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Cell culture as a test system for toxicity

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Appropriate cell culture systems provide a useful additional method of screening for toxicity, in spite of the obvious problems of relating *in vitro* effects of test compounds at the cellular level to their effects in the whole animal. It is shown that a wide range of chemically disimilar molecules have a reversible inhibitory activity on the growth of primary cultures of monkey kidney cells. The potency of these compounds correlates with their lipid solubility, suggesting that the cell membranes may be their main site of action. Support for this is obtained by the correlation of inhibitory activity and the ability of the same compounds to stabilize the erythrocyte membrane against hypotonic haemolysis which is known to be a direct effect of interaction with the erythrocyte membrane. It is suggested that the ability of the food additive butylated hydroxytoluene to act as a potent inhibitor of cellular growth may account for its reported ability to prolong the life span of mice.

Toxic effects with a low level of incidence, or associated with long term dosage are often established only after extensive usage. They are not readily detected by the conventional *in vivo* studies and it is recognized (e.g. WHO Report, 1967) that additional methods of assessing toxicity are required.

This paper suggests that *in vitro* toxicity studies of freshly isolated cells in culture can provide a valuable source of additional information. Potential toxicity may be expressed as any significant change in the growth and function of treated cells compared with untreated controls. It is possible to establish the dose-response, time course, and reversibility of any significant changes resulting from treatment of primary cells in culture, and studies of this kind on cell monolayers (Milner, 1967) and liver explants (Kirkby, 1964) have been reported. The advantage of these *in vitro* systems is that they are amenable to wide a range of microscopic and biochemical studies in a short time.

Not every significant change in treated cells need be toxic in the whole animal, but ideally the onus of proof should be to show that changes at the cellular level in culture do not result in toxicity *in vivo*. To illustrate the use of cell culture in this context the effects of a range of lipid-soluble molecules on primary cultures of Rhesus monkey kidney (MK) epithelial cells have been examined. In particular the relation between the metabolic inhibition produced in this *in vitro* system by the food additive butylated hydroxytoluene (BHT), and the *in vivo* effects of the same compound reported recently by Harman (1969) is considered.

METHODS

The experimental methods for culturing this system have been reported elsewhere (Milner, 1967). Briefly, monolayer cultures of MK cells were subcultured and grown in Eagles 199 tissue culture medium containing 10% calf serum and antibiotics. After 24 h growth the cells were exposed to the required concentration of test compound dissolved in growth medium containing 1·0% dimethyl suphoxide (DMSO) or 1·0%

dimethyl formamide (DMF). Some control cultures included 1·0% of either solvent, and others were maintained in growth medium alone. In addition to using solvents, BHT was also dissolved in growth media directly. A solution of the antioxidant was evaporated to dryness on a rotor evaporator to leave a thin film in a round-bottomed flask. This film was then taken up into solution by swirling growth medium in the flask (solubility was enhanced by the presence of calf serum). Cell growth was measured both by mitotic indices and by cell numbers using the method of Eagle & Foley (1958). The rate of synthesis of DNA, RNA and protein was followed by the rate of uptake of radio-labelled precursors.

The effect of test compounds on the haemolysis of erythrocytes was followed by the method of Seeman & Weinstein (1966). Where the compound was first dissolved in DMSO, an equivalent concentration of DMSO was also added to the control erythrocyte suspensions.

RESULTS

Solvent effects

Where drugs and additives are insoluble in water a suitable solvent is required that must not in itself affect cell growth nor alter the properties of the solute. Low doses of DMSO tested *in vivo* and *in vitro* have little or no biological effect (e.g. Brown, 1963; Kligman, 1965; Worthley & Schott, 1966; Hollman, Farrelly & Martin, 1967; and *Ann. N.Y. Acad. Sci.*, 1967)., whereas ethanol was discarded as a possible solvent when it was found that low concentrations (<0·1%) caused an increase in cell growth rate over control cultures. Diamond (1965) found that a large variety of cell cultures grown in the presence of 1·0% DMSO did not differ in growth rate from control cells, and the present results with MK cells are compatible with this. Diamond also showed that DMSO did not alter the molecular organization of hydrocarbons and Dixon, Adamson & others (1965) found no difference between *in vivo* effects of various water soluble drugs dissolved in DMSO or in saline. The possibility that the oxidizing activity of DMSO (Muset & Martin-Esteve, 1965) might affect the antioxidant action of BHT is improbable since BHT was equally potent as an inhibitor of cell growth when dissolved in either DMSO or the non-oxidizing solvent DMF. Finally, the inhibitory effect of BHT added in DMSO solution was indistinguishable from its activity when prepared as a solution directly from a thin film of BHT.

Drug effects

At concentrations up to 30 mg BHT/g cells there were no visible cytopathological effects in the MK cells examined by phase contrast microscopy. However this dose was found to inhibit the rate of MK cell growth as indicated by mitotic indices and cell counts and the inhibition was dose-dependent (Fig. 1). Within 30 min of exposure to BHT there was a decrease in DNA, RNA and protein synthesis, with a similar time course for the decrease of each component (Fig. 2), suggesting that the inhibition of growth by BHT is non-specific in this repect. The inhibition is fully reversible within 1 h and the inhibition and reversal can be repeated several times on the same cells.

A comparison of the effects of analogues of BHT at the same test concentration suggested that the degree of inhibition might be related to the lipid solubility of the analogues, since the more lipid-soluble analogues caused a greater degree of inhibition

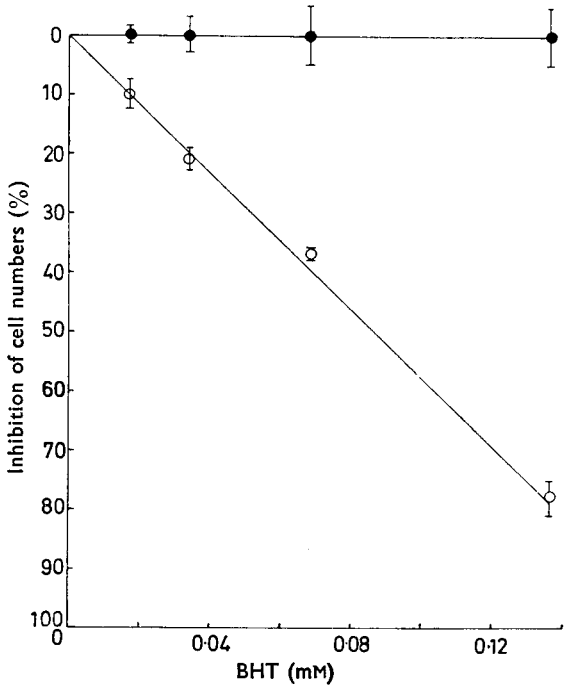


FIG. 1. ○ The dose-dependent inhibition of MK cell growth caused by BHT dissolved in growth medium containing 1·0% DMSO. The percentage inhibition is calculated by the method of Eagle & Foley (1958). ● The growth of cells exposed to 1·0% DMSO alone did not differ from control cultures.

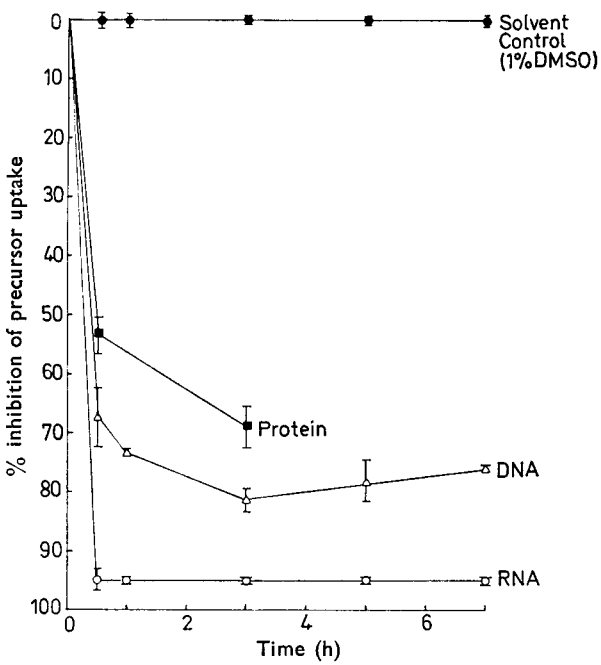
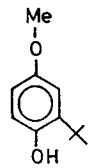
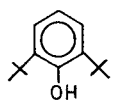
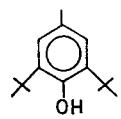
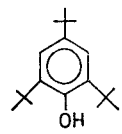


FIG. 2. The inhibition of radiolabelled precursor incorporated into cellular components in the presence of 0·136 mM BHT. ■ [^{14}C]Leucine incorporation into protein; △ [^{3}H]thymidine incorporation into DNA; ○ [^{3}H]uridine incorporation into RNA. ● Cells grown in the presence of 1·0% DMSO had unaltered rates of protein, DNA and RNA synthesis compared with controls.

Table 1. *The relation between the percentage inhibition of MK cell RNA synthesis and the lipid-solubilities of a range of BHT analogues.* The test concentration of each drug was 0·136 mM and inhibition was measured after 3 h. The lipid solubility (π) is expressed as $\log_{10}$ of the partition coefficient relative to phenol (Gilbert & others, 1967).

	 I	 II	 III	 IV
% Inhibition of RNA synthesis	0	35	58	70
Lipid solubility π	1·88	3·66	3·98	5·00

I 2-t-butyl-4-methoxyphenol.
II 2,6-di-t-butylphenol.
III 2,6-di-t-butyl-4-methylphenol.
IV 2,4,6-tri-t-butylphenol.

(Table 1). In fact it has been possible to show that a wide range of chemically dissimilar structures produce the same type of metabolic inhibition as BHT at concentrations related to their lipid solubilities (Metcalfe & Metcalfe, 1971).

Taken together these results support the conclusion that the inhibitory effect is caused by a non-specific mechanism which depends primarily on the concentration of the perturbing agent within a hydrophobic environment, and hence on the lipid solubility of the agent. An obvious candidate for the site of localization is the cell membranes, and I have taken as a working hypothesis that cellular metabolism is depressed by structural perturbation of the cell membranes in the presence of extraneous molecules. Perturbation may then result in decreased permeability of the membrane to essential metabolites. It is difficult to rigorously exclude specific sites of interaction as the effective inhibitory mechanism, but this is extremely unlikely in view of the diverse range of chemical structures producing the effect. The data are analogous to the relation of anaesthetic potency to oil/water partition coefficients demonstrated for anaesthetics by Overton and Meyer. More recently Seeman (1966) has produced evidence which strongly supports this relation in a model system. He has demonstrated that the concentration of agents required to stabilize the erythrocyte membrane against hypotonic haemolysis is linearly related to their anaesthetic potency over a concentration range of a million fold. This led me to make an analogous study of the relation of metabolic inhibition in MK cells to erythrocyte stabilization. For the limited number of compounds tested I find that there is a linear relation between MK cell inhibition and erythrocyte stabilization (Fig. 3). The strong inference is that metabolic inhibition is also determined to a good approximation by the concentration of the agent in the membrane and is not greatly dependent on the chemical nature.

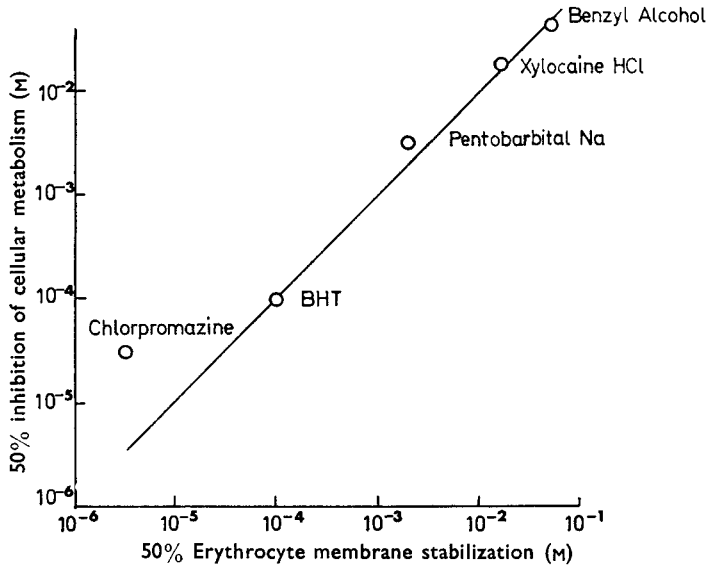


FIG. 3. The relation between metabolic inhibition of MK cells and the stabilization of the erythrocyte membrane against hypotonic haemolysis by a range of chemically dissimilar drugs. The concentration of drug required to produce 50% inhibition of RNA synthesis in MK cells is taken as the inhibitory activity, and is plotted against the concentration of the same drug required to produce 50% stabilization of the erythrocyte membrane (Seeman, 1966).

DISCUSSION

The mechanism of metabolic inhibition caused by lipid-soluble agents is discussed in detail elsewhere (Metcalfe & Metcalfe, 1971). Here, the usefulness of cell culture systems as a screening test for toxicity is considered. The main conclusion is that it is possible in cell culture systems to characterize effects of chemical agents that are not easily detected or analysed *in vivo*. By simple biochemical techniques the type of interaction involved is defined by the absence of chemical specificity, and the cell membrane can be implicated as the site of action. The obvious limitation of the technique is that there is no immediate reason to suppose that the inhibitory effects described for the cell culture system imply toxicity in the whole animal. This is to be contrasted with those specific interactions of drugs with particular subcellular components which may lead to well-defined toxic effects both *in vivo* and *in vitro* (e.g. aflatoxin, heavy metals, fluoroacetate etc.). In practical terms, however, these cases are clear-cut and cell culture experiments simply serve to confirm and define more precisely the causes of acute toxicity.

For this reason it is worth considering the relation of the cell inhibitory effects of BHT to the various *in vivo* studies of the food additive. Extensive evidence supports the safety of BHT at appropriate dose levels (Gilbert & Goldberg, 1965; Ladomery, Ryan & Wright, 1967; Frawley, Kay & Calandra, 1965; Daniel, Gage & Jones, 1968; Clegg, 1965). A report by Johnson (1965) indicated that a dietary level of 0·5% BHT (w/w) reduced the mean weight of offspring of mice, but disputes earlier work by Brown, Johnson & O'Halloran (1959) which suggested that BHT was teratogenic in mice.

It is at least plausible that long term changes in cellular metabolic rates could lead to significant changes in overall physiological function; the changes of course need not be

adverse. In this context it is of interest that Harman(1969) reported that the life span of mice was increased by 44% by the addition of BHT to their diet. It was suggested that the antioxidant properties of BHT might be implicated in the prevention of "cellular deterioration" associated with aging. The dose required per day to produce this effect was 0·5% of the weight of the animal. This is very approximately the equivalent of a dose of 20 mg BHT/g cells in the cell culture experiment. In other words the dose required for metabolic inhibition *in vitro* is of the same order of magnitude as the dose required to produce the *in vivo* effect. The immediate question, which cannot be answered directly from the data available, is whether the two effects are in any way causally related. The cell culture experiments offer as a hypothesis that a general reduction in metabolic rate might be associated with an increased life span. This is susceptible to experimental test, since it would be expected that other (non-toxic) compounds with inhibitory activity would have similar *in vivo* effects. It should also be possible to establish whether antioxidant activity is necessary to increase the life span. Many of the compounds which inhibit MK cell metabolism have little or no antioxidant activity. On the other hand the *in vivo* effect might depend on both metabolic inhibition and antioxidant activity.

Data for the induction in rates of membrane-bound drug metabolizing enzymes by analogues of BHT (Gilbert & Goldberg, 1967) do show a direct relation with the *in vitro* effect, since the more potent enzyme inducers are also the more lipid-soluble analogues. This suggests that enzyme induction is also dependent on the concentration of drug in the membrane, although little is known about the induction mechanism.

These examples of the relation of a well-defined *in vitro* effect to the possible *in vivo* consequences illustrate the potential usefulness of the system. Obviously the choice of cell type is important. Although many workers have used cell lines or cell strains to test foreign compounds *in vitro* (e.g. Gabliks, Bantug-Jurilla & Friedman, 1965) the use of freshly isolated primary cultures for toxicity testing is essential since these often retain the ability to respond to agents that are active *in vivo* (e.g. Milner & Villee, 1970; Michaelides & Coons, 1963; Gillette & Goulian, 1966; Nebert, 1970). This minimizes the problems of alteration in cellular characteristics associated with prolonged culture (Eagle, 1965). It is also preferable to choose cells from the tissue most likely to be affected *in vivo*. For example it is possible that during lipid absorption the rate of cell division in the small intestine may be inhibited by BHT where the epithelial cell lining of the small intestine has perhaps the fastest rate of cell turnover in the body (Creamer, 1967). Hepatic cells are an obvious choice as they are often the most sensitive to toxic action resulting from the accumulation of foreign compounds in the liver. Techniques for maintaining hepatic cells are becoming simpler (Kirkby, 1964; Zuckerman, Tsiquaye & Fulton, 1967) and it may soon be feasible to perform analogous experiments on primary hepatic cell cultures.

Clearly a major problem of *in vivo* testing is the test animal, and the monkey kidney cells used here have the advantage of belonging to the same group of primates as man. This may be preferable to the more common use of rodents, dogs and pigs for metabolic studies *in vivo*. Ultimately it should prove possible to investigate tissue-specific responses by the appropriate choice of a range of cell culture systems.

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