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MASTER

RADIATION BIOPHYSICAL STUDY OF BIOLOGICAL MOLECULES

Progress Report

Donald J. Fluke

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PROGRESS REPORT, CONTRACT NO. E-(40-1)-3631

Summary of Progress (Seventh contract period)

The work of this contract period involves nine sections, items published, and items publishable.

1) The work presented in preprint last year, on host strain differences in survival of irradiated T_1 bacteriophage, is shortly to appear in Radiation Research (August issue). The paper was extensively re-written and expanded in response to referee suggestions, and is represented here in revised form, from galley proof. One referee suggested that an oxygen effect rather than a water effect might be involved in the fast electron (dry phage) and X-ray (wet phage) work. There is an interesting difference in the extent of excision repair depending on oxygen for wet irradiation, which clearly is relevant to this work. There is as yet no adequate basis for deciding between a potential excitational component of ionizing radiation damage as the subject of excision repair, or a component involving chemical reaction with oxygen during irradiation, wet or dry.

2) The second section is one of several involved in our continuing study of error-prone (induced) repair. A number of manifestations of the possible single repair process operating at this level can be tested for coordinate expression. The paper by Pollard, Person, Rader, and Fluke (accepted for Radiation Research, included here in preprint form) shows that an inducible process can be unfolded from the basic fluence-squared dependence of UV mutagenesis, and that this induction process is reasonably indistinguishable from those for induced radiation resistance and for induced inhibition of post-irradiation DNA degradation. Some additional evidence of an inducible process within UV mutagenesis involves split-dose experiments with intervening incubation, X-ray induction of UV mutagenesis enhancement, and wavelength-shift, split-dose work between 265-nm induction and 313-nm development. An abstract from the San Juan Photobiology meeting presents a more complete and elaborate UV mutagenesis curve, based on chiefly my work this past winter, and bringing the test of all three inducible phenomena into the same experimental apparatus and dosimetry. The abstract is amplified by inclusion of figures and comment.

3) A preprint by Pollard and Fluke, submitted for publication in the Biophysical Journal, is included, together with a published abstract of the same work reported at the February 1977 meeting of the Biophysical Society. This work shows that inducible repair, demonstrated as UV-induced resistance toward subsequent X-ray damage, is not expressed in strains carrying a λ-lysogen. For non-inducible lysogens the extent of induced radiation resistance was that normal for the non-lysogens.

4) In further work with induction of phage λ it has become evident that the induction curve for this now-classical process cannot be superposed on those involved in UV mutagenesis and the other two coordinate expressions of induced repair. There are differences or intervening steps between induced repair and lysogen induction, if the latter is as believed related to the former. The current state of this work is represented by an abstract (SUAM-C2) from the San Juan meeting of the American Photobiology Society in May.

5) A fifth unit of work is included by abstract Ei-5 from the San Juan meeting of the Radiation Research Society, also in May. The abstract is supplemented by figures and comment on them. The chief emphasis of this work is at 313 nm. It is shown that UV mutagenesis at 313 nm is qualitatively different from that at 265-nm, in addition to a 15,000-fold sensitivity difference. Pre-irradiation at 265 nm, or by X-rays, easily induces additional UV mutagenesis by a subsequent 313-nm exposure. The effect holds out toward high revertant levels, in some contrast from induced enhancement of 265-nm (or 254-nm) mutagenesis. Pre-irradiation at 313-nm induces only marginal enhancement of mutagenesis from 265-nm damage, and the induction saturates much more markedly than for X-rays or for UV. Damage at 313-nm is readily antecedent to revertant mutation, but a poor explicit inducer. It is possible that the strong fluence-squared dependence of 313-nm mutagenesis is not as closely associated with induced repair as that seen at 265 nm.

6) A short study, not yet prepared for publication or publically reported, involves dilution effects in scoring leucine revertants in UV mutagenesis. This separate report section includes several figures. The rising and falling sections of the overall mutagenesis-fluence curve behave differently with respect to number of cells plated, and hence the number and size of the microcolonies within which revertants can arise. Unirradiated cells can substitute reasonably well for irradiated cells in establishing the competition for limiting leucine on the plates. In addition, background revertant expression has been extensively examined as a function of number of cells plated. The background revertant level is neither invariant with nor directly variant with cell dilution as a mimic of cell survival. This result will allow empirical correction of the upper ends of our mutagenesis-fluence data. Net UV revertants over background diluted less than linearly, as approximately the 0.85 power of the fluence.

7) A major effort this part summer has gone into a definitive action spectrum for UV mutagenesis, scoring leucine revertants in an E. coli uvr$^-$, leu$^-$, tyr$^-$ B/r. These very extensive data will require some time to analyze and present in definitive form, but a short preliminary write-up is included. The action spectrum is generally consistent with nucleic acid as chromophore, as might be expected, but the upper ends of the curves give some wavelength-dependent variety of behavior. There may be relative differences in inducing and primary damage efficiencies for revertant expression at various wavelengths. The work includes equally extensive data on colonial survival after UV damage, with extrapolation numbers and possible UV stimulation of cell division. Eleven wavelengths are included in this study, running from 234 nm to 313 nm. At 265-nm the effect of 10-fold difference in intensity was also studied, and found to be essentially nil.

8) A section on W-reactivation (the reactivation of damaged phage by UV to the host cells, discovered by Weigle) is also included as a preliminary write-up of much work this summer. Much effort went into technical progress in defining the phenomenon. We did achieve far more complete adsorption of phage than in last year's report. The induction curve by 265-nm irradiation of the host cells appears reasonably superposable with those for the three other induced repair manifestations (UV mutagenesis, radiation resistance, inhibition of post-irradiation DNA degradation). There are some still puzzling differences based on bacterial strain and on UV source for

inactivation of the phage. The possible effect of a post-UV incubation of the cells in growth medium before phage attachment (in attachment medium) is still equivocal. We cannot directly demonstrate as yet a metabolic dependence for the inducibility of W-reactivation.

We have one good preliminary run-through on the action spectrum for the induction, appearing probably consistent with nucleic acid as chromophore.

Perhaps our most interesting finding, dimly indicated in last year's report, is that ionizing radiation damage to phage λ is partly repairable by the W-reactivation system. The effect is seen both for heavily protected (5x nutrient broth) and more dilute X-ray irradiation media for the phage. This work continues our interest (under item 1, above) in overlap between ionizing and exciting radiations at the level of repair.

9) The direct action target work (with Dr. K. B. Storey) on phosphofructokinase, reported last year, has now advanced to the manuscript stage. Storey and I, as authors, still have revisions to work out between us before deciding whether and where to publish.

Experiments on Weigle-Reactivation of Irradiated Phage λ (Mr. Stephen Douglass assisted in this work.)

This past summer's work has developed some technical confidence in the W-reactivation (Weigle phenomenon: repair of phage damage by UV irradiation of the host cells) system in our hands. We can add a number of results, extensions, and confirmations to our report of last year.

1) At the strictly technical level we have reconfirmed the necessity for maltose as the major carbon source in growing host cells, and the necessity of shifting to a non-growth medium for phage attachment to form complexes. It is alright to UV the cells in a casamino acid C-minimal salts maltose growth medium (sufficiently UV transparent), but they must be transferred before phage attachment. We use 0.01 M $MgSO_4$ in 0.01M Tris·HCl, pH7.3. Complexes formed in growth medium progressively lose plaque survival, at a rate too high for careful work. We have demonstrated that this instability does not vary notably with holding temperature for the complexes or with plating agar temperature.

2) Also at the technical level we now routinely find 90-95% adsorption of phage to our UV'd cells in fifteen minutes exposure to phage. Adsorption of irradiated phage is slower than for unirradiated, but still reaches that desirable level. Further, we have a good routine for preparing UV-λ phage, involving two calibrated irradiations through the bottom of a Vycor flask, with a two-hour incubation in between. E. coli AB1157λ cells are grown to about 4×10^8/ml in casamino acids glucose, and 10-ml portions are transferred to 250-ml Vycor flasks. Induction is by 10-second irradiation over a UVS-11 low-pressure mercury arc, at about $8 Wm^{-2}$. Two hours incubation at 37°C yields clear inductates titering $2\text{-}4 \times 10^{10}$ plaques/ml on E. coli Q_1 as host. A second UV exposure in the same flask is for eighty seconds over an R-51 mineralight (40 to 45 Wm^{-2}), giving inactivation factors of $2\text{-}5 \times 10^5$.

3) We found good inductions (10 to 20-fold or better) for UV on E. coli AB1157 or Q_1. Induction in a uvr^- strain (AB1886) was not evident, and only marginally so in another uvr^- strain (AB1886). Prior work (e.g., Keneser et al., Virology 27, 213-221, 1965) has indicated W-reactivation in uvr^- strains, and the difference is not yet evident.

4) Our induction curves for 265-nm irradiation of the cells fit reasonably well with those for induction of radiation resistance, induction of induced inhibition of post-irradiation DNA degradation, and the induction inferred in UV mutagenesis. Again, the induction curves are normalized to the same maximum value, and the half-maximal fluences are compared.

5) We have some data bearing on the action spectrum of W-reactivation, for the UV to the cells. One culminating run comparing nine wavelengths is reasonably consistent with a nucleic acid chromophore. The comparison has not yet been more than superficially analyzed, however.

6) There may be an unusual wavelength dependence for the UV inactivating the phage. We consistently got more induction for the R-51 Mineralight on the phage rather than the UVS-11, after adjusting exposure times to give the same plaque survival. There could be an intensity effect, but wavelength differences between the light sources could also be involved.

7) We repeatedly looked for an effect of incubation of cells in growth medium after UV induction. ; As noted above, it made no evident difference whether UV was given in growth medium or attachment medium (after correcting fluences for depth distribution), provided attachment of phage was in the proper medium. An effect of incubation could indicate protein synthesis or other metabolic involvement within W-reactivation, strengthening the case for it as another manifestation of induced (error-prone) repair. Our results were equivocal. It was indicated in some experiments, counter-indicated in none, but equivocal in our later and most careful tests. Failure to demonstrate does not contra-indicate induced repair, of course, since incubation on the plates may always be sufficient to saturate such dependence anyway.

8) Our most interesting finding, very weakly suggested in last year's report, is that X-ray damage to phage λ is indeed repairable in the context of W-reactivation. We have it now both for X-ray irradiation in a heavily-protected medium (5x-concentrated nutrient broth) and for a more dilute medium (casamino acids C-minimal salts glucose medium diluted 1/30 in the 0.01M $MgSO_4$ in 0.01 M Tris·HCl attachment medium). Both irradiated solutions were aerated, by magnetic stirring and by micro-air bubbling respectively. The doses to get to the 10^{-4} level of inactivation were 900 and 190 krads, respectively. The reactivations noted were 1.4 to 2-fold, for both kinds of irradiated phage, for UV exposures of 2 to 10 Jm^{-2} to the cells.

9) There is a striking difference between the UV-phage and the X-rayed phage, simply upon forming complexes with unirradiated host cells in attachment medium. We always lost plaques for the UV phage, from 2 to 3 or 4-fold. We gained plaques by 2-fold or so for X-ray'd phage. The comparison here is for phage attachment in the plating agar, and does not depend on AB1157 <u>vs</u> Q_1 host cell difference.

The Wavelength Dependence of UV Mutagenesis

The major individual research effort of D.J. Fluke during this contract period has involved ultraviolet-induced reversion to leucine independence in a *uvr*$^-$, *leu*$^-$, *tyr*$^-$ strain of *E. coli* B/r. Two helpers have contributed very careful support assistance in these studies. Ms. Juliana Mulroy, a fourth-year Botany Ph.D. candidate, was available part-time from October until March. (Her employment was by federal work-study funding, with the 20% match from a small internal grant at Duke, since this contract was without new funding until later.) She helped with the 313-nm work and its interactions with 265-nm and X-ray irradiations, reported above, and also with the effect of plating different amounts of cell material.

For ten weeks in the summer of 1977, Mr. John Douglass was employed full-time for continuation of this work. He had been involved in some of the earlier UV mutagenesis reported from this project, and was again available after his first college year. Except for the dilution work which extended the studies of varied plating amounts, his efforts this summer were entirely directed toward an action spectrum for UV mutagenesis.

We studied ten wavelengths with the double monochromator, in addition to the standing work at 313 nm. Irradiations at 265 nm were repeated for internal consistency of all the work. One extensive run at 265 nm tested for any effect of a ten-fold intensity difference, but none was evident. At all the wavelengths an effort was made to develop the whole mutagenesis curve, from lowest fluences where revertant score is appreciable over background levels, to the highest fluences at which any revertants could still be scored. Much of our attention in the latter weeks was directed toward the upper fluence range, trying to space points closely and avoid overrunning the upper-fluence limit for scoring revertants. We also routinely tested all irradiations both for colonial survival as well as for revertants, so that much survival data has accumulated, and the survival denominators did not have to be interpolated.

A chief objective of this work was to see whether UV induction curves can be developed at other wavelengths, as at 265 nm, by dividing R/S by F, where R is net revertants/ml over background, S is colonial survival in 10^8/ml, and F is UV fluence exposure in Jm^{-2}. At 265 nm this procedure yields a plausible induction curve, at least in form (the sensitivity parameter for UV damage by the fluence divided out remains an unknown multiplying variable). The process is less clear at 313 nm, where most of the revertant curve is fluence-squared.

At all wavelengths tested there is a strong fluence-squared dependence of net revertant yield at the lower fluences. There is also some form of deviation below the fluence-squared line at higher fluences. In the far UV midregion of 254 nm through 276 nm, the three phases do all roughly appear, although not entirely consistently from run to run for a clear third phase. Outside these limits there were no clear indications of a third phase, that is, a steep climb in revertants per survivor at highest fluences. In spite of this difference, it should be possible to develop an induction curve, in form, at all wavelengths tested, even 313 nm.

The data are thus far represented in three series of plots: a collection of survivals at each wavelength, a log-log plot of uncorrected revertant yield *vs* fluence, and a log-log plot of net revertants per 10^8 surviving

colonies vs fluence. These plots are too extensive and too ephemeral for full inclusion here. The intention is to rework all these data by an empirical correction for background revertant dependence in relation to survival, based on the dilution work on revertant background. This correction will affect the interesting upper portions of the R/S vs F plots mostly, and will be vital to their interpretations in relation to induction curves.

In addition, before committing to action spectra, corrections are needed for average lateral intensity of the ultraviolet exposures. Depth averaging is done in initial figuring of fluences, but lateral average depends on fifteen-position samplings of the lateral field distribution by thermopile. All these data are in hand and have been developed as lateral averages over the circular area of the dish, by computer. There is a wavelength dependence in lateral averaging factor, and there was one significant shift mid-summer when I refocussed the UV monochromator. Hence, a comprehensive correction must be worked out according to calendar date of irradiation and with a smoothed wavelength dependence. After this process is complete the lateral displacements of fluence-squared trends in the data on the log fluence scale will be analyzed to develop a basic action spectra.

An action spectrum should also be available from the relative shifts in uncorrected revertant yields against the corrected log fluence scale. Any wavelength dependence of induction curves should also be evident from R/SF plots vs F, but these may not represent readily as an action spectrum. The two indications of mutagenesis action spectra will be compared with our best available absorption spectrum for DNA.

The nine figures which follow show results at three of the eleven wavelengths studied. Figs. 1, 2 and 3 are at 234.5 mm. Figs. 4, 5 and 6 are at 260 mm. Figs. 7, 8 and 9 are at 289.4 mm. Figs. 1, 4 and 7 are raw revertant yield data as a function of fluence, in log-log plot since the dynamic range of both dimensions is high. Figs. 2, 5 and 8 are survival curves. Figs. 3, 6, and 9 are dose-action curves for net revertants per survivor as a function of fluence. The log-log plot not only accommodates the considerable dynamic range but shows the extent of fluence-squared dependence by inspection.

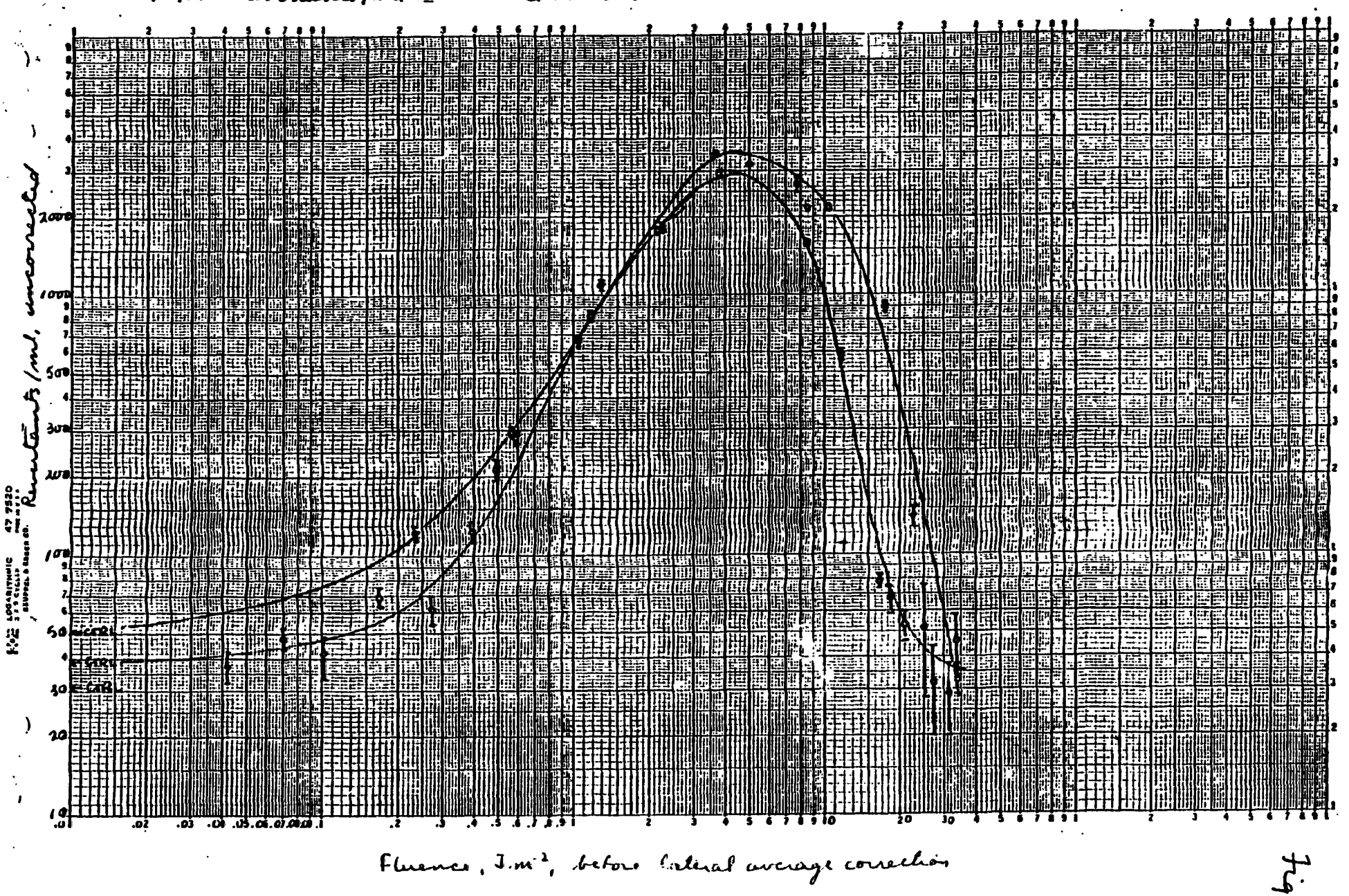

7/10/77 Revertants/ml vs fluence at 234.5 nm
○ 7/6/77 • 7/26 ◐ 7/13
Revertants/ml, uncorrected
Fluence, J.m⁻², before lateral average correction

Fig. 1

7/22/71 Survival of E.coli WU3610-89 at 234.5 nm • 7/26

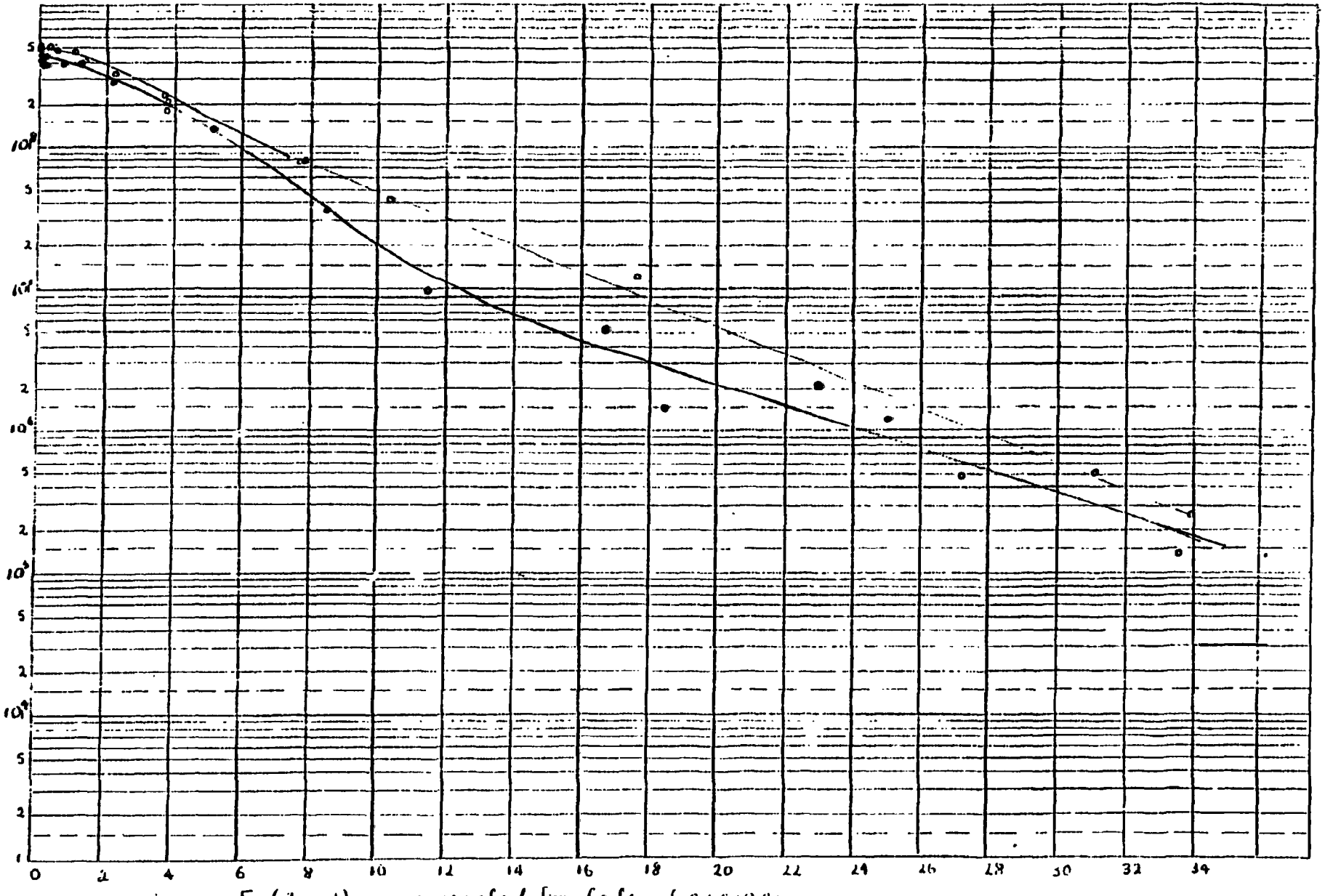


Fig. 2

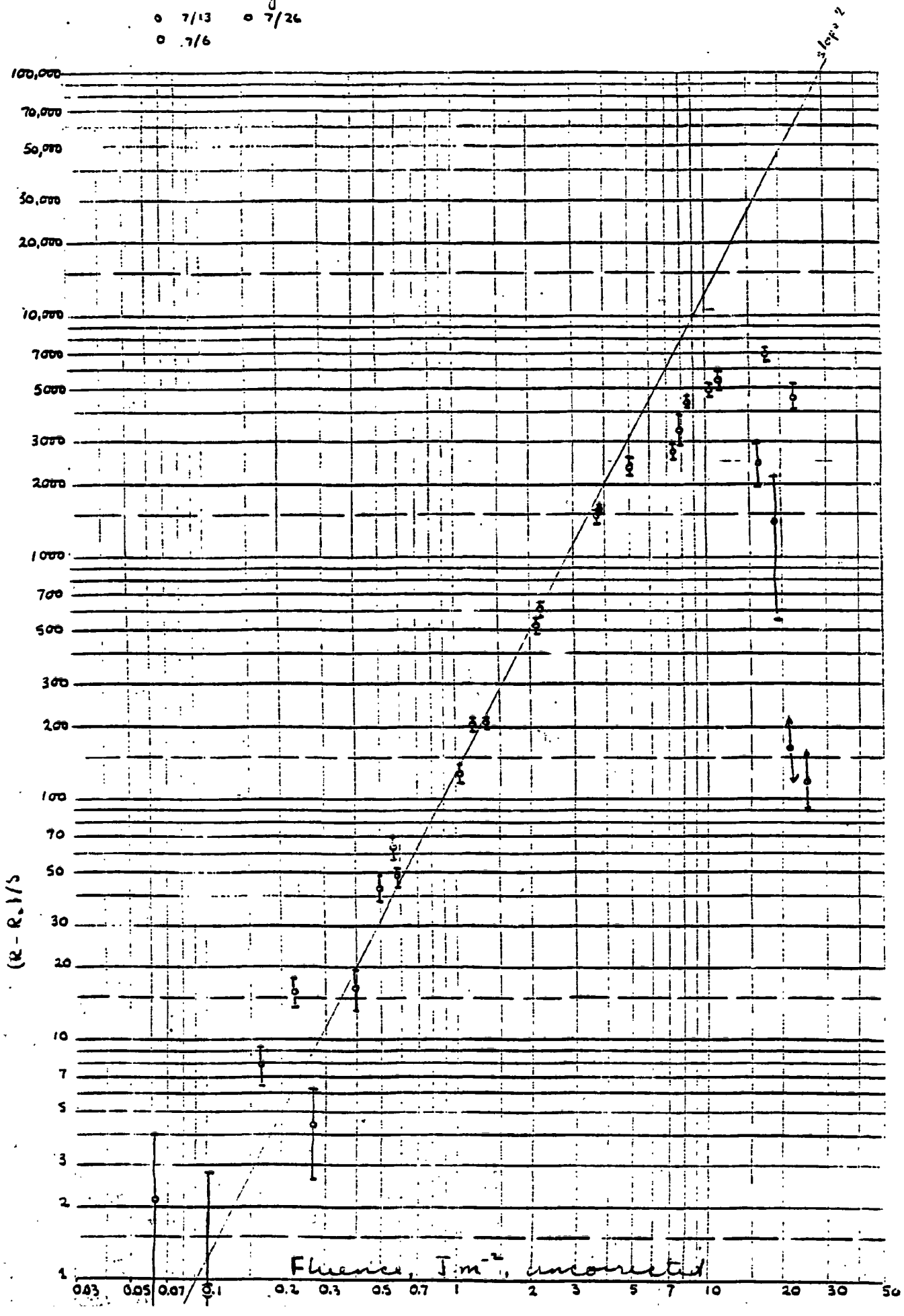
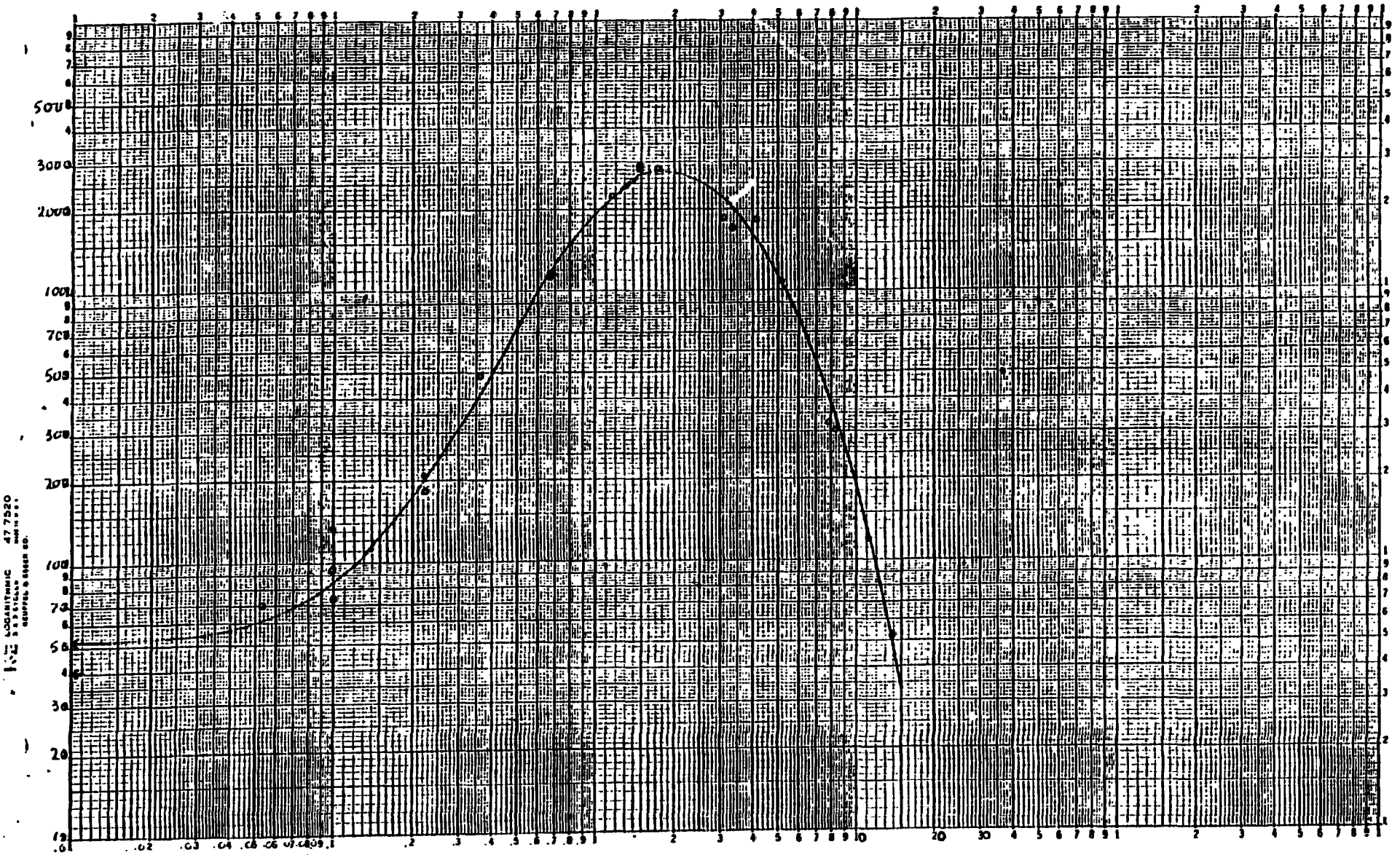

7/10/77 Mutagenesis at 254.5 nm
o 7/13 o 7/26
o 7/6
slope 2
100,000
70,000
50,000
30,000
20,000
10,000
7000
5000
3000
2000
1000
700
500
300
200
100
70
50
30
20
10
7
5
3
2
1
(R - R.)^1/2
0.03 0.05 0.07 0.1 0.2 0.3 0.5 0.7 1 2 3 5 7 10 20 30 50
Fluence, J m^-2, uncorrected

Fig. 3

8/3/77 Revertants/ml vs fluence at 260 nm ○ 7/28 ○ 7/15



Fluence (J m^{-2}) below Carbowax 400 filter

Fig. 4

8/3/77 Survival of E. coli WU3610-89 at 260 nm o 7/28

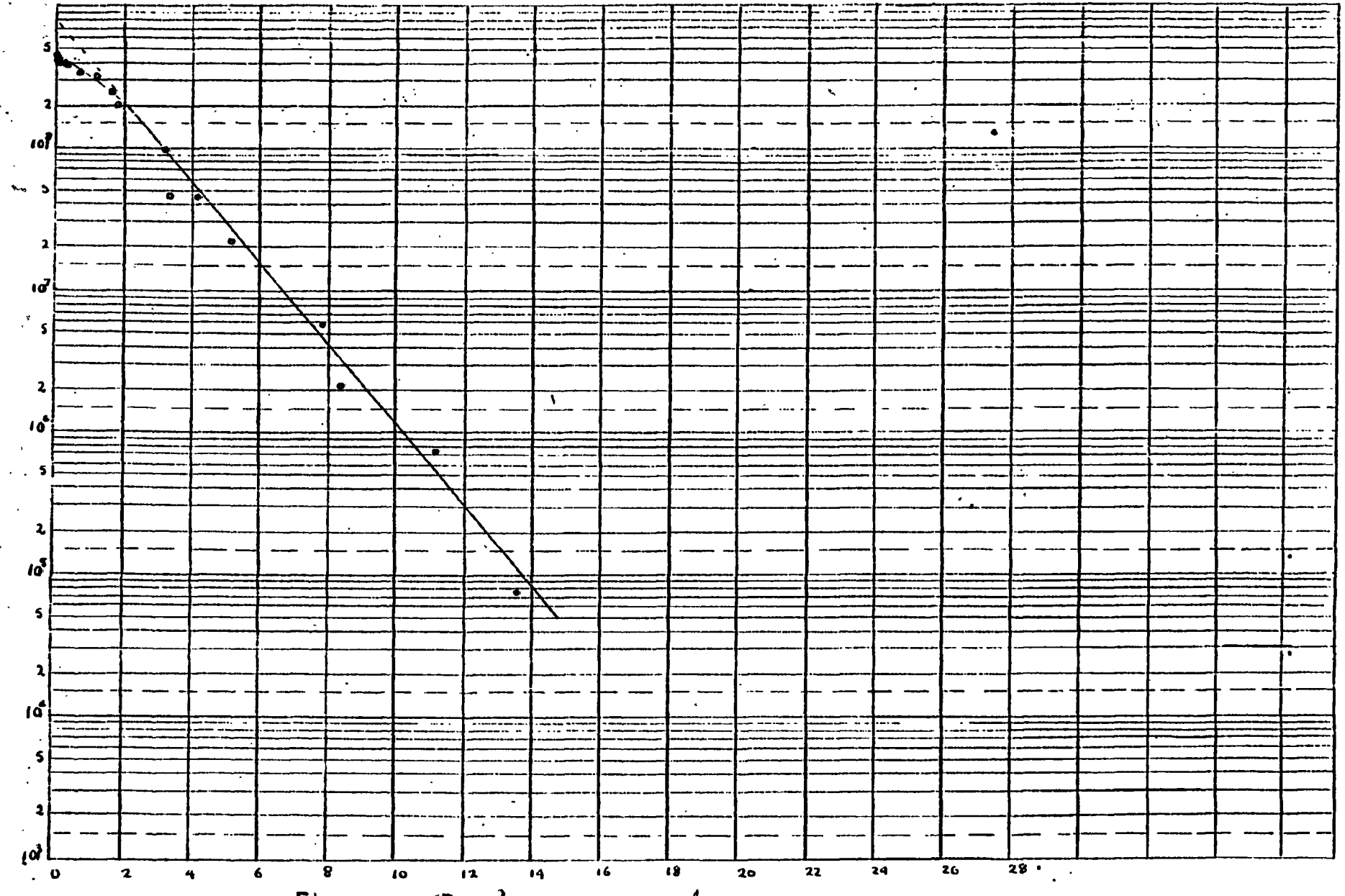


Fig. 5

8/3/77 Mutagenesis at 200 nm

E. coli WU 3610-89

○ 7/28

● 7/15

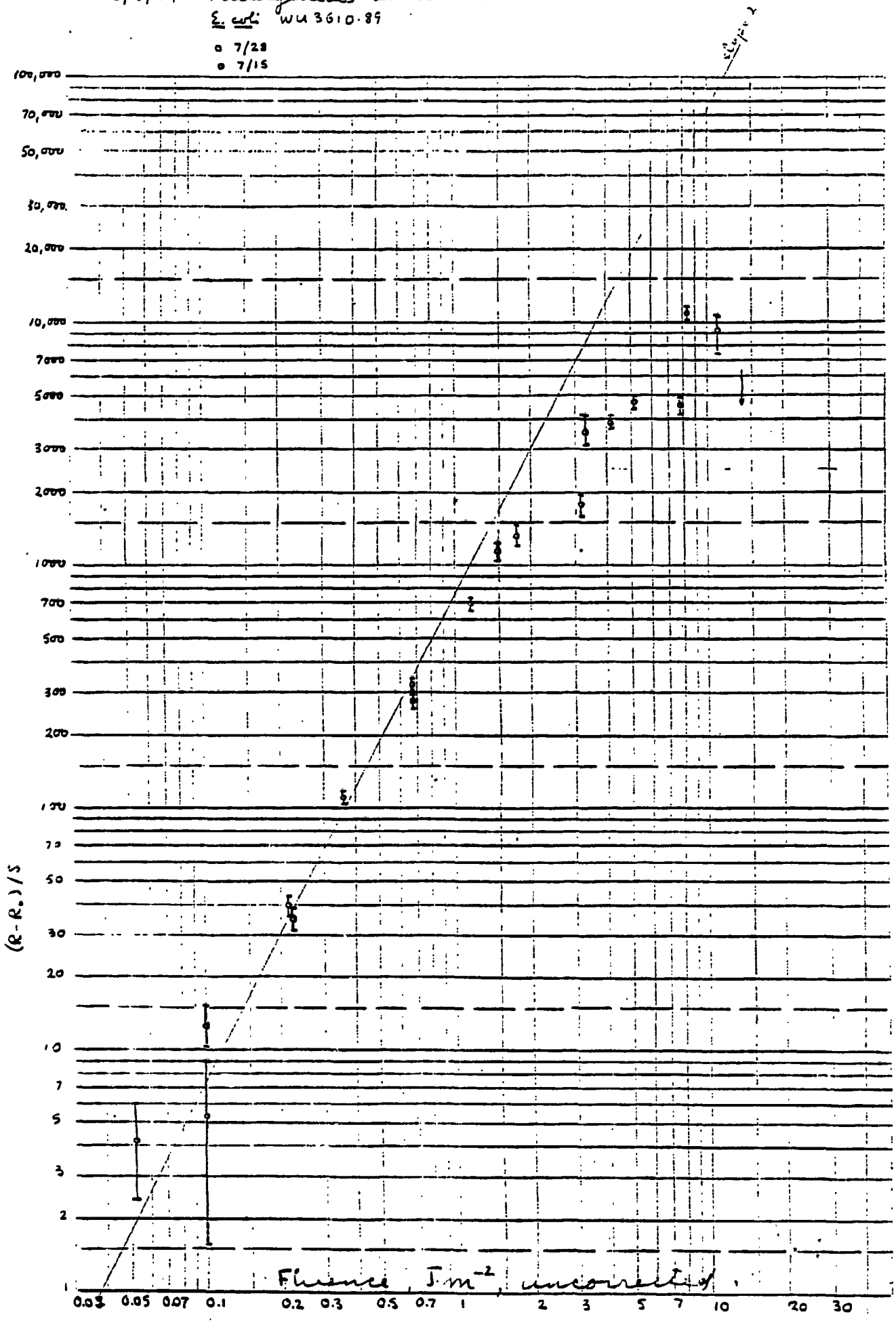


Fig. 6

7/10/77 Revertants/ml vs. fluence at 289.4 nm

• 7/6/77 • 8/3/77
○ 7/11/77

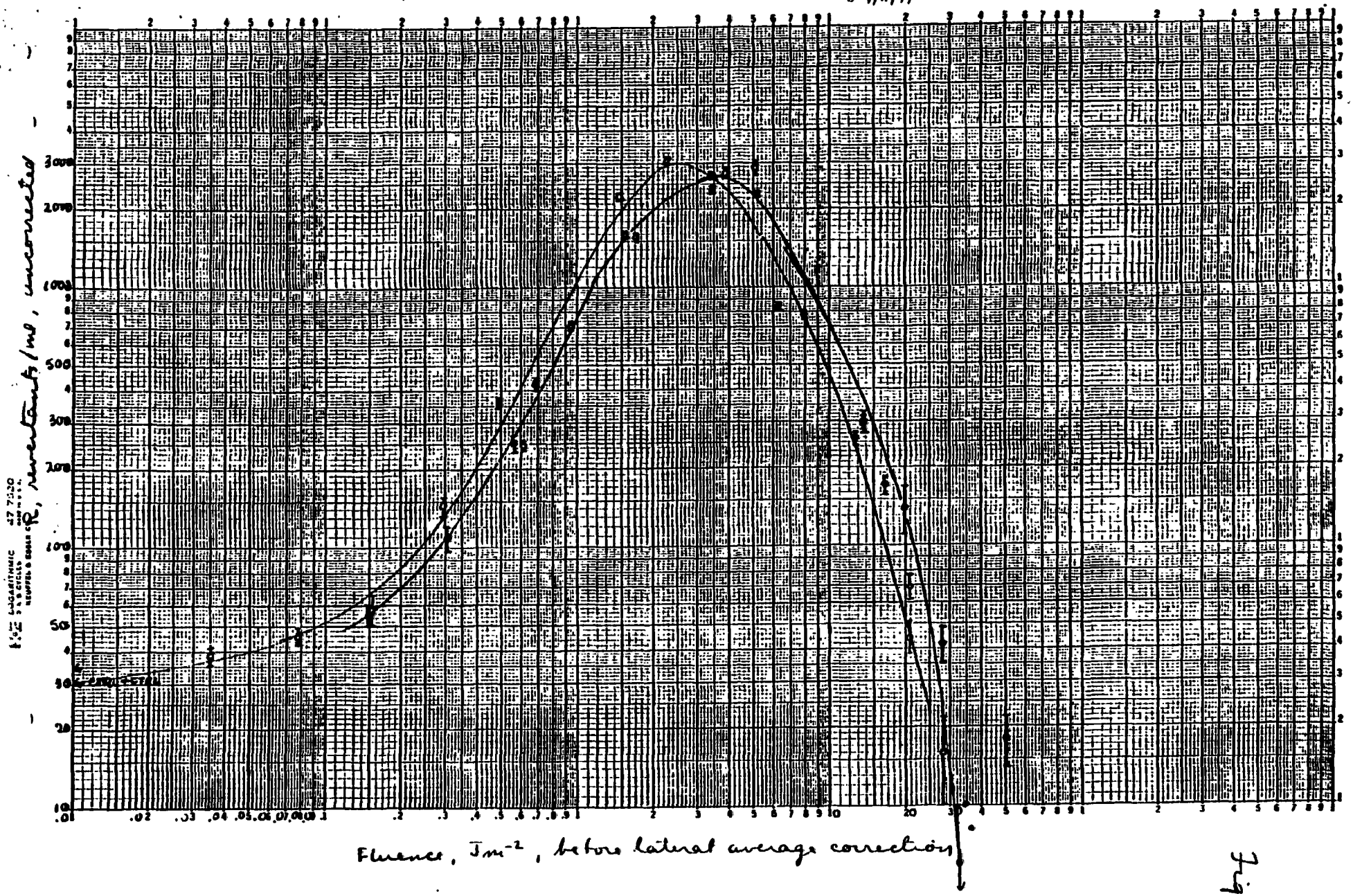


Fig. 3

7/22/77 Survival of E.coli WU3610-89 at 289.4 nm ○ 7/11 ● 8/3

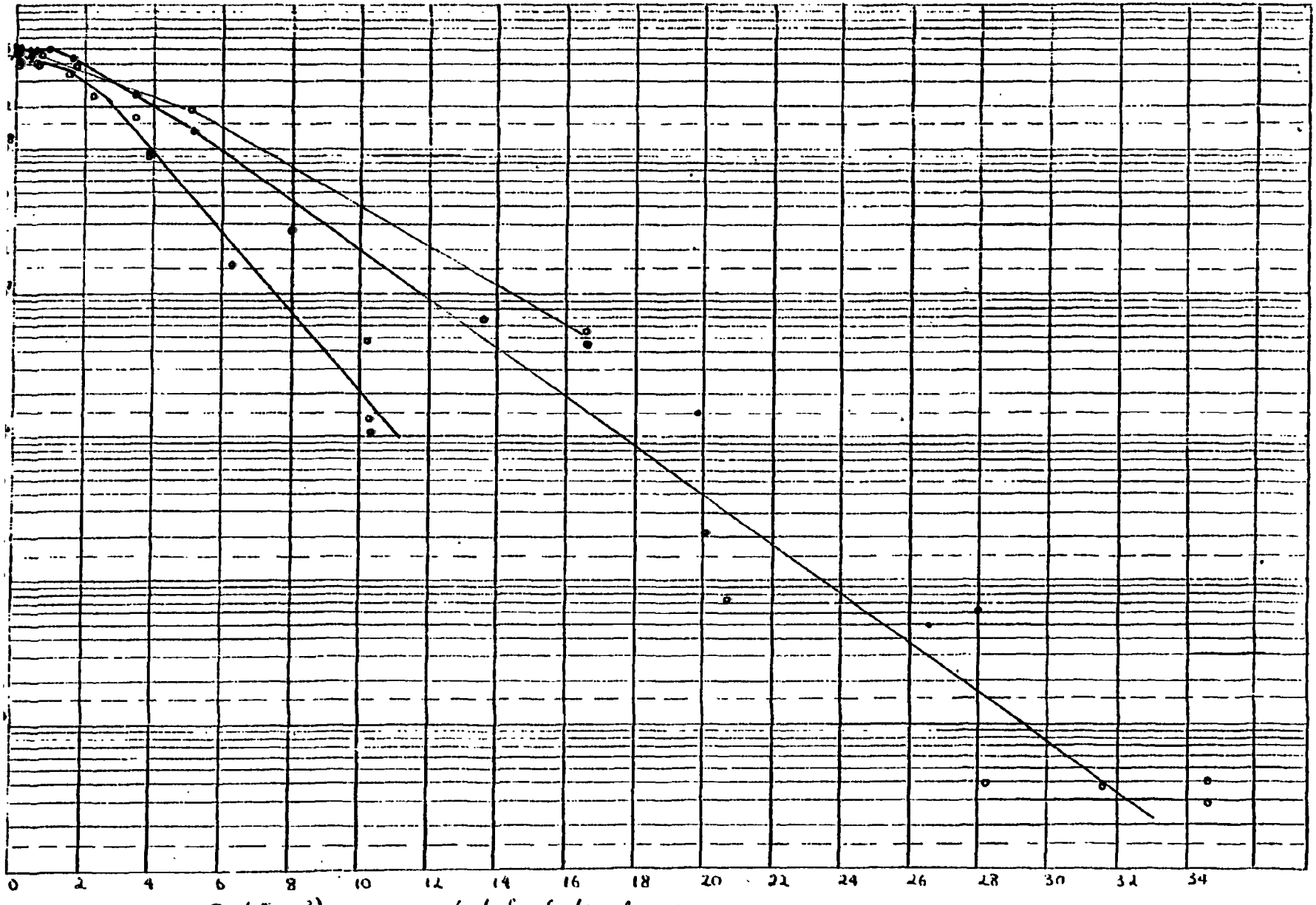


Fig. 8

7/16/77 Mutagenesis at 284.4 nm
E. coli WU 3610-89
○ 7/11 ○ 8/3
○ 7/6

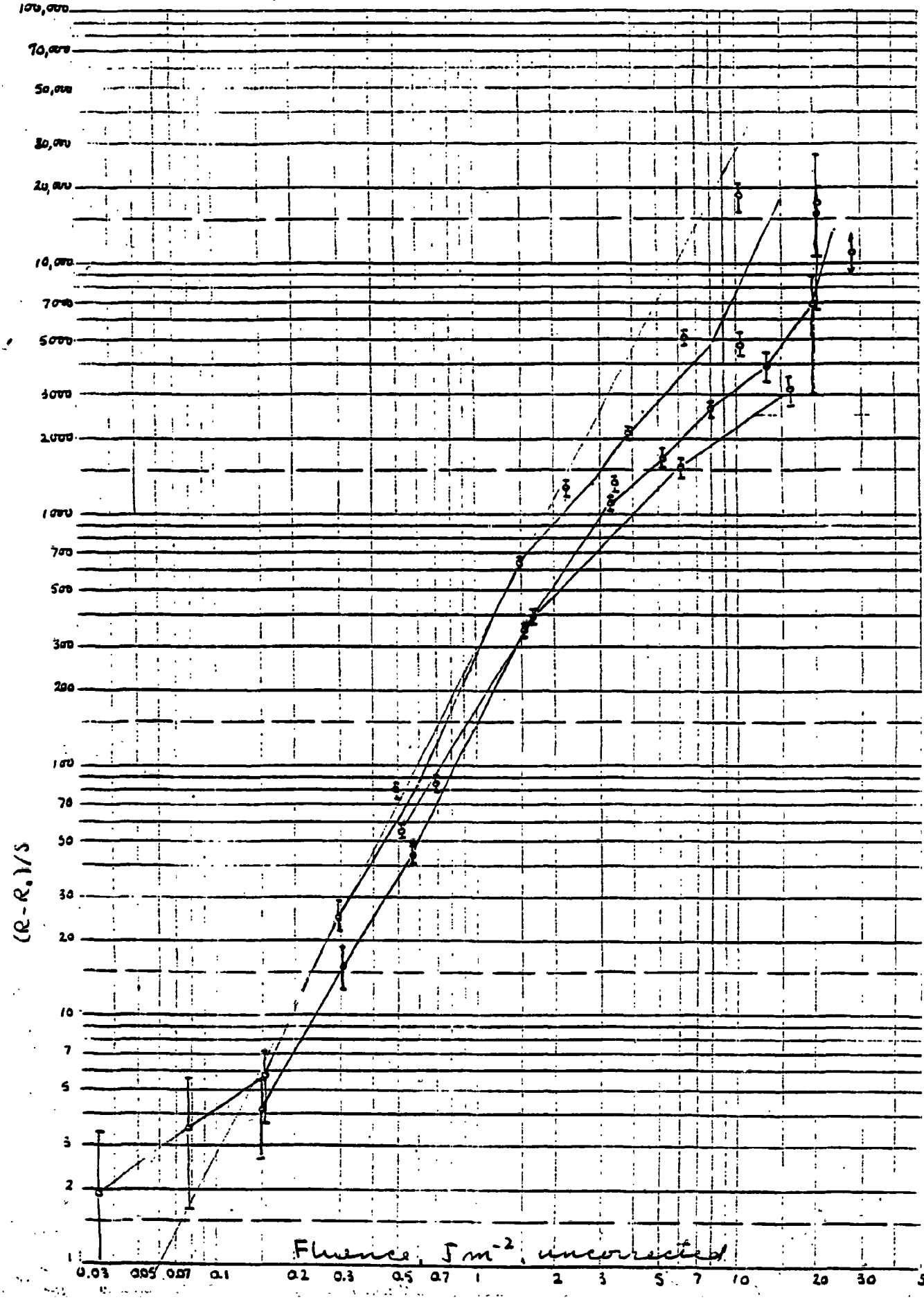


Fig. 9

The Effect of Various Numbers of Cells Plated Upon Revertant Yield

In measuring leucine revertant yield as a function of UV fluence in the E. coli WU3610-89 system, it is conventional to plate equal numbers of cells on all plates in testing revertant yield. In spite of the inconvenience of having thousands of revertant colonies to count at the highest yields, reducing the amount of cell culture material on a plate does not give equivalent reduction in countable revertant colonies. A plausible explanation is that fewer cells make fewer but more populous microcolonies within the limited leucine available; the additional growth within the micro-colonies allows more opportunity for revertant expression for each cell plated.

A specific series of experiments was undertaken in order to test this general explanation at the level of whole uncorrected mutant yield curves. In such testing it is necessary to hold the leucine content of the revertant plating medium more or less constant. Since the volume of cell culture plated contributes about half the casamino acids (hence leucine) on the plate, the following four types of platings were done:

1) 0.5 ml of irradiated cells in casamino acids medium without further addition (this is the standard plating),
2) 0.1 ml of irradiated cells plus 0.4 ml of unirradiated cells,
3) 0.1 ml of irradiated cells plus 0.4 ml of supernatant from unirradiated cell culture, and
4) 0.1 ml of irradiated cells plus 0.4 ml of sterile culture medium.

Hence, all platings had 0.5 ml total of the cell culture medium, some after growth of cells in it and some not.

The open circles in Figure 1 show a typical uncorrected revertant yield curve for 265-nm exposures. The filled circles show essentially the same yield curve for 0.1 ml of irradiated cells plus 0.4 ml of unirradiated cells. On a per-ml basis, holding the number of plated cells constant gives a reasonable dilution development for the unirradiated portion throughout the fluence range. It is possible that at low fluences the fewer irradiated cells still show some enhancement, and that at higher fluences the unirradiated microcolonies suppress revertant expression in those from irradiated cells. That is a reasonable result if there is a division delay by irradiation, for example, so that unirradiated microcolonies get a larger share of the limiting leucine. However, these differences are within the repeatability of such curves, run to run, and are suggestive more than conclusive.

In Figure 2 the normal 0.5-ml plating is shown as a dotted line, and the other two platings are shown by data points. Supplementation by 0.4 ml of either culture supernatant or sterile medium causes a 2 to 3-fold enhancement of revertant yield on the ascending limb of the curve. It appears that prior cell growth in the medium does not decrease leucine content sufficiently for notable limitation in mutant yield. The 5-fold reduction in microcolonies per plate, with resultant greater cell numbers per microcolony, clearly enhances revertant yield. However, the effect is not seen on the descending limb of the curve. Once the fluence-squared phase of mutagenesis is passed, and also when overall colonial survival decreases, the number of microcolonies per plate and the difference in their ultimate sizes have less effect.

The descending limb of the uncorrected revertant vs fluence curve represents the second and third plases of the revertant per 10^8 survivor vs fluence curves. The early portion of the descent is more rapid than decline in survival; the latter portion falls off less rapidly than survival. The lack of enhancement by dilution runs through both.

Figure 3 shows the three classes of curves (which are possibly distinguishable among the four overall) in log-log plot of revertants per 10^8 survivors vs fluence. The three phases are evident for all three curves, irrespective of cell numbers and competition effects per plate. It does appear that the lower cell number overall (heavy dashed curve) is below the standard curve (0.5 ml plated) by the third phase.

These experiments have been extended somewhat by plating serial dilutions of both irradiated and unirradiated cells. In all of the corrections of revertant yields for background revertant count it has been assumed that background revertant count is invariant with survival. Largely, this is true, but there is a steady logarithmic decline by about 50% over the first three decades of dilution. In the fourth decade and beyond it falls more sharply toward zero. These data will be used in an empirical correction of our R/S vs F (net revertants per 10^8 survivors vs fluence) curves.

A similar dilution curve for UV-induced revertants at the mid-range of the ascending yield curve gave much stronger dilution dependence; R varied as $S^{0.85}$, where R is revertant colonies/ml, and S is total colonies/ml. This dependence is much more nearly linear than the 1 to 5 dilution difference reported above, and the difference is as yet unresolved. Since all dilutions were three-fold in the casamino acids glucose growth medium, no variation in plating leucine concentration was involved.

Preprints + Preprints Remarks

Effect of Plating Amount

Figure 1

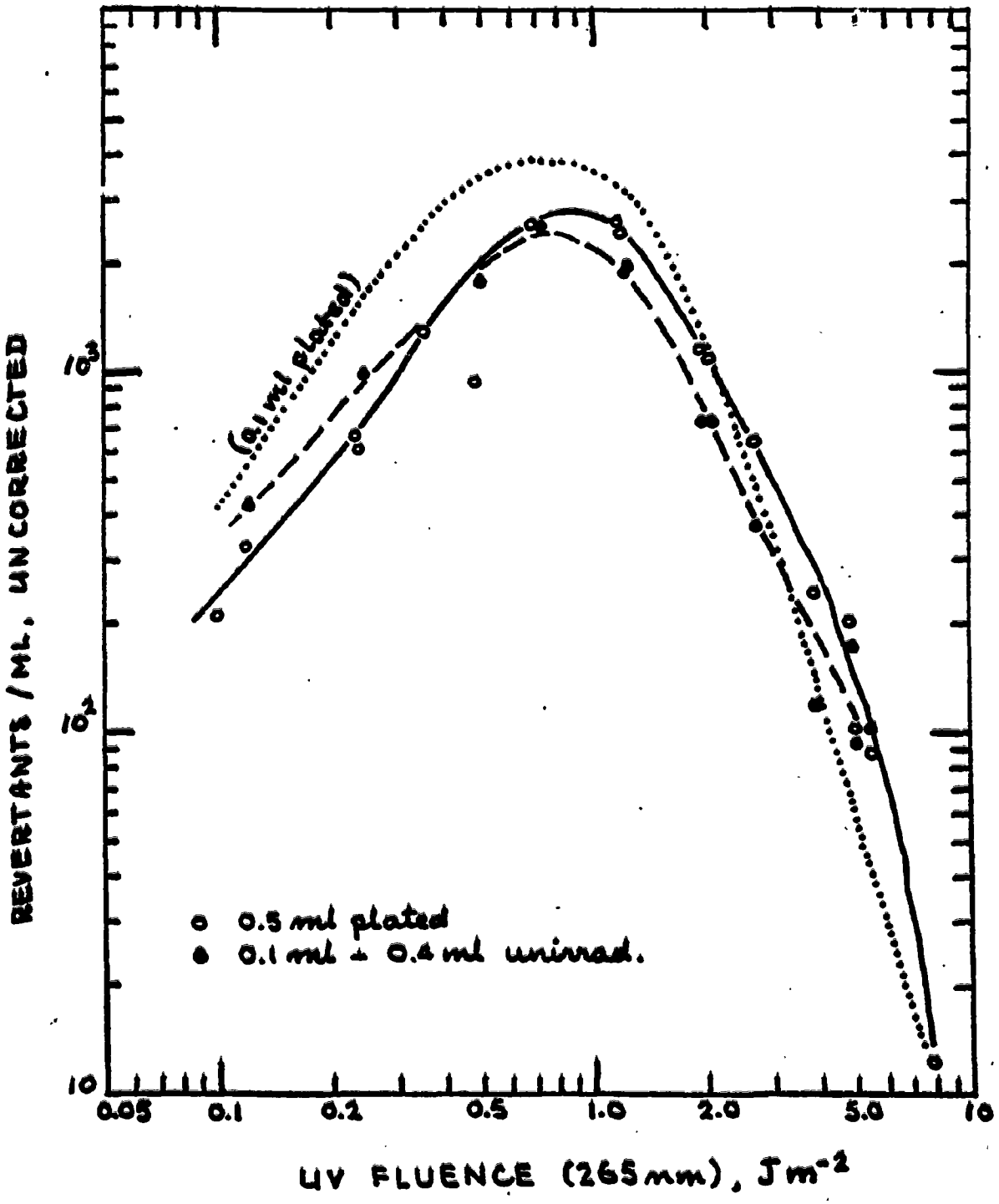


Figure 2

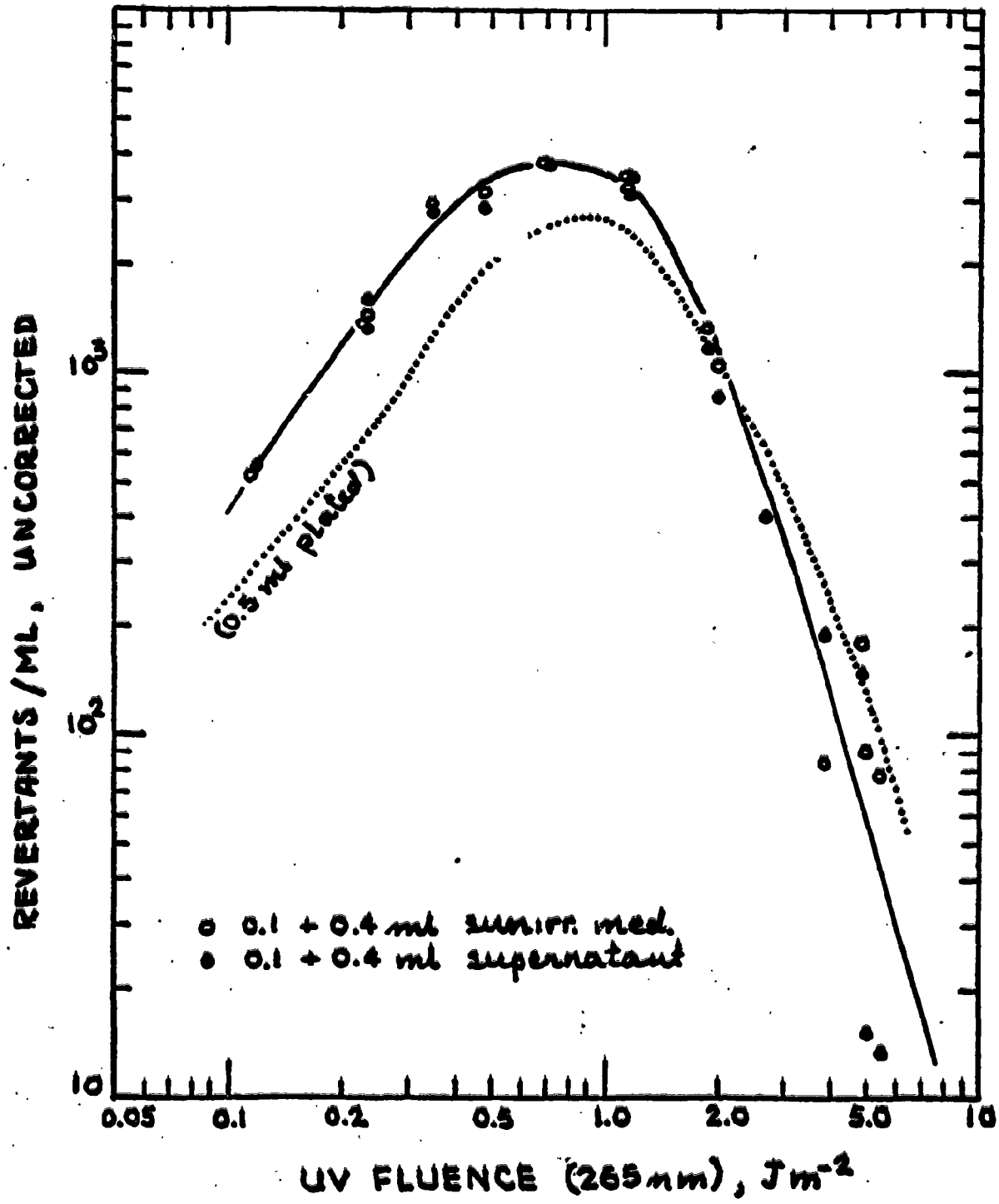

REVERTANTS/ML, UNCORRECTED
10^3
10^2
10
(0.5 ml plate)
○ 0.1 + 0.4 ml sunlight med.
● 0.1 + 0.4 ml supernatant
0.05
0.1
0.2
0.5
1.0
2.0
5.0
10
UV FLUENCE (265nm), Jm^{-2}

Figure 3

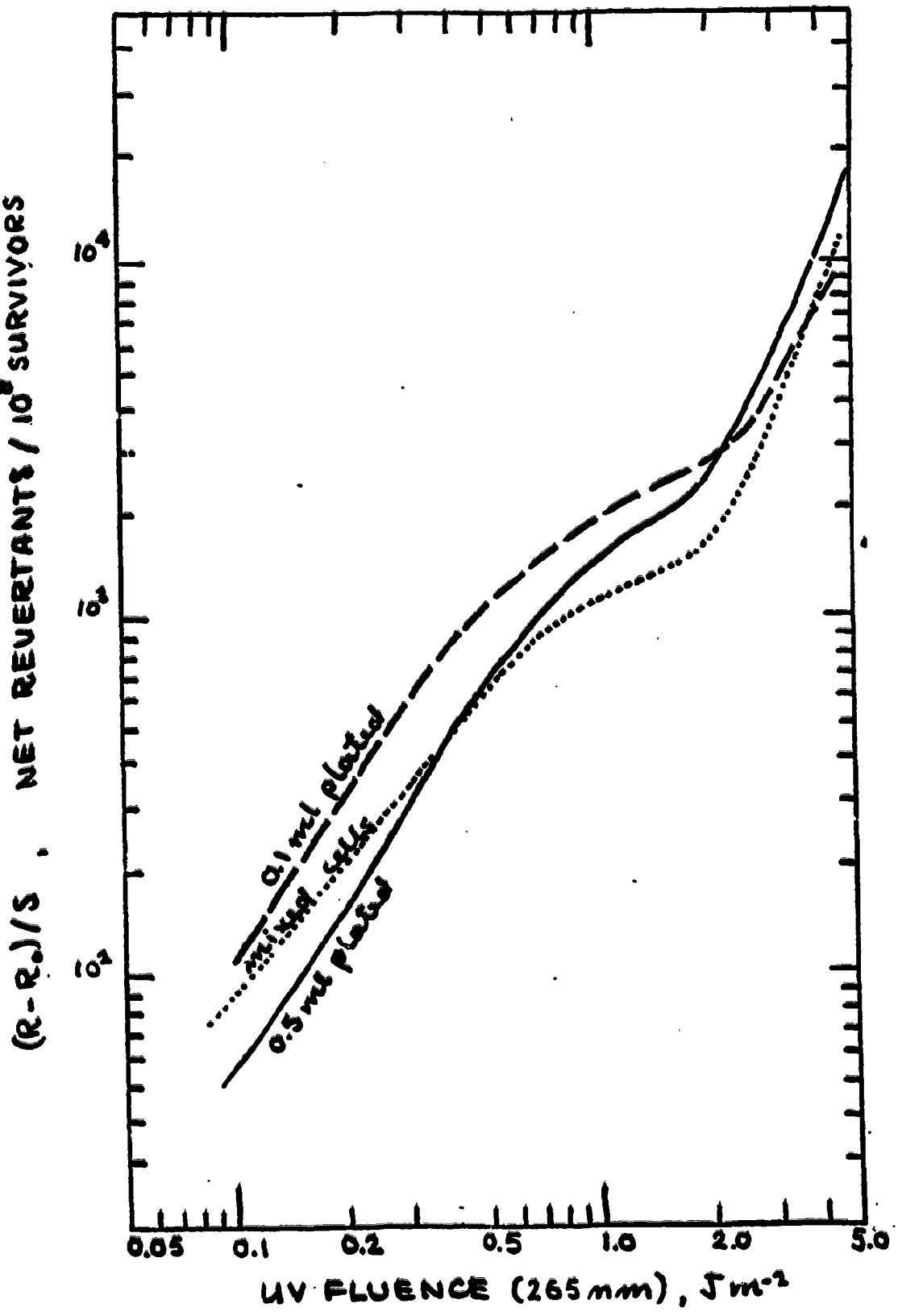
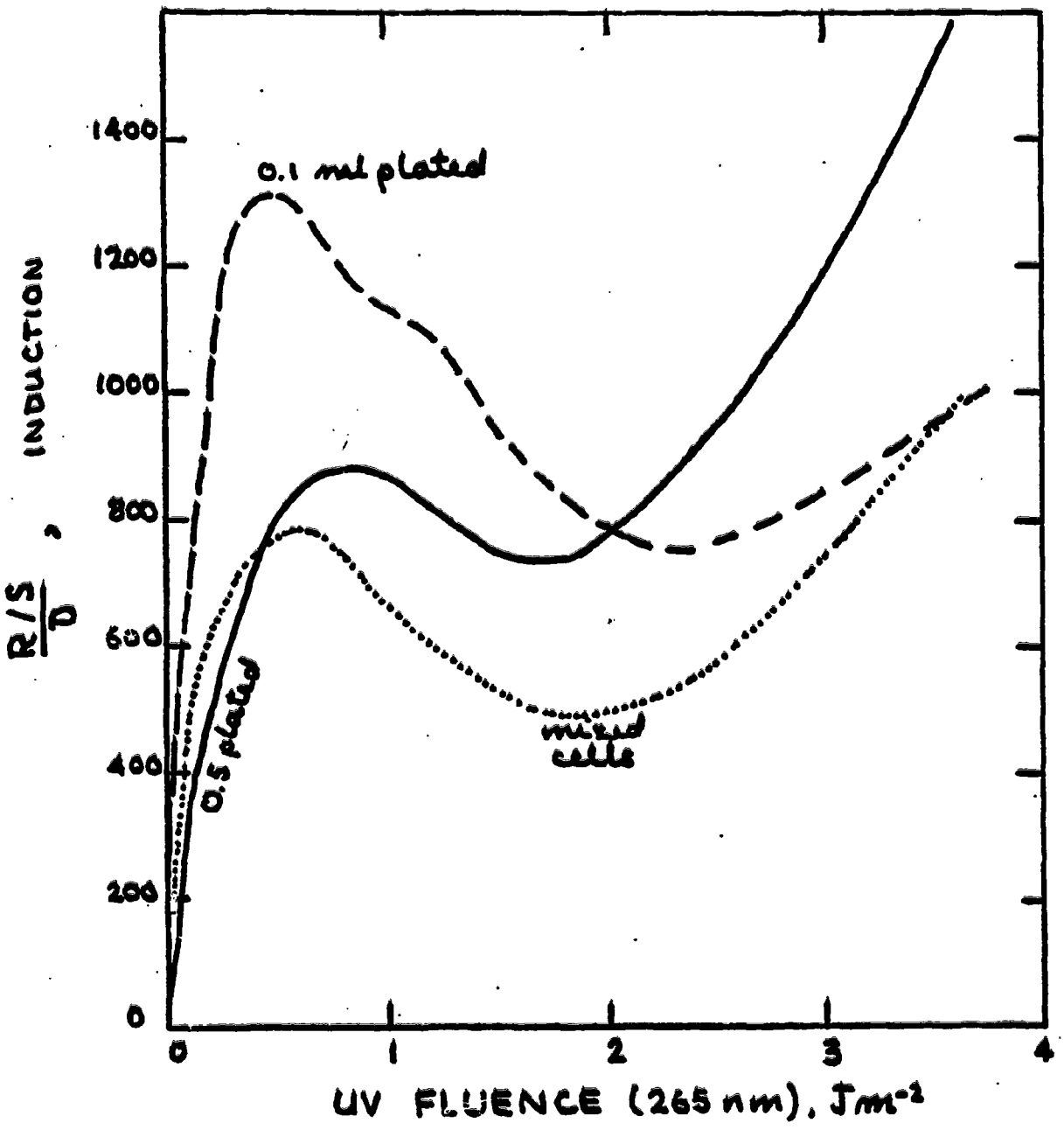

(R-R₀)/S . NET REVERTANTS / 10⁸ SURVIVORS
10⁴
10³
10²
0.1 ml plated
mixed
spots
0.5 ml plated
0.05
0.1
0.2
0.5
1.0
2.0
5.0
UV FLUENCE (265 nm), J m⁻²

Figure 4


1400
1200
1000
800
600
400
200
0
0
1
2
3
4
0.1 ml plated
0.5 plated
mixed cells
INDUCTION
R/S / D
UV FLUENCE (265 nm), Jm⁻²