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A double-isotope method for the measurement of ketone-body turnover in the rat

Effect of L-alanine

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The synthesis of 4-^{3}H-labelled ketone bodies, and their use along with ^{14}C-labelled ketone-body precursors, is employed using an '*in vivo*' rat infusion model to measure ketone-body turnover. The use of two isotopes is necessary to measure ketone-body turnover when ketogenesis may occur from more than one precursor such as glucose and fatty or amino acids. Requirements of isotopic equivalence in terms of metabolic similarity, valid stoichiometry and the lack of differences in the kinetics of relevant enzymes is demonstrated for the 4-^{3}H- and ^{14}C-labelled ketone bodies. The hypoketonaemic effect of L-alanine is shown by two distinct phases after the administration of L-alanine. During the first 12 min after alanine administration there was a 50% decrease in acetoacetate and a 30% decrease in 3-hydroxybutyrate production, with no significant change in the utilization of either compound. The hypoketonaemic action of alanine during the following 16 min was primarily associated with an uptake of 3-hydroxybutyrate that was somewhat greater than the increase in its production. There were essentially equivalent decreases in production and utilization of acetoacetate, resulting in no significant net change in the level of this ketone body in the blood.

Various measurements of ketone-body turnover *in vivo* have been described (Keller *et al.*, 1978; Balasse, 1979; Barton, 1980). In each case a single-isotope technique has been the basis of these estimates. This approach is generally useful for measurement of the overall turnover of ketone bodies; however, in specific cases the addition of a second isotope has distinct advantages. In order to measure as separate events the production and utilization of ketone bodies when one or more substrate or precursor is involved, two isotopes are virtually indispensable. An appropriate example is simultaneous lipogenesis and ketogenesis while glucose and fatty acids are contributing to both processes. Recently, Cobelli *et al.* (1982) emphasized the desirability of a double-isotope technique as an appropriate basis for such kinetic evaluation. The use of a hydrogen isotope, ^{3}H, in addition to ^{14}C, allows information regarding the identity, location and stoichiometry of the isotopes in the products of ketone-body metabolism to be obtained. Accordingly, a procedure for the use of two isotopes had to be developed. Such a double-isotope technique imposes certain requirements of isotopic equivalence among which are: metabolic similarity, valid stoichiometry, and a lack of significant difference in the kinetics of key enzymes. The present paper describes the chemical synthesis and use of 4-^{3}H-labelled ketone bodies along with ^{14}C-labelled ketone-body precursors to study the effect of alanine on their turnover.

Experimental

Materials

The [1-^{14}C]octanoic acid, [3-^{14}C]ethyl acetoacetate and D-3-hydroxy[3-^{14}C]butyrate were purchased from New England Nuclear Corp. Boston, MA., U.S.A. The hydrolysis and purification of ethyl acetoacetate was essentially as described by Barton (1980) and Ozand *et al.* (1978). Somatostatin, other chemicals and enzymes were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A., except succinyl-CoA, which was purchased from P-L Biochemicals, Milwaukee, WI, U.S.A.

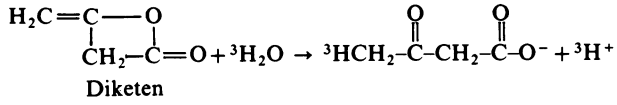
Animals and perfusion procedure

The procedure for '*in vivo*' infusion of 48h-starved 5–6-week-old (weight range 70–130g) rats has been previously described (Ozand *et al.*, 1977, 1978). When alanine was infused, somatostatin was included in order to suppress, at the same time, the secretion of insulin and glucagon (Nosadini *et al.*, 1980; W. D. Reed, P. J. Baab, R. L. Hawkins & P. T. Ozand, unpublished work). The initial dose of somatostatin was 130μg/kg, and 3μg/min per kg from then on. When the continuous infusion was interrupted to inject alanine, 30μg of somatostatin/min per kg was included.

Synthesis

The preparation of [4-^{3}H]acetoacetate and D-3-hydroxy[4-^{3}H]butyrate was according to the following reaction sequence:

[^{3}H]Acetoacetate synthesis:



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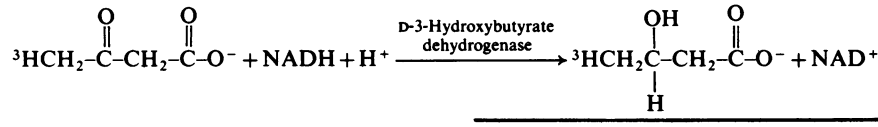
D-3-Hydroxy[^{3}H]butyrate synthesis:



The [4-^{3}H]acetoacetate was prepared by adding 100μl (5.5mmol) of ^3H_2O (500mCi) to 5.5mmol of diketene and 5.5mmol of $KHCO_3$. The mixture was stirred for 4h in an ice/water bath. The synthesis of the [4-^{3}H]acetoacetate was carried out in a semi-hermetically-closed system. The reaction was termined by injecting 10mmol of HCl in 20ml of water. The [4-^{3}H]acetoacetate was extracted in diethyl ether. The ether was separated from the aqueous phase and then evaporated under vacuum. The resultant oily residue was resuspended in 2M-HCl and the extraction step was repeated. The final residue was dissolved in water and stored at −70°C until it was chromatographed on Dowex-1 resin (Von Korf, 1969). The loss of [4-^{3}H]acetoacetate was ≃3% when kept under these conditions for 15 days.

The D-3-hydroxy[4-^{3}H]butyrate was prepared enzymically from [4-^{3}H]acetoacetate as shown above and was further purified on Dowex-1 (Von Korf, 1969). The conversion of the [4-^{3}H]acetoacetate into 3-hydroxy[4-^{3}H]butyrate was accomplished by incubating 100mmol of [4-^{3}H]acetoacetate in 30ml of 30mM-triethanolamine hydrochloride, 10mM-$MgCl_2$, 2.5mM-EDTA, 20mM-nicotinamide, 8mM-NADH, and 17 units of D-3-hydroxybutyrate dehydrogenase at pH7.3 and at 37°C for 30min (1unit = 1μmol of D-3-hydroxybutyrate oxidized/min at 37°C). The conversion was 97%. The synthesis of D-3-hydroxy[4-^{3}H]butyrate required the use of highly pure NADH (>99%) and nicotinamide. Under these conditions, with the use of purified NADH, the AMP contamination of D-3-hydroxy[4-^{3}H]butyrate was ≤2%. No loss was detected after a period of 18 months when the D-3-hydroxy[4-^{3}H]butyrate was kept at −70°C.

The acidic solutions of D-3-hydroxy[4-^{3}H]butyrate, and [4-^{3}H]acetoacetate were neutralized to pH7.4 and freeze-dried. Neither ^{3}H-labelled ketone body was contaminated to any detectable degree as judged by rechromatography on DEAE-cellulose paper using methanol/formic acid/water (10:3:27 or 1:1:1, by vol.) On rechromatography of the purified D-3-hydroxy[4-^{3}H]butyrate the specific radioactivity was 5Ci/mol. The analyses of [4-^{3}H]acetoacetate and D-3-hydroxybutyrate were made by the formation of their Denigés salts before and after treatment with D-3-hydroxybutyrate dehydrogenase as described by McGarry & Foster (1969). The isotope recovery was ≃65%. An assay of a standard was performed in each experiment to determine the recovery and correct the calculations.

Measurement of enzyme activities

The succinyl-CoA:3-oxo-acid CoA-transferase (EC 2.8.3.5) was purified from pig heart as described by Hersh & Jencks (1969) up to the step of DEAE-cellulose pH8 chromatography. This last step was replaced by P_i-cellulose chromatography as described for the purification of β-ketothiolase (acetyl-CoA acetyltransferase, EC 2.3.1.9) (Reed *et al.*, 1977; chromatogram not shown). The specific activity of the final purified product was comparable with that described by Hersh & Jencks (1969). The kinetic constants were measured (Hersh & Jencks, 1969) in a reaction mixture containing 0.1M-Tris/HCl, 15mM-$MgCl_2$, 0.7mM-succinyl-CoA (92.5% pure) and enzyme protein (10μg/ml), pH8.4, at 37°C. The acetoacetate concentration ranged from 4.5μM to 4.5mM.

The D-3-hydroxybutyrate dehydrogenase (EC 1.1.1.30) was from *Pseudomonas lemoignei* (Sigma; type IV). The activity was measured essentially as described by (Delafield & Doudoroff (1969) and

Krebs *et al.* (1969). For the forward reaction the incubation medium contained 50mM-triethanolamine hydrochloride, 15mM-$MgCl_2$, 0.15mM-NADH and enzyme protein (0.3μg/ml), pH7.0 at 38°C. The acetoacetate concentration ranged from 90μM to 3mM. In the reverse reaction the incubation medium contained 0.1M-Tris, 10mM-$MgCl_2$, 0.1mM-EDTA, 2mM-NAD^+ and enzyme protein (0.3μg/ml), pH8.2 at 30°C. The D-3-hydroxybutyrate concentration ranged from 50μM to 6mM.

The β-ketothiolase (EC 2.3.1.9) was prepared from rat liver mitochondria as described previously (Reed *et al.*, 1977). The incubation medium contained 0.1M-Tris/HCl, 20mM-$MgCl_2$, 0,3mM-dithiothreitol, 0.06mM-CoA and 0.5–5μM-[4-^{3}H]acetoacetyl-CoA. The [4-^{3}H]- and [3-^{14}C]-acetoacetyl-CoA were prepared enzymically as follows: 10mM-Tris/HCl, 4mM-$MgCl_2$, 0.6mM-succinyl-CoA, [4-^{3}H]- or [3-^{14}C]-acetoacetate (0.4mM) in 5ml final volume and purified succinyl-CoA:3-oxo-acid CoA-transferase (as described above) were incubated at 37°C for 10min. The incubation was terminated by adding HCl (final concn. 100mM). The labelled acetoacetyl-CoA was separated from CoA and succinyl-CoA by DEAE-cellulose chromatography as described for the preparation of L-3-hydroxybutyryl-CoA (Reed & Ozand, 1980). The purified preparation of succinyl-CoA:3-oxo-acid CoA-transferase used in the synthesis did not contain significant L-3-hydroxybutyryl-CoA dehydrogenase (less than 0.1%), nor any deacylases (Reed & Ozand, 1980).

The L-β-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35) activity was measured essentially as described by Lynen & Wieland (1955). The incubation medium contained 0.1M-Tris/HCl, 10mM-$MgCl_2$, 0.1mM-NADH, L-β-hydroxyacyl-CoA dehydrogenase (Sigma; pig heart type IV) (12.5ng of enzyme protein/ml), pH7.4, at 38°C. The concentration of acetoacetyl-CoA ranged between 5 and 50μM. The ^{3}H- and ^{14}C-labelled acetoacetyl-CoA species were prepared from the corresponding ^{3}H- and ^{14}C-labelled acetoacetates, CoA and L-3-hydroxybutyryl-CoA ligase. The latter was obtained as described elsewhere (Reed & Ozand, 1980).

Acetoacetyl-CoA synthase was assayed with a modification of the procedure of Buckley & Williamson (1973). The formation of acetyl-CoA was measured by CoA cycling after 5–10min of incubation at 38°C (Allred & Guy, 1969; Kato, 1975). The supernatant fluid of a 21-day-old-rat brain homogenate was the source of the enzyme. The results are the averages for duplicate experiments.

Calculations

The statistical analyses, the method of least squares, and correction for Denigés salts were as described previously (Ozand *et al.*, 1977, 1978). When a ^{14}C-labelled ketone-body precursor was used, the 1- and 3-positions of the ketone body were equally labelled; however, the 1-position was lost during the formation of the Denigés salt. Therefore an additional correction was made to calculate the ^{14}C for ^{3}H- and ^{14}C-labelled ketone bodies. The observed d.p.m. values for ^{3}H- and ^{14}C-labelled ketone bodies as the Denigés salts were at least six times the background.

The rate of ketone-body production was calculated according to the equation proposed by Steele *et al.* (1956), the application of which is discussed by Balasse *et al.* (1978). The following formula was used:

$$R_a = [I-(C \times V \times dSR/dt)]/SR$$

where R_a is the rate of appearance for the individual ketone body; I is the infusion rate of the isotope-labelled compound, C is the average concentration over the period of study, dt (4–16min), V is 35% of the body weight and dSR/dt represents the change in the specific radioactivity (SR) during the time interval dt. The rate of utilization (R_d) was calculated by using the following equation:

$$R_d = R_a-(dc/dt) \times V$$

where dc/dt represents the change in concentration (c) over the study period.

The rate of [1-^{14}C]octanoic acid disappearance was calculated as described for ketone bodies. At 0, 15 and 30min and at the end of the experiment, haematocrit values were obtained, to correct the calculation of the blood octanoate concentrations. In most experiments the blood octanoate concentration was 30–60μM. Since the amount of endogenous octanoic acid is very low in the laboratory rat, the specific radioactivity of blood octanoic acid was taken to be equal to that of the infused [1-^{14}C]octanoic acid. The rate of ^{14}C incorporation from 1-^{14}C octanoic acid into individual ketone bodies was calculated by multiplying the R_a with that segment of ketone-body pool stoichiometrically derived from the labelled octanoate.

Results

Metabolic similarity

The ^{3}H-labelled ketone bodies were metabolically equivalent to the commercially available ^{14}C-labelled acetoacetate or D-3-hydroxybutyrate. When [4-^{3}H]acetoacetate plus [3-^{14}C]acetoacetate were injected as the tracers, and concentrations of D-3-hydroxybutyrate and acetoacetate in the blood remained essentially constant (Fig. 1*a*), the kinetics of the disappearance of the ^{3}H- and the ^{14}C-

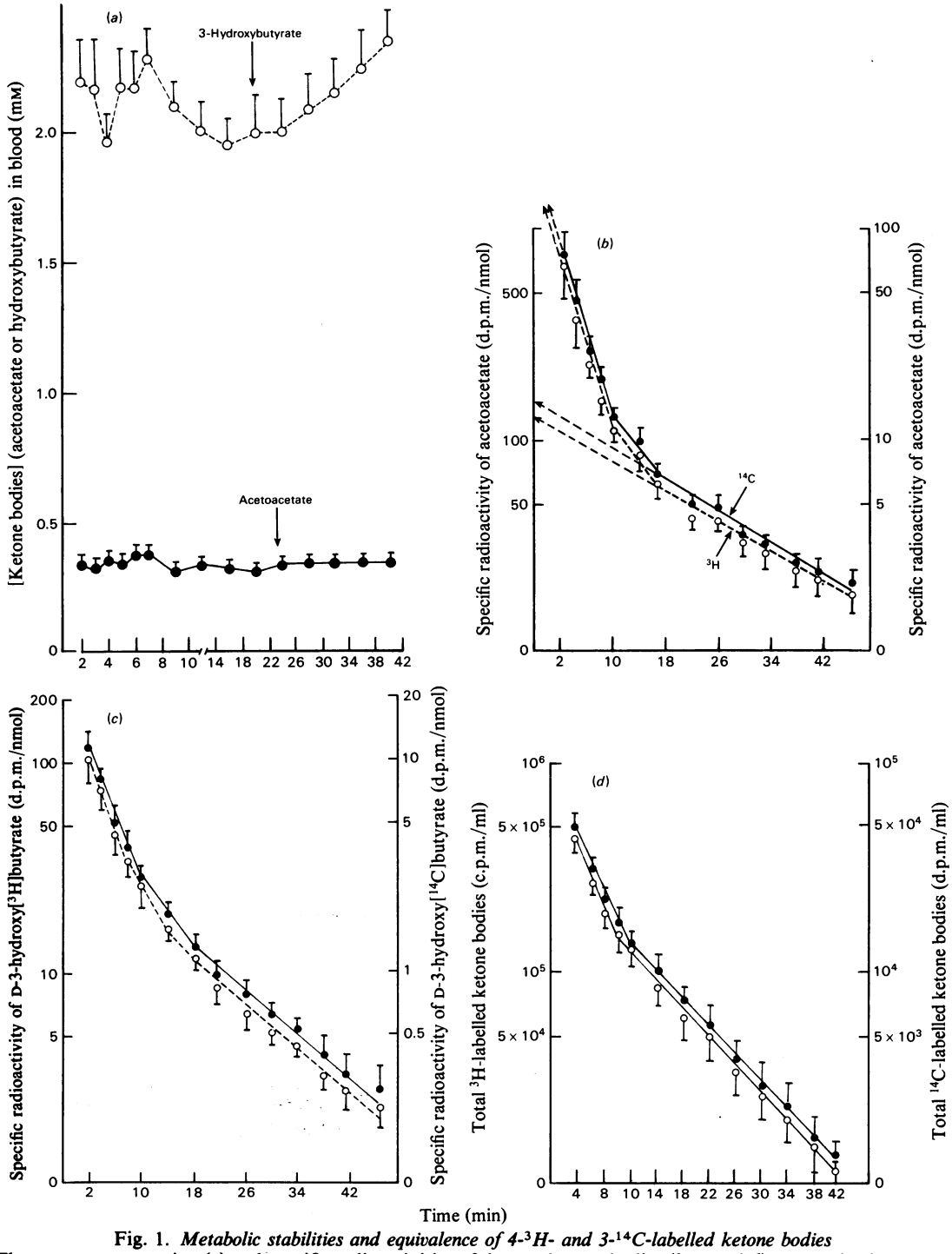


Fig. 1. *Metabolic stabilities and equivalence of 4-^{3}H- and 3-^{14}C-labelled ketone bodies*

The mean concentration (*a*) and specific radioactivities of the two ketone bodies (*b*, *c*, and *d*) $\pm$S.E.M. in six rats are presented. Each animal (average body weight 98 ± 6g) was injected with 9μCi of [4-^{3}H]acetoacetate + 1.06μCi of [3-^{14}C]acetoacetate in 0.5ml of saline (0.9% NaCl). The animals were then continuously given saline at 1.2ml/h. The volumes of blood samples removed were 50μl for 0–10min and 100μl from then on. (*a*) The concentration of acetoacetate (●) and D-3-hydroxybutyrate (○). (*b*) Specific radioactivity of acetoacetate: ^{3}H (○) and ^{14}C (●). The ratio of ^{3}H/^{14}C was 8.75 ± 0.33 at all time points. The theoretical ratio was 8.38 ± 0.16 as determined experimentally from the solution injected. (*c*) Specific radioactivity of D-3-hydroxybutyrate: ^{3}H (○) and ^{14}C (●). The ratio of ^{3}H/^{14}C was 8.64 ± 0.40 at all time points. (*d*) Specific radioactivity of total ketone bodies.

labelled ketone bodies were found to be identical (Fig. 1). This was true for the rates of disappearance of ^3H and ^{14}C measured in acetoacetate (Fig. 1*b*) or D-3-hydroxybutyrate (Fig. 1*c*), and therefore in total ketone bodies (Fig. 1*d*). It was also observed that these isotopes appeared in the alternate ketone body, i.e., in this case in D-3-hydroxybutyrate, as early as 2min after the injection of [4-^3H]acetoacetate plus [^{14}C]acetoacetate (Fig. 1*b*). The immediate appearance of ^3H and ^{14}C in acetoacetate was also seen when D-3-hydroxy[4-^3H]butyrate plus D-3-hydroxy[3-^{14}C]-butyrate were the infused tracers (results not shown). The specific radioactivities of the two ketone bodies never attained equality (results not shown). In all cases when the isotope was given as a single injection, the disappearance of labelled ketone bodies from the circulation exhibited biphasic kinetics. Each phase was first-order when expressed semi-logarithmically (Fig. 1). These data confirm that 4-^3H- and 3-^{14}C-labelled ketone bodies are metabolically similar. Therefore, by providing either a ^{14}C- or a ^3H-labelled ketone body as the tracer, it is possible to estimate temporal differences in production and utilization of acetoacetate and 3-hydroxybutyrate.

The rapid ketone-body metabolism (Fig. 1) necessitates an unrealistically large single dose of isotope if a 'pulse-label' technique is chosen. However, if a continuous infusion of the labelled ketone bodies is selected, a substantially smaller amount ($\simeq 10\%$) of isotope is required. In an experiment (Table 1) similar to Fig. 1 except that 3-

Table 1. *Turnover of ketone bodies: continuous infusion of [3-^{14}C]acetoacetate + 3-hydroxy[4-^3H]butyrate*

The rats were injected at zero time with 0.2ml of prime dose which contained 0.1μCi of [3-^{14}C]acetoacetate and 0.5μCi of D-3-hydroxy[4-^3H]butyrate in saline (0.9% NaCl). They were then given a perfusion solution (1.2ml·h^{-1}·100g^{-1}) that contained [3-^{14}C]acetoacetate (0.5μCi/ml) and D-3-hydroxy[4-^3H]butyrate (2.5μCi/ml) in saline. Blood samples were removed every 4min and were used to measure the specific radioactivities (SR) of ketone bodies. The equation of Steele *et al.* (1976) was used to calculate the metabolic rate for 8–36min of the experiment using the ^3H and ^{14}C specific radioactivities of ketone bodies. The results are the means ± S.E.M. for four animals. Calculations were as described in the Experimental section.

	Production or utilization (μM/min per kg)
Acetoacetate	
Utilization using SR of:	
^{14}C	6.0±1.0
^3H	4.5±1.0
Production using SR of:	
^{14}C	7.5±0.8
^3H	7.4±0.8
D-3-Hydroxybutyrate	
Utilization using SR of:	
^{14}C	13.2±2.3
^3H	10.6±2.6
Production using SR of:	
^{14}C	11.1±2.3
^3H	8.1±2.8

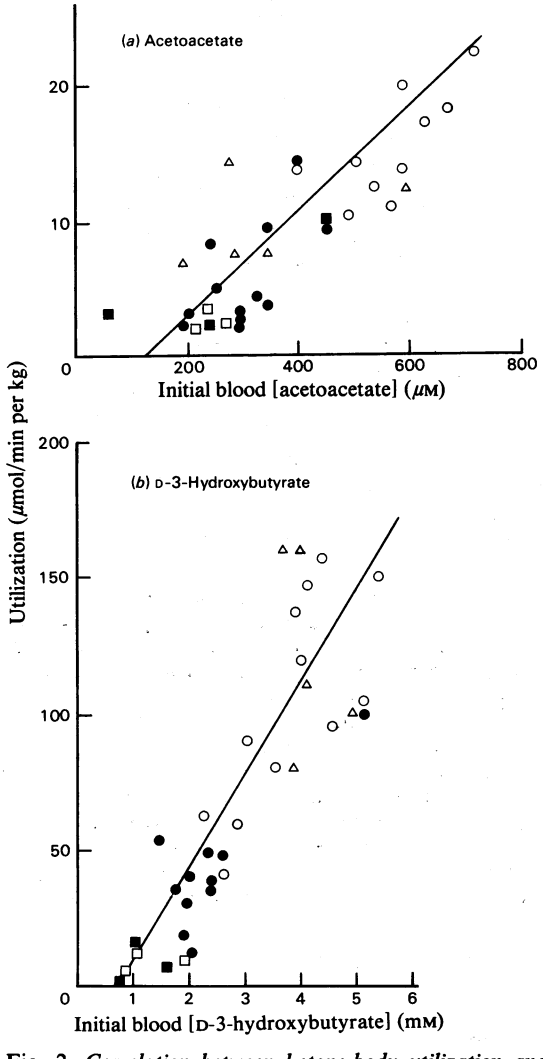


Fig. 2. *Correlation between ketone-body utilization and concentration*

The results of five different experiments (different symbols) are shown. The rate of ketone-body utilization was calculated as described in the Experimental section and was plotted against the initial blood acetoacetate (*a*) and D-3-hydroxybutyrate concentrations (*b*). The r^2 for the line of regression was $\geqslant 0.9$.

Table 2. *Effect of isotopes on enzymes of ketone-body metabolism*

All kinetic constants were calculated by linear-regression analysis of the Lineweaver–Burk plots, and the coefficient r^2 was always ≥ 0.90. The results are the averages for duplicate Lineweaver–Burk plots, with 8–20 observations each. The specific radioactivities of the labelled substrates were as indicated.

	K_m (mM)	$V_{max.}$ (μmol·min^{-1}·mg^{-1})
(1) Succinyl-CoA : 3-oxo-acid CoA-transferase		
Acetoacetate	0.35	1.5
[3-^{14}C]Acetoacetate (1.4 Ci/mol)	0.29	1.4
[4-^{3}H]Acetoacetate (4.2 Ci/mol)	0.29	1.7
(2) D-3-Hydroxybutyrate dehydrogenase		
Acetoacetate	0.39	33
[3-^{14}C]Acetoacetate (1.4 Ci/mol)	0.45	35
[4-^{3}H]Acetoacetate (4.2 Ci/mol)	0.36	32
D-3-Hydroxybutyrate	2.4	117
D-3-Hydroxy[3-^{14}C]butyrate (0.03 Ci/mol)	2.3	117
D-3-Hydroxy[4-^{3}H]butyrate (0.1 Ci/mol)	2.3	109
(3) 3-Ketothiolase	K_m (μM)	
Acetoacetyl-CoA	1.8	8.4
[3-^{14}C]Acetoacetyl-CoA (0.3 Ci/mol)	1.8	7.1
[4-^{3}H]Acetoacetyl-CoA (3.6 Ci/mol)	2.3	7.7
(4) L-3-Hydroxyacyl-CoA dehydrogenase		
Acetoacetyl-CoA	15	532
[3-^{14}C]Acetoacetyl-CoA (0.3 Ci/mol)	26	493
[4-^{3}H]Acetoacetyl-CoA (3.6 Ci/mol)	20	556
(5) Acetoacetyl-CoA synthetase		
Acetoacetate	—	2.1*
[3-^{14}C]Acetoacetate (0.14 Ci/mol)	—	1.9*
[4-^{3}H]Acetoacetate (0.42 Ci/mol)	—	1.8*

* The rate was measured in place of $V_{max.}$ and expressed as nmol of acetyl-CoA formed/min per mg of protein; the result is an average for two experiments.

Table 3. *Effect of alanine on ketone-body turnover and octanoate conversion into ketone bodies*

The results were calculated from the specific radioactivities of ketone bodies in the experiment shown in Fig. 3, using the equation of Steele *et al.* (1956) as described in the Experimental section. Linear-regression analysis was used to assign the best-fit line for each experimental animal for three different experimental periods. The results are reported for three separate periods of the experiment. Period 1, before the injection of L-alanine; period 2, first 12 min after injection of L-alanine, a period during which prominent changes occurred in blood ketone-body and alanine concentrations; period 3, next 16 min, when a new metabolic steady state was reached. At 0, 15, 30 and 58 min the hematocrit values were obtained to correct the plasma concentration of octanoate, which was 32 ± 6 μM at 15 min and 40 ± 8 μM at 30 min; minimal changes were observed after 30 min. The blood [1-^{14}C]octanoate concentration, its specific radioactivity and rate of injection permitted calculation of overall octanoic acid disappearance and conversion into ketone bodies as described in the text. The results are the means ± S.E.M. for eight animals. The Student *t* test was used to calculate the *P* values; * denotes ≤ 0.05. The *P* values were obtained by comparing either period 1 with period 2 or period 1 with period 3.

Period ...	1	2	3
Ketone-body turnover (μmol/min per kg)			
Acetoacetate			
Utilization	7.1 ± 1.5	5.8 ± 2.7	4.3 ± 0.9*
Production	7.2 ± 1.5	3.8 ± 1.2*	5.7 ± 2.0*
D-3-Hydroxybutyrate			
Utilization	82.1 ± 12.8	84.9 ± 29.2	109.2 ± 15.9*
Production	82.1 ± 12.8	59.5 ± 20.3*	94.2 ± 10.9
Fatty acid utilization (nmol/min per kg)			
Octanoate			
Converted into acetoacetate	49.5 ± 13.6	27.3 ± 7.5*	30.8 ± 7.0
Converted into hydroxybutyrate	109.1 ± 28.4	42.4 ± 10.7*	68.1 ± 19.1
Utilized	572 ± 165	593 ± 173	560.6 ± 160
Octanoate converted into total ketone bodies (%)	30 ± 6	11 ± 6*	14 ± 2

^{14}C- +4-^{3}H-labelled ketone bodies were infused continuously, results were calculated by using the equation of Steele *et al.* (1956). The ketone-body turnovers (i.e. production and utilization), when quantified, were not significantly different for acetoacetate or for 3-hydroxybutyrate with either isotope, although the turnover of the latter was somewhat faster.

Further indirect evidence for the similarity between the 4-^{3}H- and 3-^{14}C-labelled ketone bodies are the correlations presented in Fig. 2. Numerous other studies with 3-^{14}C-labelled ketone

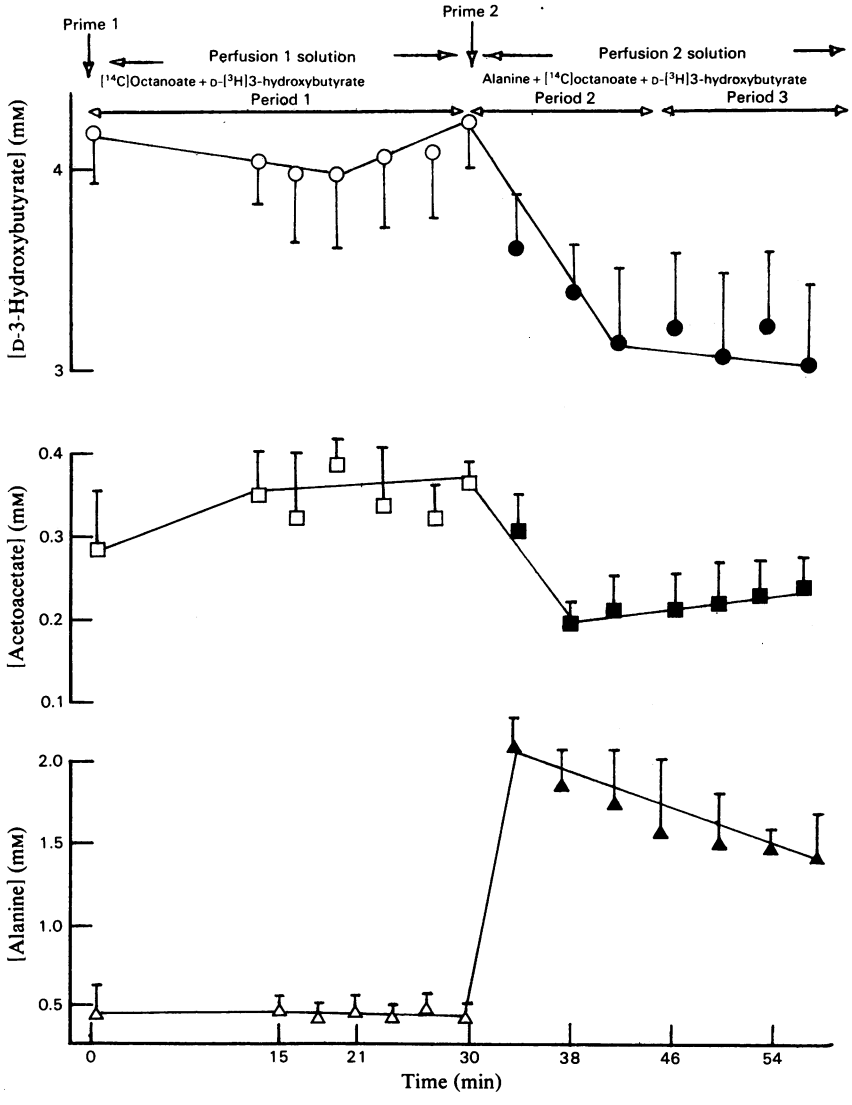


Fig. 3. *Hypoketonaemic effect of alanine*

Eight 48h-starved rats (5–6 weeks old, weight range 61–104g) were injected with 0.2ml of prime no. 1 {sodium octanoate (6.7mM), bovine serum albumin (25mg/ml), sodium [1-^{14}C]octanoate (3.3μCi) and D-[4-^{3}H]3-hydroxybutyrate (2.8μCi) in saline}. This was followed by the perfusion solution no. 1 (1.2ml/h per 100g) {sodium octanoate (4mM), albumin (25mg/ml), sodium [1-^{14}C]octanoate (10μCi/ml) and D-[4-^{3}H]3-hydroxybutyrate (1.9μCi/ml)}. At 30min, 12mg of L-alanine in 0.2ml of perfusion solution no. 1 was injected (prime 2), and the rats were infused with perfusion solution no. 2, which was similar to perfusion solution no. 1, but also contained 12mg of L-alanine/ml. At indicated time intervals blood samples were obtained. The concentration and specific radioactivities of ketone bodies as well as of the blood [alanine] concentration were determined. Results are means $\pm$ S.E.M. for eight rats. ○,●, D-3-Hydroxybutyrate; □,■, acetoacetate; △,▲, alanine.

bodies indicate that the prevailing rate of ketone-body utilization during starvation is proportional to the prevailing blood ketone-body concentration (Bates, 1971; Keller *et al.*, 1978). This was also true when the utilization of 4-^{3}H-labelled ketone bodies was compared with that of the 3-^{14}C-labelled compounds. The rates calculated with either isotope, when plotted against the initial blood concentration, exhibited a linear regression (r^2) of >0.90.

Enzymes of ketone-body metabolism: comparative effect of isotopes

The effects of ^{14}C and ^{3}H on the kinetic constants of the enzymes immediately involved in the metabolism of ketone bodies are shown in Table 2. These were the same as those for the unlabelled compounds, with minor variations ($\pm 10\%$). The results support the present data, which show a metabolic similarity between 4-^{3}H- and 3-^{14}C-labelled ketone bodies. At the specific radioactivities selected, the metabolism of labelled ketone bodies appears to be the same as for the unlabelled compounds.

Alanine and ketone-body turnover

The hypoketonaemic effect of alanine (Ozand *et al.*, 1977, 1978) can be ascribed to decreased production, increased utilization or an algebraic composite of both. The entry of long-chain free (non-esterified) fatty acids for mitochondrial oxidation requires the carnitine acyltransferase system (Fritz, 1959), and malonyl-CoA has been shown to inhibit an enzyme of this system (McGarry *et al.*, 1978). Since the oxidation of octanoic acid by-passes this step, this compound was chosen as the ^{14}C-labelled precursor. Long-chain free fatty acids are efficient ketogenic substrates during starvation; therefore, by the use of a medium- and a long-chain free fatty acid, the site of alanine action may be assigned as external or internal to passage across the mitochondrial inner membrane. The increase of blood alanine concentration in the food-deprived rat is accompanied by a decrease in the concentration of ketone bodies (Ozand *et al.*, 1978) (Fig. 3). This was explained by a decreased rate of production (Table 3). After 15min, a resumption of a lower steady ketone-body concentration occurs, at which time production equilibrates with utilization. During both time periods the decrement in total ketone-body production from endogenous long-chain free fatty acids elicited by alanine was approx. one-third, as shown by the 'Ketone-body turnover' (Table 3), which includes endogenous free fatty acids in addition to the infused octanoate. The decrement was one-half to two-thirds for ketone bodies synthesized from octanoic acid. At the same time alanine had no effect on the utilization of octanoic acid. The utilization of acetoacetate declined slightly, whereas that of 3-hydroxybutyrate increased significantly in the second period.

Discussion

The simultaneous injection of 4-^{3}H- and 3-^{14}C-labelled ketone bodies as a pulse label demonstrates parallel decrements in both labelled compounds (Fig. 1). When administered continuously, the rates of ketone-body turnover calculated individually yielded similar values (Table 1). Recently it has been emphasized by Cobelli *et al.* (1982) that models with more than one compartment should include recycling of ketone bodies in liver and extrahepatic tissues. This recycling is included in our estimates of appearance and disappearance. Bates (1971) and Keller *et al.* (1978) have shown a direct correlation between the concentration of ketone bodies in blood and their rate of turnover; the data in Fig. 2 support the results of these studies. At the specific radioactivities used, the lack of a differential isotope effect on the kinetic parameters of the key enzymes of ketone-body metabolism is shown in Table 2. These results amply demonstrate the metabolic equivalence of 4-^{3}H and 3-^{14}C in either ketone body. During increased ketogenesis, Huth *et al.* (1973) demonstrated that both acetyl groups in the ketone bodies are equivalent. Taken together, the present data and the studies of Huth *et al.* (1973) validate the use of C-3- or C-4-labelled ketone bodies. In states where lower rates of ketogenesis occur, the two acetyl groups of ketone bodies might not be equivalent (Huth *et al.*, 1973).

The results in Fig. 1 demonstrate that, although some interconversion of ketone bodies occurs in 2min, the specific radioactivity of blood acetoacetate never equals that of 3-hydroxybutyrate. Radioactive-tracer studies of ketone-body turnover pose problems concerning isotopic equilibration, because ketone bodies are interconverted rapidly, but under most conditions not to the extent that their specific radioactivities become equal (Barton, 1980). Conversion of blood 3-hydroxybutyrate into blood acetoacetate, takes place primarily in the liver, whereas the converse occurs in the periphery (Barton, 1973). Additionally, when the different rates of production and utilization for each ketone body are taken into account, it is apparent that an equivalence of specific radioactivities cannot be attained. Balasse & Delcroix (1980) noted that this non-equivalence does not create artefacts in calculating the metabolic clearance rates for ketone bodies.

Work in this laboratory (Ozand *et al.*, 1977, 1978) as well as in others (Genuth & Castro, 1974; Nosadini *et al.*, 1980), has demonstrated the anti-ketogenic action of alanine. This hypoketonaemic effect was most apparent in blood 3-hydroxybutyrate concentrations, whereas the concentration of acetoacetate remained essentially unchanged (Ozand *et al.*, 1977, 1978). However, a decrease in both ketone bodies has been observed when the rat received somatostatin to suppress endogenous release of glucagon and insulin (Nosadini *et al.*, 1980). The anti-ketogenic action of alanine has been related to hepatic redox state (Ozand *et al.*, 1977, 1978), although others have suggested that increased hepatic oxaloacetate concentration, and thus citrate formation, would decrease the availability of acetyl-CoA for ketogenesis (Nosadini *et al.*, 1980).

Our previous work demonstrated that alanine inhibited the incorporation of ^{14}C from a long-chain fatty acid (i.e. oleic acid) into ketone bodies. A greater decrease of ^{14}C incorporation into 3-hydroxybutyrate was observed than the decline in its chemical concentration (Ozand *et al.*, 1977, 1978). At that time it was impossible to calculate the rate of oleate conversion into individual ketone bodies, since a second label was not used to estimate the rate of ketone-body turnover. In the experiment outlined in Table 3 an essentially similar observation was made for octanoic acid, i.e., its conversion into ketone bodies was more significantly decreased by alanine than was endogenous ketogenesis. Octanoic acid conversion into ketone bodies was decreased by two-thirds to one-half, whereas the total ketone-body production was only by one-third in period 2 and returned to the 'pre-alanine' value in period 3. It follows that regulation of ketogenesis by passage of fatty acid across the mitochondrial inner membrane is not the primary site at which alanine suppresses ketogenesis, because octanoate does not encounter a barrier at this membrane, and in these experiments it was more affected by alanine than were the endogenous fatty acids (see under 'Ketone-body turnover' in Table 3). Octanoate, therefore, could be considered to reach the site of action of alanine more rapidly. In addition, the effect of alanine may be due to increased citrate production (therefore decreased acetyl-CoA availability for ketogenesis), because octanoic acid conversion into ketone bodies was more decreased than was the endogenous ketogenesis, whereas the overall utilization of octanoate was unaffected by alanine; it appears as though the products of β-oxidation were directed towards citrate production rather than to ketone bodies. Gluconeogenesis from oxidized substrates depends on the availability of reducing equivalents in the cytosol (Williamson *et al.*, 1969). If alanine increases the efflux of reducing equivalents from the mitochondrion, the formation of 3-hydroxybutyrate from acetoacetate would be restricted (Ozand *et al.*, 1978), although the largest change shown in Table 3, besides a decrease in acetoacetate turnover, is the increase in total 3-hydroxybutyrate utilization, not production, in period 2. These considerations, together with a presumed increased in the complete oxidation of free fatty acids (thereby increased availability of ATP), would explain the stimulation of gluconeogenesis attended by alanine (Ozand *et al.*, 1977, 1978). These results also suggest that the demonstrated increase in hepatic oxalacetate concentration results in increased gluconeogenesis, but not necessarily in an increase in hepatic citrate (Nosadini *et al.*, 1980).

In conclusion, the double-isotope method of measuring ketone-body turnover provides particular advantages for the study of agents that modify ketone-body metabolism. A similar need for the use of two isotopes has recently been noted by Cobelli *et al.* (1982). This double-isotope procedure has allowed the hypoketonaemic action of L-alanine to be localized initially with ketone-body production and subsequently with utilization. A secondary action of alanine appears to be associated with the mitochondrial fatty acid catabolism.

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