Biochemistry of the Amatoxins: Preparation and Characterization of a Stably Iodinated α-Amanitin†

Paul W. Morris,* D. L. Venton, and K. M. Kelley

ABSTRACT: Iodination of α-amanitin at the 7-position in the 6-hydroxy-2-sulfoxytryptophan moiety is effected with 1 equiv of iodine monochloride in methanol. The isolated product shows a λ_{max} in methanol at 301 nm, compared with 305 nm for the parent α-amanitin; in methanolic 0.01 M NaOH the λ_{max} are 330 and 332 nm for the product and parent, respectively. Spectrophotometric titration of the phenolic hydroxyl shows a decrease in pK_a from 9.72 (α-amanitin) to 7.94 (7-iodo-α-amanitin). Appropriate spectrophotometric examination therefore distinguishes between parent and product. Proton magnetic resonance shows two aromatic protons (ν_{4H} = 7.57; ν_{5H} = 6.90 ppm; $J_{4,5}$ = 9) in the 7-iodo-α-amanitin and three aromatic protons (ν_{4H} = 7.64; ν_{5H} = 6.78; ν_{7H} = 6.94 ppm; $J_{4,5}$ = 9; $J_{5,7}$ = 2) in α-amanitin thus establishing the extent and position of iodine substitution. The 7-iodo-α-amanitin effectively inhibits RNA polymerase activity with half-maximal inhibition at 2×10^{-9} M and 10^{-4} M for the sea urchin RNA polymerases II and III, respectively. Addition of [^{125}I]-7-iodo-α-amanitin (200 Ci/mmol) to crude extracts from sea urchin blastula, MOPC 315 plasmacytoma, and adult Oregon R *Drosophila melanogaster* followed by resolution on DEAE-Sephadex demonstrates that the radioactive ligand binds stably and specifically with the RNA polymerase II in each of these extracts.

The amatoxins constitute one of three cyclopeptide classes occurring in certain mushrooms of the genus *Amanita* (Wieland & Wieland, 1972) and are biochemically interesting by virtue of the potent inhibition exerted upon eukaryotic RNA polymerase II (Lindell et al., 1970; Meilhac et al., 1970; Jacob et al., 1970). The eukaryotic RNA polymerase III also is inhibited by the amatoxins but requires inhibitor concentrations several orders of magnitude greater than required for RNA polymerase II (Weinmann & Roeder, 1974; Weil & Blatti, 1975). This differential inhibition of eukaryotic RNA polymerase classes has served to elucidate their transcriptive functions (reviewed in Chambon, 1975). Numerous physiological systems respond to stimuli with alteration of RNA synthesis; again the differential inhibition by the amatoxins allows one to elucidate, for example, enhanced RNA polymerase II activity with estrogen (Weil et al., 1976) and viral infection (Schwartz et al., 1974a) and enhanced RNA polymerase III activity with glucocorticoids (Fuhrman & Gill, 1976) and viral infection (Schwartz et al., 1974a).

The types of studies enumerated above utilize one facet of the amatoxin–RNA polymerase interaction: the inhibition of enzymatic activity. A second facet of the interaction is the tight binding of the amatoxin to the RNA polymerase. From the potent inhibition of RNA polymerase II one expects, and studies by Cochet-Meilhac & Chambon (1974) have shown, that the K_D for the amatoxin–RNA polymerase II system is a small value and the half-life of the binary complex is long. Wieland and colleagues (Meilhac et al., 1970; Wieland & Fahrmeir, 1970) have developed reactions leading to ^{3}H- and ^{14}C-labeled amatoxins that have been used to characterize the binding process between RNA polymerase II and the amatoxins (Meilhac et al., 1970; Cochet-Meilhac & Chambon, 1974). The [^{3}H]-*O*-methyldemethyl-γ-amanitin has been used to demonstrate that CHO and myoblast cell variants selected for amanitin resistance in fact contain a mutant RNA polymerase II (Somers et al., 1975a; Ingles et al., 1976). Subsequent studies with the amatoxin ligand and amatoxin resistant cell lines lead to the postulate that RNA polymerase II controls its own synthesis (Guialis et al., 1977; Somers et al., 1975b). Identification of a polypeptide at the amatoxin binding site in calf thymus RNA polymerase II is provided by carbodiimide condensation of the carboxylate moiety in [^{3}H]amanin with RNA polymerase (Brodner & Wieland, 1976).

Clearly, the ligand binding technique with a suitably derivatized amatoxin offers considerable insight into the function of the RNA polymerase II. Furthermore, RNA polymerases I and III from select species (Valenzuela et al., 1976; Schultz & Hall, 1976) are observed to be inhibited by α-amanitin; thus all three major RNA polymerase classes from eukaryotes may be amenable to ligand binding analysis with suitable amatoxin derivatives. In an effort to more fully utilize the interaction between RNA polymerases and the amatoxins, we have examined several derivative classes of α-amanitin with the goal of preparing highly radioactive compounds that retain their avid binding to RNA polymerase II. We report here the preparation and characterization of a specific iodination product of α-amanitin. A preliminary report of this material has appeared (Morris et al., 1977).

Experimental Section

Materials. The following were used throughout this study: Polyamide SC66 (Machery, Nagel), Ching-Cheng polyamide thin-layer chromatography sheets (Pierce Chemical Co.), α-amanitin (Henley and Co., Inc.), methanol (Burdick and Jackson, glass distilled grade), nucleoside triphosphates, calf thymus DNA, and dithiothreitol (P-L Biochemicals), DEAE-Sephadex A-25 (Pharmacia), [^{3}H]uridine triphosphate and ^{125}I (Amersham/Searle), ammonium sulfate (Schwartz Mann, enzyme grade), 100% D_2O and 99.6% dimethyl-d_6 sulfoxide (Aldrich). All other chemicals were analytical reagent grade unless otherwise specified. Iodine monochloride is prepared via the described procedure (Blatt, 1943); the initial distillate (bp 98–103 °C) is purged with extra dry N_2 and then redistilled under N_2 in a carefully dried still to preclude exposure to atmospheric moisture.

† From the Departments of Biological Chemistry (P.W.M. and K.M.K.) and Medicinal Chemistry (D.L.V.), University of Illinois Medical Center, Chicago, Illinois 60612. *Received July 5, 1977.* This work was supported in part by a grant from the National Institutes of Health (GM 22147).

0006-2960/78/0417-0690$01.00/0

Iodination of α-Amanitin. To 1.45 μmol of α-amanitin in absolute methanol (480 μL) is added 1.8 μmol of ICl freshly dissolved in methanol (50 μL). Immediately upon addition of the faintly yellow ICl solution, the reaction develops a more intense yellow which persists. After 17 h at 21–23 °C in the dark, the methanol is evaporated under N_2, the residue dissolved in 1-butanol:H_2O (99:1 v/v), applied to a Polyamide SC66 column (0.4 cm × 70 cm), and eluted with 1-butanol:H_2O (99:1 v/v). Fractions (0.57 mL each) containing the iodination product are pooled (see Figure 1) and taken to dryness under vacuum. The dry residue is transferred with methanol into a tared microweighing boat and vacuum dried (100 μmHg) for 24 h at 21–23 °C over P_2O_5. Recovery is 0.785 mg or 0.753 μmol (52%) based upon the assigned structure, 7-iodo-α-amanitin (see Results). In the more usual reaction using an input of 5–25 nmol of α-amanitin, both product and starting material are recovered; in such cases the summed recovery is ≤60% of the original starting material and the 7-iodo-α-amanitin:α-amanitin ratio is approximately 60:40. For the 1.45 μmol reaction described, the Polyamide SC66 column had been continuously eluted with solvent for 1 month prior to application of the sample. This precaution of extensive elution reduces the nylon contaminant in the product to <5% of the 0.785 mg recovered.

In order to introduce ^{125}I into the ICl reactant, two procedures are used. For specific activities of 100 Ci/mmol, or less, the desired aliquot (5–10 μL) of ^{125}I (catalog no. IMS30 of Amersham/Searle) is evaporated under N_2, redissolved in 50 μL of methanol, and then added to a fresh methanolic ICl solution 5 min prior to addition of the α-amanitin. The amount of ICl required is calculated from the input ^{125}I and the desired specific activity. The ICl:α-amanitin molar ratio is 1.2:1 and the total volume ≤65 μL. For specific activities > 200 Ci/mmol, 5 mCi of $^{125}I^-$ (50 μL) is mixed with 5 μL each of 12 N H_2SO_4 and 0.01 M $K_3Fe(CN)_6$. The resultant $^{125}I_2$ is extracted with three successive 100-μL aliquots of *n*-hexane (Burdick and Jackson, glass distilled). To the $^{125}I_2$ in *n*-hexane is added a methanolic ICl solution in the amount required to yield the desired specific activity. After 5 min of vigorous agitation to promote equilibrium between the *n*-hexane and methanol phases, the α-amanitin (1.2:1 ICl:α-amanitin) is added in methanol. The total methanol ≤ 25 μL. With either the low or high specific iodination reaction, admixture of the very weakly yellow ICl solution with the colorless α-amanitin solution results in the rapid appearance of a strong yellow color. After 1 h of reaction, the *n*-hexane phase is removed and backwashed with 10 μL of methanol, and the two methanol aliquots are combined, diluted with 250 μL of butanol:H_2O (99:1 v/v), and applied to the Polyamide SC66 column. The summed recovery of product plus starting material in either the low or high specific activity reaction is considerably lower than for the nonradioactive reaction and product yield typically is 5–10%, although yields as low as 1% have been obtained. The low yield is due to the reaction conditions and not to loss of product on the chromatography column.

Characterization of the Iodination Product. All electronic spectra were recorded on the Cary 15 with methanol solvent. A known aliquot of the 0.785 mg product was used for recorded spectra before and following the addition of 5 N NaOH to a final concentration of 0.01 N. Spectrophotometric titration of the phenolic hydroxyl in α-amanitin and 7-iodo-α-amanitin was done in 0.01 M Tris-HCl (pH 7.4–8.8), 0.01 M borate–0.01 M KCl (pH 8.4–10.1), or 0.01 M carbonate (pH 9.2–10.3). All buffer pH values were standardized (Bates, 1964) with a glass electrode calibrated against standard borax (pH 9.18 at 20 °C) and a pH 10.00 standard buffer (Beckman Instruments). The following spectral constants were used in calculation of the mole fraction ionization: α-amanitin, λ_{max} = 303 nm, $\epsilon_{303}^{neut} = 12.5 \times 10^3\ M^{-1}\ cm^{-1}$, $\epsilon_{331}^{neut} = 0.206 \times 10^3$; λ_{max}^{alk} = 331 nm, $\epsilon_{331}^{alk} = 13.7 \times 10^3$, $\epsilon_{303}^{alk} = 5.29 \times 10^3$; 7-iodo-α-amanitin, λ_{max}^{neut} = 301 nm, $\epsilon_{301}^{neut} = 19.4 \times 10^3$, $\epsilon_{330}^{neut} = 0.800 \times 10^3$; λ_{max}^{alk} = 330 nm, $\epsilon_{330}^{alk} = 20.0 \times 10^3$, $\epsilon_{301}^{alk} = 8.45 \times 10^3$. The superscript, alk, designates spectral data recorded in 0.01 N NaOH while the superscript, neut, refers to distilled, deionized H_2O with pH ~5.8. Only those buffers yielding between 0.2 and 0.8 mol fraction ionization were used in calculation of the pK_A. Individual buffer solutions were separated by 0.1 pH unit.

Proton magnetic resonance spectra at 60 MHz were recorded on a Varian T60A equipped with Nicolet Fourier Transform accessories. All samples were exchanged with 100% D_2O prior to recording the spectra in 100% D_2O with 1,4-dioxane as the internal reference. Analysis of aromatic proton coupling in α-amanitin was extended by Fourier transform proton magnetic resonance at 220 MHz (Varian T220) in 99.6% dimethyl-d_6 sulfoxide.

RNA Polymerase Assays. The DNA-dependent RNA polymerase assays were performed as previously described with the single exception that the enzyme reaction was allowed to proceed for 20 instead of 10 min (Morris & Rutter, 1976). The urchin RNA polymerases were partially purified from blastulae: 1.4 g of blastula plus 1.75 mL of TMED[1] (140 mM Tris-HCl (pH 7.9 at 4 °C), 14 mM $MgCl_2$, 0.28 mM EDTA, 1.4 mM dithiothreitol) were homogenized at half-speed on the Tekmar homogenizer for 30 s, then 1.75 mL of GA (66% glycerol, 0.98 M $(NH_4)_2SO_4$) was added, and the sample homogenized at full speed for 15 s. The homogenate was loaded onto a 90-mL volume DEAE-Sephadex column equilibrated at 0.10 M $(NH_4)_2SO_4$ in TGMED (50 mM Tris-HCl (pH 7.9 at 4 °C), 5 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol, 25% v/v glycerol) and eluted by upward flow with 0.45 M $(NH_4)_2SO_4$ in TGMED. This procedure is the ion filtration technique as described by Kirkegaard et al. (1972). The effluent (10 mL) containing RNA polymerase activity was concentrated to 3.4 mL by application at 0.35 M $(NH_4)_2SO_4$ to a 30-mL DEAE-Sephadex column equilibrated at 0.1 M $(NH_4)_2SO_4$ in TGMED and eluted with 0.45 M $(NH_4)_2SO_4$ in TGMED (although not previously described, this concentration method is a direct consequence of the ion filtration and gradient sievorptive elution phenomena; for full discussion of these phenomena, see Kirkegaard et al., 1972, and Kirkegaard, 1973). The effluent (3.4 mL) from the concentration column was resolved into RNA polymerases I, II, and III by gradient sievorptive elution on a 30-mL DEAE-Sephadex column as previously described (Morris et al., 1976; Morris & Rutter, 1976). Cross-contamination among the three enzymes was less than 1% (assessed by α-amanitin inhibition) except that 2–5% of the activity in the RNA polymerase II was actually due to RNA polymerase I.

Binding of [^{125}I]-7-Iodo-α-amanitin to Cellular Extracts. RNA polymerase was solubilized from both sea urchin embryos and MOPC 315 plasmacytomas as follows: 1.0 mL, packed volume, of urchin embryos or 1.0 g, wet weight, of MOPC 315 plasmacytoma plus 1.25 mL of TMED was homogenized at half-speed on the Tekmar homogenizer for 30 s until the tissue was disintegrated and the cells mostly dis-

[1] Abbreviations used: cpm, counts per minute; ^1H NMR, proton magnetic resonance; K_D, equilibrium dissociation constant; TMED, 140 mM Tris-HCl (pH 7.9 at 4 °C), 14 mM $MgCl_2$, 0.28 mM EDTA, 1.4 mM dithiothreitol; GA, 66% glycerol, 0.98 M $(NH_4)_2SO_4$; TGMED, 50 mM Tris-HCl (pH 7.9 at 4 °C), 5 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol, 25% v/v glycerol.

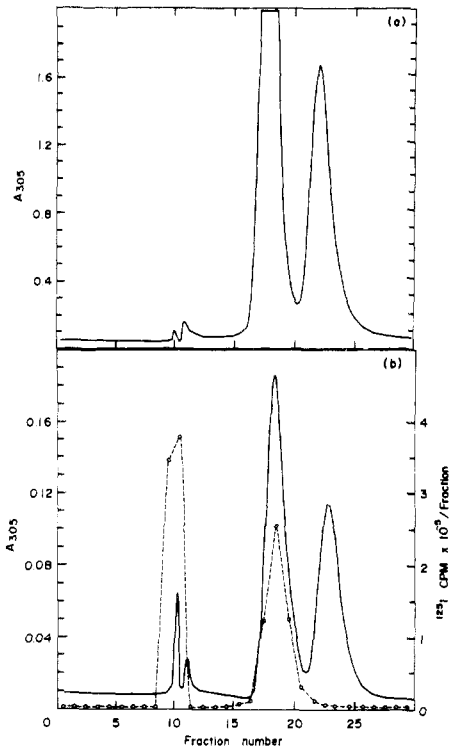


FIGURE 1: Resolution of α-amanitin and 7-iodo-α-amanitin by column chromatography (70 × 0.4 cm) on Polyamide SC66 eluted with 1-butanol:H_2O (99:1, v/v); 0.57-mL fractions are collected. α-Amanitin elutes in fractions 22–25 and 7-iodo-α-amanitin in fractions 17–19. (a) Results from iodination of 1 mg (900 nmol) of α-amanitin with ^{127}ICl. (b) Results from iodination of 36 nmol of α-amanitin with ^{125}ICl (0.3 Ci/mmol). A_{305} (—); ^{125}I cpm × 10^{-5}/fraction (-O-O-).

rupted. Then 1.25 mL of GA was added and the sample homogenized for 15 s at full speed. The MOPC 315 plasmacytoma homogenate was centrifuged for 10 min at 48 000*g* in the Sorvall SS-34 rotor; the urchin homogenate was not centrifuged. For extraction of RNA polymerase from adult Oregon R *Drosophila melanogaster*, 1 g of flies plus 2.0 mL of TMED was homogenized for 30 s at half-speed on the Tekmar homogenizer, then 2.0 mL of 1 M $(NH_4)_2SO_4$ was added, and the sample homogenized again for 20 s at full speed. The homogenate was centrifuged for 10 min at 48 000*g* in the Sorvall SS-34 rotor, the supernatant decanted through a 74-μm mesh nylon screen, and then glycerol added at the proportion of 100 μL per 300 μL of supernatant. It should be noted that these procedures, after allowance for the water in the tissue, yield a homogenate in a buffer equivalent to 0.35 M $(NH_4)_2SO_4$ in TGMED (Roeder & Rutter, 1969).

One milliliter of each extract was developed over DEAE-Sephadex (1.1 cm diameter; 38 mL bed volume) by the previously described gradient sievorptive elution technique (Morris & Rutter, 1976). For ligand binding, 10 μL of [^{125}I]-7-iodo-α-amanitin in methanol was added to the 1-mL aliquot of each extract (at 0 °C) 15 min prior to application to DEAE-Sephadex. Since the final concentration of ^{125}I-labeled ligand was 10^{-9} M and the binding reaction did not go to completion (Cochet-Meilhac & Chambon, 1974), only a fraction (<5%) of the RNA polymerase II molecules bound with the ligand. This protocol allowed us to ascertain the ligand binding in the presence of residual RNA polymerase II activity.

Results

Preparation and Characterization of 7-Iodo-α-amanitin. Reaction of the parent α-amanitin with 1 equiv of ICl in

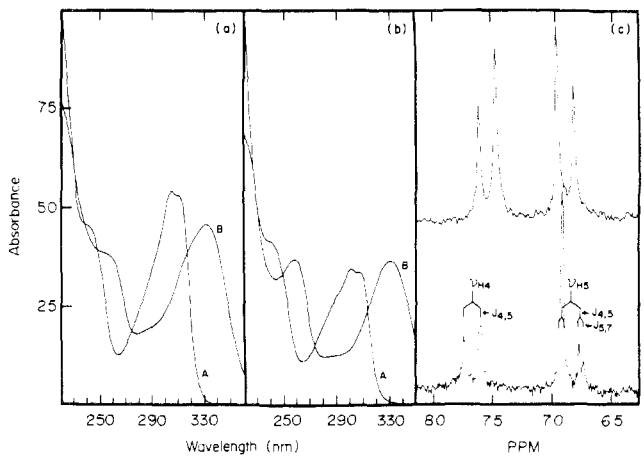


FIGURE 2: Spectral analysis of α-amanitin and 7-iodo-α-amanitin. Electronic spectra of α-amanitin (a) and 7-iodo-α-amanitin (b) recorded on the Cary 15 double-beam spectrophotometer. The solvents are methanol (spectrum A) and 0.01 M NaOH in methanol (spectrum B). Proton magnetic resonance spectra (60 MHz, Varian T60A with Nicolet pulse Fourier transform) recorded in 100% D_2O; chemical shifts are in ppm referenced to the internal standard, 1,4-dioxane, at 3.70 ppm. Lower spectrum: 1.0 mg of α-amanitin (1440 acquisitions). Upper spectrum: 750 μg of 7-iodo-α-amanitin (3120 acquisitions).

methanol proceeds rapidly at room temperature with the development of a yellowish solution. The iodinated derivative is isolated by chromatography over Polyamide SC-66 (Figure 1a) with nearly baseline resolution between the parent (eluting at fraction 22) and the product (eluting at fraction 18). In the preparation of [^{125}I]-7-iodo-α-amanitin, additional ultraviolet absorbing compounds elute early from the Polyamide SC66 column (Figure 1a, fractions 9–12); these are always minor components when using ^{127}ICl for the iodination but are more significant products in the ^{125}ICl reaction (see following paragraph). Whether the components eluting in fractions 9–12 are derived from α-amanitin is unknown; however, since the overall recovery of α-amanitin plus 7-iodo-α-amanitin is ≤60%, these components may be degraded α-amanitin. The components in fractions 9–12 give no detectable RNA polymerase inhibition. The major product eluting at fraction 18 is shown to be 7-iodo-α-amanitin on the basis of ultraviolet (Figures 2a and 2b) and proton magnetic resonance spectra (Figure 2c).

Figures 2a and 2b compare the electronic spectra of the parent and the iodinated product, respectively, in both neutral and alkaline methanol. Both the parent α-amanitin, as shown previously (Wieland & Gebert, 1966), and the iodinated product exhibit a bathochromic shift upon addition of alkali. Hence the iodinated product retains the ionizable phenolate moiety. The ultraviolet spectrum readily distinguishes between the noniodinated and 7-iodo-α-amanitin as summarized in Table I. The λ_{max} at both 301 nm (neutral) and 330 nm (alkaline) of the iodinated product exhibit a hypsochromic shift compared with the λ_{max} of the α-amanitin. For the iodinated product, the extinction coefficient of the transition at 330 nm (alkaline) is greater than the extinction coefficient at 301 nm (neutral) (Figure 2b; Table I). In the case of α-amanitin, the transition at 305 nm (neutral) has a greater extinction coefficient than that at 332 nm (alkaline) (Figure 2a; Table I). The shoulder at ~240 nm in the α-amanitin spectrum shifts to ~252 nm in alkaline solution and remains a shoulder (Figure 2a). In contrast, the shoulder at ~240 nm in the 7-iodo-α-amanitin spectrum shifts in alkaline solution to a peak with λ_{max} 258 and a distinct λ_{min} 245 nm. Thus, despite the generally similar spectra, α-amanitin and iodinated α-amanitin can

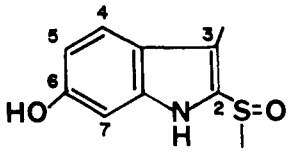
TABLE I: Physical Parameters for α-Amanitin and 7-Iodo-α-amanitin.

Parameter	α-Amanitin	7-Iodo-α-amanitin
M_r	918	1043
pK_a, phenolate	9.72	7.94
λ_{max} (nm)[a]	305	301
λ_{max} (nm), 0.01 N NaOH[a]	332	330
$\epsilon_{\lambda max}$[a]	11.9×10^3 [b]	14.3×10^3
Ratio of ϵ[c]	1.18	0.943

[a] Spectral data are given for methanolic solution and the ϵ values are in M^{-1} cm^{-1}. [b] This extinction coefficient is taken from Gebert et al. (1967). [c] This is the ratio of ϵ at the λ_{max} in neutral methanol to the ϵ at the λ_{max} in methanol containing 0.01 M NaOH.

be readily distinguished by examination of the neutral and alkaline methanolic spectra. In admixtures of the two compounds, an approximation of the amanitin content is obtainable from the ratio of the λ_{max} in neutral and alkaline solutions.

The electronic spectra strongly suggest iodination of the amanitin aromatic ring. Structure I is the 6-hydroxy-2-



I

sulfoxyindole nucleus found in α-amanitin (Wieland, 1972). While the 4,5, and 7 carbon atoms are available for reaction, the 7 position on chemical grounds is expected to be most activated toward electrophilic aromatic substitution. Proton magnetic resonance confirms these observations and establishes the position of substitution as the expected C-7 iodo derivative.

Figure 2c shows that portion of the ^1H NMR spectrum attributed to the aromatic indole protons of the parent α-amanitin and its iodinated derivative. At 60 MHz the three indole protons of amanitin approach an AMX spin system with the C-4 proton doublet (ν_{4H} = 7.64; $J_{4,5}$ = 9) being furthest downfield. The C-5 proton ($\nu_{5H} \simeq$ 6.78) appears as a broadened doublet, the lower field half of which is superimposed on the C-7 proton ($\nu_{7H} \simeq$ 6.94). At 220 MHz (spectrum not shown) the C-7 doublet (ν_{7H} = 6.94; $J_{7,5}$ = 2) and C-5 doublet of doublets (ν_{5H} = 6.78; $J_{5,7}$ = 2; $J_{5,4}$ = 9) are clearly resolved with assignments following directly from typical ortho- and meta-coupling constants.

The 60-MHz ^1H NMR spectrum in D_2O of 750 μg of iodinated α-amanitin (isolated by Polyamide SC66 chromatography as shown in Figure 1a) shows only two AB coupled protons (Figure 2c, ν_{4H} = 7.57, and ν_{5H} = 6.90, $J_{4,5}$ = 9) attributed to the C-4 and C-5 aromatic protons. Clearly aromatic substitution has occurred at the C-7 position of the indole nucleus. The signal-to-noise ratio on this ^1H NMR spectrum (Figure 2c) is adequate to eliminate the possibility of admixture with more than 5% of the isomeric substitution products. Thus these reaction and purification conditions yield a near, if not completely, homogeneous product. Examination of the ^1H NMR spectrum for the remaining 37 nonexchangeable protons in the iodinated α-amanitin shows no detectable deviation from the corresponding spectrum of the parent α-amanitin; we conclude that the aromatic substitution at the C-7 atom of the indole is most likely the only difference between the parent and the single isolated product. The isolated product thus has the chemical sequence: *cyclo*-(L-α-asparagyl-4-hydroxyl-L-prolyl-4,5-dihydroxy-L-isoleucyl-6-hydroxy-7-iodo-2-mercapto-L-tryptophanyl-glycyl-L-isoleucylglycyl-L-cysteinyl) *cyclo*-(4→8)-*S*-oxide. This is referred to as 7-iodo-α-amanitin.

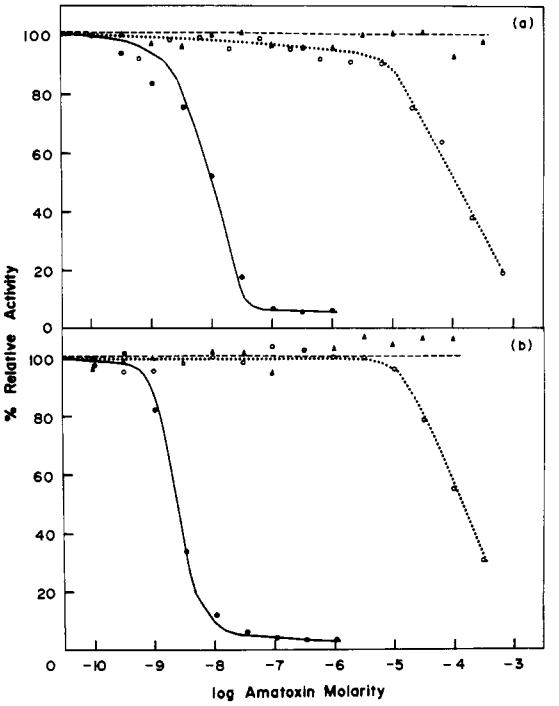


FIGURE 3: Titration of sea urchin (*S. purpuratus*) RNA polymerase I, II, and III activities with α-amanitin (a) and 7-iodo-α-amanitin (b). Aliquots (20 μL) of the respective enzymes (purified via DEAE-Sephadex, see Methods) are added to 30 μL of reaction mixture containing α-amanitin or 7-iodo-α-amanitin to give the final concentration indicated; the assays are incubated at 30 °C for 20 min. The molarity of α-amanitin is determined from the published extinction coefficient (Gebert et al., 1967) and of 7-iodo-α-amanitin from the determined value (Table I). Full activity for RNA polymerase I = 15 000 cpm (15 pmol) of UMP incorporated, II = 24 000 cpm (24 pmol), and III = 28 000 cpm (28 pmol). RNA polymerase I (Δ- - -Δ); polymerase II (●—●); polymerase III (O··· O).

Mono- and diiodinated phenols typically have pK_a values lower than the parent phenol and the decrease in pK_a is greater for diiodination than monoiodination (see pages B38-39, J195, J199 in Sober, 1970). Table I reports the pertinent pK_a values as determined by spectrophotometric titration and the pK_a of 7-iodo-α-amanitin is seen to be lower by ~1.7 pH units than that value for the parent α-amanitin. Thus the iodinated α-amanitin is similar to other phenols with respect to pK_a. A single titrable species with a discrete pK_a has a titration curve characterized by a pH span of 1.908 between 0.1 and 0.9 mol fraction ionization (the above follows from inspection of the Henderson-Hasselbach equation). Hence, a pH span = 1.908 is diagnostic for a single pK_a value. If the iodinated α-amanitin were a mixture of mono- and diiodinated species with different pK_as, then the pH span would be expected to be greater than 1.908. The observed pH span for the 7-iodo-α-amanitin titration is 1.947, a value close to that expected for a single ionizable species.

Alternative Iodination Procedures. Of the procedures tried, ICl in methanol is the only reagent that yields a stable, isolable iodo-α-amanitin. One equivalent of I_2 in methanol gives no detectable reaction; mercuration followed by treatment with I_2 (Giza & Hinman, 1964) yields a mixture from which no iodoamanitin has been isolable. Treatment of a phenol with I_2-morpholine in a 1:1:3 mole ratio is known to selectively iodinate several phenols (Chabrier et al., 1957) including a 5-hydroxybenzofuran (Giza & Hinman, 1964). With this re-

action α-amanitin yields <0.1% iodo-α-amanitin with the major product being 7-morpholino-α-amanitin isolable in yields to 90%. This reaction is the subject of a following paper (manuscript in preparation). Enzymatic iodination by lactoperoxidase is generally considered to be a very innocuous method for iodination of biological molecules with little denaturation (Morrison & Schonbaum, 1976). However, treatment of α-amanitin in aqueous solution with iodine in the +1 oxidation state abolishes the ability of α-amanitin to inhibit RNA polymerase II. Thus, lactoperoxidase is observed to catalyze the destruction, rather than the iodination, of α-amanitin. This interesting phenomenon is the subject of a separate manuscript (in preparation).

Inhibition of RNA Polymerase Activity by 7-Iodo-α-amanitin. Following preparation of the purified iodination adduct of α-amanitin, a most crucial question concerns the product's activity in inhibition of eukaryotic RNA polymerase. Figure 3a presents the control experiment where the activities of the three individual RNA polymerase classes from the sea urchin are titrated with increasing α-amanitin concentrations. The partially purified RNA polymerases of the urchin are analogous to the cognate enzymes of other higher eukaryotes with respect to α-amantin sensitivity (Lindell et al., 1970; Meilhac et al., 1970; Schwartz et al., 1974b; Weil & Blatti, 1975; Weinman & Roeder, 1974); thus RNA polymerase II is half-maximally inhibited by $\sim 10^{-8}$ M α-amanitin and RNA polymerase III by $\sim 10^{-4}$ M α-amanitin. RNA polymerase I is quite insensitive to any α-amanitin concentration up to 3×10^{-4} M; higher concentrations have not been tested on the urchin class I RNA polymerase. Whether there is any functional analogy between the urchin and the yeast polymerase I with respect to inhibition by high α-amanitin concentrations (Schultz & Hall, 1976; Valenzuela et al., 1976) is not answered by the present data.

Figure 3b demonstrates that the 7-iodo-α-amanitin is a thoroughly effective inhibitor for the RNA polymerase of the sea urchin. RNA polymerase II is seen to be half-maximally inhibited by $\sim 2 \times 10^{-9}$ M 7-iodo-α-amanitin, while the RNA polymerase III remains half-maximally inhibited at $\sim 10^{-4}$ M. Again, the class I enzyme shows no demonstrable inhibition. Since the 7-iodo-α-amanitin used in these experiments is a rather pure compound and is devoid of contamination by the parent α-amanitin, we conclude that iodination of α-amanitin at the 7 carbon of the indole moiety causes no decrease in potency. In fact, the data in Figure 3b, compared with the data of Figure 3a, indicate that the 7-iodo-α-amanitin is a more potent (~fivefold) inhibitor of RNA polymerase II than is the parent α-amanitin. For RNA polymerase III, one notes that there is no marked difference in inhibition between 7-iodo-α-amanitin and α-amanitin; this observation is of considerable interest since it suggests that various amatoxin derivatives may have differentially altered affinity for one, as opposed to another, RNA polymerase class.

Preparation and Stability of [^{125}I]-7-Iodo-α-amanitin. The preparation of [^{125}I]-7-iodo-α-amanitin is procedurally similar to preparation of the ^{127}I-labeled derivative but with a few problems unique to working with the radioactive isotope. Two procedures are used for incorporation of the isotope into the reaction and the choice between these two depends upon the desired specific activity. For 7-iodo-α-amanitin of 100 Ci/mmol, or less, the desired amount of $^{125}I^-$ in methanol is mixed with ICl in methanol for isotopic equilibrium and then the α-amanitin is added. For reactions at 200 Ci/mmol or higher, the above procedure is undesirable due to the NaOH in the Na^{125}I preparation. For high specific activities, the I^- is oxidized to I_2 with $Fe(CN)_6^{3-}$ and extracted into *n*-hexane. Methanolic ICl is then added to the I_2 in the hexane and after isotopic equilibrium the α-amanitin is added to the reaction mixture.

Figure 1b shows the results of the low specific activity procedure applied to the iodination of 36 nmol of α-amanitin with 37 nmol of ^{125}ICl at 0.3 Ci/mmol. There is an ^{125}I peak that coelutes with the ultraviolet absorbing peak due to the 7-iodo-α-amanitin (fractions 18–20); the parent α-amanitin (fractions 23–25) shows no significant ^{125}I content above that ^{125}I trailing across the column from fractions 10–11. The ^{125}I in fractions 10–11 coelutes with the unidentified ultraviolet absorbing components (cf., Figure 1a, fractions 9–12). This ^{125}I-labeled, ultraviolet absorbing component is a persistent characteristic of all radioactive iodination reactions regardless of the specific activity of the preparation. The [^{125}I]-7-iodo-α-amanitin as isolated from the Polyamide SC66 column has a 1–3% radioisotopic contamination due to the ^{125}I trailing from the peak in fractions 10–11; this contaminant is conveniently removed by filtration over a 50–100-μL bed of DEAE-Sephadex in methanol or by rechromatography over Polyamide SC66. Iodide does not elute from the Polyamide SC66 column and remains strongly bound at the top of the column; however, any $^{125}I^-$ in the preparation is very conveniently removed by the 50–100-μL bed of DEAE-Sephadex. The results obtained with the high specific activity procedure are qualitatively similar to the results presented in Figure 1b; the extent of radioisotopic contamination by the trailing ^{125}I remains <3% of ^{125}I in the 7-iodo-α-amanitin.

The iodination reaction is marked by a distinct loss of the summed α-amanitin and 7-iodo-α-amanitin chromophore. This loss has been observed to be as high as 50% in the reaction with ^{127}ICl and as high as 90% in reaction with ^{125}ICl. We observe that this loss of chromophore is correlated with the length of time that the ICl has been in solution and suggest that the disproportion reaction, $2ICl \rightarrow I_2 + Cl_2$, may possibly account for this phenomenon. The loss of chromophore cannot be attributed to loss by adsorption to the Polyamide SC66 matrix since control experiments show undetectable loss upon passage of a known quantity over the chromatography column. We therefore prepare the ICl solution only immediately prior to using the solution in the reaction.

Once the 7-iodo-α-amanitin is isolated it appears quite stable regardless of whether ^{125}I or ^{127}I is used in the preparation. Solutions of 7-iodo-α-amanitin in H_2O, pH 6.5, or in 0.01 M borate, pH 8.9, show no electronic spectra alterations or attenuations of the RNA polymerase II inhibition when stored for 2.5 months at −10 °C. These two pH conditions test for stability of both the un-ionized and ionized forms. Aqueous and methanol solutions have each been stored (−10 °C) for as long as 6 months without evidence of chemical instability. The 7-iodo-α-amanitin is also stable in aqueous solution to 7 mM 2-mercaptoethanol or pH 9.8 borate solution for at least 72 h at 4 °C.

Binding of [^{125}I]-7-Iodo-α-amanitin to RNA Polymerase II. The data in Figures 4–6 demonstrate that the [^{125}I]-7-iodo-α-amanitin functions as a ligand in binding to three eukaryotic RNA polymerase IIs. In order to test for comigration of the RNA polymerase II activity and the bound [^{125}I]-7-iodo-α-amanitin, a subsaturating concentration of the ligand was mixed with the enzyme preparation prior to resolution by gradient sievorptive elution on DEAE-Sephadex. The solubilized enzyme preparations used in these experiments deliberately were prepared with only a 3.5–4-fold dilution from the tissue volume to the final homogenate and were fractionated, at most, only by low centrifugal force prior to addition of the radioactive ligand. The homogenates retain most of the cellular

constituents in a rather high concentration; examination of the A_{280} profiles in Figures 4b, 5b, and 6 shows that the protein concentration is >4 mg/mL in many fractions. Maintenance of the high concentration of cellular constituents maximizes the probability for nonspecific binding with the ^{125}I-labeled ligand. Thus the experiments as reported here represent a compromise of high protein concentration to facilitate the detection of nonspecific binding and of low ligand concentration to assess comigration of enzyme activity and bound ligand.

Following mixture of the ligand with homogenate, the bound and free ligand can be separated by DEAE-Sephadex operated in the gradient sievorptive elution mode. In this chromatographic mode, many macromolecules, including the RNA polymerases, elute prior to those small molecules that are applied with the sample. The iodinated α-amanitin with a 1043-dalton mass could be expected to behave as a small molecule on DEAE-Sephadex A-25 and should not elute prior to fractions 48–55 (Figure 4a). Any [^{125}I]-7-iodo-α-amanitin eluting on the gradient then is indicative of binding to some macromolecule which is excluded from the pores of the matrix. Not only is the iodinated α-amanitin a small molecule that should experience gel filtration, it also interacts with the DEAE-Sephadex matrix and does not elute within 2 bed volumes after application of the sample. The nonbound ligand is quantitated by counting the ^{125}I retained by the column. The iodide anion behaves as a typical small molecule and elutes at the juncture of the gradient and the 0.35 M $(NH_4)_2SO_4$ eluate. This method thus provides a quantitation of the bound and nonbound ligand plus the iodide anion contamination.

Binding of [^{125}I]-7-iodo-α-amanitin to the urchin RNA polymerase II is shown in Figures 4a and 4b. The polymerase activity resistant to 3×10^{-7} M α-amanitin is presented in Figure 4a and shows the two enzymatic activities expected for RNA polymerases I and III in this invertebrate (Morris et al., 1976; Morris & Rutter, 1976; Roeder & Rutter, 1969). The comigration of the RNA polymerase activity and the bound [^{125}I]-7-iodo-α-amanitin is shown in Figure 4b; these two profiles are quite coincident with each other and overlap little with RNA polymerases I and III. Note that the greatest protein concentrations are in fractions 27–34; the ^{125}I content is undetectable here. Presaturation of the RNA polymerase II with 2.9×10^{-6} M α-amanitin, followed by addition of the ^{125}I-labeled ligand reduces binding to background amounts (<100 cpm/fraction). The bound [^{125}I]-7-iodo-α-amanitin coeluting with RNA polymerase II equals 12.9% of the input ^{125}I; recovery and γ counting of the DEAE-Sephadex beads account for the input radioactivity that does not elute.

Figures 5a and 5b demonstrate the binding of [^{125}I]-7-iodo-α-amanitin to RNA polymerase II from MOPC 315 plasmacytoma; the results are qualitatively similar to the sea urchin data. RNA polymerase I is the predominant activity in this plasmacytoma (Figure 5a; see also Schwartz et al., 1974a,b) and RNA polymerase III is detectable as a small activity component eluting in fractions 34–37 (Figure 5a). Again the RNA polymerase II activity and the bound [^{125}I]-7-iodo-α-amanitin co-elute in a rather symmetrical peak (Figure 5b) and coelution of radioactivity with those fractions having greatest protein concentration is minimal.

Binding to RNA polymerase II from adults of *Drosophila melanogaster,* Oregon R strain, is shown in Figure 6. The crude extracts from the whole fly are not amenable to resolution of the RNA polymerase classes as are the extracts from urchin or MOPC 315. Rather, the predominant RNA polymerase II activity totally overlaps with the RNA polymerase I and III activities and is spread over a considerable breadth

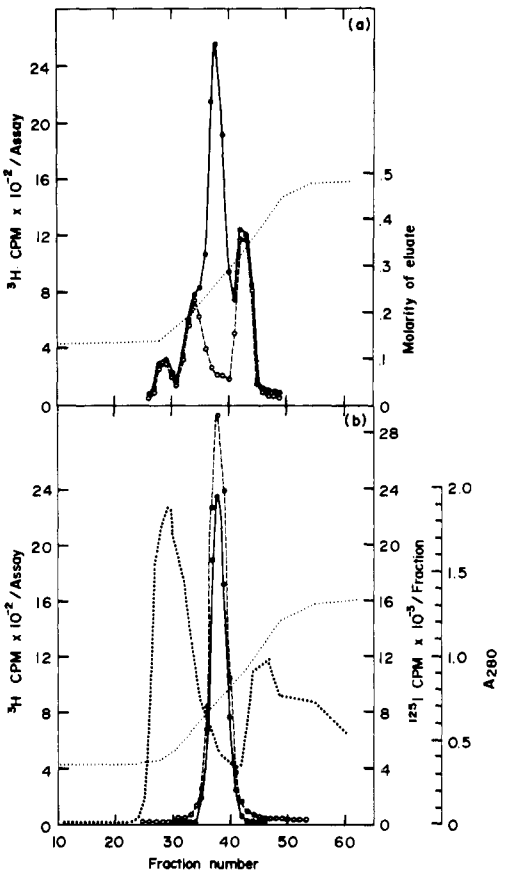


FIGURE 4: Comigration of [^{125}I]-7-iodo-α-amanitin (200 Ci/mmol) and sea urchin RNA polymerase II on DEAE-Sephadex (A-25). One milliliter of homogenate (blastula stage embryo) was incubated at 0 °C for 15 min with 10^{-9} M [^{125}I]-7-iodo-α-amanitin prior to resolution of the RNA polymerase activities by gradient sievorptive elution (Kirkegaard, 1973). (a) RNA polymerase activities in the absence (●—●) and presence (○- - -○) of 3×10^{-7} M α-amanitin in the assay. The minor activity in fractions 28–29 is not RNA polymerase (Morris & Rutter, 1976). Molarity of $(NH_4)_2SO_4$ in the eluate is indicated (• • •). (b) Comigration of RNA polymerase II activity (●—●) with the bound [^{125}I]-7-iodo-α-amanitin ligand (○- -○). The RNA polymerase II activity was calculated as the difference between the total RNA polymerase activity and the activity remaining in the presence of 3×10^{-7} M α-amanitin (data shown in a). The absorbance at 280 nm (• • •) is shown along with the eluate molarity (• • •). A single experiment is shown in a and b. Not shown is another experiment in which the RNA polymerase II was presaturated with 2.9×10^{-6} M α-amanitin prior to addition of [^{125}I]-7-iodo-α-amanitin ligand; in this case no RNA polymerase II activity was detectable and the ^{125}I eluting prior to fraction 55 was less than 150 cpm/fraction.

of the gradient. Preliminary purification prior to the DEAE-Sephadex chromatography is necessary to achieve resolution of the three polymerase classes (W. Loga:, L.-T. Lin, & P. W. Morris, unpublished data). Since there is no significant separation of the polymerase classes in these crude extracts of *Drosophila,* Figure 6 presents only the activity profile for RNA polymerase II. Again, however, the bound [^{125}I]-7-iodo-α-amanitin coincides with the activity profile except for a few fractions. Most importantly, the ^{125}I profile shows insignificant binding in those fractions containing the greatest protein concentrations.

Discussion

We report here the synthesis of monosubstituted α-amanitin useful as an isotopically tagged ligand for the study of eukaryotic RNA polymerase II. Derivatization of the toxin molecule by electrophilic substitution with ICl offers the combined advantages of a single, defined molecular structure with an

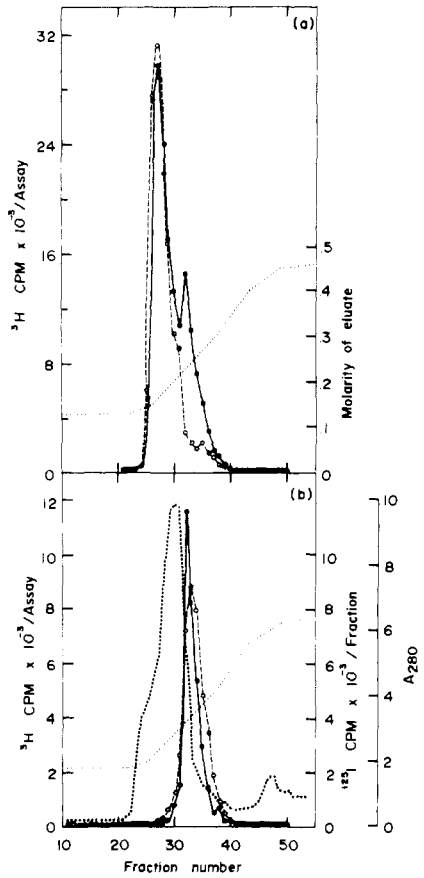


FIGURE 5: Comigration of [^{125}I]-7-iodo-α-amanitin (200 Ci/mmol) and MOPC 315 plasmacytoma RNA polymerase II on DEAE-Sephadex (A-25). Ligand binding and polymerase resolution were as described in the legend to Figure 4. (a) RNA polymerase activities in the absence (●—●) and presence (○- - -○) of 3×10^{-7} M α-amanitin. Molarity of $(NH_4)_2SO_4$ in the eluate is indicated (• • •). (b) Comigration of RNA polymerase II activity (●—●) with the bound [^{125}I]-7-iodo-α-amanitin ligand (○- - -○). The RNA polymerase II activity was calculated as described in the legend to Figure 4. The absorbance at 280 nm is shown (• • •) along with the eluate molarity (• • •). A single experiment is shown in a and b.

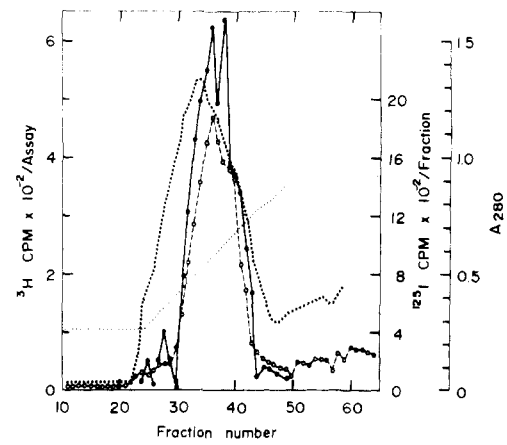


FIGURE 6: Comigration of [^{125}I]-7-iodo-α-amanitin (200 Ci/mmol) and *Drosophila melanogaster* RNA polymerase II on DEAE-Sephadex (A-25). Ligand binding and polymerase resolution were as described in the legend to Figure 4. Since the RNA polymerase II was the predominant activity and completely overlapped with polymerase I and III activities, only the RNA polymerase II activity, determined as described in legend to Figure 4, is shown. The RNA polymerase II activity (●—●) is shown with the [^{125}I]-7-iodo-α-amanitin (○- - -○). The absorbance at 280 nm (• • •) and the $(NH_4)_2SO_4$ molarity in the eluate are also shown.

inherently high specific activity (>2000 Ci/mg-atom for ^{125}I), minimized radiolytic degradation from the x-ray (as contrasted to degradation by ^{3}H β particles), enhanced inhibitory potency on RNA polymerase II, applicability to autoradiography, and quantitation by nondestructive counting on the conventional crystal detector γ counter. The combined results presented in Figures 4–6 suggest that 7-iodo-α-amanitin, like its parent, will prove to be a potent inhibitor of and a tight binding ligand for the class II RNA polymerase throughout the eukaryotic kingdom.

In view of the wide range of binding experiments possible with a high specific radioactivity amatoxin, we consider it quite important that the binding reaction should be characterized by a single association constant. A homogeneous ligand preparation is therefore essential. No evidence of multiple iodination products is discernible by the chromatography of 7-iodo-α-amanitin over Polyamide SC66. The spectrophotometric titration of the phenolate moiety finds a titration curve indistinguishable from the theoretical curve for a single ionizable group. Multiple iodination of α-amanitin would be expected to yield products with pK_a values lower than the monoiodinated product, and a mixture of mono- and diiodinated products would then yield a complex titration curve. The ^{1}H NMR spectrum quantitates the aryl protons; thus position and extent of substitution are evident from the spectrum. Despite the limitation on sample size, the signal-to-noise ratio in the ^{1}H NMR spectrum is adequate to exclude contamination (>5%) of the 7-iodo-α-amanitin with another aromatic substitution product. Thus the spectrophotometric titration and the ^{1}H NMR spectrum are positive evidence for homogeneity of the final product. Radiochemical purity, as assessed by Polyamide SC66 chromatography, is in excess of 95% in the initial isolate; rechromatography over Polyamide SC66 reduces residual radioactive contaminants to undetectable amounts. By the available assay method then the purified ^{125}I-labeled ligand is radiochemically homogeneous. Thus we expect that if any deviation from a simple binding reaction is observed for the system, [^{125}I]-7-iodo-α-amanitin plus RNA polymerase II, the deviation will be due to the enzyme preparation and not to the ligand.

The 7-iodo-α-amanitin clearly is an effective RNA polymerase inhibitor as shown by the titration of RNA polymerase II and III activities. Its inhibitory potency on RNA polymerase III is equivalent to the parent α-amanitin. Against RNA polymerase II, the iodinated product appears fivefold more potent than the parent; this observation suggests a reduction in the dissociation constant, K_D, for RNA polymerase II and the amatoxin occurs upon iodination. One must note, however, that, although a plot of enzyme inhibition vs. total amatoxin concentration (e.g., Figure 3a and 3b) has been frequently employed (Hodo and Blatti, 1977; Ingles et al., 1976; Valenzuela et al., 1976; Schultz & Hall, 1976; Cochet-Meilhac & Chambon, 1974; Schwartz et al., 1974a,b; Meilhac et al., 1970; Lindell et al., 1970) to characterize the RNA polymerase–amatoxin interaction, such an analytical technique conveys limited information about the actual dissociation constant for the binary system. For a simple ligand binding reaction at equilibrium the total ligand concentration, A_T, when half of the binding sites are saturated is given by the relation: $A_T = K_D + (E_T/2)$, where K_D is the dissociation constant and E_T is the total binding site concentration. If E_T is small with respect to K_D, then A_T approximates the K_D. For the specific situation of RNA polymerase II the binding reaction is simple with a $K_D \simeq 10^{-9}$ M and a 1:1 ratio of amatoxin binding to enzyme inhibition (Cochet-Meilhac & Chambon, 1974). Thus at 50% inhibition of the RNA polymerase activity, the total

amatoxin concentration is approximated by $A_T = K_D + (E_T/2)$. If $E_T \geq K_D$, then at 50% inhibition the $A_T > K_D$. RNA polymerase II concentrations of 10^{-10} to 10^{-8} M are calculable from the aforementioned references and hence yield a value for A_T that may be considerably larger than K_D for the system. In the present case of 7-iodo-α-amanitin, the $A_T \simeq 2 \times 10^{-9}$ M at 50% inhibition of RNA polymerase II; the actual K_D is somewhat smaller since the total RNA polymerase II $\simeq 2 \times 10^{-9}$ M. The decrease in K_D following iodination is therefore not calculable. A detailed study of the 7-iodo-α-amanitin–RNA polymerase II binding reaction is now in progress.

Substitution of the α-amanitin indole with an iodine atom increases the hydrophobic bulk around the indole as well as decreasing the pK_a of the 6-hydroxyl moiety. It is germane to consider the effect of these two phenomena on the RNA polymerase. Ionization of the 6-hydroxyl at pH 7.9 is much more extensive for the iodinated than for the parent compound; this ionization could promote electrostatic bonding with the RNA polymerase and a consequent decrease in the K_D. This seems an unlikely possibility inasmuch as the 6-hydroxyl is not crucial to amatoxin activity. Amanin lacks the 6-hydroxyl moiety (Gebert et al., 1967; Faulstich & Wieland, 1968) but is a potent toxin and inhibitor (Buku et al., 1971). Likewise, conversion of the 6-hydroxyl to its methyl ether has little effect on toxicity or binding to RNA polymerase II (Buku et al., 1971; Meilhac et al., 1970; Cochet-Meilhac & Chambon, 1974). The hydrophobic bulk of the iodine atom is a more likely determinant of increased inhibitor potency; this possibility is consonant with the observed potentiation of inhibition by the benzylamine derivative of α-amanitin (Morris et al., 1977).

Any effective radioisotopic ligand, when bound to the RNA polymerase, must dissociate slowly so as to allow separation of the bound and free ligand without appreciable dissociation. For RNA polymerase II, the half-life of the binary complex is long and varies inversely with temperature and directly with ionic strength (Cochet-Meilhac & Chambon, 1974). The half-life extends as long as 100 h (0 °C in 0.1 M $(NH_4)_2SO_4$) for calf thymus RNA polymerase II and *O*-methyldemethyl-γ-amanitin (Cochet-Meilhac & Chambon, 1974). Such a long half-life of the binary complex permits separation of the bound and free ligand by the time consuming steps of ammonium sulfate or poly(ethylene glycol) precipitation (as developed by Cochet-Meilhac & Chambon, 1974) or DEAE-Sephadex gradient sievorptive elution (present work). In the DEAE-Sephadex separation, the polymerase sample with the ^{125}I-labeled ligand is loaded at 0.35 M $(NH_4)_2SO_4$ onto the column and the ligand-enzyme complex elutes at 0.25–0.30 M $(NH_4)_2SO_4$ some 90 min later. Under these temperature and ionic conditions the half-life of the complex is likely much greater than the resolution time so that insignificant dissociation of the bound ligand occurs.

The calf thymus and the sea urchin RNA polymerase II are both quite sensitive to the amatoxin with K_D values less than 10^{-8} M. Can the ligand binding technique be extended to RNA polymerases which are much less sensitive to the amatoxin than either of these examples? Such polymerases are the yeast RNA polymerase II (half-maximal inhibition occurs at 10^{-6} M α-amanitin; Valenzuela et al., 1976; Schultz & Hall, 1976), the RNA polymerase III from organisms as sea urchin (present work) or the MOPC 315 plasmacytoma (Schwartz et al., 1974b), and the RNA polymerase I from yeast (Valenzuela et al., 1976; Schultz & Hall, 1976). A probable cause for resistance to amatoxin inhibition is an increase in the dissociation rate of the complex with a corresponding increase in the K_D (Cochet-Meilhac & Chambon, 1974); this suggestion is based on alterations in the amatoxin molecule. If the change in sensitivity to amatoxin inhibition is parallel with change in the complex dissociation rate, then an enzyme–amatoxin complex, such as the yeast RNA polymerase II, is expected to have a half-life of approximately 1 h. This half-life is too short for bound and free ligand separation by chromatography or by precipitation and will require a more rapid technique as filtration over nitrocellulose filters (Cochet-Meilhac & Chambon, 1974) or DEAE-filters (P. W. Morris, unpublished results). Ingles et al. (1976) have analyzed the amatoxin inhibition and binding to RNA polymerase II from amatoxin resistent mutants of Chinese hamster ovary cells. Half-maximal inhibition of the Ama 6 RNA polymerase II occurred at $\sim 10^{-7}$ M (0.1 μg/mL) and ligand binding to this polymerase was detectable; on the other hand, the Ama 1 RNA polymerase II was half-maximally inhibited at $\sim 2 \times 10^{-6}$ M (~2 μg/mL) and ligand binding was not detected (Ingles et al., 1976). Thus, in order to reliably quantitate amatoxin binding to any RNA polymerase characterized by half-maximal inhibition at 10^{-6} M or higher, the experimenter will need to use a rapid method for separation of the bound and free ligand. RNA polymerases I and III are probably not amenable to ligand binding studies with the 7-iodo-α-amanitin due to rapid dissociation of the complex. Extension of ligand binding analysis to these latter two enzyme types will require amatoxin derivatives with lower K_D values for the enzyme. Possible candidates are the benzylamine or hexylamine derivatives of α-amanitin (Morris et al., 1977).

Acknowledgments

We thank C. Passo and R. Litman for cheerful and skillful polymerase preparation, R. Litman for preparation of drawings, B. Rogowski for manuscript typing, Dr. N. J. Leonard, Urbana, for helpful discussion on the indole reactions, and Dr. Steve Danyluk, Argonne National Laboratory, for providing instrument time for the 220-MHz spectra.

References

Bates, R. G. (1964) *Determination of pH,* Wiley, New York, N.Y.

Blatt, A. H., Ed. (1943) *Organic Syntheses,* Collect. Vol. II, p 344. Wiley, New York, N.Y.

Brodner, O. G., & Wieland, T. (1976) *Biochemistry 15,* 3480–3484.

Buku, A., Campadelli-Fiume, G., Fiume, L., & Wieland, T. (1971) *FEBS Lett. 14,* 42–44.

Chabrier, P., Seyden-Penne, J., & Fouace, A. (1957) *C.R. Acad. Sci. 245,* 174–175.

Chambon, P. (1975) *Annu. Rev. Biochem. 44,* 613–638.

Cochet-Meilhac, M., & Chambon, P. (1974) *Biochim. Biophys. Acta 353,* 160–184.

Faulstich, H., & Wieland, T. (1968) *Justus Liebigs Ann. Chem. 713,* 186–195.

Fuhrman, S. A., & Gill, G. N. (1976) *Biochemistry 15,* 5520–5527.

Gebert, U., Wieland, T., & Boehringer, H. (1967) *Justus Liebigs Ann. Chem. 705,* 227–237.

Giza, C. A., & Hinman, R. L. (1964) *J. Org. Chem. 29,* 1453–1461.

Guialis, A., Beatty, B. G., Ingles, C. J., & Crerar, M. M. (1977) *Cell 10,* 53–60.

Hodo, H. G., III, & Blatti, S. P. (1977) *Biochemistry 16,* 2334–2343.

Ingles, C. J., Guialis, A., Lam, J., & Siminovitch, L. (1976) *J. Biol. Chem. 251,* 2729–2734.

Jacob, S. T., Sajdel, E. M., & Munro, H. N. (1970) *Biochem. Biophys. Res. Commun. 38,* 765–770.
Kirkegaard, L. H. (1973) *Biochemistry 12,* 3627–3632.
Kirkegaard, L. H. Johnson, T. J. A., & Bock, R. M. (1972) *Anal. Biochem. 50,* 122–127.
Lindell, T. J., Weinberg, F., Morris, P. W., Roeder, R. G., & Rutter, W. J. (1970) *Science 170,* 447–449.
Meilhac, M., Kedinger, C., Chambon, P., Faulstich, H., Govindan, M. V., & Wieland, T. (1970) *FEBS Lett. 9,* 258–260.
Morris, P. W., & Rutter, W. J. (1976) *Biochemistry 15,* 3106–3113.
Morris, P. W., Litman, R., & Passo, C. (1976) in *Molecular Mechanisms in the Control of Gene Expression* (Nierlich, D. P., & Rutter, W. J., Eds.) pp 255–260, Academic Press, New York, N.Y.
Morris, P. W., Venton, D. L., & Kelley, K. M. (1977) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 36,* 882 (Abstract 3222).
Morrison, M., & Schonbaum, G. R. (1976) *Annu. Rev. Biochem. 45,* 861–888.
Roeder, R. G., & Rutter, W. J. (1969) *Nature (London) 224,* 234–237.
Schultz, L. D., & Hall, B. D. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73,* 1029–1033.
Schwartz, L. B., Lawrence, C., Thach, R. E., & Roeder, R. G. (1974a) *J. Virol. 14,* 611–619.
Schwartz, L. B., Sklar, V. E. F., Jaehning, J. A., Weinman, R., & Roeder, R. G. (1974b) *J. Biol. Chem. 249,* 5889–5897.
Sober, H. A., Ed. (1970) *Handbook of Biochemistry,* 2nd ed, The Chemical Rubber Co., Cleveland, Ohio.
Somers, D. G., Pearson, M. L., & Ingles, C. J. (1975a) *J. Biol. Chem. 250,* 4825–4831.
Somers, D. G., Pearson, M. L., & Ingles, C. J. (1975b) *Nature (London) 253,* 372–374.
Valenzuela, P., Hager, G. L., Weinberg, F. & Rutter, W. J. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73,* 1024–1028.
Weil, P. A., & Blatti, S. P. (1975) *Biochemistry 14,* 1636–1642.
Weil, P. A., Sidikaro, J., Stancel, G. M., & Blatti, S. P. (1976) *J. Biol. Chem. 252,* 1092–1098.
Weinmann, R., & Roeder, R. G. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 1790–1794.
Wieland, T. (1972) *Naturwissenschaften 59,* 225–231.
Wieland, T., & Fahrmeir, A. (1970) *Justus Liebigs Ann. Chem. 736,* 95–99.
Wieland, T., & Gebert, U. (1966) *Justus Liebigs Ann. Chem. 700,* 157–173.
Wieland, T., & Wieland O. (1972) *Microbiol. Toxins 8,* 249–280.

Sodium-Dependent Binding of *p*-Nitrophenyl α-D-Galactopyranoside to Membrane Vesicles Isolated from *Salmonella typhimurium*†

Hajime Tokuda and H. Ronald Kaback*

ABSTRACT: *p*-Nitrophenyl α-D-galactopyranoside is a competitive inhibitor of sodium-dependent methyl 1-thio-β-D-galactopyranoside transport in whole cells and membrane vesicles prepared from *Salmonella typhimurium* G-30 grown on melibiose ($K_i \simeq 7$ μM in whole cells and 0.4 μM in membrane vesicles). However, the compound is not transported to a discernible extent by either intact cells or membrane vesicles. Binding of *p*-nitrophenyl α-D-[6-^{3}H]galactopyranoside to membrane vesicles has been measured by flow dialysis under various conditions. When D-lactate is added to vesicles at low sodium concentrations, ligand binds with a $K_D \simeq 3.2$ μM, and a total of about 0.2 nmol is bound per mg of membrane protein at saturating concentrations of ligand. With optimal sodium concentrations in addition to D-lactate, both the affinity and the number of binding sites observed at saturation increase ($K_D \simeq 0.6$ μM and about 0.8 nmol per mg of membrane protein). Furthermore, ligand binding can be induced by imposition of a potassium diffusion gradient ($K^+_{in} > K^+_{out}$) in the presence of valinomycin and sodium ion. Binding studies as a function of pH and titration studies with valinomycin, nigericin, and monensin indicate that binding varies with the electrochemical proton gradient ($\Delta\bar{\mu}_{H^+}$) with a bias toward the electrical component ($\Delta\Psi$). Moreover, when the effect of these ionophores on the kinetics of binding is investigated, it is clear that $\Delta\Psi$ and the sodium gradient (ΔpNa) function in different capacities with respect to ligand binding. $\Delta\Psi$ (interior negative) appears to perturb the porter directly, altering its interaction with sodium so as to allow the cation to effect an increase in binding affinity, while ΔpNa ($Na^+_{out} > Na^+_{in}$) increases the number of binding sites independent of $\Delta\bar{\mu}_{H^+}$. It is concluded that sodium binding precedes solute binding and translocation.

The use of impermeant galactoside analogues has yielded unique information regarding the dynamics of the solute-specific component of the β-galactoside transport system in membrane vesicles isolated from *Escherichia coli* (for reviews, cf. Schuldiner et al., 1976b; Schuldiner & Kaback, 1977). These studies indicate that 90% or more of the *lac* carrier protein (i.e., M protein) is cryptic to ligand unless the membrane is "energized". Oxidation of electron donors which drive transport in the vesicles leads to the generation of an electrochemical gradient of protons ($\Delta\bar{\mu}_{H^+}$) across the vesicle membrane [interior negative and alkaline] (Ramos et al., 1976; Ramos & Kaback, 1977a–c) and a dramatic increase in the number of binding sites for (*N*-dansyl)aminoalkyl 1-thio-β-D-galactopyranosides (Reeves et al., 1973; Schuldiner et al., 1975a–c, 1976a, 1977) and *p*-nitrophenyl α-D-galactopyran-

† From the Laboratory of Membrane Biochemistry, The Roche Institute of Molecular Biology, Nutley, New Jersey 07110. *Received August 16, 1977.*

0006-2960/78/0417-0698$01.00/0