based upon the oxygen consumption during 20 min. after equilibration.

	Q_{O_2} (μl. of O_2 taken up/mg. fat-free dry wt. of enzyme/hr.)							
	Liver				Brain			
Additions	No. of animals	Range	Mean	S.D.	No. of animals	Range	Mean	S.D.
None	16	4–9	6	±1	10	8–25	17	±7
Citrate (0·01 M)	12	10–26	18	±6	7	7–25	17	±7
α-Oxoglutarate (0·01 M)	11	11–24	15	±4	5	24–33	28	±3
Succinate (33 mM)	12	22–74	43	±18	9	31–48	41	±6
Fumarate (0·01 M)	5	13–25	19	±5	3	20–25	22	±3
L-Malate (0·01 M)	8	14–23	18	±3	6	27–35	32	±3
Oxaloacetate (0·01 M) + pyruvate (0·67 mM)	5	12–22	17	±4	—	—	—	—
Pyruvate (0·01 M) + L-malate (0·67 M)	6	10–15	13	±2	6	19–31	23	±5
L-Glutamate (0·01 M)	12	10–25	15	±4	5	27–37	32	±4

Table 5. *Oxidative metabolism of mitochondria*

System: standard for liver; standard + ATP mM; Co I 0·5 mM; nicotinamide 0·04 M; GSH 0·67 mM for brain. Mitochondria equivalent to 100 mg. of fresh liver and 200 mg. of fresh brain were added in 0·5 ml. of 0·25 M-sucrose, except for succinate oxidation in which the equivalents of 50 mg. and 100 mg. were added respectively. Q_{O_2} calculations are based upon the oxygen consumption during 20 min. after equilibration.

	$Q_{O_2}^{(N)}$ (μl. of O_2 taken up/mg. of enzyme N/hr.)							
	Liver				Brain			
Additions	No. of animals	Range	Mean	S.D.	No. of animals	Range	Mean	S.D.
Citrate (0·01 M)	10	47–100	78	±20	9	48–69	57	±7
α-Oxoglutarate (0·01 M)	10	99–180	129	±29	9	128–203	156	±27
Succinate (33 mM)	10	198–455	278	±79	9	192–285	233	±25
Fumarate (0·01 M)	2	89–103	96	±10	2	80–103	92	±16
L-Malate (0·01 M)	9	77–120	101	±16	9	128–183	157	±20
Pyruvate (0·01 M) + L-malate (0·67 mM)	8	97–150	132	±17	3	136–168	152	±16
L-Glutamate (0·01 M)	9	81–180	130	±34	6	166–211	183	±16

Table 6. *Oxidation of fatty acids by liver mitochondria*

System: AMP mM; ATP mM; KCl 0·05 M; $MgSO_4$ 6·7 mM; sodium potassium phosphate buffer, pH 7·4, 13·3 mM; cytochrome *c* 10 μM; $KHCO_3$, for propionate only, 6·7 mM; L-malate 0·67 mM; mitochondria equivalent to 200 mg. of fresh liver in 0·5 ml. of 0·25 M-sucrose and, for palmitate only, in 0·5 ml. of 0·15 M-KCl; water to 3 ml. final vol.; gas phase, air; temp. 38°; equilibration period, 10 min. Q_{O_2} determination is based upon oxygen uptake during 20 min.

	$Q_{O_2}^{(N)}$ (μl. of O_2 taken up/mg. of enzyme N/hr.)						
					Net fatty acid and L-malate oxidation		
Additions	No. of animals	Range	Mean	S.D.	Range	Mean	S.D.
None	4	55–97	72	±19	—	—	—
Palmitate (0·25 mM)	4	98–152	118	±25	41–55	46	±6
None	19	46–121	70	±19	—	—	—
Octanoate (1·67 mM)	19	104–228	140	±26	44–107	70	±15
None	4	46–103	66	±26	—	—	—
Butyrate (3·3 mM)	4	125–239	166	±50	70–136	100	±27
None	3	55–71	62	±8	—	—	—
DL-β-Hydroxybutyrate (0·01 M)	3	65–82	73	±8	10–13	11	±2
None	8	53–103	67	±17	—	—	—
Propionate (3·3 mM)	8	121–223	166	±33	68–137	99	±25
None	3	53–69	60	±8	—	—	—
Acetate (0·01 M)	3	67–80	74	±7	11–17	14	±3

Table 7. *Oxidation of fatty acids by kidney mitochondria*

System: as in Table 6; mitochondria equivalent to 200 mg. of fresh kidney in 0·5 ml. of 0·25 M-sucrose.

Additions	No. of animals	$Q_{O_2}^{(N)}$ (µl. of O_2 taken up/mg. of enzyme N/hr.) Range	Mean	Net fatty acid and L-malate oxidation
None	2	150–160	155	—
Octanoate (1·67 mM)	2	246–377	312	157
Propionate (3·3 mM)	2	161–206	184	29
Acetate (0·01 M)	2	215–306	261	106

Table 8. *Oxidative phosphorylation by liver and brain mitochondria*

System: standard + L-glutamate 0·01 M; glucose 33 mM; hexokinase in 0·02 ml. of 1% glucose; NaF 0·01 M; plus, for brain alone, ATP mM; Co I 0·5 mM; nicotinamide 0·04 M; GSH 0·67 mM. Mitochondria equivalent to 200 mg. of fresh tissue in 0·5 ml. of 0·25 M-sucrose; duration of experiment, 30 min., after 10 min. equilibration. Reaction was stopped by addition of trichloroacetic acid to 8% final concn.

Tissue	No. of expts.	P/O (µg. atoms of P esterified/µg. atom of O_2 consumed)	S.D.
Liver	7	2·4	±0·4
Brain	5	2·1	±0·3

Table 9. *Cytochrome oxidase activity of liver and brain*

System: sodium potassium phosphate buffer, pH 7·4, 33 mM; cytochrome *c* 80 µM; $AlCl_3$ 0·4 mM; sodium ascorbate, pH 7·0, neutralized and added just before start of experiment, 11·4 mM; liver equivalent to 8 mg. of fresh tissue was added as 2% homogenate in water; brain equivalent to 14 mg. of fresh tissue was added as 4% homogenate in water; water to 3 ml. final vol.; gas phase, air; temp. 38°; equilibration period 10 min. Q_{O_2} determination is based upon oxygen uptake during 20 min. and corrected for auto-oxidation of ascorbate.

Tissue	No. of animals	Q_{O_2} (µl. of O_2 taken up/mg. fat-free dry wt. of enzyme/hr.) Range	Mean	S.D.
Liver (adult)	8	194–290	249	±34
Brain (adult)	4	107–162	137	±23
Liver (lamb)	2	144–146	145	—

Specific enzyme systems

Cytochrome oxidase. Sheep liver and brain, in common with tissues of other species, have a large reserve of cytochrome oxidase activity (Table 9). The Q_{O_2} values are about half those for rat tissues and, as in the rat, the activity of this enzyme system is lower in the young than in the adult animal (Gallagher, 1955; Gallagher *et al.* 1956*b*).

Catalase. Ox liver is used preparatively as a rich source of catalase (Colowick & Kaplan, 1955), so it is not unexpected that ruminant liver should possess high catalase activity (Table 10). What is surprising is the disproportionately high activity of liver catalase as compared with other enzyme systems and other species. In general sheep enzyme systems are about one-third to one-half as active as rat enzyme systems (Gallagher, 1955; Gallagher *et al.* 1956*b*). By contrast sheep liver has about 20 times the catalase activity of rat liver. Sheep brain has very little, if any, catalase.

DISCUSSION

Sheep tissues possess quite high enzymic activities—in general about one-third to one-half of the activities of rat tissues. The ratio between the sheep and the rat for specific enzyme systems and substrate oxidations found in this study agrees with Krebs's (1950) figures for a more general system.

Within the limited number of animals studied no apparent effect of age, sex or nutrition was shown except a lower Q_{O_2} for cytochrome oxidase in the lamb than in the adult. A similar influence of age on the cytochrome oxidase system in the rat had been observed earlier (Gallagher, 1955; Gallagher *et al.* 1956*b*).

Points of interest are the high oxidative capacity of brain, with the notable exception of citrate oxidation, the rate of oxidation of L-malate and the distribution of enzymes as indicated by the relative Q_{O_2} values for homogenates and mitochondria.

It should be remembered when comparing the results for liver and brain that the brain mitochondria have an additional supplement of respiratory cofactors. However, the same medium is used for both tissues in the homogenate experiments, and these provide an adequate basis for comparison. Krebs (1950) recorded higher Q_{O_2} values for brain slices than for liver slices in a number of species, including sheep and rats, although Albritton (1954) published data showing the rat liver to be much more active than rat brain in oxidizing succinate. The present study supports

Table 10. *Catalase activity of liver and brain*

System: H_2O_2, 0·02N, 35 ml.; sodium phosphate buffer, pH 6·8, 0·66M, 10 ml.; enzyme, 1 ml. of 0·1% and 0·05% liver or 1 ml. of 4% and 2% brain homogenates in water; water to 50 ml. final vol.; temp. 0°; incubation period of 2 min. was terminated by discharging 5 ml. of reaction mixture into 10 ml. of N-H_2SO_4. Residual H_2O_2 was titrated with 2 mN-$KMnO_4$. Allowance was made for reducing substances in the enzyme preparation by blank incubation and titration without H_2O_2. Catalase activity is expressed as Kat.f. (Euler & Josephson, 1927), which is K/g. fat-free dry wt. of homogenate where $K = 1/t \ln (Co/Ct)$; t = time of reaction (min.); Co = concn. of H_2O_2 before addition of enzyme and Ct = concn. of H_2O_2 at time t.

Tissue	No. of animals	Kat.f. Range	Mean	S.D.
Liver	7	2200–3300	2725	±387
Brain	5	6–41	13	± 15

Krebs's findings and reveals a higher rate of oxidation by brain than by liver preparations for all substrates except succinate and citrate. Succinate is oxidized about equally fast by both tissues. The very slow oxidation of citrate by brain is in agreement with the studies of Coxon, Liébecq & Peters (1949) for pigeon brain, and Christie *et al.* (1953) and Gallagher *et al.* (1956*a*) for rat brain.

Comparing the rates of substrate oxidations by sheep brain with the rates reported by Christie *et al.* (1953) for rat brain it is apparent that L-malate is oxidized more rapidly relative to other substrates in sheep than in rat brain.

Conclusions cannot be drawn about the intracellular distribution of enzymes from these experiments. Homogenate activities are expressed on a fat-free dry-weight basis, whereas mitochondrial activities are expressed in terms of nitrogen and are therefore not strictly comparable. However, it is of interest to observe the trends shown. A good proportion of the homogenate activity for α-oxoglutarate, succinate, pyruvate and L-glutamate oxidation is present in the mitochondrial preparations. However, the rate of oxidation by mitochondria as compared with homogenates is low for citrate, fumarate and L-malate, although brain mitochondria oxidize L-malate at a faster rate compared with homogenates than do liver mitochondria. These results suggest that either a larger proportion of the enzyme system concerned is extra-mitochondrial or is lost from the mitochondria during isolation. The same pattern of enzyme distribution for rat liver was reported by Christie & Judah (1953), who found L-glutamic dehydrogenase to be entirely mitochondrial and L-malic dehydrogenase to be distributed about equally between mitochondria and supernatant.

The outstanding enzyme differences found in sheep as compared with non-ruminants are the extraordinarily high activity of sheep-liver catalase and the dependence of sheep-liver metabolism upon the oxidation of fatty acids.

The metabolic significance of catalase in the sheep is unknown but recent work suggests a possible explanation for the high activity in liver. The administration of hydrocortisone or cortisone depresses catalase activity considerably, indicating a possible regulatory role for the steroids (Troop, 1958). Dr R. L. Reid and his colleagues at the Sheep Biology Laboratory, C.S.I.R.O. (personal communication) have found the level of adrenal steroids in the plasma of normal sheep to be very low. It is, then, conceivable that the high activity of liver catalase in sheep develops as a consequence of the absence of regulatory levels of adrenocortical steroid usual in non-ruminant species. Brain, however, has hardly any catalase. Elliott (1934) reported the absence of catalase in rabbit brain.

Sheep-liver enzyme preparations cannot metabolize glucose *in vitro* unless hexokinase is added. Inability of liver homogenates to catabolize glucose anaerobically is not peculiar to sheep. Stoesz & LePage (1949) have found the same for rats and Long (1952) has shown that hexokinase activity is very low in rat-liver homogenates. However, in sheep as opposed to non-ruminants, owing to the unavailability of carbohydrate for absorption as such from the alimentary canal and the dependence upon liver gluconeogenesis to supply blood glucose and liver glycogen, failure to catabolize glucose may assume greater importance.

Glucose is not normally present in the ruminant diet and it is improbable that when present much reaches the small intestine or is absorbed from the rumen (Phillipson & McAnnally, 1942; Elsden, 1945). Even on rations very high in carbohydrate the amount which escapes ruminal fermentation and passes into the abomasum is insignificant (Weller & Gray, 1954). Large quantities of volatile fatty acids, chiefly acetic acid, propionic acid and butyric acid, are produced by fermentation of foodstuffs in the rumen and are absorbed directly from the fore-stomachs (Elsden & Phillipson, 1948). The supply of glucose for utilization by peripheral tissues, notably the central nervous system and, in the pregnant ewe, the placenta for conversion into fructose to meet foetal demands, must come from gluconeogenesis. The liver will, of course, be the major site of glucose production from non-carbohydrate sources. Consequently, it is not surprising that liver metabolism is biased towards the formation of glucose rather than its destruction. The glucokinase reaction is the most effective point for such bias to be applied. Deficiency of glucokinase, although limiting the phosphorylation of glucose,

does not much affect the reverse reaction, which is catalyzed by a different enzyme, glucose 6-phosphatase. The defect in glucose catabolism is present from birth.

Sheep liver has then an unusual pattern of metabolism, being very largely dependent upon the oxidation of fatty acids for the maintenance of tricarboxylic acid cycle activity. Under normal conditions volatile fatty acids are supplied in abundance via the portal circulation (McAnnally & Phillipson, 1942; Barcroft, McAnnally & Phillipson, 1944 and Elsden & Phillipson, 1948). Acetyl-coenzyme A is supplied by the oxidation of all fatty acids except propionic acid, which provides a source of oxaloacetate either via pyruvate and triphosphopyridine nucleotide malic dehydrogenase or, much more likely, via methylmalonyl-coenzyme A, to enter the tricarboxylic acid cycle as succinate (Lardy & Adler, 1956; Pennington & Sutherland, 1956*b*; Flavin & Ochoa, 1957). Amino acid metabolism can also provide oxaloacetate. This metabolic scheme is undoubtedly an efficient one under normal conditions. However, the unavailability of glucose, as an alternative source to fatty acids of acetyl-coenzyme A, leaves the sheep, and probably other ruminants, particularly prone to liver failure. Inhibition of the fatty acid-oxidation sequence or a fall in the supply of fatty acids to the liver will lead to a directly proportional failure of liver metabolism. Liver glycogen in the sheep will be formed essentially from non-carbohydrate sources and, if fatty acid oxidation fails, the glycogen reserve will be soon exhausted in supplying blood glucose or acetyl-coenzyme A. The glycogenic amino acids which may be available cannot much affect the issue. The possibility arises that this unusual liver metabolism with its dependence upon only one of the two usual sources of energy may be concerned in the development of certain metabolic diseases of ruminants.

SUMMARY

1. The activities of a number of enzyme systems of sheep liver, brain and kidney have been investigated.

2. Sheep tissues in general have one-third to one-half of the enzymic activities reported for rat tissues.

3. High liver catalase is the outstanding feature found in sheep.

4. Young sheep have lower cytochrome oxidase activity than adults.

5. Liver enzyme preparations are unable to catabolize glucose owing to glucokinase deficiency. The possible significance of this in relation to ruminant metabolism is discussed.

6. The dependence of sheep-liver metabolism upon the oxidation of fatty acids is suggested as a factor of possible importance in ruminant diseases.

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The Occurrence of Guanidinoacetic Acid and Other Substituted Guanidines in Mammalian Liver

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The synthesis of creatine from guanidinoacetic acid in liver was originally demonstrated by Borsook & Dubnoff (1940*a*, *b*), and these authors later concluded that guanidinoacetic acid was not a normal constituent of that organ (Borsook & Dubnoff, 1941). This conclusion was confirmed by Bodansky, Duff & McKinney (1941), who reported that no guanidinoacetic acid could be detected in normal animals. However, Melville & Hummel (1951) reported 3·3–11·0 mg. of this acid/100 g. of rabbit liver, compared with a creatine content of 4–26 mg./100 g. These authors estimated guanidinoacetic acid by a modification (Macpherson, 1942) of the Sakaguchi reaction, after destruction of the arginine present with arginase. Eden, Harrison & Linnane (1954*a*, *b*), using a method similar to that of Melville & Hummel (1951), reported appreciable amounts of guanidinoacetic acid in normal rat liver. The last-mentioned findings were at variance with the early reports as well as with some observations made in this Laboratory. In view of this, and because of the considerable error which would be introduced into the estimation of liver creatine (Ennor & Stocken, 1948) if large amounts of guanidinoacetic acid were present, the problem has been reinvestigated.

The subject-matter of this paper was presented at a meeting of the Australian Biochemical Society in Adelaide, August, 1958.

EXPERIMENTAL

All guanidine compounds used were as previously described (Rosenberg, Ennor & Morrison, 1956). All organic solvents used in chromatography were purified by fractional distillation.

Preparation of tissue extracts. Rats, starved for 16 hr., were killed by cervical dislocation and the livers removed, rinsed in water and weighed. Single or pooled livers were dispersed in a Potter–Elvehjem type of homogenizer with 5 vol. of 1·5N-$HClO_4$. The suspension was centrifuged and the supernatant filtered. The residue was re-extracted with 3 vol. of 1·5N-$HClO_4$ and the supernatant, after filtration, combined with the original extract. The final volume of the pooled extracts did not exceed ten times that of the original tissue. A small sample (5–10 ml.) was removed and adjusted to pH 7·2 by the addition of 10N-KOH. The mixture was cooled to 0° and filtered to remove $KClO_4$. This neutralized extract was used for the colorimetric estimation of total guanidine.

The remainder of the extract was then passed through a column containing Zeo-Karb 225 ion-exchange resin, H^+ form. The volume of the resin bed was about 20 ml./250 ml. of extract, and with a flow rate of 3 ml./min. the effluent was free from guanidines. The column was then washed with water and eluted with aq. 2·5N-NH_3 soln. (flow rate, 1 ml./min.). The effluent was collected in 2 ml. fractions and the first guanidine-positive fraction appeared when the pH of the effluent rose above 7. All guanidine-positive fractions were pooled and the excess of ammonia was removed by vigorous boiling under reduced pressure. The solution was then adjusted to pH 6·8 with 5N-HCl and freeze-dried.