jugular vein was catheterized, plasma samples were taken during a control period, and then salt loads were injected subcutaneously. In these two snakes the control plasma Na and Cl concentrations were lower than the mean values reported in Table 1 and the first salt load failed to stimulate salt gland secretion (Fig. 3B). A second salt load stimulated secretion of a highly saline fluid within 1.5 hours. Plasma Na and Cl concentrations increased to values above those in Table 1, and at some critical point near 200 m*M* Na and 170 m*M* Cl salt gland secretion was initiated. Thus an increase in plasma NaCl concentration (and osmotic pressure) leads to increased activity of the salt gland. This response of the gland to differing amounts of salt in the plasma suggests the presence of receptors for salt concentration or osmoreceptors.

There are approximately 50 species of sea snakes divided into two major subfamilies, the Laticaudinae and Hydrophiinae. It will be interesting to compare the structure and physiology of the posterior sublingual glands of species which differ in feeding habits and in the degree of adaptation to the marine habitat. This is especially true of the three species which have secondarily colonized freshwater lakes in the Philippine and Solomon islands and those which feed on fish eggs or invertebrates (prawns and squid) rather than fish.

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Inhibitors of DNA Polymerases of Murine Leukemia Viruses: Activity of Ethidium Bromide

Abstract. *Ethidium bromide, compared on a molar basis, was a more effective inhibitor of the DNA polymerases of the Rauscher and Moloney murine leukemia viruses than either 4-*N*-demethylrifampicin or 4-*N*-benzyldemethylrifampicin. Daunomycin inhibited the polymerases weakly, and chromomycin A_3 inhibited almost not at all. 4-*N*-Benzyldemethylrifampicin was a more active inhibitor than the 4-*N*-demethyl congener.*

The RNA-containing murine and avian oncogenic viruses have both RNA-dependent and DNA-dependent DNA polymerase activities (*1*). The RNA-dependent DNA polymerase activity has now been discovered in visna (*2, 3*) and "foamy" viruses (*3*), and in leukemic cells from patients with acute lymphoblastic leukemia (*4*). Investigation of inhibitors of the nucleic acid–synthesizing enzymes of the RNA-containing tumor viruses may be useful not only in the analysis of the mechanisms of replication of these viruses, but also may provide new drugs for and tests of their efficacy in the treatment of leukemia and other cancers. I now report the effect of several drugs on the DNA polymerase activity of two murine leukemia viruses. The drugs used were 4-*N*-demethylrifampicin (AF/AP; molecular weight, 808.94), 4-*N*-benzyldemethylrifampicin (AF/ABP; molecular weight, 899.07), daunomycin hydrochloride (molecular weight, 560), chromomycin A_3 (molecular weight, 1183; Mann Research), and ethidium bromide (molecular weight, 394; Calbiochem). The rifampicin derivatives are effective inhibitors of the DNA polymerase of the Moloney murine sarcoma virus [MSV(M)] (*5*). Daunomycin, an antibiotic of the anthracycline group, binds to DNA by intercalation (*6*) and is of interest because it is used in the treatment of acute leukemias. Chromomycin A_3 is another antibiotic that binds to DNA but not by intercalation (*6*). The trypanocide ethidium bromide binds to DNA by intercalation, and its effects on the uncoiling of the DNA double helix have been studied (*6*).

The enzyme assay was a modification of that reported by other workers (*1*). The standard enzyme assay consisted of 50 m*M* tris-HCl (*p*H 8.3), 5 m*M* $MgCl_2$, 40 m*M* KCl, 1 m*M* deoxyadenosine triphosphate (dATP), deoxycytidine triphosphate, deoxyguanosine triphosphate (Calbiochem), 20 m*M* dithiothreitol (Sigma), and 2.5 μc [^{3}H]TTP (thymidine triphosphate; New England Nuclear) in 0.1 ml (final volume) of reaction mixture. Portions (containing 20 μg of viral protein) of stock solutions of either the Rauscher [MLV(R)] or Moloney leukemia virus [MLV(M)] (both originally from Electro-Nucleonics Laboratories, and repurified by density gradient centrifugation) were added to the reaction mixture. When included, the nonionic detergent Nonidet P-40 (Shell Chemical) was added to a concentration of 0.01 percent. The mixture was incubated for 120 minutes at 39°C, and the acid-insoluble, alkali stable, macromolecular product was then isolated and counted (*1*). The drugs were added to the final concentrations indicated at the start of incubation. The stock solution of AF/ABP contained 1.25 mg/ml in aqueous ethanol (1 : 1, by volume). The stock solutions of drugs were stored in the dark at −15°C. The enzyme reaction was inhibited either by prior incubation with purified pancreatic ribonuclease or the omission of dATP from the incubation mixture, and the kinetics of [^{3}H]TMP (thymidine monophosphate) incorporation were in agreement with those described (*1*). All experiments were performed in triplicate with the same virus stocks and labeled nucleotide of the same specific activity.

Ethidium bromide was far more effective in inhibiting the DNA polymerase of MLV(R) than either daunomycin or chromomycin A_3 (Fig. 1). Inhibition of the reaction was still complete at a concentration of 20 μg per milliliter of reaction mixture of ethidium bromide (5.1×10^{-8} mole/ml); at 2.50 μg/ml (0.64×10^{-8} mole/ml) 70 percent of the reaction was inhibited. Daunomycin, which, like ethidium bromide, binds to DNA by intercalation, was still more effective than the nonintercalating DNA binder chromomycin A_3. Even at a concentration of 500 μg per milliliter of reaction mixture, chromomycin A_3 showed only 28 percent inhibition of the reaction, while daunomycin inhibited 75 percent of the reaction.

The rifampicin antibiotics inhibit bacterial and mitochondrial RNA polymerases (*7*), inhibit replication of pox and adenoviruses (*8*), block focus formation

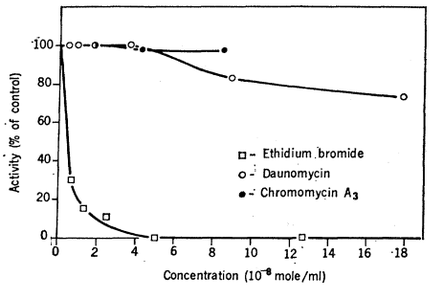

Fig. 1. Effect of chromomycin A_3, daunomycin, and ethidium bromide on Rauscher murine leukemia virus DNA polymerase. The constitution of the enzyme assay is given in the text. The reaction mixture did not contain Nonidet P-40. The control reaction incorporated 1076 count/min ([^{3}H]TMP) after 120 minutes incubation. The specific activity of the [^{3}H]TTP used was 16.5 c/mmole.

by Rous sarcoma virus (*9*), and block cleavage of the polypeptide precursor of an internal protein of vaccinia virus (*10*). The DNA polymerase of the RNA-containing tumor viruses are inhibited by rifampicin congeners (*5*). 4-*N*-Demethylrifampicin inhibits the RNA-dependent DNA polymerase of MSV(M) (*5*) and the DNA polymerases of human lymphoblasts (*4*), MLV(R), and BALB/3T3 cells (*3*). In Fig. 2 the activities of AF/AP, AF/ABP, and ethidium bromide are compared in inhibiting the DNA polymerase of MLV(M) (Fig. 2A) and MLV(R) (Fig. 2B). In agreement with an earlier report (*5*), AF/ABP was a much more active inhibitor of DNA polymerase as compared to AF/AP. However, of the three drugs ethidium bromide was the best inhibitor. While ethidium bromide at a concentration of 0.32×10^{-8} mole/ml of reaction mixture inhibited 91 percent of the activity of the MLV(M) (Fig. 2A), AF/ABP inhibited only 23 percent of the activity at a concentration of 0.29×10^{-8} mole/ml.

Another observation made during these studies was the effect of the inclusion of Nonidet P-40 in the reaction mixture on the inhibition of the DNA polymerase by the drugs. Inclusion of Nonidet P-40 in the reaction mixture enhanced the strong inhibition of the DNA polymerase of MLV(M) by ethidium bromide (Fig. 3) and the weak inhibition by daunomycin and chromomycin A_3 (Table 1). This effect was not due to a residual number of counts that were not affected by the drugs because the absolute number of counts incorporated in the assays which included detergent were lower than replicate assays which did not include the detergent. The reasons for this effect are now under study. Perhaps detergent prevents the association of the endogenous RNA template and the polymerase, allowing the inhibitory drugs to more effectively block the reaction.

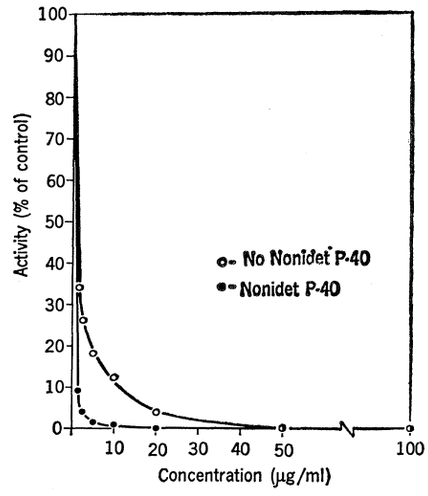

Fig. 3. Effect of detergent on the inhibition of the DNA polymerase of the Moloney leukemia virus by ethidium bromide, as judged by incorporation of [^{3}H]TMP. The control reaction mixture containing detergent incorporated 2796 count/min ([^{3}H]TMP) after 120 minutes incubation while the control containing no detergent incorporated 1043 count/min. The specific activity of the [^{3}H]TTP used was 12.7 c/mmole.

Table 1. Effect of detergent on inhibition of Moloney murine leukemia virus by daunomycin and chromomycin A_3. The amount of [^{3}H]TMP incorporated in the control reaction mixture containing Nonidet P-40 (0.01 percent) was 2796 count/min after 120 minutes incubation, and the amount incorporated in the reaction mixture containing no detergent was 1043 count/min. The specific activity of the [^{3}H]TTP used was 12.7 c/mmole.

Compound	Concentration (μg/ml)	Concentration (10^{-8} mole/ml)	Inhibition (%)	
			Detergent	No detergent
Daunomycin	100	17.9	32	20
Chromomycin A_3	100	8.5	18	10

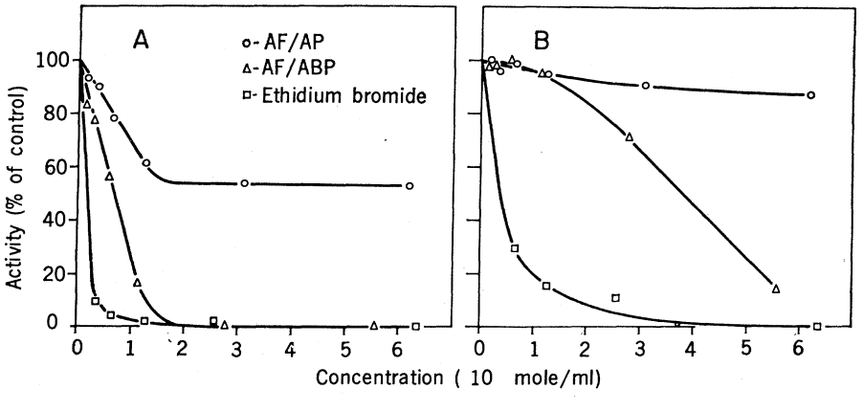

Fig. 2. Comparison of the effect of AF/AP, AF/ABP, and ethidium bromide on the DNA polymerases of (A) Moloney leukemia virus and (B) Rauscher leukemia virus. Nonidet P-40 (0.01 percent) was included in the reaction mixtures of MLV(M) and was not included in the reaction mixtures of MLV(R). The control reaction of MLV(M) incorporated 2006 count/min ([^{3}H]TMP) (specific activity of [^{3}H]TTP used was 12.7 c/mmole) after 120 minutes incubation and the control reaction of MLV(R) incorporated 1756 count/min (specific activity of [^{3}H]TTP used was 16.5 c/mmole) after 120 minutes incubation.

Of the drugs tested, ethidium bromide was the best inhibitor of the DNA polymerases of the murine leukemia viruses. Indeed, ethidium bromide has been reported to inhibit the RNA-dependent RNA polymerase of Qβ phage (*11*). The dye is believed to inhibit the DNA-dependent RNA polymerase of *Escherichia coli* by intercalation in double-stranded helical regions of DNA (*12*). It was suggested (*11*) that the dye may interact with RNA in a similar manner but that it may, on the other hand, exert a direct effect on the RNA-dependent enzyme of Qβ phage. It may be that ethidium bromide can inhibit the RNA-dependent DNA synthetic reaction of the murine leukemia viruses by binding to the RNA template and the DNA-dependent DNA reaction of the murine leukemia viruses by binding to the RNA-DNA hybrid product. The DNA polymerases of the RNA-containing on-

cogenic viruses are stimulated by double-stranded RNA and DNA but not by single-stranded RNA (*13*). Transfer RNA, which has been shown to bind ethidium bromide (*14*), stimulates the reaction (*15*). Perhaps the strong inhibition by ethidium bromide indicates that transcription of the RNA of these viruses must involve double-stranded regions. There is now evidence that ethidium bromide inhibits the growth of tumors in mice and interferes with the replication of MSV(M) in cell cultures (*16*).

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Cultivation of Borrelia hermsi

Abstract. *A medium has been developed which permits the isolation and growth of* Borrelia hermsi, *an organism that causes relapsing fever.*

Although relapsing fever borreliae may be maintained in the laboratory in experimental animals or in embryonated eggs, they have not been cultivated in artificial media (*1*). Recently in this laboratory, studies on the effects of various chemical and physical factors on the survival of *Borrelia hermsi* in vitro led to the development of a growth medium for this organism. *Borrelia hermsi* (*2*) has been maintained in continuous cultivation for 8 months (36 subcultures) and has retained its ability to infect mice.

The growth medium which evolved from the preliminary studies is shown in Table 1. The maximum yield of organisms, 3×10^7 to 5×10^7 per milliliter, was obtained after 7 days of incubation. The calculated generation time for *B. hermsi* in culture was 18 hours.

The contribution of individual components in the medium to the yield of organisms is shown in Table 2. Absence of *N*-acetylglucosamine from the medium reduced growth by 90 percent. Deletion of Proteose Peptone No. 2, Tryptone, or albumin also reduced the yield, but to a lesser extent. Eliminating gelatin from the medium did not influence the yield of organisms when tubes were inoculated with 10^5 organisms; however, growth was not obtained in the absence of gelatin when a smaller inoculum was used (10^3 organisms).

Organisms were tested at the 15th and 26th subculture for infectivity in mice by the intraperitoneal inoculation of .02 ml of medium from 5-day-old cultures. In both instances, maximum spirochetemia was observed in the blood 48 hours after inoculation. Organisms from the 26th subculture were maintained in mice through four passages and then reisolated by culturing blood from the fourth animal.

During incubation, the *p*H of the culture medium dropped from an initial value of 7.8 to 6.8 at 7 days after inoculation. In studies on the carbohydrate metabolism of purified suspensions of *Borrelia duttoni* prepared from infected rat blood, Fulton and Smith (*3*) found that glucose was rapidly metabolized to lactic acid. This was not accompanied by any detectable utilization of oxygen. In culture, *B. hermsi* was found to require high amounts of glucose, and growth was limited by acid production unless a high concentration of phosphate buffer (.05 mole/liter) was included in the medium. These culture requirements suggest a similar metabolic pathway for glucose catabolism in *B. hermsi*; the spirochete, however, would not grow in the total absence of oxygen. Culture tubes filled completely to the top with medium did not sustain growth. Conversely, growth was poor

Table 1. Composition of growth medium for *Borrelia hermsi*. Basal medium and bovine albumin solutions were stored at −20°C. The gelatin solution was autoclaved at 115°C for 15 minutes and stored at 4°C. Sodium bicarbonate solution was freshly prepared at the time of use. Borosilicate screw-cap tubes with Teflon liners (13 by 100 mm) were used as culture vessels. The tubes (9 ml, total capacity) were filled with medium to a final volume of 8.5 ml. Complete medium was prepared from the stock solutions by addition of 4 ml of sodium bicarbonate solution, 34 ml of bovine albumin (fraction V, 10 percent solution adjusted to *p*H 7.8 with NaOH), and 2 ml of distilled water to 80 ml of basal medium. The mixture was sterilized by filtration under pressure through a 0.22-μm membrane filter and dispensed in 6-ml portions per culture tube. The sterile gelatin solution (7 percent) was liquefied by immersion in warm water, and 2 ml was added to each tube. Five-tenths milliliter of sterile rabbit serum was added and the tubes were mixed by inversion. Tubes were inoculated with .05 ml of *B. hermsi* (subculture or a suspension of organisms obtained from infected mouse blood) and incubated at 35°C after the caps were securely tightened.

Compound	Stock solution (g/liter)	Final concentration (mg/ml)
Basal medium		
$Na_2HPO_4 \cdot 7H_2O$	26.52	12.40
$NaH_2PO_4 \cdot H_2O$	1.03	0.48
NaCl	1.20	0.56
KCl	0.85	0.40
$MgCl_2 \cdot 6H_2O$	0.68	0.32
Glucose	12.75	6.00
Proteose Peptone No. 2*	5.95	2.80
Tryptone*	2.55	1.20
Sodium pyruvate	1.06	0.50
Sodium citrate-dihydrate	0.47	0.22
N-Acetylglucosamine	0.53	0.26
$NaHCO_3$ (4.5 percent)		1.06
Bovine albumin		20.00
Gelatin		16.50
Pooled rabbit serum		6 percent

* Difco Laboratories.