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Measurement of the rates of acetyl-CoA hydrolysis and synthesis from acetate in rat hepatocytes and the role of these fluxes in substrate cycling

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1. Acetyl-CoA hydrolysis, acetyl-CoA synthesis from acetate and several related fluxes were measured in rat hepatocytes. 2. In contrast with acetyl-CoA hydrolysis, most of the acetyl-CoA synthesis from acetate occurred in the mitochondria. 3. Acetyl-CoA hydrolysis was not significantly affected by 24 h starvation or (−)-hydroxycitrate. 4. In the cytoplasm there was a net flux of acetyl-CoA to acetate, and substrate cycling between acetate and acetyl-CoA in this compartment was very low, accounting for less than 0.1% of the total heat production by the animal. 5. A larger cycle, involving mitochondrial and cytoplasmic acetate and acetyl-CoA, may operate in fed animals, but would account for only approx 1% of total heat production. 6. It is proposed that the opposing fluxes of mitochondrial acetate utilization and cytoplasmic net acetate production may provide sensitivity, feedback and buffering, even when these fluxes are not linked to form a conventional substrate cycle.

INTRODUCTION

Acetate metabolism in mammalian tissues is initiated by acetyl-CoA synthetase (EC 6.2.1.1), which converts acetate into acetyl-CoA. However, many tissues also hydrolyse acetyl-CoA, via a mitochondrial acetyl-CoA hydrolase (EC 3.1.2.1; Knowles *et al.*, 1974), thereby allowing substrate cycling between mitochondrial acetate and acetyl-CoA. This cycling results in the net hydrolysis of ATP which, if large, could serve to dissipate excess fuel in response to an increased blood concentration of acetate (Jessop *et al.*, 1986). However, in sheep muscle *in vivo*, the rate of this cycle is very low (approx. 0.01 μmol/min per g of muscle) and the resultant ATP hydrolysis would represent only 0.5% of the total heat production. Consequently, its regulatory and energetic significance is likely to be negligible (Crabtree *et al.*, 1987).

In contrast, the rate of acetyl-CoA hydrolysis in rat hepatocytes is quite high (1–7 μmol/min per g dry wt.) and occurs despite a net uptake of acetate by the cells, suggesting that an active substrate cycle between acetate and acetyl-CoA may operate in liver (Rabkin & Blum, 1985; Jessop *et al.*, 1986). Moreover, the rate of acetyl-CoA hydrolysis is increased by an increased acetate concentration (Jessop *et al.*, 1986), suggesting that the rate of this cycle, and hence the associated energy dissipation, may be controlled by the portal acetate concentration *in vivo*.

Although these previous investigations did not establish the precise location of this substrate cycle in liver, the results of Rabkin & Blum (1985) indicated that it is cytoplasmic. In support of this location, Crabtree *et al.* (1989) have shown that, in rat hepatocytes, most if not all of the acetyl-CoA hydrolysis is cytoplasmic, presumably catalysed by the distinct cytoplasmic acetyl-CoA hydrolase which occurs only in liver and (to a much lesser extent) in kidney and which, in contrast with its mitochondrial counterpart, is activated by ATP and inhibited by ADP (Prass *et al.*, 1980; Matsunaga *et al.*, 1985; Söling & Rescher, 1985; Jessop *et al.*, 1990). Therefore, since most of the hepatic acetyl-CoA synthetase activity is also cytoplasmic (see Groot *et al.*, 1976), it would appear reasonable to conclude that an active substrate cycle operates between acetate and acetyl-CoA in liver cytoplasm (Crabtree *et al.*, 1989), with a net uptake of acetate in this compartment and with little if any activation of acetate taking place in the mitochondria.

However, at its concentration in portal blood (approx. 1 mM; Buckley & Williamson, 1977), acetate can serve as a significant fuel for oxidation in both perfused rat liver (Snoswell *et al.*, 1982) and isolated hepatocytes (Baranyai & Blum, 1989). Therefore mitochondrial acetate activation (flux U_m, Scheme 1) is not negligible and the uptake of acetate cannot all be apportioned to the cytoplasmic acetyl-CoA synthetase (flux S, Scheme 1). Consequently, the role of the cytoplasmic cycle, and even its very existence, cannot be established until the fluxes through the two synthetases (S and U_m) have been measured and compared with that through the hydrolase (C). This paper presents methods for measuring these fluxes in rat hepatocytes and examines the existence and role of substrate cycling between acetate and acetyl-CoA.

EXPERIMENTAL

Sources of materials, animals and diets

Reagents, enzymes and radioactive substances were obtained from the sources given previously (Crabtree *et al.*, 1989).

Male rats (Hooded Lister strain, bred at the Rowett Institute) were fed with a standard laboratory chow containing 5% (w/w) fat (Oxoid, London, U.K.). Starved rats were fed on normal chow until 24 h before each experiment; food was then withdrawn, but access to water was maintained.

Preparation and incubation of hepatocytes

Rats (approx. 300 g body wt.) were killed by stunning followed by cervical dislocation at approx 11:00 h, after which a single lobe of liver was quickly removed (within 1 min) and hepatocytes were prepared as described previously (Crabtree *et al.*, 1989).

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Preparations were used only if the viability, assessed by the exclusion of Trypan Blue, was at least 85%.

Freshly prepared hepatocytes were preincubated for approx. 10 min [in the presence or absence of 7 mM-(−)-hydroxycitrate] in the medium described previously (Crabtree *et al.* 1989). Incubations were then initiated by adding either 100 μl of a solution containing ^3H_2O (750 μCi), [1-^{14}C]butyrate (6.1 μCi; 11 μmol) and non-radioactive acetate (8 μmol), or 100 μl of a solution containing ^3H_2O (750 μCi) and [1-^{14}C]acetate (1 μCi; 8 μmol). The initial concentration of acetate in all incubations was therefore approx. 1.1 mM.

Samples (2 × 0.5 ml) of the incubations with [1-^{14}C]butyrate were taken at intervals between 0 and 12 min, mixed immediately with an equal volume of 6% (w/v) $HClO_4$, and used to measure metabolite concentrations, the incorporation of ^{14}C and ^{3}H into lipids and the specific radioactivity of acetate, as described previously (Crabtree *et al.*, 1989).

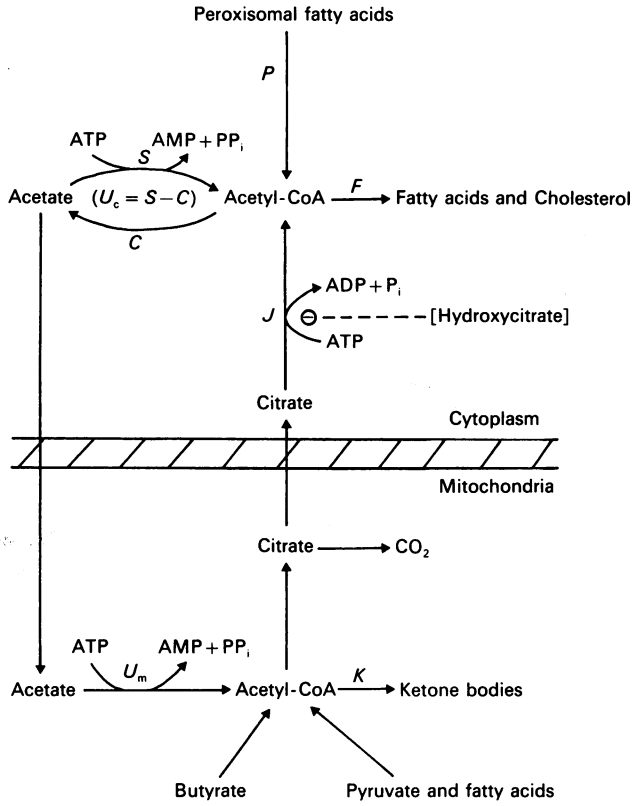
With incubations using [1-^{14}C]acetate, preliminary experiments showed that it was unnecessary to separate cells from medium before measuring the specific radioactivity of acetate; so that single (1 ml) samples were taken between 0 and 20 min and mixed immediately with 1 ml of 6% (w/v) $HClO_4$. These extracts were then used to measure acetate specific radioactivities (in addition to metabolite concentrations and the incorporation of radioactivity into lipids) as described previously (Crabtree *et al.*, 1989), except that the residues after freeze drying were dissolved in 1 ml of distilled water instead of 1 mM-butyrate, and were acidified with 100 μl of 1 M-HCl.

Measurement of fluxes

The model used to calculate the fluxes related to hepatic acetate metabolism is outlined in Scheme 1. Cytoplasmic acetyl-CoA is assumed to be derived from acetate (via flux *S*), from citrate derived from the mitochondria (via *J*) or from other cytoplasmic sources, probably peroxisomal fatty acids (via *P*). It is also assumed that cytoplasmic acetyl-CoA is either used for fatty acid and sterol synthesis *de novo* (via *F*) or hydrolysed to acetate (via the cytoplasmic hydrolase; flux *C*). These assumptions are discussed later. Mitochondrial acetyl-CoA hydrolase activity is neglected because previous investigations showed that this enzyme contributes little if anything to the hydrolysis of acetyl-CoA under the present incubation conditions (Crabtree *et al.*, 1989).

Rates of lipid synthesis *de novo* (flux *F*) and the ^{14}C specific radioactivity of cytoplasmic acetyl-CoA

The total rate of lipid synthesis *de novo* (flux *F* in Scheme 1) was calculated from the rate of incorporation of ^{3}H from ^3H_2O into the lipid fraction obtained after saponification, and the ^{14}C specific radioactivity of cytoplasmic acetyl-CoA was then calculated from this rate and the rate of incorporation of ^{14}C into the lipid fraction. Each rate was calculated from a plot of radioactivity versus time, using a best-fitting (single exponential) curve generated by the computer program MLP (see Crabtree *et al.*, 1989). Since only the total rate of lipid synthesis *de novo* (i.e. fatty acids plus sterols) was required for these experiments, the non-saponifiable lipid fraction was not separated from the saponified fatty acids before measuring the radioactivities. This should introduce no significant errors into the calculations of either total lipid synthesis or acetyl-CoA specific radioactivity, as preliminary experiments showed that the rate of sterol synthesis was only approx. 10% that of fatty acid synthesis (based on the incorporation of ^{3}H), fatty acid and sterol synthesis share a common pool of cytoplasmic acetyl-CoA (Goldberg & Brunengraber, 1980) and the factor converting ^{3}H incorporation into C_2 incorporation is approximately the same for both fatty acids and sterols (Brunengraber *et al.*, 1973).



Scheme 1. Fluxes related to acetate metabolism in hepatocytes

Only an outline of the major fluxes is presented and not their stoicheometries; cofactors are omitted, except for the adenine nucleotides at reactions referred to in the text in connection with substrate cycling. ⊖ denotes an inhibition (of ATP–citrate lyase) by hydroxycitrate. Total acetate uptake by hepatocytes, $U_t = U_m + U_c$.

Cytoplasmic acetyl-CoA hydrolysis (flux *C*)

The method for measuring cytoplasmic acetyl-CoA hydrolysis using [1-^{14}C]butyrate described previously (Crabtree *et al.*, 1989) only measures the initial rate, whereas the method outlined below can be used at any time interval. The model on which the method is based and the symbols used are as given in Scheme 1.

From the definition of specific radioactivity,

$$s_{ac} = r_{ac}/c_{ac}$$

where s_{ac}, r_{ac} and c_{ac} are the specific radioactivity, radioactivity and amount respectively of acetate. Differentiating this equation with respect to time results in the following equation:

$$ds_{ac}/dt = (c_{ac}.dr_{ac}/dt - r_{ac}.dc_{ac}/dt)/(c_{ac})^2$$

which can be simplified to:

$$c_{ac}.ds_{ac}/dt = dr_{ac}/dt - s_{ac}.dc_{ac}/dt \quad (1)$$

From the model in Scheme 1,

$$dr_{ac}/dt = C.s_{acCoA} - (S + U_m).s_{ac} \quad (1a)$$

where s_{acCoA} is the specific radioactivity of the cytoplasmic acetyl-CoA, and

$$dc_{ac}/dt = C - U_m - S \quad (1b)$$

Table 1. Acetyl-CoA hydrolysis measured using [1-^{14}C]butyrate and [1-^{14}C]acetate

The data refer to one of three similar experiments, with initial concentrations of 1.1 mM-acetate and 1.5 mM-butyrate in each incubation, details of which are given in the Experimental section. The slightly higher rates obtained with [^{14}C]butyrate may not be significant, because they were not observed consistently in the other two experiments.

Substrate	Time (min)	s_{acCoA} (μCi/mmol)	s_{ac} (μCi/mmol)	($s_{acCoA}/s_{ac}-1$)	$c_{ac}.\frac{d\ln s_{ac}}{dt}$ (μmol/min per g dry wt.)	Acetyl-CoA hydrolysis (C) (μmol/min per g dry wt.)
[1-^{14}C]Acetate	6	8.8×10^{-3}	0.086	−0.90	−1.05	1.17
	9	0.01	0.079	−0.87	−1.18	1.36
	12	0.014	0.068	−0.79	−1.32	1.67
	15	0.018	0.063	−0.71	−1.13	1.59
[1-^{14}C]Butyrate	6	0.053	0.015	2.53	4.20	1.67
	9	0.029	0.018	0.61	1.36	2.23
	12	0.023	0.020	0.15	0.44	2.93

Substituting eqns. (1*a*) and (1*b*) in eqn. (1), we get

$$c_{ac}.ds_{ac}/dt = C.s_{acCoA}-(S+U_m).s_{ac}-s_{ac}(C-S-U_m) = C.(s_{acCoA}-s_{ac}) \quad (2)$$

Dividing both sides by s_{ac} and replacing ds_{ac}/s_{ac} by $d\ln s_{ac}$, eqn. (2) becomes:

$$c_{ac}.d\ln s_{ac}/dt = C(s_{acCoA}/s_{ac}-1) \quad (3)$$

or

$$C = (c_{ac}.d\ln s_{ac}/dt)/(s_{acCoA}/s_{ac}-1) \quad (4)$$

Eqn. (4) shows that C, the rate of cytoplasmic acetyl-CoA hydrolysis, can be calculated from the slope of a plot of ln (specific radioactivity of acetate versus time after incubating hepatocytes with [^{14}C]acetate or [^{14}C]butyrate. Moreover, this method is not restricted to initial values, so that C can be calculated at any time. However, it does require the specific radioactivities of acetate and acetyl-CoA to be unequal, otherwise the denominator in eqn. (4) would be zero. This condition applies to rat hepatocytes (Table 1) and, using [1-^{14}C]acetate, the ratio s_{acCoA}/s_{ac} varied from near zero to approx. 0.2 (Tables 1–3).

With [^{14}C]butyrate the numerator and denominator of eqn. (4) are both positive, since s_{ac} increases with time and $s_{acCoA} > s_{ac}$, whereas with [^{14}C]acetate the numerator and denominator are both negative, since s_{ac} now decreases with time and $s_{acCoA} < s_{ac}$ (see Table 1). In either case, $\ln s_{ac}$ was plotted versus time and a (single exponential) curve was fitted using the computer program MLP, from which the slopes were calculated for use in eqn. (4).

The assumption that acetate is produced only from acetyl-CoA may not always be correct: for example, ethanol is converted into acetate by a pathway not involving acetyl-CoA (see Newsholme & Leech, 1983). If such a pathway existed it could not affect eqn. (1*a*), but its rate (T) would have to be added to the right-hand side of eqn. (1*b*). Eqn. (4) would then become:

$$C = \frac{c_{ac}.d\ln s_{ac}/dt}{(s_{acCoA}/s_{ac}-1)} + \frac{T}{(s_{acCoA}/s_{ac}-1)}$$

With [^{14}C]acetate the common denominator is negative (see above), so that the second term on the right-hand side is also negative. Therefore, using this radioactive substrate, C would be overestimated by neglecting T. However, with [^{14}C]butyrate the denominator is positive, so that the second term on the right-hand side is also positive. Therefore, using this radioactive substrate, C would be underestimated by neglecting T. Consequently, if there is a significant formation of acetate by pathways not involving acetyl-CoA, the value of C obtained with [^{14}C]butyrate should be less than that obtained with [^{14}C]acetate under the same conditions. However, the results in Table 1 show that the values of C with [^{14}C]butyrate were slightly (though probably not significantly) greater than those obtained with [^{14}C]acetate, suggesting that, under the present incubation conditions, acetate was produced predominantly from acetyl-CoA. This conclusion is also supported by the similar values of s_{ac} and s_{acCoA} after 12 min incubation with [^{14}C]butyrate (Table 1), indicating little if any dilution of acetate by other pathways. Furthermore, the similarity between the values of C obtained by using either radioactive substrate shows that the assumption of a uniform specific radioactivity for cytoplasmic acetyl-CoA is also satisfactory.

The results in Table 1 show that the rate of acetyl-CoA hydrolysis, C, can be measured using either [^{14}C]acetate or [^{14}C]butyrate. However, since there are fewer practical difficulties with [1-^{14}C]acetate (see above), and since this radioactive substrate must be used for measurements of cytoplasmic acetyl-CoA synthetase (see below), radioactive acetate was used to obtain the values for C given in Tables 2 and 3.

Cytoplasmic acetyl-CoA synthetase (flux S) and net utilization of acetate (flux U_c)

Applying eqn. (1) to the cytoplasmic pool of acetyl-CoA (Scheme 1) produces the following equation:

$$c_{acCoA}.ds_{acCoA}/dt = dr_{acCoA}/dt - s_{acCoA}.dc_{acCoA}/dt \quad (7)$$

Assuming that acetyl-CoA is in a metabolic steady state (so that $dc_{acCoA}/dt = 0$) and that ^{14}C enters only from acetate, so that

$$dr_{acCoA}/dt = S.s_{ac}-(C+F).s_{acCoA},$$

eqn. (7) then becomes:

$$c_{acCoA}.ds_{acCoA}/dt = S.s_{ac}-(C+F).s_{acCoA} \quad (7a)$$

whence

$$c_{acCoA}.ds_{acCoA}/dt = S.s_{ac}/s_{acCoA}-(C+F),$$

so that

$$S = (c_{acCoA}.d\ln s_{acCoA}/dt + C + F).(s_{acCoA}/s_{ac}) \quad (8)$$

Consequently,

$$U_c = S-C \text{ (see Scheme 1)} = (c_{acCoA}.d\ln s_{acCoA}/dt + C + F).(s_{acCoA}/s_{ac})-C \quad (9)$$

Therefore the rate of cytoplasmic acetyl-CoA synthesis from acetate (S) and the net rate of acetate utilization in the cytoplasm (U_c) can both be calculated from a plot of $\ln s_{acCoA}$ versus time, provided that C and F have been determined.

However, a major problem with eqns. (8) and (9) is the need to know the pool size of cytoplasmic acetyl-CoA (c_{acCoA}), which

also includes any acetylcarnitine, which equilibrates with cytoplasmic acetyl-CoA via carnitine acetyltransferase (EC 2.3.1.7, see Bieber, 1988). For technical reasons, it was not possible to measure c_{acCoA} in the present experiments. Nevertheless, its maximum value can be estimated from the total hepatic contents of cytoplasmic acetyl-CoA (approx. 0.08 μmol/g dry wt.; Siess *et al.*, 1977) and acetylcarnitine (approx. 1 μmol/g dry wt.; French *et al.*, 1985; assuming a wet wt./dry wt. ratio of 4). Since some of the acetylcarnitine is mitochondrial, the total cytoplasmic acetyl-CoA pool is therefore less than 1 μmol/g dry wt.

In the experiments summarized in Tables 2 and 3, the absolute values of $d\ln s_{acCoA}/dt$ (calculated from plots of $\ln s_{acCoA}$ versus time) were less than 0.2/min and the ratio s_{acCoA}/s_{ac} was less than 0.2. Consequently, with a pool size of 1 μmol/min per g, the first term in eqns. (8) and (9) was less than 0.04 μmol/min per g, considerably lower than the values calculated for U_c and significantly lower than most of the values calculated for S. Therefore, since this estimate of c_{acCoA} is an upper limit, it was assumed that the first term in eqns. (8) and (9) could be neglected (i.e. c_{acCoA} set to zero) without introducing any significant errors into S or U_c, which were consequently calculated as follows:

$$S = (C+F).(s_{acCoA}/s_{ac}) \quad (8a)$$

and

$$U_c = (C+F).(s_{acCoA}/s_{ac}) - C \quad (9a)$$

A further problem is that eqn. (7*a*), and hence the calculation of S and U_c from eqns. (8*a*) and (9*a*), assumes that radioactivity only enters the cytoplasmic acetyl-CoA pool from acetate. Therefore [^{14}C]butyrate cannot be used to measure S and U_c by this method, because this substrate labels the cytoplasmic acetyl-CoA via mitochondrial CoA and citrate (flux J, Scheme 1). However, ^{14}C from radioactive acetate could also label the cytoplasmic acetyl-CoA pool indirectly via this route and, if this were significant, an additional (positive) term would be needed on the right-hand side of eqn. (7*a*). Consequently, cytoplasmic acetyl-CoA synthesis (S) would be overestimated by neglecting this term when using eqn. (8*a*). However, in the experiments summarized in Tables 2 and 3, the specific radioactivity of cytoplasmic acetyl-CoA was either unaffected or slightly increased by (−)-hydroxycitrate [which inhibits the activity of ATP–citrate lyase and hence flux J (Watson & Lowenstein, 1970; Lowenstein, 1971; Brunengraber *et al.*, 1972)]. This suggests that the transfer of radioactivity from acetate to cytoplasmic acetyl-CoA via citrate is insignificant (even though the actual flux, J, may not be; see Baranyai & Blum, 1989) and therefore may be neglected.

Mitochondrial utilization of acetate (flux U_m) and ketone body formation (flux K)

The total rate of acetate uptake (U_t) was calculated from a plot of acetate concentration versus time. As with other fluxes a best-fitting (single exponential) curve was generated, using the program MLP, and was used to calculate the rates at each time interval. The mitochondrial uptake of acetate (U_m, Scheme 1) at each time interval was then calculated as

$$U_m = U_t - U_c$$

where U_c was calculated by using eqn. (9*a*).

Total ketone body (i.e. acetoacetate plus 3-hydroxybutyrate) production was calculated from plots of concentration versus time, which, in contrast with those for other fluxes, were approximately linear throughout the incubations.

Expression of results

All fluxes (except total ketone body formation) showed a distinct 'lag' period of up to 10 min (results not shown, but see Crabtree *et al.*, 1989). Therefore the rates between 10 and 20 min were used to calculate the mean values ± S.E.M. (from four experiments) given in Tables 2 and 3. All fluxes are expressed as μmol of C_2/min per g dry wt. at 37 °C, unless otherwise stated. Exponential curve fitting (using seven points between 0 and 20 min) was used because it provided a satisfactory fit to the data (judged visually and by the size of residuals). Although such curves may be inaccurate near the ends of the time range, the initial part of the curve (i.e. the lag period) was not used, and omitting the final point did not significantly affect the mean rate. These fitted curves were therefore considered to be adequate for the present investigation.

RESULTS AND DISCUSSION

Incubation conditions

The ATP content of the hepatocytes, which is a good indicator of their viability (Cornell, 1983; Pogson *et al.*, 1984), was similar to that reported previously (approx. 8 μmol/g dry wt.; Crabtree *et al.*, 1989) and showed no significant changes during the incubations. However, the initial ATP content was decreased to approx. 6 μmol/g dry wt. in cells prepared from 24 h starved animals.

The rates of net uptake of acetate (U_t) and the production of ketone bodies (K) in hepatocytes from fed rats (Table 2) were similar to those reported by other workers (Mayes & Topping, 1974; Snoswell *et al.*, 1982; Jessop *et al.*, 1986), and the rates of

Table 2. Effect of hydroxycitrate on acetate and related fluxes, and on the ratios 3-hydroxybutyrate/acetoacetate, s_{acCoA}/s_{ac} and $d\ln s_{acCoA}/dt$ in hepatocytes from fed rats

Cells were incubated and fluxes measured using [1-^{14}C]acetate, as described in the Experimental section. Fluxes (Scheme 1) are expressed as μmol of C_2/min per g dry wt. s_{acCoA} and s_{ac} are the specific radioactivities of cytoplasmic acetyl-CoA and acetate respectively, measured as described in the Experimental section. Significance was tested using the 't' test. Since the variances often differed considerably, a pooled estimate of variance was not used and the value of 't' was tested at 3 degrees of freedom (see Cochran & Cox, 1957): * signifies $P < 0.05$ versus the control.

	Control	+7 mM-Hydroxycitrate
Lipid synthesis (F)	0.85±0.31	0.23±0.07
Cytoplasmic acetyl-CoA hydrolysis (C)	1.32±0.60	1.44±0.28
Cytoplasmic net uptake of acetate (U_c)	−1.21±0.50	−1.13±0.18
Cytoplasmic acetyl-CoA synthesis (S)	0.12±0.06	0.32±0.15
Total net uptake of acetate (U_t)	0.47±0.40	1.41±0.04
Mitochondrial acetate uptake (U_m)	1.7±0.50	2.54±0.16
Ketone body production (K)	0.24±0.04	2.94±0.74*
3-Hydroxybutyrate/acetoacetate	0.46±0.11	0.70±0.12
s_{acCoA}/s_{ac}	0.06±0.01	0.07±0.01
$d\ln s_{acCoA}/dt$	−0.01±0.01	−0.01±0.02

Table 3. Effect of hydroxycitrate on acetate and related fluxes, and on the ratios 3-hydroxybutyrate/acetoacetate, s_{acCoA}/s_{ac} and $dlns_{acCoA}/dt$ in hepatocytes from 24 h-starved rats

For details see the legend to Table 2. * and *** signify $P < 0.05$ and $P < 0.001$ respectively versus fed control.

	Control	+7 mM-Hydroxycitrate
Lipid synthesis (F)	0.04±0.006*	0.03±0.005
Cytoplasmic acetyl-CoA hydrolysis (C)	1.86±0.46	1.41±0.40
Cytoplasmic net uptake of acetate (U_c)	−1.85±0.41	−1.27±0.32
Cytoplasmic acetyl-CoA synthesis (S)	< 0.003	0.14±0.09
Total net uptake of acetate (U_t)	1.28±0.10	1.18±0.26
Mitochondrial acetate uptake (U_m)	3.14±0.70	2.4±0.26
Ketone body production (K)	5.84±0.34***	5.44±0.16
3-Hydroxybutyrate/acetoacetate	0.61±0.16	0.87±0.08
s_{acCoA}/s_{ac}	0.003±0.001	0.04±0.01
$dlns_{acCoA}/dt$	−0.03±0.09	0.18±0.06

mitochondrial and cytoplasmic acetyl-CoA synthesis from acetate (U_m and S) were similar to those calculated by Baranyai & Blum (1989). The rate of lipid synthesis *de novo* (Table 2) was similar to that reported by Baranyai & Blum (1989), but was somewhat lower than that reported by Snoswell *et al.* (1982) and Brunengraber *et al.* (1973). [Direct comparisons with our previous results (Crabtree *et al.*, 1989) are not possible, because in that study the fluxes represented initial rates, and approx. 1.5 mM-butyrate was present throughout.]

Mitochondrial acetate flux

The results in Tables 2 and 3 show that the mitochondrial rate of acetyl-CoA synthesis from acetate (U_m) was usually considerably greater than that in the cytoplasm (S). This distribution, which has been reported recently by Baranyai & Blum (1989), would not have been expected from the distribution of the maximum catalytic activities of acetyl-CoA synthetase (measured *in vitro*), which shows that more than 80% of the total activity is located in the cytoplasm (see Groot *et al.*, 1976).

Moreover, since the maximum catalytic activity of acetyl-CoA synthetase in rat liver is approx. 12 μmol/min per g dry wt. at 37 °C (calculated from data in Knowles *et al.*, 1974, assuming a wet wt./dry wt. ratio of 4), and since only approx. 20% of this is mitochondrial (see above), the maximum activity of the mitochondrial enzyme is approx. 2.5 μmol/min per g. This is similar to the mitochondrial flux, U_m (Table 2), and suggests that the enzyme may be operating close to its maximum rate. This is another surprising result, because enzymological investigations *in vitro* show that the K_m of the mitochondrial acetyl-CoA synthetase for acetate is approx. 10 mM (compared with approx. 0.1 mM for the cytoplasmic enzyme; Scholte & Groot, 1975). Therefore, at the incubation concentration of approx. 1 mM-acetate, this enzyme should have been operating at well below its maximum capacity. Consequently, if the fluxes in Tables 2 and 3 are not erroneous, either the maximum catalytic activity of the mitochondrial acetyl-CoA synthetase has been greatly underestimated, or some intracellular factor increases its affinity for acetate so that it operates at near-maximum capacity *in situ*. Since enzymological predictions of the rate of the cytoplasmic hydrolase (C) *in situ* also fail to agree with that measured in hepatocytes (see Crabtree *et al.*, 1989), a comprehensive re-examination of the kinetics of liver acetyl-CoA synthetases and hydrolases is required.

Cytoplasmic acetate fluxes

The rates of cytoplasmic acetyl-CoA hydrolysis (C, Scheme 1), measured by using [1-^{14}C]acetate (eqn. 4) and shown in Tables 2 and 3, are similar to those reported by Rabkin & Blum (1985) and Crabtree *et al.* (1989), but are somewhat lower than those reported by Jessop *et al.* (1986). Neither this flux nor that of cytoplasmic acetyl-CoA synthesis (S) was significantly affected by either 24 h starvation or (−)-hydroxycitrate.

The simultaneous activities of both cytoplasmic acetyl-CoA synthesis (S) and hydrolase (C) show that a substrate cycle between acetate and acetyl-CoA does operate in rat liver cytoplasm. However, in contrast with previous proposals (see the Introduction section), the rate of synthesis (S) was much lower than that of hydrolysis (C), so that the net flux through the cycle (U_c) was in the direction of acetate production, i.e. U_c was negative (Tables 2 and 3).

If the assumption that cytoplasmic acetyl-CoA is converted only into acetate or lipids is incorrect, the rate of cytoplasmic acetyl-CoA synthetase (S) would be underestimated, and a net uptake of acetate by the cytoplasm could appear to be a net output. However, because the ratio s_{acCoA}/s_{ac} was less than 0.1 (Tables 2 and 3), any flux of cytoplasmic acetyl-CoA elsewhere, E, would have to be of the order of 10 μmol/min per g dry wt. to have any significant effect on the values calculated for S and hence U_c (see eqns. 8 and 9). Since preliminary experiments showed no detectable release of either acetyl-CoA or acetylcarnitine into the incubation medium, E would have to be an intracellular flux, and the only possibility, based on current knowledge, would be an exchange of cytoplasmic acetylcarnitine with mitochondrial carnitine. However, this system is considered to be relatively unimportant in the liver (Bieber, 1988). Moreover, if it were sufficiently active to affect the value of S and hence U_c, the transfer of ^{14}C from butyrate to lipids and acetate would not be inhibited by hydroxycitrate, contrary to what has been observed (Crabtree *et al.*, 1989).

Furthermore, a net cytoplasmic production of acetate is consistent with the results of Snoswell *et al.* (1982) who, using a perfused rat liver, found that a net uptake of acetate became a net output in the presence of high concentrations of lactate. Since lactate and acetate compete as oxidizable substrates, these results can be explained if the high concentrations of lactate inhibited the mitochondrial acetate flux (U_m), thereby allowing the cytoplasmic production of acetate ($-U_c$) to be observed as a net output by the liver. Indeed, we have found that, in the presence of 2 mM-butyrate or -propionate (which both inhibit mitochondrial acetate oxidation; see Ballard, 1972), a net acetate uptake by rat hepatocytes becomes a net output as a result of the decreased mitochondrial flux, U_m (B. Crabtree & M.-J. Gordon, unpublished work).

Since the net cytoplasmic flux of acetate (U_c) was not significantly affected by either starvation or hydroxycitrate (Tables 2 and 3), both of which inhibit ATP–citrate lyase and thus the

flux of acetyl-CoA deriving from the mitochondria (see above; Lowenstein, 1968), an extramitochondrial source of cytoplasmic acetyl-CoA must be present under these conditions. This is likely to be the peroxisomal β-oxidation of fatty acids (*P*, Scheme 1), as suggested by Mannaerts & Debeer (1981) and recently demonstrated using rat hepatocytes by Leighton *et al.* (1989). Indeed, the capacity of rat liver for peroxisomal β-oxidation, approx. 4 μmol of C_2 units produced/min per g dry wt. (Kondrup & Lazarow, 1985; assuming a wet wt. dry wt. ratio of 4), is similar to the rates of acetyl-CoA hydrolysis in Tables 2 and 3. The results in the present paper are therefore consistent with a peroxisomal source of cytoplasmic acetyl-CoA, at least during starvation or in the presence of hydroxycitrate.

Energetic significance of substrate cycling between acetate and acetyl-CoA

When the net flux through the cytoplasmic acetate/acetyl-CoA substrate cycle is in the direction of acetate formation, the 'energetically wasteful' component is the synthetase reaction (*S*), and not the hydrolase (*C*) as previously assumed. Consequently, the ATP hydrolysis by this cycle is equal to twice the rate of acetyl-CoA synthesis from acetate (*S*) (since the formation of AMP by this reaction is equivalent to the hydrolysis of two molecules of ATP), i.e approx. 0.24 μmol/min per g (see Table 2). Assuming a wet wt./dry wt. ratio of 4, a total liver weight of 10 g per animal and that the hydrolysis and subsequent resynthesis of 1 mol of ATP *in vivo* releases 75 kJ of heat (Newsholme & Crabtree, 1976), this rate of ATP hydrolysis is equivalent to a heat production of approx. 65 J/day, or approx. 0.07 % of the total heat produced by the animal. Consequently, if the rates in Table 2 apply to the whole animal, this hepatic cytoplasmic cycle is not a significant mechanism for dissipating energy.

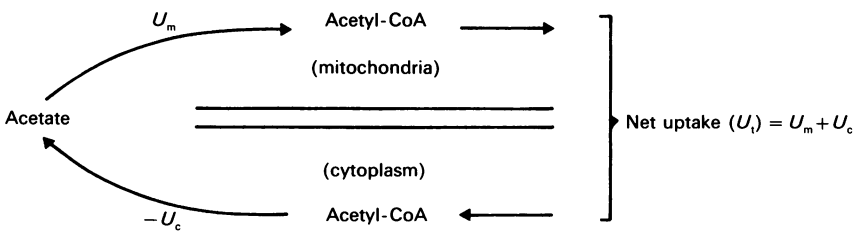
However, cytoplasmic acetyl-CoA hydrolysis could also be involved in a larger substrate cycle between the mitochondria and cytoplasm, in which acetate forms acetyl-CoA in the mitochondria (U_m) which is transferred (via citrate) to the cytoplasm (*J*) and is there hydrolysed to acetate (*C*) which returns to the mitochondria (see Scheme 1). This cycle would hydrolyse an additional molecule of ATP, in the ATP–citrate lyase reaction, so that the total amount of ATP consumed would be increased from two to three molecules per revolution. Moreover, since the rate of mitochondrial acetyl-CoA formation from acetate, U_m was greater than the rate of cytoplasmic acetyl-CoA hydrolysis, *C* (Table 2), hydrolysis would now represent the 'energetically wasteful' component of this larger cycle.

A calculation similar to that above, using the rate of acetyl-CoA hydrolysis in Table 2, shows that this cycle could account for approx. 1 % of the total heat production by the animal. Although this contribution is much greater than that calculated for the cytoplasmic cycle, it is still too low to be of energetic significance, for example to enable the cycle to dissipate a significant amount of energy in response to changes in portal acetate concentration (Jessop *et al.*, 1986). Moreover, if participation in such a cycle for energy dissipation represented the main function of acetyl-CoA hydrolysis, this flux might be expected to be decreased by treatments (e.g. starvation, hydroxycitrate) which inhibit the transfer of acetyl-CoA from mitochondria to cytoplasm, but the results in Tables 2 and 3 show that this was not the case. Therefore, although this extended substrate cycle may operate in the liver of normal fed rats, it does not appear to represent the main function of cytoplasmic acetyl-CoA hydrolysis.

Provision of sensitivity, feedback and buffering by cytoplasmic and mitochondrial acetate fluxes

In addition to dissipating energy, substrate cycles may also have regulatory roles, for example to provide feedback and extra sensitivity (Newsholme & Crabtree, 1976; Newsholme *et al.*, 1984). Consequently, the acetate/acetyl-CoA cycle could serve to increase the sensitivity of hepatic net uptake or output of acetate to regulators of acetyl-CoA hydrolase and/or synthetase (including the extracellular acetate concentration). It may also provide feedback, to allow the net uptake of acetate to be controlled by the intracellular concentration of acetyl-CoA (which would otherwise be impossible, since acetyl-CoA synthetase catalyses an irreversible reaction). Similarly, the cycle would allow the direction of hepatic acetate metabolism, i.e. net uptake versus net output, to be controlled by the extracellular acetate concentration, as has been observed with hepatocytes (Jessop *et al.*, 1986), perfused liver (Snoswell *et al.*, 1982) and liver *in vivo* (Buckley & Williamson, 1977). These regulatory effects are not mutually exclusive and, by providing a sensitive response of the net flux to extracellular acetate, the cycle can also serve as a buffer, to prevent large amounts of absorbed acetate from being passed directly into the systemic circulation (Buckley & Williamson, 1977; Snoswell *et al.*, 1982). Such buffering by the liver may be important, because elevated blood concentrations of acetate can damage essential organs such as the heart (Blaise *et al.*, 1989).

Since the regulatory effects of a substrate cycle depend on the cycling/flux ratio (Newsholme & Crabtree, 1976; Newsholme *et al.*, 1984), the cytoplasmic acetate/acetyl-CoA cycle would not appear to have any regulatory significance, as its cycling/flux ratio (S/U_c) was quite low (< 0.3; Tables 2 and 3). In contrast, the cycling/flux ratio of the larger cycle involving both mitochondria and cytoplasm ($-U_c/U_t$) was approx. 2 in fed rats (Table 2), which would be sufficient to provide significant feedback, sensitivity and buffering. Moreover, since these regulatory effects depend only on the presence of a 'reverse' step, the



Scheme 2. Sensitivity conferred by opposing fluxes of mitochondrial acetate utilization and cytoplasmic acetate production

The sensitivity, *s*, conferred by this system to [acetate] is the same as if the fluxes constituted a substrate cycle between acetate and acetyl-CoA, so that $s = 1 +$ reverse reaction/net flux. Therefore, for an initial net uptake of acetate by the liver ($U_t > 0$), $s = 1 + (-U_c)/U_t$, and, for an initial net output ($U_t < 0$), $s = 1 + U_m/U_t$.

two opposing fluxes of acetate uptake by the mitochondria (U_m) and net output by the cytoplasm ($-U_c$) do not need to form a conventional substrate cycle (i.e. to be connected via *J*). The existence of the two opposing fluxes is sufficient to provide a system (Scheme 2) which, although not a substrate cycle, is equivalent to one in providing sensitivity, feedback and buffering. Furthermore, since it does not require ATP–citrate lyase (*J*), it can operate during starvation, and, as with a conventional substrate cycle (Newsholme & Crabtree, 1976), the sensitivity, and hence the buffering capacity, can be varied (by varying the cycling/flux ratio) according to metabolic requirements.

Finally, although the present results strongly suggest that the cytoplasmic acetate/acetyl-CoA substrate cycle does not provide a cytoplasmic substrate cycle for energy dissipation, feedback, sensitivity or buffering, further investigations are required. This is because many substrate cycles are under metabolic or hormonal control (Newsholme & Crabtree, 1976; Newsholme *et al.*, 1984) and therefore the rates of cytoplasmic acetyl-CoA synthesis and hydrolysis in Tables 2 and 3 may be basal or 'resting' values. Indeed, external (possibly hormonal) control of the flux through cytoplasmic acetyl-CoA synthetase (*S*) is indicated by its extremely low rate in cells prepared from 24 h starved rats (Table 3). This is consistent with a lowered maximum catalytic activity of cytoplasmic acetyl-CoA synthetase after 48 h of starvation (Barth *et al.*, 1972) and suggests that this enzyme, and hence the rate of the cytoplasmic acetate/acetyl-CoA substrate cycle, is under hormonal control. Also, Jessop *et al.* (1986) reported higher rates of (presumably cytoplasmic) acetyl-CoA hydrolysis than those in the present paper, suggesting that factors, as yet undiscovered, may serve to control this flux and hence the activity of the cytoplasmic cycle.

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