THE INCOMPLETELY DENATURED STATE OF GLOBULAR PROTEINS

Thesis by

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CONTENTS

ABSTRACT

From the measurement of near and far ultraviolet circular dichroism, nuclear magnetic resonance of lysozyme and pepsinogen in various solution conditions, I classify the conformational states of these proteins broadly into three states, that is, native state, random coil state, and incompletely denatured state (ID state). In the ID state, the tertiary structure of the native protein is almost broken while some secondary structures are retained. Although lysozyme exhibits one-step transition and pepsinogen shows an equilibrium intermediate during guanidine hydrochloride (GuHCl) denaturation, the temperature-GuHCl state-diagrams of the two proteins are found to be almost same and consist of these three states.

I consider that the denatured protein in water could still have secondary structures, and when the concentrated denaturant such as GuHCl exists at low temperature, the secondary structures would be broken by the binding of denaturant, and the protein is unfolded to random coil state.

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I. Introduction

As shown in Fig.1 (a), many globular proteins show cooperative one-step transition from native to denatured state when the concentration of denaturant is increased (Tanford 1968ab, Privalov 1979). (Here the denaturant means the cause of denaturation of proteins such as temperature, salt, acid, alkali, alcohol, GuHCl and urea. The cooperative one-step transition means that no intermediate state appears in the equilibrium experiments of denaturation.) And the two-state model, where only the native and denatured state exist, was considered to be a good working model for describing the denaturation of globular proteins.

However, it is found that the denaturations of some proteins can not be described with the two-state model as the number of protein-denaturation data increases and the precision of the measurement is improved (Kim et al. 1982, Privalov 1982, Saito et al. 1983ab). Fig.1 (b), (c) and (d) represent the denaturation profiles of these proteins. In these figures, far ultraviolet circular dichroism (UV CD), CD at 222 nm (Chen et al. 1972), and near UV CD, CD at 280 nm (Strickland 1974), are used for good probes detecting the change of the secondary and tertiary structures, respectively. In Fig.1 (b), the secondary and tertiary structures show two transitions simultaneously,

and this profile is interpreted as the denaturation of two independent domains in order. It means that one domain unfolds at the first transition, where the tertiary and secondary structures of another domain is remained intact, and at the second transition the latter domain unfolds (Miles et al. 1982, Iwahashi et al. 1983). In Fig.1 (c), the secondary and tertiary structures are broken showing one-step transition but these transitions do not coincide, and it indicates the existence of an intermediate state, in which the tertiary structure is broken but the secondary structures are unchanged (Kuwajima et al. 1976). This profile is considered to show the hierarchy of the structures of globular proteins, and its relation to protein folding is interesting. In Fig.1 (d), the tertiary structure shows one-step transition and simultaneously the secondary structures show one transition but another transition occurs at high denaturant concentration. Pepsinogen used in this study exhibits this profile through GuHCl-denaturation (Saito et al. 1983a).

Here the tertiary structure means the structure whose fluctuation is small enough to determine the position of respective atoms in a protein by X-ray diffraction analysis, and the word is used as the almost same meaning as the ordered state of side chains of amino acid in this paper.

Several different denatured states of globular

proteins according to the variety of the denaturants have been found. While many globular proteins are unfolded to random coil state, the state without secondary or tertiary structures, in the concentrated GuHCl solution (Tanford 1968a), they retain some secondary structures in the heat-denatured state, judging from circular dichroism, opital rotatory dispersion, and laser-excited raman spectroscopy (Tanford 1968a, Tanford et al. 1970, Kugimiya et al. 1973, Chen,M.C. et al. 1973, Kato 1981, Labhardt 1982), and heat-denatured lysozyme shows transition to random coil by the increase of GuHCl concentration (Aune et al. 1967, Ahmad et al. 1983). Strictly speaking, the 'cross-linked random coil' must be used instead of 'random coil' because we discuss here the globular proteins whose S-S bond is not broken. But I use 'random coil' for 'cross-linked random coil' in this paper to avoid complexity.

Lysozyme goes to highly α-helical denatured state in methanol or ethanol solution (Ikeda et al. 1970) and to the different denatured state whose content of β-seets is rather high than native state in acetic acid solution (Kato 1981), and acid-denatured bovine carbonic anhydrase B posseses a conformational state which is quite different from the native state and random coil state (Wong et al. 1974).

On the other hand, from the precise calorimetric

measurement, no differences of enthalpy and Gibbs energy between heat-denatured state, pH-denatured (acid-denatured or alkali-denatured) state, and random coil state, was reported recently (Privalov 1979).

In order to clarify the mechanism of the denaturation of globular proteins including equilibrium intermediate state and the relation between various denatured states, the changes of the secondary and tertiary structures of lysozyme and pepsinogen in various solution conditions are investigated by the measurement of near and far ultraviolet circular dichroism and proton magnetic resonance (^{1}H-NMR). From the experimnt I present the following results and conclusions. (1) The conformational state of these proteins are classified broadly into three states, that is, native state, random coil state, and incompletely denatured state (ID state). In the ID state, the tertiary structure is almost broken while some secondary structures are retained. (2) The temperature-GuHCl state diagrams of these proteins are almost identical and consist of these three states. (3) The denatured protein could still have some secondary structures, and when the concentrated GuHCl exists at low temperature, the secondary structures would be broken by the binding of the denaturant, and the protein is unfolded to random coil.

II. Materials and Methods

(A) Materials

Porcine pepsinogen (grade I) was purchased from Sigma Chemical Co. and hen-egg-white lysozyme was from Seikagaku Kogyo Co. (Tokyo) and they were used without further purification. The purities of these proteins which were checked by SDS electrophoresis, were observed about 100% (Saito et al. 1983b).

Protein concentration was determined spectroscopically using the following extinction coefficients (absorbance of 1% solution at 280 nm): pepsinogen, A_{280} = 12.9: lysozyme, A_{280} = 26.9. The used concentration of these proteins were 0.05 - 0.1 mg/ml for circular dichroism measurement and 5 - 10 mg/ml for NMR measurement.

Guanidine hydrochloride (GuHCl) was a specially prepared grade for spectroscopic measurement by Nakarai Chemicals, Kyoto, Japan. D_2O (99.8 % deuterium oxide) and deuterated Tris (1 M solution in D_2O, MD-1049) were purchased from MSD Isotopes, Canada. Other chemicals were of reagent grade.

The concentration of GuHCl was determined by the refractive index (Nozaki 1972) for 7 - 8 M GuHCl solution.

Using buffers were, 50 mM Tris/HCl buffer pH 7 - 9 for pepsinogen, HCl/KCl buffer pH 1.7 of ionic strength 0.1 for Lysozyme, and 12.5 mM $Na_2B_4O_7$/NaOH buffer pH 9 - 10 for

pepsinogen in alkali region (Perrin et al. 1974).

pH was measured with Hitachi-Horiba F-7AD type pH meter at room temperature. The temperature dependences of pH of these buffers were followings. Tris/HCl buffer, d(pH)/dT=-0.03: HCl/KCl buffer, d(pH)/dT=-0.02: $Na_2B_4O_7$/NaOH buffer, d(pH)/dT=-0.008. I describe the pH values without temperature correction in this paper. It is probable that the midpoint of thermal transition, for example, is shifted higher of lower or the tansition-width is affected by the change of pH of the buffer. The effect is, however, expected to be so small that it may not affect the qualitative discussions of the state diagram in this paper, judging from the experiments in the solution of a little different pH.

(B) pH selection of the measurements

The pH of the measurement was selected for the following reasons.

(1) The possibility of the measurement of thermal denaturation below 70°C

It was generally known that the denaturation temperature of proteins is decreased when the pH of the solution is approached to the midpoint of the acid-denaturation or alkali-denaturation.

(2) Avoiding the aggregation during thermal denaturation

It was known that proteins tend to aggregate during

thermal denaturation near the isoelectric pH (pI) because of the decrease of the electrostatic repulsion force between protein particles. The pI value of lysozyme is 11.2. The pI value of pepsinogen is not known. The pI of pepsin is below 1 and then the pI value of pepsinogen, a precursor of pepsin, is considered to be also in acidic region.

(3) Avoiding the auto digestion of pepsinogen

This reason was only for pepsinogen. Pepsinogen was known to be activated to pepsin removing 40 amino acids, below pH 5 by auto digestion and therfore we can't measure the properties of pepsinogen in acidic region.

At neutral pH, lysozyme was thermally denatured above 80°C and then the pH value of the measurements, pH 1.7, was selected taking the pI value 11.2 into consideration. Except the thermal denaturation measurements in alkali region, the measurements of pepsinogen were done in Tris buffer (pH 7.6 at room temperature) and that of lysozyme in HCl/KCl buffer (pH 1.7 at room temperature).

(C) Circular dichroism, absorbance, fluorescence, Rayleigh scattered light intensity at 90° measurement

The apparatus used was developed to obtain simultaneously 4 spectroscopic dimensions, circular dichroism (CD), absorbance, fluorescence, and Rayleigh scattered light intensity at 90° of several wavelength (Wada et al 1980).

Far (Chen et al 1972) and near (Strickland 1974) ultraviolet circular dichroism spectra are proves for the secondary and tertially structural change, respectively.

Setting the wavelength, obtaining and saving the spectroscopic data and varying the solution condition (temperature and GuHCl concentration) were done automatically by the BASIC programs originally produced by Dr. H.Tachibana and used partially improved in this study.

The programs mainly used in the present study were followings.

(1) Program for thermal denaturation

It was programed to obtain spectroscopic data, simultaneously monitoring the temperature of the sample in the cell which was surrounded by the water bath connected to K2R Electronic type LAUDA small refrigerated thermostat whose temperature was controlled by the computer. The increasing speed of temperature was also variable and usually 0.1 or 0.2 °C/min.

(2) Program for GuHCl titration

This was programed to obtain spectroscopic data, titrating the concentrated GuHCl to the sample. The program corrected the data to subtract the absorbance of the GuHCl solution titrated in the sample and to compensate the dilution.

The GuHCl, used for titration, was usually 8 M and the measured range of GuHCl concentration was usually from 0 M

to 4 M and the usual interval of the measurements was 0.05 M.

(D) ^{1}H-NMR measurement

^{1}H-NMR spectra were measured with a Bruker 360 MHz FT NMR spectrometer and a JEOL 400 MHz FT NMR spectrometer, equipped with a temperature control unit.

The protein were deuterated by dissolving in 99.7 % D_2O and lyophilized.

50 mM deuterated Tris buffer pH 7.6 was made from purchased 1 M deuterated Tris buffer, and deuterated $Na_2B_4O_7$ which was dissolved in D_2O and lyophilized was dissolved again in D_2O to make 12.5 mM $Na_2B_4O_7$ buffer pH 9.3. The pH value was from direct pH meter reading with no correction. The concentrated GuDCl solution was made from the deuterated GuHCl which was made by dissolving GuHCl in D_2O and lyophilized. The pH of the concentrated GuDCl was adjusted after dissolved in used buffer.

The pH of the solution was adjusted by adding concentrated DCl and NaOD.

The proton chemical shifts were measured from the internal standard, sodium 2,2,3,3-tetradeutero-3-trimethylsilyl propionate (TMS).

III. Results

In all of the measurement of CD, aggregation did no occur judging from the Rayleigh scattered light intensity at 90° which is measured simultaneously in all experiments. Then it can be considered that the change of CD exhibits intra-molecular conformational change in the following results.

(A) The conformational change accompanied with the change of temperature and GuHCl concentration

(1) Lysozyme

Fig.2 shows the CD spectra of lysozyme in various solution conditions at pH 1.7. As the statement of the chapter of introduction, we can see the apparent differences of the CD spectra between heat-denatured state and GuHCl--denatured state.

The changes of CD at 222 nm of this protein are arranged in Fig.3 as varying temperature at several fixed GuHCl concentrations, or varying GuHCl concentration at several fixed temperatures. One solid line in this figure is obtained from one measurement. In Fig.3, there are three regions: (1) the plateau in the low temperature and low GuHCl concentration region, which holds the large absolute value of CD at 222 nm, (2) the region which holds

the middle absolute CD value in the high temperature (and any GuHCl concentraion) region, and (3) the region of the low temperature and high GuHCl concentration where the absolute value of CD is small. The protein is native in the first region, and it unfolds to random coil in the third region. From the magnitude of CD at 222 nm, it possesses some amounts of secondary structures in the second region. From the NMR study of thermal and GuHCl-denaturation of lysozyme, the tertiary structure of this protein is considered to be broken in both denatured state (McDonald,C.C et al. 1971).

This figure aggrees qualitatively with the 'phase diagram' presented by Tanford (Tanford 1968b).

(2) Pepsinogen

Fig.4 shows the CD spectra of pepsinogen in various solution conditions at pH 7.6.

As shown by Saito et al. (Saito et al. 1983a), pepsinogen exhibits two-step transition through the GuHCl-denaturation. At the first transition the magnitude of CD at 222 nm decreases and simultaneously the magnitude of CD at 280 nm decreases to almost zero, and at the second transition the magnitude of CD at 222 nm decreased while the CD at 280 nm exhibited no transition. This indicates that the tertially structure of the protein is almost broken accompanied with some changes of secondary

structures at the first transiotion, and the retained secondary structures are broken at the second transition.

The NMR measurement shown in Fig.5 also supports this interpretation. In the native state (27°C, 0M GuHCl), the NMR spectrum is complicated by the different environment of the inside protons of the protein. And some NH protons protected from H-D exchange are also seen in this figure in the region from 6 ppm to 10 ppm. But in random coil state (27°C, 3.8M GuHCl), the NMR spectrum is rather simplified and similar to that calculated from the spectra of the individual amino acid residues (Fig.6). Under this condition, the chemical shift of each proton is thought to be same as that of individual amino acid residues and not affected by neighboring residues. In the heat-denatured state (82°C, 0M GuHCl) and the intermediate state through GuHCl-denaturation (27°C, 1.9M GuHCl), the NMR spectra is similar to that of random coil state. This fact indicates that the tertiary structure of the native protein is almost broken in these states. A little differences of the spectra exist between these three denatured state. Whether these differences show the differences of the secondary structures or not is uncertain. Unfortunately the good proves for the secondary structural change, C_α-H proton, 4 ppm - 6 ppm, are hidden by the strong signal of HDO and then can not be observed. Considering these ambiguities above mentioned, such a model that some domains occuping

rather large part, 1/2 or 1/3, of native state still remain intact in the intermediate state, may not be adopted, judging from the similarity of the NMR spectra of the intermediate state to that of random coil state, and from the large differences from that of native state. The fact that the NH protons which are protected from H-D exchange in native state by the tertiary structure have completely disappeared in the intermediate state of GuHCl-denaturation as seen in Fig.5 supports that the tertiary structure is almost broken in the intermediate state.

The changes of the magnitude of CD at 222 nm are arranged as lysozyme in Fig.7. The thermal denaturation of pepsinogen was thought to be a one-step transition (Ahmad et al. 1978) from the measurement of absorbance at near ultraviolet (UV), but later Mateo et al. showed that the denaturation of this protein is not a one-step transition by the measurement of calorimetry (Mateo et al. 1981) and recently Wada et al. pointed out that an intermediate state existed during the thermal denaturation by the precise spectroscopic measurement of CD, absormance and fluorescence (Wada et al. 1983). As shown later, the population of the intermediate state is low in neutral pH. Then in this section about the measurement in neutral pH, the thermal denaturation of pepsinogen was considered to be a two-state transition approximately. The three distinct regions can be seen in this figure as lysozyme, native

region in the low temperature and low GuHCl concentration region which holds large absolute value of CD at 222 nm, and the region in rather high temperature which hold the middle absolute value, and random coil region in low temperature and high GuHCl concentration. Unlike lysozume the transition from the middle region to random coil region was observed. As seen in Fig.7 the two transitions through the GuHCl-denaturation become close, that is, the intermediate goes unstable at low temperature.

(B) The two-step transition through thermal denaturation

(1) Pepsinogen in alkali solution

Fig.8 shows the transition of pepsinogen through thermal denaturation measured by CD at pH 8.8 with no denaturant. Apparently this is not a one-step transition but a two-step one. At the first transition CD at 230 nm and 234 nm mainly changes and CD at 222 nm shows little change, and at the second transition, CD at 222 nm changes drasticaly and CD at 230 nm changes a little. The separation of these two transitions becomes apparent in Fig.9 measured at pH 9.3, and in Fig.10 measured at pH 10.0. The transition temperature of the first becomes apparently low as the pH is increased, while that of the second transition seems to change a little if any. The transition of the CD at 280 nm of all measurements occurs

during first transition and no transition appears at the second transition. It indicates that the tertiary structure of native protein is broken and some changes of secondary structures occur simultaneously at the first transition, and the secondary structure shows another transition as the temperature is further elevated. After the second transition, CD spectrum of the denatured protein coinsides that of heat-denatured state. While the intermediate state is stable in rahter large temperature range in alkali solution, the state is difficult to be detected by spectroscopy without precise measurement (Wada et al. 1983). Although it is intersting to know how the two transitions behave at lower pH solution, the precipitation occurs during thermal denaturation at pH 6.0 unfortunately. The relation of the behavior of these transitions and the precipitation, which is possibly a sign of occurrence of auto digestion, is also an interesting problem.

^{1}H-NMR spectra of pepsinogen in alkali region at several temperatures are shown in Fig.11. As seen in these figures, main changes occur between 20°C and 40°C, and it coincides the results of Fig.9. The observed main changes are followings (1) in methyl and ethyl, proton region (0.9 ppm - 3.4 ppm in random coil) the complicated spectra affected by the various circumstances around protons of the native structure goes to the simple peaks like that of random coil as the temperature is elevated (see the

calculated random coil like spectra of Fig.6). The two strong peaks at 1 ppm and 3.5 ppm in every spectra are from contamination, which is probably ethanol, judging from the chemical shift[5]. I used ethanol this time to substitute H_2O remained on the inner surface of NMR tube after washing.
(2) The H-D exchange of protected NH protons occurs almost completely between 20°C and 40°C as seen in Fig.11 (b). Some NH protons concealded into the stiff tertiary structures are protected from the contact with D_2O, and then the H-D exchange is good probe for the collapse of the tertiary structure. Above 40°C, the sharpening of the spectra by the thermally agitated molecular motion occurs and no drastic change can be seen. It indicates that the tertiary structure of the protein is broken at the first transition.

(2) Lysozyme in ethanol solution

Lysozyme shows two-step transition in 33 % ethanol solution at pH 1.7 when temperature is elevated (Fig.12). In this figure, while the first transition has not completed, we can see the magnitude of CD at 222 nm is increased at the first transition and decreased at the second transition. As lysozyme is denatured to the α-helical structure in the etahnol solution (Ikeda et al. 1970), it is assumed that lysozyme is unfolded toward this structure at the first transition, but as the temperature

is further elevated, the secondary structures are gradually broken to the heat-denatured state.

IV. DISCUSSION

(A) Definition of the incompletely denatured state

As mentioned in the chapter of introduction, there are various denatured states of globular proteins. The tertiary structure of proteins has been broken already in these denatured state including intermediately denatured state except in the intermediate state of Fig.1 (b), in which one domain is retained intact, and cooperative one-step transition occurs between native state and these denatured state. Even after the collapse of the tertiary structure, denatured protein possesses various secondary structures according to the solution conditions, and when the concentrated ligands such as GuHCl or urea exist in the solution, these secondary structures are broken. Random coil which possesses no secondary nor tertiary structures is called completely denatured state or completely disordered state. In contrast with the state, the incompletely denatured state is defined as the state in which the tertiary structure is broken and some secondary structures exist. It means that the incompletely denatured protein can be unfolded to completely denatured, random coil state. For example, if a globular protein, which is folded in order, changes to an α-helical lod in some solution conditions, the 'denetured state' is called incompletely denatured state. As the 'denetured state' is

considered to have highly ordered structure, it is unsuitable that the state is named (incompletely) disordered state.

In this study, the denatured state of lysozyme at high temperature (see Fig.3), and the intermediate state of pepsinogen of GuHCl denaturation and the denatured state of pepsinogen at high temperature (see Fig.7) are considered to be incompletely denateured states.

The new intermediate state through thermal denaturation of pepsinogen and lysozyme in alkali solution and ethanol solution, respectively is an incompletely denatured state in respect of the collapse of its tertiary structure and its secondary structural content. However the secondary structures of the intermediate state are different from that of denatured state at the higher temperature, and a transition are observed between these incompletely denatured states. It is important to be remarked that the incompletely denatured state does not define one paticular structure but is a general term for these denatured states.

The secondary structures were considered to be affected mainly by short range interaction from the success of predicting some secondary structures using the amino acids sequence in their neighborhood (Chen et al. 1974). On the other hand, the tertiary structure was considered to be determined under the effect of long range interaction (which means the interaction between residues positioned

long away along the polypeptide chain). Recently the prediction of the secondary structures is found to be not so sufficient for predicting the structures of unknown proteins (Nishikawa 1983). It is probable that the secondary structure of native state is affected by long range interaction to be suited to the tertiary structure. It is, therefore, interesting to study the precise differences of the secondary structures between native state and incompletely denatured state, in which the effect of the tertiary structure is considered to be much smaller than that of native state.

(B) Temperature-GuHCl state diagram

As the incompletely denatured state is defined in the preceding section, the temperature-GuHCl state diagrams of lysozyme (at pH 1.7) and pepsinogen (at pH7.6) are simplified to Fig.13. While the transition between incompletely denetured state and random coil state of lysozyme is too broad to be detected in this solution conditions, that of pepsinogen with varing GuHCl concentration is observed as a rather sharp transition. As the coil to helix transition of PBLG by temperature (Zimm et al. 1959), the shorter the length of helical region is, the broader the transition becomes. It is reasonable even if the transition is very broad, considering that the longest α-helixes of lysozyme in native state consists of only 10

residues (counted using the data from X-ray diffraction analysis). It is considered that when some concentrated ligands such as GuHCl or urea exist hydrogen bonds of proteins are cut and ligands bind to protein. Because of the decrease of entropy by binding of the ligands, they lieve from protein and hydrogen bonds recover as temperature is elevated. In Fig.3 and Fig.7, the magnitude of CD at 222 nm in the concentrated GuHCl solution changes almost lineary according to the change of temperature in this temperature range, and therefore perfect coil state has not apperared yet. But the perfect coil region will appear in these figures if the measurement in lower temperature becomes possible.

This temperature-GuHCl state diagram is considered to show the following aspects of the structural change of globular proteins. (1) The tertiary structure is destroyed showing one-step transition in spite of the kinds of denaturants. (2) Proteins whose tertiary structure is broken, that is, denatured proteins, can possess some secondary structures. (3) If the concentrated GuHCl exist at low temperature where the loss of entropy is not effective, the secondary structures of denatured protein are broken and protein is unfolded to random coil state.

Judging from the follwing results of α-lactalbumin, the protein also shows the same state diagram. (1) The far and near ultraviolet CD spectra and compactness of the

heat-denatured state are almost same as that of the intermediate state through GuHCl-denaturation, and no transition from the intermediate state to heat-denatured state is observed if the temperature is elevated (Dolgikh et al. 1981). (2) The magnitude of CD at 222 nm increases lineary in the concentrated GuHCl solution according to the elevation of temperature (Kuwajima et al. 1976).

The state diagram is expected to be a universal one among many globular proteins from the points that the heat-denatured states of many globular proteins show rather larger magnitude of far ultraviolet CD than that of GuHCl-denatured state (Saito,Y. and Wada,A. unpublished observation), and that the aspects of the structural change of lysozyme and pepsinogen is considered to be rather general ones among globular proteins than specific to these two proteins.

(C) Secondary structures and their folded structures in incompletely denatured state

The cooperative transition of secondary structures of pepsinogen after the collapse of tertiary structure through thermal denaturation in alkali solution is found in this study. Privalov et al. measured the thermal denaturation of pepsinogen by differential scanning calorimetry in the pH region from pH 6.0 to pH 8.0 and cuculated specific heat curve with two distinct peaks from measured one which

appeares to be only one peak, and they presented the model that two independent domains unfold respectively in order (Privalov et al. 1981, Privalov 1982). Though they analyzed their data without consideration of the cooperative transition of secondary structures, there is a possibility that one of the peaks they presented at higher temperature is derived from the cooperative secondary structural change. Now I am starting the precise and comparative measurements of spectroscopy and calorimetry using the same samples under the same solution conditions in the wide pH range, from pH 6.0 to 10.0.

About the changes of secondary structures according to various solution conditions, ribonuclease is investigated in detail by far ultraviolet CD spectroscopy, and the data shows that the units of secondary structures of native state can undergo indivisual transitions (Labhardt 1982). Although the tertiary structural change was not mentioned in his paper, it shows one-step transition from native to any other denatured states and no further transition of the tertiary structure can be detected by near ultraviolet CD spectroscopy (Hanai,R., Kidokoro,S. and Wada,A. unpublished observation). It indicates that the secondary structural chages between denatured states are considered as the transition in incompletely denatured state or the transition from incompletely denatured state to random coil state.

The 'molten globule state' of ∝-lactalbumin and cytochrome c was presented recently (Dolgikh et al. 1981, Ohgushi et al. 1983). In this state, the tertiary structure of the protein is broken and the fluctuation of the structure is large while its compactness is rather resemble to that of native state than that of random coil state as shown by the following results. Apparent radius of molten globule state of ∝-lactalbumin at room temperature is about 16 or 18 Å, while that of native state is about 16 Å and that of random coil state is about 24 Å from X-ray and neutron small angle scattering and quasi-elastic neutron scattering measurements (Izumi et al. 1983). The hydrodynamically equivalent radius of this state of cytochrome c is 20.1±0.4 Å while that of native state is 19.8±0.4 Å from the quasi-elastic light scattering measurement (Ohgushi et al. 1983).

To the contrary, the heat-denatured states of many globular proteins are known to show rather expanded conformation than native state, and the intermediate state of pepsinogen through GuHCl-denaturation is considered to be also an expanded state from GuHCl-gradient chromatographic measurement (Saito et al. 1983a). From the results above raised, the secondary structures of incompletely denatured state are found to show two structural types with respect to their folded structures, the compact structure as native or the expanded structure. In this context, it is also

interesting to clarify whether the intermediate state of pepsinogen through temperature-denaturation in alkali solution is the molten globular state or not, and to determine the interactions which make the compact folded forms stable.

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I would like to thank all of the members of the Wada group for their helpful discussions.

FIGURE CAPTIONS

Fig. 1. Several types of spectroscopic transition profiles of globular proteins. (a) Different probes of secondary and tertiary structure each give the identical cooperative transition. (b) Different probes of secondary and tertiary structure each give similar biphasic transition curves. (c) The secondary structure is more resistant than the tertiary structure to denaturant. (d) The secondary structure gives biphasic transition while the change of the tertiary structure is completed at the first transition. ((b) and (c) are refigured from Kimm et al. 1982)

Fig. 2. Far ultraviolet CD spectra of lysozyme: at 10°C, 0M GuHCl (native state, thick line): at 65°C, 0M GuHCl (heat-denatured state, thin line): at 10°C, 6M GuHCl (GuHCl-denatured state, dotted line)

Fig. 3. The magnitude of CD at 222 nm of lysozyme at pH 1.7 is plotted above the temperature-GuHCl concentration plane (three-dimensional figure). This figure is arranged from the measurements of thermal denaturation in 0M, 2M, 4M GuHCl, and GuHCl-denaturation at 13°C, 29°C, 34°C. The two curves of thermal denaturation in 0M GuHCl and

GuHCl-denaturation at 29°C are hatched in this figure.

Fig. 4. Far ultraviolet CD spectra of pepsinogen: at 27°C, 0M GuHCl (native state, thick line): at 65°C, 0M GuHCl (heat-denatured state, thin line): at 27°C, 1.6M GuHCl (intermediate state through GuHCl--denaturation, empty circles): at 27°C, 6M GuHCl (GuHCl-denatured state, dotted line)

Fig. 5. 360 MHz ^{1}H-NMR spectra of pepsinogen at pH 7.6. (a) native state, at 27°C, 0M GuHCl: (b) heat-denatured state, at 72°C, 0M GuHCl: (c) intermediate state through GuHCl-denaturation, at 27°C, 1.9M GuHCl: (d) GuHCl-denatured state, at 27°C, 3.8M GuHCl. The concentration of the protein is 10 mg/ml. Chemical shifts are measured from TMS. The strong peak from 4 ppm to 5 ppm is from HDO. The broad peak at 6.5 ppm of (c) and (d) is from proton of GuHCl. Amplitudes are not constant between spectra.

Fig. 6. A supperposition of the calculated spectra of free residues with the same composition of pepsinogen. Used parameters are from McDonald et al. (McDonald et al. 1969).

Fig. 7. The magnitude of CD at 222 nm of pepsinogen at pH 7.6 is plotted above the temperature-GuHCl concentration plane (three-dimentional figure).

This figure is arranged from the measurements of thermal denaturation in 0M, 1.6M, 3M GuHCl, and GuHCl-denaturation at 3°C, 17°C, 27°C. The three curves of thermal denaturation in 0M GuHCl, GuHCl-denaturation at 3°C and 27°C are hatched in this figure.

Fig. 8. Raw data of ultraviolet CD cahnge through thermal denaturation of pepsinogen at pH 8.8. (a) whole data and (b) selected data with the magnified column (arbitrary scale). Wavelengths are given by the number, in nm.

Fig. 9. Raw data of ultraviolet CD change through thermal denaturation of pepsinogen at pH 9.3. (a) whole data and (b) selected data with the magnified column (arbitrary scale). Wavelengths are given by the number, in nm.

Fig. 10. Raw data of ultraviolet CD change through thermal denaturation of pepsinogen at pH 10.0. (a) whole data and (b) selected data with the magnified column (arbitrary scale). Wavelengths are given by the number, in nm.

Fig. 11. 400 MHz ^{1}H-NMR spectra of pepsinogen at pH 9.3, 0M GuHCl. (a-1) at 10°C, (a-2) at 20°C, (a-3) at 30°C, (a-4) at 40°C, (a-5) at 50°C, (a-6) at 60°C, (a-7) at 70°C, (a-8) at 80°C, and (b) their aromatic resions. The concentration of the

protein is 5 mg/ml. The strong peak from 4 ppm to 6 ppm is from HDO. The two strong peaks at 1 ppm and 3.5 ppm are from contamination. Amplitudes are not constant between spectra.

Fig. 12. Raw data of ultraviolet CD change through thermal denaturation of lysozyme at pH 1.7 in 33% ethanol solution. Wavelengths are given by the number, in nm.

Fig. 13. Simplified state-diagram of globular proteins as a function of temperature and GuHCl concentration.

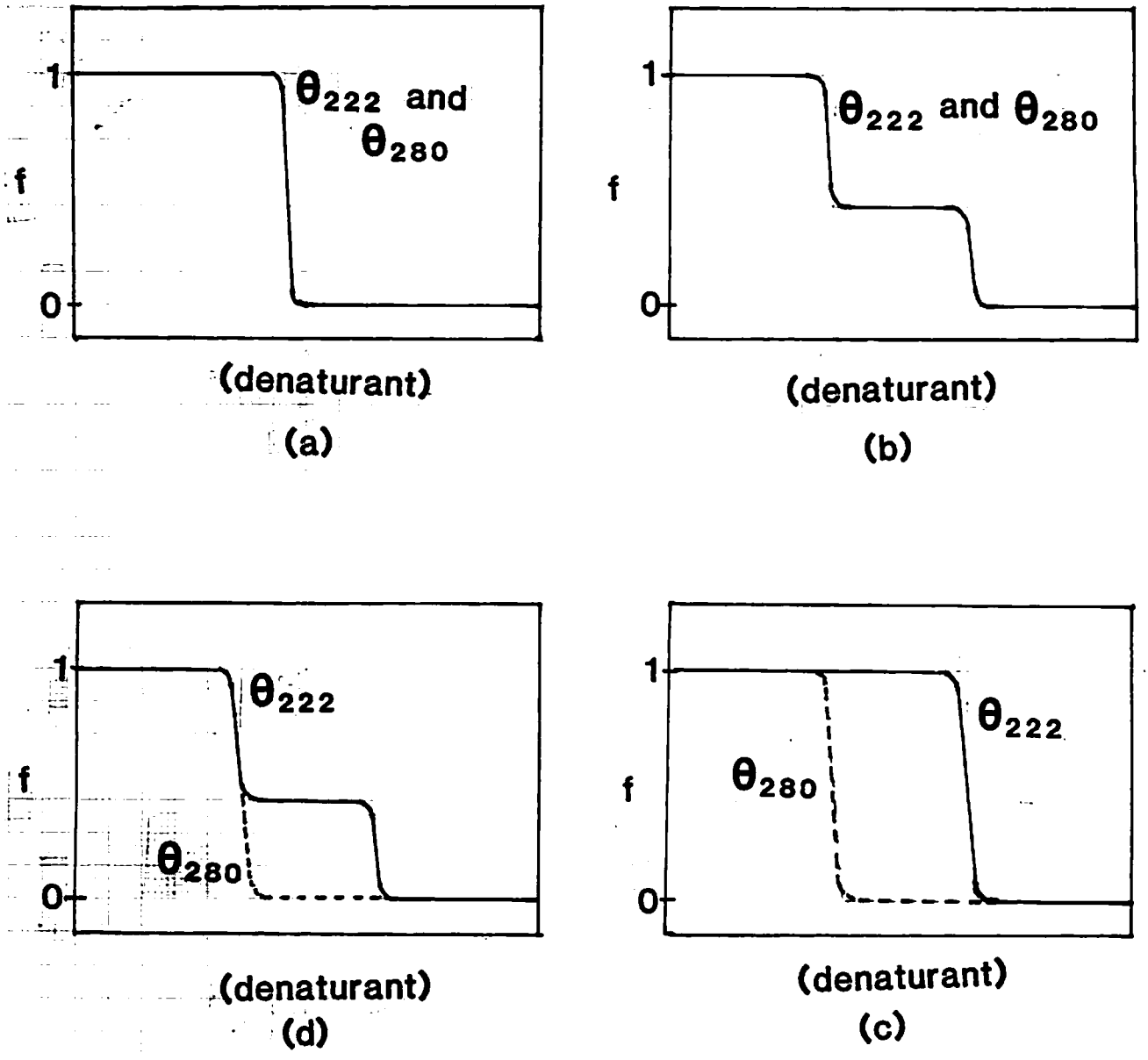

1
f
0
θ222 and θ280
(denaturant)
(a)
1
f
0
θ222 and θ280
(denaturant)
(b)
1
f
0
θ222
θ280
(denaturant)
(d)
1
f
0
θ222
θ280
(denaturant)
(c)

Fig. 1

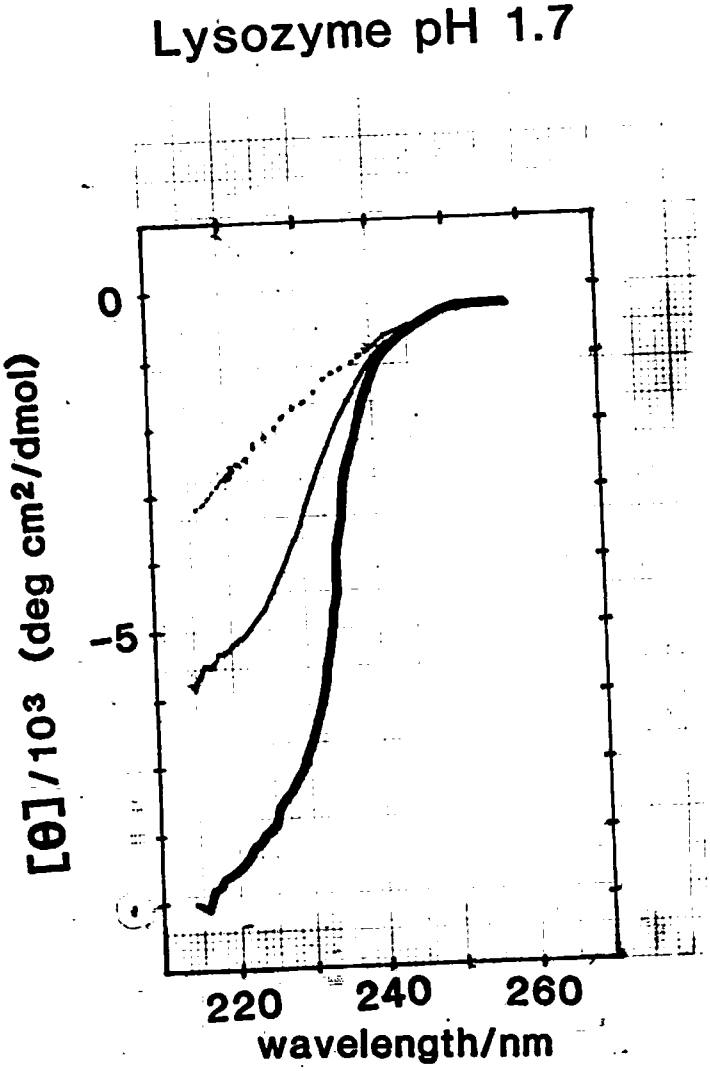


Fig. 2

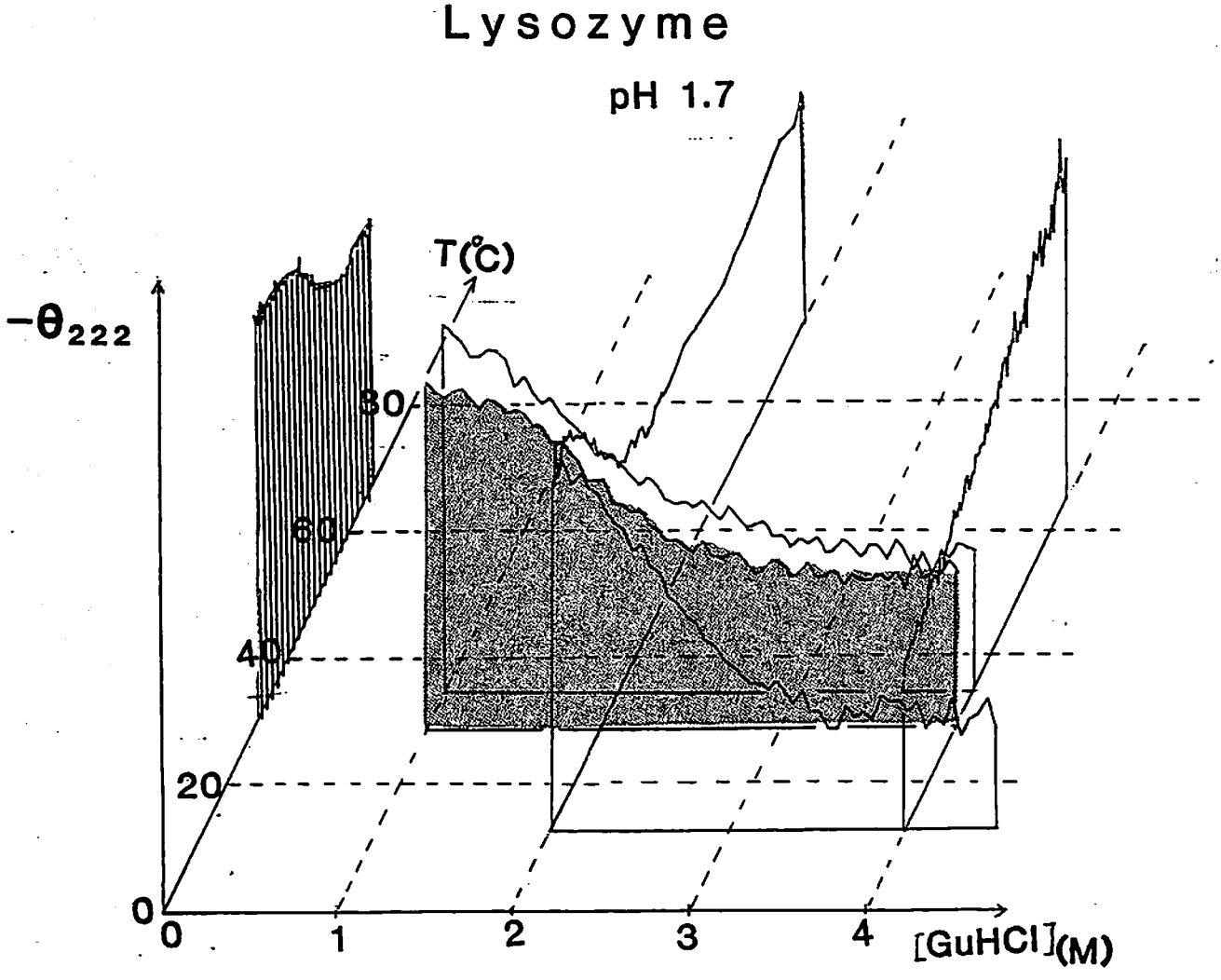


Fig. 3

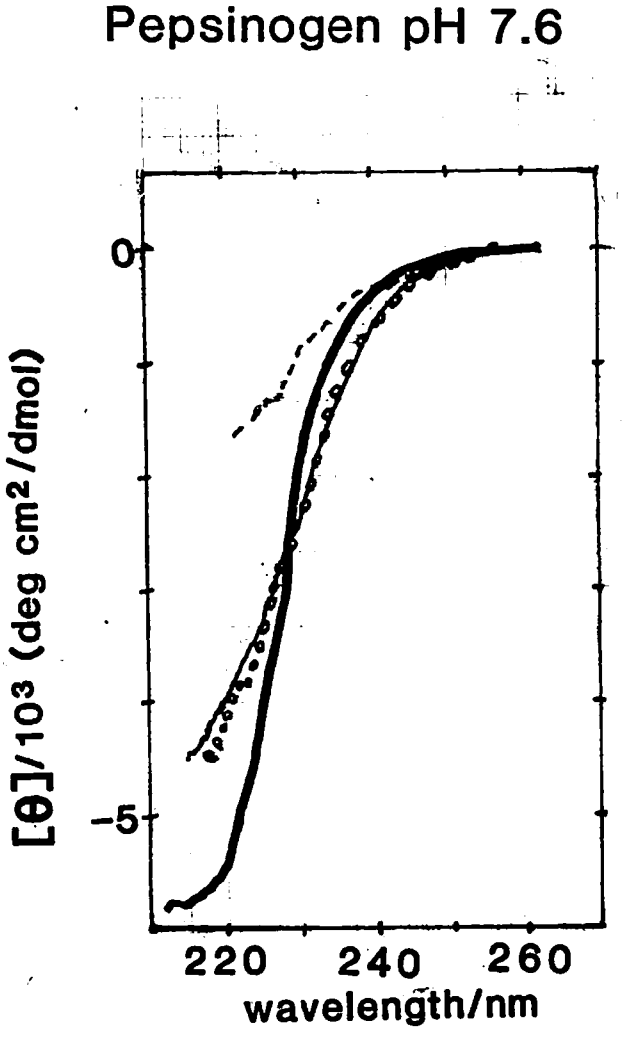

Pepsinogen pH 7.6
0
-5
[θ]/10³ (deg cm²/dmol)
220
240
260
wavelength/nm

Fig. 4

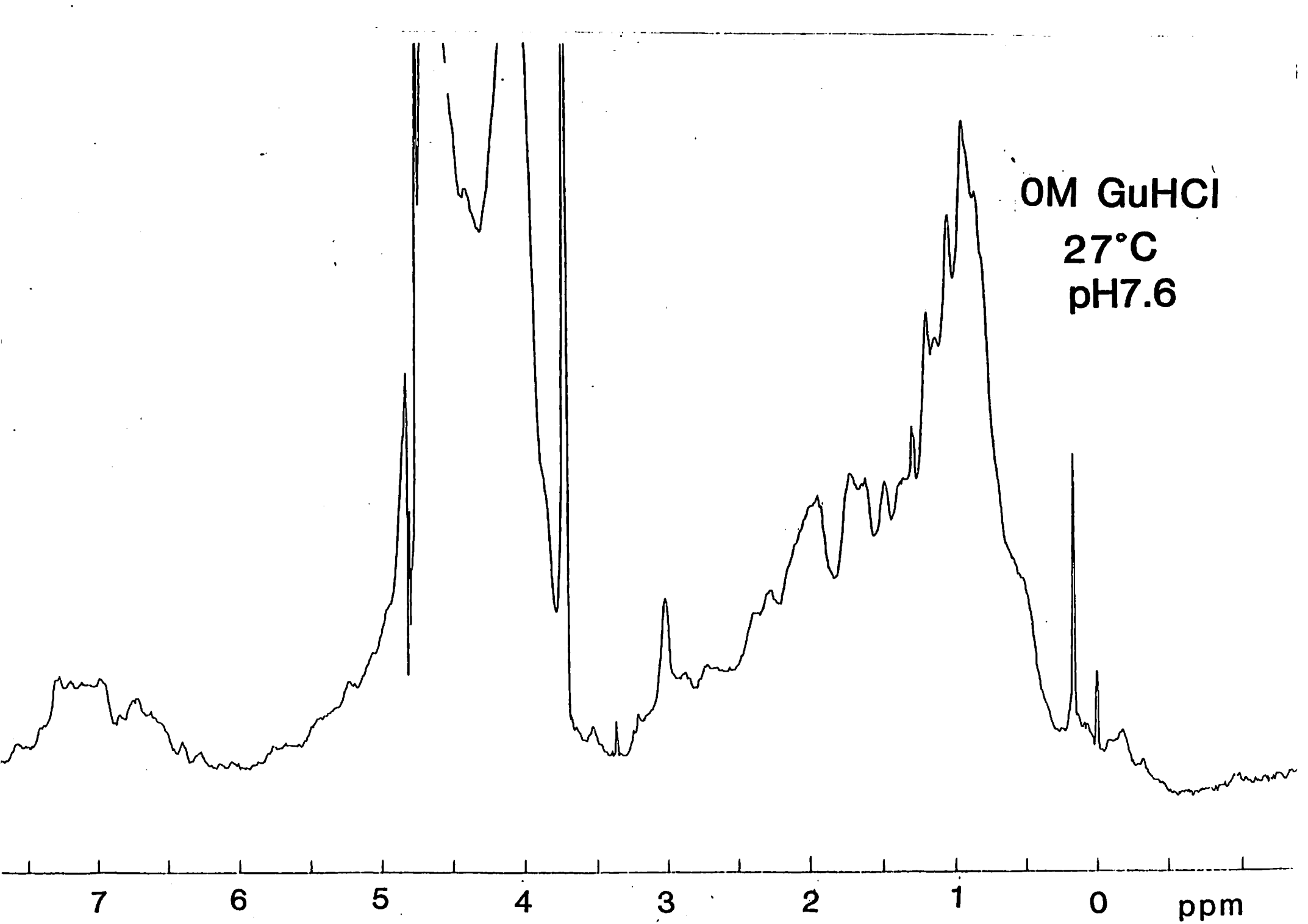
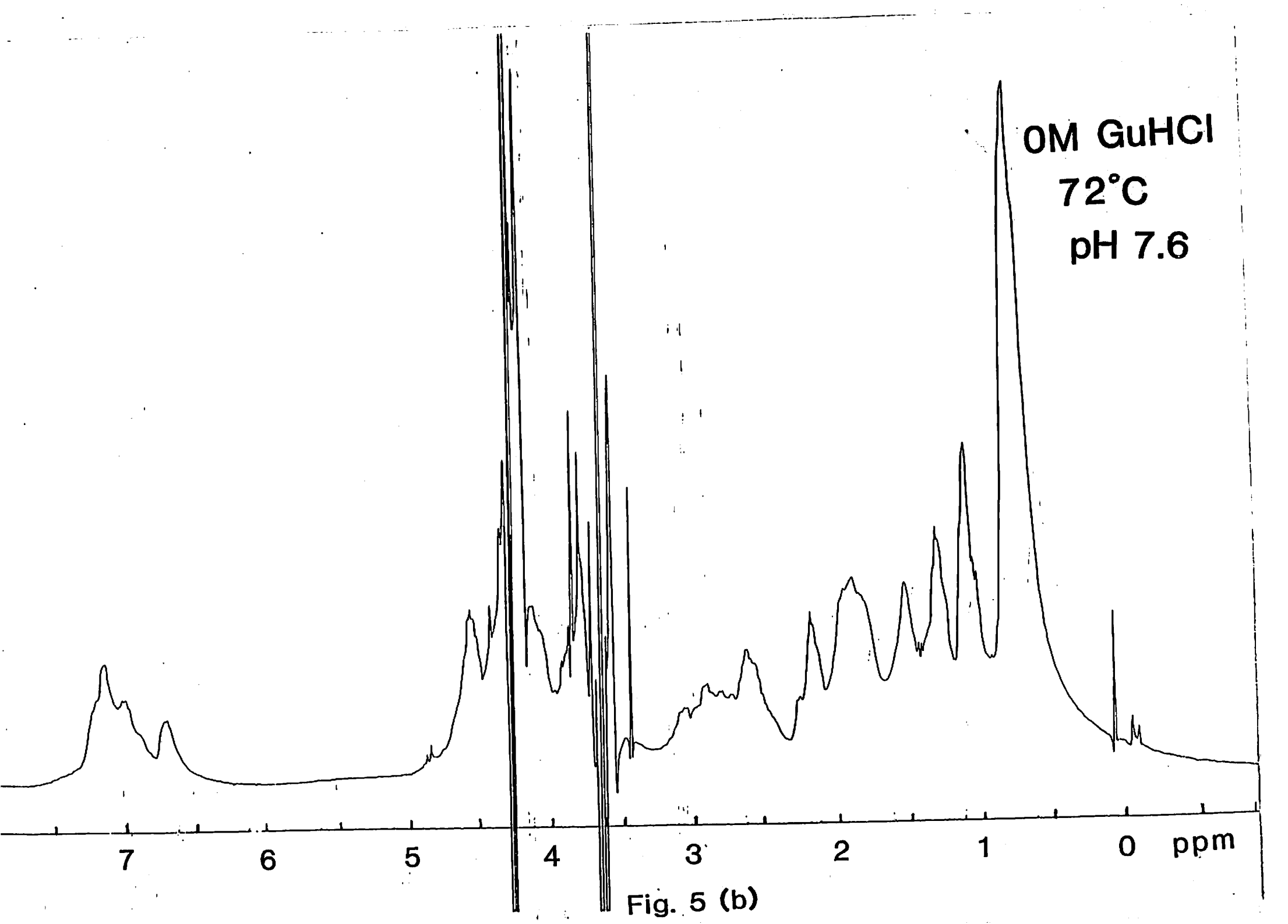


Fig. 5 (a)



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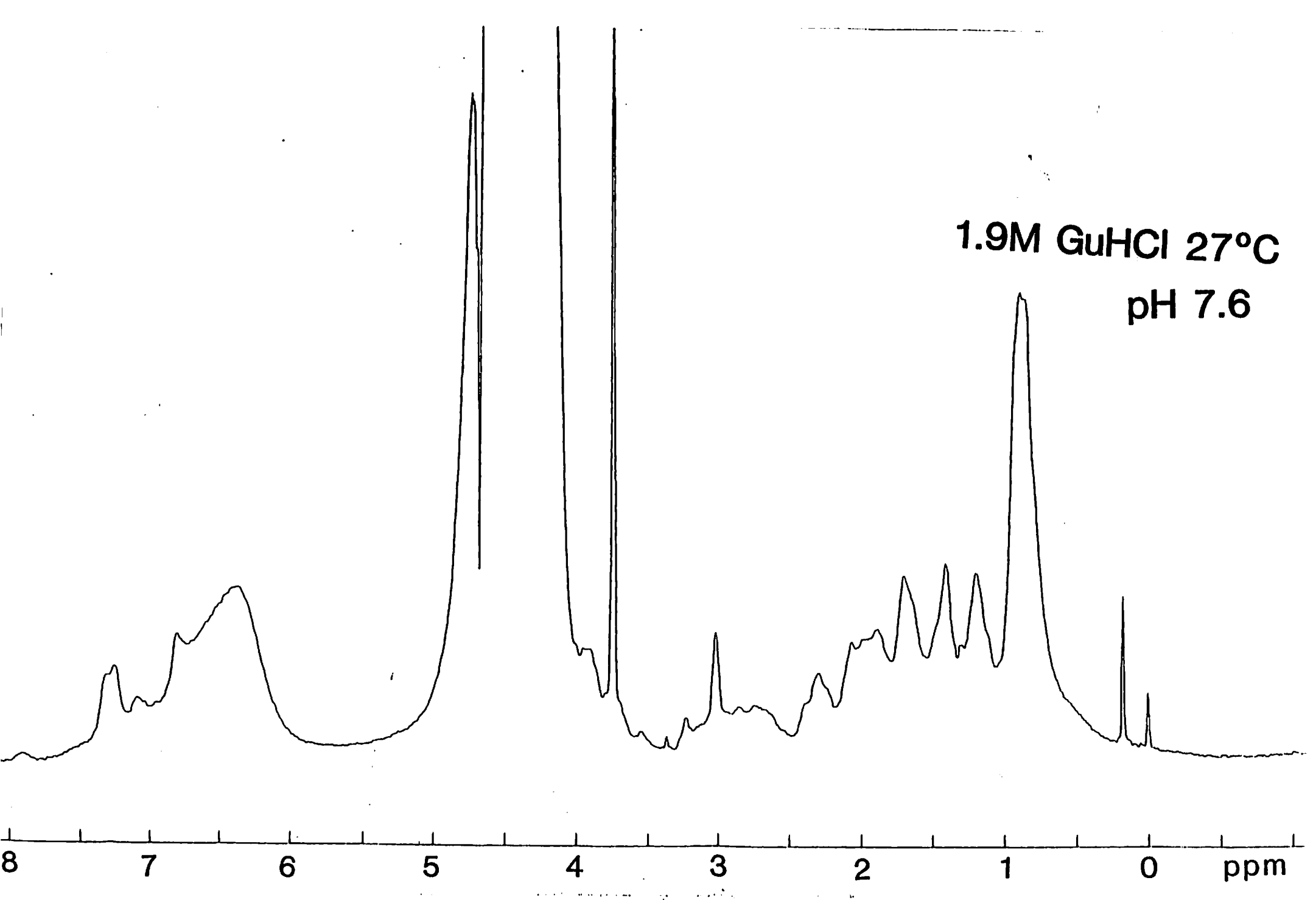
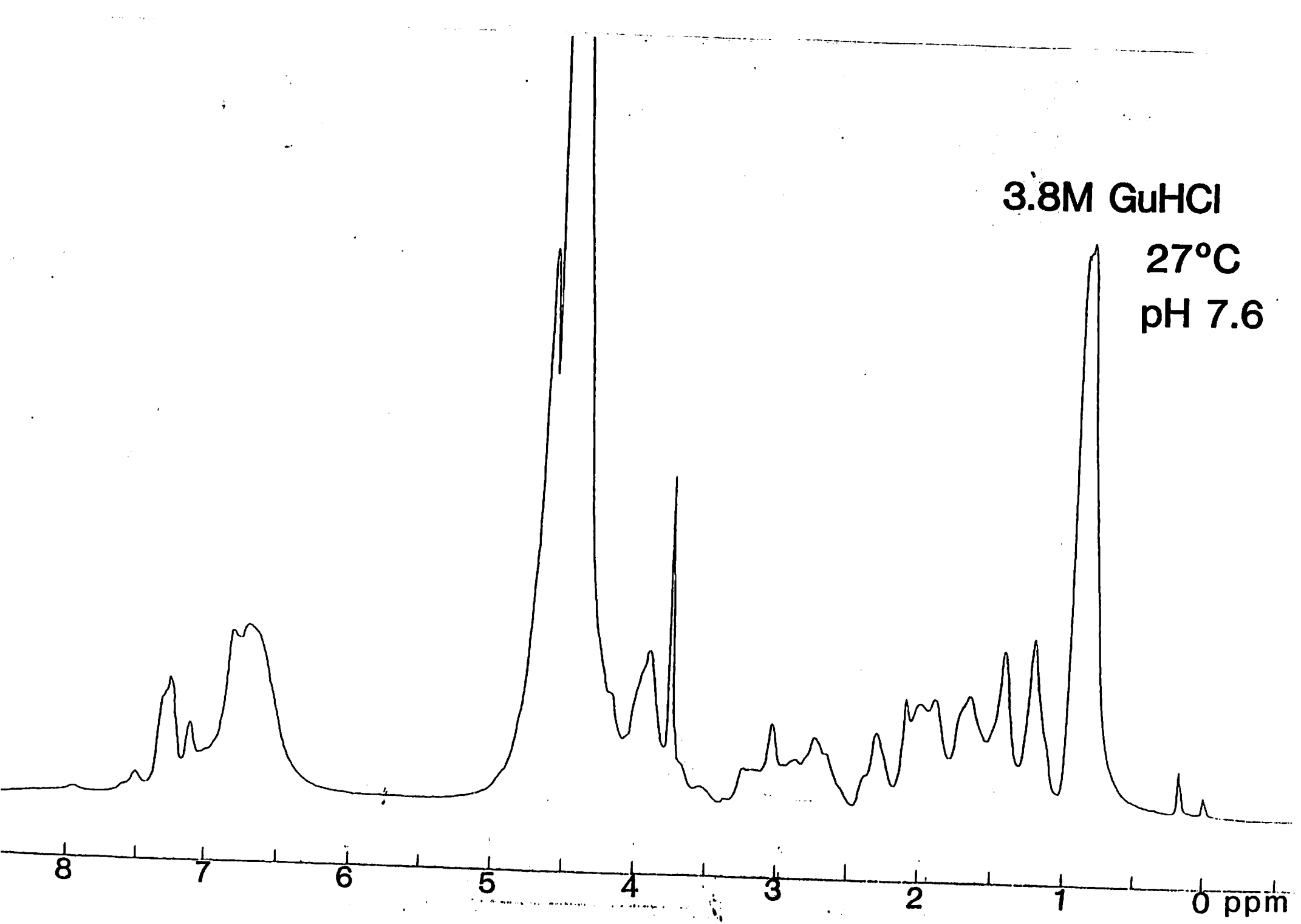


Fig. 5 (c)



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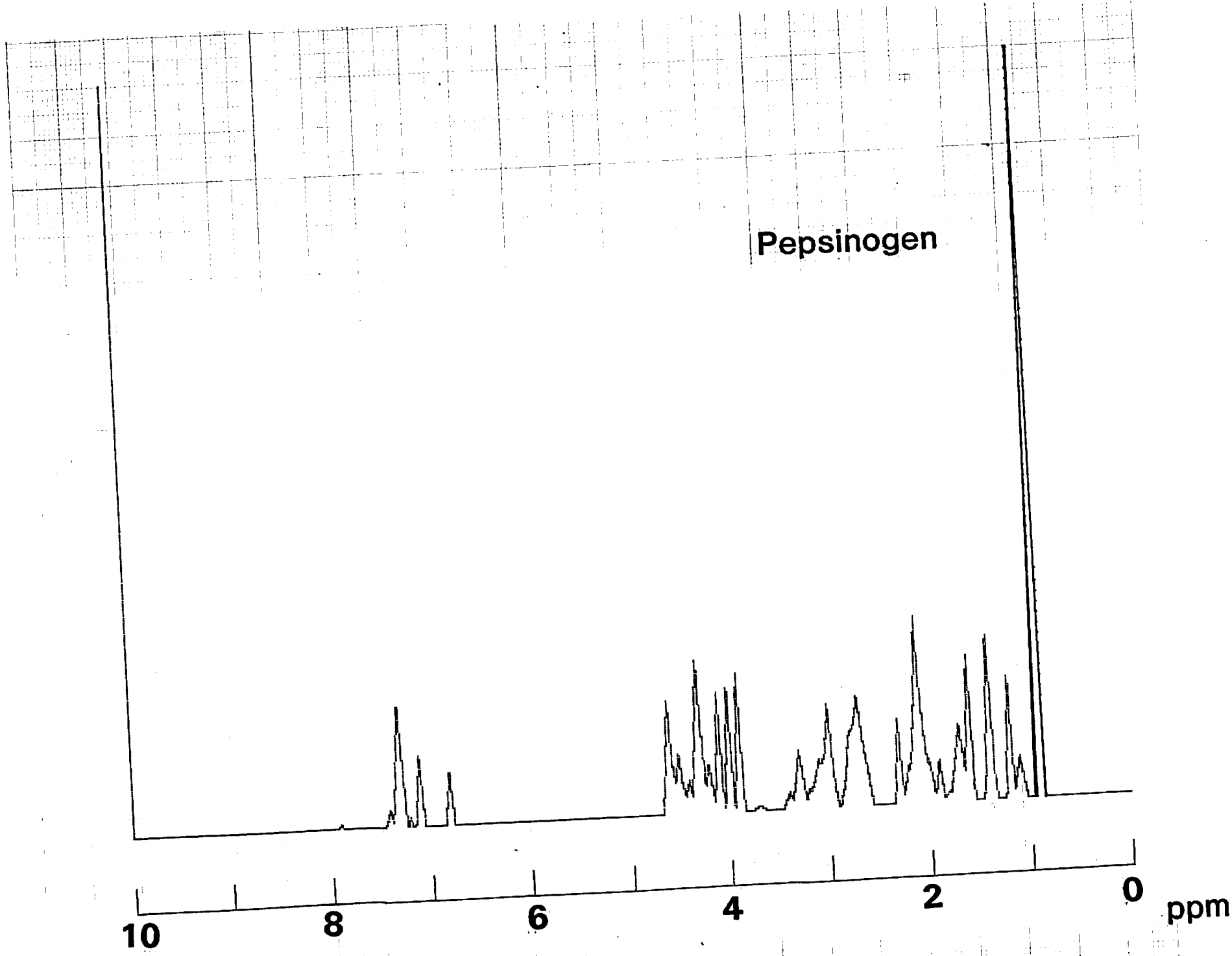

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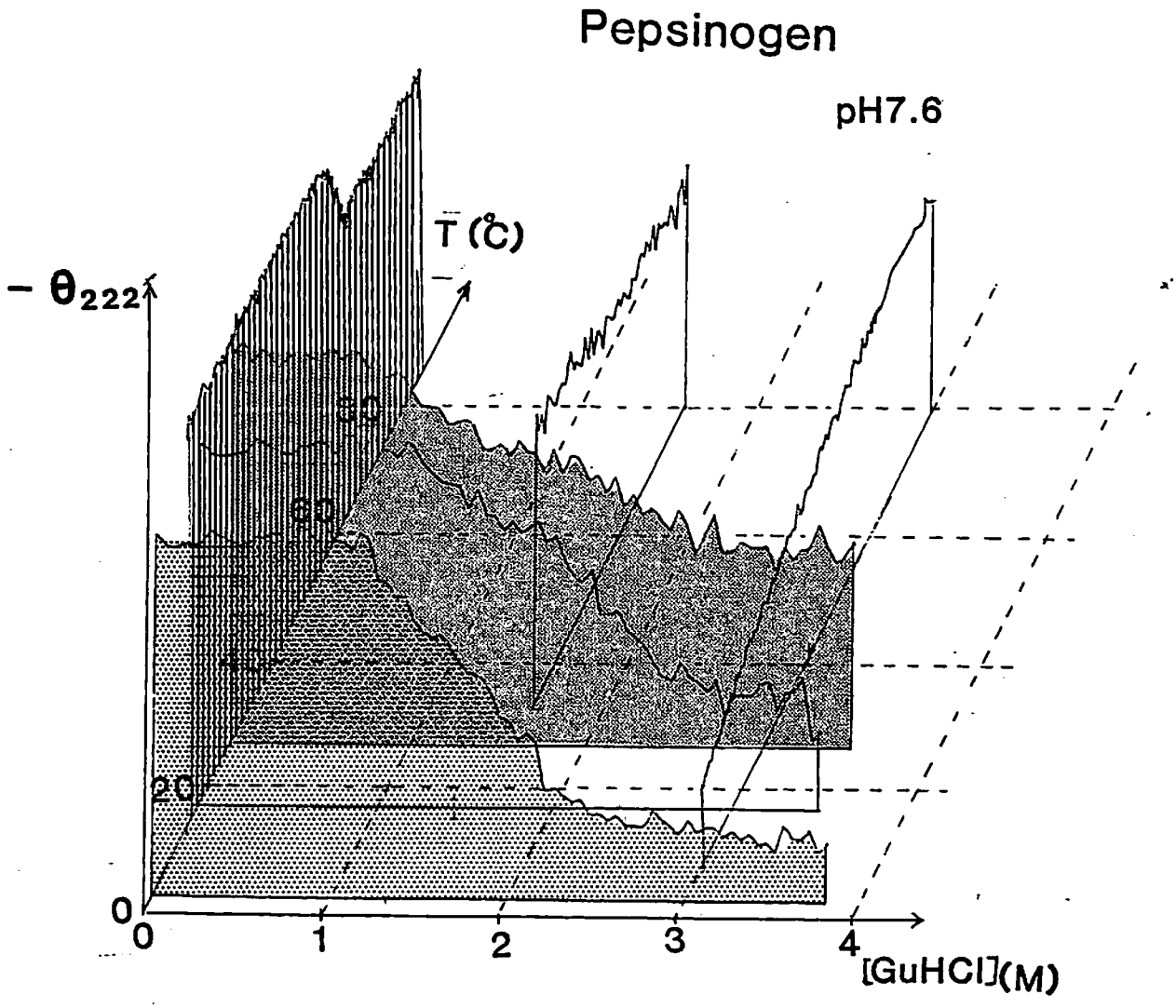

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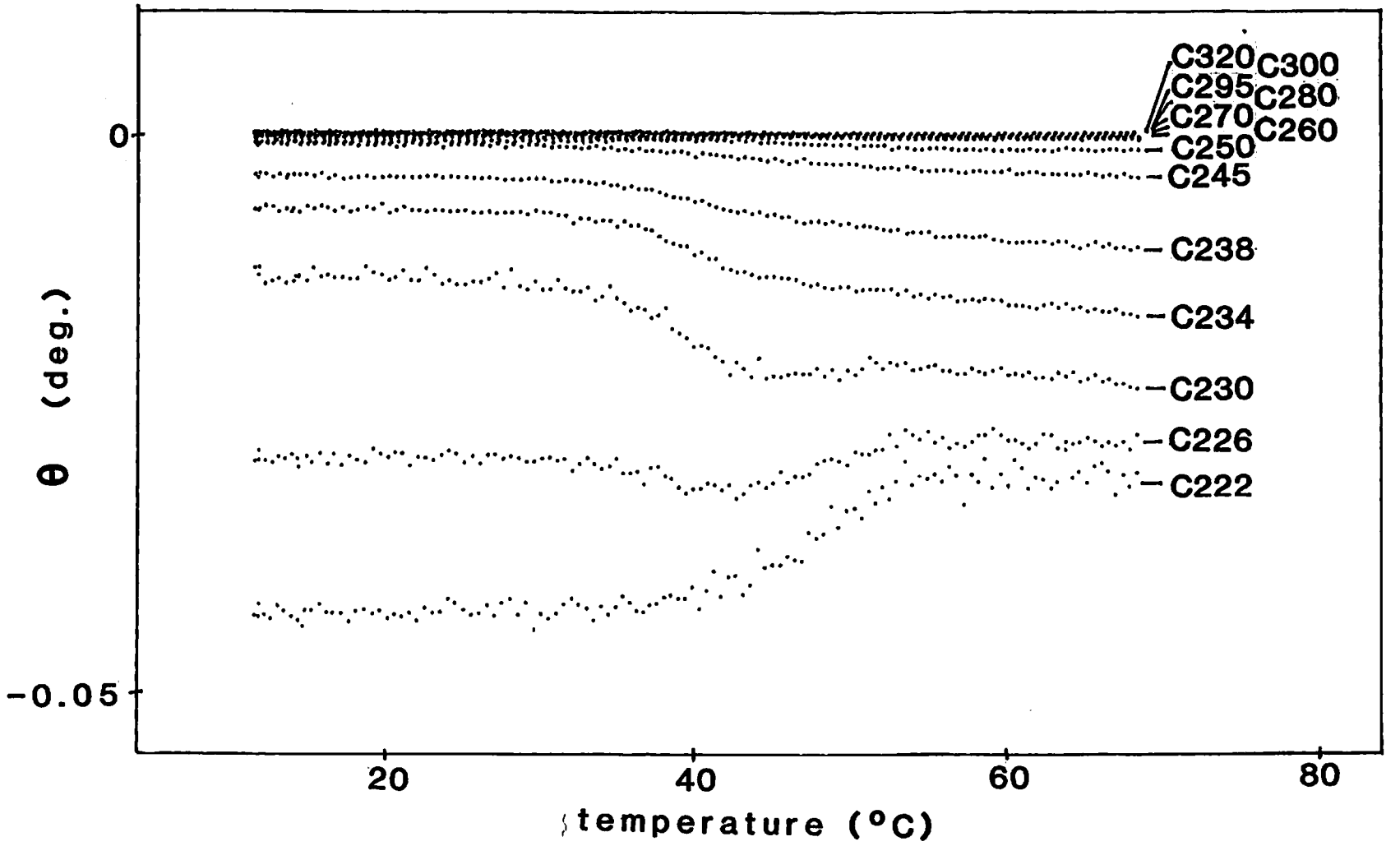
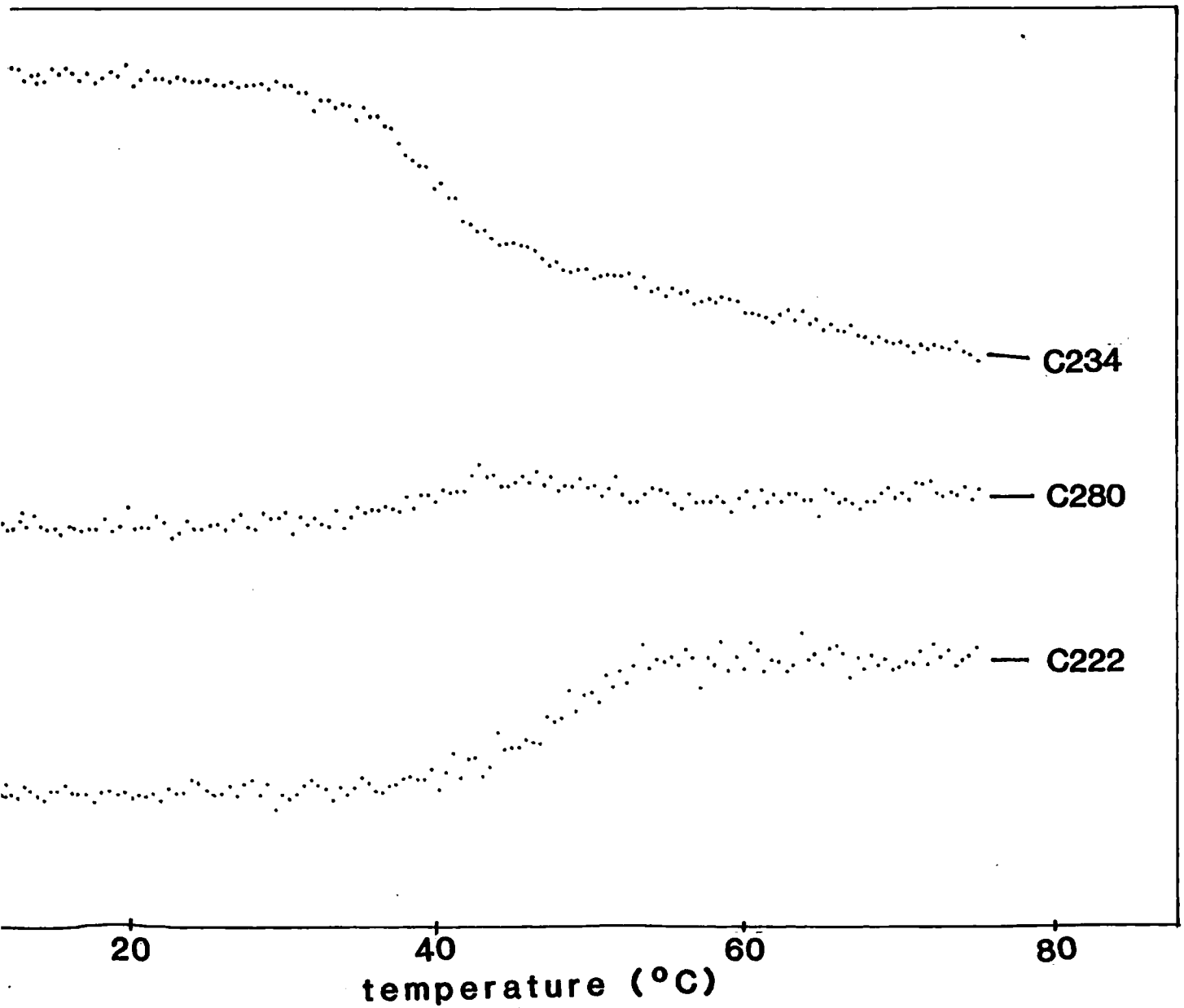


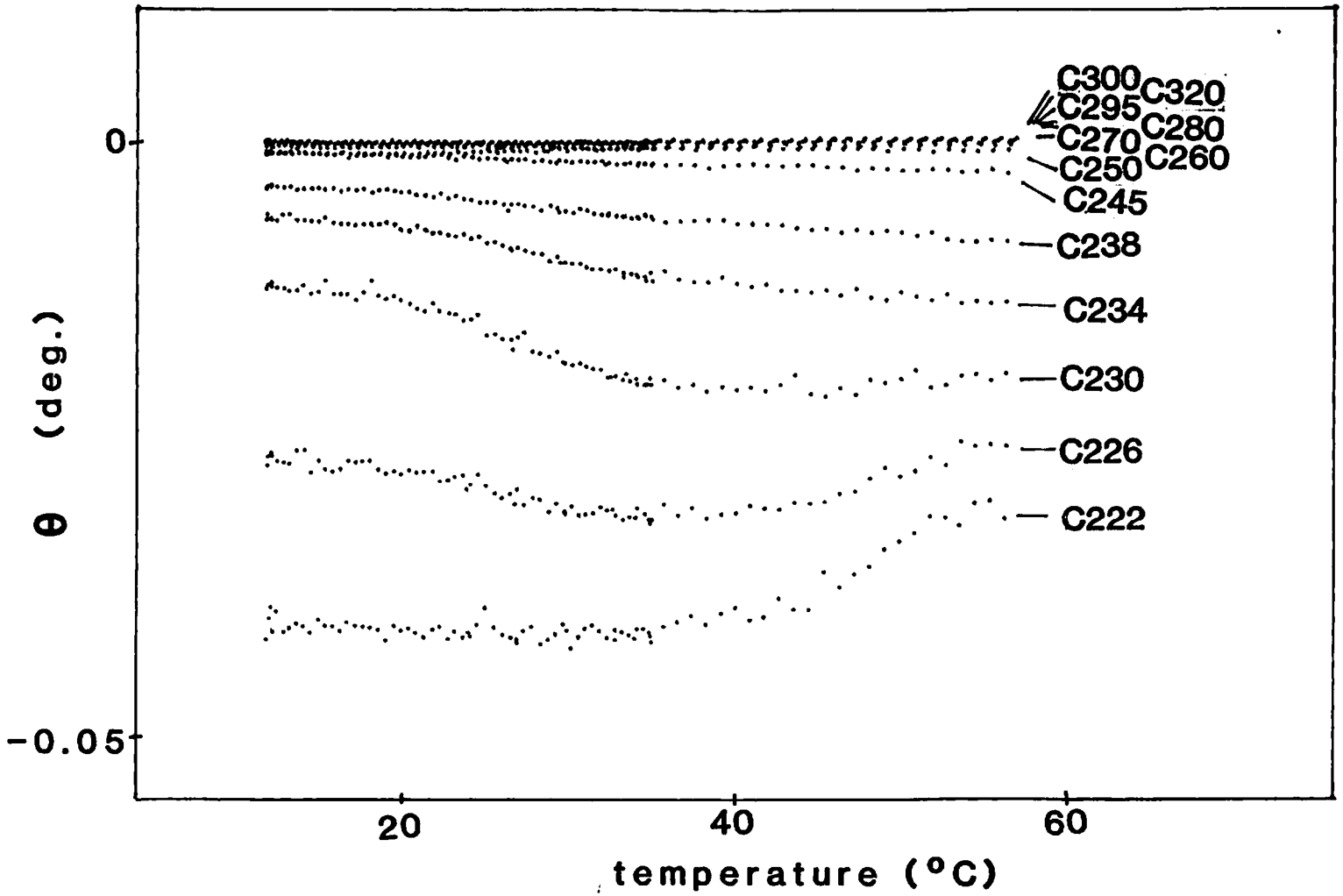
pepsinogen pH 8.8

Fig. 8 (a)



pepsinogen pH 8.8

Fig. 8 (b)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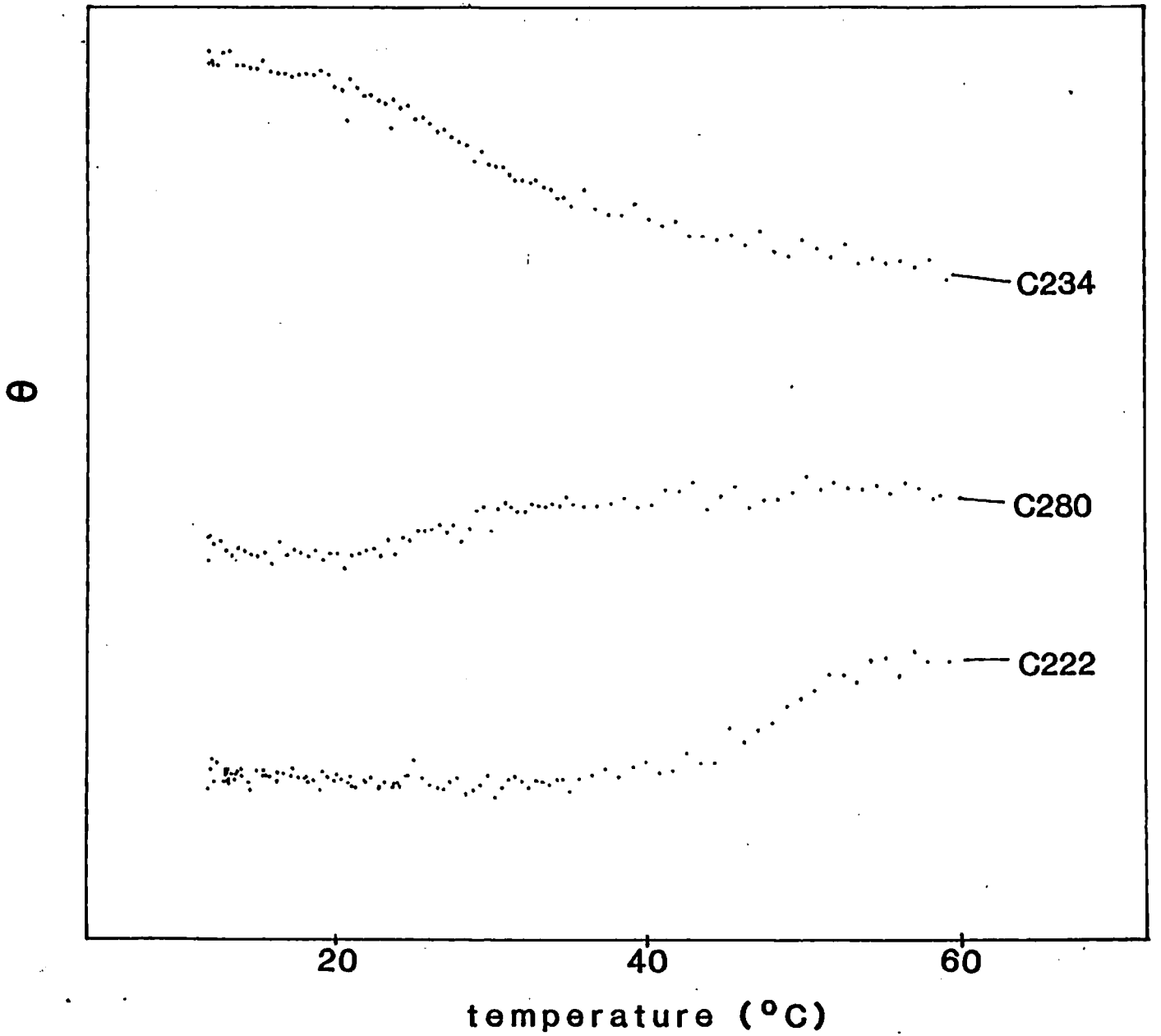
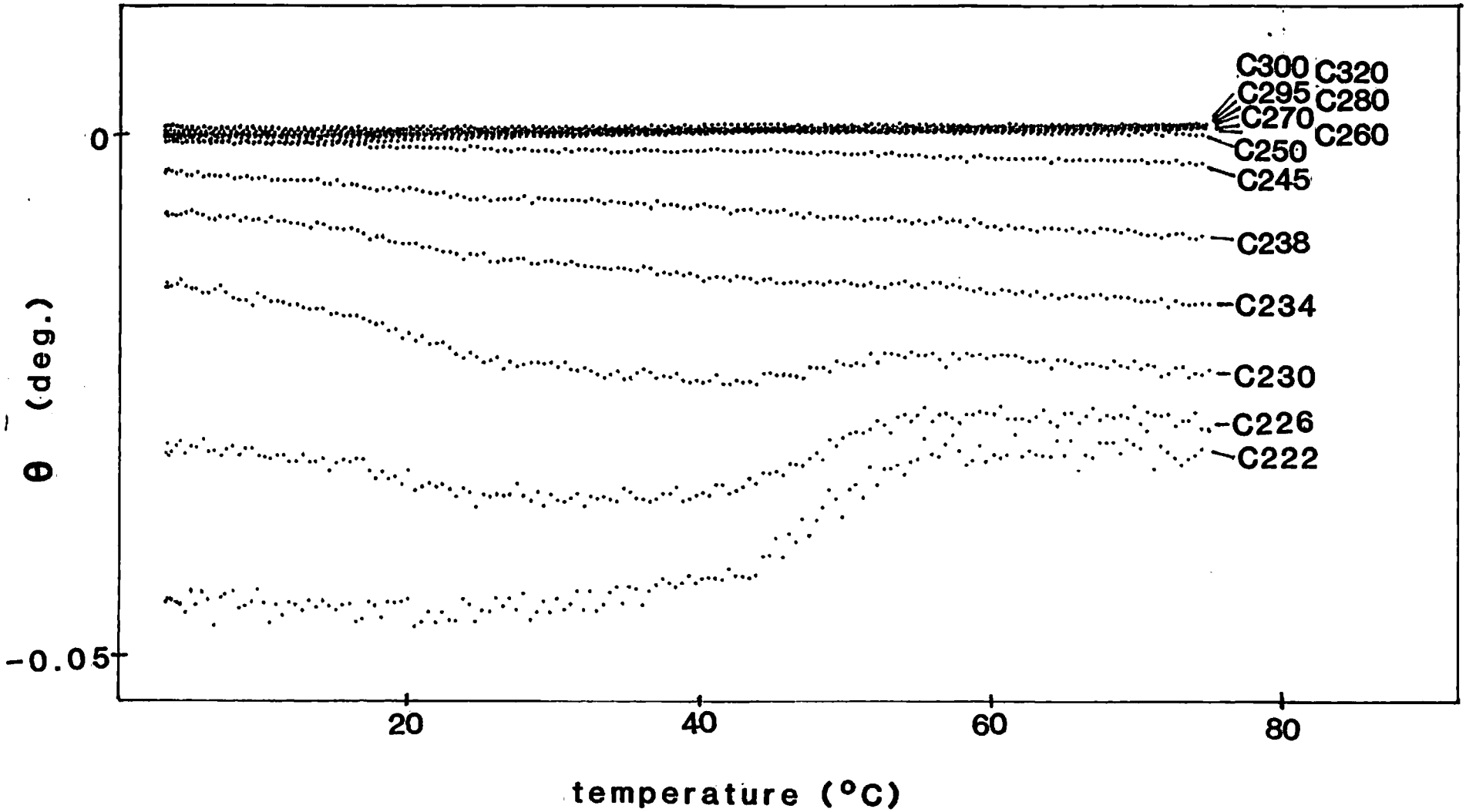
C280
C270
C260
C250
C245
C238
C234
C230
C226
C222
0
-0.05
θ (deg.)
20
40
60
temperature (°C)
pepsinogen pH 9.3

Fig. 9 (a)



pepsinogen pH 9.3

Fig. 9 (b)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C295
C280
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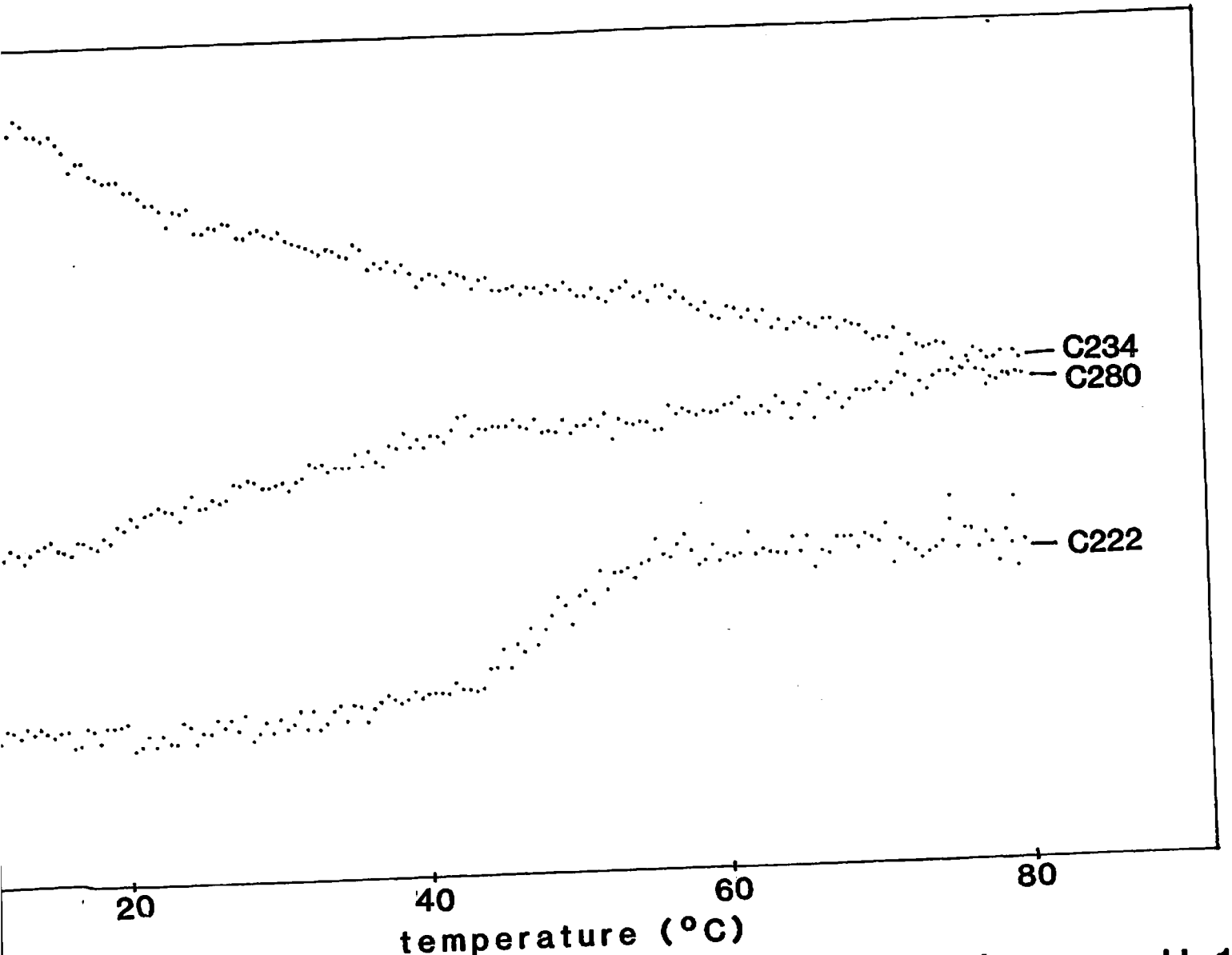
C260
C250
C245
C238
C234
C230
C226
C222
0
-0.05
θ (deg.)
20
40
60
80
temperature (°C)
pepsinogen pH 10.0

Fig. 10 (a)



pepsinogen pH 10.0

Fig. 10 (b)

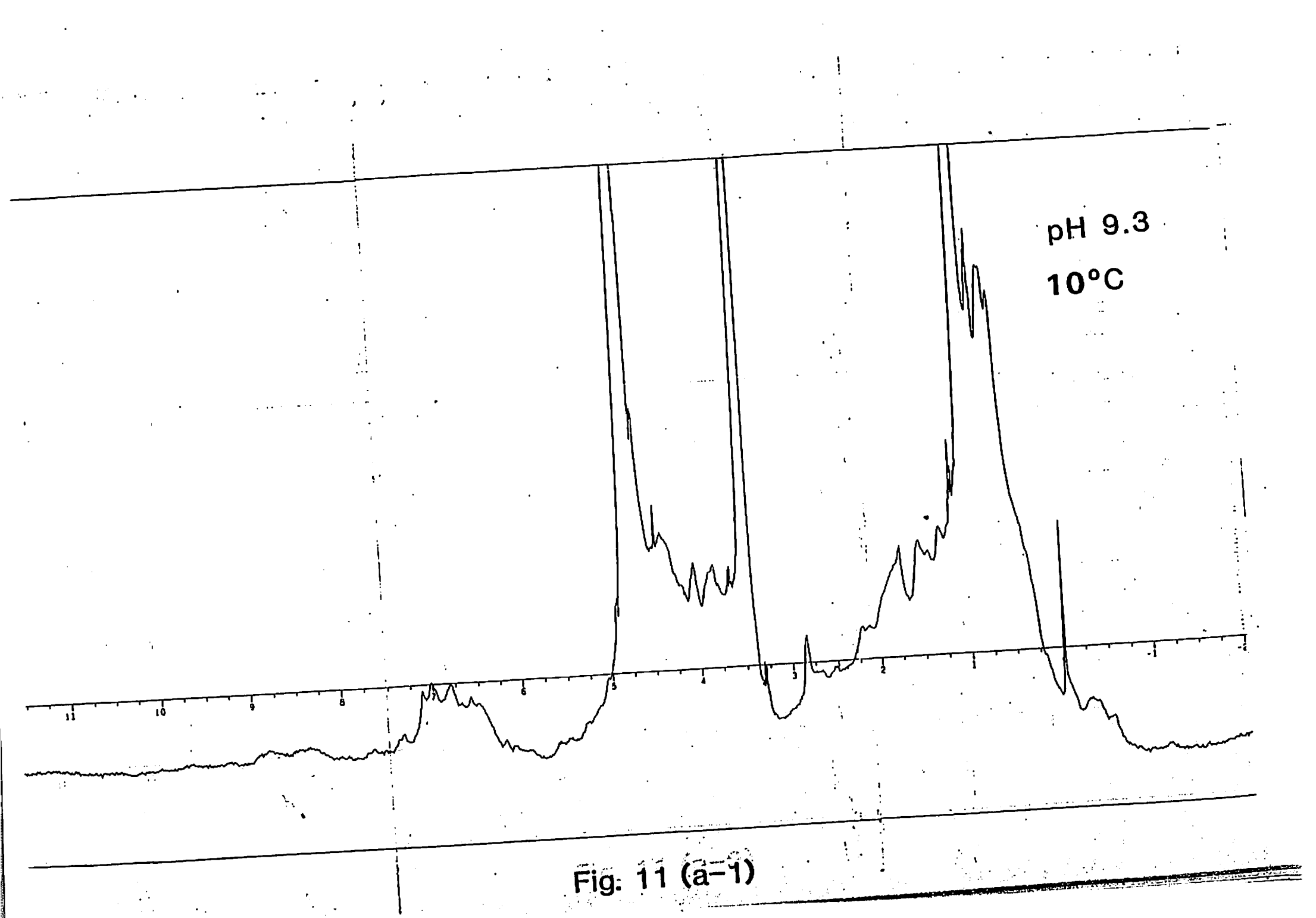


Fig: 11 (a–1)

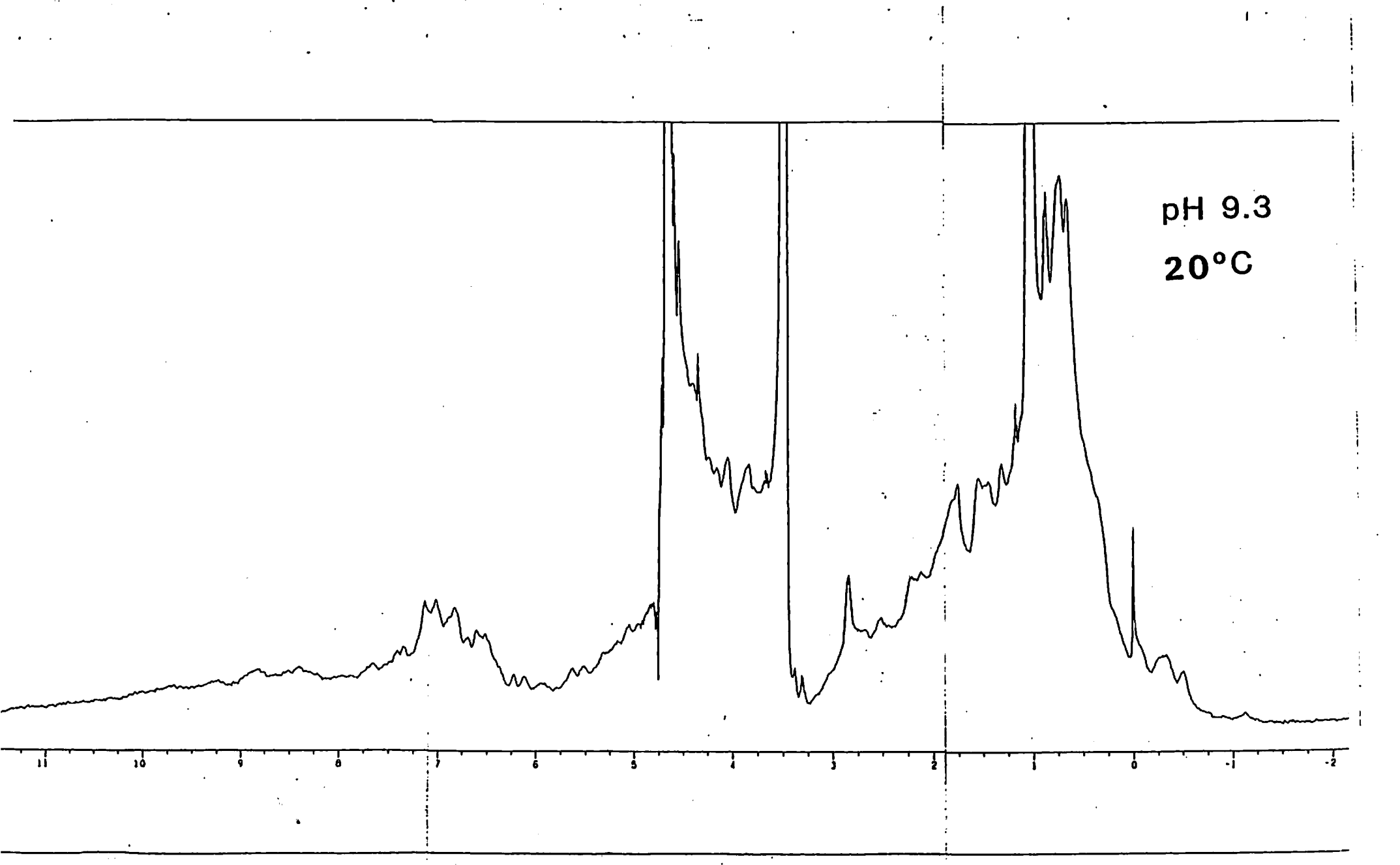


Fig. 11 (a-2)

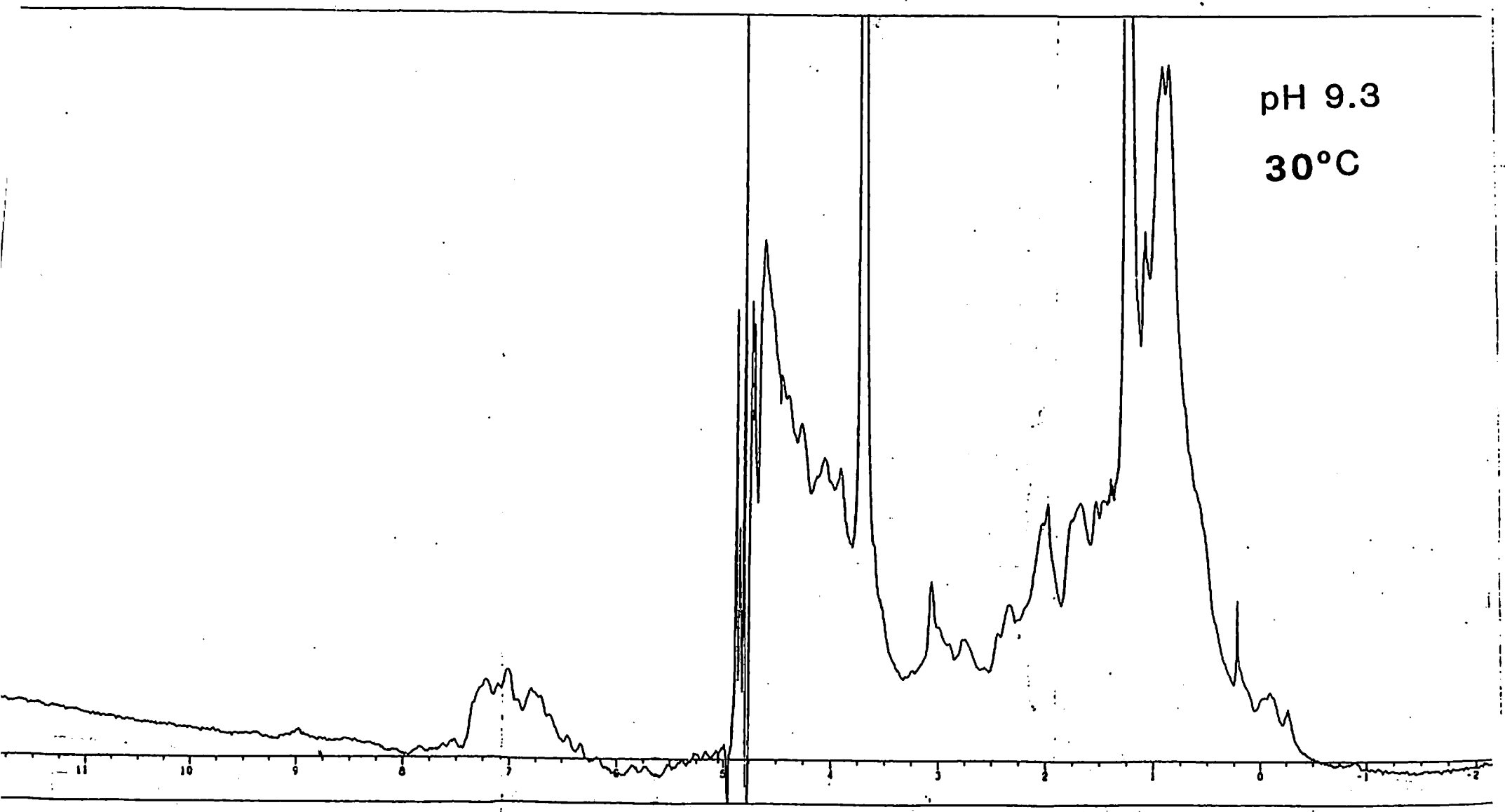


Fig. 11 (a-3)

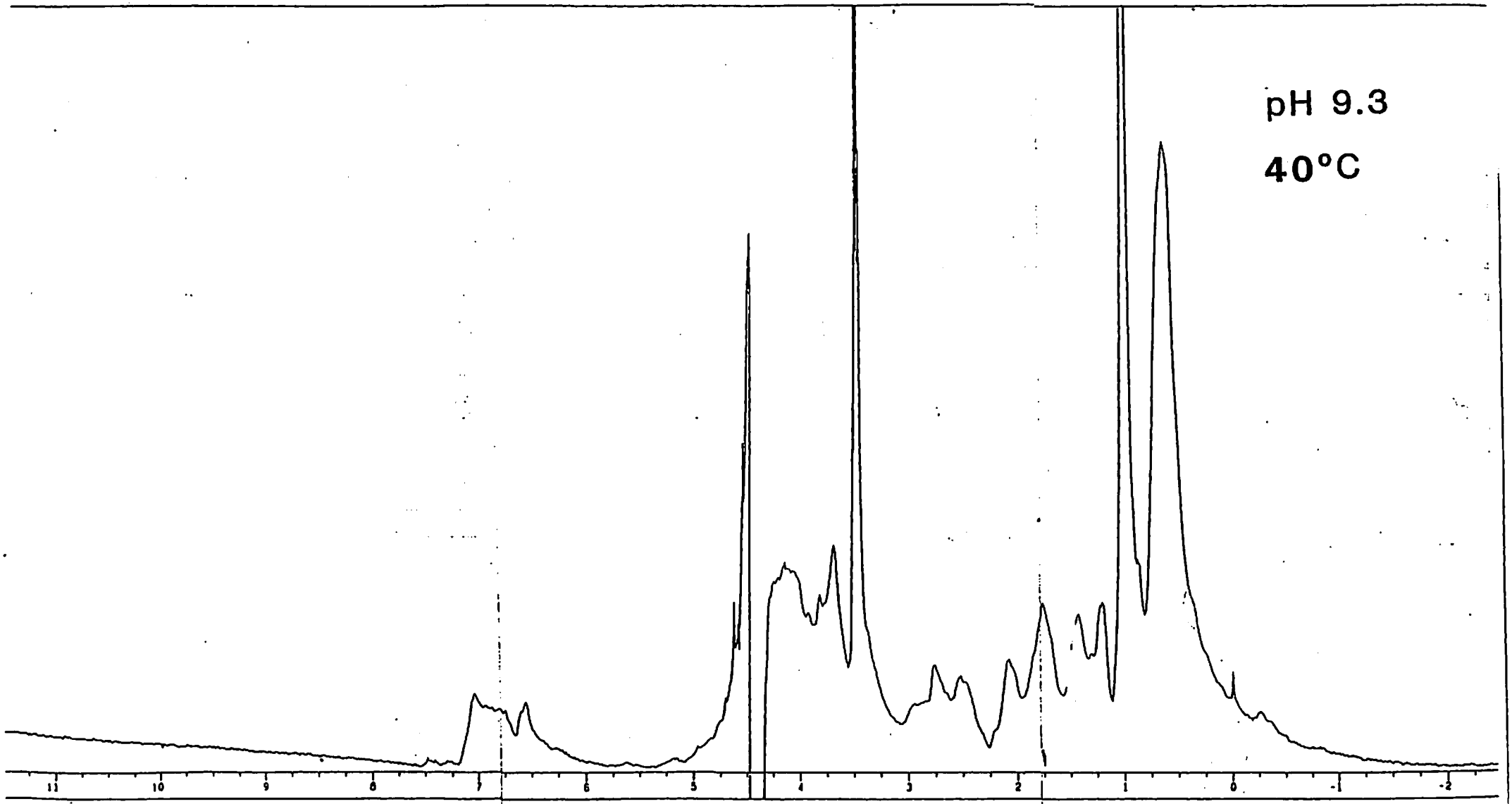


Fig. 11 (a-4)

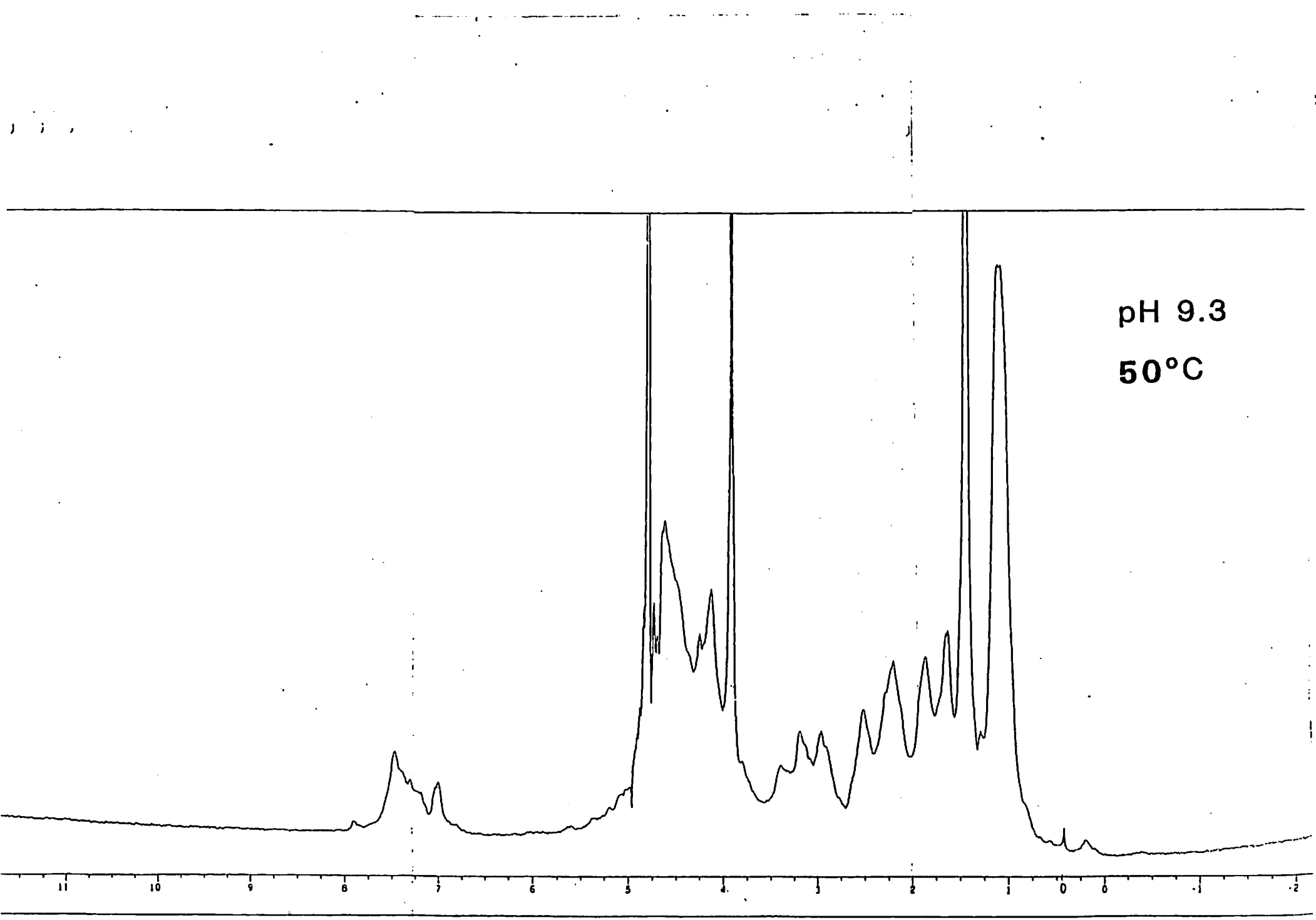


Fig. 11 (a-5)

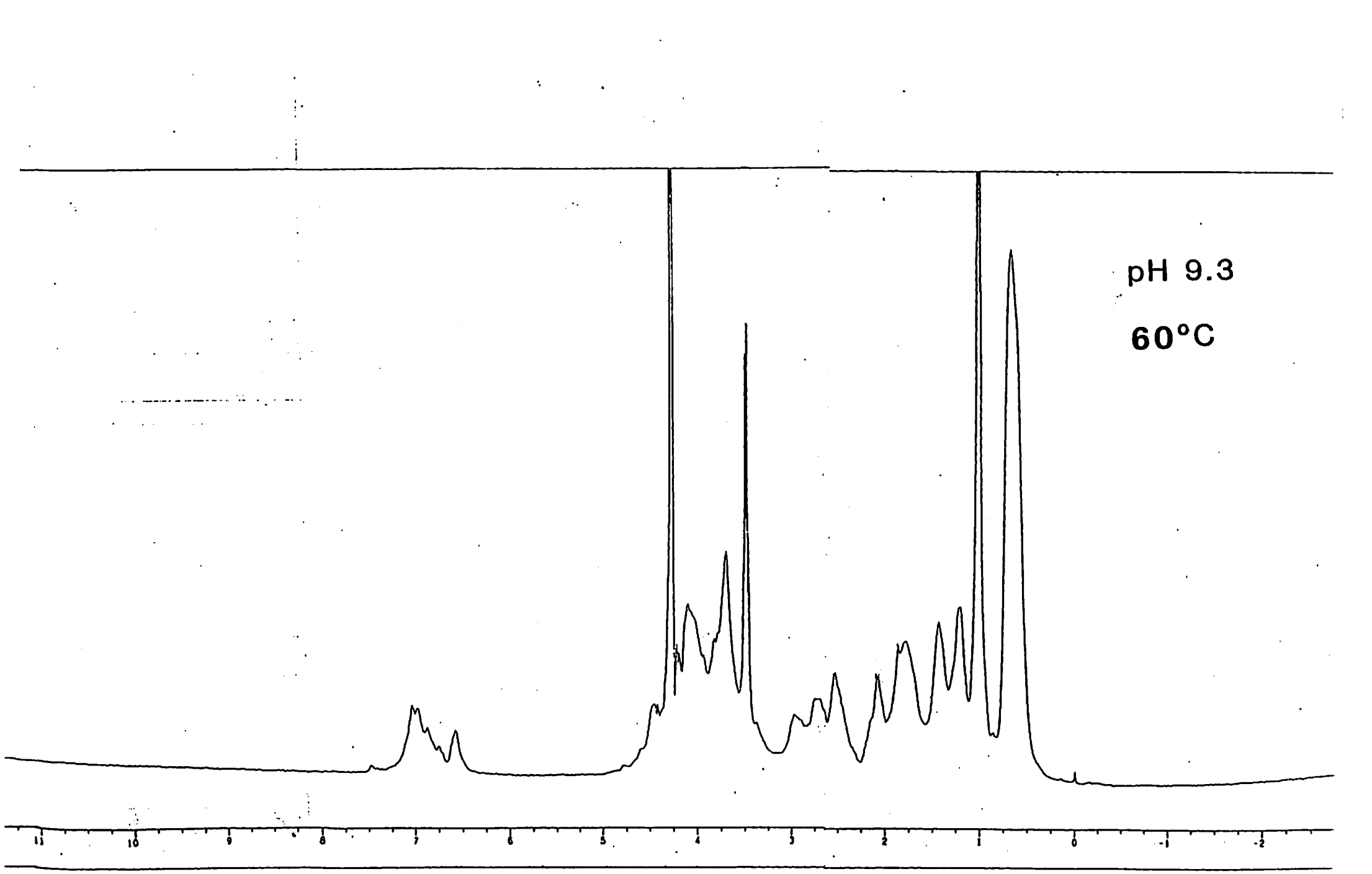


Fig. 11 (a-6)

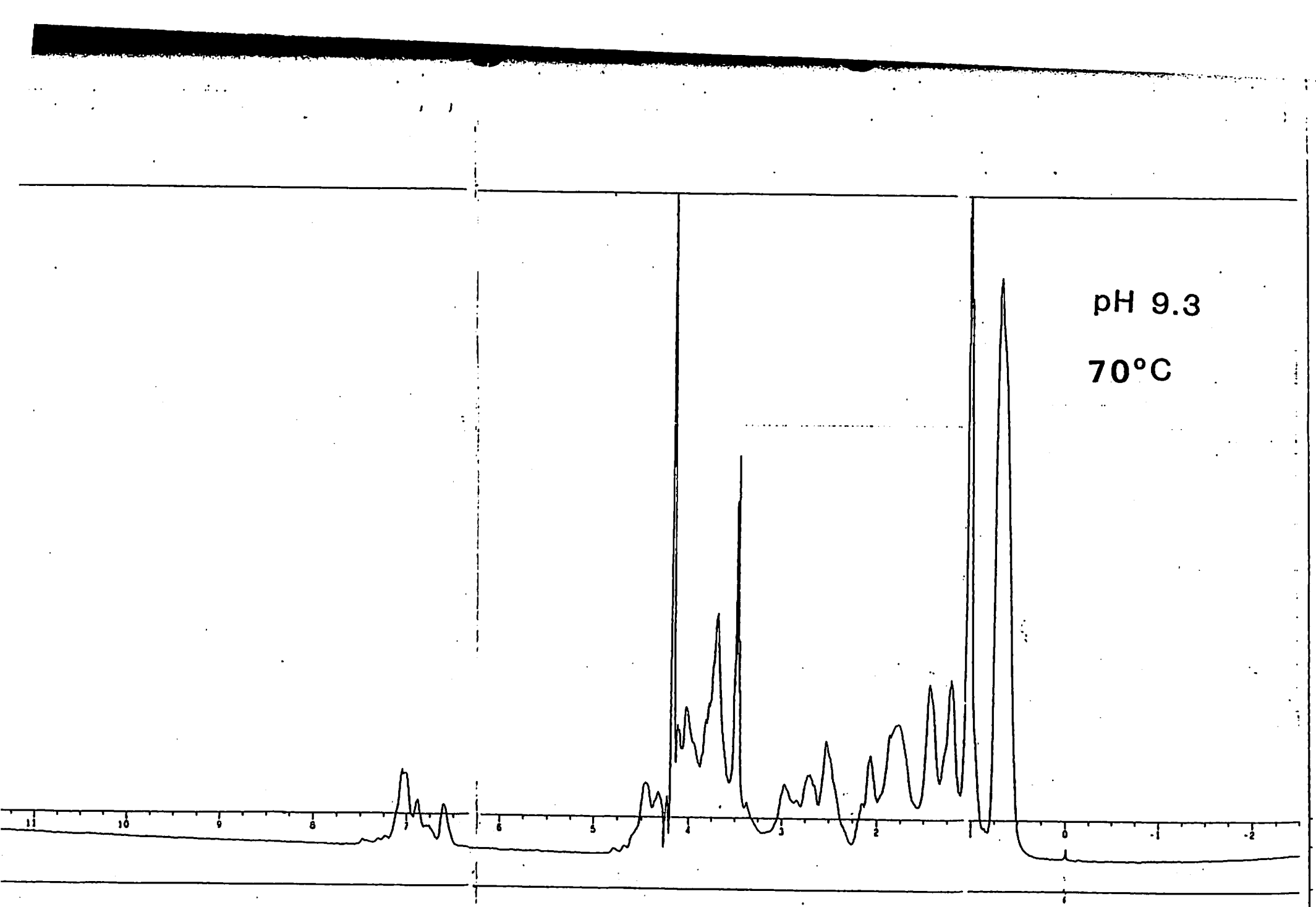


Fig. 11 (a-7)

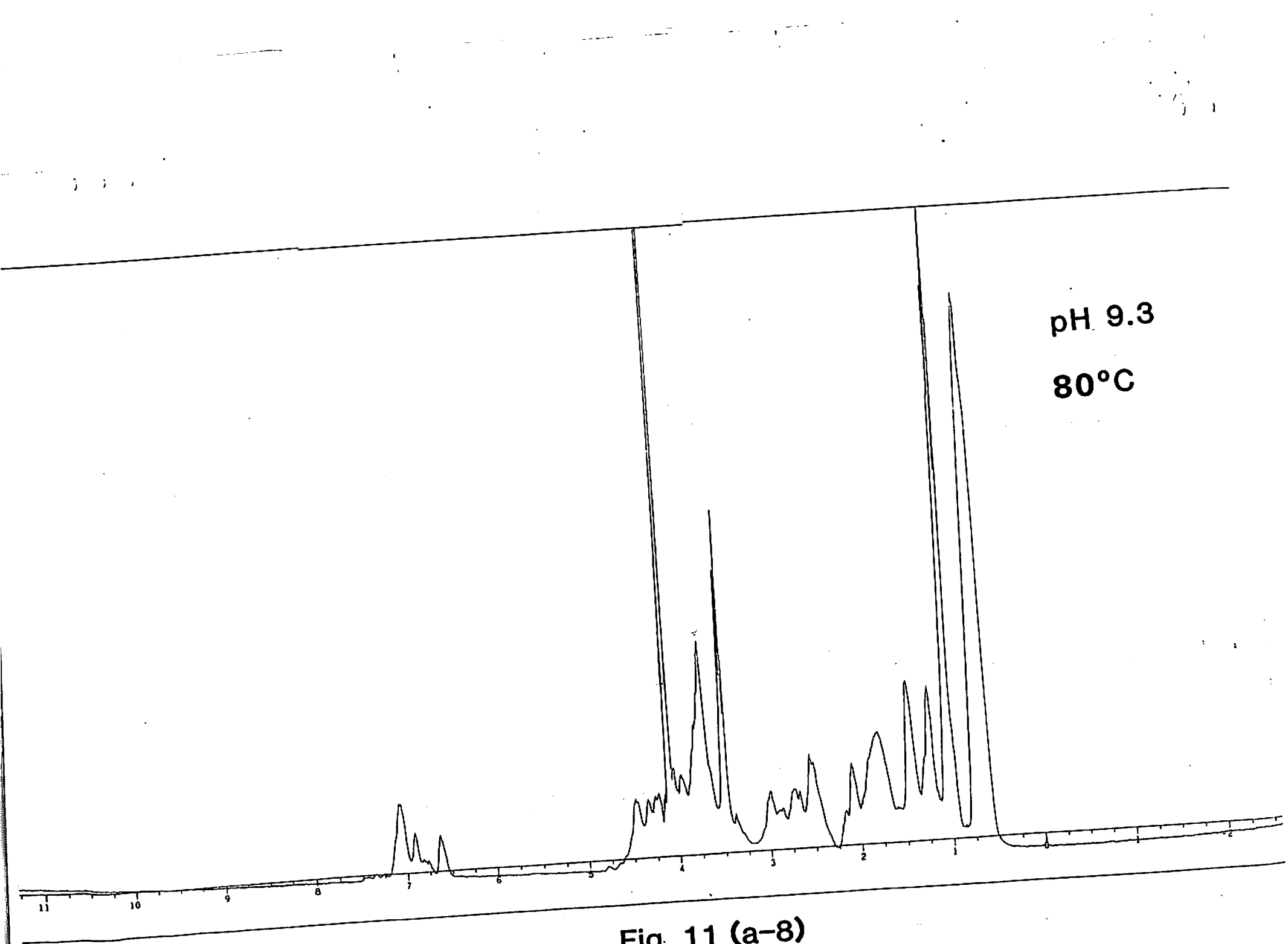


Fig. 11 (a-8)

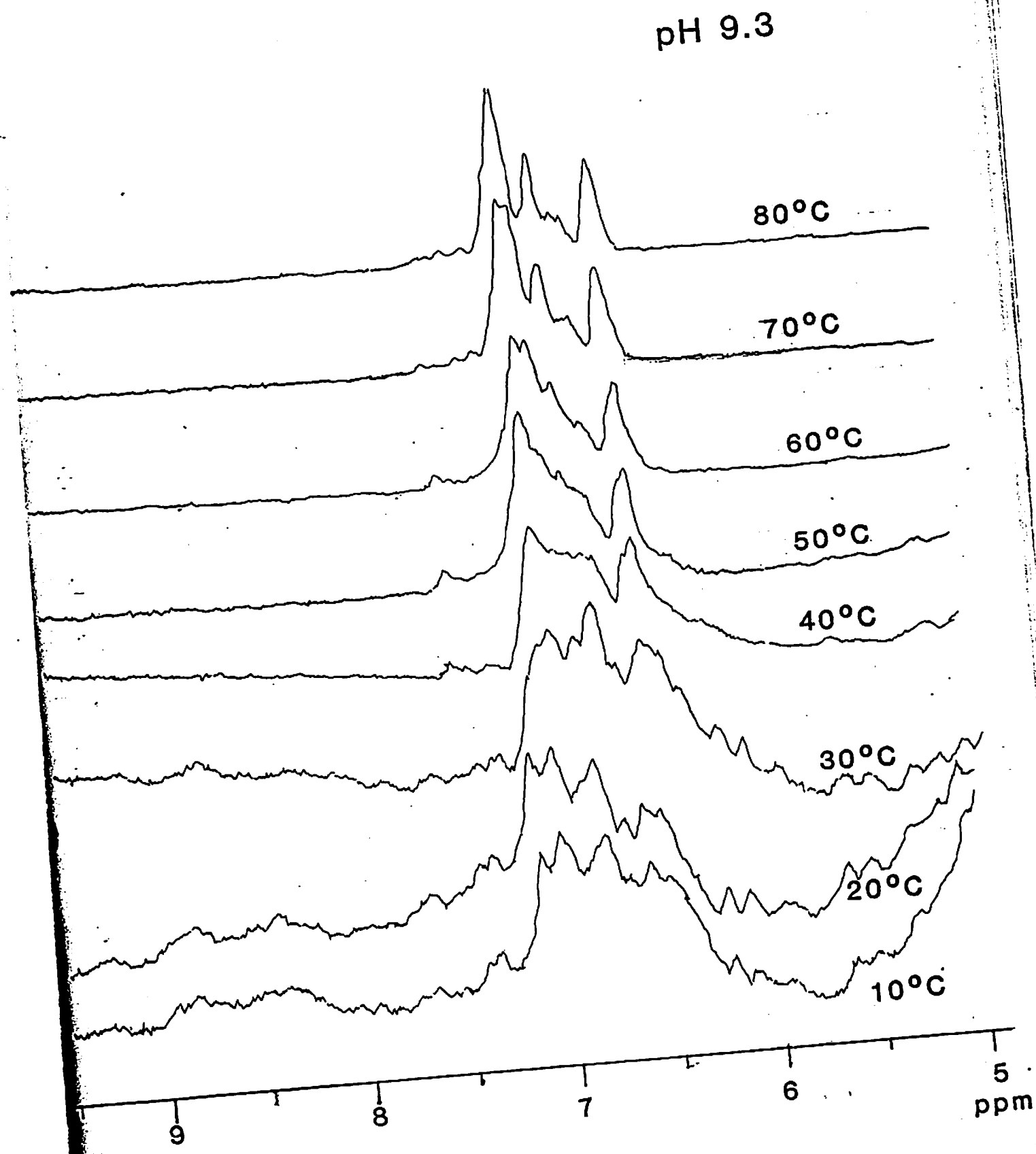
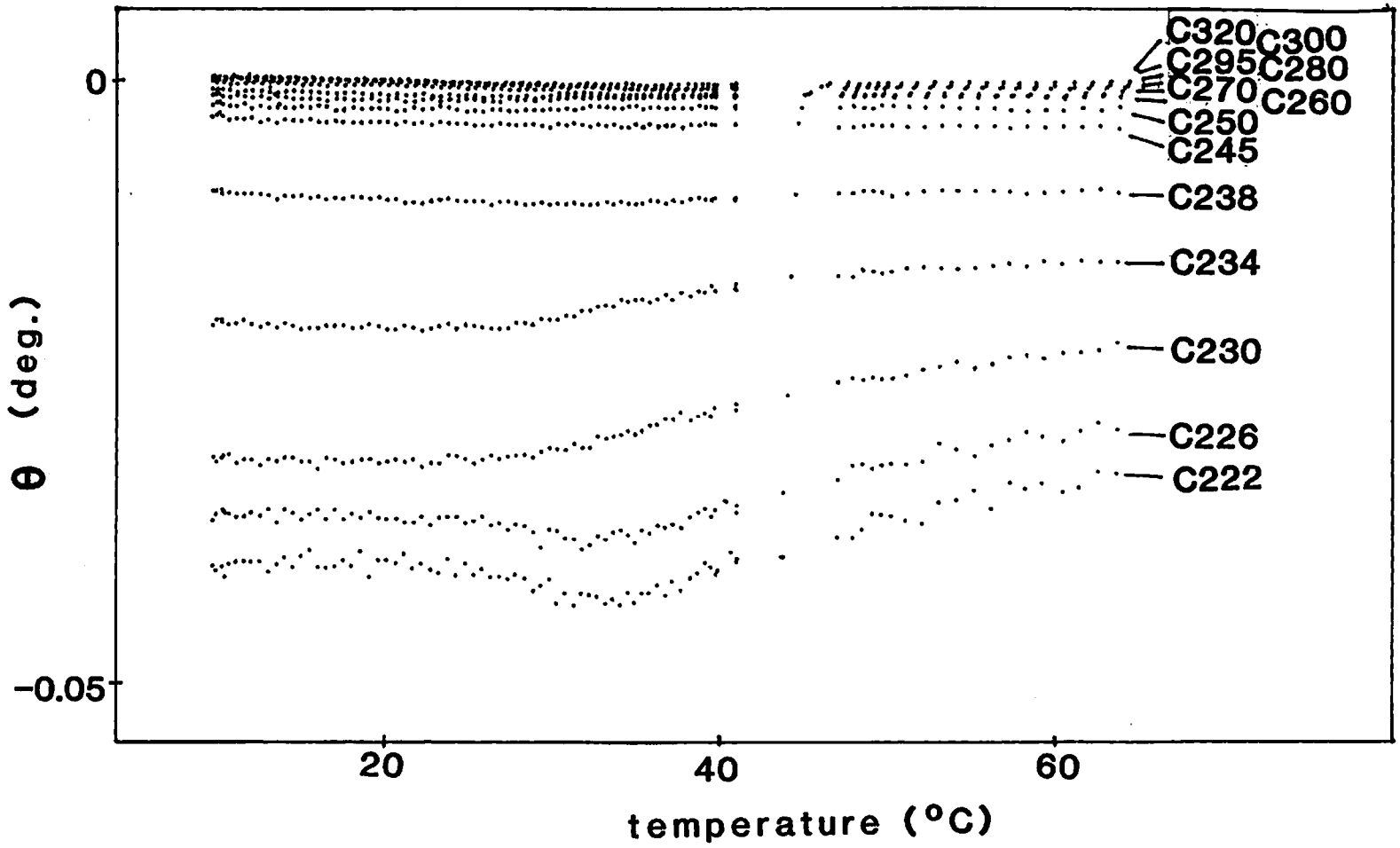


Fig. 11 (b)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−0.05
θ (deg.)
20
40
60
temperature (°C)
lysozyme pH 1.7 in 33 % ethanol

Fig. 12

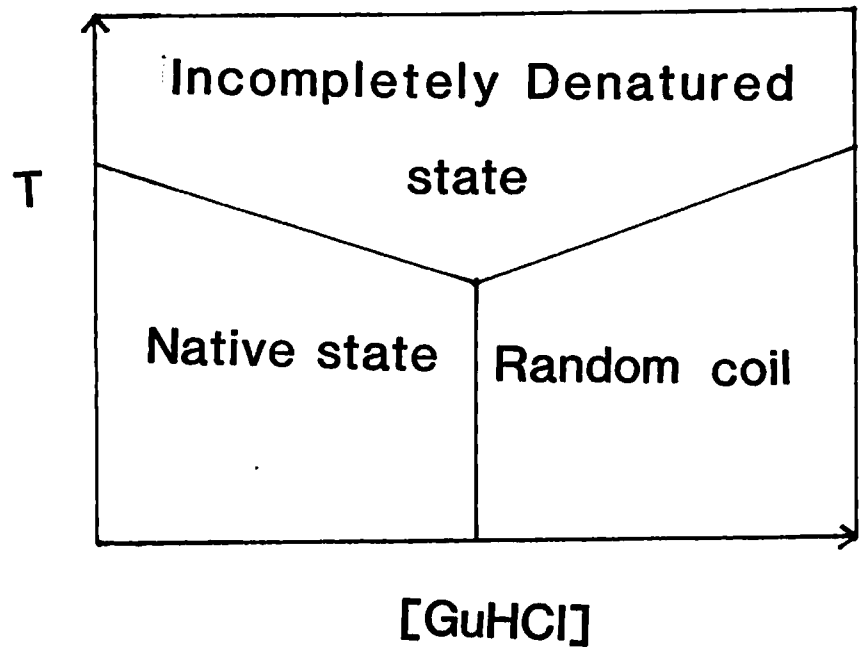
Incompletely Denatured
state
T
Native state
Random coil
[GuHCl]

Fig. 13