CONF-791059--6

REPRODUCTIVE SURVIVAL AND MACROMOLECULAR SYNTHESIS IN CULTURED MAMMALIAN CELLS EXPOSED TO SINGLE METABOLITES AND MIXTURES OF METABOLITES OF BENZO(A)PYRENE*

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Running Title: In Vitro Toxic Effects - B(a)P Metabolites

*Research sponsored by the Office of Health and Environmental Research, U. S. Department of Energy under contract W-7505-eng-26 with the Union Carbide Corporation.

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ABSTRACT

Chinese hamster V79 cells have been exposed to single metabolites and mixtures of metabolites of benzo(a)pyrene [B(a)P] and reproductive cell survival and certain aspects of cellular metabolism were measured. B(a)P trans-7,8-dihydrodiol plus B(a)P 1,6-quinone resulted in increased cell survival compared to what could be expected from treatment with either metabolite alone, the mixture thus producing a neutralizing effect. If B(a)P 4,5-oxide is mixed with 3-HOB(a)P, increasing amounts of 3-HOB(a)P do not cause any additional cell toxicity compared to the toxicity due to B(a)P 4,5-oxide alone.

Replicate cultures of V79 cells were exposed to either B(a)P 4,5-oxide or 6-HOB(a)P for 4, 12 or 48 hours, and effects on cell growth and macromolecular synthesis were determined compared to control cultures. For both metabolites, there was an exposure-time-dependent delay in cell division, and the effect was most pronounced for 48 hour exposure to 6-HOB(a)P. After the division delay, cells resumed an apparently normal exponential growth rate. The percent of cells cycling and cell multiplication rate, as determined from long periods of DNA labelling with thymidine, indicated that a larger percentage of metabolite-treated cells were cycling after treatment, than was the case with control cells, even though the exponential growth rate was similar in both sets.

RNA (probably mainly ribosomal) synthesis was found to increase above control levels after removal of the metabolite, particularly in the case of 6-HOB(a)P. Protein synthesis was inhibited by 6-HOB(a)P addition, and after metabolite removal, increased to control levels concomitantly with RNA synthesis. The results suggest that metabolite treatment causes at least a temporary

cell-cycle delay. After the metabolite is removed, protein synthesis mechanisms rebound to increase protein synthesis.

INTRODUCTION

Although it is well-known that many metabolites of B(a)P[1] exhibit varying degrees of cytotoxicity when cells are exposed to them *in vitro*, (3, 6, 13, 14), the mechanisms by which these cytotoxic effects occur are largely unknown. Many studies have shown that B(a)P metabolites, particularly the diol-epoxides, can covalently bind to intracellular macromolecules, such as DNA, RNA, and protein (1,7). What effect this binding has on subsequent functioning of the macromolecules is perhaps less well-characterized. The toxic effect *in vitro* of mixtures of B(a)P metabolites has not been extensively investigated, although for human *in vivo* exposure situations, a mixture of not only metabolites of a single chemical but multiple metabolites of several chemicals, is probably the common occurrence. Because of our task of assessing human health risks from coal-combustion processes, it became important to: (1) study the composite toxicity of mixtures of metabolites compared to the toxicity of the individual components of the mixture, and (2) to investigate cellular macromolecular effects of toxic metabolites, in an effort to better understand molecular mechanisms involved in toxicity.

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[1] The abbreviations used are: B(a)P, benzo(a)pyrene; B(a)P 4,5-oxide, benzo(a)-pyrene 4,5-oxide; 3-HOB(a)P, 3-hydroxybenzo(a)pyrene; 6-HOB(a)P, 6-hydroxy-benzo(a)pyrene; B(a)P *trans* - 7,8-dihydrodiol; *trans* - 7,8-dihydroxy-7,8-dihydrobenzo(a)pyrene; B(a)P 1,6-quinone, benzo(a)pyrene, 1,6-quinone.

MATERIALS AND METHODS

Chinese hamster V79 cells, kindly supplied by Dr. E. Huberman (Biology Division, Oak Ridge National Laboratory), were grown and sub-cultured essentially as described by Huberman et al (4), although Dulbecco's modification of Eagle's minimal essential medium was used as the culture medium. One day before an experiment was started, cells were seeded into a flask at rather low density and allowed to grow overnight. These cells were then used for the experiment. Reproductive cell survival was assessed by the ability to form colonies in 60 mm Petri dishes, and the experimental procedure followed that of Huberman et al (4) except different times of exposure to the B(a)P metabolites were used. Percent cloning efficiency for metabolite-treated cells is defined as the number of colonies in a treated dish divided by the number of colonies in an acetone control dish times 100. Metabolites of B(a)P were supplied by the IIT Research Institute through the National Cancer Institute Carcinogenesis Research Program (Dr. David Longfellow). Metabolites were dissolved in acetone for addition to the culture.

Cell counts in treated and control cultures were determined using a hemocytometer, using Trypan Blue staining to assess viable cells. Labeling of DNA, RNA and protein was accomplished using appropriate radioactive precursors (thymidine, uridine, and leucine respectively) obtained from radio-chemical suppliers (New England Nuclear and Amersham/Searle). Usually 5-10 μCi/ml of ^{3}H-labeled compound or 0.05-0.10 μCi/ml of ^{14}C-labeled chemical was added to the culture medium. The usual period of labeling was for 16-18 hours immediately before the culture was terminated, except in the case of the earliest 4 hour exposure time-point, in which case the labeling was

for 4 hours. All labeling experiments were carried out using replicate 60 mm dishes (duplicate or triplicate). Macromolecules were precipitated by cold 10% trichloroacetic acid and separated and prepared for counting by standard techniques (10, 11). Amounts of DNA, RNA, and protein in cell lysates were determined by colorimetric assays (diphenylamine [2], orcinol [8] and Lowry procedures [9] respectively). Results of the labeling studies were calculated for metabolite-treated dishes as percent of labeling in control dishes, which were treated in exactly the same manner, except no metabolite was added.

RESULTS AND DISCUSSION

A. B(A)P METABOLITE MIXTURES

Initial experiments were done to study the cytotoxic effect of varying the time the cells were exposed to a single B(a)P metabolite. The results for 3-HOB(a)P are shown in Fig. 1. There is an increasing cytotoxic effect with increasing time of exposure. There is not a direct quantitative agreement between cytotoxic effect and "dose" if "dose" is defined as concentration of toxic material multiplied by time of exposure, but a general correlation is evident. Other metabolites including B(a)P trans-7,8-dihydrodiol and B(a)P 4,5 oxide, showed similar effects, i.e. increasing toxicity with increased exposure times. Although a number of investigators (4, 13) have chosen quite brief exposure times (1-3 hrs) to measure toxicity, we selected an exposure time of 48 hours for the mixture experiments, since this may be more representative of in vivo situations. One to three hour exposure intervals would be short compared with a cell cycle time of 12 hours, and significant proportions of the total cell population may not be sensitive if the lethal damage events occur in only one or two phases of the cell cycle.

The results of representative experiments in which a relatively non-toxic B(a)P metabolite [B(a)P trans-7,8-dihydrodiol] is mixed with a rather toxic metabolite [B(a)P 1,6-quinone] are shown in Figs. 2 and 3. Fig. 2 illustrates the cytotoxic effect of an equimolar mixture of the two metabolites. The mixture is not as toxic as would be expected if the toxic effects of the individual components were the result of independent events [see the dotted lines in Fig. 2: e.g. if the toxic effects were independent, then net cell survival of mixture-exposed cells at individual component concentrations of 7.5 μg/ml should be (20%) x (75%) = 15%]. In fact, the

toxic effect produced by the mixture seems to be a large reduction in cytotoxicity compared to B(a)P 1,6-quinone alone and a small increase in cytotoxicity compared to B(a)P trans-7,8-dihydrodiol alone. Fig. 3 shows the cytotoxic effect of a mixture that contains 2 moles of B(a)P trans-7,8-dihydrodiol per mole of B(a)P 1,6-quinone. Only in the case of the highest mixture-concentration does the cytotoxicity of the mixture approach that predicted from the product of the individual cytotoxicities. At other concentrations the cytotoxicity of the mixture is less than would be predicted.

Another mixture combination was tried, in which increasing amounts of 3-HOB(a)P were added to two different concentrations of B(a)P 4,5-oxide (Fig. 4). In each case, the mixture produced the same degree of cytotoxicity as the B(a)P 4,5-oxide alone, [either 65% or 45% cell survival, depending on the concentration of B(a)P 4,5-oxide] no matter what concentration of 3-HOB(a)P was added to the mixture [up to 3 μg/ml of 3-HOB(a)P in the mixture]. These results seem to suggest that B(a)P 4,5-oxide is able to block effectively the toxic activity of 3-HOB(a)P, possibly by acting at intracellular sites that are the sensitive sites for 3-HOB(a)P, although we have no experimental proof for this conjecture.

The results with B(a)P trans-7,8-dihydrodiol and B(a)P 1,6-quinone mixtures suggest a neutralizing effect of the less cytotoxic component of the mixture ameliorating the toxicity of the more cytotoxic component. A protective effect of benzo(e)pyrene on 7,12-dimethylbenz(a)anthracene skin tumor-initiation in mice has been noted by Slaga et al (12) and these workers suggested this effect could be due to modification of metabolism of the potent carcinogen by the very weak carcinogen, benzo(e)pyrene. In the case of our

in vitro studies, these cells are not capable of further metabolizing the metabolites administered (5), so that the effects seen must occur at subcellular sites directly involved in cytotoxic mechanisms. The nature of these sites is not known to us.

B. MACROMOLECULAR EFFECTS OF TOXIC METABOLITES

Two different metabolites, B(a)P 4,5-oxide and 6-HOB(a)P were used in these studies. These metabolites differ considerably in their toxicity, with B(a)P 4,5-oxide being much more cytotoxic and also much more mutagenic (6). V79 cells were seeded at a rather low density (8×10^4) in 60 mm dishes, and the cells were exposed to the 2 aforementioned metabolites for varying periods of time. The cell number and uptake of radioactive precursors into certain macromolecules was determined over sequential intervals during and after the period in which the metabolite was in the culture medium.

The effect of varying times of exposure to 6-HOB(a)P on subsequent cell growth is shown in Fig. 5. The effect of B(a)P 4,5-oxide is very similar to 6-HOB(a)P except in the case of 48 hour exposure, in which case the subsequent cell growth very closely parallels the growth of cells exposed for 24 hours to 6-HOB(a)P. The fact that the growth curves for metabolite-treated cells, after an initial delay whose duration depends upon length of exposure, all become more or less parallel to the growth curve for untreated cells, suggests that exponential phase growth rates for metabolite-treated cells are not grossly different from control values. One possible explanation for the shape of the cell-growth curves is that the B(a)P metabolite induces a delay in the cell cycle and that after the metabolite is removed, the cells eventually recover the normal cycle kinetics. The difference in hours between the exponential growth phases of control and treated cultures might provide an

estimate of such a delay. For B(a)P 4,5-oxide, tentative delays of 6, 12, and 18 hours can be estimated for exposure times of 4, 12 and 48 hours, respectively. For 6-HOB(a)P, delays of 4, 6, 18, and 36 hours can be estimated for exposure times of 4, 12, 24 and 48 hours.

Another effect must also be taken into account in interpreting these growth curves. Treatment with metabolite results in some cytotoxicity, the degree increasing with increasing exposure times. Reproductive cell survival measurements were made as a part of this experiment and the percent cell survival associated with varying exposure times is given in Fig. 5 (Corresponding cell survival values for 4, 12 and 48 hour exposure to B(a)P 4,5-oxide were 73%, 58%, and 28% respectively). If the time required for the metabolite to kill and lyze cells is short relative to the doubling time of the cells (approximately 12 hours) then the viable cells remaining might be in exponential growth a short time after (or even during) the cytotoxic period. The apparent cell growth plateau from the start of the metabolite addition to the next cell count might not be a genuine plateau but could consist of a rapid decrease in cell number, followed by exponential growth of the surviving cells. We have not determined such intermediate cell counts on the longer exposure times, and so cannot confirm or disprove this interpretation. In any case, this latter explanation cannot fully account for the results with respect to the 6-HOB(a)P-treated growth curves as the cell growth plateau at the 48 hour exposure is so long in duration that some delay in cell cycle time must be invoked to explain the data.

In order to further investigate the cell-cycle kinetics of the metabolite-treated cells, and to study the effects of metabolite treatment on certain macromolecular synthesis processes, radioactive precursors for DNA,

RNA, and protein were used. The results of these experiments for DNA are shown in Fig. 6. The long labeling times were chosen to give incorporation of label that is proportional to the percent cells cycling and the cell multiplication rate and will not provide information about the rate of DNA synthesis. It can be seen from Fig. 6 that several different effects are produced, depending on both length of exposure and the metabolite used. B(a)P 4,5-oxide shows no initial effect (4 hour exposure), an increased uptake compared to controls (12 hour exposure) and an inhibition (48 hour exposure). The results for the shorter exposure times suggest that metabolite treatment causes an increase in percent of cells cycling at some time after the removal of the metabolite (see data points at 72 hours). Since both 4 and 12 hour-exposed cultures are in apparently normal exponential growth at this time, the increase in DNA labeling compared to controls is difficult to explain otherwise. Forty-eight hour exposures, on the other hand, result in an inhibition of DNA labeling initially (see 72 hour data point), followed by an increase to well above control levels. These data are consistent with a slowing or delay of the cell cycle during the long metabolite exposure.

The data for 6-HOB(a)P (Fig. 6) show that this metabolite has different effects on the cell-cycle kinetics than does B(a)P 4,5-oxide. The most dramatic difference is in the uptake of precursor into DNA by the 4 hour-exposed cultures. In the case of 6-HOB(a)P, there is an initial inhibition, followed by a greatly increased uptake. These results again suggest a delay or slowing of the cell cycle, followed by a rebound which causes an increase in percent cells cycling compared to controls.

Surprisingly, 48 hour-exposed cultures show increases of uptake into DNA at a time (see 72 hour data point) when the cell count seems to be

static, as compared to the control cultures, which are in exponential growth. Such an effect might occur if the metabolite induced a mitotic block. It may be that some of the uptake into DNA which we have measured is occurring as a result of DNA repair, but it is difficult to distinguish this repair incorporation from incorporation into newly-synthesized DNA in growing cultures.

The results of labeling RNA are shown in Fig. 7. The long labeling time used will most likely result in the bulk of the label being incorporated into ribosomal RNA, rather than the rapidly synthesized and degraded messenger RNA. Of course, transfer RNA will also be labeled, but this is only 10-12% of the quantity of ribosomal RNA. Rather dramatic differences are evident when the results from the two metabolites are compared (see the 4 hour and 48 hour exposure curves, particularly). The increases in incorporation into RNA, compared to control cultures, suggest increases in synthesis of ribosomal RNA, and the most logical reason for this response to be elicited is in preparation for increased protein synthesis.

Indeed, measurements of protein synthesis (Table 1) indicated that protein synthesis was increasing during the same time intervals as increased RNA synthesis was seen. For both 12 and 48 hour 6-HOB(a)P exposures, protein synthesis was initially inhibited following metabolite removal and gradually increased toward control levels as the cells continued to grow. The observed inhibition of protein synthesis could possibly be due to slowed protein synthesis associated with the slowing of the cell cycle, or it could be a consequence of fewer cells in the treated cultures (although for the 12 hour exposure, the cell killing is only 25%), or multiple factors may be involved.

These studies have indicated that the effects of B(a)P metabolite addition to cells in culture are complex, affecting many intracellular processes in different ways. Interpretation of how complex mixtures of chemicals react with cells will remain obscure until the most significant intracellular sites and processes for chemical interaction are identified.

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Table 1

Protein, RNA and DNA Synthesis in V79 Cells Exposed to 6-HOB(a)P for 12 or 48 Hours

	% Precursor Incorporation Compared to Control Cultures[1]					
	12 hour Exposure			48 hour Exposure		
	A[2]	B	C	A[2]	B	C
Protein	60	70	80	50	55	100
RNA	140	170	210	190	150	315
DNA	150	200	130	150	230	235

1. Data derived from duplicate dishes. The coefficient of variation was 5-15%.

2. A, B and C refer to time intervals after removal of 6-HOB(a)P from the culture medium of 12, 36, and 60 hours and 0, 24, and 48 hours for the 12 hour exposed and 48 hour exposed cultures, respectively.

FIGURE LEGENDS

Fig. 1. The Effect of Increasing Exposure Time on the Cytotoxicity of B(a)P Metabolites. Reproduction Survival is defined in Materials and Methods. Error bars indicate one coefficient of variation.

Fig. 2. Cellular Cytotoxicity Caused by a Mixture of B(a)P Metabolites. Dotted lines show the cytotoxicity of the individual components of the mixture. Symbols (•, Δ, ■) indicate the overall concentration of the combined metabolites in the mixture, and the concentration of each individual metabolite that went into making up the sum total: e.g. for the ■ symbol, the cell survival produced by 15 μg/ml of the mixture was 45%, while the cell survival caused by 7.5 μg/ml of each of the two components contributing to the mixture was 73% and 20%, respectively.

Fig. 3. Cellular Cytotoxicity Caused by a Mixture of B(a)P Metabolites. See Legend for Fig. 2.

Fig. 4. Cellular Cytotoxicity Caused by a Mixture of B(a)P Metabolites. Cells were exposed to either 0.6 or 0.8 μg/ml of B(a)P 4,5-oxide (associated cell survivals were 60 or 45%, respectively) and increasing concentrations (1, 2 or 3 μg/ml) of 3-HOB(a)P. Dotted lines indicate the cytotoxic effect that might be anticipated if these increasing amounts of 3-HOB(a)P were added to cells which had already been subjected to the level of cytotoxicity produced by the two concentrations of B(a)P 4,5-oxide.

Fig. 5. Cell Growth Following Treatment with a B(a)P Metabolite. Duplicate plates were exposed for the times indicated. Control plates received acetone. All cell counts indicate viable cells as counted by Trypan Blue Staining. Reproductive cell survivals associated with exposure times of 4, 12, 24, and

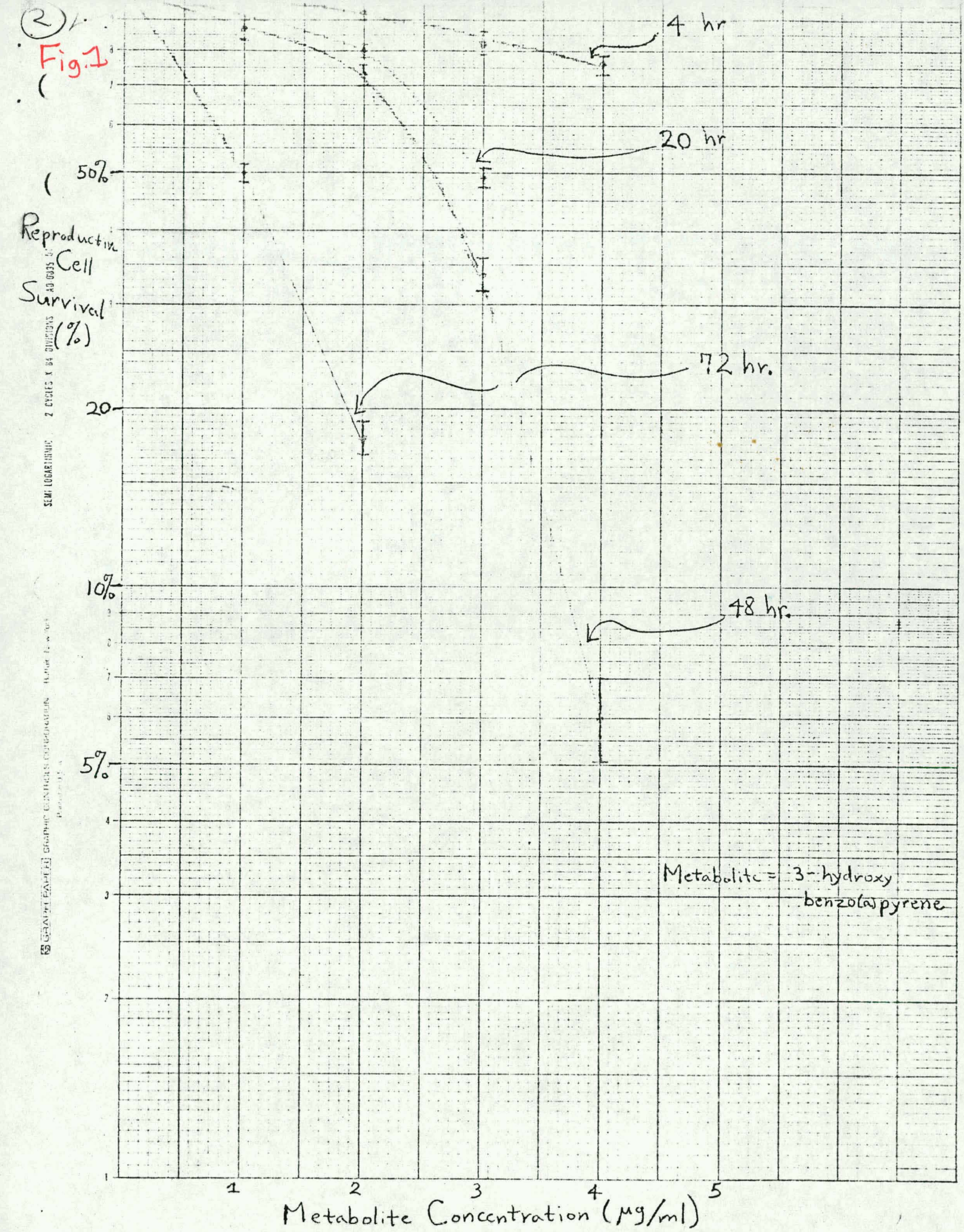
48 hours were 87%, 75%, 51%, and 31% respectively.

Fig. 6. Incorporation of ^{14}C-Thymidine into DNA of Cells Exposed for Varying Times to B(a)P Metabolites. The labeling time, in all cases, was for 16-18 hours except in the case of the first time point of 4 hour exposure, in which case the labeling time was 4 hours. All data points were calculated as cpm per μg of DNA, and normalized by dividing by the corresponding incorporation in control cultures. The * indicates the point at which metabolite-treated and control cultures were separated to start the experiment.

Fig. 7. Incorporation of ^{3}H-Uridine into RNA of Cells Exposed for Varying Times to B(a)P Metabolites. See Legend to Fig. 6 for details. All data points were calculated as cpm per μg of RNA, and normalized by dividing by the corresponding incorporation in control cultures.

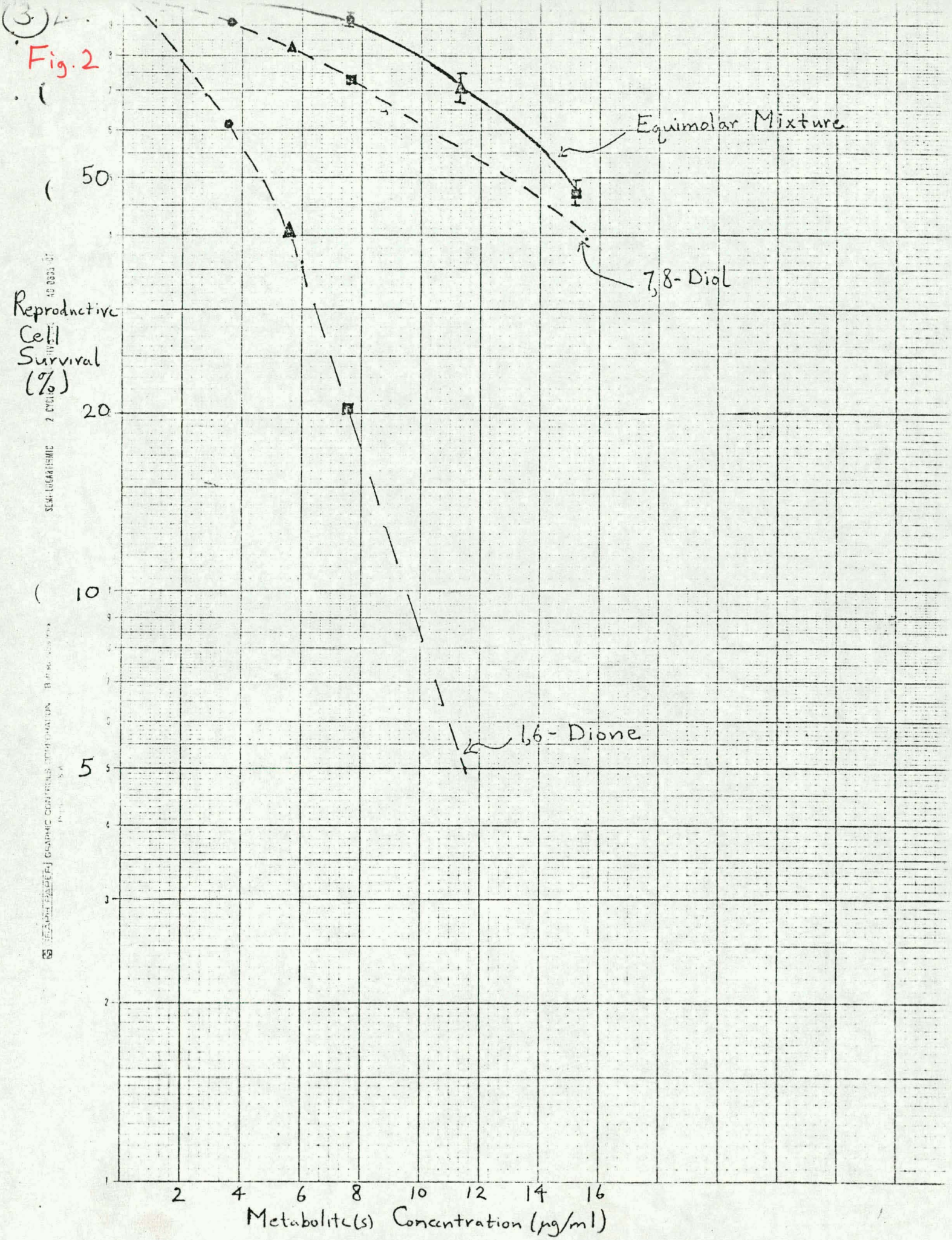
(2)

Fig. 1


4 hr
20 hr
72 hr.
48 hr.
50%
20
10%
5%
Reproductive Cell Survival (%)
Metabolite = 3-hydroxy benzo(a)pyrene
1
2
3
4
5
Metabolite Concentration (µg/ml)

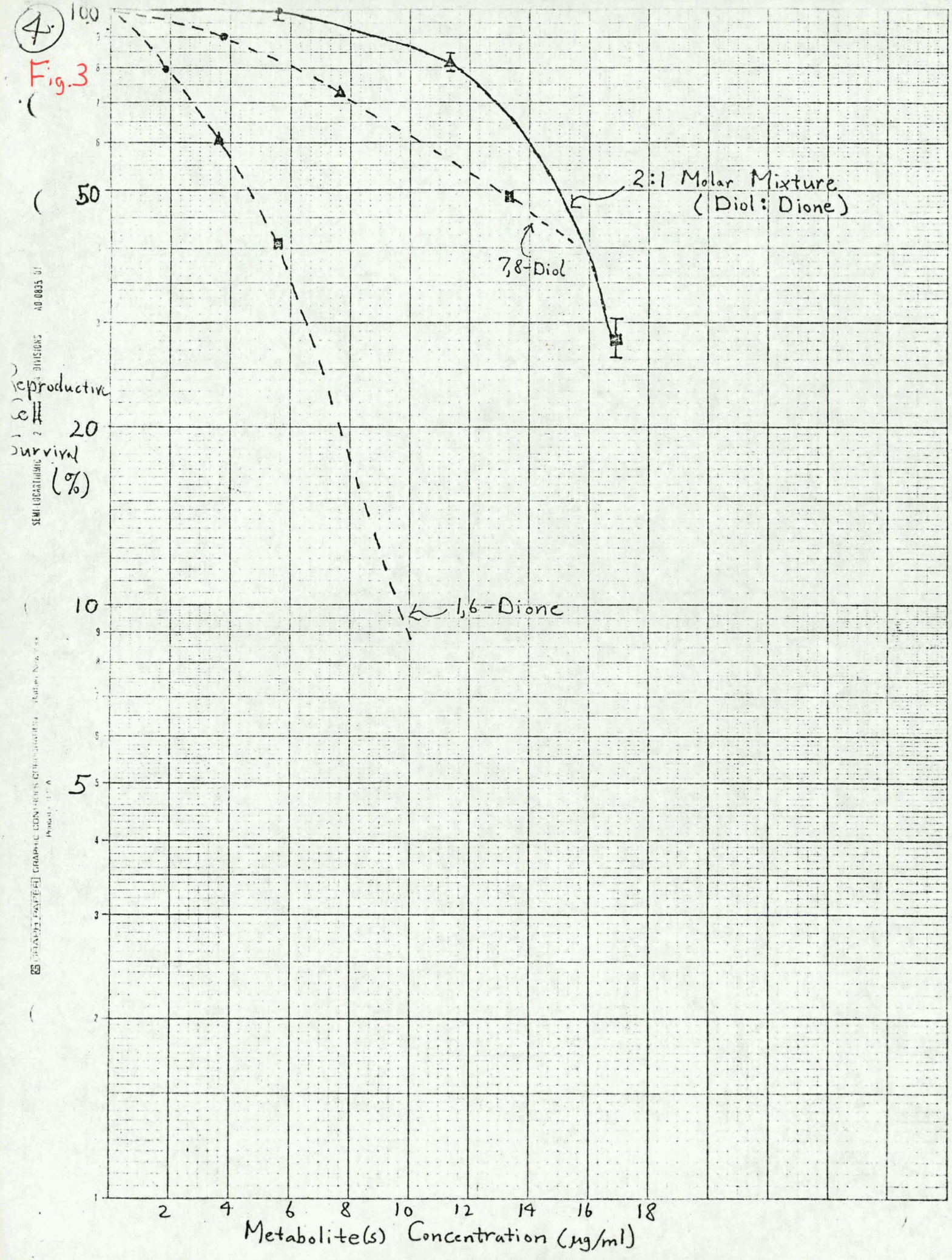
(3)

Fig. 2


Equimolar Mixture
7,8-Diol
1,6-Dione
Reproductive Cell Survival (%)
50
20
10
5
2
4
6
8
10
12
14
16
Metabolite(s) Concentration (μg/ml)

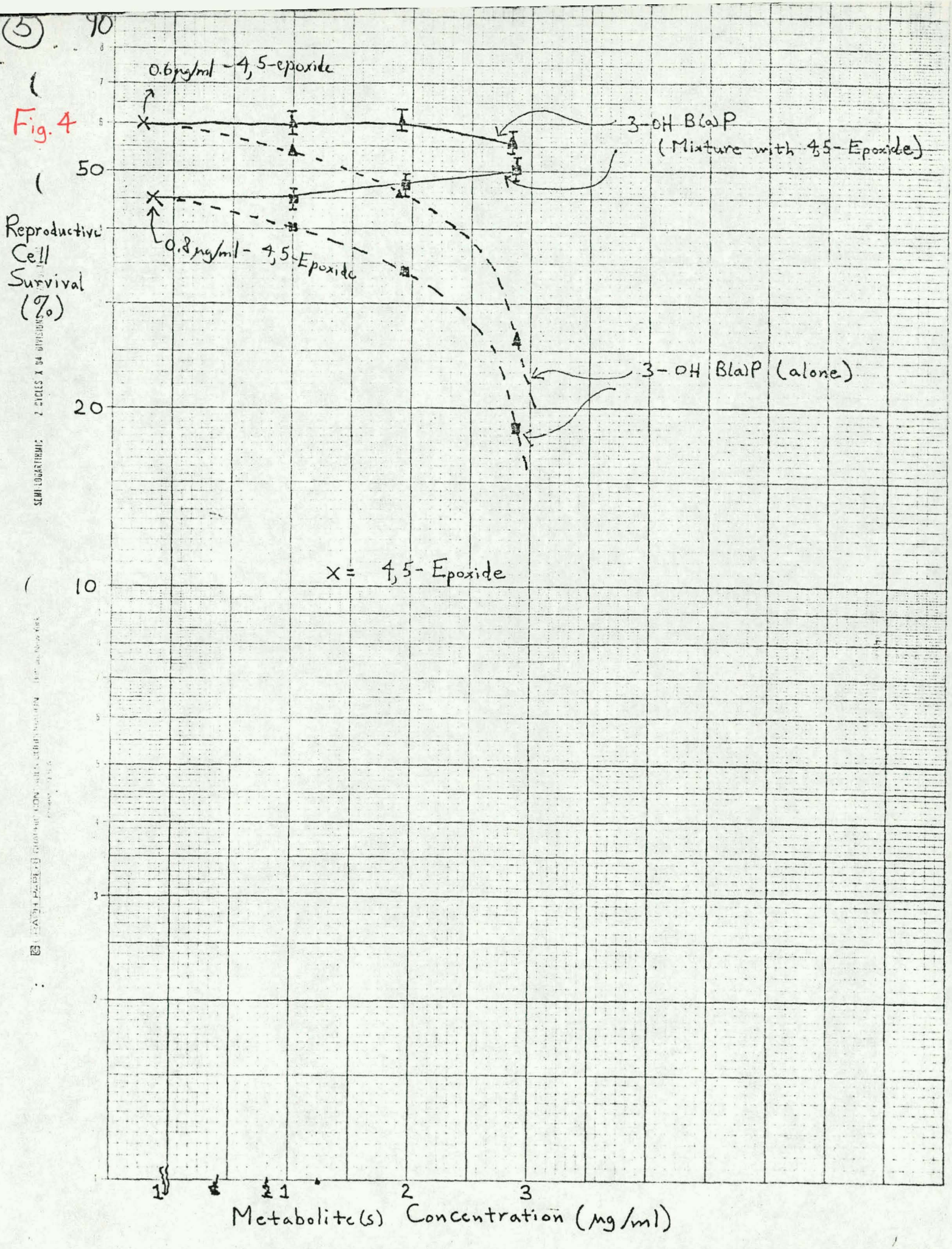
4.

Fig. 3


100
50
20
10
5
Reproductive cell survival (%)
2:1 Molar Mixture (Diol : Dione)
7,8-Diol
1,6-Dione
2
4
6
8
10
12
14
16
18
Metabolite(s) Concentration (μg/ml)

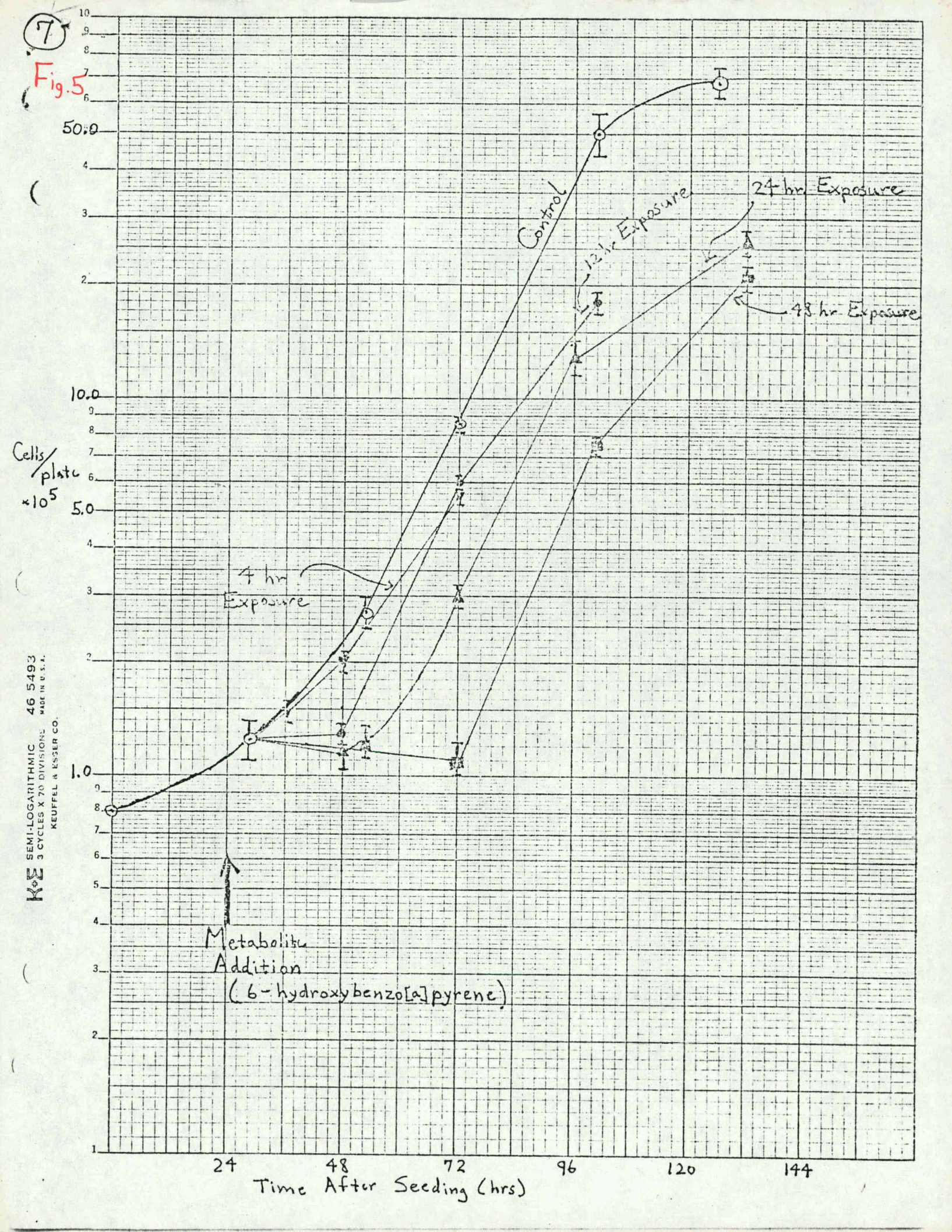
(5)

Fig. 4


0.6 μg/ml - 4,5-epoxide
3-OH B(a)P
(Mixture with 4,5-Epoxide)
0.8 μg/ml - 4,5-Epoxide
3-OH B(a)P (alone)
X = 4,5-Epoxide
Reproductive Cell Survival (%)
90
50
20
10
1
2
3
Metabolite(s) Concentration (μg/ml)

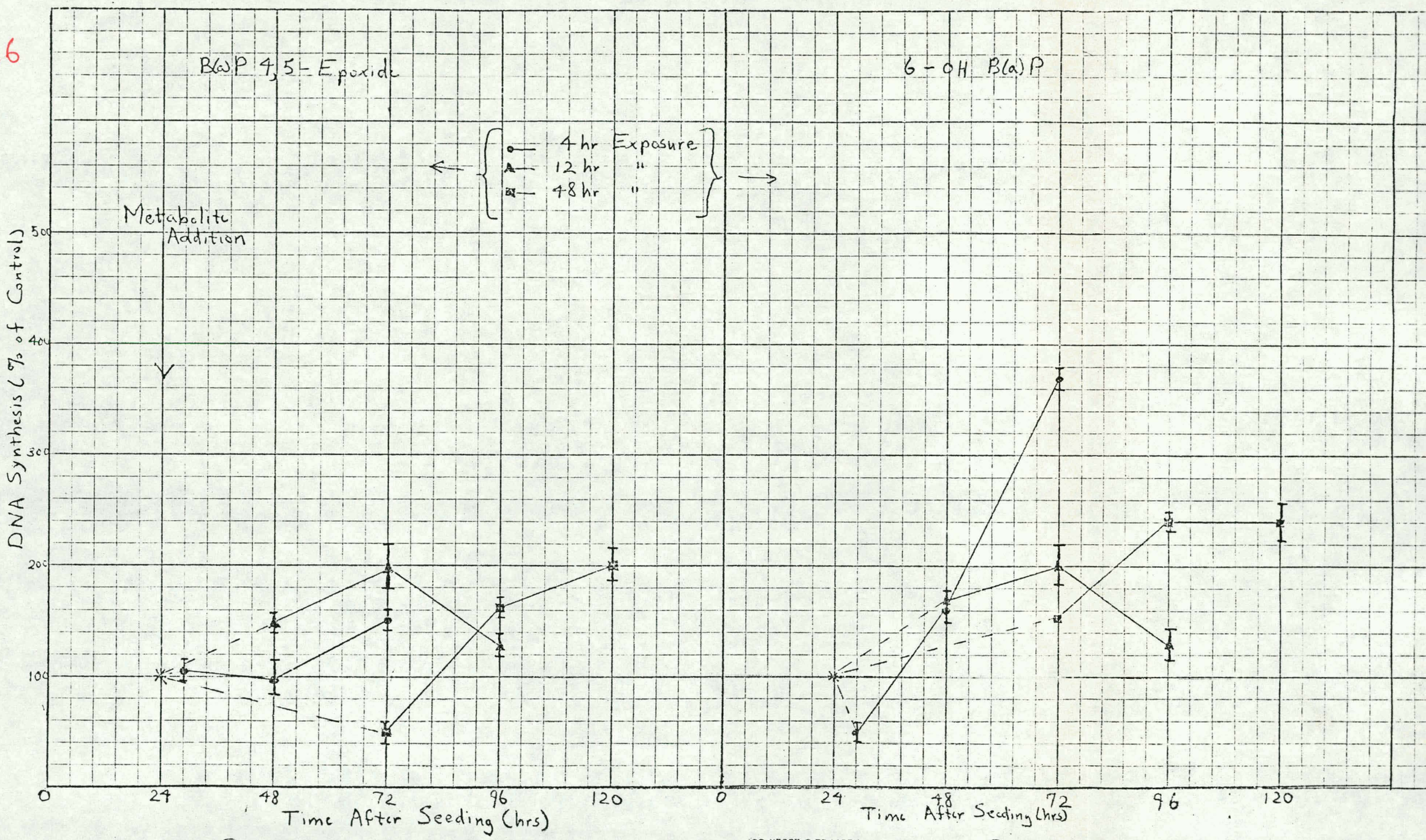
(7)

Fig. 5


Control
12 hr Exposure
24 hr Exposure
48 hr Exposure
4 hr Exposure
Metabolite Addition (6-hydroxybenzo[a]pyrene)
Cells/plate ×10^5
Time After Seeding (hrs)
24
48
72
96
120
144
50.0
10.0
5.0
1.0
SEMI-LOGARITHMIC 46 5493
3 CYCLES X 70 DIVISIONS MADE IN U.S.A.
KEUFFEL & ESSER CO.

(6)

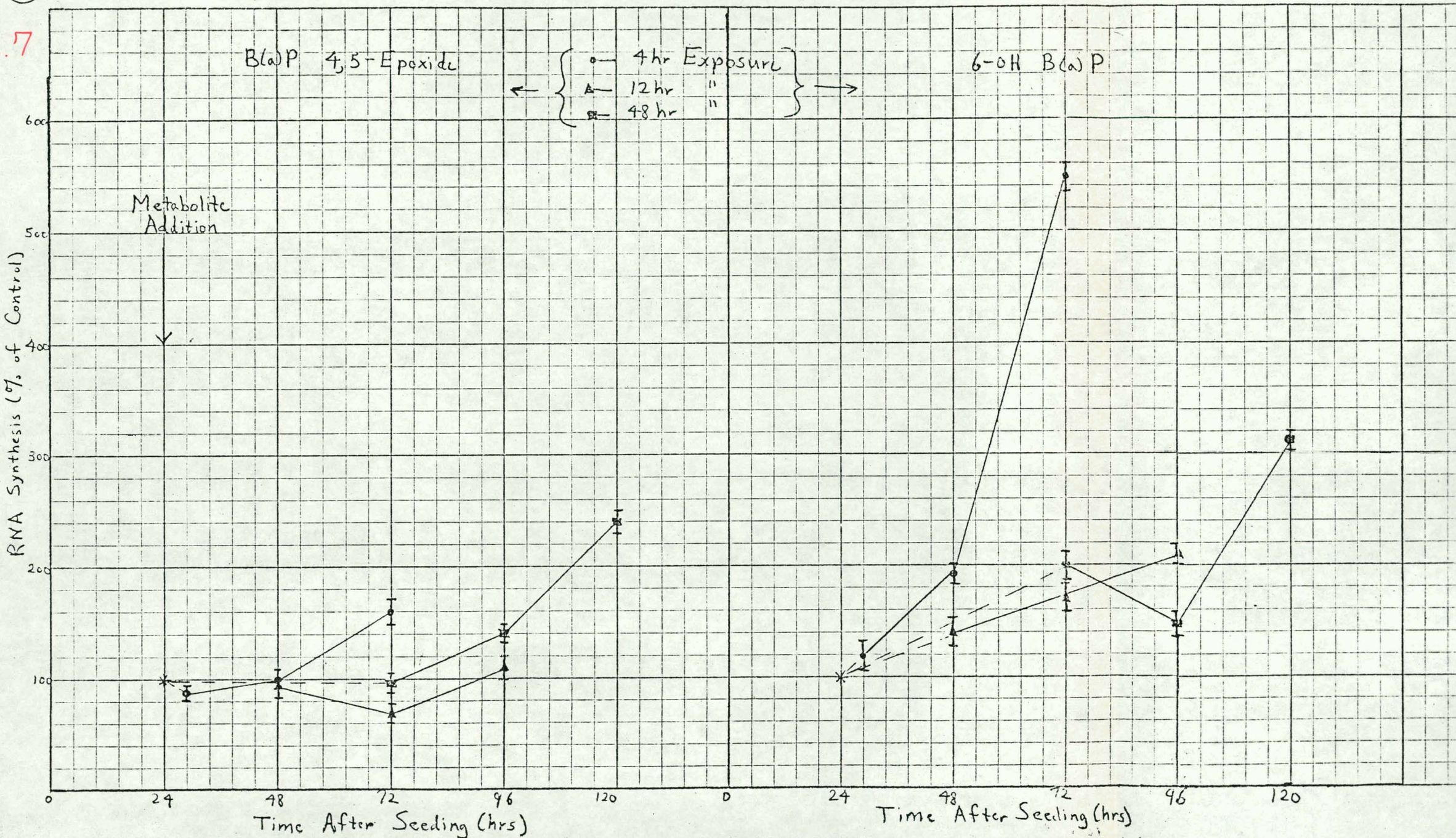
Fig. 6



KEUFFEL & ESSER CO.
MADE IN U.S.A.
5 X 5 TO THE INCH 46 0413
7 X 10 INCHES

(4)

Fig. 7



KEUFFEL & ESSER CO.
MADE IN U.S.A.
7 X 10 INCHES
5 X 5 TO THE INCH 46 0413