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2-Alkenal Reductase
Isolation, Properties and Specificities

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Summary: A 2-alkenal reductase has been isolated from the supernatant fraction ($100000 \times g$) of rat liver cells. This soluble enzyme is specific for long chain 2-*trans*- and 2-*cis*-alkenals with a chain length optimum around 14 to 16 C-atoms. The enzyme is NADPH-dependent. The hydride ion is transferred to the double bond from the A-side of the pyridine nucleotide ring. Deuterium from A (pro *R*) $NADP^2H$ is transferred to carbon atom 3 of the substrate 2*t*-hexadecenal yielding [^{2}H]palmitaldehyde. This was reduced with $NaBH_4$ to hexadecanol, which was subjected to mass spectroscopy. Typical fragments containing the deuterium were obtained. The reaction is reversible. Sulfhydryl groups apparently are not involved in the enzymatic reaction.

The affinity of 2-*trans*-alkenals is higher than of 2-*cis* unsaturated aldehydes of the same chain length. The corresponding long-chain 2*t*- and 2*c*-unsaturated alcohols are not reduced by this enzyme. The distribution of the 2-alkenal reductase in different organs of the rat has been determined.

Its importance in the reutilization of 2*t*-hexadecenal originating from 4*t*-sphingenine 1-phosphate during sphingosine catabolism for plasmalogen synthesis has been discussed.

2-Alkenal-Reduktase. Isolierung, Eigenschaften und Spezifitäten

Zusammenfassung: Eine 2-Alkenal-Reduktase wurde aus der $100000 \times g$-Überstandsfraktion der Rattenleber isoliert. Dieses lösliche Enzym ist spezifisch für langkettige 2-*trans*- und 2-*cis*-Alkenale mit einem Kettenlängenoptimum von 14 bis 16 C-Atomen. Das Enzym ist NADPH-abhängig. Das Hydridion wird von der A-Seite des Pyridinnucleotidrings auf die Doppelbindung übertragen. Durch Isocitrat-Dehydrogenase stereospezifisch reduziertes A (pro *R*) $NADP^2H$ überträgt das

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Enzymes:

2-Alkenal reductase, 2-alkenal:NADPH oxidoreductase (EC 1.3.1.?; not yet listed)

Sphingosine*-1-phosphate aldolase (lyase), sphingosine-1-phosphate alkanal-lyase (EC 4.1.2.?; not yet listed)

Sphingosine kinase, ATP:sphingosine 1-phosphotransferase (EC 2.7.1.?; not yet listed)

Glucose-6-phosphate dehydrogenase, D-glucose-6-phosphate:$NADP^{\oplus}$ 1-oxidoreductase (EC 1.1.1.49)

Lactate dehydrogenase, L-lactate:$NAD^{\oplus}$ oxidoreductase (EC 1.1.1.27)

Alcohol dehydrogenase, alcohol:$NAD^{\oplus}$ oxidoreductase (EC 1.1.1.1)

Isocitrate dehydrogenase, *threo*-D_S-isocitrate:$NADP^{\oplus}$ oxidoreductase (decarboxylating) (EC 1.1.1.42).

* Sphingosine is a generic name for all naturally occurring long-chain bases.

Abbreviation:

EGS = ethylene glycol succinate polyester.

Deuterium auf C-3 des Substrats 2*t*-Hexadecenal. Dies konnte durch Massenspektroskopie des Hexadecanols, das aus dem Reaktionsprodukt Palmitinaldehyd durch $NaBH_4$ dargestellt wurde, bewiesen werden. Die Reaktion ist reversibel.
Die Affinität für 2-*trans*-Alkenale ist größer als für 2-*cis*-isomere Aldehyde mit gleicher Kettenlänge. Die entsprechenden 2*t*- und 2*c*-ungesättigten langkettigen Alkohole werden nicht reduziert. Sulfhydrylgruppen scheinen nicht an der Reduktionsreaktion beteiligt zu sein.
Die Verteilung der 2-Alkenal-Reduktase in den verschiedenen Organen der Ratte wurde bestimmt. Die Bedeutung des Enzyms für die Weiterverwertung des im Katabolismus des Sphingosins entstehenden 2*t*-Hexadecenals für die Plasmalogen-Biosynthese wird diskutiert.

The degradation of long-chain sphingosine bases is initiated by the phosphorylation of their primary alcohol groups in an ATP-requiring kinase reaction[1–6]. In the subsequent pyridoxal phosphate-dependent lyase reaction, the phosphate esters are cleaved between carbon atoms 2 and 3, and phosphorylethanolamine and 2*t*-hexadecenal, palmitaldehyde and 2-hydroxypalmitaldehyde are produced from 4*t*-sphingenine, sphinganine and 4*D*-hydroxysphinganine 1-phosphates respectively[1,7–9]. The most abundant long-chain base occurring in complex sphingolipids, particularly of animal cells, is 4*t*-sphingenine. Studies *in vivo* in this laboratory have shown that 4*t*-sphingenine labelled in the alkyl chain is rapidly degraded and that C-3 to C-18 appear as palmitoyl residues in ester lipids[10]. On the other hand, sphinganine degradation supplies the alkyl ether and alkenylether biosynthesis with hexadecanol, as shown in rat brain and *Tetrahymena pyriformis*[11,11a].

In this paper we report on the occurrence of a 2-alkenal reductase in the organs of the rat, its isolation from rat liver, its properties and specificities.

Results

1) Localization of 2-alkenal reductase in rat liver

The availability of chemically synthesized, labelled 2-*trans*- and 2-*cis*-hexadecenal[12] allowed an easy and rapid assay of the different cell fractions of rat liver. It could be demonstrated that only the cytosol fraction (100000 × *g* supernatant) contained alkenal reductase activity. The incubation of this cell fraction with 2*t*-[2,3-$^3H_2$]hexadecenal in the presence of NADPH produced a mixture of four radioactive products in the lipid extract of the fraction, Fig. 1. It consists of the following labelled products: 1. the trimer of palmitaldehyde, 2. hexadecanol, 3. 2*t*-hexadecenal, 4. hexadecanal (palmitaldehyde). The labelled bands cochromatographed with authentic samples. They were further characterized by radio gas chromatography. The trimer of palmitaldehyde was depolymerized by refluxing with 5% methanolic HCl to form palmitaldehyde dimethylacetal, which again cochromatographed with the authentic dimethylacetal on thin-layer plates (solvent system: dichloroethane) and radio gas chromatography. No fatty acid methyl ester could be detected. Since hexadecanol and hexadecenol are not sufficiently separated by thin-layer chromatography (solvent system: dichloroethane), radio gas chromatography was the method of choice (Fig. 2).
It is evident from the analytical data that alcohol dehydrogenase interfers with the alkenal reductase reaction, particularly because an optical test is desired. However, alcohol dehydrogenase is inactivated if the 100000 × *g* supernatant is dialyzed against Tris-maleate buffer (0.05M) pH 5.7 for 40 h.

[1] Stoffel, W., Sticht, G. & LeKim, D. (1968) *this J.* **349**, 1745–1748.
[2] Stoffel, W., Sticht, G. & LeKim, D. (1969) *this J.* **350**, 63–68.
[3] Stoffel, W., Assmann, G. & Binczek, E. (1970) *this J.* **351**, 635–645.
[4] Stoffel, W., Heimann, G. & Hellenbroich, B. (1973) *this J.* **354**, 562–566.
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[6] Keenan, R. W. (1972) *Biochim. Biophys. Acta* **270**, 383–396.
[7] Stoffel, W. & Assmann, G. (1970) *this J.* **351**, 1041–1049.
[8] Stoffel, W. & Assmann, G. (1972) *this J.* **353**, 965–970.
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[10] Stoffel, W. & Sticht, G. (1967) *this J.* **348**, 941–943.
[11] Stoffel, W., LeKim, D. & Heyn, G. (1970) *this J.* **351**, 875–883.
[11a] Stoffel, W., Bauer, E. & Stahl, J. (1974) *this J.* **355**, 61–74.
[12] Stoffel, W. & Melzner, I. (1973) *this J.* **354**, 1626–1632.

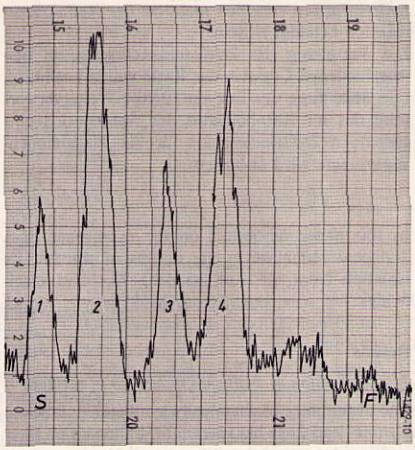


Fig. 1. Radio thin-layer chromatogram of lipid extract of *in-vitro* incubation mixture of 2*t*-[2,3-$^3H_2$]hexadecenal with 100000 × *g* supernatant of rat liver.

The incubation mixture contained in a total volume of 2.0 ml : 0.1 M Tris-HCl buffer, pH 6.8, 0.1 μmol 2*t*-[2,3-$^3H_2$]hexadecenal, 1 mg Triton X-100, 0.3 μmol NADPH and 2.0 mg protein (100000 × *g* supernatant), 37°C, 60 min.

Solvent system: dichloroethane; silica gel H plates. 1 = trimer of palmitaldehyde; 2 = hexadecanol; 3 = 2*t*-hexadecenal; 4 = palmitaldehyde; S = start; F = front.

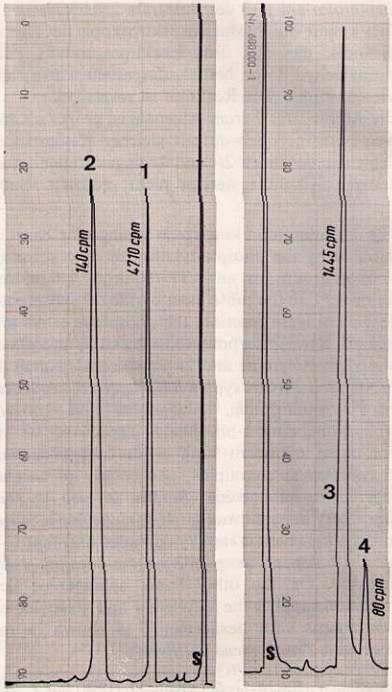


Fig. 2. Radio gas chromatography of radioactive bands 2 and 4 of Fig. 1 together with authentic compounds.

Column conditions: 15% EGS on Kieselguhr, 120 cm, 170°C, 60 ml/min argon. Discontinuous sampling of radioactivity. S = start; 1 = hexadecanal; 2 = 2*t*-hexadecenal; 3 = hexadecanol; 4 = 2*t*-hexadecenol.

The dialyzed protein solution had retained the full reductase activity but lost the alcohol dehydrogenase activity, Fig. 3. The loss is not due to the removal of NADH by dialysis, because this coenzyme does not restore alcohol dehydrogenase activity. NADPH is required for reductase activity, and NADH does not serve as coenzyme.

2) Enzyme assay

The enzyme assay by the optical test (decrease of NADPH absorption at 340 nm) proved reasonable, particularly since the absorption changes very accurately paralleled the control by thin-layer separation and elution of the radioactive bands. In this assay the substrate 2*t*-hexadecenal is injected into the enzyme test solution as an alcohol solution (0.1 μmol/20 μl ethanol).

3) Enzyme properties

Ammonium sulfate fractionation led to the isolation of the reductase activity in the fraction precipitating between 30 and 65% $(NH_4)_2SO_4$ saturation. The specific activity of this fraction is 2.7 nmol × min^{-1} × (mg protein)$^{-1}$. This enzyme preparation served for all studies on the properties and specificities of the 2-alkenal reductase.

The reaction rate increases proportionally to the protein concentration, when the substrate and NADPH concentrations are kept constant (Fig. 4).

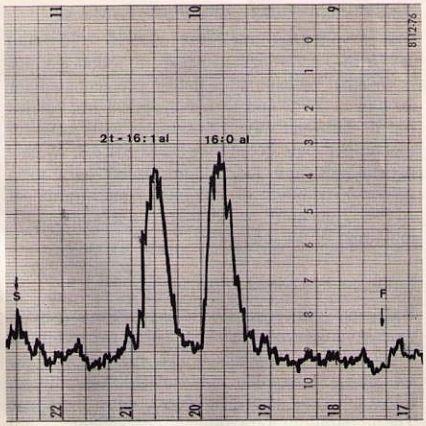


Fig. 3. Radio thin-layer analysis of the extract of an incubation mixture of 2*t*-hexadecenal with dialyzed 100000 × *g* supernatant.

Denaturation of alcohol dehydrogenase is apparent. Incubation as described under Fig. 1.

Solvent system: dichloroethane; silica gel H plates.

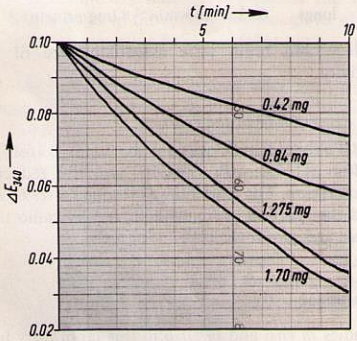


Fig. 4. Proportionality of reaction rate and enzyme concentration.

The complete incubation mixture contained in a 1 cm quarz cuvette in a total volume of 2.0 ml: 0.1 M Tris-HCl buffer, pH 6.8, 0.3 μmol NADPH, 0.1 μmol 2*t*-hexadecenal (added in 20 μl ethanol) and enzyme solution. The amount of protein added is indicated beside the curves. The decrease of absorption at 340 nm was recorded automatically.

The pH optimum is relatively sharp between 6.6 and 7.0. The Michaelis-Menten constant for 2-*trans*-hexadecenal is 1.3×10^{-5} M and for 2-*cis*-hexadecenal 2.6×10^{-5} M.

4) Chain length and substrate specificities

In order to determine the chain length specificity, 2*t*-octenal, 2*t*-decenal, 2*t*-tetradecenal, 2*t*-hexadecenal and 2*t*-octadecenal were incubated under identical conditions and the kinetics of their reduction followed spectrophotometrically. Fig. 5 demonstrates that the chain length optimum is around C_{14} to C_{15}. When the corresponding 2-*trans*-alkenols were used as substrates, no reduction of the double bond could be observed.

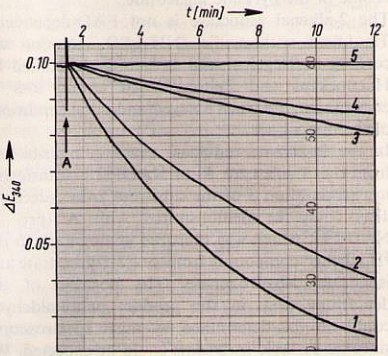


Fig. 5. Chain length specificity of 2-alkenal reductase. Substrates: 1. 2*t*-tetradecenal; 2. 2*t*-hexadecenal; 3. 2*t*-octadecenal; 4. 2*t*-decenal; 5. 2*t*-octenal. Conditions of the assay are those given in the legend of Fig. 4.

5) Stereospecificity of the reduction

A(pro*R*)-NADP^{3}H was prepared biosynthetically according to Cleland *et al.*[13] using [^{3}H]isocitrate [14] (specif. radioactiv. 1.6×10^7 dpm/μmol) and isocitrate dehydrogenase. After the complete reduction of the coenzyme, the pH was adjusted to 6.8, enzyme and 2*t*-hexadecenal added, and the reaction continued for 45 min. The reaction was

[13] Cleland, W. W., Thompson, V. W. & Barden, R. E. (1969) *Methods Enzymol.* **13**, 30–33.

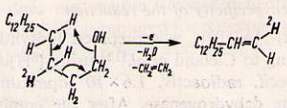
[14] Lowenstein, J. M. (1963) *Methods Enzymol.* **6**, 874–876.

then stopped in the usual way, the aldehydes extracted and analyzed by radio thin-layer and gas chromatography.

B(pro*S*)-NADP^{3}H, labelled according to Pastore *et al.*[15] and Palm *et al.*[16] by the combined hexokinase:glucose-6-phosphate dehydrogenase reaction with [1-^{3}H]glucose as substrate, had a specif. radioactiv. of 1.1×10^9 dpm/μmol. This coenzyme was used in the enzymatic reaction under the conditions described for A-NADP^{3}H. The result was as follows: 40 – 50% of the theoretical amount of ^{3}H was transferred within 30 min from the A side of NADP^{3}H to yield [^{3}H]palmitaldehyde, but only 0.1 – 0.2% of the ^{3}H-radioactivity from the B side. Therefore it must be concluded that the hydride transfer occurs stereospecifically from the A-side of the pyridine nucleotide.

The 2-alkenal reductase is not FAD-dependent. Treatment with acidic $(NH_4)_2SO_4$ solution according to Negelein and Brömel[17], modified by Huennekens and Felton[18] led to no loss of enzymic activity when assayed under the conditions of the optical test.

In the 2*t*-alkenal reductase reaction hydride ion from the A side (pro *R*) of NADPH is transferred to carbon atom 3 of the substrate 2*t*-hexadecenal. This could be demonstrated with A (pro *R*) NADP^{2}H, which was prepared analogously to the ^{3}H labelled compound from DL-[2-*D*]isocitrate and isocitrate dehydrogenase. The position of the deuterium atom in the product palmitaldehyde could not be determined by mass spectroscopy, since no suitable fragments could be expected. We therefore reduced palmitaldehyde with $NaBH_4$ to hexadecanol, which was subjected to mass spectroscopy. Typical fragments were obtained: *m/e* (*M* – 18) 225 and *m/e* [*M* – 18 – 28 ($H_2C=CH_2$)] 197 according to the following underlying fragmentation scheme:



[15] Pastore, E. J. & Friedkin, M. (1961) *J. Biol. Chem.* **236**, 2314 – 2316.

[16] Palm, D., Rambeck, W. & Simon, H. (1968) *Z. Naturforsch.* **23b**, 881 – 882.

[17] Negelein, E. & Brömel, H. (1939) *Biochem. Z.* **300**, 225.

[18] Huennekens, F. M. & Felton, S. P. (1957) *Methods Enzymol.* **3**, 950 – 959.

6) Reductase activity and sulfhydryl reagents

The enzyme preparation was made 10^{-5}, 10^{-4} and 10^{-3} M in *p*-chloromercuribenzoate, iodoacetamide or *N*-ethyl maleinimide. It was preincubated for 20 min at 37°C and then the optical test carried out in the usual way. The samples with *p*-chloromercuribenzoate became turbid at pH 6.8, *N*-ethyl maleinimide and iodoacetamide led only to a maximal inhibition of 15% at a concentration of 10^{-3} M. This must be regarded as a non-specific inhibition of the reductase.

7) Organ distribution of the enzyme

Rat liver, kidney, spleen, lungs, heart and brain were homogenized according to the wet weight in 9 vol. of 0.1 M Tris-HCl buffer, pH 6.8, and the $100000 \times g$ supernatant was isolated and dialyzed. Equal amounts of protein were used in the assay. The preparations were routinely checked for alcohol dehydrogenase activity, but none of the enzyme preparations contained any remaining activity after the usual dialysis.

The specific activities were determined as follows:

liver	1.7 nmol × min^{-1} × (mg protein)$^{-1}$
heart	0.91 nmol × min^{-1} × (mg protein)$^{-1}$
kidney	0.29 nmol × min^{-1} × (mg protein)$^{-1}$
lungs	0.12 nmol × min^{-1} × (mg protein)$^{-1}$

Spleen and brain were essentially free of this activity.

8) Reversibility of the reaction

[^{3}H]Palmitaldehyde was incubated in 0.16 M Tris buffer pH 7.4 with NADP$^{\oplus}$ and the reductase preparation. The reaction was stopped after 60 min and the reaction products analyzed by radio thin-layer and gas-liquid chromatography.

Discussion

Studies *in vivo* and *in vitro* in this laboratory have unambigiously proven that the long-chain sphingosine bases are degraded in an aldolase type lyase reaction of the phosphorylated bases, yielding phosphorylethanolamine and the alkane chain from carbon 3 to the terminal methyl group as aldehyde. This enzyme is highly specific for the 2*S*,3*R*-configuration[19]. The most abundant long chain base

[19] Stoffel, W. & Bister, K. (1973) *this J.* **354**, 169 – 181.

in animal tissues is 4*t*-sphingenine. It yields 2*t*-hexadecenal. This aldehyde must be transformed into palmitic acid, because studies *in vivo* demonstrated that sphingenine labelled in the alkyl chain is recovered as palmitate and its elongation product, stearate, from ester lipids (triglycerides, phospholipids) and amide groups (ceramide, sphingomyelin *etc.*). Also, the well documented reutilization of palmitaldehyde, released during the degradation of sphinganine, for alkylglyceryl ether and plasmalogen biosynthesis suggested the investigation of the sequence of events in the utilization of 2*t*-hexadecenal in the cell.

The chemical synthesis of labelled 2-*trans*- and 2-*cis*-alkenals[12] enabled us to study this reaction *in vitro*. It became obvious from rat liver fractionation studies that the supernatant ($100000 \times g$) fraction contained two enzymes acting on these substrates, *e.g.* 2*t*-hexadecenal, which was studied particularly because of its relation to 4*t*-sphingenine metabolism. These enzymes are a 2-*trans*-alkenal reductase and an alcohol dehydrogenase. The latter reduces long chain aldehydes and has been described previously[20].

The 2*t*-alkenal reductase, which was isolated in the 30 to 65% $(NH_4)_2SO_4$ fraction, has an absolute requirement for NADPH as hydrogen donor. NADH is completely inactive. It reduces the 2-*trans* double bond to the alkanal. Although its pH optimum lies around 6.8, it is rather active at pH 7.4.

The alcohol dehydrogenase is associated with the reductase, and reduces the carbonyl group to the alcohol group unless it is eliminated by a simple treatment such as dialysis at slightly acidic pH. All attempts to find FAD as an additional coenzyme participating in the alkenal reduction were unsuccessful (see Experimental), Furthermore, sulfhydryl groups are not essential for the reduction. Therefore the binding of the aldehyde as thiohemiacetal seems highly unlikely. Further studies regarding the purification of the enzyme might give insight into the binding mechanism.

That the transfer of the hydride ion from NADPH occurs stereospecifically from the A-side of the ring system.

The 2-alkenal reductase exhibits a chain-length specificity around C_{14} and C_{15}, which also supports its immediate physiological relevance for the metabolism of sphingosine bases.

[20] Stoffel, W. & LeKim, D. (1971) *this J.* **352**, 501–511.

2-*trans*-Alkenols do not serve as substrates for the reductase.

The K_m determinations revealed that 2-*trans*-alkenals have a higher affinity for the enzyme than the 2-*cis* isomers. The highest enzymic activity was determined in rat liver, followed by heart, kidney and lungs. Surprisingly, the brain and spleen $100000 \times g$ supernatant fractions and their homogenates showed no reductase activity, an observation which requires further studies.

We gratefully acknowledge the financial support by the *Deutsche Forschungsgemeinschaft*.

Experimentals

Labelled and unlabelled 2-*trans* and 2-*cis* long-chain aldehydes and alcohols were synthesized as described in the preceding paper[12]. Specific activity of 2*t*-hexadecenal was 1.9 mCi/mmol. The enzyme assays were carried out as follows:

a) Radio thin-layer analysis: 0.1 μmol 2*t*-hexadecenal dissolved in 2.0 m*l* 0.1M Tris-HCl buffer, pH 6.8, containing 1 mg Triton X-100 was incubated with 0.3 μmol NADPH and 2.0–2.5 mg protein at 37°C for 60 min. The reaction was stopped by the addition of 3.0 m*l* of a mixture of chloroform/methanol 2:1. Extraction was completed by vigorous shaking. The lower phase was filtered and concentrated to dryness in a stream of nitrogen at 35°C. The complete sample was chromatographed in dichloroethane. The radioactive bands were recovered from the thin-layer plates immediately after their separation by the method of Goldrick and Hirsch[21]. The silica gel was eluted with chloroform/methanol 2:1 and the fractions analyzed by radio gas chromatography (15% EGS on Kieselguhr, 2 m column at 170°C). Hexadecenal separated well under these conditions from palmitaldehyde, and hexadecenol from hexadecanol (Fig. 2).

b) Optical assay: The incubation mixture consisting of 2.0 m*l* 0.1M Tris-HCl buffer pH 6.8, 0.3 μmol NADPH and 1.0–2.0 mg protein was preincubated for 15 min at 37°C. The reaction was started by the injection of 0.1 μmol 2*t*-hexadecenal dissolved in 20 μ*l* 96% ethanol and the decrease of the extinction at 340 nm automatically registered with a PMQ II spectrophotometer, Zeiss. The change in the extinction corresponded within 1% to the radioactivity recovered from the palmitaldehyde band (including the trimer at the origin) in thin-layer chromatography from the same incubation. The correlation between enzyme concentrations and reaction rates was established at protein

[21] Goldrick, B. & Hirsch, J. (1963) *J. Lipid Res.* **4**, 482–483.

concentrations of 0.425, 0.85, 1.28 and 1.70 mg/2 ml and constant concentrations of 2*t*-hexadecenal (0.1 μmol) and NADPH (0.3 μmol).

Cell fractionation was carried out according to Hogeboom[22]. Alcohol dehydrogenase was inactivated by dialysis of the 100000 × *g* supernatant fraction against 0.05M Tris-maleate buffer, pH 5.7, for a period of 40 h. This loss of enzymic activity is not due to dialysis of $NAD^{\oplus}$/NADH, because the addition of NADH did not restore the alcohol dehydrogenase activity. The pH optimum was determined with Tris-maleate buffers at pH 8.6, 8.0, 7.4, 7.0, 6.8, 6.6 and 6.4.

Radioactive A-NADP^{3}H was prepared according to Cleland *et al.*[13] using *threo*-D_SL_S-isocitrate, $NADP^{\oplus}$ and pig heart isocitrate dehydrogenase. [2-^{3}H]Isocitrate was synthesized according to J. M. Lowenstein[14]. The chemical synthesis yields four stereoisomers, of which only [2-^{3}H]*threo*-isocitrate is the substrate.

B-NADP^{3}H was obtained following the procedure of Pastore *et al.*[15] and Palm *et al.*[16] using the combined hexokinase:glucose-6-phosphate dehydrogenase reactions with [1-^{3}H]glucose as substrate.

The distribution of 2*t*-alkenal reductase in the organs of the rat was studied with the 100000 × *g* supernatant of liver, heart, lungs, spleen, brain and kidney. The tissue mince was homogenized with a Potter homogenizer and immediately centrifuged at 100000 × *g*. Protein was determined with the Lowry procedure[23], radioactivity in a Tricarb Scintillation Counter, Packard, model 3380, thin-layer plates scanned with a Berthold, model LB 2722 or Packard thin-layer scanner, model 7201.

The reversibility of the reaction was studied under the conditions of the optical assay at pH 7.4 using $NADP^{\oplus}$ and palmitaldehyde. The reaction product 2*t*-hexadecenal was characterized by radio thin-layer chromatography and gas-liquid chromatography.

[22] Hogeboom, G. H. (1955) *Methods Enzymol.* **1**, 16–19.

[23] Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.