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CHARACTERIZATION OF IRON-SULFUR CLUSTERS IN RAT LIVER SUBMITOCHONDRIAL PARTICLES BY ELECTRON PARAMAGNETIC RESONANCE SPECTROSCOPY

ALTERATIONS PRODUCED BY CHRONIC ETHANOL CONSUMPTION

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Summary

Iron-sulfur clusters present in rat liver submitochondrial particles were characterized by ESR at temperatures between 30 and 5.5 K combined with potentiometric titrations. The spectral and thermodynamic characteristics of the iron-sulfur clusters were generally similar to those previously reported for pigeon or bovine heart submitochondrial particles. Clusters N-1a, N-1b, N-2, N-3 and N-4 of NADH dehydrogenase had midpoint oxidation-reduction potentials at pH 7.5 of —425, —265, —85, —240 and —260 mV, respectively. Clusters S-1 and S-3 of succinate dehydrogenase had midpoint potentials of 0 and +65 mV, respectively. The iron-sulfur cluster of electron-transferring flavoprotein-ubiquinone oxidoreductase exhibited the g_z signal at $g = 2.08$ and had a midpoint potential of +30 mV. This signal was relatively prominent in rat liver compared to pigeon or bovine heart.

Submitochondrial particles from rats chronically treated with ethanol (36% of total calories, 40 days) showed decreases of 20—30% in amplitudes of signals due to clusters N-2, N-3 and N-4 compared to those from pair-fed control rats. Signals from clusters N-1b, S-1, S-3 and electron-transferring flavoprotein-ubiquinone oxidoreductase were unaffected. Microwave power-saturation behavior was similar for both submitochondrial particle preparations, suggesting that the lower signal amplitudes reflected a lower content of these particular clusters. NADH dehydrogenase activity was significantly decreased

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

(46%), whilst succinate dehydrogenase activity was elevated (25%), following chronic ethanol consumption. The results indicate that chronic ethanol treatment leads to an alteration of the structure and function of the NADH dehydrogenase segment of the electron transfer chain. This alteration is one of the factors contributing to the lower respiration rates observed following chronic ethanol administration.

Introduction

Iron-sulfur clusters, formerly referred to as centers, of the mitochondrial respiratory chain have been extensively characterized by low-temperature ESR in several biological systems including bovine and pigeon heart, and yeast [1—7]. Studies with isolated mitochondria, submitochondrial particles and preparations of electron transfer complexes have identified multiple iron-sulfur clusters present in both NADH dehydrogenase (designated by the prefix, N-) and succinate dehydrogenase (designated by the prefix, S-) [8,9]. These iron-sulfur clusters have been distinguished on the basis of their respective oxidation-reduction midpoint potentials as well as by spectral parameters of their ESR signals such as *g* values, line shapes and temperature dependence. The presence of similar iron-sulfur clusters in rat liver has been reported [10—12], but their oxidation-reduction characteristics have not been determined.

Rat liver is of particular interest in relation to the problem of alcoholic liver disease because it provides an experimental system which is relevant as a model for this pathologic condition. Chronic ethanol consumption produces fatty liver in rats [13,14] as well as in as primates [15] and in man [16,17]. It also causes alterations in the ultrastructural appearance of hepatic organelles, especially mitochondria. These are often enlarged and display unusual shapes [18,19]. Several studies have found that liver mitochondria, isolated from rats chronically fed with ethanol, have lower specific respiration rates than those from control rats [20—22]. Recently, we have developed a procedure for preparing submitochondrial particles from rat liver which catalyze oxidative phosphorylation efficiently and exhibit a membrane sidedness opposite to that of intact mitochondria [23]. Our studies with these submitochondrial particles have established that chronic ethanol consumption leads to decreased respiratory activities at the level of the electron transfer chain itself [23]. Chronic ethanol consumption also results in a lower content of certain cytochrome components of the electron transfer chain, particularly cytochrome oxidase [23].

A previous study with pigeon heart submitochondrial particles has shown that low concentrations of ethanol in vitro produce modifications in the ESR spectra of certain iron-sulfur clusters [24]. The spectra of clusters in the NADH dehydrogenase region, particularly N-1b, N-2, N-3 and N-4, and also cluster S-3 of succinate dehydrogenase, are readily modified. Ethanol in vitro causes both a loss of signal amplitude and line-shape alterations in the spectra of these clusters. The spectra of clusters S-1 and S-2 of succinate dehydrogenase are unaffected by the presence of ethanol in vitro.

In order to understand further the molecular alterations contributing to

the decreased respiratory activities produced by chronic ethanol consumption, we have studied the effects of chronic ethanol treatment on the ESR spectra of iron-sulfur clusters in rat liver submitochondrial particles. Interpretation of these results required that we also establish the basic spectral and thermodynamic characteristics of the iron-sulfur clusters in this system. This study also provides a basis for relating the previously reported effects of ethanol on mitochondrial iron-sulfur clusters in vitro to the physiological changes produced as a result of chronic ethanol consumption.

Experimental procedures

Materials. Rats were purchased from Charles River Breeding Laboratories. Rat diets were obtained from Bio-Serv, Inc., Frenchtown, NJ.

Animals. Male Sprague-Dawley rats, initially weighing 150—160 g, were fed a nutritionally adequate totally-liquid diet in which ethanol provided 36% of total calories, protein 16%, fat 35% and carbohydrate the remainder [14]. Pair-fed control rats were fed a similar diet, except that carbohydrate isocalorically replaced ethanol. Rats used in this study were maintained on the diets for 40 days. Ethanol consumption averaged 14.0 ± 0.7 g/kg body wt. per day (±S.D.). Similar rats fed a standard chow diet and water ad libitum were used for experiments concerned with the basic characterization of the iron-sulfur clusters.

Preparations. Submitochondrial particles were prepared from freshly-isolated rat liver mitochondria [25] by sonication of mitoplasts in a medium containing $MgCl_2$, ATP and substrates, exactly as previously described [23], and were stored at −20°C. For redox titration experiments requiring a large amount of material, several preparations of submitochondrial particles from chow-fed rats were combined.

Analytical procedures. Samples for ESR spectroscopy were prepared by incubating submitochondrial particles (20—40 mg protein/ml) in a medium containing sucrose (250 mM), Hepes-Na^+ (50 mM), pH 7.5, and CCCP (2.5 μM, added as a dimethylsulfoxide solution, final dimethylsulfoxide concentration 0.3%) with either NADH (4 mM) or succinate (10 mM) in a total volume of 0.35 ml. After addition of substrate, the samples were immediately transferred to quartz ESR tubes (approx. 3 mm internal diameter), incubated for 3 min at room temperature and then frozen at 81 K [5]. Potentiometric titrations were conducted under an argon atmosphere using the apparatus [26] and procedures [5,27] previously described. ESR tubes having matched diameters were used for all experiments involving comparison of pairs of submitochondrial particle preparations from ethanol-treated and control rats as well as for potentiometric titrations. The ESR tubes were calibrated with a standard Cu_2SO_4/EDTA solution. Samples were prepared at identical protein concentrations for all experiments comparing submitochondrial particles from ethanol-treated and control rats. ESR spectra were recorded with a Varian E-109 spectrometer as previously described [5]. The observed g values were corrected using the free radical signal of a weak pitch standard (g = 2.0028) recorded under the same operating conditions.

NADH dehydrogenase activity was measured spectrophotometrically with

ferricyanide (0.1—2 mM) as electron acceptor [28] at 25°C in a medium containing sucrose (250 mM), Hepes-Na^+ (20 mM), pH 7.5, $MgCl_2$ (5 mM), KCN (1 mM), NADH (0.4 mM) and submitochondrial particles (0.1 mg protein/ml). Succinate dehydrogenase activity was measured spectrophotometrically using phenazine methosulfate (0.1—2 mM) and dichlorophenolindophenol (0.07 mM) as intermediate and terminal electron acceptors [29] at 25°C in a similar medium. Prior to the assay of succinate dehydrogenase, submitochondrial particles were incubated with succinate (10 mM) for 15 min at 25°C for activation of succinate dehydrogenase [30]. Protein concentration was determined by a cyanide biuret procedure [31] using bovine serum albumin as standard.

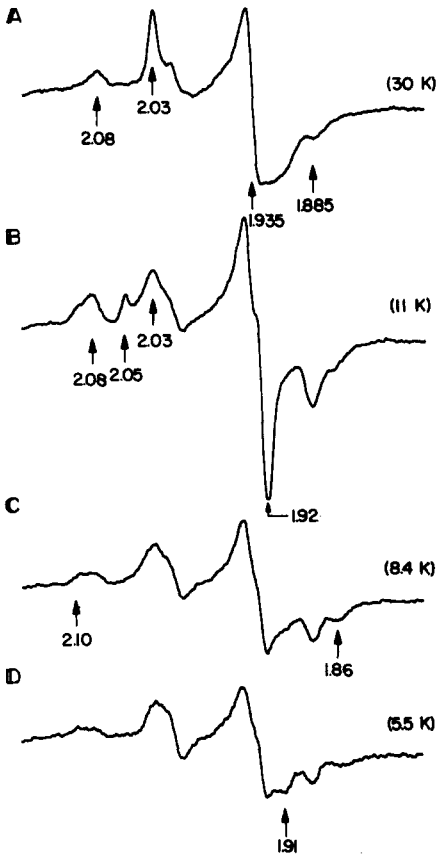
Results

Characterization of iron-sulfur clusters in rat liver submitochondrial particles

Fig. 1 illustrates typical ESR spectra observed at different temperatures with rat liver submitochondrial particles reduced by both NADH and dithionite. The spectra were generally similar to those previously reported for other submitochondrial particle preparations [2—7]. Potentiometric titration of g = 1.935 at 30 K, using a mediator system which did not include methyl viologen and benzyl viologen, indicated that it was composed of two single electron transfer components with $E_{m7.5}$ values of —20 and —265 mV (Fig. 2). These probably correspond to S-1 and N-1 (presumably N-1b), respectively, since the $E_{m7.5}$ values are similar to those observed in other systems [5—7]. When methyl viologen and benzyl viologen were included as mediators, an additional third component of the $g = 1.935$ signal was observed at very low potentials, which presumably corresponded to cluster N-1a [5,7]. The $E_{m7.5}$ of this component could not be determined accurately because of uncertainty about the endpoint of the titration, but was estimated to be approx. —425 mV. Spectra at representative redox potentials of 45, —157 and —407 mV, designated as A, B and C on the redox titration curve of Fig. 2, are presented in Fig. 3. At 45 mV, the residual $g_y = 1.935$ signal was probably due to the electron transferring flavoprotein-ubiquinone oxidoreductase * iron-sulfur cluster which also showed characteristic signals at $g_z = 2.08$ and $g_x = 1.88$ [32,33]. At —157 mV, the spectrum was dominated by that of S-1 and showed the characteristic g_z resonance of S-1 at $g = 2.03$. At —402 mV, the combined spectrum of N-1b, S-1 and electron transferring flavoprotein-ubiquinone oxidoreductase was observed and was similar to the spectrum obtained upon reduction with NADH plus dithionite (Fig. 1). Comparison of the relative amplitudes of the $g = 1.935$ signal at these potentials suggested that S-1 contributed approx. 60% of the total derivative signal amplitude under conditions of reduction by NADH plus dithionite. The presence of the Rieske iron-sulfur cluster [34] was suggested by a broad absorbance in the high-field region observable at 30 K. However, the E_m of this cluster was not determined.

At 11 or 12 K, the $g = 2.05$ signal characteristic of N-2 was observed. In addition, the central resonance showed a minimum peak position at $g = 1.92$, which is also characteristic of N-2 [3,5]. Potentiometric titration of the $g =$

* In earlier publications, this cluster has been called 'center 5' or 'center-bc_2' (see Ref. 7 for a discussion).



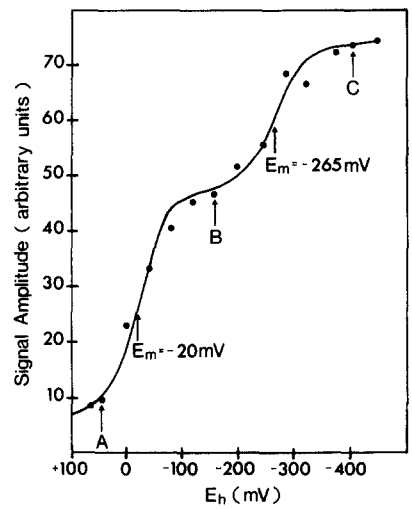


Fig. 1. ESR spectra of rat liver submitochondrial particles at different temperatures. Rat liver submitochondrial particles (46 mg protein/ml) in a medium containing sucrose (250 mM), Hepes-Na^+ (50 mM) and CCCP (2.5 μM) at pH 7.5 were reduced with NADH (1 mM) and dithionite (1 mM). First derivative ESR spectra were subsequently recorded at the indicated temperatures. ESR operating conditions were: modulation frequency, 100 kHz; modulation amplitude, 12.5 G; microwave power, 5 mW; microwave frequency, 9.11 GHz; time constant, 0.25 s; scanning rate, 500 G/min. The arrows designate g values.

Fig. 2. Redox titration of the $g = 1.935$ signal at 30 K. Rat liver submitochondrial particles (21 mg protein/ml) were titrated anaerobically in a medium containing sucrose (250 mM), Hepes-Na^+ (50 mM) and CCCP (2.5 μM) at pH 7.5. Redox mediators present were: duroquinone, indigo tetrasulfonate, indigo disulfonate, 2-hydroxy-1,4-naphthoquinone, 1,4-naphthoquinone disulfonate and phenosafranine, each at 40 μM; and pyocyanine (20 μM). Peak-to-peak amplitude of the $g = 1.935$ signal was measured from spectra recorded at 30 K of samples trapped at various E_h values. Theoretical $n = 1$ titration curves are drawn. ESR operating conditions were the same as in Fig. 1. The designations, A, B and C, refer to spectra reported in Fig. 3.

2.05 signal at 12 K indicated an $E_{m7.5}$ of −85 mV for N-2 in this system. At 11 or 12 K, additional signals were evident at $g = 2.10$ and 1.88. The signal at $g = 2.08$ was particularly prominent in the rat liver system relative to the $g = 2.10$ or 2.05 signals from clusters of NADH dehydrogenase. During potentiometric titration, the $g = 2.08$ signal titrated simultaneously with a portion of the $g = 1.88$ signal and exhibited an $E_{m7.5}$ of approx. +30 mV.

At 8.4 K, the $g = 2.05$ signal was no longer observed, but signals at $g = 2.10$, 2.08, 1.88 and 1.86, as well as the central resonance at $g = 1.935$ remained.

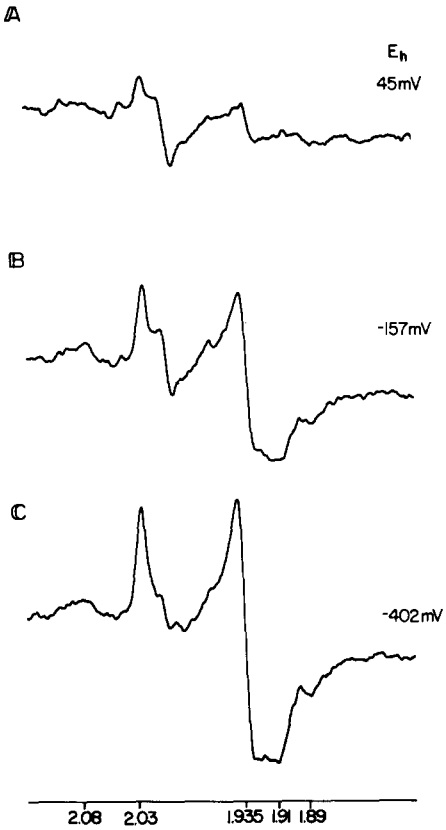


Fig. 3. ESR spectra of rat liver submitochondrial particles poised at various redox potentials. Experimental conditions are as described in the legend of Fig. 2. A, B and C correspond to spectra observed at the representative redox potentials of 45, —157 and —402 mV, respectively. The scale at the bottom indicates *g* values.

The $g = 1.86$ signal was not saturated and became more pronounced at higher power, as has been observed for the signal of cluster N-3 in pigeon heart submitochondrial particles [5,7]. At 8 K, the observed spectrum was probably mainly due to N-3 and N-4. However, potentiometric resolution of these signals was difficult because of their low derivative signal amplitudes. Titration of the $g = 1.86$ signal at 8 K, measuring the signal amplitude relative to the high field baseline, suggested an $E_{m7.5}$ of approx. —240 mV for cluster N-3. Titration of the $g = 2.10$ signal, measuring the amplitude relative to the low field baseline, indicated an $E_{m7.5}$ of approx. —260 mV for cluster N-4. An anomaly, however, was observed in the titration of clusters N-3 plus N-4 at 8 K. Upon lowering the redox potential below —300 mV, the signals at $g = 1.935$, 1.88 and 1.86 all diminished. The signal intensities reappeared as the potential was further lowered to —470 mV. Thus, the titration curves all exhibited a trough at approx. —350 mV (Fig. 4). At —470 mV, the $g = 1.86$ and 1.88 signals were restored to amplitudes similar to those observed at —300 mV. However, the $g = 1.935$ signal amplitude was greater than that at —300 mV, possibly reflecting a contribution from another N-1 type cluster. Such a trough was not

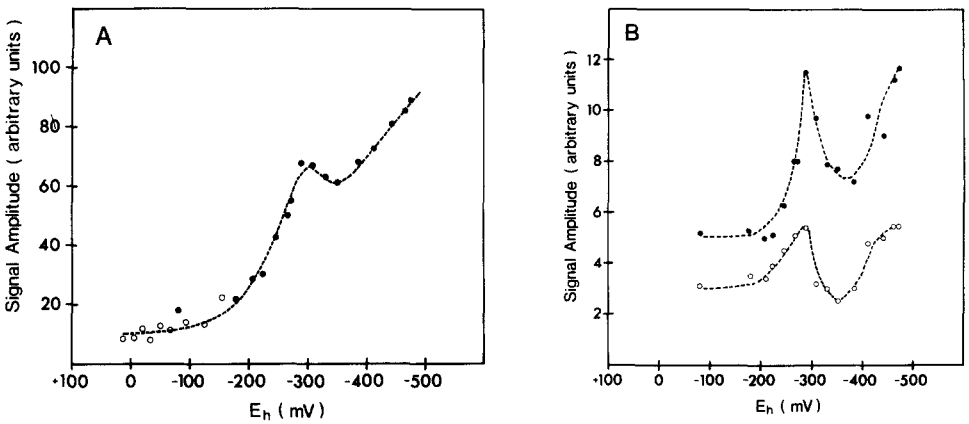


Fig. 4. Redox titration of the $g = 1.935$, 1.88 and 1.86 signals at 8 K. Rat liver submitochondrial particles (18 mg protein/ml) were titrated as described in the legend of Fig. 2. Redox mediators present were: indigo disulfonate, dihydroxynaphthoquinone, phenosafranine and safranine T, each at 40 μM; and pyocyanine, benzyl viologen and methyl viologen, each at 20 μM. A, peak-to-peak amplitude of the $g = 1.935$ signal measured from spectra recorded at 8 K in the presence of mediators described above (•) and from an overlapping titration conducted in the presence of the mediators as listed in the legend of Fig. 5 (○). B, amplitude of the $g = 1.88$ (•) and 1.86 (○) signals measured relative to the high-field baseline at 8 K in the presence of the mediators listed above. ESR operating conditions were the same as in Fig. 1.

observed in titration of the $g = 2.10$ signal, although its small amplitude may have limited the detection of such a phenomenon. A similar anomaly has been noted previously in the titration of the $g = 1.86$ signal of cluster N-3 in complex I, though not for the titration of the $g = 1.88$ signal [7].

At higher redox potentials, a spectrum characteristic of S-3 centered at $g = 2.01$ was observed at 8 K [35]. Additional signals at $g = 2.04$ and 1.99, characteristic of a spin-spin interaction of a ubisemiquinone pair [36—39], were observed as 'wings' on the S-3 signal at potentials where S-3 was only partially reduced (Fig. 5). Potentiometric titration indicated an $E_{m7.5}$ of +65 mV for S-3 in this system. The amplitudes of the spin-spin interaction signals reached a maximum at a potential around +80 mV.

Potentiometric titration of rat liver submitochondrial particles at 30 K, using a mediator system, as described for Fig. 5 which did not produce any mediator free radical signals detectable under these ESR operating conditions, indicated the formation of a stabilized free radical giving a signal at $g = 2.00$ which had maximum amplitude at a redox potential around 0 mV. The amplitude of this signal decreased at both higher and lower redox potentials. The $g = 2.00$ signal had a line width of 17 G and was probably due to both ubiquinone and flavin free radical species.

At 5.5 K, an additional spectral minimum at $g = 1.91$ was resolved (Fig. 1). However, no $g = 2.06$ resonance, characteristic of N-5 [5,7], was observed. The $E_{m7.5}$ of this signal was not determined.

Table I summarizes the resonance peak positions, temperatures of observation and $E_{m7.5}$ values of the iron-sulfur clusters of rat liver submitochondrial particles observed in this study.

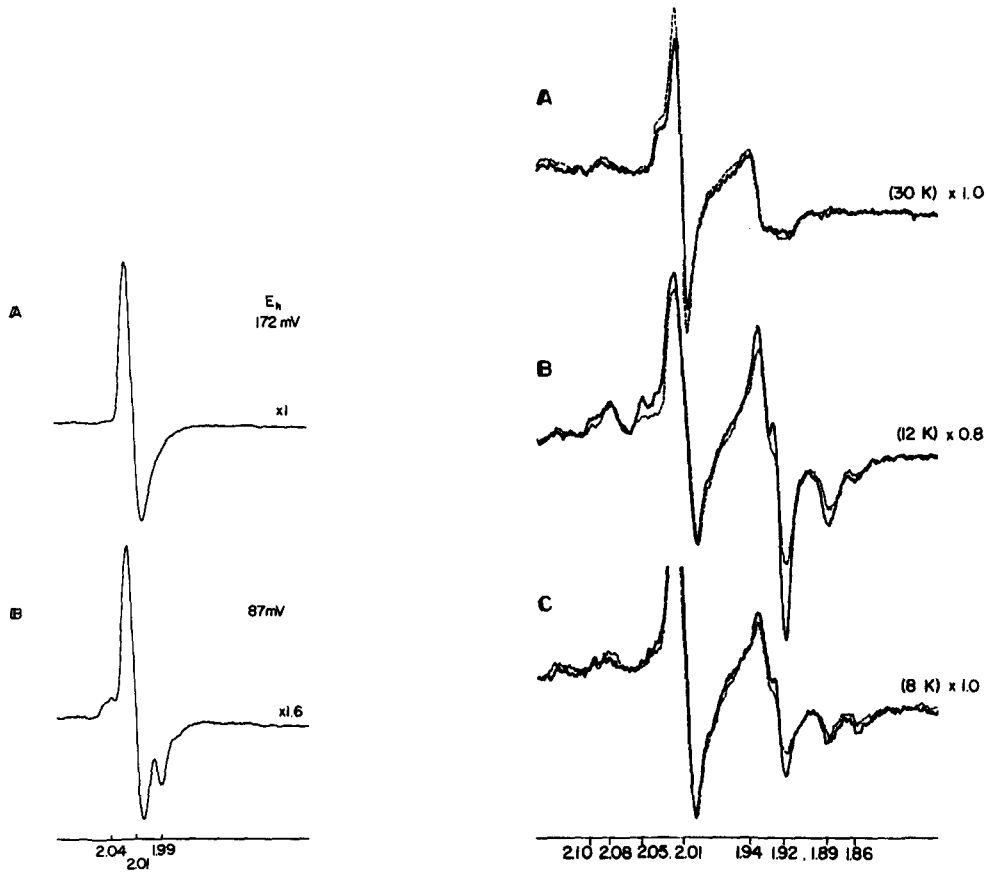


Fig. 5. EPR spectra of rat liver submitochondrial particles poised at different redox potentials. Rat liver submitochondrial particles (18 mg protein/ml) were titrated as described in the legend of Fig. 2. Redox mediators present were: duroquinone, indigo disulfonate, 1,2-naphthoquinone, 1,4-naphthoquinone, 1,4-naphthoquinone disulfonate, dihydroxynaphthoquinone and phenosafranine, each at 40 μM; and pyocyanine (20 μM). Spectra of samples poised at 172 mV (A) and 87 mV (B) were recorded at 8 K using ESR operating conditions similar to those described in Fig. 1. The scale at the bottom indicates *g* values. Relative gain factors are lised at the right-hand side of each spectrum.

Fig. 6. Representative EPR spectra illustrating the effects of chronic ethanol-treatment on iron-sulfur clusters of rat liver submitochondrial particles. EPR spectra of submitochondrial particles (22 mg protein/ml) reduced with NADH (4 mM) as described in Experimental procedures are shown for a pair-fed control rat (solid lines) and the corresponding chronic ethanol-treated rat (broken lines). Both sets of spectra were recorded under identical ESR operating conditions similar to those described in Fig. 1, except that the microwave power was 1 mW at 30 K, and 5 mW at 12 and 8 K. Relative gain factors are listed for comparison of the spectra at different temperatures. The scale at the bottom indicates *g* values.

Effects of chronic ethanol consumption on ESR spectra of rat liver submitochondrial particles

Our approach for studying the effects of chronic ethanol consumption on iron-sulfur clusters has involved the preparation of samples of submitochondrial particles from ethanol-treated and control rats at identical protein concentrations and the comparison of ESR spectra recorded under identical conditions. We have employed 30, 12 and 8 K as temperatures appropriate for resolving clusters N-1, N-2 and N-3 plus N-4, respectively, in NADH-reduced samples.

TABLE I

SUMMARY OF OXIDATION-REDUCTION MIDPOINT POTENTIALS OF IRON-SULFER CLUSTERS IN RAT LIVER SUBMITOCHONDRIAL PARTICLES

Midpoint potentials were determined from redox titrations conducted in the presence of uncoupler at pH 7.5. The data were fitted to $n = 1$ titration curves in all cases. ETF-UQO, electron- transferring flavoprotein-ubiquinone oxidoreductase.

Cluster	Temperature of observation (K)	Characteristic g values	$E_{m7.5}$
N-1a	30	2.03, 1.94	—425 mV
N-1b	30	2.03, 1.94, 1.91	—265
N-2	12	2.05, 1.93	—85
N-3	8	(2.04), (1.93), 1.86	—240
N-4	8	2.10, (1.94), 1.88	—260
S-1	30	2.03, 1.93, 1.91	—20 to +20
S-3	8	2.01	+65
EFT-UQO	12	2.08, 1.93, 1.88	+30

Representative spectra at these temperatures illustrating the effects of chronic ethanol consumption are shown in Fig. 6 and the results of several experiments are summarized in Table II. At 30 K, almost similar spectra, characteristic of cluster N-1b, were observed. However, at 12 K, lower signal amplitudes at several g values were evident in submitochondrial particles from the ethanol-treated rat, compared to those from the control rat. The line shapes of the signals were generally similar in both samples. Decreased signal amplitudes were most evident at $g = 2.05$ and the central resonance around $g = 1.93$ characteristic of N-2. At 8 K, the amplitudes of signals due to clusters N-3 and N-4, particularly the central resonance around $g = 1.93$, were slightly lower.

Spectra at a given temperature were recorded at identical microwave power settings (routinely, 1 mW at 30 K, 5 mW at 12 and 8 K) for submitochondrial particles from ethanol-treated and control rats. At these power settings, most signals were partially saturated. However, both submitochondrial particle

TABLE II

EFFECT OF CHRONIC ETHANOL TREATMENT ON ESR SPECTRA OF RAT LIVER SUBMITOCHONDRIAL PARTICLES REDUCED WITH NADH

Derivative signal amplitudes at the indicated g values were measured from ESR spectra of NADH-reduced submitochondrial particles from ethanol-treated and control rats. The spectra were recorded under identical conditions at the same protein concentration. The relative signal amplitude was calculated as the ratio of signal amplitude of the sample from the ethanol-treated rat to that from control rat for each pair. The mean values of the relative signal amplitudes determined from analyses of samples from nine pairs of rats are listed. The S.E. was less than 0.1 in all cases.

Temperature (K)	g							
	2.10	2.08	2.05	2.03	2.00	1.935	1.88	1.86
30	—	1.1	—	1.0	1.1	1.0	0.9	—
12	0.9	1.0	0.7	—	1.0	0.8	0.8	0.8
8	0.8	1.0	—	—	1.0	0.8	0.8	0.8

preparations exhibited similar power-saturation behavior. For example, cluster N-2 began to saturate at approx. 2 mW power in both cases. Comparison of spectra recorded at 1 and 5 mW indicated differences of similar magnitude between submitochondrial particles from ethanol-treated and control rats.

A notable feature of the spectra observed with hepatic submitochondrial particles from both control and ethanol-treated rats (and also chow-fed rats) was the large $g = 2.00$ free radical signal. The amplitude of this signal was similar in both preparations of submitochondrial particles. It was observed consistently with either NADH- or succinate-reduced samples. The origin of this free radical signal was probably not incomplete anaerobiosis, because increasing the incubation period at room temperature prior to trapping the samples at 81 K did not diminish the amplitude of the signal. This signal was not observed in dithionite-reduced samples. Spectra at higher resolution indicated that this $g = 2.00$ signal was similar to that which was observed at a redox potential of about 0 mV during potentiometric titrations and its line width suggested that it probably arose from stabilized ubiquinone and flavin free radical species.

The pattern of results described above was generally observed throughout analyses of several pairs of submitochondrial particle preparations. Table II summarizes results from nine pairs of rats. Of these, two pairs exhibited no difference in ESR signal amplitude. The overall results indicated a decrease of 20 to 30% in amplitudes of signals due to clusters N-2, N-3 and N-4, but no difference in amplitude of signals due to cluster N-1b. The variation observed in the extent of the decreases produced by chronic ethanol consumption between different pairs of rats appeared to be biological in origin, because similar variation was also noted in the extent of the decrease of NADH oxidase specific activity produced by chronic ethanol consumption. This variation most probably reflects differing susceptibility to the effects of chronic ethanol consumption between individual rats.

Similar experiments were conducted with succinate-reduced samples to determine effects on iron-sulfur clusters of succinate dehydrogenase (Table III). At 26 K, the amplitudes of signals around $g = 1.93$ and at $g = 2.03$, characteristic of cluster S-1, were similar. At 12 K, a lower amplitude of the $g = 2.05$ signal of cluster N-2, which is partially reducible by succinate [3,5], was observed, confirming the results obtained with NADH-reduced samples.

TABLE III

EFFECT OF CHRONIC ETHANOL TREATMENT ON ESR SPECTRA OF RAT LIVER SUBMITOCHONDRIAL PARTICLES REDUCED WITH SUCCINATE

Relative signal amplitudes were determined as described in the legend of Table II from spectra of succinate-reduced samples. The mean values of the relative signal ampltudes determined from four pairs of rats are listed. The S.E. was less than 0.1 in all cases.

Temperature (K)	*g*					
	2.08	2.05	2.03	2.00	1.93	1.88
26	1.0	—	1.0	1.2	1.0	1.0
12	1.1	0.8	—	1.1	1.0	1.0

TABLE IV

EFFECT OF CHRONIC ETHANOL TREATMENT ON DEHYDROGENASE ACTIVITIES

Dehydrogenase activities were measured as described in Experimental procedures. *V* values were determined by extrapolation of Lineweaver-Burke plots to infinite acceptor concentration. The values indicate the mean ± S.E. for seven pairs of rats. The Student's *t*-test for paired samples was used for the determination of *P*.

	V		% change	*P*
	Control	Ethanol-treated		
NADH dehydrogenase	12.7 ± 1.5 *	6.9 ± 0.8	−46	<0.01
Succinate dehydrogenase	0.6 ± 0.1	0.8 ± 0.1	+25	<0.02

* μmol acceptor reduced/min per mg protein.

In order to determine the effect of chronic ethanol consumption on cluster S-3, which is paramagnetic in the oxidized state [35—38], NADH-reduced samples were thawed, treated with ferricyanide (6 mM), refrozen and spectra recorded again at 9 K for observation of the S-3 signal at $g = 2.01$. The mean relative signal amplitude observed for six pairs of samples was 1.12 ± 0.05 (ethanol-treated/control), indicating little change in cluster S-3 as a result of chronic ethanol consumption.

Effects of chronic ethanol consumption on dehydrogenase activities of rat liver submitochondrial particles

In order to evaluate further the significance of the ESR observations, which indicated decreases in signals due to some of the iron-sulfur clusters of NADH dehydrogenase, but no change in signals due to clusters of succinate dehydrogenase, we measured the effect of chronic ethanol consumption on dehydrogenase activities (Table IV). The *V* for NADH dehydrogenase was decreased 46% by chronic ethanol consumption. The apparent K_m for ferricyanide was unaltered, approx. 2 mM. In contrast, the *V* for succinate dehydrogenase activity was actually increased 25% for submitochondrial particles from ethanol-treated compared to control rats. The apparent K_m for phenazine methosulfate was approx. 0.5 mM in both cases.

Discussion

Characterization of iron-sulfur clusters in rat liver submitochondrial particles

The spectral and potentiometric characteristics of the iron-sulfur clusters in rat liver submitochondrial particles are generally similar to those of clusters in bovine or pigeon heart submitochondrial particles. This was expected in view of the similar activities of the various preparations, and provides further evidence that the preparative method we have devised [23] produces submitochondrial particles which are comparable to those from other sources. The purity of this preparation is further indicated by the absence of ESR signals characteristic of an outer mitochondrial membrane iron-sulfur cluster which shows signals at $g = 2.01$, 1.94 and 1.89 [11]. The $g = 1.88$ region signal observed in rat liver submitochondrial particles can be accounted for by contri-

butions from clusters of NADH dehydrogenase and the electron transferring flavoprotein-ubiquinone oxidoreductase. This finding agrees with previously reported biochemical data indicating the absence of monoamine oxidase and rotenone-insensitive NADH-cytochrome *c* reductase activities, which are markers for the outer membrane, in this preparation of rat liver submitochondrial particles [23].

The anomalous trough in the titration curves of clusters N-3 and N-4 at approx. −350 mV is similar to that previously observed during titration of cluster N-3 in complex I [7]. In the latter, the trough was observed only for the $g = 1.86$ signal but not the $g = 1.88$ signal. It was associated with the appearance of a half-field signal at approx. $g = 3.9$, which reached maximum amplitude at the potential where the g 1.86 signal was minimized, namely −380 mV in pigeon heart [7,40]. It was suggested that this phenomenon might arise from interaction of a paramagnetic intermediate redox state of an $n = 2$ electron carrier, possibly FMN [7,40]. Although we have not studied half-field signals in rat liver submitochondrial particles, the redox titration behavior suggests that both clusters N-3 and N-4 may be involved in such an interaction. Alternatively, overlap of the $g = 1.88$ signal of cluster N-4 with the $g = 1.86$ signal of cluster N-3 may account for the trough in the titration of the $g = 1.88$ signal, particularly since such a trough was not observed during titration of the $g = 2.10$ signal which also arises from cluster N-4. Recently, a very similar trough at −360 mV has been observed during titration of cluster N-3 with NADH/NAD^+ in bovine heart submitochondrial particles [41].

A notable feature of the ESR spectra of rat liver submitochondrial particles compared to those of bovine or pigeon heart submitochondrial particles is the prominence of the $g = 2.08$ signal relative to signals at $g = 2.10$ or 2.05. The spectra characteristics and redox titration behavior suggest that this signal arises from the iron-sulfur cluster of electron transferring flavoprotein-ubiquinone oxidoreductase, since similar characteristics have been observed for the isolated protein [32,33]. The $E_{m7.5}$ value, approx. +30 mV, agrees well with results for pigeon heart submitochondrial particles [42]. A similarly prominent $g = 2.08$ signal is observed in brown fat mitochondria [43].

The thermodynamic properties of the iron-sulfur clusters of succinate dehydrogenase in rat liver submitochondrial particles are essentially identical to those found in other systems. The $E_{m7.5}$ of cluster S-1 is estimated to be in the range of −20 to +20 mV, because some variation was observed with different preparations. As in other submitochondrial particle preparations, cluster S-2 could not be resolved from clusters of NADH dehydrogenase [5] and was not studied. The $E_{m7.5}$ of cluster S-3, approx. +65 mV, is similar to that observed in isolated succinate-cytochrome *c* reductase [9,35,39], ubiquinone-extraced bovine heart submitochondrial particles [44] and some plant mitochondria and submitochondrial particles [45]. The latter systems all show little or no spin-spin interaction signals from a ubisemiquinone pair [7,39,44,45]. In contrast, cluster S-3 exhibits an E_m of +130 mV in pigeon heart and bovine heart submitochondrial particles which display strong spin-spin interaction signals [7,38,39]. The spin-spin interaction signals are of intermediate amplitude in rat liver submitochondrial particles compared to these other systems, but the E_m of cluster S-3 is the same as that found in systems

which show little spin-spin interaction. Thus, the possible role of a ubisemiquinone pair in controlling the midpoint potential of cluster S-3 remains to be clarified [7].

The results of the characterization studies confirm the identity of the observable ESR signals as due to known iron-sulfur clusters and facilitate an interpretation of the spectral alterations caused by chronic ethanol consumption.

Effect of chronic ethanol consumption on iron-sulfur clusters of rat liver submitochondrial particles

Comparison of ESR spectra indicates that signals due to clusters N-2, N-3 and N-4 are decreased after chronic ethanol feeding. The similar power saturation behavior, however, indicates that these clusters have similar relaxation properties and suggests that their molecular environment is not substantially changed. Thus, the lower signal amplitudes most likely reflect a lower content of these particular iron-sulfur clusters after ethanol feeding. It is interesting that cluster N-1b, which is also a component of NADH dehydrogenase, is not affected, however.

The pattern of effects on ESR spectra with NADH- and succinate-reduced samples correlates, at least qualitatively, with the results of assays of dehydrogenase activities. Variation in the susceptibility to ethanol-induced changes between individual rats, however, limits this structure-function correlation to a qualitative agreement. The greater susceptibility of clusters of NADH dehydrogenase compared to those of succinate dehydrogenase toward alterations as a result of pathologic conditions has also been observed in studies of tumor mitochondria [10]. The ESR signal of cluster N-2 was decreased in hepatoma mitochondria and the extent of the decrease correlated positively with the growth rate of the hepatoma from which the mitochondria were derived.

The effects of chronic ethanol consumption on mitochondrial iron-sulfur clusters differ somewhat from those of ethanol in vitro [24]. The spectra of clusters N-2, N-3 and N-4 are modified by ethanol in vitro as well as by chronic ethanol consumption. However, clusters N-1b and S-3 are not affected by chronic ethanol treatment, although the spectra of these clusters are readily altered by ethanol in vitro [24]. Cluster S-1 is not changed by either treatment. In addition, line-shape alterations caused by ethanol in vitro are not evident following chronic ethanol consumption. These observations provide further evidence that the alterations of mitochondrial function produced by chronic ethanol consumption represent persistent changes at a molecular level and are not due to the presence of residual ethanol or acetaldehyde.

The selectivity observed in the effects of chronic ethanol consumption on iron-sulfur clusters may indicate a differential sensitivity of the various individual proteins. Indeed, a similar selectivity has been observed previously for the cytochromes, since cytochrome oxidase content is decreased much more than that of *b*- or *c*-type cytochromes following chronic ethanol consumption [23]. Our studies of the repiratory chain thus indicate two major regions which are affected by chronic ethanol consumption. These are cytochrome oxidase [23] and, as the present results indicate, NADH dehydro-

genase. Decreases in the content of electron transfer chain components at these sites contribute to the lower respiration rates observed following chronic ethanol feeding with both isolated liver mitochondria [20—22] and submitochondrial particles [23]. The alteration at the level of NADH dehydrogenase may explain the apparently greater effect of chronic ethanol treatment on oxidation of NAD^+-linked substrates compared to succinate [20,23]. However, the change in the relative stoichiometries of respiratory chain components with respect to one another may alter the overall kinetic characteristics of the electron transfer chain leading to lower respiration rates with a variety of substrates.

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