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Internal standards in the estimation of acetyl-CoA in liver extracts

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SUMMARY The necessity for adding an internal standard to liver extracts during fluorimetric estimation of acetyl-CoA by the malic dehydrogenase-citrate synthase reaction is demonstrated. Addition of acetyl-CoA completely compensates for the inhibitory action of some tissue components. Values for hepatic acetyl-CoA in fed and fasted rats are given.

KEY WORDS acetyl-CoA · fluorimetric assay · rat · liver · fasting

APPRECIATION THAT the "steady-state" concentration of acetyl-CoA in tissues may influence the traffic along alternative metabolic pathways (1–3) has stimulated efforts to obtain measurements of this metabolite. Most have been based on the citrate synthase reaction coupled to the reduction of NAD, as proposed by Wieland and Weiss (2).

$$\text{Malate} + \text{NAD}^+ \rightleftharpoons \text{NADH} + \text{oxaloacetate}$$
$$\text{Oxaloacetate} + \text{acetyl-CoA} \rightarrow \text{citrate} + \text{CoA} \quad (4)$$

However, Pearson has emphasized (5) that the coupled assay may seriously underestimate acetyl-CoA since reduction of NAD will be equivalent to the formation of citrate only if the concentration of oxaloacetate is low with respect to that of NADH prior to the addition of citrate synthase. He has derived a curve relating the amount of NAD reduced to the actual amount of acetyl-CoA reacting in the system, and has suggested that the published values for acetyl-CoA should be corrected appropriately.

To offset the limitations of partial reactions and arbitrary "correction factors" we have applied the principle of "internal standards," as recommended by Estabrook and Maitra (6) for the enzymatic estimation of many other metabolites. Internal standards have enabled us to measure acetyl-CoA in tissue extracts directly, despite failure of the citrate synthase reaction to go to completion and variable inhibitions by other tissue components.

Methods. Studies were performed with rats (240–280 g; Charles River Farm Breeding Laboratory, Brookline,

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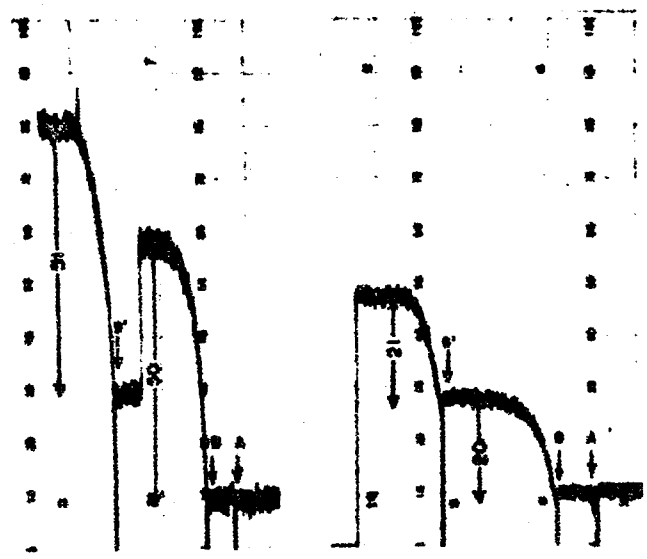

FIG. 1. Reaction of citrate synthase with internal standard (acetyl-CoA) alone. *A* denotes addition of citrate synthase (50 μg); *B* and *B'* denote repetitive additions of acetyl-CoA (2.58 mμmoles). Total volume, 3 ml.

Mass.) fed with Purina Chow, and permitted unlimited access to water when fasted. Animals were stunned by a blow on the head, and the livers were excised in toto and introduced into liquid N_2 in less than 18 sec. Portions of frozen livers were ground in a mortar that was continuously cooled with liquid N_2, and powdered aliquots were weighed by introducing them into tared beakers containing chilled 6% $HClO_4$. Thereafter, the mixtures (in proportions of 3 ml of 6% $HClO_4$ per g of frozen powder) were homogenized in chilled, ground glass homogenizers. Homogenates were centrifuged for 15 min at 4°C (100,000 *g*; Beckman Spinco model L-2) to remove the turbidity due to glycogen. Aliquots of clear supernate were neutralized in an ice bath to pH 6.8 with 1.6 N KOH and sufficient K_2HPO_4-KH_2PO_4 buffer (pH 6.8) to yield 0.05 M buffer in the final mixture. The solutions were allowed to settle for 30 min prior to removal of precipitated $KClO_4$ by 10 min of centrifugation (800 *g*; International Centrifuge PR-2).

Assay for acetyl-CoA was performed on the same day that the animals were killed. An Eppendorf photometer with a fluorimeter attachment (Netheler and Hinz, Hamburg, Germany) was employed. A Haake thermostat for maintaining constant temperature in the cuvette holders and a Honeywell recorder (Electronik 18) for recording small changes in fluorescence were attached to the fluorimeter. A filter of 313 + 366 mμ was utilized to give a mercury vapor line as the source of activating light; emitted light was filtered by the 380–3,000 mμ filter accompanying the instrument.

The following reaction mixture, in a final volume of 3 ml, was introduced into each cuvette: 0.2 μmole of NAD; 0.2 μmole of sodium malate; 0.5 μmole of HCl-Tris buffer (pH 8.0); 100 μg of malic dehydrogenase; and the extract from 50–150 mg of fresh, powdered liver. All chemicals were obtained from Sigma Chemical Co. (St. Louis, Mo.) and enzymes from Boehringer und Soehne (Mannheim, Germany; distributed by Calbiochem, Los Angeles, Calif.). Cuvettes were maintained at 34°C in the fluorimeter, and fluorescence was recorded continuously after appropriate adjustment of voltages and sensitivities to provide a measurable range of deflections. 50 μg of citrate synthase (EC 4.1.3.7) was added when the malic dehydrogenase reaction had come to equilibrium, and the subsequent increment in fluorescence was recorded until a new plateau had been established. Internal standards were then employed to translate the changes in fluorescence to absolute values, as follows. To each cuvette, 25 μl of a standard solution of acetyl-CoA (i.e., usually equivalent to about 2.5 mμmoles of acetyl-CoA) was added, and the augmentation of fluorescence was noted. When a new plateau had been reached, we added another 25 μl of the acetyl-CoA standard to establish that the initial addition of internal standard had not disrupted any of the rate-limiting equilibria in the coupled assay. The unknown amount of acetyl-CoA in the tissue extract was calculated by ratio. The concentrations of acetyl-CoA in standard solutions were verified each day by addition of phosphotransacetylase to concentrated stock solutions in the presence of potassium arsenate and determination of the decrease of optical density at 232 mμ (7).

Results and Discussion. Representative fluorimeter tracings with internal standards in the coupled assay are shown in Figs. 1 and 2. Fluorescence was not affected when citrate synthase was added to simple reaction mixtures of malate, NAD, and malic dehydrogenase (Fig. 1). However, the presence of excess malate and NAD profoundly influenced the increase in fluorescence caused by the subsequent addition of acetyl-CoA. As shown in Fig. 1, 2.58 mμmoles of acetyl-CoA elicited less than half as much of an increment in fluorescence in reaction mixtures containing 0.4 rather than 0.2 μmole of malate and NAD. Fluorimetric responses to added acetyl-CoA could also be reduced by "loading" the system with NADH; equilibrium was reached more slowly under such circumstances.

The above phenomena may have contributed to the variable increases in fluorescence that were observed when constant quantities of acetyl-CoA were introduced as internal standards in the presence of tissue extracts.

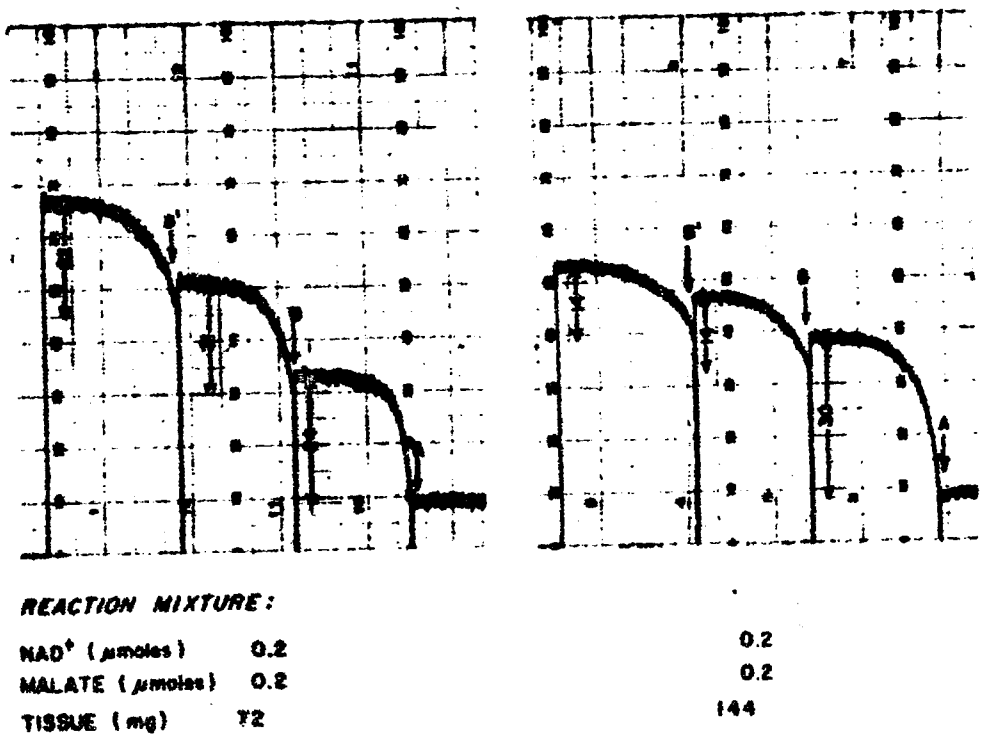

FIG. 2. Effect of variation in tissue mass on the fluorimetric response to acetyl-CoA. Tissue extract equivalent to 72 or 144 mg of fresh liver from a fed male rat was included in the reaction mixture. *A*, *B*, *B'* denote additions of citrate synthase and acetyl-CoA internal standards as in Fig. 1.

or the liver of any one animal, the magnitude of response to the internal standard decreased as the amount of tissue extract included in the reaction mixture increased (Fig. 2). However, this did not compromise analytical accuracy; simple proportional calculation gave, for the two halves of this experiment, 40.0 and 39.8 mμmoles/g of liver, respectively.

Further efforts were directed towards defining optimum conditions for the preparation of tissue extracts. Whole livers from one set of animals were rapidly excised and then frozen in liquid N_2 as described under Methods, while livers from a second set were frozen in situ by immersion of individual lobes in aluminum cups that were filled continuously with liquid N_2. Samples frozen by both techniques were broken into small pieces and either homogenized directly in chilled perchloric acid or after first being pulverized in mortars as described in Methods. Values for tissue levels of acetyl-CoA were not significantly different for excision-freezing as opposed to freezing in situ, but the subsequent handling of frozen pieces proved to be critical. About 60% of the acetyl-CoA in tissues was lost when frozen pieces were homogenized without preliminary pulverization. The loss presumably can be ascribed to destruction of intracellular acetyl-CoA during the thawing of the pieces before the perchloric acid had penetrated throughout, since the recovery of extracellular acetyl-CoA, added to frozen tissue prior to homogenization and extraction, ranged from 97.2 to 102.1%.

TABLE 1 EFFECT OF FASTING* ON "STEADY-STATE" CONCENTRATIONS OF ACETYL-CoA IN RAT LIVER

	Days of fasting		
	0	1	2
n	5	5	5
Body weight, g	264.0 ± 6.2	238.4 ± 5.2†	219.8 ± 6.3‡
Liver weight, g	10.6 ± 0.3	7.2 ± 0.5§	6.8 ± 0.3§
Acetyl-CoA: mμmoles/g liver (wet wt)	32.9 ± 3.2	47.0 ± 1.9†	52.7 ± 4.8‡
mμmoles/liver	348.7 ± 33.9	338.4 ± 13.7	358.4 ± 32.6

* Male rats were deprived of food and permitted unlimited access to water.

Findings after 1 or 2 days of fasting are compared to values in fed animals; n denotes number of animals in each category. Significant changes are denoted by:

† ($P < 0.05$);
‡ ($P < 0.01$);
§ ($P < 0.001$).

In view of the above, excision-freezing and pulverization of tissues were incorporated into the standard analytical technique. The procedure was applied to measure "steady-state" concentrations of acetyl-CoA in the livers of male rats.[1] Hepatic concentrations in fed animals (Table 1) increased significantly, after 1 or 2

[1] Garland, Shepherd, and Yates have reported that acetyl dephospho CoA may be included spuriously in the estimation of acetyl-CoA by the citrate synthase reaction (8), but since they were unable to demonstrate any acetyl dephospho CoA in liver the objection does not seem to be relevant to this tissue.

days of starvation, but since liver weights were significantly reduced during this same interval, total hepatic acetyl-CoA was not altered significantly by fasting.

Previously published estimates of acetyl-CoA concentrations in the livers of fed rats have ranged from 17.2 to 35 mμmoles/g wet weight (2, 3, 9); the present findings are therefore in concord with the higher values of others.

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