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Determination of Molecular Weight, Isoelectric Point, and Glycoprotein Moiety for the Principal Skin Test-Reactive Component of Histoplasmin

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A purified component designated HPD (histoplasmin-purified derivative) dII was isolated from two different crude histoplasmin lots by a combination of gel filtration and polyacrylamide disc electrophoresis (Sprouse, 1969). A 0.05-μg portion of HPD dII was reactive and specific in detection of delayed hypersensitivity in guinea pigs experimentally infected with *Histoplasma capsulatum*. The objective of this study was to characterize this skin test-reactive component for (i) homogeneity, (ii) molecular weight, (iii) isoelectric point, and (iv) composition. Sephadex chromatography, polyacrylamide disc electrophoresis, sucrose density gradient ultracentrifugation, acid and heat denaturation, and immunoelectrophoresis indicate that HPD dII is (i) homogeneous, (ii) of approximately 12,000 molecular weight, and (iii) a glycopeptide of approximately 60% carbohydrate and 40% proteinaceous composition. The marked acid and heat stability exhibited by the compound probably is attributable to the prominent carbohydrate moiety in the molecule. Isoelectric focusing indicated an isoelectric point of 5.68. This would suggest that dII is an acidic compound with either predominance of acidic amino acid residues in the molecule or, more probably, an abundance of electron donors in the carbohydrate moiety. In summary, HPD dII appears to be a glycopeptide of approximately 12,000 molecular weight, reactive in elicitation of delayed hypersensitivity of histoplasmosis.

Previous investigation (18) has resulted in isolation of a multicomponent histoplasmin-purified derivative (HPD) from crude histoplasmin using gel filtration. A subsequent report (17) also indicated isolation of two principal glycoproteins (dII and dIII) from HPD using discontinuous polyacrylamide electrophoresis. The more acidic component, dII, detected specific delayed hypersensitivity in guinea pigs experimentally infected with *Histoplasma capsulatum*.

The potential significance of such an antigenic component warranted additional chemical characterization. Although the carbohydrate in this preparation appeared to be firmly bound to the proteinaceous moiety, no direct evidence was available to indicate the sugar moiety was covalently linked to the peptide chain and, thus, that HPD dII is a true glycoprotein. Usually, evidence for a true glycoprotein is provided by heat or acid coagulation tests if it demonstrates removal of the carbohydrate from the solution concomitant with precipitation of the heat- or acid-denatured protein.

This investigation also involved the determination of isoelectric point (pI) and molecular weight of HPD dII essential to preparatory isolation in quantities necessary to total structural elucidation. Therefore, the specific objectives of this study were to ascertain homogeneity, molecular weight, pI point, and glycoprotein moiety of HPD dII.

MATERIALS AND METHODS

Antigens. HPD dII was obtained from crude histoplasmin HKC-43 and M-2 by a combination of Sephadex column chromatography and polyacrylamide disc electrophoresis as previously described (17, 18). All antigens were sequentially dialyzed against 0.5, 0.05, and 0.01 M NaCl solutions and distilled water, lyophilized, and stored at 4°C for subsequent examination.

Gel filtration. Sephadex G-100, G-50, G-25, G-15, and G-10 were equilibrated against elution buffers with pH 9.3 (0.2 M $NaHCO_3$-0.03 M Na_2CO_3), pH 7.2 (0.072 M Na_2HPO_4-0.028 M $NaH_2PO_4 \cdot H_2O$), and pH 2.5 (0.135 M glycine-0.065 M HCl). Columns (1.5 by 60 cm) were packed with equilibrated gel and re-equilibrated against elution buffer before addition of samples. All columns were developed at 25°C using

descending chromatography. The effluent stream was held constant by peristalsis and continuously monitored at 280 nm with a Beckman DB-GT spectrophotometer and flow-through cuvette. Consecutive 0.5-ml aliquots were collected for protein (10) and carbohydrate (3) analysis.

Disc polyacrylamide electrophoresis. Aliquots (20 to 50 μl) of HKC-43, M-2, HPD, and HPD dII were subjected to electrophoresis in 7.5% acrylamide-bisacrylamide columns (0.5 by 6 cm) using a temperature-regulated analytical electrophoresis apparatus (Buchler Instruments, Inc., Fort Lee, N.J.). Electrophoresis was completed in either an anionic system with running pH 9.3 [upper buffer: 0.043 M tris(hydroxymethyl)aminomethane-0.045 M glycine; lower buffer: 0.12 M tris(hydroxymethyl) aminomethane-0.06 M HCl] or cationic systems with running pH 4.3 (upper buffer: 0.35 M beta alanine-0.15 M acetic acid; lower buffer: 0.12 M KOH-0.75 M acetic acid) or pH 2.7 (upper buffer: 0.25 M glycine-0.05 M acetic acid; lower buffer: 0.75 M acetic acid-0.12 M KOH). After initial equilibration for 15 min at 2.5 mA/columnal cross section, the current was increased to 6.5 mA/columnal cross section for the remainder of the electrophoretic run (30 min). The gels were removed from the glass columns, fixed in 7% aqueous acetic acid, and stained with nigrosin or amido black 10B for protein, a modified periodic acid-Schiffs stain for compounds containing adjacent diol groups (carbohydrate), and Sudan IV for lipoidal compounds. Densitometric tracings were completed using a model 542 Densicord recording electrophoresis densitometer (Colab Laboratories, Inc., Chicago Heights, Ill.).

SDS electrophoresis. The above antigens were subjected to electrophoresis in 5, 7.5, 10, and 12.5% acrylamide-bisacrylamide gels containing 8 M urea and 1% sodium dodecyl sulfate (SDS) as described by Ullman and Monod (19). Upon completion of electrophoresis, gels were removed from the columns, fixed in 7.5% aqueous acetic acid, and stained with 1% Coomassie brilliant blue as described by Fairbanks et al. (4).

Isoelectric focusing. Isoelectric focusing was performed according to the method of Catsimpoolas (2). The gels were strained by the method of Griffith and Catsimpoolas (7) using mercuric chloride-bromophenol blue. Each run consisted of gels containing samples of unknown pI's and ampholyte, samples of known pI values (chymotrypsinogen A, pI = 9.1: ribonuclease A, pI = 7.8; *Escherichia coli* alkaline phosphatase, pI = 4.5; Worthington Chemical Corp., Freehold, N.J.) was ampholyte, and reference gels containing only ampholytes of either wide (3 to 10) or narrow (3 to 5, 5 to 8) pH range. After equilibrium electrophoresis, the reference gels were sliced into 1-cm sections, respective sections were eluted in 1 ml of distilled water, and the pH of each aliquot was determined using a conventional pH meter and combination microelectrode. Corresponding pI values for proteinaceous compounds in the stained gel were estimated by superimposing the pH gradients ascertained for the sectioned reference gels and reading appropriate pH values.

Ultracentrifugation. Sedimentation ratios for HPD dII in 5 to 20% and 1 to 10% continuous sucrose density gradients were compared with those for compounds of known molecular weight. Aliquots (20 μl, 0.5 to 1 mg) of each compound were layered onto the surface of continuous sucrose gradients. Centrifugation was performed at 50,000 rpm, using a Beckman L2-65B centrifuge equipped with a type 50.1 swinging-bucket rotor. The 5 to 20% sucrose gradients were centrifuged for 6 h, and the 1 to 10% sucrose gradients were centrifuged for 12.5 h. All temperatures were maintained at 5°C. Upon completion of centrifugation, tubes were punctured, the effluent was continuously monitored at 220 nm using a Beckman DB-GT spectrophotometer equipped with a flow-through cuvette, and consecutive 0.375-ml aliquots were collected for subsequent spectrophotometric reanalysis and colorimetric (3, 10) analysis.

Acid and heat denaturation. Aliquots of HPD dII and compounds of known composition were subjected to acid or heat denaturation. Acid-treated samples were prepared by adding equal volumes of 20% trichloroacetic acid, homogenized, held at −15°C for 10 min, and then refrigerated overnight at 5°C in Teflon-sealed vials. The heat-treated samples were brought to volumes equivalent to the acid-treated samples with distilled water and sealed prior to exposure at 112°C for 120 min in a constant-temperature sand bath. They also were stored at 5°C overnight. All samples were centrifuged at 2,000 × *g* for 60 min, and protein (10) and carbohydrate (3) were determined for respective supernatants.

Immunoelectrophoresis. Immunoelectrophoresis was performed by a microtechnique (6) on glass slides (1 by 3 inches [2.54 by 7.62 cm]) coated with 1.5% agar (Noble, Difco) in veronal buffer (0.026 M $C_8H_{11}N_2O_3Na$, 0.03 M $NaC_2H_3O_2$, 0.21 M HCl; I 0.05, pH 8.6) at 6 V/cm. Antigens were subjected to electrophoresis for 90 min, and lateral basins were removed and filled with known reactive, human, hyperimmune serum from a patient with chronic cavitary histoplasmosis. Slides were incubated for 3 to 5 days in moist chambers at 25°C before analysis.

Spectral analysis. Samples of known protein concentration were prepared in 0.01 N NaOH (pH 12), placed in 1-cm-path quartz cuvettes, and read against reference standards of 0.01 N NaOH, sterile asparagine medium, and distilled water. A programmed scan from 400 to 200 nm was completed for each sample using a Beckman DB-BT spectrophotometer with programmed scanner.

RESULTS

Column chromatography. Figure 1 indicates no apparent separation of protein and carbohydrate moieties for dII from Sephadex G-25 superfine at various hydrogen ion concentrations. The dII glycopeptide was increasingly retarded in the Sephadex G-50 and G-100, respectively, moderately inhibited in the Sephadex G-25 matrix, and essentially eluted in the void volume (V_0) for G-15 and G-10. A marked loss of resolution was apparent for all gels equilibrated with

the carbonate (pH 9.3) and glycine-HCl (pH 2.5) buffers.

Gel filtration chromatography indicated V_e/V_0 ratios for dII and an insulin fragment with reported molecular weight of 12,000 (16) to be identical. This would suggest a similar molecular weight for HPD dII.

Figure 2 shows respective densitometric trac-

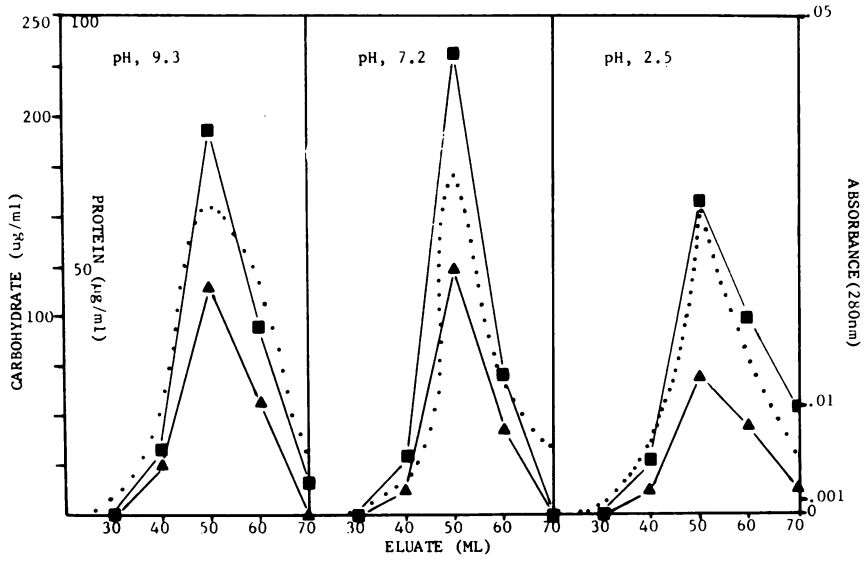

FIG. 1. *Descending column (1.5 by 60 cm) chromatography of 1 mg of dII on Sephadex G-25 equilibrated and eluted with carbonate (pH 9.3), phosphate (pH 7.2), and glycine-HCl (pH 2.5) buffers, respectively. Symbols: (■) carbohydrate concentration; (▲) protein concentration; (●) absorbancy (at 280 nm).*

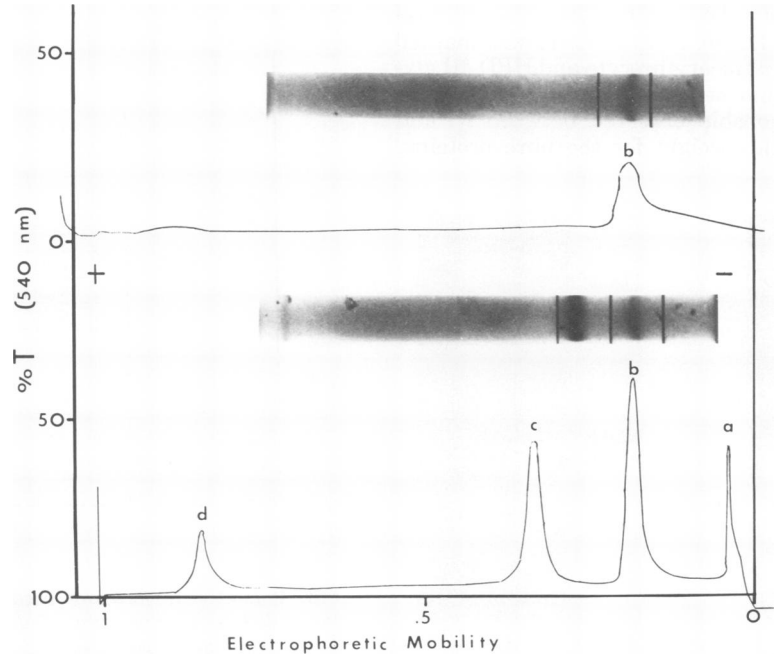

FIG. 2. *Densitometric tracings and 7.5% polyacrylamide disc gels of HPD (lower) and dII (upper) subjected to electrophoresis at pH 9.3. Gels were stained with amido black 10B. (b) and (c) indicate dII and dIII, respectively. (d) indicates the tracking dye marker added to HPD.*

ings for HPD and its dII component subjected to electrophoresis in 7.5% acrylamide-bisacrylamide at running pH 9.3. The tracings indicate that HPD consists of two major components and several minor components, and dII appears to be homogeneous. The multicomponent HPD and dII were markedly inhibited in more concentrated polyacrylamide gels. Maximal resolution was obtained in the 7.5% gels. Maximal migrational velocity for dII occurred at running pH 4.3. However, optimal resolution of all components of HPD was obtained at running pH 9.3. The electrical field was reversed for electrophoresis in the cationic systems. This also decreased in resolving time for dII from HPD since it migrated ahead of dIII and other more anodal components.

Figure 3 shows duplicate samples of HPD in 7.5% acrylamide-bisacrylamide simultaneously subjected to electrophoresis at running pH 9.3. Respective dII bands reacted with basic leucofuchsin and nigrosin, suggesting a unit molecule of carbohydrate and proteinaceous moieties or unjoined carbohydrate and proteinaceous moieties of similar charge, size, and electrophoretic mobility (R_f). The latter possibility would seem improbable.

SDS electrophoresis indicated an apparent molecular weight (MW) of less than 30,000 for HPD dII in comparison to erythrocyte glycoprotein (MW 53,000), lysozyme (MW 14,400), trypsin inhibitor (MW 14,300), and insulin fragments (MW 12,000; 24,430). There was marked retardation of the erythrocyte and HPD dII glycoproteins, and neither appeared to follow the linear relationship observed between R_f and $\log_{10}$ molecular weight for the pure proteins.

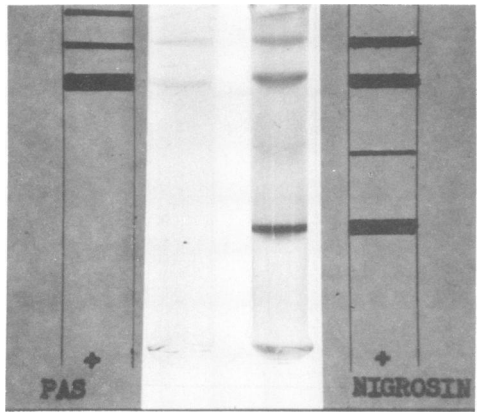

FIG. 3. *Polyacrylamide disc gels (7.5%) of HPD subjected to electrophoresis at pH 9.3 and stained with nigrosin or basic leucofuchsin. The top two nigrosin bands represent dII and dIII, respectively.*

Erythrocyte glycoprotein reportedly contained 60% carbohydrate (11), similar to that for HPD dII. The 20 to 50% correction of apparent molecular weight experimentally determined for the erythrocyte glycoprotein (15) would indicate a real molecular weight of 15,000 to 24,000 for HPD dII.

Specific pI's were determined by equilibrium electrophoresis of 50-μg samples dissolved in suitable ampholyte carrier solutions. Figure 4 indicates pI's determined using samples of crude histoplasmin HKC-43 with ampholyte focusing range of pH 3 to 10. Specific pI's of 6.19 and 6.50 were indicated for the composite dII and dIII sample. Those values subsequently were redetermined to be pH 5.68 and 6.35, respectively, for purified samples of dII and dIII (Fig. 5) using limited-range ampholyte carriers

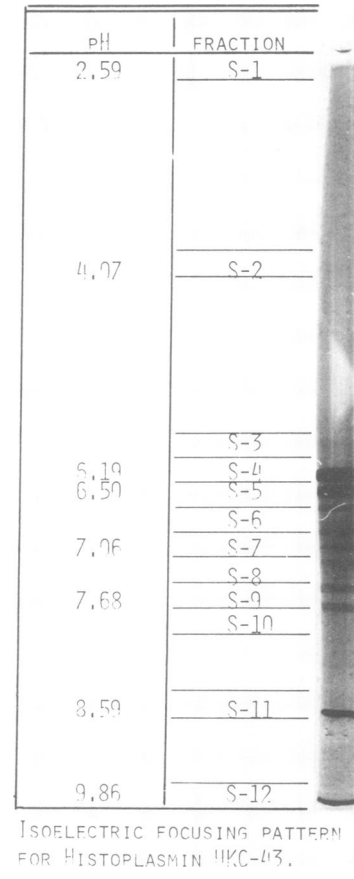

FIG. 4. *Isoelectric focusing pattern for HKC-43 with ampholyte focusing range of pH 2.59 to 9.86 (S) indicates equivalent positions for 1-cm sections of reference gel on which pH was determined. (S-4) and. (S-5) indicate the positions of dII and dIII, respectively.*

(pH 5 to 8) and ribonuclease A of known pI (pI 7.8). Concentrated samples (1 mg) of thrice polyacrylamide-electrophoresed dII and dIII also were examined individually for homogeneity using wide-range ampholytes (pH 3 to 8). No contamination was detectable by densitometric analysis for either antigen.

The sedimentation behavior of HPD dII and dIII is expressed as the ratio of the distance from the meniscus to the middle of the protein peak to the total distance from the meniscus to the bottom of the tube. The high-molecular-weight marker (insulin, MW 24,430) completely sedimented the 1 to 10% sucrose gradient and neither of the low-molecular-weight markers (lipase, MW 6,669; elastin, MW 6,830) cleared the meniscus of the 5 to 20% or 1 to 10% gradients. However, a coefficient of 0.39 was observed for a low-molecular-weight insulin fraction (MW 12,000), between values of 0.44 and 0.34 for dII and dIII, respectively.

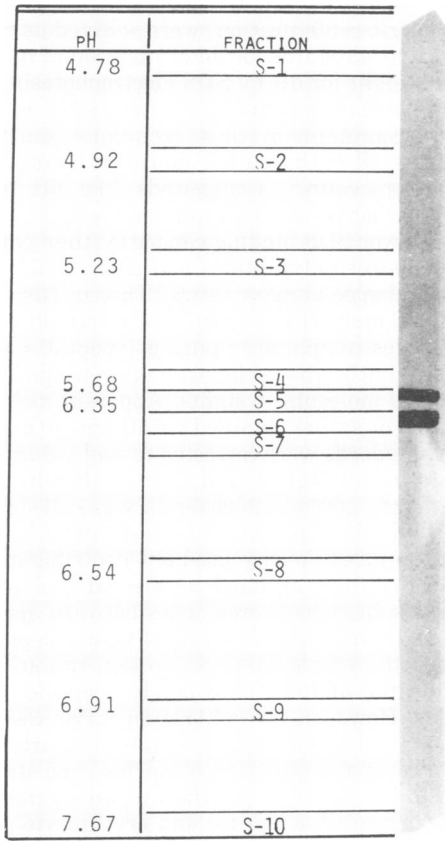

FIG. 5. *Isoelectric focusing pattern for dII and dIII with ampholyte focusing range of pH 4.78 to 7.67. (S) indicates equivalent positions for 1-cm sections of reference gel on which pH was determined. (S-5) and (S-6) indicate the positions of dII and dIII, respectively.*

Centrifugation time for the 1 to 10% gradients was more than double that of the 5 to 20% gradients. Some diffusion had occurred, and consequently peaks were not as sharp. Both dII and dIII adequately cleared the meniscus, traversed almost half the length of the gradient, and were symmetrical, allowing accurate determination of the middle of the peak. Peak symmetry, in addition to spectrophotometric, protein (9), and carbohydrate (3) analyses, suggested dII was homogeneous and of approximately 12,000 molecular weight.

Denaturation and sedimentation of the proteinaceous moiety of HPD dII simultaneously removed a proportional amount of the carbohydrate moiety, suggesting a covalent link between the two entities (Table 1). Trichloroacetic acid denaturation was more severe than heat coagulation for all the samples examined. Comparison of the heat coagulation data obtained for insulin and the insulin-sucrose mixture would suggest that the carbohydrate conveyed a protective effect for the proteinaceous entity. The erythrocyte glycoprotein and ovomucoid denaturation data corroborate the observation and also indicte that the greater the ratio of carbohydrate to protein in the molecule, the greater the protective effect. Similar analysis of dII and dIII denaturation properties suggest that both are homogeneous and exhibit strong bonding between the carbohydrate and proteinaceous moieties indicative of a glycoprotein.

Figure 6 shows the immunoelectrophoretic patterns for HKC-43 and HKC-43 HPD dII. Aliquots containing 261 μg of protein of the former and 1,000 μg of protein of the latter were subjected to electrophoresis. Analogous antisera were used against both antigens in the subsequent immunodiffusion step. The crude antigen elicited four precipitin lines (a, b, c, d), whereas the purified dII component elicited a single, strong discrete line of precipitation. This and similar data for M-2 HPD dII suggest that both are homogeneous, ubiquitous to crude histoplasmin, and reactive in immunodiffusion.

Spectral studies in the ultraviolet region indicated a maximal extinction (E^1_1) of 1.19 at 219 nm, with a shoulder between 248 and 300 nm for HPD dII (Fig. 7). The peak at 219 nm accounts for the major portion of the glycopeptide, whereas the shoulder suggests a small amount of contamination by nucleic acid or protein, probably dIII, or some polydispersion.

DISCUSSION

Several investigators (1, 9, 12, 14, 17, 20) have suggested that the skin test-active anti-

TABLE 1. *Acid and heat denaturation studies of HPD dII and other compounds*

Compound	Carbohydrate (g/g of protein)	Method of denaturation	Concn in supernatant after denaturation (%)	
			Protein	Carbohydrate
Erythrocyte glycoprotein	1.5	Acid	13	13
		Heat	18	21
Ovomucoid	0.31	Acid	7.0	6.0
		Heat	9.0	11
Insulin fragment	0.03	Acid	<1	0
		Heat	4	0
Sucrose	0	Acid	0	80
		Heat	0	97
Insulin fragment and sucrose	1	Acid	<1	84
		Heat	17	99
dIII	1.2	Acid	36	43
		Heat	43	48
dII	1.7	Acid	27	27
		Heat	36	37

gens of *H. capsulatum* yeast, hyphae, or filtrate are carbohydrate-protein complexes. Some investigators also have presumptuously reported carbohydrate and amino acid compositions without substantiating either homogeneity or glycoprotein moiety. Evidence presented in this study indicated that dII, the principal skin test-reactive component of histoplasmin, was homogeneous, of approximately 12,000 molecular weight, and a glycopeptide with a protein-to-carbohydrate ratio of 1:1.5.

Similarity in composition, size, and charge also suggested that dII and dIII could result from dissociation of a single entity in the electrophoretic field and therefore are not unique glycopeptides. Salvin (14), in studies completed several years ago, suspected that similar electrophoretic dissociation reflected the heterogeneity observed in yeast cell wall antigens that were homogeneous by ultracentrifugation. However, the occurrence of dII and dIII as unique electrophoretic entities, in various concentrations, has been demonstrated for more than 30 different histoplasmin lots (Sprouse, unpublished data). One might rationalize their separate occurrence among the various lots since all were examined by the same technique, polyacrylamide disc electrophoresis, but this fails to explain the concentration variations in dII and dIII which lend credulity to a separate existence.

If indeed this omnipresence of dII and dIII in histoplasmin lots is valid, then one would presume that all exposed hosts have been sensitized and would react to the monovalent or unit antigen, dII. This should offer numerous advantages over the polyvalent or, more correctly, multiple-isolate antigens currently used for skin test and serological evaluation.

Glycoproteins of similar carbohydrate composition and size, although not necessarily of similar steric configuration, were selected as comparative standards for determination of molecular weight for dII by SDS electrophoresis and gel filtration. Estimation of molecular weight for glycoproteins by these techniques must be interpreted with caution and corraborated by other procedures. Compounds like dII with high carbohydrate-to-protein ratios apparently have lower SDS binding capacities than carbohydrate-free polypeptides and thus exhibit reduced charge-to-mass ratios. This in turn results in marked retardation of electrophoretic mobilities compared to pure polypeptides and, consequently, discrepancies between apparent and real molecular weights. Apparent molecular weight values obtained for dII and glycoprotein standards were corrected to real values, as suggested by Segrest et al. (15). Real molecular weight of 15,000 to 24,000 for dII was still significantly higher than the 12,000 obtained by gel filtration and density gradient centrifugation.

Gel filtration, although unaffected by charge, also is subject to discrepancies between apparent and real molecular weight values depending on the carbohydrate-to-protein ration and the degree of branching of glycopeptide side chains. However, the calibration curve was obtained with compounds containing carbohydrate ranging from 100% for lactose and stachyose to less than 3% for the insulin fragment. The porcine ribonuclease and erythrocyte glycoprotein were prepared in our laboratory and reported by other investigators (11, 13) to have molecular weights and carbohydrate compositions of 17,000 to 21,000 and 20 to 35% and 53,000 and 60%, respectively, The curve was reasonably linear, suggesting minimal devia-

tion for the real and apparent molecular weight of 12,000 obtained for dII.

Data presented in Table 1, corroborated by differential Sephadex chromatography (Fig. 1) and differential staining of polyacrylamide disc electrophoretic gels (Fig. 3), offer convincing evidence for a covalent bond between the carbohydrate and proteinaceous moieties of dII. Both acid and heat denaturation of proteinaceous material, and subsequent removal from the dII solution, simultaneously removed proportionate amounts of carbohydrate. These data also confirm homogeneity and suggest considerable stability for the molecule.

Comparison of the 17% protein in the insulin-sucrose supernatant with the 4% remaining in the insulin supernatant, after heat treatment, also suggested that the sugar conveyed some protective effect to its proteinaceous counterpart. Similar examination of residual supernatant protein for insulin fragment (4%), ovomucoid (9%), and erythrocyte glycoprotein (18%) also indicated that protective effect was proportional to increase in the carbohydrate-to-protein ratio. The 43% protein remaining in the dII supernatant after the heat treatment would indicate a similar protection of the dII peptide. This protective phenomenon by the sugar moi-

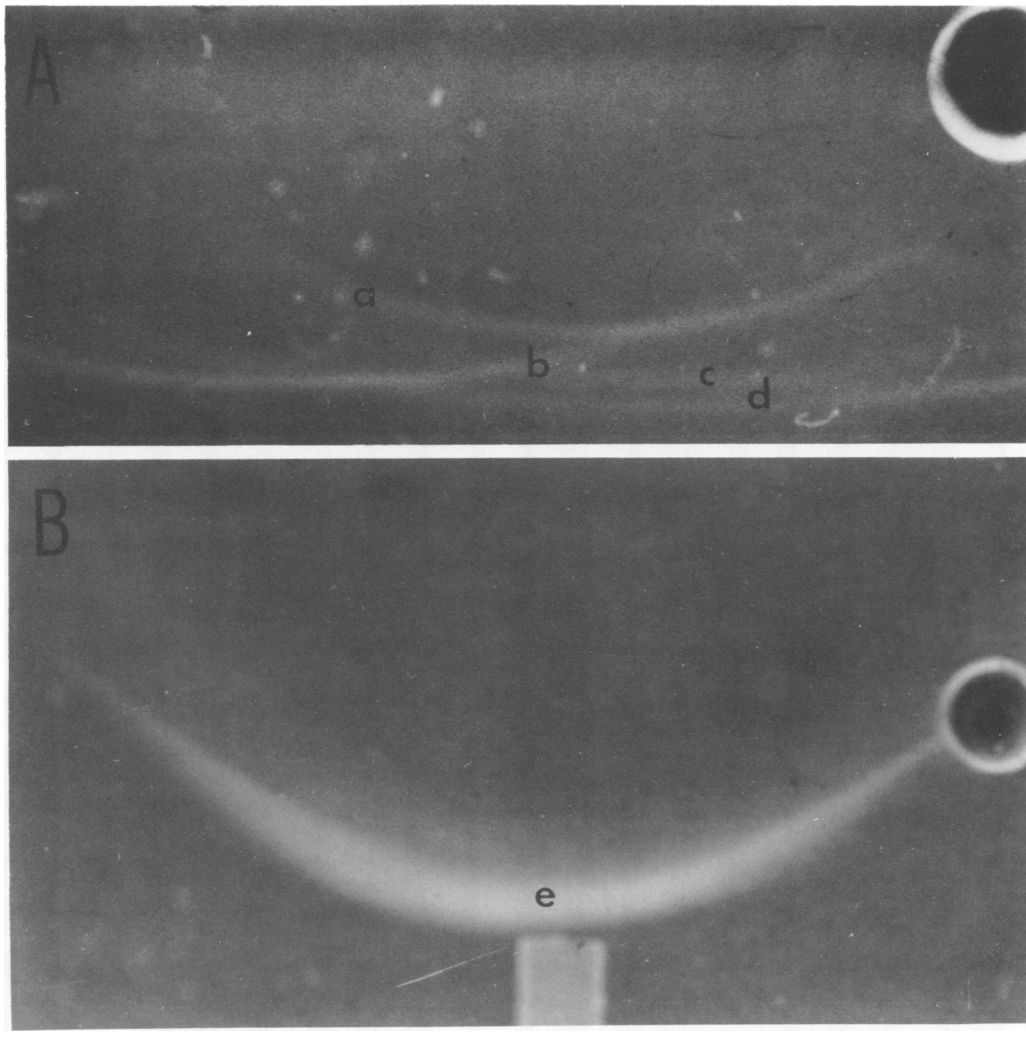

FIG. 6. *Immunoelectrophoretic patterns for (A) HKC-43 and (B) dII. Both antigens were subjected to electrophoresis and then exposed to the same polyvalent antiserum. (a), (b), (c), and (d) show the multiple lines of precipitation for crude histoplasmin. (e) indicates a single line of precipitation for dII. (a) and (e) probably indicate lines of identity. This was not confirmed by immunodiffusion.*

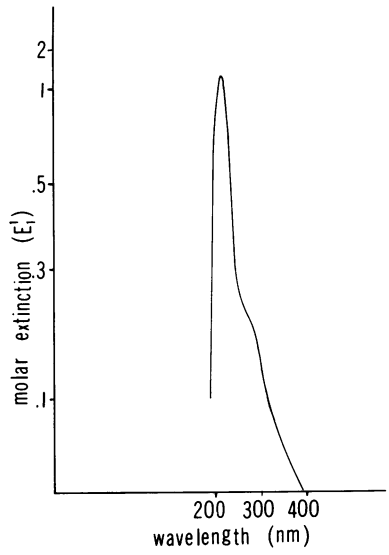

FIG. 7. *Spectral scan of dII in the ultraviolet region, at pH 12. (E_1^1) of 1.19 was observed at 219 nm, with a shoulder at 248 to 300 nm.*

ety may also explain the prolonged shelf life and heat stability observed for histoplasmin and unconfirmed reports of residual skin test and serological activity in autoclaved lots of histoplasmin with inherent high concentrations of free glucose.

The immunoelectrophoretic studies were completed only to confirm homogeneity. No attempt was made to evaluate immunogenicity since an insufficient quantity of the dII glycopeptide was available to undertake such studies. However, completion of such evaluation is potentially important if the report by Kaufman et al. (8) implicating alteration of serodiagnostic values by previous skin testing is valid. Ideally, dII would preclude this problem by eliciting the desired delayed hypersensitive reaction without simultaneously sensitizing the host.

In conclusion, a previous report indicated that 0.05 μg of dII, isolated from crude histoplasmin by a combination of gel filtration chromatography and polyacrylamide disc electrophoresis, was reactive and specific in detection of infection allergy in guinea pigs experimentally infected with *H. capsulatum* (17). The potential significance of this antigen, with its apparent omnipresence in histoplasmin lots, as a standard unit for human testing and future immunochemical studies precipitated this detailed biochemical characterization. These studies indicated that dII was a homogeneous, relatively heat-stable glycopeptide with a pI of 5.68 and molecular weight of approximately 12,000. These data also suggested that the greater than 60% carbohydrate moiety accounted for the stability of the compound and the proteinaceous moiety, consisting of 35 to 40 amino acid residues, conveyed the antigenicity.

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