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Bovine serum albumin decreases 4-methyl-2-oxovalerate utilization by isolated rat hepatocytes

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1. Binding of 4-methyl-2-oxo[1-^{14}C]valerate to defatted bovine serum albumin inhibited the utilization of this 2-oxo acid by fed-rat hepatocytes *in vitro*. 2. With 0–50g of albumin/l in the presence of 0.05mM 2-oxo acid or on increasing the 2-oxo acid concentration from 0 to 2mM in the presence of 26g of albumin/l, the extent of inhibition was essentially dependent on the change in the free 2-oxo acid concentration. 3. Intrahepatocyte 4-methyl-2-oxo[1-^{14}C]valerate concentrations were similar to extracellular free 2-oxo acid concentrations, suggesting equilibration so that the plasma membrane appears not to be rate-limiting for the utilization of this substrate by the isolated liver cells.

Branched-chain 2-oxo acids are intermediary products in the catabolism of the essential amino acids L-leucine, L-valine and L-isoleucine, and are released into the circulation by skeletal muscle of the fed rat for further metabolism in the liver (Livesey & Lund, 1980). In the circulation the 2-oxo acids are transported both in association with serum albumin and free in solution (Livesey & Lund, 1982). 4-Methyl-2-oxovalerate, the 2-oxo acid analogue of L-leucine, is readily taken up by rat hepatocytes *in vitro* with the production of ketone bodies (Lund, 1978; Williamson *et al.*, 1979), and binding of this 2-oxo acid by defatted bovine serum albumin has been characterized (Livesey & Lund, 1982). The present paper describes some effects of defatted albumin on the utilization of 4-methyl-2-oxovalerate by hepatocytes.

Experimental

Materials

4-Methyl-2-oxo[1-^{14}C]valerate was prepared from L-[1-^{14}C]leucine (The Radiochemical Centre, Amersham, Bucks., U.K.) with 99% radiochemical purity (Livesey & Lund, 1982) and diluted with sodium 4-methyl-2-oxovalerate from Sigma. Bovine serum albumin (fraction V; Miles Laboratories) was defatted by the charcoal method (Chen, 1967). Hepatocytes were prepared (Cornell *et al.*, 1973; Krebs *et al.*, 1974) from 180–250 g fed male Wistar rats.

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Methods

Erlenmeyer flasks (25ml) containing hepatocytes suspended in 1.5ml of Krebs & Henseleit (1932) saline alone and flasks containing this saline with both defatted albumin and 4-methyl-2-oxo[1-^{14}C]valerate dissolved at twice their final concentrations were each preincubated for 10–20min at 37°C under O_2/CO_2 (19:1) with shaking. Experiments were initiated by adding the hepatocytes to the albumin–2-oxo acid mixture. The flasks were regassed, Suba-sealed and incubated at 37°C with shaking (3.4cm) at 90 strokes/min. The weight of hepatocytes and the concentrations of albumin and 2-oxo acid were as given in the Tables and Figures.

When oxidation of 4-methyl-2-oxo[1-^{14}C]valerate to $^{14}CO_2$ was measured, incubations were terminated by injection, through the seal, of 0.2ml of 30% (w/v) $HClO_4$; this was done immediately after injection of 0.2ml of 1M-NaOH into a plastic tube that had been placed in the flask before the start of the incubation for the collection of $^{14}CO_2$. The $^{14}CO_2$ was collected for 2.5h at room temperature (18–22°C) with shaking and quantitatively transferred to a liquid scintillant for radioactivity counting (Livesey & Lund, 1982). Replacement of hepatocytes with saline alone during the incubation period provided blank measurements of $^{14}CO_2$ production.

When the intracellular 4-methyl-2-oxo[1-^{14}C]valerate concentration was determined, incubations were terminated by placing 0.6ml of incubation mixture over 0.6ml of Dow–Corning silicone fluid [sp. gr. 1.03 for 0–3% (w/v)-albumin/saline mixtures and 1.05 for the 5% albumin/saline

mixture], which had been layered over 0.3ml of 4% $HClO_4$ in 10% (v/v) glycerol. The three layers were immediately centrifuged at 30000*g* for 60s in an Eppendorf Microfuge. The 4-methyl-2-oxo[1-^{14}C]valerate remaining in the supernatant and that associated with the acidified cell pellet were determined by chemical decarboxylation. Immediately after the centrifugation, 0.4ml of saline supernatant was acidified with 0.2ml of 30% $HClO_4$ and metabolic $^{14}CO_2$ was allowed to evolve over 2.5h at room temperature with shaking. The 1-^{14}C-labelled 2-oxo acid was decarboxylated with 0.2ml of 15% (w/v) H_2O_2 (Odessey & Goldberg, 1979), with collection of $^{14}CO_2$ in 0.2ml of 1M-NaOH for scintillation counting. After resuspension of the acidic cell pellet, metabolic $^{14}CO_2$ was allowed to evolve for 2.5h with shaking, and the protein was partially redissolved for 2h at room temperature by adding 0.1ml of 5M-NaOH (which improved subsequent recovery of the 2-oxo acid as $^{14}CO_2$). The cell-associated 1-^{14}C-labelled 2-oxo acid was decarboxylated with acidic H_2O_2, and $^{14}CO_2$ was collected as before for scintillation counting.

Inulin[^{14}C]carboxylic acid and ^3H_2O were used to determine the volumes of extracellular and total water space respectively associated with the cells after centrifugation (Sainsbury *et al.*, 1979). The intracellular 1-^{14}C-labelled 2-oxo acid concentration was calculated (Sainsbury *et al.*, 1979) by assuming all the cell-associated 4-methyl-2-oxo[2-^{14}C]valerate to be unbound.

Results and discussion

Effect of albumin on rates of 2-oxo acid utilization

When hepatocytes were incubated with 4-methyl-2-oxo[1-^{14}C]valerate in albumin-free Krebs–Henseleit saline, the rate of decarboxylation, measured as $^{14}CO_2$ production, increased as the 2-oxo acid concentration in the medium increased from 0.05 to 2.0mM (Table 1). The presence of a physiological concentration of defatted bovine serum albumin (26g/l) decreased the decarboxylation at all 2-oxo acid concentrations, with strongest inhibition at the lower, physiological, concentrations of the 2-oxo acid. Transamination of the 2-oxo acid to L-[1-^{14}C]leucine (measured as H_2O_2-stable radioactivity; Odessey & Goldberg, 1979) was less than 10% of the rate of $^{14}CO_2$ formation at all 4-methyl-2-oxo[1-^{14}C]valerate concentrations in both the presence and the absence of albumin (results not shown). Therefore increased transamination does not explain the decreased rate of decarboxylation in the presence of albumin.

Metabolic $^{14}CO_2$ production from the ^{14}C-labelled 2-oxo acid increased linearly with time between 0 and 5min in the presence and absence of albumin, permitting the data (Table 1) to be analysed by using

Table 1. *Effects of defatted bovine serum albumin on the rates of 4-methyl-2-oxo[1-^{14}C]valerate utilization by hepatocytes*

Hepatocytes (approx. 25mg wet wt.) were incubated in 3ml of medium in the absence and presence of 26g of albumin/l as indicated under 'Methods'. Values are means ± S.E.M. for three independent determinations.

Initial 4-methyl-2-oxovalerate concn. (mM)	Rate of $^{14}CO_2$ production (μmol/h per g wet wt. of cells)		Inhibition of decarboxylation (%)
	No albumin	+ Albumin	
2.00	44.7 ± 3.9	40.7 ± 3.9	9 ± 1
1.40	45.7 ± 4.9	39.9 ± 4.1	13 ± 1
0.80	45.7 ± 5.0	38.1 ± 4.3	17 ± 1
0.40	38.9 ± 4.8	27.1 ± 4.1	29 ± 2
0.20	28.5 ± 4.2	10.7 ± 2.1	63 ± 4
0.14	20.8 ± 2.9	7.1 ± 1.6	65 ± 4
0.10	14.2 ± 2.7	5.2 ± 1.1	63 ± 6
0.05	7.8 ± 1.4	3.5 ± 0.8	55 ± 4

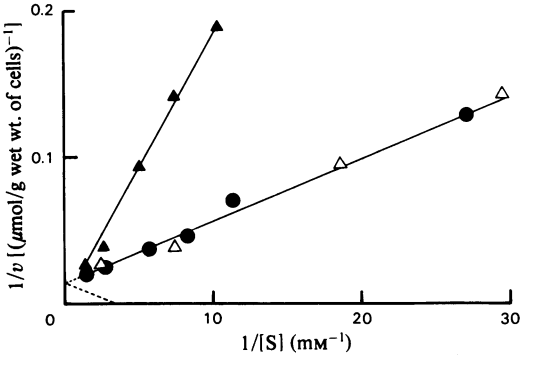

Fig. 1. *Effect of bovine serum albumin on the reciprocal plot for 4-methyl-2-oxo-valerate decarboxylation by fed male Wistar-rat hepatocytes in vitro*

Data are from the experiment shown in Table 1. Symbols: ●, no albumin; ▲, 26g of albumin/l, where [S] = total 2-oxo acid concentration; △, 26g of albumin/l where [S] = free 2-oxo acid concentration. For values of [S], the average concentration over the 5min incubation period was used.

Lineweaver–Burk reciprocal plots (Fig. 1). In the absence of albumin, the decarboxylation showed simple Michaelis–Menten kinetics, with a K_m of 250μM and $V_{max.}$ of 62.5μmol/h per g wet wt. of cells. However, in the presence of albumin, the kinetics, although giving an apparently linear reciprocal plot, did not appear simple; the Michaelis–Menten plot (v versus [S]) was slightly sigmoidal in the presence of albumin. After calculat-

ing the reciprocal of the free concentration of 4-methyl-2-oxo[1-^{14}C]valerate in the presence of the defatted albumin from the albumin binding constants $k_1 = 8.7 \times 10^3$ M^{-1}, $k_2 = 0.09 \times 10^3$ M^{-1}, and the apparent numbers of binding sites, $n_1 = 1$, $n_2 = 5$ (Livesey & Lund, 1982), and replotting, a linear curve was obtained which coincided with that in the absence of albumin (Fig. 1). This would indicate that it is by lowering the free 2-oxo acid concentration available to the hepatocytes that albumin decreases the rate of decarboxylation.

As indicated previously (Livesey & Lund, 1982), varying the 2-oxo acid concentration over a physiological range in the presence of a constant albumin concentration has little effect on the proportion of the total 2-oxo acid that is bound. Consequently, at physiological 2-oxo acid concentrations below the K_m for decarboxylation by hepatocytes (250μM; Fig. 1) the extent of inhibition of $^{14}CO_2$ production is expected to be independent of the total 2-oxo acid concentration. The correctness of this assertion is evident in Table 1, which shows that the percentage inhibition increases progressively as the 2-oxo acid concentration decreases from 2 to 0.2mM, but is little different between 0.2 and 0.05mM.

Changes in albumin concentration in the physiological range at a constant physiological 2-oxo acid concentration were also indicated to be important in determining the proportion of the total 2-oxo acid bound by albumin (Livesey & Lund, 1982). At an initial total concentration of 50μM-4-methyl-2-oxo[1-^{14}C]valerate, increasing the albumin concentration from 0 to 50g/l progressively decreased the rate of 2-oxo acid utilization (Table 2). The extent of the decrease, as a percentage of the rate of utilization in the absence of albumin, was almost identical with the proportion of 2-oxo acid bound by the albumin. Again, this would indicate that albumin decreases the rate of 2-oxo acid utilization in accordance with the decrease in the free concentration of 2-oxo acid available to the hepatocytes.

Intracellular 2-oxo acid concentrations

Intrahepatocyte concentrations of 4-methyl-2-oxo[1-^{14}C]valerate appear to increase in proportion to the change in the free 2-oxo acid concentration in the incubation medium in both the absence and the presence of 26g of defatted bovine serum albumin/l as shown in Fig. 2. The slope of the curve relating the concentration of the 2-oxo acid in the two compartments was 0.97 ± 0.03 (S.E.M.), with an intercept close to zero (1nM). The experiment with increasing concentrations of albumin in the incubation medium also showed evidence of parallelism between the free 2-oxo acid concentration in the medium and its apparent intracellular concentration, though errors were large at these low substrate concentrations (Table 2). These observations suggest that the 2-oxo acid concentrations in the two compartments readily equilibrate and imply that 4-methyl-2-oxovalerate easily permeates the plasma membranes of isolated hepatocytes.

General discussion

The apparent K_m for the decarboxylation of 4-methyl-2-oxo[1-^{14}C]valerate by the fed hepatocytes (250μM; Fig. 1) is similar to that for 48h-starved (female) hepatocytes (260μM; Livesey, 1981) and to the K_m for the transport of this substrate into rat liver mitochondria [135μM (Mackay & Robinson, 1981); 0.2–0.5mM (Williamson *et al.*, 1979)], but is an order of magnitude greater than the K_m for the decarboxylation by the intramitochondrial branched-chain 2-oxo acid dehydrogenase (Parker & Randle, 1978; Danner *et al.*, 1978; Pettit *et al.*, 1978). This, together with the present observation showing similarity in the intracellular and extracellular free 2-oxo acid concentrations, is compatible with mitochondrial transport being the rate-limiting step in the oxidation of 4-methyl-2-oxovalerate by the isolated cells. The rate of oxidation of this branched-chain 2-oxo acid is

Table 2. *Effects of defatted bovine serum albumin concentration on the utilization of 4-methyl-2-oxo[1-^{14}C]valerate by hepatocytes*

Cells (approx. 35mg wet wt./ml of medium) were incubated as indicated under 'Methods' with 50μM-4-methyl-2-oxo[1-^{14}C]valerate initially before being sampled to determine the rate of 2-oxo acid utilization and intracellular concentration at 5min. The mean rate of 4-methyl-2-oxo[1-^{14}C]valerate utilization in the absence of albumin was 8.1μmol/h per g wet wt. of cells. Free and bound 2-oxo acid concentrations were derived from total concentrations by using the constants $n_1 = 1$, $n_2 = 5$, $k_1 = 8.7 \times 10^3$ M^{-1}, $k_2 = 0.09 \times 10^3$ M^{-1} (Livesey & Lund, 1982).

Albumin concn. (g/l)	Inhibition of 2-oxo acid utilization (%)	Proportion of medium 2-oxo acid bound by albumin (%)	Total 2-oxo acid concn. in medium at 5min (μM)	Free 2-oxo acid concn. in medium at 5min (μM)	Intracellular 2-oxo acid concn. at 5min (μM)
0	0	0	25 ± 2	25	30 ± 3
10	48 ± 5	53	37 ± 2	17	18 ± 7
20	68 ± 9	70	42 ± 3	12	8 ± 9
30	76 ± 7	78	44 ± 2	9	5 ± 11
50	88 ± 5	86	47 ± 1	7	9 ± 11

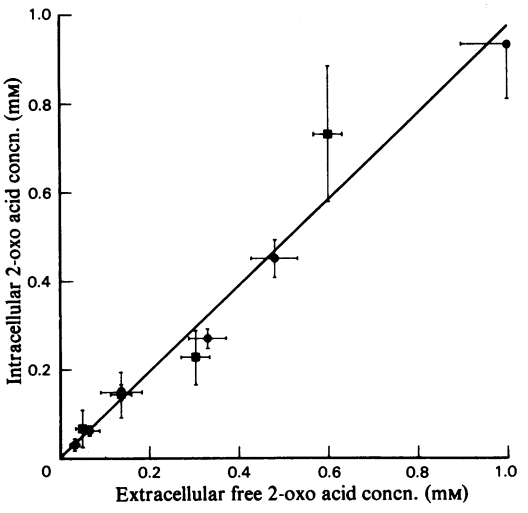

Fig. 2. *Relationship between intrahepatocyte and incubation-medium 4-methyl-2-oxo[1-^{14}C]valerate concentrations*

The experiment (Table 1) was repeated to measure extracellular and intracellular 4-methyl-2-oxo[1-^{14}C]valerate concentrations as indicated in the Experimental section. Defatted bovine serum albumin was either absent (●) or present at a concentration of 26 g/l (■). Observations ($n = 5$–7) were performed on two batches of hepatocytes: the vertical bars show the S.E.M. for the intracellular concentration and the horizontal bars the S.E.M. for the associated extracellular free 2-oxo acid concentration. The combined data gave the regression line shown, with slope of 0.97 ± 0.03 and intercept 1 nM.

further limited in the presence of albumin, in accordance with the decrease in free concentration of 2-oxo acid available to the cells and to the mitochondria. However, the equilibrium between the 2-oxo acid and albumin (Livesey & Lund, 1982) still makes all the 2-oxo acid in the medium available for metabolism.

The present observations with isolated cells are contrary to what was expected from observations on branched-chain 2-oxo acid concentrations between tissues and plasma *in vivo* (Hutson & Harper, 1981; Livesey & Lund, 1980). The large difference in 2-oxo acid concentration between plasma and liver, the main tissue metabolizing plasma branched-chain 2-oxo acids was suggested to be due to poor permeability of the plasma membrane to the 2-oxo acids (Livesey & Lund, 1980). This is certainly not the case with the isolated cells, but the reason for this is obscure. Possibly the permeability properties of the plasma membranes of the isolated cells differ from those *in vivo*, or perhaps the hepatocytes *in vitro* present a significantly larger surface area for 2-oxo acid uptake. Whatever the reason, it appears the isolated cell system may not be suitable for an investigation of factors which affect branched-chain 2-oxo acid utilization when rates of metabolism are concentration-dependent. Moreover, although defatted bovine serum albumin was used in the present work (to maximize binding of 2-oxo acid), it cannot be considered to reproduce physiological conditions in the fed rat, because rat plasma protein binds considerably less branched-chain 2-oxo acid than does defatted bovine serum albumin (Livesey & Lund, 1982). Whether binding to albumin *in vivo* limits the utilization of the branched-chain 2-oxo acids, as in the present work, or whether it simply expands the plasma pool size for these 2-oxo acids remains to be determined. This question is more relevant to man, since the bound-to-free concentration ratio is greater in human than in rat plasma (Livesey & Lund, 1982) and because this ratio is affected by changes in the unesterified fatty acid concentration (Nissen *et al.*, 1982).

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