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SHORT-CHAIN ALIPHATIC ALCOHOLS INCREASE RAT-LIVER MICROSOMAL MEMBRANE FLUIDITY AND AFFECT THE ACTIVITIES OF SOME MICROSOMAL MEMBRANE-BOUND ENZYMES

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n-Butyl and isoamyl alcohols decrease the steady-state fluorescence anisotropy of 1,6-diphenyl-1,3,5-hexatriene and enhance the efficiency of pyrene excimer formation when these probes are incorporated in rat-liver microsomal membrane, suggesting an increase in rotational and translational mobilities. Neither alcohol modifies NADH-ferricyanide reductase activity but both increase NADH-cytochrome c reductase activity. This was interpreted as an increase in the rate of lateral diffusion of the proteins cytochrome b_5 and cytochrome b_5 reductase as a consequence of the enhanced membrane lipid phase fluidity. Microsomal Δ^9 and Δ^6 desaturase activities in the presence of isoamyl alcohol were also studied. This alcohol decreases Δ^9 desaturation when it is measured at a low substrate concentration (13 μM palmitic acid), but it is not modified when it is measured at a high substrate concentration (66 μM palmitic acid). Δ^6 desaturation is diminished by isoamyl alcohol when it is measured with both 13 μM and 66 μM linoleic acid. The influence of isoamyl alcohol on the glucose-6-phosphatase system activity was also studied. in non-detergent-treated microsomes, isoamyl alcohol enhances glucose-6-phosphatase activity. However, if microsomes are previously treated with 0.1% Triton X-100 isoamyl alcohol does not modify this activity. The enhancement of the gluocse 6-phosphate transport rate is not due to membrane permeability barrier disruption, since isoamyl alcohol does not modify mannose-6-phosphohydrolase latency. This would suggest that an increase in membrane lipid phase fluidity specifically activates glucose 6-phosphate transport across the membrane.

Introduction

The membrane 'fluidity' has been considered an important factor in regulating some membrane functions [1] and it has been designated 'viscotropic regulation' [2,3]. This may be particularly important in the endoplasmic reticulum membrane, since it synthesizes its own lipid components. For this reason, we are interested in studying 'in vitro' the effect of membrane 'fluidity' upon some microsomal enzymes and complementing previous 'in vivo' studies [4]. The term 'fluidity' is somewhat ambiguous, since membranes are anisotropic and its meaning may change with the method and probe used. Here, the dynamic structural changes were checked by the steady-state fluorescence anisotropy of 1,6-diphenyl-1,3,5-hexatriene and the pyrene excimer formation, which measure the rotational and translational mobility, respectively.

In these experiments, we have used the short-chain n-butyl and isoamyl alcohols to increase microsomal lipid phase mobility, and their effects were studied on the glucose-6-phosphatase system and the fatty acid desaturases with their associated electron transport chain.

Aliphatic and aromatic alcohols can modify some properties of lipid bilayer as phase transition temperature and certain structural and mobility parameters as was shown by ESR and NMR measurements [5–7]. Benzyl alcohol and some short-chain *n*-alkanols have been also employed to examine the effects of altering lipid phase fluidity on biological membrane activities [8–12]. The relationship between membrane lipid phase dynamics and fatty acid desaturation is particularly interesting, since this enzymatic system might possibly regulate the lipid fatty acid composition and the membrane 'fluidity'. The rate-limiting step in the desaturase reaction at high saturating substrate concentration is the hydrogen abstraction for the double-bond formation [13,14]. However, it is possible that, at low substrate concentration, the rate might depend on the fatty acyl-CoA or enzyme lateral diffusion. For this reason, we have investigated the effects of short-chain alcohols on Δ^6 and Δ^9 desaturase activities at both low and high substrate concentrations.

The desaturation reactions are associated to the electron transport system constituted by the cytochrome b_5 and the cytochrome b_5 reductase flavoprotein [15–17]. The effect of these alcohols was also studied on the flavoprotein activity and the electron transfer from NADH to the exogenous electron acceptor cytochrome *c* through flavoprotein and cytochrome b_5.

Arion et al. [18–23] have presented strong evidence that at least three components of the microsomal membrane participate in the glucose 6-phosphate hydrolysis process: (a) a specific glucose 6-phosphate transporter which translocates it across the membrane [24]; (b) a relatively non-specific phosphohydrolase-phosphotransferase catalytic component which is bound to the luminal surface of the microsomal membrane; and (c) a second translocase that mediates efflux of phosphate [25]. Strong evidence has been gathered [18–26] that glucose 6-phosphate translocation is the rate-limiting step of the overall process in 'intact' microsomal vesicles, in spite of some objections [27]. Therefore, the hydrolytic component activity can be measured in microsomes disrupted with detergents or other agents [23]. The effect of isoamyl alcohol was studied on both non-detergent-treated and 0.1% Triton X-100-treated microsomes to examine the influence of lipid phase 'fluidity' on both steps: the translocation and hydrolysis of glucose 6-phosphate.

Materials and Methods

Microsome preparation

Livers of 2-month-old male Wistar rats, feeding on a standard diet (Purina chow) were homogenized in a solution of 0.25 M sucrose/1 mM EDTA (pH 7.0), except when Δ^9 and Δ^6 desaturation reactions were measured. In this case, the homogenizing solution was 0.25 M sucrose/0.15 M KCl/5 mM $MgCl_2$/1.4 mM *N*-acetyl cysteine/0.1 mM EDTA/62 mM phosphate buffer (pH 7.4). The microsomal fraction was separated by differential ultracentrifugation as already described [28]. The microsomal pellet was resuspended in the corresponding homogenizing solution (1 : 2, v/v) and the protein content was measured by the biuret reaction [29]. Microsomes were stored at −80°C under N_2 atmosphere for not longer than 1 week.

1,6-Diphenyl-1,3,5-hexatriene labeling of microsomes and steady-state fluorescence anisotropy determinations

The fluorescence probe 1,6-diphenyl-1,3,5-hexatriene was purchased from Aldrich Chemical Co. One volume of a diluted microsomal suspension (0.1 mg protein/ml) in 0.25 M sucrose/1 mM EDTA (pH 7.0) was mixed with a volume of 0.5 μM probe dispersion and incubated for 1 h at 25°C. The penetration of the probe in the membrane was monitored by following the enhancement in fluorescence intensity, which levels off after about 50 min. Suspensions of unlabeled microsomes at the same concentration were used as reference blanks which served to discount the excitant light scattering and other contributions to the fluorescence signal. The final label concentration was 0.25 μM and the final lipid concentration was approx. 20–25 μM. At this concentration of microsomes, the depolarization of fluorescent light due to scattering is practically negligible, since subsequent dilution had no effect on the measured anisotropy. The energy transfer between probe molecules and effects of the probe on microsomal membrane fluidity were also negligible at this

probe-to-lipid ratio, since when a microsomal suspension (50 μg of protein/ml) was labeled with increasing amounts of probe, the fluorescence anisotropy was unchanged at a probe concentration as high as 1 μM.

The fluorescence anisotropy measurements (352 nm excitation, 435 nm emission) were done in an Aminco-Bowman spectrofluorimeter equipped with two glan prism polarizers. They were corrected by substraction of the corresponding blank value. Although a 2.0 M $NaNO_2$ solution placed between the emission monochromator and the photomultiplier was used as a cut-off filter for wavelengths below 390 nm, the blank corrections were necessary. The steady-state fluorescence anisotropy was calculated using the equation:

$$r_s = \frac{I_{\parallel} - GI_{\perp}}{I_{\parallel} + 2GI_{\perp}}$$

where $G = I_{hh}/I_{hv}$ is a correction factor arising from instrumental factors, I_{hh} and I_{hv} are the fluorescence intensities detected with the excitation polarizer in horizontal position and the analyzer in horizontal or vertical position, respectively.

Apparent 'microviscosity' and order parameter, S_{DPH}

The extent of depolarization of the fluorophore emission in the membrane would be dependent on the rotational diffusion of the probe, thought to reflect the viscous drag imposed by the local apolar environment of the probe. An 'apparent microviscosity' of the membrane interior can be estimated by comparing the fluorescence anisotropy in the membrane system with that observed in a reference solvent of known viscosity. The apparent 'microviscosity' of the membrane interior ($\bar{\eta}$) was calculated using Perrin's equation [30] as proposed by Shinitzky and Barenholz [31] using the fluorescence anisotropy (r_s) data in:

$$\bar{\eta} = \frac{2.4 \cdot r_s}{r_0 - r_s}$$

where r_0 is the limiting fluorescence anisotropy of the probe in a freeze solvent in absence of rotation.

The application of these equations has been criticized by reason of the anisotropy of membrane [32–34]. Additionally, it has been shown [32–34] that the fluorescence anisotropy can be resolved into a fast-decaying component (r_f) and an infinitely slow-decaying component (r_∞). The last one is determined by the molecular packing and the ratio r_∞/r_0 was recognized as the square of the orientational order parameter, S_{DPH}, of the lipid bilayer [32–35]. The r_∞ and S_{DPH} were calculated in the present work using the empirical plots of Van Blitterswijk et al. [34]. The theoretical value of 0.4 was taken for r_0 to estimate microviscosities and order parameters.

Pyrene excimer formation measurements

Pyrene excimer formation was used as a parameter of the lateral diffusion in the membrane [36]. Pyrene was purchased from Fluka. A microsomal suspension (0.5 mg of protein/ml) was prepared in the deaerated 0.25 M sucrose/1 mM EDTA (pH 7.0). Different amounts of a solution of pyrene in acetone (5 mM) were added to the microsomal suspension and stirred for 1 h at 25°C. Final concentrations of pyrene were between 2 and 6 μM. Different aliquots of isoamyl alcohol were added and stirring was continued for 5 min. Samples were always under a N_2 atmosphere. Samples were divided into two fractions. In one fraction, the fluorescence spectrum was obtained directly. The other fraction was centrifuged at $100\,000 \times g$ for 1 h and the spectrum of the supernatant was recorded. It was used to correct for the contribution to the fluorescence signal by that pyrene not incorporated in the microsomes. Parallel samples without pyrene were prepared which served to discount the light scattering and intrinsic fluorescence contribution to the fluorescence signal. Before the spectra were recorded, the samples were purged with N_2 for 5 min and N_2 was also flushed into the cell compartment while the spectra were being recorded. The emission spectra were obtained at 25°C in an Aminco-Bowman spectrofluorimeter with an Aminco *X-Y* recorder. The excitation wavelength was 320 nm. The fluorescence intensity ratio of excimer to monomer (I_e/I_m) was calculated from the ratio of fluorescence intensities at 472 nm and 392 nm after correcting the spectra for light scattering and $100\,000 \times g$ supernatant contributions. In the $100\,000 \times g$ precipitate, the

pyrene-to-protein ratio was determined as a measure of the pyrene concentration in the microsomal membrane. the determination of the protein content in the 100 000 × *g* supernatants indicated that isoamyl alcohol did not increase the solubilization of microsomal proteins. Therefore, the pyrene-to-protein ratio in the 100 000 × *g* precipitate was an adequate estimation of the pyrene concentration in the microsomal membrane. For pyrene determinations, an aliquot of the 100 000 × *g* precipitate was diluted in methanol and after centrifugation at 3000 × *g* for 20 min, the absorbance at 334 nm was measured [36]. Isoamyl alcohol produced a small change in the pyrene partitioning between the aqueous phase and the membrane.

Enzymatic measurements

NADH-ferricyanide reductase and NADH-cytochrome *c* reductase activity were determined spectrophotometrically as previously described [37]. Δ^9 and Δ^6 desaturation reaction rates were determined in the microsomes by measuring the conversion of [1-^{14}C]palmitic and [1-^{14}C]linoleic acids to [1-^{14}C]palmitoleic acid γ-[1-^{14}C]linolenic acids, respectively, in a gas-liquid radiochromatograph as already described [37]. Glucose-6-phosphatase activity was determined as described [28] following the method of Baginsky et al. [38] in both non-detergent-treated microsomes and microsomes treated with 0.1% Triton X-100. For this purpose, 1 vol. 1% Triton X-100 was added to 9 vol. of a microsomal suspension (5 mg of protein/ml) and it was incubated at 4°C for 10 min. In order to examine the effects of short-chain aliphatic alcohols on the microsomal enzymatic activities, the alcohols were added in different concentrations directly to the incubation medium in presence of microsomes. After 3 min equilibration, the reactions were started by adding the corresponding substrate.

Mannose-6-phosphatase latency

Intact microsomal vesicles cannot hydrolyze mannose 6-phosphate at low concentrations (1 mM). This activity is expressed only in disrupted microsomal vesicles that lack a permeability barrier [18]. The mannose 6-phosphatase latency has been used to assess the integrity of microsomal vesicles' permeability barrier. The latency percent is the proportion of activity that is expressed only after disruption of microsomal vesicles, and it is the proportion of enzyme molecules that are in intact sealed microsomal vesicles [18].

$$\% \text{ of latency} = \frac{A_T - A_n}{A_T} \times 100$$

where A_T is the activity of Triton X-100-treated microsomes and A_n is the activity of microsomes not treated with detergent. The mannose-6-phosphatase activity was determined in the same way as glucose-6-phosphatase activity, except that the 25 mM glucose 6-phosphate substrate was substituted with 1 mM mannose 6-phosphate. The microsomal vesicle disruption was produced in the same way as when glucose-6-phosphatase was determined.

Results

Effects of n-butyl and isoamyl alcohols on the fluorescence anisotropy of the rat liver microsomal membrane

When isoamyl alcohol (40 mM) was added to a microsomal suspension previously labeled with 1,6-diphenyl-1,3,5-hexatriene, the fluorescence ani-

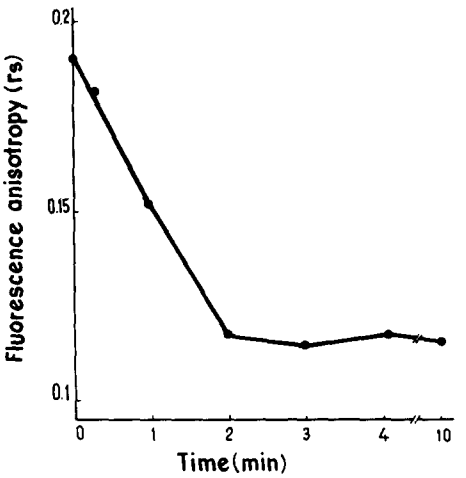

Fig. 1. Variation of fluorescence anisotropy of 1,6-diphenyl-1,3,5-hexatriene in microsomal membranes after incubation with isoamyl alcohol. To a suspension of rat-liver microsomes (50 μg of protein/ml) previously labeled with 1,6-diphenyl-1,3,5-hexatriene (25 μM), was added an aliquot of concentrated isoamyl alcohol to a final concentration of 40 mM and the fluorescence anisotropy was measured at the indicated times.

sotropy decreased rapidly, reaching a minimal value at about 2 min (Fig. 1). This effect is completely reversible, since after the recentrifugation for about 1 h at 100 000 × *g* and resuspension in an alcohol-free medium (0.25 M sucrose/1 mM EDTA (pH 7.0)), the fluorescence anisotropy in turn reached the original value (not shown data).

The probe 1,6-diphenyl-1,3,5-hexatriene is practically non-fluorescent when it is in a high-polarity medium such as water [39] (data not shown). However, it was observed that the fluorescence of this compound in the aqueous medium is increased by the addition of *n*-butyl or isoamyl alcohol, which is possibly due to a decrease in the medium polarity. Nevertheless, the recentrifugation of a suspension of microsomes labeled with 1,6-diphenyl-1,3,5-hexatriene and treated with isoamyl alcohol for 1 h at 100 000 × *g* showed that the fluorescence in the supernatant, that correspond to the probe non-incorporated in microsomes was practically negligible with respect to that of the probe incorporated in the membrane. This happened even at the highest isoamyl alcohol concentration used (100 mM) (data not shown). Therefore, these results rule out the possibility that the decrease in fluorescence anisotropy induced by alcohols is due to an increase in the fluorescence intensity (which is almost completely depolarized) of the probe that remains in the aqueous medium. In consequence, it was unnecessary to correct the data for the fluorescence of the probe in the aqueous medium.

These results also indicate that isoamyl alcohol readily partitions between the aqueous medium and the microsomes (the equilibrium is reached within 2 min at 20°C) and incorporates in the liver microsomal membrane. In Fig. 2A is shown the effect of different *n*-butyl and isoamyl alcohol concentrations on the fluorescent anisotropy of 1,6-diphenyl-1,3,5-hexatriene bound to the rat-liver microsomal membrane. At low alcohol concentration, the fluorescence anisotropy is quickly decreased, and the effect is apparently saturated at high alcohol concentrations. The effects of *n*-butyl and isoamyl alcohols were qualitatively similar, but isoamyl alcohol was more effective. Concentrations of *n*-butyl alcohol higher than those of isoamyl alcohol were required to produce the same effect. The maximal effect was reached at approx. 70 mM of isoamyl alcohol and 175 mM of *n*-butyl

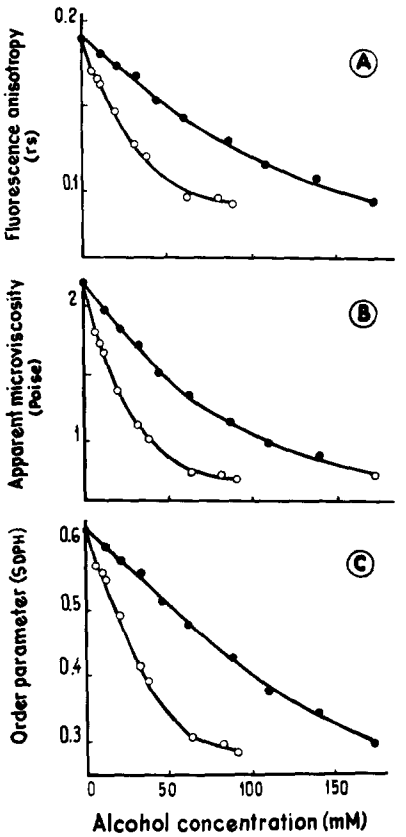

Fig. 2. Effect of short-chain aliphatic alcohols on the rotation motion of 1,6-diphenyl-1,3,5-hexatrine in microsomal membranes. To a suspension of rat-liver microsomes labeled with 1,6-diphenyl-1,3,5-hexatriene, as indicated in Materials and Methods, were added aliquots of concentrated *n*-butyl (●——●) and isoamyl (○——○) alcohols to the final concentrations indicated. After 3 min, the steady-state fluorescence anisotropy, r_s, was determined (A). With the r_s values, the 'apparent microviscosity', $\bar{\eta}$, (B) and the order parameter, S_{DPH}, (C) were calculated as was indicated in Materials and Methods.

alcohol was required to produce the same effect. The effects of the alcohols on the 'apparent microviscosity' and the other parameter, S_{DPH}, calculated from the steady-state fluorescence anisotropy as was indicated in Materials and Methods, are shown in Figs. 2B and 2C. These figures show that both apparent microviscosities ($\bar{\eta}$) and order parameter (S_{DPH}) are decreased progressively by increasing alcohol concentration reaching a constant value.

Effect of isoamyl alcohol on the pyrene excimer formation in the rat-liver microsomal membrane

In Fig. 3A, the effect of isoamyl alcohol on the plots of I_e/I_m versus pyrene concentration is shown. Only three concentrations of isoamyl alcohol are indicated to avoid superpostion of plots. The slopes of these plots were calculated and plotted versus the isoamyl alcohol concentration in Fig. 3B. The efficiency of pyrene excimer formation increased linearly with the increase of isoamyl alcohol concentration up to approx. 50 mM. At higher isoamyl alcohol concentrations, the effect is apparently saturated. A 56% of increase in efficiency of pyrene excimer formation was observed at the highest isoamyl alcohol concentrtion that was tested (92 mM). Therefore, this indicates that isoamyl alcohol also increases the rate of lateral diffusion of pyrene in the membrane, in a similar way as it increases the freedom of 1,6-diphenyl-1,3,5-hexatriene rotational mobility.

Effect of short chain aliphatic alcohols on the activity of the fatty acid desaturation system and its associated electron transport chain

The effect of different concentrations of *n*-butyl and isoamyl alcohols on the activity of microsomal cytochrome b_5-dependent electron transport system is shown in Fig. 4. NADH-ferricyanide reductase activity measures the rate of NADH oxidation by the cytochrome b_5 reductase flavoprotein, which is reoxidized by the exogenous electron acceptor ferricyanide. Neither *n*-butyl nor isoamyl alcohols had any effect on the NADH-ferricyanide reductase activity. On the contrary, NADH-cytochrome *c* reductase activity was increased by the presence of these alcohols in the reaction medium. NADH-cytochrome *c* reductase measures the rate of electron transfer from NADH to the exogenous electron acceptor, cytochrome *c*, through both flavoprotein and cytochrome *c*, which interaction is the rate-limiting step. NADH-cytochrome *c* reductase activity was rapidly increased at low alcohol concentrations and reached a plateau at higher concentrations. Isoamyl alcohol was again more effective than *n*-butyl alcohol, and concentrations of isoamyl alcohol lower than those of *n*-butyl alcohol were required to produce the same enhancement of NADH-cytochrome *c* reductase activity. At very high *n*-butyl alcohol concentrations, an inhibitory effect was observed. This is possible due to a direct effect on the proteins not mediated by the changes in membrane lipid phase dynamic strucure. Anyway, these results indicate that alcohols increase the rate of electron

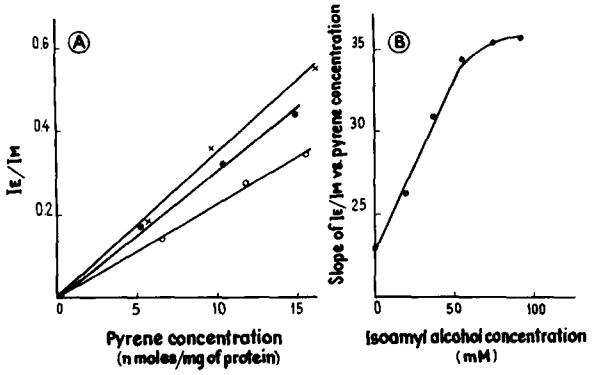

Fig. 3. Effect of isoamyl alcohol on the efficiency of pyrene excimer formation in rat-liver microsomal membranes. Microsomes labeled with different amounts of pyrene were treated with different concentrations of isoamyl alcohol (see Materials and Methods). The corrected I_e/I_m ratios were plotted versus the pyrene concentration in the membrane (A) for microsomes treated with no (○——○), 37 mM (●——●) and 74 mM (×——×) isoamyl alcohol. The slopes of I_e/I_m vs. pyrene concentration curves were plotted versus the isoamyl alcohol concentration (B).

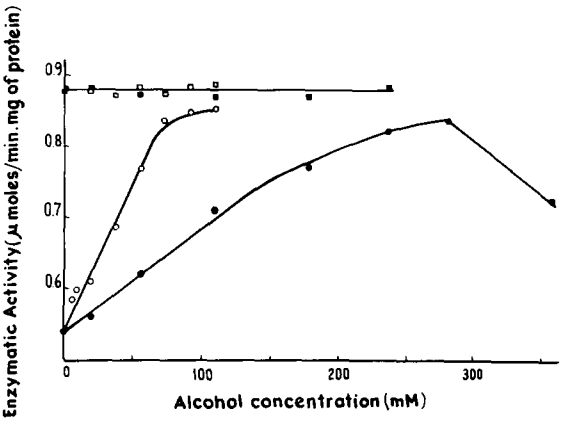

Fig. 4. Effect of short-chain aliphatic alcohols on the activity of cytochrome b_5-dependent electron transport chain of rat-liver microsomal membranes.The incubation media contained 30 μg of microsomal protein/ml, the indicated concentration of *n*-butyl (●, ■) or isoamyl (○, □) alcohol, and the corresponding substrates in Tris-acetate buffer (pH 8.1), NADH-ferricyanide reductase (■, □), NADH-cytochrome *c* reductase (●, ○). Each point is the mean of two determinations.

transport between cytochrome b_5 reductase and cytochrome b_5, but they do not modify the flavoprotein activity when it is measured with water-soluble substrates. The effect of isoamyl alcohol on Δ^9 desaturation measured with low and high substrate concentrations is shown in Fig. 5. Isoamyl alcohol in concentrations as high as 75 mM, did not modify Δ^9 desaturase activity at a high saturating substrate concentration. However, when the desaturation reaction was measured with a non-saturating low substrate concentration, it was seen to be inhibited by the addition of isoamyl alcohol to the incubation medium. The decrease in low substrate concentration Δ^9 desaturation rate compares fairly well to the decrease in fluorescence anisotropy.

In Fig. 6 is shown the effect of isoamyl alcohol on the Δ^6 desaturase reaction measured at low and high substrate concentrations. At a low substrate concentration, the effect of isoamyl alcohol was similar to that on Δ^9 desaturase reactions. However, Δ^6 desaturation was also inhibited by isoamyl alcohol at high substrate concentration, although the effect was a little lower than at low substrate concentration. The effect of 75 mM isoamyl alcohol on the partition of [1-^{14}C]palmitic acid between the aqueous phase and the membrane was tested using microsomes inactivated by heating for

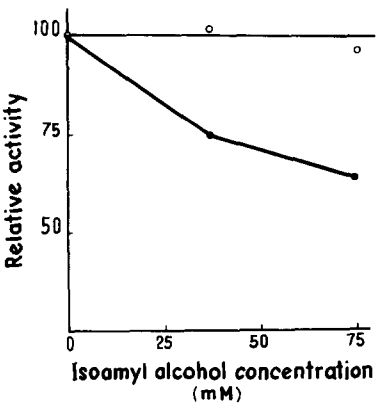

Fig. 5. Effect of isoamyl alcohol on the Δ^9 desaturation of palmitic acid by rat-liver microsomes. The incubation media contained 1.7 mg of microsomal protein/ml, the indicated concentration of isoamyl alcohol, cofactors and 13 μM (●———●) or 66 μM (○———○) palmitic acid in phosphate buffer (pH 7.4). Each point is the mean of two ddeterminations.

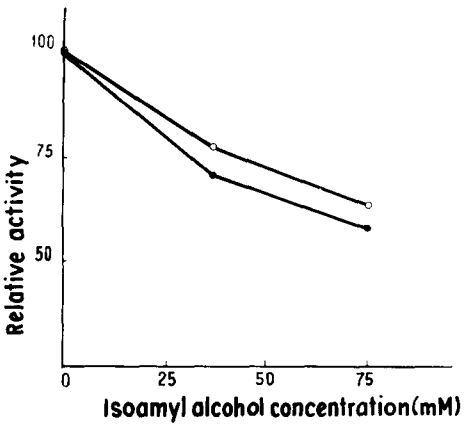

Fig. 6. Effect of isoamyl alcohol on the Δ^6 desaturation of linoleic acid by rat-liver microsomes. The incubation media contained 1.7 mg microsomal protein/ml, the indicated concentration of isoamyl alcohol, cofactors and 13 μM (●———●) or 66 μM (○———○) linoleic acid in phosphate buffer (pH 7.4). Each point is the mean of two determinations.

10 min at 60°C. No effect of isoamyl alcohol was observed.

Isoamyl alcohol effects on the activity of the glucose-6-phosphatase system

Glucose-6-phosphatase activity was measured in presence of different concentrations of isoamyl alcohol in non-detergent-treated microsomes and in microsomes previously treated with 0.1% Triton X-100. It has been determined that treatment of microsomes with detergents or equivalent agents [18,21] that fully disrupt microsomes destroys selective permeability of the membrane, allowing free access of ionic substrates to enzymes. In the case of glucose-6-phosphatase, they eliminate factors that constrain the maximal potency of the enzyme and determine its substrate specificity [18,23]. Other microsomal enzymes like UDPglucuronyl transferase are also activated by detergents [40]. In Fig. 7 it is shown that the glucose-6-phosphatase has a maximal activity and that isoamyl alcohol has no effect on the enzyme activity in microsomes disrupted with 0.1% Triton X-100. These results indicate that isoamyl alcohol does not modify the activity of the glucose-6-phosphatase when the lipid bilayer constraint has been eliminated. That is, no effect of isoamyl alcohol-induced 'fluidity' changes on the glucose-6-phos-

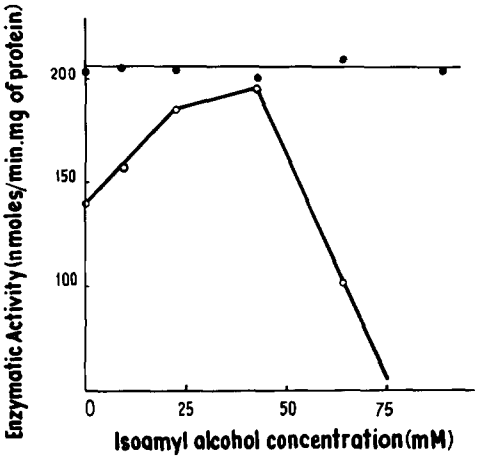

Fig. 7. Effect of isoamyl alcohol on the activity of rat-liver microsomal glucose-6-phosphatase system. The incubation media contained the indicated concentration of isoamyl alcohol, 50 μg protein/ml of non-detergent-treated (O——O) or Triton X-100-disrupted (●——●) microsomes, and 25 mM glucose 6-phosphate in cacodylate buffer (pH 6.5). Each point is the mean of two determinations.

phatase was observed in disrupted microsomes in which, according to Arion's works [18–23,26], the enzyme would be exposed and have free access to the substrate.

In non-detergent-treated microsomes, in absence of alcohol, the glucose-6-phosphatase activity is lower than in 0.1% Triton X-100-treated microsomes, but the activity is increased by isoamyl alcohol concentration in a manner similar to the decrease in fluorescence anisotropy and the increase in pyrene excimer formation. At approximately 50 mM isoamyl alcohol, the activity of non-detergent-treated microsomes reached approximately the same value as that of 0.1% Triton X-100-treated microsomes. However, above 50 mM isoamyl alcohol, the activity of non-detergent-treated microsomes was suddenly inhibited. This inhibition was not observed in 0.1% Triton X-100-treated microsomes and, using Arion's model, it can be explained by a direct effect of the alcohol on the glucose 6-phosphate transporter component [24], not related to the alcohol-induced changes in the membrane structure.

Using the same model, the increase in the glucose-6-phosphatase activity of non-detergent-treated microsomes produced by isoamyl alcohol at concentrations lower than 50 mM can be explained either by an increase in the rate of glucose 6-phosphate translocation effected by an increase in the membrane 'fluidity' or by increased disruption of the microsomal vesicles as an effect of the alcohol. The second possibility is improbable, since high alcohol concentrations only inhibited the activity of non-detergent-treated microsomes and did not modify the activity of 0.1% Triton X-100-disrupted microsomes. However, to confirm that isoamyl alcohol does not disrupt the microsomal vesicles, the effect of the alcohol on the percent of latency of mannose-6-phosphatase activity was studied. In Table I, the mannose-6-phosphatase

TABLE I

EFFECT OF ISOAMYL ALCOHOL ON THE PERMEABILITY OF MICROSOMAL VESICLES TO MANNOSE 6-PHOSPHATE

The incubation media contained the indicated concentration of isoamyl alcohol, 50 μg of protein/ml of disrupted microsomes or 200 μg of protein/ml of non-detergent-treated microsomes and 1.0 mM mannose 6-phosphate in cacodylate buffr (pH 6.5). Each value is the mean of two determinations.

Isoamyl alcohol concentration (mM)	Mannose-6-phosphatase activity (nmol/min per mg protein)		Latency (%)
	Non-detergent-treated microsomes	0.1% Triton X-100-treated microsomes	
0	1.68	15.3	89
9	1.83	14.1	87
23	1.46	16.2	91
44	1.56	15.6	90
65	1.91	14.7	87
90	1.82	15.2	88

activities of rat liver microsomes before and after treatment with 0.1% Triton X-100, in the presence of several concentrations of isoamyl alcohol in the incubation medium, are shown. The percentage of latency was calculated as indicated in Materials and Methods. Isoamyl alcohol did not have any effect on mannose-6-phosphatase latency even at concentrations as high as 90 mM. This rules out the possibility that alcohol can disrupt microsomal vesicles. Therefore, the increase in the glucose-6-phosphatase activity in non-detergent-treated microsomes produced by isoamyl alcohol at low concentrations may be due very probably to an increase in the activity of the glucose 6-phosphate transporter. The effect of isoamyl alcohol at low concentrations on the glucose 6-phosphate transporter is possibly mediated by the changes in the membrane lipid phase dynamic structure, as detected by flurescence anisotropy and pyrene excimer formation, since both effects compare quite well.

Discussion

Effect of isoamyl alcohol and n-butyl alcohol on rotational and translational diffusion of microsomal membrane

These experiments show that *n*-butyl and isoamyl alcohols, when added to rat-liver microsomal suspension, partition between aqueous and lipid phase and increase the rotational diffusion of the probe 1,6-diphenyl-1,3,5-hexatriene. This is in accordance with the effect described for short-chain alcohols on model [6] and biological membranes [12]. The greater efficiency of isoamyl alcohol compared to *n*-butyl alcohol in decreasing fluorescence anisotropy is possible due to its lower water solubility and its more favourable partition in the lipid phase or to its branched chain.

Isoamyl alcohol also increases the efficiency of pyrene excimer formation which is dependent on the rate of translational diffusion of pyrene in the plane of the membrane that determines the frequency of the collisions [36,41].

Effect on fatty acid desaturation enzymes

Cytochrome b_5 reductase, cytochrome b_5 and fatty acid desaturase are intrinsic proteins localized in the external face of the microsomal membrane [42,43]. It has been postulated [16,44–46] that the flavoprotein and the cytochrome b_5 are randomly distributed in the plane of the bilayer. The results here obtained indicate that the flavoprotein activity measured with water-soluble substrates (NADH-ferricyanide reductase) is not affected by the alcohol-induced 'fluidity' changes. Besides, it has been shown that this activity is not affected by lipid phase transition in artificial membranes [47] and in microsomal membranes modified by exogenous lipid incorporation [48]. This flavoprotein presents its catalytic centre exposed to the water phase [43] and the substrates have free access to it from the aqueous phase. Thus, the lipid-phase properties would not influence the flavoprotein catalytic properties.

Conversely, the rate of electron transference between flavoprotein and cytochrome b_5 (NADH-cytochrome *c* reductase) would be facilitated by an alcohol-induced increase in the lipid-phase mobility (Fig. 4). It has been postulated that the electron transfer between the reductase and the cytochrome b_5 would be limited by the lateral diffusion of these proteins in the bilayer [45–47]. Thus, the isoamyl alcohol-induced changes in the dynamic structure of the microsomal membrane would increase the translational diffusion of proteins in addition to aromatic hydrocrbon molecules like pyrene. In brain microsomes and synaptosomes, the rate of ganglioside hydrolysis was also increased by short-chain *n*-alkanols, and this was explained by the enhancement of lateral diffusion of substrate and enzyme [12]. In addition, it has been found in dimiristoylphosphatidylcholine vesicles with the purified proteins [47] and in microsomes enriched in dimiristoyl or dipalmitoyl choline [48] that the NADH-cytochrome *c* reductase activity decreases suddenly when the membrane lipids undergo a fluid-to-ordered transition. Therefore, these results show that the cytochrome b_5 reduction by the flavoprotein wuld be modulated by 'fluidity' changes without involving drastic structural changes as those occurring along an order-disorder transition which are less feasible to occur 'in vivo'.

Conversely to NADH-cytochrome *c* reductase, isoamyl alcohol inhibited Δ^6 and Δ^9 desaturase reactions (Figs. 5 and 6). Thus, these reactions cannot be limited by the lateral diffusion of

enzymes or substrates, even at low substrate concentration. Especially in the case of the Δ^9 desaturase reaction (Fig. 5), the alcohol effect was dependent on substrate concentration and was not shown at saturating concentrations. This could be explained if the amount of substrate available to the enzyme or the enzyme affinity for the substrate were decreased by the alcohol. However, we have shown in a previous work [49] that niether octanol, linoleyl alcohol nor α-linolenyl alcohol competitively inhibited the Δ^6 desaturase. If the amount of substrate available to the enzyme is decreased, it is not due to an altered partition of the fatty acid between the membrane and the aqueous phase.

If the changes in membrane 'fluidity' were the determining factor in the decreased rate of desaturation, this would indicate that it is a negative modulator. Thompson's group have presented evidence [50–52] that in *Tetrahymena pyriformis* the fatty acid desaturation activity is enhanced by a decrease in the membrane 'fluidity' independent from enzyme induction. These reactions may function as a self-regulating mechanism of the phospholipid unsaturated-chain content and thus the membrane 'fluidity'.

The fatty acid composition and desaturation activity of microsomes undergo circadian variations [53] and are also modified by essential fatty acid deficiency [4,54]. However, no good correlation has been found in these cases between the fatty acid desaturase activity and the unsaturated chain content or mobility parameters obtained from ESR measurements. These apparent differences may be due to the complexity of factors modulating these reactions in the whole animal.

Effect on glucose-6-phosphatase activity

These experiments showed that isoamyl alcohol incorporation in microsomes is a suitable method for studying the influence of lipid phase properties on the glucose-6-phosphatase activity, since it does not alter the 'intactness' of the membrane assessed by the maintenance of the latency of mannose-6-phosphatase activity.

Glucose-6-phosphatase activity is maximal and isoamyl alcohol has no effect on it in disrupted microsomes in which the substrate has free access to the enzyme. Since isoamyl alcohol activates glucose 6-phosphate but not mannose 6-phosphate hydrolysis in intact membranes, it apparently alters a specific mechanism for the first substrate. This mechanism may be the activation of the glucose 6-phosphate transporter postulated by Arion's group [19–23] and isolated by Zoccoli et al. [24]. Very possibly, the activation would be dependent on membrane 'fluidity'. This might be a general mechanism, since the transport rate of β-glucosides and β-galactosides across *E. coli* membrane is also affected by membrane 'fluidity' [55].

Although we cannot rule out the possibility that some of the effects of alcohol are not be mediated by changes in lipid phase 'fluidity', the present results suggest that reactions apparently requiring lateral diffusion or vertical transport across the membrane as NADH-cytochrome *c* reductase and glucose-6-phosphohydrolase in intact microsomes, respectively, could be accelerated by an increse of the membrane 'fluidity'. Conversely, reactions using water-soluble substrates such as NADH-ferricyanide reductase and glucose-6-phosphatase in permeabilized microsomes are apparently independent from the physical properties of the lipid phase.

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