sodium[13] is that the sodium and the α-aminoisobutyric acid are bound to the same carrier.

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An electron spin resonance signal in brain microsomes

Microsomal preparations from liver and adrenal cortex have been examined by ESR spectroscopy[1–5]. Studies on brain microsomes with this technique have not been reported.

Active cation transport and ATPase activity have been studied extensively, in brain as well as in other tissues[6]. A connection between microsomal electron transport and the active transport of ions has been suggested (*e.g.* refs. 6–8), but experimental evidence has been lacking. Previous work[9,10] using frog skin suggested that unpaired electrons participate in active cation transport. We report here preliminary findings showing that a free radical is in some way connected with microsomal ATPase.

Rats were killed by decapitation after light ether anesthesia. (One preparation from rats that received no ether was also examined; we observed no difference in the signal.) The brains were quickly removed and chilled. The microsomal pellet was

Abbreviation: ESR, electron spin resonance.

prepared by a modification of earlier methods (*e.g.* ref. 11). The homogenate, in 0.25 M sucrose–0.1 M Tris buffer (pH 7.5), was centrifuged at 10 000 × *g* for 15 min, and the supernatant carefully decanted. To insure complete removal of mitochondria, the supernatant was centrifuged again at 10 000 × *g* for 5 min. The resulting supernatant was then centrifuged at 105 000 × *g* for 1 h, and the pellet was suspended in 100 mM Tris buffer, pH 7.5, by gentle homogenization. All operations were done at 4°. Control experiments showed that, in the range of protein concentrations used in the experiments in Table I (28–35 mg/ml), the $(Na^+ + K^+ + Mg^{2+})$-ATPase activity was about 0.12 μmole P_i per min and mg protein, and the $(Na^+ + K^+ + Mg^{2+})/Mg^{2+}$ activity ratio about 1.5. The same assay system (at 36°) described in Table I was used for these measurements. Protein and inorganic phosphate were determined by standard methods[12,13].

For ESR measurements, samples were pipetted into quartz tubes of 3 mm internal diameter and then frozen in liquid nitrogen. A Varian V-4502 X-band spectrometer equipped with 100 kcycles/sec field modulation and with a Fieldial for regulation of the magnetic field was used. Measurements were made at approximately 0.3 mW of microwave power, −185° (Varian variable temperature dewar), field modulation of about 12 Gauss and a sweep speed of 20 Gauss/min.

MASON and co-workers observed an ESR signal in liver microsomes which they attributed to a low-spin ferric hemoprotein which they have called microsomal Fe_x (ref. 1). Microsomal Fe_x and the microsomal CO-binding pigment P-450 appear to be related[2,3,5,14]. Microsomes from bovine adrenal cortex had a weak Fe_x signal[4], and those from pig thyroid did not show this Fe_x signal[15]. Similarly, we have not observed the Fe_x signal in brain microsomes.

We did, however, observe a signal presumably due to free radicals. In freshly prepared brain microsomes we observed an ESR signal (Fig. 1) at $g = 2.0039 \pm 0.0002$ and with a peak-to-peak width of 11 ± 1 Gauss. The signal saturated readily as the microwave power was increased. In performing our measurements, we had used optimal power conditions because the signal was weak. We were apparently operating under slight saturation even at 0.3 mW. For instance, when ESR measurements were made between −185° and −100° the signal was unaltered, and it increased slightly in amplitude at −50°. At −185°, the signal was almost totally saturated at 30 mW. No change in the signal was detectable after storage of the microsomes at −60° or in liquid nitrogen for at least one week. Heating the preparation to 90° for 10 min completely abolished the signal. By comparison with a standard of pitch ($6 \cdot 10^{12}$ spins)

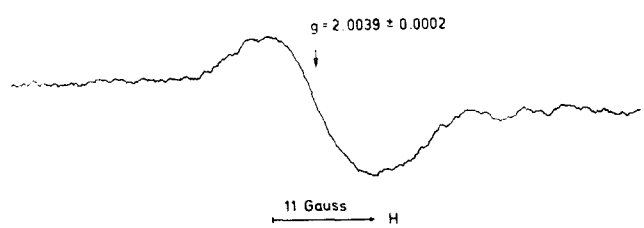

Fig. 1. ESR signal of rat brain microsomes in 250 mM Tris buffer, pH 7.5. Protein concentration was 51 mg/ml. The *g*-value was determined by accurately measuring the field strength with a proton resonance probe and the proton resonance and klystron frequencies with a Hewlett-Packard frequency meter.

TABLE I

EFFECT OF ADENINE NUCLEOTIDES ON FREE RADICAL IN BRAIN MICROSOMES

All samples contained microsomes in 100 mM Tris buffer, pH 7.5. Where indicated, 100 mM NaCl, 10 mM KCl, 10 mM $MgCl_2$, 10 mM ATP, 10 mM ADP, 10 mM AMP and 2 mM ouabain were added. Amplitude is expressed in arbitrary units. Samples incubated for 2.5 min (to insure zero-order kinetics) at 36°, are denoted by +; samples kept at 4°, with no incubation, by −. The pH of each sample was checked at the conclusion of the ESR measurements; all were in the range 7–7.5. Appropriate blanks were run and no signals were observed. Each number represents one sample. In Expt. 1, each spectrum was recorded 3–5 times; in Expt. 2, 5–6 times. The mean of the amplitude was then calculated. Minor corrections were applied for protein concentrations and tube calibrations.

Expt. No.	*Additions*	*Amplitude*	
		−	+
1	None	106	100
	$Na^+ + K^+ + Mg^{2+}$	—	112
	ATP	114	114
	ATP + $Na^+ + K^+ + Mg^{2+}$	90	73
	ATP + $Na^+ + K^+ + Mg^{2+}$ + ouabain	110	98
	ADP + $Na^+ + K^+ + Mg^{2+}$	104	92
	AMP + $Na^+ + K^+ + Mg^{2+}$	114*	106
2	None		100
			91
			109
	ATP + $Na^+ + K^+ + Mg^{2+}$		60
			60
			64

* Signal broadened slightly.

we estimate the concentration of free radicals to be of the order of picomoles per mg of microsomal protein.

MASON and co-workers, working at a microwave power of 25 mW, observed a signal at $g = 2.0$ in liver microsomes[2,3] and at $g = 2.00$ (ref. 4) (or at $g = 2.03$, ref. 1) in dithionite-reduced liver microsomes. MASON *et al.*[2] have also reported a free radical signal ($g = 2.003$, width 26 gauss) that appeared when reduced microsomes were re-oxidized with oxygen. In a recent study, MURAKAMI AND MASON[5] found that treatment of liver microsomes with *p*-chloromercuriphenylsulfonate led to several changes in the original signal, including the appearance of a signal at $g = 2.004$. The relationship of any of these signals to the one we observe in brain microsomes has not been investigated.

A possible connection between microsomal electron transport and the active transport of ions has been suggested[6–8], but the detailed mechanism of such a process is at present difficult to visualize. The ESR signal in brain microsomes decreased in amplitude in the presence of ATP + $Na^+ + K^+ + Mg^{2+}$, but not with ATP alone or ions alone (Table I). Ouabain, a specific inhibitor of the active transport mechanism, reversed this effect. Neither ADP nor AMP had any effect. Thus, we observe a decrease in the signal under optimal conditions for ATPase activity and a return of the signal to the original when the inhibitor is present.

Our initial experiments had suggested that proper control of pH is critical, but we have not investigated this point in detail.

We hasten to point out that the original signal in the microsomes is very weak

(signal-to-noise ratio about 5) and the differences we observe are rather small. A large amount of sample was required for each ESR measurement; this precluded running large numbers of samples to establish statistical significance. However, the data in Table I (Expt. 2) do demonstrate that the decrease due to ATP + Na^+ + K^+ + Mg^{2+} is real. The experiments with frog skin[9,10] had suggested a direct, not an inverse, relationship between amount of unpaired spins and extent of active cation transport. In addition, this work required lyophilization of samples, a procedure known sometimes to cause formation of free radicals.

In summary, we have observed with ESR spectroscopy a free radical signal in microsomal preparations from rat brain at $g = 2.0039 \pm 0.0002$ and with peak-to-peak width 11 ± 1 gauss. The signal amplitude was decreased by ATP in the presence of $Na^+ + K^+ + Mg^{2+}$. We suggest that a possible connection between the free radical, microsomal ATPase, and active cation transport be further investigated.

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