NUCLEAR MAGNETIC RESONANCE STUDIES OF ANAESTHETIC INTERACTIONS WITH HAEMOGLOBIN

R. W. BARKER, F. F. BROWN, R. DRAKE, M. J. HALSEY AND R. E. RICHARDS

SUMMARY

The use of a 270 MHz Fourier Transform nuclear magnetic resonance (NMR) spectrometer, combined with signal processing techniques to improve resolution, enabled proton resonances from the individual aromatic residues of haemoglobin to be distinguished. In the presence of clinical concentrations of the general anaesthetic drugs halothane and methoxyflurane, specific changes in the NMR spectrum can be distinguished which probably reflect local changes of conformation. When higher concentrations of anaesthetic are used, extensive changes in the NMR spectrum occur which are consistent with non-specific binding of the anaesthetic to the hydrophobic parts of the haemoglobin molecule.

It is well known that the potency of inhaled general anaesthetic drugs correlates with lipid solubility, implying that anaesthetic activity involves interaction with a hydrophobic centre. The site of activity might well be the hydrophobic part of a protein, and Östergren (1944) proposed that general anaesthesia may be related to a reversible alteration in protein structure. A number of different hypotheses about the mechanism of general anaesthesia have been associated with protein perturbations including (a) the biochemical theories which postulate that anaesthesia is the result of the depression of a number of energy-producing biochemical reactions in the neuron; (b) the hypothesis that certain proteins in membranes may be the critical sites of action of anaesthetic agents, which could explain why anaesthesia has very specific effects in the whole body; (c) the hypothesis that anaesthetic agents affect motile proteins associated with neurotransmitter release.

It has been demonstrated already that certain enzymes and enzyme systems exhibit specificity in their interaction with inhaled anaesthetic agents. For example, at least five enzymes in the Embden-Meyerhof pathway are unaffected by high concentrations of halothane, whereas glutamate dehydrogenase, another enzyme related to the Krebs cycle, is reversibly depressed by both halothane and methoxyflurane (Brammall, Beard and Hulands, 1973). The luciferase enzyme system of luminescent bacteria has been shown to be particularly sensitive to anaesthetic drugs although it now appears that it is only certain intermediates within the system that are affected (Halsey and Smith, 1970; White, Wardley-Smith and Adey, 1973).

Haemoglobin also has been used as a model for anaesthetic-macromolecule interactions, although Verworn's original hypothesis (1912) that anaesthesia resulted from the suppression of "oxygen carriers" is no longer tenable. Haemoglobin has a number of advantages over most proteins as a model in that it is readily available, relatively easy to prepare and its molecular structure is well known. There is evidence from solubility measurements (Gregory and Eger, 1968), x-ray crystallography (Schoenborn, 1965), and optical rotatory dispersion (Laasberg and Hedley-Whyte, 1971), that anaesthetics interact with haemoglobin although there is no marked effect on the oxygen-binding capacity (Millar, Beard and Hulands, 1971; Weiskopf, Nishimura and Severinghaus, 1971). However, the lack of effect on this particular function of haemoglobin simply indicates that the molecular interactions are not close or critical to the binding of oxygen with the iron atom in the haem group.

Small changes in structure or conformation of proteins can be studied by nuclear magnetic resonance (NMR). The technique has already been applied to a wide variety of haemoglobins and has been used to study the effects of the interaction of 100% cyclopropane and xenon with the haem-
shifted residues in myoglobin (Shulman, Peisach and WylUDA, 1970). With the advent of resolution enhancement techniques (Campbell et al., 1973), we can now study the complex central region of the haemoglobin spectrum. We have therefore chosen solutions of haemoglobin for a study, by NMR, of the interaction of a well characterized protein with anaesthetic drugs at concentrations down to those used in clinical medicine.

METHODS
Fresh packed human red cells were washed thoroughly in 0.9–1.0% NaCl solutions and then lysed with water. All stromal protein was removed by repeated centrifugation and the haemoglobin purified by ammonium sulphate precipitation techniques. It was then dialysed exhaustively against sodium phosphate/potassium chloride buffers, first prepared in water and finally in heavy water, until less than 1% normal water remained. (This removes the majority of the proton signal originating from the water, which would otherwise mask the protein spectrum.) The pH (or pD, when considering the deuterium ion of heavy water) was regulated by a buffer which matched conditions in the red cell (Altman, 1961) as far as chloride and phosphate concentrations were concerned, so as to provide better grounds for comparison with whole red cell measurements. The choice of buffer should have little bearing on the immediate interpretation of the results, since all samples containing anaesthetic were compared with the same sample before the anaesthetic was added. Appropriate oxygen/anaesthetic gas mixtures were equilibrated with haemoglobin solutions using glass syringes rotated in a water bath.

The nuclear resonance measurements were made on a 270 MHz Bruker Physik spectrometer with an Oxford Instrument Co. superconducting magnet. Convolution difference techniques (Campbell et al., 1973) were used to improve the resolution of the peaks. All measurements were made at 37°C. The pD of a sample was obtained with a normal pH meter standardized with buffers prepared in water, and the meter readings “corrected” by the addition of 0.4 units.

RESULTS
Figure 1 shows a broad region of the haemoglobin spectrum. In subsequent figures attention is confined to that part of the spectrum on the low field side of the peak from the 1% residual water, which
contains most of the resonances from protons in the aromatic residues.

Figure 2 shows a set of four spectra at different pD values and at chloride and phosphate concentrations similar to those found in the red cell; indeed, the spectra are qualitatively similar to those we have obtained from intact cell preparations. Three regions can be distinguished. On the left are peaks mainly from the histidine C$_2$ protons and in the centre are peaks from the histidine C$_4$ protons. These regions, especially the central, will also contain peaks from phenylalanine and tryptophane ring protons while the righthand region will be mainly those due to tyrosine. It should be emphasized that these spectra show only the relatively well resolved peaks; there is a large underlying mass of completely unresolved resonances which are eliminated by the data-processing technique and make only sporadic contributions to the spectrum as the resonances from certain residues sharpen under particular conditions and then broaden again.

As the pD changes, there are large shifts of the C$_2$ histidine resonances, and smaller shifts of the C$_4$ protons, as the histidine ring deprotonates. These are superimposed on smaller changes from the conformational alterations which also accompany the pH change.

Figure 3 shows a similar set of spectra taken with samples prepared in more dilute buffer solution; the effects are both qualitatively and quantitatively different from the effects of pD alone, and probably arise from conformational changes associated with both changes in ionic strength and binding of buffer ions. The titration curves for many of the resonances depend markedly on the buffer concentrations, probably because the pK of the histidine residues are affected by binding of buffer ions.

Other factors which affect the spectra are temperature and oxygen content. Therefore all measurements were made at 37°C and the haemoglobin was fully oxygenated. The concentration of haemoglobin, the H$_2$O:D$_2$O ratio and the invariably small 2,3-diphosphoglycerate content had relatively little effect on the spectra. Figure 4 illustrates one of the spectra with clinical concentrations of halothane at pD 6.8; only one or two peaks are specifically affected. Similar experiments with methoxyflurane also demonstrate specific changes at clinical concentrations. However, when about five times the clinical partial pressure of anaesthetic is used (fig. 5) there are extensive changes all across the “aromatic” region of the spectrum, dependent on the different agents being studied, which indicate that a general and non-specific “binding” of the anaesthetic to the hydrophobic regions of the molecule may be occurring.

**DISCUSSION**

The shifts shown in figure 4 might arise from small conformation changes of the protein or from a shift of the pK of one of the histidine residues arising
FIG. 4. The spectrum of haemoglobin equilibrated with 0.005 atm of halothane compared with its control. Only two resonances have apparently shifted, indicated by the arrows at —1.8 and —3.35 p.p.m. The pH was selected at 6.8 to optimize the visible spectrum changes with low concentrations of anaesthetics. The changes observed are different from those associated with pH effects. Likely, thus the two most conspicuous shifts, marked in figure 4, are in opposite directions and could be associated with alterations in plane and perpendicular ring shifts from a third aromatic group. The downfield shifting peak titrates like a histidine resonance and the upfield peak is possibly a tyrosine resonance. There are a number of possible sites which would be consistent with this evidence, such as the hydrophobic area bounded by cystein 112 and tryptophane 15 in the chain.

These studies are being extended to a wide range of clinically used anaesthetic agents, and to other proteins. In the meantime, these preliminary results have four implications, namely:

(a) Anaesthetic drugs at clinical concentrations do interact with haemoglobin in solution and by inference are capable of interacting with other proteins, such as those associated with synaptic transmission in the central nervous system.

(b) At clinical concentrations, the interactions with haemoglobin of halothane and methoxyflurane are localized and specific.

(c) At higher than clinical concentrations the
interactions are of a more general, non-specific type, although they are still reversible. Unlike the implications of some of the earlier experiments (Laasberg and Hedley-Whyte, 1971) the effects observed are related to the partial pressure of the anaesthetic agent.

(d) The conformational changes produced by high concentrations of halothane, methoxyflurane and diethyl ether are dependent on the agent as well as on the concentration.

ACKNOWLEDGEMENT
We thank Mr R. Edwards for help with the haemoglobin samples.

REFERENCES

LES ÉTUDES DE RESONANCE MAGNETIQUE NUCLÉAIRE DES INTERACTIONS DE L'ANESTHESIE AVEC L'HÉMOGLOBINE

RESUME
L'emploi d'un spectromètre (NMR) de résonance magnétique nucléaire Fourier Transform de 270 MHz, associé à des techniques de traitement des signaux visant à améliorer la résolution, a permis de distinguer les résonances protoniques des divers résidus aromatiques de l'hémoglobine. En présence des concentrations cliniques des médicaments d'anesthésie générale comme l'halothane et le méthyfluorane, il est possible de distinguer des changements particuliers du spectre NMR, ces changements reflètent probablement des changements locaux de structure. Lorsqu'on utilise des concentrations d'anesthésique plus élevées, des changements extensifs du spectre NMR se produisent, lesquels correspondent à la liaison non spécifique de l'anesthésique avec les éléments hydrophobiques de la molécule d'hémoglobine.

NUCLEARE RESONANZ-UNTERSUCHUNGEN ZWISCHEN ANÄSTHETIKUM UND HÄMOGLOBIN

ZUSAMMENFASSUNG

ESTUDIOS SOBRE LA RESONANCIA MAGNETICA NUCLEAR DE LAS INTERACCIONES CON LA HEMOGLOBINA

SUMARIO
El empleo de un espectómetro (NMR) de resonancia magnético nuclear Fourier Transform de 270 MHz, combinado con técnicas de procesos de señales para mejorar la resolución, permitió distinguir las resonancias de protones de los residuos aromáticos individuales de hemoglobina. En presencia de concentraciones clínicas de las drogas anestésicas generales halotano y metoxiflurano, se pueden distinguir cambios específicos en el espectro NMR que reflejen probablemente cambios locales de conformación. Cuando se utilizan mayores concentraciones de anestésico, tienen lugar grandes cambios en el espectro NMR que son consistentes, sin ligazón específica del anestésico con las partes hidrofóbicas de la molécula de hemoglobina.