Studies on lipid peroxidation in normal and tumour tissues

The Novikoff rat liver tumour

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A study has been made of the factors that contribute to the decreased rates of lipid peroxidation under different pro-oxidant conditions in intact Novikoff tumour cells, and in microsomal suspensions prepared from Novikoff tumour cells, compared with isolated normal rat hepatocytes and microsomal suspensions prepared from normal rat liver. The pro-oxidant conditions were the addition of either NADPH, NADPH + ADP + iron, NADPH + CCl_4 or ascorbate + iron to the experimental systems used, or exposure to γ -radiation. Contributory factors to the lower rates of lipid peroxidation observed include: (a) a significant decrease in the polyunsaturated fatty acid content of Novikoff cells or Novikoff microsomes; the decreases are especially marked for the $\text{C}_{20:4}$ and $\text{C}_{22:6}$ fatty acids; (b) a very marked reduction in NADPH–cytochrome *c* reductase; and (c) no detectable content of cytochrome *P*-450. Another, and in our opinion critical, contribution to the diminished rate of lipid peroxidation in the tumour material is the substantial increase in α -tocopherol relative both to total lipid and to methylene-interrupted double bonds in fatty acids. Moreover, the α -tocopherol is the major contributor to lipid-soluble chain-breaking antioxidant in lipid extracts of normal liver and of Novikoff tumour material.

INTRODUCTION

Many studies have concluded that lipid peroxidation is significantly decreased in rat tumour cells and tissues compared with the corresponding normal tissues (see Utsumi *et al.*, 1965; Lash, 1966; Fonnesu *et al.*, 1966; Ugazio *et al.*, 1968; Burlakova, 1975; Burlakova *et al.*, 1980; Bartoli & Galeotti, 1979; Player *et al.*, 1979; Ahmed & Slater, 1981; Dianzani *et al.*, 1984; Sharma *et al.*, 1984). However, it should be noted right at the outset that it is often difficult to obtain a completely satisfactory sample of the 'corresponding normal tissue'; indeed, with some tumours it is even unclear what is the corresponding normal tissue. Nonetheless, at least in the case of liver, the differences in lipid peroxidation between liver tumours in general and normal liver are so marked that it is clear that major disturbances exist in free radical reactions in liver tumour cells compared with normal liver.

Some contributions to such differences have been identified to be (i) a decreased content of highly unsaturated fatty acids in tumours compared with normal tissue (Utsumi *et al.*, 1971); (ii) a decreased concentration in tumours of cytochrome *P*-450 (Adamson & Fouts, 1961; Strobel *et al.*, 1978; Saine *et al.*, 1978) that normally can participate in the initiation of lipid peroxidation; (iii) a decreased content of NADPH in tumours (Glock & McLean, 1957; Ross *et al.*, 1982); (iv) changes in antioxidant status and protective enzymes such as superoxide dismutase (Oberley, 1983) and catalase (Tisdale & Mahmoud, 1983). However, no systematic study has been done of the factors favouring peroxidative reactions (pro-oxidant stimuli) versus

antioxidative influences. Moreover, no comprehensive investigations have been carried out of liver tumour cell behaviour in the aspects mentioned above compared with normal proliferating liver cells.

We have started a wide-ranging collaborative programme in which lipid peroxidation will be studied in detail in a variety of animal and human tumours in relation to normal non-proliferating and proliferating tissues. Our objectives are (i) to identify the major factors contributing to changes in lipid peroxidation in tumours, using whole cells and microsomal suspensions for comparative purposes; (ii) to decide whether the changes in lipid peroxidation are an important feature of malignant cells; (iii) to evaluate the significance of the changes in lipid peroxidation with respect to cell division.

In this initial study we have used an extreme example (see Weber, 1983) of an experimentally-induced liver tumour (the Novikoff hepatoma; Novikoff, 1957) in order to define the range of the overall problem and to develop appropriate methodology.

METHODS

Chemicals

Collagenase (type IV), bovine plasma albumin and ADP (sodium salt) were purchased from Sigma; cytochrome *c* and NADPH were obtained from Boehringer; $^{14}\text{CCl}_4$ was obtained from The Radiochemical Centre, Amersham; phenyl-*t*-butylnitron and malonaldehyde bis(diethyl acetal) were from Aldrich Chemical Co.; 6-hydroxy-2,2,5,7,8-pentamethylchroman (PMHC), 2-*t*-butyl-4-hydroxyanisole (2-BHA), 2,2'-azo-

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bisobutyronitrile (AIBN) and α -tocopherol were obtained as described previously (Burton & Ingold, 1981; Burton *et al.*, 1983a). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (TROLOX-C) was a gift from Dr. J. W. Scott (Hoffmann-La Roche, Nutley, NJ, U.S.A.).

Animals

The rats used were adult males of the Wistar strain. They were purchased from the Charles River Co. (Margate, Kent, U.K.) and were fed on a standard laboratory diet (Expanded Breed Diet No. 3; Special Diet Services, Witham, Essex, U.K.) and water *ad libitum* until 18 h before killing, at which time the food was removed.

Novikoff tumour cells

These cells were kindly provided by Dr. Thea Connell, Institute for Cancer Research, Fulham Road, London. The Novikoff cells (10^7 /rat) were implanted intraperitoneally into male Wistar rats as a suspension (1 ml) in phosphate-buffered saline. Samples of ascitic fluid were withdrawn daily for 10 days to measure cell viability by Trypan Blue exclusion, and cell numbers by using a Coulter counter. The percentage of no-takes was 27%, and cell viability was generally greater than 90% except on day 3 after implantation when viability was 84%. The doubling time of the cell line used was calculated to be 21.6 h.

Isolated hepatocytes

These were prepared by a collagenase perfusion procedure as described by Poli *et al.* (1981).

Liver microsomal suspensions

The procedure of Slater (1968) was used. Microsomal pellets were stored at -20°C until required; they were then suspended in 0.15 M-KCl.

Enzyme assays

NADPH-cytochrome *c* reductase was measured in microsomal suspensions by the method of Slater & Sawyer (1969). The content of cytochrome *P*-450 and the activity of aminopyrene demethylase were measured by the procedures described by Slater & Sawyer (1969) and Slater & Sawyer (1971) respectively. The metabolic activation of $^{14}\text{CCl}_4$ to yield covalently bound ^{14}C was determined by the method of Cheeseman *et al.* (1981). The metabolic activation of CCl_4 to $\text{CCl}_3\cdot$ that forms a spin adduct with phenyl-*t*-butylnitron was followed by the procedure described by Albano *et al.* (1982) with a Bruker 209ED e.s.r. spectrometer.

Lipid peroxidation

NADPH-dependent and CCl_4 -stimulated lipid peroxidation in microsomal suspensions were measured by the methods of Slater & Sawyer (1971). NADPH + ADP + iron-stimulated lipid peroxidation was measured in microsomal suspensions by the method of Slater (1968), and in cell suspensions by the method of Poli *et al.* (1985). Ascorbate + iron-stimulated lipid peroxidation was determined as described by Beswick *et al.* (1981). Peroxidation initiated by γ -radiation was performed using the ^{60}Co irradiation unit at Brunel University, and as described by Garner *et al.* (1986). Where appropriate, malonaldehyde was measured by the procedure of Slater & Sawyer (1971).

Protein determinations

Protein was measured by the procedure of Lowry *et al.* (1951) with bovine plasma albumin as a standard.

Determination of vitamin E and total lipid-soluble radical-trapping antioxidant capacity (TOLRAC) of lipid extracts

Lipid extracts were prepared by using chloroform/methanol (2:1, v/v; at least 20 vol./g wt; Folch *et al.*, 1957). The chloroform phase was separated, concentrated by rotary evaporation at reduced pressure and low temperature (15°C) and stored in evacuated glass bulbs at -20°C until required. Samples for analysis were concentrated by evaporation with a stream of N_2 and then made up to 5 ml in volumetric flasks with chloroform. Aliquots (2 ml) were removed, the solvent removed under a stream of nitrogen and the residues redissolved in 2 ml of *n*-heptane. The analysis of tocopherols by h.p.l.c. was performed essentially as described before (Burton *et al.*, 1985); 6-Hydroxy-2,2,5,7,8-pentamethylchroman and 2-*t*-butyl-4-hydroxy-anisole were used as non-interfering standards. The measurements of *O*-acyl total lipid, fatty acid methyl esters and of cholesterol were also performed essentially as described before (Burton *et al.*, 1983a, 1985). The quantity and composition of the fatty acids in the *O*-acyl fraction of the lipid extract and the quantity of cholesterol were each determined by g.c. analysis after duplicate transesterification of the lipid extract (Christie, 1973). The measurement of TOLRAC relies on the production of peroxy radicals at a constant known rate; it was performed using the inhibited autoxidation of styrene as described previously (Burton & Ingold, 1981; Burton *et al.*, 1983a).

It is assumed that each molecule of antioxidant in the lipid extract terminates two radical chains (i.e. $n = 2$), as is the case for the tocopherols (Burton & Ingold, 1981) and most other phenols (Boozar *et al.*, 1955; Burton & Ingold, 1981; Mahoney *et al.*, 1978). The volume of lipid extract used was 50–100 μl .

Direct oxidation of tumour and normal liver lipid

This was performed using the same apparatus as for the inhibited autoxidation of styrene measurements. Autoxidation was again initiated by the thermal decomposition of 2,2'-azobisisobutyronitrile. However, in this case the lipid itself, dissolved in chlorobenzene (1 ml), served as the oxidizable substrate, there being no added styrene or other oxidizable material. The results have already been briefly reported (Burton *et al.*, 1983b; Cheeseman *et al.*, 1984).

RESULTS

Table 1 gives values for total lipid, individual fatty acids and cholesterol in normal rat liver, Novikoff tumour cells, and microsomal fractions prepared from normal rat liver and Novikoff tumour cells. It can be seen that the total lipid contents of Novikoff cells and of microsomes prepared from Novikoff cells are much lower than in corresponding normal liver or microsomal suspensions. Moreover, Novikoff cells and microsomes have significantly less highly unsaturated fatty acids ($\text{C}_{20:4}$ and $\text{C}_{22:6}$) than normal tissue samples. The cholesterol content per g wet wt. of Novikoff cells, and

Table 1. Total lipid, cholesterol and fatty acid contents of normal rat liver, whole Novikoff tumour cells, normal rat liver microsomal suspensions and microsomal suspensions prepared from Novikoff tumour cells ('Novikoff microsomes')

Mean values are given $\pm$ S.D. with the number of estimations shown in parentheses. The units are: total lipid, mg/g wet wt. of liver, packed cells or microsomal pellets; cholesterol, % of total lipid, fatty acids, % of total fatty acids in the lipid extract. The data for microsomal suspensions (which were considerably concentrated relative to intact liver or cells) are expressed per g wet wt. of microsomal pellets; to convert to values per g wet wt. of liver or packed cells it is necessary to divide the values given for normal microsomes by 3.3, and for Novikoff microsomes by 14.3. The percentage values given for the fatty acids can be converted to approximate amounts (mg/g wet wt.) by using the following values (means $\pm$ S.D.; number of estimations in parentheses) for $100 \times$ (ratio of total fatty acids:total lipid): normal intact liver, 90 ± 1.4 (7); intact Novikoff cells, 81.8 ± 1.5 (7); normal microsomes, 89.3 ± 1.8 (8); Novikoff microsomes, 67.8 ± 2.1 (5).

Measurement	Normal intact liver	Intact Novikoff cells	Normal Liver microsomes	Novikoff microsomes
Total lipid (mg/g)	24.5 ± 2.6 (7)	9.5 ± 1.16 (7)	21.0 ± 3.0 (8)	9.22 ± 0.26 (5)
Cholesterol (%)	9.6 ± 1.0 (7)	18.3 ± 1.6 (7)	10.7 ± 1.9 (8)	32.2 ± 2.1 (5)
Fatty acids (%)				
16:0	18.5 ± 0.2 (7)	14.4 ± 0.8 (9)	18.9 ± 1.1 (8)	13.9 ± 0.8 (5)
18:0	17.5 ± 0.5 (7)	14.5 ± 4.4 (9)	22.0 ± 3.0 (8)	19.5 ± 0.6 (5)
18:1	12.0 ± 1.0 (7)	19.8 ± 0.4 (9)	8.6 ± 1.0 (8)	20.2 ± 0.5 (5)
18:2	20.1 ± 1.2 (7)	28.2 ± 1.5 (9)	17.6 ± 0.9 (8)	25.1 ± 0.6 (5)
20:4	16.7 ± 2.4 (7)	10.0 ± 1.0 (9)	19.1 ± 2.4 (8)	10.6 ± 0.3 (5)
22:6	6.3 ± 0.2 (7)	2.0 ± 0.4 (9)	6.1 ± 2.3 (8)	1.4 ± 0.3 (5)

of microsomes prepared from Novikoff cells, is considerably elevated in comparison with the normal tissue samples. Isolated rat hepatocytes have rather similar cholesterol content ($8.7 \pm 0.5\%$ of total lipid), and similar fatty acid composition (16:0, 20.1%; 18:0, 17.6%; 18:1, 10.2%; 18:2, 21.1%; 20:4, 15.5%; 22:6, 7.2% as percentages of total fatty acids) to intact liver samples.

The data given in Table 1 and in the text above demonstrate that qualitatively similar differences occur between normal liver and Novikoff tumour samples when either intact liver, whole cells or microsomal suspensions are used for the purpose of comparison.

Table 2. Contents of protein and cytochrome P-450, the activities of NADPH-cytochrome c reductase and aminopyrene demethylase, and the covalent binding of ^{14}C from $^{14}\text{CCl}_4$ in microsomal suspensions prepared from normal rat liver and from Novikoff hepatoma cells

The normal values were obtained from four separate experiments; the values for the Novikoff microsomes represent two to four experiments. Mean values are given $\pm$ S.E.M.; for other details see the text. N.D., not detectable. The value for covalent binding of $^{14}\text{CCl}_4$ to normal microsomes is taken from Cheeseman *et al.* (1984).

Determination	Normal microsomes	Novikoff microsomes
Protein (mg/g wet wt. of liver or packed cells)	17.4 ± 0.7	2.5
Cytochrome P-450 (pmol/mg of protein)	710 ± 20	N.D.
NADPH-cytochrome c reductase (nmol/min per mg of protein)	80.5 ± 4.2	6.0
Aminopyrene demethylase (nmol/min per mg of protein)	6.4 ± 0.3	N.D.
Covalent binding of CCl_4 (nmol/min per mg of protein)	0.11 ± 0.02	N.D.

Table 2 illustrates results obtained for some enzyme activities of microsomal suspensions. Although microsomes prepared from Novikoff cells ('Novikoff microsomes') have detectable NADPH-cytochrome c reductase activity, there was no detectable cytochrome P-450 or aminopyrene demethylase activity. No activation of the hepatotoxic agent CCl_4 , as measured by covalent binding of ^{14}C , was observed with samples of Novikoff tumour cells or Novikoff microsomes.

Additional evidence that Novikoff tumour microsomes do not significantly metabolize CCl_4 to a free radical intermediate was provided by e.s.r. spin trapping experiments (results not shown). Although normal liver microsomes supplemented with an NADPH-generating system metabolically activate CCl_4 to $\text{CCl}_3\cdot$ that can be spin-trapped with phenyl-t-butyl nitron (Albano *et al.*, 1982), no significant spin adduct was observed when using Novikoff microsomes.

The relative abilities to undergo lipid peroxidation when exposed to different pro-oxidant stimuli are shown in Table 3 for normal microsomal suspensions and for Novikoff microsomal suspensions. All pro-oxidant systems tested produced much greater effects in normal liver fractions than in corresponding fractions prepared from Novikoff tumour cells; the data in Table 3 include peroxidation systems dependent on enzymic reactions (NADPH-dependent, NADPH + CCl_4 -dependent) as well as one that is essentially non-enzymic in character (ascorbate + iron-dependent).

Novikoff cells also behave similarly to Novikoff microsomes in displaying a much reduced capacity for lipid peroxidation: for example, with the NADPH + ADP + iron-dependent system normal hepatocytes produced 172 nmol of malonaldehyde/h per 10^8 cells whereas no malonaldehyde production was detectable with an equivalent number of Novikoff cells. Moreover, as shown in Fig. 1, even with the powerful pro-oxidant stimulus of γ -irradiation there was almost no lipid peroxidation in Novikoff cells over a 2 h period.

The results shown in Table 3 and Fig. 1 are indicative

Table 3. Activities of various systems associated with lipid peroxidation in normal rat liver microsomal suspensions and in microsomal suspensions prepared from Novikoff hepatoma cells

Mean values are given $\pm$ S.E.M. with the number of experiments in parentheses; N.D., not detectable.

Determination	Normal microsomes	Novikoff microsomes
NADPH-linked lipid peroxidation (pmol of malonaldehyde/min per mg of protein)	80 $\pm$ 5 (4)	N.D.
CCl ₄ -stimulated lipid peroxidation (pmol of malonaldehyde/min per mg of protein)	238 $\pm$ 18 (4)	N.D.
NADPH + ADP + iron-stimulated lipid peroxidation (pmol of malonaldehyde/min per mg of protein)	1705 (2)	150 (2)
(nmol of oxygen/min per mg of protein)	108 $\pm$ 11 (4)	N.D.
Ascorbate + iron-stimulated lipid peroxidation (nmol of malonaldehyde/min per mg of protein)	1290 $\pm$ 164 (10)	60 $\pm$ 35 (4)

of a high antioxidant content in Novikoff samples in comparison with normal liver and this is confirmed by the data for total antioxidant and α -tocopherol content in normal and tumour lipid extracts shown in Table 4. Fig. 2

Fig. 1. Effect of increasing exposure to γ -radiation on the production of malonaldehyde-like substances by isolated normal rat hepatocytes (●) and Novikoff tumour cells (▲)

After irradiation the cell suspensions were acidified with trichloroacetic acid and malonaldehyde-like substances were measured in the protein-free supernatant solution by using the thiobarbituric acid procedure as described by Slater & Sawyer (1971). The data for isolated normal hepatocytes were kindly provided for us by our colleagues Dr. A. Garner and Dr. Z. Jamal using the same strain of rat and the same radiation source.

illustrates the differences in induction time ('lag times') that were observed between normal and tumour fractions: the rates of autooxidation of lipids after the induction period and with a constant rate of chain initiation will be approximately proportional to the concentration of bisallylic methylene units. It is noteworthy therefore that the rate of autooxidation of the Novikoff lipid extract at the end of the induction period (Fig. 2a) is slower by a factor of 0.5 than that observed for the lipid extract prepared from liver which is very similar to the ratio (0.6) of bisallylic methylene hydrogens in the two samples.

An illustration of the effect of experimentally increasing the content of α -tocopherol in the microsomal fraction of normal rat liver on lipid peroxidation is shown in Fig. 3. It can be seen that pre-feeding rats with α -tocopherol greatly increases the induction time of oxygen uptake of lipid peroxidation stimulated by NADPH + ADP iron. It can also be seen in Fig. 3 that α -tocopherol added *in vitro* to microsomes was not very inhibitory, probably due to a slow rate of penetration (Cadenas *et al.*, 1984); 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (TROLOX-C), with different solubility properties, was more effective.

DISCUSSION

It is quite clear from the results presented here that there are marked differences in response to pro-oxidant stimuli between the Novikoff cells (or microsomes) compared with normal rat liver (or microsomes). Similar but far less detailed results have been reported for other types of rat liver tumour such as the aflatoxin- and ethionine-induced tumours (Gravela *et al.*, 1975; Ahmed & Slater 1981), the Yoshida tumour (Ugazio *et al.*, 1968; Sharma *et al.*, 1984) and various Morris hepatomas (Bartoli & Galeotti, 1979).

Microsomal suspensions

Novikoff microsomal suspensions have much decreased contents of the components of the NADPH-cytochrome P-450 electron transport chain compared with normal (Table 2), thereby producing less favourable conditions for the initiation of lipid peroxidation in the endoplasmic reticulum. Although the activity of NADPH-cytochrome *c* reductase was considerably lower in Novikoff microso-

Table 4. Contents of α -tocopherol and of total lipid-soluble antioxidant in normal liver, Novikoff tumour cells and in microsomal suspensions prepared from normal liver or Novikoff tumour cells

Mean values are given $\pm$ S.D., with the number of estimations shown in parentheses.

Measurement	Normal intact liver	Intact Novikoff cells	Normal liver microsomes	Novikoff microsomes
α -Tocopherol (nmol/g wet wt.)	56.1 $\pm$ 12.4 (7)	53.1 $\pm$ 10.9 (7)	33.4 $\pm$ 12.7 (8)	44.9 $\pm$ 3.7 (5)
α -Tocopherol:lipid (nmol/mg)	2.30 $\pm$ 0.53 (7)	5.62 $\pm$ 0.78 (7)	1.67 $\pm$ 0.31 (8)	4.87 $\pm$ 0.37 (5)
Total antioxidant (nmol/g wet/wt.)	69.7 $\pm$ 13.9 (7)	54.1 $\pm$ 14.5 (6)	47.5 $\pm$ 5.2 (8)	59.0 $\pm$ 17.7 (5)
Total antioxidant:lipid (nmol/mg)	2.99 $\pm$ 0.51 (7)	6.19 $\pm$ 1.18 (7)	2.30 $\pm$ 0.31 (8)	6.54 $\pm$ 1.73 (5)
α -Tocopherol:total antioxidant	0.8 $\pm$ 0.1 (7)	0.9 $\pm$ 0.2 (7)	0.8 $\pm$ 0.2 (10)	0.8 $\pm$ 0.3 (5)
α -Tocopherol:bisallylic methylene groups ($\times 10^4$)	7.6	30	5.2	33

Fig. 2. Examples of induction period obtained (a) for 2,2'-azobisisobutyronitrile-initiated autoxidation of lipid extracted from normal rat liver suspensions and from Novikoff tumour cells, and (b) with microsomal suspensions obtained from normal rat liver and from Novikoff tumour cells

In (a) the results shown are representative of studies done by the inhibited autoxidation method using the lipid extract as the sole oxidizable material; in (b) the results represent studies with the NADPH + ADP + iron system. For experimental details see the Methods section. Specific details for (a) are: upper trace, lipid extract from normal whole rat liver, total lipid 21.4 mg; bottom trace, lipid extract from Novikoff tumour cells, total lipid 24.8 mg. The lipid was dissolved in chlorobenzene (1 ml) containing 2,2'-azobisisobutyronitrile (0.145 M) and shaken in a thermostatted bath at 30 °C. In each case the uninhibited autoxidation at the end of the induction period was allowed to proceed so as to consume less than 10% of the calculated amount of bis-allylic methylene units. A solution of α -tocopherol in chlorobenzene (4 μ l; 28.8 nmol) was then added to determine the rate of peroxy radical production (49.1 nmol/h) as shown to the right hand of the upper and lower experimental traces. The oxygen uptake was measured with a pressure transducer as described by Burton & Ingold (1981). Specific details for (b): the method used was as described by Slater (1968) with a Clark-type oxygen electrode and with the stock microsome suspension maintained at 25 °C. The oxygen uptake curves show typical results for normal microsome (i) and for microsomes isolated from Novikoff cells (ii). The microsomal protein content in the final mixture was approx. 1 mg/ml in all such experiments.

mes compared with normal microsomes, it was readily measurable (Table 2); our results are thereby consistent with those of Strobel *et al.* (1978), who have purified the reductase, but differ from the findings of Reynafarje & Potter (1957) who reported no detectable NADPH-cytochrome *c* reductase activity in the Novikoff hepatoma. Like Strobel *et al.* (1978) we could not detect cytochrome *P*-450 in Novikoff microsomes by conventional difference spectrophotometry; the detection limit of this method in our hands is approx. 10 pmol/mg of protein (Benedetto *et al.*, 1981). We could not detect aminopyrene demethylase activity in Novikoff microsomes (Table 2), so that the overall activity of the drug-metabolizing

system is very much less than is present in normal liver microsomes.

Novikoff microsomes peroxidize very much less than normal rat liver microsomes when exposed to a range of pro-oxidant stimuli, both enzyme-catalysed and non-enzymic (Table 3). We have used different measures of lipid peroxidation as well as a range of stimuli to ensure that the main facets of this complex process are uncovered, and are not recognized through the use only of the simple measurement of thiobarbituric-acid reacting material. It is known that the thiobarbituric acid test gives positive reactions with many compounds other than malonaldehyde (see Slater, 1972, 1984); conversely,

Fig. 3. Oxygen uptake by a liver microsomal suspension stimulated by NADPH + ADP + iron; incubation temperature, 25 °C

Trace A, control, microsomes from normal rat liver; trace B, as for A but with 50 μ M- α -tocopherol added (in 10 μ l of ethanol) just before starting the reaction with NADPH; trace C, as for A but with 50 μ M-TROLOX-C (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) added (in 10 μ l of water) just before NADPH; trace D, microsomes isolated from rats given an intraperitoneal injection of α -tocopherol (100 mg/kg body wt., 16 h before killing). The concentrations of α -tocopherol in microsomes used for traces A and D were 2.4 nmol/mg of lipid and 32.3 nmol/mg of lipid respectively. For other details see the Methods section.

some biologically reactive aldehydes such as the 4-hydroxyalkenals that are produced during lipid peroxidation are not detected by the thiobarbituric acid reaction (Esterbauer *et al.*, 1982). For such reasons we feel it important to follow the progress and rates of lipid peroxidation by several independent methods.

A striking illustration of the difference in the rates of lipid peroxidation between normal rat hepatocytes and Novikoff cells is shown in Fig. 1 in relation to the non-enzymic impact of γ -radiation. The reasons for such

a large difference are several: a decreased content of highly unsaturated fatty acids; a decreased content of the *P*-450 haemoprotein known to be active in radical chain initiation (Svingen *et al.*, 1979), and an increased content of lipid antioxidant (Table 4). Of these features we believe the major contribution arises from the increased lipophilic chain-breaking antioxidant in the microsomal membranes. The decreased content of polyunsaturated fatty acids will certainly contribute to the overall decrease in the rate of lipid peroxidation, as discussed by Galleotti *et al.* (1984) and as illustrated in Fig. 2(a), but other liver microsomal preparations can be isolated with similar polyunsaturated fatty acid contents as Novikoff microsomes whilst showing quite significant rates of lipid peroxidation. For example, microsomal suspensions prepared from rabbit liver peroxidize reasonably rapidly compared with rat microsomes (Table 5), especially with respect to γ -radiation; the rabbit microsomes, however, have much decreased contents of $C_{20:4}$ and $C_{22:6}$ (Table 5). Moreover, rats fed on diet enriched in coconut oil have considerably decreased amounts of polyunsaturated fatty acid in liver microsomal preparations (Hammer & Wills, 1978) and yet peroxidize well. In consequence, we feel that the decreased polyunsaturated fatty acid content of Novikoff microsomes is not so important in relation to lipid peroxidation as is the content of antioxidant. In addition, the decreased rate of lipid peroxidation in normal rat liver microsomes following dietary supplementation with α -tocopherol (Fig. 3) is relevant to this point. The decreased NADPH-flavoprotein activity and cytochrome *P*-450 content will also contribute to the overall decrease in lipid peroxidation in Novikoff microsomes, but the fact that almost no peroxidation occurs even after exposure to γ -radiation points to overriding inhibitory features.

We consider that a critically important factor is the ratio of antioxidants to polyunsaturated lipid. This ratio is increased approx. 3-fold in Novikoff microsomes compared with normal microsomes. An illustration of the effect of such a substantial increase in antioxidant:polyunsaturated fatty acid is the greatly increased induction times observed for lipid extracts of Novikoff tumour samples (Fig. 2). It can be deduced from the results in Table 4 that the major lipophilic chain-breaking

Table 5. Comparative values for rabbit and rat liver microsomal suspensions

Values for peroxidative reactions are shown as means $\pm$ S.E.M. for the number of experiments in parentheses; N.D., not detectable. The γ -irradiation was a total of 108 krad over a 60 min period.

Measurement	Rat	Rabbit
NADPH + ADP + iron-stimulated lipid peroxidation		
(a) Malonaldehyde production (nmol/min per mg of protein)	1.65 $\pm$ 0.11 (30)	0.59 $\pm$ 0.06 (12)
(b) Oxygen uptake (nmol/min per mg of protein)	90.6 $\pm$ 7.5 (24)	15.8 $\pm$ 2.3 (9)
Ascorbate + iron-stimulated lipid peroxidation (nmol of malonaldehyde/min per mg of protein)	2.05 $\pm$ 0.14 (3)	1.12 $\pm$ 0.08 (3)
γ -Irradiation-stimulated lipid peroxidation (pmol of malonaldehyde/min per mg of protein)	114 $\pm$ 8 (4)	103 $\pm$ 10 (3)
Polyunsaturated fatty acids (μ g/mg of protein)		
$C_{20:4}$	102 $\pm$ 5 (24)	29 $\pm$ 3 (8)
$C_{22:6}$	24 $\pm$ 2 (24)	N.D.

antioxidant in normal liver and Novikoff tumour samples is α -tocopherol; (α -tocopherol is also the major lipophilic chain-breaking antioxidant in isolated rat hepatocytes; results not shown); these results are thus similar to the conclusions of Burton *et al.* (1983a) on human plasma and erythrocyte membranes. Thus, although β -carotene (see Burton & Ingold, 1984) and ubiquinone (see Landi *et al.*, 1984) are effectively lipophilic free radical scavengers under some conditions, they appear to be insignificant as chain-breaking antioxidants relative to α -tocopherol in the systems studied here. It is noteworthy that Swick & Baumann (1951) drew attention in a perceptive paper to the importance of vitamin E in liver tumours.

Intact liver and whole cells

Essentially the same comments about the rates of peroxidation and antioxidant contents can be made for intact Novikoff cells (in relation to intact liver samples) as made above for microsomal suspensions. Since the rate of lipid peroxidation is also greatly reduced in whole Novikoff cells (Fig. 1) as in Novikoff microsomes this implies that lipid peroxidation is inhibited in the cytosol (and in organelles such as the mitochondria) and the plasma membrane as well as in the endoplasmic reticulum. The content of lipophilic chain-breaking antioxidant (Table 4) is increased in intact Novikoff cells compared with intact liver but this measure excludes water-soluble antioxidant materials (urate, ascorbate, glutathione, etc.) that we have not investigated in this study, although Wirth & Thorgeirsson (1978) have reported a much lower content of GSH in Novikoff cells than in normal liver. The water-soluble components are of major significance in relation to free radicals formed in the aqueous phase, and it is known that there can be important interactions of the hydrophilic antioxidants with α -tocopherol (see Tappel, 1968; Packer *et al.*, 1979; Doba *et al.*, 1985). In plasma, for example, although α -tocopherol is the main lipophilic chain-breaking antioxidant, it accounts for only about 5–10% of the total chain-breaking activity present (Wayner *et al.*, 1985).

Intact Novikoff cells have a decreased proportion of $C_{20:4}$ and $C_{22:6}$ fatty acids, and an increased content of cholesterol relative to total lipid in comparison with normal intact hepatocytes (Table 1). Thus, the major differences that have been referred to above for microsomal suspensions are not masked or compensated for by the constituent contents in other compartments of the cell.

General comments

In the present paper we have described the methodology and experimental approach to the broad programme on liver tumours outlined in the Introduction. We have deliberately chosen an extreme example of a very rapidly growing liver tumour for this introductory study in order to provide an indication of the maximum ranges of deviations likely to be found in other types of liver tumour in the components under study.

As cited in the Introduction there are many reports in the literature that liver tumours have a depressed rate of lipid peroxidation relative to normal whole liver or liver fractions. This change apparently occurs at an early stage in the change from normal to malignant behaviour, since so-called pre-malignant foci have a decreased content of aldehydic products of lipid peroxidation (Benedetti *et al.*,

1984). Moreover, other changes referred to in this paper concerning Novikoff cells, the decreased content of cytochrome *P*-450 and activity of aminopyrene demethylase, have also been reported to occur in pre-malignant liver nodules (Roomi *et al.*, 1985). Thus, the changes described here for the very rapidly growing Novikoff tumour are expressed very early in the complex sequence(s) of malignant transformation. There remains the possibility that the changes reported here are reflections of a proliferating cell system rather than of features directly linked to malignant transformation. We have investigated this possibility by using the normal regenerating rat liver as a model (Slater *et al.*, 1985). Although the regenerating liver shows marked differences in lipid peroxidation and antioxidant content relative to sham-operated rats, these changes are cyclical and in phase with DNA synthesis. At present we favour the view that the changes reported here for Novikoff cells represent a combination of changes associated in varying extents with the expression of malignant transformation and of cell division. A decreased lipid peroxidation appears to be an important feature of the biochemical expression of the Novikoff tumour; this is consistent with a general hypothesis (Slater, 1976; Slater *et al.*, 1984; Morisaki *et al.*, 1984) that an increased rate of cell division is associated with a decreased rate of lipid peroxidation.

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REFERENCES

- Adamson, R. H. & Fouts, J. R. (1961) *Cancer Res.* **21**, 667–673
- Ahmed, S. M. & Slater, T. F. (1981) in *Recent Advances in Lipid Peroxidation and Tissue Injury* (Slater, T. F. & Garner, A., eds.), pp. 177–194
- Albano, E., Lott, K. A. K., Slater, T. F., Stier, A., Symons, M. C. R. & Tomasi, A. (1982) *Biochem. J.* **204**, 593–603
- Bartoli, G. M. & Galeotti, T. (1979) *Biochim. Biophys. Acta* **574**, 537–541
- Benedetti, A., Malvaldi, G., Fulceri, R. & Comporti, M. (1984) *Cancer Res.* **44**, 5712–5717
- Benedetto, C., Dianzani, M. U., Ahmed, M., Cheeseman, K., Connelly, C. & Slater, T. F. (1981) *Biochim. Biophys. Acta* **677**, 363–372
- Beswick, P. H., Cheeseman, K., Poli, G. & Slater, T. F. (1981) in *Recent Advances in Lipid Peroxidation and Tissue Injury* (Slater, T. F. & Garner, A., eds.), pp. 156–176
- Boozer, C. E., Hammond, G. S., Hamilton, C. E. & Sen, J. N. (1955) *J. Am. Chem. Soc.* **77**, 3233–3237
- Burlakova, E. B. (1975) *Russ. Chem. Rev.* **44**, 871–880
- Burlakova, E. B., Molochkina, E. M. & Pal'mina, N. P. (1980) *Adv. Enzyme Regul.* **18**, 163–179
- Burton, G. W. & Ingold, K. U. (1981) *J. Am. Chem. Soc.* **103**, 6472–6477
- Burton, G. W. & Ingold, K. U. (1984) *Science* **224**, 569–573
- Burton, G. W., Joyce, A. & Ingold, K. U. (1983a) *Arch. Biochem. Biophys.* **221**, 281–290
- Burton, G. W., Cheeseman, K. H., Doba, T., Ingold, K. U. & Slater, T. F. (1983b) *Ciba Found. Symp.* **101**, 4–18
- Burton, G. W., Webb, A. & Ingold, K. U. (1985) *Lipids* **20**, 29–39
- Cadenas, E., Ginsburg, M., Rabe, U. & Sies, H. (1984) *Biochem. J.* **223**, 755–759

- Cheeseman, K. H., Lai, M. & Slater, T. F. (1981) *IRCS Med. Sci. Compend.* **9**, 600
- Cheeseman, K. H., Burton, G. W., Ingold, K. U. & Slater, T. F. (1984) *Toxicol. Pathol.* **12**, 235–239
- Christie, W. W. (1973) *Lipid Analysis*, pp. 89–90, Pergamon, Oxford
- Dianzani, M. U., Canuto, R. A., Rossi, M. A., Poli, G., Garcea, R., Biocca, M. E., Cecchini, G., Biasi, F., Ferro, M. & Bassi, A. M. (1984) *Toxicol. Pathol.* **12**, 189–199
- Doba, T., Burton, G. W. & Ingold, K. U. (1985) *Biochim. Biophys. Acta* **835**, 298–303
- Esterbauer, H., Cheeseman, K. H., Dianzani, M. U., Poli, G. & Slater, T. F. (1982) *Biochem. J.* **208**, 129–140
- Folch, J., Lees, M. & Sloane-Stanley, G. H. (1957) *J. Biol. Chem.* **222**, 497–505
- Fonnesu, A., del Monte, U. & Olivotto, G. A. (1966) *Lo Sperimentale* **116**, 353–372
- Garner, A., Jamal, Z. & Slater, T. F. (1986) *Int. J. Radn. Biol.*, in the press
- Galleotti, T., Borello, S., Palombini, G., Masotti, L., Ferrari, M. B., Cavatorta, P., Arcioni, A., Stremmenos, C. & Zannoni, C. (1984) *FEBS Lett.* **169**, 169–173
- Glock, G. E. & McLean, P. (1957) *Biochem. J.* **65**, 413–416
- Gravela, E., Feo, F., Cannuto, R. A., Garcea, R. & Gabriel, L. (1975) *Cancer Res.* **35**, 3041–3047
- Hammer, C. T. & Wills, E. D. (1978) *Biochem. J.* **174**, 585–593
- Landi, L., Cabrini, L., Sechi, A. M. & Pasquali, P. (1984) *Biochem. J.* **222**, 463–466
- Lash, E. D. (1966) *Arch. Biochem. Biophys.* **115**, 332–336
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Mahoney, L. R., Korcek, S., Hoffman, S. & Willermet, P. A. (1978) *Ing. Eng. Chem. Prod. Res. Div.* **17**, 250–255.
- Morisaki, N., Lindsey, J. A., Stitts, J. M., Zhang, H. & Cornwall, D. G. (1984) *Lipids* **19**, 381–394
- Novikoff, A. B. (1957) *Cancer Res.* **17**, 1010–1027
- Oberley, L. W. (1983) in *Oncology Overview: International Cancer Research Data Bank Program*, U.S. Dept. of Health and Human Services, National Cancer Institute, Bethesda
- Packer, J., Slater, T. F. & Willson, R. L. (1979) *Nature (London)* **278**, 737–738
- Player, T. J., Mills, D. J. & Horton, A. A. (1979) *Br. J. Cancer* **39**, 773–778
- Poli, G., Cheeseman, K., Slater, T. F. & Dianzani, M. U. (1981) *Chem.-Biol. Interact.* **37**, 13–24
- Poli, G., Dianzani, M. U., Cheeseman, K. H., Slater, T. F., Lang, J. & Esterbauer, H. (1985) *Biochem. J.* **227**, 629–638
- Reynafarje, B. & Potter, V. R. (1957) *Cancer Res.* **17**, 1112–1119
- Roomi, M. W., Ho, R. K., Sarma, D. S. R. & Farber, E. (1985) *Cancer Res.* **45**, 564–571
- Ross, D. A., Jackson, R. C., Weber, C. & Morris, H. P. (1982) *Cancer Biochem. Biophys.* **6**, 61–64
- Saine, S. E., Fang, W.-F. & Strobel, H. W. (1978) *Biochim. Biophys. Acta* **526**, 345–358
- Sharma, S. C., Schaur, R. J., Tillian, H. M. & Schauenstein, E. (1984) *IRCS Med. Sci.* **12**, 236–237
- Slater, T. F. (1968) *Biochem. J.* **106**, 155–160
- Slater, T. F. (1972) *Free Radical Mechanisms in Tissue Injury*, pp. 1–283, Pion, London
- Slater, T. F. (1976) in *Recent Advances in Biochemical Pathology: Toxic Liver Injury* (Dianzani, M. U., Ugazio, G. & Sena, L. M., eds.), pp. 381–390, Minerva Medica, Turin
- Slater, T. F. (1984) *Biochem. J.* **222**, 1–15
- Slater, T. F. & Sawyer, B. C. (1969) *Biochem. J.* **111**, 317–324
- Slater, T. F. & Sawyer, B. C. (1971) *Biochem. J.* **123**, 805–814
- Slater, T. F., Benedetto, C., Burton, G. W., Cheeseman, K. H., Ingold, K. U. & Nodes, J. T. (1984) in *Icosanoids and Cancer* (Thaler-Dao, H., Crastes de Paulet, A. & Paoletti, R., eds.), p. 21–29, Raven Press, New York
- Slater, T. F., Cheeseman, K. H., Collins, M., Maddix, S., Milia, A., Proudfoot, K., Burton, G. W., Webb, A. & Ingold, K. U. (1985) *Philos. Trans. R. Soc. London Ser. B* **311**, 633–645
- Strobel, H. W., Dignam, J. D., Saine, S. E., Fang, W.-F. & Fennell, P. M. (1978) *Mol. Cell. Biochem.* **22**, 79–91
- Svingen, B. A., Buege, J. A., O'Neal, F. O. & Aust, S. D. (1979) *J. Biol. Chem.* **254**, 5892–5899
- Swick, R. W. & Baumann, C. A. (1951) *Cancer Res.* **11**, 948–953
- Tappel, A. L. (1968) *Geriatrics* **23**, 97–105
- Tisdale, M. J. & Mahmoud, M. B. (1983) *Br. J. Cancer* **47**, 809–812
- Ugazio, G., Gabriel, L. & Burdino, E. (1968) *Boll. Soc. Ital. Sper.* **44**, 30–33
- Utsumi, K., Yamamoto, G. & Inaba, K. (1965) *Biochim. Biophys. Acta* **105**, 368–371
- Utsumi, K., Goto, N., Kanemasa, Y., Oshioka, T. & Oda, T. (1971) *Physiol. Chem. Phys.* **3**, 467–480
- Wayner, D. D. M., Burton, G. W., Ingold, K. U. & Locke, S. (1984) *FEBS Lett.* **187**, 33–37
- Weber, G. (1983) *Cancer Res.* **43**, 3466–3492
- Wirth, P. J. & Thorgeirsson, S. S. (1978) *Cancer Res.* **38**, 2861p–2865p