BBA 3880

STUDIES ON ω-OXIDATION OF FATTY ACIDS *IN VITRO*

I. OVERALL REACTION AND INTERMEDIATE

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SUMMARY

1. ω-Oxidation of straight-chain fatty acids has been demonstrated with cell-free systems of guinea-pig liver. Cell-fractionation studies have shown that the 6000 × *g* supernatant fraction is the most active.

2. Using sorbic acid amide, octatrienoic acid amide, or capric acid amide as substrate, formation of the corresponding dicarboxylic acid monoamide has been detected by paper chromatography, following incubation with the 6000 × *g* supernatant fraction of liver homogenate, together with NAD and the $NADPH_2$-generating system.

3. ω-Hydroxy-fatty acid has been found to be the intermediate compound and the hydroxylating reaction occurs in the microsomes. When [2-^{14}C]sorbic acid amide was incubated with microsomes, the formation of ε-hydroxy [2-^{14}C]sorbic acid amide was detected. Oxidation of ε-hydroxysorbic acid amide to muconic acid amide has been observed with a supernatant fraction of 105000 × *g*.

4. The hydroxylating reaction requires $NADPH_2$ and oxygen. Catalase does not inhibit the reaction. The nature of the hydroxylating reaction is discussed.

INTRODUCTION

VERKADE[1-4] first reported the ω-oxidation of fatty acids in the human body, when the latter was fed with triglycerides of fatty acids of medium chain length. Further nutritional experiments in human and animal bodies showed that some straight-chain[5-7], branched-chain[8-16], substituted[17,18] and unsaturated fatty acids[19,20] and their derivatives were also oxidized at their terminal methyl group. Likewise studies using bacteria have also been reported recently[21]. No experiment, however, has been performed with a cell-free system and the mechanism of ω-oxidation remained obscure until a preliminary report of our work[22] appeared.

We reported briefly that ω-oxidation was observed *in vitro* using a 6000 × *g* supernatant of guinea-pig liver, supplemented with NAD and the $NADPH_2$-generating system. It was also found that ω-hydroxy-fatty acid was an intermediate which furnished experimental support for the speculation given by KUHN[8]. With the use of sorbic acid amide as a substrate, ω-oxidation reaction was separated into two

steps, hydroxylation and dehydrogenation. Hydroxylation was found to proceed in microsomes requiring $NADPH_2$ and oxygen.

The present paper gives experimental details of the work together with some information on the nature of the hydroxylation. The enzyme which is concerned with the dehydrogenation step will be reported in a future communication.

EXPERIMENTAL

Material

The following compounds were obtained from commercial sources: ATP, NAD NADP (Sigma Chemical Company), sodium isocitrate (Nutritional Biochemicals Corporation), [1-^{14}C]decanoic acid (capric acid) (Radiochemical Centre) and [2-^{14}C]-monobromoacetic acid (Daiichi Chemical Company). $NADPH_2$ was prepared by the chemical reduction of NADP. Sorbic acid amide was synthesized chemically, and muconic acid amide was isolated, from the urine of a rabbit administered with sorbic acid amide, by chromatography on a Dowex-1 column as described previously[23]. Octatrienoic acid amide was synthesized chemically according to the method of KUHN *et al.*[19] and octatrienedicarboxylic acid amide was isolated from the urine of a rabbit to which octatrienoic acid amide had been administered. Sebacic acid amide was chemically synthesized from ethyl hydrogen sebacate by its treatment with concentrated ammonia. These compounds were identified by their melting point and their absorption spectra in the infrared or ultraviolet.

Synthesis of [2-^{14}C]sorbic acid amide

A mixture of 280 mg of [2-^{14}C]bromoacetic acid, with a specific activity of 0.125 mC/mmole, 106 mg of Na_2CO_3 and 110 mg of NaCN in 1.1 ml of H_2O were heated for 1 h on a water bath. Then 0.75 ml of NaOH (12 %, w/v) was added and the mixture was heated for a further 3 h on a water bath. After removal of the ammonia by aeration, 1 ml of $CaCl_2$ (22 %, w/v) was added and the mixture was left overnight in a cold room. The calcium malonate thus obtained was dissolved in a small amount of HCl and extracted with ether, using a liquid-extractor. To 149 mg of the free malonic acid, 300 mg of freshly distilled crotonaldehyde and 0.15 ml of pyridine were added and the mixture was heated on an oil bath (120°) for 1 h. After the addition of 40 mg of "carrier" sorbic acid and 1.1 ml of sulphuric acid (7.8 %, w/v), the reaction mixture was left in a cold room for 4 h and the crystals obtained were dried on a porous plate. The crystals were added, together with 50 mg of "carrier" sorbic acid, to 0.5 ml of benzene and 0.1 ml of thionyl chloride and the mixture was refluxed for 1 h. The solvent was removed under reduced pressure and then cold, concentrated ammonia was added. The precipitate which formed was centrifuged, dried well *in vacuo*, and recrystallized from ethyl acetate. Thus 26 mg of [2-^{14}C]sorbic acid amide were obtained. On the radioautogram, a single radioactive spot was found, the R_F value of which was identical with that of the authentic compound.

Synthesis of [1-^{14}C]capric acid amide

An ethereal solution of 68 mg of [1-^{14}C]capric acid (of specific activity, 0.05 mC per mmole) was treated with excess of diazomethane. Methyl caprate, obtained by removal of the solvent and the diazomethane, was dissolved in methanol, saturated with

ammonia and the mixture was then left for 3 days at room temperature. After removal of the solvent and the ammonia, recrystallization from ethyl acetate gave 11.6 mg of [1-^{14}C]capric acid amide.

Synthesis of ε-hydroxysorbic acid amide

A solution of 135 mg of ethyl hydroxysorbate (synthesized according to the method of KARRER *et al.*[24]) in 5 ml of concentrated ammonia solution, was left at room temperature for 5 days. After removal of water under reduced pressure, the residue was recrystallized from methanol and ethyl acetate. White crystalline needles (35 mg) were obtained which melted at 148° and which had the following analysis: Found: C, 56.73; H, 7.13; N, 10.81. $C_6H_9O_2N$ requires: C, 56.69; H, 7.13; N, 10.02 %. The infrared spectrum which shows the presence of hydroxyl and amide groups is given in Fig. 1.

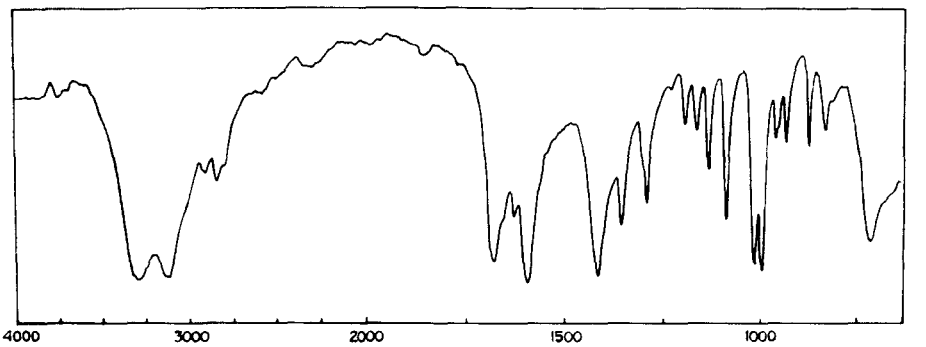

Fig. 1. Infrared absorption spectrum of ε-hydroxysorbic acid amide.

Slices, homogenates, microsomes and soluble fraction

The slices used were prepared from rabbit liver, and the homogenates from guinea-pig liver. The supernatant was obtained by the centrifugation at 6000 × *g* of a 20 % (w/v) homogenate of guinea-pig liver in Krebs–Ringer bicarbonate buffer. In the cell-fractionation study, mitochondria were obtained from the 700 × *g* to the 6000 × *g* fraction and the microsomes from the 6000 × *g* to the 105000 × *g* fraction of the homogenate. Microsomes obtained from a homogenate of 10 g of guinea-pig liver were suspended in Krebs–Ringer bicarbonate buffer and again centrifuged at 105000 × *g* for 1 h. The pellet was resuspended in 4.0 ml of 0.25 M Tris buffer (pH 7.4), and the supernatant was used as the soluble fraction.

Enzymes

Crystalline catalase was the gift of Miss YAGI of this department. Isocitric dehydrogenase was prepared from pig's heart according to the method (step 2) of OCHOA[25].

Reaction mixture and reaction conditions

The formation of muconic acid amide from sorbic acid amide was observed with the reaction mixture containing 30 μmoles of nicotinamide, 5 μmoles of ATP, 20 μmoles of $MgCl_2$, 1 μmole of NAD, 0.5 μmole of NADP, 20 μmoles of sodium isocitrate,

1.8 μmoles of $MnCl_2$, 1 μmole of [2-^{14}C]sorbic acid amide and 2 ml of a 6000 × *g* supernatant of 20 % (w/v) liver homogenate in Krebs–Ringer bicarbonate buffer, all in a total volume of 3 ml. Incubation was carried out in a Warburg vessel at 37° for 3 h in an atmosphere of oxygen.

In the experiment for the paper chromatographic detection of the reaction product when octatrienoic acid amide was the substrate, 2 ml of 6000 × *g* supernatant were incubated with 2.5 μmoles of the substrate supplemented with double the amount of NAD, NADP, nicotinamide, isocitrate and $MnCl_2$ given above, in a total volume of 7.5 ml at 37° for 1 h under air.

In the experiment to determine the amount of octatrienedicarboxylic acid amide formed, the reaction mixture consisted of 30 μmoles of nicotinamide, 0.5 μmole of substrate, 1 μmole of NAD, 0.5 μmole of NADP, 1.8 μmoles of $MnCl_2$, 20 μmoles of isocitrate and 2 ml of 6000 × *g* liver supernatant fraction in a total volume of 3 ml.

In the case of [1-^{14}C]capric acid amide, quadruple amounts of the 6000 × *g* supernatant, and of the NAD, NADP, nicotinamide, isocitrate and $MnCl_2$ given above were incubated for 3 h under air.

To study the dehydrogenation of the ω-hydroxy compound, experiments using slices or the soluble fraction were carried out. 25 slices were incubated with 2.8 mg of ε-hydroxy sorbic acid amide in Krebs–Ringer bicarbonate buffer for 3 h at 37°. When the soluble fraction was used, 6 ml of 105000 × *g* supernatant was incubated at 37° for 3 h with 1.1 mg of ε-hydroxysorbic acid amide supplemented with 3 μmoles of NAD and 70 μmoles of nicotinamide.

For the demonstration of the hydroxylation of [2-^{14}C]sorbic acid amide to [2-^{14}C]ε-hydroxysorbic acid amide, 2 ml of microsomes together with 1.9 mg of isocitric dehydrogenase were used in the mixture instead of the 6000 × *g* supernatant used in the study of [2-^{14}C]muconic acid amide formation.

Paper chromatography

The solvents used were as follows. A, butanol–1.5 N NH_4OH (10:2, v/v); B, amyl- or isoamyl formate–90 % formic acid–water (7:2:0.4, v/v/v); C, isopropanol–0.5 M citrate buffer (pH 5.0)–water (15:1:4, v/v/v); D, butanol–0.5 M citrate buffer (pH 5.0)–water (15:1:1, v/v/v); E, propanol–1.5 N NH_4OH (3:1, v/v).

For the detection of the dienoic compounds, an ultraviolet lamp of 253.6 mμ was used. For the trienoic compounds, the paper strip of Toyo Roshi No. 51 was used, and after the strip had been developed and dried, it was rendered translucent with paraffin. The absorption was then detected at 304 mμ using the Beckman spectrophotometer equipped with a suitable attachment.

For the detection of a radioactive spot of either [2-^{14}C]muconic acid amide or of [1-^{14}C]sebacic acid amide on the paper after development, the paper was cut into strips of 2 cm width. The strip was then cut into cross sections of 1 cm width each all along from the starting point to the solvent front. The radioactivity of each piece was counted in a windowless gas-flow counter.

Examination of the reaction products

The formation of muconic acid amide from sorbic acid amide was demonstrated with the reaction mixture as above, except for the omission of $MnCl_2$. After incubation,

0.5 μmole of the "carrier" muconic acid amide was added and the reaction product was isolated by the use of Dowex-1-Cl column as described in the determination of the reaction product (*vide infra*). The eluates were evaporated *in vacuo* and the residue was examined by paper chromatography. The radioactive spot with R_F value of 0.47 with solvent B was cut out and eluted with 0.1 N NH_4OH. It was again chromatographed using solvent A.

When octatrienoic acid amide was used as the substrate, the reaction mixture was deproteinized and neutralized, and the supernatant thus obtained was directly evaporated to dryness. The hot, ethanolic extract of the residue was concentrated and developed on paper, using solvent C. Spots having absorption at 304 mμ were cut out, extracted with hot ethanol and again developed using solvent C or D.

In the case of [1-^{14}C]capric acid amide, deproteinization and treatment with Dowex-1-Cl column (0.8 cm × 32 cm) was performed in a similar way, and the ethanolic extract of the evaporated eluate was developed by solvents A and E.

Examination of intermediates

After incubation of microsomes and [2-^{14}C]sorbic acid amide as described, 0.5 μmole each of carrier ε-hydroxy sorbic acid amide and muconic acid amide were added, and the reaction mixture was deproteinized, neutralized, and passed through a Dowex-1-Cl column used for the determination. The first 30 ml of effluent, which were radioactive and had strong absorption in the ultraviolet, were evaporated and extracted with ethanol. The extract was concentrated, and chromatographed on paper by solvent A.

Examination of urine

A mouse was injected intraperitoneally with 1 mg of ε-hydroxy sorbic acid amide. The urine was directly spotted on to paper and then developed with solvent A.

Determination of the reaction product

For the determination of [2-^{14}C]muconic acid amide, the reaction was stopped with addition of $HClO_4$, and the reaction mixture was neutralized with KOH. The supernatant obtained was absorbed on Dowex-1-Cl column (50–100 mesh, 0.8 cm × 14 cm). After washing with 300 ml of water, the reaction product was eluted with 50 ml of 1N HCl. The eluate (10-ml sample) was concentrated and dried on a glass planchet; its radioactivity was counted in a windowless gas-flow counter. The infinite thinness of the material thus prepared was checked and certified. In one experiment, a liquid scintillation counter was used for measurement, and a similar result was obtained.

For the determination of octatrienedicarboxylic acid amide, the same Dowex-1-Cl column was used. The column, which adsorbed the deproteinized reaction mixture, was first washed with 100 ml of 20% (v/v) tetrahydrofuran. This was followed by washing with water, and then eluting with 125 ml of 1N NaCl. The ultraviolet absorption of the eluate was measured at 304 mμ.

RESULTS

Demonstration of reaction product with cell-free system

In these experiments a 6000 × *g* supernatant fraction of guinea-pig-liver homogenate was used. The reaction mixture was composed as described in experimental

methods above. Three kinds of fatty acid amide, *i.e.* [2-^{14}C]sorbic acid amide, octatrienoic acid amide, and [1-^{14}C]capric acid amide were used independently as a substrate, and the formation of the corresponding dicarboxylic acid monoamide was detected by paper chromatography together with either its radioactivity or characteristic ultraviolet absorption. The R_F values were found to coincide with those of the corresponding authentic substances (Table I). The formation of monocarboxylic acid by amidase did not occur in these systems.

TABLE I

IDENTIFICATION OF REACTION PRODUCTS BY PAPER CHROMATOGRAPHY

Solvent A, butanol – 1.5 N NH_4OH (10:2, v/v); solvent B, amyl or isoamyl formate – 90% formic acid – water (7:2:0.4, v/v/v); solvent C, isopropanol – 0.5 M citrate buffer (pH 5.0) – water (15:1:4, v/v/v); solvent D, butanol – 0.5 M citrate buffer (pH 5.0) – water (15:1:1, v/v/v); solvent E, propanol – 1.5 N NH_4OH (3:1, v/v).

Substrate	*R_F values of*			
	Substrate	*Reaction product*	*Authentic sample**	*Solvent*
[2-^{14}C]Sorbic acid amide	0.89	0.08	0.08	A
	1.0	0.47	0.47	B
Octatrienoic acid amide	1.0	0.58	0.60	C
	0.93	0.07	0.07	D
[1-^{14}C]Capric acid amide	1.0	0.30	0.31	A
	0.95	0.63	0.63	E

* Authentic samples of the reaction products are the corresponding dicarboxylic acid monoamides, *i.e.* muconic acid amide, octatrienedicarboxylic acid amide and sebacic acid amide, respectively.

Cell-fractionation study

[2-^{14}C]Sorbic acid amide was used as a substrate in these experiments and the activity of each fraction of the homogenized liver-cells was compared under the conditions described above. As shown in Table II, the 6000 × *g* supernatant fraction was found to be the most active one when compared with the mitochondrial or nuclear fractions. Each fraction was diluted to the same volume so that the content of each component is the same as in the original homogenate.

Studies on intermediates

When chemically synthesized ε-hydroxysorbic acid amide was administered intraperitoneally to a mouse, excretion of muconic acid amide in the urine was demonstrated on paper chromatogram as shown in Fig. 2. Spots corresponding to the substrate administered and to the intermediate aldehyde were not found.

When liver slices, or the 105000 × *g* supernatant fraction of liver homogenate, were incubated with ε-hydroxysorbic acid amide, as described in the experimental procedure, the formation of a large amount of muconic acid amide was observed by its ultraviolet absorption (after the reaction mixture had been deproteinized, adsorbed on to and eluted from a Dowex-1-Cl column). Identification of the products was also confirmed by paper chromatography. The intermediate ω-hydroxy-fatty acid seemed to be very easily oxidized within the cell.

Direct evidence for the formation of the ω-hydroxy compound was also obtained by incubating [2-^{14}C]sorbic acid amide with the microsomal fraction of guinea-pig liver. When the reaction product was chromatographed with solvent A as described in the experimental procedure, a large spot, with strong ultraviolet absorption, and

TABLE II

ω-OXIDATION ACTIVITY OF THE FRACTIONATED CELLS

The reaction mixture contained 30 μmoles of nicotinamide, 5 μmoles of ATP, 20 μmoles of $MgCl_2$, 1 μmole of NAD, 0.5 μmole of NADP, 20 μmoles of isocitrate, 1.8 μmoles of $MnCl_2$, 1 μmole of [2-^{14}C]sorbic acid amide and 2 ml of homogenate or fractionated cells in a total volume of 3 ml. Incubation was carried out at 37° for 3 h under oxygen.

Fraction	*Counts/min in Muconic acid amide*
Homogenate	175
Nuclei	83
Mitochondria	64
6000 × *g* supernatant	205
None	41

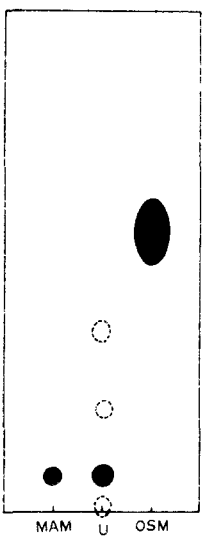

Fig. 2. Paper chromatographic detection of muconic acid amide excreted in the urine of a mouse injected intraperitoneally with 1 mg of ε-hydroxysorbic acid amide. Solvent A was used and the black spot indicates the absorption of ultraviolet rays at 253.6 mμ. The dotted spot was fluorescent. MAM, U and OSM denote muconic acid amide, urine, and ε-hydroxysorbic acid amide, respectively.

small radioactivity, appeared at R_F 0.4–0.6. This spot corresponded to the spot for ε-hydroxysorbic acid amide (and probably nicotinic acid amide also, which was not clearly separated from the former). This spot was cut out and eluted with 0.1 N NH_4OH and again chromatographed by use of solvent B. Two spots appeared, one of which showed a strong ultraviolet absorption but no radioactivity: the R_F value (0.47) of the other smaller spot coincided with that of authentic ε-hydroxysorbic acid amide and possessed weak radioactivity as well as absorption in the ultraviolet. Since

the radioactivity was found in the ε-hydroxysorbic acid amide, ω-oxidation of the [2-^{14}C]sorbic acid amide had clearly taken place.

Nucleotide requirements

As shown in Table III, no decrease in the radioactivity of the muconic acid amide formed was observed when ATP and $MgCl_2$ were removed from the reaction mixture, whereas considerable decrease was found when NAD or the $NADPH_2$-generating system was omitted. Table IV shows that the omission of isocitrate and $MnCl_2$ also decreases the radioactivity, which provides evidence for the participation of $NADPH_2$.

Oxygen requirement

When the complete system was incubated under nitrogen, little activity was found (Table V).

TABLE III

NUCLEOTIDE REQUIREMENT OF ω-OXIDATION

The complete reaction mixture contained 30 μmoles of nicotinamide, 5 μmoles of ATP, 20 μmoles of $MgCl_2$, 1 μmole of NAD, 0.5 μmole of NADP, 20 μmoles of isocitrate, 1.8 μmoles of $MnCl_2$, 1 μmole of [2-^{14}C]sorbic acid amide and 2 ml of 6000 × *g* supernatant in a total volume of 3 ml. Incubation was carried out at 37° for 3 h under oxygen.

System	*Counts/min in muconic acid amide*
Complete	188
NADP, isocitrate and Mn^{2+} omitted	60
NAD omitted	118
ATP and Mg^{2+} omitted	202
No enzyme	43

TABLE IV

EFFECT OF $NADPH_2$-GENERATOR

The complete reaction mixture contained 30 μmoles of nicotinamide, 1 μmole of NAD, 0.5 μmole of NADP, 20 μmoles of isocitrate, 1.8 μmoles of $MnCl_2$, 1 μmole of [2-^{14}C]sorbic acid amide and 2 ml of 6000 × *g* supernatant in a total volume of 3 ml. Incubation at 37° for 3 h under oxygen.

System	*Counts/min in muconic acid amide*
Complete	232
Isocitrate and Mn^{2+} omitted	157
No enzyme	41

TABLE V

OXYGEN REQUIREMENT OF ω-OXIDATION

The conditions were the same as in Table IV. Thunberg tube was used and air in the tube was replaced with nitrogen carefully three times with cooling after each evacuation. Nitrogen was free from oxygen by use of reduced vanadium oxide solution.

Gas phase	*Counts/min in muconic acid amide*
Oxygen	215
Nitrogen	25

Other factors investigated

As shown in Table VI, when the $NADPH_2$-generating system was substituted with $NADPH_2$, no difference in the activity was observed. Addition of catalase had no effect, which excluded the peroxidase activity or the participation of hydrogen peroxide. The decrease caused by the addition of vitamin K_3 probably came from the menadione reductase which oxidized $NADPH_2$. Electron-transport inhibitors such as cyanide or antimycin also gave no effect. Although EDTA did not show any inhibitory effect, the reaction was markedly inhibited by *o*-phenanthroline.

TABLE VI

EFFECT OF ADDITION OR SUBSTITUTION OF SUBSTANCES RELATED TO OXIDATION

The formation of octatrienedicarboxylic acid amide in the standard reaction mixture was taken as 100%. The standard reaction mixture contained 30 μmoles of nicotinamide, 1 μmole of NAD, 0.5 μmole of NADP, 20 μmoles of isocitrate, 1.8 μmoles of $MnCl_2$, 0.5 μmole of octatrienoic acid amide and 2 ml of 6000 × *g* supernatant in a total volume of 3 ml. Incubation was carried out at 37° for 1 h in air. NADP, isocitrate, and $MnCl_2$ were omitted when 0.8 μmole of $NADPH_2$ was added. In other experiments either of the following compounds was added: 0.97 mg of catalase, 0.3 μmole of vitamin K_3, 60 μmoles of cyanide, 8 μg of Antimycin, 3 μmoles of Atebrin, 30 μmoles of ethylenediaminetetraacetate, 3 μmoles of *o*-phenanthroline or 3 μmoles of reduced glutathione.

Added	*Dicarboxylic acid amide formed (%)*
NADPH	104
Catalase	107
Vitamin K_3	20
Cyanide	94
Antimycin	88
Atebrin	67
EDTA	114
o-Phenanthroline	58
Reduced glutathione	74

DISCUSSION

Since the report of experiments on ω-oxidation within the human body in 1933, only nutritional or perfusion studies have been carried out. It was realized early in the present investigation that the reaction diminished markedly when a cell-free system was used, and it was difficult to demonstrate its presence until substrates with radioactivity or with peculiar ultraviolet absorption were used. ω-Oxidation was observed clearly, *in vitro*, when such substrates were incubated with the supernatant fraction of liver homogenate together with NAD and the $NADPH_2$-generating system.

Sorbic acid amide had previously been reported to suffer ω-oxidation to the extent of 32% in experiments on the animal body[19]. Sorbic acid was, however, completely metabolized in the animal body probably by way of β-oxidation. It had also been reported that sorbic acid was readily oxidized when incubated with mitochondria, supplemented with ATP[26]. Introduction of an amide group seemed to augment the ω-oxidation. Furthermore, the use of amide derivatives permitted easy separation of

reaction product from the substrate and they were accordingly used as a substrate. However, because such unsaturated fatty acids are rather peculiar substrates, a doubt arose as to whether this would also hold in the case of simple, straight-chain fatty acids. It was found that the system used here was also effective in the case of capric acid amide.

On paper chromatography, the cleavage of the amide group and the formation of the monocarboxylic acid was not observed. It was reported that some dicarboxylic acids[27–30] were hardly metabolized in the animal body, although their derivatives[7,31] could be metabolized, probably by way of β-oxidation. There is still a possibility that the ω-carboxy-fatty acid amide formed under the conditions studied might be further oxidized by β-oxidation and its detection becomes more difficult. As the homogenate was fractionated and mitochondria which contain the enzymes of β-oxidation were eliminated by differential centrifugation, the identification of the reaction product became easier. The ω-oxidation of sorbic acid amide under the condition reported here thus proceeds to the extent of about 20 %. The oxidation of more highly unsaturated octatrienoic acid amide proceeded to a larger extent which is in accordance with the nutritional experiment of KUHN[19]. This substrate was only used, in the present study, however, in the experiment shown in Table VI, owing to difficulties in the identification of the intermediate, ω-hydroxy derivatives.

ε-Hydroxysorbic acid amide, the supposed intermediate in the ω-oxidation, was synthesized. The enzyme which dehydrogenates this compound was found in the 105000 × *g* supernatant. It was purified and found to be NAD-specific as reported previously[22], and this fact will be elaborated in the following paper. The separation of the reaction which effects hydroxylation of the terminal methyl group from that which effects the succeeding dehydrogenation step was achieved by fractionation of the liver cells into microsomes and supernatant.

From the nucleotide requirement of the overall reaction, it was shown that the dual requirement of $NADPH_2$ and NAD indicated the role of $NADPH_2$ for the hydroxylation, because the successive dehydrogenation of ω-hydroxy compound required only NAD. The properties of the hydroxylating enzyme, which is present in microsomes and requires both oxygen and $NADPH_2$, resemble those of aromatic hydroxylase[32–34], steroid hydroxylases[35,36] and enzymes which are concerned with dealkylation[37] and *N*-oxide formation[38]. Attempts were made to solubilize and to purify this hydroxylating enzyme but without success. Methods reported by IMAI AND SATO[39] for the solubilization of aromatic hydroxylase failed in the present case. Therefore, it is still not clear whether the enzyme concerned is identical with aromatic hydroxylase or other hydroxylating enzymes. Detailed properties of the hydroxylase in the ω-oxidation, which seemed to be one of mixed function oxidases, must await further investigation.

A large-scale experiment using molecular ^{18}O with sorbic acid amide as a substrate was carried out. The muconic acid amide isolated, however, did not contain any significant amount of ^{18}O when measured as CO_2 after pyrolysis. The possible loss of the incorporated ^{18}O by exchange is now under further investigation. Recently, BLOCH[40,41] reported that the biosynthesis of unsaturated fatty acid was also catalysed by the hydroxylating enzyme. It is of interest that the hydroxylation occurs at the C-9, or the C-10, position, which is also a suitable carbon number for the ω-oxidation of straight-chain fatty acids.

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