Identification of the Amatoxin-Binding Subunit of RNA Polymerase B by Affinity Labeling Experiments. Subunit B 3—the True Amatoxin Receptor Protein of Multiple RNA Polymerase B†

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ABSTRACT: Crude calf thymus DNA-dependent RNA polymerase, RNA polymerase B (ribonucleoside triphosphate: RNA nucleotidyltransferase, EC 2.7.7.6), was incubated with the tritium labeled, potent inhibitor [^{3}H]amanin, in order to form the enzymatically inactive [^{3}H]amanin-polymerase complex ([^{3}H]A-P complex). Subsequent purification procedures for the [^{3}H]A-P complex were based on radioactive assays. Phosphocellulose chromatography separated two radioactive components: PCI, the previously reported amatoxin binding protein, ABP (Brodner and Wieland, 1976), and PC II, the [^{3}H]A-P complex. Sodium dodecyl sulfate gel electrophoresis of the complex showed the presence of a new heavy band very close to subunit B 1 and a decreased intensity of subunit band B 3. These were the only differences noted in the subunit structure of RNA polymerase B. [^{3}H]Amanin was covalently coupled to the enzyme, affinity labeling, by a water-soluble carbodiimide and the resultant conjugate submitted to sodium dodecyl sulfate gel electrophoresis. The profile of radioactivity showed one main peak (>2000 cpm) coinciding with the 550-nm absorption peak of subunit B 3 on a stained parallel gel. Since no other protein band contains any significant radioactivity, the binding site for [^{3}H]amanin and most probably for all amatoxins is localized on the B 3 subunit SB 3.

Amatoxins, the poisonous ingredients of the mushroom *Amanita phalloides,* were shown to act at the molecular level as extremely potent inhibitors of the DNA-dependent RNA polymerases (Stirpe and Fiume, 1967; for review see Fiume and Wieland, 1970; Wieland, 1972).[1] α-Amanitin, at the very low concentrations of 10^{-8}–10^{-9} M, inhibits the Mn^{2+}-dependent nucleoplasmic polymerases but it does not inhibit the Mg^{2+}-dependent class A polymerases found in the nucleolus (Seifart and Sekeris, 1969; Jacob et al., 1970; Novello et al., 1970). The class C cytoplasmic polymerases are inhibited by amatoxins at higher concentrations of 10^{-5}–10^{-4} M (Seifart et al., 1972; Amalric et al., 1972). RNA polymerase B or II (ribonucleoside triphosphate:RNA nucleotidyltransferase, EC 2.7.7.6) has been found in a wide variety of eukaryotes (Chambon, 1974; Jacob, 1973) and it has been extensively purified from rat liver and calf thymus (Roeder and Rutter, 1969, 1970, Kedinger et al., 1972; Gissinger and Chambon, 1972). RNA polymerase B is an oligomeric enzyme composed of subunits, a structural feature common to other RNA polymerases (Chambon et al., 1972). At least two amatoxin-sensitive RNA polymerase B isozymes, RNA polymerases B I and B II, have been found to be present in calf thymus in nearly equal concentrations. Although the properties and subunit structure of RNA polymerase B have been well studied, little is known about its active site and less is known about its amatoxin binding site and mechanism of inhibition.

Amatoxins bind to the RNA polymerase B isozymes with a very high affinity, forming a 1:1 stoichiometric complex (Chambon et al., 1970). The binding constant for formation of the amatoxin-polymerase complex (A-P complex)[2] has been determined to be about 10^{-9} M. Chambon and coworkers (1971) suggest that amatoxins exert their inhibitory effect, i.e., prevention of phosphodiester bond formation and RNA synthesis, by binding to a subunit common to both isozymes B I and B II.

We have succeeded in isolating the amatoxin-binding subunit of RNA polymerase B through the use of affinity labeling. Also, in the purification procedure we took advantage of the high-binding constant of the A-P complex. Since the enzyme is inactivated by amatoxin binding, our assay procedure for the purification of the A-P complex could not be based upon enzymatic activity. After incubating crude enzyme with tritium labeled amanin, we followed protein-bound radioactivity of the [^{3}H]amanin-RNA polymerase B ([^{3}H]A-P) complex. However, under the denaturing conditions used in sample preparation for sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the polymerase subunits dissociate with simultaneous release of [^{3}H]amanin. Consequently, the labeled [^{3}H]amanin must be covalently coupled to the polymerase before sodium dodecyl sulfate-gel electrophoresis.

A water-soluble carbodiimide was successfully used to produce a covalently bound [^{3}H]A-P complex that could be dissociated into its constituent subunits upon sodium dodecyl sulfate-gel electrophoresis. Only a single protein band was found to contain significant radioactivity; all other subunit bands were equal to background levels.

Materials and Methods

[^{3}H]Amanin (1.4 Ci/mmol) (Wieland and Brodner, 1976) was especially prepared for these affinity-labeling experiments by hydrogenolysis of 6-(1-phenyl-5-tetrazolyloxy)-β-amanitin (Buku and Wieland, 1975) with 20 Ci of tritium and further chromatography on a Sephadex LH-20 column in methanol/

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[1] Amanin, a carboxylic acid, is a member of the family of the amatoxins whose most prominent representative is α-amanitin, a carboxamide. All of them are very potent inhibitors of RNA polymerase B (II), (see Wieland, 1972).

[2] Abbreviations used are: A-P, amanin-polymerase; SB, subunit band; DEAE, diethylaminoethyl; ECDI, 1-ethyl-3-(dimethylaminopropyl)-carbodiimide; EDTA, (ethylenedinitrilo)tetraacetic acid; ABP, amanitin binding protein.

H_2O (1:1). DEAE (De 23) and phosphocellulose (P-11) were obtained from Whatman (London), hydroxylapatite from Clarkson Chemical Co. (Williamsport, Pa.), Sephadex G-25 and LH-20 from Pharmacia (Uppsala), Bio-Gel A 1.5 from Bio-Rad (Richmond, Calif.), materials for sodium dodecyl sulfate-polyacrylamide gel electrophoresis from Serva (Heidelberg), ECDI (1-ethyl-3-(dimethylaminopropyl)carbodiimide) and all other chemicals were reagent grade from Merck (Darmstadt).

The following buffer systems were employed. Buffers I_5, I_{30}, and I_{50} contained Tris-HCl (0.05 M, pH 7.9), 5 mM β-mercaptoethanol, 0.25 mM EDTA, and 5, 30, and 50% (w/v) glycerol, respectively. Buffer II contained Tris-HCl (0.05 M, pH 7.9), 5 mM β-mercaptoethanol, 0.25 mM EDTA, 0.5 M NaCl, and 30% (w/v) glycerol. Buffers III_{10}, III_{40}, III_{110}, III_{150}, and III_{250} were composed of 5 mM β-mercaptoethanol, 30% (w/v) glycerol, and 10, 40, 110, 150, 250 mM sodium phosphate, pH 7.5, respectively. Buffer IV contained 0.01 M phosphate, pH 7.1, 1% sodium dodecyl sulfate and 1% β-mercaptoethanol. Buffer V was also 0.01 M phosphate, pH 7.1, with 0.1% sodium dodecyl sulfate, and 0.1% β-mercaptoethanol.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out at pH 7.1, in the presence of 0.1% sodium dodecyl sulfate and 0.1% β-mercaptoethanol according to the method of Weber and Osborn (1969) on a 5% acrylamide-gel system conducting 7 mA/gel. Better resolution of smaller subunits was achieved with a gel containing 10% acrylamide in the lower half and 5% acrylamide in the upper half mixed 5%, 10% gel system.

Protein was estimated by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

RNA polymerase B activity was assayed by in vitro system of Seifart et al. (1972).

A crude preparation protein fraction DCB, of amatoxin-sensitive RNA polymerase B, separated from the amatoxin-insensitive RNA polymerase A, was isolated from 1200 g of calf thymus tissue using the method described by Kedinger et al. (1972).

Incubation with [^3H]Amanin. One-hundred milliliters of protein fraction DCB (3 mg/ml) in buffer I_{50} was incubated for 30 min at 4 °C with 50 nM, 70 μCi, of purified [^{3}H]amanin dissolved in MeOH/H_2O (1:1). The amanin concentration was intentionally low, 5×10^{-7} M, in order to eliminate, as nearly as possible, any nonspecific amanin binding. The incubation mixture was then loaded on a Sephadex G-25 (5 × 40 cm) column previously equilibrated with buffer III_{10}. The column was eluted with buffer III_{10} and 15 ml fractions were collected. In addition to continuous monitoring at 280 nm for location of protein peaks, aliquots were taken from each fraction and counted in a liquid scintillation counter. The protein peak (200 ml) isolated by this procedure contained 6×10^7 dpm and was well separated from unbound [^{3}H]amanin.

Purification with Hydroxylapatite. The solution, 200 ml of protein-bound radioactivity, obtained from the Sephadex column was treated with 100 ml of hydroxylapatite for 30 min at 4 °C in a batch procedure. Subsequently, the centrifuged adsorbate was successively eluted with three 60-ml washes each of buffer III_{40}, III_{110}, III_{150}, and III_{250}. Although Kedinger and Chambon (1972) report that RNA polymerase B is eluted with buffer III_{110}, we found protein-bound radioactivity in fractions eluted with buffers III_{110} and III_{150}, as well as protein in buffer III_{250} fraction. We did not observe any adsorption of free unbound [^{3}H]amanin. The eluates obtained with buffers III_{110} and III_{150} were pooled as fraction HA, 350 ml.

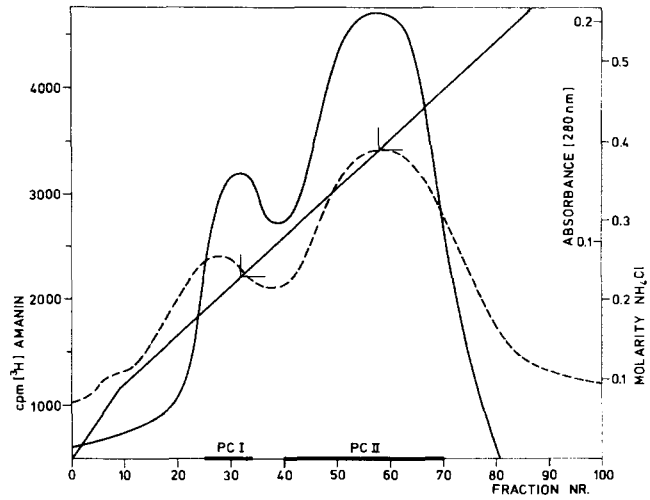


FIGURE 1: Chromatography of fraction DCB of calf thymus extract on phosphocellulose after addition of [^{3}H]amanin. Elution with increasing concentration of NH_4Cl. Radioactivity, solid line; absorbance at 280 nm, hatched line.

Fraction HA contained 10^7 dpm that represented 17% recovery of protein-bound radioactivity.

Phosphocellulose Chromatography. In order to reduce the salt concentration, fraction HA was placed on a Sephadex G-25 column and eluted with buffer I_{30}. The resulting 600 ml of fraction HA were pumped with a flow rate of 80 ml/h onto a phosphocellulose column (3.5 × 15 cm). The column was eluted at a flow rate of 60 ml/h, 9-ml fractions, with a linear NH_4Cl gradient (Figure 1). Two peaks of protein-bound radioactivity were obtained at 0.23 and 0.39 M NH_4Cl concentrations. The fractions 24–35 (PC I, 90 ml) and 40–70 (PC II, 270 ml) contained 0.6×10^6 dpm and 4×10^6 dpm, respectively. The further purification of PC I resulted in the discovery of a novel amanitin binding protein (ABP) and is reported elsewhere (Brodner and Wieland, 1976).

Fraction PC II was precipitated by adding solid $(NH_4)_2SO_4$ to a final concentration of 50% saturation (31 g of $(NH_4)_2SO_4$/100 ml of PC II) and gently stirring for 1 h. The precipitate was collected by centrifugation at 180 000*g* for 1 h and dissolved in 5 ml of buffer I_{30}. This solution, which contained about 2×10^6 dpm, indicating a considerable loss of [^{3}H]amanin during $(NH_4)_2SO_4$ precipitation, was applied at a flow rate of 4 ml/h to a Bio-Gel A-1.5 column (0.8 × 90 cm). The column was eluted with buffer II at the same flow rate and 2.7-ml fractions were collected. Fractions 10–14 contained the main peak of radioactivity. This sample, designated fraction BG, was stored in liquid nitrogen and served as starting material for all further experiments.

Affinity Labeling of Fraction BG, Formation of the [^3H]A-P Conjugate. The optimal conditions for affinity labeling were established to be as follows: fraction BG, 0.4 ml, containing 0.2 mg/ml of protein, pH adjusted to 7.0, was treated with ECDI, 3 mg, for 5 h at 4 °C. The reaction was stopped by adding two drops of glacial acetic acid to the solution. The sample was then prepared for sodium dodecyl sulfate-gel electrophoresis by one of two alternate procedures. Either the [^{3}H]A-P conjugate was precipitated by adding 0.4 ml of ice-cold 5% trichloroacetic acid and redissolved in 0.2 ml of buffer IV after washing with water to remove excess Cl_3CCOOH; or 0.2 ml of buffer IV was added directly to the sample. Subunit dissociation, i.e., protein denaturation, was accomplished either by heating at 90 °C for 2 min or by leaving the sample at room temperature overnight. After addition of

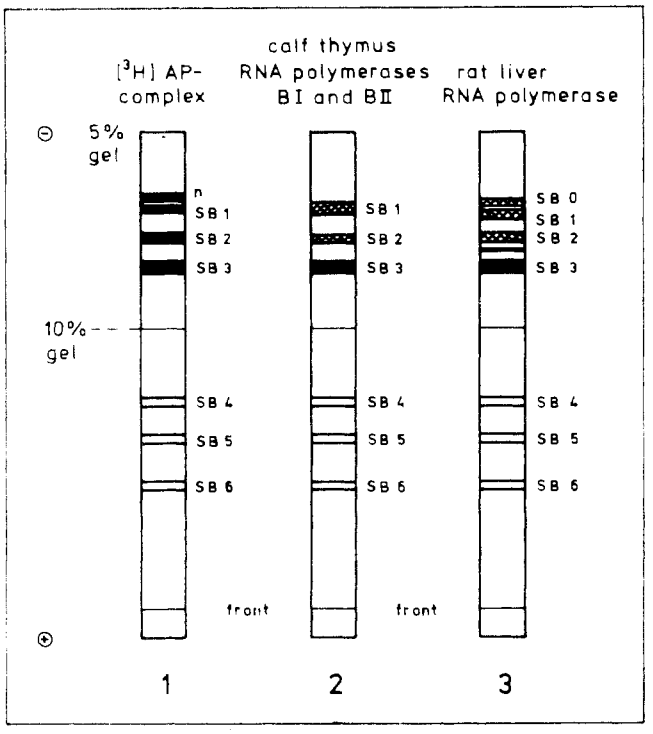


FIGURE 2: Sodium dodecyl sulfate-gel electrophoresis of the [^{3}H]A-P complex (1), RNA polymerase B I and B II from calf thymus (2), and RNA polymerase from rat liver (3).

0.8 ml of buffer V to the solution of the precipitate, aliquots were removed from the sample and subjected to sodium dodecyl sulfate-gel electrophoresis.

As a control, fraction BG, 0.5 ml, was treated with 10% trichloroacetic acid, 0.5 ml, for 10 min at 0 °C. After centrifugation and repeated washes with water to remove excess Cl_3CCOOH, the precipitate was dissolved in buffer IV, 0.2 ml, and denatured in an analogous manner as the [^{3}H]A-P conjugate. Buffer V, 1 ml, was then added and control sodium dodecyl sulfate gels prepared from aliquots of this resultant solution.

Sodium Dodecyl Sulfate Gel Analysis. Parallel gels were subjected to electrophoresis. After migration, one of the gels was stained for 4 h with 0.25% Coomassie brilliant blue, 40% methanol, 7.5% acetic acid, destained in 40% methanol, 7.5% acetic acid, and scanned at 550 nm in a Leitz PMQ 3 spectrophotometer equipped with a K 4 disk scanning accessory. The other gel was immediately cut into 1-mm slices and the slices were prepared for scintillation counting. Either the individual slices were placed directly in counting vials and eluted with 0.5 ml of buffer V for 48 h at 20 °C with shaking, and then szintigel, 15 ml, was added, or they were dried and combusted with pure oxygen in a Tricarb Sample Oxidizer 306 (Packard). Differences in length between parallel gels were corrected by the method of Weber and Osborn (1969).

Results and Discussion

The early formation of an [^{3}H]amanin-polymerase complex, [^{3}H]A-P complex, was advantageous for several reasons: the isolation procedure was accelerated, other amatoxin-binding proteins would be detected, and perhaps the enzyme-bound amatoxin might exert a stabilizing effect on the quaternary structure of the protein.

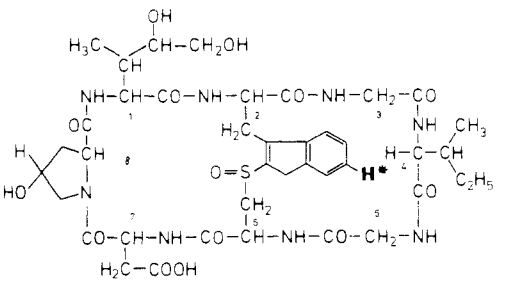
The efficacy of this approach is shown in Figure 1. Phosphocellulose column chromatography readily separated the crude [^{3}H]amanin-polymerase preparation into two fractions, PC I and PC II. Further purification of PC I resulted in the isolation of a novel amatoxin-binding protein, ABP (Brodner and Wieland, 1976). PC II contained the [^{3}H]A-P complex that was isolated in a high degree of purity by $(NH_4)_2SO_4$ precipitation followed by chromatography on a Bio-Gel A 1.5 column.

Figure 2 demonstrates the remarkable similarity of protein subunit band patterns among the [^{3}H]A-P complex (gel 1), control calf thymus RNA polymerase B isozymes (gel 2), and rat liver RNA polymerase B (gel 3). In comparison to calf thymus RNA polymerase B, the protein distribution pattern of the [^{3}H]A-P complex showed a high degree of conformity. Not only does this indicate that there is no loss of subunit structure or proteolysis evoked by our isolation procedures, it also permits easy identification of the subunit bands.

From their relative positions on the gel (Figure 2) we identified the three heavy subunits B 1, B 2, and B 3, in the upper (5%) part of the gel and the small subunits B 4, B 5, and B 6, in the lower (10%) part. The main difference in gel patterns between the [^{3}H]A-P complex and calf thymus RNA polymerase B was the appearance of an intensely stained band n, migrating a little slower than the largest subunit B 1. The molecular weight of this newly appearing protein, n, was estimated to be about 220 000. The subunit structure of rat liver RNA polymerase B, gel 3, Figure 2 (Chambon et al., 1972), also possesses a heavy subunit BO of 220 000 daltons that seems to be identical to subunit n of the [^{3}H]A-P complex. A second difference between the subunit pattern of the [^{3}H]A-P-complex and calf thymus RNA polymerase B was also observed. The subunit band B 3 was present in a relatively lower concentration, i.e., less intensely stained which differs from the subunit stoichiometry of calf thymus RNA polymerase B reported by Chambon et al. (1970).

Our interpretation of these differences in subunit composition is based on our supposition that amatoxins, when associated with RNA polymerase B, exert a strong stabilizing effect on subunit structure, as well as quaternary structure of the enzyme. The presence of subunit band n and lessened intensity of subunit B 3 may indicate that band n is a precursor molecule of SB-3.

The Covalent Coupling Reaction between [^{3}H]Amanin and RNA Polymerase B. We chose the well-established carbodiimide peptide coupling method as the means to introduce our affinity label. β-Amanitin, also an acidic amatoxin, had already been successfully covalently linked to bovine serum albumin by a carbodiimide (Cessi and Fiume, 1969). The covalent coupling of [^{3}H]amanin to RNA polymerase B, by the carbodiimide method offered us the following advantages: (1) [^{3}H]Amanin is not a semisynthetic amatoxin compound but a native poison. In contrast to the fourfold less effective tritium-labeled *O*-methyl-demethyl-γ-amanitin (Wieland, 1972) [^{3}H]amanin inhibits the RNA polymerases B and C nearly as



[^{3}H] AMANIN

effectively as α-amanitin. (2) The β-carboxyl group of the aspartyl residue (see structure) present in [^{3}H]amanin can be covalently coupled by the efficient methods of peptide chemistry. It also can function to a certain degree as a spacer molecule. (3) The carbodiimide method permitted us to purify the relatively stable enzyme-inhibitor complex, [^{3}H]A-P-complex, before affinity labeling. Other affinity labeling methods would have required the use of reactive but stable activated derivatives of [^{3}H]amanin, and necessitated after coupling the purification of the amatoxin-polymerase conjugate.

Since the coupling reaction had to occur in the aqueous environment of buffer II, we chose the water soluble carbodiimide 1-ethyl-3-(dimethylaminopropyl)carbodiimide, ECDI. The presence of glycerol and β-mercaptoethanol in the buffer apparently did not disturb the reaction.

Conditions of the carbodiimide reaction had to be such that the reaction would be relatively selective. Covalent bond formation between enzyme subunits had to be avoided as far as possible while successful affinity labeling between [^{3}H]amanin and enzyme was achieved. Since the binding constant for formation of the A-P complex is about 10^{-9} M (Chambon et al., 1970), dissociation of [^{3}H]amanin from its receptor site within the complex, even in high dilution, should be negligible. The [^{3}H]amanin aspartyl carboxyl group should be favorably situated, in 1:1 stoichiometry, with respect to a functional group of the receptor for convenient carbodiimide coupling. Consequently, amount of ECDI, reaction time, temperature, and pH were varied to establish the best conditions for affinity labeling.

Excessive amounts of ECDI produced coupling between subunits. The resulting conglomerates became immobile on the electrophoretic gels and contained randomly-bound radioactivity. Reduction of pH, prolonged reaction time, and elevated temperatures influenced the subunit gel pattern in a similar manner. Under optimal conditions, 3 mg of ECDI/0.4 ml of fraction BG, pH 7.0, 5 h, 0 °C, reproducible affinity labeling with distinct subunit band separation was obtained.

The gel pattern of the affinity labeled [^{3}H]A-P complex was very comparable to the pattern of untreated [^{3}H]A-P complex (Figure 2, gel 1). Identification of all protein bands was thus facilitated.

Gels scanned for radioactivity showed a single peak containing more than 2000 cpm (Figure 3). This peak clearly coincides with the subunit B 3 peak, when projected on the densitometric spectrum of the stained and scanned, 550 nm, parallel gel. All other protein bands were essentially void (<40 cpm) of radioactivity, especially the smaller, faster moving subunits. Noncovalently linked [^{3}H]amanin was detectable in the anode buffer. These results were reproducible, regardless of the gel system used for electrophoresis, i.e., 5% homogeneous or mixed 5% upper half, 10% lower half polacrylamide gel, whether the gel slices were dried, combusted, and counted, or eluted and counted.

Unstained parallel control gels, untreated with ECDI, were devoid of radioactivity. Even under the most mild denaturing conditions, just sufficient to dissociate subunits (0.05% sodium dodecyl sulfate, 30 min at room temperature), [^{3}H]amanin left its receptor subunit.

These findings strongly suggest that the amatoxin binding site is localized on subunit B 3 (mol wt 140 000). SB 3 represents the true amatoxin receptor of the RNA polymerase B.

[^{3}H]-Labeled amanitin was found in both RNA polymerase isozymes B I and B II during electrophoresis under nondenaturing conditions (Mandel and Chambon, 1971). This means that the amatoxin-binding subunit must be common to both

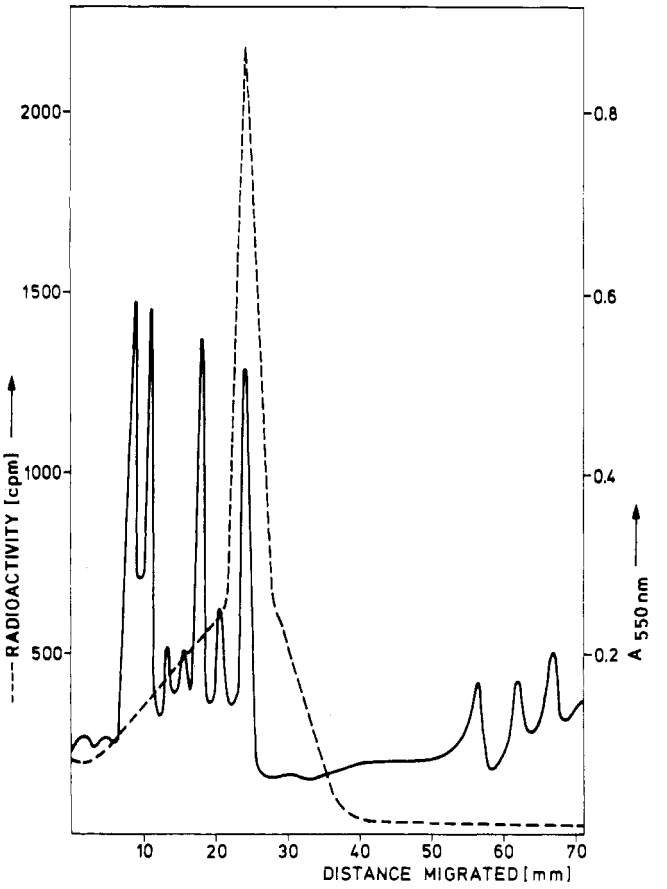


FIGURE 3: Sodium dodecyl sulfate-gel electrophoresis after covalent coupling of [^{3}H]amanin to RNA polymerase II from calf thymus. Profile of absorbance (at 550 nm) after staining with Coomassie brillant blue, solid line; radioactivity in a parallel gel, hatched line.

isozymes. This is true for all small subunits SB 4, SB 5, SB 5′, and SB 6 (a + b). Of the heavy subunits SB 1, SB 2, and SB 3, only SB 3 is common to both enzymes.

Only little can be said about the structure of the amatoxin receptor, but it is an interesting finding (Martial et al., 1975) that RNA polymerase B is inhibited by pyridoxal 5′-phosphate, although in concentrations which are 10^6-fold higher than these of the completely inhibiting amatoxins. The aldehyde forms a Schiff base with an ϵ-amino group of a lysine residue. We can speculate that in our affinity labeling reaction, the aspartyl β-carboxy group of amanin also forms a peptide bond with the ϵ-amino group of a lysine residue.

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Chemical Characterization and Subunit Structure of Human *N*-Acetylhexosaminidases A and B†

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ABSTRACT: Human hexosaminidases A and B were purified from placentae, using two stages of affinity chromatography, to a high degree of purity. Each enzyme was purified 5000–6000-fold, and isolated in 25–40% yield. Enzyme preparations appeared homogeneous in the analytical ultracentrifuge and by acrylamide gel electrophoresis. Hexosaminidase A contained 1.65 residues of sialic acid per molecule, whereas no sialic acid was present in hexosaminidase B. The molecular weights of the A and B isozymes as determined by gel filtration and sedimentation equilibrium are 100 000 and 108 000, respectively. In 5 M guanidine-HCl each of the enzymes yielded a 50 000-dalton species, which can further be dissociated into 25 000-dalton polypeptide chains by reduction and alkylation. The hexosaminidase B yielded one type of polypeptide chain, denoted β, whereas the product from hexosaminidase A could be separated by ion-exchange chromatography into two species of chains, denoted α and β, in equal amounts. The amino acid compositions of the separated α and β chains were determined, and were found to correlate well with those of the intact enzymes. These findings enable the construction of a plausible model for the molecular structure of both enzymes. According to this model hexosaminidase A is composed of two subunits, α_2 and β_2, in which the two polypeptide chains are linked by a disulfide bridge. The structure of hexosaminidase B is, in parallel, $\beta_2\beta_2$. The suggested model is discussed in view of the accumulated information about the interrelationships between hexosaminidase A and B and the genetic metabolic disorders with which they are involved.

Hexosaminidase[1] exists in human tissues in two major isozymic forms A and B; deficiency in one of the isozymes or both results in severe inherited metabolic disorders, which are manifested as GM_2 gangliosidoses, e.g., Tay-Sachs or Sandhoff–Jatzkewitz diseases (Okada and O'Brien, 1969; Sandhoff et al., 1968; O'Brien, 1969). A large body of evidence is available on the biological properties of these isozymes, including their substrate specificity, as well as genetic aspects of their expression in various conditions (reviewed in Tallman, 1974; Neufeld et al., 1975). However, only limited information exists as to their fine molecular structure, or to the molecular relationship between the A and B isozymes, which is based on direct chemical characterization of the pure isoenzymes.

Various hypotheses were suggested to fit the relevant biological and genetic phenomena. The earliest hypothesis was suggested by Robinson and Stirling (1968) stating that hexosaminidase B may be the asialo form of hexosaminidase A, since a conversion from A to "B" was observed upon treatment with neuraminidase. This suggested model was ruled out lately

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[1] 2-Acetamido-2-deoxy-β-D glucoside-acetamidodeoxyglucohydrolase, EC 3.2.1.30.