THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 246, No. 3, Issue of February 10, pp. 629-632, 1971
Printed in U.S.A.

Effect of (−)-Hydroxycitrate on Fatty Acid Synthesis by Rat Liver *in Vivo**

(Received for publication, August 7, 1970)

JOHN M. LOWENSTEIN
From the Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02154

SUMMARY

Incorporation of ^{3}H from ^3H_2O was used to measure the rate of fatty acid synthesis in rat liver. (−)-Hydroxycitrate strongly inhibits fatty acid synthesis *in vivo*.

It was shown previously that (−)-hydroxycitrate[1] is a powerful inhibitor of citrate cleavage enzyme (1). The present paper shows that (−)-hydroxycitrate is a highly effective inhibitor of fatty acid synthesis by rat liver *in vivo*. Fatty acid synthesis was measured by the incorporation of tritium from water.

EXPERIMENTAL PROCEDURE

Methods—Rats were obtained from the Charles River Breeding Laboratories, North Wilmington, Massachusetts. Upon receipt the animals were placed on a scheduled diet consisting of Purina Laboratory chow (0.5-inch Checkers), Ralston Purina Company, St. Louis, Missouri, given to one animal per cage, from 9 a.m. to 12 noon. Water was made available *ad libitum*. The animals were kept in an artificially lighted room, with the light on from 6 a.m. to 6 p.m. The room temperature was 24°. Although rats are normally nocturnal feeders, the above schedule of light and food yielded very reproducible rates of fatty acid synthesis. I am indebted to Dr. O. Neal Miller for informing me in advance of publishing his own work that this regimen leads to much more reproducible rates of fatty acid synthesis than are obtained when the animals receive food *ad libitum*. After 7 to 10 days on the chow diet the rats were fasted for 45 hours—that is to say, they missed one scheduled feeding—and they were then fed a scheduled diet high in fructose or glucose (2) for 10 to 15 days. Weights of the animals at the time of use are indicated in the legends.

Unless otherwise indicated, experiments with the rats were started 3.5 to 4 hours after the animals started their last feeding schedule. When indicated rats were injected with 2 to 20 mM sodium (−)-hydroxycitrate, pH about 7.4, in amounts indicated in the legends. Controls received an equal volume of isotonic NaCl. Most of these injections were given intraperitoneally, but some administrations were made into the tail vein for comparison. After 45 min each rat was given 0.2 ml of a solution containing about 1 mCi of ^3H_2O by injection into a tail vein. The solution had a pH of about 7.4, and was made approximately isotonic by addition of sodium chloride. The rats were killed by decapitation 45 or 60 min after administering ^3H_2O, blood was collected, and the liver was excised quickly and weighed. Immediately thereafter the liver was subjected to one of two treatments. It was either dropped into 19 ml per g of liver of chloroform-methanol (3:1, by volume) and homogenized with a Sorvall Omni-Mixer homogenizer (an overhead type of blender), or it was wrapped into aluminum foil and pressed quickly between two blocks of Dry Ice. In the latter case the liver was stored at −20° and homogenized in chloroform-methanol at a later date. Duplicates of either 10 or 20 ml of the clear chloroform-methanol extract were evaporated to dryness and saponified with 2 ml of 5 N NaOH at 90° for 2 hours. Acidification and extraction were carried out as described previously (3). The extracted fatty acids were counted in 0.4% 2,5-diphenyloxazole and 0.01% 1,4-bis[2-(5-phenyloxazolyl)]-benzene in toluene. The specific radioactivity of the body water of each animal was determined by counting a suitably diluted aliquot of its plasma. Aqueous samples were counted in the scintillator described by Bray (4); the results so obtained were converted to the all toluene scintillator mentioned above. In several experiments the radioactivity of the nonsaponifiable fraction was examined. It contained less than 5% of the total radioactivity incorporated into fatty acids.

The ^3H_2O method for studying fatty acid synthesis described above is based on work of Fain and Scow (5). According to Jungas (6), the ^3H_2O method yields an average of 0.87 atom of ^{3}H incorporated per carbon atom incorporated into palmitate. The isotope effects, exchange reactions, and the metabolic pathways which lead to this particular fraction have been discussed in detail by Jungas (6), and need not be repeated here. Suffice it to say that the isotope effects and exchange reactions which occur are approximately constant and are not of practical consequence to the present paper. In what follows results are expressed as micromoles of ^3H_2O incorporated into long chain fatty acid per g of fresh weight of liver per hour. This can be converted to micromoles of acetyl group incorporated by dividing by 0.87 (or multiplying by 1.15).

Estimates of rates of fatty acid synthesis *in vivo* have been obtained in a number of ways. Most commonly, the rate of incorporation of a labeled precursor such as ^{14}C-acetate or ^{14}C-

* This work was supported by a grant from the National Science Foundation. It is Publication 751 from the Graduate Department of Biochemistry, Brandeis University. The technical assistance of E. Gorin in part of this work is acknowledged with thanks.

[1] (−)-Hydroxycitrate is $L_s L_g$-hydroxycitrate, or its equivalent *erythro*-L_s-hydroxycitrate.

This is an Open Access article under the CC BY license.

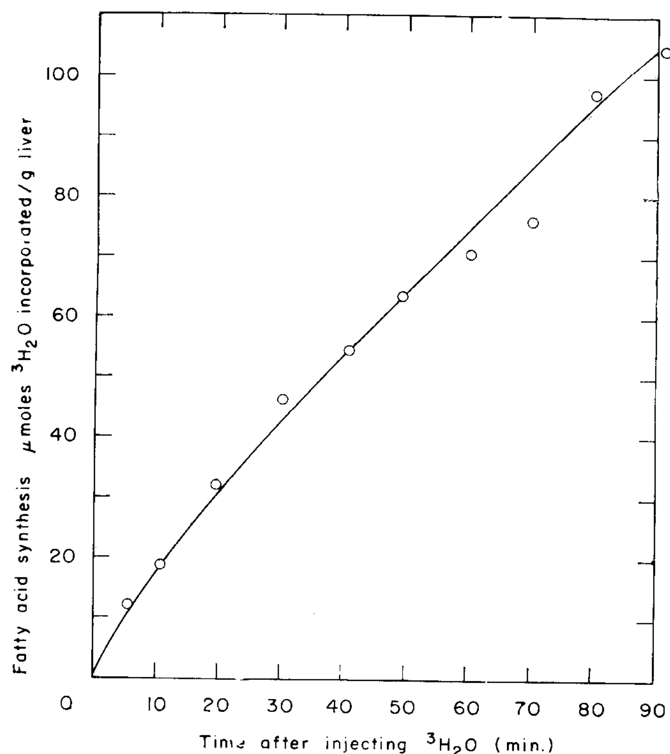

FIG. 1. Fatty acid synthesis by rat liver *in vivo* as a function of time. The animals received a scheduled diet high in glucose for 10 days as described under "Methods". The weight of the animals at the time of use was 148.2 g (S.E. = ±5.6, number of animals = 10). The results shown are from a single experiment. Each rat received 0.2 ml of ^3H_2O in isotonic NaCl solution into a tail vein 3.5 to 5 hours after starting the last feeding schedule.

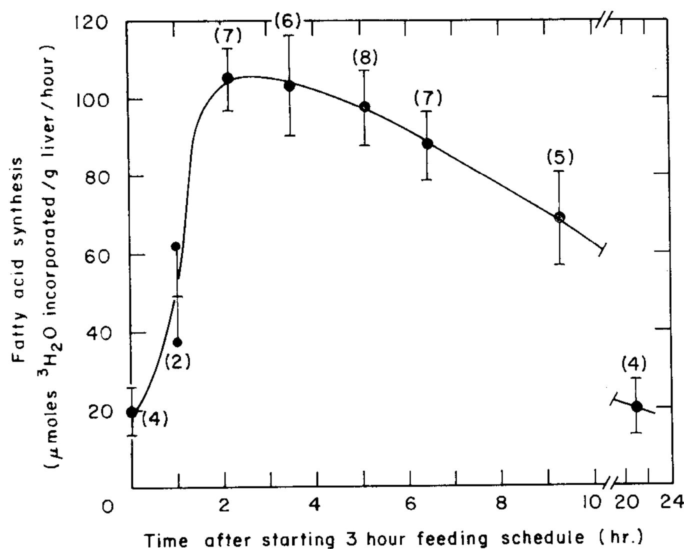

FIG. 2. Fatty acid synthesis by rat liver *in vivo* in relation to the start of feeding. The animals received a scheduled diet high in fructose as described under "Methods". The weight of the animals at the time of use was 87.4 g (S.E. = ±1.64, number of animals = 39). The results shown were obtained in four separate experiments over a period of 20 days. The points show the mean ±S.E. The number of animals in each group is in parentheses. (The *points* at 1 hour show the results obtained with two animals and the mean.)

glucose is measured. This approach is open to serious criticism because it provides only a measure of the rate at which a particular radioactive precursor is incorporated into fatty acids. This is a fraction of the total rate, a fraction which may vary depending on the pool size of the precursor in question and on the relative rates of removal of the precursor via other metabolic pathways. By far the best estimate of the total rate of fatty acid synthesis is obtained by the use of tritium-labeled water as radioactive precursor (4–8), since the incorporation of this precursor is largely independent of the source of the acetyl groups which are incorporated into fatty acids.

Materials—I am greatly indebted to Mr. Y. S. Lewis for a generous gift of (−)-hydroxycitric lactone. This material was prepared as described previously (9). The lactone was hydrolyzed to hydroxycitrate by heating with 3 eq of NaOH at 80° for 30 min.

RESULTS

In the experiment shown in Fig. 1 the rate of fatty acid synthesis in rat liver was measured 3.5 to 5 hours after starting the last feeding schedule. It is seen that the rate is proportional to time for at least 90 min after injecting ^3H_2O. The rate reaches a maximum within 2 hours after the start of the 3-hour feeding schedule. It remains at or near maximum for the following 3 hours and then slowly decreases (Fig. 2).

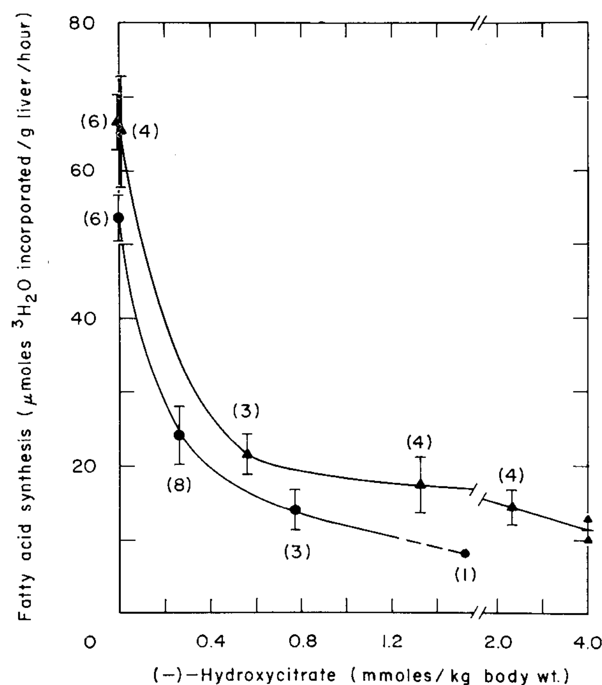

FIG. 3. Inhibition of fatty acid synthesis by (−)-hydroxycitrate. The inhibitor was given intraperitoneally 45 min before injecting ^3H_2O. Animals received a diet high in glucose (●) or fructose (▲) as described under "Methods". The weight of the animals at the time of use was 137.2 g (S.E. = ±4.3, number of animals = 18) and 158.8 g (S.E. = ±4.3, number of animals = 21) on the diets high in glucose and fructose, respectively. The *points* show the mean ±S.E. The number of animals per point is in parentheses. (The *point* (●) at 1.53 mmoles of hydroxycitrate per kg of rat represents a single animal; the *points* (▲) at 4.0 mmoles of hydroxycitrate per kg of rat represent two rats and the mean.)

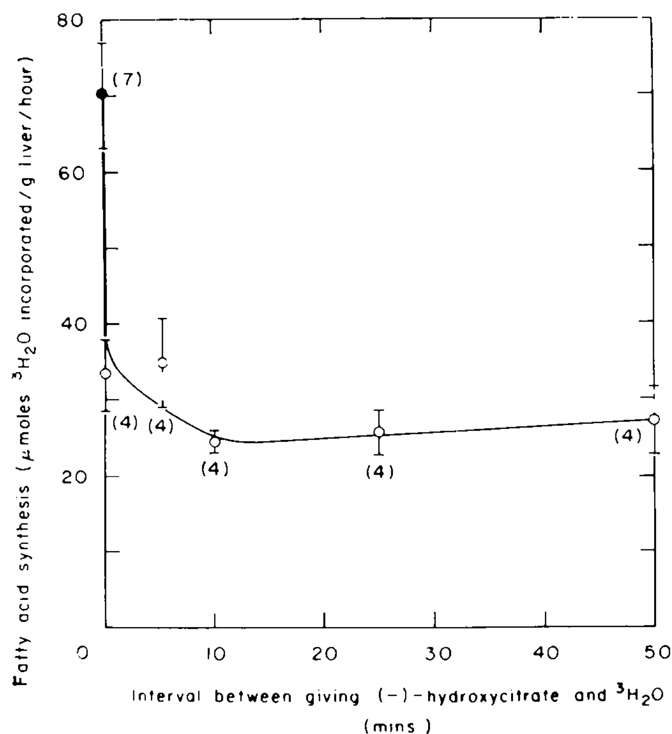

FIG. 4. Relation between rate of fatty acid synthesis in liver of rat and the time of intraperitoneal administration of (−)-hydroxycitrate (720 μmoles per kg of body weight). The animals received a scheduled diet high in glucose for 15 days as described under "Methods". The weight of the animals before their last meal was 145.2 g (S.E. = ±2.1 g, number of animals = 27). The results shown are from three separate experiments. The *points* show the mean, the standard error, and the number of animals per point. The *solid point* denotes control animals which received isotonic NaCl in place of (−)-hydroxycitrate. The *hollow points* show animals which received hydroxycitrate. In the case of animals which received inhibitor and ^3H_2O at zero time, the animals were first given (−)-hydroxycitrate intraperitoneally and then ^3H_2O intravenously within 30 to 60 sec.

Hepatic fatty acid synthesis is inhibited strongly by sodium (−)-hydroxycitrate. An intraperitoneal dose of as little as 0.1 mmole per kg of body weight inhibits fatty acid synthesis by 25 to 30%. This is equivalent to about 2.9 mg of hydroxycitrate per 150-g rat. Fifty per cent inhibition is obtained with 0.28 mmole per kg of body weight (Fig. 3). A possible explanation for the results shown in Fig. 3 is that hydroxycitrate causes the removal of newly synthesized fatty acid from the liver without inhibiting fatty acid synthesis. To test this possibility the carcass less the liver was analyzed for ^3H_2O incorporation into fatty acids in the presence and absence of (−)-hydroxycitrate. The results showed that fatty acid synthesis is inhibited to about the same extent in the carcass as in the liver.

The inhibiting effect of hydroxycitrate comes into play quite quickly, being at or near maximum within a matter of minutes after (−)-hydroxycitrate is given intraperitoneally (Fig. 4). In the experiment shown, fatty acid synthesis was measured over a period of 45 min. This suggests that immediately after giving hydroxycitrate the rate of fatty acid synthesis may have been normal, but that it must then have decreased rapidly as the inhibitor was taken up by the liver cells. The longest interval between administering hydroxycitrate and starting the measurement of fatty acid synthesis shown in Fig. 4 was 50 min. Here too, the measurement of the rate of fatty acid synthesis lasted for 45 min. This means that the inhibition of fatty acid synthesis remained strong between 50 and 95 min after administering hydroxycitrate. Four additional experiments also showed that the inhibition of fatty acid synthesis remains strong 2 to 3 hours after giving hydroxycitrate.

DISCUSSION

(−)-Hydroxycitrate is a very strong inhibitor of citrate cleavage enzyme (1) and of fatty acid synthesis *in vitro* (10). The present paper shows that (−)-hydroxycitrate is also an excellent inhibitor of fatty acid synthesis *in vivo*.

The K_i of (−)-hydroxycitrate in the reaction catalyzed by citrate cleavage enzyme is between 0.2 and 0.6 μM depending on conditions (1). The amount of (−)-hydroxycitrate required for 50% inhibition of fatty acid synthesis *in vivo*, namely 0.28 mmole per kg of body weight, is roughly 700 times larger than the K_i for citrate-cleavage enzyme. It seems likely that one of the factors limiting the effectiveness of (−)-hydroxycitrate *in vivo* is its passage across the cell membrane.

A possibility which was discussed previously (10), and which should not be overlooked here, is that the inhibition of citrate cleavage enzyme by (−)-hydroxycitrate *in vivo* switches on a pathway for the generation of extramitochondrial acetyl-CoA which does not involve citrate. Such a pathway would circumvent the inhibited citrate cleavage reaction and this would manifest itself as an apparently lower effectiveness of (−)-hydroxycitrate than might otherwise be expected. There is at present no evidence favoring such an alternate pathway in nonruminant mammals.

Another possibility which should be considered is that (−)-hydroxycitrate inhibits the transfer of citrate from the mitochondrial matrix into the cytoplasm. Experiments in which the excretion of citrate formed in liver mitochondria was measured in the presence and absence of (−)-hydroxycitrate suggest that this type of inhibition does not occur, or if it occurs that it cannot be large (10). Measurements by Drs. G. R. Williams and B. H. Robinson show that (−)-hydroxycitrate does not exchange with intramitochondrial citrate, and that it does not inhibit the egress of citrate from mitochondria previously loaded with labeled citrate.[2]

When the dose of (−)-hydroxycitrate was 1 mmole per kg of body weight the inhibition of fatty acid synthesis was about 75% (Fig. 3). The control rate of fatty acid synthesis was about 60 μmoles of ^3H_2O incorporated into fatty acids per g of liver per hour. This is equivalent to 69 μmoles of acetyl group incorporated per g of liver per hour, or 8 μmoles per liver per min (assuming the liver to weigh 7 g). An inhibition of 75% of this rate corresponds to 6 μmoles of acetyl group per liver per min being prevented from going into fatty acids. In other words, this amount of carbon must be channeled into alternative products. The nature of these products is presently under investigation.

REFERENCES

1. WATSON, J. A., FANG, M., AND LOWENSTEIN, J. M., *Arch. Biochem. Biophys.*, **135**, 209 (1969).

[2] G. R. Williams and B. H. Robinson, personal communication.

2. Kornacker, M. S., and Lowenstein, J. M., *Biochem. J.*, **94**, 209 (1965).
3. Fang, M., and Lowenstein, J. M., *Biochem. J.*, **105**, 803 (1967).
4. Bray, G. A., *Anal. Biochem.*, **1**, 279 (1960).
5. Fain, J. N., and Scow, R. O., *Amer. J. Physiol.*, **210**, 19 (1965).
6. Jungas, R. L., *Biochemistry*, **7**, 3708 (1968).
7. Windmueller, H. G., and Spaeth, A. E., *J. Biol. Chem.*, **241**, 2891 (1966).
8. Windmueller, H. G., and Spaeth, A. E., *Arch. Biochem. Biophys.*, **122**, 362 (1967).
9. Lewis, Y. S., in J. M. Lowenstein (Editor), *Methods in enzymology, Vol. 13*, Academic Press, New York, 1969, p. 613.
10. Watson, J. A., and Lowenstein, J. M., *J. Biol. Chem.*, **245**, 5993 (1970).