ENZYMATIC REACTIONS IN REVERSED MICELLES

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Proefschrift

ter verkrijging van de graad van
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Aan mijn ouders

STELLINGEN

1. Hoewel Martinek et al. erkennen dat tengevolge van de insluiting van alco-
   holdehydrogenase in omgekeerde micellen de substraatconcentratie in de
   micro-omgeving van het enzym verandert, onderkennen zij niet dat dit de
   oorzaak is van de verandering in „substraatspecificiteit" van het enzym.
   - Martinek, K., Khmel'nitskii, Yu.L., Levashov, A.V. en Berezin, I.V.
     (1982) Dokl.Akad.Nauk. SSSR 263, 737-741.
   - Martinek, K., Levashov, A.V., Khmelnitsky, Yu.L., Klyachko, N.L. en
     Berezin, I.V. (1982) Science 218, 889-891.
2. Uit economische overwegingen is het nuttiger om hydrogenase te gebruiken
   voor de ontleding dan voor de produktie van waterstofgas.
   - „Photochemical, Photoelectrochemical and Photobiological Processes"
     Solar Energy R&D in the European Community, Series D, Vol. 2.,
     (Hall, D.O., Palz, W. en Pirrwitz, D., eds.) D. Reidel Publishing
     Company, Dordrecht (1983).
3. De door Boicelli et al. met behulp van ^{31}P-NMR gemeten pH-afhankelijkheid
   van de chemical shift van P_i is niet alleen onjuist, bovendien realiseren
   de onderzoekers zich niet dat in plaats van het pH-profiel van fosfaatbuf-
   fer in omgekeerde micellen dat van de lecithine-fosfaat gemeten is.
   - Boicelli, C.A., Conti, F., Giomini, M. en Giuliani, A.M. (1982)
     Spectrochimica Acta 38A, 299-300.
   - Gadian, D.G., Radda, G.K., Richards, R.E. en Seeley, P.J. (1979) in
     „Biological Applications of Magnetic Resonance", Shulman, R., ed.
     Academic Press, New York p. 463.
4. Omdat de Michaelis konstante (K_m) van een enzymreaktie beïnvloed wordt door
   pH, ionsterkte en temperatuur, verdient het aanbeveling de standaardom-
   standigheden bij de bepaling ervan nauwkeurig te definiëren.
   - Enzyme Nomenclature (1972) Elsevier, Amsterdam.
5. Hoewel het gebruik van log P waarden geen thermodynamische rechtvaardiging
   heeft, is toch de voorspellende waarde ervan duidelijk.
   - Dit proefschrift.
6. De populaire opvatting „waar biotechnologie is, is geld", die de oorzaak is
   van de exorbitant hoge deelnemersprijzen van bijeenkomsten op biotechnolo-
   gisch gebied, leidt tot oneigenlijk gebruik van de voor biotechnologisch
   onderzoek beschikbaar gestelde fondsen.

7. Willen de mogelijkheden van genetische manipulatie ten volle benut worden in de resistentieveredeling, dan moet het resistentieonderzoek zich meer dan nu het geval is, richten op het leren kennen van genprodukten die betrokken zijn bij plant-parasiet interakties.
    - Keen, M.T. (1982) Advances in Plant Pathology, 1, 35-82.
    - Staskawicz, B.J. (1983) in „Biochemical Plant Pathology", Callow, J.A., ed. John Wiley and Sons, New York, p. 199-213.

8. In het verleden is in ontwikkelingslanden bij de economische planning gericht op maximale welvaart, de aandacht al te exclusief gericht op de produktiefaktor kapitaal. Dit werd gerechtvaardigd met het argument dat kapitaal een bij uitstek schaarse produktiefaktor is, welke, in tegenstelling tot andere, overvloedige produktiefaktoren een beperkende invloed heeft op het maximaal te bereiken nivo van de produktie. Deze redenering gaat voorbij aan het eigen karakter van arbeid, waarvoor in tegenstelling tot andere produktiefaktoren, ook in een situatie van onderbenutting en overschot, toch een „prijs" betaald moet worden, namelijk de kosten van levensonderhoud. Dit houdt in dat, afgezien van andere, sociale, overwegingen, het bereiken van een maximale materiële welvaart afhankelijk is van de produktieve bijdrage van een zo groot mogelijk deel van het arbeidspotentieel.
    - Edwards, E.O. (1974) in „Employment in Developing Nations" Report on Ford Foundation Study, Edwards, E.O., ed., Columbia University Press, New York, p. 1-46.

9. Het wordt tijd dat de dierenbescherming een aktie start tegen het om zeep helpen van huishoud- en lichaamsbakteriën door het ongecontroleerd gebruik van ontsmettende middelen in het dagelijks leven.

10. Bij het beoordelen van het maatschappelijk nut van het verhogen van het lysinegehalte in granen dient men zich te realiseren dat ondervoeding zelden wordt veroorzaakt door een gebrek aan eiwit, maar veeleer door een ontoereikende hoeveelheid voedsel en dat het wereldvoedselprobleem niet in relatie staat tot de hoeveelheid voedsel maar tot de verdeling ervan.

11. Uit het feit dat het aantal mannen in „vrouwenfunkties" sterk stijgt en dat, ondanks een aktief stimuleringsbeleid, het aantal vrouwen in „mannenfunkties" nauwelijks toeneemt, blijkt een opwaardering van „vrouwenwerk", doch niet van de vrouw zelf.

Riet Hilhorst
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VOORWOORD

Er zijn weinig proefschriften die het resultaat zijn van het werk van één persoon. Ook aan dit proefschrift hebben meerdere mensen een bijdrage geleverd. Wie de moeite neemt het hele proefschrift door te lezen komt diverse namen tegen van personen die eraan meegewerkt hebben. Omdat de meeste lezers niet verder komen dan het voorwoord en omdat niet alle bijdragen een tastbaar wetenschappelijk karakter hebben dat vermelding in een artikel rechtvaardigt, wil ik hier een aantal mensen bedanken.

Op de allereerste plaats is dat Ton, die zorgde voor de stabiliteit van het systeem door alle dieptepunten en hoogtepunten te relativeren. Ook Willem, Marleen, Bertus, Willem, Jolan en Paul wil ik bedanken voor het geduldig aanhoren van mijn verhalen.

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Johan van Leeuwen, Anita van Berkel-Arts, Kees van Dijk, Hans Grande en Sjef Boeren, met wie ik dagelijks te maken had op lab 1 en 2, hebben ieder op hun eigen wijze meegewerkt, variërend van het bedenken van formules tot het afstaan van enzym. In dit kader moeten ook Hein Kroft en Raymond Verhaert genoemd worden; van de resultaten van hun doctoraalonderzoek heb ik dankbaar gebruik gemaakt.

Van de gesprekken met Prof. J.Th.G. Overbeek en Fred Verhoeckx van de afdeling Fysische en Kolloïdchemie in Utrecht en met Thonie van de Boomgaard en Willem Norde van de afdeling Fysische en Kolloïdchemie in Wageningen heb ik veel geleerd.

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Uiteraard zijn er nog veel anderen die me geholpen hebben met het uitvoeren van de meest uiteenlopende dingen. Het zou te ver voeren om iedereen hier te noemen, maar toch wil ik ook hen bedanken.

CONTENTS

LIST OF ABBREVIATIONS AND DEFINITIONS

a_0	: molar ratio of hexanol to CTAB in the interphase
Aerosol OT	: sodium bis(2-ethylhexyl)sulphosuccinate
bistris	: bis(2-hydroxyethyl)imino-tris(hydroxymethyl)methane
CTAB	: cetyltrimethylammonium bromide
H_2ase	: hydrogenase (EC 1.12.2.1)
HEPES	: N-2-hydroxyethylpiperazine N'-2-ethanesulfonic acid
HPLC	: high performance liquid chromatography
HSDH	: 20β-hydroxysteroid dehydrogenase (EC 1.1.1.53)
interface	: the surface between the water pool and the continuous phase that forms their common boundary
interphase	: a layer existing as a distinct phase in a heterogeneous system
K_m	: Michaelis constant
$K_{m,app}$	: Michaelis constant for apolar substrates related to overall concentrations
$K_{m,ov}$	: Michaelis constant for water-soluble substrates related to overall concentrations
$K_{m,wph}$	: Michaelis constant related to water phase concentrations
k_{cat}	: turnover rate at infinite concentration of substrate
lipDH	: lipoamide dehydrogenase (EC 1.6.4.3)
MeV^{2+}	: see MV^{2+}
MV^{2+}	: methyl viologen (1,1'-dimethyl-4,4'-bipyridinium ion)
MV^{+}	: methyl viologen radical
NAD^{+}	: oxidized nicotinamide adenine dinucleotide
NADH	: reduced nicotinamide adenine dinucleotide
ODS	: octadecyl silyl
P	: partition coefficient, defined as the concentration of solute in the organic phase divided by the concentration in the aqueous phase
$P_{c,i}$	: partition coefficient over the continuous phase and interphase
$P_{w,i}$	: partition coefficient over the water phase and interphase
log P	: logarithm of partition coefficient in an octanol-water two-phase system
$\log P_i$	: log P value for the interphase

$\log P_{cph}$	: $\log P$ value for the continuous phase
$\log P_s$	: $\log P$ value for the steroid
$Ru(bipy)_3^{2+}$	: tris(2,2'-bipyridine) ruthenium (II)
surfactant-Ru^{2+} complex	: [N,N'-di(1-hexadecyl)-2,2'-bipyridine-4,4'-dicarboxamide]-bis(2,2'-bipyridine)ruthenium (II)
Tris	: Tris (hydroxymethyl)-amino-methane
V_{max}	: rate of reaction at infinite concentration of substrate
V'_{max}	: rate of reaction at infinite concentration of one substrate but non-saturating concentration of another substrate
w_0	: molar ratio of water to CTAB
$Zn(Ph)_4Por$	: Zinc tetraphenylporphyrin
$Zn(SPh)_4Por^{4-}$	: Zinc tetra-p-sulfonatophenylporphyrin
θ	: volume fraction of reversed micelles
φ	: volume fraction of water

1. INTRODUCTION

1.1 General Introduction

In living organisms almost all chemical reactions are carried out by enzymes. Over 2000 different enzymes are known; each of them catalyses a specific kind of chemical reaction. Enzymes have extraordinary catalytic power, for they accelerate reactions by several orders of magnitude, they operate under mild conditions of temperature and pH and do not produce side products. Furthermore, enzymatic reactions can be both site- and stereospecific.

Man seldom makes use of enzymes to perform chemical reactions of his interest, even though enzymes have several advantages over traditional methods of chemical synthesis. In industry, only about ten enzymes are used. All of them, with the exception of glucose isomerase, belong to the class of hydrolases, thus cleaving chemical bonds with the aid of water.

There are several reasons for the limited application of enzymes in industry: the lability of the enzymes, the expense of isolation and the fact that many enzymes need a cofactor. Because of the high cost of cofactors, application of such enzymes is economically feasible only when the cofactor is regenerated from cheap reagents. Several methods for cofactor regeneration in aqueous media have been described [1-6].

Another limitation to the application of enzymes is imposed by the fact that they function preferentially in aqueous media, whereas many chemically interesting reactions involve compounds that are not, or are poorly water-soluble. To overcome this problem, either the solubility of the substrate in the aqueous solution should be increased or the enzyme should be made to function in a non-aqueous medium while retaining its catalytic activity. Solubilisation of reactants that are poorly water-soluble, with the aid of surfactants [7-9], polymers [10], or water-miscible organic solvents [11-14] are examples of the first approach. The second approach is much more interesting from the biotechnological point of view, for systems in which the non-aqueous phase constitutes a significant proportion of the total volume have several advantages: the volume needed to perform the conversion is greatly reduced, the recovery of product and biocatalyst is facilitated, the reaction equilibrium can be shifted towards the desired direction and other compounds can be used to replace water as donor or acceptor of chemical groups [15-17].

When enzymes are dissolved in organic solvents non-miscible with water, they loose their catalytic activity, because of the disruption of the hydrophobic interactions that play a dominant role in maintaining their native structure. This can be prevented by using a two-phase system, where the substrate has to diffuse into a water phase where the enzyme is located [18-21], or by suspending porous hydrophilic particles, that are impregnated with an aqueous enzyme solution, in an organic medium [17,22]. However, in such systems mass transport is a rate-limiting factor, because of the small interfacial area and the low diffusion rate of apolar compounds in hydrophilic media. One way of dealing with this problem might be the use of reversed micelles.

1.2 Reversed Micelles

Reversed micelles are tiny water droplets that are dispersed in an organic solvent with the aid of surfactants. Because of their amphiphilic nature, the surfactant molecules are located at the interface with their polar headgroups directed towards the waterpool and their tails sticking out in the continuous oil phase (Fig. 1).
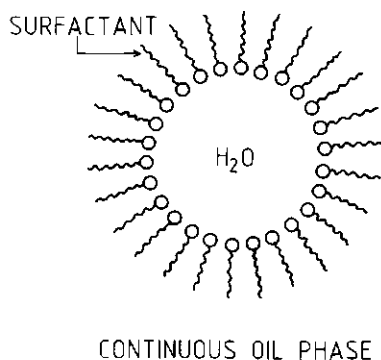

SURFACTANT

H_2O

CONTINUOUS OIL PHASE

Fig. 1. Schematic representation of a reversed micelle.

Reversed micelles are so small that no visible light is scattered, giving the solution an optically clear appearance. Hoar and Schulman [23] were the first to give a model for this type of aggregates. They proposed the name 'oleopathic

hydro-micelles' but nowadays names like 'reversed micelles' and 'microemulsion' are generally used. Up till now, no terminology has been agreed upon to distinguish between reversed micelles and microemulsions. Microemulsion tends to be a more general term that can be applied to both oil-in-water and water-in-oil dispersions, stabilized by surfactants [24,25]. In an aqueous medium, small microemulsion particles are called „swollen micelles", in an organic medium they are referred to as „reversed micelles" [23]. Upon addition of dispersed phase, a continuous transition from a thermodynamically stable microemulsion to an instable macroemulsion takes place [24,26]. This is accompanied by an increase in turbidity of the solution. In this work only optically clear, thermodynamically stable solutions with organic solvents as the continuous phase were used and are referred to as reversed micellar media.

A reversed micellar medium consists of at least three components i.e. water, a water-immiscible organic solvent and a surfactant. A prerequisite for the formation of reversed micelles is that the interfacial tension between the dispersed water phase and the continuous oil phase must be almost zero. This is achieved by the addition of a surfactant. If this condition is not met, the system will minimize its interfacial area, which leads to the formation of a two-phase system. A zero interfacial tension implies that the concentration of surfactant in the continuous phase (in case of an oil-soluble surfactant) or in the water pool (in case of a water-soluble surfactant) is below or equal to the critical micelle forming concentration. Especially when dealing with a water-soluble detergent, it is reasonable to assume that all but a small fraction of the surfactant is located in the interface [24]. This results in an enormous interfacial area; at high surfactant concentrations this can be as much as 200 m^2/ml. The surfactant molecules are anchored fairly firm in the interface and rarely exchange with the water pool, though their lateral motility is high [27].

Some surfactants are not able to lower the interfacial tension sufficiently to reach values of almost zero. In these cases the addition of a cosurfactant is necessary to lower the interfacial tension further [23,24]. Depending on its polarity, the cosurfactant partitions between the water pool, the interphase and the bulk organic phase. Exchange between the phases takes place at a nanosecond timescale [27 and references therein], and slows down with increasing alcohol chain-length. This results in a more rigid interface with a higher degree of order [28]. A less flexible interface will restrict the mobility of the water in the water pool. It was found that with long chain alcohols like octanol and

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decanol as cosurfactant, water is restricted to certain domains of the micro-emulsion [29]. With short chain alcohols water diffuses much more freely. The chain length of the surfactant itself has no influence on the mobility [29]. This implies that the degree of organization of a microemulsion depends to a large extent on the chain length of the cosurfactant.

An important parameter for reversed micellar media is the amount of water present per surfactant molecule. This parameter has been named w_o [26,30–33], ω [34,35], R [36–41], n [42] or μ [43]; in this thesis the expression w_o is used.
The minimal amount of water necessary for the formation of a reversed micellar solution depends on the type of surfactant used and the composition of the aqueous solution, as does the maximal amount that can be solubilised. At low water content all water in reversed micelles is bound to the surfactant head groups, resulting in properties greatly differing from those of bulk water [34,44–47]. With increasing water content, the water begins to reassume the properties of bulk water with respect to relaxation time, freezing point, partial specific volume, viscosity, dielectric constant etc. [44,47–49].

The area in the phase diagram where reversed micelles are stable depends on the ionic strength and pH of the aqueous solution that is used. In some cases salt increases the area where reversed micelles are formed, in other cases it leads to a decrease [25,44,50–52]. The interpretation of ionic strength and pH inside reversed micelles is not a trivial problem. The dissociation constants of salts, surfactants and water are not necessarily the same as in bulk water. This implies that a pK or pH value inside a reversed micelle does not have the same meaning as in an aqueous solution. No information is available on ionic strength in reversed micelles, but several attempts have been undertaken to determine effective pH values in reversed micelles. The groups of Fujii [53,54] and Luisi [55] used ^{31}P-NMR with this objective, whereas in another approach water soluble dyes were used as indicators [38,39,56]. These methods are not straightforward because neither for the ^{31}P-chemical shift [57], nor for the pK of the dyes can an absolute value be obtained that is related to values in aqueous media; but nevertheless these methods are useful to indicate tendencies. Considering that at low water contents the properties of water inside reversed micelles deviate most from those of ordinary water, the largest differences between the pH of the buffer solution before injection and the pH in the water pool are expected to occur at a low water content. Indeed this has been confirmed [37,55]. Furthermore, evidence has been

obtained for the existence of a pH gradient from the centre of the water pool
towards the surfactant head groups [39,56]. The course of this gradient depends
on the type of surfactant, type of buffer and buffer capacity [56,58]. Much
more research remains to be done though, before absolute pH values can be
assigned.

As mentioned before, the water in a reversed micellar medium is confined
to water pools, surrounded by surfactant molecules. In contrast to micelles in
aqueous solutions, reversed micelles are electrically neutral, so they do not
repel each other, and collisions can easily occur. Menger [59] was the first
to suggest that the water pools could communicate with each other, either by a
rapid merging and separation of the water pools or by transport via the continu-
ous phase. Eicke et al. [60] showed that the latter possibility could be ex-
cluded and suggested that transport takes place via a water channel formed
during collision of two micelles. For this situation they calculated that the
time required for the diffusion of one molecule from one waterpool to another
was only 10 ps, whereas the contact time between two reversed micelles would
be in the order of 100 ps. However, not all collisions that take place, lead
to exchange, because few micelles have sufficient kinetic energy to overcome
the barrier of interfacial energy. Approximately one in every thousand colli-
sions is effective [61,62], resulting in a $k_{exchange}$ of 10^6-10^7 $M^{-1}s^{-1}$ depend-
ing on the composition of the medium. Additives have been shown to have a
strong effect on the rate of exchange. Neutralisation of the charge of the
surfactant head groups by adding oppositely charged surfactants results in a
more rigid interface and a lower exchange rate, whereas the opposite effect is
observed with surfactants bearing the same charge [61].

1.3. Enzymes in reversed micelles

Some ten years after the first model for reversed micelles was presented
[23], enzyme activity in a reversed micellar medium was described [63], but
some twenty years went by before a more general interest in "micellar enzy-
mology" awoke and the possible applications of such systems were realised. The
groups of Martinek [64-73] and Luisi [30-32,74-76] broadened the area of re-
search by incorporating several water-soluble enzymes in reversed micelles of
artifical surfactants, differing from the phospholipids that had been used at
first to solubilize proteins in organic solvent [50,77].

Even multi enzymatic reactions can be carried out in reversed micellar
media as was shown by Martinek [65] and will be shown more extensively in chap-

ter 3 of this thesis.

A question encountered when trying to understand enzymic behaviour in re-
versed micelles, is the localization of enzymes. Several possibilities can be
imagined: either the protein can be dissolved in the organic phase, be loca-
lized in the interphase or it can reside inside the water pool (Fig. 2).
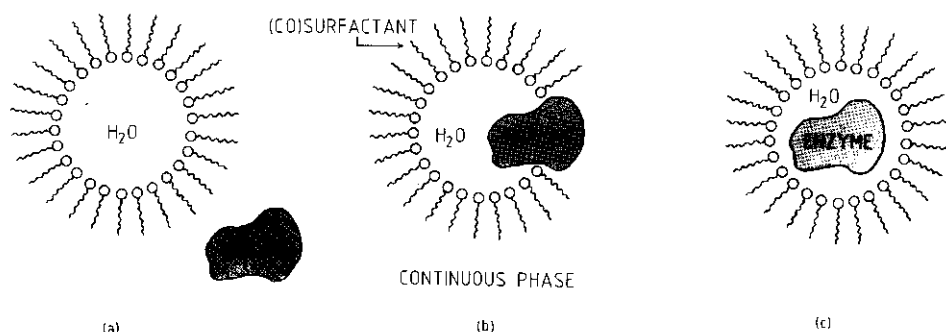

Fig. 2. Possible locations for a protein in a reversed micellar solution:
(a) in the continuous phase, (b) in the interphase and (c) in the water pool of
the reversed micelle.

The question has not been answered satisfactorily yet, as mostly water-soluble
enzymes have been the subject of research. For trypsin, chymotrypsin, cyto-
chrome b_5 and alcohol dehydrogenase the circular dichroism, ultraviolet or
fluorescence spectra differ only slightly from those in an aqueous solvent,
indicating that these enzymes are located most likely in the water pool [30,32,
76,78,79]. Ultracentrifuge studies with several other proteins indicated that
those proteins were also probably located inside the reversed micelle [31,67,
69], but the occurrence of interactions with the interface in case of membrane
bound proteins cannot be excluded [78,79]. In fact, this must be the explana-
tion for the behaviour of glucagon in reversed micelles, for the observed re-
sults [30] are consistent with the data about the conformation in aqueous mi-
celles that were recently obtained with two-dimensional NMR-studies [80].

If enzymes in reversed micelles are located mainly in the water pool, the
question then arises as to how reversed micelles can accommodate such relative-
ly large molecules. Both the groups of Martinek and Luisi showed by ultracen-
trifuge studies that the difference in size between empty and filled micelles
is largest at low water/surfactant ratio's, and decreases as this ratio

increases. At low water contents the radius of the enzyme exceeds the radius
of the water pool; the enzyme creates a new bigger water pool around itself by
increasing the number of surfactant molecules and the number of water molecules.
At high water content, the radius of the water pool exceeds the radius of the
enzyme and hardly any increase in size is observed upon entrapment of a protein
[31,69,70].

In the latter situation the enzyme would be surrounded by a water layer
of 5-20 Å thickness [32], allowing some motional freedom. In small reversed
micelles motion is restricted by the limited space available [72]. This might
also result in changes in the conformation of the protein. Circular dichroism
proved to be a useful method to study the effects of encapsulation in reversed
micelles on the protein conformation. Grandi et al. [75] showed that lysozyme
in reversed micelles contained 48% of α-helix, compared with 34% in an aqueous
solution. The helical content increased with decreasing amount of water in the
system. A high percentage of helix is indicative of a more rigid structure,
so the smaller the reversed micelle, the more the protein is folded. The same
tendency was found for chymotrypsin and alcohol dehydrogenase [31,32,76], but
not for myoglobin [78,79]. Here the content of α-helix decreased from 77% in
aqueous solution to 20-30% in reversed micelles.

This restricted motional freedom has a stabilizing influence on enzymes in
reversed micelles. The more rigid the structure of an enzyme is made, the more
inactivation by uncoiling is prevented [17]. Barbaric [76] showed that the sta-
bility of α-chymotrypsin in reversed micelles, particularly at a low water con-
tent, is much higher than in aqueous solution. Another explanation for the in-
creased stability of proteolytic enzymes is that intermolecular autolysis is
prevented by the separation of the molecules. It is interesting to note that
such effects are also observed upon immobilization of proteins in matrices [17].

It has been mentioned earlier that the properties of water in reversed
micelles differ from ordinary water in several respects. These changed proper-
ties could influence the enzyme. This line of thought has been pursued by
Menger and Yamada [37] and Luisi et al. [75,76,32]. They observed a large shift
of the optimum pH of chymotrypsin, ribonuclease and alcohol dehydrogenase cata-
lysis respectively. Though it is not a trivial problem to determine the actual
pH in reversed micelles, this shift could not be explained by a change of the
buffer pH upon injection in reversed micelles, but was attributed to a change
in pK of a group at the active site, caused by an increased hydrophobicity of
the waterpool [75] or by stabilization of this group by the charge of the sur-

factant head groups [37].

These studies are of interest because enzymes in reversed micelles can serve as model systems of enzyme function in vivo or those associated with cell membranes. Nowadays, biochemists become more and more aware of the fact that in cells many enzymatic reactions take place at interfaces. This environment could influence enzyme activity, substrate flux to the enzyme and, connected with that, reaction specificity, or it could shift the thermodynamic equilibrium by spatial separation of substrate and product. All these are factors that cannot be studied in aqueous solution, but could be very important to understand events occurring in living cells. In chapter 4 of this thesis, the influence of the composition of the interphase on the reaction rate and reaction specificity will illustrate this.

In order to gain insight in such factors, an understanding of the effect of enclosure in reversed micelles on the kinetic behaviour of enzymes is necessary. Both the groups of Martinek [65] and Luisi [32] have derived expressions to relate the parameters of the Michaelis-Menten equation in aqueous solution to those obtained in reversed micellar media. It was realized that the volume fraction of aqueous solution and the partition of substrate over the aqueous phase and the organic phase are important parameters. Martinek predicted a linear relationship between K_m and the volume fraction of water, and obtained results in agreement with this model. However, large discrepancies still exist between the actual values obtained for this parameter as found in reversed micelles and in aqueous media.

Cryoenzymology is another area of research where reversed micelles could be of great value. As was pointed out before, water inside reversed micelles does not behave the same as bulk water with respect to freezing point. When the temperature is lowered below 0^oC, the fraction of water that most resembles free water, freezes first. Then gradually the rest of the water solidifies till at about -40^oC all bound water is frozen. Douzou et al. [81,82] made use of the lowered freezing point of the water in reversed micelles in an elegant way. Whereas in aqueous solutions enzyme reactions cannot be studied at temperatures far below zero, reversed micelles offer the opportunity to work as low as -40^oC, slowing down the reaction enough to enable the study of labile intermediates of enzymatic reactions and to obtain kinetic and thermodynamic information. Until now, only preliminary studies have been reported [81,82].

1.4 Outline of this thesis

In this thesis, both applied and fundamental aspects of „enzymes in re-
versed micelles" are presented.
Chapter 2 and 3 show possible ways to produce reducing equivalents in reversed
micellar media.
In chapter 2, a photochemical system enables vectorial transport of electrons
and protons from a donor in the continuous oil phase to the waterpool. In this
model system hydrogenase consumes the protons and electrons, resulting in the
production of hydrogen gas.
Chapter 3 describes a combined enzyme system for the conversion of apolar ste-
roids in reversed micellar media. In this system, hydrogen gas is used as the
source of reducing equivalents.
Chapter 4 provides some more insight in factors that regulate the rate of enzym-
atic conversion of apolar steroids in reversed micelles. It is shown that the
hydrophobicity of the interphase and continuous phase, as compared to the hydro-
phobicity of the substrate, determine the local substrate concentration and
thus enzyme activity. In Chapter 5 some aspects of micellar enzyme kinetics are
discussed and a kinetic model is presented for enzymatic reactions with apolar
compounds in reversed micellar media.
To illustrate the potential of reversed micellar systems, the patent applica-
tion that was a consequence of this work, is added as an appendix.

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2. PHOTOSENSITIZED PRODUCTION OF HYDROGEN BY HYDROGENASE IN REVERSED MICELLES

(solar energy)

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ABSTRACT   Hydrogenase (hydrogen:ferricytochrome c_3 oxidoreductase, EC 1.12.2.1) from *Desulfovibrio vulgaris* was encapsulated in reversed micelles with cetyltrimethylammonium bromide as surfactant and a chloroform/octane mixture as solvent. Reducing equivalents for hydrogenase-catalyzed hydrogen production were provided by vectorial photosensitized electron transfer from a donor (thiophenol) in the organic phase through a surfactant–Ru^{2+} sensitizer located in the interphase to methyl viologen concentrated in the aqueous core of the reversed micelle. The results show that reversed micelles provide a microenvironment that (*i*) stabilizes hydrogenase against inactivation and (*ii*) allows an efficient vectorial photosensitized electron and proton flow from the organic phase to hydrogenase in the aqueous phase.

It has been well established that surfactant molecules dissolved in organic solvents aggregate to reversed micelles in the presence of small amounts of water. Reversed micelles are of multiple interest for they create a microenvironment that provides a unique reaction medium.

An area of active current research is the photochemical investigation of these organized structures with the aim of obtaining structural characteristics (1–3) and of modeling natural processes such as photosynthesis (4–6). The latter objective is of particular interest because it includes potential applications such as solar energy conversion and storage. Essential for efficient solar energy conversion and storage is the separation of photoproducts formed in photosensitized electron transfer reactions. Recently, Willner *et al.* (5) showed that effective separation of photoproducts can be achieved in a reversed micellar system by vectorial photosensitized electron transfer from a donor in the organic phase to an acceptor in the water pool and vice versa.

In addition, the application of reversed micelles in enzyme catalysis increases. Reversed micelles have been shown to provide a microenvironment for enzymes that protects them from the unfavorable action of organic solvents by means of surfactants. Hence, the study of structural and catalytic properties of enzymes can be extended to organic media. To date, several enzymes, such as trypsin, α-chymotrypsin, lactate dehydrogenase, peroxidase, and lysozyme have been encapsulated in the aqueous core of reversed micelles (7–9).

In this study we entrapped hydrogenase (hydrogen:ferricytochrome c_3 oxidoreductase, EC 1.12.2.1) from *Desulfovibrio vulgaris* in a reversed cetyltrimethylammonium bromide micelle; the objective was to obtain a highly organized system for an efficient coupling between hydrogenase and a photochemical system that produces reducing equivalents and protons necessary for hydrogenase action.

MATERIALS AND METHODS

Chemicals. Cetyltrimethylammonium bromide was supplied by Baker; thiophenol, by Aldrich; methyl viologen, by Sigma; and octane and chloroform, by Merck. The photosensitizers tris(2,2'-bipyridine)ruthenium(II) [Ru(bipy)$_3^{2+}$], zinc tetraphenylporphyrin (ZnPh$_4$Por), and zinc tetra-*p*-sulfonatophenylporphyrin [Zn(SPh)$_4$Por^{4-}] were from Strem Chemical Company (Newburyport, MA); [*N,N'*-di(1-hexadecyl)-2,2'-bipyridine-4,4'-dicarboxamide]-bis(2,2'-bipyridine)ruthenium(II) (surfactant-Ru^{2+} complex), 1,1'-diheptyl-4,4'-bipyridinium dibromide (heptyl viologen), and 1-octadecyl-1'-propylsulfonate-4,4'-bipyridinium bromide were gifts of M. Calvin.

Hydrogenase. Hydrogenase from *Desulfovibrio vulgaris* strain Hildenborough NCIB 8303 was purified as described by Van der Westen *et al.* (10). The preparation used in this paper was a side fraction of the hydroxylapatite column and contained 230 μg of protein per ml and 156 units/ml as determined manometrically by the standard hydrogen production assay of Chen and Mortenson (11), with dithionite as electron donor and methyl viologen as electron carrier.

Preparation of Reversed Micelles. Reversed micelles were prepared by injecting 240 μl of an aqueous solution into 3 ml of a Vortex-stirred 0.3 M solution of cetyltrimethylammonium bromide in chloroform/octane, 6:5 (vol/vol). Stirring was continued until the solution became clear. The aqueous solution contained hydrogenase (78 units/ml) in 50 mM Tris·HCl, pH 8.0/10 mM methyl viologen, unless stated otherwise in the text; pH 8.0 was found to be optimal for light-driven hydrogen production because hydrogenase activity decreases with increasing pH, whereas the rate of electron transport to methyl viologen increases. The photosensitizers were added to a final concentration of 50 μM with respect to the total volume. At this concentration, more than 90% of the incident light is absorbed. After the addition of the electron donor thiophenol (final concentration, 0.1 M), the solution was deaerated by six cycles of 30-sec evacuation/15-sec flushing with scrubbed argon.

Illumination. The micellar solution was placed in a Cary 14 spectrophotometer supplemented with a side-illuminator. The sample holder was irradiated with blue light (420–480 nm) with a 150-W Xenon lamp (Varian, VIX-150F), a cupric sulfate solution, and a band-pass filter K45 (Balzers). The temperature was 30°C, and the incident photon flux was 1.5×10^{-5} einstein·min^{-1} as determined by Reinecke salt actinometry (12). During illumination, the production of methyl viologen radical

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(MeV‡) was monitored at 602 nm; 13,600 M^{-1}cm^{-1} was used as the extinction coefficient at 602 nm (13). The quantum yield for hydrogen production was calculated by dividing the maximum rate of hydrogen production by the flux of light quanta absorbed (φ_{H_2}).

Miscellaneous. Hydrogen production was determined by gas chromatography (Pye Unicam GCD chromatograph equipped with a catharometer detector device). The Fe^{2+}/S^{2-} solution (10 mM sodium citrate/1.5 mM (NH$_4$)$_2$Fe(SO$_4$)$_2$/1.5 mM Na$_2$S/ 50 mM Tris·HCl, pH 8.0) was prepared anaerobically. The stock solution was diluted until a final concentration of 0.25 mM Fe^{2+} and 0.25 mM S^{2-} was obtained in the water pools of the reversed micellar system.

RESULTS AND DISCUSSION

Several authors have reported that enzymes can be dissolved in organic solvents with the aid of surfactants while retaining their activity (7–9). According to recent models (14, 15), the protein is confined to the water pool in the reversed micelle. The polar heads of the surfactant molecules are directed towards the inside of the micelle. A water layer, its thickness depending on the water content of the system, separates the protein from the surfactant molecules, thus preventing its denaturation.

Here we solubilized hydrogenase from *Desulfovibrio vulgaris* in a chloroform/octane mixture with cetyltrimethylammonium bromide as surfactant. Introductory experiments revealed that the rate of hydrogen production in this reversed micellar system, as measured in the standard assay with dithionite as electron donor and methyl viologen as electron carrier, is the same as in bulk water. This is in agreement with the findings of Martinek *et al.* (7) and Barbaric and Luisi (9), who reported that the activity of enzymes in reversed micelles remains the same or is sometimes even enhanced.

A schematic representation of hydrogenase entrapped in a cetyltrimethylammonium bromide-reversed micelle is shown in Fig. 1. Also shown is a photochemical system used to generate reducing equivalents with a sufficiently low potential for hydrogen production. The components of this system were chosen in such a way that an efficient photosensitized electron transfer was obtained from a donor in the bulk organic phase to hydrogenase in the aqueous pool. An efficient combination proved to be thiophenol as electron donor, a surfactant–Ru^{2+} complex as photosensitizer, and methyl viologen as electron acceptor in the aqueous phase. In the absence of hydrogenase, illumination

of this photochemical system results in the formation of MeV‡ as measured by its absorption at 602 nm (see Fig. 3, curve d). This can be rationalized as follows. The surfactant–Ru^{2+} complex is located in the interphase and, upon illumination, transfers an electron to methyl viologen that is concentrated in the aqueous phase. Consequently, the photoproducts are confined to different phases, so back-electron-transfer reactions of the intermediate photoproducts are hindered. The resulting surfactant–Ru^{2+} complex is reduced by thiophenol and, thus, the sensitizer is recycled. Evidence for the localization of the surfactant–Ru^{2+} complex at the water/oil boundary was obtained by absorption spectroscopy (Fig. 2). Ru(bipy)$_3^{2+}$ derivatives have a marked solvent dependency of their optical absorption and emission spectra (16). Consequently, the polarity of the environment of the chromophore could be assessed from the shape of the spectrum. The absorption spectrum observed in our reversed micellar system shows the characteristics of a chromophore in an environment of intermediate polarity. Therefore, we conclude that the surfactant–Ru^{2+} complex is located in the interphase.

Some other sensitizers have been tested for their ability to transfer electrons from thiophenol in the organic phase to methyl viologen in the aqueous phase (Fig. 3). The results clearly indicate that the rate of viologen reduction with a sensitizer located in the interphase is enhanced 5-fold compared to a sensitizer located in the organic phase [ZnPh$_4$Por] and 3-fold compared to those located in the aqueous pool [Ru-(bipy)$_3^{2+}$ and Zn(SPh)$_4$Por^{4-}]. The higher rate of viologen reduction obtained with the water-soluble sensitizers Zn(SPh)$_4$-Por^{4-} and Ru(bipy)$_3^{2+}$ compared to ZnPh$_4$Por might be explained by the fact that thiophenol is able to penetrate into the interphase and to come into contact with the aqueous interior of the micelle. Recently, Willner *et al.* (6) observed viol-
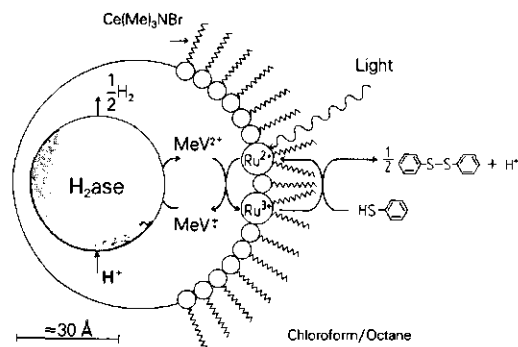

FIG. 1. Scheme for photosensitized production of hydrogen by hydrogenase in a reversed micelle. The diameter of the micelle is estimated to be about 60 Å from literature data. MeV^{2+}/MeV‡, methyl viologen redox couple; Ru^{2+} and Ru^{3+}, reduced and oxidized form of the surfactant sensitizer, respectively; Ce(Me)$_3$NBr, cetyltrimethylammonium bromide.
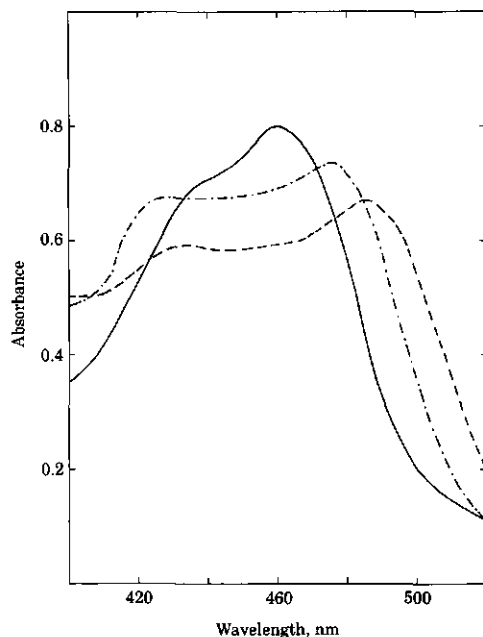

FIG. 2. Absorption spectra of surfactant–Ru^{2+} complex dissolved in chloroform (——), in a cetyltrimethylammonium bromide/chloroform/octane micellar system (–·–), and in an aqueous detergent solution (– – –).

Table 1. Quantum yield for hydrogenase-catalyzed hydrogen formation with different sensitizers

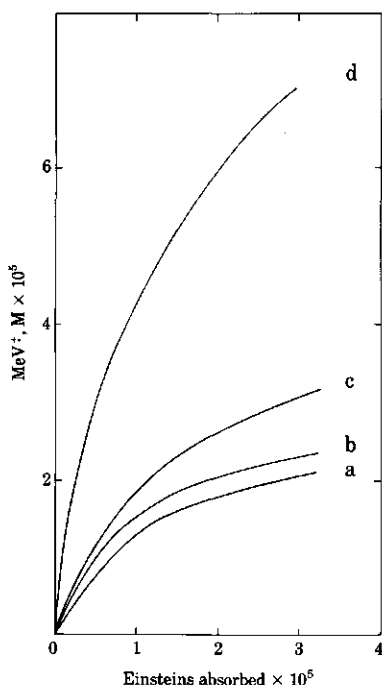| Pigment	Localization	φ_{H_2}, %
$ZnPh_4Por$	Organic phase	0.26
$Zn(SPh)_4Por^{4-}$	Aqueous phase	0.41
$Ru(bipy)_3^{2+}$	Aqueous phase	0.43
Surfactant–Ru^{2+} complex	Interphase	1.34

FIG. 3. Effect of different photosensitizers on the rate of methyl viologen reduction. Experiments were performed without hydrogenase as described. The concentration of the sensitizers was such that >90% of the incident light was absorbed. Curves: a, $ZnPh_4Por$; b, $Zn(SPh)_4Por^{4-}$; c, $Ru(bipy)_3^{2+}$; and d, surfactant–Ru^{2+} complex.

ogen reduction with the water-soluble sensitizers $Ru(bipy)_3^{2+}$ and $Zn(SPh)_4Por^{4-}$ in a system consisting of water droplets stabilized by dodecylammonium propionate in toluene. We expect that, as in our system, a significant improvement of the rate of viologen reduction will be observed when an interfacial sensitizer is used instead of a water-soluble one.

When hydrogenase was present in the aqueous interior of the micelle, methyl viologen was recycled and hydrogen was produced in half-molar amounts. In the absence of methyl viologen, no hydrogen could be detected. The maximum initial rate of hydrogen production in the complete system was 0.5 ml·min^{-1} per mg of hydrogenase. The stoichiometry of the overall reaction is such that all protons that are liberated during oxidation of the donor are consumed during the hydrogenase-catalyzed production of hydrogen, provided that an efficient proton transfer from the organic phase to the aqueous phase occurs. In Table 1 the quantum yields for hydrogen production are listed for the different sensitizers. The maximum quantum yield obtained was 1.3%.

The oxidation of MeV^{+} by hydrogenase was very efficient because hardly any MeV^{+}, as measured by its absorbance at 602 nm, could be detected when the complete system was illuminated. Usually photochemical systems that produce hydrogen through electron carriers, such as viologen, turn deep blue upon illumination. This blue color scavenges light away from the sensitizer, resulting in a drop of the efficiency of the system. This is not the case in our reversed micellar system containing hydrogenase, probably due to the organization and the flexibility of the system. As was suggested by Menger *et al.* (17), reversed micelles are not rigid but are dynamic entities that are able to

exchange their contents at a time scale of $1{-}10 \times 10^{-7}$ sec (18–20). This implies that the surfactant–Ru^{2+} complex can also be quenched by other methyl viologens than those located in the same reversed micelle and that virtually all MeV^{+} is available as a substrate for hydrogenase. In literature (15), the aggregation number for reversed micelles of ionic surfactants ranges from 300 to 1,200. This implies that routinely one hydrogenase, ≈600 surfactant–Ru^{2+} complexes, and ≈20,000 MeV^{2+} were present per 3,000–10,000 micelles. Therefore, the observation that, under these circumstances, hardly any MeV^{+} could be detected during steady-state illumination indicates that the photochemical components of the system are rapidly exchangeable.

We tried to increase the quantum yield for hydrogen production by using viologen derivatives that are known to be more efficient in quenching the excited state of the surfactant–Ru^{2+} complex than methyl viologen (21). For this purpose, the surfactant viologens 1,1'-diheptyl-4,4'-bipyridinium dibromide and 1-octadecyl-1'-propylsulfonate-4-4'-bipyridinium bromide were used. In both cases, the rate of viologen reduction was enhanced ≈6-fold as compared to methyl viologen. However, in the presence of hydrogenase, no hydrogen was produced. A reasonable explanation for this phenomenon is that these surfactant viologens become apolar upon reduction and, therefore, are extracted into the continuous organic phase. Attempts to direct the electron flow in the opposite direction to the aqueous phase by adding methyl viologen to the system were unsuccessful. Apparently the rate of extraction into the organic phase is rapid compared to the rate of electron transfer to methyl viologen.

Beside thiophenol, other donors [mainly tertiary amines such as tri(alkyl)amines and di(alkyl)anilines] were tested for their ability to donate electrons from the organic phase to the oxidized sensitizer located in the interphase. Unfortunately, none of them were active. As pointed out by Willner *et al.* (6), this might be attributed to the unique properties of thiophenol: (*i*) its slightly polar nature allows it to enter the water/oil interphase and (*ii*) thiophenol becomes more apolar upon oxidation (diphenyldisulfide) and will be extracted into the bulk organic phase. Consequently, separation of photoproducts is achieved, and back-electron-transfer reactions are hindered.

Another important feature is that the enzyme stability in reversed micelles is sometimes greater than in aqueous solution (7, 9). This was also the case in our system with hydrogenase from *D. vulgaris*. In an aqueous medium, hydrogenase loses 90% of its activity as measured by the standard assay with dithionite and methyl viologen after 10 days of storage in air at 22°C (10). Under similar circumstances hydrogenase in a reversed micelle had lost only 20% of its activity (Table 2). This shows that entrapment in the aqueous core of a reversed micelle is more favorable for hydrogenase than freedom in aqueous solution. Apparently reversed micelles provide a microenvironment for hydrogenase that stabilizes its hydrogen-producing activity against inactivation. Table 2 further shows that the rate of photosensitized hydrogen production is less than the dithionite activity. This clearly indicates that, under our experi-

Table 2. Effect of storage on the activity of hydrogenase encapsulated in reversed micelles

Storage time, days	Hydrogen production, %	
	Light driven	Dithionite driven
0	30 ± 4	100
3	30	100 ± 20
10	33	80
24	30	68

Hydrogenase (150 μg of protein per ml) in 50 mM Tris·HCl (pH 8.0) was injected into 0.3 M cetyltrimethylammonium bromide in a chloroform/octane mixture until 6% (vol/vol) water content was attained. The tubes were sealed with Suba seals and stored in the dark at 22°C under air. Before measuring light-driven hydrogen production, MeV^{2+}, Zn(SPh)$_x$Por^{4-}, and thiophenol were added to final concentrations. Afterwards dithionite was added to the system. The dithionite-driven activity at $t = 0$ is 100%.

mental conditions, the capacity of the light system limits the rate of hydrogen production.

Furthermore, it should be mentioned that the presence of thiophenol in the solution during storage resulted in a complete loss of activity within 1 day. Sulfur compounds like thiophenol are known to damage the iron-sulfur clusters of hydrogenase (22). A significant protection against inactivation by thiophenol could be offered by adding a solution of chelated Fe^{2+}/S^{2-} to the system. After 4 days of storage in the air at 22°C, only 50% of the activity was lost.

The stability of the system during illumination is another point of interest. Under continuous irradiation with visible light, hydrogen was produced in the complete system for over 16 hr (Fig. 4, curve a). This indicates that hydrogenase is provided with sufficient protons from thiophenol during catalysis.
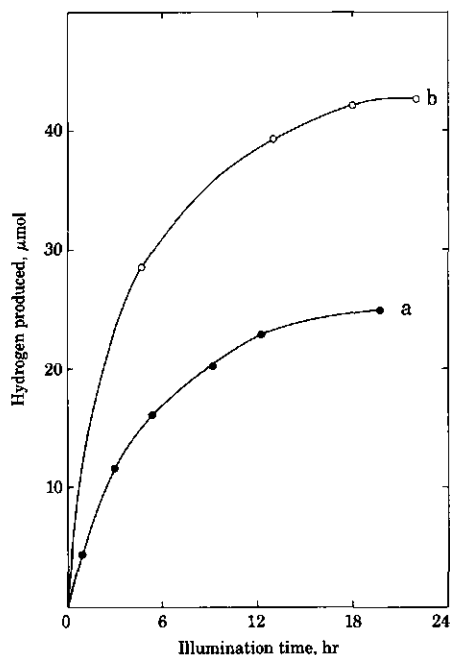

FIG. 4. Time course of photosensitized hydrogen production. Experiments were performed with the surfactant–Ru^{2+} complex as photosensitizer. Curves: a, complete system; b, Fe^{2-}/S^{2-} added.

If not, hydrogen production in this system would have ceased shortly after the onset of the photochemical reactions due to depletion of protons in the aqueous core. Hence, upon illumination, both protons and electrons from thiophenol are transferred from the organic phase to hydrogenase in the aqueous phase. However, the rate of hydrogen production gradually decreased in time. After 18 hr of continuous illumination, neither flushing with argon to remove the hydrogen produced nor addition of dithionite to the system resulted in renewal of hydrogen production. This indicates that hydrogenase was inactivated. In the presence of Fe^{2+} and S^{2-}, higher rates were observed (Fig. 4, curve b). The maximum rate thus obtained was 0.7 ml·min^{-1} per mg of hydrogenase with a quantum yield of 2%. However, no long-term stabilization was achieved.

In conclusion, reversed micelles provide a microenvironment that (i) stabilizes hydrogenase as compared to an aqueous medium and (ii) allows efficient coupling between hydrogenase and a photochemical system that produces reducing equivalents for hydrogenase. As a result, a relatively efficient ($\varphi_{H_2} = 1.3\% - 2.0\%$) light-driven system that produces hydrogen in an organic medium has been obtained.

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3. ENZYMATIC CONVERSION OF APOLAR COMPOUNDS IN ORGANIC MEDIA USING AN NADH-REGENERATING SYSTEM AND DIHYDROGEN AS REDUCTANT

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A combined enzyme system, consisting of hydrogenase, lipoamide dehydrogenase and 20β-hydroxysteroid dehydrogenase has been enclosed in reversed micelles. This system catalyzes the stereo- and site-specific enzymatic reduction of apolar, poorly water-soluble ketosteroids to their corresponding 20β-hydroxyform using an in situ NADH-regenerating enzyme system and H_2 as ultimate reductant.

Enzymatic steriod conversion   Reversed micelle   NADH regeneration

1. INTRODUCTION

Enzymes possess several properties that make them suitable as catalysts in synthetic organic chemistry. They catalyze, under mild conditions, site- and/or stereo-specific reactions that are otherwise difficult or impossible to perform and furthermore, produce pure compounds. Despite these obvious merits, the application of enzymes to organic synthesis is seriously limited by two factors.

Firstly, enzymes preferentially function in aqueous environments, while in case of apolar compounds chemical reactions are carried out in non-polar solvents that are usually harmful for enzymes. Several approaches to overcome this problem have been reported. Enzymatic reactions have been carried out at the interface of a two-phase system, one of the phases being water, the other a water-immiscible solvent. Furthermore, mixtures of water and water-miscible solvents have been studied [1-3]. To protect enzymes against inactivation by the organic solvent in these systems, they have been immobilized in gels or on solid supports [4-7]. A relatively new approach is entrapment of enzymes in reversed micelles [8-12].

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Secondly, certain classes of enzymic reactions of general interest involve cofactors which are expensive and are consumed during catalysis. Hence, the cofactor has to be regenerated from inexpensive reagents to make an enzymic process economically feasible. To date, several cofactor regenerating systems have been described, but only for aqueous media [13-18].

This paper describes an NADH-regenerating system that functions in a reversed micellar medium in combination with an enzyme that uses NADH for the conversion of an apolar steroid.

2. MATERIALS AND METHODS

2.1. Chemicals

Cetyltrimethylammonium bromide (CTAB) was from Serva; steroids, methyl viologen (MV^{2+}) and NAD^+ from Sigma and all organic solvents from Merck.

2.2. Enzymes

Hydrogenase (H_2ase, EC 1.12.2.1.) from *Desulfovibrio vulgaris* strain Hildenborough NCIB 8303 was purified as in [19] Pigheart lipoamide dehydrogenase (lipDH, EC 1.6.4.3.) and 20β-hydroxysteroid dehydrogenase (HSDH, EC

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4. RULES FOR THE REGULATION OF ENZYME ACTIVITY IN REVERSED MICELLES, ILLUSTRATED BY THE CONVERSION OF APOLAR STEROIDS BY 20ß-HYDROXYSTEROID DEHYDROGENASE

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4.1 Abstract

20ß-Hydroxysteroid dehydrogenase was enclosed in reversed micellar media consisting of cetyltrimethylammonium bromide (CTAB), hexanol, organic solvent and HEPES buffer. The influence of the composition of these media on the enzymatic reduction of the apolar steroids progesterone and prednisone was investigated by varying the water content, concentration of hexanol and type of organic solvent. By changing the water content and the type of organic solvent, the hexanol to CTAB ratio in the interphase can be varied. This ratio was determined by phase boundary titrations. It was found that the higher this ratio, the higher the rate of steroid conversion. From variations of the hexanol content it was concluded that the rate of steroid conversion is determined by the hydrophobicity of the steroid relative to the hydrophobicity of the continuous phase and the hydrophobicity of the interphase. The hydrophobicity of the phases was expressed in log P-values. This enabled us to derive the following relations between the hydrophobicity values for the substrate ($\log P_s$), for the interphase ($\log P_i$) and for the continuous phase ($\log P_{cph}$): $|\log P_i - \log P_s|$ must be minimal to ensure a high steroid concentration in the interphase and $|\log P_{cph} - \log P_s|$ must be large to keep the steroid concentration in the continuous phase low. With these considerations, for any given apolar compound, a medium can be composed that gives optimal enzymatic conversion.

4.2 Introduction

Reversed micelles (or water-in-oil-microemulsions) have attracted a lot of attention from physical chemists during the last forty years. This was not only because reversed micelles are interesting as scientific objects, but also because of their potential application in tertiairy oil recovery. It has resulted

solution vs. the volume of organic solvent, the slope represents the amount of hexanol in the continuous phase and the intercept on the Y-axis represents the amount of hexanol in the interphase and in the water pool. But, as hexanol is very poorly soluble in water, the latter amount is neglegible.

A drawback of this method is that it makes use of a phase transition. This implies that information about the composition of an interphase can be obtained at a phase boundary only, and not in the middle of the reversed micellar area of the phase diagram.

The sum of the amount of hexanol in the interphase and the amount in the continuous phase gives the minimal amount needed for the formation of a clear reversed micellar solution. This allows the preparation of a solution with a known composition of both the interphase and the continuous phase.

Preparation of a reversed micellar solution.

Reversed micellar solutions were prepared by injecting the appropriate amount of 50 mM HEPES, pH 7.6, in a 0.2 M solution of CTAB with respect to the organic solvent, containing the desired amount of hexanol.
A typical solution of w_0 = 10 contained 54 μl 50 mM HEPES, pH 7.6, 0.11 g CTAB, 1.5 ml octane and 0.16 ml hexanol.

Determination of HSDH-activity.

Prior to use HSDH was diluted to a concentration of 1.8-2.2 mg protein/ml and dialyzed to remove ammoniumsulphate. 30 μl of a freshly prepared 50 mM solution of steroid in chloroform was pipetted into a cuvette. After evaporation of the chloroform, 1.5 ml of a micellar solution was added together with 5 μl 25 mM NADH in 50 mM HEPES pH 7.6. The reaction was started by the addition of 5 μl HSDH and monitored spectrofotometrically at 340 nm. The reaction and injection temperature was 25°C.

For the determination of the maximal enzyme activity under non-saturating conditions with respect to NADH (V'_{max}) in aqueous solutions of 50 mM HEPES, pH 7.6, the steroid concentration was varied, whereas the overall NADH concentration was kept constant at 83 μM.

Determination of partition coefficients.

Partition coefficients are defined as concentration of the solute in the organic phase divided by the concentration in the aqueous phase.

To determine the partition coefficients of prednisone and progesterone,

1 μmole of steroid was shaken overnight at 25°C in a two-phase system consisting of 1 ml alcohol and 1 ml 50 mM HEPES, pH 7.6. The steroid concentration in both phases was determined afterwards by HPLC (HP 1081 B Liquid Chromatograph), equipped with a 5 μ ODS column. Detection wavelength was 240 nm. The eluens consisted of acetonitril/water, 70:30 v/v for progesterone and 35:65 v/v for prednisone.

The partition coefficient for CTAB was determined in an octanol-water system, with 0.1 mM CTAB with respect to the water phase. The CTAB concentration in the water phase was determined with conductivity measurements (Conductometer TH 27, El Hama Instruments).

4.4 Results and discussion

Phase diagram

To gain insight into the way the composition of reversed micelles influences the activity of enzymes in their water pool, a physical picture of the system is desired. Such a reversed micellar system consists of at least three components that can be mixed in numerous ratio's; one yields a liquid crystalline phase, another micelles and a third one reversed micelles. A phase diagram is a helpful tool to see which ratio's of components allow the formation of reversed micelles.

The system employed in this study consists of four components, an aqueous solution, a water-immiscible organic solvent, cetyltrimethylammonium bromide (CTAB) as surfactant and the aliphatic alcohol hexanol as cosurfactant. To describe such a system, a quaternary phase diagram is needed. An often-used simplification to obtain a pseudo-ternary phase diagram is obtained when the ratio of two components is kept constant. This implies that two components can be varied freely, whereas the ratio of the other two is kept constant. Because the water and alcohol content of the system are the most interesting parameters to vary, when studying enzyme activity, the concentration of CTAB with respect to organic solvent was kept constant at 9.4% (w/w) (0.2 M). This allowed variation of both the water and hexanol content within the plane of the phase diagram.

The area in the phase diagram where reversed micelles are present was determined as described in Materials and Methods (Fig. 1). The corners of the triangle represent the "pure" compounds, i.e. buffer, 9.4% w/w CTAB in octane, and hexanol. The weight percentage of a component is plotted along the adjacent sides. Thus, on any line drawn parallel to the opposite site, the weight percentage of a component is constant.

Fig. 1. Pseudo-ternary phase diagram for the system CTAB, octane, hexanol and 50 mM HEPES pH 7.6. The phase diagram was determined as described in Materials and Methods. All quantities are expressed as % of total weight. The concentration of CTAB was 9.4% w/w with respect to octane (0.2 M). Keeping this amount constant allowed determination of enzyme activities in this plane of the phase diagram while varying water and hexanol content. The shaded area represents the area where equilibrium in the presence of prednisone or progesterone, NADH and 20β-hydroxysteroid hydrogenase was attained rapidly, <u>i.e.</u> within mixing time. In this area initial enzyme activities were measured. Furthermore, the lines 4.4% (w/w) H_2O (w_o = 10) and 10% (w/w) hexanol are drawn. Variation of the concentration along these lines allows the largest change of the water and hexanol concentration in the shaded area where enzyme activity was measured. o-o, area where reversed micelles are formed within 4 hours.

Fig. 1 shows an area in which the four-component solution was clear and stable. At low water and hexanol content clear solutions are established within a few seconds, but at higher water and/or hexanol content, the time needed to establish equilibrium can be several hours. As a consequence initial enzyme activities could only be measured in those solutions where equilibrium with steroid, NADH and HSDH present in the reversed micellar system, was attained within the mixing time. This area is also shown in Fig. 1. Furthermore, the lines 10% hexanol and 4.4% water are drawn in Fig. 1. Following the line 10% hexanol from the right to the left, the weight fraction of hexanol with respect to the CTAB-octane mixture remains constant at 10%, but the amount of water increases from 0% to 100%.

Similarly, along the 4.4% water line, the hexanol concentration is varied at a constant weight fraction of 4.4% water with respect to the CTAB-octane mixture, or, expressed differently, at a constant molar water to CTAB ratio of 10 (<u>i.e.</u> w_o = 10). In Fig. 1 it can be seen that these lines allow the widest variation of water and hexanol concentrations in the shaded area where HSDH activities were measured.

<u>Variation of w_o</u>

Most reversed micellar systems that have been studied consist of three components. In those systems, the properties of the interphase are determined by the surfactant. In quaternary systems, a fourth component, mostly an alcohol, is present. This cosurfactant is distributed between the continuous phase and the interphase. The properties of the interphase will not only depend on the surfactant, but also on the amount of cosurfactant.

The most obvious parameter to vary in reversed micellar media is the water content of the system, which is generally expressed as w_o, <u>i.e.</u> the molar H_2O/ CTAB ratio. Because the water content can influence the composition of the interphase, the molar ratio of hexanol to CTAB in the interphase (a_o) at the lower phase boundary was determined by the titration method for the 0.2 M CTAB in octane/hexanol/HEPES system with variation of w_o. The results are shown in Fig. 2A. It can be seen that a_o decreases slightly with increasing w_o, whereas the amount of hexanol in the continuous phase is hardly influenced by changes in w_o.
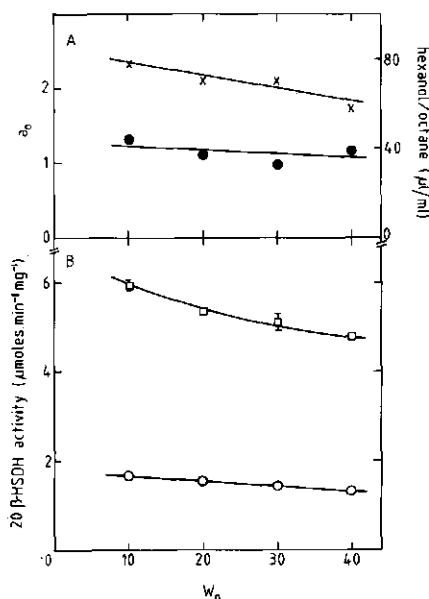

Fig. 2. The effect of variation of w_o on the composition of the continuous phase, on a_o, and on 20β-hydroxysteroid dehydrogenase activity.
Titration experiments and HSDH activity measurements were carried out as described in Materials and Methods, in systems containing CTAB in octane. Activity measurements were performed at the w_o indicated, in media containing the minimal amount of hexanol required to form reversed micelles.
A: x-x, a_o; •-•, hexanol/octane. B: ◻-◻, HSDH activity with prednisone, o-o, HSDH activity with progesterone.

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As described in Materials and Methods, these data were used to calculate the minimal amount of hexanol needed to obtain a reversed micellar solution that is just clear, i.e. very close to the lower phase boundary in the phase diagram. In such a solution the composition of the interphase is known, which makes it possible to study the effect of the composition of the interphase on enzyme activity.

The activity of HSDH was measured in micellar media containing the minimal amount of hexanol needed to obtain a clear solution. Prednisone and progesterone were used as substrates. The results are shown in Fig. 2B. Enzymatic activity with prednisone was about three times higher than with progesterone. In aqueous solutions the rate of conversion is about the same [19]. Determination of V'_{max} yielded for prednisone a value of 3.0 $\mu mol.min^{-1}.mg^{-1}$ and for progesterone 3.4 $\mu mol.min^{-1}.mg^{-1}$. This means that the activity of HSDH in reversed micelles is higher than in aqueous solutions. Similar observations have been reported for α-chymotrypsin [9,11,15]. Compared to progesterone, prednisone is much more polar and therefore the micellar concentration around the enzyme will be higher, resulting in different HSDH-activities. With both substrates, enzyme activity decreases slightly but significantly with increasing w_o. It could be envisaged that with increasing w_o, the size of the micelle increases [20] resulting in a larger distance between the enzyme and the interface. As a consequence the total steroid concentration around the enzyme might be lower and hence enzyme activity. Moreover, the amount of hexanol/CTAB in the interphase could be correlated with enzyme activity. The possibility that at higher w_o the cofactor concentration in the water pool becomes limiting because of a dilution effect can be ruled out, for a higher cofactor concentration does not yield higher enzyme activities.

Variation of hexanol concentration

The influence of a_o on enzyme activity was investigated further by varying the hexanol concentration in the system. To gain insight in the difference between a_o at the upper and the lower boundary, a_o had to be determined. However, as pointed out before, with the titration method the composition of an interphase can only be determined at a phase boundary. From Fig. 1 it can be seen that no phase transition at the upper boundary takes place at $w_o = 10$ (4.4% w/w water), which made it necessary to perform the titration at $w_o = 20$.

Application of the titration method at the lower phase boundary gives reliable results, because an almost instantaneous transition from turbid to clear takes place. Results at the upper phase boundary are less accurate because the

phase transition is a gradual event and, for the opalescent solutions obtained, the interpretation of the clear to turbid transition is very subjective. Even the application of light scattering methods did not lead to unequivocable results.

For a_0 at the upper phase boundary a value of approximately 6 was obtained. At the lower phase boundary, a_0 values were 2.3 for $w_0 = 10$ and 2.1 for $w_0 = 20$, making it clear that the amount of hexanol in the interphase increases with increasing alcohol content. At $w_0 = 20$, the amount of hexanol in the continuous phase increased from 37 to 146 $\mu l/ml$.

Although titrations were performed at $w_0 = 20$, enzyme activities were determined in 0.2 M CTAB in octane at $w_0 = 10$, because this allowed the investigation of a wider range of alcohol concentrations, as can be seen from Fig. 1.

Fig. 3 shows that HSDH activity decreases with increasing percentage of hexanol, $\underline{i.e.}$ increasing a_0, with both substrates. However, during the variation of w_0, a totally opposite relation between a_0 and enzyme activity was observed: in Fig. 2 a decrease in a_0 was accompanied by a decrease in enzyme activity.
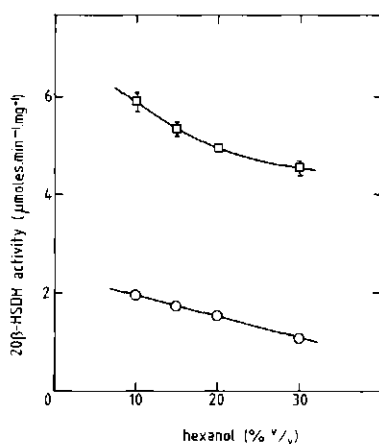

Fig. 3. The effect of variation of hexanol concentration on 20β-hydroxysteroid dehydrogenase activity.
Enzyme activities were measured in duplicate in reversed micellar medium consisting of 0.2 M CTAB in octane and $w_0 = 10$.
Other conditions were as described in Material and Methods.
□-□, HSDH-activity with prednisone; o-o, HSDH-activity with progesterone.

Upon variation of the hexanol concentration, the increase in a_0 could affect the substrate localization or influence the enzyme itself. A possible explanation is that an a_0 value of about 2 is optimal and that higher concentrations of hexanol in the interphase lead to inactivation of the enzyme.

This possibility was checked in the following way: micellar solutions containing different percentages of hexanol were prepared. By mixing equal volumes of two such solutions, a solution containing an intermediate concentration of hexanol can be prepared. Inactivation of HSDH in a solution with a high concentration of hexanol would result in a lower enzymatic activity after mixing such reversed micelles with a micellar solution with a lower alcohol content, compared to the control where the enzyme has not been exposed to a high hexanol concentration but resides in a micellar solution of the intermediate hexanol content. The results presented in Table I make clear that the enzyme activity depends on the hexanol content during the assay. The enzyme activity is not affected by the concentration of hexanol in the micelle during injection. These results exclude that irreversible inhibition of HSDH is the cause for lower enzyme activity at higher hexanol concentrations.

Table I. Effect of different hexanol concentrations on 20β-hydroxysteroid dehydrogenase activity. HSDH was injected into 0.75 ml of a CTAB-octane reversed micellar solution with w_0 = 10 at the hexanol concentration indicated. After 5 sec of mixing this solution was added to 0.75 ml of a reversed micellar solution, w_0 = 10, containing NADH (166 μM overall) and progesterone (2 mM overall) and the concentration of hexanol indicated, to bring the mixture to the desired final hexanol concentration. The enzyme was always injected into the solution containing the highest percentage of hexanol. As a control, enzyme activities were measured by adding the enzyme to a micellar solution with the final hexanol concentration of the mixture.

% hexanol during assay	prepared with	added to	HSDH-activity ($\mu mol.min^{-1}.mg^{-1}$)	
			sample	control
10%	10%	10%	2.38	2.45
15%	15%	15%	2.25	2.33
	20%	10%	2.15	
20%	20%	20%	1.85	1.85
	25%	15%	1.97	
	30%	10%	1.93	
25%	25%	25%	1.63	1.68
	30%	20%	1.57	
30%	30%	30%	1.37	1.28

Variation of organic solvent

To obtain more information on the effect of a_o on the activity of HSDH, the type of organic solvent was varied in the CTAB/hexanol/HEPES system. The system contained 0.2 M CTAB with respect to the organic phase and w_o was kept constant at 10. Determination of a_o at the lower phase boundary revealed that this parameter increases going from aromatic to aliphatic solvents with increasing chain length. The hexanol content in the continuous phase increased slightly. Also in these reversed micellar media with different organic solvents, HSDH activity was determined with progesterone and prednisone as substrate in systems of which the composition of the interphase is known. The data for a_o and the composition of the continuous phase were used to calculate the minimal amount of hexanol required to form a clear solution. The activity was plotted as a function of a_o (Fig. 4). For both substrates, activity increases with increasing a_o, although activity with prednisone levels off at high values of a_o.
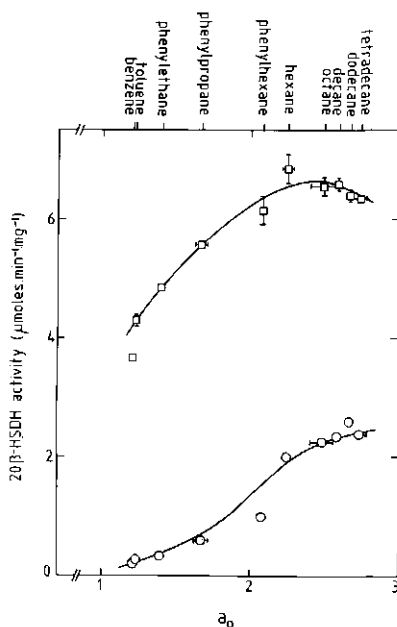

Fig. 4. 20β-hydroxysteroid dehydrogenase activity as a function of a_o at the lower phase boundary in reversed micellar media differing in organic solvent. HSDH-activities were measured in media containing 0.2 M CTAB with respect to organic solvent at w_o = 10. The amount of hexanol present was the minimal amount needed to obtain a clear solution and was calculated from the a_o value at the lower phase boundary and the composition of the continuous phase as described in Materials and Methods. Other conditions were as described in Materials and Methods.
□–□, HSDH-activity with prednisone; o–o, HSDH-activity with progesterone.

The data obtained with phenylhexane and hexane deviate from the curve. A possible cause is that these media differ in viscosity compared to the others [unpublished results].

With these media with different starting values of a_0, the effect of an increase of hexanol content on the enzyme activity was studied and related to a_0. In determinating the a_0 values for the upper phase boundary in several solvents, the same problem as described before for the CTAB/octane/hexanol/HEPES system, was encountered. For instance with toluene as organic solvent, no phase transition occurs at $w_0 = 10$, so a_0 at the upper phase boundary had to be determined at $w_0 = 15$. There, an a_0 value of 2.7 was found, whereas a_0 at the lower phase boundary was 1.2. This increase in a_0 was accompanied by an increase in the amount of hexanol in the continuous phase from 39 to 129 μl/mol. These results indicate that in systems with toluene or octane as organic solvent, both a_0 and the amount of hexanol in the continuous phase increase with increasing hexanol content.

In the CTAB/toluene/hexanol/HEPES system, HSDH-activity was measured at varying hexanol content with $w_0 = 10$. This w_0 value was adopted for the reasons mentioned before. Determination of HSDH-activity revealed a decrease in activity with increasing percentage of hexanol. Similar results were obtained with phenylpropane as organic solvent (Table II).

Table II. Effect of different hexanol concentrations on 20β-hydroxysteroid dehydrogenase activity in micellar media with 0.2 M CTAB in toluene, phenylpropane or octane as organic solvent. Enzyme activities were measured as described in Materials and Methods with $w_0 = 10$ and the amount of hexanol as indicated. $a_{o,lb}$ = a_0 at lower phase boundary, at $w_0 = 10$. $a_{o,ub}$ = a_0 at upper phase boundary, at w_0 indicated.

Organic solvent	% hexanol	HSDH activity ($\mu mol.min^{-1}.mg^{-1}$) prednisone	progesterone
toluene	6.7	4.30	0.30
$a_{o,lb}$ = 1.2 (w_0 = 10)	10	4.30	0.20
$a_{o,ub}$ = 2.7 (w_0 = 15)	15	3.90	0.18
	20	3.30	0.15
	30	2.27	0.06
phenylpropane	8	5.57	
$a_{o,lb}$ = 1.7 (w_0 = 10)	10	5.55	0.47
$a_{o,ub}$ = n.d.*	15	5.10	0.35
	20	4.90	0.28
	30	3.85	0.19
octane	10	5.90	1.99
$a_{o,lb}$ = 2.3 (w_0 = 10)	15	5.30	1.75
$a_{o,ub}$ = 6 (w_0 = 20)	20	4.97	1.56
	30	4.55	1.06

*
n.d. = not determined.

From the results presented here, it is clear that similar a_o and w_o values do not necessarily give equal enzyme activities. This implies firstly that the amount of hexanol in the interphase does not cause enzyme inhibition, neither reversible nor irreversible and secondly that not solely the composition of the interphase determines the rate of steroid conversion.

It can be concluded that the a_o value at the lower phase boundary determines the maximal enzymatic activity. The higher this value, the higher the activity. But, although for a given organic solvent, a higher hexanol content results in a higher a_o, this increase in a_o is accompanied by a decrease in enzyme activity.

Hydrofobicity

In a previous paper [17] we have remarked that changes in the composition of a reversed micellar medium influence the enzyme activity strongly. However, it could not be excluded that the enzyme _per se_ was affected by these micro-environmental changes. In the present study it is shown that variations in composition of the reversed micellar medium are accompanied by variations in the composition of the interphase, _i.e._ a_o. It is clear that the dualistic effect of a_o on the enzyme activity cannot be explained by inhibition of HSDH by the amount of hexanol present in the interphase. Furthermore, the cofactor supply does not become rate-limiting. These facts suggest that the steroid concentration in the micro-environment of the enzyme limits the rate of HSDH catalysis.

From the observations that neither water nor octane are good solvents for steroids, in contrast to hexanol, it can be concluded that the more hexanol is present in the interphase, the higher the local steroid concentration, and hence the higher HSDH activity.

This conclusion is supported by the positive correlation between the a_o value and enzyme activity, as found for the w_o-variation (Fig. 2) and between a_o of the lower phase boundary and enzyme activity as observed while varying the organic solvent (Fig. 4). Under these circumstances the composition of the continuous phase remains almost constant. This is not the case when the hexanol content of the system is varied. Here, the negative correlation between a_o and enzyme activity (Fig. 3) indicates that the composition of the continuous phase contributes considerably, but that the polarity of the organic solvent cannot be neglected either (Table II). To compare polarity of solvents, a measure is needed, and the hydrophobicity parameter appears to be a suitable one. Hydrophobicity can be expressed in log P values, _i.e._ the logarithm of the partition coefficient of a compound in the octanol- water two-phase system. The higher

its log P value, the more hydrophobic a compound is. Unknown log P values can be calculated from hydrophobic fragmental constants as determined by Rekker [21]. Rekker used experimentally determined partition coefficients of series of homologous compounds to calculate the effect of the addition of one functional group on the partition coefficient. This yielded hydrophobic fragmental constants. In such a way the log P value for any compound can be calculated by summation of the hydrophobic fragmental constants of its constituents.

Deviations between calculated and experimental values occur for more complicated structures like steroids. For these components, experimentally determined log P values were preferred. For prednisone we determined in an octanol-water two-phase system a value of 1.2 and for progesterone 2.8 (Table III),

Table III. Comparison of Rekker log P values for alcohols with experimentally determined partition coefficients for prednisone and progesterone in alcohol-water two-phase systems.
Partition coefficients were determined as described in Materials and Methods.

| alcohol | log P value[1] | log (partition coefficient) | |
		prednisone	progesterone
butanol	0.88	0.8	2.6
pentanol	1.40	1.5	2.7
hexanol	2.03	1.5	2.8
heptanol	2.59	1.4	2.8
octanol	3.15	1.2	2.8
decanol	3.98	1.1	2.8

1) Values calculated from hydrophobic fragmental constants as given by Rekker [21].

reflecting the more hydrophobic nature of the latter. Such differences in hydrophobicity will also cause differences in solubility. When studying the partition of these steroids over different alcohol-water systems, a correlation was found between the log P of the steroid (log P_s) and the log P of the alcohol in which the steroid was preferentially solubilized (Table III). Prednisone with a log P value of 1.2, solubilizes preferentially in those alcohols that have similar Rekker log P values. The partition coefficient in a butanol-water two-phase system will be too low, because of the solubility of butanol in water. Progesterone, with a log P value of 2.8 is preferentially localised in alcohols with related Rekker log P values like heptanol and octanol. From these observations it was concluded that in such systems similar log P values lead to a good mutual solubility. When log P values can be attributed to both the continuous phase and the interphase, information can be obtained about the localisation of

Comparison of log P values and HSDH activity. For A, conditions were as described for Fig. 2. For B, conditions were as described for Fig. 3 and Table II. For C, conditions were as described for Fig. 4. Unless stated otherwise, a_o and the composition of the continuous phase were determined at the lower phase boundary. In 50 mM Hepes pH 7.6, V'_{max} = 3.0 $\mu mol.min^{-1}.mg^{-1}$ for prednisone and 3.4 $\mu mol.min^{-1}.mg^{-1}$ for progesterone.

	Interphase			Continuous phase		HSDH activity ($\mu mol.min^{-1}.mg^{-1}$)	
	$\log P$[1]	a_o	$\log P_i$[2]	μl hexanol/ml	$\log P_{cph}$[3]	prednisone	progesterone
A. variation w_o							
$w_o = 10$		2.34	1.22	44	4.42	5.9	1.66
20		2.12	1.17	37	4.44	5.3	1.57
30		2.12	1.17	32	4.45	5.1	1.46
40		1.73	1.04	39	4.43	4.8	1.36
B. variation percentage hexanol							
toluene $a_{o,1b}$, $w_o = 10$		1.21[4]	0.82	39	2.61	4.30	0.30
$a_{o,ub}$, $w_o = 15$		2.7	1.31	129	2.56	2.27	0.06
octane $a_{o,1b}$, $w_o = 20$		2.12[4]	1.17	37	4.44	6.25	1.99
$a_{o,ub}$, $w_o = 20$		6.2	1.66	146	4.10	4.55	1.06
C. variation organic solvent							
benzene	2.06	1.21	0.81	39	2.06	3.67	0.19
toluene	2.63	1.23	0.82	40	2.61	4.30	0.25
phenylethane	3.16	1.40	0.90	48	3.11	4.85	0.36
phenylpropane	3.69	1.67	1.03	46	3.61	5.57	0.59
phenylhexane	5.27	2.08	1.16	53	5.02	6.15	1.00
hexane	3.50	2.25	1.19	40	3.44	6.85	2.00
octane	4.56	2.49	1.27	42	4.43	6.55	2.27
decane	5.62	2.59	1.28	43	5.37	6.60	2.35
dodecane	6.15	2.67	1.30	45	5.82	6.40	2.6
tetradecane	6.68	2.74	1.30	46	6.26	6.35	2.37
hexanol	2.03						
CTAB	-0.66						
prednisone	1.20						
progesterone	2.84						

1) log P for organic solvents and hexanol were calculated from hydrophobic fragmental constants [21].

2) $\log P_i = \dfrac{a_o}{a_o+1} \log P_{hexanol} + \dfrac{1}{a_o+1} \log P_{CTAB}$.

3) $\log P_{cph} = {}^oX_1 \log P_{hexanol} + (1-X_1) \log P_{organic\ solvent}$, with $X_1 = \dfrac{\mu l.M\ (hexanol)}{\mu l.M\ (organic\ solvent) + \mu l.M\ (hexanol)}$.

4) These values were used to estimate $\log P_i$ (for explanation see text).

the steroids within the reversed micellar system, by comparing the log P values.

In case of two compounds a log $P_{mixture}$ can be defined as the quotient of the concentrations in the octanol phase and the concentrations in the aqueous phase. This log $P_{mixture}$ can be calculated from log P values of the individual components with the formula log $P_{mixture} = X_1 \log P_1 + X_2 \log P_2$. X_1 and X_2 are the mole fractions of components 1 and 2 respectively. With this formula log P for the continuous phase can be calculated. For the organic solvents a log P can be derived from the hydrophobic fragmental constants. Attribution of a log P value to CTAB is more complicated. The value cannot be calculated and experimental determination has its drawbacks. In an octanol-water two-phase system, CTAB can either form micelles in the aqueous phase or reversed micelles in the organic phase. The first can be prevented by using concentrations below the critical micelle forming concentration, but the latter will occur. Even at low concentrations of surfactants aggregates are formed in organic solvents [1-3,22]. The aggregation phenomenon favours partition in the organic phase, resulting in too high a log P value. From partition experiments in an octanol-water two-phase system we obtained a value of 0.19.

As was mentioned before, small differences in log P indicate a good mutual solubility, provided no special type of interaction is favoured. As CTAB does not form aggregates in methanol and methanol is the best solvent for CTAB in the range methanol, ethanol etc., it seems reasonable to attribute to CTAB a log P value equal to the one for methanol, i.e. -0.66. The fact that this value is lower than the experimentally determined value is an indication that this approximation is acceptable.

With the formula given before log $P_{continuous\ phase}$ (= log P_{cph}) and log $P_{interphase}$ (= log P_i) can be calculated for the reversed micellar media of known composition. All data are listed in Table IV, as are the enzyme activities that were observed in those systems. Interpretation of the kinetic data is facilitated by this approach. From log P_i values it can be seen that the prednisone concentration in the interphase will be higher than the progesterone concentration, because of the smaller difference between the log P_i and log P (prednisone) values.

Variation of w_o results in a decrease in log P_i, whereas log P_{cph} remains constant. As the difference between log P_s and log P_i increases, the concentration of the steroids in the interphase will decrease, and so will the HSDH activity.

With respect to the variation of hexanol concentration, it has to be remarked again, that the data given in section B of Table IV do not reflect the

actual situation in the micellar solutions that were used to measure enzyme activities. But the tendency that a_o increases with increasing hexanol percentage will be true for all w_o values and also that the amount of hexanol in the continuous phase increases with increasing hexanol concentration. From the data in Table IVB it follows that the difference between $\log P_i$ and $\log P_{cph}$ decreases, which will result in a more even distribution of the steroids over the medium. Even though a_o has increased, less steroid is present in the interphase and hence the enzyme activity decreases.

When varying the organic solvent of the micellar system, an increase in a_o results in a relatively strong increase in enzyme activity. From Table IVC it becomes clear that both $\log P_i$ and $\log P_{cph}$ increase. Both effects favour a high steroid concentration in the interphase. Comparison of the curves of enzyme activity with prednisone and progesterone in Fig. 4, shows that the activity with prednisone levels off within the alkane series, whereas for progesterone activity continues to increase. Prednisone, with a log P of 1.2 shows maximal solubility in the interphase when $\log P_i$ is approximately 1.2, i.e. with hexane as organic solvent. From these results it can be concluded that a minimal $|\log P_i - \log P_s|$ will result in a maximal steroid concentration in the interphase. In addition, $|\log P_{cph} - \log P_s|$ must be large to keep the steroid concentration in the continuous phase as low as possible.
As can be seen in Table IVC, for progesterone $|\log P_i - \log P_s|$ continuous to decrease, while $|\log P_{cph} - \log P_s|$ continuous to increase. Both effects favour an increase in HSDH-activity, as can be seen from Fig. 4. For progesterone maximal activity is expected when $\log P_i = 2.8$, but in the systems that have been investigated, lower values were obtained. This can explain the lower activity with progesterone as compared to prednisone, in reversed micelles, whereas in aqueous solution equal activities are observed with those steroids.

For prednisone, Table IVC shows that $|\log P_i - \log P_s|$ increases for the alkanes longer than hexane. This influences the enzyme activity negatively. $|\log P_{cph} - \log P_s|$ increases, which stimulates enzyme activity. But the net result is that the enzyme activity decreases with longer alkanes in the continuous phase. This implies that the small differences in $\log P_i$ have a stronger effect than the increased hydrophobicity of the continuous phase.

Log P proves to be a very useful tool in analysing the effect of the composition of the interphase and continuous phase on the localisation of prednisone and progesterone and hence on the enzyme activity. It shows that HSDH activity is limited by the substrate available in its micro-environment. This concentra-
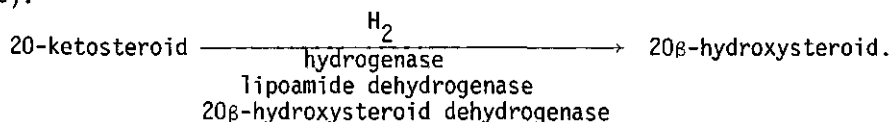
5. DISCUSSION

This thesis deals with the applied and fundamental aspects of enzymatic reactions in reversed micellar media. The reactions that are described are:
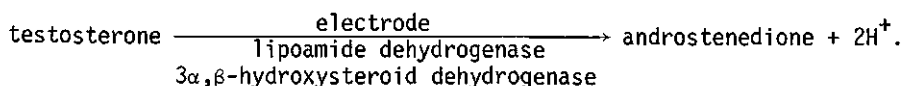
1. The photochemical production of H_2 via hydrogenase in CTAB/octane/chloroform reversed micelles (Chapter 2).

$$2 \text{ thiophenol} \xrightarrow[\text{hydrogenase}]{\text{light}} H_2 + \text{diphenyldisulfide}.$$
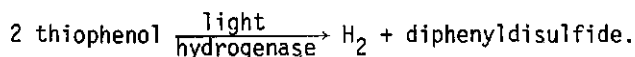
2. The reduction of apolar steroids by 20β-hydroxysteroid dehydrogenase using an NADH-regenerating enzyme system and H_2 as the ultimate reductant (Chapter 3).

$$20\text{-ketosteroid} \xrightarrow[\substack{\text{hydrogenase} \\ \text{lipoamide dehydrogenase} \\ 20\beta\text{-hydroxysteroid dehydrogenase}}]{H_2} 20\beta\text{-hydroxysteroid}.$$

3. The electrochemical oxidation of apolar steroids with 3α,β-hydroxysteroid dehydrogenase (Appendix).

$$\text{testosterone} \xrightarrow[\substack{\text{lipoamide dehydrogenase} \\ 3\alpha,\beta\text{-hydroxysteroid dehydrogenase}}]{\text{electrode}} \text{androstenedione} + 2H^+.$$

These reactions are catalysed by enzymes located in the water pools of the reversed micelles. In the preceding chapters, little attention has been paid to changes in the kinetic behaviour of enzymes in reversed micelles in relation to changes in the micro-environment imposed through enclosure in reversed micelles. These aspects will be discussed for HSDH in section 5.1.
For redox enzymes to function, transport of reducing equivalents to or from the enzyme is required. In this thesis, photo-, electro- and biochemical methods for the transport of reducing equivalents have been applied. In section 5.2 this aspect will be evaluated whereas in section 5.3 the conversion of apolar compounds will be discussed. In addition, the potential of reversed micelles for technological applications will be compared to other methods for the enzymatic conversion of apolar compounds.

5.1 Influence of the micro-environment on the kinetic behaviour of enzymes in reversed micelles

It has been reported that enclosure of enzymes in reversed micelles can

$K_{m,mic}$ = K_m expressed in micellar concentrations

Θ = volume fraction of reversed micelles

P_s = concentration in reversed micelles divided by concentration in continuous phase.

In this approach the reversed micellar medium is treated macroscopically as a two-phase system, implying that all water pools are in very fast exchange, and that the diffusion time from one pool to another can be neglected. It is assumed that the partition equilibrium is not affected during catalysis. This is only true when the rate of transfer of the substrate across the interphase is rapid compared with the rate of enzyme catalysis. Furthermore, Martinek et al. do not consider the substrate concentration in the interphase. However in Chapter 4 of this thesis it is shown that in reversed micelles, the concentration of progesterone in the interphase is a very important factor that dictates the rate of enzyme activity.

When the concentration in the interphase is taken into account and when it is not a priori assumed that the partition equilibrium does not change due to enzyme catalysis, the following expression for $K_{m,app}$ can be derived for a macroscopic two-phase system (see appendix 5.5 for the derivation):

$$K_{m,app} = K_{m,wph} \cdot \Psi \cdot \left\{ \frac{k_w k_2 + k_1 (V_{max} - v)}{k_i k_2} \right\} \qquad (8)$$

Ψ relates the substrate concentration in the interphase to the overall substrate concentration (eq. 21 in 5.5).

$K_{m,app}$ is a function of v, resulting in non-linear kinetics. If $v \approx V_{max}$ and $S_i \gg ES$, the relation simplifies to

$$K_{m,app} = K_{m,wph} \cdot \Psi \cdot \frac{k_w}{k_i} \qquad (9)$$

For HSDH catalysis in CTAB/octane/hexanol reversed micelles it can be envisaged that either the enzyme is able to convert the steroid at the interface or that the steroid has to diffuse out of the interphase into the water pool in order to be converted. In the first case the actual concentration of substrate reacting at the interface with the enzyme is higher than the overall concentration in the continuous phase, leading to apparently higher saturation of the enzyme. Assuming that the properties of HSDH do not change due to enclosure in a reversed micelle -an assumption that will be justified in section 5.1b- a decrease in $K_{m,app}$ would be expected, as compared to an aqueous medium.

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However, the $K_{m,app}$ for progesterone in reversed micelles is 400 μM, compared to 5-10 μM in an aqueous solution, so the enzyme must react with a lower progesterone concentration and hence HSDH-catalysis must take place in the water pool. From studies on HSDH-activity in two-phase systems Cremonesi et al. [9,13] concluded that in this system too the steroid has to diffuse into the water phase prior to conversion.

b. Changes in the intrinsic rate parameters of the enzyme

Studies on the influence of enclosure of enzymes in reversed micelles have revealed considerable differences between their kinetic behaviour is such media and in aqueous solution [1-8]. For α-chymotrypsin [4,6] and ribonuclease [5] the catalytic activity increases upon enclosure in reversed micelles. Furthermore, it has been observed that the pH optimum of several enzymatic reactions in aqueous solution differs from the optimum found in a reversed micellar medium [3,4,6,7]. Although the pH of the aqueous phase in a reversed micelle deviates from the pH of the original solution, evidence has been obtained that these changes are too small to account for the shift in pH optimum [4,11]. It has been suggested that changes in pK-values for groups at the active site are responsible for this behaviour, a hypothesis supported by studies with phenol red [4], bromocresol green and maleic acid [12] in reversed micelles, in which changes in pK have been observed. Another hypothesis for the changed kinetic behaviour is that the conformation of the enzyme is distorted by the enclosure in a reversed micelle. Circular dichroism, ultraviolet and fluorescence spectra were compared for enzymes in aqueous solutions and in reversed micelles [6-8]. The differences were too small to be conclusive. Luisi and Wolf [13] give the opinion: „It is likely that the conformational changes do not affect the active site but rather some remote domain".

The intrinsic rate parameters of an enzyme are influenced by several effects among which conformational effects and electrostatic effects are the most important. As the conformational effects that are observed upon enclosure of enzymes in reversed micelles are minor and do not seem to affect the active site, these factors are not considered further.

In this context, electrostatic effects include all effects connected with pH, ionic strength and electric charge. These effects are very difficult to study in reversed micelles, because neither pH nor ionic strength nor the surface potential at the interface are easily defined in the water pools. The problem of how the micro-environment influences the kinetic behaviour of an

enzyme is also encountered in the field of immobilized enzymes. The influence of electrostatic effects on the rate parameters of enzymes immobilized in charged matrices has been investigated [14-17]. It is of interest to draw a parallel with the behaviour of enzymes in reversed micelles, as in the water pools a high surface potential is present near the surfactant head groups.

In a series of very interesting papers, Goldstein and co-workers studied the effect of a high local charge density on the kinetic behaviour of α-chymotrypsin [14-17] with neutral substrates. Comparison of the results obtained in aqueous solution [14-17] with the data for α-chymotrypsin in reversed micelles [3,4,6,7] shows very interesting parallels. In their studies, Goldstein et al. used derivatives of α-chymotrypsin containing a covalently bound polyanionic or polycationic side chain. When studying the pH dependence of the enzyme activity, for the polyanionic derivative a shift of the pH-optimum to higher values was found, whereas for the polycationic derivative a shift in the opposite direction was observed. For the polyanionic derivative, protons are attracted by its negative charge, leading to a higher local proton concentration thus a lower local pH. This explains the shift of the optimum to higher pH values. Like all electrostatic effects, this is abolished at high ionic strength.

A similar shift of the pH-optimum to higher values was observed upon enclosure of α-chymotrypsin in reversed micelles of the anionic surfactant Aerosol OT [3,4,6], and this effect was less pronounced at high ionic strength [6]. The shift in pH optimum was explained as either arising from the influence of the surfactant head groups on the enzyme [3], or related to a change in pK of a group at the active site [4,6]. It can therefore be concluded that, in analogy with the results presented by Goldstein, the enzyme in an Aerosol OT reversed micelle will experience a lower local pH, reflecting itself in an apparently lower pH. optimum.

Furthermore, Goldstein reported a twofold increase in k_{cat}, the maximal turnover rate of the enzyme, for the polyanionic derivative with a neutral ester as a substrate [17]. With the same substrate, k_{cat} for α-chymotrypsin in Aerosol OT reversed micelles also increases twofold [4]. Barbaric and Luisi [6] reported a fivefold increase in k_{cat} in Aerosol OT reversed micelles with an anilide substrate, and this agrees very well with the almost fivefold increase reported by Goldstein for amide- and anilide substrates in water. The increase in k_{cat} is caused by the fact that a catalytically significant step in the catalysis of α-chymotrypsin involves two positively charged groups. This explains the decrease in activity that was observed with polycationic derivatives and the in-

crease observed for polyanionic derivatives. The differences between ester substrates and amide or anilide substrates stem from the fact that different steps in catalysis are rate limiting.

The agreements between the data of Goldstein and those presented for reversed micelles are striking and it can be concluded that mainly electrostatic effects are responsible for the observed changes in the kinetic behaviour of α-chymotrypsin.

It was investigated to what extent electrostatic effects influence the kinetic behaviour of HSDH in reversed micelles. Some preliminary results are reported here. First, a pH profile was determined. In aqueous solutions the optimum pH under non-saturating concentrations of NADH, is 6.3, but in CTAB/octane/hexanol reversed micelles HSDH catalysis is pH independent in the range 5.5-7.5, and the activity observed is the same as in an aqueous solution of pH 7. This implies that CTAB acts as a buffer around pH 7. From literature such effects are known and they are most pronounced for the circumstances used here, i.e. a low water content and a low buffer concentration [10,18,19]. These observations explain the difference in activity for HSDH between aqueous solutions and reversed micellar media that was reported in Chapter 4. In aqueous solution pH 7.6 is not optimal, whereas in reversed micelles the pH is shifted to approximately 7, closer to the optimum pH for HSDH.

At a saturating progesterone concentration, $K_{m,ov}$ for NADH and k_{cat} were determined in aqueous solution and in CTAB and Aerosol OT reversed micelles (Table I). Compared to an aqueous solution, k_{cat} increases in CTAB reversed micelles and decreases in Aerosol OT reversed micelles. For CTAB reversed micelles, the increase is not caused by a shift in pH of the water pool, for in aqueous solutions of pH 7.0 and 6.3 the same values for k_{cat} are obtained. Upon enclosure in reversed micelles, $K_{m,ov}$ for NADH increases slightly when CTAB is used as surfactant, but considerably with Aerosol OT. Considering that NADH is negatively charged and CTAB positively, it can be envisaged that NADH is associated with the interface, hence the effective concentration available to HSDH will be lower, resulting in an increase in $K_{m,ov}$. A possible explanation for the increase in $K_{m,ov}$ in Aerosol OT reversed micelles is the occurence of product inhibition by non-effective removal of NAD^+, due to binding to the Aerosol OT head groups in the micelle where it is produced by the enzyme. In CTAB reversed micelles the differences in $K_{m,ov}$ and k_{cat} decrease at high ionic strength indicating that the changes in k_{cat} and $K_{m,ov}$ are caused by electrostatic effects imposed by the charge of the head groups of the surfactant in the reversed micelle.

In conclusion it can be said that the intrinsic rate parameters of HSDH are hardly affected by enclosure in reversed micelles and that the enzyme ex-

Table I. Michaelis-Menten parameters for HSDH in aqueous and reversed micellar media. Experiments were performed in 50 mM Bistris, pH 6.3, or in reversed micellar solutions of 0.2 M CTAB in octane, 10% hexanol, w_o = 10, or 0.2 M Aerosol OT in octane, w_o = 10, containing 50 mM Bistris, pH 6.3 in the water pool. Similar solutions were prepared with 50 mM Bistris, pH 6.3 containing 500 mM NaCl. The progesterone concentration was kept constant at 50 μM in aqueous solution and 5 mM in reversed micellar medium. These values are at least ten times $K_{m,app}$ for progesterone in those media. Temperature was 25°C. K_m values are expressed in overall concentrations ($K_{m,ov}$).

medium	NaCl (mM)	$K_{m,ov}$ (μM)	k_{cat} (s^{-1})
Bistris, pH 6.3	-	3.6	10.9
Bistris, pH 6.3	500	3.4	6.2
CTAB reversed micelles	-	11.0	14.4
CTAB reversed micelles	500	3.7	7.6
AOT reversed micelles	-	60 *	5.5 *
AOT reversed micelles	500	nd	nd

* nd = not determined since NADH was converted spontaneously in these solutions.

periences an essentially aqueous environment. Furthermore, the kinetic behaviour of enzymes is influenced by the charge of the surfactant head groups. A careful choice of the surfactant can increase the intrinsic rate parameters of an enzyme and enhance the efficiency of enzyme catalysis.

5.2 Transport of reducing equivalents

For enzymatic redox reactions, reducing equivalents have to be transported to or from the compound that is to be converted, either directly or via one or more mediators such as NADH, electron-transfer proteins or viologens. In a reduction reaction the ultimate source of reducing equivalents is the electron donor, and for an oxidation reaction an electron acceptor is needed. To be applicable for practical purposes, the donor or acceptor must fulfill certain conditions: it must be cheap or yield a valuable product, it should not leave any harmful by-products, it should not interfere with the reaction or with the isolation procedure of the desired product and the supply during the course of the reaction must be sufficient.

At the time the investigation described in this thesis was started, many elegant systems transporting reducing equivalents to or from enzymes had been

designed (for a review see [20,21]), but none for reversed micellar media. In the Chapters 3, 4 and in the Appendix, three possible ways to transport reducing equivalents in reversed micellar media are described.

In Chapter 2 a photochemical system transports electrons and protons vectorially from a donor in the organic phase to a mediator in the water pool. The reducing equivalents thus generated yield hydrogen in the presence of hydrogenase. Other reactions can be envisaged as well. An interesting reaction in this respect is the recycling of NAD^+ or $NADP^+$. This requires the use of lipoamide dehydrogenase or ferredoxin- $NADP^+$ reductase instead of hydrogenase. In general the use of photochemical systems as a source for reducing equivalents has some disadvantages. Firstly, it is not reversible. This implies that different systems have to be devised for oxidation and reduction reactions. Secondly, an electron donor or acceptor has to be introduced into the system. In the photochemical process these compounds are often converted into harmful photoproducts that either contaminate the water pool or inactivate the enzyme(s) directly. Another drawback is that extra compounds in the medium could interfere with the isolation procedure of the desired product.

Such problems are avoided when the donor is consumed completely. This is the case with hydrogen gas, that only produces protons and electrons. An additional advantage of hydrogen gas is that it dissolves much better in organic solvents than in aqueous solutions. A system that generates reducing equivalents from hydrogen gas is described in Chapter 3. Here, the reducing equivalents from hydrogen are transported <u>via</u> NADH to 20β-hydroxysteroid dehydrogenase and used for the conversion of apolar steroids. A similar hydrogen consuming system has been described by Wong <u>et al</u>. [22] for aqueous media. In our system two enzymes are required for the regeneration of NAD^+. This can be reduced to one, when the hydrogenase from <u>Alcaligenes eutrophus</u> is used, which is able to reduce NAD^+ directly with hydrogen [23]. A restraint for the use of this enzyme is that the organism from which it is isolated needs hazardous growth conditions <u>e.g.</u> the simultaneous presence of O_2 and H_2. Other factors that limit the application of the hydrogen-driven system are the instability of hydrogenase in the presence of oxygen [24,25] and the fact that this system is only applicable for reduction reactions.

From this discussion it has become clear that both a photochemical and a biochemical system for the generation of reducing equivalents have some negative aspects. Yet reducing equivalents can also be generated electrochemically. A beneficial property of an electrochemical system is that the electrode potential can be adjusted to any desired value, whereas the potential that can be

obtained in the other systems depends on the nature of the sensitizer and the donor. Other advantages are that electricity is relatively cheap and that both oxidation and reduction reactions can be performed without adjustment of the system. However, an electrode produces or takes up only electrons, whereas both electrons and protons are involved in redox reactions. This problem can be solved by performing simultaneously an oxidation and a reduction reaction. The electrons that are generated at the oxidation site are pumped via an external circuit to the reduction site and the protons that are liberated during the oxidation are consumed during the reduction. An example of an oxidation reaction is given in the Patent Application (Appendix) and a combined system is shown in Fig. 1.
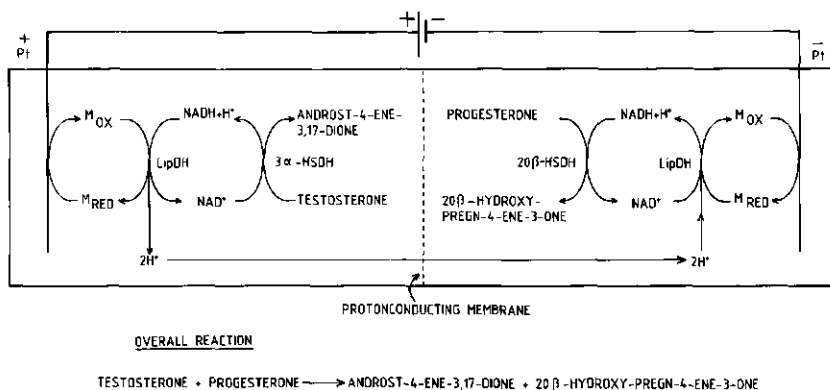

OVERALL REACTION

TESTOSTERONE + PROGESTERONE ⟶ ANDROST-4-ENE-3,17-DIONE + 20β-HYDROXY-PREGN-4-ENE-3-ONE

Fig. 1. Schematic representation of an electrochemical system for the oxidation and reduction of apolar steroids in a reversed micellar medium.

5.3. Enzymatic conversion of apolar compounds

Although most papers on enzymes in reversed micelles emphasize the potential of these media for the conversion of apolar compounds, only in three cases -apart from the one described in this thesis- successful conversion of apolar compounds has been achieved. Hanahan [26] and Misiorowski and Wells [27] reported the degradation of phospholipids that also served as surfactants. Furthermore Martinek reported the conversion of apolar alcohols by alcohol dehydrogenase [28,29] and a preliminary report on the oxidation of poly-unsaturated fatty acids by lipoxygenase has appeared [30]. In all these cases, only initial enzyme activities have been measured. For a system to be practically applicable, the reaction has to proceed for many hours.

Chapter 3 of this thesis describes the conversion of apolar steroids and shows that 20β-hydroxysteroid dehydrogenase is active for more than nine hours when encapsulated in reversed micelles. The gradual decline in activity appears to be caused by the inactivation of hydrogenase and not of 20β-hydroxysteroid dehydrogenase or lipoamide dehydrogenase. When performing long-lasting enzymatic reactions, not only enzyme stability is an important factor to consider, but in addition care should be taken that in the course of time the reaction rate does not decrease due to changes in pH or because of product accumulation in the water pools. The pH can change when hydrogen ions are produced or consumed and product accumulation occurs when the product is more polar than the substrate. In case the product is more apolar than the substrate the product diffuses away, resulting in a lower local concentration and hence a shift in the overall reaction equilibrium. Such effects are of major importance when the enzyme is subject to product inhibition or when the equilibrium lies to the substrate side. In our system, the 20β-hydroxysteroid that was produced, is slightly more apolar than the ketosteroid.

In the long-term experiments described in Chapter 3, the conditions were such that the rate limiting step was at the level of HSDH. It was observed that the rate of conversion depends on the composition of the medium. Chapter 4 deals with the relationship between the composition of a reversed micellar medium and the rate of conversion of apolar steroids. These results were generalised to guidelines that make it possible to predict the optimal composition of a reversed micellar medium for the conversion of a given apolar compound. Such guidelines will prove very valuable for the application of reversed micelles in technology, but can also be useful in membrane research.

5.4 Comparison of media for the enzymatic conversion of apolar compounds

Enclosure of enzymes in reversed micelles is one method for the conversion of apolar compounds. To date, several other methods have been described, most of which deal with enzymes in mixtures of water and water-miscible organic solvents. Such systems can never have a very apolar character and will therefore be less suitable for the conversion of highly water-insoluble compounds. Furthermore, it must be remarked that in general enzyme activity in such systems is lost and substrate specificity disappears when the percentage of water-miscible organic solvent exceeds 10-40%. However, in a few cases enzymatic conversions were possible at higher percentages. For example, immobilized trypsin

has been reported to retain its activity in solutions containing 90% ethanol or propanol [31], or 90% of a mixture of ethanol and glycerol [32]. For a more extensive discussion of such systems see references 33-36.

Here, attention will be focussed on those systems that consist for a major proportion of a non-water-miscible organic solvent. Advantages of such media for the conversion of apolar compounds are [35,36]:

1. the volume needed to perform the conversion is greatly reduced compared to aqueous solutions;
2. the recovery of product and enzyme is facilitated;
3. the reaction equilibrium can be shifted towards the desired direction;
4. other compounds can be used to replace water as a donor or acceptor during substrate conversion;
5. microbial contamination is avoided.

The design of such systems is not easy, for some conditions have to be fulfilled that are often contradictory. To make enzymes function in apolar media, a minimal amount of water is required [33] and the enzyme must be protected against denaturation caused by the organic solvent. Furthermore the substrate supply to the enzyme must not be rate-limiting. That these conditions are often contradictory can be illustrated with the studies carried out by the group of Cremonesi in a two-phase system [9,10,37]. In such a system the enzyme is located in the aqueous phase and the apolar compound in the organic phase. Diffusion of this compound into the aqueous phase is necessary for the reaction to proceed. Cremonesi et al. [10] showed that free 20β-HSDH activity was retained for at least 72 hrs in such an environment. The rate of conversion was very much dependent on the rate of shaking or stirring, indicating that the reaction rate is limited by diffusion of the substrate into the aqueous phase. This is to be expected with such a small interfacial area. At higher rates of stirring, enzyme activity decreases because of denaturation of HSDH. This can be prevented by immobilization, and it was shown that 20β-HSDH immobilized on Sepharose 4B retained 60% of its original activity after 60 days of continuous use in an ethylacetate-phosphate buffer two-phase system [37].

It should be realised however, that stabilization of enzymes by immobilization in a hydrophilic matrix imposes a barrier to the transport of apolar substrates. The larger the distance that apolar substrates have to cover, the slower conversion will be. In addition, the more apolar a substrate is, the more pronounced this effect will be. The diffusion problem can be reduced

either by reducing the distance that the substrate has to diffuse [38], or by using a more hydrophobic matrix [39]. However, the more hydrophobic a matrix is, the less water will be retained, making it more difficult for enzymes to function properly. In conclusion it can be said that for all systems a compromise between enzyme stability and substrate supply must be found.

In reversed micellar media, the enzymes are stabilized against denaturation by the organic solvent [1-8,28-30] and the amount of water present is sufficient to make them experience an aqueous environment. Furthermore, reversed micellar media possess an enormous interfacial area compared to all other systems, and the distance between the interface and the enzyme in the water pool is almost as small as possible, so the effect of diffusion limitation is greatly reduced. These properties give reversed micelles more potential for the conversion of apolar compounds, as is illustrated by the fact that Cremonesi et al. [10] did not observe any reduction of progesterone in their two-phase system, whereas it was readily converted in a reversed micellar medium.

Reversed micellar media are versatile and can easily be adapted to the special requirements imposed by a specific reaction, because their composition is easily varied. Furthermore, their dynamic nature enables an efficient contact between the constituents of a multi-enzyme system. These properties make enzymes in reversed micelles useful tools for the conversion of apolar compounds, since it seems that enzymes in such media work much more efficiently than in the other systems that have been designed.

In Chapter 3 a batch-like procedure for the isolation of the apolar product from a reversed micellar medium is described. However for industrial applications a process in which the product is removed continuously from the system via a membrane seems much more feasible. This can be achieved by designing a membrane that selects between the surfactant and the product.

5.5 Appendix

When the rate of exchange between the water pools in a reversed micellar medium is rapid compared to the enzymatic conversion, the system can be treated as a pseudo two-phase system. Here, an expression is derived for the rate of enzymatic conversion of apolar compounds in such a system. It is assumed that the reaction can be described by eq. 1 and takes place in the water phase only. Before reaction can take place, the substrate has to diffuse from the interphase into the water phase.

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Method for the enzymatic conversion of a water-insoluble
or substantially water-insoluble organic substrate.

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       The invention relates to a method of converting a
water-insoluble or substantially water-insoluble organic
substrate under the influence of a water-soluble enzyme
requiring a cofactor, by performing the conversion in a
micellar system, comprising an organic solvent which is
not or substantially not water-miscible and in which the
enzyme is solubilized via reversed micelles, which are
stabilized by one or more surfactants. Water-insoluble or
substantially water-insoluble is to be understood to mean
herein a solubility of at most approximately 3 g per litre
of water at room temperature. An organic substrate is to
be understood to mean herein an organic compound which can
be converted specifically or aspecifically by the enzyme.

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       Such a conversion is known from literature. For
example Meier and Luisi (J. Solid-Phase Biochem., Vol. 5,
No. 4, 1980, 269-282) have reduced decanal enzymatically
in a system having a hydrocarbon as a solvent; a steroid
containing a ketone function can be reduced in the same
manner. In this non-water-miscible organic solvent tiny
waterpools, stabilized by surfactants, are present,
so-called reversed micelles. The enzyme is present in the
micellar phase. Solubilized via reversed micelles is hence
to be understood to mean herein that the enzyme is present
in the organic solvent in the micellar phase (i.e. water-
pool plus interface). The total system of enzyme, micellar
phase and organic solvent is termed micellar system. Most
enzymes are not soluble in organic solvents and are more-
over easily denatured by the solvent. Therefore, such a
system of reversed micelles is often necessary to enable
an enzymatic process in an organic solvent.

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       Meier and Luisi have described an enzymatic re-
duction of decanal which is catalysed by the enzyme liver
alcohol dehydrogenase with NADH as a cofactor. The enzyme

described by said authors, like many other enzymes, re-
quires a cofactor for its enzymatic activity. Cofactors,
also termed co-enzymes, for example NADH and NADPH for
reduction reactions and NAD^+ and $NADP^+$ for oxidation
reactions, however, are very expensive organic compounds
and, in solution, are often unstable (see, for example,
Wong and Whitesides, J. Am. Chem. Soc. 103, 1981,
4890-99). If such an enzymatic process should be economic-
ally feasible it is hence necessary to regenerate the co-
factor.

The regeneration of cofactors is known, for ex-
ample, from a survey article by Wang and King in Adv.
Biochem. Eng. 12. 1979, 119-146. More recently, several
articles have been published, for example, by Wong c.s.,
which all relate to the regeneration of the NAD(P)H cofac-
tor: J. Org. Chem. 46, 1981, 4622-23, J. Am. Chem. Soc.
103, 1981, 4890-99 and 6227-28. Enzymatic methods are pre-
ferred over chemical methods in regenerating cofactors, in
particular because the regeneration occurs with a compara-
tively small efficiency when a chemical method is used.
Such an enzymatic regeneration of a cofactor is known only
in aqueous systems.

On the basis of the above it would be possible in
principle after the enzymatic conversion of the organic
substrate to isolate the cofactor to be regenerated from
the micellar system and subsequently to regenerate the
cofactor in a known manner in an aqueous medium. The re-
generated cofactor would then be available again for the
desired conversion.
It will be obvious that such a separate regeneration has
important drawbacks. A separate reaction step is expen-
sive, requires a much higher cofactor concentration and is
time-consuming. Moreover the isolation of the cofactor to
be regenerated from the micellar system is usually ex-
tremely difficult.

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It is the purpose of the invention to perform the enzymatic process mentioned in the opening paragraph in an economically acceptable manner.

According to the invention this object is achieved in that the cofactor is regenerated in situ by means of one or more second enzymes, which are also solubilized via reversed micelles in the organic solvent.

In this way the laborious isolation of the cofactor to be regenerated succeeded by a separate regeneration process can be avoided.

In US patent 3,926,726 of Antonini et al. a method for carrying out enzymatic reactions by using a biphasic aqueous-organic system is described; the cofactor can be regenerated in situ. This known biphasic system differs fundamentally from the micellar system of the invention, in which system the enzymes are solubilized via reversed micelles. So the system of the present invention should be considered as a solution and not as a biphasic system. Although in some experiments apparently high conversions could be obtained, the method described in the above US patent is generally less broadly applicable. This is obvious from e.g. a publication of the same Antonini and co-workers in Biotechn. Bioeng. Vol XVII, pp. 1101-1108 (1975), wherein is stated, that in reducing progesterone poor results were obtained in spite of the relative high specificity of 20β-HSDH for this substrate. As will be clear from the Examples, progesterone could be reduced in a high yield and specificity to 20β-hydroxypregn-4-ene-3--one, when the method of the invention was used. Therefore the method of the present invention has considerable advantages over the above-mentioned known reaction in a biphasic system.

To gain a better insight into the method of the present invention, the micellar system used in the invention is described in more detail hereafter. The re-

the micellar system. The process is preferably carried out
at room temperature or a slightly elevated temperature.
The ratio of the quantity of water and the other ingre-
dients of the micellar system is rather critical and de-
pends on the nature and the properties of these ingre-
dients. In general it holds that optimum results can be
achieved with approximately that quantity of water, that
gives maximal hydration of all the hydrophilic
head- groups of the surfactant or surfactants.

In evaluating the invention it should be con-
sidered that a system of reversed micelles is an extremely
subtle reaction medium in which small changes in the com-
position may have a dramatic influence on the stability of
the system, and may hence empede or prevent the conversion
of the substrate. In fact, prime requirements for a
successfully occurring conversion are both the stability
of the micellar system and the accessibility of the sub-
strate in the organic solvent for the enzymes in the re-
versed micelles. It is therefore an achievement of the
invention that one has succeeded to satisfactorily perform
the enzymatic regeneration of the cofactor in this subtle
micellar system without adversely influencing the desired
enzymatic conversion, namely due to a correct choice of
the nature and the composition of the ingredients of the
micellar system.

Various conversion reactions, for example, oxida-
tions, reductions and hydroxylation reactions may be car-
ried out by means of the method according to the inven-
tion. It is a characteristic of these enzymatic conver-
sions, that the reactions can be performed with a high
site- and stereospecificity.

When the method is used to oxidise a water-
-insoluble or substantially water-insoluble organic sub-

strate, NAD^+ or $NADP^+$ is preferably used as a cofactor
which is also solubilized via reversed micelles in the or-
ganic solvent. In such an oxidation reaction, an electron
acceptor is usually present in the micellar system. If the
reaction is not carried out while excluding air, the air
oxygen may serve as an electron acceptor. However, it may
also be desirable to use an artificial electron acceptor,
preferably a viologen, a phenazine derivative or an acri-
dine derivative, such as methyl viologen, benzyl viologen,
methylene blue or phenazine methosulphate, usually in the
presence of molecular oxygen, or to introduce oxygen into
the system. If the oxidation reaction is carried out in
the absence of oxygen,the artificial electron acceptor may
be oxidised electrochemically, so by using an oxidative
voltage source. Suitable second enzymes for such an oxi-
dation reaction are catalase in the presence of superoxide
dismutase, NAD(P)H-oxidase and NAD(P)H-dehydrogenase.
These enzymes can efficaciously catalyse the regeneration
of the above cofactors.

When the method according to the invention is
used for reducing a water-insoluble or substantially
water-insoluble organic substrate, NADH or NADPH which,
like the enzymes, is solubilised via reversed micelles in
the organic solvent, is preferably used as a cofactor. In
such a reduction reaction an electron donor should prefer-
ably be present in the system. An electron donor suitable
for this purpose is a reduced viologen, preferably reduced
methyl viologen (MV^+) or benzyl viologen. For regene-
rating the cofactors NADH or NADPH used in the reduction
lipoamide dehydrogenase or ferredoxine-$NADP^+$ oxidoreduc-
tase as a second enzyme have proved to be excellently
suitable.
Reduced methyl viologen (MV^+) or benzyl viologen is pre-
ferably obtained by subjecting methyl viologen (MV^{2+}) or

The invention will be described in greater detail
with reference to the following specific examples.

EXAMPLE I

A steroid, namely progesterone (pregn-4-ene-3,20-
-dione) was reduced as follows.

1.5 ml of an 0.2 M solution of cetyl trimethyl ammonium
bromide (CTAB) in a mixture of octane and hexanol in a
volume ratio of 4 : 1, in which in addition 1 mM of pro-
gesterone had been dissolved, was mixed, while stirring
intensively, with 60 μl of a buffer solution of 50 mM of
N-2-hydroxyethylpiperazine N'-2-ethane sulphonic acid
(HEPES)in dilute sodium hydroxide solution (HEPES-buffer)
with a pH of 7.6. A 1 M solution is to be understood to
mean herein a 1 molar solution which is a solution of 1
grammolecule of substance in 1 litre of solvent. The buf-
fer solution comprised 1.0 mM of NAD^+, 25 mM of methyl
viologen, 5 μg of lipoamide dehydrogenase (EC 1.6.4.3) and
19 μg of 20β-hydroxysteroid dehydrogenase (EC 1.1.1.53).
The micellar system thus obtained was made oxygen-free
with argon, after which the argon was replaced by hydro-
gen. In all the subsequent treatments the system was then
kept under hydrogen. Then 10 μl of a solution of 29 μg/ml
of hydrogenase, isolated from Desulfovibrio vulgaris as
described in the already mentioned Netherlands Patent
Application 7801517, in HEPES-buffer were added to the
system while stirring, after which the reaction system was
incubated at 25°C. At intervals a sample was taken from
the reaction system and, after removing CTAB by precipita-
tion with acetonitril, analysed by means of HPLC (high
performance liquid chromatography). The results of these
analyses are shown graphically in Figure 1. In graph B the
yield of 20β-hydroxypregn-4-ene-3-one (Y) in mg per 1.5 ml
is plotted against the time in hours. Only the β-isomer,

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and not the corresponding α-isomer, viz. 20α-hydroxypregn-
-4-ene-3-one, was found in the reaction mixture. So pro-
gesterone was reduced site- and stereospecifically. The
ratio hexanol/CTAB in the interface of the reversed
micelles was under these conditions 2.5.

When instead of 1.0 mM of progesterone 0.2 mM or
5.0 mM of progesterone was used as a starting material,
under the same conditions the results presented in curve A
or curve C were obtained.

The _in situ_ formation of reduced methyl viologen
may also take place photochemically or electrochemically
instead of enzymatically. The photochemical reduction was
carried out as described in an article by Hilhorst et al.
in Proc. Natl. Acad. Sci. USA, 79 (1982), 3927-3930. The
electrochemical reduction was carried out as described in
Example III.
Both the electrochemical and the photochemical reduction
yielded the same results as the enzymatic reduction de-
scribed above.

EXAMPLE II

Influence of cosurfactant on conversion of pro-
gesterone.
The reduction of progesterone according to Example I was
repeated with various cosurfactants. Under the same reac-
tion conditions as described in Example I 1mM of progeste-
rone was reduced to 20β-hydroxypregn-4-ene-3-one in the
presence of 180 μl of propanol, 220 μl of butanol, 250 μl
of pentanol, 300 μl of hexanol and 375 μl of octanol, res-
pectively as cosurfactants. In each of these experiments
the organic phase was supplied with octane up to a total
volume of 1.5 ml.

CLAIMS

1.   A method of converting a water-insoluble or sub-
stantially water-insoluble organic substrate under the
influence of a water-soluble enzyme requiring a cofactor,
by performing the conversion in a micellar system, com-
prising an organic solvent which is not or substantially
not water-miscible and in which the enzyme is solubilized
via reversed micelles, which are stabilized by one or more
surfactants, characterized in that the cofactor is regene-
rated in situ by means of one or more second enzymes,
which are also solubilized via reversed micelles in the
organic solvent.

2.   A method as claimed in Claim 1, characterized in
that the micellar system in addition comprises a cosurfac-
tant, wherein the substrate is well soluble.

3.   A method as claimed in Claim 1 or 2, charac-
terized in that an optionally halogenated hydrocarbon or a
mixture of optionally halogenated hydrocarbons is used as
an organic solvent and that the reversed micelles consist
of tiny waterpools, approx. 2 to 200 nm in diameter and
stabilised by one or more ionic surfactants.

4.   A method as claimed in Claim 3, characterized in
that as an organic solvent is used benzene optionally sub-
stituted with one or more chlorine atoms or alkyl groups,
a chlorinated aliphatic hydrocarbon having 1-14 carbon
atoms, an alkane or dialkyl ether having 6-12 carbon
atoms, or a mixture of two or more of these solvents.

5.   A method as claimed in any of Claims 2-4, charac-
terized in that the cosurfactant is an aliphatic or cyclo-
aliphatic alcohol having 3 to 10 carbon atoms, an amine,
or a fatty acid or a derivative thereof.

6.   A method as claimed in any of Claims 2-5, charac-
terized in that the micellar system comprises the cosur-
factant in an amount of 5-40% v/v, and that the molecular
ratio between cosurfactant and surfactant in the interface
of the reversed micelles is 0.1 to 20, preferably 1-6.

and not the corresponding α-isomer, viz. 20α-hydroxypregn-
-4-ene-3-one, was found in the reaction mixture. So pro-
gesterone was reduced site- and stereospecifically. The
ratio hexanol/CTAB in the interface of the reversed
micelles was under these conditions 2.5.

When instead of 1.0 mM of progesterone 0.2 mM or
5.0 mM of progesterone was used as a starting material,
under the same conditions the results presented in curve A
or curve C were obtained.

The in situ formation of reduced methyl viologen
may also take place photochemically or electrochemically
instead of enzymatically. The photochemical reduction was
carried out as described in an article by Hilhorst et al.
in Proc. Natl. Acad. Sci. USA, 79 (1982), 3927-3930. The
electrochemical reduction was carried out as described in
Example III.
Both the electrochemical and the photochemical reduction
yielded the same results as the enzymatic reduction de-
scribed above.

EXAMPLE II

Influence of cosurfactant on conversion of pro-
gesterone.
The reduction of progesterone according to Example I was
repeated with various cosurfactants. Under the same reac-
tion conditions as described in Example I 1mM of progeste-
rone was reduced to 20β-hydroxypregn-4-ene-3-one in the
presence of 180 μl of propanol, 220 μl of butanol, 250 μl
of pentanol, 300 μl of hexanol and 375 μl of octanol, res-
pectively as cosurfactants. In each of these experiments
the organic phase was supplied with octane up to a total
volume of 1.5 ml.

The results are shown graphically in Figure 2. In
this graph the yield (Y) of 20β-hydroxypregn-4-ene-3-one
in mg per 1.5 ml, formed after a reaction time of 4 hours,
is plotted against the chain length (C_n) of the alcohol
used as a cosurfactant. From this graph it is clear, that
hexanol and pentanol are most suitable as cosurfactants
for the desired conversion.

EXAMPLE III

Oxidation of testosterone to androstenedione;
electrochemical in situ formation of benzyl viologen.
The following ingredients were mixed throroughly: 0.06 g
of tetraheptyl ammonium bromide, 0.18 g of cetyl trimethyl
ammonium bromide (CTAB), 250 μl of hexanol, 2 ml of oc-
tane, 10 ul of a solution of 50 mM benzyl viologen in 200
mM HEPES-buffer (pH 7.6), and 20 μl of 25 mM NAD$^+$ in 200
mM HEPES-buffer (pH 7.6). To the micellar system thus ob-
tained was added 5 μmoles of testosterone. This mixture
was introduced into the anodic part of an electrochemical
cell. Into the same cell but separated from this mixture
by a dialysis membrane another mixture was introduced.
This latter mixture comprised the same ingredients as the
former, however without NAD$^+$ and testosterone. A platina
electrode was used; the reference electrode was a Ag/AgBr
electrode. The system was made oxygen-free with argon,
after which the system was kept under argon. After the
potential of the anode has been adjusted to +800 mV, 10 μl
of lipoamide dehydrogenase (2mg/ml) and 30 ul of 3α,β-hy-
droxysteroid dehydrogenase (30 mg/ml)(EC 1.1.1.52) were
added. The addition of 30 μl of 3α,β-hydroxysteroid dehy-
drogenase was repeated after 3 and 6 hours. The reaction
mixture was analyzed as described in Example I.

The results are shown graphically in Figure 3. In
graph A the yield of androstenedione (Y) in mg per 2.5 ml
is plotted against the time in hours.

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The ratio hexanol/CTAB in the interface of the reversed
micelles was 2.5.

When instead of 250 μl of hexanol and 2 ml of
octane 500 μl of hexanol and 1.75 ml of octane was used
curve B in Figure 3 was obtained.
The ratio hexanol/CTAB in the interface of the reversed
micelles was in this case greater than 2.5, but the parti-
tion coefficient of the substrate was increased relative
to the value in graph A due to the presence of more hexa-
nol in the organic phase.

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CLAIMS

1.   A method of converting a water-insoluble or sub-
stantially water-insoluble organic substrate under the
influence of a water-soluble enzyme requiring a cofactor,
by performing the conversion in a micellar system, com-
prising an organic solvent which is not or substantially
not water-miscible and in which the enzyme is solubilized
via reversed micelles, which are stabilized by one or more
surfactants, characterized in that the cofactor is regene-
rated in situ by means of one or more second enzymes,
which are also solubilized via reversed micelles in the
organic solvent.

2.   A method as claimed in Claim 1, characterized in
that the micellar system in addition comprises a cosurfac-
tant, wherein the substrate is well soluble.

3.   A method as claimed in Claim 1 or 2, charac-
terized in that an optionally halogenated hydrocarbon or a
mixture of optionally halogenated hydrocarbons is used as
an organic solvent and that the reversed micelles consist
of tiny waterpools, approx. 2 to 200 nm in diameter and
stabilised by one or more ionic surfactants.

4.   A method as claimed in Claim 3, characterized in
that as an organic solvent is used benzene optionally sub-
stituted with one or more chlorine atoms or alkyl groups,
a chlorinated aliphatic hydrocarbon having 1-14 carbon
atoms, an alkane or dialkyl ether having 6-12 carbon
atoms, or a mixture of two or more of these solvents.

5.   A method as claimed in any of Claims 2-4, charac-
terized in that the cosurfactant is an aliphatic or cyclo-
aliphatic alcohol having 3 to 10 carbon atoms, an amine,
or a fatty acid or a derivative thereof.

6.   A method as claimed in any of Claims 2-5, charac-
terized in that the micellar system comprises the cosur-
factant in an amount of 5-40% v/v, and that the molecular
ratio between cosurfactant and surfactant in the interface
of the reversed micelles is 0.1 to 20, preferably 1-6.

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7.   A method as claimed in any of the preceding
Claims, characterized in that a substrate, comprising one
or more aldehyde-, ketone- or alcohol groups, is oxidized,
while NAD^+ or $NADP^+$, being used as a cofactor, is re-
generated in the presence of an electron acceptor, and the
resulting product is then isolated.

8.   A method as claimed in Claim 7, characterized in
that a viologen, a phenazine derivative or an acridine
derivative, in the presence of oxygen or an oxidative vol-
tage source, is present in the micellar system as an elec-
tron acceptor.

9.   A method as claimed in Claim 7 or 8, charac-
terized in that catalase in the presence of superoxide
dismutase, NAD(P)H-oxidase or NAD(P)H-dehydrogenase is
used as a second enzyme.

10.   A method as claimed in any of the Claims 1-6,
characterized in that a substrate, comprising one or more
aldehyde- or ketone groups, is reduced, while NADH or
NADPH, being used as a cofactor, is regenerated in the
presence of an electron donor, and the resulting product
is then isolated.

11.   A method as claimed in Claim 10, characterized in
that a reduced viologen, preferably reduced methyl or ben-
zyl viologen is used as an electron donor.

12.   A method as claimed in Claim 10 or 11, charac-
terized in that lipoamide dehydrogenase or ferredoxine -
$NADP^+$ oxidoreductase is used as a second enzyme.

13.   A method as claimed in Claim 11 or 12, charac-
terized in that reduced methyl or benzyl viologen is used
which has been obtained by subjecting methyl or benzyl
viologen in situ to an electrochemical reduction, a photo-
chemical reduction or a reduction with hydrogen gas under
the influence of the enzyme hydrogenase.

14.   A method as claimed in any of the Claims 7-13,
characterized in that an alcohol dehydrogenase or a hy-
droxysteroid dehydrogenase is used as the enzyme requiring
a cofactor.

ABSTRACT:

　　　　　The invention relates to a method of converting a
water-insoluble or substantially water-insoluble organic
substrate under the influence of a water-soluble enzyme
requiring a cofactor. The conversion is carried out in a
micellar system, comprising an organic solvent which is
not or substantially not water-miscible and in which the
enzyme is solubilized via reversed micelles, stabilized by
one or more surfactants, while the cofactor is regenerated
in situ by means of one or more second enzymes also
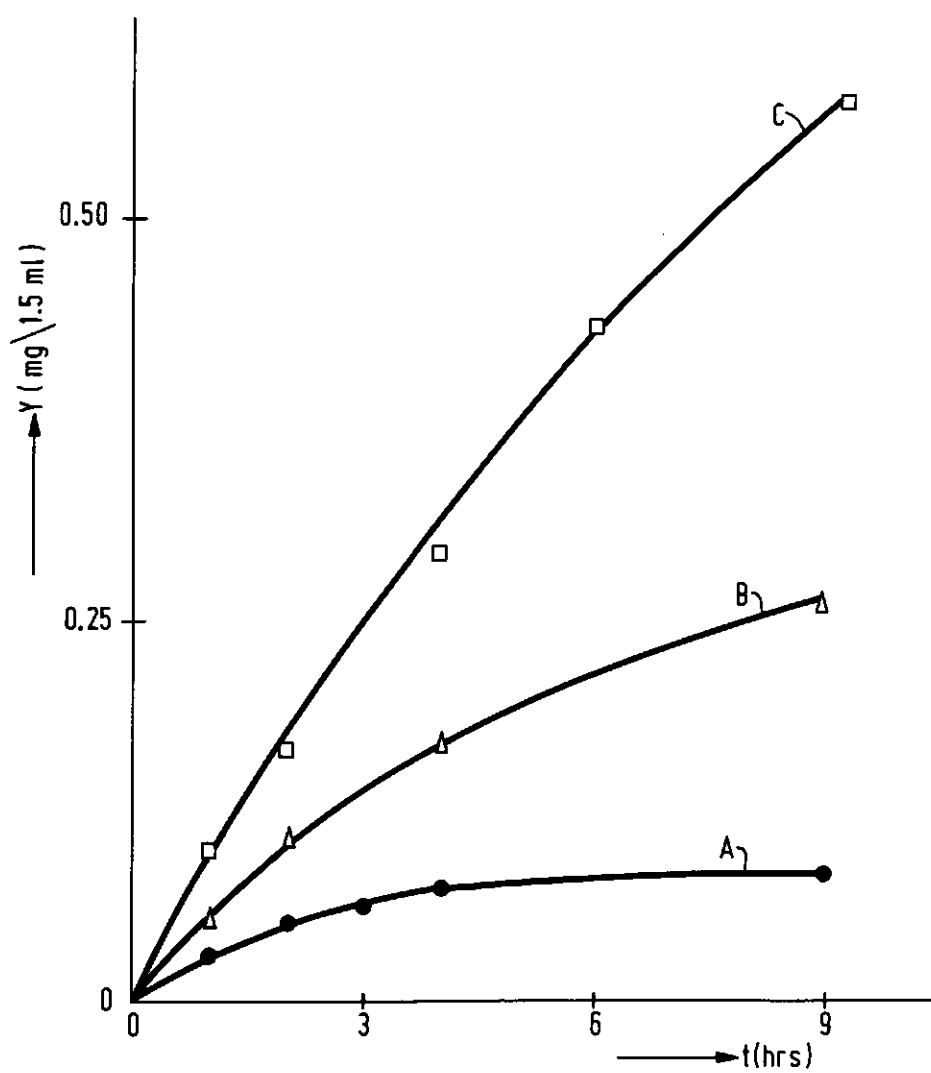solubilized via reversed micelles in the organic solvent.

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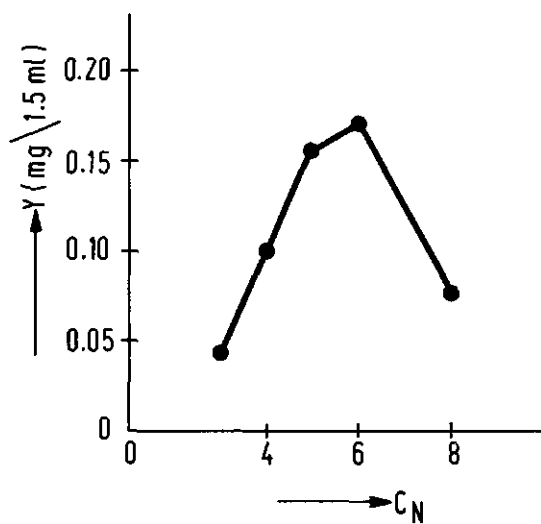35

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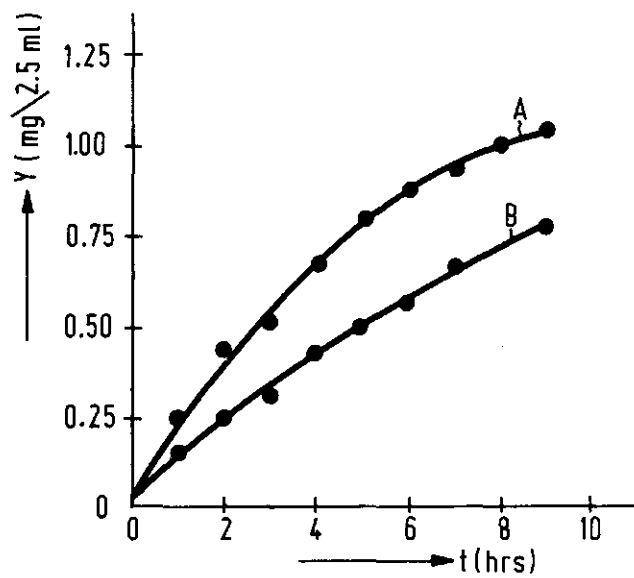**FIG. 1**

1 – II – DIR 0339

FIG.2

FIG.3

2-Ⅱ-DIR 0339

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ABSTRACT

It has been recognised that enzymes in reversed micelles have potential for application in chemical synthesis. Before these expectations will be realised many problems must be overcome. This thesis deals with some of them.

In Chapter 1 the present knowledge about reversed micelles and micellar enzymology is reviewed.
Encapsulation of enzymes in reversed micelles enables the enzymatic conversion of apolar compounds. In the literature only a few cases have been reported of conversions of apolar compounds, and only initial enzyme activities were measured. In Chapters 3 and 4 of this thesis, the conversion of the apolar steroids progesterone and prednisone by 20β-hydroxysteroid dehydrogenase is described. In Chapter 3 it is shown that the reaction proceeds for at least nine hours, indicating that the steroid dehydrogenase used here is fairly stable in a reversed micellar environment under operational conditions. The stability of hydrogenase in reversed micelles was even higher than in an aqueous solution (Chapter 2).

In order to function for longer periods of time, redox enzymes require a continuous and sufficient supply of reducing equivalents. Several systems have been described that provide reducing equivalents in aqueous solutions, but no such a system was known for reversed micellar media. In this thesis three methods for the generation of reducing equivalents in reversed micellar media have been applied. Chapter 2 describes a photochemical system for the vectorial transport of electrons from a donor in the continuous phase to an acceptor in the water pool of the reversed micelle. The spatial arrangement of the components is an important factor in determining the efficiency of such systems. The reducing equivalents thus generated can be converted to hydrogen by hydrogenase located in the water pool. In Chapter 3 a combined enzyme system consisting of hydrogenase and lipoamide dehydrogenase converts hydrogen gas into reducing equivalents in the form of NADH that are consumed by 20β-hydroxysteroid dehydrogenase during the conversion of apolar steroids. Based on the results of these two chapters, patent applications were filed in several countries, e.g. in Europe. A copy of this patent application is added as an Appendix. There, a third method is mentioned, i.e. the electrochemical regeneration of NADH and NAD$^+$.

In Chapter 4 it is investigated how the composition of a reversed micellar medium affects the rate of conversion of apolar steroids by 20β-hydroxysteroid dehydrogenase. Evidence was obtained that the steroid concentration in the interphase dictates the rate of conversion. This concentration depends on the

hydrophobicity of the substrate as compared to the hydrophobicity of the inter-phase and the hydrophobicity of the continuous phase. These observations are generalized to guidelines indicating that the difference between the hydropho-bicity of the substrate and the interphase must be minimal to ensure a high substrate concentration in the interphase and that the difference between the hydrophobicity of the substrate and the hydrophobicity of the continuous phase must be maximal to keep the substrate concentration in the continuous phase as low as possible. These guidelines will prove useful to predict the optimal composition of a medium for enzymatic conversion of apolar compounds.

Not only the composition of a reversed micellar medium with respect to organic solvent and cosurfactant is important, but also the charge of the sur-factant used. In Chapter 5, evidence is presented that the charge of the sur-factant head groups influences the kinetic parameters K_m and k_{cat} of enzymes in reversed micelles, either resulting in an increase or a decrease of activity. Furthermore, an expression is derived for the initial reaction rate of the enzymatic conversion of an apolar substrate in a pseudo two-phase system where the partition equilibrium of the substrate over the two phases can be shifted due to enzyme catalysis.

In another section of the Discussion the advantages and disadvantages of reversed micellar media for the enzymatic conversion of apolar compounds are compared with those of other systems that have been proposed for that purpose.

In conclusion, in a reversed micellar medium:
- a highly organised photochemical system can be created (Chapter 2)
- enzymes can be immobilized while retaining their activity (Chapters 1-5)
- enclosure of enzymes can lead to enhanced stability (Chapter 2)
- enzymes experience an essentially aqueous micro-environment (Chapter 5)
- enzymatic activity can be higher than in aqueous solution (Chapter 4 and 5)
- apolar compounds can be converted enzymatically (Chapters 3, 4 and Appendix)
- multi-enzymatic reactions can be performed for longer periods (Chapter 3)
- cofactors can be regenerated with hydrogen and electricity (Chapter 3 and Appendix)
- enzyme activity can be regulated by changing the composition of the inter-phase and continuous phase (Chapter 4)
- the optimal composition for enzymatic conversion of any given apolar compound can be predicted (Chapter 4)
- the thermodynamic equilibrium can be shifted in the desired direction (Chapter 5)
- the product can be isolated while the other components can be recycled (Chapter 3).

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SAMENVATTING

Het insluiten van enzymen in omgekeerde micellen biedt mogelijkheden om enzymen te gebruiken voor chemische synthese van apolaire verbindingen. Voordat deze constatering verwezenlijkt zal zijn moeten er nog veel problemen opgelost worden. De inhoud van dit proefschrift heeft betrekking op enkele van die problemen.

In hoofdstuk 1 is een overzicht gegeven van de huidige kennis op het gebied van omgekeerde micellen en micellaire enzymologie.
Enzymen die zijn ingesloten in omgekeerde micellen zijn in staat om apolaire verbindingen om te zetten. Uit de literatuur zijn maar enkele gevallen van dergelijke omzettingen bekend. In al deze gevallen werden alleen initiële enzymactiviteiten gemeten. In hoofdstuk 3 en 4 van dit proefschrift wordt de omzetting van de apolaire steroiden progesteron en prednison door 20β-hydroxysteroid dehydrogenase beschreven. In hoofdstuk 3 wordt getoond dat de reactie gedurende tenminste 9 uur verloopt, hetgeen een aanwijzing is dat het hier gebruikte steroid dehydrogenase onder operationele omstandigheden redelijk stabiel is in omgekeerde micellen. Ook hydrogenase is stabieler in omgekeerde micellen dan in een waterig milieu (Hoofdstuk 2).

Willen redoxenzymen gedurende langere tijd functioneren, dan is een voortdurende toevoer van reductie-equivalenten nodig. Er zijn diverse systemen beschreven die in waterig milieu reductie-equivalenten produceren maar geen ervan kan toegepast worden in omgekeerde micellen. Bij het onderzoek dat beschreven is in dit proefschrift, zijn op drie verschillende manieren reductie-equivalenten geproduceerd. Hoofdstuk 2 beschrijft een fotochemisch systeem dat vectorieel electronen transporteert van een donor in de continue fase naar een acceptor in het waterhart van de omgekeerde micel. De ruimtelijke ordening van de componenten blijkt erg belangrijk te zijn om een efficiënt werkend systeem te creëren. De reductie-equivalenten die met behulp van dit systeem geproduceerd zijn, kunnen door hydrogenase in een omgekeerde micel omgezet worden in waterstofgas. In hoofdstuk 3 staat beschreven hoe een gecombineerd enzymsysteem, bestaande uit hydrogenase en lipoamide dehydrogenase, waterstofgas omzet in reductie-equivalenten in de vorm van NADH. Dit laatste wordt verbruikt door 20β-hydroxysteroid dehydrogenase tijdens de omzetting van apolaire steroiden. Naar aanleiding van de resultaten beschreven in deze hoofdstukken werden octrooiaanvragen ingediend in verschillende landen, b.v. in Europa. Een kopie van deze octrooiaanvrage is toegevoegd als Appendix. In deze Appendix staat een methode voor de electrochemische terugvorming van NADH en NAD^+ beschreven.

In hoofdstuk 4 wordt ingegaan op de vraag hoe de samenstelling van een oplossing met omgekeerde micellen de omzettingssnelheid van apolaire steroiden door 20β-hydroxysteroid dehydrogenase beïnvloedt. Deze snelheid bleek bepaald te worden door de steroidconcentratie in het grensvlak, die weer afhangt van de hydrofobiciteit van het substraat in relatie tot de hydrofobiciteit van het grensvlak en de hydrofobiciteit van de continue fase.
Uit deze waarnemingen werden algemene richtlijnen afgeleid die zeggen dat het verschil in hydrofobiciteit tussen het substraat en het grensvlak zo klein mogelijk moet zijn om een hoge substraatconcentratie in het grensvlak te krijgen en dat het verschil in hydrofobiciteit tussen het substraat en de continue fase zo groot mogelijk moet zijn om de substraatconcentratie in de continue fase zo laag mogelijk te houden. Deze richtlijnen zijn van belang om die samenstelling van een oplossing met omgekeerde micellen te voorspellen, die het beste resultaat zal geven voor de enzymatische omzetting van een gegeven apolaire verbinding.

In een oplossing van omgekeerde micellen zijn niet alleen het gebruikte organisch oplosmiddel en de cosurfactant van belang, maar ook de lading van de gebruikte zeep. In hoofdstuk 5 wordt aangetoond dat de kinetische parameters K_m en k_{cat} van enzymen in omgekeerde micellen beïnvloed worden door de lading van de kopgroepen van de gebruikte zeep. Dit kan zowel een afname als een toename in activiteit tot gevolg hebben.
Ook wordt een formule afgeleid die de initiële reactiesnelheid voor de enzymatische omzetting van apolaire verbindingen beschrijft in een pseudo twee-fasen systeem waar de ligging van het verdelingsevenwicht over de twee fasen kan verschuiven ten gevolge van de enzymreactie.

In een ander deel van de Discussie worden de voor- en nadelen van oplossingen van omgekeerde micellen als media voor de omzetting van apolaire verbindingen vergeleken met die van andere systemen die voor dit doel ontwikkeld zijn.

Er kan geconcludeerd worden dat in oplossingen van omgekeerde micellen:
- een georganiseerd fotosysteem samengesteld kan worden (Hoofdstuk 2)
- enzymen geïmmobiliseerd kunnen worden met behoud van hun activiteit (Hoofdstuk 1-5)
- het opsluiten van enzymen kan leiden tot verhoogde stabiliteit (Hoofdstuk 2)
- enzymen zich bevinden in een hoofdzakelijk waterige micro-omgeving (Hoofdstuk 5)
- de enzymactiviteit hoger kan zijn dan in waterig milieu (Hoofdstuk 4 en 5)

- apolaire verbindingen enzymatisch omgezet kunnen worden (Hoofdstuk 3, 4 en Appendix)
- een gecombineerd enzymsysteem gedurende langere tijd actief is (Hoofdstuk 3)
- cofactoren teruggevormd kunnen worden met waterstofgas en electriciteit (Hoofdstuk 3 en Appendix)
- de enzymactiviteit gereguleerd kan worden door de samenstelling van het grensvlak en de continue fase te veranderen (Hoofdstuk 4)
- de optimale samenstelling voorspeld kan worden voor de enzymatische omzetting van een willekeurige apolaire stof (Hoofdstuk 4)
- het thermodynamisch evenwicht verschoven kan worden in de gewenste richting (Hoofdstuk 5)
- het product geïsoleerd kan worden terwijl de overige bestanddelen opnieuw gebruikt kunnen worden (Hoofdstuk 3).

CURRICULUM VITAE

De schrijfster van dit proefschrift werd op 2 augustus 1955 geboren in Hoogland. Middelbaar onderwijs werd genoten aan het Eemland College in Amersfoort waar in 1973 het diploma gymnasium β behaald werd. In datzelfde jaar werd begonnen met de studie Moleculaire Wetenschappen aan de Landbouwhogeschool in Wageningen. De doctoraalfase omvatte de hoofdvakken Biochemie en Moleculaire Fysica en het bijvak Fytopathologie. De praktijktijd werd doorgebracht op de afdeling Biochemie van de Universiteit van Otago, Dunedin, in Nieuw Zeeland, onder begeleiding van dr. P.A. Sullivan. De studie werd in januari 1980 met lof afgerond.

In 1980 was de schrijfster betrokken bij het Community Development Program van de Lutherse Wereld Federatie in Nasir, Zuid Soedan. Op 1 juli 1981 trad zij in dienst van de Landbouwhogeschool als onderzoeksassistent.