Biochimica et Biophysica Acta, 1023 (1990) 274–282
Elsevier

BBAMEM 74793

Incorporation of spin-labeled fatty acids into bovine brain clathrin coated vesicles

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(Received 18 December 1989)

Key words: Clathrin; Coated vesicle; Lipid–protein interaction; ESR; Spin label; (Bovine brain)

Stearic acids with a nitroxide radical at selected positions have been incorporated in the phospholipid bilayers of clathrin coated vesicles, uncoated vesicles and sonicated liposomes made from the lipids extracted from the uncoated vesicles. The extent of incorporation was found minimum for stearic acids labeled on C-12 and for bilayers of uncoated vesicles. The ESR spectra of the spin-labeled fatty acids incorporated in the bilayers showed a pronounced temperature dependence (without discontinuity) and a decrease in the hyperfine splitting as the nitroxide group was inserted deeper in the hydrophobic core of the membranes. An abrupt phospholipid phase transition or a phase separation could be excluded. The presence of the external proteins (the clathrin coat) on the membranes was not found to noticeably influence the gradient of flexibility of the fatty acid chains of the phospholipids. The influence of the internal proteins embedded in the bilayers was evidenced by a detailed analysis of the ESR spectra of (7,8)SA in terms of two components: one component arising from the labels surrounded exclusively by phospholipids, the other component arising from labels of reduced mobility perturbed by the vicinity of the proteins. These results support the persistence of lipidic domains in the endocytic vesicles despite the accumulation of receptors which follows their formation.

Introduction

Clathrin coat proteins are involved in the receptor mediated endocytosis process [1,2] and in the transfer of newly synthesized proteins from the Golgi apparatus to secretory vesicles [3]. They form a polyhedral lattice adhering to the cytoplasmic side of the coated pits. The complete budding of these specialized regions leads to coated vesicles in which the extrinsic proteins are organized in three successive shells around the phospholipid bilayer [4]. The outermost shell is made of clathrin itself (heavy and light chains), the intermediate shell of the N-terminal domain of clathrin heavy chains and the inner shell of several proteins named assembly proteins which may constitute the link between clathrin and the membrane receptors [5].

Clathrin coated vesicles are thus multimolecular complexes for which the study of the phospholipid dynamics is of particular interest.

One reason is that the driving forces leading to the budding of the coated pits and the scission of the vesicles are not yet clear. It has been suggested that the receptors and coat components could coassemble and that the curvature of the membrane could be the result of the progressive increase in the receptors concentration [6]. This reorganization may create sterical constraints and may change the packing density of the phospholipids, their lateral diffusion and the flexibility gradient of their fatty acid chains. Alternatively, coated pits could simply be formed by the selfassembly of the coat which is known to naturally produce closed shells, even in absence of phospholipids.

It is also of interest to determine the extent of lipid–protein interactions which contribute to the stability and the specificity of the system. Namely, do extrinsic coat proteins interact exclusively with the cytoplasmic side of membrane receptors, or do they interact also with the underlying phospholipid bilayer? This question is not raised because it has been demonstrated that purified clathrin can bind to protein-free phospholipid bilayers [8] and induce their fusion [8,9]. This

Abbreviations: Mes, 2-(*N*-morpholino)ethanesulfonic acid; Tris, 2-amino-2-hydroxymethylpropane-1,3-diol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; DMPC, L-α-dimyristoylglycerophosphocholine; (*m,n*)SA, (*n* + 2)-doxylstearic acids.

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could be meaningless since in these conditions the natural hexagonal network of clathrin is not restored around the vesicles of synthetic phospholipids [10]. But it has been found that the lateral diffusion of charged spin labels included into small unilamellar vesicles of pure phospholipids is lowered when clathrin and assembly proteins are added. This 'anchoring' effect could be a factor of selection explaining the enrichment of coated vesicles in specific polar heads or even in specific polyunsaturated phospholipids [11,12].

Physico-chemical studies on isolated coated vesicles can provide useful elements in the understanding of the questions exposed above. Particularly, all the methods reporting on the microviscosity inside the membranes can help in determining if some constraints on the phospholipids are released when the vesicles shed their coat. This was first studied by inserting pyrene and parinaric acid in the phospholipid bilayer of bovine brain coated vesicles and by measuring the fluorescence parameters of these dyes [7]. This has been reexamined here, by inserting paramagnetic labels at different depths of the membranes and by using ESR spectroscopy.

Materials and Methods

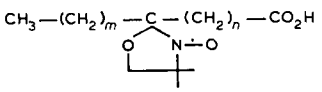
Coated vesicles purification. Coated vesicles were purified according to a procedure derived from Nandi et al. [13] and reported elsewhere [10]. Bovine brains were stored on ice and treated one hour after animals killing in the slaughterhouse.

The stock of purified samples was kept under nitrogen at 4° C and used during the month following the preparation.

Considering the tendency of clathrin coated vesicles to aggregation, samples were submitted to a low speed centrifugation before starting any experiment.

Clathrin coat proteins purification. Clathrin coat proteins were extracted from clathrin coated vesicles by dialyzing one night against 1 M Tris (pH 7.3), 1 mM EDTA and 0.1% β-mercaptoethanol. Stripped vesicles were centrifuged for 50 min at $100\,000 \times g$. It was ascertained by SDS-polyacrylamide gel electrophoresis that in these conditions and according to Wiedenmann et al. [14], the supernatant contains most of heavy and light chains and significant amounts of proteins of 100 and 50 kDa.

Incorporation of spin labels into the membrane. The stearic acid spin labels (m,n)SA were obtained from



General formula of (m,n)SA.

Molecular Probes and used without further purification. The membrane suspensions were labeled for ESR at a maximum level of 2% for the probe/phospholipid molecular ratio. An appropriate organic solution of spin label was distributed over the surface of a roundbottomed flask by rotatory evaporation and then dried under high vacuum. Finally the membrane suspensions were added to the film for fatty acid incorporation, shaken gently and incubated for 12 h. The unincorporated label remaining on the glass was not quantified and considered to be low.

Lipid extraction. Uncoated vesicles were prepared by extracting clathrin coat proteins from native coated vesicles as described above. The stripped vesicles were centrifuged for 50 min at $100\,000 \times g$ and the pellet resuspended in water.

The natural phospholipids were then extracted according to Folch et al. [15]. Typically, 18 ml of diluted native coated vesicles (2.6 mg/ml of total proteins) gave a pellet of uncoated vesicles resuspended in 11 ml of water. The phospholipids were extracted twice with 40 ml $CHCl_3/CH_3OH$ (2:1, v/v). The organic phase was washed twice with 30 ml $CHCl_3/CH_3OH/H_2O$ (3:48:47, v/v) and then evaporated in a round bottom flask. The yield of extraction was around 50%. The lipids were characterized with two systems of TLC ($CHCl_3/CH_3OH/NH_4^+$ (65:25:5, v/v) and $CHCl_3/CH_3OH/CH_3COOH/H_2O$ (75:45:12:6, v/v)). The mean molecular mass of the extracted phospholipids was taken as 800 dalton.

Liposomes were prepared by sonicating with an instrument manufactured by OSI France at an amplitude of 6 μm peak to peak and a frequency of 21 kilocycles/s.

ESR measurements. The spectra were recorded at 9.3 GHz on a Bruker ER200D spectrometer equipped with a field frequency lock and a temperature control unit. The temperature of the sample was measured inside the cell with a copper-constantan thermocouple. The sweep field was 100 gauss at 100 kHz modulation frequency and 1.0 gauss modulation amplitude (0.5 gauss in the case of (1,14)SA). In order to increase the signal to noise ratio, the spectra were accumulated in a Bruker BNC12 computer and transferred to an Olivetti M24 computer through a home-built interface. The resolution was of 4000 points/100 gauss. Spectral substractions, smoothing and double integrations were performed using an interactive routine written in BASIC.

Different physical parameters were obtained from the ESR spectra according to the type of label studied and to its motion. When the paramagnetic *N*-oxyloxazolidine ring of the stearic acid spin-label experienced rapid ($\tau_c < 3 \cdot 10^{-9}$ s) and essentially isotropic reorientational motions ((1,14)SA), an indicative correlation time was calculated following Keith et al. [16]:

$$\tau_c = (6.5 \cdot 10^{-10}) W_0 \cdot [(h_0/h_{-1})^{-1/2} - 1]$$

where W_0 is the peak to peak width, h_0 the height of the central line and h_{-1} is the height of the high-field line.

For labels of slow motion, the results were reported using the separation of the outer extrema ($2A_{\parallel}$) as an index of the mobility of the fatty acid chains in the lipid bilayers.

Results

Incorporation of spin labels into the membranes

The incorporation of spin labeled fatty acids (m,n)SA into coated vesicles, uncoated vesicles and small unilamellar vesicles of extracted phospholipids was studied at equilibrium after one night of incubation at 20 °C in 10 mM Mes (pH 6.5).

The resulting ESR spectra were clearly the superposition of two types of signals: one type arising from labels incorporated into the membranes, and the other from labels free in solution. This was considered to be due to a simple partition of the labels between the aqueous phase and the organic phase [17]. This was quantified by spectral substraction.

The extent of incorporation was found drastically dependent on the position of the nitroxide radical along the hydrocarbon chain of the stearic acid.

Fig. 1 shows typical spectra of spin labeled fatty acids in the presence of liposomes made with phospholipids extracted from the coated vesicles. ESR experiments were performed at 7 °C in 10 mM Mes (pH 6.5). While (12,3)SA was nearly completely incorporated, only little incorporation could be obtained with (5,10)SA. The corresponding concentrations of free labels in presence of identical values of phospholipids are summarized in Table I. The given values are only indicative since the double integration necessary to their estimation was realized on spectra of rather poor signal to noise ratio. Particularly the concentration of free (12,3)SA was too low and the concentration of free (5,10)SA too high. Table I and Fig. 1 illustrate however how determinant is the position of the doxyl group.

TABLE I

Incorporation of the different spin labels into the membranes

Temperature: 7 °C. 10 mM Mes. Lipid concentration: 10^{-3} M. Label concentration: $2 \cdot 10^{-5}$ M. Values marked with an asterisk have been obtained at a lipid concentration of $3 \cdot 10^{-3}$ M.

Label	% Free label		
	extracted phospholipids	uncoated vesicles	coated vesicles
(12,3)SA	<1	–	–
(10,5)SA	1 *	–	–
(7,8)SA	15.5	22.0	17.5
	6.4 *	–	–
(5,10)SA	> 50 *	–	–
(1,14)SA	2 *	–	–

The same study was applied to the incorporation of (7,8)SA into coated and uncoated membranes containing the same lipid concentration estimated by phosphorus determination (10^{-3} M, Table I). The experiments were repeated varying the concentration of the

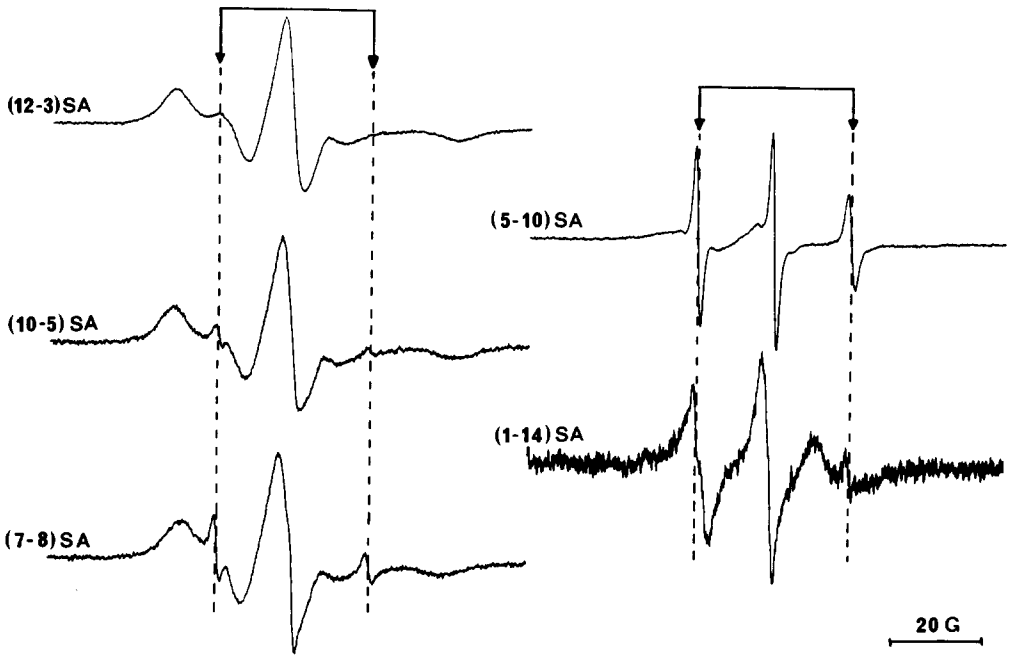


Fig. 1. Spectra of stearic acid spin labels in liposomes made of lipids extracted from bovine brain uncoated vesicles in 10 mM Mes (pH 6.5). Temperature: 7 °C. The arrows indicate the resonance lines arising from the labels free in solution.

phospholipids in order to reach enough precision in the measurement of the distribution coefficient of the label, K, defined as:

$$K = \frac{n_1 \cdot [H_2O]}{n_w \cdot [\text{lipid}]}$$

where n_1 is the number of moles in the lipid phase and n_w the number of moles in the aqueous phase. At 7 °C, we found $K = 3.1 \cdot 10^5$ for the liposomes made from the extracted lipids, $K = 2.5 \cdot 10^5$ for the coated vesicles and $K = 2.0 \cdot 10^5$ for the uncoated vesicles. The presence of the clathrin coat thus led to a slight increase of the incorporation of the spin labels. This slight increase could be the result of the binding of a few labels to the extrinsic proteins. Such a binding has been observed in preliminary experiments with purified extrinsic proteins reassembled into empty coats. It has to be noticed that the ESR spectrum of (7,8)SA adsorbed to the purified coat proteins can easily be identified since it is characterized by an hyperfine splitting approx. 10 gauss higher than that observed for the labels embedded in the liposomes made with the phospholipids extracted from the coated vesicles (Privat, J.P. et al., to be submitted).

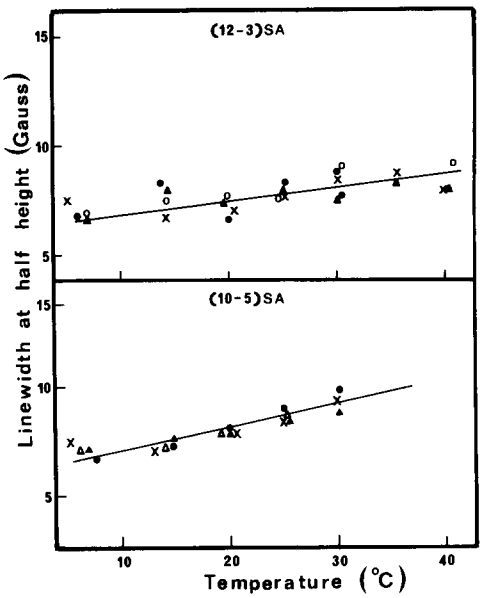


Fig. 2. Width at half-height of low-field extremum of the spin-labeled stearic acid spectra as a function of temperature. ●, Labels incorporated into liposomes of extracted lipids (100 mM Mes (pH 6.5)). ▲, Labels incorporated into liposomes of extracted lipids (10 mM Mes (pH 6.5)). ×, Labels incorporated into coated vesicles (100 mM Mes (pH 6.5)). ○, Labels incorporated into coated vesicles (10 mM Mes (pH 6.5)). △, Labels incorporated into uncoated vesicles (10 mM Mes (pH 6.5)).

ESR spectra of incorporated spin labels

Some important features appeared from the comparison of the spectra of the labels incorporated in the different membranes.

First, the homogeneity of the environment of the labels has been tested. Fig. 2 and Fig. 3 report the analysis of the linewidth of the ESR resonance at low magnetic field.

This linewidth was found independent from the type of membrane in which (12,3)SA and (10,5)SA were incorporated. It is striking that it increased with the temperature, while the mobility of the labels was also increased (as deduced from the concommitant decrease in the hyperfine coupling). Particularly for (10,5)SA, the linewidth at low magnetic field was of the same magnitude in the different membranes as in small unilamellar vesicles of egg PC at pH 5.0 where the stearic acid is not ionized [18]. These observations indicate an heterogeneity in the environment of the labels giving rise to a single broad spectrum (see discussion). The same study was applied to (7,8)SA (Fig. 3). Its linewidth at low magnetic field showed a biphasic dependence when the label was incorporated into coated or uncoated vesicles. This is exactly the behaviour which can be expected from two spectra whose overlapping is progressively reduced, i.e. at temperatures over 30 °C, two distinct contributions could be distinguished. As this was not the case when (7,8)SA was incorporated into the extracted lipids, this effect can be attributed exclusively to the presence of the intrinsic proteins (it was verified by electrophoresis that extrinsic proteins are completely absent in our preparations of uncoated vesicles). This was analyzed in terms of perturbation of the boundary lipids in the way described by Marsh [19] (see below the study of the hyperfine coupling). The linewidth measurements could not be reproduced with (5,10)SA. In this case, the 'liquid lines' associated to the non incorporated spin labeles were too strong and the underlying spectra of the incorporated spin labels could not be

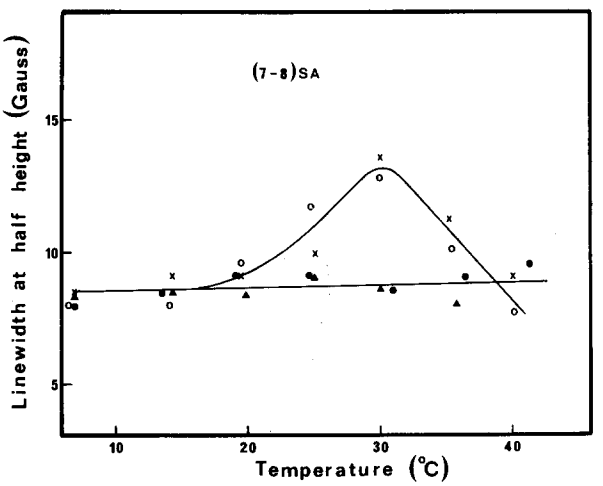


Fig. 3. Width at half-height of low-field extremum of (7,8)SA incorporated into the different phases. Same symbols as in Fig. 2.

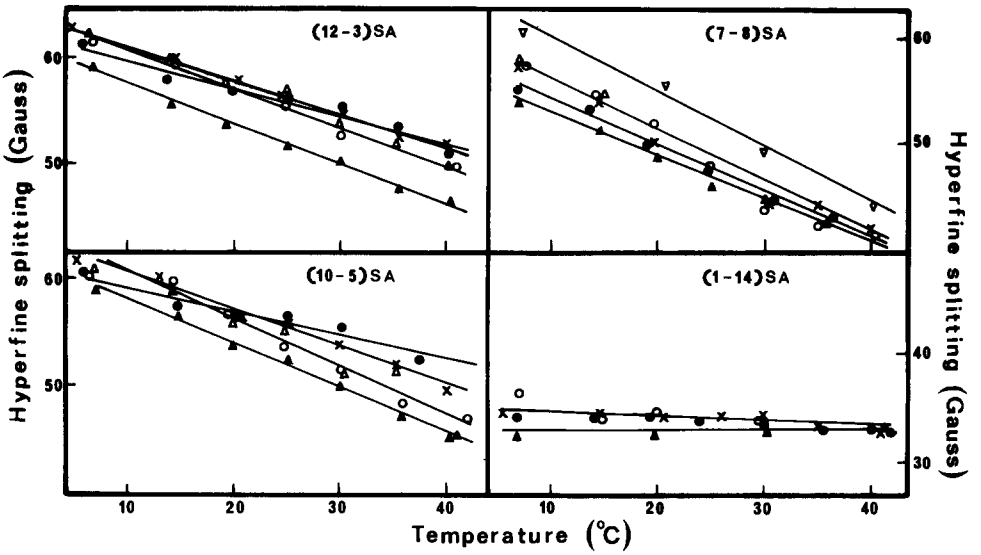


Fig. 4. Temperature dependence of the hyperfine splitting $2A_{||}$ of the spin-labeled stearic acids incorporated in the different phases. Same symbols as in Fig. 2. ▽, Labels incorporated into uncoated vesicles (100 mM Mes (pH 6.5)).

studied with accuracy. The mobility of the *N*-oxyloxazolidine ring of (1,14)SA was such that its spectra were analyzed in terms of correlation times (see below).

Secondly, the precise analysis of the motion of the labels would require a simulation of the ESR spectra not undertaken here. The variation of the linewidth at low magnetic field revealed that the heterogeneity of the environment would complicate such a study. For our purpose, the decrease of the overall hyperfine splitting $2A_{||}$ was used to illustrate the flexibility of the acyl chains of the phospholipids in the membranes. In Fig. 4 is thus reported the hyperfine splitting $2A_{||}$ as a function of the ionic strength and the temperature for the different labels and the different phases, except for (5,10)SA. The partition of (5,10)SA between the lipids and water excluded the precise determination of the hyperfine splitting of the incorporated form. For (7,8)SA, the values reported are associated with the component of higher mobility (i.e., the smaller $2A_{||}$) which dominates the ESR spectra.

A general observation is that in all phases the hyperfine coupling of all spin labels were gradually decreased with no abrupt changes over the temperature range 10–40 °C. No evidence was found for a lipid phase transition in any of the samples investigated.

The (12,3)SA carries the *N*-oxyloxazolidine ring close to the polar region of the membranes. Increasing the ionic strength from 10 mM to 100 mM Mes decreased the mobility of the doxyl ring incorporated into the liposomes of the extracted lipids. This effect of the ionic strength has been reported on other systems [20]. It could be the result of an increase of the packing of the polar heads due to a screening of the repulsive forces. It could also be the result of a change of the pK_a of the spin labels followed by the modification of the depth at which their doxyl group was anchored.

It is apparent that, independently of the ionic strength, the packing of the polar heads was the same in both coated and uncoated vesicles. The extrinsic proteins thus do not modify the environment of the ring which is essentially determined by the intrinsic proteins. As compared to the pure lipid phase, the intrinsic proteins define a lipid state which is more sensitive to the temperature (particularly in 0.1 M Mes) and less sensitive to the ionic strength.

Ionic strength and temperature effects detected by (10,5)SA were very similar, except that its ring was located deeper in the membranes and that its mobility increased slightly in all the phases as compared with that of (12,3)SA. When the label was inserted into coated or uncoated vesicles, however, some sensitivity to the ionic strength was recovered.

For (7,8)SA, the present study of the linewidth at low magnetic field demonstrated the occurrence of two distinct components in the ESR spectra of labels incorporated into coated and uncoated vesicles. For two reasons the component of higher mobility in the spectra of (7,8)SA can be attributed to the labels located in the unperturbed fluid lipid phase. Firstly, above 30 °C the hyperfine coupling of (7,8)SA in coated and uncoated vesicles is identical to that of (7,8)SA incorporated into liposomes of extracted lipids (in 10 mM Mes). Secondly, Fig. 5 shows that the result of the appropriate mathematical substraction of a component of restricted mobility (spectrum number 1) to the experimental spectrum of (7,8)SA incorporated into uncoated vesicles in 100 mM Mes at 30 °C (spectrum nb 2 in which the liquid lines have been eliminated) leads to a spectrum identical

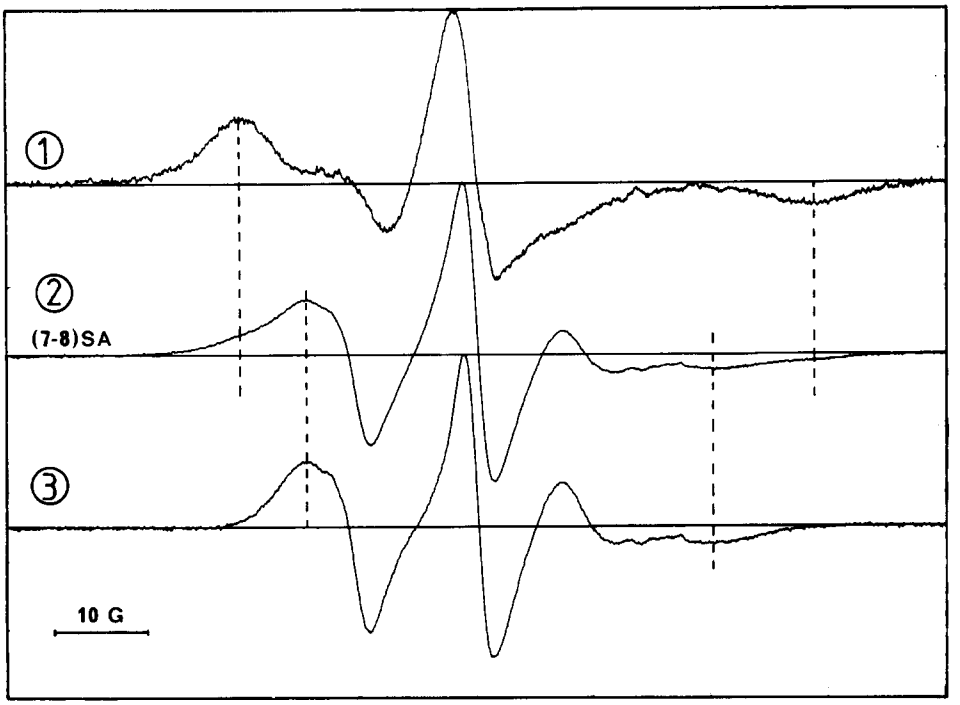


Fig. 5. Mathematical analysis of the spectrum of (7.8)SA incorporated into uncoated vesicles (100 mM Mes (pH 6.5)) at 30 °C. (1) (7,8)SA in uncoated vesicles (100 mM Mes (pH 6.5)) at 6.5 °C. Spectrum taken as the component of restricted motion. (2) (7,8)SA in uncoated vesicles at 30 °C. (3) Spectrum obtained after substraction of the contribution of the component of restricted motion to the spectrum nb 2. The hyperfine splitting $2A_{||}$ in the spectrum nb 3 is identical to that found for (7,8)SA incorporated into the extracted lipid liposomes (100 mM Mes (pH 6.5) at 30 °C).

with that of the label at 30 °C in the lipids extracted from the system (spectrum nb 3). This method of substraction has been used for the estimation of the lipids located in the close vicinity of the intrinsic proteins. This estimation can hardly be done here because the limit form defining the state of the labels of restricted motion is not known. It was taken a priori as the spectrum of (7,8)SA incorporated into the uncoated vesicles at 6.5 °C. The symmetric operation leading to subtraction with the spectrum observed in the extracted lipids should overcome this problem. But the preponderance of this spectrum is such that the operation gives a too high imprecision. Moreover, the repartition of the labels does not necessarily reflect the repartition of the different phospholipids. It is clear, however, that a large amount of labels is not influenced by the presence of the intrinsic proteins.

As the *N*-oxyloxazolidine ring went deeper in the hydrocarbon region, its mobility increased and its sensitivity to both the temperature and the ionic strength decreased for all the phospholipid phases studied. For (1,14)SA the motion of the probe was sufficiently rapid to allow (despite slow-motional contributions) a rough estimation of a correlation time at all the temperatures. This estimation was made on computed spectra obtained after substraction of the lines due to the non incorporated label. These correlation times are increased in the coated vesicles (For example, $\tau_c = 1.4$ ns in extracted lipid liposomes and $\tau_c = 1.6$ ns in coated vesicles at 20 °C (10 mM Mes, pH 6.5)). As the correlation time is a function of the microviscosity, this can be taken as the sign of an increase of the microviscosity of the hydrocarbon core.

Discussion

This work is the first study of the incorporation of spin-labeled fatty acids into the lipid phase of clathrin bovine brain coated and uncoated vesicles and into vesicles made from their extracted lipids. At a given lipid concentration, the amount of incorporation was found dependent on both the type of the lipid phase studied and the position of the nitroxide group. We verified that the presence of the free label in solution was due to a true partition process: the label can be completely extracted from clathrin-coated vesicles by chromatography on Sephacryl S-1000.

The incorporation of spin-labeled fatty acids is dependent on the nitroxide position

Among all the compounds tested, (5,10)SA was founded the less lipid-soluble. The same result was reported by Tran [21] in the study of the incorporation of spin-labeled stearic acids into Duck erythrocyte membranes and by Vachon et al. [22] in the study of their incorporation into DPPC vesicles. The characteristic sharp three lines spectrum of (5,10)SA is also visible

in presence of egg PC liposomes [17], in presence of membranes of normal 3T3 and transformed SVT2 mouse fibroblasts [23], in presence of sarcoplasmic vesicles [24] or in mitochondria of brown adipose tissue of cold-adapted rats [25]. Some exceptions were reported, however, as in presence of XC sarcoma, Vero and *Aedes aegypti* plasma membranes [26].

The minimum of incorporation found for (5,10)SA does not correlate with the extremum of a known physical property of the lipid phase itself. It is evident, for example, that this does not correlate with a discontinuity in the flexibility gradient of the phospholipid chains. Thus, the minimum of incorporation is most probably the result of an intrinsic property of (5,10)SA itself. Vachon et al. [22] proposed that the carboxylic part of this label could interact with the rather hydrophilic doxyl group. It can effectively be imagined that, according to the location of the doxyl group, a more hydrophilic molecular conformation could be formed. This hypothesis has not been demonstrated, however, and may not be verified since liquid lines can also be detected when the methyl ester of (5,10)SA is used in presence of erythrocyte membranes [27].

The clathrin network does not inhibit the spin-labeled fatty acids incorporation

Inside the lipid phases tested, several physical factors should determine the amount of incorporation of a given spin label. The partition of TEMPO [28], for example, was dependent on the microviscosity. As evidenced by the measurement of the correlation times of (1,14)SA, large changes of microviscosity in the inner core of our phases cannot be invoked. Many other factors such as the lateral pressure, the accessible volume or the surface potential may also play an important rôle.

The clathrin network itself might create an impenetrable barrier around the coated vesicles. On the contrary, the incorporation of the labels was apparently slightly greater in coated vesicles than in uncoated ones. We got many evidences that the major part of the labels was really embedded in the hydrophobic core of the membranes of coated vesicles. Particularly, the evolution of their spectral parameters (in function of the temperature or in function of the doxyl position along the chain of the fatty acids) was characterizing an hydrocarbon like region. The same situation prevailed in our previous work [10]. It has to be noticed that few labels could be adsorbed to the clathrin coat (when present) since it has been found that the spin labels can bind to the isolated coat proteins (Privat, J.P. et al., to be published). Our preliminary results, however, do not allow to determine if all the binding sites offered by the isolated proteins organized in empty coats are still accessible when the coats are organized around the membranes.

Fluctuations in the location of the incorporated labels

Once incorporated into the membranes, the labels can be located in different environments and can migrate from one place to another. This is of particular importance in the analysis of the physical parameters of the ESR spectra and is susceptible to induce either a line broadening or a line splitting.

Firstly, transversal fluctuations can occur which are due to fast changes in the depth at which the nitroxide groups are embedded in one layer of the membranes. These fluctuations are amplified in the inner core of the lipid phases [29]. The work of Sanson et al. [30] has evidenced a transversal fluctuation specific of the labeled fatty acids. Their two ionic forms are differently anchored (the acid form is anchored deeper in the membranes) and this is responsible for different hyperfine couplings clearly visible in the ESR spectra at temperatures above 40 °C. Below this temperature is found a line broadening. This is in complete agreement with our linewidth study of (12,3)SA in function of the temperature. The pH choosen here (pH 6.5) corresponds to the pK_a of the labels embedded in egg PC vesicles. The two ionic forms of the labels were probably present even if the pK_a of the labels in our system might have been shifted in a way which has not been determined in order to avoid the work at 40 °C and the possible denaturation of our membranes.

An other important contribution to transversal fluctuations is due to the interbilayer exchange. As our labels were incompletely ionized, the non-ionized form was susceptible to migrate between the outer and the inner phospholipid layer of the vesicles. Furthermore this migration could easily take place during the 12 h of the incorporation process. No attempt has been made here in order to characterize the interbilayer partition. It is evident, however, that this partition is not only susceptible to broaden the spectra but also to minimize the effect of clathrin, probably more important on the outer monolayer.

The second type of fluctuations is due to the lateral diffusion of the phospholipids and of the labels. This can induce an additional spectral broadening by spin–spin interactions. The concentration of labels used in this work was sufficiently low to avoid it. Other lateral fluctuations can be related to a phase separation (possible in the vesicles of extracted lipids), to the presence of intrinsic proteins (boundary lipids) or to anchoring effects by extrinsic proteins (the clathrin network).

Restriction on the motion of the labels located around the intrinsic proteins

The uncoated vesicles are known to be rich in intrinsic proteins (60% by weight according to Alfsen et al. [7]). Around the proteins, the boundary labels were clearly evidenced with (7,8)SA. Indeed, the restriction on the motion must occur for all the labels tested,

independently from the position of the labeled carbon. But the difference between the two lipid environments must be sufficient to induce two resolved spectra. This has been stressed in the review of Devaux and Seigneret [33]. In first analysis, according to our numerical treatment of the spectra, the boundary labels appear to have an hyperfine splitting of 57 gauss which seems to be temperature independent. This is less than the 'rigid limit', i.e., this is smaller than what would be expected from completely immobilized spin labels inserted into protein aggregates [31,32]. This value indicates, however, that the lifetime of the label/protein interactions is long enough to avoid spectral mixing during the time scale of the ESR measurements.

Use and limits of the of the ESR study

Spin-labeled stearic acids allow to probe different depths of the membranes in which they have been inserted. In this respect, they differ from fluorescence measurements using pyrene or even parinaric acids whose emission arises from a less precise location [34]. It is of particular interest, however, to compare the two types of results in order to sum up the picture emerging from them.

It is first evident that the clathrin coat proteins do not greatly perturb the inner core of the membranes. The lateral diffusion of pyrene at 25°C showed an increase of 26% upon the release of clathrin, i.e., a slight decrease in the microviscosity [7]. With (1,14)SA, we also found a slight decrease (15%) of the correlation time of the label in the liposomes made from the extracted lipids as compared with that in the coated vesicles. This is a very slight perturbation, taking into account that the ESR signal, in the case of (1,14)SA is the mean value between the signals arising from the labels close to the proteins and the other ones.

The second important feature is that both fluorescence of parinaric acids and ESR of spin-labeled stearic acids clearly resolve 'fluid' and 'restricted' lipids. ESR experiments also indicate that the state of the 'fluid' lipids is very close to that observed with liposomes made from extracted lipids devoid of extrinsic and intrinsic proteins as well. This indicates that large lipid domains exist whose organization is not drastically altered by the clathrin coat proteins. We have previously found that the binding of clathrin coat proteins to uncharged synthetic phospholipids devoid of intrinsic proteins lowers the rate of motion of their polar head groups and reduces the lateral diffusion of charged labeles. In the inner core of the membranes, the flexibility of the hydrocarbon chains and the rotational diffusion of free labels were not perturbed. Thus, clathrin coat proteins establish interactions at the membrane surface exclusively. Any penetration of the proteins inside the hydrophobic core of the phospholipids can clearly be excluded. The same situation prevails in the natural system. The opposite conclusion was drawn out by Steer et al. [35]. By infrared spectroscopy, these authors associated an increase of the number of *gauche* chain conformers to the presence of the clathrin coat. These results, however, can easily be reconciled with ours and with our conclusion if this effect is not attributed globally to all phospholipids but to an increase of boundary lipids.

In conclusion, these results support the persistence of lipid domains in the endocytotic vesicles despite the accumulation of receptors which follows their formation. In these domains, the structural organization is identical to that of lipids devoid of proteins. More work must be undertaken in order to determine if the coat proteins interact either with the exposed area of intrinsic proteins exclusively, or with phospholipids or both.

Acknowledgements

The authors wish to thank R. Lévêque whose participation to the experimental part of this work has been greatly appreciated and A. Houssin who performed the electrophoresis of coated and uncoated vesicles.

References

1 Goldstein, J.L., Anderson, R.G.W. and Brown, M.S. (1979) Nature 279, 679–685.
2 Brown, M.S., Anderson, R.G.W. and Goldstein, J.L. (1983) Cell 32, 663–667.
3 Pfeffer, S. and Rothman, J.E. (1987) Annu. Rev. Biochem. 56, 829–852.
4 Vigers, G.P.A., Crowther, R.A. and Pearse, B.M.F. (1986) EMBO J. 5, 2079–2085.
5 Pearse, B.M.F. (1985) EMBO J. 42, 2457–2460.
6 Pearse, B.M.F. and Crowther, R.A. (1987) Annu. Rev. Biophys. Chem. 16, 49–68.
7 Alfsen, A., De Paillerets, C., Prasad, K., Nandi, P.K., Lippoldt, R.E. and Edelhoch, H. (1984) Eur. Biophys. J. 11, 129–136.
8 Blumenthal, R., Henkart, M. and Steer, C.J. (1983) J. Biol. Chem. 258, 3409–3415.
9 Maesawa, S., Yoshimura, T., Hong, K., Düzgünes, N. and Papahadjopoulos, D. (1989) Biochemistry 28, 1422–1428.
10 Privat, J.P., Egret-Charlier, M., Labbé, H. and Ptak, M. (1989) Biochim. Biophys. Acta 979, 257–267.
11 Bomsel, M., De Paillerets, C., Weintraub, H. and Alfsen, A. (1986) Biochim. Biophys. Acta 859, 15–25.
12 De Paillerets, C., Bomsel, M., Weintraub, H., Pépin, D. and Alfsen, A. (1987) FEBS Lett. 219, 113–118.
13 Nandi, P.K., Irace, G., Van Joarsveld, P.P., Lippoldt, R.E. and Edelhoch, H. (1982) Proc. Natl. Acad. Sci. USA 79, 5881–5885.
14 Wiedenmann, B., Lawley, K., Grund, C. and Branton, D. (1985) J. Cell Biol. 101, 12–18.
15 Folch, J., Lees, M. and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497–509.
16 Keith, A., Bulfield, G. and Snipes, W. (1970) Biophys. J. 10, 618–629.
17 Butler, K.W., Tattrie, N.H. and Smith, I.C.P. (1974) Biochim. Biophys. Acta 363, 351–360.
18 Egret-Charlier, M., Sanson, A., Ptak, M. and Bouloussa, O. (1978) FEBS Lett. 89, 313–316.

19 Marsh, D. (1985) ESR spin-label studies of lipid–protein interactions, in Progress in Protein–Lipid Interactions, Vol. 1 (Watts, A. and De Pont, J.J.H.H.M., eds.), pp. 143–172, Elsevier, Amsterdam.
20 Görisssen, H., Marsh, D., Rietveld, A. and De Kruijff, B. (1986) Biochemistry 25, 2904–2910.
21 Tran, P.L. (1979) Eur. J. Biochem. 93, 559–563.
22 Vachon, A., Lecomte, C., Berleur, F., Roman, V., Fatome, M. and Braquet, P. (1987) J. Chem. Soc. Faraday Trans. 1, 83, 177–190.
23 Gaffney, B.J. (1975) Proc. Natl. Acad. Sci. USA 72, 664–668.
24 Seelig, J. and Hasselbach, W. (1971) Eur. J. Biochem. 21, 17–21.
25 Cannon, B., Polnaszek, C.F., Butler, K.W., Eriksson, L.E.G. and Smith, I.C.P. (1975) Arch. Biochem. Biophys. 167, 505–518.
26 Bales, B.L. and Leon, V. (1978) Biochim. Biophys. Acta 509, 90–99.
27 Butterfield, D.A., Roses, A.D., Cooper, M.L., Appel, S.H. and Chesnut, D.B. (1974) Biochemistry 13, 5078–5082.
28 Gaffney, B.J. and Chen, S.-C. (1977) Spin-label studies of membranes, in Methods in Membrane Biology, Vol. 8, (Korn, E.D., ed.), pp. 291–358, Plenum Press, New York.
29 Meraldi, J.P. and Schlitter, J. (1981) Biochim. Biophys. Acta 645, 193–210.
30 Sanson, A., Ptak, M., Rigaud, J.L. and Gary-Bobo, C.M. (1976) Chem. Phys. Lipids 17, 435–444.
31 Jost, P., Libertini, L.J., Hebert, V.C. and Griffith, O.H. (1971) J. Mol. Biol. 59, 77–98.
32 Watts, A., Davoust, J., Marsh, D. and Devaux, P.F. (1981) Biochim. Biophys. Acta 643–676.
33 Devaux, P.F. and Seigneuret, M. (1985) Biochim. Biophys. Acta 822, 63–125.
34 Sklar, L.A., Hudson, B.S. and Simoni, R.D. (1977) Biochemistry 16, 819–828.
35 Steer, C.J., Vincent, J.S. and Levin, I.W. (1984) J. Biol. Chem. 259, 8052–8055.