Effect of lipophilic vitamins on the erythrocyte membrane

^{31}P NMR and fluorescence studies

V. Viti, R. Cicero[+], D. Callari*, L. Guidoni, A. Billitteri* and G. Sichel[†]

Laboratorio di Fisica, Istituto Superiore di Sanità, Viale Regina Elena, 299,00161 Roma,
[+]Istituto di Biologia Generale, Facoltà di Medicina, Università di Bari, Bari,
**Istituto di Patologia Medica, II Cattedra, Facoltà di Medicina, Università di Catania, Catania* and
[†]Istituto di Biologia Generale, Facoltà di Medicina, Università di Catania , Catania, Italy

Received 26 April 1983

Structural changes induced by the fat-soluble vitamins A, D_3, E and K_1 in natural membranes were studied by ^{31}P NMR and fluorescence anisotropy on erythrocyte ghosts; the occurrence of cell fusion following vitamin addition was detected by optical microscopy of intact cells. Vitamins A, E and K_1, which produce cell fusion, also induce the formation of configurational phases other than the bilayer and increase, at a different extent for the different vitamins, membrane fluidity. Vitamin D_3, on the contrary, induces aggregation without fusion, paralleled by bilayer phase stabilization and microviscosity increase. A correlation between fusion phenomena and structural variations in the bilayer organization is therefore suggested.

Lipophilic vitamin *Erythrocyte membrane* *Cell fusion* *^{31}P-NMR* *Fluorescence anisotropy* *Membrane fluidity*

1. INTRODUCTION

Interest in changes produced in cell membranes by lipophilic vitamins was first elicited by studies on the toxic effects of high doses of vitamin A. High concentration of retinol affects the stability of plasma membranes [1–4]. Evidence that fat-soluble vitamins and steroids may exert their functions, at least in part, by regulating the lysosome permeability, provoked speculations about their mode of action [1,5]. In [6–8] we demonstrated that all lipophilic vitamins at high doses affect membrane permeability to different extents.

However, there have been relatively few studies that elucidate molecular events in the fusion process. This kind of phenomena has been related to configurational changes of lipid constituents of the membranes [9], but it has been also postulated that at least myoblast fusion is associated with a decrease in membrane viscosity [10,11]. While the fusogenic activity of retinol is well demonstrated [1–4], the tocopherol has fusogenic activity on erythrocytes [12] but inhibits platelet aggregation [13–15]. With the aim to gain more insight on fusion and permeabilization phenomena, we correlate here morphological modifications produced by lipophilic vitamins on erythrocyte membranes, chosen as a model for natural membranes, with changes in static and dynamic structure of the lipid bilayer. We observe that vitamins A, E and K_1 produce analogous effects, opposite to those induced by vitamin D_3.

2. MATERIALS AND METHODS

2.1. *Tested vitamins and reagents*

Vitamin A (all-*trans* retinol) was purchased by Sigma (Saint Louis MO); vitamin E (α-tocopherol), obtained by de-esterification of acetate, vitamin K_1 (3-phytylmenadione) and vitamin D_3

Published by Elsevier Science Publishers B.V.
00145793/83/$ 3.00

(colecalciferol) were purchased by Merck AG (Darmstadt). Vitamins A and E were quantitated spectrophotometrically, and vitamins K_1 and D_3 by weight. Vitamins, dissolved in absolute ethanol (Merck AG, Darmstadt) were added to samples at 1%.

DPH (1,6-diphenyl-1,3,5-hexatriene) was obtained from Sigma (Saint Louis MO). D_2O was purchased by Merck AG (Darmstadt).

2.2. *In vitro cell fusion*

The experiments were performed on human (A group, Rh^+) and hen erythrocytes. Blood treated with 3.7 μM sodium citrate (3:1) was centrifuged at $100 \times g$ for 10 min at 4°C. Erythrocytes were then washed 3 times with a modified Eagle's salt solution (pH 5.6) buffered with sodium cacodylate, according to [12].

The erythrocyte suspension was pretreated with an Eagle's salt solution containing 8% dextran 60°C for 5 min at 37°C. The erythrocyte suspension (3×10^8 cells/ml) was resuspended in the latter solution containing 0.5 mM vitamin A, D_3, K_1 and E or 1.0 mM vitamin K_1 and E and incubated at 37°C. After 15, 30 and 45 min small aliquots were mounted on glass slides and immediately observed with a phase-contrast microscope (Ziess).

2.3. *Sealed ghost erythrocyte preparation*

Human erythrocytes (A group Rh^+) were used in all experiments. Erythrocytes were isolated from citrate-treated blood by centrifugation for 10 min at $100 \times g$, 5°C; cells were washed 3 times in a buffer solution (Tris, 20 mM; NaCl, 150 mM, pH 7.4) at 4°C and then hemolysed in 80 vol. Tris, 20 mM (pH 7.4) for 30 min. After centrifugation for 15 min at $16000 \times g$, the pellet was washed with the hemolysing buffer until white ghosts were obtained. The packed membranes were then suspended in 0.15 M NaCl, 0.05 M KCl (pH 7) solution containing, for NMR measurements, 15% D_2O. In ghost suspensions, the lipid concentration was tested as in [16] and cholesterol concentration as in [17].

For NMR measurements, samples of ghost preparations at the total lipid concentration of 4 μM were incubated with the vitamins for 30 min at 37°C and then centrifuged at $16000 \times g$. After centrifugation, erythrocytes were resuspended and incubated for another 30 min at 37°C before measurements were taken. The last two steps were necessary to enhance vitamin effects, as in [18]. The final vitamin concentration was 315 μM (vitamins A, D_3, E, K_1) and 740 μM (only vitamins E and K_1). These conditions provided a vitamin: lipid ratio of 1:12 (vitamins A, D_3, E, K_1) and 1:6 (vitamins E and K_1).

For fluorescence measurements the ghost preparation was diluted with buffer to a final total lipid concentration of 5 μM.

2.5. *Fluorescence*

Fluorescence anisotropy was measured using a MPF 44 Perkin Elmer spectrofluorimeter equipped with a polarizing accessory. Polaroid polarizing filters were inserted in the excitation and emission channels. DPH fluorescence was excited at 360 nm and observed at 430 nm. The slit bandwidths were 8 and 12 for excitation and emission, respectively. The emitted light beam was passed through an interference filter to cut off wave-lengths below 390 nm. Fluorescence anisotropy was measured as in [19] method, by measuring $I_\parallel$ and $I_\perp$ (fluorescence intensity polarized parallel and perpendicular to the polarization plane of the excitation light beam). The degree of anisotropy polarization r is defined by the equation:

$$r = (I_\parallel - GI_\perp)/(I_\parallel + 2GI_\perp)$$

where G is the grating transmission factor. Sample temperatures were controlled within 0.1°C. The measurements were performed after 5 min to allow for sample equilibration.

2.6. *NMR measurements*

The ^{31}P NMR spectra were obtained with a Varian XL-100 instrument operating in Fourier transform mode at 40 MHz. The instrument was equipped with deuterium lock and proton decoupling. The spectra were obtained with presence of high power proton decoupling (10 W). Accumulated free induction decays were obtained from 20000 transients.

3. RESULTS

3.1. *Cell fusion*

From fig.1 it may be seen that lipophilic vitamins produce two different sets of phenomena on

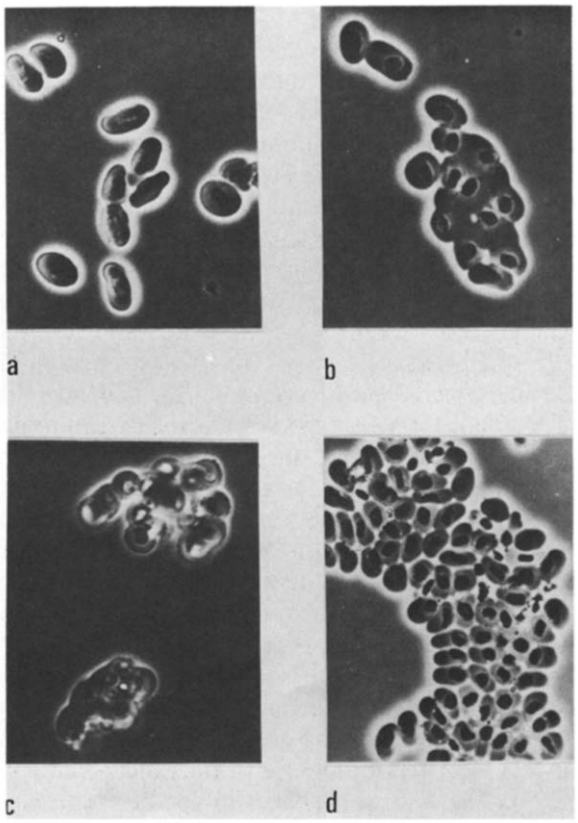

Fig.1. Phase contrast micrographs of hen erythrocytes after incubation with vitamins for 30 min, at 37°C: (a) control sample; (b) sample treated with 0.5 mM vitamin A; (c) sample treated with 0.5 mM vitamin K_1; (d) sample treated with 1 mM vitamin D_3.

hen erythrocytes depending on vitamin type: vitamin A-, E- or K_1-treated erythrocytes exhibit similar patterns which are totally unlike the pattern for vitamin D-treated erythrocytes. Vitamins A and E first cause aggregation and then syncytia formation as also reported in [2]. Fusion is rapid at our concentrations. The effect produced by vitamin A after 30 min incubation is reported in fig.1b. Incubation with vitamin K_1 produces phenomena similar to those observed with vitamin A, but only at a much higher concentration (fig.1c). On the contrary, cell aggregation with no subsequent cell fusion was observed when cells were incubated with vitamin D_3, at least for the first 30 min (fig.1d).

A systematic study of morphological phenomena in human and hen erythrocytes in the presence of vitamins was beyond the purpose of this paper and will be the subject of a following paper.

3.2. *^{31}P NMR*

The influence of lipophilic vitamins on the polymorphic behaviour of the lipid portion of resealed erythrocyte ghosts is reported in fig.2. Besides the predominant peak of the extended bilayer phase, control sample I exhibits (fig.2a) a small low-field peak in the region of high isotropic head-group mobility. This latter peak is sometimes present in ghost preparations and is probably due to the presence of lipid particles (inverted micelles) formed during ghost preparation. In control sample II no such signal was observed (fig.2a′).

Vitamin A addition to a first aliquot of sample I induces an increase in isotropic peak intensity. In the meantime, a second peak in the hexagonal phase region can be observed (fig.2b).

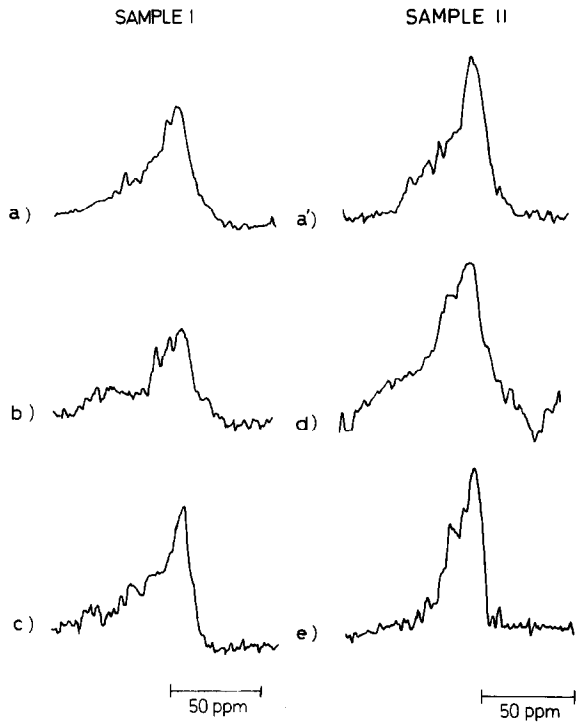

Fig.2. ^{31}P NMR spectra of resealed human erythrocyte ghosts at 37°C: (a,a′) control samples; (b) sample I treated with 0.315 mM vitamin A; (c) sample I treated with 0.315 mM vitamin D_3; (d) sample II treated with 0.315 mM vitamin E; (e) sample II treated with 0.665 mM with K_1.

The opposite effect is observed when vitamin D_3 is added to a second aliquot of sample I. In this case, the peak corresponding to isotropic motion completely disappears (fig.2c).

Vitamin E addition produces an isotropic peak in sample II where it had not been observed before (fig.2d). Besides the bilayer phase peak, the isotropic peak alone remains even when vitamin concentration is increased.

Incubation with vitamin K_1 of a second aliquot of sample II produces the same type of modifications in the ^{31}P NMR spectrum as those produced by vitamin A (fig.2e). This effect is observable only at higher vitamin doses.

3.3. *Fluorescence*

Variations in membrane fluidity following vitamin treatment were examined by measuring anisotropy r of the DPH probe (as in section 2) in view of the fact that r relates to membrane 'fluidity'.

Addition of vitamin A from 8×10^{-7} M produced a noticeable decrease in membrane microviscosity parameter (fig.3). An equivalent effect was obtained with the addition of vitamins E and K_1 but at 8×10^{-5} M (fig.3).

On the contrary, increasing low concentrations of vitamin D_3 do not produce an appreciable variation of membrane fluidity parameter, but the microviscosity does suddenly increase at the higher vitamin D_3 concentrations (fig.3).

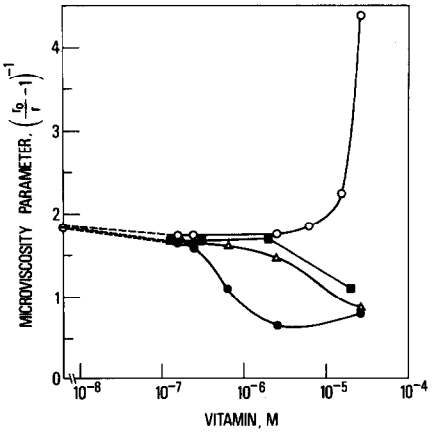

Fig.3. Microviscosity parameter $(r_0/r - 1)^{-1}$ of DPH in human erythrocyte ghosts as a function of vitamin concentrations; (●) vitamin A; (△) vitamin K_1; (■) vitamin E; (○) vitamin D_3.

4. DISCUSSION

Thus, it appears that all vitamins tested produce structural changes in the erythrocyte membranes chosen as a model for natural membranes, not all the vitamins examined act in the same way. Vitamins A, E and K_1 produce cell fusion; cell fusion is paralleled by the formation of configurational phases other than the bilayer and by the increase of membrane fluidity to different extents. Vitamin D_3 exhibits a totally different behavior: an extended aggregation phenomenon with no evidence of cell fusion is paralleled by bilayer phase stabilization and microviscosity increase. These results then substantiate observations in [18] that fusogenic activity is paralleled by phase transition of bilayer.

Furthermore, a clear correlation of fusion phenomena with increase of fluidity of membrane appears, supporting suggestions derived from experiments of fusion of myoblasts that fusion phenomena are related to an increase in membrane fluidity [20,21].

In our case the long vitamin A isoprenic chain and vitamin E and K_1 hydrocarbon chains interact directly with the molecular constituents of the membrane and are responsible for lipid segregation and resultant configurational phases other than the bilayer. The isoprenic chain has a stronger effect probably due to the presence of $-C=C-$ *cis* double bond. This interpretation agrees with the observation that the fusion is favoured by unsatured fatty acids for the increasing proportion of hydrocarbon chains in a relatively liquid state [22]. This hypothesis is supported by the observations on vitamin D effects. The increase in bilayer rigidity and the bilayer phase stabilization observed in vitamin D-treated membranes prevent fusion phenomena to be effective.

The results in [6–8] and here, correlate therefore fusogenic activity with permeability changes and the two phenomena with variations of the structure of hydrophobic core and polar head region of bilayer.

Finally the presence of agents capable of increasing membrane fluidity and of inducing configurational changes supports the idea that fusion phenomena take place with a low energy path. In fact configurational phase changes are also not accompanied by substantial energy variations [9].

REFERENCES

[1] Dingle, J.T. and Lucy, J.A. (1962) Biochem. J. 84, 611–621.

[2] Lucy, J.A. and Dingle, I.T. (1964) Nature 204, 156–160.

[3] Fell, B.H. (1964) Proc. Biochem. Soc. 35–38.

[4] Goodall, A.H., Galloway, M.I., Fisher, D. and Lucy, J.A. (1979) Biochem. Soc. Trans. 7, 937–939.

[5] Weissmann, G. (1964) Fed. Proc. FASEB 22, 1038–1041.

[6] Callari, D., Cicero, R., Sichel, G., Abate, G. and Billitteri, A. (1978) Acta Vitamin. Enzym. 32, 21–40.

[7] Callari, D., Guidoni, L., Viti, V., Cicero, R., Billitteri, A. and Sichel, G. (1981) Fis. Med. 3, 59–69.

[8] Callari, D., Abate, D. and Billitteri, A. (1978) Boll. Soc. It. Biol. Sper. 54, 1533–1539.

[9] Cullis, P.R. and De Kruijff, B. (1979) Biochim. Biophys. Acta 559, 399–420.

[10] Bischoff, R. (1978) in: Membrane Fusion Cell Surface Reviews (Poste, G. and Nicolson, G.L. eds) vol. 5, pp. 127–129, Elsevier, Amsterdam, New York.

[11] Van der Bosch, I., Schudt, C. and Pette, D. (1973) Exp. Cell. Res. 82, 433–438.

[12] Ahkong, R.F., Fisher, D., Tampon, W. and Lucy, J.A. (1973) Biochem. J. 136, 147–155.

[13] Kurokawa, I., Yoshiyuki, K., Nagai, T. and Kamimura, M. (1971) J. Vitaminol. 17, 181–184.

[14] Steiner, M. and Anastasi, I. (1976) J. Clin. Invest. 57, 732–737.

[15] Fong, I.S.C. (1976) Experientia 32, 639–641.

[16] Zilversmit, D.B. (1950) J. Lab. Clin. Med. 35, 155–160.

[17] Roschlan, P. (1974) Z. Klin. Chem. Klin. Biochem. 12, 403–407.

[18] Hope, M.J. and Cullis, P.R. (1981) Biochim. Biophys. Acta 640, 82–90.

[19] Shinitzky, M. and Barenholz, Y. (1974) J. Biol. Chem. 249, 2652–2656.

[20] Herman, B.A. and Fernandez, S.M. (1978) J. Cell. Physiol. 94, 263–264.

[21] Prives, I. and Shinitzky, M. (1977) Nature 268, 761–763.

[22] Ahkong, Q.F., Cramp, F.C., Fisher, D., Howell, J.I., Tampion, W., Verrinder, M. and Lucy, J.A. (1973) Nature New Biol. 242, 215–217.