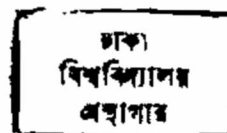


NEUTRON SCATTERING STUDIES
OF CONDENSED MATTER WITH
APPLICATION TO BIOPHYSICS



A Thesis Submitted to the University of Dhaka
for the Fulfilment of the Degree of
Doctor of Philosophy

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BY
FARID UDDIN AHMED

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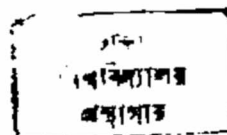
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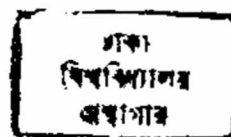
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Abstract

Some biological specimens have been studied using different techniques of neutron scattering. Small angle neutron scattering (SANS) measurements have been carried out on aqueous solutions of a neurotoxin, a cytotoxin and a cytotoxin analog isolated from different cobra venoms. The radii of gyration of neurotoxin and cytotoxin analog were found to be ≈ 12 Å. The radius of gyration of cytotoxin was found to be ≈ 11.5 Å. The shapes of the toxin molecules have been considered arbitrary and the actual shapes have been found from least square fits of these arbitrary shapes to the data. SANS data and analytical gel filtration experiments both indicate that the proteins exist as monomers in solution, at least up to 8 g/l. The gross size and shape of the neurotoxin in solution is compared with its structure as determined by X-ray diffraction from crystals.

Neutron diffraction measurements have been utilized to study the effects of Δ^9 -tetrahydrocannabinol (Δ^9 -THC), Δ^8 -tetrahydrocannabinol (Δ^8 -THC) and cholesterol incorporated in phospholipid membranes of dipalmitoylphosphatidylcholine (DPPC). These cannabinoids induced changes in interlamellar spacing similar to those produced by cholesterol. The location and orientation of Δ^8 -THC and Δ^9 -THC were deduced to be similar to those found for cholesterol in DPPC by comparison with measurements using cholesterol. The observed diffraction intensities suggest that the interaction of all three additives

with membranes is similar.

Inelastic neutron scattering has been used to study the low frequency spectra of alpha-chymotrypsin (alpha-C), chymotrypsinogen A and hemoglobin at frequencies below 3.5 THz ($\approx 117 \text{ cm}^{-1}$). Neutron scattering measurements for alpha-C show a peak in the low frequency spectrum. For alpha-C gaussian fits yield values of 0.93 ± 0.05 , 0.86 ± 0.04 , 0.81 ± 0.05 and 0.87 ± 0.06 THz for the peak position at wave vector transfer Q of 1.0, 1.4, 1.85 and $3.00 \text{ \AA}^{-1}$ respectively at 77 K. Measurements at room temperature (298 K) for alpha-C show a peak at about 0.6 THz for Q values of 1.0, 1.4, 1.85 and $4.3 \text{ \AA}^{-1}$. Measurements at 5 K and 80 K show a peak at about 0.8 THz for Q values of 2.7 and $4.3 \text{ \AA}^{-1}$. For chymotrypsinogen A a room temperature measurements were carried out for Q values of 1.0, 1.4 and $1.85 \text{ \AA}^{-1}$ and no well defined peak was observed in these measurements. In the measurements for hemoglobin at a temperature 298 K a very weak peak at about 0.8 THz was observed for Q values of 1.85, 2.7, 3.6 and $4.3 \text{ \AA}^{-1}$. At a low temperature 5 K a weak peak at about 1 THz was observed for hemoglobin for Q values of $4.3 \text{ \AA}^{-1}$. The data are interpreted in terms of the allowed oscillations of a large globular protein treated as an elastic sphere. Assuming a Raman active oscillation at 0.9 THz it is shown that a peak in the neutron scattering response at 0.6 THz may arise from rotational shear mode of the chymotrypsin molecule.

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CHAPTER 1

INTRODUCTION

The application of various radiations in studying condensed matter involves an extensive description of atomic position and motions. The relevant data may be obtained from analysis of radiation scattering experiments. The different types of radiations have different properties, but a common function is measured in all such experiments. In these experiments a monochromatic beam irradiates a sample and radiation scattered through an angle θ is studied. This scattered radiation represents the properties of the sample and sometimes the scattering process involves the transfer of momentum between the radiation and sample. The scattered intensity is proportional to the probability $S(Q, \omega)$ that the sample takes an energy $\hbar\omega$ from the radiation. The usefulness of different radiations depends on their wavelengths and energy. The wavelength region of greatest interest is around 3 Å because that wavelength is comparable to a typical spacing between the atoms of condensed matter. The difference in energy between the incident and scattered neutron should correspond to the allowed energy levels in the specimen. These allowed energy levels are 0.01 to 0.1 eV in typical cases. The neutrons meet both the above-mentioned requirements while the X-ray only provides a suitable wavelength range and light only provides a suitable energy range.

Variation of isotopic ratios (e.g. H/D) is a powerful technique for isolating particular modes of motion because the

scattering factor $S(Q, \omega)$ alters without altering the chemical nature of the sample. This fact is open to neutron scattering because neutrons interact with the nuclei, in contrast to the other cases like X-ray and light where the interaction is through the electrons or electric charge. The scattering from hydrogen by using X-ray or electron is almost negligible while this is very significant for neutron scattering. A useful technique is the relative enhancement of the scattering from a particular group in a molecular system. In the case of incoherent inelastic neutron scattering this can be done by deuterating those parts of the organic molecule which are not desirable to observe. In this method scattering from the remaining hydrogenous groups (e.g. CH_3 -) can be examined. This method is useful for study of molecular dynamics but not for structure work [1]. An analogous technique can be used in X-ray structural studies for those cases where a heavy element can be placed at given points in a molecule. Because of the relatively large scattered X-ray intensity from heavy elements compared to light elements the positions of such points are obtained. In such cases use of this technique with neutron or X-ray is complementary, one providing dynamical and the other structural information.

Of the many physical techniques that have been used to study the condensed matter, the use of thermal neutron beam has been one that has been most successfully applied so far. Today one of the very interesting areas in such studies is that of biological materials such as proteins, membranes and many other biological molecules.

A large variety of physical techniques are at present being used in studies of the structure and functions of living matter at the molecular level. Most of the techniques were devised originally by physicists. At some stage in their development they have passed into the hands of the biophysicist, biochemist or molecular biologist. Subsequently they have often undergone considerable adaptation and refinement before becoming part of the impressive array of tools with which it is possible now-a-days to probe complex molecular systems.

The principle of neutron diffraction is very similar to those for X-rays, but some important differences exist which are due to the differences in properties of the two radiations. In case of X-rays the scattering centres are the electrons of the atoms and every electron has the same X-ray coherent scattering amplitude of the order of 0.28×10^{-12} cm for $\theta = 0$ (θ is the scattering angle). Obviously the density of the coherent scattering amplitudes is proportional to the electron density of the structure. For neutrons the scattering centres are the nuclei of the atoms and each nucleus has a characteristic neutron coherent scattering amplitude. Due to the difference in scattering centres for X-ray and neutron, it is seen that there is no regularity of the neutron amplitudes as there is with X-ray. This is because neutron scattering is the result of nuclear forces and is dominated by process known as resonance scattering. In this process the incident neutron and target nucleus combine together momentarily as a compound nucleus. The scattering amplitudes at

this stage are determined by the energy level of the compound nucleus and obviously there is little regularity with Z number of the elements. In the absence of the resonance scattering, the amplitude is determined by the potential scattering which corresponds to the size of the nucleus and scattering amplitude is more regular with Z number. In case of resonance scattering the scattering amplitude can be negative, as for hydrogen but there is no negative scattering amplitude for X-ray scattering process.

The large difference between the coherent scattering amplitude of hydrogen and deuterium for the case of neutron scattering process is particularly useful in studies of biological materials using neutron beam. An important fact is that the thermal neutron scattering amplitudes do not decrease with the scattering angle as it happens with X-ray. This is because the dimensions of the nuclei, i.e., the scattering centres, are much smaller than the wave lengths of the incident thermal neutrons. In case of X-rays the dimension of the electron clouds of the atoms and the molecules, i.e., the scattering centres are comparable to the X-ray wavelengths, and due to that reason the scattering amplitudes decrease with increasing scattering angle θ .

Two types of incoherent scattering namely, isotopic incoherence and spin incoherence, occur in neutron scattering which do not occur with X-rays. The element which consists of

more than one isotope randomly distributed in the sample at the atomic positions of the element produces isotopic incoherence. The coherent scattering amplitude of the element is the average of amplitudes of each isotope, weighted by its abundance. According to Bacon [2] there is also an isotopic incoherent scattering, $\sigma_{inc} = 4\pi(\overline{b^2} - \bar{b}^2)$, proportional to the difference between the average squared amplitude and squared average amplitude. The spin incoherence occurs due to the intrinsic angular momentum (spin) of neutrons. If the target nucleus has intrinsic spin, then the coherent scattering amplitude has two values, one for spin parallel and other for spin antiparallel. In case of biological experiments with neutrons, the intrinsic spins of both the incident neutrons and target nuclei have random orientations, i.e., unpolarized beam and target. Therefore, the coherent scattering amplitude is the weighted average of the two values. The incoherent scattering cross-section is again proportional to the difference between the average squared amplitude and squared average amplitude. Both isotopic and spin incoherent scattering are the consequences of the random distributions of the different values of the atomic scattering amplitude. If there is only one isotope of each element present in the sample and these isotopes have no nuclear spin, then there is no incoherent scattering. The large incoherent scattering cross-section of hydrogen is particularly troublesome in diffraction experiment because it produces an isotropic background of scattered neutrons upon which the coherent diffraction peaks are observed. The large scattering cross-section of hydrogen is useful for dynamical measurements and it can be exploited for

studying the molecular motions of hydrogenous materials.

Now many problems in biology have been solved by using small angle neutron scattering technique. In recent years radiative probes are being used increasingly in biological work and they are transforming the discipline from a study involving visual observation and classification, to one of quantitative measurement. Among these probes we find X-ray, electron, laser light and even positron.

The trouble of shooting X-ray and electron at real biological systems is that the biological systems are usually in an aqueous environment and, in fact, often incorporate substantial amount of water molecules into their structures. When ionizing radiation like X-ray or electron impinges on aqueous solution it creates large amounts of chemically active radical (OH) which attacks dissolved organic molecules and is responsible for a large fraction of the damage to any protein and nucleic acid which may be present.

Damage is often observed during the course of fast neutron scattering (diffraction) experiment. In a reactor, however, neutrons may be reduced in speed by successive collisions and they are then called thermal neutrons. When slow-moving thermal neutrons having no charge impinge on atoms they cause little ionization unless they are absorbed by nuclei. If the atoms in the system have a high ratio of probability for scattering to probability for absorption for thermal neutrons, then such

incident neutrons will undergo gentle collisions. We say that such atoms have good scattering properties. From small changes in the neutron momentum associated with gentle collisions, the shape and spatial extent of biomolecules can be determined. For a particular wavelength the X-ray has much higher energy than that of neutron. Due to that reason X-ray ionizes the biological materials more than does neutron and hence X-ray is more damaging to biological materials.

Neutron scattering is a relatively newcomer among biophysical techniques. Despite an auspicious start in 1966 with crystallographic work with vitamin B₁₂ at Harwell by Moore, Willis and Hodgkin [3] and inelastic scattering on polypeptides, and polymers in the USA by Boutin et al. [4], Trevino et al. [5], Gupta et al. [6], it was only during the 1970s, after the establishment of research centres with high flux reactors at Brookhaven and Grenoble, that bio-neutron studies began to make an impact [7,8]. These were almost exclusively devoted to structural work i.e. the analysis of small angle interference patterns produced by coherently scattered neutrons. From a purely biophysical and biochemical point of view, all such applications of neutron scattering represent straightforward extension of X-ray diffraction, the principal new features being the emphasis on light elements and the scattering contrast between hydrogen and deuterium. From a neutron physicist's perspective, however, diffraction studies exploit only part of the information of the scattered radiation field. Any energy changes due to incoherent or coherent scattering from thermal motions, or changes in spin

states of scattered neutrons, are not analysed in conventional diffraction work. If one considers that experiments of this kind are quite expensive, whatever the particular set-up, it seems worthwhile even in the case of complex biomolecular samples to go beyond diffraction and explore the full potential of neutron scattering techniques.

During the past two decades neutron scattering has developed into an important tool for the determination of the structure of biologically important compounds. Biological neutron experiments fall into two categories: (1) at high resolution, neutrons are used to determine the placement of hydrogen atoms in biological molecules and (2) at lower resolution these experiments exploit the difference in sensitivity of neutron radiation to hydrogen and to deuterium to determine the location of molecular components in large structure. One of the more interesting results obtained with low-resolution experiments so far, is the location of protein sphere in a section of chromosome [9]. The properties of organism that identify them as living, such as motility and reproduction, are to a large extent manifestations of chemical events occurring within them. Many biochemical studies have shown that these chemical events, in turn, reflect the properties of the macromolecular components an organism contains: its proteins, nucleic acids, lipids and polysaccharides. The intimate involvement of macromolecular materials in life process has made these polymeric substances the object of intensive study for many decades.

The three dimensional structure of biological macromolecules have their biologically important properties. The important objective is to understand what these three dimensional structures are and how the physiological properties of these molecules arise from them. The earlier investigation of this kind has produced many substantial impacts. Among them a most striking case was the discovery of the structure of DNA in 1950s by J. Watson and F. Crick. The structure of DNA immediately suggested how the molecule worked as a storage device for genetic information and how it replicated its two key functions. The molecular biologists have explored the implications of these findings for the last three decades and the end is not still in sight.

The investigation of the structure of biological molecules is very important because of its extreme importance in biology. In the late 1960s when the current generation of high flux beam reactors was commissioned the study of biological materials became much viable for experiments and experimenters got access to beam time of the high flux reactors. It was supposed that the neutron beams would produce important information about the structure and properties of biological materials. It was also hoped that the new high flux reactors would produce enough thermal fluxes and that would make possible to do the experiments with these weakly scattering biological materials. One of the most valuable instruments to the experiments is the small angle neutron spectrometer at the high flux reactor of the Institut Laue-Langevin in Grenoble, France.

The objective of the high-resolution studies was to measure the crystal structure of proteins and the experimenters started biological work with neutrons at high-resolution in the late 1960s. A number of factors stimulated the high-resolution neutron scattering studies. The first was concerned with the existence of hydrogen bonds which in part are responsible for the stabilization of three dimensional structure of proteins [10]. The hydrogen bonds in protein structure are not readily visible in X-ray scattering process. In neutron scattering this is clearly observable. The ability to visualize hydrogen bonds is very important to those interested in the way enzymes work. The catalytic configurations of those molecules commonly involve complex-hydrogen-bonded networks. Often the alterations in proton chemistry accompany enzyme action. The second issue, related to the first is concerned with the solvent structure. The water structure around the macro-molecules in detail is more distinctive in case of neutron scattering than that in X-ray scattering. Because X-ray can see only oxygen and hydrogen is almost invisible to it but the neutrons can see all three atoms in H_2O . In study of some biological molecules containing water the incoherent hydrogen scattering contributes a large background to scattering or diffraction measurement. This problem has been overcome by replacing H_2O in the solvent with D_2O .

The replacement of H_2O with D_2O produces a new interesting phenomenon. Many of the protons in biological molecules are chemically labile, and these begin exchanging with deuterium in the water. It is also observed at the same time that the some

groups are not chemically capable of doing exchange, they are protected from exchange by their position in the molecule. The pattern of protection is more interesting than that expected from simple steric hindrance consideration based on the molecular structure [11]. Some groups, apparently well protected from solvent, do exchange; others do not. Thus the exchange pattern gives evidence about the dynamic properties of the molecule because flexibility is clearly required to permit exchange at interior groups. Neutron data collected in D_2O clearly distinguish exchanged from unexchanged locations and thus give valuable information on the long-time limit of the exchange process. There are some other techniques which are used to obtain information on these processes. One of such techniques is the tritium exchange experiments. Some work has been done for years [12] by using this technique. The tritium exchange method can give good kinetic data but it is hard to discover which groups are exchanging by this method [13], because the information sought tends to be lost during analysis in this method. Nuclear magnetic resonance (NMR) can also be used for studies of this kind. For small macromolecules with molecular weights of less than 10,000 NMR can identify the slowly exchanging groups and measure their exchange rates [14]. The neutron method is not limited to small molecules, unlike the NMR technique, but at present it can not give any kinetic data.

In case of neutron scattering the O and N are easily distinguishable because of their reasonable difference in scattering lengths [15]. In case of X-ray scattering it is very hard to distinguish the carbonyl side of an amide group from its

amine side which are the compounds of the residues in proteins. This is because of the small difference in the scattering lengths for O and N and also the negligible scattering length of the attached H. Thus the neutron scattering can readily place the correct groups in space.

For early stage of neutron scattering from biological materials the flux of the neutron sources was low and it is not easy to collect neutron data from protein crystals. To study the protein crystal the size of the crystals were made large to obtain high-data collection rates. The structure of myoglobin was determined using a crystal of volume 24 mm^3 [16]. In last ten years, substantial progress has been made in increasing the flux of the neutron sources, development of instruments and techniques used for collecting neutron scattering data. This fact reduces the need for unusually large crystals. Now the 1 mm^3 volume crystal can yield useful data in an acceptable length of time. At Brookhaven, the National Bureau of Standards and the Institut Laue-Langevin about six proteins have been studied [8]. A plant protein crambin molecule was crystallized and studied [17] at high resolution at $1.4\text{\AA}$.

It is to be noted that no one has developed the methods for solving the macromolecular structures using neutron data alone. The only protein crystals are studied by neutrons have already been solved using X-ray scattering; the neutron structure is obtained by refinement. Neutron analysis is in fact a method to obtain supplementary information on a protein crystal structure

that has been solved by X-ray methods.

In the literature of the past 15 years one can find experimental results which describe the use of neutrons to study structures at lower resolution. Many of the works during this period were done at higher resolution and these higher resolution works exploit the difference in scattering lengths between H^1 and H^2 in one way or another. In a low-resolution experiment the individual atoms are not visible. In such experiments the average scattering properties can be characterized in terms of scattering length densities. There are many biological objects which consist of two different chemical species and in those cases the scattering length density within their volumes vary significantly. In this low-resolution experiment one can obtain the information on disposition of the two materials, for example, shape, size, radius of gyration, separation etc. by using "contrast variation method" [18]. Many experimenters use the contrast variation experiments because they are easy to implement biochemically and because the data collection in this method is very straight-forward. By using this method some successful studies have been done on nucleosomes [19,20]. Nucleosomes, which consist of DNA and protein, are the unit of structure in chromosomes. Bently et al. [21] had done such a study which can be mentioned as an example. These and other studies done on nucleosomes in solution have led to a low-resolution model for this structure, which has now been confirmed [22] crystallographically at much higher resolution. The contrast variation method has also been used to investigate the viruses.

At high-resolution study the structures of some crystallized viruses [23] have been obtained. A shortcoming of the structures investigated is that only the viral protein coat is crystallizing. The nucleic acid contained in the coat is not ordered with respect to it, and so while the crystal structures are very informative about the protein coat, they inform us next to nothing about the placement of the nucleic acid within it. To investigate the placement of nucleic acid the contrast variation has been used [24] at low-resolution study. Baldwin et al. [25] used the contrast variation method extensively for studying the structure of chromatin which consists of chromosomal DNA complexed with histone proteins. This method was also used for studying the organization of the nucleic acid vis-a-vis the protein shell in viruses [26] by Jacrot. It was possible for them to determine the extent of penetration of the protein shell into the interior of the virus and to deduce a low-resolution model for its structure. Engelman and Moore [27] studied RNA and proteins to understand their mutual arrangements by using the method of triangulation by selecting deuteration. It was possible for them to measure the intercomponent distances in large biomolecular assemblies using selective deuterium labelling in SANS measurements for the first time. By using X-ray small angle scattering technique Ingrid Pilz et al. [28] studied the molecular size and shape of the phenylamine specific transfer RNA in 1970. Before 1970s the scattering studies of sample was limited to X-ray scattering technique.

In recent years some structural measurements have been performed at Chalk River Nuclear Laboratories [29,30,31]. Small angle scattering has been used to investigate the physical characteristics of human transferrin [29], structure of native and irradiated superoxide dismutase in aqueous solution [30]. Small angle and wide angle neutron scattering were used to study the self-association of nucleosides in aqueous solution [31].

Li et al. [32] at the Institut Laue-Langevin (ILL) have obtained strong evidence for the existence of electrostricted layer of water around the RNA molecules by using the contrast variation method. Most of the low resolution neutron experiments involve selective deuteration of the object under investigation. Localized deuteration experiments are low-resolution analogues of the isomorphous replacement experiments that are used to obtain macromolecular crystal data. In X-ray experiments, the analysis of isomorphous replacement of data determine the placement of a heavy atom in a crystal. In the low-resolution neutron experiments, the atoms whose positions are determined are the deuterium atoms used as labels. A series of experiments have been done [33] to position lipid molecules in membrane bilayers in this way. Some macromolecular aggregates have been or now being investigated by specific deuteration methods to determine the position of molecular subunits within these structures. A full study has been completed on the enzyme RNA [34] polymerase, a five subunit structure from *Escherichia coli*. The 30s ribosomal subunit from *E. coli*, containing 22 macromolecular components have been studied by similar method.

It is reported that 19 of its components have been located [35]. The 50s subunit of the ribosome was being examined [36] in a set of experiments getting under way at the ILL in 1985s.

Since 1970s a considerable number of experiments have been performed by using neutron diffraction technique for studying biological membranes and membrane components. Instrumentation for small angle diffraction and small angle scattering is particularly important for studying biological membranes. The use of long wave lengths (4 - 20 Å) is required for this purpose. Small angle diffractometers for membrane studies have been built at Harwell [37] and at Brookhaven [38]. The small angle neutron scattering facility of the Institut Max von Laue-Paul Langevin in Grenoble is described by Schametz et al. [39]. Experimental instruments for neutron quasi-elastic and inelastic scattering measurements are described by Stirling [40] and Brugger [41].

Neutron diffraction studies of bilayer of membranes have been continuing at the PLUTO reactor, Harwell, since 1972. Detailed studies on structural analysis of lamellar and related phases of membrane lipids have been done as pursued with X-rays [42,43]. Neutron diffraction measurements have been done from oriented bilayer samples of synthetic L- α dimyristoyl and L- α dipalmitoyl lecithins [44]. To perform the measurements a simple neutron diffractometer was used which selected 4.73 Å neutrons [37]. Lamellar neutron diffraction was measured from hydrated multilayers of a mixture of egg lecithin and cholesterol

oriented on glass substrates [45]. In the same year neutron diffraction was measured from bilayers of egg lecithin at Harwell [46]. Structure in regions of the phase diagram designated C, $L\alpha$ and $L\beta$ by Tardieu et al. [46] was studied. Many studies have been made of the lecithin-cholesterol-water system by using X-rays. These studies could not define the distribution of water and did not entirely agree on the position of cholesterol in the bilayer. We have studied the neutron diffraction from cholesterol in membranes to compare its position in the membranes with the positions of 8 -THC and 9 -THC in the membranes [47]. The membrane of nerve myelin was the first biological membrane studied by neutron diffraction [48]. For mammalian nerve myelin in D_2O Ringer solution, only a single prominent peak at an equivalent Bragg spacing of 90 Å was observed for poor wavelength monochromaticity and poor angular resolution. From a rabbit sciatic nerve in D_2O Ringer solution seven diffraction orders of a 180 Å periodicity were obtained by using an improved diffractometer [49,50]. Phase assignments have been made to the neutron diffraction by assuming a simple model for the myelin membranes based upon interpretation of the electron density profile [51]. These phase assignments were used to calculate a neutron Fourier profile of 26 Å resolution [52,53]. The intensities of the second and fourth order reflections were measured at several isotopic compositions of the water between D_2O and H_2O [53]. Neutron diffraction measurements were made at Harwell from dog sciatic nerve myelin in D_2O Ringer solution [37].

Several neutron diffraction studies have been performed from model membranes [54,55]. The trans-membrane location of retinal in the purple membrane of *Halobacterium halobium* was measured by low-angle neutron scattering studies on aqueous dispersion of the membranes [56]. Neutron diffraction measurements were performed for analysis of the structure of rod photoreceptor membranes in intact retinas [57]. Small angle neutron scattering of aqueous dispersion of lipids and lipid mixtures was carried out by using contrast variation method [58].

Neutron quasi-elastic and inelastic scattering have been done on biological materials since 1970. Experimental techniques for neutron time-of-flight spectroscopy were described by Stirling [40] and Brugger [41] and the principles of applications to problems of molecular motion in the liquid state are described by Powles [59], Sjolander [60] and Larsson [61].

Some theoretical investigations on biological materials were carried out during past decade. Lovesey and Schofield [62] have described the inelastic coherent neutron scattering by small particles. Theoretical calculations have been done by Suezaki and Gō [63] on breathing mode of conformational fluctuations in globular proteins. Go et al. [64] discussed the dynamics of a small globular protein in terms of low frequency vibrational mode.

The dynamics of interlamellar water molecule was first measured by neutron time-of-flight spectroscopy in studies of layered silicate clay minerals [65,66]. Similar measurements on the diffusive motions of water in the ammonium perfluorooctanoate/water system was made [67]. Measurements of the anisotropy of water diffusion was made [67] using a MARX spectrometer [68] orienting the momentum transfer vector parallel and perpendicular to water layers. The results indicated less anisotropy than was expected even for thin water layer (9 Å). The neutron scattering from the desired component can be expected to be larger than from other components of a biological system. To achieve this, one is to replace the hydrogen by deuterium or fluorine. Using this substitution method, the diffusive motion of octanol in the three component system ammonium perfluorooctanoate-water-10% octanol was studied [68]. Developments of instrumentation with increasingly better energy resolution [69] and improvements in time-of-flight data analysis [70] are contributing considerably to these neutron scattering studies of the dynamical properties of membrane components.

In the past few years some work has been carried out [71,72] on inelastic neutron scattering in biological polymers. The data indicate that the proteins have inelastic mode corresponding to whole-molecule vibrations. These low frequency modes of vibration have connected these measurements and the theoretical area of molecular dynamics. The comparison of data with theory in the field of inelastic neutron scattering area would be extremely helpful. We looked for the large scale mode

of vibrations of globular proteins experimentally and studied some vibrational spectra of such molecules [73,74].

The neutron beam has been shown to provide useful information about the biological materials with the following type of measurements:

1. Small angle neutron scattering from aqueous solution of biological molecules.
2. Small angle neutron diffraction from biological samples for structural analysis.
3. Quasi-elastic and inelastic neutron scattering measurements for studying dynamical property of biological molecules.

The present Ph.D. programme has been chalked out in a way that it enables one to acquire skill in different techniques which are involved in studying the various properties of biological materials. It will also help one learn techniques associated with data analysis using the existing computer codes and develop necessary computer programmes for analysis of data collected from different measurements. The study of various properties of different sets of biological samples will provide adequate experiences. On the basis of above philosophy the Ph. D. programme includes (1) the study of molecular shape, size and volume of biological materials, (2) study of structure of biological materials, (3) study of dynamical properties of biological molecules.

To perform the above mentioned studies on biological materials a triple-axis spectrometer is a suitable instrument provided this spectrometer can be used in a double-axis mode. A

schematic diagram of a triple-axis spectrometer has been shown in Fig. 1.1. The beam of neutrons from the reactor has an approximately Maxwellian distribution of velocity i.e. wavelength. The incident neutron beam is collimated to the desired angular divergence before it falls upon monochromator so that the unwanted neutrons from reactor are kept minimum. The direction of the incident neutrons beam can be altered by altering the Bragg angle of crystal monochromator. In this way the wavelength of incident beam can be altered. The resolution of the incident wavelength is determined by the angular spread of the beam and the crystal mosaic according to the relation, $\Delta\lambda/\lambda = \Delta\theta/\tan \theta$, where θ is the Bragg angle. The Bragg angle of the analyzer crystal can be varied to analyze the different wavelengths of the neutrons scattered by the sample, and send it into detector. A data point can then be determined as a ratio of the detected intensity to the beam monitor intensity. An instrument of this type provides a measurement of intensity at a given point in $(\omega-Q)$ space. By changing three out of four parameters θ_M , θ_A , ϕ and ψ (two Bragg angles, angle of scattering and sample orientation) another locus in $(\omega-Q)$ space can be achieved. In this manner a selected locus may be followed point by point. For this reason a triple-axis spectrometer is especially suitable for the study of vibrational spectra of molecules and lattice vibrations etc. For a given vibrational mode there will be an intensity peak at one point of $(\omega-Q)$ space only. For structural analysis scanning of scattered neutrons by the sample at different positions will give different peaks for different order of Bragg reflections.

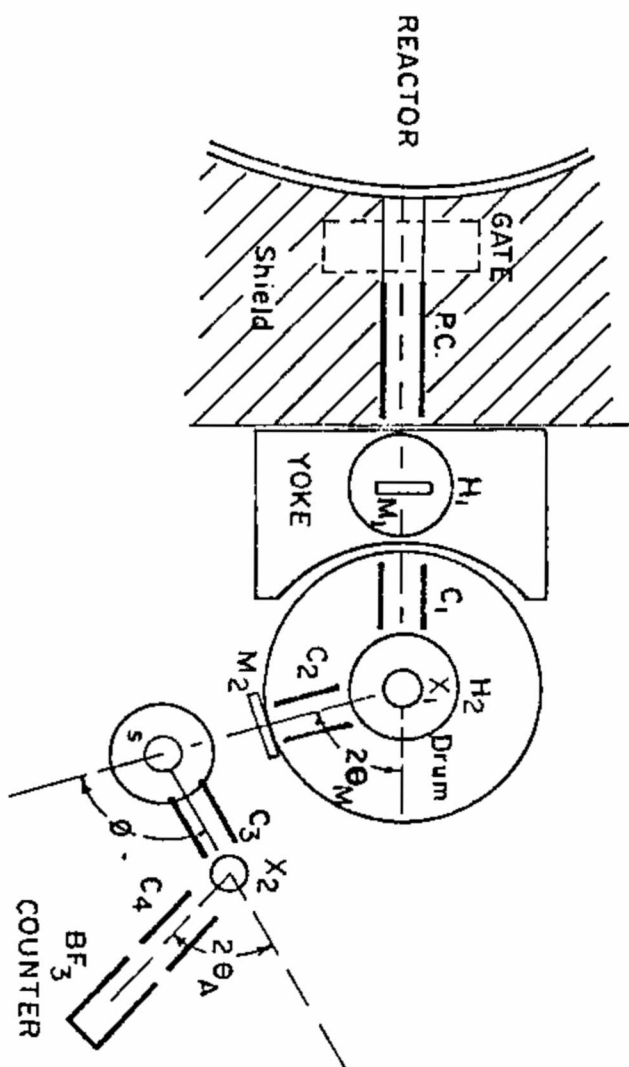


FIG.1.1 SCHEMATIC DRAWING OF A CHALK RIVER TRIPLE AXIS SPECTROMETER.

We have studied the following biological materials using different techniques of neutron scattering.

(1) Study of the small angle neutron scattering (SANS) from neurotoxin, cytotoxin and cytotoxin analog in aqueous solution. This has been described in chapter 2.

(2) Study of the small angle neutron diffraction from Δ^8 -THC, Δ^9 -THC and cholesterol in phospholipid membranes described in chapter 3.

(3) Study of the vibrational spectra of globular proteins, namely alpha-chymotrypsin, chymotrypsinogen A and hemoglobin by using inelastic neutron scattering technique. This study has been described in chapter 4.

The last chapter of the thesis namely chapter 5 contains the conclusions derived from our experiments and the possible future course of work in the field of neutron scattering applied to biological problems.

CHAPTER 2

SMALL ANGLE NEUTRON SCATTERING FROM BIOLOGICAL MOLECULES

2.1 Introduction

Small angle neutron scattering (SANS) technique allows to study the size, shape, volume, radius of gyration and monomeric or dimeric condition of macromolecules of biological materials. Using this technique we have studied the three types of cobra toxins.

Cobra toxins are of interest not only in themselves but also because of their potential use as probes for the study of membranes and synaptic processes. Thousands of people die by snake-bites in the Indian subcontinent. So the study of cobra toxins by using small angle neutron scattering is of particular interest because this study may provide valuable information about cobra toxins. Amongst the active biomolecules in cobra venoms are the cytotoxins and neurotoxins. The former render the cell membrane permeable, while the latter block transmission of nerve impulses at the neuromuscular junctions. Certain cobra venoms also contain cytotoxin analogs which are very similar to cytotoxins with respect to the aminoacid residues they contain and to their disulphide bridging pattern [75]. These analogs apparently express their toxic function in a manner different from both the neurotoxins and cytotoxins [76]. Due to the diverse effects of the three afore-mentioned venom constituents, it was of interest to compare the size and shape of these molecules in

aqueous solution. A few cobra toxins have been crystallized and their shapes are determined. The present measurements may serve as a point of reference for such studies since the active forms of toxins are those in aqueous solution. To ascertain the diversity of these cobra toxins we are interested in studying neurotoxin 3 from Naja naja naja siamensis venom [77], Cytotoxin B from Naja naja naja venom [78], and cytotoxin analog 9B from Hemachatus haemachatus [75,79] by using small angle scattering technique.

In addition analytical gel-filtration experiment was carried out and data are presented indicating that the cobra toxins (proteins) exist as monomers in solution under conditions similar to those used in the SANS measurements.

2.2 Theory

2.2.1 Small Angle Neutron Scattering

Detailed theory of small angle neutron scattering (SANS) has been given by Guinier and Fournet [80]. Small angle scattering refers to scattering at very small value of wave vector transfer $Q (= 4\pi \sin \theta / \lambda)$. In this technique Q should be small compared to the smallest reciprocal lattice vectors in crystalline substances, or to the Q value corresponding to the

first maximum of $S(Q)$ (scattering function) in non-crystalline materials. The properties of the Fourier transforms show that the scattering at low Q values will not give structural details in real space over distances smaller than $D_{min} = 2\pi/Q_{max}$, where Q_{max} is the maximum Q value accessed in the experiment. For example $Q_{max} = 0.3 \text{ \AA}^{-1}$ corresponds to $D_{min} \sim 20 \text{ \AA}$. Such low spatial resolution is inadequate for locating individual atomic positions. Consequently, the discrete arrangement of scattering centres in condensed matter can be considered as continuous distribution of scattering length density averaged over a volume $(D_{min})^3$. The fluctuations of the average scattering length densities over distances larger than D_{min} will give rise to small angle scattering in the wave vector transfer region $Q < Q_{max}$. This fact enables the study of structural inhomogeneities with linear dimension of $10 - 100 \text{ \AA}$ by analysis of SANS measurements in the wave vector transfer range $10^{-4} < Q < 0.1 \text{ \AA}^{-1}$. The structural inhomogeneities in condensed matter occur due to the fluctuation of composition and defects.

Considering the nuclear scattering only, one can write the coherent scattering cross-section per atom at scattering vector Q , for locally averaged scattering length density $\rho(r)$, r being the continuous variable position vector as

$$d\sigma/d\Omega = 1/N \left| \int \rho(r) e^{+iQ \cdot r} d^3r \right|^2 \quad (2.1)$$

$\rho(r)$ may vary over distances of the order of D_{min} or more, and it is more convenient to express it in terms of $\bar{\rho}$, the average

scattering length density of the sample over volumes much larger than $D_{\max}^3 = (2\pi/Q_{\max})^3$. Hence, one can write

$$\rho(r) = \rho + \Delta\rho(r) \quad (2.2)$$

and we get

$$\begin{aligned} d\sigma/d\Omega &= 1/N \left| \int_V (\rho + \rho(r)) e^{+i\mathbf{q} \cdot \mathbf{r}} d^3r \right|^2 \\ &= 1/N \left| \int_V (\Delta\rho(r)) e^{+i\mathbf{q} \cdot \mathbf{r}} d^3r \right|^2 \end{aligned} \quad (2.3)$$

where the Fourier transform of ρ , being a delta function has been omitted. $\Delta\rho(r) = (\rho(r) - \rho)$ denotes the contrast in locally averaged scattering length density due to structural or compositional inhomogeneities.

Suppose a sample contains N_p homogeneous particles of scattering length density $\rho_p = b_p/V_p$, embedded in a homogeneous matrix of scattering length density $\rho_m = b_m/V_m$ (where b_p , V_p , and b_m , V_m are the average scattering length and atomic volume in the particle and the matrix respectively). Then from equation (2.1)

$$d\sigma/d\Omega = 1/N (\rho_p - \rho_m)^2 \left| \int_{V_t} e^{+i\mathbf{q} \cdot \mathbf{r}} d^3r \right|^2 \quad (2.4)$$

where, the integral extends over the volume V_t occupied by all particles. In general equ.(2.4) will include the spatial and orientational correlations between particles, the effects due to their size distributions etc. If the particles are non-homogeneous ρ_p will have to be replaced with $\rho_p(r) = \rho_p + \rho_F(r)$,

where $\rho_F(r)$ denotes the density fluctuations within the particles. These situations are not uncommon in polymeric and biological materials, where one frequently encounters aggregation states of multicomponent systems.

For N homogeneous particles each with volume V_p , cross-section can be re-written as

$$d\sigma/d\Omega(Q) = \{V_p^2 N_p / N\} (\rho_p - \rho_m)^2 |F_p(Q)|^2 \quad (2.5)$$

where $F_p(Q)$ denotes the single particle form factor

$$F_p(Q) = (1/V_p) \int_V e^{iQ \cdot r} d^3r \quad (2.6)$$

$$\text{such that } F_p(0) = 1. \quad (2.7)$$

Equation (2.5) is valid for very dilute systems only, as the contributions from interparticle interference effects have been ignored. For non dilute systems, one has to include an interference term, $\psi(Q)$, which is a Fourier transform of the static pair-correlation function

$$\psi(Q) = (1/N_p) \sum_i e^{iQ \cdot (r_{0i} - r_{0j})} \quad (2.8)$$

where r_{0i} and r_{0j} are the position vectors of the centres of particles i and j respectively. With combination of equations

(2.5) and (2.8)

$$d\sigma/d\Omega(Q) = (V_p^2 N_p/N) \{\rho_p - \rho_m\} |F_p(Q)|^2 |1 + \gamma(Q)|^2 \quad (2.9)$$

Eqns. (2.5) and (2.9) are also valid for a system of monodisperse anisotropic particles, if the particles are identically oriented. When the particles are randomly oriented $|F(Q)|^2$ will have to be averaged over the orientational distribution.

The scattering function $\phi(Qr)$ is defined by the square of single particle form factor:

$$\phi(Qr) = |F_p(Q)|^2 \quad (2.10)$$

For a system of identical spherical particles with radius R_s , the scattering function is given by Rayleigh formula

$$\phi_s(QR_s) = [3\{(\sin(QR_s) - QR_s \cos(QR_s))/(QR_s)^3\}]^2 \quad (2.11)$$

The function has a maximum value of 1 at $QR_s = 0$ followed by a series of sinusoidal peaks at values of $QR_s = (n+1)\pi$, with their peak heights diminishing in the proportion $(n+1)^{-4}$. The type of scattering patterns are commonly observed from biological particles such as viruses.

For ellipsoids of revolution with axes $2a$, $2a$, $2av$ the scattering function has the form [81]:

$$\phi_e(Q) = \int_0^{\pi/2} \phi_s[Qav/(\sin^2\alpha + v^2\cos^2\alpha)]^{1/2} \cos\beta \, d\beta \quad (2.12)$$

where $\alpha = \arctan(v \tan\beta)$; and ϕ_s has the form defined in eqn.(2.11).

For thin rod like cylinders with radius r and length $2L$, the scattering function in the region $Qr < 1 \ll QL$ can be approximated as

$$\begin{aligned} \phi_{cy1}(Q) &= (\pi/2QL) e^{-Q^2 r^2 / 4} \\ &= (\pi/2QL) e^{-(Q^2 R_c^2 / 2)} \end{aligned} \quad (2.13)$$

where $R_c^2 = r^2/2$, is known as the cross-sectional radius of gyration. For the infinitesimally thin rods $\phi_{cy1}(Q) = \pi/2QL$ for $QL \gg 1$.

For flat disc like cylinders ($r \gg 1$):

$$\phi_{cy1}(Q) = \{2/(Qr)^2\} e^{-(QL)^2 / 3} \quad \text{when } QL \ll Qr. \quad (2.14)$$

The small angle neutron scattering technique is used to know the shape and dimension of biological molecules and is a very popular field of research today. The theory of neutron scattering from biological molecules in aqueous solution is analogous to the X-ray scattering. The advantages and disadvantages of neutron scattering over X-ray scattering have been described in chapter 1.

Let us consider a particle irradiated by a beam of neutrons; the neutrons are then scattered by the all nuclei of the particle. The scattered neutrons are all in phase, when the direction of the scattered neutron is same as that of the incident neutron. When the scattering angle increases the difference in phase between the various scattered neutrons also increases. The amplitude of the resultant scattered wave then decreases with increasing angle because of increasing destructive interference. It becomes zero when there are as many waves with phases between 0 and π as there are between π and 2π . This will occur for a scattering angle of the order of $2\theta = \lambda/D$, where λ is the wavelength of the incident neutron and D is the average dimension of the particle. This demonstrates how the study of the continuous central scattering offers a method for obtaining particle's dimension.

This method is applicable only for particles whose sizes lie within certain limits. If D is too large the scattering is limited to angles so small as to be inaccessible to experiment. On the other hand we see that if λ is larger, D can be larger but larger λ of X-ray has a very large absorption cross-section with matter and that gives a limitation of X-ray scattering. Comparatively, the neutron scattering is more useful to find out the dimension of large particle because of its less absorption cross-section than that of X-ray. If D is too small, of the order of several wavelengths, scattering is widely spread but too weak to be observable. In this respect the neutron scattering is more

advantageous over X-ray scattering, because, the thermal neutron scattering amplitudes do not decrease with the scattering angle as the case with X-rays. This is because the dimensions of the nuclei, the scattering centres for neutrons are much smaller than the wavelengths of incident neutrons, on the other hand the scattering centres for X-rays are the electron clouds of the atoms and molecules have dimension comparable to the X-ray wavelengths and therefore the scattering amplitudes decrease with increasing angle. To show exactly on which factors the small angle scattering depends, let us consider a small particle of scattering length density $\rho(x)$. Let us define a form factor of this particle, $S(x)$, that has the value 1 when the vector lies within the particle and the value 0 when it lies outside the particle. The amplitude $A(Q)$ of radiation which is the transform of $\rho(x)$ at the point defined by the vector Q in reciprocal space is given [80] by:

$$A_1(Q) = \int \rho(x) S(x) e^{-iQ \cdot x} dx \quad (2.15)$$

where $Q = (2\pi / \lambda)(k - k_0)$, λ is the wavelength of radiation and k_0 and k are unit vectors in the direction of the incident and scattered rays respectively. The general theorem related to the operation of folding in the theory of Fourier transformations states that if $A(Q)$ and $T(Q)$ are respectively the transforms of $\rho(x)$ and $S(x)$, then;

$$A_1(Q) = \int A(y) T(Q-y) dy \quad (2.16)$$

where y is a variable of integration.

If the dimension of the region in which $S(x)$ is different from zero, its transform $T(Q)$ is fully determined and if the particle has dimensions of several tens or several hundreds of atomic diameters, $T(Q)$ will be different from zero only for very small values of Q .

If we assume that the sample is of constant scattering length density, $\rho(x) = k$, the transform $A(Q)$ acts as Dirac delta function. It becomes zero everywhere except at $Q = 0$, where it is infinite. For the case of more homogeneous body whose neutron scattering length density shows periodicities only on an atomic or molecular scale, the transform $A(Q)$ shows a large number of peaks. However, all these peaks except the one for $Q = 0$ are product for values of Q well outside the domain in which $T(Q)$ has a non-zero value.

Under these circumstances, since $A(y)$ is essentially a Dirac delta function about $y = 0$, it may be predicted that around the origin of the reciprocal space the amplitude $A_1(Q)$ is proportional to $T(Q)$, the function $\rho(x)$ is not intervening [80]. The Dirac delta function mentioned above has the properties as follows:

$$\begin{aligned} \delta(x) &= 0 & \text{for } x \neq 0 \\ \delta(x) &= \infty & \text{for } x = 0 \\ \int \delta(x) dx &= 1 \end{aligned} \tag{2.17}$$

The above discussions imply that the scattering around the centre is practically independent of the short range order of the atoms, depending on the exterior form and dimension of the particle. Thus the SANS has become a means of studying the dimensions and form of colloidal particles and this technique has already been exploited.

The macro molecules in aqueous solution are capable of motion. This motion can always be described as the sum of the translation and rotation. A translation may be defined by a vector V , which introduces a multiplicative factor $e^{-iQ \cdot V}$ in the expression for scattered amplitude, but this has no effect on scattered intensity [80]. The rotations intervene in the calculation of an average intensity. As the molecules have the different orientations in the aqueous solution, the scattering intensity calculation requires the orientational average [82,83] relative to their wave vector transfer Q .

The scattering profiles observed for a dilute solution of macro molecules $I(Q)$ is given [80,84,85] by:

$$I(Q) = \int_0^{\infty} P(r) (\sin(Qr)/Qr) dr \quad (2.18)$$

where $Q = 4\pi \sin\theta / \lambda$ and $P(r)$ is the distribution of lengths of interatomic vectors within individual molecules.

The interpretation of small angle scattering data is mainly

based on the comparison of experimental data with model function [83]. This comparison can be made in reciprocal space with smeared scattering curves or desmeared scattering curves. In any case one has to know the corresponding model functions. The model functions of a sphere can be calculated analytically, the scattering amplitudes of simple triaxial bodies like ellipsoids, prisms and cylinders can be calculated for any special orientation. The spatial average for orientation has to be done numerically. The error of these integrations can be minimised by introducing an arbitrary small limit by a sufficient decrease of the increment of integration. Such kinds of semi-analytical calculations have been performed for a large number of different models by Porod [86], Mittelbach and Porod [87] and Mittelbach [88]. But there are many molecules under investigations that can not be approximated by simple triaxial models. The semi-analytical method has been extended to models composed of some triaxial bodies [89]. The number of necessary integrations intervals for the spatial average increases rapidly for larger scattering angles if the model is elongated. The computing time can be order of several hours. There are some other semi-analytical methods but those methods need spatial averaging and those are very time consuming. A powerful method proposed by Glatter [82] is known as "sphere method". The problem of spatial averaging does not exist in this method because this step can be performed analytically for an aggregate of small spheres. It involves filling any arbitrary shape with large number of overlapping small spheres whose total volume is equal to the volume of the model and calculating the scattering by means of

the Debye equation [90]. At wave vector transfer Q the intensity of the coherently scattered neutrons, $I(Q)$, from an aggregate of n spheres can be computed by the Debye formula:

$$I(Q) = \sum_{i=1}^n W_i^2 S_i^2(Q) + 2 \sum_{i=1}^{n-1} \sum_{j=i+1}^n W_i W_j S_i(Q) S_j(Q) \sin(l_{ij}Q) / l_{ij}Q \quad (2.19)$$

where $S_i(Q)$ is the structure factor of i th sphere. l_{ij} is the distance between the centres of the i th and j th spheres.

$$S_i(Q) = 3[\sin(R_i Q) - (R_i Q) \cos(R_i Q) / (R_i Q)^3] \quad (2.20)$$

where R_i is the radius of the small spheres and which is given by on the basis of assumption of simple cubic packing with fraction $(\pi/6)$.

$$R_i = D/2 (6/\pi)^{1/3} \quad (2.21)$$

In equation (2.21), D is the distance between the centres of two neighbouring small spheres.

$$W_i = \rho_i V_i = \rho_i 4\pi R_i^3/3 \quad (2.22)$$

where ρ_i is the neutron scattering length density of the i th sphere.

The results of most of the scattering experiments are affected by the resolution functions of the measuring instruments. Due to this unwanted instrumental effects we require

to correct the data. A clear knowledge of resolution functions can help us for optimizing the instrumental parameters and experimental procedure. The resolution functions of a crystal diffraction spectrometer are anisotropic and produce a pronounced effect at the detector position due to focussing effects. We have considered the effects of resolution functions in the horizontal and vertical directions from a point in the detector aperture.

There are two philosophies for analyzing SANS data, one is to deconvolute the experimental data with resolution functions and the other is to convolute the theoretical values with resolution functions and compare with the experimental data [91]. We follow the second approach. This leads to

$$J(Q) = k \iint \{ I[(Q - y)^2 + z^2]^{1/2} \} W_h(y) W_v(z) dy dz \quad (2.23)$$

where, y is the horizontal and z is the vertical coordinate of a point in the detector aperture in the same units as Q ; W_h and W_v are normalized Gaussian weighting functions that specify the horizontal and vertical resolution functions respectively. k is a scale factor. The width W_h can be determined experimentally by scanning the detector in 2θ across the direct beam. For a neutron diffractometer W_h has been found to be very nearly gaussian. The W_v function is well approximated by a gaussian whose width can be calculated from the known collimations. The gaussian approximation for W_h and W_v permit integration by means of a Gauss-Hermite numerical algorithm.

At small Q , Guinnier [92] has shown that $J(Q)$ is given by:

$$J(Q) = J(0) \exp(-Q^2 R_g^2 / 3) \quad (2.24)$$

where R_g is the radius of gyration of randomly oriented molecules, independent of molecular shape and $J(0)$ is the intensity at $Q = 0$. This functional form can be substituted into equation (2.23) and a resolution-corrected $J(Q)$ can then be extracted. A plot of the logarithm of $J(Q)$ versus Q^2 is a straight line at sufficiently small Q and slope of this is related to R_g by the equation [93]

$$R_g = 0.4183 \lambda (-\alpha)^{1/2} \quad (2.25)$$

where λ is the wave length of the incident neutron and α is the slope of the straight line. If the plot $I(Q)$ versus Q^2 at small Q is not a straight line, this indicates that the target sample is a mixture of different molecules of different sizes or it may be a mixture of monomer and dimer of the same molecule.

The sphere method which has been employed to calculate convoluted scattering function also enable one to calculate the R_g for any arbitrary shape of molecule. R_g is calculated from the expression:

$$R_g^2 = \frac{\sum_{i=1}^n W_i r_{oi}^2}{\sum_{i=1}^n W_i} \quad (2.26)$$

where $l_{oi}^2 = (X_o - X_i)^2 + (Y_o - Y_i)^2 + (Z_o - Z_i)^2$.

The X-coordinate, X_o , of the centre of scattering amplitude density is given by:

$$X_o = \frac{\sum_{i=1}^n W_i X_i}{\sum_{i=1}^n W_i} \quad (2.27)$$

Similar expressions are used for Y_o and Z_o . The coordinates of the centre of the small spheres are X_i , Y_i and Z_i .

In the fitting procedure, the fitting of experimental data with convoluted theoretical values in sphere method, the quality of fit parameter is defined as:

$$\chi^2 = \frac{\sum_{i=1}^N M_i (J(Q_i)_{cal} - J(Q_i)_{exp})^2}{(N - P)} \quad (2.28)$$

where $J(Q_i)_{cal}$ and $J(Q_i)_{exp}$ are the calculated and experimental values respectively at the point i , N is the number of data points and P is the number of parameters which are varied. The weighting factor is $M_i = (1 / J(Q_i)_{exp})^2$. The program developed (COBRA) [94] by us for analysis of SANS data is able to vary the all possible model parameters according to the user's choice to minimize χ^2 .

2.2.2 Analytical Gel-Filtration

A typical experiment of Gel filtration is able to indicate the monomeric and dimeric conditions of the biological molecules in solution. The Guinnier plot of SANS function also gives the

same information. To perform this experiment the sample is dissolved in sodium phosphate buffer containing sodium azide. The resulting solution is then applied to Sephadex G-50 or G-70 superfine column equilibrated and eluted with the same phosphate azide buffer used to dissolve the sample.

When the elution volume, void column and total column volume are V_e , V_o and V_t respectively, the distribution coefficients K_{av} are then determined from the expression as follows:

$$K_{av} = (V_e - V_o) / (V_t - V_o) \quad (2.29)$$

The plot of K_{av} versus known molecular weight of some molecules will supply the calibration curve. After measuring the K_{av} for some molecules of known molecular weights and comparing these with calibration curve the monomeric and dimeric condition of the molecules are determined.

2.3 Experimental Methods

2.3.1 SANS technique

SANS technique allows one to measure the average scattering function of the target material. When the target material has no layered structure scattering function provides the information about the size and shape of the target materials. The reactor-

neutron beam is collimated as desirable for the experiments. The small angle neutron scattering experiments can be performed by using a double-axis crystal diffractometer which can scan the scattered neutrons very precisely. A monochromator crystal diffracts a particular neutron-wavelength obeying the Bragg law. The neutrons of selected wavelength irradiate the sample and are scattered elastically around the sample. The scattered neutrons are scanned with a neutron detector at a very small interval of scattering angle. Usually scattering fluctuation is a function of $Q (= 4\pi \sin\theta / \lambda)$, where 2θ is the scattering angle of neutrons scattered by the sample. The scattering function is dependent on the nature, shape and size of target material. The monoenergetic neutrons selected by monochromator and neutrons scattered by the sample are collimated for better resolution in measurements. Constant monitor counts are used in this method, so that reactor power fluctuation does not affect the measurements. This technique can be applied to know the impurities in any species of molecules by comparing the scattering function of pure and impure samples. It allows one to know the monomeric and dimeric state of the molecules in aqueous solution and the coexistence of monomer and dimer of the same molecules in aqueous solution.

Now the important aspect of SANS experiment is to analyze the data and to confirm the expected results. A good guess about the dimension of the molecules can be achieved by finding the radius of gyration (R_g) of the molecules through a least-square analysis data.

The scattering function of a regular-shaped-body can be calculated analytically. With a variation of the different parameters of the regular-shaped-body and comparing the resulting values with experimental data through least square analysis the molecular shape and size of the regular-shaped-body can be found very easily.

Today the model calculations can be performed for arbitrary shape of molecules. Computer codes [82,94] are available for analysis of the small angle neutron scattering data from molecules of arbitrary shape.

2.3.2 Materials for SANS Measurements:

Lyophilized crude venoms were obtained from the following sources; Naja naja (hooded cobra) lot 702446 from Calbiochem (personal correspondence with Mr. Gene Kritchevsky, Technical Services Supervisor for Calbiochem, confirmed that the venom was from Naja naja siamensis species cobra); Naja naja naja venom from Bioactive Inc.; and Hemachatus haemachatus no. HH34SL - 5 from Miama Serpentarium Laboratories.

Carboxymethyl cellulose (CM - 52), Sephadex G-50 and G-75 Superfine, and Biorex-70 cation exchange resin (200 - 400 mesh) were of standard commercial quality.

Alpha chymotrypsinogen A, 6X crystallized type II lot 1118 - 8170 was from Sigma Chemical Co. as was Dextran blue no. D-5751 lot 90F-0266; Ribonuclease no. 3433 B37E832 from Worthington. All other chemicals were of reagent grade.

2.3.3 Isolation of the Toxins from crude venom

Cytotoxin B was isolated from one gram of Naja naja naja venom by ion-exchange chromatography as in [95]. Final purification of the toxin was achieved by rechromatography on CM-52 using a linear gradient (2 X 2000 ml) from 50 mM to 600 mM ammonium bicarbonate. Unless otherwise stated, all chromatographic procedures were performed at 4°C.

Neurotoxin 3 was isolated from Naja naja siamensis venom by the same procedure used for the isolation of cytotoxin B. Final purification was done by gel filtration on Sephadex G-50 superfine as in [77] This step results in removal of a minor "short" neurotoxin, 3C, which co-elutes with neurotoxin 3 upon ion exchange chromatography.

Cytotoxin analog 9B was isolated from Hemachatus haemachatus venom as in [96]. The toxin was rechromatographed on Biorex-70 resin using a linear gradient (2 X 1000 ml) from 200 mM to 7000 mM ammonium acetate PH 7.3.

Henceforth, these venom proteins will be referred to simply as neurotoxin, cytotoxin and cytotoxin analog.

2.3.4 Criteria for Purity of Toxins

Samples isolated from crude venom were judged to be pure on the basis of SDS polyacrylamide gel electrophoresis performed as in [97], and comparison of their amino acid compositions (determined from acid hydrolysates with a Durrum D-500 amino acid analyzer) with the values reported in the literature.

2.3.5 Preparation for SANS Measurements

Specimens were shipped to Chalk River Nuclear Labs., Canada in lyophilized state, where they were dissolved in 100 mM phosphate buffer of D₂O, PH 7.01. All samples were dissolved to a concentration of 8 g/l so that interference effects (small for this concentration) would be similar for all three samples. After a series of measurements the solution was frozen at -13°C. Subsequently measurements to improve counting statistics were carried out on thawed samples and these yielded the same results within experimental errors.

2.3.6 Experimental Procedure and Set-up for SANS Measurements.

The measurements for SANS from neurotoxin, cytotoxin and cytotoxin analog were made at 23°C on the L₃ triple-axis spectrometer, operated in two-axis mode (Fig.2.1) at NRU reactor, Chalk River Nuclear Labs., Canada. The spectrum was filtered by 30 cm of quartz. A germanium (111) monochromator was used to obtain a monochromatic beam with wavelength, λ of 2.5 Å and a wavelength spread $\Delta\lambda/\lambda$ of 0.014. The monochromatic beam was analyzed by Fermi chopper and was found to contain negligible higher order contamination. The incident and scattered beams were defined by slits of rectangular cross-section which yielded angular divergence of 1/3 deg. The sample chamber was quartz glass, wall thickness of 1mm and the specimen was 1.5 mm thick. Measurements were made by scanning the scattering angle, 2θ , in the step of 0.1 deg. A typical scan contained 301 steps and required 20 hours running time. Repeat measurements indicated no specimen deterioration over this period.

2.3.7 Experimental Procedure and Setup for Gel Filtration

A typical experiment consisted of the following; the sample (8 mg) was dissolved in 1.0 ml 100 mM sodium phosphate buffer PH 7.01 containing 0.02 percent sodium azide. The resulting solution was then applied to a Sephadex G-50 Superfine column (1.5 X 126 cm) equilibrated and eluted with the same phosphate azide buffer used to dissolve the sample. Column elution was at 12 ml/hr at ambient temperature. Elution volumes (V_e) of the proteins were determined by monitoring the absorbance at 280 nm of the elute.

Column calibration was performed using alphachymotrypsinogen A (MW 25000), Ribonuclease (MW 13700) and neurotoxin 3 from Naja naja siamensis venom (MW 7820) and which is shown in Fig. 2.1. These three proteins are known to exist as monomers in solution and to behave ideally upon gel filtration on Sephadex columns under similar conditions as those described earlier [98,77]. The void column (V_0) was determined using Dextran Blue. The total column volume (V_t) was determined by chromatographing 1.0 ml column buffer containing 5M sodium chloride and monitoring the elute by conductivity measurements. The molecular weights of neurotoxin (7820), cytotoxin (6479) and cytotoxin analog (7166) were determined from their amino acid composition.

2.4 Data Analysis for SANS Measurements

The spherical modification of the Debye method proposed by Glatter [82] has been programmed to analyze SANS data [94] from three types of cobra toxins. The model shape for different molecules have been filled up with a large number of overlapping small spheres which fill the space occupied by the molecules. The experimental data finally obtained as follows with respect to our experimental setup:

$$J(Q) = ([S(Q) - C(Q)]/t_s - [E(Q) - C(Q)]/t_E) - \delta([R(Q) - C(Q)]/t_R - [E(Q) - C(Q)]/t_E) \quad (2.30)$$

where S, R and E are the observed intensities (normalized to

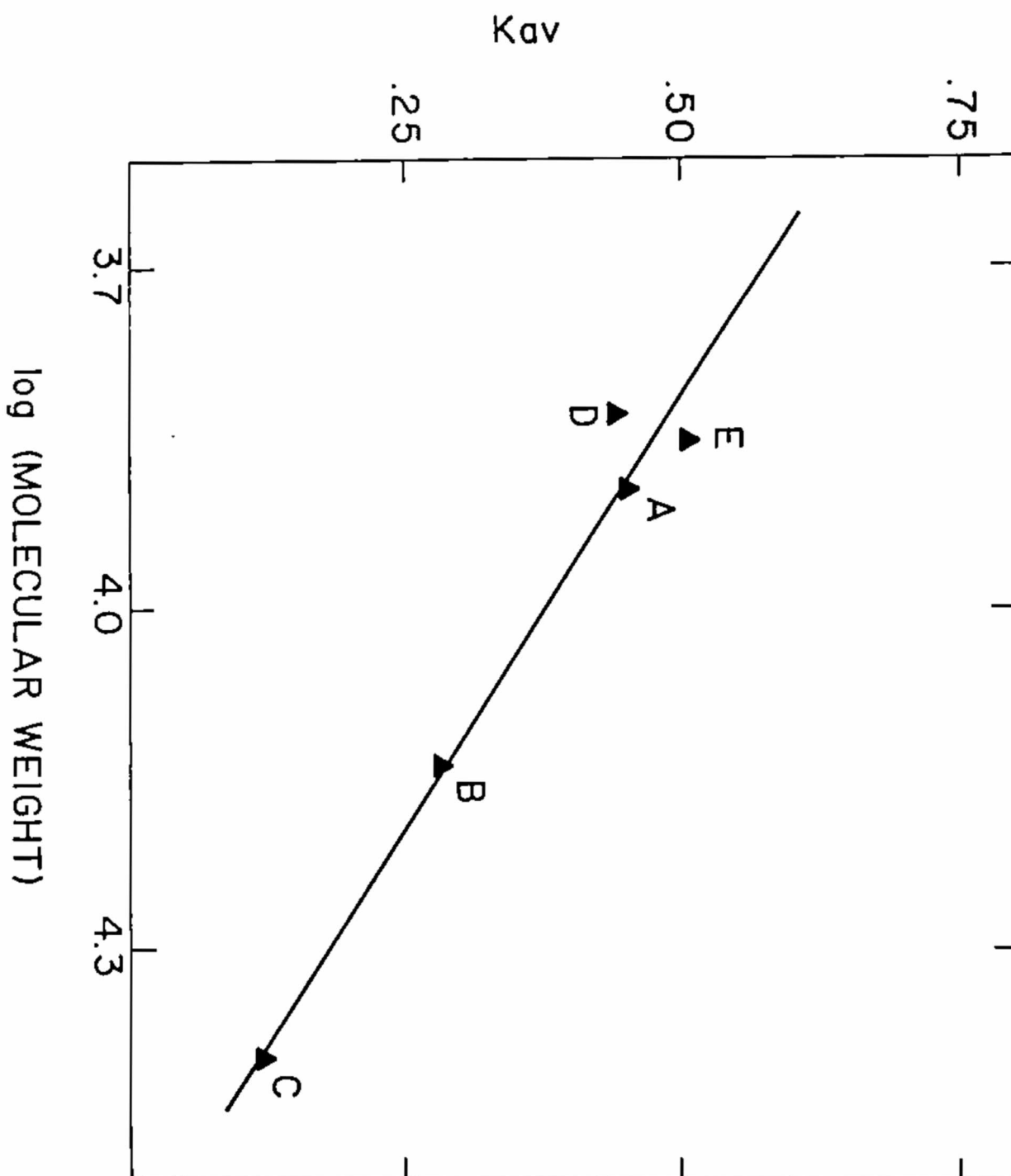


Fig. 2.1 Analytical gel filtration of the venom proteins on Sephadex G-50 superfine. The column was calibrated by using Alpha Chymotrypsinogen A (C), Ribonuclease (B) and neurotoxin 3(A) as standards. The best straight line for these standards was determined by linear regression. D: cytotoxin B from *Naja naja* venom, E: cytotoxin analog 9B from *Hemachatus haemachatus* venom.

constant monitor) of the sample plus sample container, solvent plus sample container and empty sample container respectively; t_s , t_R and t_E are the corresponding measured transmission; C is the observed intensity with incident beam blocked by cadmium; δ is the volume fraction of solvent in solution. These data were fitted to a resolution broadened model scattering function. The program automatically varies selected model parameters and normalization factors to obtain the best fit (minimum χ^2) of the experimental data to calculated values. At each stage of the iterative procedure the radius of gyration is computed. The program itself determines the coordinates of the centres and radii of the small spheres. It can also minimize the boundary effects. The performance of the model has been tested. Using this method the scattering intensity for different models and its comparison with analytical calculations are shown in Figs. 2.2, 2.3, 2.4 and 2.5. The radii of gyration of different shapes calculated by using the present method are compared with analytical values in Table 2.1. The program allows one to calculate the scattering function for mixture of two different molecular species, a possibility when partial dimerization occurs. We approximated the cobra toxins as shown in Fig. 2.6. The overlapping of the subunits (which may be sphere, ellipsoid and cylinder) for approximation of a model is allowed in our calculations. The combination of two or three regular shaped body are used to approximate the model only to assign the coordinates of the small spheres inside the model. In this method we are careful to cover the boundary of the model with the boundaries of the two or three regular shaped bodies but the overlapping of the

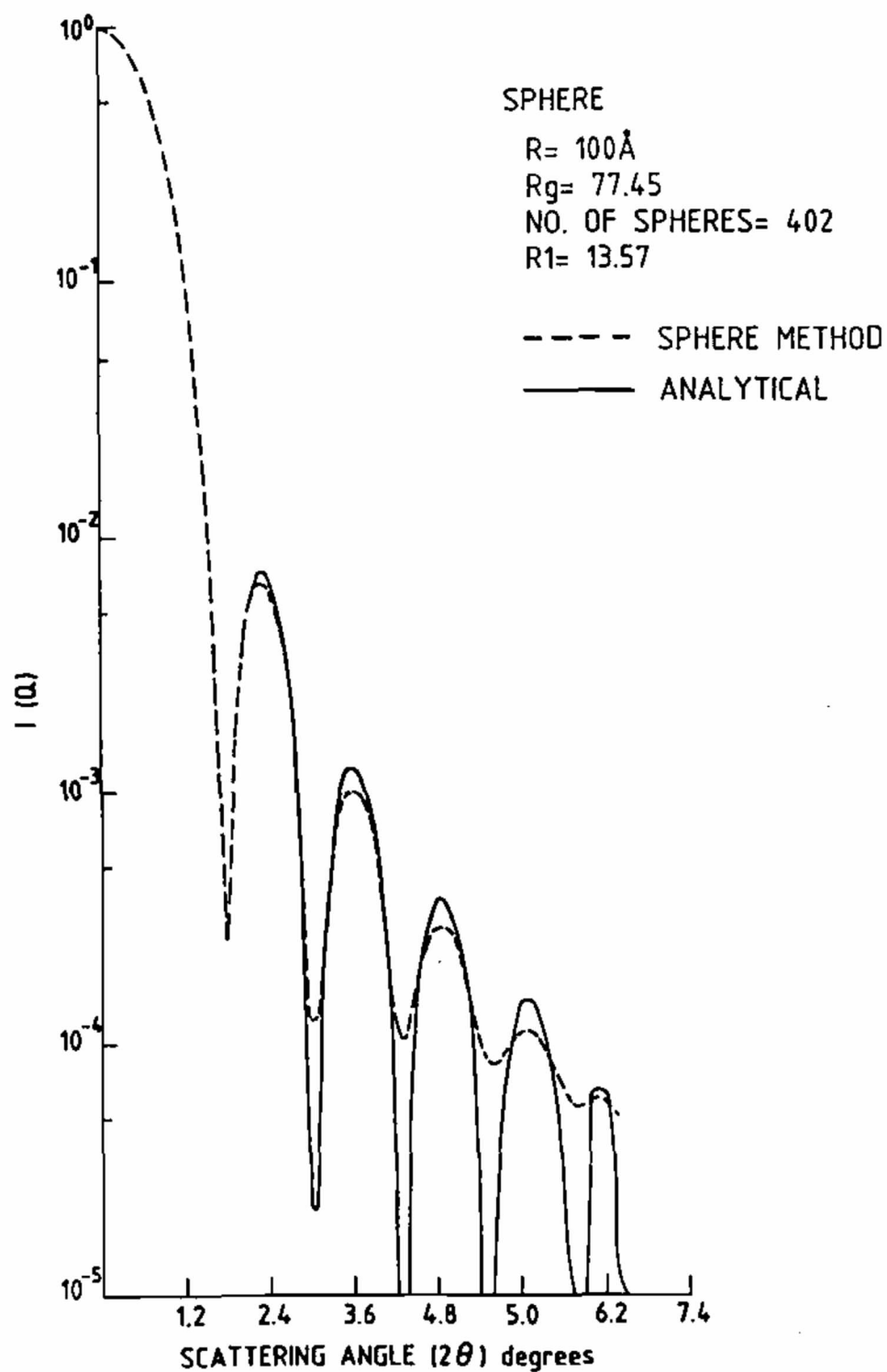


Fig. 2.2

Scattering intensity from a sphere with a radius $R = 100 \text{ \AA}$.
 The radius of the small spheres, R_1 , was $13.57 \text{ \AA}$.

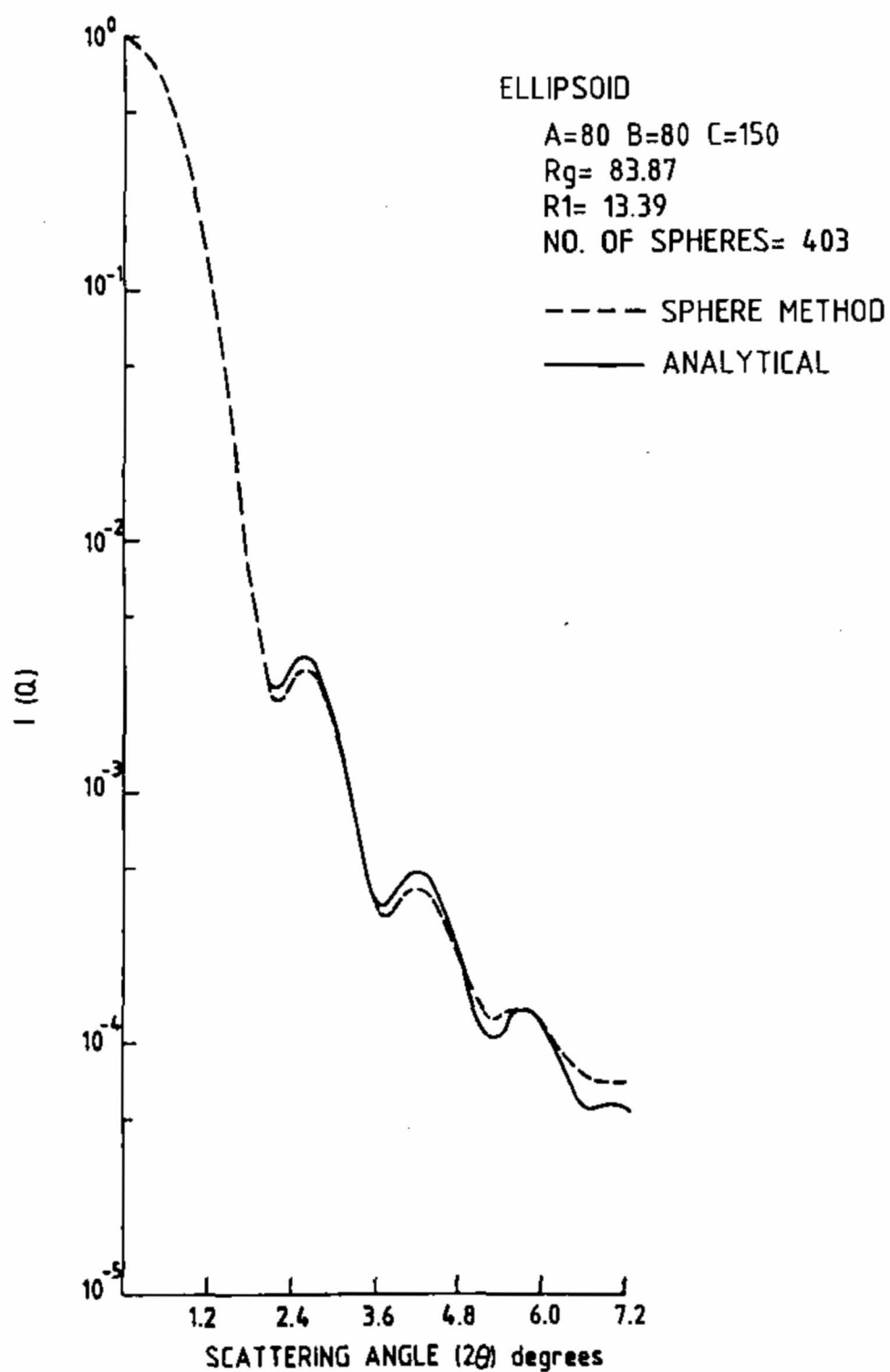


Fig. 2.3

Scattering intensity from an ellipsoid

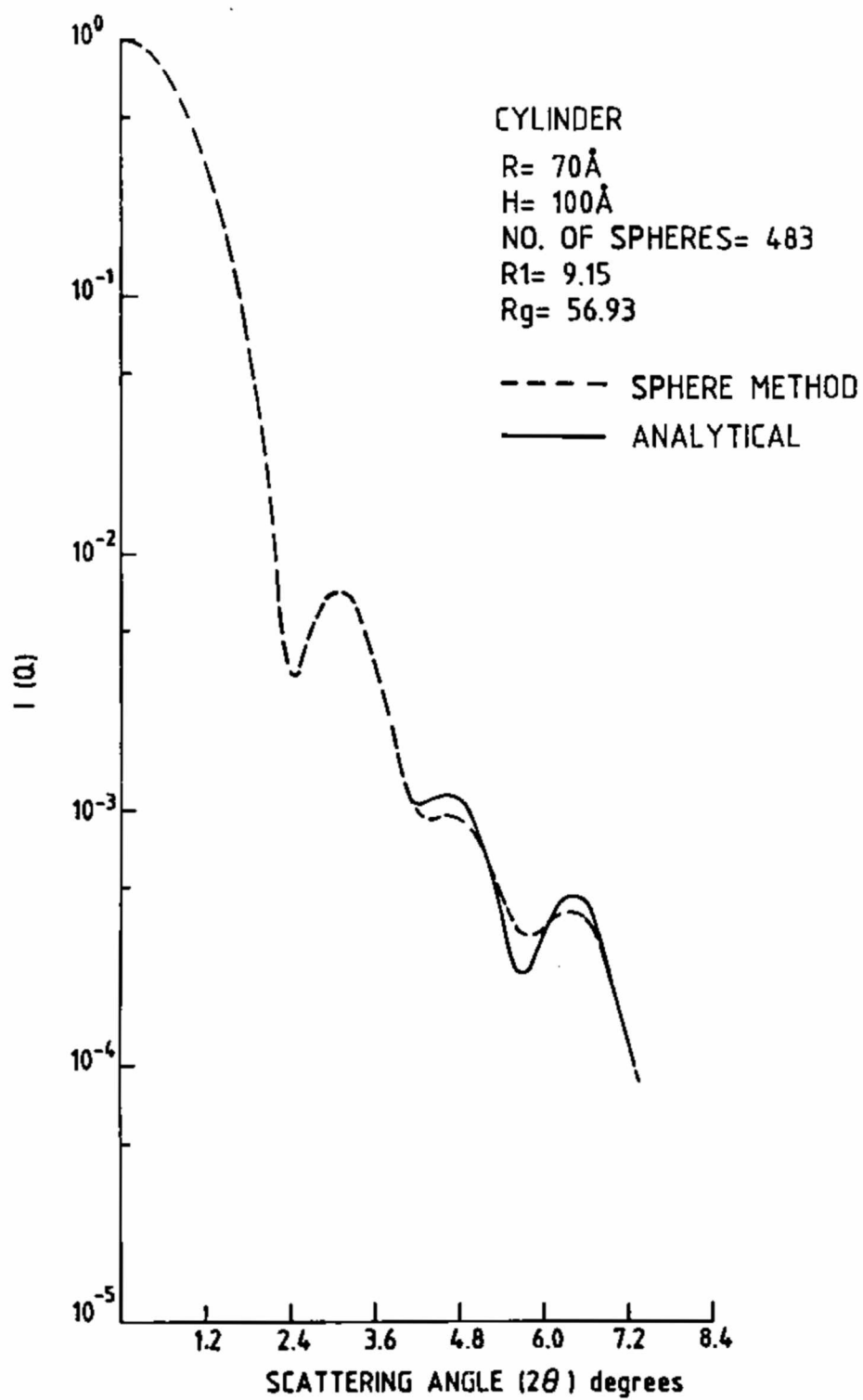


Fig. 2.4

Scattering intensity from a cylinder

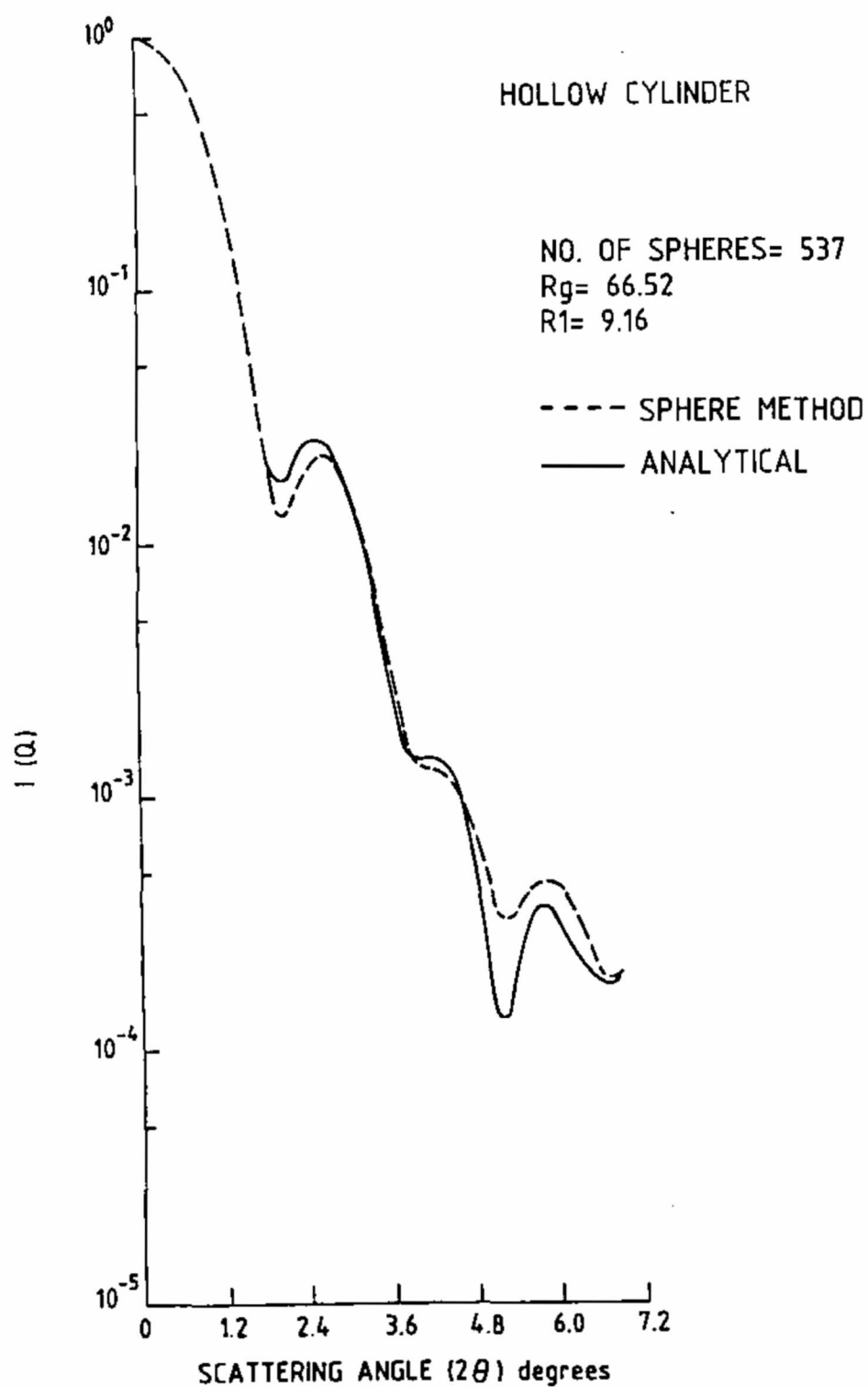


Fig. 2.5

Scattering intensity from a hollow cylinder of outer radius $R_1 = 80 \text{ \AA}$, inner radius $R_2 = 30 \text{ \AA}$ and height $H = 100 \text{ \AA}$.

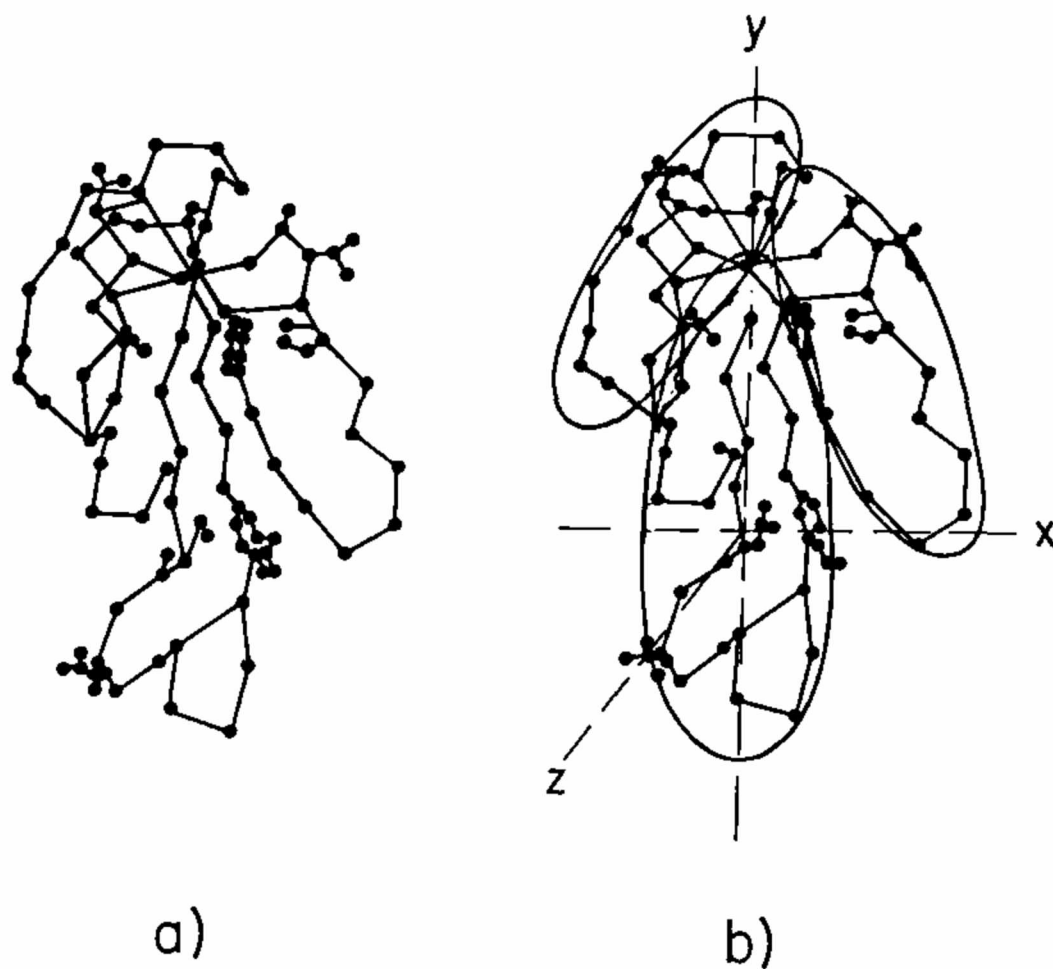


Fig. 2.6 (a) Structure of neurotoxin as determined by X-ray diffraction (Ref.100) from a single crystal.
(b) Our triple ellipsoid model approximation for neurotoxin.

two or three regular shaped bodies inside the model does not affect our calculations. In Glatter's method of calculations, he approximated the model with a large number of subunits. We did

Table 2.1

Comparison between analytical and sphere method calculations.

Model	Model parameters	Radius of gyration	
		Analytical	Sphere method
	A	A	A
Sphere	R = 100	77.46	77.45
Ellipsoid	A = 80		
	B = 150	84.02	83.87
	C = 80		
Cylinder	R = 70		
	2H = 100	57.30	56.93
Hollow cylinder	R ₁ = 80		
	R ₂ = 30	66.95	66.52
	2H = 100		

not find any more precision in Glatter's calculations over our calculations for these particular models calculations. The scope of overlapping of subunits (not small spheres) in our method has compensated for the precision of the results produced by Glatter's large number of subunits concept, where he had no provision of overlapping the subunits to approximate the arbitrary models.

The time involved in summing the Debye formula (eqn. 2.19) goes up very fast as the number of small spheres increases. In order to illustrate this and to provide an idea of running times on Cyber 175 computer, the program was used to fit scattering from superoxide dismutase holoenzyme measured at a concentration of 9.84 g/l. The scan data comprised 301 points. The model chosen was an ellipsoid as suggested by recent single crystal X-ray diffraction measurements [99]. The least square fitted result without convolution required 355 sec for convergence with $n=20$ (n = number of small spheres). For $n=40$ the time was 895 sec and for $n=60$, 3680 sec were required. This calculation includes the automatic variation of model parameters. By comparing the X^2 values for the different n 's with a calculation using an analytic scattering function [96], $n=40$ was found to give an adequate fit. The calculation with analytic function required only 14.2 secs. When we calculate the convoluted scattering function it takes a time factor of 36 and 25 for $n=40$ and $n=20$ respectively. These results suggest that calculations should be followed without

convolution until approximate values for model parameters are obtained. Final values can then be obtained by setting the mode for calculation as convoluted. If we do not want to vary the parameters of the model which can affect the scattering function except background-scattering factor, or some other parameters which are associated with the fitting procedure then we can switch on an option which will reduce the iteration time by a factor 30. By doing this a series of trial and error method estimates of model size can be quickly obtained.

A valuable feature of calculations using our program is the calculation of R_g at every iteration; this is specially desirable where complicated molecular shapes are being generated.

The accuracy of the model calculations of sphere method increases with increasing number of small spheres inside the model i.e. the smaller is the distance "d" between two small spheres, the better is the accuracy of calculations upto certain Q values. The resolution limit for the scattering curve Q_{max} depends on number of small spheres. The theoretical limitations of calculations of the scattering curve is as follows as prescribed by Glatter [82]:

$$Q_{max} < 2\pi/d \quad (2.30)$$

This limit suggests that using smaller d one can calculate the scattering curve for higher Q values. We followed this limit in our calculations.

2.5 Results and Discussions

The method of data analysis which has been used here is the sphere method discussed earlier [94]. Model calculations for neurotoxin, cytotoxin and cytotoxin analog were carried out assuming that those molecules are well approximated with three overlapping ellipsoids as shown in Fig. 2.6. The final shapes of the molecules of neurotoxin, cytotoxin and cytotoxin analog have been shown in Figs. 2.7a, 2.7b, 2.7c; 2.8a, 2.8b, 2.8c and 2.9a, 2.9b, 2.9c. In the fitting procedure we varied the different parameters of the three ellipsoids including the three centroids of the three ellipsoids until the best values have been achieved. The computer program developed by us [94] convoluted the appropriate scattering function with spectrometer resolution in order to determine best values. The scattering functions for those arbitrary shape of molecules have been successfully calculated using the sphere method. An overall scaling parameter was also varied in this calculations. Considering the spherical shape of the three toxin molecules, the results for the best computer fits are shown in Figs. 2.10, 2.11 and 2.12. The zero angle of the scattering occurs at increment number 151. The spherical model yielded R values of 14.1 ± 0.3 , 14.1 ± 0.3 and 13.9 ± 0.3 Å for neurotoxin, cytotoxin and cytotoxin analog respectively. The fits are plotted as dashed lines in Figs. 2.10, 2.11 and 2.12. The best fits from the ellipsoidal models have been depicted as thin solid lines; these fits are clearly better

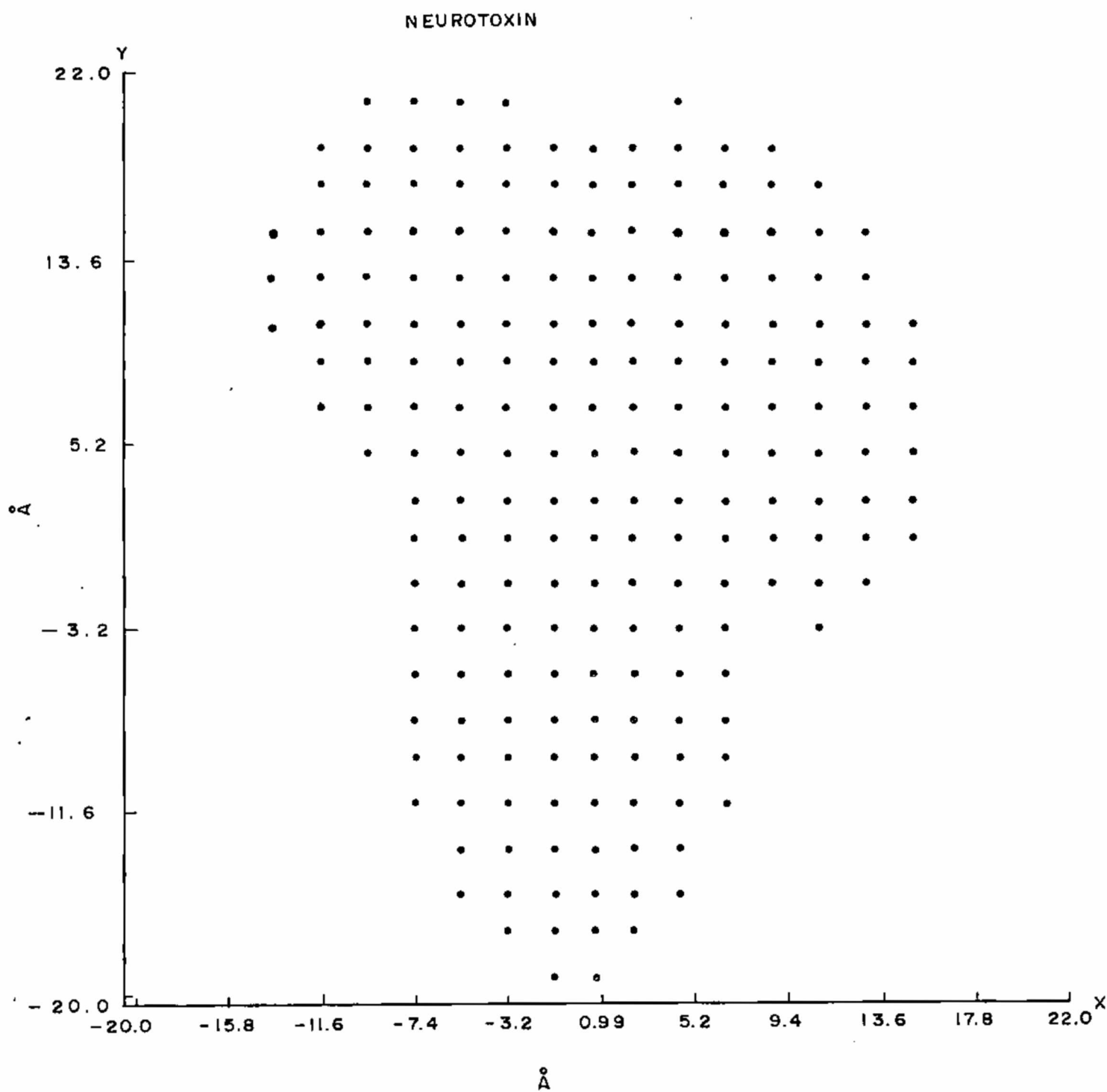


Fig. 2.7a Shape of the neurotoxin molecule obtained from "sphere method" of data analysis (Y-X plot).

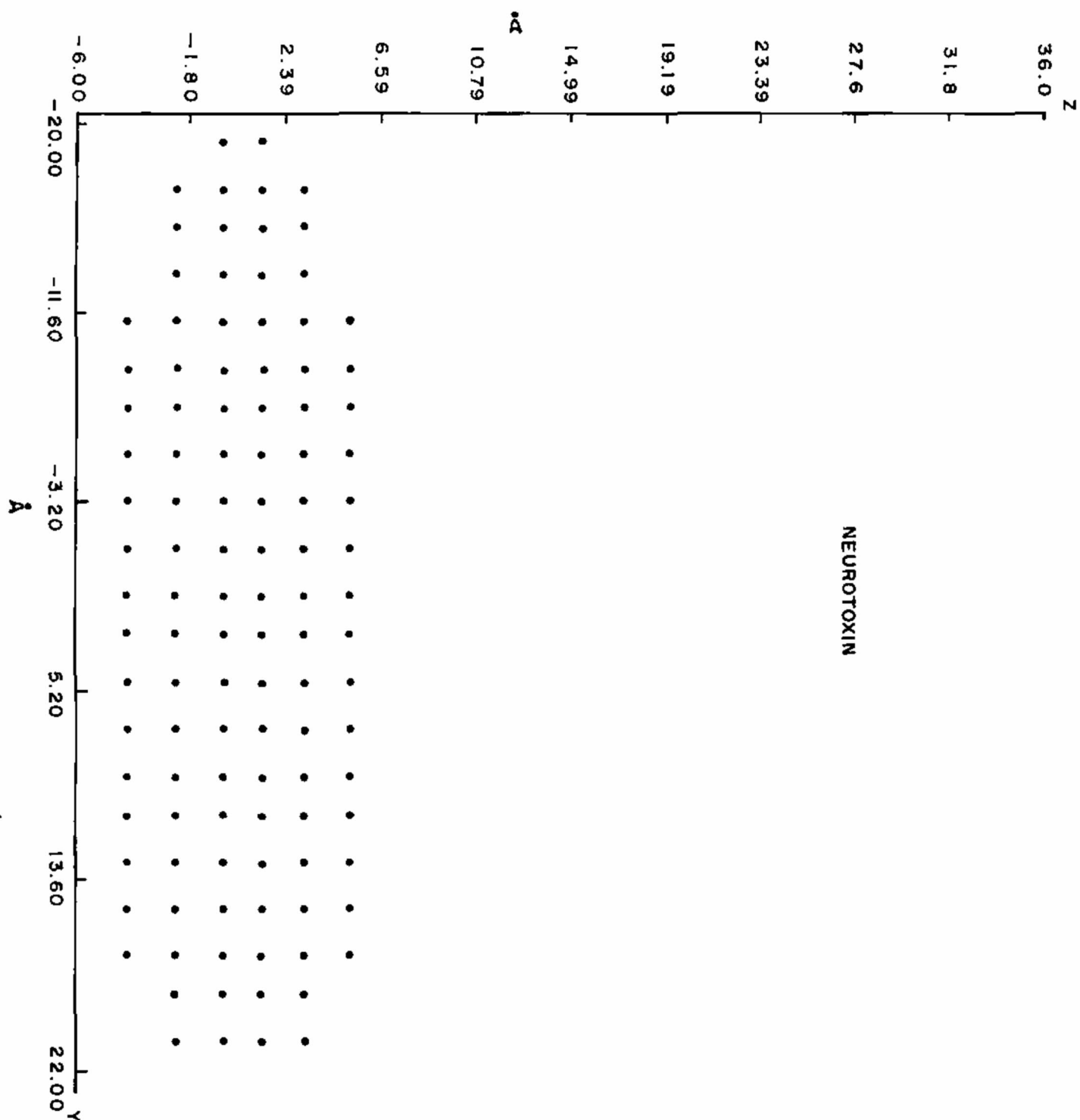


Fig. 2.7b Shape of the neurotoxin molecule obtained from "sphere method" of data analysis (X-Z plot).

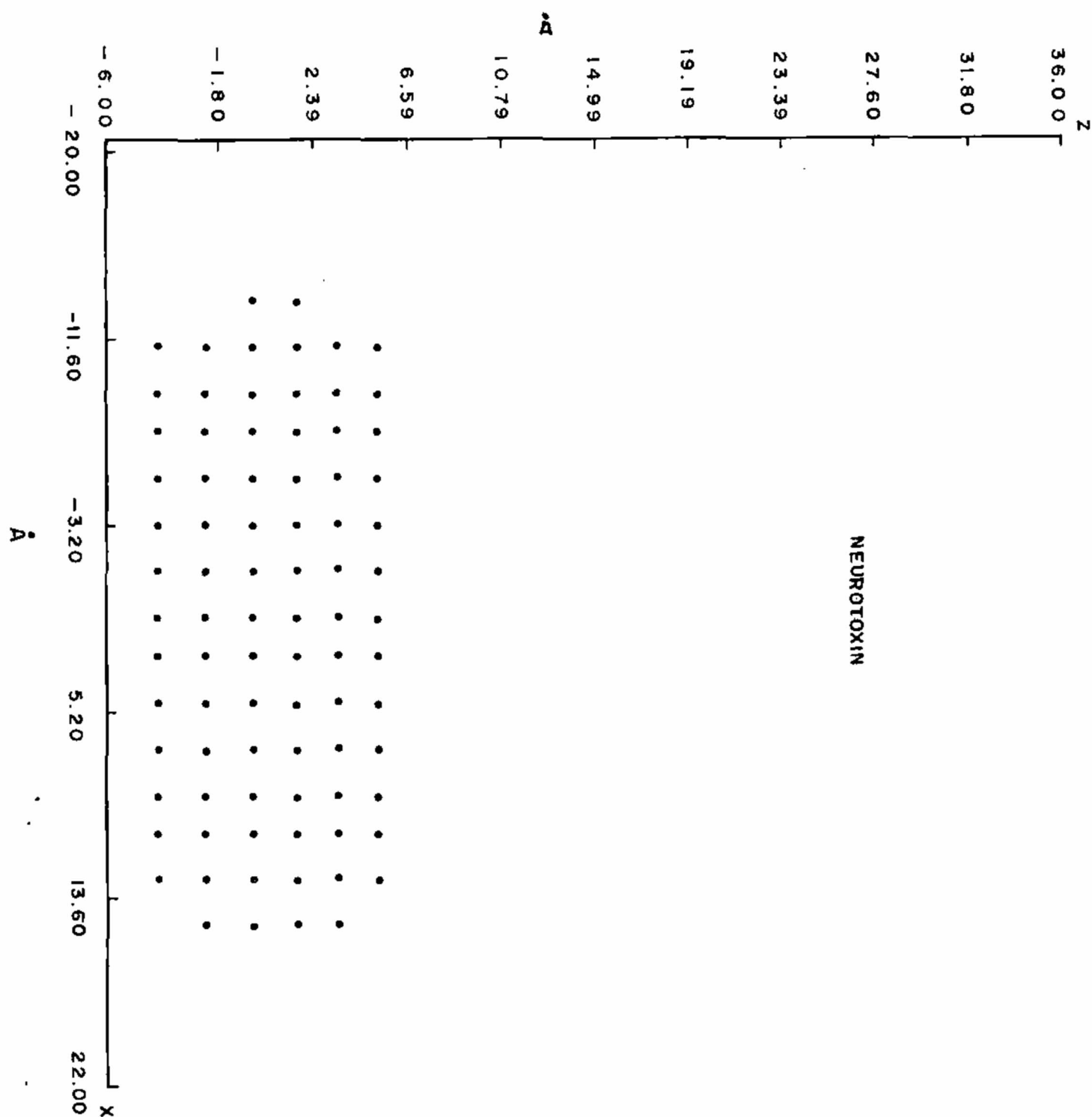


Fig. 2.7c Shape of the neurotoxin molecule obtained from "sphere method" of data analysis (Z-X plot).

CYTOTOXIN

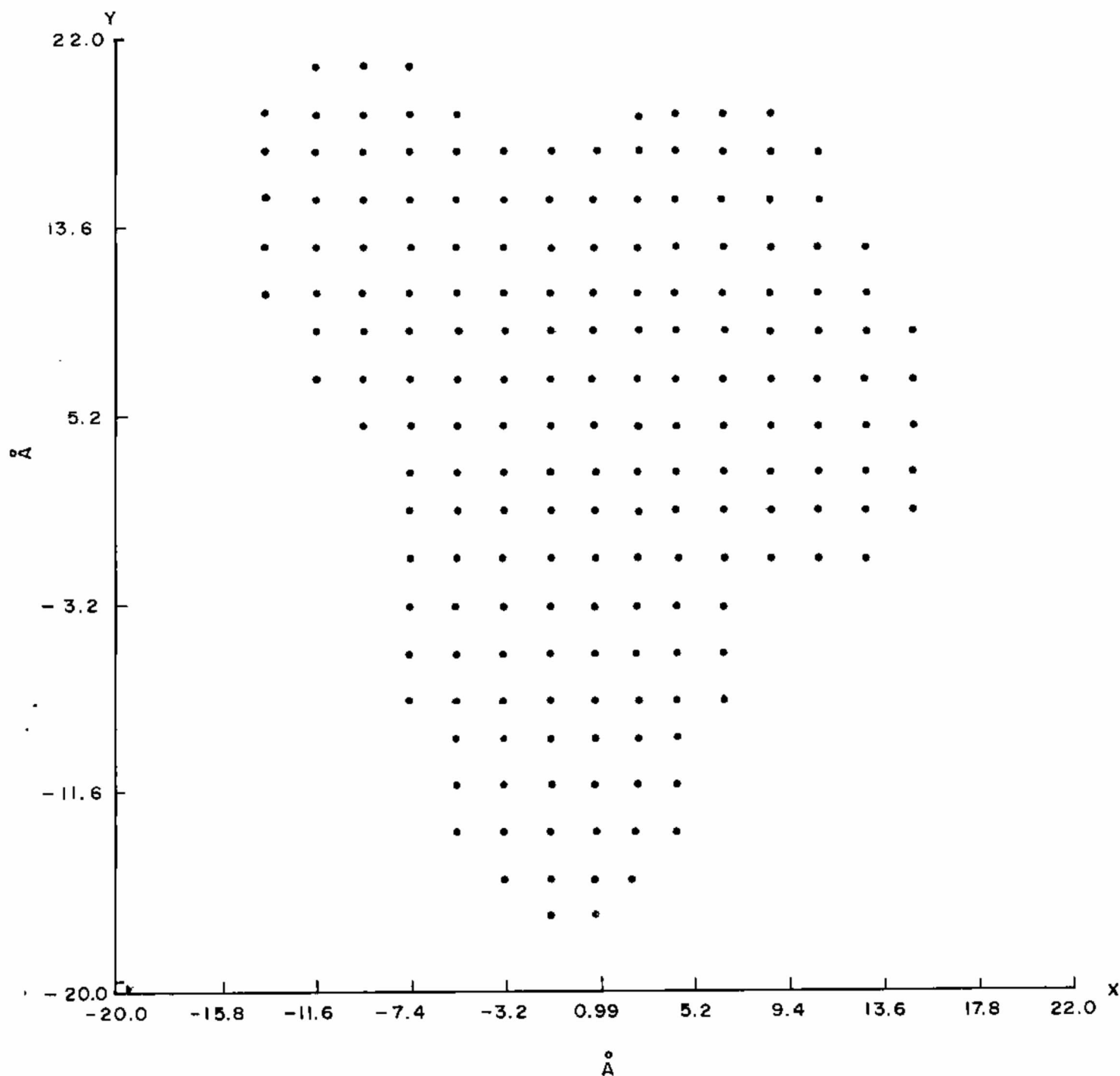


Fig. 2.8a Shape of the cytotoxin molecule obtained from "sphere method" of data analysis (Y-X plot).

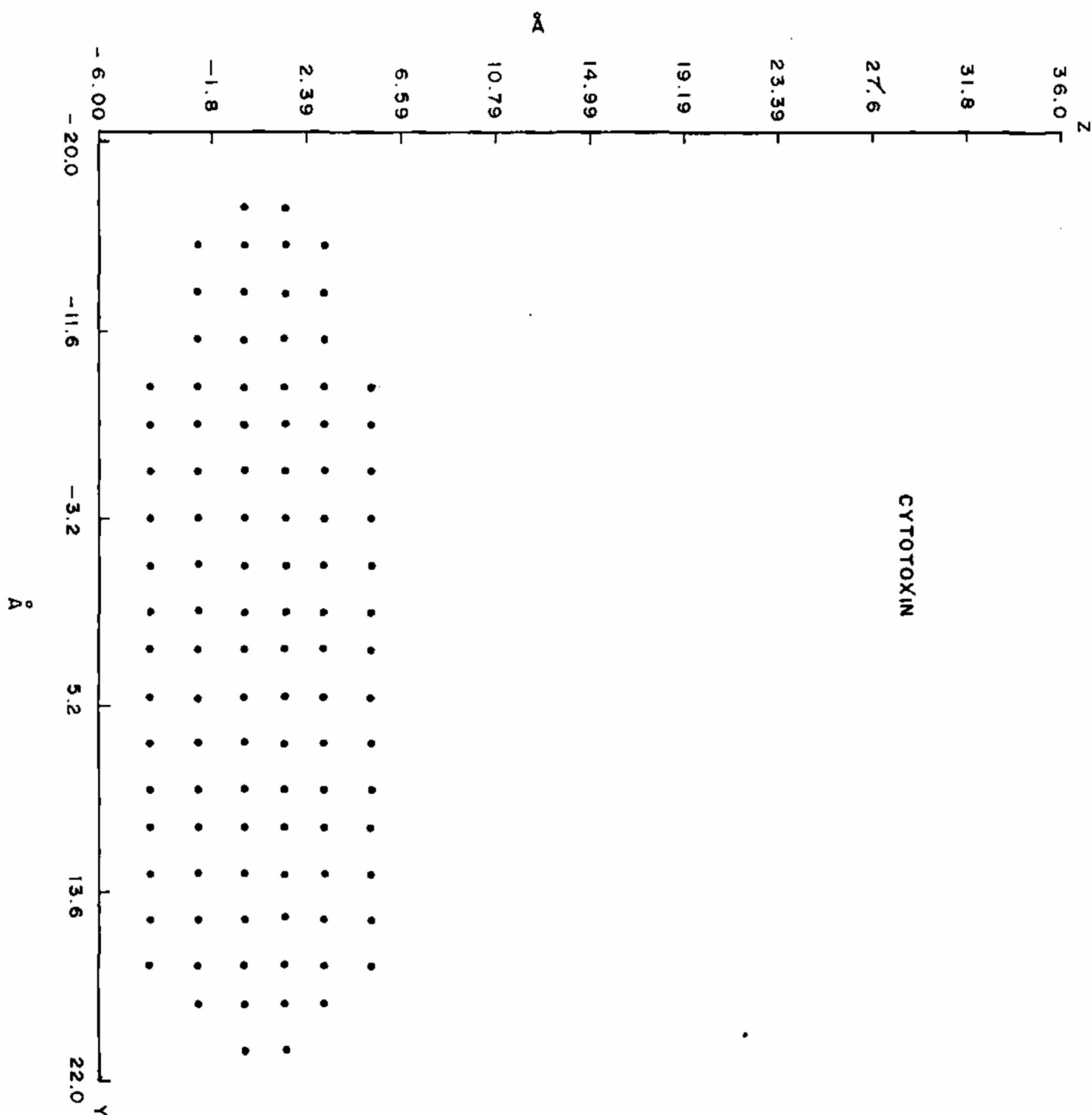


Fig. 2.8b Shape of the cytotoxin molecule obtained from "sphere method" of data analysis (X-Z plot).

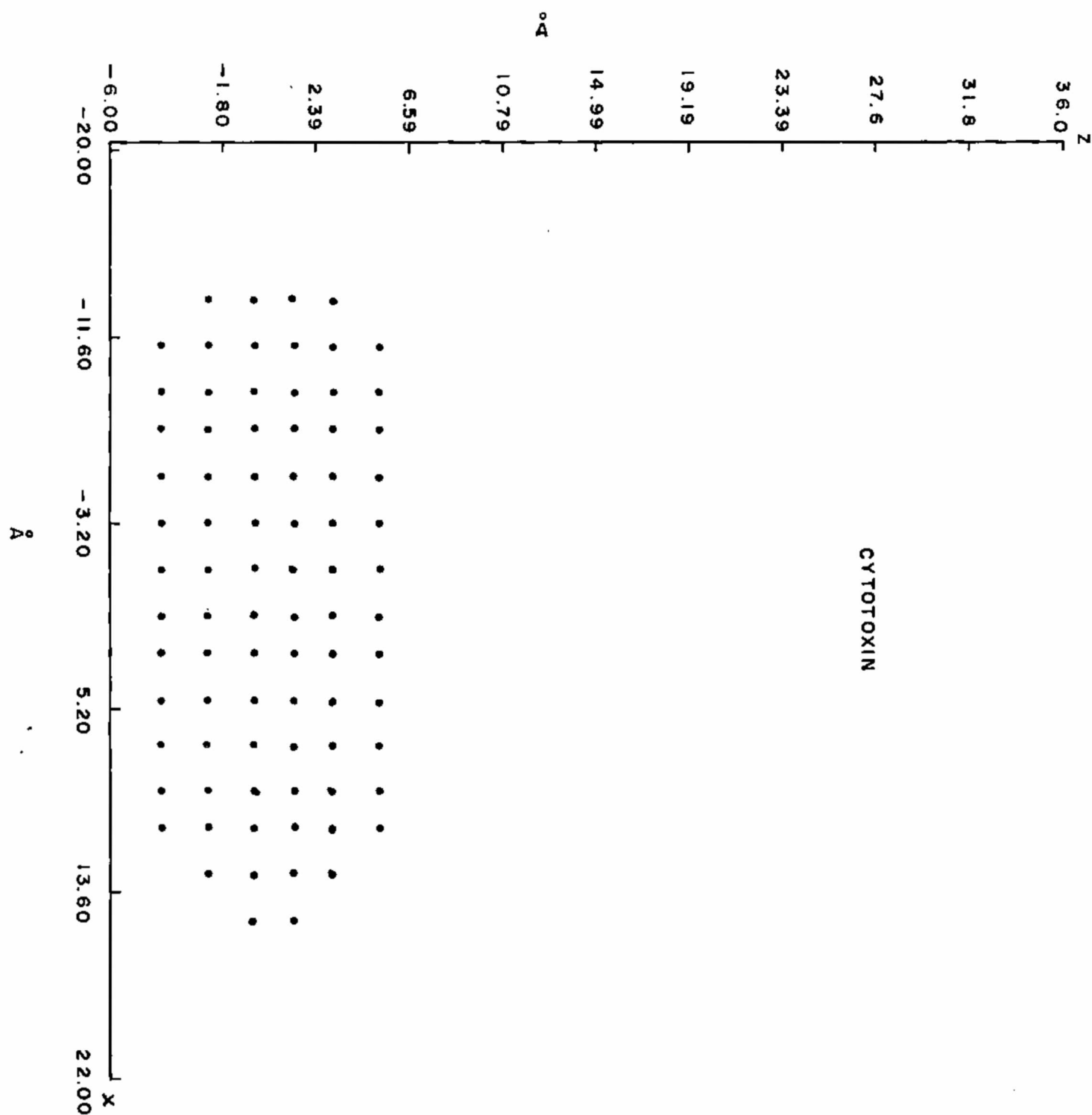


Fig. 2.8c Shape of the cytotoxin molecule obtained from "sphere method" of data analysis (Z-X plot).

CYTOTOXIN ANALOG

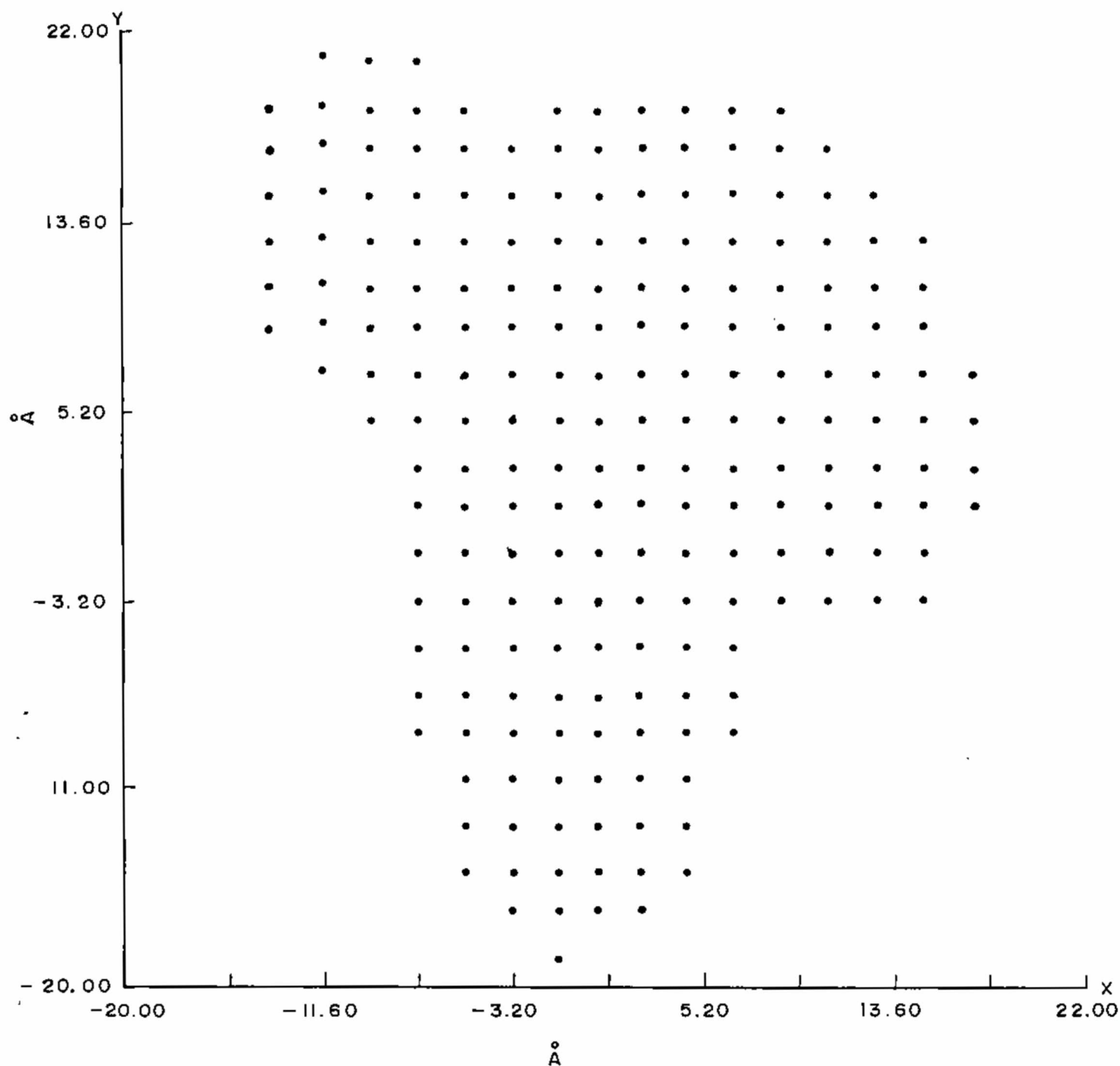


Fig. 2.9a Shape of the cytotoxin analog molecule obtained from "sphere method" of data analysis (Y-X plot).

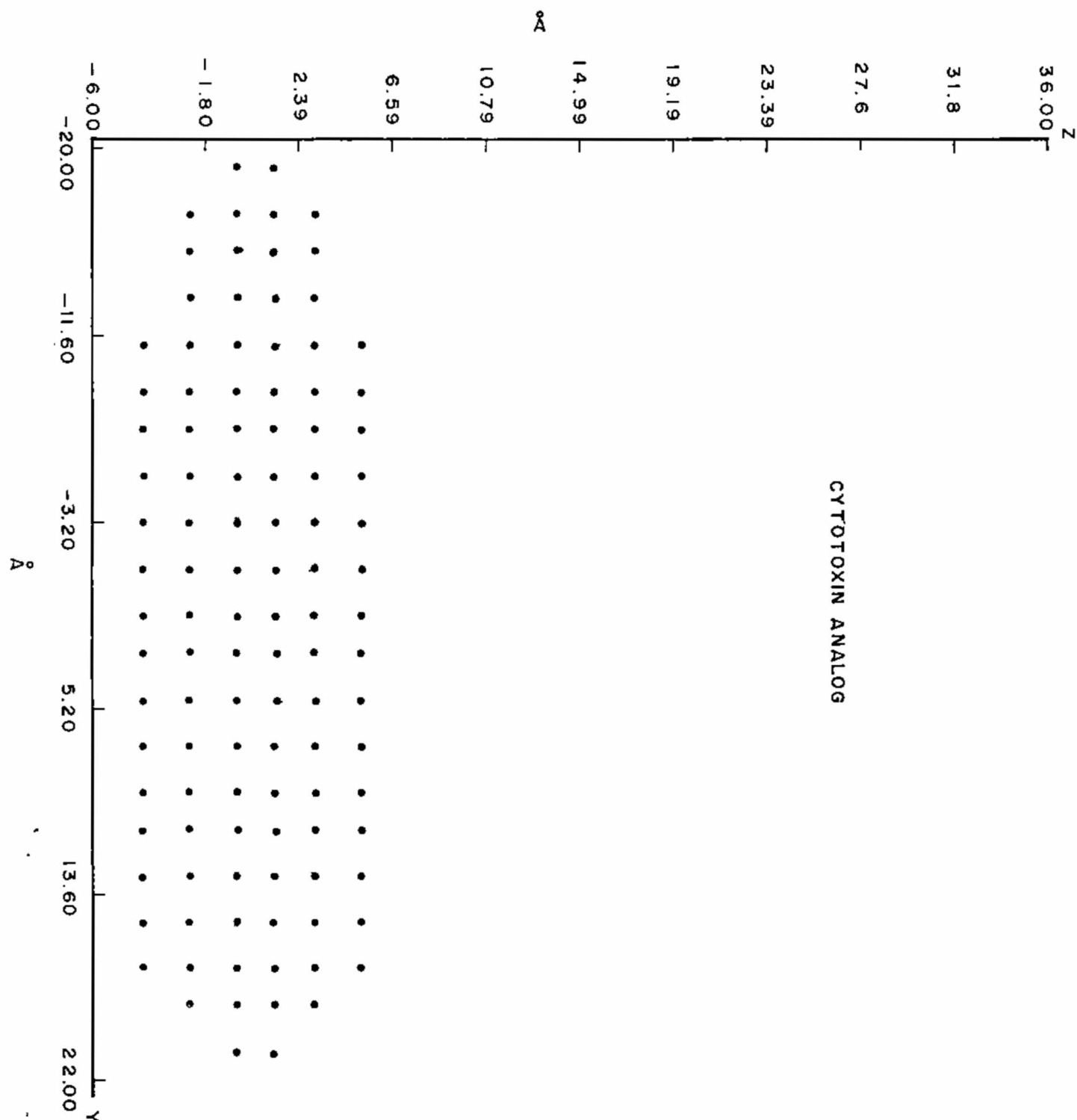


Fig. 2.9b Shape of the cytotoxin analog molecule obtained from "sphere method" of data analysis (X-Z plot).

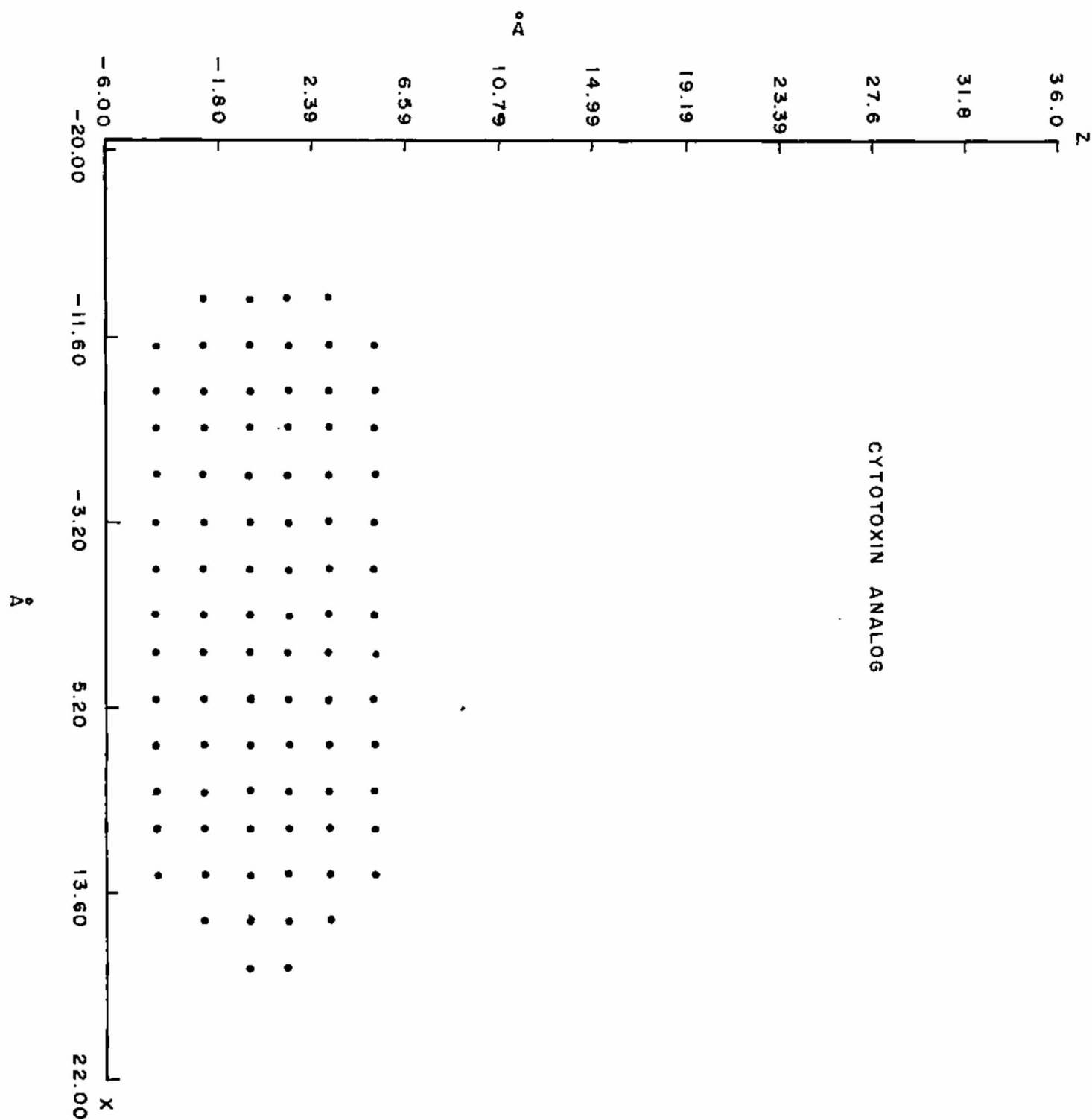


Fig. 2.9c Shape of the cytotoxin analog molecule obtained from "sphere method" of data analysis (Z-X plot).

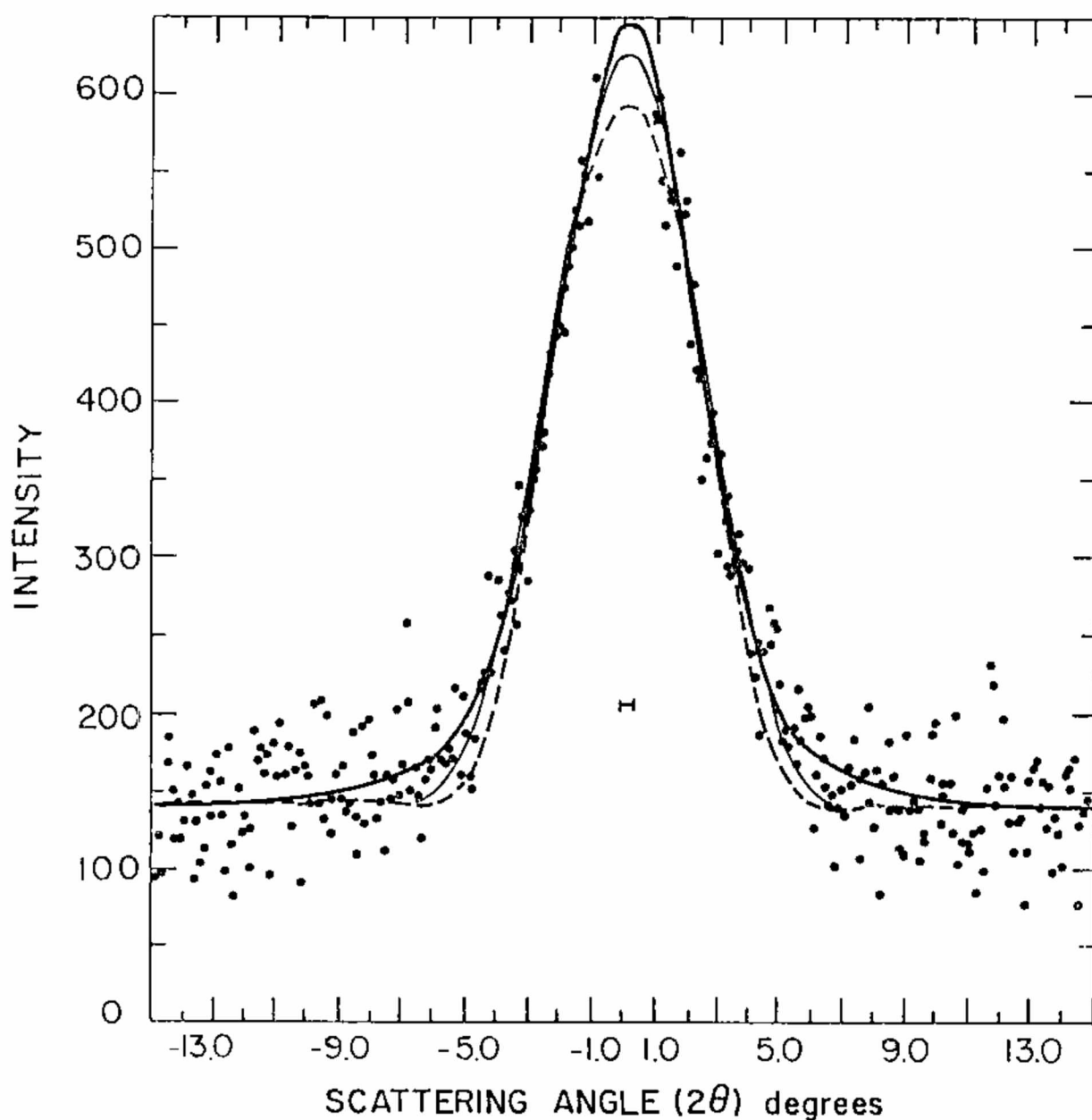


Fig. 2.10 SANS data for neurotoxin. The zero angle scattering occurs at angle increment number 151. Increments in the scattering angle, 2θ , were 0.1° . The full thick, full thin and dashed lines represent a computer fit assuming an arbitrary, ellipsoidal and spherical shapes for the molecule respectively.

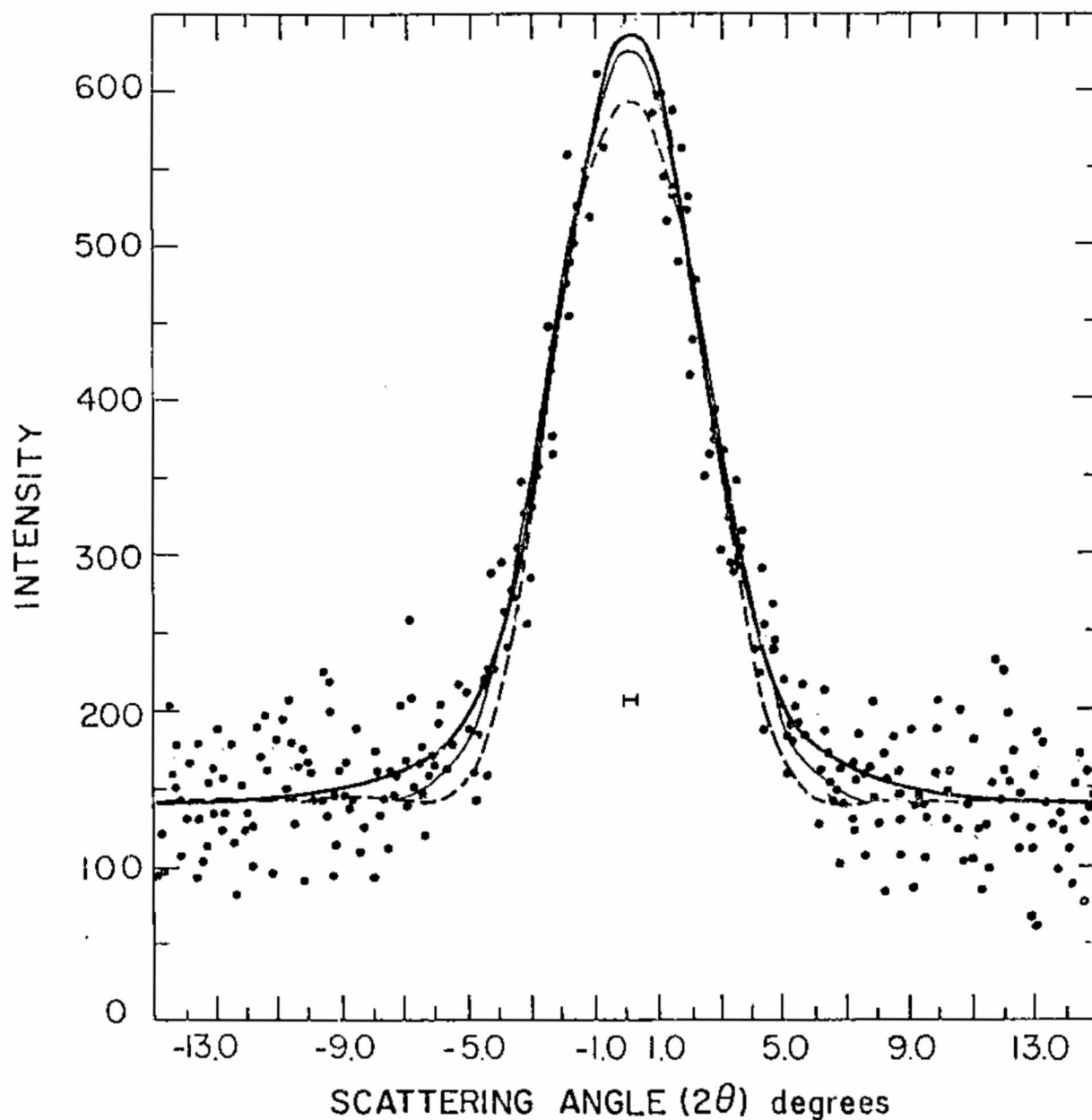


Fig. 2.11 SANS data for cytotoxin. The zero angle scattering occurs at angle increment number 151. Increments in the scattering angle, 2θ , were 0.1° . The full thick, full thin and dashed lines represent a computer fit assuming an arbitrary, ellipsoidal and spherical shapes for the molecule respectively.

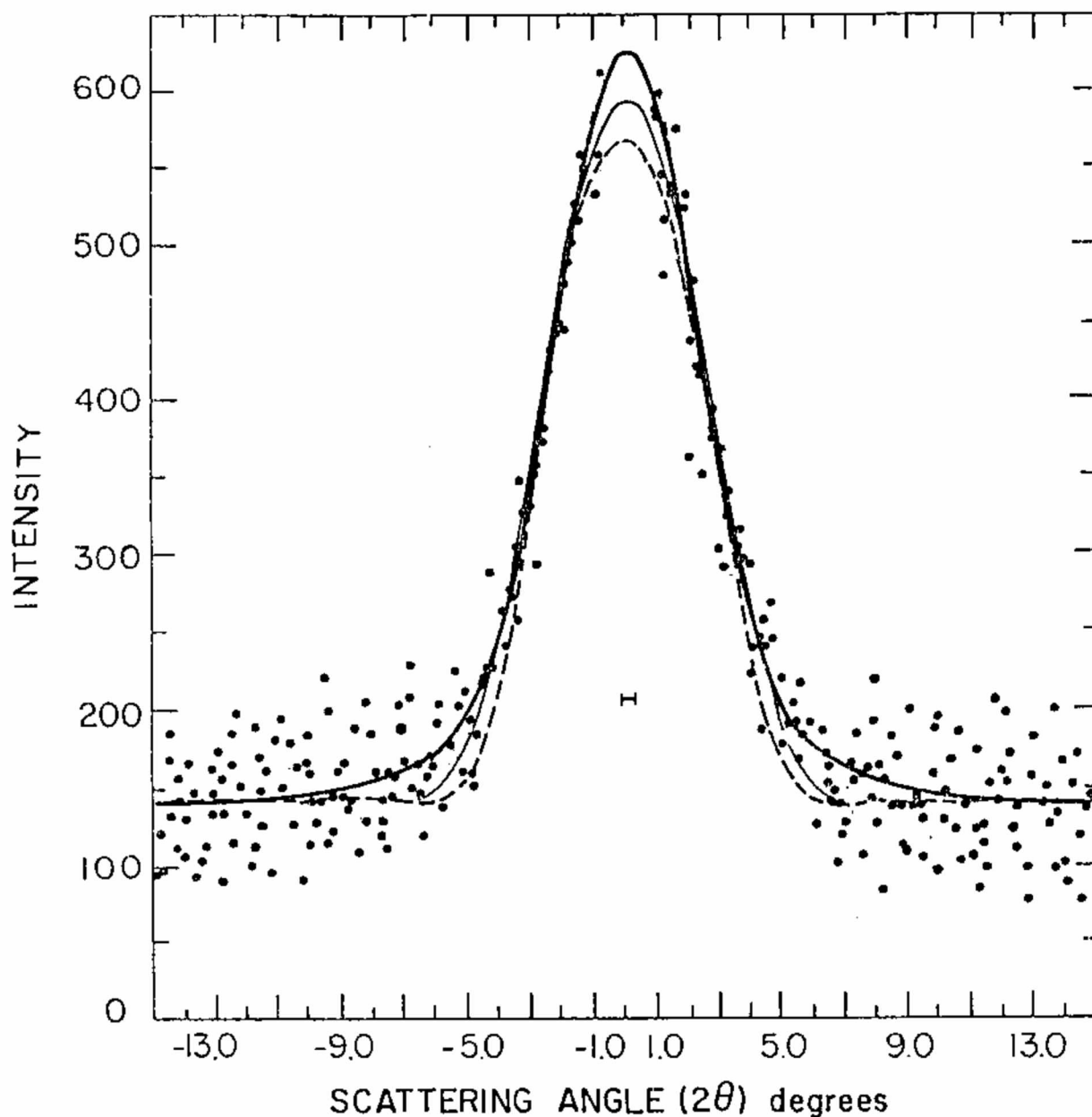


Fig. 2.12 SANS data for cytotoxin analog. The zero angle scattering occurs at angle increment number 151. Increments in the scattering angle, 2θ , were 0.1° . The full thick, full thin and dashed lines represent a computer fit assuming an arbitrary, ellipsoidal and spherical shapes for the molecule respectively.

than spherical model. The semi-axes for all three molecules are given in Table 2.2, where it is seen that cytotoxin and cytotoxin analog are essentially oblate spheroids of revolution with one very small semi-axis. Neurotoxin also has one very small semi-axis, but the other two, although much larger, are not equal to one another. Three unequal semi-axes for neurotoxin have also been found from the "Richards box" approximation to its crystallographic structure [100]. These values were 15, 10 and 20 Å. Our values for ellipsoidal model for neurotoxin are: 14.8 ± 0.9 , 6.8 ± 0.5 and 22.5 ± 0.8 Å. It gives some differences in the molecular shape. Actually the molecules have no regular shape like sphere or ellipsoid, but analysis shows that these are more ellipsoidal than spherical. The molecules of neurotoxin, cytotoxin and cytotoxin analog have their arbitrary shapes. It has been discussed earlier that a computer program [94] has been developed for analysis of neutron small angle scattering for molecules of arbitrary shape. This method of analysis enabled us to find out the arbitrary shapes of the toxin molecules. The goodness of fits are better than those of spherical and ellipsoidal models. The fitted values for arbitrary shape of molecules have been depicted as thick solid lines in Figs. 2.10, 2.11 and 2.12.

A model independent analysis can be done by the Guinnier plots as shown in Fig. 2.13. These data were analyzed by using the Guinnier expression [92], where $J(0)$ is the intensity at $Q=0$ and R_g is the radius of gyration which was mentioned in section 2.2.1 in chapter 2. If the data are plotted as in Fig. 2.8 then

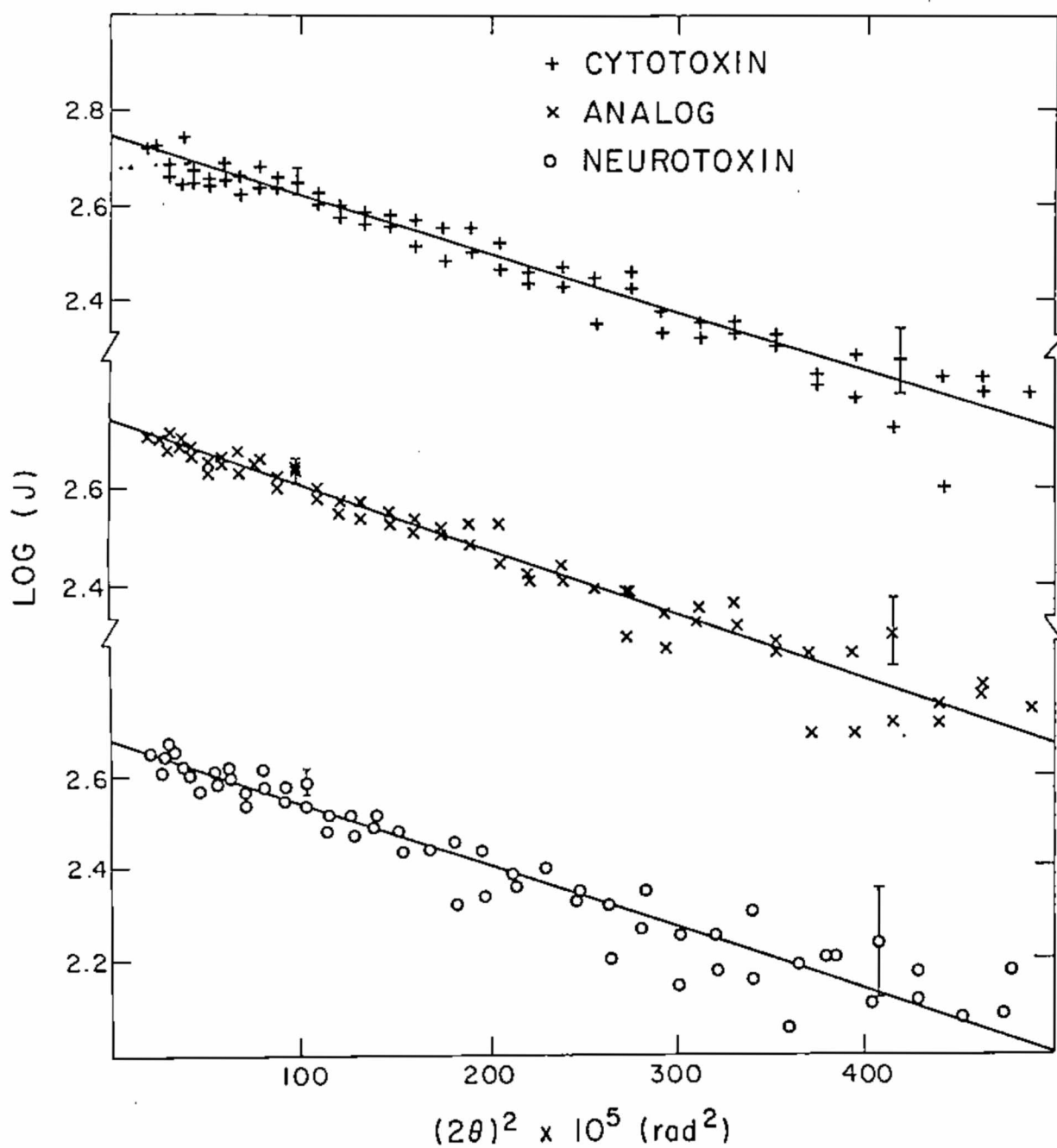


Fig. 2.13 Guinier plot of data for the neurotoxin, cytotoxin and cytotoxin analog. The straight lines represent least squares fits. J represents observed intensity.

$R_g = 0.4183 \lambda (-\alpha)^{1/2}$, where α is the slope of the fitted straight line through the data [27]. From a least square analysis for ellipsoidal shape, R_g 's for neurotoxin, cytotoxin and cytotoxin analog were found to be 12.4 ± 0.2 , 11.7 ± 0.2 and 12.1 ± 0.2 Å respectively (Table 2.2). From the least squares analysis for arbitrary shape as shown in Figs. 2.7a - 2.9c R_g 's for neurotoxin, cytotoxin and cytotoxin analog were to be 12.1 ± 0.2 , 11.6 ± 0.2 and 12.1 ± 0.2 Å respectively (Table 2.3). These results show that cobra toxin molecules are more or less ellipsoidal in shape but the actual shapes can only be shown through the analysis of sphere method, considering that the molecules are arbitrary in shape. Before developing the method for analysis of the SANS data for molecules of arbitrary shape, the experimenters could only say that the toxin molecules are ellipsoidal in shape with different three semi-axes but the present sphere method of analysis enables us to obtain results much nearer to the actual shape of the toxin molecules as in Figs. 2.7a - 2.9c. The analysis shows that the cytotoxin has a significantly lower R_g in keeping with the fact that it is a less elongated molecule than the analog. It also has a smaller molecular weight. From Table 2.3 we also see that the measured R_g 's agree within experimental error with those calculated from arbitrary shape of model.

Table 2.2

Parameters from least squares fits assuming ellipsoids having semi-axes a , b , c , and their resultant radii of gyration R_g' , compared with measured values, R_g . The R_g' values are calculated from "best fit" values for a , b , c . All dimension are in Å.

Molecule	a	b	c	R_g'	R_g
	Å	Å	Å	Å	Å
-----	---	---	---	----	-----
Neurotoxin	14.8 ± 0.9	6.8 ± 0.5	22.4 ± 0.8	12.4	12.2 ± 0.2
Cytotoxin	17.8 ± 0.7	6.7 ± 0.5	17.9 ± 0.7	11.7	11.6 ± 0.2
Cytotoxin A	18.6 ± 0.5	6.0 ± 0.4	18.6 ± 0.5	12.1	12.1 ± 0.2

Table 2.3

Parameters from least squares fits assuming arbitrary shapes (approximated with three ellipsoids) and their resultant radii of gyration R_g , compared with measured values, R_g .

Molecules	Shape and Size	R_g	R_g
		$\text{\AA}$	$\text{\AA}$
Neurotoxin	as in Figs. 2.7a - 2.7c	12.1 ± 0.2	12.2 ± 0.2
Cytotoxin	as in Figs. 2.8a - 2.8c	11.6 ± 0.2	11.6 ± 0.2
Cytotoxin A	as in Figs. 2.9a - 2.9c	12.1 ± 0.2	12.1 ± 0.2
	Volume ($\text{\AA}^3$)	χ^2 (without convol.)	χ^2 (with convol.)
Neurotoxin	6917.89	0.4823	0.4719
Cytotoxin	6850.65	0.4612	0.4455
Cytotoxin A	7387.96	0.4631	0.4478

As noted earlier, the crystallographic results of Walkinshaw et al. [100] indicate that the "long" neurotoxin of *Naja naja siamensis* may be roughly approximated by an ellipsoid with semi axis of 15, 10 and 20 $\text{\AA}$ but our present analysis for arbitrary shape of molecules allowed us to show the arbitrary shape of the toxin molecules as Figs. 2.7a - 2.9c. The radius of gyration of

such a body is 12 Å, a value which is very close to our measured value of 12.2 ± 0.2 Å. Using atom positions supplied by these authors [100] we calculated a mechanical radius of gyration of 13.4 Å. This radius of gyration weighted by neutron scattering lengths is found to be ≈ 13.5 Å. The sources of discrepancy between these various R_g 's are not all known. It may be that, peripheral atoms in the crystallographic structures are not seen by the low resolution SANS measurements. In a sense "Richard box" determination ($R_g = 12.0$ Å) is a low resolution result. At our concentrations (8 g/l) interference effects due to molecular proximity are considered to be much too small to account for the difference R_g 's of 12.2 and 13.5 Å.

Previous gel filtration measurements on the neurotoxin [77] indicated that up to 10% of the molecules could be dimerized upon lyophilization of ion exchange fractions. Earlier report of gel preparation studies of the cytotoxin were contradictory. Reed-Larsen [95] indicated that it was a dimer while Takechi and Hayashi [101] stated that it was monomer. However, because the low angle data are well fitted by a single gaussian, with R_g in fair agreement with crystallographic data, we conclude that these three constituents of cobra venom do not associate to form dimers or higher order aggregates in solution. This conclusion is reinforced by the fact that the intensity of SANS varies as the square of the particle volume. Similarly analytical gel filtration experiments also indicate that the three cobra toxins exist as monomers in solution (Fig. 2.1). K_{av} is plotted in Fig. 2.1 as a function of molecular weight. The distances of D

(cytotoxin) and E (cytotoxin analog) from the regression line are considered not to be great enough to imply aggregation. This result is important since the small size of these molecules leads to low intensities of SANS and hence a comparison study is best made at fairly higher concentration (in this experiment, 8 g/l).

In conclusion, it appears that in spite of the diverse functions of the three cobra toxins studied, their overall sizes and shapes are not substantially different. The shape of the three toxins are almost the same. It has been earlier mentioned that neurotoxin and cytotoxin analog molecules are longer than cytotoxin molecule. Nevertheless, because the neurotoxin appears to have a shape different from that of other two toxins, it seems that a "lock and key" mechanism may be involved at some stage in toxin-substrate interactions. There are other indirect evidences including NMR [102], circular dichroism [103] and laser Raman [104] spectra results which indicate that the secondary and tertiary structures of the neurotoxins and cytotoxins are quite similar. However, slight differences in overall form could lead to initial identification of possible receptor sites for the different toxins. After initial identification by form, specific local modifications probably give functional specificity in the manner described by Walkinshaw et al. [100] for acetylcholine receptor binding by the neurotoxin.

CHAPTER 3

SMALL ANGLE NEUTRON DIFFRACTION FROM BIOLOGICAL MATERIALS

3.1 Introduction

Small angle neutron diffraction technique is utilized to study the structure of biological materials such as membranes [54,55,57] and location of some substances incorporated with membranes [45,56]. We have studied the location of Δ^8 -tetrahydrocannabinol (THC), Δ^9 -THC and cholesterol embedded in membranes [47].

There are many reports in the literature concerning the physiological effects of cannabis sativa, Indian hemp. Some of the reportedly deleterious effects of these substances include lymphocytic transformation and modifications in spermatogenesis [105], interference with ovarian cell production of progesterone [106], and possible changes in the psyche [107,108]. There are also reports of beneficial effects such as prevention of extreme nausea in patients undergoing cancer therapy and of intraocular pressure in glaucoma [108]. Plainly, substances which have such a wide variety of physiological effects must interact with a cell component which is common to most cells. Although fatty tissue is known to harbor metabolites of Δ^9 -THC (the main psychoactive component of cannabis) for up to eight days [108] only a limited number of investigations [109,110] have seriously considered a fatty constituent of all cells, namely their lipid membrane

walls, as a possible repository for cannabinoids. This appears to be so in spite of the fact that the primary action site of "soft" drugs and anesthetics is generally thought to be the cell membrane [110,111]. However, electron spin resonance studies [109] have indicated that Δ^9 -THC has the ability to disorder liposome bilayers and measurements on the inhibition of the synaptosomal enzyme acyltransferase [110] indicate that cannabinoids are lipophilic to the extent that they strongly affect this membrane-bound protein.

This first report of neutron diffraction from cannabinoids in membranes indicates that they can embed themselves in the hydrocarbon region of phospholipid membranes. Our conclusions are based in large part on a comparison study using cholesterol, whose location in membrane bilayers has been determined in previous experiments [112,113] on membrane systems similar to dipalmitoylphosphatidylcholine (DPPC).

3.2 Theory

3.2.1 Small Angle Neutron Diffraction

The principles of neutron diffraction are very similar to those for X-rays but some important differences which are due to the differences in properties of two types of radiations exist. This has been discussed earlier.

In diffraction experiments, measurement is made of the intensity of radiation of a particular wavelength, scattered at an angle 2θ with respect to the direction of the incident beam. If the scattering specimen consists of layered repeating structures of regular interval of spacing d , then the scattered radiation experiences interference which is constructive only in directions given by the Bragg relation:

$$n\lambda = 2d \sin\theta \quad (3.1)$$

where n is an integer referred to as the order of the one dimensional diffraction.

Let us consider the scattering expected from a periodic sample like crystal lattice or biological membranes. The scattering potential appropriate for isotropic scattering in Born approximation is best represented by the Fermi pseudo-potential

$$V(\mathbf{r}) = (2\pi\hbar^2/m)b \delta(\mathbf{r}-\mathbf{R}) \quad (3.2)$$

where $\mathbf{R}$ is the position of the nucleus with neutron scattering length b . For an array of nuclei at $\mathbf{R}_j$ having scattering amplitudes b_j

$$V(\mathbf{r}) = (2\pi\hbar^2/m) \sum_j b_j \delta(\mathbf{r}-\mathbf{R}_j) \quad (3.3)$$

with this $V(\mathbf{r})$ the master formula for the scattering cross-section is

$$\frac{\partial^2 \sigma}{\partial \Omega \partial E} = k/k_0 \sum_{A_0 \sigma_0} P_{A_0} P_{\sigma_0} \sum_{A \sigma} |\langle \sigma A | \sum_j b_j \exp[i(\mathbf{Q} \cdot \mathbf{R}_j)] | \sigma_0 A_0 \rangle|^2 \times \delta(\hbar\omega + E_A - E_{A_0}) \quad (3.4)$$

where $|A_0\rangle$ are the initial scatterer states with population P_{A_0} and $|\sigma_0\rangle$ are the initial neutron spin states with population P_{σ_0} . For elastic scattering $d\sigma/d\Omega$ will be

$$\begin{aligned} d\sigma/d\Omega &= \sum_{A_0 \sigma_0} P_{A_0} P_{\sigma_0} \sum_{j_0} \exp[i\mathbf{Q} \cdot (\mathbf{R}_{j_0} - \mathbf{R}_j)] \langle \sigma_0 A_0 | b_j^* b_{j_0} | \sigma_0 A_0 \rangle \\ &= \sum_{j_0} \exp(i\mathbf{Q} \cdot (\mathbf{R}_{j_0} - \mathbf{R}_j)) \overline{b_j^* b_{j_0}} \end{aligned} \quad (3.5)$$

$$\text{where } \overline{b_j^* b_{j_0}} = \sum_{A_0} P_{A_0} \langle A_0 | b_j^* b_{j_0} | A_0 \rangle \quad (3.6)$$

$$\begin{aligned} \overline{b_j^* b_{j_0}} &= \overline{b_j^*} \overline{b_{j_0}} = |\overline{b}|^2 \quad \text{for } j=j_0 \quad \text{and} \\ \overline{b_j^* b_{j_0}} &= \overline{b_{j_0}} \overline{b_j} = \overline{b} \overline{b}^* \quad \text{for } j \neq j_0 \end{aligned} \quad (3.7)$$

In general we can write

$$\overline{b_j^* b_{j_0}} = |\overline{b}|^2 + \delta_{jj_0} (\overline{b^2} - |\overline{b}|^2) \quad (3.8)$$

The expression for $d\sigma/d\Omega$ contains information about both the coherent and incoherent scattering

$$d\sigma/d\Omega = (d\sigma/d\Omega)_{\text{coh}} + (d\sigma/d\Omega)_{\text{incoh}} \quad (3.9)$$

where

$$(d\sigma/d\Omega)_{coh} = \left| \sum_j b_j \exp(iQ \cdot R_j) \right|^2 \quad (3.10)$$

and

$$(d\sigma/d\Omega)_{incoh} = [\overline{|b|^2} - |\bar{b}|^2] \quad (3.11)$$

The coherent scattering cross-section is maximum for $Q=0$ or $Q =$ any point in reciprocal lattice i.e.

$$(d\sigma/d\Omega)_{coh} = N(2\pi)^3/v_o \sum_h \delta(Q-h) |F_N(h)|^2 \quad (3.12)$$

which in fact Bragg reflection condition for h , and N is the number of unit cell and v_o is the volume of the unit cell. Here $F_N(h)$ is

$$F_N(h) = \sum_j b_j \exp(2\pi i h \cdot R_j) \quad (3.13)$$

where h is the reciprocal lattice vector.

The structural analysis of biological materials consists in finding a structure whose diffraction spectra match the observed set. The structure factors are dependent on structure parameters of the specimen. To study the biological materials which have the layered structures such as membranes, we have to calculate the structure factors $F(n)$ for order n . The structure factors $F(n)$ in different relations give lot of information about the structure of the sample. Our aim was to know the structure of phospholipid membranes containing Δ^8 -THC, Δ^9 -THC and cholesterol inside the

phospholipid membranes. A centrosymmetric membrane was assumed as in Fig 3.1 and the neutron scattering length density $\rho(x)$ is given by

$$\rho(x) = F(0)/d + 2/d \sum_{h=1}^N F(h) \cos(2\pi hx)/d \quad (3.14)$$

where $F(h)$ is the structure factor of the h th peak and d is the repeating space. By Fourier inversion of equ.(3.14) it follows that

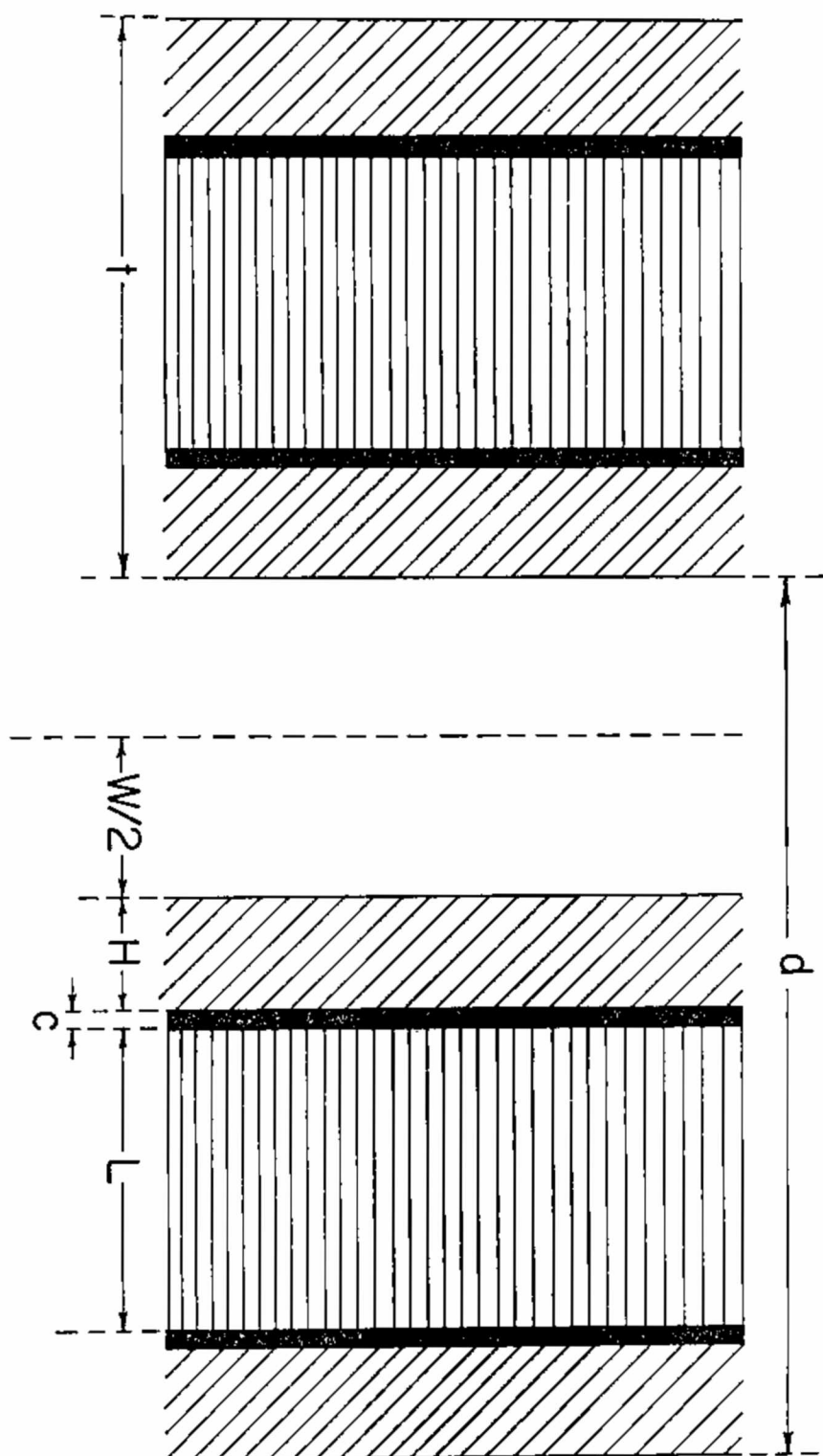
$$F(h) = 2 \int_0^{\frac{d}{2}} \rho(x) \cos(2\pi hx/d) dx \quad (3.15)$$

According to Fig.3.1 d is given by

$$\begin{aligned} d &= W + 2H + 2C + L \\ d/2 &= W/2 + H + C + L/2 \end{aligned} \quad (3.16)$$

where W , H , C and L are the lengths for D_2O , head group, cannabinoid and lipid region respectively. Accordingly $\rho(x)$ can be identified as

$$\rho(x) = \begin{cases} \rho_w, & 0 < x < (W/2) \\ \rho_h, & (W/2) < x < (W/2+H) \\ \rho_c, & (W/2+H) < x < (W/2+H+C) \\ \rho_p, & (W/2+H+C) < x < (W/2+H+C+L/2) \end{cases} \quad (3.17)$$



$$d = t + W = 2H + 2C + L + W$$

Fig. 3.1 A schematic diagram of two lipid bilayers separated by an aqueous region of thickness t . The Bragg repeat spacing observed by diffraction is labelled d . The polar head group region is of thickness H , and the hydrocarbon tail region is of thickness L . A possible site of cannabinoid or cholesterol inclusion in the hydrocarbon tail region is labelled C .

If we substitute the above values for d and $\rho(x)$ in equ.3.15 we get for $F(n)$

$$F(n) = 2 \int_0^{W/2} \rho_W(x) \cos(2\pi nx/d) dx$$

$$+ 2 \int_{W/2}^{W/2+H} \rho_H(x) \cos(2\pi nx/d) dx$$

$$+ 2 \int_{W/2+H}^{W/2+H+C} \rho_C(x) \cos(2\pi nx/d) dx$$

$$+ 2 \int_{W/2+H+C}^{W/2+H+C+L} \rho_L(x) \cos(2\pi nx/d) dx$$

$$F(n) = 2 \rho_W(x) \int_0^{W/2} \cos(2\pi nx/d) dx$$

$$+ 2 \rho_H(x) \int_{W/2}^{W/2+H} \cos(2\pi nx/d) dx$$

$$+ 2 \rho_C(x) \int_{W/2+H}^{W/2+H+C} \cos(2\pi nx/d) dx$$

$$+ 2 \rho_L(x) \int_{W/2+H+C}^{W/2+H+C+L} \cos(2\pi nx/d) dx$$

(3.18)

The integrated intensities of the Bragg reflections obtained in a diffraction experiment are proportional to the square of the structure factor $F(n)$. The magnitude of $F(n)$ is determined experimentally from integrated intensity $I(n)$ of the n th peak by relation

$$F(n) = [(\sin\theta_n \cdot \sin 2\theta_n) \cdot I(n)]^{1/2} \quad (3.19)$$

where $2\theta_n$ is the Bragg angle of the n th order diffraction and $(\sin\theta_n \cdot \sin 2\theta_n)$ is the reciprocal Lorentz correction factor for cylindrical sample [114]. The sample Δ^8 -THC, Δ^9 -THC and cholesterol in membranes obviously have no preferred orientation in cylindrical quartz tubes. At this stage the diffraction phenomenon shows a cylindrical symmetry around the incoming monoenergetic beam. Each volume element in the sample diffracts the neutrons that are scattered by a given set of constituent planes of membranes on a cone, diffraction cone with semi-angle equal to the Bragg angle $2\theta_n$. In most of the experimental techniques, all the intensity in the diffraction cone is not collected. A part of intensity is sampled and this collected part is related to the total diffraction intensity through a geometrical factor which takes into account the fraction of the sample material that is in reflection orientation for particular reflection. This factor can be called as a "sample factor" and it is seen to be $(\sin\theta_n)^{-1}$. The factor which is related to the sampled intensity with total cone intensity is strongly dependent on the method of data collection and it may be denoted as "detection factor". For our neutron diffraction experiment the

data were collected around the cylindrical sample perpendicular to the incoming neutron beam, the data were proportional to a factor $(\sin 2\theta_n)^{-1}$ related to the Bragg angle $2\theta_n$. The complete reciprocal Lorentz factor for such an experiment thus becomes $(\sin \theta_n \cdot \sin 2\theta_n)$.

The unique advantage of neutron diffraction for the elucidation of biological structure is the large difference in scattering length between hydrogen and deuterium [115]. This phenomenon is used to enhance the contrast in a biological membrane. The scattering density, ρ , of a molecular component is given by

$$\rho = \left(\sum_{i=1}^n a_i b_i \right) / V \quad (3.20)$$

where a_i is the number of i th nuclei in the component, b_i is the scattering length of the i th nucleus, and V is the molecular volume [116]. The overall enhancement in contrast, clearly favors the neutron diffraction experiment conducted in heavy water, D_2O . Of particular value is the enhanced contrast between the membrane molecular components obtained by using neutron diffraction rather than X-ray diffraction. For instance, the contrast between D_2O and lipid hydrocarbon, with neutron irradiation, is 8-fold greater than water-hydrocarbon contrast with X-rays. Another powerful technique involves contrast-matching of particular molecular species. Since the densities of the membrane components are intermediate between those of H_2O and of D_2O , the appropriate D_2O concentration can be chosen to match the scattering density

of one of the components with that of the solvent.

The scattering density profiles $\rho(x)$, equation (3.14) reflect the structure of the pure membrane and membrane containing Δ^8 -THC, Δ^9 -THC, and cholesterol. The differences in density profiles of pure membrane and membrane containing Δ^8 -THC, Δ^9 -THC, and cholesterol ensure the inclusion of Δ^8 -THC, Δ^9 -THC, and cholesterol inside the membrane.

One dimensional Patterson analysis may be employed to find out the differences in structures of the pure membrane and membrane containing Δ^8 -THC, Δ^9 -THC, and cholesterol. One dimensional Patterson maps are calculated by Fourier transformation of the reflection intensities $I(n)$

$$P(x) = 2/d \sum_{n=1}^N I(n) \cos(2\pi nx/d) \quad (3.21)$$

where $P(x)$ is the value of Patterson function at a real space distance x and d is the repeat spacing.

The experimental values of structure factors have been compared with the theoretical values, obtained by using equation (3.18). This comparison was made by using least square fitting procedure which fits the different lengths of the constituents of membranes. This structural analysis shows the structure of pure membranes and membranes containing Δ^8 -THC, Δ^9 -THC and cholesterol and ensure the location of Δ^8 -THC, Δ^9 -THC and cholesterol inside the membranes.

3.2.2 Phase Determination of Structure Factors

For the case of centrosymmetric structure, the Fourier expression for the coherent scattering amplitude density becomes a simple Fourier cosine series, shown in equation (3.14). A plus or minus sign for structure factor is used in the summation depending on whether the phase of the reflection is 0 or π . It is very important to determine phase in obtaining accurate structural information for membranes and lamellar structures of membrane components.

There are several methods for phase determination of neutron diffraction studies of membranes [117]. One method is to change the water content between bilayers so that the repeat spacing increases and the continuous Fourier transform of the centrosymmetric unit cell of amplitude of the scattered radiation $F(s)$, where $s = 2\pi \sin\theta/\lambda$

$$F(s) = \int \rho(x) \cos(2\pi sx) dx \quad (3.22)$$

is sampled at different values of s . The minimum wavelength principle of Bragg and Perutz [118] suggests that, the maxima and minima of $F(s)$ are separated by a minimum increment in s which is comparable to the reciprocal of the largest spacing. The sign of $F(s)$ is determined in this way. In studies of lipid bilayers, the water content is usually varied by changing the relative humidity

in the sample container. This method will be valid provided the structure does not change as the water content changes. Although this requirement is not always satisfied, this method retains some use if the structural changes are small and gradual or gradual.

Another method of phase assignment in neutron diffraction involves changing the scattering amplitude of the water in the structure by H₂O-D₂O exchange. The structure factors of the Bragg reflections are linear functions of the isotopic composition of water for a centrosymmetric structure. With simple assumptions about the distribution of water in the bilayers these functions may be interpreted in terms of phase assignments. It becomes very simple when the water is located only at the origin of the repeat unit between adjacent lamellar structures. Due to the large difference between the coherent scattering amplitudes of hydrogen and deuterium, the change of H₂O to D₂O at this position is similar to the X-ray procedure of introducing a heavy atom at the origin of centrosymmetric structure [119]. The Fourier transform of this D₂O-H₂O "heavy atom" is a position constant which is additive to the structure factors of the rest of the structure. On the replacement of H₂O by D₂O depending on whether the structure factors are positive or negative, the diffraction intensities will increase or decrease respectively. An alternative procedure is to calculate the difference Patterson function

$$P(x) = \sum_n (F^2_{D_2O} - F^2_{H_2O})_n \cos(2\pi nx/d) \quad (3.23)$$

which is the convolution of D₂O-H₂O heavy atoms at the centre of symmetry with the rest of the structure. Therefore P(x) gives the bilayer structure. This direct method of structure determination was discussed by Speakman [120].

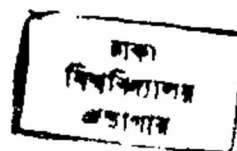
It is not always restricted that the membrane structure is centrosymmetric. In that case above idealized analysis is modified for reasonable assumption that the structure consists of a hydrophobic region. The difference Patterson function becomes

$$P(x) = \sum_n (F_{D_2O} - F_{H_2O})^2_n \cos(2\pi nx/d) \quad (3.24)$$

and the Patterson function is for water distribution. The Patterson coefficients $(F_{D_2O} - F_{H_2O})^2_n$ are determined by measuring structure factors F(n) with three different isotopic compositions of water (H₂O, D₂O, 50%D₂O-50%H₂O). The linearity of F(n) with isotopic composition determines $(F_{D_2O} - F_{H_2O})^2_n$. Considering that there is a hydrophobic region which contains no water, the Patterson function can be interpreted to give an approximate water distribution and which in turn is used to determine phase assignments.

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Another method to determine the phases of the structure factors is to use the chemical knowledge of the membrane structure. We have determined the phases of the structure factors by using the chemical knowledge of the membrane components. We make simple assumptions about the structure and select phase combinations from 2ⁿ (n is the order of structure factor)



possibilities which give Fourier profiles consistent with these assumption [121]. If the chemical and the other structural knowledge is sufficient to propose a model for the structure, then the structure factors can be calculated according to equation (3.18). The phases of the calculated $F(n)$ can then be applied to the experimental $F(n)$ and comparison made between the experimental and model profiles of the scattering amplitude density. According to our calculations the phases of the structure factors $F(0)$, $F(1)$ and $F(2)$ were all found to be +.

3.3 Experimental Methods

3.3.1 Description of the Technique

Usually neutron diffraction technique is used for structural analysis of crystal, biological materials like membranes etc. Cold and thermal neutrons are useful for this case for their right order of wavelengths. For structural analysis the scattered neutron energy is not required. Hence, the crystal spectrometer is used in its double-axis mode for this technique. The reactor neutron beam is collimated as desired by using soller collimators. The background level is maintained very low using sufficient shielding around the monochromator and detector. The higher order contamination is avoided by using filter crystal. In this technique the neutrons scattered by the sample are scanned

with a neutron detector around the sample. The angular step should be very small like 0.05 or 0.01 degree for precisional measurements. In this technique the Bragg spectrum of the sample is detected and this reveals the structure of the sample. The scattered neutrons are detected in the same manner as described in SANS technique in chapter 2. In powder diffraction technique the resolution should be very high. This technique allows the collection of high accuracy data upto high scattering angles. In powder diffraction technique usually the structures of polycrystals are investigated and it needs higher resolution than that in single crystal diffraction measurements. In this technique the detector detects many Bragg angles and through proper analysis the structure factors for each peak are determined.

To investigate the structure of the sample by using the neutron diffraction technique the following stages should be done properly.

- (1) Proper selection and preparation of samples.
- (2) Determination / information of unit cell and space group of crystals and biological materials like membranes.
- (3) Accurate intensity data collection.
- (4) Conversion of intensities to structure factors with proper application of the relevant corrections such as Lorentz correction, background, higher order contamination etc.
- (5) Building of a model in which the arrangement of atoms / layer of atoms is consistent with the data collected:

Fourier synthesis.

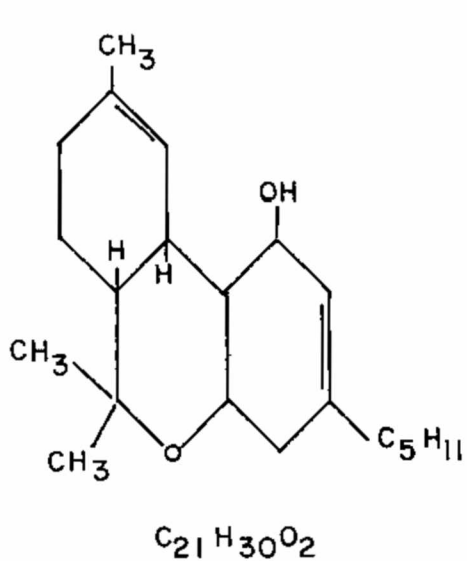
(6) Least square fitting of model for refinement of structure along with tests for the corrections of the structure, the accuracy of coordinates etc.

3.3.2 Materials for Neutron Diffraction Studies

The membrane structure has been shown in the Fig. 3.1, contains lipid, proteins and water. The cell membranes play a vital role for intercellular activities. Membranes are permeable to water and bound the cells of the biological body.

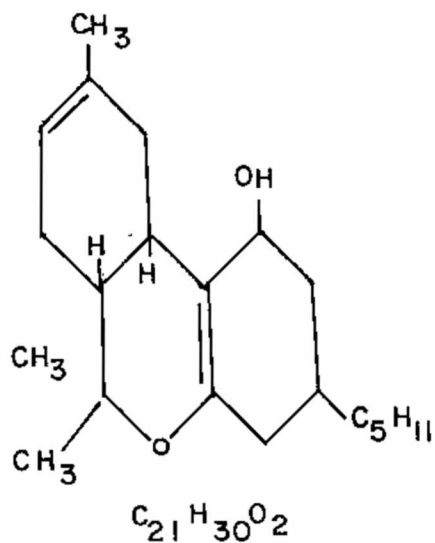
Δ^8 -THC (tetrahydrocannabinoid) and Δ^9 -THC are dangerous drugs. A growing body of evidence from both animal and human studies and from chemical observations attests to their deleterious effects on behaviour, performance and functioning of various organ systems. The chemical structure of Δ^8 -THC and Δ^9 -THC are shown in Fig. 3.2a and 3.2b.

Perhaps cholesterol is the most important sterol. It is widely distributed and has been known and studied for many years. Its chemical structural formula is shown in Fig. 3.2c. Since there are eight asymmetric carbons, theoretically 256 stereoisomers are possible. The hydroxyl group on carbon-3 is in the position; therefore it is shown with a solid line bond. Cholesterol is probably a constituent of all animal cells. The corpus luteum and the adrenal cortex are particularly rich in



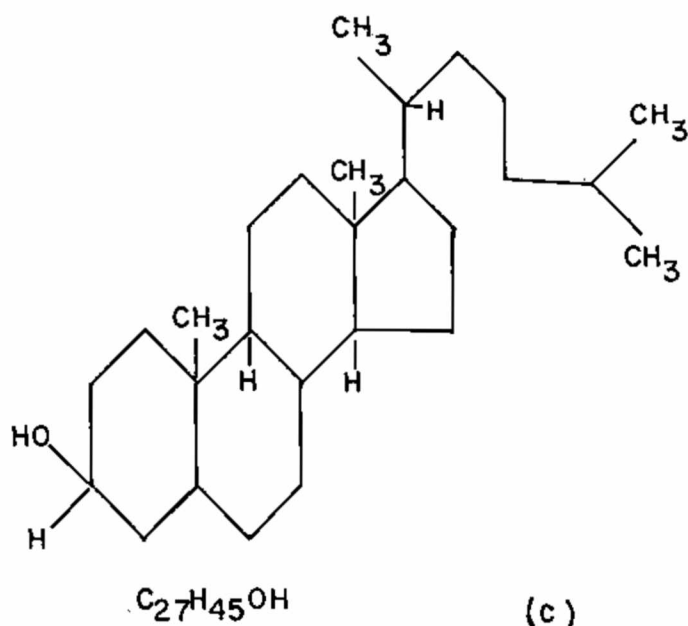
Δ^9 TETRAHYDROCANNABINOL (Δ^9 -THC)

(a)



Δ^8 TETRAHYDROCANNABINOL (Δ^8 -THC)

(b)



(c)

CHOLESTEROL

Fig. 3.2a-3.2c The chemical structure of Δ^9 , Δ^8 and cholesterol respectively.

this lipid. Cholesterol is present in blood and bile and is usually a major constituent of gallstones, from which it was first isolated.

Dipalmitoylphosphatidylcholine (DPPC) was obtained from Avanti Polar Lipids Inc., Birmingham, Alabama, USA, (catalog number 850355) and was found to have the same profile for differential scanning calorimetry as that reported previously by Janiak et al. [122]. Cholesterol (Chol) was obtained from Sigma Chemical Co. of St. Louis, Missouri, USA, and Δ^8 -THC and Δ^9 -THC were provided by the Health Protection Branch of Health and Welfare Canada in Ottawa. Both cannabinoids were found to be at least 99% pure by flame analysis spectroscopy. Various mixtures were prepared gravimetrically and all compositions are expressed as weight fractions. The method used for preparing aqueous dispersions was that of Janiak et al. [122]. Known weights of DPPC, with and without additives were dissolved in chloroform-methanol-ethanol solution (85:10:5) and poured into a glass tube with a narrow constriction at the centre. The solvent was removed by drying in vacuo and appropriate amounts of deuterated water (D_2O) added. The tube was then sealed and its contents centrifuge through the constriction at least 10 times at a temperature of approximately 50°C. Two specimen types were prepared, one containing 50% D_2O and the other, 67% D_2O . The six specimens

studied had the following composition

- I : DPPC_{0.45}(Δ^9 -THC)_{0.05}(D₂O)_{0.50}
- II : DPPC_{0.30}(Δ^8 -THC)_{0.03}(D₂O)_{0.67}
- III : DPPC_{0.30}(Δ^9 -THC)_{0.03}(D₂O)_{0.67}
- IV : DPPC_{0.45}(chol)_{0.05}(D₂O)_{0.50}
- V : DPPC_{0.50}(D₂O)_{0.50}
- VI : DPPC_{0.33}(D₂O)_{0.67}

3.3.3 Experimental Set-up for Neutron Diffraction Studies

The neutron diffraction experiments were carried out by using the triple-axis spectrometers identified as N5 and C5 at N.R.U. reactor (200 MW) at Chalk River Nuclear Laboratories (Fig. 1.1). For these measurements the analyser crystal had no use and the instrument was operated in a double-axis mode. Computer system was coupled with spectrometers and it was able to handle the spectrometer for measurements of many kinds of experimental set-ups. For neutron diffraction studies for above mentioned six samples the experimental set-ups for N5 and C5 triple-axis spectrometers were as follows:

Table 3.1

Experimental set-ups for neutron diffraction measurements.

Spectrometer	Monochromator	Filter	Angular divergence of	
			soller collimators	
			Before	After
			sample (C ₁)	sample (C ₂)
C5	Silicon(111)	Beryllium	1/3°	1/3°
N5	Pyrolitic	Beryllium	1/3°	1/3°
	graphite(002)			

The monitors M₁ and M₂ for both the spectrometes were fission counters. The detectors for both the spectrometers were ³He.

The measurements were carried out on samples contained in sealed 1.5 mm I.D. cylindrical quartz tubes maintained at 30°C. The neutron wave lengths were either 3.99 or 4.08 Å. The higher order beam contamination was removed with a beryllium filter. The transmissions of the specimens were all in excess of 80%. The neutron diffraction pattern was measured as a function of scattering angle $2\theta = 10^\circ$ with a steplength in scattering angle of 0.05°. Two orders of reflection were observed, a strong first order peak and much weaker second order reflection. Because of the weak intensity of the latter, it was measured at ten times

the monitor value used for the first peak. In these measurements we used the constant monitor counts. The sample was rotated at the rate of 30 revolutions / minute to get the average counts of the diffracted neutrons by the sample.

3.4 Data Analysis for Neutron Diffraction Experiments

The neutron diffraction measurements were carried out on samples contained in sealed 1.5 mm I.D. cylindrical quartz tubes. For the cylindrical samples a Lorentz correction $L^{-1} = (\sin\theta_n \cdot \sin 2\theta_n)$ (2θ is the scattering angle) was applied to all data. The background counts for sample container, quartz tube and for fast neutrons were subtracted from the data. A small angle background of the form $\exp(-Q^2 T^2)/Q^2$ was subtracted in the region of the first peak; it is depicted by the break lines in Figs. 3.2a-3.2l. This functional form (where $Q=(4\pi/\lambda) \cdot \sin\theta$ and T is the bilayer thickness) accounts for scattering from isolated bilayers [123,58] which may not have been aligned with other bilayers. Initial calculations showed that the low-angle asymmetry of the peak was predominantly due to resolution function effects. Two gaussians were used to describe the lineshape of the first order peak, one to represent the main peak and the second to represent the low-angle tail. The integrated intensities $I(n)$ under these gaussians plus the small angle background discussed above were determined by a least squares fitting procedure. At the second peak position, a $1/Q^3$ background was assumed as prescribed [123] for larger angles, and data were fitted to a single gaussian. The

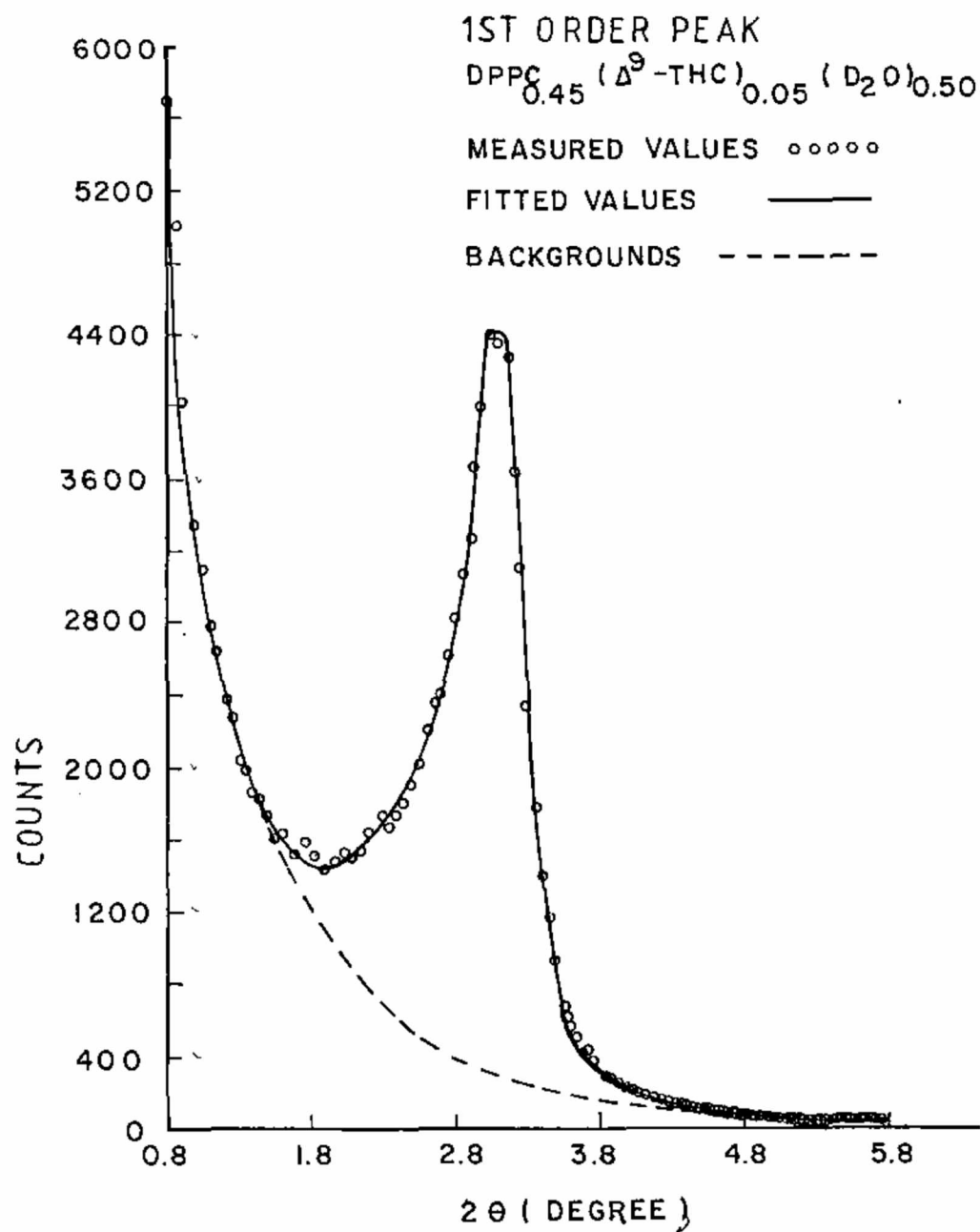


Fig. 3.3a The observed first order reflection with fitted values and fitted backgrounds from $\Delta^9\text{-THC}$ (50% D_2O) embedded in DPPC.

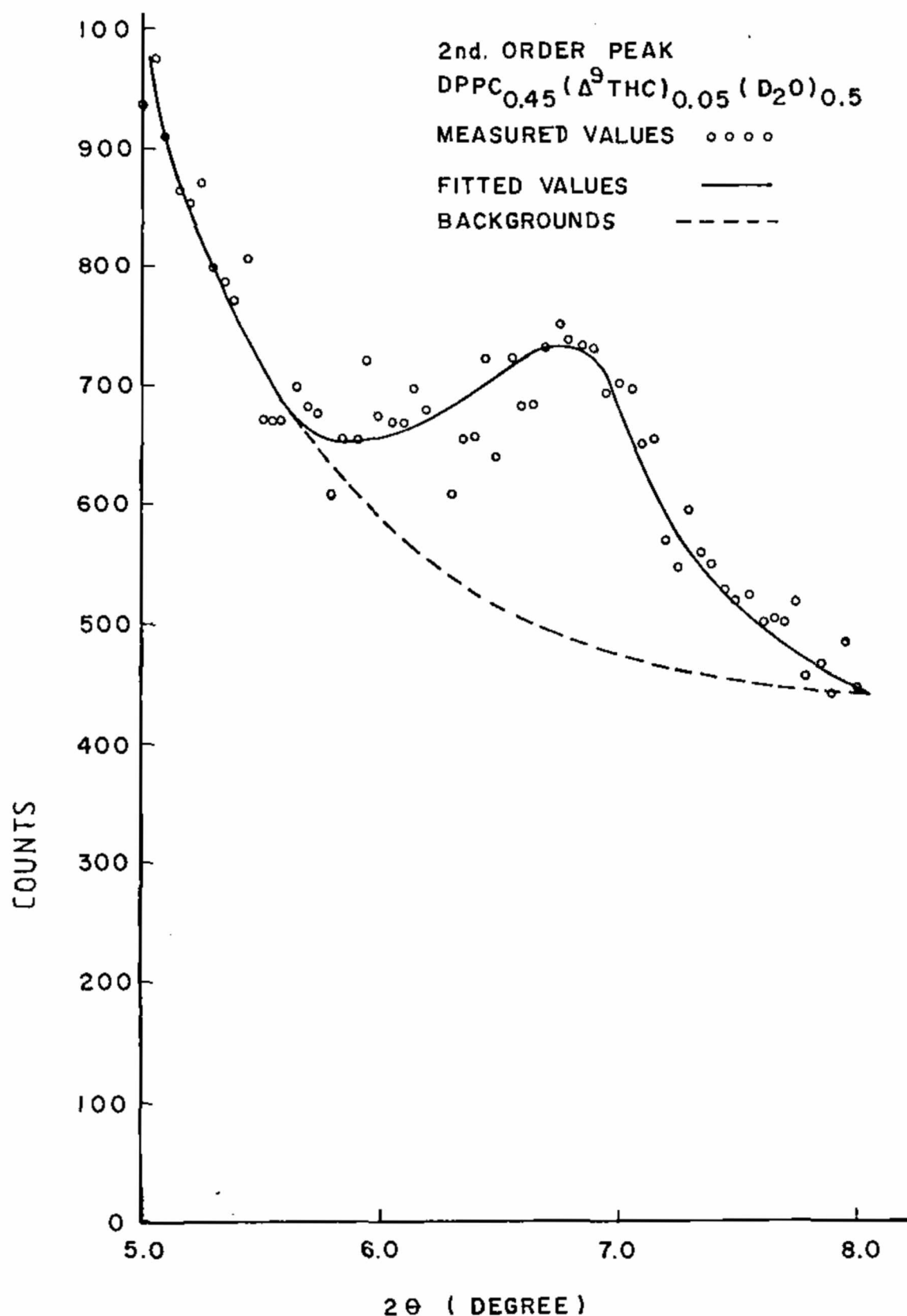


Fig. 3.3b The observed second order reflection with fitted values and fitted backgrounds from Δ^9 -THC (50% D_2O) embedded in DPPC.

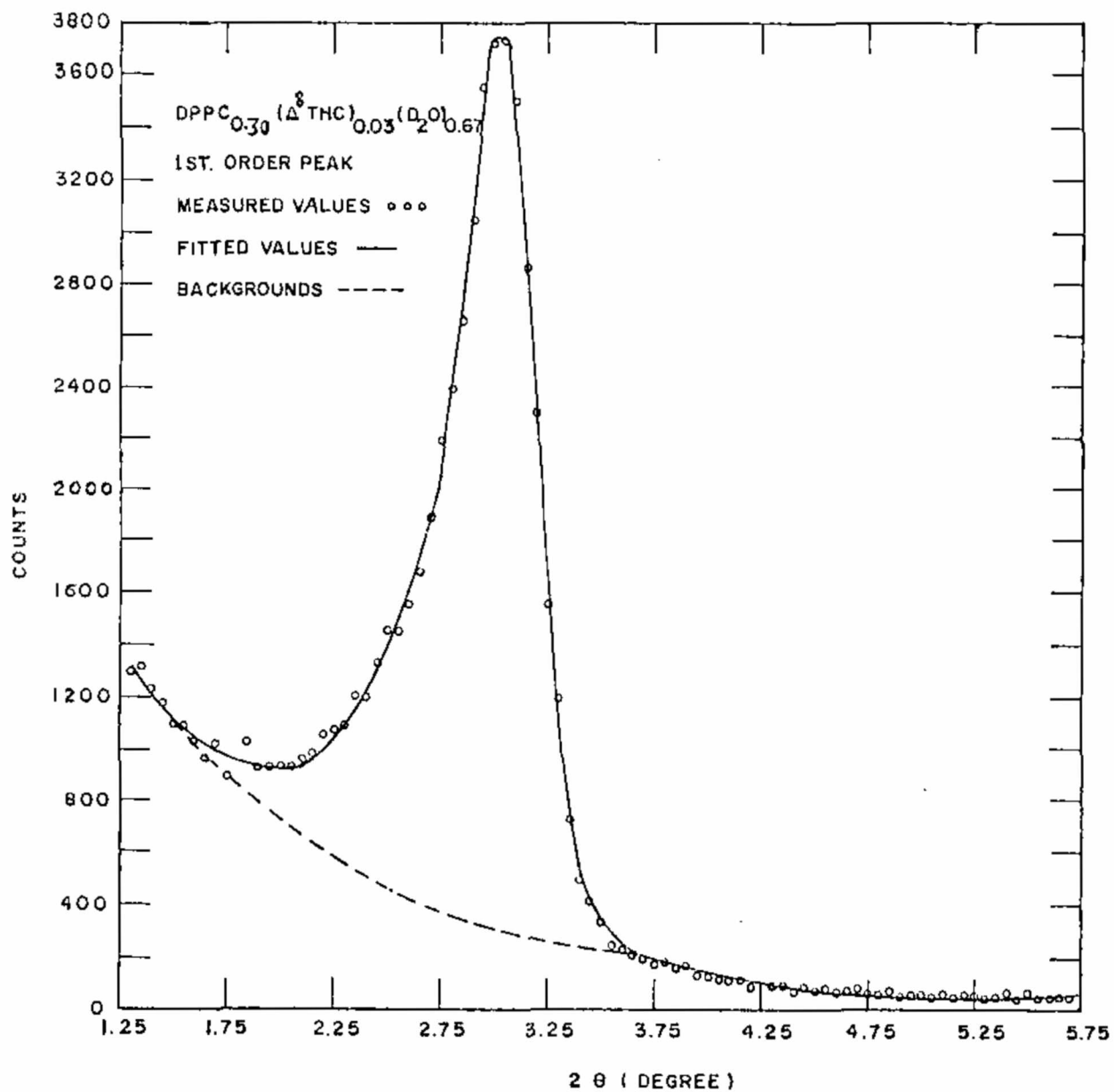


Fig. 3.3c The observed first order reflection with fitted values and fitted backgrounds from Δ^8 -THC (67% D₂O) embedded in DPPC.

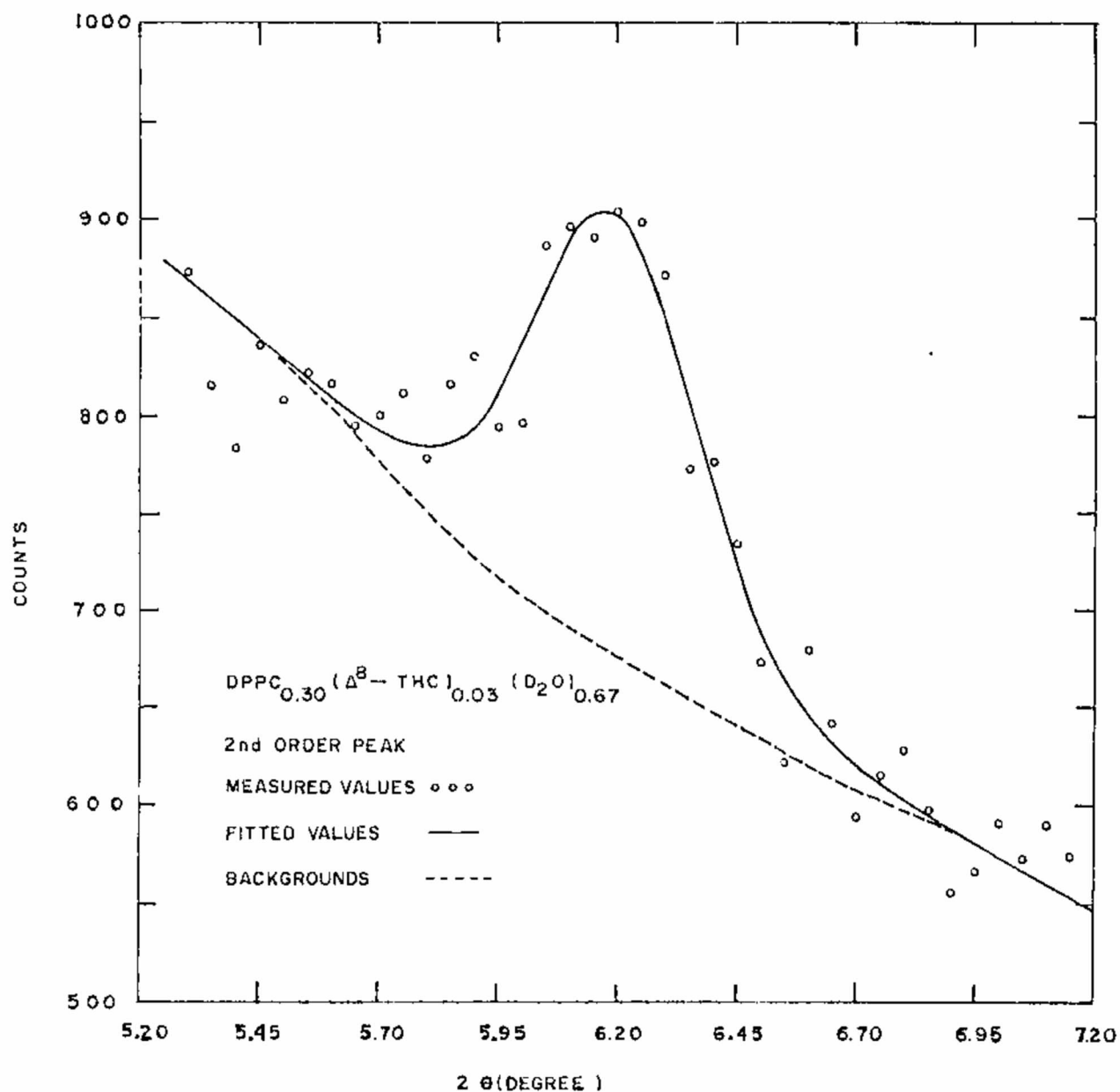


Fig. 3.3d The observed second order reflection with fitted values and fitted backgrounds from Δ^8 -THC (67% D_2O) embedded in DPPC.

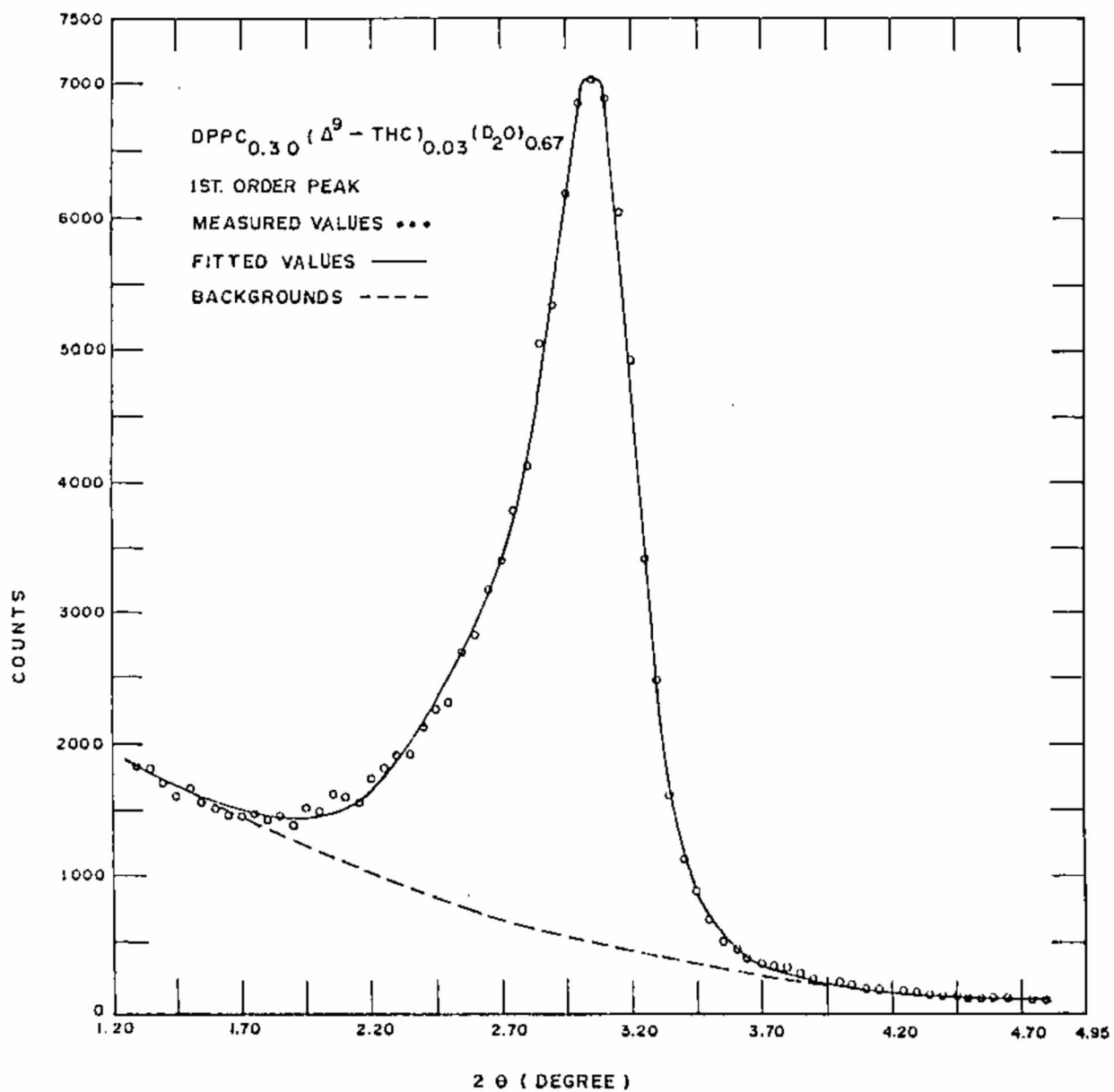


Fig. 3.3e The observed first order reflection with fitted values and fitted backgrounds from Δ^9 -THC (67% D₂O) embedded in DPPC.

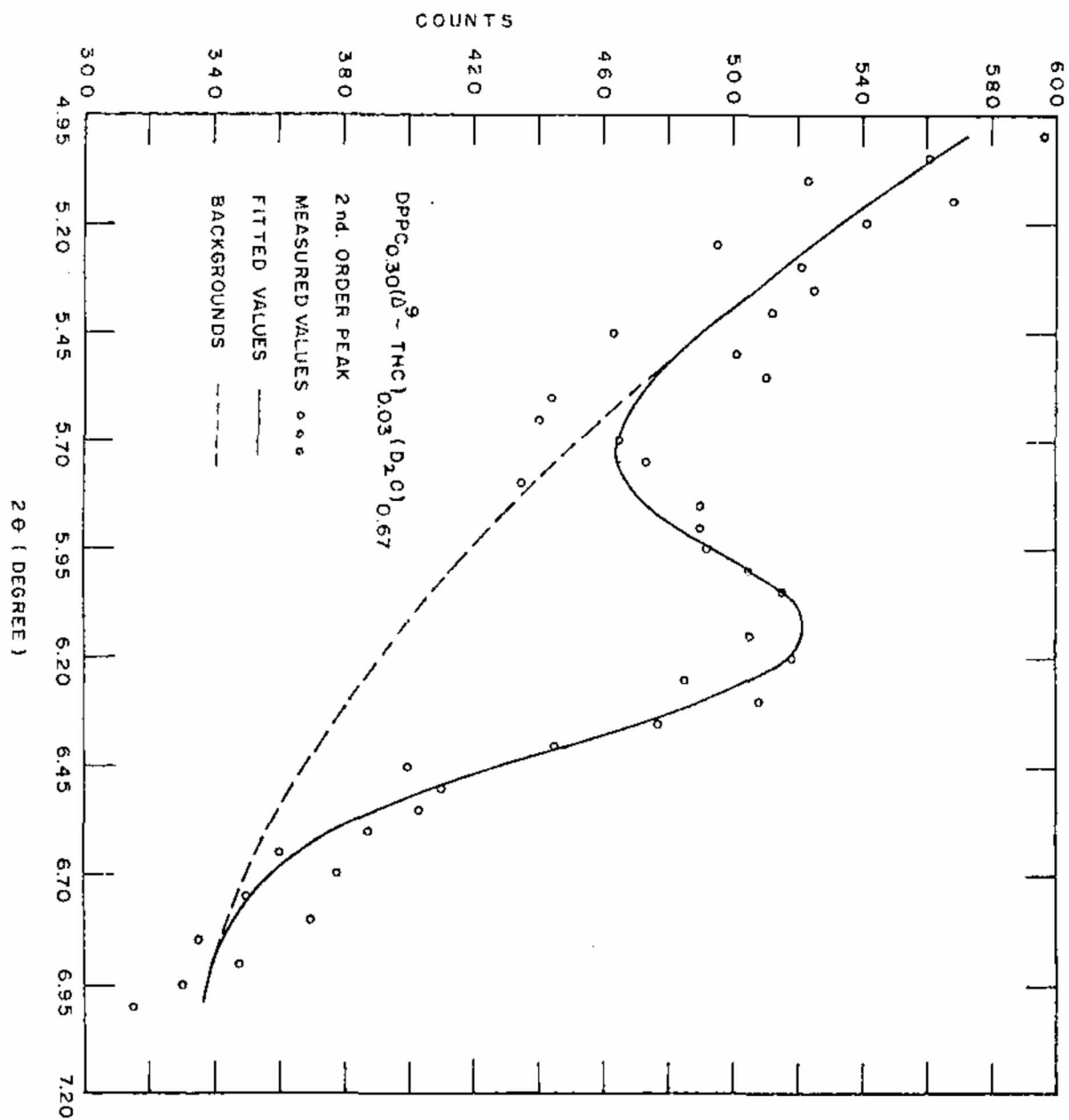


Fig. 3.3f The observed second, order reflection with fitted values and fitted backgrounds from Δ^9 -THC (67% D_2O) embedded in DPPC.

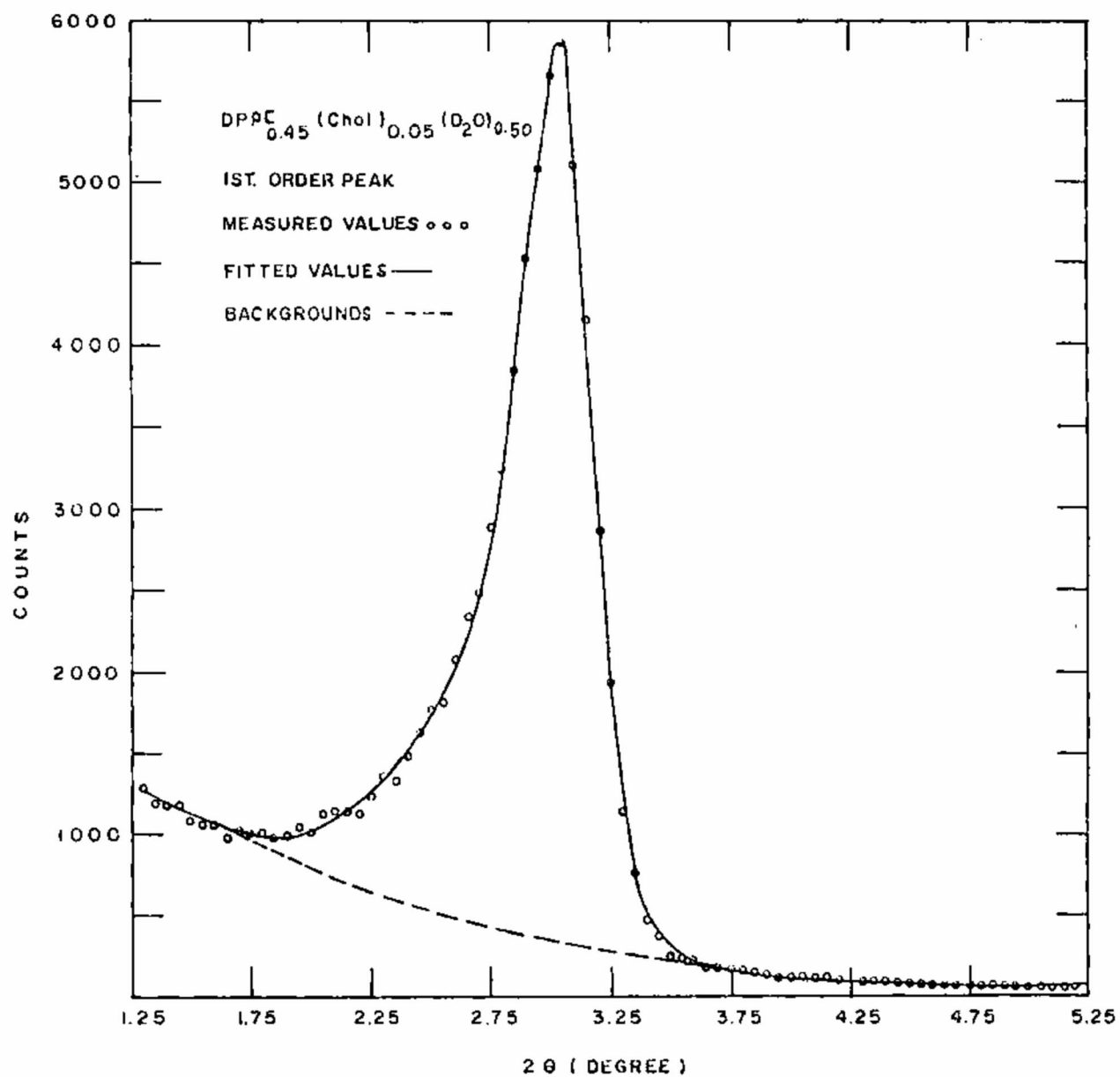


Fig. 3.3g The observed first order reflection with fitted values and fitted backgrounds from cholesterol (50% D₂O) embedded in DPPC.

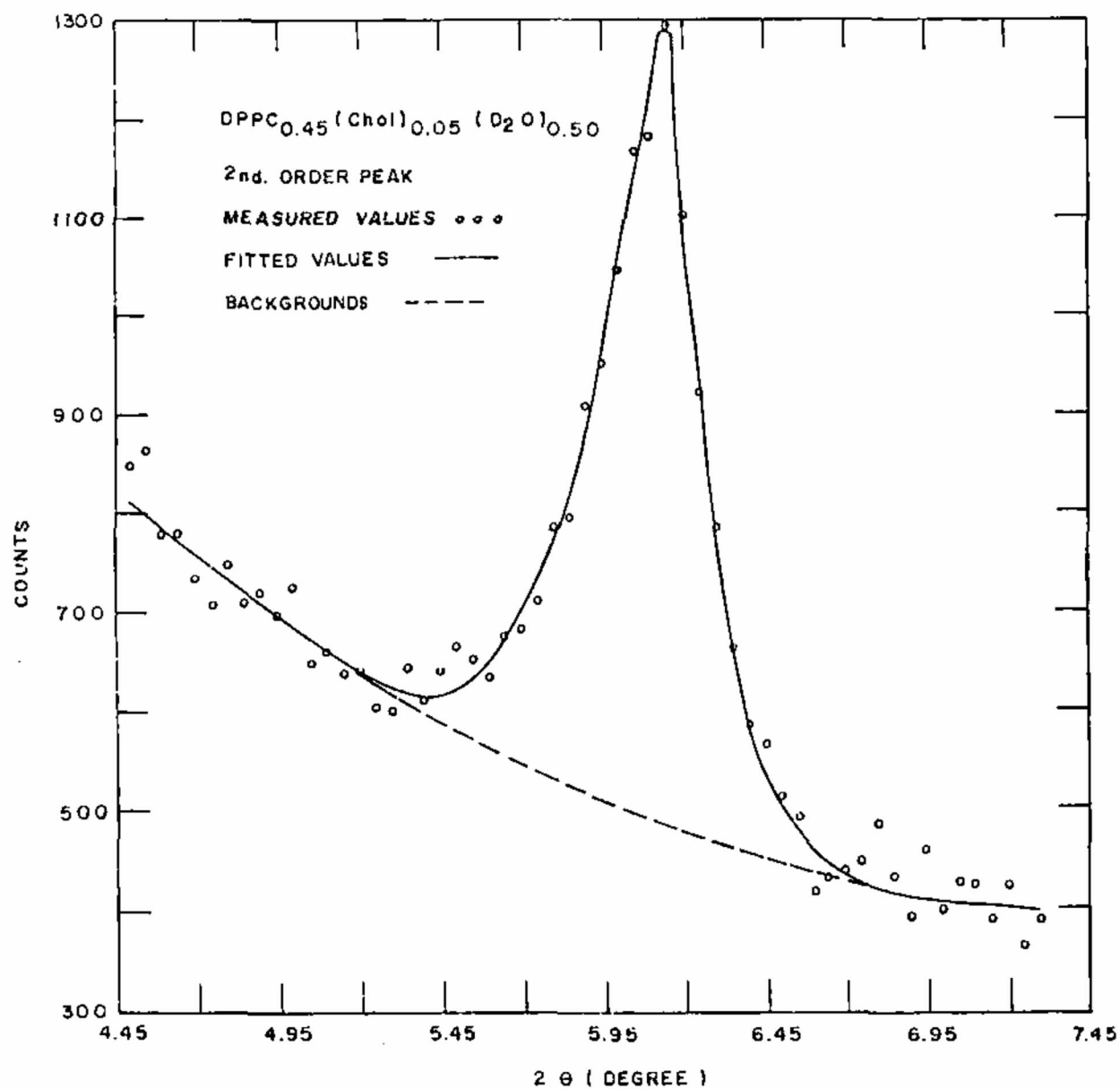


Fig. 3.3h The observed second order reflection with fitted values and fitted backgrounds from cholesterol (50% D₂O) embedded in DPPC.

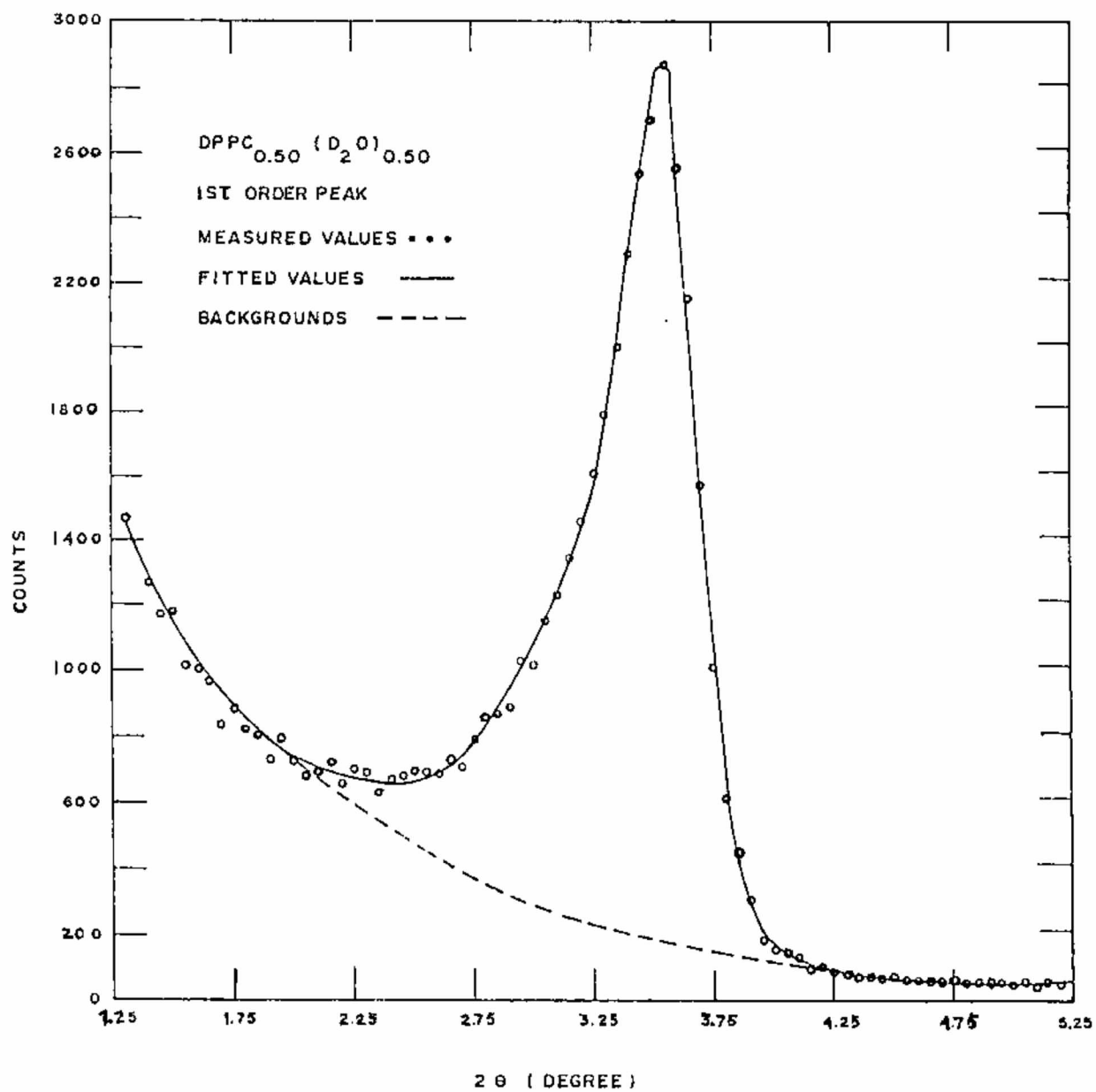


Fig. 3.3i The observed first order reflection with fitted values and fitted backgrounds from pure DPPC (50% D₂O).

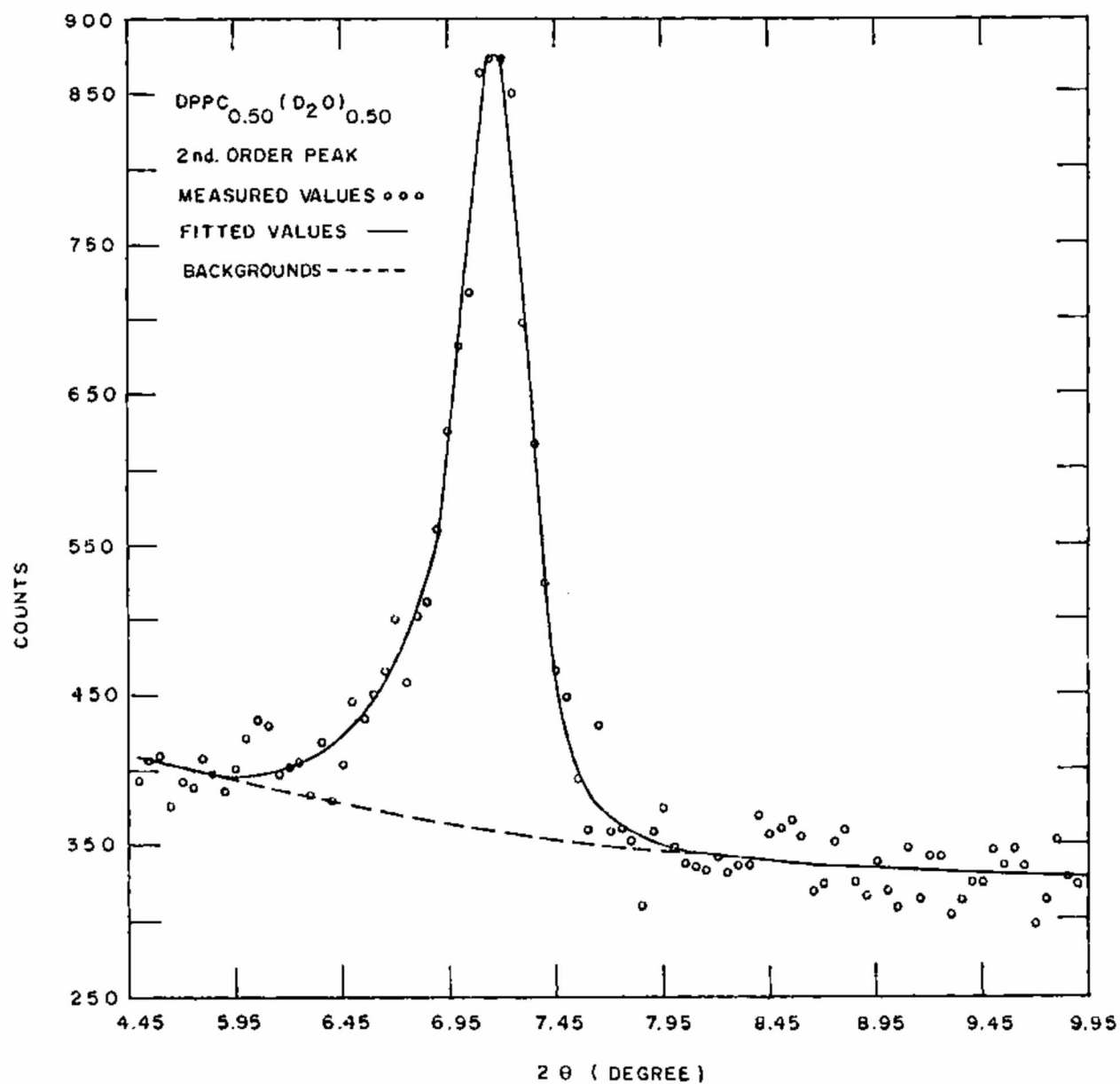


Fig. 3.3j The observed second order reflection with fitted values and fitted backgrounds from pure DPPC (50% D₂O).

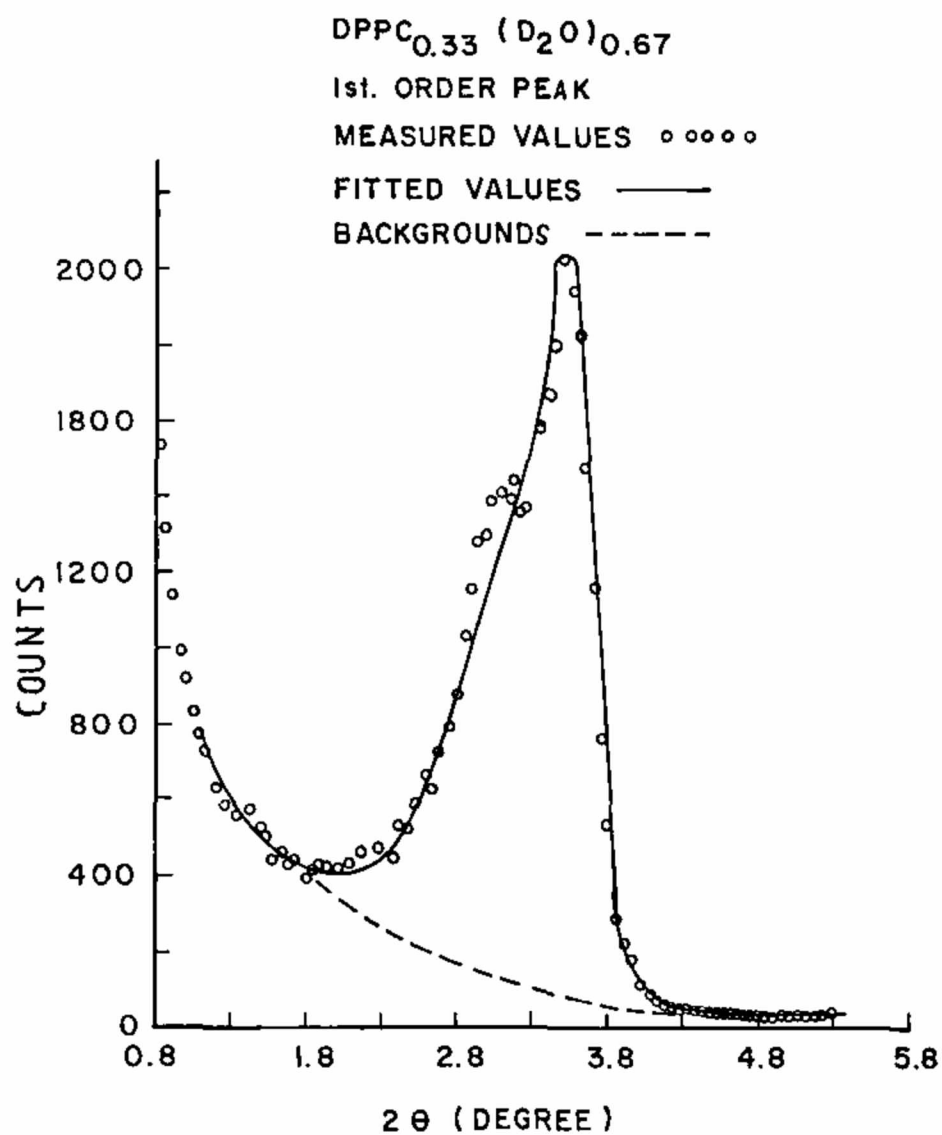


Fig. 3.3k The observed first order reflection with fitted values and fitted backgrounds from pure DPPC (67% D₂O).

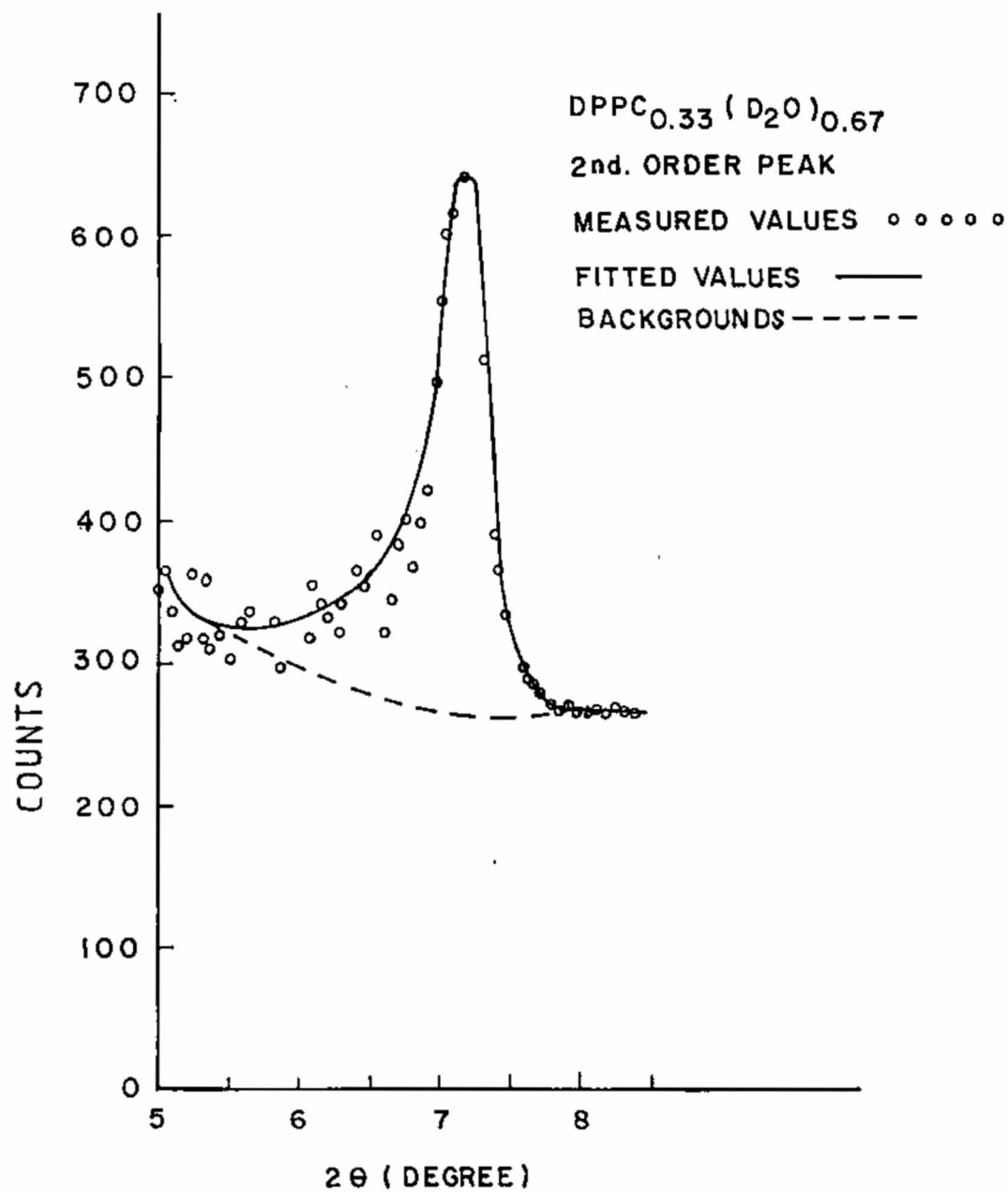


Fig. 3.31 The observed second order reflection with fitted values and fitted backgrounds from pure DPPC (67% D_2O).

integrated intensity of the peak above this fitted background was then determined by numerical integration and used in equation (3.20) to determine the structure factors of neutron diffraction by phospholipid membrane and phospholipid membrane containing Δ^8 -THC, Δ^9 -THC and cholesterol. The structure factors were also calculated by using equation (3.15) and compared with the experimental values by a least square fitting procedure where some of the parameters of the model for theoretical calculations were kept constant as known values. This fitting procedure enable us to determine the unknown parameters of the model and ensure the structure of the phospholipid membranes containing Δ^8 -THC, Δ^9 -THC, and cholesterol.

The data was analyzed in terms of step function model [57,124] with spatial parameters as defined in Fig. 3.1. It was earlier mentioned that we assumed the centrosymmetric membranes. The one dimensional Patterson maps and scattering density profiles were also calculated by using equation (3.22) and (3.14) respectively for both pure membranes and membranes containing Δ^8 -THC, Δ^9 -THC, and cholesterol to find out the location of cannabinoids in phospholipid membranes. The differences in Patterson maps and scattering density profiles ensure that the cannabinoids were embedded in membranes. Our model calculations suggest the location of cannabinoids as in Fig. 3.1.

The phases of the structure factors were determined through analysis by using the chemical knowledge of the membrane components. These calculations involve the selection of phase

combinations from 2^n possibilities which gave Fourier profiles consistent with our assumptions. The phases of the calculated $F(n)$'s have been applied to the experimental $F(n)$'s and by comparing the experimental and model profiles of scattering amplitudes density, we determined the phases (signs) of the structure factors. The structure factors were used in some of our calculations for analysis of data for neutron diffraction studies on pure membranes and membranes containing Δ^8 -THC, Δ^9 -THC and cholesterol.

The CDC Cyber 175 computer at Chalk River Nuclear Labs. and IBM 4341 Computer at Atomic Energy Research Establishment, Bangladesh were used for analysis of data for neutron diffraction experiments. The already existing computer programs and some programs developed by the candidate were used for data analysis.

3.5 Results and Discussions

A summary of the results obtained after analysis of six data sets is shown in Table 3.1. The Bragg repeat spacing, d , was determined by the peak profile function (PPF) least square fitting procedure. It can be seen from Table 3.1 that Bragg repeat spacing, d , increased by similar amounts when either cholesterol or cannabinoids were added. The d value obtained for pure DPPC in D_2O is in agreement with previous measurements involving high solvent solute ratios [122,125].

The ratio, R , of the structure factors $F(1)$ and $F(2)$ for the first and second order peaks is seen to increase when cannabinoids are added to DPPC. The increase is not as great when cholesterol is added because of the difference in the scattering amplitude densities of the cannabinoids and cholesterol (which we shall indicate below). It is found that within experimental errors R is the same for both cannabinoids.

Table 3.1

Experimental results for six specimens. The interlamellar repeat distance is listed as d . $F(0)$ is the structure factor for zero order peak and it is obtained from model calculation. $F(1)$ and $F(2)$ are the structure factors of the first and second order diffraction peaks. They are proportional to the square root of the calculated PPF intensities (see text). $R = F(1)/F(2)$.

Sample	d	$F(0)$	$F(1)$	$F(2)$	R
-----	---	-----	-----	-----	-----
I	70.2	14.61 ± 0.14	10.1 ± 0.13	1.09 ± 0.1	9.27 ± 0.8
II	73.8	17.13 ± 0.12	11.8 ± 0.1	1.19 ± 0.1	10.38 ± 0.4
III	73.2	16.69 ± 0.11	11.7 ± 0.1	1.26 ± 0.1	9.28 ± 0.43
IV	73.3	14.29 ± 0.11	9.87 ± 0.1	1.81 ± 0.1	5.95 ± 0.32
V	62.4	11.36 ± 0.12	8.31 ± 0.1	1.93 ± 0.1	4.3 ± 0.24
VI	62.5	9.41 ± 0.11	6.86 ± 0.11	1.44 ± 0.1	4.76 ± 0.25

Calculations were carried out with the bilayer model shown in Fig. 3.7 to compare the intercalation sites of cannabinoids with those of cholesterol. Utilizing the assumptions discussed below, values for the widths, C , of the aromatic cannabinoid regions and the interbilayer water thicknesses were derived and are listed in Table 3.2.

Table 3.2

Different parameters obtained from model calculations.

ρ 's are in the unit of $\times 10^{-12}$ cm/ $\text{\AA}^3$.

Sample	H($\text{\AA}$)	W($\text{\AA}$)	L($\text{\AA}$)	C($\text{\AA}$)	$\rho(H)$	$\rho(W)$	$\rho(L)$	$\rho(C)$
I	5.5	28.2	17	7	0.029	0.064	-0.003	-0.0023
II	5.5	31.8	17	7	0.025	0.064	-0.003	-0.0023
III	5.5	31.2	17	7	0.025	0.064	-0.003	-0.0023
IV	5.5	31.3	13	9	0.022	0.064	-0.003	-0.0016
V	5.5	33	18.4	-	0.069	0.064	-0.003	----
VI	5.5	33	18.5	-	0.069	0.064	-0.003	----

In all, the model comprised eight parameters, seven of which were obtainable from previous experiments reported in the literature. Four of the eight parameters are the phospholipid headgroup width H , the inter-bilayer water thickness W , the phospholipid hydrocarbon thickness L , and the width of a mixed

region C, in the phospholipid hydrocarbon region where the additives are assumed to reside; the other four parameters are the corresponding neutron scattering amplitude densities designated $\rho(H)$, $\rho(W)$, $\rho(L)$ and $\rho(C)$. The parameter $\rho(H)$ is known for dry membrane, but in the presence of excess water its value changes because water molecules are attracted to the polar headgroup layer [45]. Since D_2O has a large ρ , it is particularly suitable for monitoring changes in $\rho(H)$ due to the addition of water molecules.

With a headgroup thickness H of 5.5 Å [126] it follows from the data of Lis et al. [125] that the hydrocarbon width, L , for the pure phospholipid in excess water is ≈ 33 Å. In the presence of membrane additives this latter value was taken to be ≈ 31 Å by interpolation of the data of Lis et al. for 8:1 and 1:1 ratio of DPPC:chol. Our ratio is about 5:1. The thickness of the water strip, W , were obtained by Lis et al. [125] and were 28.2, 31.8, 31.2, 31.3, 33.0 and 33.0 Å for sample I to VI respectively. These values are in good agreement with Lis et al. [125].

The ρ -values for the W and L are 0.064×10^{-12} cm/Å³ and -0.003×10^{-12} cm/Å³ respectively [125,127]. In our model the aromatic parts of the cannabinoids are assumed to lie in the phospholipid "L" regions in the manner previously found for the aromatic part of cholesterol [113]. Furthermore, the hydrocarbon portions of the additives are assumed to blend in with the host hydrocarbons so that their contributions to the scattering can be almost neglected. The length, C , of the aromatic portions of the

cannabinoids and cholesterol are estimated to be $\approx 7 \text{ \AA}$ and $\approx 9 \text{ \AA}$ in accord with their known structures [128]. The value of $\rho(C)$ can then be calculated by the method of Rand and Luzzati [112], where the "molecules" included in the calculation contain appropriate fractions of steroid or cannabinoid. By this method $\rho(C)$ values for cholesterol and cannabinoid-containing membranes were found to be $-0.0023 \times 10^{-12} \text{ cm/\AA}^3$ and $-0.0016 \times 10^{-12} \text{ cm/\AA}^3$ respectively.

The only parameter left was $\rho(H)$, which was then determined by minimization procedure involving all six data sets. For pure DPPC, $\rho(H)$ was found to be $0.069 \pm 0.005 \times 10^{-12} \text{ cm/\AA}^3$, while for specimens containing cannabinoids and cholesterol the value was $0.032 \pm 0.007 \times 10^{-12} \text{ cm/\AA}^3$. The dry value for $\rho(H)$ is $\approx 0.011 \times 10^{-12} \text{ cm/\AA}^3$ [127]. The implication of our results for DPPC membranes in "excess water" [125] is that the headgroup region of the pure phospholipid becomes permeated with water. The addition of cholesterol and cannabinoids expels some of this water which then goes into interbilayer region, W , and increases its thickness.

It should be noted that these model calculations will not yield absolute values for the different $\rho(H)$'s; the calculations merely serve as a means of correlating results from the six samples. However, even if some of the parameters such as H or L are given different values, the same trend is observed in the changes which the additives bring about in $\rho(H)$.

The scattering density profiles and one-dimensional Patterson maps were calculated for six samples. The neutron scattering density profiles have been calculated using the three orders of reflections including the zero order reflection. The zero order reflection was calculated from least square analysis for model calculations. The zero order reflection has no effect on the nature of the density profiles curves except for an additive or subtractive factor at every point of x in real space. The density profiles curves for pure membranes and membranes containing Δ^8 -THC, Δ^9 -THC and cholesterol are shown in Fig. 3.4a and 3.4b. The separation between the low density troughs increases from 31 Å in pure DPPC to 37 Å in DPPC with cannabinoids. Cholesterol, and Δ^8 -THC, Δ^9 -THC with DPPC have similar density profiles apart from that of pure DPPC. This indicates that the Δ^8 -THC, Δ^9 -THC and cholesterol are similarly located as inclusion in the membrane interior just beneath the phospholipid headgroups. This also indicates that the W region increases remarkably in presence of cannabinoids as that of cholesterol.

The one dimensional Patterson maps have been shown in Fig. 3.5a and 3.5b. These show their similar nature as that of density profile curves. These curves are obtained by using equation (3.14) and we have used the intensities of three reflections including the intensity of zero order reflection. The broad correlation at 62 Å for pure DPPC has been shifted to longer distances at 74 Å for DPPC containing Δ^8 -THC, Δ^9 -THC and cholesterol.

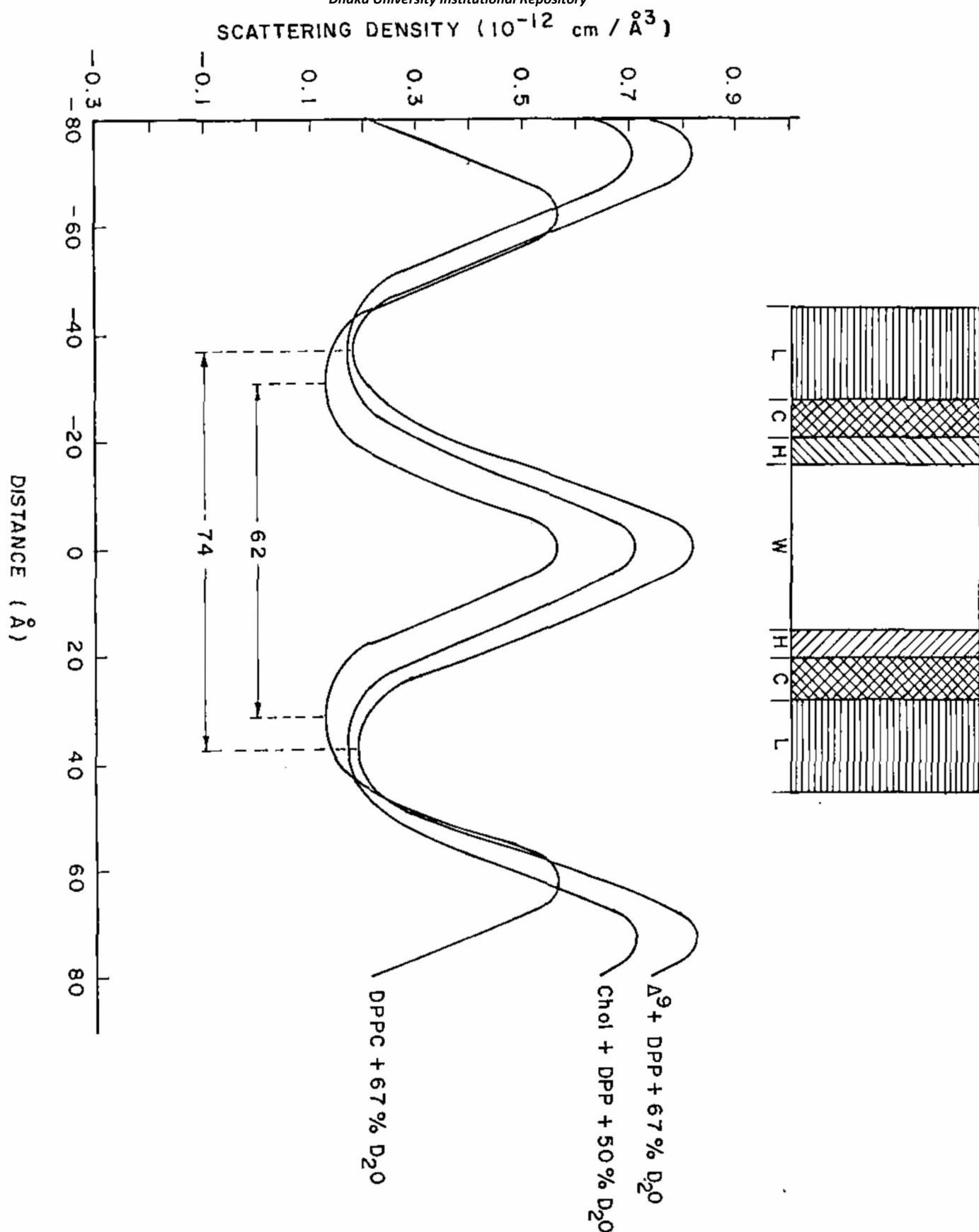


Fig. 3.4a Scattering density profiles in real space for Δ^9 -THC (67% D_2O), and cholesterol (50% D_2O) in DPPC and for pure DPPC (67% D_2O).

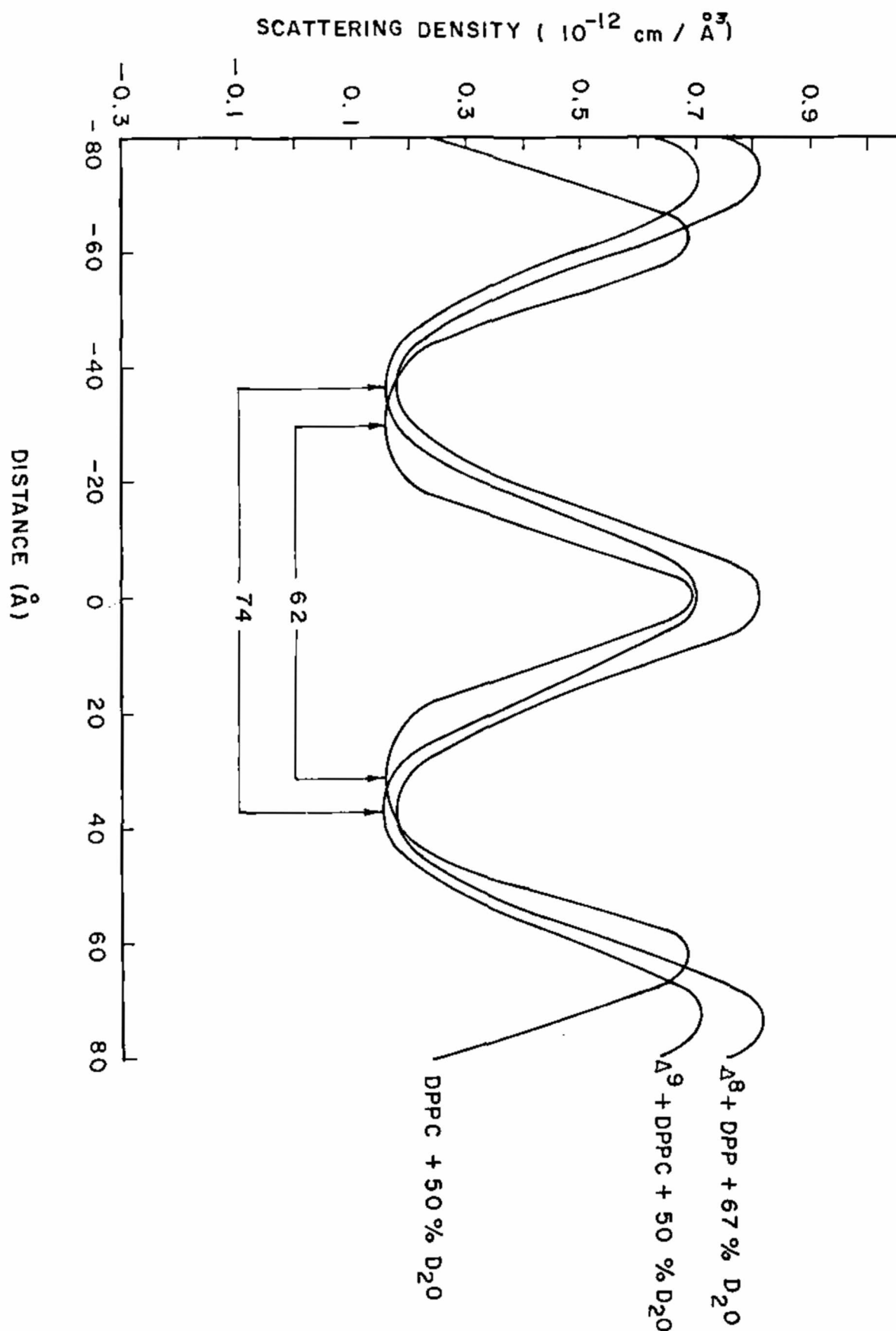


Fig. 3.4b Scattering density profiles in real space for $^9\text{-THC}$ (50% D_2O), and $^8\text{-THC}$ (67% D_2O) in DPPC and for pure DPPC (50% D_2O).

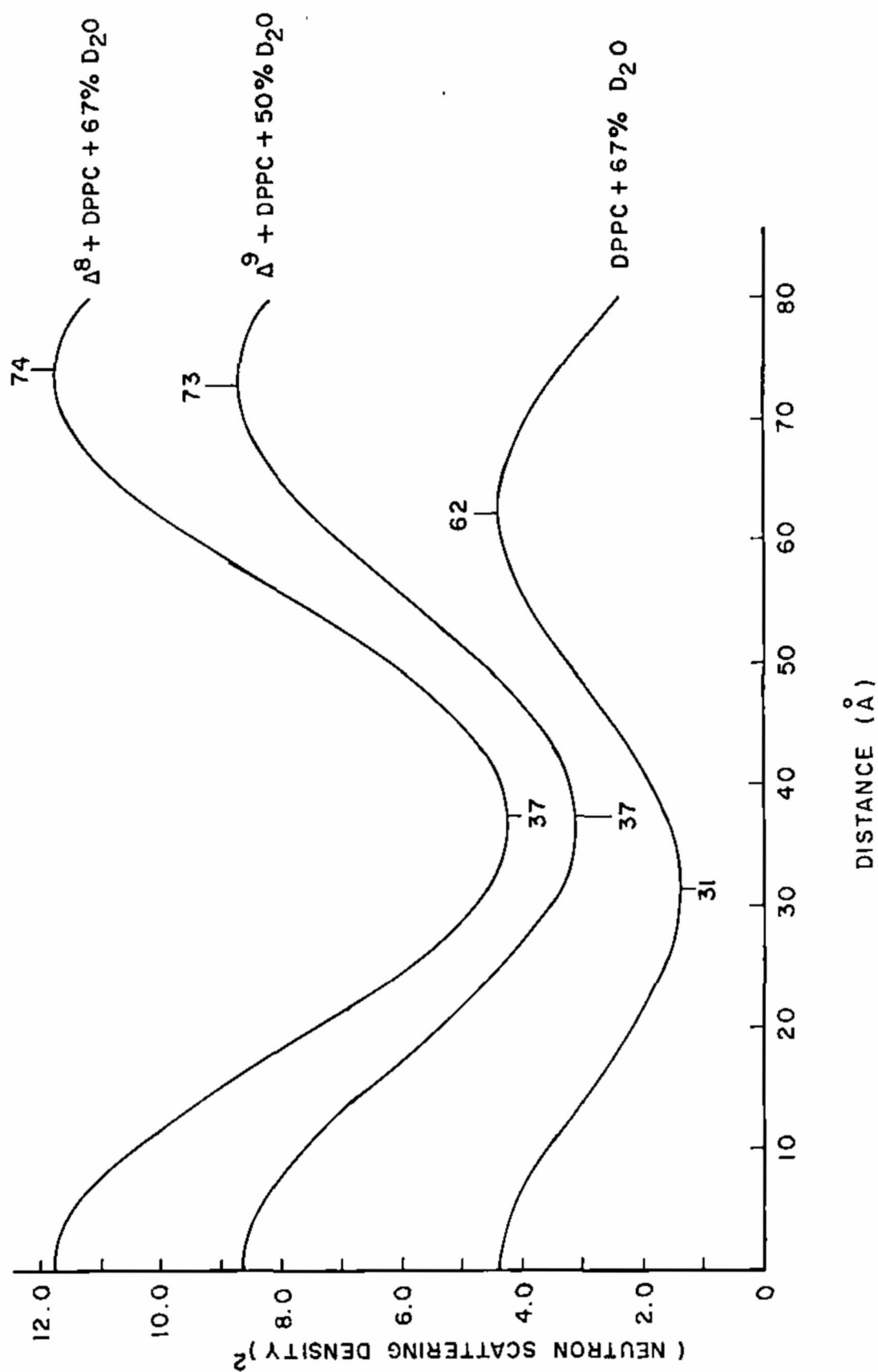


Fig. 3.5a Patterson maps in real space for Δ^9 -THC (50% D_2O), and cholesterol (50% D_2O) in DPPC and for pure DPPC (50% D_2O).

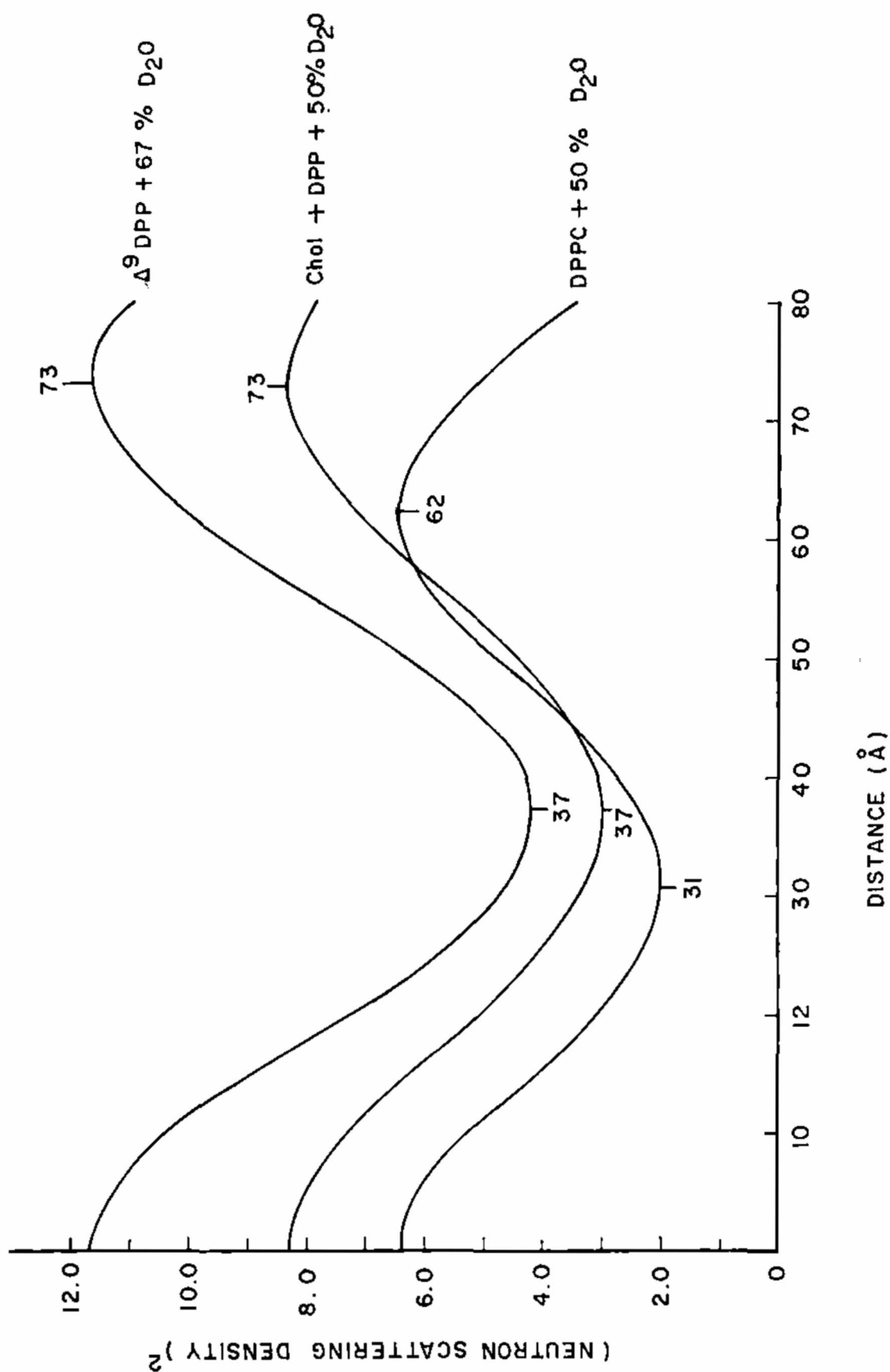


Fig. 3.5b Patterson maps in real space for Δ^9 -THC (67% D_2O) and Δ^8 -THC (67% D_2O) in DPPC and for pure DPPC (67% D_2O).

Fig.3.6a and 3.6b show the wide angle neutron diffraction profiles of pure DPPC and Δ^8 -THC in DPPC, and pure DPPC and Δ^9 -THC in DPPC. For these measurements the samples were contained in cylindrical quartz chambers having inner diameters ≈ 5 mm. A neutron wave-length of 1.452 Å was employed and the angular range was $1.2 < 2\theta < 27^\circ$ in steps of 0.1° . The profiles of pure DPPC differs from that of Δ^8 -THC in DPPC and Δ^9 -THC in DPPC at an angle $2\theta \approx 17^\circ$.

Above discussions imply that Δ^8 -THC, Δ^9 -THC and cholesterol embedded in DPPC and Δ^8 -THC and Δ^9 -THC are similarly located as that of cholesterol in the membrane interior just beneath phospholipid headgroups.

Our model is almost certainly correct in assuming a significant increase in water spacing, W , when cannabinoids are present. By means of X-ray diffraction Lis et al. [125] have also observed a large increase in W due to cholesterol. Cholesterol itself seems to be similarly located in human erythrocyte membranes [112] and model membranes [113], such as we have studied here. Our analysis is seen to be consistent with the assumption that cannabinoids and cholesterol are similarly located as inclusions in the membrane interior just beneath the phospholipid headgroups.

The neutron results also suggest that Δ^8 -THC and Δ^9 -THC produce similar effects on membranes. This result is in accord with work by Greenburg et al.[110] who found little difference in

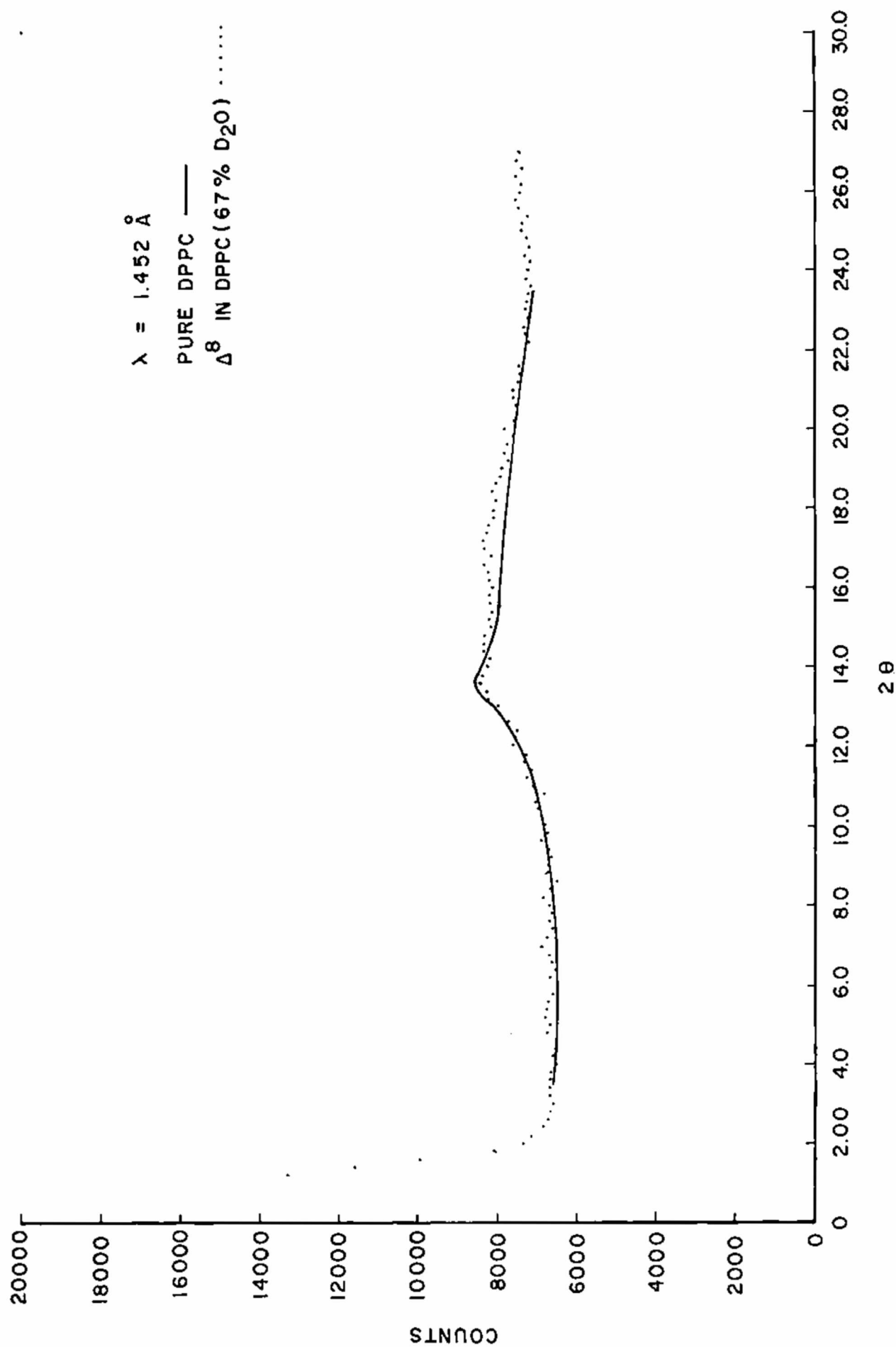


Fig. 3.6a The wide angle neutron diffraction profiles of pure DPPC (67% D₂O) and Δ^8 -THC in DPPC (67% D₂O).

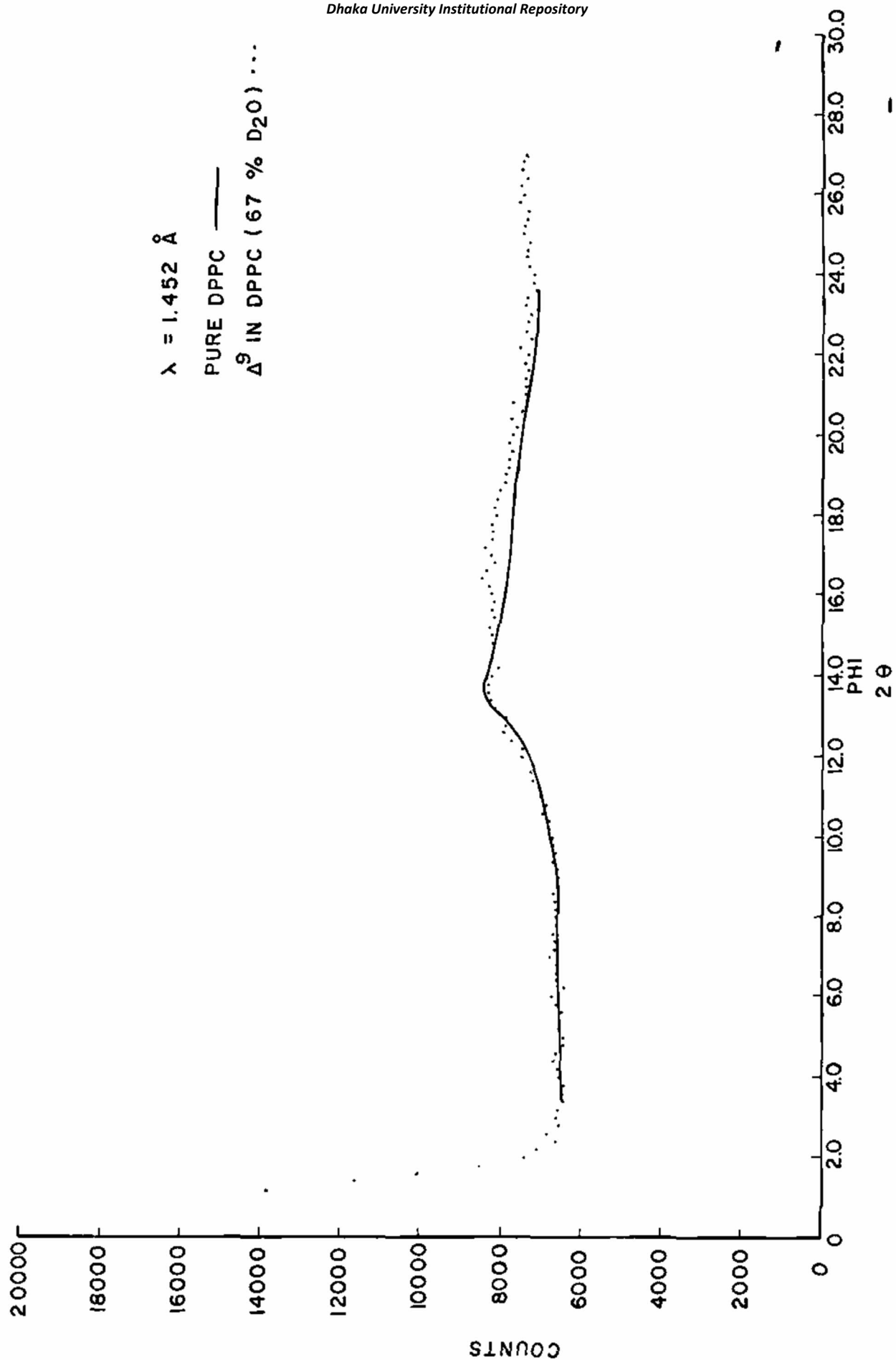


Fig. 3.6b The wide angle neutron diffraction profile of pure DPPC (67% D₂O) and Δ⁹-THC in DPPC (67% D₂O).

the membrane-expanding capacity of Δ^8 -THC and Δ^9 -THC using a parachoric technique. But Δ^9 -THC is known to be much more effective than Δ^8 -THC in inhibiting synaptosomal enzyme; it is also much more psychoactive [110]. Therefore, the psychoactive effect of Δ^9 -THC is probably due to its interaction with membrane associated proteins, specifically those parts of the protein embedded in the lipid bilayer.

CHAPTER 4

***INELASTIC NEUTRON SCATTERING
FROM GLOBULAR PROTEINS***

4.1 Introduction

In recent years considerable effort has been expended in computer studies designed with an ultimate view to understanding how the dynamical motion of proteins can affect enzymatic activity [129,130]. It is becoming widely recognized that more than a static "lock and key" mechanism is involved in enzyme-substrate interactions. Conformational changes in the enzyme probably occur just prior to activation. Furthermore, it is reasonable to assume that such conformational changes are allied to some large-scale resonance mode of the enzyme whose amplitude may be small in inactive state because of damping by internal constraints such as interpeptide bonding, e.g. thiolic bonding, or external constraints such as a modified aqueous environment. Although Raman scattering [131,132,133,134] appears to be a very effective tool for studying low frequency vibrations in proteins, nevertheless, molecular dynamics studies might benefit greatly from other measurements to serve as bench marks for theoretical calculations. Modes of vibrations which involve large fractions of enzyme volume are likely to be of low frequency and therefore amenable to study by inelastic neutron scattering. It is measurement of this kind that we wish to present here.

Another feature of our measurements which should be considered, is lowering of specimen temperature to liquid helium temperatures. Besides reducing multi-quanta process in the neutron scattering data [135] the value of such measurements lies in the fact that they serve as a check for anharmonic effects which one might expect for modes of vibration in a large heterogeneous molecule. If there were a large scale oscillation present, it could affect the frequencies of higher and more localized vibrations if the atom separations associated with the higher frequency motions were changed. An example might be the removal of local steric hindrance. On the other hand parabolic and hence harmonic potentials are not unknown for large scale oscillations [129]. Such potentials are possible because they effectively average over much faster local oscillations. As a result the temperature dependence of large scale and small scale motions may be quite different.

Here we present some data from an experimental program designed to explore the dynamical properties of globular proteins by neutron scattering in the low frequency region. In particular the low frequency vibrational spectra of alphachymotrypsin (α -C), chymotrypsinogen A and hemoglobin will be compared in detail. A comparison of the first two is of interest because they have similar amino acid compositions and one (chymotrypsinogen A) is a precursor (a zymogen) of the other.

It has recently been shown that neutron spectroscopy offers a means of acquiring experimental data for comparison with

molecular dynamics calculations on proteins [136]. Detail calculations of the low frequency motion in bovine pancreatic trypsin inhibitor (BPTI) indicated that several sharp peaks should be observed in the neutron scattering spectrum, but none were observed [137]. Here we present results which show a well defined peak for another enzyme, alpha-C, for which detail calculations have not as yet been carried out. In comparison to the peak of alpha-C we got much weaker peak for chymotrypsinogen A and hemoglobin. We will try to explain the reasons in the section 4.5 of this chapter. BPTI has a molecular weight of 6,500 and is composed of 58 amino acid residues. Even when most of the hydrogen atoms are not explicitly included in the calculations, BPTI is found to have some 300 modes of vibration below 4.5 THz [136]. Alpha-C has 241 residues and molecular weight of 25,000. A molecule of alpha-C contains over 3,000 atoms and hence more than 9,000 modes of atomic vibration are possible. Thousand of modes of vibration are possible for hemoglobin molecule. There is evidence based on computer calculations that atomic motions of large proteins are collective [137] and involve correlated motions of distant parts of the molecule. Such motions could be associated with fluctuations involved in enzyme function.

Neutron spectra might be affected by the presence of a solvent that can contribute a large background component to the scattering. For this reason, we have followed previous authors [131] and have made measurements on dry proteins. As Peticolas [133] has pointed out, when large molecules are in solution, the viscosity of the solvent will dampen the low frequency

oscillations of the molecules and, if there are strong random fluctuating forces due to Brownian motions, these low frequency oscillations may acquire a stochastic character. However, damping is not too severe, one might expect the resultant oscillations to have very nearly the same spatial displacements as their harmonic counterparts. Some changes in the dynamics of BPTI have been calculated for side-chain atoms in contact with the solvent [138] but the backbone atoms appear to be much less affected. Solvent effects should be smaller for a large molecule like alpha-C because the ratio of volume to surface area is greater. Consequently, it is expected that the density of the vibrational states, $g(\omega)$, will not differ significantly from that in solution.

Alpha-C, chymotrypsinogen A and hemoglobin belong to a class of proteins which are globular in shape. The low frequency dynamics of alpha-C molecules has previously been interpreted [136] in terms of collective vibrations associated with the modes of deformation of spheroids.

4.2 Theory

4.2.1 Inelastic Neutron Scattering

The phenomenon of inelastic scattering of neutrons from a system involves exchange of neutron energy and momentum with the system. A thermal neutron can exchange a part of its energy and

momentum with an elementary excitation in a solid and biological materials etc. This fact implies that if we can measure the energy and momentum of the neutrons both before and after the scattering event, the data produced in this experiment can be analyzed to obtain the spectrum of the excitations in our system.

The inelastic scattering cross-section is directly proportional to the dynamical structure factor $S(Q, \omega)$ modified by the scattering properties of the constituents. This $S(Q, \omega)$ factor is a characteristic of the system. $\hbar Q$ and $\hbar \omega$ are momentum and energy transfer variables respectively. If we ignore the modification in $\hbar \omega$, at that stage the $S(Q, \omega)$ is the double Fourier transform of the space-time correlation function of the constituents of the system. This includes the quanta of thermal (phonon) and magnetic excitations (magnon) respectively. The peaks in the spectrum $S(Q, \omega)$ usually correspond to these elementary excitations [139,140,141,142].

Mainly there are two kinds of excitations namely collective and individual excitations. The collective excitations are result from the cooperative motions of atoms and molecules and on the other hand the individual excitations result from the individual motions of the atoms and molecules. All of the inter-atomic and inter-molecular interactions are relevant to both the class of motions. The individual motions are viewed as the motions of individual particles due to an average influence of the surrounding and due to the inter-molecular interactions in case of the internal vibrations of molecules.

The coherent inelastic neutron scattering technique is employed to study the collective excitations of the molecules, on the other hand the incoherent neutron spectroscopy is employed to study the individual particle motions. The incoherent scattering technique is also used to sample the entire spectrum of the collective excitations while viewed as the spectrum of motions of individual particles.

There are a variety of spectra in different systems that can be studied by neutron inelastic scattering. In our context, we are interested in vibrational spectra of biological molecules such as globular proteins.

Inelastic neutron scattering technique has a unique contribution to molecular spectroscopy. Neutrons are able to probe those excitations that can not be observed optically due to specific selection rules and weak intensities due to specific electrical properties of the molecules. The biological molecules contain hydrogen atoms and neutron scattering is particularly suitable for these molecules sometimes partially and sometimes fully deuterated and that very significantly varies the coherent and incoherent neutron scattering cross-sections corresponding to excitations of some molecular groups like CH_3 or NH_3 groups in a large molecular system.

With the help of neutron spectrometers capable of very high energy resolution at small wave-vector transfer, Q , it is possible to make useful measurements on a variety of

macromolecular systems [142]. We can explore the potential of such experiments by calculating the Van Hove scattering function for coherent scattering $S(Q, \omega)$, for realistic models of some macromolecular systems. Though the basic quantity to be measured in an inelastic scattering experiment is the differential scattering cross-section as a function of energy change and angle of scattering $d^2\sigma/d\Omega dE$, it is not always necessary or possible to measure this for all angles and energies. We need the quantity $S(Q, \omega)$ where Q and ω are two variable parameters. These two parameters Q and ω follow the conservation laws of energy and momentum for neutron scattering from atomic vibrations

$$\begin{aligned} E_0 - E &= \hbar\omega \\ k_0 - k &= Q \end{aligned} \quad (4.1)$$

where h is Planck's constant, E_0 and E are the incident and scattered energy respectively, and k_0 and k are respectively the wave vector of the incident and scattered neutrons. The frequency, ω , was scanned at selected neutron momentum transfers Q . The quantity $S(Q, \omega)$ is related to the differential cross-section [143] by the relation

$$d^2\sigma/d\Omega dE = A S(Q, \omega) \quad (4.2)$$

The scattering function $S(Q, \omega)$ is given by

$$S(Q, \omega) = 1/2\pi\hbar \int dr d\tau \exp(i(Q \cdot r - \omega\tau)) G(r, \tau) \quad (4.3)$$

where $G(r, \tau)$ is the space-time correlation function and A is given by

$$A = Nb^2k/k_0 \quad (4.4)$$

where N is the number of particle present and b is the scattering length. Equations (4.3) to (4.4) apply to a monotonic system.

An outline of the theory applicable to our experiment has been given by Smith et al. [136]. These authors describe scattering in terms of the incoherent scattering function $S_{inc}^{vib}(Q, \omega)$

$$S_{inc}^{vib}(Q, \omega) = \sum_{L=1}^N b_{L, inc}^2 \exp[2W_L(Q)] \prod_{\lambda=1}^{3N-6} \exp[n \hbar \omega / 2K_B T] I_{n\lambda} \times [\hbar |Q \cdot C_L^\lambda|^2 / \{2m_L \omega_\lambda \sinh(\hbar \omega / 2K_B T)\}] \times \delta(\omega - \sum_{\lambda=1}^{3N-6} n_\lambda \omega_\lambda) \quad (4.5)$$

where $b_{L, inc}$ is the incoherent scattering length of the L th atom, $W_L(Q)$ is the Debye-Waller factor for nucleus L

$$W_L(Q) = \sum_{\lambda=1}^{3N-6} (\hbar / 4m_L \omega_\lambda) |Q \cdot C_L^\lambda|^2 \coth(\hbar \omega_\lambda / 2K_B T) \quad (4.6)$$

where ω_λ is the angular frequency of the λ th mode, K_B is the Boltzmann constant, T is the temperature and m_L is the mass of the L th nucleus, n is the number of energy quanta of the λ th mode transferred to the system, $I_{n\lambda}$ [---] is the n th order modified Bessel function, C_L^λ is the normalized mass-weighted amplitude vector for the L th atom along the λ th normal mode; the

C_l^λ are defined in a molecule fixed coordinate system and δ is the function which expresses energy conservation and shows that energy is exchanged only in discrete amounts corresponding to combinations of vibrational energy quanta. The superscript "vib" indicates that the scattering is assumed to arise from atomic vibrations. Q and ω , are respectively, the wave-vector transfer and frequency transfers. The applicability and usefulness of this function is based on three assumptions:

- (1) The large incoherent scattering of hydrogen will dominate the neutron scattering response. This follows because of the atoms in protein are hydrogen and the incoherent cross-section of hydrogen is an order of magnitude greater than that of heavy atoms (principally carbon and oxygen).
- (2) At low frequencies the hydrogen atoms follow the motion of the heavy atoms to which they are attached. This permits a calculation of the dynamics in terms of the "extended atom" approximation [136] in which hydrogen atoms are assumed to be rigidly attached to their heavy atoms neighbors.
- (3) The displacements of all "extended atoms" are the same. For low frequency modes, all atomic displacements tend to be similar. This is confirmed by the calculations for BPTI [137], which indeed indicate that the amplitudes of the atomic motions are similar at low frequencies.

If these assumptions are valid, the density of vibrational states, $g(\omega) = \sum_{\lambda=1}^{3N-6} \delta(\omega - \omega_\lambda)$ at low frequencies is proportional to $S_{inc}^{vib}(Q, \omega)$ and can be obtained to a good approximation from $S_{inc}^{vib}(Q, \omega)$ under certain limiting conditions [136]. On the other

hand, if atomic displacements vary greatly, $S_{inc}(Q, \omega)$ is proportional to the amplitude-weighted density of states $G(\omega)$ [136]

$$G(\omega) = \sum_{\lambda=1}^{3N-6} \sum_{L=1}^N (b_L^2 \sin^2 \theta_L |C_L^\lambda|^2 / m_L) \delta(\omega - \omega_\lambda) \quad (4.7)$$

Unlike the time-of-flight method used previously for proteins [137] the present work employed a neutron scattering technique which yields $S_{inc}^{vib}(Q, \omega)$ directly. This technique is called the constant-Q, variable incident energy method of neutron scattering [141]. It utilizes a triple-axis spectrometer with fixed analyzer energy and an incident beam monitor whose sensitivity is directly proportional to neutron wavelength. Incident and scattered neutron energies are determined from Bragg angles of monochromator and analyzer crystals. The scattering of neutrons from modes of atomic vibrations is governed by the conservation laws of energy and momentum as shown in equation (4.1).

Sometimes for certain samples one needs to measure the quantity $S(Q, \omega)$ with one parameter variable and the other kept constant. The advantage of the triple-axis spectrometer is that such selective experiments can be performed while in other methods like chopper or time-of-flight experiment both the parameters vary. The mechanical design of the triple-axis spectrometer makes it possible to vary the parameters like incident neutron energy E_0 , scattered neutron energy E , the angle of crystallographic orientation of the sample ψ and the

scattering angle ϕ continuously. The automation of a triple-axis spectrometer which can vary the above mentioned parameters play a major part for enabling specialized experiments like the "constant Q" method for performing many inelastic neutron scattering experiments like phonon dispersion relations measurements.

4.2.2 Constant Q Method

The constant Q method was introduced by Brockhouse [144] in 1960. In this method Q is kept constant, the energy allowed to vary so that, the conservation conditions may be satisfied for some value of the energy change. In the constant Q experiments if k_0 or k is allowed to vary over a range of energy within which the energy transfer equals the energy of the vibrational energy of lattice (phonon) or macromolecules, represented by Q then we are sure to observe a neutron group which will have the required wave-vector q of vibrational energy (phonon) of lattice or macromolecules.

The wave-vector transfer Q may be resolved in the plane of the spectrometer into the two components Q1 (vertical) and Q2 (horizontal). Then the conservation conditions [143] follow as

$$\begin{aligned} Q1 &= -k_0 \sin \psi + k \sin(\phi + \psi) \\ Q2 &= k_0 \cos \psi - k \cos(\phi + \psi) \end{aligned} \quad (4.8)$$

Now it is possible to hold the given values of Q_1 and Q_2 constant by keeping fixed k_0 ($2\theta_M$) and computing the other three parameters k , ψ and ϕ or keeping fixed k ($2\theta_A$) and computing k_0 , ψ and ϕ . $2\theta_M$ or $2\theta_A$ is allowed to vary in equal increments to cover the range within which the vibrational energy of lattice (phonon) or vibrational energy of molecules should occur. These types of experiments are possible to perform, by using the programmable automatic triple-axis spectrometer.

The constant Q method has many advantages. Phonons of predetermined q can be obtained in this method with required information by saving time. On the other hand, the changed value of q gives a certain extent of the observed width of the neutron distribution in the earlier methods. In other words, a region in reciprocal space is sampled by the distribution which is dependent on factors like the angular collimation of the incident and outgoing neutrons as well as the gradient of the (ω, q) relation at the value of q . In the constant Q method, every time the momentum conservation condition is satisfied the Q value is predetermined and as a result the phonons are usually sharper. In specialized experiments, for example those concerning the temperature variation of phonon frequency, this method certainly allows to observe always the phonon for the same q and hence the widths are readily comparable, but in the earlier methods this is not possible. In general, one can fix Q at any value and obtain all the values of ω to different polarizations easily, provided the intensity factor is sufficient [143]. All those advantages have made the triple-axis spectrometer very suitable for phonon

studies even though the pulsed time-of-flight instrument has higher rate of data collection capacity.

4.2.3 Frequency Equations for Vibrating Sphere

When the motion of every particle of a body is simple harmonic and the period $2\pi/p$, the displacement is given by the formulae [149] of the type

$$\begin{aligned} u &= A u' \cos(pt+\epsilon) \\ v &= A v' \cos(pt+\epsilon) \\ w &= A w' \cos(pt+\epsilon) \end{aligned} \quad (4.9)$$

where u' , v' and w' are the functions of x , y and z , and A is the amplitude of the vibratory motion. When the body is vibrating freely, the equations of motion and boundary conditions can be satisfied only if p is one of the roots of the frequency equations. In general the quantities u' , v' and w' are treated here as components of displacements.

The equations of small motion of the body [145] are given by

$$(\lambda + \mu)(\partial \Delta / \partial x, \partial \Delta / \partial y, \partial \Delta / \partial z) + \mu \nabla^2(u, v, w) = \rho(\partial^2 u / \partial t^2, \partial^2 v / \partial t^2, \partial^2 w / \partial t^2) \quad (4.10)$$

where $\Delta = \partial u / \partial x + \partial v / \partial y + \partial w / \partial z$, ρ is the mass density and μ is

defined as $\mu = \rho V^2$ (V is the velocity of waves of distortion). When u , v and w are proportional to $\cos(pt+e)$ then we obtain the equations by using formulae (4.9)

$$(\lambda + \mu)(\partial \Delta / \partial x, \partial \Delta / \partial y, \partial \Delta / \partial z) + \mu \nabla^2(u, v, w) + \rho p^2(u, v, w) = 0 \quad (4.11)$$

Differentiating the left hand side of these equations with respect to x , y and z respectively and adding the results, we obtain an equation which is given by

$$(\nabla^2 + h^2)\Delta = 0 \quad (4.12)$$

$$\text{where } h^2 = p^2 \rho / (\lambda + 2\mu) \quad (4.13)$$

If we put $k^2 = p^2 \rho / \mu$ equations (4.11) become

$$(\nabla^2 + k^2)(u, v, w) = (1 - k^2/h^2)(\partial \Delta / \partial x, \partial \Delta / \partial y, \partial \Delta / \partial z) \quad (4.14)$$

If we consider that Δ is determined so as to satisfy equation (4.12), then one solution (u_1, v_1, w_1) of the equations (4.14) is

$(u_1, v_1, w_1) = -1/h^2(\partial \Delta / \partial x, \partial \Delta / \partial y, \partial \Delta / \partial z)$, more complete solution is obtained by adding to these values for u_1, v_1, w_1 , complementary solutions (u_2, v_2, w_2) of the system of equations

$$(\nabla^2 + k^2)u_2 = 0, (\nabla^2 + k^2)v_2 = 0, (\nabla^2 + k^2)w_2 = 0 \quad (4.15)$$

and

$$\partial u_2 / \partial x + \partial v_2 / \partial y + \partial w_2 / \partial z = 0 \quad (4.16)$$

when these functions are determined the displacement can be written in the form

$$(u, v, w) = A(u_1+u_2, v_1+v_2, w_1+w_2) \cos(pt+\epsilon) \quad (4.17)$$

Taking χ_n and ϕ_{n+1} to be any two solid harmonics of degrees indicated by their suffixes, we have solutions of equations (4.15), (4.16)

$$u_2 = \sum [\Psi_n(kr)(y\partial\chi_n/\partial z - z\partial\chi_n/\partial y + \partial\phi_{n+1}/\partial x) - ([n+1]/[n+2])\Psi_{n+2}(kr)k^2r^{2n+5} \partial/\partial x(\phi_{n+1}/r^{2n+3})] \quad (4.18)$$

The corresponding forms of v_2 and w_2 are obtained from here by cyclical interchange of the letters x , y and z . In equation (4.17) Δ is represented as follows

$$\Delta = \sum \omega_n \Psi_n(kr) \quad (4.19)$$

where Ψ_n is a spherical solid harmonic of positive degree n , the summation refers to different values of n , $\omega_n(x)$ is the function determined by the equation

$$\Psi_n(x) = (1/x \, d/dx)^n (\sin(x)/x) \quad (4.20)$$

We have to apply this analysis to the problem of the free vibrations of a solid sphere. In this case we need to calculate the traction across a spherical surface with its centre at the origin. The components X_r , Y_r , Z_r of this traction are expressed by the formulae of the type [145]

$$r X_r/\mu = (\lambda/\mu)\Delta + \partial/\partial x(ux+vy+wz) + r\partial u/\partial r - u \quad (4.21)$$

Here Δ has the form given in equation (4.19) and u , v , w have such form

$$u = -1/h^2 \partial\Delta/\partial x + \sum [\Psi_n(kr)(Y \partial X_n/\partial z - Z \partial X_n/\partial y + \partial\phi_n/\partial x) - (n+1)/(n+2) \Psi_{n+2}(kr)k^2 r^{2n+5} \partial/\partial x (\phi_{n+1}/r^{2n+3})] \quad (4.22)$$

The coordinate of the several harmonic functions , , to the above expressions have been shown by Love [145]. Complete expressions for the tractions X_r , Y_r , Z_r can be written in accordance with equation (4.21) and the conditions that these tractions vanish at the surface of sphere $r=a$ in forms of which the type is

$$[p_n(y\partial X_n/\partial z - z\partial X_n/\partial y) a_n\partial\omega_n/\partial x + b_n r^{2n+3} \partial/\partial x(\omega_n/r^{2n+1}) + c_n\partial\phi_n/\partial x + d_n r^{2n+3} \partial/\partial x(\phi_n/r^{2n+1})] = 0 \quad \text{-----} \quad (4.23)$$

where p_n , a_n , b_n , c_n , d_n are constants. The expressions for these constants are shown by Love [145]. There are two other equations like equation (4.22) which are to be obtained by cyclical interchange of the letters x , y , z . These equations hold at the

surface $r=a$. From equation (4.23) it was shown by Love that the vibrations fall into two classes. In the first class ω_n and ϕ_n vanish, and frequency is given by the equation

$$p_n = 0 \quad (4.24)$$

In the second class X_n vanishes and the frequency is given by the equation

$$a_n d_n - b_n c_n = 0 \quad (4.25)$$

The normal modes of oscillations of a freely vibrating sphere of compressible material are given by Love [145]. For isotropic material where Lamé's elastic moduli are equal and Poisson's ratio is $1/4$ the lowest three modes of vibration occur in the frequency ratios: 0.84 : 1.41 : 1.83. The spherical Bessel function orders associated with these vibrations are 2, 0 (second class of vibration) and 1 (first class of vibration) respectively. For $n=2$ the vibrations involve shear and sphere is distorted into an ellipsoid of revolution becoming alternately prolate and oblate according to the phase of the motion. Such a mode would not be Raman active. For $n=0$, the motion is entirely radial and symmetric and Raman active. For $n=1$ the mode involves a shear motion of successive shells and would not be Raman active.

Yukio Suezaki et al. [63] mentioned the breathing mode of motion of globular proteins. In this type of conformational

fluctuation, the relative distance between various parts of a protein fluctuates with small amplitudes, maintaining the topological connectivity between them. According to them [63] for low frequency motions in which wavelengths are longer than the size of individual residues, globular proteins would behave as continuous bodies. The globular proteins were considered as homogeneous elastic bodies of spherical shape. The spectroscopy of earth dynamics [146] show that the angular frequency of the vibration of an elastic sphere is given by

$$\omega = 2\pi\nu = (\pi^2 E/\rho r^2)^{1/2}$$

and

$$\nu = 1/2(E/\rho r^2)^{1/2} \quad (4.26)$$

where E is Young's modulus of the elastic material and, ρ and r are the mass density and radius of the sphere respectively.

Gō et al [64] described the dynamics of a small globular protein. According to them the dynamics of a protein molecule is determined by both the kinetic and potential energy functions in terms of dynamic variables. The potential energy was referred to as conformational energy for analysis of conformations of biopolymers. It was described in a paper by Noguti et al. [147] that they examined the shape of the conformational energy surface of an isolated BPTI molecule near a minimum point corresponding to a X-ray crystal structure [148]. Very near to the minimum, the energy surface is a multi-dimensional parabola characterized by the second derivative matrix F at the minimum. They observed

[147] that the energy surface was a good parabola within the range of thermal fluctuations along 80% of orthogonal directions that diagonalize the second derivative matrix F . Along the remaining 20% of orthogonal directions moderate anharmonicities of the energy surface were observed. This indicates that the dynamic structure of the protein molecule in terms of collective variables may be useful.

The conformational energy E is given by them [64]

$$E = 1/2 \sum_{\mu\nu} f_{\mu\nu} \Delta\theta_{\mu} \Delta\theta_{\nu} \quad (4.27)$$

where $\Delta\theta_{\mu}$ is the deviation of the μ th dihedral angle from that at the minimum point and $f_{\mu\nu}$ is an element of matrix F . The coefficient matrix $H = (h_{\mu\nu})$ in an expression for the kinetic energy T ,

$$T = 1/2 \sum_{\mu\nu} h_{\mu\nu} \dot{\Delta\theta}_{\mu} \dot{\Delta\theta}_{\nu} \quad (4.28)$$

of the internal motions in the protein molecule is calculated by the authors [64]. The normal modes of vibration and their frequencies are obtained by solving the generalized eigen value equation

$$F_j \Delta\theta_i = (2\pi\nu_i)^2 H \Delta\theta_i \quad (4.29)$$

where ν_i is the frequency of the i th normal mode and $\Delta\theta_i$ is the vector whose μ th component $\Delta\theta_{\mu}$ is the amplitude of variations

in dihedral angle θ_μ in the i th normal mode.

4.3 Experimental Methods

4.3.1 Description of the Technique

The inelastic neutron scattering measurement technique involves the selection of monoenergetic neutrons and measurement of energy or momentum of the neutrons scattered by the sample. In inelastic scattering of any radiation from a system involves exchange of energy and momentum with the system. In our context, a thermal neutron can exchange part of its energy or momentum with an elementary excitation in a solid. It implies that if we can measure the energy and momentum of the neutrons both before and after the scattering with sample, these data can then be analyzed to determine the spectrum of the excitations in our system.

A triple-axis spectrometer is a versatile instrument which can measure a series of E_0 , k_0 , E , k (incident neutron energy, incident neutron wave-vector, scattered neutron energy and scattered neutron wave-vector) in such a way that all the time either $Q(=k_0-k)$ or $\omega(=(E_0-E)/h)$ is held constant. Any peak in the neutron counts in such a scan (the series of measurements) would correspond to a peak in $S(Q,\omega)$ with constant Q or constant ω . A large number of such measurements of peaks in the

$S(Q, \omega)$ can be used, for example, to identify several points on the phonon dispersion relation $\omega(q)$ (q is the reduced wave vector in the first Brillouin zone).

It is a matter of elementary vector algebra to obtain the analytic algorithm for the angular settings of a spectrometer corresponding to the various points on the scan. These algorithms are the part of the control electronics of the triple-axis spectrometer.

For any scan in a given X-Y scattering plane the unknown parameters to be determined are ω , Q_x and Q_y . In an inelastic neutron scattering experiment, the variable parameters are k_0 , k , ϕ and ψ (incident wave vector, scattered wave vector, scattering angle and sample orientation respectively). In constant Q or ω method out of four variables one variable can be held constant using a triple axis spectrometer. Usually the fixed variable, for convenience is k_0 or k . For carrying out the scan in the three parameters-space of (Q_x, Q_y, ω) , the variable may only be i.e. constant Q or only the magnitude of Q in fixed ω and the direction of Q . Other type of scans are also possible. This kind of scan procedure is particularly suitable for the experiments on a triple-axis spectrometer, where one has complete freedom over both k_0 and k in the given range (for a particular spectrometer) for angular ranges available and the crystals used as the monochromator and analyser for a spectrometer. In other special instruments there may be certain restriction. For example the filter detector spectrometer uses an analyser, a crystal filter

which transmits neutrons of below a certain fixed energy. Thus E is distributed between zero and a cut-off $E^{\max}$, not as an approximate gaussian peak at an average energy, but more like a triangular or a truncated gaussian curve. In spite of the drawback of filter detector spectrometer, it has the advantage of measuring larger energy phonons for the same incident E_0 since E is now smaller than that on a conventional triple-axis spectrometer. Moreover, the intensity on a filter detector spectrometer is increased, though at the cost of resolution, since it uses a larger spread in E . It is also mentioned that the measurements would be usually preferred with the filter at room temperature than at low temperature [149] since that gives a smaller spread in E and hence a better resolution.

With the use of an analyzer based on the time-of-flight method, data can be collected for E over a large range of values for each position of the scattering angle. Such a large amount of data is obtained at the cost of much smaller duty-cycle for the incident neutrons than on a triple-axis spectrometer (on a steady state reactor) due to the requirement of pulsing the beam. Also, the large data may not be easily related to a systematic (constant Q or a constant ω type) scan for the search of desired phonon. It is for this reason that the time-of-flight analyzer is usually not used for measurement of phonon or magnon dispersion relation on a steady state reactor. But time-of-flight method is useful for measurement of only the entire spectrum due to the total density of the states.

4.3.2 Materials for Inelastic Neutron Scattering Experiments

Bovine alpha-chymotrypsin and bovine chymotrypsinogen A are two protein molecules. Both of them have the similar amino acid compositions and chymotrypsinogen A is a precursor of alpha-chymotrypsin. Human hemoglobin is the iron content of human blood. These molecules are supposed to be globular proteins.

Lyophilized samples of hemoglobin(Sigma, type IV), bovine alpha-chymotrypsin (Sigma,type II) and bovine chymotrypsinogen A (Sigma, type II) were used without further purification. Henceforth these three specimens are referred to as alpha-C, chymotrypsinogen A and hemoglobin. Most of the samples were quickly transferred to quartz on vanadium cassettes and sealed within minutes. Some samples were dried overnight in a dessicator containing phosphorus pentoxide. No changes in the scattering were detected as a result of this drying procedure. Typical samples utilized in our experiments weighed approximately one gram.

4.3.3 Experimental Set-up for Inelastic Neutron Scattering Experiment

In this experiment we used the N5 triple-axis spectrometer, operated in triple-axis mode, at NRU reactor (120 MW), Chalk River Nuclear Labs. The experimental conditions are tabulated in Table 4.1. The incident and scattered beam were defined by slits

of rectangular cross-section which yielded angular divergence of $1/2^\circ$.

Table 4.1

Experimental conditions for inelastic neutron scattering experiment. (1 THz = 33.3 cm^{-1})

Run	Monochromator	Analyser	Analyser energy (THz)	Resolution (THz)
---	-----	-----	-----	-----
I	Silicon (111)	Silicon (111)	2.5	0.24
II	Silicon (111)	Silicon (111)	4.5	0.3
III	Germanium (111)	Graphite (002)	4.2	0.28
IV	Germanium (113)	Graphite (002)	3.4	0.2
V	Silicon (111)	Silicon (111)	2.5	0.2

The incident beam was filtered by 5 cm of sapphire in order to suppress short wavelength components in the incident neutron beam. The specimen shapes in run I and II were solid cylinders while a cylindrical shell of protein was utilized in runs III, IV, and V. All measured spectra were corrected for fast neutron background. Elastic scattering measurements were carried out to check for evidence of crystallinity (i.e. Bragg reflection) but none was found, although visual examination of individual grains suggested an ordered structure. In all cases, the specimens were rotated in the beam at a rate of at least 20 turns per counting

period in order to average out effects, which might arise from residual preferential orientation. For room temperature measurements the samples were contained in fused quartz while for low temperature measurements a thin-walled vanadium cassette was used. All samples were characterized by measuring transmissions through their centre sections. These measurements are referred to in experiment as TTC (transmission through centre) measurements.

4.4 Data Analysis for Inelastic Neutron Scattering Experiment

By fitting structure factor amplitudes calculated from lattice dynamical models to observe neutron scattering intensities it is possible to account for the scattering from single crystals of simple biomolecules [150]. However, the protein molecules investigated here have a large number of atoms in them. For example in alpha-C there are 3000 atoms per molecule and hence the number of vibrational modes is in excess of 9000. Unless a molecular dynamical calculation were undertaken it would be difficult to produce theoretical structure factors to describe the scattering. Therefore, in searching for peaks in our data we have relied on computer analysis based on a cubic spline fitting procedure [151] to indicate as objectively as possible whether or not such peaks exist. In the computer program the number of splines (or third order polynomial sections) decreases progressively from a initial over-estimate while maintaining the scatter of the data points about a smooth curve at a statistically significant level. In our analysis all data points

were given a weight inversely proportional to their intensity. This method has a tendency to ignore high frequency noise. It is essentially a smoothing procedure which searches out large scale periodicities in the data.

4.5 Results and Discussions

Figure 4.1 shows some high resolution measurements at 77 K for alpha-C. At all four values of Q , a peak about 0.8 THz is evident. From its lack of strong Q dependence we interpret this to be a peak in $g(\omega)$ [136]. The solid lines through the data points in Fig. 4.1 are the results of Gaussian fits. The fits yielded values for the mean peak frequency of 0.93 ± 0.05 , 0.86 ± 0.04 , 0.81 ± 0.05 and 0.87 ± 0.06 THz at Q values of 1.0, 1.4, 1.85 and $3.0 \text{ \AA}^{-1}$ respectively. The corresponding width (FWHM) were found to be: 0.62 ± 0.15 , 0.61 ± 0.13 , 0.71 ± 0.18 and 0.83 ± 0.2 THz. The values for the experimental widths include a component due to instrumental resolution of 0.2 THz. The experimental conditions were those listed as run I in Table 4.1. In addition to the results reported above, measurements were carried out on alpha-C at $Q = 2.3 \text{ \AA}^{-1}$ with one half the monitor value used in obtaining the other data. The mean peak frequency was found to be 0.77 ± 0.3 THz and the corresponding width (FWHM) 1.4 ± 1.2 THz. The large uncertainties in these results are largely attributable to poor statistics. The experimental conditions were those listed as run II in Table 4.1.

If one neglects multiphonon scattering and Debye-Waller

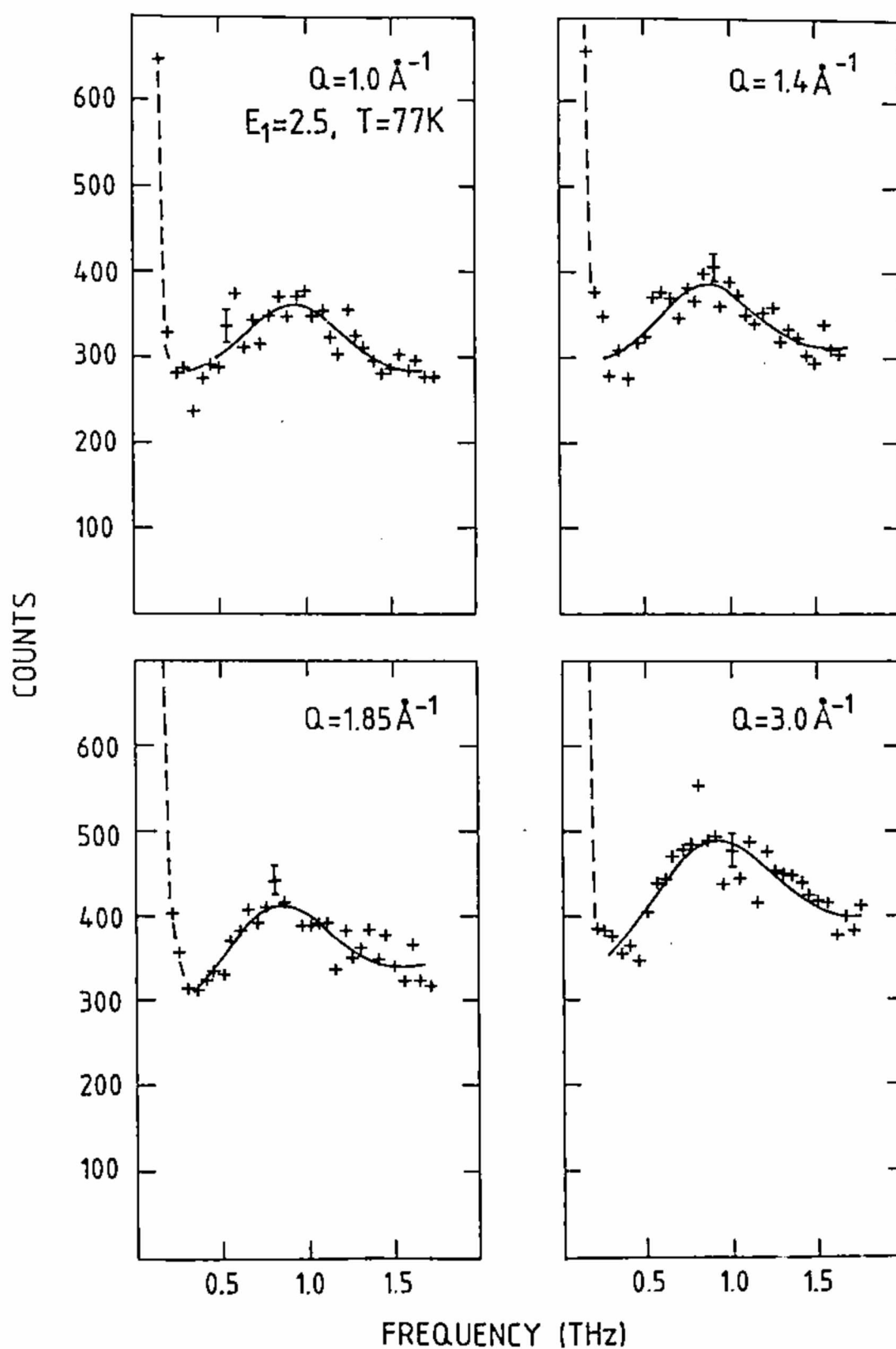


Fig. 4.1 Inelastic scattering groups obtained at four wave vector transfers, Q . The solid lines represent regions over which Gaussian fits were carried out. The specimen temperature was 77 K.

effects [136], the intensity of the peaks might be expected to increase approximately as Q^2 , the variation observed for simple molecular solids with predominantly translational phonons. The peak for $Q = 3.0 \text{ \AA}^{-1}$ would then be predicted to be about 4.6 times as intense as the peak for $Q = 1.4 \text{ \AA}^{-1}$. The ratio of the fitted intensities for the $Q = 3.0$ and $1.4 \text{ \AA}^{-1}$ peaks is found to be 2.1 ± 1.2 . The intensity of the $Q = 1.0 \text{ \AA}^{-1}$ peak is the same as that of $Q = 1.4 \text{ \AA}^{-1}$, within experimental error. It should be noted, however, that a Q^2 variation in intensity is expected to be a gross oversimplification for a large protein. According to theory [136], at small Q -values the intensity can be independent of Q .

A wider scan on alpha-C at coarser resolution (mentioned above) is depicted in Fig. 4.2. Here again, a solid line indicates the region where a gaussian fit was made. This fit indicated a centroid frequency of $\approx 0.8 \text{ THz}$. There is no indication of other peaks at higher frequency. The intensity merely decreases monotonically, approximately as $1/\omega$.

Some room temperature measurements on alpha-C are shown in Fig. 4.3. For all wave vectors, the data indicate the existence of a weak peak at $\approx 0.6 \text{ THz}$ and the overall structure is similar. When the spectra for Q values of 1.0 , 1.4 , and $1.85 \text{ \AA}^{-1}$ are combined (top curve) there is a clearer indication of a peak at about 0.6 THz . There is a suggestion of a second peak at $\approx 1.2 \text{ THz}$, but this may be attributable to two-phonon processes which increase at higher temperature [136]. The lines through the data

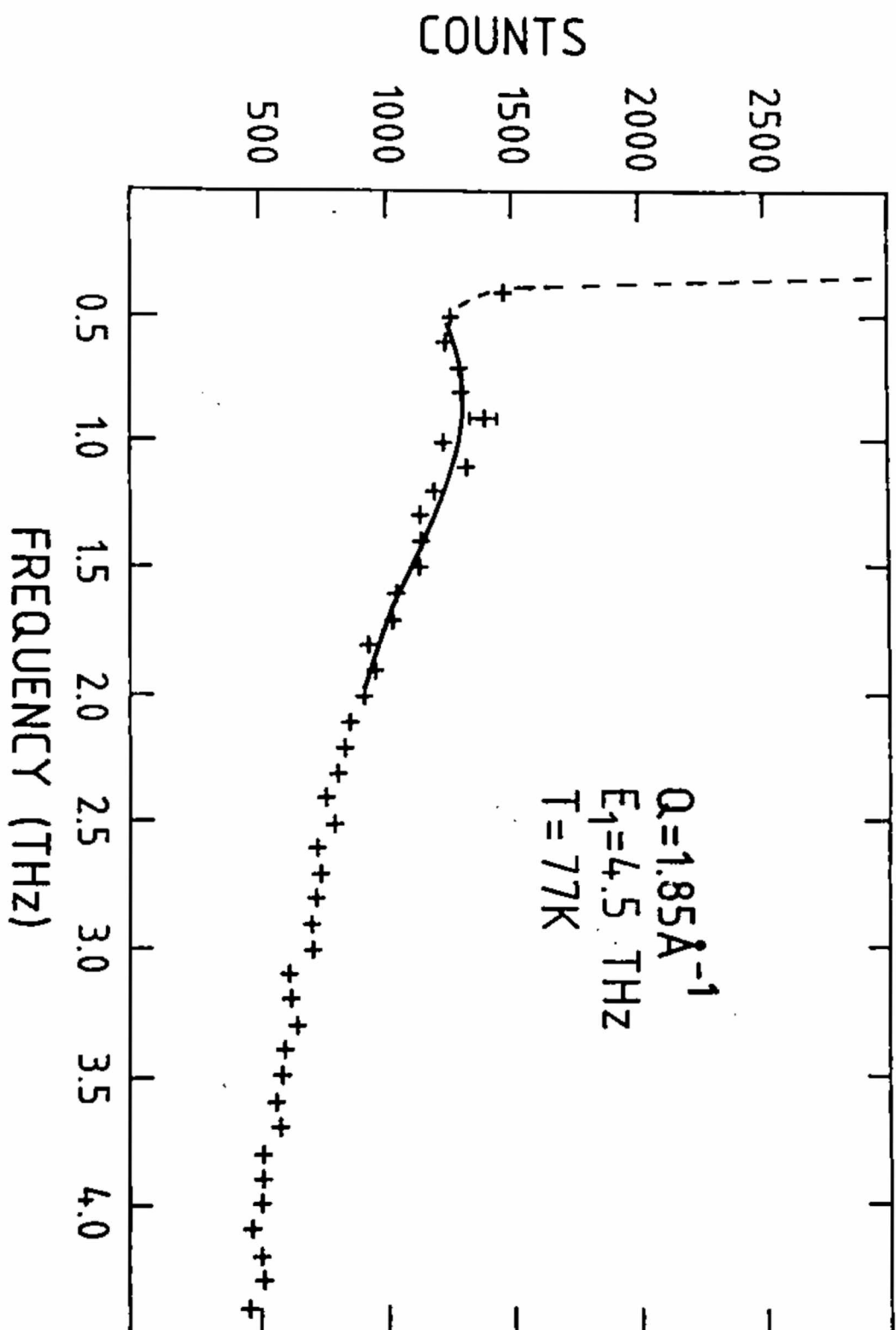


Fig. 4.2 An extended scan at low resolution for $Q = 1.85 \text{ \AA}^{-1}$. The specimen temperature was 77 K. The solid line represents the region over which a Gaussian fit was carried out (see text).

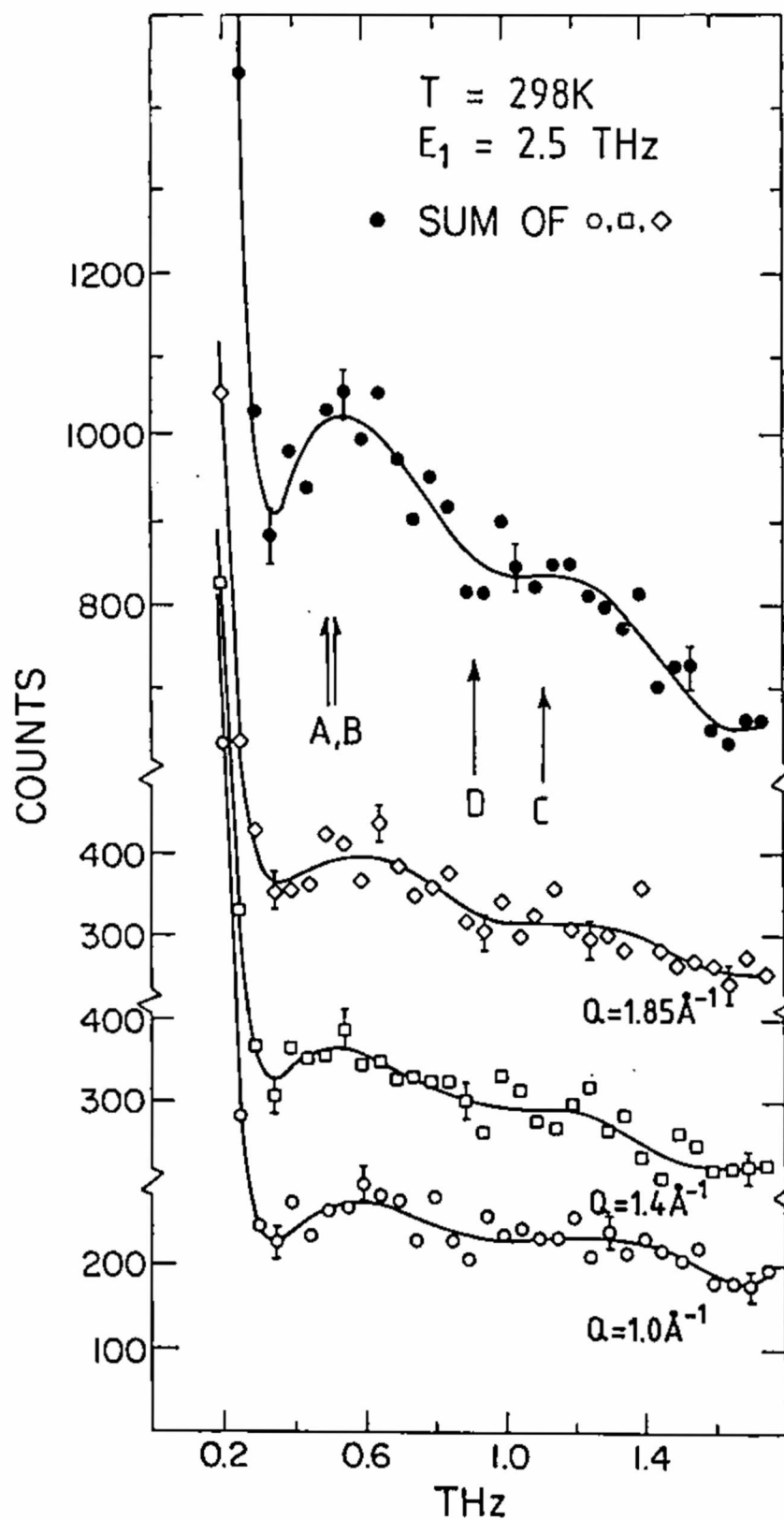


Fig. 4.3 Inelastic scattering observed at 298 K. The experimental resolution was the same as that utilized in Fig. 4.2. Q values corresponding to three lowest curves are 1.0, 1.4 and 1.85 $\text{\AA}^{-1}$. The upper curve is the sum of the three lowest data sets. A, B, C, and D mark the frequencies expected for global oscillations of the molecule as a whole as defined in Fig. 4.9.

points in these figures are merely guides to the eye. Room temperature measurements for Q 's of 1.85 and 4.3 $\text{\AA}^{-1}$ on α -C are shown in Fig. 4.4. A comparison of the data for the Q 's suggests that there may be an increase in frequency as Q increases. The data for $Q = 1.85 \text{ \AA}^{-1}$ indicate a peak near 0.6 THz. The width of the peak (FWHM) is little more than the resolution 0.28 THz and, therefore, compatible with the narrow peak width of < 0.25 THz observed by Raman scattering [131]. at a frequency of ≈ 9.0 THz. The reason for the difference in frequencies will be discussed below. Both sets of data in Fig. 4.4 were obtained with the same incident beam monitor setting. The generally higher scattering intensity from the $Q = 4.3 \text{ \AA}^{-1}$ scan may be due in part to multi-phonon scattering [152] which can be expected to increase by a factor of at least $(4.3/1.85)^2$ from its value at $Q = 1.85 \text{ \AA}^{-1}$. This higher general scattering level and possible broadening of the inelastic scattering peak near 1 THz make a definite frequency assignment difficult, but the fitting indicates a weak peak centred near 0.8 THz for $Q = 4.3 \text{ \AA}^{-1}$. This shows a Q dependent intensity at room temperature but which was not strong in the measurement at low temperature 77 K for Q values of 1.0, 1.4, 1.85 and 3.0 $\text{\AA}^{-1}$.

Room temperature measurements were also carried out on chymotrypsinogen A, the precursor of α -C. The inactive precursor is synthesized in the pancreas and is converted to chymotrypsin in the duodenum. Fully formed α -C is indicated in composition to chymotrypsinogen A except for the removal of four amino acids (serine, arginine, threonine and asparagine) from the

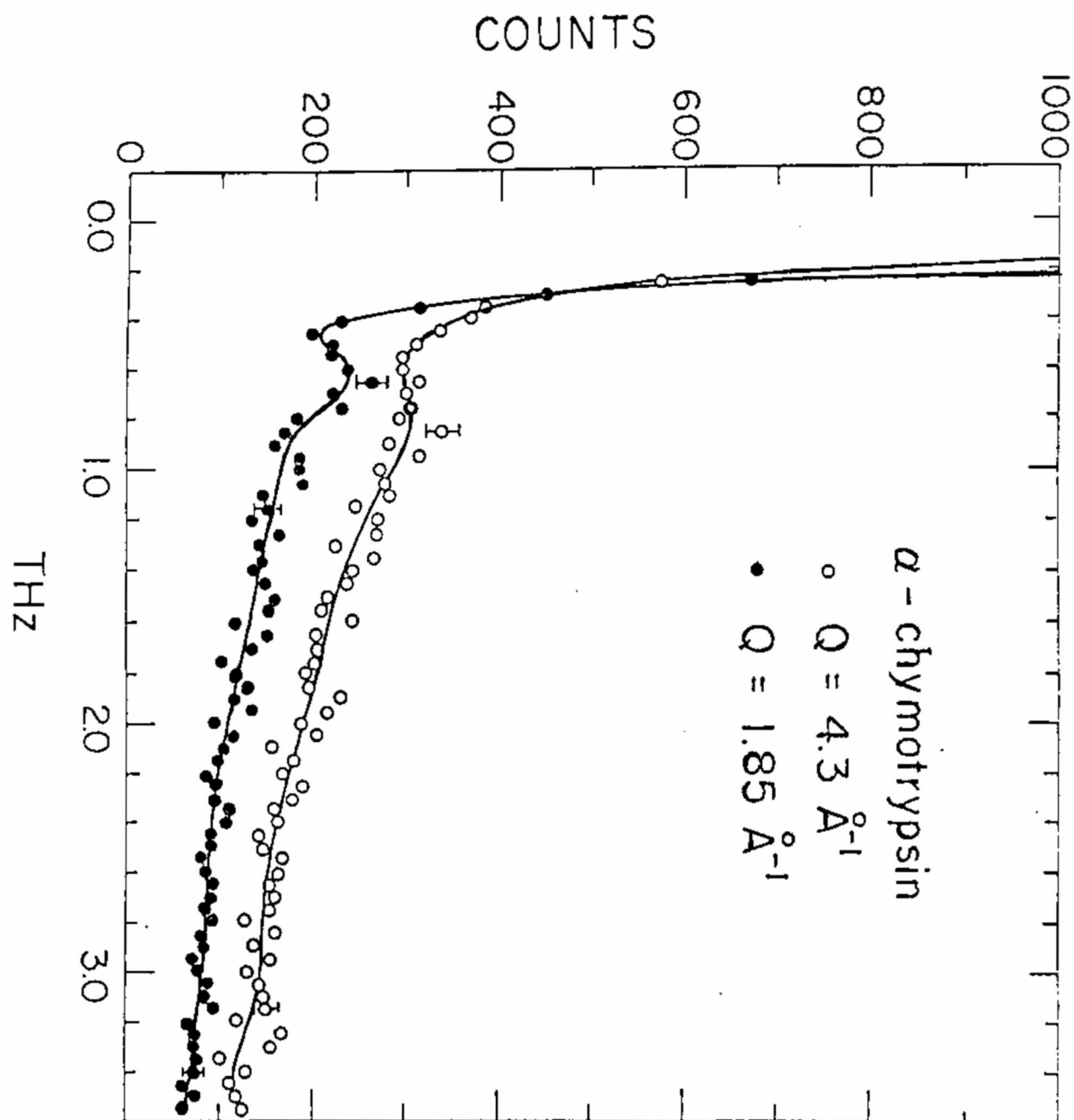


Fig. 4.4 Neutron scattering at $Q = 1.85$ and $4.3 \text{ \AA}^{-1}$ from α -C. The lower data were obtained with $Q = 1.85 \text{ \AA}^{-1}$. The temperature was 298 K.

latter's complement of 245. The four amino acids are removed during two cleavages of the precursor to form fully active and stable alpha-C. An illustration which is helpful in visualizing the structural changes can be found in paper by Mathews et al. [153]. It would appear from Fig. 4.5 that these cleavages can have a profound effect on the low frequency vibrational structure.

The data labelled O in Fig. 4.5 are for alpha-C and are the same as the Fig. 4.4, but replotted on a different scale. The data labelled E and F represent measurements on two different annular specimens of chymotrypsinogen A. The TTC values for E and F were 88% and 79% respectively as in the case of D, the data in E and F are each the sums of intensities for three values of Q (1.0, 1.4 and 1.85 $\text{\AA}^{-1}$). All results in Fig. 4.5 were obtained using the experimental conditions listed for run V in Table 4.1. Hints of some extra intensity near 0.6 THz appear in E and F but unlike the result in D, the spline fitting procedure does not indicate a well defined peak at this frequency. No well defined peak could be discerned in data for chymotrypsinogen A at individual values of Q (i.e. any of $Q = 1.0, 1.4$ or $1.85 \text{ \AA}^{-1}$).

Inelastic scattering measurements were also carried out from human hemoglobin at various Q values. In some cases there was a hint of peak near 0.8 THz but in no case there was any sharp intensity comparable to that observed for alpha-C. A comparison of the scattering from hemoglobin (TTC = 48%) and alpha-C (TTC = 55%) appears in Fig. 4.6. The experimental

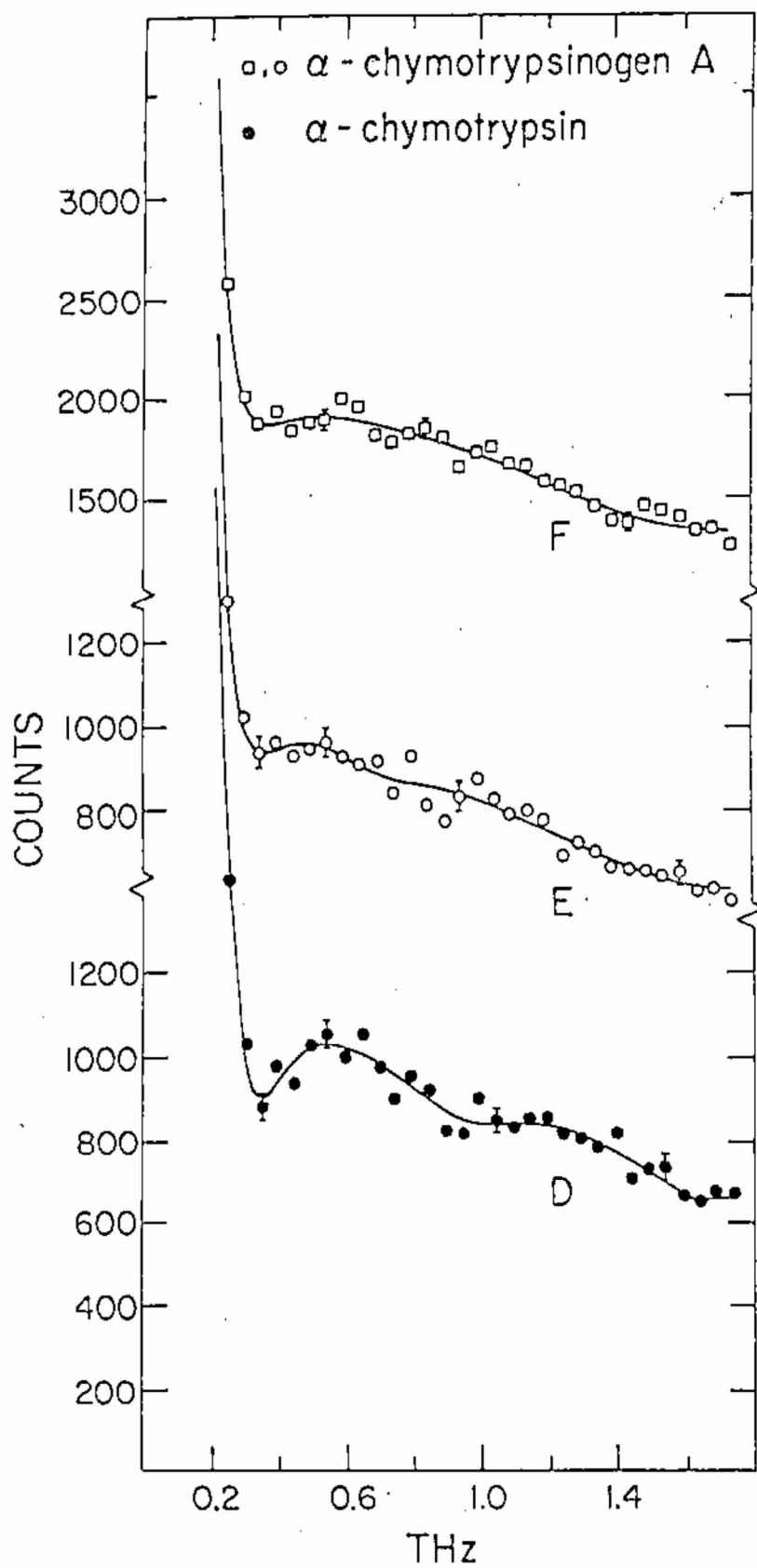


Fig. 4.5 Summed neutron scattering spectra for $Q = 1.0, 1.4$ and $1.85 \text{ \AA}^{-1}$. The samples were: α -chymotrypsinogen A (curve E) and α -chymotrypsinogen A (curve F). The temperature was 298 K .

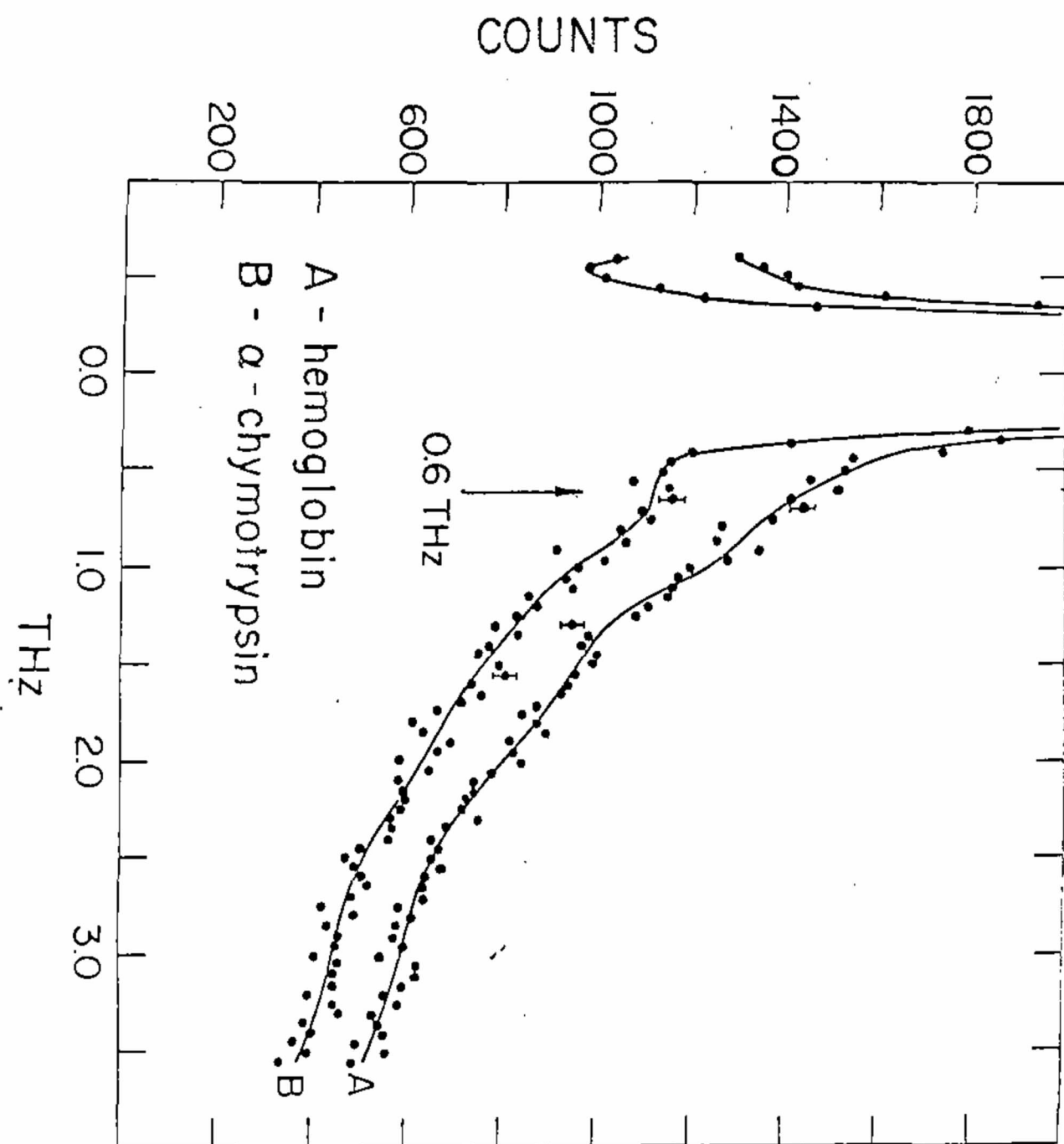


Fig. 4.6 Combined neutron scattering spectra from measurements at $Q = 1.85, 2.7, 3.6$ and $4.3 \text{ \AA}^{-1}$ for hemoglobin (curve A) and alpha-C (curve B).

conditions were those listed as run III in Table 4.1. In order to improve the statistics, scans for Q 's of 1.85, 2.7, 3.6 and 4.3 $\text{\AA}^{-1}$ were added up for each material and they are labelled as A (hemoglobin) B (alpha-C). As before, the solid lines represent cubic spline fits to the data. The presence of a shoulder is evident in the case of alpha-C; in contrast, hemoglobin seems to have a more uniform rate of increase as the frequency decreases below 1 THz.

In another series of measurements (run IV) hemoglobin and alpha-C were compared again at lower temperature and at high $Q = 4.3 \text{ \AA}^{-1}$. The counting times involved in these measurements were five times greater than in any of the previous measurements from run I to V, and the specimens had high TTC's. The weights of the hemoglobin and alpha-C samples were 1.1 and 0.9 gm respectively and their respective TTC's were 75% and 77%. It was, therefore, expected that scattering from these two samples would be similar in intensity provided their frequency distributions were not greatly different in the low frequency region studied. The upper panel of Fig. 4.7 represents the spectra observed at 5 K where multi-phonon effect should be minimal. The open circles represent hemoglobin and the filled circles represent alpha-C data at $Q = 4.3 \text{ \AA}^{-1}$. It is to be noted that the scale for hemoglobin is on the right and that it has been displaced upwards by 100 counts from that for alpha-C. Taking account of this displacement it can be seen that intensity levels of these two data sets are similar. It can also be seen that the alpha-C spectrum exhibits a peak at ≈ 0.9 THz which we presume to have the same origins as the

room temperature peak. If one excludes this peak and assumes the intensity variation under it given by dashed line, there is an indication of a broad peak in intensity centred at about 1.4 THz. The hemoglobin data seem to hint at a weaker peak near 1.0 THz but this structural feature is much less pronounced than the peak at 0.9 THz in alpha-C.

The lower panel of Fig. 4.7 depicts some results obtained on heating alpha-C from 5 K to 80 K. The same specimen was examined at both temperatures and at the same incident monitor setting. The lower data ($Q = 2.7 \text{ \AA}^{-1}$) in this panel, and to a lesser extent, the upper data ($Q = 4.3 \text{ \AA}^{-1}$) are consistent with a low frequency peak near 0.8 THz. There is also a hint of additional intensity near 1.4 THz for the $Q = 2.7 \text{ \AA}^{-1}$ data. In these and other measurements at or above 80 K, it has been found that the low frequency structure observed at high Q 's is always less well resolved from the quasi-elastic scattering near zero frequency than it is at low Q .

A comparison of the $Q = 4.3 \text{ \AA}^{-1}$ data for alpha-C in the upper and lower panels of Fig. 4.7 shows how important the thermal effects can be in determining the scattering distributions at low frequency. A large part of the low frequency intensity increase observed on changing temperature from 5 K to 80 K can be accounted for by the Boltzmann factor associated with neutron loss. This factor tends to contribute heavily to the intensity as the frequency decreases and complicates experimental attempts to extract low frequency peaks when the sample temperature is high.

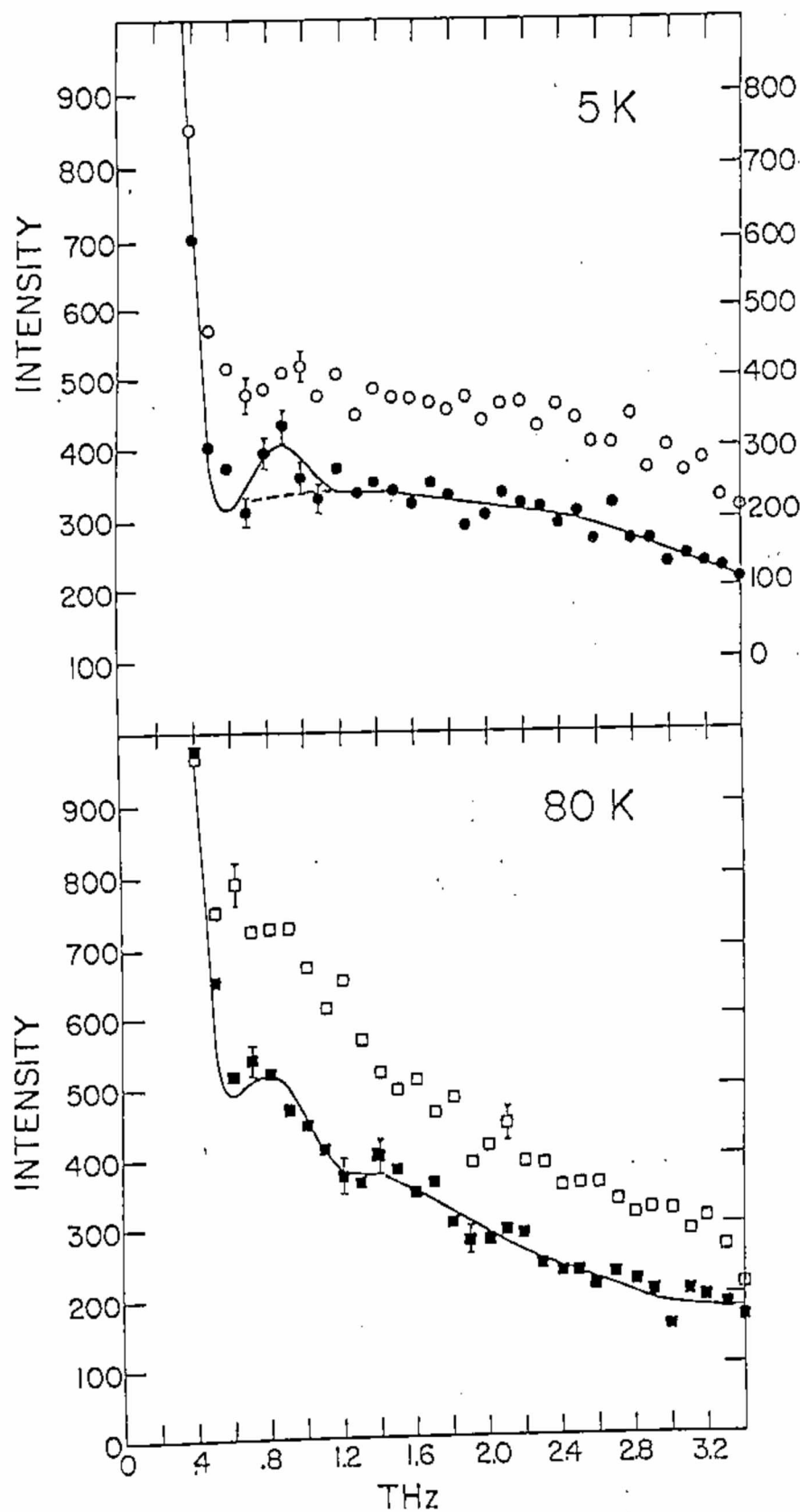


Fig. 4.7 Neutron scattering spectra of hemoglobin and alpha-C at low temperatures. Upper panel: data at 5 K. Open circles are hemoglobin data ($Q = 4.3 \text{ \AA}^{-1}$) displaced upward by 100 counts (intensity scale at right), solid circles are alpha-C data with $Q = 4.3 \text{ \AA}^{-1}$. Lower panel measurements on alpha-C at 80 K. Open squares represent $Q = 4.3 \text{ \AA}^{-1}$ data and solid squares data at $Q = 2.7 \text{ \AA}^{-1}$.

Some years ago Brown et al. [131] carried out Raman scattering measurements on alpha-C at room temperature. These measurements indicated a peak at a frequency of ≈ 0.9 THz. An explanation of this low-lying mode was subsequently advanced by Suezaki and Go [63]. Their model assumes that a globular protein would have a breathing mode involving conformational fluctuations akin to the radial pulsations possible in a sphere [145]. More recently, molecular dynamics calculations have indicated that the low frequency motions in proteins "resemble the global twisting and bending motions expected of globular elastic bodies" [137]. Alpha-C and other two molecules are not perfect spheres. For example the alpha-C is ellipsoidal with principal axes of 45, 35, and 38 Å [153]. It may be instructive to examine their large scale internal modes of vibration in terms of the normal modes of a freely vibrating sphere [145]. For an isotropic elastic material where the elastic moduli of Lamé are equal and Poisson ratio is $1/4$, the lowest four modes of vibration have the frequency ratio, $1.00 : 1.06 : 1.72 : 2.23$. The motions associated with these modes are labelled A, B, D and C respectively in Fig. 4.8.

Of the four modes depicted in Fig. 4.8, all can, in principle, be observed by neutron scattering, but only D is symmetric and Raman active. The ratio of the observed Raman and neutron scattering frequencies is ≈ 1.5 at room temperature, similar to that of the frequencies for the D and B modes which is 1.6. The frequency of mode C is approximately twice that of A and

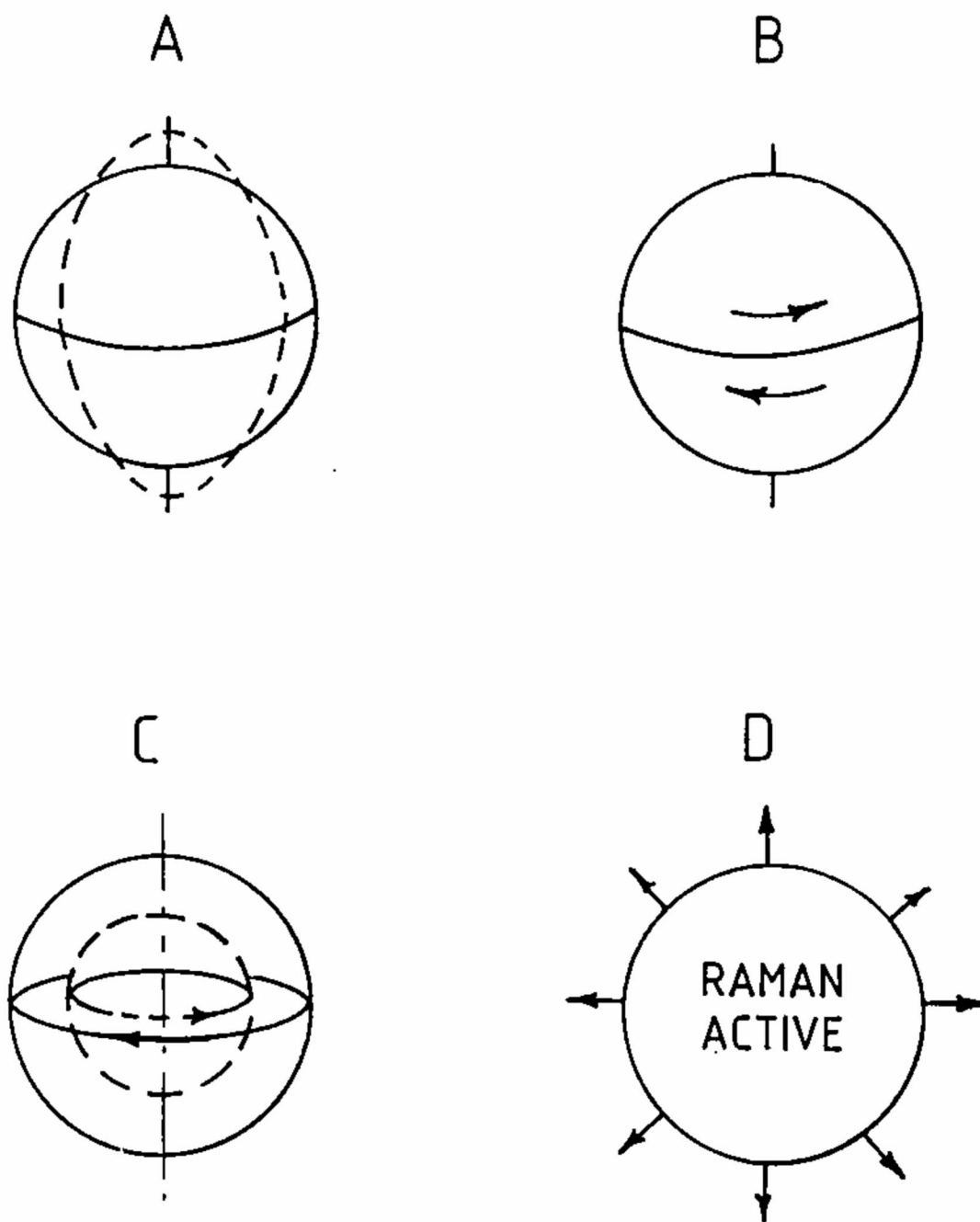


Fig.4.8 Four low frequency modes of a freely vibrating sphere obtained by reference to Love's theory of elasticity [145]. Mode D is raman active and was observed by Brown et al. [131]. The low frequency peak in the neutron scattering is tentatively identified as mode B (see text).

$$A:B:C:D = 1.00 : 1.06 : 2.23 : 1.72$$

IN FREQUENCY

B and this might account for the hint of extra intensity which occurs near 1.2 THz in Fig. 4.3. The results in Fig. 4.1 provide no additional evidence for this mode. This may be due to lack of proper statistics because of thermal effects. Applying Boltzmann statistics and assuming harmonic vibrations, the intensity level at ≈ 1 THz should increase by a factor of 3 on going from 77K to 298 K. In other measurements (not shown) the observed increase was 2.2 ± 0.3 .

It is not clear why the D mode would not be observed by neutron scattering. We suggest that we have observed mode B and Peticolas et al. [136] have observed mode D. Our choice of mode B is admittedly somewhat arbitrary since mode A is sufficiently close in frequency to mode B that we can not rule out the possibility that the peak in the neutron scattering response is attributable to mode A or combination of A and B. Molecular dynamics calculations will be necessary to adequately describe the atomic motions, their supposed relationship to these globular deformations and the shift in $S_{inc}^{Vib}(Q, \omega)$ as a function of temperature.

An obvious objection to the globular protein model arises because of the difference in the scattering between alpha-C and chymotrypsinogen A. These two molecules have approximately the same overall shape. It may be, however, that breakage of amino acid chain at two places in chymotrypsinogen A results in a much more labile molecule with a substantially reduced resistance to shear forces. The resultant "floppiness" of the molecule after

cleavage would be compatible [154] with the large scale motions thought to be involved in biological function. A feature of the Lovesey and Schofield calculations (fluid in a shell model) [62] is that the intensity and peak shape of the neutron scattering is a very sensitive function of kinematic viscosity. In the case of hemoglobin the possibility of a damped mode of slightly higher frequency (Fig. 4.7 data, upper panel) would be compatible with the lower molecular weight (≈ 16000) of each of its two slightly different subunits, whose closeness might lead to a damping out of their motion. One should note that the spherical vibrational model can not be more than a rough approximation to the actual physics of the system. The results of more sophisticated theoretical calculations are needed to decide the true situation.

According to our suggestion for well defined peak of alpha-C, we can conclude that it is interesting to speculate how a mode like B might be helpful in enzyme function. Alpha-C is a digestive enzyme whose function is to cleave polypeptide chains. The catalytic activity of alpha-C depends on the reactivity of serine and histidine residues located in the cleft in the molecular structure. When a polypeptide enters the cleft it is held near the serine and histidine by multiple hydrogen bonding. If this cleft is located near the equatorial plane, then a mode like B which generates strong shear forces would aid in the cleavage of the confined polypeptide. The absence of well defined peaks for chymotrypsinogen A (the precursor of alpha-C) in comparison to the alpha-C may be explained as (1) chymotrypsinogen A has a different composition with absence of four amino acids

(serine, arginine, theonine and asparagine) from alpha-C, and structural changes can be found according to Mathews et al. [157]. The cleavages can have a profound effect on the low frequency vibrational structure; (2) alpha-C and chymotrypsinogen A have approximately the same overall shape. It may be, however, that breakage of amino acid chains at two places in chymotrypsinogen A results in a much more labile molecule with a substantially reduced resistance to shear forces.

Hemoglobin is an oxygen carrier molecule. Oxygen is dissolved in blood due to the presence of hemoglobin and it oxydizes the biological cells and warm up the biological body. A mode like B i.e. shear forces of the hemoglobin molecules could be associated with conformational changes involving the transport of oxygen in blood e.g. the opening and closing of path ways to permit transport of oxygen to and from the heme groups in hemoglobin.

CHAPTER 5

CONCLUSIONS

Applications of neutron scattering to study biological materials using high-flux neutron beams have significantly increased. During the past decade neutron scattering technique has proved itself to be an important tool for the determination of the behaviour and structure of biologically important compounds.

Our study includes three categories of measurements using different techniques of neutron scattering. SANS measurements have been carried out on aqueous solutions of a neurotoxin, a cytotoxin and a cytotoxin analog from three cobra venoms. The overall sizes of these three toxins are not substantially different inspite of their diverse functions. Slight differences in overall shape have been found for these three toxins molecules. These differences in shapes could lead to initial identification of possible receptor sites for the different toxins. A little difference in shape of the neurotoxin from the other two toxins leads to an apparent conclusion that a "lock and key" mechanism may be involved at some stage in toxin-substrate interactions. Many species of cobra exist in the world. The toxins of different cobras have different interactions with biological systems. By studying of many other toxins of cobra venoms other than the mentioned three toxins above might lead to a conclusion for actual causes of diverse functions of toxin molecules. Due to the different interactions of different toxins with biological systems, it has been found that a snake-bite victim takes different period of time to be cured or die. More

investigations on cobra toxins of different species are required to find out their diverse functions in biological systems and it might help to treat the snake-bite victims to give them new lives.

Neutron diffraction measurements have been carried out to study the effects of Δ^8 -THC, Δ^9 -THC and cholesterol incorporated in phospholipid membranes of DPPC. Our analysis shows that the cannabinoids and cholesterol are similarly located as inclusions in the membrane interior just beneath the phospholipid head groups. The results obtained from neutron scattering suggest that Δ^8 -THC and Δ^9 -THC produce similar effects on membranes. The increase of interlamellar thickness of DPPC is same within experimental error for Δ^8 -THC, Δ^9 -THC and cholesterol. Δ^9 -THC is known to be much more effective than Δ^8 -THC in inhibiting synaptosomal enzyme; it is also much more psychoactive. Our suggestion is that the psychoactive effect of Δ^9 -THC is due to its interaction with membrane-associated proteins, specifically those parts of the protein embedded in the lipid bilayer. In these measurements we got two orders of reflections only. For scanning the second order reflection we used ten times monitor value than that for first order reflection. Inclusion of higher order of reflections could help model calculations. We have calculated the scattering density profiles and Patterson maps by using three orders of reflections including zero order reflection. The zero order reflection calculated from least square analysis for model calculations has no effect on the nature of the density profiles except for an additive or

subtractive factor. To get sufficient confidence on these results higher order reflections are required. To have third order reflection and so on, a very high-flux reactor of neutron-flux at the order of $\approx 10^{18}$ n.cm⁻².sec⁻¹ is required. We used the NRU (120 MW) reactor at Chalk River Nuclear Labs. for these measurements which has the neutron flux of the order of $\approx 10^{14}$ n.cm⁻².sec⁻¹. It was not feasible to carry out the measurements using very high monitor-value of the neutron wave length ≈ 4 Å at Chalk River Nuclear Labs., because to complete the measurements for 8 - 10 samples (some of our samples did not give good results and those results were rejected) it would take several months. More measurements are required using high-flux reactor to have higher order of reflections from above mentioned samples to predict a model with a higher degree of confidence.

Inelastic neutron scattering measurements have been carried out to investigate the low frequency vibrational modes of alpha-C, chymotrypsinogen A and hemoglobin. The analysis of different spectra of alpha-C for different Q values suggests that the well defined peak of alpha-C is the result of the vibrational mode B. The results of chymotrypsinogen A do not show any well defined peak like alpha-C, although it is the precursor of alpha-C. The reasons for this difference have been explained elsewhere in the present thesis. Alpha-C is a digestive enzyme. A mode like B might be helpful in enzyme function. The digestive enzyme alpha-C has the function to cleave polypeptide chains. The catalytic activity of alpha-C depends on the reactivity of serine and histidine residues located in a cleft in the molecular structure.

If this cleft is located near the equatorial plane, then a mode like B which generates strong shear forces would aid in the cleavage of the confined polypeptide. Hemoglobin is an oxygen carrier molecule. From the spectra of hemoglobin we found a weaker peak than that of alpha-C and we suggest that this is the result of the vibrational mode B. A mode like B i.e. shear forces of the hemoglobin molecules could be associated with conformational changes involving the transport of oxygen in blood e.g. the opening and closing of path ways to permit transport of oxygen to and from the heme groups in hemoglobin. Another question which remains open in our results is that whether the peak is due to mode B only or due to a combination of mode A and B. The frequency of mode A is close to mode B. The resolutions of peaks were not sufficient to rule out the possibility that the peak is attributable to mode A or a combination of A and B. In this regard we can suggest that molecular dynamics calculations will be necessary to sufficiently describe the atomic motions, their supposed relationship to these globular deformations and the shift in $S_{inc}^{vib}(Q, \omega)$ as a function of temperature.

Neutron scattering studies of biological materials will require the installation of reactors with much higher flux than are presently available. Till today the experimenters have exploited a very small fraction of knowledge in this field. In future more experimenters will be fascinated to be engaged in this field to add further valuable information about biological systems.

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