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Competition Binding Assay Using *o*-Methyl-[^{3}H]-Demethyl-γ-Amanitin for Study of RNA Polymerase B (40292)

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The understanding of RNA synthesis and processing, and the enzymes and control mechanisms involved is of central importance in biology. The simplest and most reliable criterion for classification of eukaryotic RNA polymerases is their sensitivity to the fungal toxin α-amanitin. α-Amanitin and other naturally occurring amatoxins, as well as their synthetic derivatives, are of particular interest as molecular probes in the study of transcription. They bind very tightly to the polymerase molecule at a site separate from that which binds to the template DNA and product RNA (1, 2). This interaction does not affect the stability of the transcription complex formed between the enzyme and template nor does it interfere with the binding of precursor nucleotide triphosphates (1, 2).

A radioactive derivative of α-amanitin was synthesized by Wieland and Fahrmeir for use in their structural studies of the molecule (3). The method of synthesis as reported used large amounts of the parent compound and employed various destructive analytic techniques to study placement of functional groups in non-radioactive intermediate compounds and in the labeled end product. To permit the synthesis of a radioacive derivative of α-amanitin from a small amount of commercially available starting material their procedure was modified, and several new methods for analyzing the unlabeled intermediate compounds and the end product were introduced. This should enable more biologists to avail themselves of this powerful tool.

To demonstrate radiochemical purity and ensure reactivity of the labeled end product, a competition assay was developed. The assay demonstrates that the labeled derivative, *o*-methyl-[^{3}H]-demethyl-γ-amanitin, binds to the same site as α-amanitin when reacted with either purified or crude preparations of RNA-polymerase B. Since this technique establishes that the labeled and unlabeled compounds are essentially interchangeable, it allows the study of amatoxin binding over wider ranges of ligand concentration than heretofore possible when radioactive material was used alone.

Methods and materials. To synthesize the first intermediate, *o*-methyl-α-amanitin, 5 mg of α-amanitin were dissolved in 4 ml of anhydrous methyl alcohol. This was added to 3 ml of an etheric solution of diazomethane generated from *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) in the outer vessel of an MNNG diazomethane generator. The vessel was stoppered immediately and the reaction mixture was kept at room temperature for 2 hr. The solvents were then evaporated *in vacuo*, the remaining residue was resuspended in a small volume of methanol/H_2O (1:1) and chromatographed on a column of Sephadex LH-20 (1.8 × 100 cm) with methanol/H_2O (1:1) (3). Column effluants were monitored at 310 nm with an Isco UA-5 uv monitor. Fractions with uv absorbing material were collected, solvents evaporated *in vacuo*, and the remaining residues redissolved in 1 mg H_2O. Concentration of the products was determined spectrophotometrically (1, 3, 4).

The second non-radioactive intermediate, *o*-methyl-aldoamanitin, was synthesized via periodate oxidation of *o*-methyl-α-amanitin (3). This was accomplished by the addition of 0.48 mg sodium periodate dissolved in 1 ml water to 2.08 mg of *o*-methyl-α-amanitin dissolved in 1.5 ml H_2O. The mixture was stirred for 5 min at room temperature followed by reduction of the excess periodate by the dropwise addition of 1.1 ml of 0.09 *N* sodium bisulfite. This mixture was chromatographed on a column of Sephadex LH-20 (1.8 × 100 cm) using H_2O as the solvent.

Synthesis of *o*-methyl-[^{3}H]-demethyl-γ-amanitin was accomplished by reduction of

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o-methyl-aldoamanitin with sodium boro-[^{3}H]-hydride (3). One and two tenths mg of sodium boro-[^{3}H]-hydride (209 mCi/mg) was added to 0.94 mg *o*-methyl-aldoamanitin dissolved in 1.5 ml of H_2O. The reaction mixture was continuously stirred at 0° for 90 min at which time the mixture was acidified by the addition of 1 ml of 0.1 *N* HCl. After an additional 15 minutes the mixture was neutralized with 0.1 *N* NaOH. The reaction mixture was chromatographed on a Sephadex LH-20 column using methanol/H_2O (1:1) as described above. Fractions found to have both a proper uv-spectrum (4) and containing radioactivity were rechromatographed on a column of Sephadex G-50 (0.9 × 15 cm) using H_2O as the solvent. Peak fractions were collected, uv-absorbance and counting rates were determined and the specific activity of *o*-methyl-[^{3}H]-demethyl-γ-amanitin was calculated. The specific activity was verified by saturation of the labeled derivative with purified wheat germ RNA polymerase in a binding assay described below.

Thin layer chromatography of α-amanitin and derivatives. In order to identify various reaction products and assess their purity, aliquots of peak fractions were concentrated and then studied by thin layer chromatography on Silica Gel-OF plates. Two solvent systems were employed, selected for their ability to separate the intermediate compounds. Chromatograms were visualized by staining with Erlich's solution or transcinnamaldehyde/HCl (3, 5). α-Amanitin was used as a reference standard against which the migration of the intermediates was compared. Radioactive products were located by scraping 0.5 cm squares from moist plates following chromatography. The resultant material was then digested overnight in Nuclear Chicago Solubilizer (NCS) at 45° and counted in nonaqueous, toluene based scintillant.

Infra-red spectrophotometry of α-amanitin and non-radioactive derivatives. As an additional proof of the proper placement of functional groups in the amanitin molecule infrared spectra were obtained for α-amanitin, *o*-methyl-α-amanitin and *o*-methyl-aldoamanitin. Small amounts of each compound (ca. 0.1 mg) were dissolved in H_2O and lyophilized. KBr pellets were prepared for each sample using a Wilk's mini press. The pellets were scanned from 4000 to 600 cm^{-1} on a Beckman Acculab 4 Infra-red Spectrophotometer. The reference beam was attenuated to permit adjustment of the baseline. Chromatographically pure α-amanitin was used as a reference compound.

Amatoxin competition binding assay. The method used to demonstrate the binding of [^{3}H]γamanitin was based on the procedure of Cochet-Meilhac *et al.* (1, 2). Purified wheat germ RNA polymerase or the enzyme present in crude homogenates of baby mouse kidneys was used as a substrate for binding the radioactive ligand. Crude homogenates were prepared by grinding whole kidneys of baby mice (8-10d) in a Potter-Elvehjem tissue grinder in homogenizing buffer (50 m*M* Tris HCl pH 7.4; 0.1 m*M* EDTA; 0.1 m*M* dithiothreitol and glycerol 30% v/v). Aliquots of 100 μl of the crude homogenates or 100 μl of the purified enzyme in binding buffer (1.63×10^{-8} *M*) were incubated in an assay mixture containing 500 μl binding buffer (80 m*M* Tris HCl pH 7.9; 0.1 m*M* EDTA; 0.1 m*M* dithiothreitol; 150 m*M* $(NH_4)_2SO_4$; 0.2 mg/ml bovine serum albumin; 0.4 mg/ml rabbit gamma globulin and 30% (v/v) glycerol), 10 μl [^{3}H]-γ-amanitin (9.13×10^{-6} μmoles ca. 2.0 Ci/mmole) and 10 μl of unlabeled α-amanitin in varying concentrations. Controls for [^{3}H]-γ-amanitin binding contained 10 μl binding buffer in place of α-amanitin. Samples were incubated at 4° for 18 hr. After 18 hr 1 vol of $(NH_4)_2SO_4$ solution, saturated at 4°, was added to the reaction mixture and samples were kept at 4° for an additional hour. Free and unbound amanitin were then separated by centrifugation at 39,000*g* for 20 min. The supernatant was discarded and the pellet was redissolved in 1 ml of binding buffer; an equal volume of saturated $(NH_4)_2SO_4$ was again added and the samples were incubated at 4° for 30 min, at which time they were recentrifuged as described above. This suspension-reprecipitation step was repeated two additional times. Finally the pellet was dissolved in 200 μl of H_2O, digested overnight in NCS at 45° and counted in non-aqueous toluene based scintillant. Counting efficiency was approximately 85% of that obtained for unquenched samples. Values obtained with the highest

concentration of α-amanitin were found to correspond to the background samples containing no RNA polymerase when the purified enzyme was used. When the assay was done using the crude homogenate as a source of RNA polymerase the values obtained at the highest concentrations of α-amanitin were assumed to represent nonspecific binding of the labeled derivative. This value did not exceed 6% of the total label bound and was used to correct experimental values obtained with crude homogenates.

[^{3}H] Amanitin saturation assay. To verify the specific activity of the [^{3}H]-γ-amanitin as determined by the ratio of radioactivity/absorbance at 310 nm an experiment was done to ascertain the amount of purified wheat germ RNA polymerase required to saturate a fixed amount of the radioactive ligand. Each sample contained [^{3}H]-γ-amanitin (1.47×10^{-8} *M*) and variable concentrations of RNA polymerase from 2.63×10^{-9} *M* to 5.26×10^{-8} *M*. Concentration of [^{3}H]-γ-amanitin at saturation was based on the 50% end point. Conditions for the assay are identical to those described above for [^{3}H]-γ-amanitin binding controls.

Materials. The materials used for these experiments were obtained from the following suppliers: *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, *p*-dimethyl-aminobenzaldehyde and MNNG diazomethane generator, Aldrich Chemical Co. Milwaukee, WI; α-Amanitin, Boehringer-Mannheim Biochemicals Indianapolis, IN; Nuclear Chicago Solubilizer (NCS) and sodium boro-[^{3}H]-hydride, Amersham-Searle Co., Arlington Heights, IL; transcinnamaldehyde, Eastman Organic Chemical Rochester, NY; wheat germ RNA polymerase, Miles Laboratories, Elkhart, IN; Sephadex LH-20 and G-50, Pharmacia Fine Chemicals, Piscataway, NJ; Silica Gel OF TLC plates, New England Nuclear, Boston, MA; rabbit IgG and ultra pure ammonium sulfate Schwartz Mann, Orangeburg, NY; sodium periodate and sodium metabisulfate, Sigma Chemical Co., St. Louis, MO.

Results. Synthesis of a radioactive derivative of α-amanitin. Chromatography of the methylation product of α-amanitin on Sephadex LH-20 resulted in two peaks absorbing at 310 nm (Fig. 1). Thin layer chromatography of the material in fraction 13 in butanol/acetone/H_2O (30/3/5) yielded a band co-migrating with the α-amanitin marker (R_f = 0.34). With methanol/H_2O (4:1) as the solvent an effective separation resulted with α-amanitin migrating further than the methylated derivative (R_f = 0.86 vs. 0.82).

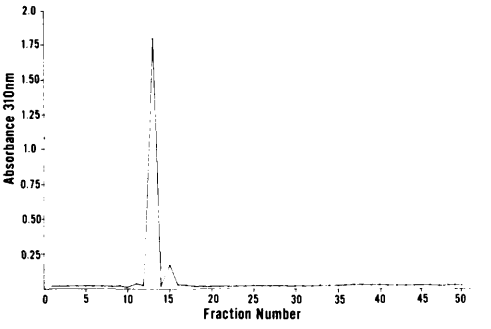

FIG. 1. Products of methylation of α-amanitin were chromatographed on a column of Sephadex LH-20 (1.8 × 100 cm) using methanol/H_2O 1:1. The column effluent was monitored at 310 nm. Fractions of approximately 7.5 ml were collected at 40 min intervals.

Infra-red spectrophotometry substantiated the differences in the methylation product and the parent compound. The slower migrating methyl derivative (an aryl ether of the phenolic hydroxyl group of the tryptophan moiety) was expected to exhibit differences in the regions of 1300–1180 cm^{-1} and 1125–1000 cm^{-1}. The comparison spectra of α-amanitin and *o*-methyl-α-amanitin (Fig. 2a and b) demonstrate changes between 1270–1230 cm^{-1} and 1125-1025 cm^{-1}.

The results of Sephadex LH-20 chromatography of the periodate oxidation product of *o*-methyl-α-amanitin are presented in Fig. 3. Only the major peak was found to have a typical uv-spectrum for an amatoxin (4). Thin layer chromatography using butanol/acetone/H_2O and stained with cinnamaldehyde/HCl showed that the reaction product migrates slower than the α-amanitin marker (R_f = 0.30) and stains reddish brown rather than violet. Infra-red spectrophotometry showed changes at 2800 cm^{-1} and 1350 cm^{-1} consistant with the introduction of an aliphatic aldehyde group into the amatoxin molecule (Fig. 2c).

Following reduction of *o*-methyl-aldo-amanitin with sodium boro-[^{3}H]-hydride the reaction products were separated on Sephadex LH-20. Three peaks absorbing at 310 nm were eluted (Fig. 4). The material in the leading peak did not possess a characteristic

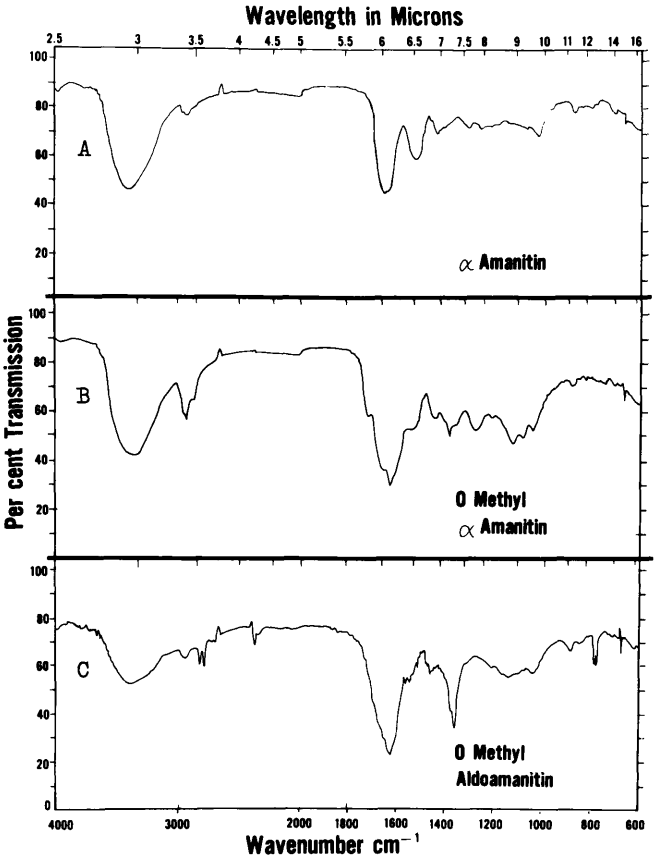

FIG. 2. A comparison of the infra-red spectra of α-amanitin (2a)*o*-methyl-α-amanitin (2b) and *o*-methyl-aldo-amanitin (2c). Samples were prepared as KBr pellets and scanned at slow speed using the normal slit program of a Beckman Acculab 4 Infra-red Spectrophotometer. The reference beam was attenuated to obtain a suitable base line.

uv-spectrum of an amatoxin and while the material in the center peak did resemble an amatoxin by uv spectrophotometry the relative amount of incorporated radioactivity was very low. Only the material in the trailing peak had both an amatoxin uv spectrum and a significant amount of incorporated label. The major peak of radioactivity was unassociated with any amatoxin containing fraction and was assumed to be unreacted. Fractions 39 and 40 were pooled, concentrated *in vacuo* and re-chromatographed on a column of Sephadex G-50 to ensure complete removal of any unreacted radioactivity. The column profile is presented in Fig. 5. The major portion of radioactivity coincided with the peak ov uv-absorbance in fraction 11. Very little contaminating radioactivity was present.

Purity of the material eluted from Sephadex G-50 was verified by thin layer chromatography in butanol/acetone/H_2O. The migration of the radioactive derivative was compared to the marker, α-amanitin, which was detected by staining with Erlich's reagent. The results of the thin layer chromatography are presented in Fig. 6. The radioactive derivative migrates as a single band ($R_f = 0.40$) ahead of the marker ($R_f = 0.34$). Neither infra-red spectrophotometry nor destructive analytic techniques were employed to verify the chemical structure of the end product because of the small amount recovered. The end product is assumed to be *o*-methyl-[^{3}H]-demethyl-γ-amanitin since only the aldehyde formed in the previous step would be available for borohydride reduction.

Saturation of wheat germ RNA polymerase with [^{3}H]-γ-amanitin. The specific activity of [^{3}H]-γ-amanitin was determined by two independent methods. Based on the uv absorption and counting rates of several small samples, the material in fraction 11 (Fig. 5) was

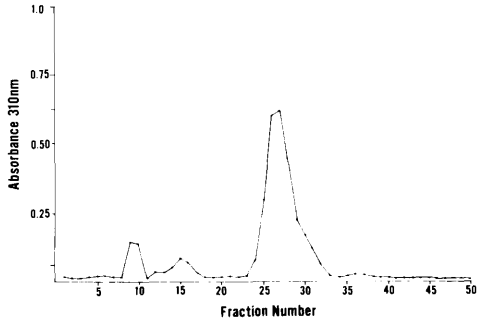

FIG. 3. Products of sodium periodate oxidation of *o*-methyl-aldoamanitin were chromatographed on a column of Sephadex LH-20 (1.8 × 100 cm) using H_2O as the solvent. Fractions contained approximately 6.25 ml. Column monitoring and fraction collection were accomplished as described in Fig. 1.

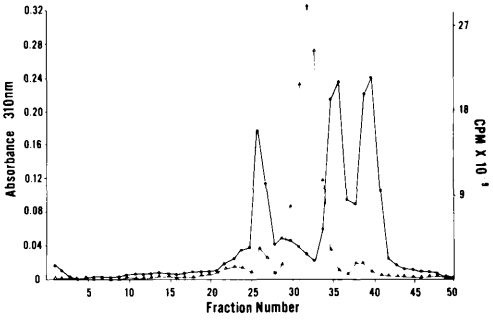

FIG. 4. The reaction products of sodium boro-[^{3}H]-hydride reduction of *o*-methyl-aldoamanitin were chromatographed on a column of Sephadex LH-20 (1.8 × 100 cm) using methanol/H_2O (1:1) as the solvent. Flow rate, column monitoring and fraction size are described in Figure 1. 50 μl aliquots of each fraction were used to approximate the total radioactivity. (●——●) absorbance 310 nm; (△——△) [^{3}H] cpm.

estimated to contain 7.25×10^{-3} μmoles/ml *o*-methyl-[^{3}H]-demethyl-γ-amanitin with a specific activity of 2.50 Ci/mmole. Fraction 12 was found to contain 7.23×10^{-3} μmoles/ml, and have a specific activity of 2.16 Ci/mmole. Specific activity estimates based on the saturation of [^{3}H]-γ-amanitin with wheat germ RNA polymerase agreed well with those obtained by instrumental methods. The fifty percent maximum binding of RNA polymerase was found to occur at 7.35×10^{-9} *M*. Assuming that the reaction was at equilibrium, had a very small K_D (approximately 10^{-11} *M*, see ref. 1, 2) and that the purified enzyme contained a single binding site, the concentration of [^{3}H]-γ-amanitin was calculated to be 1.47×10^{-5} *M* and the specific activity 1.88 Ci/mmole. Saturation data are presented in Fig. 7.

Amatoxin competition assay. A competition assay was designed to test the hypothesis that [^{3}H]-γ-amanitin bound to the same site as α-amanitin and with approximately the same affinity. The concentration of the radioactive derivative was constant at 1.47×10^{-8} *M* (approximately 12,000 cpm) and the concentration of the competing, unlabeled α-amanitin was varied from 8.27×10^{-11} to 2.62×10^{-5} *M*. The assays were carried out at four to five times the concentration of [^{3}H]-γ-amanitin required to saturate the amount of RNA polymerase present. Controls for non-

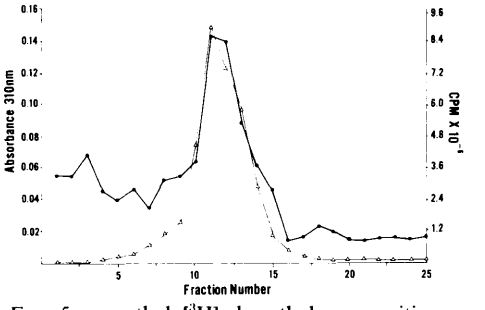

FIG. 5. *o*-methyl-[^{3}H]-demethyl-γ-amanitin contained in fraction 39 (Fig. 4) was chromatographed on a column of Sephadex G-50 (0.9 × 15 cm) using H_2O as the solvent. Each fraction contains 0.75 ml; flow rate 0.38 ml/min. Five microliter aliquots of each fraction were assayed for radioactivity (●——●) absorbance 310 nm; (△——△) [^{3}H] cpm.

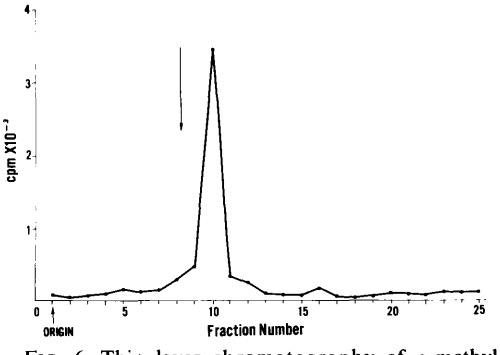

FIG. 6. Thin layer chromatography of *o*-methyl-[^{3}H]-demethyl-γ-amanitin. A 5 μl aliquot of the peak fraction eluted from Sephadex G-50 was chromatographed in butanol/acetone/H_2O (30:3:5). Migration of α-amanitin indicated by arrow. Each point represents a migration of 0.5 cm.

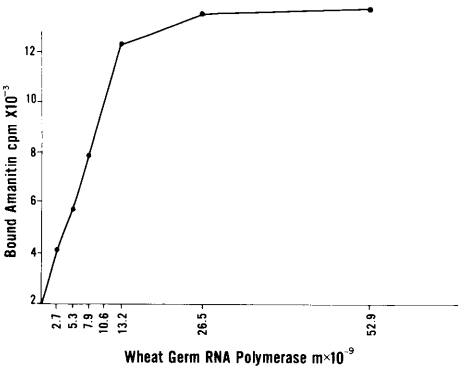

FIG. 7. Saturation of wheat germ RNA polymerase with *o*-methyl-[^{3}H]-demethyl-γ-amanitin. *o*-methyl-[^{3}H]-demethyl-γ-amanitin (1.47×10^{-8} *M*) was incubated for 18 hr in the presence of increasing concentrations of wheat germ RNA polymerase (2.63×10^{-9} *M* – 5.26×10^{-8} *M*). Assay mixture was the same as described for the competition assay except unlabeled α-amanitin was omitted.

specific binding did not contain the enzyme and bound less than 0.8% of the total input. The results of competition assays for both purified wheat germ RNA polymerase and the enzyme present in crude homogenates are presented in Fig. 8. The percent bound [^{3}H]-γ-amanitin was determined by calculating the proportion of counts bound for each concentration of α-amanitin to the counts bound in samples containing no unlabeled competing α-amanitin. The ideal curve is based on the decrease in specific activity of the total amatoxin concentration at saturation, assuming that a single binding species is present and that both compounds compete equally for the binding site. The experimental results for both enzyme preparations closely approximate the ideal curve.

Discussion. Previous studies have demonstrated *o*-methyl-[^{3}H]-demethyl-γ-amanitin to be a powerful tool in studying the eukaryotic nucleoplasmic RNA polymerase (1, 2, 6, 7). However, the unavailability of this compound or of large enough amounts of α-amanitin to synthesize this derivative by the previously reported method has restricted the widespread application of this technique.

The procedures presented in this paper enables the synthesis of small amounts of [^{3}H]-γ-amanitin from readily available quantities of starting materials by the introduction of new nondestructive analytic techniques to ensure proper placement of functional groups in nonradioactive intermediates. Use of shorter columns of Sephadex LH-20 did not affect the desired resolution and the introduction of a short column of Sephadex G-50 ensures complete removal of any unreacted radioactivity in the end product. This is verified by thin layer chromatography of the radioactive product and further substantiated by the saturation curve of [^{3}H]-γ-amanitin with purified wheat germ RNA polymerase B in which less than 3% of the total input remained unbound. The competition assay conclusively demonstrates that the final product binds to RNA polymerase in essentially the same manner as the unreacted parent compound and at the same site.

Although the competition assay was designed as a test for reactivity and radiochemical purity of the final product, the usefulness of this assay exceeds this purpose. Currently, studies of amanitin resistant RNA polymerase B have relied on the inhibition of enzyme activity by various concentrations of amatoxin to characterize wild type or mutant enzymes (6, 7). This technique cannot be applied to crude cell homogenates as the normally resistant RNA polymerases A and C as well as RNase would complicate the kinetic analysis.

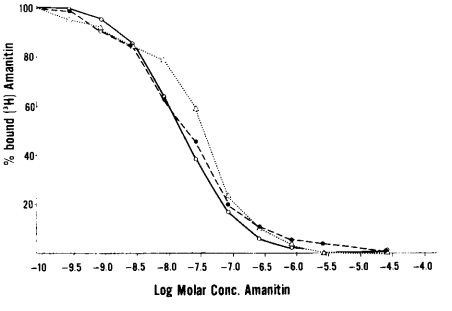

FIG. 8. Competition binding assay. The ideal curve (○——○) represents the percent of the total amatoxin present as *o*-methyl-[^{3}H]-demethyl-γ-amanitin. The assay was carried out in the presence of a constant amount of *o*-methyl-[^{3}H]-demethyl-γ-amanitin (1.47×10^{-8} *M*) previously determined to be in excess required to saturate either the purified wheat germ RNA polymerase (2.63×10^{-9} *M*) or the enzyme present in 0.0189 g of mouse kidney homogenate. Percent saturation was calculated from the total radioactivity bound in samples containing no unlabeled α-amanitin; wheat germ RNA polymerase (△···△); crude mouse kidney homogenate (●– – –●).

The amanitin competition assay could provide a new method for studying the interaction of amanitin with resistant RNA polymerase B enzymes. The assay is essentially free from interference by other RNA polymerases and is unaffected by RNase, therefore, crude homogenates as well as purified enzyme preparations can be studied. In addition, the assay provides a means of direct measurement of dose-response over a wide range of concentration and could provide additional insight into possible mechanisms of amanitin resistance.

Summary. An improved method permitting the synthesis of a radioactive derivative of α-amanitin from a small amount of the commercially available parent compound has been developed. The labeled derivative was used in an amatoxin competition binding assay designed to detect eukaryotic RNA polymerase B in either purified form or in crude homogenates. Both compounds are shown to compete for the same binding site and with approximately the same affinity. The competition assay proves to be both sensitive and highly selective for RNA polymerase B and provides a new, direct method for studying the enzyme–amanitin interaction over a much broader range of concentration than previously reported.

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