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Metabolism of Sphingosine Bases, VIII[1–7]

Distribution, Isolation and Properties of D-3-Oxosphinganine Reductase

Stereospecificity of the NADPH-dependent Reduction Reaction of 3-Oxodihydrosphingosine (2-Amino-1-hydroxyoctadecane-3-one)

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Summary: The distribution and specific activities of the NADPH-dependent 3-oxosphinganine reductase in rat liver, brain, heart muscle, skeletal muscle, kidney and spleen has been determined. The enzyme is present in the microsomal fraction and has a lipoprotein structure. It has been purified 84fold from beef liver microsomes.

The MICHAELIS constant for C_{18}-3-oxodihydrosphingosine and for C_{20}-3-oxodihydrosphingosine is $1{,}5 \cdot 10^{-5}$M and $3 \cdot 10^{-5}$M, respectively. The reduction rate of the C_{18}-3-keto compound is almost twice that of the C_{20}-homologue.

Experiments with A-NADP^{3}H and B-NADP^{3}H conclusively proved, that only the B-hydrogen from NADPH is transfered to the carbonyl carbon of 3-oxodihydrosphingosine.

The configuration of the reaction product dihydrosphingosine has been determined. Only the D-isomer of 3-oxodihydrosphingosine is reduced by the B-NADPH dependent reductase yielding D-*erythro*-dihydrosphingosine(2*S*,3*R*-2-amino-1,3-dihydroxyoctadecane). This isomer, specifically labelled by tritium transfer from B-NADP^{3}H to C-3 has been isolated as D-*erythro*-dihydrosphingosine-D-glutamate with 93% of the total [^{3}H]radioactivity. On the basis of the specificity of the hydrogen transfer to C-3 of 3-oxodihydrosphingosine we suggest that the name of the reductase be D-3-oxosphinganine reductase (D-3-oxosphinganine:B-NADPH oxidoreductase).

Zusammenfassung: *Stoffwechsel der Sphingosinbasen, VIII: Verteilung, Isolierung und Eigenschaften der* D-*3-Oxo-sphinganin-Reduktase. Stereospezifität der NADPH-abhängigen Reduktion von 3-Oxo-dihydrosphingosin (2-Amino-1-hydroxy-octadecanon-(3).* Die Verteilung und die spezifischen Aktivitäten der

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Enzymes: D-3-Oxosphinganine reductase, D-3-oxosphinganine:NADP oxidoreductase (EC 1.1.1.? not yet listed); Alcohol dehydrogenase, alcohol:NAD oxidoreductase (EC 1.1.1.1);

Glucose-6-phosphate dehydrogenase, D-glucose-6-phosphate:NADP oxidoreductase (EC 1.1.1.49);

Lactate dehydrogenase (L-lactate:NAD oxidoreductase (EC 1.1.1.27).

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[3] III. Commun.: W. STOFFEL and G. STICHT, this Journal **348**, 1561 [1967].
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NADPH-abhängigen 3-Oxosphinganin-Reduktase in Leber, Gehirn, Herz- und Skelettmuskel, Niere und Milz der Ratte wurden bestimmt. Das Enzym ist strukturgebunden in der microsomalen Fraktion und hat Lipoproteid-Struktur. Es konnte in Lösung gebracht und 84fach angereichert werden. Die MICHAELIS-Konstanten für C_{18}-3-Oxo-dihydrosphingosin sind $1{,}5 \cdot 10^{-5}$M, für C_{20}-3-Oxo-dihydrosphingosin $3 \cdot 10^{-5}$M. Die Reduktion der C_{18}-3-Ketoverbindung verläuft fast doppelt so schnell wie die von C_{20}-3-Oxo-dihydrosphingosin.

Experimente mit spezifisch markiertem A-NADP^{3}H und B-NADP^{3}H haben eindeutig bewiesen, daß nur der B-ständige Wasserstoff von NADPH auf das Carbonyl-Kohlenstoffatom des 3-Oxo-dihydrosphingosins übertragen wird.

Die Konfiguration des Reaktionsproduktes Dihydrosphingosin wurde bestimmt. Nur das D-Isomere des 3-Oxo-dihydrosphingosins wurde durch die B-NADPH-abhängige Reduktase zu D-*erythro*-Dihydrosphingosin (2*S*,3*R*-Dihydrosphingosin) umgesetzt. Dieses Isomere, das spezifisch durch Tritium-Übertragung von B-NADP^{3}H auf C-3 durch die Reduktion markiert wurde, konnten wir als D-*erythro*-Dihydrosphingosin-D-Glutamat mit 93% der Gesamt-Tritiumaktivität isolieren.

Aufgrund der Spezifität der Wasserstoff-Übertragung von B-NADPH auf C-3 des 3-Oxo-dihydrosphingosins schlagen wir als Bezeichnung für die Reduktase vor: D-3-Oxosphinganin-Reduktase (D-3-Oxosphinganin:B-NADPH-Oxydoreduktase (EC 1.1.1.?).

Previous work from this laboratory[1–7] has indicated that the hitherto postulated mechanism of the synthesis of dihydrosphingosine and sphingosine should be discounted. BRADY and his coworkers[8, 9] postulated the condensation of palmitaldehyde and serine to yield dihydrosphingosine and CO_2. Our results[5], however, clearly indicated that palmitoyl-CoA and serine are condensed to yield 3-oxo-dihydrosphingosine. Its structure proved to be identical with the authentic synthetic compound. This intermediate is subsequently transformed into dihydrosphingosine and sphingosine as proven by our *in vivo*[4]—and *in vitro* studies[5]. 3-Oxodihydrosphingosine has been isolated and characterized.

At an early stage of these studies we derived the conclusion that the 3-oxodihydrosphingosine is the key intermediate in the biosynthesis of the long-chain bases. Unfortunately the details of our studies[1–7] escaped the attention of other authors, who also study the degradation and biosynthesis of these long-chain bases[10, 11]. BRAUN and SNELL[11] identified the condensation product of palmitoyl-CoA and serine indirectly by means of the *N*-acetyl-3-oxodihydrosphingosine which confirms our previous results.

The biosynthesis of dihydrosphingosine proceeds by a two step mechanism: a) the condensation of a long-chain acyl-CoA with serine to yield 3-oxo-dihydrosphingosine and b) the reduction of this 3-oxo derivative in a NADPH-dependent reductase-reaction to yield dihydrosphingosine. This dihydrosphingosine proved to have the *erythro* configuration[5].

In continuation of these studies this paper describes a) the distribution, b) the isolation and c) the properties of the reductase and finally d) the stereospecificity of the NADPH-dependent reductase-reaction.

a) Distribution of 3-oxosphinganine reductase

It is well known that sphingolipids, and among them particularly sphingomyelin, are ubiquitous lipid components of membranes of subcellular particles. No information however is available whether these lipids and in particular their long-chain bases are synthesized *in loco* or distributed from synthetically active cell types. We therefore studied the reductase activity in different organs of the rat. Our previous studies indicated that this enzyme is present in the microsomal fraction of rat liver and *Hansenula ciferrii*[5], a yeast well known to produce large amounts of acetylated long-chain bases[12, 13].

The reductase is a NADPH-dependent enzyme which is bound to the membranes of the endo-

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plasmic reticulum. The reduction can be followed a) spectrophotometrically (Fig. 1), and b) by quantitative radio thin-layer chromatography (Fig. 2).

We applied the combination of these two analytical procedures to the determination of the specific activity of 3-oxosphinganine reductase in the following organs of the rat: liver, spleen, muscle, heart muscle, lungs, brain and kidney.

The distribution of the reductase has been investigated by cell fractionation studies in liver and also in heart and brain tissue. By far the highest enzymatic activity has always been proved to reside in the microsomal fraction although we encountered difficulties in obtaining pure brain mitochondrial fractions. As an example the distribution of the reductase in rat heart muscle cells is given in Table 2.

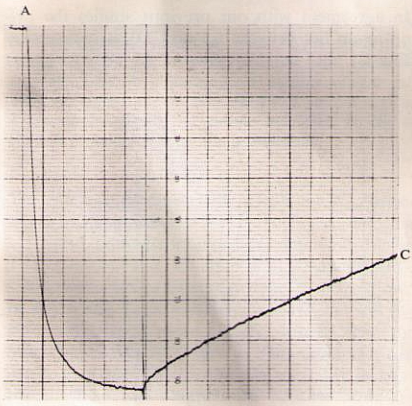

Fig. 1. Spectrophotometric analysis of the 3-oxosphinganine reductase reaction. A: Addition of [1-^{3}H]D-glucose; B: Addition of 3-oxodihydrosphingosine. Chart speed AB: 60 cm/h. BC: 12 cm/h, λ = 340 mμ. The incubation mixture contains in a volume of 2.0 ml: 200 μmoles Tris buffer pH 7.8, 0.1 μmole $MgCl_2$, 0.4 μmole $NADP^{\oplus}$, 0.7 μmole ATP, 0.2 μmole substrate, 0.15 μmole [1-^{3}H]D-glucose, 20 μg hexokinase, 2 μg glucose-6-phosphate dehydrogenase and 80 μg of 3-oxosphinganine reductase.

The distribution studies indicate that the reductase is present in all organs investigated here with highest activity in liver, muscle, brain and lung tissue.

Table 2. Subcellular distribution of 3-oxosphinganine reductase in heart muscle cells.

Cell fraction	Mitochondria	Microsomes	Supernatant
Conversion [%]	4.0	21	3.0

b) Large scale isolation and purification of 3-oxosphinganine reductase

From these screening studies on the distribution of the reductase in rat tissues, liver proved to be the most suitable enzyme source. We therefore isolated from large scale preparations the microsomal fraction of beef liver as described under Experimental.

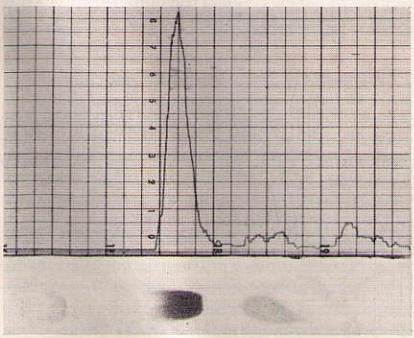

Fig. 2. Radio thin-layer chromatographic analysis of the 3-oxosphinganine reductase reaction, using B-$NADP^3H$ and 3-oxodihydrosphingosine as substrates. Solvent system: chloroform/methanol/water 65:25:4.

A: Dihydrosphingosine (131000 dpm); B: 3-Oxodihydrosphingosine (12200 dpm); C: Front (7300 dpm). The incubation mixture is the same as that given in the legend to Fig. 1.

Table 1. Distribution of 3-oxosphinganine reductase in different organs of the rat. Specific activity is defined in units. 1 mU equals 1 nmole dihydrosphingosine formed by 1 mg of protein per minute.

Organ	Liver	Spleen	Muscle	Heart muscle	Lungs	Brain	Kidney
Spec. act. [mU/mg]	0.73	0.25	0.50	0.48	0.43	0.46	0.125

Table 3. Purification of 3-oxosphinganine reductase.

Purification steps	Protein [mg]	Spec. act. U/mg Protein	Total activity	Yield	Purification factor
Microsomal fraction	12150	0.3	3745	100	1
Triton X-100 Supernatant (105000 × *g*, 60 min.)	7540	0.5	3780	100	1.7
$(NH_4)_2SO_4$ 20—40% Saturation	3230	1.0	3230	89	3.3
Calcium apatite adsorption	320	7.5	2400	66	25
Density gradient	37	25	930	25	84

The stability of the enzyme against the nonionic detergent Triton X-100 facilitated its further purification (see Fig. 3) Triton X-100 clears the incubation mixtures and therefore NADPH oxidation can be followed spectrophotometrically. The enzyme has been enriched 84fold with regard to the activity in the microsomal fraction by the following steps, summarized in Table 3.

c) Properties of 3-oxosphinganine reductase

The enzyme is surprisingly stable even in the presence of high concentrations of the nonionic detergent Triton X-100. Fig. 3 shows the relationship between enzyme activity and concentration of Triton X-100.

In agreement with our previous studies the pH-optimum lies between 6.5 and 7.0 as indicated in Fig. 4.

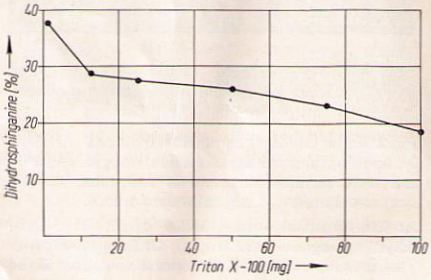

Fig. 3. Effect of Triton X-100 on the activity of 3-oxosphinganine reductase. Each incubation mixture contains in a volume of 2 ml: 200 μmoles phosphate buffer pH 6.8, 2 μmoles $MgCl_2$, 2 μmoles thioglycol, 0.1 μmole substrate, 1 mg liver microsomal protein, 0.5 μmole $NADP^{\oplus}$, 10 μmoles glucose-6-phosphate, 10 μg glucose-6-phosphate dehydrogenase and Triton X-100 as indicated. 37°C, 2 h.

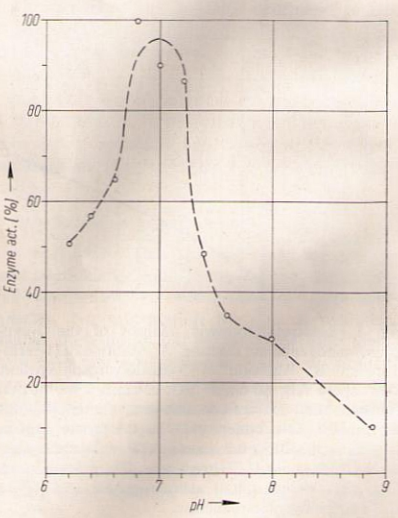

Fig. 4. pH-Optimum of 3-oxosphinganine reductase. Activity expressed as % of the enzymatic activity at pH 6.8. Each incubation mixture contains in a volume of 2,0 ml: 200 μmoles phosphate buffer and Tris buffer, pH 8.9, 0.1 μmole $MgCl_2$, 0.1 μmole substrate, 0.2 μmole NADPH and 80 μg of 3-oxosphinganine reductase.

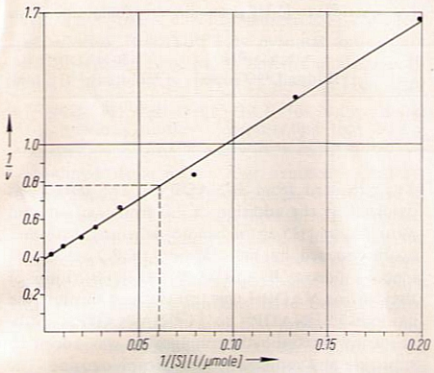

Fig. 5. Determination of the MICHAELIS constant of 3-oxosphinganine reductase with C_{18}-3-oxodihydrosphingosine by the LINEWEAVER-BURK plot. Each incubation mixture contains in a volume of 2.0 ml: 200 µmoles phosphate buffer pH 6.8, 2 µmoles $MgCl_2$, 2 µmoles thioglycol, 0.2 µmole NADPH, 80 µg of enzyme protein and substrate as indicated.

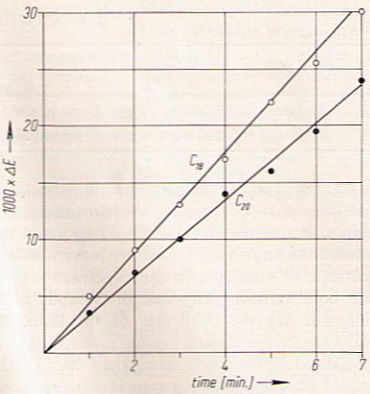

Fig. 6. Differences in reaction velocity between C_{18}- and C_{20}-3-oxodihydrosphingosine. Each incubation mixture contains in a volume of 2.0 ml: 200 µmoles phosphate buffer pH 6.8, 2 µmoles $MgCl_2$, 2 µmoles thioglycol, 0.2 µmole NADPH, 0.1 µmole substrate and 80 µg of enzyme protein.

The MICHAELIS-constant at pH 6.8 for C_{18}-3-oxodihydrosphingosine is $1.5 \cdot 10^{-5}$M (Fig. 5) and that of C_{20}-3-oxodihydrosphingosine around $3.0 \cdot 10^{-5}$M. The kinetics of the reduction of C_{18}-3-oxodihydrosphingosine and C_{20}-3-oxodihydrosphingosine shown in Fig. 6 and Table 4 clearly indicate that the C_{18} keto-derivative is more rapidly reduced than the homologue with 20 C-atoms. This difference can not be explained by the different solubility properties of these two compounds, because both are solubilized in the same manner, but express the different affinities to the enzyme, depending on the different chain lengths of the substrates. The reaction rate was followed spectrophotometrically over a period of 30 min. (Fig. 6) and the reaction products of the two incubations were isolated after 2 h. Table 4 summarizes the analytical data.

Table 4. Analytical data for 3-oxodihydrosphingosines. The reaction rate of C_{20}-3-oxodihydrosphingosine is taken 1.

Substrate concentration	µmoles of dihydrosphingosine formed		ratio of reaction rates
	C_{18}	C_{20}	C_{18}/C_{20}
0.1 µmole	29.1	18.5	1.57
0.2 µmole	22.0	12.6	1.74

d) Stereospecificity of the NADPH-dependent 3-oxosphinganine reductase reaction

Dihydrosphingosine possesses the 2*S*,3*R*-2-amino-octadeca-1,3-diol structure[14–19]. The asymmetric carbon atom 2 of the sphingosine bases is derived from L-serine. On consideration of the absolute configuration of carbon atom 2, theoretically an inversion of the asymmetric C-atom 2 should occur during the decarboxylation condensation. The NADPH-dependent reduction of the carbonyl-group at C-3 of 2*S*-2-amino-1-hydroxyoctadecane-3-one catalyzed by the 3-oxodihydrosphinganine reductase leads to the introduction of the second asymmetric C-atom with *erythro* configuration[5].

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Table 5. Transfer of ^3H from [4-^3H]A-NADP^3H and [4-^3H]B-NADP^3H to [3-^{14}C]3-oxodihydrosphingosine in the 3-oxosphinganine reductase reaction.

	A-NADP^3H		B-NADP^3H	
	^3H [dpm]	^{14}C [dpm]	^3H [dpm]	^{14}C [dpm]
Total radioactivity (dpm) in long-chain bases of the incubation	$1{,}6 \cdot 10^5$	$1{,}675 \cdot 10^4$	$9{,}9 \cdot 10^6$	$1{,}445 \cdot 10^4$
% of total ^3H and ^{14}C-radioactivity in dihydrosphingosine	2,8	31	95	39

However the *erythro* configuration still permits the possibilities of D- or L-*erythro*-dihydrosphingosine structure. It was therefore necessary to prove the absolute configuration of the *in vitro* biosynthesized dihydrosphingosine, to determine whether the D- or L- or both optical antipodes of 3-oxodihydrosphingosine are the substrates of the reductase yielding the corresponding *erythro* compound.

As indicated before never more than 50% of the racemic 3-oxodihydrosphingosine is reduced. However, if the condensation of palmitoyl-CoA and serine is carried out in the presence of NADPH, the condensation product is completely reduced to dihydrosphingosine[5,20]. These observations strongly indicated already that not only does the reduction proceed stereospecifically yielding an *erythro* compound, but also that the condensation leads to the formation of an optically active carbon atom 2 in 3-oxodihydrosphingosine. The following experiments prove that this condensation product, which becomes the substrate for the 3-oxo-sphinganine reductase, possesses D- or *S*-configuration.

Almost all NAD$^\oplus$- and NADP$^\oplus$-linked dehydrogenases and reductases exhibit absolute stereospecificity for either the A or B hydrogen of NADPH or NADPH.

We studied the stereospecificity of the reduction of 3-oxodihydrosphingosine with specifically labelled [4-T]A- and [4-T]B-NADP^3H. From the studies of VENNESLAND *et al.*[21] it is well known, that glucose-6-phosphate dehydrogenase catalyzes the transfer of hydrogen from C-1 of glucose-6-phosphate to the A-side of NADP$^\oplus$. We combined the hexokinase reaction using [1-^3H]glucose and the glucose-6-phosphat dehydrogenase reaction and transferred by this procedure ^3H from [1-^3H]glucose-6-phosphate to the B-side of NADPH[22,23]. 4α-NADP^3H was prepared from B-NADP^3H. The latter was oxidized by the addition of 2.0 μmoles of sodium pyruvate and 25 μg of lactate dehydrogenase and again reduced by the addition of 0.2 μmoles of inactive glucose. By this procedure the B-tritium of the starting NADPH was transformed through the oxidized [^3H]NADP$^\oplus$ to [4-^3H]A-NADPH. Subsequently 3-oxodihydrosphingosine was added as substrate and reduced to dihydrosphingosine. The reactions were followed spectrophotometrically at 340 mμ.

These two stereospecific forms of labelled reduced NADP^3H coenzymes were then tested as hydride donors in the 3-oxosphinganine reductase reaction.

Only the tritium of the B-position of NADP^3H was transfered to the 3-keto-group of 3-oxodihydrosphingosine. Table 5 summarizes these experiments with A- and B-NADP^3H and synthetic [3-^{14}C]3-oxodihydrosphingosine as substrates.

For the generation of B-NADP^3H and the subsequent reduction of 3-oxodihydrosphingosine, the incubation mixtures contained in a total volume of 2.0 ml: 150 μmoles of Tris-buffer pH 7.8, 0.1 μmole of $MgCl_2$, 0.4 μmole of NADP$^\oplus$, 0.1 μmole of [1-^3H]glucose ($4.84 \cdot 10^7$ dpm), 0.7 μmole of ATP, 2 μg of glucose-6-phosphate dehydrogenase, 2 μg of hexokinase, 40 μg of enzyme and 0.1 μmole of [3-^{14}C]3-oxodihydrosphingosine. The mixtures were maintained for 2 h at 37 °C.

Table 5 represents the typical results of one of the repeated experiments. It is obvious from this table, that only the hydrogen atom from the B-side of reduced NADP is transfered stereospecifically to the carbonyl group of 3-oxo-dihydrosphingosine. Since the specific activity of [3-^{14}C]3-oxo-dihydrosphingosine is $2.70 \cdot 10^5$ dpm/μmole and that of [1-^3H]glucose $4.84 \cdot 10^8$ dpm/μmole theoretically one would expect a $^3H/^{14}C$-ratio of 1795:1. After the transfer of ^3H from B-NADP^3H to [3-^{14}C; 3-^3H]3-oxodihydrosphingosine, dihydrosphingosine had a $^3H/^{14}C$ ratio of 1770:1.

When A-NADP^3H is used as labelled coenzyme only a minute ^3H-activity (2.8%) is found in di-

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hydrosphingosine. This results from the incomplete oxidation of B-NADP^{3}H by pyruvate and lactate dehydrogenase. Spectrophotometric analysis indicates that 3% of B-NADP^{3}H remains even after prolonged incubation.

Our previous studies[5] demonstrated that 3-oxodihydrosphingosine is exclusively reduced to *erythro*-dihydrosphingosine. Our synthetic substrate 3-oxodihydrosphingosine is racemic with regard to carbon atom 2. The *erythro* form is the only isomer formed upon enzymatic reduction. The yields of the reduction reaction with DL-3-oxodihydrosphingosine as substrate never exceeded 50%, although optimal reaction conditions have been explored extensively. This indicates, that probably only one isomer of the racemate is reduced. We proved the configuration of the dihydrosphingosine formed in the reductase reaction in the following way:

D- and L-*erythro*-dihydrosphingosine can be separated via the diastereomeric glutamates[24]. The [3-^{3}H]dihydrosphingosine obtained by the enzymatic reduction of DL-3-oxodihydrosphingosine with B-NADP^{3}H was diluted with synthetic radioactive *erythro*-DL-dihydrosphingosine to give a specific activity of 280000 dpm/μmole.

Upon the addition of D-glutamic acid the D-*erythro*-dihydrosphingosine D-glutamate crystallized (mp. 165—176 °C with decomposition) 1. Recrystallization: spec. activity: 489000 dpm/μmole; 2. Recrystallization: spec. activity: 521000 dpm/μmole. The combined mother liquors of these crystallizations were treated with potassium carbonate and the free base isolated. In the presence of L-glutamic acid the L-*erythro*-dihydrosphingosine L-glutamate (mp. 165 to 174 °C with decomposition) crystallized from a 90% ethanol/water mixture. The specific activity of this L-isomer was 24000 dpm/μmole. Therefore the D-form is 22 times more active than the L-form. These data conclusively prove that only the D-isomer of DL-3-oxo-dihydrosphingosine is reduced in the reductase reaction, yielding D-*erythro*-D-dihydrosphingosine, the naturally occurring isomer of dihydrosphingosine. On the basis of our results, with regard to the specificity of the enzyme we name this enzyme D-3-oxosphinganine reductase (D-3-oxosphinganine: NADPH oxidoreductase (EC 1.1.1.? not yet listed).

We gratefully acknowledge the support of this work by the Deutsche Forschungsgemeinschaft.

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Experimental

All coenzymes were purchased from Boehringer, Mannheim GmbH, [1-^{3}H]D-glucose from the Radiochemical Centre, Amersham, Great Britain; [3-^{14}C]C_{18}- and C_{20}-dihydrosphingosines and their respective *N*-acetyl-derivatives were synthesized in this laboratory[3, 25].

Rat liver or beef liver were homogenized in 9 volumes of 0.25M sucrose or 0.1M phosphate buffer, pH 7.0. Cell detritus and mitochondria were removed at 10000×*g*. Microsomes were sedimented at 105000×*g* for 90 min. in a preparative ultracentrifuge. All operations were carried out at 2—4 °C. The microsomal fraction was treated with Triton X-100 at a concentration of 0.5% in a Potter-Elvejhem homogenizer and centrifuged briefly after 15 min. at 105000×*g* for 60 min. The precipitate obtained between 25 and 40% of $(NH_4)_2SO_4$ saturation was collected by centrifugation at 14000×*g* for 15 min., dissolved in phosphate buffer (pH 6.8, 0.1M) and dialyzed against a 0.002M thioglycol solution. The protein solution (9.4 mg/ml) was then treated with calcium apatite. Protein adsorbed onto three parts of calcium apatite (W/W) was discarded after centrifugation. The enzyme of the supernatant (2 mg of protein/ml) was then adsorbed onto six parts of calcium apatite. This was centrifuged and washed with 0.1M phosphate buffer pH 6.8. The enzyme was desorbed with 0.3M phosphate buffer pH 6.8. The enzyme solution (4 mg/ml) was layered on top of a continuous sucrose gradient (density between 1.035 and 1.18) and centrifuged at 65000×*g* for a period of 10 h. The gradient was analyzed and the activity band found at a density of 1.095. The reductase was isolated according to the scheme given in Table 3.

In general the incubations were carried out either with constant shaking in Warburg-vessels or in cuvettes for spectrophotometric analysis. The long-chain bases were extracted with 2—4 ml chloroform/methanol mixtures 2:1, the combined extracts were taken to dryness and directly analyzed or the residue treated with an acetic anhydride-methanol mixture[26]. The *N*-acetyl derivatives of the reaction products were analyzed by thin-layer chromatography. The solvent system for dihydrosphingosines and 3-oxodihydrosphingosines was: chloroform/methanol/water 65:25:4, that for the *N*-acetyl derivatives was: chloroform/methanol 8:1. The radioactivity bands were localized by radio-thin layer chromatography and quantitated by transferring the radioactive bands into counting vials containing scintillator solution.

In general the incubation mixtures contained in a total volume of 2.0 ml: 0.1 μmole of substrate, 0.5 μmole NADPH-generating system (10 μmoles of glucose-6-phosphate, 5 μg of glucose-6-phosphate dehydrogenase,

[25] W. Stoffel, G. Sticht and D. LeKim, to be published.

[26] H. E. Carter and R. C. Gaver, J. Lipid Res. **8**, 391 [1967].

200 μmoles of phosphate buffer, 2 μmoles of $MgCl_2$ and 2 μmoles of thioglycol.

Determination of the configuration of the reduced substrate: DL-3-oxodihydrosphingosine (3 μmoles) was reduced in the presence of B-NADP^{3}H (0.3 μmole, $1.45 \cdot 10^8$ dpm) and 3-oxosphinganine reductase (2 mg) as described before. Dihydrosphingosine ($2.8 \cdot 10^7$ dpm) was isolated and diluted with inactive *erythro*-DL-dihydrosphingosine to give a specific activity of 280000 dpm/μmole. 30 mg (100 μmoles) dihydrosphingosine was dissolved in 1 m*l* of absolute ethanol and 15 mg (100 μmoles) of D-glutamic acid, dissolved in 1 m*l* 50% ethanol was added, the mixture was taken to dryness and the residue dissolved in 2 m*l* of hot 90% ethanol and filtered. The D-*erythro* form of dihydrosphingosine-D-glutamate crystallized at room temperature and was isolated by centrifugation. Two recrystallizations yielded a product with mp. 165—176°C (with decomposition). Spec. activity: 521000 dpm/μmole. The mother liquors were treated with a 10% K_2CO_3 solution and the free base extracted with ether. The ether solution was dried over Na_2SO_4, concentrated and treated with L-glutamic acid as described for the D-isomer.

The L-*erythro* dihydrosphingosine-L-glutamate (mp. 165—174°C with decomposition) had a specific activity of 24100 dpm/μmole).