17. M. Torres, D. de Prost, J. Hakim and M. A. Gougerot, *Eur. J. clin. Invest.* **9**, 209 (1979).
18. J. Hakim, E. Cramer, P. Boivin, H. Troube and J. Boucherot, *Eur. J. clin. Invest.* **5**, 215 (1975).
19. E. Feliu, M. A. Gougerot, J. Hakim, E. Cramer, C. Auclair, B. Rueff and P. Boivin, *Eur. J. clin. Invest.* **7**, 571 (1977).
20. R. R. Strauss, B. B. Paul and J. S. Sbarra, *J. Bact.* **96**, 1982 (1968).
21. C. O. Solberg, *Acta path. microbiol. scand. Sect. B* **82**, 258 (1974).
22. J. A. Whittaker, H. R. Hugues and M. Khurshid, *Br. J. Haemat.* **29**, 273 (1975).
23. J. A. Fee and J. S. Valentine, in *Superoxide and Superoxides Dismutases* (Eds. A. M. Michelson, J. M. McCord and I. Fridovich), p. 19. Academic Press, New York (1977).
24. P. Herzer and E. M. Lemmel, *Immunobiology* **157**, 78 (1980).

Biochemical Pharmacology, Vol. 32, No. 18, pp. 2822–2825, 1983.
Printed in Great Britain.

0006-2952/83 $3.00 + 0.00

Chemiluminescence of the *in situ* rat liver after acute ethanol intoxication—effect of (+)-cyanidanol-3

(*Received* 15 *September* 1982; *accepted* 14 *March* 1983)

The aerobic catabolism of several exogenous compounds in mammalian cells is associated with the formation of reactive oxygen species such as superoxide radical, hydroxyl radical, hydrogen peroxide and singlet oxygen [1]. These species are likely to exert toxic effects in biological systems, including lipid peroxidation, enzyme inactivation, mutagenicity and carcinogenicity [1–3]. The cytotoxicity of lipoperoxidation depends on the free radical-induced oxidative breakdown of polyunsaturated fatty acids [1–3]. Since these fatty acids constitute the major components of cell membranes, an enhanced lipid peroxidation would alter essential membrane functions and lead to cell injury [1–3].

Acute ethanol ingestion has been shown to produce an increased lipid peroxidation in the liver as assessed by different experimental procedures. Several invasive and destructive techniques such as malondialdehyde production [4–6], conjugated lipid diene formation [7, 8], and chemiluminescence [9] have been used in liver homogenates and in microsomal and mitochondrial preparations. However, the estimations of ethanol-induced lipid peroxidation by malondialdehyde formation [10–12] and by electron spin resonance spectroscopy [13] have produced conflicting results. An enhancement of hepatic lipid peroxidation by acute ethanol treatment has also been reported by measuring the *in vivo* exhalation of hydrocarbons such as ethane [14, 15], *n*-pentane [15, 16], propane, *n*-butane and isobutane [15].

Although hydrocarbon exhalation constitutes a truly non-invasive assay for detecting lipid peroxidation, it lacks organ specificity. Recently, this problem was overcome by the use of perfused rat liver, in which the addition of 44 mM ethanol markedly increased the production of ethane [17]. In view of these observations and of the role that lipid peroxidation could play in alcoholic liver disease in man [18], we report in this paper the effect of acute ethanol intoxication on the rate of lipid peroxidation in the liver *in vivo*, evaluated by measuring the low-level chemiluminescence of the intact organ [19]. This technique constitutes a specific assay which is non-invasive for the tissue and is related to the steady-state level of oxidative free radicals involved in the process of lipoperoxidation [19]. The results with the chemiluminescence measurements are compared with those obtained by the determination of malondialdehyde and diene conjugates, and the effect of the antioxidant flavonoid (+)-cyanidanol-3 [20] on the ethanol-induced chemiluminescent response of the liver was studied.

Male Long–Evans or Wistar rats (200–220 g) fasted overnight (16–18 hr) were given 5 g of ethanol/kg [as a 30% (v/v) solution in saline] or isovolumetric amounts of saline intraperitoneally. Experiments with (+)-cyanidanol-3 (Zyma S.A., Nyon, Switzerland, obtained through Ciba-Geigy, Chile) were performed in a separate group of animals that received 400 mg/kg of (+)-cyanidanol-3 (as a 80 mg/ml solution in saline) or isovolumetric saline subcutaneously, 1 hr before the administration of ethanol. Studies were carried out after 6 hr of ethanol treatment, and the animals were kept in a warm environment (28–30°).

Liver chemiluminescence was measured with a Johnson Foundation photon counter (Johnson Research Foundation, University of Pennsylvania, Philadelphia, PA, U.S.A.) specially adapted for organ chemiluminescence as described by Boveris *et al.* [19]. An EMI 9658 red-sensitive phototube with an applied potential of −1·3 kV was used. Details of the apparatus are depicted in Fig. 1. Rats were anesthetized with Nembutal (50 mg/kg in control rats and 15 mg/kg in animals intoxicated with ethanol, i.p.), and the liver was exposed. The abdominal cavity and skin were covered with aluminium foil in which a window specially cut for each rat allowed exposition of only the liver (Fig. 1). Chemiluminescence is expressed as counts per second (cps)/cm^2 of liver surface. The thiobarbituric acid assay for malondialdehyde production [21] and the determination of diene conjugates [22] in liver homogenates were carried out in a separate group of animals. Proteins were measured as described by Lowry *et al.* [23]. Significance between mean values was assessed by Student's *t*-test for unpaired results.

A typical determination of the spontaneous chemiluminescence of the *in situ* liver of a fasted rat that had been given ethanol for 6 hr is presented in Fig. 2. The *in situ* liver of an alcohol-intoxicated rat showed an enhanced light signal (about 60%) compared to that of a control rat. Furthermore, this significant increase in the low-level chemiluminescence of the *in situ* rat liver caused by acute ethanol administration was found to occur in two different strains of animals, when compared to the corresponding control rats which were given saline (Table 1). Since this organ-specific assay is associated with the steady-state level of oxidative free radicals that could lead to the peroxidation of polyunsaturated fatty acids [19], the results support the

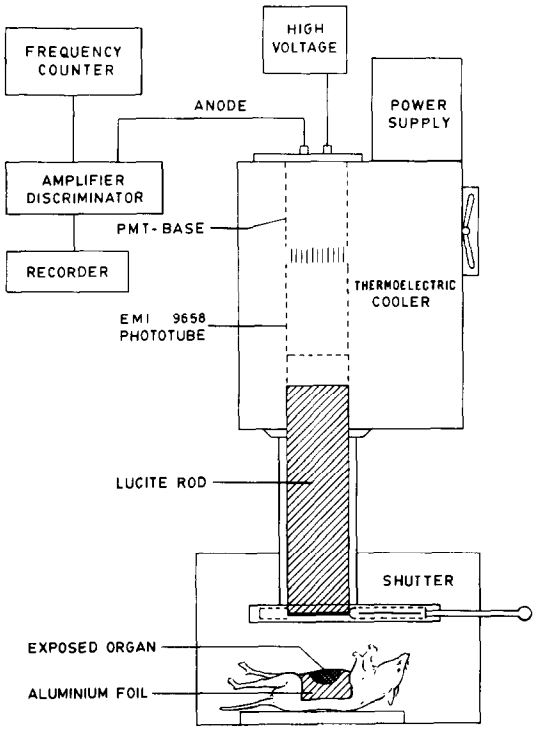

Fig. 1. Scheme of the photon counter used for the measurement of liver chemiluminescence.

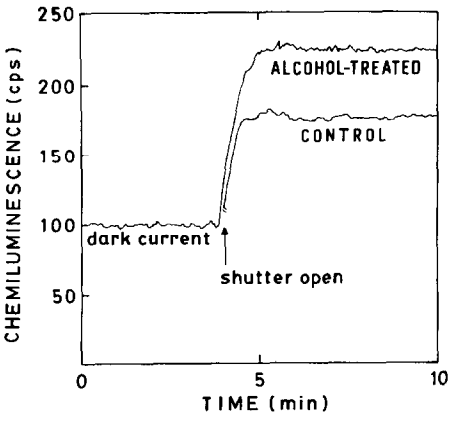

Fig. 2. Chemiluminescence of the liver of an acute-ethanol intoxicated rat and of a control rat. The exposed areas were 3.0 cm^2 and 3.2 cm^2 for the alcohol-treated and control rats respectively.

contention that acute ethanol ingestion enhances hepatic lipoperoxidation. This view is further supported by the increases in malondialdehyde and diene conjugate formation observed in liver homogenates (Table 1). The two latter destructive procedures measure different steps of the lipoperoxidation process [24].

Discrepancies in the reports on liver lipid peroxidation after ethanol ingestion could conceivably be due to differences in experimental designs. Malondialdehyde formation [10–12] and electron spin resonance spectroscopy measurements [13] were carried out in animals given 6 to 7.5 g of ethanol/kg. These doses would produce high concentrations of ethanol in the liver, which are likely to exert a better free radical scavenging action [25] than lower concentrations. Furthermore, acute ethanol intoxication has been found to impair body temperature regulation, leading to hypothermia [26]. In this situation, a metabolic depression, that includes endogenous lipoperoxidation, might occur if intoxicated animals are not kept in a warm environment. Since the extent of lipid peroxidation in the liver seems to be closely related to its content of reduced glutathione (GSH) after acute ethanol ingestion [6], the period of ethanol intoxication might be important in the assessment of this process. In fact, assays measuring hepatic lipoperoxidation in ethanol-treated animals have been carried out either when liver GSH levels were not diminished significantly [11–13] or when they had already recovered [10, 11] after maximal decrease [6]. Finally, the lack of peroxidative effect of ethanol reported in isolated microsomal and mitochondrial preparations [10, 13, 27] could possibly be due to the absence of GSH and of the enzymatic GSH-regenerating system [28], lost during the isolation procedure.

(+)-Cyanidanol-3 is a powerful free radical scavenger in *in vitro* systems [20]. The livers from rats treated with (+)-cyanidanol-3 showed a chemiluminescence similar to that of control animals given saline (Table 2). However, when the flavonoid was given 1 hr prior to ethanol, it was able to completely abolish the increase in chemiluminescence induced by ethanol (Table 2). Similar results had been obtained when lipid peroxidation was assessed by the formation of diene conjugates [29]. Thus, these results support both the scavenging properties of (+)-cyanidanol-3 *in vivo* and the generation of oxidative free radicals in the liver in the presence of ethanol [30].

In conclusion, the experimental data presented in this work indicate that ethanol ingestion enhanced hepatic lipoperoxidation, as assessed by the increased spontaneous chemiluminescence of the *in situ* rat liver. This is in agreement with the elevated malondialdehyde formation found

Table 1. Effect of acute ethanol intoxication on rat liver chemiluminescence and on malondialdehyde and diene conjugate formation*

		Lipid peroxidation assay		
Strain	Treatment	Chemiluminescence (cps/cm^2)	Malondialdehyde (nmoles/mg protein)	Diene conjugates (nmoles hydroperoxide/mg protein)
Long–Evans	Saline	26 ± 1 (8)		
	Ethanol	40 ± 2†(8)		
Wistar	Saline	24 ± 1 (8)	91 ± 5 (6)	119 ± 11 (6)
	Ethanol	39 ± 4†(8)	135 ± 10‡(6)	196 ± 8†(6)

* Values are means ± S.E.M., with the number of animals indicated in parentheses.
† $P < 0.001$, using Student's *t*-test for unpaired results.
‡ $P < 0.005$, using Student's *t*-test for unpaired results.

Table 2. Effect of (+)-cyanidanol-3 on the ethanol-induced chemiluminescence of the *in situ* rat liver*

Treatment	Chemiluminescence (cps/cm²)	Effect (%)	P
(A) Saline	24 ± 1 (10)		
(B) (+)-Cyanidanol-3	24 ± 1 (10)	0	NS
(C) Ethanol	41 ± 2 (10)	+69	< 0.001
(D) (+)-Cyanidanol-3 + ethanol	21 ± 2 (10)	−13	NS

* Values are means ± S.E.M., with the number of animals indicated in parentheses. Male Wistar rats were used in this experiment. The percentage effect and the P values shown for the different treatments are referred to the saline control group (A). Significance studies: C vs D, $P < 0.001$.

† Not significant.

in liver biopsy specimens from alcoholic patients as compared to that observed in non-drinkers [18]. Low GSH levels were found in liver biopsy samples from alcoholic subjects after a short period of abstinence (< 5 days) [31], compared to the reported values for the human liver [32]. Also, low GSH levels and increased diene conjugate formation were reported in chronically alcohol-fed baboons after acute alcohol ingestion [33]. Thus, GSH depletion and an increased lipid peroxidation in the liver seem to be associated with excessive alcohol consumption in man, but further studies are needed to assess their actual pathogenic importance in alcoholic liver disease.

Acknowledgements—This work was supported by grants from the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina, from the Cherny Foundation, Argentina, and from the Departamento de Desarrollo de la Investigación (B-1162-822-3), Universidad de Chile. CONICET also gave financial support for Dr. L. A. Videla's visit to Buenos Aires.

LUIS A. VIDELA*

Unidad de Bioquímica
Divisíon de Ciencias Médicas Occidente
Facultad de Medicina, Casilla 10455—Correo Central, Universidad de Chile
Santiago, Chile

CESAR G. FRAGA†
OSVALDO R. KOCH‡
ALBERTO BOVERIS†

†*Departamento de Química Biológica*
Facultad de Farmacia y Bioquímica
Universidad de Buenos Aires
Junín 956, 1113 Buenos Aires, and
‡*Departamento de Patología*
Facultad de Medicina, Universidad de Buenos Aires
Uriburu 956, 1114 Buenos Aires, Argentina

REFERENCES

1. B. Chance, H. Sies and A. Boveris, *Physiol. Rev.* **59**, 527 (1979).
2. H. Kappus and H. Sies, *Experientia* **37**, 1233 (1981).
3. A. L. Tappel, *Fedn Proc.* **32**, 1870 (1973).
4. N. R. Di Luzio and F. Costales, *Expl molec. Path.* **4**, 141 (1965).
5. M. Comporti, A. Benedetti and E. Chieli, *Lipids* **8**, 498 (1973).
6. L. A. Videla, V. Fernández, G. Ugarte, A. Valenzuela and A. Villanueva, *Fedn Eur. Biochem. Soc. Lett.* **111**, 6 (1980).
7. A. Valenzuela, N. Fernández, V. Fernández, G. Ugarte, L. A. Videla, R. Guerra and A. Villanueva, *Fedn Eur. Biochem. Soc. Lett.* **111**, 11 (1980).
8. N. R. Di Luzio, *Expl molec. Path.* **8**, 394 (1968).
9. N. R. Di Luzio and T. E. Stege, in *Alcohol and the Liver* (Eds. M. M. Fischer and J. G. Rankin), p. 45. Plenum Press, New York (1977).
10. R. Scheig and G. Klatskin, *Life Sci.* **8**, 855 (1969).
11. M. Pesh-Iman and R. O. Recknagel, *Toxic. appl. Pharmac.* **42**, 463 (1977).
12. M. V. Torrieli, L. Gabriel and M. U. Dianzani, *J. Path.* **126**, 1 (1978).
13. F. Keller, A. B. Snyder, F. J. Petracek and K. M. Sancier, *Biochem. Pharmac.* **20**, 2507 (1971).
14. U. Koster, D. Albrecht and H. Kappus, *Toxic. appl. Pharmac.* **41**, 639 (1977).
15. H. Frank, T. Hintze, D. Bimboes and H. Remmer, *Toxic. appl. Pharmac.* **56**, 337 (1980).
16. R. E. Litov, D. H. Irving, J. E. Downey and A. L. Tappel, *Lipids* **13**, 305 (1978).
17. A. Muller, P. Graf, A. Wendel and H. Sies, *Fedn Eur. Biochem. Soc. Lett.* **126**, 241 (1981).
18. T. Suematsu, T. Matsumura, N. Sato, T. Miyamoto, T. Ooka, T. Kamada and H. Abe, *Alcoholism Clin. expl. Res.* **5**, 427 (1981).
19. A. Boveris, E. Cadenas, R. Reiter, M. Filipowsky, Y. Nakase and B. Chance, *Proc. natn. Acad. Sci. U.S.A.* **77**, 347 (1980).
20. T. F. Slater and M. N. Eakins, in *New Trends in the Therapy of Liver Diseases* (Ed. A. Bertelli), p. 84. Karger, Basel (1975).
21. J. A. Fee and H. D. Teitelbaum, *Biochem. biophys. Res. Commun.* **49**, 150 (1972).
22. J. A. Buege and S. D. Aust, *Meth. Enzym.* **52**, 302 (1978).
23. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
24. G. L. Plaa and H. Witschi, *A. Rev. Pharmac. Toxic.* **16**, 125 (1976).
25. M. Ambar and P. Neta, *Int. J. appl. Radint. Isotopes* **18**, 493 (1976).
26. G. Freund, in *Biochemistry and Pharmacology of Ethanol* (Eds. E. Majchrowicz and E. P. Noble), Vol. 2, p. 439. Plenum Press, New York (1979).
27. R. J. Bloom and W. W. Westerfield, *Archs. Biochem. Biophys.* **145**, 669 (1971).
28. L. A. Videla and A. Valenzuela, *Life Sci.* **31**, 2395 (1982).
29. L. A. Videla, V. Fernández, A. Valenzuela and G. Ugarte, *Pharmacology* **22**, 343 (1981).
30. A. I. Cederbaum, E. Dicker and G. Cohen, in *Alcohol*

*Author to whom all correspondence should be addressed.

and Aldehyde Metabolizing Systems-IV (Ed. R. G. Thurman), p. 1. Plenum Press, New York (1980).
31. L. A. Videla, H. Ituriaga, D. Bunout, A. Valenzuela, M. E. Pino and G. Ugarte, *Gastroenterology* **82**, 1248 (1982).
32. D. Rollins, A. Larson, B. Steen, K. Krishnaswamy, L. Hagenfeldt, P. Moldeus and A. Rane, *J. Pharmac. exp. Ther.* **217**, 697 (1981).
33. S. Shaw, L. Yayatilleke, W. A. Ross, E. R. Gordon and C. S. Lieber, *J. Lab. clin. Med.* **98**, 417 (1981).

Biochemical Pharmacology, Vol. 32, No. 18, pp. 2825–2827, 1983.
Printed in Great Britain.

0006-2952/83 $3.00 + 0.00

Ethanol-induced alterations in rat hepatic ammonia metabolism

(*Received* 18 *January* 1983; *accepted* 21 *April* 1983)

Alterations in the structure and physiology of experimental alcoholic liver injury have been the subject of many studies [1–3]. Nevertheless, questions on other toxic effects during ethanol metabolism remain to be answered. The scope for the formation of other toxic agents during ethanol injestion cannot be ruled out and more so, recent reports indicate, without definite conclusions, the interference of ammonia causing neurotoxicity, especially in severe alcoholic liver damage [4] besides alterations in hepatic amino acid and protein metabolism [5]. Hence in the present study we report some of the changes in the metabolic profiles of ammonia as a consequence of repeated loading of high doses of ethanol. This sort of study may help in understanding the chronic damage inflicted on the liver and the possible interplay of synergistic toxic agents like ammonia during ethanol ingestion.

Wistar strain, male albino rats aged 3 months were subjected to a 5-day course of injections i.p. of ethanol (5 g/kg body wt in 10% dilutions). Control animals were treated similarly with physiological saline. The animals were sacrificed by decapitation after the fifth day (24 hr after the last dose of ethanol administration) since withdrawal signs were witnessed from the seventh day. The livers were excised rapidly at a temperature of 0° and homogenates (10% wt/vol.) in required media were prepared, spun at 4000 *g* for 20 min, and the clear cell-free extracts were saved for enzyme assays. Unless specificed, all the steps were carried out at 0–3°. Sucrose homogenates were utilized for assaying lactate dehydrogenase (EC 1.3.99.1) by the method of Nachlas *et al.* [6], asparate aminotransferase (EC 2.6.1.1) as suggested in the Sigma Technical Bulletin [7], glutamate dehydrogenase (EC 1.4.1.2) by the method of Lee and Lardy [8], and glutamine synthetase (EC 6.3.1.2) by the method of Rowe *et al.* [9]. Distilled water homogenate was used for the estimation of AMP deaminase (EC 3.5.4.6) activity by the method of Setlow *et al.* [10] as modified by Wegelin *et al.* [11]. Homogenates prepared in 0.1% cetyltrimethylammonium bromide were used for assaying ornithine transcarbamylase (EC 2.1.3.3), argininosuccinate synthetase (EC 6.3.4.5) by the method of Huggins *et al.* [12], and arginase (EC 3.5.3.1) activity by the method of Beruter *et al.* [13]. To determine the concentration of ammonia, the liver tissue immediately after isolation was immersed in ice-cold 10% trichloroacetic acid, and ammonia concentration was estimated as described by Ward *et al.* [14]. Urea was assayed by the method of Natelson [15] in perchloric acid extracts, while glutamine was estimated by the acid hydrolysis method of Wilcox [16]. The protein concentration in different enzyme sources was analysed following the method of Lowry *et al.* [17] using crystalline bovine serum albumin as the standard. All estimations performed in eight samples were expressed as mean of ± S.D. The statistical analysis of the significance of difference between control and experimental samples was made by the Student's *t*-test. Changes in the basic profiles of animals at the end of the experimental period are shown in Table 1. The body weight and food intake were reduced significantly while the liver gained weight. Recent reports also indicated similar results [18]. These changes associated with declined activities of lactate dehydrogenase and succinate dehydrogenase reflect the reduced energy production and hepatic enlargement. Histological studies showed inflammation of hepatocytes, presence of giant mitochondria, Mallory bodies, bundles of collagen between swollen fatty hepatocytes and depositions of IgA. All these histological features of ethanolic liver have also been reported by earlier workers [19, 20].

Formation of ammonia. Aspartate aminotransferase, NAD-dependent glutamate dehydrogenase and the AMP deaminase were elevated significantly, implying that ammonia formation was stepped up (Table 2). In consonance with the increased activities of these enzymes, ammonia concentration in the ethanolic liver was also increased. Elevated aspartic acid [21] and other dicarboxylic acids [22] in ethanolic liver might have been responsible for transamination of aspartic acid. A similar rise in aspartate aminotransferase induced by ethanol was reported recently [23]. The increased NAD-dependent glu-

Table 1. Basic profiles of animals after the 5-day course of ethanol treatment

Sample	Body weight (g)	Liver weight (g/100g)	Food intake (kcal/day)
Control (8)	193.4 ± 2.7	4.3 ± 0.1	47 ± 0.7
Ethanol-treated (8)	189.2 ± 3.3*	4.8 ± 0.2†	44 ± 0.9†

Values in parentheses show the number of animals taken.
* $P < 0.01$.
† $P < 0.001$.