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July 20, 1979

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FOLDER 40-636-77 NIH

Department of Energy
Oak Ridge Operations
Attention: Mr. J. A. Lenhard, Assistant Manager
for Energy Research and Development
Post Office Box E
Oak Ridge, Tennessee 37830

Gentlemen:

Renewal Interagency Agreement Proposal to the NCI (DOE 40-636-77, NCI Y01 CP 70222)
"Malignant Cell Transformation and Mutagenesis Induced by Carcinogenic Metals"

Enclosed for your review and approval is a renewal research proposal being submitted to the National Cancer Institute. This proposal extends the period of performance for three years beginning September 27, 1979, and requests total funding of $967,900. The initial twelve-month funding will require $308,500.

The following information is provided to assist in your review and approval of this renewal agreement:

a. This research effort is described in the approved ORNL Institutional Plan on page 52, "Work for Others (Excluding NRC)." The personnel required are included in the direct FTE's in the "Summary of Resources" on page 53.

b. E. Huberman will continue to serve as the principal investigator and devote 50% of his effort to this project. He will continue to devote 50% of his time to EPA.

c. Existing space and resources are available to conduct this research without interfering with ongoing DOE efforts.

d. This research continues to serve the DOE objectives in activity GK 01 02 02 1, "Health Effects Research in Biological Systems - Carcinogenesis." Based upon this programmatic relation, it is requested that this research be continued on an actual cost basis, depreciation and DOE added cost factor waived.

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After your approval and approval of the Office of Health and Environmental Research, please forward 15 copies of the Summary Document, Technical Proposal, and Annual Progress Report, and 6 copies of the Business Management Cost Proposal to Mr. Joe Federline, Blair Building, Room B-16, Carcinogenesis Contracts Section, RCB National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205.

If you have any questions concerning this proposal, please contact C. R. Richmond, ext. 4-4332.

Sincerely yours,

Herman Postma

Herman Postma
Director

HP:VBI:kh
Enclosure

cc: J. E. Carr
R. J. M. Fry
R. F. Hibbs
E. Huberman
J. N. Maddox, DOE-GTN
W. R. Ragland
C. R. Richmond
J. B. Storer
File - RC

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ANNUAL PROGRESS REPORT

Name and Address of Institution:

Biology Division
Oak Ridge National Laboratory
P. O. Box Y
Oak Ridge, TN 37830

Address of Principal Investigator:

Biology Division
Oak Ridge National Laboratory
P. O. Box Y
Oak Ridge, TN 37830

Title of Project:

Malignant Cell Transformation and Mutagenesis Induced by Carcinogenic Chemicals.

NCI Interagency Agreement No.:

Y01 CP 70222

Principal Investigator:

Eliezer Huberman, Ph.D.

Date of Submission: July 6, 1979

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SCIENTIFIC PROGRESS

1. Mutagenicity to mammalian cells in culture by (+) and (-) Trans-7,8-dihydroxy-7,8-dihydrobenzo(a)pyrenes and the hydrolysis and reduction products of two stereoismeric benzo(a)pyrene 7,8-diol-9,10-epoxides.

Benzo(a)pyrene (BP), a very common polycyclic aromatic hydrocarbon (PAH) in our environment, is carcinogenic for experimental animals and may also be involved in the induction of some human malignancies. However, in common with other carcinogenic PAH's, BP has to be metabolized by mixed-function oxidases and related enzymes in order to exert its biological properties. In view of the relationship between chemical carcinogenesis and mutagenesis, it is important to identify the metabolites that are responsible for the mutagenic effect of carcinogens, since these metabolites may also be responsible for carcinogenicity.

We previously demonstrated that of 15 BP synthetic derivatives, (+)r-7,t-8-dihydroxy-7,8-dihydrobenzo(a)pyrene [(±)trans-7,8-diol] (Fig. 1), upon further metabolism, was more mutagenic in mammalian cells than BP and that the further metabolic oxidation product r-7,t-8-dihydroxy-t-9-,10-oxy-7,8,9,10-tetrahydrobenzo(a)pyrene (diol-epoxide I), was the most potent direct mutagen in mammalian cells of any tested BP metabolite or derivative. The trans-7,8-diol formed metabolically from BP was subsequently found to be an optically pure (-) enantiomer. We extended these studies by testing the mutagenicity in mammalian cells of two enantiomeric trans-7,8-diols as well as two triols and four tetrols that are products of NADPH-catalyzed reduction and aqueous-medium hydrolysis of the two stereoisomeric 7,8-diol-9,10-epoxides. Mutagenesis by these metabolites was tested in Chinese hamster V79 cells, and mutation was

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characterized by resistance to ouabain. The V79 cells used in these studies do not contain detectable mixed-function oxidase, the enzyme necessary to metabolize PAH's. Mutagenesis by PAH's that require metabolic activation was tested in the V79 cells that were co-cultivated with normal Golden hamster cells capable of metabolizing BP (cell-mediated assay). Mutagenesis was, therefore, tested in the V79 cells devoid of PAH-metabolizing enzymes and in the cell-mediated assay. Our results indicated that the (±)trans-7,8-diol, (-)trans-7,8-diol, (+)trans-7,8-diol, (7/8,9)-triol, (7,9/10)-triol, (7,10/8,9)-tetrol, (7/8,9,10)-tetrol, (7,9/8,10)-tetrol and (7,9,10/8-tetrol) (Fig. 1) were not mutagenic to the V79. However, in the presence of cells capable of metabolizing PAH in the cell-mediated assay, the trans-7,8-diols exhibited mutagenic effects (Table 1). The (-)trans-7,8-diol, which is made metabolically from BP by liver microsomes, was the most active for V79 cells in the cell-mediated assay, while the (+)trans-7,8-diol, which is not a metabolite of BP, was at least six times less active at equimolar concentrations. These results are of importance in view of the experiments indicating that the (-)trans-7,8-diol leads predominantly to the formation of the diol-epoxide I, which we and others have shown to be the most active mutagenic BP derivative, while (+)trans-7,8-diol, which is less active in the cell-mediated assay, has not been detected as a metabolite of BP. None of the triols and tetrols which are hydrolysis and reduction products of diol-epoxides I and II were mutagenic to V79 cells either in the presence or in the absence of polycyclic-hydrocarbon-metabolizing cells. This indicates that these products of diol-epoxide hydrolysis or reduction are not biologically active.

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These results are in agreement with the suggestion that mutagenicity by BP is mainly due to diol-epoxide I which is produced via the metabolically formed (-)trans-7,8-diol. The data suggest that the tetrols and triols obtained from either diol-epoxide I or diol-epoxide II are detoxification products.

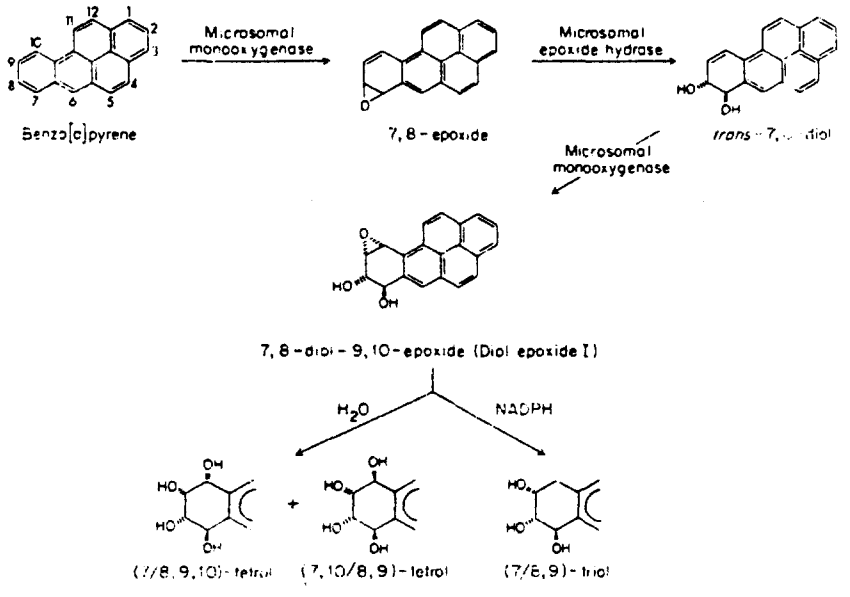

FIGURE 1. Metabolic pathway of benzo(a)pyrene that leads to the production of the major mutagenic and transforming metabolite, diol-epoxide I, and its detoxification via its corresponding triol and tetrols.

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TABLE 1

MUTAGENICITY OF BENZO(a)PYRENE DERIVATIVES IN THE CELL-MEDIATED ASSAY

Hydrocarbon	Concentration (μM)	Cloning efficiency	No. of ouabain-resistant mutants per 10^5 survivors
Control	—	74	0.5
BP	0.4	64	17
	1.3	39	32
	4.0	25	138
(±)*trans*-7,8-Diol	0.05	64	8
	0.15	58	26
	0.4	32	98
(−)*trans*-7,8-Diol	0.05	60	13
	0.15	48	50
	0.4	28	165
(+)*trans*-7,8-Diol	0.15	68	2
	0.4	46	27
	1.2	28	52
(7,10/8,9)-Tetrol	3.1	83	0.6
(7/8,9,10)-Tetrol	3.1	75	0.6
(7/8,9)-Triol	3.3	74	0.7
(7,9/8,10)-Tetrol	3.1	76	0.7
(7,9,10/8)-Tetrol	3.1	69	0.6
(7,9/10)-Triol	3.3	76	1.1

After treatment for 2 days with BP and its metabolites there were 1.0—1.6 × 10^6 V79 cells per Petri dish with 1.3—4 μM BP, 0.15—0.4 μM (−) and (±)*trans*-7,8-diol, and 1.2 μM (+)*trans*-7,8-diol. In all other cases there were 1.8—2.8 × 10^6 V79 cells per Petri dish.

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2. Mutagenicity of benz(a)anthracene dihydrodiol.

Based on our previous experiments and those of others, it was possible to identify the non-K-region diol of benzo(a)pyrene (BP), the 7,8-dihydrodiol as its proximate mutagenic and carcinogenic metabolite. It was recently suggested that similar types of non-K-region diols may be the proximate mutagenic and presumably carcinogenic metabolites of other polycyclic aromatic hydrocarbons (PAHs). We have, therefore, undertaken the testing of diols of the weak tumor promoter, benz(a)anthracene (BA) (Fig. 2), for their mutagenic effect in Chinese hamster V79 cells. Mutagenesis of BA and its diols was tested in the direct and cell-mediated assay. For comparison, we have included the potent carcinogen BP. The BA dihydrodiols tested included (±)-1,2-dihydroxy-1,2-dihydrobenz(a)anthracene, (±)-3,4-dihydroxy-3,4-dihydrobenz(a)anthracene (3,4-dihydrodiol), (±)-trans-5,6-dihydroxy-5,6-dihydrobenz(a)anthracene, and (±)-trans-8,9-dihydroxy-8,9-dihydrobenz(a)anthracene (Fig. 2). The results indicated that neither BA, nor BP, nor any of the four derivatives were mutagenic for ouabain resistance in V79 cells in the direct assay at concentrations of about 4 μM. Also, none of the tested compounds was cytotoxic at the above concentrations. However, in the cell-mediated assay BP and BA - 3,4-dihydrodiol were cytotoxic and mutagenic with the mutagenic response increasing as a function of the hydrocarbon concentration (Table 2). However, although BA 3,4-dihydrodiol was about 10 times more potent a mutagen than BA, it still had much less mutagenic activity than BP. The other dihydrodiols of BA had less activity than the parent hydrocarbon. These results and those recently reported by others indicate that BA 3,4-dihydrodiol may represent the proximate mutagenic form of BA. This is in agreement with the theory proposed by

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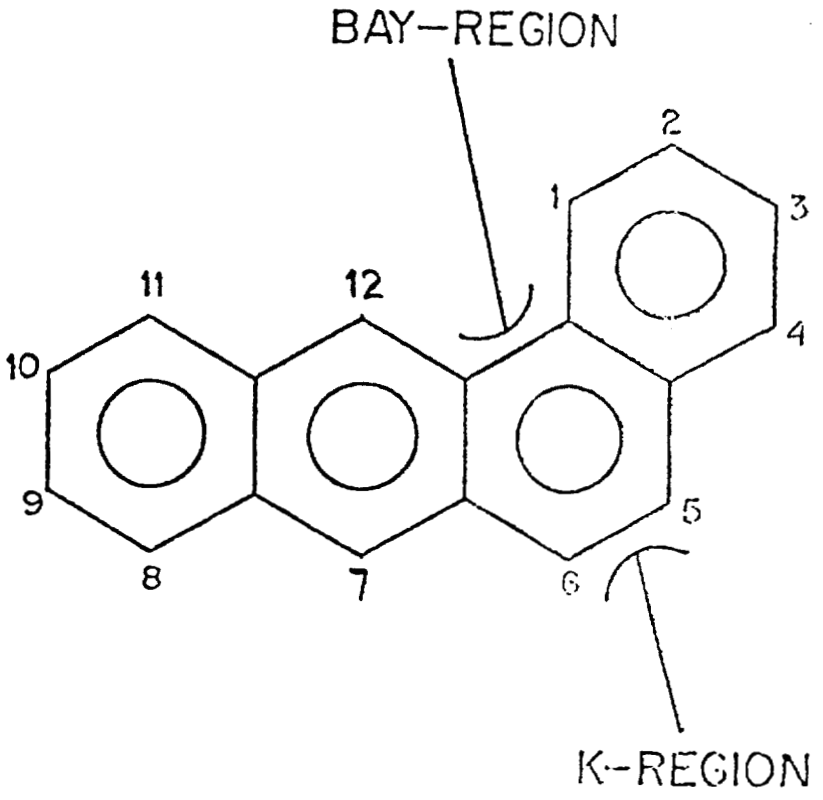

FIGURE 2. Structure of Benz(a)anthracene.

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Dr. Jerina (National Cancer Institute) which predicts that diol-epoxides in the bay-region are the principal determinants of mutagenicity and carcinogenicity. A bay-region occurs in a PAH such as BA when an angularly fused benzo ring is present (Fig. 2).

TABLE 2

INDUCTION OF MUTAGENICITY IN THE CELL-MEDIATED ASSAY BY BP AND BA AND ITS TRANS-DIHYDRODIOLS

Hydrocarbon	Concentration (μM)	Cloning efficiency	No. of ouabain-resistant mutants/10^6 survivors[a]
Control		79	0.7
BA	4.4	73	0.9
	44.0	74	2.1
BA 1,2-dihydrodiol	4.1	76	0.8
	41.0	77	0.9
BA 3,4-dihydrodiol	1.2	65	1.0
	4.1	60	2.3
	12.0	52	8.2
	41.0	31	23.0
BA 5,6-dihydrodiol	4.1	64	0.8
	41.0	65	0.9
BA 8,9-dihydrodiol	4.1	71	0.9
	41.0	61	1.2
BP	0.4	59	11.0
	1.3	38	25.0
	4.0	23	99.0

The polycyclic hydrocarbons tested were added 5 hr after 3×10^5 V79 cells were seen on 2×10^6 irradiated golden hamster cells. Two days after treatment, the cells were dissociated and seeded to determine the cloning efficiency of the V79 cells and the frequency of ouabain-resistant mutants. At the time when the cells were seeded for cloning efficiency and selection of mutation, there were in the control and in the hydrocarbon-treated cultures from 1.5 to 2.7×10^6 V79 cells/Petri dish.

[a] The maximum percentage of S.D. was less than 25%.

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3. Mutagenicity of fluorinated 7,12-dimethylbenz(a)anthracenes.

7,12-Dimethylbenz(a)anthracene,(DMBA), one of the most potent carcinogenic polycyclic aromatic hydrocarbons (PAH's), (Fig. 3), like other such carcinogens including, benzo(a)pyrene and benz(a)anthracene, has to be metabolized by mixed-function oxidases and related enzymes in order to exert its mutagenic effect in mammalian cells. As previously discussed, the oxidation product of the "bay-region" diol of a PAH, including DMBA (Fig. 3), the diol-epoxide (3,4-diol-1,2-epoxide) is presumably responsible for the PAH's mutagenic effect. It would be of interest to determine whether DMBA is indeed activated into a mutagen via this route. Therefore, four different fluorinated derivatives of this potent carcinogen were tested for mutagenesis in Chinese hamster V79 cells. The use of fluorinated substitution at a given ring carbon is expected to reduce electron density at that position, thus preventing the enzymic attack there. A reduction in the mutagenic and carcinogenic activity of the specific fluorinated DMBA would imply that this fluorinated carbon is involved in the metabolic activation of DMBA. The derivatives used include those fluorinated at position 1 or 2 (bay-region), at the 5 position (K-region), and the 11 position, which does not involve these two susceptible sites (Fig. 3).

The results indicated that DMBA and its four derivatives were not mutagenic to V79 cells. However, after their metabolism in the cell mediated assay, DMBA's fluroinated at either positions 1,2 or 5, were dramatically less mutagenic than DMBA, while fluorination at position 11, which is remote from the bay region, did not reduce the mutagenic activity of DMBA (Table 3 and Fig. 4). These results are in agreement

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with the suggestion that a diol-epoxide at the bay region (Fig. 3) may represent the ultimate mutagenic metabolite of DMBA. However, our results showing that fluorination at the 5 position of the K-region of DMBA brings about a dramatic reduction in mutagenicity (Table 3) may imply that this position is also a necessary prerequisite in the metabolic attack by the mixed-function oxidases. It is possible, however, that this position has to be free so that it can interact with the appropriate enzyme in a way that in turn allows oxidation at the bay region. These results on the 5-fluoro-DMBA indicate that either the metabolic activation of DMBA into a mutagen may involve more than one metabolic pathway or that substitution of DMBA with fluorine may alter the natural pathway of DMBA activation.

It is also interesting to note that, as in the case of benz(a)-anthracene and benzo(a)pyrene derivatives, we found, in collaboration with Dr. T. Slaga, both a quantative and a qualitative correlation between the tumor-initiating activity of DMBA and the four fluorinated derivatives in mouse skin and their mutagenic activity in V79 cells co-cultivated in the cell-mediated assay.

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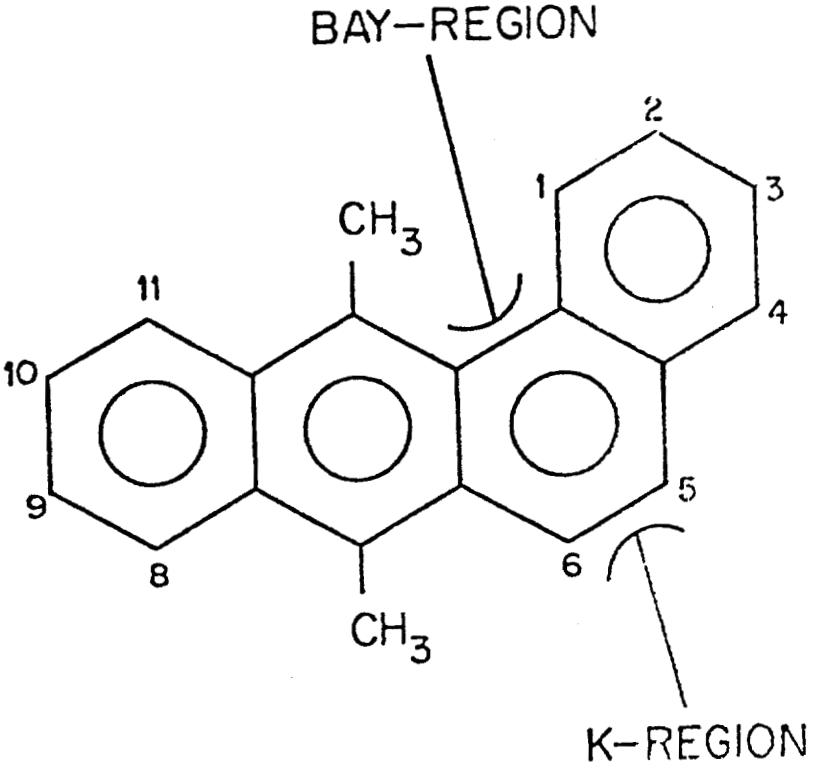

FIGURE 3. Structure of 7,12-dimethylbenz(a)anthracene

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TABLE 3

MUTAGENICITY OF DMBA AND ITS FLUORINATED DERIVATIVES IN THE CELL-MEDIATED ASSAY

Hydrocarbon	Concentration (μM)	Cloning efficiency (%)	No. of ouabian-resistant mutants per 10^6 survivors
None	—	76	0.7
DMBA	0.01	56	7
	0.04	45	24
	0.13	18	52
	0.4	2	69
1-fluoro-DMBA	3.8	80	1
	12	62	3
	38	59	6
2-fluoro-DMBA	3.8	67	0.9
	12	71	0.7
	38	66	1.0
5-fluoro-DMBA	3.8	59	2
	12	55	4
	38	43	10
11-fluoro-DMBA	0.01	66	5
	0.04	36	19
	0.12	8	37

DMBA and its derivatives were added 5 hr after 3×10^5 V79 cells were seeded on 2×10^6 irradiated embryonic golden hamster cells. Two days after treatment, the cells were dissociated and seeded to determine the cloning efficiency of the V79 cells and the frequency of ouabian-resistant mutants. At the time when the cells were seeded for cloning efficiency and selection of mutation, there were in the control and in the hydrocarbon-treated cultures from 0.9 to 3.0×10^6 V79 cells per Petri dish.

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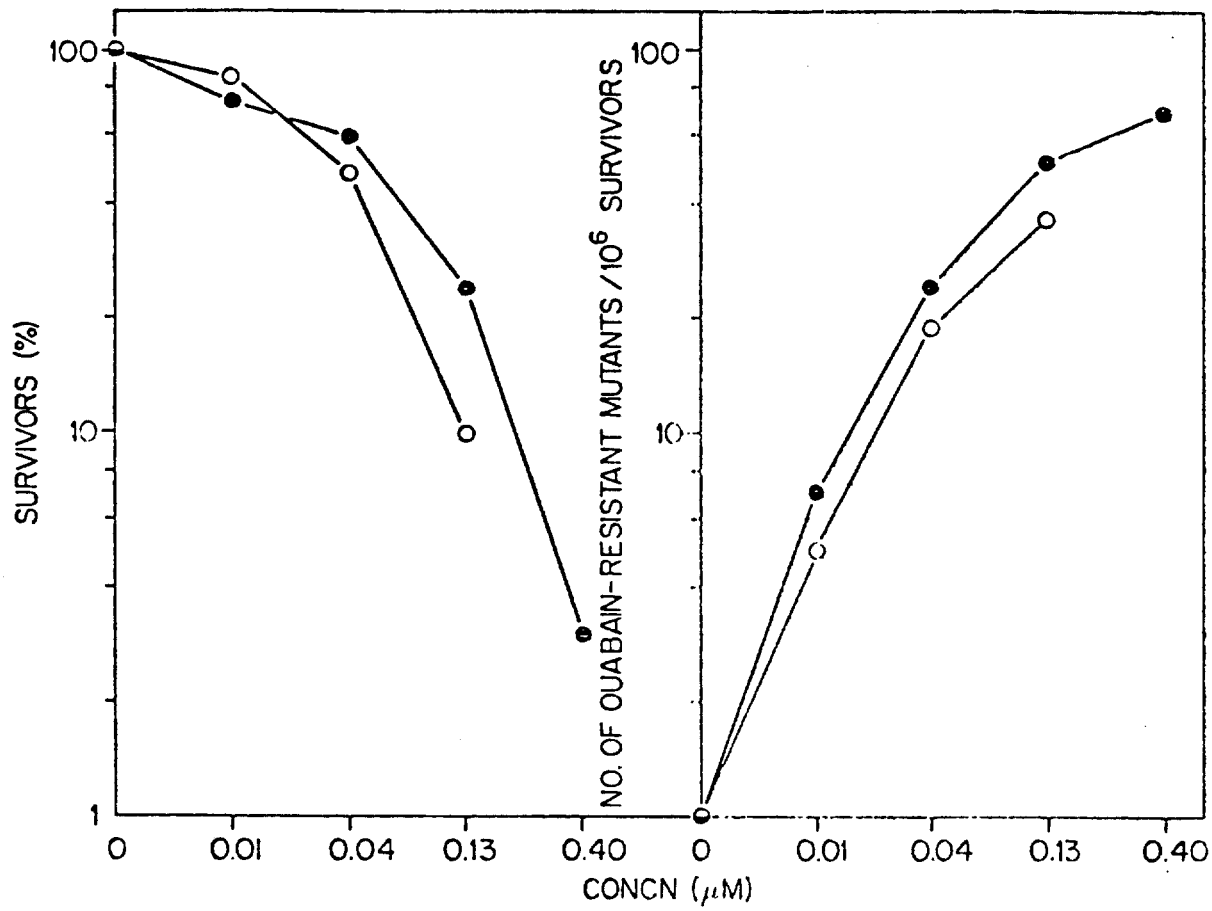

FIGURE 4. Mutagenicity of DMBA and its 11-fluoro derivative. DMBA and 11-fluoro-DMBA were added 5 hr after 3 x 10^5 V79 cells were seeded on 2 x 10^6 irradiated embryonic golden hamster cells. Two days after treatment, the cells were dissociated and seeded to determine the cloning efficiency of the V79 cells and the frequency of ouabain-resistant mutants. Left, Percent surviving colonies after treatment with the hydrocarbons as a function of the hydrocarbon dose. Right, Mutation frequency as a function of hydrocarbon dose. ●, DMBA; O, 11-fluoro-DMBA.

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4. Identification of 7,12-dimethylbenz(a)anthracene metabolites that lead to mutagenesis in mammalian cells.

The rapid progress in recent years in the understanding of the metabolic pathways of polycyclic aromatic hydrocarbons (PAH's) that lead to carcinogenicity has stemmed from efficient separation of metabolites by high-pressure liquid chromatography (HPLC), their DNA binding, and mutagenesis studies. This approach led us and others to identify benzo(a)pyrene-7,8-diol-9,10-epoxide (Fig. 1) as the major mutagenic metabolite of the potent carcinogen benzo(a)pyrene. Other studies have implicated similar types of metabolites as the major mutagenic metabolites of several other PAH's.

7,12-Dimethylbenz(a)anthracene (DMBA) is extensively studied in view of the finding that it is a PAH which can induce not only sarcomas, but also lung and mammary carcinomas, which are common malignancies in the human population. DNA fluorescence studies and studies with fluorinated derivatives have implicated the 1,2,3,4-ring of DMBA (Fig. 3) in the metabolic activation of this potent carcinogen. However, the studies with fluorinated derivatives have suggested the involvement of additional metabolic routes, not necessarily the bay region, in the metabolic activation of DMBA (see Section 3).

We have, therefore, undertaken to identify, by the use of DMBA metabolites that are formed enzymatically instead of model compounds, the metabolic pathway or pathways which lead to its mutagenic activity in mammalian cells. For this determination 11 DMBA metabolites isolated from *in vitro* metabolism of DMBA and its methyl-hydroxylated derivates with rat liver microsomes were tested for mutagenesis in the Chinese

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hamster V79 cells. The metabolites included the trans-3,4-diols of DMBA, 7-OHM-12-MBA, and 7-M-12-OHMBA; the trans-5,6-diols of DMBA and 7-OHM-12-MBA; the trans-8,9-diols of DMBA, 7-M-12-OHMBA, and 7-OHM-12-MBA; 7-OHM-12-MBA; 7-M-12-OHMBA; and 7,12-diOHMBA. Our results indicated that none of these compounds were mutagenic in the V79 cells, which do not metabolize PAHs. However, after metabolism in the cell-mediated assay which allows PAH metabolism, the trans-3,4-diols were more active than their parent hydrocarbons, whereas the trans-5,6- or 8,9-diols were either weak or not mutagenic (Fig. 5). The metabolism to active mutagens in the cell-mediated assay from the trans-3,4-diols was inhibited by the mixed-function oxidase inhibitor, 7,8-benzoflavone. This suggests that these trans-3,4-diols are further oxidized to reactive mutagenic metabolites (Table 4).

Since liver microsomes do not necessarily mimic the metabolite profile produced in intact cells, we have, in collaboration with Drs. S. Yang and M. Chou, analzyed the DMBA metabolites produced by intact cells. This metabolic study indicated that the DMBA-trans-3,4-diol, which is more active than DMBA itself in inducing mutation in mammalian cells, is also one of the major metabolites (Fig. 6). Based on this result and those with fluorinated DMBA as well as DNA binding, we suggest that DMBA-trans-3,4-diol-1,2-epoxide may be a major mutagenic and presumably the tumorigenic metabolite of this potent carcinogen (Fig. 7). Similar metabolic pathways from the further metabolism of 7-OHM-12-MBA and 7-M-12-OHMBA, which are metabolites of DMBA in intact cells, as well as the oxidative process which leads to methyl hydroxylation may also contribute to the mutagenic effects of DMBA.

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(ABBREVIATIONS: DMBA, 7,12-dimethylbenz(a)anthracene; 7-M-12-OHMBA, 7-methyl-12-hydroxymethylbenz(a)anthracene; 7-OHM-12-MBA, 7-hydroxymethyl-12-methylbenz(a)anthracene; 7,12-diOHMBA, 7,12-dihydroxymethylbenz(a)anthracene; 7-CHO-12-MBA, 7-formyl-12-methylbenz(a)anthracene; 7-M-12-CHOBA, 7-methyl-12-formylbenz(a)anthracene; 7,12-DMBA-trans-3,4-diol, trans-3,4-dihydroxy-3,4-dihydro-7,12-DMBA; other dihydrodiol metabolites are similarly abbreviated.)

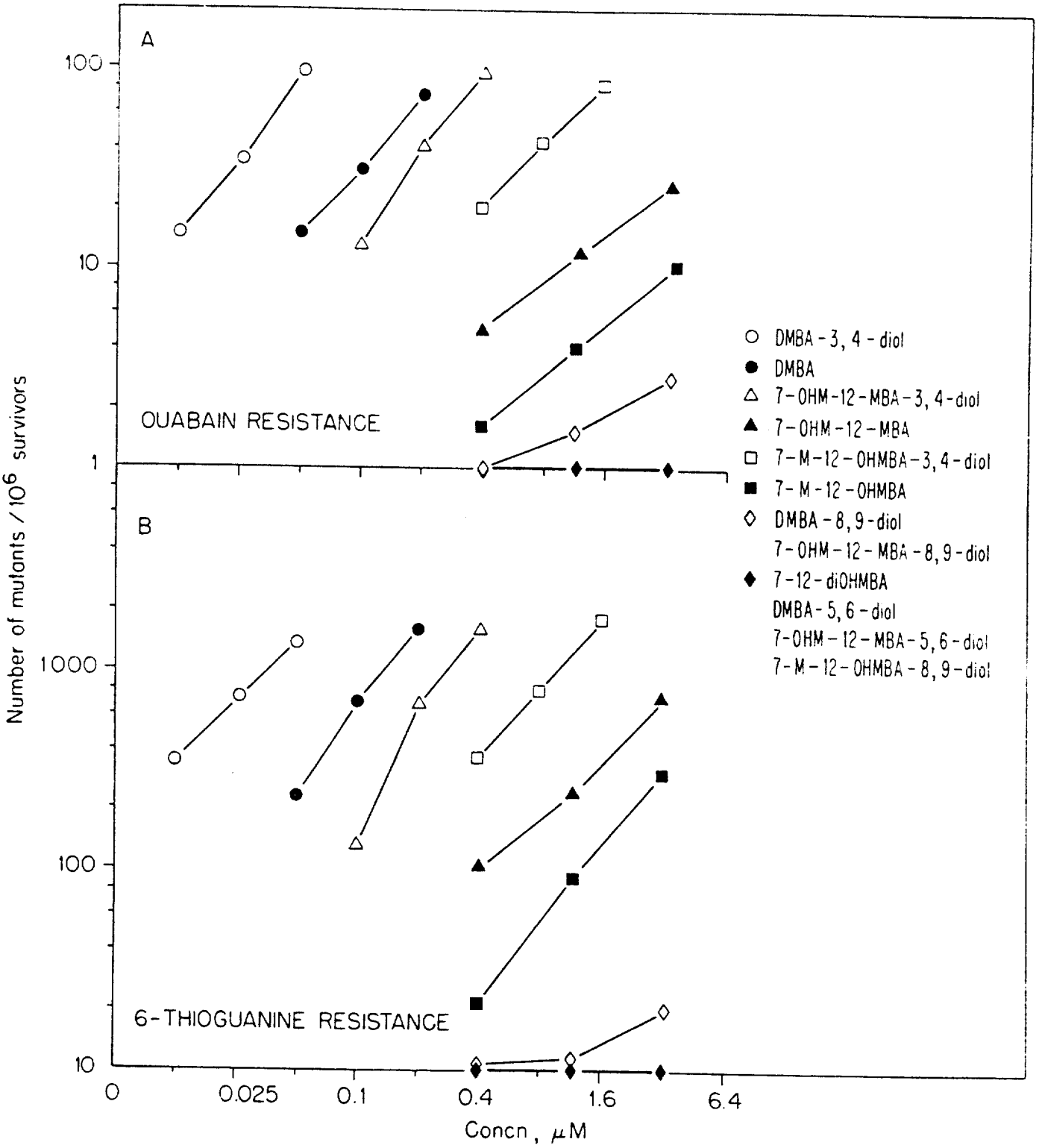

FIGURE 5. Induction of ouabain-resistant (A) and 6-thioguanine-resistant (B) mutants in Chinese hamster V79 cells cocultivated with normal golden hamster embryo cells after treatment with DMBA and 11 of its metabolites. At 2 days after treatment with DMBA and the trans-3,4-diols there were 0.8-1.5 x 10^6 V79 cells/Petri dish; these cells gave cloning efficiencies that ranged from 3 to 40%. In all other cases there were 1.6 -3.0 x 10^6 V79 cells/Petri dish which gave cloning efficiencies that ranged from 35-80%.

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Table 4. Mutagenicity of DMBA and BP and their trans-dihydrodiols to V79 cells cocultivated with golden hamster cells after pretreatment without (-) or with (+) BF

Hydrocarbon	Concentration, μM	Cloning efficiency of the V79 cells, %		No. of ouabain-resistant mutants /10^6 survivors		No. of 6-thioguanine-resistant mutants /10^5 survivors	
		-	+	-	+	-	+
Control	—	75	69	1	1	0.4	0.8
DMBA	0.05	30	65	13	1	17	1
	0.10	8	66	25	2	55	1
	0.20	2	63	89	4	162	3
DMBA-trans-3,4-diol	0.01	40	68	22	2	37	3
	0.03	21	70	44	3	64	6
	0.05	4	58	106	7	127	11
7-OHM-12-MBA-trans-3,4-diol	0.10	56	69	8	1	13	1
	0.20	38	68	41	2	60	1
	0.40	11	54	103	4	130	5
7-M-12-OHMBA-trans-3,4-diol	0.40	28	63	28	2	36	2
	0.80	24	51	51	3	77	2
	1.60	7	44	101	4	172	6
BP	4	23	70	76	6	NT	NT
BP-trans-7,8-diol	0.4	27	72	109	3	NT	NT

After cells were treated for 2 days with DMBA, BP, or their metabolites in the absence of BF, there were 0.8–1.4 X 10^6 V79 cells/Petri dish. In all other cases there were 1.4–2.8 X 10^6 V79 cells/Petri dish. NT = not tested. The BP-trans-7,8-diol used was the (-) enantiomer.

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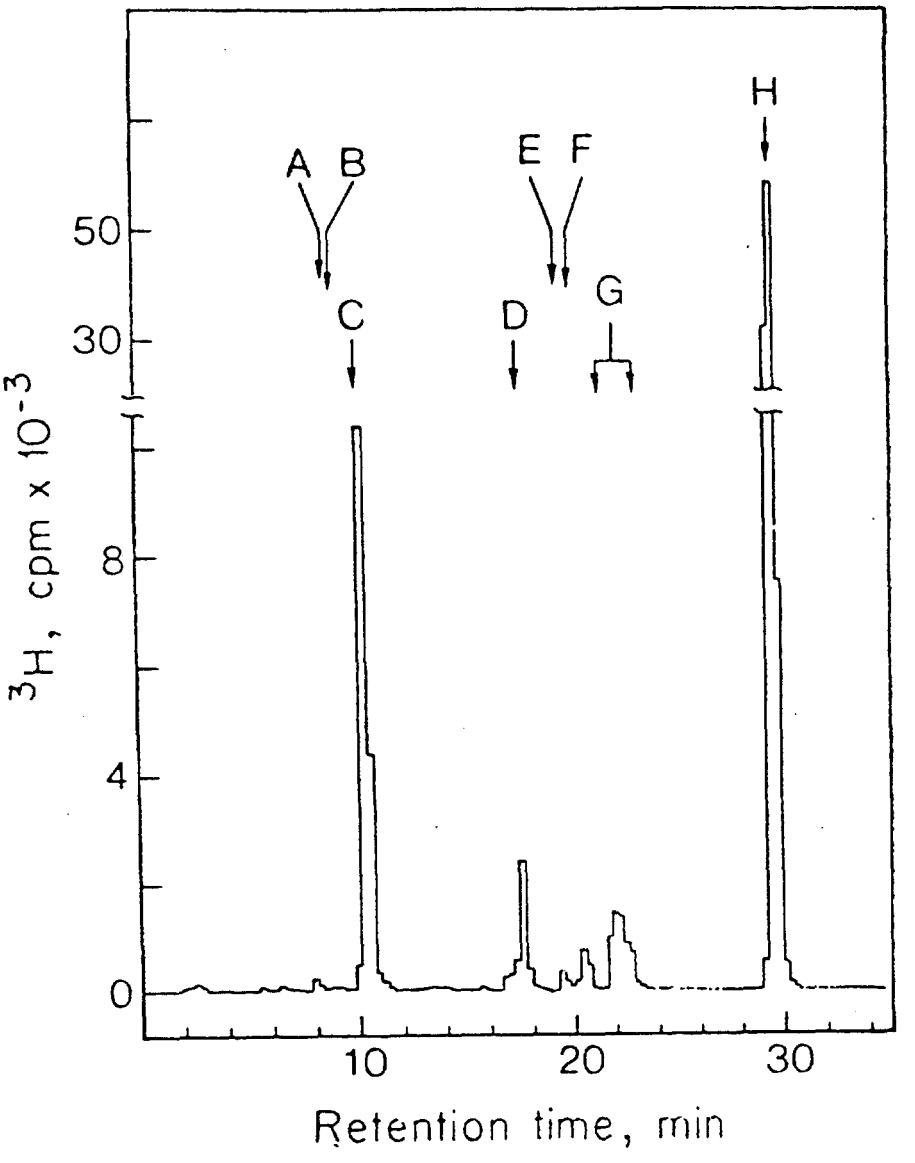

FIGURE 6. HPLC analysis of ethyl-acetate-extractable metabolites from cultured golden hamster embryonic cells cocultivated with Chinese hamster V79 cells treated with [^{3}H]DMBA for 24 hr. Known metabolites that are cochromatographic as UV markers are indicated by arrows. They are: (A) 7-M-12-OHMBA-trans-3,4-diol and 7-OHM-12-MBA-trans-3,4-diol; these two trans-3,4-diols are separable on another HPLC system. (B) DMBA-trans-5,6-diol. (C) DMBA-trans-8,9-diol. (D) DMBA-trans-3,4-diol. (E) 7-OHM-12-MBA. (F) 7-M-12-OHMBA. (G) A mixture of DMBA phenols. (H) DMBA. Through extensive isolation procedures by different columns and solvent systems, the radioactivity cochromatographed with DMBA-trans-3,4-diol was found to be free of any other dihydrodiol and phenolic metabolites of DMBA, 7-M-12-OHMBA, and 7-OHM-12-MBA . A similar metabolite profile from DMBA was obtained from the medium of the golden hamster cells which were cultured in the absence of the V79 cells.

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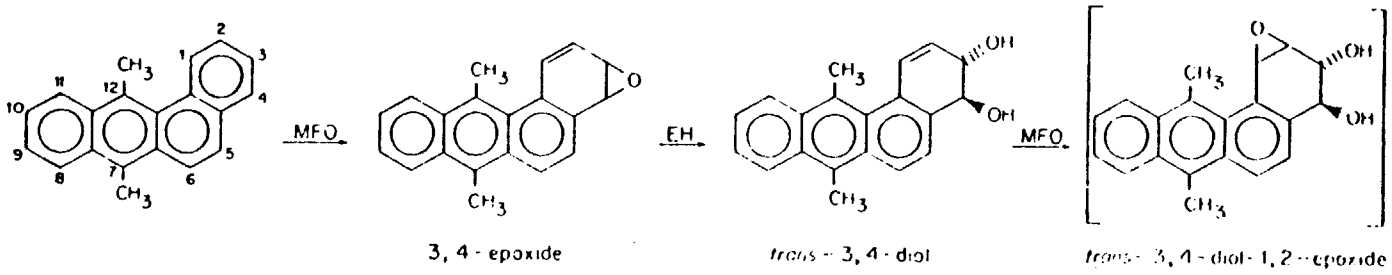

FIG. 7. Metabolic pathway of 7,12-DMBA that may lead to the formation of the trans-3,4-diol-1,2-oxide. MFO, mixed-function oxidase; EH, epoxide hydrase.

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5. Liver cell-mediated mutagenesis of mammalian cells by liver carcinogens.

The cell-mediated mutagenesis assay that we have used in the past and those mentioned in the previous studies uses fibroblasts to activate the carcinogen and mutable V79 cells which are incapable of activating most carcinogens. By using this approach, a correlation between the mutagenic activity and the *in vivo* carcinogenic activity with polycyclic hydrocarbons has been achieved. Furthermore, this system has been used, as mentioned in previous sections, to identify metabolites that are responsible for the mutagenic effect of some carcinogenic PAHs. However, cultured fibroblasts are unable to activate certain classes of compounds, including those which cause cancer of the liver.

Therefore, to increase the spectrum of compounds that can be studied in the cell-mediated mutagenesis system, we have used primary cultures of rat liver cells to activate two known classes of liver carcinogens and V79 cells to detect their mutagenic metabolites. The liver cells were prepared by standard perfusion techniques. Conversion from ouabain susceptibility to ouabain resistance, a mutation associated with the surface membrane Na^+/K^+ ATPase, was used as the genetic marker. The basic procedure for the liver cell-mediated mutagenesis assay as described in Fig. 8 was similar to the fibroblast-mediated assay. Co-cultivation of V79 cells with primary liver cells up to 3 days in the absence of carcinogen did not increase the mutation frequency for ouabain resistance. However, when V79 cells and primary liver cells were cocultivated in the presence of 1.4 mM dimethylnitrosamine (DMN), the carcinogen-induced ouabain-resistant mutants and the mutation

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frequency increased from 48 to 84 mutants per 10^6 survivors after 24 and 48 hr of carcinogen exposure, respectively. By 72 hr there had been extensive V79 cell replication and the cell cultures were degenerating. Therefore, in all of the following experiments, we used the cocultivation time of 48 hr after addition of the chemical.

When the number of attached liver cells was increased from 0.5 to 2.0×10^6 cells in the cocultivation experiments in the presence of 1.4 mM DMN, the mutation frequency increased from 7 to 96 mutants per 10^6 survivors (Fig. 9). Increasing the number of liver cells plated, in order to use more than 2.0×10^6 attached liver cells, resulted in a reduction in V79 cell replication during the 48 hr cocultivation period and decreased the cloning efficiency of the V79 cells. To eliminate this cytotoxic effect of the liver cells on the V79 cells, all further experiments were carried out by seeding 10^7 liver cells, which gave about 2×10^6 cells attached.

To determine whether mutagenesis by DMN was dose-dependent, the V79 cells cocultivated with liver cells were treated with DMN at concentrations up to 13.5 mM (Fig. 10). After treatment the frequency of ouabain-resistant mutants increased exponentially with increasing DMN concentrations up to 1.4 mM. At 1.4 mM DMN the number of mutants was about $90/10^6$ survivors. Above 1.4 mM DMN the mutation frequency increased more slowly with increasing DMN concentrations and at 13.5 mM about 180 mutants per 10^6 survivors were obtained. These results indicated that DMN was mutagenic to V79 cells in the presence of liver cells that metabolize this carcinogen to mutagenic metabolite(s) and that the degree of mutagenesis was dependent on the number of liver cells as well as on the dose of the carcinogen.

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In addition to DMN we have included in our studies two other potent liver carcinogens, diethylnitrosamine (DEN) and aflatoxin B_1 (AFB_1) as well as their inactive analogues, nitrosomethyl-tert-butylamine (MBN) and aflatoxin G_2 (AFG_2). Treatment of V79 cells cocultivated with the liver cells with the above chemicals indicated that both carcinogens DEN and AFB_1 were mutagenic and their mutagenic response was dose-dependent while their analogues were inactive (Tables 5 and 6). These results show that in the presently described liver cell-mediated assay we have demonstrated a relationship between in vivo carcinogenicity and cell-mediated mutagenicity for two classes of liver carcinogens and their noncarcinogenic analogues. Furthermore, our liver cell-mediated mutagenesis may be more sensitive than liver microsome-mediated mutagenesis. In the present studies with 2×10^6 attached liver cells from noninduced animals, a DMN concentration of only 1.4 mM was required to enhance the mutation frequency of V79 cells about 90-fold. In other studies with microsomes from induced animals, 50 mM or more DMN was needed to obtain a similar increase in mutation frequency of V79 cells. Experiments with bacteria also required higher DMN concentrations. The liver cell-mediated assay may therefore be used as a sensitive assay for screening of chemicals hazardous to humans. This approach could be extended to cocultivation of cells from other organs with mutable mammalian cells.

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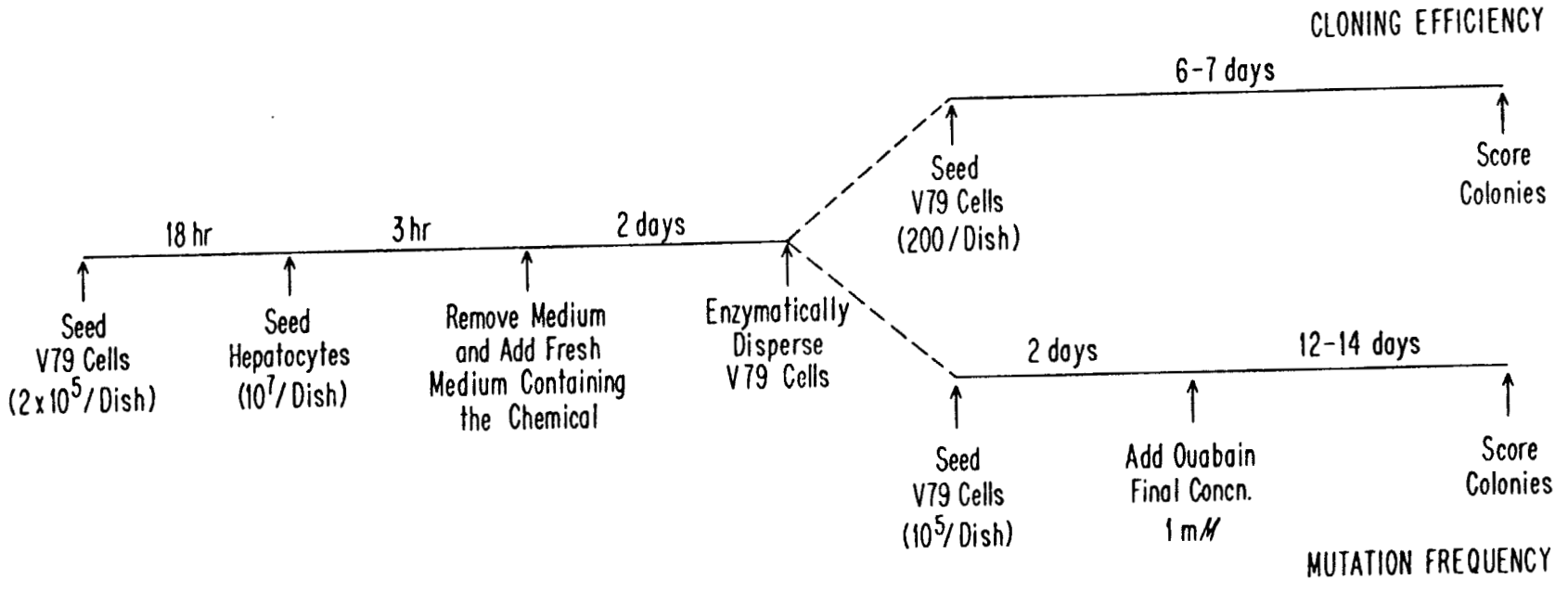

FIGURE 8. Experimental protocol for the liver cell-mediated mutagenesis assay.

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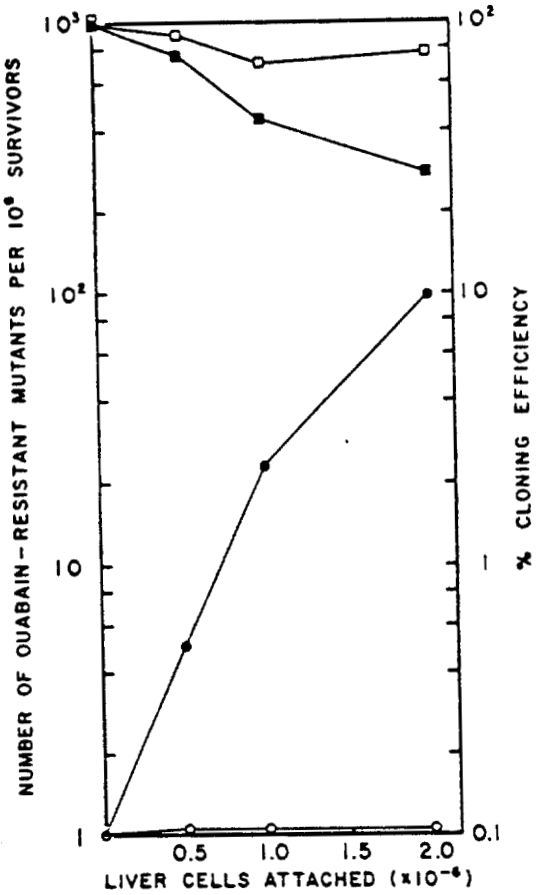

FIGURE 9. Induction of ouabain-resistant mutants in V79 cells cocultivated with different numbers of liver cells. The two cell types were cocultviated for 2 days in the presence of 1.4 mM DMN or without DMN before being reseeded to measure cloning efficiency (■, with DMN; □, without DMN) and mutation frequency (●, with DMN; O, without DMN).

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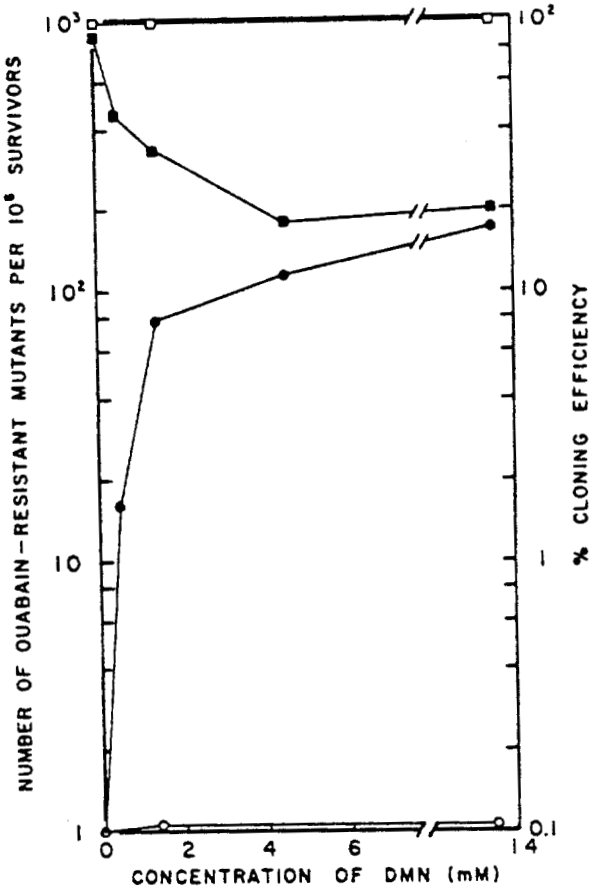

FIGURE 10. Induction of ouabain-resistant mutants after treatment with different doses of DMN. V79 cells were treated for 2 days in the presence or absence of liver cells before being reseeded to measure cloning efficiency (■, with liver cells; □, without liver cells) and mutation frequency (●, with liver cells; O, without liver cells).

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TABLE 5.

INDUCTION OF OUABAIN-RESISTANT MUTANTS BY NITROSAMINES.

Treatment*	Concentration (mM)	Cloning efficiency (%)	Ouabain-resistant mutants/10^6 survivors
Control		88	1
DMN	4.5	18	115
	13.5	20	171
DEN	0.5	94	2
	4.5	56	16
	13.5	44	30
	45.0	38	50
MBN	0.5	88	1
	4.5	84	1
	13.5	85	1

*The number of V79 cells/T-flask was 0.6×10^6 when 10^7 liver cells were seeded. The cells were treated for 2 days with the chemicals, at which time they were trypsinized and reseeded to determine cloning efficiency and mutation frequency. At this time the V79 cell number/T-flask ranged from 1.2×10^6 to 3.3×10^6.

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TABLE 6.

INDUCTION OF OUABAIN-RESISTANT MUTANTS BY AFLATOXINS.

Treatment*	Concentration (μM)	Cloning efficiency (%)	Ouabain-resistant mutants/10^6 survivors
Control		82	1
AFB_1	0.2	64	7
	1.4	40	14
	3.2	26	19
	9.6	5	67
AFG_2	0.2	89	1
	0.3	89	1
	1.4	79	1
	3.2	85	1

*See legend to Table 5.

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6. Cell specificity in metabolic activation of the potent carcinogens aflatoxin B_1 and benzo(a)pyrene to mutagens for mammalian cells.

Many environmental chemical carcinogens exhibit an organ-specific effect in experimental animals. More specifically, some carcinogens will malignantly transform liver cells while others may cause tumors derived from connective tissue cells. In view of the relationship between mutagenesis and carcinogenesis, it was of interest to study such a cell specificity in *in vitro*. To test this, we have compared the abilities of two different cell types, fibroblasts and liver cells derived from rats, to metabolize two different carcinogens to water-soluble products, to intermediates which bind to cellular DNA, and to intermediates which are mutagenic to Chinese hamster V79 cells. The two chemicals studied were benzo(a)pyrene (BP), a potent skin and lung carcinogen which can also produce fibrosarcomas and aflatoxin B_1 (AF), a potent liver carcinogen. Ouabain resistance was used as the genetic marker in V79 cells. The results indicated that BP was a potent mutagen in the V79 cells cocultivated with fibroblasts (fibroblast-mediated assay) and not when cocultivated with liver cells (liver-cell-mediated assay) (Fig. 11). The liver carcinogen AF exhibited an inverse relationship; it was a potent mutagen in the liver-cell-mediated assay but not in the fibroblast-mediated assay (Fig. 11).

The differences observed in the mutagenic activities of BP and AF in the hepatocyte- and fibroblast-mediated assays suggest that these two carcinogens are metabolized differently in each of the two cell types. Therefore, the extent of BP and AF metabolism to water-soluble metabolites by hepatocytes and fibroblasts was compared. The results indicated that

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liver cells metabolize both BP and AF to water-soluble products, while fibroblasts metabolize BP but not AF (Figs. 12 and 13). For both cell types, the metabolism of BP to water-soluble products was proportional to the cell number, the dose of the hydrocarbon, and the time of the incubation up to 48 hr. After 48 hr, BP metabolism by liver cells reached a plateau, probably due to cell degeneration, while in the fibroblasts, BP metabolism continued for an additional 24 hr. The metabolism of AF by liver cells was also proportional to the cell number, the dose of the carcinogen, and the time up to 24 hr. The liver cells showed only a limited increase in AF metabolism after 24 hr, presumably due to the cytotoxic effects of AF metabolites and degeneration of the hepatocytes in culture. The fibroblasts did not metabolize AF, and thus can explain the absence of AF mutagenicity in the fibroblast-mediated assay.

Therefore, even though on a per-cell basis, liver cells were able to metabolize BP to water-soluble products 4-5 times more effectively than fibroblasts; BP was not activated by them to a mutagen. These findings raised the question of production and/or release of mutagenic BP metabolites by the liver cells. To estimate the relative production of reactive BP intermediates by the two cell types, the binding of BP metabolites to DNA was measured. At 1 μg BP/ml and after 24 hr exposure, approximately three times as much BP was bound to the DNA of the fibroblasts as was bound to the DNA of the liver cells (Table 7). Thus, even though the liver cells metabolized four to five times as much BP to water-soluble products at 1 μg/ml, they had substantially fewer DNA-bound BP products than the fibroblasts. These findings indicate that either

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liver cells metabolize BP to less reactive intermediates as compared with fibroblasts, or that if such reactive metabolites are formed, they are rapidly detoxified.

The mutagenic activity of BP in the fibroblast-mediated assay but not in the hepatocyte-mediated assay and the inverse situation with AF are in agreement with the *in vivo* activities of these two carcinogens. However, "S-9" subcellular fractions from liver tissue have been reported to activate both carcinogens to intermediates which are mutagenic for both *Salmonella typhimurium* and V79 cells. These differences in the metabolic activation may result from the disruption in the liver subcellular preparations of the normal balance of pathways by which BP and AF are activated and detoxified in the intact liver.

Our results indicated that a cell-type specificity in chemical carcinogenesis can be investigated by the cell-mediated assay. Furthermore, while the metabolism of procarcinogens is required to manifest *in vitro* mutagenic activity, overall metabolism alone is not sufficient to predict the activity of a carcinogen in a given cell type. The discrepancy between the metabolic activation of the same chemical carcinogens by subcellular fractions as opposed to intact cells and the loss of cell specificity by the former leads us to suggest that the cell-mediated assay may provide a more useful means of screening environmental chemicals and may also be used to study tissue specificity of chemical carcinogens *in vitro*.

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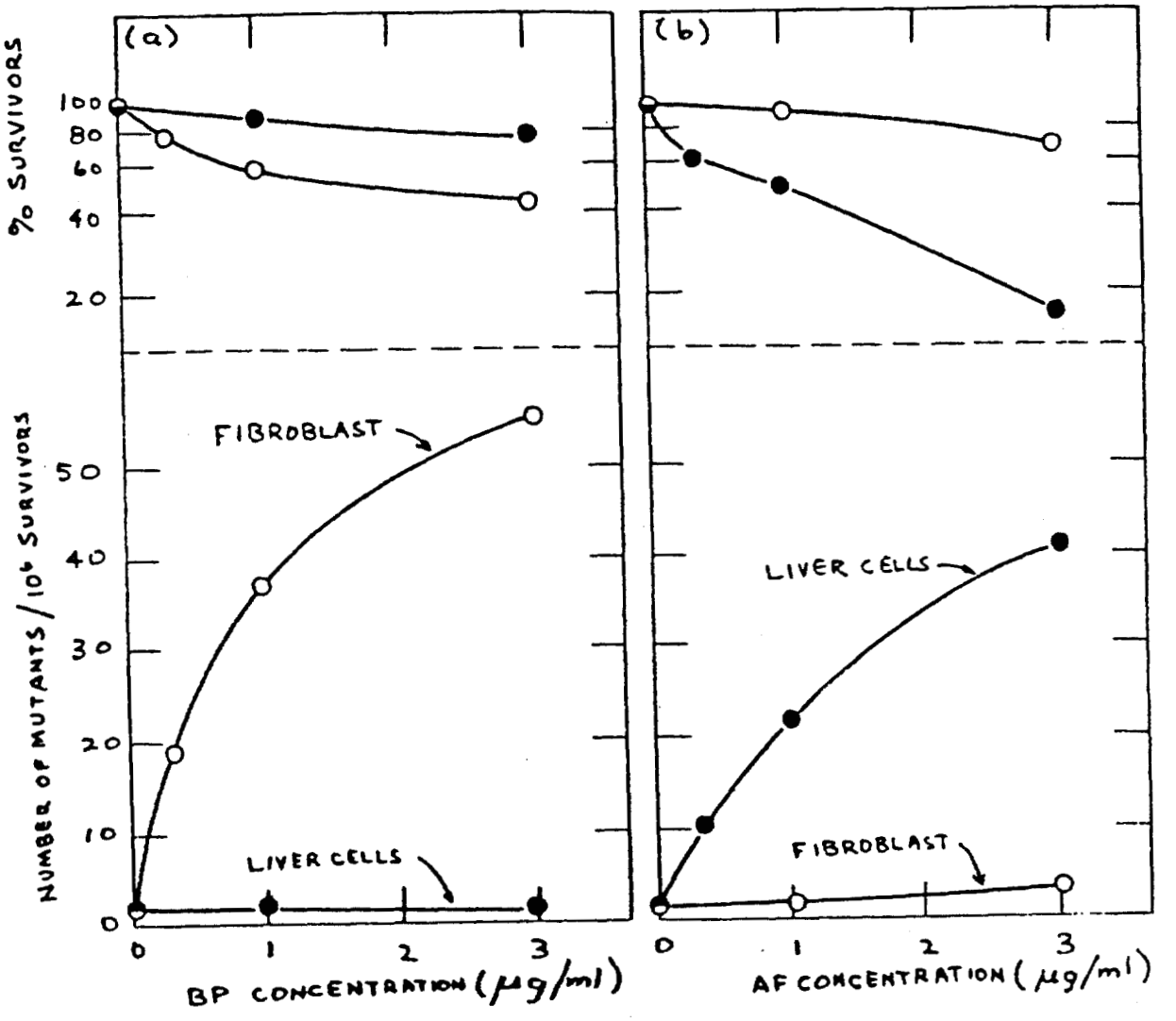

FIGURE 11. Fibroblast- and liver-cell-mediated mutagenesis by the carcinogens BP (a) and AF (b). Mutation to ouabain resistance was determined in Chinese hamster V79 cells after cocultivation with either liver cells or fibroblasts derived from Sprague-Dawley rats. The liver cells were obtained after *in vivo* collagenase perfusion of a liver from a 6- to 10-week-old male rat. V79 cells were seeded at 10^5 cells per 25-cm^2 T-flask. After 18 hr either 2 x 10^6 rat embryo fibroblasts (lethally irradiated with 5000 R) or 10^7 primary rat hepatocytes (80-90% viable by trypan blue dye exclusion and plating efficiency of 20%) were seeded on the V79 cells. After 3 hr the medium was changed and the carcinogens were added in 8 ml of fresh medium. 48 hr later the cells were dispersed with 0.05% trypsin and 0.02% EDTA, and the V79 cells were seeded at 200 cells per 50-mm Petri dish in 4 ml medium to determine cloning efficiency and at 10^5 cells per 50-mm Petri dish to determine the number of ouabain-resistant colonies. The cloning efficiency of V79 cells co-cultivated with liver or fibroblast cells was approximately 60%. After cocultivation the number of V79 cells per T-flask ranged from 1.2 to 2.8 x 10^6 cells. ●, Liver cells; O, fibroblasts.

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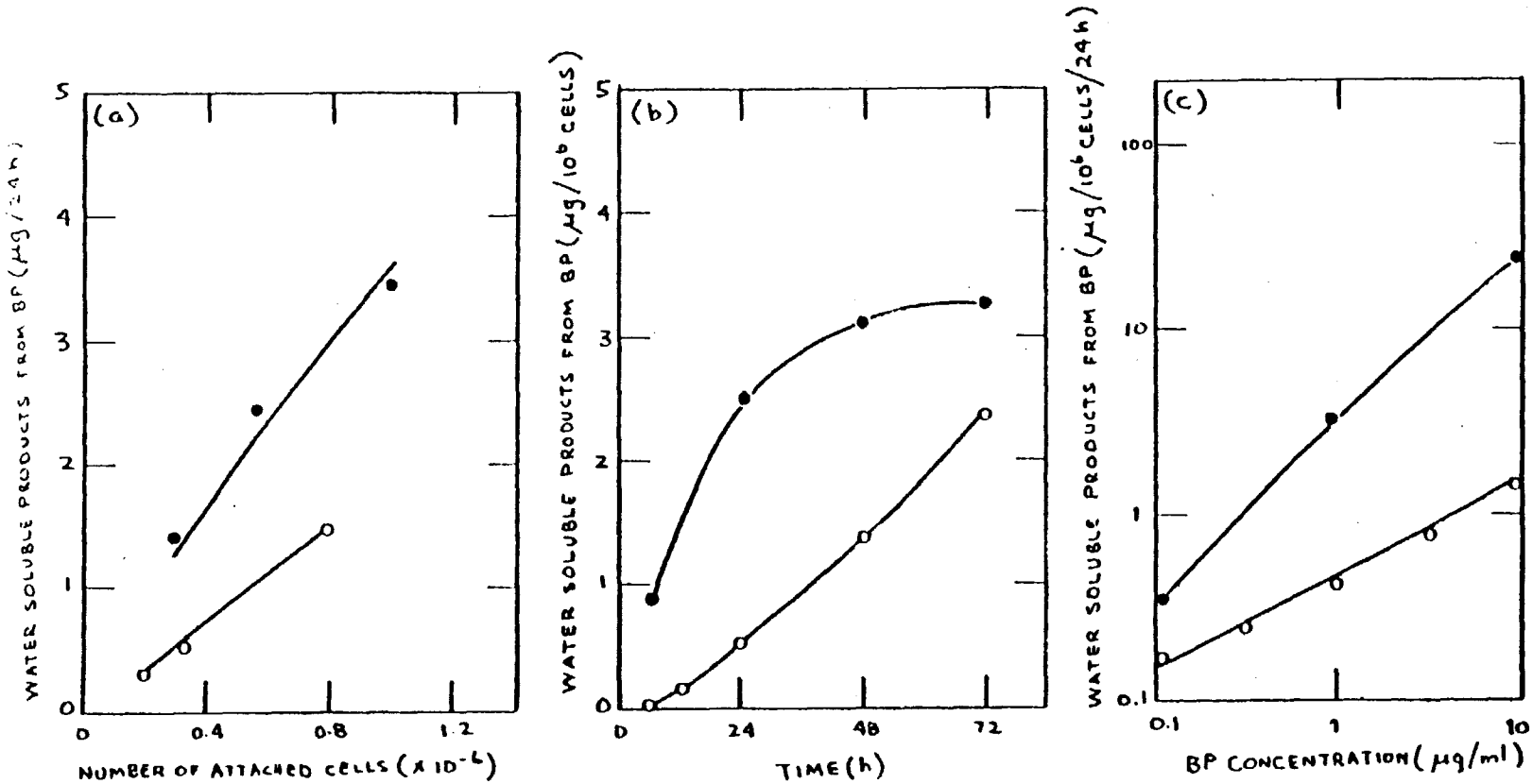

FIGURE 12. Metabolism of BP by liver cells and fibroblasts. Liver cells (plating efficiency 20%) and lethally-irradiated (5000 R) rat embryo fibroblasts (plating efficiency 80%) were seeded in 5 ml of medium into 25-cm^2 T-flasks. At 3 hr after seeding the medium was changed and 5 ml medium containing the [^{14}C]AF or [^{14}C]BP was added. The reaction was terminated by addition of 5 ml of a 1% SDS solution to each T-flask. The contents of the flask were then mixed with a pipette to ensure that all cells lysed, and 5 ml was removed and mixed with nine volumes of chloroform-methanol (2:1). The solution was vigorously mixed and then centrifuged. Duplicate 0.5-ml aliquots of the aqueous (upper) phase were removed and added to 10 ml Aquasol for counting in a Beckman Model B scintillation counter. In the absence of cells, 0.3-0.6% of the [^{14}C]BP and 3.0-4.5% of the [^{14}C]AF were soluble in the aqueous phase; this was considered as background. ●, Liver cells; 0, fibroblasts.

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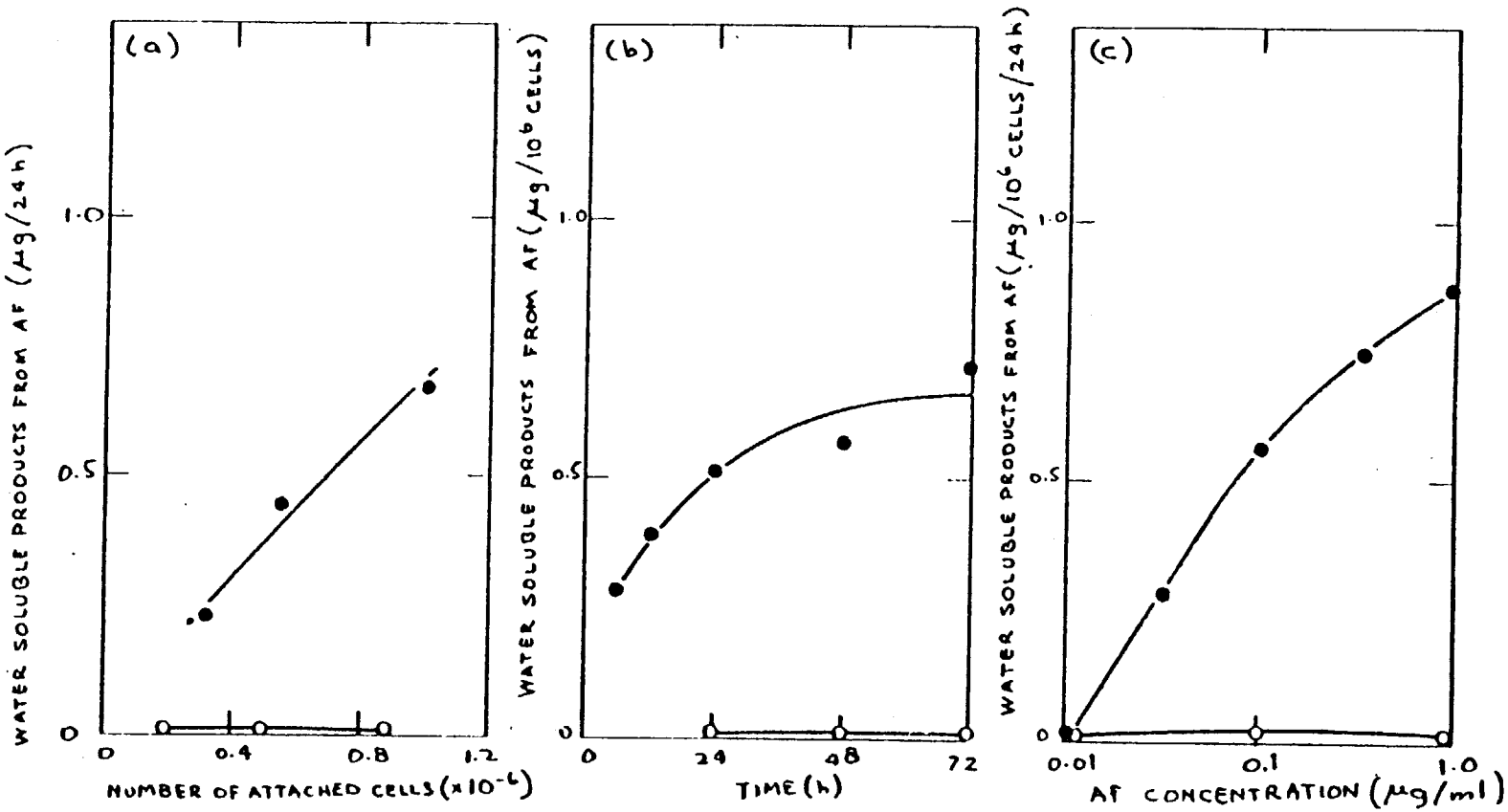

FIGURE 13. Metabolism of AF by liver cells and fibroblasts. For details see the legend to Figure 12. ●, Liver cells; 0, fibroblasts.

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TABLE 7.

BINDING OF BP TO THE DNA OF FIBROBLASTS AND LIVER CELLS.

	BP Binding (ng bound BP/mg DNA) after treatment with:	
Cell Type	1.0 μg/ml	0.1 μg/ml
Fibroblasts	8.9 ± 3.6	2.0 ± 0.8
Liver Cells	3.2 ± 0.4	1.0 ± 0.2

The binding of BP to cellular DNA was determined 24 hr after treatment with BP of 10^7 fibroblasts and 7×10^6 liver cells grown in 10 ml of medium per 100-mm Petri dish. For isolation of the DNA-BP products, the treated cells were trypsinized, washed extensively with phosphate-buffered saline (pH 7.4), and then lysed in buffer containing 10 mM Tris-EDTA, 10 mM NaCl, and 1% SDS (pH 7.4). The lysates were extracted twice with an equal volume of distilled phenol, once with an equal volume of 1 part phenol to 1 part chloroform-isoamyl alcohol (v/v 24:1), and a further three times with chloroform-isoamyl alcohol (24:1), resulting in a clear interface. The nucleic acids were precipitated from the aqueous phase with 2.5 volumes of absolute ethanol and centrifuged at 1000 g for 10 min at 4°C. The precipitate was dissolved in 0.1 SSC (0.015 M NaCl-0.0015 M sodium citrate), and the RNA was digested with 50 U ribonuclease A/ml for 40 min at 37°C. Phenol and chloroform extractions were repeated as described above, and the DNA was precipitated with 2.5 volumes of absolute ethanol in the presence of 0.1 M NaCl. The precipitate was dissolved in H_2O, and the DNA was determined quantitatively by absorbance at 260 μm. The ratio of absorbance at 260 μm to that at 280 μm was 1.95 ± 0.05. The BP bound to DNA was determined by dissolving an aliquot in scintillation solution and counting the samples in a Tri-Carb liquid scintillation spectrometer. Both ^{14}C-labeled BP (62.3 mCi/mmol) and ^{3}H-labeled BP (4.4 Ci/mmol) were used in the binding studies.

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7. <u>γ-Glutamyl transpeptidase and malignant transformation of cultured liver cells.</u>

<u>In vitro</u> mammalian cell culture systems are useful for studying the mechanism of chemical carcinogenesis as well as for identifying potential carcinogens in our environment. In such experiments, the detection of malignant cell transformation is studied frequently in fibroblasts, even though the most prevalent human tumors are derived from epithelial cell origin. The fibroblasts are used mainly because of their ease of cultivation and because only a few reproducible and objective criteria are available for predicting tumorigenicity in cultured epithelial cells.

γ-Glutamyl transpeptidase (GGT), an enzyme presumably involved in the transport of neutral amino acids across cell membranes, was found to be present in hepatomas and preneoplastic liver nodules but absent in adult liver. It was, therefore, of interest to determine whether this enzyme might also be related to malignant transformation in cultured liver cells. In recent years, a series of methods has been developed for the isolation and long-term maintenance of epithelial cells derived from liver. Once established, such cells can be transformed <u>in vitro</u> both spontaneously and by different chemical carcinogens. GGT activity was analyzed in nine such cultured epithelial cell lines derived from rat liver to determine a possible relationship between this enzyme activity and malignant transformation of liver cells in culture. For comparison, eight different normal and transformed hamster and mouse fibroblastic cell lines, which are commonly used in chemical carcinogenesis studies, were also included. The presence or absence of GGT activity was determined cytochemically. GGT activity was prominent (25 to 90% of cells) in three of five malignant

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epithelial liver cell lines. None of the four nonmalignant epithelial cell lines exhibited GGT activity (Table 8).

These studies indicated that transformation of cell lines derived from rat liver is associated with acquisition of GGT activity in a considerable proportion of cell cultures. Nontransformed and transformed fibroblasts derived from hamsters and mice were uniformly negative for GGT activity. The liver cell findings are especially noteworthy, in view of observations that, *in vivo*, most rat hepatocellular carcinomas and preneoplastic hepatocellular nodules are positive for GGT activity.

We, therefore, suggest that this simple stain for enzymatic activity, in conjunction with other cellular markers, may help in identifying tumorigenic cells in epithelial liver cultures.

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TABLE 8.

GGT in malignantly transformed cells *in vitro*

Cell line	Time in culture when tested for GGT (wk)	GGT-positive cells (%)	Intensity of GGT in positive cells	Time in culture when tested for tumorigenicity (wk)	Tumors/inoculated animals	Tumor type[a]
Derived from rat liver						
IAR-6	26	0	-	23	0/ 4	
IAR-6-1	65	90	+++	35	6/ 6	6 EP
IAR-6-1-RT7	12	25	++	12	15/15	15 EP
IAR-20A	40	0	-	35	0/ 5	
IAR-20B	32	0	-	31, 37	0/11	
IAR-20-PC1-3	21	0	-	21	0/ 7	
IAR-20-PC1-3	54	1	+	30	6/ 6	4 EP, 1 MX, 1 AT
IAR-27	73	80	+++	63	3/ 3	3 EP
IAR-2-19	74	0	-	51	2/ 3	1 EP, 1 MX
Derived from Golden hamster embryos						
Ham normal	1	0	-	1	0/ 5	
Ham MCA1	35	0	-	35	5/ 5	
Ham MCA1 Tu	8	0	-	8	5/ 5	
Ham MCA2	38	0	-	38	5/ 5	
Ham MCA2 Tu	12	0	-	12	5/ 5	
Ham NQO	42	0	-	42	5/ 5	
Derived from BALB mouse embryos						
3T3	?	0	-	NT[b]	-	
3T3 SV40	?	0	-	NT	-	

[a]EP, epithelial tumors; MX, mixed tumors (epithelial and anaplastic); AT, anaplastic tumor. The tumors observed with hamster embryo cells are all mesenchymal tumors.

[b]NT, not tested.

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8. Cell Susceptibility of Chinese hamster V79 cells to the cytotoxic and mutagenic effect of carcinogenic metals.

Metals are common contaminants in our environment as a result of coal combustion, as by-products of a variety of industries and as ingredients of human and veterinary medicines. Epidemiological studies have implicated some metals as being carcinogenic to humans. In spite of this information, we have poor knowledge of the possible mechanism of action of such carcinogenic metals. We have, therefore, studied the susceptibility of V79 cells to the cytotoxic and mutagenic effect of carcinogenic metals as well as their ability to induce cell transformation in normal embryonic hamster cells. Cell susceptibility to the cytotoxic effect was determined by the ability of the V79 cells to form cell colonies after pretreatment for 4 hr with different metal concentrations. The metals could be divided into four groups with respect to the Do, a dose that results in a survival fraction of 37% colony forming cells. One group included $CaCrO_4$ and $CdCl_2$ with a Do of 13 $\pm$ 0.5 μM. The second group included only Na_2HAsO_4 with a Do of about 0.15 mM. The third group included $CoCl_2$ and $NiCl_2$ with a Do of 0.62 $\pm$ 0.2 mM, while the fourth group included $BeCl_2$ and $MnCl_2$ with a Do of 6 $\pm$ 2 mM.

Mutagenesis by carcinogenic metals (Be^{++}, Ni^{++}, and Cr^{+6}), as well as by the noncarcinogenic ones (Mg^{++} and Ca^{++}) were tested in the V79 cells. Resistance to ouabain and 6-thioguanine was used as the genetic markers. None of the tested metals induce ouabain resistance. Induction of up to fivefold higher than the untreated controls was obtained with 6-thioguanine resistance after treatment with $CaCrO_4$ but not with the other carcinogenic metals.

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In addition to the above, we have tested carcinogenic metals for their ability to induce cell transformation in vitro. Morphological transformation was determined after treatment of secondary golden hamster embryo cells seeded for colony formation on a feeder layer of X-irradiated rat embryo cells. Transformed colonies were obtained after treatment with 0.1-3 μM of $CaCrO_4$ with a frequency of up to about 1%. No such morphologically transformed colonies could be detected after treatment with 0.1 - 5 mM $BeCl_2$.

These results suggest that only in the case of $CaCrO_4$ can somatic mutation be considered as a possible mechanism for its carcinogenic effect.

9. Induction of Terminal Differentiation in Human Promyelocytic Leukemia Cells by Tumor-Promoting Agents.

Our present state of knowledge indicates that carcinogensis is a multistage process that may require a step or steps beyond the initial mutational event (as presented in previous parts of this report). Thus it is possible that some environmental causative agents may act at the later stages of the carcinogenic process in a mechanism related to tumor promotion. Many tumor promoters are devoid of mutagenic activity and may therefore act via an epigenetic event. An example of such an event is the process of differentiation. It is, therefore, of interest to study the effect of tumor-promoting agents on cell differentiation.

Established cell lines with appropriate markers for cell differentiation offer simple models for study of the control of cell growth and differentiation. These cell culture systems also allow the identification and study of the mode of action of chemicals that may control cell differentiation. Phorbol-12-myristate-13-acetate (PMA) and other related phorbol esters,

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which are tumor promoters, were recently found to inhibit spontaneous and induced cell differentiation in avian myoblasts and in murine erythroleukemia, neuroblastoma, and adipose cells. These experiments suggest that tumor-promoting agents may mimic natural cellular agents that are involved in the regulation of cell growth and differentiation. Therefore it was important to determine whether this class of chemicals can also affect cell growth and differentiation in human cells. In view of this, we have undertaken to examine the response of human HL-60 promyelocytic leukemia cells to a series of different tumor-promoting agents. These human cells were used because they display distinct biological and morphological commitments towards differentiation. Our studies with the phorbol diesters indicated that these tumor-promoting agents can efficiently induce terminal differentiation in the HL-60 cells. Differentiation in the HL-60 was determined by an increase in the percent of myelocytes, metamyelocytes and other mature myeloid cells as well as by an increase in the percent of phagocytizing cells (Figs. 14 and 15). Induction of differentiation could be determined after 2 days of treatment with phorbol-12-myristate-13-acetate at a dose as low as 6×10^{-11} M. A correlation was found between reported tumor-promoting activity of a series of phorbol esters and their ability to induce myeloid differentiation and to inhibit cell growth. We suggest that tumor-promoting agents
differentiation at extremely low concentrations in these cells
an approach to study the control of cell growth, cell differentiation, and malignancy in human leukemic cells.

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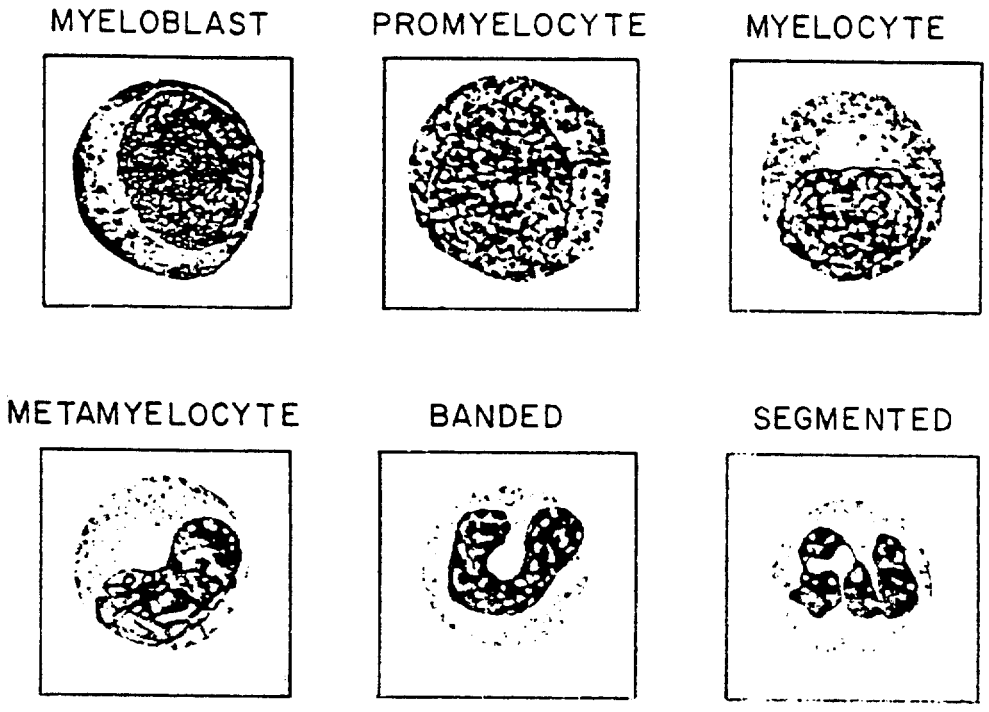

FIGURE 14. Scheme for myeloid differentiation.

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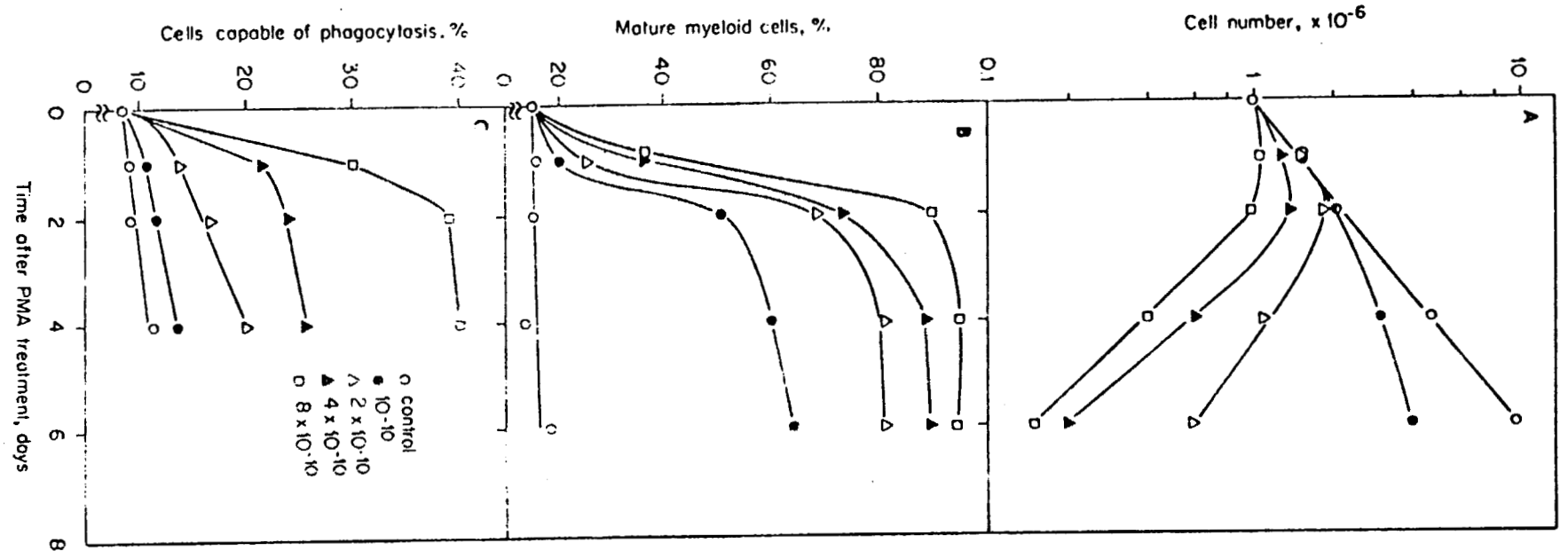

FIGURE 15. Cell growth(A), differentiation (B), and phagocytosis (C), in the myeloid leukemia HL-60 cells at different times after treatment with various concentrations of the tumor promoter PMA. The cultures were treated 1 day after 5 X 10^5 cells were seeded in 5 ml of growth medium in 60-mm Petri dishes. Cells were counted to determine cell growth and stained with Wright-Giemsa stain to determine the percent of mature myeloid and phagocytizing cells. The mature myeloid cells were composed mainly from myelocytes and metamyelocytes. A small fraction of banded and segmented neutrophils was also observed. Phagocytosis was determined after the HL-60 cells were incubated for 30 min with 4 x 10^6 per ml of the diploid Saccharomyces cerevisiae strain XY 664.

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10. Stimulation of Differentiated Functions in Human Melanoma Cells by Tumor-Promoting Agents and Dimethylsulfoxide.

Our studies with the promeylocytic leukemia cells (see chapter 9) imply that either myeloid leukemia cells respond differently from other cell types or that some human cells, including these leukemia cells, respond differently from most tested mouse and chicken cells to the phorbol diesters. In view of this, it was important to determine whether this class of chemicals could also induce cell differentiation in other human cells. We examined the response of human HO melanoma cells to a series of tumor-promoting agents. These melanoma cells offer a readily quantifiable marker of differentiation (melanin formation) as well as a morphological marker (dendrite-like structure formation). Both of these markers are characteristic of normal differentiating melanocytes. In addition to the tumor-promoting agents, we treated HO cells with dimethyl sulfoxide (DMSO), which has been found to be an efficient inducer of differentiation in some murine and human cells. Our studies indicate that both the tumor-promoting agents and DMSO can efficiently stimulate terminal differentiation in the treated melanoma cells.

Treatment of the HO melanoma cells with phorbol-12-myristate-13-acetate (PMA) at (5×10^{-10})-(5×10^{-7}) M resulted in a dose related inhibition of growth and a stimulation of differentiated functions. These included melanin synthesis and formation of dendrite-like structures. Higher doses of phorbol dibutyrate, a less potent tumor promoter, were required to produce an effect comparable to that of PMA for dendrite induction. Phorbol and two other phorbol esters which lack tumor-promoting activity were either inactive or elicited a poor response. In addition to morphological changes, treatment

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with PMA altered glucosamine incroporation into membrane gangliosides. After PMA treatment, glucosamine incorporation increased 8- to 10-fold in the GM_3 ganglioside and decreased 2-fold in the GM_1 ganglioside, as compared to phorbol or untreated control. Inhibition of cell growth and stimulation of melanin synthesis were also observed after treatment of the HO cells with DMSO. Unlike the tumor-promoting agents, DMSO did not induce the formation of dendrite-like structures in the cells. These findings indicate that stimulation of differentiated functions can also be observed in HO melanoma cells after treatment with tumor-promoting agents such as phorbol-diesters.

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Problem Areas

a) In order to be able to use the cell-mediated mutagenesis assay as a sensitive screen for potential carcinogens, the performance of the carcinogen metabolizing liver cells has to be improved. The present perfusion techniques for obtaining primary hepatocytes are not suitable enough to give a population of cells with an ability to activate some classes of chemical carcinogens such as the nitrosamines at low dose levels.

b) There is the possibility that humans may exhibit a cell susceptibility to some carcinogens which is different from that of rodents, due to the difference in carcinogen metabolism and/or DNA repair. It is, therefore, important to develop a human cell-mediated mutagenesis assay for screening of chemicals hazardous to humans.

c) Although some types of mammalian DNA repair after carcinogen insults are known, their exact relationship to cytotoxicity and mutagenicity is not clear.

d) Several unique biological properties of tumor-promoting phorbol esters have been demonstrated in cultured human cells, including inhibition of growth and induction of differentiation, but the significance of these effects with respect to the mechanism of malignant cell transformation and tumor promotion remains to be elucidated. We also do not understand the relationship between the effect of tumor-promoting agents on membrane associated functions and their ability to induce terminal cell differentiation in the human cells.

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A. SUMMARY DOCUMENT

Name and Address of Institution:

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Oak Ridge National Laboratory
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Oak Ridge, Tennessee 37830

Address of Principal Investigator:

Biology Division
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P.O. Box Y
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Title of Project:

Malignant Cell Transformation and Mutagenesis Induced by Carcinogenic Chemicals

NCI Interagency Agreement No.
Y01 CP 70222

Principal Investigator
Eliezer Huberman (50%)

Total Hours of Other Support Personnel: 140 Hours/Week

Date of Submission: July 6, 1979

1078713

B. AGREEMENT WORKSCOPE

1. Induction of malignant cell transformation in cells in culture by chemical carcinogens as well as the effect of mixtures of chemicals on cell transformation.
2. Induction of mutations at different genetic loci by chemical carcinogens. The endpoints to be used are resistance to ouabain and 8-azaguanine or 6-thioguanine and temperature sensitivity.

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C. TECHNICAL SUMMARY

It is now well recognized that our environment is contaminated with many unknown hazardous chemicals, some of which may be responsible for a significant proportion of human cancers. These carcinogenic chemicals represent a fraction of the man-made chemicals which are present in the human environment. It is therefore important to develop fast and accurate pre-screening methods which are inexpensive relative to the current *in vivo* test systems. Mammalian, including human, cell culture systems are extremely useful for such purposes. In the present proposal, we shall introduce cell culture systems which may be useful for the screening of chemicals that initiate or promote tumor formation. These systems may also be helpful in the elucidation of the mode of action of such potentially hazardous chemicals.

Our proposed experiments will, therefore, be concerned with the:

1. Improvement of the performance of the liver cell-mediated mutagenesis assay.
2. Isolation of mutagen-resistant cells for mutagenesis and DNA repair studies.
3. Use of human cells in mutagenesis studies.
4. Induction of different markers of differentiation in human malignant cells by tumor promoting agents.
5. Alterations in lipid biosynthesis in differentiating human tumor cells.

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D. ANNUAL REPORT SUMMARY

a) Scientific Progress

We have, in the last couple of years, developed a cell-mediated mutagenesis assay for testing the mutagenic effect of chemical carcinogens. In this assay, Chinese hamster V79 cells, which do not exhibit mixed function oxidase activities and therefore do not activate many chemical carcinogens, are co-cultivated with non-dividing cells - namely primary hepatocytes or X-irradiated fibroblasts that contain the enzymes required for metabolic activation. Using this cell-mediated mutagenesis system, it was possible to demonstrate a relationship between carcinogenicity and mutagenicity for polycyclic hydrocarbons, nitrosamines and aflatoxins, to identify the mutagenic metabolites of benzo(a)pyrene, benz(a)anthracene and 7,12-dimethylbenz(a)anthracene, and to show that the metabolic activation of carcinogens to mutagens is cell specific.

However, many of the short-term assays, including this mammalian cell-mediated mutagenesis assay, are designed to detect carcinogens-mutagens, and therefore, are not suitable for studying promoting agents, many of which are devoid of mutagenic activity. Tumor promoters may act via an epigenetic event in a process resembling cell differentiation. Indeed, our studies with human myeloid leukemia and melanoma cells indicate that tumor-promoting phorbol esters are capable of inducing differentiation. Induction of differentiation in these cells was directly related to the tumor promoting activity of a series of phorbol esters. Thus, these cell systems may help in identifying potential hazardous chemicals and allow the study of promotion, control of cell growth, differentiation and malignancy.

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Another approach to studying the mechanism of chemical carcinogenesis is by using *in vitro* malignant cell transformation assays. Cell transformation is studied frequently in fibroblasts, even though the most prevalent human tumors are derived from epithelial cell origin. The fibroblasts are used mainly because only a few reproducible and objective criteria are available for predicting tumorigenicity in cultured epithelial cells.

Our studies indicated that transformation of cell lines derived from rat liver is, in many cases, associated with the acquisition of γ-glutamyl transpeptidase (GGT) activity, as measured by a simple staining procedure.

It is, therefore, possible that by using this simple stain for GGT activity, in conjunction with other cellular markers, to identify tumorigenic cells in epithelial liver cell cultures, after treatment with chemical carcinogens.

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b) Problem Areas

1) In order to be able to use the cell-mediated mutagenesis assay as a sensitive screen for potential carcinogens, the performance of the carcinogen metabolizing liver cells has to be improved. The present perfusion techniques for obtaining primary hepatocytes are not suitable enough to give a population of cells with an ability to activate some classes of chemical carcinogens such as the nitrosamines at low dose levels.

2) There is the possibility that humans may exhibit a cell susceptibility to some carcinogens which is different from that of rodents, due to the difference in carcinogen metabolism and/or DNA repair. It is, therefore, important to develop a human cell-mediated mutagenesis assay for the screening of chemicals hazardous to humans.

3) Although some types of mammalian DNA repair following carcinogen insults are known, their exact relationship to cytotoxicity and mutagenicity is not clear.

4) Several unique biological properties of tumor-promoting phorbol esters have been demonstrated in cultured human cells, including inhibition of growth and induction of differentiation, but the significance of these effects with respect to the mechanism of malignant cell transformation and tumor promotion remains to be elucidated. We also do not understand the relationship between the effect of tumor-promoting agents on membrane-associated functions and their ability to induce terminal cell differentiation in the human cells.

1078718

E. SUPPORT DATA

List of Publications (NCI Interagency Agreement No. YO CP 70222)

E. Huberman

1. Huberman, E. Cell transformation and mutability of different genetic loci in mammalian cells by metabolically activated carcinogenic polycyclic hydrocarbons. In: Polycyclic Hydrocarbons and Cancer: Chemistry, Molecular Biology and Environment. H. V. Gelboin and Ts'o, P.O.P., eds., Academic Press, Inc., Vol. 2, pp 161-174, 1978.

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4. Huberman, E. Mutagenesis and cell transformation of mammalian cells in culture by chemical carcinogens. J. Environ. Pathol. Toxico., 2, 29-42, 1978.

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6. Huberman, E., and Langenbach, R. Cell-mediated mutagenesis and cell transformation by chemical carcinogens. In: Tumors of Early Life in Man and Animals. L. Severi, ed. Institute of Morbid Anatomy and Histology, Perugia, Italy pp. 745-759, 1978.

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9. Huberman, E., Montesano, R., Drevon, C., Kuroki, T., St. Vincent, L., Pugh, T. D., and Goldfarb, S. γ-Glutamyl transpeptidase and malignant transformation of cultured liver cells. Cancer Res. 39, 269-272, 1979.

10. Huberman, E. and Slaga, T. J. Mutagenicity and tumor-initiating activity of fluorinated 7, 12-dimethylbenzo(a)anthracenes. Cancer Res. 39, 411-414, 1979.

11. Huberman, E., Chou, M. W. and Yang, S. Identification of 7,12-dimethylbenz(a)anthracene metabolites that lead to mutagenesis in mammalian cells. Proc. Natl. Acad. Sci. USA 76, 862-866, 1979.

12. Huberman, E. and Callaham, M. Induction of terminal differentiation in human promyelocytic leukemia cells by tumor promoting agents. Proc. Natl. Acad. Sci. USA 76, 1293-1297, 1979.

13. Slaga, T. J., Huberman, E., DiGiovanni, J. and Gleason, G. The importance of the "bay region" diol-epoxide in 7,12-dimethylbenz(a)anthracene skin tumor initiation and mutagenesis. Cancer Letters 6, 213-220, 1979.

14. Huberman, E., Heckman, C. and Langenbach, R. Stimulation of differentiated functions in human melanona cells by tumor-promoting agents and dimethylsulfoxide. Cancer Res. 39, 2618-2624, 1979.

15. Huberman, E. Induction of mutagenesis and alterations in gene expression by tumorigenic chemicals. In: Short-Term Tests for Prescreening of Potential Carcinogens. L. Santi and S. Parodi, eds. Instituto Scientifico Per Lo Studio E La Cura Dei Tumori, Genova, pp. 110-117, 1979.

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Direct Costs Proposed in this Renewal

	Direct Labor	Materials and Supplies	Other Costs	Total
First Year	$82700	$69000	$ 8800	$160500
Second Year	88500	76000	10000	124500
Third Year	94700	83700	11200	189600

Financial Support to Professional Personnel

E. Huberman: Chemical Carcinogenesis of Cell *In Vitro*

EPA Interagency Agreement Number: 79-D-X0533

October 1, 1978 - September 30, 1979

1078721

F.

DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE

PROTECTION OF HUMAN SUBJECTS ASSURANCE/CERTIFICATION/DECLARATION

☐ ORIGINAL ☐ FOLLOWUP ☐ REVISION

☐ GRANT ☒ CONTRACT ☐ FELLOW ☐ OTHER

☐ NEW ☐ RENEWAL ☐ CONTINUATION

APPLICATION IDENTIFICATION NUMBER *(If known)*

STATEMENT OF POLICY: Safeguarding the rights and welfare of subjects at risk in activities supported under grants and contracts from DHEW is primarily the responsibility of the institution which receives or is accountable to DHEW for the funds awarded for the support of the activity. In order to provide for the adequate discharge of this institutional responsibility, it is the policy of DHEW that no activity involving human subjects to be supported by DHEW grants or contracts shall be undertaken unless the Institutional Review Board has reviewed and approved such activity, and the institution has submitted to DHEW a certification of such review and approval, in accordance with the requirements of Public Law 93-348, as implemented by Part 46 of Title 45 of the Code of Federal Regulations, as amended, (45 CFR 46). Administration of the DHEW policy and regulation is the responsibility of the Office for Protection from Research Risks, National Institutes of Health, Bethesda, Md 20014.

1. TITLE OF PROPOSAL OR ACTIVITY

Malignant Cell Transformation and Mutagenesis Induced by Carcinogenic Chemicals

2. PRINCIPAL INVESTIGATOR/ACTIVITY DIRECTOR/FELLOW

Eliezer Huberman

3. DECLARATION THAT HUMAN SUBJECTS EITHER WOULD OR WOULD NOT BE INVOLVED

☒ A. NO INDIVIDUALS WHO MIGHT BE CONSIDERED HUMAN SUBJECTS, INCLUDING THOSE FROM WHOM ORGANS, TISSUES, FLUIDS, OR OTHER MATERIALS WOULD BE DERIVED, OR WHO COULD BE IDENTIFIED BY PERSONAL DATA, WOULD BE INVOLVED IN THE PROPOSED ACTIVITY. (IF NO HUMAN SUBJECTS WOULD BE INVOLVED, CHECK THIS BOX AND PROCEED TO ITEM 7. PROPOSALS DETERMINED BY THE AGENCY TO INVOLVE HUMAN SUBJECTS WILL BE RETURNED.)

☐ B. HUMAN SUBJECTS WOULD BE INVOLVED IN THE PROPOSED ACTIVITY AS EITHER: ☐ NONE OF THE FOLLOWING, OR INCLUDING: ☐ MINORS, ☐ FETUSES, ☐ ABORTUSES, ☐ PREGNANT WOMEN, ☐ PRISONERS, ☐ MENTALLY RETARDED, ☐ MENTALLY DISABLED. UNDER SECTION 6. COOPERATING INSTITUTIONS, ON REVERSE OF THIS FORM, GIVE NAME OF INSTITUTION AND NAME AND ADDRESS OF OFFICIAL(S) AUTHORIZING ACCESS TO ANY SUBJECTS IN FACILITIES NOT UNDER DIRECT CONTROL OF THE APPLICANT OR OFFERING INSTITUTION.

4. DECLARATION OF ASSURANCE STATUS/CERTIFICATION OF REVIEW

☐ A. THIS INSTITUTION HAS NOT PREVIOUSLY FILED AN ASSURANCE AND ASSURANCE IMPLEMENTING PROCEDURES FOR THE PROTECTION OF HUMAN SUBJECTS WITH THE DHEW THAT APPLIES TO THIS APPLICATION OR ACTIVITY. ASSURANCE IS HEREBY GIVEN THAT THIS INSTITUTION WILL COMPLY WITH REQUIREMENTS OF *DHEW Regulation 45 CFR 46,* THAT IT HAS ESTABLISHED AN INSTITUTIONAL REVIEW BOARD FOR THE PROTECTION OF HUMAN SUBJECTS AND, WHEN REQUESTED, WILL SUBMIT TO DHEW DOCUMENTATION AND CERTIFICATION OF SUCH REVIEWS AND PROCEDURES AS MAY BE REQUIRED FOR IMPLEMENTATION OF THIS ASSURANCE FOR THE PROPOSED PROJECT OR ACTIVITY.

☐ B. THIS INSTITUTION HAS AN APPROVED GENERAL ASSURANCE (DHEW ASSURANCE NUMBER _____) OR AN ACTIVE SPECIAL ASSURANCE FOR THIS ONGOING ACTIVITY, ON FILE WITH DHEW. THE SIGNER CERTIFIES THAT ALL ACTIVITIES IN THIS APPLICATION PROPOSING TO INVOLVE HUMAN SUBJECTS HAVE BEEN REVIEWED AND APPROVED BY THIS INSTITUTION'S INSTITUTIONAL REVIEW BOARD IN A CONVENED MEETING ON THE DATE OF _____ IN ACCORDANCE WITH THE REQUIREMENTS OF THE *Code of Federal Regulations on Protection of Human Subjects (45 CFR 46).* THIS CERTIFICATION INCLUDES, WHEN APPLICABLE, REQUIREMENTS FOR CERTIFYING FDA STATUS FOR EACH INVESTIGATIONAL NEW DRUG TO BE USED (SEE REVERSE SIDE OF THIS FORM).

THE INSTITUTIONAL REVIEW BOARD HAS DETERMINED, AND THE INSTITUTIONAL OFFICIAL SIGNING BELOW CONCURS THAT:

EITHER ☒ HUMAN SUBJECTS WILL NOT BE AT RISK; OR ☐ HUMAN SUBJECTS WILL BE AT RISK.

5. AND 6. SEE REVERSE SIDE

7. NAME AND ADDRESS OF INSTITUTION

Oak Ridge National Laboratory
Biology Division, P.O. Box Y
Oak Ridge, TN 37830

8. TITLE OF INSTITUTIONAL OFFICIAL

Principal Investigator

TELEPHONE NUMBER

(615) 574-0698

SIGNATURE OF INSTITUTIONAL OFFICIAL

E. Huberman

DATE

HEW-596 (Rev. 4-75)

ENCLOSE THIS FORM WITH THE PROPOSAL OR RETURN IT TO REQUESTING AGENCY.

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G. CURRICULUM VITAE

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Marital Status: [redacted]

[redacted]

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1971 Visiting Scientist in the Etiology Branch
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1973 - 1976 Senior Scientist in the Department of Genetics
Weizmann Institute of Science
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1976 Associate Professor in the Department of Genetics
Weizmann Institute of Science
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1976 - pres. Group Leader, Carcinogenesis Program
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1978 - 1981 Member of the National Institutes of Health's
Carcinogenesis Program Scientific Review Committee

Eliezer Huberman

List of Scientific Publications

1. Huberman, E. and Sachs, L. Cell susceptibility to transformation and cytotoxicity by the carcinogenic hydrocarbon benzo(a)pyrene. Proc. Natl. Acad. Sci. U.S.A., 56, 1123-1129, 1966.

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1078731

A. TECHNICAL PROPOSAL

Name and Address of Institution:

Biology Division
Oak Ridge National Laboratory
P.O. Box Y
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Address of Principal Investigator:

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Title of Project:

Malignant Cell Transformation and Mutagenesis Induced by Carcinogenic Chemicals

NCI Interagency Agreement No.
Y01 CP 70222

Principal Investigator
Eliezer Huberman (50%)

Total Hours of Other Support Personnel: 140 Hours/Week

Date of Submission: July 6, 1979

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C. INTRODUCTION

Medical observations and retrospective epidemiological surveys as well as experiments on animals provide evidence that some man-made and natural chemicals in the environment may be responsible for a significant proportion of human cancers (Higginson, 1972). A major concept in carcinogenesis is the somatic mutation hypothesis which implies that malignancy is the consequence of an alteration in the genetic material of a treated cell. This hypothesis is supported by the elegant experiments of the Millers which indicated that electrophylic metabolites are generated from many chemical carcinogens by the action of mixed function oxidases. These reactive intermediates are capable of binding to cellular macromolecules, including DNA, to presumably initiate a mutagenic and/or carcinogenic event (Miller and Miller, 1974; Miller, 1978).

Based on these and other studies, it was possible, by determining mutagenic acitivity, to devise simple *in vitro* systems for the identification of potential chemical carcinogens. Recently, Ames and his associates have developed a simple assay for testing mutagenicity by chemical carcinogens using their *Salmonella typhimurium* as the target organism for mutagenesis and liver homogenates for carcinogen activation (Ames *et al.*, 1973; McCann *et al.*, 1975). Based on a consistent finding that the majority of the tested carcinogens were mutagenic, Ames has proposed that the assay be used to screen potentially hazardous compounds (McCann, *et al.*, 1975). However, liver subcellular fractions readily activate a wide spectrum of compounds into potent mutagens including those which are not necessarily carcinogenic for the liver. In addition, subcellular preparations differ from intact cells in the profile of the metabolites and DNA adducts formed after metabolism of various potent carcinogens such as aflatoxin B_1, benzo(a)pyrene and 7,12-dimethylbenz(a)anthracene (Newbold, *et al.*, 1977; Selkirk, 1977; Decad, *et al.*, 1977; Bigger, *et al.*, 1978).

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To circumvent these difficulties we have devised a system of mammalian cell-mediated mutagenesis (Huberman, 1974,1975, 1976, 1977; Huberman and Sachs, 1974, 1976; Langenbach, et al., 1978a, 1978b). In this assay, Chinese hamster V79 or Chinese hamster ovary (CHO) cells, which do not exhibit mixed function oxidase activities and therefore do not activate many chemical carcinogens, are co-cultivated with non-dividing cells - namely primary hepatocytes or X-irradiated fibroblasts that contain the enzymes required for metabolic activation. Using this cell-mediated mutagenesis system, it was possible to demonstrate a relationship between carcinogenicity and mutagenicity by polycyclic hydrocarbons (Huberman and Sachs, 1976) and to show that metabolic activation of carcinogens to mutagens is cell specific (Langenbach, et al., 1978b). Therefore, studying the mutagenicity of chemical carcinogens in mammalian cells may:

(1) help elucidate the mechanism for malignant transformation,

(2) be predictive of carcinogenic potential,

(3) be indicative of the organ specificity of a carcinogen and

(4) indicate the magnitude of potential threat to human health posed by a particular chemical.

However, our present state of knowledge indicates that carcinogenesis is a multi-stage process (Foulds, 1969) that may require a step(s) beyond the initial mutational event. Thus, it is possible that many environmental causative agents may act at the later stages of the carcinogenic process in a mechanism related to tumor promotion (Berenblum, 1954). A tumor promoter is a co-carcinogen that is effective only when administered frequently after an initial treatment with a low dose of a carcinogen-mutagen and not when the sequence is reversed (Boutwell, 1974; Hecker, 1975, 1978; Van Duuren, 1969; Weinstein, et al., 1977). Since certain initiating agents are presumably ubiquitous in the environment, the rate-limiting determinants in cancer

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causation in humans may be exposure to tumor promoters and other co-carcinogens. Indeed, there are suggestions that tumor promotion is the rate-limiting step in the development of human lung cancers which are associated with cigarette smoking. There is evidence, for example, that cigarette smoke condensates contain tumor promoters and that the tobacco leaf contains a compound which resembles the phorbol esters which are known to be classical tumor-promoting agents in the mouse skin two-stage model (Weinstein, 1978). Recent studies suggest that compounds such as saccharin and some barbiturates may act as tumor-promoters in experimental bladder and liver cancer, respectively (Hicks, et al., 1978; Peraino, et al., 1978).

In view of these facts, it is important both to elucidate the mode of action and to identify environmental chemicals which may act as tumor promoters. At the present time, the in vivo tests which can be used to study and to identify such chemicals are both time-consuming and require large numbers of experimental animals. It would, therefore, be of importance to substitute or supplement such experimental animal systems with simple in vitro cell systems. However, many of the short-term assays including Ames' Salmonella typhimurium-S9 tests and the mammalian cell-mediated mutagenesis assay (McCann, et al., 1975; Huberman and Sachs, 1974) are designed to detect carcinogens-mutagens and, therefore, are not suitable for studying promoting agents of which many are devoid of mutagenic activity. Tumor promoters act most likely via an epigenetic event. An example of such an event is the disturbance of terminal cell differentiation suggested years ago by Berenblum (1954, 1969). Indeed, in a series of publications from the laboratories of Holtzer, Diamond, and Weinstein, it was shown that tumor promoters such as the phorbol diesters can inhibit terminal differentiation in a number of murine and avian cells. These

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include myogenesis, chondrogenesis, and melanogenesis as well as erythroid, and neurite differentiation (Cohen et al., 1977; Rovera et al., 1977; Yamasaki et al., 1977; Diamond et al., 1977; Pacifici and Holtzer, 1977; Ishii et al., 1978; and Mufson et al., 1978). However, studies with human myeloid leukemia and melanoma cells indicated that the tumor-promoting phorbol esters induce rather than inhibit differentiation. Induction of differentiation in these cells was directly related to the tumor promoting activity of a series of phorbol esters (Huberman and Callaham, 1979; Huberman et al., 1979; Rovera et al., 1979). These results implied that tumor-promoting phorbol diesters may act in some cell types as promoters of the malignant phenotype while in other cell types (including some human myeloid leukemia and melanoma cells) they may suppress this phenotype. In view of this, it is important to study the effect of tumor promoters in such human cell culture systems since they may be helpful in understanding promotion as well as the control of cell growth, cell differentiation and malignancy in human cells. With the present proposal, we intend taking advantage of our developed cell culture systems for testing mutagenesis and induction of cell differentiation, in order to expand our basic knowledge of the cellular and biochemical effects of chemicals that initiate and/or promote tumors.

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D. TECHNICAL SUMMARY

It is now well recognized that our environment is contaminated with many unknown hazardous chemicals, some of which may be responsible for a significant proportion of human cancers. These carcinogenic chemicals represent a fraction of the man-made chemicals which are present in the human environment. It is therefore important to develop fast and accurate pre-screening methods which are inexpensive relative to the current *in vivo* test systems. Mammalian, including human, cell culture systems are extremely useful for such purposes. In the present proposal, we shall introduce cell culture systems which may be useful for the screening of chemicals that initiate or promote tumor formation. These systems may also be helpful in the elucidation of the mode of action of such potentially hazardous chemicals.

Our proposed experiments will, therefore, be concerned with the:

1. Improvement of the performance of the liver cell-mediated mutagenesis assay.
2. Isolation of mutagen-resistant cells for mutagenesis and DNA repair studies.
3. Use of human cells in mutagenesis studies.
4. Induction of different markers of differentiation in human malignant cells by tumor promoting agents.
5. Alterations in lipid biosynthesis in differentiating human tumor cells.

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1) Improvement of the performance of the liver cell-mediated mutagenesis assay

Chemical carcinogens are capable of binding to the DNA of susceptible mammalian cells (Miller and Miller, 1966; Brookes and Lawley, 1964; Essigman et al., 1977; Jeffrey et al., 1977) and may thereby induce mutations, some of which could involve the genes which control the expression of malignant transformation (Todaro and Huebner, 1972; Temin, 1974; Huberman et al., 1976). Therefore, studying the mutagenic activity of carcinogens in mammalian cells should provide an important approach for detecting cancer-causing agents, and possibly for elucidating the mechanisms of carcinogenesis. Many carcinogens are chemically non-reactive and have to be enzymatically activated before they can exert their biological effects (Miller, 1970; Heidelberger, 1975). These non-reactive carcinogens are not mutagenic in microorganisms or in mammalian cell lines used for mutagenesis which cannot metabolize the carcinogen (Corbett et al., 1970; Miller and Miller, 1971; Gelboin et al., 1969; Huberman et al., 1971). In such systems, mutagenesis is only elicited by supplying the chemically reactive metabolites (Huberman et al., 1971; Ames et al., 1972; Huberman et al., 1972) or a microsomal system (Ames et al., 1973; Krahn and Heidelberger 1977; Kuroki et al., 1977) which can activate chemically unreactive carcinogens. However, microsomes or S-9 fractions are prepared by disrupting cells and such subcellular fractions may not metabolize the chemicals as do intact cells (Selkirk, 1977). Therefore, a system which utilizes viable normal mammalian cells to activate the chemical may have advantages over cellular enzyme preparations. We have developed a cell-mediated mutagenesis system (Huberman and Sachs, 1974; 1976) which uses various types of fibroblasts to activate the carcinogen, and mutable V79 cells which are incapable of activating most carcinogens. Using this approach, a correlation between the mutagenic activity and the in

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vivo carcinogenic activity with polycyclic hydrocarbons has been achieved (Huberman and Sachs, 1976). Furthermore, this system has been used to quantify the potency of several mutagenic metabolites of benzo(a)pyrene (Huberman *et al.*, 1976). However, cultured fibroblasts are incapable of activating many classes of chemical carcinogens, including most liver carcinogens (Magee and Barnes, 1967; Wogan, 1976). Therefore, to expand the use of the cell-mediated mutagenesis system and allow study of liver carcinogens, we developed a system for co-cultivating primary liver cells and V79 cells. In this system, liver carcinogens such as the nitrosamines and aflatoxins, were metabolized by the liver cells to intermediates which were mutagenic to the V79 cells. In this liver cell-mediated assay the nitrosamines were metabolized to mutagens more efficiently than in any other microbial or mammalian cell system where subcellular fractions were added. However, the potent liver carcinogens, dimethyl- or diethyl-nitrosamines, exhibited activity in the millimolar range which seemed high when compared with other carcinogen classes. The sensitivity of the cell-mediated assay is determined to a large extent by the efficiency of the activating system employed, in this case hepatocytes. Therefore, a careful study of the techniques by which viable hepatocytes may be isolated and the conditions under which this viability may be maintained in culture may lead to the development of highly sensitive mutagenesis assays. In order to accomplish this, we plan to test various methods for liver preparations to determine the simplest and most economic means of obtaining good preparations of viable hepatocytes.

Several techniques have been developed to isolate primary liver cells for growth or maintenance in culture. One method for obtaining functional hepatocytes from adult animals is based on perfusion of the liver with collagenase or hyaluronidase plus collagenase. Williams and his associates

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(1974) refined the collagenase perfusion technique to the extent that a large portion of the hepatocyte population from an adult rat liver can be isolated as individual viable cells. This method was previously adopted by us to be used in the cell-mediated assay (Langenbach et al., 1978a).

While methodologies have improved for isolating large numbers of viable liver cells, the long-term maintenance of liver cells in vitro with retention of liver functions including efficient metabolism of nitrosamines, has been difficult to achieve. A number of studies on the expression of liver function in long term cultures have been reported (Coon, 1968; Gann et al., 1976; Iype, 1971; Kaighn and Prince, 1971; Weinstein et al., 1975; Williams and Gann, 1974; Bonney et al., 1974; Gann et al., 1976; Laishes and Williams, 1976). The presence in the medium of hormones, such as insulin or glucagon, has enhanced the plating efficiency of hepatocytes (Laishes and Williams, 1976), aided in maintaining normal hepatocyte ultrastructure and prolonged glycogen synthesis and hepatocyte survival (Bernaert et al., 1977; Gerschenson and Casanello, 1968; Leffert et al., 1977). Long-term culture of liver cells with ultrastructural characteristics of liver cells in vivo and with measurable levels of cytochromes b_5, P-450 and inducible tyrosine aminotransferase was achieved by maintaining cells on floating collagen membranes (Michalopoulos et al., 1976; 1978). However, this method may have limitations for mutagenesis studies due to shrinkage of the collagen gel.

To further the use of primary liver cell cultures for mutagenesis studies, we are at present, testing a methodology whereby primary hepatocytes can be maintained in culture for long periods of time. In this approach, primary hepatocytes from adult rat liver are cultured on a layer of

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irradiated C3H/10T1/2 cells (Reinwald and Green, 1975). The level of the liver-specific enzyme, tyrosine aminotransferase, and the metabolism of the liver carcinogen acetylaminofluorene to water-soluble products are higher than in hepatocytes maintained in the absence of the C3H/10T1/2 cells. Another approach to improve the performance of cultured primary hepatocytes involves slicing of the livers with a microtome blade followed by digestion with collagenase and hyaluronidase instead of using perfused livers (Fry et al., 1976). This method has an extra advantage over the perfusion method since it is readily applicable to species in which liver perfusion is difficult to perform (e.g. mice) or impossible (e.g. human biopsy samples which may not contain a vessel for perfusion). This method can also be applied to other organs such as the kidney. Therefore, using this method we may be able to develop an assay with the capacity to study species and organ differences in the activation of carcinogens. Preliminary experiments with these techniques indicate that dimethylnitrosamine and diethylnitrosamine can be activated into mutagens in the cell-mediated assay at doses as low as 1 μM. We shall try to improve these methodologies in order to be able to test efficiently the mutagenicity of a series of nitrosamines and other classes of chemical carcinogens. For comparison, other methodologies such as the Ames' Salmonella S-9 assay, require doses as high as 10-100 mM for optimal detection of nitrosamines. In view of this, the new hepatocyte mediated mutagenesis assay may be extremely useful for detecting potential carcinogens in the environment. This assay may also allow determination of potential carcinogenic potencies if a relationship between the degree of carcinogenesis and mutagenesis can be established.

The cell-mediated mutagenesis assay will be performed as follows:

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Chinese hamster V79 or cells susceptible to mutagenesis will be seeded at 3×10^5 cells in 4 ml of medium in a 60 mm Petri dish together or on top of primary hepatocytes cultured either directly onto the surface of the plastic Petri dish or on top of a layer of C3H 10T1/2 cells. Chemicals to be tested will be added in 1 ml of medium 5 hr later. Twenty-four to forty-eight hours after addition of the chemical, the V79 or other target cells, will be dissociated with trypsin-EDTA, counted with a hemocytometer, and reseeded into 60-mm Petri dishes at 200 cells/dish for determination of cloning efficiency, at 10^5 cells/dish for selection of ouabain-resistant colonies or at 2×10^4 cells/dish after an appropriate expression time for selection of 6-thioguanine resistant colonies. The mutation frequency for resistance to ouabain or 6-thioguanine will be calculated per 10^6 survivors, based on cloning efficiency and number of cells seeded for mutation selection.

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2. Isolation of mutagen-resistant cells for mutagenesis and DNA repair studies

Mutant cells with altered cell susceptibility to the cytotoxic and mutagenic effect of chemical carcinogens-mutagens may be of value in analyzing the mechanism of repair of DNA damage caused by such chemicals. In addition, cells with enhanced susceptibility to mutagenesis may be extremely useful in improving the sensitivity of the cell- or microsomal-mediated mutagenesis assay to environmental chemicals.

There are three commonly used methodologies for the isolation of mutant cells. One is to allow mutant cells which do not replicate due to non-permissive conditions to survive after the whole culture has been treated with a chemical that kills dividing cells. A typical example of this procedure is the isolation by Kao and Puck (1968, 1971) of auxotrophic mutants using a procedure which involves 5-bromodeoxyuridine incorporation followed by irradiation with visible light. Randtke et al. (1972) has applied this technique in attempts to isolate UV-sensitive cells from Hela and Chang liver cells. However, the inherent limitation of this technique is that the mutant cells, particularly those which are sensitive to the killing agent, do not readily survive under the non-permissive conditions. In addition, this procedure requires isolation of a large number of colonies since these mutants are small in number relative to the thymidine kinase and transport mutants which result from the same selective procedure. Another procedure is the replica plating technique, like those which have greatly facilitated the isolation of bacterial mutants. At present two replica plating methods are available for mammalian cells: replica plating of cells growing in parallel in the wells of microplates (Goldsby and Zisper, 1969; Suzuki and Horikawa, 1973, Suzuki et al., 1972) and Lederberg style

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replica plating using agar plate cultures (Kuroki, 1975; Kuroki et al., 1977).

Materials and Supplies:

Includes cost of non-capital equipment, tissue culture (media, sera, antibiotics, plasticware, filters, special glassware, liquid nitrogen, etc.) and substores supplies (glassware, general chemicals, alcohol, surgical gloves, instruments, paper towels, etc.), chemicals for enzyme assays, and labeled substrates.

Craft Maintenance Service:

For repair and service of existing laboratory facilities and instruments.

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DEPARTMENT OF
HEALTH, EDUCATION AND WELFARE
PUBLIC HEALTH SERVICE

National Institutes of Health
National Cancer Institute
INTERAGENCY AGREEMENT

21670222

GSA CONTRACT NO. Y-OB-[illegible]	PAGE 1 OF 6 PAGES
NEGOTIATED PURSUANT TO P.L. 92-218 and 41 USC 252(c) (15)	TYPE OF CONTRACT Cost-Reimbursement

ISSUING OFFICE

National Cancer Institute
Cancer Cause and Prevention Section
Research Contracts Branch, OD
Blair Building, Room B-16
Bethesda, Maryland 20014

CONTRACT FOR

Malignant Cell Transformation AND Mutagenesis ~~and Infectious Virus and Cell~~ Induced by Carcinogenic Metals

~~CONTRACTOR~~ AGENCY (Name and Address)

U. S. Energy Research and Development Administration
~~Washington, D. C. 20545~~
OAK RIDGE, TN 37830

ACCOUNTING AND APPROPRIATION DATA

CAN
Req. No.
Subobject Class 2531
Doc. N1 CP 70222
EIN 1-530197006-A1

PLACE OF PERFORMANCE

Oak Ridge National Laboratories, Tennessee

CONTRACT AMOUNT

MAIL VOUCHERS TO

See Billing Instructions

SPONSOR

Division of Cancer Cause and Prevention, In Vitro Program

EFFECTIVE DATE

EXPIRATION DATE

CONTRACTOR REPRESENTS

1. That it [ ] is, [ ] is not, a small business concern. If he is a small business concern and is not the manufacturer of the supplies to be furnished hereunder, he also represents that all such supplies [ ] will, [ ] will not, be manufactured or produced by a small business concern in the United States, its possessions, or Puerto Rico. (A small business concern for the purposes of Government procurement is a concern, including its affiliates, which is independently owned and operated, is not dominant in the field of operation in which it is contracting and can further qualify under criteria concerning number of employees, average annual receipts, or other criteria, as prescribed by the Small Business Administration. See Code of Federal Regulations, Title 13, Part 121, as amended, which contains detailed definitions and criteria and procedures.)
2. That it is a [ ] REGULAR DEALER IN, [ ] MANUFACTURER OF, the supplies covered by this contract.
3. That it is an [ ] INDIVIDUAL, [ ] STATE OR LOCAL AGENCY, [ ] PARTNERSHIP, [ ] JOINT VENTURE, [ ] NONPROFIT, [ ] EDUCATIONAL INSTITUTION, [ ] CORPORATION organized and existing under the laws of the state of _____

The Contractor agrees to furnish and deliver all the supplies subject to and in accordance with all the terms and conditions set forth in the attached General Provisions, Special Provisions, for the consideration stated herein. The rights and obligations of the parties to this contract shall be subject to and governed by the Special Provisions and the General Provisions. To the extent of any inconsistency between the Special Provisions or the General Provisions and any specifications or other provisions which are made a part of this contract, by reference or otherwise, the Special Provisions and the General Provisions shall control. To the extent of any inconsistency between the Special Provisions and the General Provisions, the Special Provisions shall control.

IN WITNESS THEREOF, the parties hereto have executed this contract on the day and year last specified below.

NAME OF ~~CONTRACTOR~~ AGENCY	UNITED STATES OF AMERICA
BY _____ SIGNATURE OF AUTHORIZED INDIVIDUAL	BY _____ SIGNATURE OF CONTRACTING OFFICER
_____ TYPED NAME	Keith L. Born, Contracting Officer, Carcinogenesis Contracts Section, RCB, NCI — TYPED NAME
TITLE _____	_____ DATE
_____ DATE	

HEW-804 (7/70)

3-73

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THIS AGREEMENT CONSISTS OF:

1. COVER PAGE HEW-554

2. CONTENTS OF CONTRACT HEW-555

3. SPECIAL PROVISIONS HEW-556

ARTICLE I	SUBSTANCE OF THE AGREEMENT
ARTICLE II	DESCRIPTION OF WORK
ARTICLE III	PERIOD OF PERFORMANCE
ARTICLE IV	CONSIDERATION
ARTICLE V	TECHNICAL REPORTING
ARTICLE VI	PROJECT OFFICER
ARTICLE VII	KEY PERSONNEL
ARTICLE VIII	BILLING PROCEDURES

ARTICLE I
SUBSTANCE OF THE AGREEMENT

A. GENERAL

WHEREAS, ~~the Oak Ridge National Laboratory, ERDA,~~ ENERGY RESEARCH & DEVELOPMENT ADMINISTRATION hereinafter referred to as the "Agency" and the Division of Cancer Cause and Prevention (DCCP), National Cancer Institute (NCI), National Institute of Health (NIH), hereinafter referred to as the "sponsor", desire to enter into an agreement to enhance our knowledge and understanding of the interactions between chemical compounds which may lead to adverse toxicological effects, and;

INSERT * WHEREAS, the work and services to be conducted by the ~~Agency~~ ORNL under this agreement will be require approximately two(2) years to complete.

B. OBJECTIVE - Is to investigate the biological and biochemical properties of carcinogenic metals in mammalian cells in order to provide information on the mechanisms by which metals induce malignancy.

NOW, THEREFORE, the parties do agree as follows:

ARTICLE II
DESCRIPTION OF WORK

A. SCOPE - The ~~agency~~ ORNL shall exercise its best efforts to perform the work and services hereinafter set forth and as described more fully in the ~~Agency~~ ORNL's Proposal entitled "MalignantCell Transformation, Mutagenesis and Infidelity During DNA Synthesis Induced by Carcinogenic Metals", AS MODIFIED BY THE ORNL REVISED BUDGET DATED MARCH 28, 1977. The ~~contractor~~ ORNL shall exert its best efforts to:

1. Induce malignant cell transformation in hamster embryo fibroblasts by carcinogenic metals and the effect of such metals on cell transformation induced by other classes of chemical carcinogens.

2. Induction of mutations at different genetic loci by carcinogenic metals and their effect on mutations induced by other classes of chemicals carcinogens. The endpoints to be used are resistance to ouabain and 8-azaguanine or 6- thioguanine and temperature sensitivity.

B. Human Subjects - It is hereby understood and agreed that research involving human subjects shall not be conducted under this ~~contract~~ AGREEMENT and that no material developed, modified, or delivered by or to the Government under this ~~contract~~ AGREEMENT or any subsequent modification of such material, will be used by the ~~contractor~~ ORNL or made available by him for use by anyone other than the Government, for experimental or therapeutic use on humans without the prior written approval of the Contracting Officer.

* WHEREAS, THE AGENCY HAS CONTRACTED WITH THE UNION CARBIDE CORPORATION TO OPERATE AND MANAGE THE AGENCY'S OAK RIDGE NATIONAL LABORATORY, HEREINAFTER REFERRED TO AS THE "ORNL", AND;

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C. Level of Effort

1. During the performance of work under this Interagency Agreement, the ~~Contractor agrees to~~ ORNL WILL utilize personnel in the following categories for the time indicated:

Category	Man-Months Each Year
Scientific	1.5
Other Technical	1.0

2. The ~~Contractor agrees to~~ ORNL WILL use his best efforts to accomplish all the work outlined or referenced above. His obligation will be deemed complete if the work is performed in accordance with high standards of scientific and professional skill and the approximate level of effort has been diligently applied; except, however, all other requirements must be met including delivery of reports and materials as may be required under the Interagency Agreement.

ARTICLE III
PERIOD OF PERFORMANCE

Performance of this Interagency Agreement shall begin on and shall not extend beyond , unless the period is extended by amendment of the Interagency Agreement.

ARTICLE IV
CONSIDERATION

A. Estimated Cost

For performance of this Interagency Agreement, the ~~Government~~ SPONSOR agrees to pay reimbursement to the ~~Contractor~~ AGENCY in the estimated amount of $385,000.

B. Total funds currently available for reimbursement and allotted to this Agreement is $ 184,000 . Further allocation of funds hereunder will be accomplished at the option of the sponsor, depending upon the progress and results of the work and services performed and consistent with the time that approval to proceed has granted by the sponsor.

C. It is estimated that the amount of funds currently allotted under B above will cover performance for the period through September 30, 1977.

ARTICLE V
TECHNICAL REPORTING

A. The ~~agency~~ ORNL shall submit to the sponsor's Project Officer four(4) copies of a quarterly progress report.

B. The ~~agency~~ ORNL shall also submit to the sponsor's Project Officer four(4) copies of a comprehensive annual and final report upon completion of this agreement.

C. In all publications resulting from research performed under this agreement the ~~agency~~ ORNL shall acknowledge support of the NCI.

ARTICLE VI
PROJECT OFFICER

The Project Officer representing the ~~Government~~ SPONSOR for the purpose of this ~~contract~~ AGREEMENT is, Dr. Virginia Dunkel, In Vitro Program, DCCP, National Cancer Institute.

The Project Officer is responsible for: (1) Monitoring the ~~Contractor's~~ ORNL'S technical progress, including the surveillance and assessment of performance and recommending to the Contracting Officer changes in requirements; (2) interpreting scope of work; (3) performing technical evaluation as required; (4) performing technical inspections and acceptances required by this ~~contract~~ AGREEMENT, and (5) assisting the ~~contractor~~ ORNL in the resolution of technical problems encountered during performance. The Contracting Officer is responsible for directing or negotiating any changes in the terms or conditions, or amounts cited in the ~~contract~~ AGREEMENT.

ARTICLE VII
KEY PERSONNEL

The individuals cited below are key personnel and are necessary for the successful performance of the work under this Interagency Agreement. The ~~contractor agrees to~~ ORNL WILL assign such personnel to the performance of the work under this ~~contract~~ AGREEMENT, and shall not reassign or remove any of them without the consent of the Contracting Officer. Whenever, for any reason, one or more of the aforementioned personnel is unavailable for assignment for work under the ~~contract~~ AGREEMENT, the ~~contractor~~ AGENCY shall immediately notify the Contracting Officer to that effect and shall, subject to the approval of the Contracting Officer without formal modification to this Agreement, replace such personnel with personnel of substantially equal ability and qualifications.

Name	Title
Dr. E. Huberman	Project Director

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ARTICLE VIII
BILLING PROCEDURES

The Agency shall submit vouchers for cost reimbursement on Government Standard Form 1080 Voucher and Schedule of Withdrawals and Credits, on a monthly basis. An original and five(5) copies of all such vouchers shall be sent to the following address:

Contracting Officer
Division of Cancer Cause and Prevention
Research Contracts Branch, OD
National Cancer Institute
Blair Building, Room B-21
National Institutes of Health
Bethesda, Maryland 20014

OAK RIDGE NATIONAL LABORATORY
OPERATED BY
UNION CARBIDE CORPORATION
NUCLEAR DIVISION

POST OFFICE BOX X
OAK RIDGE, TENNESSEE 37830

OFFICE OF THE DIRECTOR

December 16, 1976

U.S. Energy Research and Development
Administration
Oak Ridge Operations
Attention: Mr. J. A. Lenhard, Director
Research and Technical Support Division
Post Office Box E
Oak Ridge, Tennessee 37830

Gentlemen:

Chemical Carcinogenesis Program Proposal to NCI

Attached for your review and forwarding to J. L. Liverman, Director of the Division of Biomedical and Environmental Research, ERDA, is a research proposal on "Malignant Cell Transformation, Mutagenesis and Infidelity During DNA Synthesis Induced by Carcinogenic Metals" by Dr. E. Huberman, Biology Division, ORNL.

This proposal is the result of discussions between Dr. Virginia Dunkel, NCI, and Dr. Huberman.

If this proposal is funded, it would become part of Interagency Agreement 40-5-63 (NCI Y01 CP 50200) which has recently been renewed. The proposal is being submitted in two parts, a "Technical Proposal" and a "Business Proposal."

If DBER approves this proposal, please ask them to forward twenty (20) copies of the technical proposal and twenty (20) copies of the business proposal to Dr. Virginia C. Dunkel, Coordinator, In Vitro Carcinogenesis Program, National Cancer Institute, Landow Building A306, Bethesda, Maryland 20014.

If you have any questions concerning any aspect of this proposal, please contact C. R. Richmond (3-1477).

Sincerely yours,

Herman Postma

Herman Postma
Director

HP:REC:sv
Attachment
cc: F. L. Culler
R. A. Griesemer
R. F. Hibbs
W. R. Ragland
C. R. Richmond
J. B. Storer
Files - RC

1018112

-12-3 (NIH)
40-636-77

BUSINESS PROPOSAL

MALIGNANT CELL TRANSFORMATION, MUTAGENESIS AND INFIDELITY DURING DNA SYNTHESIS INDUCED BY CARCINOGENIC METALS

Proposed Addition to

Chemical Carcinogenesis Component
Biology Division, Oak Ridge National Laboratory

(Interagency Agreement ERDA No. 40-5-63)
(NCI Agreement No. Y01 CP 50200)

Proposal Period January, 1977 — December, 1977

1078773

MALIGNANT CELL TRANSFORMATION, MUTAGENESIS AND INFIDELITY
DURING DNA SYNTHESIS INDUCED BY CARCINOGENIC METALS

(Huberman)

FY 1977 Proposal for 12 Months Beginning 1/1/77

	FY 1977 01		FY 1978 02	FY 1979 03
	MY	$	$	$
E. Huberman	0.5			
Research Associate	1.0			
Total Ph.D.	1.5	38,000		
Total Technicians	2.0	23,000		
Direct Costs				
1) Total Salaries	3.5	61,000	66,500	72,500
2) Fringe Benefits 26%		15,900	17,300	18,900
3) Materials & Supplies		52,000	57,200	62,900
4) Maintenance Material & Shops		5,300	5,800	6,400
5) Travel		2,400	1,800	1,900
6) Animals		8,000	8,800	9,700
7) Glassware Washing		11,000	12,000	13,100
Total Direct Costs	3.5	155,600	169,400	185,400
Indirect Costs				
8) Division Administration 26%		15,900	17,300	18,900
9) Utilities		10,200	11,100	12,100
19) ORNL G & A 31%		56,300	61,300	67,100
Total Indirect Costs		82,400	89,700	98.100
GRAND TOTAL COSTS	3.5	238,000	259,100	283,500

Note: Line 2, Fringe Benefits is 26% of Line 1. Line 8, Division Administration is 26% of Line 1. Line 10, ORNL G & A, is 31% of total of Lines 1-9 and is calculated in conformance with the Federal Cost Account Standards Board Procedure. Years 02-03 do not represent any expansion of program. Salaries have been escalated at ∿9% per year, supplies and other expenses, ∿10%.

1078774

Budget Comments: Malignant Cell Transformation, Mutagenesis and Infidelity During DNA Synthesis Induced by Carcinogenic Metals

a. Personnel. Doctorate level personnel and their primary responsibilities are:

E. Huberman — Principal Investigator

Research Associate — To be appointed

b. Materials & Supplies. Includes costs of non-capital equipment, glassware, isotopes, media, serum, and chemicals.

c. Maintenance Material & Shops. These services are provided and charges are made as required to repair instruments, laboratory facilities, etc.

d. Animals. Costs associated with animal use are distributed to the Central Animal Facility on a usage basis in terms of mouse cage equivalents.

e. Utilities. Cost of electricity, heat, air conditioning, etc., including monitoring and maintenance of these utilities are charged on a square foot basis.

f. Travel. Domestic — $900 — To attend scientific meetings and to consult with personnel at the National Cancer Institute

Foreign — $1500 — Invitations attached. Two Conferences to be attended consecutively.

Sixth International Perugia Cancer Conference, June 30-July 5, 1977, Perugia Italy
Invited paper.

Second International Conference on Environmental Mutagens, July 11-15, 1977, Edinburgh, Scotland
Co-Chairman of Priority Contributed Paper session entitled, "Metabolic Activation of Mutagens"

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PERUGIA QUADRENNIAL INTERNATIONAL CONFERENCES ON CANCER
THE SIXTH: TUMOURS OF EARLY LIFE IN MAN AND ANIMALS
PERUGIA UNIVERSITY MEDICAL SCHOOL 30TH JUNE TO 5TH JULY 1977 06100 PERUGIA, MONTELUCE LECTURE HALL OF PATHOLOGY

11th November,

Prof. E. Huberman,
Department of Genetics,
Weizmann Institute of Science,
REHOVOT

Dear Professor Huberman,

PERUGIA CANCER CONFERENCE
30th June to 5th July, 1977

1957 MAMMARY CANCER

1961 THE MORPHOLOGICAL PRECURSORS OF CANCER

1965 LUNG TUMOURS IN ANIMALS

1969 IMMUNITY AND TOLERANCE IN ONCOGENESIS

1973 MULTIPLE PRIMARY MALIGNANT TUMOURS

You may be aware that this Division of Cancer Research has a tradition of sponsoring the Perugia Quadrennial International Conferences on Cancer, and that each of these deals with a different aspect of the cancer problem.

I am, at the moment, in the process of organizing the Sixth Conference, scheduled to take place from 30th June to 5th July, 1977, which has as its topic

TUMOURS OF EARLY LIFE IN MAN AND ANIMALS.

The programme will be drawn up on the broad lines of the preliminary break-down attached.

It is expected that such a programme will bring together human pathologists, experimental pathologists, clinicians, paediatricians, veterinary pathologists, oncologists, cytologists, geneticists, immunologists, electronmicroscopists and virologists.

As I am sure that this topic is of interest to you, I should be pleased to receive any suggestions you may have for improving the programme and I should, also, like to invite you to contribute to the Perugia 1977 Conference. The invitation, of course, includes your associates engaged on similar research work.

Should you have any scientific colleagues whom you think would like to take part in this conference, I should be pleased to write to them if you would be kind enough to forward me their addresses.

Yours sincerely,

Prof. Lucio Severi,
Chairman

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INSTITUTE OF PATHOLOGICAL ANATOMY AND HISTOLOGY DIVISION OF CANCER RESEARCH
P. O. B. 327 06100 PERUGIA, MONTELUCE (ITALY)

Second International Conference on ENVIRONMENTAL MUTAGENS

Edinburgh, July 11–15, 1977

Patron: H.R.H. The Duke of Edinburgh, K.G., K.T.

Hon. President: Charlotte Auerbach *Hon. Vice-Presidents:* A. Hollaender, I.A. Rapoport, Y. Tazima, K. Sundaram

DS/JHR

24 November 1976

Dr E Huberman
Biology Division
Oak Ridge National Laboratory
Oak Ridge
Tennessee 37830
U S A

Dear Dr Huberman

At the Second International Conference on Environmental Mutagens next July there will be a Priority Contributed Paper session entitled " Metabolic activation of mutagens." On behalf of the Chairman of the Programme Committee, Professor Sobels, I am writing to invite you to act as a co-chairman of this session.

As an invited contributor you will not be required to pay a registration fee. If you have already done so the money will be refunded at the Conference by our treasurer.

Please let me know as soon as possible if you are able to accept this invitation. I enclose a registration form for your convenience.

Yours sincerely

David Scott

David Scott PhD
Secretary of Programme Committee
(Address below)

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CONFERENCE OFFICERS:
President: B.A.Bridges, MRC Cell Mutation Unit, Universtiy of Sussex, Brighton, BN1 9QG England
Secretary General: P. Brookes, Pollards Wood Research Station, Nightingales Lane, Chalfont St. Giles, Bucks. HP8 4SP England
Treasurer: B.J.Dean, Tunstall Laboratory, Shell Research Limited, Sittingbourne Research Centre, Kent ME9 8AG England
Finance Committee Chairman: A. Hollaender, Associated Universities Inc., Suite 603, 1717 Massachussetts Avenue, NW, Washington DC 20036
Programme Committee Chairman: F.H.Sobels, Laboratory of Radiation Genetics and Chemical Mutagenesis, Wassenaarsweg 72, Leiden, Netherlands.
Local Committee Chairman: B.J.Kilbey, Institute for Animal Genetics, West Mains Road, Edinburgh EH9 3JN Scotland
Programme Committee Secretary: D.Scott, Paterson Laboratories, Christie Hospital, Manchester M20 9BX England

MALIGNANT CELL TRANSFORMATION, MUTAGENESIS AND INFIDELITY
DURING DNA SYNTHESIS INDUCED BY CARCINOGENIC METALS

(Huberman)

FY 1977 Proposal for 12 Months Beginning 1/1/77

	FY 1977 01		FY 1978 02	FY 1979 03
	MY	$	$	$
E. Huberman	0.5			
Research Associate	1.0			
Total Ph.D.	1.5	38,000		
Total Technicians	2.0	23,000		
Direct Costs				
1) Total Salaries	3.5	61,000	66,500	72,500
2) Fringe Benefits 26%		15,900	17,300	18,900
3) Materials & Supplies		52,000	57,200	62,900
4) Maintenance Material & Shops		5,300	5,800	6,400
5) Travel		2,400	1,800	1,900
6) Animals		8,000	8,800	9,700
7) Glassware Washing		11,000	12,000	13,100
Total Direct Costs	3.5	155,600	169,400	185,400

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TECHNICAL PROPOSAL

MALIGNANT CELL TRANSFORMATION, MUTAGENESIS AND INFIDELITY DURING DNA SYNTHESIS INDUCED BY CARCINOGENIC METALS

Proposed Addition to

Chemical Carcinogenesis Component
Biology Division, Oak Ridge National Laboratory

(Interagency Agreement ERDA No. 40-5-63)

(NCI Agreement No. Y01 CP 50200)

Proposal Period January, 1977 — December, 1977

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MALIGNANT CELL TRANSFORMATION, MUTAGENESIS AND INFIDELITY DURING DNA SYNTHESIS INDUCED BY CARCINOGENIC METALS

Principal Investigator: E. Huberman

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I. INTRODUCTION, BACKGROUND AND SCOPE:

Medical observation as well as experiments on animals provide evidence that some man-made and natural chemicals in the environment may be responsible for a significant proportion of human cancers (Higinson, 1972). In view of the complexity in studying chemical carcinogenesis in whole animals, it is useful to use simple mammalian cell cultures for such studies. Cell culture systems may also be useful for the identification of potential hazardous chemicals, since one must presume that there are many environmental chemicals whose potential carcinogenic hazard has not yet been established.

Malignant transformation of cells in culture has been achieved in either normal cells or permanent cell lines (Berwald and Sachs, 1965; Heidelberger, 1973). Using these *in vitro* systems it was also established that transformed cells are induced rather than selected by the carcinogens (Huberman and Sachs, 1966, 1968; Mondal and Heidelberger, 1970). In these transformation assays "control" fibroblast cells which have an oriented pattern of cell growth, a limited life span *in vitro* and are not tumorigenic *in vivo*, are converted after carcinogen treatment into cells with a hereditary random pattern of cell growth, an ability to grow continuously in culture and to form tumors *in vivo*. Studies with a variety of chemical carcinogens have shown a corrleation between the frequency of colonies with a hereditable random pattern of cell growth (transformed colonies) and the degree of carcinogenicity in animals (Berwald and Sachs, 1965; DiPaolo *et al*., 1972; Heidelberger, 1973). These results and the observations that carcinogens bind to cellular macromolecules including DNA (Brookes and Lawley, 1967; Kuroki and

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appear to be transmitted from the metabolizing cells and induce mutations in the V79 or CHO cells. Mutations can be determined with different markers that affect the surface membrane (e.g., ouabain resistance), nucleic acid synthesis (e.g. 8-azaguanine resistance) and protein synthesis (temperature sensitivity) (Huberman and Sachs, 1976).

Using this mammalian somatic cell mutagenesis system we were able to demonstrate a relationship between carciogenicity and mutagenicity by polycyclic hydrocarbons having different degrees of carcinogenicity (Huberman and Sachs, 1976). In addition, we identified a diol-epoxide isomer as a metabolic intermediate of benzo(a)pyrene. This diol-epoxide was the major mutagenic metabolite in this somatic cell mutagenesis assay (Huberman et al., 1976).

In view of these results, malignant cell transformation and somatic cell mutagenesis systems should be useful for the identification of hazardous cehmicals as well as for studies on their mode of action.

Metals are common environmental contaminants as by-products of industry, and some are ingredients of human and veterinary medicines. Metals, in general, have received comparatively little experimental study in mammalian cell culture systems, although whole animal studies and epidemiological data have implicated some metals as potent carcinogens (IARC, Publications 1 and 2). The mechanism whereby such metals may produce biological effects including cancer may be through chelation. A chelate may result from an interaction of a metal ion with a series of organic molecules which have at least two electron rich functional groups such as $C = \overset{\shortparallel}{O}$, $C - \overset{\shortparallel}{O}H$, $C - \overset{\shortparallel}{N}H_2$ or $C - \overset{\shortparallel}{S}H$ which are present in proteins and nucleic acids (Furst, 1963). Although

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II. TECHNICAL PROPOSAL:

This project as planned will be concerned with three areas of metallo-cell and enzyme interaction.

1. Induction of malignant cell transformation by carcinogenic metals and their effect on cell transformation induced by other classes of chemical carcinogens.

2. Induction of mutations at different genetic loci by carcinogenic metals and their effect on mutations induced by other classes of chemical carcinogens.

3. Induction of infidelity during *in vitro* DNA synthesis by chemical carcinogens including carcinogenic metals.

1. Induction of malignant cell transformation by carcinogenic metals and their effect on cell transformation induced by other classes of chemical carcinogens.

Transformation will be studied in the well-defined hamster embryo fibroblast culture system which has been routinely used for testing chemical carcinogens by the principle investigator. Minced whole embryos of Golden hamsters will serve as a source of normal cells. The metals will include berillium, nickel, platinum, copper, silver, zinc, cadmium, lead, arsenic and selenium. The metals will be in the form of water soluble chloride, and acetate salts. Treatment with the metals will be performed on sparce and confluent secondary cultures (growing vs. non-growing cells) seeded at 0.5×10^6 - 4×10^6 cell per 50 mm

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be selected from a mixed population of normal and transformed cells (Huberman and Sachs, 1968; Huberman et al., 1968).

e. Enchanced agglutinability of the transformed cells by the carbohydrate binding proetin Conconavalin A (Inbar et al., 1971).

f. Tumor production after cell inoculation into newborn and adult Golden hamsters.

Transformation studies will also be performed after treatment of cells with a combination of metals and other classes of carcinogens such as polycyclic hydrocarbons. Carcinogenic metals may not induce transformation directly but rather enhance transformation induced by other carcinogens. In addition, cytotoxicity studies with carcinogenic metals on normal and spontaneously or chemically induced tumor cells will be performed in order to determine whether the transformed cells are more resistant to the cytotoxic effect of these metals. These experiments will be of importance since it is not clear whether carcinogenic metals induce or select for preexisting spontaneously transformed cells (Fradkin et al., 1975).

Results from such experiments will determine whether carcinogenic metals transform normal into malignant cells directly or by some other mechanisms.

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The direct mutagenesis assay will be performed as previously described (Huberman et al., 1971, 1972, 1976). Briefly, CHO or V79 cells will be seeded at 200 cells per 50 mm Petri dish to determine cloning efficiencies, at 2 x 10^4 cells and 10^5 cells per 50 mm Petri dish for selection of 8-azaguanine and ouabain-resistant mutants, respectively. The metals and other mutagens will be added separately or together (to monitor potentiation) at different intervals for three hours at 16-18 hr after cell seeding, and the medium with the added chemical will then be replaced by fresh medium without the chemicals. For selection of mutants a final concentration of 0.2 mM 8-azaguanine or 1 mM ouabain will be added two days after treatment with the chemicals. In the experiments with 8-azaguanine, the medium will be replaced every two days with a medium containing fresh 0.2 mM 8-azaguanine. Colonies will be counted after staining with Giemsa at 6-8 days for cloning efficiency, 12-14 days for expression of 8-azaguanine resistance and 16-18 days for expression of ouabain resistance.

The cell-mediated assay will be performed as described (Huberman, 1976; Huberman and Sachs, 1974, 1976). V79 and CHO-temperature sensitive cells will be seeded at 3 x 10^5 cells in 4 ml of medium on a layer of lethally irradiated metabolizing cells and the chemicals will be added 5 hrs later in 1 ml medium. The treated V79 and CHO-temperature sensitive cells will be incubated for 2 and 3 days, respectively. The cultures will then be dissociated with 0.25% trypsin solution, seeded at 200 cells per 50 mm Petri dish for cloning efficiency, 2 x 10^4 cells per 50 mm Petri dish for selection of 8-azaguanine resistant mutants, and 10^5 cells per 50 mm Petri dish for selection of ouabain or tempera-

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result from their interaction with cellular polymerases. It is therefore important to study the interaction of these carcinogens with purified polymerases as well as polymerases obtained from control and carcinogen-treated cells to determine the alteration in the activity of the carcinogen-treated polymerase.

Commercial mammalian α, β, and γ polymerases as well as polymerases purified from Golden hamster embryos and cell cultures will be used in order to determine whether carcinogenic metals including [Be^{++}] and other classes of carcinogens can induce infidelity during DNA synthesis. For these experiments artificial templates such as poly dT oligo dA or poly dG oligo dC will be used and the incorporation of noncomplementary nucleotides will be measured. In addition we shall determine the activity of polymerases isolated from cells treated with different carcinogens using untreated as well as UV and carcinogen arylated templates.

The DNA polymerases will be isolated after the procedure of Weisbach (Weisbach et al., 1971; Fry and Weisbach, 1973). The treated and untreated embryos or cell cultures will be collected, homogenized and/or lysed in the appropriate buffer containing glycerol under reducing conditions by the presence of dithiotheritol. After removal of cell debris the lysate or homogenate will be passed through a DEAE-cellulose column and eluted with [KPO_4] concentration gradient. The α-polymerase will be collected at its major activity area. The activity peaks of the β and γ polymerases, which elute close together will be collected and passed through a gel filtration column. This will separate both activities, so that the three polymerase activities will be independent of each other.

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III. PROPOSED WORK DURING THE FIRST 12 MONTHS

During the first twelve months of this research project we intend to begin the three sections of the proposal using mainly three carcinogenic metals: beryllium, nickel and cobalt. These metals will be tested directly as transforming agents in cell culture and for their effect on malignant transformation induced by other carcinogens such as benzo(a)pyrene, and its reactive metabolite, diol-epoxide. Beryllium, nickel and cobalt will be tested for their mutagenic effect in the direct assay as well as for their effect on the cell-mediated mutagenesis induced by benzo(a)pyrene. The third section will deal with the induction of infidelity during DNA synthesis by the three carcinogenic metals and other carcinogens such as nitrosamides and reactive forms of carcinogenic polycyclic hydrocarbons [e.g. benzo(a)pyrene diol-epoxide] using commerically available polymerases (e.g. calf thymus alpha-polymerase). Misincorporation into synthetic polynucleotide by polymerases previously treated with these carcinogens will be investigated. Similar studies will be performed using polynucleotides methylated by nitrosoguanidine or arylated by benzo(a)pyrene diol-epoxide since arylated DNA is believed to be involved in the genesis of cancer by chemicals.

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IV. RELATIONSHIP TO ONGOING CANCER RESEARCH PROJECTS AT ORNL

The Carcinogen Metabolism and Chemical Carcinogenesis Group in the Biology Division has several programs strongly committed to the study of the mechanism of action of chemical carcinogens. Dr. J. K. Selkirk's laboratory is studying the mechanism of action and metabolism of polycyclic aromatic hydrocarbons, the enzyme kinetics of the aryl hydrocarbon hydroxylase system, and the interaction of noncarcinogenic and carcinogenic isomers of benzo(a)pyrene with cellular macromolecules. This laboratory possesses the personnel, instrumentation and expertise necessary to advise on the analytical methods for isolating and quantitating the activated carcinogen metabolites. The laboratory of Dr. T. J. Slaga is committed to understanding the mechanism of phorbol ester cocarcinogenesis in mouse skin and the effects of other chemical carcinogens and steroids on skin tumorigenesis. The laboratory of Dr. R. W. Tennant is developing the host-mediated assay for chemical carcinogenesis as developed by Dr. J. DiPaolo using chemical carcinogens which are injected into pregnant female hamsters, then cell cultures are prepared from the embryos and scored for formation of malignantly transformed colonies. In addition, there is a long standing research program under the direction of Dr. Paul Nettesheim, Respiratory Carcinogenesis Group, which studies the interaction of chemical carcinogens in the respiratory tract of rodents and is currently utilizing both *in vivo* and *in vitro* models to study the interaction of chemical carcinogens with respiratory epithelial cells and tissues and their progression from initiation to frank malignancy or *in vitro* transformation in cell culture.

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V. LABORATORY SPACE AND FACILITIES

This program has available 2,200 square feet of laboratory space, including four Biogard hoods, two high efficiency fume hoods altered to meet NCI standards for working with chemical carcinogens, and seven CO_2 incubators, in addition to generally available standard laboratory equipment.

VI. CURRICULUM VITAE

See following pages.

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CURRICULUM VITAE

Name: Eliezer Huberman

Date of Birth: [redacted] Poland

Nationality: Israeli

Marital Status: [redacted]

1969 - 1971	University of Wisconsin, Madison, Wisconsin Postdoctorate in the Department of Oncology with Prof. C. Heidelberger.
1971	Visiting Scientist in the Etiology Branch Etiology Branch, National Cancer Institute Bethesda, Maryland (4 months)
1971 - 1973	Scientist in the Department of Genetics Weizmann Institute of Science Rehovot, Israel
1973 - 1976	Senior Scientist in the Department of Genetics Weizmann Institute of Science Rehovot, Israel
1976	Associate Professor in the Department of Genetics Weizmann Institute of Science Rehovot, Israel
1976 - pres.	Group Leader, Carcinogenesis Program Biology Division, Oak Ridge National Laboratory Oak Ridge, Tennessee

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A disadvantage of this procedure is that the required mutants must be present at very high frequencies since the replica plating itself has no selective capacity. A third method involves selecting drug resistant cells by exposing the cells to a cytotoxic agent which kills wild type cells but not the mutants. We have chosen for our procedure this last method due to its simplicity. As it would be useful to have information on DNA repair in cells that are commonly used in mutagenesis studies, we used the V79 cells as the target cells for the isolation of such mutants. In preliminary studies, we treated 10^7 V79 cells with a cytotoxic dose of 2 μg/ml of N-methyl-N-nitro-N'-nitrosoguanidine (MNNG) (Bhuyan et al. 1976). A week later the cells which grew as colonies were subcultured, allowed to recover and once they reached a cell number of about 10^7 cells, the treatment with MNNG was repeated. After seven such treatments we have isolated a number of MNNG resistant colonies. Cells from one of these colonies, designated VS43, which had a near diploid karyotype, exhibited an increased resistance towards the cytotoxic effects of MNNG and nitrosomethylurea. At an equal cytotoxic level the VS43 cells yielded, relative to the wild type V79 cells, a higher mutation frequency of ouabain and 6-thioguanine resistant mutants. No such differences between the VS43 and V79 could be observed after treatment with N-ethyl-N-nitro-N'-nitrosoguanidine (ENNG), ethylmethylsulfonate, methyl methane sulfonate or x-rays. These results suggest that VS43 cells exhibit, biologically, an enhanced error prone repair towards MNNG.

The major classes of DNA repair in mammalian cells after their treatment with chemical reactive agents are excision repair and post-replication repair (Hanawalt 1977, Hanawalt and Setlow 1975, Higgins et al. 1976). We have therefore analyzed both types of repair after treatment with MNNG and ENNG

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using alkaline sucrose gradients (D'Ambrosio and Setlow 1976). Our preliminary experiments indicate that the difference between the VS43 and V79 cells may be related to the post-replication repair after MNNG damage. These results appear to support the idea that post-replication repair is an error prone repair system in mammalian cells. We shall continue to analyze this and other mutagen resistant colonies, including those resistant to the cytotoxic effect of ENNG and benzo(a)pyrene diol epoxide. Since the repair processes require DNA synthesis, it is probable that some of these mutants may have altered DNA polymerases. We have therefore established a procedure for the purification of DNA polymerase α and β from V79 cells based on a modification of the Weisbach (1977) method. In this method Chinese hamster V79 or mutant cells are harvested by trypsinization, washed with PBS, homogenized in low salt Tris buffer pH 8.0, sonicated, and centrifuged at 40K RPM for 1 hour. The supernatant is collected and applied to a 45 ml DEAE-cellulose column. A gradient of 0.02 M KCl buffer to 1M KCl buffer is applied and one ml fractions are collected. DNA polymerase β elutes in the run through, while DNA polymerase α elutes at 0.17 M KCl. DNA polymerase β is pooled and applied directly to a 10 ml phosphocellulose column. The enzyme elutes at 0.54 M KCl using the same gradient as above. DNA polymerase α is pooled and removed from the solution with ammonium sulfate at 70% saturation. The precipitate is collected and dissolved in low salt Tris buffer pH 8.0 and dialyzed. The dialyzed enzyme is then applied to a 10 ml phosphocellulose column and eluted at 0.31 M KCl using the same gradient as above.

In addition, those cell variants which exhibit alteration in the degree of excision repair by the alakline sucrose procedure will be analyzed for the removal rate of methyl, ethyl or diol epoxide DNA adducts formed after

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treatment with the appropriate mutagen/carcinogen. These experiments will improve our knowledge of the relationship between specific types of DNA repair after carcinogen-mutagen insult and the corresponding cytotoxic and/or mutagenic event. It will also allow us to use in the cell-mediated assay, a repair mutant or a combination of such mutants that are less susceptible to the cytotoxic effects of potential carcinogens but are highly susceptible to their mutagenic effect.

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3. The use of human cells in mutagenesis studies.

The ultimate objective of *in vitro* mutagenesis assays for preventive purposes, is to predict the possible danger of hazardous chemicals in our environment. Most such *in vitro* assays employ either bacteria or rodent cells as target organisms for mutagenesis. Rodent cells are also used as the main source for the enzymes required for the metabolism of many pre-mutagens or carcinogens into reactive metabolites which mutate the target cells. Some chemicals in the environment may affect human cells in a manner which is different from that of rodent or bacterial cells. Thus human cells *in vitro* should provide a more appropriate source for the enzymes involved in the metabolic activation of hazardous chemicals and as target cells for mutagenesis. However, in spite of this, their use in such assays is limited. Maher and Wessel (1975) and Maher *et al*. (1976) have studied the induction of 8-azaguanine resistant mutants in human fibroblast strains derived from healthy and xeroderma pigmentosum patients. Using direct acting mutagens, they achieved quantitation in inducing mutation at the hypoxanthine-guanine phosphoribosyl transferase (HGPRT) locus. However, since normal human fibroblasts exhibit low saturation density and low plating efficiency, this approach requires a large number of tissue culture vehicles, prolonged incubation times and frequent medium changes to achieve appropriate quantitation. Normal human fibroblasts have an additional disadvantage in that they can be subcultured for only about 50 cell generations. Thilly and his associates (1976) have overcome some of the above problems by employing human lymphoblastoid cell lines. These cells require less technical handling and can be cultured in larger numbers in suspension and grown as colonies in soft agar medium. However, like many other cell lines, this lymphoid

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cell line lacks the enzymes required for the activation of many environmental chemicals. Therefore, it is necessary to supplement them with the appropriate activation system. However, since they grow in suspension or soft agar, they are not suitable for use in the cell-mediated mutagens assay which requires contact between the target cells for mutagenesis and the cells used for the metabolism of the tested chemical (Kuroki and Drevon, 1978).

In view of this, we are at the present time in the process of isolating clones with a high plating efficiency (60-90%) from a human teratoma cell line which grows attached to the surface of Petri dishes (Zeuther et al., 1979). These teratoma cells are characterized by a short generation time (12-15 hr) leading to the production of large colonies within 8-10 days. Such characteristics will make the isolated clones extremely useful for mutagenesis studies. Some of these clones will be tested for their susceptibility to various direct acting mutagens such as nitrosamides and diol-epoxides of polycyclic hydrocarbons. The main genetic markers to be used will include ouabain and 6-thioguanine resistance. Once appropriate quantitation is achieved with the direct acting mutagens, we shall test these cells in the cell-mediated mutagenesis assay with a variety of chemical carcinogens that require metabolic activation including nitrosamines, aflatoxins and polycyclic aromatic hydrocarbons.

In addition, we shall employ some breast tumor cells (obtained from the Naval Biosciences Laboratory) in which we demonstrated an ability to metabolize benzo(a)pyrene, as carcinogen metabolizing cells in a cell-mediated assay with the teratoma cells as the target cells for mutagenesis. We shall also test human liver tumor cells for their ability to metabolize aflatoxins and nitrosamines. The use of these fast-growing teratoma cells as target cells

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for mutagenesis in combination with other human cells either from normal origin as described by Harris and his associates (Hsu *et al.*, 1978) or from some human tumors may represent an important step toward developing a reliable human cell assay for the identification of chemicals potentially hazardous for humans.

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4. Induction of different markers of differentiation in human malignant cells by tumor promoting agents.

Phorbol-12-myristate-13-acetate (PMA) and related phorbol diesters (Hecker, 1978), which are tumor promoters in a two stage mouse skin carcinogenesis system (Boutwell, 1978), were recently found to alter cell differentiation in some avian and murine cells (Cohen, et al., 1977; Rovera et al., 1977; Yamasaki et al., 1977; Ishii et al., 1978; Diamond et al., 1977; Miao et al., 1978). These experiments suggest that tumor promoting agents may mimic natural cellular agents that are involved in the control of cell growth and differentiation. In view of this, we have initiated studies to determine whether PMA and related chemicals can also affect cell growth and differentiation in human cells. To determine this, we have examined the response of human HL-60 and K562-4 myeloid leukemia cells (Collins, et al., 1978; Lozzio et al., 1976) to a series of tumor promoting phorbol-diesters. These human cells were used since they display distinct biological commitments towards differentiation and can be efficiently induced to differentiate into mature cells by dimethylsulfoxide and related compounds. Our preliminary studies with the tumor promoting phorbol-diesters indicated that these chemicals inhibited rather than induced terminal differentiation in these human cells. Differentiation in the leukemia cells was determined by an increase in the percentage of myelocytes, metamyelocytes and other mature myeloid cells as well as by an ability of the cells to phagocytize yeast cells. Differentiation could be induced by PMA at a dose as low as 10^{-10} M which is significantly lower than those used to give a biological response in avian or murine cell culture system. Induction of terminal differentiation in these cells was related to the activity of the phorbol-esters in the mouse skin.

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We plan to extend these studies to include additional markers for differentiation in the myeloid leukemia cells. They will include measurement of myeloperoxidase activity (Kaplow, 1965), the production of Fc receptors (Rabellino and Metcalf, 1975), and lysozyme activity (Sachs, 1978). This last enzyme is extremely useful since it can be easily quantified and may offer a simple biochemical marker of differentiation.

For the determination of lysozyme activity, control and treated cells will be harvested, washed with phosphate buffered saline, centrifuged, and the cell pellets will be lysed for approximately 10 min at 37°C in potassium phosphate buffer pH 6.25 that contains 0.2% Triton-X 100. Cell debris will be removed by centrifugation. The growth medium from which the cells are harvested will also be assayed for lysozyme activity. Lysozyme activity will be determined by measuring the lysis of Micrococcus lysodeikticus as visualized by a decrease in turbidity of the bacterial cell suspension at an optical density of 540 nm at 25°C. The μg equivalents of lysozyme activity will be estimated by a comparison with a standard activity curve using purified lysozyme from hen egg white.

In addition to the myeloid leukemia cells, we plan to include the human HO melanoma cells (Giovanella et al., 1976) which can also display a distinct biological commitment towards differentiation. These melanoma cells offer a readily quantitative marker of differentiation, namely, melanin, as well as a morphological marker, dendrite formation (Hirobe, 1978; Kreider et al., 1975; Kitano, 1976). Our preliminary studies indicated that as in the case of the myeloid leukemia cells, the human HO melanoma cells can be efficiently stimulated to differentiate by PMA and related tumor promoting phorbol-diesters. Differentiation with PMA could be detected at doses as low as 10^{-10} M. We shall extend these studies to include an additional quantitative marker, dopa decarboxylase (McCaman et al., 1972), an enzyme involved in melanin biosynthesis. To

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determine whether induction of terminal differentiation by tumor promoting phorbol esters is unique to the myeloid leukemia HL-60 and K-562 cells and the HO melanoma cells,we shall include additional leukemic and melanoma cells as well as other tumor cell types which will be obtained from the Cell Culture Department of the Naval Biosciences Laboratory, Naval Supply Center, Oakland, California.

Studying the induction of various endpoints of terminal differentiation in these human tumor cells will help in elucidating the cellular controls involved in the induction of terminal differentiation. If some or all of the tested human cells will display terminal differentiation after treatment with PMA, we will be able to suggest that either myeloid and melanoma cells differ from other cell types in their response to phorbol-diesters or that human cells in general may differ from rodent and avian cells in response to the PMA treatment. Thus, these agents may be extremely useful in controlling malignant cell growth in susceptible cells. In connection with this we shall attempt to determine whether tumor formation by human myeloid leukemia and melanoma cells in athymic nude mice can be inhibited by PMA and related agents. To perform such experiments we shall attempt to determine doses of PMA which will not be cytotoxic to the host (nude mouse) but which will be able to induce terminal differentiation. Once tumors are observed with the leukemia and/or melanoma cells, we shall treat the mice with appropriate doses of PMA or related compounds to inhibit tumor growth and observe for tumor regression. In addition to these experiments,we shall test whether other chemicals suspected of acting as tumor-promoting agents such as saccharin, anthraline, barbiturates, and chlorinated hydrocarbons can be effective in inducing or perhaps inhibiting terminal differentiation in the different myeloid and melanoma cells. Such experiments will indicate whether induction of terminal cell differentiation in the human cells is unique to phorbol-diesters or is characteristic of other classes of tumor-promoting agents.

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5. Alterations in lipid biosynthesis in differentiating human tumor cells.

Cell-cell interactions play an important role in the control of cell growth and differentiation in multicellular organisms. Signals expressed between cells during such interactions would appear to be due to changes in the surface membrane organization (Pardee et al., 1974; Robbins and Nicolson, 1975; Edelman, 1976). Major components of cellular membranes are the phospholipids and glycolipids (Rekonen et al., 1972; Lee and Snyder, 1973; Bretscher and Raff, 1975; Hildebrand et al., 1975). It would therefore be of interest to determine whether alterations in phospholipid and glycolipid metabolism which occur after PMA treatment (preliminary data) can be detected prior to initiation of cell differentiation. In view of this we shall undertake to study alterations in phospholipid and glycolipid metabolism in the myeloid leukemia and melanoma cells that can be induced to undergo terminal differentiation after treatment with tumor promoting phorbol-diesters.

For phospholipid analysis cells will be labeled with [^{32}P]orthophosphate or [1,2-^{14}C]choline chloride for 4 hr. at different times following PMA treatment. They will be then harvested, washed with cold phosphate-buffered saline (pH 7.2), and the lipids will be extracted by a modified method of Bligh and Dyer (1959), in which the methanol contains 2% glacial acetic acid. Aliquots of total lipid will be analyzed directly for ^{32}P by liquid scintillation spectrometry. Phospholipids will be fractionated on Silica Gel 60 HR (layers will be made basic with 1 mM Na_2CO_3) in a solvent system containing chloroform:methanol: glacial acetic acid:water (50:25:8:4, v/v). Identification of specific phospholipid classes will be performed by comparing R_f values with authentic standards. This will be also checked using an alternate solvent system, chloroform:methanol: ammonium hydroxide (60:35:8, v/v). The distribution of radioactivity along the

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chromatogram will be determined by zonal profile scanning (Snyder and Kimble, 1965). Phosphorous content will be determined after first localizing the lipids by H_2SO_4-charring, followed by scraping the individual spots into test tubes for direct analysis of phosphorous (Rouser et al., 1966). Total phospholipid phosphorous will be quantitated in an identical manner. Glycolipid extractions will be performed similar to the phospholipids. After labelling the cells for 4 hr with [^{14}C]glucosamine at different times after treatment with PMA, the labeled gangliosides will be separated on a thin layer of silica gel G-coated plate in a solvent system that contains chloroform:methanol: ammonia:water (60:35:1:7 v/v) and will be identified by their co-chromatography with authentic standards. The different gangliosides will be determined quantitatively after the appropriate bands are scraped from the thin-layer chromatogram and collected in scintillation vials, and the radioactivity will be determined in a scintillation counter.

Alterations in phospholipid and/or glycolipids turnover observed prior to the detection of markers of cell differentiation will suggest that PMA affects cell growth and differentiation by altering the turnover of some phospholipids and/or glycolipids which are major components of cellular membranes. Moreover, this may suggest that the tumor-promoting agents may alter the lipid composition of the surface membrane which in turn can alter the regular behavior of receptor molecules that bind hormone-type growth or differentiation factors present in the serum used for cell cultures (Lee and Weinstein, 1978a, 1978b, 1978c; Shoyab et al., 1979).

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E. DIRECT COST BUDGET

Beginning 9/27/79

	MY	Year 1 $	Year 2 $	Year 3 $
Personnel:				
Scientific	1.5			
Technicians	1.5			
Laboratory Aid (Glassware Washing)	.5			
Total man years	3.5			
Direct Costs:				
Personnel Salaries and Wages		82700	88500	94700
Travel		1600	1700	1800
Materials & Supplies		69000	76000	83700
Craft Support		2200	2300	2400
Animal Care		5000	6000	7000
Total Direct Cost		160500	174500	189600

Budget Comments

Personnel:

Eliezer Huberman, Ph.D., Principal Investigator
Carol Jones, Ph.D., Postdoctoral Fellow
Joseph Friedman, Ph.D., Postdoctoral Fellow

Travel:

Funds are requested to support participation in meetings of the Environmental Mutagen Society (EMS), American Association for Cancer Research, and other related scientific workshops. Participation in International meetings such as the International EMS Meeting, upon approval by the contract officer.

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