The Biochemical Journal, Vol. 122, *No.* 4

G. K. RADDA

Biochem. J. (1971) **122**, 385–396
Printed in Great Britain

Enzyme and Membrane Conformation in Biochemical Control

THE SEVENTH COLWORTH MEDAL LECTURE

BY G. K. RADDA*

Delivered at a Meeting of the Biochemical Society on 17 December 1970 at the Royal Postgraduate Medical School, Hammersmith Hospital, London W.12, U.K.

It is appropriate on an occasion like this to begin by expressing my gratitude to all my collaborators who throughout several years contributed so effectively to the work I am going to describe. There are many others whose contribution I will not be able to discuss but value equally.

Conformational changes have been assumed to be important in many biochemical processes: e.g. enzyme catalysis (Koshland, 1959), allosteric control (Monod, Wyman & Changeux, 1965), energy conservation (Green, Asai, Harris & Penniston, 1968), nerve action (Tasaki, Watanabe, Sandlin & Carnay, 1968) and so on. However, the term is used very readily to describe phenomena that we do not clearly understand. We all know that the definition of conformation is concerned with the spatial relationship of groups, side chains and the backbone of macromolecules. To demonstrate changes in conformation therefore we have to show that the distances and angles between these groups have altered in some way. This is where the difficulty lies. None of the methods available for the study of proteins (let alone membranes) in solution gives us direct information about the exact position of individual atoms or groups in the macromolecule. The question therefore is this: when we observe a change in some chemical or physical property of the system how justified are we in assigning such a change to a conformational transition? Crystallographic studies have told us that often small movements of specific groups occur as a result of ligand binding (Blake *et al.* 1967; Reek *et al.* 1967), which in some cases could be amplified by subsequent rearrangements in more distant parts of the molecule (Perutz, 1970). Because the changes may be relatively localized we have concentrated on the use of probes. These are molecules or ions that can be introduced into an enzyme or membrane at specific sites and, mainly through their spectroscopic properties, are able to give us information about their environment, and about relatively minor changes around the probe-binding site. I shall describe the use of three types of probes. The method of observation for these involves fluorescence, chemical reactivity and the n.m.r. property known as the spin–lattice relaxation time.

* Address: Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, U.K.

Theoretical background

Let us look at fluorescence first. When a molecule is excited by light of appropriate wavelength the upper electronic excited state is reached so rapidly that the only changes occurring are those that involve movement of electrons. This may result in an altered charge distribution in the molecule and a change in the polarization of the solvent molecules around the chromophore. This is the Franck–Condon principle and the excited state reached after absorption is the Franck–Condon excited state. Now before emission of light occurs (about 1–10ns) several events can happen. (1) Because of the altered electron distribution in the excited state the solvent molecules around it have time to rearrange to form the more stable 'equilibrium excited state'. The extent of interaction between the excited state and the solvent depends on the magnitude of the dipole-moment change on excitation and the polarity and polarizability of the solvent molecules. Therefore the position of the emission maximum can be used to monitor the polarity of the probe's environment, particularly when there is a large change in dipole moment on excitation. (2) In response to the new charge distribution the geometry of the excited state may change. When this occurs the potential-energy profile for the equilibrium excited state will be very different from that of the ground state, with the result that the absorption and emission spectra will be well separated (Fig. 1*b*), in contrast with the case where the two geometries are rather similar (Fig. 1*a*). There are two experimental consequences of case (*b*) that are particularly important for our purposes. These have been discussed by Thomson (1969) and others (El-Bayoumi, Dalle & O'Dwyer, 1970). One is that the fluorescence quantum yield is low and the other is that the intrinsic excited-state lifetime calculated theoretically by using the

Strickler & Berg (1962) relation is different from intrinsic lifetime, τ_0, derived from the relation:

$$\tau_0 = \tau_{measured}/\text{quantum yield}$$

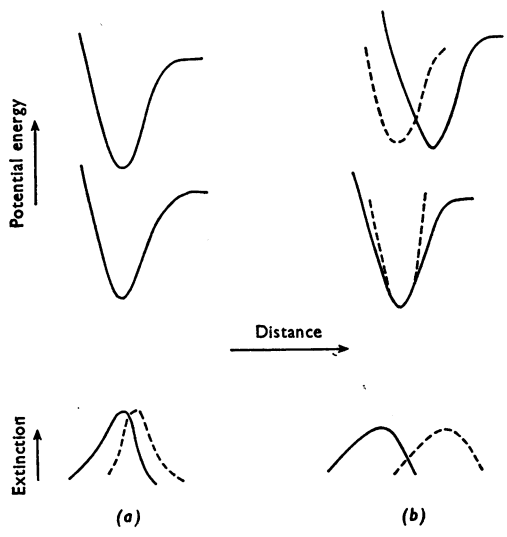
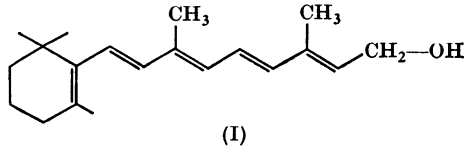


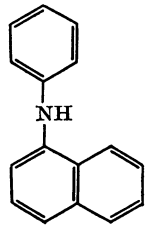
Fig. 1. Schematic potential-energy diagrams for ground and excited states and for the associated absorption and emission spectra. (*a*) Ground and excited states with the same geometry; (*b*) ground and excited states with different geometries. ——, Absorption; ----, fluorescence.

Thus the failure of the Strickler–Berg relation to give the correct value for τ_0 may be taken as an indication of a change in geometry in the excited state. If we now constrain the environment of the molecule so that we do not allow changes in geometry (broken lines in Fig. 1*b*) we approach the situation in (*a*) with a consequent increase in the fluorescence quantum yield and an increased adherence to the Strickler–Berg relation. Thus molecules in this class will have fluorescence properties that reflect 'environmental constraint'. A good example for this kind of behaviour is retinol (I) (Thomson, 1969). For this compound the calculated intrinsic lifetime is 3ns and the measured τ_0 in hexane as solvent is 600ns (Thomson, 1969).

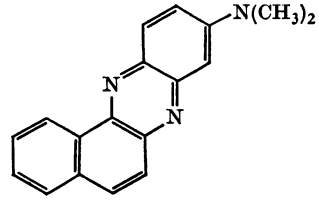
I would like to show in a little more detail how one can approach the problem of investigating the various factors that contribute to environmental sensitivity of fluorescence by referring to the properties of *N*-1-naphthyl-*N*-phenylamine (II), which, together with some of its sulphonated derivatives, we have used as a probe in some of our membrane studies. Although the effects in this molecule are more complex than in retinol (Dalle & Rosenberg, 1970), I shall use it as an example for the kinds of study necessary to separate the various factors. The absorption spectrum of this molecule in hexane–benzene mixtures is red-shifted as the polarizability of the solvent is increased (i.e. as the proportion of benzene is increased). By using a modified theory (Bayliss, 1950) of solutions proposed by Onsager (1936) we can calculate from the



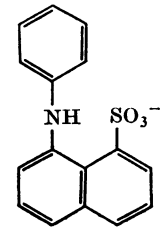
(I)



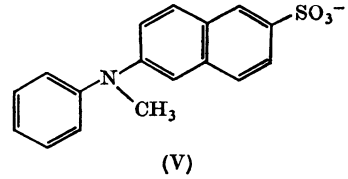
(II)



(III)



(IV)



(V)

Table 1. *Effect of solvent on the spectroscopic properties of* N-1-*naphthyl*-N-*phenylamine*

Solvent	$\lambda_{max.}$ (absorption) (nm)	$\lambda_{max.}$ (emission) (nm)	Fluorescence quantum yield	Fluorescence lifetime (ns)
Methanol	340	424	0.22	4.8
Benzene	339	395	0.53	7.4
Hexane	337	376	0.47	3.6
Water	—	459	0.02	—

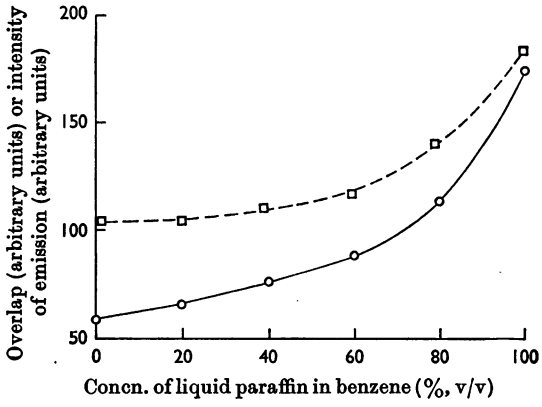


Fig. 2. Variations of the spectral properties of *N*-1-naphthyl-*N*-phenylamine. ○, Overlap between absorption and emission; □, intensity of emission.

red-shift that the change in dipole moment in going from the ground state to the Franck–Condon excited state is about 2.5 Debye units. This accounts for the large solvent-dependence of the absorption and emission spectra of *N*-1-naphthyl-*N*-phenylamine (Table 1). If we now take a solvent mixture in which we keep solvent polarity and polarizability constant and change only the viscosity we see that the overlap between the emission spectrum and the absorption spectrum increases with increasing viscosity, resulting in an enhancement in the fluorescence quantum yield (Fig. 2). This suggests that the geometries of the ground state and the excited state are different and that environmental constraint also affects the fluorescence. The change in geometry suggested above is confirmed by the observation that the calculated and measured τ_0 values do not agree (Table 2). Interestingly this table also shows that when *N*-1-naphthyl-*N*-phenylamine binds to bovine serum albumin or erythrocyte membranes (or at high viscosities) the measured τ_0 approaches the theoretical value of 20ns, indicating that binding results in a constrained environment. To simplify the discussion no distinction is made here between 'orientation' and 'packing' constraint (Becker, 1969).

The molecule 3-dimethylaminonaphtheurhodine (III) is essentially only a probe for polarity. Fig. 3 shows how the emission maximum (expressed as transition energy) and intensity depend on solvent polarity.

So far we have three types of fluorescent probes: retinol, which measures environmental constraint, naphtheurhodine, which senses polarity, and *N*-1-naphthyl-*N*-phenylamine, which is sensitive to both. We can add to this list derivatives of *N*-1-naphthyl-*N*-phenylamine such as 1-anilinonaphthalene-8-sulphonate (IV) and 2-(*N*-methylanilino)naphthalene-6-sulphonate (V), which spectroscopically are like *N*-1-naphthyl-*N*-phenylamine but contain a charged group and a hydrophobic group and are thus amphiphilic, in contrast with the first three probes, which have very low solubilities in water but dissolve in hydrocarbon phases.

Chemical reactivity. Let us turn to the second approach that I shall use, namely chemical reactivity. Useful information is obtained if the reagent satisfies a number of requirements: (*a*) the reaction should be easy to follow, preferably continuously, so that the kinetics can be resolved accurately; (*b*) the reagent should be specific for a particular type of side chain and preferably for a group located at a particular site of the enzyme. A reagent that satisfies these criteria is 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole. It is specific, at neutral pH, for thiol groups (Birkett, Price, Radda & Salmon, 1970*b*) and has the additional advantage that although the reagent is not fluorescent its product from reaction with a thiol has a convenient fluorescence that is environmentally sensitive (Fig. 4). So here we have the possibility of attaching an environmentally sensitive fluorescent chromophore covalently to a particular group on a protein or membrane.

Proton relaxation enhancement. The third approach I shall be using essentially involves a way of looking at the hydration sphere of paramagnetic metal ions by measuring the spin–lattice relaxation time, T_1, of water protons (Cohn, 1963) by pulsed n.m.r. (Mildvan & Cohn, 1970). When a metal ion such as Mn^{2+} is bound to a macromolecule it has an

Table 2. *Fluorescence properties on* N-1-*naphthyl*-N-*phenylamine in different systems*

τ_0 calculated by using the Strickler–Berg relation is 20 ns.

System	Quantum yield	$\lambda_{max.}$ (emission) (nm)	$\tau_{measured}$ (ns)	τ_0 calculated from quantum yield and $\tau_{measured}$ (ns)
Hexane	0.47	376	3.6	7.7
Erythrocyte stroma (pH 7.0, iso-osmotic NaCl)	0.34*	400	7.3	21.5
Bovine serum albumin	0.4*	410	9.6	24.0

* These values were obtained by extrapolation to infinite protein concentration. The titrations were done with constant *N*-1-naphthyl-*N*-phenylamine concentration (4 μM) and various amounts of protein or membrane.

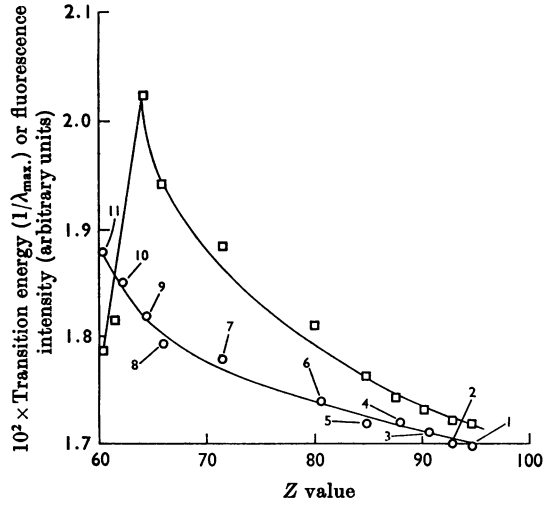


Fig. 3. Fluorescence properties of 3-dimethylamino-naphtheurhodine. ○, Transition energy of emission maximum; □, fluorescence intensity. 1, Water; 2–5, water–ethanol mixtures; 6, ethanol; 7, 2-methylpropan-2-ol; 8, acetone; 9, dichloromethane; 10, benzene; 11, cyclohexane.

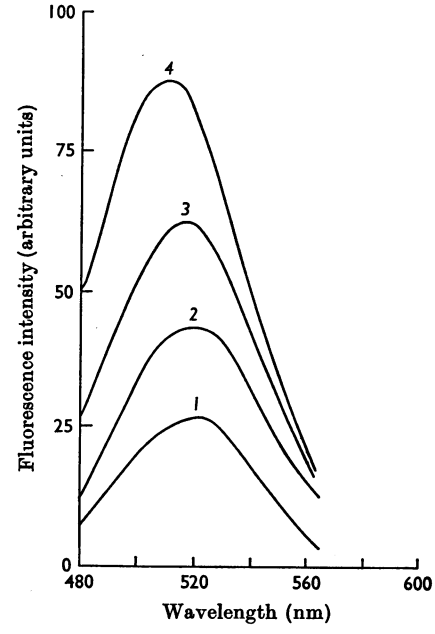


Fig. 4. Fluorescence of the *N*-acetyl-*S*-4-nitrobenzo-2-oxa-1,3-diazole derivative of cysteine in ethanol–water mixtures. Curve *1*, 0% ethanol; curve *2*, 20% (v/v) ethanol; curve *3*, 40% (v/v) ethanol; curve *4*, 60% (v/v) ethanol.

enhanced effect on the proton relaxation rates, the enhancement being defined as:

$$\epsilon^* = \frac{1/T_1^* - 1/T_{1(0)}^*}{1/T_1 - 1/T_{1(0)}}$$

where the asterisk indicates the presence of the macromolecule, $1/T_1^*$ and $1/T_1$ are the observed spin–lattice relaxation times of the solution in the presence of Mn^{2+} and $1/T_{1(0)}^*$ and $1/T_{1(0)}$ those of the same solutions in the absence of Mn^{2+}. The enhancement parameter, ϵ^*, depends on a number of variables, which are (*a*) the number of water molecules in the hydration sphere of the metal ion, (*b*) the electron-spin correlation time of the paramagnetic ion, τ_s, (*c*) the 'residence' time of the water molecules on the metal ion, τ_M, and (*d*) the rotational correlation time, τ_r, which represents the motion of the metal ion and its hydration sphere. It is clear that some or all of these may be affected when the paramagnetic metal ion interacts with the protein and that the detailed interpretation of the results is quite complex. For our present purposes it is sufficient to say that we are using ϵ_b (the enhancement parameter when all the metal ion is bound to the macromolecule) as a characteristic spectroscopic property of the metal-ion-binding site. [The detailed interpretations and arguments have been presented elsewhere (Birkett, Dwek, Radda, Richards & Salmon, 1971).]

Probes for allosteric enzymes

We demonstrated some years ago (Dodd & Radda, 1967) that when molecules like 1-anilinonaphthalene-8-sulphonate interact with glutamate dehydrogenase not only is their fluorescence enhanced and blue-shifted but the fluorescence properties of the bound chromophore reflect ligand-induced structural changes in the enzyme (Dodd & Radda, 1969; Brocklehurst & Radda, 1970). In this particular case the transition could also be observed by optical rotatory dispersion (Bayley & Radda, 1966).

Today I would like to describe our studies on rabbit muscle phosphorylase *b*, an enzyme quoted by Monod *et al.* (1965) in relation to their allosteric model. It contains two subunits of molecular weight 92500 and is active only in the presence of the regulatory ligand AMP or after an enzyme-catalysed phosphorylation of one serine residue/subunit. Activation by AMP does not produce any detectable changes in the optical-rotatory-dispersion or circular-dichroism properties of the enzyme.

Now phosphorylase *b* interacts with 2-(*N*-methylanilino)naphthalene-6-sulphonate, enhancing and shifting the probe fluorescence (Fig. 5). When AMP is added to this system it induces a further fluorescence enhancement, particularly in the presence of bivalent metal ions such as Mg^{2+} or Mn^{2+} (Fig. 5) (Birkett, Freedman, Price & Radda, 1970*a*). The simplest explanation might have been that the probe detects a conformational change on ligand binding. Detailed kinetic studies on the fluorescence change, however, have shown that the rate is low and second-order with respect to protein concentration (Birkett, Radda & Salmon, 1970*c*) ($k = 3.1 \times 10^5 \text{l} \cdot \text{mol}^{-1} \cdot \text{min}^{-1}$). This indicates that the probe follows a ligand-induced aggregation of the enzyme into a tetrameric structure. Comparison with sedimentation-velocity measurements confirms this conclusion. The probe can be used to follow aggregation even at low enzyme concentrations where other methods are not sufficiently sensitive. By the use of this probe we have been able to separate ligand-induced conformational changes (described below) from the aggregation that appears to be a consequence of the structural transitions.

Although phosphorylase *b* has no requirement for bivalent metal ions, Mn^{2+} interacts with the enzyme in an apparently specific manner in that there are two independent and equivalent metal-ion-binding sites/enzyme molecule with a dissociation constant of 170 μM. This interaction can be followed by e.s.r. (electron spin resonance) (the bound Mn^{2+} does not contribute to the e.s.r. signal) or by proton-relaxation enhancement. The enhancement parameter, ϵ_b, characteristic of the fully bound enzyme–Mn^{2+} complex can now be used to monitor changes at the Mn^{2+}-binding site. For example, it has been observed that the enzyme activity and AMP binding show an anomalous temperature-dependence in that there is an inflexion in the Arrhenius plots at about 13°C. It is probable that this inflexion is a result of a temperature-dependent conformational equilibrium. The proton-relaxation enhancement for the enzyme–Mn^{2+} complex is indeed able to detect this transition (Fig. 6).

We have another way of looking at this problem. Phosphorylase *b* reacts with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole. If the reaction is carried out under pseudo-first-order conditions (i.e. with excess of reagent) the reaction proceeds with the modification of three thiol groups/subunit. The intrinsic

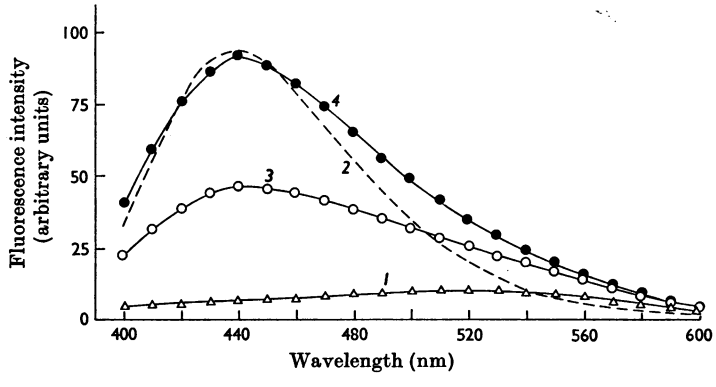


Fig. 5. Fluorescence emission spectra of 2-(*N*-methylanilino)naphthalene-6-sulphonate. Excitation was at 420nm. Curve *1*, in buffer; curve *2*, in ethanol; curve *3*, with phosphorylase *b* (1 mg/ml); curve *4*, with phosphorylase *b* + 1 mM-AMP + 10 mM-$MgCl_2$.

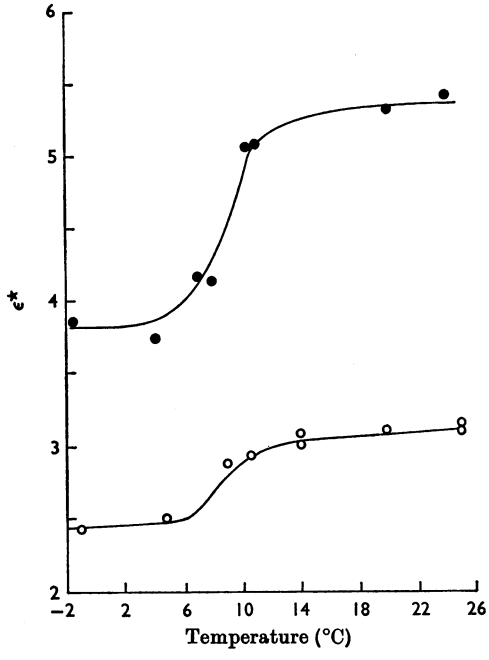


Fig. 6. Variation of proton-relaxation enhancement with temperature. The concentration of phosphorylase *b* was 90 μM and that of Mn^{2+} was 100 μM, in 50 mM-tris–HCl buffer, pH 7.0 (○) or pH 8.5 (●).

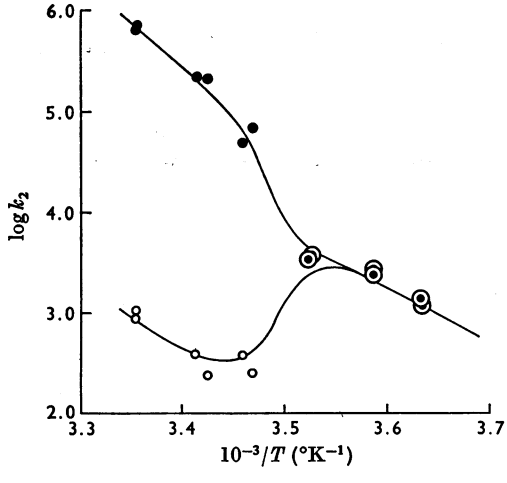


Fig. 7. Effect of temperature on the reactivity of the thiol groups of phosphorylase *b* towards 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole. ●, Rapidly reacting thiol group; ○, slowly reacting thiol groups; ◎, rapidly and slowly reacting thiol groups.

reactivity of these three groups differs sufficiently to resolve them by a mathematical procedure previously outlined (Freedman & Radda, 1968) into one rapidly reacting group ($k = 1001 \cdot mol^{-1} \cdot min^{-1}$) and two more slowly reacting ones ($k = 101 \cdot mol^{-1} \cdot min^{-1}$). This reactivity pattern is dramatically altered by changing the temperature (Fig. 7), so that below 13°C two groups react at a comparable fast rate whereas the reactivity of the third is considerably diminished. This observation, together with the proton-relaxation-enhancement data, demonstrates convincingly that a temperature-dependent conformational equilibrium exists.

Effect of ligands. We are now in the position to study the effect of AMP (the activator) and glucose 1-phosphate (a substrate) on the properties of the enzyme. Table 3 summarizes the effect of these ligands on the ϵ_b, K_D for Mn^{2+} and reactivity of thiol groups of the enzyme. Addition of AMP increases ϵ_b from 10.9 to 14.0 and decreases the reactivity of one (or two) of the slowly reacting thiol groups without affecting the rate of modification of the rapidly reacting one. Although this decrease in reactivity of the thiol group could be a consequence of a direct 'masking' by AMP, the most reasonable explanation of the increase in the proton-relaxation enhancement, ϵ_b is that the metal-ion-binding site has undergone some rearrangement. This conformational change around the Mn^{2+}-binding site is then reflected in a change in τ_f, τ_s or τ_M. The AMP effect on the proton-relaxation enhancement is clearly sigmoidal (Fig. 8), suggesting that there are interactions between the AMP-binding sites. The proton-relaxation enhancement can be taken as a measure of the conformational equilibrium at different ligand concentrations (the $\bar{R}$ function in the terminology of Monod *et al.* 1965). That the effect of AMP on the proton-relaxation enhancement is not a result of enzyme aggregation is supported by several lines of evidence: (1) under conditions where AMP does not change the fluorescence of 2-(*N*-methylanilino)naphthalene-6-sulphonate bound to phosphorylase *b* it does increase ϵ^*; (2) glycogen, which is known to prevent the ligand-induced aggregation (Kastenschmidt, Kastenschmidt & Helmreich, 1968), has very little effect on the AMP-induced proton-relaxation enhancement (Fig. 8); (3) the ligand-induced increase can be observed over a wide range of enzyme concentration and the observed enhancements can all be predicted by using single values for the limiting enhancement and dissociation constants in the ternary complex (i.e. in the enzyme–Mn^{2+}–AMP complex).

In contrast the decrease in ϵ_b on addition of glucose 1-phosphate (Table 3) could be explained on the basis of direct ligand binding at the Mn^{2+}-binding site, leading to a displacement of one or two water molecules from the co-ordination sphere of the bound metal ion. At the same time the most reasonable explanation for the increase in the

reactivity of one thiol group is that the environment of that group has undergone some rearrangement. We may therefore conclude that glucose 1-phosphate also changes the conformation of the enzyme. In fact it is likely that the decrease in ϵ_b is also an indirect ligand effect, since Mn^{2+} does not affect the activity of the enzyme as might have been expected had it been bound at the glucose 1-phosphate-binding (i.e. active) site. [Further arguments to support this have been presented elsewhere (Birkett, 1970; Birkett *et al.* 1971).] If we accept these arguments we must then conclude that in the presence of both ligands the enzyme is in yet another conformation, since addition of AMP to the enzyme in the presence of saturating concentrations of glucose 1-phosphate again gives an additional enhancement in ϵ_b (Table 3).

One can extend these observations now in several directions. First, the thiol group that appears to be in a conformationally mobile region of the enzyme (on the basis of its chemical reactivity) is 'essential' to activity, i.e. the rate of its modification under all conditions parallels the rate of loss of enzyme activity. Secondly, IMP, which is also an activator of the enzyme, has no effect on the proton-relaxation enhancement. The main kinetic difference between AMP and IMP is that, whereas activation by the former involves a change in both K_m and $V_{max.}$ for glucose 1-phosphate in the reaction (Wang & Tu, 1970), the latter alters only $V_{max.}$. We may thus conclude that the proton-relaxation enhancement detects changes that are associated with changes in K_m but not in $V_{max.}$. Thirdly, since modification of the rapidly reacting thiol group by 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole does not affect the activity of the enzyme, by using an excess of AMP we were able to prepare a covalently labelled enzyme (modified at two thiol groups/molecule of enzyme) that is fully active. The fluorescence of this label will also follow ligand binding. In particular the change in fluorescence of the 4-nitrobenzo-2-oxa-1,3-diazole group on AMP addition very closely parallels the changes observed by proton-relaxation enhancement (Fig. 9).

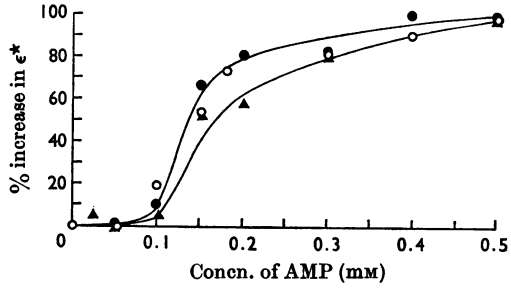


Fig. 8. Effect of AMP on the proton-relaxation enhancement of phosphorylase *b* (1 mg/ml) with Mn^{2+} (1.0 mM). Additions: ▲, none; ○, glucose 1-phosphate (5 mM); ●, glycogen (0.5%).

Several of the changes in probe properties thus reflect conformational changes. This interpretation is strengthened by the observation that ligand binding, in addition to changes in the quaternary structure of the enzyme, results in effects on the physical and chemical properties of the enzyme at three apparently different sites, namely the Mn^{2+}-binding site, the rapidly reacting thiol group and the essential thiol group. Naturally one would like to know how 'extensive' these structural changes are. All the methods we have used are sensitive to relatively minor alterations around the probe, so that the question must reduce to one of relating the different 'sites' for the probes. There are a number of ways to approach this problem, but until now we have looked at only one of these. Fluorescence can be quenched by transition-metal ions (Linschitz & Pekkarinen, 1960). For example, the fluorescence of DNS-glycine is quenched by chelation with Cu^{2+}, Co^{2+}, Ni^{2+} and Mn^{2+}, the quenching efficiency decreasing in that order. We have therefore studied the effect of Mn^{2+} binding on the fluorescence of the covalently labelled 4-nitrobenzo-2-oxa-1,3-diazole derivative of the enzyme (Fig. 10). Mn^{2+} quenches the fluorescence of the 4-nitro-2-oxa-1,3-diazole group linked to phosphorylase *b* by about 12%, whereas it has no effect,

Table 3. *Effects of ligands on phosphorylase* b

Conditions and measurements as described by Birkett (1970) and Birkett *et al.* (1971).

Ligand	ϵ_b	K_D for Mn^{2+} (mM)	Thiol-group reactivity (rate constants): Rapidly reacting group ($l \cdot mol^{-1} \cdot min^{-1}$)	Slowly reacting groups ($l \cdot mol^{-1} \cdot min^{-1}$)	
None	10.9	178	80	10	10
AMP	13.8	152	80	0	0
Glucose 1-phosphate	5.9	91	92	92	3.8
AMP+glucose 1-phosphate	7–10	90	70	0	0

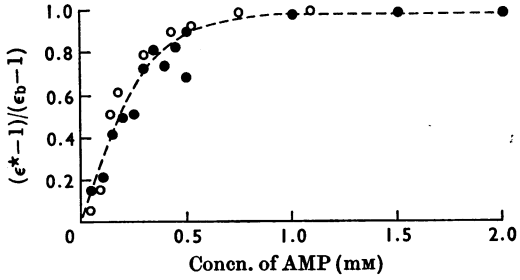


Fig. 9. Effect of AMP on the proton-relaxation enhancement and fluorescence. ○, % of maximal decrease in fluorescence of the 4-nitrobenzo-2-oxa-1,3-diazole derivative of phosphorylase *b*; ●, % of maximal increase in proton-relaxation enhancement.

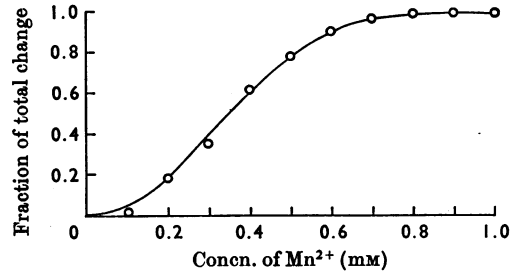
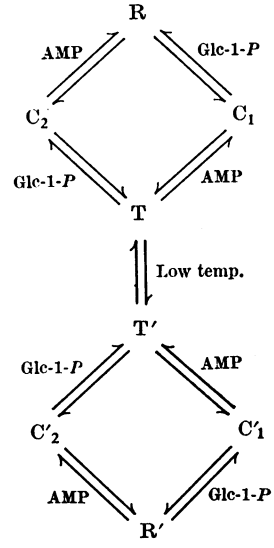


Fig. 10. Quenching of fluorescence of the 4-nitrobenzo-2-oxa-1,3-diazole derivative of phosphorylase *b* by Mn^{2+}. Phosphorylase contains one 4-nitrobenzo-2-oxa-1,3-diazole group/subunit.

even at very high concentrations, on the fluorescence of the 4-nitrobenzo-2-oxa-1,3-diazole derivative of cysteine in solution. Although we do not yet understand in detail the mechanism of this quenching, it probably involves a short-range interaction (due to electron exchange or spin–orbit coupling). This indicates that the Mn^{2+} is bound close (say 5–7 Å) to the rapidly reacting thiol group, which in turn cannot be close to either of the ligand-binding sites, otherwise its reactivity would have been also altered by the ligands. This provides further support for our conclusion that Mn^{2+} does not directly interact with either of the ligands.

On the basis of similar measurements we have shown that the 'low-temperature' form of phosphorylase *b* has a similar set of conformational states superimposed on a different basic form of the enzyme. The conformational equilibria, then, represent a range of ligand-induced states (Scheme 1).



Scheme 1. Schematic diagram for the different conformations of phosphorylase *b*.

Probes for membranes

Mainly as a result of our work on glutamate dehydrogenase involving the use of many different fluorescent probes in detecting ligand-induced conformational changes (Brocklehurst & Radda, 1970), it became apparent that the sensitivity and relative simplicity of the fluorescence method could be exploited in studying more complex biochemical phenomena. In particular the use of probes such as 1-anilinonaphthalene-8-sulphonate offered the possibility of testing whether conformational changes are involved in the energy-transducing system in mitochondria.

1-Anilinonaphthalene-8-sulphonate interacts with erythrocyte and mitochondrial membranes giving a large fluorescence enhancement (Freedman & Radda, 1969; Azzi, Chance, Radda & Lee, 1969; Brocklehurst *et al.* 1970). For our discussion here I would like to begin with the observation that the fluorescence of 1-anilinonaphthalene-8-sulphonate can be used to follow 'energization' of fragmented mitochondrial membranes (Azzi *et al.* 1969; Brocklehurst *et al.* 1970). This is illustrated in Fig. 11 (taken from Lee & Radda, 1970), where it can be seen that energy can be supplied to the electron-transfer chain by several different substrates or by ATP. In each case the fluorescence of the probe is enhanced, provided that the membrane preparation is coupled (e.g. carbonyl cyanide *p*-trifluoromethoxyphenylhydrazide abolishes these responses). Thus the 1-anilinonaphthalene-8-sulphonate response can be said to be energy-dependent. The following facts are necessary for our subsequent discussion [the evidence for these has been presented in detail elsewhere (Brockle-

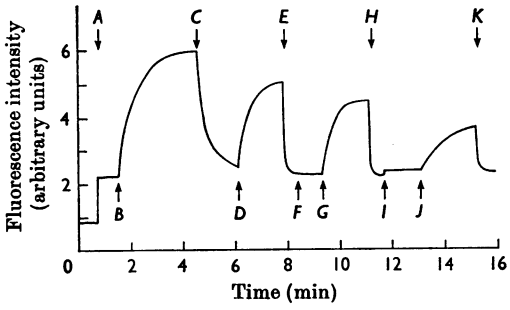


Fig. 11. 1-Anilinonaphthalene-8-sulphonate responses in submitochondrial particles energized by different substrates in 170mM-sucrose in 30mM-tris–acetate buffer, pH7.5. Additions: *A*, 1-anilinonaphthalene-8-sulphonate (5μM); *B*, NADH (0.4mM); *C*, rotenone (3.3μM); *D*, tris succinate (5mM); *E*, antimycin A (3μg); *F*, ascorbate (10mM); *G*, phenazine methosulphate (1.7μM); *H*, KCN (5mM); *I*, $MgSO_4$ (6.7mM); *J*, ATP (3mM); *K*, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazine (1μM). [Taken from Lee & Radda (1971); I am most grateful to Professor C. P. Lee for permission to use this figure.]

hurst *et al.* 1970)]. (1) The energy-linked fluorescence change is partly a result of a twofold increase in fluorescence quantum yield of the bound probe and partly due to increased binding of the probe to the energized membrane. (2) There are at least two kinetically distinguishable binding sites for 1-anilinonaphthalene-8-sulphonate on the mitochondrial membrane: those to which the probe binds in less than 10ms (the 'fast' sites) and those that are less exposed ('slow' sites). All the energy-dependent 1-anilinonaphthalene-8-sulphonate response is associated with the 'slow' sites. (3) The polarization of fluorescence of 1-anilinonaphthalene-8-sulphonate in membranes is about 0.2, suggesting that the probe has considerable rotational mobility in the 'bound' state. (4) Electronic energy transfer from tryptophan residues in the membrane to the probe can be observed. By measuring this at a series of probe concentrations we can derive, by extrapolation, the average tryptophan–1-anilinonaphthalene-8-sulphonate distance at saturating probe concentrations. This distance was found to be 18Å. This indicates that at least some probe-binding sites are close to the membrane protein. (5) By following the kinetics of the decrease in fluorescence of the probe after the addition of an uncoupler to the energized membranes it is possible to separate two processes. The initial fast phase has a half-time of 2–3s and is likely to be associated with a change around the binding site for the probe. The slow phase is a result of the effusion of the probe from the membrane (Brocklehurst *et al.* 1970; Radda, 1971).

Fluorescent probes in lipids. In order to gain further insight into the types of structural changes

Table 4. *Interaction of* Mn^{2+} *with lipid micelles followed by proton-relaxation enhancement*

Dispersions were in iso-osmotic NaCl, buffered at pH7.0 with 10mM-tris–HCl.

Lipid	K_D (mM)	ϵ_b
Ovolecithin	1.4	2.7
(Dipalmitoyl) lecithin	3.2	30
(Dipalmitoyl) lecithin + cholesterol (7:3 molar ratio)	1.8	8

in membranes that we can detect by the use of fluorescent probes we have studied a variety of lipid micelles (Radda & Smith, 1970). Here I would like to illustrate some of our conclusions and hypotheses by asking a simple question: what can fluorescence probes tell us about the effect of cholesterol on a lipid micelle?

The fluorescence of retinol is enhanced by lipids [e.g. ovolecithin (phosphatidylcholine) micelles] and membranes (erythrocyte stroma). The enhancement is larger when cholesterol is added to ovolecithin (Radda & Smith, 1970). On the basis of the theory I presented above we can say that the 'environmental constraint' increased when cholesterol was present. Since retinol is only slightly soluble in water but is soluble in hydrocarbons we can say that the hydrocarbon region of the lipid micelle became more rigid. This was an unexpected finding because of the surface-viscosity measurements made by Joos (1970) and because of our own observations on the proton-relaxation enhancement of water molecules by lipid-bound Mn^{2+}. We found that the limiting enhancement, ϵ_b, falls when cholesterol is added to a lipid micelle (ϵ_b is also very sensitive to the nature of the micelle) (Table 4). We interpreted these observations by assuming that the bound Mn^{2+} was more mobile in the presence of cholesterol. This would imply an expansion of the micelle in the presence of cholesterol. To resolve these apparent discrepancies we have measured the fluorescence spectra, intensities, lifetimes and polarizations of a series of probes in ovolecithin dispersions with and without cholesterol. The results are summarized in Fig. 12. Retinol shows an enhanced quantum yield when cholesterol is present and a decreased rotational mobility. 3-Dimethylaminonaphtheurhodine, which is a lipid-soluble 'polarity' probe, shows no significant change in its fluorescence intensity or spectrum, but also exhibits a decreased rotational mobility after addition of cholesterol. The most interesting observation is the contrasting behaviour of *N*-1-naphthyl-*N*-phenylamine and 1-anilinonaphthalene-8-sulphonate, both of which can be regarded as probes for polarity as well as for constraint. *N*-1-Naphthyl-*N*-phenylamine, which is also lipid-soluble, shows a behaviour similar to that of retinol,

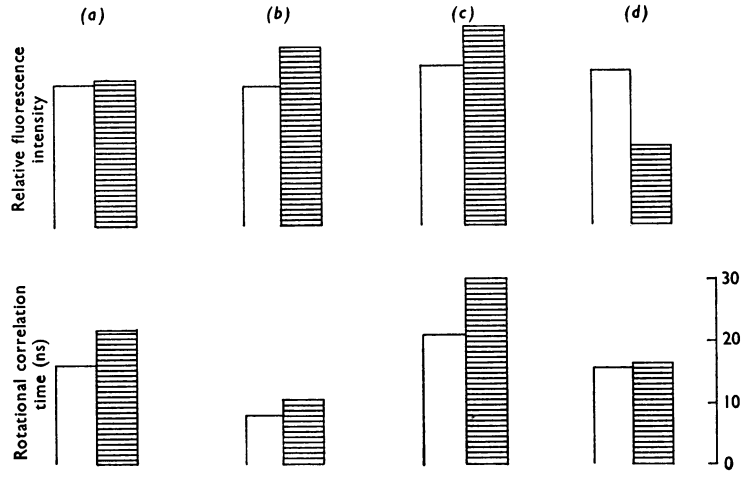


Fig. 12. Intensities and rotational correlation times of different probes in lecithin micelles with and without added cholesterol. (*a*) 3-Dimethylaminonaphtheurhodine; (*b*) *N*-1-naphthyl-*N*-phenylamine; (*c*) retinol; (*d*) 1-anilinonaphthalene-8-sulphonate. ▤, Lecithin+cholesterol (7:3 molar ratio); □, lecithin alone. Rotational correlation times, ρ, were calculated from the expression:

$$\frac{1}{p} - \frac{1}{3} = \left(\frac{1}{p_0} - \frac{1}{3}\right)\left(1 + \frac{3\tau}{\rho}\right)$$

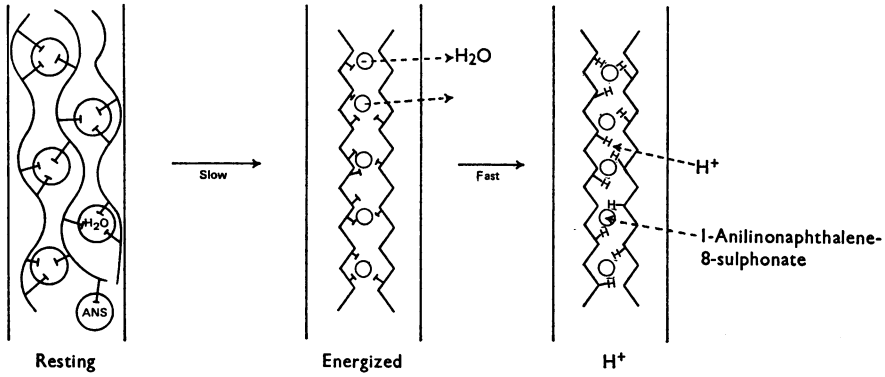
Fluorescence polarizations and lifetimes were measured as described by Radda & Smith (1970).

whereas the fluorescence intensity and lifetime of 1-anilinonaphthalene-8-sulphonate decreases by 30% in the presence of cholesterol without any measurable change in its emission spectrum or rotational mobility. The most self-consistent interpretation of all these observations is as follows. Addition of cholesterol does indeed lead to a decreased mobility in the hydrocarbon region of the lipid micelle but not to a significant change in the polarity of that environment. At the same time the interface region of the micelle is opened up so as to allow more water molecules to penetrate this part of the structure. This causes a decrease in the fluorescence intensity of 1-anilinonaphthalene-8-sulphonate by collisional quenching, which will not affect the emission spectrum of this probe. Thus our previous conclusion (Brocklehurst *et al.* 1970) that an amphiphilic molecule like 1-anilinonaphthalene-8-sulphonate probes events at polar/non-polar interfaces is reinforced.

Model for the energy-dependent 1-anilinonaphthalene-8-sulphonate response. I would like to propose a model that accounts for the energy-dependent probe response in submitochondrial particles and that also takes into account our observations with lipid micelles. The energy-dependent 'slow' 1-anilinonaphthalene-8-sulphonate-binding sites are at a polar/non-polar interface inside the membrane and this interface contains 'membrane-bound' water (possibily in a structured form) (Scheme 2). Now, if energization leads to a conformational change in the membrane proteins leading to a more ordered structure (i.e. the number of protein–protein internal hydrogen bonds, as opposed to hydrogen bonds with water, increases) water will be released from the membrane. Such loss of water would change the pK_a of some negatively charged groups (e.g. $-CO_2^-$) at the interface so that H^+ will move in (the net result is OH^- moving out). Because of the neutralization of some of the membrane negative charge more 1-anilinonaphthalene-8-sulphonate will move in slowly as a result of increased affinity for this negative probe by the membrane. The loss of water from the interface will result in an increase in the quantum yield of fluorescence by 1-anilinonaphthalene-8-sulphonate.

We can now test this model in several ways.

(1) The fluorescence properties of the amphiphilic molecule pyrene-3-sulphonate are very different from those of 1-anilinonaphthalene-8-sulphonate in that they are polarity-insensitive but do depend on the concentration and mobility of the molecule. This property arises because of the long fluorescence lifetime (80ns), which allows the formation of short-lived dimers between a molecule in its excited state and one in its ground state (excimers). Whereas the monomer emits maximally at 420nm, the excimer emission is at 500nm. Such excimer emission is observable when pyrene-3-sulphonate



Scheme 2. Proposed scheme for energy-dependent 1-anilinonaphthalene-8-sulphonate response.

Table 5. *Solvent isotope effects on the fluorescence of different probes*

Lecithin micelles were in 10mM-tris–HCl, pH or pD 7.0, in H_2O or D_2O. Submitochondrial particles were buffered as described by Brocklehurst *et al.* (1970). (In D_2O solutions the buffering salts and particles used were not exchanged with deuterium before the solutions were made up. The values for the solvent isotope effect therefore represent a lower limit only.) Abbreviations: ANS, 1-anilinonaphthalene-8-sulphonate; MNS, 2-(*N*-methylanilino)naphthalene-6-sulphonate.

System	Solvent isotope effect [(fluorescence intensity in D_2O)/(fluorescence intensity in H_2O)]	% 'exposure' to water
ANS in water	2.5	100 (by definition)
MNS in water	2.2	100
N-1-Naphthyl-*N*-phenylamine in water	2.0	100
ANS in lecithin micelles	1.9	60*
MNS in lecithin micelles	1.4	34*
N-1-Naphthyl-*N*-phenylamine in lecithin micelles	1.05	7*
ANS in resting submitochondrial particles	1.6	—
ANS in energized submitochondrial particles	1.5	—

* Allowance was made in these calculations for changes in the quantum yields on interaction with the lipid.

interacts with submitochondrial particles and is enhanced (at the expense of monomer emission) in the energized state (Brocklehurst *et al.* 1970). This enhancement is again partly a result of increased binding of the probe and partly due to increased excimer emission. Detailed arguments have been presented why the latter part of the enhancement can be taken as a result of a decrease in volume within a restricted region of the membrane that is only slowly accessibly to the probe. Such a decrease in volume is entirely consistent with our hypothesis involving water movement.

(2) We can also test the question whether membrane-bound 1-anilinonaphthalene-8-sulphonate molecules come in contact with water. The fluorescence of 1-anilinonaphthalene-8-sulphonate, 2-(*N*-methylanilino)naphthalene-6-sulphonate and *N*-1-naphthyl-*N*-phenylamine is enhanced about twofold in D_2O compared with H_2O. (The exact mechanism of quenching by H_2O is immaterial to our arguments.) Table 5 shows that the fluorescence intensities of 1-anilinonaphthalene-8-sulphonate and 2-(*N*-methylanilino)naphthalene-6-sulphonate have large solvent isotope effects even when they are bound to lipid micelles or submitochondrial particles and that the energy-dependent enhancement is also solvent-sensitive. On the other hand *N*-1-naphthyl-*N*-phenylamine (which shows no energy-dependent response) is not exposed to water either in the lipid or in the membrane. These observations provide further support for the conclusion that the amphiphilic molecules are at an aqueous/lipid interface but the neutral molecule is buried inside the non-polar region of the membrane. A further corollary is that water is present at the energy-dependent 1-anilinonaphthalene-8-sulphonate-binding sites in mitochondrial fragments.

Some further speculations and problems. It is perhaps a sad trend in modern scientific writing that stringent editorial policies and demand on journal space do not allow the publication of much

speculative material. No editor these days would allow Kekulé to publish his dreams about oscillating double bonds in benzene, and science would be the worse for it. It is only on pleasant occasions like this that one can allow oneself to present some speculations. Our ideas about the energized membrane must be regarded as a working hypothesis. There are many problems. We do not know if the probe only penetrates the membrane up to a permeability barrier. This would be necessary to account for the sidedness in probe response observed by others (Azzi, 1969). We cannot exclude the possibility at present that the probe moves within the membrane from one region to another, as has been suggested for the energy-dependent changes in Bromothymol Blue spectra (Mitchell, Moyle & Smith, 1968). We have several arguments against this on the basis of our previous work (Brocklehurst *et al.* 1970) and on the basis of the solvent isotope effect. Do we regard the energized state as detected by the probes as an intermediate in oxidative phosphorylation? Not at present. It is a state in which coupling can occur more readily as would be required by the Williams (1969) hypothesis. The role of water in ATP formation has recently been emphasized in a detailed account on 'squiggle-H_2O' by George *et al.* (1970). So, there are many problems, but what we have learnt is that we are now able to design probes for enzymes and membranes to answer specific questions (Radda, 1971).

The results on phosphorylase were obtained by Dr Birkett, Dr Dwek and Mr Salmon. Those on membranes and models were obtained by, in addition to the authors quoted in the references, Mr J. A. Taylor and Mr S. G. Ballard. I cannot thank individually all the people who so ably collaborated in the work I have described, but in my view joint authorship is the best way to give equal credit to all participants. I would like to express my thanks to Professor Britton Chance; our collaboration with the Johnson Research Foundation has been most interesting. I am also very grateful to Dr R. E. Richards for his help and advice on the n.m.r. experiments. Much of the work described has been supported by the Science Research Council.

REFERENCES

Azzi, A. (1969). *Biochem. biophys. Res. Commun.* **37**, 254.
Azzi, A., Chance, B., Radda, G. K. & Lee, C. P. (1969). *Proc. natn. Acad. Sci. U.S.A.* **62**, 612.
Bayley, P. M. & Radda, G. K. (1966). *Biochem. J.* **98**, 105.
Bayliss, N. S. (1950). *J. chem. Phys.* **18**, 292.
Becker, R. S. (1969). *Theory and Interpretation of Fluorescence and Phosphorescence*, p. 35. New York: Wiley–Interscience.
Birkett, D. J. (1970). D.Phil. Thesis: University of Oxford.
Birkett, D. J., Dwek, R. A., Radda, G. K., Richards, R. E. & Salmon, A. G. (1971). *Eur. J. Biochem.* (in the Press).
Birkett, D. J., Freedman, R. B., Price, N. C. & Radda, G. K. (1970*a*). *Biochem. Soc. Symp.* no. 31, p. 147.
Birkett, D. J., Price, N. C., Radda, G. K. & Salmon, A. G. (1970*b*). *FEBS Lett.* **6**, 346.
Birkett, D. J., Radda, G. K. & Salmon, A. G. (1970*c*). *FEBS Lett.* **11**, 295.
Blake, C. C. F., Johnson, L. N., Mair, G. A., North, A. C. T., Phillips, D. C. & Sarma, V. R. (1967). *Proc. R. Soc.* B, **167**, 378.
Brocklehurst, J. R., Freedman, R. B., Hancock, D. J. & Radda, G. K. (1970). *Biochem. J.* **116**, 721.
Brocklehurst, J. R. & Radda, G. K. (1970). In *Probes for Membrane Structure and Function*. Ed. by Chance, B., Lee, C. P. & Yonetani, T. New York: Academic Press Inc. (in the Press).
Cohn, M. (1963). *Biochemistry, Easton*, **2**, 623.
Dalle, J. P. & Rosenberg, B. (1970). *Photochem. Photobiol.* **12**, 151.
Dodd, G. H. & Radda, G. K. (1967). *Biochem. biophys. Res. Commun.* **27**, 500.
Dodd, G. H. & Radda, G. K. (1969). *Biochem. J.* **114**, 407.
El-Bayoumi, M. A., Dalle, J. P. & O'Dwyer, M. F. (1970). *J. Am. chem. Soc.* **92**, 3494.
Freedman, R. B. & Radda, G. K. (1968). *Biochem. J.* **108**, 383.
Freedman, R. B. & Radda, G. K. (1969). *FEBS Lett.* **3**, 150.
George, P., Witonsky, R. J., Trachtman, M., Wu, C., Dorwart, W., Richman, L., Shurayh, F. & Lentz, B. (1970). *Biochim. biophys. Acta*, **223**, 1.
Green, D. E., Asai, J., Harris, R. A. & Penniston, J. T. (1968). *Archs Biochem. Biophys.* **125**, 684.
Joos, P. (1970). *Chem. Phys. Lipids*, **4**, 162.
Kastenschmidt, L. L., Kastenschmidt, J. & Helmreich, E. (1968). *Biochemistry, Easton*, **7**, 3590.
Koshland, D. E., jun. (1959). *J. cell. comp. Physiol.* **54**, 235.
Lee, C. P. & Radda, G. K. (1970). In *Energy Transduction in Respiration and Photosynthesis* (*Colloquium on Bioenergetics, Pugnochioso*). Bari: Adriatica Editrice (in the Press).
Linschitz, H. & Pekkarinen, L. (1960). *J. Am. chem. Soc.* **82**, 2411.
Mildvan, A. S. & Cohn, M. (1970). *Adv. Enzymol.* **33**, 1.
Mitchell, P., Moyle, J. & Smith, L. (1968). *Eur. J. Biochem.* **4**, 9.
Monod, J., Wyman, J. & Changeux, J.-P. (1965). *J. molec. Biol.* **12**, 88.
Onsager, L. (1936). *J. Am. chem. Soc.* **58**, 1486.
Perutz, M. F. (1970). *Nature, Lond.*, **228**, 726.
Radda, G. K. (1971). *Curr. Topics Bioenerget.* **4**, (in the Press).
Radda, G. K. & Smith, D. S. (1970). *FEBS Lett.* **9**, 287.
Reek, G. N., Hartsuck, J. A., Ludwig, M. L., Quiocho, F. A., Steitz, T. A. & Lipscomb, W. N. (1967). *Proc. natn. Acad. Sci. U.S.A.* **58**, 2220.
Strickler, S. J. & Berg, R. A. (1962). *J. chem. Phys.* **37**, 814.
Tasaki, I., Watanabe, A., Sandlin, R. & Carnay, L. (1968). *Proc. natn. Acad. Sci. U.S.A.* **61**, 883.
Thomson, A. J. (1969). *J. chem. Phys.* **51**, 4106.
Wang, J. H. & Tu, J.-I. (1970). *J. biol. Chem.* **245**, 176.
Williams, R. J. P. (1969). *Curr. Topics Bioenerget.* **3**, 79.