STUDIES ON THE FATTY ACID OXIDIZING SYSTEM OF ANIMAL TISSUES

IX. STEREOSPECIFICITY OF UNSATURATED ACYL CoA HYDRASE*

by

SALIH J. WAKIL

Institute for Enzyme Research, University of Wisconsin, Madison, Wis. (U.S.A.)

The problem of the stereospecificity of the enzymes involved in the fatty acid oxidation sequence is encountered only in connection with three steps of the series. These steps are the dehydrogenation of the acyl CoA, the hydration of the α-β unsaturated acyl CoA and the oxidation of the β-hydroxyacyl CoA. GREEN, MII, MAHLER AND BOCK[1] have shown that the product after butyryl CoA dehydrogenase action on a saturated fatty acyl CoA can reduce DPN** in the presence of unsaturated acyl CoA hydrase and L(+)β-hydroxyacyl CoA dehydrogenase. Since the behavior of the product paralleled that of crotonyl CoA, they concluded that the product is probably crotonyl CoA.

LEHNINGER AND GREVILLE[2] and WAKIL, GREEN, MII AND MAHLER[3] have shown that a DPN-linked dehydrogenase isolated from liver is specific for the L(+)β-hydroxyacyl CoA*** derivatives. With the recent discovery of D(—)β-hydroxybutyryl CoA dehydrogenase by WAKIL[7] and of β-hydroxybutyryl CoA racemase by STERN and his associates[8] it was possible to reinvestigate the stereospecificity of unsaturated acyl CoA hydrase. WAKIL AND MAHLER[9] have concluded that hydrase acts upon CoA (*trans* isomer) but not *iso*crotonyl CoA (*cis* isomer). These authors have linked the hydrase reaction with the L(+)β-hydroxyacyl CoA dehydrogenase reaction and used this coupled system as a basis of their assay as shown in the following reactions:

(1) $R\text{-}CH=CH\text{-}COSCoA + H_2O \rightleftharpoons$ L(+)$R\text{-}CHOH\text{-}CH_2COSCoA$

(2) L(+)$R\text{-}CHOH\text{-}CH_2COSCoA + DPN^+ \rightleftharpoons R\text{-}\overset{O}{\overset{\|}{C}}\text{-}CH_2\text{-}COSCoA + DPNH + H^+$

(3) Sum of reactions 1 and 2

$$R\text{-}CH=CH\text{-}COSCoA + H_2O + DPN^+ \rightleftharpoons R\text{-}\overset{O}{\overset{\|}{C}}\text{-}CH_2COSCoA + DPNH + H^+$$

* Supported by a grant-in-aid from the American Cancer Society (on recommendation of the committee on growth of the national research council).

** The following abbreviations will be used: Unsaturated acyl CoA hydrase (hydrase); diphosphopyridine nucleotide (DPN), reduced DPN (DPNH); Tris(hydroxymethyl)aminomethane (tris); 2-amino-2-methyl-1,3-propandiol (diol); adenosine monophosphate (AMP), Coenzyme A (CoASH or CoA).

*** Since the configurational relationships of the β-hydroxybutyric acids to the lactic acids have been established, the author is using this convention throughout this paper[4,5,6]. In the biochemical literature the names *d* and *l* β-hydroxybutyric acids have been used to designate the L(+) and D(—)β-hydroxybutyric acids respectively.

References p. 504.

STERN *et al.*[8] have reported that hydrase* hydrates both *cis*- and *trans*-crotonyl CoA and that the product of hydration has been identified as L(+)β-hydroxybutyryl CoA by enzymic assay. Using the direct assay method described by LYNEN AND OCHOA[11], we were able to show that hydrase does indeed hydrate *trans*-crotonyl CoA as well as *cis*-crotonyl CoA, but that only the product of hydration of *trans*-crotonyl CoA is reactive in the L(+)β-hydroxyacyl CoA dehydrogenase system[2,3]. The present communication deals with the identification of the product of hydration of *cis*-crotonyl CoA. A possible mechanism for the action of hydrase is suggested.

MATERIALS AND METHODS

Crotonic acid

The commercial product obtained from Eastman Kodak Co. was crystallized twice from water and once each from petroleum ether and diethyl ether. The crystalline product had a melting point of 72° which is identical to values reported in the literature[12].

Isocrotonic acid

This compound was prepared by the procedure of HATCH AND NESBITT[13]. The acid was further purified by alcohol fractionation of the sodium salt according to MICHAEL AND SCHULTHESS[14]. The acid was recovered from the sodium salt and recrystallized several times from petroleum ether. The crystalline product melted at 13 to 14°. Infra-red examination of this preparation showed the expected bands due to COOH, $-C=C-$ and CH_3 groups. But not the characteristic band due to *trans* double bond in the region of 970 cm^{-1} which is shown by crotonic acid (*cf.* Fig. 1). The possible contamination of the *iso*crotonic acid with crotonic acid is within the limits of the errors of the infra red determination, *i.e.*, less than 5%.

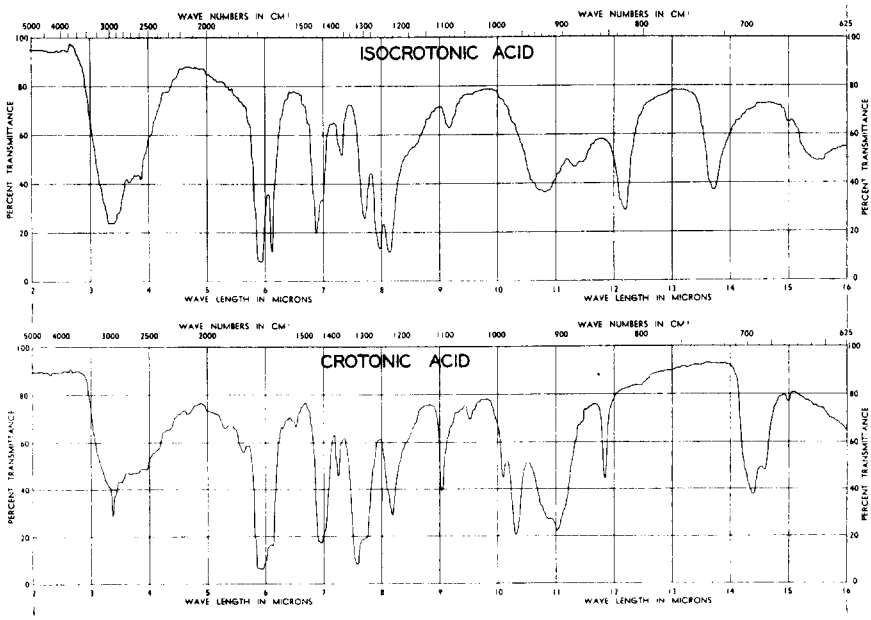

Fig. 1. Infrared spectra of *iso*crotonic and crotonic acid. Sample form used was liquid film for *iso*crotonic acid and solid film for crotonic acids, and a salt plate as reference cell. The characteristic band for the *trans* double bond at 970 cm^{-1} is present in crotonic acid while it is absent in the *iso*crotonic acid spectrum.

* This enzyme was also isolated by STERN *et al.*[10] and was called crotonase.

References p. 504.

Preparation of crotonyl CoA and isocrotonyl CoA

Crotonyl CoA and *iso*crotonyl CoA were prepared by the reaction of the corresponding acyl thiophenol with COASH according to the procedure of WIELAND AND KÖPPE[15].

The CoA used was prepared from yeast according to the method of BEINERT *et al.*[16].

Chemicals

All the reagents were commercial preparations. The D(—) and L(+)β-hydroxybutyric acids were prepared by resolving the racemic mixture as described by MCKENZIE[17].

Enzymes

Unsaturated acyl CoA hydrase and L(+)β-hydroxyacyl CoA dehydrogenase were prepared by published procedures[9,3], while the D(—)β-hydroxybutyryl CoA dehydrogenase was prepared as described elsewhere[7]. Racemase[8] was prepared from beef liver mitochondria[7]. The author is indebted to Dr. D. E. GREEN for the preparation of D(—)β-hydroxybutyric dehydrogenase[18].

Isolation of D(—) and L(+)β-hydroxybutyric acids

When the action of hydrase on crotonyl CoA and *iso*crotonyl CoA was completed (*cf.* Table II), the reaction mixtures were adjusted to pH 13 to 14 with alkali and the mixtures were incubated at 38° for ten minutes; this treatment was enough to assure complete hydrolysis of the CoA derivatives[19]. At the end of the incubation the mixture was adjusted to pH 3 with H_2SO_4 and enough solid $(NH_4)_2SO_4$ was added to saturate the solution. The samples were transferred to liquid-liquid extractors and the β-hydroxyacids were extracted with diethyl ether for 12 to 15 days. Under these conditions the recovery of the free acids was 70% as measured with known amounts of the authentic acids, and this correction was used in the calculations of the acids obtained. The ether layer was dried over anhydrous Na_2SO_4 and the ether was then evaporated under vacuum. The residue was dissolved in water, neutralized and assayed with D(—)β-hydroxybutyric acid dehydrogenase.

RESULTS

1. *Optical properties of isocrotonyl CoA*

Studies of the model compound S-crotonyl-N-acetyl thioethanolamine have led SEUBERT AND LYNEN[20] to the discovery of an absorption band at 263 μm due to the binding of the α-β unsaturated acid to sulfur in the thio esters. The difference sprectum of crotonyl CoA before and after alkaline hydrolysis of the thioester bond shows two absorption maxima[20], namely at 224 mμ and 263 mμ. *Iso*crotonyl CoA, however, shows a shift of the 224 mμ band to a longer wave length, *i.e.* maxima at 233 and 263 mμ. The molar extinction coefficients of the difference spectra of crotonyl CoA and *iso*crotonyl CoA at 263 mμ obtained as described above are $6.4 \cdot 10^6$ mole $^{-1} \times$ cm^2 and $5.5 \cdot 10^6$ mole $^{-1} \times$ cm^2 respectively.

2. *Hydration of isocrotonyl CoA*

The action of unsaturated acyl CoA hydrase on crotonyl CoA and *iso*crotonyl CoA could be followed spectrophotometrically[11, 20] by measuring the decrease in absorption

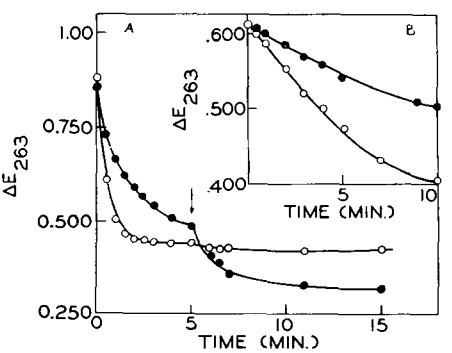

Fig. 2. Hydration of crotonyl CoA and *iso*crotonyl CoA by hydrase. A. In each cuvette the following were used: 2. μmole of versene, 0.4 mg of albumin, 20 μM of tris buffer at pH 7.5 and H_2O to 0.4 ml 0.035 μM of *iso*crotonyl CoA was added to the first cuvette (-●-●-curve) and 0.034 μM of crotonyl CoA to the second (-o-o-curve). The optical density was read against a blank containing AMP instead of the substrate. The reaction was started by addition of 0.5 μg of hydrase. At 5 minutes additional 2.5 μg of hydrase was added to reach equilibrium. B. Same as A. except that the reaction was started by addition of 0.01 μg of hydrase.

References p. 504.

at 263 mμ. Using the conditions described by LYNEN AND OCHOA[11], it was possible to show that the rate of hydration of *iso*crotonyl CoA by hydrase is about 40% the rate with crotonyl CoA as shown in Fig. 2. The equilibrium, however, is more toward the hydrated product in the case of *iso*crotonyl CoA than in that of crotonyl CoA. The equilibrium constants for the two reactions as expressed by WAKIL AND MAHLER[9] are equal to $3.6 \cdot 10^{-2}$ mole^{-1} and $7.5 \cdot 10^{-2}$ mole^{-1} for the hydration of crotonyl CoA and *iso*crotonyl CoA respectively. The ratios of β-hydroxybutyryl CoA to crotonyl CoA and *iso*crotonyl CoA are 2.0 and 4.3 respectively.

3. *Stoichiometry and product of hydration*

On studying the stoichiometry of the hydrase reaction, a marked difference was noted in the behavior of the product of hydration of *iso*crotonyl CoA as compared to that of crotonyl CoA in the L(+)β-hydroxyacyl CoA dehydrogenase system. The product of hydration of crotonyl CoA gives an immediate formation of DPNH while the reaction with *iso*crotonyl CoA proceeded only to about 10% of theory* (*cf.* Fig. 3). In both cases the product was identified as acetoacetyl CoA by citrate formation in the presence of CoASH, cleavage enzyme[21] oxalacetic acid an condensing enzyme[22].

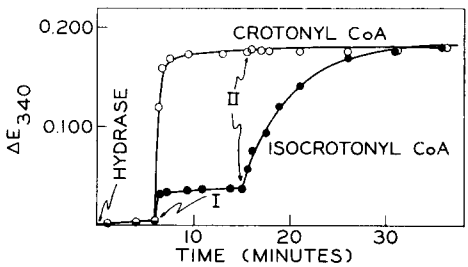

Fig. 3. The product of hydration of crotonyl CoA and *iso*crotonyl CoA as assayed by the specific dehydrogenases. Each reaction mixture contained 2 μ*M* of DPN, 20 μ*M* of diol buffer at pH 9.0 and H_2O to 0.4 ml. 0.015 μ*M* of acyl CoA (prepared enzymically[29]) were added and the reaction started by addition of 1 μg of hydrase at time zero (this was enough to give complete hydration at 3 minutes). At arrow I 0.2 units of L(+)β-hydroxyacyl CoA dehydrogenase was added and at arrow II 100 μg of D(—)β-hydroxybutyryl CoA dehydrogenase was added.

Table I contains the results of the complete stoichiometry of such studies. In this experiment the acyl CoA derivatives were prepared synthetically. The results show that only 30% of the hydrated product of *iso*crotonyl CoA is L(+)β-hydroxybutyryl CoA while the remaining 70% is D(—)β-hydroxybutyryl CoA. This is in agreement with results obtained using the enzymically prepared *iso*crotonyl CoA (*cf.* Fig. 3). In contrast the product of hydration of crotonyl CoA is 100% L(+)β-hydroxybutyryl CoA. Equivalent amounts of citrate were obtained from both derivatives which is indicative of the formation of acetoacetyl CoA.

In order to further prove this point the products of hydration were isolated as the free acids after hydrolysis of the CoA derivatives. Synthetically prepared *iso*crotonyl CoA and crotonyl CoA were subjected to the action of hydrase and the extent of hydration was measured by the disappearance of the band at 263 mμ. At the end of the reaction aliquots were taken for the measurement of L(+) and D(—)β-hydroxybutyryl CoA and the remainder was used for the isolation of the free acids (*cf.*

* In the experiments described in Fig. 3, the *iso*crotonyl CoA and the crotonyl CoA were prepared using the fatty acid activating enzyme free of both hydrase and racemization systems of WAKIL[7] and STERN *et al.*[8]. In this method of preparation *iso*crotonyl CoA is contaminated with crotonyl CoA to the extent of 10% while in the chemical method of WIELAND AND KÖPPE[15] the contamination is of the order of 20 to 30%. The remaining 90% of the product of hydration of *iso*crotonyl CoA can be accounted for only after addition of the D(—)β-hydroxybutyryl CoA dehydrogenase[7] or racemase[8].

References p. 504.

TABLE I

STOICHIOMETRY OF THE HYDRATION OF CROTONYL CoA AND *iso*CROTONYL CoA BY UNSATURATED ACYL CoA HYDRASE

Each test tube contains 2.0 mg of albumin, 2.0 μM of versene, 200 μM of tris at pH 7.5 and 0.23 μM of crotonyl CoA in the first tube, 0.24 μM of *iso*crotonyl CoA in the second and no substrate in the third. Water was added to a volume of 2.0 ml. A sample was withdrawn to determine absorption at 263 mμ[11]. The reaction was started by addition of 250 μg of hydrase and the disappearance of the unsaturated acyl CoA was followed spectrophotometrically. When equilibrium was reached, the reaction was stopped by heating, and the denatured protein was centrifuged. 0.4 ml was used for the assay of L(+)β-hydroxybutyryl CoA as described by WAKIL *et al.*[3]. The same sample was used for the assay of citrate[21,22]. Another 0.4 ml was pipetted in a cuvette, 1.0 μM of DPN and 1 μM of Mg^{++} were added, pH adjusted to 9.0 with alkali 100 μg of D(—)β-hydroxybutyryl CoA dehydrogenase was added, and the reaction was followed both at 340 and 303 mμ. Since the L(+)β-hydroxacyl CoA dehydrogenase is a contaminant of the D(—)β-hydroxybutyryl CoA dehydrogenase, the amount of DPNH formed represents the total amounts of both the L(+) and D(—)β-hydroxybutyryl CoA. By subtracting the value of L(+)β-hydroxybutyryl CoA from the total amount of β-hydroxybutyryl CoA, the amount of the D(—) isomer was obtained. Similar results were also obtained using racemase instead of D(—)β-hydroxybutyryl CoA dehydrogenase. The same samples were used for citrate determination[21,22].

	Crotonyl CoA	*Isocrotonyl CoA*
	μmole	μmole
Total amount of acyl CoA used*	0.23	0.24
Acyl CoA hydrated**	0.16	0.19
L(+)β-hydroxybutyryl CoA formed***	0.16	0.058
Citrate formed from L(+)β-hydroxybutyryl CoA§	0.27	0.13
D(—)β-hydroxybutyryl CoA formed§§	0.02	0.14
Citrate formed from D(—)β-hydroxybutyryl CoA§	0.01	0.22

* Based on total hydroxamic acid formed and on the amount of –SH released on hydrolysis.

** Based on the disappearance of the band at 263 mμ.

*** Assayed with L(+)β-hydroxyacyl CoA dehydrogenase and DPN, by measuring the absorption at 340 and 303 mμ due to the formation of DPNH and acetoacetyl CoA respectively. The acetoacetyl CoA formed was converted to citrate in a multi-enzyme system[21,22].

§ Assayed by the method of NATELSON *et al.*[28].

§§ Assayed by DPNH and acetoacetyl CoA formation in both the D(—)β-hydroxybutyryl CoA dehydrogenase and racemase systems. The citrate was formed as in § and was assayed by NATELSON's method.

methods). Samples of the free acids were assayed using the β-hydroxybutyric dehydrogenase and DPN[18]; the reaction was followed by observing DPNH formation as measured at 340 mμ. After this reaction had reached equilibrium, the protein was precipitated with trichloroacetic acid and the product, acetoacetate, was determined colorimetrically by WALKER's procedure[23]. The results (Table II) show that only the free β-hydroxybutyric acid obtained from the hydration of *iso*crotonyl CoA reacts with the β-hydroxybutyric acid dehydrogenase, and since this enzyme is specific for the D(—)β-hydroxybutyric[18], the nature of the product of *iso*crotonyl CoA is indicated.

DISCUSSION

From the above observation we can conclude the following: (1) That unsaturated acyl CoA hydrase acts upon both trans- and cis-crotonyl CoA. (2) The rate of hydration of the *cis* isomer is 40% of the rate of hydration of the *trans* isomer. (3) The product of hydration of *trans*-crotonyl CoA is L(+)β-hydroxybutyryl CoA while that of *cis*-crotonyl CoA is D(—)β-hydroxybutyryl CoA.

References p. 504.

TABLE II

FORMATION OF D(—)β-HYDROXYBUTYRIC ACID FROM *iso*CROTONYL CoA

Two flasks each contain 1000 μM of Tris pH 7.5, and water to 7.4 ml. 35 μM of *iso*crotonyl CoA was added to the first flask and 40 μM of crotonyl CoA to the second. The reaction was started with the addition of 1.0 mg of hydrase. After 30 minutes at room temperature the reaction was stopped by heating and samples were taken for analysis of L(+) and D(—)β-hydroxybutyryl CoA as described in Table I. The remaining mixture was used for the isolation of the free β-hydroxybutyric acids as described in methods. The residue after ether evaporation was neutralized with KOH and 0.02 ml (containing about 0.05 μM of the β-hydroxy acid) was pipetted into a cuvette containing 1.0 μM of DPN, 40 μM of Diol pH 9.0, 2.0 μM of mg^{++}, 20 μM of cysteine and H_2O to 0.4 ml. The reaction was started by addition of 0.01 ml of D(—)β-hydroxybutyric acid dehydrogenase and the absorption at 340 mμ was recorded. A blank with no substrate was used and standard D(—) and L(+)β-hydroxybutyric acids were also run. The values in the table were corrected for blank. At the end of the reaction, the protein was precipitated by trichloroacetic acid and the acetoacetate was determined according to WALKER[23].

Substrate	*Amount used*	*β-hydroxybutyryl CoA formed** D(−)	L(+)	*DPNH formed from D(—)β-hydroxybutyric acid*	*Acetoacetate formed***
	μmole	μmole	μmole	μmole	μmole
*iso*Crotonyl CoA	35	20	7.0	21 ***	20 ***
Crotonyl CoA	40	0.5	25	0.74 ***	0.30 ***

* Assayed in the same way as in Table I.
** Assay according to the procedure of WALKER[23].
*** The values are corrected for 70% yield on extraction with ether (*cf.* methods) and another 70% for yield in the D(—)β-hydroxybutyric acid dehydrogenase system under the conditions used.

WAKIL AND MAHLER[9] have reported that the unsaturated acyl CoA hydrase does not catalyze the hydration of *iso*crotonyl CoA. Their conclusion was based on the fact that an insignificant amount of DPNH was formed when *iso*crotonyl CoA was assayed in the presence of both hydrase and L(+)β-hydroxyacyl CoA dehydrogenase. According to the data presented here, the observation of WAKIL AND MAHLER can be explained by the fact that the product of hydration cannot be expected to react in L(+)β-hydroxyacyl CoA dehydrogenase system in the absence of a racemizing system. The data presented here are in disagreement with the statements of STERN *et al.* that the product of hydration of *iso*crotonyl CoA is L(+)β-hydroxybutyryl CoA.

The L(+)β-hydroxybutyryl CoA formed (10% from enzymically prepared *iso*crotonyl CoA or 20 to 30% from the synthetic CoA derivatives) cannot be derived from *iso*crotonyl CoA as such,

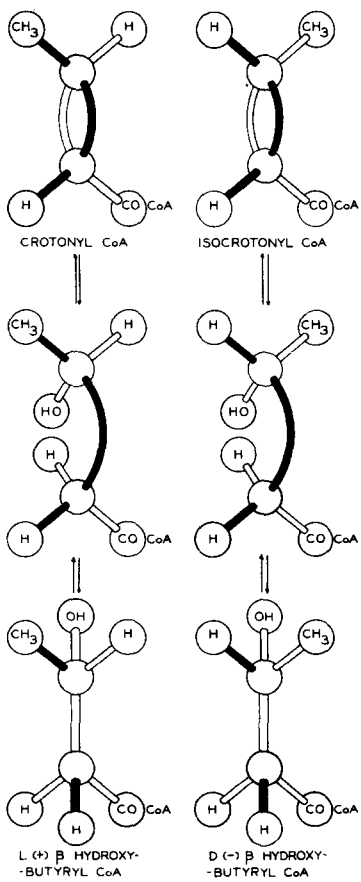

Fig. 4. A three-dimensional diagramatic representation of the reaction of unsaturated acyl CoA hydrase on crotonyl CoA and *iso*crotonyl CoA.

References p. 504.

since under conditions whereby the hydrase reaction is allowed to go almost to completion, there is no increase in the percentages mentioned above. The only time conversion of *iso*crotonyl CoA to crotonyl CoA could be obtained is in the presence of D(—)β-hydroxybutyryl CoA dehydrogenase or racemase.

The reaction catalyzed by unsaturated acyl CoA hydrase represents another example of the ability of an enzyme to distinguish between chemically identical groups. Such specificity has been demonstrated in the dehydration of citric acid[24], the oxidation and reduction of DPNH by alcohol dehydrogenase[25] and others. In order to explain this discrimination a threepoint attachment of the substrate to the enzyme has been proposed[26]. Fig. 4 shows graphically how such a mechanism would apply in the present case. The following postulates are made: (a) a three-point attachment is necessary for the linking of the substrate to the enzyme, (b) the addition of the elements of water is always from the same side; in this case the attack leads to the opening of the lower ("Light") bond, (*cf.* Fig. 4) and (c) in the reverse direction the same hydrogen of the α-carbon atom is removed as H_2O. Similar postulates have been advanced by KREBS[27] to account for the formation of L-malic acid from fumaric acid by fumarase. The special position of the methyl group seems to be important in the rate of the reaction. Whether this effect will be enhanced in the cases of the higher analogues of unsaturated fatty acids awaits further experiments.

ACKNOWLEDGMENTS

The author is indebted to Dr. D. E. GREEN for his interest and encouragement during this work. Mrs. PATRICIA HERSCHER provided valuable technical assistance. Oscar Mayer and Co. generously provided the tissues used in this investigation. The author wishes to thank Mr. SANFORD M. ARONOVIC for the infrared analysis.

SUMMARY

Unsaturated acyl CoA hydrase catalyzes the hydration of both *trans*- and *cis*-crotonyl CoA. The *cis* isomer is hydrated with 40% of the rate with which the *trans* isomer is hydrated. The product of hydration of *trans*-crotonyl CoA is L(+)β-hydroxylbutyryl CoA, while the product of hydration of *cis*-crotonyl CoA is D(—)β-hydroxybutyryl CoA. A mechanism is proposed for the reaction catalyzed by unsaturated acyl CoA hydrase.

RÉSUMÉ

L'hydrase de l'acyl CoA non saturé catalyse l'hydratation à la fois du *trans*- et du *cis*-crotonyl CoA. La vitesse d'hydratation de l'isomère *cis* est égale à 40% de celle de l'isomère *trans*. Le produit d'hydratation du *trans*-crotonyl CoA est le L(+)β-hydroxybutyryl CoA, tandis que le produit d'hydratation du *cis*-crotonyl CoA est le D(—)β-hydroxybutyryl CoA. Un mécanisme pour la réaction catalysée par l'hydrase de l'acyl CoA non saturé est proposé.

ZUSAMMENFASSUNG

Die Hydratase der ungesättigten Acylderivate des CoA katalysiert die Hydratation von *cis*- und *trans*-Crotonyl CoA. Die Hydratation der *trans*-Verbindung läuft mit 2½ mal grösserer Geschwindigkeit ab als die der *cis*-Verbindung. Das Produkt der Hydratation von *trans*-Crotonyl CoA ist L(+)β-Oxybutyryl CoA und das Produkt der Hydratation von *cis*-Crotonyl CoA ist D(—)β-Oxybutyryl CoA. Ein Mechanismus für die Wirkungsweise der Hydratase wird vorgeschlagen.

References p. 504.

REFERENCES

1 D. E. GREEN, S. MII, H. R. MAHLER AND R. BOCK, *J. Biol. Chem.*, 206 (1954) 1.
2 A. L. LEHNINGER AND G. D. GREVILLE, *Biochim. Biophys. Acta*, 12 (1953) 188.
3 S. J. WAKIL, D. E. GREEN, S. MII AND H. R. MAHLER, *J. Biol. Chem.*, 207 (1954) 631.
4 P. KARRER AND W. KLARER, *Helv. Chim. Acta*, 8 (1925) 393.
5 P. A. LEVENE AND H. L. HALLER, *J. Biol. Chem.*, 67 (1926) 329.
6 A. MAGNUS-LEVY, *Arch. Exp. Path. Pharmacol.*, 45 (1901) 389.
7 S. J. WAKIL, *Biochim. Biophys. Acta*, 18 (1955) 314.
8 J. R. STERN, A. DEL CAMPILLO AND A. L. LEHNINGER, *J. Am. Chem. Soc.*, 77 (1955) 1073.
9 S. J. WAKIL AND H. R. MAHLER, *J. Biol. Chem.*, 207 (1954) 125.
10 J. R. STERN AND A. DEL CAMPILLO, *J. Am. Chem. Soc.*, 75 (1953) 2277.
11 F. LYNEN AND S. OCHOA, *Biochim. Biophys. Acta*, 12 (1953) 299.
12 C. BULK, *Ann.*, 139 (1866) 62.
13 L. F. HATCH AND S. S. NESBITT, *J. Am. Chem. Soc.*, 72 (1950) 727.
14 A. MICHAEL AND O. SCHULTHESS, *J. prakt. Chem.*, 46 (1892) 236.
15 T. WIELAND AND H. KOPPE, *Ann.*, 581 (1953) 1.
16 H. BEINERT, R. W. VON KORFF, D. E. GREEN, D. A. BUYSKE, R. E. HANDSCHUMACHER, H. HIGGINS AND F. M. STRONG, *J. Biol. Chem.*, 200 (1953) 385.
17 A. MCKENZIE, *J. Chem. Soc.*, 81 (1902) 1402.
18 D. E. GREEN, J. G. DEWAN AND L. F. LELOIR, *Biochem. J.*, 31 (1937) 934.
19 H. BEINERT, *J. Biol. Chem.*, 205 (1953) 575.
20 W. SEUBERT AND F. LYNEN, *J. Am. Chem. Soc.*, 75 (1953) 2587.
21 D. S. GOLDMAN, *J. Biol. Chem.*, 208 (1954) 345.
22 S. OCHOA, J. R. STERN AND M. C. SCHNEIDER, *J. Biol. Chem.*, 193 (1951) 691.
23 P. G. WALKER, *Biochem. J.*, 58 (1954) 699.
24 V. R. POTTER AND C. HEIDELBERGER, *Nature*, 164 (1949) 180.
25 H. R. FISCHER, E. E. CONN, B. VENNESLAND AND F. H. WESTHEIMER, *J. Biol. Chem.*, 202 (1953) 687.
26 A. G. OGSTON, *Nature*, 162 (1948) 963.
27 H. A. KREBS, *Harvey Lectures*, 44 (1948) 165.
28 S. NATELSON, J. K. LUGOVOY AND J. B. PINCUS, *J. Biol. Chem.*, 170 (1947) 597.
29 H. R. MAHLER, S. J. WAKIL AND R. BOCK, *J. Biol. Chem.*, 204 (1953) 453.

Received July 20th, 1955