Free Rad. Res. Comms., Vol. 14, No. 2, pp. 81-89
Reprints available directly from the publisher
Photocopying permitted by license only

EFFECTS OF 4-HYDROXYNONENAL, A PRODUCT OF LIPID PEROXIDATION, ON CELL PROLIFERATION AND ORNITHINE DECARBOXYLASE ACTIVITY

GIUSEPPINA BARRERA, OLGA BROSSA, VITO MICHELE FAZIO[1], MARIA GIULIA FARACE[2], LUCIANA PARADISI, ENRICO GRAVELA and MARIO UMBERTO DIANZANI

Dipartimento di Medicina e Oncologia Sperimentale, Sezione di Patologia Generale, Corso Raffaello 30, Torino, Italy; [1]Istituto di Medicina Sperimentale, CNR, Roma; [2]Università Tor Vergata, Roma, Italy

(Received June 3, 1990)

4-hydroxynonenal (HNE) is one of the major breakdown products of cellular lipid peroxidation. Its effects on proliferation, ornithine decarboxylase (ODC) activity and DNA synthesis have been investigated in leukemic cell lines. The cells were incubated for 1 hour with different aldehyde concentrations, then washed and resuspended in medium with fresh foetal calf serum. HNE concentrations ranging from 10^{-5} to 10^{-6} M significantly inhibited ODC activity when induced by addition of fresh foetal calf serum both in K562 and HL-60 cells. ^{3}H-Thymidine incorporation in K562 cells was also inhibited from 6 to 12 hours after the treatment. The same HNE concentrations did not inhibit ODC activity when added to cytosol, thus a direct action on the enzyme can be excluded. Moreover, HNE did not affect the half-life of ODC, so that a specific effect on ODC synthesis may be supposed. These data indicate a reduction of proliferative capacity of the cells and are consistent with the possibility that HNE, at concentrations close to those found in normal cells, plays a role in the control of cell proliferation.

KEY WORDS: 4-hydroxynonenal, leukemic cells, ornithine decarboxylase, DNA synthesis.

INTRODUCTION

4-Hydroxynonenal (HNE) is one of the major diffusible products formed during lipid peroxidation of cell membranes.[1] It reacts with thiol groups of low molecular weight compounds and proteins and affects, by this mechanism, several enzymatic activities.[2] It has been demonstrated that HNE inhibits cell proliferation and DNA synthesis of Ehrlich ascites tumour cells,[3] human lymphocytes[4] and K562 cells.[5] Wawra *et al.*[6] suggested that HNE could affect DNA synthesis inhibiting of DNA polymerases, so blocking the cell cycle progression in the S phase. However, HNE exhibits this effect only at concentrations higher than 50 μM, whereas the normal level of HNE is in the range of a few nanomoles per gram of tissue or body fluid.[7] Moreover DNA synthesis inhibition may result from a block of some signaling events that are necessary for progression through the G1 phase. In fact recent data indicate that HNE, added to

The correspondence should be sent to: Dr. Giuseppina Barrera, Dipartimento di Medicina e Oncologia Sperimentale, Sezione di Patologia Generale, Corso Raffaello 30, 10125 Torino (Italy). Phone: 011/6527763-751.

cultures of actively dividing cells, blocks proliferation and leads to an arrest of most cells in the G1 phase.[8]

Ornithine decarboxylase (ODC) is the first regulatory enzyme in the biosynthesis of polyamines; it plays a key role in proliferation both of normal and neoplastic cells[9] and its induction seems to be an universal mechanism of growth stimulation by hormones, drugs, and tumour promoters.[10] Moreover it has been reported that the inhibition of ODC activity is associated with a block cell cycle progression in the G1 phase.[11] In the present study, we have examined the effect of HNE on ODC activity and on DNA synthesis in human leukemic cell lines K562 and HL-60 cells, in order to establish if, by this mechanism, HNE could affect cell proliferation.

MATERIALS AND METHODS

Cells and culture conditions

K562 and HL-60 human leukemic cells (kindly provided by Prof. Santoni, Department of Experimental Medicine, Rome, Italy) were cultured at 37°C in a humidified atmosphere of 5% CO_2-air in RPMI 1640 medium supplemented with 2 mM glutamine, antibiotics and 10% foetal calf serum (Flow Laboratories). Growth rate and cell viability, as determined by the trypan blue dye exclusion test, were monitored daily in all experiments.

HNE treatment

Cells were washed and resuspended at the concentration of 10^6 cells/ml in RPMI medium without foetal calf serum to avoid the binding between HNE and the thiol group of serum proteins. The incubation with HNE was performed for 1 hr. After this period, the cells were washed and resuspended in medium with 10% fresh foetal calf serum at the concentration of 500,000 cells per ml.

^3H Thymidine incorporation

Cells were taken from cell cultures at various time points, distributed into 96-well microtiter plates (2×10^5/sample point) and pulsed with 1 μCi ^3H thymidine for 1 hr. The incorporation of the radioactive nucleotide was measured after harvesting the cells with a Flow cell harvester (Gelman Instruments, Gelaire).

Cycloheximide treatment

K562 cells were incubated with HNE 10^{-5} M for 1 hr as previously described. Cycloheximide (1 mg/l) was added to controls and HNE treated cells 6.30 hr after foetal serum supplementation. Samples of cells were harvested at time 0 and after 15 and 30 min.

Subcellular fractionation

Aliquots of cells, ranging from 10×10^6 to 15×10^6 cells were washed twice by centrifugation (1000 RPM for 10 min) at 4°C in a medium containing 0.25 M sucrose, 50 mM phosphate buffer, pH 7.2, 40 μM pyridoxal phosphate. The final cell pellet was

resuspended in 1 ml of the same medium. Cells were lysed by 3 cycles of freeze-thawing and centrifuged at 100,000 g for 60 min to obtain the cytosolic fraction.

Determination of ornithine decarboxylase activity

Ornithine decarboxylase activity was determined by measuring the liberation of $^{14}CO_2$ developed from 0.2 μCi of L-(1-^{14}C) ornithine (Amersham International plc) Incubations were for 30 min at 37°C in 8 ml tubes equipped with rubber stoppers and polypropylene center wells (Kontes, Vineland, N.J.) The assay reaction (200 μl) contained 170 μl of cytosol (0.5–1 mg of protein), 10 μl of dithiothreitol (final concentration 1 mM) and 20 μl of labeled plus unlabeled L-ornithine (final concentration 0.5 mM). The center wells contained 0.2 ml of tissue solubilizer (BTS-450, Beckman), adsorbed on a small role of filter paper (1.5 × 1.5 cm) as CO_2 trapping agent. After incubation, the reaction was stopped by addition of 0.25 ml of 10% trichloroacetic acid injected through the rubber stopper. The rolls of paper were then transferred to liquid scintillation vials together with 5 ml of counting cocktail (Ready organic, Beckman); radioactivity was determined after 12 hr.

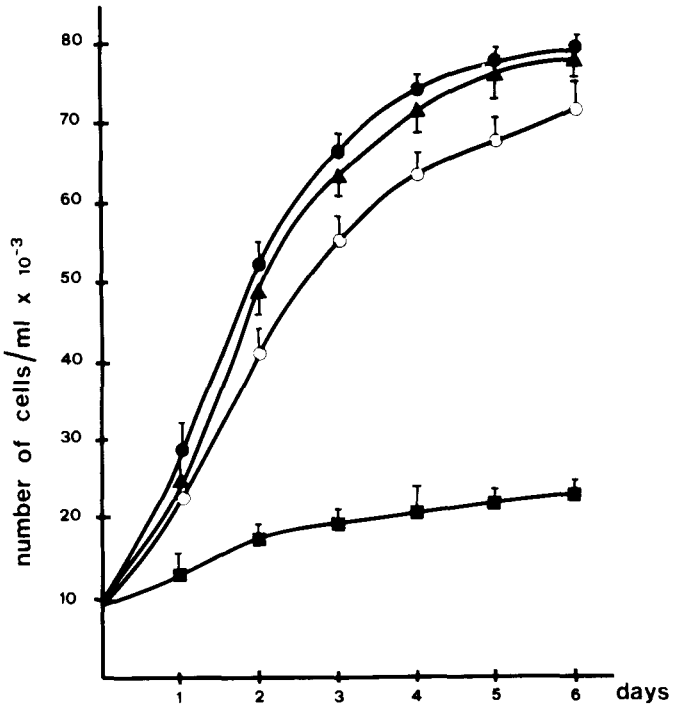

FIGURE 1 Growth of K562 cells. Cells were treated for 1 hour with different HNE concentrations in medium without foetal calf serum, then washed and resuspended in fresh medium with 10% foetal calf serum. Symbols: ● control, ▲ 10^{-6} M HNE, ○ 10^{-5} M HNE, ■ 10^{-4} M HNE. The values are means + S.D. of at least three separate experiments.

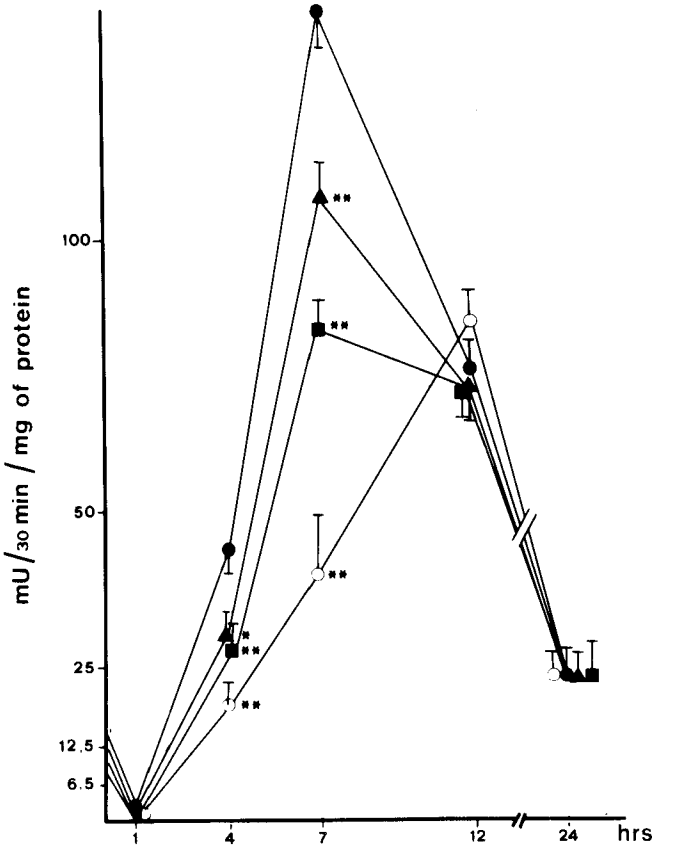

FIGURE 2 Kinetics of ODC activity in K562 cells at various time points. Treatments are performed as reported in Figure 1. Symbols: ● control, ▲ 10^{-6} M HNE, ■ 5.10^{-6} M HNE. ○ 10^{-5} M HNE. The values are means + S.D. of four separate experiments. Significance by the analysis of variance: * $P < 0.05$, ** $P < 0.01$.

Protein determination

Protein was determined in cytosolic fractions as described by Hartree,[12] by using crystalline bovine serum albumin as a standard.

RESULTS

The HNE effect on K562 cell growth is shown in Figure 1. After incubation with 10^{-5} M and 10^{-6} M HNE, cell growth was similar to the control growth, whereas it was strongly inhibited by 10^{-4} M HNE. This HNE concentration in our system was toxic to the cells and gave 30% loss of cell viability, thus it has been excluded from following experiments.

ODC activity in K562 cells is shown in Figure 2. Incubation for 1 hr without foetal calf serum reduced ODC activity to 10% of the activity value found in the initial cell

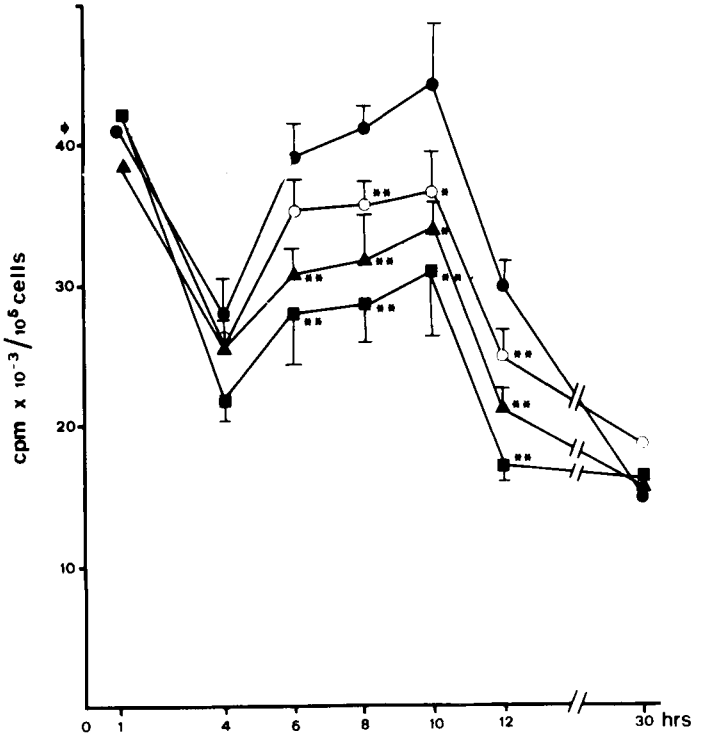

FIGURE 3 Kinetics of [^{3}H] thymidine incorporation into K562 cells treated with HNE as reported in Figure 1. Symbols: ● control, ○ 10^{-6} M HNE, ▲ 5.10^{-6} M HNE, ■ 10^{-5} M HNE. The values are means + S.D. of four separate experiments. Analysis of variance * $P < 0.05$, ** $P < 0.01$. Arrow indicates the value observed in the initial cell suspension.

suspension, both in control and in treated cells. Resuspension of the control cells in medium with fresh foetal calf serum, resulted in an induction of ODC activity that peaked six hours later.

In cells treated with 10^{-5} M HNE ODC activity peaked at 12 hr; at 4 and 7 hr ODC activity was reduced by 57% and 71%, respectively as compared with control cells. With lower HNE concentrations the inhibition of ODC induction was less marked, i.e. at 4 and 7 hr, inhibitions by 42% and 40% after incubation with 5.10^{-6} M HNE and by 35% and 25% after 10^{-6} M HNE were observed. After 24 hr the values of ODC activity in all treated cells reached values similar to control. Since the inhibition of ODC induction seems to indicate that some cells could have been blocked in the G1 phase, a decrease in thymidine incorporation may be expected at time points subsequently to the treatment. Thymidine incorporation into K562 cells was studied 1, 4, 6, 8, 10, 12 and 30 hr after preincubation with HNE. The absence of foetal calf serum for 1 hr induced a decrease of thymidine incorporation 4 hrs later both in the control and in the treated cells. Then, DNA synthesis increased and peaked at 10 hr (Figure 3). 10^{-6} M HNE significantly inhibited thymidine incorporation, even though only 20–30%, at 8, 10 and 12 hr. Higher HNE concentration inhibited thymidine incorporation even at 6 hr. After 24 hr this effect disappeared. Its reversibility may

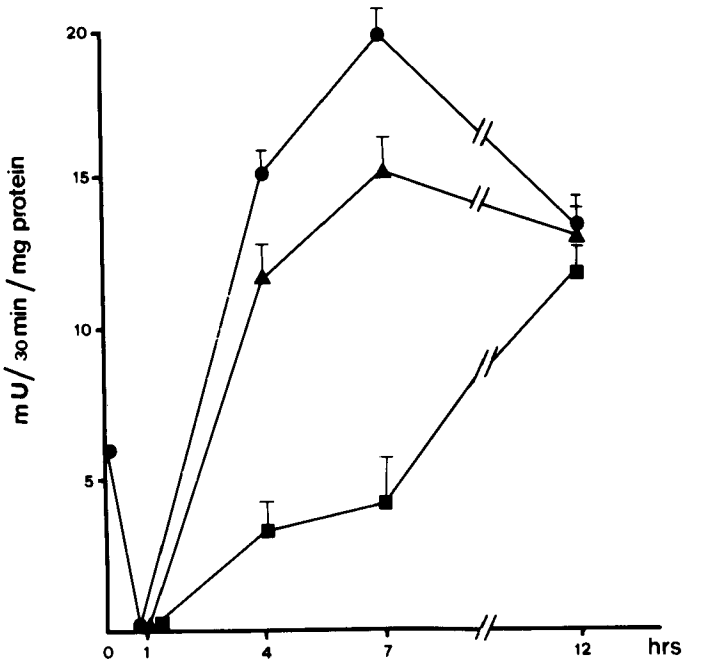

FIGURE 4 Kinetics of ODC activity in HL-60 cells at various time points after HNE treatments. The experiments were carried out as described in Figure 1. Symbols: ● control, ▲ 10^{-6} M HNE, ■ 10^{-5} M HNE. Values are means + S.D. of three separate experiments.

justify the poor inhibitory action on cell growth observed at the days following the treatment.

In order to see if the decrease of ODC activity, induced by HNE, was due to enzyme inactivation the aldehyde has been added to cytosol of K562 cells just before ODC determination. HNE at concentrations previously reported to inhibit ODC activity when added to the cultured cells did not inactivate the enzyme. A higher aldehyde concentration (10^{-4} M) inhibited ODC activity by 40% only when tested without dithiothreitol (data now shown).

The effect of HNE on ODC activity was also evaluated in another human leukemic cell line (HL-60). The experimental conditions used in the case of K562 cells were maintained also in these experiments. Figure 4 shows that both 10^{-6} M and 10^{-5} M HNE inhibited the increase of ODC activity in HL-60 cells when induced by re-suspension of the cells in medium with fresh foetal calf serum. The percent inhibition values at 4 to 7 hr were 34% and 35% after incubation with 10^{-6} M HNE and 78% and 81% after 10^{-5} M HNE. Finally, some experiments have been performed to test if inhibition of ODC activity observed in HNE-treated cells could depend on reduction of the enzyme half life. New synthesis of ODC was blocked with cycloheximide in control and HNE-treated K562 cells, 6.30 h after enzyme induction through serum supplementation.

Studies on ODC activity decay reported in Figure 5 showed that apparent active life spans were similar in control and HNE-treated cells.

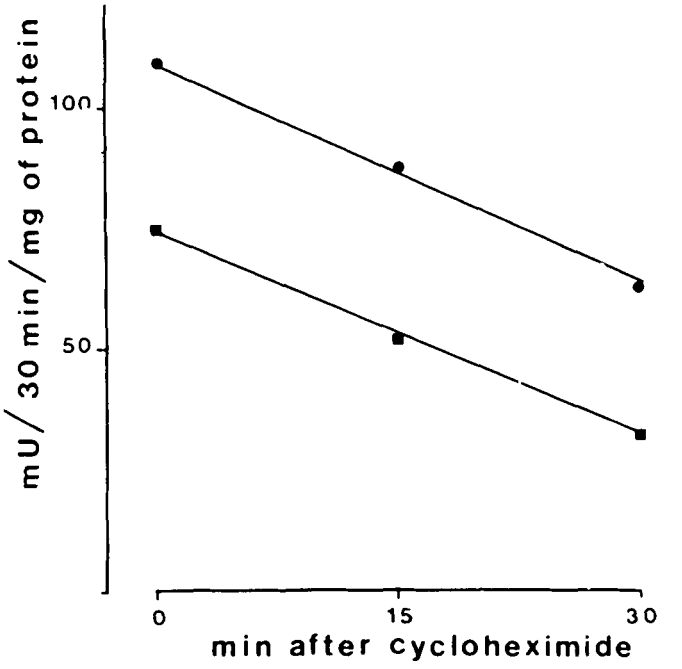

FIGURE 5 Decay of ODC activity in K562 cells after cycloheximide administration. ODC activity has been determined in the presence of 1 mM DTT; each point is mean of 3 experiments. Symbols: ● control, ■ 10^{-5} M HNE.

DISCUSSION

HNE is a highly reactive electrophilic reagent which easily combines with -SH groups of low molecular weight thiols and proteins.[7] The binding of HNE to proteins occurs predominantly through attack on the thiol groups in cysteine residues of the polypeptide chain.[13] It has been shown that the loss of SH groups is correlated with the number of aldehyde molecules covalently bound to the protein.[13] ODC is a thiol-dependent enzyme and its aminoacid sequence contains 12 cysteine residues;[14] thus, it is a potential target for HNE. This enzyme, however, is a very minor component of the total soluble cell proteins.[15,16] Thus, we can hypothesize that the high concentrations of thiol reducing agents in the cytosol combined with the low number of ODC molecules with respect to the total cytosol proteins may explain the poor inhibitory effect of HNE on cytosolic ODC. On the other hand, low HNE concentrations strongly reduced the ODC activity stimulation occurring in K562 and HL-60 cells after fresh serum supplementation. In many cell types a good correlation has been found between amount of ODC protein and enzyme activity and the regulation is chiefly exercized through changes in the rate of synthesis or degradation of this protein.[17] In the present work the possibility that HNE affects ODC protein degradation in K562 cells has been excluded. The second possibility is that HNE prevented the increase in ODC activity by blocking ODC synthesis. From this point of view, since a major part of the increase rate of synthesis occurs by virtue of a large rise in the amount of ODC mRNA,[18] HNE may act by reducing ODC mRNA transcription.

It has been recently demonstrated that HNE, at concentrations used in this work, does not inhibit RNA or protein synthesis[19] but specifically blocks transcription of mRNA for the c-myc gene in K562 cells (Fazio *et al.* submitted). The possibility exists, however, that the inhibition of c-myc expression may condition the expression of ODC gene. In fact a close correlation between the two gene transcriptions occurs when stimulated by TPA.[20] However, a direct action of HNE on the ODC gene expression cannot be excluded. Our results point to ODC synthesis as a specific target for HNE. In fact, other cellular activities involved in the control of cell proliferation, such as DNA polymerases[6] and microtubule assembly,[21] are inhibited by HNE concentrations higher than 10^{-5} M.

The inhibition of DNA synthesis observed between 6 and 12 hr points out the antiproliferative effect that low HNE concentrations exert on the cells. The fact that both the inhibitions of ODC activity induction and DNA synthesis exhaust themselves within few hours, may justify the poor or no effect on cell growth in the days following the experiments. The reversibility of the effect may be due to HNE metabolization in K562 or HL-60 cells. In fact, when 10^{-5} M was added to each of the two cell lines (10^6 cell/ml), the aldehyde, as measured by HPLC, disappears from culture media within 1 hr (Barrera *et al.* submitted). Its influence on some cellular processes leading to cell proliferation is, however, not rapidly cancelled by this disappearance. So HNE behaves like a messenger or signal and its effects are produced by a short-time contact. Moreover, in normal cells, HNE is continuously produced during the peroxidative degradation of cell membrane phospholipids and catabolized by some aldehyde-inactivating systems, so its concentrations represent a steady-state amount.[22] The fact that tumour cells exhibit quite low levels of lipid peroxidation[23] and the effects described are obtained by utilizing HNE concentrations similar to those measured in normal cells[24,25] are consistent with the hypothesis that lipid peroxidation plays a role in the control of normal and neoplastic cell proliferation,[26] through the formation of HNE.

Acknowledgements

Work supported by Association for International Cancer Research, U.K., Consiglio Nazionale delle Richerche, Roma (Special Project Oncology).

References

1. H. Esterbauer (1985) Lipid peroxidation products: Formation, chemical properties and biological activities. In: Free radicals in liver injury (eds. G. Poli, K.H. Cheeseman, M.U. Dianzani and T.F. Slater), I.R.L. Press, Oxford-Washington D.C. pp. 29–47.
2. H. Esterbauer, H. Zollner and J. Lang (1985) Metabolism of the lipid peroxidation product 4-hydroxynonenal by isolated hepatocytes and by liver cytosolic fraction. *Biochemical Journal*, **228,** 363–373.
3. S. Hauptlorenz, H. Esterbauer, W. Moll, R. Pumpel, E. Schauenstein and B. Puschendorf (1985) Effects of the lipid peroxidation product 4-hydroxynonenal and related aldehydes on proliferation and viability of cultured Ehrlich ascites tumor cells. *Biochemical Pharmacology*, **34,** 3803–3809.
4. L. Tessitore, L. Matera, G. Bonelli, F.M. Baccino and M.U. Dianzani (1987) Aliphatic aldehydes inhibit the proliferation response of human peripheral blood lymphocytes to phytohemagglutinin and alloantigens. *Chemico-Biological Interactions*, **62,** 217–226.
5. G. Barrera, S. Martinotti, V. Fazio, V. Manzari, L. Paradisi, M. Parola, L. Frati and M.U. Dianzani (1987) Effect of 4-hydroxynonenal on c-myc expression. *Toxicologic Pathology*, **15,** 238–240.
6. E. Wawra, H. Zollner, R.J. Schaur, H.M. Tillian and S. Schauenstein (1986) The inhibitory effect of

4-hydroxy-nonenal on DNA polymerases alpha and beta from rat liver and rapidly dividing Yoshida ascites hepatoma. *Cell Biochemistry and Functions*, **4**, 31–36.
7. H. Esterbauer, H. Zollner and R.J. Schaur (1988) Hydroxyalkenals cytotoxic products of lipid peroxidation. *Isi Atlas of Science*, **1**, 311–317.
8. M. Poot, H. Esterbauer, P.S. Rabinovitch and H. Hoehn (1988) Disturbance of cell proliferation by two model compounds of lipid peroxidation contradicts causative role in proliferative senescence. *Journal of Cellular Physiology*, **137**, 421–429.
9. C.W. Tabor and H. Tabor (1984) Polyamines. *Annal Review Biochemistry*, **53**, 749–790.
10. D.H. Russel (1988) Ornithine decarboxilase—a key regulatory enzyme in normal and neoplastic growth. *Drug Metabolism Reviews*, **16**, 1–88.
11. M.K. Haddox, K.F. Frasier Scott and D. Haddock Russell (1979) Retinol inhibition of ornithine decarboxylase induction and G1 progression in chinese hamster ovary cells. *Cancer Research*, **39**, 4930–4938.
12. E.F. Hartree (1972) Determination of protein: a modification of the Lowry method that gives a linear photometric response. *Analytical Biochemistry*, **48**, 422–427.
13. H. Esterbauer (1975) Aldehydic products of lipid peroxidation. In: Free Radicals, lipid perodixation and cancer (D.C.H. McBrien and T.F. Slater eds.) Academic Press, London pp. 101–128.
14. M. Gumpta and P. Coffino (1985) Mouse ornithine decarboxylase. Complete amino acid sequenced deduced from cDNA. *The Journal Biological Chemistry*, **260**, 2941–2944.
15. A.E. Pegg (1986) Recent advances in the biochemistry of polyamines in eukaryotes. *Biochemical Journal*, **234**, 249–262.
16. J.E. Seely, H. Poso and A.E. Pegg (1982) Measurement of the number of ornithine decarboxylase molecules in rate and mouse tissues under various physiological conditions by binding of radiolabelled alpha-difluoromethylornithine. *Biochemical Journal*, **206**, 311–318.
17. J.E. Seely and A.E. Pegg (1983) Changes in mouse kidney ornithine decarboxylase activity are brought about by changes in amount of ornithine decarboxylase protein as measured by radioimmunoassay. *The Journal of Biological Chemistry*, **258**, 2496–2500.
18. F.G. Bergher, P. Szymanski, E. Read and G. Watson (1984) Androgen-regulated ornithine decarboxylase mRNAs of mouse kidney. *The Journal of Biological Chemistry*, **259**, 7941–7946.
19. J.S. White and K.R. Rees (1984) The mechanism of action of 4-hydroxynonenal in cell injury. *Chemico-Biological Interactions*, **4**, 233–241.
20. S. Rose-John, G. Furstemberer, P. Krieg, E. Besemfelder, G. Rincke and F. Marks (1988) Differential effect of phorbol esters on c-fos, c-myc and ornithine decarboxylase gene expression in mouse skin in vivo. *Carcinogenesis*, **9**, 831–835.
21. L. Gabriel, A. Miglietta and M.U. Dianzani (1985) 4-hydroxyalkenals interaction with purified microtubular protein. *Chemico-Biological Interactions*, **56**, 201–21.
22. G. Poli, M.U. Dianzani, K.H. Cheeseman, T.F. Slater, J. Lang and H. Esterbauer (1985) Separation and characterization of the aldehydic products of lipid peroxidation stimulated by carbon tetrachloride or ADP-iron in isolated rat hepatocytes and rat liver microsomal suspension. *Biochemical journal*, **227**, 629–638.
23. M.A. Rossi and G. Cecchini (1983) Lipid peroxidation in hepatomas of different degrees of deviation. *Cell Biochemistry and Function*, **1**, 49–54.
24. F.J.G.M. Van Kuijk, D.W. Thomas, R.J. Stephens and E.A. Dratz (1986) Occurrence of 4-hydroxyalkenals in rat tissues determined as pentafluorobenzyl oxime derivatives by gas chromatography-mass spectrometry. *Biochemical Biophysical Research Communications*, **139**, 144–149.
25. I. Tomita, K. Yoshino and M. Sano (1987) Formation of aliphatic aldehydes in the plasma and liver of vitamin E deficient rats. In: Clinical and nutritional aspects of vitamin E (eds. D. Hayaish and M. Mino), Elsevier, Amsterdam, pp. 277–280.
26. T.F. Slater, C. Benedetto, G.W. Burton, K.H. Cheeseman, K.U. Ingold and J.T. Nodes (1984) Lipid peroxidation in animal tumours: a disturbance in the control of cell division? In: 'Icosanoids and Cancer' (Eds. H. Thaler-Dao, A. Crastes de Paulet and R. Paoletti) pp. 21–29, Raven Press, New York.

Accepted by Prof T.F. Slater