Biochimica et Biophysica Acta, 600 (1980) 263—269

BBA 78810

EFFECTS OF *n*-ALKANOLS ON THE MEMBRANE FLUIDITY OF CHICK EMBRYO HEART MICROSOMES *

GEORGE B. ZAVOICO and HOWARD KUTCHAI

Department of Physiology, University of Virginia School of Medicine, Charlottesville, VA 22908 (U.S.A.)

(Received November 19th, 1979)

Key words: n-Alkanol; Membrane fluidity; Fluorescence depolarization; Anesthetic; Diphenylhexatriene; (Chick embryo heart)

Summary

The *n*-alkanols from butanol through octanol are membrane perturbing agents that fluidize the microsomal membranes of 20-day-old chick embryo hearts as measured by the fluorescence depolarization of 1,6-diphenylhexatriene. In terms of the aqueous concentrations of *n*-alkanols the fluidizing effect increases with increasing number of carbons per *n*-alkanol. In terms of the membrane concentrations of *n*-alkanols the fluidizing effect is roughly equivalent for all the *n*-alkanols studied.

Introduction

The normal alcohols (*n*-alkanols) have commonly been used as membrane perturbing agents [1]. Like most other anesthetics they expand the membrane surface area and cause a fluidization or disordering of the membrane lipids [2]. For example, the surface area of red blood cells increases on addition of *n*-alkanols which affords protection from osmotic hemolysis [2]. The lipid disordering effect has been shown with the use of steroid spin labels [3,4] and stearic acid spin labels [5] that become increasingly mobile in the presence of *n*-alkanols. Similarly, the fluorescent probes perylene and 9-vinylanthracene [6] and 1-anilinonaphthalene-8-sulfonate [5] show increases in probe rotation rates in the presence of *n*-alkanols. In artificial membranes a broadening and a lowering of the phase transition [7—9], an increase in the amount of lipids in the liquid-crystalline state [10], and an increased rate of penetration of water into

* A preliminary report of this work appeared in (1978) Fed. Proc. 37, 516.

unsonicated liposomes [11] are all induced by *n*-alkanols. The fluidizing effect has also been demonstrated in *Escherichia coli* [12]. When grown in the presence of *n*-alkanols (C_5—C_{10}), the *E. coli* modify the fatty acid composition of their membrane phospholipids by incorporating more saturated fatty acids (primarily palmitic acid) and fewer unsaturated fatty acids (primarily vaccenic acid) to compensate for the increased membrane fluidity induced by the *n*-alkanols.

The *n*-alkanols perturb the membrane by inserting themselves within the bilayer [1]. They are amphipathic molecules that are likely to partition into the membrane by intercalating among the lipids with their hydrophilic hydroxyl moiety anchored near the water/hydrocarbon interface. In this paper we report on the effects of *n*-alkanols on the lipid fluidity of a microsomal membrane preparation from 20-day-old chicken embryo heart. The fluidity is estimated from the depolarization of the fluorescence of 1,6-diphenylhexatriene. Increasing the aqueous concentration of the series of *n*-alkanols studied, butanol to octanol, increases membrane fluidity. In terms of calculated membrane concentrations, the *n*-alkanols are roughly equipotent in increasing membrane fluidity.

Materials and Methods

The *n*-alkanols used were of reagent grade obtained from various sources and used without further purification. Diphenylhexatriene was of 'Puriss' grade from Koch-Light Laboratories, Ltd. Fertilized and incubated eggs were obtained from Grassy Knoll Hatchery (Standardsville, VA) and kept in a commercial farm incubator (Leahy Manufacturing Co.) until the eggs had been incubated 20 days. The hearts were dissected away from the embryos, minced, homogenized (20% w/v suspension in a Willems Polytron, PT 20 ST head at half maximal speed for 15 s), and centrifuged (Sorvall RC-2B, SS-34 rotor at 48 246 $\times g$ for 75 min). The supernatant was discarded, and the pellet resuspended for a differential centrifugation (SS-34 rotor for 20 min at 10 000 $\times g$, followed by 10 min at 20 000 $\times g$) yielding the microsomal fraction in the supernatant. The microsomes were stored at −20°C in 0.25 M sucrose. The microsomal fraction was predominantly membranous material, with some unidentifiable cellular debris as revealed by electron microscopy. Compared to the homogenate the microsomes were enriched 5.5-fold in the plasma membrane marker 5'-nucleotidase assayed according to Solyom and Trams [13] and depleted about 5-fold in the mitochondria marker succinic dehydrogenase assayed according to Ells [14].

The protein content of the microsomal fraction was determined by a modified biuret method [15]. The absorbance at 350 nm of tubes containing 1 ml of reagent and a variable amount of sample was measured after mixing and then standing for 10—15 min. Bovine serum albumin standards were used. The phospholipid content was measured by a modification of the method described by Bartlett [16].

Fluorescence depolarization measurements were performed on an Aminco Fluoro-colorimeter (Model J4-7439). The excitation filter was a Baird-Atomic 10-36-0 mercury line filter with a central transmittance at 365 nm and the

emission filter was a Wratten 2A that transmits light with wavelengths greater than 415 nm. The exciting light was polarized by passage through a Polacoat filter (3M Corp.) and the emitted light was analyzed after passage through polaroid filters (Edmund Scientific), one of which was oriented parallel, the other perpendicular to the excitation filter. Fluorescence depolarization measurements were made at 37° C as follows: 100 μl of the crude microsomal membranes were added to 2 ml of an *n*-alkanol solution in water or to water in 10 × 75 mm culture tubes. The tubes were vortexed and placed in a water bath at 37° C. Scattering was measured for each tube before 2 μl of a 1 mM diphenylhexatriene in tetrahydrofuran solution was added and the suspension vigorously vortexed. After 2 to 3 h at 37° C to insure incorporation of diphenylhexatriene into the membranes, the fluorescence intensity of the emitted light polarized parallel ($I_{/}$) and perpendicular ($I_{\perp}$) to the exciting light was recorded 3 times approx. 20 min apart to be certain stable values were reached. The diphenylhexatriene to lipid molar ratio was calculated to be 1 : 130.

The anisotropy of fluorescence (r) is calculated from the measured intensities as follows [17]:

$$r = \frac{(I_{/} - I_{/}^{s}) - (I_{\perp} - I_{\perp}^{s})}{(I_{/} - I_{/}^{s}) + 2(I_{\perp} - I_{\perp}^{s})}$$

where the superscript 's' refers to the scattered light contribution. Anisotropy is used to estimate microviscosity by a form of the Perrin equation [17]:

$$\frac{r_0}{r} = 1 + \frac{C(r)\, T\tau}{\eta}$$

where r_0 is the limiting anisotropy in a highly viscous system, T is the absolute temperature, τ is the fluorescence lifetime, η is the microviscosity, and $C(r)$ is a function of r that depends on the shape of the fluorophore. The Perrin equation can be modified to:

$$\left(\frac{r_0}{r} - 1\right)^{-1} = \frac{\eta}{C(r)\, T\tau}$$

where it is clear that the term $((r_0/r) - 1)^{-1}$ varies directly with η. This relationship provides a useful method for comparing fluidity in membrane systems [18].

In view of the recent criticism of using fluorescent probes to estimate the microviscosity of membranes by steady state fluorescence depolarization, it is useful to briefly justify our methodology. The criticism focuses on some basic assumptions used in the evolution of this technique [17,19]. This method depends upon a calibration curve of the fluorescence of the probe being used in a reference oil of known viscosity. Hare and Lussan [20] have demonstrated that the polarization behavior of a given probe can be quite different in different reference oils of the same viscosity. Hence the microviscosity values obtained from membranes depends critically on the reference oil being used in the calibration curve. This method also assumes that mono-exponential decay of fluorescence occurs. The methods used to measure fluorescence decay have yielded ambiguous results, however it appears certain the fluorescence decay of diphenylhexatriene in most membranes is at least bi-exponential [21,22]. The

implication here is that the rotation of the fluorophore is not isotropic, as it is in a reference oil, but depends upon the orientation it assumes in the bilayer. The question that arises is how can a microviscosity value be assigned to a membrane if the probe sees a different microviscosity depending on how it is rotating. This point is further illustrated by measuring the microviscosities of a membrane using different methods. Lackowicz et al. [23] measured microviscosity in dimyristoyl phosphatidylcholine and dioleoyl phosphatidylcholine vesicles using steady state anisotropy with diphenylhexatriene, then by the rotational rate of diphenylhexatriene by oxygen quenching anisotropy measurements and finally by the diffusivity of oxygen. All of these methods resulted in different values for microviscosity.

The conclusion to be drawn from this information is that the absolute microviscosity cannot be estimated in biological membranes by fluorescence depolarization since the lipid organization results in complex and restricted modes of motion for any probe or substance within the membrane. Hence the measured microviscosity depends initially on what probe is being used to measure microviscosity. However, as the nature of the motion of the more popular probes in bilayers become better characterized, other important information can be gained from studying this motion. Meanwhile, estimating changes in microviscosity or in the parameters used to obtain microviscosity (such as anisotropy, $((r_0/r)-1)^{-1}$, or the polarization of fluorescence) remain useful in quantifying the effects of perturbing agents as long as the same membrane preparation is used. It is in this context that our results are presented.

Results and Discussion

The series of *n*-alkanols from butanol to octanol was studied, and the resulting data is plotted in Fig. 1 where the fluidizing effect as shown by decreasing values of $((r_0/r)-1)^{-1}$ is approximately linear with the aqueous concentration

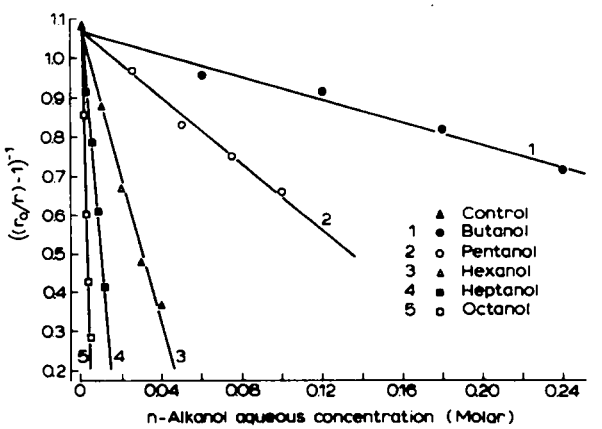

Fig. 1. The effect of different concentrations of the *n*-alkanols, butanol through octanol, on $((r_0/r)-1)^{-1}$. Anisotropy (r) was calculated from $I_{/\!/}$ and $I_{\perp}$ after correction for light scattering. r_0 was taken to be 0.362. Experiments were done at 37°C. The following slopes, *y*-intercepts and regression coefficients, respectively, were calculated: butanol, —1.45, 1.07, —0.992; pentanol, —4.23, 1.07, —0.994; hexanol, —18.30, 1.06, —0.994; heptanol, —54.67, 1.09, —0.998; octanol, —158.35, 1.05, —0.994.

of the alkanols. It is evident that increasing the aqueous concentrations of each *n*-alkanol fluidizes the membranes. Note that the longer chain alkanols are more effective in that they exert their effect at lower aqueous concentrations.

The aqueous concentrations of the *n*-alkanols are not really the relevant parameters. The concentrations of the *n*-alkanols in the membrane, calculated with known membrane/water partition coefficients, are more relevant to the function of the *n*-alkanols as membrane perturbing agents. Seeman et al. [24] measured the partition coefficients of *n*-alkanols in human red blood cell ghosts. The partition coefficients for butanol and hexanol were not given but we calculated these values from a linear plot of the log of the partition coefficients of the other *n*-alkanols studied versus the number of carbons in the *n*-alkanol. Roth and Seeman [25] measured the partition coefficients for a variety of anesthetics in nerve and muscle membranes (guinea-pig brain and rabbit gastrocnemius) and found them to be quantitatively similar to the partition coefficients of human erythrocytes. This similarity between various biological membranes supports the use of the partition coefficients for human erythrocyte membranes in our calculations with chick embryo heart microsomes.

The partition coefficients calculated by Seeman et al. [24] are in units of (mol/kg dry membranes)/(mol/liter). The molar ratios of *n*-alkanol to lipid are of more interest so conversion to (mol/mol total lipid)/(mol/liter) is desirable. The phospholipid content of the microsomal membranes was calculated to be 0.69 μmol/mg protein, and the average molecular weight of a phospholipid was assumed to be 750. The cholesterol/phospholipid ratio in purified 20-day chick embryo heart membranes is 0.63 [26]. With these values the conversion of the partition coefficients given by Seeman et al. requires multiplication by a factor of 1.49 from which the mole ratio of *n*-alkanol to total lipid (phospholipid plus cholesterol) can be obtained. The partition coefficients determined by Seeman et al. and those used here are listed in the legend of Fig. 2.

The relationship of $((r_0/r) - 1)^{-1}$ to the membrane concentrations of the *n*-alkanols studied is illustrated in fig. 2. The data are fit fairly well by a single straight line. This implies that the *n*-alkanols, butanol to octanol, are approximately equipotent in decreasing the fluorescence depolarization of diphenylhexatriene. This result can be interpreted if the expected locations of the *n*-alkanols and of the diphenylhexatriene in the membrane are compared. As previously described the *n*-alkanols might be expected to partition into the membrane such that the hydroxyl moiety is adjacent to the head groups of the phospholipids with their hydrocarbon chains extending into the bilayer parallel to the acyl chains of the phospholipids. The majority of these acyl chains in 20-day chick embryo heart are palmitic acid (30%) and stearic acid (25%) [26] so that the longest of the *n*-alkanols studied, *n*-octanol, would extend less than halfway down the acyl chains of the membrane phospholipids [9]. On the other hand, diphenylhexatriene, a highly hydrophobic molecule, would partition into the entire hydrophobic portion of the membrane in a more or less random distribution with respect to the acyl chains of the phospholipids [22,27,28]. Consequently when the *n*-alkanols partition into the membrane, and expand the surface area, the phospholipids are pushed apart

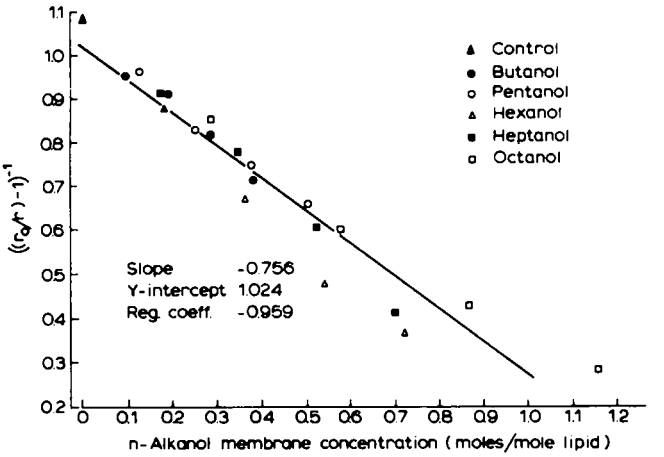

Fig. 2. The effect of calculated membrane concentrations of the *n*-alkanols on $((r_0/r) - 1)^{-1}$. The membrane concentrations were calculated using partition coefficients measured by Seeman et al. [24] that are as follows (the starred values were calculated from a plot of the log of the partition coefficients vs. the number of carbons): butanol, 1.07*; pentanol, 3.39; hexanol, 12.10*; heptanol, 39.01; octanol, 151.84 (mol/kg membrane)/(mol/liter). The partition coefficients were modified as described in the text to: butanol, 1.59; pentanol, 5.05; hexanol, 18.03; heptanol, 58.13; octanol, 226.24 (mol/mol membrane lipid)/(mol/liter).

creating vacancies deeper in the bilayer where the hydrocarbon chains of the *n*-alkanols do not reach. The Van der Waal's interactions are decreased, thereby reducing the molecular attractions between the terminal methyl and methylene groups allowing greater lipid chain mobility in the region of the bilayer where the vacancies are created [7]. It has been demonstrated that in regions of increased lipid chain mobility the angle over which the fluorophore can rotate increases due to the creation of vacancies within the lipid bilayer [29]. Steady state fluorescence depolarization measures the average rotation of the fluorophore in the bilayer so that all of the diphenylhexatriene molecules need not be affected by the perturbation of the membrane. Nevertheless, with a proportion of the diphenylhexatriene exhibiting greater motional freedom in the region where vacancies are created, it would appear that the average motional freedom of the diphenylhexatriene would increase thereby decreasing the anisotropy observed. The calculated value for $((r_0/r) - 1)^{-1}$ would also decrease implying an overall decrease in membrane microviscosity.

Acknowledgements

This research was supported by NIH Grant GM 24168 and by a Research Career Development Award HL 00014 to H.K. The authors wish to thank Susan Purdy for assistance with electron microscopy.

References

1 Jain, M.K., Gleeson, J., Upreti, A. and Upreti, G.C. (1978) Biochim. Biophys. Acta 509, 1–8
2 Seeman, P. (1972) Pharmacol. Rev. 24, 583–655

3 Grisham, C.M. and Barnett, R.E. (1973) Biochim. Biophys. Acta 311, 417—422
4 Paterson, S.J., Butler, K.W., Huang, P., Labelle, J., Smith, I.C.P. and Schneider, H. (1972) Biochim. Biophys. Acta 266, 597—602
5 Lenaz, G., Bertoli, E., Curatola, G., Mazzanti, L. and Bigi, A. (1976) Arch. Biochem. Biophys. 172, 278—288
6 Jacobson, K. and Wobschall, D. (1974) Chem. Phys. Lipids 12, 117—131
7 Eliascz, A.W., Chapman, D. and Ewing, D.F. (1976) Biochim. Biophys. Acta 448, 220—233
8 Lee, A.G. (1977) Biochemistry 16, 835—841
9 Jain, M.K. and Wu, N.-M. (1977) J. Membrane Biol. 34, 157—201
10 Lee, A.G. (1976) Biochemistry 15, 2448—2454
11 Jain, M.K., Touissant, D.G. and Cordes, E.H. (1973) J. Membrane Biol. 14, 1—16
12 Ingram, L.O. (1976) J. Bacteriol. 125, 670—678
13 Solyom, A. and Trams, E.G. (1972) Enzyme 13, 329—372
14 Ells, H.A. (1959) Arch. Biochem. Biophys. 85, 561—562
15 Zamenhof, S. (1957) Methods Enzymol. 3, 702
16 Bartlett, G.R. (1959) J. Biol. Chem. 234, 466—468
17 Shinitzky, M., Dianoux, A.-C., Gitler, C. and Weber, G. (1971) Biochemistry 10, 2106—2113
18 Shinitzky, M. and Barenholz, Y. (1978) Biochim. Biophys. Acta 515, 367—394
19 Shinitzky, M. and Barenholz, Y. (1974) J. Biol. Chem. 249, 2652—2657
20 Hare, F. and Lussan, C. (1977) Biochim. Biophys. Acta 467, 262—272
21 Kawato, S., Kinosita, K., Jr. and Ukegami, A. (1977) Biochemistry 16, 2319—2324
22 Chen, L.A., Dale, R.E., Roth, S. and Brand, L. (1977) J. Biol. Chem. 252, 2163—2169
23 Lackowicz, J.R., Prendergast, F.G. and Hogen, D. (1979) Biochemistry 18, 520—527
24 Seeman, P., Roth, S. and Schneider, H. (1971) Biochim. Biophys. Acta 225, 171—184
25 Roth, S. and Seeman, P. (1972) Biochim. Biophys. Acta 255, 207—219
26 Kutchai, H., Ross, T.F., Dunning, D.M., Martin, M. and King, S.L. (1978) J. Comp. Physiol. 125, 151—156
27 Lentz, B.R., Barenholz, Y. and Thompson, T.E. (1976) Biochemistry 15, 4521—4528
28 Andrich, M.P. and Vanderkooi, J.M. (1976) Biochemistry 15, 1257—1261
29 Badley, R.A., Martin, W.G. and Schneider, H. (1973) Biochemistry 12, 268—275