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Daniel W. Foster

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Research Article

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Ketosis of fasting was shown to be reversible within 5 minutes by the injection of glucose or insulin. This effect was due to a prompt cessation of acetoacetate production by the liver. The possibility is raised that the ketosis of fasting is due to a direct activation of acetoacetate-synthesizing enzymes secondary to a starvation-induced depression of insulin secretion by the pancreas.

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Studies in the Ketosis of Fasting *

DANIEL W. FOSTER †

(From the Department of Internal Medicine, The University of Texas Southwestern Medical School at Dallas, Dallas, Texas)

Summary. A series of experiments was performed during the induction of starvation ketosis and in the acute reversal of the ketotic state. In contrast to the predictions of two widely held theories of ketogenesis, control of acetoacetate production by the liver appeared to be unrelated to changes in fatty acid mobilization from the periphery, fatty acid oxidation, fatty acid synthesis, or the acetyl coenzyme A concentration in the liver.

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Introduction

It is generally accepted that the accumulation of ketone bodies in the blood during relative or absolute carbohydrate deprivation is due to overproduction of acetoacetic and β-hydroxybutyric acids by the liver (1). The mechanism of ketone overproduction has not been well defined, though most theories attribute the increased rate of synthesis to enhanced oxidation of long chain fatty acids by the liver. According to this hypothesis, carbohydrate deprivation causes mobilization of free fatty acids from peripheral fat depots with subsequent uptake by the liver. In the liver the fatty acids are activated to long chain coenzyme A derivatives which are then oxidized at an increased rate to acetyl coenzyme A, the latter compound accumulating in the hepatic cell (2–4). In addition, elevation of long chain acyl CoA levels is postulated to contribute to the expanded acetyl CoA pool by blocking utilization of acetyl CoA in lipogenesis and the tricarboxylic acid cycle through inhibition of the acetyl CoA carboxylase (5) and citrate synthase reactions (6, 7). The direct stimulus for accelerated ketogenesis in this formulation is considered to be the increased concentration of acetyl CoA in the liver cell, which then secondarily activates acetoacetate synthesis (8, 9).

A second major theory of ketosis differs from the above in emphasizing decreased lipogenesis rather than increased fatty acid oxidation as the primary factor in initiating ketosis (10, 11). Here, too, the immediate stimulus to increased ketone production is considered to be an increased concentration of acetyl CoA in the liver.

It is clear that fully developed ketosis is accompanied by both increased fatty acid oxidation and decreased fatty acid synthesis. The question to be considered is whether alterations in these lipid pathways *initiate* the accelerated hepatic ketogenesis found during starvation.

In the studies to be described below an attempt has been made to determine the relationship between fatty acid oxidation, fatty acid synthesis, and ketone body production in starvation at varying time intervals after the onset of fasting. Similar studies were performed during recovery from the fasted state. The role of nonesterified fatty acids

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Address requests for reprints to Dr. Daniel W. Foster, Dept. of Internal Medicine, University of Texas Southwestern Medical School at Dallas, 5323 Harry Hines Blvd., Dallas, Texas 75235.

in the blood and of hepatic acetyl CoA concentrations in the control of acetoacetate synthesis by the liver has also been assessed. The results indicate that under the conditions of these experiments hepatic ketogenesis during starvation can be altered independently of changes in fatty acid synthesis, fatty acid oxidation, nonesterified fatty acid levels in the blood, and acetyl CoA concentrations in the hepatic cell. They thus provide evidence that the ketosis of fasting may be causally unrelated to changes in lipid metabolism and suggest that current theories of ketosis may have to be modified.

Methods

Treatment of animals

Male Holtzman rats, weighing approximately 200 g and maintained on a balanced diet containing 60% carbohydrate by weight,[1] were used in all experiments. The animals were allowed to eat ad libitum until 48 hours before use, at which time tube feeding was initiated to assure a uniform caloric intake. Immediately after the last feeding control rats were killed, and additional groups of animals were killed at varying intervals thereafter as indicated in the Figures. In the recovery studies a similar procedure was followed except that rats were fasted for 48 hours and killed at precisely timed intervals after the administration of 5 ml of a solution containing 7.5 g of the 60% carbohydrate diet by stomach tube or 0.4 ml of 50% glucose intravenously. In some experiments glucagon-free insulin[2] was given intravenously alone or combined with glucose.

In vivo–in vitro experiments

Venous blood was obtained for chemical determinations at time of death, and the liver was quickly removed and placed in iced buffer. Slices of 0.5 mm thickness were prepared with an automatic tissue slicer, and samples were taken for measurement of glycogen and fatty acid content. Five hundred mg of slices was incubated in 25-ml center well flasks containing 3.0 ml of Krebs bicarbonate buffer, pH 7.4, and the isotopic substrate. For studies of fatty acid oxidation, palmitate-1-^{14}C was prepared as the potassium salt and dissolved in 1% bovine serum albumin freed of fatty acid by the method of Goodman (12). One-tenth of a ml of this preparation, containing 1.0 μc of radioactivity and 0.1 μmole of palmitate, was added to each flask. The incorporation of radioactivity from the albumin-bound palmitate-1-^{14}C into CO_2 was utilized as the indicator of fatty acid oxidation. In the studies of acetoacetate and fatty acid synthesis acetate-2-^{14}C was used as the radioactive substrate. Five μmoles of acetate and 1 μc of radioactivity were present in each flask. All determinations were performed in duplicate, and slices from the same rat liver were used with each isotope.

At the end of the incubation period the reaction was stopped by the addition of 0.25 ml of 10 N H_2SO_4, and radioactive CO_2 was collected into 1 N NaOH in the center well after shaking 30 minutes in ice (13). The incubation mixture was then decanted, and the slices were washed twice with 2-ml vol of water. The washes and incubation mixture were combined and centrifuged, and the clear supernatant was utilized for isolation of radioactive acetoacetate. The protein pellet remaining from the centrifugation of the latter was dissolved in water and added back to the washed slices in the center well flasks for determination of fatty acid radioactivity.

Acetoacetate was isolated, with minor modifications, by the method of Van Slyke (14) as described by Weichselbaum and Somogyi (15). The insoluble Denigès salt was collected by centrifugation and washed twice with 20 ml of water. The precipitate was dissolved in 1.0 ml of 4 N HCl, and a 0.2-ml aliquot was taken for assay of radioactivity. Control experiments without tissue showed no activity in the Denigès salt from acetate or palmitate alone.

For determination of fatty acid radioactivity 0.5 ml of 90% KOH was added to the incubation flask, and the contents were saponified for 1 hour at 15 pounds pressure. Nonsaponifiable lipid was extracted with petroleum ether and discarded, and the total fatty acids were isolated and washed after acidification according to the method of Siperstein and Fagan (13).

Glycogen was isolated after homogenization of samples of the tissue slices according to Stetten and Boxer (16) and hydrolyzed by the method of Good, Kramer, and Somogyi (17) for assay by glucose oxidase (18). Total fatty acids were measured gravimetrically after saponification and extraction of fatty acids as described above (13).

Nonesterified fatty acids in the liver were determined by a modification of the method utilized by White and Engel (19). Livers were frozen in liquid nitrogen and ground to a powder in a mortar and pestle cooled in liquid nitrogen. One-half g of frozen powder was then homogenized with 10 ml of the fatty acid extraction mixture of Dole (20). Titration of free fatty acids was carried out by the Trout, Estes, and Friedberg modification of the latter method (21). With a ratio of extraction mixture to tissue of 20 : 1, recovery of palmitic acid added to the powdered tissue was complete (for example, in a typical experiment 0.5 g of liver contained 1.02 μmoles of nonesterified fatty acids, whereas 0.5 g of liver plus 3.96 μmoles of palmitate yielded 4.94 μmoles of nonesterified fatty acids, a recovery of 99%).

Blood sugar was determined by the glucose oxidase method (18). Total blood ketones were measured by the method of Lyon and Bloom (22).

In separate experiments acetyl CoA concentrations in the liver were measured during the onset of fasting and 15 minutes after the reversal of ketosis by the iv adminis-

[1] General Biochemicals, Chagrin Falls, Ohio.

[2] Glucagon-free insulin was obtained from the Lilly Research Laboratories, Indianapolis, Ind., through the courtesy of Dr. W. R. Kirtley.

tration of glucose and insulin. Acetyl CoA was assayed spectrophotometrically utilizing citrate-condensing enzyme [3] as described by Ochoa, Stern, and Schneider (23). Preparation of the samples for assay was carried out as described by Wieland and Weiss (4) except that rats were anesthetized with pentobarbital rather than ether since the latter anesthetic causes a prompt and sustained fall in blood ketone concentrations. As has been pointed out by Pearson (24) and Buckel and Eggerer (25), the coupled assay for acetyl CoA underestimates the concentration of this compound because of a shift in the equilibrium of the malate dehydrogenase reaction during the assay. The actual values obtained in these experiments have been corrected according to the formula of the latter authors (25). Ninety-five per cent of standard acetyl CoA added to the frozen liver powder was recovered in the perchloric acid extracts in control experiments. The recovery could be increased to about 99% by a single additional perchloric acid wash of the precipitated protein. In view of the very small increased yield this wash was omitted in the experiments shown. Blood was obtained at the time the liver was removed for measurement of acetoacetate by the method of Walker (26) modified according to Kalnitsky and Tapley (27).

In the experiments concerned with the oxidation of acetoacetic acid-3-^{14}C various tissues and organs were removed from the animals as indicated, and 100 mg of slices was incubated with 10 μmoles of sodium acetoacetate containing 1 μc of radioactivity. The hemidiaphragm was not sliced but used intact. The rate of oxidation of ketone bodies was assayed by $^{14}CO_2$ production from acetoacetate-3-^{14}C after a 48-hour fast and 10 minutes after the iv injection of 200 mg of glucose.

In vivo experiments

Fatty acid synthesis. For studies of *in vivo* fatty acid synthesis 10 μmoles of sodium acetate-2-^{14}C containing 2 μc of radioactivity was injected intraperitoneally in 1 ml of isotonic saline. After 30 minutes the animals were killed and the livers perfused with 20 ml of ice-cold phosphate buffer, 0.1 M, pH 7.4. The tissue was then minced into a 5-ml vol of water to which was added 1 ml of 90% KOH. The livers were saponified and extracted for fatty acids as described above.

Fatty acid oxidation. Fatty acid oxidation was measured in the whole animal by collection and assay of expired CO_2 for ^{14}C content after the iv injection of palmitic acid-1-^{14}C. Vena caval and arterial cannulas were placed as described in the next section, and the animal was positioned in a glass metabolic cage through which CO_2-free air was drawn by vacuum. The expired air was collected in bubbling towers containing sodium hydroxide (28). Palmitate-1-^{14}C was prepared by dissolving the sodium salt in isotonic saline containing 2.0% bovine serum albumin such that 1.0 ml contained 5 μmoles of palmitate and 50 μc of radioactivity. This solution was diluted 2 : 1 with plasma from rats fasted 48 hours (28). Six-tenths ml of the final clear solution was injected intravenously and $^{14}CO_2$ collected for six 10-minute periods. The initial study in each rat was done after a 48-hour fast. When expired radioactivity had become negligible, 3 to 6 hours later, ketosis was reversed by the iv injection of 0.05 U of insulin and 0.1 ml of 50% glucose. The latter was included to avoid the possibility of hypoglycemia with insulin alone. Ten minutes later palmitate-1-^{14}C was again injected, and $^{14}CO_2$ was collected. Arterial blood ketone levels were measured at intervals throughout the experiment.

In a parallel set of experiments rats were injected with palmitic acid-1-^{14}C exactly as above except that $^{14}CO_2$ was not collected. Five minutes after the injection of isotope, blood was rapidly drawn from the vena cava for determination of the specific radioactivity of the plasma free fatty acids. Fatty acids were extracted and titrated as described previously (20, 21), and a portion of the extracted sample was counted for radioactivity.

Plasma nonesterified fatty acids in fasting and recovery. Rats were tube fed as described above and fasted for 48 hours. At the end of the 48-hour fast glucagon-free insulin was given intravenously in the doses indicated in the Figures. All animals not treated with insulin were given a control injection of saline to eliminate any differences due to the injection technique alone. The animals were anesthetized with pentobarbital given intraperitoneally 5 minutes before blood was drawn. Nonesterified fatty acids and acetoacetate were measured in the same sample.

The isotopic steady state. Rats were anesthetized with pentobarbital, and no. 10 polyethylene catheters were placed in the femoral artery on one side and the inferior vena cava through the femoral vein on the other. In addition a similar catheter was placed in the tail vein. The animals were then placed in individual restraining cages and allowed to awaken from the anesthesia. One to two mg of heparin was given intravenously to each animal before the experiment.

After collection of samples of arterial blood at 0 time, a constant infusion of sodium acetoacetate-3-^{14}C was started through the vena caval catheter utilizing a microperfusion pump. The specific activity of the acetoacetate was 2.8 μc per μmole, administered so that approximately 25 μmoles was given per hour. An isotopic steady state was rapidly obtained, usually by 10 minutes after the start of the infusion. At this point glucose or insulin was given intravenously, and changes in the specific activity of the blood acetoacetate were followed.

One-tenth–ml samples of the arterial blood were collected directly into calibrated micropipettes and transferred immediately into 0.4 ml of 6.25% trichloroacetic acid. After centrifugation the supernatant solution was analyzed for acetoacetate content by the method of Walker (26). One-tenth ml of the supernatant was neutralized by the addition of 0.1 ml of 1.4 M sodium acetate, and 1 ml of the diazo reagent was added. The reaction was allowed to proceed for 30 minutes at room temperature and was then stopped by the addition of 0.25 ml of 4 N HCl. After standing for 8 minutes to destroy any oxalacetate present (27), the solution was extracted with

[3] Crystalline citrate-condensing enzyme from pig heart was the generous gift of Dr. Paul A. Srere.

TABLE I

*Hepatic glycogen and fatty acid concentrations and blood glucose levels with the onset of fasting and during recovery**

Determinations	Hours after onset of fasting				Hours after refeeding	
	0	12	24	48	6	12
Blood glucose	151 ± 18	107 ± 11	90 ± 6	87 ± 8	160 ± 9	188 ± 4
Liver glycogen	42.1 ± 7.5	6.8 ± 1.9	3.7 ± 0.6	5.9 ± 0.4	13.7 ± 1.8	19.5 ± 0.9
Liver fatty acids (total)	34.2 ± 2.2	31.6 ± 3.0	36.0 ± 1.6	40.6 ± 2.2	26.6 ± 3.4	25.2 ± 1.6

* Experimental details are given in the text. Results for glycogen and fatty acid are given as milligrams per 1,000 mg wet weight of liver and are tabulated as means and standard errors of the means for nine animals at each time interval. Blood glucose is given as milligrams per 100 ml whole blood.

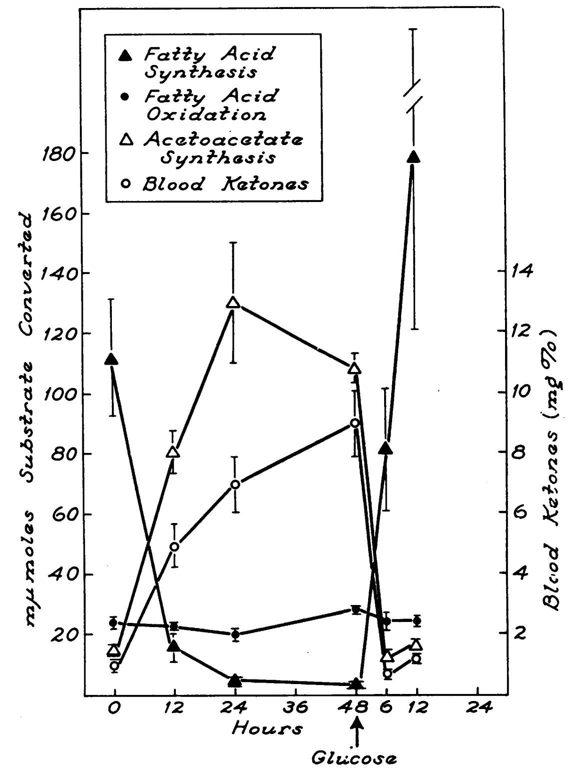

Fig. 1. Studies of fatty acid synthesis, fatty acid oxidation, and acetoacetate synthesis in liver slices with the onset of fasting and during recovery. Details of the experimental procedure are outlined in the text. Groups of rats were killed at the indicated times after the last tube feeding. At the end of 48 hours of fasting the remaining rats were refed the high carbohydrate diet by stomach tube. All experiments were performed in duplicate, and each point represents the mean and standard error of the mean of the results in nine rats. The results in the liver are tabulated as millimicromoles of substrate converted to product by 500 mg of slices in a 1-hour period. Points for the fatty acid oxidation experiments have been multiplied by 10 for easier visualization. Total blood ketones are tabulated as acetone equivalents.

1 or 2 ml of ethyl acetate, and absorbancy was measured at 450 mμ in a Beckman DU spectrophotometer. The remaining portion of the sample was used for isolation and assay of the radioactive acetoacetate as described above. In some experiments acetoacetate and β-hydroxybutyrate concentrations were measured enzymatically by the method of Williamson, Mellanby, and Krebs (29). Acetoacetate levels were comparable when measured by the two methods, and specific activities of acetoacetate and β-hydroxybutyrate were identical in the steady state.

Assay of radioactivity

Samples to be assayed for radioactivity were prepared as follows: $^{14}CO_2$. The NaOH contained in the center well of the flasks was diluted 1:10 with water, and 1.0 ml was added to 15 ml of the solution described by Bray (30). *Fatty acid-*^{14}C. The washed pentane extract containing the long chain fatty acids was evaporated to dryness under nitrogen and dissolved in 10 ml of toluene containing 400 mg of 2,5-diphenyloxazole and 5 mg of 1,4-di[2-(5-phenyloxazolyl)] benzene per 100 ml. *Acetoacetate-*^{14}C. Two-tenths ml of the acid solution containing the solubilized Denigès salt was added to 3.8 ml of ethanol together with 16 ml of the toluene phosphor solution.

All samples were counted in a liquid scintillation spectrometer with and without internal standards added to each vial to correct for differential quenching in the separate solvent systems. The results were adjusted to the fatty acid solvent system where counting efficiency was approximately 50%.

Materials

All materials were obtained from commercial sources and were of the highest available grade.[4] Purity of the ethyl acetoacetate-3-^{14}C used for preparation of the sodium salt was determined by gas chromatography. A single symmetrical peak with the precise retention time of authentic ethyl acetoacetate was obtained. This peak contained all of the injected radioactivity. The ethyl ester

[4] Radioactive substrates were obtained from the New England Nuclear Corp., Boston, Mass. The D(−)-β-hydroxybutyric acid dehydrogenase was obtained from Boehringer and Sons through Calbiochem, Los Angeles, Calif.

TABLE II

*CO_2 and acetoacetate production from acetate-2-^{14}C and palmitate-1-^{14}C in fasting**

Time fasted	Acetate-2-^{14}C		Palmitate-1-^{14}C	
	CO_2	Acetoacetate	CO_2	Acetoacetate
hours	*mμmoles†*		*mμmoles × 10†*	
0	290 ± 32	15 ± 1.0	24 ± 1.8	0.96 ± 0.07
12	326 ± 30	81 ± 6.8	23 ± 1.0	9.2 ± 3.3
24	285 ± 20	130 ± 20.0	20 ± 2.1	10.5 ± 3.8
48	330 ± 25	108 ± 4.8	28 ± 1.5	7.2 ± 1.1

* The conversion of acetate-2-^{14}C and palmitate-1-^{14}C to CO_2 and acetoacetate is listed for the indicated period of fasting in nine animals. Results are given as millimicromoles of substrate converted to product per 500 mg of slices per hour. Slices from the same animals were used with each isotope under the experimental conditions listed in Figure 1.

† Direct quantitative comparison of the rates of oxidation of acetate and palmitate cannot be made from these data because of differences in the amount of substrate added and differences in tissue pool sizes. Approximately 6% of the added acetate-2-^{14}C was recovered as $^{14}CO_2$; the figure for palmitate-1-^{14}C was 2 to 3%.

was hydrolyzed in 1 N NaOH, evaporated to dryness under vacuum, and neutralized before use. Sodium acetoacetate solutions were assayed enzymatically (29).

Results

Changes in the liver with the onset of fasting. Changes in blood sugar, liver glycogen, and total hepatic fatty acids in fasting and recovery are recorded in Table I. The blood sugar and glycogen concentrations decreased in the expected fashion with the onset of starvation. As has been previously noted, total fatty acids of the liver were unchanged by fasting (31, 32). This is in contrast to the situation in experimental diabetes where fat content of the liver does increase and appears to be correlated with the degree of ketosis (3).

With the onset of fasting after a high carbohydrate diet, profound changes occurred in the patterns of fatty acid and acetoacetate synthesis by rat liver slices. These changes are shown graphically in Figure 1. By 12 hours after the final tube feeding fatty acid synthesis had fallen almost tenfold from 111 mμmoles per hour to 16 mμmoles per hour. At the same time a fivefold rise of acetoacetate synthesis occurred, from 15 to 81 mμmoles per hour. Simultaneously blood ketones increased from 1.0 to 5.0 mg per 100 ml, continuing to rise to 9.0 mg per 100 ml at 48 hours. At 24 hours fatty acid synthesis had fallen almost to zero and remained at this level at 48 hours. In contrast to the striking changes in fatty acid and acetoacetate synthesis, fatty acid oxidation was unchanged during the first 24 hours, rising slightly but with no statistical significance at 48 hours.

It should be emphasized that the indicator of fatty acid oxidation in these experiments was $^{14}CO_2$ production from palmitate-1-^{14}C. Two factors could conceivably interfere with the adequacy of this procedure. First, since palmitate oxidation to CO_2 requires breakdown of the long chain fatty acyl CoA to acetyl CoA and subsequent oxidation of acetyl CoA to CO_2, a depression of the latter reaction might mask increased oxidation of palmitate to acetyl CoA. Second, the radioactive palmitate added to the slices might be diluted by an expanded hepatic fatty acid pool with the same effect. The data of Table II indicate that $^{14}CO_2$ production from acetate-2-^{14}C was not impaired by a 48-hour fast though acetoacetate production increased sevenfold. An essentially identical pattern was found in slices from the same liver when palmitate-1-^{14}C was the substrate as would be expected from the fact that acetyl CoA is an obligatory intermediate in CO_2 and acetoacetate production from long chain fatty acids. Since $^{14}CO_2$ production from acetate was unimpaired, it is

TABLE III

*Nonesterified fatty acids in the liver during fasting and recovery**

Time fasted	Hepatic nonesterified fatty acids
hours	*mμmoles/g*
0	524 ± 13
24	1,324 ± 115
48	717 ± 139
48 + 15 minutes treated	642 ± 87

* Nonesterified fatty acids in the liver were measured as described in the text. Results represent the means and standard errors of the means from duplicate determinations in four animals at each time interval and are given as millimicromoles of nonesterified fatty acid per gram of liver. Treated animals were given 0.05 U of insulin and 0.4 ml of 50% glucose intravenously 15 minutes before being killed.

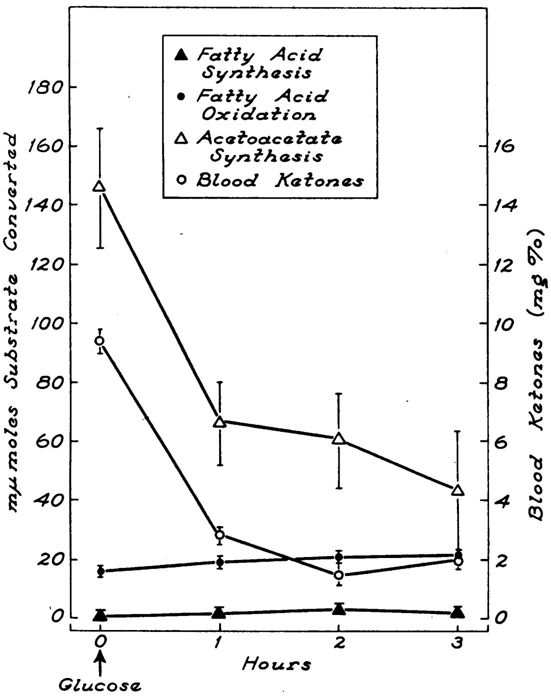

FIG. 2. STUDIES OF FATTY ACID SYNTHESIS, FATTY ACID OXIDATION, AND ACETOACETATE SYNTHESIS IN LIVER SLICES DURING RECOVERY FROM FASTING. At the end of a 48-hour fast animals were refed by stomach tube (time 0). Each point represents the mean and standard error of the mean in duplicate experiments from six animals.

clear that an increased oxidation of palmitate-1-^{14}C to acetyl CoA would be reflected by an increased recovery of radioactivity in CO_2 as well as acetoacetate provided that isotope dilution in an expanded fatty acid pool had not occurred. When hepatic free fatty acid levels were measured, as shown in Table III, a definite increase was found at 24 hours that decreased to near fed levels at 48 hours. Correction of the $^{14}CO_2$ recoveries from palmitate listed in Table II by these data indicates a maximal increase of palmitate oxidation at 24 hours of about twofold and at 48 hours of 1.6-fold. The increase in fatty acid oxidation was thus considerably smaller than the tenfold changes occurring in acetoacetate and fatty acid synthesis in the same time interval.

On the basis of these experiments, it appeared that accelerated ketogenesis by the liver and a rise in blood ketones occurred without equivalent change in fatty acid oxidation by hepatic tissue. Since fatty acid synthesis fell concomitantly with the rise in acetoacetate production and to equivalent degree, the results were compatible with the viewpoint that decreased lipogenesis might initiate overproduction of ketones by the liver. Studies performed at earlier intervals after the onset of fasting were unsuccessful in differentiating these two effects.

Changes in the liver with recovery from fasting. In view of the fact that fatty acid synthesis and acetoacetate production varied simultaneously and in reciprocal fashion with the onset of fasting, even at the earliest time intervals, these pathways were next studied in the recovery from fasting in an attempt to see whether an increased synthesis of fatty acids could be demonstrated before a fall in acetoacetate synthesis. Such a sequence would be expected if decreased fatty acid synthesis did, in fact, initiate acetoacetate overproduction in the liver.

In the initial experiments, shown in Figure 1, fasting was terminated after 48 hours by administration of 5 ml of the high carbohydrate diet by stomach tube. Fatty acid synthesis, fatty acid oxidation, acetoacetate synthesis, and blood ketones were measured at 6 and 12 hours. At the 6-hour period blood ketones and acetoacetate synthesis had both returned to normal, and fatty acid synthesis had increased from barely detectable levels to 80 mμmoles per hour. By 12 hours lipogenesis had increased to above normal levels as is typical for refeeding after starvation (33). Fatty acid oxidation was not significantly changed. Thus reversal of ketosis was accompanied by reversal of the changes in fatty acid and acetoacetate synthesis in the liver, but again the two pathways appeared to change simultaneously.

To study this relationship further, experiments were next performed at earlier intervals during the recovery from fasting. As before, rats were fasted for 48 hours and then tube fed the high carbohydrate diet. Studies were carried out at 1, 2, and 3 hours after refeeding with the results shown in Figure 2. At 1 hour blood ketones fell from 9.5 to 2.7 mg per 100 ml, and acetoacetate synthesis decreased from 145 to 65 mμmoles per hour. Surprisingly, however, no increase in fatty acid synthesis occurred, even up to 3 hours. These findings indicate that changes in acetoacetate synthesis in the liver were not dependent upon changes in fatty acid synthesis.

TABLE IV

*Acetyl coenzyme A levels in the liver during fasting and recovery from fasting**

Determination	0	12 hours	24 hours	48 hours	15 minutes treated
Acetyl CoA, *mμmoles/g liver*	34.4 ± 2.8 (6)	65.9 ± 4.0 (6)	70.5 ± 4.9 (6)	50.5 ± 3.2 (8)	47.1 ± 3.1 (8)
p		<0.001	NS	<0.005	NS
Acetoacetate, *mg/100 ml*	1.3 ± 0.3 (6)	2.9 ± 0.4 (6)	4.4 ± 0.7 (6)	8.3 ± 1.4 (8)	1.2 ± 0.4 (8)
p		<0.025	NS	<0.005	<0.001

* Rats were anesthetized with pentobarbital and the livers were frozen in liquid nitrogen. The frozen livers were powdered in a mortar and pestle cooled in liquid nitrogen and homogenized with 6% perchloric acid. Acetyl CoA was measured in the protein-free supernatant. The reaction mixture, in a volume of 1 ml, contained 100 μmoles Tris-HCl buffer, pH 7.0, 2 μmoles NAD, 5 μmoles potassium malate, 25 μg of malic dehydrogenase, and the sample to be measured. After equilibrium of the malate-oxalacetate system had occurred, the reaction was started by the addition of 10 μg of citrate-condensing enzyme, and the increase in absorbancy at 340 mμ was measured. In the recovery studies fasting was terminated by the iv injection of glucose and insulin, and determinations were made 15 minutes later. Blood was obtained for acetoacetate determination at the time of the removal of the livers. Results are recorded as means ± standard error of the means. p values are listed as not significant if greater than 0.05.

Acetyl coenzyme A concentrations in the liver during fasting. Since both the theory of increased fatty acid oxidation and that of decreased fatty acid synthesis suggest that the stimulation of acetoacetate synthesis in the liver is the result of an expanded acetyl CoA pool, the hepatic concentration of acetyl CoA was measured during the onset of fasting and in reversal of the ketotic state. If either theory were correct, a direct relationship should exist between acetyl CoA concentrations in the liver and blood acetoacetate levels; conversely, if ketosis, as suggested above, is not initiated by changes in fatty acid synthesis or oxidation, such a direct relationship would not be found. The results of studies to define this relationship are shown in Table IV. During the first 12 hours of fasting acetyl CoA levels approximately doubled in the liver and continued to increase slightly to 24 hours.[5] At the 12-hour period acetoacetate concentrations in the blood also doubled and increased further by 24 hours. At 48 hours, however, acetyl CoA levels in the liver showed a sharp and unexpected fall, whereas blood acetoacetate continued to increase to about twice the concentrations found at 24 hours. More importantly, 15 minutes after the administration of glucose and insulin, when acetoacetate levels in the blood had fallen from 8.3 to 1.2 mg per 100 ml, acetyl CoA concentrations in the liver remained completely unchanged. The results indicate that the rate of acetoacetate synthesis in the liver cannot be related primarily to acetyl CoA concentration.

Time course of reversal of ketosis and the role of insulin. As noted above blood ketones were observed to return to near normal levels within 1 hour (Figure 2) after reversal of ketosis by tube feeding the high glucose diet and within 15 minutes (Table IV) after the administration of glucose and insulin intravenously. Studies were

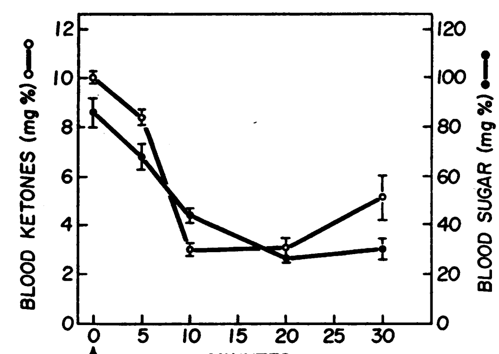

FIG. 3. ACUTE REVERSAL OF STARVATION KETOSIS BY INSULIN. After a 48-hour fast 0.025 U of insulin was given intravenously. Each point represents the mean and standard error of the mean for duplicate experiments in six animals.

[5] The acetyl CoA concentrations reported here are higher than those given by Wieland and Weiss (9), Tubbs and Garland (34), and Bortz and Lynen (35), who found 19.9, 17.2, and 16.1 mμmoles, respectively, per g of wet tissue in fed animals. Their results were uncorrected for the underestimation of the coupled assay for acetyl CoA (24, 25). The uncorrected value in these experiments was 19.2 mμmoles per g of wet tissue in the fed state.

TABLE V

Fatty acid synthesis in vivo *in the reversal of ketosis**

Condition	Fatty acid synthesis
	mμmoles substrate converted
Fed	735 ± 27
48-hour fast	55 ± 6.4
1 hour retreated	43 ± 10.2
3 hour retreated	97 ± 32

* Experimental conditions are described in the text. The results are given as the means and standard errors of the means in six animals at each time interval.

therefore undertaken to determine how rapidly reversal of ketosis might occur. The results of such experiments are shown in Figure 3. Rats were fasted 48 hours and then rapidly injected with 0.025 U of glucagon-free insulin intravenously. Samples of blood were drawn in the fasted state and at 5, 10, 20, and 30 minutes after treatment. Blood ketones began to fall by 5 minutes and at 10 minutes had decreased from the fasting mean of 10.0 to 3.0 mg per 100 ml. At the 20-minute period no further fall was noted, and at 30 minutes a definite increase in ketosis occurred. The rebound in acetoacetate concentration appeared to follow the development of hypoglycemia of about 30 mg per 100 ml. Reversal of ketosis also began within 5 minutes when insulin was replaced with 0.3 ml of 50% glucose.

The fact that insulin alone reversed the ketosis of fasting suggests that the response to glucose in the previous studies was mediated totally or in part through physiologic stimulation of insulin secretion by the pancreas.

Fatty acid synthesis and oxidation in vivo during recovery from fasting. The data of Figure 2 indicated that fatty acid synthesis and oxidation were unchanged for up to 3 hours after the reversal of fasting ketosis when measured in the liver slice *in vitro*. Although close parallel existed between *in vitro* acetoacetate synthesis and blood ketone levels and although the changes in fatty acid synthesis were those expected with fasting, it was possible that the time course of these changes might be different *in vivo*. A comparison of fatty acid synthesis in the intact animal in the fed and fasting state is shown in Table V. Forty-eight hours after initiation of the fast, fatty acid synthesis fell from 735 to 55 mμmoles per half hour, a decrease of 93% from the rate in the fed animal. No change in the rate of synthesis was seen at 1 and 3 hours after refeeding.

The rate of oxidation of palmitic acid-1-^{14}C to $^{14}CO_2$ was measured in five animals before and after reversal of ketosis. The results were similar in all. A typical experiment is shown in Figure 4. In the fasted state 24% of the injected radioactivity was recovered in CO_2 in 60 minutes. Three hours later the animal was treated with insulin and glucose. Acetoacetate concentration in the blood immediately before reversal of ketosis was 11.6%. Eight minutes after the administration of insulin and glucose, acetoacetate decreased to 4.6 mg per 100 ml in the expected fashion. At 10 minutes palmitate-1-^{14}C was injected. The cumulative recovery curve of isotope in CO_2 was almost superimposable on the pretreatment curve, and again 24% of the injected radioactivity was collected in the 60-minute period. Acetoacetate concentration at the end of the experiment was 1.6 mg per 100 ml. Measured specific activity of unesterified fatty acids in the blood 5 minutes after the injection of the radioactive palmitate was 3,120 ± 64 cpm per μmole in four fasted animals and 2,520 ± 99 cpm per μmole in four animals treated with glucose and insulin.

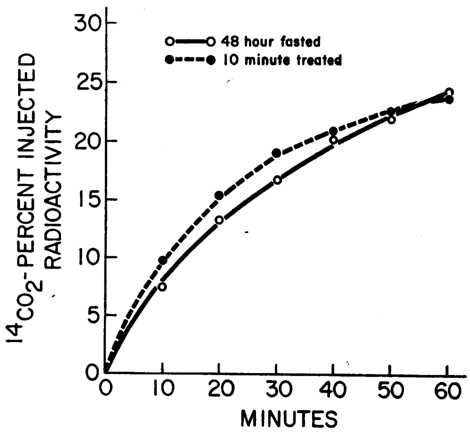

FIG. 4. PALMITATE-1-^{14}C OXIDATION TO $^{14}CO_2$ IN VIVO IN THE REVERSAL OF KETOSIS. Experimental details are outlined in the text. Fasted and treated curves were obtained in the same animal. Acetoacetate concentration in the blood immediately before the injection of insulin and glucose was 11.6 mg per 100 ml. Eight minutes after insulin acetoacetate was 4.6 mg per 100 ml, and at the end of the experiment the level was 1.6 mg per 100 ml. Palmitate-1-^{14}C in the treated curve was administered 10 minutes after insulin and glucose.

These experiments support the conclusions drawn from *in vitro* data and indicate that under circumstances where ketosis has been reversed *in vivo* no change occurs in fatty acid synthesis or oxidation.

Plasma nonesterified fatty acids in fasting and recovery. The relationship of ketosis to plasma nonesterified fatty acids was next studied. In the initial experiments, acetoacetate and nonesterified fatty acids were measured in inferior vena caval blood during the onset of fasting and 10 minutes after the iv injection of 0.05 U of insulin. The results are shown in Figure 5. By 12 hours free fatty acids had increased from 113 μEq per L to 421 μEq per L. From 12 to 48 hours a more gradual increase occurred, the peak level being 530 μEq per L. Acetoacetate concentrations rose only slightly during the first 12 hours but by 48 hours had increased about fivefold from a beginning level of 1.2 mg to a final concentration of 6.6 mg per 100 ml. Ten minutes after the injection of insulin both the ketoacid and nonesterified fatty acids had clearly decreased.

In an effort to dissociate the two phenomena, additional experiments were performed. As shown previously in Figure 3, it had been observed that

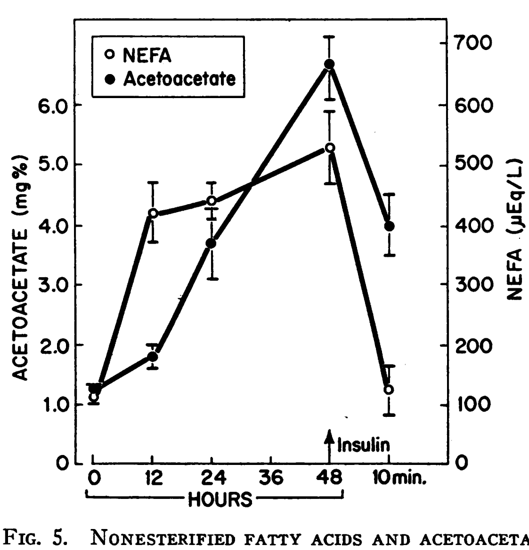

FIG. 5. NONESTERIFIED FATTY ACIDS AND ACETOACETATE IN THE BLOOD IN FASTING AND RECOVERY. Nonesterified fatty acids and acetoacetate were measured in inferior vena caval blood. After a 48-hour fast 0.05 U of glucagon-free insulin was given intravenously. Animals not injected with insulin received a control injection of saline. Each point represents the mean and standard error of the mean of the results obtained in six animals.

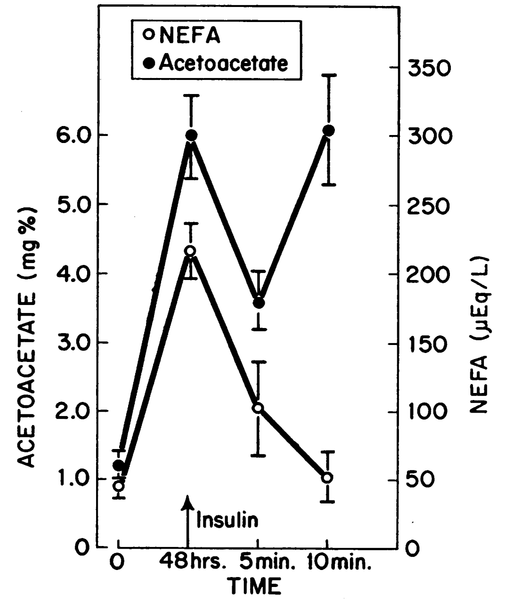

FIG. 6. NONESTERIFIED FATTY ACIDS AND ACETOACETATE IN THE BLOOD WITH INSULIN TREATMENT (0.5 U). Nonesterified fatty acids and acetoacetate were measured in blood obtained from the abdominal aorta. After a 48-hour fast 0.5 U of insulin was given intravenously. Each point represents the mean and standard error of the mean of the results obtained in eleven animals except for the fed controls, where six animals were used.

the administration of insulin caused a rapid drop in the concentration of acetoacetate in the blood until hypoglycemia supervened, at which time a rebound in ketosis occurred. To exaggerate this response a group of fasted animals were given 0.5 U of insulin intravenously, a dose ten times greater than that used in the experiment of Figure 5. The results are shown in Figure 6. Five minutes after the injection of insulin the mean acetoacetate level in the blood decreased from 6.0 to 3.6 mg per 100 ml ($p = < 0.005$). Plasma nonesterified fatty acids also fell from 216 to 103 μEq per L ($p = < 0.01$). The mean blood sugar at this time was 64 mg per 100 ml. At 10 minutes acetoacetate rose sharply to 6.1 mg per 100 ml ($p = < 0.05$) as the blood sugar fell to 41 mg per 100 ml. In contrast, plasma nonesterified fatty acid continued to decrease, reaching a low of 53 μEq per L.

Additional evidence that the ketosis of fasting may be unrelated to an elevation of free fatty acids in the blood is provided by the data of Figure 7. In this experiment animals fasted for 48 hours

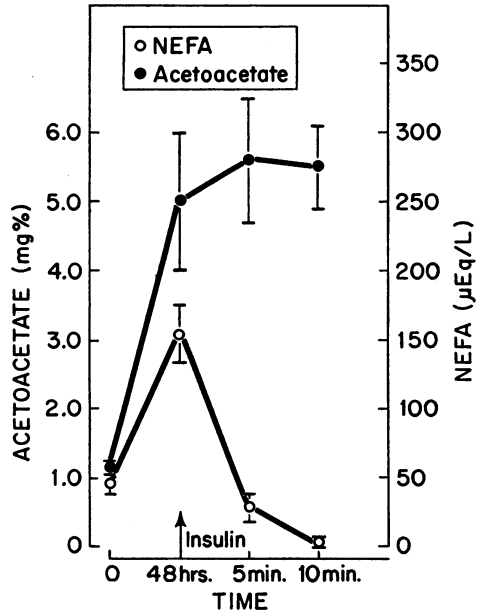

FIG. 7. NONESTERIFIED FATTY ACIDS AND ACETOACETATE IN THE BLOOD WITH INSULIN TREATMENT (0.01 U). Experimental details are as listed for Figure 6 except that 0.01 U of insulin was injected at the end of the 48-hour fast. Each point represents the results from six animals.

were treated with 0.01 U of insulin intravenously. Under these circumstances no change occurred in acetoacetate concentrations in the blood despite the fact that nonesterified fatty acids decreased promptly at 5 minutes and at 10 minutes were near 0.

These experiments demonstrate that acetoacetate and nonesterified fatty acid concentrations in the blood can be varied independently and support the conclusion that ketogenesis need not be causally related to changes in long chain fatty acid mobilization and oxidation.

Recovery from ketosis in the isotopic steady state. The very rapid fall in blood ketones after the administration of insulin or glucose raised the question of the mechanism of this reversal. Was it possible that cessation of hepatic overproduction of ketones could occur within 5 minutes after treatment, or did the reversal of the ketotic state represent an increased peripheral oxidation of ketone bodies? To answer this question, rats were fasted for 48 hours and then infused with acetoacetate-3-^{14}C until a constant specific activity of blood acetoacetic acid had been obtained. Because of the rapid turnover of acetoacetate in the intact rat, this could be accomplished without a primer dose of acetoacetate-3-^{14}C. When the isotopic steady state was reached, the animal was treated with glucose or insulin intravenously through the tail vein catheter. Since the specific activity of the blood acetoacetate under these circumstances is the equilibrium product of nonradioactive ketones synthesized by the liver and the radioactive material being infused, it is clear that cessation of hepatic synthesis would result in a rise in specific activity of the blood, whereas an increased peripheral utilization would cause no change. A typical example of such an experiment is shown in Figure 8 where it can be seen that the injection of insulin was followed by a prompt fall in arterial blood acetoacetate concentration and that concomitant with this fall a sharp rise occurred in its specific activity. Identical results were obtained when glucose was given alone. Thus recovery from fasting ketosis is accompanied by a marked diminution of acetoacetate production by the liver.[6]

Acetoacetate oxidation by various tissues before and after treatment with glucose. Although the *in vivo* experiments reported above suggested that decreased hepatic synthesis was the major mecha-

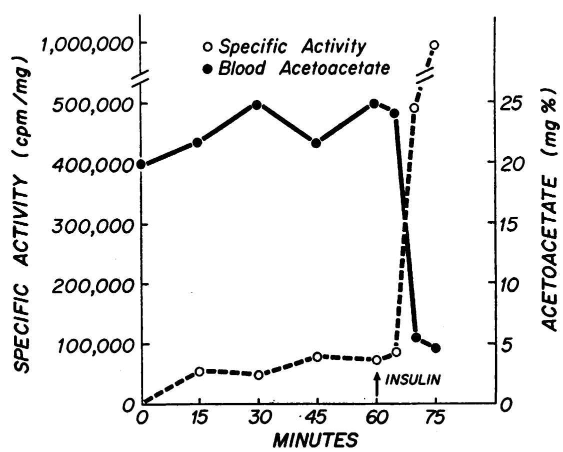

FIG. 8. THE REVERSAL OF KETOSIS IN THE ISOTOPIC STEADY STATE. The rat was fasted 48 hours and acetoacetate-3-^{14}C administered as described in the text. At the indicated time 0.025 U of insulin was given intravenously. Specific activity is recorded as counts per minute per milligram of acetoacetic acid. (For conversion of acetoacetate concentrations to acetone equivalents, multiply by 0.58.)

[6] An analysis of the kinetics of acetoacetate metabolism will be presented in a separate report.

TABLE VI

*Oxidation of acetoacetate-3-^{14}C by various tissues of the fasted rat before and after glucose administration**

Tissue	Untreated	Treated	p value
Diaphragm	4.0 ± 0.70	4.1 ± 0.79	NS
Skeletal muscle	1.1 ± 0.14	1.4 ± 0.19	NS
Kidney	2.5 ± 0.08	2.4 ± 0.01	NS
Adipose tissue	0.28 ± 0.05	0.28 ± 0.07	NS
Heart	5.6 ± 0.44	5.8 ± 0.85	NS
Blood ketones, *mg/100 ml*	8.9 ± 1.1	3.6 ± 1.0	<0.01

* Rats were fasted for 48 hours and the various tissues removed as indicated. One hundred mg of slices was incubated with 10 μmoles of sodium acetoacetate-3-^{14}C (1 μc) for 30 minutes at 37° C in 3 ml of Krebs bicarbonate buffer except that the diaphragms were not sliced and used intact as the hemidiaphragm. $^{14}CO_2$ was collected in 1 N NaOH as described in the text, evaporated in a drying oven to remove any traces of acetone, and dissolved in the counting solution. The treated animals were given 0.4 ml 50% glucose intravenously at the end of 48 hours and killed 10 minutes later. Results are tabulated as micromoles acetoacetate converted to CO_2 per 100 mg tissue in 30 minutes and are the means ± standard errors of the means in duplicate experiments from six animals.

nism whereby glucose and insulin reversed ketosis, it had been previously reported by Beatty, Peterson, Bocek, and West (36) that insulin added to skeletal muscle *in vitro* increased the oxidation of acetoacetate to CO_2. Because of this report and the fact that a small increase in peripheral oxidation of ketones would have been missed in the studies on reversal of ketosis in the isotopic steady state, acetoacetate oxidation to CO_2 was measured in skeletal muscle and several other organs from ketotic animals before and 10 minutes after treatment with glucose as shown in Table VI. All tissues studied actively produced radioactive CO_2 from acetoacetate-3-^{14}C. There was, however, no change in the rate of oxidation in any tissue after treatment with glucose despite the fact that blood acetoacetate fell as expected from 8.9 to 3.6 mg per 100 ml at the 10-minute period.

Discussion

The present studies were undertaken to describe the events occurring in the liver of the rat during the development of ketosis in fasting and to attempt to differentiate at the cellular level and *in vivo* the sequential changes in fatty acid oxidation, fatty acid synthesis, and acetoacetate synthesis that occur with starvation and its reversal. These parameters were chosen for study since, as indicated previously, most theories of ketosis emphasize the role of either increased fatty acid oxidation or decreased fatty acid synthesis as initiating factors in the overproduction of ketone bodies by the liver (1, 37). In either case it has been assumed that accelerated acetoacetate synthesis in ketosis is a secondary phenomenon, the result of increased concentrations of acetyl coenzyme A in the liver cell (8, 9).

The first point of note in the present experiments is that acetoacetate synthesis increased seven to ten times in liver slices with the onset of fasting, whereas palmitate-1-^{14}C oxidation to $^{14}CO_2$ was only minimally increased, even when corrected for changes in the hepatic free fatty acid pool. In this respect liver appears to conform to the pattern found in rat heart by Opie, Evans, and Shipp (38) and rat skeletal muscle by Fritz and Kaplan (39) where palmitate oxidation was not increased by a 48-hour fast.

In contrast, fatty acid synthesis *in vitro* was markedly depressed by fasting and to a degree compatible with the increased acetoacetate synthesis found in the slice. Since changes in the two processes could not be separated in studies of the onset of starvation at intervals shorter than 12 hours and since after refeeding, both fatty acid synthesis and acetoacetate synthesis had returned to normal by 6 hours, the possibility that a fall in fatty acid synthesis initiated ketosis, as suggested by Siperstein (11), seemed very attractive. However, when attempts were made to confirm this by studies at earlier time intervals after refeeding or the administration of glucose, it became clear that ketone levels in the blood and acetoacetate synthesis in the liver both decreased before any change in hepatic lipogenesis.

On the basis of the *in vitro* studies it was considered unlikely that increased acetoacetate synthesis in starvation was initiated by changes in fatty acid oxidation or fatty acid synthesis, though alterations in these pathways, particularly the latter, did occur with fasting. Strong confirmation of this conclusion was obtained by studies in the intact animal. The oxidation of albumin-bound palmitate-1-^{14}C to $^{14}CO_2$ was unchanged 10 minutes after the injection of glucose and insulin at a time when blood acetoacetate concentration had dropped to less than 50% of the original fasting level. Although some caution must be exercised in the interpretation of these results because of lack of knowledge of the specific activity of the tissue fatty acid pools, the fact that the measured specific activity of the unesterified fatty acids in the blood of fasted and treated animals was essentially the same [7] and the observation that the liver free fatty acid pool was unchanged with treatment (Table III) suggest that the results may accurately reflect absolute rates of fatty acid oxidation.

Fatty acid synthesis *in vivo* likewise was unchanged on reversal of ketosis. Significant increases in lipogenesis did not occur until at least 3 hours after refeeding. This is in contrast to studies by Fain, Scow, Urgoiti, and Chernick (41) in the pancreatectomized diabetic rat *in vivo* where fatty acid synthesis increased in 30 minutes after the injection of 12 U of insulin. The reason for the difference in time required for restoration of lipogenesis in fasting and diabetes is not known.

The results of both *in vitro* and *in vivo* studies thus indicated that ketosis could be interrupted without changes in either fatty acid oxidation or synthesis. If, as seems likely, recovery from ketosis represents a reversal of the processes initiating ketosis, then the overproduction of acetoacetate by the liver cannot be considered to be the secondary consequence of alterations in these lipid pathways.

It should be noted that the increased fatty acid oxidation theory of ketosis requires two phases: increased mobilization of free fatty acids from peripheral fat stores and increased oxidation of these fatty acids in the liver. To study the mobilization phase, nonesterified fatty acids and acetoacetate concentrations in the blood were compared during the onset of fasting and in recovery from the fasted state. These experiments indicated that both components increased with starvation, nonesterified fatty acids showing the greater percentage rise at 12 hours. On retreatment with 0.05 U of insulin, both acetoacetate and free fatty acid levels decreased at 10 minutes in near parallel fashion. When the insulin dose was increased to 0.5 U, a different pattern was observed. At 5 minutes acetoacetate fell sharply as expected and was accompanied by a fall in nonesterified fatty acids. At 10 minutes, however, a definite dissociation occurred. Acetoacetate concentration increased in the characteristic rebound phenomenon of hypoglycemia, whereas plasma nonesterified fatty acids continued to decline. When the dose of insulin was decreased to 0.01 U, blood acetoacetate levels did not fall despite a marked decrease in free fatty acids over the 10-minute period. If ketosis were dependent on mobilization of fatty acids from the periphery, a depression of free fatty acid concentrations in the blood would have to be accompanied by a decrease in acetoacetate levels during this period in view of the repeated observation that ketones fall by 5 minutes with adequate insulin. It thus appears, in confirmation of the fatty acid oxidation data, that no necessary relationship exists between nonesterified fatty acids in the blood and starvation ketosis.

As discussed previously, current theories that invoke either increased fatty acid oxidation or decreased fatty acid synthesis as initiating steps in the development of ketosis consider the increased acetoacetate synthesis of starvation to be the result of an expanded hepatic acetyl CoA pool. Sequential measurements of acetyl CoA indicated that concentrations of this compound increased at 24 hours to levels about double those of the fed state, a result compatible with a controlling role for acetyl CoA in acetoacetate synthesis. On the other hand, at 48 hours the acetyl CoA concentration decreased, whereas acetoacetate concentrations in the blood increased still further. The critical point, however, is that the administration of glucose and insulin to animals fasted for 48 hours caused a rapid fall of acetoacetate in the blood without decrease in the concentration of acetyl CoA in the liver. This course makes unlikely the thesis that accelerated ketogenesis in the liver is primarily related to an expanded acetyl CoA pool.

[7] As noted in Figures 5 to 7 free fatty acids in the blood decreased promptly on administration of insulin. Since the injected palmitate exchanges rapidly with a pool of fatty acid about 100 times larger than that of the blood (28, 40), the fall in concentration of nonesterified fatty acids in the blood would affect the specific activity of the tissue pool to a negligible extent.

It is of interest that the concentrations of both free fatty acids and acetyl CoA increased in the liver at 24 hours and decreased at 48 hours. Since nonesterified fatty acids in the blood continued to rise throughout the 48-hour fast, it seems likely that the major source of fatty acid contributing to the acetyl CoA pool in the liver is hepatic rather than peripheral triglyceride.[8]

In regard to the metabolism of acetyl CoA, the present studies showed no impairment of acetate-2-^{14}C oxidation to $^{14}CO_2$ during 48 hours of starvation. This observation in the intact cell, together with the recent demonstration that citrate synthase activity measured directly in liver extracts is unchanged or slightly increased in fasting (43), lends no support to the concept (6, 7, 9) that palmityl CoA–induced inhibition of the latter enzyme plays a significant role in starvation ketosis.

The rapidity with which ketosis was reversible by the administration of insulin or glucose was surprising. Amatruda and Engel (44) had previously shown that insulin reversed the ketosis of fasting in 1 hour, and Fain and associates (41) reported that blood ketones in the diabetic rat decreased 30 minutes after the injection of insulin. In the studies reported here, however, acetoacetate levels began to decline within 5 minutes after treatment with insulin or glucose. Moreover, the results of experiments in the isotopic steady state clearly showed this decrease to be due to an abrupt cessation of ketone body production by the liver.

The fact that changes in acetoacetate synthesis can be dissociated from changes in fatty acid mobilization from the periphery, fatty acid oxidation, fatty acid synthesis, and acetyl CoA concentration in the liver by kinetic studies raises the possibility that the increased acetoacetate production accompanying starvation is a primary rather than a secondary event. That is, it seems possible that starvation produces an activation of acetoacetate-synthesizing enzymes independent of changes in lipid metabolism leading to alteration in acetyl CoA production or utilization. This would not, of course, imply that increased fatty acid oxidation and decreased fatty acid synthesis do not play a role in assuring substrate for acetoacetate synthesis in ketosis, only that such changes do not initiate the ketotic state.

The mechanism by which a primary stimulation of acetoacetate synthesis might be accomplished is unknown. The observation that the injection of insulin alone reverses the ketosis of fasting within minutes by causing cessation of ketone synthesis in the liver suggests the possibility that the control of acetoacetate synthesis may be mediated by insulin itself. The corollary of this possibility would be that the ketosis of fasting may represent a primary activation of acetoacetate-synthesizing enzymes as a result of starvation-induced depression of insulin secretion by the pancreas.

[8] Repeated attempts were also made to measure long chain fatty acyl CoA levels in the liver as described by Tubbs and Garland (34) and Bortz and Lynen (35). Although the former authors state that recovery of added palmityl CoA was complete, recoveries of palmityl CoA in these experiments were only 10 to 40% utilizing their conditions for isolation, hydrolysis, and assay of the long chain CoA esters. Attempts to measure the protein-bound fatty acyl CoA with pork brain palmityl CoA deacylase of high specific activity prepared according to Srere, Seubert, and Lynen (42) were also unsuccessful.

Acknowledgments

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