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Production and Utilization of Acetate in Mammals

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1. In an attempt to define the importance of acetate as a metabolic precursor, the activities of acetyl-CoA synthetase (EC 6.2.1.1) and acetyl-CoA hydrolase (EC 3.1.2.1) were assayed in tissues from rats and sheep. In addition, the concentrations of acetate in blood and liver were measured, as well as the rates of acetate production by tissue slices and mitochondrial fractions of these tissues. 2. Acetyl-CoA synthetase occurs at high activities in heart and kidney cortex of both species as well as in rat liver and the sheep masseter muscle. The enzyme is mostly in the cytosol fraction of liver, whereas it is associated with the mitochondrial fraction in heart tissue. Both mitochondrial and cytosol activities have a K_m for acetate of 0.3 mM. Acetyl-CoA synthetase activity in liver was not altered by changes in diet, age or alloxan-diabetes. 3. Acetyl-CoA hydrolase is widely distributed in rat and sheep tissues, the highest activity being found in liver. Essentially all of the activity in liver and heart is localized in the mitochondrial fraction. Hepatic acetyl-CoA hydrolase activity is increased by starvation in rats and sheep and during the suckling period in young rats. 4. The concentrations of acetate in blood are decreased by starvation and increased by alloxan-diabetes in both species. The uptake of acetate by the sheep hind limb is proportional to the arterial concentration of acetate, except in alloxan-treated animals, where uptake is impaired. 5. Acetate is produced by liver and heart slices and also by heart mitochondrial fractions that are incubated with either pyruvate or palmitoyl-(−)-carnitine. Liver mitochondrial fractions do not form acetate from either substrate but instead convert acetate into acetoacetate. 6. We propose that acetate in the blood of rats or starved sheep is derived from the hydrolysis of acetyl-CoA. Release of acetate from tissues would occur under conditions when the function of the tricarboxylic acid cycle is restricted, so that the circulating acetate serves to redistribute oxidizable substrate throughout the body. This function is analogous to that served by ketone bodies.

A large proportion of the energy requirements of herbivorous mammals is derived from the oxidation of acetate that has been formed by microbial fermentation in the rumen or in the caecum (Blaxter, 1962). The turnover of blood acetate is rapid in these species (Annison & Armstrong, 1970), presumably because tissues that have the potential to utilize acetate have high activities of acetyl-CoA synthetase (EC 6.2.1.1):

$$\text{ATP} + \text{acetate} + \text{CoA} \rightleftharpoons \text{AMP} + \text{PP}_i + \text{acetyl-CoA}$$

Acetate is probably significant in non-herbivores also, since it is rapidly cleared from the blood if injected *in vivo* or generated from precursors such as ethanol (Ciaranfi & Fonnesu, 1954; Smyth, 1947; Krebs & Perkins, 1970). Further, acetate is present in the blood of fed or starved non-herbivores at concentrations that are in the range 0.1–0.5 mM, values that are not much lower than those found in herbivores (Annison & Armstrong, 1970; Ballard, 1972). The source of this acetate in non-herbivores or in all starved mammals is not known, although long-chain fatty acids or pyruvate are converted into acetyl-CoA, which may be hydrolysed to acetate by acetyl-CoA hydrolase (EC 3.1.2.1):

$$\text{Acetyl-CoA} + \text{H}_2\text{O} \rightleftharpoons \text{acetate} + \text{CoA}$$

In the present work we have investigated the distribution and adaptability of acetyl-CoA synthetase and acetyl-CoA hydrolase in sheep and rat tissues in an attempt to locate the potential sites of acetate formation and utilization. Further experiments with heart and liver tissue from these species incubated *in vitro* show that acetate can be formed from both pyruvate and fatty acids, particularly under conditions where the oxidation of acetyl-CoA is restricted.

Materials and Methods

Animals

The adult rats used were 7-week-old male Wistar animals bred in the Division under specific pathogen-free conditions. They were fed on a high-carbohydrate

diet *ad libitum*, except during starvation experiments. Details of the exposure of 2-day-old rats to anoxia and the method for obtaining freeze-clamped liver samples from these animals have been described previously (Ballard, 1971*a*).

Sheep were 1-year-old Merino wethers that were given 1kg of wheaten hay chaff–lucerne hay chaff (3:1, w/w) daily.

Diabetic animals were used 4–6 days after the intravenous (sheep) or intramuscular (rats) injection of 60mg of alloxan/kg body wt. The concentration of blood glucose of all diabetic animals was greater than 15mM.

Chemicals

Citrate synthase (EC 4.1.3.7), malate dehydrogenase (EC 1.1.1.37), acetate kinase (EC 2.7.2.1), lactate dehydrogenase (EC 1.1.1.27), pyruvate kinase (EC 2.7.1.40) and 3-hydroxybutyrate dehydrogenase (EC 1.1.1.30) were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, W. Germany; ATP, CoA, acetyl-CoA, NADH, NAD^+ and phosphoenolpyruvate were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A.; palmitoyl-(−)-carnitine was a gift from Dr. Alan Snoswell of the University of Adelaide.

Preparation of tissue extracts for enzyme assays

For the measurement of total enzyme activities, tissues were homogenized in 9vol. of 0.25M-sucrose at 0°C and sonicated twice for 10s each time by using a Soniprobe (Dawe Instruments Ltd., London W.3, U.K.) operated at maximum energy. The homogenate was kept in an ice–salt mixture during sonication.

The intracellular distribution of acetyl-CoA hydrolase was determined by the differential extraction procedure developed by Pette (1966). Briefly, minced tissue was stirred with 20vol. of 0.25M-sucrose for 10min followed by centrifugation at $100000g_{av.}$ for 10min. The supernatant was collected and the extraction repeated two additional times, after which the pellet was freeze-dried and then suspended in 20vol. of 0.1M-potassium phosphate buffer, pH7.0.

Enzyme assays

Acetyl-CoA synthetase. This was measured by a modification of the method described by Stacey *et al.* (1964). Eppendorf centrifuge tubes containing 33mM-Tris buffer, pH8.0, 3.3mM-ATP, pH8.0, 6.7mM-$MgCl_2$, 1.3mM-dithiothreitol, 3.3mM-potassium acetate containing 0.5μCi of potassium [1-^{14}C]-acetate (The Radiochemical Centre, Amersham, Bucks., U.K.), 17mM-potassium oxaloacetate, 4μg of citrate synthase, 0.8mM-CoA and enzyme extract in a total volume of 0.3ml were incubated for 0, 10 or 20min at 37°C. The reaction was stopped by the addition of 50μl of 10% (w/v) trichloroacetic acid. After centrifugation at $15000g_{av.}$ for 2min, 50μl portions of the supernatant were placed on filter-paper strips and placed over a steam bath for 30min to remove unused acetic acid by distillation. The paper strips were dried and the radioactivity determined in vials containing 10ml of a solution of 4g of 2,5-diphenyloxazole and 100mg of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene per litre of toluene. CoA was omitted from controls. The enzyme activity was expressed as nmol of acetate converted into non-volatile products/s (nkat). The addition of excess of oxaloacetate and citrate synthase to the assay removed acetyl-CoA as citrate and prevented product inhibition. Under these conditions the rate of reaction was linear with time up to 20min and with enzyme for up to 0.25nkat of acetyl-Co Asynthetase. It was usually necessary to purify the radioactive potassium acetate by steam distillation to remove non-volatile impurities.

Acetyl-CoA hydrolase. Activities were measured as the rate of CoA formation from acetyl-CoA. Eppendorf tubes containing 100mM-potassium phosphate buffer, pH7.0, 4mM-acetyl-CoA and tissue extract in a volume of 0.25ml were incubated for 0, 5, 10 or 15min at 37°C. The reaction was stopped by the addition of 200μl of 9% (w/v) $HClO_4$ and after centrifugation at $15000g_{av.}$ for 2min the supernatant was neutralized by the addition of 100μl of 3M-$KHCO_3$. The solution was centrifuged again and free CoA measured in 200μl portions of the supernatant by the nitroprusside method with GSH as standard (Grunert & Phillips, 1951). The enzyme activity was linear with time for 15min and with enzyme for up to 0.03nkat (nmol of CoA formed/s) of acetyl-CoA hydrolase. In some experiments the enzyme activity was also measured by the rate of removal of acetyl-CoA (Hanson & Ballard, 1967), or by the rate of acetate formation (Ballard *et al.*, 1972). Good stoicheiometry was found with the three methods.

The activities of NAD^+–malate dehydrogenase (Ochoa, 1955*b*), citrate synthase (Ochoa, 1955*a*), glutamate dehydrogenase (EC 1.4.1.2) (Ballard, 1971*b*) and lactate dehydrogenase (Kornberg, 1955) were measured at 37°C and the activities were expressed as the percentage of the total activity extracted.

Measurements of acetate in blood and liver

Blood or freeze-clamped liver was added to 5vol. of 6% (w/v) $HClO_4$ at 0°C and the precipitate removed by centrifugation. After re-extraction of the precipitate with 2vol. of 6% (w/v) $HClO_4$ followed by centrifugation, the combined supernatants were neutralized with 3M-$KHCO_3$, cooled to 0°C, and the precipitated $KClO_4$ was removed by centrifugation.

Acetate was measured in these extracts by the rate of ADP production in the presence of acetate kinase. Spectrophotometer cuvettes contained 125mM-Tris–HCl buffer, pH7.8, 12.5mM-ATP (adjusted to pH7 with KOH), 25mM-$MgCl_2$, 3.1mM-phosphoenolpyruvate, 0.6mM-NADH, 20μg of lactate dehydrogenase, 20μg of pyruvate kinase and extract in a volume of 0.8ml. After incubation at 25°C for 5min to remove any ADP or pyruvate, the concentration of acetate was measured as the rate of NADH oxidation at 340nm on addition of 20μg of acetate kinase. Under these conditions there was a linear rate of NADH oxidation for 3min when extracts or standards containing from 5 to 100nmol of acetate were used. The rate of absorbance change was linear with up to 60nmol of acetate. This kinetic assay was used in preference to a measurement of the total amount of NADH oxidized (Ballard *et al.*, 1972) because spontaneous hydrolysis of the acetyl phosphate formed produced a gradual decrease in absorbance that became faster with high initial concentrations of acetate. This was particularly evident with measurements on $HClO_4$ extracts of liver.

Incubation of tissue slices

Liver or heart slices of 0.3mm uniform thickness were cut with the aid of a grooved plastic block (Ballard & Oliver, 1964). Approx. 300mg fresh weight of slices was incubated with shaking in 3ml of Krebs–Ringer bicarbonate medium (Umbreit *et al.*, 1959) for 30, 60 or 90min at 37°C. The reaction was stopped with 1ml of 20% (w/v) $HClO_4$. After homogenization of the tissue plus incubation mixture, the homogenate was centrifuged. The supernatant was neutralized with 3M-$KHCO_3$ and $KClO_4$ was removed by a second centrifugation. The concentration of acetate was measured in the clear supernatant and changes expressed as the production or utilization of acetate during the 90min period.

Preparation and incubation of mitochondria

Portions of liver or heart were chilled in 0.15M-KCl at 0°C and homogenized in 4vol. of a solution containing 100mM-KCl, 50mM-Tris, 1mM-ATP and 5mM-$MgSO_4$ at pH7.4 in a Teflon–glass coaxial homogenizer. The homogenate was centrifuged at $700g_{av.}$ for 10min to remove nuclei and cell debris and then at $10000g_{av.}$ for 15min to sediment the mitochondrial fraction. The mitochondrial fraction was washed twice by suspension in 0.25M-sucrose followed by centrifugation at $10000g_{av.}$ for 15min, and finally suspended in 0.25M-sucrose to give a concentration of approx. 15mg of protein/ml.

Mitochondrial suspension (1ml) was mixed with 19mM-potassium phosphate buffer, 25mM-$NaHCO_3$, 80mM-KCl, 4.4mM-$MgCl_2$ and 44mM-sucrose. The pH was 7.4 and the final volume was 8.0ml. Other additions are indicated in the individual experiments. The mixture was equilibrated with O_2+CO_2 (95:5) and incubated with shaking at 37°C. At various times 1ml portions were removed, inactivated with $HClO_4$, and a neutralized extract was prepared as described above for acetate measurements. The concentration of acetoacetate (Mellanby & Williamson, 1965), 3-hydroxybutyrate (Williamson & Mellanby, 1965), pyruvate (Bücher *et al.*, 1965) and acetate were measured in the mitochondrial extracts.

Protein measurements

Protein was determined in tissue extracts by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Surgical techniques

After infiltration of local anaesthetic under the skin on the inside of the thigh of a sheep, an incision was made over the underlying saphenous artery and vein. These two vessels were exposed and a polyvinyl chloride tube (1.2mm external diam., 0.8mm internal diam.) was inserted into each vessel and passed cranially into the femoral artery and vein for a distance of about 14cm. Blood from these cannulae thus gave an arterial sample and a sample of femoral venous blood predominantly draining the muscles of the hind limb. The cannulae were flushed daily and filled with sterile 0.9% (w/v) NaCl solution containing 100 units of heparin/ml (Evans Medical Australia, Pty., Ltd., Boronia, Vic., Australia).

Results

Tissue and intracellular distribution of acetyl-CoA synthetase and acetyl-CoA hydrolase

Acetyl-CoA synthetase is present at high activities in heart and kidney cortex of both rats and sheep as well as in rat liver and in the sheep masseter muscle (Table 1). Since activities are low in rat muscles and in the other sheep muscles tested, it is probable that the high activity in the masseter muscle is a specific adaptation to the continual masticating action of the sheep jaw.

Acetyl-CoA hydrolase is present in all tissues tested (Table 2), with the highest activity in liver, where the enzyme has approximately twice the activity of acetyl-CoA synthetase, as measured in our assays. In heart, kidney cortex and adipose tissue of both sheep and rats and in the sheep masseter muscle there is substantially more acetyl-CoA synthetase activity than acetyl-CoA hydrolase, perhaps reflecting that acetate utilization is more important in these tissues than acetate production.

Table 1. *Tissue distribution of acetyl-CoA synthetase*

Acetyl-CoA synthetase activities [in nmol converted/s (nkat)] were measured in sonicated homogenates as described in the Materials and Methods section. Values are the means ± S.E.M. for measurements on between four and six animals.

Tissue	Acetyl-CoA synthetase activity (nkat/g wet wt. of tissue)	(nkat/g of tissue protein)
Rat		
Liver	42.5 ± 4.3	223 ± 27
Heart	49.5 ± 14.4	440 ± 163
Kidney cortex	66.3 ± 23.3	422 ± 125
Brain	7.7 ± 1.8	72 ± 17
Leg muscle	2.2 ± 0.7	23 ± 7
Epididymal adipose tissue	3.0 ± 1.2	235 ± 60
Sheep		
Liver	12.5 ± 2.2	67 ± 8
Heart	69.0 ± 14.7	829 ± 82
Kidney cortex	70.2 ± 6.8	545 ± 32
Brain	4.7 ± 1.2	47 ± 14
Gastrocnemius muscle	4.7 ± 2.2	33 ± 10
Soleus muscle	4.0 ± 2.0	32 ± 14
Masseter muscle	67.5 ± 5.8	750 ± 63
Perirenal adipose tissue	1.0 ± 0.5	262 ± 90

Table 2. *Tissue distribution of acetyl-CoA hydrolase*

Acetyl-CoA hydrolase activities [in nmol converted/s (nkat)] were measured in sonicated homogenates by the procedure described in the Materials and Methods section. Values are the means ± S.E.M. for measurements on between four and six animals. Tissue samples were from the same animals used in Table 1.

Tissue	Acetyl-CoA hydrolase activity (nkat/g wet wt. of tissue)	(nkat/g of tissue protein)
Rat		
Liver	80.6 ± 4.5	397 ± 27
Heart	11.2 ± 1.3	92 ± 8
Kidney cortex	13.4 ± 1.5	85 ± 10
Brain	8.2 ± 1.2	78 ± 13
Leg muscle	3.0 ± 0.8	32 ± 10
Epididymal adipose tissue	0.8 ± 0.2	77 ± 12
Sheep		
Liver	30.0 ± 4.0	167 ± 13
Heart	4.8 ± 0.7	57 ± 10
Kidney cortex	8.5 ± 1.2	62 ± 7
Brain	3.3 ± 0.7	35 ± 7
Gastrocnemius muscle	11.8 ± 2.2	120 ± 23
Soleus muscle	12.4 ± 1.5	113 ± 17
Masseter muscle	11.6 ± 1.7	122 ± 18
Perirenal adipose tissue	0.3 ± 0.2	60 ± 23

Initial experiments on the intracellular distribution of acetyl-CoA synthetase and acetyl-CoA hydrolase were performed by differential centrifugation of homogenates in 0.25M-sucrose (Schneider & Hogeboom, 1950). Although the particulate activities of the two enzymes were associated with the mitochondrial marker enzymes citrate synthase and glutamate dehydrogenase, substantial proportions of these mitochondrial enzymes were found in the supernatant obtained by centrifuging the homogenate at $100000 g_{av.}$ for 30min. This was presumably due to mitochondrial breakage during preparation or treatment of the homogenates. Subsequent experiments on the intracellular distribution of the enzymes of acetate metabolism were carried out by using the extraction method of Pette (1966). The marker enzymes used for liver were lactate dehydrogenase for cytosol and glutamate dehydrogenase for mitochondria, with malate dehydrogenase included as an example of an enzyme with bimodal distribution. In rat liver, acetyl-CoA synthetase was as rapidly extracted as lactate dehydrogenase, an indication of a cytosol location for the enzyme (Fig. 1*a*). Sheep liver, on the other hand, contained only about 60% of the acetyl-CoA synthetase activity in a form that was extractable with sucrose, the remainder of the activity being particulate as defined by the Pette (1966) procedure (Fig. 1*b*). In livers of the two species approx. 80% of the acetyl-CoA hydrolase activity was particulate, although the remaining activity had mostly been released in the first sucrose wash (Figs. 1*a* and 1*b*).

Studies on the intracellular distribution of acetyl-CoA synthetase and acetyl-CoA hydrolase in heart were made by the Pette (1966) procedure with lactate dehydrogenase, citrate synthase and malate dehydrogenase included as markers (Fig. 2). In rat heart (Fig. 2*a*) most of the acetyl-CoA synthetase and acetyl-CoA hydrolase activities were localized in a particulate fraction, with possibly 10% of the acetyl-CoA hydrolase and 15% of the acetyl-CoA synthetase of cytosolic origin. In sheep heart, acetyl-CoA synthetase has a similar distribution to citrate synthase, whereas 30% of the acetyl-CoA hydrolase activity was in the cytosol and 70% particulate.

Changes in the concentration of acetate and in the activities of acetyl-CoA synthetase and acetyl-CoA hydrolase during metabolic alterations

The concentration of acetate in blood from the aorta of fed rats is 200 μM, a value approximately one-third of that found in sheep (Tables 3 and 4). Starvation decreased the acetate concentration in rat blood, but even after 72h acetate was present at 70% of the concentration in fed animals. Alloxan-diabetic rats showed a slight increase in the concentration of blood acetate, to 245 μM. Although some of the acetate found

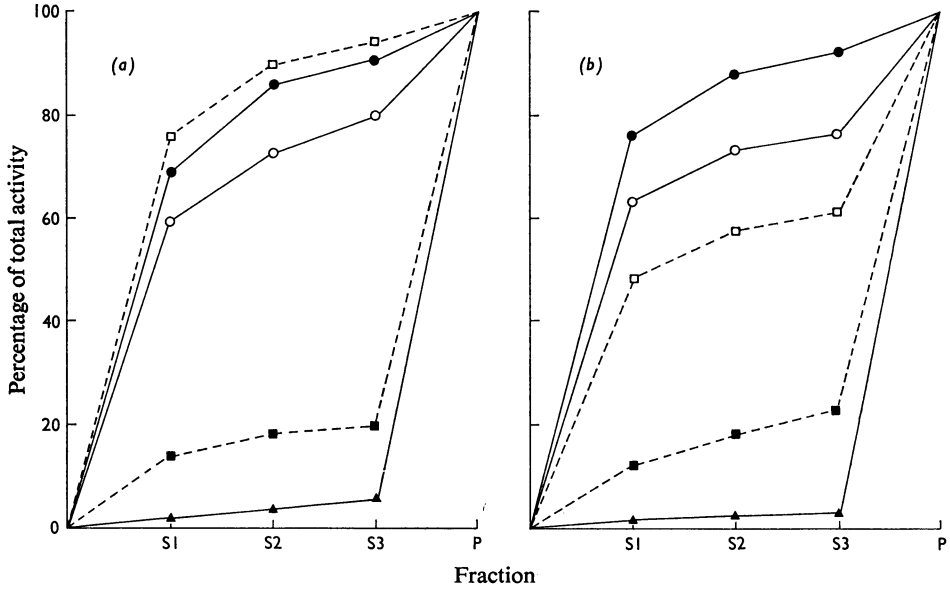

Fig. 1. *Intracellular distribution of acetyl-CoA synthetase and acetyl-CoA hydrolase in liver from rats (a) or sheep (b)*

Tissue was minced and extracted three times sequentially by stirring for 10min with 0.25M-sucrose giving fractions (S1), (S2) and (S3) or with 0.1M-potassium phosphate buffer, pH7.0, followed by freeze-drying to give fraction P. The sucrose extracts were obtained by centrifuging the stirred solution at 100000$g_{av.}$ for 10min. Enzymes measured are lactate dehydrogenase (●), glutamate dehydrogenase (▲), malate dehydrogenase (○), acetyl-CoA synthetase (□) and acetyl-CoA hydrolase (■). The values are means of experiments on extracts from two animals and are expressed as percentages of total extracted activity.

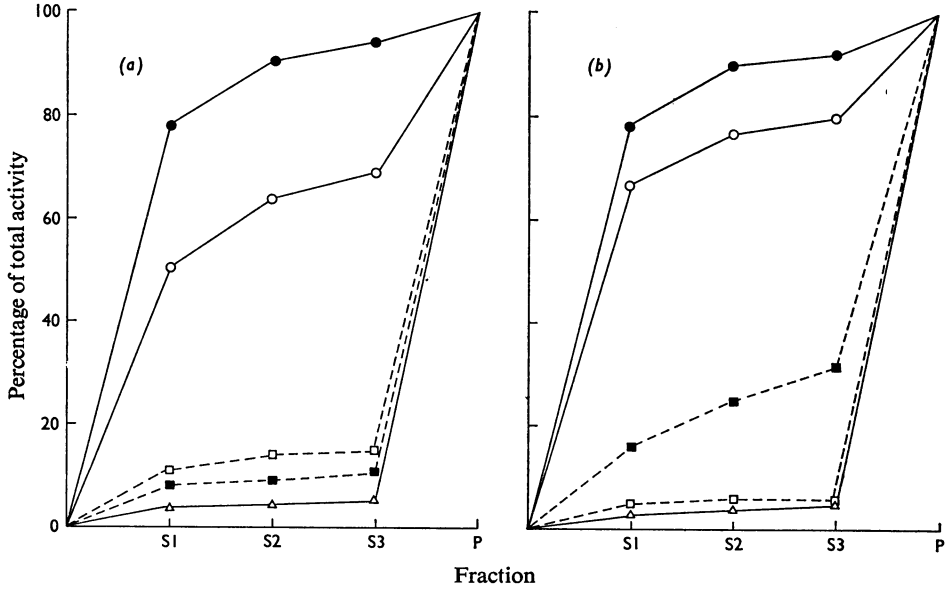

Fig. 2. *Intracellular distribution of acetyl-CoA synthetase and acetyl-CoA hydrolase in heart from rats (a) or sheep (b)*

Details and symbols used are the same as in Fig. 1 except that citrate synthase (△) replaced glutamate dehydrogenase as a mitochondrial marker enzyme. Tissues were from the same two animals as those used in Fig. 1.

Table 3. *Effect of diet and alloxan-diabetes on acetate concentrations in blood and liver of adult rats*

Samples of blood from the aorta or portions of freeze-clamped liver were extracted in $HClO_4$ and the concentration of acetate was measured as described in the Materials and Methods section. Values are the means±S.E.M. for measurements on between six and eight animals.

Treatment of animals	Blood acetate (μM)	Liver acetate (nmol/g wet wt.)
Fed	200±13	628±53
Starved for 24h	173±14	—
Starved for 24h, then re-fed for 24h	184±18	—
Starved for 72h	141±10	519±58
Alloxan-diabetic	245±12	745±82

Table 4. *Effect of diet and alloxan-diabetes on blood acetate concentrations and acetate uptake by the sheep hind limb*

Blood samples were deproteinized with $HClO_4$ and the concentration of acetate measured as described in the Materials and Methods section. Values are the means ±S.E.M. for measurement on eight to ten animals.

	Acetate concentration	
Treatment of sheep	Artery (μM)	Artery–femoral-vein concentration difference (μM)
Fed	630±108	321±60
Starved for 48h	101± 22	35± 8
Starved for 120h	90± 11	25± 9
Starved for 120h, then re-fed for 48h	352± 78	148±48
Alloxan diabetic	2471±151	123±94

in liver would be due to the extracellular acetate of plasma and interstitial fluid, the amount of acetate in freeze-clamped rat liver, 628 nmol/g wet wt., is several-fold higher than blood concentrations, and comparably higher than would be expected if all the acetate was extracellular. Presumably therefore the decrease in the concentration of hepatic acetate to 519 nmol/g wet wt. on starvation and the increase to 745 nmol/g wet wt. that follows alloxan administration represent changes in the concentration of acetate in parenchymal cells of the liver (Table 3).

The dietary changes and the effect of alloxan-diabetes on the concentration of blood acetate in sheep, although qualitatively similar to those found in rats, are of much greater magnitude. Thus withdrawal of food from sheep for 120h decreased the concentration of blood acetate from 630 to 90μM, whereas diabetes produced an increase in the concentration of blood acetate to 2470μM (Table 4). There is consequently a 27-fold difference in the concentration of

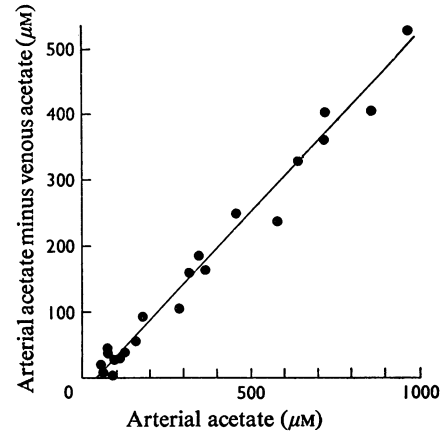

Fig. 3. *Correlation between the concentration of acetate in the femoral artery of fed, starved or starved-re-fed sheep and the femoral artery–femoral vein concentration difference*

The regression, calculated by the method of least squares, has a slope of 0.542±0.019 (S.E.M.) and an intercept on the artery–vein axis of −22.2μM. The correlation coefficient is 0.989. The data used are that given in Table 4.

acetate between the 120h-starved and the alloxan-diabetic sheep. In addition to the measurements of arterial concentrations in sheep, simultaneous samples of femoral-vein blood were taken to measure the difference in acetate concentration across the hind limb. These values for artery–femoral vein (Table 4) indicate a marked decrease in the concentration difference from 321μM in fed sheep to 25μM after starvation for 120h. On the other hand, the large increase in acetate concentrations in the blood of diabetic sheep is accompanied by a decrease in the artery–femoral vein concentration difference. When the arterial concentrations and the artery–femoral vein concentration differences for acetate are plotted for the data on fed, starved and re-fed sheep, a clear relationship is evident (Fig. 3), with a correlation coefficient of 0.989. An extrapolation of the linear regression line predicts that if the arterial acetate concentrations become very low, an output of acetate from the hind limb may occur.

The activities of hepatic acetyl-CoA synthetase are not altered by starvation, or by starvation followed by re-feeding of rats, whereas the enzyme in sheep liver is not changed by starvation or by diabetes (Table 5). In both rat and sheep liver the activity of acetyl-CoA hydrolase increases when the animals are starved, to activities that are approx. 50% higher than in the fed animal. There is thus an increase in the ratio of activities of acetyl-CoA hydrolase to acetyl-CoA synthetase during starvation, a change that should favour acetate production by liver.

Table 5. *Nutritional effects on hepatic acetyl-CoA synthetase and acetyl-CoA hydrolase activities*

Enzyme activities are expressed as nmol converted/s (nkat) with the assays described in the Materials and Methods section. Values are means±S.E.M. for determinations on five animals in each group.

Animal	Treatment	Acetyl-CoA synthetase (nkat/g wet wt.)	Acetyl-CoA hydrolase (nkat/g wet wt.)
Rat	Fed	42.5±4.3	79.0±4.5
	Starved for 24h	35.5±7.7	105 ±4.2
	Starved for 24h, then re-fed for 24h	36.7±6.3	80.5±7.3
Sheep	Fed	12.5±2.2	30.0±4.0
	Starved for 120h	14.2±3.2	47.5±2.5
	Alloxan diabetic	13.8±1.8	33.3±6.0

Table 6. *Michaelis constants for acetate and acetyl-CoA*

Michaelis constants were determined for acetate in the acetyl-CoA synthetase reaction and for acetyl-CoA in the acetyl-CoA hydrolase reaction by using the enzyme assays described in the Materials and Methods section. Mitochondrial and cytosol extracts were fractions P and S_1 respectively (Figs. 2 and 3). Values are the means of determinations on three animals.

Source of enzyme	Michaelis constants for: Acetate (mM)	Michaelis constants for: Acetyl-CoA (mM)
Rat liver cytosol	0.28	—
Rat liver mitochondrial fraction	—	0.76
Rat heart mitochondrial fraction	0.31	—
Sheep liver cytosol	0.39	—
Sheep liver mitochondrial fraction	—	0.66
Sheep heart mitochondrial fraction	0.39	—

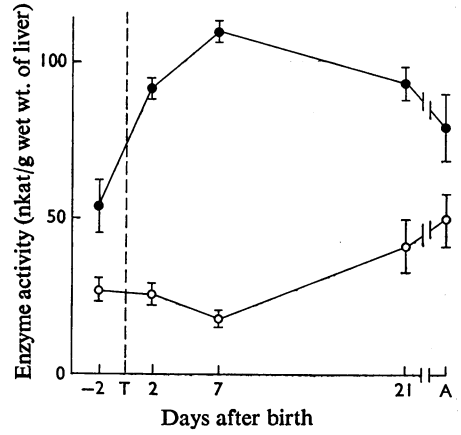

Fig. 4. *Effect of age on the activities of acetyl-CoA synthetase* (○) *and acetyl-CoA hydrolase* (●) *in rat liver*

Values are the means±S.E.M. for determinations on between four and six animals at each age. T, term; A, adult.

The Michaelis constants for acetate in the acetyl-CoA synthetase reaction and for acetyl-CoA in the acetyl-CoA hydrolase reaction were determined by using crude enzyme preparations from liver and heart. Based on the Lineweaver–Burk (1934) graphical method, the Michaelis constant for acetate in the acetyl-CoA synthetase reaction was between 0.28 and 0.39mM, with no marked differences between species, tissue or the intracellular location of the enzyme activity (Table 6). Acetyl-CoA had a K_m of 0.66–0.76mM when tested as substrate for the mitochondrial acetyl-CoA hydrolase from either rat or sheep liver.

Changes in the concentration of acetate, and in the activities of acetyl-CoA synthetase and acetyl-CoA hydrolase in developing rats

The decreased concentrations of blood acetate and the increase in the activities of hepatic acetyl-CoA hydrolase during starvation (Tables 3, 4 and 5) may be the result of an adaptive response which would partially restore acetate concentrations when the exogenous source of acetate is removed. If such a mechanism was operating in liver, it would occur in parallel with ketone-body formation, since this pathway is also increased during starvation (Krebs, 1966). To test a possible relationship of acetate production to ketogenesis, we have measured acetate concentrations and hepatic activities of acetyl-CoA synthetase and acetyl-CoA hydrolase during development in the rat. This comparison was made because ketogenesis does not occur in the foetal rat but increases after birth to activities that are higher than those found in the adult animal (Drahota *et al.*, 1966; Lee & Fritz, 1971). The mean acetate concentration of 214±17μM (S.E.M.) in blood from adult rats was found to be higher than in foetal animals [133±28μM (S.E.M.)] but there are no marked changes associated with birth or the suckling period. Both acetyl-CoA synthetase and acetyl-CoA hydrolase are present in foetal liver at lower activities on a wet-weight basis than found in adults, but by 1 week after

Table 7. *Effect of anoxia on acetate concentrations and on the activities of enzymes of acetate metabolism*

Rats (2 days old) were exposed to N_2 for 20min, after which samples of liver and blood were taken for measurement of the concentrations of acetate, as well as the activities of hepatic acetyl-CoA synthetase and acetyl-CoA hydrolase. Values are means±S.E.M. for determinations on six animals in each group.

Measurement	Normal	Anoxic
Blood acetate (μM)	146±12	245±16
Liver acetate (nmol/g wet wt.)	207±23	337±41
Acetyl-CoA synthetase (nkat/g wet wt.)	30.3±4.2	32.3±2.5
Acetyl-CoA hydrolase (nkat/g wet wt.)	108±4.2	118±4.3

birth hepatic acetyl-CoA synthetase activity has fallen from 27nkat/g wet wt. to 18nkat/g wet wt. (significant by Student's *t* test at the 5% confidence level) while there has been an increase in acetyl-CoA hydrolase activity from 53nkat/g wet wt. to 110nkat/g wet wt. (Fig. 4). The ratio of activities of hydrolase to synthetase thus changes from 2.0 in foetal liver to 6.0 by 1 week after birth, before falling to the adult ratio of 1.6.

Although acetate production from long-chain fatty acids or carbohydrate requires the continual oxidation of NADH, and thus would be restricted under hypoxic conditions, it is possible that the sensitivities of the production and utilization reactions may be different. To test this hypothesis, acetate concentrations were measured in liver and blood of normal 2-day-old rats and animals of the same age that had just been exposed to a 100% N_2 atmosphere for 20min. Anoxia was found to result in a marked increase in the concentrations of both blood and liver acetate, whereas the activities of hepatic acetyl-CoA synthetase and acetyl-CoA hydrolase were unaffected (Table 7).

Production of acetate in vitro

The changes in the concentration of blood acetate and in the activity of liver acetyl-CoA hydrolase that accompany development or nutritional alterations could be produced by alterations in the rate of acetate production. Although the major source of this acetate in ruminants would be the gut, it is likely that an additional formation of acetate occurs in some tissues, presumably via acetyl-CoA hydrolase. Further, acetate production may occur when tissues are incubated *in vitro*, and the control of acetate production may be demonstrable under these conditions. We have incubated slices of rat liver and heart in the presence of various concentrations of acetate to see whether the uptake or release of acetate is concentration-dependent. These two tissues were chosen since they differ markedly in their activities of acetyl-CoA hydrolase. Table 8 shows that acetate is produced by both heart and liver slices in the absence of added acetate. Increasing the concentration of acetate in the medium first decreases the production of acetate and then results in net acetate utilization.

Table 8. *Effect of acetate on acetate formation in tissue slices*

Total concentrations of acetate were measured in slices plus incubation medium after incubation of the tissue for 90min in Krebs–Ringer bicarbonate in the presence of various concentrations of added acetate. Values are the means of triplicate incubations on a pool of slices from three animals. A negative value represents acetate uptake. Acetate formation or utilization was approximately linear during the 90min incubation period.

Initial concentration of acetate in the incubation medium (mM)	Acetate formation (μmol/g wet wt. per 90min) in slices from rat: Liver	Heart
0	1.84	1.97
0.4	1.15	0.10
0.8	−0.96	−0.76
1.5	−3.46	−4.88
3.0	−12.4	−9.82

Table 9. *Acetate production by tissue slices*

Total acetate concentrations were measured in slices plus incubation medium at zero time and after 90min incubation in Krebs–Ringer bicarbonate medium. Values are the means±S.E.M. for duplicate determinations on slices from four animals in each group. Acetate formation was approximately linear during the 90min incubation in each experiment.

Animal	Tissue	Acetate formation (μmol/g wet wt. per 90min)
Rat	Liver	1.89±0.17
	Heart	2.39±0.42
Sheep	Liver	0.27±0.17
	Heart	1.34±0.12
Alloxan-diabetic sheep	Liver	0.71±0.05
	Heart	2.36±0.42

A comparison of acetate production by liver and heart slices from rats, fed sheep and diabetic sheep shows that acetate is formed in all cases (Table 9). However, acetate is produced at a lower rate in sheep liver than in rat liver, and alloxan-diabetes results in an increase in acetate production by liver and heart slices.

Since both heart and liver slices produce acetate, and since the activity of acetyl-CoA hydrolase in each

Table 10. *Acetate and acetoacetate production by rat liver and rat heart mitochondrial fraction*

Mitochondrial fractions (2mg of protein/ml) were incubated for various times under the conditions described in the Materials and Methods section. At various time-intervals acetate, acetoacetate, 3-hydroxybutyrate and pyruvate were measured in $HClO_4$ extracts. The initial concentrations of substrates and other additions were sodium pyruvate, 1mM; palmitoyl-(−)-carnitine, 100μM; sodium fluorocitrate, 20μM; and rotenone, 10μM. Values shown are the means of three experiments on different mitochondrial preparations and represent 30min incubation periods. No detectable 3-hydroxybutyrate was formed.

Tissue	Additions	Concentration change (mM)		
		Pyruvate	Acetoacetate	Acetate
Liver	Pyruvate	−0.40	+0.06	0
	Pyruvate, fluorocitrate	−0.56	+0.15	0
	Pyruvate, fluorocitrate, rotenone	−0.07	0	0
	Palmitoylcarnitine	0	+0.15	0
	Palmitoylcarnitine, fluorocitrate, rotenone	0	0	0
Heart	Pyruvate	−0.88	+0.02	+0.40
	Pyruvate, fluorocitrate	−0.87	+0.01	+0.43
	Pyruvate, fluorocitrate, rotenone	−0.11	0	+0.07
	Palmitoylcarnitine	0	+0.02	+0.07
	Palmitoylcarnitine, fluorocitrate, rotenone	0	+0.01	+0.04

tissue is localized in mitochondria, it would be expected that mitochondrial fractions isolated from these tissues would form acetate when incubated with an appropriate substrate. We have used two substrates, palmitoyl-(−)-carnitine, which will give rise to acetyl-CoA by β-oxidation, and pyruvate, which is converted into acetyl-CoA by pyruvate dehydrogenase. Incubation of rat liver mitochondrial fractions showed acetoacetate production from either pyruvate or palmitoyl-carnitine, but no acetate formation even when fluorocitrate was added to restrict the tricarboxylic acid-cycle flux. Heart mitochondrial fractions formed acetate from palmitoyl-(−)-carnitine and especially from pyruvate. Only marginal amounts of acetoacetate were formed by heart mitochondrial fractions, and 3-hydroxybutyrate was not formed in any of the experiments shown in Table 10.

Discussion

A comparison between the metabolism of acetate in rats and sheep must take account of the exogenous supply of acetate from the rumen of the sheep. This acetate will be produced continuously so long as cellulose is being fermented and will thus lead to high concentrations of acetate in portal blood. Rats presumably obtain little acetate from fermentation in the gut, so that the synthesis and release of acetate from rat tissues would be expected to provide most of the circulating acetate. Such 'endogenous' acetate production may also be important in ruminants, although estimates of the relative contribution of endogenous or exogenous sources to the overall metabolism of acetate vary widely (Annison & Armstrong, 1970).

In the present work we have measured acetate concentrations and the activities of acetyl-CoA synthetase and acetyl-CoA hydrolase in various tissues from rats and sheep, and during different nutritional conditions. The coupling of such a broad approach to experiments *in vitro* was chosen since there is little information on the importance of acetate metabolism other than that acetate has a very short half-life in mammalian blood, especially in non-ruminants (Smyth, 1947; Ciaranfi & Fonnesu, 1954; Annison & Armstrong, 1970) and in ruminants before the development of mature rumen function (Jarrett & Filsell, 1960). Experiments with intact animals and with tissues incubated *in vitro* have shown that acetate is metabolized by many tissues, including liver, heart, adipose tissue, kidney and muscle (Mayfield *et al.*, 1966; McCarthy *et al.*, 1958; Regen & Terrell, 1968; Randle *et al.*, 1970; Hanson & Ballard, 1967; Ballard, 1972). The generality of these metabolic capabilities is reflected in the distribution of acetyl-CoA synthetase in all tissues examined, but with relatively high activities in liver, heart, kidney and the sheep masseter muscle. In liver, the activity of acetyl-CoA synthetase was mainly localized in the cytosol fraction as determined by the Pette (1966) differential extraction procedure. This conclusion agrees with other studies where the enzyme has been measured with acetate as substrate (Barth *et al.*, 1971; Snoswell & Koundakjian, 1972). Since enzymes that acylate propionate and butyrate are located in the mitochondria of liver (Aas, 1971; Aas & Bremer, 1968), it is likely that the acetate-activating enzyme in the cytosol is a distinct protein. The location of hepatic acetyl-CoA synthetase in the cytosol obviates the need to transport acetate into mitochondria, since the enzymes involved

in a major fate of acetate in liver, fatty acid synthesis *de novo*, are also localized in the cytosol. On the other hand, acetyl-CoA synthetase occurs in the mitochondria of heart tissue, where it presumably functions to generate acetyl-CoA for oxidation in the citric acid cycle. Perhaps the dual location of acetyl-CoA synthetase between the cytosol and mitochondria reflects the existence of distinct enzymes, a result that has been confirmed with several other enzymes that are found in the two compartments (Ballard & Hanson, 1969; Wada & Morino, 1964; Henderson, 1968).

Although acetyl-CoA synthetase is present at low activities in adipose tissue and in all muscles tested except the sheep masseter, these tissues are known to use large amounts of acetate. Sheep leg muscle, for example, contains only 5% of the acetyl-CoA synthetase activity of heart tissue, although half of the acetate presented to the tissue is removed in a single passage of blood (Fig. 4), except in alloxan-diabetic animals. This situation presumably occurs because more of the acetyl-CoA synthetase present is catalytically active at the physiological concentrations of acetate, than occurs in heart or liver. The block in acetate utilization by the sheep hind limb and possibly other tissues during alloxan-diabetes leads to a large increase in the concentration of blood acetate. This effect has been noted previously and is probably due to a restriction of glucose entry into muscle in the diabetic animal since acetate utilization is dependent on glucose availability (Jarrett *et al.*, 1974).

Acetyl-CoA hydrolase activity is widely distributed in sheep and rat tissues, where it is often present at higher assayed activities than acetyl-CoA synthetase. However, the Michaelis constant for acetyl-CoA in the acetyl-CoA hydrolase reaction is higher than that for acetate metabolism by acetyl-CoA synthetase, whereas the concentration of acetate in cells is probably severalfold greater than that of acetyl-CoA (Snoswell & Koundakjian, 1972; Weland *et al.*, 1964). Although the Michaelis constants have been determined on crude preparations and are therefore less rigorous than measurements with purified enzymes, we interpret the values as indicating that acetate will be utilized rather than produced under most conditions. In both liver and heart acetyl-CoA hydrolase activity is predominantly localized in mitochondrial fractions (Figs. 2 and 3; see also Quraishi & Cook, 1972), and it is this organelle that is the intracellular site of acetyl-CoA formation from either pyruvate or long-chain fatty acids. We have found that acetate is produced by liver or heart slices when acetate was not added to the incubation medium, implying that these tissues can form acetate *in vivo*. Indeed Palmquist (1972) demonstrated that acetate could be produced from palmitate in intact sheep or cattle, and during starvation the proportion of acetate derived from palmitate increased about tenfold. Although the work of Palmquist (1972) did not delineate the tissue site of acetate production, it may be inferred that any tissue that has the capacity to form acetyl-CoA from palmitate may produce acetate, so long as acetyl-CoA hydrolase is present. It may therefore be possible to reconcile the finding that both heart and liver slices can form acetate, even though heart contains a much lower activity of acetyl-CoA hydrolase, since heart has a high capacity for fatty acid oxidation (Garland & Randle, 1964).

Oxidation of pyruvate or palmitoyl-(−)-carnitine by liver mitochondria does not lead to acetate production, and the acetyl-CoA formed is diverted to acetoacetate. Heart mitochondria do not form acetoacetate, so that an increase in acetyl-CoA formation leads to release of acetate. This formation of acetate by heart mitochondria has been noted previously (Davis, 1968; Von Korff, 1967) and is especially favoured under conditions where the tricarboxylic acid cycle is restricted by the availability of ADP or pyruvate. These situations could lead to an increase in acetyl-CoA concentration since the ability to convert acetyl-CoA into acetylcarnitine will be limited by the availability of carnitine. Thus acetyl-CoA hydrolase would function to form acetate and thus relieve 'acetyl pressure' (Pearson & Tubbs, 1967; Snoswell & Koundakjian, 1972). A further example of the restriction of the tricarboxylic acid cycle is the results of the anoxia experiments described in Table 8. The increase in the concentrations of blood and liver acetate that accompanied anoxia probably reflects an inhibition of the tricarboxylic acid cycle while acetyl-CoA formation continues.

We propose that acetate in the blood of non-herbivores or starved herbivores is derived from acetyl-CoA and that acetate functions metabolically in a similar way to the ketone bodies, acetoacetate and 3-hydroxybutyrate. All three compounds would be important in the redistribution of carbon substrates throughout the body.

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