Lipid peroxidation and hepatic antioxidants in alcoholic liver disease

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Abstract

The generation of hepatic liver peroxidation by free radicals has been proposed as a mechanism for ethanol induced hepatotoxicity. To investigate this hypothesis, lipid extracts from hepatic needle biopsy specimens from alcoholic subjects were examined for evidence of lipid peroxidation by measuring total conjugated dienes by derivative spectroscopy and, after hydrolysis of hepatic lipid extract and reverse phase high performance liquid chromatography, the molar ratio between a diene-conjugated linoleic acid isomer (18:2 (9,11)) and the parent linoleic acid isomer (18:2(9,12)). Changes were related to hepatic histology, iron deposition, glutathione and vitamin E values. Derivative spectroscopy minima suggestive of diene conjugation were identified at 233 and 242 nm and correlated weakly, suggesting these two minima may represent different classes of lipid dienes. There was a weak relation with inflammatory histological changes in the biopsy specimen but no correlation with hepatic iron grade, glutathione, or vitamin E lipid ratio. The proportion of 18:2(9,11) linoleic acid in hepatic lipids correlated significantly with inflammatory histological features and inversely with hepatic glutathione. Furthermore, hepatic glutathione was lower in biopsy specimens with greater iron staining. The ratio of vitamin E to lipid was not related to histological group, inflammation, or iron grade. These findings suggest that excess alcohol consumption leads to hepatic inflammation and lipid peroxidation.

The mechanism by which ethanol consumption leads to hepatotoxicity is unknown. The generation of hepatic lipid peroxidation by highly reactive free radicals was first proposed as a mechanism by di Luzio and Hartman¹ and later by other workers,²⁻⁶ and led to further studies which indicated that hepatic concentrations of the antioxidant tripeptide glutathione may be reduced by ethanol, either by increased utilisation⁷ or diminished production.⁸ A role for the antioxidant vitamin E in this process was also suggested.⁹

In studies in the rat, extremely high doses of ethanol are required to produce hepatotoxicity with the characteristics of free radical induced lipid peroxidation, when given acutely,¹⁰ though the changes may be potentiated by chronic dosing.¹¹ In the baboon, however, smaller acute doses may lead to similar changes which may be prevented by pretreatment with methionine, a precursor for the antioxidant glutathione.¹¹ Human evidence for this type of process is

limited and centres on the detection of markers of free radical attack on polyunsaturated fatty acids. Shaw *et al*⁷ found increased values of one such marker (diene conjugation) in hepatic tissue taken from 16 alcoholics with different stages of liver injury, when compared with a control group with liver damage unrelated to alcohol. Similarly, an increase of malonaldehyde in liver biopsy specimens also suggested hepatic lipid peroxidation in alcoholics.¹²

Free radical attack on polyunsaturated fatty acids characteristically causes a rearrangement of the double bonds to generate diene conjugated lipids. This change is detected by the development of absorbance at 233-245 nm in lipid extracts.¹³ The nature of diene conjugation in human plasma and some other tissues has been attributed to the presence of non-peroxide conjugated diene stereoisomer of linoleic acid, 18:2 (9cis,11trans) linoleic acid (9,11 LA), which may be generated in vitro by ultraviolet irradiation of 18:2 (9,12) linoleic acid (9,12 LA) in the presence of protein.¹³ The proportion of plasma phospholipid esterified diene-conjugated 9,11 LA is increased acutely in alcoholics who are still drinking and falls after ethanol withdrawal, supporting the suggestion that ethanol induces free radical generation in the liver.¹⁴

Diene conjugation can also be detected and quantified using derivative spectroscopy.¹⁵ Using this technique, derivative spectroscopy minima at 242 nm and 233 nm (equivalent to absorption maxima in normal absorption spectroscopy) have been found in hepatic lipid extracts from animals poisoned with the hepatotoxin carbon tetrachloride and were attributed to a mixture of cis,trans and trans,trans lipid diene hydroperoxides.¹⁶ From the order of appearance of these minima, it was suggested that the relative proportion of the different stereoisomers reflects the cellular redox status within the liver.¹⁶

To examine the inter-relation between lipid peroxidation and antioxidants in ethanol induced hepatic injury, we have measured diene conjugation and the hepatic antioxidants glutathione and vitamin E in hepatic needle biopsy specimens from heavy drinkers. Since reactive oxygen species are also generated in the inflammatory process¹⁷ and iron may play a catalytic role in free radical reactions,¹⁸⁻²⁰ these measures have been correlated with histological appearance, degree of hepatitis, and iron loading.

Subjects and methods

Forty three patients were studied. All underwent liver biopsy as part of a programme of assessment and treatment for alcohol abuse and gave informed consent. The study was approved by

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the local ethical committee. Each patient had been drinking at least 100 g ethanol a day for a minimum of one year. All were inpatients and had abstained from alcohol for 72 hours before the biopsy. Only tissue in excess of that required for routine histological purposes was available for study. The portion of liver available thus varied between subjects and not all analyses could be performed on all samples. The available tissue was immediately placed in a container of liquid nitrogen by the bedside and remained under liquid nitrogen until immediately before analysis. Laboratory reagents were purchased from Sigma unless otherwise stated.

HEPATIC LIPID EXTRACTION

Where necessary biopsy specimens were divided into two samples (each of approximately 5 mg) immediately after thawing. One sample was refrozen at -20°C for further analysis for glutathione and protein (see below). Lipids were extracted by the method of Burton *et al.*²¹ Tissue was homogenised in 100 μl ice cold 0.1 mmol/l sodium dodecyl sulphate, vortexed for one minute with 200 μl absolute ethanol (Spectrosol, BDH, UK) and then with 800 μl heptane (high performance liquid chromatography (HPLC) grade, Rathburn Chemicals) for a further minute. The mixture was centrifuged for 10 minutes at 2000 rpm in a bench top centrifuge and the upper heptane layer was removed.

TOTAL DIENE CONJUGATION AND TOTAL LIPID ASSAYS

Total conjugated dienes were measured by second derivative spectroscopy of the heptane lipid extract. A Shimadzu double beam scanning spectrophotometer with capability for derivative spectroscopy was used. Operating conditions were as follows: an absorbance range was selected between ± 0.02 –0.2 absorbance units (aufs), scanning was started at 250 nm and ended at 220 nm, recorded on a scale of 5 nm/cm, using a 'fast' scan speed and a 2 nm slit width. Sample and reference cells were first scanned over the operating spectrum and a background correction was 'memorised' to correct for any minor differences between cuvettes. Heptane extracts were scanned for absorbance and the second derivative spectra were obtained. Minima at 233 nm and 242 nm were identified and quantified in mm by measurement for minima to adjacent maxima at the higher wavelength¹⁵ on a printout. All values were adjusted to a detector sensitivity of ± 0.02 aufs. The ratio of peak heights at minima 233 nm and 242 nm was calculated.

ASSAY OF TOTAL LIPIDS

Conjugated dienes were corrected for the lipid concentration in the heptane extract ($\mu\text{mol/l}$) assuming a molecular weight of 750 for hepatic lipids. Total lipids were measured as follows using the method of Snyder and Stevens.²² Stock reagent was prepared by dissolving 5 g ferric perchlorate in 10 ml 71% (w/v) perchloric acid and 10 ml deionized water, and 100 ml ice cold absolute ethanol were then added. Each day of

assay, 4 ml of the stock reagent was added to 3 ml perchloric acid and 100 ml ice cold ethanol (working reagent 1).

Alkaline hydroxylamine was prepared daily by taking two aliquots each of 2.5 ml deionized water; to one was added 2 g hydroxylamine-HCL (BDH Chemicals, UK) and into the second 4 g sodium hydroxide. Each preparation was subsequently dissolved in 50 ml ice cold ethanol. The two resultant solutions were combined and centrifuged at 2000 rpm for 10 minutes. The supernatant (working reagent 2) was used in the assay.

A total of 200 μl of heptane lipid extract was dried under nitrogen and resuspended in 300 μl of working reagent 1. The samples were incubated in stoppered tubes in a water bath at 65°C for two minutes and cooled in ice cold water, 750 μl working solution 1 was added, vortex mixed, and allowed to stand for 20 minutes. Optical density was measured at 530 nm against an air blank. A daily standard curve was prepared using 1–100 μl 2 $\mu\text{mol/l}$ glycerol trioleate in spectroscopic grade hexane (1 μl = 1.82 μg lipid).

HEPATIC VITAMIN E

Hepatic vitamin E was measured using 20 μl of the heptane lipid extract as described by Thurnham *et al.*²³ using normal phase HPLC on a 250×4.6 mm column of 5 μm particles of Lichrosorb Si60. The mobile phase was heptane: isopropanol (99:1). Peak detection was by a fluorescence detector (Perkin Elmer LS-1) with two interference filters; ex 280 nm, em 330 nm. Peak areas were quantified (CI-10 computing integrators, Laboratory Data Control) using response factors derived from freshly prepared external standards in heptane (α tocopherol 23.2 $\mu\text{mol/l}$; Sigma, UK). Standards were injected after every fourth sample and gave a coefficient of variation of 6.2% within batch. Values were expressed as the ratio of vitamin E to lipid ($\mu\text{mol/mmol}$).

LINOLEIC ACID ISOMERS

The remaining heptane lipid extract was blown to dryness under nitrogen, hydrolysed for 30 minutes at 65°C with 1 ml methanolic potassium hydroxide (2 mol/l) in stoppered glass tubes under nitrogen, and the pH was adjusted to 7.4 with 3 mol/l hydrochloric acid. The extract was poured onto 3 cc C18 solid phase extraction columns (Bond Elut, Analytichem Int). Free fatty acids were eluted from the column using 1 ml 2:1 propan-2-ol: acetonitrile (HPLC grade 'S', Rathburn Chemicals, Peebleshire, UK).

Samples of 20 μl were injected onto a 250×4.6 mm column of 5 μm particles of Spherisorb ODS2 using a 50×4.6 mm pelli-cular silica precolumn (Technicol Ltd). The mobile phase (acetonitrile:methanol:acetic acid, 90:10:0.1) was pumped at 2 ml per minute and ultraviolet absorbance was monitored by twin ultraviolet detectors set in series to measure conjugated dienes and native polyunsaturated fatty acids at 234 nm and 205 nm respectively.²⁴ Response factors and peak identification were

determined using external standards of 18:2 (9,12) LA (Sigma, UK) and 18:2 (9,11) LA (a gift from Dr T Dormandy, Department of Chemical Pathology, Whittington Hospital, London) prepared in propan-2-ol: acetonitrile (2:1) and allowed peak identification. Peak areas were quantified using CI-10 computing integrators (Laboratory Data Control). The percentage molar ratio, R; [18:2 (9,11) LA] × 100/[18:2 (9,12) LA] was calculated.

TOTAL HEPATIC GLUTATHIONE

Total glutathione was measured by an adaptation of the method of Beutler *et al.*²⁵ Hepatic tissue (5 mg) was homogenised in 200 µl 0.5 mol/l Tris-HCl, pH 7.4. A 20 µl aliquot was removed for protein assay (see below). The remaining homogenate was vortex mixed in 200 µl 0.5 mol/l perchloric acid to precipitate protein. After centrifugation, 300 µl of the supernatant was added to 600 µl 0.3 mol/l Na₂HPO₄ and 150 µl Ellman's reagent prepared by combining 200 mg 5,5'-dithiobis-2-nitrobenzoic acid and 5 g sodium citrate in 1 litre HPLC grade water (Rathburn Chemicals). After incubation at 37°C for 10 minutes, the yellow colour formed was measured against a reagent blank at 412 nm in a Pye Unicam double beam spectrophotometer (SP1800). Standardisation was against a daily standard curve prepared with fresh glutathione (1–10 µmol/l). The coefficient of variation for this method was 3.5% within batch at the midpoint of the standard curve. Hepatic glutathione was corrected for protein, which was measured using 20 µl of the original homogenate and a commercially available kit (Biorad protein assay; Wako Chemicals) using human serum albumin (5–25 mg/l) as a standard (Sigma).

HISTOLOGICAL EXAMINATION

Standard paraffin sections were stained with haematoxylin and eosin, Orcein, and Perle's stain. All slides were examined by the same experienced hepatic pathologist (JG). In addition to the formal report, the degree of inflammation was recorded as 1, 2, or 3 (2 and 3 representing moderate and severe inflammation respectively) and the degree of iron staining in the biopsy specimen as 1 or 2 (2 representing increased iron staining). Grade 1 in each category corresponded to the normal appearance. Reanalysis with the observer blind to the previous score showed excellent agreement with the initial score.

TABLE 1 Hepatic conjugated dienes and antioxidants

Variate	No	Median	IQ range	Minimum	Maximum
D ₂₃₃ (mm/µmol)*	39	0.51	0.26–0.72	0.13	4.76
D ₂₄₂ (mm/µmol)*	39	0.65	0.40–1.22	0.029	2.87
D _{242/233} †	39	2.05	1.0–2.47	0.016	3.2
Vitamin E (µmol/mmol)	39	0.46	0.28–0.91	0.04	2.56
Glutathione (µmol/g protein)	41	30.9	25–41.9	7.8	78
9,11 LA × 100/9,12 LA; *R‡	21	2.7	1.97–4.25	1.21	6.1

*Diene conjugation (DC) measured using derivative spectroscopy (see text for full details of method). Absorbance minima at 233 nm (D₂₃₃) and 242 nm (D₂₄₂) in second derivative spectroscopy represent absorbance maxima in the ultraviolet spectrum. Results are expressed after adjustment to a detector scale of ±0.02 au/s and corrected for lipid measurement by the method of Snyder and Stephens.²² †The ratio of DC measurements at D minima 242 and D minima 233 nm. ‡9,11 LA (9,11 linoleic acid) measured by HPLC after hydrolysis of lipid extract (for method see text). Results expressed as the percentage molar ratio [9,11 LA] × 100/[9,12 LA]; R.

STATISTICS

All data were analysed using non-parametric statistics with the SPP package for personal computers (Timberlake Clarke, UK). Medians with interquartile range are quoted throughout. Grouped data were compared using non-parametric one way analysis of variance (ANOVA). Post hoc pairwise testing (Scheffe) was used to test for differences between groups. Correlations between variates were assessed by Spearman's method or linear regression analysis after normalisation of data by logarithmic transformation. Multiple linear regression was also performed using log transformed data. Significance is quoted for p<0.05.

Results

The liver biopsy specimens from 41 patients were graded as follows: fatty liver (23 patients), mild hepatitis (nine patients), severe hepatitis (six patients), and cirrhosis (three patients). Inflammation in the biopsy specimen was graded 1 (normal), 2, and 3. Numbers in each group were 25, 11, and five respectively. Tissue iron was graded 1 (normal) or 2. Nine of the 41 biopsy specimens were judged to fall into the category with greater iron staining. Insufficient tissue was available for all measurements to be performed on all biopsy specimens and the numbers in each category are indicated where appropriate. There was a weak positive correlation between inflammation grade and the degree of iron staining (R_s 0.355, n=41, p<0.02).

Table I summarises the results for hepatic conjugated diene and antioxidant measurements. The subsequent analysis investigated the relations between hepatic morphology, antioxidant concentration, and conjugated dienes.

CONJUGATED DIENE MEASUREMENTS

Typical second derivative spectra of the lipid extracts of 2 representative liver biopsy speci-

Figure 1: Typical second derivative spectra of heptane extracts from hepatic needle biopsy tissue.

The scans illustrate the minima (arrows) at 233 nm and 242 nm which correspond to absorption maxima in the ultraviolet spectrum. Measurements were made from minima to the adjacent maxima at the higher wavelength adjusted to a detector scale expansion of 0.02 au/s. Scan (A) represents a predominance of absorption at 242 nm (D₂₄₂) while for scan (B) absorption at 233 nm predominates (D₂₃₃).

Figure 2: The relation between conjugated diene signals in the second derivative spectra of hepatic lipid extracts from heavy drinkers, after logarithmic transformation (log D₂₄₂ and log D₂₃₃). There was a significant correlation between the two variates ($r=0.433$, $n=39$, $p<0.006$) expressed by the following equation; $\log D_{242}=0.433 \times \log D_{233}+1.46$.

mens are shown in Figure 1(A) and (B). Minima in the second derivative spectra (which correspond to absorption maxima in the ultraviolet spectra) are seen at 233 nm (D₂₃₃) and 242 nm (D₂₄₂) and are similar to those found in rat liver extracts after carbon tetrachloride and ethanol poisoning.^{16,26} Figure 1(A) and (B) illustrates the differences in relative proportions of the two diene peaks in different biopsy specimens. The two peaks have been interpreted to represent different classes of diene hydroperoxides and their relative proportions may be determined by the cellular redox status.¹⁶ There was a significant correlation between peak height of minima at 233 nm and 242 nm after logarithmic transformation ($R=0.433$, $n=39$, $p<0.006$; Fig 2). The peak height at 242 nm tended to be less when values of the corresponding peak at 233 nm were in the higher range, suggesting a curvilinear relation.

TABLE II Hepatic total dienes (minimum 233 nm) in relation to hepatic morphology

Histological group	No	Total dienes (min 233 nm)		Anova H	p<	Scheffe	p<
		Median*	IQ range				
Fatty liver	22	0.47	0.25-0.68	1.09	NS		NS
Mild hepatitis	8	0.65	0.36-0.81				
Severe hepatitis	6	0.44	0.31-0.81				
Cirrhosis	3	0.35	0.25-4.31				
Inflammation grade:				5.88	0.055	2>1 3<2	0.081 0.017
1	24	0.42	0.25-0.65				
2	10	0.7	0.52-2.28				
3	5	0.37	0.25-0.46				
Iron grade:				0.97	NS		
1	30	0.53	0.29-0.73				
2	9	0.37	0.15-0.74				

*Units; mm/μmol hepatic lipid/l, detector scale expansion 0.02 aufs.

TABLE III Hepatic total dienes (minimum 242 nm) in relation to hepatic morphology

Histological group	No	Total dienes (min 242 nm)		Anova H	p<	Scheffe	p<
		Median*	IQ range				
Fatty liver	22	0.51	0.34-1.02	4.28	NS	2>1 4>1	NS 0.074 NS 0.061
Mild hepatitis	8	1.06	0.53-1.85				
Severe hepatitis	6	0.76	0.41-0.96				
Cirrhosis	3	0.79	0.78-2.87				
Inflammation grade:				4.18	NS	2>1 3>1	NS 0.041 0.091
1	24	0.51	0.35-0.85				
2	10	1.06	0.58-1.85				
3	5	0.68	0.62-0.97				
Iron grade:				0.12	NS		
1	29	0.67	0.44-1.26				
2	9	0.61	0.38-1.4				

*Units; mm/μmol hepatic lipid/l, detector scale expansion 0.02 aufs.

After correction for lipid, the heights of the two diene absorption peaks were studied in relation to hepatic histology, inflammation grade, and iron grade (Tables II and III).

The diene signal at 233 nm (D₂₃₃) showed no significant differences between groups when analysed by histological group (Table II; H 1.09, NS). When analysed according to inflammation grade, differences between groups just failed to reach significance (H 5.88, $p=0.055$). Values in the group with moderate inflammation (grade 2), $n=10$, median (interquartile range) 0.7 (0.52-2.28) mm/μmol tended to be greater than those in the severe (grade 3) and absent (grade 1) inflammation groups ($p<0.02$ and $p=0.081$ respectively). Total conjugated dienes were not related to iron grade (Table II).

The diene signal at 242 nm (D₂₄₂) showed a similar pattern of results to those obtained for the D₂₃₃ signal (Table III). D₂₄₂ in hepatic biopsy specimens with cirrhotic changes and those with mild hepatitis tended to be greater than those with fatty liver ($p=0.074$ and $p<0.061$ respectively, NS). Compared with biopsy specimens without inflammatory changes (grade 1) those with inflammation (grades 2 or 3) tended to have higher D₂₄₂ values ($p<0.05$ and NS $p=0.091$ respectively).

The ratio $[9,11 \text{ LA}] \times 100/[9,12 \text{ LA}]$ (R) was measured in 21 biopsy samples (Fig 3). Numbers in each category are shown in the figure. When analysed by histological group (Fig 3(A)) significant differences emerged (H=11.44, $p<0.01$). The two groups with hepatitis differed significantly from those with fatty liver (severe hepatitis, median (interquartile range) 4.3 (3.37-5.25), $p<0.01$; mild hepatitis, 4.35 (3.36-5.38), $p<0.005$; fatty liver, 2.12 (1.73-2.7)). Similarly for inflammation grade (Fig 3(B)) a difference between the groups emerged overall (H=8.95, $p<0.02$). Values were significantly greater in the two groups with a significant inflammatory component compared to those without (inflammation grade 3, 4.4 (4.3-6.1), $p<0.005$; inflammation grade 2, 3.4 (2.94-4.8), $p<0.05$; inflammation grade 1, 2.12 (1.78-3.17)). No significant differences were noted with regard to iron grade (Fig 3(C)).

HEPATIC GLUTATHIONE

When analysed by histological grade, the hepatic glutathione content (μmol/g protein) did not differ significantly between the groups (fatty liver, 35.22 (26.1-44), $n=23$; mild hepatitis, 30.92 (23.8-36.9), $n=9$; severe hepatitis, 27.25 (11.9-41.0), $n=6$; cirrhosis, 28.82 (19.5-46.1), $n=3$; H=2.15, NS). For inflammation grade (Fig 4(A)) overall no intergroup differences emerged (inflammation grade 3, 28.82 (19.5-46.1), $n=5$; inflammation grade 2, 30.45 (18.6-33.5), $n=11$; inflammation grade 1, 35.22 (26.9-44.7), $n=25$; H=3.31, NS), though the grouped data showed a significant trend to declining glutathione with increasing inflammation ($p<0.05$). Hepatic glutathione was significantly lower in the group with greater iron staining (grade 2, 21.13 (12.9-30.4), $n=9$; grade 1, 33.5 (27.9-45), $n=32$; Z=2.95, $p<0.05$, Fig 4(B)).

Figure 3: Conjugated dienes; $\frac{9,11 \text{ LA} \times 100}{9,12 \text{ LA}}$ (R) in hepatic biopsy specimens by histological grade and iron grade.
*Measured by reverse phase high performance liquid chromatography after hydrolysis of hepatic biopsy lipid extract (for conditions see text).

HEPATIC VITAMIN E ($\mu\text{MOL/MMOL}$)
When analysed by histological group, inflammation grade, and iron staining no significant differences emerged between the groups (Table IV). In 35 of 39 biopsy specimens concentrations of vitamin E fell below a value of $1.11 \mu\text{mol/mmole lipid}$ (0.8 mg/g) regarded as indicative of vitamin E deficiency in serum²³

CORRELATIONS
Multiple linear regression analysis after logarithmic transformation of data with either D_{233} , D_{242} , or $D_{233/242}$ as the dependent (y) variate and hepatic glutathione and vitamin E as independent variates showed no significant relations between hepatic antioxidants and total conjugated dienes. Hepatic glutathione correlated inversely with $R \frac{9,11 \text{ LA} \times 100}{9,12 \text{ LA}}$; $R_s = -0.521$ $p < 0.02$.

Discussion
This is the first study to use derivative spectroscopy to identify conjugated dienes as indirect

TABLE IV Hepatic vitamin E:lipid ratio in relation to hepatic morphology

Histological group	No	Vitamin E:lipid ratio		Anova H	p<	Scheffe
		Median*	IQ range			
Fatty liver	22	0.45	0.28–0.96	2.82	NS	NS
Mild hepatitis	8	0.57	0.23–0.99			NS
Severe hepatitis	6	0.37	0.28–0.63			NS
Cirrhosis	3	0.81	0.80–1.85			NS
Inflammation grade:						
1	24	0.46	0.31–1.04	0.55	NS	NS
2	10	0.33	0.23–0.87			NS
3	5	0.6	0.32–0.77			NS
Iron grade:						
1	30	0.46	0.28–1.03	0.35	NS	NS
2	9	0.44	0.27–0.81			NS

*Units; $\mu\text{mol vitamin E/mmole lipid}$.

markers of hepatic lipid peroxidation in alcoholic liver disease in man. Using this technique, we identified two minima at 233 nm and 242 nm in the second derivative spectrum of hepatic lipid extracts, which correspond to absorbance maxima in the conventional ultraviolet spectrum. Similar diene absorption spectra have been identified in hepatic microsomal lipid extracts from rats poisoned with carbon tetrachloride, which is known to generate hepatic lipid peroxidation.^{16,26} In those studies, the signal at 233 nm was found to increase in a linear fashion up to three hours after poisoning.²⁶ Twenty four hours after ethanol poisoning in rats similar changes were also observed.²⁶ These two minima have been attributed to mixtures of trans, trans and cis, trans hydroperoxydiene lipids and it is suggested that their relative proportions reflect the cellular redox status since the 233 nm signal appeared after the signal at 242 nm.¹⁶ Furthermore, the appearance of the signal at 242 nm after carbontetrachloride poisoning could be prevented by pretreatment with the antioxidant vitamin E.¹⁶ In this study, a considerable variation was seen in the proportion of the two diene signals, with a suggestion that at higher levels of the D_{233} signal, the D_{242} signal diminished (Fig 1), though we could detect no evidence of a relation between these signals (or their ratio) and hepatic antioxidants. These findings suggest that for hepatic tissue the two minima represent absorption spectra of different species of conjugated diene lipids whose generation during lipid peroxidation in hepatic tissue does not conform to a strict stoichiometric pattern. The studies using alkaline hydrolysis and HPLC

Figure 4: Glutathione* in hepatic biopsy specimens by inflammation grade and iron grade.
*For method see text. Results are expressed as $\mu\text{mol/g liver protein}$.

indicated that a proportion of diene conjugation within hepatic lipids exists as 9,11 LA, the fatty acid that accounts for >90% of diene conjugation in serum, bile, and duodenal juice,^{13 14 24} however, a number of other more polar ultraviolet absorbing peaks were detected at 234 nm, which could be hydroperoxides. Their precise nature was not determined in the present study and the possibility that these peaks were generated in vitro during hydrolysis cannot be discounted. Future studies might therefore use phospholipase hydrolysis at 25°C.²⁷

We attempted to correlate changes in hepatic total dienes (D_{233} and D_{242}) with hepatic morphology. There was no clear relation with histological grade, though D_{233} and D_{242} signals tended to be greater in those biopsy specimens with moderate inflammation (Tables II and III).

For the 21 biopsy specimens for which measurements of the ratio R ; $[9,11 \text{ LA}] \times 100 / [9,12 \text{ LA}]$ were made a clearer picture emerged. Higher values for R were correlated with the presence of hepatitis and inflammation (Fig 3), although neither total dienes nor R correlated with the degree of iron deposition in the biopsy specimens (Tables II and III, Fig 3). Transition metals may play a catalytic role in free radical reactions, stimulating the decomposition of hydroperoxides to promote further lipid peroxidation, thus generating a wide variety of aldehydes including malonaldehyde which can react with thiobarbituric acid.^{28 29} Thiobarbituric acid reactive products have been previously detected in human hepatic biopsy material from alcoholics.¹² In the present study a weak correlation between hepatic iron and inflammation grade was observed ($R_s 0.355$, $n=41$, $p<0.02$). The lack of a correlation between iron and R or total dienes may indicate that iron deposition is a consequence of the ethanol induced hepatic injury rather than a cause.

Hepatic antioxidant status may play a critical role in the defence against oxidative stress. Glutathione functions with the enzyme glutathione peroxidase to reduce free lipid hydroperoxides and hydrogen peroxide.³⁰ Furthermore, it is a cosubstrate for the peroxidation inhibiting protein which is capable of destroying hydroperoxide fatty acids located in phospholipids.³¹ Shaw *et al* detected increased hepatic diene conjugation and a reduction in glutathione in heavy drinkers,⁷ though other workers failed to confirm the findings for glutathione.³² In the present study, hepatic glutathione concentrations showed a trend to decrease with increasing inflammation grade (Fig 4) and increased iron grade was also associated with reduced hepatic glutathione ($p<0.05$; Fig 4), findings compatible with either diminished production or increased utilisation. Furthermore, there was a significant inverse correlation between R and concentrations of the antioxidant glutathione ($R_s = -0.52$, $n=20$, $p<0.02$). Additional information in respect of hepatic redox state might have been provided by utilising an enzymic method of glutathione determination which may also be used to determine oxidised glutathione³³ and in view of the present findings this may be considered in future studies.

Since vitamin E represents the major source of

chain breaking antioxidant activity in membranes³⁴ and our previous studies have indicated impaired vitamin E status in alcoholics,²³ we assessed hepatic vitamin E status. Concentrations varied considerably between biopsy specimens (median $0.46 \mu\text{mol}/\text{mmol}$, range 0.04 – 2.56 , Table I), and in 35 of the 39 biopsy specimens fell below $1.11 \mu\text{mol}/\text{mmol}$ (0.8 mg/g), a value which has been quoted as the threshold for vitamin E deficiency in serum²³ and is associated with increased peroxide fragility of red cells. However, the applicability of such figures to hepatic lipids is unknown. We could detect no significant differences in respect of hepatic vitamin E and any of the morphological characteristics we assessed (Table IV).

The hypothesis that ethanol can induce hepatic injury through a mechanism involving free radical induced lipid peroxidation is not new.¹ Debate continues over a possible mechanism for free radical generation in response to ethanol. Slater² has proposed that ethanol is converted to a hepatotoxic ethoxy radical ($\text{C}_2\text{H}_5\text{O}\cdot$), whilst Reitz³ implicated the microsomal enzyme system. When ethanol ingestion is high it has been suggested that an alternative pathway for the metabolism of acetaldehyde involving mixed function cytochrome P_{450} oxidase activity may generate free radicals.⁴ Lewis and Paton proposed that the superoxide radical could be generated within areas of relative hypoxia in the liver from acetaldehyde via xanthine oxidase.⁵ Alternatively, lipid peroxidation might result from the effects of reactive oxygen species liberated by infiltrating inflammatory cells¹⁷ as a result of hepatitis. Our data suggest that heavy alcohol consumption leads to hepatic inflammation and lipid peroxidation. Though the precise mechanism for its generation could not be determined, the correlation with inflammatory histological features and the relation with hepatic glutathione raise the possibility that the process of ethanol induced hepatic injury may be modulated either by agents to stimulate hepatic glutathione regeneration or by strategies involving antioxidants. A trial of this treatment in alcoholic hepatitis would be one possibility.

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