

Studies on lipid peroxidation in normal and tumour tissues

The Yoshida rat liver tumour

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Reduced rates of lipid peroxidation have been observed in Yoshida hepatoma cells and microsomes when compared with appropriate control tissue (normal rat liver) under the same pro-oxidant conditions. The pro-oxidant conditions used were incubation with NADPH + ADP + iron or ascorbate + iron or exposure to γ -irradiation. As previously shown with the Novikoff hepatoma, the relative concentrations of α -tocopherol and polyunsaturated fatty acids are important in conferring resistance to lipid peroxidation in the Yoshida hepatoma. Furthermore, NADPH-cytochrome *c* reductase and the NADPH-cytochrome *P*-450 electron transport chain, which are involved in the initiation and propagation of certain types of lipid peroxidation, are found at very much reduced levels in the Yoshida hepatoma. The relative importance of these aberrations are discussed.

INTRODUCTION

One of the characteristic biochemical changes known to occur in rat liver tumours and in tumour fractions is a highly significant reduction in lipid peroxidation when exposed to pro-oxidant conditions compared with normal liver (Utsumi *et al.*, 1965; Lash, 1966; Fonnesu *et al.*, 1966; Ugazio *et al.*, 1968; Burlakova, 1975; Gravela *et al.*, 1975; Bartoli & Galeotti, 1979; Player *et al.*, 1979; Ahmed & Slater, 1981; Dianzani *et al.*, 1984; Sharma *et al.*, 1984; Cheeseman *et al.*, 1986a).

We have previously shown that lipid peroxidation is significantly decreased in the highly un-differentiated, rapidly proliferating Novikoff hepatoma, when compared with normal rat liver tissue (Cheeseman *et al.*, 1986a). Work reported by Borello *et al.* (1985) suggests that in a slow-growing hepatoma (Morris 9618A) the changes in lipid peroxidation are not so large, although still significant. Two mechanisms have been proposed to explain the reduction in lipid peroxidation exhibited by the range of hepatomas studied. One mechanism is based on an observed reduction in the NADPH-cytochrome *P*-450 electron transport chain; this enzyme system has been implicated in the production of propagating and initiating radicals (Svingen *et al.*, 1979; White & Coon, 1980). The second mechanism is based on the view that the primary reason for reduced lipid peroxidation in hepatomas exposed to oxidative stress is an increase in the lipid soluble antioxidant (α -tocopherol) of intracellular and plasma membranes, accompanied by reductions in cytochrome *P*-450 content and in the content of polyunsaturated fatty acids (Cheeseman *et al.*, 1984). The present paper on the Yoshida hepatoma continues our programme in which we are studying lipid peroxidation in a variety of human and animal tumours in order to (i) identify the major factors contributing to the changes in lipid peroxidation in tumours exposed to oxidative stress, (ii) to assess whether these changes are consistent and significant features of malignant cells and (iii) to evaluate whether lipid peroxidation is of importance in relation to cell division.

MATERIALS AND METHODS

Chemicals

Collagenase (type IV), ADP (sodium salt), cholesterol oxidase, cholesterol esterase, horseradish peroxidase and α -DL-tocopherol were obtained from Sigma. Cytochrome *c* and NADPH were purchased from Boehringer. All other reagents were of AnalaR grade or better.

Yoshida tumour cells

The Yoshida hepatoma was maintained in male Wistar rats (200–300 g body weight), obtained from Charles River Ltd. (Margate, Kent, U.K.), which were allowed access to food (Expanded Breeder Diet no. 3: Special Diet Services, Witham, Essex, U.K.) and water *ad libitum*. The cells were passaged at approx. 7 day intervals by taking 1 ml of ascitic fluid, containing approx. 10^7 cells, and injecting this into the peritoneum of the recipient rat. The cells were collected at appropriate times and suspended in a lysing buffer (17 mM-Trizma/140 mM-NH₄Cl, pH 7.4), followed by centrifugation to remove red blood cells and to wash off the contaminating haemoglobin. Cell viability was always greater than 90% as measured by Trypan Blue exclusion.

Isolated hepatocytes

These cells were prepared by the collagenase perfusion *in situ* technique of Poli *et al.* (1981) and always had a viability of greater than 85% as measured by Trypan Blue exclusion. Cells were suspended in the indicated media to give final experimental concentrations of not greater than 4×10^6 cells/ml.

Microsomal suspensions

Microsomes were prepared from control livers and hepatoma cells as previously described (Slater, 1968), without prior starvation of the rats. Pellets were stored at -70°C until required; they were resuspended in 0.15 M-KCl such that 1 ml of suspension contained 15–20 mg of protein.

Enzyme assays

NADPH-cytochrome *c* reductase was measured in microsomal suspensions by the method of Slater & Sawyer (1969). An index of cytochrome *P*-450-dependent drug metabolism was obtained by measuring the activity of 7-ethoxycoumarin de-ethylase in microsomal suspensions by using the procedure of Prough *et al.* (1978). Cytochrome *P*-450 was measured by the technique of Omura & Sato (1962).

Lipid peroxidation

NADPH + ADP + iron-stimulated lipid peroxidation was measured in microsomal suspensions as oxygen uptake by the method of Slater (1968) and in cell suspensions as malondialdehyde production by the method of Poli *et al.* (1985). Ascorbate + iron-induced lipid peroxidation was measured in both microsomal suspensions and isolated cells by the method of Beswick *et al.* (1981) using 500 μM -ascorbate, 5 μM -ferrous sulphate and an incubation time of 15 min. Peroxidation in isolated cells, initiated by γ -irradiation, was according to the method described by Garner *et al.* (1986) using the ^{60}Co irradiation unit at Brunel University. Irradiation of cells was performed in culture medium (medium 'C' in Poli *et al.*, 1979) or phosphate-buffered saline solution with continuous bubbling of $\text{N}_2\text{O}/\text{O}_2$ (4:1) to prevent sedimentation of cells, prevent anoxia and promote the production of hydroxyl radical (HO^\bullet). No differences in cell viability were observed during the course of experimental procedures using these media. Cell suspensions were irradiated for 20 min at a dose rate of 11.1 $\text{Gy} \cdot \text{min}^{-1}$ (final dose 222 Gy) following which they were incubated at 37 °C. Where appropriate, malondialdehyde was measured as thiobarbituric acid ('TBA')-reactive material by the procedure of Slater & Sawyer (1969). In this paper we use malondialdehyde synonymously with 'TBA-reactive material', since in all the liver microsomal systems we have studied these two parameters, measured independently, have been quantitatively identical (see Esterbauer *et al.*, 1982, 1984).

Determination of α -tocopherol and total lipid-soluble radical-trapping antioxidant capacity

Lipid extracts were prepared from microsomes and isolated cells by the addition of 1 vol. of *n*-heptane, 2 vol. of ethanol and 1 vol. of 25 mM-SDS to 1 vol. of cell preparation or by using chloroform/methanol (2:1, v/v; at least 20 vol./g wet wt.; Folch *et al.*, 1957). The analysis of α -tocopherol was by h.p.l.c. as described by Burton *et al.* (1985). Aliquots (100 μl) of the heptane extract were injected onto a 25 cm \times 0.5 cm 5 μm Spherisorb CN or Lichrosorb CN column (Baird & Tatlock, Dagenham, Essex, U.K.) and eluted with hexane/propan-2-ol (99:1, v/v) at a flow rate of 1.5 $\text{ml} \cdot \text{min}^{-1}$. Analysis of column eluent was by on-line fluorimetry (excitation, 295 nm; emission, 325 nm) giving a lower limit of detection of approx. 25 pmol of α -tocopherol. Quantification of α -tocopherol was performed using an external standard calibration with authentic α -DL-tocopherol. The total lipid-soluble radical-trapping antioxidant capacity of the lipid extract was performed using the inhibited autoxidation of styrene as described previously (Burton & Ingold, 1981; Burton *et al.*, 1983). It is assumed that each molecule of antioxidant in the lipid extract terminates two radical chains, as is the case for the tocopherols

(Burton & Ingold, 1981). The volume of lipid extract used was 50–100 μl .

Determination of lipid and cholesterol

The analysis of total lipid from microsomes and isolated cells was performed on lipid extracts using the method of Zollner & Kirsch (1962); samples (200 μl) of heptane extract were evaporated to dryness under N_2 and redissolved in 200 μl of ethanol. These were then heated to 100 °C for 10 min with 4 ml of conc. H_2SO_4 ; 200 μl of this mixture was added to 5 ml of vanillin reagent (14 M-orthophosphoric acid/13 mM-vanillin). Absorbance at 530 nm was measured after incubation at 25 °C for 30 min. Inclusion of a lipid standard (10 $\text{mg} \cdot \text{ml}^{-1}$; Boehringer) allowed calculation of sample lipid concentration. Cholesterol in chloroform/methanol extracts was determined according to the procedure of Gamble *et al.* (1978) in which cholesterol oxidase generates H_2O_2 and horseradish peroxidase catalyses the reaction of H_2O_2 with *p*-hydroxyphenylacetic acid to yield a stable fluorescent product.

Determination of fatty acids

The quantity and composition of the fatty acids in the *O*-acyl fraction of lipid extracts was determined by g.c. analysis after transesterification of the lipid extract according to the method of Christie (1973). Heptadecanoic acid was used as a non-interfering internal standard.

Determination of protein

The protein content of cell preparations was measured by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard.

RESULTS

The results given in Table 1 show that the total lipid content of Yoshida hepatoma cells, and microsomes prepared from Yoshida cells, is lower than the level found in corresponding normal rat liver cells and liver microsomal suspensions. In addition to this, the data in Table 1 show that Yoshida cells and microsomes contain different proportions of fatty acids to normal liver and liver microsomes. In both Yoshida cells and microsomes, there is a significant reduction in $\text{C}_{20:4}$ and $\text{C}_{18:0}$ fatty acids accompanied by a significant increase in the $\text{C}_{18:1}$ fatty acid fraction (in each instance $p < 0.001$; Student's *t*-test). An additional deviation from normal liver fatty acid content in Yoshida hepatoma cells and microsomes is the increased content of $\text{C}_{22:5}$. The cholesterol content (as a proportion of total lipid) of the Yoshida hepatoma cell and microsomes is approx. 2-fold that of the appropriate controls.

Table 2 shows that microsomal suspensions prepared from Yoshida hepatoma cells have very much lower NADPH-cytochrome *c* reductase activity and no detectable 7-ethoxycoumarin de-ethylase activity, when compared with microsomal suspensions prepared from normal tissue. There was no detectable cytochrome *P*-450 in Yoshida microsomes (Table 2). The limit of detection for this procedure is approx. 10 pmol of *P*-450/mg of protein (Benedetto *et al.*, 1981); since normal microsomes have a *P*-450 content of approx. 800 pmol/mg of protein (Table 2) it can be estimated that the *P*-450

Table 1. Contents of total lipid, α -tocopherol and fatty acids in normal liver, Yoshida hepatoma cells and in microsomal suspensions prepared from normal liver and Yoshida hepatoma cells

Fatty acids are expressed as a percentage of total fatty acids in the control extract. Where fatty acid values are not shown, these accounted for less than 1%. Mean values \pm S.D. are given, with the number of estimations shown in parentheses. N.D., not determined.

Measurement	Normal intact liver	Intact Yoshida cells	Normal liver microsomes	Yoshida microsomes
Protein (mg/10 ⁶ cells)	N.D.	0.133 \pm 0.008 (4)	N.D.	0.028 \pm 0.002 (4)
Total lipid (mg/mg of protein)	0.41 \pm 0.08 (7)	0.26 \pm 0.9 (6)	0.37 \pm 0.03 (8)	0.21 \pm 0.05 (10)
Cholesterol (μ g/mg of lipid)	96.0 \pm 8.1 (7)	215.2 \pm 6.2 (6)	110.0 \pm 12.3 (8)	266.85 \pm 13.5 (10)
Fatty acids (%)				
16:0	18.5 \pm 0.2 (7)	18.7 \pm 2.0 (6)	18.9 \pm 1.1 (8)	18.5 \pm 0.5 (10)
18:0	17.5 \pm 0.5 (7)	13.3 \pm 1.1 (6)	22.0 \pm 3.0 (8)	13.7 \pm 0.2 (10)
18:1	12.0 \pm 1.0 (7)	21.5 \pm 0.8 (6)	8.6 \pm 1.0 (8)	18.1 \pm 0.3 (10)
18:2	20.1 \pm 1.2 (7)	23.9 \pm 1.0 (6)	17.6 \pm 0.9 (8)	21.6 \pm 0.4 (10)
18:3	—	—	—	1.3 \pm 0.3 (10)
20:4	16.7 \pm 2.4 (7)	8.7 \pm 0.7 (6)	19.1 \pm 2.4 (8)	9.6 \pm 0.8 (10)
22:5	—	2.9 \pm 0.1 (6)	—	2.4 \pm 0.1 (10)
24:0	—	1.2 \pm 0.1 (6)	—	2.9 \pm 0.3 (10)
22:6	6.3 \pm 0.2 (7)	5.2 \pm 0.6 (6)	6.1 \pm 0.3 (8)	5.3 \pm 0.4 (10)

Table 2. Activities of NADPH-cytochrome *c* reductase and 7-ethoxycoumarin de-ethylase in microsomal suspensions derived from normal rat liver and Yoshida hepatoma cells

Values shown are mean \pm S.D. with the number of determinations shown in parentheses. n.d., not detectable.

Measurement	Normal liver microsomes	Yoshida hepatoma microsomes
NADPH-cytochrome <i>c</i> reductase (nmol/min per mg)	80.5 \pm 4.2 (4)	9.13 \pm 1.4 (6)
7-Ethoxycoumarin de-ethylase activity (nmol/min per mg)	0.14 \pm 0.04 (4)	n.d. (6)
Cytochrome <i>P</i> -450 (nmol/mg)	0.81 \pm 0.06 (6)	n.d. (4)

Table 3. Lipid peroxidation in normal rat liver microsomal suspensions and in microsomal suspensions prepared from Yoshida hepatoma cells

Ascorbate + iron-induced lipid peroxidation was measured as malondialdehyde production and NADPH + ADP + iron-stimulated lipid peroxidation was measured as oxygen uptake, as described in the Materials and methods section. Values shown are means \pm S.D. with number of determinations shown in parentheses.

Pro-oxidant	Peroxidation (nmol/min per mg)	
	Normal microsomes	Yoshida microsomes
Ascorbate + iron	1.29 \pm 0.16 (10)	0.24 \pm 0.10 (7)
NADPH + ADP + iron	82.4 \pm 9.21 (6)	1.95 \pm 0.51 (7)

Table 4. Malondialdehyde production stimulated by NADPH + ADP + iron (2.5 mM-ADP/0.1 mM-iron) in suspensions of isolated normal rat hepatocytes and Yoshida hepatoma cells

Values shown are means \pm S.D. with the number of determinations shown in parentheses.

Incubation time (min)	Malondialdehyde production (nmol/10 ⁷ cells)	
	Isolated hepatocytes	Yoshida hepatoma cells
5	11.6 \pm 6.51 (4)	0.010 \pm 0.002 (6)
30	27.93 \pm 10.32 (4)	0.08 \pm 0.01 (6)
60	37.87 \pm 9.26 (4)	0.91 \pm 0.02 (6)

content of microsomes prepared from Yoshida cells is less than 2% of normal.

The results in Tables 3 and 4 and Figs. 1(a) and 1(b) illustrate the differences in lipid peroxidation observed when Yoshida cells or Yoshida microsomal suspensions are subjected to a variety of pro-oxidant stimuli and compared with corresponding normal hepatocytes or microsomal suspensions. In all cases there is a significant reduction in lipid peroxidation when observed either directly (O₂ uptake, Table 3) or indirectly (malondialdehyde determination, Tables 3 and 4) in hepatoma preparations. Furthermore, these differences remain significant regardless of the type of pro-oxidant stimulus employed. The differences are apparent using the enzymic NADPH-dependent ADP + iron assay (Tables 3 and 4), as well as when using the essentially non-enzymic ascorbate + iron assay (Table 3); moreover, they are apparent following exposure of cell preparations to the strongly peroxidizing influence of γ -irradiation that is independent of enzyme or haemoprotein mechanisms (Figs. 1a and 1b).

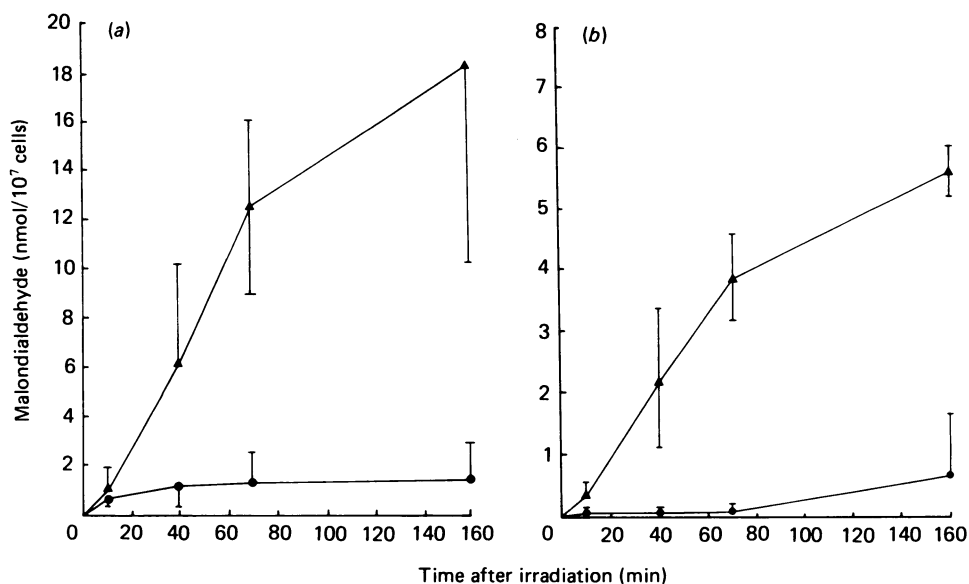


Fig. 1. Production of malondialdehyde by normal rat hepatocytes (▲) and Yoshida hepatoma cells (●) induced by γ -irradiation

In (a) the results shown were obtained by incubating cells in phosphate-buffered saline solution; the results shown in (b) were obtained by incubating cells in an amino-acid-enriched medium. All cell suspensions were given a dose of 222 Gy before incubation at 37 °C. Aliquots of cell suspension were removed at the time points indicated and acidified with trichloroacetic acid. Following centrifugation, the protein-free supernatant was assayed for the presence of malondialdehyde by using the thiobarbituric acid procedure of Slater & Sawyer (1971). Points shown are means \pm S.D.; for normal hepatocytes $n = 4$, for Yoshida hepatoma cells $n = 6$.

Table 5. Content of α -tocopherol relative to total lipid and bis-allylic methylene units in normal liver, Yoshida hepatoma cells and in microsomal suspensions prepared from normal liver and Yoshida hepatoma cells

Values given are means \pm S.D. The numbers of determinations are shown in parentheses. N.D., not determined. The values for normal whole liver and microsomes are reproduced from our previous paper (Cheeseman *et al.*, 1986a).

Measurement	Normal intact liver	Yoshida hepatoma cells	Normal microsomes	Yoshida microsomes
α -Tocopherol:lipid (nmol/mg)	2.30 \pm 0.53 (7)	4.16 \pm 0.63 (6)	1.67 \pm 0.31 (8)	3.06 \pm 0.42 (10)
α -Tocopherol: bis-allylic methylene groups ($\times 10^4$)	7.6	31.8	5.2	38.6
Total antioxidant:lipid (nmol/mg)	2.99 \pm 0.5 (7)	4.21 \pm 0.31 (4)	N.D.	N.D.

α -Tocopherol is found in higher quantities in Yoshida cells and microsomes than in normal liver or normal liver microsomal suspensions (Table 5). In addition, Table 5 shows that this increase becomes highly significant when the α -tocopherol content is expressed as a ratio of the readily peroxidizable moieties of lipid membranes (bis-allylic methylene groups). As with the Novikoff tumour (Cheeseman *et al.*, 1986a), α -tocopherol is the major, and possibly only, lipid soluble chain-breaking free radical scavenger in the Yoshida hepatoma, since it accounts almost completely for the total antioxidant capacity of lipid extracts (Table 5).

DISCUSSION

The Yoshida AH130 ascites hepatoma was established in 1951 following treatment of rats with diaminoazobenzene (Yoshida *et al.*, 1951). Many previous studies

have reported lower rates of lipid peroxidation in rat liver tumour cells under pro-oxidant conditions (see the references cited in the Introduction). Results presented here show unequivocally that lipid peroxidation, stimulated and measured by a variety of systems, occurs in the Yoshida hepatoma at rates far below those observed in normal liver under identical conditions. Similar, although far less detailed, results were reported from the Yoshida hepatoma by Ugazio *et al.* (1968) and later by Sharma *et al.* (1984).

This study has identified three possible reasons for the resistance to lipid peroxidation exhibited by the Yoshida hepatoma cells and microsomes prepared from them. Firstly, Yoshida microsomal suspensions have markedly decreased NADPH-cytochrome *c* reductase activity (Table 2) when compared with normal microsomal suspension levels and this enzyme is responsible for

catalysing NADPH + ADP + iron-dependent lipid peroxidation. Additionally, the hepatoma microsomes have no detectable cytochrome *P*-450 when assayed by 7-ethoxycoumarin de-ethylase activity or by its difference spectrum following exposure to CO (Table 2). Overall, therefore, major components of the NADPH-cytochrome *P*-450 electron transport chain are lacking in or absent from the Yoshida hepatoma microsomes and these factors would most certainly produce less favourable conditions for the initiation and/or propagation of lipid peroxidation in these preparations (Svingen *et al.*, 1979). The observation, however, that lipid peroxidation does not occur to a large extent even when Yoshida hepatoma cells are exposed to a flux of hydroxyl radicals produced by γ -irradiation (Figs. 1*a* and 1*b*) suggests very strongly that there is a more significant over-riding inhibitory mechanism operating than electron transport chain deficiency alone, as suggested by Borrello *et al.* (1985) for their observations on the slow-growing Morris 9618A rat hepatoma.

Lipid extracts of both Yoshida cells and microsomal suspensions contain fatty acids in differing proportions from those observed in corresponding control tissues (Table 1). It is apparent that the levels of polyunsaturated fatty acids are significantly reduced, particularly C_{20:4} and to a lesser extent C_{22:6}. This observation would certainly account for a decreased rate of lipid peroxidation, as discussed by Galleotti *et al.* (1984). It is known, however, that microsomal suspensions from rabbit liver (Cheeseman *et al.*, 1986*a*; Proudfoot, 1986) and from the livers of rats fed on a coconut oil enriched diet (Hammer & Wills, 1978) peroxidize well even though these preparations contain markedly decreased quantities of C_{20:4} and C_{22:6}. We therefore feel that the limited availability of the polyunsaturated fatty acids that are the substrates for lipid peroxidation cannot by itself account for the very low rates of lipid peroxidation found in the Yoshida hepatoma.

We have shown here significantly increased levels of α -tocopherol in lipid extracts of Yoshida hepatoma cells and microsomal suspensions (Table 5). These results show an approx. 2-fold increase in α -tocopherol content of hepatoma cells and microsomes over corresponding controls when expressed as the concentration of the antioxidant in total lipid. This increase is even more significant when expressed as a ratio to bis-allylic methylenes (Table 5). This is a better way to express the data since the susceptibility of the membrane to lipid peroxidation will be inversely proportional to the α -tocopherol:bis-allylic methylene ratio. In the Yoshida hepatoma there is an approx. 5-fold increase in this ratio. We have previously shown that α -tocopherol is the major lipophilic chain-breaking antioxidant of normal liver and the Novikoff hepatoma; a similar conclusion was reached for human plasma and erythrocytes (Burton *et al.*, 1983). Here we find that in Yoshida hepatoma cells α -tocopherol again accounts for practically all of the total lipid soluble radical-trapping antioxidant capacity (Table 5).

Increasing the α -tocopherol content of rat livers by intraperitoneal administration produces liver microsomes whose resistance to lipid peroxidation is correlated with their α -tocopherol content (K. H. Cheeseman, S. Emery & T. F. Slater, unpublished work). Normal rat liver cells in regenerating liver exhibit reduced rates of peroxidation and increased α -tocopherol content (Slater *et al.*, 1985; Cheeseman *et al.*, 1986*b*). We suggest that,

as in the Novikoff hepatoma, the increased α -tocopherol content is a major factor, in combination with the reduced levels of polyunsaturated fatty acids and of cytochrome *P*-450, which reduces the rates of lipid peroxidation in the Yoshida hepatoma when compared with control tissue.

A large body of work over many years has established that an increased rate of cell division is often associated with a decreased rate of lipid peroxidation, and this has led to the hypothesis that lipid peroxidation may be involved in the same mechanisms that influence cell division (Slater, 1976; Burlakova *et al.*, 1980; Slater *et al.*, 1984; Morisaki *et al.*, 1984; Cheeseman *et al.*, 1986*a,b*). The data presented here do not conflict with that general hypothesis.

We are grateful to the Cancer Research Campaign (K. H. C. and S. E.), the National Foundation for Cancer Research and the Association for International Cancer Research for generous support.

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Received 29 May 1987/13 August 1987; accepted 23 October 1987