THE EFFECTS OF ADDED AGAR ON THE RADIATION SENSITIVITY OF TRYPSIN IN SOLUTION

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ABSTRACT

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by Wendell E. Holladay

The radiation sensitivity of different biological molecules has been studied by numerous workers in an attempt to determine the mechanism by which cells are affected by ionizing radiation. This present research is an attempt to determine the effects of ionizing radiation upon one specific system which may simulate certain conditions within the cell: the effect on the radiation sensitivity of the enzyme trypsin when a gel-forming material, agar, is added.

Only small concentrations of both, trypsin and agar were used to avoid difficulties involved in the measurement of residual activity, and the possible masking of radiation effects by a preponderance of one component or the other. The residual enzymic activity after irradiation was determined using the method of Anson (4) which involves the digestion of hemoglobin by the remaining active trypsin and measurement of the peptide residues split off during digestion which are not precipitated by 5% trichloroacetic acid. The analysis of the data was performed using a modification of the method of Augenstein (7). In this method the portion of enzyme grossly affected by the agar was discounted from

the calculation by resolution of the logarithmic curve obtained when log residual activity was plotted against dose absorbed. The ratio of that portion least affected by the agar to recovarable activity is taken as the fraction of the initial trypsin concentration which is neither complexed nor inactivated by the agar. The activity remaining in this "unbound portion" after a given dose is then compared with the total recoverable activity (before irradiation) to determine the effect of the added agar upon the radiation sensitivity of the "unbound portion."

The results of the analysis indicate that the presence of agar exerts a definite protective effect upon "unbound" trypsin molecules. Others have demonstrated that agar does not complete for radicals which react with methylene blue (15), and that methylene blue does complete effectively for radicals which inactivate trypsin (5). It is possible, however, that some radicals are capable of inactivating trypsin but not methylene blue.

The results of these experiments indicate that several factors are involved in determining the sensitivity of uncomplexed molecules; these include local concentration effects occurring when the solutions of agar and trypsin are mixed before irradiation, length of time which trypsin and agar are allowed to interact, the amount of agar and trypsin present, and whether or not the trypsin remains biologically active when absorbed to agar. The pertinence of these

experiments to those performed by other investigators in similar model systems and also in cells is discussed. Also, it is concluded that the protective effect exhibited by the agar toward the unbound fraction of trypsin is the result of competition for radiation-produced water radicals.

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By

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INTRODUCTION AND SURVEY OF LITERATURE

Early in the study of radiation effects on biological organisms it was found that the living cell is approximately a thousand times more sensitive to ionizing radiation than *in vitro* preparations of critical cellular constituents.

There are a number of potential explanations for this thousand-fold disparity:

a. radiation destroys certain critical cellular components of which there is only "one-of-a-kind" per cell (i.e. genes);
b. radiation-produced disturbances in the delicately balanced cellular metabolism become amplified;
c. radiation either produced poisonous products or else modifies internal structures and cellular membranes so as to allow critical compounds to leak out or poisonous compounds in;
d. the mechanism of energy-interchange within biological systems is such that absorbed energy is funneled to critical sites.(1)

Two types of inactivation are possible when a substance is exposed to ionizing radiation. Direct inactiviation occurs when the radiation produces a primary event such as an ionization within a portion of the molecule itself. Indirect inactivation is the result of reaction between solute molecules and radicals which are produced by the interaction of incident radiation with solvent molecules. In aqueous solutions the following scheme is thought to represent the manner in which ionizing radiation produces radicals during interaction with water molecules (3):

$$\xrightarrow{h\nu\ \sim} HOH \longrightarrow HOH^{+} + e^{-}$$

$$HOH^{+} + (H_2O)_n \longrightarrow (H_{2n+1}O_n)^{+} + OH^{0}$$

$$HOH + {}^{-} \longrightarrow HOH^{-} \longrightarrow OH^{-} + H^{0}$$

The OH^0 is an oxidizing radical and the H^0 a reducing radical. These may react with solute molecules to produce inactivation or with each other. The further reaction of H^0 and OH^0 with other solute molecules or other radicals can produce other inactivating entities such as H_2, HO_2, and H_2O_2. Theoretically both direct and indirect action occur. However, it was first shown by Dale (12, 13) that very pure preparations of enzyme in solution could be inactivated by 10^4r instead of the 10^6r or greater which is usually necessary for direct inactivation of impure solutions (22, 32), or dry (31) preparations. Barron (11) further found that sulfhydryl enzymes in solution could be inactivated by as low as 10^2r. Thus, in dilute aqueous solutions the probability of inactivation from direct effects is so low that normally it may be neglected. The mechanisms proposed to explain radiation inactivation are summarized in a review by Augenstein (8).

The complex physical and chemical nature of the cell makes it very difficult to investigate a specific adsorbed system in its natural setting. The radiation sensitivity of adsorbed substances has been known by many investigators to depend upon the nature of the adsorption and the amount

of water present. It has been found that the effects of radiation upon adsorbed substances vary widely depending upon the character of the system used. For example, DNase adsorbed on cellulose or ion-exchange resins and then dried is about ten times more sensitive to radiation than is DNase in non-adsorbed mixtures or when dried with its substrate (16, 17, 30). This indicates that in some cases the energy absorbed by the substrate material may be transferred to the biologically active molecule resulting in inactivation. According to a hypothesis by Augenstein (8) inactivation is the result of specific modifications in molecular conformation. These modifications occur in a cluster of weak bonds including disulfide and other intramolecular bonds which are involved in maintaining the unique topography of the active site which is calculated to be approximately 100-500A^{02} in area. Inactivation is presumed to result from localization of enough absorbed energy in the clusted to disrupt all crucial bonds. This model is primarily concerned with the role of structural S-S bonds and does not relate to free sulfhydryl groups which might be necessary for the activity of some enzymes.

Protection of a given substance in solution from the effects of ionizing radiation was first described by Fricke and Washburn (18). However, it was Dale (13) who first demonstrated that the yield for inactivation of carboxy-peptidase was 85% greater in pure solution than when its substrate was

present. Further, he showed (14) that the addition of protein, sugars, amino acids and/or some organic compounds to solutions of d-amino acid-oxydase could prevent radiation damage by competing with the enzyme for radiation produced radicals.

Alexander (1) has postulated another mechanism of the protection of substances from irradiation on the basis of studies of the mortality of mice and the degradation of polymethacrylic acid in aerated water. He suggests that the action of chemical protectors is not to reduce initial damage, but to enhance repair processes. That is, he proposes that x-rays destroy a "regeneration factor" which stimulates repair of damage in cells, and chemicals protect this factor from destruction from x-ray produced radicals.

After measuring the oxidation-reduction potentials of HO_2^o, OH^o and H_2O_2 Garin (21) arrived at the conclusion that any reducing substance would serve a protective function. It was later shown by Holmes et al. (23) that chemically generated OH^o radicals can produce inactivation typical of that from x-rays.

The type of protection observed by Dale is commonly termed competitive protection, because the added solutes compete with the enzymes for the radiation-produced entities present in the water. A second type of radiation protection occurs when the added substrate reacts with the enzyme to produce a radiation-insensitive product. Barron (1) also

reported that the presence of electronegative moieties may produce steric hinderances to the oxidation of nearby groups so that their rate of reaction with the radiation-produced oxidizing or reducing agents is altered.

In pursuing this type of investigation it is an advantage to simplify the system as much as possible, and yet retain essential characteristics of the property being investigated. The system chosen for this experiment was agar and trypsin. The enzyme trypsin was chosen because it is possible to assay its activity accurately when present in only uM quantities. Agar was chosen as the adsorbing substrate because it is a polysaccharide, and therefore, insensitive to trypsin digestion, and also aqueous gels composed of agar have an atomic number similar to that of tissue (15). There is one possible disadvantage to the use of agar. Kersten and Dwight (27) have found that after irradiation in the dry state agar gives solutions of lower viscosity and pH. This indicates that irradiation does affect the agar molecule to some extent. Since the doses of radiation used in this experiment were relatively small we have neglected the damage produced in agar by radiation.

The object of this study was to investigate how the radiation-sensitivity of trypsin is changed when it is placed in solution with a substance such as agar with which it can apparently form some type of complex or adsorption mixture. Initially it was shown by Gevantman (20) that trichloroethylene

and potassium iodide are inactivated to a greater extent in agar gels than in pure solution. This established that the agar does not compete effectively for radicals which can react with these substances, and that the gel may lead to a more effective utilization of the absorbed energy. The work of Day and Stein (16) showed that agar does not compete for radicals responsible for the inactivation of methylene blue. E. S. Augenstein (5) has concluded that since trypsin and methylene blue are co-competitors for radiation produced radicals, the same radical species must be involved in inactivating both. Thus the agar should not compete for all kinds of the radicals which can inactivate trypsin, and therefore, should have a limited effect upon the number of radicals available for reaction with the enzyme.

In previous work, it has been found that agar normally has a micelle structure in solution (33), and that at a pH of 4.5 it has a net negative charge (34). E. S. Augenstein (5) found that in agar concentrations from 0.4 to 2.0% (agar forms a solid gel above 0.2%) there was no observable inactivation of trypsin. She postulated that trypsin molecules were surrounded by particles of agar, and so tightly bound that even if sufficient energy were present to cause incipient inactivation of the molecule it would be physically restrained in its active configuration by the agar cage. A second possibility advanced was that the trypsin had adsorbed to the agar in such a way that an "umbrella" had formed over the active site which blocked reactions necessary for inactivation.

A method has been developed by L. G. Augenstein (7) for the accurate analysis of the protective effects of various substances at low enzyme concentrations. As stated in (7) this analysis is based on four propositions:

a. Radiation produces activated solvent molecules, including ions as well as oxidizing and reducing radicals.

b. Inactivation, if it occurs, is the result of a single collision with a radiation produced radical (this excludes those situations where multiple collisions either with additional radicals or other solute radicals play a major role).

c. Inactivation is produced solely by collisions with radicals (the theory applies only where direct inactivation is negligible).

d. This method utilizes the following equation:

$$\frac{1}{Y_{tr,j}} = \frac{1}{S_{tr,j}} + \left[\frac{\sum_{i=2}^{n} z_{ij} R_{ij} N_i}{z_{tr,j} R_{tr,j} S_{tr,j}} \right] \frac{1}{N_{tr}} \quad (1)$$

where:

N_i = the number of solute molecules of kind i in unit volume

$Y_{tr,j}$ = number of trypsin molecules inactivated per j^{th} radical produced, the "yield"

$S_{tr,j}$ = the probability that a collision which results in radical elimination will cause inactivation of the trypsin molecule

$R_{i,j}$ = the probability that a collision between an i^{th} solute molecule and a j^{th} radical will destroy the radical

$z_{i,j}$ = probability that any given collision involves a specific one of the j^{th} radicals and a specific molecule of solute i.

If the reciprocal of $Y_{tr,j}$ is plotted against the reciprocal of N_{tr} then the resulting straight line will have a slope indicated by the value in the brackets, and an intercept equal to the reciprocal of $S_{tr,j}$. Since this is a reciprocal plot the intercept represents the yield at infinite concentration. This method of analysis allows the separation of the $S_{tr,j}$ and $R_{tr,j}$ parameters, and therefore, provides a means of distinguishing between two different types of protector effects--competitive and reactive protection.

A low agar concentration was chosen for this study to facilitate the separation of a presumed cage effect from the effects of simple complex formation. Low agar concentrations make possible the investigation of the radiation sensitivity of both the "unbound" and "bound" portions of trypsin. The low concentration of agar also reduces the difficulties of assaying trypsin activity by increasing the amount of active enzyme which can be recovered.

EXPERIMENTAL PROCEDURES

Since radiation produced radicals react with any contaminants present as well as trypsin, it is necessary for all containers and solutions to be clean and pure as possible. All solutions were made up with water distilled twice in a pyrex still. Trypsin solutions were handled only in silicone-coated glassware. Solutions were prepared at about 6:00 A.M. to reduce contamination from airborn materials to a minimum.

Cleaning Equipment

The glassware used in these experiments was washed for thirty minutes in 10% NaOH, rinsed six times with distilled water and coated with "Siliclad" (Clay-Adams glassware coating compound). This procedure provided a non-wetting surface which minimized the adsorption of small concentrations of trypsin on to the glass.

The polystyrene vials used in the irradiation (7dr capped vials obtained from McKesson-Robbins) were washed with Hemosol detergent solution, rinsed six times in distilled water, and once in double distilled water. They were dried under cover to reduce the possibility of dust contamination. Polystyrene was chosen as the irradiation container material on the bases of work carried out by L. G. Augenstein (7). The vials were placed in Hemosol solution immediately after the assay was completed.

Preparation of Solutions

Trypsin.--A stock 25μM solution was made up in pH 4.5 sodium phosphate buffer. Sixty mg. of trypsin (2X crystallized, Worthington Biochemical Co.) were dissolved initially in 30 ml of 0.01M H_3PO_4 and diluted to 100 ml with 0.01M NaH_2PO_4. The concentration of the stock solution was determined with a Beckman DU spectrophotometer at 280mu. (Extinction coefficient of trypsin is 3.4×10^4). The solution was made fresh just prior to each experiment.

Hemoglobin-Urea Solution.--The Hb-urea solution was made up as follows:

a. Sixty-six grams of Hb were added slowly to 234ml of water. The mixture was stirred constantly to avoid lumping, allowed to stand twenty minutes and then strained to remove undissolved Hb.

b. In another container 80ml of 0.1N NaOH, 720 ml H_2O and 360 gm urea were combined; 100ml of the Hb filtrate from (a) was added and the solution was left for 25 minutes without stirring.

c. To (b) a solution of 40 gm of urea dissolved in 100 ml of 1M KH_2PO_4 was added and then the whole mixture was refrigerated in a closed plastic container.

Agar.--0.01 gm Difco Special Nobel Agar was dissolved in 100 ml NaH_2PO_4 at 90°C. The temperature was then held constant for 30 minutes to assure complete solution of the

agar after which 10ml of this solution were diluted to 100ml with NaH_2PO_4 at 90°C and allowed to cool to about 55°C. The pure agar solution showed no absorption in the 280 mu range.

Irradiation

A General Electric Maximar 250 KVP, 15 ma x-ray unit designed for continuous operation was used as the radiation source. Twelve vials containing the samples were placed around the circumference of a one-inch thick lucite disc mounted on a one-half inch brass plate. The samples were covered with a one-fourth inch bakelite disc held on with bolts at three points. Two one-eighth inch brass fins were attached to the bottom of the brass plate to facilitate temperature regulation. The temperature of the samples was held at about 4°C during irradiation by placing the sample holder in an ice-water bath. The water bath and sample holder were rotated under the x-ray beam at 33-1/3 rpm using a standard phonograph turntable.

Using the ferrous sulfate dosimeter of Fricke (19) the absorbed dose was found to be 130 rad/min. This dose rate remained constant throughout the 400 min. irradiation period. One ml samples of the trypsin-agar solutions were irradiated at doses of 0, 2.6, 5.2, 7.8, 10.4, 13.0, 15.6, 18.5, 26.0, 32.5, 39.0, 45.5, and 52.0 x 10^3 rads.

Hemoglobin Assay in Solution

After the irradiation of all samples was completed the enzymatic activity remaining was determined by the method of

Anson (4). The assay was carried out in the plastic irradiation containers to eliminate errors introduced by transfer manipulations. Five ml of Hb-urea solution at room temperature were added to the irradiated sample and allowed to react for a time calculated by the following empirical relation:

$$\text{conc. } (\mu M) \times \text{time (min)} = 40$$

At the appropriate time the digestion was stopped by the addition of 10ml of trichloroacetic acid (5% w/v) and allowed to stand thirty minutes. The mixture was then filtered through Whatman No. 3 filter paper, and the optical density of the filtrate measured at 280 mu with a Beckman DU spectrophotometer.

The TCA percipitated out all the protein from the solution so that only peptide fragments split off by the enzyme remained in the filtrate. The optical density of the filtrate is linearily correlated with the enzymatic activity present in the irradiated sample.

Calculation of $1/Y_{tr}$

Taking into account the various constants involved, the reciprocal of the Y_{tr} parameter (Eq. 1) may be calculated by:

$$\frac{1}{Y_{tr}} = \frac{(2.92)\ D_{37}}{10^3\ N_{tr}} \qquad (2)$$

where D_{37} is the dose in rads needed to inactivate 63% of the trypsin and N_{tr} is in μM/liter.

Detailed Procedure of One Experiment

To five, ten ml portions of 0.01% agar were added appropriate amounts of trypsin and 0.01M NaH_2PO_4 to make 20 ml portions of 0, 0.7, 1.0, 1.7, and 5 μM trypsin. These solutions were allowed to stand one hour before one ml aliquots of individual solutions were placed in the polystyrene vials for irradiation. This time was thought to be sufficient for equilibration of the anticipated enzyme-agar complex. Irradiation was carried out as described above.

The assay included the following blanks:

a. Unirradiated trypsin-agar solution.

b. Solution containing buffer, agar and Hb-urea. The DU was zeroed against this blank to eliminate any error due to autohydrolysis of the Hb-urea during the assay.

c. A solution of buffer and trypsin to which the Hb-urea and agar were added simultaneously. This blank was used to determine the total trypsin activity which could be recovered in the presence of agar under conditions where the formation of trypsin-agar complexes should be minimal.

RESULTS AND DISCUSSION

Trypsin Alone

The data shown in Figure 1 are typical of plots of the log residual enzyme activity remaining vs dose delivered for different concentrations of irradiated trypsin. This is in agreement with results of previous workers (7) and implies:

$$\frac{N_D}{N_0} = e^{-\alpha D} \tag{3}$$

or equivalently:

$$\ln N_D = \ln N_0 - \alpha D \tag{3a}$$

where the number of active molecules N_D remaining after a dose D is proportional to the optical density observed with the Hb assay.

For each line of Figure 1 the radiation dose was determined at which 37% of the original activity remains, i.e.,

$$OD_{37} = 0.37\ (OD_0) \tag{4}$$

The parameter D_{37} is a convenient measure to use because:

$$e^{-1} = 0.37 = e^{-\alpha D_{37}} \tag{5}$$

so that:

$$\alpha D_{37} = 1 \tag{6}$$

$$\alpha = 1/D_{37} \tag{7}$$

The following table shows the parameters derived from the lines in Figure 1 in which the data have been normalized to a common point to better demonstrate the relationship of the slopes.

TABLE 1.--Parameters calculated from Figure 1 in which various concentrations of trypsin were irradiated and equation (2) applied to the resulting data.*

$N_{tr}(\mu M)$	0.4	0.7	1.0	1.7	5.0
$1/N_{tr}$	2.25	1.43	1.0	0.59	0.2
D_{37} (rads)	1610	3010	3330	3550	14940
D_{37}/N_{tr}(rads/μM)	3620	4320	3330	2090	2950
$1/Y_{tr}$	8.6	6.0	9.7	12.5	11.7

*Similar tables corresponding to Figures 2a, 2b, 5, and 7 are found in Appendix B.

These data are plotted in Figure 6 (0% agar) as $1/Y_{tr}$ vs $1/N_{tr}$. The $1/S_{tr}$ ($1/Y_{tr}$ value at the intercept $1/N = 0$) agrees with the values of 8.4 previously reported for trypsin by L. G. Augenstein (7) and E. S. Augenstein (6).

The reactive capacity of the contaminants in the water can be equated to the reactive capacity of an amount of trypsin which can be designated N^{o}_{tr}. This equivalence is obtained from Equation (1). For that concentration at which $1/Y_{tr}$ is equal to $2(1/S_{tr})$ then:

$$z_{c,j} R_{c,j} N_c = z_{tr,j} R_{tr,j} N_{tr}^{c}$$

in which the superscript (c) denotes anything besides trypsin which can react with the j^{th} radicals. From the slope of the line in Figure 2 it was determined that when $1/Y_{tr} = 2(1/S_{tr})$ the value of $1/N_{tr}$ would be 4.9. Thus, N_{tr}^{c} is only 0.2 μM/liter.

Trypsin in Agar

Initially trypsin was irradiated in two different concentrations of agar 0.0025 and 0.005% with relatively small doses of x-rays. Figures 2a and 2b show the log residual activity vs dose plots of this data. It was expected that the $1/Y_{tr}$ vs $1/N_{tr}$ plot would result in three straight lines of differing slopes with a common intercept. This would indicate that the agar was providing competitive protection to the trypsin. The plot in Figure 3 does not display the expected results. The characteristic of the curves is that as the concentration of trypsin decreases, and the concentration of agar increases there is a marked increase in the slope of the curve. This would appear to be inconsistent with the basic Equation (1). To correct this apparent inconsistency, the following model was postulated on the basis of more extensive experiments: when agar is placed in solution with trypsin there are three forms of trypsin present--

a. an unbound fraction which reacts with radiation products similarly to trypsin in "free" solution with little competitive material present;

b. a complexed fraction available for enzymic assay but relatively very radiation resistant;
and

c. a fraction which is unavailable for assay either due to binding in agar micelles or due to destruction of enzyme activity during the initial exposure to agar.

If fraction (c) were constant for all ratios of agar to trypsin then no difficulty would be encountered in a plot such as shown in Figure 3. If, however, the proportion of (c) increases or decreases with a change in the agar plus trypsin ratio, this would result in a change in the amount of trypsin available for assay and consequently a change in the effective trypsin concentration and inactivation yield. In particular, if fraction (c) is appreciable the values of N_{tr} used in Figure 3 could be much too large.

In order to overcome this difficulty, it was necessary to modify the analytical method slightly. Figure 4 illustrates the method of analysis used to determine the effective concentration of trypsin present.

The quantity B-C is presumably unbound trypsin in solution which competes with the agar and bound trypsin for the radicals present. The A value is obtained by adding the Hb-urea and agar to the trypsin at the same time. This is the total amount of activity recoverable when presumably the agar-trypsin complex does not form.

The solutions irradiated in the experiments represented by Figures 1, 2, and 5 were all made by adding trypsin to the buffer-agar solution. Another series of experiments (Figure 7) were run in which the solutions for irradiation were made by adding agar to the buffer-trypsin mixture. A typical result of these experiments is shown in Figure 5. A comparison of the curves in Figure 6 labeled "0.005% agar + trypsin" (trypsin added to agar) and "trypsin + 0.005% agar" demonstrates that when agar is added to trypsin the nature of the complex is changed so that the modified method of analysis (Figure 4) no longer gives linear $1/Y_{tr}$ vs $1/N_{tr}$ plots as would be expected: apparently in this instance this method is no longer appropriate for determining the amount of trypsin available for assay and/or inactivation. The general effect observed when trypsin is diluted with agar is an increase in the quantity of residual activity remaining after a given dose of irradiation. This discrepancy indicates that local concentration gradients may play an important role in determining the extent of adsorption and/or the irradiation yield which is observed. It is important to emphasize that the curve in Figure 6 labeled 0.005% agar + trypsin is linear, and its intercept compares favorably with the values found previously for trypsin in solution alone (6, 7).

A longer experiment was attempted, the object of which was to standardize the data by making all irradiation

solutions from the same agar and trypsin. An attempt was made to irradiate the usual trypsin concentrations in 0, 0.0025, and 0.005% agar. This experiment necessitated the storage of the samples for approximately forty-eight hours due to the extended time required for irradiation. It was found that the analysis did not follow the previously observed pattern. This indicates that when trypsin and agar are allowed to interact for long periods of time the nature of the complex, and the components of the solution change in such a way that their behavior when exposed to radiation is unpredictable. The results obtained from this experiment may be due to a change in the state of aggregation of both trypsin and agar, or even simply to an aging of the trypsin itself. When a tryspin solution is allowed to stand for a period of time the original activity is known to decrease. This is attributed to auto-digestion of the trypsin. An explanation of the mechanism of auto-digestion might provide some insight into the problem of cell differentiation.

It is possible to determine the concentration of a trypsin solution using the optical density and the molar extinction coefficient of the trypsin. When this is attempted with the trypsin-agar solution the calculated concentration is 50 to 100% greater than the known amount of trypsin added. This suggests that the formation of the trypsin-agar complex is, in some way increasing the ability of trypsin to absorb ultraviolet light at its characteristic wavelength. An

absorption spectrum run on a Carey Recording Spectrophotometer showed that there is an increase in absorbance at 280 and 250 mu in proportion to the agar concentration present. The explanation of this observation is not obvious.

Hutchinson (24, 25, 26) has carried out a series of experiments in which he irradiated yeast cells in both a wet and dry state in an attempt to determine the radiation sensitivity of the enzymes invertase, alcohol dehydregenase, and co-enzyme A in intact cells. He defines two parameters in his experiments: the yield and the distance which the reaction intermediates diffuse before reacting with an enzyme molecule. He measured inactivation yields by standard enzyme assays, and the distance of diffusion was calculated from the difference between the inactivation in the wet and dry situations. In particular, he assumed that the sensitivity of the enzymes remains the same whether the molecule is wet or dry, and that recovery of enzymic activity is not modified by irradiation. On the basis of the information presented here it appears he is not justified in assuming that there is any simple relationship between water and other constituents of the cell. In the present results it is clear that concentration effects can alter radically the behavior of the radiation sensitive entities with respect to inactivation. Most importantly he made no correction for the proportion of enzyme which may be within the cell bound in a fairly permanent condition and thus unavailable to radiation inactivation.

Previous studies have also shown that irradiation can "liberate" active enzyme from solid gels (6). The present results also clearly show two fractions of trypsin having widely different radiation sensitivities, but both having D_{37}'s much less than "dry" tryspin.

In another series of experiments Fletcher and Okada (16, 17, 30) have studied the effect of added adsorbing materials on the radiation inactivation of deoxyribonuclease. They recognized only two fractions of enzyme in solution which are both assayable; a bound and unbound portion. The present results indicate that either the adsorbents and solute molecules in their systems behave differently toward radicals than do trypsin and agar, or else they may have ignored a significant portion of the enzyme present in making their calculations and assumptions. Furthermore, they postulate that the only plausible mechanism to explain the protective effect of adsorbents such as cellulose on DNAse I (17) is the effective reduction of the water content in the region of the molecules concentrated at the interface. Comparison of the two curves in Figure 6, labeled "0.005% agar + trypsin (with correction)" and "0% agar" shows that there is a definite protective effect exhibited when agar is present in solution. The $1/N_{tr}$ vs $1/N_{tr}$ plots just mentioned show the protective effect on fraction (a) is competitive in nature. The trypsin in fraction (b) is also protected but to a much greater extent. This portion has been subtracted out from

the data in Figure 6 by the method of analysis used (Figure 4). It appears unlikely that the bound trypsin is actually the protective agent which functions to absorb radicals inasmuch as the corrected agar line in Figure 6 is linear. Thus, presumably the extent of radiation damage to fraction (a) is limited by the constant amount of agar added. The constancy of the intercepts in the $1/Y_{tr}$ vs $1/N_{tr}$ plots indicate that there is no reactive protection provided to fraction (a) by fraction (b). If interaction between the two fractions of trypsin existed, then a shift in the intercept ($1/S_{tr}$) should be observed. The competitive nature of the protection implies that the agar can destroy radicals potentially capable of inactivating trypsin molecules in fraction (a).

The D_{37}'s for fraction (b) molecules is ca 5×10^4 rads. This value is 10^2 - 10^3 less than the D_{37}'s reported for dry trypsin preparations. Thus, even though the fraction (b) molecules are presumably bound to the agar, they are not in a virtual state of dryness. If as suggested by Fletcher and Okada, the decrease in radiation sensitivity of adsorbed materials is primarily a concentration effect, then calculating from the D_{37}'s for fraction (b) the materials in that fraction have an effective concentration of 20 μM trypsin.

CONCLUSION

This work has shown that there exists in a trypsin solution, which contains agar, at least three fractions of enzyme. One of these fractions is almost as sensitive to radiation as trypsin in "free" solution. A second fraction requires inactivating doses ten times larger, but 10^2 -10^3 times less than those required to inactivate "dry" preparations. The sensitivity of this fraction in a 0.7 μM trypsin solution is characteristic of trypsin molecules in a 20 μM trypsin solution with no agar present. Presumably this decrease in sensitivity reflects the formation of agar-trypsin complexes. The third fraction has an undertermined sensitivity due to its lack of availability for assay.

The inactivation of the most sensitive fraction is decreased by the presence of the agar. This protection results from competition between agar and trypsin for radiation produced radicals rather than a reaction between agar and trypsin molecules included in this fraction.

The data presented indicate the difficulty in interpreting the *in vivo* effects of radiation upon specific cellular constituents which numerous authors have reported. The results of these experiments show that even in such a relatively simple system as agar-trypsin mixtures, the interactions between the two elements profoundly change the

radiation response, and in fact the nature of the interactions of these two compounds depend upon such a simple factor as the order in which they are mixed.

APPENDIX A

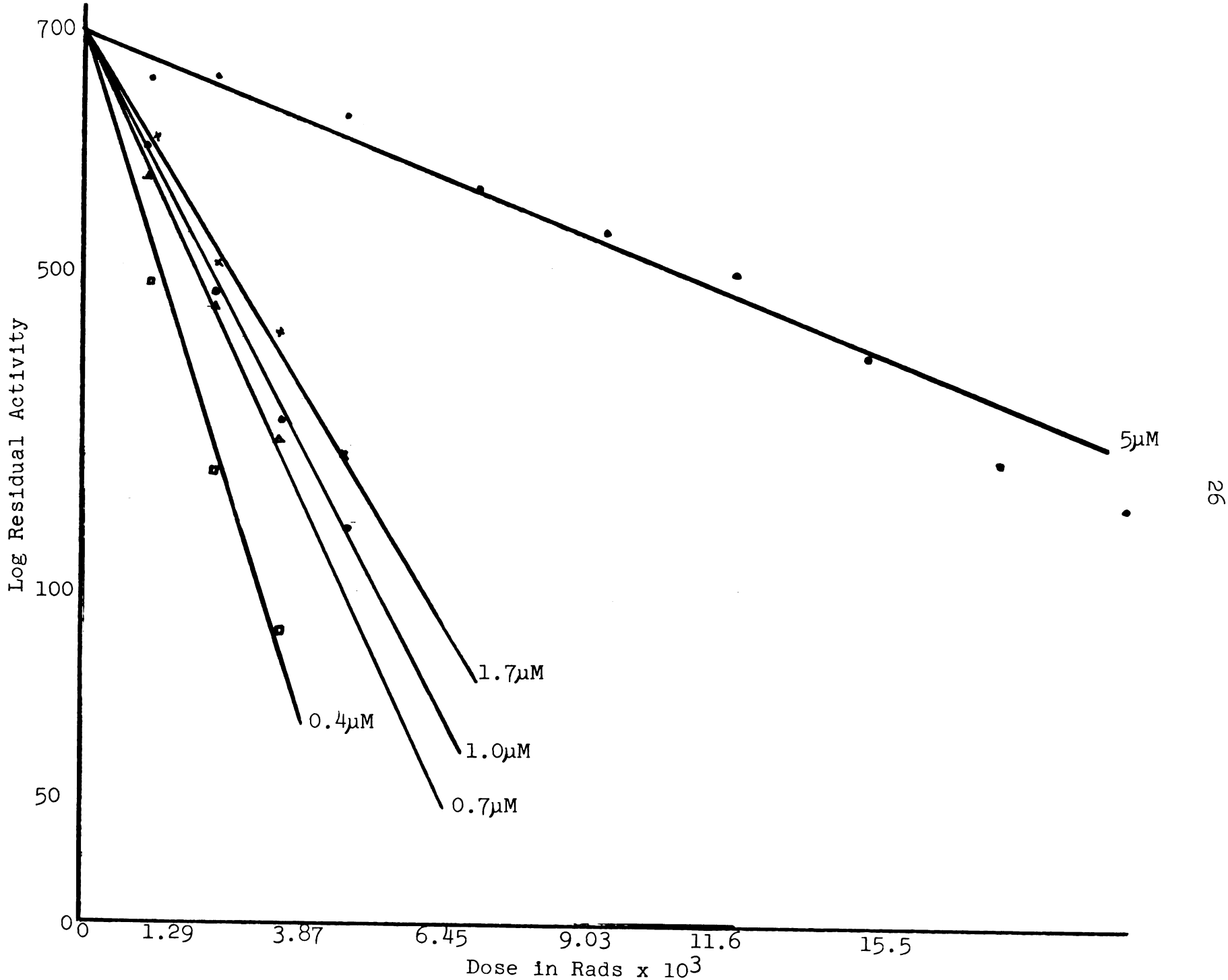
700
500
100
50
0
Log Residual Activity
0
1.29
3.87
6.45
9.03
11.6
15.5
Dose in Rads x 10^3
5μM
1.7μM
1.0μM
0.7μM
0.4μM

Fig. 1

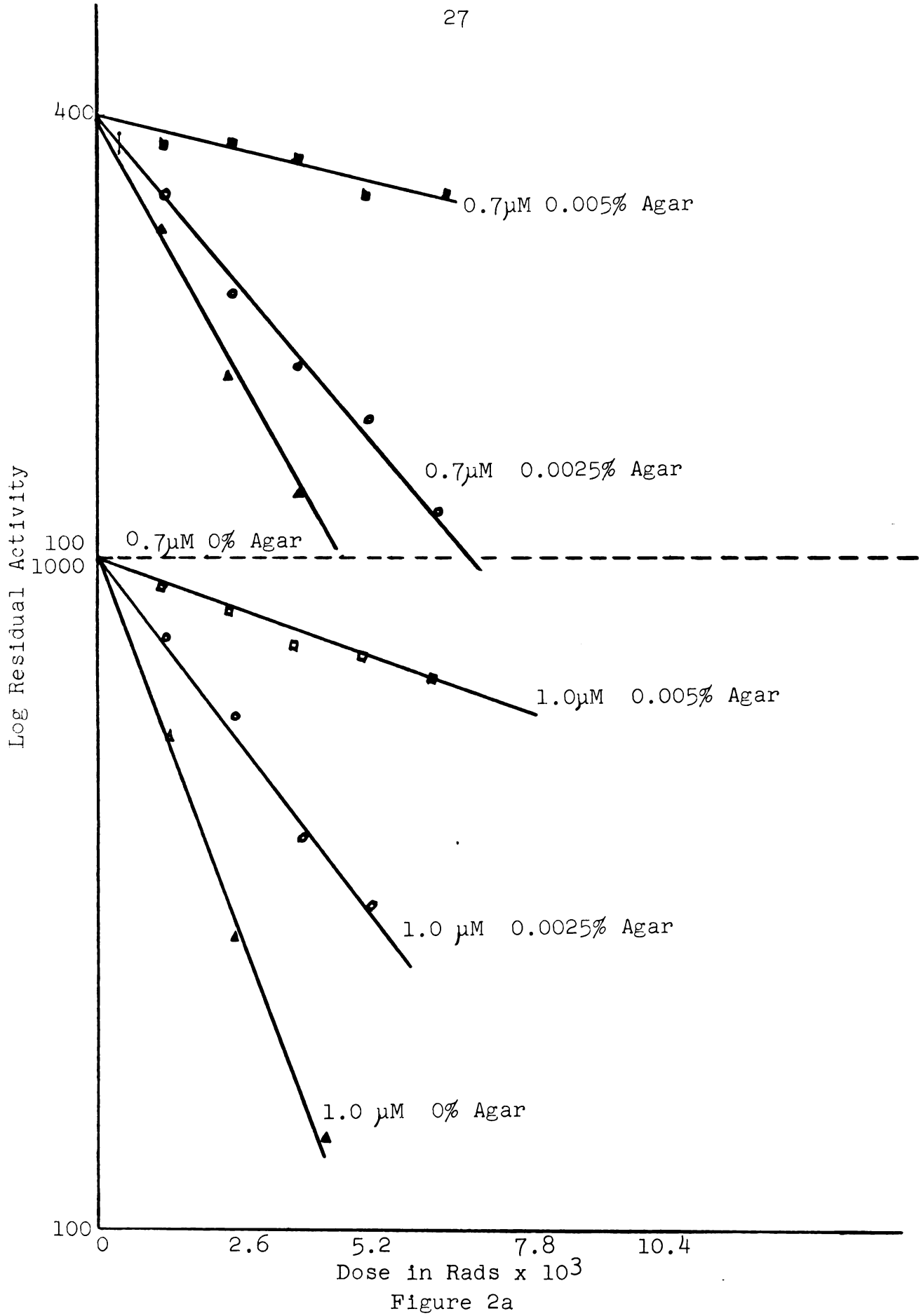
400
100
1000
100
Log Residual Activity
0.7μM 0.005% Agar
0.7μM 0.0025% Agar
0.7μM 0% Agar
1.0μM 0.005% Agar
1.0 μM 0.0025% Agar
1.0 μM 0% Agar
0
2.6
5.2
7.8
10.4
Dose in Rads x 10³

Figure 2a

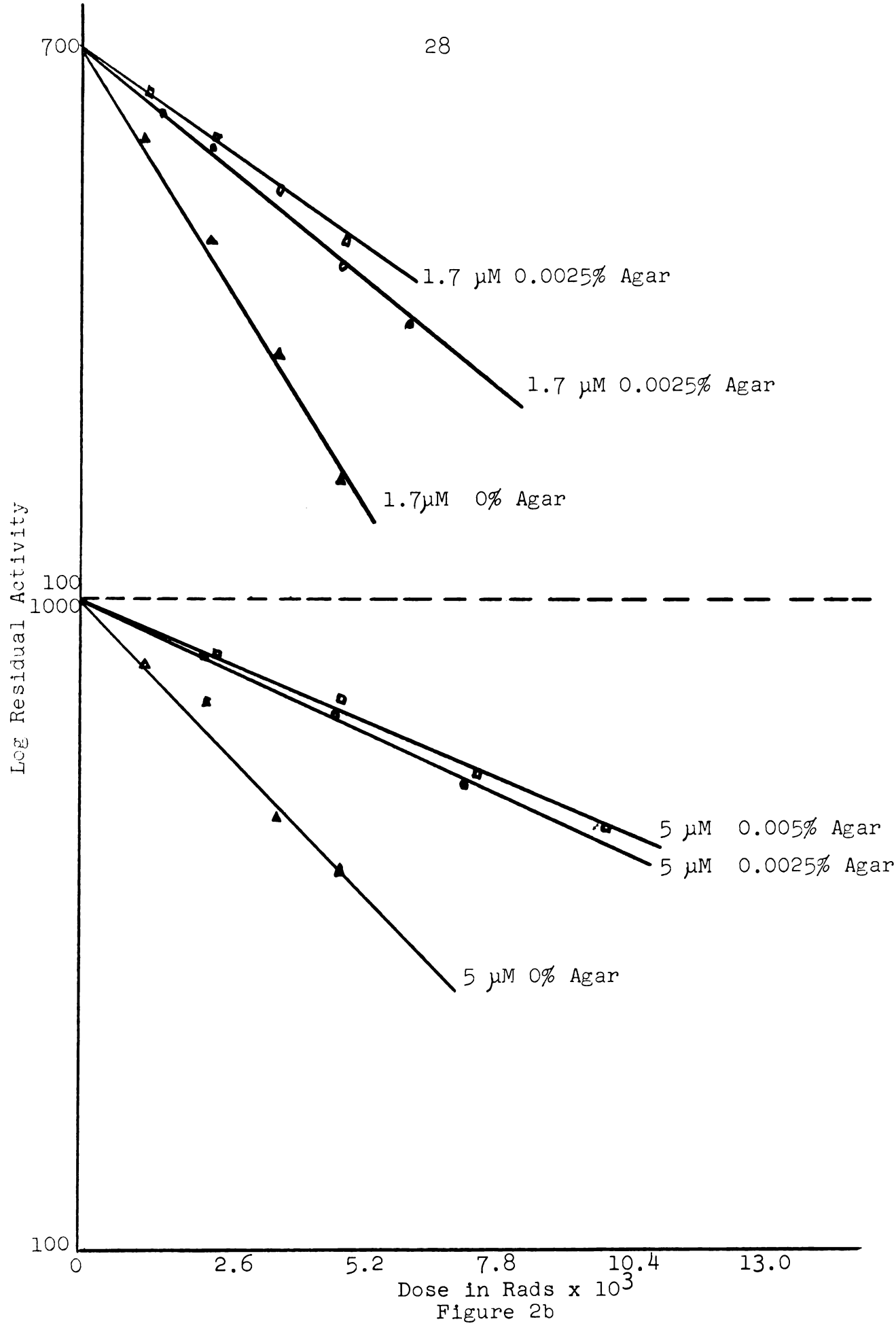
700
1.7 µM 0.0025% Agar
1.7 µM 0.0025% Agar
1.7µM 0% Agar
100
1000
Log Residual Activity
5 µM 0.005% Agar
5 µM 0.0025% Agar
5 µM 0% Agar
100
0
2.6
5.2
7.8
10.4
13.0
Dose in Rads x 10^3

Figure 2b

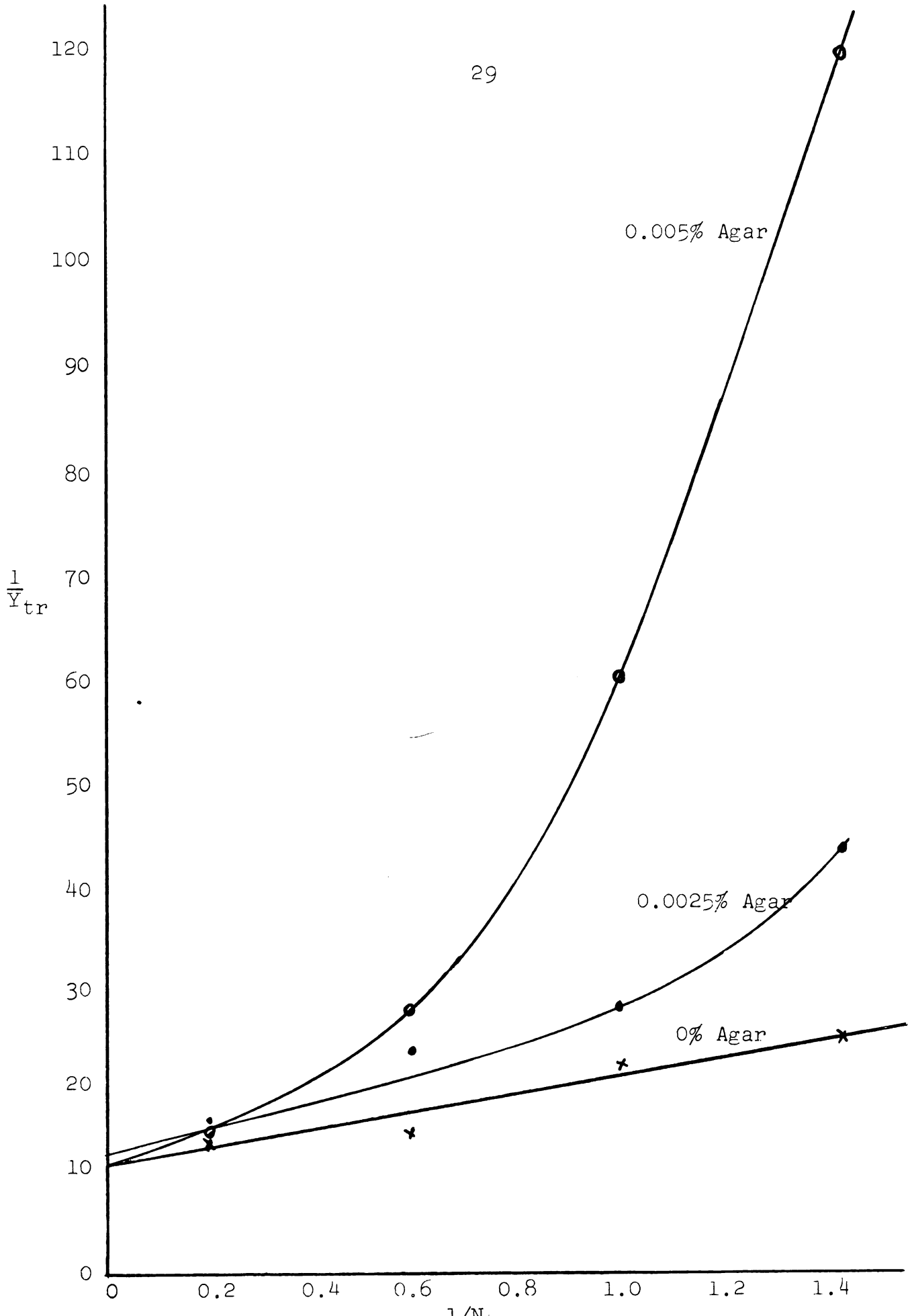
120
110
100
90
80
70
60
50
40
30
20
10
0
$\frac{1}{Y_{tr}}$
0.005% Agar
0.0025% Agar
0% Agar
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
$1/N_{tr}$

Figure 3

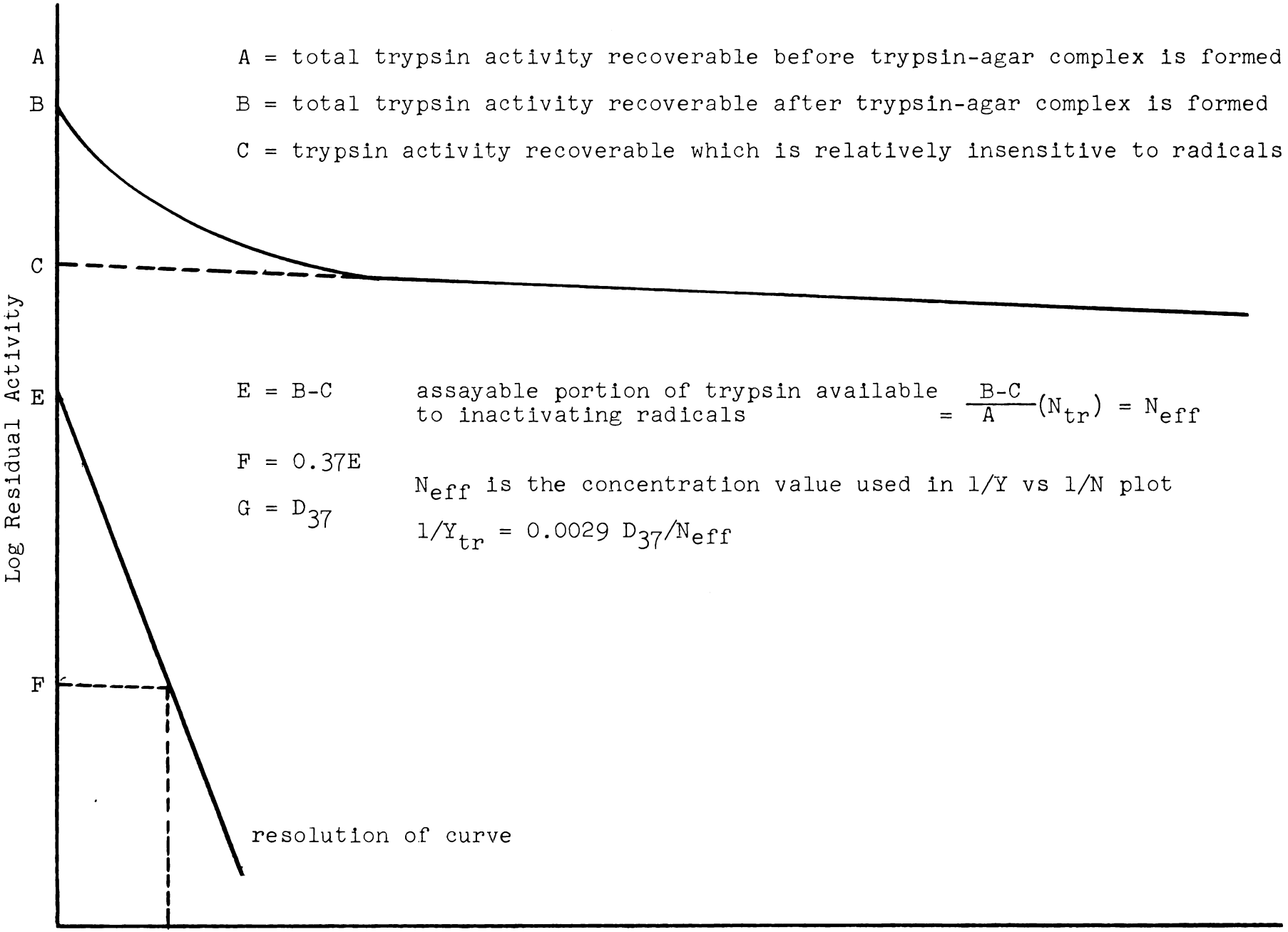
A
B
C
E
F
G
Log Residual Activity
A = total trypsin activity recoverable before trypsin-agar complex is formed
B = total trypsin activity recoverable after trypsin-agar complex is formed
C = trypsin activity recoverable which is relatively insensitive to radicals
E = B-C
assayable portion of trypsin available to inactivating radicals = $\frac{B-C}{A}(N_{tr}) = N_{eff}$
F = 0.37E
G = D_{37}
N_{eff} is the concentration value used in 1/Y vs 1/N plot
$1/Y_{tr} = 0.0029\ D_{37}/N_{eff}$
resolution of curve
Dose in Rads

Figure 4

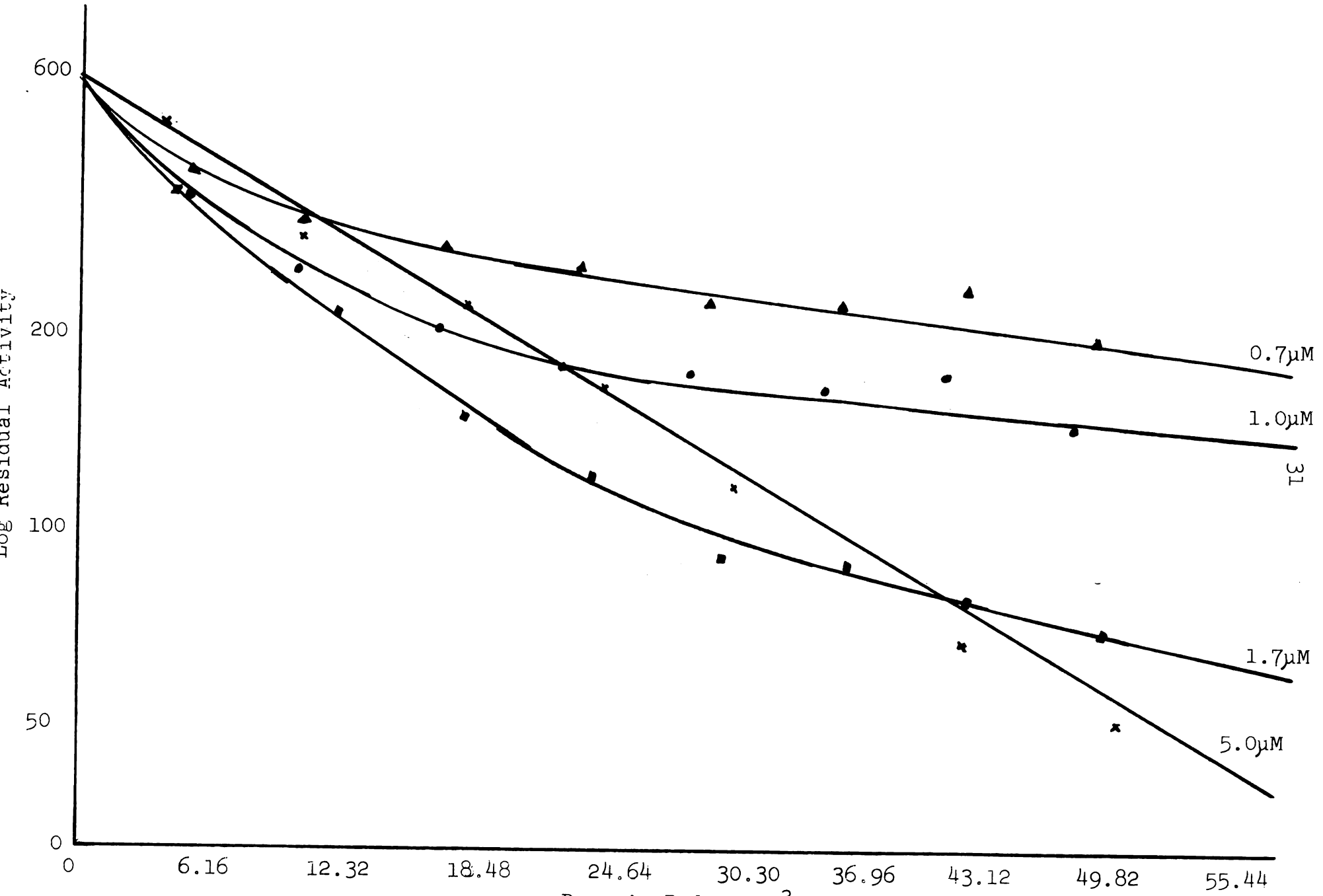
600
200
100
50
0
Log Residual Activity
0
6.16
12.32
18.48
24.64
30.30
36.96
43.12
49.82
55.44
Dose in Rads x 10^3
0.7μM
1.0μM
1.7μM
5.0μM
31

Figure 5

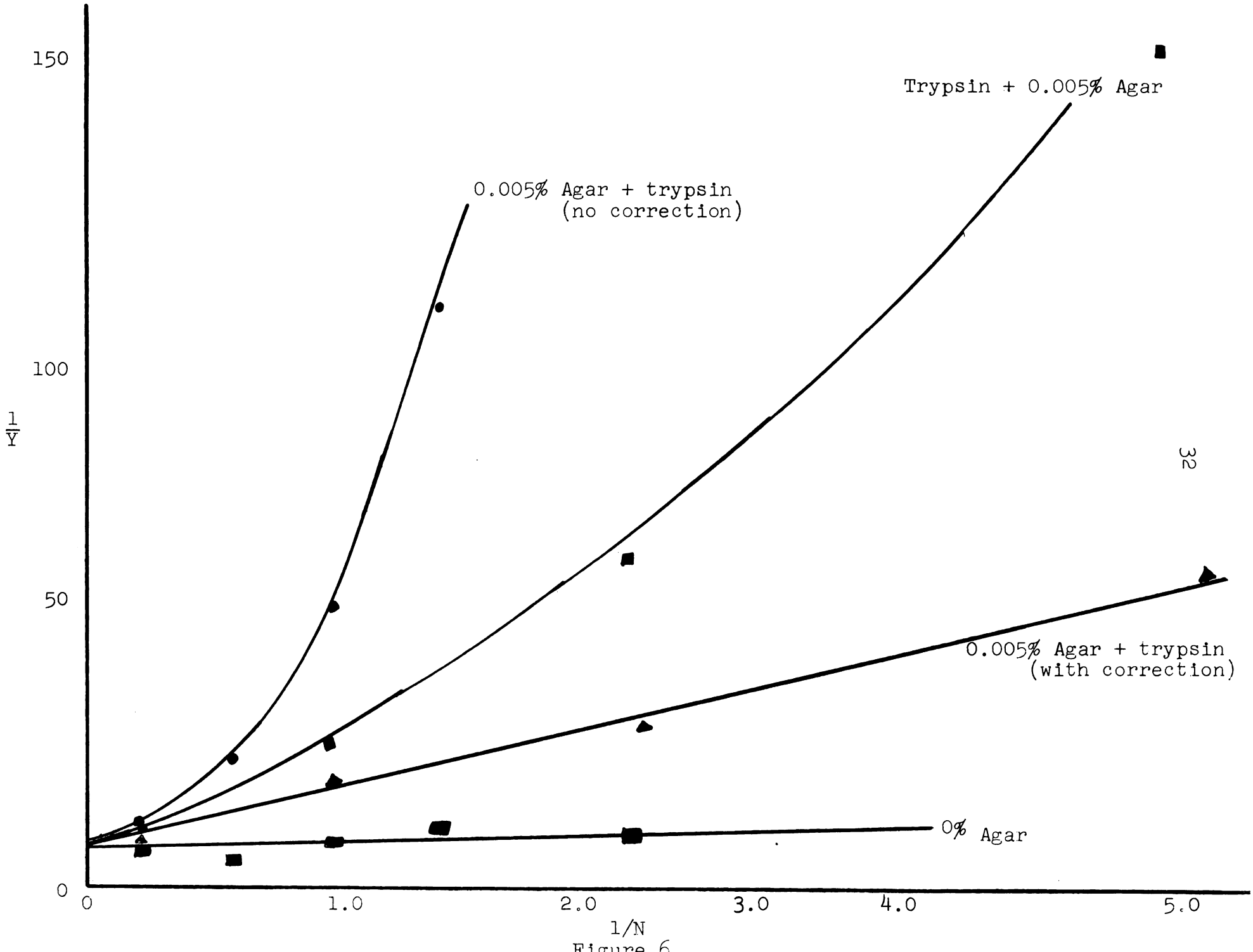
150
100
50
0
1/Y
0
1.0
2.0
3.0
4.0
5.0
1/N
Trypsin + 0.005% Agar
0.005% Agar + trypsin
(no correction)
0.005% Agar + trypsin
(with correction)
0% Agar

Figure 6

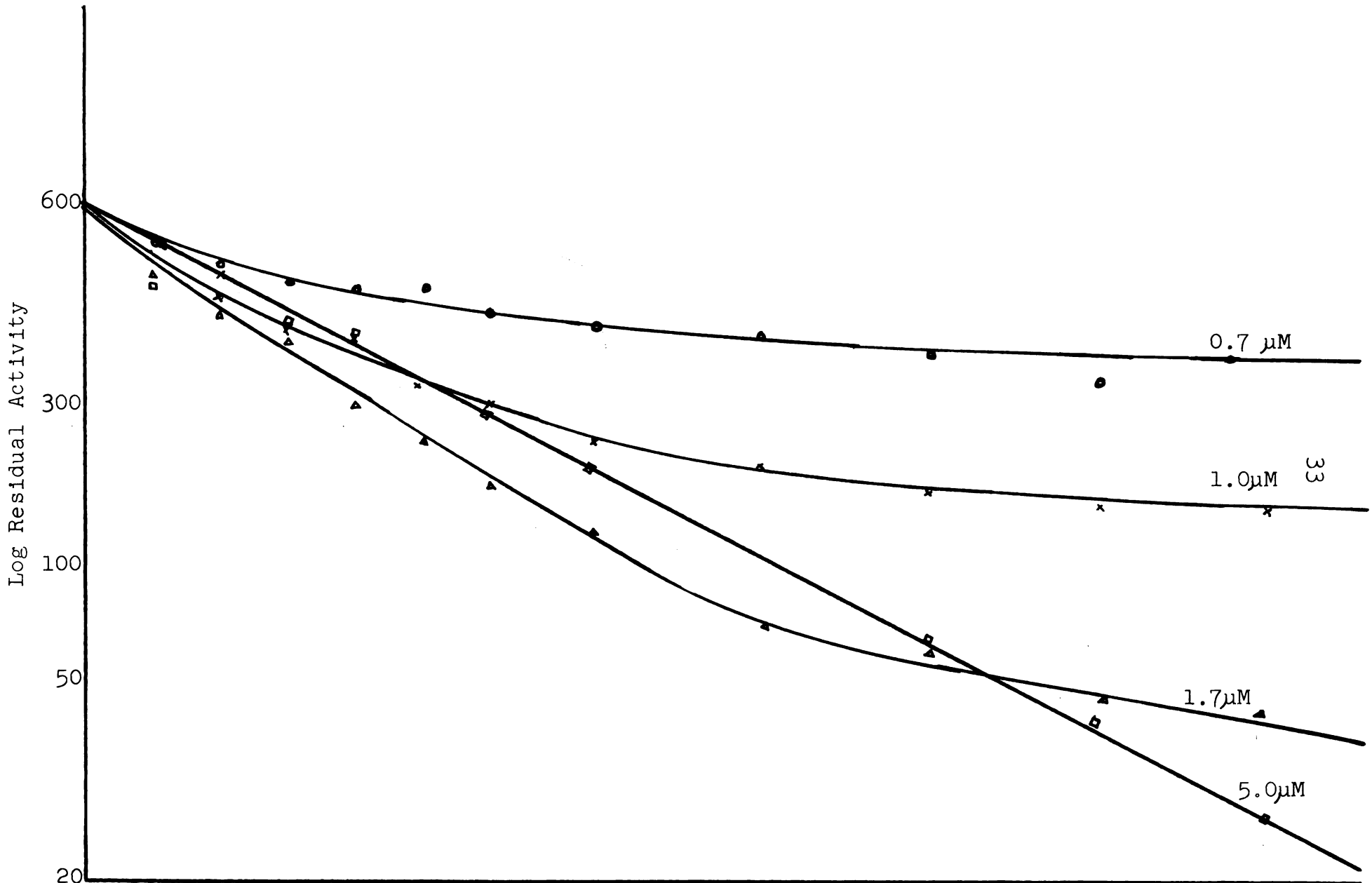
600
300
100
50
20
Log Residual Activity
0.7 µM
1.0µM
1.7µM
5.0µM
0
5.2
8.4
15.6
20.8
26.0
31.2
36.4
41.4
46.6
Dose in Rads x 10³

Figure 7

APPENDIX B

TABLE 2.--Calculations from Figures 2a, b Using Equation 2

	0% Agar			
$N_{tr}(\mu M)$	0.7	1.0	1.7	5.0
$1/N_{tr}$	1.43	1.00	0.59	0.2
D_{37}(rads)	5460	6900	7800	20,800
D_{37}/N_{tr}(rads/μM)	7820	6900	4600	4160
$1/Y_{tr}$	22.7	20.0	13.3	12.1
	0.0025% Agar			
$N_{tr}(\mu M)$	0.7	1.0	1.7	5.0
$1/N_{tr}$	1.43	1.00	0.59	0.20
D_{37}(rads)	10,300	8850	12,600	24,700
D_{37}/N_{tr}(rads/μM)	14,720	8850	7440	4950
$1/Y_{tr}$	42.6	25.7	21.6	14.3
	0.005% Agar			
$N_{tr}(\mu M)$	0.7	1.0	1.7	5.0
$1/N_{tr}$	1.43	1.00	0.59	0.2
D_{37}(rads)	27,600	20,000	14,900	23,900
D_{37}/N_{tr}(rads/μM)	39,050	20,000	8,800	4,800
$1/Y_{tr}$	113	58.0	25.5	18.7

TABLE 3.--Calculations from Figure 5 Using Equation 2

	0.005% Agar			
$N_{tr}(\mu M)$	0.7	1.0	1.7	5.0
$N_{eff}(\mu M)$	0.2	0.44	1.01	5.0
$1/N_{eff}$	5.0	2.28	1.0	0.2
D_{37}(rads)	5240	6780	8780	22,800
D_{37}/N_{eff}(rads/μM)	26,200	15,450	8780	4560
$1/Y_{tr}$	76.0	44.8	25.5	13.2

TABLE 4.--Calculations from Figure 7 Using Equation 2

	0.005% Agar			
$N_{tr}(\mu M)$	0.7	1.0	1.7	5.0
$N_{eff}(\mu M)$	.08	.45	.98	5.0
$1/N_{eff}$	12.5	2.22	1.02	.2
D_{37}(rads)	11,700	10,500	10,400	21,100
D_{37}/N_{eff}(rads/μM)	146,300	23,300	10,600	4240
$1/Y_{tr}$	42.5	67.5	30.8	12.3

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