Catecholamines in the adrenal gland was also decreased after oudenone administration. A maximum reduction was observed between 8 and 11 hr after the administration (Fig. 2). The time course of changes of catecholamine levels in the adrenal gland was similar to that of tyrosine hydroxylase activity. Norepinephrine level in the heart was decreased after oudenone administration. A maximum reduction of norepinephrine was observed between 9 and 11 hr after the injection (Fig. 3). Dopamine and norepinephrine levels in the brain showed a slight decrease at 11 hr after oudenone administration (Fig. 4).

Since tyrosine hydroxylase activity in the adrenal gland was found to be inhibitied *in vivo* after the administration of oudenone, the decrease in the catecholamine levels may be attributed to the inhibition of catecholamine synthesis at the tyrosine hydroxylase stage. The hypotensive effect of oudenone could be attributed to the inhibition of tyrosine hydroxylase *in vivo* and the resultant reduction of tissue catecholamines.

Acknowledgement—The capable technical assistance of Miss Yuko Nishikawa and Miss Yumiko Shibahara is gratefully acknowledged.

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Addendum—The structure of oudenone has been reported to be: (S)-2-[4,5-dihydro-5-propyl-2 (3H)-furylidene]-1,3-cyclopentanedione. M. OHNO, M. OKAMOTO, N. KAWABE, H. UMEZAWA, T. TAKEUCHI, H. IINUMA and S. TAKAHASHI, *J. Am. chem. Soc.* **93**, 1285 (1971).

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Biochemical Pharmacology, Vol. 20, pp. 2507–2511. Pergamon Press, 1971. Printed in Great Britain

Hepatic free radical levels in ethanol-treated and carbon tetrachloride-treated rats

(*Received* 15 *June* 1970; *accepted* 9 *October* 1970)

THE OBSERVATION by Gallagher[1] that the antioxidants, α-tocopherol (vitamin E), sodium selenite and *N*,*N*′-diphenyl-*p*-phenylenediamine (DPPD), afforded rats protection against the lethal effects of carbon tetrachloride (CCl_4) and ameliorated histological damage to the liver has given impetus to a large volume of studies designed to elucidate the protective and toxic mechanisms involved. Carbon tetrachloride hepatotoxicity has been the subject of a recent comprehensive review.[2]

Di Luzio[3,4] has demonstrated a similar protective action of antioxidants in lessening the hepatotoxic effects of ethanol. In a study with Costales,[5] the findings of Gallagher[1] for the CCl_4 model were reconfirmed and, in view of the protection afforded by antioxidants in both of these syndromes, a common hepatotoxic mechanism involving lipoperoxidative membrane damage was suggested. In attempting to gain chemical proof for the establishment of lipoperoxidation as the major toxic mechanism in these instances, methods heretofore employed in the study of the auto-oxidation of unsaturated fats have been employed, namely iodine titration of peroxides,[6] spectral measurements of conjugated dienes formed by rearrangement of polyenoic fatty acids as a consequence of free radical attack,[7] and measurement of malondialdehyde (MDA) production as a colored thiobarbituric acid (TBA) complex.[8] The MDA has been postulated as arising from triply or higher unsaturated fatty acids by free radical attack at a methylene bridge between two centers of unsaturation and eventual cleavage of a three-carbon fragment.[8] All of the artifacts being measured by the foregoing techniques are formed by mechanisms involving lipid-free radical intermediates generated in the case of CCl_4 by the attack of its metabolic homolytic cleavage products ($CCl_4 \rightarrow .CCl_3 + .Cl$) or in the case of ethanol by the presumed attack of a $CH_3\dot{C}{=}O$ radical derived from the major metabolic product, acetaldehyde.

The results obtained through use of the above analytical methods in attempting to establish a chemical basis for the lipoperoxidation-hepatotoxicity hypothesis have varied in the hands of different investigators, and their interpretation forms the basis for considerable controversy in the current biochemical literature.[9–15] The MDA–TBA results suffer, since they are based upon changes in fatty acids of the linolenic-arachidonic type, which are mainly exogenous in origin and constitute only a minor portion of cell wall lipids. Further serious doubt has been raised as to their validity in the current application, since the results are subject to variance due to the size and age of experimental animals and to the possibility of interfering color reactions arising from interaction with protein or carbohydrate fragments, particularly when the reagent is used with whole liver homogenates.[12,13] The conjugated diene measurements have the common shortcoming of measuring changes in only a small proportion of the target molecules (those containing two or more double bonds) and there is also the added complication of different chromophores being generated, depending upon the degree of unsaturation in the substrate as well as the difficulty of obtaining small difference measurements in an area of the spectrum where tailing end absorption may interfere.[11] The iodine titration method would appear to offer the advantages of recording "total peroxide" values without regard to degree of unsaturation of the substrate; however, in the case of rat liver lipids, the sample size is sufficiently small so that doubts have been raised as to the validity of these titration values.[11]

Gordis,[16] in an elegant investigation utilizing radiolabeled CCl_4, has demonstrated the incorporation of the trichloromethyl radical into liver lipid to form unnatural branched-chain chlorinated lipids. This study indicated another pathway to free radical mediated toxicity, namely, that of direct addition of a radical to a center of unsaturation not necessarily involving a peroxide intermediate. The experiment was not carried out in such a way as to indicate the magnitude of this route, but does seem to confirm its existence.

A common feature of both the branched-chain lipid and lipoperoxidation routes was the free radical nature invoked for the intermediates. The considerable controversy mentioned above[9–15] made necessary a search for an alternative method of analysis, which would have the features of ease and reliability of operation, independence of degree of unsaturation of the substrate, specificity of the parameter being measured (freedom from extraneous interference), and finally, which would be amenable to quantitation, thus indicating the magnitude of the process.

The use of electron spin resonance (ESR) spectroscopy appeared to offer a means of satisfying the above criteria and by specifically detecting free radicals would afford a method of viewing the "transition" or intermediate states postulated for the toxic mechanisms involved.

ESR has previously been used to monitor signal changes associated with liver neoplasms[17–20] and in irradiated tissue,[21] and this prior art indicated that the method would be applicable to the present problem.

The ESR instrument used in these experiments was a Varian X-band spectrometer equipped with a 12-in. magnet and a Fieldial, and utilizing a dual cavity (TE_{104}) operated from the low power mode of the microwave bridge. Field modulation of 10^5 Hz at 15 Oe was applied to the sample cavity, and of 400 Hz to the accessory cavity which contained a sample of 0·1% pitch in KCl (Varian) for determining g value and relative ESR sensitivity. Absolute spin density was determined from a first-moment analysis with the 0·1% pitch sample as a spin standard. The signal-to-noise ratio of the sample cavity with a quartz liquid nitrogen dewar and the standard sample was 18 for normal operating conditions. A time-averaging computer (Varian, C1024) was employed to improve the signal-to-noise ratio, and the instrumental sensitivity was sufficient to enable detection of free radicals at levels as low as 10^{-6} to 10^{-7} M.

A low temperature technique, which was a modification of that employed by Swartz and Molenda,[18] was used. The advantages over measurements at room temperature are that a significantly better signal-to-noise ratio and a narrower line width are obtained, and the steady state concentration of free radicals resulting from enzymatic processes in living tissue can be preserved. Initial studies using a room temperature liver slice technique similar to that employed by Vithayathil *et al.*[19] gave variable results with slices (0·5 × 7 × 15 mm) obtained from the same liver lobe with a plastic-coated, chrome-steel razor blade. The ESR spectra at room temperature of four consecutive slices are shown in Fig. 1 for an incident microwave power level of about 200 mW; the variations among the spectra were much less at a lower microwave power. These variations were obviated by use of a tissue press (Harvard Apparatus Company, No. 303 stainless steel, No. 141) to give a random average sample which was easily reproduced. The sieved liver tissue was sucked into a quartz tube (4·0 ± 0·1 mm o.d. and 0·5 mm wall) to form a column 5 cm high and submerged in liquid nitrogen, where it was stored until ESR spectra could be measured. For the measurement, the quartz tube with the frozen liver was transferred to the quartz dewar containing liquid nitrogen in the ESR cavity. This technique for quantitative analysis of free radical concentration did not suffer from the disadvantages of using the tissue cell at room temperature, where the positioning of the cell (Varian No. E-226, quartz tissue cell) is very critical and difficult to reproduce, the sample size is difficult to control, the liver tissue has little nutrient to sustain its activity during measurement since the liquid content of necessity must be maintained at a low value, and water tends to evaporate from the cell and cause shifts in the baseline of the ESR spectrum.

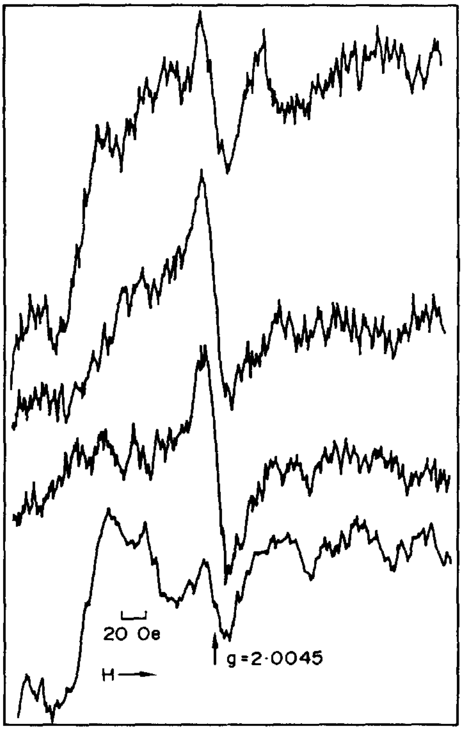

FIG. 1. ESR spectra of four consecutive liver slices measured at room temperature (200 mW microwave power; gain decreased by X2 for bottom spectrum).

The ESR spectrum measured at liquid nitrogen temperature consists of a resonance with a g value of 2·0045 ± 0·0002 and a line width of 18 Oe, which is presumably due to free radicals arising from enzymatic processes, and a complex set of lines which have been ascribed[18] to paramagnetic trace elements. The $g = 2{\cdot}0045$ line is easily power saturated while the complex of lines is not. Therefore, as the microwave power was decreased, the intensity of the $g = 2{\cdot}0045$ line increased and that of the complex of lines decreased. These data are in essential agreement with those found by previous investigators.[18,19] For microwave power levels below about 25 mW, the $g = 2{\cdot}0045$ line predominates. Most of the experimental results were obtained at 12 mW, where the intensity of the $g = 2{\cdot}0045$ line is about 90 per cent of its maximum value for the lowest practical microwave power usable.

Using such low microwave power, it is expected that most organic free radicals will be detectable; however, there is a remote possibility that a free radical may not be detected if its resonance is very broad, i.e. its spin relaxation time is very small.

In the present study, the rats (Holtzmann females) were dosed by oral intubation with CCl_4 (0·25 ml/100 g) in a 1:1 mixture with mineral oil, ethanol (6 g/kg) in a 50% aqueous solution, or DPPD (10 mg/100 g) in mineral oil (1 ml). The nonfasted animals were sacrificed after a sharp blow to the head 15 min after dosing, and the fasted animals were similarly sacrificed after 8 hr. The time elapsed from sacrifice to preparation of the frozen liver sample was approximately 7 min. For the unfasted rats, one control was performed with mineral oil and in one case the CCl_4 dose was given 2 hr before sacrificing the animal. For the fasted rats, all controls were treated with mineral oil. The mode of dosing and times of sacrifice were determined from previous studies[9–11] wherein peak lipid peroxide levels had been indicated by other less direct measurements.

TABLE 1. EFFECT OF DOSING RATS ON THE RELATIVE INTENSITY OF ESR LINE AT $g = 2{\cdot}0045$*

Dose	Unfasted	Fasted
Control	165 ± 20 (10)	194 ± 6 (4)
CCl_4	187 ± 23 (10)	186 ± 20 (4)
Ethanol	178 ± 19 (9)	192 ± 29 (4)
DPPD	—	201 ± 9 (4)

* The test data, when analyzed by a factorial design analysis of variance, showed no significant linear effects in any of the factors at the 95 per cent level.

The relative intensity data in Table 1 were obtained from the peak–peak intensities of the first derivative curves after time averaging (25 times) a region 250 Oe scanned in 0·5 min with an instrumental time constant of 0·1 sec. A typical time-averaged ESR spectrum is shown in Fig. 2.

A comparative analysis of the shape of 20 representative ESR curves, in the form of positive transparencies which could be superimposed, showed no significant difference in line shape. The absolute spin density associated with this line was estimated to be equivalent to a free radical concentration in the liver tissue of about 10^{-5} M.

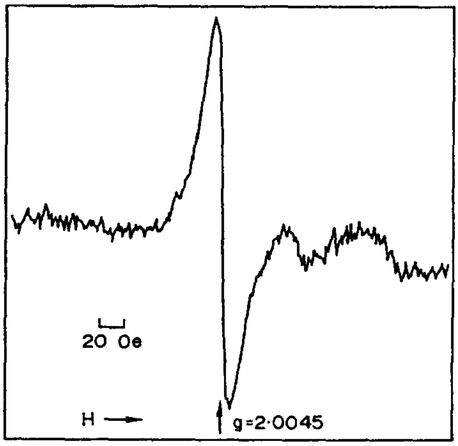

FIG. 2. ESR spectrum of sieved liver tissue at 77° K (microwave power, 12 mW; spectrum time averaged 25 times).

In an attempt to determine whether the presence of more than one type of free radical contributed to the $g = 2{\cdot}0045$ line, the effect of microwave power at two levels was evaluated for several samples and no significant differences were found.

The reproducibility of the ESR measurement of free radical concentration was within ±5 per cent for several samples from different livers stored in liquid nitrogen and re-examined 1 or 2 days after the initial measurements. The reproducibility was ±3 per cent for four samples of the same rat liver examined in different tubes.

The above data indicate that liver free radical levels in rats treated with CCl_4, ethanol and DPPD do not differ from the normal metabolic levels associated with oxidative enzymatic processes. The apparent ability of the liver to accommodate to a normal free radical level as high as 10^{-5} M would seem to argue against a toxicity induced by any added small amount of free radicals *per se*, and would require that the nature of these radicals and their locus play a more important role in eliciting any observed toxic symptoms than has previously been postulated.

In the absence of any discernible rise in liver free radical levels, these findings would tend to refute the premise that lipoperoxidation is a major hepatotoxic route in CCl_4- and ethanol-treated rats and confirm the conclusion of other workers[12–15] that lipoperoxidation does not play an important role in eliciting these syndromes.

Acknowledgement—We thank R. Kinnison, Biostatistician, Riker Laboratories, Inc., for the statistical analysis.

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