Acetoacetyl Coenzyme A Deacylase Activity in Liver Mitochondria from Fed and Fasted Rats*

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ABSTRACT: Evaluation of either the β-hydroxy-β-methylglutaryl-CoA pathway or the acetoacetyl coenzyme A (acetoacetyl-CoA) pathway for the production of free acetoacetate is fraught with many difficulties since acetoacetate is a product and acetoacetyl-CoA is an intermediate in both pathways. Since Stern and Miller's original observation (Stern, J. R., and Miller, G. E. (1959), *Biochim. Biophys. Acta 35*, 576) that both β-ketothiolase and β-hydroxy-β-methylglutaryl-CoA condensing and cleavage enzymes were completely inhibited by preincubation with iodoacetamide, but acetoacetyl-CoA deacylase was not affected by this treatment, a tool has been available to evaluate the effect of various ketogenic regimens on acetoacetate production *via* acetoacetyl-CoA deacylase without interference from the β-hydroxy-β-methylglutaryl-CoA pathway. Disappearance of acetoacetyl-CoA was determined spectrophotometrically in triplicate by using β-hydroxyacyl-CoA dehydrogenase and reduced diphosphopyridine nucleotide (DPNH). Nonenzymatic deacylation was also determined in triplicate and substracted from all experimental values before any calculations were made. The disappearance of acetoacetyl-CoA corresponded very closely to the appearance of free acetoacetate, thus validating this type of assay in whole mitochondria. Addition of acetyl-CoA or coenzyme A to mitochrondria which had been preincubated with iodoacetamide failed to increase the disappearance of acetoacetyl-CoA whereas similar additions to mitochondria which had not been preincubated with iodoacetamide resulted in a marked increase in the amount of acetoacetyl-CoA disappearing. Thus, iodoacetamide appears to be an effective inhibitor of β-ketothiolase and β-hydroxy-β-methylglutaryl-CoA condensing enzyme in intact mitochondria. Acetoacetyl-CoA disappearance increased 42% in iodoacetamide-treated liver mitochondria from rats fasted for 24 hr when compared to fed rats. This effect was seen in rats of all ages and was still apparent after a 48-hr fast. Thus, free acetoacetate production *via* the acetoacetyl-CoA deacylase pathway is enhanced with fasting and acetoacetyl-CoA available for synthetic processes would thereby be effectively reduced.

Two major pathways have been proposed for the synthesis of free acetoacetate from acetoacetyl-CoA. Lynen *et al.* (1958), using a partially purified system from acetone powders of ox liver, showed that acetoacetate production involved condensation of acetoacetyl-CoA with acetyl-CoA. The product of this reaction, β-hydroxy-β-methylglutaryl-CoA (HMG-CoA),[1] is then cleaved to yield free acetoacetate and acetyl-CoA. Stern and Miller (1959), using sonicated rat liver mitochondria, and Drummond and Stern (1960), using a solubilized system from ox liver purified in a different manner than that of Lynen *et al.*, showed that another pathway may be involved in free acetoacetate production. This pathway, acetoactyl-CoA deacylation, gave rise to free acetoacetate and coenzyme A directly from acetoacetyl-CoA.

Relatively little effort has been expended in studying the effect of known ketogenic regimens on HMG-CoA cleavage enzyme (EC 4.1.3.4) and/or acetoacetyl-CoA deacylase (EC 3.1.2). Although Wieland *et al.* (1960) studied the HMG-CoA pathway in rat liver preparations during starvation and alloxan-induced diabetes and Segal and Menon (1961) studied the HMG-CoA pathway and the acetoacetyl-CoA deacylase pathway in rat liver preparations during starvation, alloxan-induced diabetes, and various hormonally induced states, it is extremely difficult to evaluate their data in view of the fact that both the HMG-CoA and acetoacetyl-CoA deacylase pathways were operative. The same objection can be raised with the work of Caldwell and Drummond (1963) who studied the HMG-CoA pathway in partially purified beef extracts obtained according to the method of Stern *et al.* (1960).

This paper shows that, in a system where the HMG-CoA pathway has been inhibited with iodoacetamide, acetoacetyl-CoA deacylation occurs in whole mitochondrial preparations. This finding is in keeping with Stern and Miller's (1959) and Drummond and Stern's (1960) work using solubilized systems. In addition, we show that there is a significant difference in acetoacetyl-CoA deacylase activity in rat liver mitochondria obtained from fed and 24-hr fasted rats.

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[1] Abbreviation used: HMG-CoA, the coenzyme A ester of β-hydroxy-β-methylglutaric acid; DPNH, reduced diphosphopyridine nucleotide.

Experimental Procedure

Reagents. Acetoacetyl-CoA was synthesized from diketene and reduced coenzyme A (Wieland and Rueff, 1953). Coenzyme A and the lithium salt of acetyl-CoA were purchased from P & L Laboratories, Milwaukee, Wis. DPNH and β-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35) were purchased from Boehringer Mannheim Corp., New York, N. Y.

Methods. Free acetoacetate was determined by the method of Walker (1954). Acetoacetyl-CoA was purified on an Ecteola-cellulose column according to the method of Sauer and Erfle (1966). Acetoacetyl-CoA was assayed spectrophotometrically with β-hydroxyacyl-CoA dehydrogenase, according to the method of Decker (1963). Protein was determined by the method of Lowry *et al.* (1951).

Preparation of Animals. Male rats of the Wistar strain were purchased from Albino Farms, Red Band, N. J. All rats used in these studies weighed between 200 and 400 g unless otherwise indicated. The animals were fed Purina rat pellets *ad libitum.* Food was removed from animals which were to be fasted 24 hr prior to an experiment unless otherwise indicated. Fed animals ate from 15 to 25 g in the 18 hr preceding an experiment.

Isolation of Mitochondria. The animals were killed by a blow to the head and bled thoroughly. The liver was removed, minced, and then homogenized with a loose-fitting Potter-Elvehjem homogenizer for less than 30 sec with three volumes of 0.25 M sucrose. Mitochondria were obtained by differential centrifugation (Burch *et al.*, 1964), washed once with one-fourth the original volume of 0.25 M sucrose, and subsequently diluted with 0.25 M sucrose to a final protein concentration of 7–15 mg/ml. The preparation was kept at 0° until used. A gram of liver gave an average yield of approximately 4.5 mg of mitochondria from both fed and fasted animals.

Experimental. Within 2 hr after isolation of the mitochondria, unless otherwise indicated, triplicate 0.1-ml samples were preincubated for 10 min at room temperature with iodoacetamide (10^{-3} M final concentration), except where this was purposely excluded, in the presence of 100 μmoles of Tris buffer (pH 7.9) and 5 μmoles of $MgCl_2$. Purified acetoacetyl-CoA (0.02 ml) in the amount of 0.10–0.21 μmole was then added and the mixture was incubated for 20 min at 37°; final volume was 2.0 ml. In those experiments where iodoacetamide was purposely omitted, the final volume was also 2.0 ml. Reactions were stopped by the addition of 0.2 ml of 25% trichloroacetic acid. The tubes were kept in ice for 3 min. The pH was adjusted to 7.0 by adding a predetermined volme of 1.0 N KOH. After centrifugation one-half the final volume of the reaction mixture was transferred to a cuvet and the amount of acetoacetyl-CoA remaining was assayed spectrophotometrically with β-hydroxyacyl-CoA dehydrogenase (Decker, 1963). The results of the triplicate assays were averaged. Assay of acetoacetyl-CoA with and without iodoacetamide gave identical results indicating that β-hydroxyacyl-CoA dehydrogenase was not inhibited by iodoacetamide. A nonenzymatic control was also analyzed in triplicate and averaged for determination of nonenzymatic deacylation, and this amount was subtracted from all enzymatic reactions before any calculations were made.

In those experiments where free acetoacetate was determined, quadruplicate samples were taken through the trichloroacetic acid step. Three of these samples were assayed for disappearance of acetoacetyl-CoA as described above. The fourth sample was centrifuged and an aliquot of the supernatant was assayed for free acetoacetate by the Walker (1954) method.

Purity of Acetoacetyl-CoA. The elution pattern of impure acetoacetyl-CoA chromatographed on Ecteola-cellulose according to the method of Sauer and Erfle (1966) was identical with the pattern observed by those authors. The absorption of aliquots of the effluent was determined at pH 7.9 in the presence of Mg^{2+} at 303 mμ. Purified acetoacetyl-CoA was eluted from the column between 120 and 150 ml. These samples were pooled, adjusted to pH 6, lyophilized, and the purified acetoacetyl-CoA was extracted exactly as described by Sauer and Erfle. In order to show that the purified acetoacetyl-CoA was not contaminated with glutathione the sample was again placed on an Ecteola-cellulose column and eluted according to the method of Sauer and Erfle. The rechromatographed material contained only one absorption peak which was eluted between 120 and 150 ml. No increase in absorbancy over the base line was found in the fractions from 30 to 60 ml which normally would contain acetoacetylglutathione. To further verify that no glutathione was present as a contaminant, the coenzyme A obtained by alkaline hydrolysis of purified acetoacetyl-CoA was chromatographed on 3MM paper in isopropyl alcohol–ethyl alcohol–formic acid–water (47:23:2.5:28). The R_F of the purified coenzyme A was 0.25; this corresponded to the R_F of the main spot of impure coenzyme A which also had two small spots with an R_F of 0.33 and 0.50. Authentic glutathione had an R_F of 0.50. The purified coenzyme A contained no detectable glutathione even with amounts which were equivalent to double the amount of purified acetoacetyl-CoA normally used in our studies. Purified coenzyme A was also chromatographed in ethyl alcohol–*t*-butyl alcohol–formic acid–water (60:20:5:15) where its R_F was 0.04. Again this corresponded to the R_F of the main spot of impure coenzyme A which also had two additional minor spots with an R_F of 0.2 and 0.35. Authentic glutathione had an R_F of 0.34. Purified coenzyme A contained no detectable glutathione. Chromatograms were sprayed with nitroprusside reagent for visualization of the compounds.

Results

Bucher *et al.* (1960) showed that drastic treatment of mitochondria was necessary to demonstrate maximal activity of HMG-CoA cleavage and condensing (EC 4.1.3.5) enzymes. Similarly, Bendall and de Duve (1960) showed that drastic treatment was necessary to demonstrate maximal activity of other mitochrondrial enzymes. Consequently numerous attempts were made to enhance acetoacetyl-CoA deacylase activity by either solubilizing or injuring the mitochondria. The methods

TABLE I: Effect of Added Acetyl-CoA and Coenzyme A on Acetoacetyl-CoA Disappearance Using Untreated and Iodoacetamide-Treated Mitochondria.[a]

Expt	Contents	Acetoacetyl-CoA Disappearing (mμmoles/mg of protein per 10 min)	
		Untreated Mitochondria	Iodoacetamide-Treated Mitochondria
I	Complete system	105	27.5
	Complete system + acetyl-CoA	133	28.7
	Complete system + CoASH	133	29.8
II	Complete system	98	21.5
	Complete system + acetyl-CoA	144	15.2
	Complete system + CoASH	158	22.8
III	Complete system	115	24.6
	Complete system + acetyl-CoA	174	24.6
	Complete system + CoASH	200	27.9

[a] Experiments were performed as described under Experimental Procedure and this is designated as the complete system. Untreated mitochondria were used after standing 10 min at room temperature. Iodoacetamide-treated mitochondria were preincubated 10 min with iodoacetamide in a concentration of 10^{-3} M at room temperature. Where indicated, 100 mμmoles of either acetyl-CoA or reduced coenzyme A was added after the addition of acetoacetyl-CoA. Incubations were for 10 min. In all instances the total volume was 2 ml. Nonenzymatic disappearance of acetoacetyl-CoA was 32, 23.3, and 27.6 mμmoles in expt I, II, and III, respectively.

utilized were treatment with concentrations of deoxycholate ranging from 0.05 to 2.5%, storage overnight with glycerol at −20°, repeated freezing and thawing, storage overnight at −20 and 0°, and ageing for periods up to 6 hr at 0°. In no instance was deacylase activity enhanced and frequently the treatment resulted in a marked diminution or complete loss of activity.

The data shown in Table I indicate that the disappearance of acetoacetyl-CoA is four times greater when mitochondria have not been preincubated with iodoacetamide than when the same mitochondria have been pretreated with iodoacetamide for 10 min. Addition of 100 mμmoles of acetyl-CoA to untreated mitochondria stimulates the disappearance of acetoacetyl-CoA, whereas addition of the acetyl-CoA to the same mitochondria which had been preincubated with iodoacetamide did not stimulate the disappearance of acetoacetyl-CoA. These results show that a 10-min preincubation of the mitochondria completely inhibited the HMG-CoA condensing enzyme. This finding is in keeping with Stewart and Rudney's (1966) results using a partially purified preparation from yeast and is in accord with the results of Stern and Miller (1959) using sonicated rat liver mitochondria. Since HMG-CoA condensing enzyme is inhibited by preincubation of the mitochondria with iodoacetamide, one would anticipate that the thiolase enzyme (EC 2.3.1.9) was also inhibited since Stewart and Rudney (1966) found the yeast thiolase to be far more sensitive to iodoacetamide than the HMG-CoA condensing enzyme. The addition of 100 mμmoles of coenzyme A to an incubation utilizing untreated mitochondria is also shown in Table I. Acetoacetyl-CoA disappearance is again enhanced over control values without coenzyme A indicating that the thiolase enzyme is quite active. Addition of coenzyme A to incubations utilizing the same mitochondria which had been preincubated with iodoacetamide failed to increase the disappearance of acetoacetyl-CoA indicating that the thiolase enzyme had been inactivated by the iodoacetamide treatment. However, to demonstrate that all the added coenzyme A was not reacting with excess iodoacetamide, free sulfhydryl was determined at the end of an incubation by the method of Grunert and Phillips (1951). When 100 mμmoles of free coenzyme A was added to iodoacetamide-treated mitochondria, 55 mμmoles was present at the end of the 10-min incubation.

The final step in the validation of this assay in intact mitochondria consisted in showing that the amount of acetoacetyl-CoA disappearing in a system utilizing iodoacetamide-treated mitochondria corresponded to the amount of free acetoacetate formed and was thus a true measure of acetoacetyl-CoA deacylase activity. As shown in Table II the amount of acetoacetate formed in this system corresponded very closely with the amount of acetoacetyl-CoA which disappeared. Therefore, it is apparent that measurement of the disappearance of acetoacetyl-CoA in this system gives a true measure of acetoacetate production through the deacylation of acetoacetyl-CoA.

Next the effect of fasting on deacylase activity was evaluated. Table III compares acetoacetyl-CoA deacylase activity in liver mitochondria obtained from fed rats and rats fasted for 24 hr. The data presented in this

TABLE II: Acetoacetyl-CoA Disappearance and Acetoacetate Formation Using Iodoacetamide-Treated Mitochondria.[a]

Expt	Contents	Acetoacetyl-CoA Disappearing (mμmoles/mg of protein per 20 min)	Acetoacetate Formed (mμmoles/mg of protein per 20 min)
I	Complete system	46.2	42.9
	Complete system + acetyl-CoA	33.2	31
	Complete system + CoASH	30.3	28.6
II	Complete system	38.9	35.9
	Complete system + acetyl-CoA	35.8	31.2
	Complete system + CoASH	32.6	31.2

[a] Experiments were performed as described under Experimental Procedure and this is designated as the complete system. Where indicated, 100 mμmoles of either acetyl-CoA or reduced coenzyme A was added after the addition of acetoacetyl-CoA. Incubation were for 20 min. In all instances the total volume was 2 ml. Nonenzymatic disappearance of acetoacetyl-CoA was 45.2 and 44 mμmoles of free acetoacetate was formed.

table represent eight separate experiments in which the normal animal is compared with the fasted animal. In comparing individual experiments the smallest increment of increase with fasting was 25% and the largest increment was 67%. The average increase with starvation in the eight experiments was 42%. Identical experiments were carried out with younger animals weighing 72–200 g and with older animals weighing 400–600 g. No significant difference could be found with fasting between these two groups and the group of experiments herein reported. Similarly, in evaluating the effect of the length of fasting on deacylase activity, animals fasted for 48 hr had increased activity in the same range as the animals shown in Table III. At the end of 72 hr of fasting deacylase activity had reverted to normal levels or lower.

TABLE III: Acetoacetyl-CoA Deacylase Activity in Iodoacetamide-Treated Mitochondria from Fed and 24-hr Fasted Rats.[a]

Status of Animals	Acetoacetyl-CoA Disappearing (mμmoles/mg of protein per 20 min)
Fed (8)	39.8 ± 4.8
24 hr fasted (8)	56.6 ± 7.0

[a] Experiments were performed as described under Experimental Procedure. The values shown represent the average plus and minus the standard deviation. Incubations were for 20 min. The numbers in parentheses represent the number of experiments performed in triplicate. In eight experiments nonenzymatic deacylation was 43 ± 8.7 mμmoles.

Discussion

Weiland *et al.* (1960) have studied the effect of starvation and diabetes on the HMG-CoA condensing and cleavage enzymes in rat liver. Their studies indicate that the increased production of acetoacetate in diabetes is due to increased HMG-CoA condensing enzyme activity. Although starvation of nondiabetic animals was associated with increased acetoacetate production, there was no associated increase in HMG-CoA condensing enzyme activity. Caldwell and Drummond (1963) have presented evidence for the primacy of the HMG-CoA pathway using a partially purified extract from normal beef liver. Their preparation was isolated according to the method of Stern *et al.* (1960). In the studies of Lynen *et al.* (1958), Weiland *et al.* (1960), and Caldwell and Drummond (1963) an extract of *Clostridium kluyveri* containing phosphotransacetylase (EC 2.3.1.8) and β-ketothiolase was used to generate acetoacetyl-CoA from acetyl phosphate and coenzyme A. Since enzymatic activity was measured by the appearance of free acetoacetate, it was impossible in all of these studies to delineate the amount of free acetoacetate produced by deacylation of acetoacetyl-CoA.

Segal and Menon (1961) attempted to study both the HMG-CoA cleavage and acetoacetyl-CoA deacylase pathways for acetoacetate production using frozen-thawed or Triton-treated rat liver mitochondria. They claimed to show that free acetoacetate was produced primarily by the acetoacetyl-CoA deacylase pathway. In those instances where they generated acetyl-CoA from acetyl phosphate using an extract of *C. kluyveri*, it is not possible to tell how much of the acetoacetate is derived from cleavage of HMG-CoA which has been formed during the course of incubation. In studies wherein Segal and Menon utilized chemically synthesized acetoacetyl-CoA, no valid conclusions can be drawn since measurement of free acetoacetate in a system which contains both pathways for the synthesis of this

product gives no indication of which pathway is involved and to what extent. Sauer and Erfle (1966) have recently shown that chemically synthesized acetoacetyl-CoA contains large amounts of acetoacetylglutathione and an unknown contaminant, in addition to acetoacetyl-CoA. Thus, the possibility of acetoacetylglutathione hydrolase (EC 3.1.2.8) contributing significant amounts of the free acetoacetate measured by Segal and Menon further invalidates their findings. Finally, their studies which excluded the HMG-CoA pathway for free acetoacetate synthesis by incubating their frozen-thawed mitochondria with [^{14}C]acetyl-CoA and an unlabeled pool of acetoacetyl-CoA could be interpreted in favor of the HMG-CoA pathway since information about the final specific activity of the [^{14}C]acetyl-CoA is lacking. The studies reported here seem to have circumvented the difficulties described above. The possibility of interference with the deacylase reaction by other substances present in chemically synthesized acetoacetyl-CoA has been lessened considerably by using acetoacetyl-CoA purified according to the method of Sauer and Erfle. Both β-ketothiolase and HMG-CoA condensing enzymes have been inhibited by preincubating the mitochondria with iodoacetamide. This is shown in the studies with and without iodoacetamide, wherein the addition of coenzyme A or acetyl-CoA markedly enhances the disappearance of acetoacetyl-CoA in untreated mitochondria but does not increase the disappearance of acetoacetyl-CoA in iodoacetamide-treated mitochondria. Thus, it appears that only acetoacetyl-CoA deacylase is being evaluated in these studies.

Sauer and Erfle (1966), using an ammonium sulfate precipitate of sonicated liver mitochondria obtained from pregnant, fasted guinea pigs, were able to show that acetoacetate was produced exclusively by the HMG-CoA pathway without involvement of acetoacetyl-CoA deacylase. Their findings do not detract from the results we report since the ammonium sulfate fractionation of their enzyme could easily have removed any acetoacetyl-CoA deacylase which might have been present. The values presented here are considerably less than those of Stern and Miller who used sonicated rat liver mitochondria. We can offer no explanation for this discrepancy between their results and ours. However, the possibility that the acetoacetyl-CoA deacylase exists in an inactive form in the mitochondria or that the substrate, acetoacetyl-CoA, is not coming in contact with all of the enzyme because we are using intact mitochondria must certainly be kept in mind. These possibilities are not likely in this system in view of the fact that solubilization of the mitochondria failed to enhance deacylase activity. The values presented here compare quite favorably with those obtained by Drummond and Stern (1960), using a solubilized ox liver preparation. Therefore, it appears that this is a valid system which gives a true picture of acetoacetyl-CoA deacylase activity in intact mitochondria and that this activity is enhanced 42% with fasting in these studies.

Weinhouse (1952), utilizing isotopic methods, showed that rat liver slices from fed animals produced 9.6 g of acetoacetate/kg per day and liver slices from fasted rats synthesized 22 g of acetoacetate/kg per day. Drummond and Stern (1960) assumed that liver was 15% protein and recalculation of their data yields a value of 12.3 g of acetoacetate synthesized/kg per day. If we assume that liver is 15% protein and that mitochondria constitute 32% of the total protein (Schneider *et al.*, 1948) then the data in Table III yield values of 14.9 g of acetoacetate/kg per day for the fed animals and 21.3 g of acetoacetate/kg per day for the fasted animals.

Since acetoacetyl-CoA is an important intermediate in mitochondrial fatty acid synthesis, cholesterol synthesis, and free acetoacetate production, the data presented offer an interesting and intriguing possible explanation for the fact that acetoacetate is elevated in starvation and both fatty acid synthesis and cholesterol synthesis are depressed. Such a mechanism, which could regulate the amount of acetoacetyl-CoA available for synthetic processes by a direct conversion of this intermediate into free acetoacetate and coenzyme A, could certainly be considered as a control mechanism.

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