Biochem. J. (1973) **135**, 331–341
Printed in Great Britain

Studies by Electron Paramagnetic Resonance on the Catalytic Mechanism of Nitrogenase of *Klebsiella pneumoniae*

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(*Received* 6 *March* 1973)

The properties and catalytic reactions of the enzyme nitrogenase purified from *Klebsiella pneumoniae* were studied by electron-paramagnetic-resonance (e.p.r.) spectroscopy at temperatures down to 8°K. The two protein fractions, Kp1 (the iron–molybdenum protein) and Kp2 (the iron protein), were examined alone and in steady-state mixtures and also in pre-steady-state experiments, by using the rapid-freezing method. Kp1 protein in dithionite solution shows a rhombic type of spectrum with g_1 4.32, g_2 3.63, g_3 2.009 at pH 6.8 (0°C). Small changes in the spectrum produced by protons ($pK = 8.7$ at 0°C) or by acetylene indicate binding of these oxidizing substrates to this protein fraction. Kp2 protein shows a rhombic spectrum with g_1 2.053, g_2 1.942, g_3 1.865, which integrates to about 0.45 electron/molecule. Binding of ATP, with a dissociation constant of 4×10^{-4} M, changes the spectrum to an axial form with $g_{\parallel}$ 2.036, $g_{\perp}$ 1.929, thus indicating a conformation change of Kp2 protein. The Kp2 protein spectrum disappears reversibly on cautious oxidation. The signals of both proteins are diminished in their steady-state mixtures, obtained in the presence of ATP and dithionite (with an ATP-generating system and Mg^{2+} ions) and with protons, N_2 or acetylene as oxidizing substrate. At the same time as dithionite is consumed in such reactions, the Kp1 protein signal is gradually restored and the Kp2 protein signal diminishes to zero. In rapid-freezing experiments the signals from the two proteins decreased at indistinguishable rates ($t_{\frac{1}{2}}$ about 10ms), then they remained constant. Results are interpreted in terms of a scheme in which reducing equivalents pass from dithionite to Kp2 protein, then, in an ATP-dependent reaction to Kp1 protein, this being finally reoxidized by N_2 or another oxidizing substrate. In this scheme Kp1 protein cycles between its signal-giving state and a very highly reduced signal-free state.

The enzyme nitrogenase has been purified and studied extensively in a number of laboratories [see Mortenson *et al.* (1973*a*) and Eady *et al.* (1973) for recent summaries]. Nitrogenase from *Klebsiella pneumoniae*, like that from other sources, consists of two proteins. The smaller of these, referred to as Kp2 protein, contains four iron and four acid-labile sulphur atoms per molecule, but the larger, Kp1 protein, has a much higher content of iron and labile sulphur and contains molybdenum also (Eady *et al.*, 1972). In preliminary studies by e.p.r.* spectroscopy (Smith *et al.*, 1972), we succeeded in demonstrating rapid electron transfer from Kp2 protein to an oxidized form of Kp1 protein and we proposed that the mechanism of action of the enzyme may be represented by the electron-transfer scheme shown in Scheme 1.

We now report more detailed e.p.r. work supporting this mechanism, including further pre-steady-state studies by the rapid-freezing method. Very recently, details of closely related independent work have been published (Orme-Johnson *et al.*, 1972*a*; Zumft *et al.*, 1972, 1973; Mortenson *et al.*, 1973*a*, *b*). These publications refer to nitrogenase from different sources from ours but the similarity of the results implies that differences between the enzymes must be small. Though both the above groups of workers propose basically the same electron-transfer scheme as we do in Scheme 1, there are nevertheless important differences.

$$\text{Dithionite} \rightarrow \text{Kp2} \xrightarrow{\text{ATP}} \text{Kp1} \rightarrow \begin{cases} N_2 \\ H^+ \\ C_2H_2 \end{cases}$$

Scheme 1. *Proposed electron transfer scheme*

For discussion, see the text.

* Abbreviations: e.p.r. electron paramagnetic resonance; Hepes, 2-(*N*-2-hydroxyethylpiperazin-*N'*-yl)ethanesulphonic acid. G, gauss; $1\,G = 10^{-4}$ T.

Oxidation states of Kp1 protein

It is generally agreed that the iron- and molybdenum-containing nitrogenase protein, as it is normally isolated in dilute dithionite solution, gives, at low temperatures, a characteristic e.p.r. signal with resonances at g values of about 4.3, 3.7 and 2.01 (Davis *et al.*, 1972; Palmer *et al.*, 1972; Smith *et al.*, 1972; M. C. W. Evans, personal communication). Smith *et al.* (1972) showed that Kp1 protein can exist in various oxidation states and they denoted the one giving rise to the above signal as state S. They reported that this could be oxidized reversibly, without loss of enzymic activity, either to state III or to state II. State III was obtained on treatment with dye, Lauth's Violet, and had no detectable e.p.r. signals. State II, obtained with ferricyanide, gave complex signals particularly in the region $g = 1.6$–2.0. Palmer *et al.* (1972), working on the enzyme from *Clostridium pasteurianum*, also found that dyes, particularly Methylene Blue, removed the signal, i.e. in our nomenclature, converted state S into state III. They studied the signals obtained with ferricyanide (state II) in some detail. However, since Smith *et al.* (1972) concluded that neither state III nor state II is involved in the catalytic cycle of nitrogenase, we think it is not important to study these species further at present. Smith *et al.* (1972) proposed that an additional highly reduced state of Kp1 protein, state IV, is generated in the catalytic reaction. State IV, like state III, gives no e.p.r. signals. They reported that under steady-state conditions Kp1 protein was converted largely into state IV. Clearly, however, in studies on the mechanism of nitrogenase action, it is of the utmost importance, and a matter of some difficulty, to distinguish between states III and IV. Though Orme-Johnson *et al.* (1972*a*), working with the enzyme from *C. pasteurianum*, also deduced reduction to state IV in the steady state, they seem to have been unaware of the existence of the oxidized signal-free state (state III). Therefore, particularly because they did not include pre-steady-state studies, their results cannot be regarded as fully definitive. In contrast, Mortenson and co-workers (Mortenson *et al.*, 1973*b*; Zumft *et al.*, 1972) preferred to interpret their results on the *Clostridium* enzyme in terms of the oxidative rather than the reductive disappearance of the e.p.r. signal, and consequently arrived at a reaction mechanism substantially different from ours.

Materials and Methods

Nitrogenase preparations

The enzyme was purified as described by Eady *et al.* (1972). Kp1 protein samples had specific activities of between 850 and 1500nmol of C_2H_4 produced/min per mg of protein. Kp2 protein activities varied from 600 to 1500nmol of C_2H_4 produced/min per mg. These variations may be associated with changes in the growth and harvesting conditions employed for the bacteria used in different preparations.

pH values

For experiments on the effect of pH on the e.p.r. spectrum of Kp1 protein (Fig. 2), pH measurements were made at 21°C on the contents of the e.p.r. tubes after the spectra had been recorded, by using a microglass reference electrode from Activion Glass Co. Ltd., Kinglassie, Fife, U.K. Since it is likely that pH values at 0°C are more relevant to frozen samples than are those at 21°C, the results were then corrected to 0°C by using the temperature coefficients of Good *et al.* (1966). All pH values in the present paper have been so corrected.

Preparation of samples and e.p.r. measurements

Strict precautions to exclude contamination of solutions with O_2 were essential and unless otherwise stated $Na_2S_2O_4$ was always present in them. Preparation of samples by manual mixing for e.p.r. measurements was as described previously (Eady *et al.*, 1972; Smith *et al.*, 1972). For experiments in which a full nitrogenase system was present an ATP-regenerating system of creatine phosphate and creatine kinase was added to prevent accumulation of ADP. The ratios of the components of the reaction mixture (including ATP) used in these experiments were those of Eady *et al.* (1972). Such mixtures of ATP with the regenerating system will be referred to as 'the ATP system'. In manual-mixing experiments the ATP system was employed at 20mM-ATP and in rapid-freezing experiments it was used at 10mM-ATP (after mixing).

The rapid-freezing technique of Bray (1961) and Bray & Pettersson (1961) has recently been improved in this laboratory (see Bray *et al.*, 1973, for details). An important point for the present anaerobic work was that thick-walled nylon tubing was used to replace the original polyethylene, since the former is much less permeable to O_2. Also, whereas in the original method the reaction mixture was shot through an air gap from the jet to the surface of the cold isopentane, in the present work this gap was flushed continuously with N_2. Rapid-freezing experiments were of two types. In the first Kp1 protein plus Kp2 protein in one syringe was made to react with the ATP system in the other. It was found here that reproducible results were obtained only when the two protein fractions had been in contact with one another at 20–25°C for not more than 1–1.5h. In the second type of experiment, Kp1 protein plus the ATP system was made to react with Kp2 protein. Here, at least with some Kp1 protein preparations, it was found that preincubation of the protein with the ATP system for 0.5–1h improved reproducibility.

E.p.r. measurements were made on a Varian E9 spectrometer with a helium-gas-flow temperature-

control system, as described previously (Lowe *et al.*, 1972). The g values were determined by comparison with diphenylpicrylhydrazyl and the Cr^{3+}-containing crystals of an ammonium–cobalt–EDTA complex (Aasa *et al.*, 1966). The spectrum of the latter compound was measured at room temperature, since its g values were temperature-dependent.

Integration of signals for absolute quantitative measurement was performed under non-saturating conditions with Cu^{2+}–EDTA as standard and results were corrected for the difference in g values between the standard and unknown (Aasa & Vänngård, 1970). For relative quantitative measurement, signal amplitudes of the g_2 features of Kp1 or Kp2 proteins, measured under standard conditions, were employed. In such work, changes in the form of the Kp1 protein signal (cf. Fig. 2) were ignored but complex-formation between Kp2 protein and ATP (Fig. 3) was corrected for in the following manner. Spectra corresponding to mixtures of free Kp2 protein and its complex with ATP were simulated by adding together the two spectra of Fig. 3 in various proportions, by using an ICL 1904 A computer with a Calcomp plotter. Comparison of experimental spectra with these simulated spectra was then used to deduce the extent of complex-formation. For measurement of Kp2 protein signal intensity changes in steady-state experiments, the appropriate amplitude correction could then be made from a graph of relative amplitude of the simulated signal against percentage of complex-formation. With mixtures of Kp1 and Kp2 proteins, another type of correction must also be considered. Our Kp1 protein samples frequently contained impurities giving weak resonances in the $g = 1.94$ region (cf. Fig. 1). In the most unfavourable cases the amplitude of this signal approached half of that of the signal of the added Kp2 protein. Since it is not certain what changes, if any, would take place to the impurity signal on putting the enzyme into a steady state, no attempt was made to subtract this signal from the Kp2 protein signal. In any case such corrections to our results would not affect our main conclusions significantly.

Results

E.p.r. signal from state S of Kp1 protein

The spectrum of Kp1 protein in state S (Fig. 1, Table 1) was unaffected by variations in the concentration of dithionite up to 0.25M. At 8°K the signal was about 20% saturated at 2mW and about 50% saturated at 200mW. The signal broadened rapidly on raising the temperature and at 15°K, the amplitude, measured under non-saturating conditions and normalized for the Boltzmann effect, was about 50% of that at 8°K. Palmer *et al.* (1972) have proposed that the signal is due to a system of interacting iron atoms

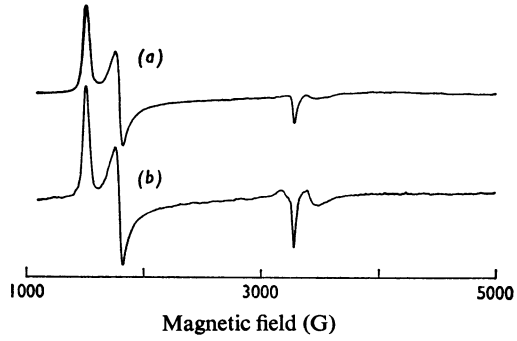


Fig. 1. *E.p.r. spectra of nitrogenase fraction Kp1 in state S*

The sample had a specific activity of 1250nmol of C_2H_4 produced/min per mg and was at a concentration of 12mg/ml in 250mM-Hepes–NaOH buffer, pH8.1 (0°C), containing 10mM-dithionite. The ATP system (see the Materials and Methods section) was also present but other experiments showed that this did not influence the spectrum. Spectra were recorded at 8.1°K and at a microwave frequency of 9.1GHz. The microwave powers were: (*a*) 200mW; (*b*) 2mW. The gain for spectrum (*b*) was 8 times that for spectrum (*a*).

Table 1. *g values of nitrogenase proteins*

The parameters of the high-pH spectrum of Kp1 protein are extrapolated values (cf. Fig. 2). g_2 and $g_\perp$ values were measured at the point where the spectrum crossed the baseline. The g values quoted are correct to ±0.003, except for those in the region of 3.7 and 4.3, where the errors are ±0.02. Errors of the shifts in g values with pH are smaller than on absolute g values.

Protein	g_1 or $g_\parallel$	g_2 or $g_\perp$	g_3
Kp1, state S, low-pH form	4.32	3.63	2.009
Kp1, state S, high-pH form	4.27	3.73	2.018
Kp2, reduced, free (50mM-Mg^{2+})	2.053	1.942	1.865
Kp2, reduced, ATP complex (50mM-Mg^{2+})	2.036	1.929	

with a net spin of 3/2. In agreement with this work we found that the signal from state S of Kp1 protein was unchanged when protein from bacteria grown on medium 97% enriched in ^{95}Mo was used. Since molybdenum hyperfine splittings would be expected to be too large to escape detection the signal cannot be due to this metal. Further, though growth on medium 87% enriched in ^{57}Fe yielded Kp1 protein with the e.p.r. spectrum little changed, the sharpest feature, that at g_3, did seem to be broadened slightly (about 5G). This further supports the conclusion that the signal is due to iron.

Preliminary examination of an unenriched sample at 35GHz gave g values differing little from those observed at 9GHz. No new transitions were observed.

The sample of Kp1 protein in Fig. 1 apparently contains a small amount of an impurity giving a weak signal in the $g = 1.94$ region. The impurity signal saturates more readily than does the main signal (cf. Davis *et al.*, 1972; see also Palmer *et al.*, 1972). Its form is very like that from the ATP complex of Kp2 protein (see below). Meaningful integration of a signal of the type given by state S is obviously difficult (cf. Palmer *et al.*, 1972) and we made no attempt to do so. Also, we made no attempt at precise comparison of the amplitudes of samples of Kp1 protein of differing specific activities.

Modification of the e.p.r. signal from state S by protons and by acetylene

The precise g values and line widths of the e.p.r. signal given by state S of Kp1 protein depend on the pH of the medium, as shown in Fig. 2 and Table 1. All changes occurred together and appeared to correspond to an acid dissociation with a pK of 8.7 at 0°C. The effect was most noticeable at the g_2 feature, which moved to lower field as the pH was increased.

Since the proton is a substrate for nitrogenase (cf. Scheme 1) the above pH effects could reflect, for example, a conformational change influencing the e.p.r. chromophore and resulting from the binding of a proton as substrate at the active site of Kp1 protein. It was therefore of the greatest interest to see whether other substrates affected the e.p.r. signal. We found (Fig. 2) that when Kp1 protein was incubated with C_2H_2+Ar (87.5:12.5) at pH8.2 the ratio of the high- and low-pH forms of the e.p.r. signal of state S was displaced in favour of the high-pH form, indicating that acetylene does compete with protons for the binding site.

E.p.r. signals from Kp2 protein

Mortenson and co-workers (Zumft *et al.*, 1972, 1973) and Orme-Johnson *et al.* (1972*a*) have both reported plant ferredoxin-like signals from the iron-containing protein from *C. pasteurianum* that changed their form in the presence of ATP. The former workers found an integrated signal intensity corresponding to 0.2 electron/molecule (Palmer *et al.*, 1972; Zumft *et al.*, 1973) whereas the latter found about 0.8 electron/molecule (Orme-Johnson *et al.*, 1972*a*). We have consistently found signals from Kp2 protein in the presence of dithionite (cf. Eady *et al.*, 1972), as shown in Fig. 3. The form of the signals changed from the rhombic to the axial type on addition of ATP in the presence of Mg^{2+} (see also Table 1). Intensity of

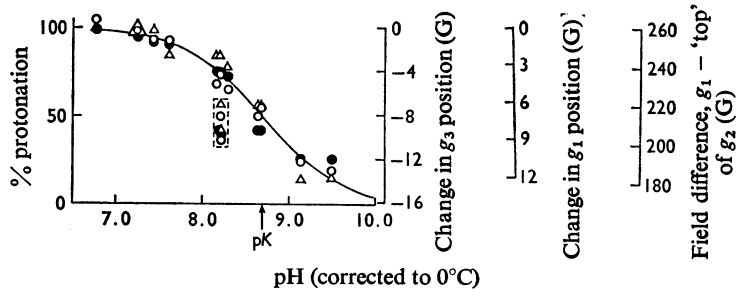


Fig. 2. *Variation with pH of the signal form of nitrogenase fraction Kp1 in state S*

The samples were prepared in 250mM-Tris–HCl (above pH8.5) or 250mM-Hepes–NaOH (below pH8.5) buffer. The parameters plotted are: change in g_1 position in G (△); change in g_3 position in G (○) and field difference in gauss between the 'top' of the g_2 resonance and g_1 (●). The theoretical curve of % protonation versus pH is calculated for a pK of 8.7. The points within the box refer to measurements on duplicate samples at pH8.2 (0°C), which had been incubated with an atmosphere of C_2H_2+Ar (87:5:12:5). The protein had a specific activity of 1500nmol of C_2H_4 produced/min per mg and was used at a concentration of 7.5mg/ml. The e.p.r. spectra were recorded at 12.0°K at a microwave frequency and power of 9.1GHz and 100mW, respectively. pH values are corrected to 0°C (see the Materials and Methods section).

the e.p.r. signals (measured in the absence of ATP) was somewhat variable in the earlier stages of our work, possibly because of faulty anaerobic technique. More recently, on each of five different highly purified samples of Kp2 protein, we found signals that integrated to 0.45±0.07 (S.D.) electron/molecule. These samples, however, showed specific activities, measured by acetylene reduction, varying from 600 to 1500 nmol/min per mg. Integrated signal intensity was not influenced by variation of the concentration of Kp2 protein in the range 1.2–40 mg/ml, by increasing the concentration of $Na_2S_2O_4$ up to 0.25 M, or by addition of the redox dye Methyl Viologen. It therefore seems that association or dissociation of the protein (cf. Thorneley & Eady, 1973) is not the cause of the low integration value and that also it is not due to incomplete reduction. The integration has to be compared with other four-iron ferredoxins, for example, that from *Bacillus polymyxa* which gives 1 electron/molecule (Orme-Johnson *et al.*, 1972*b*). The significance of these findings is discussed below.

The Kp2 protein signal was fully sharpened at 12°K and at this temperature was 5–10% saturated at 1 mW and about 50% saturated at 100 mW. At somewhat higher temperatures the signal began to broaden. At 25°K the amplitude, normalized for the Boltzmann effect and measured under non-saturating conditions, was about 50% of that at 8°K. Effects of power and temperature on the spectrum were similar in the presence or absence of ATP (with Mg^{2+}).

The change in signal induced by ATP and Mg^{2+} is presumably due to complex-formation between Kp2 protein and the nucleotide. Indeed, Zumft *et al.* (1972, 1973) have indicated tight binding in such a complex for the protein from *Clostridium*. Addition of ATP had little effect on the integrated intensity of the Kp2 protein signal, and we studied the form of the signal as a function of the ATP concentration, in the presence of Mg^{2+}. Comparison with computed spectra (see the Materials and Methods section) confirmed that only the two species shown in Fig. 3 were present at intermediate ATP concentrations. Though ATP decreased the overall signal amplitude (see Fig. 3), a graph of amplitude against percentage complex-formation, from the simulations, was non-linear, owing to the position of the maximum shifting, and showed a progressive amplitude decrease to about 30% complexing, but little further change beyond this point. Thus amplitude changes could not be used to measure the extent of complex-formation, and comparison of the overall forms of experimental and simulated spectra was used instead to obtain a dissociation constant of about 4×10^{-4} M for the complex (Fig. 4). Since Zumft *et al.* (1973) apparently used uncorrected amplitude measurements, their data on ATP binding are likely to be in error.

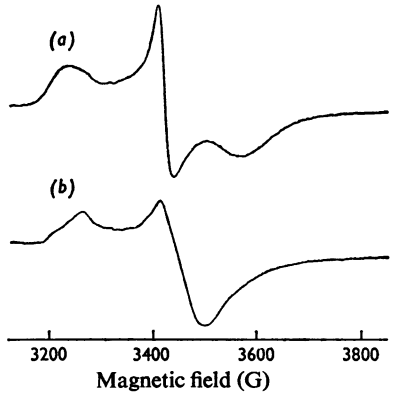


Fig. 3. *E.p.r. spectra of nitrogenase fraction Kp2*

Kp2 protein was prepared in the presence of 50 mM-Mg^{2+} (*a*) alone and (*b*) in the presence of 1.9 mM-ATP. The protein had a specific activity of 700 nmol of C_2H_4/min per mg and a concentration of 11 mg/ml. The buffer was 25 mM-Tris–HCl, pH 8.1 (at 0°C), and 1 mM-dithionite was present. The e.p.r. spectra were recorded at about 18°K at a microwave frequency and power of 9.1 GHz and 20 mW respectively.

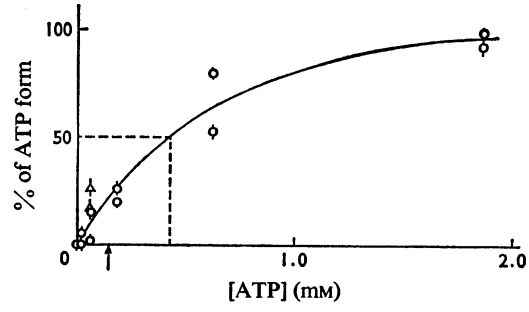


Fig. 4. *Titration of nitrogenase fraction Kp2 with ATP*

Percentage of ATP form (i.e. extent of complex-formation) is plotted against ATP concentration and the arrow shows the Kp2 protein concentration. The extent of complex-formation was estimated by comparing the form of the spectrum with that of simulated spectra (see the Materials and Methods section). Error bars indicate the limits of such estimates made by different observers. Samples of Kp2 protein were incubated with various concentrations of ATP for 2 min, then frozen for e.p.r. measurements at about 20°K. The protein had a specific activity of 700 nmol of C_2H_4 produced/min per mg and a concentration of 11.2 mg/ml (○) or 5.6 mg/ml (△). The experiment was carried out in 35 mM-Tris–HCl buffer, pH 8.1 (0°C), with Mg^{2+} at a concentration of 50 mM.

In the absence of ATP, some relatively small changes in the form of the Kp2 protein signal were also noted on varying the concentration of Mg^{2+}. As this was increased (up to 200mM) the line widths increased and the g_2 and g_3 features moved a little closer together.

By analogy with the plant ferredoxins it would be expected that an oxidized, signal-free state of Kp2 protein should also exist. We were able to confirm this directly, by removing excess of dithionite by means of gel filtration, then exposing the Kp2 protein very cautiously to traces of O_2. However, limited stability of the oxidized form was indicated in these experiments, because re-addition of dithionite only partially restored the signal and because there was some loss of activity.

E.p.r. signal changes in steady-state experiments

Smith *et al.* (1972) reported that, when the complete nitrogen-fixing system was present under presumed steady-state conditions, i.e. when Kp1 and Kp2 proteins and ATP with Mg^{2+} were mixed in the presence of dithionite, with either N_2 or protons available to act as oxidizing substrate, then there was a substantial diminution in the e.p.r. signal from Kp1 protein. We did not report our observations on the Kp2 protein signal then, as we were still in doubt about its origin, because of low and variable integrations. Other workers have confirmed our findings, reporting that for the *Clostridium* enzyme the signal from the iron–molybdenum protein decreased in the steady state (Zumft *et al.*, 1972; Orme-Johnson *et al.*, 1972*a*; Mortenson *et al.*, 1973*b*). Zumft *et al.* (1972) also noted steady-state decreases in the intensity of the iron-protein signal.

We have carried out a large number of experiments of this type and typical results are presented in Figs. 5 and 6. Fig. 6 shows that both the Kp1 protein state-S signal and the reduced Kp2 protein signal were substantially diminished 2.8min after initiation of the reaction by addition of ATP and Mg^{2+} (with an ATP-regenerating system). At longer reaction times the Kp1 protein signal gradually increased whereas the Kp2 protein signal decreased. After 10–15min the former signal was almost fully restored and the latter had virtually disappeared. In this experiment we presume that these relatively slow signal-intensity changes correspond to the gradual exhaustion of dithionite by the combined acetylene reductase and hydrogenase activity of the system, according to Scheme 1. The changes in the form of the feature at $g = 3.7$ (Fig. 5) seem to be due at least in part to pH changes (cf. Fig. 2). The latter are presumably due to ATP hydrolysis, with an inadequate buffer concentration. An adequate supply of ATP was assumed to be present since the ATP plus creatine phosphate available was 25 times in excess of the available

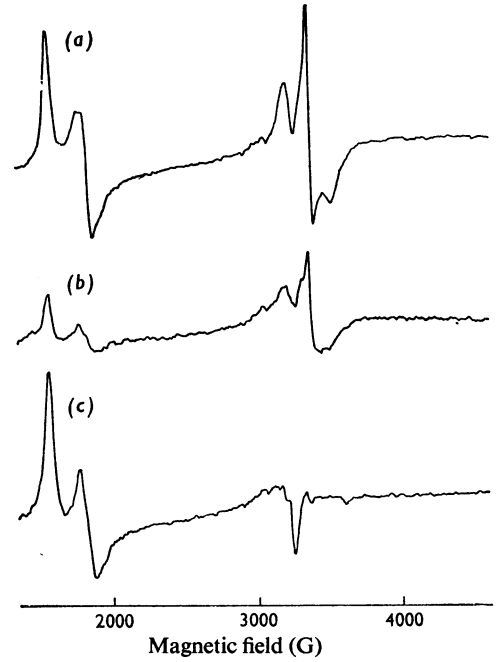


Fig. 5. *E.p.r. signals from the nitrogenase system: (a) before initiation of the reaction, (b) during steady-state turnover initiated by addition of ATP and (c) after exhaustion of dithionite*

Reaction times before freezing in (*b*) and (*c*) were respectively 2min and 120min at about 20°C. The initial dithionite concentration was 4mM. The buffer was 150mM-Hepes–NaOH, pH8.1 (0°C). The reaction was initiated by addition of the ATP system to the proteins under an atmosphere of C_2H_2+Ar (80:20). The specific activity and concentration of Kp1 protein were 1100nmol of C_2H_4 produced/min per mg and 5mg/ml, and of Kp2 protein 850nmol of C_2H_4 produced/min per mg and 1.7mg/ml, respectively. E.p.r. spectra at 9.1GHz were recorded at about 10°K with a microwave power of 20mW. Gain settings were the same for all samples.

$S_2O_4^{2-}$, and the Kp2 protein signal was present partly in its ATP form throughout.

Calculations of the expected time for dithionite exhaustion, based on the maximum turnover number of Kp1 protein (Eady *et al.*, 1972), gave much shorter times than those indicated by Fig. 6. However, in this experiment (Fig. 6) the Kp1/Kp2 protein ratio was such (about equimolar) that the Kp1 protein would be expected (cf. Eady *et al.*, 1972) to exhibit only about 25% of its maximal activity. Acetylene reduction assays at the protein and ATP-system concentrations used in the e.p.r. experiments showed that activity was further diminished to about

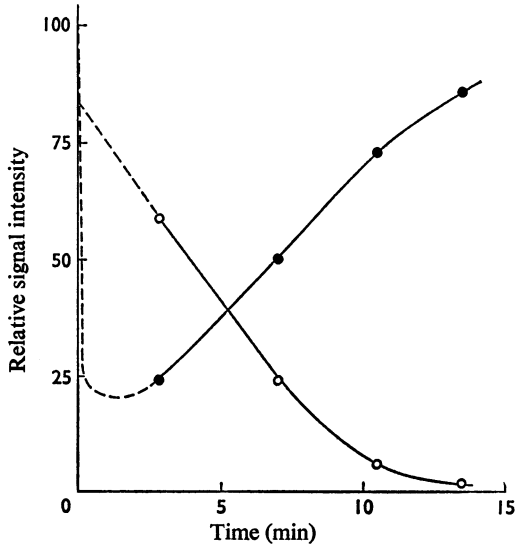


Fig. 6. *Intensities of the e.p.r. signals from the nitrogenase system during steady-state turnover and as dithionite becomes exhausted*

The signals from Kp1 (●) and Kp2 (○) proteins were measured and corrected as described in the Materials and Methods section. Signal intensities are expressed as a percentage of those before initiation of the reaction, by adding the ATP system (see the Materials and Methods section) under an atmosphere of C_2H_2+Ar (50:50). The buffer was 150mM-Hepes–NaOH, pH8.1 (0°C), and the initial dithionite concentration was 2mM. The specific activity and concentration of Kp1 protein were 1250nmol of C_2H_4 produced/min per mg and 5.5mg/ml and of Kp2 protein were 1100nmol of C_2H_4 produced/min per mg and 2.5mg/ml respectively. The dashed lines linking the signal intensities before reaction with those measured after 2.8min are the expected reaction curves deduced from the rapid-freezing data.

50% of that expected in dilute assay mixtures. This inhibition was presumably due to the high buffer concentration present (150mM). In addition the incubations for e.p.r. experiments were carried out at room temperature (20–22°C) whereas the above assays were at 30°C. This would lower the activity by a further factor of about 2.5 (R. R. Eady, personal communication). Thus the predicted turnover number for Kp1 protein in these experiments would be about 5% of the maximum when saturated with $S_2O_4^{2-}$.

Fig. 6 shows a progressive, almost linear change in the signal intensities with time which seems reasonably consistent with an apparent K_m for dithionite of 2mM for acetylene reduction (R. R. Eady, personal communication). That the intensity changes with time were indeed due to exhaustion of dithionite and not to some other cause was confirmed by setting up further experiments in which samples with differing initial dithionite concentrations were examined after a fixed (short) incubation period. In two separate experiments, doubling the dithionite concentration caused a significant decrease in the Kp1 protein signal together with a significant increase in the Kp2 protein signal.

About twenty separate experiments of the above type have been carried out, employing various proportions of the constituents and with nitrogenase preparations of different specific activities. Some experiments were done under N_2 and others under C_2H_2. In every case, at the shortest reaction time examined (1–2min), a decrease in signal intensity was observed for Kp1 protein (60–90%), and in almost all cases there was also a decrease for Kp2 protein (up to 60%). Precise analysis of the various factors controlling the magnitudes of these decreases has not yet been possible. However, in experiments in which the concentration of Kp2 protein decreased systematically, with all other variables kept constant, it was found that Kp1 protein bleaching (e.p.r. signal bleaching) also tended to decrease. Thus, in one case changing from a Kp2/Kp1 protein molar ratio of 1.6:1 to 0.4:1 decreased bleaching from 77 to 64%. In another experiment, however, bleaching was less influenced (decrease from 66 to 61%) on decreasing the Kp2 protein concentration to the low molar ratio of 0.15:1. In contrast, acetylene-reduction-activity measurements, carried out at the enzyme concentration used in the e.p.r. work and at a fixed Kp1 protein concentration, showed dramatic decreases of overall activity on decreasing the Kp2 protein concentration, as expected from previous work (Eady *et al.*, 1972). The relative smallness of the e.p.r. effects produced by changes in Kp2 protein concentration has a parallel in work on the enzyme from *C. pasteurianum* (Mortenson *et al.*, 1973*b*) and is discussed in subsequent sections.

E.p.r. signal changes in pre-steady-state experiments

Particularly in view of some of the results in the paragraph above, it seemed important to determine by means of the rapid-freezing method whether or not the signal-intensity changes observed in manual experiments were taking place within the turnover time of the enzyme. Results of typical rapid-freezing experiments on nitrogenase are shown in Figs. 7 and 8.

In Fig. 8(*a*), the reaction was initiated by mixing Kp2 protein from one syringe of the apparatus with Kp1 protein plus the ATP system from the other syringe. In Figs. 7 and 8(*b*) the two proteins were combined and placed in one syringe and the other syringe contained the ATP system. In both cases decreases in intensities of the signals from Kp1 and Kp2

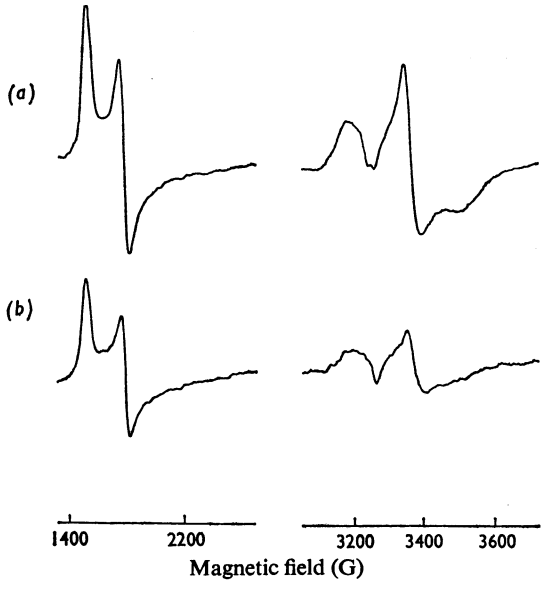


Fig. 7. *E.p.r. signals from the nitrogenase system obtained by the rapid-freezing method: (a) before initiation of the reaction and (b) 16 ms after addition of ATP*

The spectra were recorded at 9.1 GHz and 12.1°K, at microwave powers of 100 mW for the low-field regions and 1 mW for the high-field regions. Gain settings were the same for both samples. In (*a*) a mixture of Kp1 and Kp2 proteins in one syringe was treated with buffer in the second syringe and frozen by the rapid-freezing method. In (*b*) the buffer was replaced by the ATP system (see the Materials and Methods section) and the reaction time was 16 ms at 23°C. Specific activities and concentrations after mixing were as follows: Kp1 protein: 900 nmol of C_2H_4 produced/min per mg and 9.2 mg/ml; Kp2 protein: 850 nmol of C_2H_4 produced/min per mg and 3.7 mg/ml; dithionite: 5 mM. The buffer was 50 mM-Hepes–NaOH plus 25 mM-Tris–HCl, pH 8.1 (0°C).

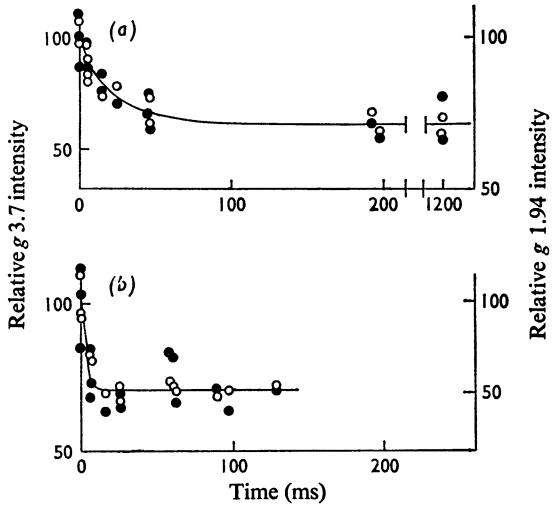


Fig. 8. *Intensities of the e.p.r. signals from the nitrogenase system as a function of time in rapid-freezing experiments*

The signals from Kp1 (●) and Kp2 (○) proteins were measured and corrected as described in the Materials and Methods section. In (*a*) a mixture of Kp1 protein with the ATP system (see the Materials and Methods section) in one syringe was treated with Kp2 protein in the other. In (*b*) Kp1 and Kp2 proteins together in one syringe were treated with the ATP system in the other. The reactions were carried out at 23°C and samples were frozen by the rapid-freezing method at the times indicated after mixing. Signal intensities are expressed as percentages of those before initiation of the reactions. The zero-time points were obtained in (*b*) by replacing the ATP with buffer and in (*a*) by replacing first one protein, then the other, with buffer and adding the spectra so obtained. In both experiments, the buffer was 50 mM-Hepes–NaOH plus 25 mM-Tris–HCl, pH 8.1 (0°C). The dithionite concentration after mixing was 10 mM in (*a*) and 5 mM in (*b*). Specific activities and concentrations of the proteins after mixing were: in (*a*) Kp1 protein, 850 nmol of C_2H_4 produced/min per mg and 12.6 mg/ml; Kp2 protein: 850 nmol of C_2H_4 produced/min per mg and 3 mg/ml; in (*b*) Kp1 protein: 900 nmol of C_2H_4 produced/min per mg and 9.2 mg/ml; Kp2 protein: 850 nmol of C_2H_4 produced/min per mg and 3.7 mg/ml.

proteins took place rapidly and with indistinguishable time-courses. In the experiment with the proteins in separate syringes the half-time for the decrease was about 15 ms and when the proteins were pre-mixed it was about 5 ms, i.e. close to the limit of the time-resolution of the rapid-freezing method. In both cases, too, complex-formation of Kp2 protein with ATP took place very rapidly indeed. After the initial signal decreases, their intensities remained constant at least up to about 1 s (Fig. 8*a*).

Several experiments similar to those in Figs. 7 and 8 have been done. In some of these more active Kp1 protein, which had no significant impurities giving signals in the $g = 1.94$ region, was used. Though the extents of signal bleaching were somewhat variable, in no case could the time-course of bleaching of Kp1 protein be distinguished from that of Kp2 protein. The extent of Kp2 protein bleaching (20–50%) in rapid-freezing experiments was comparable with that in experiments that used manual mixing. On the other

hand, the Kp1 protein bleaching was generally smaller, ranging from 30 to 50%.

Discussion

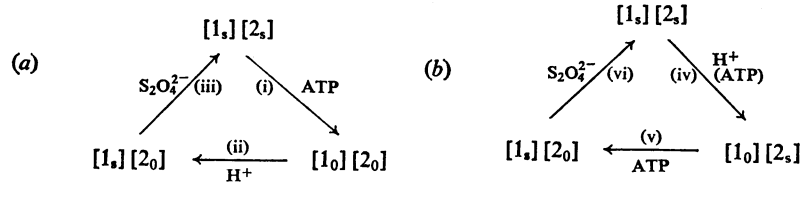
Origin of the e.p.r. signals

Other workers have not discussed in detail the relation between e.p.r. signal intensities and specific activities of the nitrogenase protein fractions. However, there seems to be no reason to doubt that the e.p.r. spectrum of state S of Kp1 protein preparations represents a functional nitrogenase protein. On the other hand, the signals in reduced Kp2 protein preparations did not always parallel enzymic activity and integrated to only 45% of the minimum theoretical value. In steady-state and pre-steady-state experiments, as discussed below, intensities of both signals changed in the general manner that would be predicted from Scheme 1, suggesting that they are indeed due to functional enzyme components. Nevertheless, it might be postulated that the Kp2 protein signal was due to an impurity in our preparations (maybe, for example, degraded enzyme molecules), capable of rapid equilibration with the system and functioning merely as an indicator of the overall redox potential. Although this hypothesis is not rigorously excluded it is rendered improbable by the relatively high and constant integration of the signal, implying that this represents a major constituent present in constant amount in all Kp2 protein samples. Constancy of the signal from apparently homogeneous samples differing by as much as 2- or 3-fold in specific activity implies, however, that it must be given both by functional and non-functional molecules. The latter might represent, for example, Kp2 protein with a damaged binding site for the other protein but with its e.p.r. chromophore intact. The behaviour of such material in steady-state mixtures is not readily predictable, but it is possible that it would equilibrate with the system, if only slowly.

Mechanism of the reaction

In the light of the above considerations, it seems reasonable to presume that at least those e.p.r.-signal-intensity changes that we have observed within the turnover time of nitrogenase in rapid-freezing experiments are due to oxidation–reduction reactions of the functional enzyme components. Our results are fully in agreement with the mechanism of Scheme 1, with Kp2 protein cycling between its reduced signal-giving form and the oxidized signal-free form, whereas Kp1 protein cycles between the very highly reduced signal-free state IV and the more oxidized signal-giving state S (Scheme 2*a*). Both rapid-freezing and manual mixing results support this scheme. Thus, in rapid freezing, both signals decreased together, as expected for reaction (i) of Scheme 2(*a*). (Incomplete bleaching could be due to reversibility of this reaction.) Similarly, when the supply of dithionite ran out in manual experiments, so slowing reaction (iii), then both proteins became progressively more oxidized. The conformation change in reduced Kp2 protein (cf. Zumft *et al.*, 1972, 1973) produced by ATP and Mg^{2+} ions and indicated by the change in e.p.r. spectrum presumably somehow facilitates the transfer of reducing equivalents between the proteins (reaction i). The small effects we have seen of oxidizing substrates on the form of the Kp1 protein signal further confirms that these substrates interact with this protein (reaction ii).

Much further work would be needed to establish rate constants for all the reactions of Scheme 2(*a*). However, the rate of the decrease in the signals of Kp1 and Kp2 proteins in the experiments of Fig. 8(*a*) would imply a minimum second-order rate constant



Scheme 2. *Alternative forms of Scheme* 1

Kp1 cycles between ...	Oxidized form	Reduced form
(*a*)	State S	State IV
(*b*)	State III	State S

Reaction pathways. In the reaction schemes above, $[1_s]$ and $[2_s]$ refer to the signal-giving forms of Kp1 and Kp2 proteins respectively. The corresponding symbols with the subscript 0 refer to signal-free forms. Thus, $[2_0]$ is oxidized Kp2 protein and $[1_0]$ is Kp1 protein in state IV for (*a*) but in state III for (*b*). For simplicity, a long-lived complex between the proteins is indicated, but this is not obligatory.

for reaction (i) of the order of $10^6 M^{-1} \cdot s^{-1}$. The still faster rate seen when pre-mixed Kp1 and Kp2 proteins were treated with ATP (Fig. 8*b*) is presumably due to association between the proteins being slower than electron transfer within a complex between them. Smith *et al.* (1972) suggested that reaction (ii) might be rate-limiting in enzyme turnover. However, it is obvious that in a complex mechanism, possibly involving several second-order reactions, no one step will remain rate-limiting under all conditions.

Though the general picture from steady-state experiments performed with manual mixing techniques is consistent with Scheme 2(*a*), quantitative aspects are less readily interpreted. On our simple theory, decreasing the Kp2/Kp1 molar ratio within the stoicheiometric range should decrease the bleaching of Kp1 protein signal substantially. The magnitude of the observed effects was small, however. The explanation of this and the fact that greater Kp1 protein signal bleachings were observed in manual than in rapid-freezing experiments is not certain. Part of the answer might lie in the purity of the preparations (cf. integration of the Kp2 protein signal). Additionally, slow equilibration of 'surplus' Kp1 or Kp2 protein with a long-lived functional complex between stoicheiometric amounts of the two proteins could readily explain the slow changes. In any detailed mechanism the possible roles in the observed phenomena of additional abortive complexes among the proteins in their various oxidation states must also be considered (cf. inhibition of acetylene reduction assays at high Kp1/Kp2 ratios). Though abortive complexes could readily be accommodated into various kinetic models for the enzyme, it seems premature to do so until further work has been carried out.

Reasons for rejection of an alternative reaction mechanism

As noted above, our data are compatible with Scheme 2(*a*), which is the one proposed by Smith *et al.* (1972) and Orme-Johnson *et al.* (1972*a*). In contrast, Mortenson and co-workers (Zumft *et al.*, 1972; Mortenson *et al.*, 1973*a*,*b*) have favoured a more complicated mechanism, which we have simplified to Scheme 2(*b*); the schemes shown in Scheme 2 are the two simplified ones. Deciding unequivocally between them is surprisingly difficult, largely because an oxidizing substrate for nitrogenase (the proton) is always present in all experiments.

First, we must consider the experiments of Smith *et al.* (1972). In these experiments the reduction of Kp1 protein in state III by reduced Kp2 protein was studied in the presence of ATP and Mg^{2+}. This is reaction (v) of Scheme 2(*b*). According to Scheme 2(*a*), reaction (i), the Kp1 protein ought to be reduced by this system, through state S, on, at least in part, to state IV. Unfortunately the original experiments, which were only semi-quantitative and semi-anaerobic, did not determine the precise extent of conversion into state S (or state IV). However, Mortenson *et al.* (1973*b*) suggested that step (v) of Scheme 2(*b*) was rate-limiting, whereas Smith *et al.* (1972) followed this reaction directly and reported a half-time of the order of 10 ms. This clearly means that reaction (v) is not rate-limiting and to this extent the mechanism of Mortenson *et al.* (1973*b*) must be in error.

We must now consider whether other variants of Scheme 2(*b*) are acceptable. Concerning more recent rapid-freezing data, our results in Fig. 8 may be reconcilable with Scheme 2(*b*), by making assumptions about the rates and reversibility of the various reaction steps. Nevertheless, there are still problems in reconciling our data with those of Mortenson and also in reconciling Mortenson's own data with his scheme (see Mortenson *et al.*, 1973*b*). Scheme 2(*b*) becomes consistent with our observed rapid and simultaneous decreases in both e.p.r. signals (Fig. 8), if a fast reaction (iv) is followed by an even faster and reversible reaction (v), leaving reaction (vi) as the rate-limiting step of the turnover. Smith *et al.* (1972) showed that reaction (v) was rapid but a more precise rate determination would be needed to decide whether or not the reaction is sufficiently fast, in relation to step (iv), to account for simultaneous decreases of the signals. However, Zumft *et al.* (1972) and Mortenson *et al.* (1973*b*) reported, for the enzyme from *Clostridium*, not simultaneous decreases of the signals as we found, but faster disappearance of the signal of the iron protein than of that from the iron–molybdenum protein. This is the reverse of what would be expected from Scheme 2(*b*), assuming now that step (v) is not faster than step (iv). The significance of this work, in relation either to our work or to Scheme 2(*b*), is therefore not clear.

Another point against Scheme 2(*b*) is that it postulates that the normally stable state S of Kp1 protein (which must have a higher redox potential than dithionite; see Smith *et al.*, 1972) can be induced to reduce protons (or N_2) by entering into complex formation with Kp2 protein and ATP. This, too, may seem improbable since the e.p.r. chromophore of Kp1 protein is quite uninfluenced either by Kp2 protein or by ATP on their own. If, in combination, they produce so drastic an effect on the reactivity of Kp1 protein, then at least one of them might have been expected to affect its chromophore, if only slightly.

Finally, against Scheme 2(*b*) we have the inherent improbability that led Smith *et al.* (1972) to propose Scheme 2(*a*). This improbability stems from the experimental finding that, as the supply of dithionite becomes exhausted, so Kp1 protein gradually reverts to state S. Scheme 2(*b*) would require that Kp1 protein should become fully reduced as reaction (vi)

slowed to zero, whilst Kp2 protein became fully oxidized. Increasing reduction of any component as the supply of dithionite, the ultimate source of reducing equivalents, becomes exhausted, seems unusual. In sharp contrast to our findings on this point, Mortenson and co-workers (Zumft *et al.*, 1972; L. E. Mortenson, Joint Meeting of the Biochemical Society Molecular Enzymology Group and the British Biophysical Society, Oxford, September 1972, unpublished work) originally stated that the state S signal from the enzyme from *Clostridium* remains largely bleached when dithionite becomes exhausted from the system. However, this observation, though obviously compatible with Scheme 2(*b*), is apparently not correct (L. E. Mortenson, personal communication).

There are no difficulties, comparable with the above, in accepting Scheme 2(*a*). Our kinetic findings in both manual mixing (Fig. 6) and rapid-freezing experiments (Fig. 8) are simply and obviously consistent with Scheme 2(*a*). The only problem is that the very highly reduced state of Kp1 protein, state IV, has not so far been reached by non-enzymic means (see below). However, Mössbauer-effect spectroscopy (B. E. Smith & G. Lang, unpublished work; see also Eady *et al.*, 1973) provides additional support for its existence.

Chemistry of the reactions

The precise chemical reactions involved in nitrogenase action and the reaction mechanisms are in no sense understood. It seems that ATP in the presence of Mg^{2+} produces a conformation change in the reduced Kp2 protein (cf. Zumft *et al.*, 1972), which in some manner permits it to reduce state S of Kp1 protein to the highly reduced form, state IV. This reduction step cannot be achieved by dithionite alone and has not been demonstrated by any chemical means, though it is possible that strong reducing agents, e.g. the hydrated electron, might prove capable of carrying it out. Clearly, however, much further work remains to be done, since even the point in the catalytic cycle at which ATP is hydrolysed is entirely unknown.

Since the e.p.r. signal from Kp1 protein that changes in intensity during the catalytic cycle is due to iron rather than to molybdenum, this implies that iron is involved in electron transfer to the nitrogen molecule during nitrogenase action. Although the form of the signal is also modified slightly by acetylene and protons, nevertheless, by analogy with other iron–sulphur proteins, oxidizing substrates are probably not bound to iron in Kp1 protein. The role of molybdenum in the enzyme remains problematical. In view of the finding of Chatt *et al.* (1972) that dinitrogen bound to molybdenum can be reduced to bound N_2H_2, this metal might help in some way to stabilize the binding of the nitrogen molecule and then its intermediate reduction products, until all six reducing equivalents have been transferred to it, via iron systems.

Work by R. C. B. and D. J. L. was supported by a Programme Grant from the Medical Research Council. We thank Professor J. R. Postgate for comments on the manuscript and Miss Alison McGechie and Mrs. Anne Funnell for technical assistance.

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