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The Effect of Phenobarbital on the Submicrosomal Distribution of Uridine Diphosphate Glucuronyltransferase from Rat Liver

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1. The detergent Triton X-100 activates UDP glucuronyltransferase from rat liver *in vitro* six- to seven-fold with *p*-nitrophenol as substrate. The enzyme activity when measured in the presence of Triton X-100 is increased significantly by pretreatment of male rats with phenobarbital for 4 days (90mg/kg each day intraperitoneally). If no Triton X-100 is applied *in vitro* such an increase could not be shown. In all further experiments the enzyme activity was measured after activation by Triton X-100. 2. The K_m of the enzyme for the substrate *p*-nitrophenol does not change on phenobarbital pretreatment. 3. When the microsomal fraction from the liver of untreated rats is subfractionated on a sucrose density gradient, 47% of the enzyme activity is recovered in the rough-surfaced microsomal fraction, which also has a higher specific activity than the smooth-surfaced fraction. 4. Of the increase in activity after the phenobarbital pretreatment 50% occurs in the smooth-surfaced fraction, 19% in the rough-surfaced fraction and 31% in the fraction located between the smooth- and rough-surfaced microsomal fractions on the sucrose density gradient. 5. The latency of the enzyme *in vitro*, as shown by the effect of the detergent Triton X-100, is discussed in relation to the proposed heterogeneity of UDP glucuronyltransferase.

The enzyme UDP glucuronyltransferase (UDP-glucuronate glucuronyltransferase; EC 2.4.1.17) catalyses the transfer of a glucuronyl group from UDP-glucuronic acid to various endogenous and exogenous substrates in the liver. As I am interested in the effect of phenobarbital on the metabolism of drugs in the liver and their excretion in the bile, I chose UDP glucuronyltransferase for study, because it metabolizes drugs and at the same time seems to hold a key position in the excretion of its substrates in the bile (Hargreaves & Lathe, 1963).

Lueders & Kuff (1967) have shown that the detergents Triton X-100 and deoxycholate activate UDP glucuronyltransferase *in vitro* five- to ten-fold. Their results suggested that the greater part of the enzyme is latent *in vitro*, or at any rate not accessible to the substrate(s) *p*-nitrophenol and UDP-glucuronic acid. Experiments on the effect of phenobarbital on UDP glucuronyltransferase have been performed; however, no detergent was used in these studies. Thus Zeidenberg, Orrenius & Ernster (1967) showed that this enzyme activity in the rat liver microsomal fraction increases by about 50% after pretreatment with phenobarbital for 4 days, with onset of this increase at the third day of treatment. Gram, Hansen & Fouts (1968) separated smooth- and rough-surfaced microsomal fractions from rabbit liver and compared the specific activities of UDP glucuronyltransferase for different substrates in these two microsomal fractions. They found the highest specific activity for the substrates *p*-nitrophenol and *o*-aminophenol in the rough-surfaced fraction and for phenolphthalein equal specific activities in the rough- and smooth-surfaced fractions. In their experiments UDP glucuronyltransferase activity towards all three substrates failed to show any response to phenobarbital pretreatment. However, they reported only specific activities of UDP glucuronyltransferase in their smooth- and rough-surfaced fractions without recovery data and data on the distribution of protein.

As neither Zeidenberg *et al.* (1967) nor Gram *et al.* (1968) used a detergent in their studies they may have measured only a part of the enzyme activity present in their preparations. Further investigation therefore seemed desirable and rat liver was chosen since it is known to respond to phenobarbital (Zeidenberg *et al.* 1967).

MATERIALS AND METHODS

Animals. Male rats (Wistar, Organisatie voor Toegepast Natuurwetenschappelijk Onderzoek, Zeist, The Netherlands) weighing 190–250g and having free access to food and water were used. Phenobarbital was injected intraperitoneally in a dose of 90mg/kg, once daily at 4p.m., for

4 days. In the microsomal subfractionation experiments the animals were used after being deprived of food for 24h; the phenobarbital-pretreated group received its fourth injection during this starvation period. The animals were decapitated between 8 and 9 a.m. on the day after their last phenobarbital injection.

Postmitochondrial supernatant in 0.154M-*potassium chloride.* Some of the initial experiments were performed on the postmitochondrial supernatant of a 20% (w/v) rat liver homogenate in 0.154M-KCl, which was prepared by centrifuging the homogenate for 10min at 1470*g*. For measurement of free UDP glucuronyltransferase activity in the absence of Triton X-100 this supernatant was not diluted; after activation by 0.25% (v/v) Triton X-100 the supernatant was diluted fourfold with 0.154M-KCl.

Microsomal subfractionation. Smooth- and rough-surfaced microsomal subfractions were prepared from liver homogenates by the method of Dallner (1963). For every fractionation homogenates of six livers were pooled. After decapitation the rat was bled and the liver was immediately removed and put into ice-cold 0.25M-sucrose, pH 7.0. All further manipulations were performed at 0–4°C. A 20% homogenate in 0.25M-sucrose was made by using a Potter–Elvehjem homogenizer with a Teflon pestle. Unbroken cells and nuclei were removed by centrifugation at 670*g* for 10min (nuclear fraction). The complete supernatant was decanted and centrifuged in the SW25 rotor of the Spinco L-2 ultracentrifuge at 15000*g* for 15min to sediment the mitochondria. The resultant supernatant was adjusted to 15mM with respect to CsCl. In each of eight centrifuge tubes of the Ti50 rotor of the Spinco centrifuge, 5ml was layered above 4.5ml of 1.30M-sucrose (pH 7.0) that was also 15mM with respect to CsCl. Thus the postmitochondrial supernatant of 12.4g of liver was divided between eight centrifuge tubes. After centrifugation at 150000*g* for 105min a pellet was present at the bottom of the tubes and a double band at the interface of the sucrose layers. The 0.25M-sucrose layer of the gradient, including the upper band at the interface, was removed with a pipette (fraction 1). The lower band was removed similarly (fraction 2). The remaining 1.30M-sucrose layer above the pellet was decanted (fraction 3) and the pellet (fraction 4) was resuspended gently in 0.25M-sucrose.

Ribosomes could not be detected by electron-microscopic examination in fraction 2, whereas fraction 4 was rich in these organelles. Therefore fraction 2 is tentatively called the smooth-surfaced fraction and fraction 4 the rough-surfaced fraction. I have not classified fraction 3 as rough or smooth because it contains ribosomes and its RNA/protein ratio and the characteristics of its response to phenobarbital (see the Results section) suggest that it may occupy an intermediary position between these two. Fraction 1 is the non-particulate cellular supernatant.

UDP glucuronyltransferase activity was measured in the presence of the detergent Triton X-100 (Sigma Chemical Co., St Louis, Mo., U.S.A.), added to the fractions before further dilution. Optimum activating concentrations were 0.50% (v/v) in the homogenate, 0.63% in the resuspended nuclear fraction, 0.25% in the resuspended mitochondrial fraction and the postmitochondrial supernatant and 0.20% in the gradient fractions.

Enzyme assays. UDP glucuronyltransferase was assayed essentially as described by Hollmann & Touster (1962) with *p*-nitrophenol (spectrophotometric grade; Sigma Chemical Co.) as substrate. The incubation medium contained (final concns.) 1.7mM-*p*-nitrophenol and 1.5mM-UDP-glucuronic acid (disodium salt; C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany) in 50mM-sodium phosphate buffer, pH 7.3; the total volume was 2ml. To economize in UDP-glucuronic acid, while giving a sufficiently high and constant rate of activity in the incubation medium, a final concentration of 1.5mM was used. Though the rate was some 50% of the maximum rate (the K_m for UDP-glucuronic acid was 1.4mM in our incubation medium with the postmitochondrial supernatant in 0.154M-KCl as enzyme preparation), the enzyme activity was linear with enzyme concentration up to a 30% conversion of *p*-nitrophenol. Incubations were performed at 37°C for 15min and were terminated by the addition of trichloroacetic acid (final concn. 3%, w/v). The decrease of E_{405} was measured spectrophotometrically against blanks that contained no UDP-glucuronic acid in the incubation mixture but were otherwise treated identically. When there was no Triton X-100 in the enzyme preparation the E_{405} value of blanks incubated for 15min was much lower than of those that were not incubated. When the detergent was present, however, there was only a slight difference. This difference may be caused by a higher co-precipitation of *p*-nitrophenol in the blanks that had been incubated. The amounts of enzyme protein in the incubation medium used were, expressed as equivalents of mg of bovine serum albumin: for the homogenate, the nuclear fraction, the mitochondrial fraction and the postmitochondrial supernatant 7–10mg; for fraction 1 15mg; for the other gradient fractions 2–4mg. One unit of UDP glucuronyltransferase catalyses the conversion of 1 μmol/min in this incubation medium.

Glucose 6-phosphatase was measured by the method of de Duve, Pressman, Gianetto, Wattiaux & Appelmans (1955) as modified by Bouma (1966). One unit catalyses the conversion of 1 μmol of glucose 6-phosphate/min.

Other determinations. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) as described by Layne (1957), with bovine serum albumin (Poviet, Amsterdam, The Netherlands) as standard. RNA was measured by the orcinol method of Schneider (1957) as modified by Slater (1958), with ribose as standard.

RESULTS

Effect of Triton X-100 in vitro. When Triton X-100 is added to the postmitochondrial supernatant in 0.154M-potassium chloride, there is a six- to seven-fold increase in UDP glucuronyltransferase activity (Fig. 1). Concentrations higher than 0.35% (v/v) produce a smaller activation, which may be due to an inhibitory action of Triton X-100 at high concentrations. These results agree with those of Lueders & Kuff (1967), who, however, added the detergent to the incubation mixture, whereas I add it to the undiluted supernatant, because the enzyme preparation retains its activity after dilution of the Triton X-100 concentration once it has been activated. Like these authors I was unable to analyse this detergent-induced increase

in activity by means of kinetic measurements because of the low activity without detergent.

In contrast with its effect on UDP glucuronyltransferase Triton X-100 up to a concentration of 0.25% (v/v) in the undiluted supernatant had either no effect or a small inhibitory effect on the activity of glucose 6-phosphatase, another microsomal enzyme.

Effect of phenobarbital on UDP glucuronyltransferase. The effect of pretreating the rats with phenobarbital on the UDP glucuronyltransferase activity in the Triton X-100-treated postmitochondrial-supernatant enzyme preparation was tested. Both enzyme activity/g of liver and the liver weight/body weight ratio significantly increased after 4 days of phenobarbital treatment (Table 1). This increase occurred in the latent detergent-activated enzyme activity; the low free UDP glucuronyltransferase activity (0.06 unit/g of liver), measured in the absence of detergent, did not increase. In kinetic experiments I found no significant difference in the K_m values for *p*-nitrophenol of the enzyme from treated and untreated animals (Table 1). The K_m value is in good agreement with those reported in the literature (Isselbacher, Chrabas & Quinn, 1962; Lueders & Kuff, 1967; Halac & Reff, 1967).

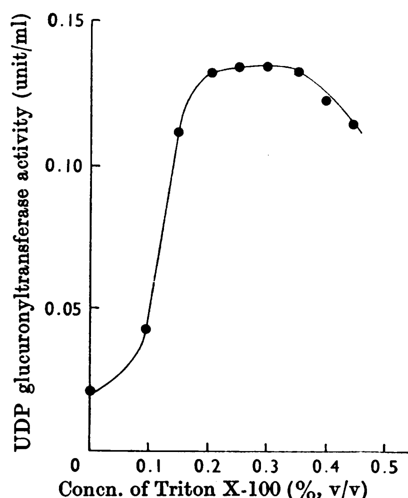

Fig. 1. Effect of Triton X-100 *in vitro* on UDP glucuronyltransferase activity. Various concentrations of Triton X-100 were added to the undiluted postmitochondrial supernatant in 0.154M-KCl. The UDP glucuronyltransferase activity is expressed as unit/ml of undiluted supernatant. The activity at zero Triton X-100 concentration was measured in the undiluted supernatant, at a concentration of 0.1% (v/v) Triton X-100 a 50% (v/v) supernatant, diluted with 0.154M-KCl, was used and at the higher concentrations of Triton X-100 a 25% supernatant was used.

Submicrosomal distribution of UDP glucuronyltransferase. The distributions of protein, UDP glucuronyltransferase and glucose 6-phosphatase are shown in Table 2. There is no difference in distribution and recovery between treated and untreated rats. About 46% of the UDP glucuronyltransferase activity and 36% of the glucose 6-phosphatase activity are recovered in the postmitochondrial supernatant in 0.25M-sucrose. The remainder of the activity is mainly in the nuclear fraction. The cause of this high percentage of both enzyme activities in this fraction is probably the presence of unbroken cells owing to the fact that the nuclear sediment was not washed and rehomogenized.

To test whether the Cs^{+} ions present in the submicrosomal distribution experiments might affect UDP glucuronyltransferase activity various concentrations of caesium chloride were added to the incubation mixture up to a concentration of 5mM, the highest to be expected in the experiments. It had no effect on UDP glucuronyltransferase activity.

The postmitochondrial supernatant of livers from untreated and phenobarbital-treated rats was applied to the sucrose density gradient and centrifugation distributions of RNA, protein and UDP glucuronyltransferase were obtained as given in Table 3. It is clear that whereas in untreated rats the rough-surfaced microsomal fraction contains the greater amount of the UDP glucuronyltransferase activity, the greater part of the increase in activity after the phenobarbital treatment is located in the smooth-surfaced fraction. As in both

Table 1. *Effect of phenobarbital pretreatment on UDP glucuronyltransferase activity*

The treated rats received phenobarbital pretreatment for 4 days (90mg/kg intraperitoneally daily). The postmitochondrial supernatant in 0.154M-KCl was used as enzyme preparation; enzymic activity was measured after activation with Triton X-100 *in vitro*. Results are given as means±S.E.M. with the numbers of animals used in parentheses. Statistical analysis was carried out by the procedure of Wilcoxon (1945).

Rats	Liver wt./body wt. ×100	Activity (unit/g of liver)	K_m for *p*-nitrophenol (mM)	$V_{max.}$ for *p*-nitrophenol (μmol/min per g of liver)
Untreated	4.45±0.14 (8)	0.58±0.01 (8)	0.62±0.10 (4)	1.07±0.10 (4)
Phenobarbital-treated	5.15±0.19 (8)	0.96±0.01 (8)	0.55±0.10 (4)	1.94±0.20 (4)
	$P<0.02$	$P<0.001$		

Table 2. *Distribution of protein, UDP glucuronyltransferase activity and glucose 6-phosphatase activity in the subcellular fractions*

A 20% homogenate in 0.25M-sucrose containing 32g of liver from six rats was fractionated as indicated in the Materials and Methods section. The absolute amounts of protein (expressed as equivalents of mg of bovine serum albumin), glucose 6-phosphatase (units) and UDP glucuronyltransferase (units) are given. UDP glucuronyltransferase was measured in the presence of Triton X-100. The recovery is the percentage of the protein or enzyme activity in the fractions recovered from the amount originally present in the homogenate. The treated rats received phenobarbital pretreatment for 4 days (90mg/kg intraperitoneally daily). All results are the means of three fractionations.

	Untreated rats			Phenobarbital-treated rats		
Fraction	Protein (mg)	UDP glucuronyltransferase (units)	Glucose 6-phosphatase (units)	Protein (mg)	UDP glucuronyltransferase (units)	Glucose 6-phosphatase (units)
Homogenate	7840	23.5	575	8130	45.3	410
Nuclear fraction	4170	10.3	325	4100	18.3	220
Mitochondria	720	1.8	80	765	4.8	50
Supernatant	2570	10.4	220	2890	20.2	150
Recovery	95%	95%	109%	96%	96%	103%

Table 3. *Submicrosomal distribution of protein, RNA and UDP glucuronyltransferase activity of the liver of untreated and phenobarbital-treated rats*

The postmitochondrial supernatant in 0.25M-sucrose corresponding to 12.4g of liver was subfractionated over a discontinuous sucrose density gradient in the presence of 15mM-CsCl. Four microsomal subfractions were obtained from the gradients. Absolute amounts of protein (expressed as equivalents of mg of bovine serum albumin), RNA (μg of ribose) and UDP glucuronyltransferase (units) per fraction are given. UDP glucuronyltransferase activity was measured in the presence of Triton X-100. All results are the means of three fractionations and for each fractionation six rat livers were pooled. The treated rats received phenobarbital for four days (90mg/kg intraperitoneally daily).

	Untreated rats			Phenobarbital-treated rats		
Fraction	RNA (μg of ribose)	Protein (mg)	UDP glucuronyltransferase (units)	RNA (μg of ribose)	Protein (mg)	UDP glucuronyltransferase (units)
Postmitochondrial supernatant	6290	1000	4.04	5740	1110	7.73
Fraction 1	700	600	0.18	500	600	0.20
Smooth-surfaced microsomes	400	104	0.82	660	177	2.86
Fraction 3	770	77	0.79	960	118	2.06
Rough-surfaced microsomes	4140	139	1.61	3060	129	2.36
Recovery	95%	92%	89%	90%	92%	97%

cases microsomes from 12.4g of liver were applied to the gradients, it is obvious that the amounts of microsomal protein and the enzyme activity/g of liver were increased; in contrast, the amount of RNA decreased. Of the increase in enzyme activity after the phenobarbital treatment 50% occurred in the smooth-surfaced microsomal fraction, 31% in fraction 3 and 19% in the rough-surfaced microsomal fraction. The increase in protein was mostly localized in the smooth-surfaced fraction and to a smaller extent in fraction 3; in the rough-surfaced fraction there was no increase at all. This explains the fact that, although there was a 3.5-fold increase in UDP glucuronyltransferase activity in the smooth-surfaced fraction against only a 1.5-fold increase in the rough-surfaced fraction, the specific activity of the enzyme in the smooth-surfaced fraction is only slightly more increased than in the rough-surfaced fraction. Compared with the specific activity of UDP glucuronyltransferase in the homogenate a

fourfold purification of the enzyme has been achieved in the rough-surfaced fraction.

All gradient fractions from untreated and treated rats were also tested for free UDP glucuronyltransferase activity in the absence of detergent. Though there was a low activity present (less than 3% conversion of *p*-nitrophenol in 15min), in none of these fractions was the free UDP glucuronyltransferase activity increased after phenobarbital treatment.

DISCUSSION

So far UDP glucuronyltransferase and NADPH-neotetrazolium oxidoreductase (Dallner, 1963) seem to be the only microsomal enzymes whose activation by detergents *in vitro* has been demonstrated. That this activation is not common to all microsomal enzymes is demonstrated by the lack of effect of Triton X-100 on glucose 6-phosphatase.

The values I have found for total UDP glucuronyltransferase activity are mostly higher than those reported in the literature. This difference is probably due to the use of Triton X-100 in the measurement of enzyme activity, whereas other authors seem to have measured an unknown fraction of the activity. However, Halac & Sicignano (1969), who activated their enzyme preparations by dialysis against EDTA–mercaptoethanol, obtained activities comparable with those reported here. On the other hand the published values for the free activities, measured in the absence of detergent, are much higher than mine. This may be caused by the repeated rehomogenization of the microsomal pellet that is used in most studies as the enzyme preparation. Thus White (1966) found unexpected activation of UDP glucuronyltransferase during her fractionation experiments. Moreover, the fact that Zeidenberg *et al.* (1967) found a distinct effect of phenobarbital pretreatment on UDP glucuronyltransferase activity from rat liver without using a detergent *in vitro* whereas I find no effect in that case might be explained by a Triton X-100-like effect of their repeated rehomogenization of the microsomal pellet. Because such varied procedures as treatment with detergents, ageing of microsomal preparations (Lueders & Kuff, 1967), EDTA treatment (Halac & Reff, 1967), ultrasonic vibration (Henderson & Dewaide, 1969), treatment of the microsomal fraction with snake venom (Isselbacher *et al.* 1962) and probably rehomogenization of the microsomal pellet seem to have the same activating effect on UDP glucuronyltransferase, the common mechanism may well be unfolding of the microsomal membranes, by which means the formerly latent enzyme becomes accessible to the substrates. If this is the case it would seem advisable to use a detergent, because slight variations in the homogenization procedure may cause variations in the degree of latency of UDP glucuronyltransferase *in vitro*.

Comparison between my results and those of Gram *et al.* (1968) is difficult because the rabbits they used did not respond to a phenobarbital treatment with respect to UDP glucuronyltransferase, because they reported their distribution data incompletely and because they did not use a detergent. However, they also found a higher specific activity of UDP glucuronyltransferase for the substrate *p*-nitrophenol in the rough-surfaced microsomal fraction from rabbit liver than in the smooth-surfaced fraction.

The decrease in the amount of RNA in the rough-surfaced microsomal fraction and the increase of that in the smooth-surfaced fraction after phenobarbital treatment found in the present work are well known to occur (Remmer & Merker, 1963; Dallman, Dallner, Bergstrand & Ernster, 1969), though in my experiments the RNA/protein ratio for the smooth-surfaced fraction remained constant after phenobarbital treatment. Thus the distribution of RNA over the gradient fractions changed remarkably after the treatment. According to Moulé (1968) some RNA is always attached to the smooth membranes in the liver cell, so it would not be unexpected that, when there is a very great increase in the smooth membranes in the liver cells (Stäubli, Hess & Weibel, 1969), more RNA becomes associated with the smooth-surfaced fraction.

Orrenius (1965) found that within 24h after the first phenobarbital injection some microsomal proteins, e.g. NADPH–cytochrome *c* reductase and cytochrome *P*-450, show an increase in activity. UDP glucuronyltransferase was shown to react only after the second injection, 48h after the first, with an increase in activity that is very small compared with the effect on the oxidative enzymes (Zeidenberg *et al.* 1967). This suggests that it may be caused by a mechanism different from that involving the oxidative enzymes. If the latency of the enzyme shown to exist *in vitro* by the effect of Triton X-100 were also present *in vivo*, the increase after phenobarbital treatment could be caused in some way by a detergent-like effect of the phenobarbital treatment *in vivo*. My finding that the increase in activity resided in the part of the enzyme that is latent *in vitro* makes such an explanation unlikely. It is difficult to explain the lack of effect of phenobarbital on the free UDP glucuronyltransferase activity; it could be a consequence of the very low activity of this enzyme fraction, that is at the limit of sensitivity of the method.

Whereas Howland & Burkhalter (1969) showed a decrease in the K_m of UDP glucuronyltransferase for *o*-aminophenol after 3-methylcholanthrene pretreatment of rats, I was unable to find an effect

of phenobarbital pretreatment on the K_m of UDP glucuronyltransferase for *p*-nitrophenol, thus giving no indication of altered enzyme characteristics as a result of phenobarbital pretreatment.

It is as yet impossible to decide whether the latency of UDP glucuronyltransferase *in vitro* is also present *in vivo* or whether it is an artifact of the homogenization procedure. However, it seems possible that many data on UDP glucuronyltransferase must be greatly modified by the use of detergents in the measurement of the enzyme activity, as exemplified by the effect of Triton X-100 and the effect of the carcinogen diethylnitrosamine on UDP glucuronyltransferase activity from the liver of Gunn rats towards *o*-aminophenol. Whereas enzyme preparations from the livers of these rats have a very low *o*-aminophenol-glucuronidating activity, this can be increased by diethylnitrosamine *in vitro* to the same value as that in the diethylnitrosamine-activated enzyme from normal rat liver (Stevenson, Greenwood & McEwen, 1968). Moreover, Halac & Sicignano (1969) who activated their enzyme preparations by dialysis against EDTA–mercaptoethanol, could not find the previously described (Inscoe & Axelrod, 1960) sex difference in the glucuronidation of *p*-nitrophenol and bilirubin in homogenates from male and female rat livers.

According to the results of Dutton (1966), Halac & Reff (1967), Catz & Yaffe (1968), Gram *et al.* (1968) and Howland & Burkhalter (1969) there should be a great number of different UDP glucuronyltransferases distinguishable by their substrate specificities. An attractive alternative to this would be that the difference in substrate specificity is not caused by different enzymes but by a different membrane environment in which the enzyme is located. This environment could well influence the K_m for a substrate, and different distributions of the enzyme activity between rough- and smooth-surfaced microsomal fractions for different substrates (Gram *et al.* 1968) could reflect differences in the membrane environment for the enzyme in the subfractions. Conversely, for a particular substrate the enzyme distribution would mean a difference in accessibility for this substrate rather than a real enzyme distribution. Of course there might be a limited number of different transferases with different membrane environments as accompanying specificity-determining phenomena, thus giving rise to a large number of seemingly different transferases. Moreover, one could postulate that a specificity-determining membrane environment is an essential part of an enzyme and consequently that the same enzyme molecule in different membrane environments could be called different enzymes. This remains to be investigated further.

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