A dynamic characterization via relaxation techniques of the shell membranes of the domesticated fowl (gallus/gallus (L.))

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The shell membrane is made up of a unique three-layered structure which consists of outer, inner and limiting membranes. The structure is capable of adsorbing water in excess of 2.5 times its weight. The water containing species give rise to dielectric and n.m.r. relaxations associated with tightly bound and loosely bound water. An additional relaxation which appears to be intrinsic to the biomolecule is observed at low temperatures. The intensity and effectiveness of this relaxation is governed by the amount of water present. The facility of the membrane to interact with water enables the molecular reorientational process of the membrane itself to take place with a greater ease, with the result that the structure changes its deformation characteristics from that of a brittle to a low modulus elastomeric material. Thermal treatment of the membrane alters its relaxation properties. The membrane's ability to adsorb water is drastically reduced and the molecular reorientational processes do not occur as readily. As a result, the change from brittle fracture to low modulus yield that is observed for a saturated, non-heat treated material is not found, and the water-saturated, heat treated material fails in a brittle manner. It appears that the irreversible changes that take place on heat treatment do so gradually above room temperature reaching an appreciable rate above 410K. No changes corresponding to those found in the relaxation, differential scanning calorimetry (d.s.c.) and mechanical measurements are observed in the scanning electron microscopy (s.e.m.) data of the heat treated material.

INTRODUCTION

The shell membrane of the domesticated fowl is a two-ply opaque, white envelope which lies on the inside of the shell and fits snugly about the outer layers of the egg albumin. The inner membrane (*membrana putaminis*) surrounds the albumin. This membrane is in contact with the outer liquid albumin in all but the polar regions of the egg where some of the mucin fibres of the albuminous sac penetrate the membrane to form the *ligamenta albuminis*. The outer surface of the inner membrane is firmly attached to the inside of the outer membrane (*membrana testae*), except at the blunt end of the egg where the space between the two membranes is occupied by the air cell. The outer membrane lies between the inner membrane and the shell, and it is attached to the shell through the mammillary knobs on the inside surface of the shell.

Both the inner and outer shell membranes are made up of a network of fibres which lie parallel to the surface of the egg, and which are randomly oriented[1–4]. The diameter of the fibres vary from approximately 0.4 to 3.6 μm and most fibres are found to be of uniform thickness throughout their lengths[3]. Bellairs and Boyd[3] have also reported that the fibres of the inner membrane seldom exceed 2 μm in diameter, whereas those of the outer membrane are usually of the order of 3.6 μm.

Each fibre is composed of a medulla (core) and a cortex (mantle). The medulla can be regarded as the fibre proper while the cortex appears to be a thick coating material that binds the fibres together[3,4]. The medulla is homogeneous and extremely electron dense and contains small cavities of widely varying diameters. Some of these cavities appear empty while others have a moderately dense content[4]. The cortex which surrounds individual fibres and small groups of fibres is a moderately electron-dense substance and is thought to have the same composition as the protuberance on the fibres[3].

A limiting membrane separates the inner shell membrane from the albumin. It is a layer of homogeneously dense material in which the innermost fibres of the shell membrane appear to terminate. The deep surface of this lining is coated with a flocculent material that is less dense than the lining itself and varies in width from 0.25 to 0.75 μm. Similar material is also found in the interstices of the shell membranes and may represent a less dense condensed form of the cortical material of the shell membrane fibres[4].

The chemical composition of the shell membrane fibres is not fully established. Many reports have claimed the fibres to be composed of keratinous material[1,5–7]; however, Hoffer[4] points out that these conclusions were based upon outmoded techniques and old concepts and, as such, all that can be said is that the shell membrane, based on histochemical observations, contains both protein and carbohydrate[4]. By similar techniques, the cortex of each fibre has been reported to consist of mucopolysaccharides[5,8].

The shell membranes which serve both a protective and a regulatory function for the egg are made up of a unique structure. This structure performs its regulatory function efficiently, allowing the passage of metabolic reactants and products in and out while preventing the passage of bacteria into the egg. As a result, we have attempted to characterize the shell membranes by a series of physical techniques and

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to determine some of the changes in properties of the material following thermal treatment, and on interaction with water.

EXPERIMENTAL

The shell membranes used in this study were obtained from freshly laid eggs of the leghorn domestic fowl [*Gallus gallus* (*L.*)]. Differential scanning calorimetry (d.s.c.) measurements were carried out on a Perkin–Elmer DSC II. The heating rate was 10 K/min. Scanning electron microscopic (s.e.m.) examination of the membranes was made on an International Scientific Instrument Mini SEM. Membrane samples were coated with gold to a thickness of ~200Å.

The mechanical measurements were made on a table model Instron Tensile Tester with a crosshead speed of 0.02 in./min.

Nuclear magnetic spin–lattice (T_1) and spin–spin (T_2) relaxation times were measured on a Bruker SXP spectrometer operating at 90 MHz. T_1 was determined by the 180–τ–90 pulse technique[9]. The values of T_1 were derived from a least squares analysis of plots of $\ln [A(\infty) - A(\tau)]$ as a function of time interval τ between the 180° and 90° pulses in accordance with the equation:

$$\ln[A(\infty) - A(\tau)] = \ln[2A(\infty)] - \tau/T_1 \qquad (1)$$

where $A(\tau)$ is the amplitude of the free induction decay following the 90° pulse at time, τ. $A(\infty)$ is the limiting value of $A(\tau)$ for infinite time between the 180° and 90° pulses. No significant deviations from exponential behaviour were observed, and the slope of the straight line was given by $1/T_1$. T_2 was taken as $t_{1/2}/\ln 2$, where $t_{1/2}$ is the time for the free induction decay to fall to one-half its original value after the 90° pulse[10,11]. The FID following the 90° pulse was exponential at all temperatures and moisture contents studied, and it showed no signs of a second component. The temperature was controlled by a gas flow thermostatically controlled system to an accuracy of ±1K. Relaxation data were obtained on samples which were stored in water, air dried and subsequently evacuated at 10^{-5}mmHg for 4 h at 303K. After completion of measurements, the samples were dried under vacuum at 373K to remove the excess water, and the moisture content determined. These samples contained approximately 5.8% water by wt. Relaxation data were also obtained on samples which were evacuated at 10^{-5}mmHg for 24h, 4h of which the samples were heated to 333K. Measurements were made on these samples from 160 to 440K. At the high temperatures there is evidence, through fog formation on the top of the tube, of water being driven from the samples. These samples were cooled to room temperature and allowed to sit for a few days so that the water could re-equilibrate itself into the sample. The relaxation times were then redetermined. Through further drying at 373K under vacuum these samples were found to contain approximately 0.8% H_2O. The moisture adsorbing capability of the untreated and heat treated membranes were determined by soaking the membranes in water for a week, removing from the water, pat drying with a paper towel to remove excess water and weighing. The weighing was performed as a function of time and the weight of water was determined by extrapolating to zero time.

The dielectric data was obtained with an automated dielectric system that has been described elsewhere[12,13]. The system incorporates a General Radio 1683 RLC automatic bridge and Doric Scientific DS-100 digital thermocouple with a multiplex system built by Data Graphics Corporation. Samples were scanned at a rate of 0.9 K/min in a Delta Design Environmental Oven. Capacitance and tan δ were recorded on tape at 1 min intervals as a function of temperature. The data were transferred to a computer for processing. The dielectric data were taken at a frequency of 120 Hz. For these experiments the membrane was placed in a spring loaded dielectric cell quenched to liquid nitrogen temperature and scanned. Sample I was dried at 333K under a vacuum of 10^{-3}mmHg for 48 h. Sample II was stored in an atmosphere of 20% relative humidity (r.h.) of water for 48 h prior to measurement. Sample III is a membrane sample which was heat treated to 440K and equilibrated in a 20% r.h. atmosphere before scanning.

RESULTS AND DISCUSSION

Scanning electron microscopy shows the shell membrane to consist of inner and outer layers of randomly oriented fibres which run in the plane of the membrane[1–4]. Although the inner and outer membranes are firmly attached to each other, there does appear to be a natural plane of cleavage between these two membranes. *Figure 1* is a view looking down onto the membrane. In the right half of the micrograph, the outer shell membrane (*OSM*) is cut away revealing the inner shell membrane (*ISM*). The similarities between these two structures (*OSM* on the left, *ISM* on the right) are clear. No essential differences are found in the structure and arrangement of the fibres in any part of the inner and outer membranes. In both membranes, the fibres lie parallel to the surface of the egg. Individual fibres are randomly oriented and no fibre is observed to travel from one level of the membrane to the other. At higher power (*Figure 2*) both membranes exhibit large spaces between the fibres. The individual fibres are found to consist of a central core (medulla) (M) covered by a less dense cortex (mantle) (C). A broken fibre with these respective components is observed in *Figures 2* and *3*. In some areas where fibres cross or run alongside each other, the cortical material may be communal so that individual identification throughout the length of the fibre is not possible. On the inner membrane, the cortical

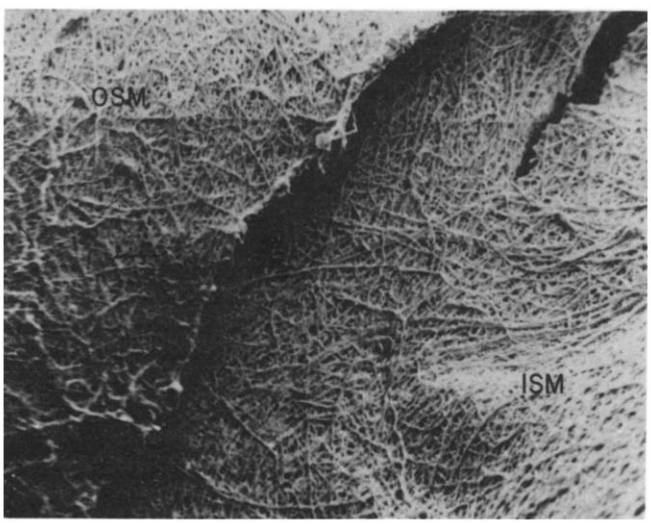

Figure 1 S.e.m. of shell membranes at 100 X. View looking down onto membrane with right half of membrane cut away to reveal *ISM*. *OSM* on the left, *ISM* on the right

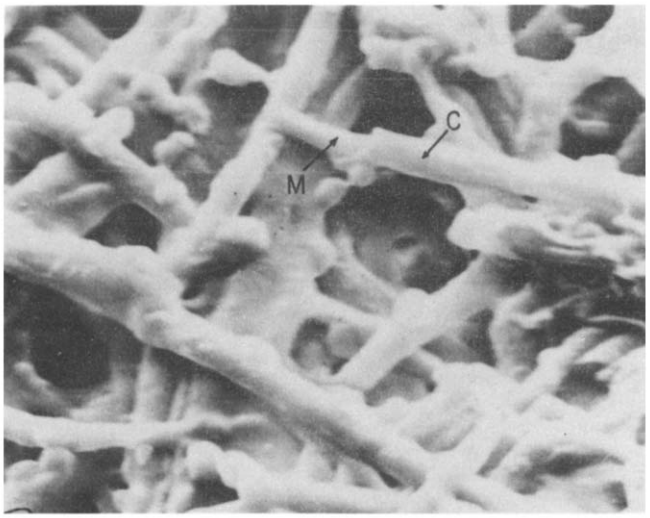

Figure 2 S.e.m. of *OSM* at 3000 X. Note large spaces between the fibres. Individual fibres consist of a central core (medulla) (M) and a less dense cortex (mantle) (C). A broken fibre with these components is indicated

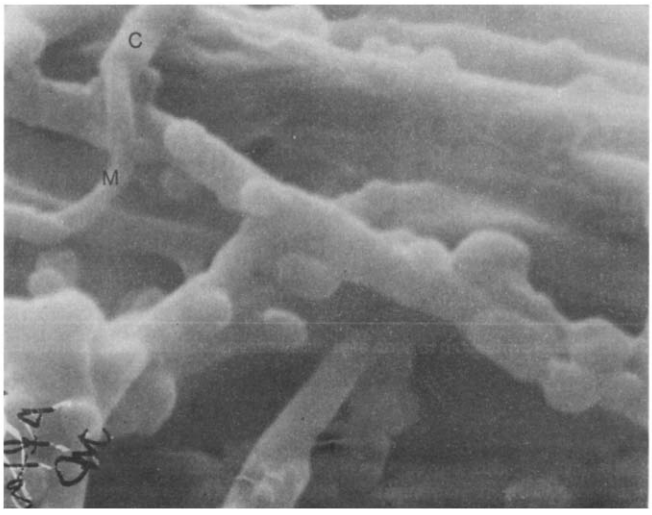

Figure 3 S.e.m. of individual fibres at 5000 X. The oval protuberences are observed to appear in a random fashion both individually and in groups

material is also found interwoven between the fibres (see *Figure 2*). In *Figure 3*, at high magnification, the oval protuberances are observed on the fibres. They appear in a random fashion both in groups and individually on the fibres of the *OSM* and *ISM*.

On the inside of the inner membrane next to the albumin is the limiting membrane whose structure is seen in *Figure 4*. It is a flocculent material which is not as thick as the *OSM* or *ISM*. Impressions of fibres of the *ISM* can be seen underneath this structure. It has been suggested that its composition may be similar to the material found in the intersticies of the *ISM* or it is a less condensed form of the cortical material[4].

The spin–lattice (T_1) relaxation data for the shell membrane is given in *Figure 5*. All three samples exhibit two well-defined minima. Sample A, containing 5.8% water, exhibits the most efficient T_1 relaxation. Its low temperature T_1 minimum of 320 msec is at 216K while the high temperature minimum is slightly more intense, having a minimum of 270 msec at 376K. Sample B with 0.8% water relaxes slightly more slowly. T_1s at the minima are 380 and 355 msec, respectively, and both minima occur some 18K higher in temperature. In Sample C, drastic changes are observed. The low temperature minimum is much sharper and the T_1s are longer than those of samples A and B. The minimum occurs at 202K with a T_1 of 450 msec. The high temperature minimum also occurs at the lower temperature of 380K and has a T_1 of 750 msec.

Two transitions are also observed in the spin–spin relaxa-

Figure 4 S.e.m. of limiting membrane at 2000 X. It is a flocculent material. In this micrograph an impression of a fibre of the *ISM* can be seen underneath this structure

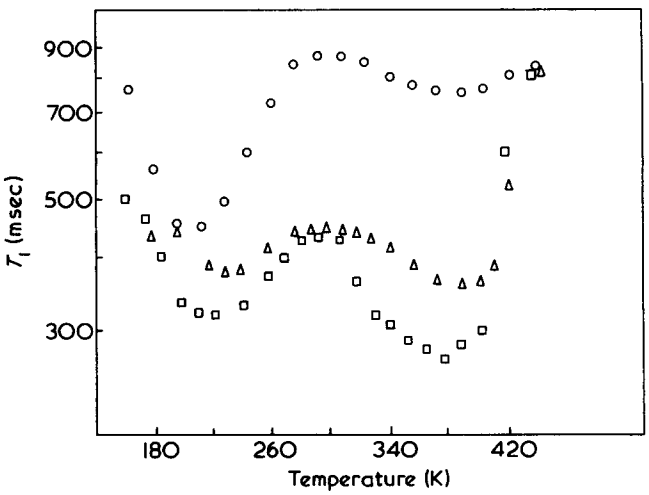

Figure 5 Plot of T_1 *versus* temperature for the shell membrane. Sample A (□), evacuated at 10^{-5}mmHg for 4h at 303K – water content ~5.8%. Sample B(△), evacuated at 10^{-5}mmHg for 24h, 4h of which sample was heated to 333K – water content ~0.8%. Samp C (○), Sample B re-equilibrated in sealed tube for four days with original water content after measuring T_1 to 440K

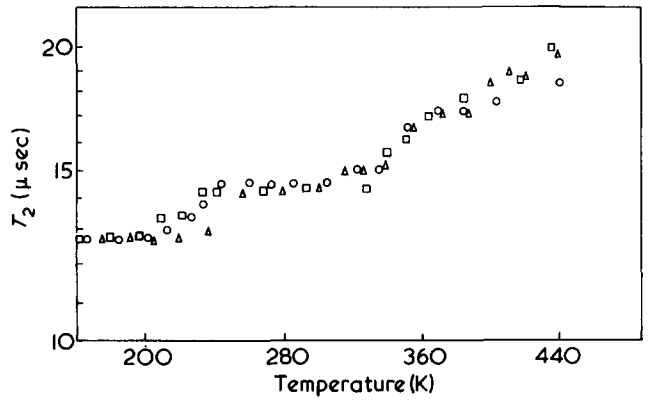

Figure 6 Plot of T_2 *versus* temperature. Sample A (□), Sample B (△), Sample C (○)

tion (T_2) data (*Figure 6*). The first occurs at approximately 220K and is rather minor, involving a change in T_2 of about 1.5 μsec. The second and more intense transition occurs at 320K and involves a change in T_2 which is fairly gradual, increasing by a factor of 1.5 over a temperature range of 120K. Unlike the T_1 data, the T_2 process is less sensitive to water content and thermal treatment. No discernable changes (within experimental error) are observed in going from samples A to C. Both T_1 and T_2 processes are described by a single exponential at these moisture contents.

Plots of tan δ for the shell membrane are given in *Figure 7*. Sample I, which was dried at 330K for 4h, shows two maxima in tan δ at approximately 260 and 330K, respectively. These relaxations are not very intense. When a similar sample was exposed to an atmosphere of 20% r.h. (Sample II), the material exhibited higher dielectric loss factors over the whole temperature range. The low temperature transition broadened, now encompassing a range from 120 to 260K, while the high temperature transition also broadened (from 270 to 373K) and became more intense. Tan δ increased through two orders of magnitude.

Relaxations due to tightly bound and loosely bound water are observed in collagen. A β_1 relaxation at 260K (1 Hz), decreases in intensity as water is removed while the temperature at which it occurs remains unchanged. This relaxation has been assigned to a loosely bound water phase. A tightly bound water relaxation appears as a shoulder on the lower temperature side of β_1 and shifts to lower temperature as water is removed[18,19]. Sorbed water up to 2% produces a β process at 200K in nylon. Loosely bound water has little additional effect on the relaxation behaviour and no β_1 analogue of collagen is found in nylon-6[20]. Poly(L-glutamic acid), poly(L-leucine) and copolymers of the two exhibit a β_1 relaxation at 260K with the β_2 process appearing as a shoulder on the low temperature side of β_1. At low moisture contents only the β_2 process is observed, and it increases in intensity up to 1% water, above which the β_1 peak increases until the specimen is saturated[21]. The low temperature transitions in T_2 and tan δ of the *OSM* are observed in the same temperature range as the relaxations for tightly and loosely bound water[18–21]. The dielectric relaxation is fairly broad and asymmetric, and may possibly result from the reorientation of water molecules which are attached with varying degrees of binding to the biomolecule.

The high temperature T_2, tan δ and T_1 relaxations are very sensitive to moisture content. Various degrees of bound water as well as free water have been observed in cellulosic systems by n.m.r.[14,15]. For such systems very tightly or primary bound water is found to exist at concentrations up to roughly 10% moisture by wt. The concentration of secondary or less tightly bound water increases up to approximately 25 to 30% moisture. Simultaneously the dramatic build-up of multilayers of free water begins to take place at the transition between the primary and secondary bound water phases. A consequence of such a build-up of multilayers is that the water molecules which are far away from the surface of the cellulose will take on properties approaching that of bulk water. As a result the loosely bound or free water tends to be driven from the system as the temperature is increased above 273K[14,15]. In being driven off the water molecules experience increased motional freedom, contributing in a large part to the intense high temperature relaxations. Supportive evidence of the removal of water from the biomolecule is rendered by the d.s.c. data (*Figure 8*) which shows a very intense endotherm centred at 360K and covering a range of 100K. As a result the high temperature dielectric and n.m.r. transitions are probably due to the motion of very loosely bound of free water trapped in the system.

The low temperature relaxation in T_1 giving rise to the minimum at approximately 216K broadens and becomes more efficient with increasing water content. For the heat treated samples, the minimum sharpens up exhibiting much steeper slopes for the sides of the minimum. Step-wise changes in T_2 occur at a frequency of approximately 10^4 Hz (the frequency of reorientation at the transition is given by $\upsilon \sim 1/\pi T_2$ and $\upsilon \sim 1.4\omega_0$ at the T_1 minimum) while the T_1 minimum is observed at frequencies of $\sim 10^8$ Hz. Con-

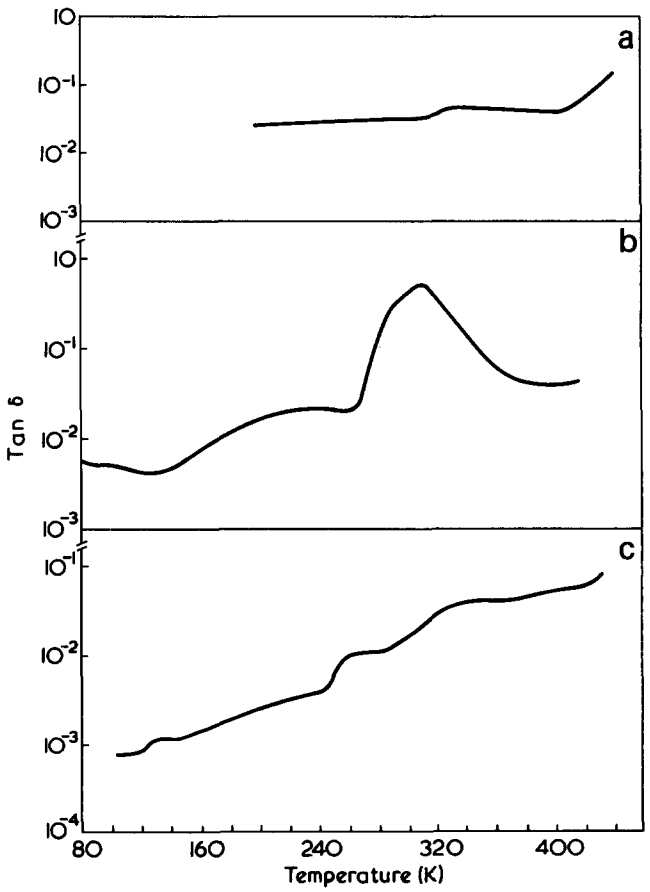

Figure 7 Plot of tan δ *versus* temperature at 120 Hz. (c) Sample I, dried at 333K under vacuum of 10^{-3} mmHg for 48 h. (b) Sample II, stored in 20% r.h. atmosphere for 48 h prior to measurement. (a) Sample III, Sample I measured to 440K, then re-equilibrated at 20% r.h. for 48 h

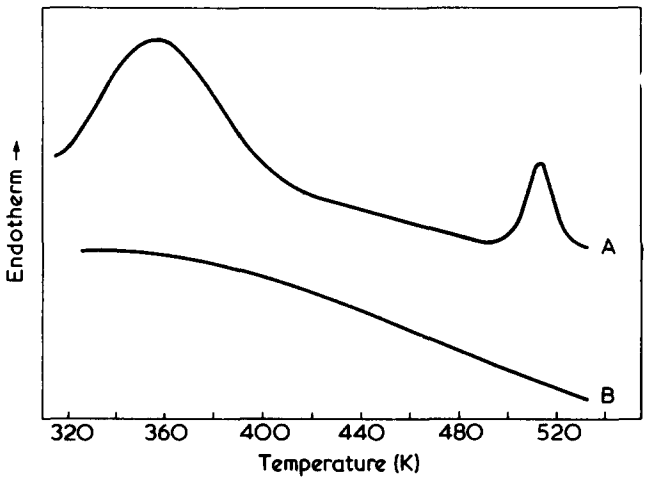

Figure 8 D.s.c. trace of shell membrane. First cycle, heating of air dried membrane. Second cycle, reheating after scanning to 530K and allowing to re-equilibrate at room temperature

sequently, if the motional processes involved are Arrhenius activated, the transitions in T_2 and tan δ corresponding to the T_1 minimum would have to occur at much lower temperatures. As a result, they are not observed in the temperature range studied. The low temperature T_1 relaxation, therefore, is probably related to some intrinsic process of the biomolecule which is assisted by the presence of water. Since the exact structure of the biomolecule is not known, it is not possible at this time to identify the exact nature of the reorientation. However, the ease of occurrence of the relaxation is facilitated by the presence of water which gives rise to a more efficient T_1 process. Such assistance lends itself to a broader distribution of correlation times associated with the reorientation and results in a flattening out of the T_1 minimum[16]. Curtis[17] and Illers[18] have studied the effect of sorbed water on the low temperature dielectric relaxations in polyurethanes and have explained their data in terms of a water/polymer complex which enhances the intensities of these relaxations. In fact, Illers suggested that the complex was due to hydrogen bonding of the water molecules to the carbonyl of the polyurethane[18]. This membrane is certainly proteinaceous, and the i.r. spectra reveal the presence of N–H and carbonyl groups which are capable of hydrogen bonding with water. It is not unreasonable, therefore, that below room temperature the sorbed water in the system would probably remain in its local environment and enhance the n.m.r. and dielectric response of any molecular motion.

On thermally cycling the material, changes occur in the relaxations. Although little change is observed in the T_2 data for the heat treated material, the T_1 data of Sample C are vastly different from Sample B which has the same moisture content. The relaxation processes in Sample C appear to be slightly less efficient. This is consistent with the almost featureless tan δ trace of Sample III. It is clear that on heat cycling the ability of the membrane to readsorb and, therefore, interact with water has changed drastically. A d.s.c. trace showing two endotherms in the first cycle is observed in *Figure 8*. The endotherm at 360K is due to the removal of water from the sample. That at 510K may be due to some exothermic process which involves a chemical alteration of the biomolecule. As a matter of fact, the T_1 and tan δ data indicate that it is not necessary to subject the material to temperatures as high as 510K to obtain these irreversible changes. Above 410K, the T_1s appear to increase sharply. This might reflect the procedure of the chemical change to a sufficient extent to be observed in the n.m.r. and dielectric data. It is possible that the irreversible changes in the membrane take place slowly at temperatures above room temperature, but that its rate only becomes appreciable above 410K, reaching a maximum at 510K. As a result of these changes, the membrane's ability to bind water tightly is severely impaired and no endotherm is observed when the material is reheated on the d.s.c. The water adsorption properties of a membrane heat treated to 453K for 6h under vacuum was greatly reduced from the untreated material. Heat treating for that length of time reduced its adsorption capability from 2.33 to 0.76 g H_2O/g membrane. Despite the changes observed in relaxation data on heat treatment no changes were found in the scanning electron micrographs of the material. Thus, the physical structure of the material remained unimpaired. Hints of small amounts of hydrogen sulphide given off when the membrane is heated in air may suggest some type of crosslinking process in which thermal treatment may destroy some of the sites on the molecule capable of hydrogen bonding with water.

Many of the changes observed in the n.m.r. and dielectric data are relfected in mechanical deformation experiments. *Figure 9* gives a plot of force *versus* percent elongation for various samples of the membrane. An air dried sample breaks in a brittle fashion after an elongation of less than 2%. When the membrane is saturated with water, it behaves more like a low modulus elastomeric material yielding some

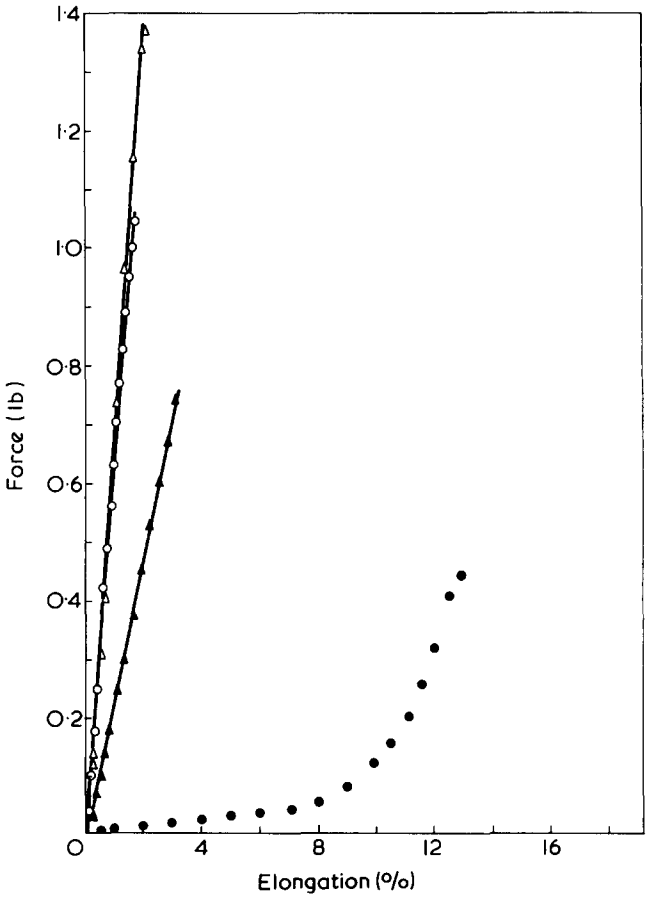

Figure 9 Plots of force *versus* percent elongation for (○) air dried membrane, (●) untreated membrane saturated with water, (△) membrane heat treated at 453K for 6h, (▲) heat treated membrane saturated with water

13% before breaking. The presence of water in the material clearly facilitates molecular reorientation of the biospecies. A sample which was heat treated for 6 h at 453K broke in a brittle fashion similar to the air dried material. However, when the heat treated material was saturated with water it also broke in a brittle fashion, having yielded only 3% before fracture. The saturated heat treated material clearly does not show the elastomeric tendencies of the untreated membrane and this finding is consistent with its reduced ability to bind water tightly as reflected in the relaxation measurements and the adsorption data.

CONCLUSIONS

The shell membrane is made up of a unique three-layered structure which consists of outer, inner and limiting membranes. The structure is capable of adsorbing in excess of 2.5 times its weight. The water containing species give rise to dielectric and n.m.r. relaxations associated with tightly bound and loosely bound water. An additional relaxation which appears to be intrinsic to the biomolecule is observed at low temperatures. The intensity and effectiveness of this relaxation is governed by the amount of water present. The facility of the membrane to interact with water enables the molecular reorientational process of the membrane itself to take place with a greater ease, with the result that the structure changes its deformation characteristics from that of a brittle to a low modulus elastomeric material.

Thermal treatment of the membrane alters its relaxation properties. In the thermally treated material, the reorientational processes do not occur as readily. The membrane's ability to adsorb water is drastically reduced. As a result, the change from brittle fracture to low modulus yield that is observed for saturated, non-heat treated material is not found, and the water saturated heat treated material fails in a brittle manner. It appears that the irreversible changes that take place on heat treatment do so gradually above room temperature reaching an appreciable rate above 410K. No changes corresponding to those found in the relaxation, d.s.c. and mechanical measurements are observed in the s.e.m. data of the heat treated material.

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