Biochem. J. (1982) **204**, 265–272
Printed in Great Britain

Binding of branched-chain 2-oxo acids to bovine serum albumin

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(*Received 5 November 1981/Accepted 18 January 1982*)

1. Binding of branched-chain 2-oxo acids to defatted bovine serum albumin was shown by gel chromatography and equilibrium dialysis. 2. Equilibrium-dialysis data suggest a two-site model for binding in Krebs–Henseleit saline at 37°C with $n_1 = 1$ and $n_2 = 5$. Site association constants were: 4-methyl-2-oxovalerate, $k_1 = 8.7 \times 10^3\,M^{-1}$, $k_2 = 0.09 \times 10^3\,M^{-1}$; 3-methyl-2-oxovalerate, $k_1 = 9.8 \times 10^3\,M^{-1}$, $k_2 = 0.08 \times 10^3\,M^{-1}$; 3-methyl-2-oxobutyrate, $k_1 = 1.27 \times 10^3\,M^{-1}$, $k_2 = <0.05 \times 10^3\,M^{-1}$. 3. Binding of 4-methyl-2-oxovalerate to defatted albumin in a phosphate-buffered saline, pH 7.4, gave the following thermodynamic parameters: primary site $\Delta H_1^0 = -28.6\,kJ \cdot mol^{-1}$ and $\Delta S_1^0 = -15.2\,J \cdot mol^{-1} \cdot K^{-1}$ ($\Delta G_1^0 = -24.0\,kJ \cdot mol^{-1}$ at 37°C) and secondary sites $\Delta H_2^0 = -25.4\,kJ \cdot mol^{-1}$ and $\Delta S_2^0 = -46.1\,J \cdot mol^{-1} \cdot K^{-1}$ ($\Delta G_1^0 = -11.2\,kJ \cdot mol^{-1}$ at 37°C). Thus binding at both sites is temperature-dependent and increases with decreasing temperature. 4. Inhibition studies suggest that 4-methyl-2-oxovalerate may associate with defatted albumin at a binding site for medium-chain fatty acids. 5. Binding of the 2-oxo acids in bovine, rat and human plasma follows a similar pattern to binding to defatted albumin. The proportion bound in bovine and human plasma is much higher than in rat plasma. 6. Binding to plasma protein, and not active transport, explains the high concentration of branched-chain 2-oxo acids leaving rat skeletal muscle relative to the concentration within the tissue, but does not explain the 2-oxo acid concentration gradient between plasma and liver.

The liver is an important site for the further metabolism of the branched-chain 2-oxo acids that are released from skeletal muscle of the rat on transamination of leucine, isoleucine and valine. However, the liver content of these 2-oxo acids is extremely low (<5 nmol/g) compared with the plasma concentration of approx. 40 nmol/ml (Hutson, 1980; Livesey & Lund, 1980; Hutson & Harper, 1981). This led us (Livesey & Lund, 1980) to suggest that the rate of transfer of the 2-oxo acids across the plasma membrane may be rate-limiting for their metabolism by the liver. Alternatively, binding of the 2-oxo acids to plasma albumin could be a factor contributing to the apparent gradient between plasma and tissues. We have examined the latter possibility, and find that binding accounts for a large proportion of branched-chain 2-oxo acids in bovine, rat and human plasma. The present paper is concerned with the characteristics of the phenomenon when plasma is replaced by defatted bovine serum albumin.

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Materials and methods

Materials

Dialysis tubing (0.6 cm inflated diameter) was from Gallenkamp and Co. Sephadex G-25-40, lysozyme (grade 1), egg albumin (grade V) and human serum albumin (fraction V) were products of Sigma Chemical Co. Casein was from BDH Chemicals, trypsin from Worthington Biochemical Corp. and bovine serum albumin from Miles Laboratories. Defatted albumins were prepared as described by Chen (1967). The sodium salts of L-2-hydroxy-4-methylvaleric acid, L-2-hydroxy-3-methylbutyric acid, 3-methyl-2-oxovaleric acid ('oxo isoleucine'), 3-methyl-2-oxobutyric acid ('oxo valine'), 4-methylthio-2-oxobutyric acid and phenylpyruvic acid, prepared as described by Walser *et al.* (1973), were a gift from Dr. M. Walser. Acetoacetate was prepared from ethyl acetoacetate as described by Williamson *et al.* (1962). L-[1-^{14}C]-Leucine, L-[U-^{14}C]isoleucine, L-[1-^{14}C]valine and inulin[^{14}C]carboxylic acid were from The Radiochemical Centre. All other compounds were obtained from Sigma Chemical Co., BDH Chemicals,

0306-3283/82/040265-08$01.50/1

Boehringer Corp., Cambrian Chemicals or Fluorochem.

Preparation of radiolabelled 2-oxo acids

4-Methyl-2-oxo[1-^{14}C]valerate, [U-^{14}C]3-methyl-2-oxovalerate and 3-methyl-2-oxo[1-^{14}C]butyrate were prepared from L-[1-^{14}C]leucine, L-[U-^{14}C]isoleucine and L-[1-^{14}C]valine respectively as described by Lund (1978). Each radiolabelled 2-oxo acid was separated from its parent amino acid by ion-exchange chromatography on an Amberlite IR-120 (H^+ form) resin column (180mm × 8mm) eluted wtih water. 2-Oxo acids were found to be at least 99% pure radiochemically by t.l.c. analysis on polysilicate-gel-impregnated glass fibres (Lund, 1978). Treatment of the 1-^{14}C-labelled 2-oxo acids with acidic H_2O_2 (Odessey & Goldberg, 1979) evolved 98–99% of the radiolabel as $^{14}CO_2$, confirming the high radiochemical purity of these 2-oxo acids.

Determination of branched-chain 2-oxo acid binding to bovine serum albumin

The binding of branched-chain 2-oxo acid (0.02–20mM free concentration) to bovine serum albumin was measured by equilibrium dialysis (Lipsett *et al.*, 1973). Freeze-dried defatted albumin was dissolved in Krebs & Henseleit (1932) saline (2.6%, w/v, unless otherwise specified). Portions (0.5ml) were tied into dialysis tubing (previously washed with nitric acid; Warner & Weber, 1953) to form sacs, which were washed at room temperature (20–22°C) for 30min against 40vol. of gassed Krebs–Henseleit saline. The sacs were then dialysed against 4ml of Krebs–Henseleit saline, containing a radiolabelled 2-oxo acid (5–20nCi) and other additions as specified in Tables and Figures, for 5h at 37°C with shaking under O_2/CO_2 (19:1).

Where the effect of certain fatty acids (Fig. 3) on 2-oxo acid binding by albumin was investigated, the albumin was dissolved in the 4ml of Krebs–Henseleit saline external to the dialysis sac with 0.5ml of the saline alone inside. Fatty acids and radiolabelled 2-oxo acid were added to the albumin solution. In experiments on the effect of temperature on 2-oxo acid binding to albumin the Krebs–Henseleit saline was replaced by phosphate-buffered saline (50mM-KH_2PO_4/K_2HPO_4 buffer, pH7.4, containing 154mM-NaCl). Incubations were for 5h at 42 and 37°C and for 16h at 25, 17 and 3°C.

The solutions on each side of the dialysis membrane were sampled (0.05–0.2ml made up to 0.2ml with water) for scintillation counting of radioactivity in 10ml of a mixture of 3 litres of toluene, 2 litres of 2-methoxyethanol, 300g of naphthalene and 28g of Packard Permablend III. Counting of radioactivity was in an LKB Rackbeta instrument, with the use of an external standard, with an efficiency of >90% for ^{14}C.

Concentrations of radiolabelled 2-oxo acids were calculated from the specific radioactivity determined in each experiment. Albumin binding was determined by difference. Bovine serum albumin concentrations were determined spectrophotometrically, by using an absorption coefficient ($A_{1cm}^{1\%\,(w/v)}$) at 280nm of 6.6 (Cohn *et al.*, 1947).

Analysis of binding data

The stoicheiometric binding constant, K', for 2-oxo acid binding to albumin was calculated from the equilibrium concentrations of bound ligand, b, free ligand, c, and albumin, p, in accordance with the expression:

$$K' = b/c(p-b)$$

This calculation assumes the binding of one molecule of 2-oxo acid by one molecule of albumin, which is found in the present study to be essentially correct when $b \ll p$. Binding data were analysed by the method of Scatchard (1949) in accordance with the equation:

$$r/c = nk - rk$$

where r is the fraction of albumin to which ligand is bound (i.e. b/p), n is the number of binding sites per albumin molecule and k is the site association constant. Values of n and k were obtained from plots of r/c against r; curved plots were resolved by the method of Rosenthal (1967). Binding ratios (r) were calculated from a molecular weight of 69000 for bovine serum albumin (McMenamy & Oncley, 1958).

Sephadex G-25 chromatography

A Sephadex G-25-40 column (180mm × 8mm) and 0.5ml of 2.6% (w/v) defatted bovine serum albumin were equilibrated at 20–22°C with pregassed (O_2/CO_2, 19:1) Krebs–Henseleit saline containing 50μM-4-methyl-2-oxo[1-^{14}C]valerate. The albumin solution was added to the column and eluted with the saline/radiolabelled 2-oxo acid mixture at a flow rate of 0.3ml/min. Fractions (approx. 0.3ml) were collected and assayed for 2-oxo acid (scintillation counting of radioactivity) and albumin (A_{280}).

Binding of branched-chain 2-oxo acids in plasma

Fresh blood was obtained from the antecubital vein of fed adult male humans, from the aorta of fed adult male rats under ether anaesthesia and from cows at slaughter. Samples were incubated with 4-methyl-2-oxo[1-^{14}C]valerate, [U-^{14}C]3-methyl-2-oxovalerate or 3-methyl-2-oxo[1-^{14}C]butyrate (10μl containing 5–10nCi/ml of blood) at 37°C under O_2/CO_2 (19:1). When distribution of radiolabel

reached plateau (5 min), the mixtures were centrifuged at room temperature and a plasma sample (0.1–0.2 ml) of each was counted for radioactivity. The remainder of the plasma was re-incubated at 37°C for 5 min before ultrafiltration through an Amicon Centriflo 224-CF-50 membrane cone at 37°C in an MSE High Spin-21 centrifuge at 800*g* for 10 min. The ultrafiltrate was assayed for radioactivity, and the amount of protein-bound 2-oxo acid was calculated by difference.

For determination of the distribution of 2-oxo acid in whole blood, the plasma volume was determined with inulin[^{14}C]carboxylic acid.

Results and discussion

Association of branched-chain 2-oxo acids with albumin

Binding of 4-methyl-2-oxo[1-^{14}C]valerate to albumin was shown by equilibrium dialysis. A time course showed equilibrium to be established within 3.5–4 h after the addition of 225 nmol of 4-methyl-2-oxo[1-^{14}C]valerate to the dialysis system. At 5 h, the bound/free concentration ratio (b/c) of 2-oxo acid was 2.6 with 100.9 ± 0.9% recovery of radiolabel (mean ± S.E.M., $n = 27$). During this period of dialysis the albumin concentration remained essentially unchanged at 98.5 ± 0.7% (mean ± S.E.M., $n = 26$) of the initial concentration. Transfer of dialysis sacs containing albumin equilibrated with 4-methyl-2-oxo[1-^{14}C]valerate (as above) to 200 vol. of 2-oxo acid-free medium showed more than 99% of the radioactivity to be removed from the albumin solution within 4 h.

When either [U-^{14}C]3-methyl-2-oxovalerate or 3-methyl-2-oxo[1-^{14}C]butyrate replaced 4-methyl-2-oxo[1-^{14}C]valerate in the above experiments, similar reversible associations were observed with essentially unchanged time courses. At 5 h (equilibrium) for [U-^{14}C]3-methyl-2-oxovalerate b/c was 2.7 with 98.4 ± 1.9% (mean ± S.E.M., $n = 7$) recovery. 3-Methyl-2-oxo[1-^{14}C]butyrate associated less than the other two branched-chain 2-oxo acids, with a b/c at equilibrium of 0.4 and a recovery of 101.1 ± 1.9% (mean ± S.E.M., $n = 5$).

Under the above conditions, trypsin, lysozyme, casein or egg albumin bound very little or no 4-methyl-2-oxo[1-^{14}C]valerate ($b/c < 0.04$), but human serum albumin bound amounts ($b/c = 2.3$) similar to those bound by bovine serum albumin. Association of 2-oxo acid with the bovine albumin was confirmed by the Sephadex G-25 chromatography experiment (see the Materials and methods section), which showed 4-methyl-2-oxo[1-^{14}C]valerate to be eluted well in advance of its expected elution position, coincidentally with the albumin.

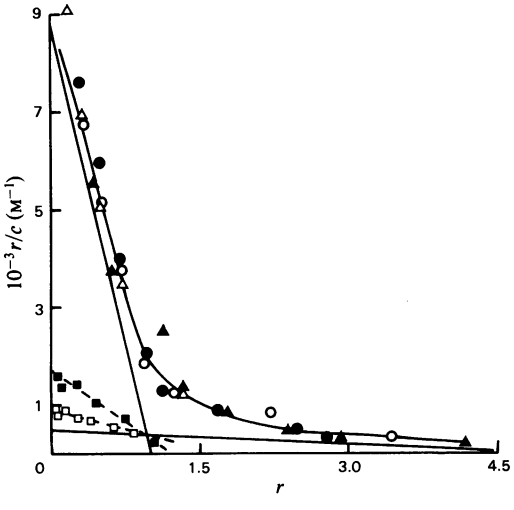

Fig. 1. *Binding of 4-methyl-2-oxo[1-^{14}C]valerate to defatted bovine serum albumin*

Each point shows the average of duplicate observations at 37°C at pH 7.4 with four albumin concentrations in Krebs–Henseleit saline: ▲, 5.0%; ●, 2.6%; ○, 1.0%; △, 0.5% (w/v). The continuous curved line was computed from $n_1 = 1$, $k_1 = 8.7 \times 10^3\,M^{-1}$, $n_2 = 5$, $k_2 = 0.09 \times 10^3\,M^{-1}$, i.e. values that describe the two linear components shown. The broken lines show 4-methyl-2-oxo[1-^{14}C]valerate binding to 2.6% (w/v) defatted albumin in the presence of 2 mM-3-methyl-2-oxovalerate (□) and of 5 mM-3-methyl-2-oxobutyrate (■).

Scatchard analysis of branched-chain 2-oxo acid binding to albumin

Binding data, obtained at several concentrations (0.05–20 mM) of 4-methyl-2-oxo[1-^{14}C]valerate and a 10-fold range of albumin concentration (0.5–5%, w/v), showed a curved Scatchard plot (Fig. 1). The smooth curve through the points was computed from the two linear components summed along vectors of constant free ligand concentration (Rosenthal, 1967). These linear components indicate 4-methyl-2-oxo[1-^{14}C]valerate to bind to the defatted bovine serum albumin at a single primary site ($n_1 = 1$) with a site association constant (slope of the curve), k_1, of $8.7 \times 10^3\,M^{-1}$ and several other secondary sites ($n_2 = 5$) with an average site association constant, k_2, of $0.09 \times 10^3\,M^{-1}$. The binding data at each albumin concentration appear to fit the smooth curve calculated from these constants, suggesting that the constants are not a function of albumin concentration, as is the case with certain other ligands (see Bowmer & Lindup, 1980). Three different preparations of defatted bovine serum albumin had very similar values of n_1,

n_2, k_1 and k_2 (results not shown), and hence the defatting procedure yielded a consistent product.

Scatchard analysis of [U-^{14}C]3-methyl-2-oxovalerate binding (Fig. 2*a*) fitted a curve computed from two linear components with $n_1 = 1$ and $k_1 = 9.8 \times 10^3\,M^{-1}$ and with $n_2 = 5$, $k_2 = 0.08 \times 10^3\,M^{-1}$; hence binding was very similar to that for 4-methyl-2-oxo[1-^{14}C]valerate. However, Scatchard plots for 3-methyl-2-oxo[1-^{14}C]butyrate binding (Fig. 2*b*) fitted a single linear curve with $n_1 = 1.02$ and $k_1 = 1.27 \times 10^3\,M^{-1}$ (both obtained by linear regression); this suggests a lower affinity for the primary binding site with either no, or very little, affinity for the secondary binding sites. (If $n_2 = 5$, as for the other 2-oxo acids, then $k_2 = <0.05 \times 10^3\,M^{-1}$.)

Binding of 4-methyl-2-oxo[1-^{14}C]valerate was determined in the presence of 5mM-3-methyl-2-oxobutyrate and of 2mM-3-methyl-2-oxovalerate (Fig. 1). The slopes of these Scatchard binding curves are not as steep as for 4-methyl-2-oxo-[1-^{14}C]valerate alone. Binding of the ligand to the secondary binding sites in the presence of the unlabelled branched-chain 2-oxo acids does not produce substantial curvature in the binding curves. Thus the slope of each curve approximates to the apparent primary-site association constant, k'_1, which was used to calculate inhibitor constants, K_i, in accordance with the formula (Edsall & Wyman, 1958):

$$K_i = \frac{1}{I} \cdot \left(\frac{k_1}{k'_1} - 1 \right)$$

where k_1 is the primary-site association constant for 4-methyl-2-oxo[1-^{14}C]valerate ($8.7 \times 10^3\,M^{-1}$) and I is the free concentration of the inhibitor. I was set at 2mM for 3-methyl-2-oxovalerate and 5mM for 3-methyl-2-oxobutyrate because the fraction of 3-methyl-2-oxovalerate and 3-methyl-2-oxobutyrate bound in these experiments was small at all 4-methyl-2-oxo[1-^{14}C]valerate concentrations. For 3-methyl-2-oxovalerate $K_i = 8.7 \times 10^3\,M^{-1}$ and for 3-methyl-2-oxobutyrate $K_i = 1.1 \times 10^3\,M^{-1}$, values close to the primary-site association constants for these branched-chain 2-oxo acids ($k_1 = 9.8 \times 10^3\,M^{-1}$ and $k_1 = 1.27 \times 10^3\,M^{-1}$ respectively). The values of K_i will be in small error, since no account is taken of binding of ligand to the secondary sites. Nevertheless the similarities of K_i and k_1 suggest that all three branched-chain 2-oxo acids bind to the same primary site.

The independence of albumin concentration and the binding parameters for 2-oxo acid binding to albumin (Fig. 1) permits the calculation of the proportion of each 2-oxo acid bound to albumin when the concentrations of both protein and ligand are varied over physiological values. Varying the total 2-oxo acid concentration between 1 and 150μM has only a small effect on the proportion of 2-oxo acid bound to albumin at a given concentration, but varying the albumin concentration from 20 to 50g/l has a substantial effect when the total 2-oxo acid concentration remains constant (results not shown).

Although Scatchard or site-binding models may be questioned mechanistically, they are still considered valid (see Meisner *et al.*, 1980; Ferguson-Miller & Koppenol, 1981). In the present study they show that branched-chain 2-oxo acids bind to defatted bovine serum albumin in accordance with a two-site model with a single primary site and five secondary sites. The primary-site association constants for 4-methyl-2-oxovalerate and 3-methyl-2-oxovalerate are similar and about 100-fold higher than those for the secondary sites; 3-methyl-2-oxobutyrate binds less well to the primary site and only to a small extent, or not at all, to the secondary sites. These primary-site association constants (k_1) are in general slightly lower than those reported for a wide range of drugs, steroids and short-to-medium-chain fatty acids, but several orders of magnitude lower than for the binding to albumin of bilirubin or

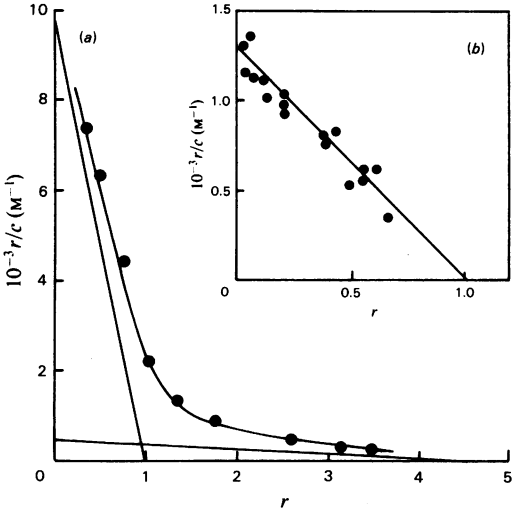

Fig. 2. *Binding of (a) [U-^{14}C]3-methyl-2-oxovalerate and (b) 3-methyl-2-oxo[1-^{14}C]butyrate to defatted bovine serum albumin*

Each point shows the average of duplicate observations at 37°C in Krebs–Henseleit saline at pH 7.4. The continuous curved line in (*a*) was calculated from $n_1 = 1$, $k_1 = 9.8 \times 10^3\,M^{-1}$, $n_2 = 5$, $k_2 = 0.08 \times 10^3\,M^{-1}$, i.e. values that describe the two linear components shown. The linear curve in (*b*) was obtained by regression analysis of results from two independent experiments and gave the values $n_1 = 1.02$ and $k_1 = 1.27 \times 10^3\,M^{-1}$.

long-chain fatty acids (Spector, 1975; Peters, 1975; Wosilait & Ryan, 1980).

Effects of temperature on 4-methyl-2-oxo[1-^{14}C]-valerate binding

Phosphate-buffered saline replaced Krebs–Henseleit saline to maintain a constant pH of 7.4 as the temperature was varied (see the Materials and methods section). 4-Methyl-2-oxo[1-^{14}C]valerate (0.02–15mM free concentration) binding to defatted bovine serum albumin (2.6%, w/v) at five temperatures from 3 to 42°C gave curved Scatchard plots, which resolved by Rosenthal (1967) analysis into two linear components. The binding of 4-methyl-2-oxo[1-^{14}C]valerate to the defatted albumin was strongly temperature-dependent, with increases in both k_1 and k_2 with decreasing temperature. The free energies of binding to the primary and secondary sites were, however, virtually constant over the temperature range tested and were more than twice as negative at the primary site ($\Delta G_1^0 = -24.0\,kJ\cdot mol^{-1}$ at 37°C) as at the secondary sites ($\Delta G_2^0 = -11.2\,kJ\cdot mol^{-1}$ at 37°C). Van't Hoff plots [$\log k$ versus $1/T\,(K^{-1})$] constructed from k_1 and k_2 at each temperature gave linear plots (as judged visually) with correlation coefficients of 0.974 and 0.996 respectively. This suggests that changes in enthalpy (heat content), ΔH_1^0 and ΔH_2^0, accompanying binding of 4-methyl-2-oxo[1-^{14}C]-valerate to the primary and secondary sites respectively are constant over the temperature range 3–42°C. Values for ΔH_1^0 and ΔH_2^0 were therefore calculated from the slopes (obtained by linear regression) of the Van't Hoff plots and were found to be similar and negative ($\Delta H_1^0 = -28.6 \pm 2.7\,kJ\cdot mol^{-1}$; $\Delta H_2^0 = -25.4 \pm 0.9\,kJ\cdot mol^{-1}$). The entropy changes, ΔS_1^0 and ΔS_2^0, accompanying binding to the primary and secondary sites respectively were independent of temperature and were calculated as the mean of the values obtained at each temperature. ΔS_2^0 was $-46.1 \pm 0.3\,J\cdot mol^{-1}\cdot K^{-1}$, about 3 times as negative as ΔS_1^0, which was $-15.2 \pm 0.9\,J\cdot mol^{-1}\cdot K^{-1}$.

Effects of carboxylic acids on the binding of 4-methyl-2-oxovalerate to defatted bovine serum albumin

The effects of increasing concentrations of various carboxylic acids on 4-methyl-2-oxo[1-^{14}C]valerate binding to 2.6% (w/v) defatted bovine serum albumin at low ligand concentrations are shown in Table 1. The inhibitory potencies of the added carboxylic acids are shown as decreases in the stoicheiometric association constant (K') for binding, since this takes into account the small changes in free ligand concentration that accompany the inhibition. Values for inhibitor constants, K_i, were not calculated, since the free concentrations of the inhibitors at equilibrium were not determined.

As already mentioned above, each branched-chain 2-oxo acid inhibited 4-methyl-2-oxo[1-^{14}C]-valerate binding. 3-Methyl-2-oxobutyrate was the least inhibitory, in accordance with its low site association constant at the primary site (see Fig. 2). Pyruvate was a poor inhibitor, but inhibition progressively increased as the chain length of the straight-chain 2-oxo acid increased. Dicarboxylic 2-oxo acids were poor inhibitors by comparison with the monocarboxylic 2-oxo acids of the same chain length. The 2-oxo acids related to tryptophan, phenylalanine and methionine were also inhibitory. The branched-chain 2-hydroxy acids were less inhibitory than were the corresponding branched-chain 2-oxo acids, but the branched-chain 2-amino acids were not inhibitory (except for a small inhibition by the highest concentration of isoleucine). The branched-chain fatty acids inhibited binding with increasing potency as the chain length increased. Several physiological carboxylic acids, including tryptophan, were not inhibitory at near-physiological concentrations.

The effect of straight-chain fatty acids of increasing chain length on the stoicheiometric association constant (K') for 4-methyl-2-oxo[1-^{14}C]-valerate binding is shown in Fig. 3. Increasing the chain length to C_6 progressively increased the extent of inhibition of binding. At 0.2mM hexanoate was the most effective inhibitor. Binding then became less inhibited as the chain length increased. The optimum inhibitory chain length may be a function of fatty acid concentration, since it appeared to be C_6 at 0.2mM, C_8 at 1.0mM and possibly longer at 10mM. Although long-chain fatty acids do not freely permeate dialysis membranes (Spector, 1975), separate experiments showed no association of 4-methyl-2-oxovalerate with long-chain fatty acids.

Structural requirements for binding of branched-chain 2-oxo acids

The site-binding model for association with bovine albumin ($n_1 = 1$; $n_2 = 5$) is similar to that for octanoate ($n_1 = 1$; $n_2 = 7$; Meisner *et al.*, 1980) and considerably different from that for long-chain fatty acids (see Wosilait & Ryan, 1980). The observations in Fig. 3 support this suggestion, but confirmation would require direct competition studies.

Binding of the 2-oxo acids to the binding sites for medium-chain fatty acids on the albumin molecule implies that hydrophobic bonding is important. Several observations confirm this. Firstly, the higher site constants for the binding of 4-methyl-2-oxovalerate and 3-methyl-2-oxovalerate than for 3-

Table 1. *Effects of carboxylic acids on the stoicheiometric association constant for 4-methyl-2-oxovalerate binding to defatted bovine serum albumin*

In calculating the stoicheiometric association constants, K', it was assumed that the ligand binds to a single site on the albumin molecule at low free ligand concentrations (see the Results and discussion section). Binding was determined as described in the Materials and methods section. The initial free concentrations of 4-methyl-2-oxovalerate was 50μM (225nmol/4.5ml). Values of K' are means ± S.E.M. for three independent observations or means for two independent observations for lactate and citrate. Values not significantly different from three matched controls at the 95% level of confidence were determined by the paired t test and are indicated by an asterisk (*). The stoicheiometric association constant for 4-methyl-2-oxovalerate binding to albumin in the absence of additions was $9.7 \times 10^3 M^{-1}$ (S.E.M. $= 0.4 \times 10^3 M^{-1}$; $n = 10$). For comparison, when the initial concentration of 4-methyl-2-oxovalerate was increased by 0.2, 1.0 and 10mM, $10^{-3}K'$ (M^{-1}) was decreased to 3.9 ± 0.1, 2.3 ± 0.2 and 0.2 ± 0.1 respectively.

	$10^{-3}K'$ (M^{-1})		
Initial free concn. ...	0.2mM	1.0mM	10.0mM
Branched-chain 2-oxo acids			
3-Methyl-2-oxobutyrate (oxo valine)	9.2 ± 0.7	5.9 ± 0.7	1.1 ± 0.1
3-Methyl-2-oxovalerate (oxo isoleucine)	5.6 ± 1.1	2.5 ± 0.4	0.3 ± 0.1
Straight-chain 2-oxo acids			
2-Oxopropionate (pyruvate)	9.1 ± 1.3*	8.2 ± 1.3*	5.9 ± 0.8
2-Oxobutyrate	7.6 ± 0.5	6.5 ± 0.7	2.3 ± 0.3
2-Oxovalerate	5.5 ± 0.7	3.0 ± 0.4	0.6 ± 0.1
2-Oxohexanoate	4.8 ± 0.5	1.9 ± 0.2	0.4 ± 0.2
2-Oxo-octanoate	2.2 ± 0.3	0.5 ± 0.2	<0.1
Dicarboxylic 2-oxo acids			
2-Oxobutanedioate (oxoglutarate)	8.6 ± 0.2	8.0 ± 0.1	4.3 ± 0.7
2-Oxohexanedioate (oxoadipate)	9.7 ± 0.2*	6.9 ± 0.1	4.0 ± 0.1
Miscellaneous 2-oxo acids			
3-Indolyl-2-oxopropionate (indolylpyruvate)	7.3 ± 0.7	4.1 ± 0.2	0.3 ± 0.1
3-Phenyl-2-oxopropionate (phenylpyruvate)	4.6 ± 0.2	1.4 ± 0.1	0.1 ± 0.1
4-Methylthio-2-oxobutyrate	6.7 ± 0.3	3.3 ± 0.4	0.7 ± 0.1
Branched-chain fatty acids			
2-Methylpropionate (isobutyrate)	7.0 ± 1.3	6.1 ± 0.7	1.5 ± 0.1
3-Methylbutyrate (isovalerate)	6.2 ± 0.4	2.7 ± 0.2	0.5 ± 0.1
4-Methylvalerate	2.9 ± 0.2	1.0 ± 0.1	0.2 ± 0.1
Branched-chain 2-hydroxy acids			
L-2-Hydroxy-3-methylbutyrate	9.5 ± 0.6	6.0 ± 0.7	2.0 ± 0.1
L-2-Hydroxy-4-methylvalerate	6.5 ± 0.5	3.7 ± 0.4	0.9 ± 0.1
Branched-chain 2-amino acids			
L-2-Amino-3-methylbutyrate (valine)	8.3 ± 0.7*	9.5 ± 0.9*	8.6 ± 0.4*
L-2-Amino-3-methylvalerate (isoleucine)	9.9 ± 0.3*	10.9 ± 0.2*	5.5 ± 0.2
L-2-Amino-4-methylvalerate (leucine)	9.9 ± 0.1*	9.9 ± 0.6*	9.9 ± 0.1*
Miscellaneous			
Acetoacetate	10.5 ± 0.9*	9.7 ± 0.4*	5.6 ± 0.2
DL-3-Hydroxybutyrate	10.4 ± 1.3*	10.7 ± 0.2	5.1 ± 0.3
Lactate	8.2	8.6	6.4
Citrate	8.7	9.4	9.1
Tryptophan	9.0 ± 0.1*	7.8 ± 0.1	6.5 ± 0.1

methyl-2-oxobutyrate (see Figs. 1 and 2) suggest that the additional hydrophobic methylene group of the former two compounds is important. Secondly, replacement of the very hydrophobic branched-chain aliphatic carbon chains by the less hydrophobic methyl group (to give pyruvate) markedly decreases the capacity to compete with 4-methyl-2-oxovalerate for binding (see Table 1), but replacement with the more hydrophobic 2-oxo-octanoate increases the capacity to compete for the binding site. Thus the 2-carbonyl-carboxylate group itself is not a major determinant for binding. Thirdly, increasing the hydrophobicity of 4-methyl-2-oxovalerate by replacing the carbonyl group with a hydrophobic methylene group improves the capacity to compete, whereas replacement with a hydrophilic hydroxymethylene group decreases it.

Although a hydrophobic residue appears to be

Table 2. *Association of branched-chain 2-oxo acids with plasma proteins*

Endogenous branched-chain 2-oxo acid was labelled by mixing a trace quantity of radioactive 2-oxo acid with whole blood. After equilibration the recovery of radioactivity in the plasma fraction and the proportion of plasma radioactivity that was bound to protein were determined at 37°C as described in the Materials and methods section. Values shown are means ± S.E.M. (*n*).

	4-Methyl-2-oxo[1-^{14}C]valerate	[U-^{14}C]3-Methyl-2-oxovalerate	3-Methyl-2-oxo[1-^{14}C]butyrate
Recovery of 2-oxo acid in plasma (%):			
Cow	99 ± 6 (6)	100 ± 8 (6)	103 ± 9 (6)
Man	94 ± 2 (5)	95 ± 2 (6)	84 ± 4 (6)
Rat	81 ± 3 (11)	84 ± 4 (6)	78 ± 4 (6)
Plasma 2-oxo acid bound to protein (%):			
Cow	69 ± 2 (6)	78 ± 1 (6)	30 ± 3 (6)
Man	61 ± 2 (5)	69 ± 2 (6)	34 ± 1 (6)
Rat	43 ± 2 (4)	44 ± 2 (4)	14 ± 1 (4)

essential for binding, other interactions must account for the energy changes accompanying binding. This is because the free-energy change (ΔG^0) accompanying hydrophobic bonding is mainly accounted for by changes in entropy ($T \cdot \Delta S^0$), but for 4-methyl-2-oxovalerate changes in enthalpy (ΔH^0) appear to account for a larger part of the free-energy changes for binding to albumin at the primary site.

Binding of branched-chain 2-oxo acids in rat, cow and human plasma

Branched-chain 2-oxo acids are recovered almost entirely in plasma when trace amounts of ^{14}C-labelled marker are equilibrated with endogenous 2-oxo acids in human or bovine whole blood (Table 2). The proportion retained in rat plasma is lower (about 80% retained; Table 2), giving a similar distribution between blood cells and plasma to that found in rat blood by Hutson & Harper (1981). The pattern of binding of the three 2-oxo acids in plasma is similar to the pattern of binding to defatted bovine serum albumin (see Figs. 1 and 2), the implication being that binding is to the albumin fraction of plasma. A larger proportion of plasma 2-oxo acid is protein-bound in bovine and human plasma than in rat plasma. Nissen & Haymond (1981) report values of over 80% binding of 4-methyl-2-oxovalerate in human and dog plasma.

The smaller degree of binding of the 2-oxo acids in rat plasma may be due either to smaller binding constants for rat albumin than for albumin from the other species or to more inhibition by endogenous monocarboxylates in rat plasma. For bovine plasma, 2-oxo acid binding to albumin is likely to be mostly uninhibited by endogenous monocarboxylates, since the proportions of each 2-oxo acid bound (Table 2) are similar to those predicted from the binding constants (Figs. 1 and 2) for physiological concentrations of albumin (30g/l) if it is assumed that the plasma concentration of each 2-oxo acid is

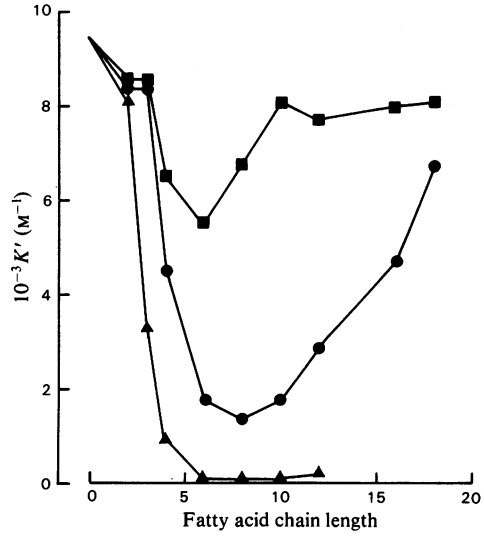

Fig. 3. *Effects of chain length on inhibition by straight-chain fatty acids of 4-methyl-2-oxo[1-^{14}C]valerate binding to defatted bovine serum albumin*

The stoicheiometric association constants (K') are means of three independent determinations calculated assuming the ligand to bind to a single site on the albumin molecule at a low free ligand concentration (see the Results and discussion section). Binding was determined as described in the Materials and methods section. The initial free concentration of 4-methyl-2-oxo[1-^{14}C]valerate was 50 μM (225nmol/4.5ml) and that of the fatty acids 0.2mM (■), 1.0mM (●) or 10mM (▲).

10–30 μM. However, a quantitative comparison of binding by bovine plasma and defatted bovine serum albumin is precluded, since the individual 2-oxo acid concentrations in bovine plasma are not known and small proportions of the 2-oxo acids might bind to other plasma proteins (Nissen & Haymond, 1981).

Physiological significance of binding of branched-chain 2-oxo acids in plasma

Some assessment of the physiological consequences of binding can be made for the rat, the only species for which tissue concentrations of the 2-oxo acids are available. Plasma concentrations in the vena cava of fed rats are reported to be 16, 12 and 10 μM for the 2-oxo acid analogues of leucine, isoleucine and valine respectively (Hutson & Harper, 1981). After correction for binding (see Table 2) these values become 9, 7 and 9 μM respectively, so that they become more similar than the total concentrations would suggest. From our measurements (Livesey & Lund, 1980) in tissues and plasma, the skeletal-muscle content, even after recalculation on the basis of tissue water, appeared to be lower than in the femoral vein, yet this appeared to be the only tissue contributing branched-chain 2-oxo acids to the circulation. Binding to plasma protein explains this paradox and eliminates the need to postulate active transport. However, binding does not explain the extremely low concentrations of the 2-oxo acids in tissues other than skeletal muscle. In these tissues, and especially liver, which oxidizes 2-oxo acids released from peripheral tissues, the rate of transfer across the plasma membrane is likely to be limiting (see Livesey & Lund, 1980).

G. L. and the work were supported by a Medical Research Council Project Grant held by P. L.

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