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# INFRARED SPECTROSCOPY OF *MICROCOCCUS LYSODEIKTICUS* MEMBRANES AND MEMBRANE FRACTIONS

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SUMMARY

As in other cell membrane systems, the principal characteristics of the infrared spectra of membranes from the bacterium, *Micrococcus lysodeikticus*, originate from the Amide I and II bands of the proteins and from the vibrations of the O–H, C–H, C=O, P=O, C–O–C and P–O–C groups of the lipids. In addition the asymmetric and symmetric methylene stretching vibrations at 2930 and 2855 $cm^{-1}$, respectively, have provided a parameter interpretable in terms of protein–lipid interactions. Lipid-depleted membrane protein fractions had substantially higher $CH_2$ asymmetric:symmetric ratios than those observed for whole membranes. The effects of lipid extraction, sonication, and dissociation with deoxycholate have been determined.

## INTRODUCTION

An understanding of the interactions between lipids and proteins within cell membranes is of primary importance for determining membrane structure. Several studies using infrared and NMR spectroscopy of membranes and membrane fractions have been reported recently[1–5] and these have clearly shown the value of this type of investigation for the determination of the conformations of lipids and proteins in membranes and for indicating the structural implications. One of the most important conclusions emerging from these studies has been the realization of the basic role of weak interactions, probably of a hydrophobic nature, in determining the structure of membranes, as opposed to a 'classical' biomolecular leaflet cemented together by ionic bonds[2–4].

In this paper an infrared spectral study of a bacterial membrane is presented. The data have been analyzed in terms of protein and lipid conformation, and the symmetry of the methylene stretching vibration is interpreted as a parameter of protein–lipid interaction as opposed to a lipid–lipid interaction. The effects of lipid extraction, sonication, and separation of the membrane into well-defined fractions by treatment with deoxycholate, and recombination of separated membrane lipids and proteins are presented and discussed.

## EXPERIMENTAL

### *Infrared spectroscopy*

A Perkin–Elmer spectrophotometer Model 421, was employed throughout. KBr pellets were prepared by compressing thoroughly mixed, dry KBr and lyophilized membrane preparations and fractions. The pellets were approx. 0.3 mm in thickness. Spectra were also taken of solid films prepared by air drying at room temperature, aqueous suspensions spread on AgCl plates[2,4] essentially as described by WALLACH AND ZAHLER[2]. Both pellets and films were dried, *in vacuo* over $P_2O_5$ before spectra were taken. The pellets gave more reproducible spectra than the films, but there were no qualitative differences in the results obtained by the two methods.

Temperature effects could not be studied very accurately because of the lack of a thermostated device on the spectrophotometer. However, low-temperature effects were examined qualitatively by immersing the pellets or films in liquid $N_2$ for about 5 min prior to recording their spectra. A series of spectra was taken over a narrow frequency range (3100–2800 or 800–700 $cm^{-1}$) until the spectra returned to that given by a control preparation at room temperature. To prevent moisture from condensing on the membrane sample, the AgCl plate method was used, with a second AgCl plate held tightly against the first, so that the film was between the two plates. Moisture was removed from the outer surfaces of the plates by a strong stream of $N_2$ gas.

The ratios of the asymmetric to symmetric methylene stretching vibrations were estimated by measuring the peak heights relative to a baseline determined by the straight line connecting the absorption spectrum at 3000 and 2700 $cm^{-1}$. This method was found to give the most reproducible results regardless of the slope of the baseline[6]. The line connecting the absorptions at 1800 and 1000 $cm^{-1}$ determined the baseline used for calculating the other ratios. The slope of this line was usually close to zero.

### *Preparation of membranes and membrane fractions*

Membranes from *Micrococcus lysodeikticus* were isolated as previously described[7] and submitted to the 'standard' wash procedure outlined by SALTON[8].

Extraction of lipids from the membranes was performed with three different solvent systems, *n*-butanol, acetone–methanol (7:2, v/v) and chloroform–methanol (3:2, v/v) and the extracted membranes were usually washed 2 or 3 times with a given solvent. The lipids extracted by the acetone–methanol procedure were also examined spectroscopically after evaporating the solvent under a stream of $N_2$ and preparing the KBr pellets or AgCl plates as described above.

Sonication of membranes was performed as previously reported[9], the preparations receiving ten 30-sec periods of exposure to ultrasound, alternating with cooling periods of 1 min.

All preparations were adjusted to an equal concentration (w/v) and dried for an equal length of time prior to recording of spectra.

Dissociation of the membranes by treatment with 1% sodium deoxycholate into deoxycholate-soluble and deoxycholate-insoluble fractions was carried out as described recently[10]. The deoxycholate-soluble fraction from which the deoxycholate was removed by extensive dialysis at 4°, was examined spectroscopically before

and after removal of the lipids by three successive extractions with *n*-butanol in the cold (0–4°). The solvent in the aqueous phase was removed by dialysis against distilled water at 4°. The deoxycholate-insoluble residues were washed on the centrifuge and dialysed against distilled water at 4°. These fractions were then lyophilized.

Recombination or reassociation of the membrane lipids and proteins was accomplished by adding together again the lipids extracted in chloroform–methanol (3:2, v/v) or in *n*-butanol and the extracted protein residues dispersed as a slurry in the same solvent. Solvents were removed by evaporation under $N_2$ and pellets were prepared from the dried fractions. That reassociation of lipids and proteins had occurred under these conditions was indicated by the behavior of the fractions on sucrose density gradient centrifugations as used in previous studies[8,10] (unpublished observations). Although the banding on gradients indicated that the fractions were not simply mixtures of free lipid and lipid-depleted protein, we have no evidence that 'membrane profiles' are reformed.

## RESULTS

The assignment of spectral peaks and the positions of the main bands follows that reported by WALLACH AND ZAHLER[2] and CHAPMAN *et al.*[4], with the following additions: the asymmetric and symmetric methylene stretching vibrations at 2930 and 2855 $cm^{-1}$, respectively; the C–O–C stretching vibration at 1170 $cm^{-1}$; the symmetric methyl deformation vibration at 1380 $cm^{-1}$ (refs. 11 and 12). Typical spectra and the identities of the principal bands for whole membranes of *M. lysodeikticus* are illustrated in Figs. 1A and 1B.

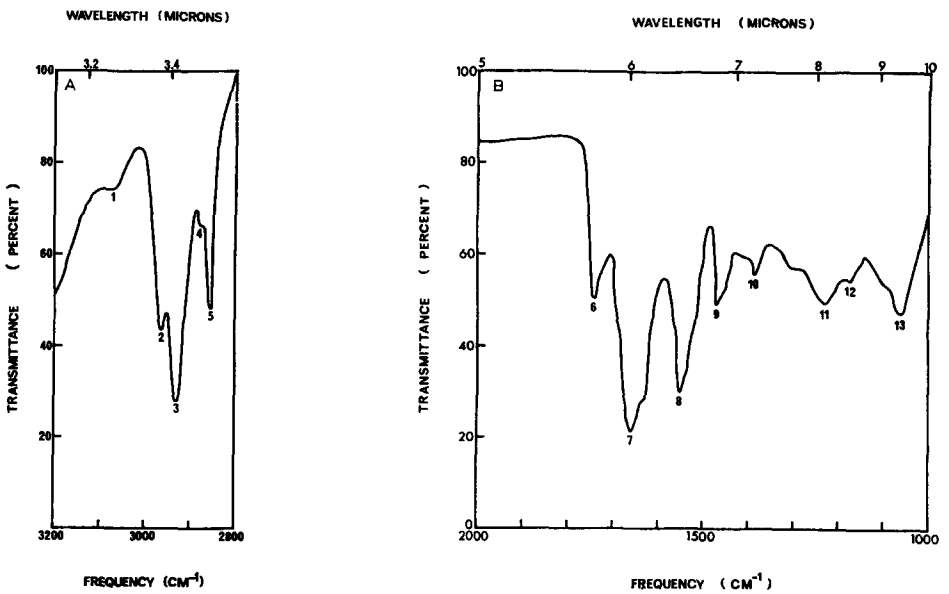


Fig. 1. A and B. Infrared absorption spectra of whole membranes of *M. lysodeikticus*. Peaks identified as following: (A). 1, C–H stretching; 2, $CH_3$-asymmetric; 3, $CH_2$-asymmetric stretch; 4, $CH_3$-symmetric; 5, $CH_2$-symmetric stretch. (B). 6, C=O stretch; 7, Amide I; 8, Amide II; 9, $CH_2$-scissoring and $CH_3$-bending; 10, $CO_2^-$-stretch; 11, P=O stretch, C–O–C stretch (asymmetric); 12, C–O–C stretch (symmetric); 13, P–O–C stretch.

It must be emphasized that no attempt was made to determine the absolute absorbance or molar extinction coefficient of any of the bands in our spectra. The difficulties involved in the quantitative analysis of solid state spectra[13,14], would in this case be overwhelming. A qualitative analysis was therefore undertaken by determining the ratios of particular band heights (percent transmission), within a given spectrum, and then comparing these ratios with correponding ones from spectra of various membrane fractions. The results, although descriptive in nature, illustrate the basic lipid–protein interactions within the membrane.

The ratios which were compared were: (1) the asymmetric to symmetric methylene stretching vibrations; (2) the Amide I to C=O stretching vibration at 1735 $cm^{-1}$. The ratio of the asymmetric to symmetric methylene absorptions is interpreted by us to indicate the relative interactions of lipids with other lipids and proteins and the differences in the intensities of the bands can be seen for whole membranes, membrane lipids and membranes extracted with acetone–methanol, as shown in Fig. 2. The significance of this aspect of the study will be discussed more fully below. The ratio of Amide I and C=O stretching vibration gives an indication of the extent of lipid extraction since the ester absorptions are due primarily to the membrane lipids. The shape of the Amide I and Amide II bands is related to the conformation of the protein.

For comparative purposes, the various ratios and properties of *M. lysodeikticus* membranes and membrane fractions as determined by infrared spectroscopy are summarized in Table I together with data for bovine serum albumin and lipid spherules. The ratios of asymmetric to symmetric methylene stretching vibrations are expressed

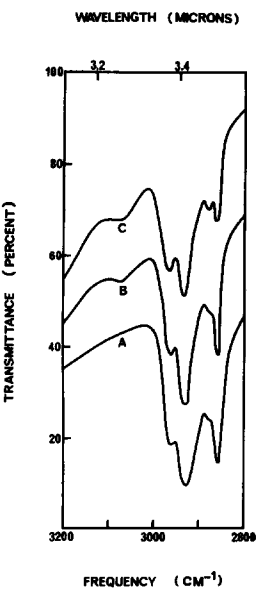


Fig. 2. Infrared absorption spectrum of total lipid fraction from *M. lysodeikticus* membranes (A), whole membranes (B) and membranes (C) of *M. lysodeikticus* extracted with acetone–methanol (7:2, v/v), showing differences in relative absorption intensities due to $CH_2$-asymmetric (2930 $cm^{-1}$) and $CH_2$-symmetric (2855 $cm^{-1}$) stretching vibrations. Spectra have been arbitrarily displaced by a unit of 10% transmittance at 3200 $cm^{-1}$.

TABLE I

SOME PROPERTIES OF *M. lysodeikticus* MEMBRANES AND MEMBRANE FRACTIONS DETERMINED BY INFRARED SPECTROSCOPY

| *Fraction* | *Protein conformation* | *Asymmetric/ symmetric methylene** (%) | *Lipid extraction*** |
|---|---|---|---|
| Whole membrane | | | |
| room temperature | $\alpha >> \beta$ | 30 | 1.7 |
| liquid $N_2$ preparation | $\alpha >> \beta$ | 0 | 1.7 |
| Recombined membrane | | | |
| chloroform–methanol | $\alpha \simeq \beta$ | 40 | 1.6 |
| *n*-butanol | $\alpha \simeq \beta$ | 50 | 1.8 |
| Lipid-extracted membrane | | | |
| acetone–methanol (7:2) | $\alpha >> \beta$ | 70 | 3.4 |
| chloroform–methanol (3:2) | $\alpha >> \beta$ | 140 | 7.3 |
| Deoxycholate-soluble fraction | $\alpha >> \beta$ | 20 | 1.6 |
| Lipid-extracted deoxycholate-soluble fraction (*n*-butanol) | $\alpha >> \beta$ | 250 | 9.1 |
| Deoxycholate-insoluble fraction | $\alpha >> \beta$ | 80 | 3.7 |
| Total membrane lipid | — | 10 | 0.5 |
| Spherules (liposomes) | — | 0 | 0.3 |
| Bovine serum albumin | $\alpha >> \beta$ | 280 | 10.0 |

* The values given are the percent increase of the ratios over that of the whole membrane at low temperature (cooled with liquid $N_2$) based on a minimum of five determinations.

** The values given are the ratios, Amide I/C=O (1735 $cm^{-1}$), which are a function of the protein to lipid ratio.

as percentage increases over the value of that for the whole membrane preparation at low temperature. As shown in Table I the former value is equal to that observed for spherule (liposome) preparations made from pure lecithin and cholesterol as described by BANGHAM *et al.*[15]. Both preparations showed a relatively strong absorption at 720 $cm^{-1}$, which indicates the presence of at least four consecutive methylene groups in an all-*trans* conformation[16].

Extraction of the lipids from the membranes resulted in marked increases in the ratio of Amide I to C=O. However, some phosphate ester absorption remained regardless of the solvent used, probably indicating the presence of residual, 'tightly bound' lipid. The frequency of absorption of the ester carbonyl of this tightly bound lipid was shifted down about 20 $cm^{-1}$ to 1715 $cm^{-1}$. This may be indicative of a relative increase in hydrogen bonding[17] of the carbonyls on the residual lipids. Acetone–methanol (7:2, v/v) extraction of lyophilized membranes was the least efficient, the residues containing approx. 5 % or more lipid. The lipid-depleted deoxycholate-insoluble residues generally contained 3–4 % residual lipid[10], while the deoxycholate-soluble fraction extracted with *n*-butanol contained less than 1 % lipid. Total lipid contents of *M. lysodeikticus* membranes range from about 25–30 % (ref. 7). In all instances, lipid extraction with organic solvents or depletion by deoxycholate extraction resulted in approx. 2–5-fold changes in the percentage increase of the ratios of asymmetric to symmetric methylene absorptions.

Upon removal of lipid from the membranes the Amide I band was slightly broadened at 1630 $cm^{-1}$ indicating a small change from $\alpha$ and/or unordered conformation to the $\beta$-conformation. A marked shift of the Amide I band to 1630 $cm^{-1}$ was

observed after extracting the deoxycholate-soluble fraction with *n*-butanol, thus indicating an increase of $\beta$-conformation of the proteins in this fraction. Spectra of the recombined membrane lipid and protein showed shoulders at 1630 $cm^{-1}$ indicating significant shifts to the $\beta$-conformation (*cf.* Figs. 3A and 3B). Transitions of membrane proteins to a $\beta$-conformation following either thermal denaturation or exposure to certain organic solvents have been noted in earlier studies[1–4]. Sonication produced no significant change in the spectra.

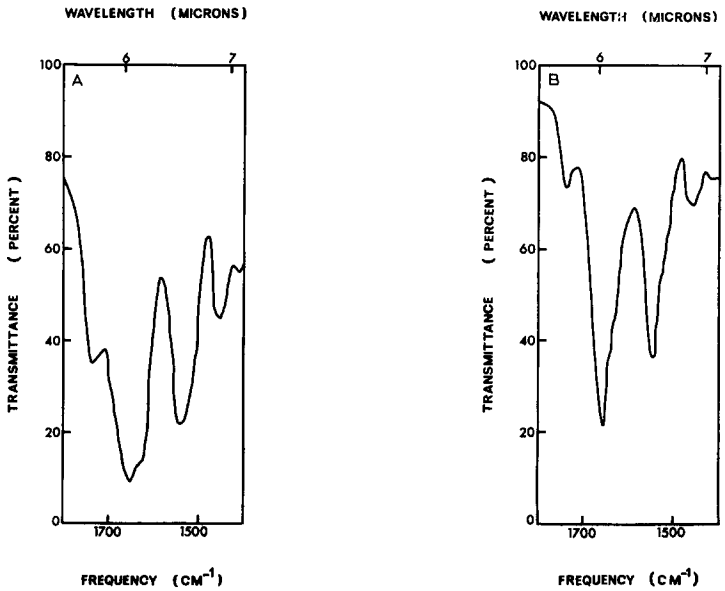


Fig. 3 Infrared absorption spectra of *M. lysodeikticus* membrane fractions. A. Recombined *M. lysodeikticus* membrane lipid and protein. B. Deoxycholate-insoluble residue from *M. lysodeikticus* membranes. Note the broadening of the Amide I band in the recombined preparation.

## DISCUSSION

Infrared spectra of the membrane isolated from the Gram-positive bacterium, *M. lysodeikticus*, are remarkably similar to those reported earlier for mammalian membrane systems including those derived from the plasma membranes and endoplasmic reticulum of Ehrlich acites carcinoma[2,3], and erythrocyte membranes[4,5]. This similarity would be anticipated as these bacterial membranes are composed largely of protein and lipid[7] so that the dominant absorption bands (see Figs. 1A and 1B) can be assigned to these two major classes of chemical constituents of the membranes. As found in the previous studies with animal cell membranes, the Amide I band of the bacterial membranes in the region of 1650 $cm^{-1}$ can be interpreted as being due to an $\alpha$-helical or random coil conformation or a combination of both conformations[1–4].

In accord with earlier studies on other cell membranes, extraction of the *M. lysodeikticus* membranes with organic solvents led to a reduction in the intensity of absorption bands due to the lipids. Complete abolition of the vibrations due to lipid constituents was not observed and the incompleteness of extraction suggested

that a certain fraction of the lipids may be more firmly bound to the membrane proteins. The existence of tightly bound lipid–protein complexes has been noted by MARINETTI AND PETTIT[18] for the interaction of $\gamma$-globulin with cardiolipin and other phospholipids. It is not known at present whether or not the residual membrane lipid is bound by ionic or configurational short range forces.

Lipid extraction of whole membranes produced only slight changes in protein conformation, but with the deoxycholate-soluble fraction, a marked shift to a $\beta$-conformation was observed. The conformational change is probably not due to the direct action of deoxycholate on the protein, since deoxycholate-insoluble material, which has a very low lipid content, retains the predominantly $\alpha$ or random-coil conformation. It thus appears likely that the conformation of the proteins in the deoxycholate-soluble fraction is more dependent upon interactions with the lipids than is the case for the deoxycholate-insoluble proteins which are still in the form of a membranous sheet[10].

Although the conformation of the membrane protein remains predominantly $\alpha$ after lipid extraction with both the chloroform–methanol and acetone–methanol solvents, it is still possible that more subtle conformational changes are induced in the protein by the solvent which determine the nature of its interaction with lipid. The study of the effects of different solvents on membrane proteins is now in progress. The fact that the removal of lipid by chloroform–methanol leaves the protein in an $\alpha$ conformation, while reassociation of lipid with protein in the same solvent results in an irreversible conformational change in some of the membrane proteins, suggests that this protein at least, exists in a metastable state, the conformation of which is extremely sensitive to the nature of the lipid–protein interaction.

Conformational changes in the protein of protein–lipid complexes, induced by changes in the binding of lipid have been studied by DEARBORN AND WETLAUFER[19], by optical rotatory dispersion (ORD) measurements. The effects of membrane lipids on membrane protein conformation require further study.

Before discussing the variation of the intensity of the symmetric and asymmetric methylene stretching vibrations, it must be recalled that the absolute intensity of absorption is directly proportional to the change in the net electric dipole moment of the molecule (the methylene group is considered to be a triatomic molecule). The dipole moment at any instant is determined by the bond length and bond angle. The bond angle is dependent on the degree of $sp$ character of the C–H bonds. Since dehybridization is possible during a vibration[20], the frequency and intensity of absorption can change when a methylene group is close to an O atom, a C = O group, or is part of a strained ring system. The basic problems of symmetry and intensity have of course been fully discussed and presented in the literature[21–23].

The spectra of a system as complex as a biological membrane can only give an average of the contributions of many different chemical groups present in the membrane. An exact analysis is thus impossible. However, one can make certain generalizations by comparing the spectra of the membranes with better defined and more homogeneous systems. Thus, the 720 $cm^{-1}$ band due to the C–H rocking vibration is present in the spectra of purified synthetic lipids (and spherules made from them), and extracted membrane lipids, but not in intact membranes at room temperature. CHAPMAN[16] has pointed out that the presence of this band indicates that at least four consecutive hydrocarbons are in an all-*trans* conformation.

Studies on long-chain hydrocarbons[24] have shown that the *trans* conformation is more extended, and lower in energy (by about 0.8 kcal/mole), than the 'gauche' conformation. Furthermore, the same workers have shown that the assumption of the *trans* conformation by long-chain hydrocarbons is a cooperative phenomenon, *i.e.* it is more likely to occur, the more the hydrocarbon chains come into close association.

Examination of space-filling models shows that when a hydrocarbon is in a 'gauche' conformation, methylene protons will come into close association with other methylene protons on the chain. Such association will alter the dipolar properties of a methylene stretching vibration, causing a net increase in the number of methylene groups showing asymmetric C–H stretching vibrations in the infrared region. This explains the decrease in the asymmetric/symmetric $CH_2$ stretching vibration for those spectra in which the 720 $cm^{-1}$ was present (*e.g.* extracted lipids, spherules, whole membranes at low temperature). Furthermore the ratio can give us some knowledge about the extent of *trans* conformation in the membrane lipids even when the 720 $cm^{-1}$ band is absent. Thus the increase in the ratio for the reassociated lipid and protein components indicates that the lipid hydrocarbons in the reassociated complex have less *trans* character than in the native membrane, and are therefore probably less ordered in the former state than in the latter.

It should be pointed out that the basis for the differences in the asymmetric/symmetric ratios cannot be due to changes in the extinction coefficient of the methylene stretching vibrations caused by variation in the average chain length of the hydrocarbons. Indeed, from the known effects of chain length on extinction coefficients[12], results opposite to those observed would have been predicted.

The fact that this ratio is higher for the whole native membrane than for extracted lipids could be due to the presence of apolar amino acid side chains on the membrane protein, which by means of hydrophobic interactions with the lipid hydrocarbons, prevents the latter from attaining the close association necessary for the *trans* conformation. The importance of hydrophobic interactions between membrane proteins and lipids has been demonstrated by NMR experiments performed by CHAPMAN *et al.*[5]. The role that bound water within the membrane plays in determining lipid conformations is presently being studied.

It is interesting that lipids in the native membrane do not show more *trans* character, since this is the conformation of lowest energy. It is tempting to speculate that the lipids may, under certain physiological conditions, assume more of a *trans* character. The configurational energy gained by doing so could be coupled to a simultaneous energonic process, *i.e.* transport. Further infrared and NMR work is being undertaken to gain a better understanding of this problem.

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