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The Catalase–Hydrogen Peroxide System

A THEORETICAL APPRAISAL OF THE MECHANISM OF CATALASE ACTION

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1. The mechanisms of catalase action advanced by Jones & Wynne-Jones (1962) and by Nicholls (1964) are compared in terms of their relative plausibilities and their utility for extension to accommodate more recent experimental information. 2. A revised formal mechanism is advanced that avoids the less satisfactory features of these mechanisms and attempts to account for the roles of catalase sub-units in both reversible and irreversible deactivation phenomena. 3. Theoretical studies of the redox chemistry of peroxides are used to provide the basis for a discussion of the mechanism of the redox act in catalatic action at the molecular level. It is suggested that an important feature of catalase action may be a mediation of the formation of a reactive intermediate by stereospecifically located acid–base functions in the active site. 4. A more detailed statement of this concept is attempted in terms of a hypothetical partial molecular model for the composition and stereochemistry of the active site of catalase. The utility of this model in describing the catalatic and peroxidatic actions of catalase is assessed.

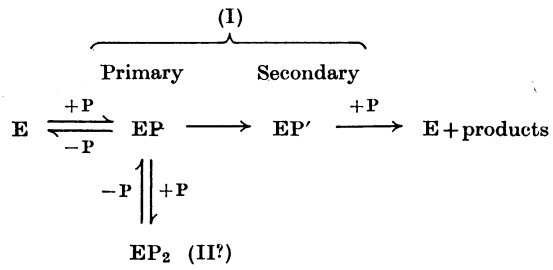
Recent experimental studies of the catalase–hydrogen peroxide system (Jones & Suggett, 1968*a*,*b*; Jones, Pain & Suggett, 1968) have revealed new phenomena for which existing theories of catalase action do not account. In particular, this work has shown that reversibly formed catalase sub-units play important roles in catalase action at physiological pH.

Jones & Wynne-Jones (1962) proposed a mechanism for catalase action that attempted to account for the data available at that time. It was shown that the mechanism could also comprehend the available information on the catalytic action of Fe^{3+} ion, both in the decomposition of hydrogen peroxide and as a mediator of coupled 'peroxidatic' oxidations. Nicholls (1964) rejected the Jones & Wynne-Jones (1962) scheme for catalase action and put forward an alternative model. Nicholls's (1964) model implies that the mechanisms by which catalase and Fe^{3+} ion bring about the mutual oxidation and reduction of two molecules of hydrogen peroxide differ qualitatively.

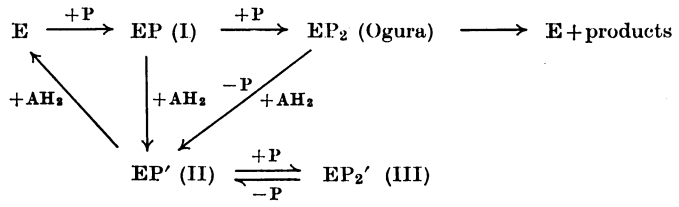
In section (i) of the Theory section of the present paper the Jones–Wynne-Jones and Nicholls schemes are compared both as mechanistic models and in terms of their implications at the molecular level. The purpose of this comparison is to assess the relative plausibilities of the two schemes and their utility for extension to accommodate more recent experimental information. In section (ii) a model is proposed that attempts to comprehend the data currently available, and tests are suggested that could be of value in resolving areas of uncertainty.

Attempts to discuss the mechanism of catalase action at the molecular level are restricted by the lack of a detailed knowledge of the structure of the enzyme itself. Although much work has been done on the formal characterization of intermediates (the catalase 'Compounds'), little theoretical attention has been paid to the fate of the two molecules of hydrogen peroxide that are involved in catalatic action. In section (iii) ideas deriving from a molecular-orbital study of the redox chemistry of peroxides (Jones & Perkins, 1967) are used to suggest a formal model for the manner in which the catalase protein may be involved in facilitating catalatic action. A more explicit molecular statement is then attempted in terms of a model for the composition and stereochemistry of the active site of catalase. Although this model cannot be regarded as definitive, it is remarkably successful in accounting for the phenomena of catalatic action and it suggests that the extraordinary catalytic efficiency of the enzyme derives from the stereospecific location of appropriate acid–base functions in the active site. In section (iv) the implications of the model in the context of 'peroxidatic' oxidations by catalase–peroxide systems are discussed.

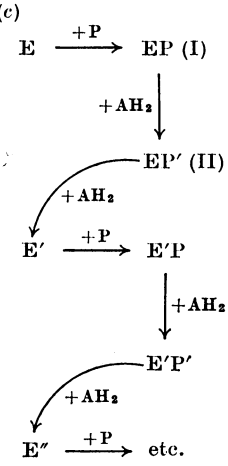
(*a*) Jones–Wynne-Jones mechanism



(*b*) Nicholls mechanism



(*c*)



Scheme 1. Comparison of mechanisms proposed for catalase action.

THEORY

(i) *Comparison of the Jones–Wynne-Jones and Nicholls mechanisms.* The two schemes are represented in Schemes 1(*a*) and 1(*b*) respectively. Before attempting a comparison, some clarifications of terminology and principle are required. Nicholls (1964) takes the view that his mechanism is an extension of the 'peroxidatic theory' of catalase action (Chance, Greenstein & Roughton, 1952), whereas the Jones–Wynne-Jones theory cannot be so described. On the contrary, both mechanisms contain the essential concept of 'peroxidatic theory', which is that the mutual oxidation and reduction of hydrogen peroxide in catalatic action is a two-electron-equivalent redox process in which a catalase–peroxide 'compound', containing both oxidizing equivalents of its parent peroxide molecule, oxidizes a second hydrogen peroxide molecule to oxygen. 'Peroxidatic' oxidations occur via a competition between hydrogen peroxide and other oxidizable species for this oxidizing 'compound'.

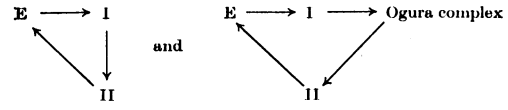
Nicholls (1964) has commented that the extension of inorganic theories to cover catalase action is dangerous. In our view it is important to enquire whether the mechanisms of catalytic decomposition of hydrogen peroxide by catalase and Fe^{3+} ion differ qualitatively or essentially quantitatively; indeed,

such enquiries are important for the development of catalytic theory. The Jones–Wynne-Jones mechanism is not an 'inorganic theory', nor is Nicholls's mechanism a 'biochemical theory'; both are (rather crude) models that must be tested both by comparison with experiment and by consideration of their inherent plausibilities.

The catalatic pathway is represented by the upper horizontal flow-line in both mechanisms in Schemes 1(*a*) and 1(*b*). Here Nicholls follows Chance *et al.* (1952) and Ogura (1955) in supposing that the key oxidizing entity (Compound I) is formed in a single step from catalase and hydrogen peroxide and that the redox step occurs in a ternary complex (Ogura complex). It is an essential feature of the scheme that both complex-formation steps occur substantially irreversibly. The Jones–Wynne-Jones scheme has a more conventional enzyme-kinetic form in supposing the reversible formation of a primary complex that undergoes an essentially irreversible transformation into a secondary complex before taking part in the redox act. Thus, in this model, 'Compound I' is regarded as a steady-state mixture of two species. Although the direct kinetic and spectrophotometric data do not exclude either formulation, important indirect support for the Jones–Wynne-Jones model comes from the detailed analysis of the spectra of catalase derivatives by Brill & Williams (1961), in which they concluded that 'Compound I' is a steady-state mixture of two species. Further support comes from the data obtained by Strother & Ackerman (1961), who found that, whereas the rate constant for the reaction of 'Compound I' with peroxide showed a normal Arrhenius temperature-dependence, the rate constant for the formation of 'Compound I' showed a complex temperature variation. This suggests that the formation of 'Compound I' is kinetically complex. The large change in absorption spectrum accompanying the formation of 'Compound I' indicates a substantial electronic rearrangement. Such a process would more plausibly occur with a high overall rate constant via a sequential pathway rather than in a single step. The Jones–Wynne-Jones formulation is therefore retained in developing a revised formal mechanism in section (ii) and in considering an interpretation at the molecular level in section (iii).

The reactions that are supposed to be responsible for reversible deactivation are represented as branches from the main catalatic pathways in Scheme 1. The Jones–Wynne-Jones mechanism has a single branch where deactivation is accounted for by the slow reversible formation of a diperoxy-catalase species from the primary complex. Nicholls's scheme is more complex and involves the formation of two inactive species, Compounds II and III. The concept of two inactive species is the more satisfactory in the light of our studies of the kinetics of the effect of reaction time on catalase kinetics at high substrate concentrations (Jones & Suggett, 1968*b*), since one must account both for the apparent change in K_m with time and for the reversible inhibition by excess of substrate in the β-activity phase of catalatic action.

However, there are serious objections to the manner in which these processes are supposed to occur in Nicholls's mechanism. The reactions by which Compound II is formed from Compound I and by which catalase is regenerated from Compound II are both supposed to be one-electron-equivalent redox steps involving an 'endogenous donor' present in the catalase molecule. The introduction of this hypothetical species superficially avoids violation of the Principle of Microscopic Reversibility in the loops represented by:

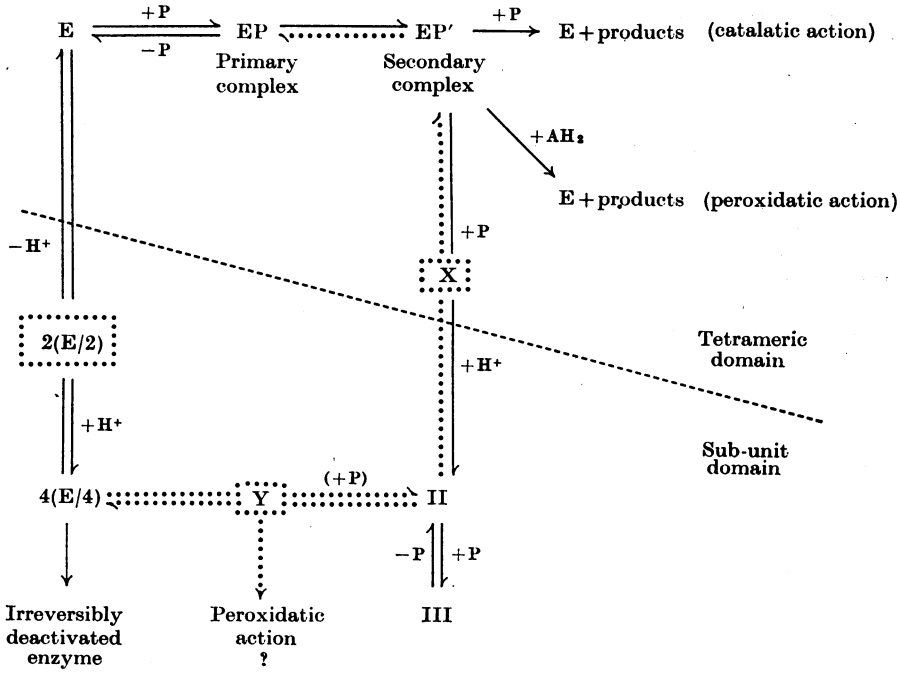


There is, however, a logical flaw in the argument. If the donor is truly 'endogenous', then native catalase cannot be regenerated by the above pathways, but the end product must involve successive steps of chemical modification of the enzyme for each pass through the 'loops'. The changes cannot be either cyclic or reversible (without further assumption) and a more accurate representation of the first 'loop' is shown in Scheme 1(*c*). Nicholls's kinetic scheme can therefore only be maintained with the aid of further assumptions of the type:

$$\begin{aligned} \mathrm{E} &\equiv \mathrm{E}' \equiv \mathrm{E}'' \equiv \ldots\ldots \\ \mathrm{EP} &\equiv \mathrm{E}'\mathrm{P} \equiv \mathrm{E}''\mathrm{P} \equiv \ldots\ldots \\ &\qquad\qquad\qquad\qquad \text{etc.} \end{aligned}$$

where the equivalence signs indicate the assumptions that successive stages of chemical modification of catalase and the reaction intermediates alter neither their kinetic behaviour nor their spectroscopic properties. It is not possible to dismiss hypotheses of this type entirely, but their plausibility diminishes sharply with increase in the weight of assumption necessary to sustain them.

At the molecular level further difficulties with this model appear. If the 'endogenous donors' are assumed to lie so close to the active site that the redox changes may occur directly, then gross changes in chemical composition very near the active site must be presumed to have no effect on catalase activity. If the 'endogenous donors' are assumed to be remote from the active site then one-electron equivalents of oxidation must be transmitted either through the protein matrix or, as free radicals, through the solution.



Scheme 2. Revised formal mechanism for catalase action.

We conclude that the 'endogenous-donor' hypothesis is unlikely to be of value in developing a revision of the mechanism of catalase action.

(ii) *Revised formal mechanism of catalase action.* The model proposed, in which Compounds I, II and III have the usual Chance connotation, is shown in Scheme 2 and has the following features:

(*a*) The main catalatic pathway is shown by the upper horizontal flow-line. The scheme follows the Jones–Wynne-Jones mechanism and assigns catalatic behaviour to the tetrameric domain of catalase behaviour (but see *b*). The species EP′ is considered to be derived from the primary enzyme–substrate complex by some intramolecular rearrangement.

(*b*) The left-hand vertical flow-line shows the protonation-induced dissociation of catalase into four sub-units with subsequent irreversible deactivation of the sub-units (Jones & Suggett, 1968*a*). A dimeric species (E/2) is tentatively indicated in the dissociation process. This suggestion meets the requirements of the studies made by Schütte, Steinbrecht & Winder (1960), whose results also suggest that a dimer may be the essential catalatic unit. In view of our results on the peroxide-induced re-formation of active catalase from sub-units (Jones & Suggett, 1968*a*) we have not included a separate catalatic pathway at the dimeric sub-unit level.

(*c*) The major reversible deactivation pathways in the presence of substrate are represented by the right-hand vertical flow-line. This represents the formation of Compound II as a transition from the tetrameric to the sub-unit domain (Jones *et al.* 1968) and as a process in which a molecule of hydrogen peroxide reacts with the tetrameric secondary complex (Jones & Suggett, 1968*b*). Direct studies of the kinetics of Compound II formation have revealed a complex situation (Chance, 1949) and these results are not accounted for by existing models for the process. Thus Nicholls (1963) has proposed a scheme in which the transition from Compound I to Compound II is described by a single rate constant, which is independent of substrate concentration in some circumstances but not in others. This is unsatisfactory. The implications of the results are either that there is a single complex pathway (more than one step), or that there are at least two independent pathways. The scheme proposed by Nicholls (1964) and shown in Scheme 1(*b*) suggests a second pathway for Compound II formation via the 'Ogura ternary complex'. The problems arising from the invocation of 'endogenous donor' in these schemes have been discussed above; it should also be pointed out that they do not comprehend the observed effect of pH.

In Scheme 2 it is suggested that, at low substrate

concentrations (as when hydrogen peroxide is generated by the glucose oxidase system), Compound II is largely formed from monomeric sub-units (E/4 species) in a reaction with peroxide at a rate that is controlled by the protonation-induced dissociation of catalase. At higher substrate concentrations the enzyme dissociation is displaced towards the tetrameric domain (Jones & Suggett, 1968*a*) and the second pathway becomes important. In the latter the rate of Compound II formation is controlled by the rate of reaction of the secondary complex with peroxide. It is suggested that this results in a tetrameric intermediate (X), which undergoes rapid protonation and dissociates to yield Compound II. The species X is, in a sense, a diperoxy-catalase derivative, in that the secondary complex contains both oxidizing equivalents and probably the components (Chance & Schonbaum, 1962) of its parent peroxide molecule. It seems possible that the second peroxide molecule is bound at a different haem site from the first and that this process destabilizes tetrameric catalase with respect to protonation-induced dissociation.

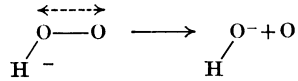
Catalase Compound III is a less well characterized species, which is reportedly formed by the reversible binding of hydrogen peroxide to Compound II, and this is formally indicated in Scheme 2. It is possible that Compound III may result from the binding of peroxide by haem groups liberated in Compound II formation. An alternative possibility is that its formation results from a reversible displacement by peroxide of the protein bound to the fifth co-ordination position of an iron atom in a monohaem sub-unit species.

It has been proposed by a number of workers that Compound II is an Fe(IV) species, the evidence deriving mainly from titration of its oxidizing power. Evidence obtained in a similar manner was held for a number of years to support the formulation of peroxidase Compound II as an Fe(IV) species. The spectra of catalase Compound II and peroxidase Compound II are strikingly similar. The reinvestigation of peroxidase Compound II by Yonetani (1965) has shown the Fe(IV) formulation to be unsound and suggests an urgent need for reappraisal of the catalase system. In the latter case experiments would undoubtedly be complicated by sub-unit interactions and it seems unlikely that a study of gross properties would yield an unequivocal result. It is perhaps worth pointing out that a diperoxy derivative of tetrameric catalase is formally an Fe(IV) species.

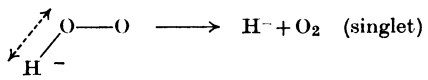
(iii) *Partial molecular model for catalase action.* The detailed molecular structure of catalase is unknown and thus the development of a definitive molecular mechanism for catalase action is precluded. Nevertheless, it is pertinent to enquire whether the available information suggests a likely molecular explanation of the extraordinary catalatic power of this enzyme. Jones & Wynne-Jones (1962) argued that there was substantial support for the thesis that catalatic action and Fe^{3+} ion catalysis are qualitatively similar processes, and more recent work (Kremer, 1965; Brown & Jones, 1968) both lends support to this argument and suggests the inclusion of haemin in this comparison. We do not propose to develop this argument here and the present discussion is restricted to experimental information deriving directly from studies of catalase action and to the results of theoretical studies of the redox chemistry of peroxides.

In a theoretical investigation, using the Pople–Segal SCF–MO method (Pople & Segal, 1965), Jones & Perkins (1967) computed the variation of electronic charge density across the ground-state energy surface of the HO_2^- ion. The results may be summarized as follows:

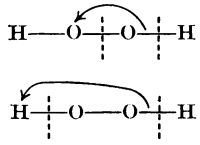
(*a*) Extension of the O–O bond in HO_2^- leads to negative charge accumulation on the OH group and ultimately to the process:



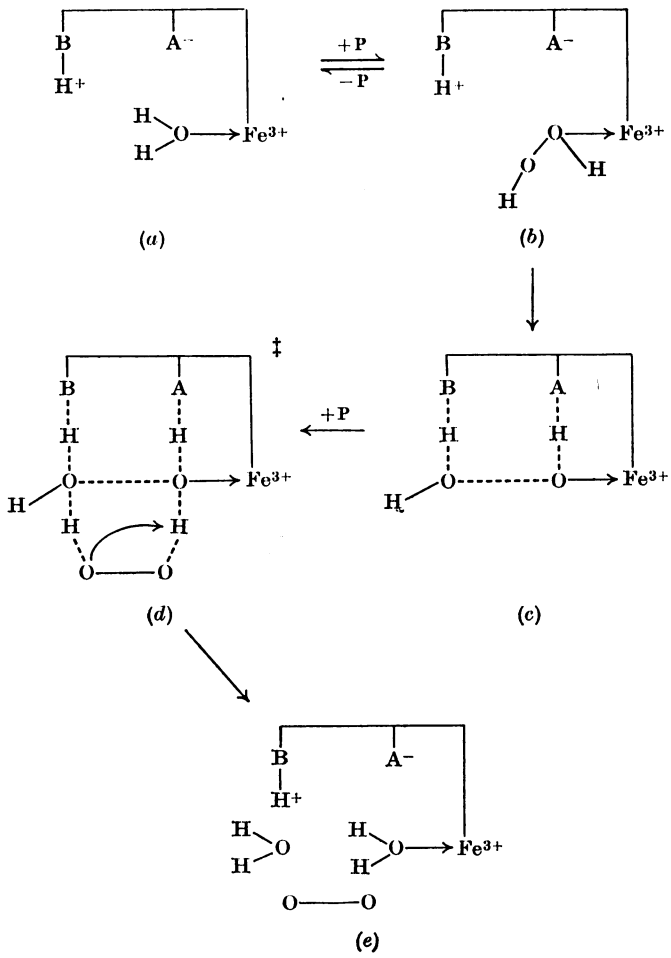
(*b*) Extension of the O–H bond in HO_2^- leads to negative charge accumulation on H and ultimately to the process:



These results suggest that HO_2^- may act as an oxygen-atom-transfer oxidizing agent and as a hydride-ion-transfer reducing agent. There is substantial support in the peroxide literature for the occurrence of both types of process (see Jones & Perkins, 1967). Although these results find direct application to Fe^{3+} ion and haemin catalysis, where reaction via HO_2^- complexes is implicated, the evidence is that catalase utilizes molecular hydrogen peroxide. For this situation Jones & Perkins (1967) suggested that a coupling with proton transfer might be involved:



Thus, in its oxidizing action, hydrogen peroxide may undergo either a two-step dehydration (via HO_2^-), or a one-step dehydration via a 'concerted' acid–base action. Edwards, Di Prête, Curci & Modena



Scheme 3. Hypothetical scheme for the role of catalase protein in facilitating catalatic action.

(1968) have elegantly demonstrated that the latter type of process occurs, in hydrogen peroxide oxidation of organic sulphides to sulphoxides, by the mediation of water molecules in cyclic transition states. In applying this concept to catalase action it would be plausible to suppose that acid–base mediation derives from the catalase molecule rather than from the solvent. In these terms a model for catalase action would have the form shown in Scheme 3, where BH^+ and A^- represent functions of suitable acid–base power, sterically oriented to facilitate the reaction. (*a*) in Scheme 3 represents the 'active site' of catalase with a water molecule bound to the ferric centre. The primary reversible binding of hydrogen peroxide to the ferric centre is shown in (*b*) in Scheme 3. (*c*) in Scheme 3 then represents the transition to the secondary complex as a locking into position of the hydrogen peroxide molecule by the formation of hydrogen bonds from BH^+ and to A^-. This process has consequential electronic effects; the coupled 'push–pull' partial proton transfers result in a clockwise flow of negative charge that produces a weakening of the peroxidic O–O bond and a development of the oxygen atom bond to iron as a powerful electrophilic centre. If the proton transfers were complete this process would result in the formation of an oxidizing species of the FeO^{3+} type. In (*c*) in Scheme 3 we accept the result of Chance & Schonbaum (1962) that the components of the parent peroxide molecule are retained in 'Compound I' (in our terminology, in the secondary complex) by indicating partial proton transfer but significant extension of the O–O bond. As a hydrogen peroxide molecule approaches the secondary complex it is polarized so that a coupled proton and hydride ion transfer, via the transition state (*d*) in

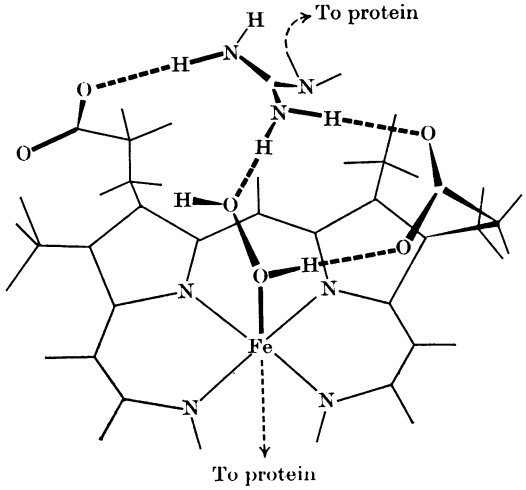


Fig. 1. Projection showing the stereospecific binding of H_2O_2 in a hypothetical partial molecular model for the active site of catalase.

Scheme 3, is facilitated, leading to the formation of products and regeneration of the active catalatic site.

A more explicit molecular statement of this model must satisfy both the experimental kinetic data for catalase action and the stereochemical implications of the processes represented in Scheme 3. Although it may be argued that such a model could not be definitive in the absence of direct structural information, the number of combinations that meet the rather stringent requirements must be severely limited and it is certainly important, at least, to demonstrate the possibility of constructing a satisfactory model with the information currently available. From an assessment of the experimental data and with the aid of molecular models we have concluded that the most satisfactory picture of the active site of catalase is obtained if BH^+ is identified with a $>C(NH_2)_2^+$ group from an arginine residue in the protein and A^- with a carboxylate group that may derive either from a propionate side chain of the protoporphyrin ring or from an aspartate or glutamate residue in the protein, or perhaps from a terminal amino acid. Fig. 1 shows a projection of a molecular model for the key step in the reaction, the stereospecific binding of hydrogen peroxide in the secondary complex, constructed on the assumption that A^- is a propionate group. The hydrogen-bond lengths required (i.e. the internuclear separations of the heavy atoms) are about 2·9 Å for a normal O–O peroxide bond length (1·48 Å) and regular tetrahedral bond angles. Partial proton transfer from arginine nitrogen would probably result in a change of its co-ordination towards a trigonal conformation, which would accommodate extension of the peroxidic O–O bond.

A key feature of the model is the hydrogen bond between the arginine and propionate residues that locks the functional components into a stereochemical array precisely suited for the binding of hydrogen peroxide and for the bifunctional 'push–pull' acid–base interactions formally envisaged in (*c*) in Scheme 3. In Fig. 1 it is also suggested that a further stabilization of the active site may arise from a hydrogen bond between the arginine residue and the second propionate side chain of the protoporphyrin ring.

In comparing this model with experiment the following important points emerge:

(*a*) Catalatic reaction via this path would be independent of pH between about pH 5 and pH10.

(*b*) Protonation of the propionate group or deprotonation of the arginine residue would disrupt the active site and lead to deactivation of catalase at the extrema of pH as observed. In this connexion we recall the observation of Agner & Theorell (1946) that a process of pK 3·8 was associated with the deactivation of catalase at low pH. At the time it was suggested that the observation related to acid ionization of a water molecule bound to the ferric centre, but later authors have not accepted this view. We suggest that this phenomenon may well be related to protonation of a propionate group in the active site of catalase. A rapid decrease in catalase activity above pH10·5 has been reported by several workers (Jones & Suggett, 1968*a*,*b*) and in our model is ascribed to deprotonation of arginine $>C(NH_2)_2^+$.

(*c*) Protonation of the propionate carboxyl group or deprotonation of arginine may induce dissociation of the enzyme, particularly if the arginine residue is primarily bound in a sub-unit other than the one in which it is involved as an active site-component. Our results (Jones & Suggett, 1968*a*) imply that protonation of catalase leads to dissociation and subsequent irreversible deactivation. The conclusion that catalase undergoes dissociation at high pH has been reached by a number of workers, and Samejima (1959*a*,*b*) reported a 'partially reversible' deactivation under these conditions.

(*d*) A 'catalatic' reaction involving only alkyl hydroperoxides is proscribed by this model, but an oxidation of hydrogen peroxide by a secondary complex formed from alkyl hydroperoxide is unimpaired. Internal oxidation–reduction of an *n*-alkyl hydroperoxide molecule bound in a secondary complex is probable (and is observed).

(*e*) The product molecular oxygen derives from a single parent hydrogen peroxide molecule (Jarnagin & Wang, 1958*a*), and the model is also consistent with observed deuterium kinetic isotope effects, both in the catalatic reaction (Jarnagin & Wang,

1958*b*) and in peroxidatic oxidations of hydrogen donors (Frei & Aebi, 1957; Nicholls & Schonbaum, 1963*b*).

(*f*) In the secondary complex there are likely to be substantial interactions between the outer peroxidic oxygen and the π-electron system of the protoporphyrin ring. These might make a significant contribution to changes in the spectrophotometric properties of the enzyme. In secondary complexes formed from alkyl hydroperoxides there may be interactions between the alkyl group and the hydrophobic region of the protoporphyrin ring, leading to quantitative differences between the spectra of these species and the secondary complex formed from hydrogen peroxide.

(*g*) The model suggests that inhibitors may act in a variety of ways. For some species (perhaps cyanide) a straightforward reversible binding of the ferric centre is likely. A more complex action may result with species that can compete successfully for the arginine–propionate hydrogen bond and bring about dissociation of the enzyme. Effects of this type may be enhanced by binding of substrate in the secondary complex and could result in irreversible deactivation. 3-Aminotriazole (Margoliash, Novogrodsky & Schejter, 1960) may belong to this category. Some inhibitors may bind both the ferric centre and the acid–base functions in the intact enzyme, a type of action that would involve substantial steric effects so that the inhibition might be rather slowly established. This could explain the difference in catalase activity in different buffers when measured by the Bonnichsen, Chance & Theorell (1947) assay procedure and that obtained when catalase is equilibrated with buffers other than phosphate (Jones & Suggett, 1968*a*,*b*).

(*h*) The model satisfies all the criteria for a successful mechanism of catalase action prescribed by Nicholls & Schonbaum (1963*a*), with the exception of those deactivation processes (see section i) that are supposed by them to involve an 'endogenous donor', and is also in accord with more recent experimental data.

(iv) *Peroxidatic oxidations.* Studies of 'peroxidatic oxidations' of reducing substrates by catalase–peroxide systems have revealed considerable phenomenological diversity (Keilin & Nicholls, 1958). The model that we have proposed has a number of implications that might go some way towards resolving these problems.

A major group of substrates contain the group:

including primary and secondary alcohols and their derivatives and formic acid. It is suggested that they compete with hydrogen peroxide for Compound I (the secondary complex in our terminology) and are oxidized by a two-electron-equivalent hydride transfer, coupled with proton transfer. This type of process is readily accommodated by our model and indeed is supported by theoretical (MO) studies of α-hydrogen abstraction in a number of species of this type (P. Jones, K. Levison & P. G. Perkins, unpublished work). These show that hydride transfer is the preferred mode of α-hydrogen abstraction from formate, methoxide and isopropoxide ions.

In (*d*) in Scheme 3 we have suggested that, in the oxidation of hydrogen peroxide, proton release is facilitated by the outer (nucleophilic) peroxidic oxygen atom in the secondary complex. It is, of course, possible that this process is mediated by another basic residue from the catalase protein, in both catalatic and peroxidatic actions.

There is a considerable body of literature indicating that the irreversible formation of sub-units from catalase produces species with greatly enhanced peroxidatic properties (Anan, 1958; Inada, Kurozumi & Shibata, 1961; Caravaca & May, 1964). It is not impossible that reversibly formed sub-units may show similar behaviour, and in Scheme 2 we have tentatively indicated this possibility. Complexities deriving from sub-unit formation may be important in the phenomena of peroxidatic oxidations, but the sub-unit domain of catalase behaviour is relatively unexplored at the present time.

Finally, our model of catalatic action suggests the possibility that the molecular oxygen produced may emerge as an excited singlet species that could itself oxidize reducible substrates present in the system. In this connexion the work of Anbar (1966) is relevant. A measurable oxidation of molecular nitrogen to nitrite and nitrate was observed when nitrogen was dissolved under a pressure of 100 atm. in water and hydrogen peroxide was either oxidized by hypochlorite (a reaction in which chemiluminescence from singlet molecular oxygen can be observed) or catalytically decomposed by catalase in these solutions.

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