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BASIC MECHANISM IN CARCINOGENESIS

Progress Report

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DISCLAIMER

PUBLICATIONS

A. Articles

1. R.J. Lorentzen, W. Caspary, S. Lesko, and P. Ts'o. "The Autoxidation of 6-Hydroxybenzo(a)pyrene and 6-Oxo-Benzo(a)pyrene Radical, Reactive Metabolites of Benzo(a)pyrene". Biochemistry, in press (1975).

2. S. Lesko, W. Caspary, R. Lorentzen, and P. Ts'o. "Enzymic Formation of 6-Oxo-Benzo(a)pyrene Radical in Rat Liver Homogenates from Carcinogenic Benzo-(a)pyrene". Biochemistry, in press (1975).

B. Abstracts

1. J.C. Barrett, L. Schechtman, S. Brefach, and P. Ts'o. "A Mammalian Cellular System for the Concomitant Study of Neoplastic Transformation and Somatic Mutation". Presented at the American Association for Cancer Research, San Diego meeting, May, 1975.

2. S.A. Lesko, W. Caspary, R. Lorentzen, and P. Ts'o. "Enzymic Formation of 6-Oxo-Benzo(a)pyrene Radical from Carcinogenic Benzo(a)pyrene". Presented at the Biophysical Society, Philadelphia meeting, February, 1975.

3. R.J. Lorentzen, W. Caspary, S. Lesko, and P. Ts'o. "Metabolic Formation and Autoxidation of 6-Hydroxy-benzo(a)pyrene and 6-Oxo-Benzo(a)pyrene Radical, Metabolites of Benzo(a)pyrene". Presented at the American Chemical Society, Atlantic City meeting, Division of Medicinal Chemistry, April, 1975.

OUTLINE

The research done in the cell biology and carcinogenesis program in the Division of Biophysics has reached a culmination this year. This progress report consists of seven items indicated in the following page as well as two preprints which are the appendix.

The titles of these two preprints are, "Enzymic Formation of 6-Oxo-Benzo(a)pyrene Radical in Rat Liver Homogenates from Carcinogenic Benzo(a)pyrene," by Stephen Lesko, William Caspary, Ronald Lorentzen, and P. O. P. Ts'o (Biochemistry, in press (1975)), and "The Autoxidation of 6-Hydroxybenzo(a)pyrene and 6-Oxo-Benzo(a)pyrene Radical, Reactive Metabolites of Benzo(a)-pyrene" by Ronald J. Lorentzen, William J. Caspary, Stephen A. Lesko, and P.O.P. Ts'o (Biochemistry, in press (1975)).

A substantial part of this research was supported by the contract awarded by AEC last year. In addition, support has been obtained from fellowships, training grants, and from the University for this achievement.

RESEARCH DONE IN THE LABORATORY OF CELL BIOLOGY
AND CARCINOGENESIS

DIVISION OF BIOPHYSICS

SCHOOL OF HYGIENE & PUBLIC HEALTH

THE JOHNS HOPKINS UNIVERSITY

1. Development of an in vitro morphological transformation assay for mechanistic study.

2. Preliminary study on the properties of 6-hydroxyl benzo(a)pyrene by the in vitro transformation system.

3. Confirmation of the tumorigenicity of the morphologically transformed cells and the establishment of the neoplastic cell lines.

4. Study on the continuous progression of the transformation process.

5. Chemistry and Metabolic Activation of Polycyclic Hydrocarbons, particularly the benzo(a)pyrene.

6. Study on the somatic mutation with the Syrian embryonic fibroblast system - development of a mammalian cellular system for the concomitant study of neoplastic transformation and somatic mutation.

7. Interaction of drugs, mutagens, and carcinogens with helical nucleic acid in solution at the atomic level as studied by nuclear magnetic resonance.

Development of an in vitro Morphological Transformation Assay for Mechanistic Study.

This assay was developed with the following characteristics:

1. Normal Syrian hamster embryo cells of early passages (3-4 passages) are used.

2. The system does not require a feeder layer.

3. The system can sustain a vigorous washing to remove all the polycyclic hydrocarbons after a given period of exposure.

4. The assay takes only 7-8 days.

The protocol for our assay system is outlined on Chart 1. Plastic Petri dishes, 100 mm in diameter, are seeded 24 hrs. before treatment is initiated with early passage Syrian hamster whole embryo cells (SHE cells) in the absence of any feeder cell layer. Control dishes are plated with 5,000 - 10,000 cells and carcinogen-treated dishes are plated with up to 20,000 cells, thereby allowing for cell loss during washing and for cytotoxic effects due to exposure to the hydrocarbon. Our effective cloning efficiency is 1-2% or from 100 to 400 clones per 100 mm plate. The solvent for all hydrocarbons is DMSO. The carcinogen in DMSO is diluted to 2X concentration in modified Dulbecco's Eagle's reinforced medium (Biolabs, Northbrook, Ill.) supplemented with 10% fetal bovine serum (Reheis Chemical Co.) in the absence of antibiotics. Tested cultures are exposed to the carcinogens for defined periods of time at 37°C. Control cultures are exposed to DMSO solvent only, $\leq$ 0.5% final concentration. Treatment is terminated by withdrawal of the incubation medium followed by five washes with complete medium, allowing further elution of unbound hydrocarbon during 10 minute incubation periods at 37° for each wash. Afterwards, the cells are permitted to clone in fresh medium for 6-7 days. The resulting colonies are then fixed in 100% methanol, stained with 10% aqueous Giemsa, and scored for morphological transformation.

Chart 1.

Morphological In Vitro Transformation Assay

A. $5.0 \times 10^3 - 2.0 \times 10^4$ cells/dish seeded without feeder layer.

B. Treatment for x hours.

C. Five washes with complete medium.

D. Incubation for cloning 6 - 7 days.

E. Score for transformation frequency:

1. Fix, stain, and examine the preserved clones.
2. Examine living clones under phase-contrast microscope, mark clones on plate, allow to grow further before isolation and subsequent clonogenic mass culture.

Of paramount importance to this assay is the effectiveness of the washing procedure. Chart 2 illustrates the efficacy of the washing procedure in rendering hydrocarbon from the 10^6 SHE cells after the monolayer has been exposed to either 10 µg [^{3}H]-B(a)P/ml or 5 µg [^{3}H]-6-OH-B(a)P/ml for the time intervals indicated. In this experiment, the cell monolayer was washed as described after carcinogen treatment, dispersed with trypsin : EDTA solution (0.25%: 0.1%), an aliquot used to determine cytotoxic effects, and the remainder lysed by freeze-thaw (4X) and assayed for [^{3}H]-hydrocarbon remaining associated with the cells or covalently linked to cellular components. Chemical binding was determined by precipitation of the cell lysate with ice cold 0.2 M perchloric acid; precipitates were trapped on glass fiber filters where they were washed exhaustively, three times with 95% ethanol, three times with benzene, and three times with hexane (5-10 ml/wash). Filters were dried and counted for radioactivity.

This extensive washing procedure carried out on the SHE cells effectively removes 99.94% of the B(a)P applied. The amount of B(a)P that becomes cell-associated increases gradually until 3% of that administered remains associated with the rinsed cell monolayer after 24 hr. The viability of these cells remains constant and only after 24 hr. of exposure are there signs of B(a)P becoming slightly cytotoxic (<6% cell death). With respect to the covalent linkage of B(a)P to cellular components, the background level of 0.02% would indicate that we find little, if any, covalent binding of B(a)P until a 24 hr. exposure.

Regarding the binding of 6-OH-B(a)P, the amount does not become noticeably high; even at 24 hr., only less than 1% of the 6-OH-B(a)P applied becomes non-washable. However, it appears that of the amount which becomes non-releasable from the cells, a steadily increasing quantity becomes covalently bound, and this linkage seems to occur

Chart 2.

Sample	[^{3}H]HC Remaining with Washed Cell Monolayer	[^{3}H]HC Covalently Linked to Cell Components (Washed PCA ppt)	Cell Survival
B(a)P (10 µg/ml)	%	%	%
0 hr (15 sec)	0.06	0.02	100
1	0.3	0.009	100
2	0.3	0.015	100
4	0.4	0.013	100
6	0.5	0.016	100
24	3.0	0.071	94.1
6-OH-B(a)P (5 µg/ml)			
0 hr (15 sec)	.02	0.004	100
1	0.1	0.022	100
2	0.2	0.053	100
4	0.3	0.095	100
6	0.3	0.117	95.9
24	0.8	0.457	37.5

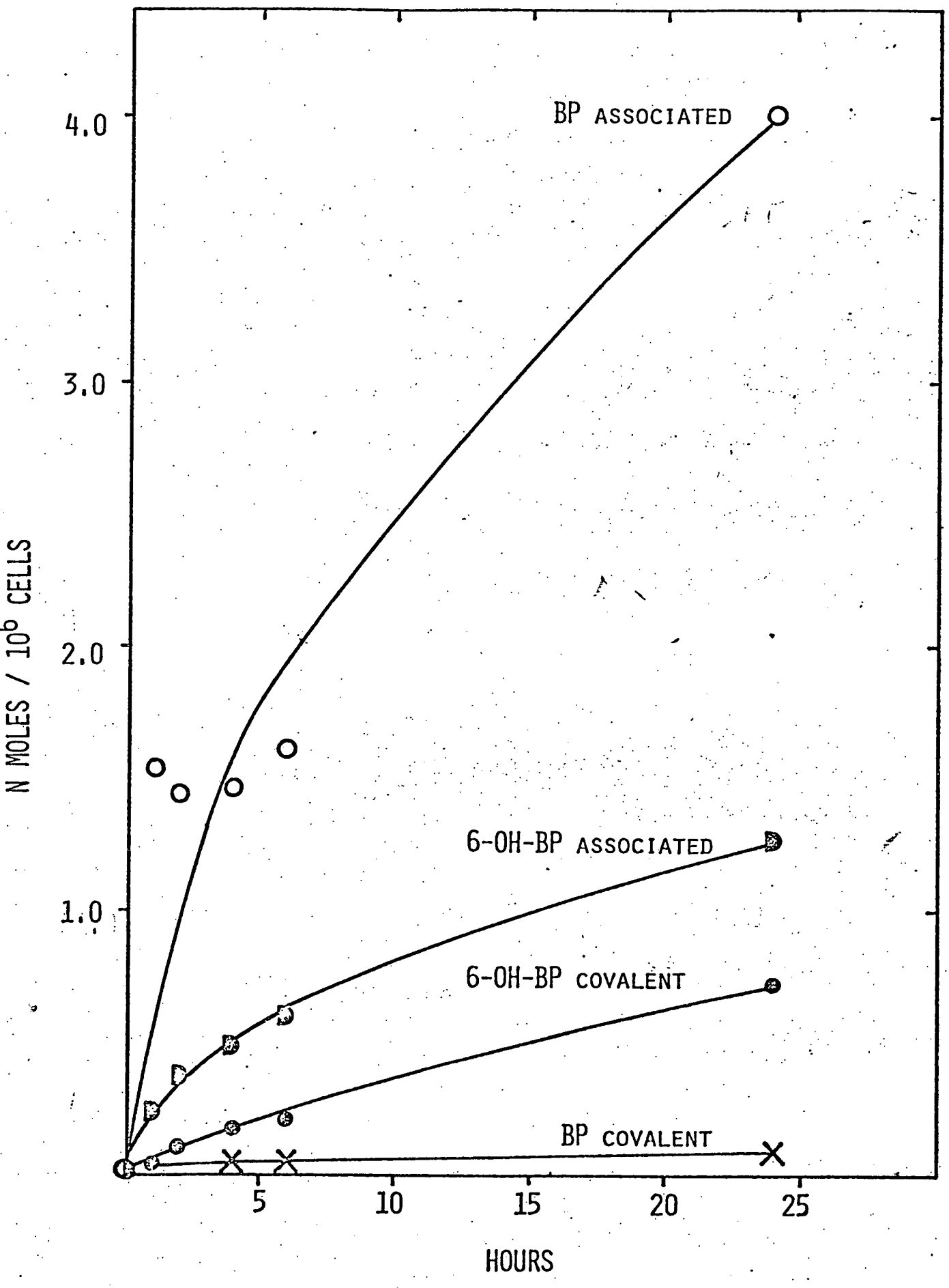
almost immediately. Furthermore, 5 µg 6-OH-B(a)P/ml already begins to show cytotoxic effects after only 6 hr. exposure and becomes extremely toxic with a 24 hr. treatment (>60% kill). When the amount of hydrocarbon bound is normalized to the number of cells present at the end of the treatment period, a direct comparison can be made between the binding of B(a)P and that 6-OH-B(a)P (Chart 3). The parent compound, B(a)P, B(a)P, physically associates with the washed cell monolayer to a greater extent than does the active metabolite. However, the level of <u>covalent</u> linkage of 6-OH-B(a)P is higher than that of B(a)P.

A series of photographs has been included (Plates 1-3) to demonstrate our classification of (1) normal, (2) B(a)P-transformed, and (3) 6-OH-B(a)P-transformed clones. The normal one illustrates a characteristic organized arrangement of cells in a streamline pattern. The transformed clone displays disorganized growth patterns and disoriented criss-cross arrangement of spindle-shaped cells. Transformation test results are scrutinized by two readers in our laboratory and the clones in the photographs have been independently confirmed as morphologically transformed by Dr. Bruce Casto of BioLabs, Inc. Northbrook, Ill.

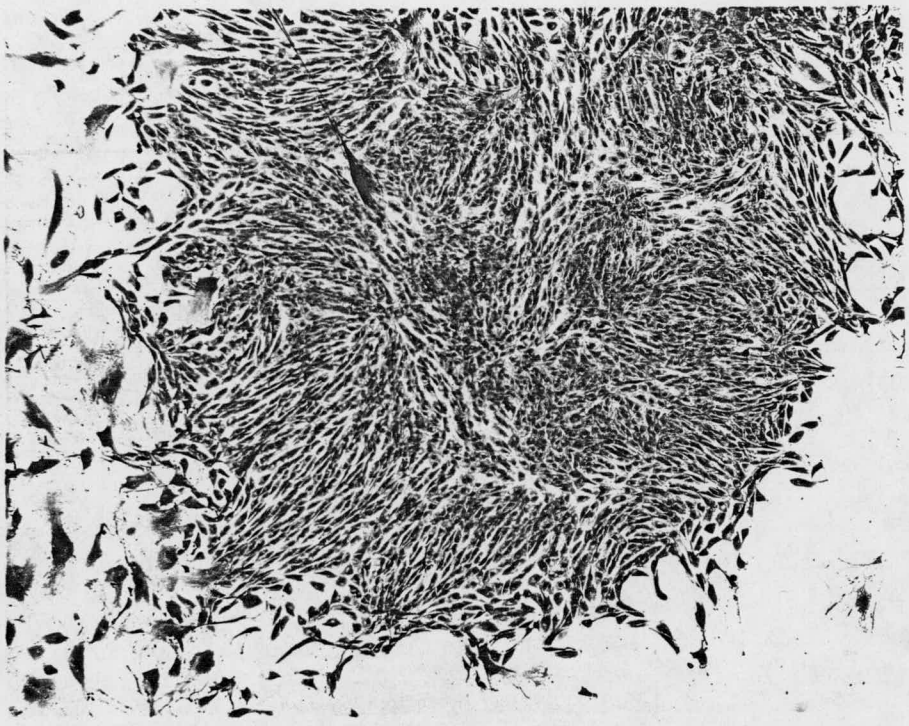
Chart 4 shows a dose-response relationship for 2 hr. and for 24 hr. exposure to B(a)P in terms of the number of transformed colonies per surviving clones (%) as a function of µg B(a)P/ml. Over 9000 clones were examined to generate the 24 hr. curve. An approximately linearly increasing transformation frequency was obtained, up to ~7%, with logarithmically increasing doses of B(a)P. The fractional numbers represent the number of transformed clones vs. the total number of clones surveyed for that time point.

The 2 hour curve seems to suggest that even with such a brief exposure period, after a critical dose is reached (e.g. >1 µg B(a)P/ml), transformation frequency may follow a similar linear pattern. However, consideration must be given to the possibility that the level of transformation obtained here might be due to a residuum of B(a)P

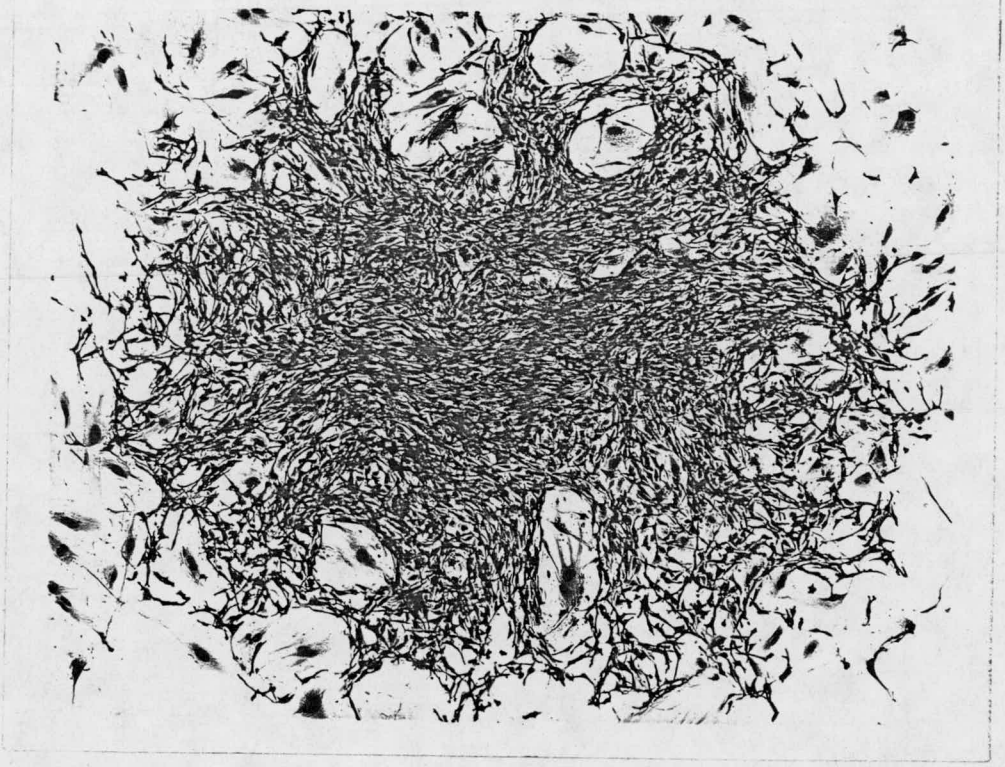
Chart 3.


BP ASSOCIATED
6-OH-BP ASSOCIATED
6-OH-BP COVALENT
BP COVALENT
4.0
3.0
2.0
1.0
N MOLES / 10^6 CELLS
5
10
15
20
25
HOURS

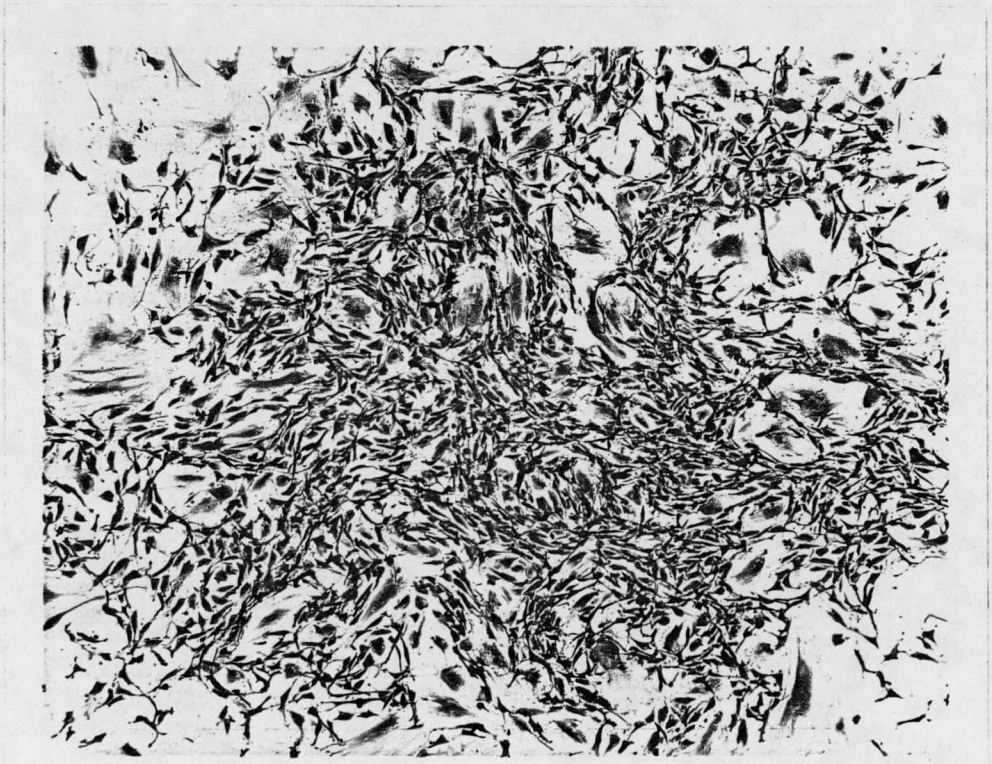
Golden Syrian Hamster Embryonic Fibroblasts (early passage), exposed to 0.1% DMSO for 24 Hours. Normal Clone as control.



Golden Syrian Hamster Embryonic Fibroblasts (early passage) Exposed to 10 μg B(a)P. Two Transformed Clones (Independently Identified by L. Schechtman from Johns Hopkins University and B. Casto, Biolab, Inc.)

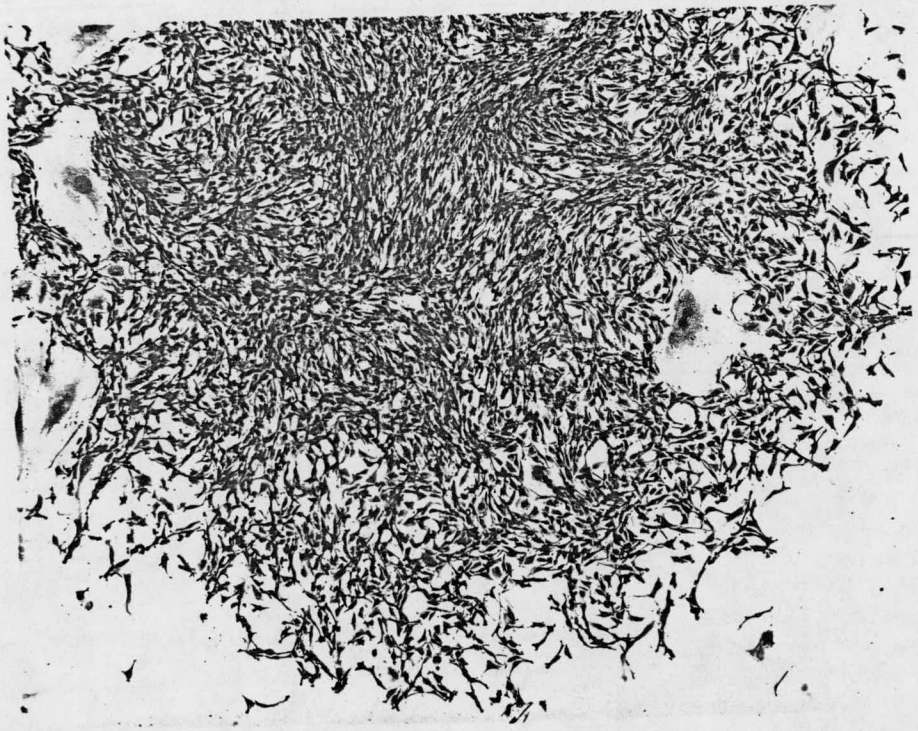


4 hours exposure

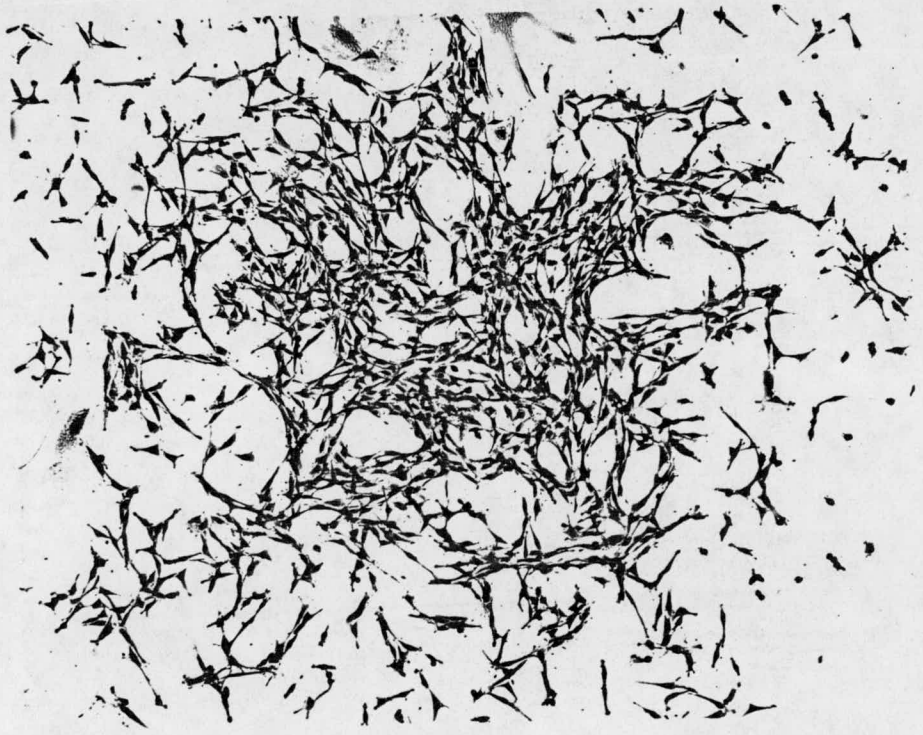


24 hours exposure

Golden Syrian Hamster Embryonic Fibroblasts (early passage)
Exposed to 3 µg 6-Hydroxy-B(a)P. Two Transformed Clones
(Independently Identified by L. Schechtman from Johns Hopkins
University and B. Casto, Biolab, Inc.)

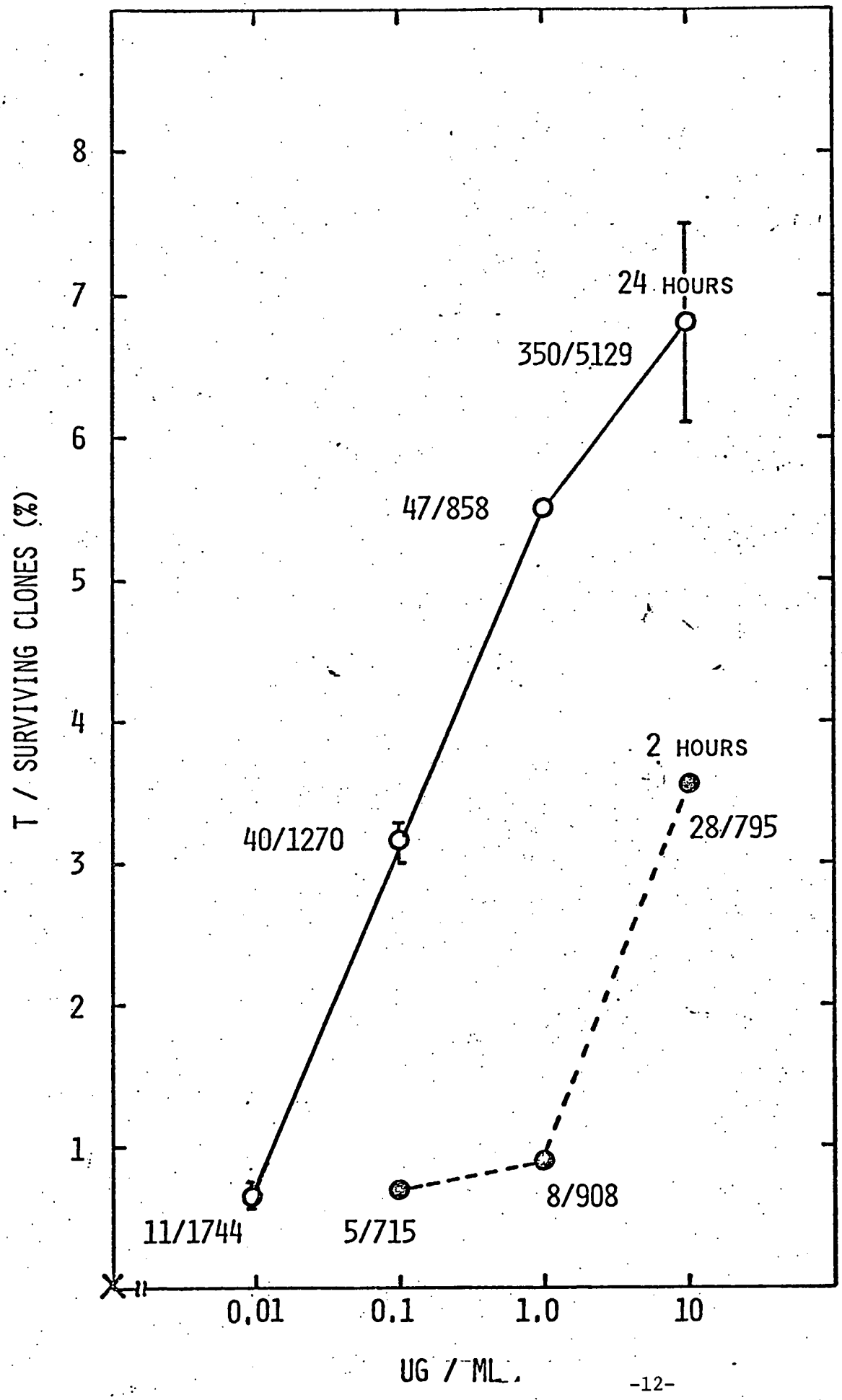


2 hours exposure



24 hours exposure

Chart 4.


T / SURVIVING CLONES (%)
8
7
6
5
4
3
2
1
24 HOURS
350/5129
47/858
40/1270
11/1744
2 HOURS
28/795
8/908
5/715
0.01
0.1
1.0
10
UG / ML.

not removed by the washing procedure. This concern is due to the finding that the percent of transformation is proportioned to the logarithm of the concentration of B(a)P. Therefore, even a reduction of 10 µg BP/ml to 0.1 µg BP/ml only reduces the percent of transformation from~6.5% to~3%.

The results presented in chart 5 represent an attempt to demonstrate exposure requirements for B(a)P at 10 µg/ml necessary to achieve transformation. As implied above, we were surprised to find that a short exposure time, such as 2 or 4 hr., could yield a significant level of transformation. Therefore, at an applied dose of 10 ug B(a)P/ml, a shorter exposure period might be all that is required to obtain morphological transformation. However, the critical point raised regarding the effectiveness of the extensive washing process becomes even more important. At this high dose of B(a)P (e.g. 10 µg/ml) part of the observed level of transformation with brief treatment periods could be attributable to residual carcinogen left behind. Since the system is so sensitive to B(a)P, it became obvious that such studies must be re-examined at lower applied does, e.g., 1-0.1 µg B(a)P/ml, such that residual amounts of non-washable hydrocarbon become insignificant. In view of these considerations, a series of studies were undertaken to investigate the effect of exposure period of B(a)P on transformation frequency, when the administered dose was lowered to 1 µg B(a)P/ml (Chart 6). Once again, as was demonstrated previously with an applied dose of 10 µg B(a)P/ml, a small but significant proportion of morphologically transformed clones could be recognized with as little as 2-5 hours exposure to the carcinogen. However, as predicted, at the lower dose, the level of transformation obtained during short exposure periods decreased. Still, the transformation frequencies obtained at 24 hours exposure were nearly the same (6.5-7%) regardless of the dose administered. To insure that residual B(a)P left after the washing procedure was not present to compli-

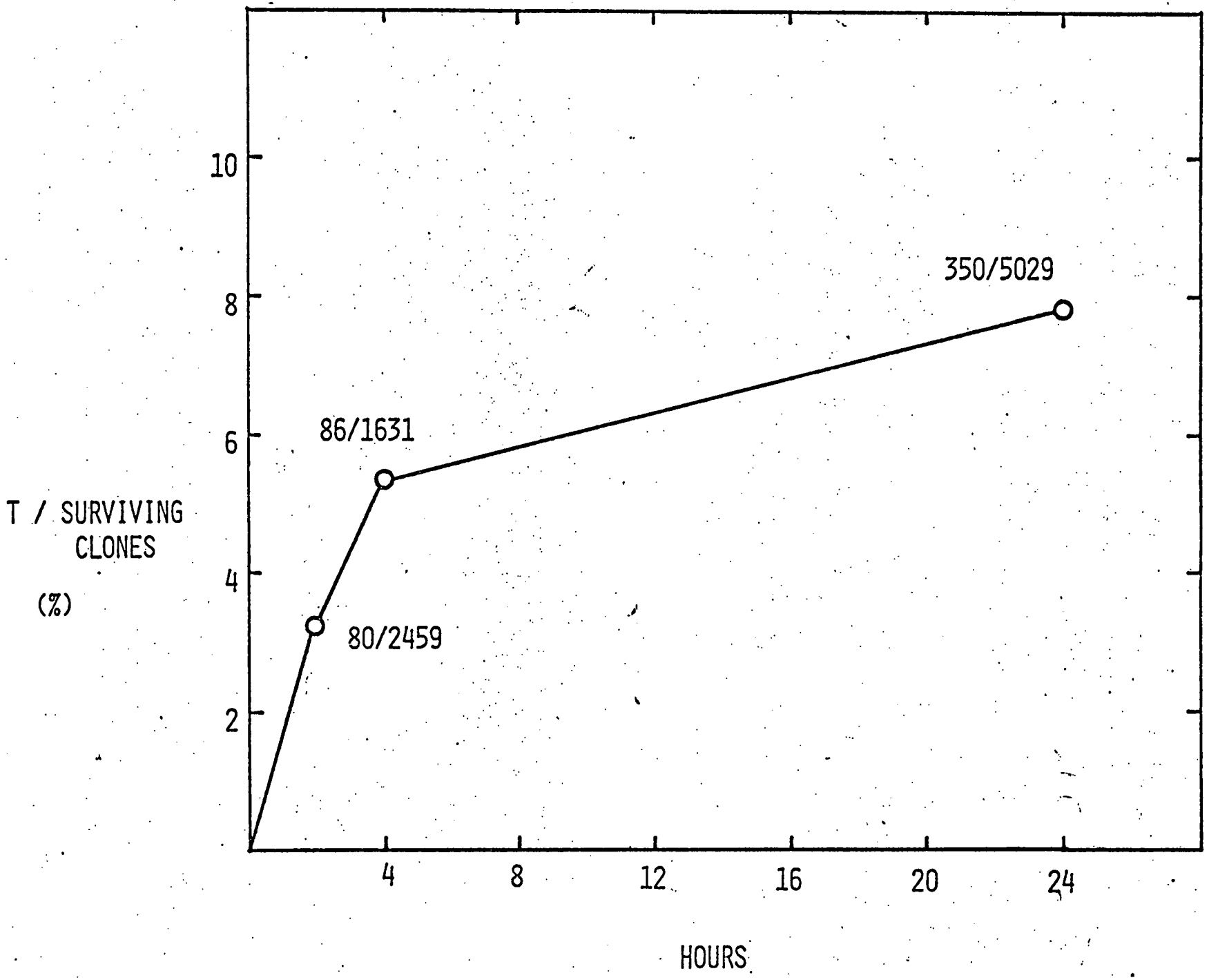

T / SURVIVING CLONES (%)
10
8
6
4
2
350/5029
86/1631
80/2459
4
8
12
16
20
24
HOURS

Chart 5.

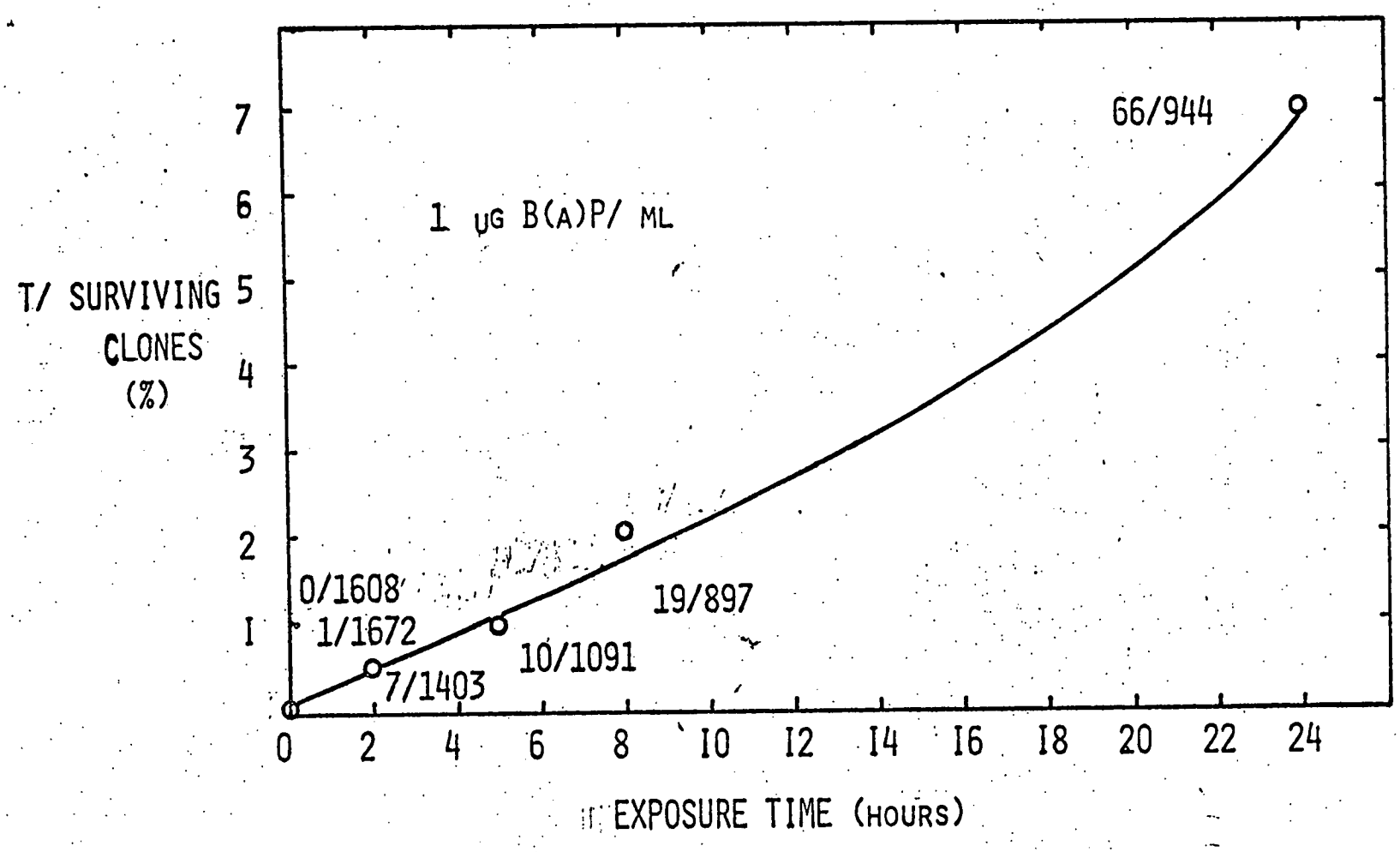

1 µG B(A)P/ ML
T/ SURVIVING CLONES (%)
EXPOSURE TIME (HOURS)
0/1608
1/1672
7/1403
10/1091
19/897
66/944
0
2
4
6
8
10
12
14
16
18
20
22
24
1
2
3
4
5
6
7

Chart 6.

cate the system, an exposure period of 30 seconds to 1 µg B(a)P/ml was included; only one clone was judged to be morphologically altered out of 1672 (i.e. 0.06% transformation). Therefore, at the lower applied dose of carcinogen, one could be confident that the transformation frequencies obtained with brief periods of treatment are genuine and are not attributable to residual non-washable carcinogen.

A more extensive examination of duration of treatment with B(a)P was then undertaken to include daily time points up to 8 days exposure to 1 µg B(a)P/ml (Chart 7). Here, the washing process was performed after the indicated treatment periods, which was followed by further incubation for clone formation until day 8, when the experiment was terminated. It is obvious that the level of transformation plateaus at 24 to 48 hours of exposure to the carcinogen. Beyond this length of treatment, the level of transformation fell steadily to a point (8 days exposure) where approximately 0.5% of the surviving clones could be judged as morphologically transformed. This decline in transformation frequency was not accompanied by a drop in the cloning efficiency since approximately the same numbers of surviving clones were found from 8 hours to 8 days of exposure to the carcinogen. Thus, the progressive decrease in per cent transformation beyond 48 hours of treatment is not attributable to cytotoxic effects of B(a)P with respect to the total number of surviving clones. However, selective cytotoxicity to transformed clones or to cells in the process of undergoing the transformation event(s) cannot be ruled out.

From these studies of varying the duration of exposure to low applied doses of B(a)P, several major observations can be made:

(1) The washing procedure is effective in eliminating erroneous results attributable to non-washable residual carcinogen.

(2) The "time of exposure" to B(a)P now takes on a meaningful definition; it can be defined as that interval of time between the point of application of the carcinogen and the completion of the final wash. Based upon results presented earlier (Charts 2 and 3), residual chemical remaining with the cells no longer presents itself as an indeterminable variable.

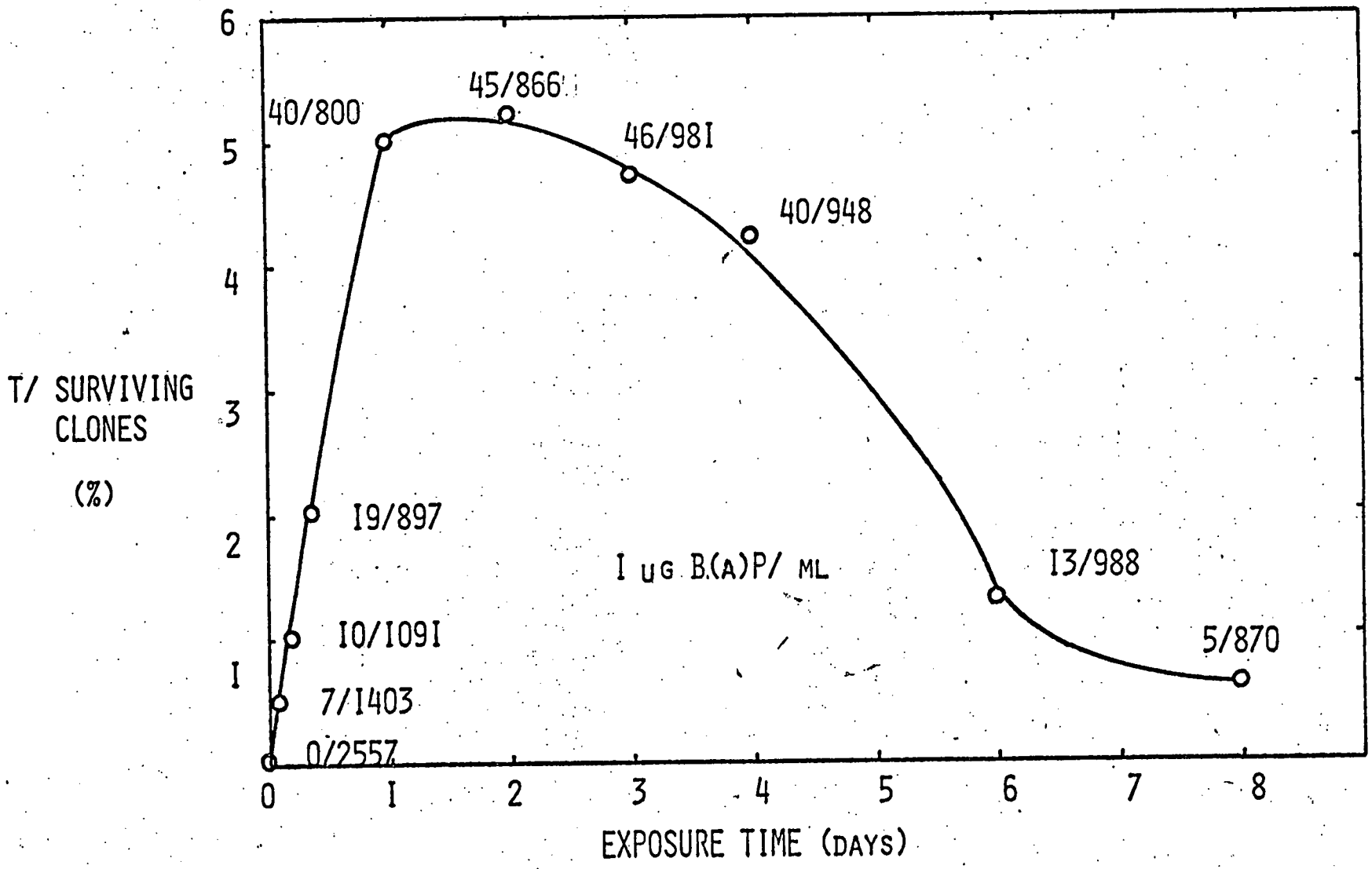

T/ SURVIVING CLONES (%)
6
5
4
3
2
I
0
I
2
3
4
5
6
7
8
EXPOSURE TIME (DAYS)
40/800
45/866
46/98I
40/948
I3/988
5/870
I9/897
I0/I09I
7/I403
0/2557
I uG B.(A)P/ ML

Chart 7.

(3) Peak levels of morphological transformation can be obtained with much briefer exposure periods than had been previously assumed.

(4) Recalling that these results were obtained in the absence of any feeder cell system, metabolic conversion of the parent hydrocarbon to a metabolically active carcinogenic form by anything other than the test cell system is eliminated as a parameter of concern.

Preliminary Study on the Properties of 6-Hydroxybenzo(a)-pyrene by the In Vitro Transformation System.

With the development of this morphological transformation assay, studies were undertaken to examine the carcinogenic potential of 6-OH-B(a)P. A definitive study of dose-response effects of 6-OH-B(a)P applied for 24 hrs. was carried out involving the examination of more than 8100 clones. Chart 8 shows that logarithmic increases in applied dose of 6-OH-B(a)P resulted in increasing frequency of transforamtion up to approximately 7%, approaching linearity between 0.1 and 3 ug 6-OH-B(a)P/ml. Beyond 3 ug/ml, 6-OH-B(a)P was found to be extremely cytotoxic and results could be interpreted only with difficulty. At lower doses (e.g. 0.01 ug/ml), the level of transformation (approximately 1%) may be a function of the rapid binding of this active metabolite in a non-washable form. Chart 9 compares the dose-response effects of B(a)P and 6-OH-B(a)P on SHE cells exposed for 24 hours. There appears to be a close similarity in the dose responses of transformation by the two compounds within the dose range of 0.01 - 3 ug/ml. However, at 10 ug/ml there is a large discrepancy between the two compounds, with a considerable decline in the percent of transformation in 6-OH-B(a)P experiment but not apparently with the B(a)P experiment. This sharp decline in transformation frequency at high applied doses of 6-OH-B(a)P may be a function of selective cytotoxic effects exerted by the metabolite to transforming SHE cells.

The studies of the effects of exposing SHE cells to 6-OH-B(a)P for various periods of time were carried out using an applied concentration of 1 ug 6-OH-B(a)P/ml in order to directly compare the results with those obtained for B(a)P at the same concentration. It was hoped in this way that it could be determined whether 6-OH-B(a)P could be a metabolically active form of the parent hydrocarbon B(a)P; if this were the case then one would predict that the transformation event should require a shorter exposure time to reach a maximal efficiency when cells are treated by

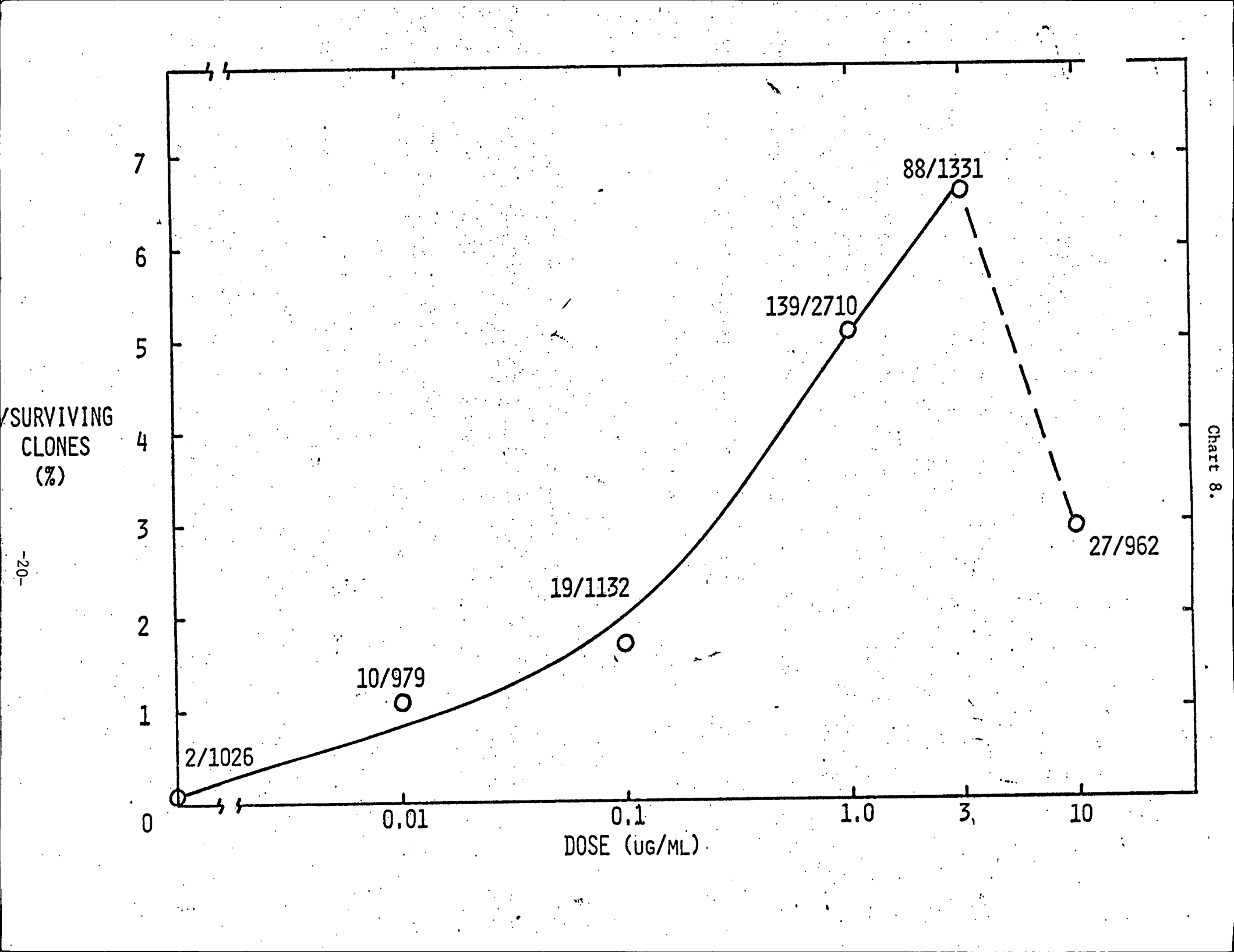

/SURVIVING
CLONES
(%)
0
1
2
3
4
5
6
7
2/1026
10/979
19/1132
139/2710
88/1331
27/962
0.01
0.1
1.0
3
10
DOSE (UG/ML)

Chart 8.

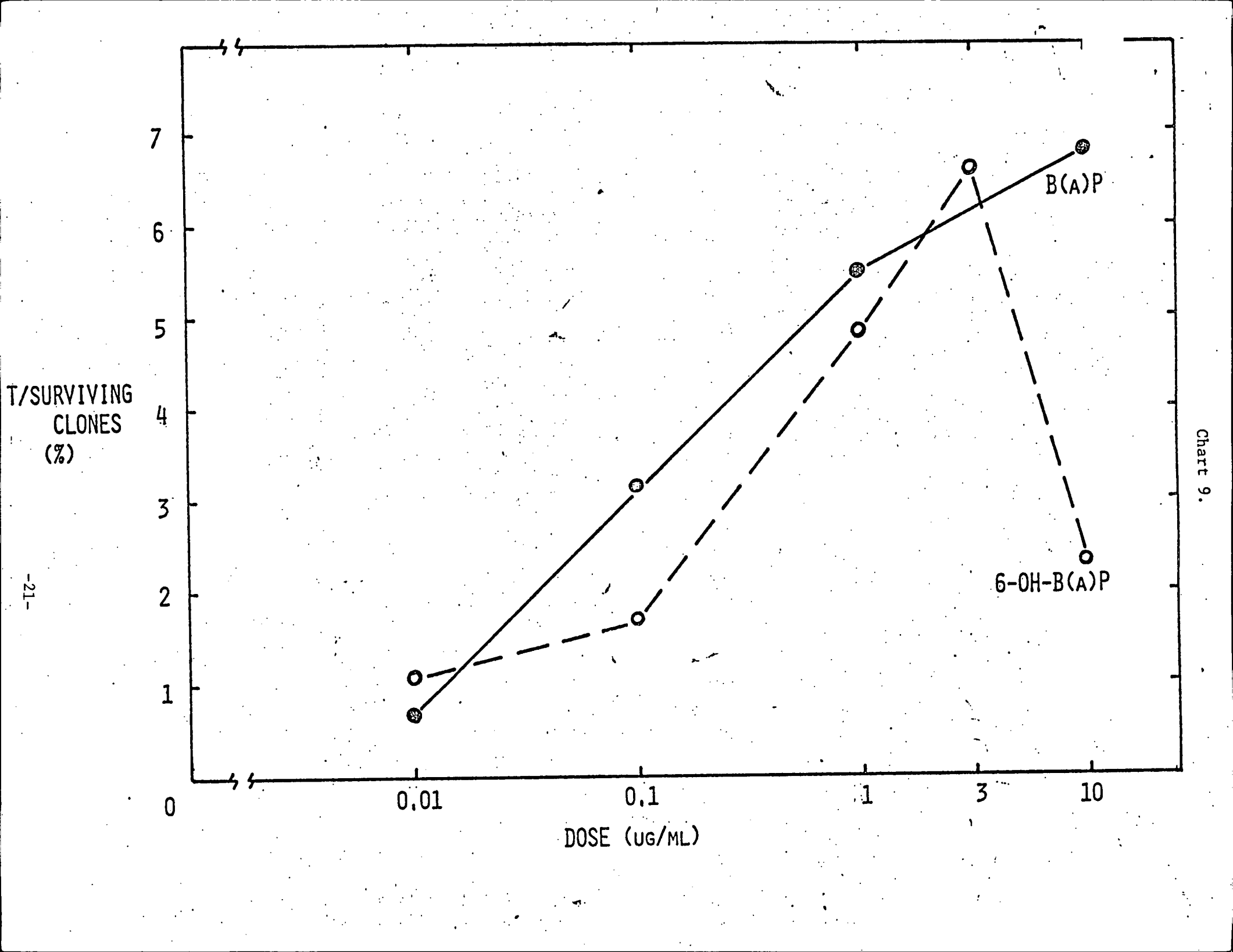

T/SURVIVING CLONES (%)
7
6
5
4
3
2
1
0
0.01
0.1
1
3
10
DOSE (UG/ML)
B(A)P
6-OH-B(A)P

Chart 9.

proximate carcinogen or ultimate carcinogen. This prediction presupposes that the metabolic conversion of the precarcinogen to ultimate carcinogen is the rate determining process during the exposure period. Chart 10 illustrates the frequency of transformation as a function of duration of exposure to 6-OH-B(a)P. There appears to be a rapid increase in percent transformation, with no apparent lag, before a plateau is reached after 2 hours exposure. Over 25,000 clones were examined to generate this curve. At the concentration studied (1 ug/ml), cytotoxic effects of 6-OH-B(a)P, with respect to the number of surviving clones, could be circumvented by adjusting the cell seeding levels upward to allow for longer exposure periods to the carcinogen.

Chart 11 is a composite of the exposure time-response curves determined for B(a)P and for 6-OH-B(a)P at the same applied dose, 1 ug/ml. When the two curves are compared, it becomes apparent that 6-OH-B(a)P treatment requires a much shorter time to reach the plateau of maximal transformation frequency. The dramatic differences between the slopes of the two curves during the shorter exposure periods seems to lend credence to the argument that 6-OH-B(a)P is an actively transforming metabolic intermediate or proximal carcinogen of B(a)P.

Chart VII.

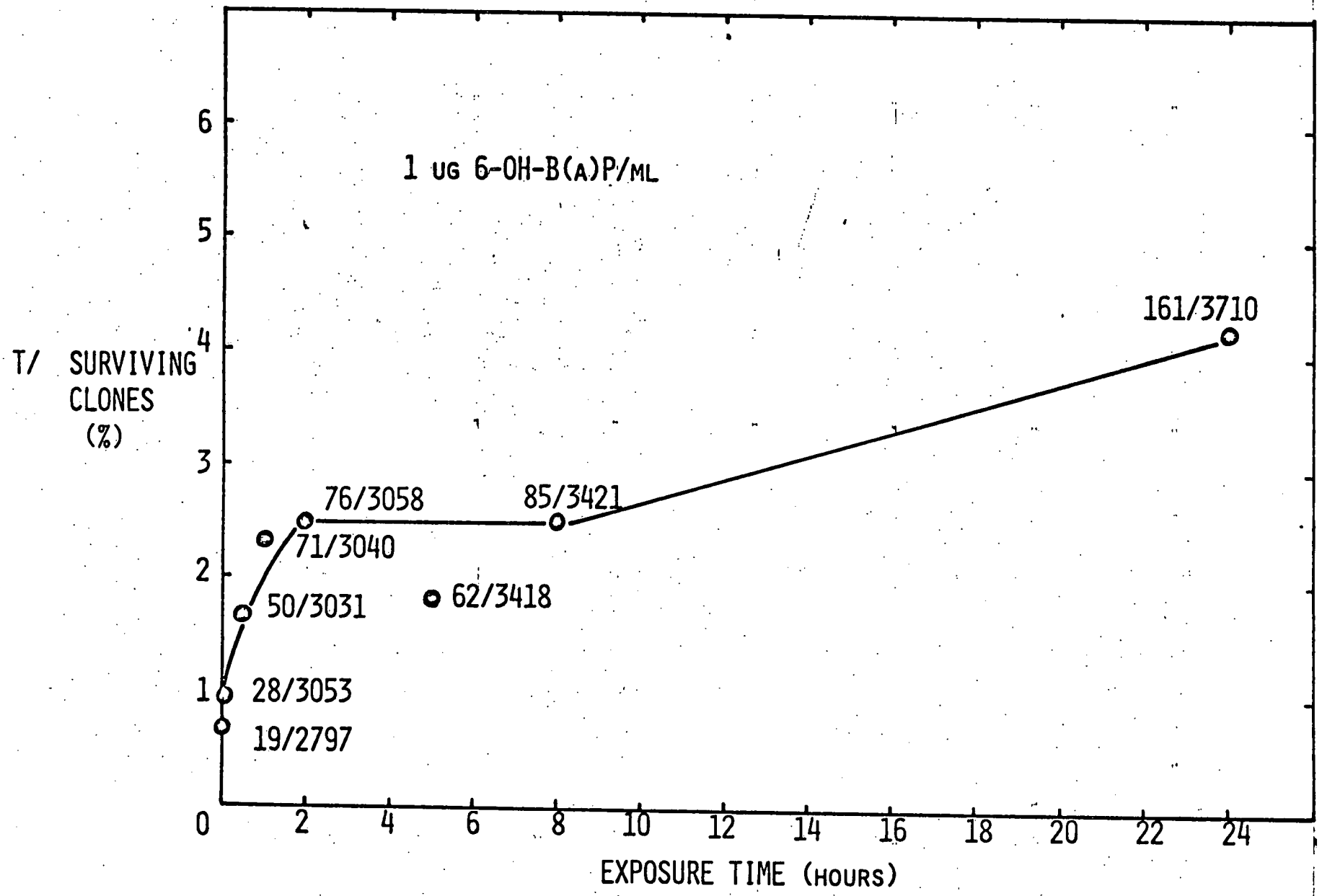
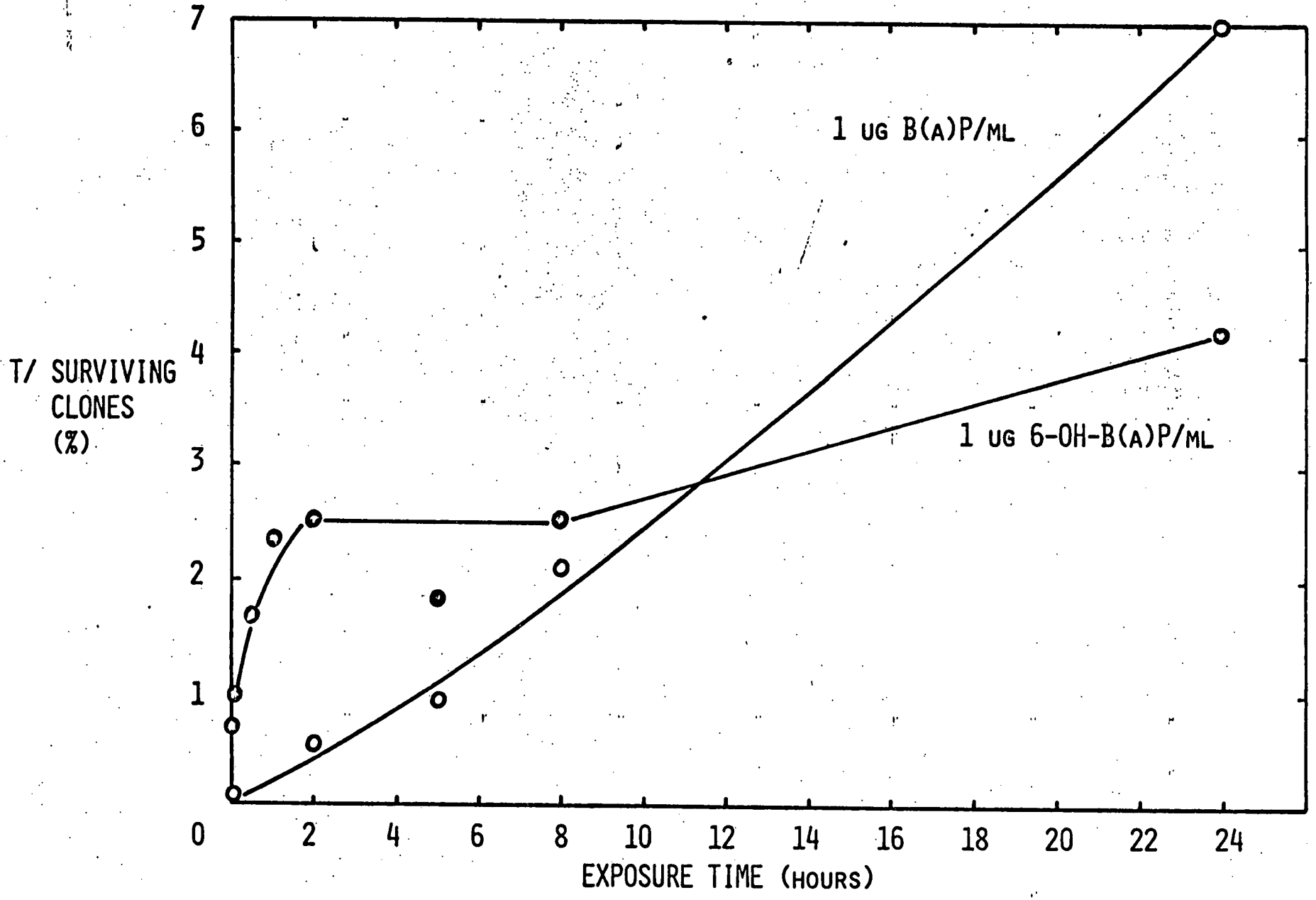

1 UG 6-OH-B(A)P/ML
T/ SURVIVING CLONES (%)
EXPOSURE TIME (HOURS)
0
1
2
3
4
5
6
2
4
6
8
10
12
14
16
18
20
22
24
161/3710
76/3058
85/3421
71/3040
50/3031
62/3418
28/3053
19/2797

Chart VIII.


T/ SURVIVING CLONES (%)
7
6
5
4
3
2
1
0
2
4
6
8
10
12
14
16
18
20
22
24
EXPOSURE TIME (HOURS)
1 UG B(A)P/ML
1 UG 6-OH-B(A)P/ML

Confirmation of the Tumorigenicity of the Morphologically Transformed Cells and the Establishment of the Neoplastic Cell Lines.

A considerable amount of effort has been devoted to confirming the tumorigenicity and to characterizing other aberrant biological properties of the morphologically transformed cells. Chart 12 is a schematic representation of the elaborate sequence of events in such confirmatory studies. The top portion of the flow chart is a reiteration of the 8 day morphological cloning transformation assay, which is now standardized to a 24 hour treatment with the polycyclic hydrocarbon at the desired dosage. At the end of the 8 day assay, representative dishes are fixed, stained, and scored for morphological transformation in the usual manner. Other plates which were concurrently run in parallel and were treated identically to the stained plates, are scored for aberrant clones in the living state under the phase microscope. The photographs in Plate 4 to Plate 6 illustrate our ability to recognize morphologically transformed clones in the living state. In each pair of photographs, 'A' is a representation of the live clone and 'B' is the identical clone oriented in the same direction, after fixing and staining. These clones were derived from SHE cells exposed to 10 ug B(a)P/ml for 24 hours and have been shown subsequently to be tumorigenic. Also included (Plate 6) is one morphologically normal clone both in living and stained condition; this clone previously was exposed to 0.25% DMSO.

Such clones, once circumscribed, are permitted to grow to increase their cell mass and are finally isolated at post-treatment passage (PTP) #1, approximately 2 weeks after scoring. Through the next two PTPs, the cells are encouraged to grow through enrichment of their growth medium to increase their numbers. In approximately 8 weeks from the time the experiment was first initiated, a sufficient number of cells is usually available to carry out other in vitro confirmatory studies and to establish the tumorigenic potential of the aberrant clonogenic populations.

The confirmation studies carried out in vitro presently include: (1) repeating the morphological transformation assay of these cells in low cell density culture, in the absence of the carcinogen, in order to confirm the morphological aberrations originally recognized for that clone at the end of the initial 8 day assay; (2) assaying the ability of the cloned cell populations to grow into colonies in semi-solid agar, and (3) karyotypic analyses for chromosomal aberrations.

At any point in time, cells can be frozen and stored indefinitely in our liquid nitrogen cell re pository for further study at some future date. Ultimately, all clonogenic established cell lines are tested for their in vivo tumorigenicity in isologous unconditioned newborn hamsters by subcutaneous inoculation of 10^7 cells per host. In vivo confirmation of neoplastic transformation is acknowledged upon tumor initiation.

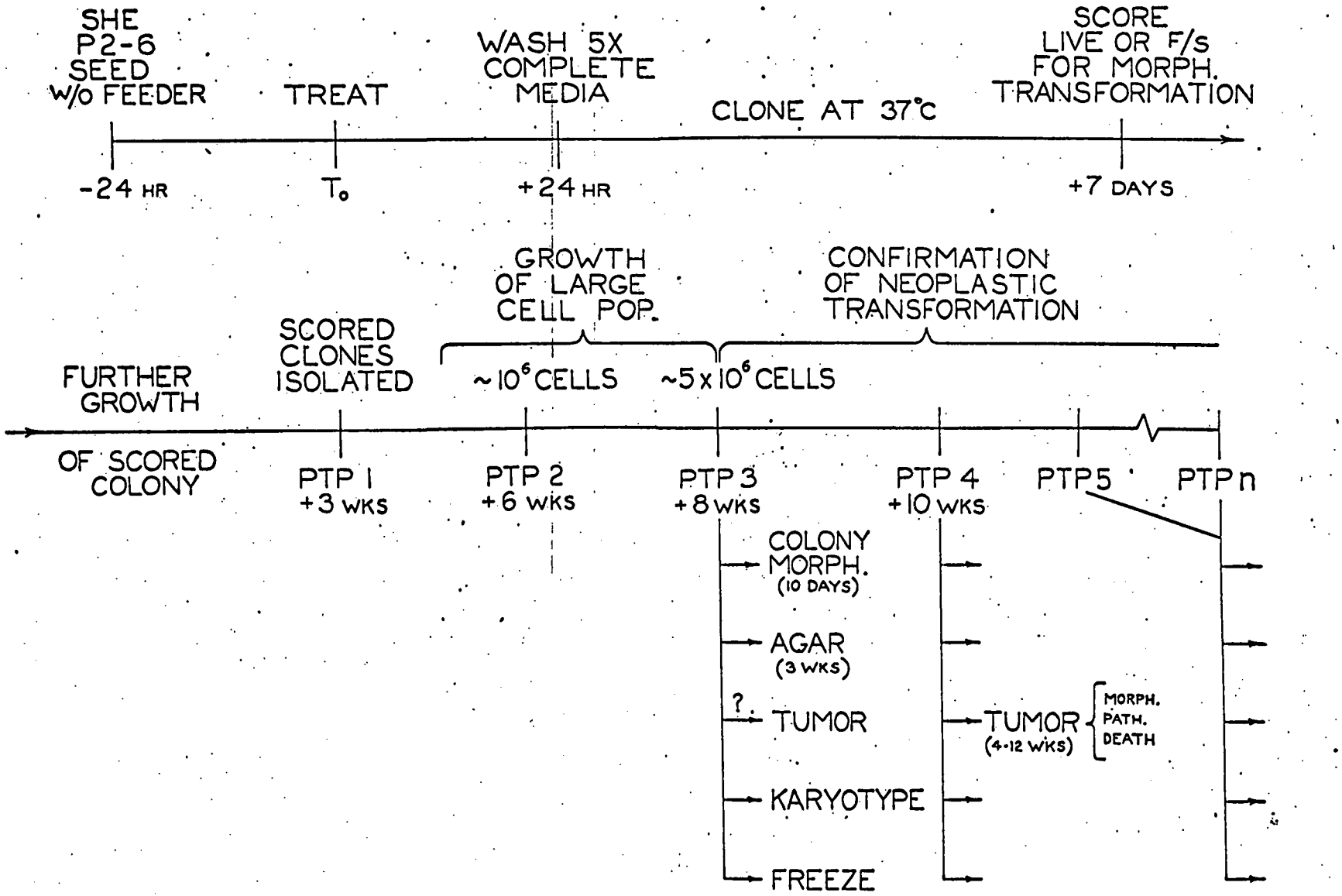
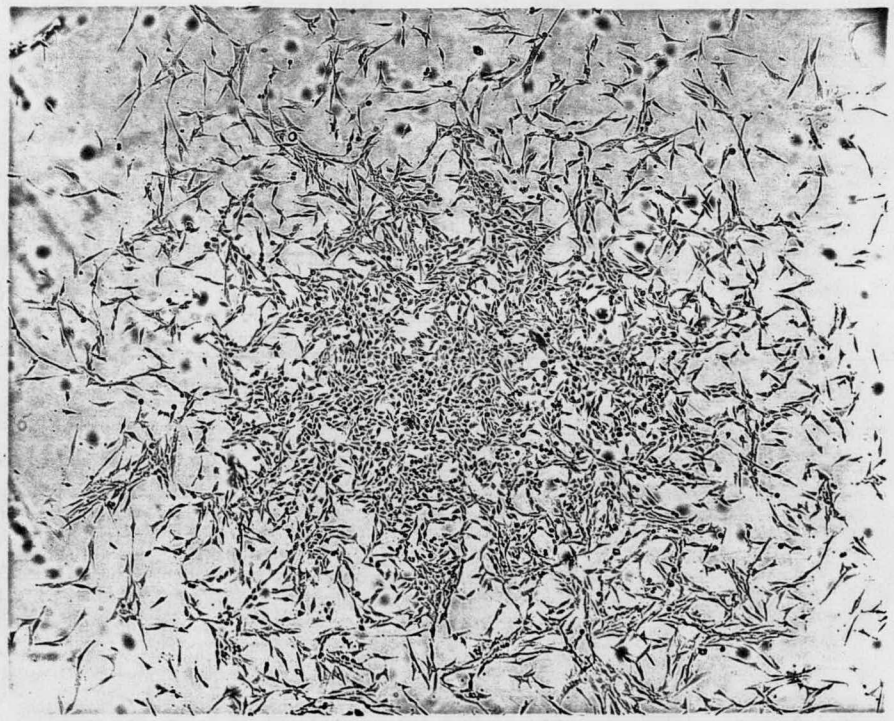

SHE
P2-6
SEED
w/o FEEDER
TREAT
WASH 5X
COMPLETE
MEDIA
CLONE AT 37°C
SCORE
LIVE OR F/s
FOR MORPH.
TRANSFORMATION
-24 HR
T₀
+24 HR
+7 DAYS
GROWTH
OF LARGE
CELL POP.
CONFIRMATION
OF NEOPLASTIC
TRANSFORMATION
SCORED
CLONES
ISOLATED
FURTHER
GROWTH
OF SCORED
COLONY
~10⁶ CELLS
~5x10⁶ CELLS
PTP 1
+3 WKS
PTP 2
+6 WKS
PTP 3
+8 WKS
PTP 4
+10 WKS
PTP 5
PTP n
COLONY
MORPH.
(10 DAYS)
AGAR
(3 WKS)
?
TUMOR
KARYOTYPE
FREEZE
TUMOR
(4-12 WKS)
MORPH.
PATH.
DEATH

PLATE 4

A



B

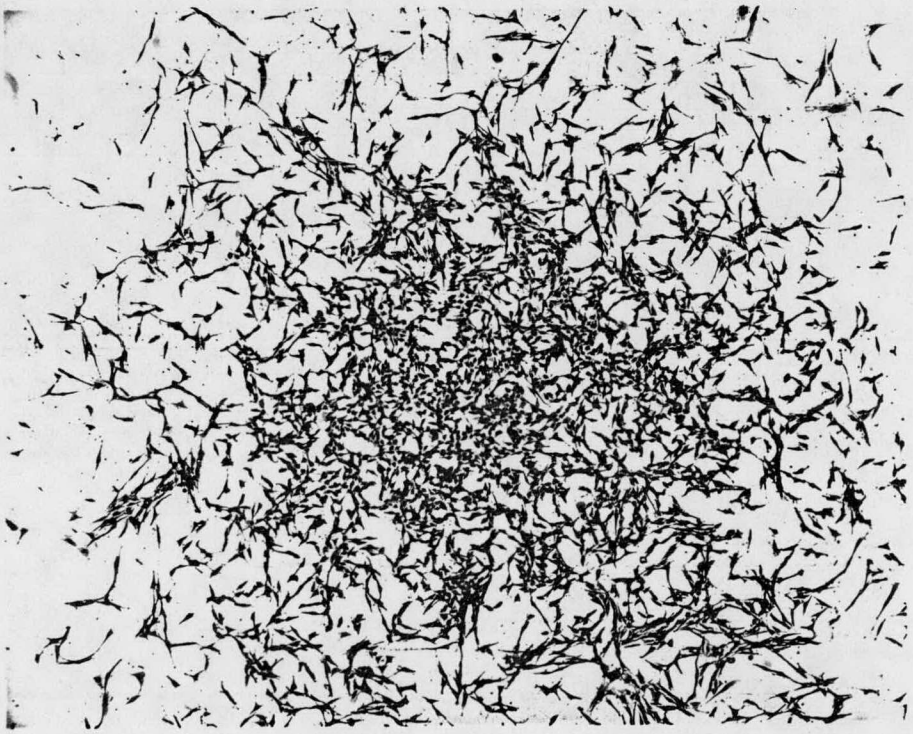
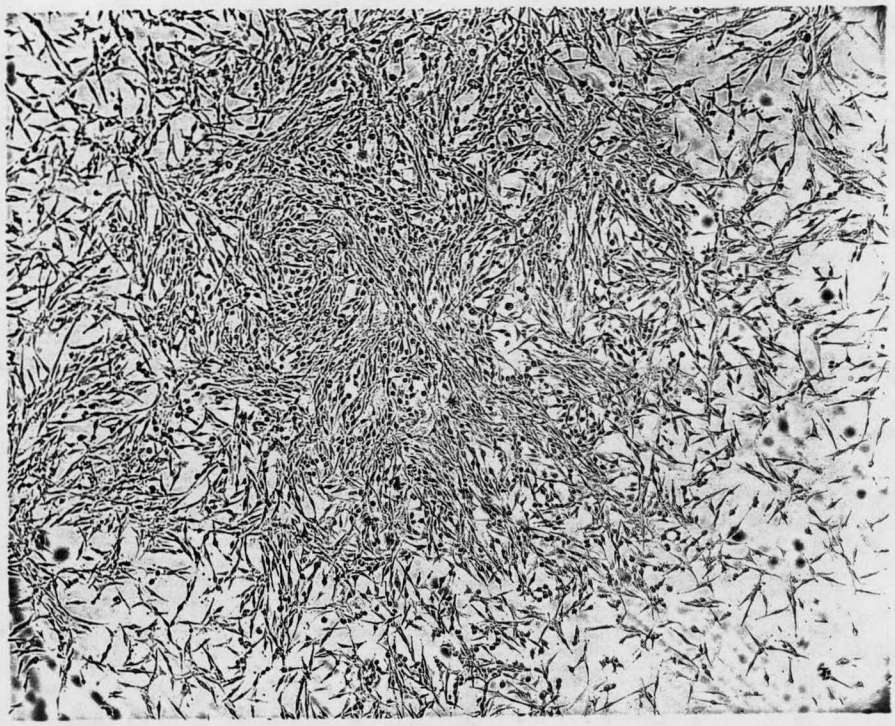


PLATE 5

A



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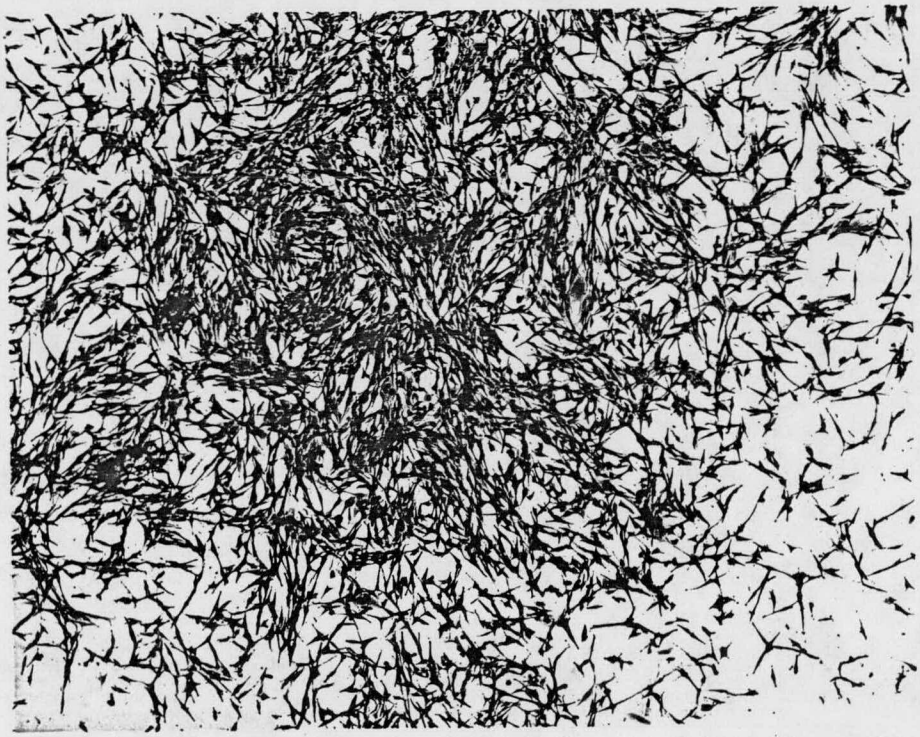
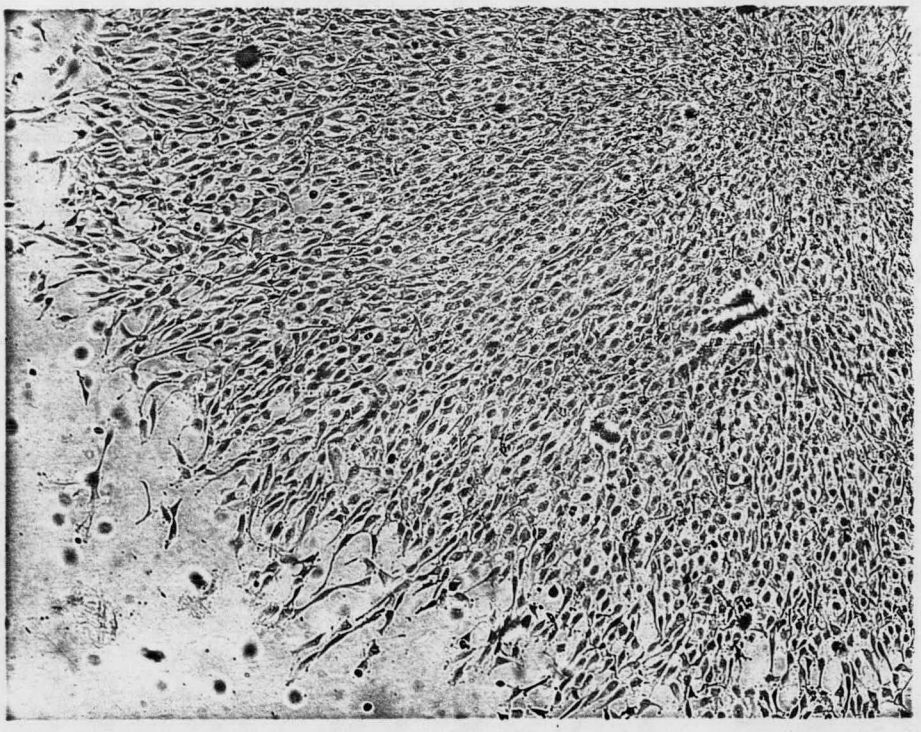


PLATE 6



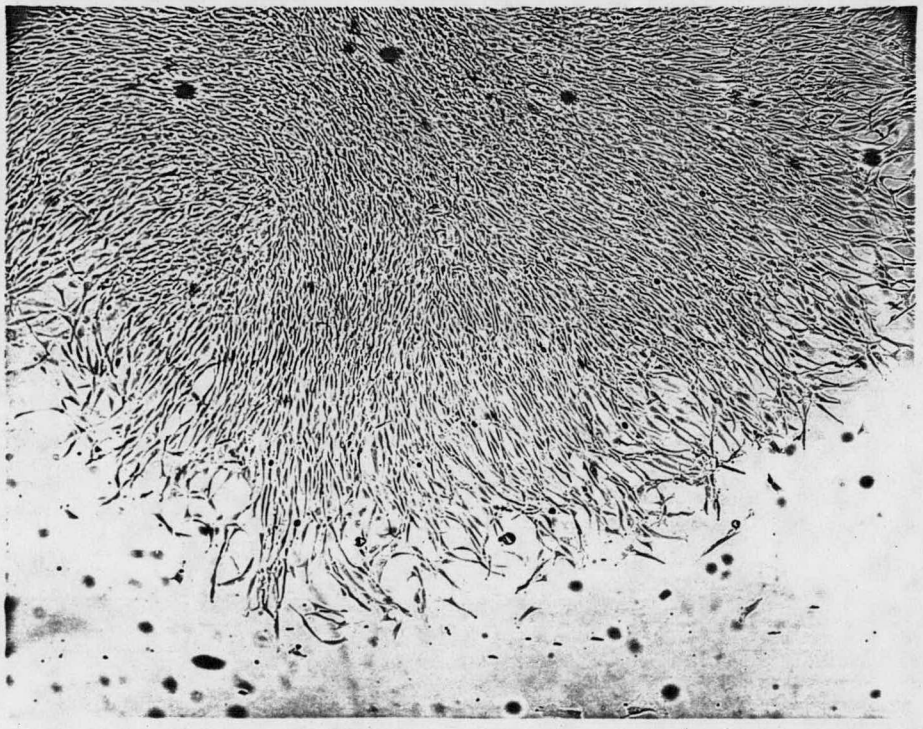


Chart 13 tabulates the results of such an in-depth study with five clonogenic B(a)P-transformed cell lines. All five transformed lines which were selected originally because of their morphologically aberrant cellular social pattern all retained the biological property of forming abnormal colonies. The normal SHE cells, even at equivalent passage levels, remained morphologically normal. All five transformed lines formed colonies in soft agar (an example of which is shown in Plate 7), while their normal counterparts failed to do so. Karyotypically, all five transformed lines were aneuploid and control SHE cells remained diploid. Cell saturation densities for each transformed cell populations increased well over that of controls. All 35 newborn hamsters inoculated with the different clonogenic transformed lines developed palpable tumors in 3-6 weeks which grew progresssively and killed their hosts; none of the 19 hamster newborns inoculated with control SHE cells displayed neoplasms regardless of the passage level of the cells at the time of the injection. Examinations of the tumors by a certified pathologist revealed that they were fibrosarcomas, as predicted. Photographic examples of some tumor-bearing hamsters have been included (Plates 8 and 9). The results of a detailed karyotypic study of one of the cloned transformed cell lines (18 Cl.10) and of cells cultured from a tumor derived therefrom (18 Cl.10 T) are also provided. In Chart 14, the karyotypes of the normal Golden Syrian Hamster embryo cells are shown. The distribution of the chromosomal numbers of fifty cells is also demonstrated in Chart 15. The results indicate that at passage 15 the chromosome distribution is very narrow for the normal cells. In chart 15, it is also shown that the transformed lines, 18 Cl. 10, tends to have subdiploid chromosomal numbers, indicating a loss of chromosomes. A more careful analysis of the karyotype of this transformed cell line, 18 Cl.10, is shown in Chart 16. There is definitely an indication of a loss of chromosomes in addition to the appearance of a particular marker (labeled as M). When this line of transformed cells was injected into the newborn hamster, the chromosomal pattern of the tumor cells was analyzed and shown in Chart 15. The data indicate that the cells in the animal have now become multiploidy (or mainly tetraploidy) with a wide spread in chromosomal numbers. However, these tumor cells can still be identified with the original transformed line, 18 Cl. 10, because of the presence of the "M" marker in the tumor cells.

As for the procedure of karyotyping, cells in mid-log phase are treated with 0.5 ug/ml Colcimid for 2-4 hours. After harvesting with trypsin, cells are allowed to swell in 0.075 M KCl for 15-20 minutes, then fixed in methanol: acetic acid (3:1). Slides are prepared by blowing the cell suspension across a clean slide and air drying. Slides are either stained directly with Giemsa stain or processed to obtain chromosome banding. Currently, G-banding (Giemsa-banding) by the ASG, urea-Giemsa, or trypsin techniques, are employed. C-banding (centromere-banding) and R and T-banding (reverse and terminal-banding) are also used if necessary.

Chart 13

CHEMICALLY TRANSFORMED ESTABLISHED CLONAL CELL LINES

Cell Line:	Normal SHE	18-C1.10	14-C1.2	8-C1.10	19-C1.7	17-C1.13
Treatment:	None	10µg B(a)P/ml 24 hours	10µg B(a)P/ml 24 hours	10µg B(a)P/ml 24 hours	10µg B(a)P/ml 24 hours	10µg B(a)P/ml 24 hours
Present P (Passage) Level:	P16	P35	P31	P21	P14	P14
Cell Saturation Density/75 cm^2 ($x10^6$):	3-7	33	23	37	13	19
P:	P2-P15	P18	P19	P20	P12	P13
% Morph. Transformed Clones/Total Clones:	1.4	96	45	90	40	84
P:	P15	P18	P14	P14	P14	P14
Karyotype:	Diploid,>99%:44	Aneuploid,84%: 38-40	Aneuploid,50%: >4N	Aneuploid,~80% Sub-tetraploid, ~20%	Aneuploid,~30%: >4N Sub-tetraploid, ~70%	Aneuploid,~20% Sub-tetraploid, ~80%
P:	P15	P25	P18	P18	P10	P10
Growth in Soft Agar:	No	Yes	Yes	Yes	Yes	Yes
P:	P15	P23	P16	P16	P11	P12
Tumorigenicity in Unconditioned Hamsters:	0/19	9/9	9/9	7/7	6/6	4/4
P:	5 at P6, 1 at P7, 3 at P13, 10 at P18	P18	P13	P21	P12	P13
Established Tumor Cell Line in Culture:		Yes:18-C1.10-T_1				
P:		P2				
		Karyotype: Sub-tetraploid, ~70% Aneuploid,~30%: >2N				

PLATE 7

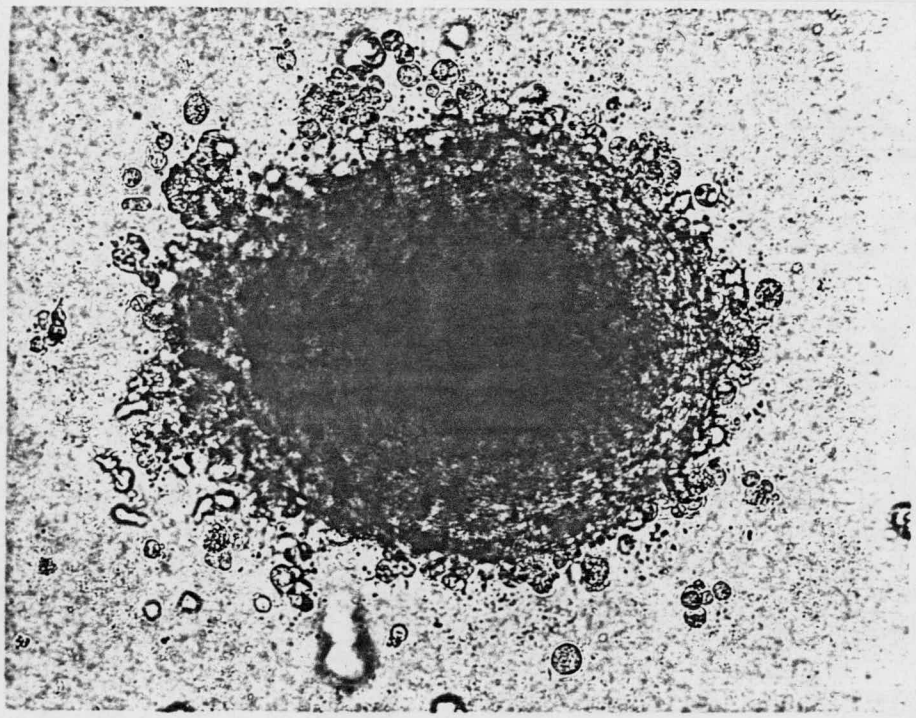


PLATE 8

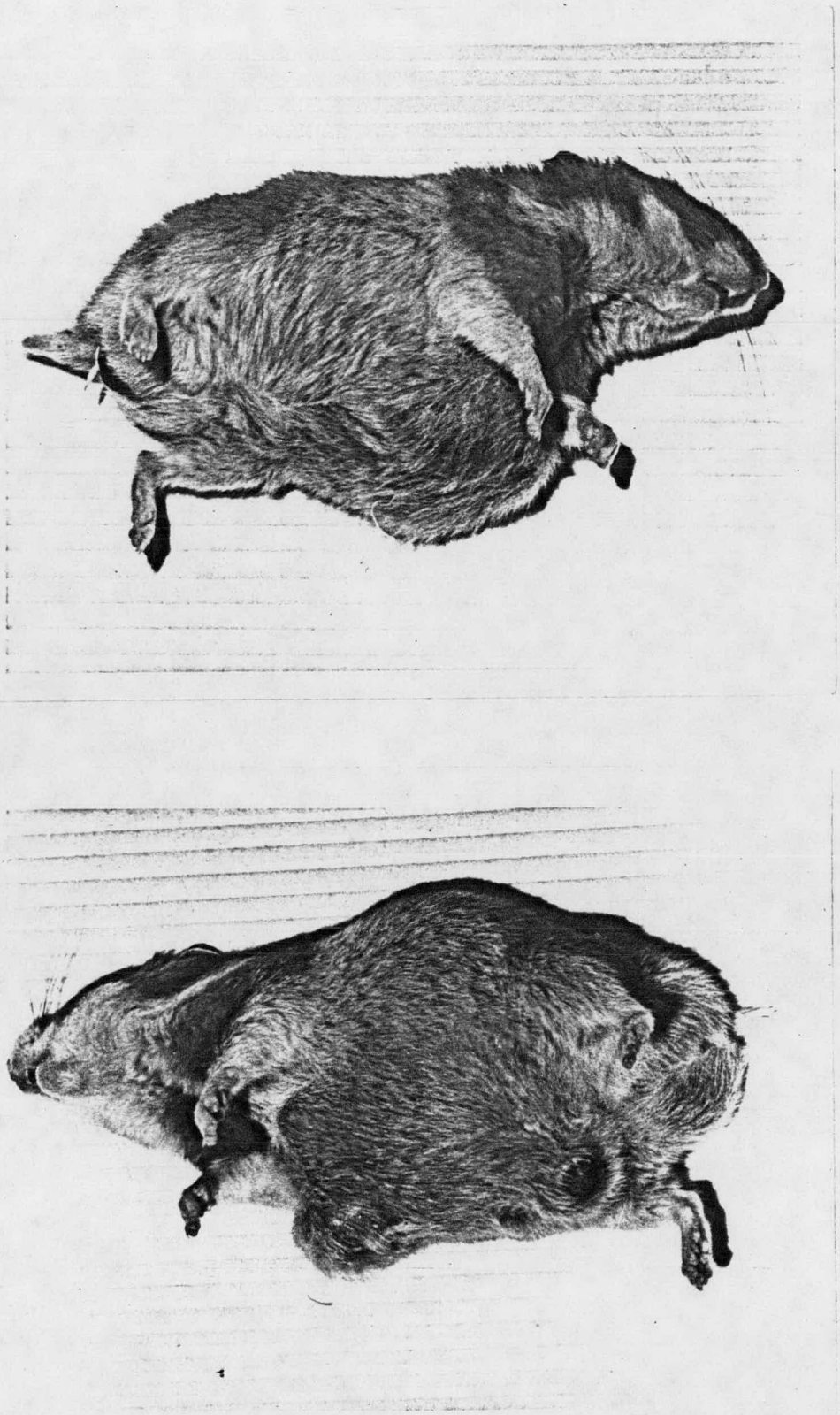


PLATE 9

CHART 14

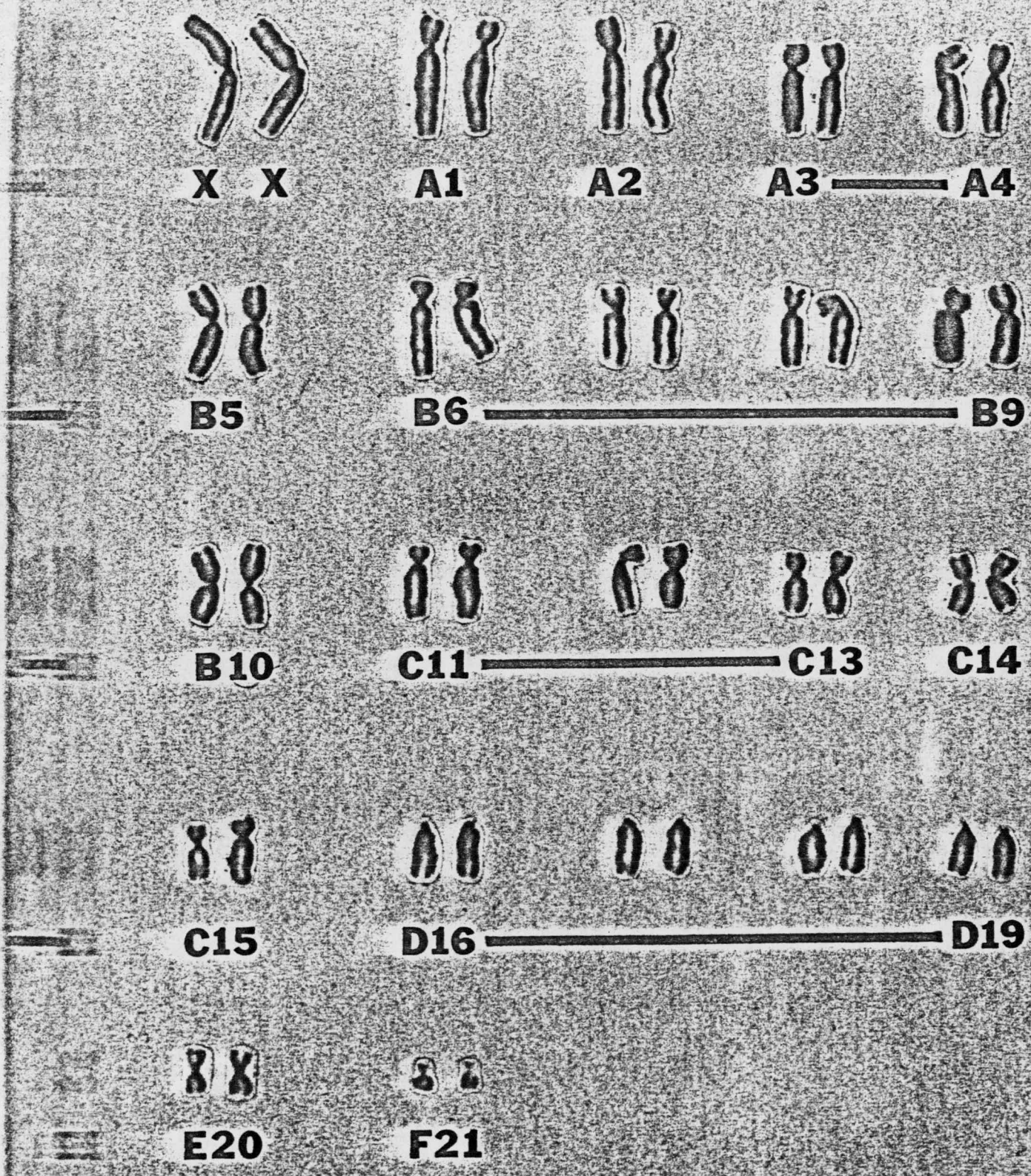

X X
A1
A2
A3
A4
B5
B6
B9
B10
C11
C13
C14
C15
D16
D19
E20
F21
GSHE Cell
2f1 P15

CHART 15

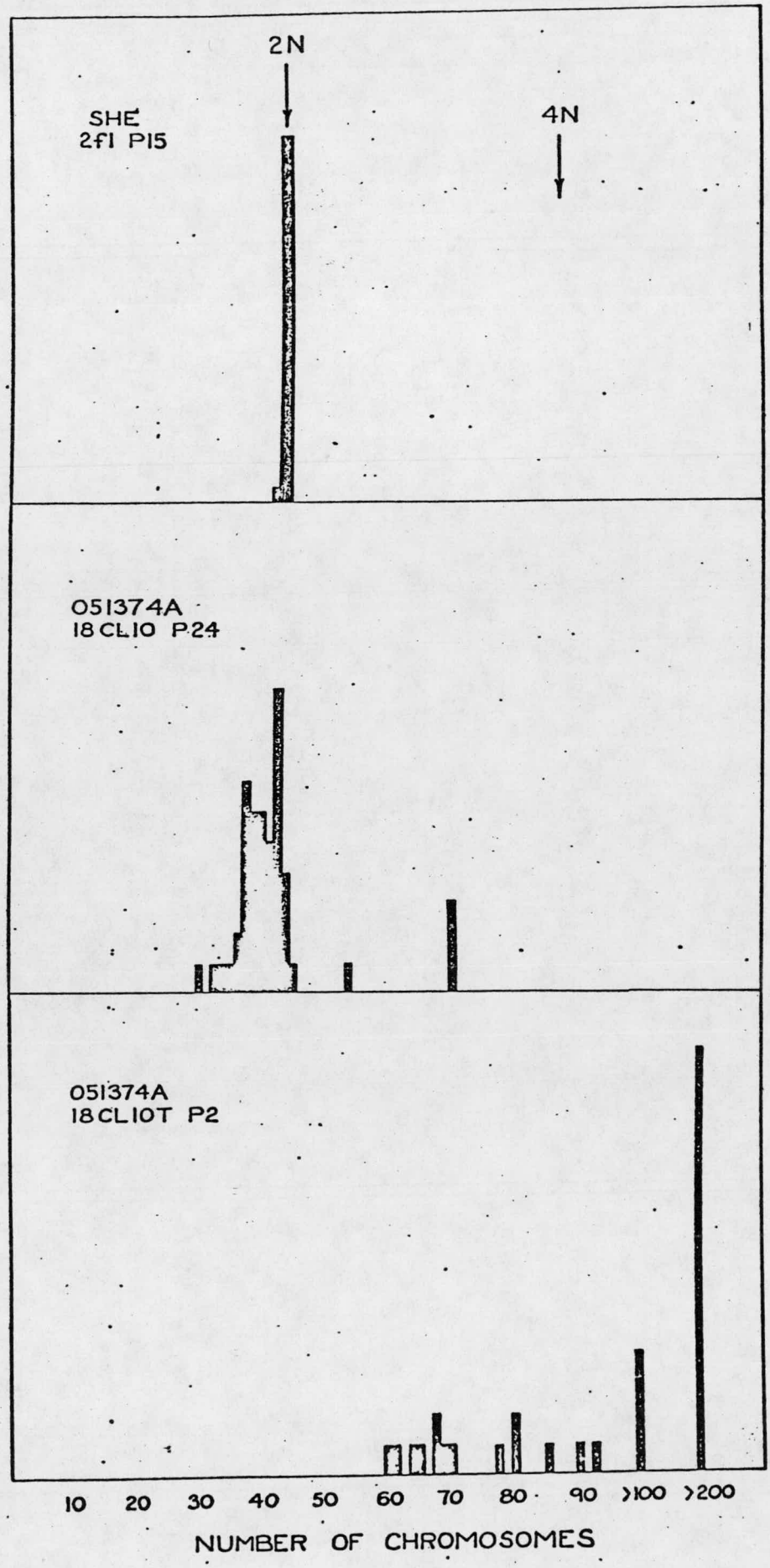
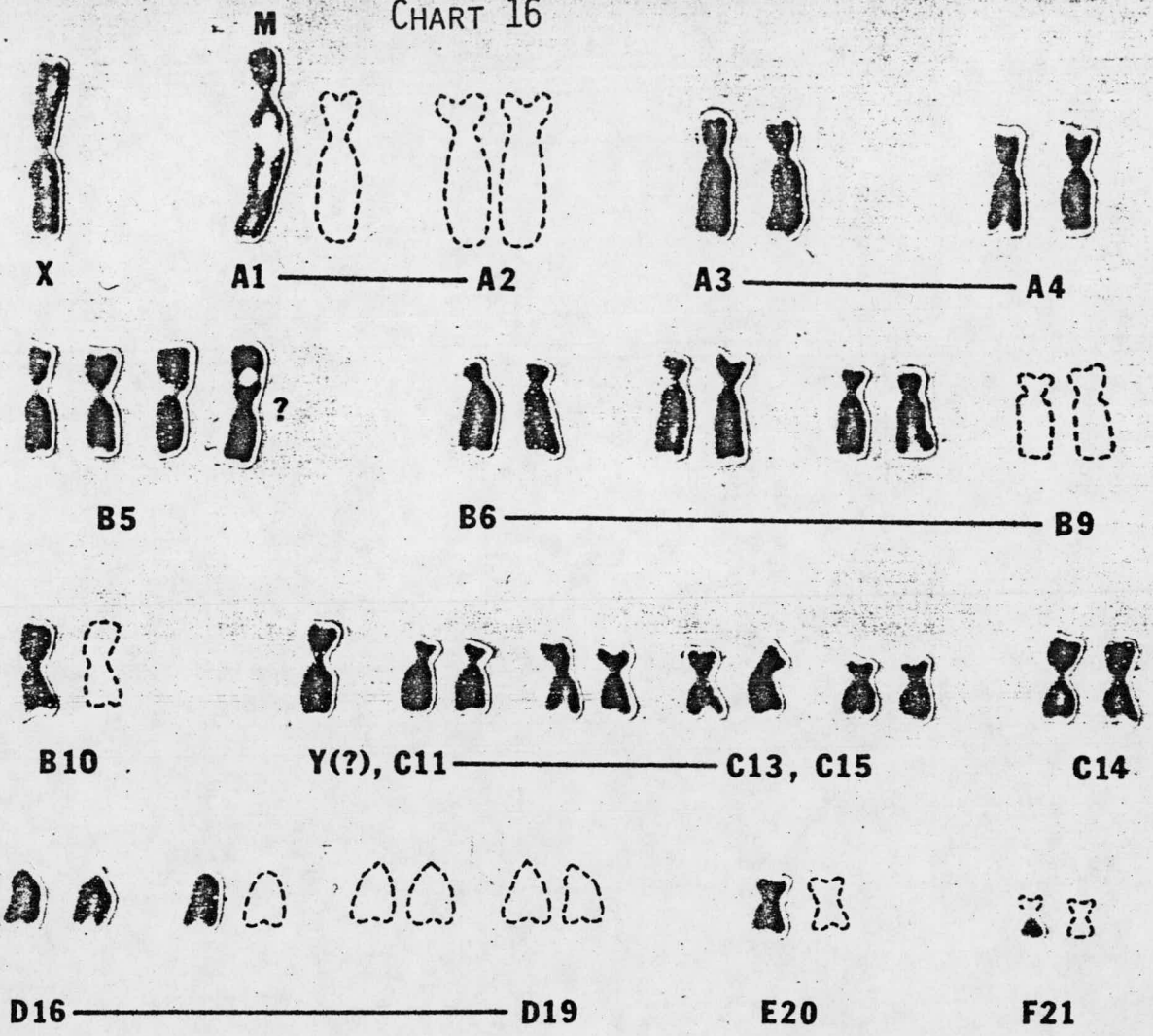
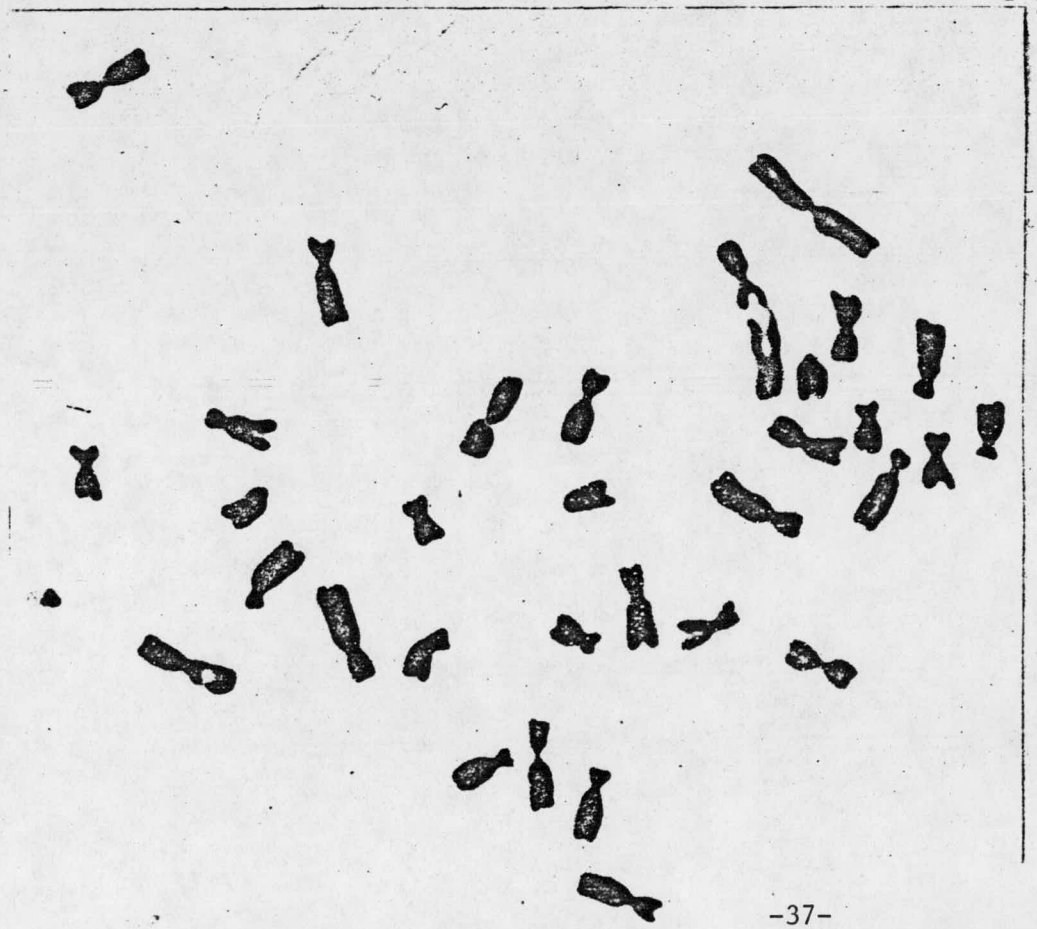

2N
4N
SHE
2f1 P15
051374A
18CL10 P24
% OF CELLS
051374A
18CL10T P2
10
20
30
40
50
60
70
80
90
>100
>200
NUMBER OF CHROMOSOMES

CHART 16





051374A
18CL10
P24

There are several major inherent problems in isolating and cultivating morphologically transformed clones for in vivo confirmation. Among these are (1) the ability to recognize morphologically transformed clones in the living state, (2) pleomorphism of transformed clones, e.g. dense conterminous vs. thin semi-contiguous colonies, (3) the sensitivity of clones to physical and chemical manipulation, and (4) the extremely slow initial rate of growth of such cellular isolates. Problems involved in identification of live aberrant clones necessitate the isolation of large numbers of individual altered clones in excess of those which are distinctly aberrant. After successful clonal isolation, and an extremely long lag period of growth, member cells of physically dispersed clones begin to populate their dishes and must undergo about 5 passages in culture before they are growing vigorously enough to be tested in vivo. To avoid unnecessary cultivation and in vivo inoculation of what may turn out to be untransformed clones, mass culturess derived from colonial isolates have been subjected to growth in enriched medium containing reduced levels (0.1 mM) of $CaCl_2$ (Freeman, A.E. et. al., 1967. J. Virology 1, 362). In such medium, non-transformed cells lose their doubling potential and ultimately die, while categorically transformed cells survive and multiply at an accelerated pace. Such has been the case with numberous clones that have survived isolation. Thus far, the five morphologically transformed clones previously discussed have survived repeated subcultivation in 0.1 mM $CaCl_2$, all of which have initiated massive subcutaneous fibrosarcomas.

Several other techniques are being examined at the present time as alternative confirmation testing programs for in vitro morphological transformation. One such assay is based upon the focus transformation assay developed by Kakunaga (int. J. Cancer 12, 463, 1973) for BALB/ 3T3 cells. In this system, zones of transformed cells referred to as "foci" and characterized by piling up of randomly oriented cells displaying criss-cross patterns appear against a background of organized contact-inhibited monolayer cells. Attempts to apply the focus transformation assay have thus far proved disappointing in the Syrian hamster embryo cell system. Numerous false positives as well as a prevalence of spontaneously transformed foci (all of which survived repeated passage in 0.1 mM $CaCl_2$) made it difficult to compare the two assays quantitatively.

Studies on the Continuous Progression of the Transformation Process.

Currently, the progressive nature of neoplasia in our cell culture system is being examined. This was prompted by the realization that:

(1) Several mass cultures derived from a pool of isolated clones originally judged morphologically transformed seemed to contain fewer numbers of morphologically aberrant colonies. With successive passages in culture, the number of aberrant colonies in these cultures became increasingly higher. At present, this phenomenon of increasing the number of morphologically transformed colonies in a mass culture can be due to a selection process or to a progression of the transformation process, and deserves much closer investigation.

(2) Several B(a)P-transformed cell lines displaying morphological aberrations failed to grow initially in soft agar. With time and subcultivation in vitro, such cells eventually acquired the ability to form colonies suspended in semi-solid agar.

(3) A pool of early PTP transformed clonal isolates failed to initiate tumors upon subcutaneous injection of 10^7 cells. This pool of cells is now being grown continuously in culture for the examination of the progressive process.

The major technical problem encountered thus far was being able to examine neoplastic progression in vitro in a relatively short period of time. It was determined that a high percentage of the individually isolated clones failed to establish, in a reasonable period of time, mass cell populations which could be systematically studied. Furthermore, in view of the considerable period of time required to establish large cell populations, it seemed reasonable to assume that one or more of the progressive events leading to the malignant state might go unnoticed because the cells had not yet achieved sufficient numbers for testing. Therefore, the approach we have elected to take in the attempt to establish a large, testable cell population in the shortest amount of time, has been the pooling of morphologically transformed clones gathered at PTP 1 (Chart 12). In this approach, we can develop mass populations of transformed cells from morphologically aberrant clones. These transformed clones have been grown in continuous cultures, with scheduled medium changes, for a considerable length of time in the absence of the cytotoxic effect of the original carcinogen treatment.

This progression process of transformation starts from the time when the cells are treated with carcinogens. The first manifestation of the transformation is the appearance of the morphologically aberrant colonies, which requires about 8 days after the carcinogen treatment for its recognition. The process is then examined for the following three events: (1) when the cells acquire the ability to clone in semi-solid agar, (2) when such cells display chromosomal aberrations, and (3) at what stage these cells become tumorigenic in vivo. Other biological

and biochemical characteristics of the cells will also be examined during this progression process. Following the scheme presented in Chart 12 as a systematic approach to study neoplastic progression. One population of pooled transformed clones (100874A: 8-Tr. Cls.) has displayed both a morphological progression and a progressive acquisition of growth in soft agar. At PTP 4, this culture had only 7.5% morphologically transformed clones; at PTP 5, 92% of the clones were morphologically aberrant. These same cells, when suspended in semi-solid agar, failed to grow at PTP 4, showed approximately 12 abortive attempts (two-cell colonies) at PTP 5, and finally at PTP 7 a majority of these cells grew positively into multicellular clones in soft agar in less than 7 days.

The most serious problem in the interpretation of the progression study on pooled populations of the transformed clones is to reach a clear distinction between the selection process and the progression process. This problem actually also exists even in the study of populations derived from one single clone. The progenies of this one cell may progress at different rates in the transformation process and the selection process may also be operative. Experimentally, we propose to study this problem, i.e. selection vs. progression, in the following manner. Cell cultures derived from pooled clones (or single clones if possible) are grown continuously in successive passages. At every passage, these cells are divided into three portions: (1) frozen and stored at liquid nitrogen temperature; (2) continuously grown in culture to the next passage; (3) testing for various properties. Those cells grown at different passages can then be mixed again into the same culture and grown together at a later time. For a hypothetical example, cells at PTP 3 only exhibit 10% of property-X (say, morphologically transformed colonies) while the same culture at PTP 6 now exhibit 100% of property-X. We can now form a mixed population of cells of PTP 3 (with 10% property-X) and cells of PTP 6 (with 100% property-X) in the same culture. The competition or selection in a mixed culture of these two cell populations collected at two different passages and possessing different degrees of property-X can be observed and analyzed. This information may allow us to distinguish the influence of the selection process vs. the influence of the progression process exerted on the cells in culture. With such a systematic approach to the study of neoplastic transformation, it is hoped that a reliable quantitative relationship can be obtained between tumor induction and <u>in vitro</u> morphological transformation.

Chemistry and Metabolic Activation of Polycyclic Hydrocarbons, Particularly the Benzo(a)pyrene.

In the last ten years considerable evidence has accumulated in support of the hypothesis that cellular metabolism is a prerequisite for the oncogenic activity of many chemical carcinogens, particularly the polycyclic hydrocarbons. The environmental carcinogen, benzo(a)-pyrene (B(a)P), is metabolized to many identified, stable products by liver homogenates (Selkirk et. al., Science 184, 169, 1974) as well as by other in vitro and in vivo systems.

In the laboratory of Ts'o and Lesko, efforts were made in two directions. In the first direction, chemical means were found to activate the B(a)P under mild and physiological conditions in order to understand the chemistry of B(a)P; in the second direction, the metabolic activation of B(a)P in rat liver homogenates was investigated in order to understand the biological significance of these chemical products from B(a)P. In both approaches, special emphasis was placed on the radical intermediates as studied by esr.

About five years ago, our laboratory (Lesko, Ts'o, and Umans, Biochemistry 8, 2291, 1969;Hoffman, Lesko, and Ts'o, Biochemistry 9, 2594, 1970) reported that B(a)P and other carcinogenic polycyclic hydrocarbons react specifically with DNA and polynucleotides in the presence of I_2 in aqueous ethanol systems. Under similar conditions noncarcinogenic analogs react with nucleic acid to a much lesser extent. It was postulated that radicals of B(a)P and other hydrocarbons may serve as reaction intermediates (Lesko et. al, 1969; Hoffman et. al, 1970). Earlier, several investigators had proposed the radical cation of B(a)P as the intermediate in the reaction of B(a)P with pyridine or nucleic acid bases in a solid-phase system activated by I_2 vapor (Rochlitz, Tetrahedron 23, 3043, 1967; Wilk and Girke, Jerusalem Symposium, Quantum Chem. Biochem. 1, 91, 1969). In 1960, Szent Györgyi et. al. (Proc. Natl. Acad. Sci. U.S. 46, 1444) reported the existence of radicals of a number of compounds including B(a)P when activated by I_2.

Electron paramagnetic resonance studies indicate the presence of benzo(a)pyrene radicals in benzene, methanol and cyclohexane solution induced by iodine. The esr spectra of the B(a)P radical and MCA radicals induced by I_2 are shown in Fig. 1. These radicals are quenched by pyrimidine, purine, nucleosides, imidazole, and other nitrogenous compounds but not by alcohol, aldehyde, or water. These results strongly support the proposal that radicals of benzo(a)pyrene are involved in the chemical reaction between the hydrocarbon and nucleic acids in the presence of iodine. The electron paramagnetic resonance studies on the steady-state radical concentration of 14 polycyclic hydrocarbons formed in the presence of iodine indicate that, in general, the carcinogenic compounds such as benzo(a)pyrene, 7,12-dimethylbenzanthracene, 3-methylcholanthrene, etc., have a much higher concentration of radicals than the non-carcinogenic compounds such as benzo(e)pyrene, benzanthrene, pyrene, naphthacene, etc. There are one or two exceptions. The steady-state radical concentrations of these compounds do not correlate well with their ionization potentials, though the compounds having low ionization potentials do tend to yield higher concentration of radicals.

As for the mechanism of the formation of B(a)P radicals, the general reaction scheme (Fig. 2) proposed by Jeftic and Adams (J.A.C.S. 92, 1332, 1970) and based on an electrochemical oxidation study on B(a)P appears to be applicable. In this scheme (Fig. 2) the oxidation of B(a)P can be conveniently thought to occur in two steps: the first step begins with formation of the radical cation, then the hydrated neutral radical, and finally results in 6-hydroxy B(a)P; the second step begins with the formation of a neutral oxo radical, which is then further oxidized to a carbonium ion, and finally ends with dihydroxyl-B(a)P. The dihydroxyl-B(a)P can be further oxidized to the quinone. All these radicals and the electrophilic carbonium ion species can potentially participate in chemical reactions with nucleic acids.

A logical step following our previous study on chemical activation of B(a)P is to search for a proximate carcinogen that may be generated inside the cell through the metabolism of B(a)P. Falk et. al. (J. Natl. Cancer Inst. 28, 699, 1962) reported in 1962 that the first metabolite detected in the bile of rat after intravenous injection of [^{14}C]B(a)P was 6-OH-B(a)P glucuronide. Casu et. al. (Tumori 37, 527, 1951) and Pihar and Spaleny (Chem. Listy 50, 296, 1956) also have identified 6-OH-B(a)P as a metabolite of B(a)P.

Chemical studies indicated that position six of the B(a)P nucleus is the most nucleophilic site on the molecule (Cavalieri and Calvin, PNAS 68, 1251, 1971) and is also the primary site of attack in ordinary chemical oxidation and electrochemical oxidation. In 1971, our laboratory reported the chemical linkage of DNA and poly G with [^{3}H]6-OH-B(a)P in a buffer-ethanol solvent system (Prog. in Mol. and Subcell. Biol., Vol. 2, 348; 1971). Independently, this observation was also made by Nagata et. al. The chemical synthesis of 6-OH-B(a)P was achieved using a modified procedure of Fieser and Hershberg (JACS 61, 4565; 1939). [^{3}H]6-OH-B(a)P with a specific activity of 3.3 X 10^{7} cpm/mmole was synthesized from [^{3}H]B(a)P to study the covalent linkage of 6-OH-B(a)P to nucleic acids.

In 0.01 M phosphate buffer, pH 7.0/95% ethanol (1:1,v/v) synthetic 6-OH-B(a)P is oxidized by air to a mixture of products, 6,12 B(a)P dione (36%), 1,6 B(a)P dione (27%), 3,6 B(a)P dione (29%) and a violet-colored, orange fluroescent material (3%), which are separated by alumina chromatography and identified by comparison of their physical and spectral data with those of the literature. The unidentified violet-colored product is paramagnetic; its esr spectrum is a stable, broad singlet and its yield was so estimated by double integration of this signal in the product mixture at the end of the reaction. Oxygen uptake studies with an oxygen electrode in closed systems indicate that the oxidizing agent is atmospheric oxygen dissolved in solution. The reaction is virtually halted in nitrogen-sparged solution. The half-life of 6-OH-B(a)P in the air saturated aqueous-ethanol solutions mentioned above is estimated to be 45 minutes at 22°.

It is also known that upon the autoxidation of 6-OH-B(a)P, a radical is formed spontaneously. This radical affords a characteristic multiplet esr spectrum centered at g=2.004 (Fig. 3), and has been identified by Nagata and co-workers (Gann 58, 289; 1968) as the 6-oxy-B(a)P radical. The radical is formed rapidly in buffer-ethanol mixture, and its concentration continues to increase during the first 100 min. and then decays slowly (8-20 hr.) to yield a stable but

unidentified singlet (g=2.004). The maximal amount of radicals formed is larger at pH 7.8 than at 6.3(by about 2.5-fold) and is also larger in the presence of poly A (by about 40%) than in its absence.

As described in the subsequent section, 6-OH-B(a)P reacts spontaneously with nucleic acid; Fig. 4 shows the kinetics of the chemical linkage of 6-OH-B(a)P to poly A, together with the kinetics of the formation and the decay of the radicals. A certain degree of correlation results from this data that suggests that the radical could be the species that reacts with the poly A.

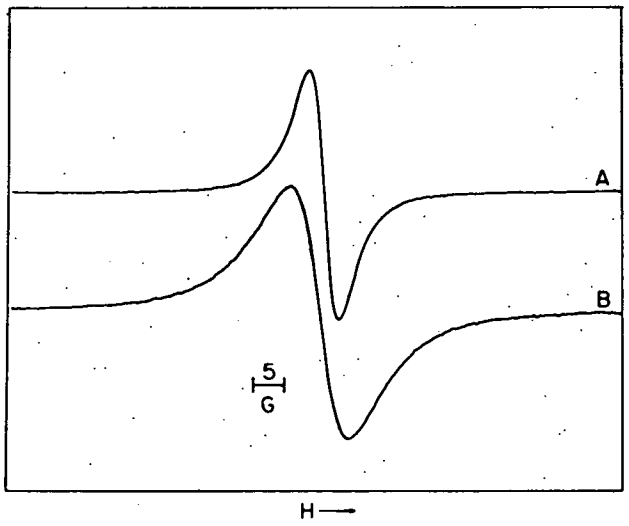


FIG. 1. EPR spectra of B(a)P (A) and MCA (B) when mixed with I_2 in benzene and quick frozen in liquid nitrogen. Spectra taken at -25° using hydrocarbon concentration of 5×10^{-4} M and I_2 concentration of 5×10^{-3} M. Modulation amplitude, 1.66 G; microwave power, 5 mW.

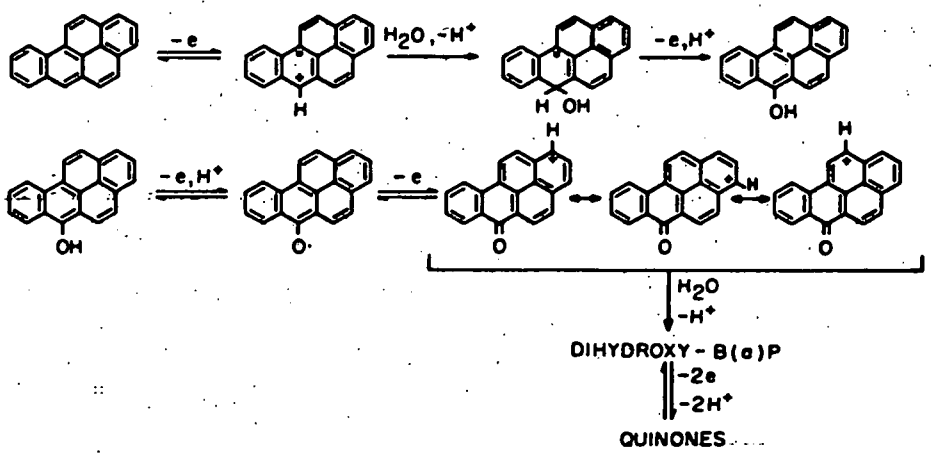


FIG. 2. General reaction scheme for electrochemical oxidation of B(a)P proposed by Jeftic and Adams

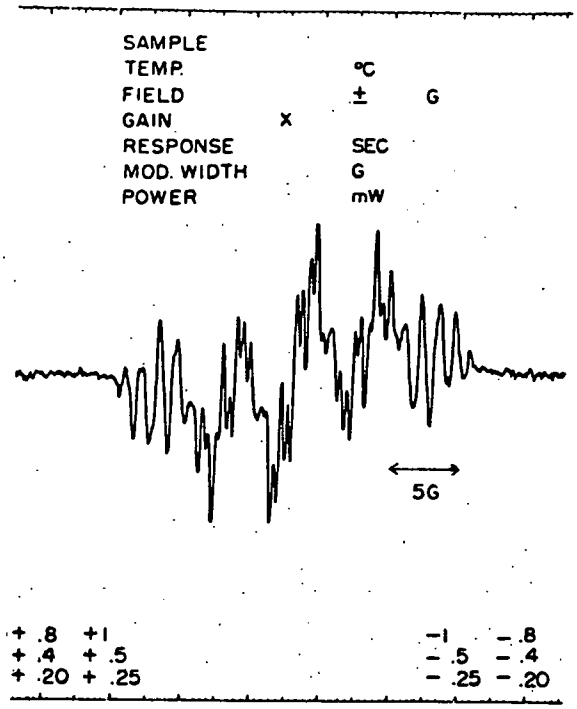


FIG. 3. EPR spectrum of 6-oxy-B(a)P radical in ethanol-Na phosphate buffer (1:1, pH 7.0). Modulation amplitude, 0.2 G; time constant, 1 sec; microwave power, 10 mW; room temperature.

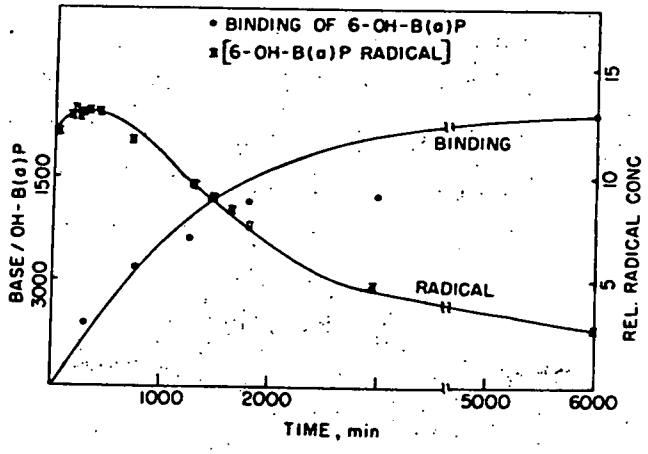


FIG. 4. Comparison of the kinetics of the 6-oxy-B(a)P radical and of the covalent binding of [^{3}H]6-OH-B(a)P to poly A at room temperature in ethanol-Na phosphate buffer (1:1, pH 7). Hydrocarbon/base, 1:1, poly A concentration, 5×10^{-4} M in nucleotide. Radical concentration is measured by comparing the peak heights of the 6-oxy radical and a standard. After 3000 min, the 6-oxy radical concentration has diminished to zero, and the radical observed shows a singlet spectrum whose peak height is measured and included in the graph. Between 2000 and 3000 min a combination of singlet and 6-oxy radical multiplet is observed.

The 6-oxo-B(a)P radical undergoes further oxidation reluctantly to diones in solvents such as benzene. Thus, 6-OH-B(a)P in benzene solutions can be selectively and quantitatively oxidized to the 6-oxo-B(a)P radical as the sole product by shaking with aqueous solutions of oxidizing agents such as $K_3Fe(CN)_6$. Once obtained, benzene solutions of pure 6-oxo-B(a)P radical can be evaporated to give relatively stable solids (under N_2) of the 6-oxo-B(a)P radical. This procedure can be conveniently and quantitatively monitored by esr signal and absorption spectrum (radical λ_{max} in benzene = 427 nm, ε: 2.71×10^4). The radical is autoxidized by air in buffer-ethanol solutions; under the identical conditions that 6-OH-B(a)P is autoxidized, the radical produces the same three B(a)P diones and violet-colored material in the same ratio as evidenced by chromatographic separation and comparison of the absorption spectra of the final product mixtures.

Partially reduced oxygen compounds are known to be produced during autoxidations where radical intermediates are found. The autoxidation of 6-OH-B(a)P in aqueous-ethanol solutions produces hydrogen peroxide. Its concentration is determined with a spectro-photometric assay using horseradish peroxidase in conjunction with o-dianisidine, an electron donor which is oxidized specifically to a highly colored dye by the peroxidase-H_2O_2 complex. During the autoxidation the concentration of H_2O_2 increases for about 1.5 hours concurrently with uptake of oxygen. It then decreases more slowly over a period of several hours. It is also likely that other closely related but more transient species, such as superoxide radical and hydroxyl radical, are produced also during the autoxidation. Hydrogen peroxide and hydroxyl radicals have well-known effects on DNA, among which is the production of DNA strand breaks (Rhaese et. al., BBA 155, 491; 1968). This effect of H_2O_2 could be the cause of the DNA strand breakage observed in the reaction of 6-OH-B(a)P with DNA in aqueous ethanol solution reported previously by our laboratory (Ts'o et. al., 1974). The toxic effects of H_2O_2 toward cells is also known and may account, at least in part, for the extremely high toxicity of 6-OH-B(a)P to cells in culture.

As for the study on metabolic activation of B(a)P, upon incubation of B(a)P in rat liver homogenates at 37°, a species with an esr signal is generated. It can be extracted into benzene, in which it is relatively stable, and is identified by its characteristic hyperfine structure as the 6-oxo-B(a)P radical. This radical is derived from 6-OH-B(a)P by one-electron oxidation. Formation of this radical is enzyme catalyzed. It is dependent on a NADPH generating system and is not observed in heated homogenates. The radical is transient, and its concentration in homogenate peaks at about 14 minutes and afterwards rapidly decays.

The amount of B(a)P metabolism preceeding through the 6-OH-B(a)P pathway is determined by comparing the rate of total B(a)P metabolism with the rate of 6-OH-B(a)P formation at 37°. The determination of the latter rate is difficult because of lability of 6-OH-B(a)P. Fortunately, 6-OH-B(a)P can be followed conveniently via the 6-oxo-B(a)P radical and corrections can be made for its lability.

Total metabolism of general-labeled [^{3}H]B(a)P in rat liver homogenate is determined by measurement of the release of [^{3}H]-water. This release follows zero-order kinetics over the first 10 minutes with rate constants per mg protein of 1.5×10^{-8} and 0.7×10^{-8} moles liter^{-1} minute^{-1} for Sprague-Dawley and ACI rat liver homogenates,

respectively. The amount of 6-OH-B(a)P produced during metabolism is determined by measuring the 6-oxo-B(a)P radical concentration in benzene extracts after all 6-OH-B(a)P has been selectively and quantitatively oxidized to the radical by shaking with an aqueous solution of 2,6-dichloroindophenol. The fate of added synthetic 6-OH-B(a)P in rat liver homogenate is also monitored by an esr technique after its conversion to the 6-oxo-B(a)P radical. This rate of disappearance of 6-OH-B(a)P is not lowered by either the lack of a NADPH generating system or heating of homogenate; therefore, the disappearance is nonenzymic in nature. It follows first order kinetics with a rate constant of 0.29 min^{-1} ($t_{1/2} \sim 3$ minutes) at 37°. After correction for the lability of 6-OH-B(a)P in homogenate, the rate constants per mg protein for 6-OH-B(a)P formation are estimated to be 2×10^{-9} and 1×10^{-9} moles $liter^{-1}$ $minute^{-1}$ for Sprague-Dawley and ACI rat liver homogenates, respectively. This represents 13% and 14% of the total B(a)P metabolism in each case.

The more rapid oxidation of 6-OH-B(a)P in rat liver homogenate ($t_{1/2} \sim 3$ minutes at 37°) also yields the three B(a)P diones as products but in a different ratio: 6,12 B(a)P dione (15%);B(a)P dione (41%); 3,6 B(a)P dione (44%). No violet-colored product has been detected in the extracts either by esr signal or chromatography. The 1,6 and 3,6 B(a)P diones are routinely reported as metabolic products of B(a)P in various metabolizing systems. More recently, the high pressure liquid chromatography technique, which has high sensitivity and resolution capabilities, has allowed tentative identification of the 6,12 B(a)P dione as well (Selkirk et. al., 1974).

No radicals were found in the liver homogenates after the addition of B(e)P, a non-carcinogenic analog.

In concluding, we believe that this data confirms that 6-OH-B(a)P is a natural product of B(a)P metabolism in rat liver homogenate. It has also been shown to be a reactive compound which is easily autoxidized by air to B(a)P diones by a mechanism involving free radical intermediates (6-oxo-benzo(a)pyrene radical) and reactive reduced oxygen intermediates (H_2O_2). The 6-oxo-B(a)P radical has been isolated and characterized. Presently we are investigating more closely the role all these species may play in the biological effects shown by 6-OH-B(a)P and B(a)P.

Study on the somatic mutation with the Syrian embryonic fibroblast system - development of a mammalian cellular system for the concomitant study of neoplastic transformation and somatic mutation.

After the development of an *in vitro* neoplastic transformation system based on the diploid Syrian embryonic fibroblast, our laboratory began to develop a somatic mutation assay with the same cell system about one year ago. The genetic marker adapter is an alteration of the enzymic activity of the hypoxanthine guanine phosphoribosyl transferase (HGPRT). The premise of this alteration is based on the analogy to the genetic disease of Lesch-Nyhan syndrome which is linked to the X-chromosome. These mutated cells are resistant to the purine analogs such as 8-azaguanine but are sensitive to a HAT medium containing hypoxanthine-aminopterium-thymidine, a medium which inhibits the normal purine synthetic pathway but supplies the cells with hypoxanthine and thymidine. We have learned a great deal about the system and are much encouraged by the preliminary results on which an abstract was submitted to and accepted by the AACR National meeting at San Diego, May 1975.

Currently, the indirect population assay is operational while the direct clonal assay is still in the developmental stage.

The protocol for the indirect population assay is as follows:

1) 1×10^6 Syrian embryonic fibroblasts of passage 2 to passage 3 (same cells used for *in vitro* neoplastic transformation) are seeded in a 75 cm^2 flask.

2) After 15 hours, the cells are treated with MNNG at a concentration of 1×10^{-6} M or 5×10^{-6} M for 2 hours, or with B(a)P at a concentration of 1 ug/ml or 10 ug/ml for 24 hours.

3) After the exposure time, the flasks treated with MNNG are washed thoroughly with phosphate buffer-saline (PBS) and the flasks treated with B(a)P are washed five times by the complete medium (containing 5% serum), the same washing procedure used in the neoplastic transformation assay.

4) The flasks are then allowed to reach confluency in 4 to 7 days.

5) The flasks at confluency are sucultured at a split ratio of 1:10 and allowed to grow to confluency. Confluency is normally obtained in 7 days with untreated cells but 5×10^{-6} M MNNG or 10 ug/ml B(a)P treated cells require 2-3 weeks to reach confluency.

6) At each passage, 10^5 cells are seeded in a 100 mm petri dish. After 15 hours, 8-azaguanine containing medium is added to the petri dish to a final concentration of 40 ug/ml.

7) The cells in the petri dish are cultured further for 3-4 weeks with intermittent changes of medium containing 40 ug/ml of 8-azaguanine.

8) At each passage, concomitantly, 1×10^4 cells from the same preparation are seeded in 100 mm petri dish. These cells are incubated for 7 days, then fixed, stained and are scored for neoplastic transformation as described in the previous sections.

The preliminary data are shown in the following Table:

Preliminary data on the somatic mutation frequency of Syrian Hamster embryonic fibroblast selected by the resistance to 8-azaguanine

Treatment	Dosage	Post Treatment Passage	Recovery Period	Mutation Frequency
Untreated	-	-	-	$<10^{-6}$
MNNG	1×10^{-6}M	1 or 2	3 days	$\sim 5 \times 10^{5}$
	5×10^{-6}M	1	5 days	$<10^{-5}$
	5×10^{-6}M	2	3 weeks	$\sim 10^{-4}$
B(a)P	10µg/ml	1	5 days	$<10^{-5}$
		2	3 weeks	$\sim 5 \times 10^{-5}$

Mutation frequency is calculated from the number of colonies which survived after 3-4 weeks of culture in 40 ug/ml of 8-azaguanine.

There are two uncertainties in the interpretation of the results inherent in the present experimental design. The first one concerns the cloning efficiency of the normal cells and the mutated cells at the seeding level of 10^5 cells per 100mm petri dish. The present calculation of mutation frequency is based on the assumption that the cloning efficiency of both types of cells at this high seeding level is the same and is 100%. It is rather unlikely that the cloning efficiency will be less then 50% for the normal cells. If the cloning efficiency is indeed only 50% and is the same for the normal cell and the mutated cell, then the mutation frequency is underestimated by about 50%. This question can be examined to a certain extent experimentally.

The second uncertainty concerns the possible competitive advantage or disadvantage which mutated cells may or may not have during the recovery period (especially when this period is long) in the complete medium without the selective agent, 8-azaguanine. This point again can be examined experimentally.

One of the alternatives to avoid this uncertainty is by the adaptation of a direct clonal assay as described below:

1) 10^5 cells of the same preparation of Syrian hamster embryonic fibroblasts are seeded in a 100mm petri dish.

2) After 15 hours, these cells in the petri dishes are treated with MNNG and B(a)P as described earlier in the indirect population assay. Parallel experiments with 2×10^4 cells/100mm petri dish are conducted to determine toxicity as measured by the decrease on cloning efficiency.

3) These cells are then washed, refed with complete medium for a recovery period of 1-3 days.

4) After the recovery period, 40 µg/ml of 8-azaguanine is added to the medium and the cells are grown in this selective medium for 3-4 weeks with refluiding before fixed, stained, and scoring for mutated colonies.

The preliminary results of this direct clonal assay procedure are very encouraging.

There are three difficulties in using normal diploid cells for a somatic mutation assay. These three difficulties are:

1) Low cloning efficiency in the culture at very low cell density. The cloning effect of less than 100 cells in the 100 mm petri dish is zero for the normal diploid cells. Therefore, the basic strategy of the selective procedure is to allow the normal cell to die slowly or to remain alive but without further dividing. These dying or undividing normal cells are then cross-feeding the mutated cells with the needed metabolites. When the mutated cells begin to form a colony, then the dependence of these cells in the colony on outside support becomes much less. Therefore, the selection procedure has to be gradual and requires a long period to work properly.

In addition, the concentration of 8-azaguanine added to the medium containing the serum has to be adjusted with different lots of serum, so that an appropriate concentration is adopted in the selection procedure.

2) Long recovery period from the insults of the mutagens and carcinogens. The normal diploid cells recover from the injury inflicted by these compounds much slower than cells from established transformed or untransformed lines. This difficulty not only prolongs the experiments but also adds uncertainty to the interpretation as we have experienced. A total recovered population from the initial injury seems to

yield a much higher frequency of surviving colonies in the selection medium. While the reason for this phenomenon is not yet certain, it is very unlikely to be due to a competitive advantage over the normal cells grown in a complete medium without the selective agent. There are at least two parameters which affect the length of the recovery time. The first parameter is the expression of the mutated phenotype which is a function of the mitotic fixation of the mutation and dilution of the normal enzyme from the cells. The second parameter is the recovery of the cloning efficiency of the treated cells which is significantly reduced after treatment with high concentrations of B(a)P and MNNG. This latter difficulty may be more pronounced with normal diploid cells than with established cell lines. The variation of the mutation frequency versus expression time has been reported and disucssed by Simons (Mutation Research 25, 219 (1974)). This variation may actually reflect the number of cell divisions rather than the length of time in culture. If the cells are too sick to divide, then the genetic damage may not be installed into the cells. Wild (Mutation Research 25, 229 (1974)) reported that even with the V79 Chinese hamster cells, reduced growth rate of cells, reduced expression rates, and low yield of mutants can be induced simultaneously by changing the serum supplement to the medium.

3) Difficulty in investigating the mutant colonies about the nature of the 8-azaguanine resistance. Since these mutated colonies are from the normal diploid cells, they are very difficult to isolate and cultivate to a large quantity for further investigation. One approach we have currently used is to let all the colonies in the same dish grow continously to confluency. We can then test these cells for their survival in the HAT medium and for the HGPRT enzyme activity assay. Isolation of these colonies individually has not been rewarding and will await a further understanding of the recovery time of the cells.

These difficulties are all due to the low cloning efficiency and low growth potential of these normal cells versus transformed cells or cell lines. We plan to repeat and compare these somatic mutation experiments with our own transformed Syrian hamster fibroblast lines which have excellent growth properties. However, we also have to suffer another uncertainty in this experiment, since the transformed cells are aneuploidy and can change their karyotype with time; currently, the effect of the change in karyotype on this somatic mutation is not well understood.

Interaction of Drugs, Mutagens, and Carcinogens with Helical Nucleic Acid in Solution at the Atomic Level as Studied by Nuclear Magnetic Resonance.

The enzyme polynucleotide phosphorylase was used to synthesize 30 mg of a hexaribonucleotide, A(1)pA(2)pG(3)pC(4)pU(5)pU(6). Under appropriate conditions, the self-complementary hexanucleotide forms a short helix in solution. The chemical shifts (δ) and $H_{1'}$-$H_{2'}$ coupling constants ($J_{1'-2'}$) of the 11 base C-H and 6 ribose $C_{1'}$-H resonances of this self-complementary oligomer have been assigned in both the helix (h) and coil (c) states in D_2O. Also the three exchangeable NH-N (N_1-H of G and N_3-H of U) resonances have been observed for the helix in H_2O. Populations of the two states were varied by controlled changes in the temperature, ionic strength, and strand concentration.

Assignments of the NH-N resonances were made by observing differential peak broadening as a function of temperature. The values correlate well with values calculated from ring current shielding effects, and the A'-RNA model determined from x-ray diffraction studies of fibers of polynucleotides. This correlation as well as other optical spectroscopic and hydrodynamic considerations confirms the existence of this oligomer in solution as a helical duplex with Watson-Crick base pairing. The linewidths of these resonances provide information about the exchange lifetime of each unique base pair; the lifetimes are 10.6, 7.2, and 4.1 msec for the G(3)·C(4), A(2)·U(5), and A(1)·U(6) pairs, respectively at 0°C, 0.1 M NaCl, 0.01 M PO_4^{3-} pH 7.6, and 10 mM strands.

The structure of this short helical RNA in solution built by the dimension specified by the x-ray diffraction study and verified by the nmr data is shown in the following figure.

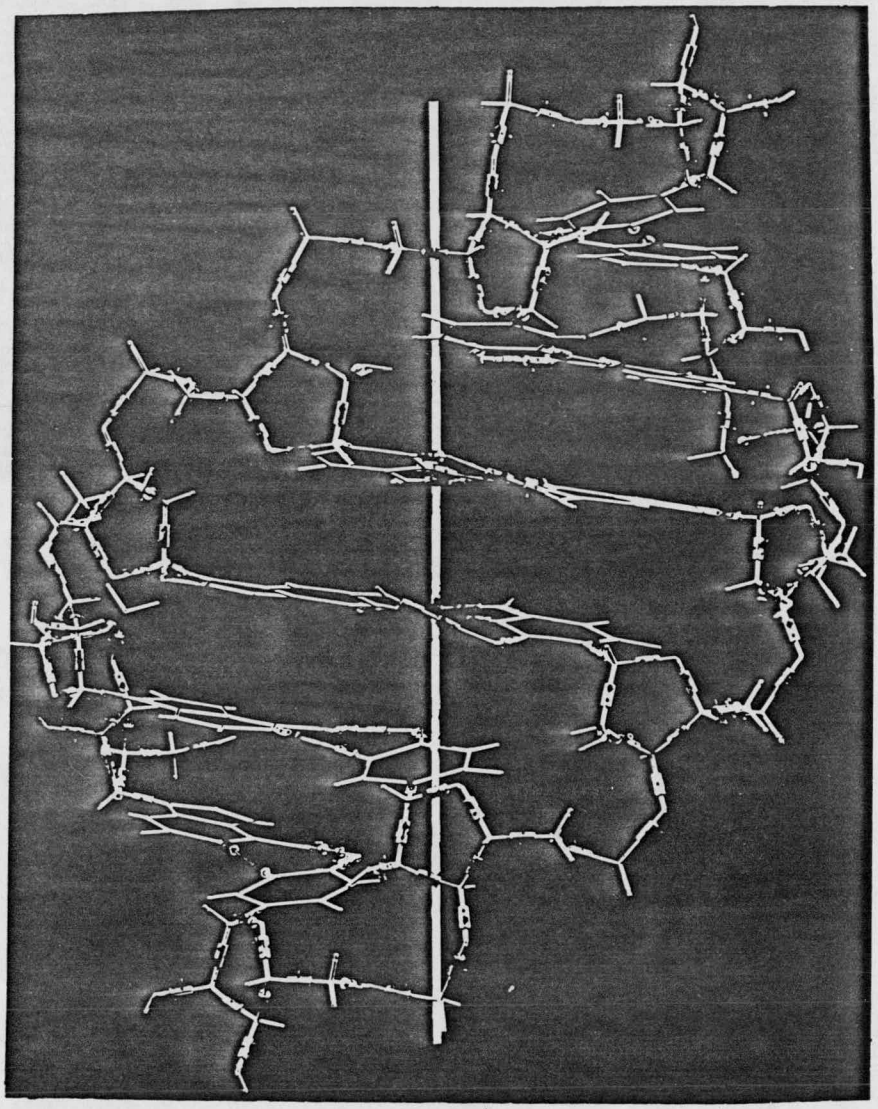
We can now adopt the short helices of nucleic acid as a model system for the investigation of the interaction of drugs (including mutagens and carcinogens) with helical nucleic acid in aqueous solution. These interactions can now be examined at the atomic level by the powerful technique of nmr.

Alterations of the geometry by intercalating agents are manifested by changes in the δ, $J_{1'-2'}$, and T_1 values. Also these values for the intercalating agent give information about the mode of intercalation. For caffeine (CF) at high ratios of h/CF (=10), the three CH_3 peaks shift upfield 0.14-0.18 ppm. The shifts of the base protons at h/CF=0.1 (downfield: 0.2 ppm for A(1)$H_{2'}$, H_8, and A(2)H_8; 0.05 ppm for A(2)H_2 and G(3)H_8; upfield: 0.05 ppm for U(6)H_3 and 0.1 ppm for U(5)H_3) suggests that caffeine intercalates between the purine bases, as well as stacked outside the helix on A(1). The data indicates that the five-membered ring of caffeine is probably stacked upon the six-membered ring of the purines. The T_1 data of h show a small general increase at h/CF=0.1. All of these results show that the h+CF$\rightleftharpoons$h·CF is in a rapid equilibrium. Additions of caffeine at high H/CF ratios have the greatest effect on the δ values of A(1)H_2, G(3)H_8, A(2)H_8, and the smallest effect on the

values of A(1)H_8. The simplest explanation of this effect is that the binding sites for interaction between the purines have higher association constants than that for the terminal-stack site of A(1). The linewidths of the NH-N signals of h at h/CF= 0.1 lead to exchange lifetimes of 8,14, and 20 msec for the A(1)·U(6), A(2)·U(5), and G(3)·C(4) pairs, respectively. The mechanism by which CF sharpens the NH-N lines is presently being investigated.

In conclusion, the pmr parameters of 20 protons in A_2GCU_2 have been extensively characterized throughout the h→c transition. The pmr parameters are correlated in a well-understood manner with structural and dynamic features of the helix and coil. Therefore, this short helix is useful as a model for interactions of RNA with various drugs and molecules. The addition of caffeine is the first study using this approach and gives much useful information about the interaction.

Currently, a deca-deoxynucleotide, D-CCAAGCTTGG, is being constructed and will be finished in 2-3 months. This decamer will form a "one-turn" DNA helix (B form) in solution. This short helix of DNA will soon be used as a three-dimensional model for the study on the interaction of drugs, mutagens, and carcinogens at the atomic level.



A view of the Kendrew model of the A_2GCU_2 duplex built in the A'-RNA geometry according to the coordinates of Arnott et. al. (1972). A view approximately along the dyad axis of the G(3)·C(4) pair which is the third base pair from the bottom. The view faces the minor (or shallow) groove of the helix with the helix axis indicated behind the base pairs.