# INHIBITION OF MAMMARY GLAND ACETYL CoA CARBOXYLASE BY FATTY ACIDS

H. Richard Levy*

The Ben May Laboratory for Cancer Research, University of Chicago, Chicago, Illinois

Received September 3, 1963

It has been suggested that accumulation of milk in the lactating rat mammary gland following weaning may contribute toward the rapid cessation of fatty acid synthesis which ensues (Levy, 1963a; 1963b). The strong inhibition of fatty acid synthesis in rat mammary extracts produced by the addition of small quantities of rat milk provides support for this concept (Levy, 1963a; 1963b). Examination of acetyl CoA carboxylase in mammary extracts revealed that this enzyme was markedly inhibited by rat milk (Levy, 1963b). The inhibitory activity was located entirely in the residue ($R_{105}$) obtained upon centrifuging the milk at 105,000 x g; none was found in the fat layer or in the clear supernatant solution (Levy, 1963b). This communication describes attempts to elucidate the nature of the inhibitor and the mechanism of inhibition of acetyl CoA carboxylase.

Experimental. The abdominal and inguinal mammary glands were excised from primiparous Sprague-Dawley rats 15-20 days post-partum. All subsequent operations were performed at approximately 4°. The glands were homogenized in a Waring blendor for 1 minute with 2 volumes of 0.1 M potassium phosphate, pH 7.3, containing 7.0 mM $\beta$-mercaptoethanol. After removal of most of the fat and debris by centrifugation at 2000 x g, the extracts were further clarified by centrifuging at 20,000 x g for 30 minutes. Crude acetyl CoA carboxylase was prepared from the supernatant solution ($Sp_{20}$) by dissolving the protein, which precipitated upon adding

*Present address: Biological Research Laboratories, Department of Bacteriology and Botany, Syracuse University, Syracuse, New York.

ammonium sulfate to 50% of saturation, in 0.05 M potassium phosphate pH 6.5 containing 7.0 mM $\beta$-mercaptoethanol, and dialyzing against this same buffer for 3 hours. The dialyzed enzyme was clarified by centrifuging at 20,000 x g, or at 105,000 x g, or by freezing, thawing and recentrifuging at 20,000 x g. The last two procedures gave enzyme preparations with the highest activity. The enzyme was stable for several weeks if kept frozen. The assay procedure was based on that of Vagelos et al (1963). The enzyme (1-7 mg of protein, determined by the method of Warburg and Christian (1941) ) was subjected to a 15 minute prior incubation at 37° in a solution containing 40 µmoles of potassium phosphate, pH 6.5; 10 µmoles of potassium citrate (except where noted); and 1.4 µmoles of $\beta$-mercaptoethanol, in a volume of 0.6 ml. The reaction was then initiated by adding 0.4 ml of a solution containing 10 µmoles of ATP; 4 µmoles of $MnCl_2$; 25 µmoles of $NaHC^{14}O_3$ (0.075 mc/mmol); and 0.15 µmoles of acetyl CoA. Acetyl CoA was omitted from the controls. After incubation for 15 minutes at 37°, the reaction was halted with 0.1 ml of 5 N perchloric acid, followed by 0.2 ml of 2 N KCl; precipitated protein and perchlorate were centrifuged off and an aliquot of the clear supernatant solution plated on steel planchets, evaporated to dryness, and counted in a windowless gas-flow counter. The reaction product was tentatively identified as malonyl CoA by paper chromatography in an ethanol-sodium acetate system (modified from Pabst Laboratories Circular OR-17, 1961). Thiolesters were visualized with nitroprusside and methanolic sodium hydroxide (Stadtman, 1957). Support for the identity of the product was obtained by chromatographing its alkaline hydrolysis product (Vagelos, 1960) in a glacial acetic acid-ether-water system (Dennison and Phares, 1952). Acids were visualized with bromocresol green and radioactive spots were located with the aid of a paper chromatogram scanner. A single radioactive spot was found which accounted for over 90% of the radioactivity of the applied sample; its $R_f$ was identical to that of malonic acid. The specific activity of $NaHC^{14}O_3$

was determined by plating suitably diluted aliquots on steel planchets containing excess barium hydroxide, drying, and counting in the gas-flow counter.

Results. When a twice-washed $R_{105}$ fraction from rat milk was extracted five times with acetone at room temperature, the clarified extract contained most of the inhibitory activity. Thus, in one experiment the following quantities of $C^{14}O_2$ were incorporated when the indicated additions were made: 43.9 (control); 29.0 (0.1 ml of rat milk); 23.9 ($R_{105}$ from 0.1 ml of rat milk); 20.5 (acetone extract from the same quantity of $R_{105}$; solvent removed by evaporation). Pressman and Lardy (1956) found fatty acids in acetone extracts of rat liver microsomes. When an acetone extract of rat milk $R_{105}$ was methylated by the procedure of Stoffel et al (1959) and then subjected to gas liquid chromatography, methyl esters of the following acids were identified by their retention time: lauric, myristic, palmitic and stearic. This suggested that the inhibition of acetyl CoA carboxylase might result from the presence of certain unesterified fatty acids, bound to particles in the milk. The effect of free fatty acids upon acetyl CoA carboxylase was therefore examined. Table 1 shows that capric, lauric, and myristic acids were potent inhibitors of this enzyme. It must be emphasized, however, that the difficulty in maintaining long-chain fatty acids in solution renders the lack of inhibition found with these acids of doubtful significance. The procedure used in experiment 2 (Table 1) proved more effective in this respect, which may account for the greater inhibition seen with myristic and palmitic acids in this experiment, compared to experiment 1b, although the quantities added were smaller.

The stimulation of latent ATPase by fatty acids observed by Pressman and Lardy (1956) does not seem to be the cause for the inhibition of $C^{14}O_2$ incorporation. ATPase was assayed by measuring phosphate liberation using King's modification (King, 1932) of the method of Fiske and

Subbarow (1925) under conditions used in the acetyl CoA carboxylase assay, but substituting tris-acetate for phosphate buffer (which had no substantial effect upon carboxylase activity). No ATPase was detected, either in the absence or in the presence of 0.5 mM caprate. Since the enzyme preparations used were free of mitochondria, no effect upon mitochondrial swelling is involved. Biotin did not protect against fatty acid inhibition.

TABLE 1

Inhibition of Acetyl CoA Carboxylase by Fatty Acids

| Fatty Acid Added | Experiment 1a* | | Experiment 1b* | | Experiment 2* | |
|---|---|---|---|---|---|---|
| | mμmoles $C^{14}O_2$ incorporated | % | mμmoles $C^{14}O_2$ incorporated | % | mμmoles $C^{14}O_2$ incorporated | % |
| None | 43.9 | 100 | 43.9 | 100 | 70.1 | 100 |
| Butyric | 48.3 | 110 | 46.9 | 107 | 69.2 | 98 |
| Caproic | 45.6 | 104 | 46.8 | 106 | 69.7 | 99 |
| Caprylic | 43.9 | 100 | 7.57 | 17 | 44.4 | 63 |
| Capric | 29.8 | 68 | 0.88 | 2.0 | 1.10 | 1.6 |
| Lauric | 17.1 | 39 | 0.59 | 1.3 | 0.96 | 1.4 |
| Myristic | 38.6 | 88 | 31.6 | 72 | 0.67 | 1.0 |
| Palmitic | 41.7 | 95 | 40.7 | 93 | 46.8 | 67 |
| Stearic | 46.5 | 106 | 47.6 | 109 | 68.7 | 98 |

* Concentration of fatty acids, if completely dissolved (see text): experiment 1a, 0.5 mM; 1b, 5.0 mM; 2, 2.0 mM. Fresh $Sp_{20}$ was used for experiments 1a and 1b; the same enzyme, kept frozen for 1 week, thawed, and clarified by centrifuging at 20,000 x g was used in experiment 2. In experiments 1a and 1b, fatty acids were added to the incubation tubes as petroleum ether solutions, the solvent evaporated, and the other components then added. In experiment 2, the acids were added as potassium salts in aqueous solution and were completely clear when added. Subsequent addition of the other reaction components led to some precipitation of the long chain fatty acids.

That the inhibition by fatty acids may involve interference with activation of acetyl CoA carboxylase by citrate or other di- and tricarboxylic acids (Martin and Vagelos, 1962; Waite and Wakil, 1962), is suggested by the experiments shown in Table 2. In experiment 1,

high concentrations of citrate were able, partly, to overcome the inhibitory effect of caprate. Experiments 2 and 3 show that preincubation of enzyme with citrate or malonate gave considerable protection against caprate inhibition. Succinate, $\alpha$-ketoglutarate, and DL-malate also provided protection but, as demonstrated by Martin and Vagelos (1962) and Waite and Wakil (1962), these acids were less effective enzyme activators.

TABLE 2

Effects of Caprate and Activating Acids on Acetyl CoA Carboxylase

| Experiment No.* | Activator µmoles/ml | $C^{14}O_2$ incorporated, mµmoles A. No Caprate | B. 0.5 mM Caprate | $\frac{B}{A} \times 100$ |
|---|---|---|---|---|
| 1 | None | 1.26 | - | - |
| | Citrate, 5 | 38.9 | 6.77 | 17 |
| | 10 | 46.1 | 13.0 | 28 |
| | 50 | 48.7 | 16.1 | 33 |
| 2 | None | 0.51 | 0.80 | 157 |
| | Citrate, 10 | 12.8 | 4.08 | 32 |
| | Malonate, 10 | 2.72 | 0.56 | 21 |
| 3 | None | 0.85 | 0.56 | 66 |
| | Citrate, 10 | 20.2 | 14.8 | 73 |
| | Malonate, 10 | 4.34 | 3.17 | 73 |

*In experiment 1 both citrate and caprate were present in the prior incubation. In experiment 2 caprate was present in the prior incubation and citrate or malonate were added with substrates, whereas in experiment 3 the activator was present in the prior incubation and caprate was added with substrates. The same enzyme was used for experiments 2 and 3; a different preparation was employed for experiment 1.

These data suggest that certain fatty acids may interfere with activation of acetyl CoA carboxylase by di- and tricarboxylic acids. The specificity of this effect and its relationship to the inhibition of fatty acid synthesis by milk is under investigation.

Acknowledgement. Gas-liquid chromatography was kindly performed by Mr. Laurence E. Frazier of the Department of Pathology, University of

Chicago. Excellent technical assistance was provided by Mr. Richard W. Harrington. Supported by grants from the United States Public Health Service and the American Cancer Society.

## REFERENCES

Dennison, F. W., Jr., and Phares, E. F., Anal. Chem., 24, 1628 (1952).

Fiske, C. H., and Subbarow, Y., J. Biol. Chem. 66, 375 (1925).

King, E. J., Biochem. J., 26, 292 (1932).

Levy, H. R., Federation Proc., 22, 363 (1963a).

Levy, H. R., Manuscript in preparation (1963b).

Martin, D. B., and Vagelos, P. R., J. Biol. Chem., 237, 1787 (1962).

Pressman, B. C., and Lardy, H. A., Biochim. et Biophys. Acta, 21, 458 (1956).

Stadtman, E. R., in S. P. Colowick and N. O. Kaplan (Editors), Methods in Enzymology, Vol. III, Academic Press Inc., New York, 1957, p. 931.

Stoffel, W., Chu, F., and Ahrens, E. H., Jr., Anal. Chem., 31, 307 (1959).

Vagelos, P. R., J. Biol. Chem., 235, 346 (1960).

Vagelos, P. R., Alberts, A. W., and Martin, D. B., J. Biol. Chem., 238, 533 (1963).

Waite, M., and Wakil, S. J., J. Biol. Chem., 237, 2750 (1962).

Warburg, O., and Christian, W. Biochem. Z., 310, 384 (1941).