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THE INTERACTION OF A LYTIC PEPTIDE, MELITTIN, WITH SPIN-LABELED MEMBRANES

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SUMMARY

The effect of the amphophilic cationic peptide, melittin, on the biophysical state of erythrocytes, their ghosts, and spherules formed from total erythrocyte lipids was studied using the stearic acid spin labels J(1.14), J(5.10), and J(12.3) incorporated into these membranes. Melittin effects a dose-dependent increase of orientation of the label J(5.10) in the case of ghosts and erythrocyte lipid membranes. The influence on the labels J(1.14) and J(12.3), however, is comparatively small. A distinct dose-dependent difference was found if the labels were incorporated into intact red cells. There is an increase of the molecular motion, particularly of the label J(5.10), in the presence of low melittin concentrations and a decrease at high concentrations. The results suggest that in erythrocyte ghosts and total lipids melittin penetrates into the membrane and its action occurs mainly by retracting the apolar region of the membrane. The effect on intact cells involves an increasing fluidity of the apolar fatty acid chains at low, and a decrease at high melittin concentrations. The influence of the surface active peptide melittin on the physical state of the membranes investigated is not comparable with that of a simple detergent like Triton X-100.

INTRODUCTION

The cationic amphipathic polypeptide melittin, a well-defined component of bee venom, disrupts biomembranes and artificial phospholipid vesicles[1,2]. The physical properties of melittin would explain its ability to increase membrane permeability. The molecular mechanisms of this process, however, are still not clear. For melittin, it is possible to derive some information about the correlations between the molecular structure of this lypolytic peptide and its effects upon the biophysical state of biomembranes. The following general mechanisms of action will be discussed[1–3]. (1) Melittin might act as a detergent to form mixed micelles with membrane lipids; (2) melittin is supposed to disrupt apolar forces between the hydrocarbon chains of phospholipids; or (3) it interacts with polar forces in the region of the head groups of phospholipids. The spin labeling technique has been shown to provide information about changes in the physical state of membrane lipids[4,5].

In the present study we have employed fatty acids with different distances between the carboxyl group and the nitroxide radical. Changes in the anisotropic or isotropic motion of the labels incorporated into intact erythrocyte membranes, their ghosts and artificial lipid spherules have been investigated in the presence of different melittin concentrations.

MATERIALS AND METHODS

Stearic acid spin labels J(1.14), J(5.10), and J(12.3) were purchased from Synvar Associates Paolo Alto, Calif. Fresh erythrocytes were obtained from 10-ml samples of citrated blood from rabbits. The cells were sedimented and washed five times with phosphate-buffered (0.05 M) physiological saline (pH 7.2 at 4 °C). The cell pellet was resuspended in 3 ml of the buffered saline. Rabbit erythrocyte membranes were prepared using the method described by Dodge *et al.*[6]. The membranes obtained from 10 ml blood were suspended in 3 ml of the buffered saline. Membranes of total erythrocyte lipids were prepared using a modified method described by Weissmann and Sessa[7]. Extraction of lipids from aliquots of erythrocyte membranes was carried out according to Folch *et al.*[8].

Spin label in a mole ratio of approx. 1 : 100 to lipid was added to 7.3 mg lipid and the mixture was dissolved in chloroform and poured into a round-bottom flask. A thin film was formed from this mixture by bringing the chloroform to evaporation in a nitrogen atmosphere using a rotary evaporator. 1 ml of NaCl–KCl solution (0.145 M total molarity) adjusted to pH 7.2 was then added and lipid spherules were formed by shaking the mixture under nitrogen on a Köttermann rotation mixer at room temperature.

Spin labeling of erythrocytes and their ghosts was carried out after an exchange from bovine serum albumin[9,10]. Separate samples of 1 ml erythrocytes and erythrocyte ghosts were suspended in 0.5 ml 5.8 % NaCl. After labeling at room temperature for 4–8 h the samples were washed five times in buffered saline and finally resuspended in 1 ml of the buffered saline. After placing the samples in micro-hematocrit tubes (a product of Clay Adams, N.Y.) electron spin resonance (ESR) spectra were obtained at room temperature using a Varian E9 spectrometer. Samples consisted of 40μl of the erythrocytes, erythrocyte membranes (both containing 5.1–6.2 mg lipid/ml) and liposomes (7.3 mg/ml suspension), respectively. Different amounts of melittin were added up to a final volume of 50μl. After incubation for 1 h at room temperature the probes were analyzed by ESR. The spin label concentration used in the experiments with erythrocytes and erythrocyte ghosts was approx. 0.02 μmole per test sample. The hyperfine splitting $2T_{||}$ and $2T_{\perp}$ of the spectra were measured within an approximation of $\pm$0.5 G.

RESULTS

Fig. 1 shows the ESR spectra of labeled ghosts to be similar to those obtained by other workers[10,11] The spectrum (a) is compared with (b) which has been recorded after adding 50 μg melittin. The labels J(12.3) and J(5.10) show anisotropic motion around the long molecular axis μ[4,12]. The different degree of anisotropy could be described quantitatively by the parameter S of the equation: $S = 0.56\ (T_{||} - T_{\perp})/a$

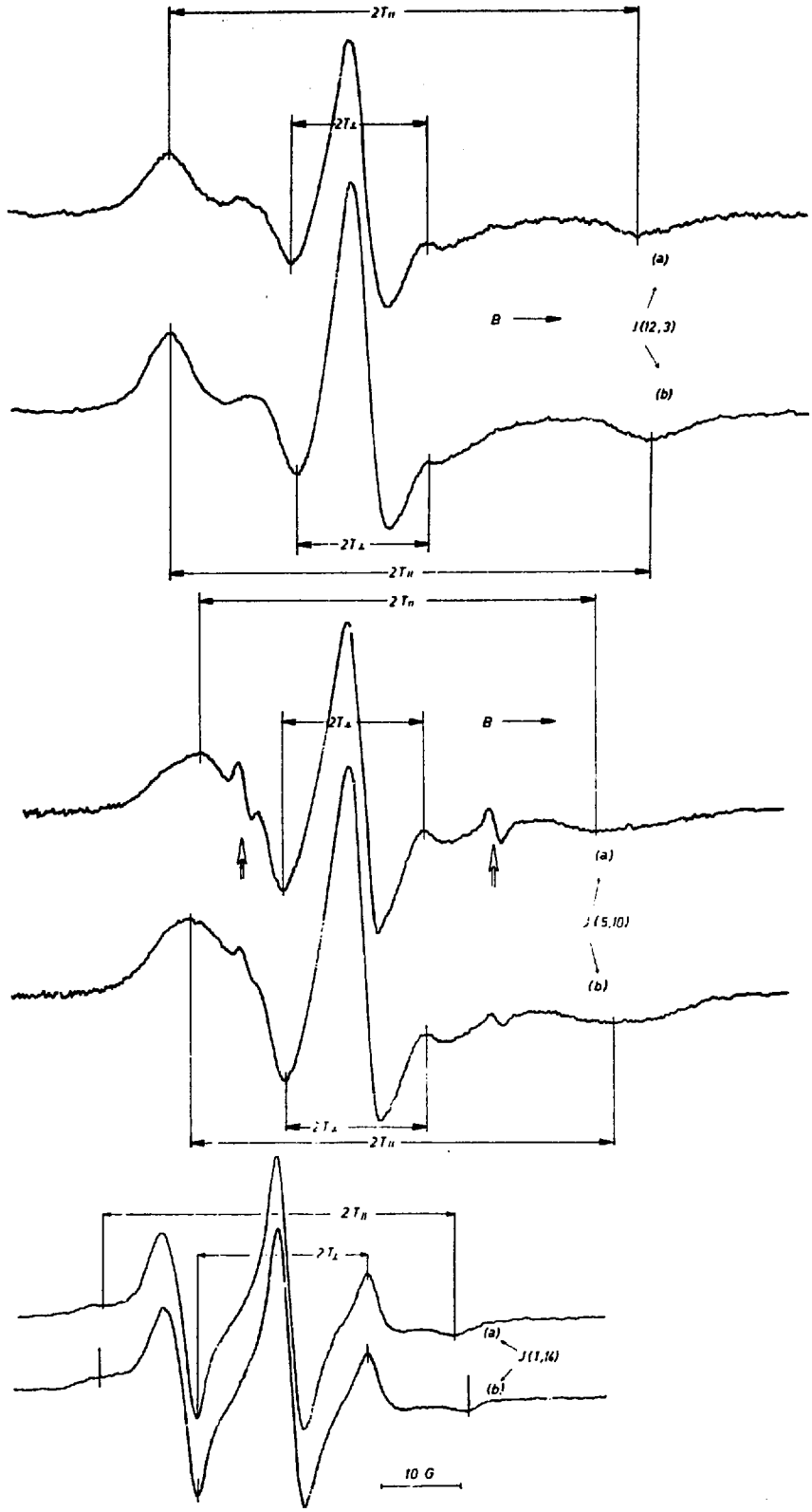

Fig. 1. First derivative of the ESR spectrum. Spectra of spin label incorporated into rabbit erythrocyte ghosts obtained from the labels J(12.3), J(5.10) and J(1.14) as indicated. (a) Ghosts (5.1–6.2 mg lipid per ml) in phosphate-buffered isotonic saline at pH 7.2. (b) The same after treatment with 50 μg melittin. $2T_{\parallel}$ and $2T_{\perp}$ correspond to the splitting of the outer and inner hyperfine extrema, respectively.

introduced by Seelig and Hasselbach[13,14]. $T_\perp$ and $T_\parallel$ represent the inner and outer hyperfine splitting, respectively. The isotropic hyperfine splitting constant a[4], which is dependent upon the polarity of the environment[5] of the spin label, was calculated using the equation: $a = 1/3\ (T_\parallel + 2T_\perp)$. A further parameter used for the analysis of the spectra is the angular deviation α between μ and the nitroxide $2p\pi$ orbital axis as described by Seelig and Hasselbach[14]. $\alpha = \arccos[(2S+1)/3]$. As shown in Table I the hyperfine splitting $2T_\parallel$ increases in the presence of melittin, indicating a decrease in the motional freedom of the spin labels. The arrows (Fig. 1) indicate a spectrum due to spin label which is not incorporated into the membrane. The intensity of this signal varied from preparation to preparation. In Fig. 2 the angular deviation of the labels incorporated in erythrocyte membranes is plotted against melittin concentrations. There is a progressive obstruction of the angular deviation of J(5.10) and J(1.14), indicating a decrease of the membrane fluidity towards the apolar region of the membrane.

TABLE I

EFFECT OF MELITTIN ON MOTION PARAMETERS OF SPIN LABELS INCORPORATED INTO ERYTHROCYTE GHOSTS (6.2 mg LIPID/ml TEST SUSPENSION)

Label	*Melittin* (μg)	$T_\parallel$ (G)	$T_\perp$ (G)	a (G)	S
J(5.10)	0	24.6	8.9	14.1	0.624
	10	24.6	8.9	14.1	0.624
	20	25.3	8.9	14.3	0.640
	30	25.5	8.9	14.4	0.646
	40	25.8	8.9	14.5	0.652
	50	26.5	8.9	14.8	0.669
	60	27.6	8.8	15.0	0.703
J(12.3)	0	29.3	8.4	15.3	0.762
	10	29.4	8.4	15.4	0.765
	20	29.4	8.5	15.5	0.756
	30	29.5	8.5	15.5	0.759
	40	29.3	8.5	15.4	0.754
	50	29.9	8.4	15.5	0.775
	60	29.6	8.4	15.5	0.769
	70	30.1	8.4	15.6	0.780

In the case of J(12.3) where the nitroxide group is located near the polar carboxyl group, only a small increase in orientation is observed at higher melittin concentrations. A more detailed analysis of the spectra is summarized in Table I. The parameter S is close to 1 and reflects an increasing degree of anisotropic motion dependent on the melittin concentration (J(5.10)). The splitting between the two outer extrema $2T_\parallel$ of the spectra increases as the motion of the label is hindered. The isopropic hyperfine splitting constant a indicates a melittin-dependent increase of the polarity of the spin label environment[13,15]. In our experiments, however, the label is partly immobilized and in this case the solvent-dependent interpretation of a is uncertain[5]. The results of the experiments with the spin labels incorporated in total lipids of erythrocyte membranes are shown in Table II. In these membranes melittin causes a slight decrease of α of the labels in the presence of concentrations higher than 10 μg.

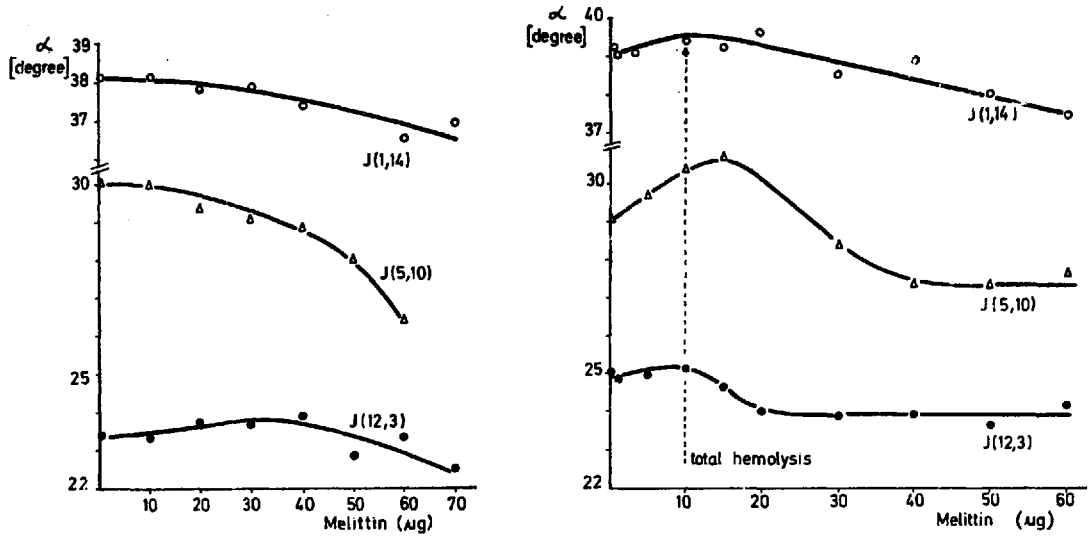

Fig. 2. The change of the calculated angular deviation of the labels (J (5.10), J (12.3) and J (1.14) in erythrocyte ghosts on increasing the melittin concentration (5.1–6.2 mg lipid/ml test suspension). Mean of two (J (5.10); J (1.14)) and four (J (12.3)) different preparations.

Fig. 3. The angular deviation α plotted against melittin concentration; intact erythrocyte suspension in phosphate-buffered isotonic saline, pH 7.2. Care has been taken that the lipid concentration of erythrocytes is comparable with their ghosts (5.1–6.2 mg lipid/ml test suspension). Mean of four (J (1.14)) and two (J (12.3); J (5.10)) different preparations.

TABLE II

EFFECT OF MELITTIN ON MOTION PARAMETERS OF SPIN LABELS INCORPORATED INTO VESICLES OF ERYTHROCYTE LIPIDS (7.3 mg LIPID/ml VESICLE SUSPENSION)

Label	*Melittin* (µg)	$T_{\parallel}$ (G)	$T_{\perp}$ (G)	*a* (G)	*S*	α
J (12.3)	0	28.25	8.25	14.92	0.751	24° 4 min
	20	28.50	8.19	14.96	0.760	23° 55 min
	50	28.75	8.13	15.00	0.770	23° 3 min
J (5.10)	0	24.50	8.88	14.08	0.621	30° 10 min
	20	24.50	8.69	13.96	0.634	29° 35 min
	50	24.81	8.63	14.02	0.647	29° 1 min
J (1.14)	0	17.72	10.63	12.99	0.306	42° 53 min
	20	17.90	10.53	12.98	0.318	42° 23 min
	50	18.06	10.50	13.02	0.325	42° 7 min

In Fig. 3 the spectra of the intact erythrocytes in the absence of melittin show a slight difference as compared with those spectra obtained from ghosts. This phenomenon has been interpreted recently by Landsberger *et al.*[16]. There is a marked difference in the angular deviation α for J(5.10) as plotted against the melittin concentration, between intact erythrocytes on one hand and their ghosts or erythrocyte lipid vesicles on the other. Up to a concentration of about 10 µg melittin (a concentration at which total hemolysis of erythrocytes occurred under our experimental conditions) it is especially the spin label J(5.10) which reflects an increase of the membrane

fluidity and at higher concentrations a decrease. The spin labels J(12.3) and J(1.14), however, show merely a slight increase in the angular deviation at melittin concentrations of up to 10 μg. Higher concentrations lead to a decrease of the membrane fluidity similar to that observed in liposomes and ghosts. Comparable experiments were

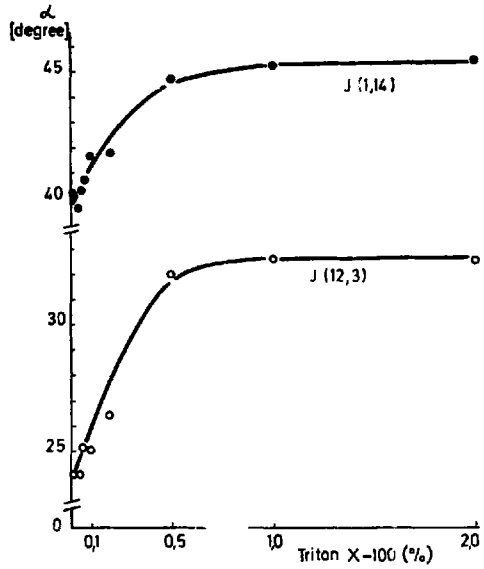

Fig. 4. The effect of Triton X-100 concentration on the angular deviation α; intact erythrocytes (5.1–6.2 mg lipid/ml suspension) in phosphate-buffered saline, pH 7.2.

TABLE III

EFFECT OF TRITON X-100 ON MOTION PARAMETERS OF LABEL J (12.3) AND J (1.14) INCORPORATED INTO INTACT ERYTHROCYTES (6.2 mg LIPID/mg TEST SUSPENSION)

Label	*Triton X-100 (% total concentration)*	$T_{\parallel}$ (G)	$T_{\perp}$ (G)	*a* (G)	*S*
J (12.3)	0	28.4	8.4	15.0	0.745
	0.01	29.0	8.3	15.2	0.766
	0.025	28.9	8.4	15.2	0.755
	0.05	28.0	8.5	15.0	0.728
	0.075	28.3	8.5	15.1	0.733
	0.1	28.1	8.5	15.0	0.731
	0.2	27.6	8.3	15.0	0.703
	0.5	24 8	9.6	14.7	0.577
	1.0	24.1	9.6	14.5	0.562
	2.0	24.6	9.8	14.7	0.566
J (1.14)	0	20.4	10.9	14.1	0.375
	0.01	20.4	10.9	14.1	0.375
	0.025	20.8	10.9	14.2	0.390
	0.05	20.7	11.1	14.2	0.376
	0.075	20.0	11.0	14.0	0.360
	0.1	19.5	11.1	13.9	0.337
	0.2	19.4	11.1	13.9	0.333
	0.5	19.3	12.6	14.8	0.253
	1.0	19.4	12.8	15.0	0.248
	2.0	19.1	12.8	14.9	0.237

carried out with phosphatidylethanolamine dispersions containing the spin labels. The results of the analysis of these spectra were qualitatively the same as listed for vesicles from total lipid extracts of red blood cells. During our experiments melittin itself might possibly interact with the spin label molecules. Experiments were thus carried out in which the spin label was dissolved in 20 % (v/v) ethanol. Melittin up to a concentration of 50 μg per test sample caused no detectable change of the spin label spectra.

It was also considered that the action of melittin on intact erythrocytes was detergent-like and at higher concentrations an aggregation would effect the broadening of the spectra. Thus labeled, intact erythrocytes were treated with increasing concentrations of Triton X-100. The angular deviation of J(12.3) as shown in Fig 4, as well as that of J(1.14), reflects an increased membrane fluidity. More detailed data of the analysis of these spectra (J(12.3), J(1.14)) are shown in Table III. Centrifugation of the samples at high speed had no effect on the spectra.

DISCUSSION

A comparison of the three membrane systems shows that higher concentrations of melittin (>10.0 μg; melittin–lipid in a molar ratio of 1:130) preferentially tend to reduce the membrane fluidity in the hydrophobic environment of the labels J(1.14) and J(5.10). On the other hand, if the nitroxide group is located near the polar head group, J(12.3), the analysis of the spectra shows only a small restriction of the molecular motion. These results lead to the assumption that the predominant effect is a melittin–lipid interaction in the hydrophobic core of the membrane. It is well known that melittin penetrates lipid films and our results support the hypothesis of Sessa *et al.*[2] which suggests that bonds between the hydrophobic tail of the melittin molecule and the acyl chains of membrane lipids hydrogen could be formed. These intermolecular bonds may be much stronger than the bonds between the hydrophobic chains of the lipids. In this way a melittin molecule inserted in the lipid could form local areas with a higher degree of immobilisation and orientation of the fatty acid chains. This could explain the net-like stuctures in different melittin-treated biomembranes observed by electron microscopy[1–3] and it furthermore explains a restriction of molecular motion of fatty acid chains. In the presence of low concentrations of melittin no spectral changes are obtained in the labeled erythrocyte ghosts and vesicles formed from lipid extracts of the red blood cells. This suggests that either no melittin penetrates between the fatty acid chains, or the few melittin molecules inserted into the lipid layer effect only local changes which are not detectable by the spin-label technique used in our experiments. In recent X-ray diffraction studies it could be shown that only higher concentrations of amphipathic molecules (ANS) penetrate in the apolar hydrocarbon core of model membranes and change the molecular arrangement in that part of the membrane[17]. In the case of melittin, it is probable that the higher the concentration the deeper is the penetration into the hydrophobic interior of the membrane.

A comparison of the motion parameters of the spin labels in intact erythrocytes and their isolated ghosts show considerable differences in the effect of melittin. Low hemolytic concentrations up to 10 μg decrease the orientation, particularly reflected by the label J(5.10). The more polar part of the membrane seems to remain uneffected

(label J(12.3)). A simple detergent, however, (Fig. 4 and Table III) increases the mobility of the polar as well as the apolar groups of membrane lipids. In the presence of higher concentrations of melittin (>10 μg, Fig. 3) the detergent-like effect is accompanied by an increasing stiffness of the fatty acid chains similar to that observed in ghosts. One possible explanation for this fact is that in the presence of small amounts of milittin an electrostatic attraction occurs in the polar region of the intact erythrocyte membrane which involves an increasing fluidity of the apolar chains. The different action on erythrocytes and their ghosts apparently supports results which show that the membrane of intact cells differ markedly from those of isolated ghosts[18].

These studies indicate that the spin-label method is appropriate for the investigation of the biophysical action of the lytic toxins on biomembranes. Further comparative experiments on lytic toxic peptides are still in progress.

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REFERENCES

1 E. Habermann, *Rev. Physiol.*, 60 (1968) 221.
2 G. Sessa, J. H. Freer, G. Colacicco and G. Weissmann, *J. Biol. Chem.*, 244 (1969) 3575.
3 D. Hegner, *Arch. Pharmak. Exp. Path.*, 261 (1968) 118.
4 H. M. McConnell and B. G. McFarland, *Quart. Rev. Biophys.*, 3 (1970) 91.
5 O. H. Griffith, L. J. Libertini and G. B. Birrell, *J. Phys. Chem.*, 75 (1971) 3417.
6 J. T. Dodge, C. Mitchell and D. J. Hanahan, *Arch. Biochem. Biophys.*, 100 (1963) 119.
7 G. Weissmann and G. Sessa, *J. Biol. Chem.*, 242 (1967) 616.
8 J. Folch, M. Lees and G. H. Sloane-Stanley, *J. Biol. Chem.*, 226 (1957) 497.
9 W. J. Hubbel and H. M. McConnell, *Proc. Natl. Acad. Sci. U.S.*, 63 (1969) 16.
10 F. R. Landsberger, J. Lenard, J. Paxton and R. W. Compans, *Proc. Natl. Acad. Sci. U.S.*, 68 (1971) 2579.
11 W. L. Hubbel and H. M. McConnell, *Proc. Natl. Acad. Sci. U.S.*, 64 (1969) 20.
12 W. L. Hubbel and H. M. McConnell, *J. Am. Chem. Soc.*, 93 (1971) 314.
13 J. Seelig, *J. Am. Chem. Soc.*, 72 (1970) 3881.
14 J. Seelig and W. Hasselbach, *Eur. J. Biochem.*, 21 (1971) 17.
15 R. Briere, H. Lemaire and A. Rassat, *Bull. Soc. Chim. France*, 32 (1965) 3273.
16 F. R. Landsberger, J. Paxton and J. Lenard, *Biochim. Biophys. Acta*, 266 (1971) 1.
17 W. Lesslauer, J. Cain and J. K. Blasie, *Biochim. Biophys. Acta*, 241 (1971) 547.
18 B. Roelofson, R. F. A. Zwaal, P. Comfurius, C. B. Woodward and L. L. M. van Deenen, *Biochim. Biophys. Acta*, 241 (1971) 925.