that about half of 175 paired pycnia produced aecia. In a later report by Craigie (*9*), more detailed data were given. In two studies with *P. helianthi*, the ratios of pycnia producing aecia to those that did not produce aecia were 108:138 and 15:33. Similarly, in two studies with *P. graminis*, ratios of 24:35 and 30:44 were obtained. Each of these do not differ significantly from a 1:1 ratio except the 15:33 ratio, which differs significantly at $P < .01$. In a further study with *P. helianthi*, Brown (*10*) observed 288 pairs of coalescing pycnia and found that 110 produced aecia and 178 did not, which differs from 1:1 at $P < .001$. In all five sets of data, the number of crosses that produced aecia was less than the number that did not. Brown does not give the source of the teliospores he used in his tests, but Craigie's *P. helianthi* teliospores were collected from a field of sunflowers (*Helianthus annuus*), and those of *P. graminis* came from wild barley (*Hordeum jubatum*). Therefore it is possible that these teliospores may have included more than one strain, in which case some of the fertilization tests carried out in Craigie's study could have been between pycnia of the same strain and others between pycnia of different strains. This would give misleading results if the mating system were not of a (+) and (−) kind.

Although the earlier studies clearly established the heterothallic nature of several rust species, they did not determine with any certainty that only two types of thalli occur. Thus the suggestion that heterothallic rust species possess a (+) and (−) mating system, which has become widely accepted, is not well supported by the present data.

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Limitations of Metabolic Activation Systems Used with in vitro Tests for Carcinogens

Abstract. *Important differences between the metabolic activation of 7,12-dimethylbenz[a]anthracene in intact cellular systems and in liver homogenates suggest that the use of homogenates in conjunction with short-term assays for carcinogens could yield misleading results.*

Percival Pott first recognized chemical carcinogens more than 200 years ago (*1*) when he examined the environmental history of cancer victims. Since recent exposures to chemical carcinogens have been discovered (*2*) in the same manner, the development of reliable and rapid means of recognizing carcinogenic chemicals before human exposure occurs is a prime objective of research in carcinogenesis. The *Salmonella* mutagenicity assay designed by Ames *et al.* (*3*) represents a major step toward this goal (*4*). Nevertheless, if the full potential of such tests in assessing risk to man is to be realized, further development of the metabolic activation system used in the assay may be necessary. At present, we feel there is a need for caution concerning the interpretation of results from this and other short-term tests that incorporate rat liver homogenates for metabolic activation of test compounds. This follows from the studies of activation of the potent carcinogen 7,12-dimethylbenz[*a*]anthracene (DMBA), which we now report, indicate that the activation in such systems differs from that of intact cellular systems and from that of a target tissue for this carcinogen.

Since the interaction of chemical carcinogens with DNA is considered a critical event in the carcinogenic process, we investigated the metabolic activation occurring in various systems by comparing the products of the binding of DMBA to DNA in these systems. The "bay region" (that is, the region between positions 1 and 12 in DMBA) diol epoxide of DMBA (DMBA-3,4-diol-1,2-epoxide) is apparently responsible for the binding of this carcinogen to DNA in mouse skin (*5–8*), and the carcinogenic potency of its precursor, the 3,4-diol (*9*), indicates that this diol epoxide is also the ultimate carcinogenic form of DMBA. Thus, the ability of an in vitro system to generate DNA adducts through this metabolite can be used to assess how accurately the system simulates target tissue activation.

DNA was isolated from mouse skin and various cells in culture exposed to [^{3}H]DMBA or recovered from incubation mixtures consisting of calf thymus DNA, [^{3}H]DMBA, cofactors, and rat liver microsomes or S9 fraction. After purification, the DNA was enzymatically hydrolyzed to hydrocarbon-deoxyribonucleoside adducts, and these were subjected to chromatography on Sephadex LH-20 columns eluted with a methanol-water gradient (*10*). This yielded chromatographic profiles of the type illustrated in Fig. 1 where hydrocarbon-deoxyribonucleoside adducts elute after 250 ml of eluant has passed through the column but the early eluting radioactivity is as yet uncharacterized (*5–7*). The DMBA-DNA adducts from mouse embryo cells exposed to [^{14}C]DMBA were included in each chromatogram in the present study since these adducts are chromatographically identical to the adducts formed in a target tissue, mouse skin (*5, 11*).

At a high ratio of DMBA to microsomal protein (for example, 320 nmole/mg) binding of DMBA to DNA (catalyzed by Aroclor-induced rat liver microsomes) occurs through DMBA-5,6-oxide rather than through the diol epoxide (*5*). However, at lower DMBA concentrations (for example, 24 nmole/mg), some adducts are generated which elute in coincidence with the ^{14}C-labeled diol epoxide adducts (Fig. 1a), although other adducts are also present (*11*). An even wider variety of adducts is generated when microsomes are replaced by the cruder S9 fraction (Fig. 1b), but the same general principle seems to apply. At high DMBA concentrations, DMBA-5,6-oxide is responsible for much of the binding; at lower DMBA concentrations (Fig. 1b), ^{3}H-labeled adducts eluting in coincidence with the ^{14}C-labeled diol epoxide adducts are present although they represent only a small fraction of the total binding (*12*). In contrast to these findings with the subcellular fractions from liver, no dose-dependent qualitative changes in adducts have been observed in mouse embryo cells in culture exposed to doses of DMBA yielding the same levels of binding obtained (up to 70 μmole of DMBA bound per mole of DNA phos-

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phorus) in the incubations with liver fractions (*6, 11*).

These observations suggest that cellular integrity may be critical for the reproduction in vitro of the activation for DNA binding that occurs in mouse skin in vivo. We therefore examined the binding of DMBA to DNA in cultured rat liver cells and, indeed, the DMBA-DNA adducts formed in this system eluted from Sephadex LH-20 chromatograms in coincidence with the mouse embryo cell adducts (Fig. 1c). Human skin cells in culture yielded essentially the same result (Fig. 1d). Thus, in four intact cellular systems—mouse skin in vivo (*5, 11*), mouse embryo cells in culture, rat liver cells in culture, and human skin cells in culture (Fig. 1, c and d)—binding of DMBA to DNA occurred through the diol epoxide route, which is responsible for the carcinogenic action of DMBA. However, with rat liver microsomes or S9 fraction, a wider variety of activation routes is operative (Fig. 1, a and b). The contributions of these various routes to adduct formation are dose-dependent (*11, 12*) such that diol epoxide adducts are formed only at low concentrations of DMBA and, even then, they represent only a small contribution to the total binding.

Since the *Salmonella* mutagenicity assay was designed to monitor interactions of carcinogens and DNA, the response of this assay to DMBA, in the presence of an Aroclor-induced S9 fraction, should reflect the metabolic generation of a range of DNA-reactive metabolites, including known mutagens such as DMBA-5,6-oxide (*13*). If such an assay were directly monitoring the carcinogenic potential of this carcinogen, it should reflect only the metabolic generation of the bay region diol epoxide. Furthermore, since the range of DNA-reactive metabolites generated by the S9 system varies with the dose of carcinogen, dose-response curves, which are frequently used to strengthen confidence in mutagenicity data, may be potentially misleading.

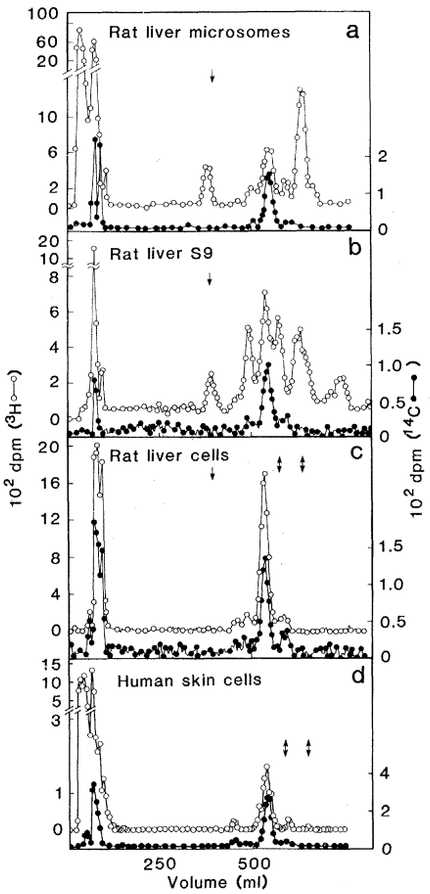

Fig. 1. Comparison of chromatography on Sephadex LH-20 of DMBA-deoxyribonucleoside adducts formed by activation of DMBA in intact cells or by liver homogenates. [^{14}C]DMBA-deoxyribonucleoside adducts (●—●), formed by enzymatic digestion of DNA isolated from mouse embryo cells treated with [^{14}C]DMBA (0.78 μM) for 24 hours (*6*), were included as markers in each analysis (a to d). The single-headed arrow (a to c) denotes the position of elution of an added ultraviolet-absorbing marker, 4-(*p*-nitrobenzyl)pyridine. The double arrow denotes the position of elution of DMBA-5,6-oxide-deoxyribonucleoside ultraviolet-absorbing markers (*5*). (a) Liver microsomes were prepared from male Sprague-Dawley rats treated with Aroclor 1254 (*5*). [^{3}H]DMBA-deoxyribonucleoside adducts (○—○) were formed by incubation of microsomes (0.25 mg/ml) with calf thymus DNA (3 mg/ml), reduced nicotinamide adenine dinucleotide phosphate (NADPH) (0.7 μmole/ml), an NADPH-generating system, and [^{3}H]DMBA (6 μM, 9.6 Ci/mmole) for 2 hours at 37°C, and by recovery, purification, and enzymatic digestion of the DNA (*11*). (b) Liver S9 fraction was prepared from rats (Sprague-Dawley) treated with Aroclor 1254 (*12*). The [^{3}H]DMBA-deoxyribonucleoside adducts (○—○) were formed by incubation of S9 fraction (0.5 mg/ml) with calf thymus DNA, cofactors, and [^{3}H]DMBA (80 μM, 0.7 Ci/mmole) for 2 hours at 37°C, and by recovery, purification, and enzymatic digestion of the DNA (*12*). (c) Sprague-Dawley rat liver cells (fifth passage) were isolated from male rat livers by collagenase-hyaluronidase perfusion and cultured in Hams F10 medium with 10 percent fetal calf serum (*18*). The [^{3}H]DMBA-deoxyribonucleoside adducts (○—○) were obtained as described for mouse embryo cells after exposure of these cells to [^{3}H]DMBA (0.078 μM) for 24 hours. (d) The [^{3}H]deoxyribonucleoside adducts (○—○) were obtained from primary cultures of human foreskin epithelial cells (*19*) grown in Dulbecco's modified Eagle's medium containing 10 percent fetal bovine serum and exposed to [^{3}H]DMBA (3.9 μM) for 24 hours. The concentrations of [^{3}H]DMBA used in the experiments resulted in similar levels of binding to DNA: 15, 20, 6, and 4 μmole of DMBA bound per mole of DNA phosphorus respectively for panels (a) to (d).

These observations are of particular concern with respect to the recommendations generated at a workshop, sponsored by the National Institute of Environmental Health Sciences, on the *Salmonella* mutagenicity assay (*14*). Workshop recommendations included the use of Aroclor-induced rat liver S9 fraction for routine testing purposes and the use of DMBA to check the activity of each batch of S9. A positive result in this assay was defined as a "reproducible dose-related increase in histidine-independent colonies" (*14*).

We have no definitive explanation for the apparently key role of cellular integrity in the activation of DMBA, but it seems reasonable that the organization and spatial orientation of relevant enzymes and the presence of various cofactors (which may be lost during cell fractionation) are important. Based on our findings, and those of others (*15*), the use of intact cells for metabolic activation of carcinogens in short-term tests would appear to have advantages over the use of liver homogenates. Intact hepatocytes have been used successfully in conjunction with the *Salmonella* mutagenicity assay in some cases (*16*) but not in others (*17*). These findings suggest that the use of intact cells for metabolism is a potential alternative to liver homogenates, but some permanent cell line (which could be grown in bulk and preserved in the frozen state) with appropriate metabolic activity would clearly be superior to primary hepatocyte preparations.

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Cell Variants Showing Differential Susceptibility to Ultraviolet Light–Induced Transformation

Abstract. *Six variant clones isolated from a subclone of BALB/3T3-A31 clone were classified into three groups according to their different susceptibilities to cell transformation by ultraviolet light irradiation: highly susceptible, intermediately susceptible, and resistant. All variant clones showed similar susceptibility to cytotoxic effects induced by ultraviolet light.*

Many hypotheses have been proposed to account for cell transformation due to chemical carcinogens or radiation. However, neither metabolism nor cellular target molecules have been unequivocally identified as factors directly involved in the induction of cell transformation. To define the factors involved, it would be very useful to isolate the cell variants that show different susceptibilities to transformation. Characterization of such variants may lead to the identification of the crucial processes leading to cell transformation.

One of the advantages of using established cell lines as target cells for a transformation assay system is the availability of cloned lines. Isolation, characterization, and use of mammalian cell variants are possible only with established lines unless the mutated cells are available as biopsies of animals or of humans. Cells are available from patients who are predisposed genetically to cancer. However, the transformation assay systems used for human cells are still far from being quantitative and rapid (*1*). There have also been reports of animal strains having different susceptibilities to polycyclic hydrocarbon–induced carcinogenesis (*2-4*). Differences in susceptibility between strains, however, have been ascribed to the differences in genetic factors that control carcinogen metabolism (*2-4*).

We now report the isolation of cell variants from a BALB/3T3-A31 clone showing different susceptibilities to ultraviolet (UV) light–induced transformation. To avoid isolating variants having either altered membrane permeability to chemical carcinogens or altered carcinogen-metabolizing activity, UV irradiation was used as the transforming agent in these experiments.

Malignant transformation was assayed by scoring transformed foci, as reported previously for A31-714 cells (*5, 6*). Actively growing cells were plated at 10^4 cells per 60-mm plastic dish for the transformation assay and 100 to 500 cells per dish for assay of plating efficiency. Eighteen hours after plating, the medium was removed and the cells attached to dishes were exposed to UV light at room temperature. The flux of the 15T8GE germicidal lamp used as a source of UV light was 1.01 W/m^2 as measured by MGL method (*7, 8*). After irradiation, the cells were reincubated in fresh medium. Plating efficiency was determined 7 days later, and transformation was scored 26 to 27 days after irradiation, as described previously (*5, 6*). Only the transformed foci that showed clear alteration in growth pattern and cell arrangement (such as abundant mitoses, crisscrossed cell arrangement in the center and at the edge of focus, and dense staining in the cytoplasm) were scored. Scoring foci showing only partial alteration of morphology (such as high cell density but no clear pattern of crisscrossed cell arrangement at the edge) resulted in a 1.3- to 1.9-fold increase in the absolute frequency of transformation compared to scoring only the clearly transformed foci, but such analysis did not affect the differences in relative susceptibility to transformation observed among the variants.

Mass cultures of A31-1 cells were used as sources for the isolation of variants. The A31-1 line was isolated from BALB/3T3-A31 clone (*9*) which was obtained from Ikawa at the National Cancer Institute. The A31-1 cells of early passage (at least up to 50 cell generations after isolation were homogeneous in cell morphology and susceptibility to transformation by UV, 3-methylcholanthrene, and benzo [*a*]pyrene. The A31-1 cells of late passage (150 to 180 cell generations after isolation) consisted of a mixture of cells varying in morphology and having variable frequency of transformation by chemical carcinogens. More than 100 subclones were isolated from mass culture of A31-1 of late passage. Among them, 22 clones that showed homogeneity in morphology and high sensitivity to density-dependent inhibition of cell growth were tested for the susceptibility

Table 1. Grouping of variant cells according to their susceptibility to UV-induced transformation.

Susceptibility to transformation	Subclone*	D_{37}† (erg/mm^2)	Transformation‡ frequency ($\times 10^5$)
Highly susceptible	A31-1-13	38	160 ± 28
Intermediately susceptible	A31-1-1	36	9 ± 0.2
	A31-1-2	34	8 ± 1.4
	A31-1-7	36	10 ± 2.0
Resistant	A31-1-8	40	< 1.6
	A31-1-15	41	1 ± 1.0

*Subclones isolated from BALB/3T3-A31-1 clone were plated at 100 and 300 cells for survival assay and 10^4 cells for transformation assay per 60-mm plastic dish containing 5 ml of Eagles minimum essential medium (Earle's salt solution) supplemented with 10 percent fetal bovine serum. Eighteen hours after cells were plated the medium was removed, and the cells attached to the substrate were exposed to UV at a dose of 75 ergs/mm^2 for transformation assay and at various doses ranging from 12.5 to 100 ergs/mm^2 for survival assay. After irradiation, the cells were reincubated in fresh medium. The dishes for assay of survival were maintained without medium change and fixed 7 days after irradiation. The cultures for transformation assay had a medium change twice a week and were fixed 25 or 26 days after UV exposure. †D_{37} was calculated from the survival curves. ‡Mean ± standard error for 12 to 27 plates, expressed as ratio of cells transformed to cells surviving UV dose of 75 ergs/mm^2.

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