Biochimica et Biophysica Acta, 462 (1977) 501–514

BBA 47406

EPR STUDIES OF HIGHER PLANT MITOCHONDRIA

I. UBISEMIQUINONE AND ITS RELATION TO ALTERNATIVE RESPIRATORY OXIDATIONS

PETER R. RICH, ANTHONY L. MOORE, W. JOHN INGLEDEW and WALTER D. BONNER, Jr.

Johnson Research Foundation and Department of Biochemistry and Biophysics, University of Pennsylvania, Philadelphia, Pa. 19174 (U.S.A.)

(Received May 2nd, 1977)

SUMMARY

An EPR investigation of the region of the higher plant respiratory chain involving ubiquinone and Center S-3 of succinate dehydrogenase is reported. At temperatures close to those of liquid helium, first derivative spectra corresponding to Center S-3 ($g_{max} = 2.017$) and a signal split around $g = 2.00$ (major features of peaks and troughs at g values of 2.045, 2.03, 1.985, 1.97 and 1.96) were observed in mung bean (*Phaseolus aureus*), *Arum maculatum* spadix, *Sauromatum guttatum* spadix and tulip bulb (*Tulipa gesnerana*) mitochondria. The split signal was small or absent in potato tuber and *Symplocarpus foetidus* spadix mitochondria.

The redox behavior of these signals in mung bean mitochondria in a variety of respiratory steady-state conditions suggested that the components giving rise to them were an integral part of the respiratory chain and were located on the substrate side of coupling Site II. The split signal could be removed by addition of hydroxamic acids in all tissues tested, although the K_s of this effect was an order of magnitude higher than the K_i of inhibition of the alternative respiratory pathway in mung bean and *Sauromatum guttatum* spadix mitochondria.

The results are discussed in relation to the current ideas on the ordering of components in the region around the classical Site II of the respiratory chain and in relation to the location of the alternative respiratory oxidase pathway of higher plants.

INTRODUCTION

The application of the technique of electron paramagnetic resonance (EPR) spectroscopy has greatly advanced knowledge upon the types, nature and number of animal mitochondrial electron transport components. A variety of iron sulfur centers have been described, being paramagnetic in their reduced (Center N-1a, N-1b, N-2 to N-5, "Center bc-2" or "Center 5", Rieske's, S-1 and S-2) or oxidized (S-3 and Ruzicka's) forms [1–6]. The species may be distinguished by utilizing differences in spectroscopic, redox potentiometric and temperature and power saturation profile

properties. Further more complex signals have also been observed, in particular a split signal centered around $g = 2.00$, which has been attributed to an interaction of quinones in the partially reduced form and HiPIP-type Center S-3 of succinate dehydrogenase in its oxidized form [7, 8].

Many higher plant mitochondria are known to possess a cyanide- and antimycin A-insensitive alternative route of substrate oxidation (for reviews see refs. 9 and 10) and suggestions of a role of iron sulfur centers in these pathways have been made. These suggestions have come from inhibition studies with metal chelators [11], hydroxamic acids [12] or piercidin A and 2-thenoyltrifluoroacetone [13]. However, EPR studies to try to find a paramagnetic component uniquely associated with this alternative pathway have been rather negative, although the finding of an elevated level of Center N-1 in some cases has been suggested to have significance in this respect [14, 15].

Since the branchpoint of the alternative respiratory pathway of higher plants is thought to involve the $QH^{\cdot}/QH_2$ couple which is associated with succinate dehydrogenase [16], the EPR signal reported to involve semiquinone species was of great interest as a possible means of studying the alternative respiratory pathway. It was found that a signal resembling, but more complex than, that observed in animal mitochondria could be observed in a variety of plant mitochondria (mung bean, tulip bulb, *Sauromatum guttatum* spadix, *Arum maculatum* spadix), although it was apparently in low amounts or absent from potato or skunk cabbage (*Symplocarpus foetidus*) mitochondria. The signal was observed in resting mitochondria, and in mitochondria in a variety of respiratory steady states and its behavior suggested a location on the substrate side of coupling Site II. Salicylhydroxamic acid (an inhibitor of several types of oxidases [12]) abolished these signals in all mitochondria tested. 2-Thenoyltrifluoroacetone also removed the signals. Similar effects of salicylhydroxamic acid upon the analogous signal in pigeon heart mitochondria were observed.

The relation of this signal and its properties to our current state of knowledge on the nature of alternative respiratory oxidations is discussed. A preliminary report of part of this work has already been presented [17].

MATERIALS AND METHODS

Plant materials. Etiolated mung bean hypocotyls (*Phaseolus aureus*) were grown for 5 days in a dark room maintained at 28 °C and 60 % relative humidity. *Symplocarpus foetidus* inflorescences were collected from swamp areas in Pennsylvania in February and March and *Sauromatum guttatum* inflorescences were grown indoors from dry corms. *Arum maculatum* inflorescences were collected from Cambridgeshire, England, during April and May. Tulip bulbs (*Tulipa gesnerana* var. Darwin) and potato tubers (*Solanum tuberosum*) were purchased locally.

Preparation of mitochondria. Mitochondria were prepared as described by Bonner [18]. In the case of *S. guttatum* and skunk cabbage spadices, EDTA concentration was doubled to 2 mM in the homogenisation medium and bovine serum albumin was increased to 0.5 % (w/v) in both homogenisation and resuspension media.

EPR measurements. Samples for EPR measurements of mitochondria in various metabolic states were frozen rapidly in an isopentane/methylcyclohexane (5 : 1, v/v) freezing mixture (81 °K), and stored in liquid nitrogen until assayed.

All spectra were obtained with a Varian E-4 or E-109 EPR spectrometer (Varian Associates). The temperature of the samples for EPR measurements was controlled with a variable temperature cryostat (Air Products Model TLD-3-110). Temperature was monitored with a calibrated carbon resistor placed in the helium flow directly below the sample and g values were corrected by reference to a weak pitch standard. Quartz glass EPR sample tubes were calibrated with a standard copper sulphate/EDTA solution and values for signal heights were corrected correspondingly.

Relative quantitation was performed by comparison of the double integrals of the appropriate signals which had normally been signal averaged. Signal averaging and double integrations were carried out with a Nicolet signal averager (NIC-1074).

Steady-state experiments. Steady-state experiments were carried out with oxygen-saturated resuspension medium A (0.3 M mannitol, 10 mM KCl, 5 mM $MgCl_2$ and 10 mM potassium phosphate at a pH of 7.2 [18]). The concentrations of reagents added were: succinate, 10 mM; NADH, 1 mM; malate, 20 mM; ATP, 0.3 mM; ADP, 1.0 mM; KCN, 1 mM; antimycin A, 1 μg/ml; salicylhydroxamic acid, 1 mM; dinitrophenol, 20 μM. Enough time was allowed (generally 10–15 s for 3 mg/ml protein) so that equilibrium had been reached but anaerobiosis had not begun. Oxygen was blown over the surface of the liquid during the incubation.

The salicylhydroxamic acid stock solution was 5 mM in medium A, dinitrophenol was 2 mM in medium A, and both were in the absence of organic solvents.

Others. Standard chemicals used were of the highest grade available commercially. Protein was measured by the method of Lowry et al. [19].

RESULTS

The nature of the novel EPR signal in a variety of higher plant mitochondria

EPR investigations at temperatures approaching that of liquid helium revealed a complex signal split around $g = 2.00$ which appeared both in oxidized and partially oxidized states. The splitting was observed in mung bean (*Ph. aureus*), *A. maculatum* spadix, *Sa. guttatum* spadix and tulip bulb (*T. gesnerana*) mitochondria, but was small or absent from potato and skunk cabbage (*Sy. foetidus*) spadix mitochondria. Fig. 1 illustrates the signals or their absence in resting mitochondria. In the first derivative spectra illustrated, features were observed at g values of 2.045, 2.03, 2.02, 1.985, 1.97 and 1.96 in mung bean, *A. maculatum* and *Sa. guttatum* mitochondria. The extreme low field features ($g = 2.045$, 2.03) appeared to be slightly modified in mitochondria of tulip bulbs. The signal centered at $g = 2.01$ and having a maximum at $g = 2.017$ was comparable to the HiPIP-type species observed in mammalian mitochondria [6] under similar conditions, and was identified as Center S-3, an iron sulfur center associated with succinate dehydrogenase. Table I illustrates that a correlation between the presence of a split signal and the presence of an alternative respiratory pathway does not exist.

It was found that the split signal was completely destroyed by addition of a small quantity of ethanol (< 0.1 %, v/v), without the appearance of any new signals at these low (8 °K) temperatures. Use was made of this to obtain the first derivative spectrum of the split signal alone in the case of *Sa. guttatum* mitochondria (Fig. 2). The spectrum is split around $g = 2.00$ and is analogous to the computer-simulated spectra obtained by Ruzicka et al. [7] for a dipolar interaction between a paramagnet-

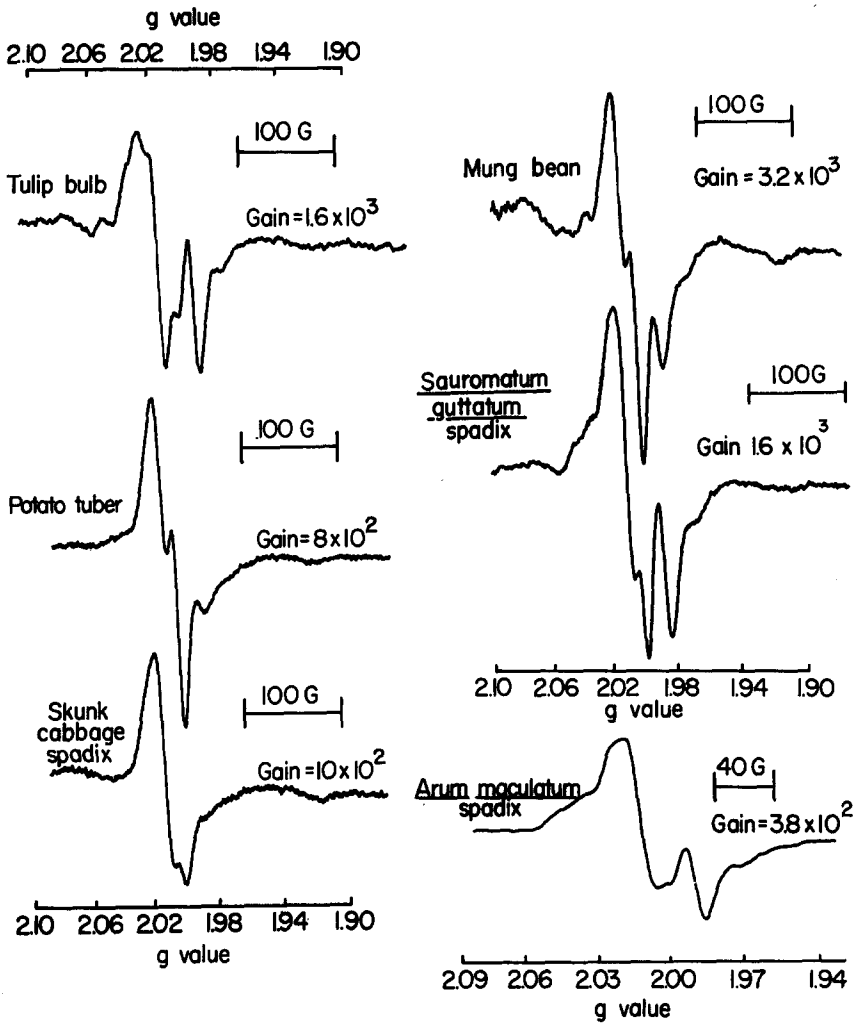

Fig. 1. First derivative EPR spectra at 8 °K of aerobic mitochondria from a variety of higher plant tissues. Mitochondria were resuspended in medium A [18] to an appropriate protein concentration, gassed with oxygen for 15–30 s and frozen rapidly in liquid nitrogen. Conditions were: microwave power, 5 mW; modulation amplitude, 12.5 G; microwave frequency, 9.18 GHz; temperature, 8 °K. The signal heights are abitrary units, but are identical at the same gain for all samples. Protein concentrations were: tulip bulb, 12.5 mg/ml; potato tuber, 15–20 mg/ml; skunk cabbage, 6 mg/ml; mung bean, 5 mg/ml; *Sa. guttatum*, 5.5 mg/ml; *A. maculatum*, 14 mg/ml.

ic species and ubisemiquinone. In the case of mung bean, *Sa. guttatum* spadix and *A. maculatum* spadix an extra peak on the low field side and a small trough on the high field side of the spectrum is also seen. Some small change in a $g = 2.00$ feature is also appearing in these difference spectra, causing some deviation from the computer-simulated spectra of Ruzicka et al. [7].

In the case of the *Sauromatum* system, the double integral of the spectrum in the presence of ethanol (presumably mostly Center S-3) compared to the double

TABLE I

A SURVEY OF DEGREE OF CYANIDE INSENSITIVITY OF RESPIRATORY ACTIVITY AND PRESENCE OF A SPLIT SIGNAL IN A VARIETY OF HIGHER PLANT MITOCHONDRIA

State 3 oxygen consumption was measured with an oxygen electrode with medium A [18], succinate (10 mM), ATP (0.3 mM), ADP (1 mM) and mitochondria at a suitable protein concentration. 1 mM KCN was then added and the residual oxygen consumption was compared to the uninhibited State 3 rate. The ratio of spins of S-3 and split signal were measured in rapidly frozen aerobic mitochondria at 8 °K as shown in the legend to Fig. 2.

Tissue	Percentage of State 3 respiratory oxygen consumption insensitive to 1 mM KCN	Center S-3 : split signal spin ratio
Mung bean (*Phaseolus aureus*)	15–25 %	1.0 : 0.6
Sauromatum guttatum spadix	100 %	1.0 : 0.9
Arum maculatum spadix	100 %	1.0 : 1.0
Symplocarpus foetidus spadix	70–95 %	1 : < 0.1
Potato tuber (*Solanum tuberosum*)	< 2 %	max. of 1 : 0.1
Tulip bulb (*Tulipa gesnerana*)	< 2 %	1.0 : 0.9

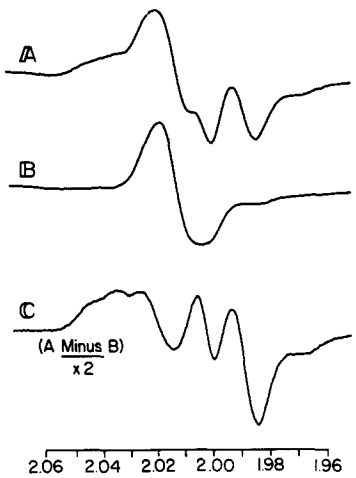

Fig. 2. First derivative EPR Spectra of Center S-3 and the split signal in *Sa. guttatum* mitochondria. *Sa. guttatum* mitochondria were suspended in medium A to a final protein concentration of 5.8 mg/ml and pure oxygen was blown over the liquid surface for 30 s. Sample A was then transferred to a quartz EPR tube and frozen rapidly in liquid nitrogen. 0.1 % (v/v) ethanol was added to the remaining sample and this was similarly frozen in an exactly matching quartz EPR tube (sample B). The spectra shown have been computer-averaged eight times. Spectrum C is the computer-derived difference spectrum of A minus B and the gain is double that of spectra A and B. Conditions for EPR measurement were: microwave power, 5 mW; modulation amplitude, 12.5 G; frequency, 9.18 GHz; temperature, 8.5 °K; scan rate, 400 G/min; gain $8 \cdot 10^2$.

integral of the split signal alone, gave a stoichiometry of about 1.0 to 0.9 for the spins of Center S-3 compared to the spins of the split signal, respectively. The spin ratios for other tissues are summarized in Table I and the maximal ratio observed was 1 : 1. It is thought that the splitting of the semiquinone which is relaxed by Center S-3 is caused by a second, closely located semiquinone which relaxes more slowly. Hence, at low (8 °K) temperature, one expects to detect only the split semiquinone spin in these experiments, i.e. only one spin per semiquinone pair. This means that a ratio of 1 : 1 indicates one semiquinone pair per one Center S-3.

The temperature dependencies of the $g = 2.045$, 2.03, 2.02, 1.985, and 1.97 signals were compared. At 5 mW of microwave power, all these signals were maximal at about 9 °K and were virtually undetectable by 25 °K. The microwave power dependencies are illustrated in Fig. 3. Since they all saturate at the same power level ($\approx$ 10 mW) at the given temperature, the species giving rise to these signals must have similar relaxation rates, as has been shown to be the case in animal systems [8, 20]. Also included in Fig. 3 is the power dependency of the $g = 1.923$ signal. This does not

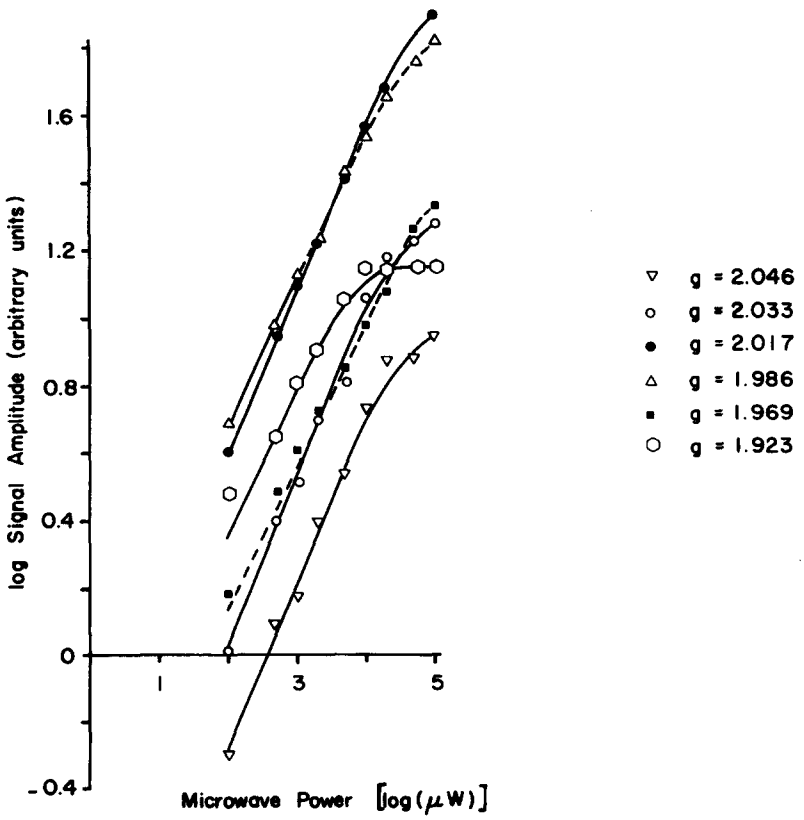

Fig. 3. Temperature power profile of the mung bean center S-3 and split features. Mung bean mitochondria were suspended in medium A [18] to a final protein concentration of 33 mg/ml. The microwave power was varied at a constant temperature of 12 °K. Other conditions for EPR measurement were: modulation amplitude, 12.5 G; microwave frequency, 9.13 GHz; scan rate, 400 G/min.

TABLE II

THE REDOX BEHAVIOR OF CENTER S-3 AND THE SPLIT SIGNAL IN MUNG BEAN MITOCHONDRIA RESPIRING IN VARIOUS STEADY STATES

Mung bean mitochondria (generally 5 mg protein/ml final concentration) were suspended in medium A [18] containing appropriate concentrations of reactants (see Materials and Methods). Pure oxygen was blown across the surface of the suspension for 15–30 s to ensure that equilibrium had been reached but anaerobiosis had not begun. Samples were then quickly transferred to quartz EPR tubes and frozen rapidly in freezing mixture at 81 °K. The redox state of Center S-3 was measured as the percentage of the $g = 2.017$ peak remaining and the split signal as the percentage of the $g = 1.985$ trough remaining.
Values are average of three experiments and are accurate to ± 15 % of aerobic (control) value.

Conditions	Percentage of aerobic signal remaining	
	Center S-3 ($g = 2.017$ peak height)	Split signal ($g = 1.985$ trough)
Control (aerobic)	100	100
+succinate/ATP	104	57
+succinate/ATP/ADP	70	99
+succinate/ATP/dinitrophenol	78	98
+succinate/ATP/KCN	95	34
+succinate/ATP/SHAM*	91	0
+succinate/ATP/SHAM*/KCN	67	0
+succinate/ATP/ADP/anaerobic	17	0
+NADH	105	6
+NADH/ADP	109	42
+NADH/dinitrophenol	119	24
+NADH/KCN	107	0
+NADH/SHAM*	109	0
+NADH/SHAM*/KCN	66	0

* SHAM, 1 mM salicylhydroxamic acid.

have the same power saturation profile as the other five signals and is therefore not part of the same species. The $g = 1.923$ signal is possibly due to Center N-2 (plus S-1), a center associated with the NADH dehydrogenase complex, which is paramagnetic in the reduced form (in contrast with HiPIP species [21]) and the $g_{\parallel}$ value corresponding to the $g_{\perp} = 1.923$ is smaller and broader than its equivalent and is at $g = 2.05$.

In all tissues tested, the split signal was very sensitive to membrane intactness. For example, the signal rapidly diminished as the mitochondria aged at 0 °C (a significant decrease could be observed in just 2 h) and it was small or absent in frozen/thawed mitochondria or in submitochondrial particle preparations.

It should be noted that the absence or loss of the split signal could have several causes, and does not necessarily imply the absence of semiquinone species. For example, any treatment which causes full oxidation or reduction of either quinone, or any treatment which moves the interacting species apart, would cause the splitting to disappear.

The behavior of the signals under a variety of steady-state conditions in mung bean mitochondria

In order to try to gain an understanding of the nature and function of these signals, mung bean mitochondria were rapidly frozen while respiring under a variety of steady-state conditions. A maximally oxidized state of the HiPIP-type Center S-3 signal was taken as that present in aerobic mitochondria in the absence of added substrate (Fig. 4A). It was noticed, however, that addition of 500 μM ferricyanide caused

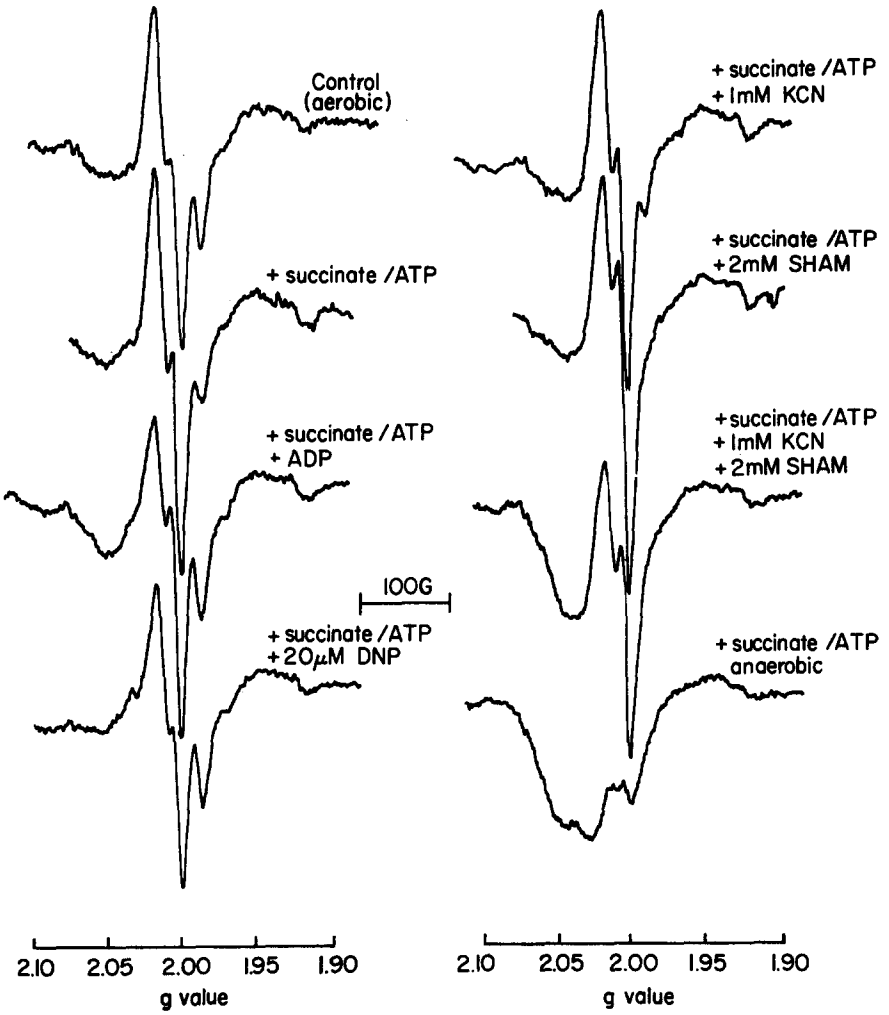

Fig. 4. EPR Spectra of mung bean mitochondria trapped in a variety of steady-state respiratory conditions. Mung bean mitochondria were suspended to an appropriate protein concentration (generally around 5 mg/ml) in medium A [18] to which had been added the appropriate reagents (see Materials and Methods for details). Oxygen was blown over the suspension for 15–30 s before it was transferred to a quartz EPR tube and frozen rapidly in a freezing mixture at 81 °K. Conditions of measurement were: modulation amplitude, 12.5 G; microwave frequency, 9.1 GHz; microwave power, 5 mW; temperature, 8 °K; scan rate. 500 G/min; gain, 10^4.

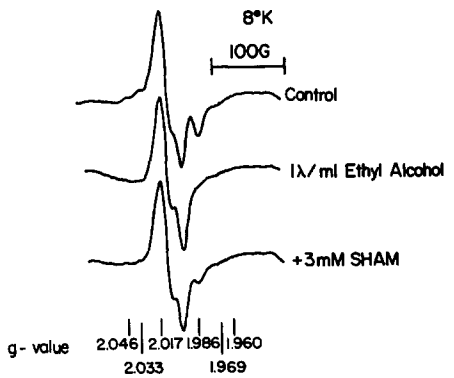

Fig. 5. The effects of ethanol and salicylhydroxamic acid on the low temperature EPR spectrum of aerobic mung bean mitochondria. Mung bean mitochondria were suspended in medium A to a final protein concentration of 15–20 mg/ml. Oxygen was blown over the sample surface for 1 min before the control sample was transferred to a quartz EPR tube and frozen rapidly in a freezing mixture at 81 °K. The other samples were prepared similarly after addition of 0.1 % (v/v) ethanol or 3 mM salicylhydroxamic acid (in the absence of ethanol). Conditions for EPR measurements were: modulation amplitude, 12.5 G; microwave frequency, 9.1 GHz; microwave power, 5 mW; temperature, 8 °K; scan rate, 500 G/min; gain, $1.25 \cdot 10^3$.

a slight increase of Center S-3 over this aerobic level. The split signals were extremely labile and were completely destroyed on addition of 0.1 % (v/v) ethanol or acetone (Fig. 5). Hence, all steady-state experiments were performed in the absence of organic solvents, and all reagents were dissolved in aqueous media.

When NADH was used as substrate (via the externally facing inner membrane dehydrogenase [22]), 90–95 % of the split signals disappeared, when mitochondria were respiring in State 4, 60 % in State 3 and 75 % in uncoupled State 3. In the presence of NADH and the respiratory inhibitor KCN, none of the split signals could be observed at all. HiPIP-type Center S-3 was not reduced in States 3 or 4, nor in the presence of KCN or antimycin A, but could be significantly reduced on anaerobiosis or in the presence of KCN plus salicylhydroxamic acid.

Succinate removed about 40 % of the split signals in State 4, but not in State 3 or the uncoupled state. This latter result is in contrast to that which we reported previously, because in this case we had no artifact from ethanol addition. The addition of 1 mM KCN with succinate as substrate caused about 65 % of the split signals to disappear. Center S-3 remained mostly oxidized in States 3 and uncoupled State 3, fully oxidized in State 4 or in the presence of KCN and 5–25 % oxidized upon anaerobiosis with succinate as substrate. A typical example of the spectra seen in these experiments with mung bean mitochondria is presented in Fig. 4.

The effects of antimycin A on the split signal were not investigated because of the problem of insolubility of this compound in aqueous media.

When malate was used as substrate, results similar to those obtained with succinate were found. Table I summarizes the redox states of Center S-3 and the component giving rise to the split signals in the various respiratory steady states described.

In highly cyanide-insensitive mitochondria, such as those of *Sa. guttatum* spadix, the split signal remained maximal even in the presence of succinate plus KCN,

and greater than 50 % oxidized with NADH plus KCN present. This indicates that the split component can be oxidized by the alternative oxidation step and therefore is a good candidate for the "branchpoint" of the main and alternative pathways.

EPR-detectable effects of salicylhydroxamic acid

It was found that salicylhydroxamic acid could be dissolved in medium A [18] to a concentration of 5 mM without the aid of organic solvents and this stock solution was used to test its effects on the signals. Addition of salicylhydroxamic acid to aerobic mitochondria resulted in a disappearance of the signals split around $g = 2.00$ (Fig. 5). The titration curve of this effect for mung bean mitochondria is illustrated in

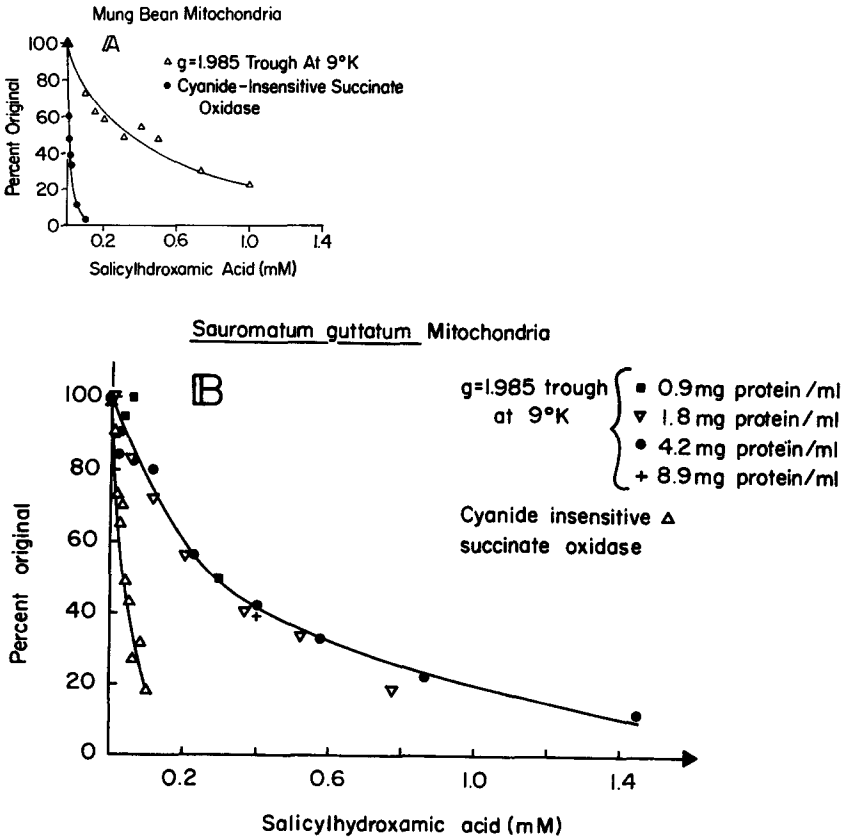

Fig. 6. A comparison of the effects of salicylhydroxamic acid on alternative respiratory oxygen consumption and on the removal of the split signal. Alternative respiratory oxygen consumption was measured with an oxygen electrode. The mitochondria were suspended in air-saturated medium A, generally to a protein concentration of around 1–2 mg/ml, in the presence of 1 mM KCN and 0.3 mM ATP. Oxygen consumption was measured after the addition of 10 mM succinate and sufficient time was allowed so that a maximal rate had been attained. Various salicylhydroxamic acid concentrations in the absence of ethanol were added before substrate. The split signal was measured as the $g = 1.985$ trough size at 8 °K. Salicylhydroxamic acid was added in the absence of organic reagents and protein concentration was either 5–10 mg/ml (mung bean) or variable (*Sauromatum*). Other EPR conditions were: modulation amplitude, 12.5 G; microwave frequency, 9.18 GHz; microwave power, 5 mW.

Fig. 6A and the concentration required for half-maximal effect was approx. 200 μM salicylhydroxamic acid. This value is rather higher than the K_i of 30 μM for inhibition of succinate-driven oxygen consumption by the alternative respiratory pathway (determined in a concurrently-run experiment). In some cases salicylhydroxamic acid did not completely remove the split signal, even at concentrations of up to 2 mM, but, this residual signal was never more than 20 % of the original amount.

A similar result was obtained with *Sa. guttatum* spadix mitochondria (Fig. 6B) and in this case it can be seen that the difference in K_s of split signal removal and the K_i of the alternative oxidase activity is not caused by the difference in protein concentration of the oxygen consumption and EPR experiments. The discrepency between the measured values of K_i and K_s may be caused by the fact that the K_i was measured under steady-state respiratory conditions whereas the K_s was measured in the aerobic (oxidized) state. Salicylhydroxamic acid inhibits phenol oxidase reactions, for example, by competitive inhibition with respect to the hydrogen-donating substrate (Rich, P. R., unpublished data). If such were the case in mitochondria, one would expect an effect of the redox state of the carrier with which it competes on the K_i of inhibition.

A further effect of salicylhydroxamic acid was observed on the redox state of mung bean Center S-3 in the aerobic steady state in the presence of cyanide. It was found that the addition of 1 mM salicylhydroxamic acid increased the level of reduction of the Center S-3 signal from about 0 to 35 % with NADH (plus 1 mM KCN) as substrate and from 10–20 % to 30–60 % with succinate or malate (plus 1 mM KCN) as substrate. These effects were much more dramatic in *Sa. guttatum* spadix mitochondria.

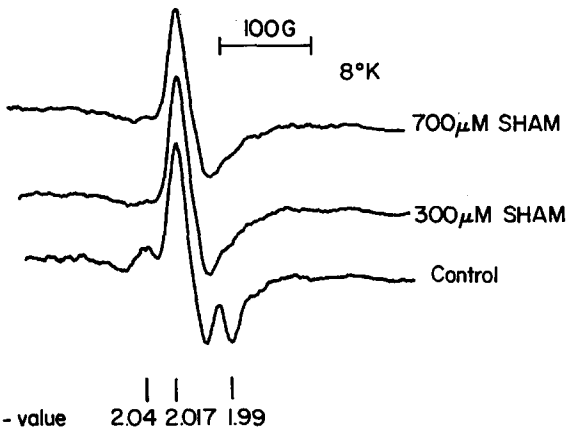

Fig. 7. The effect of salicylhydroxamic acid on pigeon heart submitochondrial particles. Pigeon heart submitochondrial particles [3] were resuspended to a final protein concentration of approx. 11 mg/ml in 100 mM sucrose, 50 mM *N*-2-hydroxyethylpiperazine-*N*-'2-ethanesulfonic acid (HEPES) and 1 mM $MgCl_2$ at pH 7.5. Anaerobiosis was attained with a flow of argon gas and in the presence of duroquinone, pyocyanine, phenazine methosulphate and phenazine ethosulphate (20–50 μM range of concentrations). The potential of the system was set at +90 mV with 2 mM sodium ascorbate. The control sample was anaerobically extracted into a quartz EPR tube and frozen rapidly in liquid nitrogen. The other samples were similarly taken after the addition of appropriate amounts of salicylhydroxamic acid (in ethanol, which does not destroy the mammalian split signal). Conditions for EPR measurement were: modulation amplitude, 10 G; microwave frequency, 9.1 GHz; microwave power, 10 mW; temperature 8.5 °K; scan rate, 250 G/min; gain, $5 \cdot 10^2$.

Salicylhydroxamic acid also removed the signals at $g = 2.04$ and 1.96 which have been observed in animal mitochondria and submitochondrial particles [7, 8] and which are considered to be the counterparts of those that we have observed in the higher plant systems. Fig. 7 illustrates this effect in pigeon heart submitochondrial particles, although the effect was also observed on the very small split signal which we found in rat liver mitochondria. The K_s of the effect was similar to that for the higher plant systems.

2-Thenoyltrifluoroacetone, which apparently has inhibitory properties both on succinate oxidations and on the alternative respiratory pathway [24–26], also removed the split signals. Ingledew and Ohnishi (unpublished data) have found that this compound removes the split signal in mammalian systems.

DISCUSSION

The work described in this paper is an EPR study of the region of the respiratory chain where succinate dehydrogenase connects to the main ubiquinone pool. The signal centered at $g = 2.01$ and peaking at $g = 2.017$ has been attributed to Center S-3 of succinate dehydrogenase [6] (the second HiPIP center, isolated by Ruzicka and Beinert [27], is apparently not present or at very low levels in mung bean and skunk cabbage spadix mitochondria, Rich, P. R., unpublished data). The partially overlapping signals at g values of 2.045, 2.03, 1.985, 1.97 and 1.96 are interpreted as a pair of ubisemiquinone radicals which split each other because of a spin-spin interaction between one of the pair and the rapidly relaxing Center S-3 of succinate dehydrogenase (cf. Ruzicka et al. [7] and Ingledew et al. [8]). This explains the experimental observations that the split signal components have the same temperature and power saturation properties as Center S-3. The fact that the maximal observable ratio of Center S-3 spins: semiquinone pairs is about 1 : 1 would also agree with this interpretation. This finding of an apparently stabilized semiquinone species which interacts with Center S-3 of succinate dehydrogenase has significance with respect to the possible functioning of a proton-motive ubiquinone cycle mechanism, as proposed by Mitchell [28]. In these schemes, the semiquinone species of ubiquinone plays a key role. Since this stabilized semiquinone species is at most stoichiometric with Center S-3, which in its turn is significantly less than stoichiometric with respiratory chains, it is not surprising that it would be overlooked in studies on the total ubiquinone pool, which is much in excess of respiratory chains [29].

The behavior of the split signal in a number of steady-state conditions has been investigated, in the hope that some light might be shed on its role in the electron transport pathway. In all cases it behaved as a single component. It could be reduced by all three substrates tested and its level of reduction in State 4 was greater than in State 3 or the uncoupled state. Hence, it appears that the component(s) giving rise to the spectrum is indeed part of, or in rapid equilibrium with, the main respiratory chain. Further, its redox behavior in various steady states is consistent with its being on the substrate side of coupling Site II and also with its being the site of the branchpoint of the alternative respiratory pathway from the main chain (refs. Bahr and Bonner [30, 31]), as we have previously suggested from more theoretical considerations [16].

It is of interest that Center S-3 remained largely oxidized in the steady-state

experiments described, being significantly reduced only when substrate was added in the presence of both cyanide and salicylhydroxamic acid, or under anaerobic conditions. This is somewhat in contrast to the results obtained with pigeon heart mitochondria (cf. Ohnishi et al. [3]) and may in part reflect a close location of Center S-3 to the "alternative oxidase", although it cannot explain why addition of salicylhydroxamic acid did not cause a much more dramatic effect on its redox state. The results may reflect the fairly slow activation of succinate dehydrogenase activity on addition of substrate, as is seen by the slow onset of maximal oxygen consumption when succinate is added to mung bean mitochondria, even in the presence of ATP. A more detailed examination of the redox behavior of Center S-3 in a variety of higher plant mitochondria will be presented elsewhere.

It would be tempting to speculate that perhaps the "alternative oxidase" itself is one of the components which is producing the observed split signal. Current data is rather negative and argues against this, however. For example, no correlation exists between the presence of the splitting and the occurrence of an alternative oxidase pathway. Further, the K_s of salicylhydroxamic acid removal of the split signal is an order of magnitude higher than the K_i of inhibition of the alternative respiratory pathway in both mung bean and *Sa. guttatum* spadix mitochondria. It was of interest, however, that this K_s of salicylhydroxamic acid effect was very similar to the K_i of inhibition of superoxide anion production by mung bean mitochondria [32]. This superoxide anion production is apparently a rather different process to the classical "alternative pathway" (Rich, P. R., unpublished data). It is thought unlikely that salicylhydroxamic acid interacts with Center S-3 directly since 2 mM salicylhydroxamic acid was found to have no detectable effect on the midpoint potential, temperature and power profiles or signal size or shape of Center S-3 (Rich, P. R., unpublished data).

Despite these results, the component causing the split signal does behave in the expected manner to be the branchpoint of the main and alternative respiratory pathways, as discussed. Furthermore, the origin of the detailed structure differences in splitting which seem to occur between cyanide-resistant and cyanide-sensitive mitochondria is unknown at present and investigations of a relation between these and the alternative oxidase may be useful.

The inherent instability of the split signal (for example, sensitivity to alcohol, acetone, dimethylformamide, membrane intactness) is in contrast to the properties of its mammalian counterpart. This has hindered detailed characterization of physical properties (e.g. redox potential determination) since addition of mediators in solvent is not feasible. It is thought that although the interaction is not a requirement for mitochondrial activity (for example, the electron transport pathway is able to work normally in a concentration of ethanol which totally removes the signal), its fortuitous presence under many conditions has given us an extra tool to study this increasingly more complex region of the respiratory chain and we hope that studies of these and related signals will give a greater insight into the nature of the "alternative pathway" and into electron transport in this region of the respiratory chain in general.

ACKNOWLEDGMENTS

We would like to thank Dr. T. Ohnishi for many useful discussions, Ms. K. Wiegand for expert technical assistance and Ms. M. Mosley for preparation of the manuscript. The work was supported by grants from the National Science Foundation and the Herman Frasch Foundation.

REFERENCES

1 Ohnishi, T. (1975) Biochim. Biophys. Acta 387, 475–490
2 Orme-Johnson, N. R., Hansen, R. E. and Beinert, H. (1974) J. Biol. Chem. 249, 1922–1927
3 Ohnishi, T., Ingledew, W. J. and Shiraishi, S. (1976) Biochem. J. 153, 39–48
4 Beinert, H., Ackrell, B. A. C., Kearney, E. B. and Singer, T. P. (1975) Eur. J. Biochem. 54, 185–194
5 Ohnishi, T., Salerno, J. C., Winter, D. B., Lim, J., Yu, C. A., Yu, L. and King, T. E. (1976) J. Biol. Chem. 251, 2094–2104
6 Ohnishi, T., Lim, J., Winter, D. B. and King, T. E. (1976) J. Biol Chem. 251, 2105–2109
7 Ruzicka, F. J., Beinert, H., Schepler, K. L., Dunham, W. R. and Sands, R. H. (1975) Proc. Natl. Acad. Sci. U.S. 72, 2886–2890
8 Ingledew, W. J., Salerno, J. C. and Ohnishi, T. (1976) Arch. Biochem. Biophys. 177, 176–184
9 Henry, M-F. and Nyns, E-J. (1975) Sub-Cell. Biochem. 4, 1–65
10 Ikuma, H. (1972) Ann. Rev. Plant Physiol. 44, 126–134
11 Bonner, Jr., W. D., Bendall, D. S. and Plesnicar, M. (1967) Fed. Proc. FASEB 26, 731
12 Schonbaum, G. S., Bonner, Jr., W. D., Storey, B. T. and Bahr. J. T. (1971) Plant Physiol. 47, 124–128
13 Wilson, S. B. (1971) FEBS Lett. 15, 49–52
14 Cammack, R. and Palmer, J. M. (1973) Ann. N.Y. Acad. Sci. 222, 816–823
15 Jenesel, S., Edwards, D. L. and Warden, J. T. (1976) Biophys. J. 16, 87a
16 Rich, P. R. and Moore, A. L. (1976) FEBS Lett. 65, 339–344
17 Moore, A. L., Rich, P. R., Ingledew, W. J. and Bonner, Jr., W. D. (1976) Biochem. Biophys. Res. Commun. 72, 1099–1107
18 Bonner, Jr., W. D. (1967) in Methods in Enzymology (Estabrook, R. W. and Pullman, M. E., eds.), Vol. X, pp. 126–133, Academic Press, New York
19 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
20 Ingledew, W. J. and Ohnishi, T. (1975) FEBS Lett. 54, 167–171
21 Ohnishi, T. (1973) Biochim. Biophys. Acta 301, 105–128
22 Douce, R., Mannella, C. A. and Bonner, Jr., W. D. (1973) Biochim. Biophys. Acta 292, 105–116
23 Tappel, A. L. (1960) Biochem. Pharmacol. 3, 289–296
24 Baginsky, M. L. and Hatefi, Y. (1969) J. Biol. Chem. 244, 5313–5319
25 Wilson, S. B. (1971) FEBS Lett. 15, 49–52
26 Rich, P. R., Moore, A. L. and Bonner, Jr., W. D. (1977) Biochem. J. 162, 205–208
27 Ruzicka, F. J. and Beinert, H. (1974) Biochem. Biophys. Res. Commun. 58, 556–563
28 Mitchell, P. (1976) J. Theor. Biol. 62, 327–367
29 Klingenberg, M. and Kröger, A. (1967) in Biochemistry of Mitochondria (Slater, E. C., Kaniuga, Z. and Wojtczak, L., eds.), pp. 11–28, Academic Press, London
30 Bahr, J. T. and Bonner, Jr., W. D. (1973) J. Biol. Chem. 248, 3441–3445
31 Bahr, J. T. and Bonner, Jr., W. D. (1973) J. Biol. Chem. 248, 3446–3450
32 Rich, P. R., Moore, A. L. and Bonner, Jr., W. D. (1977) Abstract for Biophysical Society Meeting, New Orleans, February 1977