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INVESTIGATIONS OF THERMOTROPIC PHASE CHANGES IN PERIPHERAL NERVE OF FROG AND RAT

A SPIN LABEL STUDY*

U. SCHUMMER[a], D. HEGNER[a,**], G. H. SCHNEPEL[b] and H. H. WELLHÖNER[c]

[a]*Institut für Pharmakologie und Toxikologie im Fachbereich Veterinärmedizin der Justus Liebig-Universität Giessen,* [b]*Institut für Biophysik, Strahlenzentrum der Justus Liebig-Universität Giessen and* [c]*Pharmakologisches Institut der Justus Liebig-Universität Giessen, D-63 Giessen (G.F.R.)*

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SUMMARY

The temperature-dependent fluidity of myelin of frog and rat peripheral nerve (Nervus ischiadicus) was studied using the spin label technique. In frog nerve a phase change was detected at 38 °C. In rat nerve no sharp phase change could be established, and the lipid-depleted frog and rat nerve also showed no transition. From the spectral data, it was concluded that in frog and rat nerve the lipid-protein interactions are different, i.e. species dependent. Ca^{2+}-depletion of frog nerve caused a loss of transition, while rat nerve remained unaffected. Thus it was indicated that, in frog nerve, Ca^{2+} is involved in the phase change. In the total lipid extract of frog nerve a phase change centered at 32 °C occurred, while the total lipid extract of rat nerve again showed no transition. It is suggested that a connexion exists between our results and investigations on the temperature dependence of an axonal conduction block of nerve.

INTRODUCTION

Thermotropic phase transitions which occur with phospholipids are of considerable interest with regard to biological membrane function. Myelin of the central and peripheral nervous system of mammals (e.g. beef brain and human femoral nerve) and extracted lipids have been examined for these phase transitions by Chapman and co-workers [1–3]. These studies have shown that, in wet fresh myelin from homeothermic animals, thermotropic phase transitions do not occur in a temperature range

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** Present address: Institut für Pharmakologie, Toxikologie und Pharmazie der Tierärztlichen Fakultät der Universität München, D-8000 München (Germany).

of biological interest. These transitions are present only in lipids which are not rich in cholesterol [1,4–6].

In natural membranes of homeothermic animals, cholesterol apparently controls the fluidity of the hydrocarbon chains of the lipids and provides a mixture of fluid and solid phases at physiological temperatures [7]. The fatty acids in some membranes of poikilothermic animals are to a great extent unsaturated. The degree of unsaturation depends on the temperature of the environment that the animals live in [8]. In such membranes, the degree of condensation of the phospholipids caused by cholesterol may be somewhat different from that of homeothermic animals [9, 10]. It may be assumed that the physical state of the lipids of peripheral frog nerve is important to the temperature dependence of the susceptibility to and fixation of neurotoxins [11]. The experiments described here are also of considerable interest in understanding the mechanism that is responsible for a reversible, temperature-dependent conduction block in peripheral nerves. This conduction block is dependent on species and affected by Ca^{2+} [12].

We report here a comparative spin label study on peripheral nerves of frogs and rats, which was carried out to examine the following questions. (1) Are there any thermal transitions in nerves of frogs and rats detectable by incorporated spin label? Provided that those transitions exist, (2) are they also present in the extracted lipids? And if this is true, (3) is there an electrostatic interaction which affects such a phase change between the lipids and calcium ions?

MATERIALS AND METHODS

Stearic acid spin label I (12.3) was purchased from Syva, Palo Alto, Calif., and stored at −18 °C. All reagents used were of analytical grade, and the water was double glass distilled.

The frogs used in the following experiments were of the species *Rana temporaria*. They were obtained from Tierhandlung Koch, Holzminden (Germany), and kept in a water tank. The rats were one-and-a-half-year-old white Wistar rats. Nn. ischiadici obtained from frogs and rats were stored at 4 °C in Ringer solutions for poikilothermic and homeothermic animals, respectively, and labeled by an exchange reaction from bovine serum albumin as described previously [13, 14]. Lipid was extracted (more than 95 %) from the nerves without pertubing their structure following the method of Fleischer and Fleischer [15]. Total lipid extraction was carried out according to Folch et al. [16]. Lipid analysis was carried out using the following method. Lipid phosphorus was determined with Fiske-SubbaRow reagent as described by Bartlett [17]. The total cholesterol and cholesterol ester content of the lipid was measured according to Liebermann-Buchard [18]. Methyl esters of fatty acids were prepared by heating approximately 2 mg of the lipid in 2 ml of 5 % methanolic HCl up to 70 °C under a nitrogen atmosphere. The methyl esters were extracted with pentane. The fatty acid pattern was determined using an F 20 Perkin Elmer gas chromatograph, equipped with a 5 % EGS chromosorb column (column temperature 190 °C) and a flame ionization detector. Vesicles were formed from the extracted lipids in Ringer solution as described elsewhere [19].

Calcium ions were removed from the Nn. ischiadici of frogs and rats by gently stirring the nerves for about 10 h at 4 °C in a Ca^{2+}-free Ringer solution containing

10 μmol disodium EDTA. The nerves were placed in plain micro-hematocrit tubes and centrifuged on a Clay Adams micro-hematocrit centrifuge for 5 min to avoid orientation phenomena in the spectra.

ESR spectra were recorded using a Varian E-9 spectrometer. The temperature was controlled by a Varian temperature control unit. The controller was calibrated with an iron-constantan thermocouple. The estimated accuracy was about ±1 °C.

RESULTS

The results of the analysis of the fatty acid composition of the total lipid extract of Nn. ischiadici of frogs are given in Table I. There is a remarkably high content of unsaturated fatty acids, especially oleic acid. The portion of unsaturated fatty acids is 48 mol%.

The lipid composition of the N. ischiadicus of frog is given in Table II. The values for the composition of the lipids of the peripheral nervous system of the rat were calculated from the results of Evans and Finean [20].

TABLE I

FATTY ACID PATTERN OF THE LIPIDS OF THE N. ISCHIADICUS OF FROG

Fatty acid	14 : 0	16 : 0	16 : 1	18 : 0	18 : 1	18 : 2
Content (mol %)	4	36	11	12	33	4

TABLE II

LIPID COMPOSITION (MOL%) OF Nn. ISCHIADICI OF RAT AND FROG

	Rat[a]	Frog
Cholesterol	38.3	24.1[b]
Cerebroside	15.3	26.5
Phosphatidyl-serine, -inositol	8.8	9.4[c]
Phosphatidyl-ethanolamine	17.2	10.8[c]
Sphingomyelin	9.2	6.5[c]
Unidentified fraction	—	14.0

[a] Results of Evans and Finean.
[b] Mean value of four experiments (accuracy better than 10 %).
[c] Mean value of five experiments (accuracy better than 4 %).

Fig. 1 (Curve a) shows the molecular motion of the spin label I (12.3), incorporated in frog nerve, in terms of outer hyperfine splitting, $T_{\|}$, versus temperature. The curve has a sharp discontinuity, which indicates an abrupt change of the fluidity of the myelin at a temperature of about 38 °C. The curve changes its shape when most of the Ca^{2+} is removed from the nerve by disodium EDTA (Fig. 2, Curve a). In the range from 36 to 48 °C the slope of the curve changes, but no sharp transition,

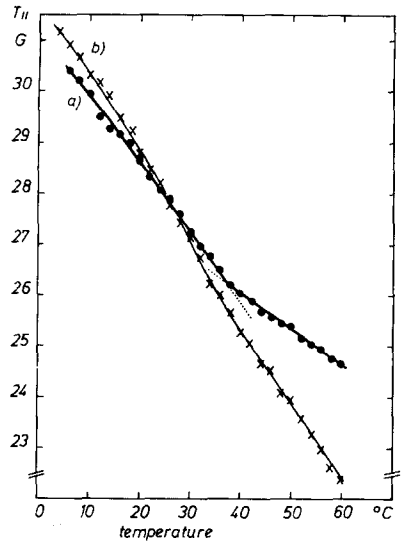

Fig. 1. The outer hyperfine splitting $T_{||}$ of spin label I (12.3) plotted vs temperature of frog nerves. (a) Untreated N. ischiadicus; (b) lipid-extracted N. ischiadicus.

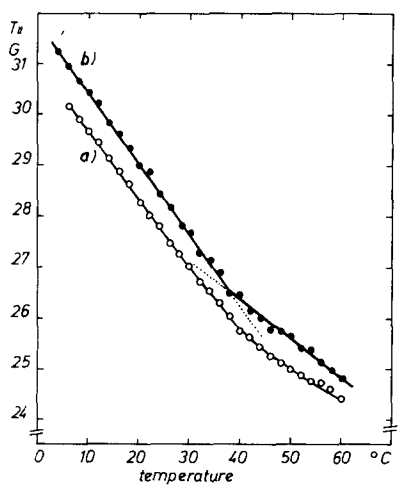

Fig. 2. The outer hyperfine splitting $T_{||}$ of spin label I (12.3) as a function of the temperature of disodium EDTA-treated Nn. ischiadici of frogs in (a) Ca^{2+}-free Ringer solution; (b) Ca^{2+}-containing Ringer solution.

similar to that found for the untreated nerve, is detectable. If the disodium EDTA-treated nerve is gently stirred in a Ca^{2+}-containing Ringer solution for about 10 h, the abrupt change in the fluidity occurs again at a temperature of 39 °C (Fig. 2, Curve b). The extraction of more than 95 % of the lipids from the nerve also causes a loss of this transition (Fig. 1, Curve b). Vesicles formed from the total lipid extract show a phase change centered at 30 °C (Fig. 3).

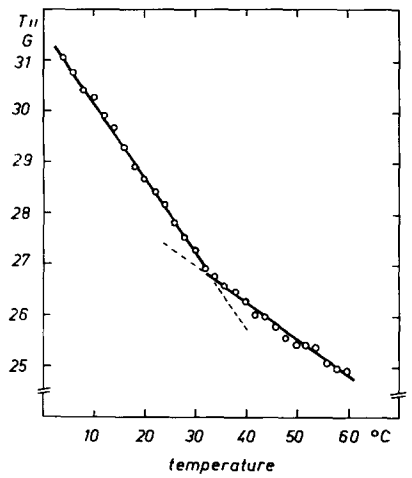

Fig. 3. The outer hyperfine splitting $T_{\perp}$ of spin label I (12.3) plotted vs temperature of spherules of the total lipid extract of Nn. ischiadici of frogs.

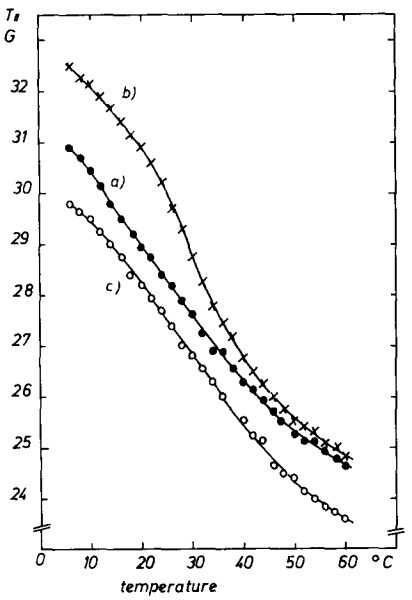

Fig. 4. The outer hyperfine splitting $T_{\perp}$ of spin label I (12.3) plotted vs temperature of Nn. ischiadici of rats and the total lipid extract of Nn. ischiadici. (a) N. ischiadicus of rat; (b) lipid-extracted N. ischiadicus of rat; (c) vesicles of the total lipid extract of Nn. ischiadici.

The results of our investigations on rat nerve are shown in Fig. 4. $T_{\perp}$ is plotted versus the temperature of the specimen. The label I (12.3) was incorporated in (a) nerve, (b) lipid-depleted nerve and (c) vesicles of the extracted lipids. The degree of mobility increases from the lipid-extracted nerve to the lipid vesicles. This indicates a remarkable decrease in the motional freedom of the label incorporated into the myelin,

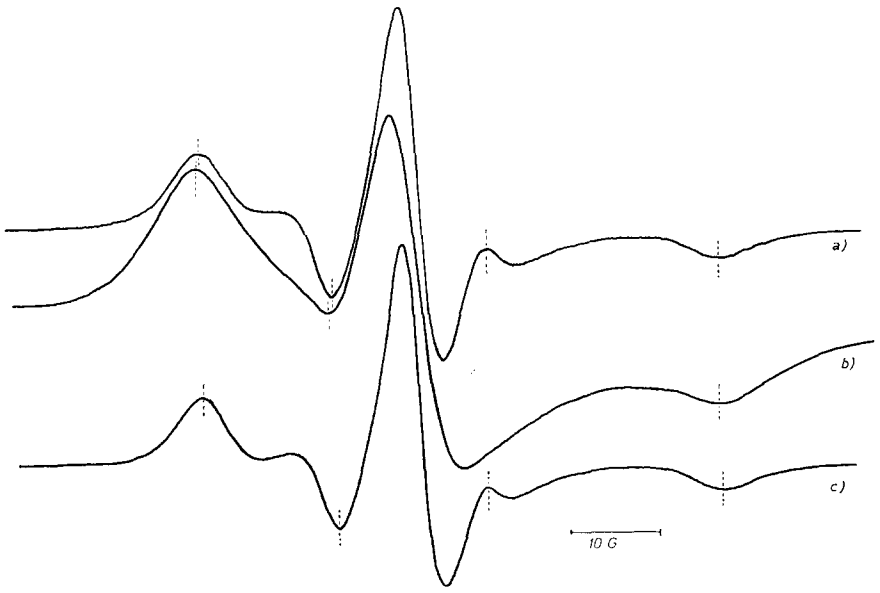

Fig. 5. First derivative of the ESR spectra of spin label I (12.3) incorporated in (a) spherules of the total lipid extract of Nn. ischiadici of frogs, (b) lipid-extracted N. ischiadicus of frog and (c) N. ischiadicus of frog. The spectra were recorded at a specimen temperature of 20 °C. Note that the outer hyperfine splitting $T_{||}$ is nearly the same for all spectra.

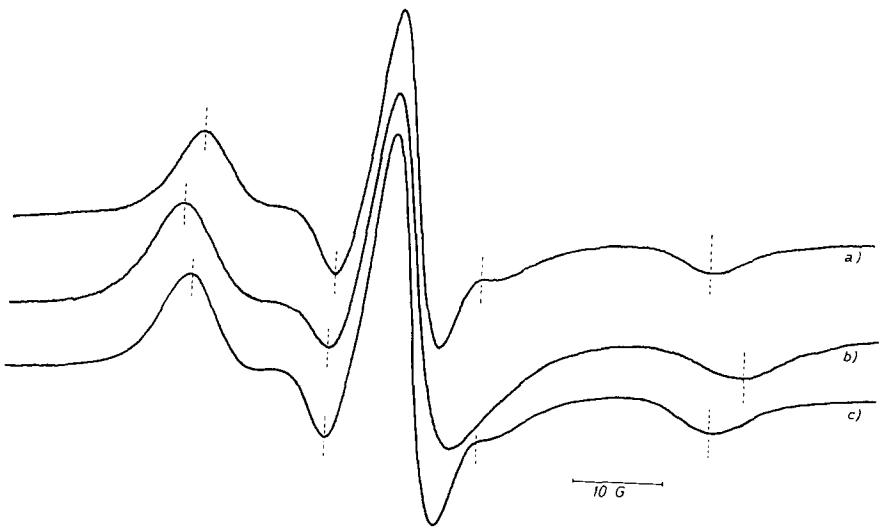

Fig. 6. First derivative of the ESR spectra of spin label I (12.3) incorporated in (a) spherules of the total lipid extract of Nn. ischiadici of rats, (b) lipid-extracted N. ischiadicus (same animal) and (c) N. ischiadicus (same animal). The spectra were recorded at a specimen temperature of 20 °C.

caused by the extraction of the lipids, in the temperature range lower than 40 °C. Within the temperature range examined, all the curves indicate no phase change.

Fig. 5 shows the first derivatives of the ESR spectra of the spin label I (12.3) obtained from the studies on frog nerve. The label was incorporated in (a) spherules of the total lipid extract of Nn. ischiadici, (b) lipid-extracted N. ischiadicus and (c) N. ischiadicus. The spectra were recorded at a specimen temperature of 20 °C. Comparison of the line shapes of (a) and (c) shows that the label molecules perform the same rapid anisotropic motion [21] in both cases and it is suggested that the environment of the fatty acid molecules is virtually the same. Quite a different situation is reflected by the label incorporated in the lipid-extracted nerve (b). Although the outer hyperfine splitting $T_{||}$ is nearly the same as for (a) and (c), the label shows a strongly hindered motion, similar to that known from spin label rigidly bound to large protein molecules [22].

The spectra of the spin labels incorporated in spherules of the total lipid extract of Nn. ischiadici of rats (a), lipid-extracted N. ischiadicus (b) and N. ischiadicus (c) are given in Fig. 6. Spectra (a) and (c) are somewhat different in their respective hyperfine splitting $T_{||}$ and $T_{\perp}$, but they are of the same type, reflecting a rapid anisotropic motion around the long molecular axis. This motion is different from that of the spin label incorporated in lipid spherules and nerves of frogs, as a comparison with Fig. 5 Curves a and c shows. This indicates that the environment of the spin label is different in the nerves of frogs and rats. It is also different for vesicles of the extracted lipids.

DISCUSSION

In this study, it is demonstrated that the stearic acid spin label I (12.3) incorporated in the frog N. ischiadicus reflects a reversible phase change centered at approximately 38 °C. The total lipid extract dispersed in Ringer solution exhibits a similar transition apart from a decrease of the transition temperature to about 32 °C (Fig. 1, Curve a and Fig. 3). Such a phase change is not observed in the lipid-depleted nerve in the whole temperature range investigated (Fig. 3, Curve b). Thus we conclude that the label indicates phase changes of the fatty acid chains of frog nerve lipids and that it does not reflect thermotropic conformation changes of membrane proteins. This is in accordance with the results of the studies of Blazyk and Steim [23] carried out on rat liver microsomes and mitochondria using the differential scanning calorimetry technique. A comparison of the results obtained from labeled rat nerve, vesicles of the total lipid extract and lipid-depleted nerve shows a considerable difference in the temperature-dependent mobility of the spin label (Fig. 5). The slopes of the Curves a and b change at approximately 42 °C and 45 °C, respectively. However, this change in the fluidity of the membrane lipids cannot be considered a phase change. As can be seen from Table II, the cholesterol content of the total lipid extract of the rat nerve myelin exceeds 38 mol%. The failure in detecting a phase transition in those lipids is in good agreement with the findings of several authors [4, 6, 23]. They have found that if the cholesterol content is greater than approximately 30 mol%, the lipids, especially the phospholipids, are prevented from undergoing phase transitions.

The lipid composition of the N. ischiadicus of frog provides a suitable fluidity

of the membranes in the temperature range occurring under living conditions. The role that cholesterol plays here was studied by Papahadjopoulos et al. [24]. There is a great amount of unsaturated lipid and a relatively small content of cholesterol in frog nerve myelin. The cholesterol content of the total lipids is about 24 mol%, which is not enough to prevent phase transitions. Actually, a phase transition occurs in the myelin at approx. 38 °C. Studies on the threshold temperature of axonal conduction block [12, 25, 26] show that in Nn. ischiadici of frogs (*Rana pipiens* and *Rana temporaria*) a complete, but reversible conduction block occurs at a temperature of 34–36 °C. Of course, we have no evidence for a direct connexion between this phenomenon and the phase change, but the coincidence of the threshold temperature and the phase change temperature is striking.

As was mentioned above, a phase change is found not only in the frog nerve but also in the extracted lipids. Therefore it is obvious that the phase change is related to the lipids rather than to the proteins. However, the phase change temperature of vesicles of the extracted lipids is about 6 °C lower than the temperature measured for the phase change in the nerve. This fact corresponds with the results of investigations on different membrane systems, which prove that electrostatic interactions between lipids and proteins can affect the gel to liquid-crystalline transition of the lipids [5, 23, 27].

The results of the studies on Ca^{2+}-depleted frog nerve (Fig. 2) indicate that Ca^{2+} is involved in the phase transition. There is some evidence that Ca^{2+} forms complexes with acidic lipids by changing their molecular arrangement [19, 28, 29]. Ca^{2+} causes a decrease of the fluidity of the hydrocarbon chains of the lipids. This is reflected by a decrease in the outer hyperfine splitting, $T_{||}$, of the spin label incorporated in the Ca^{2+}-depleted nerve. Here, the sharp phase change, detectable in the presence of Ca^{2+} (Fig. 2, Curve b), is ironed out (Curve a). The approximation of Curve a by two straight lines leads to an intersection centered at about 40 °C. If such an intersection is interpreted as a point of phase change, this phase change is shifted to higher temperatures in comparison with the change observed for the untreated nerve.

Nevertheless, we would like to emphasize that we are not sure whether the Ca^{2+}-depleted nerve shows a phase change or not. A conclusion could be reached only from additional experiments using other spin labels or probably another technique.

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