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# Dilatometry of Biological Membranes†

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ABSTRACT: Use of a precision dilatometer with a small sample size has allowed characterization of the thermal volume changes of erythrocyte ghosts, *Mycoplasma laidlawii* membranes, and vesicles made from dipalmitoyl-L-α-lecithin associated with cytochrome *c* in dilute aqueous suspensions. Erythrocyte ghosts were found to have a mass coefficient of expansion, $\epsilon_T$, which increases with temperature, going from 0.005 ml per gram (ghost) per degree at 16° to 0.014 ml per gram (ghost) per degree at 40°. These large values of $\epsilon_T$ are comparable to those of lecithin vesicles. Reproducible details also appear in the plot of $\epsilon_T$ *vs.* temperature for erythrocyte ghosts. *M. laidlawii* membranes, submitted to treatment which either denatures (heat) or removes (Pronase) the bulk of the membrane protein, have an average $\epsilon_T$ of about 0.004–0.005 ml per gram (membrane) per degree, with little temperature dependence. The $\epsilon_T$ of native *M. laidlawii* membranes exhibits a large increase (0.003 ml per gram (membrane) per degree) in the 32–50° temperature range. This hump is destroyed by treatment which destroys or denatures protein. The second feature of the $\epsilon_T$ *vs.* temperature plots of *M. laidlawii* membranes is a sharp dip in $\epsilon_T$ at 22°. This very striking phenomenon occurs over a 5° range. In fresh membrane preparations this dip extends to negative values. It is destroyed in a systematic manner by processes which denature membrane protein. Thin-walled vesicle preparations of pure synthetic phospholipids or solutions of cytochrome *c* display no dips in their $\epsilon_T$ *vs.* temperature plots. If, however, cytochrome *c* is bound to these vesicles, a sharp dip in the $\epsilon_T$ of this aggregate occurs at 22°. Calorimetric findings together with the dilatometric results indicate a highly cooperative effect. The likelihood of this being a water-related effect is diminished by membrane suspensions in $D_2O$ having 22° dips indistinguishable from those of membrane preparations in $H_2O$. The 22° dip appears to be a phenomenon involving protein, present in lipid bilayer systems acting as a class.

The development of a precision dilatometer requiring small samples (Rothman *et al.*, 1972) provides us with the opportunity of using coefficients of thermal volume change to characterize biological membranes. We have previously applied this technique to study phase transitions in phospholipid vesicles (Melchior and Morowitz, 1972) and in the present study we extend the technique to the plasma membranes of *Mycoplasma laidlawii* and human erythrocytes as well as lecithin vesicles associated with cytochrome *c*.

Our findings indicate dilatometry to be a potentially useful tool in the investigation of biological substances. Each of the systems studied was found to have a characteristic volume *vs.* temperature curve. Several distinctive features were found which should aid in increasing knowledge about biological membranes. The most notable of these bears on protein associated with lipid bilayers.

## Materials and Methods

*Preparation of Mycoplasma laidlawii Membranes. M. laidlawii B* were grown on a medium consisting of 18 g of Tryptose (Fisher, Fair Lawn, N. J.), 8 g of Yeast Extract (Fisher), 4 g of Tris, 5 g of NaCl, and 900 ml of water. After autoclaving, 100 ml of 10% sterile glucose solution and 10 ml of sterile Bacto-PPLO serum fraction (Difco, Detroit, Mich.) were added. For cell growth 1 l. of medium was inoculated with 10 ml of a 24-hr culture and incubated at 37°. The cells were harvested at late log phase and lysed in deionized water, and membranes were prepared by the method of Engelman *et al.* (1967).

*Preparation of Red Blood Cell Ghosts.* Human red blood cell membranes, "ghosts," were prepared by the method of Hoffman and Ryan (Parker and Hoffman, 1964). Fresh red blood cells were hemolyzed in 10 vol of cold $1 \times 10^{-4}$ M EDTA at pH 7.4. The resulting ghosts were washed until hemoglobin free with a solution containing 0.0153 M sodium chloride, 0.0017 M Tris, and $1 \times 10^{-4}$ M EDTA (pH 7.4).

*Cytochrome c.* Cytochrome *c* (horse heart, Miles-Seravac, Maidenhead, Berks., England) was dissolved in deionized water and the pH was adjusted to 8.0.

*Preparation of the DPL Vesicle–Cytochrome c Complex.* Synthetic DPL[1] (Calbiochem, Los Angeles, Calif.) was formed into thin-walled vesicles with diameters of 0.2–5 μ by the method of Reeves and Dowben (Melchior and Morowitz, 1972). The vesicles were brought to a concentration of about 0.2%, the pH was adjusted to 8.0, and cytochrome *c*

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[1] Abbreviation used is: DPL, dipalmitoyl-L-α-lecithin.

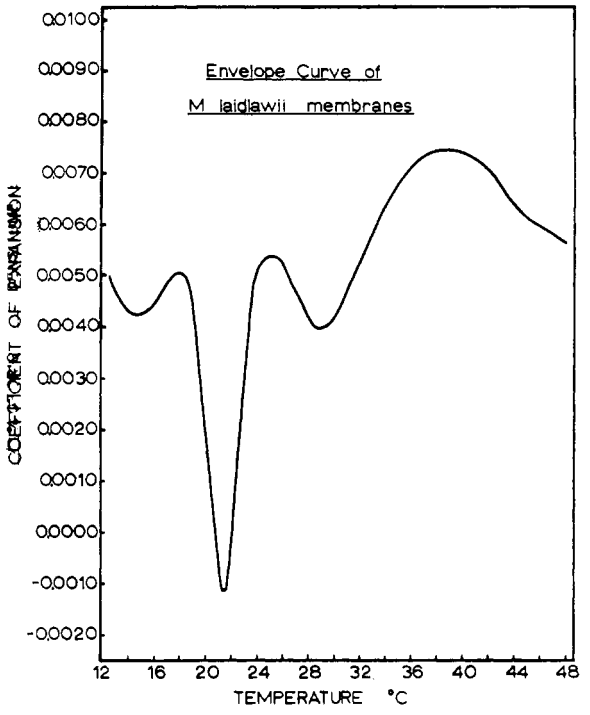


FIGURE 1: Plot of the apparent mass coefficient of expansion, $\epsilon_T$, in milliliters per gram per degree against temperature for an aqueous suspension of *M. laidlawii* membranes. This plot is drawn from $\epsilon_T$ values averaged over eight runs on fresh membrane preparations. The standard error of the scatter of experimental points about this curve is $\pm 5 \times 10^{-4}$ ml per gram per degree.

was added to a concentration of 5%. This mixture was shaken at 55° for approximately 2 hr, the vesicles were pelleted by centrifugation, and the supernatant was decanted. The pellet was washed by resuspension in deionized water, repelleting by centrifugation, and discarding the supernatant. This washing procedure was carried out several times. The resulting pellet was pink in color and individual vesicles appeared darker when viewed by phase contrast microscopy than before the addition of cytochrome *c*.

*Pronase Digestion.* Membranes at a concentration of 5 mg of protein per ml of deionized water were mixed with Pronase (Calbiochem) at a concentration of 100 mg/ml and incubated at 45° for 2 hr. The resulting suspension was centrifuged and the partially digested membranes were resuspended in distilled water.

*Dry Weights.* Dry weights were obtained by heating samples at 90° *in vacuo* (10–15 mm) for approximately 5 hr.

*Dilatometry.* The instrument used has been previously described (Rothman *et al.*, 1972). The experimental procedure as well as treatment of data are described in an earlier article (Melchior and Morowitz, 1972).

*M. laidlawii* membranes were run in deionized water and in buffer (0.39 mM NaCl–0.125 mM Tris in deionized water adjusted to pH 7.4 with HCl), red blood cell ghosts were run in a buffer consisting of 15.3 mM NaCl, 1.7 mM Tris, and 0.1 mM EDTA in deionized water at pH 7.4. Cytochrome *c* was run in deionized water at pH 8.0; the DPL vesicle–cytochrome *c* complex was also run in deionized water.

The *M. laidlawii* membranes, red blood cell ghosts, and DPL vesicle–cytochrome *c* complex concentrations were in the range of 0.5–1.5% by weight. The concentrations of cytochrome *c* were about 3%.

## Results

Dilatometer runs on membranes of *Mycoplasma laidlawii* were indistinguishable regardless of whether the membranes were suspended in buffer or deionized water. Figure 1 is a plot of the apparent mass coefficient of expansion, $\epsilon_T$, in milliliters per gram per degree of *M. laidlawii* membranes against temperature. This plot is drawn from $\epsilon_T$ values averaged over eight runs on fresh membrane preparations. The most striking feature of this curve is the large dip in $\epsilon_T$ which extends to negative values. This dip is a regular feature of fresh membrane preparations. It invariably appears centered around 22°. When newly prepared *M. laidlawii* membranes were run suspended in $D_2O$, their $\epsilon_T$ *vs.* temperature curves displayed 22° dips indistinguishable from those of membranes suspended in water.

While the dip is invariant in its temperature, its size is very sensitive to the past treatment of the membranes. Fresh membrane preparations on their first dilatometric run show the dip if not always extending to negative values, at least very close to them. If membranes are heated to 50° in the course of a run and then cooled and rerun, the dip appears with a diminished magnitude. If this procedure is repeated several more times the dip disppears altogether. Storage of membranes in the dilatometer for at least 1 week at 4° causes no diminuation of the dip.

The other feature prominent in Figure 1 is the broad diffuse peak which starts at 32° and tails off at about 50°. For a given run this hump consists of many peaks. While these peaks are fairly reproducible for a given membrane preparation, they vary somewhat, from one to another. Averaging over many preparations causes this detail to be lost and the smoothed maximum displayed in Figure 1 is what remains. Although the hump is affected by heating the membranes to 50°, it is not as sensitive to temperature as the 22° dip.

Figure 2 is a plot of $\epsilon_T$ *vs.* temperature for a series of individual runs on *M. laidlawii* membranes. In this diagram the 22° dip in the first heat is somewhat smaller than usual for a first run. The peaks in the 32–50° region are above the noise level of the instrument and should be considered significant. If after the first heat the dilatometer is brought up to 62° and the membranes left at this temperature for about 2 hr, the next run performed on this material has no indication of a dip at 22° although vestiges of the peaks in the 32–50° region remain. A further run gives the same curve with the 32–50° peaks even more reduced. Curves of $\epsilon_T$ *vs.* temperature for runs where all the prominent features have been eliminated by heating are straight lines with zero slopes and $\epsilon_T$ values of about 0.004–0.005 ml per gram per degree.

In one series of experiments fresh batches of *M. laidlawii* membranes were divided into two parts. For a given experiment one of these parts was stored at 4°; the other was digested with Pronase to remove approximately 80% of the membrane protein. This Pronase-treated sample was then run in the dilatomer. The $\epsilon_T$ *vs.* temperature curve obtained was a featureless line with zero slope and an average apparent mass coefficient of expansion of 0.004–0.005 ml per gram per degree. This curve is very similar to that obtained for *M. laidlawii* membranes after the severe thermal treatment described in the preceding paragraph. The noise level on Pronase-treated membrane curves was always high and related to the sample, not the instrument. After the runs on the Pronase-treated membranes, those parts of the membrane preparations which had been stored at 4° were loaded into the dilatometer and run. They gave the typical curves characteristic of fresh membrane preparations.

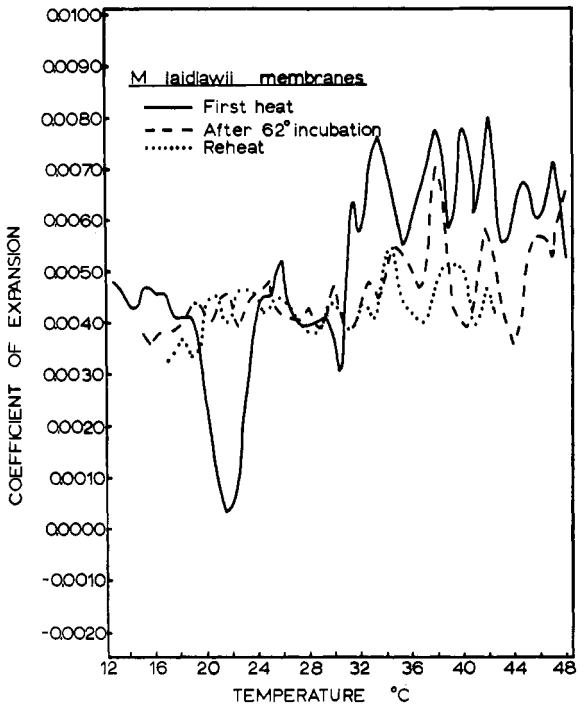


FIGURE 2: Plots of the apparent mass coefficient of expansion, $\epsilon_T$, in milliliters per gram per degree against temperature for a series of individual runs on *M. laidlawii* membranes. The solid line is the first heat. After the first heat the membranes were kept at 62° for 2 hr, cooled, and run to 50° giving the dashed line. The dotted line is the reheat of the run represented by the dashed line. The instrumental uncertainty for the values shown in these curves is $\pm 5 \times 10^{-4}$ ml per gram per degree.

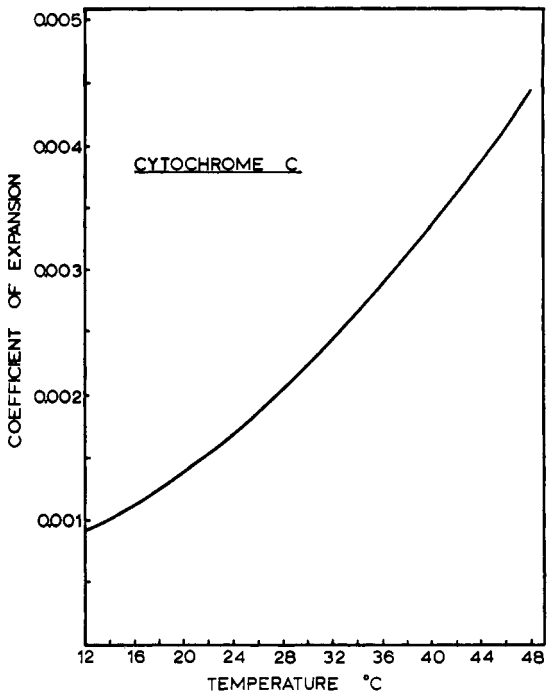


FIGURE 3: Plot of the apparent mass coefficient of expansion, $\epsilon_T$, in milliliters per gram per degree against temperature for cytochrome *c* (horse heart) in water at pH 8.0. The standard error of the scatter of experimental points about this curve is $\pm 1 \times 10^{-4}$ ml per gram per degree.

Calorimetric runs performed on these *M. laidlawii* membranes showed no new detail from those previously published (Melchior *et al.*, 1970).

A mass coefficient of expansion *vs.* temperature plot for DPL vesicles in the range of 10–30° is a straight line (Melchior and Morowitz, 1972). When cytochrome *c* is added to DPL vesicles forming a DPL vesicle–cytochrome *c* complex and this system run in the dilatometer, a dip at 22° appears identical with the one occurring in fresh *M. laidlawii* membranes. Figure 3 is a dilatometer run on a solution of cytochrome *c* at pH 8.0. There is no indication of any dip at 22° in the curve.

A temperature scan on a preparation of red blood cell ghosts is shown in Figure 4. The general shape of this curve is different than that of *M. laidlawii* membranes. There is a large upward slope in $\epsilon_T$ values ranging from about 0.005 ml per gram per degree at 16° to 0.014 ml per gram per degree at 40°. The fine structure of this curve is above the error level of the dilatometer. This detail is reproducible from run to run on a given preparation of red blood cell ghosts. In repeated runs on the same sample of ghosts a temperature of 40° was never exceeded. For a set of these runs the detail did not undergo substantial degradation.

## Discussion

In discussing the previous experiments it is important to remember that $\epsilon_T$ is an *apparent* mass coefficient of expansion. Therefore, this term represents not only the pure material's coefficient of expansion, but also such factors as solvent effects. This was demonstrated in previous dilatometric studies on lecithin (Melchior and Morowitz, 1972) where a much higher $\epsilon_T$ was obtained for the lipid in vesicle form than in dispersion. The vesicle's large apparent mass coefficient of expansion was attributed to interaction of lipid head groups with bulk water. Since $\epsilon_T$ can be a measure of such things, it is a quantity requiring cautious interpretation.

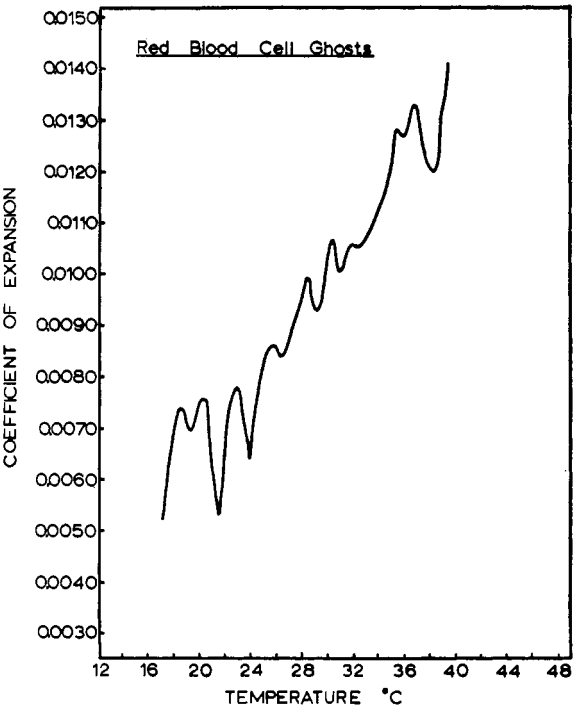


FIGURE 4: Plot of the apparent mass coefficient of expansion, $\epsilon_T$, in milliliters per gram per degree against temperature for red blood cell ghosts suspended in buffer solution (see Materials and Methods) The instrumental uncertainty for the values shown in this curve is $\pm 3 \times 10^{-4}$ ml per gram per degree.

Dilatometry on biological membranes has problems which are not encountered in the dilatometry of simpler substances. Because it is a technique very sensitive to alterations in membrane structure, the labile nature of membranes gives results whose reproducibility is considerably less than instrumental error. This necessitates careful experimental design. For example, Pronase-treated *M. laidlawii* membranes gave $\epsilon_T$ *vs.* temperature curves which appeared to have a great deal of noise. Tests were performed on the instrument before and after such runs. In all cases the instrument was found stable. Therefore, the scatter of data points was attributed to the material studied.

Numerous runs were performed on all materials and phenomena were considered significant only if reproducible or if they behaved in a consistent, systematic manner.

Figure 1 displays the two major features of an $\epsilon_T$ *vs.* temperature plot for native membranes of *M. laidlawii*. One of these features is the large, broad peak in the 32–50° range. This appears to be a protein-related effect since it is destroyed by treatment which denatures and destroys membrane protein.

The second feature found on $\epsilon_T$ *vs.* temperature plots of *M. laidlawii* membranes is the sharp dip at 22°. This very striking phenomenon is not detected by calorimetry. Since this dip's breadth is narrow, its magnitude large, and its $\Delta Q$ small, it must reflect a highly cooperative phenomenon. In order to gain a rough idea of the extent of cooperativity involved in this dip the following approach will be used. A standard enthalpy change, $\Delta H^{\circ}_{vH}$, for this process can be calculated from a plot of the volume change (subtracting out the contribution of $\epsilon_T$ over this interval) *vs.* temperature of the dip using the van't Hoff equation (Yow Tsong *et al.*, 1970)

$$\left(\frac{d\alpha}{dT}\right)_{T=T_m} = \frac{H^{\circ}_{vH}}{4RT_m^2}$$

where $T_m$ is the temperature of half-conversion and $(d\alpha/dT)_{T=T_m}$ is the slope of the volume change *vs.* temperature curve at that temperature. For a typical fresh membrane preparation the value of $\Delta H^{\circ}_{vH}$ for the dip is approximately 139 kcal/mol. Using $2.5 \times 10^{-3}$ cal/g of suspended material as the minimum $\Delta Q$ accompanying the dip which would have gone undetected by the calorimeter (Hinz and Sturtevant, 1972), a minimum value of $5.6 \times 10^7$ g/mol of substance undergoing the process is obtained.

Since runs performed on membranes suspended in $D_2O$ had dips indistinguishable from those of membranes suspended in water the likelihood that the 22° dip is a water effect is diminished. The much greater sensitivity of the 22° dip to agents which affect protein compared to the sensitivity of the 32–50° hump to these same agents implies that this dip has a delicate dependence on the state of membrane protein. The protein relatedness of the dip is confirmed by the experiments performed on suspensions of the DPL vesicle–cytochrome *c* complex. In view of these experimental results it appears that this effect is one involving proteins acting as a class in association with lipid bilayers.

Dilatometry on *M. laidlawii* membranes showed no indication of the lipid phase transition. This is not surprising. If one considers that the transition occurs over an approximate 20° interval, that the membranes are about 50% lipid, and assumes the volume change over the transition to be about what it is for lecithin, 0.025 ml/g (Melchior and Morowitz, 1972), the transition should be seen as an increase in volume of 0.0006 ml per gram (membrane) per degree over its extent. This is an order of magnitude smaller than the $\epsilon_T$ of the membrane.

In contrast to *M. laidlawii* membranes, red blood cell ghosts give a very differently shaped $\epsilon_T$ *vs.* temperature plot. The large values of $\epsilon_T$ comprising this curve are comparable to those of vesicles.

It is likely that the upward slope of the red blood cell ghost $\epsilon_T$ *vs.* temperature plot is a reflection of an effect different from those which are responsible for the fine structure of the curve. If this is so, a considerable amount of detail exists in this plot which is partially masked out by whatever causes the large slope in $\epsilon_T$. If this slope can be experimentally suppressed, a dilatometer scan of the red blood cell ghost–water system may reveal a significant amount of detail. This detail could prove quite useful in understanding the structure of the red blood cell membrane.

It should be noted that the procedure for preparing red blood cell ghosts involves washing hemolyzed red blood cells until hemoglobin free. Based on the results of the studies on *M. laidlawii* membranes, this procedure may be sufficiently violent to destroy any existing 22° dip. The slight indication of a 22° dip seen in those red blood cell ghost preparations run in the dilatometer (Figure 4) demands further study before any definite statements about it can be made.

The most striking result of these studies is the sharp unanticipated transition at 22°. The high degree of cooperativity as well as the association with undenatured lipid–protein systems renders this phase change one of major interest. It is hoped that studies now in progress will provide some information on the mechanism of this effect.

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