mounted in a saturated solution of sucrose in distilled water for phase and fluorescence microscopy.

For the chimera tadpoles cultivated for 35 days, the mature thymuses were carefully removed and fixed in Carnoy's solution for 30 min. The tissue blocks were dehydrated through absolute ethanol and embedded in Epon 812 resin. 1-μm plastic sections were cut on a Porter Blum MT-1 Ultramicrotome and stained with the fluorescence dye quinacrine as stated above. For localization purpose the sections were stained with 1% toluidine blue after fluorescence examination. A Nikon FL Fluorescence Microscope was used to examine the sections.

Results and discussion. On the basis of the staining characteristics of cell nuclei with the fluorescence dye quinacrine, each nucleus of *X. borealis* in *X. laevis- X. borealis* chimerae could be clearly identified. As described by Thiébaud[5], the nuclei of *X. borealis* exhibited a number of bright fluorescent spots against a homogeneous background, while the stained nuclei of *X. laevis* fluoresced only homogeneously (figs. 1b, 2b and 3b). Thus, this method was effective for marking migrating cells in chimera larvae. In 20 chimera larvae cultivated for 3 days after the preparation, immigration of cells of *X. borealis*-origin into the thymic rudiment was observed in 6 cases (30%), while in the remaining 14 cases immigration did not occur. Figure 1b indicated that the primary rudiment of the thymus was composed of cells of *X. laevis*-origin only, i.e. from the anterior part of chimera embryos. This agreed with the conception that the thymus arises as a pair of dorsal buddings of the epithelium from the second visceral pouches. As shown in figure 2, in all chimerae (13 cases) cultivated for 4 days after the preparation, the cells possessing bright spots, which arose from the posterior part of chimera embryos, were observed in the primary thymic rudiment. Because chimera larvae were produced from 22-h-old embryos of two *Xenopus* species, thereafter cultivated for 3 and 4 days, these corresponded to 4- and 5-day-old larvae which had developed normally. Thus it was concluded that extraneous cells immigrated into the thymic rudiment 4 days after fertilization. Except for the thymic rudiment, largest number of the cells derived from *X. borealis* were present in the vascular system.

To investigate further the differentiation course of immigrating cells, mature thymuses of chimera tadpoles cultivated for 35 days after preparation were embedded in epon resin, and compared using both quinacrine fluorescence and toluidine blue observations in the same section. According to Rimmer et al.[8], at 30 days of age larval thymus displays a clearly defined corticmedullary differentiation with an abundance of small lymphocytes. Figure 3 showed that the majority of thymic lymphocytes were progeny of the cells which had immigrated into the thymic rudiment in the early embryonic stage (4 days after fertilization). Hence, it might be expected that lymphoid precursor cells of blood island-origin enter the thymic rudiment during the early development stage.

There is still a serious discrepancy between different authors on this subject. In line with the experiments by Le Douarin and Jotereau[3] and by Tochinai[4], our data supported the immigration of lymphocyte precursor cells into the thymic rudiment. Turpen et al.[2] have, however, advocated the intrinsic origin of thymic lymphocytes, in *Rana pipiens*. If thymic lymphocytes arise in situ from the thymic rudiment itself in *Xenopus* as in *Rana pipiens*, the lymphocytes of chimera embryos in this experiments should be from the *X. laevis* side. On the contrary, the cells possessing bright spots from the *X. borealis* side were actually observed in the thymic rudiment, as shown in figure 2b. Further, figure 3 proposes that the immigrating cells differentiated into thymic lymphocytes during subsequent thymus development. The apparent discrepancy may be the result of a species difference. In the present study, the procedures using epon-embedding materials for localization provided a new possibility for electron microscopic studies of migrating cells.

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Carbon tetrachloride modulates the rat hepatic microsomal UDP-glucuronyl transferase activity and membrane fluidity[1]

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Summary. Modulations in rat hepatic microsomal UDP-glucuronyl transferase activity have been observed during carbon tetrachloride (CCl_4) poisoning, with a large decrease in the enzyme cooperativity and increase in the membrane fluidity, occurring 30 min after administration. The results strengthen the possibility that an increase in microsomal membrane fluidity may be an early key event in liver injury induced by CCl_4.

Key words. UDP-glucuronyl transferase; carbon tetrachloride; membrane fluidity; rat hepatic microsomal membrane.

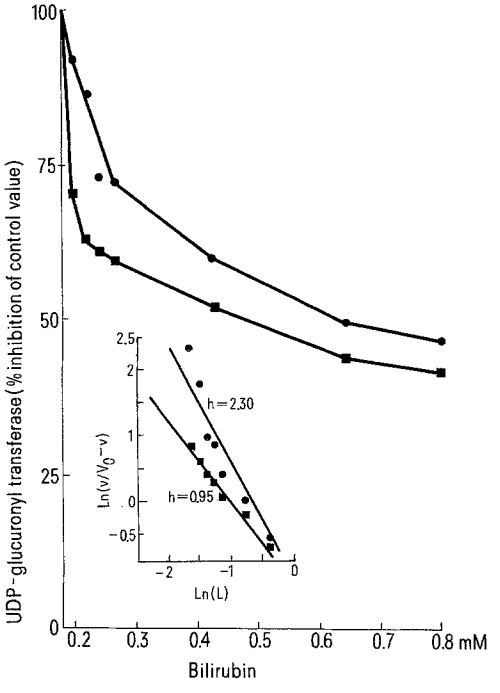
UDP-glucuronyl transferase (EC 2.4.1.17) is an integral enzyme, or group of related enzymes, of the endoplasmic reticulum membrane of liver cells catalyzing the reversible transfer of the glucuronate grouping from UDP-glucuronate to a wide variety of poorly water soluble, nucleophilic acceptors[2,3]. There is considerable evidence that the phospholipids of the microsomal membranes are important for efficient function of UDP-glucuronyl transferase[4]. Because phospholipids, depending on their composition, have variable effects on the activity of UDP-glucuronyl transferase, it is indicated that lipid-protein interactions could modulate the substrate binding and catalytic properties of the enzyme[5]. The initial events of liver injury induced by CCl_4 are thought to stem directly from carbon-halogen bond cleavage by the cytochrome P-450 mixed function oxidase system in the liver smooth endoplasmic reticulum. The free radical product of the interaction leads to the initiation of peroxidation of adjacent membrane lipids[6]. Various pathological phenomena, including disruption of the endoplasmic reticulum, loss of associated en-

zyme functions, triglyceride accumulation, and breakdown of the cell membrane, result from these initial events via unknown secondary mechanisms[7].

In our previous studies we have shown that lipid bilayer fluidity and liquid-crystal gel phase transitions play an important role in modifying the properties of many membrane bound enzymes[8-12]. Phospholipids are predominant constituents of membranes and their fluidity may depend on the fatty acid composition[13]. Changes in membrane conformation and protein-lipid interactions can be detected by physical methods[14] and also enzymatically by evaluation of changes in Hill coefficients of membrane-bound allosteric enzymes[8,9,11,15]. In the present study microscopic changes in the relationship between hepatic microsomal proteins and their associated lipids induced by CCl_4 administration in vivo were estimated, enzymatically by measuring the Hill coefficient of the UDP-glucuronyl transferase, and also by a physical method which measures the degree of fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene (DPH).

Materials and methods. Male Wistar rats (270–325 g) were used. Sodium phenobarbital (0.5 g/l) was given to the animals in their drinking water for 15 days before they were killed. Before killing, the animals were fasted for 12 h. CCl_4 (1 ml/kg) was given i.p. 30 min before killing. Animals were killed by stunning followed by decapitation and exsanguination. The liver was quickly weighed, minced with scissors and chilled on ice. Homogenates (20% w/v) were prepared in 0.25 M sucrose/0.05 Tris·HCl ph 7.8/0.01 M Na_2EDTA. The preparations were centrifuged at 15,000 × g for 20 min, at 4 °C. The supernatant was aspirated and centrifuged at 105,000 × g for 60 min at 4 °C in Beckman L5-75 Ultracentrifuge. The resulting pellet was resuspended in 0.25 M sucrose/0.05 M Tris·HCl pH 7.8 at a concentration of 10 mg of microsomal protein per ml. The protein content was determined by the Lowry method as described by Miller[16] with bovine serum albumin (Sigma) as standard. Microsomes were stored at −70 °C and used within 2 weeks. Microsomal aliquots were separated and used for estimation of membrane fluidity. Microsomes used for the determination of UDP-glucuronyl transferase activity were treated as follows: Solid deoxycholate (0.3% w/v) was added to the microsomal suspension and stirred with a magnetic stirrer for 4 h at 4 °C. Thereafter, the suspension was centrifuged at 105,000 × g for 60 min at 4 °C. The clear phase of the supernatant was used for the assay of the UDP-glucuronyl transferase activity.

Enzyme assay. UDP-glucuronyl transferase activity towards bilirubin was measured by the method of Tavoloni et al.[17]. The incubation mixture (total volume 0.80 ml) contained 0.25 ml triethanolamine·HCl buffer pH 7.9, 0.25 ml 20 mM UDP-glucuronic acid, 0.05 ml 0.2 M $MgCl_2$ and 0.25 ml of bilirubin solution at final concentration range (0.042–0.8 mM) in 2.5% bovine serum albumin pH 7.9. The reaction was started by the addition of 0.2 ml of microsomes solubilized by deoxycholate and the mixture was incubated with shaking at 37 °C for exactly 30 min. The reaction was terminated by the addition of 0.3 ml glycine-HCl buffer pH 2.6 and cooled quickly in an ice bath. After removal from the ice the samples were transferred to a water bath at 28 °C and submitted to the diazo reaction according to Van Roy and Heirwegh[18] with minor modifications. After 10 min the mixtures were placed at 28 °C in a water bath, 0.5 ml of diazo reagent were added and the diazotation was allowed to proceed for exactly 20 min. The reaction was stopped by adding 1 ml of 8% ascorbic acid solution. After 10 min 2.5 ml of extraction mixture (2-pentanon:n-butyl acetate 85:15 v/v) were added and the tubes shaked mechanically for 1 min. The extraction was continued by rotating mechanically for 30 min. The upper layer was separated by centrifugation at 3,000 × g for 15 min and its absorbance at 530 nm was measured against blanks which were treated in the same way except that 0.2 ml 0.25 M glucose/0.005 M Tris·HCl pH 7.8 was used instead of the sample. All assays were run in duplicate. For the assay of the inhibition by bilirubin of the UDP-glucuronyl transferase the reaction mixture contained increasing amounts of bilirubin as indicated in the figure.



Effect of bilirubin on the reaction rate of the UDP-glucuronyl transferase for carbon tetrachloride treated microsomes (■—■) and untreated microsomes (control; ●—●). The insert shows Hill plots of the same data. Corresponding Hill coefficients (h) are as indicated. The correlation coefficients (r^2) for the straight lines in the insert are > 0.95. V is the reaction velocity, and V^o is the rate of the reaction in which bilirubin had no inhibitory effect on the enzyme activity. Points in the curves drawn are mean values of duplicated determinations from a typical experiment which has been repeated 3 times.

Bilirubin solutions were prepared as follows: the appropriate amount of bilirubin was initially dissolved in a few drops of 0.25 M NaOH and rapidly transferred to a 2.5% solution of bovine serum albumin. Then the pH was adjusted to 7.9. The solution was freshly prepared. Diazo reagent was prepared by the addition of 0.3 ml 0.5% $NaNO_2$ (freshly prepared) to ethyl anthranilate suspension (0.1 ml ethyl anthranilate was suspended in 10 ml 0.15 N HCl) and left for 5 min. Ammonium sulphamate 17.1% (0.1 ml) was then added and the reagent used within 3 min.

Steady-state fluorescence polarization. The method used was that of Shinitzky and Barenholz[14], with some modifications. The evolution of the microsomal membrane fluidity was inferred by the evolution of fluorescence polarization (P) of 1,5-diphenyl-1,3,5-hexatriene (DPH) buried in the hydrocarbon core of the lipid bilayer.

Fluorescence labeling of DPH was carried out as follows: 2 mM DPH in tetrahydrofuran was diluted 1:1000 just before use with 50 mM Tris·HCl pH 7.4, then mixed in a 1:1 ratio with the microsomal suspension to give a final protein concentration of 50 µg/ml. The mixture was incubated at 35 °C for 45 min. Incorporation of DPH was followed by an increase of the fluorescence signal which is constant after an incubation time of 40 min. Experiments were carried out at 35 °C in an AMINCO SPF-500 Spectrofluorometer with polarization accessory for fluorescence polarization measurments. This apparatus is designed to give directly the degree of fluorescence polarization (P) following the equation:

$$P = \frac{I_{VV} - I_{VH}}{I_{VV} + I_{VH}}$$

The subscripts (V, H) of the emission intensities refer to the vertical and horizontal polarization of the excitation and emission light, respectively. The excitation and emission wavelengths were 366 and 428 nm, respectively. Control samples of DPH suspensions and of unlabeled microsomes were examined in each experiment. These light intensities, which amounted to approximately 3% of the total fluorescence, were neglected.

Results and discussion. It has been demonstrated that the lipid composition of the endoplasmic reticulum membrane is easily modified by physiological and non-physiological factors[8,19], and that changes in the microenvironment of membrane-bound enzymes can have a significant effect on enzyme activity[10,20]. Therefore, one of the most important problems concerning endoplasmic reticulum biochemistry is to understand and explain the intimate mechanism of the membrane function dependent on lipid-protein interactions. Such interactions may cause conformational changes in the proteins involved, thereby modulating the activity of enzymes and transport units.

Carbon tetrachloride is known as a hepatotoxic agent[21]. During its metabolism a reactive radical is formed which causes lipid peroxidation in the microsomal membranes. Since the activity of microsomal UDP-glucuronyl transferase is constrained by the phospholipids of the membrane[4,5,22], it was of interest to investigate the effect of CCl_4 treatment on the physical properties of microsomal membranes using as a functional parameter the activity of UDP-glucuronyl transferase.

The cooperative behavior of several membrane bound enzymes could be used as a tool to detect modifications at the cell membrane level, since variations in the Hill coefficient in cooperative membrane enzymes depend on their relationship to lipid and on the fluidity of the latter[8,9,11,15]. UDP-glucuronyl transferase activity exhibits a biphasic curve of changes at different concentrations of its substrate bilirubin. Low bilirubin concentrations (0.042–0.171 mM) evoked an increase of the enzyme activity, higher concentrations (up to 0.8 mM), however, effected a progressive allosteric inhibition (up to 55%) with respect to the maximal stimulation.

The allosteric inhibition of UDP-glucuronyl transferase activity by bilirubin was studied to detect a possible influence of the administration of CCl_4 to rats on hepatic microsomal membrane fluidity. Several preliminary experiments were conducted here to indicate that bilirubin allosterically inhibits the UDP-glucuronyl transferase and that this co-operative behavior of the enzyme could be used as a tool to detect possible modulations of the membrane fluidity. The figure shows the curves obtained when the relative rates of the enzymatic activity were plotted against different concentrations of bilirubin. The Hill coefficient h (slope of the plot) for the control (not treated) animals was $h = 2.30 \pm 0.25$ indicating the presence of co-operativity, which was reduced to $h = 0.93 \pm 0.10$ in CCl_4-treated animals, indicating a loss of co-operativity in the enzyme, consistent with a general increase of the membrane fluidity.

The physical properties of the microsomal membranes were studied by steady-state fluorescence polarization[14] using 1,6-diphenyl-1,3,5-hexatriene (DPH) probe. Although this method is now routinely performed in many laboratories, care must be taken in its application in biological membranes. The steady-state fluorescence polarization (P) of diphenyl-hexatriene-labeled membranes cannot be interpreted in terms of microviscosity, i.e., the rate rotational diffusion of the probe, but in terms of membrane lipid order (the reciprocal of lipid fluidity), reflecting the degree to which the fluorophore rotations are restricted to a cone formed by the molecular packing of the phospholipids[23]. The present results show a statistically significant decrease of the fluorescence polarization (P) values in microsomal membranes isolated from CCl_4 treated animals, $P = 0.145 \pm 0.007$ ($p < 0.01$), when compared to control microsomal membranes (animals not treated with CCl_4), $P = 0.199 \pm 0.004$. Therefore CCl_4 treatment significantly increased liver microsomal membrane fluidity as indicated by the decrease of P-values.

Microsomal aliquots were separated and further purified by the method of Glaumann et al.[24], which includes osmotic shock by suspension in water and gentle sonication. This preparation was used to check the membrane purity and absence of trapped non-membrane constituents. Fluorescence polarization studies of this highly purified membrane gave results not significantly different from those for the washed microsomes. Therefore, washed microsomes were used throughout the experiments.

In the present study, the UDP-glucuronyl transferase activity was slightly decreased (approximately 20%) by the CCl_4 (1 ml/kg), within 30 min after its administration to the rats. Moore et al.[25] have shown that the hepatic smooth endoplasmic reticulum can sequester calcium and that this ability has decreased severely within 30 min after CCl_4 administration to rats. It was suggested that disturbed endoplasmic reticulum calcium pump activity may have a critical role in the expression of CCl_4 hepatotoxicity. The time course of the disruption of the calcium pump was essentially identical to the time course of microsomal membrane fluidity alterations shown in the present study. Therefore, we suggest that the fluidity of microsomal lipid bilayer may be involved in the alteration of calcium pump activity. This conclusion is supported by our previous findings that microsomal Ca^{2+}-ATPase is an integral protein of the membrane and that cholesterol could interfere with the enzyme function[10]. Moreover, decrease of UDP-glucuronyl transferase activity by means of increase in the microsomal membrane fluidity would have implications for the glucuronidation of drugs and xenobiotics. A disturbance in glucuronidation reactions in the presence of toxigenic CCl_4 in vivo may be a key event in the resulting cellular dysfunction. The area of liver glucuronidation must be further considered in the study of mechanisms of hepatotoxicity induced by CCl_4.

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