notes on methodology

Determination of acetyl coenzyme A. Interference by a contaminant in malate dehydrogenase

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Summary Spectrophotometric determinations of acetyl CoA with malate dehydrogenase and citrate synthase are likely to overestimate the amount of acetyl CoA in solutions containing acetoacetyl CoA, since commercial preparations of malate dehydrogenase may contain thiolase.

Supplementary key words acetoacetyl CoA · thiolase

THE SPECTROPHOTOMETRIC DETERMINATION of AcCoA with CS (EC 4.1.3.7) and MDH (EC 1.1.1.37) is a well-known and useful method (1). From the differences in NADH absorbance in the reaction couple

(1) L-malate + $NAD^+ \overset{MDH}{\rightleftarrows}$ oxaloacetate + NADH + H^+

(2) AcCoA + oxaloacetate + $H_2O \overset{CS}{\rightleftarrows}$ citrate + CoA

the concentration of AcCoA can be calculated.

In our work, difficulties arose if our experimental assay mixtures contained AcacCoA in concentrations comparable to that of AcCoA. After addition of the required amounts of malate, NAD^+, and the enzymes, sometimes the absorbance due to NADH formation did not stop rising. Our experiments led us to conclude that thiolase (EC 2.3.1.9) was present as a contaminant in the commercial MDH preparation.

Experimental. All substrates, coenzymes, and enzymes used were obtained from Boehringer Mannheim GmbH, unless otherwise stated.

AcCoA solutions were prepared by incubating acetyl-DL-carnitine HCl (17.5 mM), CoA (1.4 mM), and carnitine acetyltransferase (EC 2.3.1.7, 0.6 U/ml) for 1 hr at pH 8.0 (0.05 M Tris buffer). The enzyme was precipitated with $HClO_4$. Solutions were made fresh daily and kept at pH 5–6.

AcacCoA solutions were prepared from CoA and diketene and were purified chromatographically by the method of Sauer and Erfle (2).

Abbreviations: AcCoA, acetyl CoA; AcacCoA, acetoacetyl CoA; CS, citrate synthase; MDH, malate dehydrogenase.

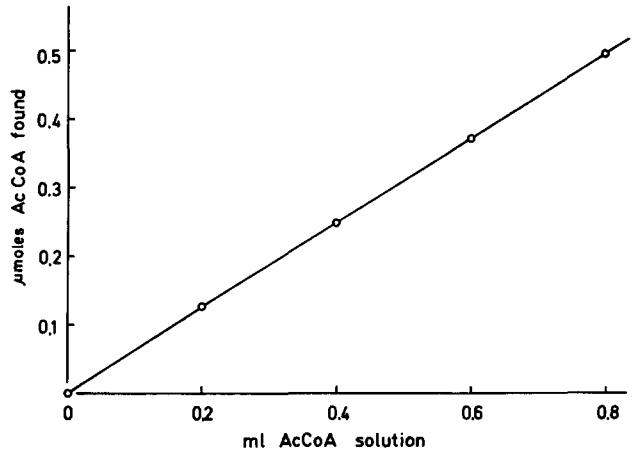

FIG. 1. AcCoA assay. Cuvettes contained 0.2 M Tris buffer, pH 8.1, 1.5 mM NAD^+, and 5 mM DL-malate. AcCoA solution (ca. 0.65 mM) was added in the amounts indicated. Total volume was 2.0 ml. Increase in absorbance at 366 nm (Eppendorf photometer, $\epsilon_{NADH} = 3.3 \times 10^6\ M^{-1}\ cm^{-1}$) was measured 3 min after addition of MDH (2.7 U; ΔE_1) and subsequently 30 min after addition of CS (0.17U; ΔE_2). The amount of AcCoA (a) found was calculated from

$$a = \frac{2.0}{3.3}\Delta E_2\left(1 + \frac{\Delta E_1}{\Delta E_1 + \Delta E_2}\right)\ \mu\text{moles}.$$

Experimental conditions of the AcCoA assay were generally as described by Decker (1).

Results and discussion. The assay method (1) proved to be satisfactory (Fig. 1). The upper part of Table 1 shows that addition of AcacCoA gave rise to higher results in the normal AcCoA determination. Sometimes (not shown in the table) in the presence of AcacCoA the rise in absorption at 366 nm after addition of CS was not completed within the usual 30 min. Increasing the MDH concentration in the assay mixture (Table 1) caused a much higher increase in absorbance after addition of CS if AcacCoA was present. Increasing the concentration of CS had no effect. With high concentrations of MDH

TABLE 1. Influence on ΔE_2 of various concentrations of AcacCoA, MDH, and CS

AcCoA	AcacCoA	MDH	CS	ΔE_1	ΔE_2 20 min	ΔE_2 30 min	ΔE_2 60 min
mM	*mM*	*U/ml*	*U/ml*				
0.12		*1.4*	*0.09*	0.085	0.308	0.312	0.313
0.12	0.04	*1.4*	*0.09*	0.083	0.311	0.316	0.322
0.12	0.20	*1.4*	*0.09*	0.083	0.320	0.323	0.325
0.12	0.60	*1.4*	*0.09*	0.084	0.347	0.356	0.356
		9	1.2	0.087	0.003	0.003	0.004
0.12		9	1.2	0.086	0.317	0.318	0.316
0.12	0.12	9	1.2	0.083	1.093	1.117	1.123
	0.12	9	1.2	0.088	0.126	0.227	0.769
	0.12	9	*0.09*	0.087	0.113	0.203	0.713
	0.12	*1.4*	1.2	0.088	0.014	0.014	0.016

AcCoA assay conditions were as in Fig. 1, unless otherwise stated. Italics indicate enzyme concentrations used in the normal AcCoA assay.

the increase in absorbance continued after 30 min if AcacCoA alone was present, and the rate of increase also increased with time.

An increasing decarboxylation of oxaloacetate could explain the observations, but this is improbable because the effect was abolished by omitting AcacCoA or by lowering the MDH concentration. Another possibility is the increasing formation of a compound that could react so as to give rise to an increasing amount of NADH. This criterion is met by the thiolytic cleavage reaction of AcacCoA into AcCoA. CoA, which is necessary for this reaction, is present in a low concentration in the initial stages of the incubation. Its concentration increases by spontaneous hydrolysis of AcacCoA and of AcCoA and by the CS reaction.

The effect of the increased absorbance in the presence of AcacCoA could be partly prevented by adding iodoacetamide (3.75 mM) to our assay mixtures (Table 2); this is in accordance with the fact that thiolase is readily inhibited by this concentration of iodoacetamide (3, 4). The iodoacetamide interferes slightly with the AcCoA determination.

After incubation of MDH or CS with AcacCoA for 1 hr at pH 8.1 and deproteinization with $HClO_4$, AcCoA could be shown to be present in the supernate from the MDH incubation but not in that from the CS incubation (Table 3). After correction for the spontaneous hydrolysis of AcacCoA to CoA and acetoacetate, the amount of AcacCoA that disappeared was half the amount of AcCoA found, as is required by the thiolase reaction. The amount of AcCoA found increased with increasing concentrations of MDH in the incubation mixture.

If MDH is to be used in reactions like the one described where thiolase might interfere, we feel that the presence of this contaminant should be checked. This is the more urgent since we found that the amount of thiolase in different lots of MDH varied considerably and, moreover, diminished with time. The same problems occurred when AcCoA determinations were made in suspensions of homogenates or cell fractions of rat liver. Usually, AcacCoA concentrations in these tissue preparations are sufficiently low to permit an undisturbed AcCoA assay. In in vitro studies of lipid or ketone body metabolism, however, AcacCoA may accumulate or may have been added on purpose. In such studies CoA is generally present, and this favors the disturbing thiolase reaction.

Inclusion of iodoacetamide in the reaction medium as a standard procedure is not recommended, as in cases of severe contamination of MDH by thiolase even a concentration of 3.75 mM of this substance is not sufficient to inhibit the thiolase reaction completely, and it also affects the AcCoA assay (Table 2). It seems advisable to select a proper lot of MDH or to remove AcacCoA from the assay medium by means of 3-hydroxyacyl CoA dehydrogenase (EC 1.1.1.35) and NADH as in the AcacCoA assay (5). The addition of NADH, however, prevents the use of the equation given in the legend to Fig. 1 to calculate the amount of AcCoA.

TABLE 2. Influence of iodoacetamide on the amount of AcCoA found in the presence and the absence of AcacCoA

			AcCoA Found			
			MDH 14 U/ml, CS 1.7 U/ml		MDH 1.4 U/ml, CS 0.09 U/ml	
AcCoA	AcacCoA	Iodoacetamide	30 min	60 min	30 min	60 min
mM	mM	mM	nmoles/ml			
0.046			46	49	43	47
0.046		3.75	43	43	38	39
0.046	0.13		246	252	60	80
0.046	0.13	3.75	79	89	41	43
			2	2	2	3
		3.75	2	2	3	4
	0.13		49	199	8	14
	0.13	3.75	10	13	4	5

AcCoA assay conditions were as in Fig. 1, except for the concentrations of MDH and CS and the time after which ΔE_2 was measured, which were as indicated.

TABLE 3. Formation of AcCoA from AcacCoA by a CS and an MDH preparation

CS	MDH	(a) AcacCoA Added	(b) AcacCoA Recovered	(c) Acetoacetate Formed	(d) AcacCoA Not Recovered in Columns b and c	(e) AcCoA Formed × 0.5
U/ml	U/ml			%		
		100	61	35	4	5
3		100	60.5	36.5	3	2
	0.7	100	55	33.5	11.5	11.5
	1.4	100	50	32	18	17
	2.8	100	40	31.5	28.5	26
	5.5	100	15	28	57	52
	11.0	100	3	24	73	63
	22.0	100	2.5	17	80.5	65

Incubation of AcacCoA (0.25 mM, equivalent to 100%, column *a*) for 1 hr with CS or MDH in a medium containing 0.05 M Tris buffer, pH 8.1, 5 mM $MgCl_2$, and 0.25 mM CoA. Total volume, 5 ml. Figures in columns *b* and *c* were calculated from the decrease in absorbance of added NADH after addition of 3-hydroxyacyl CoA dehydrogenase (5) and 3-hydroxybutyrate dehydrogenase (EC 1.1.1.30) (6), respectively. Figures in column *d* were obtained by subtracting those in columns *b* plus *c* from 100%. Figures in column *e* were obtained by the AcCoA assay (see Fig. 1).

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