Modification of membrane fluidity by changing lipid unsaturation in mammalian cells: the medical aspects

Ph.D. Thesis

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List of papers related to the subject of the Ph.D. thesis:

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2. Joó, F., **Benkő, S.**, Horváth, I., Török, Zs., Nádasdi, L., Vígh, L.: Hydrogenation of biological membranes using a polymer-anchored colloidal palladium catalyst. *React. Kinet. Catal. Lett.* **48:** 619-625 (1992)

3. Duda, E., **Benkő, S.**, Horváth, I., Galiba, E., Páli, T., Joó, F., Vígh, L.: Lipid saturation in the target cells plasma membrane blocks tumor necrosis factor mediated cell killing. *In: Advances in Psychoneuroimmunology* (Eds.: Berczi,I. and Szelenyi, J.), Plenum Press, New York, pp. 181-190 (1994)

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5. Győrfy, Zs., **Benkő, S.**, Kúsz, E., Maresca, B., Vígh, L., Duda, E.: Highly increased TNF sensitivity of tumor cells expressing the yeast Δ9-desaturase gene. *Biochem. Biophys. Res. Commun.* **241:** 465-470 (1997)

6. Horváth, I., Glatz, A., Varvasovszki, V., Török, Zs., Páli, T., Balogh, G., Kovács, E., Nádasdi, L., **Benkő, S.**, Joó, F., Vígh, L.: Membrane physical state controls the signaling mechanism of the heat shock response in *Synechocystis* PCC 6803: identification of hsp17 as a novel "fluidity gene". *Proc. Natl. Acad. Sci. USA* **95:** 3513-3518 (1998)

7. **Benkő, S.**, Horváth, I., Török, Zs., Joó, F., Vígh, L.: Selective hydrogenation of surface membrane in platelets using a polymer-anchored colloidal palladium catalyst (Manuscript, submitted).

Contents

Abbreviations

PE	phosphatidylethanolamine
PC	phospahtidylcholine
CL	cardiolipin
PS	phospatidylserine
16:0	palmitic acid
16:1	palmitoleic acid
18:0	stearic acid
18:1	oleic acid
18:2	linoleic acid
18:3	linolenic acid
20:4	arachidonic acid
22:6	docosahexanoic acid
TNF	tumor necrosis factor
DPH	1,6-diphenyl-1,3,5-hexatriene
DPH-PA	3-(p-(6-phenyl)- 1,3,5-hexatrienyl) phenylpropionic acid
TMA-DPH	trimethylammonium-1,6-diphenyl-1,3,5-hexatriene
PVPP	polyvinyl-polypirrolidone
TLC	thin layer chromatography
PLA	phospolipase A

1. INTRODUCTION

General considerations about membranes and lipids

Although the basic structure of the biological membrane has since been elucidated in great details, Singer and Nicolson proposed in 1972 the conceptually most plausible model for the organization of biological membrane called the fluid mosaic model. In this model, proteins were thought to be either superficially or integrally associated with lipids in a fluid bilayer membrane. The fluidity of the membrane is essential for many of its functions. The membrane integral proteins could be embedded into the lipid core to various extents, some completely spanning the membrane. The membrane was shown to be asymmetric regarding its lipid-protein disposition. Strictly controlled by the overall level of membrane fluidity, membrane lipids undergo a solid-to-liquid crystal (order-disorder, or "gel-to-fluid") phase transition at a particular temperature called transition temperature (T_t). The phase transition is a cooperative process and at the transition state, three phases namely solid (or "gel") , liquid crytalline (or "fluid") and "interfacial lipid" phases coexist. The phase behavior of a mixture of different lipids is very complex. With very different lipids, a lateral separation of solid and fluid lipid domains takes place at a T_t.

Though depending on their number, position and distribution between alkyl chains, introduction of double bonds, especially cis double bonds into lipids interfere dramatically with the manner of lipid packing and change in the lipid's T_t. The alkane chain length, the nature of the head groups and some other factors like the presence of negatively charged phospholipids also affect lipid order and modulate T_t. Cholesterol interacts strongly with sphyngomyelin and unsaturated phospholipids, and therefore can cause a lateral phase separation within membranes.

The integral membrane proteins are closely associated with the lipids. The closeness of this association is well reflected in the massive alteration in protein activities by a change in the physical state of the lipids. For example, if the lipids are frozen by lowering the temperature, it markedly inhibits the proteins enzymatic and transport functions. Lipids are immobilized around the integral proteins: this imaginary lipid layer has been called the boundary (or annular) lipid. The annular lipid probably does not extend much beyond one molecule in thickness. It was shown, that

the most perturbed lipids are the ones next to the proteins and the lipids least perturbed are the bulk lipids. Superficial proteins associate with membrane surfaces via electrostatic interactions.

In conclusion, a great number of factors contribute to the overall fluidity of the lipids e.g. lateral and rotational motions of the molecules, head group's conformational flexibility, frequency of "kink" formation and swaying motion of the hydrocarbon chains. The contribution of each of these factors separately to the modulation of a particular protein's action and thereby the most various membrane functions is a central subject of the current membrane biochemistry. Accordingly, there have been serious efforts to manipulate by various means the fluidity of biological membranes in order to study their specific effects on membrane functions and cell physiology. Such means include genetics (see later), use of chemical inhibitors of lipid biosynthetic enzymes, supplying cholesterol in liposomes or fatty acids in organic solvents, dietary regulation for whole animals or cultured cells. In all these cases, however, complex changes in membrane lipid composition take place, because the cell attempts to maintain its membrane's natural physical state/fluidity by various ways: e.g. altering endogeneous lipid's head group, acyl chain composition, cholesterol level or the ratio of protein-to lipid. Moreover, while the biological membranes can be directly enriched for cholesterol with sterol containing liposomes, both components of the "carrier" liposomal phospholipids and cholesterol itself will equilibrate between the exogeneous sorce and target membranes. Thus, the effects of such manipulation on the functions of membrane proteins is not easy to interpret in terms of specific alterations caused solely by altering sterol levels. Fatty acids can directly be incorporated into biological membranes by supplying them as a solution in ethanol or dimethyl sulfoxide. Again, however, the side-effect of the fatty acid solvent that also gets incorporated into the membrane is hard to discount.

A method of considerable promise, especially in light of the pitfalls of the methods discussed above, is *hydrogenation of cis-double bonds of the lipid hydrocarbon chains with specific catalysts*. Obviously, when suitable conditions are found for penetrations of the membrane by the catalysts and its removal after hydrogenation, this method is very desirable. In the present study the *in situ* hydrogenation of cis-double bonds of the lipid hydrocarbon chains of mammalian cell membranes will be introduced by using novel hydrogenation procedures.

2. OVERVIEW OF LITERATURE DATA IN THE SUBJECT OF HYDROGENATION

Over the past decades there has been intense study on the effects of altered membrane unsaturation on cell structure and function. Although much is known about the effects of unsaturation on cell membrane physical properties, the manner in which extrinsic and intrinsic membrane proteins (receptors, various elements of signal and energy transductions, immune response and transport systems, etc.) are influenced by varying degrees of polyunsaturation is still not clear. Recently, the focus has shifted to the examination of the effect of unsaturation at discrete regions of biomembranes, like protein-lipid interface, defined depth in the bilayer or in laterally separated domains.

The idea that chemical modification of membrane lipids could be achieved *in situ* was first demonstrated by Chapman and Quinn in 1976 [1]. The rationale underlying the work was that, if the unsaturated double bonds were largely responsible for the fluid character of the membrane lipid matrix, their saturation would result in a reduction in fluidity. Although simple in concept the practice required application of an entirely novel approach to the catalytic hydrogenation of lipids. It was found that conventional hydrogenation catalysts, such as Adam's catalyst, were unable to bring about hydrogenation of unsaturated lipids dispersed in aqueous systems. The solution to this problem was to employ homogeneous catalysts in which complexes of transition metal atoms were linked to suitable ligands that are able to gain access to the lipid substrate arranged in a bilayer configuration. The initial work was performed using rhodium complexes with triphenyl phosphines designed for hydrogenation and hydroformylation reactions in organic solvents [2] but, subsequently, water-soluble homogeneous catalysts [3] were found to be active against lipid substrates in aqueous dispersions. One of the objectives of performing hydrogenation reactions *in situ* was to modulate the fluidity of biomembranes and to examine the role of membrane lipid fluidity in biochemical and physiological functions. Additionally, because membrane lipids with six or more unsaturated double bonds were found to be significant components of some membranes, such as the retinal rod membranes of the eye, the hydrogenation of these lipids was thought to be a useful tool for identifying their role in these membranes. The aim of collecting literature data bellow is to review

developments since the introduction of hydrogenation techniques, with particular emphasis on the use of homogeneous catalysts in hydrogenating model and biomembrane systems and the information that has been gained about the role of lipid unsaturation/fluidity in membrane functions.

2/1. Hydrogenation of unsaturated membrane lipids

When unsaturated membrane lipids are dispersed in aqueous media they aggregate into structures that are inaccessible to conventional heterogeneous catalysts. Likewise, lipids of biological membranes arranged in bilayer configuration cannot be hydrogenated in the presence of such catalysts. Atomic complexes of transition metals such as nickel, copper, platinum, palladium and ruthenium, have been shown to be active in hydrogenation reactions in homogeneous reaction systems. Most transition metals can be formed into complexes with hydrogenation activity; however, some complexes are considerably more active than others. Furthermore, the physical properties of the catalyst can be tailored to achieve highly selective hydrogenation reactions by either influencing the interaction of catalyst with the substrate or by varying solubility in the reaction medium. The catalytic complexes consist of atomic forms of the transition metal, ensuring that all the metal atoms of the catalyst can participate in reactions, thereby making the reaction more efficient in terms of the amount of catalyst required to sustain a given rate of hydrogenation.

2/2. Homogeneous hydrogenation catalysts

Many transition metal complexes capable of activating molecular hydrogen are known [4]. Most of these complexes have been shown to catalyse the efficient reduction of unsaturated bonds, including olefinic -C=C-, =C=O and =C=N-. When using such catalysts in biological systems, however, there are a number of factors that need to be taken into account. *In the case of living organisms, for example, it is essential that the catalyst is non-toxic or at least that the level of toxicity at concentrations required to sustain a reasonable level of hydrogenation is low.* Toxicity mostly can arise by breakdown of the catalyst complex and liberation of the transition metal element and/or ligands of the complex, either of which may be toxic. Furthermore, side reactions other than hydrogenation may lead to the formation of unwanted, although not necessarily toxic, byproducts. Such reactions include ligand exchange with biomolecules resulting in complexes with altered catalytic properties. Side reactions

are potentially damaging in the case of sulphonated derivatives of Wilkinson's catalyst, for example, where the catalytically active species, $RhH(SP\varnothing_2)_3$ and $[Rh(SP\varnothing_2)_3]^+$, are known to hydrogenate =C=O functions in addition to *cis* unsaturated bonds of olefins. Reaction of biochemical compounds of a susceptible chemical configuration could have repercussions for cell viability.

Chemical catalysis is often performed under conditions of temperature etc. that are well outside the physiological range. In biological applications, *the catalyst complex must be stable under the conditions required to preserve stability of biomembranes or viability of living organisms. At the same time reasonable reaction rates must be sustained under these physiological conditions.* Ideally the presence of the catalyst in the system should not affect any properties of the membrane other than its response to the altered level of saturation of the constituent lipids. This can be achieved by removal of the catalyst complex at the completion of the hydrogenation reaction.

Because of these relatively stringent requirements there are comparatively few complexes that are suitable for biological applications. The group of complexes such as $[Co(CN)_5]_3^-$ for example, although very active under conditions appropriate for hydrogenation of biological membranes, are stable only in the presence of excess cyanide [5]. Another common ligand, 2-aminopyridine, in catalysts such as $RuCl_2(2\text{-}Ampy)_2$, although producing highly active catalysis under relatively mild conditions, is highly toxic to living cells. Another group of the type $RuCl_n(H_2O)_{6-n}$ requires high temperatures and concentrated chloride solutions to produce even modest rates of hydrogenation of unsaturated fatty acids [6). Finally, the classic group of organometallic compounds containing low-valent transition metal ions are largely unsuitable because of their unstable character in aqueous media.

2/3. Water-insoluble homogeneous catalysts

The first catalyst used in homogeneous catalytic hydrogenation of membrane lipids was Wilkinson's catalyst [7,8]. The chemistry of the hydrogenation process has been described in detail for this and related catalysts [2]. *The mechanism of catalytic hydrogenation of alkenes involves three steps: activation of molecular hydrogen; activation of the substrate; and transfer of hydrogen to the substrate to form the saturated product.* The final stage of the hydrogenation involves hydrogen transfer to

the co-ordinated alkene, most likely in a two-step process, to give an alkane. The catalytic cycle is completed when the complex takes up a further molecule of hydrogen and alkene to form an activated ternary complex. The metal catalyst therefore cleaves the H-H bond of molecular hydrogen (homolytic splitting of the hydrogen molecule) weakens the C=C bond through the formation of a coordination complex, and brings the two hydrogen atoms and alkene into sufficiently close proximity to enable the transfer reaction to occur at an efficient rate.

In adapting water insoluble catalysts for use in biological systems it is necessary to introduce the catalyst into the membrane using a solvent vector. Solvents such as tethrahydrofuran and dimethylsulphoxide have been found to be useful. The introduction of catalyst in a minimum amount of solvent, which is miscible with water, causes the insoluble complex to partition into the hydrophobic domain created by the lipid substrate. Moreover, this type of catalyst obviously cannot be removed subsequently from the substrate without destroying the integrity of the membrane. It is also important to verify that the sovent used to introduce the catalyst does not perturb the stability of the membrane. The original studies of hydrogenation of phospholipids dispersed in aqueous systems were performed using Wilkinson's catalyst introduced in a solvent vector of tetrahydrofuran [1,9,10]. It was shown that complete hydrogenation of the dispersed lipid could be achieved under relatively mild conditions of temperature, hydrogen pressure and catalyst concentration. Biophysical studies employing differential scanning calorimetry and X-ray diffraction confirmed that the solvent used to deliver the catalyst and the presence of catalyst in the lipid bilayer did not drastically alter the structural properties of the membrane. Wilkinson's catalyst has also been used to hydrogenate model membranes prepared from lipid extracts of rat liver mitochondria and microsomes and human erythrocytes [11]. It was found that the presence of cholesterol markedly influences the hydrogenation of mixed phospholipid dispersions [12]. This could be explained by the fact that cholesterol restricts partition of the catalyst into the lipid bilayer structure.

2/4. Water-soluble homogeneous catalysts

A major advance in the application of homogeneous catalytic hydrogenation methods to the modulation of lipid phase behaviour was the use of water-soluble catalysts. The need to employ solvent vectors to introduce the catalyst into the membrane can be

avoided and there is more scope for removal of the catalyst at the end of the reaction. Water-soluble complexes can be removed simply by washing, gel filtration, density gradient centrifugation and, in the case of charged complexes, by adsorption to ion-exchange resins.

Synthesis of the first water-soluble catalyst complexes was reported by Joó and Beck [3] and involved the replacement of triphenylphosphine with sulphonated triphenylphosphine. The sulphonated derivative was found to stabilize the lower oxidation states of a number of transition metals, such as Rh, Ru, Ir, Pt, Ni and Cu, in aqueous systems and these water-soluble catalysts facilitated hydrogenation of soluble substrates such as pyruvic acid. The water-soluble complexes appear to have very similar chemical properties to their non-sulphonated triphenylphosphine counterparts. The presence of the charged sulphonyl group renders the catalyst complex very soluble in neutral aqueous solutions [13], and solubility can be modulated by salt concentration or pH. The sulphonate group is not generally coordinated to the metal, and infrared spectra indicate only minor differences in electronic state of the central metal ion compared with the triphenylphosphine complexes. The synthesis and reactivity of a range of monosulphonated triphenylphosphine complexes have been reported [14, 15].

Homogeneous catalytic complexes containing triphenylphosphine ligands are generally unstable in the presence of oxygen and this places a major limitation on their use with living organisms under aerobic conditions. This problem has been largely overcome by synthesis of catalytic complexes based on sulphonated alizarine derivatives of Ru and Pd [16]. The Pd (II) alizarine complex is not only resistant to inactivation by oxygen, which renders it more stable over relatively long reaction times, but also readily soluble in water [17] (see bellow in more details). Hydrogenation of unsaturated phospholipids dispersed in aqueous systems using a water-soluble homogeneous catalyst was first reported by Madden and Quinn [18]. The catalyst was a sulphonated derivative of Wilkonson's catalyst which did not appear to affect the structure of bilayers with respect to their permeability barrier properties [19]. The catalyst was found to hydrogenate oil-in-water emulsions and two-phase oil-water systems without the need for organic co-solvents [20]. The reaction rate could be increased significantly by screening the electrostatic charge on the sulphonate groups with inorganic cations added to the aqueous phase. This

allowed the catalyst to penetrate into the substrate at the interface; partition of the catalyst from the aqueous phase into the lipid phase could not be detected.

2/5. $Pd(QS)_2$, hitherto the most efficient water-soluble catalyst for the hydrogenation of model and biomembranes

Following systematic studies with various candidates, at the early eighties it became obvious, that the palladium(II) complex of the dye alizarin red (sodium 1,2-dihydroxy-9-10-anthraquinone-3-sulfonate), if properly used, is a superb catalyst for biochemical hydrogenations (17). The chemistry and lipid hydrogenation properties of the complex was studied in details by the strong co-operation of two laboratories led by F. Joo (KLTE, Debrecen) and L. Vigh (BRC, Szeged). It has been established, that the catalyst shows a complex behavior in the action of molecular hydrogen and oxygen, giving rise to the formation of at least four products. Owing to this complexity, precise control of the raction requires pretreatment of the catalyst. When partial hydrogenation of the Pd-complex is followed by air oxidation, a catalyst solution is produced which is stable on air and maintains catalytic hydrogenation activity for several days. This form of the catalyst induces hydrogenation of unsaturated lipids with no induction period making a strict timing of the procedure possible (17). Of the several other factors affecting the outcome of membrane hydrogenation, one of the most important is the accessibility to the catalyst of particular membrane regions or lipid pools. Differences in accessibility may arise as a consequnce of different local microviscosities or their change during hydrogenation, of the appearance of distinct liquid crystalline phases, and of strong protein-lipid interactions. Obviously, in case of whole-cell hydrogenations (see also later), the accessibility is influenced by the spatial separation of the organelles, as well. Controlled hydrogenation may give valuable data regarding lipid-protein interactions and membrane structure. For instance, such experiments shed light on the presence of a specific membrane compartment in rat liver mitochondria, composed mainly of cardiolipin (33).

Careful control studies revealed, that in most of the cases the catalyst showed negligible self-effect on the integrity of cultured cells (*D.salina, Tetrahymena, A.nidulans*), on various enzymatic activities in microsomal membranes (27), on reduction rate of flash oxidized cytochrome f (56), on phospholipase A_2 activity in rat liver mitochondria (34), or on stability of light harvesting complexes and

photosynthetic electron transport (42,44,50,55). All these observations suggested, that the self-effect of the residual catalyst (if any) can be accurately taken into account.

The reactivity of $Pd(QS)_2$ has been systematically examined in multilamellar dispersions of unsaturated phospholipids [21,22]. With substrates of dioleoyl-phosphatidylcholine there is a transient appearance of *trans ω9*, but no *cis* double bonds were observed when the *trans ω9* derivative of phosphatidylcholine was used as substrate. It was suggested that hydrogenation may proceed by a *cis-trans* isomerization followed by reduction of the *trans* double bond. Hydrogenation of di-18:2 and di-18:3 derivatives of phosphatidylcholines (PC) show highly complex patterns of partially saturated molecular species including combinations of *cis* and *trans* positional isomers with little evidence of bond migration. Comparisons of the rate of hydrogenation of unsaturated molecular species of phosphatidylcholines with those of dioleoylphosphatidylethanolamine (PE) revealed that the reduced $Pd(QS)_2$ catalyst had a slight preference for PC. A preference for polyunsaturated molecular species compared with the monounsatureted molecular species of phosphatidlycholine was also observed. Differences in the accessibility of catalyst to substrates presented in bilayer form compared with those in inverted hexagonal-II (H_{II}) configuration may explain the different susceptibilities exhibited by phosphatidylcholines and phosphatidylethanolamines. These differences persisted in mixed dispersions hydrogenated at temperatures at which phase separations of bilayer and hexagonal-II structure would be expected to occur in the substrate.

The relationship between phospholipid saturation and membrane physical structure in a complex, typically highly polyunsaturated biological membrane (trout liver microsomes) has been studied recently by the graded and specific hydrogenation of polyunsaturated fatty acids (22). The Pd-complex caused rapid and effective hydrogenation, increasing the proportion of saturated fatty acids from 20-30 % up to 60 %, without loss or fragmentation. Long chain polyunsaturated fatty acids (20:5 ω3, 22:6 ω3) were rapidly converted to a large number of partially hydrogenated isomers, and ultimately to the fully saturated C20 and C22 fatty acids. C18 mono- and di-unsaturates showed slower rates of hydrogenation. Increased saturation led to highly ordered membranes exhibiting gel-to-liquid crystalline phase transition. Thus, without

direct alteration of other membrane components, the method offered unique means of assessing the influence of polyunsaturation and membrane structure and function (22).

2/6. Hydrogenation of biological membranes: the directions of applicability

We have seen that homogeneous catalyst complexes are active against unsaturated phospholipids dispersed in aqueous systems and under physiological conditions of temperature and electrolyte concentration. Although anaerobic conditions in the presence of hydrogen gas are required for hydrogenation, biological membranes and cells can tolerate such conditions, often for relatively long periods, without deleterious effects. The effects of hydrogenation of isolated biological membranes, suspensions of subcellar organelles and preparations of living cells have all been reported. The most important (though selected) studies of the effects of hydrogenation on biological membranes will be overviewed below.

Effect of lipid saturation on cation pumps

The effect of hydrogenation of lipids of rabbit hind leg muscle sarcoplasmic reticulum preparations on the activity of Ca^{2+}-ATPase pump protein has been reported about 20 years ago [23,24]. It was shown that up to 35% of the unsaturated bonds could be saturated during 5 h incubation at 9 atm H_2 in the presence of the Wilkinson's catalyst. Removal of 25% of *cis* double bonds does not affect the activity of Ca^{2+}-ATPase. This result could be interpreted in any of the following ways: (i) removal of 25% of the unsaturated double bonds does not significantly decrease membrane fluidity; (ii) activity of the enzyme is not dependent on membrane fluidity; or (iii) only a selected group or pool of lipids are hydrogenated. Selective hydrogenation may arise from preference of the catalyst for polyunsaturated fatty acyl chains. No direct methods were employed to determine the effect of hydrogenation on membrane fluidity

Membrane homeoviscous adaptation

Many organisms are known to adapt the extent of unsaturation of their constituent membrane lipids in response to environmental factors, most notably temperature [17, 25,26). The question of whether the activity of acyl chain desaturase enzymes is altered directly by the change in environmental factors, or indirectly by a change in the local microviscosity of the lipid domain in which the enzymes are integral components, has been examined by homogeneous catalytic hydrogenation. One

system that has been studied is microsome fraction prepared from potato tubers [27]. Oleoyl-CoA desaturase from potato tuber microsomes is believed to consist of four intrinsic membrane proteins [28,29]: (i) a reductase that transfers electrons from NADH to (ii) an electron carrier; (iii) a lysophosphatidylcholine-acyltransferase; and (iv) oleoylphosphatidylcholine desaturase. Cytochrome b_5 is believed to be the electron carrier involved in oleate desaturation, and NADH-cytochrome b_5 reductase [30] and cytochrome b_5 [31) from potato tuber microsomal preparations have been isolated and characterized. When membrane lipids of microsomal suspensions were hydrogenated in the presence of $Pd(QS)_2$ catalyst there was a marked rigidification of the hydrocarbon domain of the membrane as judged by electron spin resonance (ESR) probe measurements and a stimulation of NADH reductase using ferricyanide as the electron acceptor. It was suggested that the increased NADH-ferricyanide reductase activity was caused by greater accessibility of the active site of the enzyme to the electron acceptor, ferricyanide, possibly as a result of a displacement of the protein with respect to the membrane lipid matrix as the lipids become less fluid. Vertical displacement of proteins in these circumstances has also been suggested by other studies [32]. In contrast, when cytochrome *c* replaced ferricyanide as the electron acceptor it was found that NADH-cytochrome reductase as well as oleoyl residue acylation and desaturation were markedly inhibited by saturation of the membrane lipids. Similar conclusions were drawn from studies on 18:1-CoA desaturase activity in yeast microsomal membranes hydrogenated in the presence of Pd-complex [35]. In this system there was a clear indication for the dependence of lipid desaturase activity on the lipid microviscosity as judged by fluorescence probe polarization measurements.

Membrane lipid biosynthetic pathways

The possibility of modifiying the extent of saturation of membrane lipids at sites remote from their site of synthesis in living cells provides a tool for examining the factors regulating lipid homeostasis. Information on the pathways of unsaturated membrane lipid biosynthesis and processes of redistribution form the site of synthesis to the different subcellular membranes has been obtained from studies of the unicellular green alga, *Daniella salina*. This cell, in common with other plant cells, has a complex mechanism of membrane lipid biosynthesis. Some lipids are synthesized in the endoplasmic reticulum, others in the chloroplast and others partly in

both locations. Membrane lipids of *Daniella* can be hydrogenated extensively in the presence of water-soluble palladium alizarine catalyst under conditions that permit full recovery of the cells within 24 h [36]. Restoration of lipid unsaturation permits a resumption of growth as membrane functions are presumably restored. Subfractionation of hydrogenated cells showed that the plasma membrane component of the microsomal fraction was hydrogenated to the greatest extent and endoplasmic reticulum to a considerably lesser extent. Attempts to hydrogenate the outer surface of the plasma membrane of *Tetrahymena mimbres* have also been reported [37] and >20% saturation has been achieved. There was, however, loss in viability of the cells when more than trace amounts of hydrogenation were detected. The cause of this sensitivity to hydrogenation of the plasma membrane in *T. mimbres* is presently unknown.

Membrane lipid topology and function

The topology of lipids in the membranes of complex organisms or in subcellular membrane preparations can be probed by determining access to a hydrogenation catalyst. Water-soluble catalyst complexes, for example, are not readily permeable to membranes and, when added to suspensions of cells or closed vesicular structures, their action has been shown to be largely restricted to the outer monolayer, at least at short time intervals after commencement of the reaction (see [37]). Selective hydrogenation of lipid classes has also been observed. Lipid acyl bonds in isolated rat liver mitochondrial membranes (33) were gradually reduced by palladium complex-catalysed hydrogenation, and the resulting saturation was monitored by fatty acid analysis of PC, PE and cardiolipin (CL). The courses of hydrogenation of these phospholipids suggested that CL is in a membrane compartment which is less accessible to the applied catalyst. Native CL and its hydrogenation products were further characterized by analysis of their molecular diacylglycerol species. A decrease in the double bond content was accompained by an increased amount of motionally restricted lipids at the hydrophobic interface of proteins as measured by lipid spin labels. The protein immobilized fraction of spin-labelled stearic acid increased in parallel with the hydrogenation of CL rather than of PC or PE. These data are interpreted in terms of a tight association of CL with membrane proteins, which becomes looser upon double bond reduction leading to the replacement of CL by spin-labelled stearic acid in the solvation shell and well support the important functional

role of CL (33,34). Analysis of the pattern of hydrogenation during incubation of pea chloroplasts in the presence of $Pd(QS)_2$ catalyst is an other example [38].

The role of lipid unsaturation in controlling phospholipase A activity

Catalytic hydrogenation has been used to examine the mechanism of retailoring membrane lipids in the process of thermal (cold) adaptation in T. *pyriformis* [39]. Isolated cilial membranes, when hydrogenated in the presence of Pd (II) sulphonated alizarine complex, showed a marked increase in order parameter and rotational correlation time of ESR probes as the constituent lipids became saturated. This was associated with a dramatic decrease in the endogeneous phopholipase A activity of the membrane even when only a small proportion of the unsaturated bonds had been hydrogenated. The way in which an endogeneous phospholipase responds to the change in physical state of its substrate is believed to be the mechanism whereby the biochemical changes responsible for thermal adaptation are bought about (25). This has been further confirmed by studies of endogeneous phospholipase A (PLA) activity in rat liver mitochondria (34). After hydrogenation, mitochondria lost their ability to hydrolyze endogenous phospholipids in alkaline, Ca^{2+} containing medium, while PLA_2 retained full activity against exogeneous substrates, regardless of whether those substrates were hydrogenated or not. *Inhibition by hydrogenation of PLA activity correlated with the loss of polyunsaturated fatty acids, rather than with changes of the bulk membrane fluidity.* These data suggest that the level of unsaturation of membrane lipids is a key element of the mechanism of phospholipid breakdown governed by endogeneous phospholipases.

Membrane stability at extreme temperatures: testing the adaptive value of cis double bonds

Some organisms or differing strains of the same organism suffer loss in viability resulting from a sudden exposure to cold. These effects appear to be related to the extent of unsaturation in lipids of constituent membranes. Catalytic hydrogenation has proved to be an extremely useful method for investigating the molecular basis of chilling sensitivity. The phase behaviour of membrane lipids of the blue-green alga *Anacystis nidulans* is believed to underly the chilling sensitivity of this organism [40], and catalytic hydrogenation studies by $Pd(QS)_2$ have been undertaken to examine this hypothesis [41, 42]. Series of experiments supported the hypothesis that the phase

behaviour, as modulated by the degree of unsaturation of the constituent membrane lipids, is directly related to the susceptibility of the cells to chilling damage.

Adaptation of organisms to elevated temperature is often associated with a shift in the moleculatr species of membrane lipids to more saturated fatty acyl substituents. It is often argued that this change renders the membrane more stable at elevated temperatures. This concept has been examined in considerable detail in photosynthetic membranes which are ideal for hydrogenation studies because of the highly unsaturated lipids present and the dependence of the membrane on these lipids for maintaining structural stability and organization [43]. The first studies were performed using Wilkinson's catalyst [44] and it was found that decreases of up to 40% of unsaturated bonds did not alter the ultrastructural features of photosynthetic membranes. Later investigations using Pd-complex [45,46,50,53,54,55,56] showed that saturation of the lipids results in a decrease in electron tranfer between the 'primary' electron acceptor QA and 'secondary' acceptor, QB. Fluorescence-inducation kinetics indicated that there is an optimal level of lipid unsaturation for maintaining an efficient electron transfer from QA^- to the plastoquinone pool. Furthermore, the proportion of photosystem-IIb, the form of photosystem-II with complete peripheral chlorophyll *a/b* light-harvesting chlorophyll-II, increases with increased hydrogenation of the membrane lipid. Hydrogenation of the lipids of the photosynthetic membrane of higher plant chloroplasts prevents disruption of the membrane exposed to elevated temperatures. Freeze-fracture electron microscopic studies of hydrogenated chloroplasts has revealed the presence of particle-free domains within lipids [50]. When exposed to elevated temperatures (40-45°C), a treatment that results in a loss in photosynthetic electron transport [51], membrane destacking and dissociation of supramolecular protein complexes [52], hydrogenated membranes show a reduced tendency to destack and vesiculate. Chlorophyll *a* fluroescence measurements and differential scanning calorimetry suggest that the hydrogenation of thylakoid membrane lipids causes an increased thermal stability of pigment protein complexes of the photosystem-II light harvesting apparatus. Similar stabilization of photosystem-I complex subjected to heat stress has also been observed [53].

Hydrogenation of the membranes of living cells

Hydrogenation of membrane lipids of a living organism was first described by Chapman *et al.* [23], who accomplished the saturation of fatty acid residues of the membrane of *T. pyriformis* in the presence of Wilkinson's catalyst. The organism appeared to revive after partial hydrogenation of the membrane lipids but no long-term survival study was performed. Hydrogenation of lymphocytes with Wilkinson's catalyst has also been reported [57] but this catalyst was found to be highly toxic to the cells. Different cell types (algal, plant, mammalian) have been studied since then by using with much more success the $Pd(QS)_2$ catalyst, introduced above. The present Ph.D. thesis is dealing with *the first adaptation of the method of Pd-catalyst mediated hydrogenation on murine GRSL leukemia cells* (58*) and mouse L929 tumor cells* (59). The optimal conditions of the hydrogenation of the natural killer (NK) cells's target K562 erythroleukemic cells has also been accomplished, enabling us to show the striking target lipid saturation sensitivity of the action of NK-cells (Benko et al., manuscript, in preparation). In general, cells have been found to vary considerably in their ability to survive the partial hydrogenation of the plasma membrane. As noted above, the protozoan *T. nimbres* appears to be particularly sensitive [37]. All the observations suggest, however, that with careful control experiments the self-effect of the residual catalyst (if any) in most cases can accurately be taken into account, and that way the biophysical, biochemical or physiological response(s) of the cells or particular organelles to the reactions can be properly related to the saturation of their fatty acids (8,17,36, 41,42,61,63). In some rare occasions, however, the self-effect of the catalyst renders use even of the highly efficient soluble $Pd(QS)_2$ impossible. To overcome this problem, colloidal metals, preferably on a biocompatible carrier, or soluble catalysts attached onto the surface of ion-exchange beads had to be considered (64,65). As highlighted also in the present Ph.D. thesis (see later), mechanism of the activation of rat platelet by trombin could not be investigated in the presence of soluble $Pd(QS)_2$ since the catalyst itself (under N_2) blocked completely the aggregation. However, colloidal Pd on poly(polyvinylpyrrolidinone) had only a small self-effect, and the influence of hydrogenation on the aggregability could be studied by using this macroheterogeneous catalyst (60,64,65,66).

One fundamental question of membrane biochemistry is how do cells maintain the physical structure of their membrane lipid bilayers within tolerable limits. As was

highlighted above, the short answer is, that first of all fatty acid unsaturation is varied to provide the appropriate milieu for membrane function. Yet, little is known about how this is achieved and absolutely nothing about the cellular sensor concerned. By using $Pd(QS)_2$, Vigh et al. have shown, that the catalytic hydrogenation of a small pool of plasma membrane fatty acids activates transcription of specific desaturase genes in the same way as cooling thus leading to the resynthesis of unsaturated fatty acids (61). The results offered the first direct evidence that subtle changes in surface membrane structures (as could be caused also by various disease states, ageing, etc.) affects gene transcriptional activity, and point to the control of membrane lipid biosynthesis by a negative feedback loop based on lipid order (25, 62).

Despite the numerous efforts aimed at understanding how cells are capable of sensing changes in ambient temperature, the exact nature of sensors and signals participating in the heat shock response is presently unknown (25). Lending support to a model in which thermal stress is transduced into a cellular signal at the level of thylakoids, plasma membrane saturation by catalytic hydrogenation using microheterogeneous complexes developed in the presently described study (see below) did not affect temperature threshold of the heat shock protein gene's activation (63). In other words, the surface membrane directed hydrogenation allowed to measure the separate contribution of the physical states of plasma- and endomembranes to the mechanism of heat shock signalling. Learning the lessons from cyanobacterial modells, similar studies are in progress with K562 and L929 mammalian cells (Vigh, Balogh, Benko, Joo, Horvath et al. manuscript in preparation).

3. AIM OF THE STUDY

3/1. Establishing conditions for hydrogenation of plasma membrane lipids of murine GRLS leukemia cells by using $Pd(QS)_2$ catalyst: study the effects on membrane fluidity and expression of cell surface antigens

Alterations in the cholesterol content which have often been brought about by treatment of cells with cholesteryl hemisuccinate has been reported to increase immunogenicity [67,68] of tumor cells and to decrease the expression of H-2 antigens on the cell surface [69]. We considered it worthwhile to examine whether similar

effects can be obtained when the membrane fluidity is altered by another principle, that is, without introducing foreign molecules in the membrane. Our purpose in the present study was to introduce in the first time the Pd-complex mediated membrane hydogenation technique on lymphoid cells, finding conditions where maximal hydrogenation is obtained with minimal cell death, and to see whether this treatment affected the expression of various cell surface antigens. Furthermore, in view of the conflicting results that have been reported as to whether a certain change in the level of fatty acyl unsaturation may lead to a change in membrane fluidity [70], we have investigated the effect of hydrogenation on purified plasma membranes and liposomes prepared from their extracted lipids. We applied gas chromatographic analysis of the fatty acids and measured the membrane fluidity by fluorescence polarization with the probe 1,6-diphenyl-1,3,5-hexatriene [71].

3/2. Modulation of the action of tumor necrosis factor mediated cell killing by catalytic hydrogenation of the target cell's plasma membranes

TNF-α is a cytokine with anti-tumor and a variety of other biological activities. It is homotrimer of 17 kDa subunits, [72, 73] synthesized by activated mononuclear phagocytes and by some other cells. TNF causes hemorrhagic necrosis of certain tumors in expreimental animals and in cancer patients and exerts cytotoxic or cytostatic activity on many transformed cell lines *in vitro* [73-77]. The action of TNF requires specific binding to high-affinity cell surface receptors, which are expressed not only on most malignant cells but also on normal diploid cells of various tissues. [78-81]. Despite high affinity binding many tumor cells and most normal cells appear to be resistant to the cytotoxic or cytostatic action of TNF indicating that sensitivity is controlled at post-receptor level. [78-81]. Subsequent clinical trials revealed that the medical applications of TNF are jeopardized by its systemic toxicity, which is manifested in high fever, hypotension, thrombocytopenia, convulsions, intravascular coagulation, etc. Therefore, TNF cannot be administered safely, unless we can restrict its action to the malignant tissues or protect normal cells against its cytotoxicity. TNF induces apoptosis (programmed cell death) in most susceptible target cells. The exact mechanism of TNF induced cell killing is not known, though in a model system ceramide, an intermediate of the TNF signalling pathway functions as an intracellular mediator of apoptosis induced by TNF [82]. TNF is a powerful

pyrogen and the sensitivity of target cells was shown to be temperature dependent, as is the microviscosity of the cellular membranes. It is well established that TNF elicited signaling involves events which are highly influenced by the physical properties of the membrane, like clustering of receptor molecules and liberation of second messengers by various phospholipases (83-85). Phospholipid acyl chain saturation dependent parameters of the bilayer were shown to correlate with membrane protein functions (43) including G-protein coupled systems (86) and phospholipases (33).

Combining all of these findings, here we decided to explore the role of the lipid saturation and physical state of the plasma membrane of the TNF target mouse L929 tumor cells in the TNF induced cell killing. Accordingly, we adapted the method of membrane hydrogenation by using the powerful, nontoxic, water-soluble $Pd(QS)_2$ complex as catalyst.

3/3. Testing the TNF sensitivity of genetically fluidized L929 tumor cells expressing the yeast Δ^9-desaturase gene

We have shown (see above) that the selective saturation of fatty acids located in surface membrane phospholipids of L929 target tumor cells, if carried out by Pd-catalysed hydrogenation, decreased TNF sensitivity by orders of magnitudes (59). On the other hand, fluidization of the membranes with phospholipid liposomes containing a high proportion of unsaturated fatty acids lead to a significant increase of sensitivity to TNF (Kusz, E. and Duda, E. unpublished). Furthermore, both microviscosity of target cell membranes and TNF sensitivity exhibited a remarkable increase at elevated temperatures. Keeping in mind the above findings, the aim of that part of our studies was to constitutively express the yeast desaturase gene, *Ole1* (87-89) and thereby interfering with the overall level of lipid unsaturation in an opposite direction as we attained before by *in situ* hydrogenation of L929 tumor cells. Parallel with changing the composition and physical state of membranes, we intended to test the TNF sensitivity of the transformed cells.

3/4. When the use of Pd(QS)2 is impossible: colloidal metal dispersions as novel catalysts for leaflet-selective surface membrane hydrogenation of rat platelets

As we discussed above, in some cases, the mostly negligable self-effect of the catalyst renders use of the soluble $Pd(QS)_2$ impossible. To highlight such a typical

case, activation of platelet by trombin from rat could not be investigated in the presence of $Pd(QS)_2$, since the catalyst itself blocked completely the aggregation (Benko et al., unpublished). On the other hand, several biochemical findings pointed to a different involvement of the outer and inner leaflets of the asymmetric plasma membrane in most various membrane bound functions (8) and therefore it seemed important to design biocompatible catalysts capable of site-selective surface hydrogenation of living cells. As we have seen before, though a remarkable selectivity could be achieved by controlling the hydrogenation time even with the Pd-complex, the slow diffusion of the catalyst into the inner leaflet of the plasma membrane and further to the interior of the cells can not be prevented. In conclusion, at that stage of our studies our aim was to develop a new family of hydrogenation complexes that fulfills these specific requirements. As we will show, the above problems could be eliminated by the introduction of largely uniform, nanosize palladium catalysts deposited on the surface of insoluble poly(N-vinyl-2-pyrrolidone), Pd-PVPP (64,65).

There have been considerable speculations about the fundamental importance of the composition and physical state of the surface membrane lipids of platelets in their aggregation (66,90). Cholesterol-enriched platelets and platelets of hypercholesterolaemic patients show increased aggregation (90). This finding led to the hypothesis that a more rigid plasma membrane facilitates signal transducing pathways in the lipid bilayer either through increased expression of receptors for activating agents or better post-receptor signal processing. It is generally beleived, that unsaturated fatty acids alter platelet aggregation and thereby have an important role for instance at risk of cardiovascular disease. Indirect evidence exists that n-6 polyunsaturated fatty acids may exert favorable effects on thrombotic processes in vivo, but large clinical trials have failed to show benefits of linoleic or linolenic acid diets (91). It was assumed, that the great difference of microviscosity between the two halves of platelet plasma membranes which is almost completely averaged out after activation results from the unequivalence of their acyl chain composition and unsaturation. The effect of the scrambling at the time of platelet activation would be of significant biological relevance to ensure the proper fluidity of the platelet surface in the coagulation cascade (66). Model membrane data suggested, that the fatty acyl unsaturation of specific lipid molecular species could participate in the sideness of phospholipids. Namely, the asymmetry resulting from a flip-flop equilibrium which

favours the faster transverse distribution of unsaturated species of PE and PS between the two halves.

As a first step to establish a system in which the proposed causal relationship between plamamembrane fluidity, lipid unsaturation and platelet aggregation can be studied, we aimed at working out the method of Pd-PVPP catalyst-mediated hydrogenation for rat platelets.

4. MATERIALS AND METHODS

4/1. Establishing conditions for hydrogenation of plasma membranes of murine GRLS leukemia cells by using Pd(QS)2 catalyst and study the effects on membrane fluidity and expression of cell surface antigens

Membrane preparation and lipid analysis

Plasma membranes were purified from rat (R/A) liver [92] and murine leukemic GRSL cells [93,95] according to standard methods in our laboratory. Briefly, livers were homogenized in 1 mM $NaHCO_3$ (pH 7.5), using a Potter-Elvehjem homogenizer. After several low-speed differential centrifugations, plasma membranes were purified by flotation in a discontinuous sucrose gradient at the *d* 1.16/1.18 interface [92]. GRSL ascites cells were disrupted by pumping a suspension of 5 X 10^7 cells/ml in Hanks' balanced salt solution (Oxoid, London, UK) through an air-driven cell disruptor (Stansted Fluid Power Limited, Stansted, Essex, UK; model AO 612, disrupting valve 516) using an air pressure of 45 lb/inch^2. The GRSL plasma membranes were purifed by utilizing a discontinuous sucrose gradient, as described in detail before [93]. The purity of the plasma membrane preparations was ascertained routinely by electron microscopy and by marker enzyme assays, as described previously [92,93]. Total lipids were extracted from these membrane preparations and from whole cells by chloroform/methanol (2:1, v/v), followed by Folch's partition [94]. Phospholipids were isolated by standard silicic acid column chromatography in the methanol eluate. The fatty acid composition of the total phospholipids was analysed, after transesterification with H_2SO_4/methanol, by capillary gas-liquid chromatography on an open tubular glass column, 25 m x 0.21 mm, wall-coated with Silar 5 CP, as described in detail [95].

Hydrogenation procedure

Aliquots (10-50 ml) of murine GRSL cells, adjusted to a concentration of 1 x 10^6 cells/ml in Hanks' medium containing 1.2% glucose and 3.5% polyvinylpyrrolidon (M_r 40000; Sigma), were placed in high-pressure siliconized glass vessels and transferred to a thermostated (20 °C) water bath. The reaction vessels were connected to a manifold and the gas phase was evacuated and replaced by 1.5 atm hydrogen or oxygen-free nitrogen (control). The catalyst used for the hydrogenation was palladium-di-(sodium alizarine monosulphonate) abbreviated as $Pd(QS)_2$. It was prepared by F. Joó using methods described elsewhere [96]. A stock solution containing 5 mg/ml catalyst of glass distilled, deionized and degassed water was prepared and aliquots were injected into the cell suspension through a silicon rubber septum to initiate the reaction. The final catalyst concetration in the reaction medium was 100 μg/ml. The gas pressure was increased to 2 atm and the reaction vessels were rotated at 45 rev./min for the duration of the hydrogenation (45 min). At the end of the incubation period the gas pressure was reduced slowly over a period of 5 min to avoid the formation of gas bubbles in the medium. After having established the optimal hydrogenation conditions for living GRSL cells, isolated plasma membranes from GRSL cells and from rat liver, and of liposomes of the extracted total lipids of these membranes were subjected to the same hydrogenation procedure, at a membrane concentration corresponding to 70 nmol phospholipid/ml reaction mixture. Finally cells, membranes or liposomes were spun down and washed twice with fresh assay medium to remove the catalyst.

Estimation of membrane lipid fluidity

The fluorescent hydrocarbon 1,6-diphenyl-1,3,5-hexatriene (DPH) was used as a probe for measuring the degree of lipid fluidity in plasma membrane preparations and liposomes or from whole cells, by steady-state fluorescence polarization (P_{DPH}). The measurements were performed at 25 °C with an Elscint apparatus, model MV-1A (Elscint Ltd., Hifa, Israel) as described previously [93].

P_{DPH} values mainly reflect the orientational constraint of the motions of the probe, and these values, or rather the r_s values (steady-state fluorescence anisotropy) can be quantitatively converted into lipid order parameters, S_{DPH}, using semi-empirical relationships [71,98]. Membrane lipid fluidity may be defined as the reciprocal of the

lipid order parameter [71]. In the present paper we used for calculation of S_{DPH} the equations: $r_\infty = (4\ r_s/3) - 0.10$ (valid for the region $0.13 < r_s < 0.28$ or $0.18 < P_{DPH} < 0.37$) and $(S_{DPH})^2 = r_\infty/r_0$, in which r_∞ represents the limiting hindered fluorescence anisotropy, and $r_0 = 0.4$ is the theoretically maximal flurorescence anisotropy [71].

Immunofluorenscence of cell surface antigens

The following rat monoclonal antibodies were used (diluted 1/10) to measure the expression of antigens on the surface of murine GRSL cells: 30-H11, detecting a glycolipid antigen on GRSL cells [99], has been prepared and described by Ledbetter and Herzenberg [100]; 142/5 detects a glycoprotein of 95 kDa, denominated Pgp-1 by Trowbridge et al. [101]; 120C2, raised against GRSL plasma membranes, detects a 15 kDa protein which is also present on normal mouse lymphocytes and bone marrow cells (kindly provided by H. Haisma); 78H3B5F12 (kindly provided by R. Nusse) was raised against a mouse tumor viral envelope component gp52 and detects the viral precursor molecule $Pr73^{env}$, also called MLr antigen [93,102], on the surface of GRSL cells. Monoclonal antibodies from hybridoma clone H100-5/28 against the $H\text{-}2^k$ haplotype (determinant H-2.m3; Ref. 103) were purchased from Camon Labor-Service GmbH (Wiesbaden, F.R.G.); it was used in a dilution of 1/100. As secondary antibodies we used FITC-conjugated goat IgG against rat or mouse IgG, purchased from Nordic Immunological Labs. (Tilburg, The Netherlands), in a dilution of 1/20.

The cells were first incubated with the monoclonal antibodies for 30 min, then washed twice with Hanks' balanced salt solution, incubated with the second antibodies and washed again twice. All treatments were done at 4 °C.

Flow microfluorometry was carried out with a Fluorescence-Activated Cell Sorter (FACS IV; Becton-Dickinson) in which the cells were excited by an argon laser (488 nm) and fluorescence emission was measured at 520 to 550 nm. The fluorescence signal is given in channel numbers, which are logarithmic functions of its intensity. Cells were light scatter-gated for live cells [69].

4/2. *Modulation of tumor necrosis factor mediated cell killing by catalytic hydrogenation of the target cell's plasma membranes*

Cell culture:

L929 mouse fibroblast cell line was cultured as monolayer in DMEM (SIGMA Chemical Co., St. Louis, USA) medium supplemented with 10% fetal calf serum (GIBCO BRL, Gaithersburgh, USA) at 37°C in 5% CO_2 in air. For fatty acid analysis and microviscosity measurements (see later) we used subconfluent cultures of cells.

Recombinant human TNF-α

The human TNF-α gene was cloned in *E. coli* cells, TNF was produced and purified to homogeneity in our laboratory. The specific activity of the preparations was more that 20 million U/mg, with no significant endotoxin content (<30 pg/mg protein). TNF was radioiodinated by chloramine T in a multi-drop system: TNF, $Na^{125}I$ (IZINTA, Budapest) were dissolved in 100 mM K-phosphate buffer, pH 7.0, 50 µl of this solution, in the form of a drop on siliconized glass surface was exposed to chlorine produced by neighbouring drops of chloramine T solution (SIGMA, St. Louis, MO, 10 mg/ml, 50 µl each).

Hydrogenation of the living L929 cells.

The catalyst, $Pd(QS)_2$ was at that time the product of Molecular Probes, Eugene, Oregon. Mouse L929 tumor cells were grown in DMEM (SIGMA Chemical Co., St. Louis, USA), containing 5% newborn calf serum (GIBCO BRL, Gaithersburgh, USA), to near confluency. Cells were trypsinized (the action of trypsin was arrested by the addition of minute amounts of serum) and collected by centrifugation and washed free of serum proteins, suspended in 10 mM HEPES buffer (N-2-hydroxy-ethylpiperazine-N'-2-ethanesulfonic acid, SIGMA, St. Louis, MO), pH 7.4 containing isotonic solution of mannitol (hydrogenation medium). The suspension contained 5 x 10^5 cells/ml. A stock solution of catalyst (5 mg/ml) was prepared in degassed HEPES buffer (10 mM, pH 7.4). Part of the catalyst solution was activated by incubation in hydrogen atmosphere for 5 min before use, in order to convert it to its hydride (B) form [17]. Thirty-forty ml aliquots of L929 cell suspension were tranferred into custom designed reaction vessels and placed into a water bath at 37 °C, the reaction vessels were connected to a manifold and the gas phase was evacuated and replaced by hydrogen or nitrogen (control), at atmospheric pressure. To initiate the reaction, aliquots of inactive or activated forms of the catalyst were then injected into the

reaction vessels through a rubber septum. The final concentration of either form of the catalyst was 0.1 mg/ml. During the hydrogenation procedure (max. 5 min), the reaction vessels were rotated at 45 rpm. At the end of the desired incubation time L929 cells were removed, the reaction stopped by aerating the suspension with 5% CO_2 containing air. The cells were repeatedly washed to remove the catalyst, harvested for lipid extraction, ESR measurements or plated for TNF assay. Viability of hydrogenated cells was estimated by dye exclusion. Long-term survival of the cells was quantitated by plating aliquotes on Petri dishes in DMEM, 10% FCS and counting the arising clones two weeks later.

Lipid extraction and fatty acid analysis

Lipids were extracted from washed cells by the method of Bligh and Dyer. Phospholipids were separated on precoated TLC plates (Merck 5721) developed with hexan/aether/glacial acetic acid (60:40:2). For the determination of the constituent fatty acids, aliquots of the total lipids or the phospholipids were subjected to methanolysis, the resultant methyl-esters were analyzed on Hewlett Packard gas chromatograph (HP 3396A) equipped with flame ionization detector on SP2230, 30cm long capillary column (Supelco). Quantitation was made by a Hewlett Packard (HP 3396A) integrator.

ESR measurements: estimation of L929 plasma membrane fluidity

To measure the degree of lipid fluidity in the plasma membrane of control and briefly hydrogenated cells the fatty acid spin label 2-(3-carboxypropyl)-2-trydecyl-4,4-dimethyl-3-oxazolidinyloxyl-1,5-doxylsterate (Molecular Probes, Eugene, Oregon) was used. Five µl aliquots of solution of the probe (2 mg/ml in ethanol) were added to 400 µl cell suspensions (1.5×10^7 cells/ml) and vortexed occasionally for 2 min. After 5 min of incorporation of the spin probe, the cell suspension was centrifuged and the cells were transferred into 1 mm diameter quartz ESR capillaries. Experiments were performed in an X-band spectrometer (JEOL JES-PE-1X) using 100 kHz modulation frequency technique. Spectra were recorded at 37°C. The time dependent changes in the spin probe signal were monitored by setting the magnetic field of the spectrometer at the peak of the midfield line of ESR spectrum [104]. Alterations of the line height was recorded and plotted against incubation time. Data were collected, stored and

manipulated with an IBM PC computer. Orientation order parameter, S, was calculated from the position of the inner and outer splittings and converted for polarity as described by Griffith et al. [105] Following the ESR experiments, cell viability was routinely tested by dye exclusion. More than 90% of control or briefly (2 min) hydrogenated cells proved to be alive by this test.

Determination of TNF cytotoxicity.

TNF-α was quantitated in a modified bioassay using the highly sensitive mouse L929 tumor cells [106] and the metabolic indicator tetrazolium dye, MTT (3-[4,5-dimethylthiazol-2yl]-2,5-diphenyl 2H-tetrazolium bromide, SIGMA, St. Louis, MO) 3x 10^4 cells in 200 µl medium/well of microtiter plates were plated one day before the assay. MTT was added (10 mg/ml, 10 µl/well) 16 hrs after the exposure to TNF. The color of the dye rapidly changed in the presence of living cells. After 5 hrs of incubation plates were centrifuged, medium removed and 200 µl DMSO was added to dissolve the precipitated dye and measure the absorbance at 540 nm. Data were calculated as percent survival rate or as units of TNF-α, 1 U/ml being defined as the concentration that resulted in the death of 50% of the L929 cells in the presence of 0.1 g/ml actinomycin D at 37°C. For the characterization of the cytotoxic activity in our TNF preparation samples were incubated either with an excess of rabbit (anti-recombinant human) TNF-α (generously provided by Dr. K. Nielsen) or with control rabbit sera. After 2 hrs of incubation at 37°C, residual cytotoxicity was determined on L929 cells.

TNF receptor assay.

^{125}I labelled TNF was incubated with the monolayers (grown on microscopic cover slides) at different temperatures for 10, 20, 30 and 60 min in the presence or absence of 10^4 fold excess of non-labelled TNF. The cells were washed at 0°C with PBS. Non-internalized TNF was washed off with 50 mM Na-acetate buffer, pH 3.0. The amounts of receptor bound, non-specifically bound and internalized TNF were calculated on the basis of counts measured by immersing the cover slips into scintillation liquid (dried after consecutive washes with TCA and chloroform:methanol)

4/3. Testing the TNF sensitivity of L929 tumor cells overexpressing the yeast Δ^9-desaturase gene

Plasmids:

Constructs were inserted in the mammalian expression vector pRc/CMV (Invitrogen BV, Leek, The Netherlands). We replaced the original cytomegalovirus promoter by *Hin*dIII-*Sal*I digestion of the pRc vector with Δ^9-desaturase promoters derived from *H. capsulatum* (88). 2.7 kb DNA fragments (for details see ref. 88) were cloned into the *Xba*I site of the pRc plasmid, containing the mutant (Downs) *H. capsulatum* promoters and the *S. cerevisiae* desaturase (*Ole1*) gene (88,89) *LK* (L929) cell line - used as negative control - contained the promoterless pRc/OLE1 vector. The expression vectors were amplified in *DH5α E. coli* cells.

Transformation and selection of mammalian cells:

Transformations with the mammalian expression vectors were performed into L929 cell line using Lipofectamine (Gibco BRL, Gaithersburgh, USA). For each transformation 1-2 μg DNA and 10^5 cells were used. Transformant colonies were selected in the presence of 400 μg/ml G418 (SKW, Budapest, Hungary) in the culture medium. 10-10 clones of stable transfectants were choosen for further characterization from each transformation. The code of L929 cell lines containing the pRc/Downs/Ole1 vector was labeled as *LD*. Amplification of genomic DNA by PCR , Southern analysis, RT-PCR, cDNA synthesis – experimental processes in which I was not involved directly - are described in details in ref.107.
Analysis of fatty acids was made as described in Chapter 4/2.

Microviscosity determination:

Microviscosity of cell membranes were followed by measuring DPH (1,6-diphenyl-1,3,5-hexatriene) and DPH-PA (3-(p-(6-phenyl)-1,3,5-hexatrienyl) phenylpropionic acid) (Aldrich) fluorescence anisotropy. After harvesting cells were washed twice and resuspended in PBS. Concentration of the cell suspension was adjusted with PBS to OD_{360}=0.07 (~10^5 cell/ml) in 3 ml volume, labeled by the addition of DPH or DPH-PA, and dissolved in tetrahydrofuran to a final concentration of 0.1 μM. The final concentration of the organic solvent was 0.1 % (vol %). Cells were incubated with

DPH-PA for 10 min or with DPH for 30 min at 37°C. Steady state fluorescence measurements were carried out at 37°C using a Quanta Master QM-1 T-format luminescence spectrometer (Photon Technology Int. Inc., NJ, USA). Fluorescence anisotropy was calculated as described by Schlame et al. (34).

In vitro TNF cytotoxicity assay:

L929 cells were grown in 96-well plates to a density of $3x10^4$ cells/well. After 24 hour incubation at 37 °C, serially diluted TNF solution was added to the cells containing 5% of FCS with or without 1μg/ml Actinomycin D (108). After 16-24 hours of incubation viability of cells was determined using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay (109).

4/4 Surface membrane hydrogenation of rat platelets and its effect on thrombin induced aggregation.

Preparation of Pd-PVPP catalyst

The insoluble PVPP was obtained from Fluka (Plasdone XL). $[(NH_4)_2PdCl_4]$ was a product of Johnson-Matthey. All other materials were obtained from Aldrich and Sigma and were purified by recrystallization or distillation if necessary. Soybean lecithin was a Lucas Mayer (Germany) product purchased in a local pharmacy. Pd-PVPP catalyst was prepared by refluxing ethanolic solutions of $[(NH_4)_2PdCl_4]$ (42.7 mg) in the presence of PVPP (700 mg) for 2 h. The catalyst was isolated by evaporating the mixture to dryness. The resulting shiny black product could be freely dispersed in aqueous media. After cooling on ice, the ethanolic supernatant Pd-PVP sol was filtered, leaving a grey solid of Pd-PVPP, which was washed with ethanol and dried in air. Palladium content of the Pd-PPVP catalyst was determined using a SPECTROFLAME ICP AS equipment (cc. H_2SO_4/H_2O_2 digestion). Particle size distribution was determined both by transmission electron microscopy (JEOL 2000 FX II TEM) and dynamic light scattering (Brookhaven BI-200SM). The surface charge of the colloidal Pd particles was determined on a Brookhaven ZetaPlus zeta potential analyzer using Au electrodes, as we described (64,65).

Hydrogenation with Pd-PVPP

The catalyst suspended in 20 mM Na-phosphate buffer (pH=7.0) was connected to a vacuum line and placed under H_2 with 5 evacuation/refill cycles and stirred until the colour of the solid changed to dark grey. The suspension was let to settle and the supernatant was discarded under H_2 blanket. The remaining wet solid was taken up in 10 ml of fresh buffer and was ready for hydrogenation. Hydrogenation of liposomes or platelets was carried out at atmospheric H_2-pressure, essentially as described before (58).

Preparation and measuring aggregation and surface membrane fluidity of rat platelets.

Fresh rat platelets were prepared by differential centrifugation as in (110). Aggregation of platelets was followed spectrophotometrically, following activation with 2U thrombin (Sigma), also from rat. The trombocytes were labelled with the surface membrane selective fluorescence label TMA-DPH. Steady-state fluorescence anisotropy was calculated as in (34).

5. RESULTS AND DISCUSSION

5/1.Catalytic hydrogenation of fatty acyl chains in plasma membranes of GRLS leukemic cells: effect on membrane fluidity and expression of cell surface antigens

Optimal reaction conditions were established for homogeneous catalytic hydrogenation of the fatty acids in living GRSL leukemia cells. Under these conditions, specified under Methods, at least 80% of the cells remained viable, as judged by trypan blue exclusion. The additions of 1.2% glucose and 3.5% polyvinylpyrrolidon to the hydrogenation medium (($Pd(QS)_2$ containing Hanks' balanced salt solution) were found to be beneficial for cell viability. Bovine serum albumin was also good in this respect, but was found to inhibit hydrogenation significantly. The extent of hydrogenation of double bonds in the fatty acids of the phospholipids was analyzed by gas-liquid chromatography. The results are shown in Table I. Hydrogenation predominantly occurred in the polyunsaturated fatty acids 18:2, 20:4 and 22:6, resulting in a reduction of the double bond index in the cellular phospholipids of about 40%.

Having thus established the optimal hydrogenation conditions for living cells, we then subjected purified plasma membranes from these GRSL cells and liposomes prepared form their lipid extracts to the same hydrogenation procedure. Table I shows that also in these cases the polyunsaturated fatty acids become hydrogenated, to an even greater extent than in whole cells. This is understandable because the catalyst may have no or less access to the membranes of the interior of the cell, which contain the highest amounts of polyunsaturated fatty acids. In fact, evidence of selective catalytic hydrogenation of a peripherally located membrane has been reported for alga cells, where the cytoplasmic membrane lipids become hydrogenated immediately, but the inner membranes only at much later stage [21,42).

Table I

Effect of catalytic hydrogenation on the fatty acid composition (mol % of major fatty acids) of the phospholipids in living GRSL leukemia cells, their isolated plasma membranes, and of liposomes made from extracted total lipids from these membranes

Fatty Acids	Whole GRSL cells		Plasma membranes		Liposomes	
	Control (%)	H_2 + cat.	Control (%)	H_2 + cat.	Control (%)	H_2 + cat.
16:0	20.8	20.5	22.5	22.0	19.3	20.4
16:1	1.7	1.4	3.8	4.5	3.2	3.4
18:0	24.5	33.1	17.5	28.2	19.4	32.7
18:1	17.9	16.5	15.8	14.3	15.5	10.9
18:2	19.9	14.3	13.0	3.2	12.8	3.0
20:0	---	3.4	---	6.4	---	8.6
20:1	---	1.2	---	3.2	---	2.6
20:4	10.0	4.8	8.5	1.1	10.0	0.5
22:0	---	1.6	---	2.4	---	2.8
22:6	3.5	1.5	2.6	---	2.7	---
d.b.i.	1.22	0.77	1.14	0.38	1.21	0.29

Data are representatives of three experiments with essentially similar results.
d.b.i. (double bond index) = mean of double bonds per fatty acid.

Hydrogenation was also performed under the same conditions on a second type of plasma membrane, i.e. that purified from rat liver (Table II). Also in this case, hydrogenation occurred in the 18:2, 20:4 and 22:6 fatty acyl chains, and thereby the double bond index in these membranes was reduced by 60%, somewhat more than in the GRSL plasma membranes (37%; see Table I). Liposomes prepared from liver

membrane lipids exhibited the same susceptibility to hydrogenation as the native membranes, whereas liposomes from GRSL membranes were somewhat more susceptible.

Table II

Effect of catalytic hydrogenation on fatty acid composition (mol % of major fatty acids) of purified plasma membranes from rat liver and liposomes of the extracted total lipids of these membranes[a]

[a] See legend of Table I.

Fatty Acids	Plasma membranes		Liposomes	
	Control	H_2 + cat.	Control	H_2 + cat.
16:0	24.5	26.0	22.0	22.1
16:1	4.9	5.1	5.2	4.1
18:0	18.4	21.0	17.4	24.4
18:1	13.1	17.5	14.0	15.7
18:2	14.3	4.2	15.1	7.9
20:0	---	6.8	---	7.4
20:1	---	2.6	---	2.5
20:4	9.6	1.0	9.2	1.2
22:0	---	3.0	---	2.7
22:6	4.5	1.1	4.4	0.7
d.b.i.	1.25	0.50	1.29	0.53

In the latter liposomes the double bond index was reduced by 75% upon catalytic hydrogenation. It appears that membranes orliposomes with a higher degree of fluidity (see below) are more susceptible to hydrogenation, possibly due to facilitated intercalation of the $Pd(QS)_2$ catalyst in membrane lipid chains that are more loosely packed (less ordered; more fluid). It should be noted that the higher fluidity of GRSL plasma membranes as compared to liver plasma membranes is mainly due to their lower cholesterol and sphingomyelin contents, rather than to a difference in fatty acid composition (71,92,93). In a different experimental system, i.e. chloroplasts, the extent of catalytic hydrogenation of membrane lipids was found to increase in the order of the number of double bonds per lipid molecules present [38].

The structural order (reciprocal of membrane fluidity; Ref. 71) in dispersed lipid extracts of treated or untreated cells, and in treated or untreated plasma membranes or their liposomes, was determined by fluorescence polarization with the probe diphenylhexatrience (P_{DPH}) (Table III). In all membranes and liposomes there

was a small but clear effect of the catalyst by itself (with N_2 instead of H_2; non-hydrogenating conditions), indicating that some catalyst remained intercalated in the membranes, even after extensive washing (see also Table IV). However, the specific hydrogenation effect itself, by which extensive saturation of fatty acids occurred (Tables I, II), was much larger. The structural order parameters (S_{DPH}), calculated from the P_{DPH} values, were increased 17% and 25% in hydrogenated plasma membranes from liver and GRSL cells, respectively (Table III).

Table III

Catalytic hydrogenation of living GRSL leukemia cells, isolated plasma membranes from GRSL cells and from rat liver, and of liposomes of the extracted total lipids of these membranes; effect on fluorescence polarization (P_{DPH}) and lipid order parameter (S_{DPH}) at 25 °C.

	P_{DPH}			S_{DPH}		
	untreated	cat + N_2	cat + H_2	untreated	cat + N_2	Cat+ H_2
GRSL leukemia cells	0.175	0.180	0.191	0.41	0.42	0.45
Whole cells						
Plasma	0.276	0.289	0.387	0.65	0.68	0.85
membranes	0.227	0.248	0.400	0.54	0.59	0.87
Liposomes						
Rat liver						
Plasma	0.291	0.309	0.382	0.68	0.72	0.84
membranes	0.244	0.247	0.394	0.58	0.59	0.86
Liposomes						

Data for whole cells refer to a diphenylhexatriene (DPH)-labelled sonicate in phosphate-buffered saline of the total lipids extracted from the untreated or treated cells, respectively, thus elimintaing effects of proteins and of residual traces of the catalyst itself (partitioning in the water phase). In the catalyst-treated membranes or liposomes some catalyst still remained after washing, intercalated in the phospholipids and as such affected the P_{DPH} value.

Hydrogenation of the liposomes of these membranes resulted in 45% elevation in S_{DPH} values. The difference in this hydrogenation effect between plasma membranes and their liposomes may be attributed to intrinsic membrane proteins, which by themselves may impose some structural order upon the apolar regions of the lipid bilayer [58,71). The structural order parameter of membrane lipids has been shown to

increase exponentially with rigid intrinsic membrane molecules, such as cholesterol or certain proteins, towards a maximal plateau value [58,111]. In the present study the values reached in the membranes and liposomes by catalytic hydrogenation are all very similar ($0.382 \leq P_{DPH} \leq 0.400$; $0.84 \leq S_{DPH} \leq 0.87$) and very close to the maximum values that can be obtained experimentally ($P_{DPH} = 0.425$, $S_{DPH} = 0.90$) [111].

Table IV.

Catalytic effect on the fluorescence polarization (P_{DPH}) value after catalytic hydrogenation of isolated plasma membranes of GRSL leukemia cells, demonstrated by comparison of the whole membranes with liposomes of their extracted total lipids[a]

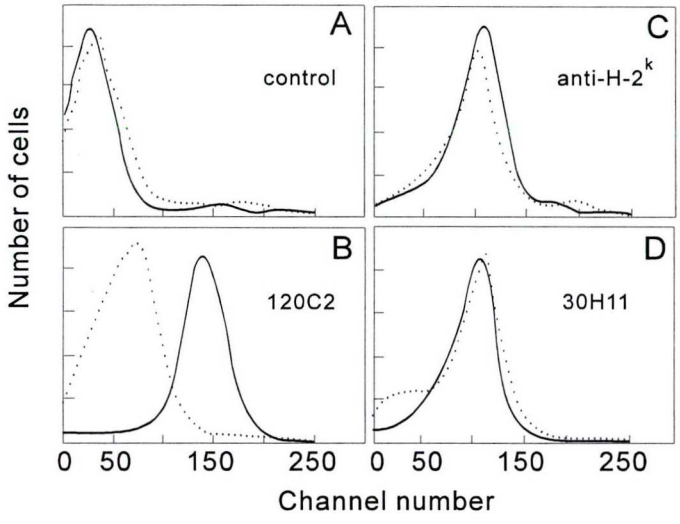
Treatment o	P_{DPH} (at 25 °C)	
Plasma membranes	plasma membranes directly[a]	liposomes of total lipids[b]
Untreated control	0.276	0.271
$Pd(QS)_2$ only	0.290	0.273
$Pd(QS)_2/N_2$ pressure	0.295	0.268
$Pd(QS)_2/H_2$ pressure	0.340	0.318

[a]In this particular experiment catalytic hydrogenation reduced the fatty acids 18:2 and 20:4 from 17% to 11.5%, and from 5.4% to 1.4%, respectively.
[b]Lipids were extracted from the membranes after the various catalyst treatments. The liposomes were devoid of catalyst.

The expression of a number of cell surface antigens was estimated by immunofluorescence in the fluorescence-activated cell sorter (FACS), using monoclonal antibodies against the antigens and secondary ('sandwich') antibodies which were conjugated to a fluorescent label (FITC). Fig. 1. shows that the monoclonal reagent 120C2, detecting a 15 kDa surface protein (the function of which is unknown yet), is exposed at a higher level on hydrogenated cells. The increase in fluorescence intensity per cell was in this case estimated to be 4-fold relative to the non-hydrogenated cells. Whether this increase represents previously unexposed (hidden) antigens or whether it is due to some lateral rearrangement leading to clustering of the antigens, is not clear. Fig. 1 further shows that the expression of two other antigens, i.e. a $H\text{-}2^k$ antigen and a glycolipid antigen (reacting with 30H11) is hardly affected. Also two other surface antigens (see Methods) were not altered in expression by hydrogenation (not shown). It should be noted that the viability of the

cells, being at least 80% after hydrogenation, could sometimes decrease to a level as low as 40% due to the subsequent manipulations (incubations with antibodies, washings etc.). The fluorescence measured in the fluorescence-activated cell sorter was, however, in all cases light scatter-gated for live cells [69].

Figure 1. Relative expression of cell surface antigens on GRSL leukemic cells as measured by the membrane immunofluorescence intensity (channel number) in the fluorescence-activated cell sorter (FACS IV).



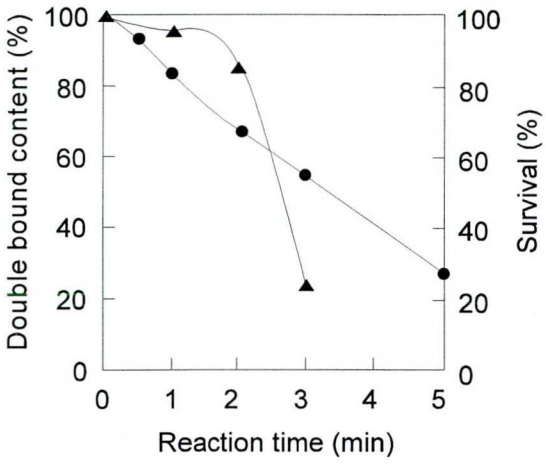
The profiles shown are representatives of three experiments with similar results. Cells were pretreated with $Pd(QS)_2$ under N_2 (dotted line) or under hydrogenation conditions (H_2; solid line). Primary monoclonal antibodies were (A) control:an immunoglobulin unrelated to the GRSL cell surface, (B) 120C2, (C) H100-5/28 against $H\text{-}2^k$, (D) 30H11.

5/2. Lipid saturation in the L929 target cell's plasma membrane blocks tumor necrosis factor mediated cell killing

Previously we observed that the exposure of L929 cells to TNF at different temperatures resulted in very different LD_{50} values. Incubation of tissue cultures with liposomes composed of different phospholipids, free fatty acids and cholesterol modified significantly the TNF sensitivity of the cells (Kusz and Duda, unpublished). To define the exact role of the physical state of the plasmamembrane in the cytotoxic action of TNF we used the method of catalytic hydrogenation. Treatment of living L929 mouse tumor cells seemed to be a useful method to alter the lipid composition of the (plasma)membranes in a rapid and reproducible way without seriously

interfering with the metabolism of the cells. The hydrogenation procedure was accomplished by incubation the cells with the active form of Pd-catalyst under

Figure 2. Changes in the viability and the level of fatty acid saturation of L929 cells during catalytic hydrogenation.



Membrane lipids of living L929 cells were hydrogenated as described in the Methods. The level of fatty acid saturation (●●●) (% decrease in total fatty acid double bonds) of L929 cells increased with time during catalytic hydrogenation. Note that virtually all cells survived, exposed to reduced catalyst up to two minutes long hydrogenation.(▲▲▲)

atmospheric presure of H_2 gas. The effects of the presence of the catalyst in its inactive (oxidized) or active (reduced) form, the hydrogenation reaction and the rotation of the reaction vessels were individually tested on living cells. When cellswere subjected to the conditions of hydrogenation in the absence of hydrogen, cell viability was not effected adversely. Brief exposures to the active catalyst in the presence of hydrogen did not decrease the number of viable cells significantly. Accordingly, about 90 to 95% of the cells tolerated 2 min hydrogenation and more than 80% survived reaction times up to 3 min. Further reduction of lipid double bonds of cellular membranes during longer periods of hydrogenation decreased the viability of the cultured cells. Fig. 2. shows time dependent saturation of phospholipids and concomitant change in the viability of L929 cells, respectively. It is worthwhile to mention that treated cells adhered to the plates much more slowly than trypsinized, untreated controls. A detailed study was made for lipid hydrogenation rates in living cells. Hydrogenation proceeded as described in previous reports [see above] with

polyunsaturated fatty acids being reduced to saturates or monounsaturates, including some unnatural trans-isomers.

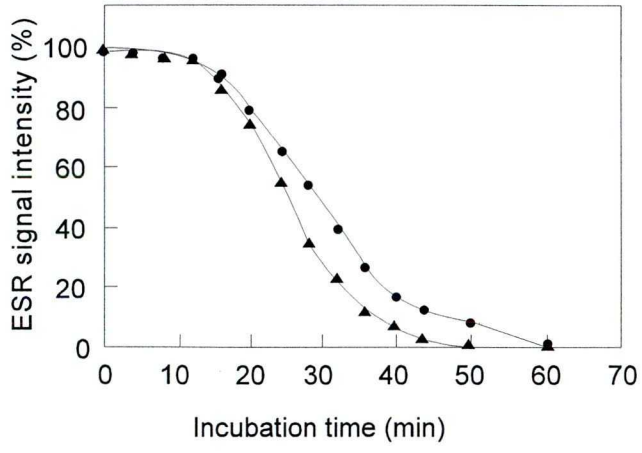
Table V. Change of fatty acid composition of total lipids and phospholipids in L929 cells after catalytic hydrogenation (mol % of major fatty acids, C: control cells; H: cells hydrogenated for 2 min).

Fatty acids	Total lipids		Phosphatidylcholine		Phosphatidyl-ethanolamine	
	C	H	C	H	C	H
16:0	17.1	20.9	18.1	22.5	10.7	10.0
16:1	3.0	1.4	4.6	2.8	2.9	1.6
18:0	18.9	26.2	14.2	27.6	29.1	32.2
18:1	53.1	46.8	59.1	45.1	50.4	50.1
18:2	1.2	0.4	0.2	0.0	1.4	0.9
18:3	3.5	2.0	2.6	0.9	1.9	1.7
20:4	2.4	2.3	1.2	1.1	3.6	3.5

Table V details the data of fatty acid compositions of lipids of the cellular membranes of control cells (treated with inactive catalyst) and for those treated with the active catalyst for 2 min (almost complete survival of treated cells, as shown on Fig. 2). Efficient reduction of the major fatty acid, oleic acid was the most striking difference found between the two samples. Hydrogenation occured also in the palmitoleic acid and in polyunsaturated C18 fatty acids as well, but these were only minor compounds. It was noteworthy, that no significant hydrogenation of 20:4, arachidonic acid was found. (Due to a variety of hydrogenation products that were distributed among several minor peaks, we could not identify saturated derivatives of arachidonic acid). A more detailed analysis revealed that certain lipid classes were virtually untouched by 2 min hydrogenation of L929 cells. Focusing on two main classes of complex phospholipids, fatty acids within PE appeared to be more resistant to hydrogenation than those esterified to PC. Preference of the catalyst for PC was not found in mixed dispersions of these isolated complex lipids (unpublished data). This finding therefore was considered to be an indication of a restricted access of the catalyst to some acyl lipids (found primarily in the inner leaflet of the membranes). Evidence for selective catalytic hydrogenation of a peripherally located membrane of intact cells was reported earlier [see this thesis]. In accordance with these findings, we assumed that

endomembranes of living L929 cells are not readily accessible to the catalyst during short hydrogenations, whereas lipids of the plasmamembrane were exposed to the effect immediately. To prove more strongly this point, we carried out experiments using nitroxy-fatty acid spin labels incorporated into L929 cells. It was reported, that nitroxid spin labels used to probe membrane dynamics are reduced by mammalian cells to the corresponding hydroxylamines (104). It appeared that reduction of doxylstearates takes place at the ubiquinon level in the respiratory chain in mitochondria. Therefore, it was suggested that the rate of the reduction of the doxyl moiety is partially controlled by the internalization of the spin probe. In view of the above considerations the kinetics of the reduction of 5-doxylstearate was determined in both control and 2 min hydrogenated cells (Fig. 3.).

Figure 3. Time dependent decrease of electron spin resonance signal in hydrogenated and control cells.

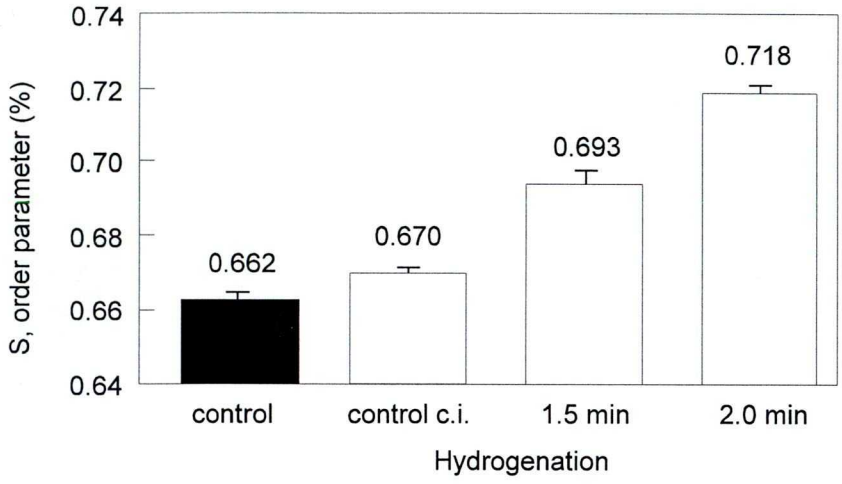


Membrane lipids of living L929 cells were hydrogenated and the ESR signal was measured as described in the Methods. All data were recorded at 37 °C. Values shown for non-hydrogenated cells were obtained using N_2 gas instead of H_2 with the catalyst.
▲▲▲ control cells, ●●● hydrogenated (2 min) cells

Time-dependent changes in the concentration of the spin label run closely parallel in both samples as shown on Fig. 3. Our results also indicated that up to 10 min the reduction rate is non-detectable. Consequently, during that period the fatty acid spin label was located in the membrane of the cell surface. In accordance with this finding, the orientation order parameters were calculated from the ESR spectra of those cells prelabelled for 5 min with 5-doxylstearate (Fig. 4.).

The structural order parameter of membrane lipids was not significantly affected by the presence of inactive catalyst (data not shown). Parallel with the progress of hydrogenation at conditions resulting in no reduction of the viability of the cells, ordering state of the plasmamembrane, however, increased markedly. The above

Figure 4. The effect of the reaction time of hydrogenation on the order parameter of fatty acid chains by using 5-doxyl stearate spin probe.



Membrane lipids of living L929 cells were hydrogenated and order parameter was measured as described in the Methods. Control represents order parameter obtained from plasma membrane of cells grown at 37°C, control c.i.. is in the presence of inactive catalyst under N_2 athmosphere.

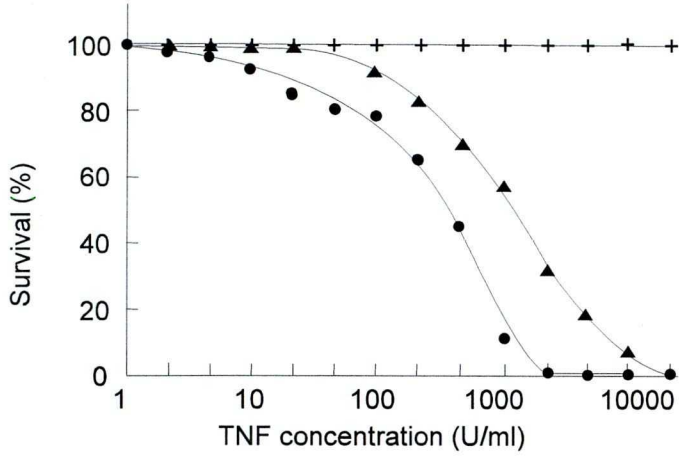
described experiments underlined the usefulness of catalytic hydrogenation in changing the acyl chain composition of the membranes of living L929 cells and also proved that the effect of brief treatments is indeed, confined to the plasma membrane.

The effect of hydrogenation on the susceptibility of treated and control tumor cells to the cytotoxic effect of TNF was then investigated. As it is shown in Fig. 5, a brief 2 min hydrogenation of L929 cells resulted in a dramatic change in their TNF sensitivity. TNF practically lost its cytotoxicity toward treated cells. In order to rule out that the effect is caused by traces of $Pd(QS)_2$, in one control experiments the catalyst was not washed out completely. The compound did not protect the cells against TNF; actually, traces of the complex even slightly enhanced the killing effect of the cytokine (Fig. 5).

Receptor binding experiments with ^{125}I labelled TNF molecules showed almost no difference between TNF binding of hydrogenated and control cells, whereas internalization of TNF-receptor complexes somewhat slowed down in hydrogenated cells (data not shown).

It is noted, that brief hydrogenations, where viability of the hydrogenated cells remains over 90%, quantitative analyses detected a remarkably low change in arachidonic acid content. Obviously, this alteration alone was hardly enough to explain a dramatic change in TNF sensitivity. In light of the above findings, we

Figure 5. The effect of hydrogenation of membrane lipids of L929 cells on TNF mediated cytotoxicity.



Membrane lipids of living L929 cells were hydrogenated and their TNF sensitivity was measured as detailed in the Methods. Saturation of the membrane lipids decreased the sensitivity of L929 cells to TNF by at least 100-fold. Non-hydrogenated cells, containing trace amounts of catalyst proved to be more sensitive to TNF than control cells. Control cells: ▲ ▲ ▲, inactive catalyst:● ● ●, 2 min. cells hydrogenated for 2 min. by $Pd(QS)_2$. ✣ ✣ ✣

assumed that the explanation of the decreased TNF sensitivity should be found elsewhere. It was shown that membrane fluidity controls PLA_2 activity. A relatively low degree of hydrogenation markedly influenced PLA catalysed arachidonic acid release, as demonstrated by using ciliary membranes of *Tetrahymena* [39] or rat liver mitochondria [34]. Arachidonic acid metabolites play a definitive role in TNF mediated cell killing, inhibitors of the cyclooxygenase and lipoxygenase pathways protect sensitive cells against the cytotoxic effect of TNF [112]. It was shown that in certain cells TNF activates the sphingomyelin signal transcduction pathway liberating ceramide as the second messenger molecule. [113,114] TNF resistance of the hydrogenated cells could therefore stem from the fact that liberation of arachidonic acid or the relase of ceramide is blocked by the elevation of the membrane microviscosity. Our previous data suggested that intramembraneous hydrogenation of lipids reduces the substrate availability, but not the enzyme activity of endogenous

PLA_2. [34] In conclusion, at that time we assumed that temporary changes in the physical state of the plasmamembrane, caused alone by the diet or the actions of the neuroendocrine and/or the immune systems can influence the sensitivity of the tumor cells. Since membrane microviscosity can be modified by hypo- or hyperthermia, pharmacological agents, local treatment of tumors might render malignant cells more sensitive to TNF action, or alternatively, sensitive healthy tissues could be protected against TNF toxicity. By increasing the sensitivity of the target cells it would be possible to lower the therapeuric doses of TNF, eliminating or significantly decreasing the systemic toxicity of this powerful cytokine.

5/3. Genetically modified L929 cells with reduced level of membrane molecular order possesses a highly increased TNF sensitivity.

L929 mammalian cells were transformed with DNA constructs in order to study the effects of the expression of the yeast Δ^9-desaturase gene under the control of a *H. capsulatum* promoter. This promoter was isolated from the temperature susceptible strain of *H. capsulatum (*Downs) (88,89). In the Downs strain of *H. capsulatum* transcription of Δ^9-desaturase gene is upregulated. This is in agreement with the previous finding that membranes of this strain contain higher level of oleic acid then wild type cell membranes (88). The coding sequences of the *H. capsulatum Ole1* alleles are virtually identical, but differences exist in specific regions of the promoters (89). Our experiments indicated that in transformed L929 clones the *S. cerevisiae* Δ^9-desaturase gene was transcribed and translated under the control of selected *H. capsulatum* promoter and the yeast enzyme was active in the mammalian cells.The presence of the transforming sequences in the animal cells were detected by PCR and Southern analysis. Yeast gene was also transcribed in transformant colonies, specific mRNA was detected by RT-PCR analysis.The introduction of "Downs" promoter resulted in an elevated expression of the *Ole1* gene in transformed cells.(see details in ref. 107).

Transformed cells exhibited altered fatty acid composition of membrane phospholipids and reduced microviscosity of their membranes. We measured fatty acid composition of total lipids and phospholipids of control vs. transfected cells. Table VI. A and B show that the values of palmitoleic acid (16:1) and especially

oleic acid (18:1) of *LD* cells are significantly higher than those of the non-transformed L929 cells. The ratio of monounsaturated fatty acids was about the same in total lipids and phospholipids in the transformed cells as well, showing that oleic and palmitoleic acids produced by the yeast gene is not stored in lipid droplets but assembled into phospholipids of cellular membranes. According to

Table VI. Total lipid and phospholipid analysis of fatty acids in transformed cells in comparison with the control L929 cells.

Fatty acid composition of total lipids									
	16:0	**16:1**	**18:0**	**18:1**	**18:2**	**20:1**	**20:4**	**22:5**	**22:6**
L929	19.5	3.4	18.2	*45.3*	2.2	1.3	4.9	2.4	2.7
LD	15.0	4.6	15.5	*53.3*	1.8	1.2	4.4	1.9	2.4
Fatty acid composition of phospholipids									
	16:0	**16:1**	**18:0**	**18:1**	**18:2**	**20:1**	**20:4**	**22:5**	**22:6**
L929	18.8	3.7	18.4	*45.7*	1.9	1.0	5.1	2.5	2.8
LD	14.9	3.7	15.9	*57.5*	1.0	1.8	2.9	1.1	1.2

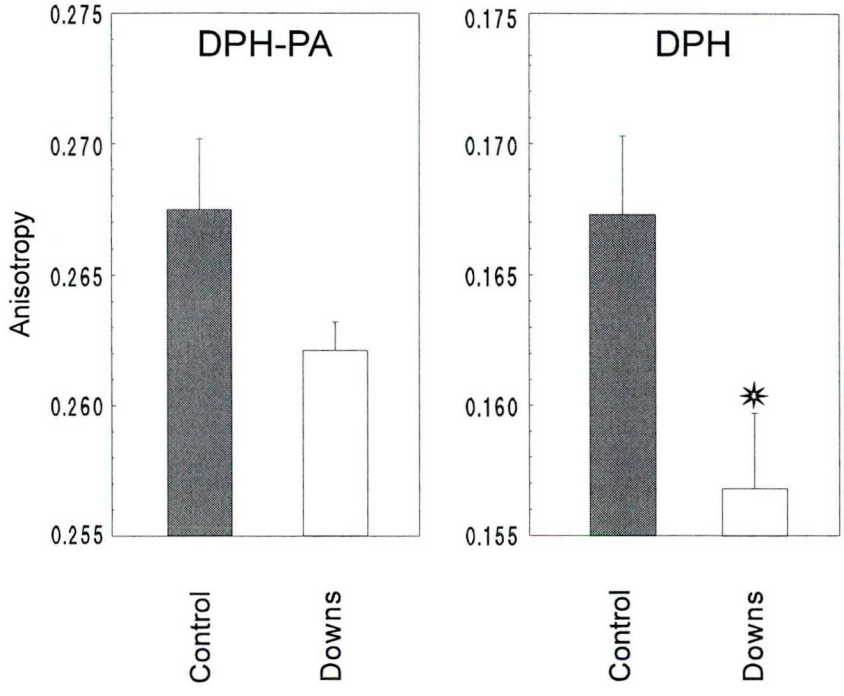
biophysical measurements using DPH and DPH-PA as probes, microviscosity of cellular membranes was significantly decreased in the case of *LD* cells compared to that of the control cells (Fig. 6.).

Our experiments proved that the yeast *S. cerevisiae* gene is expressed under the control of regulatory sequences of *H. capsulatum* in mammalian cells. At 37°C, where the animal cells are grown, the level of *Ole1* specific transcripts and the corresponding enzyme activities are higher in cells transformed with the Ole1 gene driven by Downs promoter. The fungal desaturase gene product exhibited enzymatic activity and was able to modify the lipid unsaturation of membranes in the transformed cells.

At that stage of our studies, we could state, that the system is a promising new tool to alter the fatty acid composition of phospholipids in membranes of mammalian cells and suitable to study the effect of these modifications on the mechanism of TNF-mediated cell killing. On the basis of our previous results gained by hydrogenation we expected that desaturase overexpressing L929 cells would

exhibit a significantly increased TNF sensitivity. A number of different clones from each transformation experiments and a set of isolated, non-transformed control clones were compared using the standard cytotoxicity assay, both in the presence and absence of macromolecular synthesis inhibitor Actinomycin D. The results fully justified our expectations. Clones expressing the yeast desaturase gene under

Fig. 6. Membrane fluidity changes in transformed L929 cells measured by DPH-PA and DPH probes.



Control: L929 cell line, Downs: *LD* cell line.

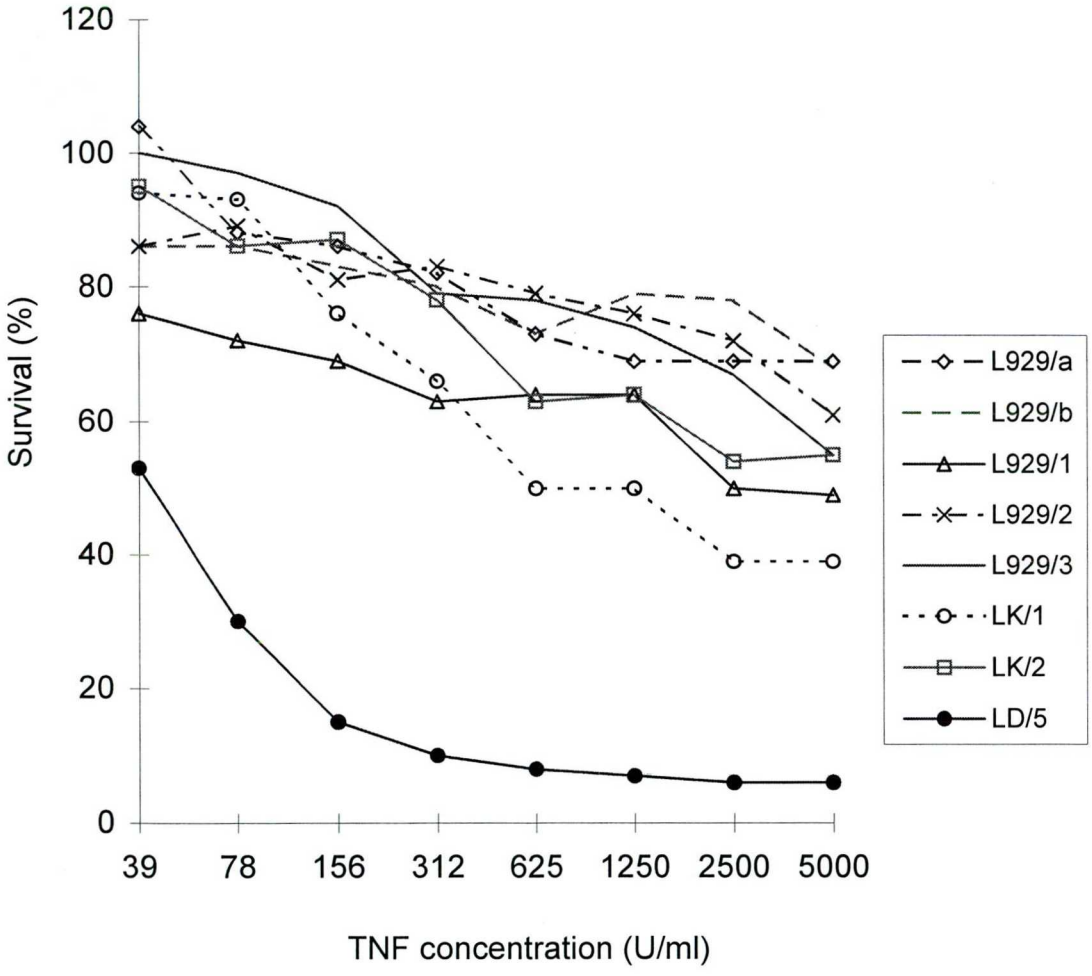
the control of constitutive promoters displayed significantly increased sensitivity to the cytotoxiceffects of TNF . Isolated clones of the parental L929 strain or colonies transformed with the promoterless construct showed slight variations in sensitivity, but all were significantly more resistant than the desaturase producing clone (Fig.7.)

Addition of actinomycin D to the assay medium highly decreased the concentrations of TNF necessary to kill 50% of the cells, but did not alter the *ratio* of the sensitivities of desaturase producing and control cells (Fig. 8.).

The cytotoxic effect of TNF on virus infected cells and tumor cell lines was shown to be increased dramatically at elevated temperatures, suggesting an evolutionary link between this fact and the pyrogenic effect of TNF (115). Exposing mammalian cells to high temperature is known to induce a rapid decrease in the alkyl chain order of membranes (25). Combining these observations (together with data based on hydrogenation studies) we assumed that thermally induced changes in the packing properties of membranes could be casually linked to increased TNF sensitivity of

Fig. 7. Survival of TNF-treated L929 clone LD/5 (containing the pRc/Downs/OLE1 plasmid) versus different negative controls without actinomycin D.

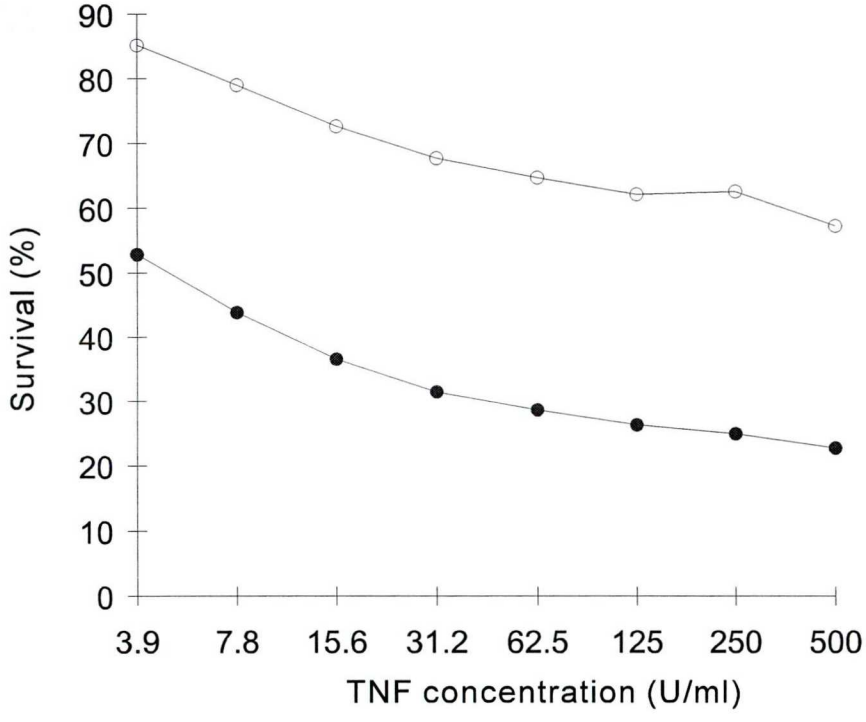
.



L9291, L9292 and *L9293* are parental L929 clones; *L929a* and *L929b* are the original L929 cells from different frozen stocks; *LK/1* and *LK/2* are L929 cell lines transformed with the promoterless, control plasmid, pRc/OLE1.

target cells at high temperature. Our previous experiments supported this assumption. As seen above (See Chapter 5/2) minor changes in the saturation level of phospholipid fatty acids, induced by catalytic hydrogenation of cell membranes, triggered dramatic changes in TNF sensitivity. Though this type of treatment was very effective in inducing *TNF resistance* in the manipulated cells, from a therapeutic point of view, methods that increase *TNF sensitivity* are obviously more desirable. **Fig. 7.** Survival of TNF-treated L929 clone LD/5 (containing the pRc/Downs/OLE1 plasmid) versus different negative controls without actinomycin D.

Fig. 8. TNF sensitivity of the transformed cell line LD/5 (containing the pRc/Downs/OLE1 plasmid) versus the control L929 with actinomycin D (1 μg/ml).



Wild type: ○ ○ ○, transformed cells: ● ● ●

In accordance with our expectations transformant clones expressing the yeast desaturase gene exhibited a highly increased TNF sensitivity. The relative low abundance of yeast desaturase transcripts together with the significant, but modest changes observed in fatty acid composition and overall level of membrane fluidity suggest again that the mechanism of TNF sensitivity is strictly controlled even by minor changes in membrane unsaturation/physical state. We assumed, that

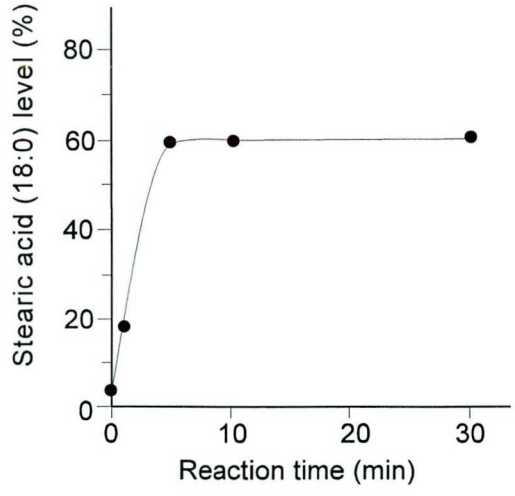
approaching the problem solely by considering membrane fluidity (59) describing the overall feature of the membrane bilayer is probably not sufficient. A parameter devised to describe the overall state of the bilayer can not take into account the existence of heterogeneous lateral and transversal regions or domains of differing lipid composition or physical state in which specific enzymes could exist. Moreover, membranes of all the organisms invariably contain considerable amounts of lipid domains which prefer to organise themselves in non-bilayer structures and under appropriate conditions, undergo a transition from the lamellar bilayer (L_α) to the inverted hexagonal (H_{II}) phase (25). The balance between lamellar or H_{II} organisation of specific lipids is precisely regulated and influenced by several factors. Amongst these factors the introduction (or removal) of *cis* double bonds into the acyl chains is known to be a major determinant (8,25,38). Basic functions of membranes appear to be connected to the H_{II} propensity of lipids. Recently a model has been presented to explain the way as non-bilayer lipids preserve the functional structure of membrane proteins by exerting high lateral packing pressure (115). It was suggested, that non-lamellar structures are required in particular events of signal transduction, such as membrane fusion, membrane association of G proteins and specific PKC isoforms (116). The activities of several membrane enzymes including phospholipases are also influenced by the non-bilayer forming lipids (116,117). Consequently, the TNF signaling mechanism may also be influenced by the nonbilayer propensity of lipids within cellular membranes. In addition, TNF-induced cytotoxicity is triggered by TNF receptor clustering (118), a process influenced by the "fluid state" of surface membranes. Furthermore, there are several enzymes (neutral and acidic sphingomyelinases, PLA_2,, PLC and PLD) involved in TNF signaling that function by liberating messenger molecules from the lipid bilayer of the membrane (see references in 83). As we documented earlier in a rat liver mitochondrial model, saturation of membrane phospholipid reduced the substrate availability of endogenous phospholipase A_2 (34). The same mechanism probably holds true for the operation of other phospholipases, as well. Furthermore, TNF induced membrane disintegration is thought to be the result of lipid peroxidation, as a consequence of increased production of free oxygen radicals in the mitochondrial compartment of TNF sensitive cells (119). Possibly, a membrane containing extra double bonds may also become more accessible to the action of free radicals.

5/4 Novel surface membrane directed hydrogenation of rat platelets by Pd-PVPP catalyst and its effect on membrane physical state and platelet aggregation

Testing the catalytic properties of Pd-PVPP by hydrogenation of liposomes

According to the palladium determinations, the Pd content of Pd-PVPP is relatively low (approximately 0.02%). However, this small amount of palladium is strongly retained on the surface and the catalyst – as shown below – proved to be useful for the hydrogenation of very small amounts of substrates (e.g. 2.0 mmol lipid/sample) usually encountered in hydrogenation of biomembranes. Hydrogenation of dioleyl-phosphatidylcholine (DOPC) liposomes with palladium-on-PVPP polymer was attempted to test first the performance of this heterogeneous catalyst. As seen in Fig. 9, a fast hydrogenation of DOPC could be achieved under very mild conditions. The experiment, under the conditions described for treatment of the platelets, showed that after 3 min, the conversion of oleic acid to stearic acid is completed and leveled off at cca. 50-60 %. The level of the conversion remained unchanged during at least 30 min. This maximum value would represent the pool of DOPC forming the outer leaflet of the highly curved vesicle membranes.

Figure 9. Hydrogenation of dioleoylphosphatidylcholine vesicles with the macroheterogenous Pd-PVPP.



20 mg $[(NH_4)_2PdCl_4]$ on 100 mg PVPP, 1 mg DOPC, 10 ml Na-phosphate buffer, pH 7.0, 25 °C (see also Methods).

The macroheterogeneous Pd-PVPP catalysts could be filtered by conventional means or be settled with gentle centrifugation which allows their separation form cell suspensions. By using pentadecanoic acid (15:0) as internal standard it could be determined that even in case of a high catalyst load (300 mg Pd-PVPP) >90% of the vesicles lecithin were recovered in the filtrate after hydrogenation. This is a very substantial improvement compared to the strong adsorption observed with the earlier Pd-PVPP preparations (64) and with heterogenized homogeneous catalysts (e.g. $Pd(QS)_2$ bound on ion-exchange resins [64]). The weak adsorption of lipids on Pd-PVPP is in accordance with the zero zeta potential of the sol.

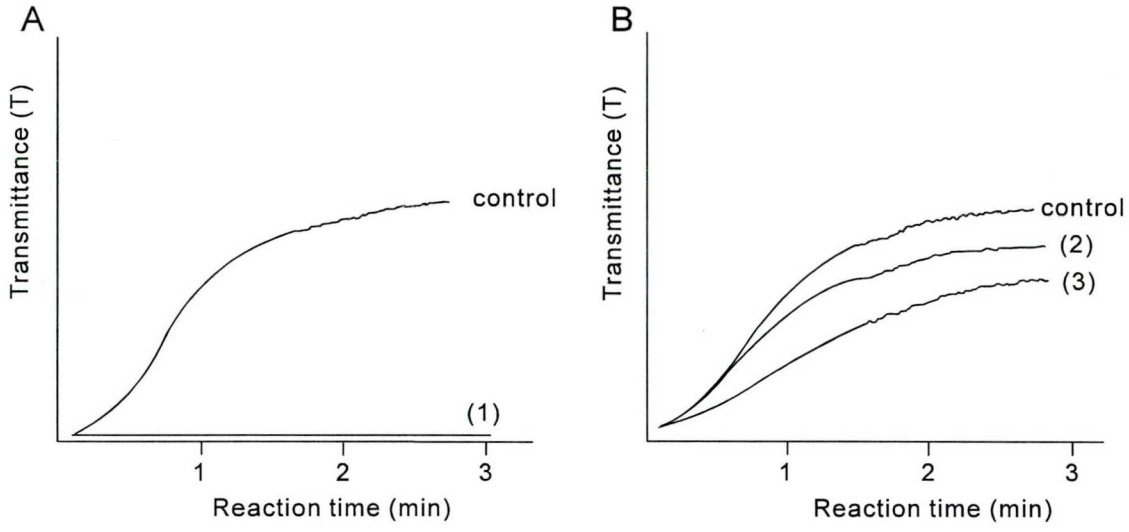
Hydrogenation of rat platelets by Pd-PVPP.

When the freshly prepared rat platelets were coincubated with the soluble $Pd(QS)_2$ complex, the aggregation ability was completely lost (Fig. 10/a). The mechanism of this inhibition is so far unknown. In contrary, the insoluble Pd-PVPP catalyst possessing zero surface charge had only a mild self effect on the thrombin-induced aggregation of platelets (Fig. 10/b) which might be a consequence of the fact, that this catalyst could be removed completely from the platelet suspension by a mild centrifugation (1200 g, 5 min). Hydrogenation for a short duration (5 min) had no effect on cell viability (data not shown). Since the plasmamembrane comprises of only about 5-10 % of the cell constituent membranes, to follow the rate of the saturation reaction through total fatty acid analysis proved to be meaningless. Instead, we applied a cell surface specific fluorescence probe (as above) and measured plasma membrane fludity *in situ*. It could be established, that hydrogenation attained by the non permeant catalyst caused a considerable increase in plasma membrane microviscosity as measured by TMA-DPH anisotropy, ie. catalytic lipid saturation proceeded according to the expectations (r_s in control: 0.2340 ± 0.003; in 5 min hydrogenated cells: 0.2450 ± 0.002). At the same time, the ability for thrombin induced aggregation was decreased remarkably if compared to the time-aggregation profile detected in the presence of Pd-PVPP under N_2. Therefore, we concluded, that this impaired aggregation response to thrombin is a direct consequence of the decreased unsaturation of the plasma membrane of resting rat platelets.

Human platelets from patients with hyperlipoproteinemia are enriched in cholesterol and respond to platelet activating agents with increased aggregation and secretion

(120). In studies with cholesterol-enriched platelets, a more rigid plasma membrane was accompained by increased expression of thrombin receptors (121), a higher phospholipase (PLA_2) activity, more liberation of arachidonic acid, and a greater rise in cytosolic Ca^{2+} (see refs. in 90). It was suggested, that post-receptor

Figure 10. Self-effect of the Pd-PVPP catalyst and effect of 5 min. hydrogenation on thrombin induced aggregation of platelets as shown by change in transmittance (T).



Conditions:3 x 10^8 cells/ml, 2U thrombin, 37°C. Platelets were incubated for 5 min with 0.1 mg/ml $Pd(QS)_2$ (**1**) or with 0.07 mmol Pd/100 mg PVPP (**2**,**3**) in 10 ml Na-phosphate buffer (pH=7) under N_2 (**1**,**2**) or H_2 (**3**), respectively. Pd-PVPP was removed from the cell suspension by centrifugation (1200 x g, 10 min).

signal processing was undisturbed, instead the hyperaggregability is caused by increased numbers of trombin receptors at elevated membrane cholesterol (90). Several methods have already been employed to modulate fatty acid saturation of platelets, including the manipulation of fatty acid synthesis, membrane delipidation or in most cases dietary supplementation, in order to link platelet functions and the level of lipid unsaturation. However, with any of these methods it seems impossible to exclude contributions from an altered protein complement or composition, altered lipid-headgroup distribution and other aspects of membrane architecture, ie. to link *directly* the effects to changing cis unsaturation level, *per se*. Even though it is well established, that dietary cis-unsaturated fatty acids modify the mechanism of platelet aggregation so far an uncertainity exists how to interpret the *in vitro* results. Because

phospholipids, in particular molecular species containing 20:4 (arachidonic acid), are substrates for various enzymes (phospolipases, transacylases, etc.), therefore the effects of dietary manipulation of lipids can not be separated from the secondary metabolic modulations. Thus, dietary supplementations with precursors of arachidonic acid may alter simultaneously the level of thromboxanes, metabolites of arachidonic acid. In addition, based on the well known principles of the homeostatic control of membrane physical state, administration of "fluidity perturbing" exogeneous lipids may also trigger compensatory responses within membrane components (8). In other words, understanding the regulatory function of dietary fatty acids in platelets is an extremely complex phenomenon, and clearly more should be understood about the association between dietary fatty acids and platelet membrane fatty acids in connection with the platelet responses to physiological stimuli and subsequent signal transduction inside the platelets.

6. CONCLUSION

6/1. *Catalytic hydrogenation of fatty acyl chains in plasma membranes of GRLS leukemic cells: effect on membrane fluidity and expression of cell surface antigens*

Optimal reaction conditions were established for hydrogenation of plasma membranes of living murine GRLS leukemia cells, using the water–soluble catalyst $Pd(QS)_2$. Under the optimalized conditions more than 80 % of the cells remained viable. GC analysis revealed that hydrogenation occurred predominantly in the 18:2, 20:4 and 22:6 fatty acyl chains of the membrane phospholipids. Hydrogenation was also performed in purified plasma membranes from GRLS cells and from rat liver, and liposomes prepared from the total lipid extracts of these membranes. Hydrogenation increased the lipid structural order parameter in the membranes, as measured by fluorescence polarization. Combining the above findings we concluded, that it is feasible to reduce the lipid fluidity of cellular membranes of living leukemia cells to a great extent by homogeneous catalytic hydrogenation using Pd-catalyst. Thus, the method provides an alternative for the membrane rigidization attained by incorporation of cholesterol or its hydrophilic esters, a treatment that has been shown to increase immunogenecity (67,68) and to alter the exposition of various cell surface

determinants (69,122). The two method, while both resulting in an elevated membrane rigidity, yielded however, different results concerning H-2 expression. Thus, it became clear, that not the *bulk* membrane fluidity, but certain *domains* of the plasma membrane with specific lipid composition which control expression of cell surface antigens. In addition, the introduction of the technique allows investigations of the most various aspects of lipid unsaturation dependent membrane processes on this system.

6/2. *Lipid saturation in the L929 target cell's plasma membrane blocks tumor necrosis factor mediated cell killing*

Similar to the GRLS leukemic cells, the method of the catalytic membrane hydrogenation by $Pd(QS)_2$ proved to be a powerful tool for changing lipid unsaturation level in a programmed manner of L929 murine fibroblasts, the well-established target cells of the cytokine, TNF. While the Pd-catalyst *per se* did not affect TNF-cytotoxicity, a brief hydrogenation confined presumably to the surface membrane of the cells blocked completely the TNF-killing. No measurable difference could be seen between TNF binding of control and hydrogenated cells. Our *in vitro* findings strongly suggest, that similar to hydrogenation any change caused by the most various means in the fatty acid unsaturation/lipid physical state of TNF-target tumor cells *in vivo* (diet, hyperthermic stress, etc.) could cause simultaneously a dramatic alteration in the TNF-mediated cytotoxicity *in vitro.*

6/3. *Genetically modified L929 cells with reduced level of membrane molecular order possesses a highly increased TNF sensitivity*

Further in line of the above reasoning and basically in order to extend the validity of our statements in respect to the lipid unsaturation control of TNF killing we established an alternative strategy, ie. transfection of L929 cells by DNA constructs containing delta-9 fatty acid desaturase gene (Ole1). Fully supporting our previous observations based on hydrogenation experiments, genetically modified cells which exhibited an *increased* ratio of monounsaturated fatty acids in their membranes possessed a highly *increased* TNF sensitivity. Combining these findings with the results obtained with hydrogenation, obviously, the actual level of lipid unsaturation of target cells plays a central role in the mechanism of TNF-mediated cytotoxicity. In addition to our previous reasoning made in conjunctions with hydrogenation, later we

suggested the involvement of the nonbilayer propensity of some membrane lipids as a potential mediator between unsaturation and cytokilling cascade. We have to emphasise, however, that at the present stage of our stuidies, we are unable to establish causal relationship between altered level of lipid saturation and one or more specific steps of the TNF-mediated cytotoxicity cascade (which fundamentally include G protein coupled activation of phospholipases, generation of free radicals, and damage to nuclear DNA, etc.). Based on the plasma membrane selectivity of hydrogenation experiments, our most important recognition is the demonstration, that *surface membrane modulation* of lipid unsaturation is the *necessary and sufficient* precondition of the alteration of the mechanism ofTNF-killing.

Various concepts operating with "fluidity" or "non-bilayer propensity" of plasma membranes have beed discussed, which may give satisfactory explanations to our findings. Our presented studies may also facilitate the rational design of pharmaceuticals that can attenuate or potentiate the action of this important cytokine.

6/4. *Novel surface membrane directed hydrogenation of rat platelets and its effect on membrane physical state and platelet aggregation*

As we outlined before, the only hitherto known direct method of manipulating lipid saturation without affecting other membrane components is the *in situ* chemical hydrogenation of unsaturated fatty acids. The method established in the present study by firstly introducing the "plasma membrane directed", non-permeating Pd-PVPP complex, provided us an appropriate tool to examine more exactly the role of lipid unsaturation *per se* in the thrombotic processes. Our data revealed, that just like when the platelets are enriched with cholesterol, Pd-PVPP mediated hydrogenations made the lipid matrix more rigid. In contrary to sterol enrichment, however, Pd-PVPP acts specifically within the outer leaflet of plasma membrane (66). Whereas platelets enriched in cholesterol responded to thrombin and other platelet activating agents with increased aggregation, reduced lipid unsaturation of a specific pool of plasma membrane brought about a significantly *decreased rather than increased* aggregability. Thus, the major finding of our study is, that the conventionally proposed and widely accepted causal link between *bulk* membrane fluidity *per se* and platelet *aggregability* is questionable. The apparent contradiction can be resolved, however, by considering the fundamental difference of the specificity and mechanism

of the two methods, employed both to raise the molecular packing order of the membranes (8) . Therefore, it is suggested, that not the overall fluidity, instead the specific lipid composition and organization of platelet membranes – together with many additional factors – which determine the mechanism of aggregation. Further studies by Pd-PVPP hydrogenation *acting on the outer leaflet lipids* of plasma membrane may bring us closer also to the clinical utility of dietary fatty acids to accomplish well-designed clinical trials with patients with defective platelet functions.

The field of the potential applications of surface and leaflet selective macroheterogeneous catalysts is fare beyond the scope of the present thesis. Recently we prepared *PVPP protected bimetallic colloids* with zero zeta potential (63), wich were sufficient for proper lipid saturation of prokaryotic model systems and could completely be removed from the outer leaflet of plasma membranes. These catalysts were exploited as novel and valuable tools to map the initial elements of those hypothetic stress induced heat shock signal cascade(s) which can be provoked by stress induced changes of membrane physical state. Their operation leads eventually to the activation of the genes encoding various stress proteins, most of them acting in the cells as molecular chaperones (25,63).

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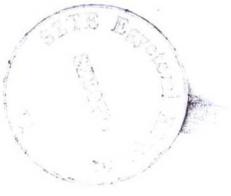

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9. ANNEX