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Interaction of an RNA-Instructed DNA Polymerase with Rifamycin Derivatives and Non-ionic Detergents*

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Running title: RIDP activity with rifamycin derivatives and detergents

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ABSTRACT: Several rifamycin derivatives were tested for their inhibition of an RNA-instructed DNA polymerase (RIDP) activity extracted from virally transformed cells. One new drivative was found to be extremely effective -- inhibiting the enzyme activity 50% at 4 μg/ml. However, the inhibition by all the rifamycin derivatives was shown to be significantly altered by small changes in the assay concentration of the non-ionic detergents used to solubilize and stabilize the RIDP activity. Evidence is presented relating decreased inhibitory effectiveness at high detergent concentration to micelle formation. The detergents were also shown to be important activators of the RIDP activity at very low concentrations.

Introduction

The discovery by Baltimore (1970) and Temin and Mizutani (1970) of a DNA polymerase which can utilize RNA as its template, and its relatively specific association with oncogenic viruses, has opened a new area of research on possible chemotherapy for virus-related cancers. Certain rifamycin derivatives serve as effective antibiotics by specifically inhibiting the bacterial DNA-dependent RNA polymerase (Wehrli and Staehelin, 1971). Such specificity for a particular polymerase has encouraged attempts to test many rifamycin derivatives for specific inhibition of the viral RNA-instructed DNA polymerase, RIDP (Gurgo et al., 1971; Yang, et al., 1972; Green, Bragdon & Rankin, 1972; Gurgo, Ray & Green, 1972). Most of this testing of rifamycin derivatives has been done in the presence of a non-ionic detergent using purified whole virus particles (MSV, FeLV or AMV) as an RIDP source.

Eight new rifamycin derivatives (Tischler, Joss & Calvin, to be published) and six previously tested rifamycin derivatives were compared for their inhibitory effect on an RIDP activity, as a function of the concentration of non-ionic detergents. The RIDP activity used was

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Abbreviations: (M), Moloney strain; MSV, murine sarcoma virus; MLV, murine leukemia virus; FeLV, feline leukemia virus; AMV, avian myeloblastosis virus; RSV, Rous sarcoma virus; dpm, disintegrations per minute; cpm, counts per minute.

easily extracted from virally transformed cells but could not be detected in uninfected, non-transformed cells. We were able to show that the non-ionic detergent is an important activator of the RIDP as well as being required for efficient solubilization and extraction of the RIDP activity. On the other hand, at detergent concentrations significantly greater than those required for full RIDP activation, we were able to show that the rifamycin derivatives lose inhibitory effectiveness. This loss of effectiveness could be correlated to the formation of detergent micelles and to the hydrophobic characteristics of the rifamycin derivatives, suggesting an extraction of the inhibitors into detergent micelles. We were able to prove this extraction qualitatively with fluorescence techniques and quantitatively with gel chromatography of a rifamycin derivative in the presence of a detergent.

Materials

Tissue Culture Cells and Viruses. Dr. A. J. Hackett (Cell Culture Laboratory, University of California, Berkeley -- PH43-63-13, Special Virus-Cancer Program) generously provided the tissue culture cells, both cell line UC1-8 (Hackett & Sylvester, in press) and cell line Balb/3T3 clone A31 (Aaronson & Todaro, 1968). When received in this laboratory, the cells had been seeded in 250 ml plastic flasks (75 cm^2) at 10^6 cells per flask. Some of the cultures had been infected with virus 24 hours later. UC1-B cells had been infected with 10^4 focus forming units of MLV(M), and clone A31 cells had been infected with 10^4 focus forming units of MSV(M). Cells were grown in Eagle's

minimal essential media with 10% fetal bovine serum and had been fluid changed on the 4th day post seeding. The cells were usually received on the 5th day and the RIDP extracted on the 7th day.

Rifamycin Derivatives. The structures for all of the rifamycin derivatives used in this work are given in Table 1. The derivatives rifamazine, rif-urea, dirifampin, rifazabicyclo-9, rifazacyclo-16, aminodesmethylrifampicin and spin-labeled rifampicin 1 were synthesized in this laboratory by Allan Tischler (to be published). The derivatives DMB, ^{3}H-DMB, DMB-oxidized, dansyldesmethylrifampicin, and spin-labeled rifampicin 2 were synthesized in this laboratory by Urs Joss (to be published). Rifamycin derivatives rifaldehyde octyloxime, rifampicin and desmethylrifampicin were provided by Gruppo Lepetit, S.p.A., Milan, Italy.

Detergents. Triton X-100 (Rohm and Haas) is the octyl phenoxy polyethoxy ethanol mixture obtained when one mole of the octylphenol is reacted with 9-10 moles of ethylene oxide.* Only 93.6% of the Triton X-100 was dialyzable in hollow fiber dialysers with a molecular weight cut off of 5,000 (Dow Chemical Company; Bio-Rad Laboratories), even when the detergent was prevented from clogging the dialysis pores by alternating positive and negative pressure. A more defined Triton X-100 was prepared by chromatographing Triton X-100 on silica gel (Biorad, Biosil A, 200-235 mesh) according to the method of Kelly

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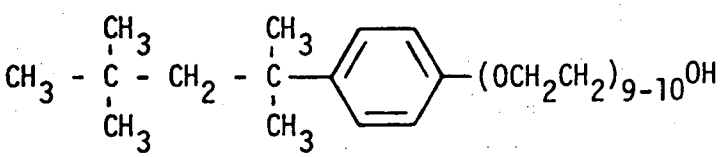
* 

(Kelly & Greenwald, 1958). Triton X-100 molecules containing 10 to 17 polyoxyethylene residues were pooled (yield = 17.6%) and are referred to in this paper as Triton X-1017. Nearly all (99.8%) of the Triton X-1017 was dialyzable. Triton DN-65 (a gift from Rohm and Haas) is a non-ionic detergent prepared by reacting one mole of a mixture of n-octyl and n-decyl alcohols with approximately seven moles of ethylene oxide and approximately two moles of propylene oxide. Brij 35 (Sigma) is the polyoxyethylene-23-lauryl ether obtained by reacting one mole of lauryl alcohol with 23 moles of ethylene oxide.

Other Reagents. Poly-rA:oligo-dT (Collaborative Research, Inc.) contains the nucleotides in molar proportions and the oligonucleotide is 12-18 nucleotides in length. The ^{3}H-dTTP was obtained from New England Nuclear Company. The 2-[p-toluidinyl]naphthalene-6-sulfonate (Sigma) was recrystallized twice from a water-ethanol mixture before use.

Methods

RIDP Assay. RIDP activity as used in this paper is defined by the following procedure. Assays were done in 100 µl which is 82-94 mM Tris-HCl (pH = 7.8), 100 mM KCl, 0.2 mM dithiothreitol (DTT), 0.02 mM ^{3}H-dTTP (1 C/mmole), 0.1 mM $MnCl_2$, 2-4% glycerol and contained 1 µg poly-rA:oligo-dT ($\sim$ 0.015 OD_{260}). All assays were started by the addition of a chosen amount of enzyme extract and were incubated for 30 min at 37°C. The rate of incorporation of ^{3}H-dTTP into acid-insoluble material was constant for 30 min but decreased for reaction times greater than 30 min., probably due to filling of available sites on the poly-rA (Baltimore & Smoler, 1971). However, the units

of activity were calculated as picomoles of ^{3}H-dTTP incorporated into acid insoluble material per hour. The acid insoluble material (in 6.7% trichloroacetic acid, TCA) was collected on 0.45 μ Millipore filters (presoaked in 0.02 M $Na_4P_2O_7$) and washed extensively with 5% TCA. After thorough drying, the filters were dissolved in scintillation fluid containing ethanol and dioxane, and fumed colloidal silica was added to form a stable gel. This gel was counted in a scintillation counter and an automatic external standard was used to obtain dpm from cpm. There was a noticeable quenching in samples from assays which contain relatively high concentrations of the rifampicin derivatives. For addition to the assays, the rifamycin derivatives were first dissolved in dimethylsulfoxide (DMSO) at 10 mg/ml and then appropriately diluted with 0.01 M Tris-HCl (pH = 7.8). Control activity assays without the rifamycin derivatives were done in the presence of corresponding amounts of DMSO. Exact assay concentrations of the derivatives, DMSO, detergent and protein were variable and are given in the text and figure legends.

Protein Assay. Protein concentrations were determined using a Lowry method (Lowry et al., 1951). The Triton X-100 and the high salt in the buffer sometimes gave a precipitate which was centrifuged out before the optical densities of the solutions were measured. Triton X-100 was checked for interference with the protein assay using crystalline bovine serum albumin as a protein source. At the concentrations usually present in our assays (20 μg/1.2 ml Lowry), the detergent produced no detectable interference. However, higher Triton X-100 concentration gave falsely high protein values.

Standard samples containing BSA and buffer A (see next section) were run with each set of determinations.

RIDP Extraction. The extraction procedure used was adapted from that proposed by Ross, et al. (1971). On the 7th day post seeding, 2 flasks of transformed cells or 3-4 flasks of non-transformed cells were used in the following extraction of the RIDP activity. The growth media was decanted, and the cells in each flask were washed three times with 25 ml phosphate-buffered saline (0.01 M sodium phosphate, pH = 7.2 and 0.9% NaCl). The cells were removed from each flask with the aid of a rubber policeman and 3 ml buffer A. Buffer A was 0.05 M Tris-HCl (pH = 7.8), 0.5 M KCl, 0.001 M dithiothreitol (DTT) and 20% glycerol. All subsequent steps were carried out at 0°C. The cells from the 2 transformed or 4 non-transformed flasks were pooled separately and centrifuged at 2500 g for 15 min. The resulting cell pellet was placed in the vessel to be used for sonication and covered with 2 ml of buffer A. The cells were then exposed to four 5-second pulses of mild sonication (the power used on a Branson Sonifier (W-185) was very near the minimum required to disperse and break the cells). Two ml of saturated ammonium sulfate in 0.05 M Tris-HCl (pH = 7.8) and 0.001 M EDTA were added to the sonicate and allowed to equilibrate for at least 30 min. The precipitate was collected by centrifugation at 27,000 g for 30 min and resuspended in 1.96 ml buffer A. Triton X-100 or Triton DN-65 was usually added to obtain a final detergent concentration of 0.1% in 2 ml. However, in some extractions the detergent concentration was varied (0-0.5%). All extracts, regardless of the detergent concentration, were then incubated for 15 min at 37°C and then centrifuged at 100,000 g for 60 min. The supernatant containing the RIDP activity

was stored at 4°C, and the RIDP activity in these extracts was stable for several months. The specific activity of transformed UCl-B extracts was usually between 200 and 300 pmol/hr/μg protein,but was occasionally as high as 500 pmol/hr/μg. The protein concentrations were typically between 0.7 mg/ml and 1.0 mg/ml.

Detergent Micelle Formation. A number of techniques exist for following micelle formation in solutions of detergents. A convenient method, applicable to aqueous solutions, is provided by 2-[p-toluidinyl]-naphthalene-6-sulfonate (TNS), one of a small number of compounds which fluoresce strongly in a non-aqueous environment while giving only a weak fluorescence in water (McClure & Edelman, 1966). Thus, below the critical micelle concentration (CMC), a detergent solution containing TNS has a weak fluorescence which is not strongly dependent on detergent concentration. Above the CMC, the fluorescence increases steadily as the detergent concentration is raised and the TNS is extracted into the micelle. The CMC of the detergent can be determined from the discontinuity in a plot of fluorescence vs. detergent concentration.

The fluorescence measurements described in this paper were made with a Perkin-Elmer MPF-2A fluorescence spectrophotometer equipped with a variable temperature cell holder. The excitation and emission wavelength used were 320 mμ and 440 mμ, respectively. Both slits were set to 10 mμ.

Results

RIDP Extraction. Significant levels of the RIDP activity extracted and assayed as described in Methods were found in tissue culture cells after infection with a transforming virus. Assays for the RIDP activity in extracts from uninfected, non-transformed cells, either A31 or UC1-B, never incorporated enough ^{3}H-TTP to give cpm significantly above background. We also found that the specific activity of the extracted RIDP is correlated with the number of foci of transformed cells in UC1-B cultures (produced either by inhibiting the viral transformation with DMB (Calvin, et al., 1971) or by infecting with fewer focus-forming units). This correlation would be expected if only transformed cells contained significant levels of RIDP activity.

The absence of RIDP activity in the uninfected cell extract was not due to low protein concentrations or the presence of an inhibitor in the extract or to accidental inactivation of the extract. By using more flasks of non-transformed cells, the volume of the cell pellets and the concentration of protein in the final extracts were nearly equal for transformed and non-transformed cells.Addition of a non-transformed cell extract caused only low levels of inhibition of the RIDP activity which could also be produced by equal amounts of BSA protein. In assays for DNA-dependent DNA polymerase (DDP) (0.10 OD_{260} activated calf thymus DNA; 0.1 mM dATP, dCTP and dGTP; 0.02 mM ^{3}H-dTTP; and 6 mM $MgCl_2$ were substituted for template, substrate, and divalent cation, respectively) the non-transformed cell extract yielded ~20% of the activity of the transformed cell extract. The five-fold difference between the DDP activity in the two extracts was probably due to the fact that the non-transformed cells were contacted inhibited at the seventh day and the transformed cells were not. However,

detection of the DDP activity in the non-transformed cell extract indicated the presence of active enzymes in that extract.

These studies on the RIDP activity found in virally transformed cells were facilitated by at least a 4-10 fold higher specific activity of the RIDP extractable from the cryptic cell line UC1-B than was extractable from the Balb/3T3 clone A31. The high levels of activity may be the result of the abnormal maturation of virus particles into cytoplasmic vacuoles (Hackett & Sylvester, in press) which could act as pockets of activity inside the cell. All of the data presented in this paper were obtained using RIDP activity extract d from UC1-B cells. However, the RIDP activities extracted from the two different cells lines were very similar, if not the same, since both RIDP activities were identical in their response to seven rifamycin derivatives (data not shown). Very little activity was found in either cell line during the first two days after infection. On the third day after infection significant levels of RIDP were detectable and increased with time until about the sixth day. By the eighth day of growth, the cells were coming loose from the cell layer especially in the A31 cell line. The extractions were normally done on the seventh day of growth, or six days after infection.

The optimum Triton X-100 concentration for solubilizing the RIDP in the extraction from transformed cells was determined to be 0.1% for protein concentrations near 1 mg/ml (Figure 1). Triton DN-65 and Triton X-1017 were also tested and were approximately as efficient in solubilizing the RIDP at concentrations of 0.1%. A large portion of the activity which is missing in the 100,000 g supernatant when low concentrations of Triton X-100 are used for solubilization can be found in the 100,000 g pellet.

RIDP Assay. Since the transformed UCl-B cells were a new source of RIDP activity, the optimum conditions for assay had to be established. The conditions given in Methods were determined by experimentation to be at or near optimum for the RIDP activity from transformed UCl-B cells with the exception of the pH. The optimum pH for this RIDP activity is between 8.3 and 8.7. However, assays done with a pH greater than 8.0 caused the rapid oxidation of the rifamycin derivatives (data not shown) and were not used for this reason. DTT concentrations between 0.2 mM and 2.0 mM yielded a constant amount of activity. KCl showed a broad optimum concentration which centered around 100 mM. Glycerol concentrations from 2-4% did not alter the activity but concentrations greater than 4% decreased the reaction rate. The poly-rA:oligo-dT and dTTP concentrations were nearly saturating and each yielded approximately 90% of the maximal activity. The principal difference between our assays and those described in the literature is the Mn^{++} concentration. The optimum Mn^{++} concentration was carefully determined and found to be between 0.05 mM and 0.13 mM (Figure 2).

The detergent concentration in the assay was also found to strongly influence the RIDP activity. As the concentration of detergent (Triton X-100, Triton DN-65, or Triton X-1017) was reduced in the assay below 0.004%, or approximately 0.05 mM, an increasing amount of activity was lost. The detergent requirement for full activity could be satisfied by Brij-35 but not by polyethylene glycol-400. The results are summarized in Figure 3. This activation by detergents was not altered by as much as a four-fold increase in the protein concentration of the assay (BSA added) or by the presence of DMSO from 0-0.4% in the assay.

Triton X-100 Effect on RIDP Activity. In order to determine if the loss in activity at the low detergent concentrations noted above was reversible two types of experiments were done. In the first experiment, an RIDP extract was diluted to 0.00085% Triton X-100 and kept at 0°C. The RIDP activity in this diluted extract was then assayed as a function of time at a constant, optimal Triton X-100 concentration (0.004%). As shown in Figure 4, about 50% of the RIDP activity was irreversibly lost after 30 min, with a half-time of approximately 10 min, followed by a slow loss of RIDP activity in the ensueing 24 hours. The lost activity could not be recovered even when the diluted extract was brought to 0.005% Triton X-100 and allowed to stand 30 min before an aliquot was taken for assay. The appropriate control, also shown in Figure 4, indicated that the loss of RIDP activity was due to the lowered detergent concentration and not to changes in glycerol or protein concentrations. The concentration of Triton X-100 in the RIDP extract could be diluted to as low as 0.0025% without loss of activity. However, dilution to 0.0015% Triton X-100 led to an irreversible loss of approximately 37% of the RIDP activity.

Since the rate of irreversible activity loss of RIDP activity had leveled off to approximately zero after 20 min, it was possible to do the second type of experiment. In this type of experiment, the activity of an RIDP extract, which had been partially (~50%) inactivated by exposure to low detergent for varying periods of time, was determined as a function of Triton X-100 concentration in the assay. As shown in Figure 5, the extent of RIDP activity in the stable but partially inactivated extract was still dependent on the detergent concentration in the

assay even though the irreversible inactivation was no longer a factor. Figure 5 also shows that the detergent activation of the partially inactivated RIDP did not change with increasing time of exposure of the RIDP to dilute detergent. Thus, by reducing the Triton X-100 concentration of the RIDP extract to 0.0007-0.0009% for 30 min and assaying at about the same concentration, approximately 80% of the RIDP activity was lost. Of that 80%, approximately 30% was recoverable by addition of detergent to the assay and the remaining 50% was not.

In addition to using aliquots of highly diluted extracts obtained with detergent solubilization, the minimal activity obtained in the 100,000 g supernatant without detergent solubilization was assayed for activation by Triton X-100. The results are shown in Figure 5. The detergent activation of this RIDP activity, extracted without solubilization, was identical to the activation of the solubilized RIDP. The above result suggests that the Triton X-100 is an activator of the RIDP activity in addition to being a solubilizing agent.

Detergent Effects on RIDP Inhibition by Rifamycin Derivatives. The quantity of a rifamycin derivative which was required to inhibit the RIDP activity from transformed UCl-B cells was also found to be dependent upon the detergent concentration present in the assays. Typical RIDP inhibition curves at two Triton X-100 concentrations are shown in Figure 6a for DMB, a commonly tested derivative which is known to be an effective viral RIDP inhibitor. In Figure 6b are shown typical RIDP inhibition curves at three Triton X-100 concentrations for rifazacyclo-16, a new derivative which was found to be a very effective RIDP inhibitor but also extremely sensitive to the variation in detergent concentration.

It is interesting to note at this point that the DMB inhibition curve at 0.005% Triton X-100 was exactly the same for RIDP extracted in the 100,000 g supernatant both with and without detergent solubilization.

Table 2 shows the amount of each of the rifamycin derivatives tested which was required to yield a 50% inhibition of the RIDP activity at three different Triton X-100 concentrations. The inhibition curves from which these half inhibition values were taken were only reproducible within a few µg. However, this reproducibility was maintained even though the RIDP extracts used were prepared on different days and contained differing amounts of protein and activity.

In order to determine more precisely the Triton X-100 concentration which would yield maximum RIDP inhibition by the rifamycin derivatives, experiments were performed at a constant derivative concentration and variable Triton X-100 concentration. The results for three derivatives are summarized in Figure 7. A Triton X-100 concentration of 0.005% allowed maximum inhibition by all three derivatives, even though their sensitivity to the detergent concentration was very different. Both lower and higher concentrations of Triton X-100 caused a reduction in the RIDP inhibition by the rifamycin derivatives. The reduced ability of the derivatives to inhibit the RIDP at low detergent concentrations may be an artifact caused by the reduced enzyme activation occurring at these low detergent concentrations. It is interesting, however, that the extent of reduced RIDP inhibition at low detergent concentrations seems to be dependent on the rifamycin derivative causing the inhibition. DMB may even activate the RIDP activity at extremely low detergent concentrations.

The reduced ability of the rifamycin derivatives to inhibit the

RIDP activity at high detergent concentrations was found to be due to extraction of the derivatives into detergent micelles. The detergent concentration range over which nearly maximal RIDP inhibition by rifamycin derivatives was obtained could be greatly expanded over the range possible with Triton X-100 by the use of Triton DN-65, or it could be contracted by the use of Brij-35. Figure 8 shows the relief of RIDP inhibition by rifazacyclo-16 and the micelle formation as a function of the concentration of three detergents (Triton X-100, Triton DN-65 and Brij-35).

The critical micelle concentration (CMC) determined from the discontinuity of the TNS fluorescence in Figure 8 was 0.013% (0.20 mM) for Triton X-100, 0.027% (0.48 mM) for Triton DN-65, and 0.006% (0.05 mM) for Brij-35. These CMCs tend to be lower and less sharply defined than the CMCs determined by the same method in distilled water; 0.015% for Triton X-100, 0.043% for Triton DN-65, and 0.005% for Brij-35. However, the CMCs obtained in distilled water for Triton X-100 and Brij-35 are in very good agreement with published data (Ross & Olivier, 1959; Hsiao, Dunning & Lorenz, 1956; Rose, 1971).

The extraction of the rifamycin derivatives into detergent micelles and away from the RIDP reaction is further suggested by two different lines of evidence. Strong evidence for the extraction of DMB into detergent micelles was provided by co-chromatography of DMB with Triton X-100 on Sephadex gel filtration columns. More than 80% of the DMB applied to a Sephadex G-50 column in buffer A containing 0.5% Triton X-100 was eluted at the exclusion volume with the detergent micelles while less than 0.1% was eluted at the exclusion volume when detergent was omitted from the DMB solution (Figure 9). The other line of evidence involves

the ability of the rifamycin derivatives to quench the TNS fluorescence which is associated with the extraction of TNS into detergent micelles. The first eight derivatives in Table 1 were very effective quenchers while rifampicin and desmethylrifampicin were much less effective and aminodesmethylrifampicin had an intermediate effectiveness. Thus the quenching efficiency roughly correlated with the inhibitory effectiveness at low detergent concentration and with the sensitivity of the derivative to suppression of inhibition by high detergent concentrations. If the ansa ring dimers and monomers are considered independently, the quenching efficiencies also correlated with the partition ratios in Table 1.

Discussion

An RIDP activity has been extracted from virally-transformed cells which could not be extracted from uninfected, non-transformed cells. However, other laboratories have reported RIDP activity in normal cells using either poly-rA:poly-dT as template with Mn^{++} (Ross, et al., 1971) or endogenous RNA with Mg^{++} (Coffin & Temin, 1971). The fact that we did not detect an RIDP activity in normal cells was probably the result of the combination of a relatively specific template, poly-rA:oligo-dT (Goodman & Spiegelman, 1971), and low concentrations of Mn^{++} as the divalent cation, a combination which does detect viral RIDP but elicits no response from non-transformed Balb cells (Ross, et al., 1971). Using this same combination of template and divalent cation, Reid and Albert (1972) were able to detect an RIDP activity in tumor tissues which was not present in comparable normal tissues. The UC1-B cells used in this study were found to be an unusually good source of the RIDP activity from virally-transformed

cells and may prove extremely useful in detecting a similar activity in cells transformed by chemical carcinogens or radiation.

Non-ionic detergents were found to be activators of the RIDP activity from virally-transformed cells. This activation by detergents

appears to be an intrinsic property of the enzyme and not an artifact introduced by detergent solubilization: the activation curve is the same whether approached from 0% detergent (Figure 5) or from greater than saturating concentrations of detergent (Figure 3). The fact that the purified Triton X-1017 activated as well as Triton X-100, Triton DN-65 and Brij-35 makes unlikely the possibility that the activation is caused by a minor component or contaminant in the three commercial detergents. The activation by detergents and not by polyethylene glycol suggests that the enzyme has a site or region which strongly prefers a hydrophobic environment. In the cell such a region might function as a membrane attachment site. If this RIDP activity were the viral RIDP, hydrophobic areas on the enzyme might also play an important role in the tight packing of the virus particle nucleoid in the aqueous environment of the cell cytoplasm.

In activating the RIDP, the detergent could bind to the hydrophobic site and allow the enzyme to assume a more active conformation. The binding of the detergent must be reversible since the RIDP activity decreased upon simple dilution of the detergent. The reversibility of the detergent-enzyme interaction is also suggested by the similar saturation characteristics of the detergent activation curves for the fully active RIDP at 0.0025% Triton X-100 (Figure 3) and for the RIDP partially inactivated by exposure to 0.00085% Triton X-100 (Figure 5). The mechanism of activation must be a molecular one since the activation by different detergents occurs at comparable molar concentrations which are as much as an order of magnitude below the critical micelle concentration of these detergents.

Several previous studies have shown that non-ionic detergents are required to obtain maximal levels of RIDP activity from RSV virions (Garapin, et al., 1970; Quintell, et al., 1971; Bishop, et al., 1971), from MSV virions (Green, et al., 1970; Peebles, Haapala & Gazdor, 1972), and from MLV virions (Scolnick, Aaronson & Todaro, 1970). The effect observed in these studies was dependent on the protein concentration of the virion preparation (Garapin et al., 1970) and was correlated to the disruption of the virus particle (Scolnick, Aaronson & Todaro, 1970; Bishop, et al., 1971). In preliminary studies, we have found that the RIDP activity obtained from detergent-solubilized (M)MLV particles shows a detergent activation similar to the one presented here for the RIDP activity extracted from transformed cells.

from transformed UC1-B cells

Once solubilized, the RIDP/undergoes a significant loss of activity in dilute solutions (6 μg protein/ml) when most of the detergent is removed in the absence of template and substrate. The template and/or substrate may bind to the RIDP and act as stabilizers of an active RIDP conformation. The irreversible loss of RIDP activity (∿ 50%) which occurs at 0.00085% Triton X-100 in the absence of template and substrate is probably due to exposure of the enzyme's hydrophobic region causing it to denature or to attach to the glass surfaces of the test tube. This irreversible loss of activity at dilute detergent concentrations would be expected to occur with dialysis of the detergents but was not found when RIDP extracts solubilized with 0.1% Triton X-100 were dialyzed against buffer A. This was apparently due to the fact that the Triton X-100 was only 93.6% dialyzable leaving the dialyzed extracts with more

than 0.006% Triton X-100. On the other hand, we observed that the RIDP extracts solubilized with 0.1% Triton X-1017 (99.8% dialyzable) lost approximately 60% of their activity upon dialysis against buffer A.

A similar inactivation in the absence of non-ionic detergents may occur in RIDP preparations from detergent-solubilized virus particles. It is a common practice in many laboratories studying the RIDP activity from a variety of viruses to maintain a certain level of detergent in the enzyme solutions to avoid a loss of enzyme activity. When detergent-solubilized RIDP is exposed to column chromatography in the absence of detergent, the yield of enzymatic activity is often very low (Duesberg, Helm & Canaani, 1971; Ross, et al., 1971; Faras, et al., 1972). The need for detergent to stabilize the enzyme activity may increase as the enzyme is purified (Klaus V.D. Helm, personal communication). However, Kacian et al. (1971) have reported a purification of the RIDP from AMV which gives high yields of enzyme activity in the absence of detergent.

The above results demonstrate the need to maintain a certain detergent concentration not only in the assays but also during purification and storage of the RIDP. However, it should be emphasized that the detergent concentrations to be used in assays which test for the inhibition of RIDP by rifamycin derivatives must be selected carefully. Assays done at detergent concentrations lower than approximately 0.06 mM involve problems due to incomplete activation of the RIDP by the detergent. Assays done at detergent concentrations approaching the CMC of the detergent involve alterations in the concentrations of the rifamycin derivatives due to extraction into detergent micelles.

Thus the best detergent concentrations for testing rifamycin derivatives as inhibitors of RIDP lie between that required for full RIDP activation and that which gives micelle formation. The range of Triton X-100 concentrations which meets these requirements is very narrow, from 0.004% to 0.006%. These same limitations on the appropriate detergent concentration would apply to Nonidet P-40 (Shell Chemical), a commonly used detergent very similar to Triton X-100 in structure and synthesis. However, Triton DN-65 is not subject to these restrictive limitations. Even though Triton DN-65 has approximately the same efficiency in solubilizing the RIDP in the extraction procedure as does Triton X-100 and activates as well as Triton X-100, it has a concentration range between full activation of the RIDP and micelle formation which is much wider than Triton X-100, 0.004% - 0.023%. In fact, studies by Becher (1959) on other similar detergents indicate that the CMC of Triton DN-65 is unusually high for this type of detergent. This may be the result of the smaller hydrophobic region of Triton DN-65.

The rifamycin derivatives used in this work showed a wide range of effectiveness as inhibitors of the RIDP extracted from virally transformed cells. The derivatives DMB, rifaldehyde octyloxime, and rifamazine* have previously been shown to be very effective viral RIDP inhibitors at 0.01% Triton X-100 (Gurgo, Ray & Green, 1972; Yang, et al., 1972). Using the RIDP extracted from virally-transformed cells and a similar Triton X-100 concentration (0.0125%) all of the first eight derivatives in Table 2 were found to be inhibitors of roughly comparable effectiveness on a μg/ml basis. However, at 0.005% Triton X-100 the eight derivatives are less comparable: the rifazacyclo-16 and the rifaldehyde octyloxime are clearly the most effective inhibitors, followed by the dansyldesmethyl-

* NCI compound #145-617 has recently been found to have the structure of rifamazine (Gruppo Lepetit, S.p.A., Milan, Italy, personal communication)

rifazabicyclo-9.
rifampicin and the / Two of the ansa ring dimers, rifamazine and di-rifampin, are approximately as effective as the latter two mentioned above when considered on a molar basis. DMB and rif-urea are the least effective of the first eight in Table 2, but are still significantly more effective than aminodesmethylrifampicin. Rifampicin and desmethyl-rifampicin are essentially inactive as inhibitors of the RIDP from virally transformed cells. It appears from this limited sample of rifamycin derivatives that increasing the size or length and the hydrophobicity of the synthetic side chain of a rifamycin derivative increases its effectiveness as an in vitro RIDP inhibitor.

Since the RIDP appears to have a hydrophobic site which prefers the environment provided by detergent molecules, the hydrophobicity of a rifamycin derivative and the degree to which it prefers the environment of the detergent micelle might be a very significant property for concentrating the derivative in close proximity to the RIDP for intracellular inhibition. At least a qualitative measure of each derivative's preference for the detergent micelle environment can be obtained by taking a ratio of the derivative concentration required for half inhibition at 0.005% Triton X-100 to the concentration required near 0.012% where micelle formation is beginning to occur. If one compares the half inhibition values at 0.005% and 0.0125% Triton X-100 in Table 2, only three or possibly four derivatives show a significantly higher half inhibition concentration at 0.0125% due to extraction into this very low concentration of micelles. The three derivatives are the rifaldehyde octyloxime, the dansyldesmethylrifampicin, and the rifazacyclo-16. DMB exhibits only a slightly higher half inhibition concentration at 0.0125%.

The first two derivatives have been shown to significantly alter the

cell growth of tissue culture cells and for that reason have not been tested for inhibition of viral transformation in tissue cultures (Hackett, unpublished results). DMB has been shown to inhibit viral transformation in tissue culture (Calvin, et al., 1971) and to inhibit chemically-induced tumors in whole animals (Joss, Hughes & Calvin, to be published). Preliminary results show that rifazacyclo-16 may be even more effective than DMB in inhibiting viral transformation in tissue culture (Hackett, personal communication).Thus, the extractability of a derivative into detergent micelles may indeed be a significant property in determining effective intracellular inhibitors of viral transformation, and the change of the concentration required for half inhibition of the RIDP from 0.005% to 0.012% Triton X-100 is a sensitive measure of that extractability. Further studies with the new rifamycin derivatives are in progress to test this hypothesis.

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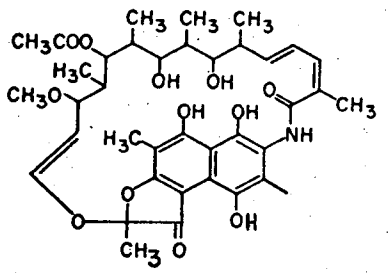
Table 1. Structures of Rifamycin Derivatives



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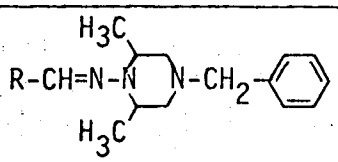
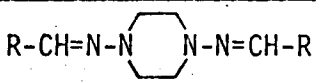
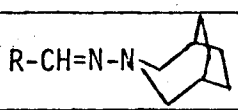
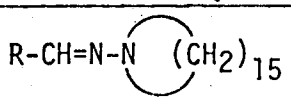
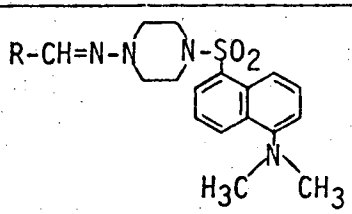
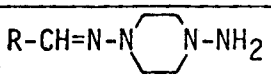
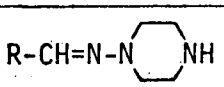
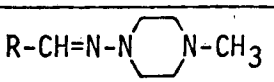
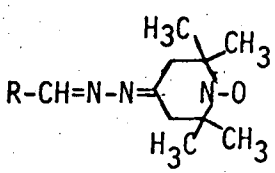
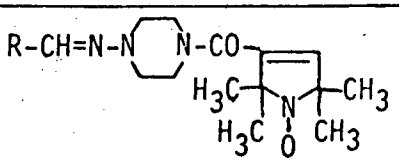
Rifamycin derivative	Molecular weight	Partition ratio*	Structure
DMB	925	1700	
Rifaldehyde octyloxime	853		R-CH=N-O-CH_2-$(CH_2)_6$-CH_3
Rifamazine	1448	34	R-CH=N-N=CH-R
Rif-urea	1506	22	R-CH=N-NH-CO-NH-N=CH-R
Dirifampin	1532	49	
Rifazabicyclo-9	848	>2000	
Rifazacyclo-16	948	>2000	
Dansyldesmethylrifampicin	1042	>2000	
Aminodesmethylrifampicin	824	730	
Desmethylrifampicin	809	15	
Rifampicin	823	14	
Spin labeled rifampicin 1	892		
Spin labeled rifampicin 2	975		

Footnote to Table 1

* Determination of the partition ratios. The rifamycin derivative was dissolved in n-octanol at 150-500 μg/ml along with 0.6-1.1 mg/ml of DTT (required to avoid oxidation of the derivatives), and the maximum optical density (OD_1) between 450 and 550 mμ was determined. 1 ml of the octanol solution was shaken with 100 ml of an octanol-saturated aqueous solution of 0.01 M Tris-HCl (pH = 7.8) and 0.1 mM DTT. After the octanol was removed and centrifuged, the maximum OD (OD_2) was again determined. The partition ratio was calculated as $100 \times OD_2/(OD_1 - OD_2)$. The value for rif-urea was increased to 60 by high ionic strength (0.09 M Tris, 0.1 M KCl); 4% glycerol in addition to high ionic strength also resulted in a value of 60.

Table 2 Concentration (μg/ml) Yielding 50% Inhibition of the RIDP

Rifamycin Derivative	Triton X-100 Concentration		
	0.005%	0.0125%	0.025%
DMB	22	25	73
Rifaldehyde octyloxime	6	16	48
Rifamazine	21	22	35
Rif-urea	27	24	60
Dirifampin	18	17	35
Rifazabicyclo-9[a]	12	12	36
Rifazacyclo-16	4	12	115
Dansyldesmethylrifampicin	11	20	85
Aminodesmethylrifampicin	55	44	135
DMB-oxidized[b]	--	100	--
Desmethylrifampicin	> 100	--	--
Rifampicin	> 100	>400	--
Spin-labeled rifampicin 1	--	--	275
Spin-labeled rifampicin 2	--	--	> 500

a Tetrahydrofuran was used instead of DMSO.

b Quinone form of DMB

Assays were done as described in Methods. DMSO was 1.0% or 1.0%/100 μg derivative, whichever is greater. Protein was 0.45 μg-2.5 μg with an activity of 200-62 pmol/hr/μg.

FIGURE CAPTIONS

Figure 1. Specific activity of the extracted RIDP as a function of the detergent concentration used for solubilization. Assays were done in duplicate as described in methods. The Triton X-100 concentration was 0.01% in all assay tubes. The protein concentrations varied between 1.2 μg and 4.5 μg per assay depending upon activity. All four extractions were normalized to 100% of maximum specific activity at 0.1% detergent solubilization.

(o) Data obtained in one extraction utilizing one resuspended $(NH_4)_2SO_4$ Pellet. Total protein = 1.18 mg/ml.

(•) Data obtained from three different extractions.

Figure 2. Divalent cation requirement. Assays were done as described in Methods except that the Mn^{++} concentration was varied as indicated in the figure. There were 2.5 μg protein and 0.025% Triton X-100 in each assay. Each point was determined from duplicate assays. Three separate experiments are included in the figure:

(o) Exp. 1 (KCl = 100 mM, DTT = 0.2 mM) using $MnCl_2$

(•) Exp. 2 (KCl = 90 mM, DTT = 2.5 mM) using $MnCl_2$

(x) Exp. 3 (KCl = 90 mM, DTT = 2.5 mM) using $Mn(acetate)_2$.

Figure 3. Effect of non-ionic detergents on RIDP activity. Assays were done as described in Methods, and the detergents were added to yield the concentrations indicated in the figure. All points on each curve were determined from at least duplicate assays. The majority of the points for Triton X-100 were determined from at least 6 assays in 3

Captions, 2

experiments. The majority of points for Triton DN-65 curve were determined from 4 assays done in 2 experiments.

(o) Added detergent was Triton X-100. The RIDP used was solubilized and stored in 0.1% Triton X-100 and diluted to 0.0032% with buffer A for addition to the assays; protein = 0.45 μg per assay and maximal activity = 200 pmol/hr/μg; DMSO = 0.25-0.40%.

(●) Added detergent was Triton DN-65. The RIDP used was solubilized and stored in 0.1% Triton DN-65 and diluted to 0.0025% with buffer A for addition to the assays; protein = 0.38 μg per assay and maximum activity = 250 pmol/hr/μg; DMSO = 0.25-0.30%.

(x) Added detergent was Triton X-1017. The RIDP used was solubilized and stored in 0.1% Triton X-1017 and diluted to 0.0025% with buffer A for addition to the assays; protein = 0.50 μg per assay and maximum activity = 266 pmol/hr/μg.

(▲) Added detergent was Brij-35. The RIDP used was solubilized and stored in 0.1% Triton DN-65 and diluted to 0.0025% with buffer A for addition to the assay. The 0.0088 mM contributed by the Triton DN-65 added with the enzyme was included in the detergent concentration given in the figure. Protein = 0.38 μg and maximum activity = 132 pmol/hr/μg.

(△) Polyethylene Glycol-400 was added in the assay instead of a detergent. The RIDP used was solubilized and stored in 0.1% Triton DN-65 and diluted to 0.0025% with buffer A

Captions, 3

for addition to the assay. The 0.0088 mM contributed by Triton DN-65 added with the enzyme was included in the detergent concentration given in the figure. Protein = 0.38 μg and maximum activity (determined from the maximum activity with Triton DN-65) = 132 pmol/hr/μg.

Figure 4. Loss of RIDP activity at 0.00085% Triton X-100. Assays were done as described in Methods. Triton X-100 was added to each assay to a final concentration of 0.004%. The same RIDP extract was used for both curves and each assay contained 0.36 μg protein.

(o) RIDP diluted 1:49:100-enzyme in buffer A containing 0.127% Triton X-100:buffer A:0.006% Triton X-100. Final detergent concentration in the enzyme solution was 0.0048%.

(•) RIDP diluted 1:49:100-enzyme in buffer A containing 0.127% Triton X-100:buffer A:water. Final detergent concentration in the enzyme solution was 0.00085%.

Figure 5. Effect of Triton X-100 on partially inactivated RIDP and on unsolubilized RIDP. Assays were done in duplicate as described in Methods. Triton X-100 was added to yield the concentrations indicated in the figures.

(o, △, □) RIDP inactivated by exposure to 0.00085% Triton X-100 for 20 minutes, 3 hours, and 24 hours, respectively; protein = 0.35 μg per assay and maximum activity = 93, 92 and 92 pmol/hr/μg, respectively.

Captions, 4

(●) RIDP extracted in the 100,000 g supernatant without exposure to detergent until added to the assay; protein = 1.51 μg per assay and maximum activity = 126 pmol/hr/μg (at≥0.005% Triton X-100).

Figure 6a. RIDP inhibition by DMB at two Triton X-100 concentrations. Assays were done as described in Methods. DMB was added as indicated in the figure. Control activity was determined from assays which omitted only the DMB.

(○) Triton X-100 = 0.005%. This curve is the average of 3 separate experiments in each of which the 4 points of lowest DMB concentration were done in duplicate. DMSO = 1.0%, protein = 0.45-0.49 μg per assay, and the control activity = 170-200 pmol/hr/μg.

(□) Triton X-100 = 0.025%. This curve is the combined data from 2 separate experiments. DMSO = 0.25 and 1.0%, protein = 0.45 and 2.5 μg per assay, and the control activity = 209 and 62 pmol/hr/μg.

Figure 6b. RIDP inhibition by rifazacyclo-16 at three Triton X-100 concentrations. Assays were done as described in Methods. Rifazacyclo-16 was added as indicated in the figure. Control activity was determined from assays which omitted only the rifazacyclo-16. Each curve is the average of 2 separate experiments, in each of which the 5 points of lowest rifazacyclo-16 concentration were done in duplicate. DMSO = 1.0%, protein = 0.45-0.49 μg per assay, and control activity = 180-200 pmol/hr/μg.

Captions, 5

(o) Triton X-100 = 0.005%

(Δ) Triton X-100 = 0.125%

(□) Triton X-100 = 0.25%

Figure 7. RIDP inhibition by three derivatives as a function of Triton X-100 concentration. Assays were done as described in Methods. Each assay contained 0.45 μg protein of the same RIDP extract (solubilized by 0.1% Triton X-100). Control activity (170-190 pmol/hr/μg) was taken as the average of at least 4 points done in duplicate at a concentration of ≥0.005% on a Triton X-100 activation curve which was run at the same time as the curve shown in the figure.

(o) 0.25% DMSO and 25 μg/ml (0.025 mM) DMB

(□) 0.40% DMSO and 40 μg/ml (0.025 mM) rifamazine

(Δ) 0.30% DMSO and 7.5 μg/ml (0.0079 mM) rifazacyclo-16 (RC-16)

Figure 8. RIDP inhibit on by rifazacyclo-16 and micelle formation as a function of three detergents. RIDP assays and TNS fluorescence measurements were done as described in Methods.

extra space here

RIDP assays contained 0.3% DMSO and 7.5 μg/ml rifazacyclo-16. Control activities were determined from assays which omitted only the rifazacyclo-16.

(Δ) Triton DN-65 was the detergent in the assays which contained 0.38 μg protein of an RIDP extracted with 0.1% Triton DN-65. Control activity (average of 18 assays containing 0.005%-0.080% Triton DN-65) was 226 pmol/hr/μg.

Captions, 6

(o) Triton X-100 was the detergent in the assays which contained 0.45 μg protein of an RIDP extracted with 0.1% Triton X-100. Control activity (average of 8 assays containing 0.005%-0.023% Triton X-100) was 186 pmol/hr/μg.

(□) Brij-35 was the detergent in the assays which contained 0.35 μg/protein of an RIDP extracted with 0.1% Triton DN-65. Control activity (average of 6 assays containing 0.008%-0.015% Brij-35) was 132 pmol/hr/μg. The 0.0088 mM contributed by the Triton DN-65 added with the enzyme was not included in the concentration indicated in the figure.

Fluorescence measurements were done in 0.1 M Tris-HCl (pH = 7.8), 0.1 M KCl, 4% glycerol, 0.3% DMSO and 1 x 10^{-6} M TNS.

(—·) Triton DN-65 added

(— —) Triton X-100 added

(——) Brij-35 added

Figure 9. Gel filgration of DMB at various initial concentrations of Triton X-100. 0.1 μg ^{3}H-DMB (1.0 C/mmole) was dissolved in buffer A and Triton X-100 was added to give the concentrations indicated below. These mixtures were applied to a Sephadex G-50 column and eluted with buffer A. The fractions contained 3 drops each. The dilution due to band broadening was 1:4 at the time of the elution of the void volume (30 min).

(Δ) 0.1 μg DMB only

(o) 0.1 μg DMB in 0.2% Triton X-100 (weight ratio, 1:2000)

(□) 0.1 μg DMB in 0.5% Triton X-100 (weight ratio, 1:5000)

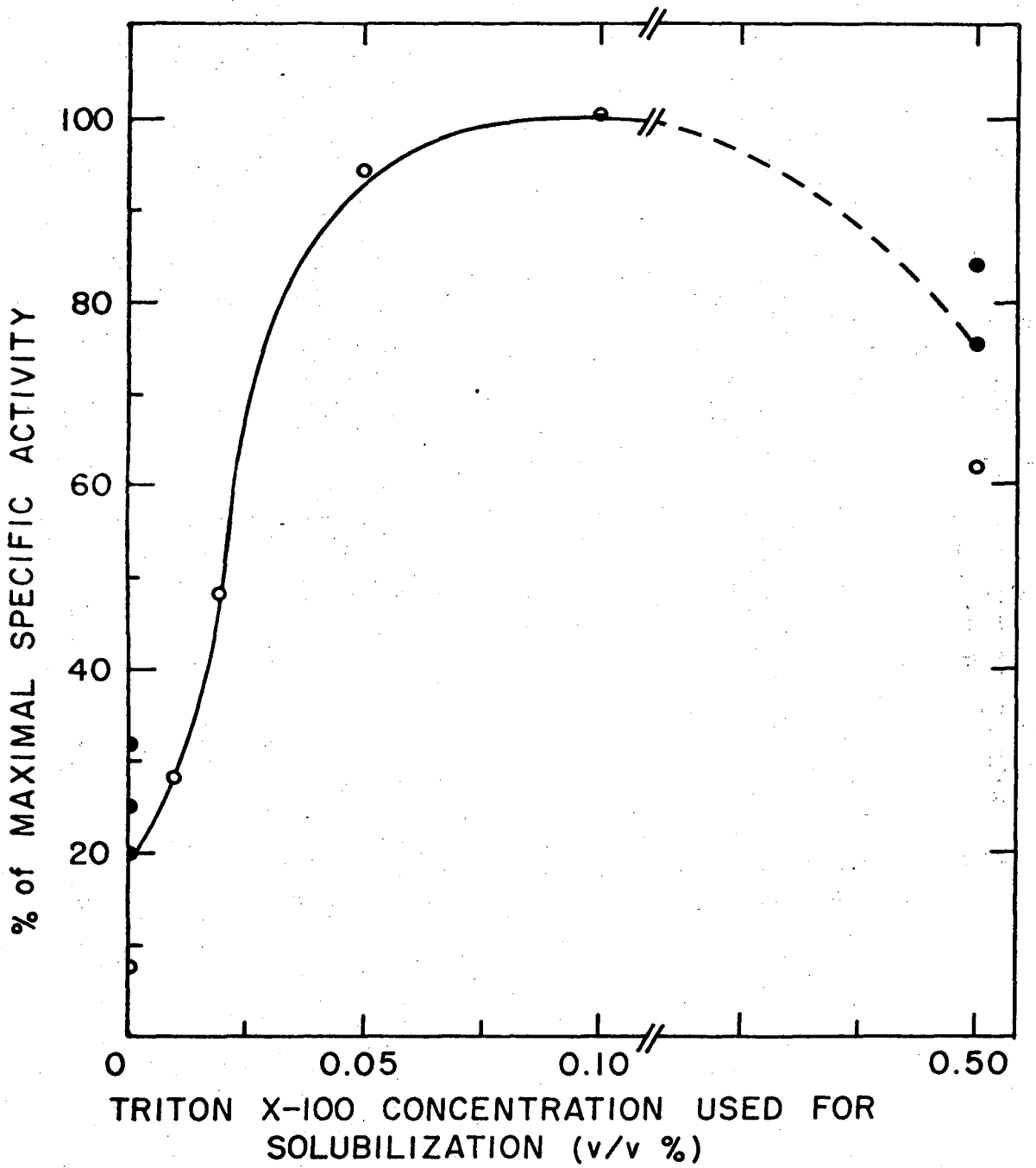


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FIGURE 1

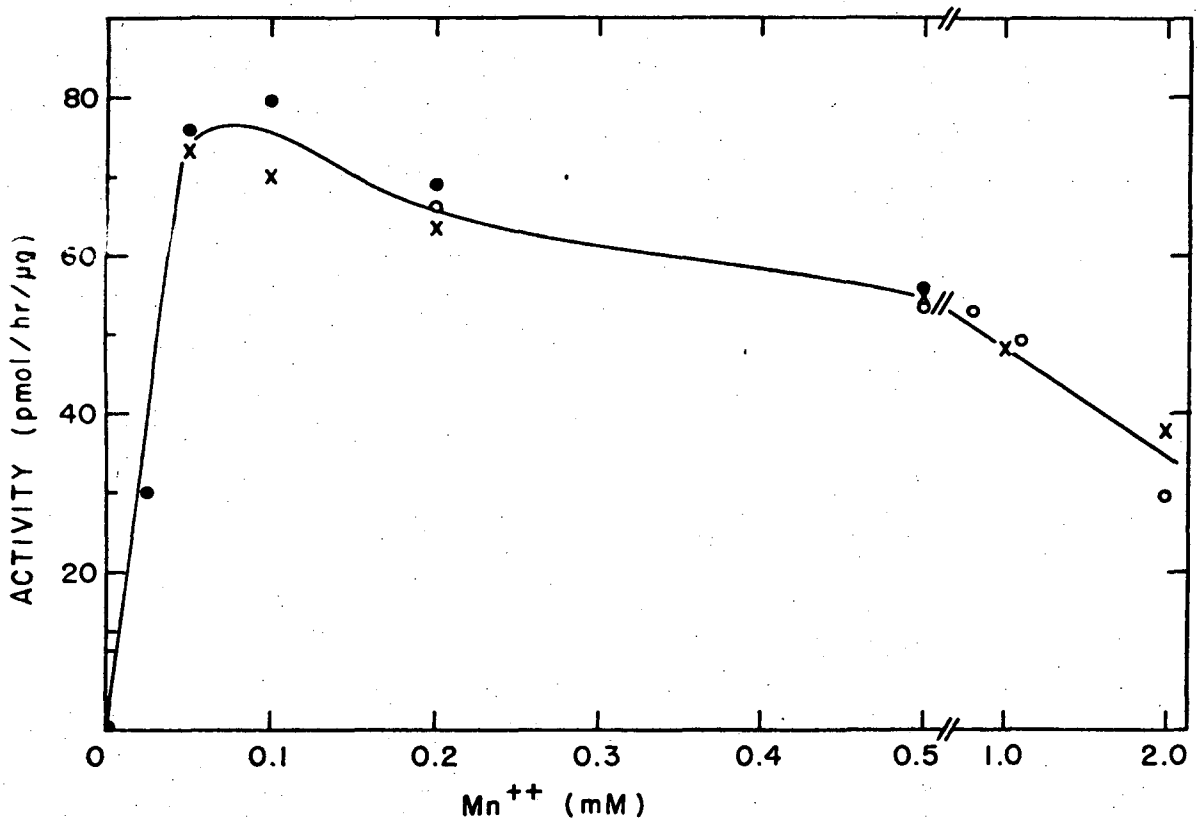

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FIGURE 2

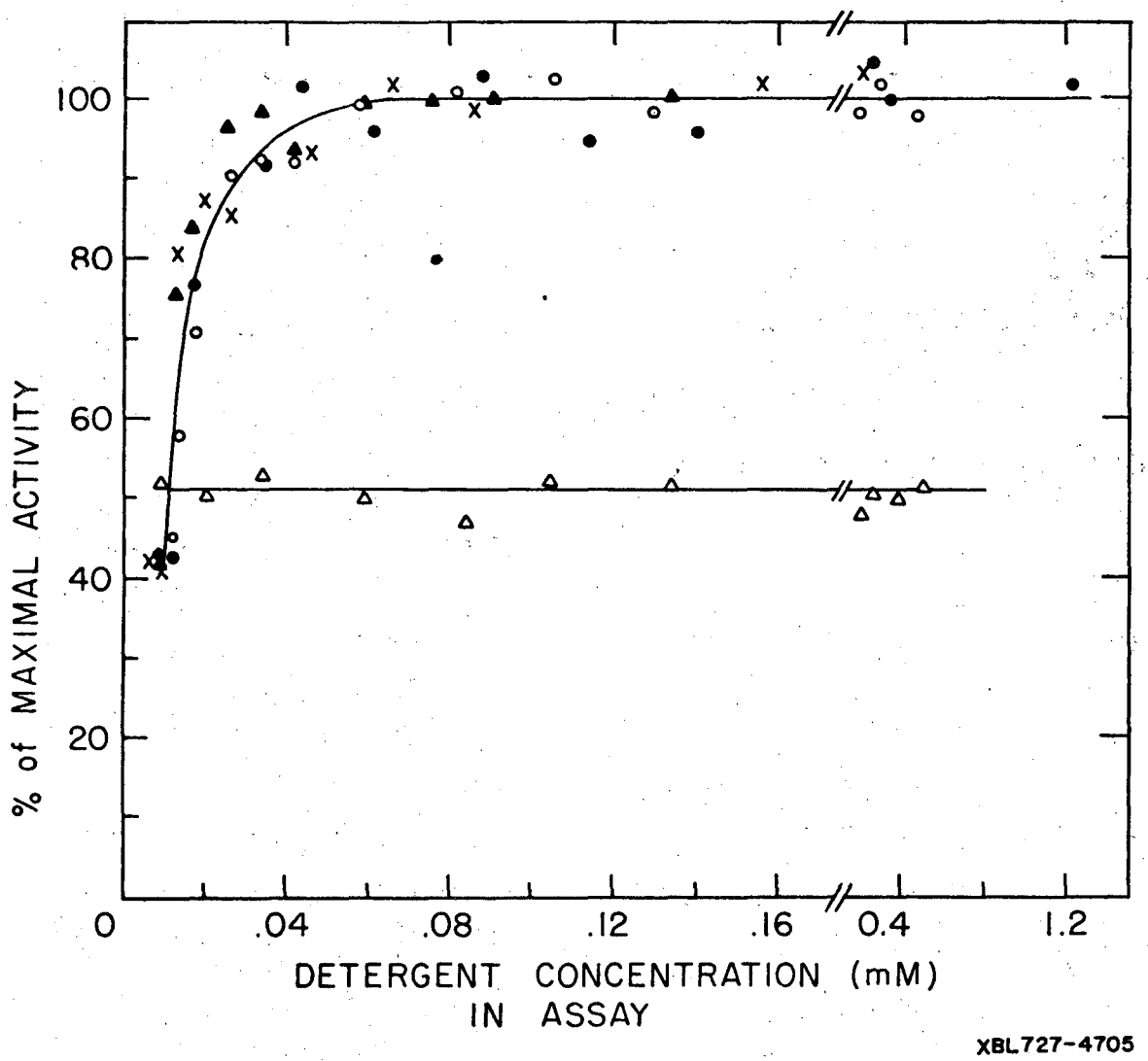

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FIGURE 3

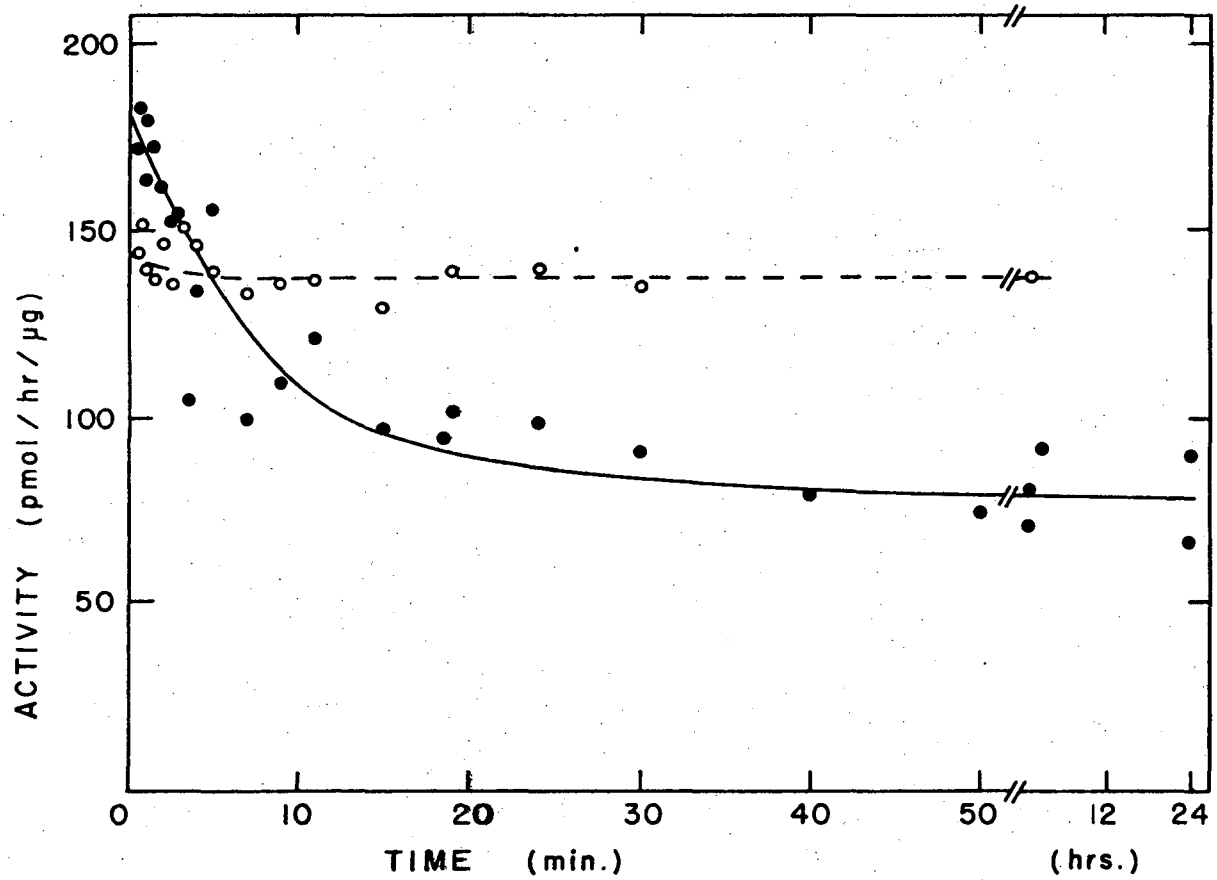

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FIGURE 4

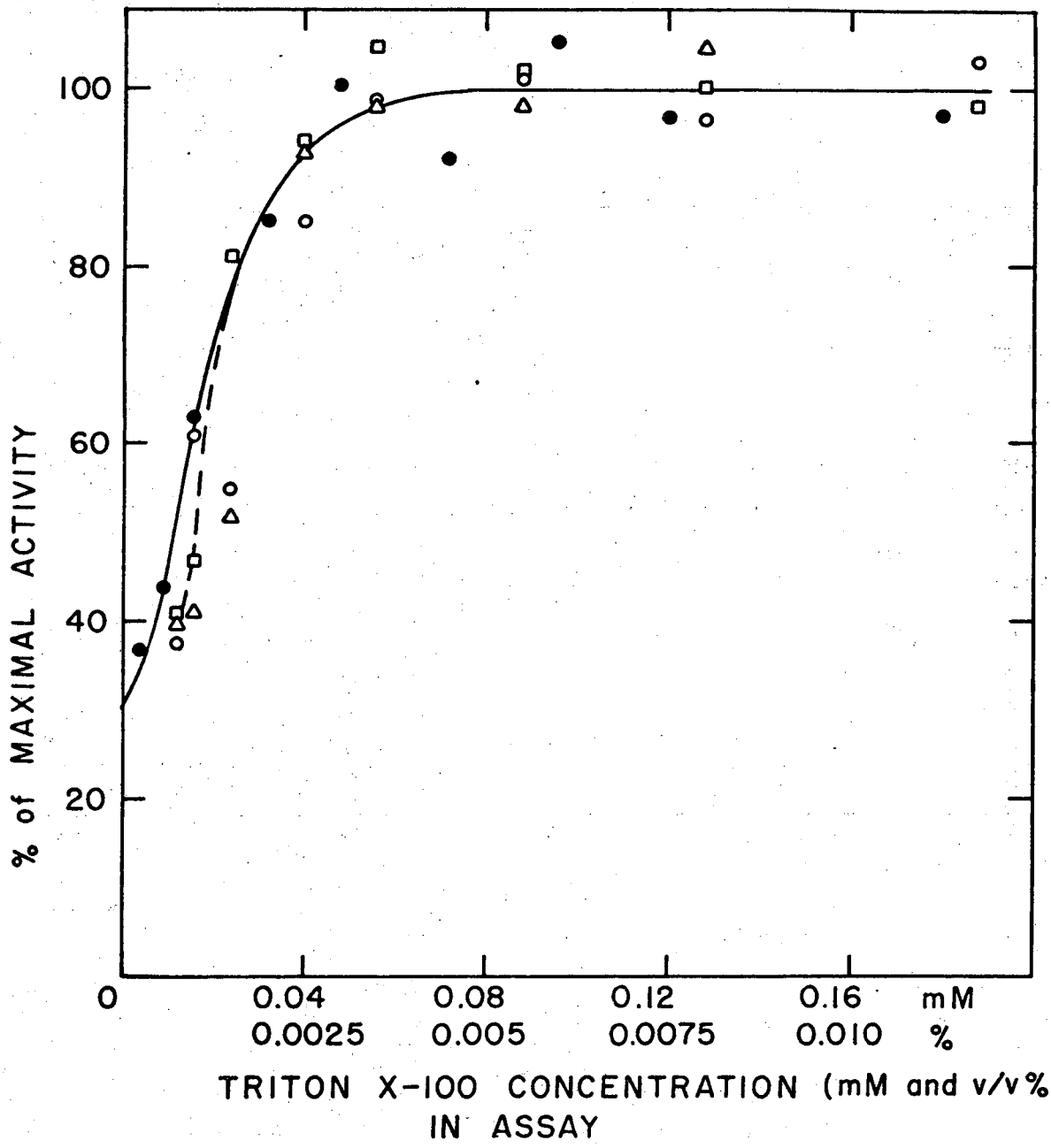

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FIGURE 5

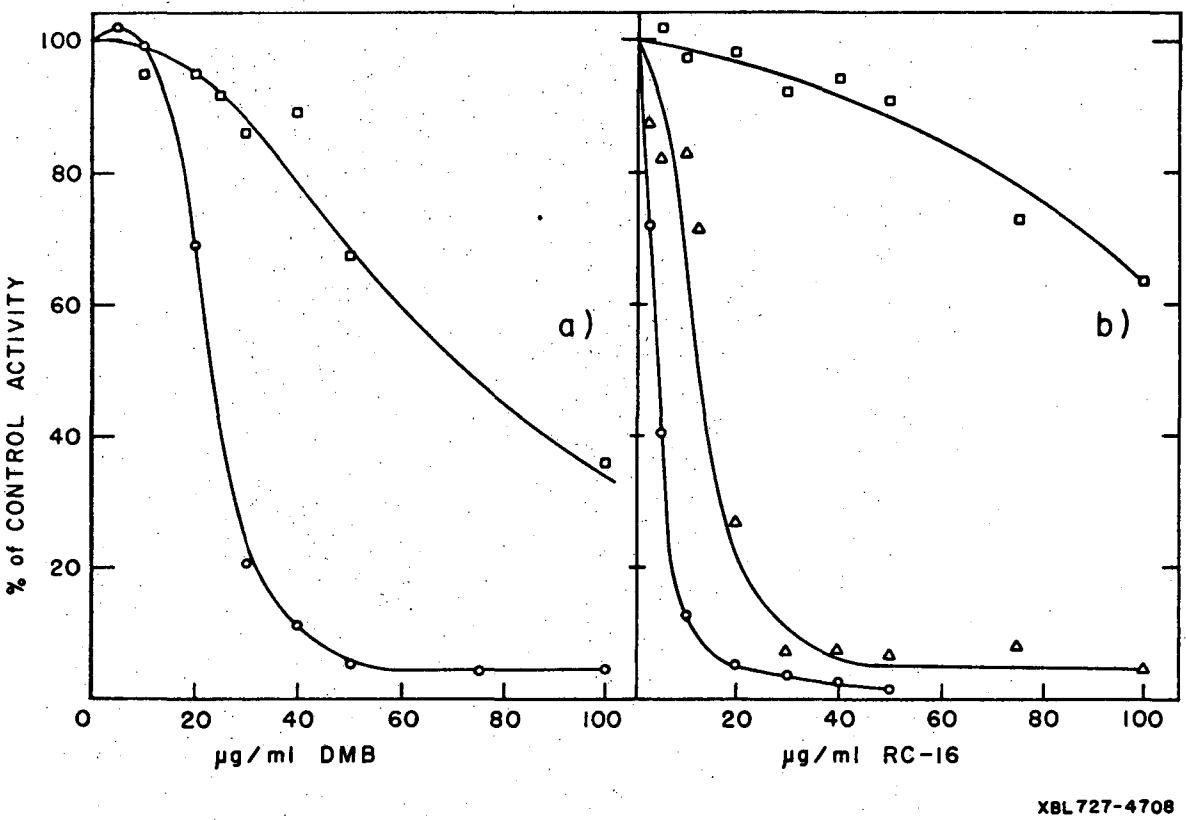

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FIGURE 6

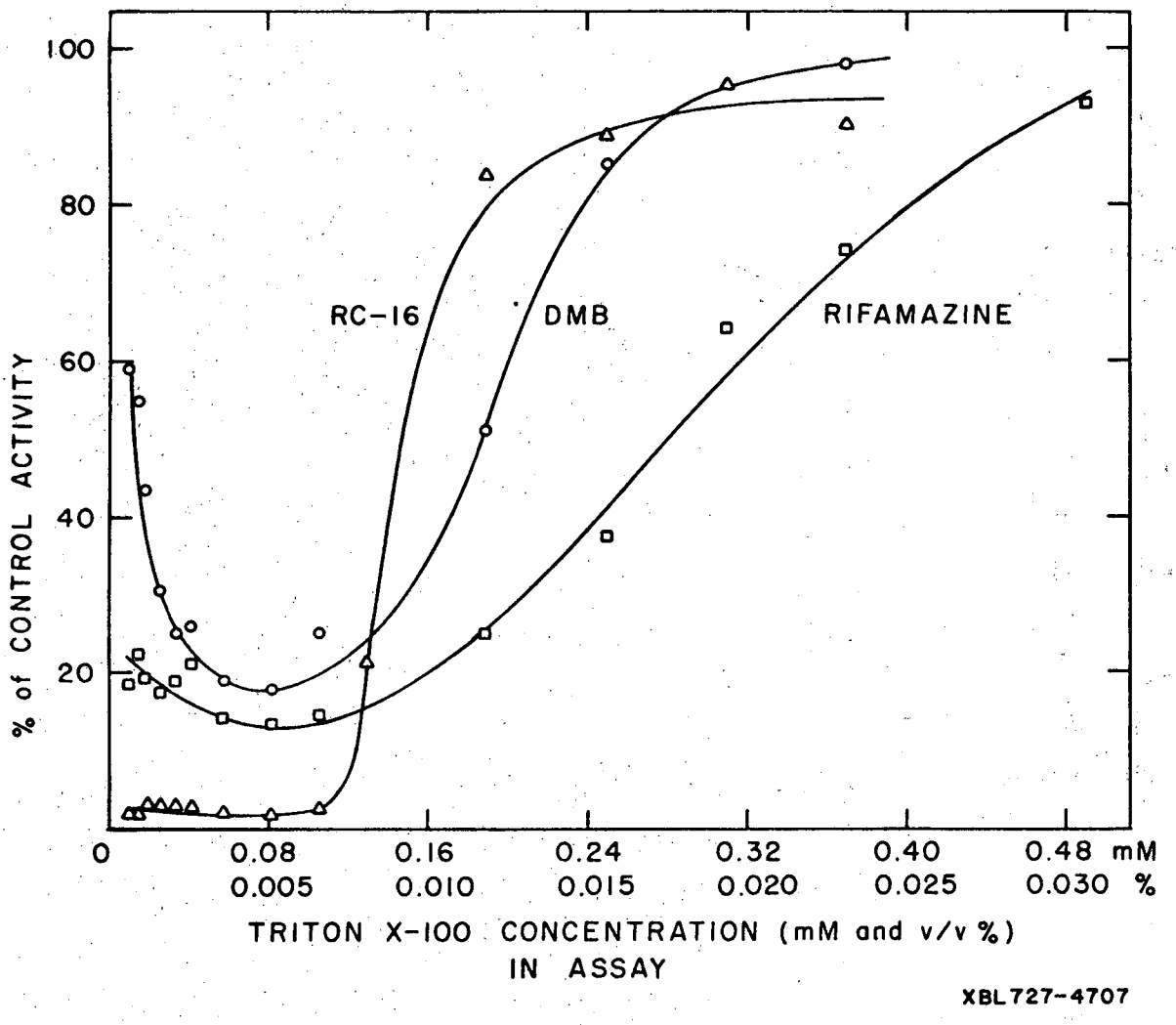

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FIGURE 7

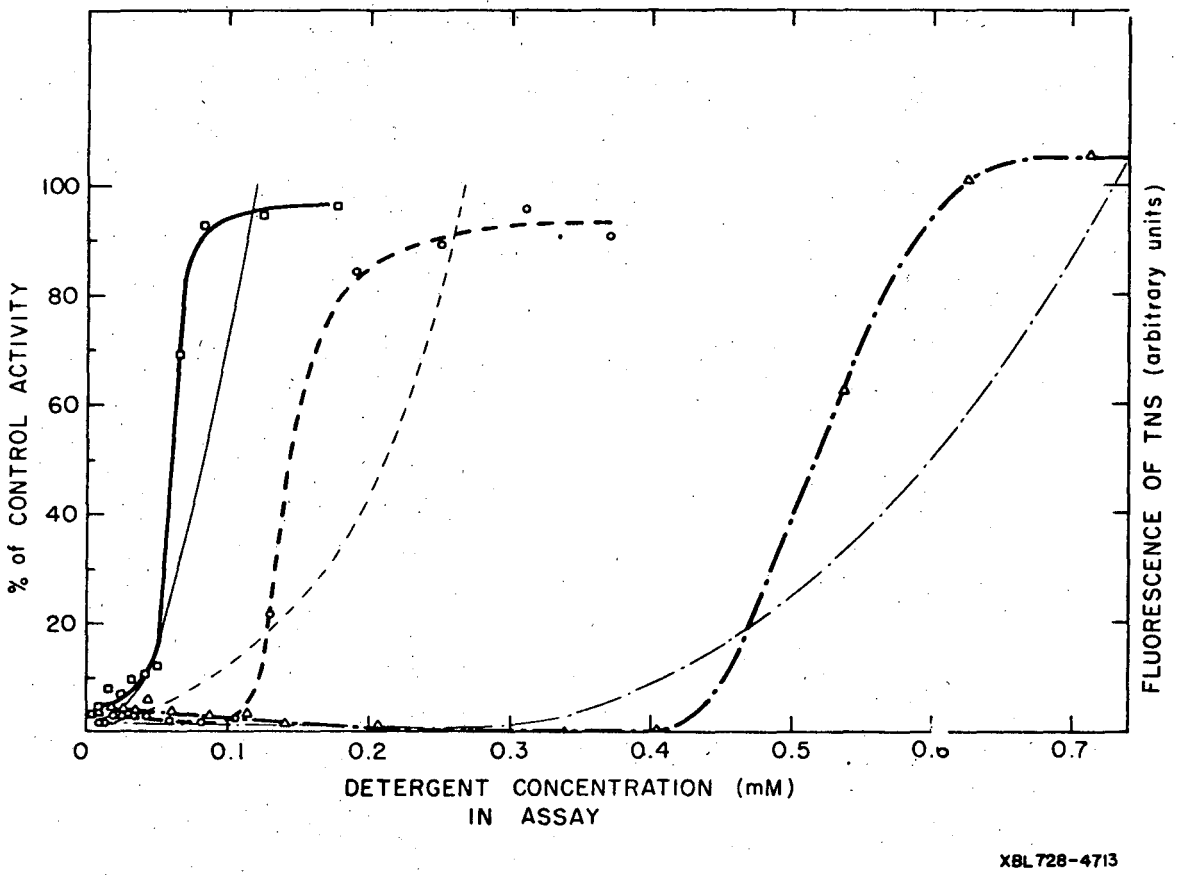

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FIGURE 8

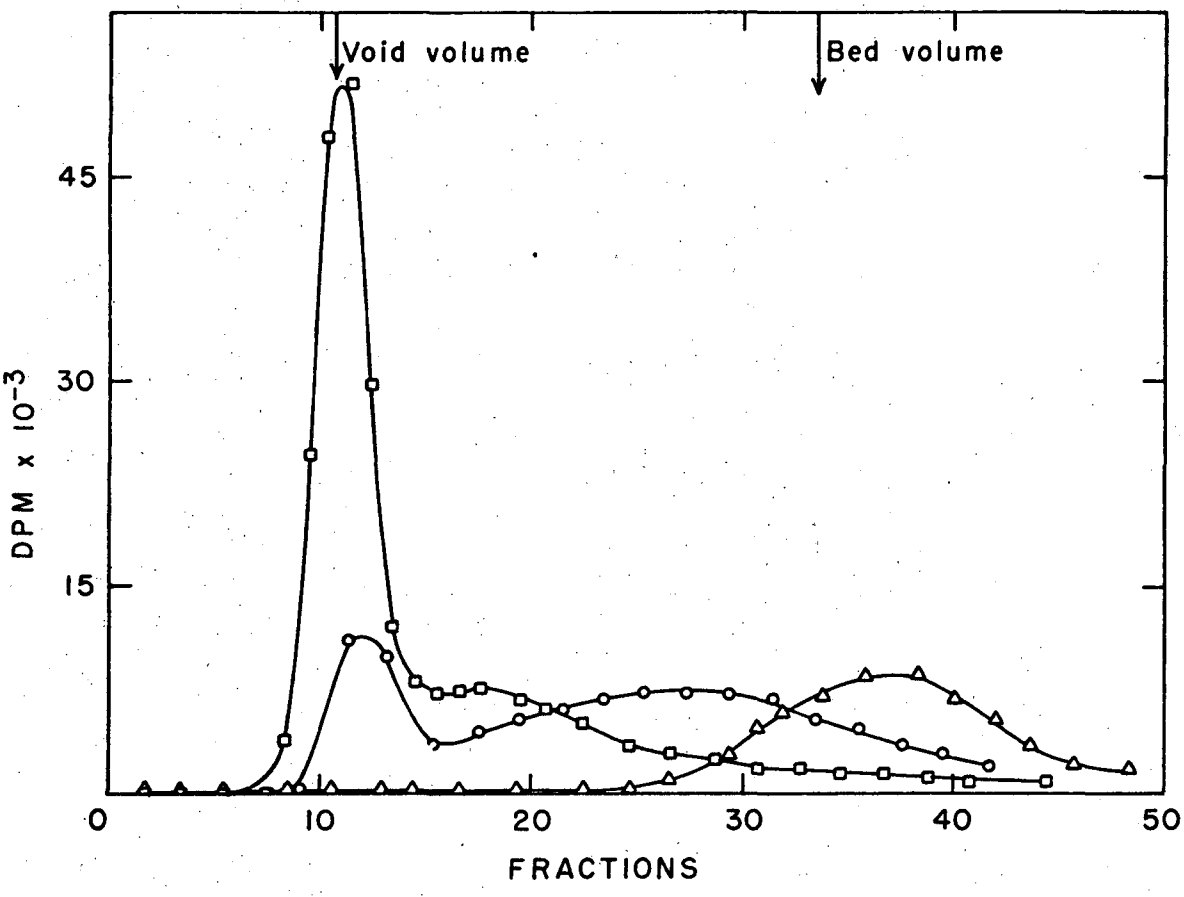

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FIGURE 9

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