Biochimica et Biophysica Acta, 1023 (1990) 230–238
Elsevier

BBAMEM 74783

Action of a homogeneous hydrogenation catalyst on living *Tetrahymena mimbres* cells

Yunbae Pak [1], Ferenc Joó [2], László Vigh [3], Agnes Katho [2] and Guy A. Thompson, Jr. [1]

[1] *Department of Botany, University of Texas, Austin, TX (U.S.A.),* [2] *Institute of Physical Chemistry, Kossuth Lajos University, Debrecen (Hungary) and* [3] *Institute of Biochemistry, Biological Research Centre, Hungarian Academy of Sciences, Szeged (Hungary)*

(Received 10 October 1989)

Key words: Hydrogation catalyst; Lipid hydrogenation; (*Tetrahymena mimbres*)

Various conditions were tested in an attempt to hydrogenate the unsaturated fatty acids of living *Tetrahymena mimbres* with the homogeneous catalyst palladium di-(sodium alizarine monosulfonate) without causing serious damage to the cells. Using a low (20 μg/ml) catalyst concentration in the external medium, hydrogenation of > 20% of surface membrane lipid double bonds were obtained, but hydrogenation of intracellular membranes was minimal. When exposed to H_2, cells preincubated with inactive catalyst for several hours and visibly loaded with the catalyst lost viability as soon as hydrogenation exceeded trace levels. Material secreted by *Tetrahymena* into their medium effectively inhibited hydrogenation of added oleic acid, normally a good substrate. Mucus secreted by the cells, soluble proteins isolated from cell homogenates, bovine serum albumin, and cysteine were also inhibitory, but the inhibition could be overcome by employing higher catalyst concentrations. Although some enzymatic retroconversion of saturated lipids back to unsaturated lipids appeared to take place, the scale of the conversion was small, and further experimentation will be required to understand the mechanism involved. The selective hydrogenation of surface membranes achieved by these methods may be especially useful to those interested in fluidity effects on plasma membrane properties.

Introduction

The adaptation of organisms to changes in their environment often depends upon their ability to adjust the fluidity of their cellular membranes [1]. Since one major mechanism for achieving this adjustment involves regulating the number of double bonds in phospholipid-bound fatty acids, it is not surprising that much research has been devoted to studying fatty acid desaturation and its modulation.

In recent years a family of homogeneous hydrogenation catalysts has become available for use in these studies [1a]. Of these catalysts, one in particular, palladium di(sodium alizarine monosulphonate) ($Pd(QS)_2$), has been extremely useful in hydrogenating unsaturated lipids in cell organelles and in living cells [1a]. This non-toxic catalyst affords an opportunity to alter membrane fluidity through hydrogenation without the physiological complications associated with the large fluctuations in culture temperature, salinity, etc. traditionally needed to effect fluidity changes.

Experiments involving catalytic hydrogenation of living cells [2–5] have for the most part been of short duration and have not been concerned with long term viability following the experiments. In one study the green alga *Dunaliella salina* was shown to recover after a treatment causing measurable hydrogenation of lipids in the plasma membrane and the microsomal fraction [5]. This initial success encouraged us to conduct a more detailed analysis of in vivo hydrogenation under conditions favoring cell survival, using as a model system the ciliated protozoan *Tetrahymena mimbres*. The regulation of membrane fluidity by *Tetrahymena* has been studied intensively, especially with regard to activity of fatty acid desaturases associated with the cell's endoplasmic reticulum [6]. In the experiments described below, we have examined the accessibility of various cellular membranes to the active catalyst under conditions preserving long term cell viability and extended response measurements.

Abbreviations: $Pd(QS)_2$, palladium di(sodium alizarine monosulphonate); THM, *Tetrahymena* hydrogenation medium; BSA, bovine serum albumin.

Correspondence: G.A. Thompson, Jr., Department of Botany, University of Texas, Austin, TX 78713, U.S.A.

Materials and Methods

Cell culture, harvesting and fractionation

Cultures of *Tetrahymena mimbres* [7] were maintained at room temperature in 500 ml Erlenmeyer flasks containing 200 ml of sterile enriched proteose peptone medium [8]. Cell density was monitored using a Coulter counter Model ZB. Cells were harvested in the middle logarithmic phase and in some instances fractionated by previously described methods [9], to obtain ciliary, pellicle, and microsomal fractions. 'Cytosolic proteins' were represented by the postmicrosomal supernatant, which was stored at $-80\,^{\circ}C$ until needed. Mucus was discharged from cells and purified by the procedure of Tucker and Wilhelm [10].

Preparation of Tetrahymena phospholipid liposomes

Lipids were extracted from whole cells by the method of Bligh and Dyer [11]. The lipid extract was chromatographed on silica gel H thin-layer plates in the solvent system petroleum ether/ethyl ether/acetic acid (70:30:1, v/v). After drying the plate under N_2, the area of the plate containing the phospholipid was scraped and eluted in chloroform/methanol (6:1, v/v). Weighed samples of phospholipids containing 0.05% (w/w) of butylated hydroxytoluene (Sigma) as antioxidant were dissolved in chloroform; the solvent was then evaporated with a stream of nitrogen, which led to the deposition of the lipids on the walls of the vessel. The lipids were then dispersed in 15 ml 2.5 mM deoxygenated potassium phosphate buffer (pH 6.0) by sonication with a probe type sonicator (Branson, Model W140) for 30 s in 10 s bursts. The resulting suspensions had a concentration of 0.1 mg lipid/ml.

Preparation and activation procedures of the hydrogenation catalyst

The catalyst, palladium di(sodium alizarine monosulphonate), $Pd(QS)_2$ was prepared according to the methods described in Ref. 13. Since in the hydrogenations catalyzed by this unmodified form of the catalyst (**A**) lengthy and poorly reproducible induction periods may be often seen, in some of the experiments we used different procedures for the activation of the catalyst prior to hydrogenation of lipids and other substrates. The following is a representative example. A stock solution containing 100–1000 μm of **A** per ml of 0.5 mM $MgSO_4$/5.9 mM NaCl/2.5 mM potassium phosphate buffer (pH 6.0), designated *Tetrahymena* hydrogenation medium (THM), was prepared. The catalyst was allowed to become fully active (bright yellow color) by bubbling with N_2 for 2 min and then H_2 for 2 min. This yellow compound, **C**, is moderately stable under H_2, and aliquots of its solution were used to hydrogenate unsaturated lipids with no induction periods. A more convenient way to prepare active catalyst solutions is to shake or bubble vigorously the solutions of **C** with air for a few minutes when an air stable pink form of the partially reduced catalyst (**B**) is obtained. Under hydrogenation conditions **B** is instantaneously reduced to **C**, and again, no induction periods are observed. Stock solutions of **B** can be stored in air without refrigeration for at least a week with no loss of catalytic activity. Though the actual chemical structure of the recently discovered **B** and **C** are presently not clear (results of our current studies will be published later) we want to stress that these ways of activation give stock solutions of catalysts allowing highly reproducible hydrogenations.

Hydrogenation of Tetrahymena phospholipid liposomes

Aliquots of lipid dispersion were placed in 100 ml custom-designed glass reaction vessels [12]. The reaction vessels were connected to a manifold and the gas phase evacuated and replaced by hydrogen or oxygen-free nitrogen (controls), each maintained at a pressure of 1 atm. A stock solution containing 400 μg **A** form of the catalyst per ml of the degassed 2.5 mM potassium phosphate buffer was activated under a hydrogen atmosphere to give **C**, aliquots of which were then injected into the lipid dispersion through a rubber septum to initiate the reaction at room temperature. The final concentration of lipid was 0.09 mg/ml and the highest final concentration of catalyst was 100 μg/ml in a total volume of 15 ml. During the hydrogenation procedure (lasting a maximum of 15 min), reaction vessels were rotated at 45 rev./min. At the end of the desired incubation period samples were removed from the vessel and the reaction was stopped by bubbling with air for 20–30 s. Lipids were then extracted using the procedure of Bligh and Dyer [11].

Hydrogenation of Tetrahymena in vivo

Hydrogenation of total lipids of intact cells was performed at room temperature and then fractionated in some instances to obtain a ciliary, a pellicle, and a microsomal fraction. After cells were harvested by centrifugation, they were washed twice and resuspended at room temperature in THM and the concentration was adjusted to $1 \cdot 10^5$ or $2 \cdot 10^5$ cells/ml. A stock solution containing 400 μg of **A** per ml of degassed THM was prepared simultaneously and the catalyst solution was converted to **C** by incubation under a hydrogen atmosphere for 2 min. Aliquots (36 ml) of *Tetrahymena* cells suspended in THM were placed in the 100 ml custom designed reaction vessels, which were connected to a manifold to evacuate the gas phase and replace by hydrogen at a pressure of 1 atmosphere. Aliquots of the preactivated form of $Pd(QS)_2$ were then injected into the cell suspension through a rubber septum to initiate the reaction. The final concentrations of the catalyst used for the hydrogenation varied from a minimum of 5

μg/ml to a maximum of 100 μg/ml. During the hydrogenation procedure (lasting a maximum of 70 min), reaction vessels were rotated at 45 rev/min. At the end of the desired incubation *Tetrahymena* cells were removed from the vessel, and the reaction was stopped by bubbling the suspension with air for 20–30 s, causing the retroconversion of the activated catalyst to its oxidized form, as revealed by a color change from brownish-green to brownish-red. After the cell suspension was centrifuged, cells were resuspended for lipid extraction or cell fractionation.

Hydrogenation of Tetrahymena preincubated with catalyst

Tetrahymena cells ($(1–2)\cdot 10^5$ cells/ml) were preincubated in THM with 10–100 μg of **A**/ml for up to 4 h prior to initiating the hydrogenation reaction. Hydrogenation of the preincubated cells was performed in the preincubation medium, without washing the cells, by introducing H_2 after evacuating air from the reaction vessels. The time of catalyst activation was detected by noting a color transition of the cell suspension from red to yellow. The rest of the hydrogenation procedure was identical to that described in the previous paragraph.

*Hydrogenation of Tetrahymena preincubated with **B**-form catalyst*

Tetrahymena cells ($10\cdot 10^5$/ml) were preincubated in THM with 100 μg of **B**-form catalyst per ml for 18 h. After the long preincubation, the cells were washed with THM twice and resuspended in THM with fresh catalyst (100 μg/ml) for a further 2 h. After the second preincubation, the cells were washed and resuspended in THM for hydrogenation. This was carried out by bubbling the suspension with N_2 for 2 min and then H_2

TABLE I

Hydrogenation of liposomes prepared from Tetrahymena phospholipids and treated with two catalyst concentrations

Fatty acid	Percentage					
	C_{cat} = 100 μg/ml			C_{cat} = 20 μg/ml		
	control	5 min	15 min	control	5 min	15 min
	of total fatty acids					
14:0	8	7	6	7	8	8
16:0	16	20	22	16	21	21
i17:0	2	4	4	2	4	4
16:1	5	–	–	5	–	–
16:2	1	–	–	1	–	–
18:0	2	41	51	2	33	35
*t*18:1	–	12	8	–	15	15
9*c*18:1	4	8	2	4	8	7
11*c*18:1	1	–	–	1	–	–
18:2	26	–	–	26	1	1
γ18:3	26	–	–	27	1	–
Minor (≤ 3% sat'd and unknown FA)	8	9	8	8	9	7
Number of d.b./100 FA	(142)	(21)	(10)	(144)	(28)	(25)
% hydrogenation [a]		85%	93%		81%	83%
	of C_{16} fatty acids					
16:0	74	100	100	74	98	99
16:1	21	–	–	21	2	1
16:2	5	–	–	5	–	–
Number of d.b./100 FA	(31)	(0)	(0)	(31)	(2)	(1)
% hydrogenation [a]		100%	100%		94%	97%
	of C_{18} fatty acids					
18:0	4	67	84	4	57	60
*t*18:1	–	19	12	–	26	25
9*c*18:1	7	13	4	7	15	13
11*c*18:1	1	–	–	1	–	–
18:2	44	1	–	43	2	1
γ18:3	44	–	–	44	1	1
Number of d.b./100 FA	(227)	(34)	(16)	(228)	(48)	(42)
% hydrogenation [a]		85%	93%		79%	82%

[a] % hydrogenation $= \frac{\text{change in d.b./100 FA}}{\text{initial d.b./100 FA}} \times 100.$

for 2 min, after which the vessel was sealed until the end of the desired incubation. After the reaction, the cell suspension was centrifuged to collect cells and the cells were resuspended for lipid extraction or cell fractionation.

Lipid analysis

GLC analyses of the fatty acids were carried out after transesterification in 5% HCl-MeOH at 80 °C for 2 h under N_2. GLC was performed with a Varian Model 3700 chromatograph using a 30 m SP-2330 fused silica capillary column and a Hewlett Packard Model 3390A integrator. The methyl esters were identified by comparing their retention times with those of authentic standards (Supelco, Bellefonte, PA, U.S.A.). All experiments were performed at least twice, and all the data presented here are the mean of three of more independent sets of experiments.

Results

Hydrogenation of purified Tetrahymena phospholipids

Before conducting experiments with living *Tetrahymena* cells, the effect of the concentration of the catalyst, $Pd(QS)_2$, was tested on liposomes prepared from phospholipids extracted from whole *Tetrahymena* cells. The multilamellar liposomes of whole cell phospholipids were very susceptible to hydrogenation in THM at room temperature under 1 atm of H_2 pressure. The rates of hydrogenation (Table I) were generally similar at both a high level (100 μg/mg) and lower level (20 μg/mg) of catalyst in 5-min reactions, and the results generally resembled those observed under similar conditions using synthetic phospholipids [12]. The major individual *Tetrahymena* phospholipids phosphatidylethanolamine, phosphatidylcholine, and 2-aminoethylphosphonolipid, tested individually, were also hydrogenated rapidly with only minor differences in rate (data not shown).

Effect of hydrogenation on survival of Tetrahymena cells

In order to test cell viability following the reaction, a series of experiments was carried out with living cells. Before exposing *Tetrahymena* to the complete hydrogenation protocol described above, cells in mid-logarithmic growth ($(1–2) \cdot 10^5$ cells/ml) were harvested, resuspended in THM, and exposed separately to each potentially harmful condition used in the hydrogenation protocol. Cell viability, as judged by comparing cell number and microscopic observations of cell shape and motility of treated and control cultures 1, 2, 4, 8, and 17 h after treatment was not affected by the presence of hydrogen or of inactive (oxidized) catalyst, or by rotation of the reaction vessel, when each of these parameters was tested individually (Fig. 1). However, when the catalyst was activated by H_2 and introduced in final

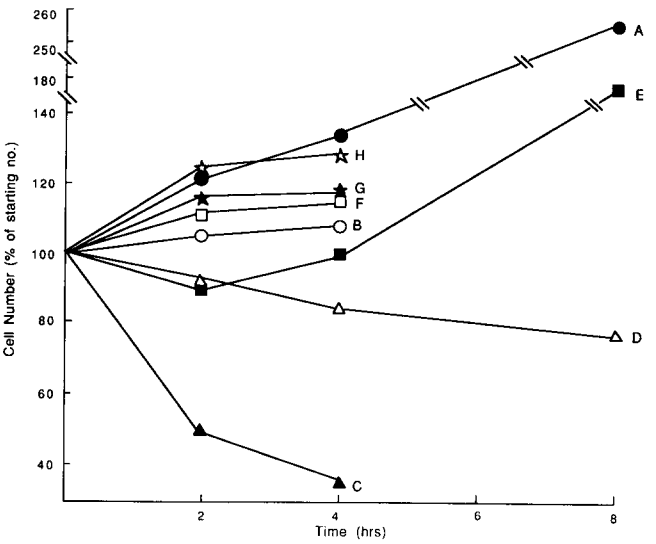

Fig. 1. Effects of hydrogenation conditions on cell survival. Cell suspensions were prepared as described in Materials and Methods and treated in the absence of catalyst (control), curve A; with 100 μg inactive catalyst/ml (no H_2), curve B; or with 100, 50, 20, 10, 8, or 5 μg active catalyst/ml in curves C, D, E, F, G and H, respectively. In curves C–H, hydrogenation continued until the first indication of cell distress was seen microscopically. These times were: C, 5 min; D, 25 min; E, 25 min; F, 70 min; G, 70 min; H, 60 min (no distress seen). Abscissa indicates time following resuspension of hydrogenated cells in fresh growth medium.

concentrations ranging from 5- to 100 μg/ml to a cell suspension maintained under H_2, cell viability was reduced markedly at higher catalyst concentrations (Fig. 1). In the presence of 100 μg catalyst/ml more than 50% of the cells died within 1 h following the hydrogenation. The survival of *Tetrahymena* was dependent upon the ratio of active catalyst to cells. Irreversible damage to cells was delayed in experiments where the catalyst was introduced in an oxidized state and reduced in situ by H_2 (see Fig. 3). Using the lower concentration of catalyst (5–50 μg catalyst/ml), the cells were capable of recovering and resuming growth at normal rates (Fig. 1).

Hydrogenation of Tetrahymena lipids in vivo

Detailed experiments were conducted to test the effects of the catalyst/cell ratio on the lipid hydrogenation rates in living cells (Fig. 2). A catalyst concentration of 20 μg, designated as mild hydrogenation conditions, was selected for extensive subsequent experimentation since that catalyst level afforded the best combination of high cell survival (Fig. 1) and significant lipid hydrogenation (Fig. 2). The extent of whole cell lipid hydrogenation following these mild conditions, although reproducible, was much less extensive than found with *Tetrahymena* phospholipid presented to the catalyst in the form of liposomes (Table I).

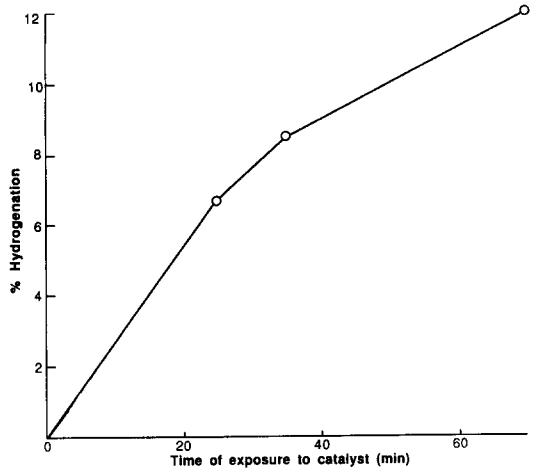

Fig. 2. Percent hydrogenation (% decrease in total fatty acid double bonds) resulting from treatment of cell suspension with 20 μg active catalyst/ml for the indicated time periods.

Hydrogenation of Tetrahymena preincubated with catalyst

The studies with living *Dunaliella* cells described in the introduction suggested that permeation of the catalyst into the cells might be relatively slower than into liposomes prepared from *Dunaliella* lipids [5]. In an effort to render the cellular lipids of *Tetrahymena* more accessible to the catalyst, cultures were preincubated with the oxidized form of the catalyst, known to be non-toxic (Fig. 1), and then the cultures were exposed to H_2 for 10 min (Fig. 3). However, even 1 h of preincubation led to a certain degree of microscopically visible indications of cell distress after the 10-min hydrogenation, and as the time of preincubation increased, irreversible cellular damage following hydrogenation became more obvious (Fig. 3).

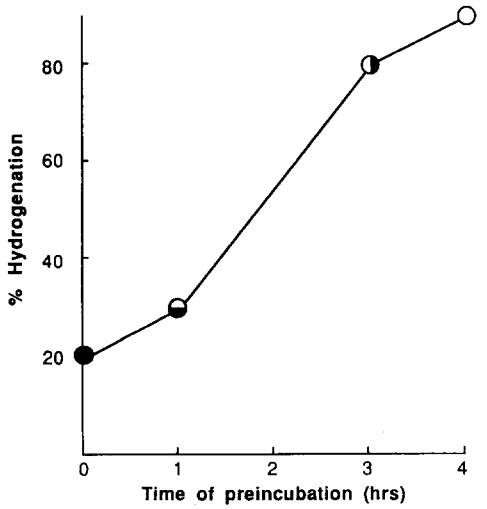

Fig. 3. Percent hydrogenation (% decrease in total fatty acid double bonds) resulting from exposing cells to H_2 for 10 min following preincubation with 100 μg inactive catalyst/ml for the times indicated. ●, cells unaffected; ⊖, cells swimming abnormally; ◑, cells not moving; ○, cells disrupted.

Other efforts to improve cell viability were conducted with reduced levels of the catalyst. However in the presence of < 10 μg catalyst/ml, an excessively long time (sometimes, more than 60 min) was needed for catalyst activation even though the reaction vessel was saturated with H_2 by repeatedly evacuating the vessel and readmitting H_2. While there was ultimately a reproducible hydrogenation, cell viability invariably dropped when hydrogenation increased.

Intracellular sites of in vivo lipid hydrogenation

Following the mild hydrogenation of living *Tetrahymena* cells ($2 \cdot 10^5$ cells/ml and 20 μg catalyst/ml) for 40 min, cilia, pellicles, and microsomes were isolated for lipid extraction. Whereas the hydrogenation of fatty acids from whole cell total lipids was only $0.7 \pm 0.5\%$, that of ciliary total lipids isolated from the same cell culture was $22.8 \pm 2.8\%$. The hydrogenation was reflected mainly in the loss of 18 : 3 and, to a lesser extent, 18 : 2, as was the case with liposomes (Table I). Pellicles were in general less effectively hydrogenated than cilia but more effectively hydrogenated than microsomes. In fact, there was no significant hydrogenation of microsomes by this mild condition (data not shown). It is possible that the complex structure of the cell's surface membrane-the cytosol is enclosed by a pellicle featuring three overlapping unit membranes [14], would retard the inward diffusion of catalyst.

Inactivation of $Pd(QS)_2$ under conditions simulating whole cell incubations

Because $Pd(QS)_2$ was relatively ineffective in hydrogenating lipids of living cells, tests were designed to determine whether catalytic activity is inhibited by material secreted from *Tetrahymena*. Liposomes were prepared from whole cell phospholipids, as described in Materials and Methods, except that the THM used to resuspend and disperse the lipid had been used immediately before to resuspend a culture of *Tetrahymena* (cell density $2 \cdot 10^5$ cells/ml). In one experiment the cells were removed from the THM by centrifugation as quickly as possible (exposure to cells approx. 7 min), yielding a '0 time supernatant'. In another case the cells were incubated in THM for 40 min before centrifugation to obtain a '40 min supernatant'.

As shown in Fig. 4, the lipids of control liposomes in untreated control THM were rapidly hydrogenated as usual. However, those in THM having been exposed to living *Tetrahymena* cells were less susceptible to hydrogenation, and the inactivation of the catalyst was greater when the THM had been exposed to cells for the longest period.

In separate experiments, the ability of a low concentration (20 μg/ml) of activated catalyst to hydrogenate *Tetrahymena* microsomes was tested. Unwashed microsomes either freshly thawed or thawed and prein-

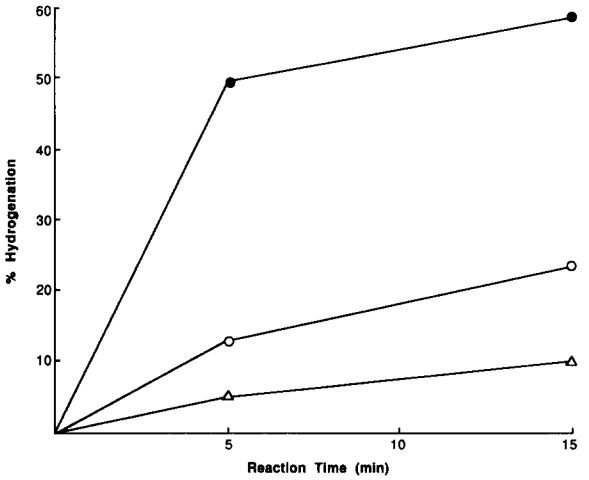

Fig. 4. Effect of secreted material on hydrogenation (% of C_{18} FA d.b. lost) in liposomes of whole cell phospholipids. ●, liposomes in fresh THM; ○, liposomes in '0 time supernatant' (see text); △, liposomes in '40 min supernatant'. Catalyst concentration was 20 μg/ml.

cubated for 10 min at 25 °C were injected into a vessel in amounts equivalent to approx. 0.09 mg lipid/ml THM. In sharp contrast to the effective hydrogenation achieved using liposomes prepared from purified phospholipids (Table I), lipids extracted from the microsomes incubated for 15 min with the catalyst had sustained an even lower amount of hydrogenation than any shown in Fig. 4.

Studies of hydrogenation inhibitors

Additional studies were conducted using a partially activated species (see Methods) of the catalyst, termed **B**, which has the advantages of being (1) stable in solution under air and (2) convertible to the fully active species almost instantaneously upon readmitting H_2. Using this more conveniently manipulated form of the catalyst, the properties of the putative inhibitory substances secreted by the cells were examined.

Initial studies measured effects of possible inhibitors on the action of **B** towards a synthetic substrate, oleic acid. Cellular supernatants were prepared by centrifugation after incubating cells ($2 \cdot 10^5$/ml) for specified times in THM alone or, to more closely simulate previous experiments with whole cells, in THM containing either 20 or 100 μg **B**/ml. When these supernatants were provided with H_2 (and 20 μg **B**/ml if it was not already present), those which had been exposed to living cells for short time periods were capable of extensive oleate hydrogenation (Table II). However, supernatants exposed to cells for longer periods were less supportive of hydrogenation. Those from cell suspensions preincubated with 20 μg **B** for 2.5 h inhibited oleate hydrogenation by nearly 50%, but increasing the content of the catalyst to 100 μg/ml partially overcame the inhibition.

TABLE II

Effect of cell secretions on the hydrogenation of oleate

907 nmol oleic acid were added in 25 μl methanol to 3 ml of the supernatants following the indicated pretreatment. Each mixture also contained 72.6 nmol $Pd(QS)_2$, form **B**. Total incubation time under H_2 was 30 min.

Pretreatment of supernatant	Product distribution (% of total FA mass)		
	18:0	*t*18:1	*c*18:1
1 h with cells	100	0	0
18 h with cells	3	8	89
1 h with cells and 20 μg cat./ml	96	2	2
2.5 h with cells and 20 μg cat./ml	51	49	0
1 h with cells and 100 μg cat./ml	100	0	0
2.5 h with cells and 100 μg cat./ml	71	29	0

It is well known that *Tetrahymena* cells secrete a variety of substances, particularly proteins, into their medium [16]. Accordingly, the effect of a test protein, bovine serum albumin, on oleate hydrogenation was measured. As shown in Table IIIa, increasing levels of BSA caused a progressive decrease in hydrogenation. The effect was most pronounced at the lower (20 μg/ml) catalyst concentration and was accompanied by a relatively high production of *trans*-18 : 1. (Note: the highest level of BSA tested, 3376 μg., was equivalent to 0.05 μmol, whereas 20 μg $Pd(QS)_2$ equals 0.03 μmol). In the presence of a larger amount of catalyst the inhibitory effect of BSA was partially overcome.

BSA contains one free cysteine residue, and its sulfhydryl group might be expected to inactivate $Pd(QS)_2$. Cysteine was indeed shown to be a potent inhibitor of

TABLE III

Effects of various inhibitors on the hydrogenation of oleate

The conditions were as in Table II, with inhibitors in 3 ml THM.

Final catalyst concentration	Additive	Composition (%)		
		18:0	*t*18:1	*c*18:1
a.	BSA(μg)			
20 μg cat./ml (0.03 μmol/ml)	167	100	0	0
	846	76	15	9
	1693	74	19	7
	3376 (0.05 μmol)	52	27	21
100 μg cat./ml	167	100	0	0
	846	100	0	0
	1693	94	3	3
	3376	92	4	4
b.				
20 μg cat./ml	$\frac{\text{Cysteine (μg)}}{880}$	2	8	90
20 μg cat./ml	$\frac{\text{Mucus (μg protein)}}{2100}$	2	8	90

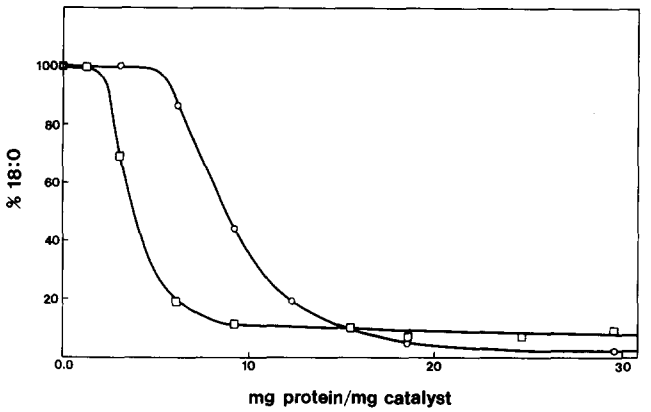

Fig. 5. Inhibition of oleic acid hydrogenation by cytosolic proteins from *Tetrahymena*. Conditions: 907 nmol oleic acid were added in 25 μl methanol to 3 ml 2.5 mM potassium phosphate buffer (pH 6.0) containing 72.6 nmol $Pd(QS)_2$, form **B**, and increasing amounts of protein as indicated. The mixtures were exposed to H_2 for 30 min. □, untreated protein solution; ○, supernatant following boiling for 10 min and centrifugation to remove proteins (abscissa for this curve reflects protein content before boiling).

the catalytic process (Table IIIb). Crude preparations of mucus discharged by *Tetrahymena* following dibucaine treatment also severely inhibited oleate hydrogenation. In fact, mucus is a likely prospect for the physiological inhibitor, since it was observed microscopically to be discharged in copious amounts following addition of the catalyst.

Bearing in mind our desire to achieve an in vivo hydrogenation of lipids situated in intracellular membranes, we measured the effect of *Tetrahymena* cytosolic proteins on the hydrogenation process. A pronounced inhibition developed as increasing amounts of protein were added (Fig. 5). However, it appears that a significant proportion of the inhibitory activity is not attributable to proteins per se, since boiling and centrifuging the coagulated cytosolic protein from the preparations did not entirely eliminate the inhibition.

Activity of the catalyst internalized by cells

In an effort to hydrogenate intracellular membranes selectively, cells were preincubated for 18 h with large amounts of **B**, washed, and then exposed to more catalyst for an additional 2 h before a final thorough washing to remove all excess catalyst. These cells, when examined microscopically, had an overall pinkish colour and contained conspicuous dark vacuoles which developed a distinct yellow colour shortly after the cells were exposed to H_2. From time to time samples were withdrawn from the cell suspension under hydrogen, and viability was checked by microscopy. Gradually the cells became more and more stressed as revealed by their round shape and decreased mobility, leading to the death of the majority of cells in 15 min in some experiments and > 45 min in others. In one of these experiments, following the period of 45 min under H_2, the cells were deciliated, and lipids from cilia and deciliated cells were analyzed separately. Neither the lipids of cilia nor deciliated cells had been measurably hydrogenated despite the obvious color change of internalized catalyst indicative of its conversion to an active form.

Retroconversion of hydrogenated lipids to their original unsaturated form

The fact that *Tetrahymena* cells subjected to mild hydrogenation can survive and resume active growth (Fig. 1) indicates that cells are capable of reversing or overcoming the inhibitory effects of the lipid alterations. To investigate how the cells respond to the reduced fatty acid unsaturation, whole cell total lipids as well as ciliary, pellicle, and microsomal lipids were prepared from cells at various times following their hydrogenation under conditions leading to a measurable reduction of double bonds while assuring survival of all the cells. The composition of whole cell total fatty acids isolated from cells incubated for up to 17 h following hydrogenation (under these conditions the cell number doubled within 8 h) had recovered and had even surpassed the degree of unsaturation found in the whole cell total lipids prior to the hydrogenation. However, comparing these data with those from non-hydrogenated controls was not straightforward since control lipids also underwent a gradual increase in their total fatty acid unsaturation. In a typical experiment the whole cell fatty acid unsaturation, expressed as % of zero time control sample double bonds/100 FA, was, at 0, 2, 4, and 6 h, as follows – control sample: 100, 98, 100, and 112; hydrogenated sample: 96, 101, 99, and 130. The initial small drop in control cell unsaturation can be attributed to the degradation of fatty acid desaturase system enzymes known [14] to occur during anaerobic conditions such as we used briefly to initiate the experiment. Response of the control cells to restore optimal membrane fluidity could lead to the elevated level of unsaturation observed at 6 h. It is not known whether the small additional increase of unsaturation observed in recovering hydrogenated cells is statistically significant. By 8 h control and hydrogenated cells had essentially equal levels of unsaturation.

Discussion

As described above, there has been some success [2–5] in using $Pd(QS)_2$ to hydrogenate lipids of living cells. Since effecting a massive hydrogenation of membrane lipids in vivo would be of obvious utility in studying the cell's own ability to regulate membrane fluidity, we tested the action of $Pd(QS)_2$ on the well characterized [15] protozoan *Tetrahymena mimbres*. Although the cells were unaffected by inactive catalyst and by other manipulations involved in the hydrogenation treatment, they were severely stressed by exposure

to the activated catalyst. Fractionating cells following a brief nonlethal hydrogenation by extracellular catalyst revealed a fairly sizeable decrease in the unsaturation of surface membrane (ciliary) lipids. But under these circumstances no significant hydrogenation of intracellular lipids was detected.

Enzymatic control of cellular fatty acid desaturation is known to be centered in the endoplasmic reticulum [17], and therefore efforts were made to hydrogenate this organelle in vivo. Cells were preincubated with inactive catalyst under conditions causing a large uptake of catalyst into food vacuoles and possibly other cellular compartments. The introduction of hydrogen appeared to activate the catalyst but caused irreversible cell damage before an appreciable fatty acid hydrogenation was accomplished. Under these and all other experimental protocols tested, membrane-bound lipids were hydrogenated at a markedly lower rate than were the same lipids extracted and presented to the catalyst in the form of liposomes.

The desired massive hydrogenation of internal cell constituents failed for at least two reasons, i.e., (1) inhibition of the catalytic action by certain compounds released from the cells to the extracellular solution, and (2) prevention of fatty acid hydrogenation by internalized catalyst despite the colour changes signalling that a reaction of **B** and H_2 did take place.

It has long been known, both in homogeneous and heterogeneous catalysis, that certain sulfur-containing compounds like thiols can have a very strong inhibitory effect [18], especially with catalysts containing metallic or divalent Pt, Pd and Ni. Coordination of an -SH group of $Pd(QS)_2$ may alter the electronic distribution around the central metal ion so as to hinder the redox changes necessary for H_2 activation, or alternatively, it can block the coordination site where substrate unsaturated acids should approach the metal prior to H-transfer. Indeed, in addition to the effect of cysteine, shown in Table IIIb, we observed the same strong inhibition when 2-mercaptoethanol or KSCN was added to the solution of $Pd(QS)_2$. An important observation is that in the presence of these inhibitors, neither **A**, i.e. the inactive, nor **B**, the partially activated form of the catalyst could be hydrogenated to the active yellow state (**C**), showing that coordination of the inhibitor molecules render the complex unreactive towards molecular hydrogen.

In microheterogeneous systems like cell suspensions, the catalyst should be highly mobile and permeant to substantially hydrogenate the unsaturated fatty acids occupying the closely packed and rather hydrophobic interior of the membranes. It has been shown [19] that water soluble catalysts containing sulphonate groups bind very strongly to cationic macromolecules, like various anion exchange polymers, and that this attachment often leads to a dramatic decrease in the rate of hydrogenation of soluble, i.e., molecularly dispersed and freely diffusing substrates, despite the fact that the catalyst on the surface of the polymer does react with H_2, as shown by characteristic colour changes.

Both of the above two mechanisms, namely, coordination of free -SH groups and immobilization of the catalyst, can be operative in an inhibition caused by proteins. In the cases of inhibition by extracellular BSA and of the internalized catalyst, however, we always observed the characteristic pink to yellow colour change under hydrogen. This shows that most probably it is immobilization of the catalyst which is responsible for the lack of hydrogenation in the *Tetrahymena* cells.

An intriguing question remains regarding the cause of cell death upon hydrogenation. In case of an 'external' catalysis one may assume that a substantial hydrogenation of cilia, as was demonstrated above, is enough stress to kill the cells. Comparable studies of detached cilia in which lipid double bonds were hydrogenated to about the same extent (25%) found a significant loss of membrane fluidity as inferred from ESR measurements of added 5-doxylstearate and 16-doxylstearate [20]. On the other hand, no hydrogenation of fatty acids was observed in the various cell fractions following hydrogenation by the internalized catalyst despite the obvious stress and death of the cells. If the hydrogenation reduced fatty acids in this latter case as well, they must be very particular ones in an almost negligible concentration but of vital importance, and we cannot identify such a particular pool in *Tetrahymena*. This opens the way for assumptions regarding less selective actions of the catalyst, affecting other kinds of vital compounds present in low concentrations. Although $Pd(QS)_2$ was shown incapable of hydrogenating plastoquinones and carotenoids in pea chloroplasts, the accessibility of a given substrate may markedly alter its susceptibility to hydrogenation [21]. Alizarin red, the quinone ligand of the catalyst itself, is known to be hydrogenated by $Pd(QS)_2$ in true solution [13].

Despite the greatly reduced hydrogenation obtained in most experiments, there was some saturation of double bonds in the surface membranes of cells briefly exposed to extracellular catalyst. These hydrogenated cells were not seriously injured and responded by an apparent induction of a temporarily supraoptimal fatty acid desaturase activity. Unfortunately, the rather lengthy (up to 70 min) exposure of cells to anaerobic conditions during this procedure induces an elevated desaturase activity even in control cells [14], and the increment separating control and hydrogenated cell values was too small to be meaningful. Thus the test of whether reduced membrane fluidity can induce a restoration of optimal fluidity through increased desaturase activity [22] was inconclusive in this instance.

The $Pd(QS)_2$ catalyst remains a promising tool for the study of phenomena involving membrane fluidity.

The present experiments serve to emphasize three important points with respect to the use of $Pd(QS)_2$ on living cells. Hydrogenation with the preactivated catalyst is confined mainly to the surface membranes. Severe physiological damage may follow treatments which cause limited but highly localized hydrogenation. And careful strategy must be devised to circumvent the inhibitory action of endogenous inhibitors if a significant hydrogenation of intracellular membranes is to be achieved.

Acknowledgements

This work was supported in part by National Science Foundation Grants DCB-8802838 and INT-8717090 and Welch Foundation Grant F-350 to G.A.T. and by the Hungarian National Scientific Research Foundation (OTKA 543) to L.V.

References

1 Cossins, A.R. and M. Sinensky (1984) in Physiology of Membrane Fluidity, Vol. 2, Shinitzky, M. ed., pp. 1–20, CRC Press, Boca Raton.
1a Quinn, P., Joó, F. and Vigh, L. (1990) Prog. Biophys. Mol. Biol., in press.
2 Vigh, L., Joó, F., and Cseplo, A. (1985) Eur. J. Biochem. 140, 241–244.
3 Vigh, L., Gombos, Z., and Joó, F. (1985) FEBS Lett. 191, 200–204.
4 Benko, S., Hilkmann, H., Vigh, L. and Van Blitterswijk, W.J. (1987) Biochim. Biophys. Acta 896, 129–135.
5 Vigh, L., Horváth, I. and Thompson, G.A., Jr. (1988) Biochim. Biophys. Acta 937, 42–50.
6 Thompson, G.A., Jr. and Nozawa, Y. (1984) in Membrane Fluidity (Kates, M. and Manson, L.A., eds.), pp. 397–432, Plenum Press, New York.
7 Ryals, P.E. and Thompson, G.A., Jr. (1987) Biochim. Biophys. Acta 919, 122–131.
8 Thompson, G.A., Jr. (1967) Biochemistry 6, 2015–2022.
9 Nozawa, Y. and Thompson, G.A., Jr. (1971) J. Cell. Biol. 49, 712–721.
10 Tucker, C. and Wilhelm, J.M. (1981) J. Biol. Chem. 256, 10475–10484.
11 Bligh, E.C. and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911–917.
12 Vigh, L., Horváth, I., Joó, F. and Thompson, G.A., Jr. (1987) Biochim. Biophys. Acta 921, 167–174.
13 Bulatov, A.V., Izakovich, E.N., Karklin, L.N. and Khidekel, M.L. (1981) Izv. Akad. Nauk USSR 9, 2032–2035.
14 Umeki, S. and Nozawa, Y. (1984) Biochim. Biophys. Acta 792, 25–32.
15 Thompson, G.A., Jr. and Nozawa, Y. (1977) Biochim. Biophys. Acta 472, 55–92.
16 Banno, Y., Sasaki, N. and Nozawa, Y. (1987) Exp. Cell. Res. 170, 259–268.
17 Lynch, D.V. and Thompson, G.A., Jr. (1988) in Physiological Regulation of Membrane Fluidity (Aloia, R.C., Curtain, C.C. and Gordon, L.M., eds.), pp. 323–360, A.R. Liss, New York.
18 Willner, I. and Mandler, D. (1989) J. Am. Chem. Soc. 111, 1330–1336.
19 Joó, F. and Beck, M.T. (1984) J. Mol. Catal. 24, 135–145.
20 Horváth, I., Vigh, L., Pali, T. and Thompson, G.A., Jr. (1989) Biochim. Biophys. Acta 1002, 409–412.
21 Szalontai, B., Droppa, M., Vigh, L., Joó, F. and Horváth, G. (1986) Photobiochem. Photobiophys. 10, 233–240.
22 Kasai, R., Kitajima, Y., Martin, C.E., Nozawa, Y., Skriver, L. and Thompson, G.A., Jr. (1976) Biochemistry 15, 5228–5233.