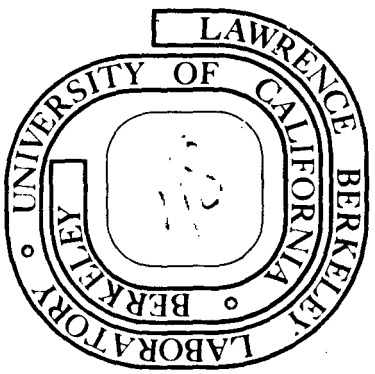
RIFAMYCINS AS INHIBITORS OF NUCLEOTIDE POLYMERASE FUNCTIONS

Allan N. Tischler
(Ph. D. Thesis)

May 1974

Prepared for the U. S. Atomic Energy Commission
under Contract W-7405-ENG-48



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DISCLAIMER

Dedication

To my parents - who got me started.

To my wife - who keeps me going.

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Contents (cont.)

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May 1974

ABSTRACT

Several new rifamycin derivatives have been synthesized and tested as inhibitors of an RNA-instructed DNA polymerase (RDP) function in an effort to determine the effect of rifamycin structure on RDP inhibition and the effect of rifamycin structure on RDP inhibitory specificity. It was found in general that RDP inhibition is favored by lipophilic tails bound to the 3-position of rifamycin SV. It was also found that RDP inhibitory specificity, compared with E. coli DNA-directed DNA polymerase I, is favored by lipophilic acyclic rifamycin derivatives. Lipophilicity was measured by a reversed phase thin layer chromatographic technique.

A few attempts were made to purify RDP from a cellular source (transformed UC1-B cells) by affinity columns to which rifamycin derivatives had been covalently bound. It was found that RDP activity could be effectively held up by the columns but in the few attempts made, the RDP was not eluted from the columns.

One of the rifamycin derivatives prepared in this program, rifazone-8_2, aside from being a potent and specific inhibitor of

RDP, was found to be a potent inhibitor of focus formation in chick fibroblast monolayers infected with Rous sarcoma virus. A large part of this phenomenon has been attributed to a pronounced toxicity of this derivative to transformed cells. The derivative was found to be nontoxic to normal chick cells at similar concentrations.

Nonionic detergents were found to stabilize RDP activity under assay conditions. The same effect was noted with two other polymerase enzymes. This effect was not found to be generalized with enzymes other than polymerases.

INTRODUCTION

The now familiar class of antibiotics, the rifamycins, was first discovered in 1957 by Sensi and coworkers from the fermentation products of Streptomyces mediterranei.[1] One of the first compounds isolated from the fermentation broth, rifamycin B, exhibited little antibacterial activity. However, it was soon noted that rifamycin B in solution was readily air oxidized to give rifamycin O, which in turn was hydrolyzed to rifamycin S and glycolic acid.[2] Treatment of rifamycin S with various mild reducing agents, such as ascorbic acid, resulted in yet another compound, named rifamycin SV. Both rifamycins S and SV were found to be relatively potent antibacterial agents.[2,3] Rifamycin SV (Figure 1), the less toxic of the two in mammals, has found clinical use in the treatment of various bacterial diseases, including tuberculosis.[4]

The structure of the rifamycins already mentioned, and others, were not known until 1963, when Prelog and coworkers determined their structures by chemical and instrumental methods.[5] Their basic apporach involved the hydrolysis of the ansa ring (Figure 1, C-15 to C-29) from the naphthalene hydroquinone chromophore, with the subsequent structure determination of each piece. As a result, the chemistry of the conversion of rifamycin B to rifamycins S and SV was elucidated. These reactions are indicated in Figure 2. Rifamycin B, a hydroquinone ether, is oxidized to the quinone α'-oxoketal, rifamycin O, which is hydrolyzed to the quinone, rifamycin S and glycolic acid. Reduction of rifamycin S yields the corresponding hydroquinone, rifamycin SV. The exact stereochemical structure of the rifamycins, which contain 9 assymetric centers,

RIFAMYCIN SV

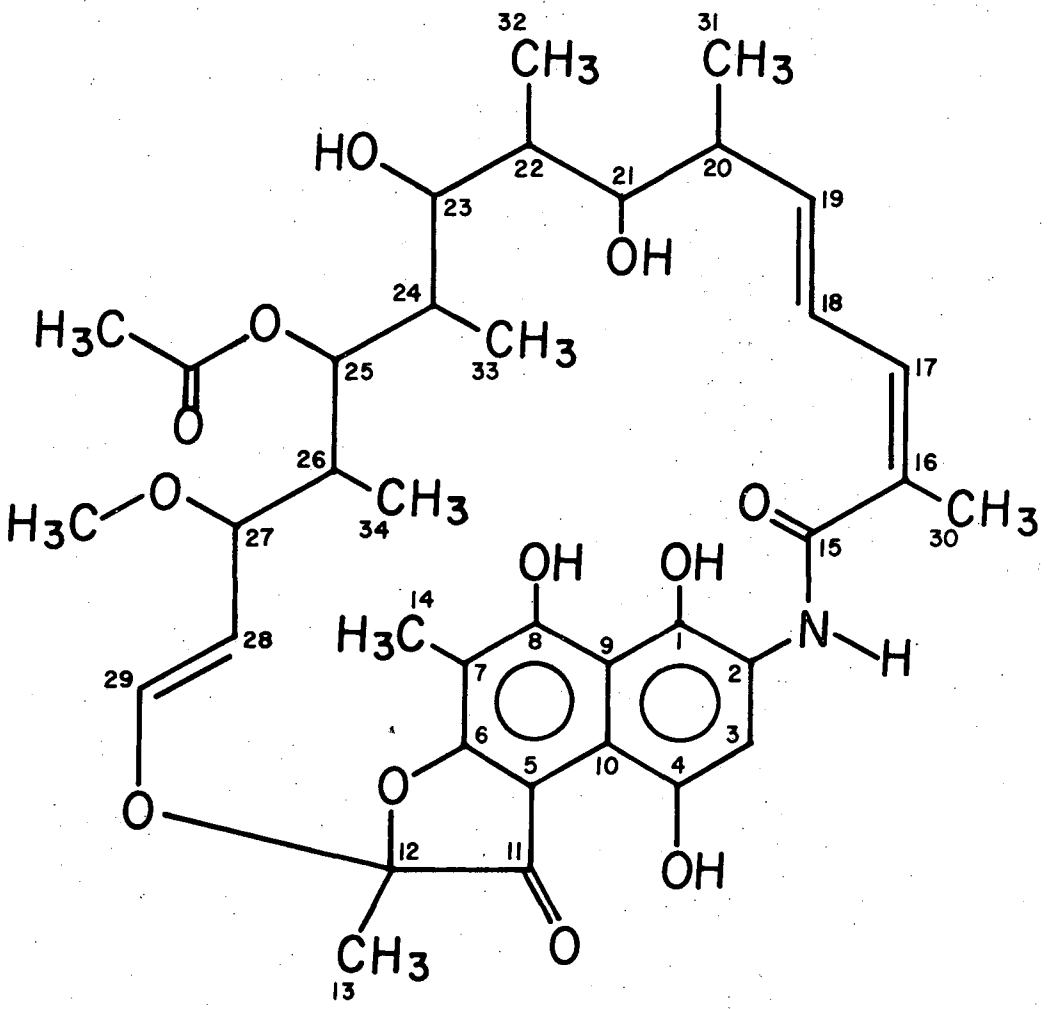

Figure 1

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Synthesis of Some Basic Rifamycin Derivatives

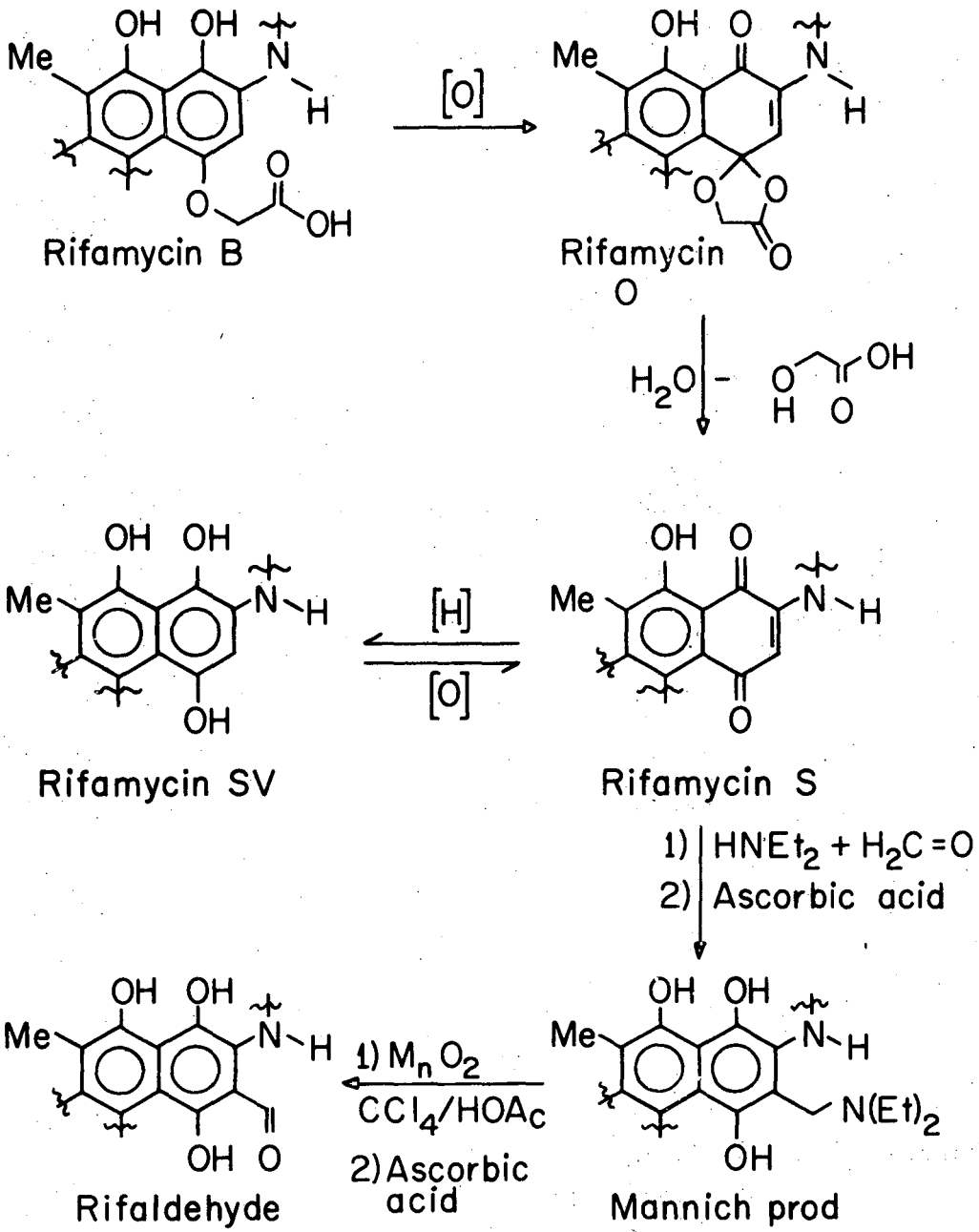

Figure 2

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was determined in 1964 by Brufani et al., utilizing X-ray crystallographic techniques.[6] They used the p-iodoaniline amide of rifamycin B.

Rifamycin SV, typical of the rifamycins, is a yellow-orange solid that decomposes above 140°. It is slightly soluble in water and is increasingly soluble in increasingly alkaline aqueous solution. It is soluble in ether and very soluble in ethanol, methanol, acetone and ethyl acetate. Rifamycin SV is a monobasic acid, pKa = 2.96.[5a] This high acidity is consistent with the hydrojuglone structure (1,4,8-trihydroxynaphthalene) which is incorporated in the rifamycin chromophore. Aqueous solutions absorb both in the UV and visible regions; 223 nm (ε = 40,900), 314 nm (ε = 22,480), and 445 nm (ε = 14,240). Changes in structure not affecting the aromatic moiety have little effect on the absorption properties.

Rifamycin SV, marketed under the name rifacin, left considerable room for improvement as an antibiotic . Firstly, it was found to be effective only against gram-positive bacteria, having little or no activity against gram-negative microorganisms.[3] Secondly, although it is quite effective for the treatment of certain bacterial diseases in humans, and significant blood levels are attained when administered subcutaneously or intramuscularly, no effect is obtained when the drug is administered orally.[7] As a direct result of these drawbacks, an ambitious campaign was undertaken to chemically alter and derivatize the basic rifamycin structure in a search for improved and more generalized antibiotic properties. To date, approximately 750 semisynthetic rifamycins have been prepared and tested. These derivatives can be grouped into the following structural classes[8]:

1) Modifications of the ansa ring. Several modifications have been made. The 3 double bonds of the ansa ring (see Figure 1) can be partially or completely reduced.[5c] The amide at C-15 of rifamycin S has been converted to an iminomethylester which has been hydrolyzed to cleave the chain at this carbon.[5a] The entire ansa ring has been removed from the chromophore by the further hydrolysis of the C-29 end of the ansa ring. The acetyl group at C-25 has been removed by hydrolysis in several rifamycins.[9] Naturally occurring rifamycins with altered ansa rings have been isolated from Streptomyces mediterranei.[10] It has been found that all modifications of the rifamycin SV ansa ring reduce antibacterial activity compared with rifamycin SV.[8]

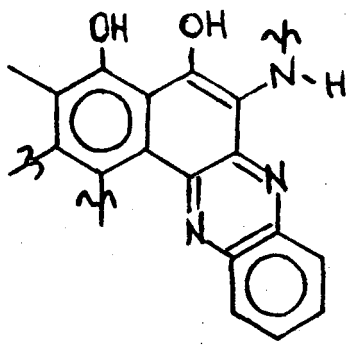
2) Glycolic acid derivatives of rifamycin B. Many amides, esters, and hydrazides have been prepared and tested.[11] On the whole, these derivatives are considerably more active than rifamycin B, but few are better than rifamycin SV. It was noted that amides derived from disubstituted aliphatic amines were more potent than nonsubstituted amides. Amides from amines that contained hydrophilic groups such as hydroxyl, amino, cyano, halogen, etc., were of diminished activity compared to amides from simple aliphatic amines.[8] While several of these derivatives were found to have improved actions compared to rifamycin SV when given orally to mice infected with S. aureus, none were as effective as would be required clinically. One derivative from this class, rifamycin B-N,N-diethylamide (rifamide), was considered the best improvement on rifamycin SV and has found some clinical application.[12]

3) Hydrazones and Schiff bases of rifamycin S. These derivatives have primarily formed by the reactions of aromatic amines, hydrazines, hydrazides, aminoquanidines, etc., with rifamycin O.[13] They were found on the average

to be active, but none were an improvement on rifamycins S or SV.[14]

4) Condensations involving both C-3 and C-4 of rifamycin S.

1,2-Aromatic diamines have been reacted with rifamycin S to give cyclic pyrazine derivatives.[9,15] The reaction can be envisioned as Schiff base formation at C-4 and Michael addition at C-3. The derivative obtained from o-phenylenediamine (rifazine) is as effective as rifamycin SV against gram-positive bacteria and shows improved activity against gram-negative bacteria.[10] Rifazine was one of the first derivatives to demonstrate reasonable antibacterial properties in mice upon oral administration.[16]



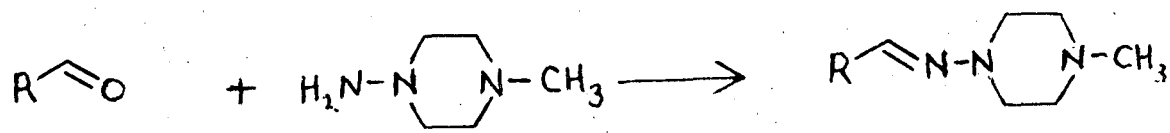
rifazine

5) Michael additions at C-3 on rifamycin S.[9] Many amines and some thiols (those with relatively low reducing potentials, i.e. aryl) have been added to rifamycin S to give derivatives of rifamycin SV substituted at C-3. While many of the resulting derivatives compare well to rifamycin SV, none possess the desired oral activity or activity against gram-negative microorganisms.

6) Mannich condensations on rifamycin S. The reaction of rifamycin S in the presence of formaldehyde and a secondary amine proceeds to yield an aminomethyl substitution on rifamycin SV at C-3.[16,17] Many of these derivatives have been prepared, and while none of them proved to be useful antibiotics in themselves, they have become important intermediates.[8]

7) Rifaldehyde and its derivatives. It was found that many of the Mannich products discussed above could readily be oxidized to yield 3-formylrifamycin SV (rifaldehyde).[18] The synthesis of rifaldehyde is included in Figure 2. From here on the structure of rifaldehyde will be referred to as R=O.

Probably more attention has been given to the derivatization of rifaldehyde than to any other rifamycin. The derivatives prepared and studied belong to various classes; acetals, imines, hydrazones, oximes, hydrazide-hydrazones, and others.[19] Many of these derivatives, especially the N,N-disubstituted hydrazones, were of interest because of their high antitubercular activity and high in vivo activity with mice infected with staphlococcus (S. aureus). As a class, these hydrazones demonstrated greatly increased oral effectiveness.[20] One particular derivative, rifaldehyde N-amino-N'-methylpiperazine hydrazone (rifampicin), to a large extent possessed the properties sought.[8,21] In the S. aureus infected mice, rifampicin's orally administered ED_{50} (0.11 mg/Kg) is as effective as when administered subcutaneously, and approximately 150 times more effective than rifamycin SV also administered subcutaneously. Rifampicin is about as effective against gram-positive organisms as are the cephlosporins and penicillin G, and against gram-negative organisms it is comparable to the cephlosporins and ampicillin.[8] Against M. tuberculosis H37RV only isoniazid is more effective. Today rifampicin is used clinically in the treatment of many bacterial diseases, especially tuberculosis.



rifaldehyde rifampicin

Of the rifamycins that are and have been used clinically, toxicity has not been much of a problem. For example, the LD_{50} for rifampicin in mice is over 600 mg/Kg.[8] That is over 100 times the therapeutic level. Rifampicin also demonstrates low toxicity in cell culture systems such as chick fibroblasts[44] and Balb 3T3.[22a] While low toxicity is common among rifamycin derivatives, it is not general. For example, two rifamycin derivatives, dimethylbenzyldesmethylrifampicin and rifaldehyde octyloxime, both of which have been of interest in cancer therapy, are known to exhibit considerable toxicities in cell culture.[22]

Since the rifamycins were discovered, much effort has been devoted toward the elucidation of its mode of action. In 1964, Frontali and coworkers[23] observed that rifamycin SV inhibited protein synthesis as measured by the uptake of ^{14}C-isoleucine in the cell-free extracts of B. subtilis. The following year, Calvori et al.[24] reported that rifamycin SV inhibits the uptake of ^{14}C-uracil in B. subtilis extracts, and that the polyuridylic acid stimulated incorporation of ^{14}C-phenylalanine into acid insoluble material in crude E. coli B extracts is not inhibited by rifamycin SV. These findings suggested that rifamycin acts directly on transcription. Hartmann and coworkers[25] reported in 1967 that rifamycins caused marked inhibition of in vitro RNA synthesis with DNA-directed RNA polymerase from E. coli in the presence of several DNA templates, natural and synthetic. They also found that in vitro DNA synthesis with DNA-directed DNA polymerase I from E. coli in the presence of the same DNA templates is not affected by the rifamycins. These experiments clearly indicate that RNA synthesis is blocked, not by the binding of the inhibitor to the template, as is the case for actinomycin and chromomycin, but by the direct action of the inhibitors on the bacterial RNA polymerase.

The rifamycins were thus the first antibiotics documented to act by such a mechanism.

Today the nature of the rifamycin-RNA polymerase interaction is well documented. Wehrli and Staehelin[26] have found that while rifamycins do not interfere with the initial binding of the DNA template to the RNA polymerase, they do interfere with the ability of this initial complex to transform, or rearrange to an activated DNA-enzyme complex, whereby the enzyme and the template change their configuration such that the enzyme now binds to specific promotor-sites on the DNA. Thus, transcription is inhibited before the binding of the first nucleotide triphosphate to the activated DNA-enzyme complex. It has been determined that the enzyme-inhibitor complex is quite strong, involving approximately 2 molecules of rifamycin per molecule of the E. coli RNA polymerase. The inhibition constant (K_I) for the interaction has been measured to be between 0.6 and 1.5 x 10^{-7}. In general the rifamycins bind to mammalian RNA polymerases only at concentrations 2 to 3 orders of magnitude higher. Wehrli[26] reported that the inhibition of the bacterial RNA polymerases by the rifamycins is relatively independent of alterations in rifamycin structure except for the ansa ring, suggesting that the observed differences in antibacterial effectiveness is due to differences in transport properties. Rifamycins, in which the ansa ring has been altered, bind to the enzyme either poorly or not at all.

Since the rifamycins exhibit such different effects on RNA polymerases from bacterial and mammalian sources, their effect on viruses and viral polymerases rapidly became a new area of investigation. One of the first viruses investigated was pox virus, a DNA virus that carries its own RNA polymerase. Different investigators[27] noted that fairly

high concentrations of rifampicin (100 ug/ml) inhibited the growth of poxvirus but had no effect on herpes virus and pseudorabies virus. Furthermore, they found that out of the several rifamycins they tested, only rifampicin showed the inhibition. When rifampicin was tested as an inhibitor of the purified vaccinia virus RNA polymerase, no inhibition was measured.[28]

The anti-poxvirus activity of rifampicin has been attributed to the inhibition of the final virus assembly.[27b,29] More specifically, rifampicin has been found to prevent the formation of a certain core polypeptide by inhibiting the cleavage of a longer precursor molecule.[30]

The effect of rifamycins on RNA tumor viruses and their polymerases has been a matter of considerable interest in the last 4 years. The effect of rifamycins on infectious, non-oncogenic RNA viruses, exemplified by vesticular stomatitis virus and reovirus, was also investigated, but no viral or viral polymerase inhibition was found.[27] The interest in the oncogenic RNA viruses can be primarily attributed to the independent discovery by Baltimore, and by Temin and Mizutani, in 1970, of an RNA-directed DNA polymerase (RDP) associated with the virions of mouse leukemia virus (MSV) and Rous Sarcoma virus (RSV).[31] The association of this enzymatic function with oncogenic RNA viruses in general has been made.[32] Because RDP catalyzes a reaction in the reverse direction of transcription, it is often referred to as reverse transcriptase. The presence of this enzyme has provided explanations for the ability of RNA viruses to express themselves genetically in host cells. Three related hypotheses of cell transformation have resulted:

1) Provirus hypothesis.[33] The virus enters the cell and, utilizing its RDP, produces its DNA copy (provirus), which then integrates via other viral or host specific enzymes into the host DNA. This integration, or closely followed acts as a consequence of integration, could then lead to the transformation of the host cell. This hypothesis of transformation involves infectious, horizontally transmitted genetic information.

2) Oncogene hypothesis.[34] The genetic information for transformation (integrated provirus?) is already part of the host DNA, although its expression is repressed, and can be carried for many generations before other factors such as viruses, radiation, or chemical carcinogens act to "turn on" the expression of this genetic information.

3) Protovirus hypothesis.[35] The genetic information for transformation (integrated provirus?) is already part of the host DNA, although it is scattered throughout the cellular genome. Transformation arises when this information is brought together in an expressible form, possibly via RDP.

As can be seen, the oncogene and protovirus hypotheses are similar in that they explain transformation as being hereditary or vertically transmitted. They do not stipulate the origin of the oncogenic material, although viral infection is a possible cause.

As to which, if any, of these hypotheses actually represents the truth and to what extent is still a matter of great controversy.[32c] Also a matter of controversy is the role that RDP plays in the transformation process and its role, if any, in the maintenance of the transformed state. To the extent that the provirus hypothesis

contributes to cancer, compounds which are RDP inhibitors could be useful agents for prophylaxis. If in addition, rapidly growing neoplastic cells, regardless of their origin require RDP, possibly as an alternate route for DNA replication or to affect needed gene amplification, RDP inhibitors could be of value in cancer therapy.

There is now evidence that the provirus hypothesis functions at least in some cases. Evidence for the existence of the provirus associated with the DNA of transformed human cells has been presented utilizing DNA-RNA and DNA-DNA hybridization techniques.[36] A radioactive provirus is prepared through the action of RDP on an endogenous viral RNA from an RNA tumor virus such as mouse mammary tumor virus. This labelled DNA can then act as a probe for locating similar DNA sequences in human DNA. Spiegelman and coworkers, who have prepared and utilized this type of probe, have found that the viral genetic information is usually found in transformed human cells and not in the corresponding normal cells.[37,39b] In one case Spiegelman reported that for 2 different sets of identical twins, in which one twin from each set was leukemic, homology with the labelled provirus was found only in the leukemic twins and not in the normal twins.[38] In many of the cases where the viral genetic information was detected by hybridization, Spiegelman also cited evidence for the presence in these cells of RDP incapsulated in an RNA particle,70 S in size - the same size and configuration associated with RNA tumor viruses.[39]

Thus, since the discovery of RDP, it became desirable to find inhibitors of RDP.[40] If good inhibitors, derived from the rifamycins or any other source could be obtained, they could be useful as a

probe in cell culture and animal systems to help determine the role of RDP and possibly, directly or indirectly, lead to useful therapeutic agents. The rifamycins seemed as likely a point of departure as any for developing RDP inhibitors. Firstly, as a class of compounds they are well documented inhibitors of a class of transcription enzymes. Could they not also be inhibitors of a new class of enzymes which catalyze reverse transcription? Secondly, there were available for testing over 200 derivatives already prepared in the aforementioned antibacterial program.

Major screenings of these derivatives plus a few new ones have been carried out in separate programs by Green and Gallo.[41] The initial screenings amounted to testing each derivative at 100 ug/ml and measuring the percent inhibition of the control RDP activity. Many of the derivatives tested showed activity at this high concentration. Some of the better inhibitors were studied in more depth. However, because of the crude way inhibition was measured very little could be concluded concerning RDP inhibition as a function of rifamycin structure.

Gallo and coworkers have loosely defined 3 classes of rifamycin derivatives, A, B, and C, according to their increasing inhibition of RDP and other cellular polymerases.[42] Class C, the most potent inhibitors of RDP, on the whole contain larger and more lipophilic side chains or "tails" than derivatives of the other two classes. Even the best of the inhibitors uncovered by Gallo and Green do not approach the level of effectiveness of the rifamycins as a class on bacterial RNA polymerases. For example DMB, which is one of the better inhibitors of RDP and the rifamycin used as a standard through-

out this thesis work, inhibits RDP only at concentrations approximately 100 times higher than it inhibits bacterial RNA polymerase.[41]

One serious question as to the value of rifamycins as RDP inhibitors was raised by Silvestri and coworkers.[43] They postulated that the so-called "Class C" rifamycins are nonspecific inhibitors of nucleotide polymerases, if not inhibitors of enzymes in general. This suggestion was based on the study on one class C rifamycin, rifaldehyde octyloxime (one of the more potent RDP inhibitors), on the inhibition of a rifampicin resistent DNA-directed RNA polymerase (DRP) isolated from a rifampicin resistent mutant of E. coli. They found that rifaldehyde octyloxime inhibited this enzyme at about the same concentration as it inhibits RDP. They point out that the octyloxime also inhibits mammalian polymerases at this same concentration, and that it even inhibits enzymes unrelated to polymerases, such as glutamate pyruvate transaminase, and alkaline phosphatase, although at higher concentrations than for the polymerases. They also found that bovine serum albumin (BSA) protects the rifampicin resistent polymerase against inactivation by rifaldehyde octyloxime, suggesting that the derivative binds even to the BSA.

Approximately concurrent with the interest in testing rifamycins as RDP inhibitors was an interest in determining if any of the rifamycins have an effect on the transformation of cell cultures innoculated with appropriate RNA tumor viruses. Here the initial assumption was simple - if rifamycins act as inhibitors of RDP, and RDP is a necessary enzyme for transformation, then rifamycins might be able to inhibit cells from undergoing transformation. Actually, the first

case of rifamycins as inhibitors of transformation, that of Diggelmann and Weissmann,[44] was reported before the discovery of RDP.

It should be noted that during the late 1960's, rifamycins, particularly rifampicin, were a widespread biochemical curiosity. As a result, they were being investigated by many laboratories for almost every activity for which they could be imagined,[26b] and perhaps even a few activities beyond imagination.

After the discovery of RDP, rifamycins as transformation inhibitors in cell cultures were taken much more seriously. In these systems, transformation inhibition is measured as the percent reduction of "foci" in the test plates compared to the controls. A monolayer of normal culture cells is inoculated with the transforming virus at a titer such that relatively few cells are actually transformed. Several days later individual colonies of piled up, neoplastic cells, or foci, are visible against a background of a monolayer of normal cells, marking the sites of transformation. A compound to be tested as a focus inhibitor is added to the test plates either before, during or shortly after viral innoculation and is generally present for the remainder of the experiment. Several reports of foci inhibition have resulted to date.[22,42,45] Rifampicin, which was found not to be an RDP inhibitor, was also found not to be an effective foci inhibitor.[45a] Partial inhibition is found only at levels at or above 80 μg/ml and this may be attributable to toxic effects alone. A few rifamycins, among which DMB is perhaps the most exemplary, were found to be reasonably inhibitory (at approximately 6 μg/ml) in cell systems such as Balb 3T3 inoculated with MSV.[22a] However, DMB is fairly toxic at this level. Amphotericin B, a compound noted for its ability to

increase cell transport of many compounds, has been found to have a synergistic effect with rifamycins in various applications.[46] Hackett and Calvin[47] found that Rifazacyclo-16,[48] a product of this thesis effort, at a level of 6 μg/ml and amphotericin B at 1 μg/ml very effectively inhibited foci in a Balb 3T3 derived line (UCl-B) with no toxicity to normal cells, even up to 3 times the effective dose. Rifazacyclo-16 alone exhibits weaker foci inhibition while amphotericin B alone has no effect.

The goal of this thesis effort has been to further and more systematically investigate rifamycins as inhibitors of RDP, for the purpose of developing useful probes to be used in the investigation of the role of RDP in the transformation process and maintenance of the transformed state. The properties of rifamycin derivatives that we have devoted ourselves directly to are lipophilicity (and size), since the earliest investigations by Green, Gallo, and ourselves suggested that these properties are of some importance. Toward this end several new rifaldehyde hydrazone derivatives have been prepared and evaluated in light of the following questions:

1) How does RDP inhibition vary as a function of the lipophilicity of the derivatives?
2) Can all rifamycin RDP inhibitions be explained in terms of their inherent lipophilicities?
3) Do any of these derivatives specifically inhibit RDP compared to other polymerase enzymes, or are they nonspecific inhibitors of enzymes in general, as has been suggested?

In addition to this central issue, a few spinnoffs and side-tracks have earmarked this thesis effort (like many thesis efforts). They will be mentioned either in the text or in separate sections as seems most appropriate.

PART I:

REVERSE TRANSCRIPTION

A. Introduction

As previously mentioned, RDP is found in the virions of RNA tumor viruses. From one source, avian myeloblastosis virus (AMV), it has been estimated that RDP comprises between 0.3 and 1% of the total viral protein.[49] In cells, RDP is generally found only in transformed cells.[32c] The best evidence concerning human cells has been presented by Spiegelman and coworkers.[39,50] They have found that RDP, in close association with a 70S RNA occurred in approximately 95% of the cells from human leukemias and other cancers. No RDP was found in corresponding normal human cells. Gallo and coworkers have reported similar results.[51] RDP is also frequently found in RNA virus transformed tissue culture cells. These cells, in addition to producing RDP, often produce whole and/or incomplete C-type viruses. Some well documented evidence for RDP in non-transformed cells is that of Crippa and Tocchini-Valentini.[52] They observed RDP activity in the growing oocytes of Xenopus laevis (African clawed frog), suggesting that gene amplification in these differentiating cells and possibly others, involve a reverse transcription mechanism. A review article on RDP by Sarin and Gallo has recently appeared.[32c]

Perhaps the best definition for an RDP is a polymerase activity which can transcribe a natural, endogenous, viral 70S RNA into DNA given all 4 nucleotide triphosphates. This distinction is important,

as much confusion has resulted in the literature distinguishing RDP from other polymerase activities. For a while it was assumed that the ability of an enzyme to synthesize DNA on any RNA template, particularly synthetic templates such as poly(rA):poly(dT) was evidence for an RDP. The main ambiguity stems from the observation that other polymerases, both bacterial and cellular, will also catalyze this reaction under the proper conditions. However, this is not to say that synthetic templates cannot be used for distinguishing between the viral polymerase and other polymerases.[32c] Like many other polymerases which use single stranded polynucleotides as templates, RDP requires primer nucleotides. Unlike these other polymerases, RDP prefers an oligo nucleotide as a primer with an RNA template. An RDP is indicated if it shows a preference for a template comprising poly(rA):oligo(dT) over poly(rA):poly(dT), and shows a relative lack of reactivity with poly(dA):oligo(dT). A much better indication of the viral polymerase yet is that in addition to the above template responses, the enzyme is also capable of using poly(rC):oligo(dG) as a template.

Although the enzyme used in this thesis work is from a cellular source, it has been shown to be an RDP by the proper responses to synthetic templates.[53] The reason for choosing a cellular source over the more common viral sources was largely a matter of convenience. UC1-B cells, a mutant cell line derived from Balb-3T3 and developed by an associate of this laboratory, is a cell line that is readily transformable by mouse leukemia virus (MLV) without the need for a sarcoma virus.[54] The resulting transformed cells exhibit an unusually high RDP activity.[53] Inhibition studies were performed primarily on

partially purified cellular extracts as outlined in Figure 3. This figure also outlines the preparation of partially purified RDP from the virus particles produced by the transformed UC1-B cells. The RDP from both sources has been shown to be identical, by template responses and by rifamycin inhibitions.[53] All of the studies that we have performed with RDP, aside from characterizing the enzyme, have been done with a poly(rA):oligo$(dT)_{12-18}$ template.

Data on the physical properties of various RDP's are now becoming available.[49,55] The enzyme from avian sources, AMV and RSV, appear to be composed of 2 subunits--an α chain of molecular weight 65,000 to 70,000 and a β chain of molecular weight approximately 110,000. It seems possible that either unit alone catalyzes reverse transcription since different reported molecular weights for RDP's other than avian fall between 50,000 to 70,000 and 100,000 to 120,000.[56]

As stated, reverse transcription requires a nucleotide primer. In the endogenous reaction, low molecular weight RNA (4S) has been found physically associated with the 70S RNA, and it is believed that these RNA species serve as primer molecules.[57] The product of the endogenous reaction includes single stranded DNA, DNA-RNA hybrids, and double stranded DNA after short reaction periods, and approximately equal amounts of single and double stranded DNA after extensive reaction periods.[58] In all cases the bulk of the DNA synthesized is comprised of small pieces of DNA, sedimenting in sucrose gradients mostly between 8 and 10S.[58,59] The predominence of the small DNA product may largely be an artifact of the in vitro reaction conditions. It appears that while some sequences of the endogenous template are

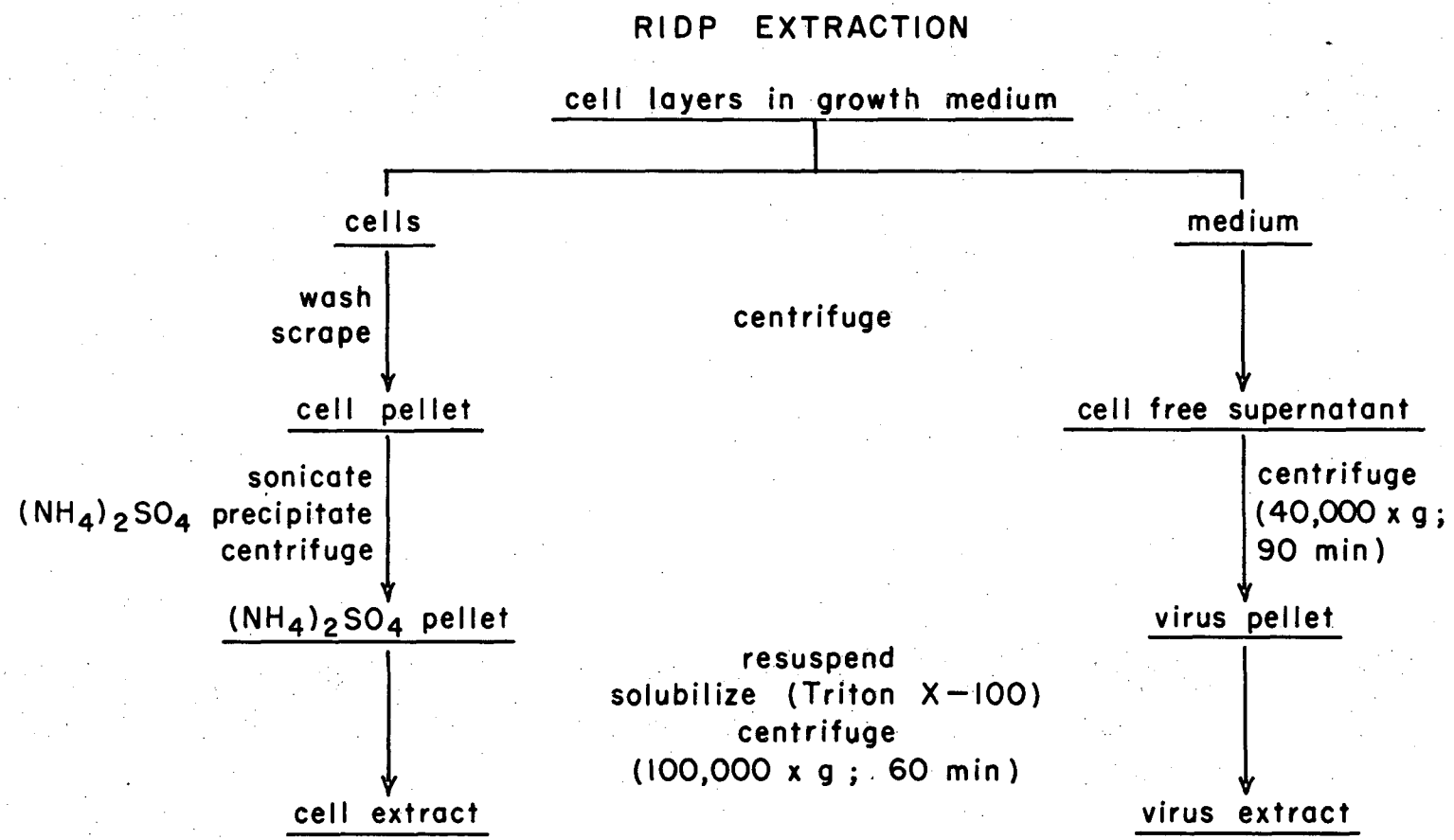


Figure 3

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copied much more frequently than others, the entire 70S RNA is transcribed to some extent.[60] When reverse transcription is performed with a synthetic poly(rA):oligo(dT) template, the product is varying lengths of poly(dT). The synthesis occurs at the 3'-OH end of the primer which is incorporated into the product.

B. The RDP Assay--Assay Conditions

Since the MLV transformed UCl-B cells were a new source of RDP activity, the optimum conditions for the assay had to be established. The conditions given in the experimental section were determined by experimentation to be at or near optimum for the RDP activity from UCl-B cells with the exception on the pH. The optimum pH for this RDP activity is between 8.3 and 8.7. However, assays done at a pH higher than 8.0 resulted in decreased inhibitions by rifamycins due to the rapid oxidation of the rifamycin hydroquinone chromophore. A pH of 7.8 and the use of dithiothreitol (DTT) as an antioxidant at 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 to 4% did not alter the activity, but concentrations greater than 4% decreased the reaction rate. The poly(rA):oligo(dT) and TTP concentrations were nearly saturating, each yielding approximately 90% of the maximal activity. The principal difference between our assays and those described in the literature is the divalent cation concentration. Maximum activity was obtained between 0.05 to 0.20 mM Mn^{+2}. The substitution of Mg^{+2} for Mn^{+2} resulted in greatly reduced activity (<5% of

optimum Mn^{+2} activity). Optimum activity with Mn^{+2} occurred at approximately 1.0 mM.

C. Detergents and Rifamycin Inhibition

It has been well established that a nonionic detergent is required in the RDP assay. Triton X-100 and Nonidet P-40 have been used most often for this purpose. The detergent concentration in our assay was also found to strongly influence the RDP activity. The effect of RDP activity as a function of concentration of Triton X-100 is shown in Figure 4. It is of interest to note that the shape of the curve very much resembles a substrate activation curve.

The quantity of a given rifamycin derivative which was required to inhibit the RDP activity from MLV transformed UCl-B cells was also found to be dependent on the detergent concentration and on the detergent used in the assay. The earliest rifamycin inhibition studies in our laboratory were performed with Triton X-100 as the detergent. In general, it was observed that a single concentration of the effective rifamycin inhibitors of RDP gave lower inhibition values as the concentration of Triton X-100 was increased. This reduced ability of the rifamycin derivatives to inhibit RDP at higher detergent concentrations was found to be due to extraction of the derivatives into detergent micelles. The detergent concentration range over which constant RDP inhibition could be observed was greatly expanded when another detergent, Triton DN-65, was used in place of Triton X-100. Triton DN-65, while as effective as Triton X-100 at activating the enzyme, forms micelles only at much higher concentrations. Figure 5, reproduced from

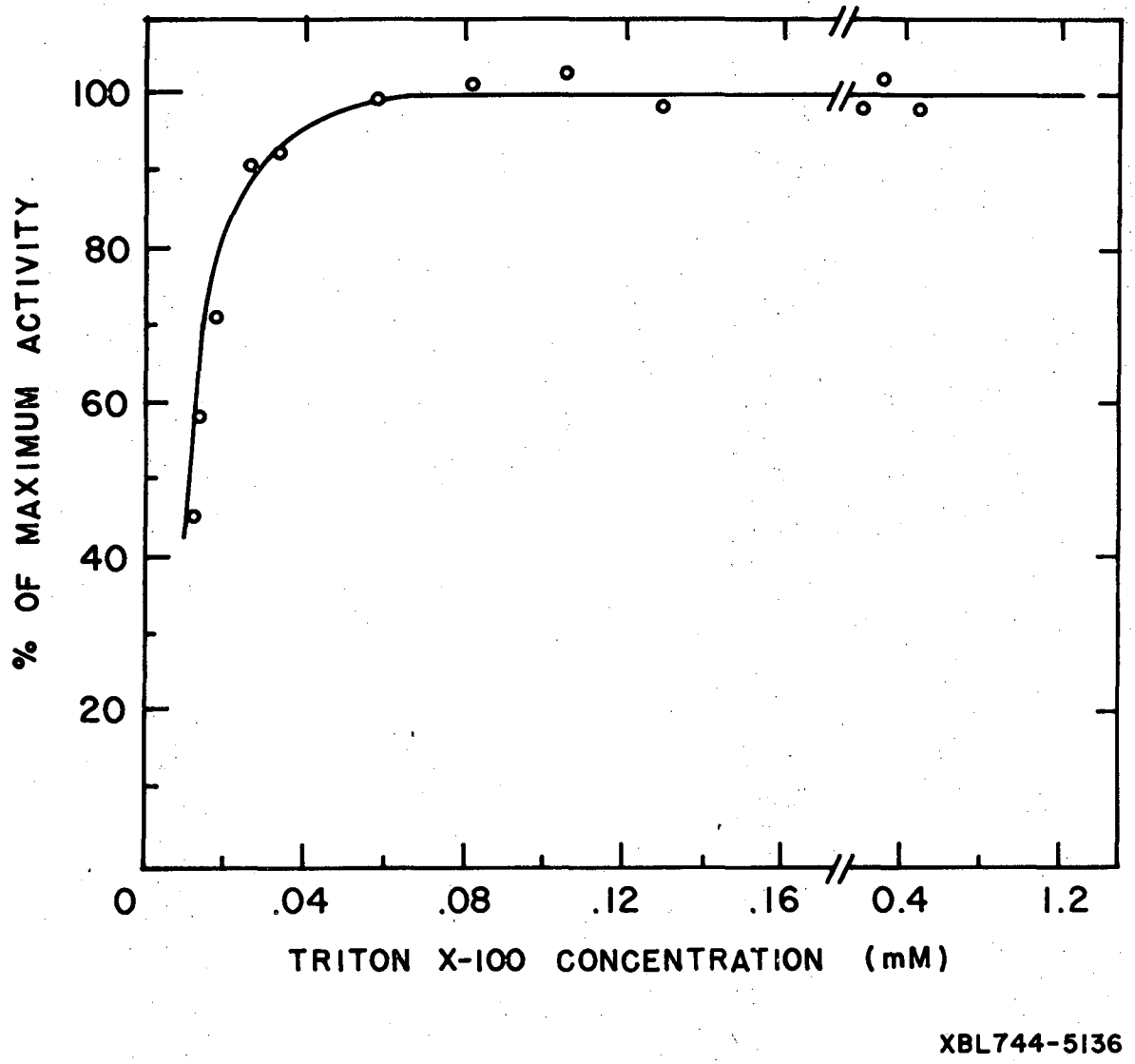


Figure 4. RDP activity as a function of Triton X-100 concentration.

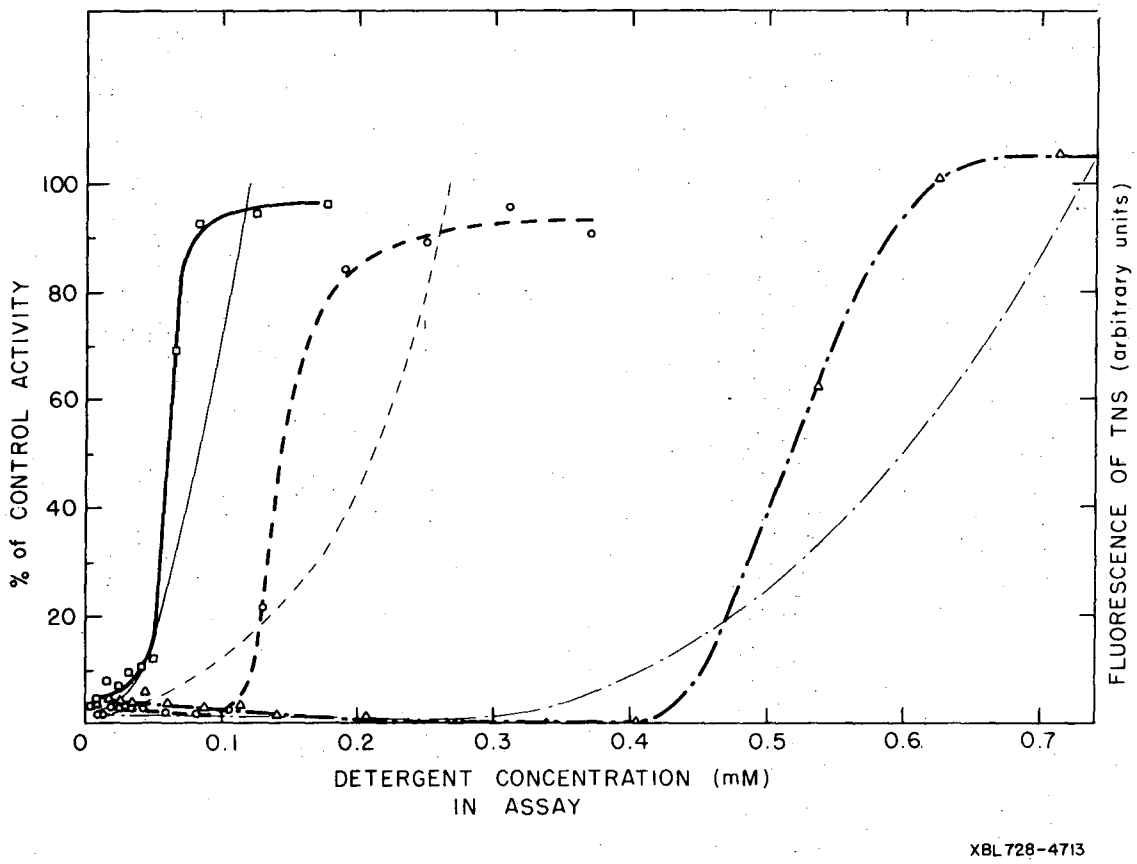


Figure 5. Inhibition of RDP by rifazacyclo-16 (7.5 g/ml) in the presence of 3 nonionic detergents (heavy lines). Micelle formation by 3 nonionic detergents as measured by TNS fluorescence (thin lines). □, Brij-35; o, Triton X-100; Δ, Triton DN-65.

a paper by Thompson et al.,[53a] demonstrates this phenomenon quite well. In this figure, the RDP inhibition of a constant concentration of the rifamycin derivative, rifazacyclo-16 (7.5 μg/ml), is represented in the presence of increasing concentrations of 3 different nonionic detergents: Brij-35, Triton X-100, and Triton DN-65. Superimposed on these inhibition curves is the fluorescence of 2-p-toluidinylnaphthalene-6-sulfonate (TNS) as a function of the same detergent concentrations in a solution which is otherwise the same as the assay solution. Since TNS only fluoresces in nonpolar environments, it provides a convenient measure of micelle concentration. The figure shows that RDP inhibition by rifazacyclo-16 falls off as the concentration of micelles increases. Because Triton DN-65 affords the widest concentration range in which rifamycin inhibition is constant, it was used in all of the inhibition measurements made in this thesis. Triton X-100 is p-(1,1,3,3-tetramethyl-)butylphenol with an average of 9-10 ethylene oxide residues reacted at the phenol oxygen, approximate molecular weight = 650. Triton DN-65 is a mixture of n-octyl and n-decyl alcohols each with an average of 7 ethylene oxide and 2 propylene oxide residues reacted at the alcohol oxygens, approximate molecular weight = 570.

The effect of the detergents on the inhibition of RDP by the rifamycins proved to be useful as a tool for studying the reversibility of rifamycin inhibition. RC-16 was added at an inhibitory level to several RDP assay tubes at a low Triton X-100 concentration, i.e., well below its critical micelle concentration. After 15 min of incubation, the Triton X-100 concentration was increased to well above

the critical micelle concentration in some of the assay tubes, and all the tubes were incubated for an additional 15 minutes. The RDP to which the additional X-100 was added regained nearly full activity, suggesting that inhibition by RC-16 is reversible.

Because of the low water solubility of most of the rifamycin derivatives, it was necessary to first dissolve the derivatives in dimethylsulfoxide (DMSO). These solutions could then be diluted with the assay buffer as required. All assays, including controls and blanks were adjusted to a constant level of DMSO. The level of DMSO used was found not to affect the RDP activity.

D. Experimental

All assays were run in a total volume of 100 µl which was 94 mM Tris-HCl (pH 7.8), 100 mM KCl, 0.4 mM dithiothreitol, 0.02 mM ^{3}H-dTTP (1 C/mmole), 0.1 mM $MnCl_2$, 4% glycerol, 10 µg/ml poly(rA):oligo(dT)$_{12-18}$ (approximately 0.015 OD_{260}/100 µl assay), 0.01% Triton DN-65, 1.0% DMSO, and 43 units of enzyme activity (0.46 µg protein). The rifamycin derivatives tested were initially dissolved in DMSO (at 10 mg/ml) and were diluted as necessary before adding to the reaction mixture.

All assays were initiated by the addition of the enzyme extract and were incubated for 30 min at 37°. A carrier RNA (0.5 ml of 5 mg/ml Torula RNA, 10 mM sodium pyrophosphate, 20 mM EDTA) was added and the material insoluble in 6.7% trichloroacetic acid was collected on 0.45 µ Millipore filters (presoaked in 0.2 M $Na_4P_2O_7$) and washed extensively with 5% trichloroacetic acid. After they were dried, the filters were dissolved in scintillation fluid containing ethanol and dioxane.

Fumed colloidal silica was added to form a stable gel. The gel was counted in a scintillation counter and dpm was calculated from the cpm by comparison with an automatic external standard.

Approximate inhibition constants were first measured based on the authors estimated potency. Once the approximate values were known for each derivative, they were measured more accurately. In these final assays at least 6 concentrations, each in duplicate, were tested for each derivative. Dimethylbenzyldesmethylrifampicin was always included as a standard.

All final inhibition values are reported as an inhibition constant, K_I. The K_I for each derivative is obtained from a plot of derivative concentration versus percent control activity, and it is defined as the concentration of the rifamycin derivative corresponding to 50% inhibition of the control RDP activity.

PART II:

SYNTHESIS OF RIFAMYCIN DERIVATIVES

A. Introduction

The author's work on the development of new rifamycin derivatives began in the summer of 1971. Until that time (from November, 1970) the author had been engaged in a different project--the synthesis of rigid binitroxyl radicals and the study of their spin interactions as measured by electron spin resonance (esr). The transition between this original project and the project which is the subject of this thesis was a gradual one. In June, 1971 Professor Calvin asked the author to devote some time to the synthesis of a nitroxyl derivative of rifaldehyde. It was thought that such a spin labeled rifamycin would be of value in the isolation and characterization of the then recently discovered RDP. It was assumed by Professor Calvin and the author that this task would only amount to a temporary diversion from the original project. However, this assumption was to be short-lived. Even though the spin labeled rifamycin turned out to be not an effective RDP inhibitor, a degradation product of this derivative, identified as the azine dimer of rifaldehyde (rifamazine), did. This led to the investigation of other simple dimers. The results of these derivatives and the results of the mass screening programs independently

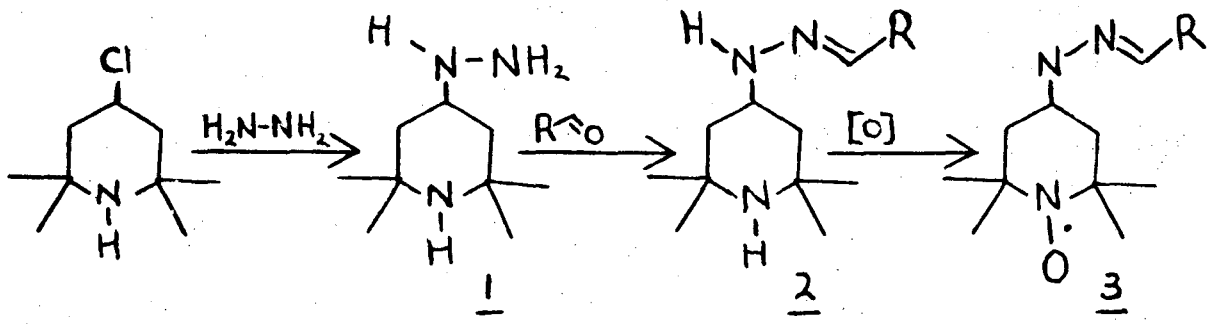
conducted by Gallo and Green, which were published at about this time, led the author to pursue the study of RDP inhibitors as a function of rifamycin lipophilicity. It was also at approximately this time that the author took an active interest in the enzymology involved in RDP inhibition and other aspects of reverse transcription.

This section, which encompasses the bulk of the thesis project, is presented for the most part in a chronological order of events in order to best represent the development of the character and the results of this project.

B. Spin Labeled Rifamycins

It was considered desirable to obtain a potent RDP inhibitor that contained a nitroxyl radical bound to rifamycin. Aside from the usual uses of labeled compounds, such as studying the kinetics of formation and the stability of an enzyme-substrate (or inhibitor) complexation, a nitroxyl label by virtue of the sensitivity of its esr signal to hindrences of rotation could possibly shed some light on the nature of this interaction, in the presence and absence of other substrates.[61] In addition, since the esr hyperfine splitting constant, a, is largely determined by the polarity of its environment, we could learn of the hydrophilicity or lipophilicity of the inhibitor binding site.[62] This technique was used by Hower et al.[61] in 1971, when they investigated the interaction of Bovine carbonic anhydrase with a spin labeled inhibitor.

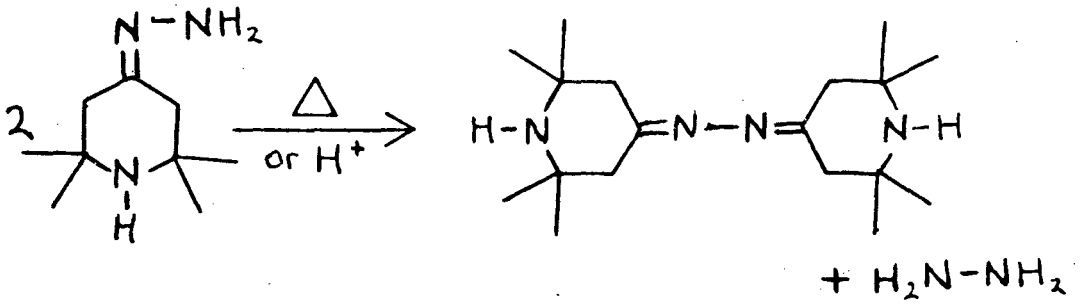
In our first approach we sought to displace the chlorine of 2,2,6,6-tetramethyl-4-chloropiperidine by hydrazine.



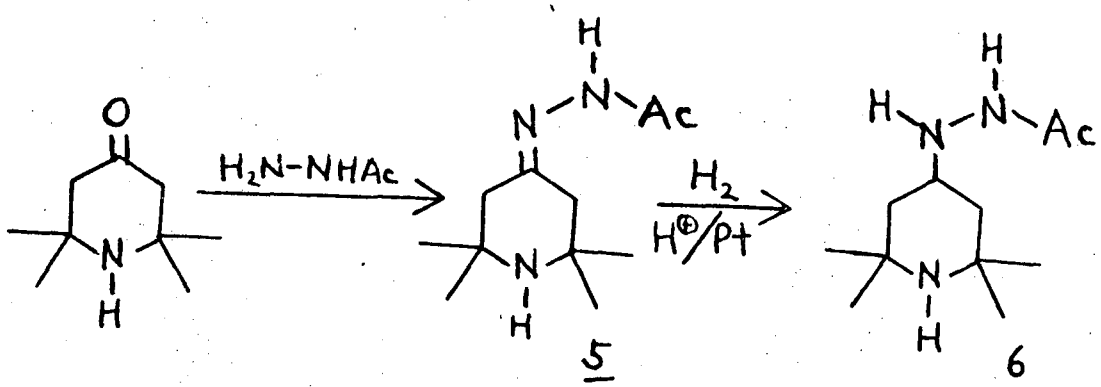
This hydrazine (1) could then be condensed with rifaldehyde to give the tetramethylpiperidine derivative (2). The oxidation of 2,2,6,6-tetramethylpiperidines to nitroxyl radicals is well known.[63] Before

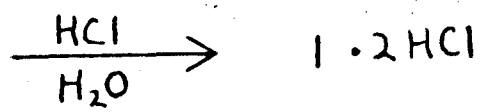
attempting this scheme, rifamycin SV was subjected to the oxidation conditions necessary for nitroxyl formation, sodium tungstate and ethylenediaminetetraacetic acid tetrasodium salt in dilute H_2O_2, and found to be stable. Therefore, the conversion of (2) to the spin labeled rifamycin (3) seemed reasonable. The chloropiperidine was prepared according to the method of Rozantsev,[63] that is the reaction of 2,2,6,6-tetramethyl-4-hydroxypiperidine with $ZnCl_2$ and HCl in a sealed tube. Unfortunately, it proved to be unreactive with hydrazine under a variety of conditions.

Another attempt at the synthesis of (1) was through the catalytic reduction of 2,2,6,6-tetramethylpiperidine-4-one hydrazone (triacetoneamine hydrazone) (4). This hydrazone is readily prepared by the reaction of triacetone amine with an excess of hydrazine.[64] The reduction was found not to take place in neutral solution at room temperature. Temperatures above room temperature were not attempted as it was known that this hydrazone readily disproportionates to di-(triacetoneamine)-azine and hydrazine. The reduction could not be accomplished in acid solution either, since it was found that acid also catalyzes the disproportionation.



The desired hydrazine, (1), was obtained in the following manner:





Triacetone amine was reacted with acetylhydrazide to give the hydrazone-hydrazide, (5), which does not undergo disproportionation. (5) was easily reduced in dilute aqueous acid in the presence of hydrogen and a platinum catalyst to yield the hydrazine-hydrazide, (6).[65] When (6) was dissolved in hydrochloric acid and allowed to react for 48 hrs, the dihydrochloride salt of (1) was obtained upon evaporation of the water, HCl, and acetic acid. As is the case for many of the larger monosubstituted hydrazines, (1) was found to be unstable in air as the free base. When (1) was dissolved in water and neutralized, the oil that separated was found to decompose with the evolution of gas. The dihydrochloride salt of (1) is quite stable. The instability of the free base proved not to be a problem as the next reaction in the sequence, the condensation with rifaldehyde, proceeds best in slightly acid solution, as does the condensation of hydrazines and aldehydes in general.[66] The desired rifamycin derivative, (2), was thus obtained in good yield.

When rifamycin derivative (2) was subjected to H_2O_2 in the presence of Na_2WO_4 and EDTA tetrasodium salt to affect nitroxyl formation, a nitroxyl triplet was formed as detected by esr, and a new rifamycin was formed as detected by tlc. However, when a small amount of the new rifamycin formed was purified by preparative tlc and looked at in the esr spectrometer, no nitroxyl signal was found, indicating that the triacetoneamine moiety had been cleaved from the rifamycin. The author suspects that the product may be 3-carboxy-rifamycin SV.

In order to avoid this oxidation problem in the future, it became obvious that it would be necessary to condense a preformed radical onto rifaldehyde. It was found that this could easily be done by utilizing an azine linkage in the product instead of a hydrazone. The successful synthesis of a spin labeled rifamycin derivative is outlined in Figure 6.

When N-oxyl-2,2,6,6-tetramethylpiperidin-4-one (7) is treated with a large excess of hydrazine in methanol, two reactions occur simultaneously: formation of the hydrazone of the keto group and reduction of the oxyl group to the hydroxylamine. The reaction proceeds with the evolution of nitrogen as a result of the latter reaction. Removal of the excess hydrazine followed by air oxidation in ethanol results in the oxidation of the hydroxylamine, yielding the oxyl hydrazone (8). Condensation of this hydrazone with rifaldehyde affords the spin labeled rifamycin. Both (8) and (9) give the expected nitroxyl triplet in the esr. The hydrazone (8) was never isolated in completely pure form. It slowly disproportionates to the azine dimer (10). This

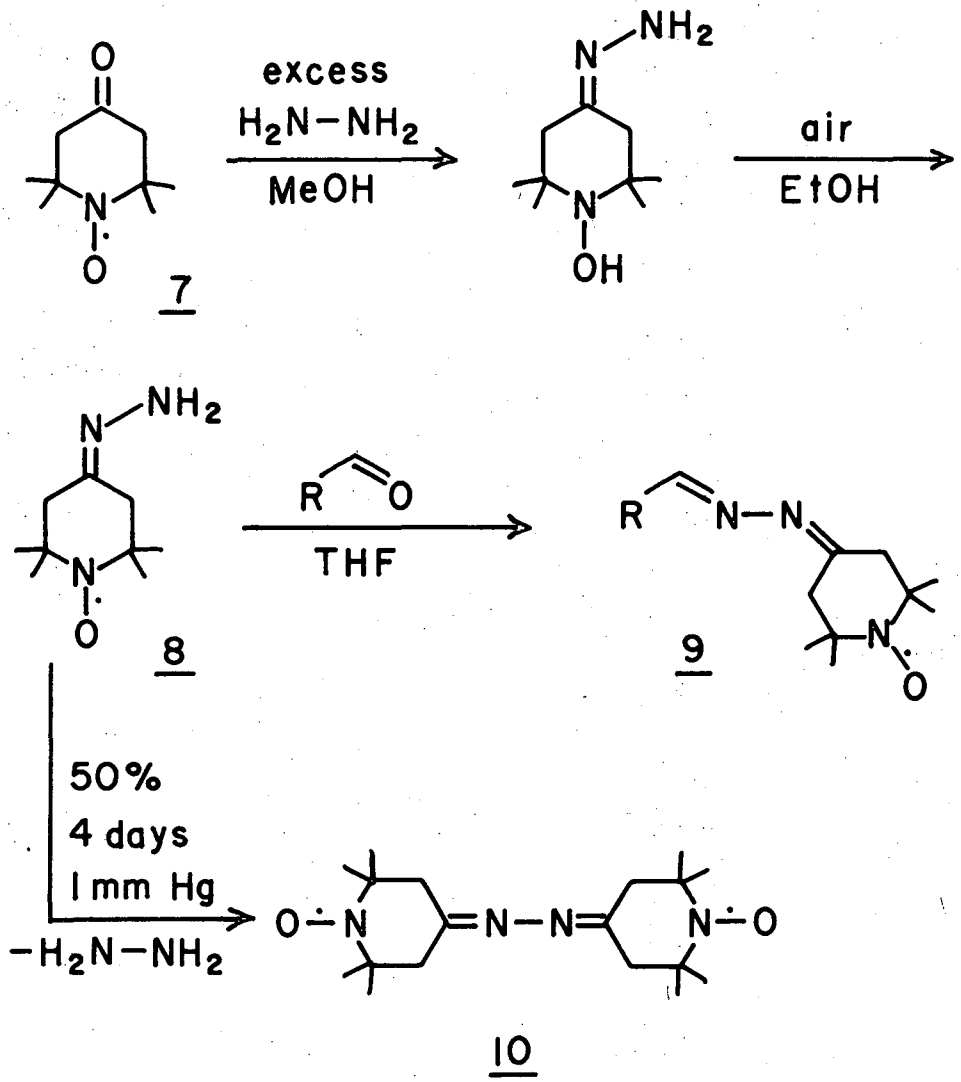
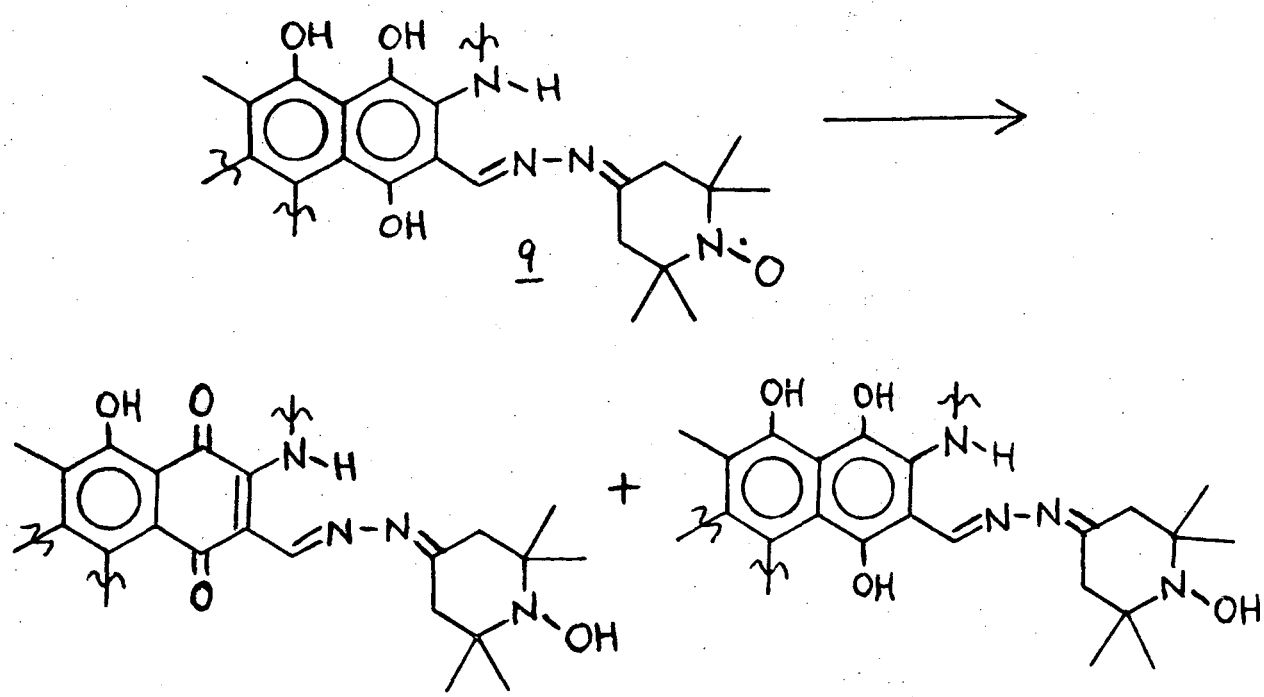


Figure 6. Synthesis of a spin labeled rifamycin.

proved to be no hindrance, as (10) does not react with rifaldehyde. When (8) is evacuated at 50° for 4 days, the azine dimer, which gives an esr consistent with a biradical, is quantitatively formed. The spin labeled rifamycin, (9), was readily separated from (8) and (10) by column chromatograph on neutral alumina.

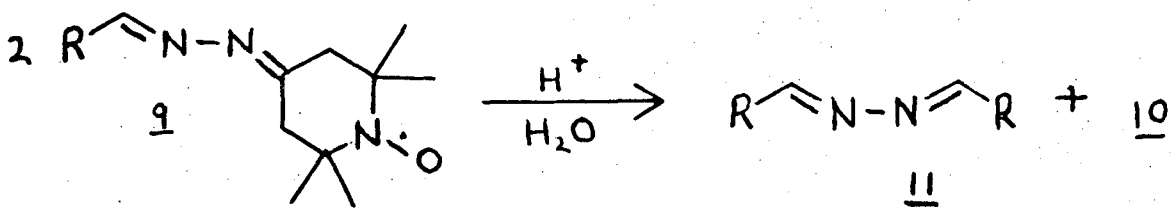
(9) was observed to slowly decompose even under nitrogen at 0°, with loss of its esr signal. Presumably, the compound undergoes a self oxidation-reduction. Nitroxyl radicals easily undergo a 1 electron reduction to hydroxyl amines,[63] and the hydroquinone of the rifamycin chromophore undergoes two 1 electron oxidations to the quinone. Therefore, the products of complete self oxidation-reduction would be an equimolar mixture of the quinone hydroxyl amine and the hydroquinone hydroxyl amine.



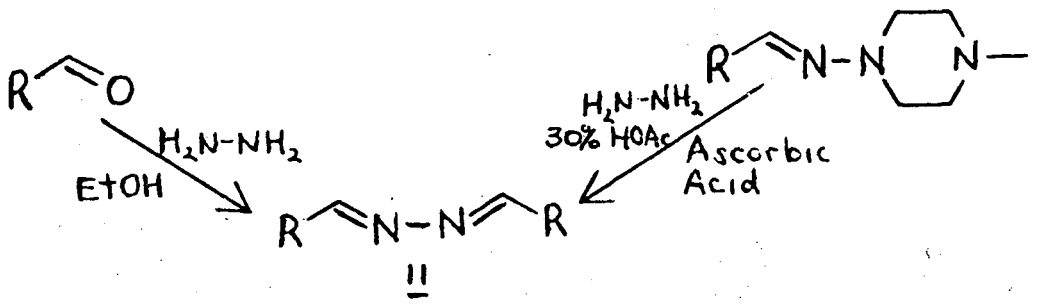
Most probably a stable nitroxyl labeled rifamycin would be obtained if (9) were oxidized to the quinone nitroxyl. This was not done since (9), which was stable over a period of days, was tested as an RDP inhibitor and was found not to be particularly effective. We have determined from studies of DMB and other rifamycins that for any given derivative the hydroquinone is a more effective inhibitor than the quinone.

C. Dimers

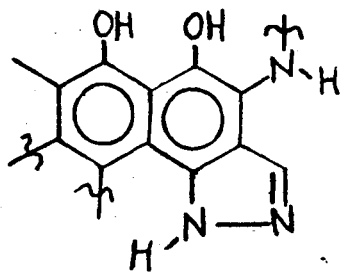
Spin label (9) was found to readily react in aqueous acid to give a bright red precipitate and a compound later identified as the azine biradical (10). The red precipitate was identified by nmr, ir, uv, and Rast molecular weight determination, as the azine dimer of rifaldehyde (rifamazine). In the uv one sees a bathochromic shift relative to rifaldehyde due to the extension of conjugation across 2 rifamycin chromophores.



Rifamazine (11) can be more conveniently prepared by the direct reaction of rifaldehyde with hydrazine or by the reaction of the more readily available rifampicin with hydrazine under hydrolyzing conditions. Both routes afford the azine dimer (11) in near quantitative yield.



The author soon after learned that rifamazine had actually been prepared before by Sensi and coworkers at Gruppo Lepetit S.p.A. in Italy, but they had assigned the structure of the product of the reaction of rifaldehyde and hydrazine as pyrazolo-[4,3-C]4-desoxy-rifamycin SV.



Clearly this could not be the structure on the basis of the uv, analysis and molecular weight determination. On the basis of a correspondence between the author and Dr. Sensi, it has been agreed that the azine dimer is the correct structure.

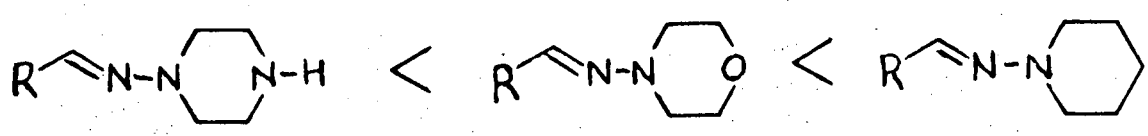
When rifamazine was tested as an inhibitor of RDP it was found to be a more potent inhibitor than our standard rifamycin, DMB (K_I's = 11 x 10^{-6} and 20 x 10^{-6} respectively).

Two other rifamycin dimers were prepared and tested as RDP inhibitors to determine if dimers as a class possess strong inhibitory qualities. The synthesis of 2 dimers, dirifaldehyde-N,N'-diaminourea

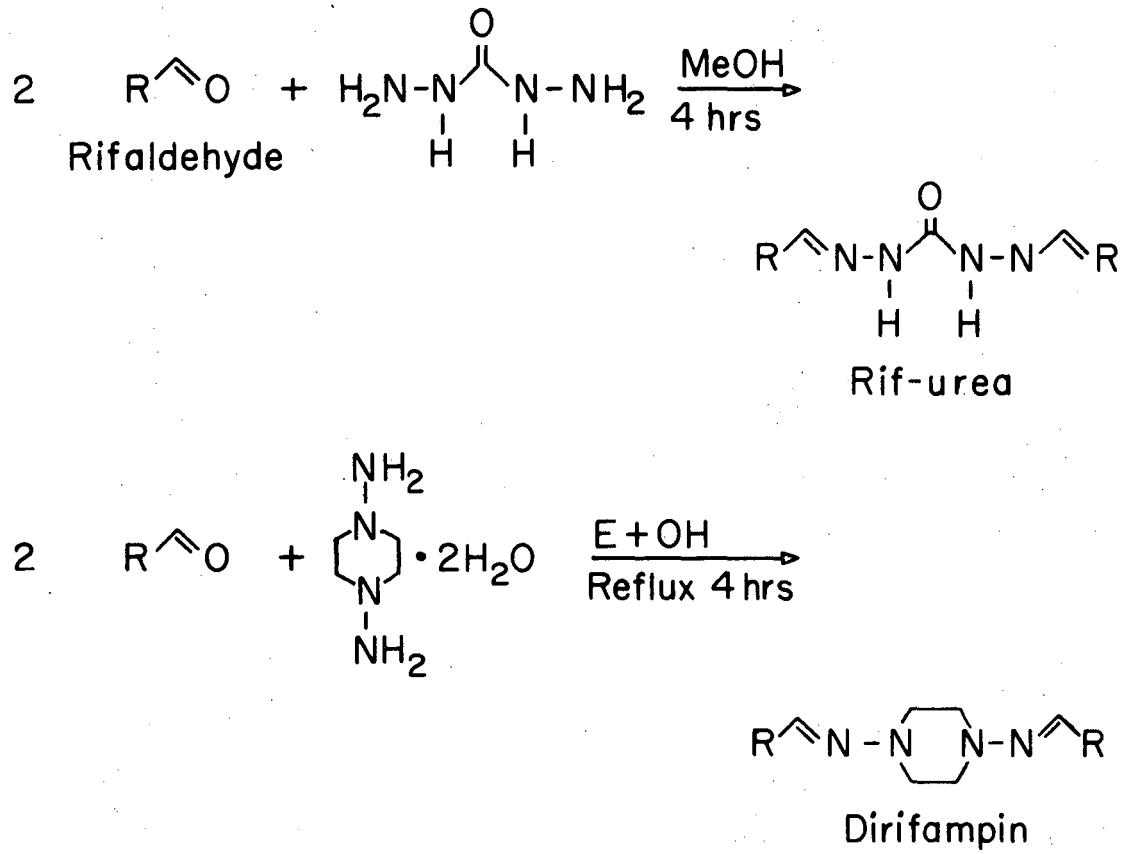
dihydrazone (rif-urea) and dirifaldehyde-N,N'-diaminopiperazine dihydrazone (dirifampin) are given in Figure 7. They were prepared simply by the reaction of rifaldehyde with N,N'-diaminourea and N,N'-diaminopiperazine respectively. Both of these derivatives were found to inhibit RDP similarly to rifamazine, indicating that there is little difference among simple dimers and that there is nothing unique about conjugation extension across 2 rifamycin chromophores.

D. Rifazacyclo-16 and Rifazabicyclo-9

The question arose as to whether the activity of the dimers is due to 2 chromophores in 1 molecule or 1 chromophore with a very bulky group bound to it. A test of the latter possibility, which seemed more reasonable to us, would involve the synthesis and evaluation of derivatives with large groups attached to the C-3 position. Such new derivatives would preferably involve cyclic "tails" to mimic the cyclic ansa structure of rifamycin. In addition, the work of Green and Gallo[41] suggested to us that substituent size being approximately equal, hydrophobic "tails" are more effective than hydrophilic. For example, when one compares the RDP inhibition of the rifaldehyde derivatives derived from N-aminopiperazine, N-aminomorpholine and N-aminopiperidine, one finds almost no inhibition for the piperazine, some inhibition for the morpholine, and better yet inhibition for the piperidine.



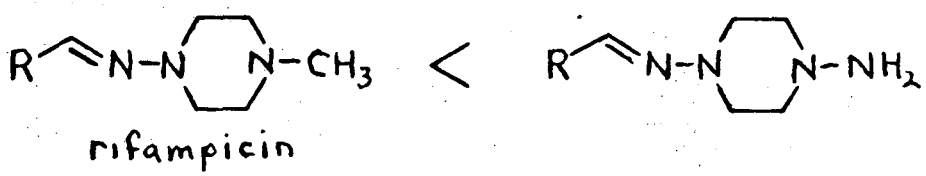
Rifamycin Dimers
Rif-urea and Dirifampin



XBL738-4003

Figure 7

The piperazine, which has a basic N in the 4' position (pK_a 10-11) is the most hydrophilic of the three derivatives, since it is charged at the pH of the RDP assay (pH 7.8). The morpholino derivative, which has an O in the 4' position, is hydrophilic due to the polarity of the C-O bonds, but not nearly as hydrophilic as the piperazine since the O does not carry a full charge. The piperadine derivative, having a C in the 4' position is of course the least hydrophilic of the three. Another interesting comparison is that of rifampicin and aminodesmethylrifampicin. Of the 2, only the aminodesmethyl derivative inhibits RDP.



rifampicin

Just as with desmethylrifampicin, the 4' N of rifampicin is charged at the assay pH. However, the 4' N of aminodesmethylrifampicin, being part of a hydrazine (pK_a 6-7) would be very largely unprotonated at the assay pH and therefore less hydrophilic.

From the above, it was inferred that in addition to being large, hydrophobicity of the rifamycin tail might also be desirable. To test this hypothesis, two new derivatives were prepared.[48] The synthesis of rifaldehyde N-aminoazacyclohexadecane hydrazone (rifazacyclo-16, or RC-16) is given in Figure 8 and rifaldehyde N-amino-3-azabicyclo-[3.2.2]nonane hydrazone (rifazabicyclo-9, or RB-9) is given in Figure 9.

Synthesis of Rifazacyclo-16 (RC-16)

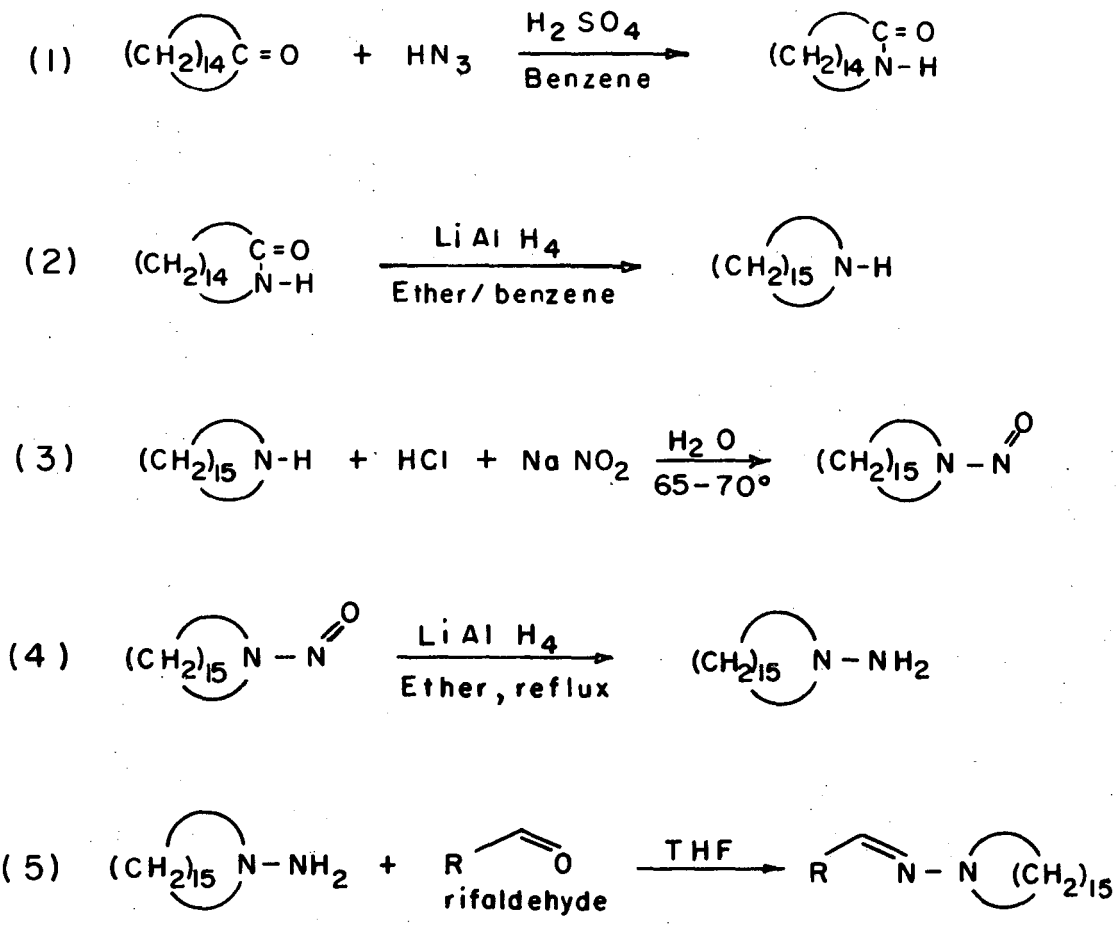


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Figure 8

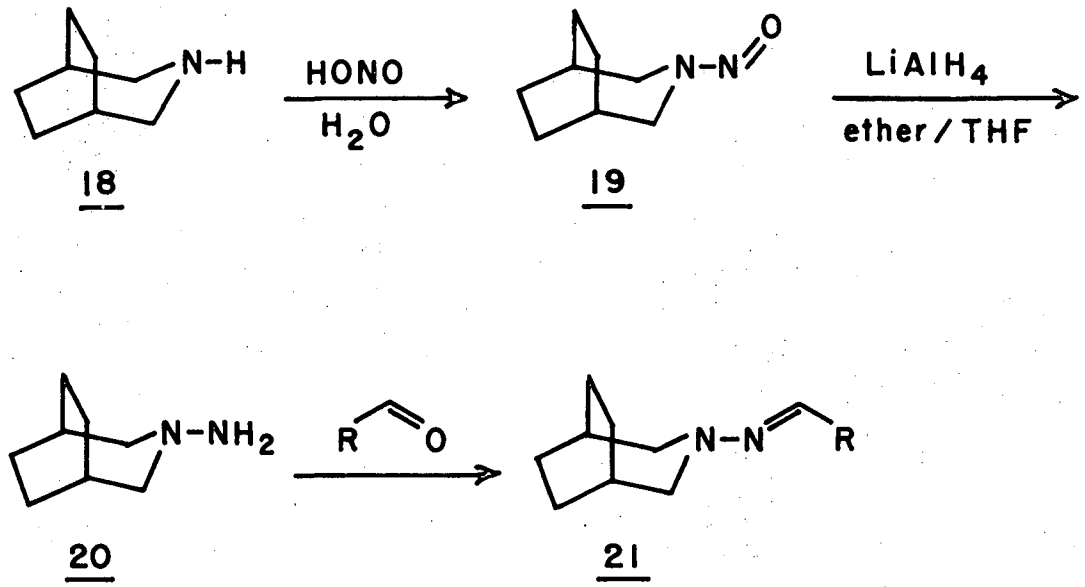

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Figure 9. Synthesis of rifazabicyclo-9.

Cyclopentadecanone (12),[67] one of the odoriferous components of musk oil (exaltone), undergoes the Schmidt reaction in benzene to give the cyclic amide (13),[68] which is readily reduced by lithium aluminum hydride to azacyclohexadecane (14). This amine, typical of secondary amines,[69] is converted to N-aminoazacyclohexadecane (16) by nitrosation to N-nitrosoazacyclohexadecane (15) followed by LiAlH reduction. Analogously, 3-azabicyclo[3.2.2]nonane (18, see Figure 9), a readily available amine prepared commercially by the metal catalyzed pyrolysis of 1,4-di(aminomethyl)cyclohexane,[70] is converted to N-amino-3-azabicyclo[3.2.2]nonane (20) by nitrosation to N-nitroso-3-azabicyclo[3.2.2]nonane (19) followed by $LiAlH_4$ reduction. These two hydrazines (16) and (20) were condensed with rifaldehyde in THF to yield the corresponding hydrazones (17) (rifazacyclo-16) and (21) (rifazabicyclo-9), respectively.

Both rifazacyclo-16 and rifazabicyclo-9 were found to be better inhibitors of RDP than DMB. Rifazacyclo-16 ($K_I = 2.1 \times 10^{-6}$) is the most potent inhibitor of all the rifamycins tested thus far.

E. Rifazacyclo and Rifazone Series

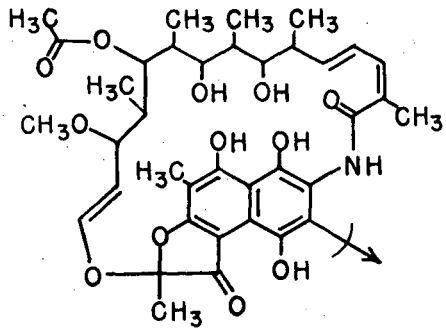
The success of rifazacyclo-16 encouraged us to initiate a detailed study of the effects of lipophilicity. RDP inhibition was not the only reason for this decision. Parallel to the synthetic program conducted by the author was a study of the better derivatives (the dimers and RC-16) as inhibitors of focus formation in both Balb-3T3 cells infected with MSV and UC1-B cells infected with MLV. Both the dimers and RC-16 were found to be ineffective compared to DMB. However, a marked increase in focus inhibition was noted for each of

these derivatives when amphotericin B was included in the focus assay. The increase in activity was most notable for RC-16. The implication is that RC-16 and other rifmaycins are poorly transported into the cells. Amphotericin B acts by increasing the general transport properties of cells, thus allowing a higher concentration of poorly transported compounds inside the cell.[47] As a result, not only were we interested in determining how RDP inhibition is affected by changes in tail size and lipophilicity, but also how cell transport and focus inhibition properties are affected by these tail parameters. It was hoped that such a study would result in the determination of the best compromise between RDP and focus inhibition.

A synthetic program, generating rifazacyclo derivatives with varying ring sizes was in order. However, in addition, lipophilic derivatives with acyclic tails were also in order since no need for cyclic tails had really been established. The synthesis of 2 homologous series has resulted. The rifazacyclo series encompasses the cyclic derivatives with ring sizes ranging from 6 to 16 atoms. The name rifazacyclo (RC) indicates this series and the number that follows indicates the number of atoms in the cyclic tail including the heterocyclic hydrazine N. For example, rifazacyclo-16 (RC-16) is thus rifaldehyde N-aminoazacyclohexadecane hydrazone and rifazacyclo-6 (RC-6) refers to rifaldehyde N-aminopiperidine hydrazone. The term rifazone (R) refers to rifaldehyde derivatives derived from N,N-di-n-alkyl hydrazines. Each number following rifazone refers to the number of C atoms in each alkyl chain. For example, rifazone-8_2 (R-8_2) refers to rifaldehyde N,N-di-n-octylhydrazone. Symmetric

rifazones containing from 8 to 16 C atoms have been prepared. The structures of the rifazacyclo and rifazone derivatives prepared are given in Figure 10. Of these, only RC-6, RC-7 and R-4_2 have been prepared before.

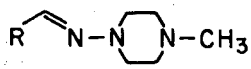
The general synthesis of the derivatives of the rifazacyclo series is outlined in Figure 11. The position in Figure 11 with which each synthesis began depended on the availability of the starting materials. For RC-6 and RC-7 the hydrazines (26, n = 6 and 7) were available, and the rifamycin derivatives were readily prepared by condensation with rifaldehyde. For RC-8, RC-9, and RC-13, the synthesis began with the lactams (23, n = 8, 9 and 13), and for RC-11 and RC-16, the synthesis started with the cyclic ketones (22, n = 11 and 16). For the most part conditions did not vary much from one substrate to another for each reaction. All of the reactions gave yields of greater than 70% once the conditions were worked out. One reaction which deviated from the norm is worth mentioning. The common conditions for the Schmidt reaction, the reaction of a ketone with NaN_3 or HN_3 and H_2SO_4 in benzene, were not suitable for cyclodecanone. The product of the reaction, 2-azacycloundecanone, is only slightly soluble in benzene. The reaction when run in benzene resulted in massive foaming, very sluggish reaction, difficult workup since most of the product forms a thick layer between water and benzene, and low yields. All of these problems were eliminated when chloroform is substituted for benzene. Both the 11 and 13 membered ring lactams were found to be rather insoluble compounds, especially when pure. All the solid intermediates were purified by recrystallization



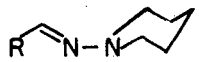
= R

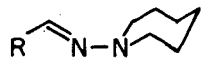
R–H
Rifamycin SV

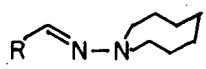

Rifaldehyde

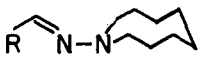

Rifampicin

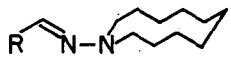
Rifazacyclo Series

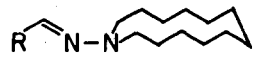

Rifazacyclo-6 (RC-6)

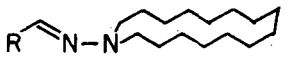

Rifazacyclo-7 (RC-7)


Rifazacyclo-8 (RC-8)

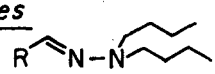

Rifazacyclo-9 (RC-9)

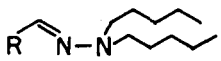

Rifazacyclo-11 (RC-11)

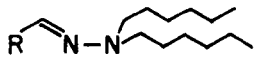

Rifazacyclo-13 (RC-13)

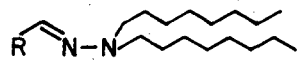

Rifazacyclo-16 (RC-16)

Rifazone Series

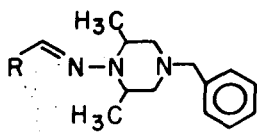

Rifazone-4_2 (R-4_2)

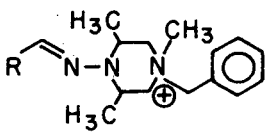

Rifazone-5_2 (R-5_2)

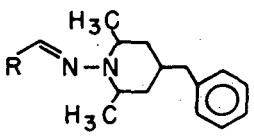

Rifazone-6_2 (R-6_2)


Rifazone-8_2 (R-8_2)

Dimethylbenzyl Series

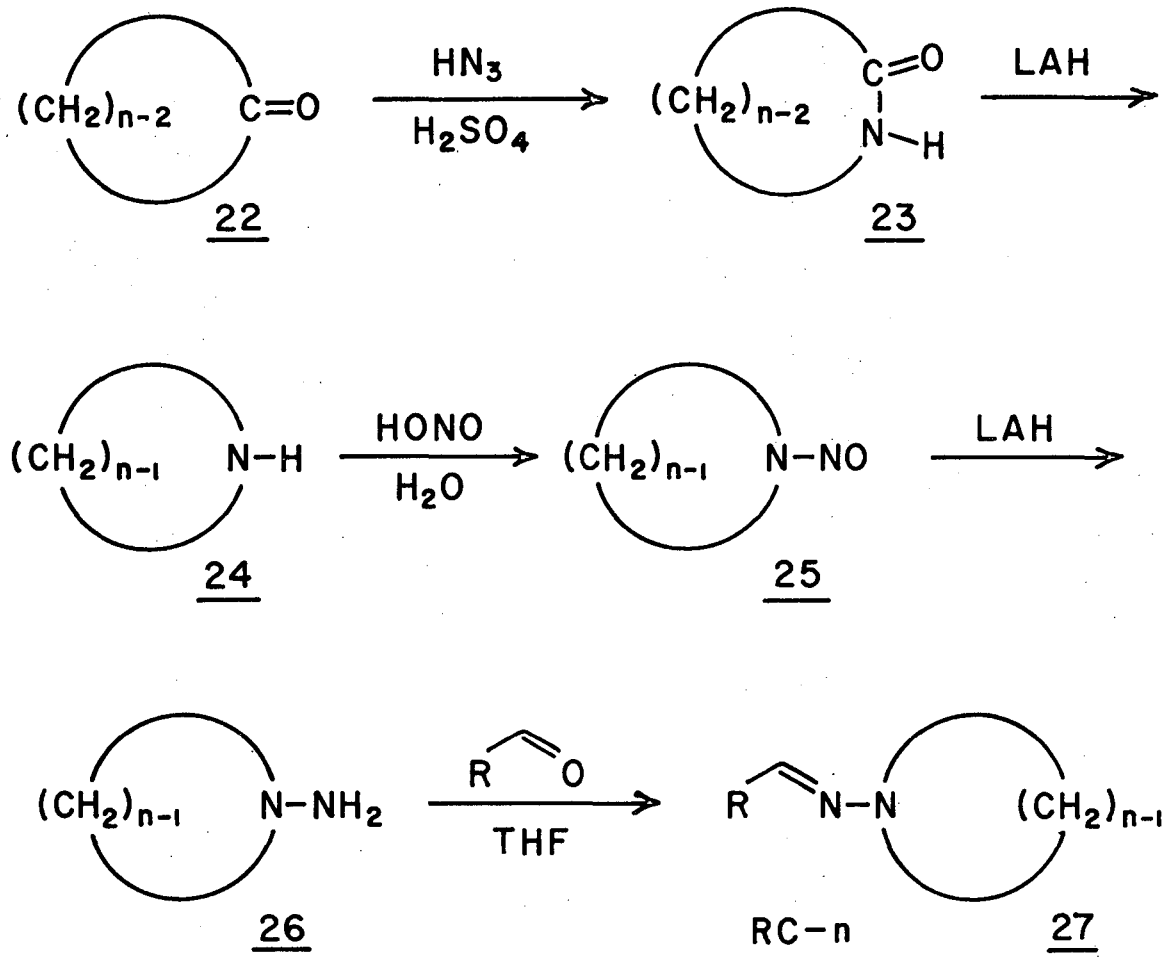

Dimethylbenzyldesmethylrifampicin
(DMB)


DMB – Methyliodide
(DMB-MI)


DMB – Analog

XBL7312-4969

Figure 10. Rifamycin derivatives.



XBL744-5138

Figure 11. Synthesis of rifamycin derivatives of the rifazacyclo series.

techniques and liquid intermediates were distilled under reduced pressure.

All of the rifaldehyde condensations were done in THF with a slight excess of the hydrazine. The reaction times, which varied from derivative to derivative (longer for the larger hydrazines) were conveniently followed by tlc in THF. The condensations all proceeded cleanly and completely. The excess hydrazine was usually separable from the powdered product by washing with hexane. Further purifications were possible either by recrystallization or column chromatography.

The starting material for each of the derivatives in the rifazone series was the appropriate disubstituted amine which was converted to the respective hydrazines by nitrosation followed by lithium aluminum hydride reduction. These reactions and the condensation of the hydrazines with rifaldehyde usually paralleled those of the rifazacyclo series. The only exception was the nitrosation of di-n-octyl amine, which did not go in water since the amine hydrochloride is insoluble. The method of Carroll and Wright,[71] which uses KNO_2 in acetic acid, was used instead.

The results of the testing of these series of rifamycin derivatives, along with others, will be presented and discussed in Part III.

F. DMB Series

The rifazacyclo and rifazone series discussed in the previous section were designed to look at both size and lipophilicity, since within each series the more lipophilic tails are also larger. It was thought to be desirable to prepare a new series to investigate

lipophilicity in a series of derivatives of approximately the same size and configuration. Such series can be picked out of the literature, as the piperazine, morpholine, and piperidine derivatives mentioned earlier, but none of them include derivatives which are potent RDP inhibitors.

The compound that we thought most desirable to build derivatives around was DMB. DMB has been until recently one of the best studied rifamycin inhibitors of RDP and inhibitors of focus formation in cell culture. It has been suspected that there is something unique about its configuration. We found that cis-dimethyl DMB (the isomer most often used) is a better inhibitor than the trans-dimethyl DMB. In spite of its activity, DMB contains a basic nitrogen in the piperazine ring. If lipophilicity is a desirable property for RDP inhibition, and there is something unique about the configuration of the tail in DMB, then the a derivative that replaces the basic 4'-N in DMB for carbon might be a particularly potent inhibitor.

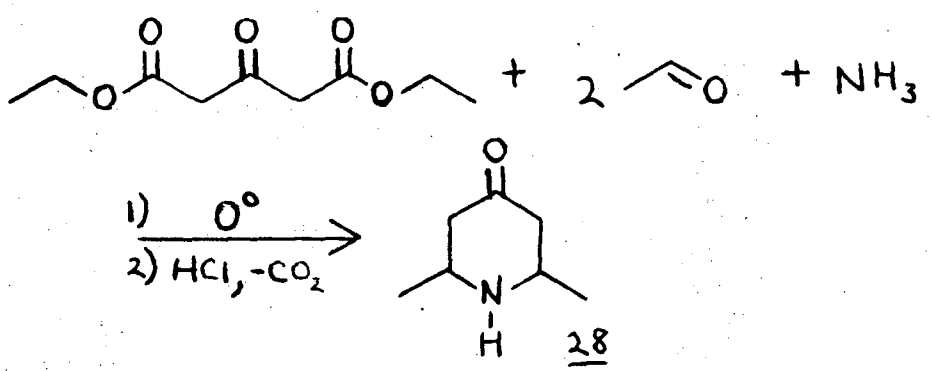
It was also suspected that the activity of DMB was due largely to unprotonated molecules. The 4' N of DMB would be expected to be considerably less basic than the 4' N of rifampicin (which does not inhibit RDP) due to increased steric bulk of the 2 methyl and 1 phenyl groups. Therefore another derivative which would be useful would be a DMB-like structure with a fixed charge on N.

The result of these considerations has been the preparation of a DMB series--the synthesis of a quaternary derivative of DMB, DMB-methl-iodide (DMB-MI), and an analog of DMB in which the 4' N is replaced with carbon (DMB-analog). Since DMB-MI has a fixed charge and DMB-

analog has no charge, we can investigate the effect of lipophilicity in a series of compounds each having approximately the same configuration. The structures of these derivatives are included in Figure 10 (p. 47).

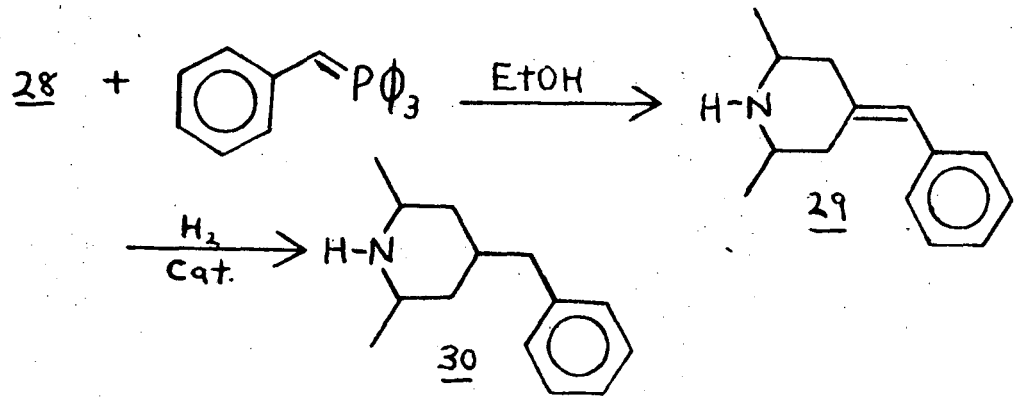
DMB-MI and rifampicin-methyl iodide (RMI) are readily prepared from their parent derivatives. When rifampicin was dissolved in methyl-iodide, RMI precipitated out within a few hours. The compound exhibits increased water solubility over rifampicin and aqueous solutions treated with $AgNO_3$ resulted in the immediate precipitation of AgI. In the nmr the N-methyl peak is replaced by a larger downfield singlet integrating as 6 protons. DMB reacted similarly except the product does not precipitate from methyliodide. The nmr of DMB-MI shows a downfield shift of the benzyl protons and the appearance of a new methyl group just upfield of the benzyl absorption.

The preparation of DMB-analog has proved to be much more difficult. It has been reported by Hall[72] that the condensation of diethylacetone-1,3-dicarboxylate with acetaldehyde and ammonia proceeds to give cis-2,6-dimethyl-4-piperidone.



28

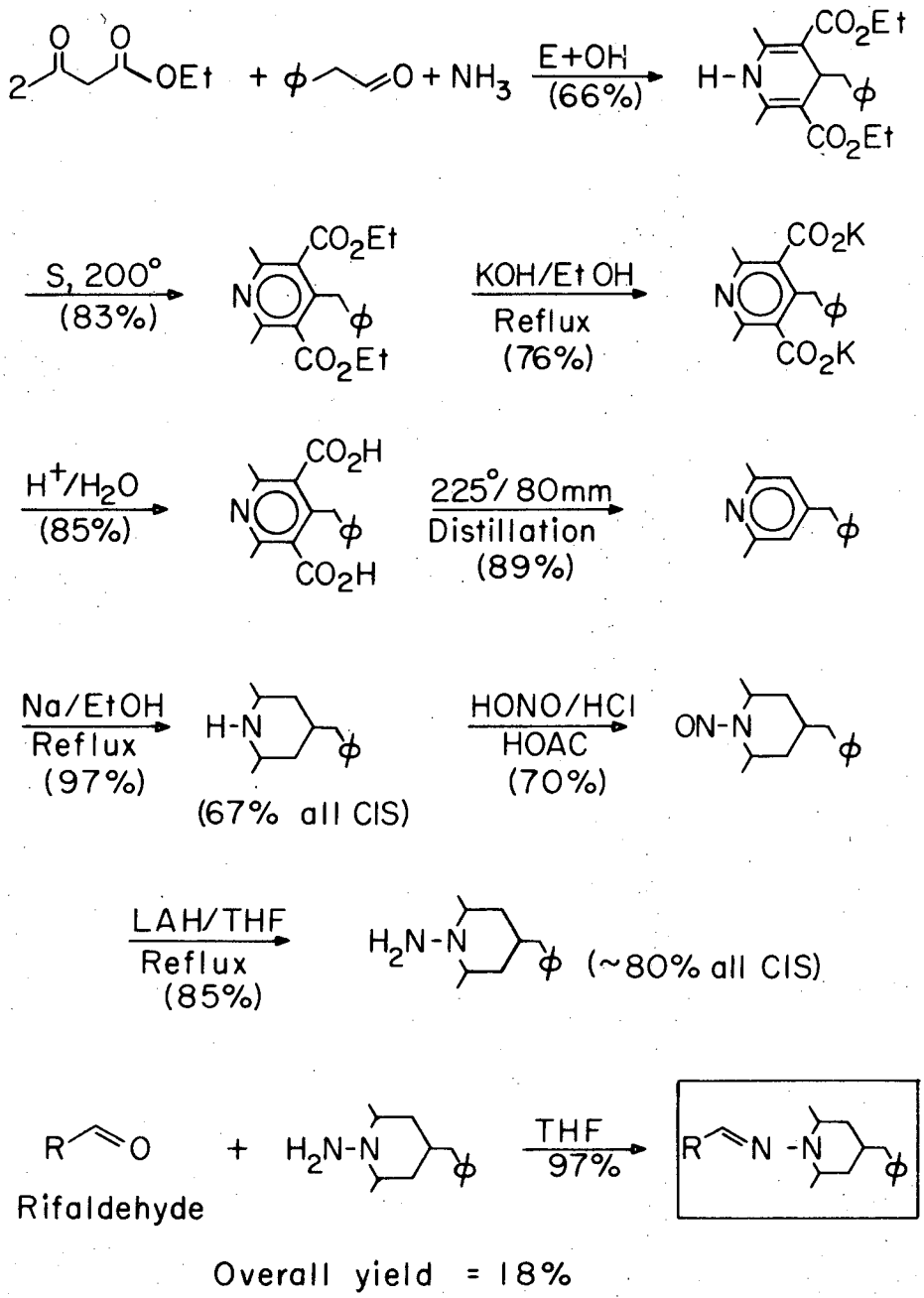
It was hoped to obtain this piperidone and to react it with a benzyl ylid to give (29). Catalytic reduction of the double bond of (29) would yield the amine from which DMB-analog could be routinely prepared.



Unfortunately, the author has not been able to repeat the preparation that yields (28)[72] after three attempts. The product in each case has been only dark brown polymers. Rather than to embark on a detailed analysis of this reaction, it was decided to attempt the synthesis of DMB-analog through another route. This alternate synthetic scheme, although it has led to the desired product, has become a longer scheme than originally anticipated. The final synthesis is presented in Figure 12.

The condensation of phenylacetaldehyde, ethylacetoacetate and ammonia in ethanol was performed according to Huntress and Shaw.[73] It was noted that unless the phenylacetaldehyde was distilled just before using, poor yields were obtained. Phenylacetaldehyde readily polymerizes at room temperature. Most of the polymer is reverted to the monomer upon distillation. It was hoped that the resulting dihydropyridine-diester, (31), could be saponified to the dihydropyridine-

Synthesis of a Lipophilic Analog of Dimethylphenylrifampicin

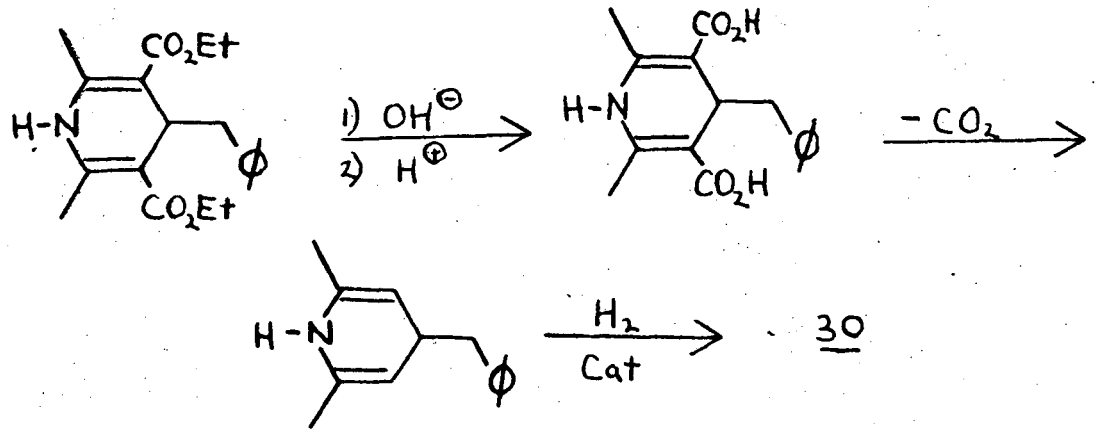


Overall yield = 18%

XBL738-3995

Figure 12

diacid. This compound could then be decarboxylated and then catalytically reduced to give the desired amine, (30).



However, when (31) was refluxed in ethanolic KOH, even for extended periods, no diacid, (39), was detected. Only the partially decomposed starting material was obtained. The saponification was also attempted in hot KOH in DMSO. It was intended that the increased nucleophilicity of hydroxide in this aprotic solvent would allow the soaponification to proceed, but again the vinylogous carbamate was stable.

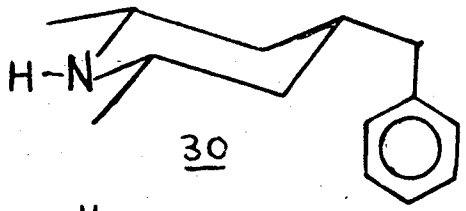
It was then decided to proceed along as indicated in Figure 12, since the pyridine diester, (32), had been both saponified and decarboxylated by Huntress and Shaw.[73] The aromatization of (31) to the pyridine-diester, (32), and the subsequent saponification of (32) to the pyridine-diacid dipotassium salt, (33), proceeded as described. The decarboxylation of (33) was also described by Huntress and Shaw. However, their pyrolysis which was run above 1000° required special glassware made from Corning 172 Pyrex instead of ordinary Pyrex. In addition, only 10 g of (33) could be decarboxylated at a time. Rather than using this procedure, we decided to investigate the decarboxylation

of the free acid, (34). It was found that (34) could conveniently be decarboxylated in quantity and high yield by placing the diacid in a vacuum distillation apparatus, and heating at 225° at 80 mm. Under these conditions the product was distilled into a collection flask. It was found that the isolation of the free acid, (34), was not necessary. The diacid dipotassium salt could be neutralized in aqueous solution with H_2SO_4 and the water evaporated under vacuum. The mixture of the diacid and K_2SO_4 could be utilized in the decarboxylation reaction directly.

We had expected to be able to carry out the reduction of pyridine, (35), by hydrogenation with Adam's catalyst. It has been reported that the reduction of pyridines occurs preferentially to benzenes, even when both aromatic moieties are present in the same molecule. However, after several attempts, we found that both rings in (35) were reduced, and when only 3 equivalents of hydrogen were allowed to be taken up, the phenyl ring took up more hydrogen than the pyridine ring. Presumably, the normally facile pyridine ring reduction is slowed due to steric factors. We found that (35) could easily be reduced to the pyridine when a modification of the Birch reduction was employed. This reaction has been used for the reduction of the parent compound, pyridine, to piperidine.[74] The reaction proceeded with the reduction of the pyridine ring exclusively.

The piperidine, (30), was analyzed by nmr to determine to what extent the thermodynamically most stable isomer, the all cis piperidine, was formed. The major component of the mixture, comprising approximately 67%, was an isomer in which the 2 methyl groups were

equivalent, and therefore cis. The equivalence of the methyl groups in the major isomer present, itself does not tell us whether the benzyl group is cis or trans. Since the reaction that led to the piperidine is nonstereoselective, we would expect the thermodynamically most stable isomer to predominate. Therefore, it is most likely that the benzyl group in the major product is cis to the methyl groups.



30

all cis isomer

Because of the low water solubility of the hydrochloride salt of (30), the nitrosation to the N-nitrosopiperidine, (36), was carried out in acetic acid in accordance with the procedure of Carroll and Wright. (36) was reduced to the hydrazine, (37). Nmr of the purified hydrazine indicated that the all cis isomer comprised approximately 80% of the isomer mixture. It is possible that this enrichment of the all cis isomer is due to the higher reactivity of the less hindered all cis piperidine, (30), upon nitrosation and subsequent reduction.

As expected, the hydrazine, (37), condensed with rifaldehyde to give the DMB-analog, (38). Although the synthesis was longer than anticipated (9 steps), all of the steps proved to be straightforward and reproducible. The overall yield based on phenylacetaldehyde was 18%.

G. Lipophilicity

In order to judge the effect of rifamycin derivative lipophilicity on RDP inhibition, it is necessary to have a quantitative measure of the lipophilicity that each "tail" contributes to the basic rifamycin SV structure. Within each homologous series, the rifazacyclo series for example, the number of carbon atoms in the tail might be sufficient for comparing lipophilicities, since each methylene that we add should contribute a constant increase in lipophilicity. However, the number of carbon atoms in the tail is of little value when comparing rifamycin derivatives from different series. For example, we can compare the lipophilicities of RC-9 and RC-11, which have 8 and 10 carbon atoms in the tail, respectively. They are both cyclic structures, and are therefore similarly restricted in their conformations. RC-11 is clearly more lipophilic than RC-9. But it is difficult to compare the lipophilicity of RC-11 with R-5_2 (also 10 carbons) or with R-4_2 (8 carbons). Both R-5_2 and R-4_2 are open chain structures and as a result possess considerably more freedom of movement--i.e., a larger number of conformations, since each carbon-carbon and carbon-nitrogen bond can rotate 360°. From this we may guess that R-5_2 is more lipophilic than RC-11, since they both have 10 carbon atoms in the tail, but what about RC-11 and R-4_2? Here it is more difficult to predict. Within the DMB series, it is quite reasonable to say that DMB-analog is more, and DMB-MI is less lipophilic, than DMB, but a priori we cannot predict by how much. It is even more difficult to predict whether a derivative such as DMB is more or less lipophilic than RC-13, let alone by how much.

It is quite clear then that a measure of tail lipophilicity is necessary. Perhaps the most useful parameter for relating the lipophilicity of a series of similar compounds, in this case derivatives of rifamycin SV, is the substituent coefficient, π, which has been defined by Hansch[75] as the free energy relationship,

$$\pi = \log (P_X/P_H) ,$$

where P_X and P_H are the experimentally determined partition coefficients for the substituted and unsubstituted compounds respectively. By cancelling the effect of the unsubstituted compound, in our case rifamycin SV, we are left with a relative measure of the lipophilicity contributed by the tail alone. The partition coefficients are obtained by an accurate determination of the relative concentration that results when a compound is allowed to reach an equilibrium between an aqueous and organic (usually n-octanol) layer. The usefulness of the π parameter as opposed to just a partition coefficient stems from the fact that in addition to the cancelling of the contribution of the unsubstituted part of the molecule, the specifics of the experimental conditions, such as temperature, nature of the organic solvent, pH, and ionic strength of the aqueous phase are, at least in theory, also cancelled as a result of the free energy derived nature of the π parameter.

We found that the technique of partition coefficients unsuitable for the more lipophilic rifamycin derivatives, primarily because of the low water solubility of these derivatives. Instead, we have resorted to techniques of reversed phase thin layer chromatography.[76]

This technique, which is especially suited for the separation of lipophilic molecules, utilizes the elution by a polar solvent system on a hydrophobic solid support. The solid support most often used is silica gel which has been coated with a silicon fluid to render the surface hydrophobic. The eluent is commonly a mixture of acetone and water saturated with the silicon fluid. The R_F values thus obtained have been shown to be related to the partition coefficients, P, by the expression[76b]

$$P = \text{constant } (1/R_F - 1)$$

By utilizing the term R_M which has been defined as log $(1/R_F-1)$,[77] we can obtain an expression for the substituent coefficient, π, in terms of data obtained by the reversed phase thin layer chromatography system.

$$\pi = \Delta R_M = R_M(\text{derivative}) - R_M(\text{rifamycin SV})$$

A photograph of a typical experimental reversed phase tlc chromatogram is given in Figure 13. No visualization was required, as all of the rifamycin derivatives are highly colored. The ΔR_M calculated from the measured R_F values for each derivative was the average of at least 5 determinations. The average ΔR_M thus obtained for each derivative of the rifazacyclo, rifazone, and DMB series, and a few others is given in Table I along with the number of carbon atoms in the tail of each derivative. Figure 14 shows that there exists a near linear relationship between ΔR_M and the number of carbon atoms in the tail for both the rifazacyclo and rifazone series. The graph

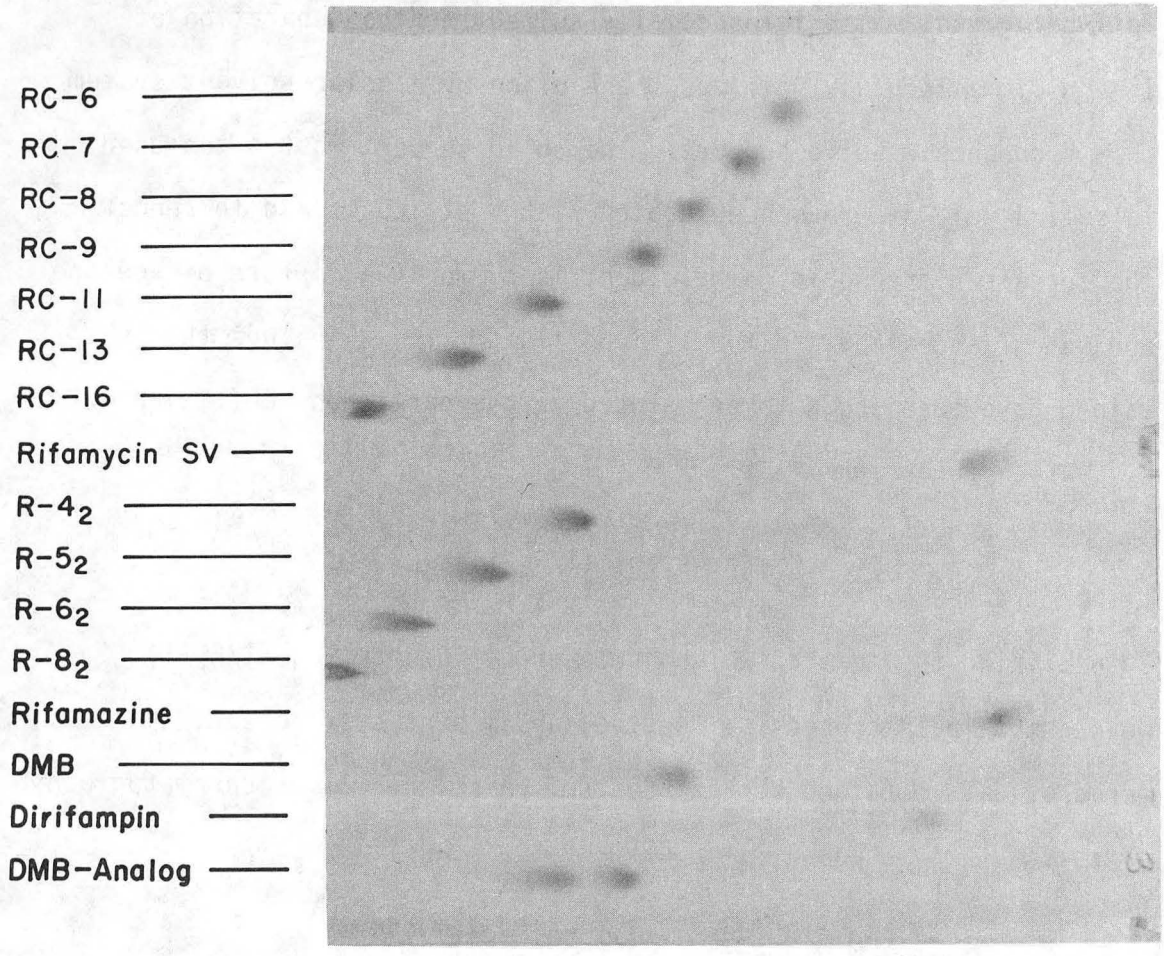


Figure 13. Reversed phase thin layer chromatograph of selected rifamycin derivatives.

Table I

Derivative	No. of Carbon Atoms in Tail	R_M
Rifazacyclo series		
Rifazacyclo-6	5	0.56
Rifazacyclo-7	6	0.68
Rifazacyclo-8	7	0.80
Rifazacyclo-9	8	0.92
Rifazacyclo-11	10	1.20
Rifazacyclo-13	12	1.49
Rifazacyclo-16	15	2.06
Rifazone series		
Rifazone-4_2	8	1.11
Rifazone-5_2	10	1.43
Rifazone-6_2	12	1.80
Rifazone-8_2	16	2.61
DMB series		
DMB	13	0.85
DMB-MI	14	0.40
DMB-analog	14	1.20 (1.01)*
Rifamazine	--	0.00
Dirifampin	--	0.15
Rifampicin	--	0.41

*Isomers other than all cis-dimethylbenzylpiperidine.

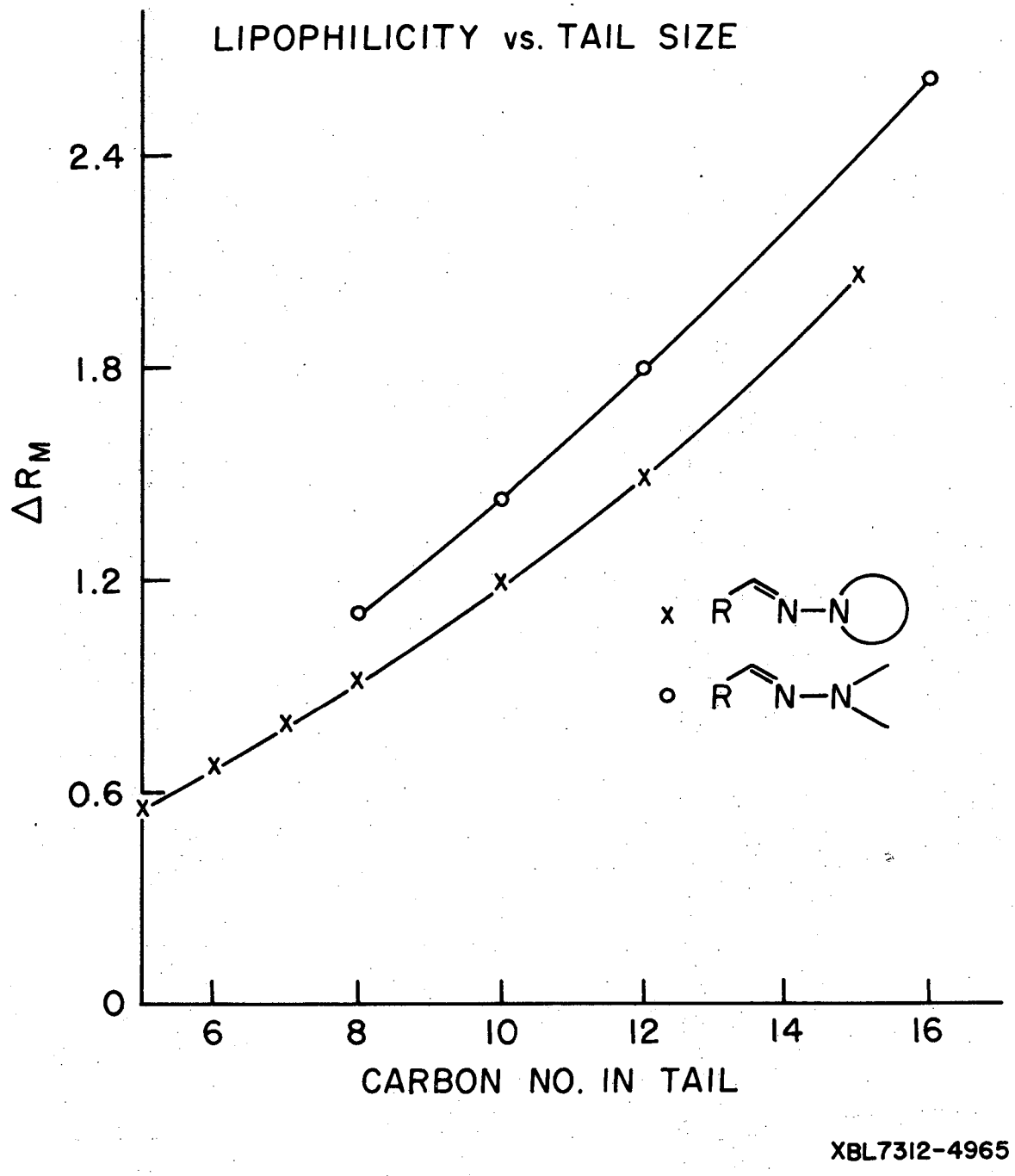

LIPOPHILICITY vs. TAIL SIZE
2.4
1.8
1.2
0.6
0
ΔR_M
6
8
10
12
14
16
CARBON NO. IN TAIL
x R N—N
o R N—N
XBL7312-4965

Figure 14

also indicates that as a class of compounds, the rifazone series is more lipophilic than the rifazacyclo series. A detailed discussion of the inhibition of RDP by these derivatives and the effect of lipophilicity is given in Part III.

Experimental Section

Ir spectra were taken on either a Perkin-Elmer Model 137 or a Beckman Model 5A grating infrared spectrometer. Nmr of all compounds except the rifamycin derivatives were recorded on a Varian Associates Model T-60 and or Model HR-220 instrument, and the rifamycin derivatives were recorded on a Varian Associates HR-220 instrument. Uv spectra were recorded on a Cary Model 14 spectrophotometer. Esr spectra were recorded on a Varian Associates spectrometer Model E-3. Tlc, except where noted, was done on Eastman Chromagram 6060 silica gel sheets. Rifamycin derivatives used as precursors were kindly supplied by Gruppo Lepetit S.p.A., Milan, Italy. Where analyses are indicated only by symbols of elements, analytical results obtained for these elements were within $\pm$ 0.4% of the theoretical values.

2,2,6,6-Tetramethyl-4-chloropiperidine. Method of Rozantzev.[63]

Triacetoneamine hydrazone (4). Method of Lutz, et al.[64]

2,2,6,6-Tetramethylpiperidine-4-N'-acetylhydrazine. Method of Joss, this laboratory, unpublished results.

2,2,6,6-Tetramethylpiperidine-4-hydrazine (1). 2,2,6,6-Tetramethylpiperidine-4-N'-acetylhydrazine (1.00 g, 0.0047 moles) was

dissolved in concentrated HCl (8 ml) at 0°. The solution was warmed to 25° and stirred for 48 hrs. Evaporation of the H_2O, HCl, and acetic acid under vacuum resulted in a light yellow viscous oil. The oil was dissolved in ethanol (30 ml) and ether (120 ml) was added dropwise to the vigorously stirred solution. Soon after the addition of ether began, a white precipitate started forming. The precipitate was collected, washed with ether, and the ethanol-ether crystallization procedure was repeated. The product was shown to be the di HCl salt of (1) by analysis and by titration against NaOH. m.p.264-265 (decomposition). Yield, 1.04 g (91%). Anal., C, H. N.

Rifaldehyde-2,2,6,6-tetramethylpiperidine-4-hydrazine hydrazone (2). Rifaldehyde (0.025 g, 3.45×10^{-5} moles) was dissolved in THF. Anhydrous $MgSO_4$ (0.25 g) was added to the solution along with hydrazine dihydrochloride (1) (0.017 g, 6.89×10^{-5} moles). One N NaOH was added dropwise to raise the pH to approximately 6, and the reaction was stirred overnight. The product was purified by column chromatography on Bio Rad Bio-Sil A silica (5 in column, 0.25 in diameter). The reaction mixture was placed on the column and was eluted with ethanol:ether, 1:1. R_F of the product on silica tlc eluted with ethanol:ether:ethylacetate, 1:1:1 = 0.22. Yield, 78%.

Oxidation of (2). Rifaldehyde derivative (2) (0.015 g, 1.7×10^{-5} moles) was dissolved in 1 ml 95% ethanol and a 1:1 mixture of EDTA tetrasodium salt and Na_2WO_4 (~1 mg) was added. 30% H_2O_2 (10 mg) was added and the reaction was placed in the dark at 25°. The reaction was monitored both by esr, which showed an increasing nitroxyl signal, and by silica gel tlc in ethanol:ether:ethylacetate, 1:1:1, which

indicated the disappearance of (2) and the appearance of a new colored product. The reaction was complete after 4 hrs. Preparative tlc of the newly formed rifamycin (R_F 0.50) provided enough pure product to look at by esr--no signal was detected, indicating that the nitroxyl formed in the reaction was not attached to the rifamycin product.

N-Oxyl-2,2,6,6-tetramethylpiperidin-4-one hydrazone (8). N-Oxyl-2,2,6,6-tetramethylpiperidin-4-one (1.00 g, 0.0058 mol) was dissolved in methanol (3 ml). The solution was cooled to 0° and hydrazine hydrate (2.90 g, 0.058 mol) was added to it dropwise. The flask was fitted with an air lock and then allowed to stand at room temperature for 5 days. All volatile material was removed under vacuum. The resultant pale yellow oil, which gave no esr signal and one spot by the thin layer chromatography (tlc) (silica gel in both dioxane and chloroform), was dissolved in 95% ethanol (15 ml) and stirred vigorously open to the air for 24 hr. Removal of the solvent afforded a bright yellow liquid (0.90 g, 84% of theory) which gave both esr and ir consistent with the proposed structure. The hydrazone was used without further purification.

Di(N-oxyl-2,2,6,6-tetramethylpiperidin-4-one)azine (10). N-Oxyl-2,2,6,6-tetramethylpiperidin-4-one hydrazone (0.50 g, 0.0027 mol) was heated at 50° under vacuum for 4 days, affording a yellow crystalline product (0.45 g), m.p. 173-177°. A degassed THF solution gave a typical binitroxyl spectrum. Ir was consistent with the proposed structure. Anal. C, H, N.

Spin-label (9). Hydrazone (8) (0.17 g, 0.0010 mol) dissolved in 95% ethanol (3.5 ml) was added to a solution of rifaldehyde (0.500 g,

0.00069 mol) in 95% ethanol (10.5 ml) and the resultant mixture was stirred at room temperature for 15 hr.

A column of alumina (activity 1) was prepared with a bed volume of 100 ml in 3:1 ethyl acetate-ethanol (by volume). The above reaction mixture was added to the column and then eluted with 3:1 ethyl acetate-ethanol until the wash gave no signal by esr. The contents of the column were then placed in a flask and extracted with three portions of 100% ethanol (100 ml each). Removal of the solvents under vacuum afforded 0.43 g of the spin-labeled drug 3. Esr, ir, and uv are consistent with the assigned structure. Tlc showed R_F 0.43 (dioxane).

Rifamazine. Method A. Rifaldehyde (0.100 g, 0.000138 mol) was dissolved in 95% ethanol (17 ml). To it was added 0.100 M hydrazine in 95% ethanol (2.80 ml, 0.00028 mol). The pH of the resultant solution was adjusted to 6.0 with 0.10 M HCl. Within 5 min a red precipitate was observed. The solution was then stirred for an additional hour, after which time water (20 ml) was added. The solution was filtered, and the red precipitate was washed with 50% aqueous ethanol and dried under vacuum. Yield, 0.098 g (100%); tlc R_F 0.28 (ether-ethanol-ethyl acetate, 1:1:1); uv (in ethanol) 228 nm (ε 54,200), 358 (33,500), 505 (ε 20,000).

Method B. Rifampicin (0.100 g, 0.000122 mol), 0.20 M aqueous hydrazine (3.0 ml, 0.00060 mol), and ascorbic acid (0.025 g) were dissolved in 30% aqueous acetic acid (25 ml). The solution was stirred in the dark for 5 days at room temperature. The resultant red precipitate was collected by filtration, washed with ethanol, and dried under vacuum. Yield, 0.085 g (97.5%).

Rifurea. Rifaldehyde (0.100 g, 0.000138 mol) and carbohydrazide (0.00585 g, 0.000065 mol) were dissolved in methanol (10 ml). After stirring 4 hr at room temperature, water (10 ml) was added dropwise to affect crystallization. The orange precipitate was collected and washed twice (50% ethanol) by centrifugation. The product was dried under vacuum. Yield, 0.058 g (67%); tlc R_F 0.33 (ether-ethanol-ethyl acetate, 1:1:1); uv (in ethanol) 232 nm (ε 51,500), 337 (38,800), 475 (20,000).

Dirifampin. Rifaldehyde (0.100 g, 0.000138 mol) and N,N'-diaminopiperazine dihydrate (0.00988 g, 0.000065 mol) were dissolved in ethanol (12 ml). The reaction vessel was fitted with a condenser and then heated to reflux for 2.5 hr. An orange precipitate was observed soon after reaching reflux. The solution was then cooled to 0° to complete precipitation. The precipitate was collected and washed (100% ethanol) by centrifugation and then dried under vacuum. Yield, 0.085 g (84%); tlc R_F 0.75 (ethanol); uv (in ethanol) 234 nm (ε 32,700), 348 (29,900), 476 (17,700).

2-Azacyclohexadecanone. Cyclopentadecanone (4.50 g, 0.0201 mol) and hydrazoic acid (16.9 ml of 1.25 M HN_3 in benzene) in benzene (30 ml) was added dropwise to an ice-cold mixture of sulfuric acid (15.5 ml) and benzene (47 ml) with stirring. The temperature was maintained below 10°. After the addition, the ice bath was removed and the reaction mixture was stirred for another 20 min. Ice water (100 ml) was then added. The benzene layer was separated and the aqueous layer was washed once with benzene (15 ml). The two benzene solutions were combined and washed once with 1.0 N KOH (50 ml) and dried over

Na_2SO_4. The benzene was removed under vacuum, affording a white crystalline solid which was recrystallized from 50% aqueous acetone. Yield, 4.30 g (89%); m.p. 131-134°.

Azacyclohexadecane. 2-Azacyclohexadecanone (4.30 g, 0.0179 mol) dissolved in benzene (12 ml) was added dropwise to a stirred mixture of $LiAlH_4$ (0.70 g, 0.018 mol) in ether (12 ml) at a rate to maintain a gentle reflux. Reflux was then maintained by heating for 15 hr. The sequence water (0.70 ml), 15% NaOH (0.70 ml), and water (2.1 ml) was then added and the reaction mixture was filtered and washed with additional benzene. The filtrate was dried over Na_2SO_4 and evaporated under vacuum, affording the product as a waxy solid. m.p. 45-47.5°; yield, 3.90 g (96%).

N-Nitrosoazacyclohexadecane. Concentrated HCl (1.35 ml, 0.0167 mol) was slowly added to a mixture of azacyclohexadecane (3.00 g, 0.0133 mol) and water (3.0 ml) at 0°. The reaction flask was then fitted with a thermometer and heated to 65°. A solution of $NaNO_2$ (1.02 g, 0.0167 mol) in water (3.0 ml) was then added dropwise at a rate which maintained the temperature between 65 and 70°. This temperature was maintained by heating for an additional 5 min after the addition. The reaction mixture was then cooled to 25° and titrated with 15% NaOH to pH 7.0. The organic layer was removed by extraction with three portions of benzene (15 ml each). The benzene solutions were combined, treated with Na_2SO_4 and decolorizing carbon, filtered, and evaporated under vacuum. The N-nitroso compound resulted as a pale yellow, low-melting solid: m.p. 32-34°; yield 3.02 g (90%. Anal. C, H, N.

N-Aminoazacyclohexadecane. N-Nitrosoazacyclohexadecane (2.95 g, 0.0116 mol) in ether (12 ml) was added dropwise to a stirred mixture of $LiAlH_4$ (0.50 g, 0.013 mol) in ether (6 ml) at a rate to maintain a gentle reflux. Reflux was then maintained by heating for 15 hr, after which time the sequence water (0.50 ml), 15% NaOH (0.50 ml), and water (1.50 ml) was slowly added dropwise. Benzene (10 ml) was then added and the reaction mixture was filtered and washed with additional benzene. The filtrate was dried over Na_2SO_4 and evaporated under vacuum, affording the product as a waxy solid: m.p. 39-41°; yield, 2.58 g (93%); hydrochloride salt, recrystallized from cyclohexane, m.p. 145-147°. Anal. C, H, N (as the hydrochloride).

Rifazacyclo-16. Rifaldehyde (0.190 g, 0.000262 mol) and N-aminoazacyclohexadecane (0.0630 g, 0.000262 mol) were dissolved in THF (12 ml) from which oxygen had been removed by bubbling in nitrogen. The solution was stirred at 25° for 48 hr, after which time the solvent was removed under vacuum. The resulting orange solid was recrystallized from petroleum ether (40 ml). Yield, 0.205 g (81%); tlc R_F 0.087 (tetrahydrofuran); uv (in ethanol) 227 nm (ε 24,100), 279 (22,300), 350 (30,500), 479 (12,900).

Acetone derivative of N-aminoazacyclohexadecane. N-Aminocyclohexadecane (0.10 g, 0.00042 mol) was dissolved in acetone (10 ml) and stirred at 25° for 48 hr. Removal, under vacuum, of the excess acetone yielded a light yellow oil which gave the expected nmr and ir for the acetone hydrazone derivative. Yield, 0.12 g (100%). Anal. C, H, N.

N-Nitroso-3-azabicyclo[3.2.2]nonane. 3-Azabicyclo[3.2.2]nonane (10.0 g, 0.080 mol) was slowly added to ice-cold concentrated HCl (8.12 ml, 0.100 mol). The flask was then fitted with a thermometer and heated to 65°. A solution of $NaNO_2$ (6.0 g, 0.10 mol) in water (18 ml) was added dropwise at a rate which maintained the temperature between 65 and 70°. This temperature was maintained by heating for 10 min after the addition. The reaction mixture was cooled to 25°. The yellow precipitate that resulted was collected by filtration, dissolved in ether (100 ml), and treated with Na_2SO_4 and decolorizing carbon. The filtered solution was evaporated under vacuum affording the N-nitroso compound as a pale yellow solid, which readily sublimes above 150°. Yield 6.0 g (49%). Anal. C, H, N.

N-Amino-3-azabicyclo[3.2.2]nonane. N-Nitroso-3-azabicyclo[3.2.2] nonane (3.00 g, 0.0195 mol) in ether (10 ml) was added dropwise to a stirred mixture of $LiAlH_4$ (0.75 g, 0.020 mol) in ether (10 ml) and THF (10 ml) at 25°. Ten minutes after the addition, the reaction was refluxed for 15 hr, after which time the sequen-e water (0.75 ml), 15% NaOH (0.75 ml), and water (2.25 ml) was added dropwise. The reaction mixture was then filtered, and the precipitated hydroxides were washed twice with ether (10 ml each). The filtrate was dried over Na_2SO_4 and evaporated under vacuum. The bicyclic hydrazine (2.5 g, 90%) was obtained as a low-melting, very hydroscopic white solid. Anal. C, H, N.

Rifazabicyclo-9. Rifaldehyde (0.100 g, 0.000138 mol) and N-amino-3-azabicyclo[3.2.2]nonane (0.0193 g, 0.000138 mol) were dissolved in THF (12 ml). The solution was stirred for 24 hr at 25°.

The solvent was then removed under vacuum. The product, which resulted as an orange solid, was recrystallized from ethyl acetate. Yield, 0.071 g (61%); tlc R_F 0.54 (tetrahydrofuran).

2-Azacycloundecanone. The cyclic ketone (5.00 g, 0.032 moles) is dissolved in chloroform (100 ml) in a flask fitted with a magnetic stirring bar and a thermometer. The solution is cooled in an ice bath to 5-10° and sulfuric acid (25 ml) is added. NaN_3 (2.23 g, 0.034 moles) is added approximately 10% at a time over a period of 1 hour. The ice bath is removed and the reaction is stirred for another hour. The contents of the reaction flask are then poured into ice and water (125 ml) and the resulting chloroform layer is separated. The water layer is washed with additional chloroform and the chloroform solutions are combined, washed with 1 N KOH (25 ml), dried over Na_2SO_4 and evaporated under vacuum to yield the product. The product is recrystallized from acetone. Yield, 4.7 g (91%); m.p. 162-165°.

Nitrosations

Method A - for water soluble amine hydrochlorides. The amine (n moles) and water (~250 n ml) are added to a flask fitted with a thermometer, magnetic stirring bar and addition funnel. The flask is placed in an ice bath and concentrated HCl (1.25 n moles) is added dropwise maintaining the temperature below 10°. After the addition the ice bath is removed and the solution is heated to 65°. A solution of $NaNO_2$ (1.25 n moles) in water (250 n ml) is added dropwise at a rate maintaining the temperature between 65 and 70°. After the

addition the reaction is stirred at temperature for another 10 min. The flask is cooled to 25° and the contents are transferred to a separatory funnel where the product is extracted with benzene. The benzene solution is dried over Na_2SO_4 and evaporated under vacuum.

Dibutylnitrosamine, n = 0.100, yield = 99%

Di-n-pentylnitrosamine, n = 0.100, yield = 98%

Di-n-hexylnitrosamine, n = 0.125, yield = 85%

N-Nitrosoazacyclooctane, n = 0.0945, yield = 57%, bp_{10} = 118°, structure by nmr, ir, m.p. = 42-47°

N-Nitrosoazacycloundecanone, n = 0.0535, yield = 86%, structure by nmr, ir

N-Nitrosoazacyclotridecanone, n = 0.0490, yield = 95%, structure by nmr, ir

Method B* - for water insoluble amine hydrochlorides. The amine (n moles) and acetic acid (1000 n ml) are added to a flask fitted with a thermometer, magnetic stirring bar, and addition funnel. The flask is placed in an ice bath and concentrated HCl (1.25 n moles) is added dropwise, keeping the temperature below 10°. The solution is then heated to 25° and a solution of KNO_2 (3 n moles) in water (∿150 n ml) is added dropwise over a period of 20 min. The reaction is stirred for another 20 min after the addition. Water (∿1500 n ml) is then added, the product is extracted with ether which, in turn, is washed with 5% Na_2CO_3, dried over Na_2SO_4 and evaporated under vacuum.

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*Solution must be kept warm (∿40°).

D-n-Octylnitrosamine, n = 0.0249, yield = 92%

N-Nitroso-2,6-dimethyl-4-benzylpiperidine, n = 0.0345, yield = 68%, structure by nmr, ir

Lithium Aluminum Hydride (LAH) Reductions

LAH was used for the reduction of amides to amines and of N-nitroso compounds to hydrazines. It was generally noted that the reaction time required for high conversions was quite sensitive to the freshness of the LAH. The use of fresh LAH resulted, in every case, in shorter reaction times, i.e., less than 4 hr reflux in THF or ether.

The compound to be reduced (n moles), either neat or dissolved in THF, ether or benzene, is added dropwise to a stirred refluxing mixture of LAH (n-2n moles) in THF or ether over a period of approximately 30 min. The reaction is maintained at reflux until the conversion is complete. (This is determined by the workup of 3 ml aliquots. Amide reduction is monitored by the loss of the carbonyl by ir. Nitrosamine reduction is monitored by the loss of the characteristic downfield α-protons by nmr.) The reaction is then cooled in a cold water bath. For x g of LAH used, a sequence of water (x ml), 15% NaOH (x ml) and water (3x ml) is added dropwise very slowly with vigorous stirring. The resulting crystalline mixed hydroxides are removed by vacuum filtration. The hydroxides are washed with additional solvent. The combined filtrates are evaporated under vacuum to yield the crude product.

Amide Reduction

Azacyclooctane-amide (n = 0.118) in THF (25 ml), LAH (0.20 moles) in THF (125 ml), yield = 99%

Azacyclononane-amide (n = 0.106) in THF (50 ml), LAH (0.21 moles) in THF (150 ml), yield = 88%

Azacycloundecane-amide (n = 0.0622) in THF (50 ml)*, LAH (0.13 moles) in THF (100 ml), yield = 88%

Azacyclotridecane-amide (n = 0.0507) in THF (100 ml)*, LAH (y .108 moles) in THF (75 ml), yield = 99%

Nitrosamine Reduction

N-Aminoazacyclooctane-nitrosamine (n = 0.0423) in THF (15 ml), LAH (0.0810 moles)in THF (60 ml), yield = 80%, bp_{21} = 67-73°

N-Aminoazacyclononane-nitrosamine (n = 0.0513) in THF (50 ml), LAH (0.103 moles) in THF (75 ml), yield = 43%, bp_{19} = 87°

N-Aminoazacycloundecane-nitrosamine (n = 0.0407) in THF (25 ml), LAH (0.092 moles) in THF (75 ml), yield = 61%, bp_{10} = 113°

N-Aminoazacyclotridecane-nitrosamine (n = 0.0400) in THF (50 ml), LAH (0.090 moles) in THF (50 ml), yield = 64%, bp_{60} = 95-99°

N,N-Dibutylhydrazine-nitrosamine (n = 0.0633) in THF (25 ml), LAH (0.132 moles) in THF (150 ml), yield = 86%, bp_{80} = 109-113°

N,N-di-n-Pentylhydrazine-nitrosamine (n = 0.0633) in THF (25 ml), LAH (0.132 moles) in THF (150 ml), yield 85%, bp_{51} = 124-130°

N,N-di-n-Hexylhydrazine-nitrosamine (n = 0.084) neat, LAH (0.168 moles) in THF (125 ml), yield (dist) 96% (74%), bp_{15} 124-128°

N,N-di-n-Octylhydrazine-nitrosamine (n = 0.0204) in THF (25 ml), LAH (0.0254 moles) in THF (30 ml), yield = 97%.

N-Amino-2,6-dimethyl-4-benzylpiperidine-nitrosamine (n = 0.0216) in THF (25 ml), LAH (0.0526 moles) in THF (50 ml), yield = 85%, $bp_{0.4}$ = 118-123°

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*Solutions must be kept warm (∿40°).

Rifaldehyde Condensations

Rifaldehyde (n moles) and the appropriate hydrazine (1.05 n moles) are dissolved in THF (~500 ml per 10 g rifaldehyde) and stirred at room temperature until the reaction is complete by silica gel tlc in THF (1 - 24 hr). The solvent is then evaporated under vacuum, and the product is pulverized, washed with n-hexane and dried again. The products can be further purified either by recrystallization from hexane or hexane-toluene on a silica gel column eluted with ethyl acetate. Yields are all above 90%.

RC-8	(n = 0.0140)
RC-9	(n = 0.0167)
RC-11	(n = 0.0132)
RC-13	(n = 0.0126)
$R\text{-}4_2$	(n = 0.0165)
$R\text{-}5_2$	(n = 0.0165)
$R\text{-}6_2$	(n = 0.0140)
$R\text{-}8_2$	(n = 0.0108)
DMB-analog	(n = 0.00917)

The reaction has been found to be near quantitative when both the rifaldehyde and the hydrazine are of high purity. The hydrazines were usually distilled prior to use. The major tool used for the confirmation of the structure of the rifaldehyde derivatives was 220 MHz nmr. The basic assignment of the nmr absorptions of the rifamycins was given by Prelog.[5a] Aside from minor changes in the spectrum observed when rifaldehyde is condensed with a hydrazine, 2 major changes occur. First, the disappearance of the aldehyde proton absorption at

10.6 δ and the appearance of a new 1 proton signal at approximately 8.0 δ due to the formation of an aldehyde hydrazone proton. The second major change in the spectrum is the appearance of new absorptions due to the protons of the hydrazine that was condensed with rifaldehyde. These absorptions, which are nearly identical with the spectrum of the hydrazine, are usually discernable among the rifamycin peaks.

2,6-Dimethyl-4-benzylpyridine. 2,6-Dimethyl-3,5-dicarboxy-4-benzylpyridine (23.0 g, 0.081 moles) was placed in a flask connected to a Vigreux vacuum distillation apparatus. The system was evacuated to 85 mm by means of an air bleed valve. The flask was heated until all the distillate, which came over at 200-204°, was collected as a ruby red liquid. The red color was found to be due to a highly colored impurity which was removed by dissolving the product in n-hexane (250 ml), cooling to 0° for 1 hr, filtering out the red precipitate and evaporating the filtrate. Yield, 14.1 g (89%).

2,6-Dimethyl-4-benzylpiperidine. 2,6-Dimethyl-4-benzylpyridine (3.24 g, 0.0164 moles) was dissolved in 100% EtOH (30 ml) in a flask fitted with a reflux condenser. Na metal (4.5 g, 0.196 moles) was added gradually in small pieces to maintain a very gentle reflux. Five min after the Na addition was complete, EtOH (15 ml) was added and the reaction was refluxed until all the Na reacted. (Note: When the Na was first added, the solution turned yellow; during the final reflux, the yellow color abruptly disappeared.) Water (20 ml) was then added dropwise. The EtOH was evaporated under vacuum, resulting in 2 phases which were transferred into a separatory funnel containing

water (15 ml). The product was obtained by extraction with ether, drying over Na_2SO_4 and evaporation under vacuum. Yield, 3.12 g (94%).

DMB-MI. DMB (0.200 g) was dissolved in methyl iodide (20 ml) and the solution was stirred at 25° overnight. Evaporation of the methyl iodide afforded the product in quantitative yield.

Rifampicin-methyliodide (RMI). Rifampicin (1.00 g) was dissolved in methyl iodide (50 ml) and the solution was stirred at 25° for 4 hrs, during which time the product precipitated. RMI was collected, washed with chloroform and dried. Yield, 97%.

Reversed phase tlc. Tlc plates were prepared with silica gel (E. Merck AG-Darmstadt) spread at a thickness of 0.25 mm (50 g to 100 ml water). The plates were activated and developed in a 5% solution of Dow-Corning 200 fluid (10 cc) in ether overnight. The ether was allowed to evaporate and then the rifamycin derivatives, dissolved in acetone (10 mg/ml), were spotted ($\sim$6 µg). The plates were developed in an acetone/water solution (2/3 v/v) saturated with the Dow-Corning fluid. No means of visualizing was required, as the spots are distinctly colored. In all cases the reported ΔR_M is the average of at least 5 determinations.

PART III:

RIFAMYCIN INHIBITION OF NUCLEOTIDE POLYMERASES

A. Introduction

Part I of this thesis described the reverse transcription assay and Part II dealt with the synthetic development of new rifamycin derivatives in an effort to systematically study RDP inhibition as a function of tail structure. This Part of the thesis deals with the results of this study. We have found that tail lipophilicity is an important but not exclusive factor in obtaining good RDP inhibition.

A second point to which this Part is devoted is the question of inhibitory specificity. We have held this question to be particularly important in light of the suggestion by Silvestri *et al.* that all lipophilic rifamycins are nonspecific binders of proteins. To investigate this possibility we have tested many of the derivatives introduced in Part II as inhibitors of other polymerase enzymes, namely *E. coli* DNA directed DNA polymerase I (DDP) and *E. coli* DNA directed RNA polymerase (DRP), and other enzymes. The result of this study has revealed that while many of these derivatives tend to be nonspecific inhibitors of polymerase enzymes, a few, particularly rifazone-8_2, tend to be specific inhibitors of RDP.

B. RDP Inhibition

Under the conditions outlined in Part I, each derivative was tested as an RDP inhibitor. Each derivative was initially tested with RDP as it became available in order to estimate their approximate effectiveness. The data obtained from these individual assays enabled us to select the proper range of concentrations for each derivative so that accurate measurements of K_I could be made. All the K_I's reported were obtained from large experiments in which each derivative was included. At least 6 concentrations were tested for each derivative, and each concentration was run in duplicate. The K_I for each derivative was obtained from a plot of percent control RDP activity versus derivative concentration. From the few derivatives that were tested on many different occasions, particularly DMB, we estimated that the error of the measured K_I is approximately $\pm$ 10%. It should be noted that the K_I reported may represent submaximal inhibition due to the interaction of the derivatives with the extraneous protein present as impurities. As the enzyme is more highly purified, the measured K_I may thus become smaller. The measured K_I for each derivative is given in Table II along with the lipophilicity parameter, ΔR_M.

A plot of inhibition (log K_I) as a function of lipophilicity (ΔR_M) is given in Figure 15 for both the rifazacyclo and rifazone series. In addition, the points for other rifamycin derivatives from Table II are plotted. In general it can be concluded that RDP inhibition is favored by tail lipophilicity. There appears to be an optimal lipophilicity for both the rifazacyclo and rifazone series

Table II

RDP Inhibition and Tail Lipophilicity

Derivative	ΔR_M	K_I
Rifazacyclo-6	0.56	58
Rifazacyclo-7	0.68	34
Rifazacyclo-8	0.80	17
Rifazacyclo-9	0.92	9.0
Rifazacyclo-11	1.20	4.9
Rifazacyclo-13	1.49	3.0
Rifazacyclo-16	2.06	2.1
Rifazone-4_2	1.11	8.8
Rifazone-5_2	1.43	4.1
Rifazone-6_2	1.80	2.5
Rifazone-8_2	2.61	3.5
DMB-MI	~0	>100
DMB	0.85	19
DMB-analog	1.15	5.9
Rifamazine	0	11
Dirifampin	0.15	16

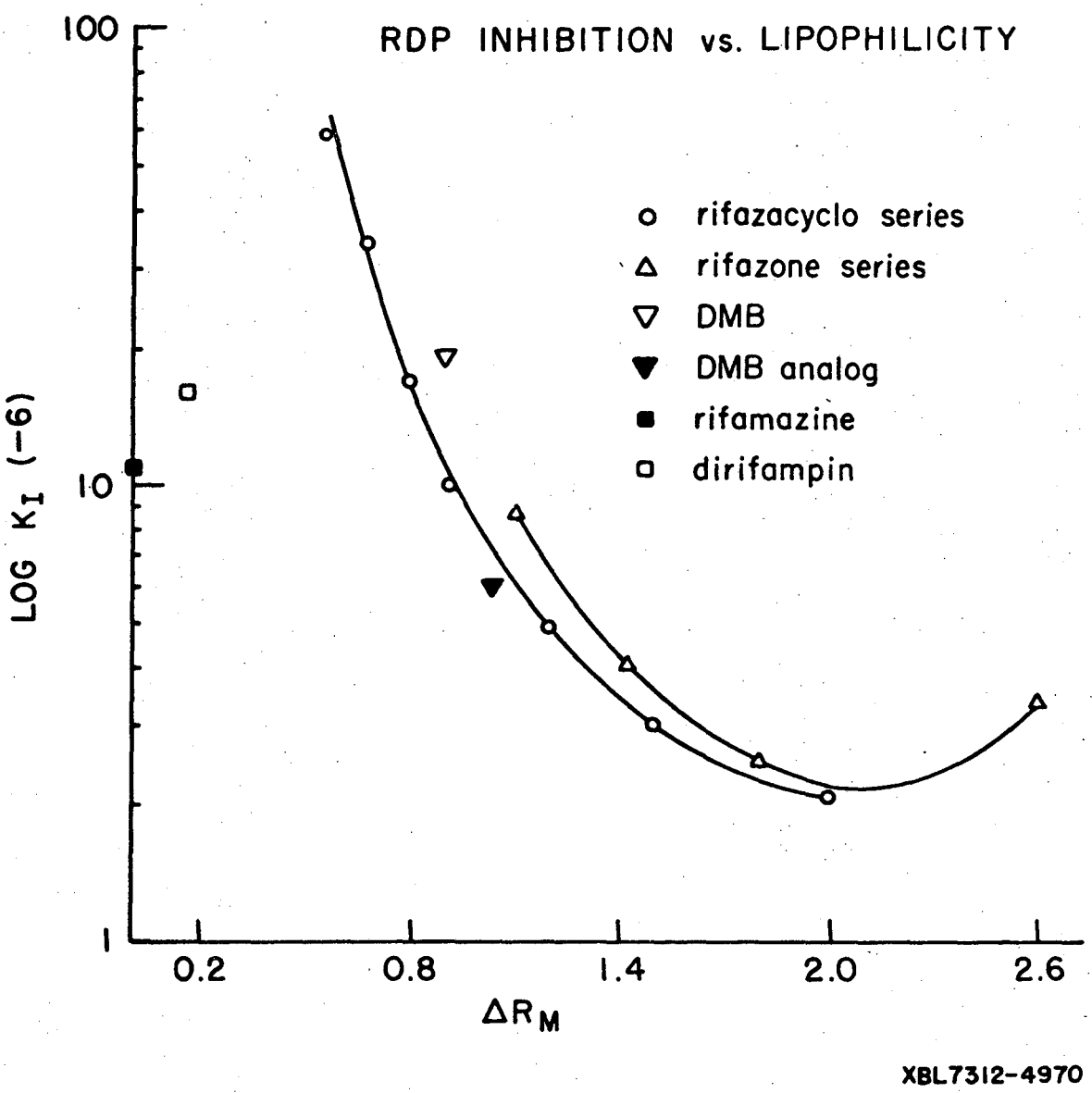
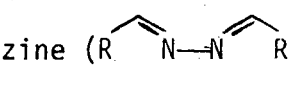

RDP INHIBITION vs. LIPOPHILICITY
100
10
1
LOG K_I (-6)
0.2
0.8
1.4
2.0
2.6
ΔR_M
rifazacyclo series
rifazone series
DMB
DMB analog
rifamazine
dirifampin
XBL7312-4970

Figure 15

at approximately $\Delta R_M = 2.1$. Accordingly, R-8_2, the only derivative with $\Delta R_M > 2.1$, is less effective an inhibitor than the less lipophilic R-6_2. It is of interest to note that while there is no large difference between the cyclic and acyclic series, the more lipophilic rifazone series is less effective than the rifazacyclo series at inhibiting RDP. This can be taken as evidence for the existence of factors other than lipophilicity for optimal RDP inhibition.

The derivatives of the dimethylbenzyl series, DMB and DMB-analog, fall in the same area of Figure 15 as the cyclic and acyclic series. This suggests to us that their inhibition is due primarily to their lipophilicity and not to their particular configuration. Also, the fact that the uncharged DMB-analog is over three times more effective than DMB, while the charged DMB-MI is ineffective, suggests that the inhibition exhibited by DMB itself, which should be largely charged at the assay pH (7.8), is due to the unprotonated form only. The actual inhibition curves for the derivatives of the DMB series are given in Figure 16. Aside from clearly indicating the effect of lipophilicity in this series, the data are indicative of the data obtained for the inhibition of RDP by rifamycin derivatives.

There is one anomalous group of derivatives to the generalized inhibition as a function of lipophilicity. These are the rifaldehyde dimers which have been described earlier. As can be seen from the positions of rifamazine () and dirifampin () on Figure 3, they are both reasonably good inhibitors in spite of the fact that they are relatively hydrophilic. Either these dimers

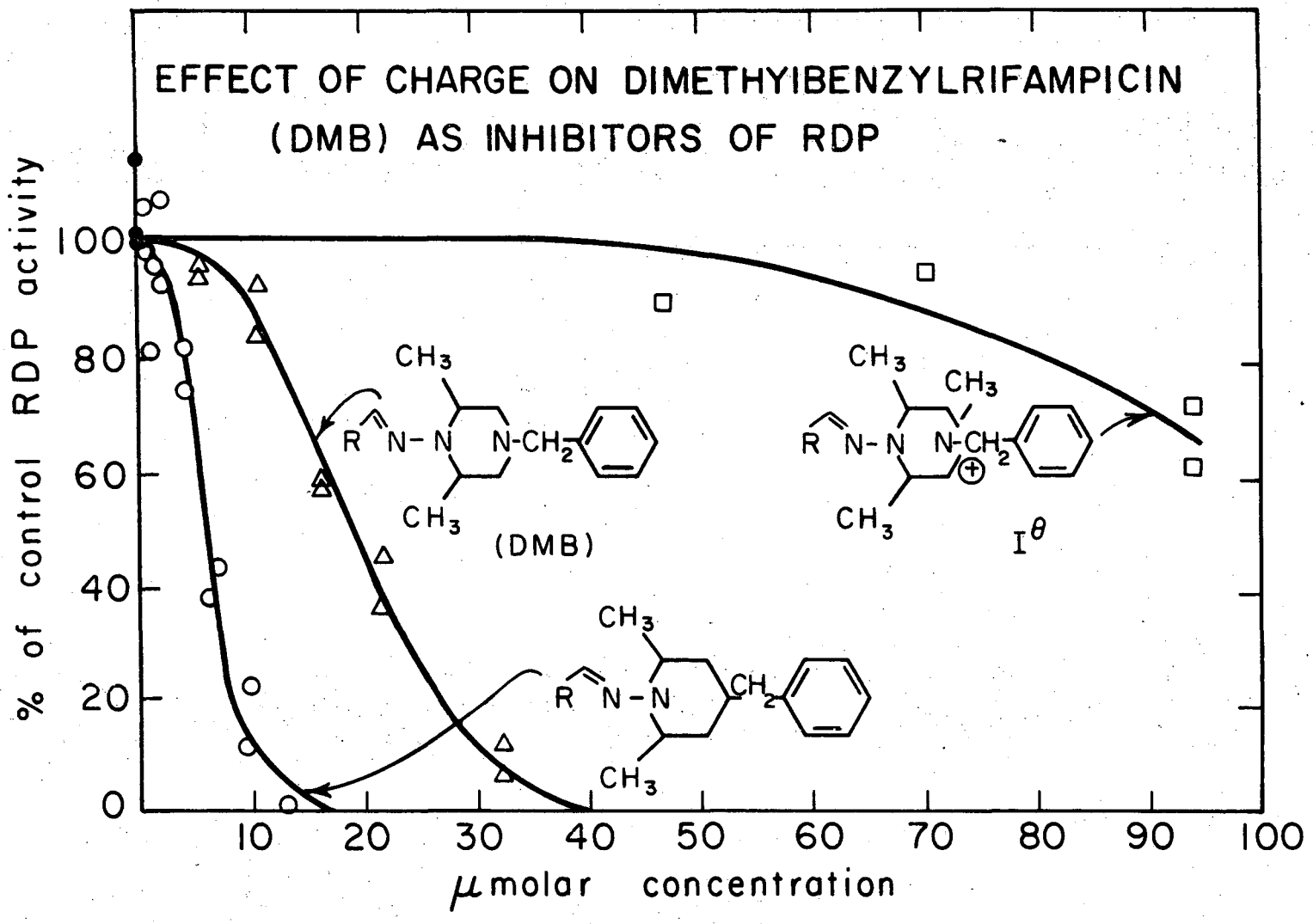


Figure 16

XBL738-3992

inhibit RDP through a different mechanism of interaction or they possess a desirable tail property not yet recognized.

We have also investigated the possibility of detergents with lipophilicities similar to the rifamycins being inhibitory toward RDP, and have found no correlation. For example, the R_M for the detergent Triton X-100 is nearly identical with R-6_2 and yet no inhibition of RDP is observed, even at concentrations as high as 0.1%.

C. Inhibition Specificity

As previously discussed, it has been our intention to investigate the suggested lack of inhibitory specificity among lipophilic rifamycin derivatives. Work in other laboratories on human lymphocyte DDP[41b,78] and calf thymus DRP[79] indicated that for both of these mammalian polymerases the inhibition by rifamycin derivatives (rifampicin, rifaldehyde octyloxime, and DMB) is nearly identical to the inhibition we obtained for *E. coli* DDP I. Since we are interested in the selectivity of the inhibitors for RDP versus mammalian polymerases, and *E. coli* DDP appears to behave toward rifamycins as do mammalian polymerases, we decided to use the readily available *E. coli* DDP as a model polymerase for comparison with RDP. A reasonable measure of the inhibitory specificity of the rifamycin derivatives for RDP can be obtained by taking the ratio of the K_I for the DDP to the RDP.

E. coli DRP was also selected for study with the rifamycin derivatives since this bacterial RNA polymerase has been shown to bind rifamycins in a well defined stoichiometric relationship.[26]

The binding constants of the E. coli DRP and the derivatives thus represent a limiting value. Table III presents the measured inhibition concentrations for several of the rifamycin derivatives with the viral RDP, E. coli DRP, and E. coli DDP.[80] In addition, the table lists the specificity parameter, $K_I(DDP)/K_I(RDP)$. It is important to note that the specificity parameters probably represent minimum values due to the fact that the E. coli DDP was more highly purified than the RDP. Assays with the DDP had a protein concentration only about 1% of that in the RDP assays. From the table it can be seen that as suggested, there is little specificity for DMB, the dimers, and the rifazacyclo series. However, the rifazone series shows consistent specificity for RDP and R-8_2 shows the highest specificity.

In order to establish that the observed specificity was dependent on the enzymes being tested and not on the template and divalent cation, the same rifamycin derivatives were tested for inhibition when the DDP was assayed on poly(rA):oligo(dT) with Mn^{+2} instead of the normal poly-d(AT) duplex with Mg^{+2} and the RDP was assayed on the poly (dA-dT) duplex with Mg^{+2} instead of the normal poly(rA):oligo (dT) with Mn^{+2}. When assayed under these abnormal conditions, the RDP and DDP had only 1-2% and 5%, respectively, of the activity assayed under normal conditions. The K_I values determined under the abnormal conditions are given in Table IV. For both enzymes, the rifamycin derivatives were more inhibitory in the abnormal assays than in the normal assays and the specificity is maintained. Thus the K_I value does not correlate to the template and the divalent

Table III

Inhibition constants ($K_I \times 10^8$) for rifamycin derivatives with various nucleotide polymerases

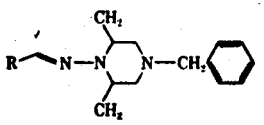
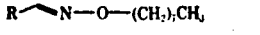
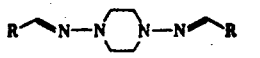
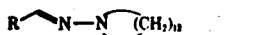
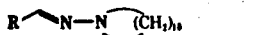
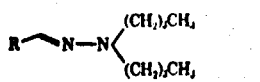
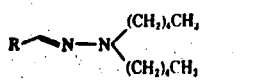
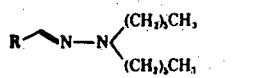
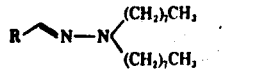
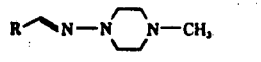
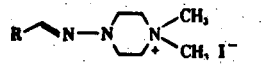
		E. coli DRP	Viral RDP	*E. coli* DDP	K_I DDP / K_I RDP
DMB		0.49	19	43	2.3
Octyloxime		0.06	2.9	21	7.2
Rifamazine		0.12	11	82	7.4
Dirifampin		0.13	16	73	4.4
RC-7		0.32	34	103	3.0
RC-11		0.11	4.9	14	2.9
RC-13		0.07	3.0	9.4	3.1
RC-16		0.25	2.1	2.6	1.2
R-4[2]		0.50	8.8	72	8.2
R-5[2]		0.12	4.1	59	15
R-6[2]		0.24	2.5	31	12
R-8[2]		1.7	3.5	114	33
Rifampicin		0.06	>120 (23)	>120 (2)	
RMI		0.03	>100 (14)		

Table IV

Inhibition constants ($K_I \times 10^6$) for rifamycin derivatives with two nucleotide polymerases on abnormal templates

	Viral RDP*	*E. coli* DDP†	$\frac{K_I \text{ DDP}}{K_I \text{ RDP}}$
DMB	7.2	39	5.4
Octyloxime	2.2	14	6.4
Rifamazine	5.2	33	6.3
Dirifampin	3.2	25	7.8
RC-7	21	46	2.2
RC-11	3.1	13	4.1
RC-13	0.99	5.8	5.9
RC-16	0.74	1.7	2.3
$R\text{-}4_2$	8.2	22	2.7
$R\text{-}5_2$	3.2	14	4.4
$R\text{-}6_2$	1.1	11	10
$R\text{-}8_2$	0.6	23	38
Rifampicin	45		

See Table 1 for chemical structures of compounds listed.

* The template was poly(dA-dT) duplex with Mg^{+2} instead of the normal poly(rA)·oligo(dT) with Mn^{+2}.

† The template was poly(rA)·oligo(dT) with Mn^{+2} instead of the normal poly(dA-dT) duplex with Mg^{+2}.

cation. Instead, the K_I is dependent on the enzyme and its ability to utilize a given template and divalent cation. The lower the enzyme's ability to utilize a given template and cation, the lower the K_I. This correlation may indicate that the binding of rifamycin derivatives is competitive with the binding of template and/or divalent cation.

While many of the lipophilic rifamycin derivatives appear to be nonspecific inhibitors of polymerase enzymes, we have found that these rifamycin derivatives do not inhibit all enzymes which bind polynucleotides. We tested 3 derivatives on a mammalian deoxyribonuclease (DNase). DMB and RC-16 do not inhibit DNase at all at 50×10^{-6} M, while at this same concentration $R\text{-}8_2$ only inhibits

about 20%. We interpret these results as further evidence against total nonspecificity.

R-8_2, which has the highest ratio of K_I's, is also an exception to the generalization that changes in rifamycin structure not involving the ansa ring have little effect on the inhibition of bacterial DRP (see Table III).[26] R-8_2 is approximately 30 times less inhibitory to E. coli DRP than rifampicin and differs from all the other derivatives we tested by almost an order of magnitude. While nearly all the other derivatives listed in Table III are over 50 times more effective on E. coli DRP than RDP, R-8_2 prefers the bacterial enzyme by only a factor of 2. We believe that higher molecular weight rifazone derivatives yet to be synthesized (such as R-9_2 or R-8,9) may even prefer the viral polymerase to the bacterial.

A plot of log K_I versus ΔR_M for the rifazone series with E. coli DRP and viral RDP is given in Figure 17. It visually shows how specificity varies with lipophilicity for the rifazone series. In addition, by extrapolating the curves beyond the ΔR_M of R-8_2, we can guess at the specificity of the as of yet not synthesized R-9_2. From Figure 14 we can make a good estimation of the ΔR_M expected for R-9_2 ($\Delta R_M \sim 3.0$) due to the linear nature of the plot. Application of this ΔR_M to the extrapolation indicated in Figure 17 gives us a rough estimation of the specificity ratio expected for R-9_2, which is over 100.

Rifampicin methyl iodide (RMI) which is included in Table III, is also an interesting new derivative in terms of its potential as

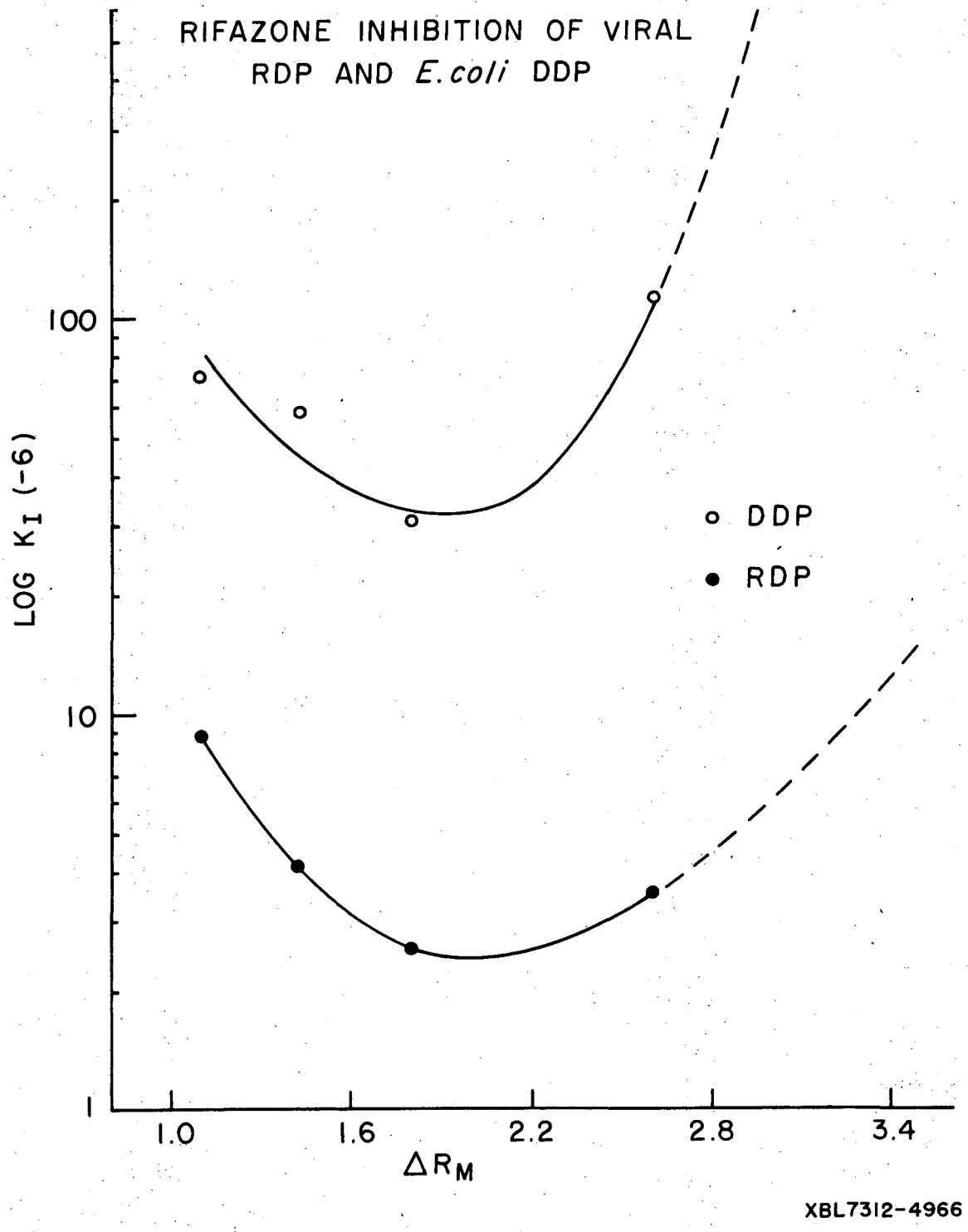

RIFAZONE INHIBITION OF VIRAL
RDP AND E.coli DDP
LOG K_I (-6)
100
10
1
DDP
RDP
1.0
1.6
2.2
2.8
3.4
ΔR_M
XBL7312-4966

Figure 17

an antibacterial agent since it is twice as inhibitory as rifampicin for the bacterial DRP and is perhaps also more specific.

D. Experimental

Enzymes. The E. coli DDP (Kornberg polymerase) was obtained in the highest available purity from Boehringer Mannheim. The E. coli DRP was obtained from Miles Laboratories. Ribonuclease-free deoxyribonuclease I from bovine pancrease was obtained from Worthington Biochemical Corp. The viral RDP was partially purified from murine leukemia virus (MLV)-transformed UCl-B cells, as previously described in Part I.

Assays. All polymerase assays were done in 100-µl total volume. There was no preincubation of enzymes with the rifamycin derivatives to enhance the inhibition since all assays were started by addition of enzyme. Activity is measured as incorporation of radioactive nucleotide triphosphates into acid-insoluble material. Rifamycin derivatives were dissolved in Me_2SO at 10 mg/ml and diluted for addition to the assays.

E. coli DDP assays were 60 mM potassium phosphate (pH 7.4), 0.01% Triton DN-65, 4% glycerol, 1% Me_2SO, 0.5 mM dithiothreitol (DTT), 0.03 mM [^{3}H]dTTP (0.5 Ci/mmol), 0.03 mM dATP, 10 µg/ml of poly(dA-dT) duplex, 6 mM $MgCl_2$, and 1.2 ng of enzyme protein.

E. coli DRP assays were 40 mM Tris·HCl (pH 7.8), 0.01% Triton DN-65, 0.1% Me_2SO, 0.1 mM DTT, 0.1 mM EDTA, 150 mM KCl, 0.15 mM each ATP, GTP, and CTP, 0.15 mM [^{3}H]UTP (0.1 Ci/mmol), 10 mM $MgCl_2$, 0.15 mg/ml of calf-thymus DNA, and 12 µg/ml of enzyme protein.

RDP assays were performed as described in Part I.

PART IV:

ATTEMPTED PURIFICATION OF RDP BY AFFINITY CHROMATOGRAPHY

A. Introduction

A relatively recent and effective technique for enzyme purification is that of affinity chromatography,[81] which has been developed largely by Cuatrecasas and Anfinsen. With this technique, one binds a substrate or inhibitor of the enzyme to be purified to an inert porous support. When a mixture of proteins is passed through a column of this material only the enzyme or enzymes that complex the fixed substrate are detained. The enzyme can later be released by an appropriate change in the nature of the eluent, such as ionic strength, pH, temperature, the addition of a denaturant, or the addition of substrate.[81] Various support materials have been used. Agarose has been used most frequently, but cellulose, polyacrylamide and others have also been used.

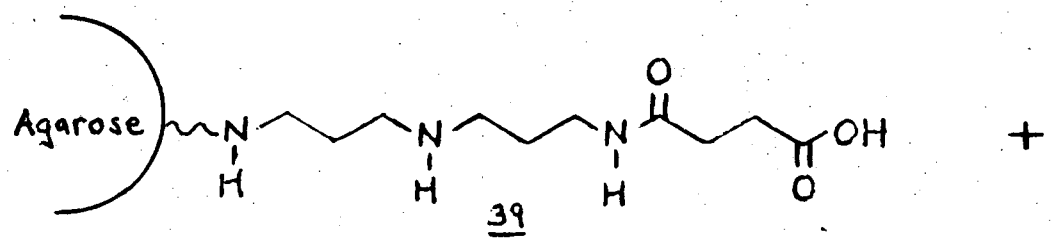
Affinity chromatography has been twice applied to the purification of RDP. In one approach by Todaro *et al.*,[82] an immunoprotein was obtained from rabbits after the injection of RDP previously purified by classical methods. This immunoprotein was then coupled to agarose and RDP from viral and cellular sources were purified in fair yield. Some drawbacks of this method include: the initial need for pure RDP, the time-consuming preparation of the immunoprotein, the instability of such a column, especially in the presence of proteinase activity.

A second affinity approach is that of Gerwin et al.[56b,83] They covalently bound oligo$(dT)_{12-18}$ to cellulose, since viral RDP shows a preference for $(dT)_{12-18}$ as a primer while other cellular polymerases only show a small affinity for the oligo polymer. Columns of this material provided a one-step purification of RDP from the detergent disrupted virions of murine leukemia virus (MLV) and an appreciable RDP enrichment from transformed mouse and human cells. The oligo dT is quite sensitive to nucleases, and therefore the column can be used only a few times.

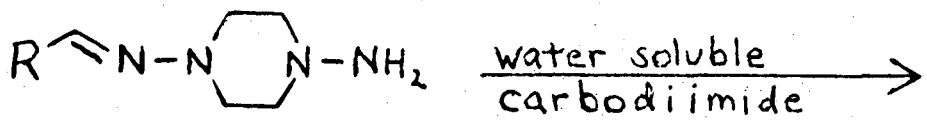
It had been our contention that an agarose column with a rifamycin bound to it could be a useful column for the purification, or at least enrichment, of RDP from both viral and cellular sources. It was also believed that such a column could also be useful for the isolation of any other proteins that possessed particular affinity for rifamycins.

B. The Synthesis and Use of Rifamycin Affinity Columns

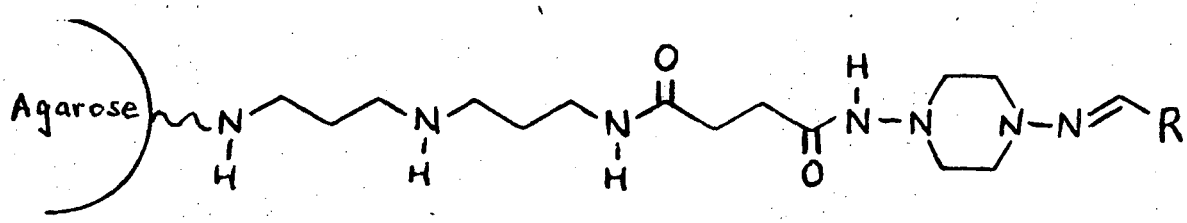
The first rifamycin affinity column (41), prepared for us by P. Rainey,[84] involved the condensation of aminodesmethylrifampicin (40) with a suitably substituted agarose bead derivative (39).



39



40



41

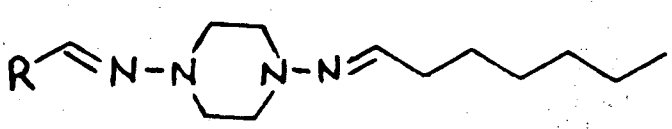
Rifamycin (40) which was prepared by the reaction of rifaldehyde with a large excess of N,N'-diaminopiperidine contained the dimer, dirifampin as an impurity. It was thought that the dimer impurity would pose no problem since it would be unreactive with the agarose derivative, (39). While this was found to be true, it was also found that a significant amount of the dimer was held by the agarose product, (41). Washing the dimer off the column proved to be a major problem since only aqueous eluents can be used in order to prevent the irreversible shrinkage of the agarose beads, and dirifampin is quite insoluble in every aqueous solution tried. The result was a very slow washing of the dimer off the beads. After several liters of wash, dirifampin was still detectable.

One attempt at RDP purification was attempted with a column of (41). The column equilibration and elution buffer was Tris-HCl (50 mM, pH 7.8), KCl (100 mM), DTT (1 mM), 20% glycerol, and Triton DN-65 (0.005%). The packed column had a bed volume of 25 ml. Crude RDP extract (0.96 ml) which contained 0.17 mg protein and 4500 units RDP activity (picomoles ^{3}H-dTTP incorporated per hour at 37°) was loaded onto the column which was maintained at 4°. Elution with buffer yielded 3 protein peaks. The first peak, amounting to an overload, eluted at the void volume. The second

peak came shortly after the first and the third peak eluted approximately 10 ml after the second. Some RDP activity was found in each peak, but the total yield was only 10%. Further elution was tried using a KCl gradient (0.1 to 0.5 M) and then a detergent gradient (0.005% to 0.10%). The detergent gradient eluted 3 additional small peaks of protein, the RDP activity of which yielded another 15% of the activity applied to the column. The slow leaking of RDP activity could be due to the slow elution of the free rifamycin dimer which was competing with the bound rifamycin for the RDP. The dimer did not interfere with the detection of RDP since the assay was run at a high detergent level (0.10%).

In order to eliminate the problem of the competition between bound and free rifamycins, it was decided to build a new column material. The synthesis of this second rifamycin affinity column (44) is outlined in Figure 18. The major advantage of this column is that rifamycin was introduced to agarose derivative (43), as rifaldehyde. The excess rifaldehyde, being much more soluble than dirifampin, was easily washed from the rifamycin agarose derivative, (44).

A simple derivative of aminodesmethylrifampicin (40) was prepared, (45), and tested as an RDP inhibitor to see if such derivatives, as were applied to both rifamycin affinity columns, were RDP inhibitors. (44) Inhibited RDP with a $K_I = 22 \times 10^{-6}$.



45

Rifamycin Affinity Column

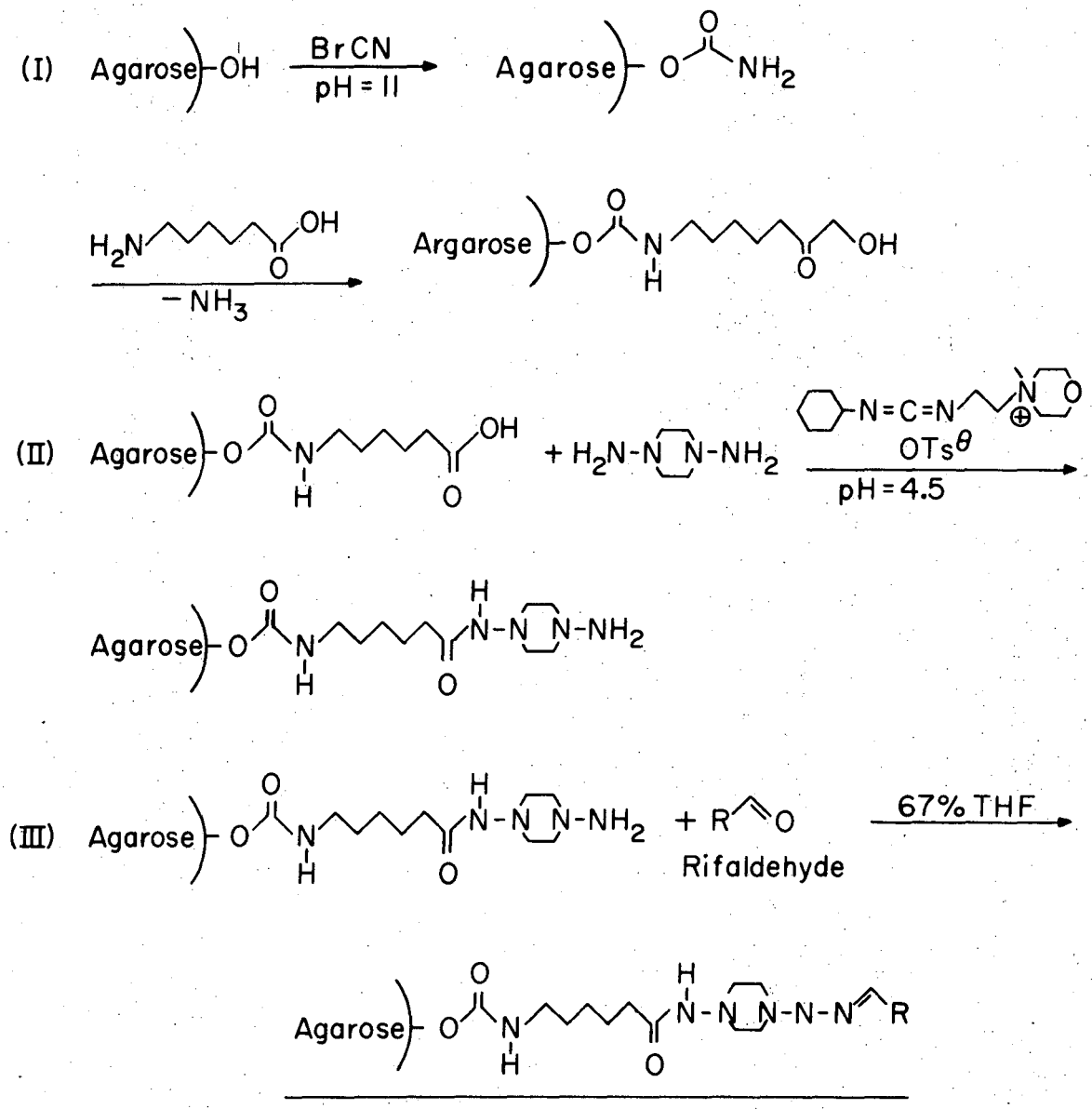


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Figure 18

The column buffer in the second RDP purification attempt contained 0.01% Triton DN-65. The 0.25 ml of RDP extract loaded onto the column contained 0.35 mg protein and 19,000 units per ml of the column buffer (except that it was 0.02% Triton DN-65). The column had a bed volume of 28 ml and a flow rate of 20 ml/hr. Elution with column buffer yielded a small peak at the void volume which had no RDP activity and a large second peak about 12 ml behind the first peak. The tailing edge of the second peak contained some RDP activity but the yield was only 5% and the specific activity was no better than in the sample applied to the column. Further elution with a detergent gradient (0.01% to 0.1%) yielded no more activity. However, application of 20 μg poly-rA per ml column buffer eluted another 2% of the applied activity.

In the third attempt, also using agarose derivative (44), the column was equilibrated with column buffer containing 0.01% Triton DN-65 but was eluted with column buffer containing only 0.005% Triton DN-65, in an effort to enhance the RDP binding. The 0.25 ml of RDP extract applied to the column contained 0.10 mg protein and 9000 units per ml of column buffer containing 0.01% Triton DN-65. The protein elution pattern with column buffer was very similar to that of the second attempt, but there was no RDP activity eluted. The elution of the RDP was then done using 8 μg poly-rA and 2 μg oligo-dT per ml of elution buffer. The nucleotides were allowed to enter the column and then left overnight (12 hr) before elution. This elution yielded approximately 20% of the activity applied to the column. Unfortunately, the activity was too low and too dilute to give very accurate yields of activity and protein.

Future attempts should probably include a divalent cation in the nucleotide mixture used to elute the RDP and/or a column with a shorter "arm" (group attached to the agarose) to reduce the RDP binding and facilitate elution.

C. Experimental

N-Amino-N-desmethylrifampicin. Rifaldehyde (1.00 g, 0.00138 mol) in THF (50 ml) was added dropwise to a stirred solution of N,N'-diaminopiperazine dihydrate (3.80 g, 0.028 mol) in water (50 ml). The addition was made over the period of 1 hr. The reaction was stirred for an additional 1 hr after the addition. Half of the reaction volume was evaporated under vacuum. The remaining portion was extracted once with chloroform (100 ml). The chloroform solution was evaporated under vacuum affording the product as an orange powder: yield 0.98 g (83%); tlc R_f 0.45 (ethanol); uv (in ethanol) 237 nm (ε 25,500), 340 (21,200), 479 (11,500).

Affinity column (41). Prepared by P. Rainey.[84]

Rifamycin derivative (45). N-Amino-N-desmethylrifampicin (1.0 g, 0.000607 moles) was dissolved in THF (30 ml) and heptaldehyde (0.17 g, 0.0015 moles) was added. Within 5 min an orange precipitate started to form. The reaction was stirred for 2 days, and then the solution was filtered. The precipitate was identified as the rifamycin dimer, dirifampin. The filtrate was evaporated and washed with hexane to yield the crude product which was purified by column chromatography on silica gel eluted with EtOH:EtOAc (1:1).

Affinity column (44). Activated agarose (10.0 g, Pharmacia) was placed in a glass filter and washed with dilute HCl (2 l of 0.001 M) over a period of 15 min. The beads were then washed with 50 ml of the coupling buffer (aqueous solution - 0.1 M $NaHCO_3$, 0.5 M NaCl, adjusted with 1.0 M NaOH to pH 9.3). A solution of 6-amino-caproic acid (1 g in 50 ml buffer, readjusted to pH 9.3) was added to the beads and the contents of the funnel were stirred and transferred to a stoppered 125 ml flask. The flask was then mechanically shaken for 2 hrs at room temperature. The beads were then transferred back to the funnel, filtered, and washed with buffer (100 ml) and distilled water (500 ml). The beads were stored at 0° in distilled water.

Gel (42) was placed in a glass filter and washed with distilled water (500 ml). A solution of N,N-diaminopiperazine dihydrate (1.5 g) and the water soluble carbodiimide (3.00 g) in distilled water was adjusted with concentrated HCl to pH 4.5 and added to the beads. The mixture was gently stirred and transferred to a glass stoppered 125 ml flask, and shaken mechanically for 12 hrs at room temperature. The mixture was then transferred back to the glass funnel and washed with distilled water. The beads were stored at 0° in distilled water.

Gel (43) was placed in a glass filter and washed with 67% THF (200 ml). The reaction solution, prepared by adding distilled water (25 ml) to a solution of rifaldehyde (1.00 g) in THF (50 ml), was added to the beads. The mixture was stirred gently, transferred to a glass stoppered 125 ml flask, and shaken mechanically at room temperature for 5 hrs. The mixture was then transferred back to

the filter and washed first with 67% THF (500 ml) and 0.05 M Tris-HCl, pH 8, until the filtrate was colorless (∿500 ml). The beads were then stored in distilled water for several days. By this time the filtrate was slightly orange and required washing with a solution that was 0.05 M Tris-HCl (pH 7.8), 0.1 M KCl, 1.0 mM DTT, 0.02% Triton DN-65 and 20% glycerol. Before use, the beads were further washed with the column buffer.

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ACKNOWLEDGMENTS

In retrospect I have found this part of my thesis the most difficult to write by far. When I sat down to write it I thought quite the opposite. I thought that thanking those whose help I have relied and depended on would be a simple task. But as the names of those involved, and the contributions made by each began to materialize in my mind, the task became increasingly difficult. For as one realizes how small a contribution he has made, independent of those who aided him, he is humbled, and setting to a task which humbles oneself, is at least for me a difficult endeavor. There are many names and many countless contributions associated with them, that must be deleted here short of turning these few pages into a major section of the thesis. I assure those that they are not forgotten and that I am indebted to them. To the following I offer my deepest thanks and humility.

To Professor Melvin Calvin - I often feel that I could not have worked under any other research director. He encouraged me to think for myself and to exercise my own initiative. He also doled out restraint and guidance when and where it was required. He served as a source of inspiration and enthusiasm when I failed to generate them myself. Lastly, and perhaps the most important, he tolerated my droll sense of humor.

To Frances Thompson, who taught me what I know of enzymology, and contributed a great deal to this thesis effort, directly and indirectly. She convinced me that the difficulties involved with working with molecules are not necessarily proportional to the square of their molecular weights, contrary to what I thought.

To Urs Joss, Louis Libertini, Jim Bartholomew, Priscilla Ross, Mina Bissell, Pete Rainey and Kurt Metzner. These people contributed time, ideas, assistance, and companionship. My additional thanks to Priscilla - together we comprised the lesser coffee club of Biodynamics.

To Jo Onffroy, Evie Litton, Bill McAllister, Ethel LeFall and Paul Hayes for their many assists and rebounds.

To Marilyn Taylor, Director of the Director, who answered at least 1000 of my questions.

To the sponsors of my research - the Atomic Energy Commission, The Dow Chemical Co., and the Elsa Pardee Foundation.

APPENDIX A

DETERGENT EFFECTS ON THREE NUCLEOTIDE POLYMERASE ACTIVITIES

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SUMMARY

Non-ionic detergents stabilize the activities of three nucleotide polymerases but have varied effects on four other enzymes.

Bovine serum albumin (BSA) has been used for many years to stabilize some enzymes in reaction mixtures and solutions which would otherwise have very low protein concentrations. More recently, non-ionic detergents and lipids have been used to solubilize, stabilize or activate enzymes (1). In particular, it is becoming apparent that non-ionic detergents effect the activity of viral reverse transcriptase.

We have previously reported that the concentration of various non-ionic detergents in the assay strongly influences the activity of an RNA-instructed DNA polymerase (RDP) function from MLV-transformed UC1-B cells (2). This RDP function was shown to be present in the cells only after viral infection and to have the template preferences characteristic of viral reverse transcriptase (3).

Some information about the detergent effect on the RDP activity can be obtained by assaying activity as a function of time. As shown in Figure 1, the initial activity was nearly independent of the detergent concentration. In the presence of 0.01% Triton DN-65, the reaction continued at a constant rate for more than 60 minutes. However, at low detergent concentrations, the activity decreased with time, and the rate of activity loss was greatest for the lowest detergent concentrations. Thus, the effect of detergent appears to be primarily one of stabilization rather than activation. Increasing the detergent concentration from 0.00067% to 0.01% after the reaction has proceeded for 60 minutes appeared to stabilize the remaining activity but did not recover any of the activity which had been lost.

As also shown in Figure 1, 30 µg/ml of BSA resulted in nearly complete stabilization of the polymerase activity.

The volume of the reaction was also found to be very important. In another experiment when the reaction at 0.00067% (0.012 mM) Triton DN-65 was run in a total volume of 1.8 ml from which 100 µl samples are withdrawn at the appropriate times, the activity remains constant for at least 60 minutes, incorporating 43 pmoles at 60 minutes. These results are very different from those shown in Figure 1 for the same detergent concentration in individual 100 µl assays where the surface-to-volume ratio is very much larger. The different results obtained by the two assay methods indicate that at least a portion of the stabilizing effect of detergents is a protection of the enzyme from irreversible surface inactivation on the glass walls of the test tube or at the air-water interface.

We also found that template and/or substrate contribute to the stability of the enzyme activity in the above experiments. Dilution of an extract to 0.014 mM detergent (in 1.36 ml) in the absence of template and substrate resulted in a rapid loss of about 50% of the polymerase activity within 30 minutes (at 0°C) followed by slight loss of the remaining activity in the next 24 hours. The lost activity was not recovered by adding detergent to the diluted extract. However, the loss could be prevented if the detergent concentration was maintained at 0.077 mM, suggesting that the detergent can substitute for the substrate/template in stabilizing the activity.

This detergent effect may, in fact, be a general phenomenon particularly for enzymes which are not always fully exposed to the aqueous environment of the cell. We have examined the effect of detergent on the stability of several other enzymes.

Three other nucleotide polymerase preparations were tested for detergent effects -- the reverse transcriptase from AMV purified through the DEAE cellulose step as described by Kacian, et al. (4), highly purified E. coli DNA-dependent DNA polymerase (DDP) and E. coli DNA-dependent RNA polymerase (DRP). As expected, the results from the AMV reverse transcriptase are very similar to those shown in Figure 1. Since the purity of the AMV enzyme was greater than for the RDP partially purified from cells, the similarity of the results indicated that the effect of detergents is not an artifact caused by the contaminants in the cellular preparations of the RDP. The results for the two E. coli polymerases are shown in Figure 2. There is a striking similarity in the non-ionic detergent effects for these two enzymes and the RDP. The only difference in the curves for RDP, DDP and DRP is in the rate of decrease of enzyme activity in the absence of additional protein or detergent. This difference is explainable in terms of the different protein concentrations in the assays -- the DDP contained only 2.3 ng/ml; the RDP, 3.1 μg/ml; and the DRP, 12 μg/ml.

Four enzyme systems which are not nucleotide polymerases were also examined for the effects of a non-ionic detergent. The results are also shown in Figure 2. Lactate dehydrogenase is not unstable at low protein concentrations and is not affected by the detergent. Hexokinase,

in a mixed assay with more than a five-fold excess of glucose-6-phosphate dehydrogenase, appears to have a much higher activity in the presence of 100 μg/ml BSA and detergent can partially substitute for the BSA. Glutamate dehydrogenase is stabilized by 100 μg/ml BSA but not by detergent. Instead, detergent is an inhibitor of the enzyme activity. Deoxyribonuclease I is stabilized by 100 μg/ml BSA and by 0.01% detergent. However, in addition to stabilizing this enzyme, detergent also inhibits its activity. These detergent effects are obviously quite different from the effects on the polymerases and also different for each enzyme.

These results demonstrate that there are no completely general non-ionic detergent effects on enzymes. The similarity of the results on the three different polymerases, however, indicates that these enzymes have some characteristics in common which the other enzymes tested do not have. It is not subunit structure since the DDP is a single subunit enzyme (5) and the DRP is a multisubunit enzyme (6). It is probably not related to the binding of polynucleotides since the deoxyribonuclease also binds polynucleotides. It may be that the polymerase enzymes have hydrophobic sites, e.g., membrane attachment sites, which are protected in aqueous solutions by the detergents. The soluble enzymes which prefer an aqueous environment would tend to be unaffected or inhibited by detergents. Guidotti has recently described work in which detergents show little binding to soluble enzymes but bind extensively to nonsoluble proteins (7). Not all nonsoluble enzymes would necessarily have their activity positively affected by detergents since some might require a more extensive hydrophobic environment (8) or the spacial orientation provided by intact membranes.

Future work in this laboratory will be directed toward separating the solubilizing aspects of the detergents from other possible effects.

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9. This work was supported, in part, by the U.S. Atomic Energy Commission. Two of us (F.M.T. and A.N.T.) were supported by the Elsa U. Pardee Foundation for Cancer Research. One of us (L.J.L.) was supported by the Damon Runyon Foundation for Cancer Research.

FIGURE CAPTIONS

Figure 1. Detergent Effect on RDP Activity. Duplicate assays were done in 100 μl total volumes which were 90 mM Tris-HCl (pH 7.8), 4% glycerol, 100 mM KCl, 0.3 mM dithiothreitol (DTT), 0.02 mM [^{3}H]dTTP (1 C/mmole), 10 μg/ml poly-rA:oligo-dT and 0.1 mM $MnCl_2$. Activity is measured as the incorporation of [^{3}H]dTTP into acid insoluble material. Each assay contained 0.31 μg (3.1 μg/ml) protein from the RDP extract. For addition to the assays, the RDP extract was diluted from 0.1% to 0.0033% Triton DN-65 with buffer A (0.05 M Tris-HCl, 1 mM DTT, 0.5 M KCl and 20% glycerol).

(△) Triton DN-65 was added to assays to yield a final concentration of 0.01%.

(□) BSA was added to assays to yield a final BSA concentration of 30 μg/ml with 0.00067% Triton DN-65.

(×) Triton DN-65 was added to assays to yield a final concentration of 0.0013%.

(○,●) Two experiments with a final Triton DN-65 concentration of 0.00067%.

(●) 1 μl 1% Triton DN-65 added to solid circle assays at 60 min

(●) 1 μl H_2O added to solid circle assays at 60 min

Figure 2. Detergent and Protein Effects on the Activities of Six Other Enzymes.

DRP (E. coli K-12 from Miles): Duplicate 100 μl assays were 40 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 0.1 mM DTT, 0.1 mM EDTA, 150 mM KCl, 0.15 mM each ATP, CTP and GTP, 0.15 mM [^{3}H]UTP (0.1 C/mmole), 0.15 mg/ml calf

thymus DNA and 12 µg/ml enzyme.

DDP (Kornberg polymerase, E. coli MRE 600, from Boehringer-Mannheim #15481): Duplicate 100 µl assays were 60 mM potassium phosphate buffer (pH 7.4), 6 mM $MgCl_2$, 0.45 mM DTT, 0.03 mM dATP, 0.03 mM [^{3}H]dTTP (0.5 C/mmole), 10 µg/ml poly-d(AT) duplex, 3% glycerol and 2.3 ng/ml enzyme.

Lactate Dehydrogenase (beef heart, type III, from Sigma): One ml assays were 25 mM Hepes (pH 7.0), 0.5 mM $NADH_2$, 1.0 mM pyruvate and 2.4 ng/ml enzyme protein.

Hexokinase (yeast, type III, from Sigma): One ml assays were 10 mM Hepes (pH 8.0), 0.5 mM NADP, 10 mM $MgCl_2$, 1.5 mM ATP, 1.5 mM glucose, 2.5 µg/ml glucose-6-phosphate dehydrogenase and 2 µg/ml hexokinase.

Glutamate Dehydrogenase (bovine liver, type II, from Sigma): One ml assays were 12.5 mM α-ketoglutarate and 12.5 mM NH_4Cl at pH 7.0, 0.5 mM $NADH_2$ and 1 µg/ml enzyme protein.

Deoxyribonuclease (bovine pancrease, type I, from Worthington): One ml assays were 25 mM Hepes (pH 7.0), 6 mM $MgCl_2$, 4 mM KCl, 0.06 mg/ml calf thymus DNA and 2 ng/ml enzyme protein.

(○) no additions

(△) 0.01% Triton DN-65 except DRP which was 0.008%.

(□) 100 µg/ml BSA except DRP which was 400 µg/ml.

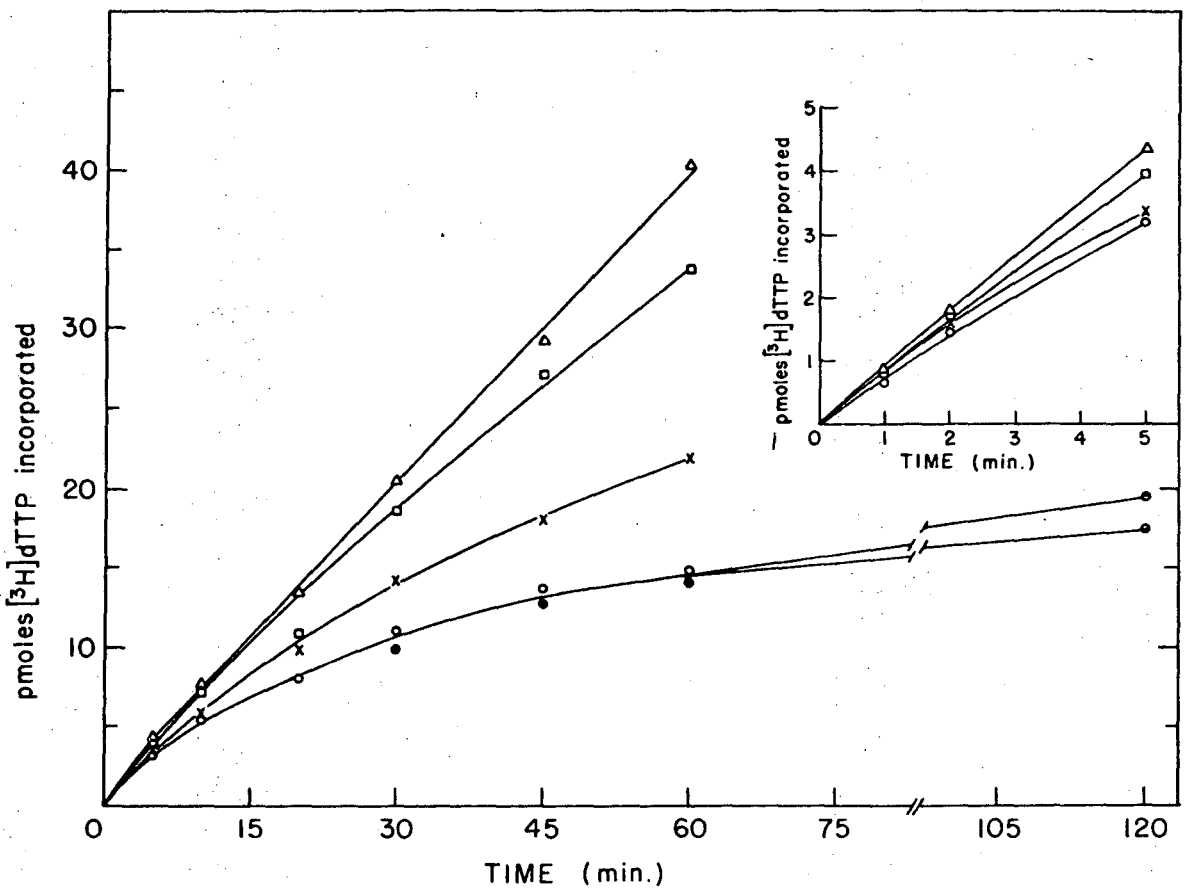
pmoles [3H]dTTP incorporated
TIME (min.)
0
15
30
45
60
75
105
120
10
20
30
40
pmoles [3H]dTTP incorporated
TIME (min.)
0
1
2
3
4
5

Figure 1

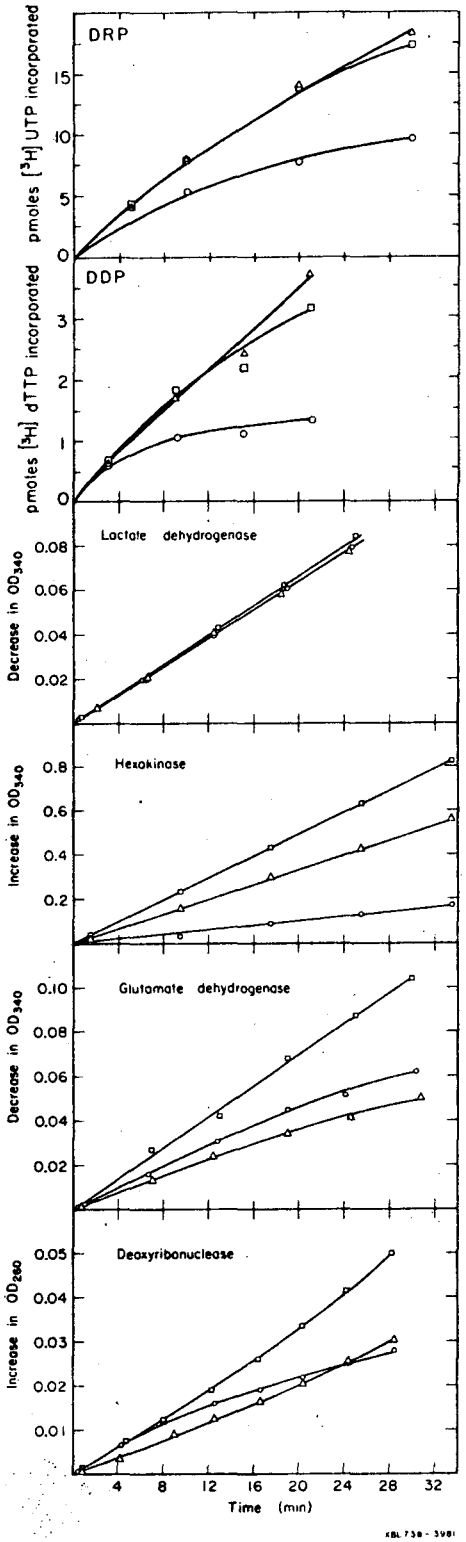
DRP
pmoles [³H] UTP incorporated
DDP
pmoles [³H] dTTP incorporated
Lactate dehydrogenase
Decrease in OD340
Hexokinase
Increase in OD340
Glutamate dehydrogenase
Decrease in OD340
Deoxyribonuclease
Increase in OD260
Time (min)

FIGURE 2

APPENDIX B

Preferential Inhibition of the Growth of Virus-transformed Cells in Culture by Rifazone-8_2, a new Rifamycin Derivative

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ABSTRACT Rifazone-8_2 (R-8_2) a new rifamycin derivative, is shown to preferentially inhibit the growth of virus-transformed chick cells in culture. Macromolecular synthesis and glucose uptake of transformed cells are also appreciably decreased in the presence of low concentrations of R-8_2 where the normal cells appear unaffected. While R-8_2 is shown to be a selective inhibitor of RNA-directed DNA polymerase *in vitro*, its action on the growth of transformed cells may involve some other mechanism.

Introduction

Rifamycin and its derivatives have been shown to inhibit focus formation by RNA tumor viruses in various cell types in culture (1-5). The *in vitro* demonstration that RNA-directed DNA polymerase (RDP) is inhibited by the action of some of these derivatives (6-13) has raised the possibility that the drugs may inhibit focus formation by inhibiting RDP activity. Smith *et al.* have shown recently that those rifamycin derivatives that are toxic to leukemic human leucocytes are also those that inhibit RDP best *in vitro* (14).

Rifampicin, a well known derivative of rifamycin has been shown to inhibit the replication of vaccinia virus in tissue culture when added at very high levels (> 100 µg/ml) (15,16) and to reduce the incidence of Adenovirus-induced tumors in male hamsters (16). In chick cells it has been argued that rifampicin a) inhibits focus formation by Rous sarcoma virus (1), b) has no effect on transformation, as it is toxic to both normal and transformed cells (17), and c) is preferentially toxic to transformed cells (18). Variation in culture conditions, serum concentration, and cell density undoubtedly play a role in such contradictory findings. For example, whether or not amphotericin B is present in culture medium, may drastically change the result of focus inhibition (4,19). Furthermore, the high concentration of rifampicin used in these experiments (20-80 µg/ml) under some conditions is quite toxic to normal cells and makes interpretation of these data very difficult. Rifampicin itself has little or no effect on RDP activity *in vitro* at concentrations used in tissue culture studies (7,17,20). We have tested several new rifamycin

derivatives synthesized in this laboratory which have been shown to be inhibitors of RDP *in vitro* and inhibitors of focus formation *in vivo* in other cell systems (4-6). In addition, we have tested Rifazone-8_2 (R-8_2), a new rifamycin which is to date the most selective inhibitor of viral RDP *in vitro* (6,21). We find that at low concentrations (3-10 μg/ml), R-8_2 can selectively inhibit the growth of transformed cells and prevent focus formation while allowing the normal cell growth and function to continue.

Materials and Methods

Growth of Cell Cultures. Primary cultures were prepared from 10-day old C/O or C/B type SPF chick embryos as described previously (23,24), except that Amphotericin B (Fungizone) was eliminated at this point (25). The cells were seeded in Medium 199, which was supplemented with tryptose phosphate broth (2%), calf serum (1%), and heated chicken serum (1%). The medium was changed on day 3. Secondary cultures were prepared 4 days after the primary seeding by trypsinization of primary cultures and were seeded at the desired cell concentration in 60 or 35 mm petri dishes. An additional 1 mg/ml of glucose was added to the medium at this time, bringing the final concentration of glucose to 11.00 mM, and calf serum concentration was raised to 2%. For studies with transformed cultures, half the cells of a single embryo were infected 4 hours after primary seeding with 4×10^5 focus forming units of SR-RSV or B-RSV. Secondary cultures were prepared as above.

Focus Assay. Assays were performed essentially as described (22), with slight modifications. We found that gentle removal of the agar

on day 4 or 5 and addition of either liquid medium or another agar overlay enhances the visibility of foci. Four hours after seeding secondary normal cells at 2.5 x 10^6 per 60 mm dishes, medium was removed and cells were exposed to the appropriate dilution of virus in 0.5 ml for 1 hr. The virus was then removed, the monolayer rinsed, and appropriate concentrations of drugs were added together with 1 µg/ml Fungizone in 0.5 ml of medium 199. Rifamycins were dissolved in dimethyl sulfoxide (DMSO) so that all cultures had a final concentration of 0.1% DMSO. Thirty minutes later the derivatives were removed and cells were overlaid with agar-medium containing the same concentration of the derivatives. In experiments where the rifamycins were added at a later time, the agar layers of control cultures were removed and replaced with agar containing DMSO at the same time. A known titre of SR virus stock accompanied all assays.

Rifamycin Derivatives. These were synthesized as previously described (20,21).

Measurements of the Rate of DNA and Protein Synthesis and Glucose Uptake. Radioactive assays were performed as previously described (25,26). All radioactive compounds were purchased from New England Nuclear.

Results

Focus Formation

When foci were scored on day 8 and 6 for SR-RSV or B-RSV respectively, there was a marked inhibition of the number of foci in the presence of rifazone-8_2 (R-8_2) (Table I). Ten µg/ml dimethylbenzyl

rifampicin (DMB) also caused appreciable inhibition with SR-RSV. Rifazacyclo-16 (RC-16), previously shown to be an inhibitor of MSV focus formation on UC1-B cells (4), was not very effective in chick cells. Rifampicin, at 20 μg/ml, had no effect on focus formation. The few remaining foci in the presence of the effective rifamycins were usually much smaller than the control foci (Fig. 1). When the foci were scored again 3 days later, the apparent inhibition had decreased in almost all cases suggesting an inhibition of the growth of the focus once it had been formed (Table I).

a. Normal Cells at High Cell Density. Under the condition of agar assay (more than 1×10^5 normal cells/cm^2), 10 μg/ml of R-8_2 had no effect on the growth properties of normal cells (Fig. 2). DMB, however, was toxic under these conditions. To avoid the complication of toxicity under assay conditions, we chose to work with R-8_2 alone. The pronounced inhibition of focus formation by R-8_2, therefore, is not due to toxicity to the normal cells in the monolayer. As a further control, the normal cells from the uninfected areas of drug treated cultures were removed from under agar and replated. They showed normal growth properties when compared to control cultures re-grown after agar removal and were morphologically normal as well.

b. Comparison of Normal and Transformed Cells at Low densities. When cells were seeded at lower cell densities (2×10^4 cells/cm^2) they were more sensitive to R-8_2 than cells at the higher cell densities (compare Figs. 2 and 3a). However, at comparable cell densities and growth rates, transformed cells were always much more sensitive

than normal cells (Fig. 3). While normal cells were unaffected by 5 μg/ml of R-8_2, the growth of transformed cells was considerably inhibited by 3 μg/ml of R-8_2 after 48 hr (Fig. 3c). Three days after addition of 5 μg/ml R-8_2, the morphology and cell number were still the same in untreated and treated normal cultures (Fig. 4A, B). The R-8_2 treated transformed cells, however, were drastically reduced in number and the remaining cells were either vacuulated or had a normal morphology (Fig. 4D). DNA synthesis as measured by [^{3}H]-thymidine incorporation (26) and uptake of glucose as measured by [^{3}H]-2 desoxyglucose (26) showed the same pattern of preferential sensitivity to R-8_2 (Table II). The soluble pool of [^{3}H]-thymidine was not affected by the presence of R-8_2. The rate of protein synthesis as measured by [^{3}H]-leucine incorporation into the acid insoluble pool was the least sensitive to the action of R-8_2, although transformed cells were still more affected (Table IIb).

Effect of R-8_2 on Previously Formed Foci

To understand to what degree this preferential toxicity to transformed cells could explain the focus inhibition, two kinds of experiments were performed. In one series of focus assays, 10 μg/ml R-8_2 was added 1 hr after infection, resulting in more than 90% inhibition of focus number after 8 days. If the action of the R-8_2 was solely on inhibiting the initiation of transformation one would expect that addition of R-8_2 2 days later would have no effect on the number of foci produced. In fact, when R-8_2 was added 2 days after infection there was still more than 40% inhibition of the foci if scored on day 8. Furthermore, the remaining foci were smaller than control (compare Fig. 1C and D), and the percent inhibition decreased to about 10% by day 11

when the foci became quite visible. This experiment indicated to us that a large proportion of the focus inhibition observed (Table I) may be attributed to the preferential toxicity of transformed cells to R-8_2 in addition to any inhibition of initiation of transformation.

In a second series of experiments B-RSV foci were allowed to develop until they were visible (5 days). The agar was then removed gently and complete medium was added with or without 10 μg/ml of R-8_2. The foci continued to grow with a measurable rate in control cultures (Fig. 5A, B, Fig. 6). The removal of the agar resulted in a "necrotic" focus as the piled up transformed cells in the center of the focus were lost to the medium (Fig. 5B). The growth of the foci after the addition of 10 μg/ml R-8_2, however, was virtually arrested (Fig. 5C, D, Fig. 6). It has been shown previously that 48 hr after seeding, the normal chicken cells seeded at high density are no longer susceptible to transformation by B-RSV (22). The focus of B-RSV, therefore, is comprised essentially of the progenies derived from the initially transformed cell. Indeed, we observed no additional foci in control cultures, despite the fact that virus is released into the medium after agar removal. Thus the lack of focus growth in the presence of R-8_2 is due to inhibition of transformed cell growth rather than an inhibition of secondary infection.

Mixed Cultures

Two experiments were performed in which 50% normal and 50% transformed cells were plated and allowed to grow in the presence and absence of R-8_2 (5 μg/ml) for 3 days. In the first experiment, where the cells were plated at a low density (1 x 10^4 cells/cm^2), cells grown in the

absence of R-8_2 appeared to be completely transformed (Fig. 7A), while cultures grown in the presence of R-8_2 appeared to be largely normal by the end of the 3-day period (Fig. 7B). In the second experiment, where the cells were plated at a higher density (5×10^4 cells/cm^2), cells grown without R-8_2 appeared to be completely transformed as expected. The cells with R-8_2 present, however, were still in a monolayer and the majority of cells were normal, although dispersed transformed cells could be seen in the culture. Consistent with previously described results, these mixed culture experiments indicate a preferential inhibition and/or killing of transformed cells. Furthermore, they might suggest an additional role of R-8_2 in preventing secondary infection by inactivating the virus itself. Whether the presence of R-8_2 in these experiments selects for normal cells by killing transformed cells, or whether it actually causes a reversion toward a normal morphology, is a question currently under investigation.

DISCUSSION

The selective inhibition and/or destruction of neoplastic cells while the normal cell growth and function continues, is a general aim of cancer chemotherapy. We have shown that rifazone-8_2, a specific inhibitor of RDP (21) also inhibits the growth of virus-transformed cells in tissue culture without appreciable side effects to normal cell growth. Preferential inhibition of transformed cells has been reported for rifampicin previously (18). However, rifamipicin does not inhibit RDP appreciably (9) and the dosage needed for focus inhibition (more than 20 μg/ml) is toxic to normal chick cells in our hands.

There are a number of possible explanations for this increased toxicity of R-8_2 to the transformed cells. 1) There is abundant evidence that cancer cells in general have altered permeability (27) and altered membrane properties (28), factors which could account for the observations described here. Once the nature of these differences is understood they may further be exploited to synthesize new derivatives which can cross the membrane of specific tumor cells more readily. 2) The drug may act partially by preventing transformation through inhibition of reverse transcriptase (12). If a continuing involvement of this enzyme in the growth of transformed cells is postulated, the additional inhibitory action of R-8_2 on the growth of transformed cells may be explained. 3) Alternatively, R-8_2 may be inhibiting an as yet unknown enzyme function(s) which might be essential to the growth of transformed cells. These possibilities are not mutually exclusive. The additional action of R-8_2 on the infectivity of the virus itself will also be investigated (12). To what extent a change in permeability of transformed cell membrane is responsible for the observed effects will be studied by use of radioactive derivatives. The effect of R-8_2 and other rifamycin derivatives in various cell systems and animals is under investigation.

Acknowledgments. The work described in this paper was sponsored, in part, by the U. S. Atomic Energy Commission and, in part, by the National Cancer Institute, Grant NCI-1RO-1-CA14828-1. One of us (A.N.T.) was supported by the Elsa U. Pardee Foundation for Cancer Research.

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Abbreviations used: R-8_2 or Rifazone 8_2, rifaldehyde-N,N-di-n-octyl-hydrazine-hydrazone; RDP, RNA-directed DNA polymerase; RSV, Rous sarcoma virus; B-RSV, Bryan high titre strain of RSV; SR-RSV, Schmidt-Ruppin sub-group A strain of RSV; Rif, Rifampicin; DMB, 2',6'-dimethyl-4'-benzyl-4'-desmethylrifampicin; DMSO, dimethyl sulfoxide.

Figure Legends

Fig. 1. Focus formation in the presence and absence of R-8_2. Focus of SR-RSV on day 8. A) in 0.1% DMSO, B) in the presence of R-8_2 (10 µg/ml) added 1 hr after virus infection, C) in 0.1% DMSO; the agar overlay was replaced after 2 days. D) in the presence of R-8_2 (10 µg/ml) added 2 days after virus infection.

Fig. 2. Growth of high density normal cells in the presence of rifamycin derivatives. Normal cells were plated at 1 x $10^5/cm^2$ in the presence or absence of the chemicals as described in Methods. Average of 4 measurements.

Fig. 3. Growth of normal and RSV-transformed cells in the presence of R-8_2. Normal and SR-transformed cells were seeded at 2 x 10^4 cells/cm^2 4 days after primary seedings. The medium contained either DMSO or R-8_2 (3, 5, 10 and 20 µg/ml). Cells were counted on successive days in triplicate cultures. Fig. 3C is the percent cells left on the dish after 48 hr (taken from 3A and 3B; the cell number in control cultures was set equal to 100).

Fig. 4. Morphology of normal and transformed cells in the presence of R-8_2. Legend as in Fig. 3, except that cells were seeded at 1.5 x 10^4 cells/cm^2 and medium was changed on day 2. Control and treated cultures (5 µg/ml R-8_2) were photographed 3 days after seeding. A and B, normal cells in the absence and presence of R-8_2. C and D, transformed cells in the absence and presence of R-8_2.

Fig. 5. Arrest of focus growth after addition of R-8_2.
Two sets of focus assay plates of B-RSV were allowed to develop until foci were visible (5 days). The agar overlay was then removed and replaced with regular medium containing 0.1% DMSO or R-8_2 (10 µg/ml in 0.1% DMSO). Visible foci were encircled with black pen and numbered and they were then photographed on successive days. Arrows indicate the boundary of focus in each case. A, control focus 1 hr after addition of liquid medium; B, the same focus as in A, 82 hr later; C, focus 1 hr after addition of liquid medium and R-8_2 (10 µg/ml); D, the same focus as in C, 82 hr later.

Fig. 6. The rate of growth of previously formed foci in the presence or absence of R-8_2.
Experiment was performed as described in Fig. 5. The area under the foci was estimated by use of a planimeter. Each curve is the average of 3 foci.

Fig. 7. Morphology of mixed cultures in the presence and absence of R-8_2. 50% normal and 50% transformed cells were seeded at a total population of 1 x 10^4 cell/cm^2. The pictures were taken 3 days after seeding. A), culture with DMSO. B), culture with R-8_2 (5 µg/ml).

Table I

Focus Inhibition - % of Control

			Schmidt-Ruppin*		Bryan+	
			Day 8	Day 11	Day 6	Day 9
Cont (DMSO)			100 (80±20)†	100 (85±20)	100 (70±20)	100 (70±20)
Rif	20	/ml	100 ± 10	100 ± 10	100 ± 10	100 ± 8
RC-16	10	/ml	75 ± 10	85 ± 10	-	-
DMB	1	/ml	50 ± 7	85 ± 8	-	-
DMB	10	/ml	0-2	10 ± 6	-	-
R-8_2	1	/ml	90 ± 10	90 ± 10	90 ± 10	95 ± 10
R-8_2	5	/ml	40 ± 10	70 ± 15	45 ± 10	70 ± 15
R-8_2	10	/ml	0-1	15 ± 10	10 ± 8	20 ± 10

50-100 focus forming units of Schmidt-Ruppin (SR) subgroup A or Bryan strain of Rous sarcoma virus were assayed under agar as described in Methods. The foci were scored on day 8 and 11 for SR and on days 6 and 9 for the Bryan strains. Amphotericin B (1 μg/ml) was present throughout the focus assay, even though at best it improved the inhibition by only 10%.

* Average of 6 experiments

+ Average of 4 experiments

† The number in parenthesis indicates the actual number of foci which was set equal to 100.

Table IIa

[^{3}H]-Thymidine Incorporation

	% of control after 48 hr µg of drug per ml					
	0	3	5	10	15	20
N	100 (38600)*	100	100	74	39	20
T	100 (44600)	70	64	51	28	4

*DPM/mg protein. Average of 3 experiments.

Procedure was as described in Fig. 3 and Methods.

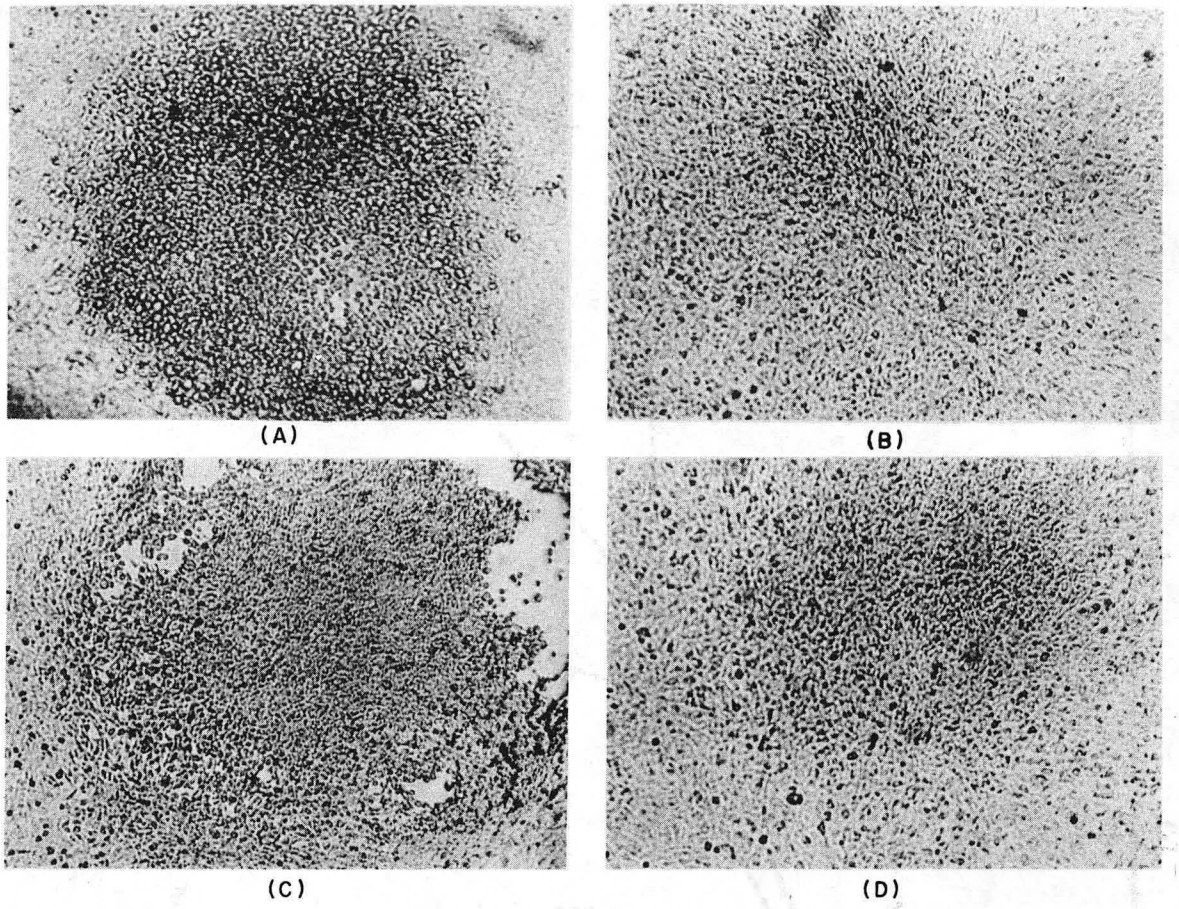
Table IIb

[^{3}H]-Leucine Incorporation and [^{3}H] 2-Deoxyglucose Uptake

	Leucine		2-DG	
	N	T	N	T
	% of control after 48 hr			
Control	100 (32100)*	100 (42900)	100 (43900)	100 (98100)
3 g/ml	100	100	108	72
5 g/ml	100	88	105	53
10 g/ml	85	- †	85	42

* The numbers in parentheses represent DPM/mg protein.

† Too few cells left for determination. Average of triplicate samples of one experiment.



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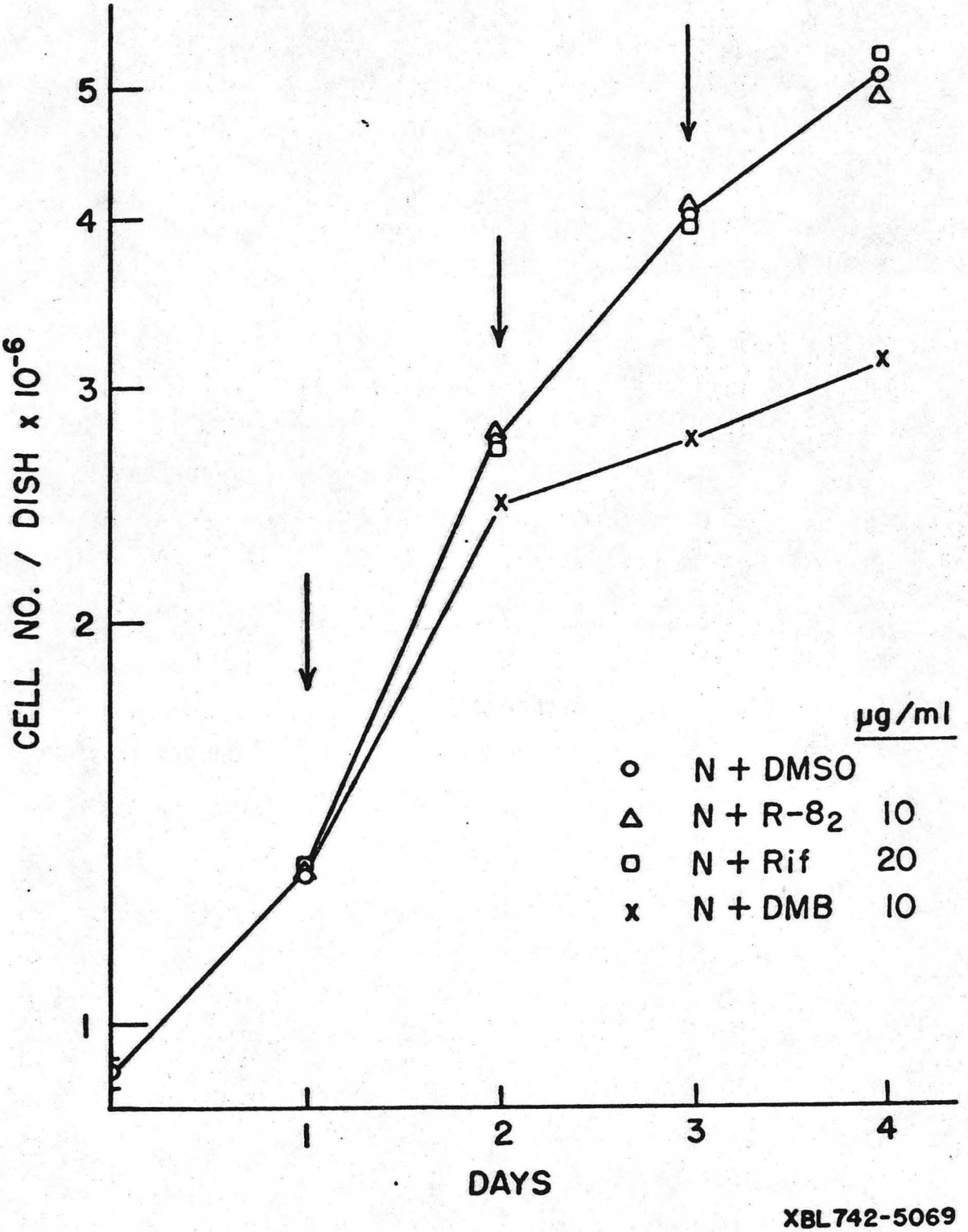
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5
4
3
2
1
1
2
3
4
DAYS
µg/ml
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N + R-8₂ 10
N + Rif 20
N + DMB 10
XBL742-5069

Figure 2,

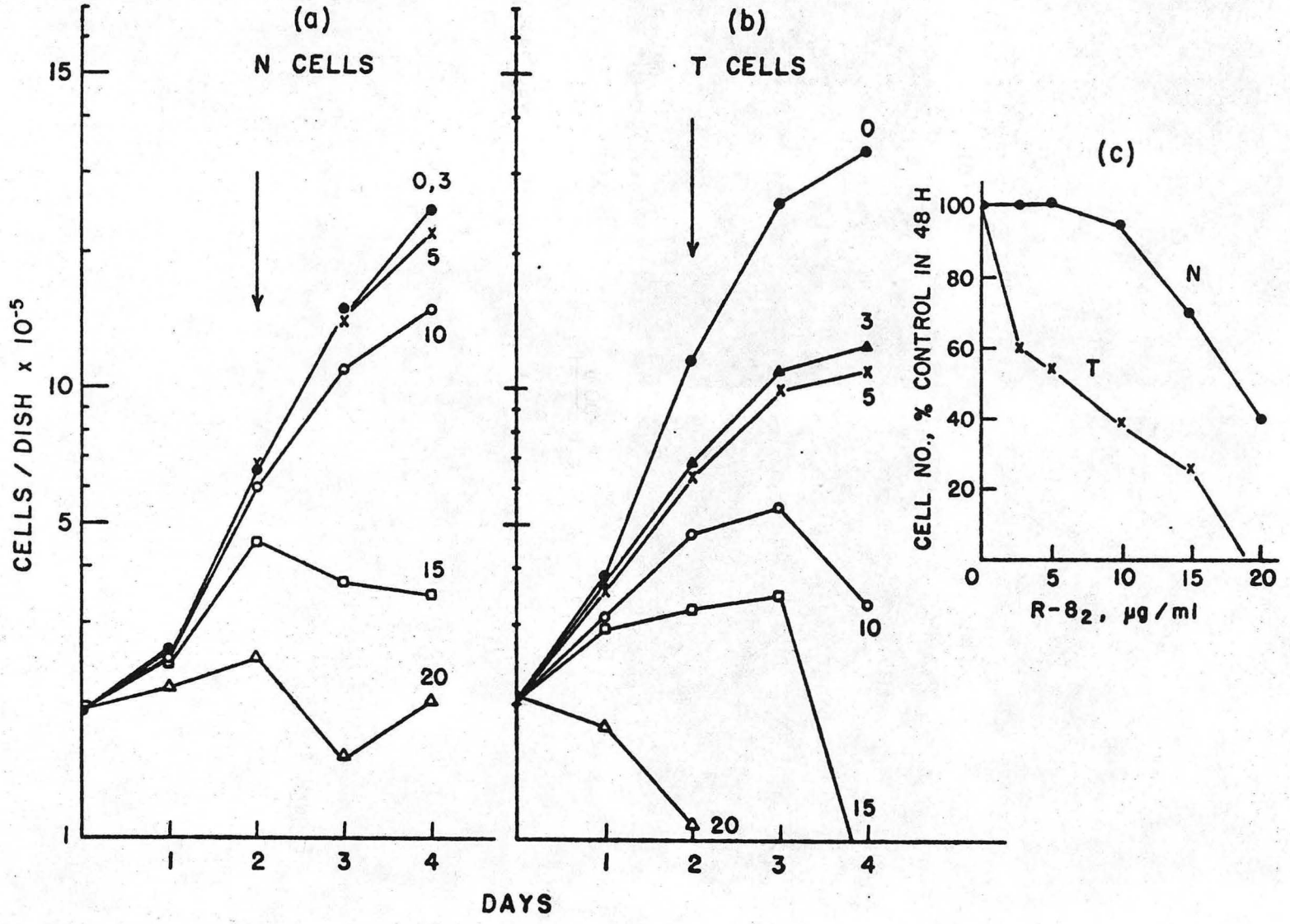

Figure 3

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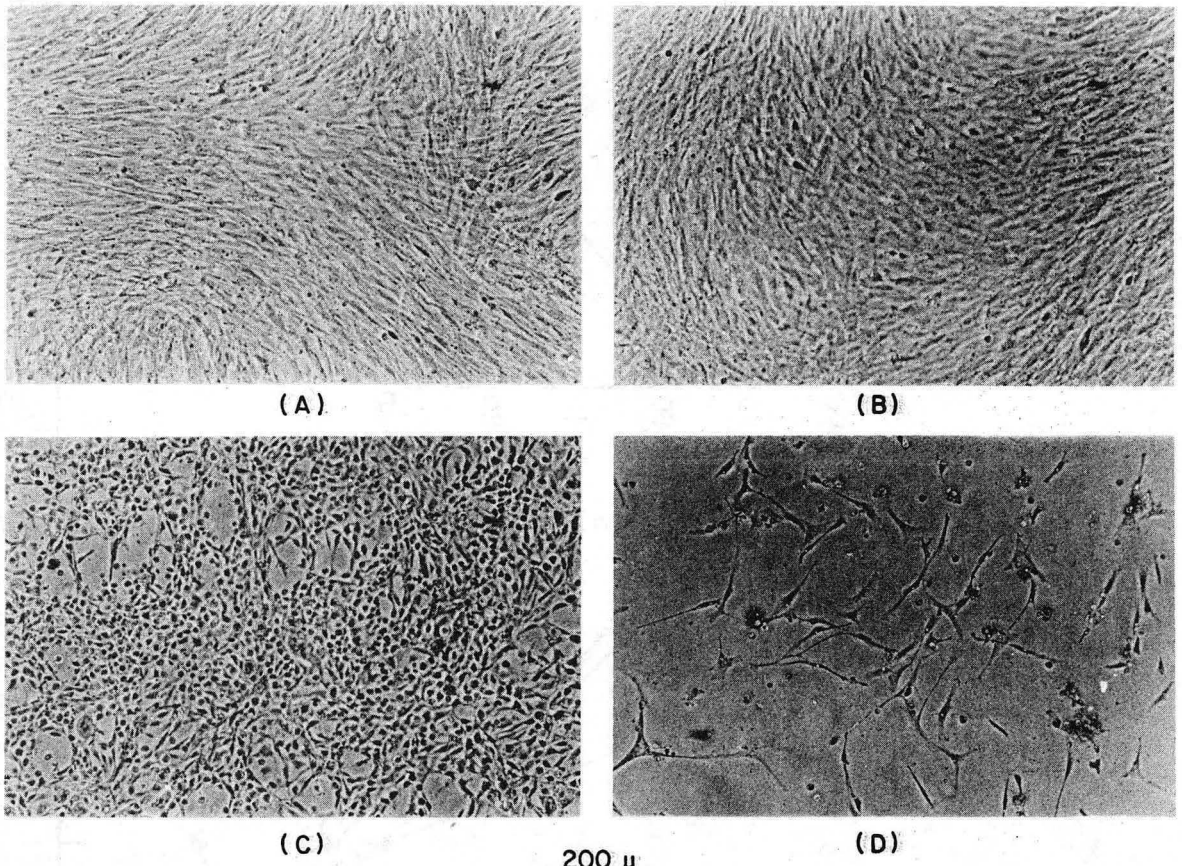

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Figure 4

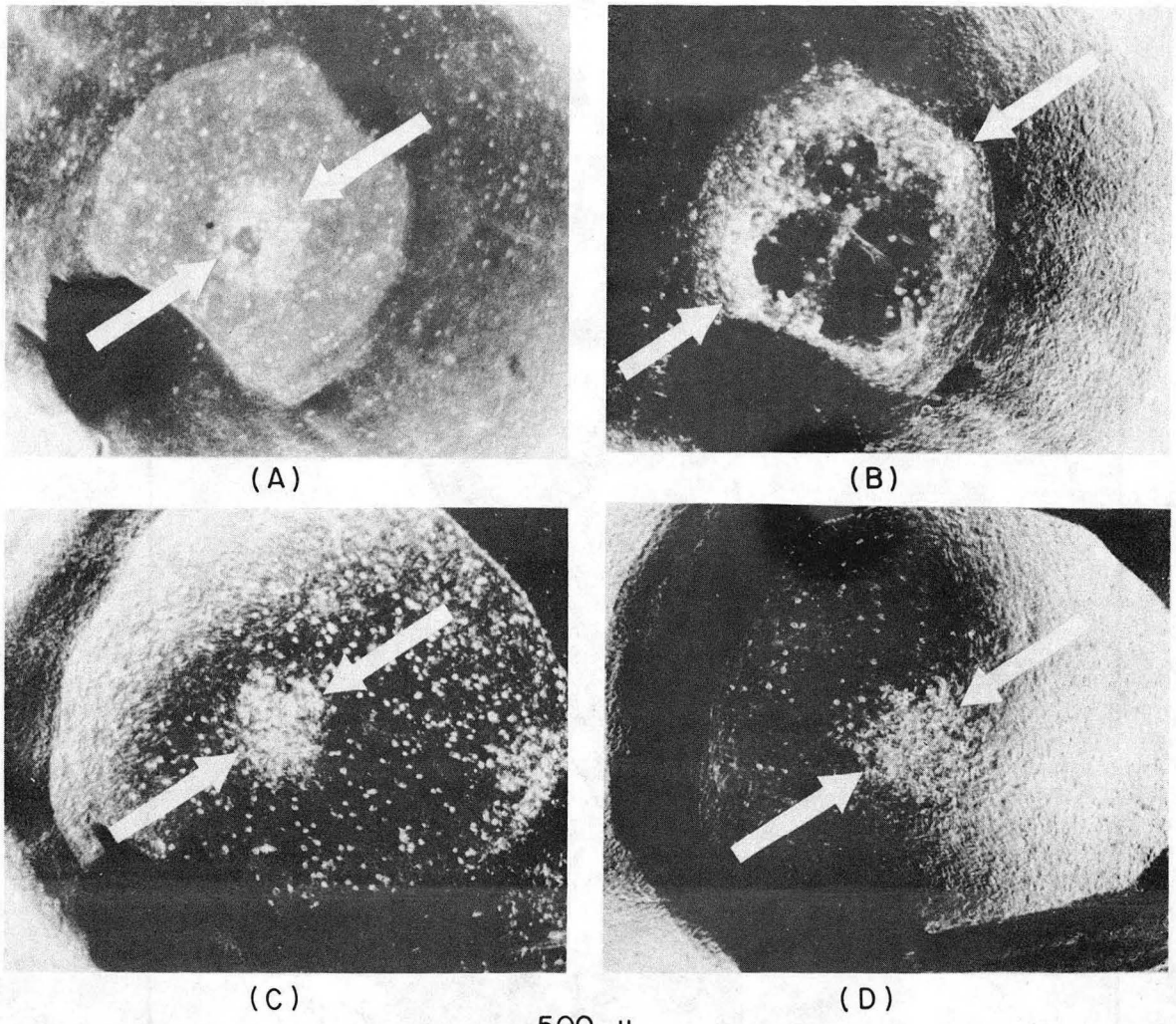

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Figure 5

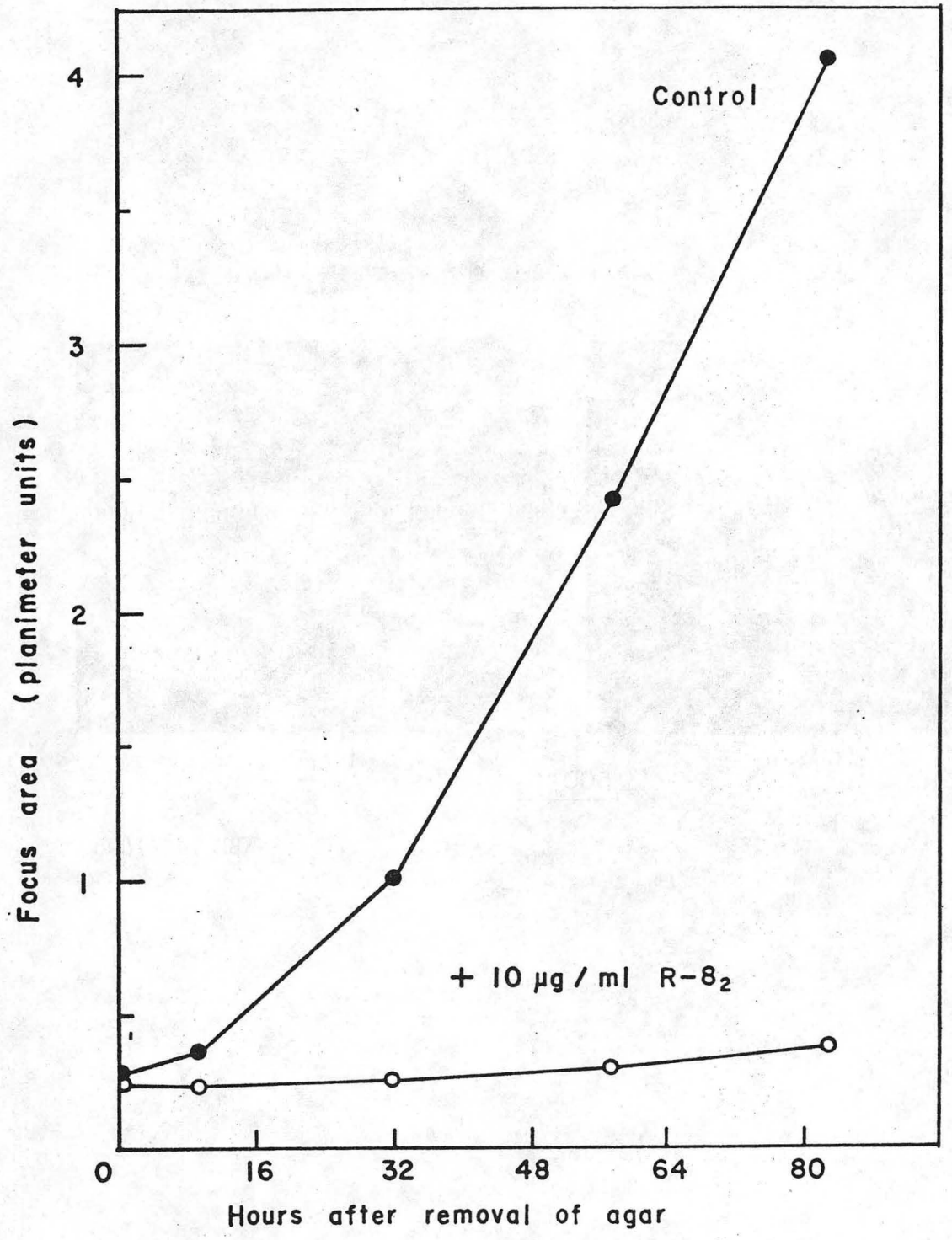
Control
+ 10 µg / ml R-8₂
Focus area (planimeter units)
Hours after removal of agar
0
16
32
48
64
80
1
2
3
4
XBL743-5080

Figure 6,

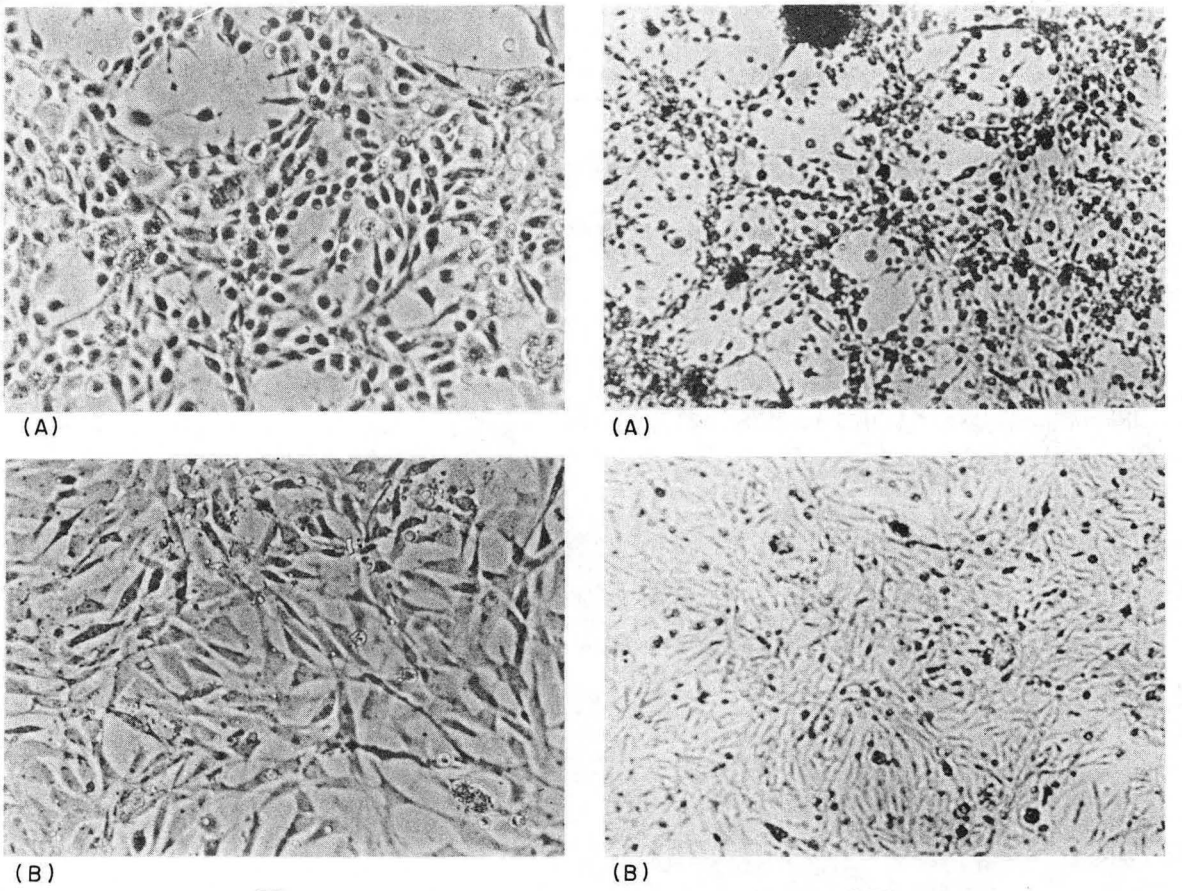

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Figure 7

APPENDIX C: ABBREVIATIONS

DDP	DNA-directed DNA polymerase
DMB	dimethylbenzyldesmethylrifampicin
DMSO	dimethylsulfoxide
DNase	deoxyribonuclease
DRP	DNA-directed RNA polymerase
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
esr	electron spin resonance
ir	infrared spectrum
K_I	inhibition constant
LAH	lithium aluminum hydride
nmr	nuclear magnetic resonance
P	partition coefficient
ϕ	phenyl, C_6H_5-
RDP	RNA-instructed (directed) DNA polymerase
R_F	distance of spot from the origin ÷ distance of solvent front from the origin
THF	tetrahydrofuran
tlc	thin layer chromatography
TNS	2-p-toluidinylnaphthalene-6-sulfonate
uv	ultraviolet spectrum

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