Br. J. Cancer (1987), **55**, Suppl. VIII, 74–86 © The Macmillan Press Ltd., 1987

Radiation and chemically induced transformation: Free radicals, antioxidants and cancer

C. Borek

Radiological Research Laboratory, Department of Radiation Oncology and the Department of Pathology/Cancer Center, Columbia University, New York, NY 10032, USA.

The carcinogenic potential of X-rays in humans was realized within the first decade after their discovery by Roentgen in 1895 (Brown, 1936). This was confirmed in later years through epidemiological data, the largest single source of information being from Hiroshima and Nagasaki (UNSCEAR, 1977). The data provided good evidence to suggest that various forms of cancer including leukaemia represent the most significant late effect when human populations are exposed to substantial doses of radiation.

While epidemiology and animal studies have yielded much information on radiation induced malignancies they have their limitation in studies concerned with the oncogenic effects of low doses of radiation and in studies addressing mechanisms in the multistep process of radiation carcinogenesis and its modulation (Borek, 1985*a*, *b*).

Cell transformation *in vitro*

Cell cultures offer a powerful tool in carcinogenesis research. In these *in vitro* systems cells are grown under defined conditions free from complex homeostatic mechanisms that prevail *in vivo*. These systems afford us the opportunity to assess at a cellular level the carcinogenic potential of physical and/or chemical agents (Borek & Sachs, 1966; Borek & Hall, 1973; Borek, 1982*a*, 1984*b*).

We expose single cells to the agent(s) and determine the ensuing short-term toxic actions on the cells. These include cell killing, damage to DNA and membranes as well as malignant transformation (Borek, 1982*a*; Borek *et al.*, 1987) (Figure 1).

Having these defined models we can identify modulating factors which enhance transformation rates (co-carcinogens, promoters) or inhibit and at best eliminate the induction and development of malignant transformation (protective systems) (Borek, 1982*a*, *b*; 1984*a*, *b*; 1985*a*).

A most significant contribution, which served as a basis for the study of radiation oncogenesis *in vitro* was the development of the clonal assay by Puck and Marcus (1956) and their demonstration of a dose-related effect of radiation on the survival of single cells. These findings made it possible in later years to assess which surviving cells had been transformed into a neoplastic state following exposure to radiation, and to determine the incidence of transformation.

The direct oncogenic effect of X-rays was first demonstrated in diploid hamster cells (Borek & Sachs, 1966*a*) by exposing hamster embryo cells to 300 rad of X-rays and transforming a fraction of them into cells which differed morphologically from untreated controls (Figure 1). The transformed cells gave rise to tumours upon injection into hamsters, whereas untreated cells showed no spontaneous transformation (Borek & Sachs, 1966*a*, 1967). The work demonstrated that in order to fix radiation transformation as a hereditary property, cell divisions must take place soon after exposure, and that subsequent additional replications are essential for expression of the neoplastic state (Borek & Sachs, 1966*a*, 1967, 1968) (Figure 2). The work also indicated that there exists among cells a differential physio-

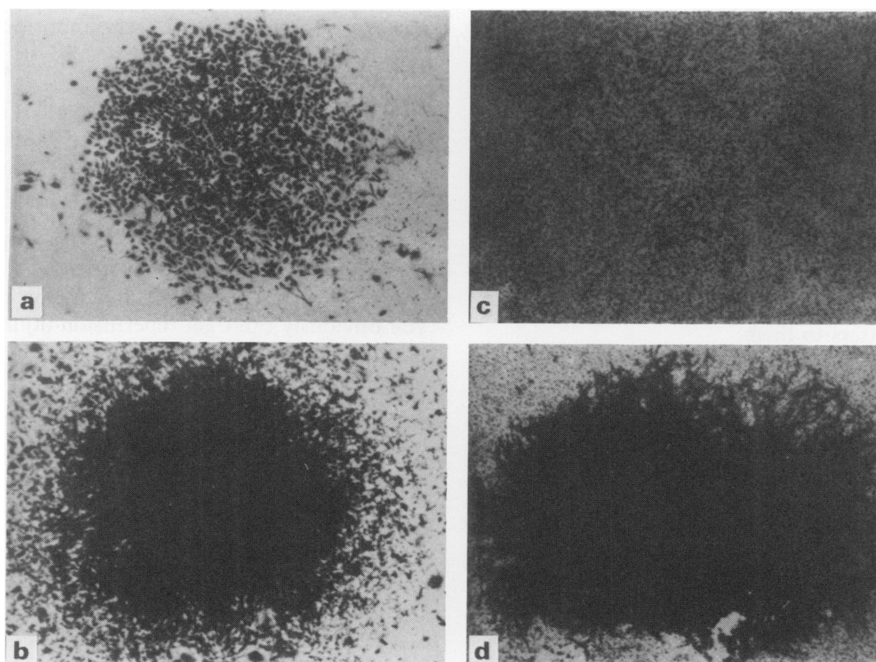

Figure 1 (a) a normal colony of hamster embryo cells; (b) an X-ray transformed colony of hamster embryo cells; (c) mouse C3H/10T-1/2 cells; (d) a focus of C3H/10T-1/2 cells transformed by 400 rad.

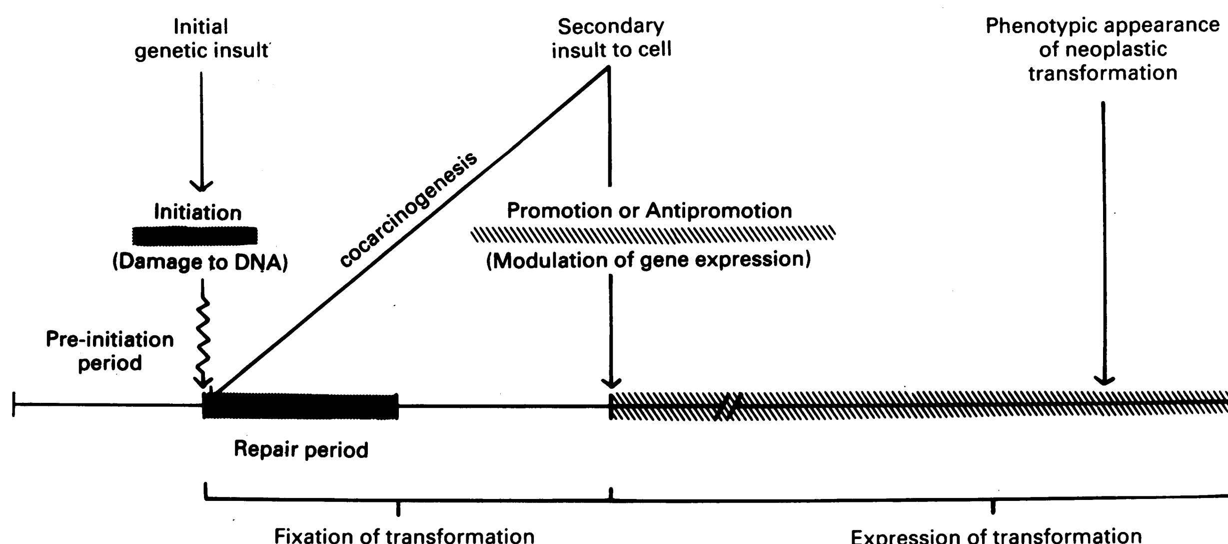

Figure 2 A schematic representation of multistage carcinogenesis *in vitro* and possible modification of the process.

logical and genetic competence to be transformed and that surface-mediated cell recognition was modified in culture upon transformation (Borek & Sachs, 1966*b*).

Transformation of mammalian cells *in vitro* by radiation was shown later in mouse cell systems (Terzaghi & Little, 1976; Miller & Hall, 1978) and in human cells (Borek, 1980; Borek & Andrews, 1983), making it possible to evaluate the effects of radiation on cells across the lines of various species (reviewed in Borek, 1982*a*, 1985*a*, *b*).

Culture systems currently used in radiation transformation studies

There are a limited number of cell systems currently used in radiation transformation studies. These are composed of fibroblast-like cells, where morphological criteria serve well in quantitative assays of transformation. Because in man the preponderance of carcinomas over sarcomas is unequivocal, there is a constant and urgent need to develop epithelial cultures to study transformation. A number of epithelial cell systems have been developed and used in studies on chemically induced transformation (reviewed in Borek, 1982*a*, 1983), but so far not applied to quantitative studies in radiation carcinogenesis. There is a particular uniformity in fibroblast-like cells that does not exist in epithelial cells, whose susceptibility to radiogenic transformation may depend on the particular differentiated qualities and on the source and age of tissue from which they are derived. Human as well as animal data have indicated that in radiation carcinogenesis, latency, age and specific organ susceptibility determine the incidence of cancer. A further difficulty with epithelial cells arises from the fact that criteria for early stages in the neoplastic state of epithelial cells are expressed phenotypically in a less consistent manner than in fibroblasts (Borek, 1982, 1985*a*).

Among the fibroblast lines there are two main cell systems used in radiogenic transformation studies; primary cultures and strains, and established cell lines (Borek, 1982*a*, 1985*a*).

Established cell lines

Established cell lines have an unlimited life span, examples are mouse 3T3 or C3H/10T-1/2 cells (for review see Borek, 1982*a*, 1985*a*). They represent cell populations which originated as primary cultures. Following a continuous and meticulous time regime of subculturing a selected population emerges that had undergone a 'crisis', enabling the cells to grow indefinitely at a constant rate. The karyotype of these cells shows various chromosomal rearrangements and heteroploidy. In contrast to primary cells, immortalized lines represent cells which have already undergone a change. Thus exposure to radiation induces transformation events which are superimposed on a genotype which has undergone an initial change towards transformation.

In normal diploid cultures, one finds subsets of cells which lose the ability to become transformed (Borek & Sachs, 1967). Thus later passages of embryonic cells show a certain resistance to transformation. These may be cell populations which have undergone differentiation (Nakano & Tsò, 1981). The process of differentiation has long been known to modulate *in vivo* cell susceptibility to oncogenic transformation. Thus, a similar situation may hold true *in vitro* where undifferentiated stem cells are the most susceptible to being transformed.

A factor which may serve as an additional modulator of transformation is cell communication (Borek *et al.*, 1969). Normal cells communicate via permeable junctions while a wide variety of transformed cells do not. Normal cells may be communicating with transformed cells and inhibit the proliferation of the transformants (Borek & Sachs, 1966*b*). Thus, in transformation studies it is important to maintain a particular seeding schedule to prevent a possible inhibition of the potentially transformed cells by the normal cells in the dish.

Stages in transformation

Initiation and expression

One can roughly delineate events in the multistage process of transformation as follows:

1. *Initiation of transformation* associated with irreversible damage to DNA and requiring cell replication soon after treatment to fix transformation, as a hereditary property of the cell (Figure 2).

2. *Expression of transformation* requiring additional cell replications to express the transformed phenotype. This expression is initially in the form of altered morphology in cell culture, and a subsequent acquisition of the ability to become anchorage independent and grow in semisolid medium, a characteristic which in cells of solid tissue is associated with a transformed phenotype (Borek & Sachs, 1966*a*; reviewed in Borek, 1982*a*, 1985*a*). In rodent fibroblasts, growth in agar appears at later stages after morphological transformation (Barret & Tso, 1978), though in human cell transformation the loss of anchorage

independence is acquired at the same time as altered morphology (Borek, 1980).

The ultimate expression of transformation and the unequivocal demonstration of the neoplastic state are tumorigenicity in appropriate hosts. One establishes the neoplastic potential of cells by isolating transformed foci or colonies, propagating them, and injecting them into the appropriate hosts; usually 10^5 to 10^6 cells are injected.

Co-carcinogenesis

Since we are constantly exposed to a variety of carcinogens, the oncogenic potential of one agent can be enhanced by other initiators (i.e. agents which are carcinogens in themselves).

The evaluation of co-carcinogenesis which may be additive or synergistic is carried out by exposing cells to an additional carcinogen at the time of initiation, and assessing transformation frequency compared to that observed with the single agent. Examples of co-carcinogens which act with radiation are food products such as tryptophan pyrolysates (Borek & Ong, 1981) acting in additive fashion, and ozone (Borek *et al.*, 1986*b*) which acts synergistically with radiation.

Promotion

In the multistage process of carcinogenesis the course and frequency of the transformation process can be enhanced by inherent agents such as hormones or external agents such as tumour promoters, which in themselves are not carcinogens. One of the tumour promoters that has been used extensively is the phorbol ester derivative TPA (12-0-tetradecanoyl-phorbol-13-acetate) (Hecker, 1971). TPA is a mitogen as well as a producer of free radical (Borek & Troll, 1983). Its action in enhancing transformation may lie in part in its mitogenic activity in enhancing the clonal population of cells, which may be affected by free radical. Teleocidin, derived of algea is a tumour promoter (Sugimura, 1982) with 100 times the activity of TPA in enhancing radiogenic transformation (Borek *et al.*, 1984*a*).

Among the hormones which have been shown to act as critical potentiators in transformation are thyroid hormones (Guernsey *et al.*, 1981; Borek *et al.*, 1983*a*) and oestradiol (Borek, 1980).

DNA transfection

The ability of genomic high molecular DNA purified from *in vitro* transformed cells to transmit the transformed phenotype to normal cells by DNA mediated transfer (Wigler *et al.*, 1979) constitutes an important criterion for the neoplastic state of the cells exposed to the carcinogen (Shilo & Weinberg, 1981; Borek *et al.*, 1984*d*; Borek, 1985*a*; Borek *et al.*, 1987). It indicates that the transformed phenotype of the cells exposed *in vitro* to the carcinogen is encoded in the DNA. This criterion more than all the others aids in the mechanistic studies of transformation by further analyzing the specific transforming genes which are activated as a result of exposure to the carcinogen and the elucidating nature of the genetic changes (Reddy *et al.*, 1982; Borek *et al.*, 1984*b*, 1987) (Figure 3).

Transforming genes in radiation in vitro *transformed cells*

Our earlier findings have long suggested that DNA is a target in radiogenic transformation. Both low LET and high LET radiation induce transformation in a dose related manner (Borek *et al.*, 1978, 1983*b*; Borek & Hall, 1973; Miller & Hall, 1978; reviewed in Borek 1982*a*, 1985*a*). DNA metabolism and replication are essential for fixation of transformation by radiation as a heritary property of the cells in both rodent (Borek & Sachs, 1967, 1968) (Figure 2) and human cells (Borek, 1980).

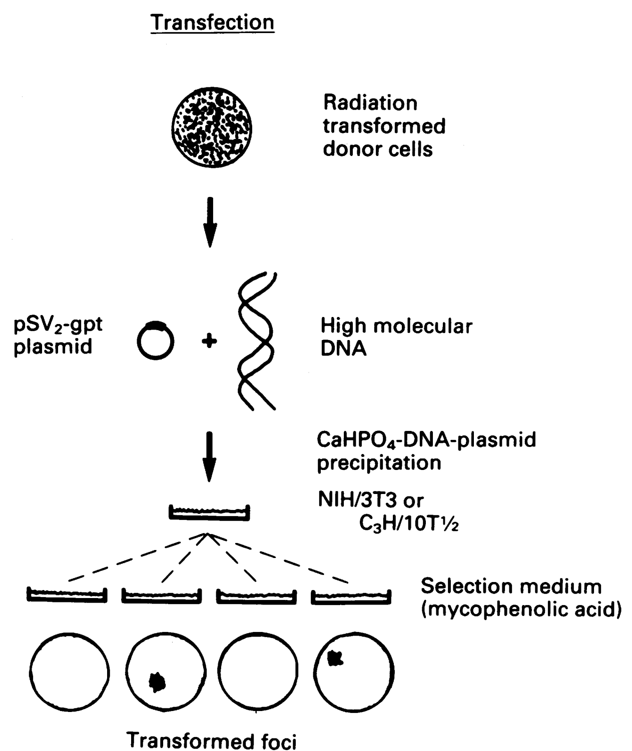

Figure 3 A schematic representation of DNA mediated gene transfer (transfection) in radiation transformation studies (Borek *et al.*, 1987).

Our recent findings using molecular probes indicate that the *in vitro* radiation induced phenotype is encoded in the DNA of the radiation transformed cells (Borek *et al.*, 1987).

In order to establish whether direct exposure of single cells to X-rays results in the activation of transforming genes, we transformed hamster embryo cells or C3H/10T-1/2 cells to 300 rad of X-rays. We found that DNAs from hamster embryo cells and mouse C3H/10T-1/2 cells transformed *in vitro* by X-irradiation into malignant cells, transmit the radiation transformation phenotype by producing transformed foci (transfectants) in three recipient lines, the NIH/3T3 and C3H/10T-1/2 and the rat-2 cells (Table I). DNAs from unirradiated cells or irradiated and visibly untransformed cells do not produce foci (Borek, 1985*b*).

The transfectants grow in agar and form tumours in nude mice ascertaining their neoplastic state. Treatment of the DNAs with restriction endonucleases prior to transfection indicated that the same transforming gene (oncogene) is present in each of the transformed mouse cells and the same in each of the transformed hamster cells. Patterns of sensitivity or resistance to endonuclease inactivation differed from DNAs of mouse and hamster origin suggesting that different oncogenes may be activated in the two species, though at present one cannot rule out an evolutionary divergence of a single locus (Borek *et al.*, 1987).

Southern blot analysis of 3T3 transfectants carrying oncogenes from radiation transformed C3H/10T-1/2 or hamster cells indicated that the oncogenes responsible for the transformation of 3T3 cells are not the K-*ras*, Ha-*ras* or N-*ras* genes nor are they the *neu*, *trk* or *raf* oncogenes. Quick blot analysis using 11 oncogene probes showed no enhanced expression of the *ras* geners in the transfectants but detected elevated transcripts of c-*abl* and c-*fms* in the 3T3 transformants containing oncogenic sequences from radiation transformed C3H/10T-1/2 cells but not in those containing hamster transforming genes (Borek *et al.*, 1987).

Thus, in contrast to the *ras* genes which appeared to be activated in some X-ray induced thymomas (Guerrero *et al.*, 1984) transformation of cells *in vitro* by X-rays results in the

Table I Efficiency of transformation by DNA of *in vitro* X-ray transformed cells

Donor DNA	*In NIH/3T3 Tr.col.* μg^{-1} *DNA*	*In C3H/10T-1/2 Tr.col.* μg^{-1} *DNA*	*In Rat-2 Tr.col.* μg^{-1} *DNA*
	<0.001	ND[a]	ND
C3H/10T-1/2 normal	<0.001	0.001	<0.001
C3H/10T-1/2 irradiated untransformed	<0.001	0.001	<0.001
C3H/10T-1/2 X-ray transformed line C_1	0.15	0.10	0.009
C3H/10T-1/2 X-ray transformed line C_2	0.18	0.13	0.011
C3H/10T-1/2 tumour induced by line C_1	0.12	0.09	0.007
HE (secondary cultures)	<0.001	<0.001	<0.001
HE irradiated, untransformed	<0.001	0.001	<0.001
HE X-ray transformed line H_1	0.23	0.20	0.012
HE X-ray transformed line H_2	0.25	0.17	0.015
HE tumour induced by line H_1	0.15	0.09	0.008

In each transfection 40 μg of DNA were cotransfected with 1 μg pSV$_2$gpt and the cells grown in selection medium containing mycophenolic acid. Transformed foci were scored 21 days later (Borek *et al.*, 1987).
[a]ND – not determined.

activation of genes which have not been described heretofore (Borek *et al.*, 1987).

Permissive and protective factors

While DNA is the target in radiogenic and chemically induced *in vitro* transformation (Shilo & Weinberg, 1981; Borek *et al.*, 1987) and defective DNA repair enhances cell susceptibility to transformation (Borek & Andrews, 1983), a variety of endogenous and exogenous factors play a determining role in modifying the onset and expression of the neoplastic process (reviewed in Borek, 1985*a*) (Figure 4). Physiological factors as well as cell contact may modify the expression of radiogenic transformation in genetically homogenous, cloned populations of cells (Borek & Sachs, 1967). Cell-cell communication between cells as well as between normal and transformed cells is modified and may lead to an inhibition of growth in the transformed cell populations by contact with their normal counterparts (Borek *et al.*, 1969). Serum factors also modify cell-cell communication among transformed cells (Borek *et al.*, 1969) and modulate their 'metastasising' phenotype *in vitro*.

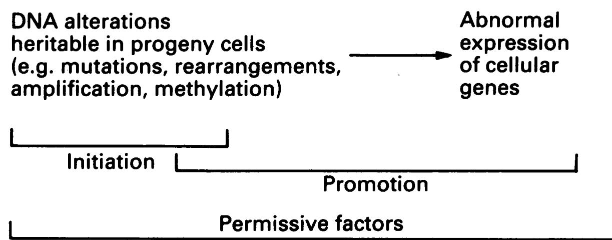

Figure 4 A schematic representation of the requirement of permissive factors (including non-optimal levels of protective factors) in oncogenic transformation.

Hormones as permissive and potentiating factors in transformation

Hormones have long been known to exert an important influence on neoplastic transformation *in vivo*, yet the underlying mechanisms at a cellular level have been hard to define. *In vitro*, where cells are grown and treated under defined conditions free from homeostatic events, we investigated the role of hormones in radiogenic transformation of rodent and human cells (Guernsey *et al.*, 1980; Borek, 1980).

Thyroid hormones Our studies, using hamster embryo cells and C3H/10T-1/2 mouse cells or NRK rat cells, show that thyroid hormones play a critical role in cellular transformation by radiation, chemical carcinogens, and tumour viruses (Guernsey *et al.*, 1980, 1981; Borek *et al.*, 1983*a*, 1985*b*).

Removal of thyroid hormones from the serum did not modify cell growth or survival, but made the cells refractory to transformation by X-rays, benzo(a)pyrene (BP), and N-methyl-N′-nitro-N-nitrosoguanidine (MNNG), and by the Kirsten murine sarcoma virus. When triodothyroine (T3) was added at physiological levels (10^{12} to 10^{10} M), cell transformation was induced in a T3-dose-dependent manner (Guernsey *et al.*, 1981; Borek *et al.*, 1983*a*, 1985*b*) (Figure 5).

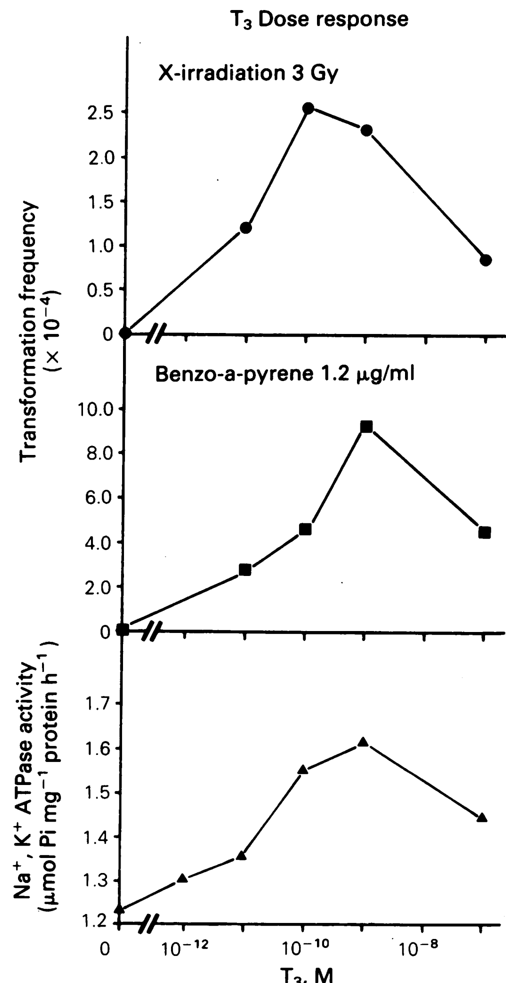

Figure 5 Thyroid hormone dose-dependence of radiation and chemically induced transformation and the enzyme Na/K ATPase (Borek *et al.*, 1983).

The induction of transformation took place within a confined window in time. Maximum transformation was observed when the hormone was added 12 h prior to exposure to the oncogenic agents, and it had no effect on transformation frequencies when added after exposure to radiation, chemicals, or the virus (Guernsey *et al.*, 1981; Borek *et al.*, 1983*a*).

The action of thyroid hormones in transformation appears to be mediated via more than one route. Our studies indicate that T3 may influence gene expression and the synthesis of a specific transformation-associated protein that may play a role in the neoplastic process (Guernsey *et al.*, 1981; Borek *et al.*, 1983*a*). This possibility is supported by the fact that the T3 dose-response relationship for transformation is similar to the T3 dose-dependence for a cellular protein, the enzyme Na/K^+ (Guernsey *et al.*, 1981; Borek *et al.*, 1983*a*) and by recent work using 2 dimensional electrophoresis where the T3 induced modulation of cellular specific proteins is observed (unpublished).

Other recent data indicate that T3 plays a role in cellular transfection by DNA from radiation-transformed cells, indicating that the hormone regulates cellular gene expression in transformation (Table II) in a similar manner to the tumour promoter TPA (Hsiao *et al.*, 1984; Borek *et al.*, 1987).

An additional mechanism for thyroid hormone regulation of transformation resides in the ability of the hormones to modify the pro-oxidant state of the cells. This possibility is suggested by the fact that superoxide dismutase (SOD) suppresses the effects of T3 in potentiating radiogenic transformation as well as inhibiting the promoting action of TPA and teleocidin (Borek *et al.*, 1984*a*), tumour-promoting agents that generate free oxygen species.

Antioxidants as protective factors in transformation

The interaction of cells with radiation, both X-ray and ultraviolet (UV) light, as well as with a variety of chemicals, results in an enhanced generation of free oxygen species and free radical products and in a modified pro-oxidant state (Pryor, 1976). The result is a loss in the optimal cellular balance between the oxidative challenge, a source of DNA damage, and the inherent mechanisms that protect the cell from excess oxidative stress. These include enzymes (SOD, catalase, peroxidases, transferases) and thiols. Also included are a variety of nutrients that directly or indirectly prevent peroxidation and autoxidation of macromolecules: vitamin A, B-carotens, vitamin C, selenium, and vitamin E (Borek *et al.*, 1986*a*; Ames, 1983; Cerutti, 1985).

In recent years, increasing evidence has implicated free radical mechanisms in the initiation and promotion of malignant transformation *in vivo* and *in vitro*. Much of the evidence has come from the fact that the agents that scavenge free radicals directly or that interfere with the generation of free radical-mediated events inhibit the neoplastic process. We have shown in hamster embryo cells that SOD inhibits transformation by radiation and bleomycin and suppresses the promoting action of TPA (Borek & Troll, 1983) (Figure 6). Catalase had no effect as an inhibitory agent in this cell system, perhaps because of the inherent high level of the enzyme in the hamster cells (Borek & Troll, 1983). SOD had a more dramatic inhibitory effect when maintained on the cells throughout the experiment suggesting that later stages in the transformation process are influenced by free radicals (Borek & Troll, 1983).

Bisulfites

Bisulfites serve as food additives and, at low levels, can act as anticarcinogens, inhibiting radiogenic and chemically induced transformation (Borek *et al.*, 1985*a*). Their action is possibly mediated via the inhibition of the free radical process.

Selenium and vitamin E

Other agents which qualify as important antioxidants are various examples of nutrients important in controlling free radical damage *viz.* selenium, a component of glutathione peroxidase, and vitamin E, a powerful antioxidant and a component of the cell membrane (Packer *et al.*, 1979; Niki *et al.*, 1984). We examined the single and combined effects of selenium and vitamin E on cell transformation induced in C3H/10T-1/2 cells by X-rays, benzo(a)pyrene, or tryptophan pyrolysate and on the levels of cellular scavenging systems and peroxide destruction. Incubation of C3H/10T-1/2 cells with 2.5 μM Na_2SeO_3 (selenium) or with 7 μM alpha-tocopherol succinate (vitamin E) 24 h prior to exposure to X-rays or the chemical carcinogens resulted in an inhibition of transformation by each of the antioxidants with an additive-inhibitor action when the two nutrients were combined (Figure 7). Cellular pretreatment with selenium resulted in increased levels of cellular glutathione peroxidase, catalase, and nonprotein thiols (glutathione) (Figure 8) and in an enhanced destruction of peroxide (Figure 9). Cells pretreated with vitamin E did not show these biochemical effects, and the combined pretreatment with vitamin E and selenium did not augment the effect of selenium on these parameters. The results support our earlier studies showing that free radical-mediated events play a role in radiation and chemically induced transformation. They indicate that selenium and vitamin E act alone and in additive fashion as radio-protecting and chemopreventing agents. Selenium confers protection in part by inducing or activating cellular free-radical scavenging systems and by enhancing peroxide break-down. Thus enhancing the capacity of the cell to cope with oxidant stress. Vitamin E appears to confer its protection by an alternate complementary mechanism.

Selenium acts as a true protector. Time course experiments indicate that the addition of selenium at various exposures to X-rays results in a suppression action which diminishes with time (Figure 10).

An important detriment in the efficiency of cellular protection by inherent antioxidants lies in the interaction between various factors. The metabolic function of vitamin E and selenium are interrelated and selenium plays a role in the storage of vitamin E (Borek *et al.*, 1986*a*). Vitamin E action is also closely related to that of vitamin C, which appears to increase its antioxidant effect (Packer *et al.*, 1979; Niki *et al.*, 1984).

Table II Thyroid hormone modulation of cellular transforming genes

	Efficiency of transformation		
Donor DNA	*Foci μg^{-1} DNA* *Recipient*	*Foci μg^{-1} DNA Thyroid-containing serum*	*Foci μg^{-1} DNA Thyroid-depleted serum*
C3H/10T-1/2	NIH/3T3	0.00	0.00
C3H/10T-1/2 X-ray transformed	NIH/3T3	0.14	0.00
Hamster embryo normal	NIH/3T3	0.00	0.00
Hamster embryo X-ray transformed	NIH/3T3	0.15	0.03

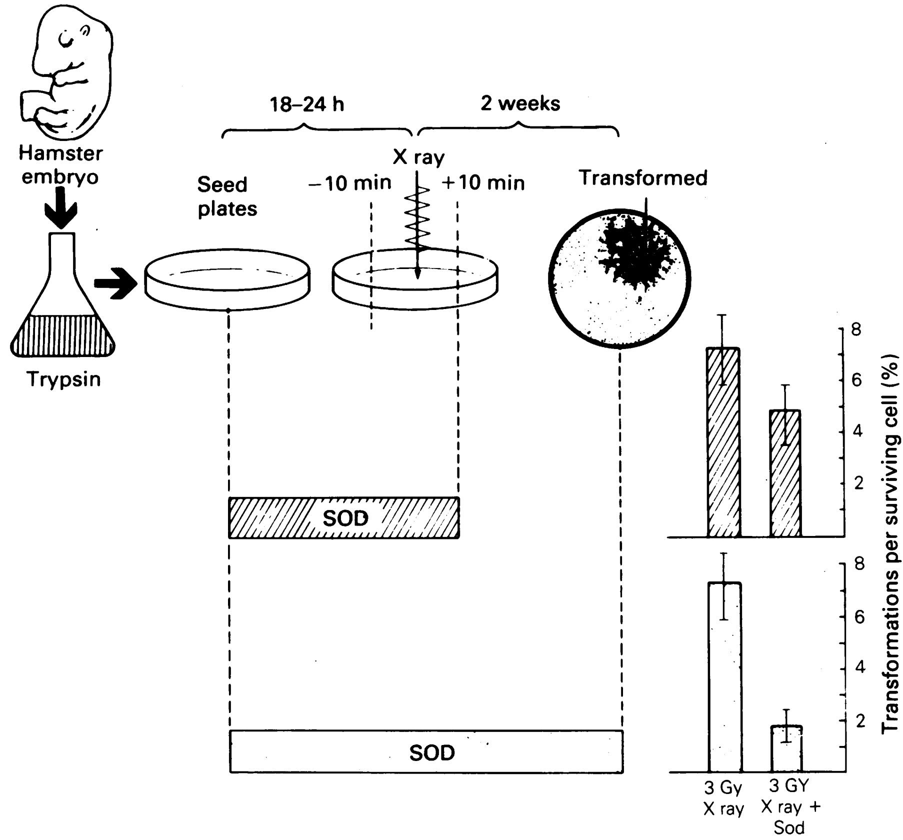

Figure 6 The inhibitory effect of SOD on radiogenic transformation of hamster embryo cells (Borek & Troll, 1983).

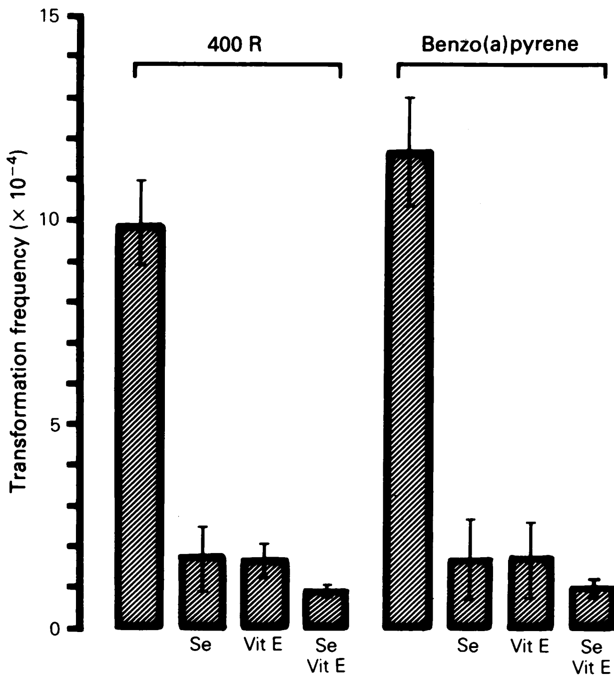

Figure 7 The inhibitory effect of Selenium (Se) and vitamin E on radiation and chemically induced transformation of C3H/10T-1/2 cells (Borek *et al.*, 1986*a*).

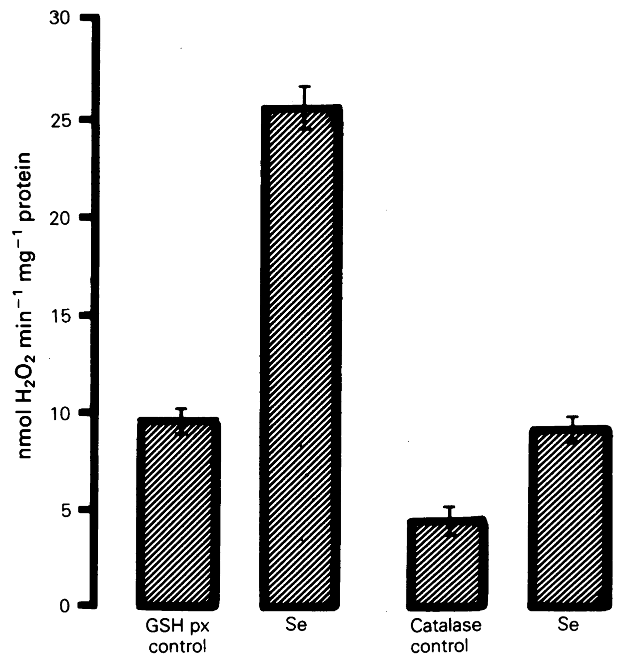

Figure 8 Enhanced levels of glutathione peroxidase (GSH px) and catalase in the presence of Selenium (Se) compared to control (Borek, 1986*a*).

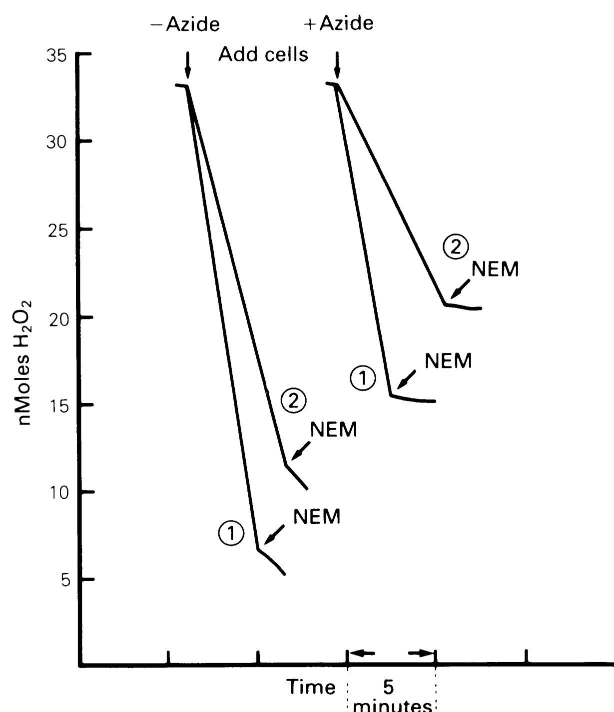

Figure 9 Decomposition of H_2O_2 by C3H/10T-1/2 using an oxidase meter in the presence and absence of Selenium. Selenium treated cells trace 1 and 3 or untreated cells trace 2 and 4 were added to the buffer mixture at 10^6 cells ml^{-1} at times indicated by upper arrows. Azide (an inhibitor of catalase) and N ethyl maleamide (NEM) (an inhibitor of peroxidase) were added to evaluate the single and combined roles of catalase and glutathione peroxidase in peroxide breakdown (Borek *et al.*, 1986*a*).

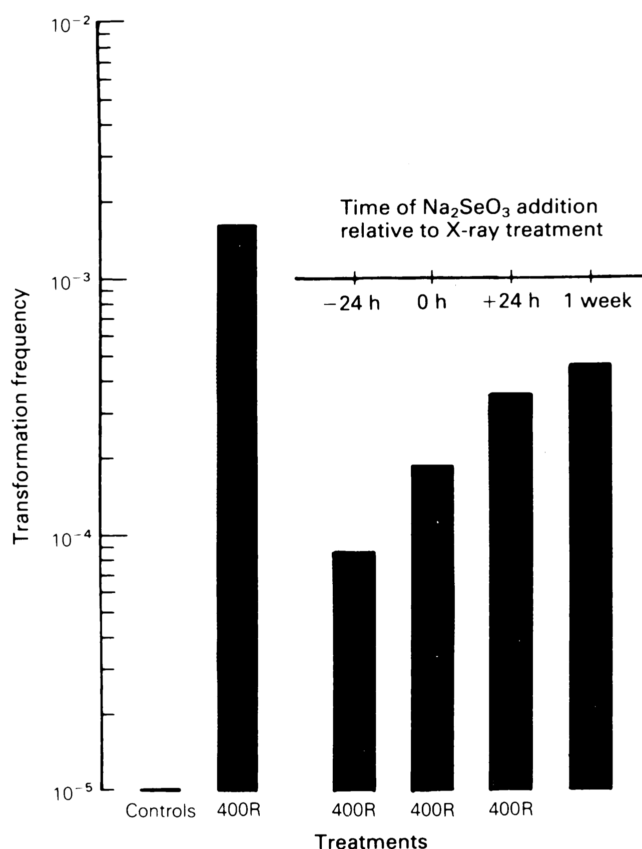

Figure 10 Time course of selenium inhibition of radiogenic transformation *in vitro*.

Different organs have a different content of inherent antioxidants such as selenium and these may vary from one species to another as well as from one individual to another. Thus, tissues and cells will vary in their response to oxidant stress. Adding external antioxidants may be effective in helping some cells mount a protective response while being ineffective in others.

Ozone as a carcinogen

The role of free radicals in the carcinogenic process can be inferred from the protective action of agents which scavenge free radicals at different stages of the oxidative process (Figure 11). However, their role can further be substantiated by the carcinogenic action of ozone, a free radical producing agent (Borek *et al.*, 1986*b*).

Ozone, a reactive species of oxygen, is not a free radical *per se*. However, it interacts with a wide range of molecules to produce free radicals (Pryor, 1983; Borek & Mehlman, 1983). Its toxic action can be counteracted by a variety of antioxidants (Figure 12) thus preventing its direct oxidation of protein or polyunsaturated fatty acids.

We have evaluated whether ozone can directly transform cells *in vitro* and whether ozone as a radiomimetic agent modulates the transforming action of ionizing radiation (Borek *et al.*, 1986*b*).

We found that treatment of hamster embryo and mouse C3H/10T-1/2 cells with 5 ppm O_3 for 5 min resulted in enhancing cell transformation compared to control untreated cells. Also shown in Figure 13 are transformation rates corresponding to a protocol where cells were first irradiated with gamma-rays and then, 2 h later, exposed to O_3. These latter results are statistically consistent with the notion that O_3 and ionizing radiation act synergistically in inducing cell transformation (Borek *et al.*, 1986*b*).

While O_3 as an oxidant may interact with a large number of molecules to produce its effects (Pryor, Borek & Mehlamn, in preparation) one of its major actions resides in its ability to peroxidize polyunsaturated fatty acids and produce malonaldehyde, which reacts with thiols, crosslinks DNA and histones and acts as an initiator in mouse skin carcinogenesis (Borek *et al.*, 1986*b*).

We tested the short term effects of 5 ppm O_3 in producing lipid peroxidation products in the hamster embryo and mouse C3H/10T-1/2 cells compared to air-treated controls. The assays were done in conjunction with the transformation experiments and used cultures parallel to the ones being exposed to O_3 for transformation assays. Lipid peroxidation as measured by the formation of thiobarbituric acid (TBA) reactive products was assayed within 10 min after O_3 exposure (Borek *et al.*, 1986*b*).

The results shown in Table III show that malonaldehyde and malonaldehyde-like products were formed at higher levels in O_3 exposed cells as compared to controls.

The finding that lipid peroxidation products are elevated in response to O_3 suggests a partial role for free radical-mediated reactions in O_3 induced neoplastic transformation. Further support comes from our recent results indicating that the antioxidant vitamin E (alpha tocopherol) inhibits O_3 induced transformation in the hamster and C3H/10T-1/2 cells (Table IV) (Borek *et al.*, submitted).

Conclusions

One of the basic conundrums in carcinogenesis evolves from our inability to unequivocally distinguish primary events

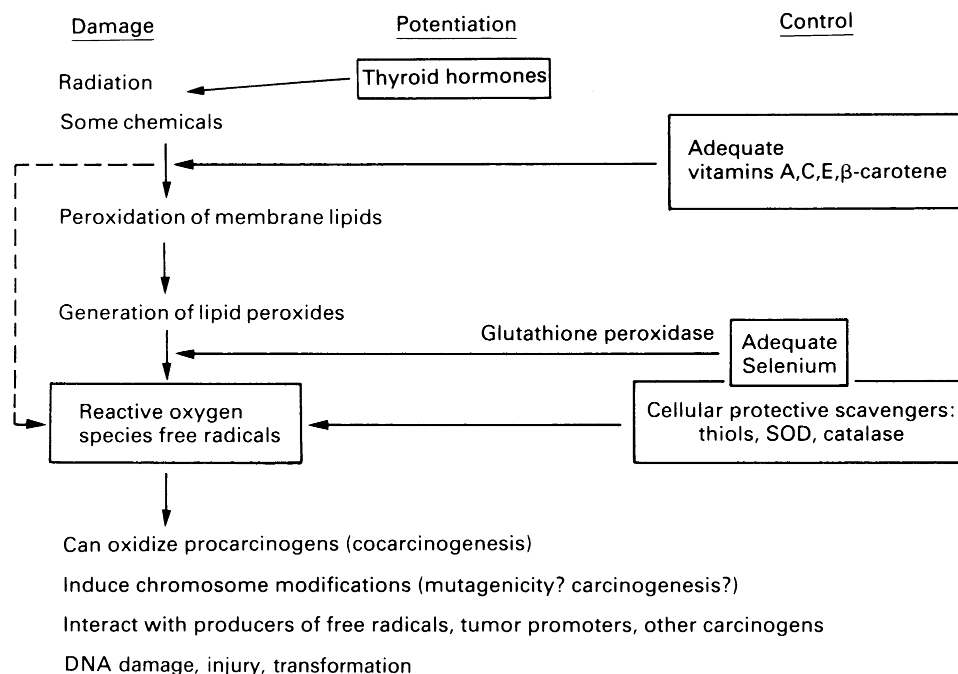

Figure 11 A scheme describing the possible actions of potentiators and protectors in modifying free radical induced toxicity and transformation.

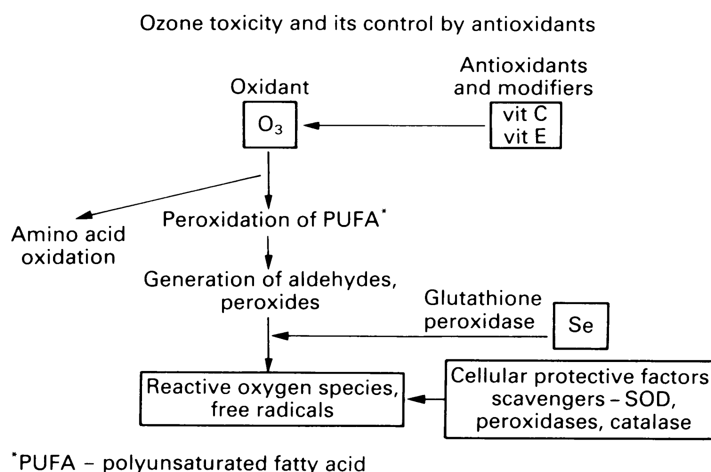

Figure 12 A scheme describing biochemical mechanisms of ozone toxicity and the inhibition of its toxicity by free radical scavengers.

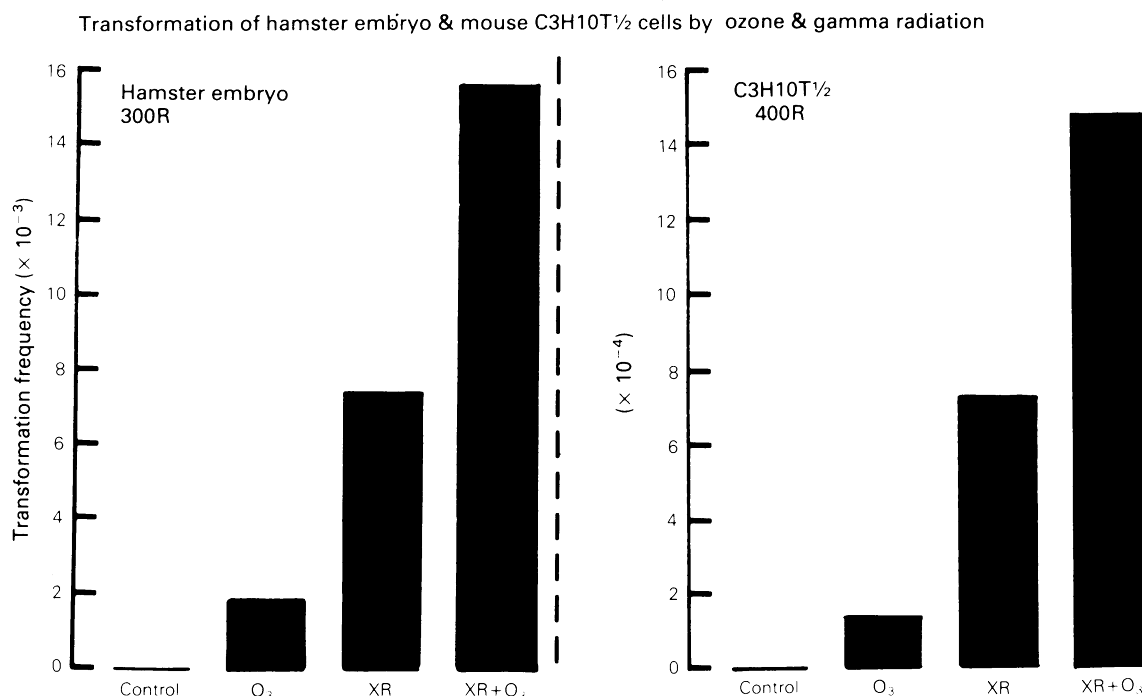

Figure 13 The single and combined action of ozone and radiation in transforming cells *in vitro* (Borek *et al.*, 1986*b*).

Table III TBA$^+$ reactive products in ozone-exposed hamster embryo and C3H/10T-1/2 cells

Experiment	Cell type	O_3 exposure	Absorbance mg^{-1} protein 450 nm	530 nm
A	C3H/10T-1/2	–	0.114	0.019
		+	0.197	0.029
B	Hamster embryo	–	0.219	0.015
		+	0.267	0.035

The TBA method measures malonaldehyde and malonaldehyde-like substances produced in peroxidized tissues. Replicate experiments indicate that the technique is reproducible within 10% (Borek *et al.*, 1986*b*).

Table IV The effect of vitamin E on ozone induced transformation in hamster embryo cells

Treatment	Transformation frequency
Control	0
Ozone (5 ppm/5 min)	1.4×10^{-3}
Vitamin E (7 μm)	0
Vitamin E + ozone	0.4×10^{-3}

associated with initiation of malignant transformation from those which function as secondary events. Thus the rule of oncogenes, mutations, gene rearrangements, amplification and other DNA alterations in transformation is yet unclear. The changes which take place give rise to abnormal expression of cellular genes.

We must always be cognizant of the fact that a variety of factors may modify the neoplastic process at its various stages of development. These constitute physiological permissive or protective factors. When permissive factors prevail, such as genetic susceptibility, optimal stage in the cell cycle, optimal hormonal control or a particular stage in differentiation, initiation of transformation will take place. By contrast if these permissive factors do not prevail, protective factors such as free radical scavengers will inhibit to varying degrees, the onset and progression of the neoplastic process. These may be inherent cellular factors or those added externally by dietary means acting as anti-carcinogens. Thus, the interplay between inherent genetic and physiological factors and lifestyle influences which either enhance or inhibit the neoplastic process, are critical determinants in the process of multistage carcinogenesis and in establishing the incidence of cancer.

This work was supported by Grant No. CA-12536 from the National Cancer Institute, and by a contract from the National Foundation for Cancer Research.

References

AMES.(1983).

BARRETT, J.C. & TS'O, P.O.P. (1978). Evidence for the progressive nature of neoplastic transformation *in vitro*. *Proc. Natl Acad. Sci. USA*, **71**, 3761.

BOREK, C. (1980). X-ray induced *in vitro* neoplastic transformation of human diploid cells. *Nature*, **183**, 776.

BOREK, C. (1982*a*). Radiation oncogenesis in cell culture. *Adv. Cancer Res.*, **37**, 159.

BOREK, C. (1982*b*). Vitamins and micronutrients modify carcinogenesis and tumor promotion *in vitro*. In *Molecular Interrelations of Nutrition and Cancer*, Arnott, M.S. *et al.* (eds) p. 337. Raven Press: New York.

BOREK, C. (1983). Epithelial *in vitro* cell systems in carcinogenesis studies. *Ann. N.Y. Acad. Sci.*, **407**, 284.

BOREK, C. (1984*a*). Permissive and protective factors in malignant transformation of cells in culture. In *The Biochemical Basis of Chemical Carcinogenesis*, Greim, H. *et al.* (eds) p. 175. Raven Press: New York.

BOREK, C. (1984*b*). *In vitro* cell cultures as tools in the study of free radicals and free radical modifiers in carcinogenesis. In *Methods in Enzymology, Volume on Oxygen Radicals in Biological Systems*, Colowick, C.P. *et al.* (eds) p. 465. Academic Press: New York.

BOREK, C. (1985*a*). The induction and control of radiogenic transformation *in vitro*: Cellular and molecular mechanisms. *Pharmac. Ther.*, **27**, 99.

BOREK, C. (1985*b*). Oncogenes and cellular controls in radiogenic transformation of rodent and human cells. In *The Role of Chemicals and Radiation in the Etiology of Cancer*, Huberman, E. (ed) p. 303. Raven Press: New York.

BOREK, C. & ANDREWS, A. (1983). Oncogenic transformation of normal, XP and Bloom syndrome cells by X-rays and ultraviolet irradiation. In *Human Carcinogenesis*, Harris, C.C. & Antrup, H. (eds) p. 519. Academic Press: New York.

BOREK, C., CLEAVER, J.E. & FUJIKI, H. (1984*a*). Critical biochemical and regulatory events in malignant transformation and promotion *in vitro*. In *Cellular Interaction by Environmental Tumor Promoters and Relevance to Human Cancer*, Fujiki, H. *et al.* (eds) p. 195. Japan Scientific Societies: Tokyo.

BOREK, C., GUERNSEY, D.L., ONG, A. & EDELMAN, I.S. (1983*a*). Critical role played by thyroid hormone in induction of neoplastic transformation by chemical carcinogens in tissue culture. *Proc. Natl Acad. Sci. USA*, **80**, 5749.

BOREK, C. & HALL, E.J. (1973). Transformation of mammalian cells *in vitro* by low doses of X-rays. *Nature*, **243**, 450.

BOREK, C., HALL, E.J. & ROSSI, H.H. (1978). Malignant transformation in cultured hamster embryo cells produced by X-rays, 430 keV monoenergetic neutrons, and heavy ions. *Cancer Res.*, **38**, 2997.

BOREK, C., HALL, E.J. & ZAIDER, M. (1983*b*). X-rays may be twice as potent as gamma-rays for malignant transformation at low doses. *Nature*, **301**, 156.

BOREK, C., HIGASHINO, S. & LOEWENSTEIN, W.R. (1969). Intercellular communication and tissue growth. IV. Conductance of membrane junctions of normal and cancerous cells in culture. *J. Memb. Biol.*, **1**, 274.

BOREK, C. & MEHLMAN, M.A. (1983). Evaluation of health effects, toxicity and biochemical mechanism of ozone. In *Biochemical Effects of Ozone and Related Photochemical Oxidants*, Lee, M. *et al.* (eds) p. 325. Senate Press: Princeton, New Jersey.

BOREK, C. & ONG, A. (1981). The interaction of ionizing radiation and food pyrolysis products in producing oncogenic transformation *in vitro*. *Cancer Lett.*, **12**, 61.

BOREK, C., ONG, A., BRESSER, J. & GILLESPIE, D. (1984*b*). Transforming activity of DNA of radiation transformed mouse cells and identification of activated oncogenes in the donor cells. *Proc. Am. Assn. Cancer Res.*, **25**, 100.

BOREK, C., ONG, A. & MASON, H. (1985*a*). Sodium bisulfite protects against radiogenic and chemically induced transformation in hamster embryo and mouse C3H/10T-1/2 cells. *Toxicol. Indus. Hlth. J.*, **1**, 69.

BOREK, C., ONG, A. & MASON, H. (1987). Distinctive transforming genes in X-ray transformed mammalian cells. *Proc. Natl Acad. Sci. USA*, **84**, 794.

BOREK, C., ONG, A., MASON, H., DONAHUE, L. & BIAGLOW, J.E. (1986*a*). Selenium and vitamin E inhibit radiogenic and chemically induced transformation *in vitro* via different mechanisms of action. *Proc. Natl Acad. Sci. USA*, **83**, 1490.

BOREK, C., ONG, A. & RHIM, J.S. (1985*b*). Thyroid hormone modulates transformation induced by Kirsten murine sarcoma virus. *Cancer Res.*, **45**: 1702.

BOREK, C. & SACHS, L. (1966*a*). *In vitro* cell transformation by X-irradiation. *Nature*, **210**, 276.

BOREK, C. & SACHS, L. (1966*b*). The difference in contact of cell replication between normal cells and cells transformed by different carcinogens. *Proc. Natl Acad. Sci. USA*, **56**, 1705.

BOREK, C. & SACHS, L. (1967). Cell susceptibility to transformation by X-irradiation and fixation of the transformed state. *Proc. Natl Acad. Sci. USA*, **57**, 1522.

BOREK, C. & SACHS, L. (1968). The number of cell generations required to fix the transformed state in X-ray induced transformation. *Proc. Natl Acad. Sci. USA*, **59,** 83.

BOREK, C. & TROLL, W. (1983). Modifiers of free radicals inhibit *in vitro* the oncogenic actions of X-rays, bleomycin, and the tumor promoter 12-0-tetradecanoylphorbol 13-acetate. *Proc. Natl Acad. Sci USA*, **80,** 5749.

BOREK, C., ZAIDER, M., ONG, A., MASON, H. & WITZ, G. (1986*b*). Ozone acts alone and synergistically with ionizing radiation to induce *in vitro* neoplastic transformation. *Carcinogenesis*, **7,** 1611.

BROWN, P. (1936). *American Martyrs of Science Through Roentgen Ray*. Thomas Springfield: Illinois.

CERUTTI, P.A. (1985). Prooxidant states and tumor promotion. *Science*, **227,** 375.

GUERNSEY, D.L., BOREK, C. & EDELMAN, I.S. (1981). Crucial role of thyroid hormone in X-ray induced transformation in cell culture. *Proc. Natl Acad. Sci. USA*, **78,** 5708.

GUERNSEY, D.L., ONG, A. & BOREK, C. (1980). Modulation of X-ray induced neoplastic transformation *in vitro* by thyroid hormone. *Nature*, **288,** 591.

GUERRERO, I., CALZADA, P., MAYER, A. & PELLICER, A. (1984). A molecular approach to leukomogenesis. Mouse lymphoma contained an activated c-*ras* oncogene. *Proc. Natl Acad. Sci. USA*, **81,** 202.

HECKER, E. (1971). Isolation and characterization of the cocarcinogenic principles from croton oil. In *Methods in Cancer Research*, Busch, H. (ed) **6,** p. 439. Academic Press: New York.

HSIAO, W.L.W., GATTON-CELLI, S. & WEINSTEIN, I.B. (1984). A tumor promoter enhances oncogene induced transformation of C3H/10T-1/2 cells. *Science*, **226,** 552.

MILLER, R.C. & HALL, E.J. (1978). X-ray dose fractionation and oncogenic transformations in cultured mouse embryo cells. *Nature*, **272,** 58.

NAKANO, S. & TS'O, P.O.P. (1981). Cellular differentiation and neoplasia: Characterization of subpopulations of cells that have neoplasia-related growth properties in Syrian hamster embryo cell cultures. *Proc. Natl Acad. Sci. USA*, **78,** 4995.

NIKI, E., SAITO, T., KAWAKAMI, A. & KAMIYA, Y. (1984). Inhibition of oxidation of methyl linoleate in solution by vitamin E and vitamin C. *J. Biol. Chem.*, **259,** 4177.

PACKER, J.E., SLATER, T.F. & WILLSON, R.L. (1979). Direct observation of a free radical interaction between vitamin E and vitamin C. *Nature*, **278,** 737.

PRYOR, W.A. (1976). The role of free radical reactions in biological systems. In *Free Radicals in Biology*, Pryor, W.A. (ed) **1,** p. 1. Academic Press: New York.

PRYOR, W.A., DOOLEY, M.M. & CHURCH, D.F. (1983). Mechanisms for the reaction of ozone: Its biological molecules, the source of the toxic effects of azone. In *The Biomedical Effects of Ozone and Related Photochemical Oxidants*, Lee, S.D. *et al.* (eds) p. 7. Princeton Scientific Publishers: Princeton, New Jersey.

PUCK, T.T. & MARCUS, P.I. (1956). Action of X-rays on mammalian cells. *J. Exp. Med.*, **103,** 653.

REDDY, E.P., REYNOLDS, R.K., SANTOS, E. & BARBACID, M. (1982). A point mutation is responsible for the acquisition of transforming properties by the T24 human bladder carcinoma oncogene. *Nature*, **300,** 149.

SHAMBERGER, R.J., ANDREONE, T.L. & WILLIS, C.E. (1974). Antioxidants and cancer. IV. Initiating activity of malonaldehyde as a carcinogen. *J. Natl Cancer Inst.*, **53,** 1771.

SHILO, B.A. & WEINBERG, R.A. (1981). Unique transforming gene in carcinogen-transformed mouse cells. *Nature*, **289,** 607.

SUGIMURA, T. (1982). Tumor initiators and promoters associated with ordinary foods. In *Molecular Interrelations Nutrition and Cancer*, Arnott, M.S. *et al.* (eds) p. 3. Raven Press: New York.

TERZAGHI, M. & LITTLE, J.B. (1976). X-radiation-induced transformation in a C3H mouse bryo-derived cell line. *Cancer Res.*, **36,** 1367.

UNSCEAR (United Nations Scientific Committee on the Effects of Atomic Radiation). (1977). Report to the General Assembly on Sources and Effects of Ionizing Radiations. United Nations: New York.

WIGLER, M., PELLICER, A., SILVERSTEIN, S. & AXEL, R. (1979). Biochemical transfer of single copy enkaryotic genes using total DNA as donor. *Cell*, **14,** 725.

Discussion

Cramp: How do you isolate small bits of DNA?

Borek: You can chop the DNA with restriction enzymes.

Cramp: You can't chop it up before you add it to the cells?

Borek: Yes, you can.

Cramp: And is it still transfecting?

Borek: Yes, when we use the calcium phosphate technique.

Cramp: The small bits of DNA?

Borek: Yes, if they contain the transforming gene. You can take an X-ray transformed colony, you can isolate the DNA, high molecular weight DNA, and transfect normal cells. You can see the effect of transformation and can then ask the question is the X-ray transformed phenotype conferred upon the normal cell when adding only discreet portions of the genome.

Cramp: But how do you get the little bits back into the cells?

Borek: Using the calcium phosphate method: it's like just taking a big block of wood splitting it up.

Cramp: What do you think are the post irradiation intermediates that react with the SOD sometime after irradiation?

Borek: There are so many possibilities: we don't know.

Alper: The results you show us are they always in terms of transformants per survival cell?

Borek: Yes.

Alper: How do you sort out the effect of all these things on the killing of the cells. How do you sort out the effects of transformation against cell killing?

Borek: We always do survival curves. I didn't bring them along because you have seen a lot of survival curves. Lets start with the various modifiers. The promoters do not modify survival. If we look at hormones, for example the thyroid hormone, to see if it modifies survival, or modifies cell growth – it does not. Therefore any result with this agent is directly related to its ability to modify transformation. Regarding other agents like SOD and selenium and vitamin E, they do not modify survival very much at the doses used. It is the transformation that seems to be affected.

Wallace: I have a technical question to ask about SOD. What happens to the SOD? How stable is it when you add it at 37°? Does it just stay in the extra cellular fluids?

Borek: It must. It doesn't go into the cell unless it is by endocytosis.

Wallace: How does it affect transformation?