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HEALTH EFFECTS OF COMBUSTION-GENERATED SOOT
AND POLYCYCLIC AROMATIC HYDROCARBONS

Progress Report
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Progress Report #1

Mutagenicity of soots and soot components in S. typhimurium.

Investigators: B.M. Andon
D.A. Kaden

Rationale: We need to know what causes soot to be mutagenic and bacterial forward mutation assay offers an economical means for primary detection of potent mutagens.

Report: Soots

Our studies of methylene chloride extracts of kerosene soot, two separate samples of diesel engine emissions, and two urban air particulate samples are summarized in Figures 1-6.

In figures 1 and 2 relative activities of the kerosene soot, two separate samples from an Oldsmobile 350 CID engine, and air particulate samples from St. Louis and Boston are compared in the presence (Fig. 1) or absence (Fig. 2) of an exogenous metabolic system. In the presence of a metabolic element (post mitochondrial supernatant of the livers of aroclor pretreated rats) the kerosene soot, and the two diesel emission samples (provided by the Inhalation Toxicology Research Group, Albuquerque, N.M. and the Exxon Research and Engineering Company, Linden, N.J.) were found to have comparable mutagenic activities, which surprisingly equaled or exceeded somewhat the activity of pure benzo[α]pyrene. The air sample extracts did not contain significant mutagenic material in the concentration ranges tested and were at least thirty times less potent than the kerosene soot extract.

Surprisingly, in the absence of a metabolic element both diesel exhaust and urban air particulate extracts demonstrated mutagenic activity.

Neither kerosene soot extract nor pure benzo[α]pyrene have mutagenic activity under these conditions. These findings stimulated much discussion as to what kinds of chemicals might be stable enough to survive collection

and extraction but reactive enough to induce mutation without microsomal metabolism.

However, resolution obviously requires more analytical studies.

The first attempt at further analysis involved elution of the methylene chloride extracts of these various samples from a silicic acid column by a series of solvents of increasing polarity. The mass balance and proportion of total mutagenic activity found in each fraction in the presence and absence of the exogenous metabolic element are summarized here:

Mutagenic Activity and Mass of Eluate Fractions
of Diesel Exhaust Particulate Extracts
[350 Oldsmobile]

	EXXON SAMPLE			ITRI SAMPLE		
	% total activity (150 μ g/ml)			%total activity (60 μ g/ml)		
	<u>wt%</u>	<u>ARO</u> <u>PMS</u>	<u>NO</u> <u>PMS</u>	<u>wt%</u>	<u>ARO</u> <u>PMS</u>	<u>NO</u> <u>PMS</u>
Alkane (hexane or pentane)	27	40	7	18	3	0
alkane/toluene 1:1	7	30	18	13	71	60
toluene	7	15	36	7	16	19
methylene chloride	14	15	24	8	9	13
methylene chloride/methanol 2:1	38	0	16	--	--	--
methylene chloride/methanol 1:2	4	0	0	--	--	--
methanol	4	0	0	54	1	7

Figures 3 and 3a report the quantitative mutation data for the ITRI generated diesel engine sample which was collected by the "hot-pipe" method on a glass filter. Figures 4 and 5 report the equivalent data for the EXXON generated diesel engine sample which was collected with the aid of a standard dilution chamber. Some remarkable biological and chemical differences may be noted. A greater alkane elutable fraction is found in the EXXON sample which was collected on a relatively cool filter. The mutagenic activity of this fraction is significant representing a major fraction of the total in the presence of a metabolic element. The highest specific mutagenic activity is found in either the alkane/toluene or toluene fractions whether or not a metabolic element is used in the assay.

In contrast, air particulate sample extracts eluted from a silicic acid column by an identical procedure demonstrated mutagenic potential only in the methanol eluant indicating activity in very polar compounds (Figures 6 and 7).

FIG 1: MUTATION TO Bac^R BY METHYLENE CHLORIDE EXTRACTS OF SOOT SAMPLES. APOLOX INDUCED RAT LIVER POST MITOCHONDRIAL SUPERNATANT(PMS) 10% V/V.

FIG 2: MUTATION TO Bac^R BY METHYLENE CHLORIDE EXTRACTS OF SOOT SAMPLES. NO PMS.

FIG 3: OLDSMOBILE 300 "HOT PIPE" SAMPLE FURNISHED BY INHALATION TOXICOLOGY RESEARCH INSTITUTE. ALBUQUERQUE, NM. PMS METABOLIZING SYSTEM PRESENT.

FIG 3A: SAME AS 3. NO PMS

FIG. 4: OLDMOBILE 350 SAMPLE PROVIDED BY EXXON RESEARCH AND ENGINEERING COMPANY, ARO PMS.

FIG. 5: SAME AS FIG. 4. NO PMS.

FIG. 6: ENVIRONMENTAL PROTECTION AGENCY STANDARD AIR PARTICULATE SAMPLE, ARO PMS.

FIG. 7: SAME AS FIG. 6. NO PMS.

LEGEND FOR FIGURES 8-17: MUTATION TO GA^R BY VARIOUS POLYCYCLIC AROMATIC HYDROCARBONS IDENTIFIED IN SOOTS. MEANS OF AT LEAST DUPLICATE EXPERIMENTS WITH 95% CONFIDENCE INTERVALS ARE PLOTTED. THE 95% UPPER CONFIDENCE LIMIT ON THE HISTORICAL SPONTANEOUS BACKGROUND (IN 350) IS PLOTTED AS A HORIZONTAL DASHED LINE. ARO PMS DENOTES 10% V/V POSTMITOCHONDRIAL SUPERNATANT OF THE LIVER OF AROCLOR PRE-INDUCED RATS. PB PMS INDICATES PHENOBARBITAL PREINDUCED PMS. NO PMS INDICATES ABSENCE OF EXOGENOUS METABOLIZING ELEMENT.

Figure 8

Figure 9

Figure 10

Figure 11

(ii) Individual Soot Components

Table A-4 summarizes our findings to date of specific mutagenicity of compounds identified in combustion effluents from various systems. Most of this data has appeared in Kaden et al., 1979 but 5 additional compounds have been examined and summarizing this data in one table is useful in itself.

Table A-1
Mutagenicity of some components to *S. typhimurium*

Compound	M.N.	Pre ^a	Significant induced mutation	Concentration ^b	Relative mutagenicity ^c
Benzene	78	A, PS ^d	++	33 mM	
Pyridine	79	A, PS	++	4 mM	4.01
Indole	117	A, PS	++	4 mM	
Naphthalene	128	A, PS	++	2 mM	
Quinoline	129	A	++	80 mM	8.70
Isoquinoline	129	A, PS	++	1 mM	
3-Methylindole	131	A, PS	++	2 mM	
1-Methylnaphthalene	142	A	++	4 mM	0.01
2-Methylquinoline	143	A, PS	++	7 mM	
4-Methylquinoline	143	A	++	90 mM	0.05
1-Methylisoquinoline	143	A, PS	++	7 mM	
3-Methylisoquinoline	143	A, PS	++	4 mM	
Acenaphthylene	152	A, PS	++	1 mM	0.07
1-Cyanoacetylnaphthalene	153	A, PS	++	1 mM	
2-Cyanoacetylnaphthalene	153	A, PS	++	1 mM	
Acenaphthalene	154	A, PS	++	400 mM	0.01
2-Phenylpyridine	155	A, PS	++	6 mM	
4-Phenylpyridine	155	A, PS	++	650 mM	0.10
2,6-Dimethylnaphthalene	156	A, PS	++	160 mM	
2,6-Dimethylquinoline	157	A, PS	++	2 mM	
2,7-Dimethylquinoline	157	A, PS	++	2 mM	
2,3-Dimethylquinoline	158	A, PS	++	3 mM	
Fluorene	166	A, PS	++	300 mM	
6-Asafluorene	167	A, PS	++	1 mM	
3H-Benz[gl]indole	167	A, PS	++	1 mM	
Carbazole	167	A, PS	++	3 mM	
Dibenz[b,d]thiophene	168	A, PS	++	300 mM	
o-Dinitrobenzene	168	A	++	2 mM	45.01
2,3,6-Trimethylnaphthalene	170	A, PS	++	600 mM	
Anthracene	178	A, PS	++	225 mM	
Phenanthrene	178	A, PS	++	300 mM	
Acridine	179	A, PS, none	++	100 mM	NA ^e
1,4-Benzquinoline	179	A	+	140 mM	0.05
5,6-Benzquinoline	179	A, PS	+	84 mM	0.11
7,8-Benzquinoline	179	A	+	280 mM	0.03
6H-Cyclopenta[def]phenanthrene	196	A, PS	-	1 mM	
2-Methylanthracene	192	A	+	80 mM	0.15
9-Methylanthracene	192	A, PS	+	75 mM	0.01
1-Methylphenanthrene	192	A, PS	+	80 mM	0.57
2-Methylphenanthrene	192	A, PS	+	60 mM	0.30
2-Phenylnaphthalene	182	A, PS	-	4 mM	
Anthrone	194	A, PS	-	520 mM	
Pyrene	202	A	+	140 mM	0.07
Fluoranthene	202	A	+	5 mM	1.00
1-Asafluoranthene	203	A, none	+	50 mM	NA
2-Asafluoranthene	203	A, none	+	60 mM	NA
3-Asafluoranthene	203	A, none	+	60 mM	NA
7-Asafluoranthene	203	A, none	+	700 mM	NA
Benzo[b]fluorene	204	A	+	25 mM	0.01
Anthraquinone	204	A, PS	-	100 mM	
3H-Benz[el]indol-2-one	210	A, PS	-	2 mM	
1-Methylpyrene	216	A	+	180 mM	0.05
Cyclopenta[ed]pyrene	226	A	+	7.3 mM	1.51
Benzo[a]anthracene	228	A, PS	+	65 mM	0.14
Thrycene	229	A, PS	+	45 mM	0.20
Triphenylene	229	A	+	44 mM	0.07
7H-Benz[de]anthracene-7-one	230	A, PS	+	100 mM	0.20
o-Terphenyl	230	A, PS	-	900 mM	
o-Terphenyl	230	A, PS	-	900 mM	
1,2-Benzodibenzo[b,d]thiophene	234	A, PS	-	500 mM	
1,7,3,5'-Tetramethylbenzidine	240	A, PS	-	470 mM	
Benzo[a]pyrene	252	A	+	6 mM	1.00
Benzo[e]pyrene	252	A, PS	+	90 mM	0.11
Perylene	252	A	+	1.1 mM	6.00
1,1'-Binaphthyl	254	A, PS	-	800 mM	
9-Phenylanthracene	254	A, PS	-	600 mM	
7,12-Dimethylbenzo[a]anthracene	256	A, PS	+	25 mM	0.44
3-Methylcholanthrene	266	A, PS	+	190 mM	0.06
Benzo[ghi]perylene	276	A, PS	+	72 mM	0.08
Anthanthrene	276	A, PS	+	40 mM	0.08
Dibenz[a,c]anthracene	278	A, PS	+	13 mM	0.77
Dibenz[a,h]anthracene	278	A	+	75 mM	0.08
Flizene	278	A, PS	-	36 mM	
Coronene	300	A, PS	-	170 mM	
Dibenz[b,e]fluoranthene	302	A	+	26 mM	0.88

^aWhere there is more than one pretreatment listed, calculations refer to untreated pretreatment. "0", no significant induced mutation; "+", significant induced mutation.

^bNumber listed is lowest concentration of significant induced mutation for positive responses.

^cHighest concentration tested for negative responses. ^dMutagenic activity relative to that of

the 80 μ M benzo[a]pyrene-positive control performed simultaneously with test compound.

^eA, Aroclor; PS, phenobarbital; NA, not appreciable. ^fIndicates upper limit of solubility.

Figures 8-26 summarizes those compounds active at concentrations of less than 300 μ M in 2 hour incubations generally with maroclor induced metabolising system.

One notes (Fig. 8) that benzo[e]pyrene a putative non-carcinogen has detectable potency as a bacterial mutagen being approximately 1/10 as active as benzo[a]pyrene. Figure 9 shows a similar strong vs. weak response between dibenz[a,c]anthracene and dibenz[a,h]anthracene. Figure 10 compares benzanthrane to the more potent mutagen 7, 12 dimethyl benzanthrane and also shows a weak mutagenic potency for 7H-benzo[de]anthracene-7-one, interesting to us as we consider polar derivatives of PAH which are mutagenic. Figure 11 compares some anthracene derivatives revealing the increased potency of the 9-methyl derivative of the apparently non-mutagenic anthracene.

Figure 12 shows a similar effect of methyl substitution on the mutagenicity of phenanthrene. Also in Figure 12 are data indicating that the three aza analogues of phenanthrene are relatively potent mutagens. Figure 13 shows differences between mutagenicity of pyrene and methylpyrene but not at low concentrations. Figure 14 summarizes similar studies of the methyl and aza derivatives of naphthalene and Figure 15 shows the anthracene aza analogue, acridine, to mutate S. typhimurium in the absence of an exogenous metabolic element.

Figure 12

Figure 13

Figure 14

Figure 15

Figures 16 and 17 show that three aza analogues of fluoranthene are mutagenic in the absence of exogenous metabolic activation while fluoranthene displays no such activity. However, fluoranthene is a more potent mutagen than the four aza analogues tested to date in the presence of the rat liver extract. *[This work is discussed in greater detail in the sections devoted to fluoranthene and the analogues.]*

Figures 18-26 show the mutagenic dose response and the structures for some PAH of which are found with alkyl and other substituents in diesel soots and thus represent starting points in considering structure activity relationships. Of particular interest is the high potency of perylene, a topic which has been explored further and is addressed in a manuscript *[Penman et al., 1980]* now in press *[Mutation Research]*.

Figure 16

Figure 17

LEGEND FOR FIGURES 16-25: MUTATION TO BAC^r BY POLYCYCLIC AROMATIC HYDROCARBONS IDENTIFIED IN SOOTS. MEANS OF DUPLICATE EXPERIMENTS ARE PLOTTED AND 95% UPPER CONFIDENCE LIMIT ON THE HISTORICAL SPONTANEOUS BACKGROUND IS SHOWN AS A HORIZONTAL DASHED LINE. VARIATION WITHIN SIMULTANEOUS DUPLICATE EXPERIMENTS WAS CONSISTENTLY SMALL AND IS NOT SHOWN.

Figure 26

Progress Report #2

Mutagenicity of soots and soot components in human lymphoblasts.

Investigator: Howard L. Liber

Rationale: We have hypothesized that the mutagenic activity of complex mixtures of soots and other combustion effluents is accounted for by the sum of the mutagenic activities of their chemical constituents. Therefore, we have begun testing various well-defined soot extracts and other less well-defined environmental samples for their mutagenic activity to human lymphoblasts. Also, we have begun testing some of the polycyclic aromatic hydrocarbon components of the soot mixtures.

Report: Soot extracts are obtained from various sources. The kerosene soots AN5 and AN33 were prepared at M.I.T. by R. Hites. Diesel soot was provided by Exxon Corporation. The St. Louis air particulate extract is a sample from the National Bureau of Standards (#1648). Individual polycyclic aromatic hydrocarbons were either purchased from commercial sources or received as gifts.

Lymphoblasts were treated for 2 or 3 hours in the presence of an exogenous metabolizing system, which would allow oxidative metabolism of compounds in the test samples by microsomal mixed function oxygenases. The metabolizing system consisted of 5% (v/v) post-mitochondrial supernatant (PMS), along with an NADPH generating system. After treatment, the cells were allowed a phenotypic expression period, and then plated to determine changes in mutant fraction.

The first set of experiments was done with AN-5, a soot obtained by burning kerosene [Skopek, T.R. *et al.*, 1979. *Mutation of Human Cells by Kerosene Soot*. J. Natl. Cancer Inst., 68: 309-312].

AN-5 induced an increase in the 6TG^R mutant fraction of HH4 in the 0-26 µg/ml range (Figure 1). Three active components of this soot that, to date, have been determined are: benzo[α]pyrene (BP), fluoranthene (FA)

and cyclopenta[c,d]pyrene (CP)(Figure 1). A fourth component, perylene was inactive (Table 2). However, these components can only account for about 16% of the total mutagenicity of AN-5 (Table 1). Therefore, there may be other mutagens of undiscovered potency in the soot extract.

Table 1

	*1. wt. % of soot	conc. in 26 µg/ml soln. *2.	*3. IMF	% total IMF
AN 5	100	26 µg/ml	120	100
Benzopyrene	1	0.26 µg/ml	0	0
Cyclopenta[c,d]pyrene	15	3.9 µg/ml	10	8
Fluoranthene	4	1.0 µg/ml	10	8
Perylene	2	0.5 µg/ml	0	0
Total of Components	22	5.7 µg/ml	20	16

*1. the percentage of each component present in AN 5 extract

*2. the calculated concentration of a particular component in the
26 µg/ml treatment

*3. the estimated IMF ($\times 10^6$) by the conc. from col. 2.

Later experiments have been done in TK6, in which two genetic loci (6TGR^R, F₃TdR^R) have been utilized. AN33, a kerosene soot similar in composition to AN5, yielded mutant fractions of 49×10^{-6} for 6TGR^R and 62×10^{-6} for F₃TdR^R, after the cells were treated with 20 µg/ml for 3 hours.

Benzo[a]pyrene and fluoranthene elicited similar responses in TK6 as they had in HH4 (Figures 2,3). Perylene, a PAH which was very mutagenic in bacteria, failed to increase the mutant fraction in human cells (Table 2). This was due to the fact that bacterial cells were treated in minimal medium, while human cells were treated in complex medium

[Penman, B.W. *et al.*, 1980. Perylene is a More Potent Mutagen than Benzo[a]pyrene for *S. typhimurium*. *Mut. Res.*, in press].

Two environmental samples were tested in TK6. A diesel soot exhaust and the St. Louis air particulate extract were active, each inducing significant increases in mutant fraction at concentrations of between 50 and 100 $\mu\text{g/ml}$ (Figures 4,5). These soots were inactive when the metabolizing element was not included (Figures 6,7).

TABLE 2
PERYLENE IS NON-MUTAGENIC TO HUMAN CELLS
IN CELL CULTURE MEDIUM

Concentration ($\mu\text{g/ml}$)	$\text{F}_3\text{TdR}^{\text{R}}$ MF $\times 10^6$ Avg			6TG^{R} MF $\times 10^6$ Avg		
0A	3.0,	2.2	3.2	6.8,	2.8	4.7
0E	3.2,	4.3		5.5,	3.9	
2.5A	2.9,	2.6	3.3	4.5,	3.0	3.8
2.5B	3.7,	4.1		2.8,	4.7	
5.0A	4.5,	3.8	3.7	5.8,	6.0	5.4
5.0B	3.4,	3.1		6.7,	2.8	
BP A	136,	128	129	119,	75	94
BP B	150,	95		84,	96	

TK6 cells were treated in duplicate with perylene for 3 hr in the presence of 5% (v/v) aroclor-induced rat liver post-mitochondrial supernatant. They were plated in microtiter dishes for $\text{F}_3\text{TdR}^{\text{R}}$ (1 $\mu\text{g/ml}$) on days 3 and 5, and for 6TG^{R} (5 $\mu\text{g/ml}$) on days 5 and 7 following treatment. Benzo[a]pyrene was a positive control, at 5 $\mu\text{g/ml}$ for 3 hr.

Figure 1. Toxicity and mutagenicity of kerosene soot (AN5) and its components to human cells.

HH4 cells were treated with AN5 or the indicated polycyclic aromatic hydrocarbon in the presence of 5% (V/V) aroclor induced post mitochondrial supernatant and plated for resistance to 5 μg/ml 6TG 7 - 10 days later. Relative survival was determined by plating in non-selective conditions immediately after treatment. Error bars are 95% confidence intervals.

Figure 2. Mutagenicity of benzo(a)pyrene to TK6.

TK6 were treated and plated for resistance to 1 μg/ml F₃TdR or 5 μg/ml 6TG. Error bars are 95% confidence intervals.

Figure 3. Mutagenicity of Fluoranthene to TK6

TK6 were treated and plated for resistance to 1 μg/ml F₃TdR. Error bars are 95% confidence intervals.

Figure 4. Mutagenicity of diesel exhaust soot to TK6 in the presence of an activating system

Error bars are 95% confidence intervals. The dashed line is the 95% upper confidence limit of the historical background mutant fraction.

Figure 5. Mutagenicity of St. Louis air particulate extract in the presence of an activating system

Figure 6. Mutagenicity of diesel exhaust soot to TK6 in the absence of an

Figure 7. Mutagenicity of St. Louis air particulate extract in the absence of an activating system

Progress Report #3

"Synthesis of Oxides and Dihydrodiols of the Most Mutagenic Polycyclic Aromatic Hydrocarbons", W.H. Rastetter.

Our initial proposal to The Department of Energy was directed toward the synthesis of cyclopenta[c,d]pyrene (acepyrylene) and its oxide and dihydrodiol. Shortly after submission of our proposal, Koreeda and Akagi announced the syntheses of the hydrocarbon and its oxide [M. Koreeda, H. Akagi, *Abstract No. 499 from the American Chemical Society/Chemical Society of Japan, Chemical Congress, Organic Chemistry Division, Honolulu, Hawaii, April 2-6, 1979*]. Subsequently, reports from two other groups have appeared on the synthesis of the hydrocarbon or its oxide [a) M. Konieczny, R.G. Harvey, *J. Org. Chem.*, 1979, 44, 2158; b) A. Gold, J. Brewster, E. Eisenstadt, *Chem. Commun.*, 1979, 903]. Given our late start on the chemistry of cyclopenta[c,d]pyrene, we abandoned our initial plans and focused on the mutagenic hydrocarbon fluoranthene. This progress report outlines our work directed at possible oxidative metabolites of fluoranthene during the period 1 May 1979 - 30 November 1979. A limited study of fluoranthene funded by the National Institutes of Health was initiated in our laboratory on 1 December 1979. Our proposed, non-overlapping effort for DOE support is detailed below.

Our efforts have been directed at both A- and B-ring functionalization of fluoranthene. We have achieved

fluoranthene

the syntheses of key intermediates which will serve as branch points for production of various A-ring and B-ring products.

A-ring Intermediates: Our targets for A-ring functionalization are discussed in the Background and Specific Aims sections on p.41 and 45 of this proposal. In particular, our targets include arene oxide 1, diol epoxide 2 (p. 42), and arene oxides 17, 18 and 19 (p. 47).

Our progress on A-ring functionalization is shown on Scheme 1 below.

Scheme 1

Acenaphthylene (1) serves as our starting material. We have developed a one step epoxidation/rearrangement mediated by *m*-chloroperoxybenzoic acid to produce ketone 2. [The known procedure for the synthesis of 2 is less convenient,

and requires three separate steps: L.S. Fieser, J. Cason, *J. Am. Chem. Soc.*, 1940, 62, 434.] A known sequence was used to convert 2 into annulated product 3 [H. Christol, D.D. Koulodo, M. Mousseron, F. Plénat, *Bull. Soc. Chem. Fr.*, 1960, 1573], however, various attempts to selectively functionalize the A-ring of 3 failed giving mixtures or over-functionalized products. A more functionalized annulated product (4) was produced via a Robinson annulation sequence. Cyclohexenone 4 has been converted to alcohol 5 and to epoxy ketones 7. Alcohol 5, in turn, via its silyl ether 6 has yielded epoxide 8.

The intermediates of Scheme 1 offer many routes to the desired A-ring oxidative products of fluoranthene. Routes utilizing ketone 2 and epoxide 8 are proposed in Schemes 3, 4 and 5 on p. 51 and 52. Alcohol 5 and the epoxy ketones 7 are potential precursors for arene oxide 1 and diol epoxide 2 (p. 42).

B-ring Intermediates: Our targets for B-ring functionalization include arene oxides 3 and 5 and their corresponding diol epoxides 4 and 6 (p. 42), as well as arene oxides 15 and 16 (p. 47). Work just initiated on the B-ring systems is outlined in Scheme 2 below.

Scheme 2

Though easily produced directly from fluoranthene, o-quinone 9 [J. von Braun, E. Anton, Chem. Ber., 1929, 62, 145] has not proven to be a useful entry to B-ring functionalization. Various attempts at reduction to trans-dihydrodiol 10 have failed producing instead a product, lacking aliphatic ¹H NMR absorptions, assigned structure 11.

The less functionalized ketone 12 [A. Campbell, S.H. Tucker, J. Chem. Soc., 1949, 2623] may be a more versatile intermediate. Its reduction to alcohols 13 proceeds cleanly. Early attempts at dehydration of 13 to olefin 14 [HOAc, HCl, heat] have given only acetates 15. A direct conversion of ketone 12 to 14 [via the tosylhydrazone and treatment with MeLi] will be attempted soon.

The further use of ketone 12 is outlined in Scheme 2 on p. 50. Olefin 14 will be used for the syntheses of arene oxide 3 and diol epoxide 4 (p. 42).

FLUORANTHENE: SYNTHESSES OF OXIDATIVE PRODUCTS

Background:

Fluoranthene occurs in various fossil fuel combustion effluents at concentrations equal to or greater than benzo[a]pyrene. Hites has found that fluoranthene comprises 2-4% of all polycyclic effluents in several combustion systems studied [G.P. Prado, M.L. Lee, R.A. Hites, D.P. Hoult, J.B. Howard, in "16th International Symposium on Combustion", pp. 649-661, Cambridge, Mass. The Combustion Institute, 1973]. Since we found fluoranthene and benzo[a]pyrene to be equipotent as mutagens for S. typhimurium [D.A. Kaden, R.A. Hites, W.G. Thilly, Cancer Research, 1979, 39, 4152], and now have found fluoranthene to be equipotent with benzo[a]pyrene as a mutagen for human lymphoblasts [manuscript in preparation], the present need for a comprehensive study of the oxidative activation of fluoranthene is clear.

To this end, one of us (W.H. Rastetter) has sought and received funding from the National Institutes of Health to synthesize three fluoranthene oxides and the corresponding diol epoxides (Figure 1).

Figure 1. Three fluoranthene oxides (1, 3 and 5) and the corresponding diol epoxides (2, 4 and 6). The diol epoxides may be derived enzymically from the parent oxides by epoxide hydration and subsequent epoxidation.

Our NIH proposal is based on analogy with the well characterized diol epoxide activation of benzo[a]pyrene [see, e.g., a) M. Koreeda, P.D. Moore, P.G. Wislocki, W. Levin, A.H. Conney, H. Yagi, D.M. Jerina, *Science*, 1978, 199, 778; b) K. Nakanishi, H. Kasai, H. Cho, R.G. Harvey, A.M. Jeffrey, K.W. Jennette, I.B. Weinstein, *J. Am. Chem. Soc.*, 1977, 99, 258; c) K.M. Straub, T. Meehan, A.L. Burlingame, M. Calvin, *Proc. Natl. Acad. Sci. U.S.A.*, 1977, 74, 5285; d) P. Sims, P.L. Grover, A. Swaisland, K. Pal, A. Hower, *Nature*, 1974, 252, 326]. However, the structural positioning of the "bay region" in the alternant

hydrocarbons such as benzo[a]pyrene, dibenzo[a,h]anthracene or 3-methylcholanthrene [see a) P.P. Fu, R.G. Harvey, F.A. Beland, *Tetrahedron*, 1978, 34, 857; b) D.M. Jerina, R.E. Lehr, H. Yagi, O. Hernandez, P.M. Dansette, P.G. Wislocki, A.W. Wood, R.L. Chang, W. Levin, A.H. Conney, in "*In Vitro Metabolic Activation in Mutagenesis Testing*", ed. F.J. de Serres, J.R. Fouts, J.R. Bend, R.M. Philpot, North-Holland Pub. Co., Amsterdam, 1976] is only roughly approximated in the non-alternant hydrocarbon fluoranthene. The oxidation of fluoranthene or the reactivity of its diol epoxides may not match that expected for the alternant hydrocarbons and their diol epoxides.

Other modes of activation may actually be operative for fluoranthene. This proposal discusses non-diol epoxide modes of activation for fluoranthene, and outlines the syntheses of several candidates for proximate or ultimate mutagens of the hydrocarbon. The biological testing of these materials may lead to important findings in the area of oxidative activation, i.e., to the identification of new, reactive structural units which can promote cellular mutagenic events.

Strategy:

Since the observation of the N.I.H. shift in the mid 1960's, a vast body of data has implicated epoxides as the initially formed metabolites formed by action of cytochrome P-450-type enzymes on unsaturated carbon frameworks. The diversity of oxidative products is large. In addition to the diol epoxides (e.g., see 2, 4 and 6), arene oxides (e.g., 9), oxepin oxides (e.g., 11), and enol ether epoxides (e.g., 13) have been implicated as oxidative metabolites. For example, the epoxides 9 [W.H. Rastetter, L. Nummy, *Tetrahedron Lett.*, 1979, 2217], 11 [a) W.H. Rastetter, *J. Am. Chem. Soc.*, 1976, 98, 6350; b) W.H. Rastetter, T.J. Richard, *ibid.*, 1979, 101, 3893], and 13 [J.M. Essigmann, R.G. Croy, A.M. Nadzan, W.F. Busby, Jr., V.N. Reinhold, G. Büchi, G.N. Wogan, *Proc. Natl. Acad. Sci. USA*, 1977, 74, 1870] are believed to be intercepted by nitrogen nucleophiles during the biogenesis of gliotoxin (10), aranotin (12) and during DNA alkylation by the epoxide of aflatoxin B₁ (13), respectively.

R.J. Lorentzen, in *"In Vitro Metabolic Activation in Mutagenesis Testing"*, ed. F.J. de Serres, J.R. Fouts, J.R. Bend, R.M. Philpot, North-Holland Pub. Co., Amsterdam, 1976].

Some chemical principles may guide our selection of several fluoranthene oxidative products as primary synthetic goals. For instance, not all epoxides should be considered as likely alkylating agents for DNA or RNA. Bruice has defined the term "nucleophilic susceptibility" to denote the relative propensity of an epoxide to undergo nucleophilic addition vs. rearrangement [see A.R. Becker, J.M. Janusz, D.Z. Rogers, T.C. Bruice, *J. Am. Chem. Soc.*, 1978, 100, 3244 and references therein]. Epoxides such as 9 do not readily undergo nucleophilic epoxide opening in *in vitro* experiments. Presumably, the biogenesis of gliotoxin proceeds with enzymic catalysis in the epoxide opening step. It is less likely that "accidental" DNA or RNA alkylation will occur with such enzymic intervention. Rather, the epoxides which serve as *in vitro*, ultimate mutagens from polycyclic hydrocarbons will give stabilized carbonium ions (i.e., those without propensity for rearrangement) which will be sufficiently long-lived to survive the cellular milieu and do genetic damage [see P.P. Fu, *et al.*, *op cit*, particularly p. 863 and D.M. Jerina, *et al.*, *op cit*, p. 168]. At the very least, the epoxides will have to react with considerable S_N^1 character, ensuring alkylation of the weakly nucleophilic genetic material [P.B. Hulbert, *Nature*, 1975, 256, 146]. The epoxides proposed for synthesis herein were carefully selected with these considerations in mind (*vide infra*).

Specific Aims:

1. We will consider possible modes of fluoranthene activation via arene oxides, oxepin oxides and enol ether epoxides. Such fluoranthene-derived materials will be synthesized and tested for activity as proximate or ultimate mutagens.

2. Proximate mutagens [those which show activity upon subsequent *in vitro* oxidation] will be further studied. In particular, identification of the ultimate mutagens will be undertaken by isolation and identification of DNA reaction products

by methods outlined in other sections of this proposal.

The immediate goals for the proposed research are the syntheses of the oxygenated fluoranthenes 15 - 19 (Figure 2). These either directly or via further activation may serve as mutagens (vide infra).

Figure 1. Possible oxidative metabolites of fluorantrene. The expected position of the arene oxide/oxepin valence tautomerization is indicated.

Our long range goal is the identification of the specific mode of activation of the fluoranthene-derived mutagen. Thus, e.g., it might be possible to generate and characterize the enol ether epoxide 20 [Enol ether epoxides will be very labile toward acid. Their generation must be accomplished under neutral to basic conditions, e.g., with the peroxy-carbonic acid reagents described by J. Rebek, Jr., S.F. Wolf, A.B. Mossman, Chem. Commun., 1974, 711] or the oxepin oxide 21 [We have developed and shown the generality of a route to oxepin oxides, see a) W.H. Rastetter, J. Am. Chem. Soc., 1976, 98, 6350; b) D.D. Haas, W.H. Rastetter, ibid, 1976, 98, 6353; c) W.H. Rastetter, T.J. Richard, Tetrahedron Lett., 1978, 2995; d) W.H. Rastetter, T.J. Richard, J. Bordner, G.L.A. Hennessee, J. Org. Chem., 1979, 44, 999; e) W.H. Rastetter, T.J. Richard, J. Am. Chem. Soc., 1979, 101, 3893] derived from 16b and 18b, respectively. These materials should be exceedingly reactive, yielding stabilized carbonium ions in aqueous media. The chemistry and in vitro behavior of these epoxides will be pursued if their precursors prove active in bacterial mutation assay.

20 21

Methods of Procedure:

Initial in vitro testing. Preliminary mutagen testing has been performed [at M.I.T. by Dr. Avi Stark according to a) B.N. Ames, J. McCann, E. Yamasaki, Mutation Res., 1975, 31, 347 and b) A.A. Stark, B. Koblé, K. Matsuo, G. Büchi,

G.N. Wogan, A.L. Demain, *Appl. Env. Microbiol.*, 1975, 36, 4121] with oxepin oxide (22) [W.H. Rastetter, *J. Amer. Chem. Soc.*, 1976, 98, 6350] and with the indane oxides 23 a,b [W.H. Rastetter, M.D. Lewis, T.J. Richard, J. Adams, *J. Org. Chem.*, 1979, 44, 3175].

22 23a, R=H23b, R-OH

The structures of 22 and 23a can be found within the larger systems 21 and 19a (*vide supra*). The qualitative results show 23 a,b to be mutagenic; the toxicity found for 22 would have masked any mutagenic effect. The results show the correct reactivity pattern toward *in vitro* systems and suggest that the more substituted derivatives (e.g., 21 and 19a) may yield longer-lived and potentially even more damaging carbonium ions. The mutagen testing of the simple systems 22 and 23 a,b, will be performed quantitatively by forward mutation assay in *S. typhimurium*. The results will provide standards for comparison with the polycyclic derived materials.

Syntheses. Potential routes to the oxygenated fluoranthenes 15 - 19 are outlined in Schemes 1-5. Chemical precedent for many of the transformations is provided in the footnotes at the end of Scheme 5. The proposed routes should yield large quantities of the needed materials; in some cases new and general entries to the oxepin/arene oxide systems will be developed.

Scheme 1. Synthesis of 15a $\rightleftharpoons$ 15b.

Scheme 2. Synthesis of 16a $\rightleftharpoons$ 16b.

Scheme 3. Synthesis of 17a $\rightleftharpoons$ 17b. Scheme 4. Synthesis of 18a $\rightleftharpoons$ 18b.

Scheme 4 (Cont'd.)

Scheme 5. Synthesis of 19a ⇌ 19b.

Footnotes (Schemes 1-5):

- (1) R.G. Harvey, D.F. Lindow, P.W. Rabideau, Tetrahedron, 1972, 28, 2909.
- (2) W.H. Rastetter, T.J. Richard, M.D. Lewis, J. Org. Chem., 1978, 43, 3163.
- (3) R.E. Lehr, M. Schaefer-Ridder, D.M. Jerina, J. Org. Chem., 1977, 42, 736.
- (4) H. Yagi, D.M. Jerina, J. Am. Chem. Soc., 1973, 95, 243.
- (5) A. Campbell, S.H. Tucker, J. Chem. Soc., 1949, 2623.
- (6) E. Winterfeldt, Synthesis, 1975, 617.
- (7) P.P. Fu, H.M. Lee, R.G. Harvey, Tetrahedron Lett., 1978, 551.
- (8) We have made this protected epoxy alcohol via a Robinson annulation route.
Large quantities are available (unpublished work, see Progress Report p. 38).
- (9) We have made this ketone by m-chloroperoxybenzoic acid epoxidation and
in situ rearrangement of acenaphthylene. Large quantities are available
(unpublished work, see Progress Report p. 38).
- (10) M.S. Newman and C.H. Chen, J. Am. Chem. Soc., 1973, 95, 278.
- (11) W.T. Norris, J. Org. Chem., 1968, 33, 4540.

Progress Report #4

Synthesis and Mutagenic Activity of the Five Isomeric Azafluoranthenes

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Rationale

Various studies have demonstrated that aza-aromatics are present in urban air particulates (Table 1). In contrast to the rapid progress achieved in studies of carcinogenic "all-carbon" polycyclics [for leading references, see D.M. Jerina and J.W. Daly, *Science*, **185**, 573 (1974); M.M. Marsh and D.M. Jerina, *J. Med. Chem.*, **21**, 1298 (1978); R.A. Becker, *et al.*, *J. Am. Chem. Soc.*, **100**, 3244 (1978); P.P. Fu, *et al.*, *Tetrahedron*, **34**, 857 (1978); G.D. Berger, *et al.*, *Tetrahedron Lett.*, 231 (1978); "In Vitro Metabolic Activation in Mutagenesis Testing", ed. F.J. de Serres, J.R. Fouts, J.R. Bend, and R.M. Philpot, North-Holland Publishing Co., Amsterdam (1976), p. 159], little attention has been paid to the chemistry and biochemistry of the aza-aromatics. In many instances, the mutagenic properties of the aza-aromatics are unknown, and in those cases where mutagenic activity has been demonstrated, little or no information is available concerning the mode of metabolic activation [A. Dipple, "Polycyclic Aromatic Carcinogens", in *Chemical Carcinogens* (ACS Monograph #173), C.E. Searle, ed., American Chemical Society, 1976, pp. 245-314; see also Y. Kitahara, *et al.*, *Chem. Pharm. Bull.*, **26**, 1950 (1978) and Y. Kitahara, *et al.*, *Heterocycles*, **8**, 363 (1977)]. We propose herein to continue our studies of the azafluoranthenes and to expand our efforts to the synthesis and biological study of the azapyrenes and the benzocinnolines. (The latter may be thought of as the aza-analogues of the mutagenic benzoquinolines.) These studies are prompted by knowledge that (i) the

Table 1. Summary Present and Previous Air Particulate NCOC Studies
in terms of ng NCOC/1000 m³ air

Compound	MW*	Rome(2)	Ottawa(3)	Composite(1)**	Antwerp(4)	New York(5)	Boston(6)
Year particulates collected		1972	1972	1963	1975	1975	1978
Quinoline	129	600			17	90	55
Isoquinoline	129				50	160	300
Methyl-	143				150	340	240
Dimethyl-	157	300			170	210	300
Trimethyl-	171				170	40	
Tetramethyl-	185				50		
Methylindole	130	200					
Carbazole	167	1100					
Benzoquinolines	179		460	500	500	90	140
Methyl-	193				670		
Dimethyl-	207				500		
Acridine	179		450		1000	40	250
Methyl-	193					7	
Phenanthridine	179				170	20	160
Methylbenzo-	243				500		
Benzocinnolines	180	1000					
Methyl-	194						240
Azafluoranthene	203	3000			2670	20	20
Azapyrene	203	13000				20	95
Methyl-	217				670		
Fluorene-carboni-							
trile	191		70				
Azafluorene	167					5	
Benzacridines	229			800	2700		
Dibenzacridines	279			120	670		
Azabenzofluoran-							
thene	253				1500		

*molecular weight.

**composite collected from 100 urban areas throughout the United States

Foot-notes

- 1) Sawicki, E., S.P. McPherson, T.W. Stanley, J. Meeker, and W.C. Elbert, Int. J. Air Water Pollution, 9, 515 (1965).
- 2) Brocco, D.A., Cimmino and M. Possanzi, "Determination of Aza-heterocyclic compounds in Atmospheric Dust by a Combination of Thin Layer and Gas Chromatography, J. Chromatography, 84, 371-377 (1973).
- 3) Lao, R.C., R.S. Thomas, H. Oja and L. Dubois, "Application of Gas Chromatograph-Mass Spectrometer Data Processor Combination to the Analysis of the Polycyclic Aromatic Hydrocarbon Content of Airborne Particulates", Anal. Chem., 45(6), 908-915 (1973).
- 4) Cautretts, W., and K. VanCauwenberghe, "Determination of Organic Compounds in Airborne Particulate Matter by Gas Chromatography-Mass Spectrometry", Atmos. Environ., 10, 447 (1976).
- 5) Hoffman, D., M.W. Dong, and D.C. Locke, "Characterization of Aza-Arenes in Basic Organic Portion of Suspended Particulate Matter", Env. Sci. Tech., 11(6), 612-8 (1977).
- 6) Hites, R.A., J.I. Liu, and R. Walters, M.I.T. Dept. of Chem. Eng., unpublished results.

azafluoranthenes, azapyrenes, and the benzocinnolines are present in the urban environment (see Table 1); (ii) that fluoranthene, pyrene and the benzoquinolines are known to be mutagenic to bacteria; and (iii) that in some cases aza-aromatics are known to be as mutagenic, or more so, than their parent "all-carbon" polycyclics [D.A. Kaden, *et al.*, *Cancer Research*, 39, 4152 (1979)]. These studies will contribute to an increased understanding of the processes by which the azafluoranthenes are metabolized and activated [to mutagens.] These studies will also increase our knowledge of the chemicals which are responsible for the biological properties of fossil fuel soot.

Progress to Date

Four of the five isomeric azafluoranthenes have been synthesized and their mutagenicities determined toward *Salmonella typhimurium*. 1-Aza-(1) and 2-azafluoranthene (2) were synthesized by known methods [1: G.M. Budger, *et al.*, *J. Chem. Soc.*, 4996 (1952); N. Cambell, *et al.*, *ibid.*, 4743 (1958); 2: J.M. Birnie, *et al.*, *ibid.*, C, 2634 (1971)].

1 (1-aza)

2 (2-aza)

3 (3-aza)

4 (7-aza)

5 (8-aza)

3-Azafluoranthene (3) was synthesized by a modified literature procedure

(Scheme 1). The literature procedure [C.F. Koelsch, *et al.*, *J. Org. Chem.*, 18, 1516 (1953); J.W. Cook, *et al.*, *J. Chem. Soc.*, 1160 (1950)] calls for the conversion of 6 into 10 as indicated in the scheme.

Scheme 1

The last step of this sequence involves the copper catalyzed decarboxylation of 9, a reaction which must be performed at 230° in the presence of quinoline. We found it more convenient to convert 6 into 10 via phosphonate 11 using phosphonate-Wittig methodology [W.S. Wadsworth, Jr., *Org. Reactions*, 25, 73 (1977)]. Amide 10 was elaborated into 3 as previously described.

A synthesis of 7-azafluoranthene (4) is in the literature [C. Jutz, *et al.*, *Annalen*, 874 (1975)]. However, preparation of β -N,N-dimethylaminoacrolein

[J. Ficini, et al., Bull. Soc. Chim. Fr., 1294 (1964)], a starting material used in the synthesis described by Jutz, et al., requires that the commercially unavailable propargaldehyde be synthesized [J.C. Sauer, et al., Org. Syn., Coll. Vol. IV, 813 (1963)]. Explosions have been reported during attempts to repeat this procedure [D.N. Crouse, Chem. Eng. News, 57(41), 4 (1979)]. Accordingly, we deemed this procedure unsafe and devised the new synthesis outlined in Scheme II. The length of this route is comparable to that described by Jutz, et al. However, the final step presently proceeds in only ~10% yield. Attempts to optimize the yield of this reaction are in progress.

Our synthesis of the previously unknown 8-azafluoranthene (5) has progressed to the stage of 12 (Scheme III).

Scheme II

Scheme III

The mutagenic activities of 1-4 are summarized in Charts 1 and 2. These results are preliminary and will be duplicated in the upcoming months.

1-Azafluoranthene (1, Chart 1) yields significant (99%) mutation to 8-azaguanine resistance in S. typhimurium at 50 μM in the presence of a 10% v/v aroclor induced rat liver postmitochondrial supernatant (ARO PMS). This compares with a minimum concentration of 5 μM for significant (99%) mutation by fluoranthene, which was used as the positive control in all of these experiments. Interestingly, 1-azafluoranthene is mutagenic ($\sim 65 \mu\text{M}$) without an added metabolic activation system while fluoranthene is without activity even at very high concentrations when tested in the absence of ARO PMS (Chart 2).

2-Azafluoranthene activity as a S. typhimurium mutagen is approximately equal to that of 1-azafluoranthene (Charts 1,2). However, under the set of conditions employed, activity with metabolic activation is somewhat less than without, although these experiments have not yet defined this difference with adequate precision.

3-Azafluoranthene (3), with or without ARO PMS, yielded significant mutation at about 225 μM (Charts 1,2). This level of activity is significantly less than for fluoranthene or for the 1- and 2-azafluoranthenes, and is approximately the same as observed for the three azaphenanthrenes studied by M.I.T. Genetic Toxicology Group: benzo[f]quinoline, benzo[h]quinoline, and phenanthridene. Similarly, 7-azafluoranthene (4) in the presence of ARO PMS shows significant mutation (dose response curve intercepts the 99% upper confidence limit) at 450 μM (Chart 1). 8-Azafluoranthene (5) is presently being synthesized.

What have these studies of the azafluoranthenes taught us at this point? First of all, we now have an example of a polycyclic aromatic hydrocarbon for which at least 4 of 5 aza analogues are less mutagenic than the parent hydrocarbon. Previously, studies by the M.I.T. Genetic Toxicology Group of naphthalene compared to quinoline, anthracene compared to acridine, and

phenanthrene compared to three of the five aza derivatives indicated a marked increase in mutagenic activity of the aza derivatives compared to the parent hydrocarbons. In one case (acridine), exogenous metabolism decreased the mutagenic activity, but in all other cases, metabolism was required for mutagenic action (quinoline, benzo[f] or [h]quinolines, and phenanthridine). One aza derivative of naphthalene, isoquinoline, was without detectable mutagenic activity even at 8 mM. However, this does not represent a decrease in activity in the aza derivative, since naphthalene itself is not mutagenic.

Clearly, we do not yet understand the meaning of these results in terms of either metabolic paths of activation or possible candidates for ultimate mutagens. Studies of the effects of varying the concentration of PMS on aza polycyclics [included in: Seixas, *et al.*, Mutation Research, accepted November 1979] has already indicated the importance of this parameter. Activation and inactivation are taking place simultaneously and total activity is not sensitive to the presence of inhibitors of epoxide hydrase. Whether such considerations are useful in understanding fluoranthene or azafluoranthene mutation remains to be seen.

Future Work

A. Initial studies.

1. Azafluoranthenes. The synthesis of 5 will be completed, and the mutagenicity of this compound will be determined against Salmonella typhimurium. The N-oxides of 1-5, potential products of metabolic oxidation, will be prepared and tested. The mutagenicity of 3 towards human cells will be determined. Studies of the activation and metabolism of the azafluoranthenes are suggested in Section 4 (see below).

2. Azapyrenes. The azapyrenes are present in the air particulates of Boston at a concentration of approximately five times that of the azafluoranthenes (Table I). The mutagenicities of the three possible isomers have not yet been

CHART 1

CHART 2

examined. One isomer (13:naphtho[2,1,8-def]quinoline) is commercially available (Eastern Chemicals). The other two isomers 14 (naphth[2,1,8-def]isoquinoline: [R. Kirchlechner, *et al.*, *Agnew. Chem., Int. Ed.*, 7, 376 (1968)]) and 15 (thebenidine: [D.H.R. Barton, *et al.*, *J. Chem. Soc., C*, 729 (1971)]) will be prepared according to the literature procedures cited. The mutagenicity of each isomer will be determined. If warranted by the results of these studies, the N-oxide derivatives of 13-15 will be prepared and studied. Attempts will be made to correlate the biological activities of these compounds with their electronic structures [J. Koutecký, *et al.*, *Coll. Czech. Chem. Commun.*, 28, 2089 (1963)].

13 14 15

3. Benzocinnolines. The benzocinnolines are present in urban soot at much higher concentrations than the azafluoranthenes or the azapyrenes (Table I). These compounds may be thought of as aza-analogues of the mutagenic benzoquinolines. Accordingly, the six isomers 16-21 will be prepared and tested for mutagenicity against *Salmonella typhimurium*.

16

(benzo[c]cinnoline)

17

(benzo[c]cinnoline)

18

(benzo[h]cinnoline)

19

(benzo[f]phthalazine)

20

(benzo[g]cinnoline)

21

(benzo[g]phthalazine)

Isomers 16 [G.M. Badger, *et al.*, *J. Chem. Soc.*, 3207 (1951)], 17, 18 and 19 [H.-H. Perkampus, *et al.*, *Tetrahedron*, 28, 2099 (1972)] will be synthesized according to the literature methods cited. Synthetic routes to 20 and 21 are suggested in Schemes IV and V, respectively. Literature references for key transformations are included.

Scheme IV - benzo[g]cinnoline (20)

1. R.E. Ireland, *J. Am. Chem. Soc.*, 81, 2907 (1959); see also G. Bram, *et al.*, *Bull. Soc. Chim. Fr.*, 945 (1964) and D.W. Brooks, *et al.*, *Angew. Chem. Int. Ed.*, 18, 72 (1979).
2. K.C. Nicolaou, *et al.*, *J. Am. Chem. Soc.*, 101, 768 (1979).
3. W. Borsche, *et al.*, *Annalen*, 548, 74 (1941).
4. G.W. Kenner, *et al.*, *J. Chem. Soc.*, 522 (1955); A.H. Goldkamp, *et al.*, *J. Med. Chem.*, 8, 409 (1965).

Scheme V - benzo[g]phthalazine (21)

1. W. Ried, et al., Chem. Ber., 89, 708 (1956); M.P. Satchell, et al., J. Chem. Soc., C, 468 (1971).

B. Long range goals.

We wish to understand the processes by which the mutagenicities of the azafluoranthenes are expressed. It is of great interest that some of the azafluoranthenes (1, 2, and 3) are mutagenic without activation by ARO PMS. Apparently, these compounds are themselves mutagenic, or else S. typhimurium is capable of activating them in an unprecedented manner. Accordingly, further studies of 1 and 2, the most active of the isomers examined to date, are warranted. These studies will be designed to shed light on the biochemical processes which activate these compounds.

Two approaches to this problem may be considered. The first would involve attempts to isolate covalent DNA adducts 1 and 2. The structures of such adducts would provide much useful information concerning the nature of metabolic activation of these compounds. This approach has proven successful in identifying metabolites of benzo[a]pyrene [K.M. Straub, *et al.*, Proc. Natl. Acad. Sci., 74, 5285 (1977); K. Nakanishi, *et al.*, J. Am. Chem. Soc., 99, 258 (1977); M. Koreeda, *et al.*, Science, 199, 778 (1978)] and aflatoxin B₁ [J.M. Essigmann, *et al.*, Proc. Natl. Acad. Sci., 74, 1870 (1977)].

The second approach would involve theoretical studies of the azafluoranthenes. Molecular orbital calculations would be performed to determine the π -electron stabilization of various azafluoranthene-derived carbonium ions relative to the parent epoxide ($\Delta E\pi$). Carbonium ions with large $\Delta E\pi$ will be appreciably stabilized relative to their parent epoxides, and will have relatively long lifetimes. The relative low nucleophilicity of DNA has led to the prediction that substrates capable of reacting through intermediates displaying appreciable carbonium ion character will have high mutagenic activity [P.B. Hulbert, Nature, 256, 146 (1975)]. Hence, the epoxides corresponding to carbonium ions with large $\Delta E\pi$ would be considered to be potential metabolites of the azafluoranthenes. Such calculations have been applied to various PAH derivatives to predict the carcinogenicity (mutagenicity) of their diol epoxides [P.P. Fu, *et al.*, Tetrahedron, 34, 857 (1978); M.M. Marsh, *et al.*, J. Med. Chem., 21, 1298 (1978)] or the

oxidative susceptibility of their dihydrodiol precursors [G.D. Berger, *et al.*, Tetrahedron Lett., 231 (1978)].

In this manner, we would predict sites of metabolic activation of the azafluoranthenes, assuming that epoxides and diol epoxides are products of metabolic activation. The appropriate metabolites would then be synthesized and their mutagenicities determined. This is the approach being pursued by Professor W.H. Rastetter with potential metabolites of fluoranthene as described elsewhere in this proposal.

One shortcoming of the theoretical approach is that the compounds prepared as a result of the M.O. calculations may not actually be in vivo metabolites. There is little doubt that epoxides and diol epoxides play an important role in the metabolism of certain polycyclic aromatic hydrocarbons. However, Jerina has cautioned that "other metabolites may be the ultimate carcinogens or play an important role in carcinogenicity in addition to [epoxides or] diol epoxides, [furthermore] diol epoxides may not form or may form in trace amounts from certain hydrocarbons [D.M. Jerina, *et al.*, "In Vitro Metabolic Activation in Mutagenesis Testing", ed. F.J. de Serres, J.F. Fouts, J.R. Bend, R.M. Philpot, North-Holland Publishing Co., Amsterdam (1976), p. 159]. Accordingly, our studies of the metabolism of the azafluoranthenes will concentrate on the isolation and structure determination of the covalent DNA adducts.

Covalent DNA adducts. Our studies in this area will initially concentrate on the metabolites of 1-aza (1) and 2-azafluoranthene (2), the most active of the four azafluoranthenes examined to date, and on fluoranthene itself. A comparison of the mode of metabolic activation of the parent versus the aza derivatives would be most interesting. In vivo and in vitro DNA binding studies will be performed using radiolabeled substrates in the presence and absence of PMS. For the in vitro studies, double stranded DNA will be incubated with tritium labeled substrates (with and without PMS). The DNA

recovered by ethanol precipitation will be hydrolyzed with 88% formic acid, and the products analyzed by high pressure liquid chromatography. Radioactive bands will be isolated, and structures assigned by chemical and spectroscopic method. This procedure is essentially that which the MIT Toxicology Group has successfully used for DNA binding studies of aflatoxin B₁ [J.M. Essigmann, *et al.*, *Proc. Natl. Acad. Sci.*, 74, 1870 (1977)]. These studies will be performed in advance of the *in vivo* experiments, since larger amounts of adducts will be available for characterization.

The *in vitro* studies of 1 and 2 in the absence of PMS is essential to probe the question of how 1 and 2 themselves are able to mutate cells.

Duplicate experiments will be performed *in vivo* using *S. typhimurium*, again with and without added PMS. Covalent DNA adducts will be isolated, and if these are different from those obtained from *in vitro* experiments, structures will be assigned. Of particular interest will be whether or not the DNA adducts formed with and without PMS are the same. If not, we may be able to make definitive statements concerning the unprecedented PAH activation performed by *S. typhimurium* on 1 and 2.

A source of radiolabeled substrates is required for these studies. Tritium labeled 2 will be prepared as outlined in Scheme VI. Compound 22 is a known

Scheme VI

precursor of 2 [J.M. Birnie, *et al.*, *J. Chem. Soc., C*, 2634 (1971)]. This substance will be transformed into the α -lithium derivative 24 by standard procedures [D.C. Owsley, *et al.*, *J. Org. Chem.*, 38, 901 (1973); M. Loy, *et al.*, *J. Med. Chem.*, 16, 549 (1973)]. Then, tritium labeled 2 will be obtained by hydrolysis of 23 with tritiated water (T_2O).

Tritium labeled samples of fluoranthene and 1-azafluoranthene will be prepared by electrophilic bromination of the parent aromatic hydrocarbons followed by lithiation with t-butyllithium and hydrolysis with T_2O . The electrophilic bromination of fluoranthene has been described [J. v. Braun, *et al.*, *Annalen*, 488, 111 (1931), see also N. Campbell, *et al.*, *J. Chem. Soc.*, 1555 (1949)].

Structures of the ultimate mutagens derived from fluoranthene and its 1- and 2-aza analogues will be suggested on the basis of the structures of the covalent DNA adducts. These metabolites will then be synthesized and studied biologically to confirm their role as the ultimate mutagens derived from these aromatic hydrocarbons. [It is possible that the putative metabolites deduced from adduct structure will already have been synthesized by W.H. Rastetter for fluoranthene. 1- and 2-azafluoranthene derived putative metabolites will require synthesis in our laboratory.]

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Progress Report #5

Mutagenicity studies in human lymphoblasts (Model Mutagens)

Investigators: staff

Rationale: These were two purposes for developing mutation assays in human lymphoblast cells. First, we wished to examine a variety of chemical mutagens, in order to determine if different types of dose response curves would be generated, depending on the compound. Second, we wanted to study mutation at several different genetic loci, in order to test our hypothesis of the generality of response of various targets to particular mutagens.

Report: Three different cell lines have been used to study mutation in human lymphoblasts. Table 1 outlines their properties.

Table 1: Cell lines used to study mutation

<u>Cell line</u>	<u>Plating Conditions</u>	<u>Loci examined</u>
MIT-2	soft agarose with feeder layer	6TG ^R
HH-4	soft agarose; microtiter	6TG ^R
TK-6	soft agarose; microtiter	6TG ^R , F ₃ TdR ^R , Oub ^R

To date, we can score mutation at three distinct loci in human cells. 6TG-resistance is based on the loss of the x-linked gene HGPRT, which is a salvage enzyme that phosphoribosylates purines. Mutants deficient in the enzyme do not metabolize the analog 6TG to a toxic nucleotide, and

thus survive in its presence. F_3 TdR-resistance is based on the similar loss of a pyrimidine salvage enzyme, thymidine kinase (TK). The major difference is that an autosomal gene codes for TK and it was necessary to isolate a TK-heterozygote to use in quantitative mutation studies (see attached publication, Skopek et al., 1979).

Ouabain is a drug which binds to and inactivates an essential enzyme, Na^+/K^+ ATPase. Ouabain-resistance is based on a minor alteration in the gene such that the enzyme maintains its function but loses the ouabain binding site. This mutation is dominant.

The cell lines described above have been used to study the mutagenicity of a variety of chemical and physical agents. Examples are listed in Table 2. Dose-response curves are shown in figures 1-16. Human lymphoblasts, as expected, respond to a wide range of compounds, including alkylating agents, base analogs, polycyclic aromatic hydrocarbons, mycotoxins, acridines, and UV light.

Dose-response curves seem to fall into two general classes: (1) those in which mutant fraction appears to increase linearly with dose, and (2) those in which there is a shoulder; i.e. a concentration below which little or no mutation occurs, and above which the mutant fraction increases more rapidly. Figures 2-6 and 8-16 are examples of apparently linear responses. For some compounds, such as ICR-191 (figure 8), a plateau was reached; above $1.5 \mu M$ x 24 hr., there was no further mutation. Similar data was

Table 2

	MIT-2 6TG ^R	HH4 6TG ^R	6TG ^R	TK6 6TG ^R	Oub ^R
nitrosomethylurea	80μM x 24hr		2.5μM x 24hr	2.5μM x 24hr	
nitrosoguanidine	0.14μM x 24hr				
β-propiolactone	90μM x 24hr				
methyl methanesulfonate	100μM x 24hr				
ethyl methanesulfonate	300μM x 24hr		90μM x 24hr	90μM x 24hr	
propyl methanesulfonate	1mM x 24hr				
butyl methanesulfonate	1mM x 24hr		1mM x 1hr	1mM x 1hr	1mM x 1hr
nitrosomethylurethane	0.5μM x 24hr				
aflatoxin B ₁		1μM x 3hr			
1CR-191	0.5μM x 24hr		0.62μM x 24hr	0.62μM x 24hr	not activ
4-nitroquindine-oxide			0.13μM x 24hr	0.13μM x 24hr	
UV light			10 ergs/mm ²	15 ergs/mm ²	44 ergs/mr
bromodeoxyuridine	50μM x 24hr	100μM x 2hr			
fluorodeoxyuridine			100μM x 4hr	100μM x 4hr	
iododeoxyuridine	100μM x 24hr				
chlorodeoxyuridine	100μM x 24hr				
benzo(a)pvrene		24μM x 3hr	10μM x 3hr	10μM x 3hr	10μM x 3h
fluoranthene		10μM x 3hr	10μM x 3hr	10μM x 3hr	10μM x 3h
cyclopenta(c,d)pyrene		18μM x 3hr			

obtained from propylmethane sulfonate (figure 5).

Figures 1 and 7 are examples of the second type of dose-response curve. Nitrosomethylurea and nitrosomethylurethane appeared to cause at least a biphasic response, and possibly one which could be considered curvilinear.

Comparisons between the responses of different cell lines at one locus, $6TG^R$, revealed that in general, MIT-2 and HH4 or TK6 are affected by similar concentrations of mutagens (table 2). For example, ICR-191 mutates MIT-2 (figure 8) and TK6 (figure 9) in the concentration range 0 to $.125 \mu M$ for 24 hr.

In comparing the three loci in TK6, we have found that mutation by a given chemical to $6TG^R$ and F_3TdR^R seem to occur at the same concentrations and to the same degree (figures 2, 6, 9, 10, 11, 16 ; table 2). When an increase in ouabain-resistance is induced, it occurs at concentrations similar to F_3TdR or $6TG$ (table 2). However, whereas ouabain-resistance is induced by base-pair substitution mutagens, such as butylmethane sulfonate, UV light or benzo(a) pyrene, the locus is not at all affected by the frameshift mutagen ICR-191 (table 2). Furthermore, all measurements at the ouabain-resistance locus (background and induced mutant fractions) are 10-50 fold lower than the $6TG^R$ or F_3TdR^P loci, because of the specificity of the change required to confer resistance.

Halodeoxyuridines are mutagenic to human lymphoblasts. In figures 12 - 14, MIT-2 is seen to respond to 24 hour exposures BUdR, ClUdR and IUdR. The curves reach a

maximum and decline, presumably because of inhibitory effects on DNA synthesis at high concentration of the analogs. These experiments were done in ambient lighting. Figure 15 is the mutagenic response of HH4 to a 2 hr. exposure to BUdR, in the dark.

The work described above has appeared in the following publications:

1. [Thilly, W.G. et al., 1976. Mutation of human lymphoblasts by methylnitrosourea; Chem. Biol. Inter. 15:33-50]
2. [Penman B.W. and Thilly W.G., 1976. Concentration-dependent mutation of diploid human lymphoblasts by methylnitrosoguanidine: The importance of phenotypic lag; Somatic Cell Genet. 2:325-330]
3. [Penman, B.W. et al., 1976. Mutagenicity of 5-halodeoxyuridines to diploid human lymphoblasts; Life Sciences, 19: 563-568]
4. [DeLuca, J.G. et al., 1977. Comparative mutagenicity of ICR-191 to S.typhimurium and diploid human lymphoblasts; Mutat. Res. 46:11-18]
5. [Hoppe, H. IV, et al., 1978. Alkylmethanesulfonate mutation of diploid human lymphoblasts and S.typhimurium; Cancer Res. 38:1595-1600]
6. [Skopek, T.R. et al., 1979. Mutation of human cells by kerosene soot; J. Natl. Cancer Inst. 63:309-312]
7. [Penman, B.W. et al., 1979. Concentration-dependent mutation by alkylating agents in human lymphoblasts and Salmonella typhimurium: N-methyl-N-nitrosourethane and β -propiolactone; J. Natl. Cancer Inst. 63:903-907]

Figure 1. Mutagenicity of nitrosomethylurea to MIT-2

Cells were treated for 24 hr and plated in agarose over a HGPRT⁻ fibroblast feeder layer 10 - 14 days later for resistance to 10 $\mu\text{g/ml}$ 6-thioguanine. Error bars are standard error of the mean.

Figure 2. Mutagenicity of nitrosomethylurea to TK6.

Cells were treated and plated for resistance to 1 $\mu\text{g/ml}$ trifluorothymidine (2 - 7 days later) or 5 $\mu\text{g/ml}$ 6-thioguanine (7 - 10 days later). Error bars are 95% confidence intervals.

Figure 3. Toxicity and mutagenicity of β -propiolactone to MIT-2

See legend to figure 1. Toxicity was determined by plating cells in non-selective conditions after treatment and calculating relative survival.

Figure 4. Toxicity and mutagenicity of nitroso-guanidine to MIT-2

See legends to figures 1, 3.

Figure 6. Mutagenicity of butyl-methane sulfonate to TK6

See legend to figure 2

Figure 5. Toxicity and mutagenicity of alkylmethane sulfonates to MIT-2

See legends to figures 1, 3. The methane sulfonates were methyl-, ethyl-, propyl-, and butyl- (MMS, EMS, PMS, BMS).

Figure 7. Toxicity and mutagenicity of nitosomethylurethane to MIT-2

See legends to figures 1, 3.

Figure 8. Toxicity and mutagenicity of ICR-191 to MIT-2

See legends to figures 1, 3.

Figure 9. Mutagenicity of ICR-191 to TK6

See legend to figure 2.

Figure 10. Mutagenicity of 4-nitroquinoline oxide to TK6

See legend to figure 2.

Figure 11. Mutagenicity of ultra-violet light to TK6

See legend to figure 2.

Figure 12. Bromodeoxyuridine

Figure 13. Chlorodeoxyuridine

Figure 14. Iododeoxyuridine

Figures 12 - 14. Toxicity and mutagenicity of 5-halodeoxyuridines to MIT-2

See legends to figures 1, 3. Solid symbols represent the compound alone. Open symbols (fig 12) were in the presence of 10 μM cytidine. All work was done in ambient light.

Figure 15. Toxicity and mutagenicity of bromodeoxyuridine to HH4

Cells were treated for 2 hr, and plated for resistance to 5 $\mu\text{g}/\text{ml}$ 6TG 7 - 10 days later. All work was done in the dark.

Figure 16. Mutagenicity of benzopyrene to TK6

Cells were treated for 3 hr in the presence of an aroclor induced 5% (v/v) rat liver post-mitochondrial supernatant. Cells were plated under selective conditions as described in the legend to figure 2.

Figure 17. Mutagenicity of fluoranthene to TK6

See legend to figure 16.

Figure 18. Mutagenicity of aflatoxin B₁ to HH4

Cells were treated for 3 hr in the presence of an uninduced 2.5% rat liver post-mitochondrial supernatant and plated 7 - 10 days later for resistance to 5 μg/ml 6TG.

Progress Report #6

Hypermutable Cell Lines

Investigator: John G. DeLuca

Rationale: Repair deficient mutants have played a key role in elucidating some of the complexities of prokaryotic and eukaryotic DNA repair systems and the mechanisms of survival and mutation. Unfortunately, few studies utilizing the various human repair deficient fibroblast strains available have examined the effect of these lesions on mutability, probably due to the large expenditure of time and resources required to perform such studies. Since suspension grown lines lend themselves to considerably more facile assay techniques, we have obtained various lymphoblast lines derived from XP patients and tested them for their cloning ability under standard assay conditions.

Results: One line derived from an XP group C patient, XPA3, has consistently displayed 10-20% plating efficiency (PE) and its toxic and mutagenic response to UV has been examined.

Figure 1 shows the level of hydroxyurea insensitive H^3 TdR incorporation after UV irradiation in these cells compared to normal cells. It can be seen that they are defective in this manifestation of excision resynthesis.

Figure 2 shows the toxic and mutagenic response to UV irradiation of both XPA3 and TK6 cells. It can be seen that the XPA3 defect potentiates mutation as well as toxicity, though to a somewhat lesser extent.

Other XP lymphoblast lines have shown less consistent plating efficiencies in our hands. GM2345 a line derived from group A patient (XP-205) now shows a 5% plating efficiency and it is hoped that with continuous culture, this will improve, as has been the case with other lines in this laboratory (unpublished observations).

An alternative approach which we have also been pursuing is to derive repair deficient and/or hypermutable lines from TK6, the normal human line with which we currently work. Since such mutants are likely to be recessive, they would be theoretically extremely rare, on the order of 10^{-10} in an unmutagenized population. In order to bring the frequency of such mutants into the range where one might realistically attempt to select them, a large population of cells must be extensively mutagenized. ICR-191 is being used for this purpose for a variety of reasons.

First, since we are interested only in surviving mutants, the mutagen must induce many mutants in the absence of significant toxicity. Treating cells with 0.5 ug/ml ICR 191 for 24 hours results in approximately 1 surviving mutant for every 10^4 cells treated. This is one of the highest "mutant yields" obtained to date. Thus, multiple low doses of ICR 191 represents an excellent technique for increasing the mutant fraction of a cell population.

Second, ICR 191 is known to produce predominantly frameshift-type mutations and these are more likely to lead to total gene inactivation than point mutations.

Finally, ICR-191 produces low levels of chromosome aberrations, a desirable feature if one wishes the derived, mutated lines to retain the diploid character of the parental line.

TK6 cells are presently being subjected to successive 0.5 ug/ml ICR-191 treatments and when last monitored (after 6 treatments) the 6TG^R mutant fraction had risen to 6.8×10^{-4} . We intend to continue to treat these cells until MF's in the vicinity of 10^{-2} are obtained. Such a highly mutagenized cell culture would be ideal populations from which to attempt selection of a wide variety of interesting mutants.

FIGURE 1: HYDROXYUREA INSENSITIVE INCORPORATION; CONTROL. BACKGROUND HAS BEEN SUBTRACTED.
 ○ - NORMAL (PARENT LINE OF TR5)
 △ - XP43

FIGURE 2: SURVIVAL:
 OPEN SYMBOLS - TR5 CELLS; NUMBERS IN PARENTHESES INDICATE THE NUMBER OF INDEPENDENT DETERMINATIONS.
 CLOSED SYMBOLS - XP43 CELLS; DIFFERENT SYMBOLS REPRESENT INDEPENDENT EXPERIMENTS.
 MUTATION TO 6780:
 OPEN SYMBOLS - TR5 CELLS
 CLOSED SYMBOLS - XP43 CELLS

Progress Report #7

Hypomutable Human Cells

Investigator: Bruce W. Penman

Rationale: By obtaining cell lines which differ only in their sensitivity to the toxic and/or mutagenic effects of mutagens one should be able to determine the specific biochemical differences which result in differential sensitivity.

Our working hypothesis for induced mutation is: mutagens interact with DNA to form adducts; these adducts are either effectively removed (repaired) or give rise to a change in DNA sequence; unrepaired adducts can also result in cell killing. To understand the mechanisms of induced mutation is to determine quantitatively which chemical and cellular processes affect and effect the steps of adduct formation, adduct removal and adduct fixation. Mutants which differ in their sensitivity to mutagens are the most facile tools for illuminating these chemical and cellular processes.

Methodology: Cell lines which are resistant to the toxic and mutagenic effects of mutagens can be obtained by screening existing lines. Alternatively, cell lines which are resistant to the toxic effects of mutagens can be isolated from relatively sensitive, clonally-derived lines after treatment with toxic concentrations of mutagens. This latter method is preferred, since the resistant sub-clones isolated presumably differ from the

relatively sensitive cells at only one or a few loci. Existing lines derived from different individuals, on the other hand, have unknown genetic heterogeneity.

In our examination of existing cell lines we were fortunate to find one line, GM 130, which was remarkably resistant to the toxic and mutagenic effects of the alkylating agent butylmethane-sulfonate [Slapikoff et al (1978) *Mut. Res.* 54: 193-196]. GM 130 is also resistant to the alkylating agents methylnitrosourea (MNU, figure 1) and methylnitrosoguanidine (MNNG, figure 2). Importantly, GM 130 is sensitive to the intercalating, frameshift mutagen ICR-191 (figure 3). Therefore, resistance is not an artifact of methodology and the resistance is apparently dependent on the mode of action of the mutagen. [Slapikoff et al. (1980), *Mut. Res.* in press].

The MNU [Thilly et al (1976), *Chem. Biol. Inter.* 15:33-50; fig. 1] and MNNG [Penman and Thilly (1976), *Som. Cell Genet.* 2:325-330; fig 2]; dose-response curves for survival suggest that small (<1%) sub-populations, which are resistant to these chemicals, exist in clonally-derived human lymphoblast lines. We have isolated a population of MIT-2 lymphoblasts after repetitive treatment with MNU. These cells are at least two orders of magnitude more resistant to MNU than the parent population (figure 4).

The MNU dose-response curve for the survival of the resistant cells is biphasic, suggesting a sub-population

of even more resistant cells. Further MNU treatment and reisolation yielded a population with no further substantial increase in MNU resistance (figure 4). The shape of the dose-response curve suggests that 50% of the population are still relatively resistant. The half-life of MNU in cell culture medium is very short compared to the doubling time (length of the cell cycle) of the lymphoblasts [Penman et al (1979), *J. Natl. Can. Inst.* 63:903-907] and the exposure is therefore a "pulse dose". The biphasic response may be a cell cycle effect in which S phase and non-S phase cells differ in their sensitivity to MNU.

We infer that the resistant cells isolated are also resistant to the mutagenic effects of MNU. Direct testing of this inference is in progress. Cells which were isolated after a lower MNU dose are as sensitive as the parent population to the toxic effects of MNU (figure 4). The MNU sensitive cells isolated showed an increase in the 6-thioquanine resistant mutant fraction relative to controls while the mutant fraction in the resistant population was equal to the controls.

Saul Slapikoff (now at Tufts University) has shown that the total methylation of MIT-2 cells, MIT-2-MNU resistant cells and GM-130 cells is the same as a function of MNU concentration. Thus the resistance mechanism does not seem to be a reduced uptake of alkylating agents.

Future Work: We intend to characterize the hypomutable cell lines obtained with regard to: (1) sensitivity to the toxic and mutagenic effects of mutagens which differ in their proposed mechanisms (e.g. UV light, alkylating agents and ICR-191); (2) cell cycle dependence of toxicity and mutagenicity; (3) adduct formation and adduct removal. We hope to eventually determine the specific biochemical change(s) responsible for the hypomutability of each line.

Fig. 1. Toxicity and mutagenicity of MNU to human lymphoblast lines. Relative survival was resuspended 24 h after addition of MNU to culture (open symbols) as determined by soft agar plating. The mutant fraction (closed symbols) was determined by outplating (MIT-2 and WI-L2) or outplating (GM-130) platings in soft agar on days 11, 13, 16, and 20 (MIT-2); days 20 and 21 (WI-L2); and days 20 and 24 (GM-130) after treatment allowing phenotypic expression of the HGPRT state. Mutant fraction is obtained by dividing the plating efficiency in the presence of 6TG by the plating efficiency in the absence of 6TG. The data for mutant fraction are expressed as mean $\pm$ s.e.m. Absence of error bars indicate the s.e.m. for that data point are within the symbol size. $\circ$ = MIT-2; $\square$ = WI-L2; $\triangle$ = GM-130.

Fig. 2. Toxicity and mutagenicity of MNU to human lymphoblast lines. Relative survival 24 h after addition of MNU (open symbols) and mutant fractions (closed symbols) were determined as in Fig. 1. Platings for mutant fraction were on days 16, 20, and 28 for all lines. $\circ$ = MIT-2; $\square$ = WI-L2; $\triangle$ = GM-130.

Fig. 3. Toxicity and mutagenicity of ICR-191 to human lymphoblast lines. Relative survival after 24 h treatment (open symbols) and mutant fractions (closed symbols) were determined as in Fig. 1. Platings for mutant fraction were on days 11, 13, 20, and 21 (MIT-2 and WI-L2); and days 19, 21, and 23 (GM-130). $\circ$ = MIT-2; $\square$ = WI-L2; $\triangle$ = GM-130.

Fig. 4. Toxicity of MNU to subpopulations isolated after previous MNU treatment. $\triangle$ control cells - no prior MNU treatment; $\bullet$ low MNU dose - no mutation induced; $\circ$ low MNU dose - mutation induced; $\blacktriangle$ cells from $\bullet$ resuspended after a further high dose MNU treatment.

Progress Report #8

Relation of DNA Adducts to Mutation

Investigators: R. Croy, H. Liber

Rationale: If DNA reaction products are premutagenic lesions then it is important to know the quantitative relationship between adduct formation and removal and mutation.

Progress: The relationship between the DNA adduct formation and forward mutation has now been measured in *S. typhimurium* [Stark, A.A, et al., Aflatoxin B. mutagenesis, DNA binding, and adduct formation in *Salmonella typhimurium*; P.N.A.S. 76:1343-1347, 1979] and revealed that the average probability of any aflatoxin DNA adduct giving rise to a mutation is on the order of 1 in 40 to 1 in 20. This seemed somewhat high intuitively since we would expect a higher efficiency of repair but it should be pointed out that the *S. typhimurium* strain used, TM677, carries the repair deficiency mutations *uvrB* and also a partial repair disfunction on the plasmid pKm 101.

Mutation in diploid human lymphoblast line MIT-2 was also compared to the frequency of DNA adducts formed with aflatoxin B. This work has been reported and discussed in two papers now in press, [Thilly, W.G., Study of Mutagenesis in diploid human lymphoblasts, in: Mammalian Cell Mutagenesis: The Mutation of Test Systems, ed. by V.K. McElheny, Cold Spring Harbor Laboratory (1979) and Wogan, G.N. et al., Mechanisms of action of aflatoxin B₁ and sterigmatocystin: Relationships of macromolecular

binding to carcinogenicity and mutagenicity in the Proceedings of the International Conference on Environmental Carcinogenesis, in press, 1980] The basic observation is summarized in figure 2.

These manuscripts represent the beginning of what promises to be a major collaboration among researchers skilled at analyzing and interpreting chemical adduct formation and researchers skilled at measuring and interpreting genetic and cellular changes.

Incidentally, the probability of an average adduct in human cells leading to a mutation is estimated to be of the order of one in two hundred indicating an efficiency of repair of greater than 99%.

Future studies involve several laboratories cooperating to speed the identification and measurement of fluoranthene adducts and relating this information to other biological experimental results.

Figure 1: The amount of aflatoxin B₁ bound to cellular DNA immediately after a 3-h incubation of MIT-2 cells in the presence of a 10% (v/v) rat-liver postmitochondrial supernatant was determined by hydrolysis of isolated DNA and separation of aflatoxin-adducted free bases by high-pressure liquid chromatography (R.G. Croy). Simultaneously, determination (following growth recovery and phenotypic lag) of the 6THR mutant fraction was made (T.R. Skopek and H.L. Liber). Vertical bars show the 95% confidence limits based on two independent experiments. [Thilly, W.G., (1979)]

Progress Report #9

Pseudomutation in human cells

Investigator: H.L. Liber

Rationale: In experiments designed to study the mutagenicity of bromodeoxyuridine (BUdR) to TK6 human lymphoblasts at the F_3TdR -resistance locus, it was observed that the F_3TdR^R mutant fraction approached 10^{-3} then inexplicably fell to the background by 10 days after treatment. This phenomenon has been investigated further, in the hopes that it might reveal some interesting aspect of the mechanism of mutagenesis.

Methodology: The locus studied in this report is F_3TdR -resistance, which measures loss of the enzyme thymidine kinase. The cell line used, TK6, is a $TK^{+/-}$ heterozygote, and F_3TdR -resistance is ostensibly conferred by a genetic change resulting in $TK^{+/-} \rightarrow TK^{-/-}$. Cells lacking TK do not convert F_3TdR to a toxic nucleotide.

Progress: TK6 cells were treated with 0 or 200 μM BUdR for 2 hrs. and plated in microtiter dishes every day for F_3TdR^R and $6TG^R$. The F_3TdR^R fraction rose to a maximum of at least 3×10^{-4} on day 2, and fell to the background level by day 7. The $6TG^R$ fraction was unaffected by the treatment (figure 1). When the data was replotted on semi-log paper (figure 2), it appeared that the F_3TdR^R fraction declined with a half-life of about 1 day.

Figure 1. Induction of transient resistance to F_3TdR by BUdR in TK6

TK6 were treated with 200 μM BUdR for 2 hr, and plated daily for resistance to 1 $\mu g/ml$ F_3TdR or 5 $\mu g/ml$ 6TG. Mutant fractions of controls have been subtracted. Error bars are 95% confidence intervals.

Figure 2. Decay of " F_3TdR -resistant" TK6 cells induced by BUdR

The data from figure 1 has been re-graphed in a semi-log plot.

Figure 3. Biological effective time of F_3TdR .

F_3TdR (1 $\mu g/ml$) was added to four different media: RPMI 1640 without serum, or with fetal calf serum (FCS), horse serum (HS), or heat-treated horse serum (HTHS). Each day, fresh TK6 cells were resuspended in an aliquot of medium, and their growth rate was determined, relative to a control bottle which had not received F_3TdR .

Figure 4. Growth rate of mutants and pseudomutants in microtiter dishes

TK6 were treated with 3 mM butylmethane sulfonate or 200 μM BUdR for 2 hr, and 2 days later plated at 4×10^4 per well in 1 $\mu g/ml$ F_3TdR on microtiter dishes. Plates were examined periodically, and positive and negative wells were counted on a Coulter counter to determine the number of cells/well.

The solid line represents the growth curve of the cultures; the dashed line is the inverse of the growth curve - the line that would be expected to best fit the decay kinetics if the non-exponential growth hypothesis were correct. However, the dotted line, which is the calculated best fit, has a steeper slope than the inverse of the growth curve, and is consistent with the non-cycling hypothesis. Solving the equation $N = N_0 2^{t(\frac{1}{T_2} + \frac{1}{R})}$ for R , using this data, one obtains a conversion rate of about 125 hr. for $G_H \xrightarrow{R} C$.

A second difference between the two hypotheses is that in A, non-exponential growth, all affected cells should start growing once the F_3TdR has been eliminated. In B, non-cycling, affected cells can convert at anytime after the F_3TdR has been degraded. Therefore, if A were correct, plates incubated longer than the normal 14 days should maintain a constant fraction of " F_3TdR^R " colonies. If B were correct, the fraction should increase with time, as more and more cells converted from $G_H \rightarrow C$. Figure 6 contains the results of an experiment in which plates incubated for 14 days were counted, reincubated, and recounted. It is clear that the " F_3TdR^R " fraction steadily increases. This is consistent with B, the non-cycling hypothesis, but inconsistent with A. Furthermore, if one interprets the sharp increase from days 14 to 16 as part of the initial burst of colony formation, and the growth from days 16 on as a reflection of the conversion rate R , then that rate is 112 hr, which is very close to the number calculated from figure 5.

The initial question raised was: were the observed colonies true mutants? A number of colonies were removed from the F_3 TdR-containing microtiter wells, and their ability to grow under various selective conditions was determined. All of the colonies were sensitive to 1 μ g/ml F_3 TdR. Therefore, it seemed that BUdR was not inducing mutation, but that it was somehow altering cellular conditions so that F_3 TdR-sensitive cells could survive and grow.

It was soon discovered that F_3 TdR^S cells were able to grow in F_3 TdR because the biological effectiveness of the selective agent disappeared in 4 days (figure 3). The BUdR-induced pseudo- F_3 TdR^R colonies were delayed in their growth while the selective agent was being degraded; once that was complete, and the medium in the microtiter dishes was free of F_3 TdR, the colonies began to form (figure 4).

Two possible mechanistic explanations were developed to explain the data.

A. Non-exponential growth hypothesis

In this hypothesis, cells containing BUdR (probably in DNA) are affected such that they no longer synthesize TK. Since TK is completely recycled each cell cycle, the enzyme level in affected cells soon falls to zero. Cell division continues to occur, resulting in one affected (BUdR containing) and one unaffected (non-BUdR containing) daughter. Therefore dilution of the effect under non-

selective conditions is due solely to division. Under selective conditions, non-exponential growth occurs; one daughter cell lives while the other dies. However, once F_3TdR is degraded, exponential growth can resume.

B. Non-cycling hypothesis

In this hypothesis, cells affected by BUdR go into a non-cycling state called G_H , in which TK, as a cyclical enzyme, is not present. Cells in G_H convert back to a cycling state C, at some constant rate. Under non-selective conditions, the effect is diluted by cell division and the rate of $G_H \rightarrow C$. Under selective conditions, G_H cells which convert back to C cells after degradation of F_3TdR has occurred can grow to form colonies.

One difference between the two hypothesis is that they predict different kinetics for dilution of the BUdR-effect. Hypothesis A, non-exponential growth, requires that the decline be due solely to cell division; therefore the equation which describes the decline would be $N = N_0 2^{-t(\frac{1}{T_2})}$ where T_2 is the doubling time of the culture. On the other hand, the non-cycling hypothesis predicts a decline due to two factors, division and conversion. The equation to describe this is $N = N_0 2^{t(\frac{1}{T_2} + \frac{1}{R})}$, where R is the rate of conversion from $G_H \rightarrow C$. However, it is not possible to distinguish whether the kinetics of decay of the effect support either hypothesis. Figure 5 plots the results of an experiment in which TK6 were treated with 50, 100 or 200 μM BUdR for 2 hr.

Finally, experiments were done to see if incorporation of BUdR into DNA could be correlated to the BUdR-effect. The dose-dependent response of TK6 to BUdR is plotted in figure 7, along with the incorporation of ^3H -BUdR into the acid-precipitable fraction of whole cells. Both curves plateau at similar concentrations, about 25 - 50 μM , implying that the rate limiting step for incorporation is the same as for the $\text{F}_3\text{TdR}^{\text{R}}$ -effect. However, there is a shoulder at low doses on the curve for " $\text{F}_3\text{TdR}^{\text{R}}$ ", while there is none for incorporation. The shoulder indicates that there is not a simple one-to-one relationship between incorporation and the observed induction of transient resistance. However, it is possible that the induction requires "multiple hits"; i.e. that more than one site must be substituted before the effect is observed. Therefore, the data is not inconsistent with the idea that incorporation of BUdR into DNA leads to the induction of transient resistance to F_3TdR .

Figure 5. Time dependent decrease of pseudomutants.

TK6 were treated with BUdR for 2 hr and plated daily for resistance to 1 $\mu\text{g}/\text{ml}$ F_3TdR . The solid line is the growth curve for all cultures. The dotted and dashed lines represent two possible decay curves (see text).

Figure 7. Dose response of TK6 to BUdR; incorporation of ^3H -BUdR into DNA and pseudomutation.

TK6 were treated with BUdR and plated for resistance to 1 $\mu\text{g}/\text{ml}$ F_3TdR 2 days later. Incorporation was studied by adding ^3H -BUdR during the incubation, and filtering the cells onto a glass fiber filter, and counting them.

Figure 6. Time dependent increase of pseudomutants

TK6 were treated and plated for resistance to 1 $\mu\text{g}/\text{ml}$ F_3TdR . The plates were counted after 14 days, and then reincubated and recounted and the " F_3TdR -resistant" fraction determined. Numbers next to the curves are the doubling times (in hours) of the " F_3TdR " fraction.

○ - BUdR treated; ● - control

Progress Report #10

Progression Effects Following Mutagen Exposure in
Human Cells.

Investigator: J. DeLuca

Rationale: The rate at which mammalian cells progress through the various phases of the cell cycle determines the cell cycle distribution of a particular population. Since the response of mammalian cells to DNA damaging agents is known to vary with cell cycle position, an interesting aspect concerns changes in cell cycle progression (and, therefore, distribution) subsequent to DNA damage. In vivo, cells are generally exposed to low levels of DNA damaging agents either repeatedly or continuously, and if small changes accumulate, this could lead to marked changes in biological response.

Results: We have used autoradiography to study changes in L.I. (labeling index, or number of cells in S-phase) with time after UV irradiation. After relatively non-lethal fluences, ($<100 \text{ ergs/mm}^2$) (P.R. #6), we see a 10-20% increase over control cells in L.I. between 6 and 10 hours after irradiation (figure 1A). This implies a transient accumulation of cells in S-phase, presumably due to the concomitant decrease in the rate of DNA synthesis.

Paradoxically, 24 hours after receiving a fluence of 44 ergs/mm^2 (95% survival), cell multiplicity is equivalent to that seen in unirradiated control cultures, despite the appearance (figure 1B) of an extended lag in the recovery of

the rate of DNA synthesis per labelled cell. In any event, the demonstration of a significant change in the kinetics of progression after a non-lethal amount of DNA damage indicates that such effects might be expected under in vivo conditions; this question certainly deserves further study.

Autoradiography, however, is of limited utility if one wishes to examine G_1 or G_2 populations; toward this end, we have been able to apply a cytofluorometric approach developed by Darzynkiewicz [Darzynkiewicz *et al.*, (1977) *Cancer Research* 37:4635-4640], to our cells. The procedure, which involves RNAase digestion of fixed cells followed by acidification and acridine orange staining, is theoretically capable of distinguishing all five cell cycle subpopulations (G_0 , G_1 , S, G_2 , M) on the basis of the varying ratios and absolute intensities of red and green fluorescence. Preliminary results appear promising.

FIGURE 1 : A) Labelling index (closed symbols) and mass culture rate of TdR incorporation (open symbols);
 B) Rate of TdR incorporation per cell
 ○● - 44 ergs/mm²; □■ - 66 ergs/mm²
 ▲△ - 132 ergs/mm²; ◇◆ - 176 ergs/mm²

Progress Report 11 Cell Cycle Effects on Mutation in
CHO Cells

Investigator: Charles Crespi

Rationale: Mitotic selection takes advantage of the phenomenon that anchorage dependent cells round up during mitosis and become less adherent to the growth surface. This technique has been used extensively to obtain highly synchronous cell populations, but is limited by a low overall cell yield. This limitation arises from the condition that only a small fraction of an exponentially growing population is in mitosis at one particular time (typically 1-6%). We have overcome this limitation by applying microcarrier technology which allow large numbers of cells to be cultured in small culture volumes. Mitotic cells may be detached from microcarriers by gentle shearing. Using these synchronous cells, we are investigating the cell cycle position dependent induction of mutation by a variety of direct acting alkylating agents and nucleotide analogs.

Progress: *[The progress summarized here has been fully reported in a manuscript submitted to Cell and Tissue Kinetics (see appendix, Ng et al.).]*

We have been able to successfully grow Chinese hamster ovary (CHO-K1) cells on positively-charged dextran microcarriers. A cell inoculation at 3×10^5 cells/ml

quickly attaches to the microcarrier surface and grows with a 14-16 hour doubling time, reaching confluence at 5×10^6 cells/ml. (see fig. 1). Mitotic cells were

Figure 1: Growth curve of CHO-K1 cells on microcarriers. Cells grew with a 14-16 hour doubling time in RPMI#1640 media supplemented with 15% fetal calf serum. Microcarriers were at a concentration of 5 mg/ml and the spinner culture stirred at 60 RPM.

detached from the microcarriers when the cell density was in the range of $2 - 2.5 \times 10^6$ cells/ml; at this point the population was still in exponential growth. To detach mitotic cells, the exponentially growing CHO-K1 culture was treated with colcemid (30 ng/ml) for 2.5 hours, and the culture stirring speed increased to 150-200 RPM for 10 minutes. The shear from the increased stirring speed detaches the mitotic cells from the microcarrier surfaces. Using this procedure we can routinely detach 4-6% of the total population, 85-95% of which are visibly arrested in metaphase.

Mitotic CHO-K1 cells were subcultured into plastic tissue culture dishes in fresh growth media, and the cell

number (figure 2), modal volume (figure 3), and incorporation of ^3H -thymidine into acid precipitable DNA (figure 4) were monitored. After subculturing, mitotic CHO-K1 under went a round of division 0.5 - 1.5 hours after plating (figure 2). The cell number then remained constant until 15 hours after plating, and then nearly doubled in the interval from 15-19 hours. The mean cell cycle time was 16 hours, which is comparable to that of

Figure 2:
Growth curve
of mitotic
cells after
subculturing
into plastic
tissue culture
dishes.

an exponentially growing culture. The Engleberg synchronization index was 90% for the first division and 60% for the second division. The modal cell volume was found to increase as cell age increased (figure 3). The

- modal volume increased from 250-260 μm^3 at 1-3 hours

Figure 3:
Modal volume
of a synchronous
CHO-K1 cells
versus cell age.

after plating to $500 \mu\text{m}^3$ at 5 hours. The beginning of S-phase was determined by incorporation of ^3H -thymidine

Figure 4: In-
corporation of
 ^3H -thymidine into
acid precipitable
DNA versus cell age
in a synchronous
CHO-K1 cell culture.

into acid precipitable DNA (figure 4). S-phase began 7-8 hours after plating. The end of S-phase could not be precisely determined because of decay in the cell synchrony.

Exponentially growing CHO-K1 cell cultures were treated for two hours with 5-bromo-2'-deoxyuridine (BUdR), a thymidine analog, or ethyl methanesulfonate (EMS), a direct acting alkylating agent. The relative surviving fractions were determined immediately after phenotypic expression. The 6-thioguanine-resistant ($6TG^R$) and ouabain-resistant (OUA^R) fractions were determined after phenotypic expression. For both mutagens, the relative surviving fraction decreased in a log linear fashion with increasing mutagen concentration (figures 5a and 6a), and the induced $6TG^R$ fraction and OUA^R fraction increased with increasing mutagen concentration (figure 5b and 6b).

Figure 5: (a) Concentration-dependent relative survival of an exponentially growing culture of CHO-K1 cells after a two hour treatment with BUdR in the dark. (b) Concentration-dependent induction of 6-thioguanine (●—●) and ouabain (○—○) resistance after a two hour treatment with BUdR in the dark. Error bars represent 95% confidence limits on each determination.

Figure 6: (a) Concentration-dependent relative survival of an exponentially growing culture of CHO-K1 cells after a two hour treatment with EMS. (b) Concentration-dependent induction of 6-thioguanine (●—●) and ouabain resistance (○—○) after a two hour treatment with EMS. Error bars represent 95% confidence limits on each determination.

Synchronous CHO-K1 cultures were also treated with the mutagens BUdR and EMS. A two hour treatment with BUdR (in the dark) caused a lower relative survival in S-phase cells as compared to G₁, G₂, or mitotic cells (figure 7a). A two hour treatment with EMS also caused a lowered survival in S-phase cells, but only at higher concentrations (figure 8a). Significant BUdR induced mutation was observed only during S-phase. 6-thioguanine resistance was induced maximally 10 hours after plating of mitotic cells, while ouabain resistance was induced maximally 10-12 hours after plating (figure 7b). Significant EMS-induced mutation was observed at all times

Figure 7: (a) Relative survival after a two hour treatment with either 0.15 mM (○—○) or 0.30 mM (●—●) BUdR in the dark versus cell age on a synchronous CHO-K1 culture. (b) Induction of 6-thioguanine (●—●) and ouabain (○—○) resistance versus cell age. Synchronous CHO-K1 cell cultures were treated with 0.15 mM BUdR for two hours in the dark. Error bars represent 95% confidence limits on each determination.

during the cell cycle. A small, but significant change in induced mutant fraction was observed for both 6-thioguanine and ouabain resistance (figure 8b); early G_1 and G_2 cells were approximately two-fold more sensitive to the mutagenic action of EMS compared to S-phase cells.

Finally, it is interesting to note that the mutant

fraction observed after mutagen treatment of an exponentially growing culture is very close to the relative cell number corrected integral mutant fraction for a synchronous population. The relative cell number corrector integral mutant fraction is an adjusted mutant fraction to correct for relative survival, mutant fraction, and total number of cells of each interval compared to an asynchronous culture. (See table)

<u>Treatment</u>	<u>Selective Systems</u>	<u>Mutant Fraction Exponential Culture</u>	<u>Corrected Integral Mutant Fraction Synchronous Culture</u>
EMS	6TG ^R	9×10^{-5}	10×10^{-5}
0.5 mM x 2 hr.	OUA ^R	35×10^{-6}	48×10^{-6}
BUDR	6TG ^R	5×10^{-5}	5.3×10^{-5}
0.15 mM x 2 hr	OUA ^R	25×10^{-6}	19×10^{-6}

This indicates that the observed mutant fraction in an asynchronous cell population can be explained via the relative sensitivity to mutation and survival of cells in each phase of the cell cycle.

We are currently investigating cell-cycle dependent mutagenic effects of three other alkylating agents, 4-nitroquinoline-1-oxide, nitroso methylurea, N-methyl-N-nitro-N-nitroso-guanidine, and the nucleotide analog 5-fluorodeoxyuridine.

Progress Report #12

Cell Cycle Dependent Mutation in Human Lymphoblasts

Investigator: Henry Hoppe IV

Rationale: The human cell mutation assay developed in this laboratory gives us the opportunity to dissect the mutagenic response of diploid human lymphoblasts to selected chemicals. Using centrifugal elutriation to reproducibly separate an asynchronous population into fractions on the basis of size, we can easily examine the cell cycle dependent toxic and mutagenic response of human cells to selected agents. Comparison of this data with data obtained from rodent cells synchronized on microcarriers (Progress Report #11) will allow detailed analysis of the similarities and differences between human and rodent cells in their ability to handle model mutagens. The synchronization technique also lends itself to an analysis of the cell cycle dependent efficiency of adduct formation, removal, and the relationship to toxicity and mutation.

Progress: In centrifugal elutriation the tendency of particles to sediment in an applied gravitational field is opposed by the flow of liquid in the opposite direction. When the centrifugal and counter flow forces are balanced, particles differing in Stokes' radii are separated. Exploiting this, we have used the Beckman JE-6 Centrifugal Elutriator System to fractionate an exponentially growing culture of human lymphoblasts on the basis of cell size, which reflects their position in the cell cycle.

Figure 1 shows the size distribution of four out of six fractions obtained from a separation of 10^9 cells. All sizing was done manually on a coulter electronic particle counter Model Z_F. The entire separation was carried out at 37°C using RPMI 1640 medium supplemented to 15% with fetal calf serum. Fraction 1 was the first population obtained, corresponding to the

smallest cells. Fraction 4 was the last population obtained, corresponding to the largest cells, and Fractions 2 & 3 are two of the intermediate populations. All populations obtained contained greater than 10^8 cells.

The growth characteristics of the separated populations (Figure 2) show that the cells are highly viable, suffer little apparent trauma, and, for at least Fractions 1 and 4 are widely separated in the cell cycle. Fraction 1 is presumably mainly G_1 and early S phase cells and does not show any signs of growth for 10 hours post separation. Fraction 2 resumes growth 4 hours post separation and Fraction 3 resumes growth 2 hours post separation (4 hours after separation of Fraction 1, as the total elapsed time for the separation is over 2 hours). Fraction 4 is presumably G_2 and late S phase cells and shows no growth delay at all. The cells immediately enter mitosis, completely double in number within 6 hours and then exhibit no further increase in cell number for at least 6 hours. This degree of synchronization is equal to or greater than that currently published in the literature [Mitchell and Tupper, *Exp. Cell Res.*, 106 (1977), 351-355; Meistrich, Meyn and Barlogie, *Exp. Cell Res.*, 105 (1977), 169-177].

The change in relative cell size as Fraction 4 grew was also followed (Figure 3). Immediately upon separation (0) the cells have a unimodal diameter of about 13.6 microns. Within one hour there has been a 25% increase in total cell number (Figure 2), and the size distribution is now bimodal with relative diameters of approximately 11 and 14 microns. By 4 hours post separation over 85% of the cells have divided and though the size distribution is still bimodal (with diameters of approximately 12 and 14.7 microns), it reflects both the continued growth of the cells and the relative amount of division which has occurred. By 7 hours post separation the cells once again have a unimodal size distribution of approximately 12.5 microns in diameter. At 10 hours post separation the cells have continued to increase in size (to about 13.4 microns average diameter), but have not yet started dividing. The larger relative

spread in size by 10 hours after the initial separation is indicative of the natural decay process inherent in any synchronization technique.

With the world shortage of fetal calf serum in the spring of 1979 it became necessary to adapt our cells to growth in available horse serum. The use of horse serum for centrifugal elutriation presented technical difficulties that we have just overcome. It was found necessary to heat-treat horse serum at 57°C for 2 hours, freeze to -20°C, thaw and then centrifuge at 9,000xg for 40 minutes to remove all precipitable material. It was also found that treatment for 1 hour at 37°C with 250 µg/ml deoxyribonuclease I (Sigma Chemical, 1880 kunitz units/mg) was necessary to remove factors which caused significant clumping in the separation chamber during operation of the elutriator. The final result is, however, a satisfactory means to synchronize human lymphoblasts grown in the presence of horse serum. The degree of resolution between the separate fractions using horse serum is as great, if not greater, than that we obtained using fetal calf serum (Figure 4). Experiments to verify the growth, viability and volume changes of the separate fractions in horse serum are presently under way. Use of an acridine orange staining technique for fixed cells and analysis on an Ortho FC48004/FC200 cytofluorograph allows us to unambiguously determine cell cycle position based on the relative amounts of single stranded versus double stranded DNA. [Z. Darzynkiewicz, F. Traganos, T. Sharpless, and M.R. Melamed; *Cancer Research*, 37(1977), 4635-4640.]

In order to examine the toxic and mutagenic effects of an agent as a function of the cell cycle, it is necessary to have an agent which is both mutagenic and not overly toxic (greater than .1 relative survival) within a short exposure. We have chosen to examine the simple alkylating agent butylmethane sulphonate (BMS) because (1) it does not need any exogenous metabolism to be mutagenic, (2) previous work with long exposure times have shown significant mutation with minimal toxicity and (3) it has a long half

life in tissue culture medium, simplifying concentration X time estimations.

In Figure 5, the toxic and mutagenic effects of a 1 hour exposure of human lymphoblasts to BMS are shown. At concentrations up to 5 mM relative survival was greater than 60%. Induced mutation at three genetic loci (6-thioguanine resistance, trifluorothymidine resistance and ouabain resistance) was an approximately linear function of BMS concentration.

We have also chosen the base analogue 5-bromo-deoxyuridine (BUdR) for our initial studies. BUdR is incorporated into DNA predominately in the place of thymidine. It is hypothesized to cause A-T to G-C base pair transitions by mispairing with guanine (acting as if it were deoxycytidine) in later replications. It may, however, rarely be incorporated into the DNA as deoxycytidine and cause G-C to A-T base pair transitions by mispairing with adenine (acting as if it were thymidine) in later replications. Thus, under appropriate exposure conditions, we should be able to obtain cell-cycle dependent mutation, corresponding to replication of the genetic locus for drug resistance. In Figure 6, the toxic and mutagenic effects of a 2 hour exposure of human lymphoblasts to BUdR are shown. At concentrations of up to 200 μ M, greater than 40% relative survival occurred with significant mutation to 6-thioguanine resistance occurring at concentrations of 100 μ M or lower.

Future work: The projects in progress or planned include:

- 1) Verification of the viability and discreteness of the separate fractions obtained from centrifugal elutriation. This will be done using growth curves post separation, following volume changes post separation, and by analysis of the DNA content per cell using acridine orange staining technique and flow cytofluorometry.

- 2) An analysis of cell cycle dependent mutation in human lymphoblasts using butyl methane sulphonate and 5-bromo-deoxyuridine as molecular probes.

- 3) An analysis of cell cycle dependent mutation in human lymphoblasts using other mutagens, (such as UV light, ICR-191, and fluoranthene, in order to test our hypotheses regarding the relation between DNA repair,

cell cycle position and probability of mutation.

4) A comparison of cell cycle dependent mutation in human lymphoblasts and rodent cells to allow an analysis of their similarities and differences in handling the toxic and mutagenic effects of mutagens.

5) Progression studies on the placement and removal of DNA lesions and how this relates to the cell cycle and mutation. This will be done by using labelled compounds in conjunction with high pressure liquid chromatography to follow the fate of lesions induced in the cellular DNA at different points in the cell cycle as the cells continue to progress through the cell-cycle.

6) An analysis of cell-cycle dependent mutation caused by mutagens contained in combustion effluent samples. This analysis will allow us to compare the action of an unknown mutagenic substance with that of the known model mutagens to obtain some assessment of its mode of action.

FIGURE 1

RELATIVE SIZE OF CELL POPULATIONS 1-4 IMMEDIATELY AFTER SEPARATION FROM AN EXPONENTIALLY GROWING CULTURE OF THE CELLS VIA CENTRIFUGAL ELUTRIATION. SIZE WAS DETERMINED USING DIFFERENTIAL THRESHOLD SETTINGS ON AN ELECTRONIC PARTICLE COUNTER (COULTER ELECTRONICS, COUNTER MODEL Z₄) CALIBRATED WITH 10.0μ MICRON LATEX SPHERES. CHANNEL WIDTH IS APPROXIMATELY 1 MICRON.

FIGURE 2

RELATIVE GROWTH OF CELL POPULATIONS 1 (○), 2 (□), 3 (△) AND 4 (◇) AFTER SEPARATION FROM AN EXPONENTIALLY GROWING CULTURE OF THE HUMAN LYMPHOBLASTS VIA CENTRIFUGAL ELUTRIATION. TIME IS EXPRESSED AS HOURS POST SEPARATION OF FRACTION 1 (○). ELUTRIATION WAS DONE WITH COMPLETE MEDIUM (RPMI 1640) SUPPLEMENTED WITH 15% FETAL CALF SERUM AT 37° C. SEPARATED POPULATIONS WERE THEN COUNTED. THE CELL CONCENTRATION ADJUSTED TO 4×10^5 CELLS/ML AND PLACED IN PRE-WARMED 250 ML SPINNERS IN A 37°C WATER ROOM. CELL COUNTS WERE MADE HOURLY USING AN ELECTRONIC PARTICLE COUNTER (COULTER ELECTRONICS).

FIGURE 3

RELATIVE SIZE OF FRACTION 4 IMMEDIATELY (0), 1, 4, 7 AND 10 HOURS AFTER SEPARATION. SIZE WAS DETERMINED USING DIFFERENTIAL THRESHOLD SETTINGS ON AN ELECTRONIC PARTICLE COUNTER (COULTER ELECTRONICS, COUNTER MODEL Z₄) CALIBRATED WITH 10.0μ MICRON LATEX SPHERES. CHANNEL WIDTH IS APPROXIMATELY 1 MICRON.

FIGURE 4

RELATIVE SIZE OF FIVE CELL POPULATIONS IMMEDIATELY AFTER SEPARATION FROM AN EXPONENTIALLY GROWING CULTURE OF HUMAN LYMPHOBLASTS GROWN IN HORSE SERUM. SIZE WAS DETERMINED USING DIFFERENTIAL THRESHOLD SETTINGS ON AN ELECTRONIC PARTICLE COUNTER (COULTER ELECTRONICS, COUNTER MODEL Z₄) CALIBRATED WITH 10.0μ MICRON SPHERES.

FIGURE 5

CONCENTRATION DEPENDENT TOXICITY AND MUTAGENICITY OF BUTYL METHANE SULPHONATE (BMS) TO THE HUMAN LYMPHOBLAST LINE TK6. THE RELATIVE SURVIVING FRACTIONS (UPPER CURVE) WERE DETERMINED BY PLATING IN MICROWELLS. THE 6-THIOGUANINE (6TG^R), TRIFLUOROTHYMINE (F₃Torr) AND OUBAIN (O^R) RESISTANT FRACTIONS (LOWER CURVE) WERE DETERMINED THREE TIMES EACH BY PLATING IN MICROWELLS.

FIGURE 6

CONCENTRATION DEPENDENT TOXICITY AND MUTAGENICITY OF 5-EPIDIO-PROXYMETHINE TO HUMAN LYMPHOBLASTS. THE RELATIVE SURVIVING FRACTIONS (UPPER CURVE) WERE DETERMINED BY PLATING IN MICROWELLS (O-O) OR BY EXTRAPOLATION OF G₀/G₁ CURVES AFTER TREATMENT (●-●). THE 6-THIOGUANINE RESISTANT (6TG^R) FRACTIONS WERE DETERMINED 7 TO 10 DAYS POST TREATMENT BY PLATING IN MICROWELLS. DATA IS THE COMPILATION OF SEVERAL EXPERIMENTS. ALL WORK WAS DONE IN THE PAST.

C. Developments In Human Cell Mutation Assay Systems.

Progress Report #13

1. Developments using traditional colony forming techniques.

Probably the most appreciated development for us in the laboratory has been the improvements achieved over the past two years in the facility which with we can use human lymphoblasts to perform quantitative assays. In 1977, it was necessary to grow lymphoblast colonies in an agarose gel over a feeder layer of human fibroblasts. Copious amounts of fetal calf serum were required in order to achieve desirable colony forming efficiencies.

Today we still use diploid human lymphoblasts but the feeder layers, the agarose gel and the fetal calf serum are gone.

In 1977, using subclones (e.g. MIT-2) of the PGLC 33 line of lymphoblasts, we were compelled to carry continuous cell cultures for 18 days after treatment in order to permit the phenotypic expression of 6-thioguanine resistance in that line. 6-Thioguanine resistance was the only marker available because of the requirement for a feeder layer itself resistant to killing by the selective conditions.

Today, by good fortune, we use subclones of the lymphoblast line WIL-2, e.g. TK6, which we found not to require a feeder layer and coincidentally, to display a phenotypic lag of only 5 days for 6-thioguanine resistance. Other markers are readily employed and we have used ouabain resistance with

facility and have even been able to isolate heterozygotes for trifluorothymidine resistance and develop a forward gene locus assay which has a phenotypic lag of only 48 hours!

This is not to imply that human cell mutation assays are easy to perform, but we think that during the course of this contract we have increased the facility of the assay to the point where we would defend the contention that there is no more facile assay for gene locus mutation in any mammalian cell type.

Rather than expand this section unnecessarily, we feel that the varied contributions by virtually every member of our research group are best summarized by our chapter now in press for Hollaender's series Chemical Mutagens: Principles and Methods for their Detection, the galley proofs for which we reproduced and included in the appendices [*Thilly et al*, *Gene locus mutation assays in diploid human lymphoblast lines in Chemical Mutagens: Principles and Methods for this Detection* ed. by A. Hollaender and F. DiSerres, Plenum Press N.Y., vol. 6, p 331-363 (1980)].

One aspect not addressed when this chapter was written was the world-wide shortage of fetal calf serum which virtually shut down our laboratory for several months in 1979. We were indeed fortunate in the work of a new graduate student from Tulane University, MS. Phaik-Mooi Leong, who tested a series of other serum sources and was successful

in adapting our WIL-2 subclone lines to rapid and clonal growth in as little as 2.5-5% horse serum which at this writing is still available in good supply. Looking to the future, Ms. Leong has reproduced the serum-free growth of MDCK cells achieved by Gordon Sato's laboratory and an important subproject will be a continuing effort to achieve satisfactory cloning efficiencies for the diploid human lymphoblasts in serum free conditions. [Taub, M., Chuman, L., Saier, Jr., M.H., and Sato, G.; Growth of Mudin-Darby canine kidney epithelial cells (MDCA) line in hormone-supplemented serum-free medium. *P.N.A.S.* 76:3338-3342 (1979)].

Progress Report #14

Human Cell Mutation Assay at the Thymidine Kinase Locus

Investigator: H.L. Liber

Rationale: This section describes the development of a gene-locus mutation assay in diploid human lymphoblasts at an autosomal locus, TK, which codes for a pyrimidine salvage enzyme, thymidine kinase. Cells which contain functional TK enzyme are sensitive to the toxic effects of the thymidine analog trifluorothymidine, while mutants without the enzyme are resistant.

There were two compelling reasons for developing this cell system. First, a screening assay which examines mutation at the TK locus should have a significant advantage over those at the HGPRT locus, because phenotypic expression for loss of TK is several generations shorter than for loss of HGPRT. Secondly, we have a means of comparing induced mutant fraction at two loci in the same cell, and can thus be more assured that the responses obtained at a given locus are representative for all genes.

Progress: Figure 1 is a block diagram outlining the general procedure which is followed in performing quantitative mutation assays. Cells are treated under defined conditions, and then incubated in non-selective medium for a time sufficient to allow phenotypic expression of the desired genetic marker. They are then plated in the presence of selective agent to determine the number of mutant cells able to form colonies, and in the absence of selective agent in order to determine the absolute fraction of cells which are able to form colonies. The fraction of mutants in a population can then be calculated.

1. Isolation of TK heterozygote.

In order to measure mutation from a TK^+ to a TK^- state, it was necessary to first isolate a TK heterozygote. This is described in

Figure 1.

GENERAL FORMAT OF MUTATION
ASSAY IN DIPLOID HUMAN LYMPHOBLASTS

Skopek, T.R. *et al.*, 1978; Isolation of a human lymphoblastoid line heterozygous at the thymidine kinase locus: Possibility for a rapid human cell mutation assay. *Bioch. Biophys. Res. Comm.*, 84: 411-416. The cell line picked for further study, TK6, was chosen because it grew well and formed large and distinct colonies.

2. Optimization of F_3TdR concentration.

The concentration of the selective agent used in a mutation assay is preferably completely toxic or inhibitory to wild-type cells, but without affect to mutants. Figure 2 shows that the dose-response of TK6 to F_3TdR plateaus at concentrations in excess of 0.3 $\mu\text{g/ml}$. Mutants are only slightly affected by much higher concentrations (10 $\mu\text{g/ml}$). The F_3TdR concentration chosen for the remainder of these studies was 1 $\mu\text{g/ml}$.

3. Phenotypic expression time of F_3TdR^R .

Phenotypic expression time (time required after mutagen treatment for full expression of induced mutant fraction) has to be experimentally determined for each locus and cell line. It depends on (1) time required to genetically "fix" the mutation (i.e., how long after insult to DNA it takes until no further healthy mRNA is produced), and (2) dilution of preexisting enzyme to the point where there is insufficient residual activity to adversely affect cell growth in the presence of the selective agent. The first point depends on the molecular mechanism of mutation, which is unknown for virtually all mutagens. The second point depends on growth rate (enzyme/cell is reduced by a factor of two with each division) and degradation rates of healthy mRNA and protein (which depend on the specific case).

The phenotypic expression time was determined by treating TK6 with a pulse dose of a mutagen, and then plating every day for resistance to either F_3TdR or 6TG. Figure 3 compares these expression times after the cells had been treated with 3 mM BMS for 2 hours. Mutant fractions for both markers plateaued at the same value, 40×10^{-6} ; the expression

Figure 2. Toxicity of F₃TdR to TK6

TK6 were plated in the indicated initial concentration of F₃TdR. Error bars are 95% confidence intervals.

time for F_3TdR^R was 2 days, while for $6TG^R$ it was 5 days.

In a similar experiment, cells were treated with 5 $\mu\text{g/ml}$ BP for 3 hours, and plated daily for F_3TdR^R . A plateau value of 120×10^{-6} was reached in 2-3 days (Figure 4).

4. Relative toxicity of BMS to $TK^{+/-}$ and $TK^{-/-}$ cells.

In order to demonstrate that treatment with a toxic mutagen is not selecting for pre-existing mutants, wild type $TK6^{+/-}$ and $TK^{-/-}$ mutants were treated with toxic doses of BMS for 24 hours, and then plated to determine the surviving fraction. Figure 5 indicates that either a mixture of mutants or a single mutant clone die with the same kinetics as the wild-type $TK6^{+/-}$. However, the most persuasive proof that what is observed is in fact induced mutation and not selectivity for pre-existing mutants is that large increases in mutant fraction can be observed after treatments which produce little or no toxicity. For example, 3 mM BMS X 2 hour yielded a 10-fold increase in mutant fraction while killing only 10% of the cells (Figure 3).

5. Cell density effects of $TK^{+/-}$ cells on mutant expression.

The calculation of MF depends on the plating efficiency of cells at low density being the same as their plating efficiency at high density. In other words, if a $TK^{-/-}$ mutant can form colonies with a 20% PE at a density of 2 cells/well in normal medium, one assumes that it also forms colonies with a 20% PE in the presence of selective agent, when surrounded by 4×10^4 dead wild-type cells.

The experiment which was done to confirm this assumption was to plate various $TK^{-/-}$ mutants on microtiter dishes in the presence and absence of F_3TdR , and in the presence of F_3TdR and 4×10^4 $TK6$ cells/well. Table 1 shows that the PE of 6 different $TK^{-/-}$ lines does not change under the different conditions. Therefore, the assumption is valid that the PE observed on a plate of low density accurately reflects the PE on a high density plate (4×10^4 cells/well).

Figure 3. Phenotypic expression time of TK6 after exposure to butylmethane sulfonate

TK6 cells were treated on day 0 and plated daily for resistance to 1 $\mu\text{g}/\text{ml}$ F_3TdR or 5 $\mu\text{g}/\text{ml}$ 6TG. Error bars are standard deviations of two independent cultures.

Figure 4. Phenotypic expression time of TK6 after exposure to benzopyrene.

TK6 cells were treated on day 0 in the presence of 5% (v/v) postmitochondrial supernatant. They were plated daily for resistance to 1 $\mu\text{g}/\text{ml}$ F_3TdR .

Figure 5. Toxicity of butylmethane sulfonate to TK6 and $\text{TK}^{-/-}$ mutants.

TK6 ($\circ, \Delta$) or a BMS induced $\text{TK}^{-/-}$ mutant ($\blacktriangle$), or a population of 6 different $\text{TK}^{-/-}$ mutants ($\bullet$) were treated with BMS for 24 hr, and plated to determine relative survival.

Table 1.

PLATING EFFICIENCIES ON MICROTITER PLATES OF TK^{-/-} MUTANTS
IN THE PRESENCE AND ABSENCE OF HIGH DENSITIES OF TK^{+/-} CELLS

MUTANT	PE IN RPMI	PE IN RPMI WITH F ₃ TDR	PE IN RPMI WITH 40,000 TK6/WELL
BMS-2	0.11	0.09	0.09
BMS-3	0.19	0.21	0.17
BMS-4	0.16	0.11	0.15
BMS-5	0.29	0.29	0.31
ICR-1	0.17	0.21	0.21
ICR-2	0.13	0.09	0.10

RPMI IS RPMI 1640 SUPPLEMENTED WITH 15% FETAL CALF SERUM.
F₃TDR CONCENTRATION IS 1 MICROGRAM/ML

6. Characterization of TK^{-/-} mutants.

Since it is presently impossible to directly examine the DNA of purported TK^{-/-} cells to confirm that they are real mutants, their character must be inferred by other means. TK^{-/-} mutants should be F₃TdR^R, CHAT^S, and deficient in TK enzyme activity. These properties should be genetically stable and thus persist after many generations of growth. Table 2 demonstrates that mutants induced by either BMS or ICR-191 behave as predicted. They are all completely F₃TdR^R, CHAT^S, and remain so after many generations in non-selective growth medium. All of the purported mutants are also lacking the TK enzyme activity of the wild-type TK6.

Table 2.

GROWTH CHARACTERISTICS OF F_3TDR^R MUTANTS

A. 20 - 30 GENERATIONS AFTER ISOLATION

<u>MUTANT</u>	<u>T₂ - RPMI</u>	<u>T₂ - F₃TDR</u>	<u>T₂ - CHAT</u>
BMS-2	18.6	18.4	NG
BMS-3	18.1	19.2	NG
BMS-4	17.3	17.7	NG
BMS-5	20.1	19.7	NG
ICR-1	18.2	18.6	NG
ICR-2	21.0	21.2	NG
TK6	17.5	NG	17.5

B. 120 - 150 GENERATIONS AFTER ISOLATION

ALL OF THE ABOVE MUTANT CLONES GROW EQUALLY WELL IN RPMI AND F_3TDR , BUT NOT AT ALL IN CHAT.

T₂'S ARE DOUBLING TIMES, IN HOURS

RPMI IS RPMI1640 WITH 15% FETAL CALF SERUM

F_3TDR CONCENTRATION WAS ONE MICROGRAM/ML

CHAT IS 10 μ M CYTIDINE, 200 μ M HYPOXANTHINE, 200 NM

AMINOPTERIN AND 17.5 μ M THYMIDINE

NG MEANS NO GROWTH OBSERVED IN THREE DAYS

Progress Report 15 Developments Using Flow Cyto-
fluorometry In Human Cell Mutation Assay.

Investigator: Deborah W. Mascioli, Ph.D.

Progress and Discussion: The purpose of this project is to develop automated, rapid measurements of mutant fractions in cultured human lymphoblasts. Presently, the determination of mutant fraction in human lymphoblasts is accomplished by determining the colony forming ability of a mutagen treated population under selective conditions. Twelve to fourteen days must elapse before the colonies have reached macroscopic size and can be counted.

Flow cytofluorometry is the measurement of fluorescent intensity of cells in suspension as they flow in single file at high speed through a laser beam. The instrument provided by this contract, (Ortho Instruments FC4800A/FC200 Cytofluorograph), is capable of making such measurements at approximately 10^5 cells/minute. Thus, the fluorescence of 10^7 cells can be measured in 100 minutes, and this is sufficient number to contain a statistically significant number of mutants at frequencies encountered in unmutagenized cell cultures.

What is needed to apply this technology to the job of counting mutants is a reliable and facile way of differentiating between non-mutants and mutants. A number of schemes have been attempted by us since acquiring our instrument in the summer of 1978. For instance, the

quenching of various DNA binding fluorochromes such as Hoechst 33258 by halogen substituted deoxyribonucleoside incorporation was quickly found to change fluorescence emission by only 2-fold and thus not enough for discerning the very small (10^{-4} - 10^{-6}) fraction of mutants in the population.

Similarly, simple staining (e.g. trypan blue,) schemes for live (mutant) and dead cells (non-mutant), under selective conditions (e.g. 6-thioguanine) while capable of differentiating the two populations, permitted too many non-mutants to be counted as unstained (mutant cells).

Despite these limitations we are confident that we can indeed reach our developmental goal. One strategy which we have pursued for the past year offers a means to achieve a fluorescent signal from mutant cells some 10 fold greater than from non-mutant cells. The basis for this approach is that cells which are non-mutant for hypoxanthine-guanine phosphoribosyltransferase (hgprt⁺) will cease to synthesize DNA after overnight culture in the presence of 6-thioguanine while hgprt⁻ mutants continue DNA synthesis. This permits us to achieve a differential in substitution of mutant cell DNA with the thymidine analog bromodeoxyuridine (BUdR) by simple addition of BUdR to cultures after DNA synthesis has been suppressed. The presence of relatively large amounts of BUdR in mutant nuclei is then detected by antibodies prepared from goats immunized with protein bound BUdR and the antibodies are in turn detected by rabbit

derived anti-goat immunoglobulin antibodies which are bonded to the fluorochrome, fluorescein and thus detectable by flow cytofluorometry.

The proposed process is outlined in this diagram:

The use of antibodies for this purpose arises from the work of Gratzner et al. [Gratzner, H.G. et al., 1975. Exp. Cell Res., 95:88-94; Gratzner, H.G. et al., 1976. J. Histochem Cytochem, 24:34-39; Gratzner, H.G. et al., 1979. J. Histochem Cytochem, 27:496-499.] who used rabbit derived antibody specific for bromodeoxyuridine (BUdR) for the immunofluorescent determination of DNA replication in single cells and chromosomes fixed on slides. We have extended this general approach to the labelling of cells in suspension, although it was necessary to turn to goats for antibody production in order to achieve

sufficient quantities of antibody which would bind bromodeoxyuridine and not thymidine.

Progress: We have found that the instrumentation in the FC4800A/FC200 cytofluorometer is sensitive enough to permit detection of minute fractions of fluorescent cells in non-fluorescent populations (10^{-6} to 10^{-3}). One must be able to measure such small fractions accurately, since these are the frequencies of mutations that are normally encountered. The results of our experiments are shown in figure 1, in which known numbers of fluorescent beads were mixed with unstained lymphoblasts and then counted on the cytofluorograph. The observed fractions were very close to the predicted fractions, even in the most dilute sample. Also, 2×10^6 unstained cells with no beads were run and no fluorescent signals were observed; i.e., there is very little machine noise present in the system to interfere with the counting of small numbers of fluorescent particles in non-fluorescent populations.

Second, we have chosen a method for selectively and fluorescently labelling the mutant fraction. As outlined above, this involves labelling the mutagenized lymphoblast cultures with bromodeoxyuridine (BUdR) after growth in the presence of 6-thioguanine (6TG). In the presence of this selective agent, wildtype cells containing functional copies of the purine salvage enzyme hypoxanthine-guanine phosphoribosyltransferase (hgprt⁺) will die and mutant cells (hgprt⁻) will live, replicate and take up BUdR.

After labelling with BUdR, cells are treated with antibody to BUdR and then with fluorescently labelled, heterospecific antibody to the BUdR antibody.

As the first step in this process, we have obtained the appropriate immunological reagents. Antibody specific to BUdR was prepared by Dr. Walter Gray of Gateway Immunosera Co., St. Louis, Missouri, using BUdR-bovine serum albumin immunized goats. Affinity chromatography was used to separate anti-BUdR antibodies from anti-thymidine (TdR), thus providing the necessary specificity for our immunoreagent. The goat derived anti-BUdR antibody is itself reacted with fluorescein isothiocyanate labelled rabbit anti-goat antibody. The fluorescein dye is excited by the 488 nm argon-ion laser in our flow cytofluorometer and emits >600 nm wavelength (green) fluorescence.

We are presently determining conditions for optimal binding of anti-nucleoside antibodies to DNA. [Gratzner *et al.*, (1975, 1976 see above), Freeman *et al.*, (Freeman, M.V.R. *et al.*, 1971 *Exp. Cell Res.*, 69:345-355) and Liebskind *et al.*, (Liebskind, D. *et al.*, 1974, *Exp. Cell Res.*, 83:399] have found that anti-nucleoside antibodies bind only to single-stranded DNA. Thus we are testing procedures which will maximize the single strandedness of DNA in samples prepared for counting.

We have found that nuclei from human lymphoblast strain WIL-2 show measured binding of antibody when (a) chromosomal proteins are removed, (b) when DNA is denatured by heating and (c) when single strand breaks are induced in DNA by gamma irradiation. Figure 2 shows the green fluorescence

distribution analyzed by one-dimensional pulse height analysis of WIL-2 nuclei labelled with goat anti-thymidine antibody and fluorescein conjugated rabbit anti-goat antibody after treatment with an acidic solution (pH 1.0) to remove histones, heating at 70°C for 10 minutes and 100,000 rads of gamma irradiation.

This signal is extremely intense and is approximately 10,000-fold greater in frequency than the non-specific labeling observed with cells without anti-thymidine. The strength of this signal which, incidentally is achieved either with fixed whole suspended cells or isolated nuclei, gives us our greatest hope for eventual success in using this approach.

We are, of course, simultaneously determining sufficient conditions for differentially labelling mutant cells. As mentioned above, our approach is based on the premise that wild type cells will cease DNA replication in the presence of the selective agent and not be able to take up BUdR. Mutant cells would continue to divide and incorporate BUdR. Two types of considerations arise here: (1) the length of time in and concentration of selective agent necessary for wildtype cells to cease DNA synthesis and (2) the conditions which will result in maximum levels of BUdR incorporation into cells and thus the conditions for maximum binding of antibody in mutants. Figure 3 illustrates our approach toward (1). ³H thymidine uptake was measured in wild type cells at varying times of exposure to 5 µg/ml 6-thioguanine. By 16 hours of exposure, non-mutant cells had ≤ 2.5% the rate of DNA synthesis observed in the absence of 6-thioguanine.

It is not presently known if this residual incorporation represents nuclear DNA synthesis; however, the 40-fold differential is sufficient for the purposes of our developmental effort.

Second, experiments with ^3H -BUdR labelling of WIL-2 cells for two hours indicates that maximal incorporation occurs at BUdR concentrations greater than 50 μM (figure 4). Preliminary experiments indicate that when WIL-2 cells grown for two hours in 200 μM BUdR are stained with anti-BUdR antibodies and fluorescent labelled rabbit anti-goat antibodies, a fluorescent signal equal in intensity to that obtained with anti-thymidine in unlabelled cells was obtained.

Future work: Future work is directed toward adjusting the selective labelling conditions in order to obtain maximum differences in the fluorescent signals emitted from mutants and non-mutants. Many different possibilities present themselves among which are: (1) Other denaturing agents such as methyl nitrosourea or sodium camptothecin [*Liebskind, D. et al.*, 1974. *Exp. Cell Res.* 69:345-355], will be used to treat cells prior to antibody labelling since these are particularly more efficient in introducing single strand DNA breaks than gamma-irradiation. (2) G_2 cell cycle blocking agents should increase the number of mutant cells containing tetraploid amounts of DNA and thus increase the amount of fluorescent antibody bound per mutant cell. G_2 blocking compounds such as adriamycin [*Barlogie, B. et al.*, 1976. *Can. Res.* 36:1975] will be

tried. (3) Two dimensional analysis for detection of the mutant fraction offers a very important possibility for improvement. The analysis thus far described is one-dimensional. We are proposing to detect as few as 50 mutants out of 10^7 cells by virtue of their green fluorescence. However, with the single antibody method for fluorescently labelling cells, certain factors may significantly limit the precision of our detection system. These include (1) non specific binding of the rabbit anti-goat antisera and (2) some binding of the anti-BUdR antibodies to thymidine in the DNA of non-mutant cells. Our efforts to maximize the intensity of our signal (i.e. move the peak to higher channels in a pulse height analysis) by denaturation (see Future Work 1) and G_2 blocking agents (see Future Work 2) may or may not overcome this problem. Figure 5 illustrates the goal of differential signal ratios necessary for detecting mutant fractions of 10^{-6} - 10^{-4} using one-dimensional analysis.

Our flow instrumentation can, however, simultaneously measure two different parameters of cells (e.g. green fluorescence, red fluorescence, light scatter and absorbance (axial light loss)), and we can use this capability to increase the sensitivity of our mutant inumeration.

For example, after 24 hours in the presence of 6-thioguanine many other cellular functions appear to be suppressed in addition to DNA synthesis. If our primary labelling approach were to have a limit of detecting only one mutant among ten thousand cells instead of the goal

of one in a million then a second independent labelling capable of detecting mutants with a sensitivity of only one in a hundred cells, would when used in combination with the primary labelling system permit counting of mutants occurring at frequencies of $1/100 \times 1/10,000$ or 10^{-6} as desired.

Among the cellular functions which may be somewhat suppressed by exposure to 6-thioguanine are

- (a) phagocytosis
- (b) exclusion of various "viability stains"
- (c) ingestion of surface bound lectins or antibodies

We intend to evaluate each of these possibilities in parallel with our attempts to maximize the sensitivity of our primary differential labelling system.

Figure 1: Determination of the fraction of fluorescent beads in an unstained lymphoblast population.

Figure 2: Fluorescence distribution of WIL-2 nuclei treated with antibodies.

Nuclei from exponentially growing WIL-2 cells were made by lysis in a hypotonic (0.1M) sodium citrate solution. The nuclei were gamma irradiated with 100,000 rads, treated with a dehistonizing solution, heated for ten minutes at 70°C, incubated with goat anti-thymidine antibodies for 30 minutes at 37°C and then with rabbit anti-goat antibodies for 30 minutes at 37°C. The green fluorescence signal was measured at a gain setting of 2.85 out of 10 and analyzed by pulse height analysis. The abscissa measures fluorescence intensity in channels and the ordinate the number of pulses (cells) per channel.

Figure 3: The effect of 6-thioguanine on DNA synthesis of *hprt*⁺ cells.

³H thymidine incorporation was measured in wild type WIL-2 cells at varying times after addition of 5 μ Ci/ml 6-thioguanine. The ordinate is expressed as percentages of the rate of uptake of ³H thymidine by *hprt*⁺ cells resistant to 6-thioguanine.

Figure 4: BUDR incorporation into WIL-2 cells.

³H BUDR incorporation was measured in WIL-2 cells after two hours of exposure at varying concentrations.

Figure 5: Ideal conditions for detection of mutant fraction with one-dimensional, pulse height analysis.

In this idealized conception, the mutant signal, while less in frequency, is ten times as intense and thus easily detected and quantitated. The non-mutant signal occurs at low intensity (channel number 0-200).