*Molecular Interactions and Activity in Proteins*

Editors: RUTH PORTER (Organizer) and DAVID W. FITZSIMONS

# Perturbations of model protein systems as a basis for the central and peripheral mechanisms of general anaesthesia

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*Abstract* Protein perturbations associated with anaesthetic interactions are relevant to: (*a*) the central molecular mechanisms of general anaesthesia; (*b*) the molecular basis of physiological selectivity and anaesthetic specificity of the many 'side-effects' of anaesthesia; (*c*) the use of anaesthetic agents as selective hydrophobic probes for the study of protein structures and activities in detail.

Small but specific protein perturbations have been studied with various nuclear magnetic resonance procedures with haemoglobin as a model protein to establish the 'ground-rules' for anaesthetic–macromolecule interactions. The correlation of one aspect of these perturbations with anaesthetic potency and hydrophobic solubility indicates that hydrophobic pockets in proteins can behave like bulk-lipid phases in terms of their solubility characteristics. Other aspects appear to depend on physical characteristics such as size, geometry, structure and composition of the individual agents. These data support the hypothesis that anaesthetic actions can be explained on a molecular basis by direct interactions with proteins in addition to lipid and aqueous effects.

Protein conformational perturbations produced by clinical concentrations of general anaesthetics are potentially relevant to three areas of investigation: first, the central molecular mechanisms of general anaesthesia which occur in the synaptic regions of the neuronal networks; second, the peripheral mechanisms of anaesthesia associated with the 'side-effects' and toxicity of specific agents; third, the use of anaesthetic agents as selective hydrophobic probes for the study of protein structures and activities in detail.

The mechanism of general anaesthesia at a molecular level is still far from clear. It may be ascribed either to changes in lipid thermotropic behaviour or to a malfunction of neuronal proteins due to direct anaesthetic interactions, or to a combination of the two. The inhalational anaesthetics are usually considered to be the archetype of non-specific drugs: that is, their central actions do not depend on their precise chemical structures interacting with an array of

spatially organized receptor sites. However, this traditional view may have to be modified to accommodate recent evidence on the selectivity and specificity of anaesthetic 'side-effects' which are superimposed on the central mechanisms of anaesthesia (Halsey 1977). This selectivity of action can be explained on a molecular level by the diversity of protein structures associated with different functions.

The general anaesthetic potencies *in vivo* of all the inhalational agents correlate better with their lipophilic solubilities than with any other physical property so far studied. This has led to the assumption that anaesthesia occurs when a critical molar, or volume, concentration is attained by hydrophobic sites of action. Thus, anaesthetic potency is thought to depend on either the concentration of the agent (mole fraction) at the site of action or the product of this concentration and the molar volume of the anaesthetic (volume fraction). The most probable solubility characteristics of the sites of action have been quantitated in terms of solubility parameters with values of $18.4 \pm 2$ and of $20.5 \pm 2$ $(J/cm^3)^{1/2}$ in the case of the critical molar and volume concentration assumptions, respectively (Miller *et al.* 1972).

These characteristics exclude a significant proportion of the anaesthetic site of action having polar characteristics and imply that anaesthetics act at hydrophobic sites in lipid bilayers or macromolecules.

Another feature of general anaesthesia is that it can be 'antagonized' by application of high pressures of the order of 100 atm. Estimates of the compressibility of the site of action ($6 \times 10^{-5}$ $atm^{-1}$) agree with those predicted from the solubility characteristics (Miller *et al.* 1973). However, quantitation of the pressure–anaesthetic interactions in rats and mice has revealed details of this phenomenon which are not consistent with the traditional unitary view that all anaesthetics act at the same type of molecular site (M. J. Halsey, B. Wardley-Smith & C. J. Green, unpublished work). It may be that future hypotheses for molecular mechanisms of anaesthesia will have to include multi-sites of action.

## ANAESTHETIC EFFECTS ON PROTEIN FUNCTION

As long ago as 1944, Östergren postulated that lipid-soluble agents could affect 'protein chain folding' and that this was related to 'narcosis'. There is now considerable evidence that the inhalational agents can interact with many proteins and produce functional changes. The effects of the environment and inhibitors on a range of different proteins give some clues to the relative importance of anaesthetic perturbations of secondary, tertiary and quaternary structures.

Anaesthetic depression of enzyme activity has been investigated in several cases. For example, glutamate dehydrogenase is reversibly depressed by halothane ($CF_3$·CHBrCl) and methoxyflurane (MeO·$CF_2$·$CHCl_2$) whereas many of the enzymes in the Embden–Meyerhoff pathway are relatively unaffected (Brammall *et al.* 1974). One enzyme system that has been extensively studied is the luminescent reaction in luminous bacteria, which is activated by low concentrations and reversibly depressed by clinical concentrations of inhalational anaesthetics (White & Dundas 1970; Halsey & Smith 1970). Detailed *in vitro* biochemistry with 'lipid-free' luciferase has identified the probable site of anaesthetic action (Middleton 1973; White *et al.* 1975). The structure of this enzyme has not been elucidated fully but includes a highly hydrophobic active centre with an associated reactive thiol group (Nicoli *et al.* 1974). Such a structure in the active centre leads to the working hypothesis that protein functions are sensitive to anaesthetics if, and only if, there are suitable hydrophobic areas available at or near the active centres. White and his colleagues reviewed the structure of those enzymes whose secondary and tertiary structures are known, and selected papain and bromelain as examples of proteins with hydrophobic active centres containing active thiol groups, in contrast with the properties of lysozyme and α-chymotrypsin. The functions of papain and bromelain are reversibly depressed by clinical concentrations of anaesthetics whereas the functions of the 'non-hydrophobic' active-centre proteins are unaffected (King *et al.* 1977).

The relationships between different anaesthetic-induced functional changes in the same protein are not always clear. Thus, the oxygen-binding function of haemoglobin does not appear to be affected by clinical inhalational anaesthetics (Millar *et al.* 1971; Weiskopf *et al.* 1971) even at the extremes of the physiological pH range (M. J. Halsey & B. Minty, unpublished work). However, high partial pressures of nitrogen appear to increase the oxygen affinity of haemoglobin (Kiesow 1974) but low partial pressures of dichloromethane affect its capacity to bind carbon monoxide (Settle 1975).

## ANAESTHETIC EFFECTS ON PROTEIN CONFORMATION

Relatively few direct investigations have been made of the underlying conformational perturbations produced by anaesthetics. X-ray crystallography has been used to locate the binding site of xenon in haemoglobin and of both xenon and cyclopropane in myoglobin (Schoenborn 1965, 1968). Optical rotatory dispersion has been used to demonstrate the tertiary structural changes produced by high concentrations of anaesthetics on bovine serum albumin and β-lactoglobulin (Balasubramanian & Wetlaufer 1966).

Similar studies have also been done on various forms of haemoglobin – including the isolated β-chains (Laasberg & Hedley-White 1971) – and on myosin, aldolase, human serum albumin, ribonuclease and lysozyme (Leuwenkroon-Strosberg 1973). The helical content of the haemoglobins, aldolase and myosin, appeared to be decreased by 3–10% on exposure to all anaesthetics used but significant effects on the secondary structure of the other proteins could not be detected.

## NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

This paper is devoted to aspects of anaesthetic perturbations of the conformation of a model protein system. To observe perturbations of this kind we need a technique that is sensitive to small structural changes in a protein which, if possible, is in its physiological state: i.e. membrane-bound proteins should be studied bound to membranes, and soluble proteins should be studied in solution. Departure from these existing conditions may alter or restrict the access of inert anaesthetic molecules which do not bind in the accepted sense but merely undergo selective 'solution' within the protein interior.

At present it is not possible to make direct detailed studies on membrane-bound proteins and there are limitations in assessing potential perturbations in membrane-bound proteins from the behaviour of soluble proteins. However, even though part of the surface of a membrane-bound protein may be covered by hydrophobic groups, the underlying concept of protein distortion by invasion of hydrophobic areas beneath the surface is probably still a valid one, although the effects of such distortions are less easily assessed as they may affect not only the conformation of the active site but the mobility of the protein within the lipid bilayer.

One of the techniques most sensitive to small structural perturbations in soluble proteins is spin-echo Fourier-Transform nuclear magnetic resonance spectroscopy (Campbell *et al.* 1975). If this technique is to be used effectively, the protein must produce a well-resolved spectrum containing at least a few lines capable of unique assignment so that the properties observed are those of one particular nucleus, even if its actual assignment is unknown. This imposes an upper limit on the molecular weight of the protein of about 70 000. At the same time it is an advantage to have a large protein with various hydrophobic pockets so as to provide an opportunity for study of site preferences determined by the size, shape and composition of the anaesthetic molecule. Furthermore, allowance must be made for the basic insensitivity of n.m.r. spectroscopy in terms of signal acquisition, so that the protein must be soluble

(e.g. > 1 mmol/l) and available in reasonably large quantities (e.g. 50 mg of a high-molecular-weight protein per sample).

A protein that fits all these requirements is haemoglobin. It has a further advantage that, being composed of subunits whose relative movement is germane to its function, it offers further scope for comparison with, for example, the acetylcholine receptor protein, whose function may also be associated with the movement of subunits.

The application of spin-echo techniques to Fourier-Transform n.m.r. spectroscopy greatly enhances the basic sensitivity of the method to small structural changes. Not only is the signal frequency sensitive to the precise magnetic environment of a nucleus within the protein framework, but the length of time taken by a particular nucleus to dissipate its spin energy either individually to another similar nucleus ($T_2$) or collectively to the lattice ($T_1$) will critically control the line width and line intensity in a given set of experimental conditions. It is these latter, more delicate, parameters to which the spin-echo experiment is particularly sensitive through its two delay variables, the interpulse spacing (sensitive to $T_2$) and the scan repetition rate (sensitive to $T_1$). These parameters will be most affected by the subtle changes in conformation and mobility caused by anaesthetic perturbations. Although such changes in the relaxation times, and therefore in the line intensity, will be small compared with the normal peak height of a perturbed resonance, it is possible, by use of fairly long delay times between the 90° and 180° pulses, to amplify these effects greatly so that these small intensity changes now become a significant fraction of the intensity observed in these conditions.

When anaesthetics interact with haemoglobin a series of perturbations occur but the ones which will be considered here are those from the C-2 protons from histidyl groups on the surface of the molecule. The reason for this is not that their perturbations are more pronounced but because their resonances are the only ones which can be clearly resolved as being derived from single protons. As implied earlier, this is very important if we are to be able to differentiate different perturbations in different parts of the molecule, even though the actual part referred to may not be known. The power of this technique can be further enhanced by the use of deconvolution procedures such as those developed by Ernst (1966) (Fig. 1).

The study of these particular resonances has the advantage that their position, which is downfield of the main bulk of the haemoglobin spectrum, is pH-dependent, depending on the state of protonation of the imidazole ring. Thus the p$K$ of the ring protonation provides a further parameter for study as well as providing facility for monitoring other details of the interaction between anaesthetic and protein.

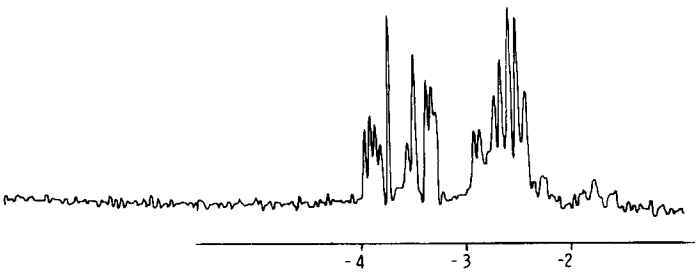


FIG. 1. A spin-echo Fourier-Transform nuclear magnetic resonance spectrum of the aromatic-residue region of haemoglobin obtained with mild resolution enhancement by a deconvolution procedure, as used by A. Ferridge (unpublished work, 1977). The left- and right- hand groups of resonances originate from histidyl H-2 and H-4, respectively.

The first n.m.r. experiments with this system (Barker *et al.* 1975) studied the effects of chloroform, halothane, ether and methoxyflurane on haemoglobin by normal Fourier-Transform n.m.r. spectroscopy. We used higher concentrations of anaesthetic than used clinically. In each case, noticeable changes in the chemical shifts of only one or two histidine resonances, out of the 12 titratable ones present, were observed.

More detailed studies by the same methods with halothane (Brown *et al.* 1976) showed that specific perturbations persisted down to clinical concentrations of anaesthetic and we attempted to determine the position in the protein where these effects originated.

The application of the spin-echo techniques has provided a much more powerful method for studying these perturbations, since a slight shift of one of the histidyl residues under observation either towards or away from one of its neighbours might not change the chemical shift much (unless that neighbour was changed) but it could alter its relaxation time considerably. This has proved to be the case. Chloroform, ether, halothane and cyclopropane were studied. These various anaesthetics once again produced a few select perturbations among the histidines, only this time they were manifest not only as changes in chemical shift but also as concentration-dependent fluctuations in intensity. These fluctuations were not, however, proportional to the changes in the chemical shift, as shown in Fig. 2.

The intensity behaviour proved to be a more discriminating perturbation than the chemical shift, although the latter is a prerequisite for this particular pattern of behaviour to be observed. This observed $T_2$-dependent variation in intensity with anaesthetic concentration is characteristic of the exchange phenomena observed for a fast exchange situation. The equations describing this process have been given by Baldo *et al.* (1975) and the results observed for

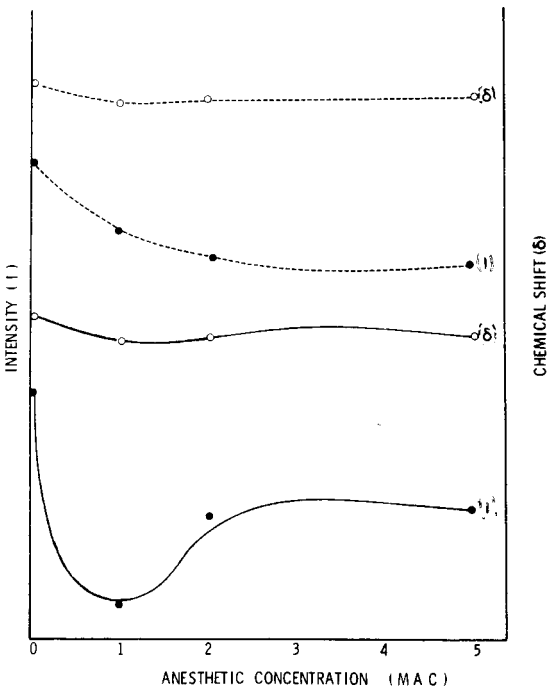


FIG. 2. Changes in chemical shift and intensity (arbitrary units) of two haemoglobin histidyl resonances as a function of diethyl ether concentration (expressed as a multiple of the minimum alveolar concentration, MAC – an index of clinical anaesthetic potency in man). The upper set of lines (---) illustrates the response of most of the histidyl resonances in the presence of anaesthetics. The decay in intensity is probably related to general surface interactions. The lower set of lines (–) illustrates the selective response of only one or two histidyl resonances and the minimum in the intensity/concentration curve is characteristic of an anaesthetic exchange process.

one of the resonances perturbed by several anaesthetics have been fitted to their curve, which describes the broadening of an n.m.r. line as a function of percentage occupancy of a binding site (Fig. 3). The broadening observed is a maximum when slightly over 40% of the binding sites are filled. If the 'binding' properties of each anaesthetic are the same, then the broadening becomes directly related to the molecular concentration of the anaesthetic.

Although the perturbations being described are observed on surface residues, it does not necessarily imply that the interaction is a surface one. Several indications suggest that it is not. It would be surprising if such a non-specific and inert set of compounds as the common anaesthetics were so selective in their interactions with surface histidines that they selected only one or two of all the surface histidines.

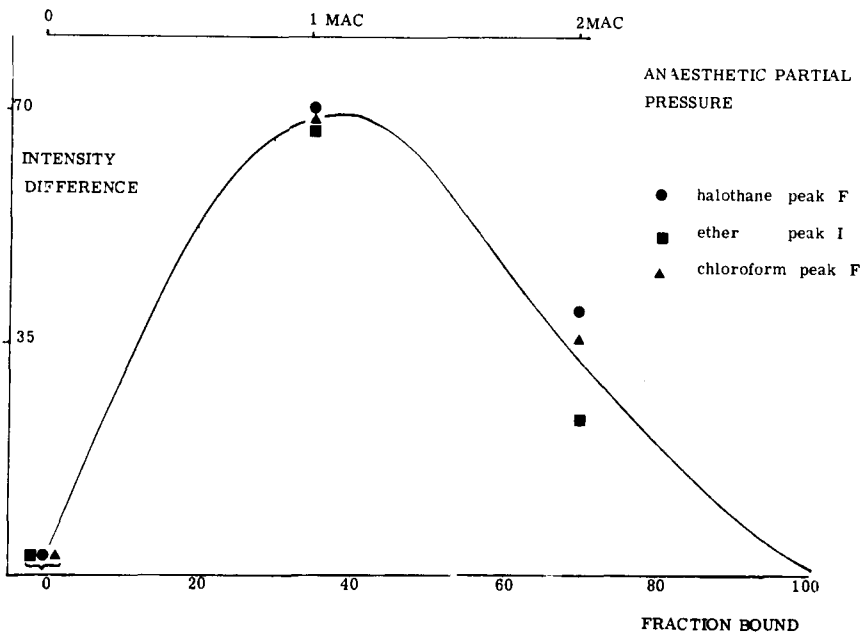


FIG. 3. A plot of the normalized intensity differences between shifted and non-shifted peaks against the percentage fraction of anaesthetic bound (bottom scale) and the anaesthetic partial pressure (upper scale) expressed as multiples of the minimum alveolar concentration (MAC). The solid line is the predicted curve derived from the equation

$$\pi\Delta\nu_{obs} = \frac{1}{T_{2(obs)}} = \frac{f_H}{T_2[H]} + \frac{f_{HA}}{T_2[HA]} + f_H^2 f_{HA}^2 (\delta_H - \delta_{HA})^2 (\tau_H - \tau_{HA})$$

which describes the fast exchange equilibrium between free haemoglobin (H), the anaesthetic (A) and the complex HA:

$H + A \leftrightarrows HA$

where $\Delta\nu$ is the line width at half height, $\tau$ is the lifetime of a component, $\delta$ is the chemical shift of that component, and $f$ is the fraction of that component.

A more plausible conclusion is that the surface perturbations monitor the expansion of hydrophobic pockets within the protein which have been invaded by the hydrophobic anaesthetic molecules. Thus the 'binding' referred to earlier becomes no more than a simple solution process within these pockets and the exchange monitored by the broadening reveals no more than the movement of 'solute' in and out of these pockets. In this case the concentrations which relate to the decrease in $T_2$ for different anaesthetics would become directly comparable. Fig. 3 demonstrates this as these broadenings turn out to be directly proportional to their anaesthetic potencies and hence to their hydrophobic molecular solubilities.

## CONCLUSION

These data provide the first direct evidence that anaesthetics can interact with hydrophobic pockets in proteins and that the site(s) appear to behave as simple bulk solvents in terms of their solubility characteristics. The conformational perturbations produced by such hydrophobic interactions can be transmitted and detected in non-hydrophobic areas of the protein. The correlation of these phenomena with lipid solubility and hence with *in vivo* anaesthetic potency is an encouraging asset for any mechanism of anaesthesia involving protein perturbations (Brown *et al.* 1977). The fact that conformational effects specific to individual agents are also observed in proteins (Barker *et al.* 1975) is relevant to the peripheral mechanisms of the side-effects, biotransformation and toxicity of anaesthetics. Proteins that could be involved in anaesthetic action include enzymes, neurotransmitter receptors, and membrane transport and motility systems. The link between the molecular and cellular effects of anaesthetics may also be mediated through calcium permeability (Allison 1974).

The purpose of the present studies has been to understand more about the 'ground rules' for molecular interactions of anaesthetics. However, the data that we have obtained also indicate that anaesthetics may have a limited role as selective hydrophobic probes for studying protein activity in general.

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# Discussion

*Roberts:* Are your data good enough to extract the information on the rate of exchange of the anaesthetic between the bound and free states which can, in principle, be obtained from these changes in line-width?

*Halsey:* The observed $T_2$-dependent variation in intensity with anaesthetic concentration is characteristic of a fast-exchange process. We have not yet quantified the rates but have observed, for example, that methoxyflurane

($MeO{\cdot}CF_2{\cdot}CHCl_2$) – in contrast to the other clinical anaesthetics so far studied – appears to be in slow exchange with the haemoglobin site(s).

*Richards:* This technique can yield some interesting structural information about proteins but I do not understand what this has to do with anaesthesia.

*Blundell:* The binding of halothane by myokinase (Sachsenheimer *et al.* 1977) leads to malignant hyperthermia, characterized by rigidity of muscles and a continuous rise in body temperature.

*Halsey:* I discussed the general relevance of protein structure and function to anaesthesia but let me make two additional points. First, investigations of anaesthetic effects on proteins form only one aspect of the many current investigations into the mechanisms of general anaesthesia. Other complementary aspects include studies on lipids, cell membranes, neuronal systems and whole animals (Halsey *et al.* 1974). Second, our choice of haemoglobin as the initial model protein for attempting to determine the 'ground rules' for anaesthetic–macromolecule interactions was governed by pragmatic reasons (as I indicated) rather than by its physiological significance in anaesthesia. We are also extending our structural studies both to an example of a membrane-associated protein whose function is already known to be affected by clinical concentrations of anaesthetics (luciferase) and also to a neuroprotein of potential physiological importance (acetylcholine receptor).

*Franks:* Both you and Professor Richards referred briefly to the effect of pressure on protein stability. Application of pressure to metmyoglobin (at low temperature) unfolds the protein but if, at high pressures, the temperature is increased, then the protein begins to fold again spontaneously (Zipp & Kauzmann 1973). This behaviour reflects the complexity of the hydrophobic effects and points to some connection between the anaesthetic effect and a conformational change which involves the hydrophobic region of the protein.

*Halsey:* Professor Gutfreund mentioned the pressure-relaxation method for investigating transient conformation changes. What pressures were applied?

*Gutfreund:* About 200 atm; the effect probably centres on disturbances of charge–charge interactions. We are at present analysing the volume changes from the amplitudes of the various processes. We developed the pressure-relaxation technique to study assembly processes such as in myosin and tubulin, their interaction with other proteins, and regulation of such processes by ligand binding. These processes depend strongly on pressure. Myosin, for example, at high ionic strength dissociates into monomers at a pressure of only 5 atm (Davis & Gutfreund 1976).

*Halsey:* Are not the volume changes in the whole membrane associated with the lipids greater than those associated with the protein?

*Franks:* Maybe so, but the existence of a pressure reversal in the unfolding indicates that initially $\Delta V > 0$ but at higher pressures (or temperatures) $\Delta V < 0$. Lipids are compressible but they will not show this reversal of pressure effects.

*Halsey:* We are referring to 'pressure reversal' in a different sense. The phenomenon observed in animals below 100 atm is that an animal already anaesthetized at 1 atm will wake up as pressure is applied either hydrostatically or with helium (a relatively 'inert' gas in pharmacological terms). This has been termed 'pressure reversal of anaesthesia' (Lever *et al.* 1971). This does not imply that there will be a reversal in sign of the pressure effects on their own. However, it is possible that at pressures above 100 atm the protein-volume-reversal effect might be relevant to the animal studies. For example, current studies on the pressure reversal of intravenous anaesthesia (M. J. Halsey, B. Wardley-Smith & C. J. Green, unpublished observations) indicate that for some agents there is an upper limit to the pressure reversal effect. It has not yet been possible to expose animals to high enough pressures at which the protein reversal effects that you describe could occur.

*Gutfreund:* Increasing the pressure should lower the permeability of the lipid – rotation of proteins would be inhibited if that depends on the mobility of the lipids. One easy way to find out about the pressure dependence of phase transitions is to measure it calorimetrically.

*Franks:* The effects of pressure on lipid monolayers are straightforward. A lipid bilayer is probably much the same as two monolayers. But no single instance of a reversal in sign has been observed with lipids, i.e. $(\delta V/\delta P) < 0$.

*Halsey:* The effects of pressure on their own are interesting physiologically and in particular are related to the high-pressure neurological syndrome, which is one of the major problems in deep-sea diving (Halsey *et al.* 1975). The fact that anaesthetics can ameliorate the syndrome is consistent with the idea that anaesthetics expand the critical molecular sites and pressure contracts them.

*Cornforth:* Has a chiral form of an anaesthetic of the halogenocarbon type been made? It ought to be possible. Perhaps one could get enantiomers equivalent in all physical properties except chirality.

*Halsey:* I am not aware of any evidence that chirality is important in general anaesthesia produced by inhalational agents. For example, there is no stereospecific activity of halothane ($CF_3$·CHBrCl) in an isolated nerve or a phospholipid-bilayer membrane model system (Kendig *et al.* 1973). There are problems in interpreting the stereochemical influences on variations in the pharmacological activities of intravenous anaesthetics but recently significant differences for the ketamine enantiomorphs have been observed in rats (Marietta *et al.* 1977).

*Williams:* You studied haemoglobin; was it in the carbonmonoxy-haemoglobin form?

*Halsey:* No, most of our work has been done with unmodified oxyhaemoglobin isolated from human red cells (as described in Brown *et al.* 1976).

*Williams:* Haemoglobin binds xenon. Is it known where it binds?

*Halsey:* Schoenborn (1965) studied the binding of xenon to crystals of horse methaemoglobin and identified two possible sites.

*Williams:* But that is probably where it binds in the deoxy form. As the deoxy form is paramagnetic, I doubt that it would have given n.m.r. spectra like the one you illustrated. So you have eliminated the specific binding problem by using a diamagnetic state and so blocking the normal site for an anaesthetic to bind in haemoglobin.

*Halsey:* I agree that there may be differences between the anaesthetic effects on the oxy- and deoxy-form (I. D. Campbell and F. F. Brown are studying this at Oxford University, Biochemistry Department). However, any of the anaesthetic sites in haemoglobin may be regarded as 'specific' and capable of being 'blocked' in the oxy-form. If this were so, anaesthetics would significantly shift the shape or position of the oxygen-dissociation curve which has been shown to be insensitive to high concentrations of anaesthetics (Millar *et al.* 1971). There is also no evidence for saturation of the haemoglobin 'solution' sites as the partial pressures of most of the anaesthetics are increased. The list of agents studied includes xenon (Schoenborn 1965) and the only known exception is cyclopropane (Gregory & Eger 1968).

*Williams:* The methyl resonances in the n.m.r. spectrum might be good indicators of the specific effects in the deoxy form.

*Halsey:* The methyl resonances associated with the haem group of ferrimyoglobin cyanide have been studied by Schulman *et al.* (1970) who observed various shifts produced by xenon and cyclopropane. Unfortunately, they used a single relatively-high concentration of the two agents. Comparison between these experiments and other anaesthetic effects on proteins is difficult.

*Lipscomb:* The binding-site of xenon in haemoglobin is far removed from the haem but it is near the haem in myoglobin (see Schoenborn 1965).

*Knowles:* But, to echo Professor Richards' point, what is the relevance of this? Correlations can be drawn between all sorts of parameters: the birth rate in England during this century correlates splendidly with the population of storks in Heligoland!

*Halsey:* Maybe our attempt to establish ground rules for the interaction between proteins and anaesthetics was overambitious but until recently few people believed that anaesthetics ever affected proteins.

*Lipscomb:* If there is a general effect on proteins, do you observe an effect in a simple model system like bacterial motility?

*Halsey:* There are anaesthetic effects on bacterial proteins such as luciferase and also on various motile proteins (for a general review see Allison 1974). Most studies have been functional rather than structural and what is now needed is clear understanding of the link between the effects.

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