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The Metabolism of Sphingosine Bases in *Tetrahymena pyriformis*

Sphingosine Kinase and Sphingosine-1-phosphate Lyase

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Summary: *Tetrahymena pyriformis* rapidly depletes the medium of labelled long chain sphingosine bases and metabolizes these bases. The degradation products of the alkyl chains (C-3 to C-18) of sphinganine and 4*t*-sphingenine appear as fatty acids (mainly palmitic acid) in ester lipids and as alkyl groups in 2-acyl-1-hexadecyl-glycero-3-phosphorylcholine and in 2-acyl-1-hexadecyl-glycero-3-aminoethylphosphonate of the phosphatidylethanolamine fraction. [^{18}O]Hexadecanol utilization by this organism for alkyl ether formation was also studied. ^{18}O is retained and the complete alcohol molecule incorporated into the aforementioned ether lipids.

Studies *in vitro* on the catabolism of labelled sphingosine bases combined with cell fractionation studies revealed a sphingosine kinase, which phosphorylates the primary hydroxy group, and a sphingosine-1-phosphate aldolase (lyase), which cleaves the phosphate ester of the long-chain bases to phosphorylethanolamine and the corresponding long-chain aldehyde. Cell fractionation studies localized these enzymes in the microsomal membrane fraction. Their specificities (enantiomeric form of substrate, coenzyme requirement) and properties (pH-optimum, K_m and susceptibility to sulfhydryl reagents) have been determined. An alcohol dehydrogenase present in the $100000 \times g$ supernatant of *Tetrahymena* reduces the long-chain aldehyde, usually palmitaldehyde, arising in the curse of degradation of the long-chain bases to the long chain alcohol, which is then available for alkyl ether synthesis. Chimyl alcohol is the only alkyl ether formed in *Tetrahymena pyriformis*.

Stoffwechsel von Sphingosinbasen in Tetrahymena pyriformis. Sphingosin-Kinase und Sphingosin-1-phosphat-Lyase

Zusammenfassung: *Tetrahymena pyriformis* nimmt aus dem Nährmedium rasch markierte langkettige Sphingosinbasen auf und metabolisiert diese Basen. Die Abbauprodukte der Alkylkette (C-3 bis C-18) des Sphinganins und 4*t*-Sphingenins erscheinen als Fettsäuren in den Esterlipiden oder als Alkylgruppen in 2-Acyl-1-hexadecyl-glycero-3-phosphorylcholin und in 2-Acyl-1-hexadecyl-glycero-3-aminoäthylphosphonat in der Phosphatidyläthanolamin-Fraktion.

Hexadecanol, das von diesem Organismus für die Alkylätherbildung verwendet wird und ein Inter-

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Enzymes:

Sphingosine kinase, ATP:sphingosine 1-phosphotransferase (EC 2.7.1.?; not yet listed)
Sphingosine-1-phosphate lyase, sphingosine-1-phosphate alkanal-lyase (EC 4.1.2.?; not yet listed)
Alcohol dehydrogenase, alcohol:NAD oxidoreductase (EC 1.1.1.1)
2-Alkenal reductase, 2-alkenal:NADPH oxidoreductase (EC 1.3.1.?; not yet listed).

Note:

Sphingosine stands for all long chain bases.

mediärprodukt im Abbau des Sphingosins und der Synthese der Alkylglycerinäther darstellt, wurde ^{18}O-*markiert* und im Hinblick auf mechanistische Studien als Substrat verwendet. Der Chimylalkohol der Alkylglycerinätherfraktion enthält das ^{18}O. Somit wird das gesamte Hexadecanolmolekül eingebaut.

In-vitro-Untersuchungen in Verbindung mit Zellfraktionierungen zum Katabolismus der markierten Sphingosinbasen ergaben, daß eine Sphingosinkinase zuerst die primäre Alkoholgruppe phosphoryliert und dann eine *Sphingosin-1-phosphat*-Aldolase (Lyase) den Ester zu Phosphoryläthanolamin und dem entsprechenden langkettigen Aldehyd, meist Palmitinaldehyd, spaltet. Es handelt sich bei beiden Enzymen um mikrosomale Enzyme. Ihre Spezifitäten (enantiomere Form der Substrate, Coenzym-Abhängigkeit) und Eigenschaften (pH-Optimum, K_m und Empfindlichkeit gegen SH-Reagenzien) wurden bestimmt.

Eine *Alkohol-Dehydrogenase*, die sich im 100000 $\times g$-Überstand von *Tetrahymena* befindet, reduziert die langkettigen Aldehyde z. B. Palmitinaldehyd, der beim Abbau der langkettigen Basen anfällt, zum Alkohol, der dann für die Alkyläthersynthese Verwendung findet. Chimylalkohol ist der einzige Vertreter.

Recent analytical studies on the chemical composition of the lipids of *Tetrahymena pyriformis* have yielded a rather detailed picture[1–3]. Regarding the phosphorus-containing lipids, two remarkable findings should be mentioned: 1) besides the ordinary phospholipids cardiolipin, phosphatidylcholine and phosphatidylethanolamine, phosphonolipids are present, in which the 3-position of glycerol is substituted with the aminoethylphosphonate group, and 2) not only the 1,2-diacylglyceryl derivatives but large amounts of 2-acyl-1-alkylglycerol species are present in the phosphatidylcholines and the phosphonates in the phosphatidylethanolamine fractions.

The only alkyl glycerol ether species is chimyl alcohol (*1-hexadecylglycerol*). Furthermore, branched chain C_{17}- and C_{19} 4*t*-sphingenines have been isolated and characterized in the ceramide and ceramide aminoethylphosphonate fractions[4]. The latter sphingolipid has also been described in marine plants[5,6].

Only a few studies on the phospholipid metabolism in *Tetrahymena pyriformis* have been carried out. Thompson[7] observed a rapid incorporation of palmitate and chimyl alcohol into the phosphatidylcholine fraction. From the slow transfer of the two into the aminoethylphosphonates he concluded that phosphatidylcholine might be the precursor for the O-P-C bond of these compounds. Smith and Law[8] suggested from their experiments with labelled *S*-adenosylmethionine that only the 1,2-diacyl-3-glycerophosphorylethanolamine fraction can be methylated.

Dennis and Kennedy[9], who studied the pathway of the serine and ethanolamine containing phospholipids in *Tetrahymena*, arrived at the conclusion that phosphatidylserine is formed by a base exchange reaction between phosphatidylethanolamine and L-serine, and that the decarboxylation of phosphatidylserine can lead to phosphatidylethanolamine.

Studies in this laboratory[10–12] with the specifically labelled long-chain bases sphinganine, 4*t*-sphingenine and 4*D*-hydroxysphinganine have demonstrated that their degradation yields palmitaldehyde, 2*t*-hexadecenal and 2-hydroxypalmital-

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[1] Thompson, G. A., Jr. (1967) *Biochemistry* **6**, 2015–2022.

[2] Berger, H., Jones, P. & Hanahan, D. J. (1972) *Biochim. Biophys. Acta* **260**, 617–625.

[3] Smith, J. D., Snyder, W. R. & Law, J. H. (1970) *Biochem. Biophys. Res. Commun.* **39**, 1163–1169.

[4] Carter, H. E. & Gaver, R. C. (1967) *Biochem. Biophys. Res. Commun.* **29**, 886–891.

[5] Rouser, G., Kritchewsky, G., Heller, D. & Lieber, E. (1963) *J. Amer. Oil Chem. Soc.* **40**, 425–454.

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[7] Thompson, G. A., Jr. (1969) *Biochim. Biophys. Acta* **176**, 330–338.

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[9] Dennis, E. A. & Kennedy, E. P. (1970) *J. Lipid Res.* **11**, 394–403.

[10] Stoffel, W., Sticht, G. & LeKim, D. (1968) *this J.* **349**, 1745–1748.

[11] Stoffel, W. & Assmann, G. (1970) *this J.* **351**, 1041–1049.

[12] Stoffel, W. & Assmann, G. (1972) *this J.* **353**, 965–970.

dehyde, all three representing carbon atoms 3 to 18 of the bases and phosphorylethanolamine (C-1 and C-2). The two degradation products are utilized in the *de novo* synthesis of phospholipids; the aldehydes are either oxidized to palmitate and incorporated in ester lipids, or reduced to hexadecanol. Palmitaldehyde is reduced directly; and 2*t*-hexadecenal is reduced to palmitaldehyde by a 2*t*-alkenal reductase and then to hexadecanol by an alcohol dehydrogenase[13]. We have also demonstrated that in myelinating brain, palmitaldehyde originating from sphinganine is reduced to hexadecanol and incorporated *via* the alkyl ether derivative into plasmalogens of the brain[14,15].

In order to study the correlation of sphingosine metabolism and phospholipid synthesis under more controlled conditions than is possible with myelinating rat brain, *Tetrahymena pyriformis* was chosen. There was evidence from analytical data of the simultaneous occurrence of sphingolipids and alkyl ether-containing phospholipids.

As in nervous tissue, the simultaneous occurrence of sphingolipids and phosphatidylethanolamine, predominantly 2-acyl-1-alkyl-glycerolaminoethylphosphonate, which is related to plasmalogens, is observed in *Tetrahymena*.

As demonstrated for myelinating brain, the association of these lipids may also reflect the metabolic relations in this organism.

In this paper we describe 1) the studies *in vivo* on the metabolism of the long-chain sphingosine bases sphinganine and 4*t*-sphingenine in *Tetrahymena pyriformis*, and 2) the degradation *in vitro* of the long-chain bases with enzyme preparations from this organism, namely the sphingosine kinase, sphingosine-1-phosphate aldolase and alcohol dehydrogenase.

Results

I. Studies in vivo on the metabolism of long-chain bases in Tetrahymena pyriformis

1) [3-^{14}C] and [3-^{3}H]D(+)-erythro-sphinganine

In order to study the metabolic fate of the long-chain sphingosine bases D(+)-*erythro*-sphinganine labelled at C-3 with either ^{14}C or ^{3}H was added as supplement to the medium of cells growing in the log-phase; after 24 h the cells were harvested and the total lipids extracted. 53% of the radioactive substrate had been incorporated into the cells. Silicic acid column chromatography separated the mixture into three fractions: a) the "neutral lipid" fraction, b) the phosphatidylethanolamine fraction, which also contained the phosphonate fraction, and c) the phosphatidylcholine fraction.

The "neutral lipid" fraction (20% of recovered total radioactivity) consisted of radioactive ceramide, hexadecanol, neutral lipids and cardiolipin. The latter two yielded, on alkaline hydrolysis, 38% of their radioactivity as fatty acids. The phosphatidylethanolamine fraction (68% of the total radioactivity) was separated into the phosphono- and phospho-derivatives by preparative thin-layer chromatography using the solvent system of Artom[16] and Kapoulas[17]. Free sphinganine was also separated from phosphatidylethanolamine in this system, but a better separation of sphinganine from phosphatidylethanolamine was achieved in the system chloroform/methanol/conc. NH_4OH/water 75:25:2.5:2.5.

The phosphatidylethanolamine fraction free of sphinganine contained 34% of the total incorporated radioactivity, and of this activity, 57% is found in the aminoethylphosphonates.

The phosphatidylcholine fraction represented 12% of the incorporated radioactivity.

Alkyl ethers were only present in the phosphonocephalin and phosphatidylcholine fractions. The analyses are based on the isolation of labelled alkylglycerol after $LiAlH_4$ reduction of the two phospholipid fractions by preparative thin-layer chromatography and are represented by the radio thin-layer analysis of the total lipid extract (Fig. 1a), the $LiAlH_4$-hydrolysate of the phosphatidylethanolamine (1b), the aminoethylphosphonate fraction (1c) and the phosphatidylcholine (1d). Chimyl alcohol and fatty alcohols were the only radioactive products. The radio thin-layer scan of the purified chimyl alcohol and hexadecanol fraction is given in Fig. 2.

The alkyl glycerol proved to be solely chimyl alcohol, characterized by radio gas-liquid chromatography and combined mass spectroscopy of

[13] Stoffel, W. & Därr, W. (1974) *this J.* **355**, 54–60.

[14] Stoffel, W., LeKim, D. & Heyn, G. (1970) *this J.* **351**, 875–883.

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

[16] Artom, C. (1964) *Biochem. Biophys. Res. Commun.* **15**, 201–206.

[17] Kapoulas, V. (1969) *Biochim. Biophys. Acta* **170**, 324–329.

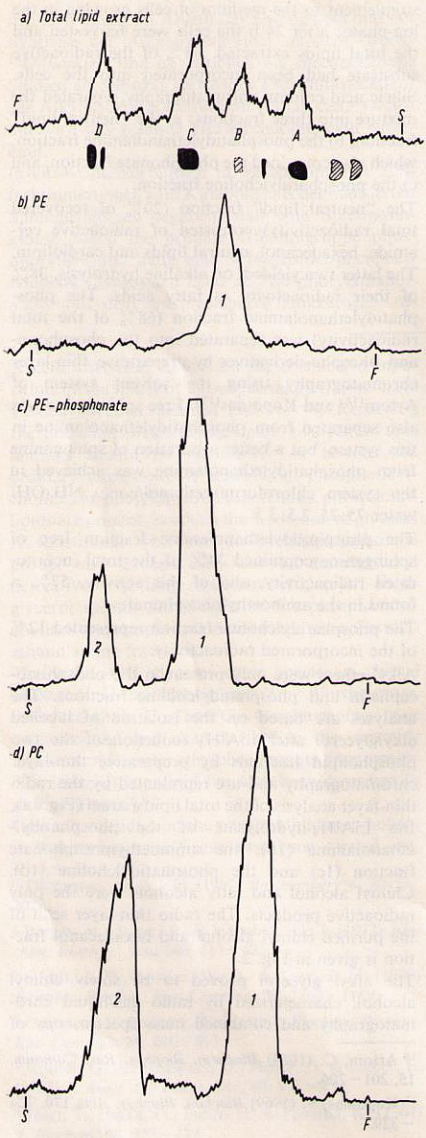

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Fig. 1a. Radio thin-layer scan of total lipid extract of *Tetrahymena pyriformis* after growth on a medium containing [3-^{3}H; 3-^{14}C]sphinganine.

A = Phosphatidylcholine fraction; B = sphinganine (substrate); C = aminoethylphosphonate and phosphatidylethanolamine; D = cardiolipin, triglyceride, palmitaldehyde; S = start; F = front. Solvent system: chloroform/methanol/water 65:25:4.

Fig. 1b–d. Reaction products after $LiAlH_4$ reduction of phospholipid fractions.

1b) phosphatidylethanolamine; 1c) phosphatidylethylphosphonate; 1d) phosphatidylcholine. 1 = Hexadecanol; 2 = chimyl alcohol. Solvent system: ether/conc. ammonia 100:0.25.

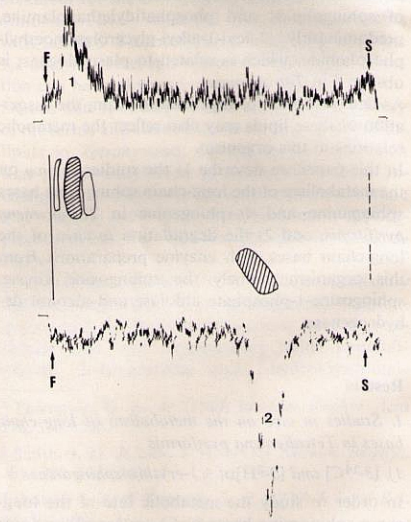


Fig. 2. Radio thin-layer scan of purified chimyl alcohol and hexadecanol isolated from phosphatidylcholine by $LiAlH_4$ reduction and purified by preparative thin-layer chromatography. 1 = Hexadecanol; 2 = chimyl alcohol.

Solvent system: ether/conc. ammonia 100:0.25.

the isopropylidene derivative with typical ions of $M^{\oplus}$ 356, *m/e* 341 (M − 15) and *m/e* 101

and the bis(trimethylsilyl) derivatives. The isotope ratio of chimyl alcohol was identical with that of the substrate, indicating that palmitaldehyde is for the most part directly reduced to hexadecanol and incorporated into alkyl ether phospho- and phosphonolipids. The complete distribution of the radioactivity in the different lipids and in chimyl alcohol is summarized in Table 1.

2) [3-^3H]D(+)-erythro-4t-sphingenine

[3-^{3}H]4*t*-sphingenine added to the growth medium of *Tetrahymena pyriformis* gave a similar pattern of incorporation into complex phospholipids. 30% (2.5×10^8 dpm) of the radioactive base was taken up from the medium and metabolized. The incorporation of its degradation products into the phospholipids is reflected in Fig. 3a. The phosphatidylethanolamine fraction, including its phosphono derivative and the phosphatidylcholine fraction, were heavily labelled. The phosphatidylethanolamine fraction does not contain the alkyl ether group. 77% of the total radioactivity of these two fractions were located in chimyl alcohol isolated from the aminoethyl phosphonates and 23% in chimyl alcohol derived from phosphatidylcholine liberated by $LiAlH_4$ reduction and purified by preparative thin-layer chromatography, Fig. 3b.

Not only the kinase and lyase but also the 2*t*-alkenal reductase must therefore be present in *Tetrahymena pyriformis*. This conclusion from the study *in vivo* was verified by the studies *in vitro*.

3) Comparative utilization of labelled D(+)*-erythro-sphinganine and palmitic acid for alkyl ether synthesis*

In order to get insight into the relative importance of the pathway of sphingosine metabolites for alkyl ether synthesis, we performed a comparative experiment with [3-^{14}C]D(+)-*erythro*-sphinganine and [7,8,10,11-$^3H_4$]palmitic acid in equimolar amounts as precursors of chimyl alcohol synthesis in *Tetrahymena pyriformis*. The $^3H/^{14}C$-ratio was 1.55. 65% of the labelled compounds were incorporated into the phospholipids. Chimyl alcohol was prepared from the phospho- and phosphonolipids by phospholipase C *(B. cereus)* hydrolysis followed by mild alkaline hydrolysis. Its isotope ratio was 1.67. Therefore it can be concluded that under the growth conditions chosen in this experiment (see Experimental), the long chain-base donates C-3 to C-18 to the biosynthesis of the alkyl ether bond as efficiently as palmitic acid, which is utilized *via* the acyl-CoA reductase reaction. In another experiment, in which [3-^{3}H]D(+)-*erythro*-sphinganine and [1-^{14}C]palmitic acid were the substrates, it

Table 1. Distribution of radioactivity in lipids of *Tetrahymena pyriformis* after growth in a medium supplemented with [3-^{3}H; 3-^{14}C]sphinganine.

Ratio of activities of $^3H/^{14}C = 4.5$. For experimental details, see Experimental.

	Radioactivity			
	^{3}H [dpm]	^{14}C [dpm]	% of fraction	$^3H/^{14}C$
1) $CHCl_3$ eluate (palmitaldehyde, hexadecanol, triglyceride)	9.7×10^6	2.5×10^6		
2) $CHCl_3/CH_3OH$ (4:1) (sphinganine, phosphatidylethanolamine, aminoethylphosphonate)	1.1×10^7	3.4×10^6		
chimyl alcohol	1.8×10^6	4.0×10^5	12	4.5
3) $CHCl_3/CH_3OH$ (3:2) (phosphatidylcholine, ceramide aminoethylphosphonate)	1.76×10^6	5.9×10^5		
chimyl alcohol	6.35×10^5	1.37×10^5	23	4.5

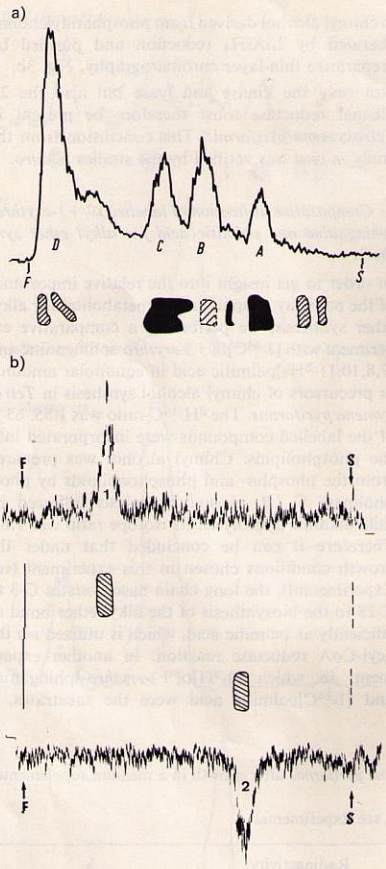


Fig. 3a. Radio scan of lipid extract of *Tetrahymena pyriformis* grown in a medium supplemented with 4*t*-[3-^{3}H]sphingenine.

A = Phosphatidylcholine; B = 4*t*-sphingenine (substrate); C = phosphatidylethanolamine and aminoethylphosphonate; *D* = *cardiolipin, triglyceride, palmitaldehyde*, hexadecanol. Solvent system: chloroform/methanol/water 65:25:4.

Fig. 3b. Radio scan of hexadecanol (1) and chimyl alcohol (2) isolated from phosphatidylcholine by $LiAlH_4$ reduction.

Solvent system: ether/conc. ammonia 100:0.25.

could be demonstrated that palmitaldehyde, liberated in the lyase reaction, is not measurably oxidized to palmitate and then reduced again *via palmitaldehyde to hexadecanol, because the* ^{3}H isotope would have been lost in the course of this reaction sequence, which is not the case. In this experiment, too, an equal utilization of both precursors could be demonstrated.

4) Incorporation of [^{18}*O*]*hexadecanol into alkyl ether lipids of Tetrahymena pyriformis*

Preliminary to future mechanistic studies on the formation of alkyl ether bonds, [^{18}O]hexadecanol was used as substrate in studies *in vivo*. In order to determine the yield of uptake and incorporation into the chimyl alcohol moiety, [1-^{14}C]hexadecanol was added as tracer (13 μmol [1-^{14}C]hexadecanol, specif. radioactiv. 2.34×10^6 dpm/μmol) to [^{18}O]-hexadecanol (50 μmol, 90% enriched).

71% of the substrate was taken up from the medium, and 40% was incorporated into the phospholipids of *Tetrahymena*. After $LiAlH_4$ reduction, 79% of the radioactivity was recovered in the fatty alcohol fraction and 21% in the chimyl alcohol fraction. The mass spectrum of the ^{14}C/^{18}O-labelled chimyl alcohol is given in Fig. 4. The isotope ratio of representative peaks at *m/e* 370/372 and 445/447 indicate an ^{18}O-enrichment of approximately 15%. *The results agree well with those of Snyder et al.*[18], who also found that the oxygen of hexadecanol is retained after incorporation into chimyl alcohol from preputial gland tumor cell lipids.

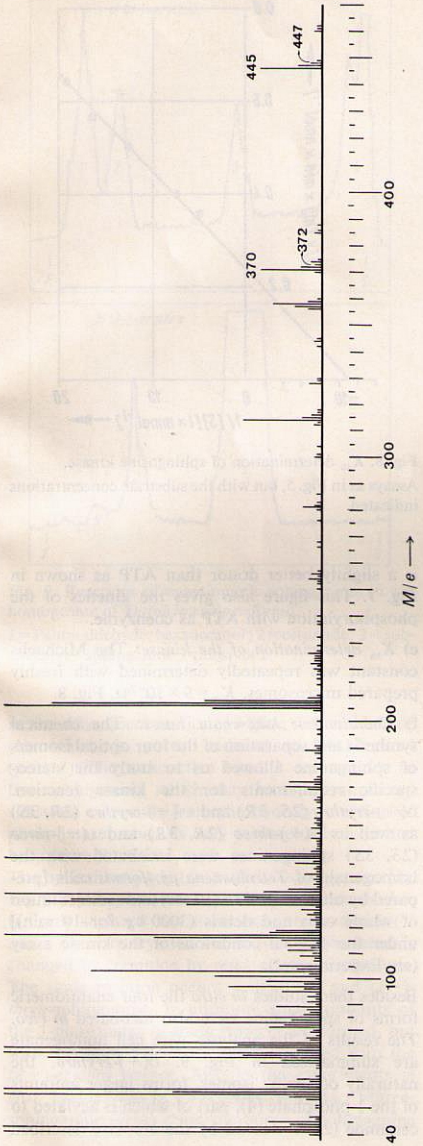
II. Studies in vitro on the degradation of long-chain bases in Tetrahymena pyriformis

1) Sphingosine kinase

a) *Cell fractionation: Tetrahymena pyriformis* cells growing in the log-phase (3 − 4 days) were homogenized and fractionated into a microsomal, mitochondrial and $100000 \times g$ supernatant fraction by the procedure outlined by Smith and Law[8]. The highest kinase activity was found to be in the microsomal fraction. The microsomal kinase is also active after lyophilization. The dependence of the *reaction rate on the concentration of protein is* given in Fig. 5.

b) *Enzyme assay:* The ion exchange chromatography described previously was applied for the

[18] Snyder, F., Rainey, W. T., Jr., Blank, M. L. & Christie, W. H. (1970) *J. Biol. Chem.* **245**, 5853 − 5856.



◀ TMS

Fig. 4. Mass spectrum of ~~isopropylidene~~ derivative of chimyl alcohol isolated from phosphatidylcholine fraction of *Tetrahymena pyriformis* isolated after growth in a medium supplemented with [^{18}O]hexadecanol.

separation of the 1-phosphate ester and the free sphingosine base[19]. Substrate and product are separated completely by this procedure.

c) *pH optimum:* The kinase reaction has been studied between pH 5.5 and 8.5 using Tris-maleate (pH 5.5–7.0) and Tris-HCl (pH 7.5–8.5). The pH optimum of the kinase proved to be between pH 7.5 and 8.5 (Fig. 6).

d) *Coenzyme requirement:* Our studies on the kinase isolated from the supernatant of blood platelets[19, 20] have demonstrated that ATP is the coenzyme of the kinase and that UTP can serve only to a limited extent as phosphate donor. We also tested the *Tetrahymena pyriformis* kinase, which is microsomal and membrane-bound, and its coenzyme requirement. However the enzyme has no specificity for any one of the nucleoside triphosphates, GTP

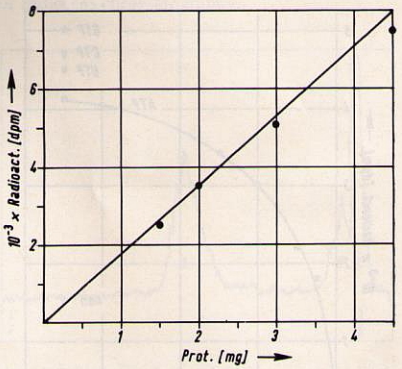


Fig. 5. Dependence of sphingosine kinase reaction rate on enzyme concentration.
Incubation time: 30 min. Ordinate: radioactivity in the product, sphinganine 1-phosphate. Assay as in Experimental.

[19] Stoffel, W., Heimann, G. & Hellenbroich, B. (1973) *this J.* **354**, 562–566.
[20] Stoffel, W., Hellenbroich, B. & Heimann, G. (1973) *this J.* **354**, 1311–1316.

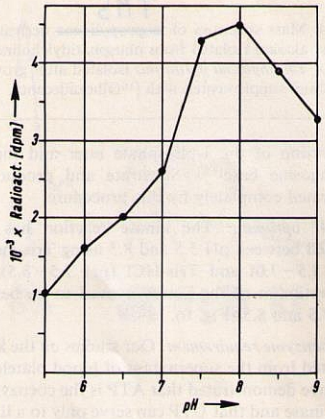


Fig. 6. pH optimum of sphinganine kinase of *Tetrahymena pyriformis*.

Assay as in Fig. 5, but with 0.1M Tris-maleate buffer between pH 5.5 and 7.0, and 0.1M Tris-HCl buffer between pH 7.5 and 8.5.

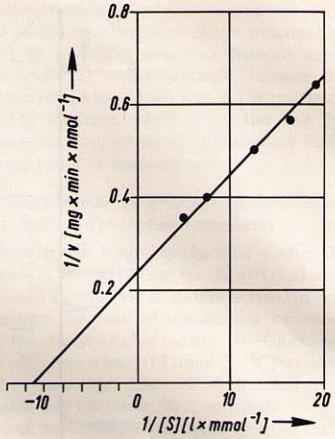


Fig. 8. K_m determination of sphingosine kinase.

Assays as in Fig. 5, but with the substrate concentrations indicated.

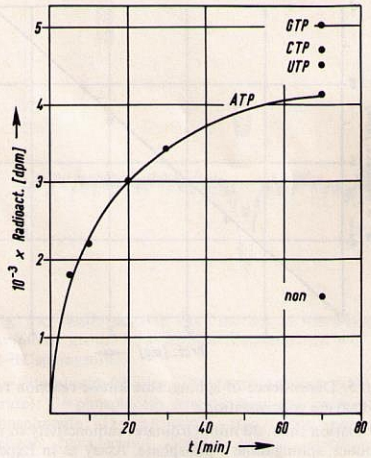


Fig. 7. Nucleoside triphosphate requirement of sphingosine kinase of *Tetrahymena pyriformis*.

The assays were similar to the kinase assays described in Experimental but with 5mM of the nucleoside triphosphate indicated or with no nucleoside.

is a slightly better donor than ATP as shown in Fig. 7. This figure also gives the kinetics of the phosphorylation with ATP as coenzyme.

e) K_m *determination of the kinase:* The Michaelis constant was repeatedly determined with freshly prepared microsomes. $K_m = 9 \times 10^{-5}$M, Fig. 8.

f) *Enantiomeric long-chain bases:* The chemical synthesis and separation of the four optical isomers of sphinganine allowed us to study the stereospecific requirements for the kinase reaction. D(+)-*erythro* (2*S*, 3*R*) and L-(−)-*erythro* (2*R*, 3*S*) as well as D(+)-*threo* (2*R*, 3*R*) and L(−)-*threo* (2*S*, 3*S*) sphinganines were incubated with the homogenate of *Tetrahymena pyriformis* cells [prepared by ultrasonication (10 sec) and sedimentation of whole cells and debris (3000 × *g* for 10 min)] under the optimal conditions of the kinase assay (see Experimental).

Besides these studies *in vitro* the four enantiomeric forms of sphinganine were also incubated *in vivo*. The results of the analyses with cell homogenate are summarized in Fig. 9. D(+)-*erythro*, the naturally occurring isomer, forms larger amounts of the 1-phosphate (4), part of which is acylated to ceramide (2) or cleaved by the aldolase described

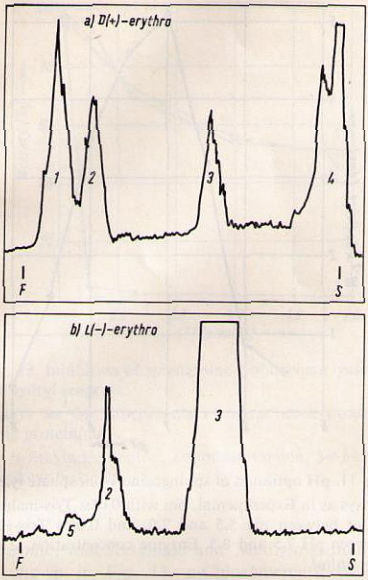


Fig. 9. Reactions of enantiomeric sphinganines in cell homogenate of *Tetrahymena pyriformis*.
1 = Palmitaldehyde, hexadecanol; 2 = ceramide; 3 = substrate; 4 = sphinganine 1-phosphate.

in the subsequent section, yielding palmitaldehyde (1), which is partly reduced to hexadecanol (peak 1). The radioactive band 3 corresponds to free sphinganine, which may be either unreacted substrate or results from the hydrolytic action of phosphatase, which is only incompletely inhibited by sodium fluoride.

The optical antipode L(−)-*erythro*-sphinganine (3), Fig. 9, is phosphorylated neither *in vitro* nor *in vivo*, but only acylated to a minor extent to ceramide (2) and a di- or triacylated derivative (5). The latter is changed to ceramide by mild alkaline hydrolysis. The same reaction occurs with D(+)- and L(−)-*threo*-sphinganine. In addition, small amounts of the 1-phosphates of these isomers are formed, but with less than 5% of the yield obtained with the D(+)-*erythro* isomer. Also in the experiments *in vivo*, besides the D(+)-*erythro*-, only L(−)-*threo*-sphinganine forms minor amounts of sphinganine 1-phosphate. Again acylation products are formed to some extent, but most of the substrate remains unchanged.

2) Sphingosine-1-phosphate aldolase (lyase)

a) Cell localization by fractionation studies

The disruption and fractionation procedures described under II, 1a, were also applied to study the localization of the sphingosine-1-phosphate lyase. D(+)-*erythro*-sphinganine 1-phosphate was used as substrate. The incubation mixture had been fortified with 2 nmol pyridoxal phosphate and the phosphatase reaction inhibited with sodium fluoride. Fig. 10 shows the lyase activity of *Tetrahymena* microsomal fraction with sphinganine 1-phosphate as substrate. The incubations were extracted with chloroform/methanol 2:1 and $CHCl_3$, the extracts concentrated to dryness and dissolved in a small volume of dichloroethane, and placed on a silicic acid column prepared in a Pasteur pipette. The product palmitaldehyde was eluted with dichloroethane. The dichloroethane fraction was concentrated after dilution with radioactive palmitaldehyde and 2*t*-hexadecenal, portion was taken for the determination of the yield, and the rest used for radio gas chromatography.

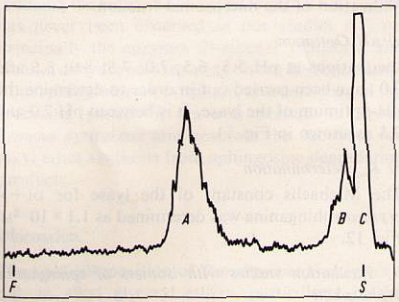


Fig. 10. Sphingosine-1-phosphate lyase reaction in the microsomal fraction of *Tetrahymena*.
Radio thin-layer scan of lipid extract of incubation mixture consisting of 2 ml 0.1M Tris buffer pH 7.4, 0.1 μmol [3-^{14}C]sphinganine 1-phosphate, 1μM pyridoxal phosphate; enzyme concentration, 2 mg protein/ml.
Solvent system: dichloroethane. A = Palmitaldehyde and 2*t*-hexadecenal; B = ceramide and traces of hexadecanol; C = substrate.

Table 2. Lyase activity in cell fractions of *Tetrahymena pyriformis*.

Cell fraction	Radioactivity in palmitaldehyde	Enz. act. [U/mg prot.]
Homogenate	137 000	56
10000 × *g* Supernatant	154 000	64
Mitochondria	6 000	2
Microsomes	144 000	60
100000 × *g* Supernatant	13 400	5

Table 2 summarizes the specific activities of the cell fractions. In the assay the amount of palmitaldehyde formed in the reaction was measured by radio thin-layer and radio gas chromatography. The methanol eluate contained ceramide, sphinganine and unreacted substrate.

In another experiment, the silicic acid column was first eluted with dichloroethane in order to isolate palmitaldehyde and hexadecanol (40%); then chloroform eluted ceramide (43%), chloroform/methanol 2:1, free sphinganine (14.5%); and methanol some residual substrate (2%).

The lyase activity remains unimpaired by lyophilization of the microsomal fraction.

b) pH Optimum

Incubations at pH 5.5; 6.5; 7.0; 7.5; 8.0; 8.5 and 9.0 have been carried out in order to determine the pH-optimum of the lyase. It is between pH 7.0 and 7.5 as shown in Fig. 11.

c) K_m determination

The Michaelis constant of the lyase for D(+)-*erythro*-sphinganine was determined as 1.1×10^{-4}M, Fig. 12.

d) Incubation studies with isomers of sphinganine 1-phosphate

The 1-phosphate esters of L(−)-*erythro*- and L(−)-*threo*-sphinganine were tested as inhibitors. No inhibition was caused by increasing concentrations of the L(−)-*erythro* optical antipode. However L(−)-*threo*-sphinganine 1-phosphate, which has the same chirality at C-2 as the natural substrate, leads to competitive inhibition. K_i has been determined to be 9.7×10^{-3}M by the method of Dixon[21].

[21] Dixon, M. (1953) *Biochem. J.* **55**, 170–171.

Fig. 11. pH optimum of sphingosine-1-phosphate lyase.
Assays as in Experimental, but with 0.05M Tris-maleate buffer between pH 5.5 and 7.0, and 0.05M Tris-HCl between pH 7.5 and 8.5. Enzyme concentration, 2 mg protein/ml.

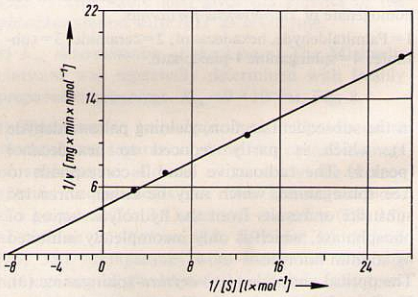


Fig. 12. K_m determination of sphingosine-1-phosphate lyase.
Assays as in Experimental, but with the concentrations of D(−)*erythro*-sphinganine indicated.

We have found in previous studies[10] that rat liver sphingosine-1-phosphate lyase is an enzyme susceptible to sulfhydryl reagents. *Tetrahymena* microsomal lyase is also inhibited by *p*-chloromercuri-

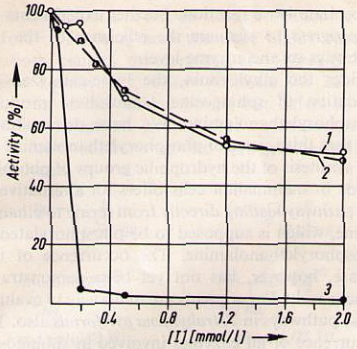


Fig. 13. Inhibition of sphingosine-1-phosphate lyase by sulfhydryl reagents.

Assays as in Experimental, enzyme concentration, 2 mg protein/ml.

1 = *N*-Ethylmaleinimide; 2 = iodoacetamide; 3 = *p*-chloromercuribenzoate.

benzoate, iodoacetamide and *N*-ethylmaleinimide as shown in Fig. 13. *p*-Chloromercuribenzoate leads to a complete inhibition at 0.2mM; iodoacetamide and *N*-ethylmaleinimide to a 50% inhibition at a concentration of 1mM.

Neither phosphorylethanolamine nor palmitaldehyde inhibits the lyase reaction, and no back-reaction between palmitaldehyde and phosphorylethanolamine could be observed.

3) 2-Alkenal reductase

The studies *in vivo* with 4*t*-sphingenine made studies *in vitro* with 2*t*-hexadecenal feasible. Incubations of 2*t*-hexadecenal with the 100000 × *g* supernatant in the presence of NADPH yielded hexadecanal, hexadecanol and hexadecenol. The saturated and unsaturated aldehydes and alcohols were separated by radio thin-layer chromatography (Fig. 14) and gas-liquid chromatography. About 30% of the radioactivity was present in palmitaldehyde and its trimer (activity at the origin), 35% of the radioactivity was present in hexadecanol. About 45% of the extracted radioactivity was located in hexadecenol. Hexadecanol and hexadecenol running together in peak 2 were separated and quantified by gas-liquid chromatography as described before[13]. The alcohol dehydrogenase

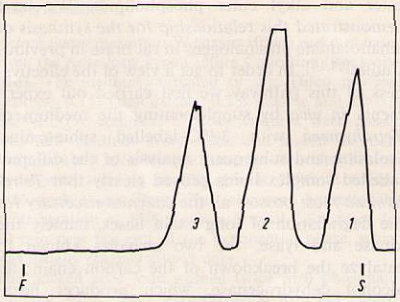


Fig. 14. Radio thin-layer chromatographic analysis of incubation of 2*t*-[4,5-$^3H_2$]hexadecenal with the 100000 × *g* supernatant in the presence of NADPH.

The incubation mixture contained in a total volume of 2 ml 0.1M Tris buffer pH 6.8: 0.1 μmol 2*t*-hexadecenal (added in 30 μl ethanol), 1 μmol NADPH, 10 mg 100000 × *g* supernatant. Incubation time: 30 min at 37°C.

1 = Trimer of palmitaldehyde; 2 = hexadecanol and hexadecenol; 3 = palmitaldehyde. Solvent system: dichloroethane.

diminishes the actual substrate concentration by reducing the substrate to hexadecenol. This alcohol has never been observed in our studies *in vivo*. Principally the enzymes 2*t*-alkenal reductase and alcohol dehydrogenase acting on the 2*t*-hexadecenal from sphingosine 1-phosphate, and described in the preceding paper[13], are also present in *Tetrahymena pyriformis* and generate the substrate for alkyl ether synthesis from sphingosine degradation products.

Discussion

The lipid composition of *Tetrahymena pyriformis* is rich in alkyl glyceryl ethers, particularly in the phosphatidylethanolamine and -choline fractions. *Tetrahymena pyriformis* also synthesizes sphingosine-containing lipids, *e.g.* ceramide aminoethylphosphonate[2,3]. The simultaneous occurrence of these two lipid classes stimulated our studies to find out whether this organism also possesses the enzymes for the degradation of the sphingosine bases, namely sphingosine kinase and sphingosine-1-phosphate aldolase, and whether it reutilizes the degradation product for the *de novo* synthesis of

ester and alkyl ether phospholipids. We have demonstrated this relationship for the synthesis of ethanolamine plasmalogens in rat brain in previous studies[14,15]. In order to get a view of the effectiveness of this pathway we first carried out experiments *in vivo* by supplementing the medium of *Tetrahymena* with 3-^{14}C-labelled sphinganine. Isolation and subsequent analysis of the different labelled complex lipids proved clearly that *Tetrahymena* must possess all the enzymes necessary for the degradation of long chain bases, namely the kinase and lyase, the two enzymes known to catalyze the breakdown of the carbon chain; an alcohol dehydrogenase, which produces hexadecanol for the alkyl glyceryl ether bond formation; and an aldehyde oxidase, which oxidizes the aldehyde to fatty acid, which is utilized for the ester bond formation in the ester lipids. The alkyl chain of the long-chain base was oxidized and reduced to a considerable extent, as could be demonstrated by $LiAlH_4$ reduction of the phospholipid fractions, which released the esters as fatty alcohols. The chimyl alcohol remains intact under these conditions.

4*t*-Sphingenine is also degraded by *Tetrahymena pyriformis*. Carbon atoms 3 to 18 are recovered as a long-chain acyl group, namely palmitic acid, from the ester lipids (triglyceride, phosphatidylcholine, phosphatidylethanolamine and its phosphono derivative) and as hexadecanol in the chimyl alcohol moiety of the alkyl ether phospholipids. Therefore, by analogy to the enzymes of rat liver catalyzing 4*t*-sphingenine degradation, it can be concluded that besides the sphingosine kinase and lyase, there must also be an alkenal reductase and an alcohol dehydrogenase in *Tetrahymena*. We approached an evaluation of the relative importance of the re-utilization of sphingosine metabolites for the alkyl ether synthesis by a simultaneous supplementation of the medium with ^{3}H-labelled sphinganine and ^{14}C-labelled palmitate and *vice versa*. The results of these experiments indicate that palmitaldehyde released from sphinganine and palmitic acid are incorporated in equal amounts. Experiments with [3-^{3}H; 3-^{14}C]sphinganine proved that palmitaldehyde is directly reduced to hexadecanol, the precursor of the hexadecylglycerol ether moiety.

It appears that *Tetrahymena pyriformis* synthesizes its alkyl ether lipids by two routes: the reduction of palmitoyl-CoA to palmitaldehyde catalyzed by a palmitoyl-CoA reductase; and the utilization of palmitaldehyde derived from the sphingosine-1-phosphate lyase reaction. Further experiments are in progress to evaluate the efficiency of the two pathways on the enzyme level.

Besides the alkyl chain, the lyase-catalyzed degradation of sphingosine 1-phosphate produces phosphorylethanolamine. We have demonstrated [22] that this source of phosphorylethanolamine for the synthesis of the hydrophilic groups of phospholipids in mammalian cells offers an alternative to the pathway leading directly from serine to ethanolamine, which is supposed to be phosphorylated to phosphorylethanolamine. The occurrence of this kinase, however, has not yet been demonstrated conclusively. Experiments are underway to evaluate these pathways in *Tetrahymena pyriformis* also. The occurrence of all enzymes involved in sphingosine degradation has been demonstrated in subcellular fractions of *Tetrahymena*. Part of our results with the kinase in *Tetrahymena pyriformis* are in good agreement with those of Keenan[23]. This is true for the pH optimum, the phosphoryl group donors ATP, GTP, CTP and UTP, and the microsomal localization of the kinase. However our results on the phosphorylation of the four enantiomeric sphinganine isomers are at variance with the preliminary studies of this author. Only the D(+)-*erythro* (2*S*, 3*R*) and L(−)-*threo* (2*S*, 3*S*) sphinganines serve as substrates. The Michaelis constants of these two acceptors were determined. The naturally occurring D(+)-*erythro* isomer has a much higher affinity than the L(−)-*threo* isomer. It is comparable with the K_m of the platelet kinase isolated in this laboratory[20].

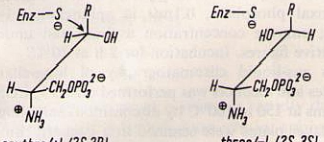
The sphingosine-1-phosphate lyase, whose presence we deduced from the degradation products of the experiments *in vivo*, was isolated in the $100000 \times g$ pellet, which represents the microsomal fraction. This particulate enzyme proved to be dependent on pyridoxal phosphate. Sulfhydryl groups obviously participate in the cleavage reaction, because SH-reagents, particularly *p*-chloromercuribenzoate, strongly inhibit the reaction at low concentrations. We have previously suggested a mechanism in which the sulfhydryl group attacks carbon atom 3 with the intermediate formation of a thiohemiacetal[24]. Experiments with the enantiomeric sphinganine 1-phosphate proved that a 2*S*, 3*R* absolute con-

[22] Stoffel, W. & Sticht, G. (1967) *this J.* **348**, 1561–1569.

[23] Keenan, R. W. (1972) *Biochim. Biophys. Acta* **270**, 383–396.

[24] Stoffel, W. (1973) Mol. Cell. Biochem. **1**, 147–155.

figuration of the 2-amino-1,3-diol system is required[25]. The enzyme is therefore highly specific for both centers of chirality. L(−)-*threo*-Sphinganine 1-phosphate, which possesses the 2*S*-configuration of the natural isomer but differs in the chirality of carbon atom 3 (see structures), is a competitive inhibitor of the sphingosine-1-phosphate lyase.



The K_m of the lyase of *Tetrahymena pyriformis* is similar to that of the mammalian liver sphingosine-1-phosphate lyase. It cleaves the saturated sphinganine 1-phosphate and also the unsaturated sphingenine 1-phosphate to palmitaldehyde and 2*t*-hexadecenal and phosphorylethanolamine. The unsaturated 2*t*-hexadecenal is reduced by a NADPH-dependent 2-alkenal reductase to palmitaldehyde. The presence of this enzyme in the $100000 \times g$ supernatant in *Tetrahymena pyriformis* could easily be demonstrated by the use of labelled 2*t*-hexadecenal as substrate and in the optical assay[13,26]; like the mammalian enzyme it is NADPH-dependent. The $100000 \times g$ supernatant fraction also contains a NADH-requiring alcohol dehydrogenase, which forms hexadecanol from the palmitaldehyde formed.

The results of the experiments summarized in this communication underline 1) the ubiquity of the sequence and its enzymes in the degradation of long-chain sphingosine bases, 2) the reutilization of its degradation products for the synthesis of ester and alkyl ether lipids as apparent in the incorporation of the alkyl chain into the chimyl ether moiety of alkyl ether phospholipids, 3) the necessity of considering additional pathways operative in the synthesis of complex phospholipids, and 4) the suitability of *Tetrahymena pyriformis* for the study of the mechanism of the alkyl ether bond formation.

This study was supported by the *Deutsche Forschungsgemeinschaft* (SFB 74).

[25] Stoffel, W. & Bister, K. (1973) *this J.* **354**, 169–181.
[26] Stoffel, W. & Melzner, I. (1973) *this J.* **355**, 1626–1632.

Experimentals

Tetrahymena pyriformis W (strain 10542) was purchased from the American Type Culture Collection (Rockville, Md). The medium consisted of 2% Difco proteose peptone, 0.1% yeast extract, 0.2% glucose and 0.003% $Fe^{3\oplus}$-EDTA complex[8]. Stock cultures were passed into new medium every week and stored at room temperature. Cultures were grown in 1 *l* round bottom flasks with swirling at 150 rpm at 28°C in a New Brunswick Water Bath. The cells were harvested by centrifugation at $8500 \times g$ in a Sorvall GSA rotor for 30 min near the end of the exponential phase, which was usually reached after 3 days.

The packed cells were washed twice with phosphate-buffered saline. They were extracted with hot chloroform/methanol 2:1 and 1:2 by refluxing for 15 min. The combined extracts were washed with water, filtered and concentrated to dryness under vacuum. The lipid mixture was separated into

a) a "neutral lipid" fraction, which contained triglycerides, free fatty acids, long-chain alcohol (hexadecanol), ceramide, ceramide aminoethylphosphonate and also cardiolipin,

b) the phosphatidylethanolamine fraction and

c) the phosphatidylcholine fraction

by silicic acid chromatography (Mallinckrodt 100–200 mesh) with increasing concentrations of methanol in chloroform. The fractions were analyzed by thin-layer chromatography on silica gel H plates. Solvent systems: chloroform/methanol/conc. NH_4OH 60:35:5 and chloroform/methanol/water/conc. NH_4OH 75:25:2.5:2.5. $LiAlH_4$ reduction[27] of fractions b and c yielded radioactive alkyl glycerol ethers and fatty alcohols, the latter arising from the fatty acyl residues of the 2-acyl-1-alkyl glycerophospho- and phosphonolipids. They were separated by preparative thin-layer chromatography on silica gel H. Solvent system: ethyl ether/conc. NH_4OH 100:0.25. Chimyl alcohol was identified as the only alkyl glycerol ether by gas-liquid chromatography as trimethylsilyl, diacetate and isopropylidene derivatives [28] on 3% SE 30 columns, 120 cm, at 210, 220 and 190°C, respectively, and compared with authentic samples. Gas-liquid chromatography-mass spectroscopy was also used. A Varian MAT CH 5 mass spectrometer was used at 70 eV.

The labelled sphinganines and their phosphate esters were synthesized in this laboratory[22,25,29,30]. The specific radioactivity of the free base was 4.5×10^6 dpm/μmol and of sphinganine 1-phosphate, 2.68×10^6 dpm/

[27] Schmid, H. H. O. & Mangold, H. K. (1966) *Biochem. Z.* **346**, 13–25.
[28] Hanahan, D. J., Ekholm, J. & Jackson, C. M. (1963) *Biochemistry* **2**, 630–641.
[29] Tschung, T. S. (1973) doctoral thesis, Cologne.
[30] Bauer, E. (1974) doctoral thesis, Cologne.

μmol. The substrates were added to the growth medium as ethanol/water (1:1) solutions (10 μmol/ml).

For the experiments reported in Table 1 and Fig. 1 and 3, the cells were grown in 500 ml batches. The medium was supplemented with either 20 μmol [3-^{3}H; 3-^{14}C]-sphinganine (specif. radioactiv. 1.04×10^6 dpm/μmol ^{14}C, 5.03×10^6 dpm/μmol ^{3}H) or 10 μmol 4*t*-[3-^{3}H]-sphingenine (specif. radioactiv. 8.4×10^7 dpm/μmol). Cells were harvested in the logarithmic phase of growth. 53% of the labelled sphinganine was incorporated into cell lipids.

For cell fractionation, *Tetrahymena pyriformis* cells were grown in 1.0 *l* cultures to the end of the log-phase (3−4 days), then harvested by centrifugation at 4°C at $5000 \times g$ for 20 min. They were homogenized in 50 ml 0.05M Tris-HCl buffer pH 8.1, 0.25M in sucrose and 10^{-3}M in thioglycol in an Ultraturrax for 1 min. The homogenate was further treated by 12 strokes in a Potter-Elvejhem homogenizer followed by centrifugation at $1000 \times g$ for 10 min. When centrifuged at $14500 \times g$ for 10 min, the supernatant yielded a pellet which was washed twice with buffer and recentrifuged at $10000 \times g$ for 10 min. It represented the mitochondrial fraction. The microsomes were pelleted from the $14500 \times g$ supernatant by centrifugation at $100000 \times g$ for 60 min.

The incubation mixtures for the *kinase assay* consisted of 2 ml 0.05M Tris-HCl buffer pH 7.4, 20mM in NaF, 1mM in thioglycol, 5mM in ATP, 10mM in $MgCl_2$; substrate 0.1mM D(+)-*erythro*-sphinganine; enzyme concentration as indicated under the figures. The *lyase assay* mixture was 2 ml 0.05M Tris-HCl buffer pH 7.4, 20mM in NaF, 1mM in thioglycol, 10mM in $MgCl_2$, 1μM pyridoxal phosphate, 0.1mM in sphinganine 1-phosphate; enzyme concentration as indicated under the respective figures. Incubation for 2 h at 20°C.

Radio gas-liquid chromatography of long-chain aldehydes and alcohols was performed on 2.5% EGS, 2 m columns at 150 to 160°C by discontinous sampling.

Thin-layer plates were scanned in a Packard thin-layer scanner, model 7201, or a Berthold scanner, model LB 2722. [^{18}O]Hexadecanol was synthesized by $LiAlH_4$ reduction of [^{18}O]methyl palmitate. [^{18}O]Palmitic acid was prepared in a Grignard reaction between pentadecylbromide and $K_2C^{18}O_3$ (purchased from Miles Laboratories).