A STUDY OF METHODS FOR RECONSTITUTING BACTERIORHODOPSIN INTO DIMYRISTOYLPHOSPHATIDYLCHOLINE VESICLES SUITABLE FOR DEUTERIUM NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

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ABSTRACT

Few studies have as yet been performed on the putative interactions between lipids and integral membrane proteins at the membrane surface. Such systems are difficult to study due to problems in the purification of intact and functional proteins and the lack of suitable physical methods for examining directly the molecular interactions at membrane surfaces. The problem is also compounded by the limited knowledge of detailed tertiary structure for membrane proteins.

Bacteriorhodopsin (bR) provides an obvious candidate for study. It is easy to purify, is the most thoroughly understood integral protein at the structural level, and is well understood in function, although the mechanism remains elusive. However no suitable reconstitution procedure is available which satisfies the criteria for deuterium nuclear magnetic resonance spectroscopy (D-NMR), i.e:- complete lipid replacement free from endogenous lipid, and large homogenous vesicles (dia. $\geqslant$ 50 nm).

In this project, the successful reconstitution of bacteriorhodopsin has been achieved into a single common phospholipid, dimyristoylphosphatidylcholine, specifically deuterated in the choline head-group (DMPC-d_9) and so suitable for D-NMR. Gel filtration to remove endogenous lipid followed by detergent dialysis to ensure large vesicle size has been used. A method for the quantitation of the detergent octyl-β-D-glucoside was devised. This allowed detailed analysis of the gel filtration to be undertaken; previously this was only possible by using a radiolabelled detergent.

Preliminary D-NMR studies on the lipid-protein interactions between the DMPC headgroups and bR have been undertaken, with the indication that at higher protein to lipid ratios, the protein decreases the

quadrupole splitting by perturbation of the orientation and/or amplitude of motion of the lipid head-group. ^{31}P-NMR confirms the bilayer structure of the vesicles produced and negative staining electron microscopy shows the size to be homogeneous with a diameter of ca. 150 nm.

INTRODUCTION

Halobacterium halobium is an extreme halophile, living best in environments containing about 5M salt. In anaerobic conditions, the bacterium is capable of synthesising specialised patches in the plasma membrane, termed purple membrane by virtue of the vivid colouration which is due to the presence of high amounts of a single species of retinal containing polypeptide, bacteriorhodopsin (bR). bR is responsible for pumping protons across the membrane using visible light (Oesterhelt and Stoeckenius, 1977).

The purple membrane can be isolated in stable form at low ionic strength. The protein exists in a two-dimensional, triclinic crystalline lattice in the membrane (Blaurock and Stoeckenius, 1971), a feature which allowed the elucidation of the three dimensional electron density map to 7 Å resolution using a novel technique of tilting samples in the electron microscope to obtain diffraction data and combining this with direct visualisation methods (Henderson and Unwin, 1975).

The ability to produce stable two dimensional arrays has permitted greater structural analysis to be undertaken for this protein than for any other integral membrane protein. Elucidation of the primary structure of bR (Ovchinnikov et al., 1979; Gerber et al., 1979) has enabled attempts to be made to predict the secondary structure. By combining electron density maps, neutron diffraction and model building, a proposed path of the bR polypeptide through the lipid membrane has been presented (Engleman and Zaccai, 1980, Engleman et al., 1980) with the finding that hydrophobic amino acid residues contact the lipids, whilst the polar amino acids are confined to the centre of the molecule

and the membrane surface.

Bacteriorhodopsin is one of three retinal containing proteins found in the membrane envelope of H. halobium. The two others are halorhodopsin, a chloride ion pump (Stoeckenius and Bogomolini, 1982), and sensory rhodopsin, a phototactic receptor (Stryer, 1984). bR is a light dependent proton pump, ejecting a proton to the external medium on activation with a photon and producing an electrochemical gradient that may reach 300 mV. This gradient is used to drive directly some metabolic processes, or is converted to Na^+ and K^+ electrochemical gradients which can be 'stored' and then used to drive energy requiring processes. The charged surfaces on the internal α-helical faces have been proposed to play a role in proton translocation.

A wide range of physical studies of lipid-protein interactions have been performed on a number of systems, e.g:- bovine rhodopsin, erythrocyte band 3, myelin basic protein, cytochrome c oxidase, Na^+ K^+ ATPase, lipophilin etc, by reconstitution methods.

Whilst procedures for the reconstitution of bR into phosphatidylcholine (PC) vesicles have been developed, the protein has not yet been the subject of lipid-protein interaction studies by magnetic resonance methods. Reconstitution of bovine rhodopsin into vesicles of a single lipid type (dimyristoylphosphatidylcholine deuterated at the polar head-group) has recently been achieved and examined by D-NMR. Since the tertiary structures of both bR and rhodopsin are reported to be very similar (despite marked disparity in the primary structure (Ovchinnikov, 1982)), it may be that the lipid-protein interactions will also be similar, despite the very different native lipid enviroments. It may also be possible to compare D-NMR data with that for other integral membrane proteins, such as

cytochrome oxidase and band 3, although no systematic reports are yet available in the literature.

The technique of D-NMR is capable of yielding similar information to electron spin resonance (ESR); both to probe the local environment within the lipid bilayer. D-NMR has the advantage over ESR in that replacement of protons by deuterons causes very little, if any, perturbation of the labelled molecule in comparison to nitroxide spin labels (e.g. TEMPO, the ring of which is bulky), although the dynamic information from both methods is complementary when it can be compared directly. D-NMR also has advantages over proton NMR;

1) A deuterium spectrum consists of only a few resonances when using a specifically labelled probe. These can be assigned unambiguously thereby allowing the 'probing' of specific sites.

2) Dipolar couplings are reduced due to the smaller magnetic moment of the deuteron.

3) Deuterium NMR can detect fast anisotropic movement ($\tau_c=10^{-4}$s).

Disadvantages of the technique are:

1) Lack of selectively deuterated molecules. The synthetic production of labelled lipids is difficult and expensive.

2) The low sensitivity requires a high level of labelling ($>$1.0 mg of deuterated lipid per sample).

The origin of the D-NMR spectrum lies in the fact that the deuterium nucleus possesses a non-spherical charge distribution, resulting in a quadrupole moment, so that the energy required to induce spin transitions of the type detected in an NMR experiment depends on the orientation of the C-D bond (see Figure 1) with respect to the external field (B_o). A singlet is observed when the C-D bond undergoes rapid isotropic motion because the orientation dependent transitions are

motionally averaged (the spectrum can be said to have collapsed). If the motions are fast, but not completely averaged in all directions, then anisotropy of the motion is revealed in a powder spectrum, which has a width given as the quadrupole splitting ($\Delta\nu_Q$). The degree of anisotropy provides a measure of the motional energy of the deuterons in the sample, and this can be measured from the change in $\Delta\nu_Q$.

For a random unoriented sample such as a lipid bilayer, in which the CD bonds are moving quickly and anisotropically, the quadrupole splitting is:-

$$\Delta\nu_Q=(3/4)(e^2q\ Q/h)S_{CD}$$

where $e^2q\ Q/h$ is the static quadrupole constant of 172 MHz, and S_{CD} is the C-D order parameter, defined as

$$S_{CD}=1/2(3\cos^2\theta_{CD}-1)$$

where θ_{CD} is the angle between the C-D bond and the molecular symmetry axis, which varies randomly but completes with time. Thus the value of S_{CD} gives a measure of the time-averaged angle θ_{CD} (Seelig 1977).

A change in $\Delta\nu_Q$ can be brought about by two factors;

1) From a change in the rate of motion of the C-D bond if it is becoming slow ($\tau_c > 10^{-4}$ sec) or,

2) By an alteration in the orientation of the C-D bond.

As T_1, the spin lattice relaxation time, is sensitive only to changes in the rate of CD bond motion, an analysis of the T_1 of the DMPC-d_9 in the vesicles should provide an indication of which effect is altering the quadropole splitting. The dependence of T_1 on S_{CD}, for $|S_{CD}| \leqslant 0.1$, is described by the following (Brown et al., 1979);

$$1/T_1=3/8(e^2qQ/h)^2\,\tau_c(1-S_{CD}^2)$$

where τ_c is the correlation time of the CD bond.

If, in a membrane, a protein has significantly immobilised the

deuterated lipid segment, then T_1 may alter. However, if the relaxation time remains the same, then the protein has not altered the fast motions which dominate T_1. T_2 determinations can give information on slow motion but these measurements cannot be done with the present instrumentation in Oxford.

The interaction between the fatty acid chains in a membrane lipid molecule and integral proteins has been extensively studied. The specificity of such interactions may be due to two factors;

1) The nature of the fatty-acyl groups, i.e:- length of the carbon chain and degree of unsaturation (governing the thickness and fluidity of the membrane); and,

2) The structure of the polar lipid head-group.

Certain membrane-bound enzymes have been shown to require specific lipids to be present if they are to have full activity, e.g:- the Na^+-K^+ and the Ca^{++}-Mg^{++} ATPases require acidic phospholipids for full activity (Stekhoven and Bonting, 1981). This phenomenon probably indicates a requirement for general properties in the macroenvironment, rather than a specific interaction. The presence of the acidic groups may confer a local pH and dielectric constant to the solvent close to the membrane and hence proteins within the bilayer. Lipid in direct contact with the protein (in the annulus) are only bound weakly, as the rate of lateral diffusion of this lipid is ca. 1/10 of that for bulk lipid (Watts, 1981).

The work carried out here is an investigation into the character of the head-group/protein interaction at the membrane surface. It is possible that ionic effects occur between the charged head-groups of certain phospholipids and the ionic functional groups of the protein in the locality of the surface of the membrane, as well as the

well-recognised hydrophobic interaction/complementation of fatty acyl chains and intramembrane surfaces of the protein. The existence of hydrogen bonding between molecules of phosphatidylethanolamine (PE) or PC has been suggested (Israelachvili, 1980) and observed by D-NMR (Sixl and Watts, 1982). If such interactions do indeed occur, this would provide a futher role for the protein selectivity of particular lipid species and for selectivity-requirements in the maintenance of protein function.

For D-NMR studies on bR, certain stringent conditions must be fulfilled :

1) The vesicle size must be large i.e. greater than 50 nm diameter or ideally even larger, to prevent the effect of fast vesicle tumbling (on the NMR time scale) averaging out the $\Delta \nu_Q$, CD bond and phosphorus anisotropy,

2) All the endogenous lipid must be removed to ensure that the spectrum produced is from a well characterised system.

The reconstitution method developed in this project meets these conditions by using detergent dialysis to produce large vesicles, and a gel filtration step to remove ca. 95% of the endogenous lipid.

These two features allow the sucessful reconstitution of bR into DMPC vesicles suitable for NMR spectroscopy.

The phospholipid used in the reconstitution complexes were synthesised in this laboratory using the method of Sixl and Watts (1982) which allows the production of DMPC specifically deuterated in the polar choline head group alone. This method ensures strong intensity signals for NMR spectroscopy.

MATERIALS AND METHODS

PROTEIN ESTIMATION: Protein was quantified colorimetrically by the procedure of Markwell (1981) using crystalline bovine serum albumin (Sigma) as standard.

ELECTROPHORESIS: Protein (0.1 mg in 20 ul) was prepared for electrophoresis by heating for 5 min. at 100 ^{o}C, in the presence of 0.225 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol and 0.001% bromophenol blue. Electrophoresis was performed under reducing conditions in 15% polyacrylamide gels at 3 mA/track for 5 hrs in apparatus thermostatted at room temperature. Protein bands were visualised by staining with 0.25% Coomassie brilliant blue in 50% methanol; 10% acetic acid. Molecular weight calibration was achieved by running protein molecular weight markers (Sigma; VII-L markers) parallel to the bR tracks.

PHOSPHATE ESTIMATION: Phospholipid concentrations, in the 0 - 50 nmole range, were determined by using either the method of Rouser (1975), or Bartlett (1959). Inorganic phosphate was used as standard (Applied Science Labs).

BACTERIAL GROWTH: Halobacterium halobium R1 was grown following one of two separate protocols. A stock of R1 received from B. Jackson (University of Birmingham), was grown using the procedure of Oesterhelt and Stoeckenius (1974), and a slope from N.C.I.M.B. Aberdeen, was grown

following the method of Payne et al. (1960). The cultures were aerated using an electric pump delivering air through a pipette, and illumination was provided by a mercury bulb positioned 100 mm from the culture vessels, which were 5 litre conical flasks containing 2.5 litres of culture. The cultures were incubated at 37 ^{o}C without additional stirring. It was ascertained that the bacteria were producing bR by pelleting out 1.5 ml aliquots in a microfuge. When purple membrane patches were present, colouration was clearly seen in the pellet. Bacteria were then harvested.

PROTEIN PURIFICATION: Purple membrane patches were prepared by the method of Oesterhelt and Stoeckenius (1974). Then the purple membrane patches were taken up in 200 ml 0.1 M NaCl and centrifuged (4 ^{o}C; 30 min.; 25000 rpm), the pellets were resuspended in a minimum volume of 30% sucrose and applied to the surface of a 30/60% sucrose density gradient. Gradients were centrifuged for a minimum of 17 hours (25000 rpm; 4 ^{o}C) on a Beckman L2-45B Ultracentrifuge using an SW27 rotor. Contrary to Oesterhelt and Stockenius' report (1974),there was no red band above the purple patches after centrifugation. Only material from the main band was removed. The membrane patches were washed by diluting to 100ml with deionised water and centrifuging (40 rotor; 30k rpm; 30 min.; 4 ^{o}C). This was repeated and the pellets taken up in a minimum volume, typically 5.0 ml of buffer (0.15 M NaCl; 0.01 M Tris-HCl; 0.025% NaN_3).

The bR was solubilised in 40 mM octyl glucoside in the dark at 4 ^{o}C for 48 hours. The patches are reported to be at least 95% solubilzed by this procedure (Dencher and Heyn 1978).

DELIPIDATION: Removal of $\geqslant$ 95% of endogenous polar lipid was carried out using the method of Huang et al.(1980). Aliquots of solubilised bR, 2.0 mg protein in 10 ml, were loaded on a G-75 Sephadex column (200 x 15mm, void volume 18ml) and eluted with buffer (0.01 M Tris-HCl; 0.15 M NaCl; 0.25% Deoxycholate; 0.025% NaN_3/pH 8.0) delivered by an LKB peristaltic pump (Varioplex 12000). The flow rate of the column was adjusted to ca. 70 ml/hr and 2.0 ml fractions collected with an LKB Ultrorac 2000 fraction collector. The delipidation was performed at room temperature. The bed volume of the column was found by running 2-^{3}H-glycerol (Amersham International) through the column and counting activity on a LKB Ultrobeta 2000 scintillation counter. A total activity of 2.5 x 10^6 cpm was added. The scintillant used contained, in one litre, 660 ml toluene, 330 ml Triton X-100, 6.0 g PPO and 200 mg POPOP.

RECONSTITUTION: Fractions containing protein were pooled and concentrated to a volume of 5.0 ml using an Amicon ultrafiltration cell, with pressure delivered from a N_2 cylinder at 15 bar. Deuterated lipid was dried from chloroform/methanol in vacuo for at least 2 hrs. The required mass of lipid was taken up in deoxycholate (DOC) solution and mixed with bR in buffer, to give a final concentration of 3.0 mM DOC, a concentration well above the critical micelle concentration (CMC). The precise quantities used depended on the lipid-protein ratio eventually required, but it was found that the amount of lipid initially used must be in excess of that required in the vesicles to counteract loss of material during dialysis. About 50% of the lipid is lost during dialysis. The lipo-protein preparation was then dialysed against 1.0mM DOC; 0.15 M NaCl; 0.01 M Tris-HCl; 0.25% NaN_3; pH 8.0; 2 litres; 4 ^oC for 48 hours (two changes of buffer), and then against 0.15 M NaCl;

0.01 M Tris-HCl; 0.025% NaN_3; pH 8.0; 1 litre; 4 ^{o}C until vesicles had formed, usually about five days.

PREPARATION OF SAMPLES FOR NMR: The dialysate was centrifuged (40 rotor; 35k rpm; 15 min.; 4 ^{o}C), and the pellet resuspended in 1.0 ml 10% sucrose, made up in the second dialysis buffer. The vesicles were centrifuged through a 10%-40% sucrose density gradient also made up in the second dialysis buffer and spun for a minimum 3.5 hours at 35k rpm, 4 ^{o}C, in an SW40 rotor. The vesicles were removed as a discrete band and washed twice by diluting in DOC free dialysis buffer and centrifuging (40 rotor; 35k rpm ;30 min.; 4^{o}C). Residual buffer was carefully removed using tissue paper and the pellet resuspended in a minimum volume of deuterium - depleted buffer (20 mM sodium borate; 50 mM NaCl; pH 8.0) and transferred to NMR tubes. The size of the vesicles was determined by negative stain electron microscopy (see below).

LIPID SYNTHESIS: DMPC-d_9 was prepared by methylation of dimyristoylphosphatidylethanolamine (DMPE) (Fluka) with C^2H_3I (Aldrich Gold Label) and purified by passage down a 70-200 mesh silica column (Merck), eluting with chloroform; methanol; ammonium hydroxide solvent (Eibl, 1980, Sixl and Watts, 1982). The purity of synthetic lipids was assessed by thin layer chromatography (TLC).

OCTYL-β-D-GLUCOSIDE ASSAY: The concentration of octyl glucoside in the fractions eluted from the delipidation column was determined by a new application of the thiobarbituric acid assay of Warren (1959). This method was able reliably to detect the elution of detergent from the column without the need to use a radiolabelled tracer. The chromophore

was extracted into acidified (5% HCl) butan-1-ol (Aminoff, 1961). Octyl glucoside was obtained from Sigma.

NMR SPECTROSCOPY: NMR spectra were obtained using a Bruker WH-300 spectrometer operating at 46.1 MHz for deuterium and 121.8 MHz for phosphorus. The spectrometer was equipped with a nitrogen gas flow variable temperature unit. Temperature could be measured to an accuracy of $\pm 0.5\ ^{o}C$. The low radio frequency power of the machine necessitated the use of large 90^{o} pulse widths (29 μs for deuterium and 20 sec for phosphorus), resulting in some degree of spectral distortion. These distortions limit the use of the WH-300 for measuring large quadrupole splittings, but Gally et al.(1981) report that the aberations, resulting from the finite pulse lengths do not affect the value of the narrow splittings of the DMPC-d^{9} N-methyl deuterons ($\leq$1.5-1.3 MHz).

T_1 measurements were made using the inversion-recovery method, with a $(180^{o}-\tau-90^{o})$ pulse sequence. Eight spectra using different delays (τ) between the inversion and observe pulses, were obtained to generate data from which T_1 values (accurate to 10-15%) were determined from semilogorithmic plots (figure 7).

ELECTRON MICROSCOPY: Small aliquots of lipoprotein vesicles from the NMR experiments, typically 0.2 ml, were taken and diluted ten fold with 2.0% aqueous ammonium acetate and then dialysed against 5 litres of 2.0% ammonium acetate. Grids, 400 mesh size, were cleaned and coated with Formvar and carbon. A specimen was placed on each grid with a drawn out pasteur pipette and negatively stained using the drop method (Hayat, 1972). Micrographs were taken using the Phillips 400 electron microscope.

RESULTS AND DISCUSSION

BACTERIAL GROWTH: Both the cultures received exhibited long lags in their growth but once replication had begun the bacteria grew to late log phase in three or four days. Futher incubation under anaerobic conditions, with strong illumination, for seven days was found to produce purple membranes. Their colouration could only be seen when cells were harvested. Bacteria were allowed to grow until the culture had reached an optical density (400 nm) of 1.5.

ELECTROPHORESIS: The gels (Figure 2) clearly show the increased purity of the bR during the isolation, and the final preparation shows with only one protein band as visualised by Coomassie staining. Purified bR runs at about 20 000 daltons, which is lower than the reported molecular weight of 26k dal. However, this anomolous behaviour is quite common for membrane proteins run on polyacrylamide gels, and is probably due to a difference in behavior between the portions of the protein normally within the lipid bilayer and soluble proteins when interacting with detergent. The increased mobility observed here may be due to the bR binding more SDS per unit length than an aqueous soluble globular protein. This would result in a higher charge per unit length which then causes the protein to move faster towards the cathode. Such behaviour has been reported for bR (Bridgen and Walker, 1976).

PROTEIN PURIFICATION: The solubilisation of bR was light sensitive, and resulted in loss of colouration due to solubilisation of the

chromophore. This is due to the presence of the octyl glucoside (Dencher and Heyn, 1978). When the octyl glucoside was replaced with DOC on gel filtration, the protein became light stable.

DELIPIDATION AND OCTYL GLUCOSIDE ASSAY: The detergent octyl glucoside decreases the stability of bR with respect to triton X-100 but was preferable in this case due to the improved resolution of detergent/lipid mixed micelles from solubilised protein in the delipidation step (figure 3). In addition, octyl glucoside has a higher CMC (23 mM) which makes the reconstitution of lipid and protein more efficient. Contamination with Triton X-100 (in protocols using this detergent for solubilisation (Huang et al., 1980)) which has a lower CMC (0.3 mM), leads to a greatly increased dialysis time. Moreover, Triton X-100 is a poor detergent in reconstitution studies in general (Reynolds, 1981). The elution profile for the column (Figure 3) compares favourably with that for Triton X-100 (Huang et al., 1980), with a much improved resolution due to the lower micellar size of the octyl glucoside. Indeed, the tail of the bR peak is not distinct from the detergent/lipid peak when using Triton X-100. This latter feature is most important with regard to choice of detergent for this system.

The measurement of octyl glucoside in this work demonstrates a new application of the sialic acid assay (Warren, 1959) using the Aminoff modification (1961). The method has been shown to be reliable and reproducible and capable of accurately measuring the quantity of the detergent in the nM range. The methodology used in this study varied from those of previous workers. It was found that incubation at room temperature in the light causes the chromophore produced at the end of the reaction to exhibit a transient increase in the optical density (OD)

at 450 nm, probably due to polymerization. Figure 9 shows the variation of the chromophore OD with time. At higher concentrations of octyl glucoside, the chromophore precipitates and adheres to the side of the cuvette when left to stand for comparatively long periods. Also the polymerization reaction results in a change in the colour of the chromophore, which will be reflected in the absorption spectrum. These factors account for the eventual tailing off of the absorbance at higher concentrations of the detergent and with longer exposure to the light. Samples kept in the dark changed at a much slower rate. From inspection of figure 9, it is clear that an incubation of 50 minutes in the light prior to obtaining OD values provides the optimum linearity of OD with respect to detergent concentration and is sensitive enough for the requirements of this work. Extrapolation back to the time of addition of the butanol to the final reaction vessels did not provide an improved calibration (data not shown).

This assay allows the quantitation of octyl glucoside by a chemical method. The procedure is easy and quick and requires the minimum of equipment. There is no need for expensive radiolabeled detergent, which is difficult to synthesise in the laboratory. By comparison, tritiated triton X-100 is cheap and thus can be used routinely.

Because the assay measures total free sugar, the applicability of the method is clearly limited and could not be used if the protein were, for example, heavily glycosylated because the initial periodate cleavage would be at the terminal saccharide of a polysaccharide moiety. However for the above system, and possibly for study of other prokaryotic membrane proteins (which are not glycosylated), this procedure provides an alternative to radiolabelling in the assay of detergents such as octyl glucoside that contain sugar groups.

Analysis of protein and lipid contents of the bR, before and after gel filtration, confirms that 95% of the endogenous lipid is removed by this treatment.

The peak of the protein elution profile, to the left of figure 3, shows a possibility of there being two components. That both the OD (depending on the bR retinal) and the total protein assay show this feature, implies that this is only due to bR. However, the possibility that this is due to different allotypic forms of the protein is unlikely in view of the evidence from gels, which show only one band and would be expected to show up such variants. It is more likely is that the heterogeneity of the mixed micelle population accounts for this observation.

RECONSTITUTION: Due to the NMR time scale, small vesicles (dia. $\lesssim$ 50 nm) tumble at a rate ($\tau_c \approx 10^{-6}$ sec) that will collapse a D-NMR spectrum produced from a bilayer and obscure the useful information. To overcome this it is necessary to slow down the vesicle tumbling rate by ensuring that vesicles are of a large diameter ($>$ 50 nm); such vesicles tumble slowly.

The method worked out to form large diameter vesicles involved dialysis of the DOC solubilised DMPC-d_9/bR mixture against a buffer containing detergent to slow down the rate of decrease in detergent concentration as the CMC was approached. (Fast dialysis usually results in small vesicles.) The concentration of DOC in the buffer outside the bag was 1.0 mM. This is just below the CMC whose value varies with ionic strength, from 2.0 mM at I = 0.0 to 0.91 mM at I = 0.15 (Helenius et al., 1979). At this concentration, vesicles could be seen to be

forming after dialysis against detergent for 48 hours (the protein mixture became turbid). Once this had occurred, dialysis against DOC-free buffer removed any remaining detergent, producing stable vesicles with lipid in the bilayer phase (see below).

NMR SPECTROSCOPY: Phosphorus-NMR powder patterns (Figure 5) confirm that the lipoprotein vesicles are composed of authentic phospholipid bilayers thus providing the bR with an environment comparable to that found in the purple membrane in vivo (Cullis and de Kruijff, 1979). But the lipid headgroup-protein interactions observed in this work may be different from those encountered in the native system because the lipid species involved are different. The lipids of the purple membrane are thought to contain dihydrophytol chains in ether linkage to the glycerol backbone, rather than esterified fatty acid. The dihydrophytol is a branched chain, the methyl groups serving the same function as unsaturated bonds in fatty acids (Kates et al., 1965, Henderson, 1977). However bR reconstituted in PC vesicles is biologically active (Huang et al., 1980) and reconstitution methods provide the only method for relating function to lipid environment, although D-NMR may not actually give any data about lipid-protein interactions in the native purple membrane. The geometry of the reconstituted system may be similar to that in the native membrane, so that those groups on the protein that interact with lipid headgroups in the purple membrane are also those participating in head-group interactions in the reconstitution.

Figure 8 shows the variation of ΔV_Q with temperature. As ΔV_Q is directly related to the order parameter, S_{CD}, assuming the axis of the CD bond is unchanged, the values of S_{CD} are not calculated, since this

axis of averaging is unknown for head-groups. However, current neutron experiments by this group may provide the answer. ΔV_Q, the measured experimental parameter, is clearly sensitive to lipid protein interactions (figure 8) and has been used to quantify these interactions (Sixl et al., 1984).

Both the orientation of the CD bond to the bilayer normal and the amplitude of motion contribute to the quadrupole splitting. For the CD bond, the theoretical maximum quadrupole splitting is for a statically orientated sample, where ΔV_Q = 250 kHz. A decrease from this value is due to averaging out of the anisotropy. Splittings for the CD bond in the terminal methyls of a PC headgroup are quite small, ie ca. 1400 Hz in pure lipid bilayers, indicative of a large amplitude of motion with respect to the bilayer normal. Because the spectra give one discrete value for ΔV_Q, it is proposed that all the lipid has the same conformational properties, possibly due to a cooperation between the headgroups forming a loose lattice-like array.

Introduction of a protein (e.g. bR) collapses ΔV_Q to a lower value (figure 8), indicating a futher decrease in residual anisotropy. In other words, some of the constraints on motion in the cooperative bulk phase are lost on interaction with the protein. The spectral features still consist of a single ΔV_Q, indicating fast exchange between the two populations of lipid, namely bulk lipid and protein associated lipid. In the fast exchange limit the observed quadropole splitting is a weighted average of quadrupole splittings of all components contributing to the spectra. Decrease in anisotropy of the amplitude of motion or a change in the orientation is consistent with the idea that the rough surface topology of the protein deforms the headgroup orientation from that found in the bulk lipid phase.

The T_1 determination of the CD_3 groups in the DMPC-d_9, (figure 6), provides a value of 45.0 msecs. This value is sufficiently close to that for pure DMPC-d_9 vesicles (ca. 50 msecs) effectively to rule out the rate of motion of the phospholipid head-group as a contributing factor to the observed variation in ΔV_Q. (It is possible that a separate unmeasured change is occurring in the slow motions of the lipid, which are undetectable by this method, or that any such changes are countered by a futher alteration to the system caused by the presence of the bR.)

Results from studies with several proteins, e.g:- band 3, rhodopsin and myelin basic protein, are consistent with the interpretation that the quadrupole splitting of the protein-associated lipid may be near zero, indicating a considerable loss of residual anisotropy in the CD bond. Addition of more protein will further decrease ΔV_Q, as the population of lipid associated with the protein will increase with respect to the bulk lipid phase because there is more protein surface area in contact with lipid. If protein aggregation were to occur at higher protein/lipid ratios, the ΔV_Q would be greater than that expected for protein at that concentration due to a decrease in the surface area available for interaction with lipid.

Futher work with different lipid species and with heterogeneous lipid mixtures should indicate possible preferences in lipid-protein associations due to a greater decrease in anisotropy with the prefered lipid with respect to other lipids in the system.

ELECTRON MICROSCOPY: Several vesicle preparations were examined, with different lipid to protein ratios. All showed mainly homogeneous populations, with a mean vesicle size of ca. 150 nm diameter (figure 10), by comparison with Influenza A particles which have a diameter of

120 nm prepared so as to prevent collapse in the EM. This confirms that detergent dialysis does indeed allow the production of lipoprotein vesicles with dimensions suitable for D-NMR.

120 nm prepared so as to prevent collapse in the EM. This confirms that detergent dialysis does indeed allow the production of lipoprotein vesicles with dimensions suitable for D-NMR.

Bacteriorhodopsin has been reconstituted into large bilayer vesicles suitable for NMR studies. Their vesicular diameter is $>$ 50nm, and they contain a single specifically-deuterated lipid species. This has been achieved by a combination of gel filtration delipidation, detergent dialysis and synthesis of lipid of a high purity.

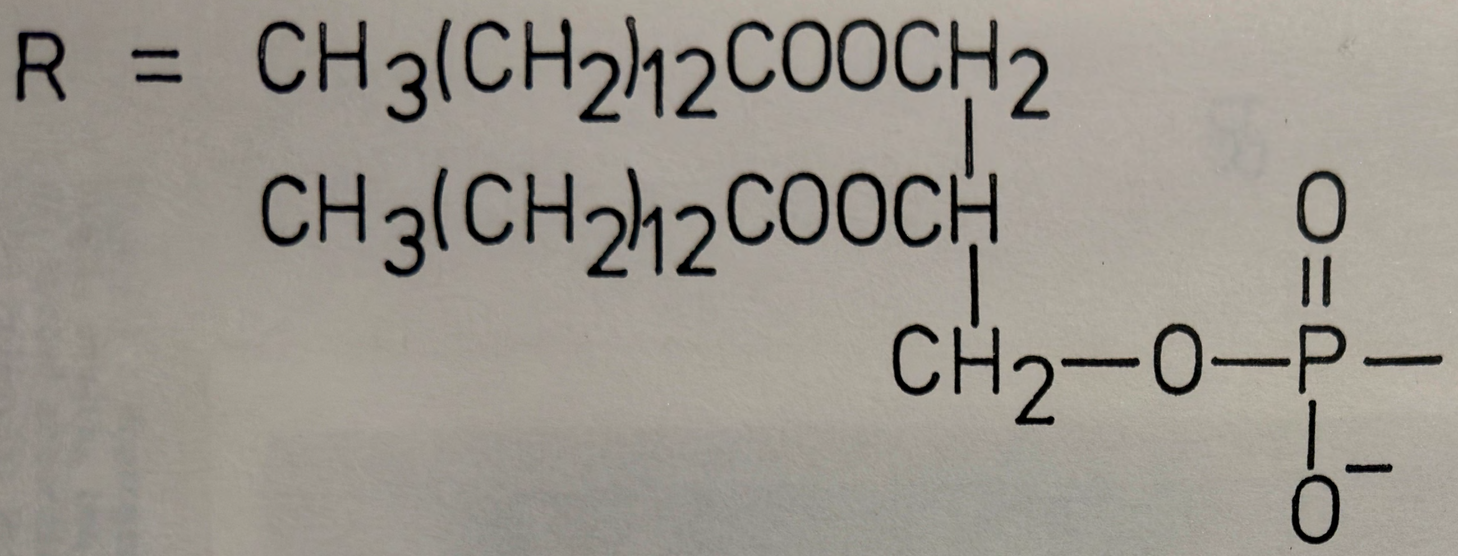
Phosphorus NMR confirms that the vesicles contain predominantly lipid in the bilayer configuration and T_1 relaxation measurements indicate that the rate of motion of the PC headgroups is not significantly affected by the presence of reconstituted protein.

D-NMR indicates that there is a decrease in ΔV_Q at lower lipid to protein ratios due to a decrease in the anisotropy of the N-methyl CD bond. It is proposed that this effect is due to the rough topology of the protein in the reconstituted vesicles causing the head groups to be disturbed from the anisotropic arrangement found in the bulk phase. Because the observed value of ΔV_Q is an average of protein-associated lipid and bulk lipid quadrupole splittings, then as more protein is added the ΔV_Q will decrease still futher due to increased contribution from the isotropic component associated with the protein. It is thought that in PC bilayers without protein, a cooperative lattice-like arrangement of headgroups may exist leading to the anisotropy in the system.

Attempts to measure light dependent proton pumping in the intact vesicles were undertaken, but due to paucity of the material and inexperience with the system, light dependent pH changes could not detected.

It is hoped that this work may be extended in the near future to analyse interactions between bR and different lipid species (e.g. dimyristoylphosphatidylglycerol (DMPG) and DMPC/DMPG mixtures) to assess the effect of factors like charge and headgroup size on the interactions observed. DMPC is zwitterionic and it may be that electrostatic interactions are important between lipids and bR. Preferential association of a lipid with the bR protein will be detectable in the D-NMR spectra, and this may in turn be important to function. Complementary spin-label ESR studies may also be of importance in deriving the time-scale of lipid-protein interactions in membranes.

Futher reconstitutions would also allow a calculation of the number of lipids in the annulus i.e:- those that are retarded in lateral diffusion with respect to the bulk lipid phase (Sixl and Watts, 1982). It is expected that some 25-50 molecules occupy the annulus simply on geometric grounds (Henderson and Unwin, 1975).



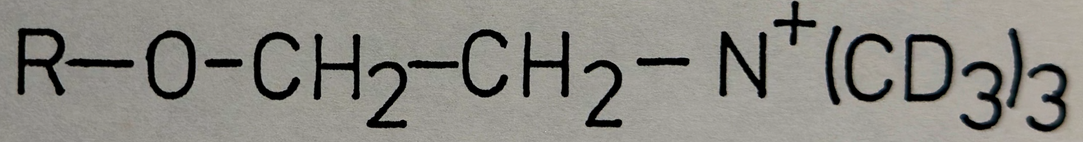


Figure 1: Structure of DMPC-d_9, showing the positions of the deuterons in the three N-methyl groups.

Figure 2 : Photograph of 15% SDS-PAGE visualised by coumassie blue stain, showing the increased purification of bR as described. The gel was loaded according to the procedure in the text. Tracks show : (A) VII-L Molecular Weight Markers (Sigma), (B) Crude purple membranes from fractionation of H.halobium, (C) Partially purified bR, following sucrose density gradient centrifugation, (D) Solubilized bR monomers in 40mM octyl glucoside, and (E) Delipidated bR after passage down a Sephadex G-75 column (see text). The gel was photographed on Kodak Safety Film 5060.

Figure 3 : Elution profile of delipidation column (Sephadex G-75) using the method of Huang et al..Key :+-+ : Optical density at 560nm, △-△ : concentration of protein (mg/ml), ×-× : concentration of octyl glucoside (mM), □-□ : cpm for [^{3}H-2] glycerol (x10^{3}), ⊙-⊙ : concentration of phospholipid (mM).

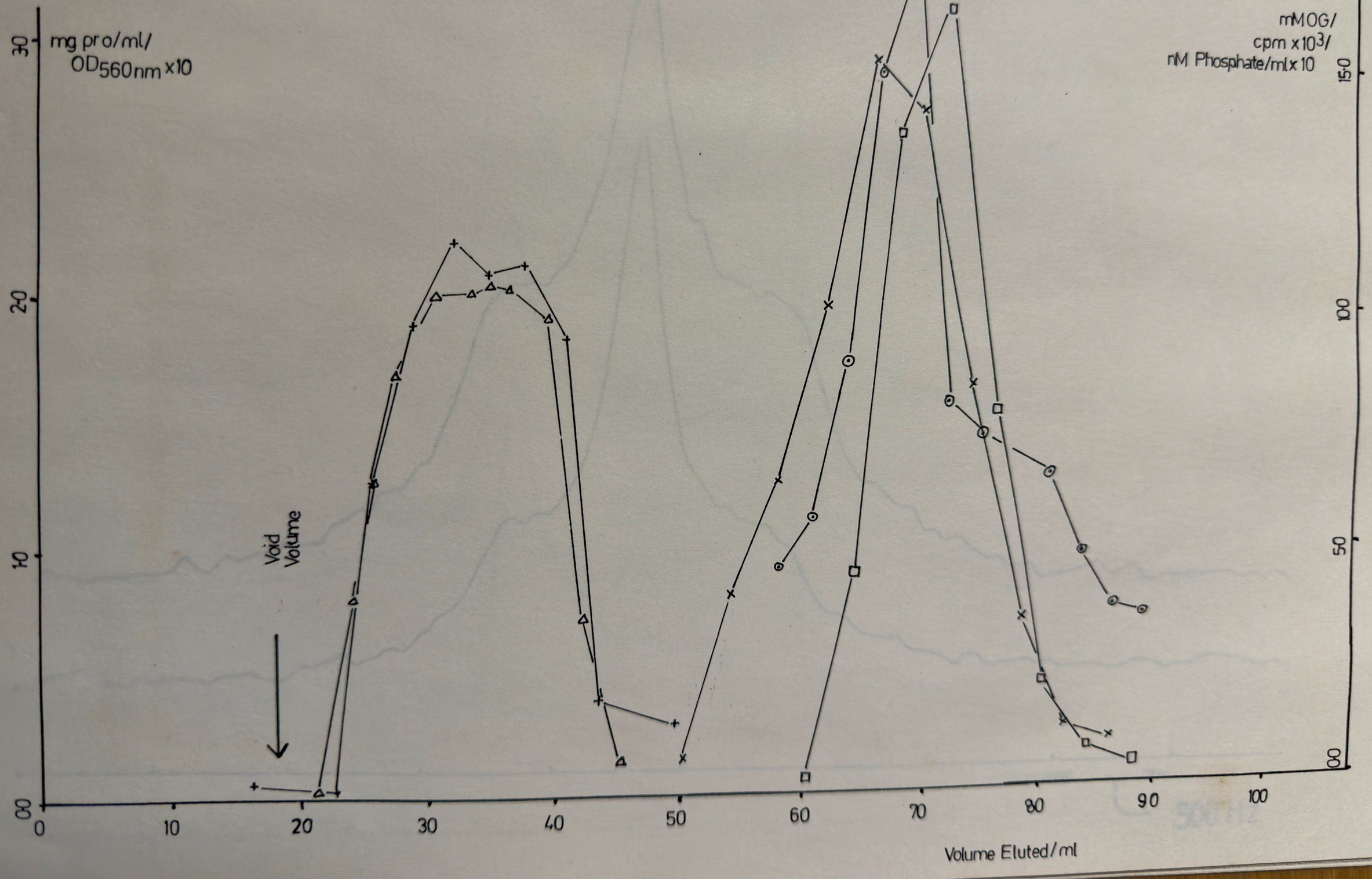


Figure 4a : Deuterium-NMR spectra (46.1MHz) of DMPC-d_9/bR vesicles for lipid:protein weight ratio of 30:1; temperature 30°C (a); 40°C (b); buffer 20mM sodium borate, 50mM NaCl, pH 8.0. 29 s (90°) pulses were applied. The number of scans was 2 000.

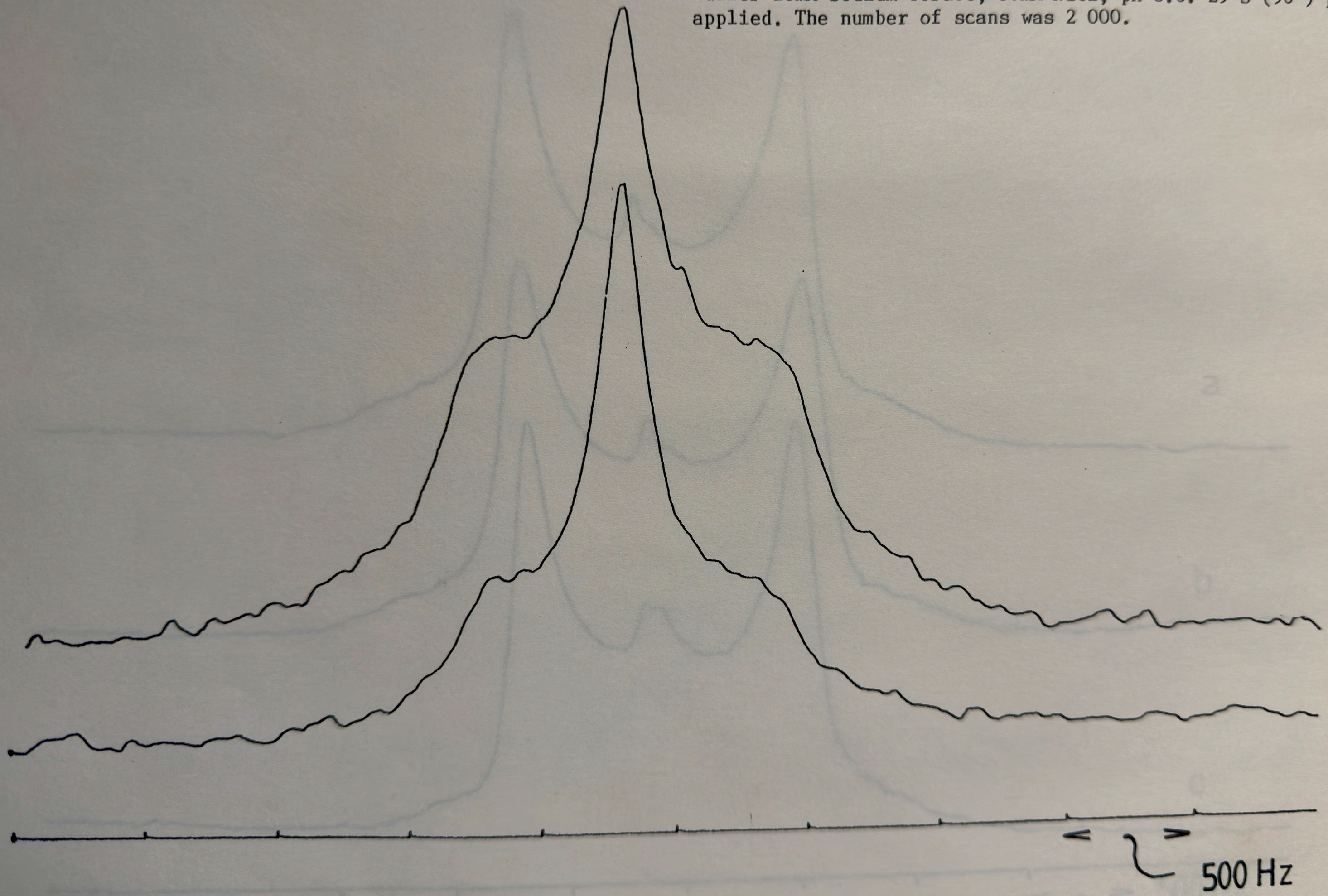


Figure 4b : Deuterium-nmr spectra (46.1MHz) of DMPC-d_9/bR vesicles for lipid protein weight ratio of 50:1. Temperatures (a) 27°C, (b) 30°C, (c) 36°C; buffer 20mM sodium borate, 50mM NaCl, pH 8.0. 29μs (90°) pulses were applied. The number of scans was 2 000.

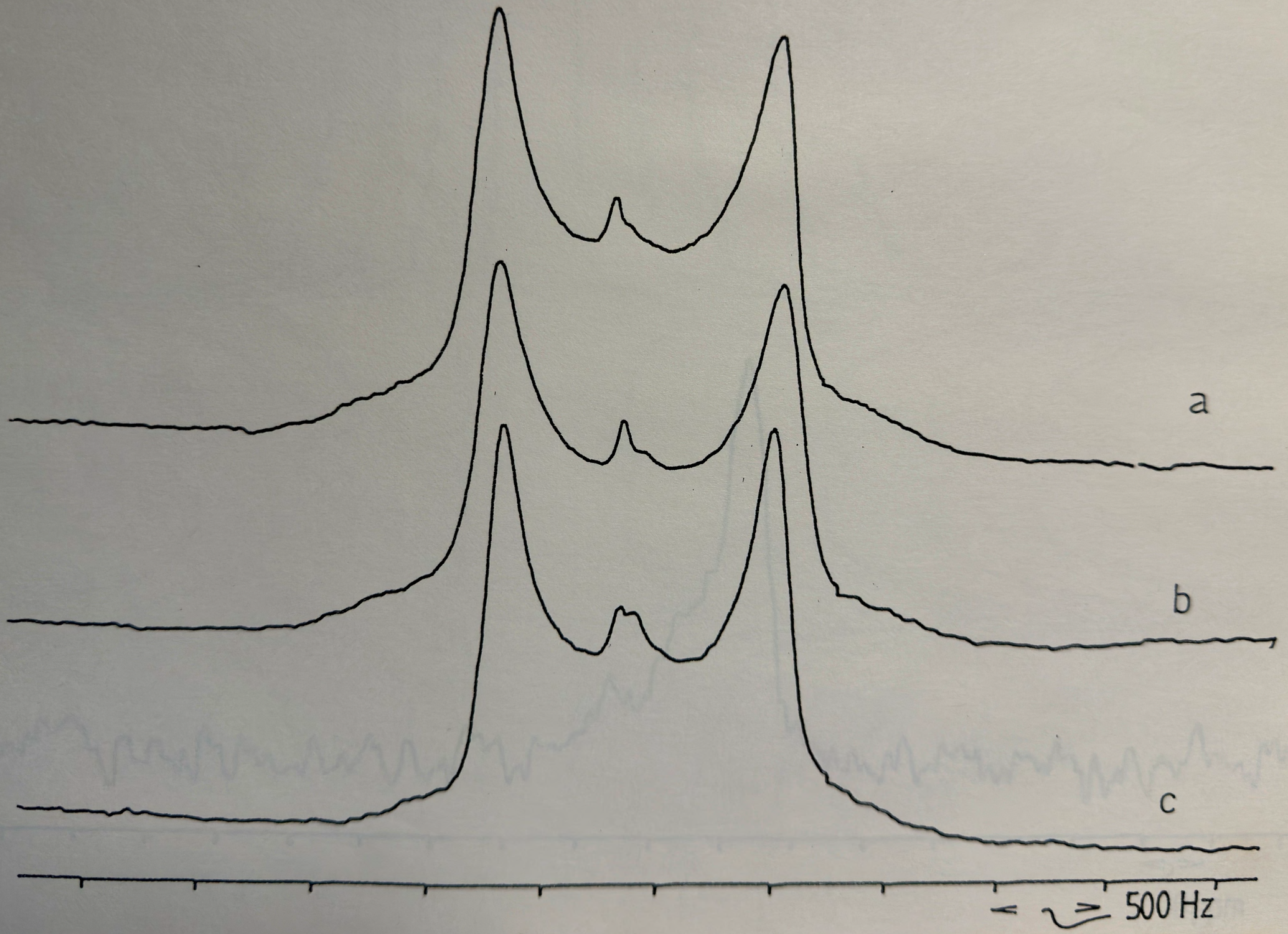


Figure 5 : Phosphorus-NMR spectrum (121.8MHz) of bR-DMPC-d_9 vesicles at 50:1 lipid:protein weight ratio. Temperature 32°C; buffer 20mM sodium borate, 50mM NaCl, pH 8.0. 20μs (90°) pulses were applied. The number of scans was 2 500.

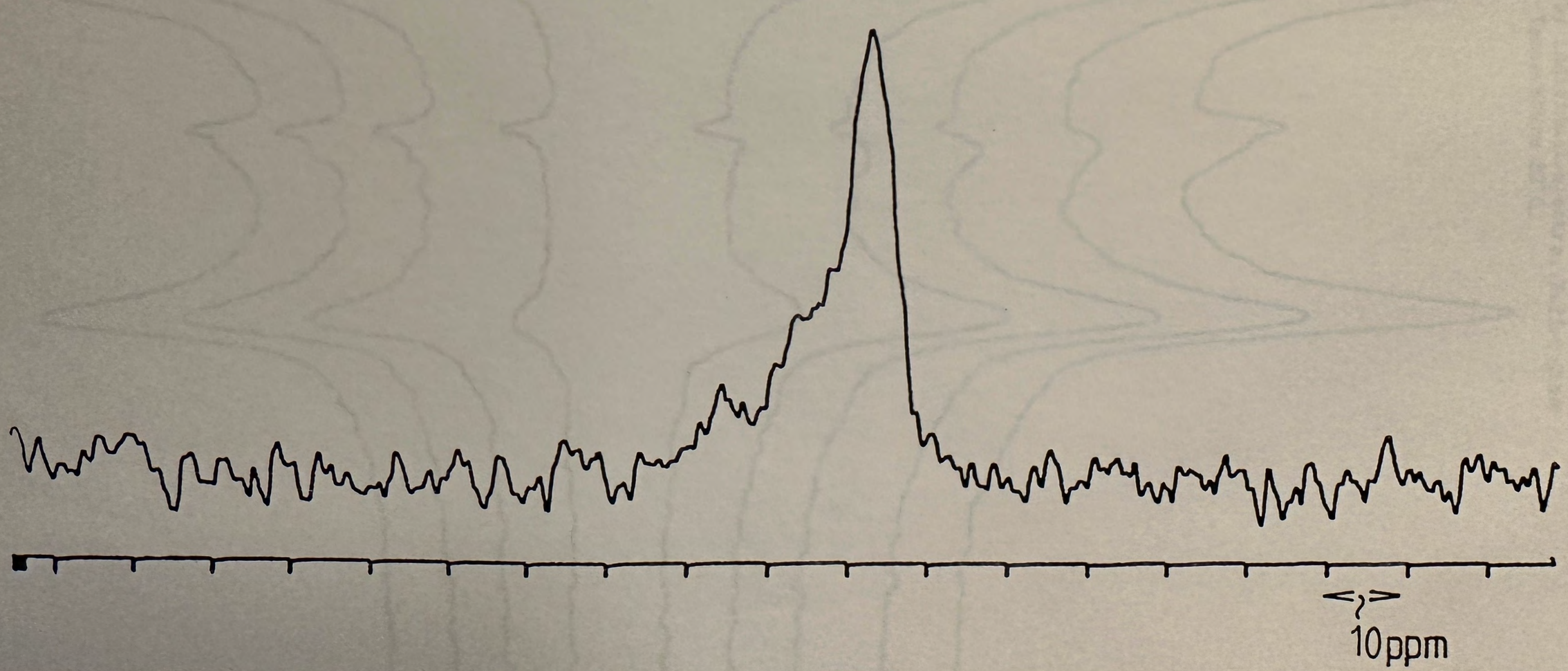


Figure 6: T_1 determination. Vesicles at 50:1 lipid:protein weight ratio were pulsed to selectively invert the deuteron peaks. The pulse durations (180°-τ-90°) are shown by individual spectra. Buffer 20mM $Na_2B_4O_7$-$10H_2O$, 50mM NaCl, pH 8.0. Temperature $32^\circ C$.

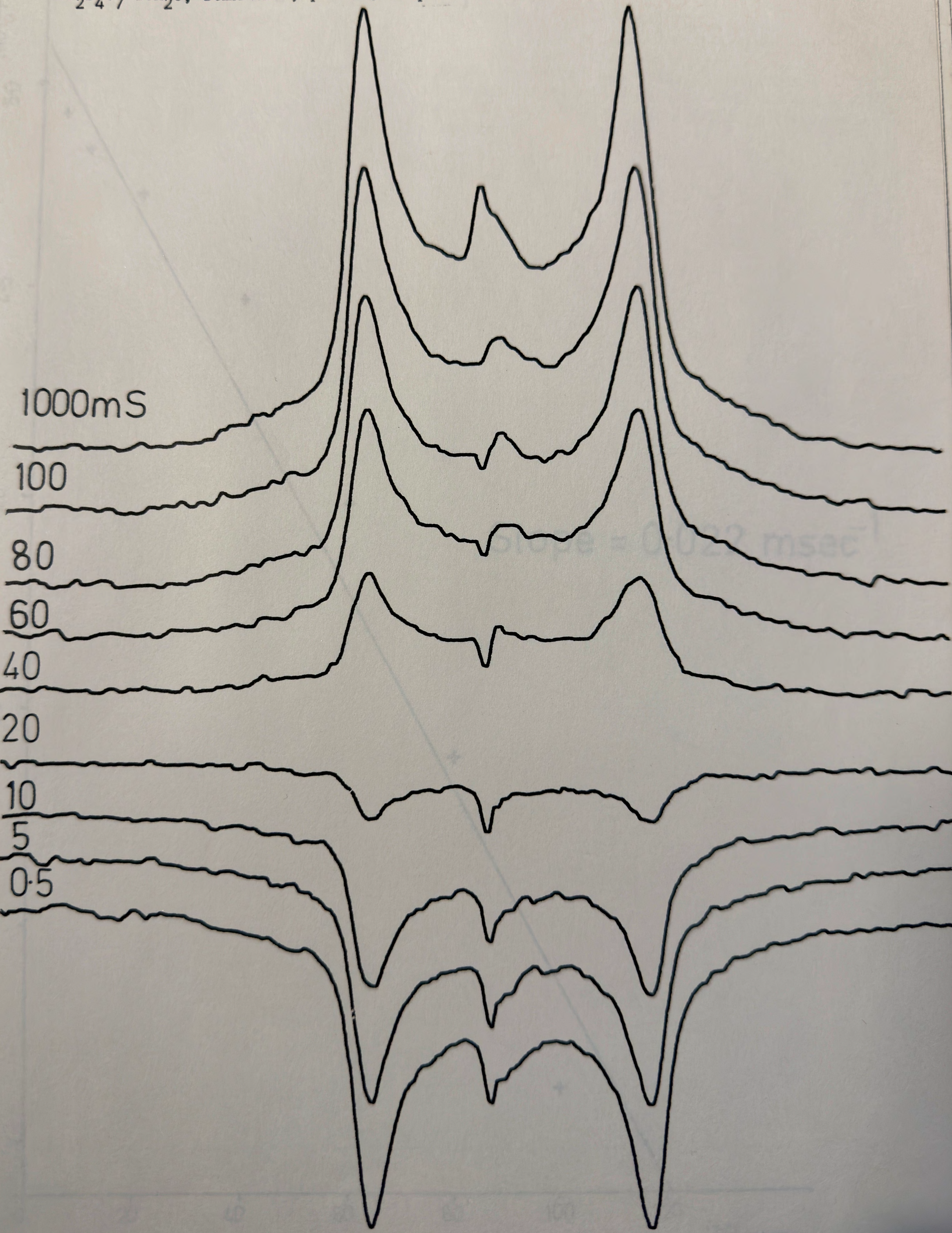
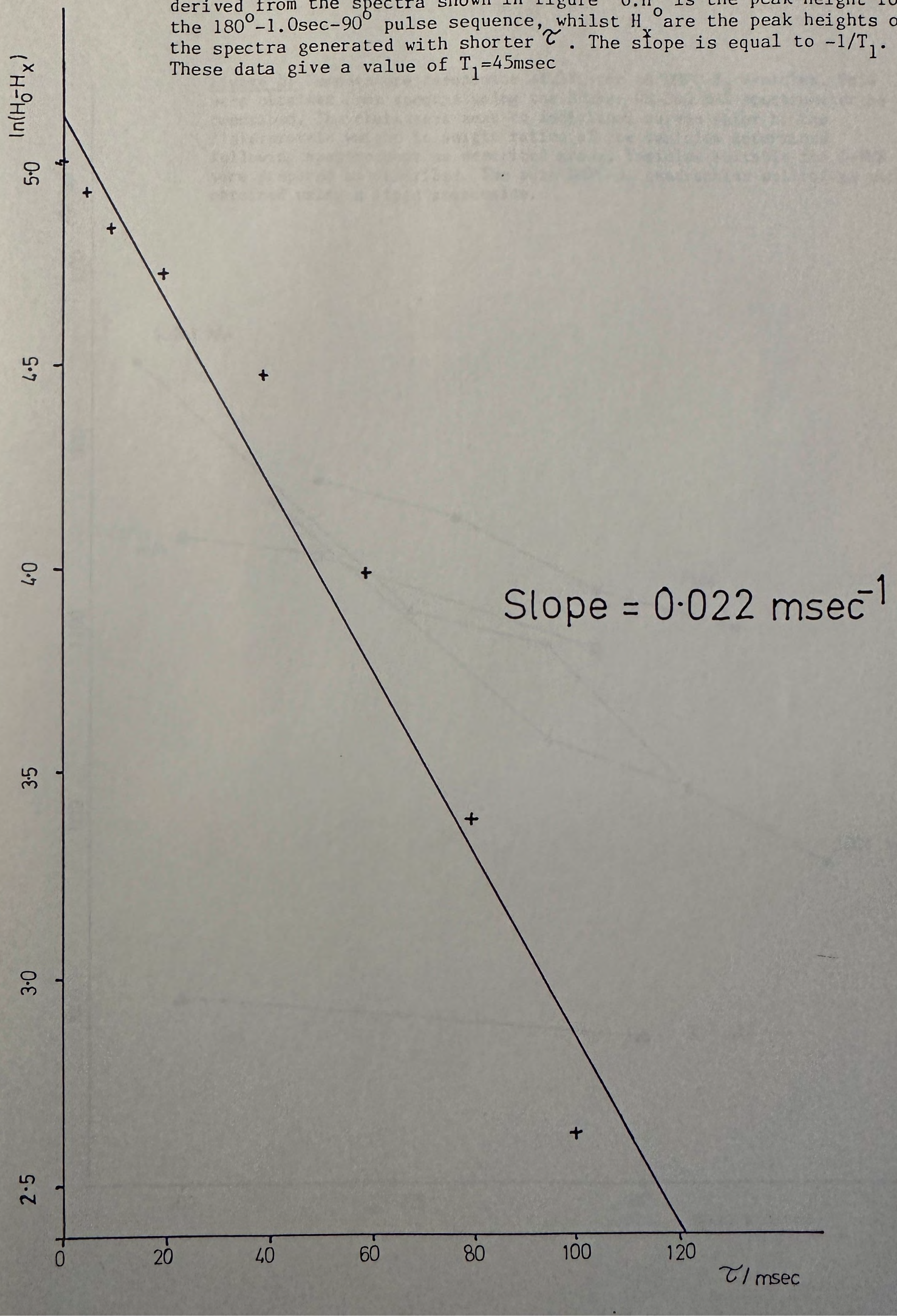


Figure 7: Plot of $\ln(H_o - H_x)$ against τ (the delay between inversion and observe pulses) for determination of CD T_1. The data in the plot are derived from the spectra shown in figure 6. H_o is the peak height for the 180^o-1.0sec-90^o pulse sequence, whilst H_x are the peak heights of the spectra generated with shorter τ. The slope is equal to $-1/T_1$. These data give a value of T_1=45msec



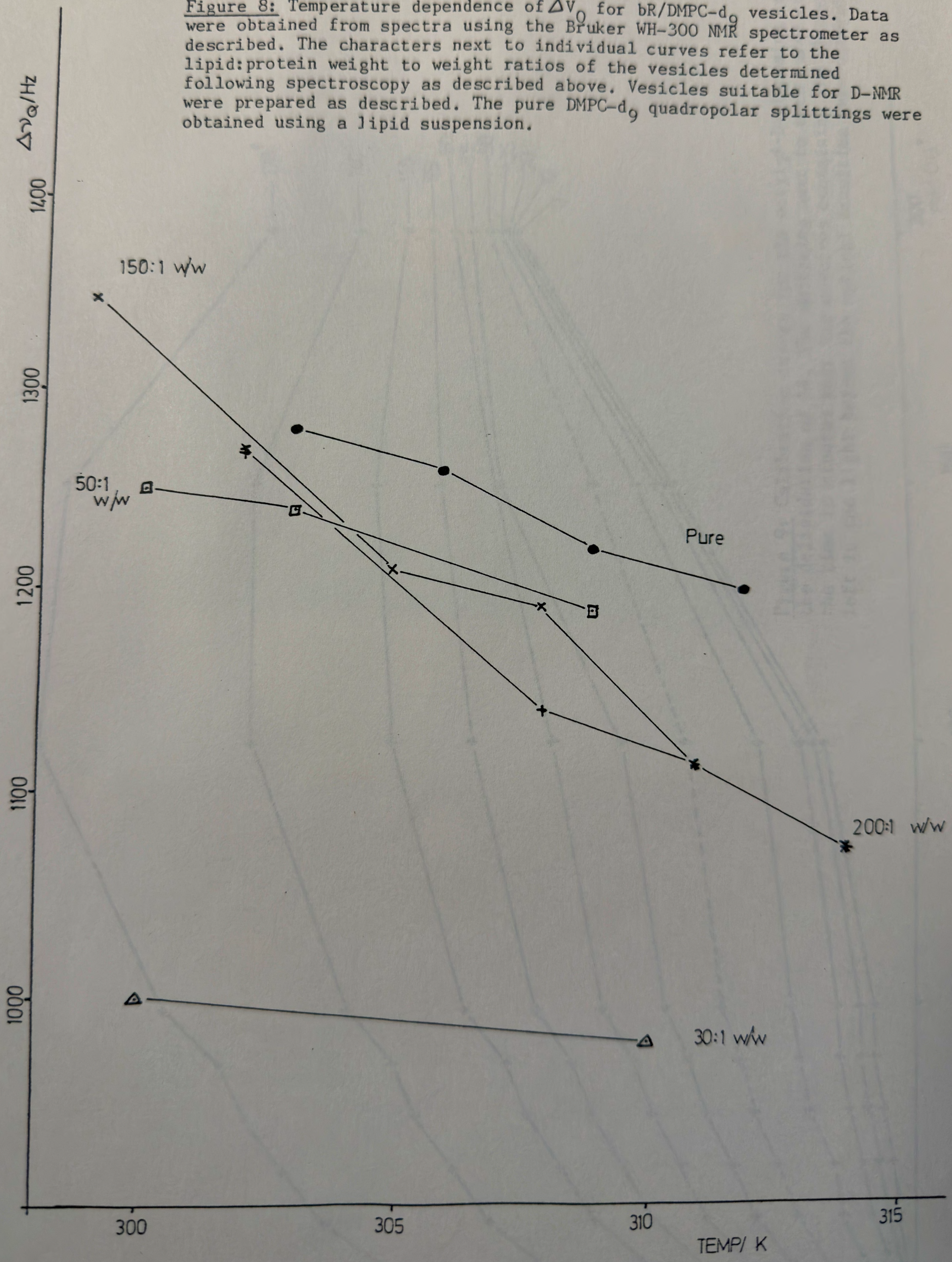


Figure 8: Temperature dependence of $\Delta\nu_Q$ for bR/DMPC-d_9 vesicles. Data were obtained from spectra using the Bruker WH-300 NMR spectrometer as described. The characters next to individual curves refer to the lipid:protein weight to weight ratios of the vesicles determined following spectroscopy as described above. Vesicles suitable for D-NMR were prepared as described. The pure DMPC-d_9 quadropolar splittings were obtained using a lipid suspension.

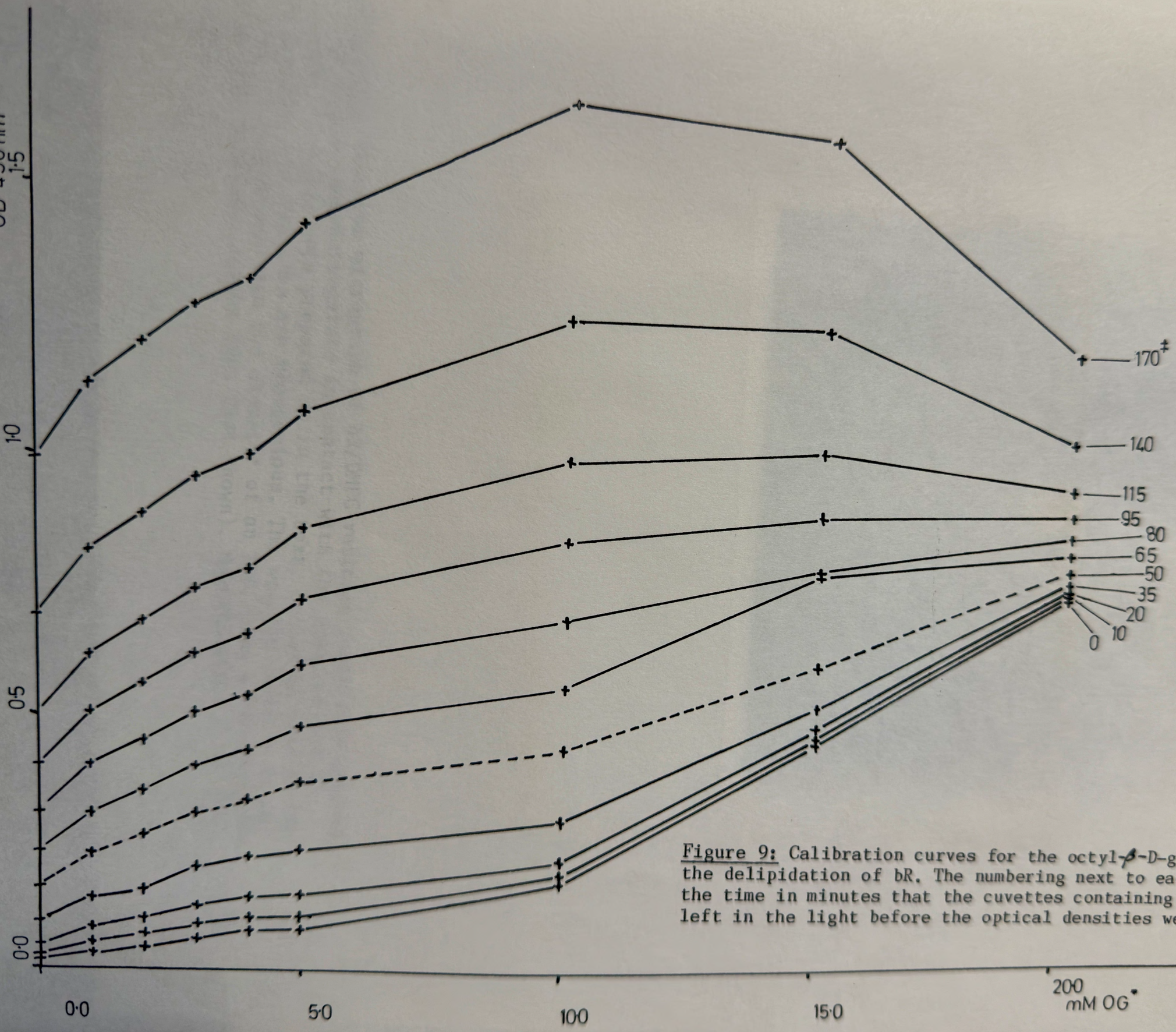


Figure 9: Calibration curves for the octyl-β-D-glucoside assay used in the delipidation of bR. The numbering next to each curve corresponds to the time in minutes that the cuvettes containing the chromophore were left in the light before the optical densities were determined.

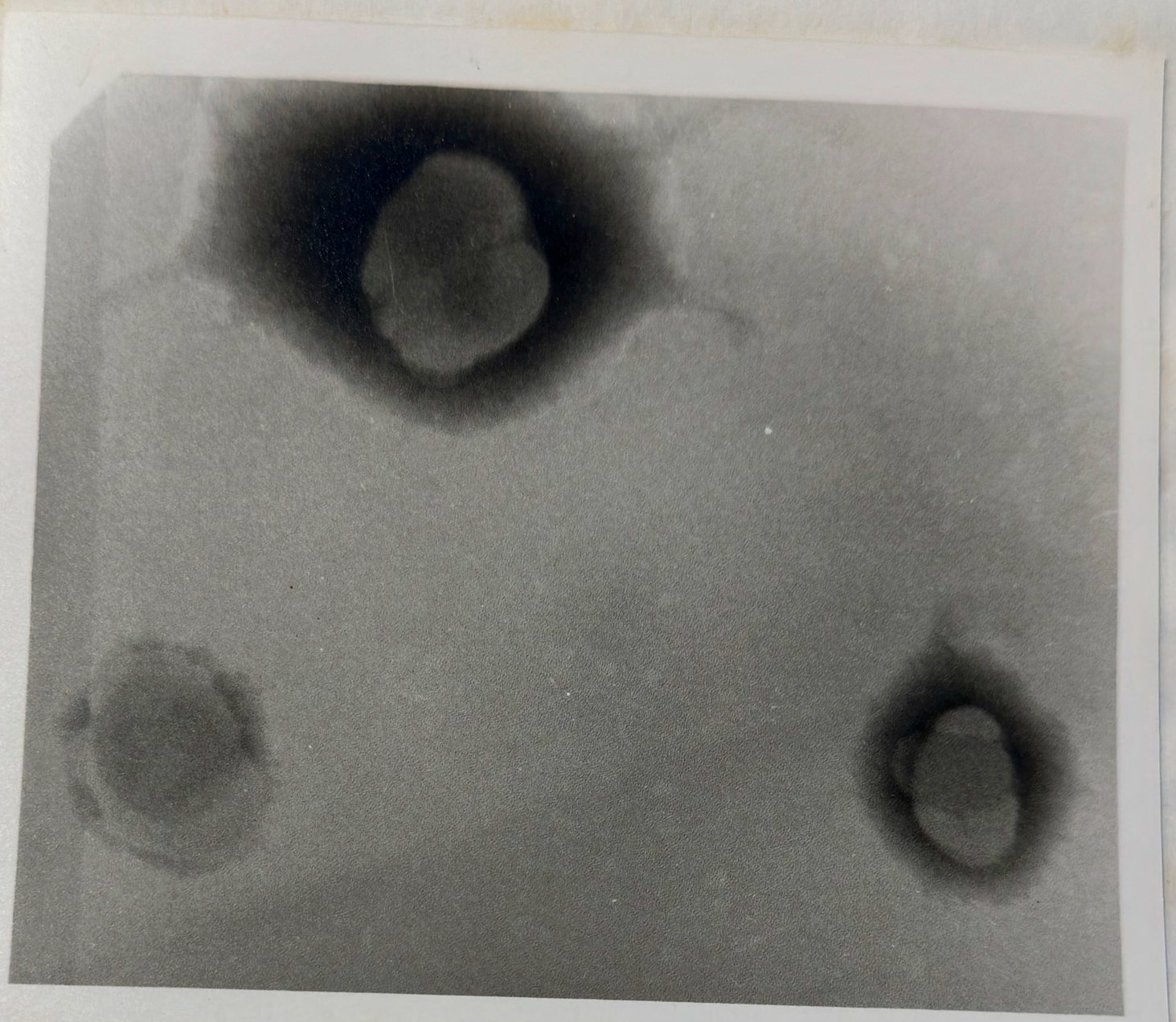


Figure 10: Electron micrograph of bR/DMPC vesicles negatively stained using sodium phosphotungstate in contact with the specimen for 45 seconds. Vesicles were prepared as in the text. As can be seen in the micrograph, the vesicles are homogenious. The vesicle diameter is ca. 150nm, by comparison with the diameter of an Influenza A Virion at the same magnification, radius 58nm (not shown). Magnification X 100 000 times.

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