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Correlation between the Biliary Excretion of Ouabain and the Lateral Mobility of Hepatocyte Plasma Membrane Proteins in the Rat—The Effects of Age and Spironolactone Pretreatment

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The biliary excretion of intravenously injected ouabain and the diffusion constant of the lateral mobility of hepatocyte plasma membrane proteins were examined in control (saline-treated) and spironolactone-treated Wistar male rats of different ages (4, 14 to 15 and 24 months old). The biliary excretion of ouabain progressively decreased with age in control rats, the first 10-min biliary recovery in 24-month-old animals being one-third that of the youngest rats (4-month-old). The oral administration of spironolactone for 4 days (10 mg per 100 gm body weight on the first day and 20 mg per 100 gm body weight for the successive 3 days) caused a marked increase in the biliary recovery of ouabain in all age groups. Similarly, the average lateral diffusion constant of hepatocyte plasma membrane proteins as measured by fluorescence recovery after photobleaching showed a linear decrease with age, as was previously observed with F-344 rats of both sexes. Markedly and significantly (30 to 40%) higher diffusion constants were observed in rats pretreated with spironolactone for all three age groups, compared with the respective control values of corresponding ages. The parallelism between ouabain excretion and protein diffusion (i.e., a decrease with age and an increase with spironolactone pretreatment) suggests that the lateral mobility of proteins in the hepatocyte plasma membrane is a candidate mechanism for regulating ouabain excretion through the liver into the bile, most probably by regulating the hepatic uptake process for ouabain.

It is generally assumed that there are at least three mutually independent pathways through the hepatocyte canalicular elimination system for organic anions, cations and neutral compounds (1, 2). The exact mechanisms of these transport pathways, however, remain largely unelucidated. It is also assumed that different carriers specific to particular materials or groups of substances of similar chemical characteristics are present in hepatocyte surface membranes (1, 2). Several candidates for these carrier proteins have been reported for bile salts and organic anion dyes (3–5). For the majority of substances, however, such a carrier (or binding) protein has not been identified. In the rat, for example, ouabain (a neutral cardiac glycoside) is known to be excreted into the bile very efficiently without any biotransformation prior to its biliary excretion (6–9). However, attempts to identify intracellular protein fractions specifically bound to ouabain have not been successful (7). Nor is it known what energy source is the specific driving force of this efficient transport system.

It has been repeatedly reported that the administration of spironolactone (Sp) and pregnenolone 16α-carbonitrile increases the biliary recovery of i.v. administered ouabain as well as the bile flow rate in the rat (6, 9). Recently, the physical-chemical characteristic of the hepatocyte plasma membrane (membrane microviscosity as assessed by a lipid probe) has been proposed as a regulatory factor for the biliary excretion of materials (10).

Interestingly, pretreatment with Sp is reported to decrease (rather than increase) the fluidity of the rat hepatocyte plasma membrane (11). This suggests that a change in lipid fluidity may not play an important role in determining the biliary excretion of ouabain or in bile formation, since an increase in fluidity should be induced by this treatment if it is to enhance the biliary excretory function. We have reported that the biliary excretion of i.v. injected ouabain progressively decreased with age in male Wistar rats of 3 to 24 months (9). Furthermore, we recently found that the diffusion constant of the lateral mobility of hepatocyte plasma membrane proteins, as assessed by fluorescence recovery after photobleaching (FRAP), progressively decreases with age in a virtually linear fashion in both male and female Fischer 344 rats (12–14). This finding is important for one theory of aging, since such a physical-chemical alteration of the cell surface membrane was predicted theoretically and found in experimental data (15, 16). Furthermore, as the lateral mobility characterization of membrane proteins has never been reported for compact tissues such as the liver, it seemed important to further clarify its physiological significance. The present study was aimed at elucidating the relationship between the physical-chem-

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ical and physiological properties of the hepatocyte plasma membrane by examining these parameters in control rats of different ages and in rats pretreated with Sp.

In examining the protein diffusion constant of the hepatocyte plasma membrane in rats of different ages, we wanted to determine first of all the reproducibility of the age-dependent decrease in this parameter that we observed previously in rats of a different strain (F-344) (12, 13). Furthermore, we hoped that the manipulation of the membrane quality by the administration of Sp would allow us to compare changes in biliary excretion of ouabain and the diffusion constant.

MATERIALS AND METHODS

Male Wistar derived rats of three different ages (4, 14 to 15 and 24 months old) were used throughout. The animals were bred and raised in the aging farm of the institute in a specific pathogen-free condition with 12-hr lighting. The husbandry conditions for these animals are the same as those described elsewhere for Fischer 344 rats (17). The 50% survival of these animals was about 26 months, and the longest survival ever recorded was 38 months. Pathologies found in the later period of their lives are also described elsewhere (18).

SP Pretreatment

Animals were brought from the aging farm to a clean, conventional animal house with a unidirected filtered air supply. Other husbandry conditions were the same as for the specific pathogen-free aging facility. Sp powder (Sigma Chemical Co., St. Louis, Mo.) suspended in distilled water (10 mg per ml) was administered orally by intubation for four successive days. On the first day, only 10 mg per 100 gm body weight were given. This was followed by 20 mg per 100 gm per day (10 mg per 100 dose, twice daily) for the following 3 days. Control rats were given distilled water of the same volume as that of the Sp suspension. All experiments for the FRAP study as well as ouabain excretion were done on the fifth day.

Ouabain Excretion

Rats were anesthetized with pentobarbital (Nembutal, Abbott Laboratories, North Chicago, Ill.). The common bile duct was cannulated with PE-50 tubing (Clay Adams, B-D, Parsippany, NJ). A femoral vein and artery were also cannulated with PE-10 and PE-50 tubings, respectively. Another catheter (PE-10) was inserted into a jugular vein for infusing physiological saline to compensate for the loss of body water by bile collection.

[^{3}H]Ouabain (NEN, Boston, Mass.) mixed with ouabain octahydrate was dissolved in distilled water at a concentration of 1 mg per ml. After obtaining a basal 10-min bile sample, the ouabain solution was i.v. injected through the femoral vein catheter at a dose of 0.1 mg per 100 gm body weight in the course of 10 to 15 sec. The femoral vein catheter was immediately flushed with a small amount of saline. Bile was collected at 10-min intervals for 60 min. Blood samples were obtained at appropriate time intervals after the injection through the femoral artery cannula. After the 60-min collection period, the rats were killed, and the liver, heart and both kidneys were removed and weighed. The ouabain concentrations in the bile and plasma samples were determined from the specific activities of samples measured by a liquid scintillation counter (9). The biliary recovery of administered ouabain was calculated from the bile concentration and bile volume which was determined by bile weight, assuming that the specific gravity of all bile samples was unity.

FRAP Experiments

The lateral diffusion constant of hepatocyte plasma membrane proteins was determined by the FRAP technique on liver specimens obtained from rats of different ages and treatment. An autofluorescence enhanced and stabilized by pretreatment with H_2O_2 (1 m*M*) for 10 min was used as described by the authors in detail (12, 13), instead of the usual method using an exogenous fluorescence label (14). Although most of the technical aspects of our FRAP system was described in detail in previous publications (12–14, 19), our technique using smeared liver tissues and an autofluorescence induced by hydrogen peroxide is rather new, so we will briefly describe it.

Specimen Preparation: Liver smears were prepared with two glass slides (one for pressing the tissue and the other for fixing the tissue) from fresh tissue removed from rats of different ages immediately after decapitation. The smears were fixed on the surface of a frosted glass slide as described previously (12, 13). The thickness of the smears (about 50 μm) was controlled (12). This smear thickness corresponds to about two cell layers. The smearing procedure was performed within a few seconds.

Development of Autofluorescence: The liver smear was placed horizontally in a Petri dish and covered with 1 ml Kreb-Henseleit bibarbonate Ringer solution of pH 7.4 containing 1 m*M* H_2O_2. The dishes were kept on the surface of a water bath of 30° to 40°C, keeping the temperature on the surface of the smear at 37°C throughout the incubation and washing procedures (12). The smears were incubated for 10 min with Kreb-Henseleit Ringer solution containing H_2O_2 and then washed with a H_2O_2-free Ringer solution 3 times (1 min each) and covered with a cover glass. The excessive liquid was then wiped away with filter paper in order to avoid the tissue movement caused by streaming which disturbs the FRAP measurements (12). The edges of the cover glass were sealed with melted paraffin. The autofluorescence thus developed and stabilized by the peroxide treatment [peroxide-induced autofluorescence (PIAF)] is yellowish-green when excited by blue laser light of 476 nm and was suitable for FRAP measurement (12).

FRAP Procedures: The lateral diffusion constants of hepatocyte plasma membrane proteins were determined by using a FRAP apparatus constructed at the laboratory. The instrumental set-up used in the present study was essentially the same as described previously (19). All measurements were performed by using an Olympus FLPL objective (× 40, NA = 0.75), giving a spot half diameter at an intensity of Ie^{-2} of W = 1.5 μm (19). A minor modification for a more sophisticated personal computer (NEC PC 9801E) with 120 Kbyte memory instead of the original TI-99/4 and the complexity of the subsequent computation of the diffusion constant using the equation described by Yguerabide et al. (20) have been described elsewhere (12–14).

The actual FRAP experiment records are shown in Figure 1. After an observation period of 100 sec monitored by a weak laser beam recording a sufficiently stable fluorescence intensity on a single cell surface membrane, the spot was automatically exposed for 0.2 sec to a strong (10,000 times higher in intensity than the monitoring beam) laser beam, efficiently bleaching the fluorescence. The bleached area was then monitored again by a weak laser beam for 100 sec and the recovery of the fluorescence intensity recorded.

The recovery curve was analyzed first by using the best-fit method to the so-called reciprocal equation (20). This procedure resulted in two important parameters in the first cycle, namely F(0), (i.e., the fluorescence intensity at zero photobleaching time) and F(inf), (i.e., the maximum recovery). In addition, the characteristic diffusion time (τ) and the diffusion constant were also calculated as described by Yguerabide et al (20). The

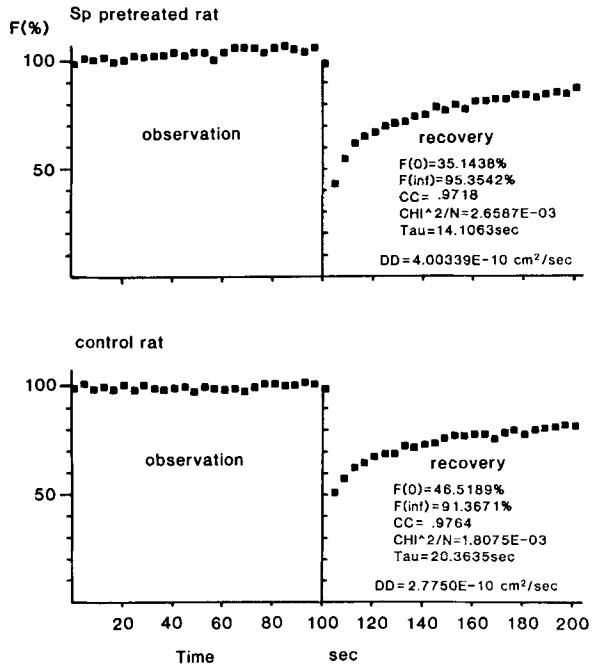

FIG. 1. Representative FRAP measurement studies on control (*lower panel*) and Sp-pretreated (*upper panel*) young rats automatically recorded by a computer. CC = correlation coefficient of fit to the reciprocal equation of Yguerabide et al. (20). $\chi^{2/N}$ indicates the goodness of the same fit.

validity of the data was then checked by using F(0) and F(inf) obtained by these calculations as described previously by ourselves (19). These two diffusion constants based on different calculations were actually very close to each other, but the average of the two was used as the diffusion constant (DD) for each individual cell. About 20 to 40 FRAP measurements were performed on each specimen, and the diffusion constants were averaged after discarding several anomalous values, if any, using the χ^2 method (12).

Analysis of the statistical differences between the values in the two sets of experiments of the same age groups with or without SP treatment was done by means of Student's t test. The parameters of rats of different age groups were analyzed by one-way ANOVA and least-squares linear regression. When the values were found to be significant with respect to age by ANOVA, Scheffe's test was used for the comparison of the two different age groups. All values in the results are expressed as mean ± S.D. P values lower than 0.05 were judged to be significant.

RESULTS

In Figure 1, representative studies for measuring diffusion constants of plasma membrane proteins using FRAP are shown for control and SP-pretreated young rats. In both studies, the degree of fluorescence recovery after photobleaching followed exactly the theoretical recovery course, yielding a very high fitness (CC in the figure).

Figure 2 illustrates the average diffusion constants of young rats. In Figure 3, all data for FRAP measurements are summarized. The diffusion constants in rats with and without Sp pretreatment showed an age-dependent decrease. Relations between the age (X, months) and the coefficient (Y, $cm^2 \cdot sec^{-1}$) obtained by the least-squares linear regression were $Y = -2.74 \cdot 10^{-12} \cdot X + 2.77 \cdot 10^{-10}$

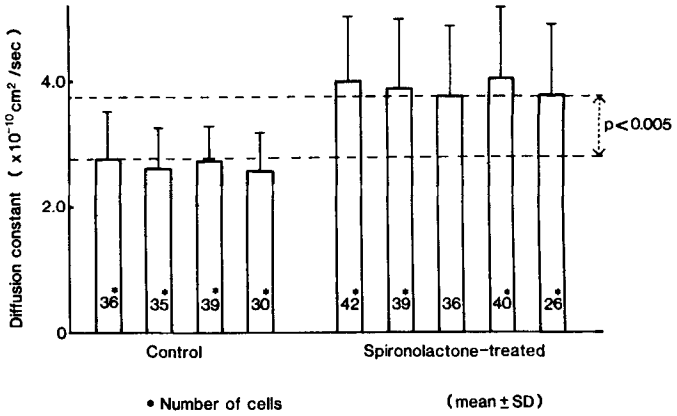

FIG. 2. Diffusion constants of hepatocyte plasma membrane proteins in young rats with and without Sp pretreatment. *Number in the column* indicates the number of cells examined. Values are expressed as mean ± S.D.

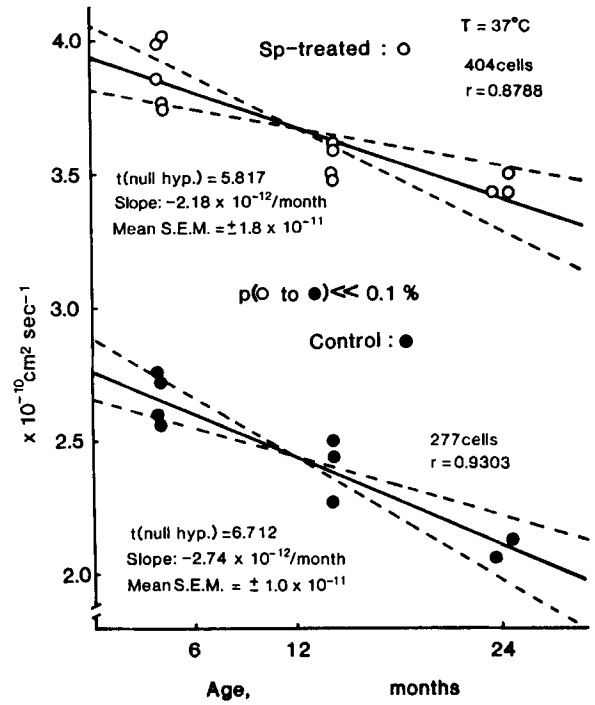

FIG. 3. Diffusion constants of hepatocyte plasma membrane protein of rats of different ages with and without Sp pretreatment. *Closed circles* indicate values of control rats, and *open circles* indicate values of Sp-pretreated rats.

for control rats and $Y = -2.18 \cdot 10^{-12} \cdot X + 3.93 \cdot 10^{-10}$ for Sp pretreated rats. In rats pretreated with Sp, the diffusion constants were significantly (30 to 40%) higher than corresponding control values.

Figure 4 shows the sequential changes in the biliary excretion of ouabain (left panel) and bile flow rate (right panel) in control and Sp-pretreated young (4 months) and old (24 months) rats. In Table 1, the results of ouabain excretion in rats of all age groups are summarized. The biliary excretion of ouabain in control rats was highest in young rats and decreased progressively with age. This was most clearly demonstrated in the first 10-min biliary recovery values, which were significantly

different with respect to rat age [$F(2, 17) = 34.05$, $p < 0.001$]. Differences among the three age groups were all significant ($p < 0.05$). The second 10-min recovery values were also significantly different with respect to age [data not shown, $F(2, 17) = 5.42$, $p < 0.015$]. For the 60-min total recovery, however, the significance with respect to age was lost [$F(2, 17) = 0.397$, $p < 0.146$]. In contrast, the bile flow rate in the basal period before ouabain injection changed little with age ($p > 0.05$).

In rats of all three age groups, Sp pretreatment caused the biliary excretion of ouabain as well as the bile flow rate to be significantly higher than control values of corresponding ages. The only exception was the bile flow rate in 24-month-old rats which was very similar for control and Sp-treated animals. Ouabain excretion in the first 10-min period in Sp-treated animals also showed an age-dependent decrease [$F(2, 14) = 144.533$, $p < 0.001$]. In rats with Sp pretreatment, the 12- and 24-month values were significantly lower than the youngest value ($p < 0.01$).

In Figure 5, the relationship between the diffusion constants and the first 10-min biliary recovery values are shown for all groups examined. Both parameters decreased with age and increased with Sp pretreatment. In control and Sp-treated groups, the two parameters of the corresponding ages showed a linear relationship.

Figure 6 shows the sequential changes in plasma ouabain levels in control and Sp-pretreated rats of three different ages. The plasma ouabain levels in the first 10 min tended to be higher as rat age increased. Sp pretreatment caused a clear decrease in plasma ouabain levels compared to control values at corresponding times.

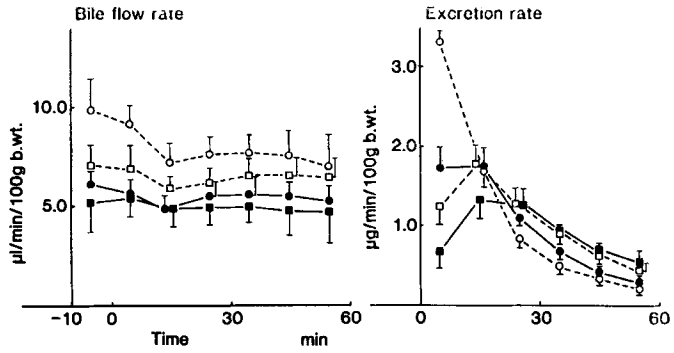

FIG. 4. Sequential changes in the biliary excretion of ouabain (*right panel*) and bile flow rate (*left panel*) of ouabain in control and Sp-pretreated young (4-month-old) and old (24-month-old) male rats. *Circles* indicate values for young rats, and *squares* indicate values for old rats. *Solid line* indicates values in control rats, and *dashed line* indicates those in Sp-pretreated rats. *Vertical bars* indicate S.D. Number of rats in each group is shown in Table 1.

DISCUSSION

The results of the present study have shown that the biliary excretion of i.v. administered ouabain progressively decreases with age in male Wistar-derived rats. This observation is quite consistent with that we ob-

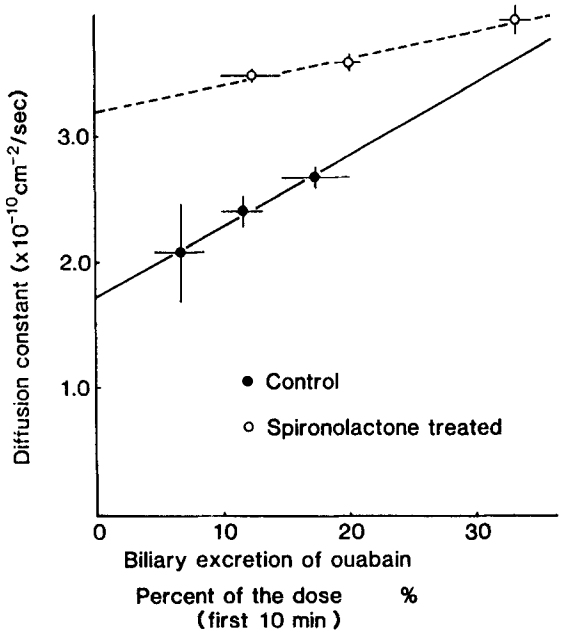

FIG. 5. Comparison of the diffusion constants of hepatocyte plasma membrane proteins and ouabain excretion into the bile (first 10-min value) in rats of different ages with and without Sp pretreatment.

TABLE 1. Basal bile flow rate (before ouabain injection) and biliary recoveries of i.v. injected ouabain for the first 10-min and total 60-min periods in rats of different ages with and without Sp pretreatment

Age	Body weight (gm)	Liver weight (gm)	Basal bile flow rate		Biliary ouabain recovery (% of the dose)	
			μl/min/100 gm b.wt.	μl/min/gm liver wt.	10 min	60 min
4 months						
Control (8)	330.0 ± 10.53	10.32 ± 0.89	6.37 ± 0.92	2.03 ± 0.16	17.28 ± 2.56	59.83 ± 4.17
Sp pretreated (4)	317.5 ± 6.45	12.11 ± 0.86[a]	9.89 ± 0.34[a]	2.59 ± 0.34[a]	33.16 ± 1.32[a]	69.48 ± 6.23[a]
14–15 months						
Control (8)	462.5 ± 9.26[b]	13.57 ± 0.84	4.89 ± 0.38	1.61 ± 0.19	11.59 ± 1.73[b]	59.96 ± 3.95
Sp pretreated (3)	433.3 ± 31.75[a]	15.01 ± 1.94	8.20 ± 0.35[a]	2.37 ± 0.07[a]	21.02 ± 1.07[a,b]	63.41 ± 2.43
24 months						
Control (4)	440.3 ± 27.45[b]	14.38 ± 2.41[a]	5.39 ± 0.79	1.70 ± 0.27	6.73 ± 2.08[b]	54.41 ± 7.02
Sp pretreated (8)	424.3 ± 41.46	17.63 ± 3.05[b]	6.89 ± 1.21[a]	1.70 ± 0.43	12.44 ± 2.40[a,b]	62.76 ± 8.24

All values are mean ± S.D. Values in parentheses indicate the number of rats. b.wt. = body weight; wt. = weight.

[a] Significantly different from respective control values ($p < .05$, t test).

[b] Significantly different from the corresponding 4-month values ($p < 0.05$, Scheffe's test).

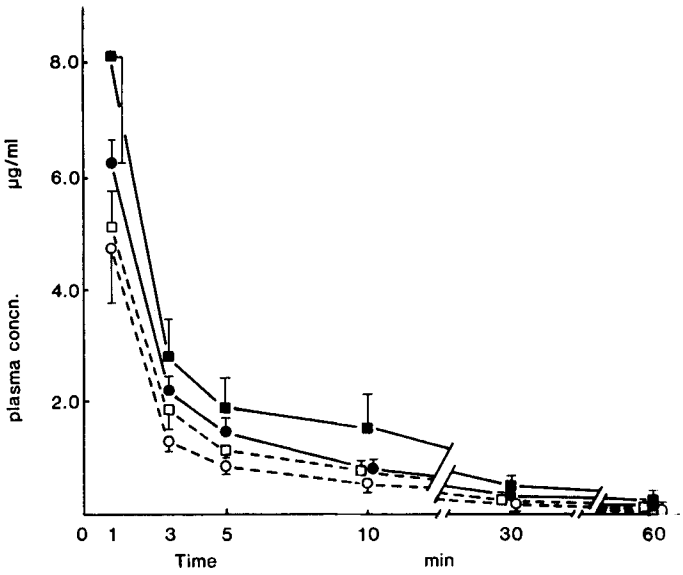

FIG. 6. Sequential changes in plasma ouabain concentrations in control and Sp-pretreated rats of different ages. Symbols are the same as in Figure 4.

served previously in the same rat strain and sex using a 4-fold higher ouabain dose (9). Furthermore, the figures also agree with our recent study using F-344 rats of both sexes with the same ouabain dose (0.1 mg per 100 gm body weight) (21), where we found ouabain excretion to decrease in a significant age-dependent manner in both sexes. From these results, one is tempted to generalize that the biliary excretion of ouabain decreases with age in rats regardless of sex, strain or test dose used. Furthermore, the significant enhancing effect of Sp pretreatment on the biliary excretion of ouabain shown in the present study also conforms to past observations on young rats by others (6) as well as on aging (16-month-old) male rats reported previously by the authors (9). Thus, it seems clear that the Sp pretreatment is effective in enhancing the biliary excretion of ouabain up to 24 months of rat age.

In parallel with the change in ouabain excretion, there was an age-dependent decrease in the lateral diffusion constant of hepatocyte plasma membrane proteins as assessed by FRAP in control rats, which parallels our study of male and female F-344 rats (12). Furthermore, it is clear from Figures 3 and 4 that pretreatment with Sp causes a significant increase in the lateral mobility of plasma membrane proteins, not only in the young rats but also in the oldest (24-month-old) rats. Our present observation of the effect of SP pretreatment on the lateral diffusion constant of hepatocyte plasma membrane proteins appears to be the first such report in the literature. Since our FRAP procedure employs several new techniques, some technical aspects of this method should be discussed before we interpret our data.

The major difference between our current method and previously reported FRAP techniques is the use of tissue smears and PIAF. The membrane integrity of tissue smears was demonstrated by an investigation using electron microscopy (12). Although we discussed the possible mechanism for its production (12), the nature of the autofluorescence (PIAF) is not completely clear. However, the following lines of evidence suggest that PIAF is specifically located in plasma membrane proteins.

1. PIAF is observed exclusively in the uppermost layer of the cells of the smear. This can be checked by comparing the focal depth in phase-contrast and epiillumination fluorescence microscopy (12). This situation is quite similar to the condition where hepatocytes were labeled with external fluorescent ligands (e.g., wheat germ agglutinin-fluorescein isothiocyanate and concanavalin A-fluorescein isothiocyanate (14).
2. The fluorescence recovery curves of PIAF fit very well the mathematical model of the lateral diffusion of intramembrane proteins (20, 22). Furthermore, the diffusion constants we measured are well within the range of reported data on the mobility of various membrane proteins determined by using standard external labels, as discussed earlier (12).
3. The PIAF in liver smears used for FRAP and the fluorescence of isolated surface membrane isolated from peroxide-treated hepatocytes both have a fluorescence characteristic having a peak emission wave length at around 520 nm when excited by a 476 nm laser beam (Nokubo et al., unpublished observation). These observations strongly suggest that the FRAP measurement using the PIAF yielded the diffusion constant of proteins located in the hepatocyte surface membrane. A FRAP study using ordinary external labels (e.g., concanavalin A-fluorescein isothiocyanate) could be done for the hepatocytes preparations used in the present study 14). However, we have found that all external fluorescent labels presently available decrease the diffusion constant in a dose-dependent manner, so the conventional procedure is not optimal for measuring such a labile surface membrane as that of hepatocytes (14). A similar phenomenon has been recently shown for mast cell membranes treated with IgE antibody (23). Furthermore, the age-dependent decline in the diffusion constant of hepatocyte plasma membrane proteins shown in the present study as well as in the previous study (12) is quite reproducible, allowing an age estimation of the liver tissue donors (13).

As is clear from the procedure, what we measured as the diffusion constant in the present study is an average value for many different kinds of membrane proteins and not that of a specific protein species. Physical-chemical alterations of cell plasma membranes with age such as K^+ permeability decrease (15, 16), the accumulation of macromolecular proteins in membranes (16) and the increase in lipid microviscosity (24) have been reported previously by different authors including ourselves, using plasma membrane preparations. All of these results are consistent with the contention that the proteins in the membrane bind more closely with each other through a cross-linking, and/or lipid or protein peroxidation by free radicals during aging (12, 16). Thus, the nature of decreased lateral mobility as shown by our

present FRAP is most likely shared by many protein species in the membranes of aged animals. Consequently, it is reasonable to assume that the as yet unelucidated ouabain receptor protein in the membrane is one of a number that are similarly affected by the aging process.

Miner and his coworkers (11) reported previously that pretreatment with Sp decreased the lipid fluidity of hepatocyte plasma membranes. A similar observation has also been reported by Smith and Gordon (24). The present results, however, have shown that the diffusion constant of plasma membrane proteins decreases with age but increases with the Sp pretreatment. The disparity between the lipid fluidity (11, 25) and protein diffusion shown in the present study with regard to the effect of Sp pretreatment is in accord with increasing evidence that lipid fluidity and protein diffusion do not necessarily change in parallel with each other (26–28).

The mechanism for the biliary excretion of ouabain through hepatocyte still remains totally unknown. Eaton and Klaassen (29) examined the subcellular bindings of ouabain in hepatocytes but could not find any specific protein fraction bound to ouabain *in vitro*. Furthermore, they (29, 30) showed that ouabain uptake by the isolated rat hepatocyte conforms to Michaelis-Menten kinetics, suggesting that ouabain uptake is a carrier-mediated saturable process. They also demonstrated that ouabain uptake is considerably enhanced by pretreatment with pregnenolone 16α-carbonitrile (30), which enhances *in vivo* ouabain excretion like Sp. We have recently found that the ouabain uptake maximal velocity of isolated rat hepatocytes in old rats (24 to 29 months) is about one-half that of the young rats (4 months), while Kd values did not significantly differ (31). Furthermore, a significant negative correlation was observed between the uptake velocity and rat age in months (31). Thus, the age-dependent decline in the biliary ouabain recovery values observed in the present study appears to be at least partly caused by the decline in the ouabain uptake velocity of hepatocyte membranes. Furthermore, a previous study that showed the markedly enhanced ouabain uptake velocity of hepatocytes in rats treated with pregnenolone 16α-carbonitrile (30) indicates that Sp's effect on the biliary excretion may also be at least partly due to its effect on hepatic uptake. The maximal velocity for a carrier-mediated transport is a function of the number of specific-binding sites and the diffusion coefficient of the receptor-ligand complex (32). Thus, the observed decrease in the maximal uptake velocity with age is explained as a result of either: (a) the decreased diffusion constant of the presumed ouabain uptake carrier in the hepatocyte membrane or (b) the loss of specific receptor protein(s) for ouabain uptake (or the combination of both). In the case of enhancement of ouabain transport by Sp pretreatment, opposite changes [i.e. (a) an increase in receptor number and (b) an increase in diffusion constant] can be conceived.

It is clear from the present results that changes in protein diffusion caused by aging and Sp pretreatment all fit in with the alteration in the biliary excretion of i.v. administered ouabain. The correlation between the biliary excretion of ouabain and the diffusion constant of plasma membrane proteins demonstrated in the present study, therefore, raises the possibility that protein diffusion in the hepatocyte plasma membrane is a candidate mechanism for regulating the biliary excretion of ouabain by modulating its hepatic uptake. At least our results on protein diffusion (i.e., increase by Sp pretreatment and decrease by aging) are changes that would be expected from alterations in the biliary excretion of ouabain, if these two parameters are causally related. Since we demonstrated only a correlation between the two parameters, however, a further direct proof is obviously required to validate our suggestion on their possible causal relationship.

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