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The role of aldehyde oxidase in ethanol-induced hepatic lipid peroxidation in the rat

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Hepatic lipid peroxidation has been implicated in the pathogenesis of alcohol-induced liver injury, but the mechanism(s) by which ethanol metabolism or resultant free radicals initiate lipid peroxidation is not fully defined. The role of the molybdenum-containing enzymes aldehyde oxidase and xanthine oxidase in the generation of such free radicals was investigated by measuring alkane production (lipoperoxidation products) in isolated rat hepatocytes during ethanol metabolism. Inhibition of aldehyde oxidase and xanthine oxidase (by feeding tungstate at 100 mg/day per kg) decreased alkane production (80–95 %), whereas allopurinol (20 mg/kg by mouth), a marked inhibitor of xanthine oxidase, inhibited alkane production by only 35–50 %. Addition of acetaldehyde (0–100 μM) (in the presence of 50 μM-4-methylpyrazole) increased alkane production in a dose-dependent manner (K_m of aldehyde oxidase for acetaldehyde 1 mM); menadione, an inhibitor of aldehyde oxidase, virtually inhibited alkane production. Desferrioxamine (5–10 μM) completely abolished alkane production induced by both ethanol and acetaldehyde, indicating the importance of catalytic iron. Thus free radicals generated during the metabolism of acetaldehyde by aldehyde oxidase may be a fundamental mechanism in the initiation of alcohol-induced liver injury.

INTRODUCTION

Evidence of lipid peroxidation and glutathione alterations induced by ethanol demonstrated in rodent models [1–8], sub-human primates [9] and in man [10] suggests the importance of ethanol-induced free radicals in the pathogenesis of alcohol-induced liver injury. However, although the effects or the metabolism of acetaldehyde have been implicated as a source of free radicals [8], the mechanism of free-radical generation induced by ethanol is unknown.

In vitro, the metabolism of ethanol by alcohol dehydrogenase to acetaldehyde and the further metabolism of the latter by xanthine oxidase generates superoxide [11,12], which can promote peroxidation [13] and mobilize ferritin iron [13,14]. However, the relatively high K_m of xanthine oxidase for acetaldehyde (> 30 mM) [15], the availability of other substrates such as xanthine and hypoxanthine with greater affinities for xanthine oxidase, and the predominance of the dehydrogenase form rather than the oxidase form of the enzyme all mitigate against its role *in vivo*. Increased nucleotide catabolism is observed during ethanol metabolism, possibly attributable to the obligatory removal of excess acetate [16]. The oxidation of purines by xanthine oxidase produces free radicals. However, acetate administration itself does not mimic ethanol-induced peroxidation, whereas acetaldehyde administration does [8,17].

Rajagopalan and colleagues characterized a cytosolic molybdenum-containing enzyme, aldehyde oxidase (EC 1.2.3.1) [18,19]. This enzyme has a lower K_m (1 mM) for acetaldehyde than xanthine oxidase, produces superoxide during the metabolism of various aldehyde-containing substrates and has a much lower affinity for purines than has xanthine oxidase [20–22]. It would thus appear to be a much more likely pathway of free-radical generation due to acetaldehyde metabolism than xanthine oxidase.

The role of aldehyde oxidase in ethanol-induced lipid peroxidation was studied by measuring ethanol- and acetaldehyde-induced alkane production in isolated hepatocytes in the presence of various inhibitors to block selectively aldehyde oxidase, xanthine oxidase or both. The extent to which catalytic iron plays a role in ethanol-induced lipid peroxidation was studied in these models by assessing the effect of iron chelation with desferrioxamine.

METHODS

Animals and diets

Male Sprague–Dawley rats were obtained from Taconic Farms, Germantown, NY, U.S.A. Studies were performed in rats fed Purina rat chow *ad libitum*, as well as in animals pair-fed nutritionally adequate liquid diets with and without tungsten supplementation. Chronic feeding of tungstate has been shown to inhibit the molybdenum-dependent oxidases xanthine oxidase and aldehyde oxidase [23]. Liquid diets (Lieber–DeCarli regular rat diet) were obtained from Dyets Inc., Bethlehem, PA, U.S.A. One diet was supplemented with 1.64 g of sodium tungstate/kg dry diet. The diets were pair-fed to the animals using the simultaneous pair-feeding system described by Israel *et al.* [24] for a period of 12 weeks. Animals consumed approx. 100 mg of sodium tungstate/day per kg.

All animals were anesthetized with Ketamine (350 mg/kg intraperitoneally) before operation for liver perfusion or being killed by decapitation.

Isolated-hepatocyte studies

Isolated hepatocytes were prepared by the method of Berry & Friend [25] as modified by Seglen [26]. Animals were studied in the fed state after withdrawal of diets at 08:00 h. Viability of hepatocytes was determined by exclusion of Trypan Blue. Incubations were carried out with cells that retained a viability of > 90 %; final cell counts in incubations were between 1×10^6 and 2×10^6 cells/ml. Cells were incubated in sealed 20 ml vials in a shaking waterbath at 37 °C. They were suspended in Hanks solution containing Ca^{2+}, Mg^{2+} and HCO_3^- (Sigma Chemical Co., St. Louis, MO, U.S.A.) plus bovine serum albumin (1 %), lactate (1 mM), pyruvate (0.1 mM), with the final pH adjusted to 7.4.

Acetaldehyde concentrations were verified by headspace-gas chromatography on a Perkin–Elmer 2000 headspace-gas chromatograph by the method of Korsten *et al.* [27]. Pentane and

ethane were measured after 90 min of incubation by analysis of headspace gas using a 5% Carbowax 80/120 Corsopack B column [1.83 m × 0.32 cm (6 ft × 0.125 in)] (Supelco, Bellefonte, PA, U.S.A.) on a Perkin–Elmer Sigma 2000 gas chromatograph as previously described [14]. Reactions were stopped by addition of 0.5 ml of 3.4% (w/v) trichloroacetic acid.

Treatment with allopurinol

Animals were given 20 mg of allopurinol/kg suspended in normal saline by gavage 90 min before preparations of isolated hepatocytes in order to inhibit xanthine oxidase. Selected animals were also studied after administration of allopurinol (100 mg/kg intraperitoneally) at both 24 and 1 h before study in order to investigate ethanol-induced alkane production when virtually complete xanthine oxidase inhibition is achieved. Some incubations of control hepatocytes were carried out with the addition of 20 μM-allopurinol as well as 20 μM-oxipurinol.

Xanthine oxidase activity

Xanthine oxidase activities in the livers of control, allopurinol- and tungsten-treated animals were determined by a modification of the method of Topham *et al.* [28]. Hepatocytes from animals were homogenized in 1.15% KCl, and the 100000 ***g*** supernatant was analysed after passage through a G-25 Sephadex column. Oxidation of xanthine to uric acid was determined spectrophotometrically by the increase in A_{295} in the absence of added NAD^+ at pH 7.5. Protein was determined by the method of Lowry *et al.* [29].

Xanthine oxidase activity was markedly inhibited by tungsten feeding: control ($n = 6$) 0.083 ± 0.023 versus tungsten-fed ($n = 6$) 0.019 ± 0.012 A_{295} min per mg of protein ($P < 0.001$). Treatment with allopurinol by gavage or by incubation of hepatocytes with 20 μM-allopurinol virtually abolished xanthine oxidase activity, addition of menadione (20 μM) had no effect. As determined by acetaldehyde disappearance using a Lineweaver–Burk plot, the apparent K_m of rat liver xanthine oxidase for acetaldehyde was approx. 30 mM.

Aldehyde oxidase activity

Aldehyde oxidase was purified from rat liver by the method of Rajagopalan *et al.* [18] as modified by Branzoli & Massey [30]. In summary the liver was perfused with iced saline (0.9% NaCl) to remove all blood and then homogenized in 0.05 M-phosphate buffer (pH 6.8) 1.0 mM-EDTA. The homogenate was maintained at 55 °C for 10 min then chilled rapidly in ice to 0.5 °C and centrifuged at 14000 ***g*** for 40 min. The supernatant was saturated 50% (w/v) with $(NH_4)_2SO_4$ and the precipitate collected by centrifugation. It was then redissolved in the harvesting buffer and passed though Sephadex G-25.

Aldehyde oxidase activity was measured by the reduction of potassium ferricyanide using acetaldehyde as an electron donor as described by Hall & Krenitsky [20] and by measuring the oxidation of acetaldehyde disappearance by using headspace chromatography. Activities were determined in the presence of 20 μM-allopurinol to inhibit any contaminating xanthine oxidase activity.

Aldehyde oxidase was markedly inhibited by tungsten feeding (all $n = 4$): control, 0.354 ± 0.027, versus tungsten-treated, 0.002 unit/g of liver at 37 °C, measured by potassium ferricyanide reduction); control, 0.270 ± 0.014 versus tungsten-fed, not detected (measured by acetaldehyde metabolism) (both $P < 0.001$). Activities of aldehyde oxidase were undetectable on addition of 20 μM-menadione, but were not affected by the addition of allopurinol (20 μM). The apparent K_m of acetaldehyde for aldehyde oxidase, as determined by acetaldehyde disappearance using a Lineweaver–Burk plot, was 1 mM.

Glutathione determinations

Hepatocyte suspensions were treated with 0.2 ml of 4% (w/v) sulphosalicylic acid to each 1 ml of incubation mixture and centrifuged 10000 ***g*** for 5 min. GSSG and GSH were determined on the filtrates by the method of Griffith [32]. Addition of acetaldehyde (500 μM) or sodium tungstate (1 mM) to filtrates had no effect on the assays for the glutathione species.

Tungsten feeding had no effect on glutathione (control versus tungsten-fed): GSH, 38.2 ± 3.4 versus 36.3 ± 4.4 nmol/10^6 cells; GSSG, 4.5 ± 0.8 versus 4.0 ± 0.4 nmol/10^6 cells).

Other enzymes

Selected pairs of animals were used for the determination of other enzymes that have been shown to be important in alcohol metabolism or possibly free-radical generation by ethanol. Livers were fractionated by differential ultracentrifugation. After perfusion with iced 1.5% (w/v) KCl they were homogenized in 4 vol. of KCl and centrifuged at 3000 ***g*** (20 min), 10000 ***g*** (30 min) (for the mitochondrial fraction) and 100000 ***g*** (60 min) (for cytosol and washed microsomal fraction).

Alcohol dehydrogenase activity. Alcohol dehydrogenase activity was determined in hepatic cytosol by the method of Bonnichsen & Brink [33].

Aldehyde dehydrogenase activity. Aldehyde dehydrogenase activity was determined for hepatic cytosol, mitochondria (extract and membrane fractions) and microsomes for both the low-K_m (propionaldehyde, 0.068 mM) and high-K_m (propionaldehyde, 13.6 mM) enzymes by the method of Greenfield *et al.* [34].

Microsomal cytochrome *P*-450. The microsomal cytochrome *P*-450 content was determined by the method of Omura & Sato [35].

Microsomal cytochrome *P*-450 reductase activity. Microsomal cytochrome *P*-450 reductase activity was determined by the method of Phillips & Langdon [36].

Microsomal ethanol oxidation. Microsomal ethanol oxidation was determined essentially by the method of Lieber & DeCarli [37], with the modification that all incubations were carried out in the presence of 20 μM-allopurinol to inhibit any contaminating xanthine oxidase.

Tungsten feeding had no significant effect (mean for two pairs of animals, tungsten-fed versus control) on alcohol dehydrogenase activity (0.0196 versus 0.0240 unit/mg of cytosolic protein), aldehyde dehydrogenase activity (cytosol, low K_m; 3.9 versus 3.9 nmol/min per mg of protein); (mitochondria, low K_m extract: 41.8 versus 41.2; high-K_m extract: 27.0 versus 36.1; low-K_m membranes: 4.6 versus 3.1; high-K_m membranes: 25.1 versus 21.0), microsomes (low-K_m: 3.4 versus 3.0; microsomes: high-K_m: 16.8 versus 15.0), microsomal cytochrome *P*-450 (0.90 versus 0.80 nmol/mg of microsomal protein), cytochrome *P*-450 reductase (40.2 versus 48.0 units/mg of microsomal protein), microsomal ethanol oxidation (4.54 versus 5.18 nmol/min per mg of microsomal protein).

Reagents

Allopurinol, Hanks solution, collagenase Type IV, NAD^+ (Type I), ethanol and glutathione (GSSG, GSH) were obtained from Sigma. Acetaldehyde was obtained from Eastman–Kodak (Rochester, NY, U.S.A.). All solutions were prepared with Chelex-treated water.

Statistical methods

Means (±S.E.M.) were calculated, and the significance of differences between groups was assessed by Student's t test.

RESULTS

The effect of inhibitors of molybdeum-containing oxidases on ethanol-induced alkane (ethane and pentane) production are shown in Fig. 1. The increase in alkane production induced by ethanol was abolished by treatment with tungsten feeding, but was only partially inhibited by allopurinol treatment (35–50%). There was no significant difference between the ethanol-induced alkane production after oral allopurinol administration and treatment with allopurinol intraperitoneally (ethane: 2.2 ± 0.3 versus 2.8 ± 0.4 pmol/h per 10^6 cells; pentane: 2.6 ± 0.2 versus 3.0 ± 0.4; both not significant).

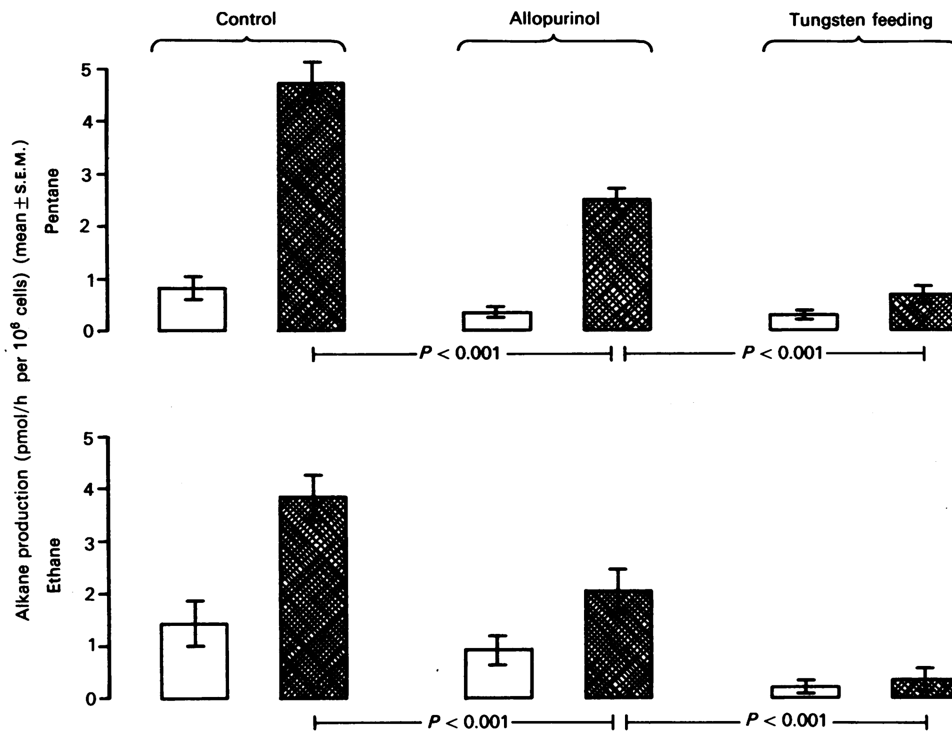

Fig. 1. Ethanol-induced alkane production: modification by tungsten feeding and allopurinol (20 mg/kg by mouth)

Tungsten feeding, which inhibited both aldehyde oxidase and xanthine oxidase activity, abolished the increase in alkane production induced by ethanol. By contrast, inhibition of xanthine oxidase activity with allopurinol only partially inhibited the effect of ethanol (all $n = 6$). ▩, Ethanol (20 mM); □, control (no ethanol).

Table 1. Ethanol- and acetaldehyde-induced alkane production in isolated control hepatocytes

Acetaldehyde added at physiological concentrations increased alkane production to rates comparable with those seen with ethanol. Alkane production induced by acetaldehyde was inhibited by menadione (an inhibitor of aldehyde oxidase), but not by allopurinol (an inhibitor of xanthine oxidase). Abbreviation: nd, not detected.

Incubation mixture	Alkane production (pmol/h per 10^6 hepatocytes)	
	Ethane	Pentane
No additions	0.85±0.03	0.84±0.04
Ethanol (20 mM)	2.02±0.22	4.30±0.24
Ethanol+allopurinol (20 μM)	1.80±0.15	3.70±0.26
Ethanol+oxipurinol (20 μM)	1.70±0.24	3.90±0.37
Ethanol+menadione (20 μM)	0.40±0.18	1.48±0.44
4-Methylpyrazole (50 μM)	nd	0.75±0.12
+Acetaldehyde		
25 μM	1.90±0.09	1.63±0.06
50 μM	2.33±0.37	3.15±0.70
100 μM	3.07±0.16	5.45±0.65
4-Methylpyrazole (50 μM)		
+acetaldehyde (50 μM)		
+Allopurinol (20 μM)	1.85±0.12	1.8±0.007
+Menadione (20 μM)	nd	0.02±0.005
+Tungsten feeding	nd	nd

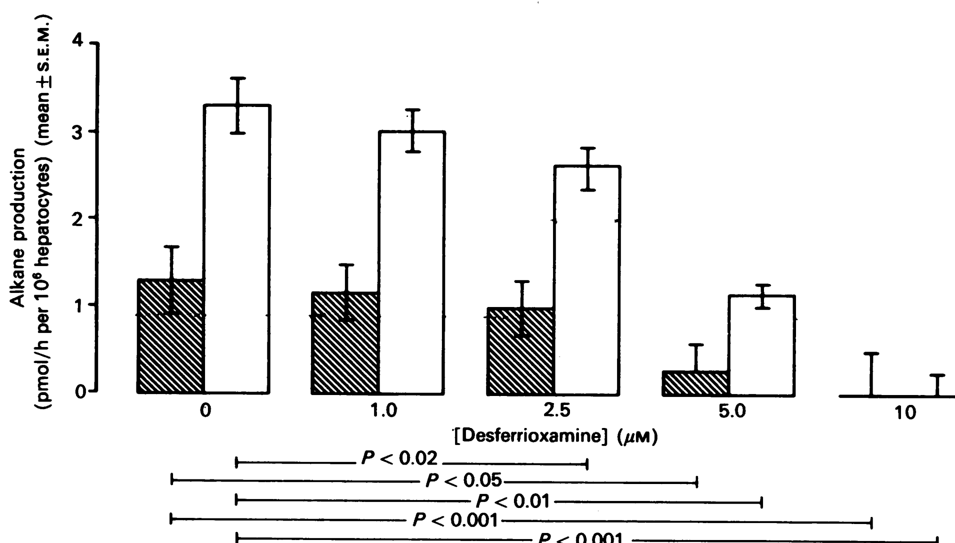

Fig. 2. Role of catalytic iron in ethanol-induced alkane production

Addition of desferrioxamine inhibited the increase in alkane production induced by ethanol in a dose-dependent manner, with virtually complete inhibition at a concentration of 10 μM (all $n = 6$). ▧, Ethane; □, pentane.

Alkane production induced by ethanol in control hepatocytes was markedly inhibited by addition of menadione (20 μM), which selectively inhibited aldehyde oxidase, but was only partially diminished by allopurinol (20 μM), which selectively inhibited xanthine oxidase (Table 1).

Incubations of control hepatocytes with acetaldehyde (25–100 μM) (in the presence of 4-methylpyrazole) revealed a dose-dependent increase in peroxidation with rates comparable with that seen with ethanol. Incubations with added menadione (20 μM) virtually suppressed alkane production, whereas addition of allopurinol had only a minimal effect (Table 1). Addition of acetate (up to 2 mM) alone had no significant effect on alkane production.

The role of catalytic iron in ethanol-induced alkane production is shown in Fig. 2. Incubation in which desferrioxamine was added at concentrations above 5 μM inhibited the increase in alkane production induced by 20 mM-ethanol. Similarly, desferrioxamine (> 5 mM) completely inhibited alkane production induced by the addition of acetaldehyde (25–100 μM) plus 4-methyl pyrazole (ethane and pentane all < 0.02 pmol/h per 10^6 cells).

DISCUSSION

These studies show that the metabolism of acetaldehyde by hepatic aldehyde oxidase is a source of free radicals, which initiate hepatic lipid peroxidation. The initiation of peroxidation was observed to occur both during the metabolism of ethanol as well as upon addition of acetaldehyde at physiological concentrations and was found to depend upon the presence of catalytic iron. Addition of acetaldehyde at concentrations which are observed under physiological conditions during the metabolism of ethanol did not produce peroxidation in cells treated with inhibitors of aldehyde oxidase. However, peroxidation was produced under the same conditions when xanthine oxidase was inhibited. The partial inhibition of ethanol-induced alkane production by allopurinol demonstrates that xanthine oxidase plays at least a secondary role in promoting alcohol-induced lipoperoxidation.

The K_m for xanthine oxidase for acetaldehyde is as high as 36 mM under some conditions [15], whereas that for aldehyde oxidase is considerably lower (1 mM) [21]. Furthermore, the ability of the enzymes to metabolize endogenous substrates differs markedly; purines such as xanthine and hypoxanthine, for example, are readily oxidized by xanthine oxidase but only very poorly, or not at all, by aldehyde oxidase [20]. Increased catabolism of nucleotides has been described after alcohol; it has been suggested that this is due to the enhanced catabolism of ATP resulting from the obligatory metabolism of excess acetate [16]. However, increased hepatic oxidative stress is observed in the perfused liver upon addition of acetaldehyde, but not acetate [8,17]. Increased nucleotide catabolism as well as xanthine/xanthine oxidase-generated free radicals are observed after periods of hypoxia, and these effects are potentiated by ethanol [38]. Increased conversion of xanthine dehydrogenase into xanthine oxidase by ethanol could be one mechanism of such injury. Increased, conversion of xanthine dehydrogenase into xanthine oxidase has been reported after acute ethanol administration [39,40]. The mechanism by which ethanol converts xanthine dehydrogenase into xanthine oxidase is unknown, but a role of acetaldehyde has been implicated [39]. The complete inhibition of ethanol- or acetaldehyde-induced alkane production by menadione and the partial effect of allopurinol are suggestive of a possible role for aldehyde oxidase-generated free radicals in the conversion of xanthine dehydrogenase into xanthine oxidase.

The metabolism of alcohol by alcohol dehydrogenase *in vitro* causes a slow mobilization of ferritin iron [13,14], and the further metabolism of generated acetaldehyde, albeit at high concentrations, by xanthine oxidase greatly enhances this effect. In these studies the addition of the iron chelator desferrioxamine markedly inhibited ethanol-induced alkane production at concentrations as low as 5 μM, and virtually abolished peroxidation at concentrations above 10 μM. These findings are consistent with an important role for catalytic iron and ethanol-induced iron mobilization in alcoholic liver injury. The role of aldehyde oxidase in ethanol-induced iron mobilization, however, remains to be demonstrated.

In conclusion, these studies suggest that the molybdenum-containing enzyme aldehyde oxidase plays a significant role in ethanol-induced hepatic lipoperoxidation. Peroxidation appears

to be mediated predominantly by the metabolism of acetaldehyde by aldehyde oxidase in the presence of catalytic iron, with xanthine oxidase playing a contributory role.

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REFERENCES

1. DiLuzio, N. R. & Kalish, G. H. (1966) Gastroenterology **50**, 392–396
2. Comporti, M., Benedetti, A. & Chieli, E. (1973) Lipids **8**, 498–502
3. MacDonald, C. M. (1973) FEBS Lett. **35**, 227–233
4. Koster, H., Albreach, D. & Kappus, H. (1977) Toxicol. Appl. Pharmacol. **41**, 639–648
5. Koes, M., Ward, T. & Pennington, S. (1974) Lipids **9**, 889–904
6. Torrielli, M. V., Gabriel, L. & Dianzini, M. U. (1978). J. Pathol. **126**, 11–25
7. Reitz, R. C. (1975) Biochim. Biophys. Acta **380**, 145–154
8. Muller, A. & Sies, H. (1982) Biochem. J. **206**, 153–156
9. Shaw, S., Jayatilleke, E., Ross, W. A., Gordon, E. R. & Lieber, C. S. (1981) J. Lab. Clin. Med. **98**, 417–425
10. Shaw, S., Rubin, K. & Lieber, C. S. (1983) Dig. Dis. Sci. **28**, 585–589
11. Kellogg, E. W. & Fridovich, I. (1975) J. Biol. Chem. **250**, 8812–8817
12. Nagano, T. & Fridovich, I. (1985) J. Free Radicals Biol. Med. **1**, 39–42
13. Shaw, S. & Jayatilleke, E. (1987) Biochem. Biophys. Res. Commun. **143**, 984–990
14. Shaw, S., Jayatilleke, E. & Lieber, C. S. (1988) Alcohol **5**, 135–140
15. Fridovich, I. (1966) J. Biol. Chem. **241**, 3126–3128
16. Faller, J. & Fox, I. H. (1982) N. Engl. J. Med. **307**, 1598–1602
17. Muller, A. & Sies, H. (1987) Alcohol Alcohol. Suppl. **1**, 67–74
18. Rajagopalan, K. V., Fridovich, I. & Handler, P. (1962) J. Biol. Chem. **237**, 922–928
19. Rajagopalan, K. V., Fridovich, I. & Handler, P. (1962) J. Biol. Chem. **239**, 2027–2035
20. Hall, W. H. & Krenitsky, T. A. (1986) Arch. Biochem. Biophys. **15**, 36–46
21. Rajagopalan, K. V. & Handler, P. (1963) J. Biol. Chem. **239**, 2027–2035
22. Rajagopalan, K. V. (1980) in Enzymatic Basis of Detoxication (Jakoby, W. B., ed.), vol. 1, pp. 295–309, Academic Press, New York
23. Johnson, J. L., Rajagopalan, K. V. & Cohen, H. J. (1974) J. Biol. Chem. **249**, 859–866
24. Israel, Y., Oporto, B. & Macdonald, B. A. (1984) Alcohol.: Clin. Exp. Res. **8**, 505–508
25. Berry, M. N. & Friend, D. S. (1969) J. Cell Biol. **43**, 506–520
26. Seglen, P. O. (1973) Exp. Cell Res. **82**, 391–398
27. Korsten, M. A., Matsuzaki, S., Feinman, L. & Lieber, C. S. (1975) New Eng. J. Med. **292**, 386–389
28. Topham, R. W., Walker, M. C. & Calisch, M. P. (1982) Biochem. Biophys. Res. Commun. **109**, 1240–1246
29. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275
30. Branzoli, U. & Massey, V. (1974) J. Biol. Chem. **14**, 4339–4345
31. Reference deleted.
32. Griffith, O. W. (1980) Anal. Biochem. **106**, 207–212
33. Bonnichsen, R. K. & Brink, N. G. (1955) Methods Enzymol. **1**, 495–500
34. Greenfield, N. J., Pietruszko, R., Lin, G. & Lester, D. (1976) Biochim. Biophys. Acta **428**, 627–632
35. Omura, R. & Sato, R. (1964) J. Biol. Chem. **239**, 2370–2375
36. Phillips, A. H. & Langdon, R. G. (1962) J. Biol. Chem. **237**, 2652–2660
37. Lieber, C. S. & DeCarli, L. M. (1970) J. Biol. Chem. **245**, 2505–2512
38. Younes, M. & Strubelt, O. (1987) Biochem. Pharmacol. **36**, 2973–2977
39. Sultatos, L. G. (1988) J. Pharmacol. Exp. Ther. **246**, 946–949
40. Oei, H. H. H., Zoganas, H. C., McCord, J. M. & Schaffer, S. W. (1986) Res. Commun. Chem. Pathol. Pharmacol. **51**, 195–203

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