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ACTIVATION ENERGY OF GLUCURONIDE BIOSYNTHESIS

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SUMMARY

1. UDPglucuronosyltransferase (*p*-nitrophenol) of rat liver was found to be resistant to temperatures up to 50 °C in native microsomes. In digitonin- and trypsin-treated membranes the resistance was lowered.

2. The apparent K_m for UDPglucuronic acid of UDPglucuronosyltransferase increased with the increasing temperature in the native microsomes, but it remained unchanged in digitonin- and trypsin-treated membranes. The apparent K_m for *p*-nitrophenol was the same in spite of the temperature change in the native membranes, but it increased with temperature in digitonin- and trypsin-treated microsomes. The V values were about 5 times higher in the digitonin- and trypsin-treated than in the native membranes at 37 °C.

3. There were no breaks in the Arrhenius plots in the temperature range studied. The activation energy of glucuronide biosynthesis catalyzed by UDPglucuronosyltransferase in the native microsomes for UDPglucuronic acid was 84 kJ/mole, and for *p*-nitrophenol a value of 78 kJ/mole was obtained. The digitonin and trypsin treatment of the membranes decreased the activation energy to 45 kJ/mole for both substrates. The lowering of the activation energy contributes to the increase in glucuronide biosynthesis after the digitonin and trypsin treatment of the microsomal membranes.

INTRODUCTION

UDPglucuronosyltransferase (EC 2.4.1.17) catalyses glucuronide biosynthesis. Recently the reaction has been shown to be reversible [1]. The enzyme is tightly bound to microsomal membranes. The measurable enzyme activity can be greatly increased by treating the membranes with surfactants [2–7], chaotropic agents [8], phospholipases [1, 9] and trypsin [6]. This suggests that the membrane environment has a great effect on the catalytic properties of the enzyme. UDPglucuronosyltransferase is both functionally and topochemically closely related to the microsomal mixed-function oxidase complex [10, 11]. Abrupt changes in K_m values in the hydroxylation of aniline and aminopyrine have been reported when the incubation temperature is increased [12]. Some enzymes in mitochondrial membranes also show marked changes in activation energy if the temperature is varied [13]. These changes have been

suggested to indicate a close relationship between the membrane lipids and respective enzymes.

In the present study the activation energy and some kinetic properties of UDP-glucuronosyltransferase were determined both in the native, surfactant and trypsin-pretreated membranes. The activation energy of the glucuronide biosynthesis has not been reported earlier.*

METHODS

About 4-month-old random-bred male Wistar rats (*Rattus norvegicus*) fed ad libitum were used. The microsomal fraction was isolated from the livers as described earlier with the difference that 0.25 M sucrose was used instead of 0.15 M KCl [3]. The microsomes were resuspended to give 35–45 mg of protein per ml. The biuret method was used in protein determinations [14]. Sodium deoxycholate (1.5%, w/v) was added to the reagent.

The digitonin treatment of the isolated liver microsomal fraction was carried out by adding 1.5 vol. of 1% aqueous digitonin (E. Merck AG, Darmstadt) solution [3]. After 30 min at 0 °C the microsomes were resedimented by centrifugation at 105 000 × *g* for 60 min at 0 °C and resuspended again in 0.25 M sucrose to give about 10 mg protein per ml.

Trypsin digestion of the membranes was carried out by adding 0.5 mg trypsin (3 times crystallized, Worthington Biochemical Co. Freehold, N.J.) to 0.5 ml of microsomal suspension in the presence of 0.10 M KCl and 7 mM potassium phosphate buffer, pH 7.0, during 30 min at 38 °C in a total volume of 0.75 ml. The reaction was stopped by adding 0.25 ml (2 mg/ml) of trypsin inhibitor (type II-0, Sigma Chemical Co., St. Louis, Mo.) in 20 mM potassium phosphate buffer, pH 7.0. The unsolubilized membrane fraction was then harvested by centrifugation at 105 000 × *g* for 60 min in a Spinco ultracentrifuge. The pellet was resuspended in 2 ml of 0.25 M sucrose.

The UDPglucuronosyltransferase (*p*-nitrophenol) activity was determined by using two different methods. When the concentration of the glucuronyl donor substrate, UDPglucuronic acid (UDPGlcUA), was varied, the activity was determined in 0.1 ml of a reaction mixture containing 0.75 mM *p*-nitrophenol, different concentrations UDPGlcUA and 10 mM EDTA (dipotassium salt) in 0.5 M potassium phosphate buffer adjusted to pH 7.0 to which the enzyme preparation was added in 25 μl (about 1 mg protein in native microsomes and about 0.2 mg in digitonin- and trypsin-treated microsomes). After incubation the reaction was stopped by adding 0.9 ml of 3% trichloroacetic acid. Before measuring the free *p*-nitrophenol, the samples were diluted with 1 ml of water. The initial reaction velocity was determined at different temperatures. Incubation times varied from 25 to 5 min depending on temperature. The same method was also used in assay in the case of the digitonin- and trypsin-treated membranes, when the *p*-nitrophenol concentration was varied in the presence of constant (3.6 mM) UDPglucuronic acid concentration. When the enzyme activity was measured from the native membranes in the presence of varying concentrations of *p*-nitrophenol, a more sensitive method with labeled *p*-nitrophenol (spec. act. 16.7

* A preliminary report has been given in the 9th International Congress of Biochemistry, Stockholm, 1–7 July, 1973.

Ci/mole, International Chemical and Nuclear Corporation, Irvine, Calif., U.S.A.) was used [15] due to difficulties in assay by the conventional method at low aglycone concentrations.

The Lineweaver and Burk's plots [16] have been calculated with the aid of a PDP 8/L computer by using the program (the least squares method) developed by Dr Antero Aitio, with the exception of the case when the labeled *p*-nitrophenol was used as substrate. For the estimation of the activation energies in the glucuronide biosynthesis Arrhenius plots have been used [17].

RESULTS

UDPglucuronosyltransferase showed considerable stability towards the increase of temperature in the native microsomal membranes isolated from the rat liver. After digitonin and trypsin treatment of the microsomes the stability was lowered, and the inactivation of the enzyme started earlier (Fig. 1). The UDPglucuronosyltransferase

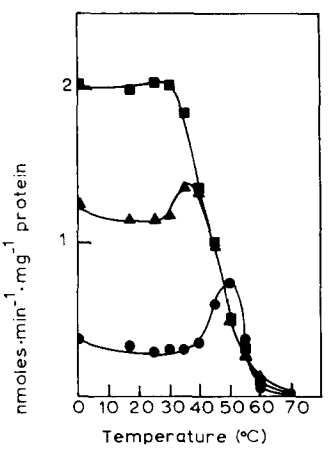
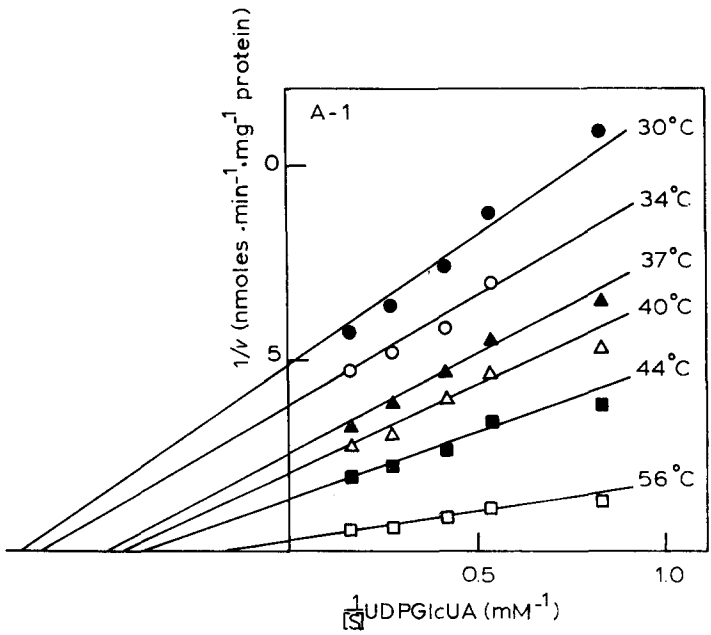


Fig. 1. The stability of UDPglucuronosyltransferase towards increasing temperatures in the native (●), digitonin- (■) and trypsin- (▲) treated membranes. The microsomal suspensions were pre-incubated at various temperatures for 10 min followed by assessment of remaining UDPglucuronosyltransferase activity under standard incubation conditions (10 min at 38 °C in the presence of 0.35 mM *p*-nitrophenol and 5 mM UDPGlcUA).

activity was measured for microsomal suspensions after pre-incubation at different temperatures for 10 min. During this time the autoactivation reached its maximum at 50 °C in untreated microsomes. A slight autoactivation was also noticed in trypsin-digested microsomes (Fig. 1).

The apparent K_m for UDPglucuronic acid of UDPglucuronosyltransferase increased linearily under the conditions used from 30 to 56 °C from a value 1.4–5.0 mM (Fig. 2A). The apparent $K_{UDPGlcUA}$, however, remained unchanged in the digitonin- (temperature range 26–44 °C) and trypsin- (temperature range 30–56 °C) treated membranes. The values obtained under the conditions used and substrate concentration range studied were 3.3 and 4.5 mM in the digitonin- and trypsin-treated microsomes, respectively (Fig. 2B and 2C).


A-1
1/v (nmoles .min-1.mg-1 protein)
0
5
30°C
34°C
37°C
40°C
44°C
56°C
0.5
1.0
1/[S] UDPGlcUA (mM-1)

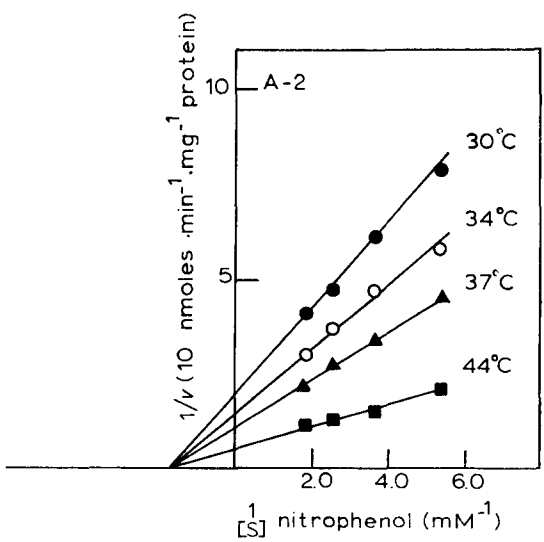
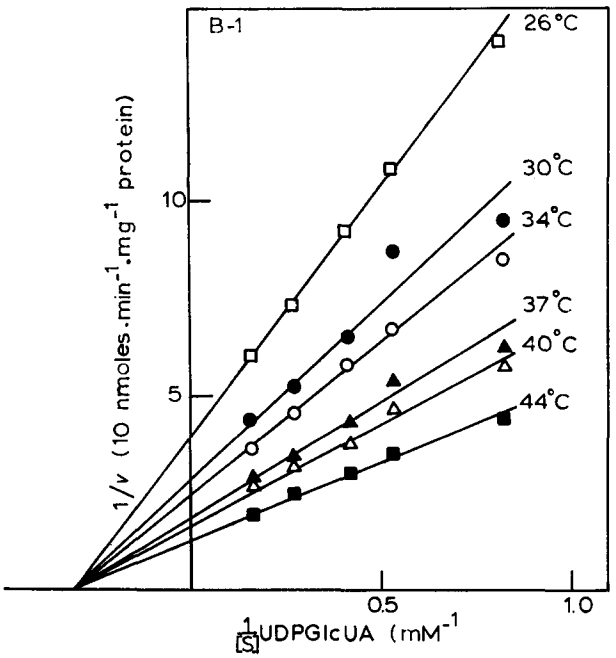

A-2
1/v (10 nmoles .min-1.mg-1 protein)
10
5
30°C
34°C
37°C
44°C
2.0
4.0
6.0
1/[S] nitrophenol (mM-1)

Fig. 2.


B-1
26°C
30°C
34°C
37°C
40°C
44°C
1/v (10 nmoles.min-1.mg-1 protein)
10
5
0.5
1.0
1/[S] UDPGlcUA (mM-1

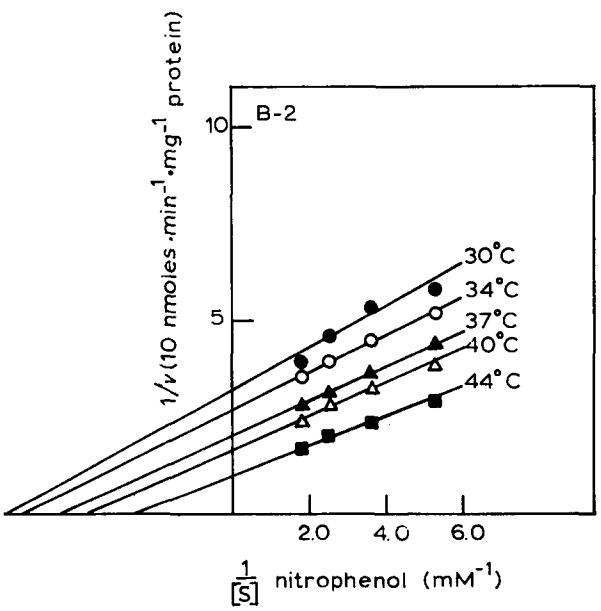
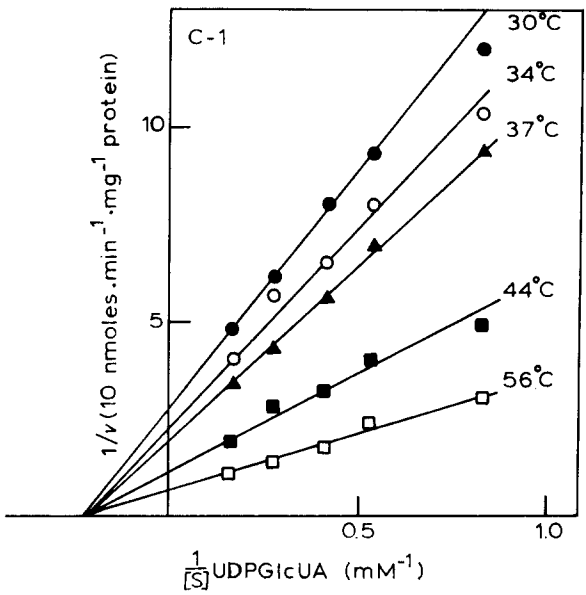

B-2
10
5
30°C
34°C
37°C
40°C
44°C
1/v (10 nmoles.min-1.mg-1 protein)
2.0
4.0
6.0
1/[S] nitrophenol (mM-1)

Fig. 2.



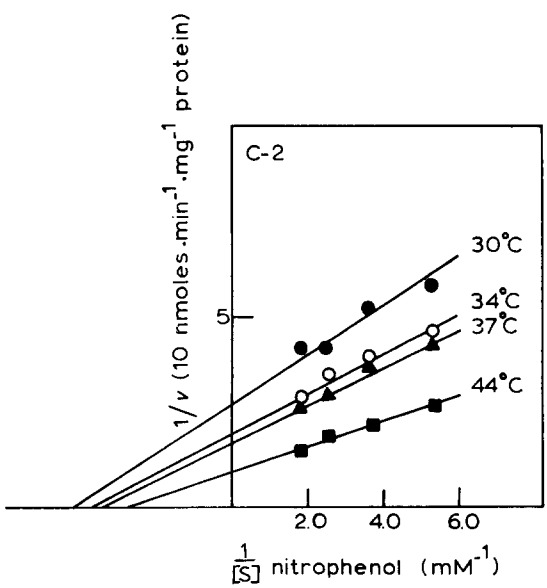


Fig. 2. The effect of varying temperatures on the *p*-nitrophenylglucuronide synthesis catalyzed by the membrane-bound UDPglucuronosyltransferase in the native (panels A-1 and A-2), digitonin-treated (B-1 and B-2) and trypsin-treated (C-1 and C-2) microsomes. The data have been plotted according to Lineweaver and Burk's method. When the kinetic constants were determined for the glucuronyl donor substrate, a fixed concentration of *p*-nitrophenol (0.75 mM) was used, and the concentrations of UDPglucuronic acid varied between 1.2 and 6.1 mM (panels A-1, B-1 and C-1). When the concentration of UDPGlcUA was fixed (3.6 mM), the concentrations of *p*-nitrophenol varied between 0.19 and 0.56 mM (panels A-2, B-2 and C-2).

The apparent K_m for *p*-nitrophenol 0.57 mM under the conditions used did not change in contrast to $K_{UDPGlcUA}$ in the native microsomal membranes, when the incubation temperature was increased from 30 to 44 °C (Fig. 2A). In the digitonin-treated membranes the apparent K_m for *p*-nitrophenol did, however, increase considerably from a value of 0.17 mM at 30 °C to a value of 0.28 mM at 44 °C (Fig. 2B). In the trypsin-predigested membranes an increase also took place, although it was smaller than in the digitonin-treated membranes. A value of 0.23 mM was observed at 30 °C and 0.35 mM at 44 °C (Fig. 2C).

The V of the glucuronide biosynthesis extrapolated from the data obtained by using the Lineweaver and Burk's plots increased exponentially with the increase in temperature both in the native and digitonin- or trypsin-treated membranes. Digitonin and trypsin treatments of the microsomes considerably increased the V values. This was especially the case at lower temperatures. The increase in V with temperature was lesser, when the digitonin- and trypsin-treated membranes were used as the enzyme source and UDPglucuronic acid was a variable substrate. For some reason the V values of the glucuronide synthesis by UDPglucuronosyltransferase in the native membranes calculated from the values obtained after a variation of the acceptor substrate are somewhat higher than from the experiments where the glucuronyl donor substrate concentration has been varied (Fig. 2A).

When the present data were used in Arrhenius plots to estimate the activation energies in the glucuronide biosynthesis under the various conditions used, the spots fitted in linear lines within the experimental error in the temperature range studied

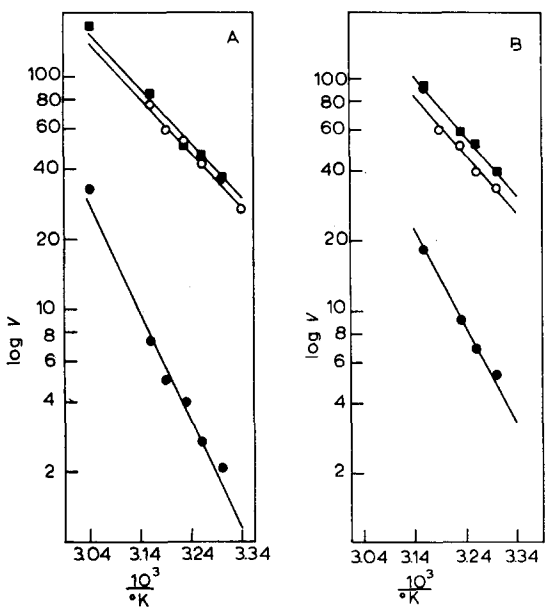


Fig. 3. The Arrhenius plots used for the calculation of the glucuronide biosynthesis. The values of V at each temperature were extrapolated from the data in the plots shown in Fig. 2. The values of the activation energy of the glucuronide biosynthesis catalyzed by UDPglucuronosyltransferase in native (●), digitonin-treated (■) and trypsin-treated (○) microsomal membranes have been depicted in panel A for the glucuronyl donor substrate and in panel B for the glucuronyl acceptor substrate.

(Fig. 3A and B). The activation energy value for the glucuronyl donor substrate was 84 kJ/mole, when the glucuronide synthesis was catalyzed by UDPglucuronosyltransferase in the native microsomes (Fig. 3A). If the membranes were treated either with digitonin or trypsin, there was a drastic decrease in the activation energy to a value 45 kJ/mole (Fig. 3A). The value of activation energy obtained for the glucuronyl acceptor substrate *p*-nitrophenol was somewhat lower than for the glucuronyl donor substrate in the reaction catalyzed by the enzyme in the native membranes (78 kJ/mole) (Fig. 3B). After the treatment of the microsomes with either the surfactant or protease, the activation energy decreased to the same value 45 kJ/mole as found for the glucuronyl donor substrate (Fig. 3B).

DISCUSSION

The stability of UDPglucuronosyltransferase towards increasing temperatures decreased when the native microsomes were treated with digitonin or trypsin. In native microsomes the spontaneous autoactivation of UDPglucuronosyltransferase takes place even at 0 °C [6]. The present results indicate that the increase of temperature appears to promote autoactivation. Digitonin, which is a cholesterol-complexing neutral surfactant, dissolves only minor amounts of microsomal proteins and no UDPglucuronosyltransferase [3, 6, 10]. It appears, however, to labilize the enzyme by interacting with the membrane or the enzyme itself. Trypsin is known to peal off certain surface proteins of the microsomes [18, 19], but it does not solubilize UDP-glucuronosyltransferase [6, 20]. The loss of protecting membrane components probably explains in this case the decrease of temperature stability of the enzyme.

The increase in temperature does not appear to affect that part of UDP-glucuronosyltransferase which binds the glucuronyl acceptor substrate in native membranes. The apparent K_m values for *p*-nitrophenol did not change with temperature. The affinity for *p*-nitrophenol did, however, decrease with increasing temperature after a digitonin and trypsin pretreatment of the membranes. Digitonin especially appeared to affect directly or indirectly the algycone binding site of the enzyme. There is evidence that the catalytic site is lipophilic or that it is behind a lipophilic barrier [21]. In contrast to aglycone binding the apparent affinity of the enzyme for the glucuronyl donor substrate decreased with the increasing temperature in the native membranes due to the changes in the catalytic site itself or in its membrane environment. The digitonin and trypsin treatments abolished the temperature sensitivity of the enzyme in this respect. Trypsin treatment probably promotes the penetration of charged glucuronyl donor substrate to the enzyme by removing the overlaying proteinous membrane components. It has been suggested that most UDPglucuronosyltransferase molecules are located in the deep layers of the membrane structure or the inner surface of the vesicles [6, 20, 21]. A diminution of the interactions of the enzyme with other membrane components is probable. Another possibility is that the hydrolysis of UDPGlcUA rapidly increases with temperature in the native membranes, but not in the treated membranes. This could lead to an apparent lessening of the affinity of UDPglucuronosyltransferase for UDPGlcUA.

The K_m plots versus temperature of the microsomal drug hydroxylation have revealed abrupt changes at temperatures between 28 and 37 °C in case of aniline and

aminopyrine oxidation [12], although there is not complete agreement with the results of some other workers [22]. In the case of UDPglucuronosyltransferase no jumps could be observed in the present study.

The treatment of microsomal membranes by digitonin or trypsin mostly increased the V values of the glucuronide synthesis as has already been previously reported [3, 6]. It has been suggested that the treatment uncovers new enzyme molecules in the membrane which are dormant in the native microsomes [6]. There are some variations in the K_m values for both the glucuronyl donor and acceptor substrates reported in the literature [5, 6, 23–26] and also transitions in the Lineweaver and Burk's plots have been described [25, 27]. An intimate relationship between the enzyme and its microenvironment exists and the values obtained are affected by the reaction conditions used and the state of the membrane. There are also data although not thoroughly convincing that more than one enzyme might catalyze the glucuronide synthesis [28]. In spite of the changes in the substrate affinity of UDPglucuronosyltransferase with temperature under the experimental conditions the Arrhenius plots obtained were linear in the temperature range studied both for the native and treated microsomes. This speaks against the presence of two or more different enzymes catalyzing the conjugation of *p*-nitrophenol, since it is not very probable that different enzymes could have similar activation energies [12, 16].

Several mitochondrial membrane activities show breaks in Arrhenius plot as Lenaz et al. [13] have shown. These breaks have been suggested to indicate an intimate relationship between the state of the membrane lipids and the respective enzyme proteins. There is no doubt about the effect of various membrane lipids and their components on UDPglucuronosyltransferase [1, 9, 29]. The partially purified UDPglucuronosyltransferase preparations also contain lipid components [30]. The relationship between UDPglucuronosyltransferase and the membrane lipids does, however, appear to be different from some other membrane enzymes.

The activation energy of glucuronic acid conjugation catalyzed by the UDPglucuronosyltransferase in the native microsomes is according to the present results similar or somewhat higher than the activation energy of drug hydroxylation reported in the literature (54–88 kJ/mole) depending on the substrate [12, 22]. The treatment of the microsomal membranes either with digitonin or trypsin appeared to diminish drastically the activation energy of the glucuronide biosynthesis, and a value lower than in drug hydroxylation was obtained. Especially in the case of the glucuronyl donor substrate the activation energy was much reduced. UDPglucuronic acid is a large and highly charged molecule and its penetration into the membrane is most probably difficult [31]. The glucuronyl acceptor substrates are lipid soluble and the affinity of the enzyme increases with their lipophility [21]. The action mechanisms of the in vitro activators of UDPglucuronosyltransferase used in the present study favor the idea that they decrease the activation energy of the glucuronide biosynthesis by interactions in the membrane structure, since the enzyme remains in the membrane without dislocation into solution. The decrease in the apparent activation energy of glucuronide biosynthesis might also be possible if the unmasked UDPglucuronosyltransferase had a much lower activation energy than the enzyme active in the native microsomal membrane.

The increase in the measurable activity of UDPglucuronosyltransferase in the microsomal membrane after digitonin and trypsin treatments is most probably to a

considerable degree due to the decrease in the activation energy in glucuronide biosynthesis.

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