ALPHA-AMANITIN-CONCANAVALIN A CONJUGATES AS INHIBITORS OF SPECIFIC CELL TYPES

By

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ALPHA-AMANITIN-CONCANAVALIN A CONJUGATES AS INHIBITORS OF SPECIFIC CELL TYPES

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Macromolecular conjugates of the fungal toxin α-amanitin and concanavalin A (Con A) were used to evaluate the ability of Con A to impart selectivity with respect to cellular uptake to α-amanitin. The efficiency of targeting to specific cellular receptors via Con A saccharide binding sites, the degree to which these receptors could facilitate entry of toxin into the cell, and determination of resultant α-amanitin mediated cytotoxicity comprise the major objectives of the study.

Covalent α-amanitin-Con A conjugates prepared with a free carboxyl group containing derivative of α-amanitin (ADGG) via reaction with carbodiimides contained an average of 3.6 moles ADGG per mole Con A. They retained a high degree of binding specificity and affinity for calf thymus RNA polymerase II <u>in vitro</u> ($K_I = 27 \times 10^{-9}$M versus 6.9×10^{-9}M for free ADGG). The ADGG-Con A conjugates

absorbed to Sephadex G-75 and eluted with specific saccharide in volumes identical to native Con A. The conjugates agglutinated red blood cells at near equivalent concentrations to Con A. Evidence from other Con A conjugates substituted with hippuric acid as an analog for ADGG indicate that the ADGG-Con A conjugates retain full lectin associated ligand binding specificities.

Exposure of H-7 CHO cells for short periods of time to ADGG-Con A resulted in 50% inhibition of cell proliferation at 2.1×10^{-7}M with respect to ADGG concentration. At that concentration, free ADGG or the equivalent amount of Con A to that present in the conjugate had no effect. Extrapolation from the maximum doses of ADGG used indicates that a minimum 50-fold enhancement of the toxicity of free ADGG is achieved by conjugation to Con A. Cell binding measurements determined that ADGG-Con A is bound by H-7 CHO cells in identical amounts to ^{125}I-Con A, that it competes for ^{125}I-Con A binding sites as efficiently as native Con A, and that its binding is inhibitable by preincubation of cells with Con A or by the presence of the Con A specific ligand, α-methyl-D-mannopyranoside (MDM). The potent toxicity of ADGG-Con A for H-7 CHO was abolished by treatment of the conjugate with MDM prior to exposure of the cells. These data strongly suggest that ADGG-Con A conjugates derive their enhanced toxicity for CHO cells from the binding to specific cell receptors with subsequent internalization of the bound α-amanitin. Confirmation of

this hypothesis was obtained from the interaction of α-amanitin resistant rat fibroblasts (LAN-2) with ADGG-Con A. LAN-2 possess an altered RNA polymerase II with greatly reduced affinity for α-amanitin. Although LAN-2 bind Con A as well as their parent cell line, they were refractory to the toxic effects of ADGG-Con A.

These experiments demonstrate that covalent conjugates of Con A and α-amanitin retain the biological activity of both portions of the conjugate. Furthermore, the data clearly indicate that Con A permits efficient and specific targeting of amanitin to cells as a function of interaction with specific saccharide receptors on the cell surface.

SECTION I
INTRODUCTION

Objectives

The overall objectives of this research were to assess the potential of selected proteins for targeting of inhibitors to specific cells and to evaluate the ability of cell membrane receptors to mediate uptake of these protein-inhibitor conjugates. The specific objectives defined for accomplishing this were: 1) synthesis and biochemical characterization of conjugates of α-amanitin and bovine serum albumin (BSA) and concanavalin A (Con A), 2) determination of the degree to which Con A conjugates retain the biological properties of each component of the conjugate, and 3) the use of α-amanitin-Con A conjugates to investigate the effectiveness of Con A as a targeting vehicle for α-amanitin based on the numbers and/or affinities of Con A receptor sites present on the target cell. The effectiveness of Con A for imparting selectivity with respect to cellular uptake to α-amanitin and the ability of cell surface receptors to mediate the entry of bound toxin and thereby be specifically killed, were used as criteria for assessing the degree to which these objectives were fulfilled.

Background

Alpha-amanitin is one of a group of fungal peptide toxins isolated principally from members of the fungal genus *Amanita* (Wieland, 1968). The structures of the amanitins have been rigorously established as bicyclic octapeptides containing two moles glycine, and one mole each of hydroxytryptophan, cysteine, hydroxyproline, asparagine (or aspartic acid), isoleucine and a hydroxylated isolencine, which varies depending on the derivative (Wieland and Wieland, 1972; Fiume and Wieland, 1970; Wieland and Faulstich, 1978). The sulfur atom of the cysteine residue is connected to the indol moiety of the tryptophan ring via a sulfoxide bridge dividing the molecule into two rings. Alpha-amanitin, the most common derivative, contains asparagine and dihydroxyisoleucine and because of its availability is the most thoroughly investigated of the amanitins.

The amanitins have been proven to possess extreme toxicity for mammalian cells, *in vitro* and *in vivo* (Wieland and Wieland, 1959; Fiume and Wieland, 1970; Sekeris and Schmidt, 1972). Amanitins derive their toxicity from the ability of the toxins to bind with high affinity to eucaryotic DNA-directed RNA polymerase II (Stirpe and Fiume, 1967; Seifart and Sekeris, 1969; Lindell at al., 1970; Jacob et al., 1970). The specificity and high affinity of the interaction of amanitins with polymerase is reflected by the

inhibition constant (K_I) which was determined for calf thymus RNA polymerase II to be 3-10 x 10^{-9}M (Cochet-Meilhac and Chambon, 1974). The amanitin mediated inhibition of RNA polymerase II results in cell death due to the lack of messenger RNA (mRNA) synthesis. RNA polymerases II are those polymerases identified as being responsible for most of the mRNA synthesis in eucaryotic cells (Chambon, 1975). Evidence for this is derived from several observations. The synthesis of poly-adenosine containing heterogenous nucleoplasmic RNA (hnRNA) by HeLa cells is inhibited by low concentrations of α-amanitin (Zylber and Penman, 1971). HnRNA is a precursor in eucaryotic cells to those species of RNA that have been identified as functional mRNA (Brawerman, 1974). Low concentrations of α-amanitin have also been shown to inhibit the synthesis of adenovirus mRNA in human KB cells (Ledinko, 1971) but do not affect the synthesis of mRNA by viruses which use virus-coded RNA-dependent RNA polymerases that are amanitin resistant (Wieland and Faulstich, 1978).

Mammalian cell lines resistant to the effects of α-amanitin have provided additional verification of the molecular target of α-amanitin. Isolation and purification of RNA polymerase II from α-amanitin resistant Chinese hamster ovary (CHO) cells yielded an enzyme resistant to α-amanitin (Chan et al., 1972). Amanitin resistant mutants of BHK-T6 hamster cells (Amanti et al., 1975), rat myoblasts (Somers et al., 1975), mouse myeloma MOPC 104 E cells

(Wulf and Bautz, 1976) and human diploid fibroblasts (Buchwald and Ingles, 1976) have all demonstrated similarly resistant polymerase II molecules as being responsible for the observed α-amanitin resistance. Thus α-amanitin can be seen to be a structurally well characterized toxin that possesses an extremely specific mechanism of action. The properties of high specificity, known mechanism of action and low concentration at which it is inhibitory make α-amanitin an ideal inhibitor for examining targeting of inhibitor conjugates to specific cell receptors.

Conjugates of the amanitins and macromolecules were first prepared as an attempt to produce antibodies to β-amanitin as a haptenic substituent linked to rabbit serum albumin (RSA) (Cessi and Fiume, 1969). Beta-amanitin,which contains a free carboxyl group, was directly coupled to RSA by reaction with water soluble carbodiimides. The conjugates not only failed to elicit antibody production, but were found to be approximatley 10-fold more toxic to mice than free β-amanitin (Cessi and Fiume, 1969). The conjugated β-amanitin retained its specificity of interaction with RNA polymerase II but with a reduced binding affinity (Fiume et al., 1971; Derenzini et al., 1973). Furthermore the enhanced *in vivo* toxicity of the β-amanitin conjugates was determined to result from increased uptake by the sinusoidal cells of the liver and the proximal tubule cells of the kidney, presumably because of the protein portion of the conjugate (Fiume, 1969; Fiume et al., 1969). Cultured

cells with high rates of protein uptake, e.g. macrophages, were preferentially killed by conjugated β-amanitin (Barbanti-Brodano and Fiume, 1973; Barbanti-Brodano and Fiume, 1974; Fiume and Barbanti-Brodano, 1974). Further coupling of β-amanitin-albumin conjugates to fluorescein enhanced the conjugate toxicity for hepatocytes, cells known to possess fluorescein receptors (Fiume et al., 1971). Although very qualitative in nature, this work demonstrated that certain conjugates of β-amanitin were preferentially taken up by cells on the basis of the macromolecular portion of the conjugate. Subsequent cell death resulted presumably as a result of transport of the conjugate, or minimally, the amanitin portion to the nucleus where inhibition of mRNA synthesis occurred (Faulstich et al., 1975). However, it is likely that the enhanced toxicity of these β-amanitin conjugates is a relatively non-specific phenomenon based upon pinocytosis by cells with a high rate of protein uptake. The carbodiimide procedure used for coupling can result in protein cross-linking and ester formation, increasing the molecular weight of the conjugate and decreasing the stability of the linkage (Carraway and Koshland, 1972; Timkovich, 1977). Increased molecular weight and decreased stability of binding of the inhibitor and carrier protein would be apt to favor pinocytic uptake and degradation of the conjugates.

Other conjugates of β-amanitin and proteins have been synthesized by formation of the hydroxysuccinimide ester

of β-amanitin which is then directly linked to amino groups on proteins (Faulstich et al., 1975). This procedure should not have the carbodiimide associated side reactions and would lead to well defined, covalently linked conjugates. To date, no additional work beyond the initial publication has been presented to define the characteristics of these conjugates. Furthermore, β-amanitin is a relatively minor component of the naturally occurring amatoxins and is not readily available.

Conjugates of the more widely available α-amanitin were first prepared by the modification of α-amanitin to a derivative containing a free amino group which can be coupled to proteins via carbodiimides (Faulstich and Trischmann, 1973). These derivatives when conjugated to BSA were shown to be equally as toxic *in vivo* as free α-amanitin. *In vitro* inhibition of calf thymus RNA polymerase II by the α-amanitin-BSA conjugates was 20-fold less than free α-amanitin. These studies as well as the previously described investigations with β-amanitin conjugates pointed out the feasibility of using amanitin-protein conjugates for exploring the targeting of inhibitors to specific cells. They also underlined the need for careful, quantitative evaluation of the interaction of a well characterized conjugate with the cellular target.

Investigations of drug targeting with inhibitors other than amanitins have primarily centered on increasing the specificity of uptake of an inhibitor by conjugation with

immunoglobulins directed against cell membrane constituents. The inhibitors used are generally non-specific in their mode of action and without some means for increasing the selectivity of uptake they are unable to inhibit a specific subpopulation of cells within a larger population. Without selectivity, they are of limited use for differentially inhibiting the growth of transformed cells, the primary goal of drug targeting research. Various low molecular weight inhibitors have been coupled to protein carriers. Most of these inhibitors have as the basis for their toxicity interaction with nucleic acid structure or synthetic processes. 5-Flurodeoxuridine (FUDR) and albumin were coupled (Barbanti-Brodano and Fiume, 1974), producing conjugates that inhibited transformed 3T3 fibroblasts *in vitro* almost as well as free FUDR but did not inhibit non-dividing macrophages. High molecular weight conjugates of methotrexate and albumin were effective in prolonging the half life of the drug *in vivo* but displayed no selectivity in uptake by cells (Chu and Whiteley, 1977). Triaziquone conjugated to γ-globulin or albumin was toxic to polyoma transformed baby hamster kidney (BHK) cells. Inhibition of pinocytosis did not affect the toxicity of the conjugates, suggesting membrane mediated uptake of the drug. However, normal BHK cells were 3-fold more susceptible to the conjugates than the transformed cell (Linford and Froese, 1978). Additionally, no actual binding of the conjugate was detectable by fluorescein labeling which would imply a distinct lack of

specificity in the uptake of conjugates. Cytosine arabinoside-albumin conjugates were effective in inhibiting viral replication within mouse liver cells, suggesting retention of toxicity after conjugation. However, the high molecular weight complexes that resulted from the synthesis, make non-specific endocytosis a likely route for uptake of these conjugates (Balboni et al., 1976). Greater specificity of uptake and apparent toxicity was obtained with conjugates of p-phenylenediamine mustard and anti-lymphoid cell antibodies (Davies and O'Neil, 1977). Other conjugates of chlorambucil and antibodies against specific cell types displayed selective toxicity and some degree of chemotherapeutic potential, *in vivo* (Ghose et al., 1972). Anti-Ehrlich ascites tumor cell antibody-chlorambucil conjugates also were effective in treatment of the neoplasia (Flechner, 1973). However, in similar studies, Rubens and Dulbecco (1974) have demonstrated a lack of covalent association of chlorambucil with the antibodies, making it difficult to ascertain the exact mechanism of the observed toxicity.

Perhaps the most effective conjugates of immunoglobulin and inhibitors that interact with DNA have been conjugates of daunomycin and anti-mouse lymphoid cell tumor antibodies (Levy et al., 1975). These conjugates were examined for both inhibition of specific tumor cell growth *in vivo* and for *in vitro* inhibition of tumor cell RNA synthesis. In comparison to daunomycin linked to non-specific immunoglobulin, the anti-tumor antibody-daunomycin

conjugates were significantly more effective in inhibiting specific tumor cell processes. These conjugates represent a considerable improvement over those previously discussed in that their synthesis is a result of the interaction of a single reactive group on daunomycin with the immunoglobulin. This results in well defined covalent conjugates that retain the targeting specificity of the immunoglobulin. The primary disadvantage to their use as chemotherapeutic agents is that they interact with DNA rather than a critical enzyme required for macromolecular replication, transcription or translation and would thus require much larger doses to achieve a given cytotoxic effect. This presents a significant problem in view of the fact that the inhibitors will act on normal as well as neoplastic cells with potentially detrimental side effects.

Conjugates of diphtheria toxin and immunoglobulins directed against cell surface antigens have proven to be selective as well as effectively cytotoxic. When coupled to immunoglobulin with specificity directed against mumps virus infected monkey kidney cells, the diphtheria toxin conjugates were selectively toxic to virally infected cultures (Moolten and Cooperband, 1970). Other conjugates of diphtheria toxin and anti-DNP antibodies exhibited selective toxicity *in vivo* against hapten (DNP) coated tumor cells in hamsters (Moolten et al., 1972). Hamster lymphoma growth was effectively suppressed by conjugates of diphtheria toxin and antibodies prepared against SV-40

transformed cells (Moolten et al, 1976). HeLa cells that had been coated with hapten (2,4,6-trinitrophenyl sulfuric acid) were killed by diphtheria toxin-antibody conjugate only when the antibody was hapten specific (Philpott et al., 1973). Mouse anti-lactate dehydrogenase antibody when coupled to diphtheria toxin resulted in a conjugate more toxic for Erhlich ascites cells than for normal mouse kidney cells due to increased expression of lactate dehydrogenase on the ascites cells (Samagh and Gregory, 1972).

Thorpi et al. (1978) coupled purified antilymphocytic globulin-chlorambucil conjugates to dephtheria toxin via an activated anhydride reaction that yielded well defined conjugates which could readily be purified by gel filtration. The conjugates were significantly more toxic to cultured lymphoblastoid cells than was the free toxin. The conjugates overcame a significant problem with the synthesis of diphtheria toxin-protein conjugates described above. Dephtheria toxin is a protein macromolecule which is readily cross-linked during most of the procedures used to produce conjugates. This results in ill-defined preparations of varying compositions and specificities.

The advantage to using dephtheria toxin resides in its mechanism of toxicity. Diphtheria toxin is a potent inhibitor of eucaryotic protein synthesis by virtue of its catalytic ADP-ribosylation of elongation factor, EF-2 (Collier, 1975).

Chang and Neville (1977) synthesized conjugates of diphtheria toxin and human placental lactogen that contained equimolar ratios of toxin to carrier protein. The conjugates bound effectively to lactogen receptors on mammary gland explants but these receptors were unable to mediate entry of the toxin to the cell as the conjugate did not inhibit protein synthesis in the target cells (Chang et al., 1977; Neville and Chang, 1978).

Although previously described conjugates using albumins and other proteins without defined cell binding activities resulted in some degree of enhanced cytotoxicity, only those conjugates that could interact with specific receptors on the target cell produced significant cell specific toxicity. One other category of receptor specific macromolecules besides antibody and hormones that may mediate the targeting of inhibitors are lectins. Lectins are proteins that possess specific binding sites for carbohydrate moieties. They have been isolated from a wide variety of plants and animals and possess a broad range of individual binding specificities. Their specific interaction with cell surface glycoproteins has been thoroughly documented and leads to an array of complex cellular responses including agglutination, mitogenic stimulation and cell toxicity (Sharon and Lis, 1972; Lis and Sharon, 1973; Nicolson, 1974).

Perhaps the most widely investigated lectin is that protein isolated from the jack bean, Concanavalin A.

Con A has been extensively studied and characterized with respect to ligand binding specificity (Goldstein et at., 1965; So and Goldstein, 1968), chemical structure and properties (Edelman et al., 1972; Sharon and Lis, 1972) and interactions with cell surfaces (Nicolson, 1974). The effects of Con A on cells are widely varied and include agglutination, induction of mitosis, alterations in cell permeability and transport phenomena as well as cytotoxicity. The mechanisms of these complex interactions of Con A are by no means clear but they all contain as a central feature the binding of Con A to cell surface glycoproteins. Since the extent of the effect of Con A on a given cell is related to the surface architecture, density and mobility of the cell surface glycoprotein and glycolipid constituents, it seems plausible that variations on Con A receptors could lead to differential toxicity of Con A-inhibitor conjugates.

The observations that Con A induces agglutination of some virally transformed cells at a concentration of lectin much lower than that required for agglutination of normal cells (Inbar and Sachs, 1969; Burger, 1969), implied that an inherent difference in membrane architecture between normal and transformed cells may exist. Attempts to quantitatively detect a difference in the number of Con A binding sites on normal cells and their virally transformed counterparts met with limited success (Cline and Livingston, 1971; Ozanne and Sambrook, 1971). Differences, if any, were extremely small

and intrepretations were complicated by variation in technique and cell lines. Using low temperature conditions, Noonan and Burger (1973a) were able to demonstrate that in the absence of endocytosis and with appropriate corrections for surface area and volume differences, certain virally transformed cells possessed 3 to 5 times the number of Con A binding sites of a normal cell. Similar results were obtained with normal cells at mitosis (Noonan et al., 1973), after brief protease treatment (Noonan and Burger, 1973b) or exposure of certain cell lines to dibutyrylcyclic AMP (Veen et al., 1976). Under these conditions slight differences in the numbers of Con A binding sites could be detected. Although the relationship of the Con A binding to increased agglutinability or the transformed state still remains undetermined, the altered surface structure of transformed cells may allow for differential interaction with Con A-inhibitor conjugates in comparison to normal cells. Kitao and Hattori (1977) tested a conjugate of Con A and daunomycin for its ability to suppress the *in vivo* development of Ehrlich ascites and L1210 cells. Prolonged survival of the host in comparison to free daunomycin was obtained following administration of the conjugate with either cell type. While characterization of this conjugate was minimal and the test results qualitative in nature, selective toxicity of Con A conjugates was implied. The use of Con A as a targeting agent would therefore seem feasible based on the known differences between cells with respect to

quantitative and qualitative variations in cell membrane receptors for the lectin. Con A would also provide a means for assessing the effects of conjugation on the biological activities of the native lectin.

Rationale

Alpha-amanitin presents several distinct advantages over most inhibitors used for investigations of drug targeting. The affinity of the interaction of α-amanitin with eucaryotic RNA polymerase II make it an extremely potent inhibitor. Moreover, α-amanitin is of low molecular weight and after modification can be coupled via a single reactive site to proteins. This will allow for reproducible production of conjugates that have minimal structural interference by the amanitin moiety. Work by other investigators has clearly demonstrated that amanitin conjugates retain inhibitory potential for RNA polymerase II. The fact that α-amanitin inhibits an enzyme present in limited quantities, approximately 10^4 RNA polymerase II molecules per cell (Cochet-Meilhac et al., 1974), that is critical to survival of the cell will allow relatively low doses of inhibitor to generate a measureable cytotoxic effect. Radioactively labeled derivatives of α-amanitin may be synthesized and can be used to quantitate the coupling to protein as well as the binding to specific cells. Therefore, the initial phase of this investigation consists of developing procedures necessary for the synthesis of α-amanitin conjugates of defined

chemical nature that retain inhibitory potential for RNA polymerase II.

For targeting of α-amanitin to specific cell receptors Con A will be used. Con A is well characterized chemically and,unlike specific immunoglobulins, is available in quantity from commercial sources. The conjugation with α-amanitin can be monitored by a number of biochemical and biological parameters for its effects on lectin activity. Specific ligands with known binding affinities for Con A are available, as are defined systems for evaluating the interaction of Con A conjugates with cells.

Following synthesis and characterization of the α-amanitin-Con A conjugates, the final aspect of the study will be to evaluate the potential of Con A for targeting α-amanitin to cells and the ability of Con A receptors to mediate uptake of bound conjugate. Although it would appear that the differences in Con A binding between normal and virally transformed cells would represent a potential system for discerning targeting differences of Con A conjugates, the conditions necessary to achieve quantitative differences in the number of cell receptors and the low magnitude of the observed differences, suggest other approaches may be more productive. A cell line known to possess a distinct number of Con A receptors, H-7 CHO, will be used to examine the cytotoxicity of Con A-α-amanitin conjugate in comparison to free α-amanitin. The study will be restricted to evaluating whether specific interaction

of the conjugate with membrane receptors for Con A occurs and if the interaction leads to endocytosis of the conjugate. The mechanism of toxicity (if any) of the conjugate will be evaluated by the use of a Con A resistant CHO mutant and an α-amanitin resistant rat fibroblast cell. These cells will differentiate Con A cytotoxicity, anticipated to be minimal with short exposure times and low concentrations, from toxicity due to the amanitin portion of the conjugate.

SECTION II
MATERIALS AND METHODS

Modification of α-Amanitin for Conjugation to Proteins

Isolation and Purification

All chemicals used throughout these investigations were of reagent quality and were used without further purification unless noted otherwise. The primary commercial suppliers were Scientific Products (Mallinkrodt) and Sigma Chemical Co. Water for all procedures was deionized and glass distilled.

The α-amanitin used for these studies was obtained primarily from specimens of *Amanita suballiacea* collected from the Gainesville, Florida,area. Individual specimens were collected, identified on the basis of gross morphological characteristics according to published descriptions (Miller, 1977; Murrill, 1941; Murrill, 1948) and pooled for extraction of α-amanitin. After thorough washing with running tap water, the specimens were coarsely chopped and combined with methanol to an approximate final concentration of 50% methanol (v/v). This crude slurry was shaken on a rotary shaker at 100rpm and 22°C for 24 hours, filtered through Whatman #1 filter paper and the crude methanolic extract was flash evaporated to a thick syrup. Ten volumes of ice cold 100% methanol were added to precipitate

polysaccharides and the mixture was filtered through scintered glass. The resulting filtrate was flash evaporated to near dryness, resolubilized in water and extracted three times with 3 volumes of anhydrous ether. The final extract was made 50% with respect to methanol (spectral grade) and subjected to chromatography on Sephadex LH-20 in 50% methanol. The α-amanitin containing fractions were identified by TLC on silica gel G with methyl ethyl ketone: methanol (1:1) and detection after spraying with 2% methanolic t-cinnamaldehyde and exposing to HCl vapors (Wieland et al., 1949; Sullivan et al., 1965). The characteristic R_f and violet color served to identify amanitin containing fractions. The peak fractions were combined, flash evaporated to dryness, resolubilized in water and chromatographed on Biogel P-2 in water. After concentration of pooled α-amanitin fractions from this step, final purification was obtained by chromatography on Sephadex LH-20 with water. The resulting α-amanitin was characterized as to purity on the basis of TLC mobility with two different solvent systems (methanol: methyl ethyl ketone (1:1), n-butanol:acetic acid:water (4:1:1), ultraviolet (uv) and visible absorption spectra, and nuclear magnetic resonance (NMR) spectroscopy. All samples prepared proved identical by these criteria to a crystalline α-amanitin standard obtained from Th. Wieland, Max Planck Institute for Medical Research, Heidelberg, Germany. These purification procedures were developed from procedures previously published (Faulstich, et al., 1973;

Wieland, 1968) by other investigators. Additional details of the experiments resulting in the final purification procedure are contained in a manuscript currently in preparation for publication (Preston et al., in preparation for submission to Lloydia).

Derivatization

The absence of either a free carboxyl or amino group on α-amanitin necessitated the development of methods for modifying the basic amanitin structure to allow direct coupling of α-amanitin to proteins. The basic concept was derived from the work of Faulstich and Trischmann (1973) in which α-amanitin was coupled via diazotization to an aromatic group linked to a six carbon spacer molecule containing a free terminal amino group. Their procedure was used with some modification to produce α-amanitin derivatives with free amino groups. A derivative containing a free carboxyl group was obtained by using the diazonium coupled spacer molecule approach with new procedures developed by J. F. Preston (Preston and Hencin, 1979). Details of both of these syntheses are presented below.

For production of α-amanitin derivatives containing terminal amino groups, α-amanitin was diazotized to N-(4-aminobenzoyl)-N'-BOC-hexamethylenediamine. Mono-BOC-1,6-diaminohexane was prepared according to procedures described by Faulstich and Trischmann (1973). N-(4-nitrobenzoyl)-N'-BOC-hexamethylenediamine was prepared by acylation of mono-BOC-1,6-diaminohexane with 4-nitrobenzoyl chloride. Catalytic

reduction with hydrogen and palladium/$BaSO_4$ of N-(4-nitrobenzoyl)-N'-BOC-hexamethylenediamine yielded N-(4-aminobenzoyl)-N'-BOC-hexamethylenediamine which was subjected to diazotization with α-amanitin. In a typical reaction 34mg of $NaNO_2$ were added to 112mg of N-(4-aminobenzoyl)-N'-BOC-hexamethylenediamine in 7ml of 50% acetic acid (5°C) and incubated 10 minutes at 5°C. This diazonium cation containing solution was combined with 65mg of α-amanitin dissolved in 4.5ml of pyridine (5°C). After 15 minutes of reaction at 5°C, the mixture was flash evaporated to dryness (40°C with vacuum for rapid removal of pyridine and other solvents), resolubilized in spectral grade methanol and applied to a column of Sephadex LH-20 in methanol (4.25 x 95cm). The α-amanitin derivative, α-amanitin-diazobenzoyl-N-N'-BOC-hexamethylenediamine (ADBH) was identified as a reddish-purple band eluting in 0.54 column volumes by its typical mobility during TLC in methanol: methylethylketone (Faulstich and Trischmann, 1973). The ADBH was further characterized by absorption and NMR spectra and stored at room temperature, dried, in darkness until used (Hencin and Preston, 1979).

Free carboxyl group containing α-amanitin derivatives were produced by diazotization of α-amanitin with p-aminobenzoylglycylglycine (Preston and Hencin, manuscript in preparation). p-Aminobenzoylglycylglycine was prepared by catalytic hydrogenation of p-nitrobenzoylglycylglycine with Pd/$BaSO_4$. For coupling to α-amanitin, 30mg of p-aminobenzoylglycylglycine dissolved in 1.0ml of 50% acetic acid

were cooled to 0.2°C and 10mg of $NaNO_2$ were added. Following 10 minutes of occasional shaking, 18.5mg of α-amanitin in 2.0ml cold pyridine were added. After 10 more minutes at 4°C the reaction mixture was dried in vacuo, resolubilized in 4.0ml 80% methanol and chromatographed on Sephadex LH-20 in 80% methanol. Identification of the resulting product, α-amanitin-diazobenzoylglycylglycine (ADGG) was achieved by TLC, uv and NMR spectroscopy.

A tritium labeled derivative of ADGG used in these studies was prepared and purified by Dr. J. F. Preston. The derivative, ^{3}H-demethyl-ADGG (^{3}H-DM-ADGG) was synthesized by oxidation of ADGG with sodium periodate and subsequent reduction with ^3H-$NaBH_4$. Purification by column chromatography on Sephadex LH-20 with various buffers to isolate the product and remove exchangeable tritium resulted in a product with a specific activity of 7.4×10^8dpm/μmole. The ^{3}H-DM-ADGG was determined to be essentially free from contaminants detectable by TLC and fluorography according to the methods of Randerath (1970). The absorption spectra and inhibition obtained for calf thymus RNA polymerase II as well as TLC mobility indicated that it is identical to ADGG.

Synthesis of α-Amanitin-Protein Conjugates

ADH-BSA

Conjugation to bovine serum albumin (BSA) was performed by modifications of the methods presented by Faulstich and Trischmann (1973). The first step of the conjugation was

removal of the t-BOC group from ADBH by dissolving 6mg of ADBH in 5ml of dry trifluoroacetic acid at room temperature, swirling for 1 minute followed by immediate evaporation of the acid at 40°C in vacuo. The resulting compound, α-amanitin-diazobenzoylhexamethylenediamine (ADH), contains a free amino group that may be coupled directly to free protein carboxyl groups by reaction with water soluble carbodiimides (Carraway and Koshland, 1972). Twenty milligrams BSA dissolved in 2ml water with 200mg of N-ethyl-N'(dimethylaminopropyl)-carbodiimide HCl (EDC) were added to the dried ADH in a round bottom flask. After 24 hours at room temperature with occasional mixing, the reaction mixture was adjusted to 0.05% with respect to NH_4HCO_3 by addition of solid NH_4HCO_3, applied to a Sephadex G-75 column (2.5 x 30cm) pre-equilibrated with 0.05% NH_4HCO_3, and eluted with the same buffer. Conjugated ADH-BSA eluted as a single peak followed by unreacted ADH which was pooled and concentrated for reuse. The peak of protein conjugate was analyzed for protein content by uv absorbance at 280nm ($E^{1\%}_{1cm}$ = 6.61) and by the Lowry method (Lowry et al., 1951), for α-amanitin content as indicated from absorption of the diazo linkage at 384nm (E^{384} = 14000cm^2/mMole) and by inhibition of calf thymus RNA polymerase II. After analysis, individual fractions were pooled, lyophilized and stored at -20°C.

ADH-Con A

Conjugates of α-amanitin and concanavalin A (Con A) were prepared in a similar fashion to the ADH-BSA conjugates.

Five milligrams of dried ADBH were dissolved in 4ml dry trifluoroacetic acid, swirled for 1 minute and evaporated to dryness at 40°C. Twenty milligrams of Con A (Sigma, grade IV) dissolved in 2.5ml of water with 200mg EDC were added to the ADH and reaction was carried out at room temperature with intermittant mixing. A fine precipitate was formed after 2 hours that settled out of the reaction mixture. After 24 hours the reaction mixture was adjusted to 0.05% with respect to NH_4HCO_3 and applied to a Sephadex G-75 column with reasonable care taken to leave the precipitate in the reaction vessel. The conjugate ADH-Con A eluted with 0.05% NH_4HCO_3 as a single peak followed by the unreacted ADH. Analysis of the individual fractions by absorption at 280nm ($E^{1\%}_{1cm}$ = 1.14) and 384nm and analysis of the pooled ADH-Con A peak by inhibition of calf thymus RNA polymerase II was performed.

ADGG-BSA

Conjugation of α-amanitin to free amino groups on BSA was undertaken with the ADGG derivative by procedures similar to those used to couple ADH. ADGG (3.34mg) was dissolved in 2.0ml of water along with 16.2mg of BSA. The pH was adjusted to 7.2 with 0.1N NaOH and 200mg of EDC were added. After 24 hours of reaction at room temperature the product ADGG-BSA was isolated by gel filtration on Sephadex G-75 with 0.05% NH_4HCO_3 as eluant. The conjugate peak was analyzed by absorption at 280nm and by the Lowry assay to determine protein content. Alpha-amanitin content was determined from the peak absorption of the diazonium moiety which for ADGG

is at 395nm (E^{395} = 1400cm^2/mMole). Inhibition of calf thymus RNA polymerase II was determined for the peak fractions and the ADGG-BSA conjugate was lyophilized and stored at -20°C.

ADGG-Con A

The optimal reaction conditions for production of ADGG-Con A conjugates were determined from a series of experiments utilizing ^{14}C-hippuric acid (^{14}C-HA) as a free carboxyl group containing analog for ADGG. The optimal conditions for conjugation with Con A were verified in studies with ^{3}H-DM-ADGG. Details of the ^{14}C-HA and ^{3}H-DM-ADGG reactions are presented in later sections. The procedure described below represents a typical synthesis used for the production of ADGG-Con A conjugate.

The Con A used for reaction with ADGG or hippuric acid was purified by affinity chromatography on Sephadex G-100 followed by exhaustive dialysis versus water and lyophilization as described by Agrawal and Goldstein (1965). Purified, lyophilized Con A was stored at -20°C until used. Prior to conjugation, Con A was dissolved to an approximate concentration of 10mg/ml in 0.01M phosphate buffer, pH 5 and allowed to remain at room temperature for 1 hour. The turbid solution was then clarified by centrifugation at 10,000rpm (Beckman J-21B centrifuge; JA-20 rotor) for 30 minutes. The supernatant was filtered immediately prior to use through a 0.2μ Millipore filter and the concentration determined by absorbancy at 280nm. Reactants were added in the following order to achieve final concentrations as listed: 0.01μmoles/ml

Con A, 1.0μmole/ml ADGG and sufficient 0.1M phosphate buffer, pH 5 to give a final concentration of of 0.01M phosphate. The reaction was initiated by addition of 50μl of 1mmole/ml EDC in water per milliliter of reaction mix final volume. The reaction was allowed to proceed at room temperature for 12 hours after which time it was adjusted to 0.15M with respect to NaCl and applied to a small column of Sephadex G-100 in 0.15M phosphate buffered saline, pH 7.4 containing 0.1mM maganese and calcium (PBS^+). The column bed volume was generally 2 to 2.5 times the reaction mix volume. The reaction mixture was allowed to enter the column at a rate of approximately 0.1ml/minute. Unreacted ADGG emerged from the column first as essentially 100% of the Con A as ADGG-Con A conjugate bound to the gel. After elution with 10 column volumes of PBS^+, 0.1M D-glucose was added to the eluant causing the displacement of ADGG-Con A in a single well-defined band. The conjugate was characterized by absorption at 280 and 395nm and dialyzed extensively against 0.15M PBS^+, pH 7.4 at 3-5°C. ADGG-Con A conjugates were stored at 3°C and were used within three weeks of their synthesis. During this period of time essentially no change in protein concentration or absorbance spectra were noted.

<u>^{3}H-DM-ADGG-Con A</u>

Verification of the optimal conditions for conjugation of ADGG and Con A and production of a tritium labeled Con A conjugate for cell binding studies were performed with ^{3}H-DM-ADGG. Details of the individual experiments are

presented in the figure legends. The general format for determining the extent of conjugation over a period of time was to first establish the specific reaction conditions desired. The different parameters investigated included buffer concentration and pH, Con A, ^{3}H-DM-ADGG and EDC concentrations. All reactions were initiated by the addition of carbodiimide. At the desired sampling point, duplicate 50 or 100µl samples were removed to small tubes containing 200µg each of RNA and BSA in 0.02M $Na_2P_2O_7$ in 0.1ml and placed on ice. Cold 10% TCA, 2.0ml, was immediately added and after 15 minutes on ice the precipitates were collected on GF/C glass fiber discs and processed for scintillation counting as described by Preston et al. (1975).

Production of ^{3}H-DM-ADGG-Con A for cell binding was accomplished by reaction of 0.0032µmoles of Con A with 0.22µmoles of ^{3}H-DM-ADGG (specific activity = 7.4 x 10^8dpm/µmole) with 75µmoles of EDC in 1.5ml of 0.01M phosphate buffer pH 5. After 12 hours of reaction, the preparation was applied to a column of Sephadex G-75 (0.2ml bed volume) pre-equilibrated with 0.15M PBS^+, pH 7.4 and eluted with the same buffer. Following elution of 80ml, 0.1M D-glucose was added to the eluting buffer. Individual 1.0ml fractions were sampled for determination of total radioactivity in Bray's solution and for TCA precipitable activity as described above. The ^{3}H-DM-ADGG-Con A peak was pooled after analysis and dialyzed versus three two liter volumes of PBS^+ at 5°C.

^{14}C-HA-Con A

Evaluation of the optimal reaction conditions for carbodiimide mediated coupling of free carboxyl containing derivatives of α-amanitin to Con A was performed with the use of ^{14}C-hippuric acid as an analog. The reaction of ^{14}C-HA and Con A was examined under a number of different conditions. Reactions were generally carried out in a final volume of 1.0ml at room temperature. Procedures for sampling the reaction at various time points were those previously described for ^{3}H-DM-ADGG reactions. The reaction buffer was either 0.1M sodium phosphate, pH 5, 6 or 7, or pH 7.2 NaCl, 0.01 or 0.1M containing either 0.1 or 0.01M $CaCl_2$ and $MnCl_2$, respectively. Con A, (0.001μmole/ml) and 1.0μmole/ml of ^{14}C-carboxyl hippuric acid (8.5 x 10^5dpm/μmole, ICN Chemical and Radioisotope Division) were reacted with either 10 or 100μmoles of EDC in the buffer systems described above.

HA-Con A

Conjugates of hippuric acid and Con A without ^{14}C label were prepared for analysis of the retention of lectin characteristics by the HA-Con A conjugates. Two different reaction conditions were chosen for these studies. Reaction mixtures containing 0.001μmole/ml Con A, 0.1μmole/ml HA and 10μmole/ml EDC were prepared in either 0.1M phosphate buffer, pH 5 or 0.1M NaCl, pH 7. The reactions were incubated at room temperature for 2 hours followed by overnight dialysis against 0.15M PBS^+ at 5°C to remove unreacted components. The resulting HA-Con A conjugates designated HA-Con A (PO_4) and HA-Con A (NaCl) were used without further purification.

Biochemical Characterization of Conjugates

Spectroscopy

As previously mentioned all conjugates were analyzed for protein content on the basis of their uv absorption at 280nm and for their α-amanitin content at 384nm (ADH conjugates) or 395nm (ADGG conjugates). A Beckman dual beam spectrophotometer and recorder were used for spectral studies with standard 1cm pathlength quartz cuvettes. Measurements were made with aqueous solutions unless noted otherwise.

Protein Determination

Protein determinations were made by minor modification of the Lowry-Folin assay (Lowry et al., 1951). Crystalline BSA (Sigma, lyophilized, crystallized) or affinity purified Con A were used as standards. The assay was performed at 20-22°C with 0.1ml of protein sample containing between 20 and 300μg of protein per milliliter. Results were obtained by sample absorption at 600nm, a wavelength at which no interference from the chromogenic diazo linkage of the conjugates was found. Duplicate or triplicate determinations were made for each assay point and standard.

Analysis of Conjugate Linkages

Conjugate bond formation. The nature of the chemical bond formed between the protein (BSA or Con A) and the EDC coupled moiety was examined with radioactively labeled ^{14}C-HA-Con A and ^{3}H-DM-ADGG-Con A conjugates. The stability of the bond to hydrolysis by hydroxylamine was determined by exposure of the conjugates to 0.5M hydroxylamine at 37°C

for 12 hours (Carraway and Koshland, 1972). The remaining TCA precipitable activity in comparison to a nonhydroylzed control sample was used as an indicator of covalent bonding.

Protein-protein cross-linking. The extent of protein to protein cross-linking induced during the carbodiimide conjugation was evaluated for ADH-BSA and ^{14}C-HA-Con A conjugates by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Weber and Osborn (1969). Electrophoresis was performed with 5% acrylamide disc gels run at 8 milliamp per gel. Visualization of the bands was achieved by staining with Commassie brilliant blue (0.1%) in 50% TCA for 1 hour at 37°C (Laemmli, 1970). Diffusion destaining with multiple changes of 7% acetic acid was carried out to remove dye not associated with protein.

Inhibition of Calf Thymus RNA Polymerase II

Calf thymus RNA polymerase II was purified through DEAE cellulose chromatography according to the method of Kedinger, et al. (1975) and stored in liquid N_2. A single preparation of enzyme used for all studies presented here proved to be extremely stable in liquid N_2 over a four year period. Assay of the inhibition of calf thymus RNA polymerase II was performed according to the procedures developed by Preston, et al. (1975). The reaction mixture used was that described by Cochet-Meilhac and Chambon (1974). The inhibition assays were performed as follows: To the bottom of a new 10 x 75mm test tube was added 0.01ml of each inhibitor concentration

being tested followed by 0.03ml of enzyme. The reaction was initiated by adding 0.06ml of reaction mix containing 5-^{3}H-uridine triphosphate (^{3}H-UTP). After 10 minutes at 37°C the reaction was stopped by addition of 0.1ml of 0.02M $Na_2P_2O_7$ with 2.0mg/ml each of RNA and BSA and placed on ice. Ice cold TCA/$Na_2P_2O_7$ (7% TCA, 0.02M $Na_2P_2O_7$) was added(2.0ml/tube) and the tubes allowed to stand on ice for 15 minutes. Precipitates were collected on GF/C glass fiber discs pre-washed with TCA/$Na_2P_2O_7$ on a vacuum manifold. Collected precipitates were washed three times with TCA/$Na_2P_2O_7$, three times with ethanol:ether (3:1), two times with ether, air dried at 80°C and counted in a toluene based scintillation cocktail.

Estimation of the apparent inhibition constant for each inhibitor was performed determining the inhibition of ^{3}H-UTP incorporation obtained by a series of increasing concentrations of inhibitor for a constant quantity of enzyme and a known concentration of labeled substrate (^{3}H-UTP). The same assay was repeated with two or three different concentrations of substrate. These results when plotted according to the method of Dixon (Dixon and Webb, 1958) yield a value for the apparent inhibition constant, K_I.

Determination of Lectin-Associated Properties

Binding to Sephadex. Ligand binding activity of the conjugates was examined on the basis of their interaction with Sephadex G-75. Preparations of conjugates were equilibrated with 0.15M PBS$^+$, pH 7.0 by dialysis and applied to a

column of Sephadex G-75 (2.4 x 40cm). After elution of one column volume of PBS^+, 0.1M D-glucose was added to the eluant. Monitoring of fractions for 280nm absorbancy was used to determine the degree of binding to the gel matrix in reference to native Con A.

Ligand affinity constant determination. The apparent affinity constant for the ligand binding of selected conjugates was examined spectrophotometrically by the method of Bessler et al. (1973). A chromogenic ligand, p-nitrophenyl α-D-mannopyranoside (PNPM) generates a uv difference spectrum following interaction with Con A the magnitude of which is proportional to the affinity constant. A series of dilutions of PNPM in 0.15M PBS^+ were measured for absorbance at 317nm as was the solution of Con A or Con A conjugate being examined. A constant amount of the protein was then mixed with the PNPM dilutions and 317nm absorbance redetermined. From these data, the following parameters can be defined: P_t, concentration of protein in protein and PNPM mixtures: D_t, concentration of ligand (PNPM); $\Delta A_{317} = [A_{317}\ (\text{protein}) + A_{317}\ (\text{ligand})] - A_{317}\ (\text{protein + ligand})$. A plot of $\frac{1}{\Delta A}$ versus $\frac{1}{[D_t]}$ for values of $\frac{1}{[D_t]}$ much greater than $[P_t]$ yields a y-intercept the reciprocal of which equals ΔAmax or the change in absorbancy when all ligand binding sites are saturated. From this value the affinity constant can be determined by first calculating the concentration of protein-ligand complex, [PD] from the relationship $[PD] = \frac{\Delta A}{\Delta Amax}\ [P_t]$. The concentration of free ligand, [D], is then calculated from $[D] = [D_t] - \frac{\Delta A}{\Delta Amax}\ [P_t]$.

A plot of $\frac{[P_t]}{[PD]}$ versus $\frac{1}{[D]}$ generates a straight line with a slope equal to the reciprocal of the association constant, K_a.

Hemagglutination. The hemagglutinating ability of various Con A conjugates was examined with human type A red blood cells. Standard microtiter plates (Cooke Engineering) and dilutions were used throughout. Agglutinations were determined for serial two-fold dilutions of protein in 0.15M PBS^+, pH 6.8 and red blood cells at 1×10^8 cells per milliliter. After complete mixing and suspension of cells, hemagglutination plates were allowed to stand at room temperature until a control well containing only red blood cells in PBS^+ had formed a well defined pellet. Agglutination titers were read by visual inspection and are expressed as the reciprocal of the largest dilution yielding positive agglutination.

Interaction of Conjugates with Cultured Cells

Maintenance of Cell Cultures

Cultured mammalian cells used for these studies were maintained at 37°C in a 5% CO_2:95% air humidified atmosphere. Tissue culture media, sera and antibiotics were obtained from Flow Laboratories, Rockville, MD or International Scientific Industries, Avon Park, IL. All media were supplemented with 10% fetal calf serum (FCS), pennicillin (100 units/ml) and streptomycin (100μg/ml). Stock cultures were maintained in $25cm^2$ or $75cm^2$ disposable plastic culture

ware (Corning). Transfer of cell stocks and experimental procedures were performed in a laminar flow hood isolated from other laboratory activities.

Human amnionic cells, AV3, were obtained from Dr. G. Gifford, Department of Immunology and Medical Microbiology, University of Florida, Gainesville, Florida. AV3 cells were grown in Eagles MEM with Earle's balanced salts, 10% heat inactivated FCS (56°C, 30 minutes) and penicillin-streptomycin. They were routinely passaged every two days at a density of 1.25×10^4 cells/cm^2. For transfer of cells, the cell monolayer (approximately 80% confluent) was rinsed two times with 0.15M PBS containing 0.05mM EDTA. One milliliter of PBS containing 0.02% trypsin and 0.05M EDTA (trypsin-EDTA) was added and the cells were allowed to detach from the culture vessel, generally within one to two minutes. Four milliliters of fresh 37°C media was added and cell counts were made with a hemocytometer. Cells were transferred to new flasks with fresh media after dilution and incubated.

Chinese Hamster Ovary cells, CHO, were obtained from Dr. K. D. Noonan, Department of Biochemistry, University of Florida, Gainesville, Florida. Three CHO lines were used, M-7, H-7 and H-7Wcr. M-7 and H-7 differ primarily in their response to dibutryl cyclic adenosine monophosphate (db-cAMP) whereas H-7Wcr is a Con A resistant line derived from H-7. CHO cells were maintained in McCoy's 5A (modified) medium with 10% FCS, penicillin and streptomycin. They were

routinely transferred every two days at a plating density of 1×10^4 cells/cm^2 by trypsinization as described above.

Mouse lymphocytic lukemia cells, EL4, were obtained from the Salk Institute for Biological Studies, La Jolla, CA. EL4 cells were grown as suspension cultures in RPMI 1640 with 10% FCS,penicillin and striptomycin. They were transferred every three days at a final density of 1×10^4 cells/ml.

Rat fibroblast cells, A-9, and an α-amanitin resistant mutant of the A-9 line, LAN-2, were obtained from Dr. J. Eisenstadt, Institute of Human Genetics, Yale University, School of Midicine, New Haven, CT. The LAN-2 amanitin resistant line contained RNA polymerase II activity several fold more resistant to inhibition by α-amanitin *in vitro* compared to the analogous enzyme from the parent line A-9, (R. Bryant, personal communication). Both lines were grown in Eagles MEM with Earle's salts, 10% heat inactivated FCS, penicillin and streptomycin. Stocks of A-9 and LAN-2 were transferred every three days at a final density of 1×10^4 cells/cm^2 by trypsinization as previously described.

Inhibition of Cell Growth

Inhibition of cellular proliferation by amanitin-protein conjugates was measured by determining cell numbers with an electronic particle counter (Celloscope, Particle Data, Inc.). For inhibition by ADH-BSA conjugates, cells were grown in sterile glass scintillation vials. Prior to each experiment AV3 and M-7 CHO cells were harvested by

trypsinization, resuspended at a density of 0.5 to 1.0 x 10^5 cells/ml in the appropriate media and added in 1.0ml aliquots to scintillation vials. After 12-16 hours of growth 100µl of each inhibitor concentration (in culture media) being tested were added to triplicate cultures to achieve the desired final concentration of inhibitor. Growth was allowed to proceed for 48 hours after which time the cells were harvested for counting. Cultures were gently rinsed two times with 2.0ml cold balanced salt solution (Gey's A without Ca^{2+} or Mg^{2+}) and treated with 1.0ml trypsin-EDTA for 5 minutes. After detachment from the glass, 9.0ml of Gey's A solution was added to each vial and cells were counted directly.

EL4 cells were grown in suspension in scintillation vials for 12-16 hours, exposed to inhibitor for 48 hours and counted directly by dilution with Gey's A.

Inhibition of CHO H-7 and H-7Wcr cell growth by ADGG or α-amanitin was also examined by cell number determination but the cells were grown in plastic multiwell tissue culture plates (Corning). CHO cells H-7 and H-7Wcr were plated at 1 x 10^4 cells/cm^2 in 1.0ml 10-12 hours prior to the addition of inhibitor. Forty-eight hours after addition of 50µl of inhibitor, the cells were rinsed two times with cold PBS and removed from the vessel surface by two minutes of treatment with 1.0ml trypsin-EDTA. Aliquots were removed directly into 10ml PBS in a siliconized scintillation vial and immediately counted with the celloscope. Care was taken to ensure that cell clumps were well

dispersed by pasteur pipetting with a silicone treated pipette. A single plate containing 18 individual wells was processed at a time with the entire procedure requiring less than 30 minutes.

Inhibition of H-7 and H-7Wcr cell growth by Con A, ADGG or ADGG-Con A conjugates was measured by exposure of established cell monolayers to the protein for 15 minutes. Cells were plated at a density of 1×10^4 cells/cm^2 and allowed to establish growth for 12 hours. The media was carefully aspirated and the cells gently rinsed two times with 0.15M PBS, pH 7.4 at room temperature. Dilutions of each inhibitor were added in 1.0ml of PBS^+ to triplicate wells for each concentration used. After 15 minutes of exposure, the protein solution was aspirated, the cells rinsed twice with PBS and 1.0ml fresh media added to the well. After 48 hours of growth, the cells were processed for counting as described above.

A-9 and LAN-2 cells were treated as described for CHO H-7 and H-7Wcr cells for determination of their sensitivity to free or conjugated α-amanitin.

Inhibition of ^{3}H-Thymidine Incorporation

Incorporation of [methyl-^{3}H]-thymidine (^{3}H-TdR) was measured for AV3, CHO M-7 and EL4 cells as an additional method for assessing inhibition of cellular functions by ADH-BSA conjugates. A procedure similar to that of Ball, et al. (1973) was used. Labeled precursor (Schwartz-Mann, 5mCi/mmole) was added as a 100μl addition to give a final

concentration of 1μCi/ml 47 hours after addition of inhibitors to cultures. The assay was performed in scintillation vials as described for inhibition of cell number by ADH-BSA conjugates. After 1 hour at 37°C, incorporation by AV3 and CHO M-7 cells was stopped by addition of 10ml of ice cold Gey's A. Cultures were washed three times with 5.0ml of 1.5% perchloric acid, once with 95% ethanol and drained. The vials were heated for 40 minutes at 80°C after addition of 1.0ml/vial of 5% perchloric acid. This treatment served to hydrolyze the nucleic acids for scintillation counting which was done following addition of 10ml/vial of a Triton X-100 based scintillation cocktail with a Beckman L5-133 counter.

Measurement of ^{3}H-TdR uptake by EL4 cells was accomplished with a 1 hour exposure to the labeled nucleoside. Collection of the entire culture on GF/C glass fiber filters (prewashed with 1.5% perchloric acid) was performed after stopping incorporation with 1.0ml 3% perchloric acid. The collected precipitates were washed once with 1.5% perchloric acid, three times with ether:ethanol (1:3), twice with ether, dried at 100°C and counted in a toluene based scintillation cocktail.

Measurement of Pinocytosis

Synthesis of ^{125}I-RSA. Measurement of the pinocytic activity of AV3, CHO M-7 and EL4 cells by uptake of ^{125}I-BSA was performed by modification of the method of Steinman, et al. (1974). ^{125}I-BSA was prepared by iodination with

carrier free ^{125}I (New England Nuclear, carrier free, sodium salt, 50mCi/ml) and solid state lactoperoxidase. The lactoperoxidase (Sigma Chemical Co.) was coupled to Sepharose 4B activated by cyanogen bromide according to David and Reisfeld (1974). BSA was iodinated to a specific activity of 1μCi/μg and purified by gel filtration on Sephadex G-25 in 0.01M phosphate buffer, pH 7.

Determination of pinocytic activity. For determination of pinocytic uptake of ^{125}I-BSA, AV3 and CHO cultures were grown in scintillation vials to near confluence. Each culture received 50μl of ^{125}I-BSA containing approximately 10^7cpm. After 24 hours the media was removed and the monolayers washed six times with 20ml of serum free medium. Trypsin-EDTA (0.5ml/culture) was used to detach the cells after which the cells were removed to a plastic tube for counting in a gamma counter (Nuclear Chicago). The culture vessel was washed an additional two times with 1.0ml medium which were added to the cells. Prior to counting, the cells were separated from the media by centrifugation (10 minutes at 1500rpm) and the cell pellet and supernatant were counted independently. In all cases, negligible activity was found in the supernatant fraction. Duplicate cultures which received 50μl of ^{125}I-BSA 5 minutes prior to harvesting were processed as above. The activity associated with the cell pellet for these cultures was subtracted from the 24 hour culture values as a control for nonspecific surface absorption. Pinocytosis by EL4 cells was determined by similar methods with the

addition of a centrifugation step after each wash to pellet the suspended cells.

Cell Binding

Synthesis of ^{125}I-Con A. Evaluation of the number of membrane binding sites present on cultured cells for Con A was acheived by using Con A labeled to a high specific activity with ^{125}I. The chloramine T method developed by Cuatrecasas (1973) was used with minor modification. To 400μg of Con A in 0.05ml of 0.15M PBS$^+$, pH 7.4 was added 1mCi of ^{125}I (New England Nuclear, sodium salt, carrier free) contained in 0.1ml of 0.1M sodium phosphate buffer, pH 7.4. Freshly prepared chloramine T (100μg in 0.025ml water) was quickly added and mixed for 50 seconds. Sodium metabisulfate (200μg in 0.025ml water) was added to stop the reaction followed by addition of 0.30ml PBS$^+$. The entire mixture was applied to a 2.5ml column of Sephadex G-100 pre-equilibrated with PBS$^+$. The column was washed with 25 volumes of PBS$^+$ after which time negligible activity eluted from the gel. D-glucose (0.3M) was added to the elution buffer and the bulk of the ^{125}I-labeled Con A was collected in a single 1.0ml fraction. The ^{125}I-Con A was dialyzed versus two three liter changes of PBS at 5°C over a 48 hour period. The labeled protein was analyzed for total activity, TCA precipitable activity and total protein by the fluorometric assay of Bohlen et al. (1973). Following analysis the preparation was diluted with native Con A to a final specific activity of 2 x 10^5cpm/μg and stored at -20°C in Nunclon

freezing vials (Vanguard International) in 0.5ml aliquots (210μg/vial). The labeled Con A was thawed one time only and the preparation was used within 18 days of its synthesis. It should be noted that successful labeling was acheived only with ^{125}I batches used within five days after their arrival.

Cell binding measurement. Methods for the determination of ^{125}I-Con A binding to cultured CHO H-7, H-7Wcr and rat A-9, LAN-2 cells were generally adapted from those presented by Noonan and Burger (1973a). Cells were grown to approximately 80% confluency in Linbro multiwell plates with 30mm diameter wells (Linbro Scientific). For binding, cultures were removed to room temperature for 5 minutes, media were removed by aspiration washed gently two times with 0.15M PBS, pH 7.4 and 1.0ml of the desired concentration of ^{125}I-Con A (2x 10^4cpm/μg) in PBS^+ was added to duplicate cultures. After 15 minutes incubation the Con A was removed followed by 5 washes of 2.0ml PBS. PBS containing 0.05M EDTA (1.0ml/well) was added and the cells were allowed to remain at room temperature for 30 minutes. Trypsin-EDTA (0.5ml) was added to each well and the plate incubated at 37°C for 15 minutes. A final addition of 1.0ml 20% Triton X-100 was made and the plates heated at 40°C for 60 minutes. Duplicate 0.5ml aliquots were removed from each well to 10ml of Brey's solution and the activity determined by counting in a Beckman LS-133 liquid scintillation counter set on the narrow tritium window. Each experiment

contained controls for background adsorption to empty wells, inhibition of ^{125}I-Con A binding by 30 minutes of pre-incubation with Con A (100μg/ml), specific ligand (α-methyl-D-mannopyranoside (MDM), 2mM or non-specific protein (BSA, 100μg/ml).

Identical procedures to those described above were used for determination of binding of ^{3}H-DM-ADGG-Con A conjugates. For these experiments the specific activity was 3.46×10^{3}cpm/μg and additional controls for the interference of ADGG with the binding were performed.

SECTION III
RESULTS

ADH-BSA Conjugates

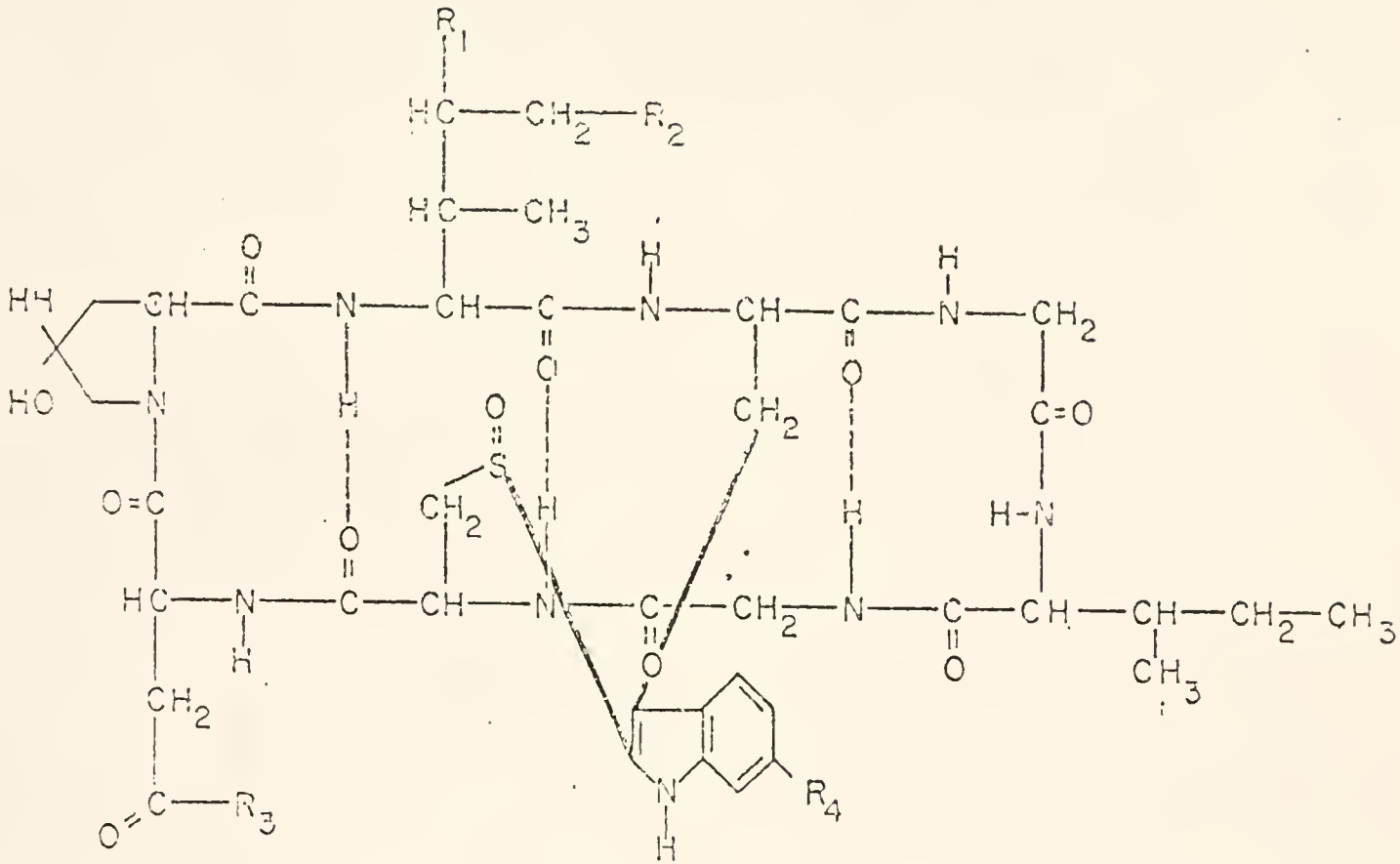
Synthesis

Preliminary investigations of the targeting of α-amanitin to specific cells by macromolecular carriers were patterned after the work of Faulstich and Trischmann (1973). The initial objective was to synthesize α-amanitin-protein conjugates which would then be evaluated on the basis of their biochemical properties and *in vitro* toxicity for selected cell types. Work by other investigators (Wieland, 1968) and NMR studies by Preston and Gabbay (unpublished results, 1977) had demonstrated that α-amanitin contains a hydroxytryptophan moiety (Figure 1) that is available for chemical cross-linking via diazotization to other aromatic groups. A procedure was developed (Figure 2) from the synthesis reported by Faulstich and Trischmann (1973) that allowed for the formation of an α-amanitin derivative, ADH, containing a free amino group.

Synthesis of compounds leading to the formation of N-(4-aminobenzoyl)-N'-BOC-hexamethylenediamine was performed by Dr. J. F. Preston. This compound was then coupled to the hydroxytryptophan moiety of α-amanitin via a diazo bond and the resulting derivative, ADBH, purified

Figure 1. STRUCTURE AND SELECTED PROPERTIES OF AMATOXINS

The structure presented was derived from NMR studies by Preston and Gabbay (unpublished results, 1977) and from published data by Wieland (1968). Toxicity data as reported for mice and inhibition constants for calf thymus RNA polymerase II were adapted from Buku et al. (1971).



	R_1	R_2	R_3	R_4	LD_{50}	K_I
α-Amanitin	OH	OH	NH_2	OH	0.30	3.1×10^{-9}
β-Amanitin	OH	OH	OH	OH	0.35	——
γ-Amanitin	CH	HH	NH_2	OH	0.15	4.3×10^{-9}
Amanin	OH	OH	OH	H	0.50	——
Amanullin	H	H	NH_2	OH	non toxic	9.1×10^{-9}

Figure 2. SYNTHETIC SCHEME FOR ADH-PROTEIN CONJUGATES

Generalized scheme for derivation of α-amanitin and carbodiimide mediated conjugation to protein carboxyl groups.

α-AMANITIN

+

$H_2N-C_6H_4-CO-NH-(CH_2)_6-NHBOC$

$\downarrow$ HNO_2

$AMANITIN-N=N-C_6H_4-CO-NH-(CH_2)_6-NHBOC$

(ADBH)

$\downarrow$ $F_3C\ COOH$

$AMANITIN-N=N-C_6H_4-CO-NH-(CH_2)_6-NH_2$

(ADH)

R'N=C=NR" (CARBODIIMIDE) $\downarrow$ HOOC-PROTEIN

$AMANITIN-N=N-C_6H_4-CO-NH-(CH_2)_6-NH-CO-PROTEIN$

(ADH-PROTEIN)

by LH-20 chromatography in methanol. Yields were generally 69% or greater. The purified ADBH chromatographed as a single spot in TLC in methanol:methyl ethyl ketone (1:1) with an R_f of 0.67. NMR spectroscopy by Drs. E. Gabbay and W. Brey, Jr. of the Department of Chemistry, University of Florida, Gainesville, Florida indicated that the hydrogen atom resonance spectra of the ADBH were consistant with the proposed structure. The uv and visible spectra presented for ADBH in Figure 3 show ratios of extinctions at 384nm to 304nm of 0.85, identical to the ratio of the extinction coefficient for the azo dye at 384nm, 14000cm^2/mmole to that of free α-amanitin at 304nm, 16400cm^2/mmole. This would imply a stoichiometric relationship between α-amanitin and the diazo linked spacer molecule.

Bovine serum albumin was chosen for the initial conjugate studies based on reports in the literature of selective toxicity of BSA-β-amanitin conjugates for macrophages (Fiume and Barbanti-Brodano, 1974). Following removal of the BOC group from ADBH and carbodiimide coupling to BSA, the conjugate was purified from the reaction mixture by chromatography on Sephadex G-75 (Figure 4).

Characterization

The conjugate peak eluted in the void volume of the G-75 column and contained significant absorption at 384nm as well as 280nm (Figure 3) indicative of covalent association of the diazo compound and BSA. The ADH-BSA contained a molar ratio of α-amanitin (as azo compound) to BSA of 1.2

Figure 3. UV AND VISIBLE ABSORPTION SPECTRA OF ADBH AND ADH-BSA CONJUGATE

The absorption spectra of ADBH (solid line) dissolved in methanol (33 μg/ml), ADH-BSA (dashed line) in 0.05% NH_4HCO_3, pH 8.0 (1 mg/ml) and BSA (dotted line) in 0.05% NH_4HCO_3, pH 8.0 (1 mg/ml) were determined with a Beckman model 24/25 spectrophotometer at room temperature and a 1.0 cm light path.

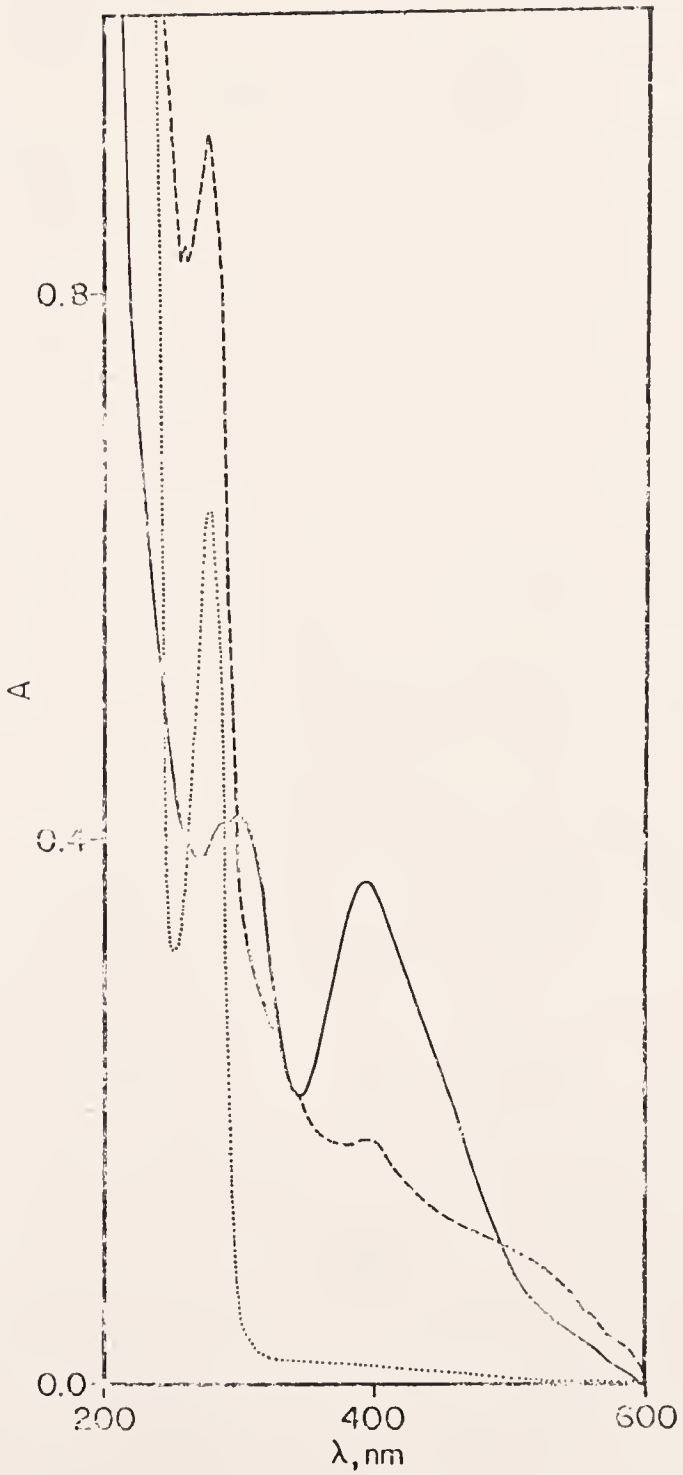
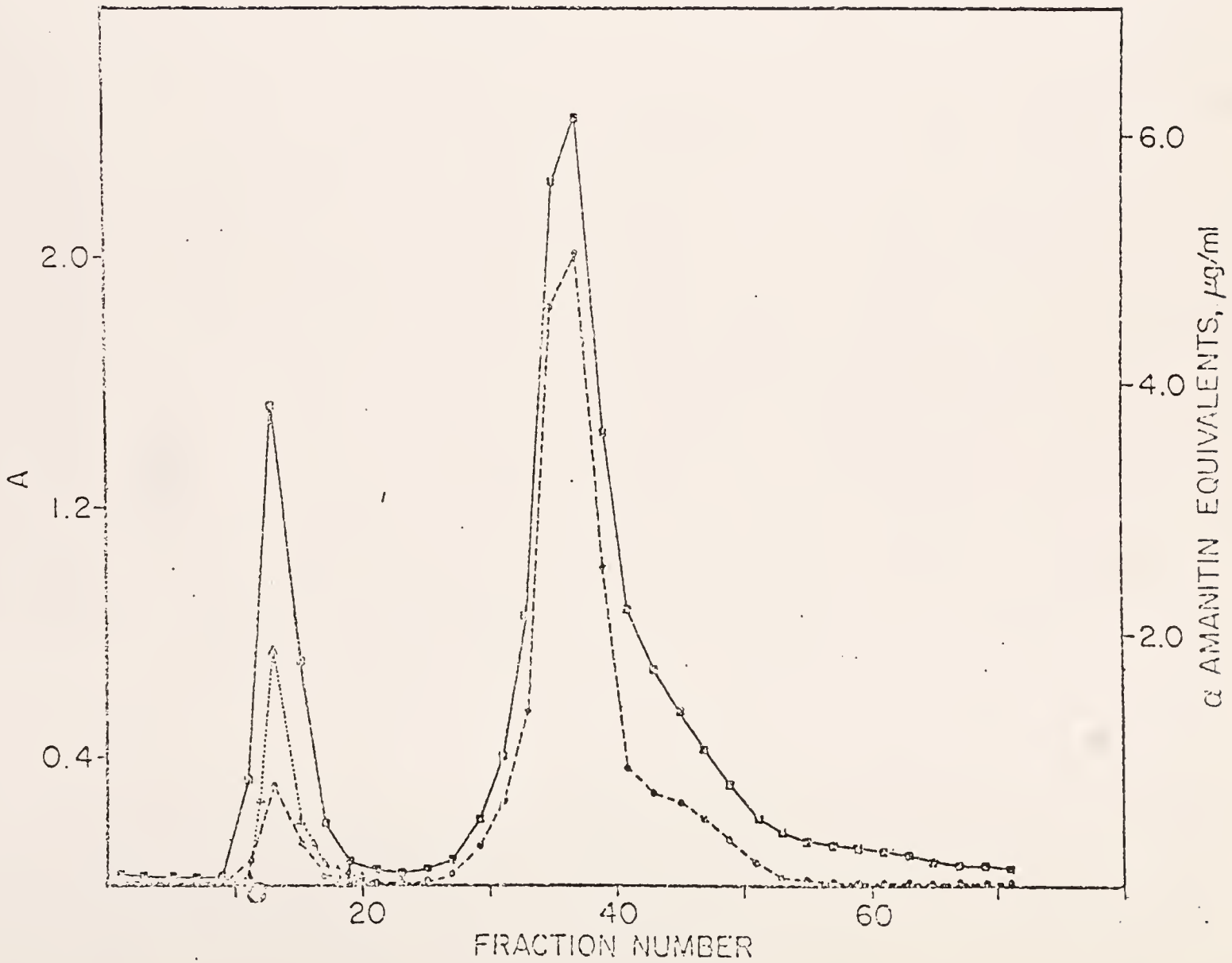

0.8
0.4
0.0
A
200
400
600
λ, nm

Figure 4. SEPHADEX G-75 CHROMATOGRAPHY OF ADH-BSA CONJUGATE

The reaction mixture from EDC mediated conjugation of ADH and BSA was chromatographed on Sephadex G-75 in 0.05% NH_4HCO_3. The squares (■) represent absorbancy at 280nm, the circles (●) absorbancy at 384nm and the triangles (▲) depict inhibition of calf thymus RNA polymerase II in amanitin equivalent units (1 unit = 1 μg α-amanitin/ml).


A
2.0
1.2
0.4
20
40
60
FRACTION NUMBER
6.0
4.0
2.0
α AMANITIN EQUIVALENTS, μg/ml

as determined from the absorption spectra. Lowry protein analysis of the conjugate yielded slightly lower values for the amount of protein present than those derived from the 280nm absorbance. Since no interference with the Lowry assay was noted from ADH added to known quantities of BSA, it is presumed that the ADH conjugation induces a slight increase in the 280nm absorption of the BSA. A factor of 1.18 times the 384nm absorbance was subtracted from the 280nm value to empirically correct for these differences. Inhibition of calf thymus RNA polymerase II by the individual fractions obtained for G-75 chromotography gave additional confirmation of the association of α-amanitin with BSA for the ADH-BSA conjugate.

The extent of protein to protein cross-linking resulting as a side reaction from carbodiimide coupling was estimated by SDS-PAGE of the ADH-BSA. The relatively high protein concentrations (10mg/ml) and high EDC concentration (100mg/ml) used for conjugate synthesis led to significant cross-linking, shown in Figure 5. Estimation of the area under each peak of the scan of gel 1 indicated approximately 50% of the protein was in the monomeric form. Molecular weight calibration with known protein standards (Figure 6) demonstrated the cross-linked material to be in multiples of BSA molecular weights (66,000 daltons) which would imply that no fragmentation of the protein occurs during conjugation.

Figure 5. SDS-PAGE OF ADH-BSA CONJUGATES

Duplicate 50 μg samples of ADH-BSA (gels 1 and 2) were electrophoresed on 5% acrylamide at 8 ma/gel. A mixture of different molecular weight proteins was run for calibration purposes on gel 3. The spectrophotometric (at 600nm) tracing of gel 1 identifies the monomeric (1), dimeric (11), trimeric (111) and larger (IV) peaks obtained from the carbodiimide conjugation.

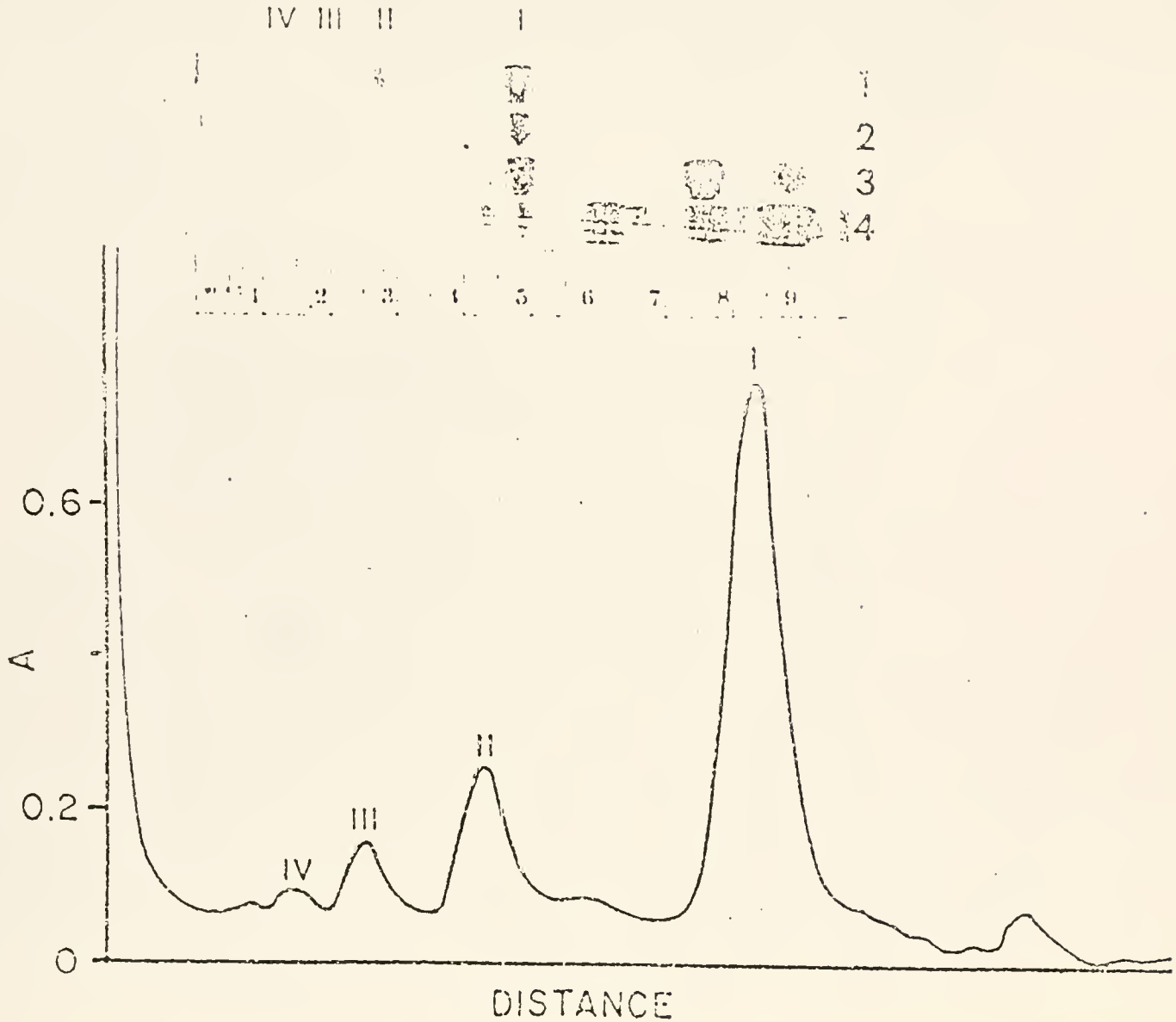
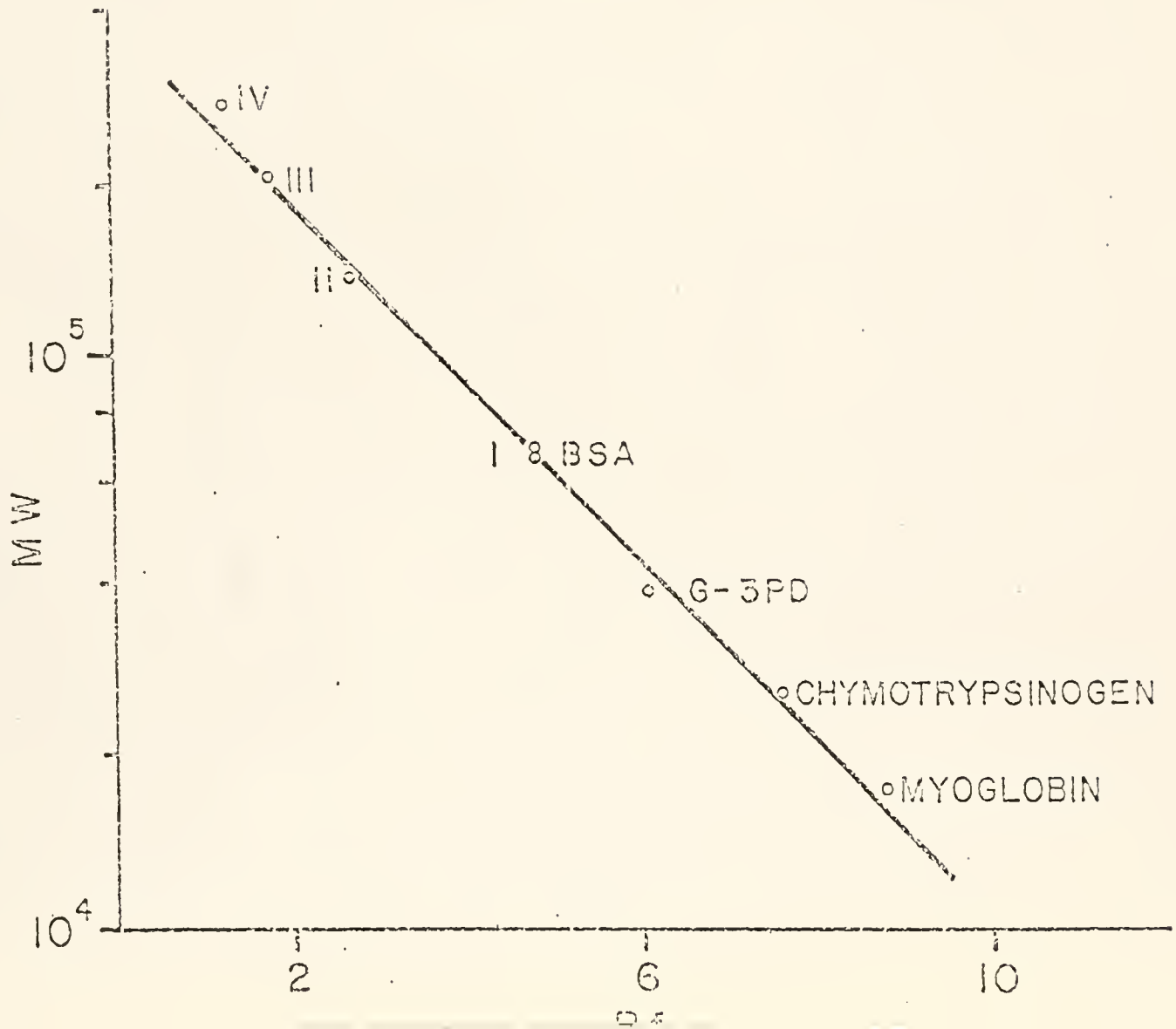

IV III II I
1
2
3
4
1 2 3 4 5 6 7 8 9
0.6
0.2
0
A
IV
III
II
I
DISTANCE

Figure 6. LOG MOLECULAR WEIGHT VERSUS ELECTROPHORETIC MOBILITY

The log of molecular weight for several known protein standards electrophoresed in gel 3 of Figure 5 is plotted in relationship to electrophoretic mobility. The roman numerals correspond to the protein peaks observed in ADH-BSA (gel 1) electrophoresis.


IV
III
II
I
BSA
G-3PD
CHYMOTRYPSINOGEN
MYOGLOBIN
MW
10^5
10^4
2
6
10

The inhibitory potential of the ADH-BSA conjugate for calf thymus RNA polymerase II was quantitatively compared to that of free α-amanitin or ADH by determining the apparent inhibition constant (K_I) for each compound. The value of 1.8×10^{-9}M obtained for α-amanitin agrees with published values (Chochet-Meilhac and Chambon, 1974) and does not differ significantly from that for ADH (K_I=3.0×10^{-9}M). Both inhibitions were noncompetitive with respect to UTP. ADH-BSA inhibition deviated from the strictly noncompetitive inhibition seen for α-amanitin and ADH with an average of the x-intercepts giving an apparent inhibition constant of 69×10^{-9}M. Conjugation to BSA resulted in a 38-fold decrease in the affinity of bound α-amanitin for calf thymus polymerase.

Interaction with Cells

The toxicity of conjugated ADH-BSA was examined in three cultured cell lines, CHO M7, AV3 and EL4. Table 1 presents the effects of continuous 48 hour exposure to ADH-BSA or free α-amanitin on cellular proliferation and ^{3}H-thymidine incorporation of CHO M-7 cells. Comparing equivalent molar concentrations of free or conjugated α-amanitin, conjugated α-amanitin was found to be slightly less effective an inhibitor of cell growth than free α-amanitin for CHO cells. Greater inhibition is seen when examining ^{3}H-TdR incorporation, with conjugated α-amanitin inducing 60% of the inhibition obtained at 1×10^{-6}M concentration in comparison to free α-amanitin.

Figure 7. INHIBITION OF CALF THYMUS RNA POLYMERASE II BY ADH-BSA, ADH AND α-AMANITIN

Results are expressed in a Dixon plot of the reciprocal of velocity expressed as μμmole ^{3}H-UMP incorporated per minute versus concentration of inhibitor for three different substrate (UTP) concentrations: 0.004 mM (●), 0.008 mM (▲) and 0.016 mM (■). Least squares analysis was used to obtain the best fit for each line. For ADH and ADH-BSA, the concentrations of α-amanitin were determined from the diazo linkage extinction coefficient.

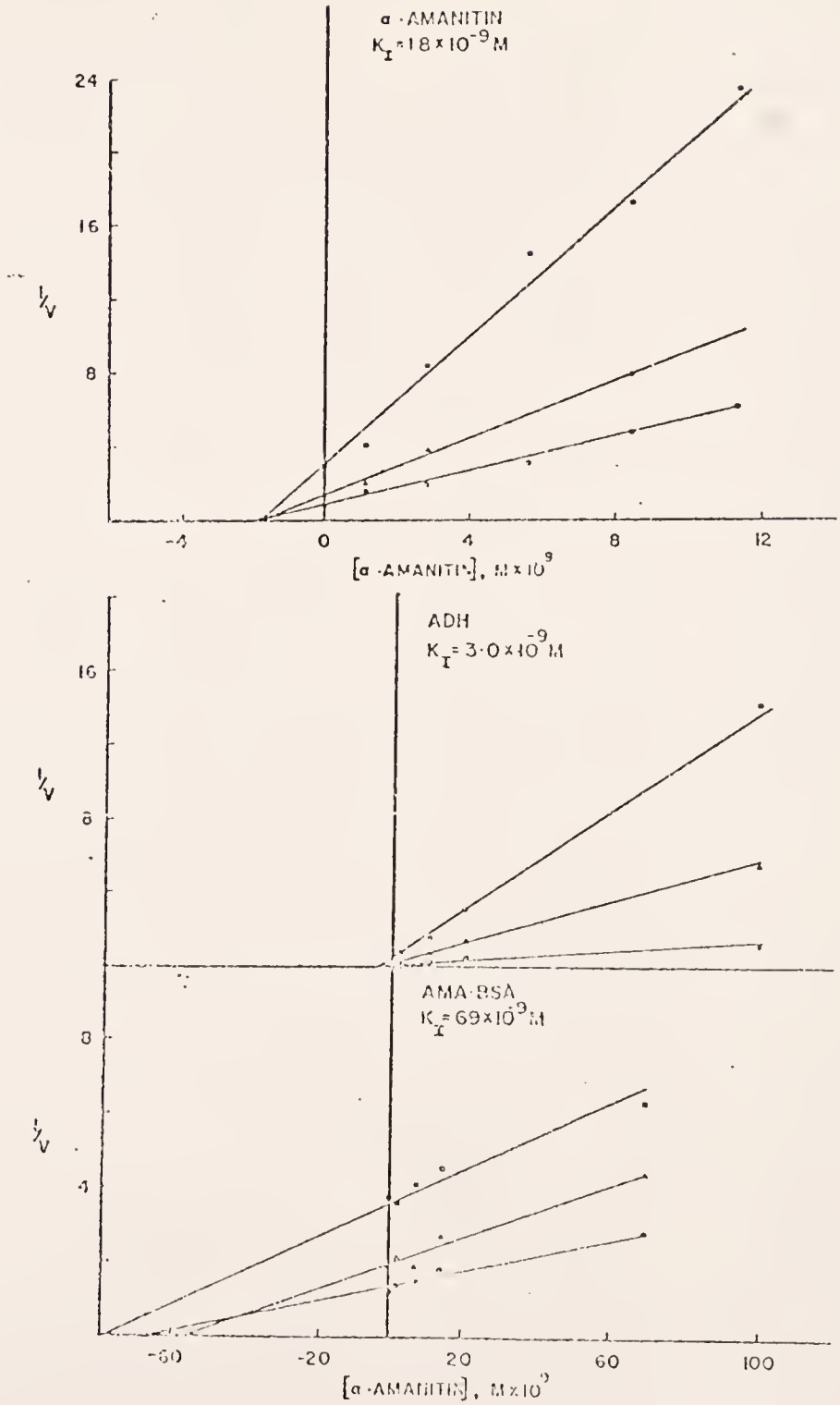

α-AMANITIN
$K_I = 1.8 \times 10^{-9}$ M
24
16
8
1/v
-4
0
4
8
12
[α-AMANITIN], M x 10^9
ADH
$K_I = 3.0 \times 10^{-9}$ M
16
8
1/v
AMA-BSA
$K_I = 6.9 \times 10^{-9}$ M
8
4
1/v
-60
-20
20
60
100
[α-AMANITIN], M x 10^9

TABLE 1

CHINESE HAMSTER OVARY CELLS (CHO): EFFECTS OF α-AMANITIN AND ADH-BSA CONJUGATE ON CELL PROLIFERATION AND ^{3}H-THYMIDINE INCORPORATION

		Cell Number		^{3}H-TdR Incorporation	
Inhibitor	(α-AMA) M x 10^{-7} [a]	Total Cells x 10^{-5}	Percent Inhibition	CPM x 10^{-4}	Percent Inhibition
None	0	1.67	0	2.75	0
α-AMA	1.09	1.49	10.8	2.33	15.4
α-AMA	2.18	1.48	11.4	1.76	36.0
α-AMA	5.45	1.37	18.0	1.36	50.5
α-AMA	10.90	1.30	22.2	0.75	72.8
None	0	1.67	0	2.62	0
ADH-BSA	1.09	1.48	11.4	2.29	12.9
ADH-BSA	2.18	1.47	12.0	2.32	11.5
ADH-BSA	5.45	1.47	12.0	1.77	32.5
ADH-BSA	10.90	1.41	15.6	1.43	45.7

[a]Molarity for ADH-BSA refers to concentration of bound α-amanitin

The same comparisons are presented for EL4 cells in Table 2. As was observed for CHO cells, free α-amanitin is more effective in inhibiting cell growth than is conjugated α-amanitin with about three fold greater inhibition seen at 5×10^{-6}M α-amanitin for nonconjugated toxin. EL4 cells were approximately five times less sensitive to α-amanitin in either form than were CHO cells. Although the ^{3}H-TdR data presented parallels,the cell number data with α-amanitin being more inhibitory than ADH-BSA at equivalent concentrations, EL4 cells did not incorporate exogenous thymidine to any great extent under the conditions examined. The data is presented only for comparative purposes.

Table 3 contains similar inhibition data for AV3 cells. Like EL4 cells, AV3 did not incorporate exogenous thymidine to a significant extent but the inhibition obtained paralleled the cell number data. For AV3 however, inhibition of cell proliferation by ADH-BSA was strikingly different than that observed in CHO and EL4 cells. Although AV3 cells are equally as sensitive to free α-amanitin as are CHO cells, the AV3 cells were eight times more susceptible to conjugated α-amanitin in comparison to an equivalent molar dose of free α-amanitin. This would imply a preferential uptake of ADH-BSA by AV3 and/or modification of the conjugate to a more toxic derivative after uptake by AV3 cells.

Figure 8 depicts cell size distributions of AV3, CHO and EL4 cells after 48 hours of exposure to ADH-BSA or α-amanitin. The results clearly indicate that no shift in

TABLE 2

MOUSE LYMPHOCYTIC LEUKEMIA CELLS (EL4): EFFECTS OF α-AMANITIN AND ADH-BSA CONJUGATE ON CELL PROLIFERATION AND ^{3}H-THYMIDINE INCORPORATION

		Cell Number		^{3}H-TdR Incorporation	
Inhibitor	(α-AMA) M x 10^{7}*	Total Cells x 10^{-5}	Percent Inhibition	CPM x 10^{-4}	Percent Inhibition
None	0	3.65	0	85	0
α-AMA	1.09	--	--	65	--
α-AMA	2.72	3.31	9.3	--	23.3
α-AMA	5.45	--	--	64	24.2
α-AMA	10.90	2.83	22.4	--	--
α-AMA	27.20	1.74	52.3	--	--
α-AMA	54.50	0.53	85.4	43	49.8
None	0	3.65	0	85	0
ADH-BSA	2.72	3.41	6.6	--	--
ADH-BSA	5.00	--	--	65	23.5
ADH-BSA	10.90	3.38	7.4	63	26.1
ADH-BSA	27.20	2.63	28.0	--	--
ADH-BSA	54.50	1.68	54.0	50	41.1

*Molarity for ADH-BSA refers to concentration of bound α-amanitin

TABLE 3

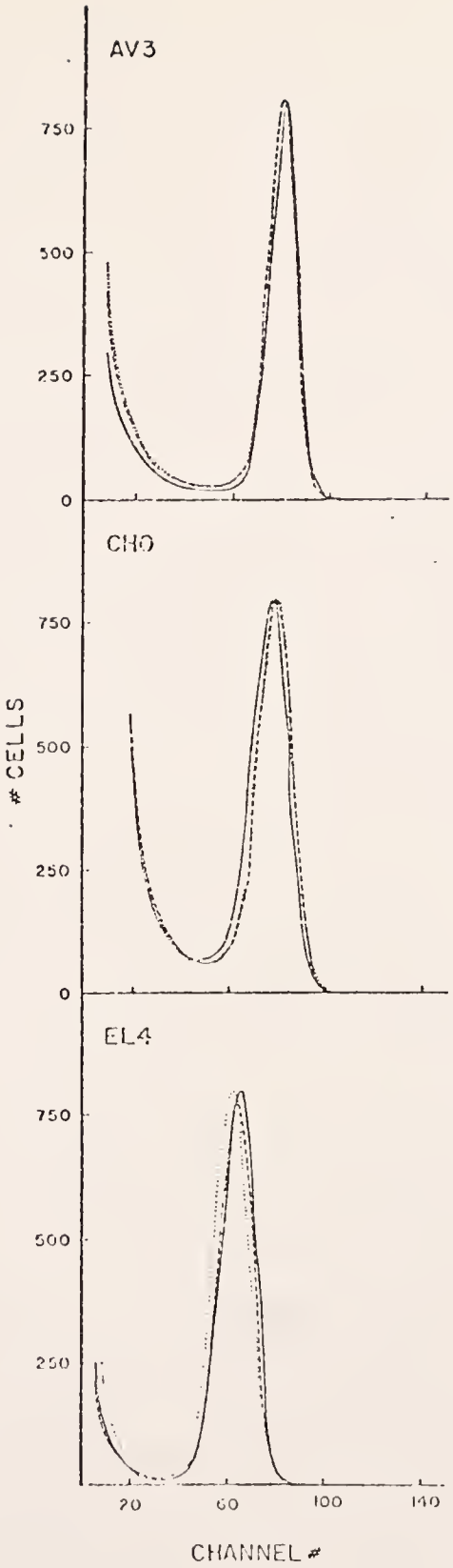
HUMAN AMNION CELLS (AV3): EFFECTS OF α-AMANITIN AND ADH-BSA CONJUGATE ON CELL PROLIFERATION AND ^{3}H-THYMIDINE INCORPORATION

		Cell Number		^{3}H-TdR Incorporation	
Inhibitor	(α-AMA) M x 10^{7}*	Total Cells x 10^{-5}	Percent Inhibition	CPM	Percent Inhibition
None	0	8.0	0	123	0
α-AMA	5.4	---	---	89	28.1
α-AMA	10.9	---	---	83	33.1
α-AMA	24.0	2.1	73.8	---	---
α-AMA	48.0	0.65	91.9	---	---
α-AMA	54.5	---	---	76	38.5
α-AMA	96.0	0.15	98.0	---	---
None	0	8.0	0	123	0
ADH-BSA	0.56	7.1	11.6	---	---
ADH-BSA	1.10	4.5	43.8	---	---
ADH-BSA	2.20	3.8	52.7	---	---
ADH-BSA	2.95	---	---	75	39.5
ADH-BSA	5.89	---	---	72	41.7
ADH-BSA	11.80	---	---	69	44.2

*Molarity for ADH-BSA refers to concentration of bound α-amanitin

Figure 8. CELL SIZE DISTRIBUTIONS OF CHO, AV3 AND EL4 CELLS EXPOSED TO ADH-BSA AND α-AMANITIN.

Following 48 hours of exposure to either ADH-BSA (dotted line) or α-amanitin (dashed line) as described in Materials and Methods, cells were compared to normal cells (solid line) on the basis of size by a 110 channel celloscope particle counter.


AV3
750
500
250
0
CHO
750
500
250
0
EL4
750
500
250
CELLS
20
60
100
140
CHANNEL #

mean cell size has occurred as a result of exposure to either inhibitor which might have occurred in a growing but nondividing population of cells or in one that was dividing in the absence of DNA synthesis.

Differential uptake of ADH-BSA could possibly occur by means of receptor mediated uptake or enhanced pinocytotic uptake by susceptible cells. In order to explore the latter possibility, AV3, CHO and EL4 cells were examined for their relative rates of pinocytosis. Uptake of ^{125}I labeled BSA over a 24 hour period was used as a measure of pinocytosis (Steinman et al., 1974). Table 4 presents a summary of the pinocytosis rates in comparison to the molar concentrations of free versus conjugated α-amanitin required for 25% inhibition of cell growth and ^{3}H-TdR incorporation for all three cell lines. The amount of pinocytosis observed with each cell line was in direct correlation to the relative sensitivity of the cell line to conjugated α-amanitin. AV3 cells were 3.5 times more active in the pinocytic uptake of ^{125}I-BSA than were CHO cells, whereas EL4 cells took up negligible amounts under similar conditions. These data indicate that the increased sensitivity of AV3 cells to conjugated α-amanitin is a direct function of increased pinocytotic uptake of the conjugate relative to the other cell lines tested.

The results of the ADH-BSA experiments pointed out the necessity for using a macromolecular carrier for which specific cellular receptors are known to exist to further

TABLE 4

SUMMARY: INHIBITION BY α-AMANITIN AND ADH-BSA CONJUGATE

Cell Line	α-AMA Cell Number	α-AMA ^{3}H-TdR Incorporation	ADH-BSA Cell Number	ADH-BSA ^{3}H-TdR Incorporation	Pinocytosis of ^{125}I-BSA μg/Cell
AV3	8.5	3.5	1.0	1.0	2.83×10^{-10}
CHO	4.0*	1.6	9.6*	4.4	0.78×10^{-10}
EL4	23.0	7.0	53.0	9.0	$<0.2 \times 10^{-10}$

Inhibition data presented as the molar concentration x 10^{7} required for 25% inhibition

*15% inhibition

clairfy the role of receptor mediated uptake of α-amanitin conjugates. For this reason conjugates of α-amanitin and the plant lectin, Concanavalin A, were synthesized.

ADH-Con A Conjugates

Synthesis

Similar conditions to those used for the synthesis of ADH-BSA were used to prepare ADH-Con A conjugates. The conjugates were purified after carbodiimide coupling by chromatography on Sephadex G-75 (Figure 9). The ADH-Con A eluted in the void volume of the G-75 column and contained substantial absorbance at 384nm and at 280nm (Figure 10). From the molar extinction coefficients of Con A and the azo moiety it was determined that all of the Con A applied to the column eluted in a single peak and contained a molar ratio of α-amanitin to Con A of 0.67.

Characterization

The ADH-Con A conjugates were first characterized with respect to their inhibition of calf thymus RNA polymerase II. The results presented in Figure 11 generate an inhibition constant of 186 x 10^{-9}M for calf thymus polymerase. The high K_I observed implies that a substantial reduction in the affinity of α-amanitin for calf thymus RNA polymerase II has occurred as a result of conjugation to Con A. Furthermore, as evidenced by the lack of binding of the ADH-Con A to Sephadex G-75, these conjugates possessed little or none of the ligand binding specificity of the native lectin.

Figure 9. SEPHADEX G-75 CHROMATOGRAPHY OF ADH-CON A CONJUGATES

The reaction mixture from EDC mediated conjugation of ADH and Con A was chromatographed in 0.05% NH_4 HCO_3. The squares (□) represent absorbancy at 384nm and the circles (○) represent absorbancy at 280nm.

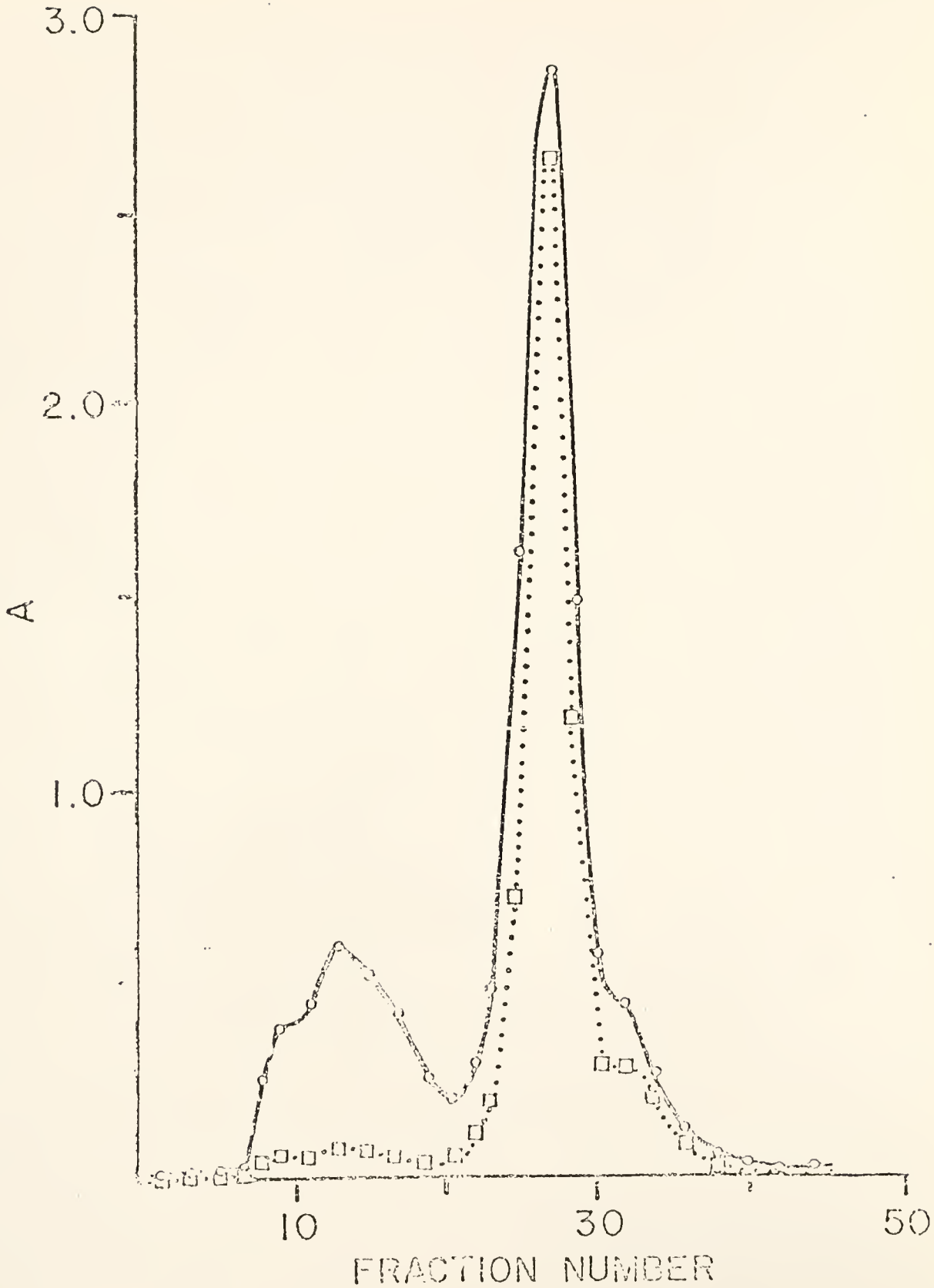

3.0
2.0
1.0
A
10
30
50
FRACTION NUMBER

Figure 10. UV AND VISIBLE ABSORPTION SPECTRA OF ADH-CON A

The absorption spectra of ADH-Con A conjugate was determined in 0.05% NH_4 HCO_3, pH 8.0.

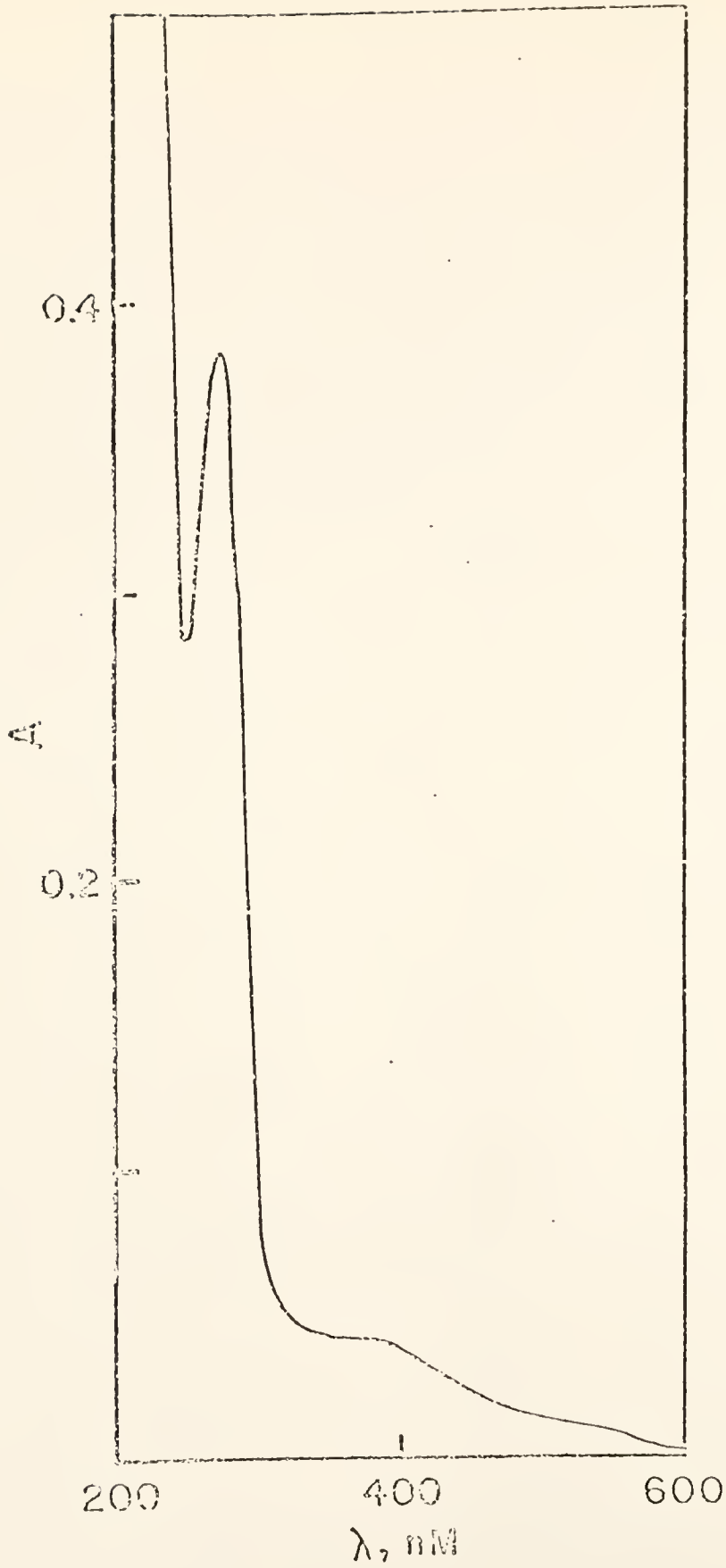
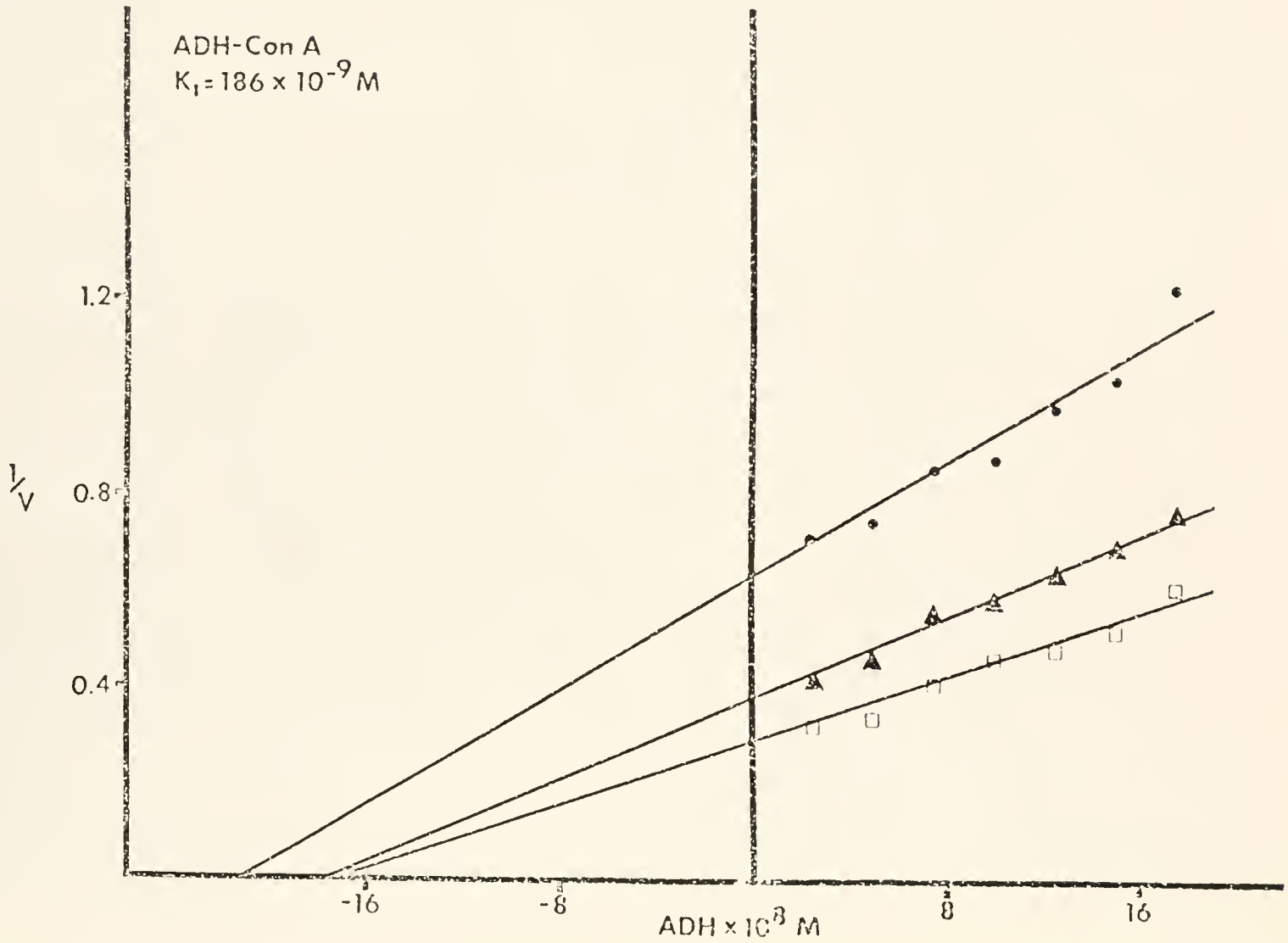

0.4
0.2
A
200
400
600
λ, nM

Figure 11. INHIBITION OF CALF THYMUS RNA POLYMERASE II BY ADH-CON A CONJUGATES

Results are expressed as the reciprocal of velocity (μμmole ^{3}H-UMP incorporated per minute)$^{-1}$ versus concentration of inhibitor for three different substrate (UTP) concentrations: 0.004 mM (□), 0.008 mM (▲) and 0.016 mM (●). Least squares analysis was used to obtain the best fit for each time.


ADH-Con A
$K_I = 186 \times 10^{-9}$ M
1.2
0.8
0.4
1/V
-16
-8
8
16
ADH × 10^8 M

Other preparations in which Con A and ADH were coupled in the presence of saturating amounts of specific ligand and under varying salt and ion concentrations also failed to absorb to Sephadex. Since it appeared likely that conjugation to carboxyl groups on Con A resulted in a loss of ligand binding activity, investigations of these ADH-Con A conjugates were discontinued.

ADGG-BSA Conjugates

The results of the ADH-Con A studies demonstrated the need for an α-amanitin derivative containing a free carboxyl group. Such a derivative could be coupled to free protein amino groups and possibly result in a derivative of Con A that retained ligand binding properties as well as the specificity of interaction of α-amanitin and RNA polymerase II.

Synthesis

The synthetic pathway for one derivative of this type is shown in Figure 12 as developed by Dr. J. F. Preston. The synthesis follows the same general pattern as that for ADH conjugates. It results in the production by diazotization of α-amanitin and an aromatic containing carbon chain spacer molecule, p-aminobenzoylglycylglycine, of a free carboxyl containing α-amanitin derivative, α-amanitin-diazobenzoylglycylglycine (ADGG). The ADGG was purified from the diazotization reaction by chromatography on Sephadex LH-20 in 80% methanol. The product was judged to be

Figure 12. SYNTHETIC SCHEME FOR ADGG-PROTEIN CONJUGATES

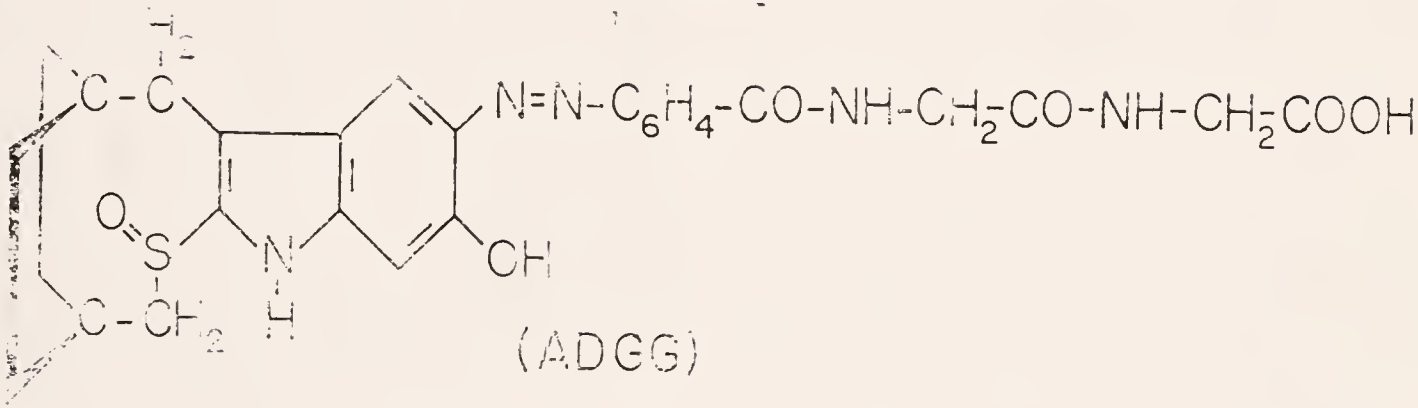
Generalized scheme for derivation of α-amanitin and carbodiimide mediated conjugation to protein amino groups.

α-AMANITIN

+

$H_2N-C_6H_4-CO-NH-CH_2-CO-NH-CH_2-COOH$

$\downarrow HNO_2$



$R'N=C=NR''$ (CARBODIIMIDE) $\downarrow H_2N$-PROTEIN

$AMA-N=N-C_6H_4-CO-NH-CO-CH_2-NH-CH_2-\overset{O}{\overset{\|}{C}}-\overset{H}{\overset{|}{N}}-PROTEIN$

(ADGG-PROTEIN)

pure on the basis of its appearance as a single spot with R_f of 0.57 after TLC in the systems described for ADBH. Concentrations were determined for its absorption at 395nm with an extinction of 14000cm^2/mmole.

ADGG was coupled to BSA for production of a conjugate that could be directly compared with the previous work on ADH-BSA conjugates. After EDC mediated conjugation, the ADGG-BSA conjugate was isolated by chromatography on Sephadex G-75 (Figure 13). The ADGG-BSA eluted as a single peak followed by unreacted ADGG. The elution profile contains significant absorbance at both 384 and 280nm indicating covalent association of the ADGG and BSA. Individual fractions were further analyzed by inhibition of calf thymus RNA polymerase II. The inhibition profile closely parallels the absorbance at 384nm demonstrating retention of inhibiting activity of α-amanitin after conjugation.

Characterization

Figure 14 shows the absorption spectra for ADGG and ADGG-BSA. The peak absorbancy at 395nm for the azo moiety was used to determine α-amanitin concentration whereas the protein concentration was obtained from Lowry protein analysis. No interference with the Lowry assay was noted by ADGG. The ADGG-BSA conjugate prepared contained 2.9 moles of ADGG per mole of BSA.

The inhibition of calf thymus RNA polymerase II by ADGG and ADGG-BSA is shown in Figure 15. Free ADGG compared favorably to α-amanitin with respect to inhibition of

Figure 13. SEPHADEX G-75 CHROMATOGRAPHY OF ADGG-BSA CONJUGATE

The reaction mixture from EDC mediated conjugation of ADGG and BSA was chromatographed in 0.05% NH_4HCO_3. The dashed line represents absorbancy at 395nm, the solid line represents absorbancy at 280nm and the circles (○) represent inhibition of calf thymus RNA polymerase II in amanitin equivalent units (1 unit = 1 μg α-amanitin/ml).

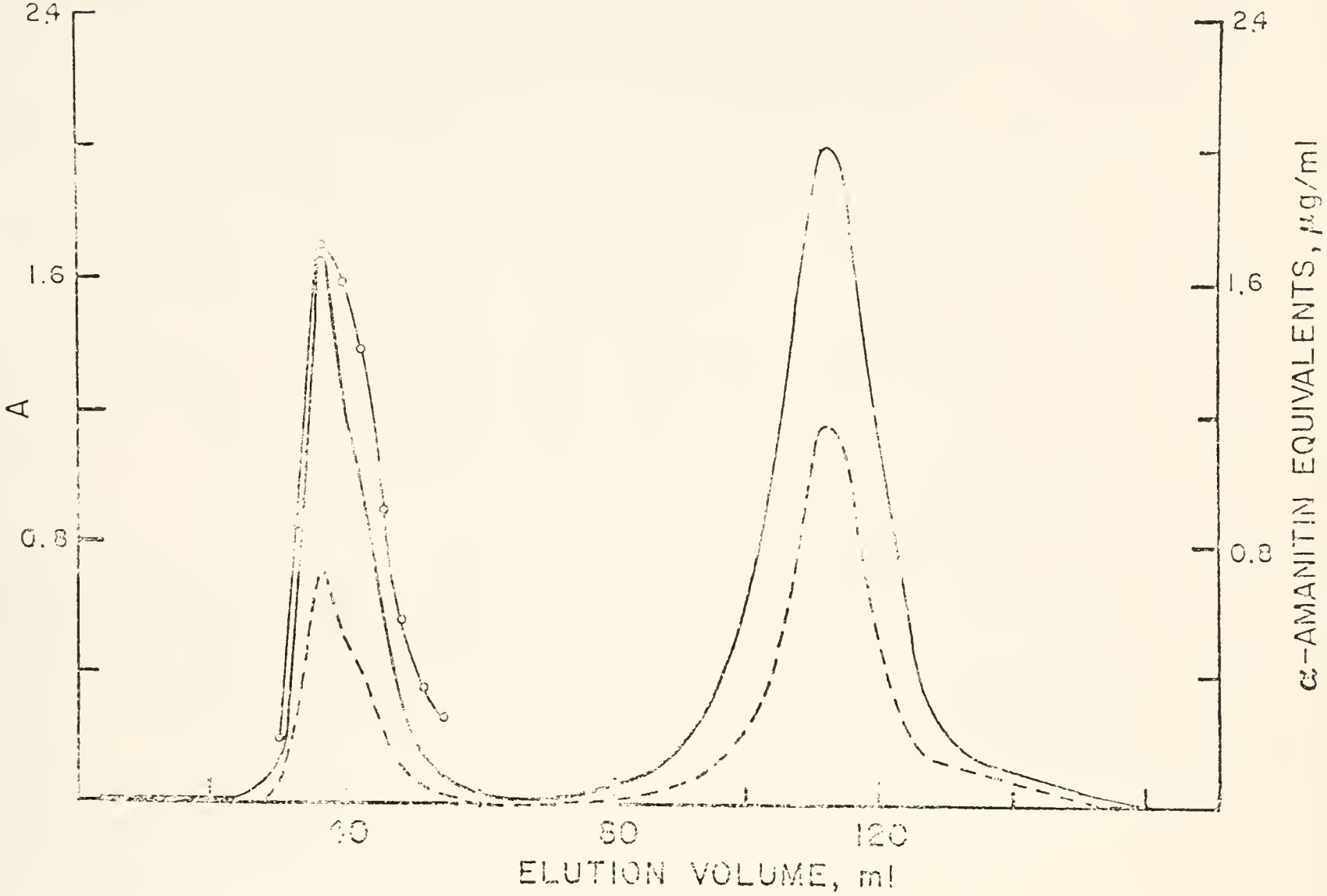

2.4
1.6
0.8
A
2.4
1.6
0.8
α-AMANITIN EQUIVALENTS, μg/ml
40
80
120
ELUTION VOLUME, ml

Figure 14. UV AND VISIBLE ABSORPTION SPECTRA OF ADGG-BSA CONJUGATES

Lyophilized ADGG-BSA from the peak eluting from Sephadex G-75 (Figure 13) was dissolved in water and diluted to 199 μg/ml of BSA in 0.0001 M Tris HCl pH 7.0.

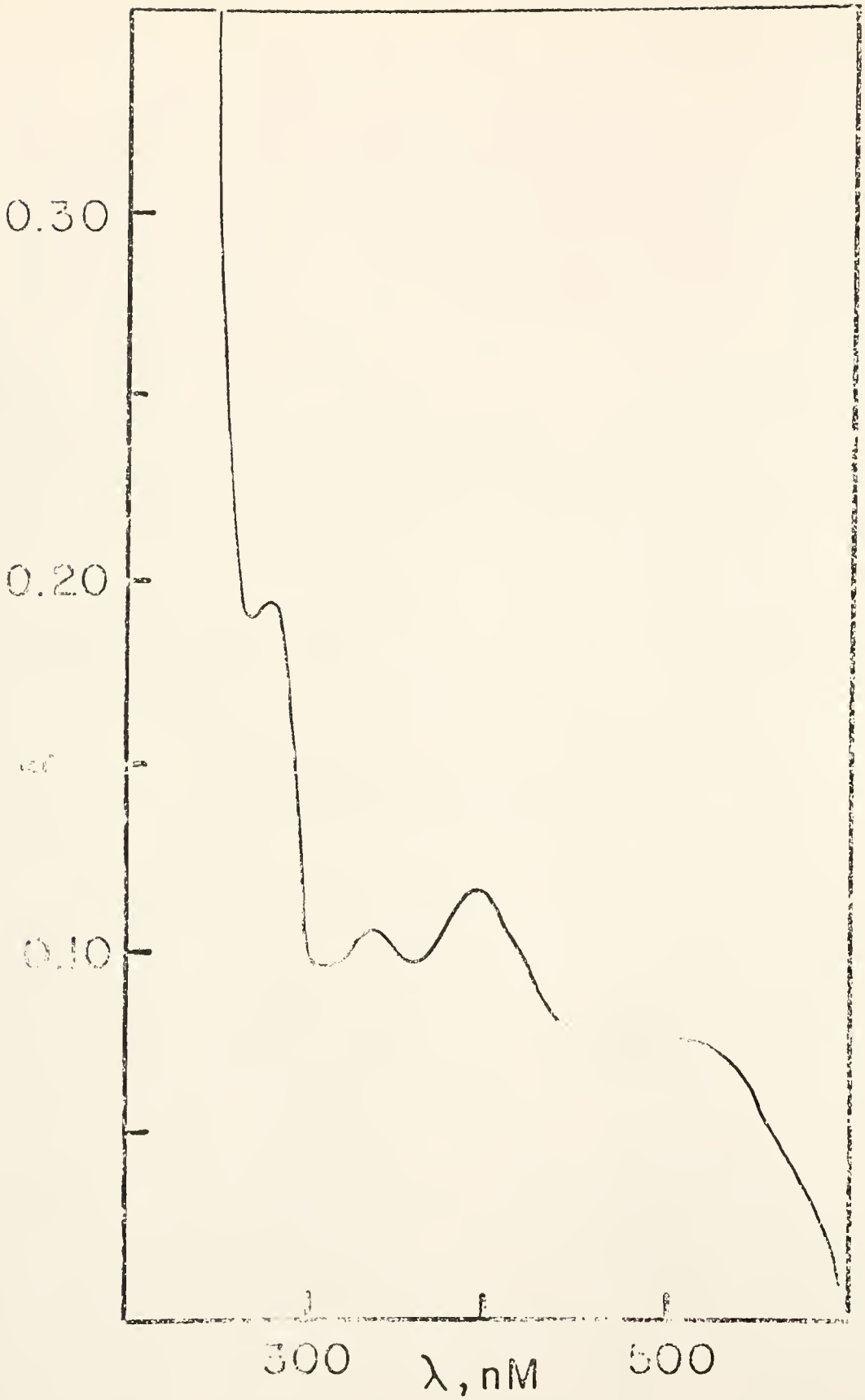
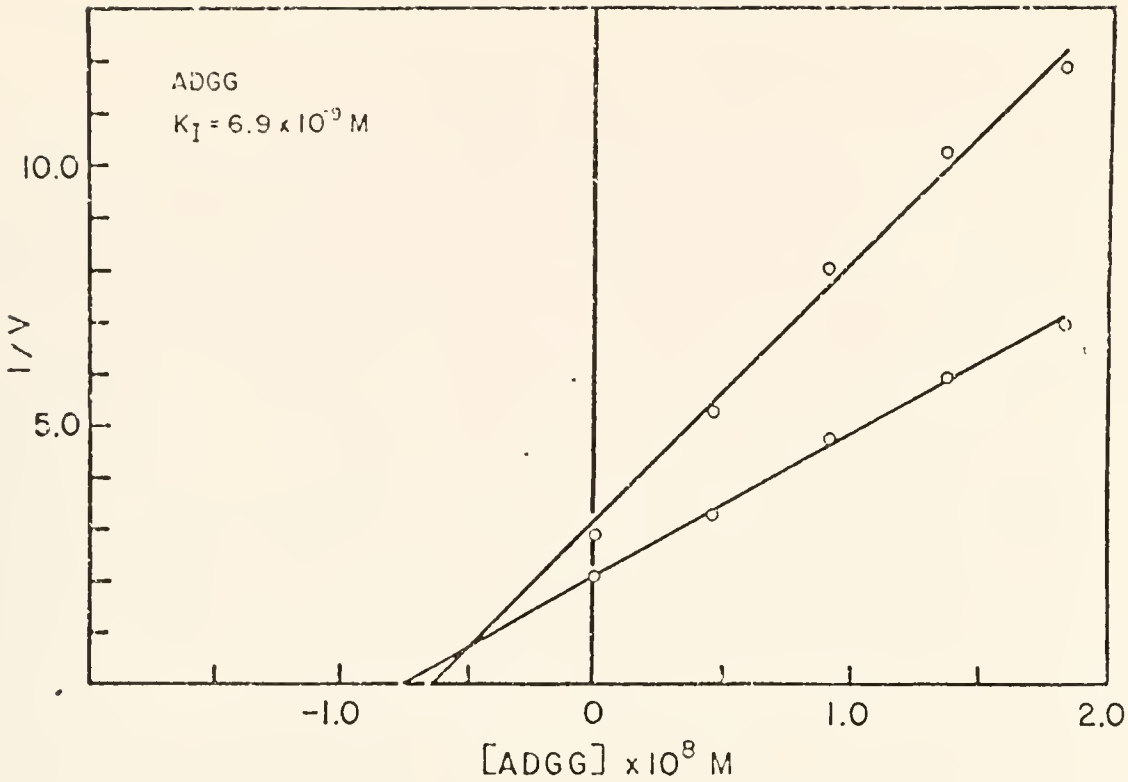
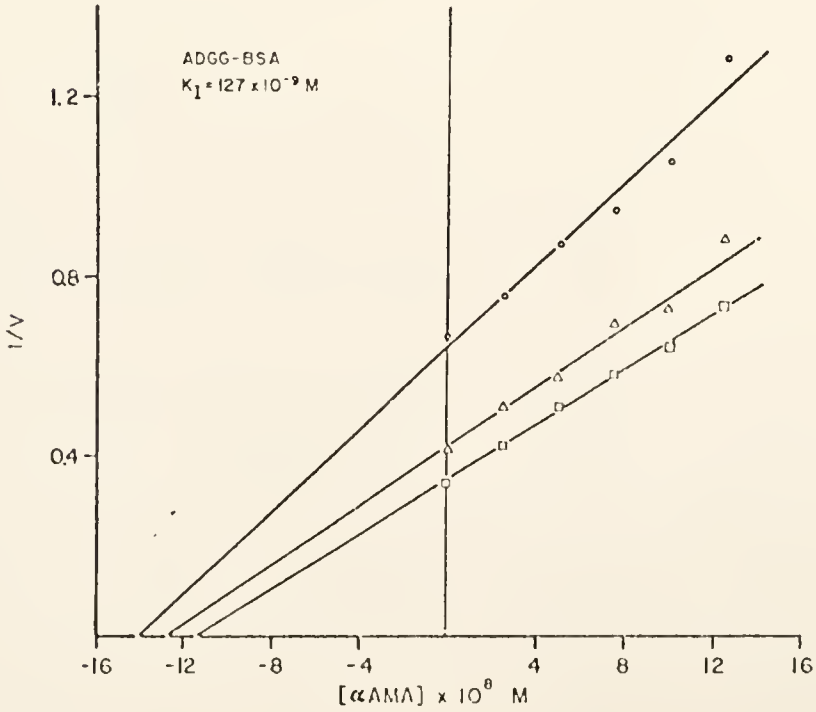

0.30
0.20
0.10
300
λ, nM
500

Figure 15. INHIBITION OF CALF THYMUS RNA POLYMERASE II BY ADGG AND ADGG-BSA

Results are expressed as the reciprocal of velocity ($\mu\mu$mole ^{3}H-UMP incorporated per minute)$^{-1}$ versus concentration of inhibitor for different concentrations of substrate (UTP). For ADGG (Figure 15a) the squares (□) are 0.008 mM ^{3}H-UTP and the circles (○) 0.016 mM. For ADGG-BSA (Figure 15b) the concentrations of ^{3}H-UTP are 0.004 mM (□), 0.008 mM (▲) and 0.016 mM (○). Lines were drawn as the best fit for least squares determination of the linear regression.


ADGG
$K_I = 6.9 \times 10^{-9}$ M
10.0
5.0
1/V
-1.0
0
1.0
2.0
[ADGG] x 10^8 M


ADGG-BSA
$K_I = 127 \times 10^{-9}$ M
1.2
0.8
0.4
1/V
-16
-12
-8
-4
4
8
12
16
[αAMA] x 10^8 M

polymerase and possessed an apparent K_I of 6.9×10^{-9}M. The inhibition seen with ADGG-BSA clearly deviated from the strictly noncompetitive, as was the case with ADH-BSA conjugates. An average of the three x-intercepts resulted in an apparent K_I for ADGG-BSA of 127×10^{-9}M.

These studies verified the retention by ADGG of the inhibitory potential of free α-amanitin and demonstrated that its carbodiimide mediated conjugation to proteins was feasible. From the previously described attempts at conjugation of ADH and Con A, the need for a system to evaluate the conditions under which successful conjugation of ADGG to Con A could be obtained became apparent. The optimal conditions for production of conjugate that retained both α-amanitin and lectin associated properties required definition. For this reason and to conserve ADGG, the kinetic analysis of the EDC mediated binding of ^{14}C-hippuric acid and Con A was undertaken.

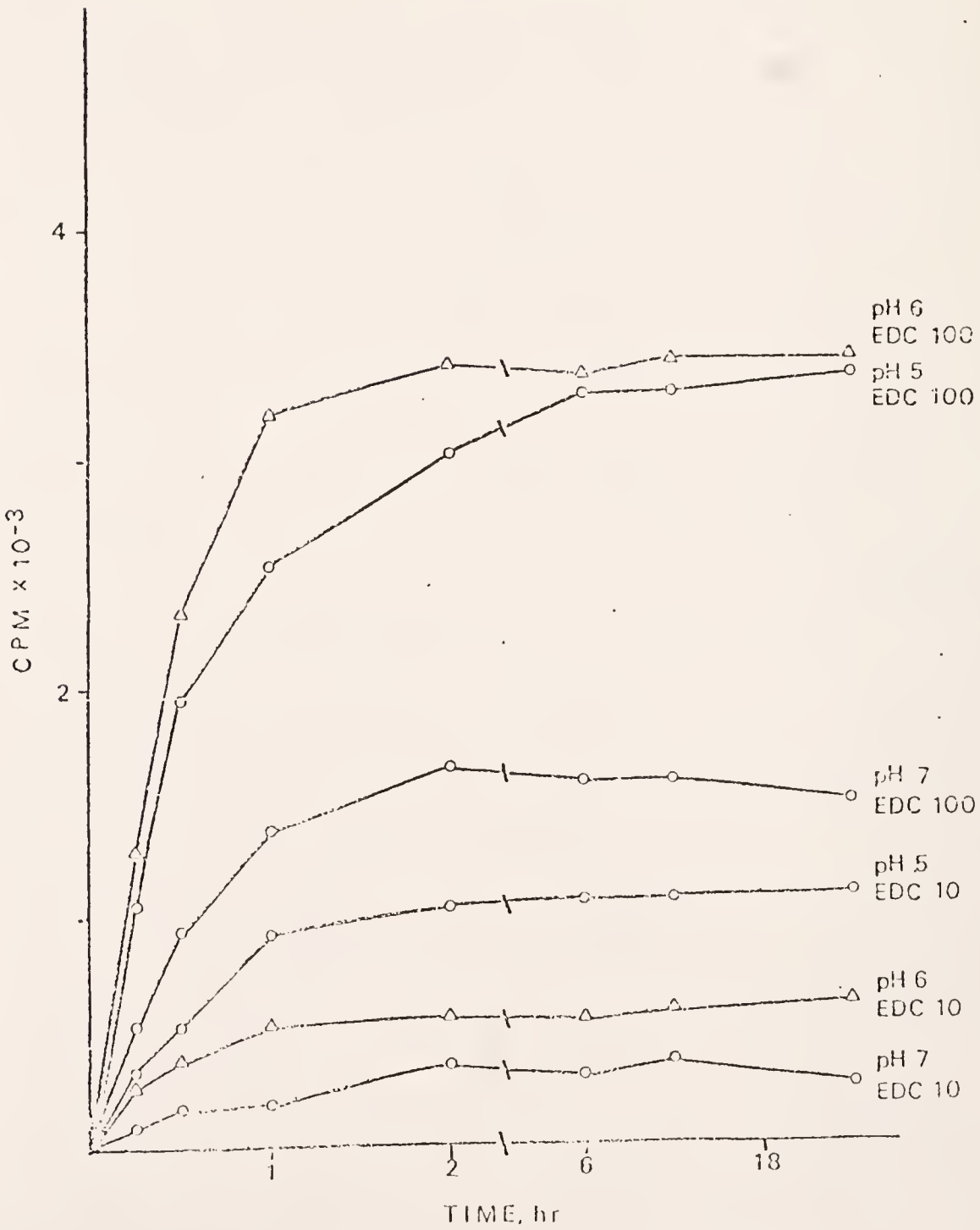
Hippuric Acid-Con A Conjugates

Synthesis

The conditions which would allow for the introduction of a defined number of ^{14}C-labeled hippuric acid residues onto Con A with a minimum of protein cross-linking were determined by measuring the amount of ^{14}C activity precipitated by TCA and collected on GF/C glass fiber filters as described in Materials and Methods. Figure 16 presents the results of an experiment in which the effect of varying pH

Figure 16. EFFECT OF PH AND EDC CONCENTRATIONS ON ^{14}C-HA-CON A CONJUGATION

Reactions were carried out in 1.0 ml volumes of 0.1 M sodium phosphate buffer containing 1.0 μmoles of ^{14}C-HA (8.5×10^5 dpm/μmole), 10 or 100 μmoles of EDC and 0.001 μmoles of Con A. Results are expressed as the total activity of the TCA precipitable material collected from duplicate 0.05 ml samples on GF/C glass fiber discs as described in Materials and Methods.

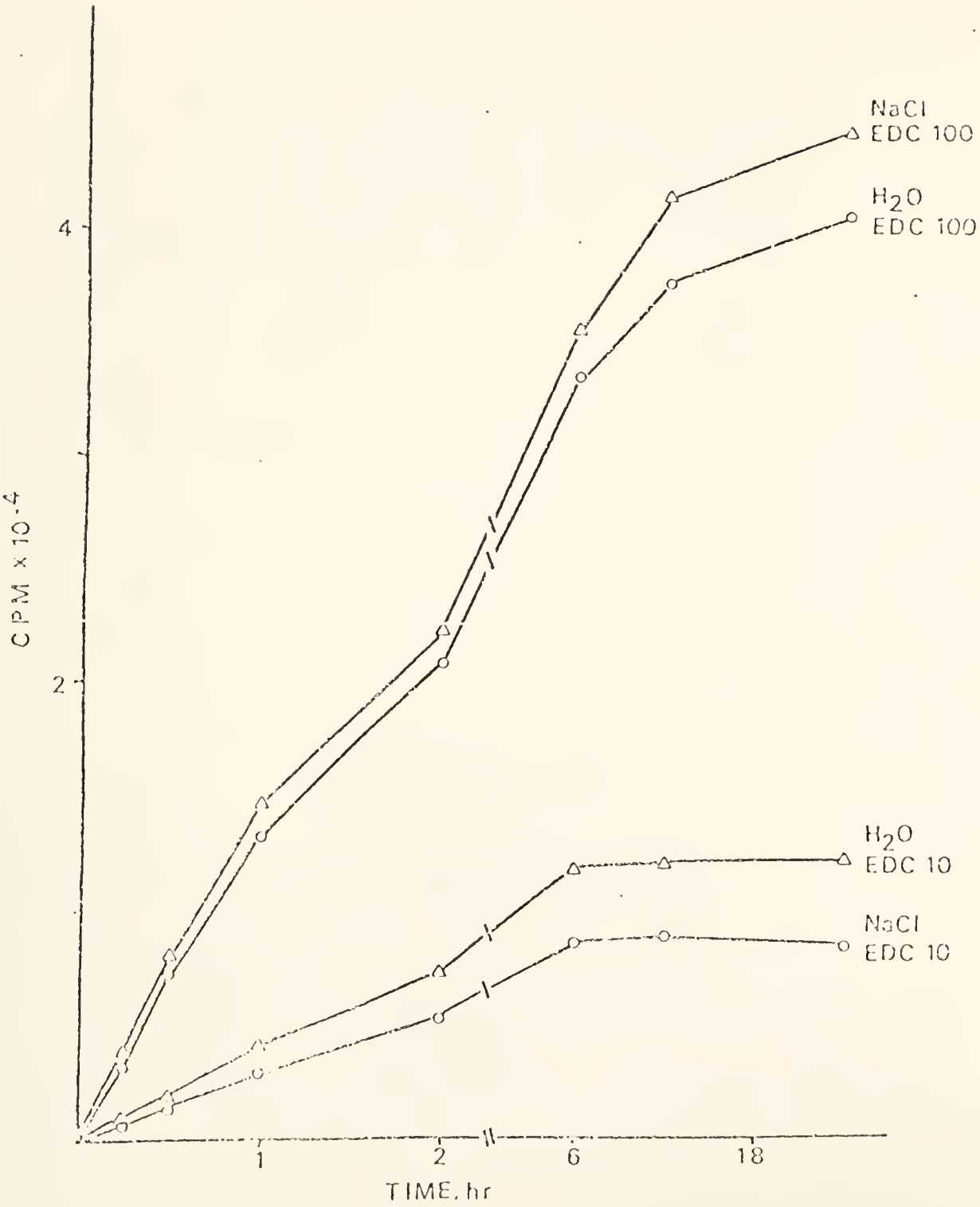

4
2
CPM x 10-3
pH 6
EDC 100
pH 5
EDC 100
pH 7
EDC 100
pH 5
EDC 10
pH 6
EDC 10
pH 7
EDC 10
1
2
6
18
TIME, hr

and EDC concentrations were examined. Carbodiimide coupling was performed in the presence of 0.1M phosphate buffers and in all cases higher concentrations of EDC (100μmole/ml versus 10μmole/ml) gave greater incorporation. Both the rate of reaction and maximal amount of labeling were increased. For a given concentration of EDC, the lowest pH examined, pH 5, proved optimal with an EDC concentration of 10μmole/ml resulting in the incorporation of 10^3cpm per 0.001μmole Con A. An EDC concentration of 100μmole/ml yielded a maximum of 3.3 x 10^3cpm per 0.001μmole Con A at the same pH. Increasing the pH to 6 or 7 caused a decrease in the rate and maximum level of incorporation. Under all conditions examined here, the reactions were essentially complete by 2 hours.

The effects of the presence of NaCl, an important parameter affecting the confirmations of Con A, at two different EDC concentrations are presented in Figure 17. No effect on the rate or extent of reaction was observed in the presence of 0.1M NaCl in comparison to the aqueous reaction. Increasing the EDC concentration 10-fold resulted in an approximate 5-fold increase in the maximum amount of incorporation as well as an increase in the reaction rate. The reactions in the absence of phosphate buffer took much longer to go to completion (18-24 hours versus 2 hours) and attained 10-fold greater maximal values than for the corresponding reaction at pH 5 in phosphate buffer. These data would argue that phosphate is providing a controlled situation

Figure 17. EFFECT OF NACL AND CONCENTRATION OF EDC ON ^{14}C-HA-CON A CONJUGATION

Reactions were carried out with concentrations of reactants identical to those described for Figure 16. The buffer systems for this experiment were 0.1 M NaCl and 0.01 M NaCl pH 7.2 containing 0.1 mM and 0.01 mM $CaCl_2$ and $MnCl_2$, respectively.


NaCl
EDC 100
H_2O
EDC 100
H_2O
EDC 10
NaCl
EDC 10
4
2
CPM x 10-4
1
2
6
18
TIME, hr

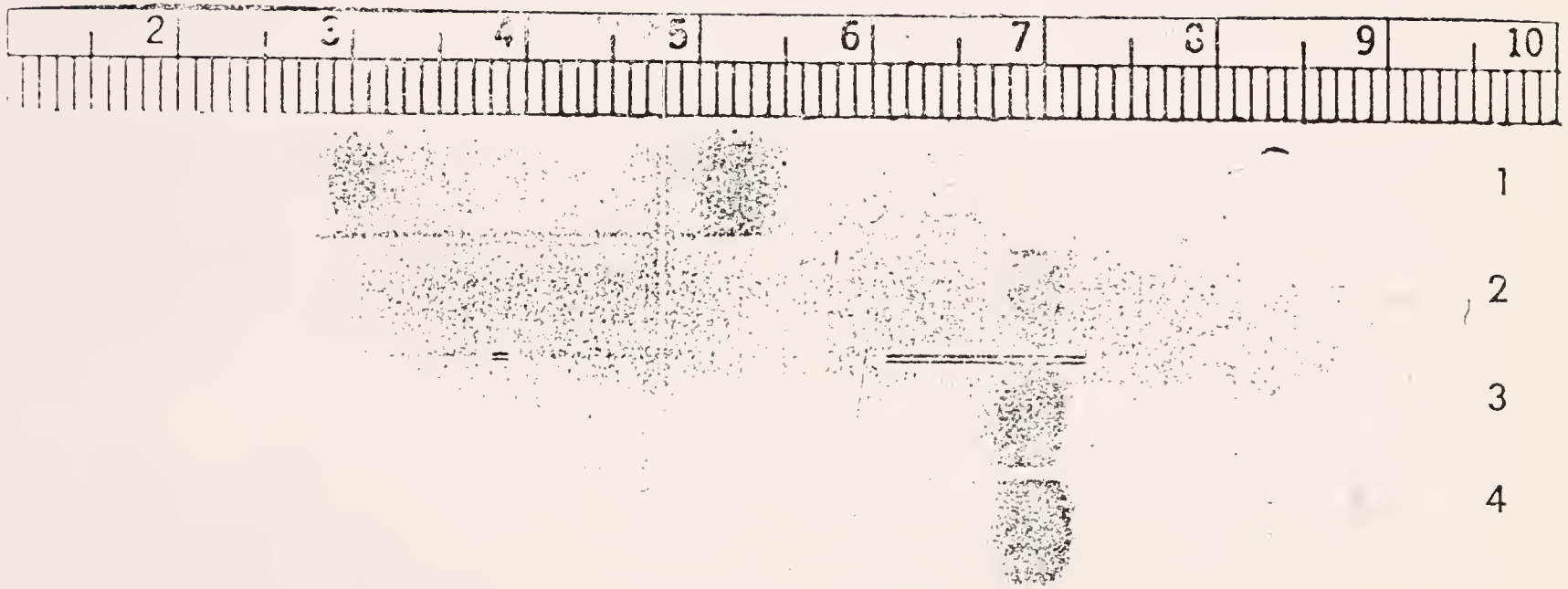
under which a defined number of acid residues may be introduced onto Con A by EDC.

Characterization

For examination of the extent to which hippuric acid conjugates of Con A retain lectin associated properties, two different conjugates were prepared utilizing the same concentration of ^{14}C-HA described above. One was prepared in the presence of 0.1M phosphate buffer, pH 5 and the other in 0.1M NaCl, pH 7.2. Both were reacted with 10μmole/ml of EDC for 1 hour. After reaction they were dialyzed and subjected to SDS-PAGE. The results are shown in Figure 18. The upper gel contains molecular weight standards ranging from 53,000 to 265,000 daltons. The next gel (2) is a sample of native Con A and shows the typical 24,000 dalton subunit with a small amount of the naturally occurring fragments of Con A. Gel 3 contained ^{14}C-HA-Con A conjugate prepared in phosphate buffer, pH 5. This conjugate preparation contained 2.4 residues of hippuric acid per mole of Con A. The electrophoresis shows a lack of material with a molecular weight greater than the Con A subunit. This would indicate that no cross-linking of the protein subunits has occurred. The bottom gel contained ^{14}C-HA-Con A conjugate from the NaCl reaction. The molar ratio of hippuric acid moieties to Con A for this conjugate was 9.6. This conjugate contained some degree of cross-linked material, estimated to be less than 8% of the total by scanning the gels at 600nm on a recording spectrophotometer. Both

Figure 18. SDS-PAGE OF ^{14}C-HA-CON A CONJUGATES

Samples of ^{14}C-HA-Con A conjugates prepared in 0.1 M phosphate buffer, pH 5 (gel 3) and ^{14}C-HA-Con A conjugates prepared in 0.1 M NaCl, pH 7.2 (gel 4) were electrophoresed on 5% acrylamide in comparison to native Con A (gel 2) or molecular weight standards ranging from 53,000 to 265,000 daltons (gel 1).


2
3
4
5
6
7
8
9
10
1
2
3
4

reaction conditions, and pH 5 phosphate buffer in particular, seem to preserve the native subunit structure of Con A.

The nature of the chemical bond between the hippuric acid residue and Con A was examined by exposure of the conjugates, prepared as described above, to hydroxylamine. The conditions of exposure were such that hydrolysis of ester type bonds, that may form as a side reaction during carbodiimide coupling (Carraway and Koshland, 1972; Timkovich, 1977), would be complete. The conjugate prepared in phosphate buffer was 88% stable to hydroxylamine and the preparation from the NaCl reaction contained 65% stable bonds. These would presumably represent covalent peptide linkages.

The ligand binding activity of the two conjugates as determined from the ability to bind to Sephadex G-75 and be eluted with specific saccharide ligand is shown in Figure 19. Complete retention of saccharide binding activity by this criterion was demonstrated for both conjugates. They adsorbed to the gel and were eluted by the Con A specific ligand, 0.1M D-glucose, in volumes identical to native Con A.

A more quantitative determination of the ligand binding activity of the HA-Con A conjugates was made by determining the apparent association constant (K_a) of each conjugate for the chromogenic ligand, PNPM. Details of the method are described in Materials and Methods and the results are presented in Figure 20. At 22°C and under the conditions of the assay used, native Con A had an affinity constant of

Figure 19. SEPHADEX G-75 ABSORPTION CHROMATOGRAPHY OF HIPPURIC ACID- CON A CONJUGATES

HA-Con A conjugates from the phosphate pH 5 reaction and the NaCl pH 7 reaction were compared to Con A for binding to Sephadex G-75 in 0.15 M PBS^+, pH 7.0 after overnight dialysis in the same buffer. D-glucose (0.1 M) was added to elution buffer after one column volume had been eluted.

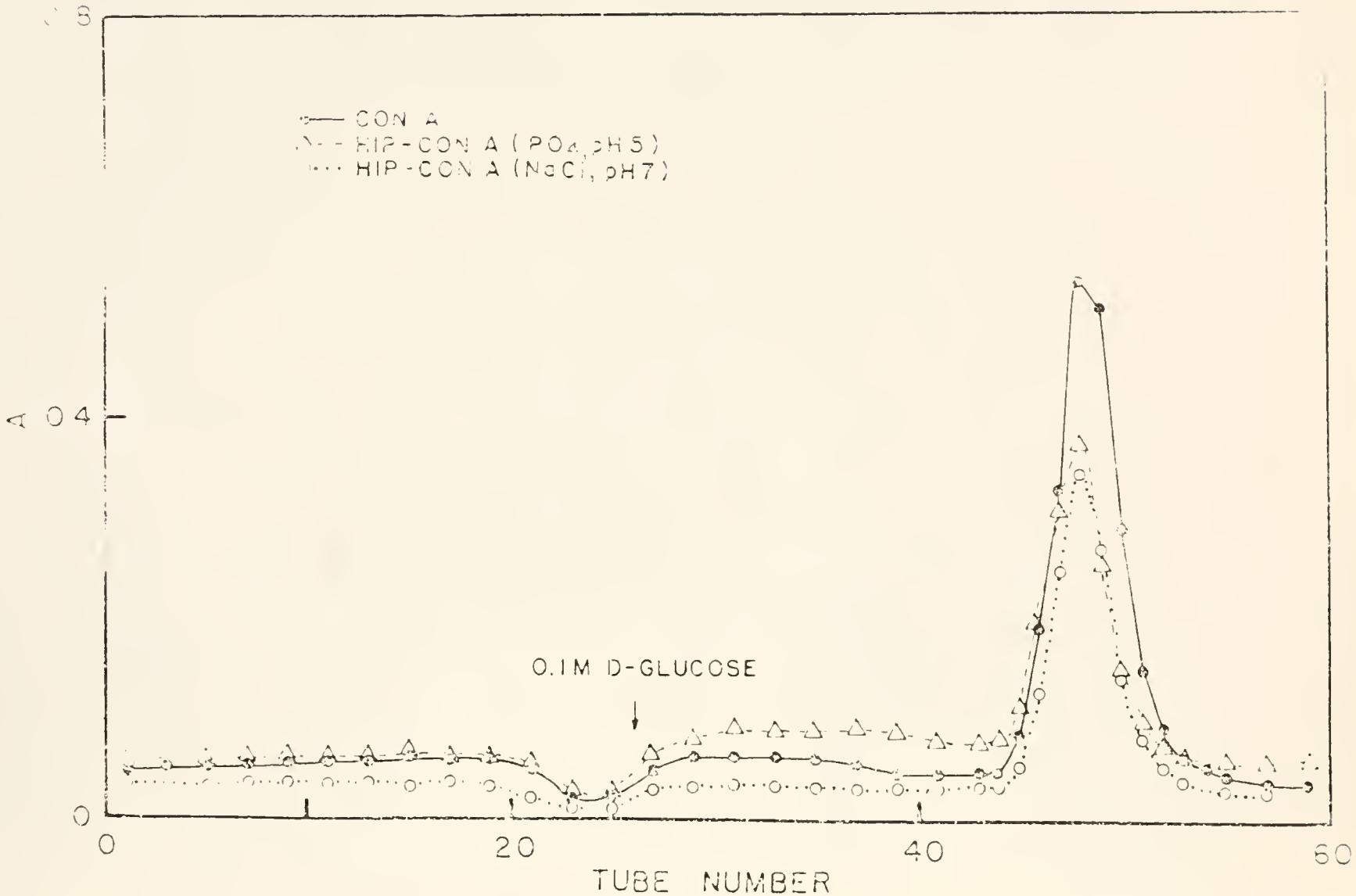
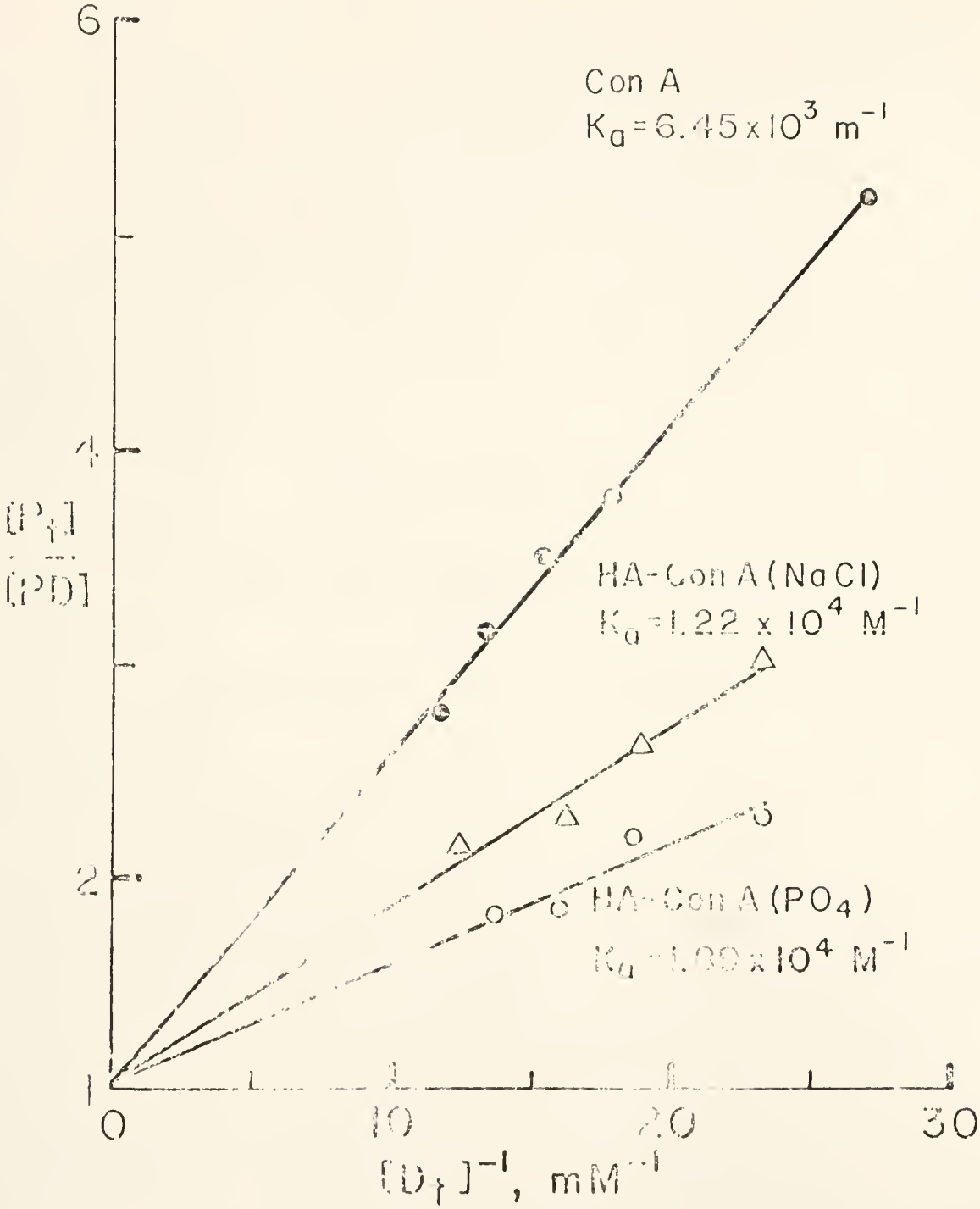

CON A
HIP-CON A (PO4, pH5)
HIP-CON A (NaCl, pH7)
0.1M D-GLUCOSE
A 0.4
0
0
20
40
60
TUBE NUMBER

Figure 20. SPECTRAL DETERMINATION OF HA-CON A CONJUGATE SACCHARIDE AFFINITY CONSTANTS (K_a)

Affinity constants determined as described in Materials and Methods for the chromogenic ligand, PNPM. Con A concentrations were 1 mg/ml and PNPM concentrations varied from 0.012 μmole/ml to 0.56 μmole/ml.


Con A
$K_a = 6.45 \times 10^3$ m^{-1}
HA-Con A (NaCl)
$K_a = 1.22 \times 10^4$ M^{-1}
HA-Con A (PO_4)
$K_a = 1.09 \times 10^4$ M^{-1}
$\frac{[P_t]}{[PD]}$
$[D_t]^{-1}$, mM^{-1}
0
10
20
30
1
2
4
6

$6.45 \times 10^3 M^{-1}$. This value compares with the value of 1.5×10^4 (at 27°C) presented by Bressler et al. (1973). The HA-Con A conjugate prepared in phosphate buffer exhibited a slightly greater association with $K_a = 1.22 \times 10^4 M^{-1}$ and the conjugate prepared in NaCl had a K_a slightly larger still ($K_a = 1.89 \times 10^4 M^{-1}$). Although the reason for the differences in the association constants is not immediately apparent, the values clearly point to the retention of ligand binding affinity by the HA-Con A conjugates.

In addition to the determination of binding affinity, the specific interaction of HA-ConA conjugates and saccharide moieties was further examined by the agglutination of red blood cells. Human type A red blood cells were used in a standard hemagglutination assay to determine the minimal concentration of each conjugate that would induce hemagglutination. The results are presented in Table 5 along with a summary of the physical parameters previously discussed for HA-Con A conjugates. Native Con A caused agglutination at a minimal concentration of 4.5μg/ml whereas HA-Con A (PO_4) agglutinated at 3.1μg/ml and HA-Con A (NaCl) at 6.2μg/ml. Both conjugates agglutinated red blood cells as effectively as native Con A within the limits of error for serially diluted agglutination assays (±50%).

TABLE 5

PROPERTIES OF HIPPURIC ACID-CON A CONJUGATES

Conjugate	Substitution Mole HA / Mole Con A	Percent Peptide Bond[a]	Ligand K_a	Hemagglutination[b] Minimum Concentration, μg/ml
HA-Con A (PO^4)	2.4	88	1.89×10^4	3.1
HA-Con A (NaCl)	9.6	65	1.22×10^4	6.2
Con A	---	--	6.45×10^3	4.25

[a] 0.5M hydroxylamine, pH 7.0, 12 hour, 37°C
[b] 1×10^8 RBC/ml, 0.15M PBS^+, pH 6.8, 22°C

ADGG-CON A Conjugates

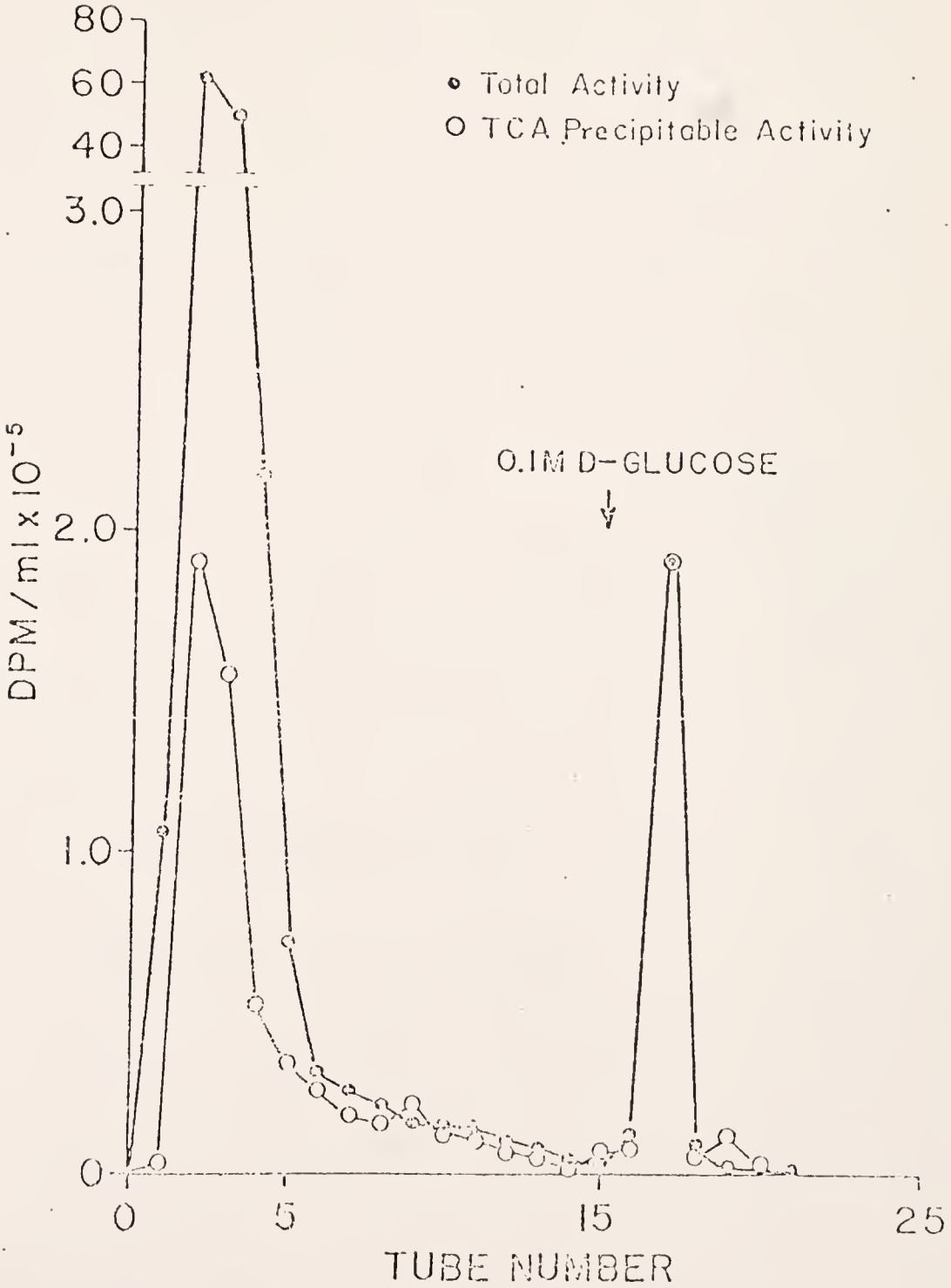
Synthesis

The results of the ^{14}C-hippuric acid studies indicated that reaction at pH 5 in phosphate buffer with relatively low concentrations of protein and carbodiimide would result in formation of Con A conjugates with a defined number of substitutions and minimal perturbation of the native lectin structure. These conditions were used for the synthesis of ^{3}H-DM-ADGG-Con A conjugates in order to verify the reaction conditions and to produce a labeled ADGG-Con A conjugate for cell binding experiments.

After completion of the synthesis, the ^{3}H-DM-ADGG-Con A containing reaction mixture was applied to a Sephadex G-75 column and isolated by absorption to the gel. Figure 21 shows the chromatographic profile as determined by total radioactivity eluted and TCA precipitable acitvity. The majority of the radioactivity emerged from the column as a nonprecipitable fraction in a single peak. Following addition of 0.1M D-glucose to the eluant, ^{3}H-DM-ADGG-Con A conjugate was eluted in a single peak that was 100% precipitable by TCA. This peak contained greater than 90% of the total lectin present in the reaction. Similar reaction conditions were used to produce other conjugates of ADGG and Con A that were used for examining the interaction of ADGG-Con A conjugates with cultured cells. The ADGG-Con A conjugates were isolated by absorption chromatography to Sephadex G-75.

Figure 21. SEPHADEX G-75 ABSORPTION CHROMATOGRAPHY OF OF ^{3}H-DM-ADGG-CON A CONJUGATES

The reaction mixture from EDC conjugation of ^{3}H-DM-ADGG and Con A was adjusted to 0.15 M with respect to NaCl and pH 7 and chromatographed in 0.15 M PBS^{+} pH 7. Results as shown determined as total ^{3}H activity (●) and TCA precipitable activity (o) from 0.01 ml samples of fractions eluting from the column.


80
60
40
3.0
2.0
1.0
0
DPM / ml x 10^-5
• Total Activity
O TCA Precipitable Activity
0.1M D-GLUCOSE
0
5
15
25
TUBE NUMBER

The conjugates contained an average of 3.6moles of ADGG per mole of Con A as determined from spectral absorption at 395nm and Lowry protein determination. The absorption spectrum was similar to those presented for ADGG-BSA conjugates and contained absorption peaks at 280nm and 395nm characteristic of covalent association of the protein and ADGG components.

Characterization

The ^{3}H-DM-ADGG-Con A conjugates were analyzed for stability to hydroxlyamine under neutral conditions. By this criterion they were found to contain a minimum of 78% stable covalent bonds comprising the ^{3}H-DM-ADGG lectin linkage.

The hemagglutinating ability of the ^{3}H-DM-ADGG-Con A and ADGG-Con A conjugates was determined with human type A red blood cells. ADGG-Con A conjugates would cause agglutination at a minimal concentration of 9.5μg/ml. The effect of conjugation of ^{3}H-DM-ADGG to Con A and a number of other lectins with varying ligand specificities was compared after the synthesis at pH 5 in phosphate buffer. Table 6 presents the results of the agglutinations and the molar ratios of ^{3}H-DM-ADGG to lectin that resulted from conjugation. In all cases significant binding of ^{3}H-DM-ADGG to lectin occurred. Comparison of the hemagglutination titers of the ^{3}H-DM-ADGG-lectin conjugates with the control value for each lectin shown in parenthesis, demonstrates that the hemagglutination titers of Con A, castor bean type I (CB-I),

TABLE 6

RETENTION OF HEMAGGLUTINATING ACTIVITY BY ^{3}H-DM-ADGG-LECTIN CONJUGATES

Lectin	Lectin Concentration μmole/ml x 10^3	μmole ^{3}H-DM-ADGG Bound/ml x 10^3	μmole ^{3}H-DM-ADGG / μmole Protein	Hemagglutination Titer (Control)
Lentil	2.08	2.48	1.19	2 (4)
SBA	0.83	1.38	1.66	1 (8)
PHA	0.67	1.64	2.44	1 (4)
WGA	4.26	4.55	1.07	8 (16)
CB-I	0.83	2.11	2.55	2 (4)
CB-II	1.67	2.04	1.22	4 (8)
Con A	0.94	2.14	3.34	4 (8)

Reactions performed in 0.01M PO_4, pH 5 at final concentrations of: lectin, 100 μg/ml; ^{3}H-DM-ADGG, 0.1 μmole/ml (S.A. $\cong$ 9.6 x 10^7 dpm/μmole): EDC, 40 μmole/ml. After 22 hours of reaction, 50 μl samples were precipitated with TCA for determination of ^{3}H-activity. The remaining material was adjusted to Ph 6.8 and 0.15 M with respect to NaCl for hemagglutination assay.

castor bean type II (CB-II), WGA and lentil lectin were only slightly decreased. Soybean agglutin (SBA) and phytohemagglutin (PHA) demonstrated significant reduction in hemagglutination titer after conjugation. With the exception of SBA and PHA, all of the ADGG-lectin conjugates retained essentially equivalent agglutinating activity to that of the native lectin.

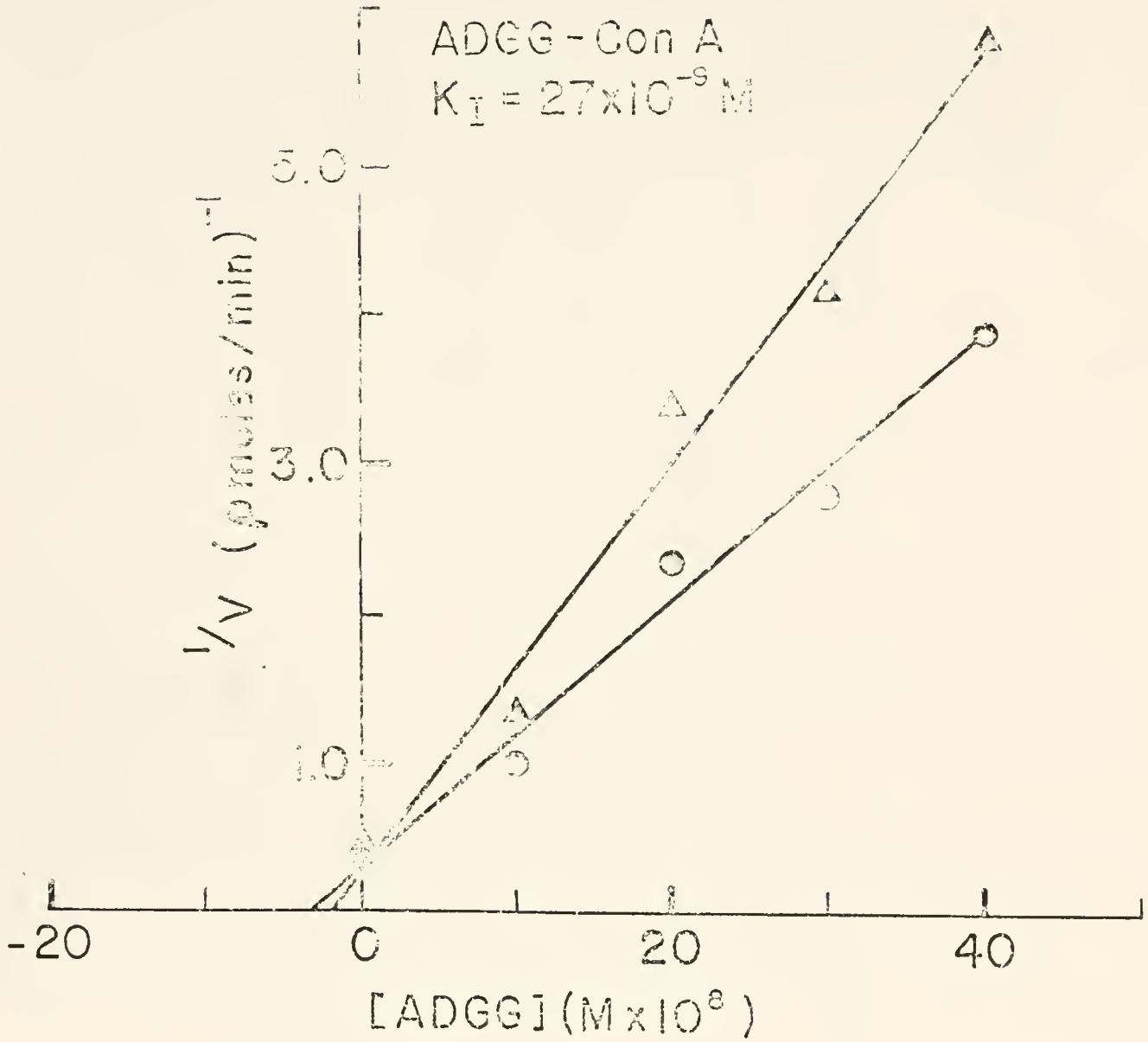
Retention of the amanitin associated ability to interact with RNA polymerase II was examined for ADGG-Con A conjugates. The determination of the inhibition constant for calf thymus RNA polymerase II is shown in Figure 22. This preparation, containing 3.6 moles ADGG per mole of Con A, had an inhibition constant K_I, of 25×10^{-9}M. Under the conditions used to establish this value, native Con A at equivalent concentrations to that present with the conjugate did not cause inhibition of polymerase activity. The inhibition by ADGG-Con A was unaffected by the presence of 0.1M D-glucose further establishing the specificity of the inhibition as being derived for the amanitin portion of the conjugate.

Interaction with Cells

The binding of ADGG-Con A conjugates to cell surface glycoproteins present on cultured cells was investigated by competition experiments with ^{125}I-labeled Con A. The amount of ^{125}I-Con A bound by CHO cells was first determined for a 15 minute exposure at 22°C. The results of this binding for CHO H-7 cells and H-7 Wcr cells, a line derived from

Figure 22. INHIBITION OF CALF THYMUS RNA POLYMERASE II BY ADGG-CON A CONJUGATES

Results are expressed as the reciprocal of velocity (μμmole ^{3}H-UMP incorporated per minute)$^{-1}$ versus concentration of inhibitor for two different concentrations of substrate (UTP): 0.008 mM (●) and 0.016 mM (▲). Least squares analysis was used to obtain the best fit for each line.


ADGG - Con A
$K_I = 27 \times 10^{-9}$ M
5.0
3.0
1.0
$1/V$ (μmoles/min)$^{-1}$
-20
0
20
40
[ADGG] (M x 10^8)

H-7 that is resistant to Con A induced cytotoxicity, is presented in Figure 23. During the 15 minute exposure the H-7 cells bound a maximum of 16 x 10^6 molecules Con A per cell whereas the H-7Wcr bound a maximum of 14.8 x 10^6 molecules per cell. Furthermore, the binding by H-7Wcr reached a plateau at 37μg/ml Con A while the H-7 cell binding did not appear to level off. The ^{125}I-Con A binding for both cells was inhibited 89% by preincubation with Con A (100μg/ml, 30 minutes) and was inhibited only 21% by prior exposure to BSA (100μg/ml, 30 minutes). Both cell lines appear, within limits of error, to bind essentially the same amount of Con A over the range of concentrations investigated.

Figure 24 depicts the binding of ^{125}I-Con A by rat fibroblast cells, A-9, and their amanitin resistant subline, LAN 2. Both cell lines were exposed to ^{125}I-Con A for 15 minutes at 22°C. The results indicate that both cell lines bound an equivalent number of ^{125}I-Con A molecules with a maximum of 12.6 x 10^6 molecules per cell being obtained. This amount is somewhat less than that bound by the CHO cells. In addition, the rat cells bound fewer Con A molecules over the entire range of concentrations tested than did the CHO cells. The binding was inhibited completely by pretreatment with Con A (100μg/ml, 30 minutes) and only inhibited 12% by prior exposure to BSA.

The binding of ^{3}H-DM-ADGG-Con A was examined only with H-7 CHO cells due to the limited quantities of labeled

Figure 23. BINDING OF ^{125}I-CON A TO CHO H-7 AND H-7 WCR CELLS

Duplicate 80% confluent monolayers of cells were exposed to increasing concentrations of ^{125}I-Con A (S.A. = 2 x 10^4 cpm/μg) for 15 minutes at 22°C. The binding of the maximum ^{125}I-Con A dose was 89% inhibitable by 30 minutes preincubation of the cells with Con A (100 μg/ml).

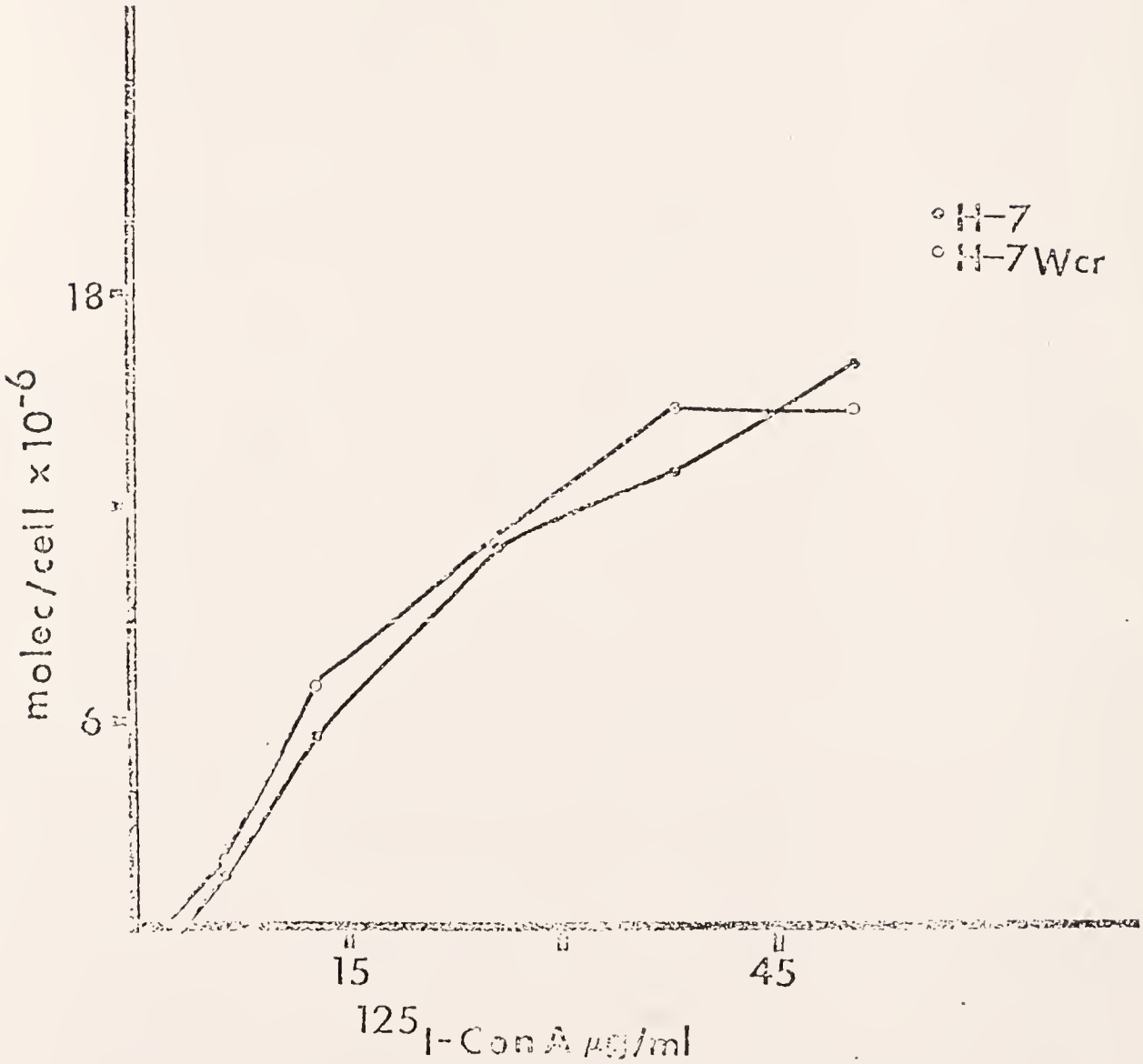
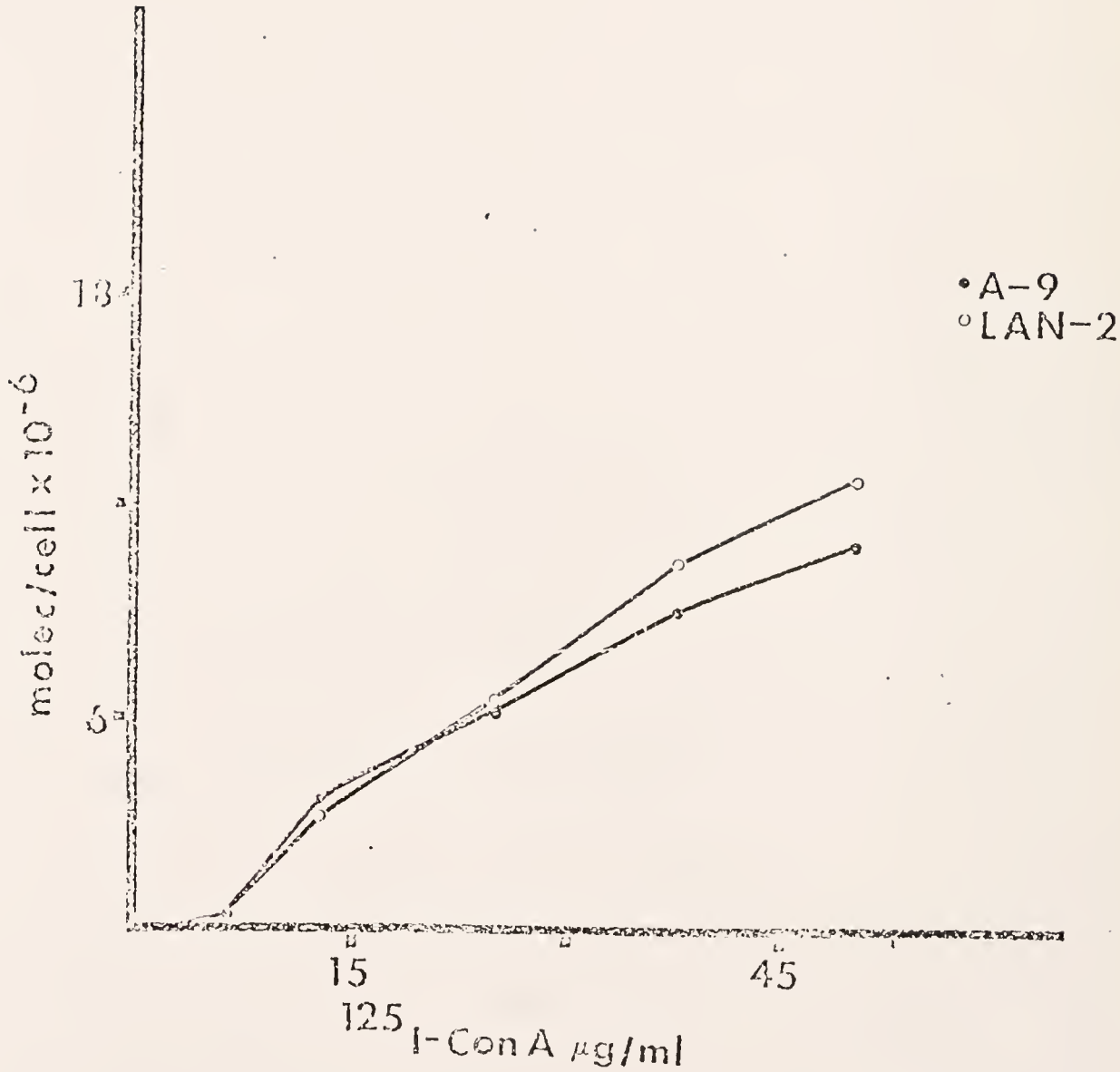

H-7
H-7Wcr
18
6
molec/cell ×10⁻⁶
15
45
125I-Con A μg/ml

Figure 24. BINDING OF ^{125}I-CON A TO A-9 AND LAN-2 CELLS

Duplicate 80% confluent monolayers of cells were exposed to increasing concentrations of ^{125}I-Con A (S.A. = 2 x 10^4 cpm/μg) for 15 minutes at 22°C. The binding of the maximum ^{125}I-Con A dose was 99% inhibitable by 30 minutes preincubation of the cells with Con A (100 μg/ml).


13
6
molec/cell x 10^-6
•A-9
°LAN-2
15
45
125
I-Con A μg/ml

ADGG that were available. The number of molecules bound over a 15 minute period at 22°C was compared to the amount of ^{125}I-Con A bound at equivalent concentrations. The results are presented in Table 7. The H-7 cells bound identical amounts of either Con A derivative (10×10^6 molecules/cell). The binding of both was 87-89% inhibitable by 30 minutes preincubation with Con A (100μg/ml) and 76-85% inhibitable by 1mM MDM. ADGG (2×10^{-6}M) did not interfere with the binding of ^{3}H-DM-ADGG-Con A or ^{125}I-Con A further establishing that the conjugates interact with cell surface glycoproteins by virtue of specific binding of the Con A portion of the conjugate.

Competition of ADGG-Con A or Con A with the binding of ^{125}I-Con A by H-7 CHO cells is presented in Figure 25. A constant amount of ^{125}I-Con A was competed with increasing amounts of ADGG-Con A or Con A. The data is presented as the fraction ^{125}I-Con A bound versus the fraction of competitor Con A of the total Con A present. The linearity of the data and the near identical results obtained for both competitors clearly identify the sites for cellular binding of ADGG-Con A as being identical with those for Con A.

The actual uptake and internalization of ADGG-Con A by H-7 CHO cells was quantitated by exposing cells under sterile conditions to ^{3}H-DM-ADGG-Con A as previously described for cell binding assays. After washing the cells as if for determination of bound material, fresh culture media was added instead and the cells returned to 37°C for 60 minutes.

TABLE 7

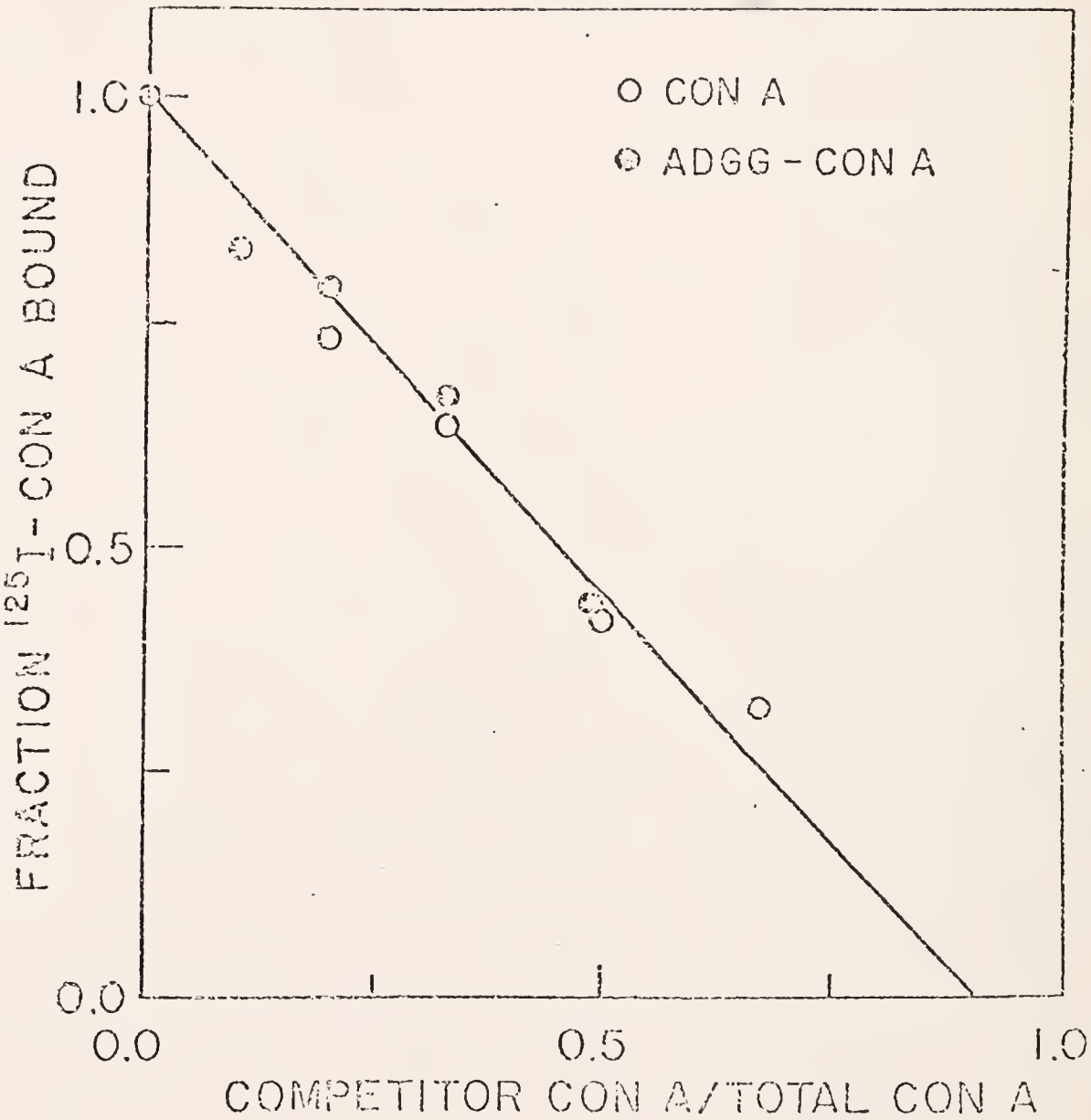
BINDING OF ^{125}I-CON A AND ^{3}H-DM-ADGG-CON A BY H-7 CHO CELLS

Derivative 2.0×10^{-4}M[a]	Molecules Bound Per Cell	Percent Inhibition	
		MDM, 10^{-3}M[b]	Con A, 9.4×10^{-4}M[c]
^{125}I-Con A	10.0×10^{6}	85	89.3
^{3}H-DM-ADGG-CON A	9.9×10^{6}	75.5	87.2

[a] Concentration of Con A
[b] Inhibition after 30 minutes preincubation with Con A derivatives; MDM present during binding
[c] Inhibition after 30 minutes preincubation with cells; Con A washed off prior to binding of derivatives

Figure 25. COMPETITION OF ADGG-CON A AND CON A WITH ^{125}I-CON A BINDING BY H-7 CHO CELLS

A constant amount of ^{125}I-Con A (10 μg/ml) was competed with increasing amounts (0 to 20 μg/ml) of either Con A (○) or ADGG-Con A (●) for a 15 minute exposure at 22°C. Cells were preincubated for 30 minutes with the competing Con A, washed and exposed to ^{125}I-Con A for 15 minutes.


O CON A
ADGG - CON A
1.0
0.5
0.0
FRACTION ^{125}I - CON A BOUND
0.0
0.5
1.0
COMPETITOR CON A/TOTAL CON A

The media was removed followed by three washes of fresh media which were pooled and counted for determination of eluted ^{3}H-DM-ADGG-Con A. Identical control cultures were processed without exposure to media for determination of the total amount of ^{3}H-ADGG-Con A that bound to the cells during 15 minutes. The cells exposed to media were processed for determination of remaining cell associated activity. This experiment allowed for estimation of the amount of conjugate that is removed by exposure to carbohydrates and glycoprotein components of the serum present in tissue culture media. Exposure to media resulted in 60% of the total activity being released from the cells. The remaining 40% (4.2×10^{6} molecules/cell) is irreversibly associated with the cell and is presumably internalized. This fraction of the conjugate would be expected to be that which causes inhibition of cellular activity.

The ability of Con A conjugates of α-amanitin to bind to specific cell receptors via the Con A moiety has been clearly demonstrated by the previous experiments. Whether this binding would result in targeting of the α-amanitin with subsequent enhanced toxicity of the conjugate in comparison to free α-amanitin was examined in the following experiments.

Twelve hour cultures of H-7 CHO cells were exposed to ADGG-Con A, Con A or free ADGG for 15 minutes at 22°C, washed and incubated at 37°C for an additional 48 hours as described in Materials and Methods. Toxicity was

quantitated by determining cell numbers as a function of increasing concentrations of each inhibitor to which the cells had been exposed. Figure 26 shows the results expressed as a percentage of the control number. Free ADGG had little effect on the growth of the cells after a 15 minute exposure to concentrations as high as 3×10^{-6}M. Con A had no effect up to 40μg/ml but at 120μg/ml resulted in 30% inhibition of cell growth. ADGG-Con A had a remarkable effect at a concentration as low as 0.5×10^{-6}M with respect to amanitin where greater than 50% inhibition of cell growth was observed. At the maximum dose tested, 3×10^{-6}M, ADGG-Con A caused 80% inhibition of cell growth. The concentration of conjugated ADGG that resulted in 50% inhibition (50% inhibitory does, ID_{50}) was 2.1×10^{-7}M. At that concentration, free ADGG or the equivalent amount of Con A to that present in the conjugate, had no effect.

In another experiment, the inhibition of H-7 cell growth obtained by an ADGG-Con A concentration of 1×10^{-6}M was prevented by exposing the cells to the conjugate in the presence of the Con A specific sugar, MDM (1.0mM). MDM by itself caused no effect, 1×10^{-6}M ADGG-Con A caused 58% inhibition and cells protected with MDM had only 6.7% inhibition.

These results were compared to the effects of continuous exposure of H-7 CHO cells to α-amanitin or ADGG for 48 hours shown in Figure 27. Alpha-amanitin resulted in 50% inhibition at a concentration of 0.5×10^{-6}M whereas

Figure 26. INHIBITION OF H-7 CHO CELLS BY ADGG, CON A AND ADGG-CON A

Inhibition of cell numbers, expressed as percent of control, was determined 48 hours after a 15 minute exposure at 22°C of H-7 cells to ADGG (●), Con A (□) or ADGG-Con A (○).

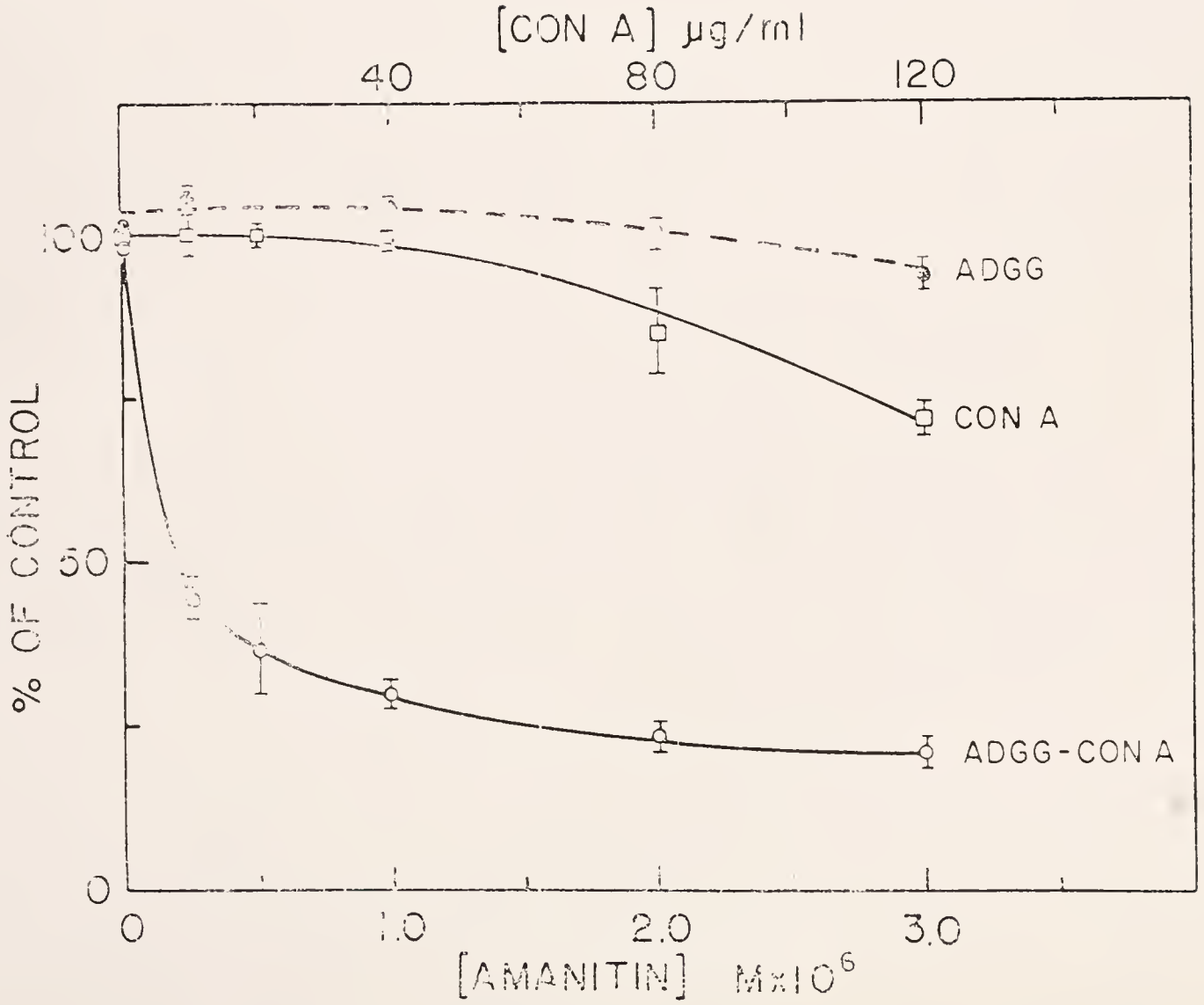
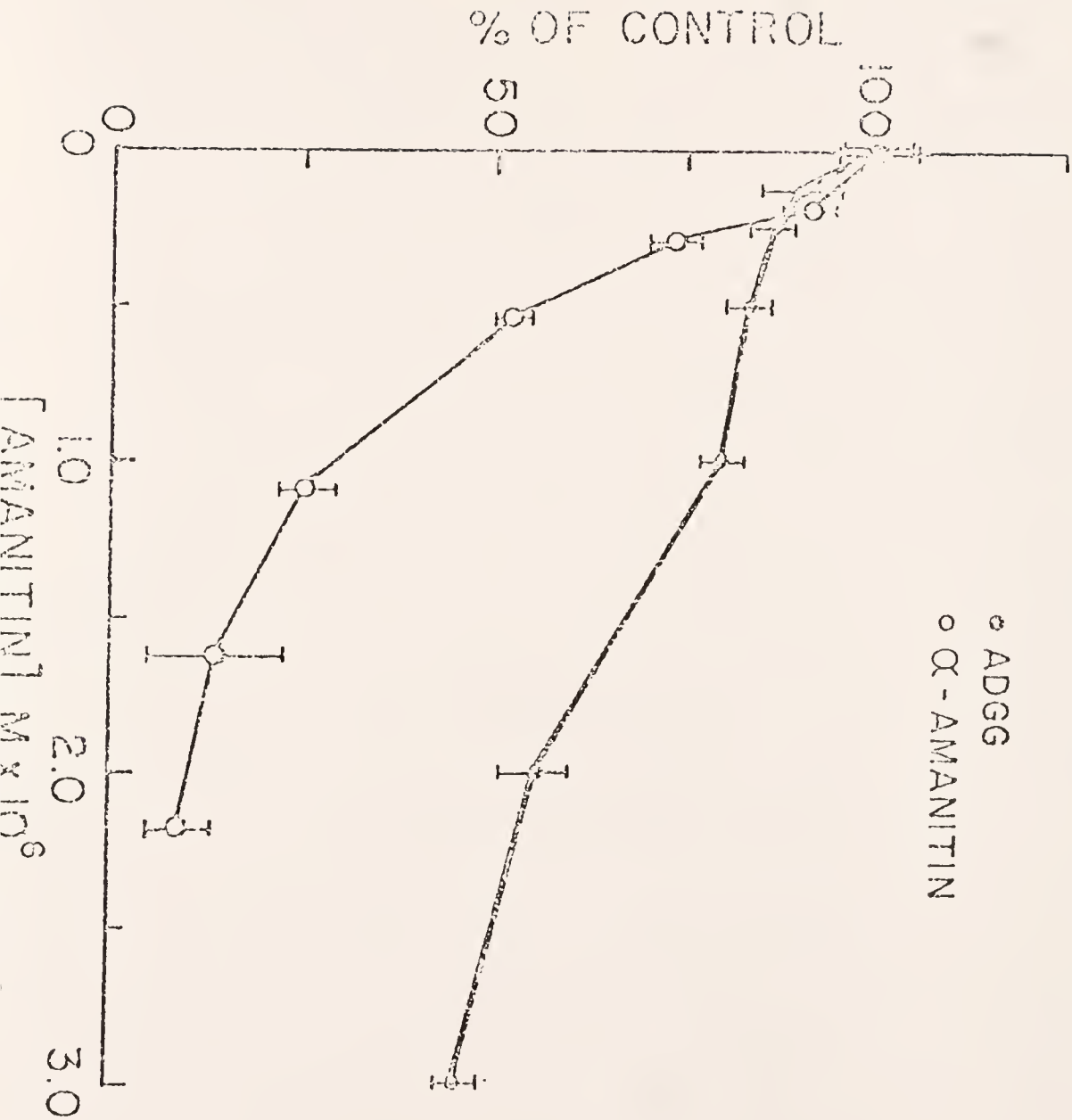

[CON A] µg/ml
40
80
120
100
50
0
% OF CONTROL
ADGG
CON A
ADGG-CON A
0
1.0
2.0
3.0
[AMANITIN] M×10⁶

Figure 27. EFFECT OF CONTINUOUS EXPOSURE TO α-AMANITIN AND ADGG ON H-7 CHO CELLS.

Inhibition of cell numbers, expressed as percent of control, was determined following 48 hours of continuous exposure of H-7 cells to ADGG (●) or α-amanitin (○).


% OF CONTROL
100
50
0
0
1.0
2.0
3.0
[AMANITIN] M x 10^6
• ADGG
○ α-AMANITIN

ADGG had an $ID_{50} = 3 \times 10^{-6}$M. ADGG is somewhat less toxic than α-amanitin which may be a consequence of its lower affinity for RNA polymerase II or of permeability differences. Both compounds are clearly toxic to the cells with long exposure times which implies that entry to the cell is restricted. The Con A portion of the ADGG-Con A conjugate appears to be providing facilitated entry of the conjugate to allow for the observed cytotoxicity with only 15 minutes of exposure.

The effects of α-amanitin and ADGG over 48 hours of incubation with H-7Wcr cells are presented in Table 8. As was found with H-7 cells, ADGG was less toxic than free α-amanitin with an ID_{50} of 1.9×10^{-6}M in comparison to 6.4×10^{-7}M for α-amanitin. These values are essentially the same as those for the parent cell line, H-7. The effects of conjugated ADGG were examined with the H-7Wcr in an attempt to explore the mechanism of ADGG-Con A toxicity. H-7Wcr cells are resistant to the toxic effects of Con A exposure but the mechanism of their resistance is not known. Although they bind Con A as well as the parent line, it was thought that the H-7Wcr may not internalize the bound lectin. Exposure of H-7Wcr to different concentrations of ADGG-Con A, Con A, ADGG and α-amanitin for 15 minutes resulted in the inhibition shown in Table 9. As was expected, Con A had minimal toxic effect on the cells up to 160μg/ml where 9% inhibition was seen. ADGG-Con A, however, was very toxic with an ID_{50} of 3.5×10^{-7}M, comparable to

TABLE 8

EFFECT OF CONTINUOUS EXPOSURE TO α-AMANITIN AND ADGG ON H-7WCR CHO CELLS

$M \times 10^6$	Percent Of Control	α-Amanitin $M \times 10^6$	Percent Of Control
0	100	0	100
0.12	102.3	0.13	94.1
0.25	99.5	0.27	79.1
0.50	98.6	0.54	55.1
1.0	74.4	1.09	27.4
2.0	47.9	1.60	17.5
3.0	39.2	2.18	13.6

Inhibitors were added to 12 hour cultures of H-7Wcr cells. After 48 hours additional incubation, inhibition was determined by counting cell numbers.

TABLE 9

INHIBITION OF H-7WCR CHO CELLS BY ADGG, CON A AND ADGG-CON A

α-Amanitin M x 10^6	Percent of Control	ADGG M x 10^6	Percent of Control	ADGG-Con A M x 10^{6a}	Percent of Control	Con A μg/ml[b]	Percent of Control
0	100	0	100	0	100	0	100
0.12	87.4	0.12	63.6	0.12	60.9	10	109.0
0.25	73.0	0.25	69.7	0.25	40.1	20	110.6
0.50	59.0	0.50	62.9	0.50	29.8	40	96.1
1.0	56.8	1.0	58.9	1.0	26.4	80	93.2
2.0	49.8	2.0	60.9	2.0	18.9	160	90.5

Inhibition determined 48 hours after a 15 minute exposure of the cells to each concentration of inhibitor as descrived in Materials and Methods.

[a] Molarity of bound ADGG
[b] Concentrations equivalent to those present in ADGG-Con A

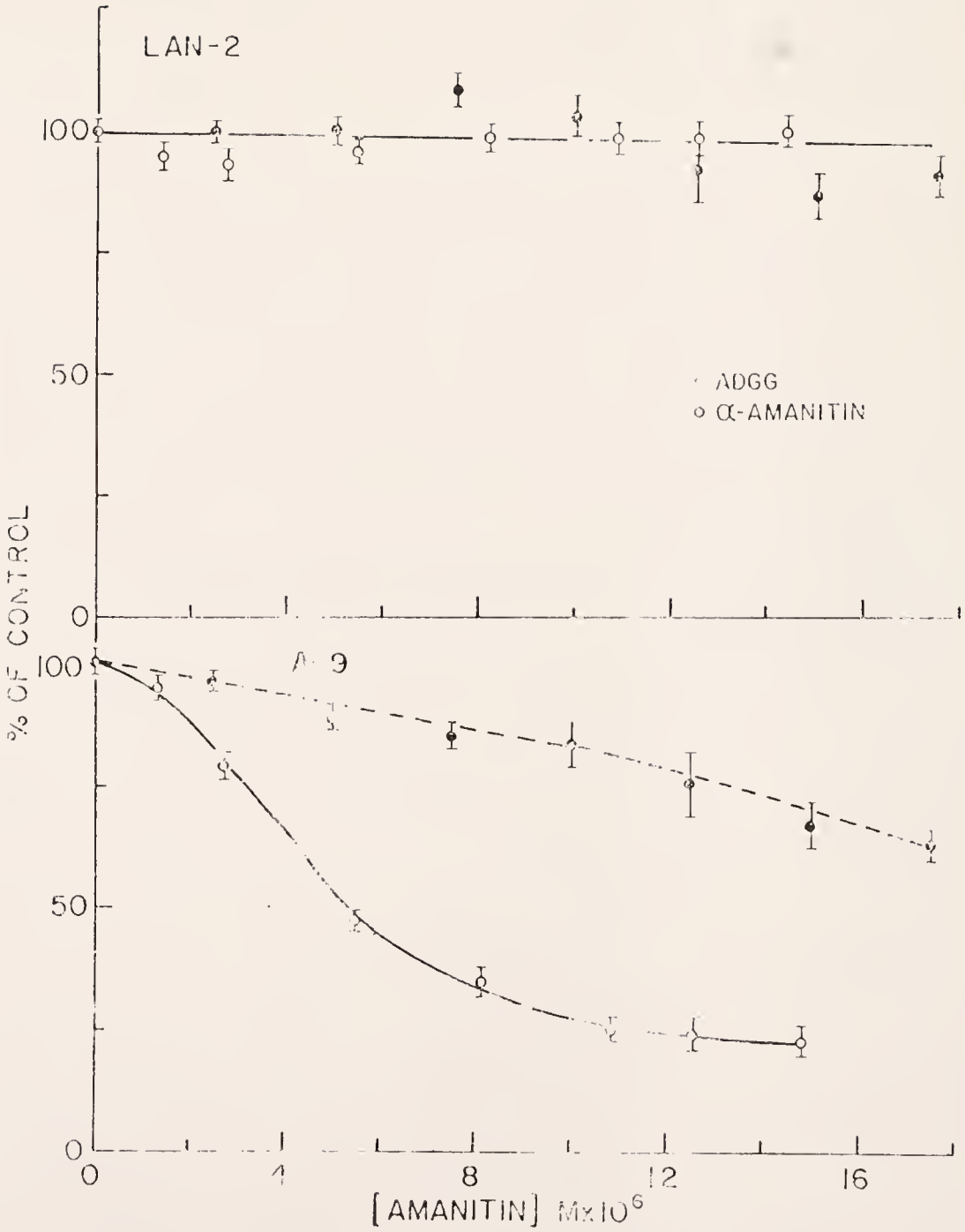
the ID_{50} of 2.1×10^{-7}M for H-7 cells. The inhibition of H-7Wcr cells obtained with free ADGG and α-amanitin was somewhat different than the minimal inhibition seen for H-7 cells and ADGG. Both ADGG and α-amanitin had a cytotoxic effect on the H-7Wcr cells after a 15 minute exposure. The inhibition was essentially the same for both compounds over the entire range of concentrations in contrast to the continuous exposure data. At the maximum dose tested, 2×10^{-6}M, ADGG gave 40% inhibition, α-amanitin 50% inhibition and conjugated ADGG- Con A gave 82% inhibition.

The ADGG-Con A clearly has more cytotoxic potential than either of the unconjugated inhibitors for both CHO cell lines. In order to verify that the conjugate toxicity is due to Con A mediated targeting of the α-amanitin portion of the conjugate and not some unique property of the conjugate itself, an α-amanitin resistant cell line, LAN-2, and its normal parent line, A-9, were tested for sensitivity to ADGG-Con A, Con A, ADGG and α-amanitin.

The effects on A-9 and LAN-2 cells of continuous exposure to α-amanitin and ADGG are presented in Figure 28. The LAN-2 cells were entirely refractory to inhibition by either ADGG or free α-amanitin up to the maximum dose tested (1.6×10^{-5}M). The parent cells, A-9, were inhibited by both ADGG and α-amanitin and as was found with CHO cells, α-amanitin ($ID_{50} = 5.4 \times 10^{-6}$M) was more toxic than ADGG (extrapolated $ID_{50} = 2.1 \times 10^{-5}$M).

Figure 28. EFFECT OF CONTINUOUS EXPOSURE TO α-AMANITIN AND ADGG ON LAN-2 and A-9 CELLS

Inhibition of cell numbers, expressed as percent of control, was determined following 48 hours of continuous exposure of A-9 and LAN-2 cells to ADGG (●) or α-amanitin (○).


LAN-2
100
50
0
ADGG
α-AMANITIN
% OF CONTROL
100
50
0
0
4
8
12
16
[AMANITIN] M x 10^6

Exposure of the two cell lines for 15 minutes to ADGG-Con A, ADGG and Con A resulted in the inhibition shown in Table 10. Con A at 40μg/ml caused approximately 10% inhibition of growth in both A-9 and LAN-2 cells. Free ADGG did not affect LAN 2 cells and at 2.5×10^{-6}M had a marginal effect on the growth of A-9 (12% inhibition). ADGG-Con A again demonstrated greatly enhanced cytotoxicity with 40% inhibition of A-9 cells occurring at 1×10^{-6}M ADGG-Con A. At the same concentration of ADGG-Con A, LAN-2 cells displayed 13% inhibition, almost an identical amount to that obtained with the same concentration of Con A alone (11% at 40μg/ml). These results strongly imply that the toxicity of ADGG-Con A is due to the specific inhibition of RNA polymerase II by the α-amanitin protein of the conjugate.

TABLE 10

INHIBITION OF A-9 AND LAN-2 CELLS BY ADGG, CON A AND ADGG-CON A

Inhibitor	Cell Type	Number of Cells x 10^{-5}	Percent of Control
None	A-9	1.40 ±.17	100
	LAN-2	1.37 ±.16	100
ADGG[a]	A-9	1.23 ±.16	87.8
	LAN-2	1.35 ±.06	98.5
ADGG-CON A[b]	A-9	0.85 ±.10	60.6
	LAN-2	1.19 ±.24	86.9
Con A[c]	A-9	1.26 ±.19	90.0
	LAN-2	1.22 ±.06	89.1

Inhibition determined 48 hours after a 15 minute exposure to the cells to each concentration of inhibitor as described in Materials and Methods.

[a] [ADGG] = 2.5×10^{-6}M
[b] ADGG-Con A: [ADGG] = 1×10^{-6}M
[Con A] = 40 μg/ml
[c] [Con A] = 40 μg/ml

SECTION IV
DISCUSSION

The primary objectives of this investigation were to assess the effectiveness of certain macromolecules, Concanavalin A in particular, to impart selectivity with respect to cellular uptake to the fungal toxin α-amanitin, and to evaluate the ability of cell surface receptors to mediate the entry of macromolecule bound toxin into the cell and thus specifically kill that cell. Achievement of these objectives required that a system be developed by which conjugates of α-amanitin and carrier macromolecules could be synthesized, characterized for retention of α-amanitin and carrier associated properties and evaluated for specificity of interaction with selected mammalian cells. The initial phase of this development was stimulated by the investigations of Fiume and coworkers with conjugates of β-amanitin and proteins (Cessi and Fiume, 1969; Fiume et al., 1969; Fiume et al., 1971; Barbanti-Brodano and Fiume, 1974; Fiume and Barbanti-Brodano, 1974).

ADH-BSA Conjugates

The work described here utilized the most widely occurring derivative of the amatoxins, α-amanitin, which is available in quantity from locally gathered specimens. The

lack of a free carboxyl group on α-amanitin required that it first be chemically modified to permit conjugation to proteins via reaction with water soluble carbodiimides. Diazotization of α-amanitin to an aromatic moiety containing a six carbon spacer molecule and a terminal free amino group (Faulstich and Trischmann, 1973) provided the ADH derivative that was readily purified by gel chromatography and identifiable by its characteristic spectral absorption and TLC mobility. Conjugation to BSA via carbodiimide reaction provided covalently linked conjugates of α-amanitin and BSA as evidenced by co-elution of protein and 384nm absorbance of the azo moiety from Sephadex G-75. Quantitation of the inhibition of calf thymus RNA polymerase II by the ADH-BSA conjugates in comparison to free ADH or α-amanitin (Figure 7) demonstrated that modification of α-amanitin to the ADH derivative had little effect on its binding to polymerase. Both ADH and α-amanitin have similar K_I values and are noncompetitive inhibitors. Conjugation to BSA, however, resulted in a 38-fold decrease in binding affinity, most likely as a result of steric hinderance rather than a conformational alteration of α-amanitin. The lack of convergence of the three lines generated with different VIP concentrations implies that conjugation to BSA not only decreases binding affinity but alters the nature of the inhibition from the strictly noncompetitive type.

Examination of the macromolecular composition of the ADH-BSA conjugates by SDS-Page showed that the relatively high concentrations of carbodiimide used for coupling caused cross-linking of BSA into higher molecular weight complexes. Covalent polymerization as a side reaction of carbodiimide coupling was also reported by Timkovich (1977) to occur at high carbodiimide and protein concentrations. The absence of a specific ligand which interacts with BSA prevented any quantitative measurement of the effects this cross-linking may have on the recognition of BSA by cells. However, the cross-linking may contribute to the reduction in binding affinity for RNA polymerase seen with ADH-BSA.

The effects of the ADH-BSA conjugates on cultured mammalian cells were examined for three different cell lines. These studies provided a comparison to the previously described investigations of β-amanitin-protein conjugates in addition to establishing the methodology and preliminary evidence necessary for examination of other α-amanitin protein conjugates. The toxicity of ADH-BSA for EL4 and M-7 CHO cells was approximately equal to that of free α-amanitin on a molar basis of amanitin. This is surprising in view of the greatly reduced affinity of ADH-BSA for RNA polymerase II. The enhanced toxicity observed of the conjugate for AV3 cells indicates that either preferential uptake of the conjugate, modification to a more toxic derivative, or some combination of the two is occurring in these cells. Since preferential uptake could result from specific

receptor mediated processes or non-specific pinocytosis, the rates of pinocytosis of the three cell lines were determined.

Uptake of ^{125}I-BSA as a measure of pinocytic capability (Chapman-Andresen, 1964; Steinman et al., 1974) showed a good correlation of sensitivity to ADH-BSA conjugate and pinocytic rate for the three cell lines. AV3 were 3.5 times more active than CHO cells in the uptake of ^{125}I-BSA whereas EL4 cells were relatively inactive under the same conditions. Pinocytic uptake followed by lysozomal digestion of the protein portion of the ADH-BSA (Ehrenreich and Cohn, 1967) with release of free α-amanitin probably contributes to the toxicity of the ADH-BSA in susceptible cells.

ADH-Con A Conjugates

The preliminary study with ADH-BSA conjugates demonstrated that conjugates of α-amanitin and proteins retain the inhibitory potential of α-amanitin for RNA polymerase II and exhibit differential cytotoxicity for cultured cells. The lack of specific, well defined cellular receptors for the protein portion of the ADH-BSA conjugate made interpretation of the cytotoxicity data difficult with respect to specific cell targeting. For this reason, Con A was chosen for conjugation with ADH as a protein carrier with well defined ligand binding and cell receptor specificities. The use of Con A for conjugation studies would also present a readily definable means for assessing the effects of conjugation to α-amanitin on the properties of the carrier protein.

Synthesis of conjugates of ADH and Con A resulted in ADH-Con A conjugates that had a molar ratio of ADH to Con A of 0.67 as determined from the absorption spectra (Figure 10). The conjugates retained the specificity of interaction with calf thymus RNA polymerase II but at a greatly reduced affinity ($K_I = 186 \times 10^{-9}$M). However, conjugation of ADH via free protein carboxyl groups had a deleterious effect on the ligand binding ability of the Con A. The failure of the ADH-Con A conjugates to bind to Sephadex G-75 implied a loss of Con A specific ligand binding. Synthesis of ADH-Con A conjugates in the presence of the specific ligand D-glucose (1M) failed to protect the Con A saccharide binding site. Synthesis in the presence of 1M NaCl to provide the optimal ionic strength for native Con A conformation also failed to produce conjugates with ligand specificity. Since it appeared likely that conjugation to carboxyl groups on Con A resulted in a loss of ligand binding activity, this approach was discontinued and derivatives of α-amanitin that couple to free amino groups on proteins were examined.

ADGG-BSA Conjugates

The synthesis and chemical characterization of an α-amanitin derivative possessing a free carboxyl group (ADGG) provided an opportunity to produce conjugates whose properties could be directly compared to the ADH-BSA conjugates already characterized. With ADGG-BSA conjugates containing 2.0 moles of ADGG per mole of BSA, there resulted a 21-fold

decrease in the affinity for calf thymus RNA polymerase for the α-amanitin portion of the conjugate (K_I = 127 x 10^{-9}M). As was seen for the ADH-BSA conjugates, conjugation also altered the nature of the inhibition from the strictly non-competitive. Since free ADGG possessed a K_I of 6.9 x 10^{-9}M, not dramatically different from free α-amanitin, it appears likely that the reduction in binding affinity of the ADGG-Con A conjugate is due to steric hindrance by the protein portion of the conjugate. In any case, the ADGG conjugated to Con A is still a potent inhibitor of RNA polymerase II *in vitro*.

Hippuric Acid-Con A Conjugates

The use of ^{14}C-hippuric acid as a free carboxyl group containing analog to ADGG was intended to define those conditions of carbodiimide mediated coupling to Con A that would result in the introduction of a defined number of acid moieties onto Con A with minimal perturbation of the basic lectin structures and ligand binding activities. All of the previous conjugations had been carried out at pH 7 in water with moderately high concentrations of EDC. The proposed mechanism of carbodiimide coupling (Hoare and Koshland, 1967; Carraway and Koshland, 1972) had as a first step the formation of a carbodiimide-carboxyl group adduct in the form of an O-acylisourea. A stable product is formed by the nucleophilic displacement of the carbodiimide group with subsequent formation of a piptide bond. The specificity of nucleophilic attack, which in this case is

by the protein amino group, should be increased by running the reaction at lower pH where amino groups would be the rate limiting factor. Reaction of ^{14}C-HA and Con A in dilute phosphate buffer solution at pH 5, 6 or 7 resulted in increased conjugation with lower pH. For a given pH, increasing the concentration of carbodiimide 10-fold increased the rate and extent of reaction. When the identical reaction was run at pH 7 in 0.1M NaCl, the extent of conjugation was increased over the equivalent reaction in phosphate buffer at pH 5 approximately 10-fold. Additionally, the reactions in phosphate buffer reached completion within 2 hours whereas without phosphate, the reaction took 18-24 hours to reach completion. These results indicate that phosphate is limiting the rate and extent of reaction and appears to provide a controlled condition for the conjugation of a limited number of acid residues to Con A.

^{14}C-HA-Con A conjugates prepared in either NaCl, pH 7 or phosphate buffer, pH 5 were analyzed for protein cross-linking by SDS-PAGE. In contrast to the ADH-BSA conjugates, a barely detectable amount of cross-linked material was observed for both ^{14}C-HA-Con A conjugates which represented less than 8% of the total material. This is undoubtedly a direct result of the reduction of carbodiimide concentration by 10-fold over that used for the ADH-BSA conjugates. The pH 5, 0.1M phosphate buffer condition with ratios of Con A to HA to EDC of 1:100:1000 seem to be optimal for production of conjugates with minimal alteration of gross molecular properties.

Retention of lectin properties by two HA-Con A conjugates, HA-Con A (PO_4) and HA-Con A (NaCl) was examined by adsorption to Sephadex G-75 (both eluted in volumes identical to native Con A following addition of 0.1M D-glucose), hemagglutination (similar titers obtained with conjugates from both reaction conditions and with native Con A) and determination of the association constant for the chromogenic ligand, PNPM. Although the K_a obtained for both HA-Con A (PO_4) and HA-Con A (NaCl) were slightly greater than native Con A (Figure 20), all of these results indicated that the HA-Con A conjugates retained most if not all lectin associated activities.

The stability of the ^{14}C-HA-Con A conjugate bond to neutral hydroxylamine treatment was examined for conjugates from both reaction conditions. Hydroxylamine under these conditions will hydrolyze any ester type linkages but will not affect peptide bonds (Carraway and Koshland, 1968). The conjugates were 88% and 65% stable to hydroxylamine for the phosphate and NaCl reactions, respectively. Since Con A is devoid of any free sulfhydryl groups which could form side reactions with carbodiimide (Carraway and Koshland, 1970), these values represent the minimal amount of ^{14}C-HA bound to Con A via peptide bonds. Table 5 summarizes the salient features of the HA-Con A conjugates in reference to native Con A. It can be concluded that these conjugates are essentially identical to native Con A with respect to their interaction with specific saccharide ligands.

ADGG-Con A Conjugates

Final verification of the procedures for EDC coupling to Con A was obtained by reaction of a radioactively labeled derivative of α-amanitin, ^{3}H-DM-ADGG, with Con A under the phosphate buffer, pH 5 condition. Since the ^{3}H-DM-ADGG had a specific activity of 7.8 x 10^{8}dpm/μmole, the ^{3}H-DM-ADGG-Con A conjugates contained sufficient activity to be used in cell binding experiments. These conjugates with molar ratios of amanitin to Con A of 1.1 were 78% stable to hydroxylamine and compared favorably with the similar values for HA-Con A conjugates of 65-88% stability to hydroxylamine. They absorbed to Sephadex G-75 and eluted with 0.1M D-glucose indicative of retention of saccharide binding affinity by the conjugate.

Reaction of ^{3}H-DM-ADGG with other lectins possessing varying ligand binding specificities was performed to evaluate the effects of ADGG conjugation on hemagglutination activity. The fact that all of the lectins tested bound significant amounts of ^{3}H-DM-ADGG (molar ratios ^{3}H-DM-ADGG: Con A greater the 1.0) and with two exceptions (SBA and PHA) retained hemagglutinating capability equal to the native lectin, clearly indicates the feasibility of binding α-amanitin to proteins with retention of the original biological activity of the protein.

Inhibition of calf thymus RNA polymerase II by an ADGG-Con A conjugate containing 3.6 moles of α-amanitin per mole of Con A yielded a K_I value for the ADGG-Con A of

27×10^{-9}M. In comparison to ADGG-BSA ($K_I = 127 \times 10^{-9}$M) the ADGG-Con A conjugate is a more potent inhibitor of RNA polymerase II. No inhibition by Con A alone was observed nor was the reaction affected by the presence of the Con A specific sugar D-glucose. This indicates that the observed inhibition is a distinct function of the α-amanitin portion of the conjugate and not due to interaction of the Con A with the enzyme or some component of the reaction mixture.

The procedures developed for the conjugation of ^{14}C-hippuric acid and ^{3}H-DM-ADGG to Con A have provided a means for obtaining specific conjugates of Con A and α-amanitin that retain the biological activities of both components of the conjugate. The binding of the Con A portion of the ADGG-Con A conjugate to a cell receptor should result in a specific cytotoxicity for that cell if the Con A receptors are able to facilitate entry of the conjugate to the cell. The interaction of ADGG-Con A conjugates with cultured CHO cells was examined to first establish whether the Con A receptor could facilitate entry of the amanitin portion of the conjugate into the cell and secondly, determine whether any such entry resulted in greater toxicity than for free α-amanitin following exposure of the cells to equivalent concentrations of free and conjugated α-amanitin.

Two CHO lines, H-7 and a Con A resistant mutant H-7Wcr, were both found to bind equivalent quantities of ^{125}I-Con A following a single 15 minute exposure at 22°C (Figure 23). The H-7Wcr line is resistant to Con A cytotoxicity at

high concentrations of Con A (in excess of 100μg/ml) but the mechanism of its resistance in unknown. If it is resistant as a result of not being able to internalize Con A, then it would be expected to be more resistant to ADGG-Con A toxicity than the parent H-7 line. If it is resistant at some step after internalization of bound Con A, then the H-7Wcr would be expected to be as sensitive to ADGG-Con A as the parent line.

Both cell lines displayed comparable sensitivity to free α-amanitin or ADGG over 48 hours of continuous exposure to either inhibitor (Figure 27; Table 8) with ADGG being less toxic than α-amanitin by a factor of six for both lines. This can be a reflection of the reduced affinity of ADGG for RNA polymerase II and/or impeded entry of the toxin to the cell. With a K_I for ADGG of 6.9×10^{-9}M and an ID_{50} for H-7 CHO cells of 3.0×10^{-6}M there is obviously a barrier to the entry of ADGG to the cell with less than 0.25% of the inhibitor entering the cell.

Exposure of H-7 cells to ADGG, Con A or ADGG-Con A for 15 minutes (Figure 26) resulted in little toxicity from free ADGG. This could be a function of low permeability of the cells to ADGG. ADGG-Con A caused a dramatic inhibition with 50% inhibition of cell growth occurring at 2.1×10^{-7}M with respect to ADGG. Neither ADGG nor Con A at concentrations equivalent to those present in the conjugate caused inhibition. This result indicates that the ADGG-Con A conjugate is minimally 50 times more toxic than the free ADGG following a 15 minute exposure.

H-7Wcr cells were unaffected by a 15 minute exposure to Con A as was expected. They were, however, very sensitive to ADGG-Con A with an ID_{50} of 3.5×10^{-7}M (Table 9). This value is comparable to that seen with H-7 cells and indicates that either H-7Wcr cells are resistant to the effects of Con A at a step after internalization of bound Con A or that ADGG-Con A is degraded on the cell surface with entry to the cell of only the amanitin portion of the conjugate.

In contrast to the effects seen on H-7 cells, free α-amanitin and ADGG did inhibit the H-7Wcr cells following a 15 minute exposure, although, at the maximum dose tested of 2×10^{-6}M neither inhibitor produced half the inhibition seen with ADGG-Con A. A likely explanation is that the mechanism of the H-7Wcr resistance to Con A involves alterations in membrane components that allow for rapid entry of the unconjugated inhibitors. If this is the case, the entry must be saturable with respect to amanitin as the continuous exposure data indicate that no more of either compound enters the H-7Wcr cell than enters the H-7 cell. The rapid plateauing of the 15 minute inhibition data after exposure to a 0.5×10^{-6}M concentration of both ADGG and α-amanitin supports this hypothesis.

In order to establish that the inhibition caused by ADGG-Con A conjugate is a function of the inhibition of RNA polymerase II by the amanitin portion of the conjugate, the toxicity of ADGG-Con A was examined in a cell line resistant to α-amanitin. LAN-2 cells possess an altered

RNA polymerase II that has a greatly reduced affinity for α-amanitin (R. Bryant, personal communication, 1979). If inhibition by ADGG-Con A is due to inhibition of polymerase, then LAN-2 cells should be much less sensitive to ADGG-Con A than the parent line A-9.

Both A-9 and LAN-2 bind equivalent amounts of Con A as determined from the binding of ^{125}I-Con A (Figure 24). LAN-2 cells were totally unaffected by either α-amanitin or ADGG following 48 hours of exposure to concentrations as high as 16×10^{-6}M. A-9 cells showed sensitivity to both inhibitors and, as was found with CHO cells, the ADGG was less toxic at equivalent doses than free α-amanitin (Figure 28).

Following 15 minutes of exposure to Con A at a concentration of 40μg/ml, neither A-9 nor LAN-2 were inhibited by more than 11%. ADGG at 2.5×10^{-6}M caused no inhibition of LAN-2 and only slight inhibition of A-9 cells (13%). ADGG-Con A caused 40% inhibition of A-9 at concentrations equivalent to those given for ADGG and Con A whereas the LAN-2 cells were only inhibited to the extent observed from Con A alone (Table 10). This experiment provides conclusive evidence in support of the proposed mechanism of toxicity for ADGG-Con A. The Con A portion of the conjugate binds to specific cell surface receptors followed by internalization of at least the α-amanitin moiety which then inhibits RNA polymerase II causing cessation of mRNA synthesis and cell death.

The remaining evidence necessary for confirmation of the above mechanism is to establish that ADGG-Con A does indeed bind to cells via specific receptors for Con A. The binding of ADGG-Con A by H-7 CHO cells was determined directly with ^{3}H-DM-ADGG-Con A and compared with the results obtained with equivalent amounts of ^{125}I-Con A. At 2 x 10^{-4}M with respect to Con A concentration, H-7 cells bound identical amounts of either Con A derivative (Table 7). The binding of each substance was equally inhibited by preincubation with excess unlabeled Con A. Exposure in the presence of 10^{-3}M MDM resulted in 85% inhibition of ^{125}I-Con A binding and 76% inhibition of ^{3}H-DM-ADGG-Con A binding. These results indicate that essentially identical numbers of Con A and ADGG-Con A molecules are bound by H-7 CHO cells and that they have the same apparent specificities of binding. A more direct comparison was made by competition of native Con A and ADGG-Con A with ^{125}I-Con A for receptor sites present on H-7 cells (Figure 25). The results, when plotted as fraction ^{125}I-Con A bound versus fraction of competitor to total Con A, yield a straight line with virtually identical points for both Con A and ADGG-Con A. This result further establishes that ADGG-Con A binds to cells by virtue of the Con A portion of the conjugate.

Specific binding of ADGG-Con A and resultant toxicity should be inhibitable by the Con A specific carbohydrate ligand, α-methyl-D-mannopyraniside. This was tested by exposure of H-7 cells to ADGG-Con A at 1 x 10^{-6}M for 15

minutes which resulted in 58% inhibition of cell proliferation 48 hours later. Exposure to the same amount of ADGG-Con a in the presence of 1mM MDM resulted in a total of 6.7% inhibition. Almost complete protection from the toxic effects of ADGG-Con A was achieved by blocking the binding of the Con A portion of the conjugate to cell receptors with saccharide ligand.

The cell binding and cytotoxicity studies strongly point to the targeting potential of ADGG-Con A for delivery of amanitin to Con A receptor bearing cells. A numerical estimation of this targeting ability was obtained by exposing H-7 CHO cells to ^{3}H-DM-ADGG-Con A for 15 minutes at room temperature followed by incubation in culture media at 37°C for 1 hour. At the end of the hour, the culture media and three PBS washes of the cells were counted for ^{3}H-DM-ADGG-Con A activity which eluted from the cells upon interaction with the glycoproteins and carbohydrates present in the serum containing medium. The cells were counted separately to determine the amount of Con A that remained irreversibly associated with the cell and was presumably internalized. A total of 60% of the activity was found in the medium leaving 40% associated with the cell or 4.21×10^{6} molecules/cell. Assuming an intracellular volume of 3.09×10^{-9}ml/cell (Noonan and Burger, 1973; volume calculated from a surface area of 2200μ^{2} given for a 3T3 cell), the intracellular concentration of ADGG achieved is on the order of 2×10^{-9}M. This value

compares favorably with the magnitude of the inhibition constant for α-amanitin ($K_I = 1.8 \times 10^{-9}$M) and for ADGG ($K_I = 6.9 \times 10^{-9}$M). On this basis, the targeting of α-amanitin by ADGG-Con A conjugates can be seen to be extremely effective in comparison to the total amount of inhibitor exposed to the cell. This can only occur as a result of the specific interaction of the Con A portion of the conjugate with cell surface carbohydrates, followed by internalization of at least the amanitin portion of the conjugate.

In conclusion, covalent conjugates of α-amanitin and concanavalin A were synthesized that retained the biological activities of both components of the conjugates: inhibition of RNA polymerase II by α-amanitin and the carbohydrate binding specificities of Con A. These conjugates resulted in receptor mediated targeting and uptake of the amanitin by interaction of the Con A with specific cell surface receptors. The resulting toxicity for CHO cells of ADGG-Con A conjugate was minimally 50 times that achieved with an equivalent concentration of free ADGG. The high toxicity and well defined binding specificity of the ADGG-Con A conjugates suggest the potential for specific inhibition of selected cells within a mixed population by these conjugates.

The amanitin-Con A conjugates investigated here possess a targeting potential that contains several advantages over those systems discussed in the introduction. The

specific inhibition of RNA polymerase II, a critical enzyme to transcription, by amanitin allows low doses of inhibitor to produce a significant cytotoxic effect. This is analogous to the inhibition produced by diphtheria toxin which is extremely toxic because of the ADP ribosylation of elongation factor 2 resulting in specific inhibition of protein synthesis. Diphtheria toxin however, is macromolecular in nature, and the procedures used for conjugation tend to produce conjugates of ill defined molecular composition due to cross-linking of the diphtheria toxin with itself and/or cross-linking of the carrier protein. Alpha-amanitin conjugates circumvent the problem of nonhomogeneity by virtue of the well-defined mechanism of conjugation via a single available site on the amanitin derivative for conjugation to proteins. Furthermore, the coupling conditions defined resulted in insignificant levels of intermolecular protein cross-linking as evidenced from the SDS-PAGE data.

Conjugates of inhibitors that interact with DNA (daunomycin, 5-FUDR, chlorambucil) tend to be more homogeneous in composition like α-amanitin conjugates as a consequence of having a limited number of points on a small molecule available for conjugation to proteins. However, their toxicity is a result of interaction with DNA and thus requires a much larger dose of inhibitor to achieve significant cytotoxicity than would be required for an inhibitor that acts on an enzyme essential to

nucleic acid replication, transcription or translation. Alpha-amanitin conjugates would appear to combine both desirable qualities, inhibition of a vital cellular enzyme present in low numbers within a cell and a well defined single mechanism for conjugation to proteins.

The second aspect of targeting inhibitor-protein conjugates is the specificity of the carrier protein. ADGG-Con A conjugates were well characterized with respect to binding specificities and resulted in targeting to specific carbohydrate moieties. In comparison to conjugates of albumins or non-immune gammaglobulins, the Con A conjugates possess a much greater specificity of action. However, because of the universal distribution of carbohydrate moieties on cells of all types, the specificity of targeting of α-amanitin should be improved by use of cell surface directed immunoglobulins. Further studies with ADGG conjugates of anti-Thy1.2 antiserum are projected. Use of this antibody from AKR/J mice will allow the synthesis of conjugates that should have minimal, if any, reactivity to the host animal. The conjugate should result in specific targeting to Thy-1 antigen present on EL4 lymphocytic leukemia cells both *in vivo* and *in vitro*. Studies similar to those of Moolten et al., (1972) with conjugates of anti-DNP immunoglobulin G and ADGG will allow for quantitative investigation of conjugate receptor affinities following the introduction of variable numbers of toxin molecules into the carrier protein. The relationship

of toxin density to cell specific cytotoxicity will be of great significance to future studies of drug targeting. The nature of the interaction between ADGG-Con A conjugates and their cell receptor can be more throughly investigated by comparing the results of this study to those obtained with conjugates of ADGG and anti-Con A receptor antibodies. These will allow a more thorough definition of the mechanisms of binding and endocytosis of lectin on the cell surface.

Alpha-amanitin would thus appear to be a logical choice for continued investigations of protein-inhibitor conjugate development and targeting of cytotoxic drugs.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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