Specificity of Long-Chain Acyl Coenzyme A Synthetase from Rat Liver Microsomes. Influence of the Position of Double Bonds in Octadecadienoic Acids†

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ABSTRACT: The activation of linoleic acid 18:2(*n*-6), and of four of its cis,cis isomers 18:2(*n*-5), 18:2(*n*-7), 18:2(*n*-8), and 18:2(*n*-9) by rat liver microsomes has been studied by a method of assay based on the insolubility of linoleyl-CoA in diethyl ether. Under optimum concentrations of cofactors for the activation of linoleic acid, the kinetics of activation of the different octadecadienoic acids were investigated. Linoleic acid had the lowest K_m (2.22 μM) and the displacement of its double bonds by one atom of carbon either toward the carboxylic group or toward the methyl group caused the K_m to increase by a factor of 10:20 μM for both 18:2(*n*-5) and 18:2(*n*-7). Further displacement of the double bonds toward the carboxylic group caused the K_m to decrease: 7.14 and 3.33 μM for 18:2(*n*-8) and 18:2(*n*-9), respectively. Thus the lowest K_m were found for 18:2(*n*-6) and 18:2(*n*-9), and the greater affinity of the enzyme for these two substrates was tentatively attributed to the Δ^9 double bond which is common to both fatty acids. The influence of the Δ^9 double bond on the specificity of long-chain acyl-CoA synthetase was further supported by the results of the inhibition study of linoleic acid activation by 18:2(*n*-7) and 18:2(*n*-9); 18:2(*n*-7), which has its double bonds in $\Delta^{8,11}$, is a mixed-type inhibitor of the reaction whereas 18:2(*n*-9), which has its double bonds in $\Delta^{6,9}$, behaves more like a competitive inhibitor.

The specificity of acyl-CoA[1] synthetase (acid:CoA ligase (AMP), EC 6.2.1.3) has been investigated (Brindley and Hubscher, 1966; Pande and Mead, 1968; Aas, 1971) but few kinetic data for long-chain fatty acid activation are available (Borgstrom and Wheeldon, 1961; Creasey, 1962; Pande and Mead, 1968). The progress made in this field was probably hindered by the limitations of the methods used for isolation of acyl-CoA: hydroxamate formation (Kornberg and Pricer, 1953), acyl-carnitine formation (Farstad *et al.*, 1967), or selective extraction (Creasey, 1962). A more sensitive assay has been recently described (Samuel and Ailhaud, 1969) which is based on the insolubility of acyl-CoA in diethyl ether.

In the present report, this method has been used to study the influence of the position of the double bonds on the kinetics of activation of octadecadienoic acids. For this purpose, synthetic 1-^{14}C-labeled octadecadienoic acids (Marcel and Holman, 1968) were used as substrates and comprised: [1-^{14}C]octadeca-10,13-dienoic, 18:2(*n*-5); [1-^{14}C]octadeca-9,12-dienoic, 18:2 (*n*-6); [1-^{14}C]octadeca-8,11-dienoic, 18:2(*n*-7); [1-^{14}C]-octadeca-7,10-dienoic, 18:2(*n*-8); and [1-^{14}C]octadeca-6,9-dienoic, 18:2(*n*-9) acids. With linoleic acid as a substrate, the kinetics of inhibition by two of its isomers, 18:2(*n*-7) and 18:2(*n*-9) which occur in mammalian tissues (Stoffel and Ahrens, 1959; Fulco and Mead, 1960; Koshimoto and Radin, 1964), and by Triton WR-1339, are also presented. In all assays for acyl-CoA synthetase activity, special attention was given to maintaining the concentration of fatty acids below their estimated critical micellar concentrations.

Experimental Section

Materials. ATP, CoA, and linoleyl-CoA were obtained from General Biochemicals, and dithiothreitol from Sigma. [1-^{14}C]Linoleic acid and [1-^{14}C]palmityl-CoA were purchased from Amersham-Searle. Other fatty acids were synthesized as previously described (Marcel and Holman, 1968; Christie and Holman, 1967) or were obtained from the Hormel Institute. Triton WR-1339 was a product of the Ruger Chemical Co., Irvington, N. J. Stock solutions of fatty acids were prepared by the addition of one volume of NH_4OH (10%) and nine volumes of Triton solution (1%). These fatty acid solutions were kept frozen under nitrogen and in the presence of dithiothreitol (3 mg/ml) for short period of time (less than 1 week).

Preparation of Microsomal Fraction. After a 12-hr fast, weanling male rats (Sprague-Dawley strain) were killed by decapitation after ether anesthesia. The blood was drained, and the livers were quickly removed and cooled in cold 0.25 M sucrose solution. All subsequent operations were done at 4°. The livers were sliced and homogenized in 0.25 M sucrose with a Potter-type homogenizer. The homogenate was centrifuged at 20,000*g* for 20 min. The supernatant was taken out and centrifuged at 105,000*g* for 1 hr. The microsomal pellets were resuspended in the same sucrose solution and were centrifuged again at 105,000*g* for 1 hr. The washed microsomes thus obtained were collected and homogenized in 0.25 M sucrose solution which contained dithiothreitol (1 mg/ml). Small aliquots of this microsomal preparation were stored under nitrogen at −20°. Under these conditions, no loss of activity of acyl-CoA synthetase was observed during storage periods of up to 1 month. Protein concentration was measured by the method of Lowry *et al.* (1951).

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[1] Abbreviations used are: ATP, adenosine triphosphate; CoA, coenzyme A.

Assays for Long-Chain Acyl-CoA Synthetase. The assay for the activation reaction was a modification of the method of Samuel and Ailhaud (1969), which is based on the insolubility of acyl-CoA in diethyl ether. The standard system for the activation of [1-^{14}C]linoleic acid or of one of its 1-^{14}C-labeled isomers 18:2(*n*-5), 18:2(*n*-7), 18:2(*n*-8), and 18:2(*n*-9) contained the following constituents which were added in the given order: a varying amount of [1-^{14}C]fatty acid (ammonium salt), 0–20 μl of 1% aqueous solution of Triton WR-1339,[2] 70 μmoles of Tris-HCl buffer (pH 7.5), 1 μmole of dithiothreitol, 2 μmoles of ATP, 0.25 μmole of CoA, 0.8 μmole of $MgSO_4$, and water to adjust to a final volume of 0.2 ml. Control experiments without CoA and ATP were systematically included in each series of incubations.

After a brief preincubation (2–3 min) at 37°, the reaction was started by addition of the microsomal fraction and the mixture was incubated in a Dubnoff shaking incubator at 37°. The microsomal fraction was diluted with 0.1 M Tris-HCl buffer (pH 7.5) which contained dithiothreitol (1 mg/ml). The assay was usually linear up to 4 min. and up to a protein content of 5 μg/assay. The reaction was stopped after 1, 1.5, or 2 min by addition of 0.6 ml of $HClO_4$ (1.5%). The remaining free fatty acid was extracted four times with diethyl ether (5 ml). The traces of ether were removed from the water phase by evaporation under a stream of nitrogen. The aqueous phase was then mixed with a solution of Bray (1960), and directly transferred to scintillation vials for radioactivity counting.

In order to identify the product of the reaction (linoleyl-CoA), large-scale incubations (600 nmoles of [1-^{14}C]linoleic acid, 0.65 nCi/nmole) were done in triplicate experiments. The procedure was identical with that described above and the mixture was incubated for 10 min. The ether extracts, which contained most of the free fatty acid, were pooled, evaporated to dryness, and the residue dissolved in Bray's solution for radioactivity counting. The pH of the water phase was adjusted to 7.4 with 4 N KOH. To this solution were added linoleyl-CoA (100 nmoles) as a carrier and an excess of hydroxylamine (1 mmole, pH 7.4). These mixtures were kept at room temperature for 30 min and were then extracted four times with ether (5 ml). The ether extracts were chromatographed on a glass-fiber sheet impregnated with silicic acid (Gelman Instrument Corp, Ann Arbor, Mich.). The developing solvent system contained chloroform–methanol–water (95:5.6:0.5) (v/v).[3] The remaining free fatty acids and the hydroxamate thus separated were visualized by exposure to iodine vapors and identified by comparison with standards. The areas of the chromatograms which corresponded to the identified products were cut and dropped into scintillation vials for radioactivity counting with Bray's solution. Alternatively, the products contained in the water phase and the ether phase were analyzed directly by paper chromatography with the solvent system 1-butanol–acetic acid–water (5:2:3, v/v) according to Seubert (1960). After identification by comparison with standards, the areas of the chromatograms corresponding to linoleyl-CoA and linoleic acid were cut and counted for radioactivity as described above.

[2] The total amount of Triton WR-1339 was kept constant (0.2 mg) in standard assay systems for two reasons. First, it was found that, in lower range of substrate concentration, the absence of Triton caused a loss in the total recovery of radioactivity (up to 80%) which has been attributed to adsorption of the fatty acid onto the glassware. This effect could be completely eliminated by homogenization of the incubation system with Triton. Second, Triton WR-1339 was found to be a noncompetitive inhibitor for the activation of linoleate. Therefore it was essential to keep the concentration of this detergent as low as possible, and constant, in the series of experiments.

[3] G. Graff, Y. L. Marcel, and R. T. Holman, unpublished observation.

TABLE I: Evaluation of the Assay for Long-Chain Acyl-CoA Synthetase and Identification of the Product of the Reaction.

	Aqueous Phase		Ether Phase	
Expt	Acyl-CoA	Free Fatty Acids	Acyl-CoA	Free Fatty Acids
1	0	1.8 ± 0.8[a]	0	98.2 ± 0.8
2	98.9 ± 0.1	0	1.1 ± 0.1	0
3	34.1 ± 0.8	7.6 ± 0.4		55.7 ± 2.0

[a] Per cent of total radioactivity recovered in both aqueous and ether phases (±SE). Experiment 1: [1-^{14}C]linoleic acid was incubated for 10 min at six different concentrations (1–30 μM) in the conditions described in the Experimental Section but in the absence of CoA and ATP. Experiment 2: [1-^{14}C]palmityl-CoA was incubated for 10 min at three different concentrations (0.5, 2, and 5 μM) in the general conditions of assay for acyl-CoA synthetase as described in the Experimental Section but in the presence of boiled microsomes. Experiment 3: [1-^{14}C]linoleic acid (600 nmoles) was incubated for 10 min in triplicate assays with 10 μg of microsomal protein as described in the Experimental Section. After extraction with ether of the unreacted linoleic acid, the water phase was treated with hydroxylamine and linoleyl-CoA was identified as linoleyl hydroxamate after separation by thin-layer chromatography.

Results

Evaluation of the Assay System. First, in order to evaluate the selectivity of the extraction procedure, [1-^{14}C]linoleic acid and [1-^{14}C]palmityl-CoA were incubated under the standard assay procedure and extracted with diethyl ether as described above. Most of linoleic acid (98.2%) was recovered in the ether phase (Table I, expt 1) and most of palmityl-CoA (98.9%) was recovered in the aqueous phase (Table I, expt 2). Since linoleic acid is more water soluble than palmitic acid, the partition of linoleyl-CoA between water and ether was expected to be more favorable than that of palmityl-CoA.

Second, in order to identify the product of the reaction (linoleyl-CoA), large-scale incubations were carried out as described in the Experimental Section (Table I, expt 3). Most of the radioactivity of linoleic acid was recovered in the ether phase, which represented the amount of unreacted fatty acid. After treatment of the aqueous phase with hydroxylamine and its subsequent extraction with either, the bulk of radioactivity in this phase (34.1% of total recovered radioactivity) was identified by thin-layer chromatography as acyl hydroxamate after comparison with synthetic standards. After substraction of the radioactivity in the aqueous phase of the control experiment (lacking CoA and ATP) from the radioactivity in the aqueous phase of the batch incubation, it was calculated that the compound tenatively identified as linoleyl hydroxamate represented 82% of the radioactivity of the water phase. The balance of radioactivity (18%) was identified by thin-layer chromatography as linoleic acid,

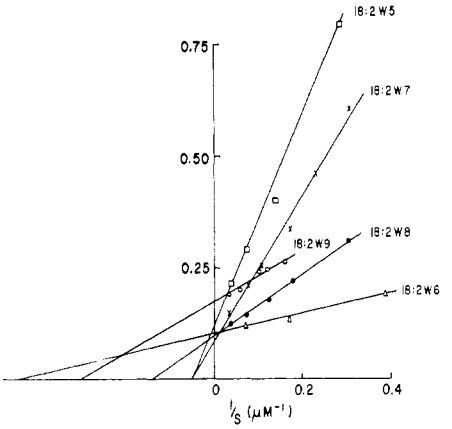

FIGURE 1: Kinetics of activation of cis,cis isomers of linoleic acid: 18:2(*n*-5) (□), 18:2(*n*-6) (△), 18:2(*n*-7) (×), 18:2(*n*-8) (●), and 18:2(*n*-9) (○). The conditions of the assay were as described in the Experimental Section and the protein concentration was varied together with the substrate concentration in order to maintain the linearity of the reaction up to 2 min. Units as *V* is expressed as μmoles of acyl-CoA formed per hr per mg of protein.

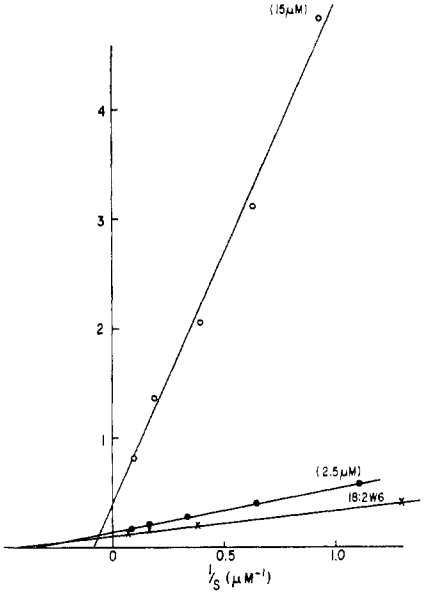

FIGURE 2: Inhibition of linoleic acid activation by 18:2(*n*-7). Standard assay conditions were used and 18:2(*n*-7) was added at two different concentrations: linoleic acid was incubated either alone (×), or in the presence of 18:2(*n*-7) [2.5 μM (●)] or in the presence of 18:2(*n*-7) [15 μM (○)]. Units as in Figure 1.

which probably results from the decomposition of linoleyl-CoA during hydroxylamine treatment. Confirmation was obtained from the results of the direct paper chromatography of the water phase: by this method, 95% of the radioactivity of the water phase was identified as linoleyl-CoA. From the criteria of solubility in water and ether, from the tentative identification by thin-layer chromatography and paper chromatography, and from the linearity of the activation reaction in the presence of ATP, CoA, Mg^{2+}, and from the absence of activation reaction in the lack of ATP and CoA, the product formed by the reaction and isolated in the water phase was identified as linoleyl-CoA.

Activation of Linoleic Acid. The optimum cofactor requirements were determined for the activation of linoleate by the microsomal preparation. Addition of ATP, CoA, and Mg^{2+} was essential and the optimum concentration for each of these cofactors was: ATP, 10 mM; CoA, 1.2 mM; and Mg^{2+}, 4 mM. Under these conditions and at two different concentrations of substrate (1.76 and 29.5 μM), the rate of activation was proportional to the amount of microsomes between 0.1 and 10 μg of protein. At low concentration of substrate (1 μM and less), the linearity as a function of time was obtained only at low protein concentrations (less than 0.5 μg) or for a short incubation period (1 min).

At low concentrations of substrate (1 μM and less), the addition of Triton was necessary to ensure homogeneous dispersion of the substrate. The effect of this detergent on the activation of linoleic acid was studied at two concentrations (0.2 and 1 mg of Triton per assay), and the double-reciprocal plots of the kinetics of the reaction gave the following results. At both concentrations of Triton, K_m remained the same (2.22 μM) whereas the maximum velocity decreased from 9.71 to 5 μmoles per hr. It was therefore concluded that Triton WR-1339 was a noncompetitive inhibitor of the activation of linoleic acid. Thereafter in the comparative study of activation of different isomers of linoleic acid, we assumed that Triton would have the same effect on each substrate and we kept the concentration of Triton constant at 0.2 mg/assay.

Effect of Double-Bond Position on the Activation of Octadecadienoic Acids. The double-reciprocal plots for the activation of linoleic acid and its isomers are illustrated in Figure 1 and the numerical values of K_m and V_{max} are summarized in Table II. Cofactor concentrations and amount of Triton were the same for each substrate. Initial velocities were determined at several levels of substrates which ranged from 0.3 to 30 μM. However in the case of 18:2 (*n*-9), which had the lowest specific activity (0.35 μCi/μmole), substrate concentration ranged from 6 to 30 μM. The amount of microsomal protein was varied with the concentration of substrate in order to maintain the linearity of the reaction to 2 min.

Inhibition of Linoleic Acid Activation by 18:2(n-7) and 18:2(n-9). Two linoleic acid isomers, 18:2(*n*-7) and 18:2(*n*-9), have been found in different rat tissues and more specially in the liver (Fulco and Mead, 1960; Koshimoto and Radin, 1964). Therefore these acids were used in the inhibition study rather than the other isomers which do not occur in mammalian tissues. The inhibitor was assayed at two concentrations (2.5 and 15 μM) and the results are shown as double-reciprocal plots in Figure 2. From the intercepts, the activation of linoleic acid in the presence of 18:2(*n*-7) (2.5 μM) has a K_m = 3 μM and a V_{max} = 7.14 μmole/hr per mg of protein, while in the presence of 18:2(*n*-7) (15 μM) it has K_m = 11.9 μM

TABLE II: Michaelis Constants and V_{max} Values of Linoleate and Its Analogs Were Calculated from Intercepts at Both Axes of Figure 1 for Each Substrate.

Substrate	Michaelis Constants (μM)	V_{max} (μmoles/hr per mg of Protein)
18:2(*n*-5)	20.00	8.33
18:2(*n*-6) (Linoleate)	2.22	9.71
18:2(*n*-7)	20.00	11.76
18:2(*n*-8)	7.14	10.20
18:2(*n*-9)	3.33	5.71

and a V_{max} = 2.50 μmoles/hr per mg of protein. It was, therefore, concluded that the effect of 18:2(*n*-7) upon the activation of linoleic acid was a mixed-type inhibition.

When the kinetics of linoleic acid activation were studied in the presence of 18:2(*n*-9), at 2.5 μM of inhibitor the intercepts of the double-reciprocal plot gave K_m = 2.5 μM and V_{max} = 7.69 μmoles/hr per mg of protein, and at 15 μM of inhibitor the intercepts gave K_m = 4.26 μM and V_{max} = 7.69 μmoles/hr per mg of protein. Hence the effect of 18:2(*n*-9) upon the activation of linoleic acid was also interpreted as that of a mixed-type inhibition. However it should be noted that this mixed-type inhibition by 18:2(*n*-9) is not as well defined as that by 18:2(*n*-7), since V_{max} was the same at both concentrations of inhibitor.

Discussion

The trapping of acyl-CoA by hydroxylamine was developed to measure acyl-CoA synthetase activity by Kornberg and Pricer (1953) who applied it to fatty acid solutions whose concentration ranged from 0.5 to 20 mM. The activation of fatty acids has been extensively studied by this method, but the requirement for the spectrophotometric assay of hydroxamate has always limited the range of substrate concentrations to the values cited above (Vignais *et al.*, 1958; Brindley and Hubscher, 1966; Pande and Mead, 1968). Mahler *et al.* (1953) found that high concentrations of hydroxylamine were inhibitory to purified acyl-CoA synthetases and described a spectrophotometric assay based on the disappearance of CoASH, but the range of substrate concentrations was the same. Farstad *et al.* (1967) have developed a method in which acyl-CoA formed is quantitatively transformed to acyl-DL-[*methyl*-^3H]carnitine which is extracted by 1-butanol. This assay has also been used in the same range of substrate concentration by these authors and by Van Tol *et al.* (1969). Similarly the assay system of Creasey (1962), in which acyl-CoA are extracted in an ethanol–water–isopropyl alcohol mixture, has been applied to the activation of stearic acid in the range 1–7 mM.

The critical micellar concentrations (cmc) of fatty acid salts and acyl-CoA have been studied by different authors (Corrin *et al.*, 1946; Sakharova and Shutova, 1964; Lhomar and Tookey, 1959; Zahler *et al.*, 1968). Although there are some differences in the value reported because of differences in assay systems, the cmc of most long-chain fatty acids range from 100 μM to 10 mM and that of palmityl-CoA is about 2 to 5 μM (Zahler *et al.*, 1968; Dorsey and Porter, 1968). Therefore most of the studies on acyl-CoA synthetase, which were based on the methods cited above, have been done with substrate concentrations above their cmc. Recently Samuel and Ailhaud (1969) have developed a simple assay method for acyl-CoA synthetase in which they assayed the activation of palmitic and oleic acids at 0.7 mM. This system, which is based on the insolubility of acyl-CoA in diethyl ether, was proven sensitive and accurate for linoleyl-CoA and palmityl-CoA (Table I). The range of substrate concentration is only limited by the specific radioactivity of the substrate: *i.e.*, linoleic acid with a specific activity of 57 μCi/μmole could be assayed at 0.3 μM. However at very low substrate concentrations, the amount of enzyme was also a limiting factor because the latter was not very stable or homogeneously dispersed at low concentration (around 0.1 μg of protein/assay). With this method the activation of linoleic acid could be studied in a wide range of substrate concentrations (0.3–30 μM), which were below the cmc of linoleic acid, which ranges from 100 to 250 μM (Corrin *et al.*, 1946; Lhomar and Tookey, 1959). Barden and Cleland (1969) have found a cmc for linoleyl-CoA of 5.5 μM. Therefore, in our assay system the yield of the reaction was kept below 5 μM of linoleyl-CoA in order to insure that the product of the reaction was below its cmc. The K_m thus obtained for linoleyl-CoA synthetase (2.22 μM) is lower by a factor of 100 to 1000 than any other K_m measured for different acyl-CoA synthetase. Borgstrom and Wheeldon (1961) found K_m of 1.3 and 4 mM for lauryl- and oleyl-CoA synthetase, respectively. Similarly Creasey (1962) obtained a K_m of 0.7 mM for stearyl-CoA synthetase. The important difference between these values and ours is probably related to the methods used. The substrate concentration in the assay of these authors was in the millimolar range whereas ours was in the micromolar range. The maximum velocity measured for linoleyl-CoA synthetase (9.71 μmoles/hr per mg of protein) is certainly lower than the real V_{max} of the reaction because of the noncompetitive inhibition by Triton whose presence in the assay was found to be necessary.

The position of the double bonds in the carbon chain of octadecadienoic acids has a determining effect on the affinity of the long-chain acyl-CoA synthetase for its substrate as measured by the K_m. Indeed, when the position of double bonds was displaced intramolecularly by one atom of carbon either toward the carboxylic group-18:2(*n*-7) or toward the terminal methyl group-18:2(*n*-5), the K_m of the reaction increased by a factor of 10 (Figure 1, Table II). It can, therefore, be concluded that linoleyl-CoA synthetase is an enzyme specific for the $\Delta^{9,12}$ position of the double bonds of linoleic acid. When the double bonds were displaced further along the carbon chain toward the carboxylic group-18:2(*n*-8) and 18:2(*n*-9), the K_m decrease again, and that for 18:2(*n*-9) is almost as low as that for linoleic acid (3.33 μM). These results are particularly interesting since 18:2(*n*-9) is the most abundant isomer of linoleic acid in mammalian tissues. It is synthesized in these organisms by desaturation of oleic acid and it has its double bonds in $\Delta^{6,9}$ position. Therefore the double bond in Δ^9 position, which is common to both 18:2(*n*-6) and 18:2(*n*-9), may be the determining factor for the specificity of the acyl-CoA synthetase. The Δ^9 double bond indeed is present in all of the most abundant unsaturated fatty acids with 16 or 18 atoms of carbon which occur in mammalian tissues, such as palmitoleic, oleic, linoleic, and linolenic acids but the kinetics of activation of these acids remains to be studied.

Among linoleic acid isomers, 18:2(*n*-7), which has its double bonds in $\Delta^{8,11}$, is the product of the desaturation and chain elongation of palmitoleic acid whereas 18:2(*n*-9), which has its double bonds in $\Delta^{6,9}$, is derived from oleic acid by desaturation. These two fatty acids were chosen for the inhibition studies because their effects on activation of linoleic acid could be more relevant under physiological conditions than that of 18:2(*n*-5) or 18:2(*n*-8). Although both isomers behave like mixed-type inhibitors, in the case of 18:2(*n*-7), V_{max} decreases from 9.71 to 7.14 to 2.50 μmoles per hr per mg of protein, while in the case of 18:2(*n*-9) V_{max} decreases only from 9.71 to 7.69 μmoles per hr per mg of protein at both concentrations of inhibitor. Therefore it appears that 18:2(*n*-9) is more of a competitive inhibitor than 18:2(*n*-7) which seems to provide more evidence for importance of the Δ^9 double bond: Because of its Δ^9 double bond, 18:2(*n*-9) competes with linoleic acid more specifically than does 18:2(*n*-7) for the active sites of linoleyl-CoA synthetase.

In conclusion, this study has demonstrated that linoleyl-

CoA synthetase is an enzyme specific for the double bonds in $\Delta^{9,12}$ position, and the Δ^{9} double bond is a main determinant of the specificity. Because we did not investigate the optimum cofactor requirement of long-chain acyl-CoA synthetase for each isomer of linoleic acid, the possible existence of different enzymes for each substrate was not excluded. However this possibility seems remote with fatty acids which do not occur, or occur only as minor constituents in rat liver.

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Studies on the Enzymatic Synthesis of the Cyclic Trimer of 2,3-Dihydroxy-*N*-benzoyl-L-serine in *Escherichia coli*†

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ABSTRACT: The enzyme system in *Escherichia coli* K_{12} responsible for the synthesis of the cyclic trimer of 2,3-dihydroxy-*N*-benzoyl-L-serine from L-serine, ATP, and 2,3-dihydroxybenzoic acid has been resolved into three separate protein components, each essentially free of the other activities. Two covalently bound enzyme intermediates have been isolated and characterized as thio ester linked L-serine and 2,3-dihydroxy-*N*-benzoyl-L-serine, representing the first two stages of the overall synthesis. In addition another enzyme has been resolved which hydrolyzes this trimer, through intermediates, to 2,3-dihydroxy-*N*-benzoyl-L-serine.

The cyclic trimer of 2,3-dihydroxy-*N*-benzoyl-L-serine (DBS)[1] and its breakdown products (Figure 1) are found in the cell-free growth medium of iron-deficient cultures of *Escherichia coli* (Brot *et al.*, 1966; Brot and Goodwin, 1968; O'Brien and Gibson, 1970a), *Salmonella typhimurium* (Pollack and Neilands, 1970) and *Aerobacter aerogenes* (O'Brien and Gibson, 1970b). That this compound may be implicated in iron transport is suggested by the observation that the enzymes responsible for the synthesis of the cyclic trimer were repressed when the organism was grown in an iron-sufficient medium (Brot and Goodwin, 1968; Bryce and Brot, 1971a). Additional evidence is provided by the isolation of iron-transport mutants which are defective in the enzymatic synthesis of *cyclo*-$(DBS)_3$ in *S. typhimurium* (Pollack *et al.*, 1970) and *Escherichia coli* (Cox *et al.*, 1970).

A previous report from this laboratory (Bryce *et al.*, 1971) showed that at least two enzymes were required for the synthesis of *cyclo*-$(DBS)_3$ from 2,3-dihydroxybenzoic acid,

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[1] Abbreviations used are: DBS, 2,3-dihydroxy-*N*-benzoyl-L-serine; *cyclo*-$(DBS)_3$, *cyclo*-tris(2,3-dihydroxy-*N*-benzoyl-L-seryl); DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DON, 6-diazo-5-oxonorleucine.