

## INHIBITION OF CHOLESTEROL SYNTHESIS BY (–)-HYDROXYCITRATE IN PERFUSED RAT LIVER. EVIDENCE FOR AN EXTRAMITOCHONDRIAL MEVALONATE SYNTHESIS FROM ACETYL COENZYME A

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### 1. Introduction

(–)-Hydroxycitrate, an inhibitor of citrate cleavage enzyme (EC 4.1.3.8) [1] inhibits fatty acid synthesis in rat liver [2]. This effect is likely to result from a shortage of cytoplasmic acetyl-CoA. Whereas fatty acid synthesis is considered an extramitochondrial process, formation of HMG-CoA serving as precursor of mevalonate may be catalyzed by mitochondrial HMG-CoA synthase (EC 4.1.3.5) or by an extramitochondrial pathway, e.g. [3].

In this paper an inhibition of hepatic cholesterol and fatty acid synthesis by (–)-hydroxycitrate in the isolated perfused rat liver is reported; acetate reversed the effect on fatty acid synthesis. In cell-free extracts of rat liver (–)-hydroxycitrate is shown not to influence cholesterol formation itself but to be a potent stimulator of fatty acid from  $^{14}\text{C}$ -acetate.

These experiments are taken as evidence of a physiologically important extramitochondrial pathway from acetyl-CoA to mevalonate in rat liver.

### 2. Methods

For measuring fatty acid [4] and cholesterol synthesis by the  $^3\text{HOH}$ -method, livers of male Buffalo rats (150 g) were perfused as described extensively elsewhere [5]. The medium contained in 100 ml Krebs–Henseleit buffer: 2.5 g albumin reconst (Behringwerke AG, Frankfurt/Main, West Germany), 0.55 mmole glucose, 4 mg ampicillin and amino acids corresponding to steady state levels observed in liver

perfusions [6]. Assays with cell-free extracts were performed according to [7]. ATP was determined after freeze-clamping of the perfused liver [8], citrate in deproteinized homogenates [9], and acetoacetate plus 3-hydroxybutyrate were analyzed in the perfusion medium [10]. Samples of (–)-hydroxycitric acid lactone were kindly supplied by Dr. Y.S. Lewis, Mysore, India, and by Dr. H. Lengsfeld (Hoffman-La Roche, Basel, Switzerland). The lactone was hydrolyzed according to [2] before use.

### 3. Results

#### 3.1. Perfusion experiments

Fatty acid and cholesterol synthesis were inhibited in a similar manner by different (–)-hydroxycitrate concentrations (fig. 1). As *in vivo* [2], this compound appears to only partially inhibit fatty acid synthesis in the perfused organ.

ATP levels of liver tissue perfused for 60 min with different concentrations of (–)-hydroxycitrate remained in the control range. In the presence of 0.44, 1.1, and 2.2 mM (–)-hydroxycitrate, ATP contents of  $2.8 \pm 0.9$ ,  $2.61 \pm 0.49$ , and  $2.65 \pm 0.07$   $\mu\text{moles/g}$  wet weight ( $\pm\text{S.D.}$ ), respectively, were found as compared to  $2.88 \pm 0.41$  at the end of the control periods. Ketone body production (acetoacetate plus 3-hydroxybutyrate) by perfused liver was not changed in the presence of 1.1 mM (–)-hydroxycitrate. It amounted to  $8.35 \pm 5.15$   $\mu\text{moles/g}$  liver, wet weight  $\times$  hr ( $\pm\text{S.D.}$ ) as compared to  $9.25 \pm 4.8$   $\mu\text{moles/g}$  liver, wet weight  $\times$  hr in control experiments.

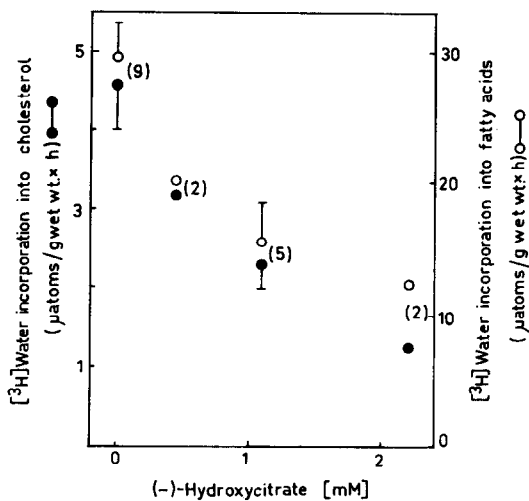


Fig. 1. Influence of (-)-hydroxycitrate on the rate of cholesterol and fatty acid synthesis in perfused rat liver. 40 min after operation (9:00 hr)  $^3\text{H}$ -water was added to the perfusion medium (final specific activity 15,000 dpm/ $\mu$ atom hydrogen). 30 min thereafter a control liver sample was taken. (-)-Hydroxycitrate was added and perfusion continued for another 60 min, after which 2 additional liver samples were taken and saponified. The non-saponifiable fraction was washed twice ( $\text{H}_2\text{O}$ ) and treated with digitonine (cholesterol). The fatty acids were extracted after acidification and washed with  $\text{H}_2\text{O}$  (1X) and 0.2 N  $\text{CH}_3\text{COOH}$  in 20% ethanol (2X). Ordinates: Controls represent averages of all control periods. The other values have been calculated from the increments of  $^3\text{H}$ -incorporation during the (-)-hydroxycitrate periods.  $^3\text{H}$ -incorporation into lipids was previously shown to be linear during 90 min. Vertical bars, S.E.M.

### 3.2. Acetate effect

Studies on the subcellular localization of acetyl-CoA synthetase (EC 6.2.1.1) revealed that acetate is a citrate-independent precursor of cytoplasmic acetyl-CoA [11]. Acetate uptake by the perfused rat liver amounted to about 50  $\mu$ moles/g liver, wet weight  $\times$  hr, if its medium concentration was higher than 10 mM [5].

Fig. 2 shows that the simultaneous addition of (-)-hydroxycitrate and 10 mM acetate increased fatty acid synthesis to 150% of the uninhibited rate. The reversion by acetate indicates that (-)-hydroxycitrate inhibition of fatty acid synthesis is mediated by a shortage of cytoplasmic acetyl-CoA. On the other hand, the inhibition of cholesterol synthesis was not relieved by acetate addition (fig. 2). Therefore, an

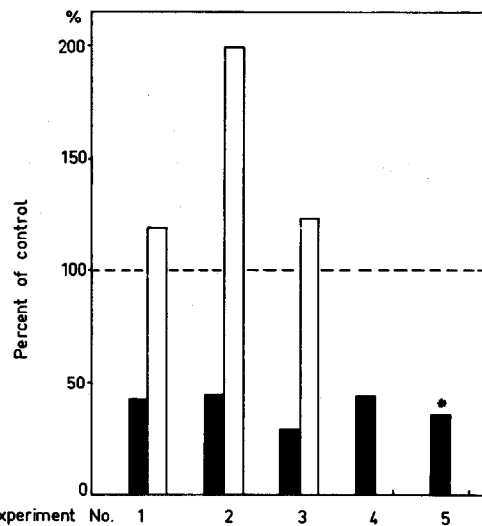


Fig. 2. Influence of acetate on (-)-hydroxycitrate-inhibition of cholesterol and fatty acid synthesis in perfused rat liver. Experimental design as in fig. 1. At 30 min, 10 mM acetate and 1.1 mM (-)-hydroxycitrate were added to the perfusion medium. This concentration of acetate was shown not to influence  $^3\text{H}$ -incorporation into liver lipids [5]. The synthetic rates after acetate  $\pm$  hydroxycitrate addition (30–90 min) are shown as percentages of control periods (0–30 min). White columns: fatty acid synthesis; black columns: cholesterol synthesis. \*20 mM acetate.

additional effect of (-)-hydroxycitrate on cholesterol synthesis had to be considered.

### 3.3. Experiments with cell-free extracts

Addition of (-)-hydroxycitrate to a 10,000 g supernatant of rat liver increased the rate of  $1\text{-}^{14}\text{C}$ -acetate incorporation into fatty acids considerably with a concomitant slight inhibition of its incorporation into cholesterol. If fatty acid synthesis was lowered by avidin to subnormal rates, however, formation of labeled cholesterol was not inhibited by (-)-hydroxycitrate (fig. 3). This was corroborated in other experiments where  $1\text{-}^{14}\text{C}$ -acetate incorporation into cholesterol in the presence of both 1.33 mM (-)-hydroxycitrate and 1 U/ml avidin ( $7470 \pm 168$  dpm/assay) was the same as in the controls ( $7776 \pm 211$  dpm/assay) ( $n = 4$ ; mean  $\pm$  S.E.M.).

Previous dialysis of the homogenate did not change the response of fatty acid synthesis to (-)-hydroxycitrate (fig. 3). As less than 0.01 mM citrate was

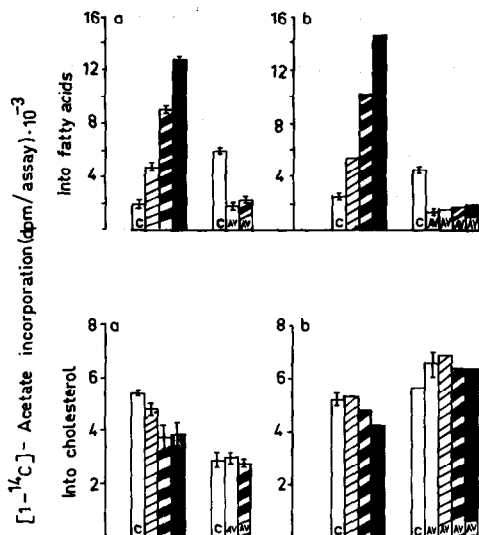


Fig. 3. Influence of (-)-hydroxycitrate and avidin on  $1\text{-}^{14}\text{C}$ -acetate incorporation into cholesterol and fatty acids by 10,000 g supernatant of rat liver. In each of the 4 experiments, livers from 2 male Wistar rats (200 g) were forced through a sieve. Homogenization: 6 strokes (300 rpm) in a loose-fitting Potter-Elvehjem homogenizer with 2.5 vol/g, wet weight of a medium, containing 81 mM potassium phosphate, pH 7.4; 5 mM  $\text{MgCl}_2$ ; 37 mM nicotinamide. Assay mixture, see Methods. Start by addition of 10 mM  $1\text{-}^{14}\text{C}$  acetate (specific activity 150,000 dpm/ $\mu\text{mole}$ ) after gassing 10 min at  $0^\circ$  with 95%  $\text{O}_2$ :5%  $\text{CO}_2$ . For analytical methods see fig. 1. Columns: AV denotes 10 min preincubation with 1 U/ml avidin.  $\square$ : controls without additions. Addition of (-)-hydroxycitrate: 0.53 mM,  $\text{▨}$ ; 1.33 mM,  $\text{▩}$ ; 2.66 mM,  $\blacksquare$ . Vertical bars: range of duplicate assays. a) 10,000 g supernatant; b) supernatant dialysed twice (30 min each) against 500 ml of the above medium + 20 mM cysteine.

present at 0 and 60 min of the incubation period, it is concluded that (-)-hydroxycitrate rather than endogenous citrate is responsible for the stimulatory effect.

#### 4. Discussion

The similarity of the (-)-hydroxycitrate effect on cholesterol and fatty acid synthesis is suggestive of a common mechanism of inhibition, e.g. lowering of the cytoplasmic acetyl-CoA level. This assumption requires that both effects be abolished by replenishment of the acetyl-CoA pool by exogenous acetate. While this was

verified for fatty acid synthesis,  $^3\text{H}$ -incorporation into cholesterol was not normalized by acetate addition. Experiments with cell-free extracts, however, demonstrated that  $^{14}\text{C}$ -acetate incorporation into cholesterol was not inhibited if acetyl-CoA consumption by fatty acid synthesis was blocked by avidin (fig. 3). Moreover, they revealed a considerable stimulation of fatty acid synthesis by (-)-hydroxycitrate; this effect may be explained most readily by an activation of acetyl-CoA carboxylase (EC 6.4.1.2) as known from other tricarboxylates [12]. The (-)-hydroxycitrate stimulation of fatty acid synthesis was also observed in the perfused liver if acetate was added (fig. 2). It appears that the stimulated fatty acid synthesis drains acetyl-CoA generated by acetyl-CoA synthetase off from other acetyl-CoA requiring reactions in the cytoplasm, e.g. mevalonate synthesis.

The reported effect of (-)-hydroxycitrate on cholesterol synthesis cannot be explained by an impairment of intramitochondrial acetyl-CoA metabolism including HMG-CoA synthesis because ketone body production was found unchanged.

These observations suggest that acetyl-CoA must be available in the cytoplasm for an unimpaired cholesterol synthesis in rat liver; this implies the existence of an extramitochondrial pathway from acetyl-CoA to mevalonate. The fact that cholesterol synthesis was not inhibited by avidin nor stimulated by (-)-hydroxycitrate does not support a pathway involving the known acetyl-CoA carboxylase reaction.

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