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Cholesterol Oxidase in Microemulsion: Enzymatic Activity on a Substrate of Low Water Solubility and Inactivation by Hydrogen Peroxide

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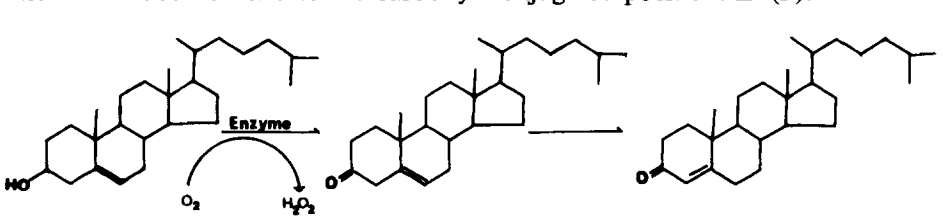
Cholesterol oxidase from *Nocardia erythropolis*, *Pseudomonas*, and *Streptomyces* species was active in microemulsion in which cholesterol is well solubilized. The activity was stable in nonionic microemulsions whereas in cationic and anionic microemulsions the activity decreased with time. The coupled activity test using horseradish peroxidase which is very stable in microemulsion, was modified. The activity at very low water concentration in nonionic microemulsions increased with the water content. The kinetic constants were determined: the Michaelis constant is in the range 10 to 28 mM in the microemulsions, compared to 10 to 28 μM in buffer. The maximum velocity was reduced by a factor of 3 to 5 compared to that in buffer. Neither substrate excess nor product inhibition was detected. The preparative oxidation of cholesterol revealed the inactivation of the cholesterol oxidase by hydrogen peroxide. In contrast to glucose oxidase, hydrogen peroxide inactivated cholesterol oxidase in the absence of substrate. Catalase provides protection during the cholesterol oxidation. Microemulsions are very good media in which to perform enzyme catalyzed reactions with substrates of low water solubility. Their use for the reproducible determination of cholesterol should be examined.

INTRODUCTION

Cholesterol oxidase performs the oxidation of the 3β-hydroxyl group of cholesterol and of related steroids to a keto group by oxygen, and catalyzes the isomerization of Δ^5 double bond to the carbonyl conjugated position: Δ^4 (*1*):



The enzyme seems to have a substrate specificity somewhat depending on its source (*2–7*). The oxidase and isomerase activity was inhibited by 5,10-seco-19-nor-5-cholestyne-3,10-dione (*1*, *7*).

The enzyme is membrane bound and by various treatments the lipophilic part may be released with no decrease of enzymatic activity (*8*). The cofactor was

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shown to be 8α-/N(1)-histidyl/FAD for the enzyme isolated from *Schizophyllum commune* (*9*). The oxidase acts on cholesterol, a substrate of low water solubility. The effects of organic solvents and of detergents on cholesterol oxidase have been extensively studied (*8, 10–13*). The oxidation of cholesterol has been carried out in a heterogeneous medium using cholesterol oxidase bound to cells (*14, 15*).

When studying the enzyme activity in microemulsions, we found that alcohol dehydrogenase acted on substrates of low water solubility (*16, 17*) and that papaïne showed good activity in these media (*18*). Cholesterol oxidase, the activity of which is not affected by some detergents and organic solvents, is an interesting enzyme to study in microemulsions in view of the low water solubility of the substrate. Besides, oxygen is definitely more soluble in organic solvents than in water (*14*). The present study was performed in three types of microemulsion: anionic, cationic, and nonionic. The enzymic interconversion of steroids in reversed micelles and in organic solvents is known (*19, 20*).

MATERIALS AND METHODS

Materials

Sodium dodecyl sulfate (Fluka) and tetradecyltrimethylammonium bromide (Fluka) were recrystallized from ethanol, washed with pentane, and dried under vacuum for 3 days. Triton X-100 (Sigma), cyclohexane for UV spectroscopy (Fluka), and 4-amino-antipyrine (Fluka) were used with no further purification. But *n*-butanol (Fluka) was distilled. The buffer used was 50 mM TES, pH 7.5, unless otherwise stated.

Cholesterol oxidase from *Nocardia erythropolis* and horseradish peroxidase Grade II were purchased from Boehringer-Mannheim and the cholesterol oxidase from *Pseudomonas* species and *Streptomyces* species were purchased from Sigma. The cholesterol oxidase from *N. erythropolis* (in ammonium sulfate) was dialyzed against buffer for 24 h and the enzyme concentration was determined by the Lowry method. The cholesterol oxidase from *Pseudomonas* (2 mg) or *Streptomyces* (0.5 mg) was dissolved in buffer (0.5 ml) and dialyzed against buffer. The protein concentration was estimated from manufacturer's indication.

Assays

Assays were conducted in buffer and in anionic and cationic microemulsions by following the absorption increase at 240 nm (*21*). Coupled assays in nonionic microemulsions were done in the presence of horseradish peroxidase by following the absorption increase at 500 nm (*6, 22*) with the reagent concentrations as indicated for microemulsion 2b. The concentration of the reagents (phenol, 4-amino-antipyrine, and peroxidase) was adjusted in cationic microemulsion 2b so that the absorption at 500 nm was half that at 240 nm. These overall concentrations were then used for the assays in the Triton X-100 microemulsions: 0.1 M phenol, 3×10^{-4} M 4-amino-antipyrine, 17 mM cholesterol and 4.6 U of peroxidase from a 0.5- to 0.7-ml assay.

Microemulsions

Microemulsions with three different surfactants were used. The composition of the microemulsions was as follows (given in % weight). The microemulsions were prepared at 20°C by adding the various constituents under constant stirring. They were filtered before use.

Microemulsion	Cyclohexane	Buffer	SDS[1]	*n*-Butanol
1	58	10	12	20
		CTAB⟶		
2a	71	5	12	12
2b	66	10	12	12
2c	61	15	12	12
2d	56	20	12	12
		Triton X-100⟶		
3a	76	0	12	12
3b	74	2	12	12
3c	71	5	12	12
3d	69	7	12	12
3e	66	10	12	12
3f	64	12	12	12

Enzyme Activity in Microemulsion

The influence of the surfactant and of the water content on the activity of the cholesterol oxidase were studied for samples of the enzyme from each of the three sources.

(*a*) *Effect of the surfactant.* Cholesterol oxidase from *N. erythropolis* (0.025 mg in buffer: 50 μl) was incubated in microemulsion 1 (5 ml) and in microemulsion 2b (5 ml). At determined times, aliquots (0.4 ml) were taken and cholesterol microemulsion 1 or 2b (36 mM, 0.3 ml) was added to the aliquot. The UV absorption at 240 nm was measured and from this the activity was calculated.

Nonionic microemulsion 3e (3 ml) containing cholesterol oxidase (0.020 mg in buffer: 40 μl) were incubated. At determined times, aliquots (0.3 ml) were taken and to these were added 26 mM cholesterol in microemulsion 3e (0.2 ml), a 1×10^{-3} M 4-amino-antipyridine and 0.33 M phenol microemulsion 3e (0.2 ml) and a peroxidase solution (25 μg; 5 μl). The increase in the absorption at 500 nm was monitored.

The activity of cholesterol oxidase from *Pseudomonas* and *Streptomyces* species was studied in the same cationic and nonionic microemulsions 2b and 3e and the activity was determined using the coupled assay described above.

(*b*) *Effect of water content.* This effect was studied on the cholesterol oxidase from *N. erythropolis* in cationic 2a–2d and nonionic microemulsions 3a, 3c. In cationic microemulsions 2a–2d, the initial activity and the time dependence of the activity were determined as above. For comparison the same parameters were determined in buffer.

[1] Abbreviations used: SDS, sodium dodecyl sulfate; CTAB, tetradecyltrimethylammonium bromide; TES, 2-[tris(hydroxymethyl)methylamino-1-ethanesulfonic acid.

For nonionic microemulsion a more extensive study was carried out. As a result enzymatic activity was found in microemulsion 3a. The activity in nonionic microemulsions whose water content was increased from 1.8 to 12.1% was determined using the coupled assay.

Michaelis Constant and Maximum Velocity

These kinetic constants were determined with the enzyme from *N. erythropolis* in buffer, in cationic and in nonionic microemulsions. The constants in buffer were determined as described in the literature (*21*) with Triton X-100 as a detergent. In cationic microemulsion 2b, the cholesterol concentration was varied from 2.5 to 20 mM with a pH of 6.8, 7.5, or 8.2. These kinetic constants were also determined after 4 h incubation of cholesterol oxidase in microemulsion 2b (loss of 25% of the activity). In nonionic microemulsions 3b, 3d, 3e, and 3f, the coupled assay was used. The final enzyme concentration was 3 μg/ml.

The kinetic constants were determined for the enzyme from *Streptomyces* species in microemulsions 2b and 3e by following the increase in absorption at 240 nm for 2b and at 500 nm by the coupled assay for 3e. The enzyme concentration was 10 μg/ml.

Inhibition by Substrate Excess and Product

In microemulsion 2b, no inhibition by substrate excess up to 0.5 M cholesterol was detected by the coupled assay with either the oxidase from *N. erythropolis* or that from *Streptomyces*. The inhibition by the product Δ^4-cholestenone was studied with cholesterol oxidase from *Streptomyces* in cationic microemulsion 2b using the coupled assay. The cholesterol concentration was 74 mM and the Δ^4-cholestenone concentrations were 28 and 56 mM.

Enzyme Inactivation during Cholesterol Oxidation

In microemulsion 2b, the time dependence of the activity was determined under three sets of conditions: the first with the enzyme alone, the second with the enzyme during cholesterol oxidation, and the third with the enzyme during cholesterol oxidation in presence of catalase.

In the first experiment, cholesterol oxidase from *Streptomyces* (0.7 mg) was incubated in microemulsion 2b (2 ml). At given times, aliquots (5 μl) were taken and diluted in microemulsion 2b of 0.2 M cholesterol (0.5 ml). The activity was determined from the UV absorption at 240 nm. The second experiment was done in the same way except that the incubation medium contained 0.26 M cholesterol.

The third experiment was done with an incubation medium containing 0.26 M cholesterol and catalase (2600 U/ml).

Inactivation by Hydrogen Peroxide

A solution of cholesterol oxidase from *N. erythropolis* (0.5 mg) or from *Pseudomonas* (2.0 mg) was incubated in microemulsion 3e (1 ml) containing hydrogen peroxide at 30 mM overall concentration. At determined times, an aliquot (30 μl)

was diluted in microemulsion 2b containing 0.13 M cholesterol (0.57 ml). The activity was determined from the UV absorption at 240 nm.

A solution of cholesterol oxidase from *Streptomyces* (0.35 mg) in microemulsion 2b (1 ml) containing hydrogen peroxide at 30 mM concentration was incubated. At determined times, an aliquot (5 μl) was diluted in microemulsion 2b containing 0.26 M cholesterol (0.4 ml). The activity was determined by the UV absorption at 240 nm.

A solution of cholesterol oxidase from *N. erythropolis* (0.5 mg), *Streptomyces* (1.0 mg), and from *Pseudomonas* (5.0 mg) was incubated in buffer solution containing hydrogen peroxide at 270 mM concentration, at a determined time an aliquot (3 μl) was diluted in 2×10^{-5} M cholesterol buffer solution and monitored at 240 nm.

RESULTS

The activity of cholesterol oxidase in microemulsions was determined by following the absorption increase at 240 nm which is the wavelength at which the final product Δ^4-cholestenone has an absorption maximum. The nonionic microemulsion with Triton X-100 containing an aromatic chromophore have a strong absorption in this region and a coupled assay with horseradish peroxidase had to be performed. This assay using 4-amino-antipyrine and phenol gives rise to a chromophore at 500 nm with an absorption coefficient ($\varepsilon = 6000$) which is half that of Δ^4-cholestenone at 240 nm ($\varepsilon = 12{,}000$). The coupled assay with the peroxidase was used for the activity determination of cholesterol oxidase. With the enzyme from *N. erythropolis* in cationic microemulsions, the concentrations of 4-amino-antipyrine and of phenol had to be adjusted in order to have the proper activity. Maximum activity was obtained with concentrations of 4-amino-antipyrine and phenol of 3×10^{-4} M and 0.1 M, respectively. These conditions were then used without further optimization in all cationic and nonionic microemulsions and for the cholesterol oxidase from other sources. The peroxidase activity seemed to be quite stable in the microemulsions tested.

Enzyme Activity in Microemulsion

We determined as a function of time the activity of cholesterol oxidase from *N. erythropolis* in microemulsions prepared with various surfactants (Fig. 1). In anionic microemulsions, the activity decreased rapidly to 25% of the initial activity after 4 h whereas in cationic microemulsions, the enzymatic activity decreased to about 75% of the initial activity in 2 h and remained at that value for 40 h. In nonionic microemulsions, the activity remained constant for at least 8 h.

Cholesterol oxidase from *Pseudomonas* and *Streptomyces* species was assayed in the same cationic 2b and nonionic 3e microemulsions. The activity of both enzymes was quite stable for 24 h in the nonionic microemulsion 3e. In cationic microemulsion 2b, the activity of the enzyme from *Streptomyces* was stable for at

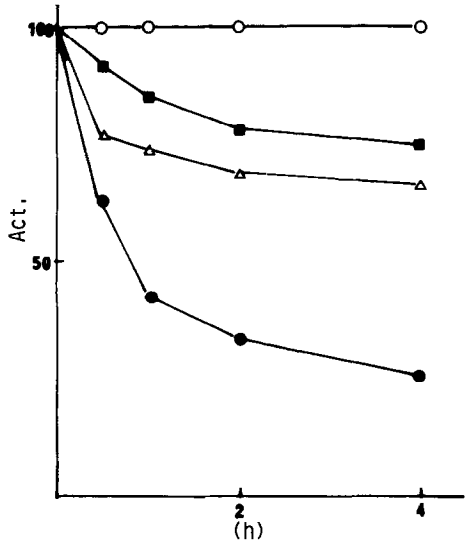

FIG. 1. Cholesterol oxidase activity in microemulsion as a function of time. Cholesterol oxidase from *Nocardia erythropolis*, *Pseudomonas*, and *Streptomyces* in nonionic microemulsion 3e, from *Streptomyces* in cationic microemulsion 2b (○), from *N. erythropolis* in cationic microemulsion 2b (■), from *Pseudomonas* in cationic microemulsion 2b (△), from *N. erythropolis* in anionic microemulsion 1 (●).

least 24 h whereas the activity of the enzyme from *Pseudomonas* was reduced in 3 h to 70% of the initial activity.

Effect of the Water Content in Microemulsions

This study was done with the enzyme from *N. erythropolis*. In cationic microemulsions 2a–2d, the initial activity did not vary with a water content of 5 to 20%, however, the enzymatic activity decreased with time more rapidly at lower water contents (Table 1). At a water content of 20%, the activity decreased at the same rate as in the buffer.

In nonionic microemulsion 3a where the only buffer present was that from the enzyme solution enzymatic activity could be detected. The enzymatic activity was stable during the time of observation (24 h). An increase of 10% in the water content in this system led to a doubling of the activity. The activity was not increased further at water contents above 10%.

Whether or not this variation in activity was related to the effect of the water content on the assay or to the activity of the cholesterol oxidase was not investigated.

However, the activity of the enzyme in a medium of low water content was remarkable.

Michaelis Constant and Maximum Velocity

The results with cholesterol oxidase from *N. erythropolis* and from *Streptomyces* species are presented in Tables 2 and 3. No substrate excess inhibition was

TABLE 1

ACTIVITY OF CHOLESTEROL OXIDASE FROM *Nocardia erythropolis* IN FUNCTION OF TIME AND WATER CONTENT IN MICROEMULSION AND IN BUFFER

Medium	Water content (%)	Time (h)					
		0	½	1	2	4	8
2a	5	100	83	76	74	74	70
2b	10	100	90	84	77	75	71
2c	15	100	93	86	79	77	73
2d	20	100	96	93	89	86	80
3a	0	62	63	61	62	62	63
3c	5	100	102	98	100	102	100
Buffer	100	100	95	93	90	86	81

observed at the higher concentration of cholesterol (0.5 M) with both oxidases. After 4 h incubation of the enzyme from *N. erythropolis* in microemulsion 2b, only the maximum velocity was affected. Product inhibition of the cholesterol oxidation with the enzyme from *Streptomyces* was not detected at 56 mM concentration of Δ^4-cholestenone.

Enzyme Inactivation during Cholesterol Oxidation

The cholesterol oxidase from *Streptomyces* showed great stability in microemulsions. Its enzymatic activity remained at the same value for 24 h. In the

TABLE 2

MICHAELIS CONSTANT OF CHOLESTEROL (K_m) AND MAXIMUM VELOCITY (V_{max}) OF CHOLESTEROL OXIDASE FROM *Nocardia erythropolis* IN MICROEMULSION AND BUFFER

Microemulsion	K_m (mM)	V_{max}	(mM, mn^{-1}, $mg^{-1}\varepsilon$)
2b	10	2.2	pH 6.8
2b	14	2.7	pH 7.5
2b	10	1.38	After 4 h incubation
2b	12	2.3	pH 8.2
3b	16	2.1	pH 7.5
3d	16	3.1	pH 7.5
3e	16	3.5	pH 7.5
3e	12.5	2.4	pH 6.8
3e	12	2.3	pH 8.2
3f	16	3.7	pH 7.8
Buffer	0.012	7.3	pH 7.5

TABLE 3

MICHAELIS CONSTANT OF CHOLESTEROL (K_m) AND MAXIMUM VELOCITY (V_{max}) OF CHOLESTEROL OXIDASE FROM *Streptomyces* IN MICROEMULSION AND BUFFER

Microemulsion	K_m (mM)	V_{max} (mM, mn^{-1}, $mg^{-1}\varepsilon$)
2b	28	0.9
3e	28	1.0
Buffer	0.028	3.7

presence of cholesterol, the activity decreased quite rapidly and 50% of the activity was lost after 3 h and about 12% of the cholesterol was oxidized (see Fig. 2). If the molecular weight of cholesterol oxidase is 32,000 as determined for the enzyme from *Brevibacterium sterolium* (*4*), taking this figure it was calculated that about 4000 mol of cholesterol had been oxidized per mol of enzyme when the enzyme activity had fallen to half of its initial value. If the cholesterol oxidation was performed in the presence of catalase, the cholesterol oxidase activity had decreased by 5% after 5 h and all of the cholesterol was oxidized in about 20 h. The cholesterol oxidase activity could not be determined after 5 h reaction, since the absorption due to the Δ^4-cholestenone was too strong. The catalase activity was high and stable for 24 h in the microemulsion (results not shown).

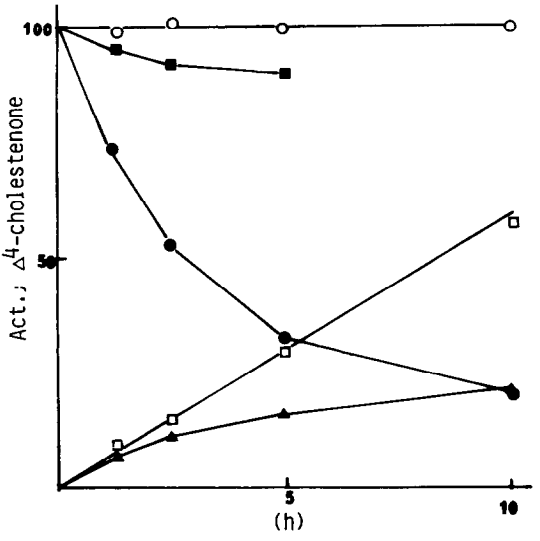

FIG. 2. Inactivation of cholesterol oxidase from *Streptomyces* during cholesterol oxidation in cationic microemulsion 2b and Δ^4-cholestenone appearance. Activity is expressed in percentage of initial activity and Δ^4-cholestenone in percentage of initial cholesterol. Activity in microemulsion 2b (○); activity in presence of cholesterol (0.26 M) (●), activity in presence of cholesterol (0.26 M) and catalase (2600 U/ml) (■), transformation of cholesterol to Δ^4-cholestenone (▲) in presence of catalase (□).

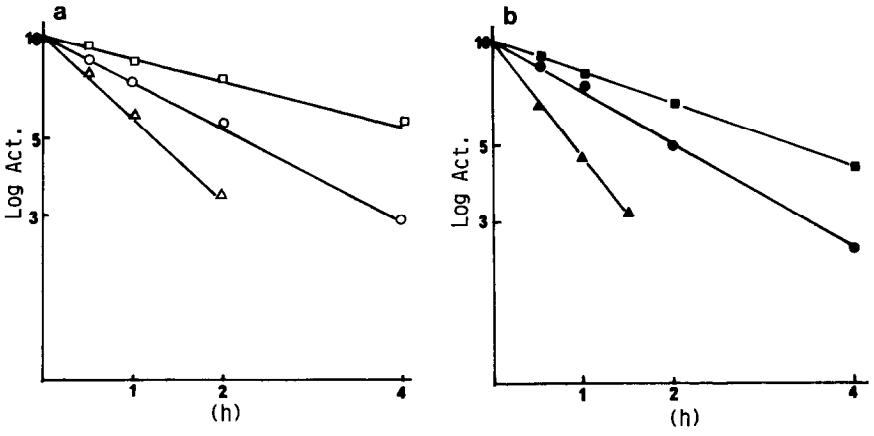

FIG. 3. Inactivation of cholesterol oxidase by hydrogen peroxide. (a) In buffer, 270 mM hydrogen peroxide: cholesterol oxidase from *Pseudomonas* (□), from *N. erythropolis* (△), and from *Streptomyces* (○). (b) In nonionic microemulsion 3e, 30 mM hydrogen peroxide: cholesterol oxidase from *Pseudomonas* (■) and from *N. erythropolis* (▲) and in cationic microemulsion 2b, 30 mM hydrogen peroxide: cholesterol oxidase from *Streptomyces* (●). Activity is expressed in percentage to the initial activity.

Inactivation by Hydrogen Peroxide

Cholesterol oxidase from the three sources studied here was inactivated by hydrogen peroxide in microemulsion (Fig. 3b) and in buffer (Fig. 3a). The inactivation rates in the two media were very close, the hydrogen peroxide concentration being adjusted to be the same in the buffer phase in microemulsion as in the buffer. The enzyme from *N. erythropolis* and *Pseudomonas* was incubated in microemulsion 3e, but the activity test was performed in microemulsion 2b, because the coupled assay was perturbed by the hydrogen peroxide and microemulsion 3e had a strong absorption in the 240-nm region. The rapid inactivation of the cholesterol oxidase by hydrogen peroxide, one of the products of cholesterol oxidation, explains the limited extent of the cholesterol oxidation. If catalase is added to the microemulsion, the cholesterol oxidation goes to completion.

DISCUSSION

Cholesterol in spite of its low solubility in cyclohexane, was found to be very soluble in the microemulsions studied here (about 300 mg/ml). It is puzzling to find that the microemulsions in which high levels of cholesterol can be dissolved, retain their normal properties especially since cholesterol is known to influence membranes properties (*23*).

For the determination of the activity of cholesterol oxidase the direct assay which relies on the UV absorption of the product Δ^4-cholestenone at 240 nm was used for cationic and anionic microemulsions. The nonionic microemulsions ab-

sorb strongly at 240 nm (*13*) therefore for these a coupled test based on hydrogen peroxide, the other reaction product was used. By coupling the cholesterol oxidase with horseradish peroxidase, phenol, and 4-amino-antipyrine an absorption at 500 nm could be followed (*6, 22*). It was found that because of the partition of the reagents between the water and the oil their concentrations had to be adjusted.

In anionic microemulsions, the activity of cholesterol oxidase decreased rapidly. The inactivating effect of anionic microemulsions has been detected with alcohol dehydrogenase (*16, 17*) and papaïne (*18*). For the nonionic microemulsions, the enzymic activity remained stable for a long period of time. For the enzyme from *N. erythropolis* and from *Pseudomonas* in cationic microemulsions, the activity decreased during the first hours and then it remained constant.

The water content influenced the enzymic activity for the nonionic microemulsion. At low water contents, the activity of cholesterol oxidase from *N. erythropolis* was reduced. At 10% water content the activity was the same as that found in buffer and did not increase further with the increasing water content. In nonionic microemulsions, the activity of cholesterol oxidase was detectable at low water contents and as in cationic microemulsions, the initial activity increased with the increasing water content. This is similar to the behavior of alcohol dehydrogenase where a redox catalysis was detected in microemulsions of low water content (*17*).

The kinetic constants were then determined in respect to the microemulsion composition and pH for cholesterol oxidase from *N. erythropolis* and less extensively for the enzyme from *Streptomyces*. For the enzyme from *N. erythropolis*, the Michaelis constant of cholesterol had a value in the range 10 to 16 mM compared to 0.012 mM in buffer with detergent. The partition of the substrate between the oil and water influences the Michaelis constant (*19*). The maximum velocity was reduced in the microemulsion by a factor of 3 to 5 compared to the maximum velocity in buffer. At pH 7.5, the Michaelis constant and the maximum velocity seemed maximum. The optimum pH has been reported to be 7.0 for cholesterol oxidase from *N. rhodochnous (8)*.

For cholesterol oxidase from *Streptomyces*, the Michaelis constant in microemulsion was increased by the same factor as for the enzyme from *N. erythropolis*. The maximum velocity was reduced by the same factor.

The increase of the Michaelis constant of cholesterol for both cholesterol oxidases in microemulsion compared to that in water can be explained in two ways. The cholesterol oxidation is performed in buffer by the enzyme and the cholesterol in water is bound with the enzyme. Then the partition of cholesterol between oil phase and water has to be taken in account. Or the enzyme acts in the oil phase. The binding of the substrate dissolved in oil to a binding site of the enzyme occupied by oil will be different to that observed with the enzyme and substrate in water. For hydrophobic substrates, an increase of Michaelis constant is to be expected when the reaction medium is changed from water to a hydrophobic phase.

Neither substrate excess, not product inhibition were detected in the enzyme from *Streptomyces*. The absence of substrate excess inhibition means that the substrate transfer rate in the microemulsion was not reduced by a high concentra-

tion of cholesterol. Since the enzyme from this source showed the greatest stability in microemulsions, a preparative oxidation of cholesterol was attempted with this enzyme, but no more than 12% of the cholesterol could be oxidized. The cause of the reaction halt was investigated. No product inhibition by Δ^4-cholestenone was detected, but it was found that the cholesterol oxidase was inactivated during the reaction. In the presence of catalase, the oxidation of cholesterol was completed and therefore inactivation by hydrogen peroxide was suspected. The enzyme from each of the three sources was indeed inactivated by hydrogen peroxide in the absence of cholesterol indicating that hydrogen peroxide modified the enzyme with a loss of activity. In a preliminary experiment, it was shown that the UV spectrum of the enzyme–coenzyme was not modified during the inactivation. This inactivation contrasts with that observed in glucose oxidase which performs the same reaction of glucose. Glucose oxidase is also inactivated during the substrate oxidation, but hydrogen peroxide in the absence of glucose does not inactivate the enzyme. It has been proposed that the inactivation occurs during the oxidation by oxygen of the reduced coenzyme (*24, 25*). The hydrogen peroxide inactivation of cholesterol oxidase does not seem to have been previously reported.

The use of a substrate of very low water solubility such as cholesterol for an enzymatic reaction has now been illustrated in microemulsions. The use of such media will enable enzymes to be used in organic synthesis and should provide a reproducible method for the determination of cholesterol (*13*).

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REFERENCES

1. SMITH, A. G., AND BROOKS, C. J. W. (1977) *Biochem. Soc. Trans.*, 1088–1090.
2. IKAWA, S., TAKITA, M., AND OGURA, M. (1979) *J. Biochem.* **85,** 1447–1452.
3. SMITH, A. G., AND BROOKS, C. J. W. (1975) *Biochem. Soc. Trans.*, 675–677.
4. UWAJIMA, T., YAGI, H., AND TERADA, O. (1974) *Agr. Biol. Chem.* **38,** 1149–1156.
5. KERENYI, G., SZENTIRMAI, A., AND NATONEK, M. (1975) *Acta Microbiol. Acad. Sci. Hung.* **22,** 487–496.
6. SMITH, A. G., AND BROOKS, C. J. W. (1976) *J. Steroid Biochem.* **7,** 705–713.
7. SMITH, A. G., AND BROOKS, C. J. W. (1977) *Biochem. J.* **167,** 121–129.
8. SASAKI, I., GOTOH, H., YAMAMOTO, R., TANAKA, H., TAKAMI, J., YAMASHITA, K., JAMASHITA, J., AND HORIO, T. (1982) *J. Biochem.* **91,** 1555–1561.
9. KENEY, W. C., SINGER, T. P., FUKUYAMA, M., AND MIYAKE, Y. (1979) *J. Biol. Chem.* **254,** 4689–4690.
10. MCGUINNESS, E. T., BROWN, H. D., CHATTOPADHYAY, S. K., AND CHEN, F. (1978) *Biochim. Biophys. Acta* **530,** 247–257.
11. UWAJIWA, T., AND TERADA, O. (1978) *Agr. Biol. Chem.* **42,** 1453–1454.
12. ABE, T., NIHIRA, T., TANAKA, A., AND FUKUI, S. (1983) *Biochim. Biophys. Acta* **749,** 69–76.
13. WILLIAMS, W. M. (1980) *Clin. Chim. Acta* **101,** 77–84.
14. BUCKLANS, B. C., DUNNILL, P., AND LILLY, M. D. (1975) *Biotechnol. Bioeng.* **17,** 815–826.

15. FUKUI, S., AND TANAKA, A. (1981) *Acta Biotechnol.* **1,** 339–350.
16. SAMAMA, J. P., AND BIELLMANN, J. F., in preparation.
17. LEE, K. M., SAMAMA, J. P., AND BIELLMANN, J. F., in preparation.
18. WIRTH, P., AND BIELLMANN, J. F., in preparation.
19. HILHORST, R., SPRUIJT, R., LAANE, C., AND VEEGER, C. (1984) *Eur. J. Biochem.* **144,** 459–466.
20. LEGOY, M. D., AND THOMAS, D. (1980) *Bull. Soc. Chim. Fr.* **II,** 98–100.
21. SMITH, A. G., AND BROOKS, C. J. W. (1974) *J. Chromatogr.* **101,** 373–378.
22. ALLAIN, C. C., POON, L. S., CHAN, C. S. G., RICHMOND, W., AND FU, P. C. (1974) *Clin. Chem.* **20,** 470–475.
23. LAWACZECK, R. (1984) *Biophys. J.* **45,** 491.
24. GREENFIELD, P. F., KITTRELL, J. R., AND LAURENCE, R. L. (1975) *Anal. Biochem.* **65,** 109.
25. MALIKKIDES, C. O., AND WEILAND, R. H. (1982) *Biotechnol. Bioeng.* **24,** 2419–2439.