Factors influencing the utilization of ketone bodies by mouse adipose tissue

RICHARD W. HANSON* and ZIGMUND Z. ZIPORIN

U. S. Army Medical Research and Nutrition Laboratory, Fitzsimons General Hospital, Denver, Colorado

ABSTRACT Factors influencing the utilization of ketone bodies by mouse adipose tissue in vitro were studied. Epididymal fat pads can oxidize DL-β-hydroxybutyrate-3-^{14}C and acetoacetate-3-^{14}C to $^{14}CO_2$ as well as convert these compounds to fatty acid-^{14}C. An increased output of $^{14}CO_2$ from β-hydroxybutyrate-3-^{14}C was noted in response to glucose plus insulin, succinate, oxaloacetate, L-asparate, and L-malate. Fatty acid synthesis from β-hydroxybutyrate was enhanced by glucose plus insulin, L-aspartate, L-malate, oxaloacetate, and citrate.

Nicotinamide stimulated the oxidation of β-hydroxybutyrate but not of acetoacetate to CO_2, and did not affect fatty acid synthesis from either ketone body. Nicotinamide increased NAD^+ and $NADP^+$ levels in epididymal fat pads without affecting the concentration of NADH and NADPH. "Superlipogenesis" caused by fasting the mice for 48 hr and re-feeding them for 24 hr sharply enhanced CO_2 output and lipogenesis from β-hydroxybutyrate. The activities of glucose-6-phosphate dehydrogenase, 6-phosphogluconic dehydrogenase, NADP-malic dehydrogenase, and citrate cleavage enzyme from mouse adipose tissue were increased during "superlipogenesis." Free fatty acid release by epididymal fat pads in vitro was slightly increased by β-hydroxybutyrate. The relationship of ketone body metabolism and lipogenesis in adipose tissue is discussed.

KEY WORDS adipose tissue · ketone bodies · metabolism · oxidation · conversion to fatty acid · lipogenesis · enzymes · NAD · NADP · nucleotide coenzymes · nicotinamide · free fatty acid release · mouse

The metabolism of ketone bodies by muscle and liver has been the subject of numerous recent reports (1–4). However, ketone body metabolism by adipose tissue has received little attention. Krebs, Eggleston, and D'Alessandro (2) measured acetoacetate conversion to β-hydroxybutyrate with concomitant oxygen consumption by rat epididymal fat pads and estimated that acetoacetate, when added as the sole substrate, may account for more than 50% of the cellular respiration. Hanson (5), using acetoacetate-3-^{14}C, has demonstrated the oxidation of this ketone body to $^{14}CO_2$ by adipose tissue. This report also showed a stimulatory effect of acetoacetate on the conversion of glucose-U-^{14}C to $^{14}CO_2$, fatty acid-^{14}C and glycogen-^{14}C, and a slight depression in the formation of glyceride-glycerol-^{14}C. No effect of acetoacetate on glucose metabolism was demonstrated in the absence of insulin.

The present study is an attempt to investigate the factors which influence the utilization of ketone bodies by adipose tissue. The results indicate an increased β-hydroxybutyrate oxidation to CO_2 and conversion to fatty acid under conditions of rapid lipogenesis. The role of the Krebs cycle and possible points of control of ketone body metabolism by adipose tissue are discussed.

MATERIALS AND METHODS

Male A strain mice, approximately 8 months old, maintained on Purina Laboratory Chow, were used throughout these experiments. The animals were killed by cervical dislocation and the epididymal fat pads were removed, bisected laterally, and weighed on a rapid-weighing torsion balance. The tissue was transferred to 3.0 ml of Krebs-Ringer bicarbonate buffer, pH 7.4, containing 3% bovine serum albumin (Fraction V) in an Erlenmeyer flask constructed with a 1.5 × 1 cm glass center well. The center well contained a 2 × 2 cm piece of filter paper. The flasks were stoppered with thin-walled rubber cups and the incubation was carried out in an atmosphere of 95% O_2–5% CO_2 at

Abbreviation: FFA, free fatty acid.

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* Present address: Fels Research Institute, Temple University School of Medicine, Philadelphia, Pennsylvania 19140.

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38° in a metabolic shaker at 95–100 strokes/min. After a 3 hr incubation period, 0.1 ml of 15% KOH was introduced into the center well and 0.5 ml of 1 N H_2SO_4 into the main compartment of the flask by injection through the rubber stopper, to insure complete liberation of CO_2. Substrate concentrations, as well as the specific activity of the labeled intermediates, are noted in each Table. Control flasks containing buffer without tissue were included in each experiment to measure the spontaneous breakdown of β-hydroxybutyrate-3-^{14}C and acetoacetate-3-^{14}C to $^{14}CO_2$ during the incubation. After the incubation, the $^{14}CO_2$ that had been evolved was measured by determining the ^{14}C in the filter paper by the method of Buhler (6). The filter paper was dried in air, aligned in a scintillation vial and counted in 10 ml of toluene containing, per liter, 4 g of 2,5-diphenyloxazole (PPO), 0.015 g of 1,4-bis-[2-(5-phenyloxazolyl)] benzene (POPOP), and 230 ml of absolute ethanol. Using known concentrations of standard $Na_2{}^{14}CO_3$ treated with H_2SO_4 in a closed vessel, this system was found to have an efficiency of 18–20% for determining $^{14}CO_2$ and all counts were corrected to 100% efficiency on this basis.

Adipose tissue was removed from the incubation medium, rinsed several times in 0.9% NaCl, and extracted in 15 ml of chloroform–methanol 2:1 for 6 hr. The extraction process was shown by gravimetric analysis of the defatted pad to be 98–99% complete. The chloroform–methanol-extracted lipids were washed three times with 0.05% $CaCl_2$ and saponified in methanolic KOH (95 ml of methanol–3 g of KOH in 5 ml of water) at 50° for 1 hr. Completeness of saponification under these conditions was verified by thin-layer chromatography. Unsaponifiable lipid was removed by extraction with petroleum ether (bp 30–60°). The total saponifiable lipid was then acidified with HCl and extracted with petroleum ether and portions were taken for radioactivity measurements in the scintillation medium described above. The counting efficiency, as determined by internal standards, was 50–60%. The counts were corrected on this basis. Radioactivity was measured in a Nuclear-Chicago ambient temperature liquid scintillation spectrometer.

The release of FFA in vitro was determined by the method of Dole and Meinertz (7). The β-hydroxybutyrate used in the incubation (Table 5) contributed less than 0.5% to the titratable acidity of the medium.

Potassium DL-β-hydroxybutyrate-3-^{14}C was purchased from New England Nuclear Corporation, Boston, Mass. and ethyl acetoacetate-3-^{14}C from Nuclear-Chicago Corporation, Des Plaines, Ill. Acetoacetate and acetoacetate-3-^{14}C were prepared as lithium acetoacetate by the method of Hall (8). The purity of the salt was checked by absorption spectra in water, NaOH, and HCl and by paper chromatography (9). Before use, sodium acetoacetate was prepared by ion exchange on Amberlite IR-120 charged with NaCl. Highly purified porcine insulin was generously supplied by Dr. W. W. Bromer of the Eli Lilly & Company, Indianapolis, Ind.

Enzyme assays were carried out on extracts of adipose tissue prepared by homogenizing epididymal fat pads in 0.15 M KCl, and centrifuging for 15 min at 1,000 × *g* in plastic tubes. The homogenate separated into three layers, an upper fat cake, a lower button containing cell debris and fibrous stroma, and an intermediate layer which was used for the assay of the enzymes. The intermediate layer was recentrifuged at 22,000 × *g* for the determination of citrate cleavage enzyme. Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (EC 1.1.1.44) were measured separately by the method of Horecker and Smyrniotis (10) and NAD- and NADP-malic dehydrogenase (EC 1.1.1.37 and 1.1.1.40, respectively) as described by Ochoa (11). Citrate cleavage enzyme (EC 4.1.3.6) was determined as outlined by Srere (12). Enzyme activities were measured at 30° using a Beckman DK2A recording spectrophotometer equipped with constant temperature cell holder.

Pyridine nucleotide coenzymes were determined by the method of Lowry, Roberts, and Kapphahn (13) as modified by Lindall and Lazarow (14). Recoveries from extracts of adipose tissue were[1] NAD^+ 95–98%, NADH 87–90%, $NADP^+$ 99–103% and NADPH 82–90%. The nitrogen content of the fat pads was determined by micro-Kjeldahl digestion followed by nesslerization (15).

Differences between groups were tested for significance using the "*t*" test.

RESULTS

The conversion of β-hydroxybutyrate-3-^{14}C to $^{14}CO_2$ and fatty acid-^{14}C in the presence of various metabolic intermediates was studied (Table 1). Glucose plus insulin, succinate, oxaloacetate, L-aspartate, and L-malate stimulated $^{14}CO_2$ output from β-hydroxybutyrate-3-^{14}C. The ketone body was converted to fatty acid, a process enhanced by glucose plus insulin, L-aspartate, L-malate, oxaloacetate, and citrate. Ketone body oxidation to $^{14}CO_2$, markedly stimulated by succinate, could be depressed 50% by malonate, but returned to normal when malonate plus succinate were included in the incubation medium.

Adipose tissue from animals fasted for 48 hr and refed for 24 hr had an increased capacity to utilize β-

[1] *Convention for coenzyme nomenclature.* NAD^+ and $NADP^+$ refer to the oxidized, NADH and NADPH to the reduced forms of the dinucleotide coenzymes. NAD and NADP are used for the coenzymes in general, without reference to their state of oxidation.

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hydroxybutyrate for oxidation to CO_2 and for lipogenesis (Table 2). The measurement of adipose tissue enzymes involved in lipogenesis also revealed a greater activity of glucose-6-phosphate dehydrogenase, 6-phosphogluconic dehydrogenase, NADP-malic dehydrogenase, and citrate cleavage enzyme in mice undergoing rapid lipogenesis induced by re-feeding after a prolonged fast.

The effect of nicotinamide on the pattern of utilization of acetoacetate-3-^{14}C and β-hydroxybutyrate-3-^{14}C by adipose tissue is shown in Table 3. Nicotinamide significantly increased the oxidation of β-hydroxybutyrate to CO_2 but did not affect CO_2 output from acetoacetate or lipogenesis from either of the ketone bodies. The concentrations of NAD^+ and $NADP^+$ found in epididymal fat pads after incubation with nicotinamide were increased. No change in the levels of NADH and NADPH could be demonstrated (Table 4).

Increasing the concentration of β-hydroxybutyrate in the incubation medium over a range of from 3 to 240 μmoles per 3.0 ml stimulated both the oxidation of the ketone body to CO_2 and its incorporation into lipid (Fig. 1). Succinate increased β-hydroxybutyrate oxidation to CO_2 at ketone body concentrations higher than 15 μmoles, but had no effect on lipogenesis.

The effect of β-hydroxybutyrate on the release of FFA from epididymal fat pads in vitro is shown in Table 5. FFA release was depressed by the addition of glucose and insulin to the incubation medium. However, the addition of β-hydroxybutyrate, either alone or in combination with glucose and insulin, caused a slight but significant ($P < 0.05$) rise in FFA release.

TABLE 1 Factors Influencing Utilization of DL-β-Hydroxybutyrate-3-^{14}C by Adipose Tissue in Vitro

Metabolite Measured	Additions to Incubation Medium Containing β-Hydroxybutyrate-3-C^{14}: None	Glucose + Insulin	Succinate	Malonate	Malonate + Succinate	L-Aspartate	L-Malate	Oxaloacetate	Isocitrate	Citrate	α-Ketoglutarate
	μmoles β-hydroxybutyrate-3-^{14}C utilized/g tissue in 3 hr										
$^{14}CO_2$	0.343 ±0.024 (8)	0.545* ±0.051 (8)	1.516* ±0.308 (8)	0.180* ±0.008 (4)	0.341 ±0.053 (5)	0.619* ±0.074 (8)	0.501* ±0.108 (8)	0.409* ±0.023 (8)	0.296 ±0.056 (4)	0.371 ±0.073 (4)	0.394 ±0.023 (6)
Fatty acid-^{14}C	0.024 ±0.004 (8)	0.515* ±0.118 (8)	0.049 ±0.014 (12)	0.018 ±0.001 (4)	0.038 ±0.014 (4)	0.302* ±0.081 (5)	0.536* ±0.209 (5)	0.298* ±0.039 (8)	0.067 ±0.015 (4)	0.339* ±0.040 (4)	0.023 ±0.003 (3)

Mean values ± SEM for the number of animals shown in parentheses.
* Values that are significantly different at the 5% probability level from those representing incorporation of β-hydroxybutyrate-3-^{14}C into $^{14}CO_2$ and fatty acid-^{14}C without added substrate (column 1).
Substrate concentrations per 3.0 ml of Krebs-Ringer bicarbonate buffer, pH 7.4, were 30 μmoles of glucose, 0.1 U of insulin, 30 μmoles of DL-β-hydroxybutyrate, 0.25 μc of DL-β-hydroxybutyrate-3-^{14}C, and 10 μmoles of succinate, malonate, L-aspartate, L-malate, oxaloacetate, isocitrate, citrate, and α-ketoglutarate.

TABLE 2 Effect of Re-feeding after Fasting on Utilization of DL-β-Hydroxybutyrate-3-^{14}C and on Activity of Selected Enzymes in Adipose Tissue

Metabolic State of the Animal	Utilization of β-Hydroxybutyrate-3-^{14}C by Adipose Tissue in Vitro: $^{14}CO_2$	Fatty Acid-^{14}C	Enzyme Activity: G-6-PD	6-PGD	NAD-MD	NADP-MD	Citrate Cleavage
	μmoles β-hydroxybutyrate-3-^{14}C utilized/g tissue in 3 hr						
Normal fed	0.343 ±0.024 (8)	0.024 ±0.004 (12)	1.090	0.273	19.475	0.156	0.049
Fasted 48 hr; re-fed 24 hr	1.884 ±0.282 (7)	4.948 ±0.886 (7)	1.764	0.458	17.086	0.498	0.306
P	<0.001	<0.001					

Enzymes were determined on an extract of 14 epididymal fat pads, prepared as described in Methods.
Mean values ± SEM for the number of animals shown in parentheses. Values for normal fed mice taken from Table 1.
Substrate concentrations of β-hydroxybutyrate-3-^{14}C utilization study were 30 μmoles of β-hydroxybutyrate, 0.5 μc of β-hydroxybutyrate-3-^{14}C in 3.0 ml of Krebs-Ringer bicarbonate buffer, pH 7.4. Enzymes assayed were glucose-6-phosphate dehydrogenase (G-6-PD), 6-phosphogluconic dehydrogenase (6-PGD), NAD-malic dehydrogenase (NAD-MD), NADP-malic dehydrogenase (NADP-MD), and citrate cleavage enzyme (citrate cleavage).

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TABLE 3 Effect of Nicotinamide on Utilization of DL-β-Hydroxybutyrate-3-^{14}C and Acetoacetate-3-^{14}C by Adipose Tissue In Vitro

Metabolite Measured	Additions to Incubation Medium					
	AcAc-^{14}C	AcAc-^{14}C Nicotinamide	*P*	BOH-^{14}C	BOH-^{14}C Nicotinamide	*P*
	μmoles of labeled substrate utilized/g tissue in 3 hr					
$^{14}CO_2$	3.276 ±0.420 (5)	2.205 ±0.468 (5)	N.S.*	0.421 ±0.041 (8)	0.898 ±0.070 (9)	<0.001
Fatty acid-^{14}C	0.452 ±0.228 (5)	0.625 ±0.135 (5)	N.S.	0.056 ±0.014 (8)	0.082 ±0.043 (9)	N.S.

Mean values ± SEM for the number of animals shown in parentheses.
* Not significant.
Substrate concentrations per 3.0 ml of Krebs-Ringer bicarbonate buffer, pH 7.4, were: 30 μmoles of DL-β-hydroxybutyrate, 30 μmoles of acetoacetate, 0.5 μc of DL-β-hydroxybutyrate-3-^{14}C (BOH-^{14}C), 0.5 μc of acetoacetate-3-^{14}C (AcAc-^{14}C), and 15 μmoles of nicotinamide.

DISCUSSION

If the utilization of β-hydroxybutyrate by adipose tissue is similar to that found in mammalian muscle (16), the initial metabolic steps would convert D-β-hydroxybutyrate to acetyl CoA. Acetyl CoA in adipose tissue could then be oxidized to CO_2 or serve as a source of carbon for fatty acid synthesis. On the basis of this reasoning, it was expected that those factors which stimulated utilization of acetate would also affect utilization of ketone bodies. In an effect similar to that on acetate (17), glucose plus insulin increased lipogenesis from β-hydroxybutyrate. Moreover citrate, which is known to enhance fatty acid synthesis from acetyl CoA (18, 19), stimulated lipogenesis from β-hydroxybutyrate.

There was a marked increase in both $^{14}CO_2$ output and fatty acid-^{14}C synthesis from β-hydroxybutyrate-3-^{14}C by epididymal fat pads from mice fasted for 48 hr and re-fed for 24 hr ("superlipogenesis"). As has been found for rat adipose tissue (20, 21), re-feeding after fasting elevated the activities of glucose-6-phosphate dehydrogenase, 6-phosphogluconic dehydrogenase, NADP-malic dehydrogenase, and citrate cleavage enzyme in mouse adipose tissue. NAD-malic dehydrogenase, although not affected by re-feeding, has a level of activity 10–20 times higher than any of the other enzymes measured. The pattern of enzyme activity noted in mouse adipose tissue during "superlipogenesis" is consistent with the (citrate cleavage)–(NADP-NAD-malic dehydrogenase) system described by Young, Shargo, and Lardy (20) for adipose tissue from fasted and re-fed rats. The concomitant increase in β-hydroxybutyrate-3-^{14}C incorporation into fatty acids-^{14}C during this period of rapid lipid synthesis suggests the involvement of this pathway in the conversion of the ketone body to fatty acids. The addition to the incubation medium of L-aspartate (which would be converted to oxaloacetate), oxaloacetate, or malate also stimulated lipogenesis from β-hydroxybutyrate. It is possible that the conversion of these intermediates to pyruvate via the reaction sequence discussed by Young et al. (20) provides NADPH for fatty acid synthesis.

The Krebs cycle from α-ketoglutarate to citrate has been shown to function in the "reverse" direction in rat adipose tissue (22). However, only 20% of the α-ketoglutarate was shown to be converted to citrate by "reversal" (23). The effect of citric acid on the conversion of β-hydroxybutyrate to lipid could be caused by a stimulation of acetyl CoA carboxylase activity (18, 19) and (or) a generation of NADPH by the NAD-NADP-malic dehydrogenase system using as a substrate the oxaloacetate formed from citrate cleavage.

The reason for the elevated $^{14}CO_2$ output from β-hydroxybutyrate-3-^{14}C by epididymal fat pads caused by aspartate, malate, and oxaloacetate but not by α-ketoglutarate, isocitrate, and citrate is not clear. Since the first three intermediates could increase the intracellular concentration of oxaloacetate, the stimulation of CO_2 formation may be due to increased synthesis of citrate-^{14}C via the condensation of oxaloacetate and acetyl-^{14}C CoA. It should be noted, however, that the

TABLE 4 Effect of Nicotinamide on the Concentration of Pyridine Nucleotide Coenzymes in Adipose Tissue in Vitro

Pyridine Nucleotide Coenzyme Measured	Additions to Incubation Medium	
	None	Nicotinamide
	mμmoles coenzyme/mg tissue N	
NAD^+	2.903	6.547
NADH	0.203	0.212
$NADP^+$	0.707	1.519
NADPH	0.358	0.306

Nucleotide coenzyme levels were measured on 8 epididymal fat pads after incubation for 3 hr in Krebs-Ringer bicarbonate buffer, pH 7.4. Nicotinamide concentration was 60 μmoles per 20 ml of buffer.

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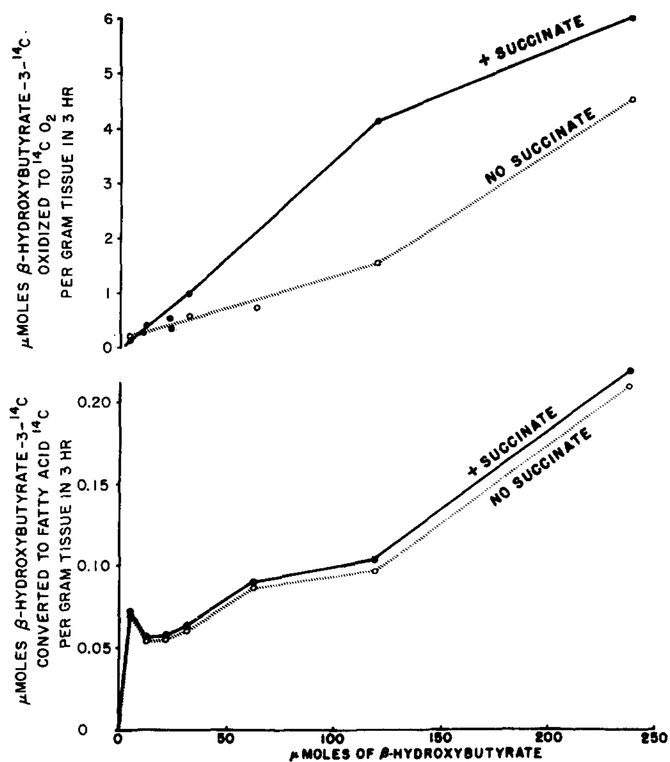

Fig. 1. The effect of increasing concentrations of DL-β-hydroxybutyrate with and without succinate on conversion of β-hydroxybutyrate to CO_2 and fatty acids by epididymal fat pads. Each point is the mean for two experiments performed as outlined in Table 1. The concentrations of β-hydroxybutyrate employed were 3, 6, 12, 30, 60, 120, and 240 μmoles per 3.0 ml of buffer. Succinate was present at a concentration of 10 μmoles/3.0 ml.

absence of an effect on ketone body metabolism by any of the intermediates used may be due to an impermeability of adipose tissue to these intermediates.

In the present study, it was noted that succinate, at concentrations greater than 15 μmoles/3.0 ml of incubation medium, sharply increased the output of CO_2 from β-hydroxybutyrate without affecting lipogenesis. Succinate has been shown to affect β-hydroxybutyrate dehydrogenase of rat liver mitochondria (24), presumably by increasing the concentration of NADH at the site of action of the electron transport chain (25, 2). It is possible that a similar mechanism underlies the action of succinate on β-hydroxybutyrate oxidation in adipose tissue.

In view of the importance of pyridine nucleotide coenzymes as regulators of metabolism (26, 27), it is of interest to note that nicotinamide is known to increase the concentration of NAD^+ in liver and brain (28–30). We have demonstrated an elevation of NAD^+ and $NADP^+$ levels in adipose tissue and a stimulation of β-hydroxybutyrate-3-^{14}C oxidation to $^{14}CO_2$ in the presence of nicotinamide in vitro. No effect could be demonstrated on acetoacetate oxidation to CO_2 or conversion of either of the ketone bodies to fatty acid. An increase in intracellular NAD^+ could stimulate β-hydroxybutyrate oxidation to CO_2 by increasing its conversion to acetoacetate, yet not affect acetoacetate oxidation. Since both acetoacetate and β-hydroxybutyrate share the same oxidative pathway (except for the initial oxidative step), and since nicotinamide does not affect acetoacetate oxidation to CO_2, it is probable that the increase in NAD^+ affected β-hydroxybutyrate dehydrogenase activity. Thus, the availability of NAD^+ may act to limit the rate of β-hydroxybutyrate oxidation by adipose tissue by limiting its conversion to acetoacetate.

The decreased synthesis of glyceride-glycerol from glucose in the presence of ketone bodies noted in a previous study (5) suggested an effect on the rate of FFA release. Madison and Mebane (31), using dogs infused with β-hydroxybutyrate, have observed a decrease in plasma FFA levels. This decrease was attributed to an effect of the ketone body on insulin output. The present paper shows that β-hydroxybutyrate can affect adipose tissue directly, increasing the rate of FFA release in vitro, perhaps as a consequence of the decreased availability of α-glycerophosphate (5).

In discussing the results of this study, we have left unanswered the larger question of ketone body metabolism by adipose tissue in diabetes. It seems noteworthy that lipogenesis from β-hydroxybutyrate can be stimulated by intermediates other than glucose. The increased fatty acid synthesis from ketone bodies in the presence of aspartate, as well as when higher concentrations of ketone bodies are present in the incubation medium (Fig. 1), may mean that in diabetes, ketone bodies can serve as a source of carbon for lipogenesis. Such an interpretation must remain speculative, however, until studies on diabetic animals are carried out.

TABLE 5 Effect of DL-β-Hydroxybutyrate on Release of FFA by Adipose Tissue in Vitro

Additions to Incubation Medium			
None	Glucose + Insulin	Glucose + Insulin + β-Hydroxybutyrate	β-Hydroxybutyrate
μmoles FFA released/100 mg tissue in 3 hr			
0.906	0.719*	1.287*	1.310*
±0.079	±0.045	±0.075	±0.096
(8)	(9)	(9)	(9)

Mean values ± SEM for number of animals shown in parentheses.

* Mean values significantly different at 5% probability level from that of column 1.

Substrate concentrations per 3.0 ml of Krebs-Ringer bicarbonate buffer, pH 7.4, were: 30 μmoles of glucose, 30 μmoles of DL-β-hydroxybutyrate, and 0.1 U of insulin. The tissue was pre-incubated in 3.0 ml of buffer for 30 min, then transferred to 3.0 ml of fresh buffer for the 3-hr incubation in an atmosphere of 95% O_2–5% CO_2.

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The principles of laboratory care as promulgated by the National Society for Medical Research were observed.

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