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TAHA, Bariah Hamdy, 1934-
ISOLATION AND CHARACTERIZATION OF SKIN TEST ACTIVE COMPONENTS OF HISTOPLASMIN.

The University of Oklahoma, Ph.D., 1969
Chemistry, general

University Microfilms, Inc., Ann Arbor, Michigan

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THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

ISOLATION AND CHARACTERIZATION OF SKIN TEST ACTIVE COMPONENTS OF HISTOPLASMIN

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

BY

BARIAH HAMDY TAHA

Norman, Oklahoma

1969

ISOLATION AND CHARACTERIZATION OF SKIN TEST

ACTIVE COMPONENTS OF HISTOPLASMIN

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ACKNOWLEDGMENT

I would like to express my sincere appreciation to Dr. Leon S. Ciereszko and Dr. Howard W. Larsh for their advice and guidance throughout the course of this work.

I am deeply grateful to the Microbiology Department for provision of facilities, equipments and material which made this study possible.

A note of gratitude is also extended for the assistance extended by numerous colleagues and technical personnel.

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ISOLATION AND CHARACTERIZATION OF SKIN TEST ACTIVE COMPONENTS OF HISTOPLASMIN

CHAPTER I

INTRODUCTION

Histoplasmosis, a lung disease, was first recognized by Darling (1,2), and was considered a rare and fatal disease. In the two decades since 1945 histoplasmosis has come to be regarded as a common rather than rare, but rarely fatal disease. There is no evidence of its being a communicable disease, but like most other mycoses, histoplasmosis may simulate any of several other diseases, particularly tuberculosis.

The disease and its etiological agent, Histoplasma capsulatum, have attracted the attention of both epidemiologist and mycologist. Emmons (3) has isolated H. capsulatum from several groups of feral animals. The fungus has been isolated from soils in Tennessee, Indiana, and Oklahoma by Ajello, et al. (4), Ziedberg, et al. (5), Graystone, et al. (6), and Furcolow and Larsh (7). The same fungus has been isolated from histoplasmosis patients.

The antigen histoplasmin, so widely used in skin testing surveys, is the crude antigenic filtrate of liquid culture media in which H. capsulatum has been grown for varying periods of time. It was first prepared by Zarafonetis and Lindberg (8), and was made available for large scale surveys by Christie and Peterson (9), Emmons (10), and Howell (11). A cutaneous reaction to histoplasmin could be demonstrated in an infected human patient or experimentally infected animal.

Numerous epidemiologists have studied the geographic distribution of histoplasmosis utilizing histoplasmin as the major tool for their surveys. Christie and Peterson (9), and Palmer (12), have been successful in mapping areas of high incidence of histoplasmosis in the United States.

Various names have been given to lots of histoplasmin prepared on a large scale, for example H-3, H-15, H-40, and H-42 which have been used by the United States Public Health Service. Histoplasmin lot H-42 is available now and most widely used today. However the supply is limited, will be depleted and a new preparation will be needed.

Despite the fact that histoplasmin has been used extensively, several questions concerning specific antigenicity and relative potency remain unanswered. The resolution of these questions may reside in improvement and standardization of the preparation of histoplasmin. Presently much remains to be understood concerning the

chemical and biochemical aspects of different components which are known to comprise the culture filtrate.

Active skin substance has been isolated by various methods. Van Pernis et al. (13) precipitated a polysaccharide complex from histoplasmin with acetone, others (14, 15,16) have used precipitation with ethanol. Green et al. (17) have been able to isolate two active components on DEAE-cellulose columns. Fadula and Larsh (18) isolated active skin test components by ion exchange chromatography using H. duboisii culture filtrate. Gel filtration (Sephadex 25) also has been employed to isolate the active skin test components from H. capsulatum culture filtrate by Sprouse and Larsh (19).

The importance of histoplasmin skin test as the major tool to determine the occurrence of histoplasmosis is obvious. However until the antigens are fully evaluated, their usefulness in diagnostic tests must remain subject to question.

This study is designed to give some information concerning the production of the active component, the influence of the origin of the isolate, and of the age of culture. Identification of the various components by disc electrophoresis, as well as the isolation of skin test components on preparative polyacrylamide gel columns was attempted. Since H-42 is now the antigen most widely used by the United States Public Health Department and is considered

to be a very potent skin test material, a detailed study was made of the material as a basic step for further improvement of the preparation of a reliable histoplasmin.

CHAPTER II

GENERAL CHEMICAL CHARACTERIZATION OF HISTOPLASMIN

Variation of the potency of histoplasmin has been reported by many investigators. Emmons (10) prepared histoplasmin H-3, and standardized it by determining its skin test potency by intradermal inoculation of guinea pigs sensitized to H. capsulatum. Shaw, Howell, and Weiss (20) prepared histoplasmin H-15, standardized against histoplasmin H-3. Their method of standardization was the basis for the skin testing procedures adopted as the minimal standard, and which depend primarily on size of skin reaction. A procedure for producing a standardized antigen is desirable. The production of histoplasmin apparently depends upon several factors which have to be studied before an appropriate procedure for its preparation can be worked out. Some of the important variables neglected in the preparation of and testing of histoplasmin include:

1. Isolate used for preparation of histoplasmin.
2. Optimal harvest time of culture.

3. Quantity and nature of material used for skin test.

4. Medium in which skin test is administered.

These items, as well as others, should be evaluated. The following chapter deals with the investigation of these items.

Materials and Methods

Isolates of Histoplasma capsulatum used for antigen preparation

Isolate name	Source
Scritchfield	Missouri State sanatorium (sputum)
Grand Island	Soil from Grand Island, Nebraska
NCDC No. 267	Culture collection NCDC laboratory, Atlanta, Georgia
District	Soil from park, Washington, D.C.

Preparation of histoplasmin

Histoplasmins were prepared by quantitative inoculation of sterile Smith's asparagine medium (21) with a mycelial suspension of H. capsulatum. Aliquots (500 ml) of homogeneous inoculated medium were dispensed into 1 liter cotton-plugged Erlenmeyer flasks. The flasks were incubated at 27°C and approximately 75% relative humidity to minimize dehydration. Histoplasmins were harvested after successive periods of time, starting at one week and stopping at 12 months, by addition of (1:10,000) merthiolate solution and double filtration through a Seitz filter. Crude histoplasmins

were dispensed into 100 ml antigen bottles, sealed, labeled and stored at 4°C until used.

Skin testing with histoplasmin

Albino guinea pigs were used. All animals were tested initially with histoplasmin (H-42) and those exhibiting a negative reaction (less than 5 mm) were used. The negative animals were sensitized by intraperitoneal inoculation of a viable mycelial suspension of H. capsulatum in a total volume of 1 ml. Viability was determined by quantitative inoculation of mycelial suspension onto the surface of Sabouraud's dextrose agar plates. Crude histoplasmins were assayed for infection allergy by intradermal inoculation. Erythema and induration were measured and recorded at 24 and 48 hours after inoculation. However, only 48 hour induration readings were used in the interpretation of results.

Chemical analysis

Protein was determined quantitatively by the modified Folin phenol method of Lowry (22) using crystalline bovine serum albumin as standard. Carbohydrates were determined with anthrone reagent (23), and with the phenol sulfuric acid technique (24), using D-glucose as standard. Reducing sugars were determined colorimetrically using the arsenomolybdate and copper reagent of Somogyi (25). Total sugar was taken as the average of both methods and done on the untreated filtrate. Total protein was assayed after

dialysis for 96 hours against repeated changes of distilled water and lyophilization. Dry weight was obtained on the non-dialyzable material.

Results

Effect of isolate and harvest time

The wide variation of protein and carbohydrate concentration observed among the isolates is shown in Fig. 1. Histoplasmin from Scritchfield isolate showed little variation in carbohydrate and protein during the 12 months period of study. Histoplasmin from the other three isolates showed a rise in protein content corresponding to decrease in reducing sugars as well as in the total sugars. However the age at which the change occurred was different, ranging from 8 months for Grand Island, 4 months in NCDC, to 2 months for District.

Concentration of skin test material

Dry weight/ml of crude filtrate varied among the isolates as well as for the different ages of the same isolate. The ratio of protein to total solids represented in Table 1, also showed variations.

Although Scritchfield culture filtrate showed a slight increase in total solids, the ratio of protein/total solids remained constant (which agreed with the growth curve where no change in protein or carbohydrate was detected).

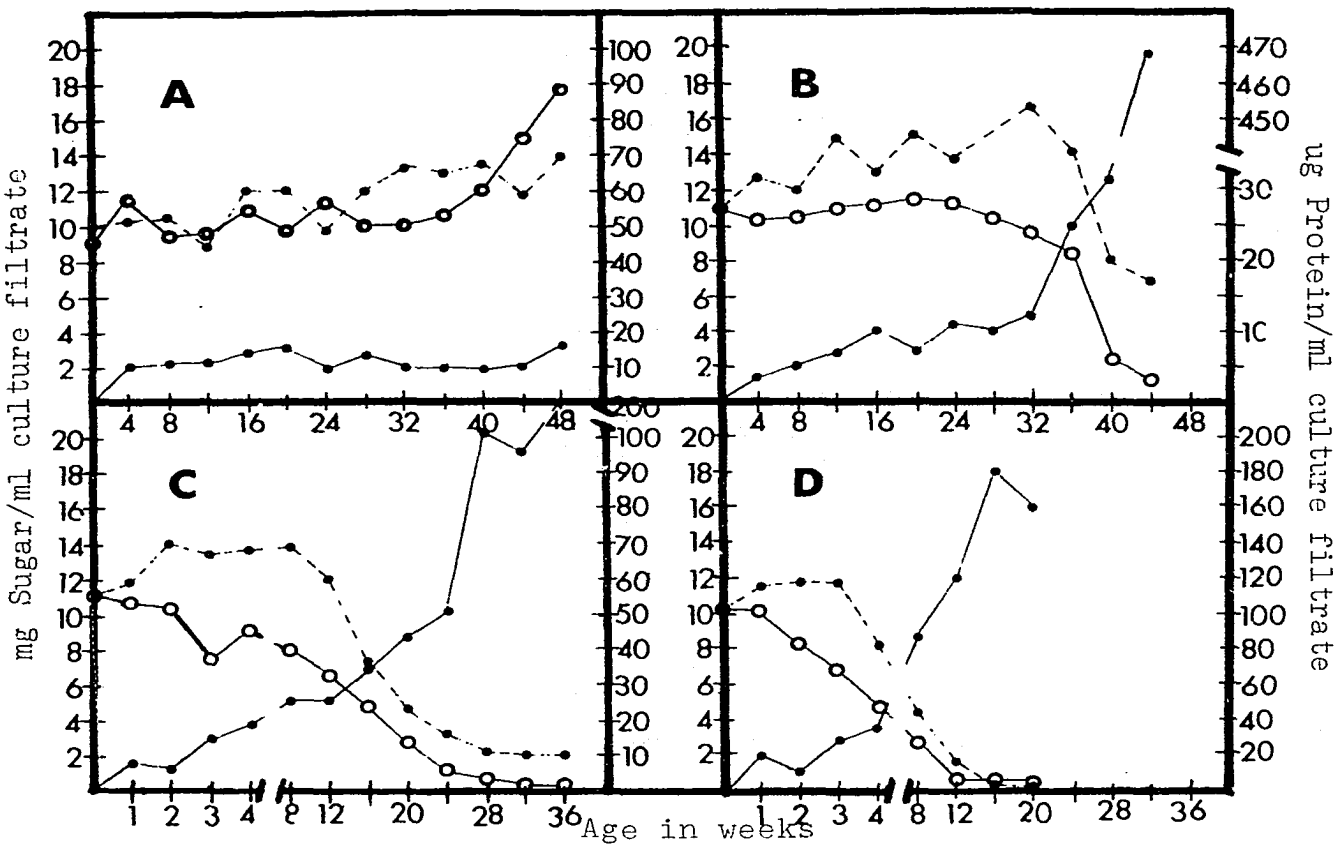


Fig. 1.--Protein and carbohydrate as affected by culture age.
.——. = total protein ; .- - -. = total carbohydrate ;
o——o = reducing sugar .
A. Scritchfield C. NCDC #267
B. Grand Island D. District

TABLE 1

DRY WEIGHT OF NONDIALYZABLE MATERIAL FROM DIFFERENT CULTURE FILTRATES

Isolate No.	Age in months	Name	mg nondialyzable solids / ml culture	ug protein / ml filtrate	% protein / total solids
HT-1	1	Scritchfield	0.65	22.6	3.5
HT-1	11	Scritchfield	0.943	32.0	3.4
HT-2	1	Grand Island	0.365	58.3	1.6
HT-2	11	Grand Island	0.897	243.3	27.0
HT-3	1	NCDC #267	0.083	4.0	4.8
HT-3	9	NCDC #267	0.61	166.6	27.0
HT-4	1	District	0.093	13.3	14.0
HT-4	4	District	0.29	160.6	55.0

In the case of the other three isolates the change in protein/total solids corresponds to the change in protein and carbohydrate detected previously in growth curve. The activity of the isolates was compared as shown in Fig. 2 by injecting a known quantity of histoplasmin protein. Scritchfield showed no difference in reaction at three or eleven months Fig. 2 A and B, or with the 400-fold increase in dosage. Grand Island showed an increase from

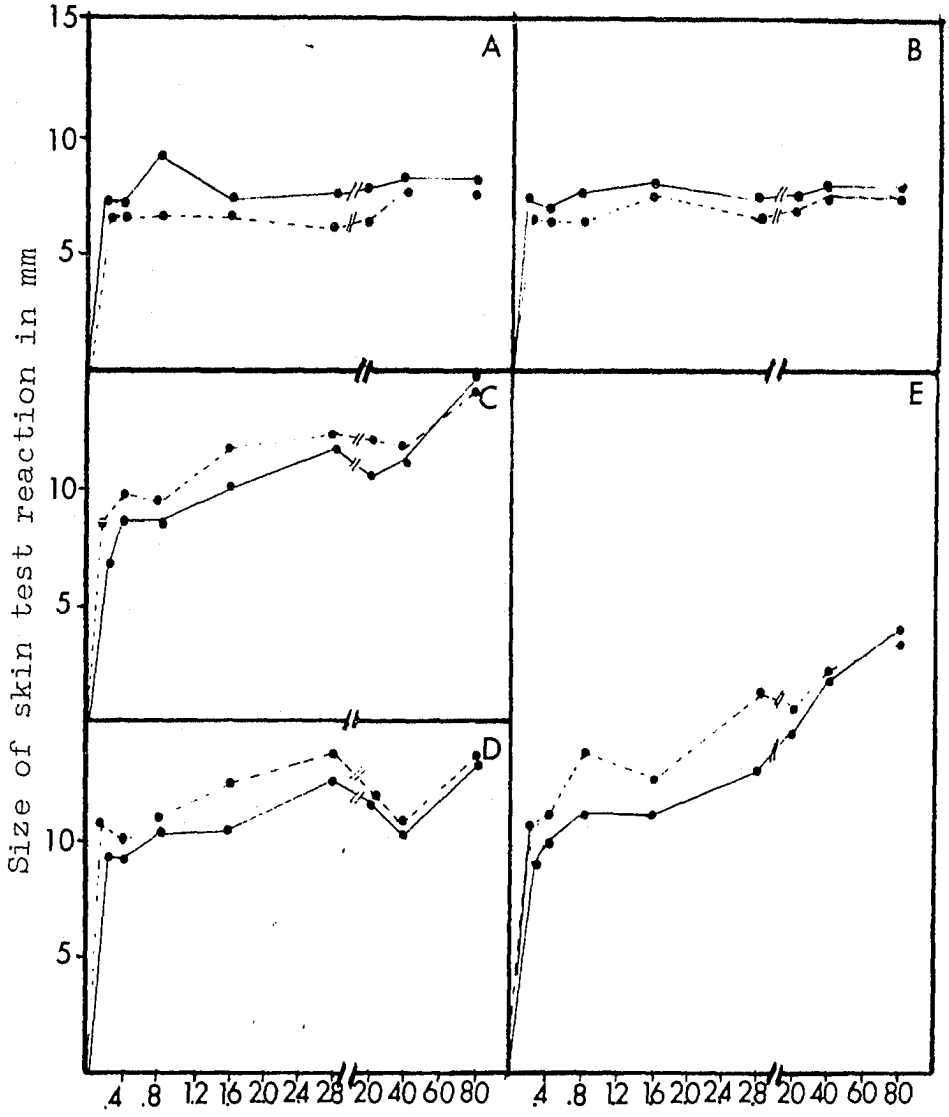


Fig. 2. Skin test reaction as affected by protein concentration.
.——. = induration; .- - -. = erythyma
A. Scritchfield 3 month old
B. Scritchfield 11 month old
C. Grand Island 11 month old
D. NCDC #267 11 month old
E. District 8 month old

approximately 10 mm at 0.2 ug to about 18 mm at 80 ug, Fig. 2 C. Similar results were obtained with the other two isolates, Fig. 2 D and E. However 0.2 ug protein was sufficient to give a reasonable reaction size, in all isolates tested.

Effect of medium on skin test reaction size

Histoplasmin from nine month old culture of NCDC #267 was chosen for this study. Solutions of various concentrations of histoplasmin were tested using asparagine medium, saline, Sam's buffer, and water as solvents. Results are shown in Fig. 3 A,B,C and D. Differences were detected only at high concentrations of histoplasmin.

Discussion

From the previous results it is clear that there are differences in the antigenic activity of the histoplasmins obtained from the various isolates of Histoplasma capsulatum, although morphologically they are identical. Goodman and Larsh (26), showed that an increase in skin test activity parallels the increase of protein content. A good skin test reaction was reported in culture filtrates at two weeks. Whether the increase in skin reaction with age is due to increase of specific antigen or due to increase of non-specific proteins still has to be determined. Whether the four isolates produce the same antigen or a closely

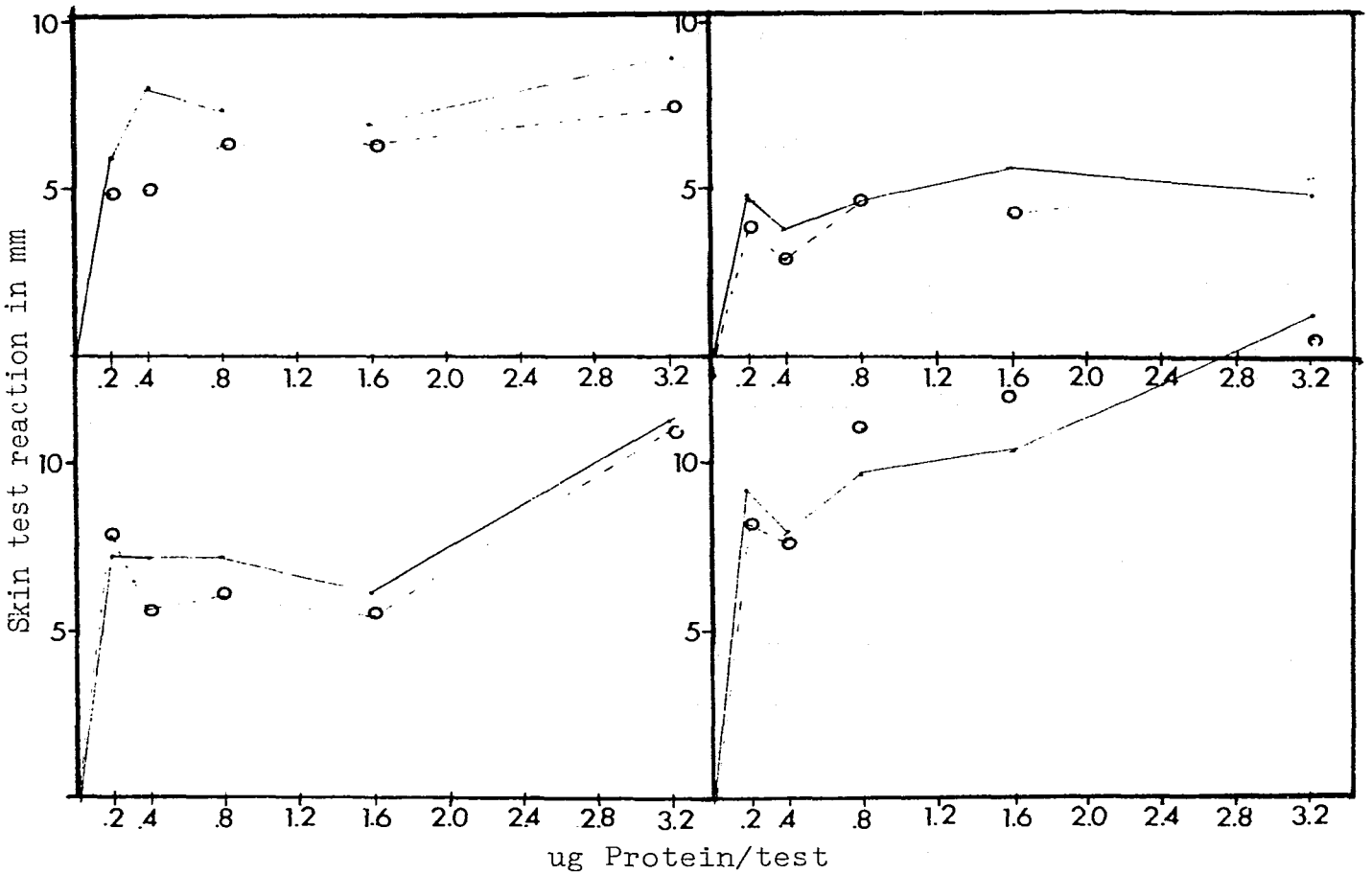


Fig. 3.--Skin test reaction as affected by medium (NCDC #267).
.——. = induration; o- - -o = erythyma
A. Asparagine; B. Saline; C. Sam's buffer; D. Water

related group of antigens is also not known, and it is necessary to specify the isolate used and age of culture filtrate in histoplasmin preparation.

In the second experiment, there was an increase in skin reaction with some isolates, and a positive reaction was detected with 0.2 ug protein. This could lead to the conclusion that the culture filtrate could be harvested after one or two months, when a very small amount of the material is needed and when it is more likely to be free from contamination by non-specific protein.

The skin reaction to histoplasmin in a buffer solution such as Sam's buffer or asparagine medium showed no variation over a wide range of concentration, while water or saline gave increases in skin test reaction with higher concentration of material. At a dose of 0.2 ug of histoplasmin protein all gave a measurable reaction of almost the same size; at low concentration any medium could be used safely.

CHAPTER III

ELECTROPHORETIC PATTERNS OF HISTOPLASMIN PREPARATIONS USING POLYACRYLAMIDE GEL

The comparative disc gel electrophoresis technique for identification and further characterization of culture filtrate components was used. The technique was introduced by Ornstein et al. (27), and was employed previously in similar studies which demonstrated a high degree of resolution. Recent application of this technique by Shechter et al. (28) proved to be useful in the comparative study of dermatophytes.

Material and Methods

Preparation of the culture filtrate protein

Histoplasmin obtained from the four isolates mentioned previously was used. In addition culture filtrates from several other genera of fungi, Aspergillus, Penicillium, Beauveria and Spicaria, were collected and subsequently compared with those from Histoplasma isolates. The age of cultures of these fungi ranged from 1-12 months. Every culture was grown in asparagine medium as described

previously. The culture filtrate was dialyzed against repeated changes of 100 volumes of distilled water for a period of 96 hours at 4°C. The dialyzed material was next lyophilized and finally redissolved in 40 ul of distilled water. The protein content of each sample was determined by the Lowry method (22). Twenty ul of the concentrated histoplasmin corresponding to 20 ml of original filtrate, and 200 ug of protein from each of the other organisms were used in each test.

Procedure

Analytical disc electrophoresis in polyacrylamide gels was performed as described by Buchler instruments manual (29), using 3.5% spacer gel and 7.5% separating gel columns in a tris-glycine, tris-HCl, buffer system at pH 9.3 at a current of 2.5 ma applied for a period of 35 minutes. The gels were then stained with Amido Schwarz for one hour, followed by partial destaining in 7% acetic acid for one hour to yield qualitative protein identification. Duplicate samples were stained with Schiff's reagent and with Sudan black B for determination of carbohydrate and lipid content respectively. A Canalco Model F microdensitometer was used to scan the resultant gel.

Results

A distinctly characteristic electrophoretic pattern for the protein was obtained from each culture filtrate.

Sterile asparagine medium processed in a similar manner along with a blank tube in each run yielded a clear gel after staining. Human serum was included in each run as a reference sample which yielded reproducible patterns with consistent RF values. All tests were conducted in replicate to determine reproducibility for the system. Histoplasmin from Scritchfield and District isolates which had been under refrigeration since 1961 were tested to determine whether changes in the filtrates had occurred due to storage. All samples derived from the same isolate showed a high degree of similarity regardless of storage time.

Semi-quantitative and qualitative differences were seen not only among species but also among isolates. Of primary interest is the variation noted in the filtrate obtained from the same isolate but harvested at different time periods.

Fig. 4 shows the results obtained from the Scritchfield isolate. Only one protein band (RF 0.7) was visible during a 12 month period. The same band also appears with carbohydrate staining. Because this culture filtrate demonstrated skin test activity we can assume that the antigen is a glycoprotein. The Grand Island isolate exhibited one band (RF 0.7) which corresponds to the one in Scritchfield. However, a difference is the presence of several other bands occurring by the eleventh month as shown in Fig. 5, which also includes the results of carbohydrate staining and the

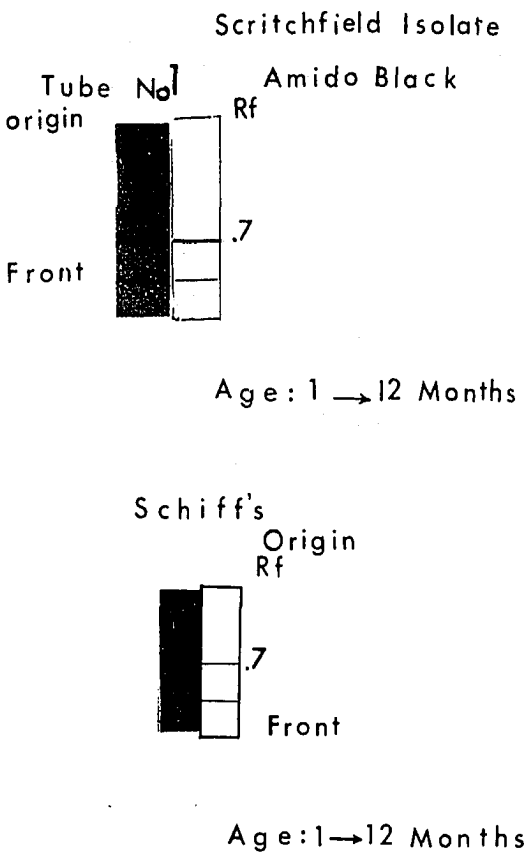
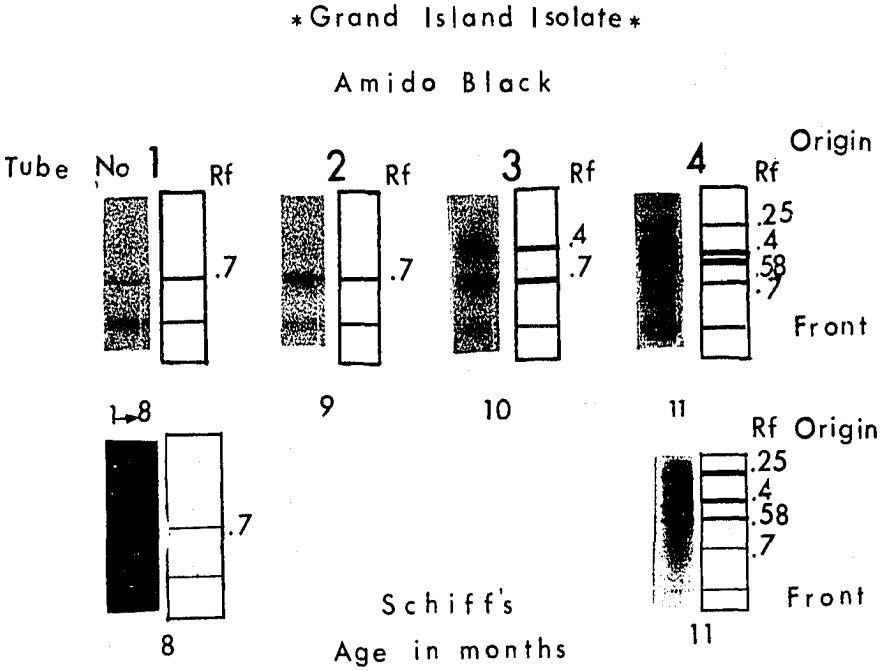


Figure 4. --Histoplasma capsulatum Scritchfield isolate. Electrophoretic patterns in acrylamide gel from culture filtrate.



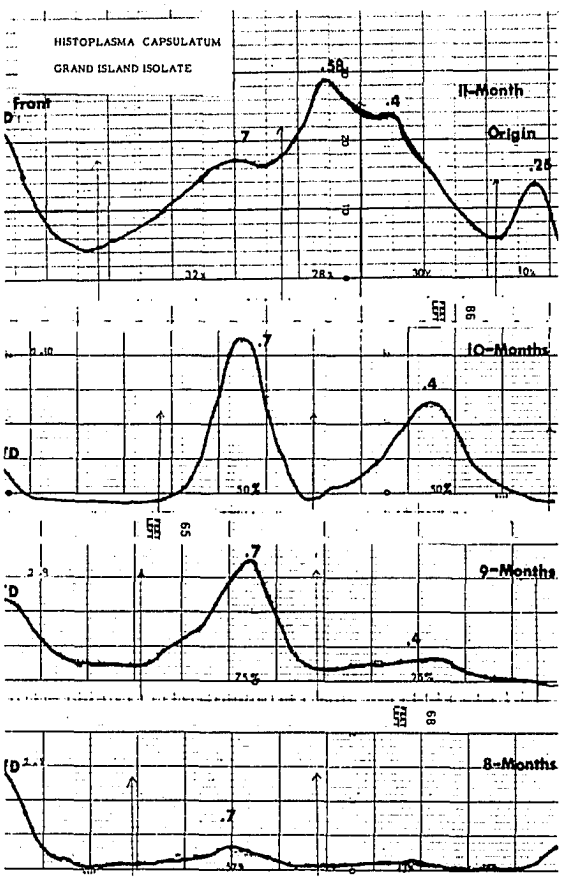
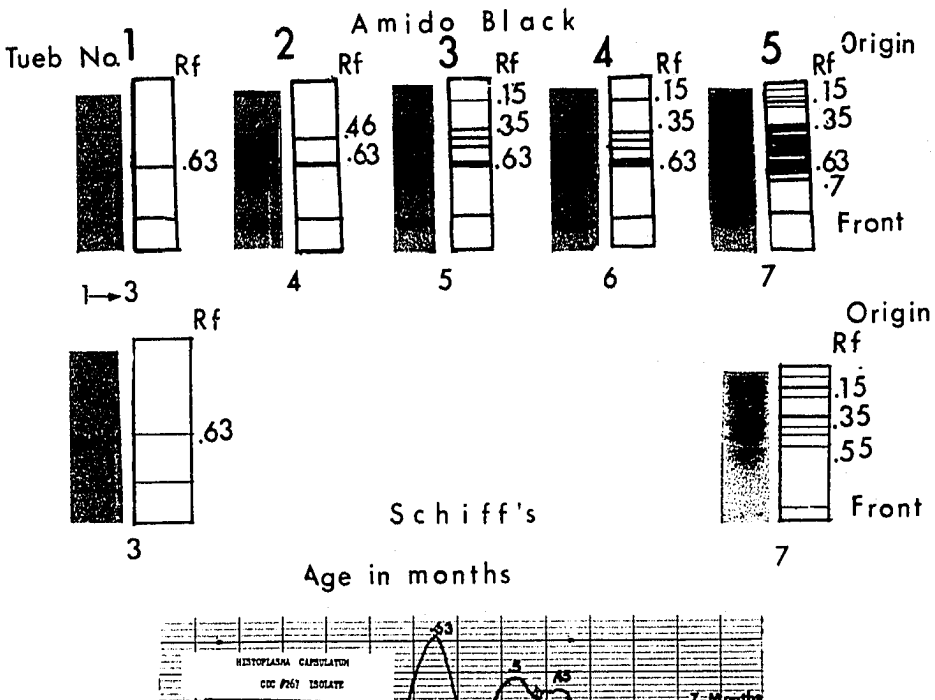


Figure 5. --Histoplasma capsulatum Grand Island isolate. Electrophoretic patterns in acrylamide gel from culture filtrate.

densitometer tracing for four different ages of the culture filtrate. NCDC #267 exhibited a pattern distinctively different from those of the two previous isolates as shown in Fig. 6. The District isolate gave a more abundant growth in culture and subsequently further differences were noted at early culture ages in the electrophoretic pattern of the protein bands (Fig. 7). Fig. 8 shows the characteristic disc electrophoretic pattern as well as the reproducibility of the method for Penicillium 5052. Aspergillus, as did H. capsulatum isolates, exhibited a definite difference among species. The two species used in this study were A. terreus and A. fumigatus. The electrophoretic patterns of the two species of Aspergillus are shown in Fig. 9. Tubes 1, 2, 3 refer to A. terreus and tube 4 to A. fumigatus.

Similarity among the isolates of Beauveria is shown in Fig. 10. Spicaria samples exhibit slight differences as depicted in Fig. 11. None of the samples tested proved positive for lipids, which suggests that no correlation exists between skin test reactivity and the presence of lipid materials. All the other species showed patterns different from those of Histoplasma capsulatum.



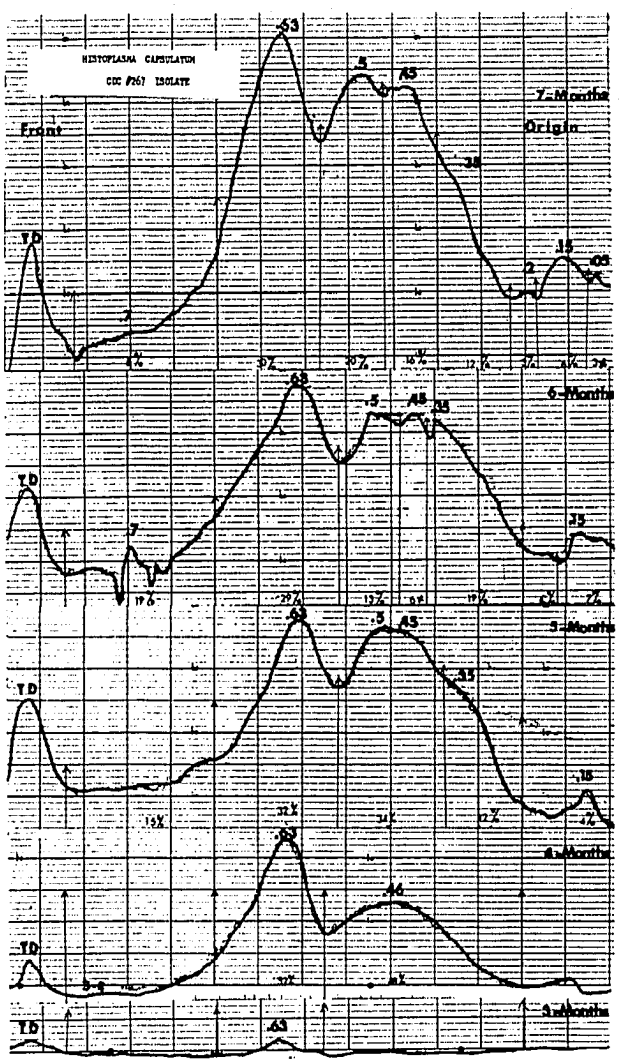
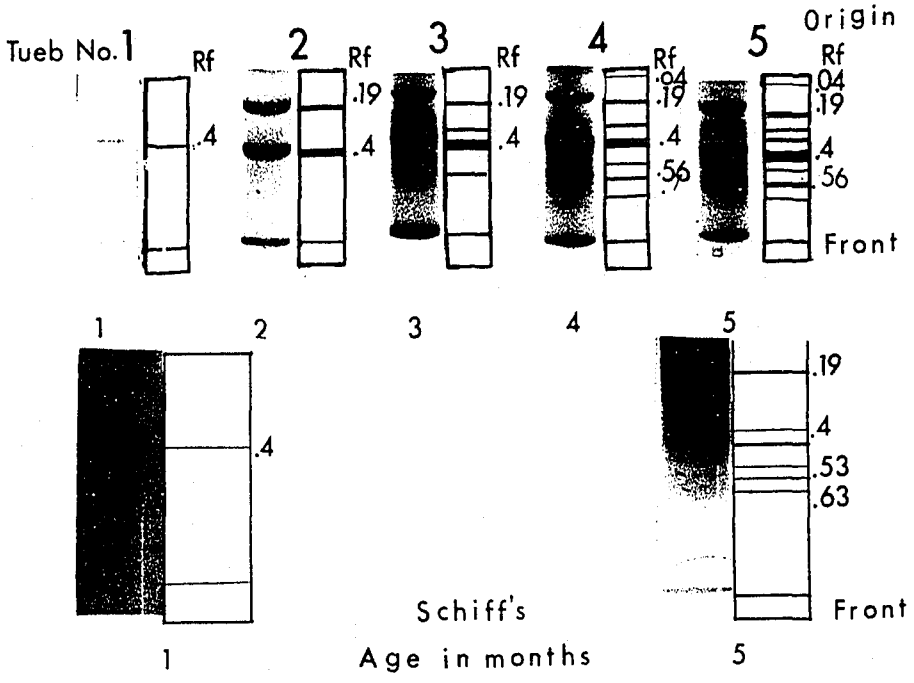


Figure 6. --Histoplasma capsulatum NCDC #267 isolate. Electrophoretic patterns in acrylamide gel from culture filtrate.



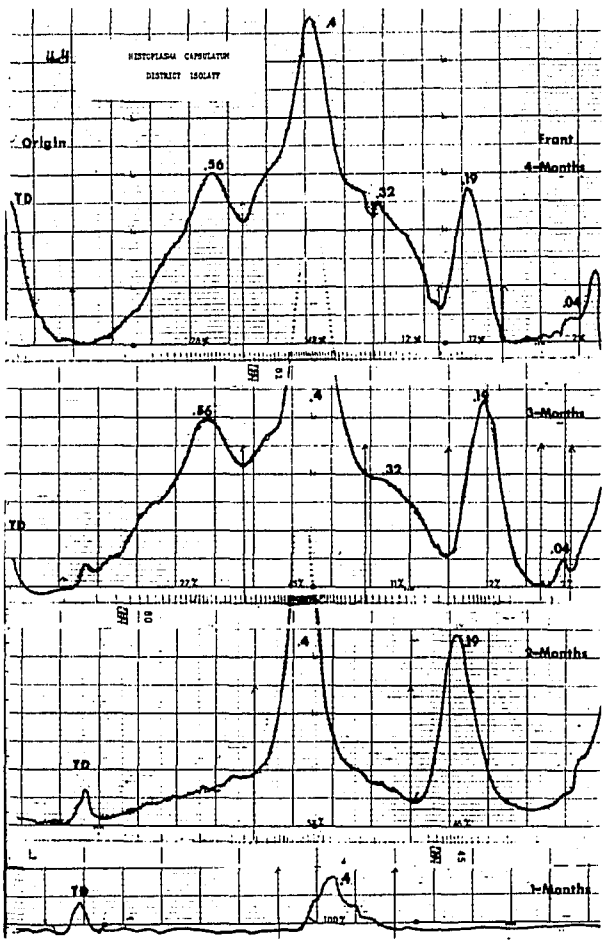
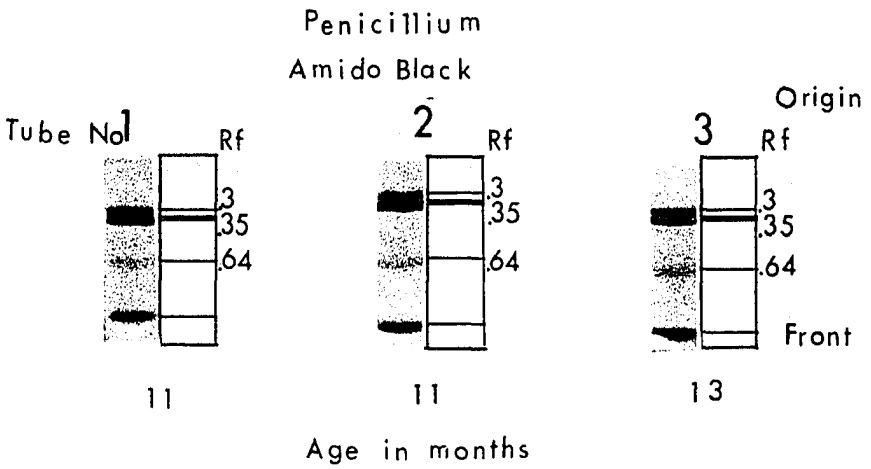


Figure 7.--Histoplasma capsulatum District isolate. Electrophoretic patterns in acrylamide gel from culture filtrate.



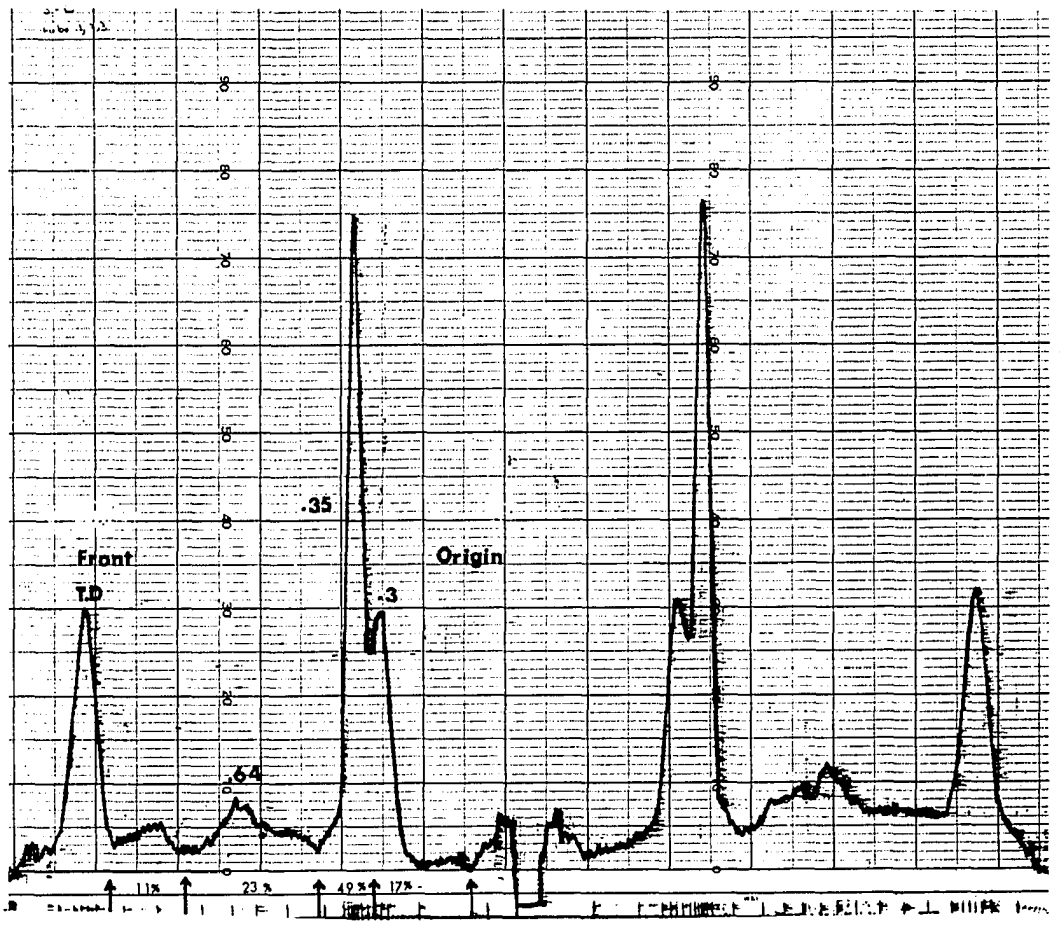


Figure 8. --Penicillium 5052. Electrophoretic patterns in acrylamide gel from culture filtrate.

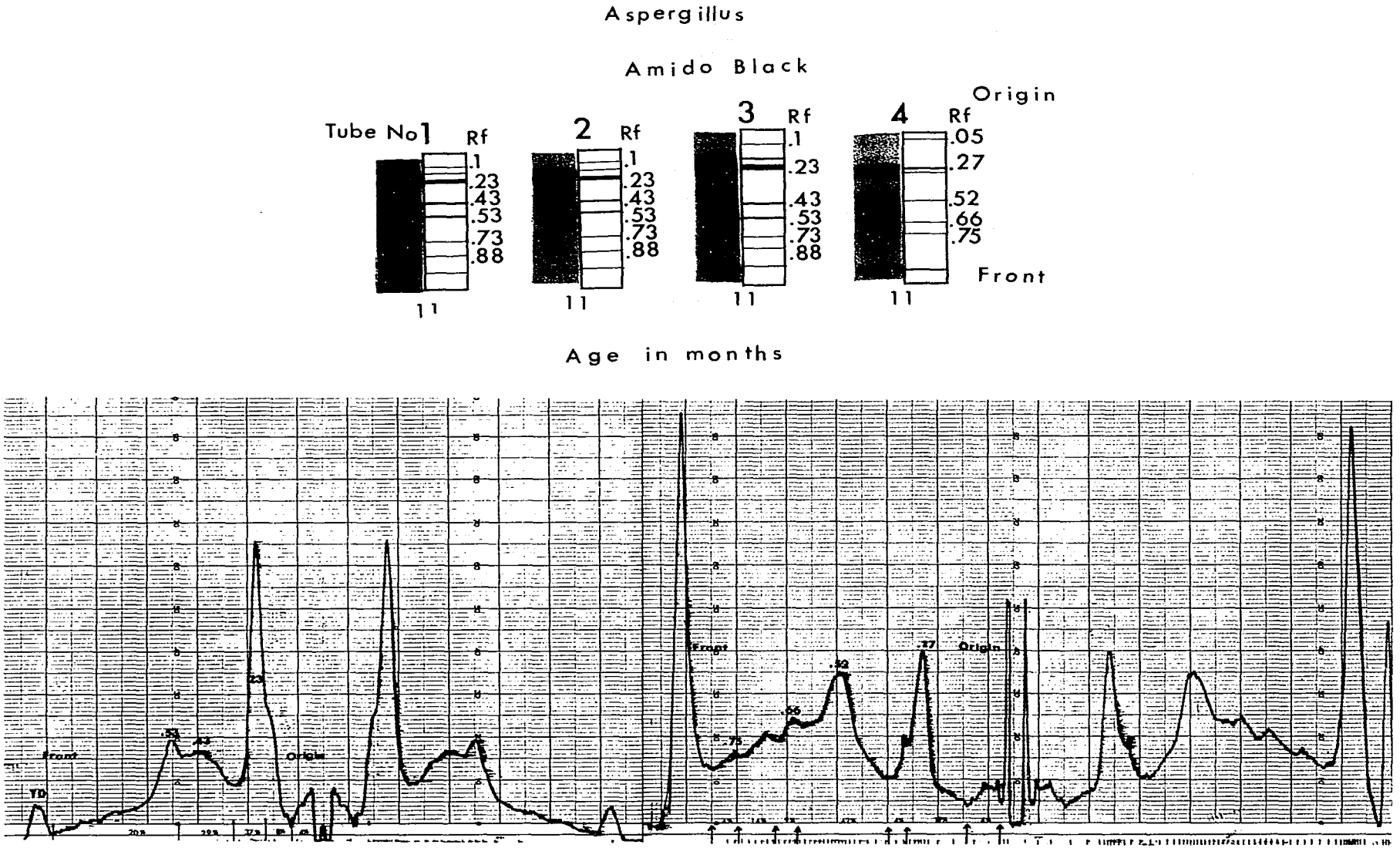
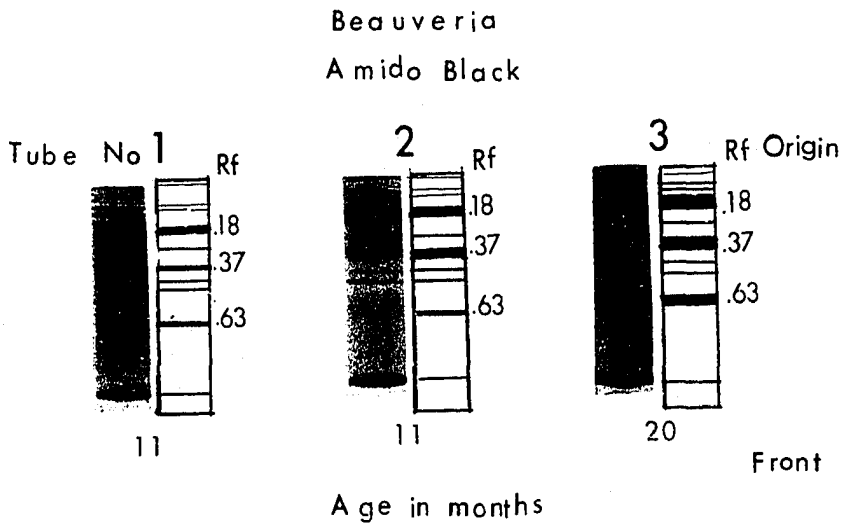


Figure 9.--Aspergillus Electrophoretic patterns in acrylamide gel from culture filtrate.
Tube No. 1, 2, 3 Aspergillus terrus
Tube No. 4 Aspergillus fumigatus



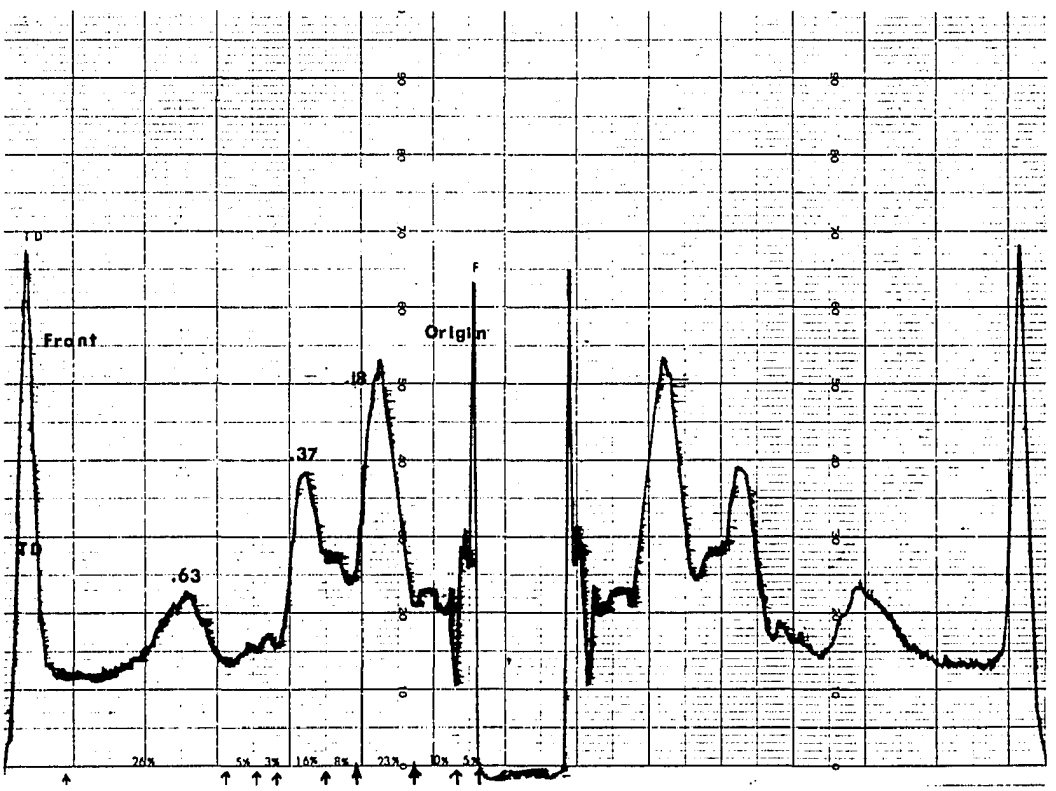
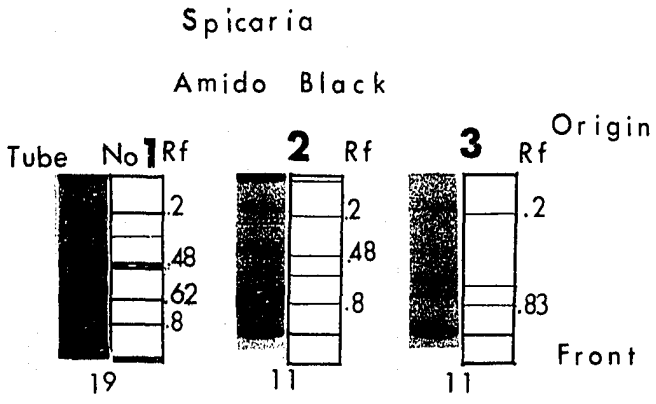


Figure 10--Beauveria Electrophoretic patterns in acrylamide gel from culture filtrate.
Tube No. 1, 3 Beauveria 1460
Tube No. 2 Beauveria 3235



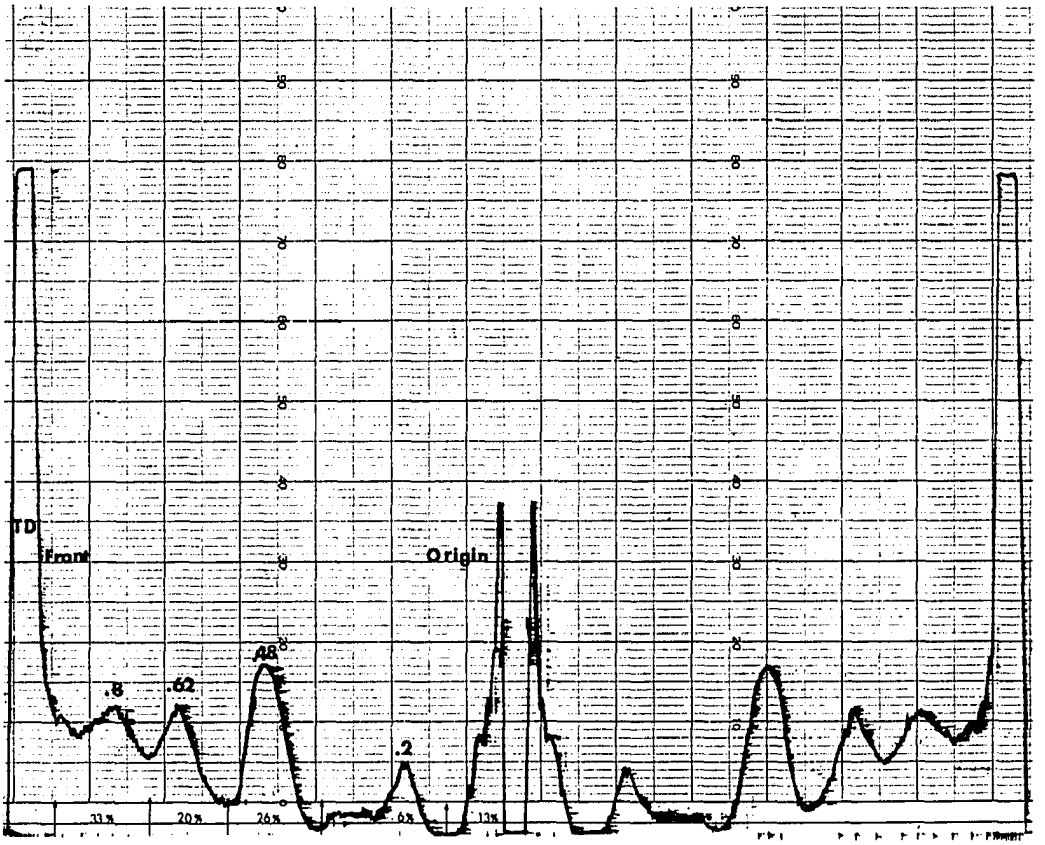


Figure 11--Spicaria Electrophoretic patterns in acrylamide gel from culture filtrate.

Tube No. 1 Spicaria 6296
Tube No. 2 Spicaria 2736
Tube No. 3 Spicaria violacale

Discussion

Comparison of the electrophoretic patterns shows that quantitative and qualitative differences in the proteins exist among species, among isolates as well as among the cultures of different ages. No difference was apparent among samples taken from identical materials which had been stored for varying lengths of time. In samples taken from the early stages of histoplasmin production and stained with Amido Schwarz, each isolate showed one band with a different and characteristic RF value. Therefore, differences in the composition of culture filtrates, based on electrophoretic mobilities, do exist even in the early stages.

Schiff's reagent stained the same band as did Amido Schwarz, suggesting that carbohydrate may exist bound with the protein as glycoprotein. No staining with Sudan Black was found suggesting that there is no lipoprotein in the filtrate.

Culture filtrates from all four isolates demonstrated skin test activity even as early as one month, and showed at least one glycoprotein band, even though this band had different electrophoretic mobility in the various isolates. At later stages of incubation, several protein components became easily recognized but the potency and specificity of these individual components is still to be determined.

Previous study has demonstrated an increase in skin test reactivity parallel to the increase in total protein

(26). The final evaluation of this fact requires a detailed investigation of the different protein fractions present in the filtrate.

CHAPTER IV

FRACTIONATION AND ISOLATION OF SKIN TEST COMPONENTS OF HISTOPLASMIN BY PREPARATIVE DISC ELECTROPHORESIS COLUMN

Qualitative gel electrophoresis showed several different components as well as characteristic bands for the different isolates, therefore it was adapted for quantitative separation of the different components. The technique is fairly new, and has been used only a few times (30,31,32).

Material and Methods

Column

Poly-acrylamide preparative gel column 10 cm (Canalco model P D 2/70), as shown in Fig. 12 was used.

Reagents for Prep Disc Electrophoresis

Stock solutions were mixed and working solutions freshly made for every column as described by Canalco manual.

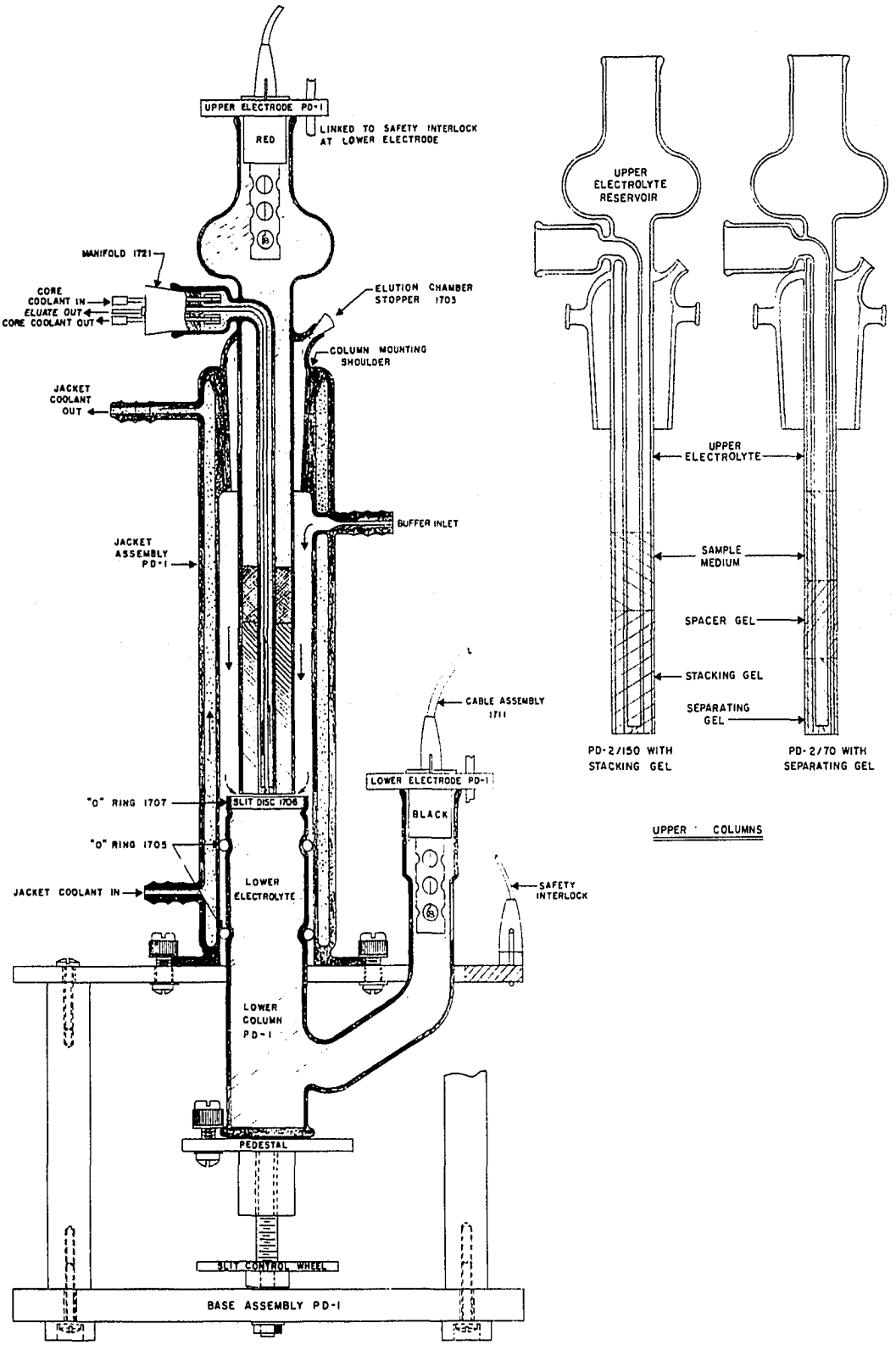


Figure 12--Prep-Disc electrophoresis. column.

Procedure

The method used was that described by the Canalco instruction manual (33). Current regulated power supply was used to maintain steady progress of the sample as the resistance changed in the column. A current of 10 ma was used for each column. Elution time varied between 3 and 4 hours; peaks were detected by absorption at 254 mu using a monitor (Canalco model D). The dialyzed, lyophilized sample was dissolved in 1 ml of 40% sucrose and introduced to the column by layering it on the top of the gel using a long needle. Fractions were collected with the aid of an automatic fraction collector using timed flow. Elution buffer flow was regulated at 3 ml/min. Consecutive aliquots with peaks were pooled.

The pooled aliquots were dialyzed against distilled water and dried under vacuum, then redissolved in sufficient distilled water to bring the samples to the original volume of the culture filtrate taken. Similar samples containing 200 ug were used for the analytical electrophoresis, processed under identical conditions, and stained with Amido Schwarz. Densitometer tracings were made with a Canalco densitometer model F.

Results

Crude histoplasmins were subjected to fractionation on several kinds of gel: 5%, 7%, and 10% stacking gels as well as 10% separating gel. The 7% stacker gel showed good

separation in a reasonably short time. Table 2 summarizes the size and dilution of histoplasmin samples applied to the columns and amount recovered. Figures 13, 15, 17, 19 show the elution patterns of the different histoplasmin preparations mentioned in Table 2, as well as the pattern on the analytical gel and the densitometer tracing of the gel. The arrows indicate the pooled fractions; the numbers show the pool number as well as the corresponding band in the analytical column and peak in the densitometer tracing. The automatic shift of the recorder from 1X to 3X depends on the amount of absorption of 254 mu of the specific material. Fig. 13 shows the pattern for Scritchfield isolate at the age of 11 months (HT-1 A-11). Four fractions were separated although on the gel and densitometer only three could be seen, probably because of the poor separation of fraction 3 and 4 on the analytical column or because one of the two fractions was too small to stain. It should be noted that only one band was detected on 7.5% separator gel in Chapter III. Fig. 14 shows the amount of protein, carbohydrate, and the induration response. Only fraction 2 showed the presence of carbohydrate. The four fractions showed almost the same skin test reaction. If we consider that the size of reaction depends on the quantity of protein, fraction 3 would be more potent than the other three fractions. Fig. 15 shows the pattern for Grand Island isolate at the age of 8 months (HT-2 A-9). Here also only three

TABLE 2

FRACTIONATION OF HISTOPLASMIN ON PREPARATIVE GEL ELECTROPHORESIS

Isolate name	Age	Vol. used (ml)	Final vol. (ml)	Total protein (mg)	mg protein/column	mg recovered	% recovery
Scritch-field (HT-1)	11	200	0.1	3.4	3.4	3.34	98.23
Grand-Island (HT-2)	9	200	1.0	10.0	2.0	1.58	79
NCDC #267 (HT-3)	9	100	1.0	16.0	4.0	3.72	93
District (HT-4)	4	100	1.0	11.25	2.25	1.435	63

TABLE 2--Continued

Fraction no.	Total ug prot./pool	Total ug carb./pool	Skin test* ug/test	Skin test		
				Ery. mm.	Ind. mm.	mm/ug
1	1000	0.0	0.5	8.9	6.7	13.4
2	1140	160.0	0.57	8.8	6.5	11.4
3	340	0.0	0.11	8.5	6.9	40.6
4	860	0.0	0.43	9.8	6.4	14.9
1	365	30.0	0.93	2.38	2.38	2.6
2	765	82.5	1.91	4.46	3.9	2.06
3	150	42.5	0.37	6.46	6.23	16.6
4	50	12.5	0.125	0.93	0.69	5.6
5	250	62.5	0.65	2.9	2.5	4.0
1	76	0.0	0.38	7.83	5.1	13.4
2	76	0.0	0.38	7.1	4.66	12.3
3	740	40.0	3.7	7.28	5.0	13.5
4	2740	280.0	13.7	12.66	8.16	0.6
1	185	345.0	0.925	2.1	2.2	2.4
2	250	25.0	1.25	1.4	1.5	1.2
3	650	180.0	3.25	16.6	13.8	4.3
4	185	125.0	0.93	12.0	11.0	11.8
5	100	60.0	0.5	10.6	9.6	19.2
6	65	50.0	0.32	4.1	4.3	13.4

*For skin test the volume was restored to its original volume.

bands were clearly seen on the analytical gels, and four were separated on the preparative column, but if we consider the relative amount of protein in each fraction, it is obvious that fraction 4 has too small quantity to show with the stain. Fig. 16 shows the amount of protein, carbohydrate, and size of induration. Fraction 2 has the highest quantity of both protein and carbohydrate but the highest skin test reaction was given by fraction 3, suggesting the relative specificity of this fraction. NCDC #267 isolate at 9 months (HT-3 A-9) showed poorer separation due to the large number of protein components (Fig. 17). Fraction 4 (Fig. 18) showed the largest quantity of protein and carbohydrate. It also showed a higher reaction, but if we consider the quantity of protein injected, fraction 4 will have the lowest activity. District isolate 4 month old (HT-4 A-4) showed a high degree of resolution as shown in the pattern in Fig. 19. The quantitative analysis of the components (Fig. 20) showed that fraction 3 is the highest in proteins while fraction 1 has the highest amount of carbohydrates. Fractions 3, 4, 5 showed a reasonable skin test reaction, but again considering the amount of proteins fraction 5 has the highest activity.

Discussion

Polyacrylamide gel showed a satisfactory resolution of the components of different kinds of histoplasmin preparations. Preparative electrophoresis proved to be promising.

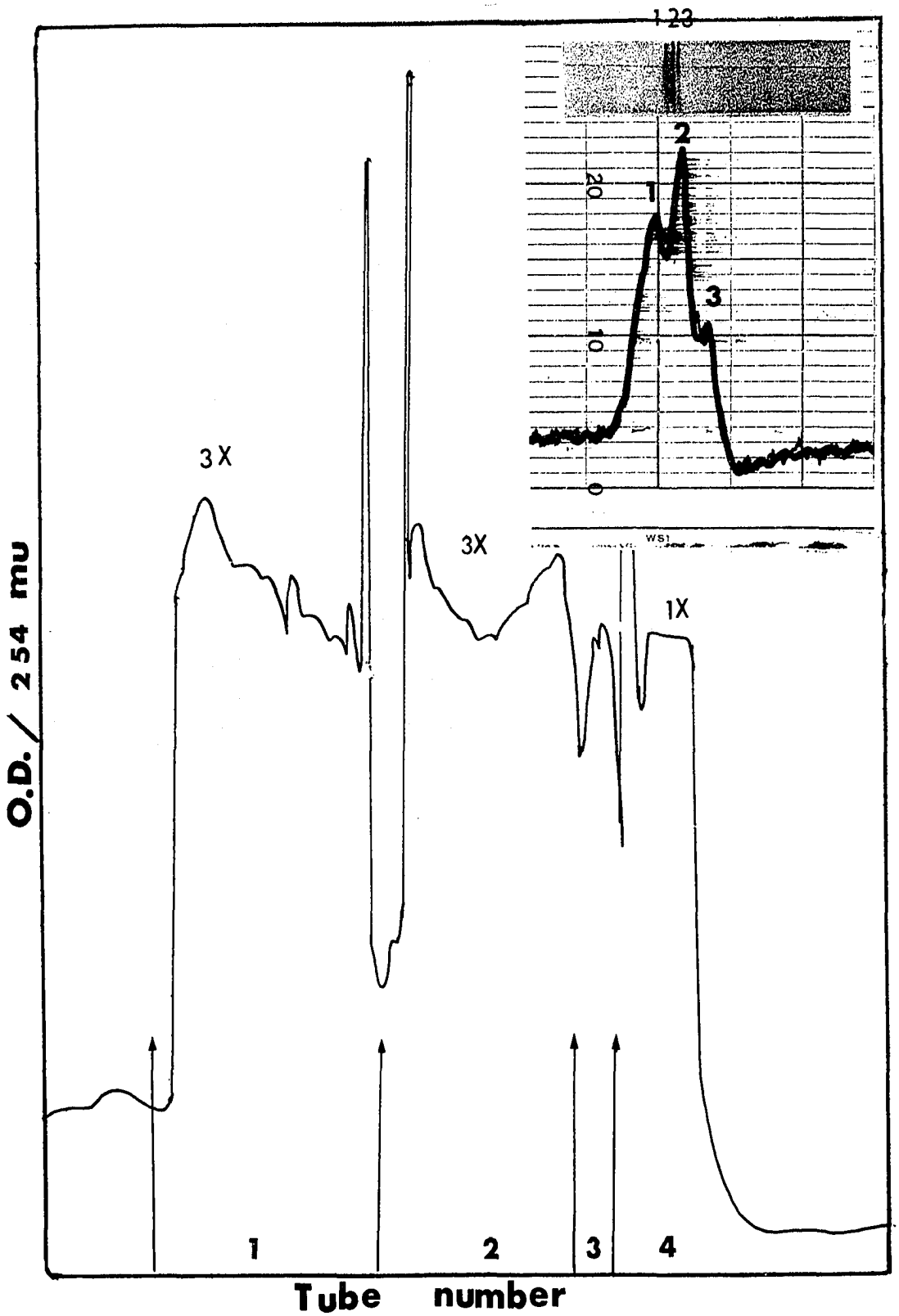


Fig. 13.--Electrophoretic elution pattern of histoplasmin (Scritchfield isolate HT-1 A-11) on 7% stacker acrylamide gel. Left: Elution pattern from preparative column. Upper right: Analytical gel pattern and densitometer tracing.

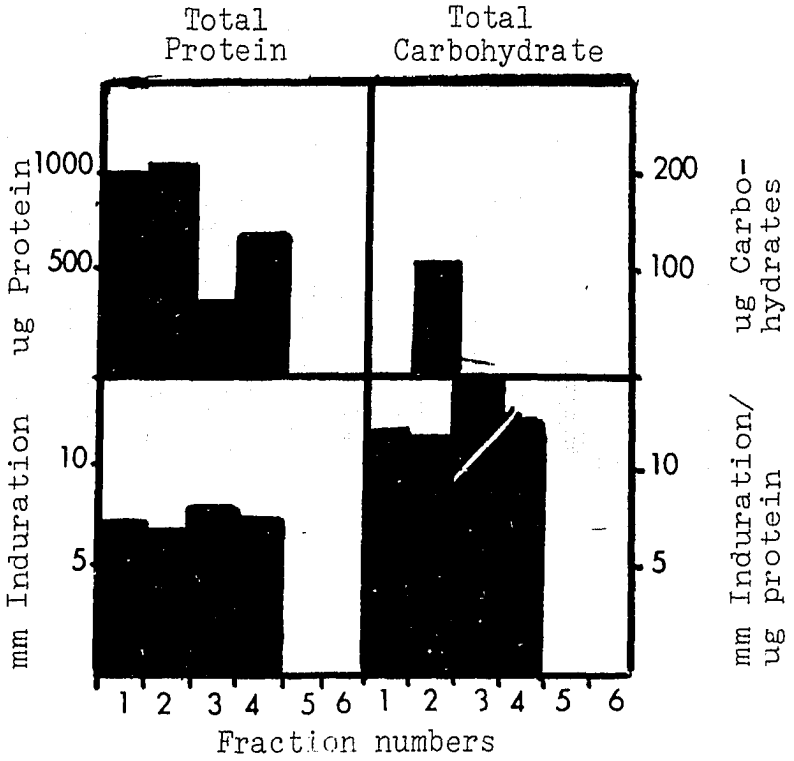


Fig. 14.--Chemical characterization of histoplasmin (Scritchfield isolate HT-1 A-11) fractions.

A. Total protein
B. Total carbohydrate
C. mm induration
D. mm induration/ug protein.

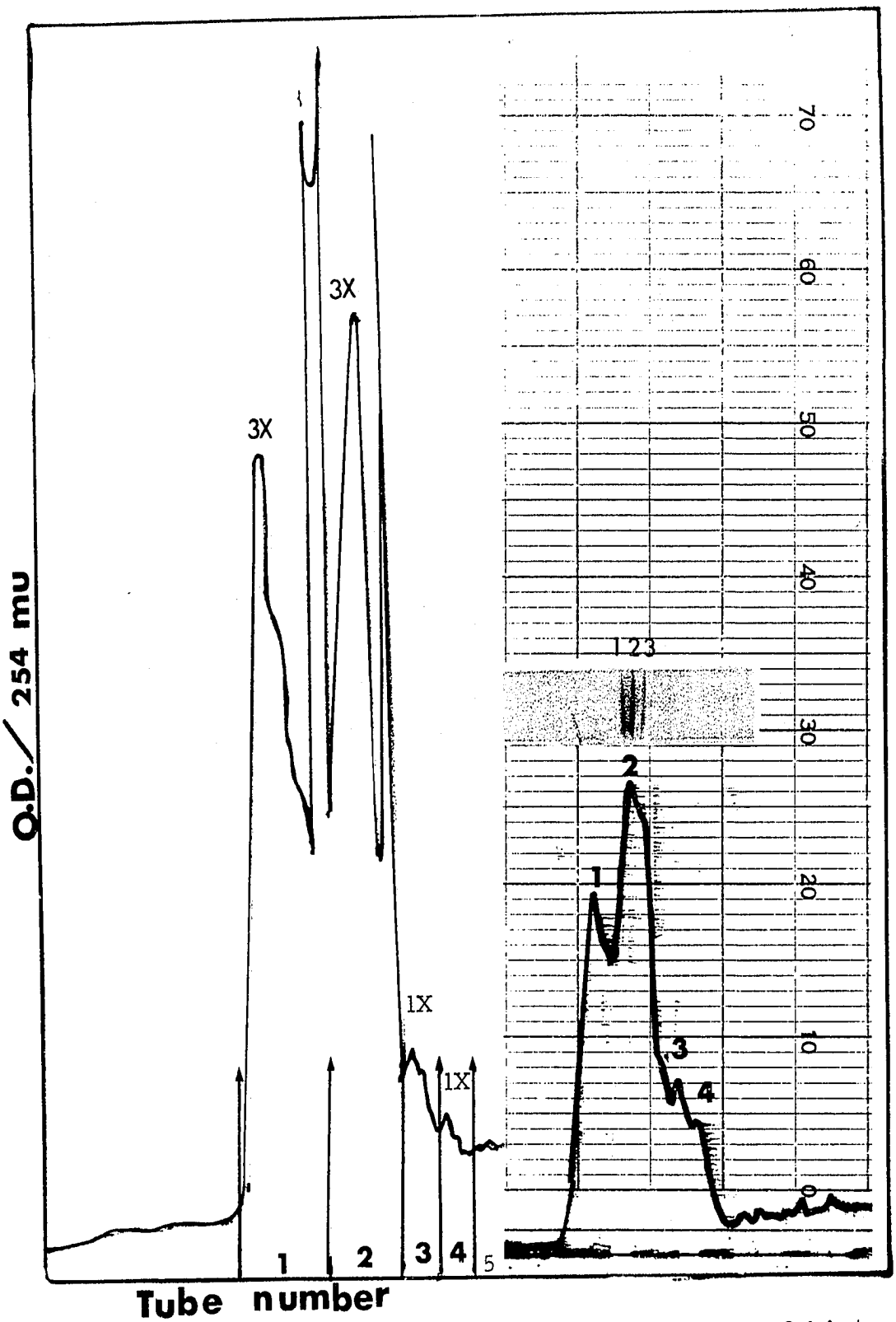


Fig. 15.--Electrophoretic elution pattern of histoplasmin (Grand Island isolate HT-2 A-9) on 7% stacker acrylamide gel. Left: Elution pattern from preparative column. Right: Analytical gel pattern and densitometer tracing.

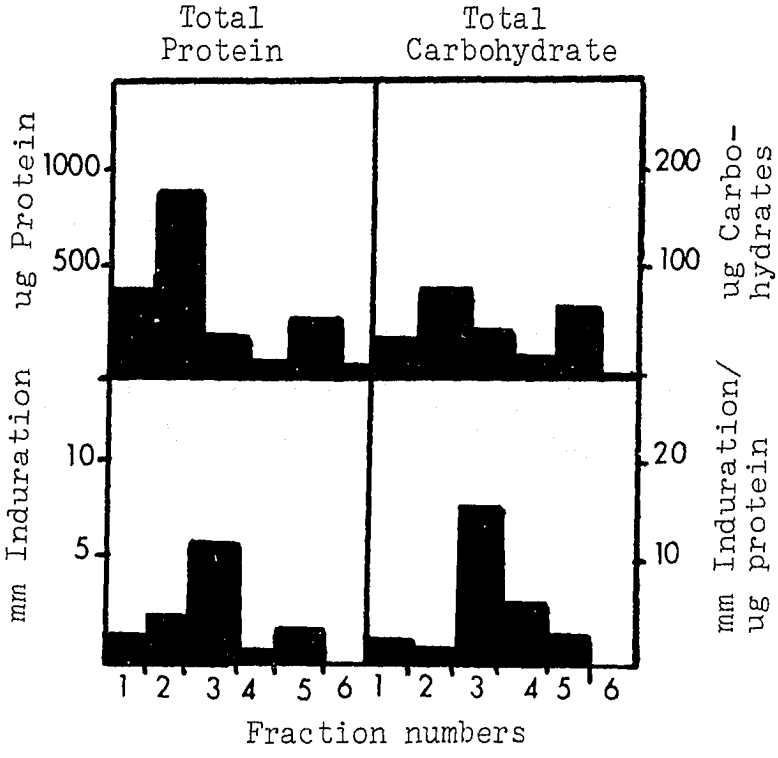


Fig. 16.--Chemical characterization of histoplasmin (Grand Island isolate HT-2 A-9) fractions.

A. Total protein
B. Total carbohydrate
C. mm induration
D. mm induration/ug protein.

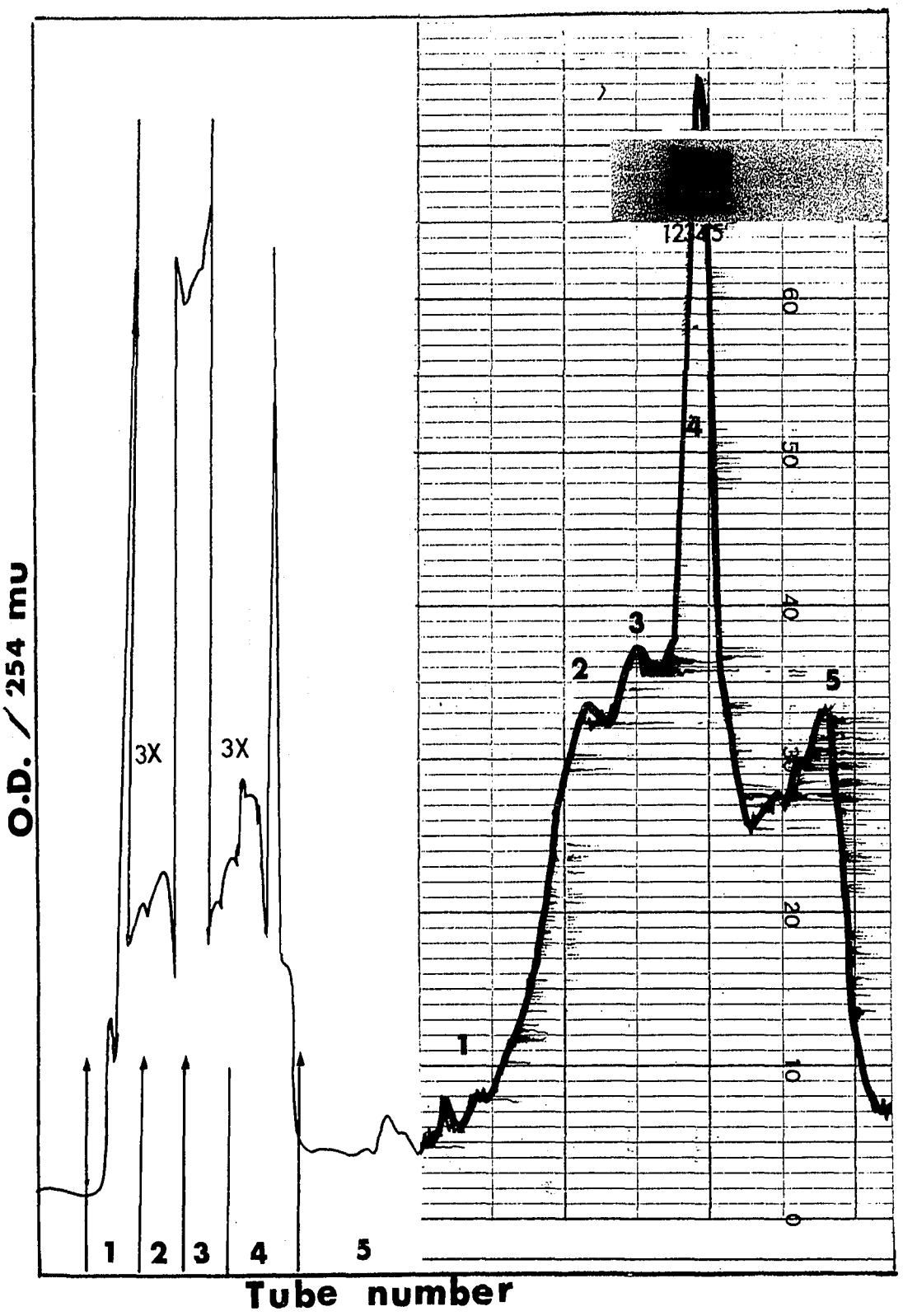


Fig. 17.--Electrophoretic elution pattern of histoplasmin (NCDC #267 isolate HT-3 A-9) on 7% stacker acrylamide gel. Left: Elution pattern from preparative column. Right: Analytical gel pattern and densitometer tracing.

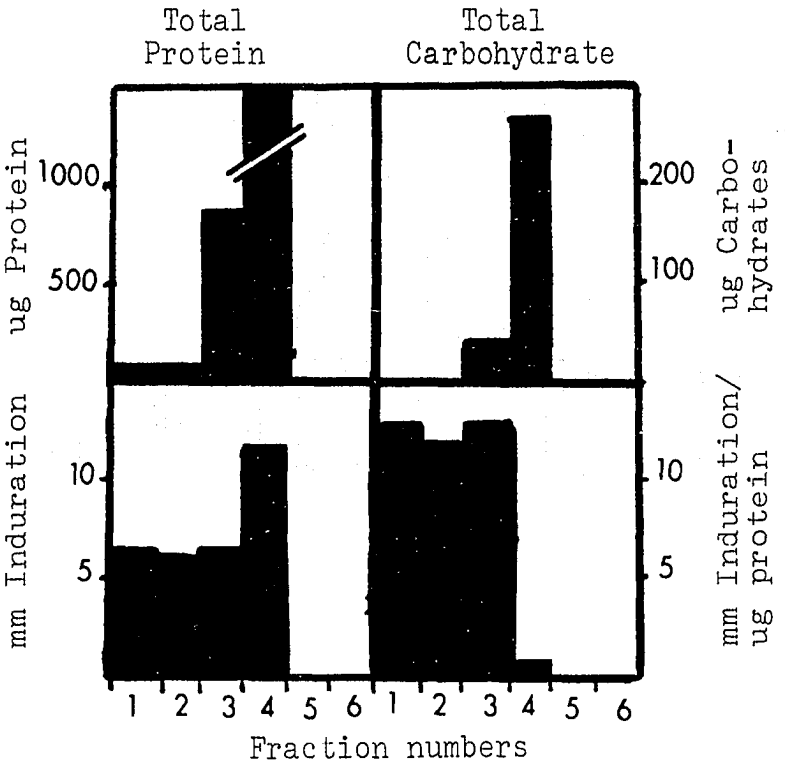


Fig. 18.--Chemical characterization of histoplasmin (NCDC #267 isolate HT-3 A-9) fractions.

A. Total protein
B. Total carbohydrate
C. mm induration
D. mm induration/ug protein

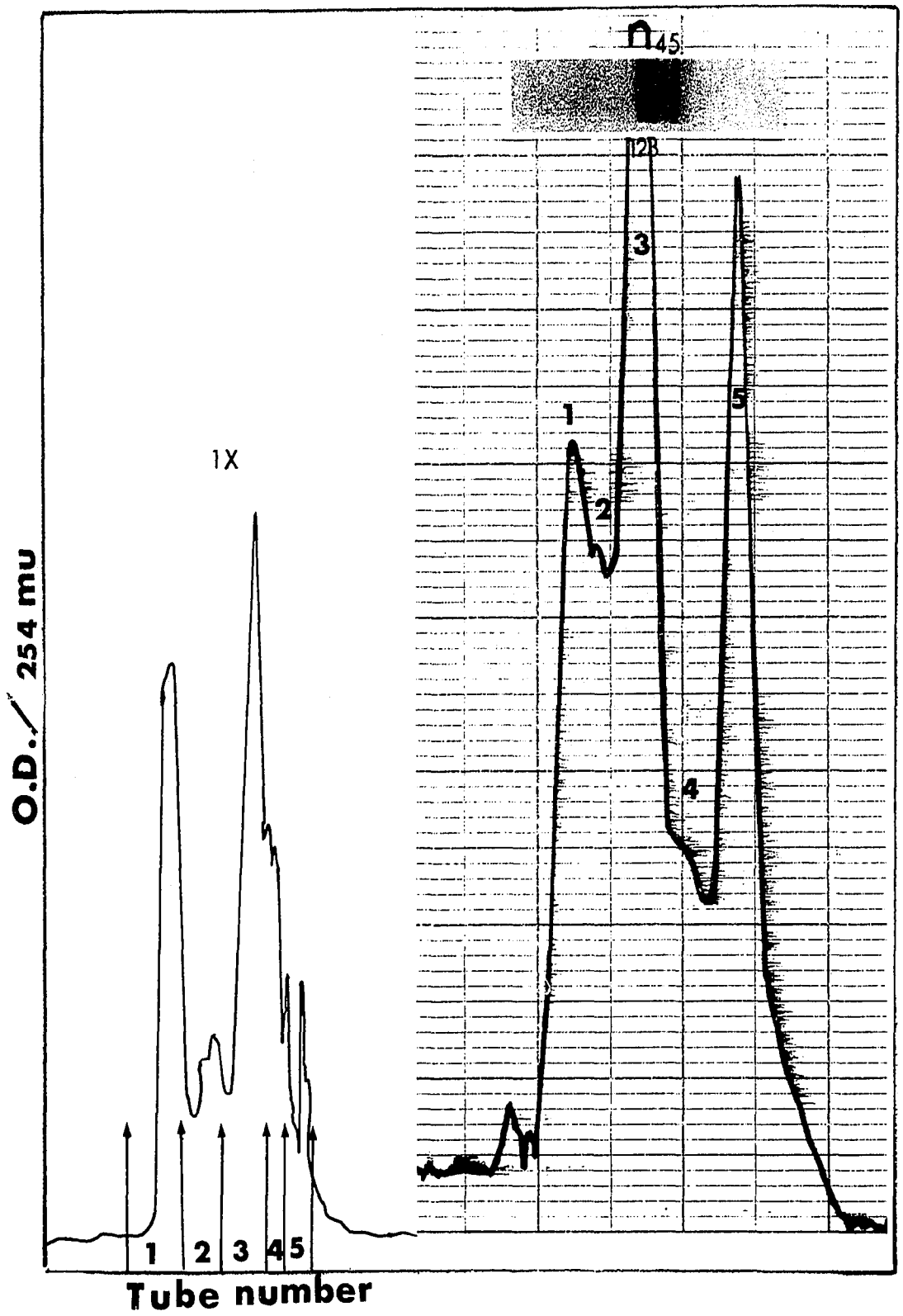


Fig. 19.--Electrophoretic elution pattern of histoplasmin (District isolate HT-4 A-4) on 7% stacker acrylamide gel. Left: Elution pattern from preparative column. Right: Analytical gel pattern and densitometer tracing.

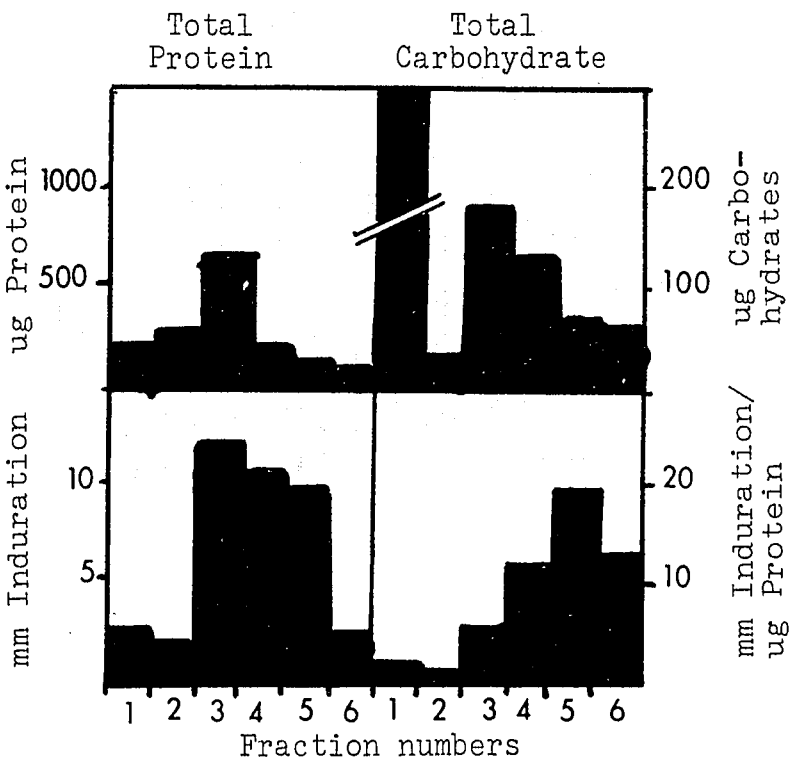


Fig. 20.--Chemical characterization of histoplasmin (District isolate HT-4 A-4) fractions.

A. Total protein
B. Total carbohydrate
C. mm induration
D. mm induration/ug protein.

The use of 7% stacker gels showed to be excellent for fractionation of the crude histoplasmin giving fast clear peaks in most cases with no loss in activity. A considerable purification step is achieved through this process which can be used for further studies. The use of 7.5% separatory gel in the analytical runs gave a good indication of the differences between the isolates as well as the ages of the same isolate. Use of 7.5% separatory gel on the preparative columns required a long time, making the procedure a slow process. Use of 7% stacker gel on preparative columns required between three and four hours for good separations depending on the type of culture filtrate. The elution buffer contains a very low concentration of salts which makes it easier to handle and requires shorter dialysis time. One should note the different patterns between stacker and separator columns. The method is extremely sensitive and variations in pattern occur with slight changes in conditions such as pH, current, polymerization time and length of column. The use of two different criteria, absorbance at 254 mu in the column, and staining with Amido Schwarz in the analytical runs showed a discrepancy in the size of peaks in some cases, but not all. For example, HT-1 shows high peaks in the elution but not in the analytical gel.

The fractions showed variation in everything, amount of protein, carbohydrate, and activity. The skin reaction does not seem to be related to the amount of carbohydrate,

as shown in HT-4, HT-2, and HT-1. In all of these the highest peaks of carbohydrates do not correspond to the maximum reaction. Activity also does not seem to be correlated with the quantity of proteins as shown by HT-1, although it does in HT-4, HT-3. It seems that some fractions are more specific than others. Whether the size of the reaction of one protein is affected by the quantity of the total protein needs to be clarified. In previous studies with the crude histoplasmin a slight increase in activity was observed which was not linear with the quantity of protein used. However, these crude materials are now clearly a combination of specific, nonspecific, and nonreactive proteins which all contribute to the erythema and induration reactions.

CHAPTER V

CHARACTERIZATION OF HISTOPLASMIN H-42

Histoplasmin H-42 is now the product most widely used for skin testing. However the supply is limited and a new preparation will be needed. H-42 was selected after testing several proposed lots. It was chosen by the United States Public Health Service because of its high potency. H-42 was chosen in this study as an example of histoplasmin. The analyses previously described and more detailed studies were conducted on this culture filtrate, hoping to get a better understanding of histoplasmin antigen for comparison with future preparations. A 50 ml sample of concentrated H-42 was supplied by the United States Public Service. The limited supply made it difficult to obtain a complete characterization.

Material and Methods

Protein and carbohydrate were estimated on the crude filtrate, dialyzed material and fractions. Nitrogen was estimated using the microdetermination of Kjeldahl (34). Analytical electrophoresis was done on 7.5% separator and

7% stacker gels, as described previously. Preparative electrophoresis was also carried out on 10% separator gels corresponding to 7.5% separator on the analytical gels and 7% stackers. Preparative columns of 7% stacker were made exactly as described in Chapter IV. The 10% separator columns were 5 mm long. Tris buffer was used in anode and cathode vessels with a current of 3.5 ma per column. Elution fractions were collected in tubes every minute at a flow rate of 2 ml/min. Fractions corresponding to each peak were pooled, dialyzed against water, lyophilized and tested on 7.5% analytical columns.

Acid hydrolysis for amino acids (35)

Heavy walled Pyrex tubes washed several times with acid and stored in an evacuated desiccator were used. Lyophilized proteins were suspended in 5.6 N double glass distilled HCl. The tubes were frozen, evacuated to a pressure of 50 microns, the solutions were thawed until vacuum was 60 microns, the tubes were immersed again in the freezing solution, letting the air bubbles out. This process was used to degas the hydrolysis mixture. The tubes were shaken gently during the freezing and thawing. The tubes were sealed at 50 microns. The hydrolysis was conducted at 110 ± 1°C for 24 hours. After the tubes had cooled to room temperature liquid on the sides was spun down by gentle centrifigation. HCl was removed on the rotary evaporator,

the residue redissolved in water and evaporated to dryness three times.

Acid hydrolysis of carbohydrates (36)

Dry samples of histoplasmin and its fractions were hydrolyzed for 20 hours in 0.25N H_2SO_4 in a sealed tube using a process similar to that described for amino acids. The hydrolysis was conducted at 105 ± 1°C. After hydrolysis the solutions were neutralized with $BaCO_3$ to pH 7, using pH paper. The precipitate was removed by centrifugation. Supernatant was decanted, evaporated in the rotary evaporator, and redissolved in water. Amino acids, peptides and proteins were removed by passing the solution through columns of Dowex-50-H^+ (5 mm x 5 cm). The columns were washed with 1 ml of water, and the washing was added to the eluate, which was evaporated to dryness and redissolved in 20 ul of water.

N-terminal amino acid residues

Reaction with dinitrofluorobenzene (DNFB).--The procedure of Levy and Li (37), and Sanger (38) was used. The dry sample was dissolved in 0.05N aqueous KOH at 40°C., and the pH was adjusted to 8 (addition of 0.05N KOH). DNFB was added and the mixture was stirred vigorously in darkness, while the pH and temperature were kept constant by addition of KOH in an incubator. The reaction was terminated when no further base was consumed. The solution was extracted

three times with ether and acidified. The precipitated DNP-compounds were centrifuged, washed with water, acetone, and ether, then dried over phosphorus pentoxide.

Total hydrolysis of dinitrophenyl protein (38).--The DNP-protein was hydrolyzed with the 100 fold quantity of double distilled 5.7N HCl at 105 ± 1°C for 16 hours (in a sealed tube under vacuum). The hydrolysate was diluted to a HCl concentration of about 1N. The hydrolysate was extracted three times with peroxide free ether.

C-terminal amino acid residues (39,40,41)

Hog pancreas carboxypeptidase B, a Sigma product already treated with DFP (diisopropylphosphofluoride) was used as an aqueous suspension containing 7.6 mg protein/ml and an activity of 116 umole Hippuryl-L arginine/min. The enzymatic reaction was carried out at 25°C in 0.2 M phosphate at pH 7. The reaction was terminated by the addition of 1N HCl at various intervals of time. The mixture was then passed through Dowex 50 H^+ column (5 mm x 10 cm), which had been washed with water to remove residual proteins and peptides. The free amino acids were eluted with 5 M NH_3, dried in evacuated desiccator over H_2SO_4, redissolved in water and identified by thin layer chromatography.

Qualitative estimation of amino acids

Silica gel was used for two dimensional thin layer chromatography, with n-butanol/glacial acetic acid/water 80/20/20 by volume as the first solvent and phenol/water 70/30 by volume as the second solvent. Ninhydrin (0.3 g ninhydrin + 100 ml n-butanol + 3 ml glacial acetic acid) was used to detect amino acids.

Qualitative estimation of carbohydrate

A. Descending paper chromatography.--Whatman No. 1 filter paper was developed in ethyl acetate-pyridine-water (10/5/6) by volume and sprayed with benzidine (0.5 gm benzidine, 10 ml glacial acetic acid, 10 ml of 40% trichloacetic acid, 80 ml ethanol).

B. Ascending chromatography.--The chromatograms were developed in isopropanol/acetic acid/water (3/1/1) and sprayed with aniline oxalate reagent.

Qualitative estimation of DNP-derivatives

Silica gel was used for ascending thin layer chromatography and 22 amino acids derivatives prepared as described by Chung (37) were included as standards. Benzene/pyridine/glacial acetic acid (80/20/20) and chloroform/methanol/glacial acetic acid (95/5/1) were used for the ether soluble derivatives as described by Brener et al. (42). Identification of water soluble DNP-derivatives was done on silica gel

developed in n-propanol/34% ammonium hydroxide (7/3) (43).

Quantitative estimation of amino acid

A Beckman/Spinco Amino Acid Analyser Model 120 was used. 100 ug (A-2) sample hydrolysate, dissolved in 0.5 cc of buffer and 10 ug of internal standard was included.

Results

General chemical characterization of H-42 histoplasmin

H-42 was dialyzed against water (100 fold), lyophilized and was subjected to various chemical determinations as shown in Table 3.

Electrophoretic patterns were obtained on 7.5% separator gel. Because of the very low concentration of proteins in the nondialyzed filtrate, electrophoresis was omitted. On staining with Amido Schwarz five bands could be demonstrated, two of them were sharp (RF 0.7 and RF 0.38). The band with RF 0.2 was fairly sharp but those with RF 0.05 and 0.64 were very light. Schiff's reagent gave bands with RF 0.7 and 0.38. None of the bands stained with Sudan Black.

Separation on preparative columns

Two types of acrylamide gel were used, 7% stacker and 10% separator. In the 7% stacker gel all proteins migrate mainly according to their charge regardless of molecular weight. In this gel a thin layer of concentrated protein is formed, which runs rapidly through the gel without

TABLE 3

CHEMICAL ANALYSIS OF HISTOPLASMIN H-42

	Sample	
	H-42 Nondialyzed	H-42 Dialyzed
Total protein/ug/ml (Lowry)	850	350
Total carb./ug/ml (anthrone)	1450	290
Total carb./ug/ml (phenol)	1650	340
Reducing sugar/ug/ml	600	100
N ug/ml	2500	130
Total protein/ug/ml estimated from N	--	825
RF disc gel electrophoresis	--	0.05 0.2 0.38 0.64 0.7
Skin test*		
Erythema	16	11
Induration	10	8

*0.1 ml of 1:25 dilution is usually used in the animal test and 0.1 ml of 1:100 in human.

spreading, the large molecules running just as fast as the small ones of like charge. The disadvantage of this column is that most substances run very close with a poor separation. The advantage is that it is fast, and the elution buffer required is very dilute and easy to eliminate. An elution pattern on a 840 ug sample corresponding to 2.4 ml of the concentrated H-42 or 60 ml of 1:25 H-42 is represented in Fig. 21. Pool 1 showed the highest peak with highest absorption at 254 mu. The pools were tested for

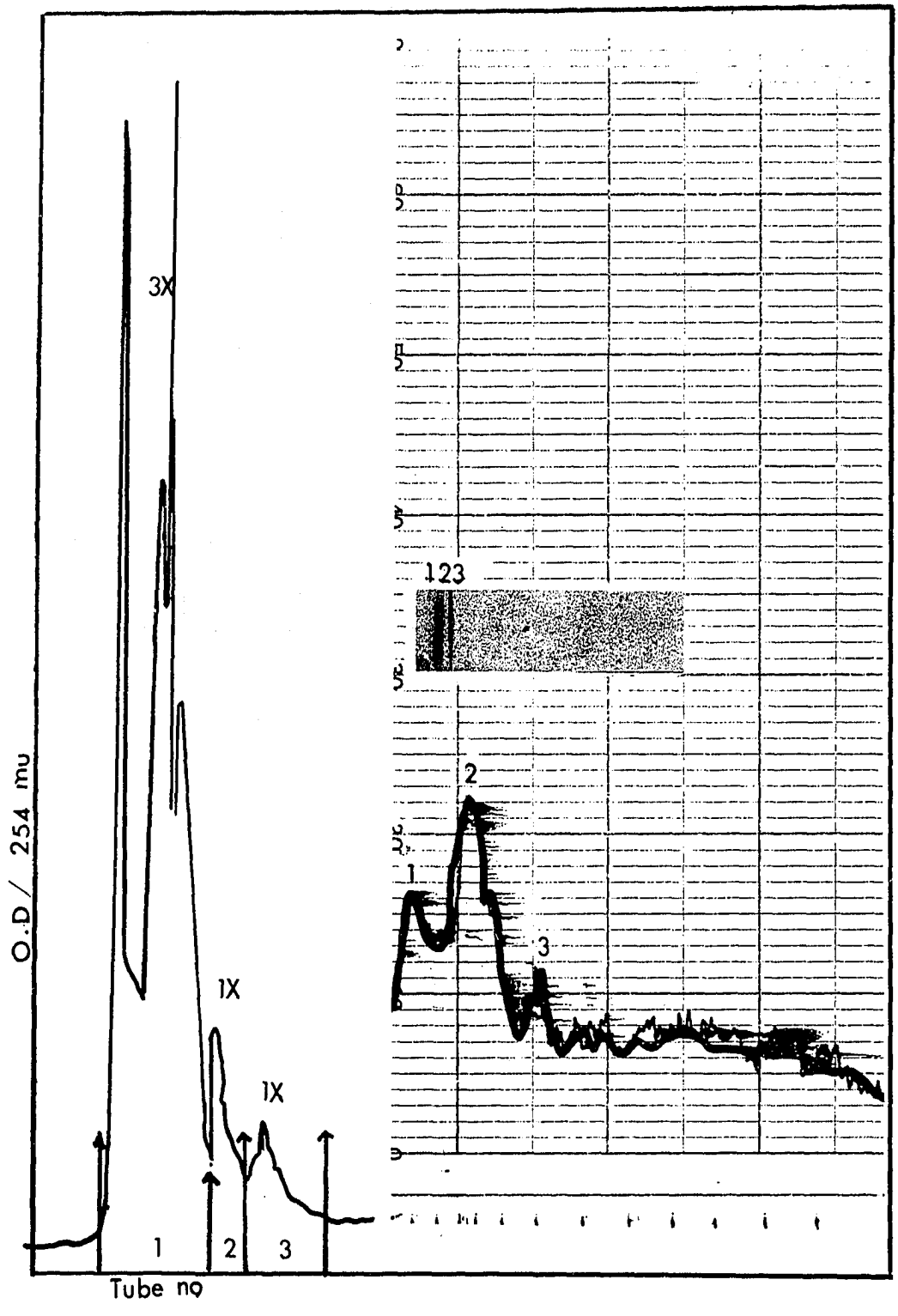


Fig. 21.--Electrophoretic elution pattern of histoplasmin H-42 on 7% stacker acrylamid gel.
Left: Elution pattern from preparative column.
Right: Analytical gel pattern and densitometer tracing.

purity by running 100 ug of each on the 7.5% analytical columns. All fractions showed the presence of the band with RF 0.7, pool 1 had a very light band. Only pool 4 showed a band with RF 0.38. All fractions were tested for protein and carbohydrate content as shown in Table 4.

TABLE 4

CHEMICAL ANALYSIS OF HISTOPLASMIN H-42 FRACTIONS ELUTED FROM PREPARATIVE ACRYLAMIDE GEL COLUMN 7% STACKER

	Fraction number					
	1	2	3	4	Total/ original	% Re-covery
Total protein ug/pool	180	365	185	150	880/840	103
Total carb. ug/pool	40	145	100	150	495/696	69
Reducing sugars	+	+	+	+		
Total N ug/pool	108	150	123	-		
Skin test dilution	60	60	60	60		
Skin test						
Erythema	0	11.8	7.4	3.6		
Induration	0	9.8	6.4	3.6		

Since a reasonable separation was shown on the 7.5% separating analytical gel it was chosen for the preparative column. The advantage of this type of gel is that it gives better separation and pure fractions. The disadvantage is that protein migrates so slowly that it cannot be eluted in a reasonable time in the same gel, and spreading of the

bands occurs. A sample of 2800 ug corresponding to 8 ml of the concentrated H-42 or 100 ml of 1:25 was used. The elution pattern is represented in Fig. 22. All three pools were examined on the 7.5% analytical gel Fig. 22. Pool 1 representing fractions 1-30 has the highest peak, pool 2 (fractions 31-70) has a very small peak which in some other columns is represented by a very small hump. Pool 3 never showed a peak and it is a pool of fractions 71-120. When these pools were tested with the analytical column (100 ug/ column), pool 1 showed no band while pool 2 showed protein with RF 0.7. The last pool had the band with RF 0.38 and in most cases contaminated with the 0.7 band. The minor components with RF 0.64, 0.2 and 0.05 were not recovered.

Carbohydrate and protein content of the fractions is shown in Table 5.

Pools from stacking gel are designated by S-1, S-2, S-3, and those from the separator A-1, A-2, A-3. Only fractions 1 and 2 along with H-42 were hydrolyzed for amino acids and carbohydrates, and only A-2 which has one protein (RF 0.7) was subject to end group determination. Neutral sugars from carbohydrate hydrolysis were identified by paper chromatography. The following standards were used for comparison: D-glucose, D-galactose, D-mannose, L-fucose, L-rhammnose.

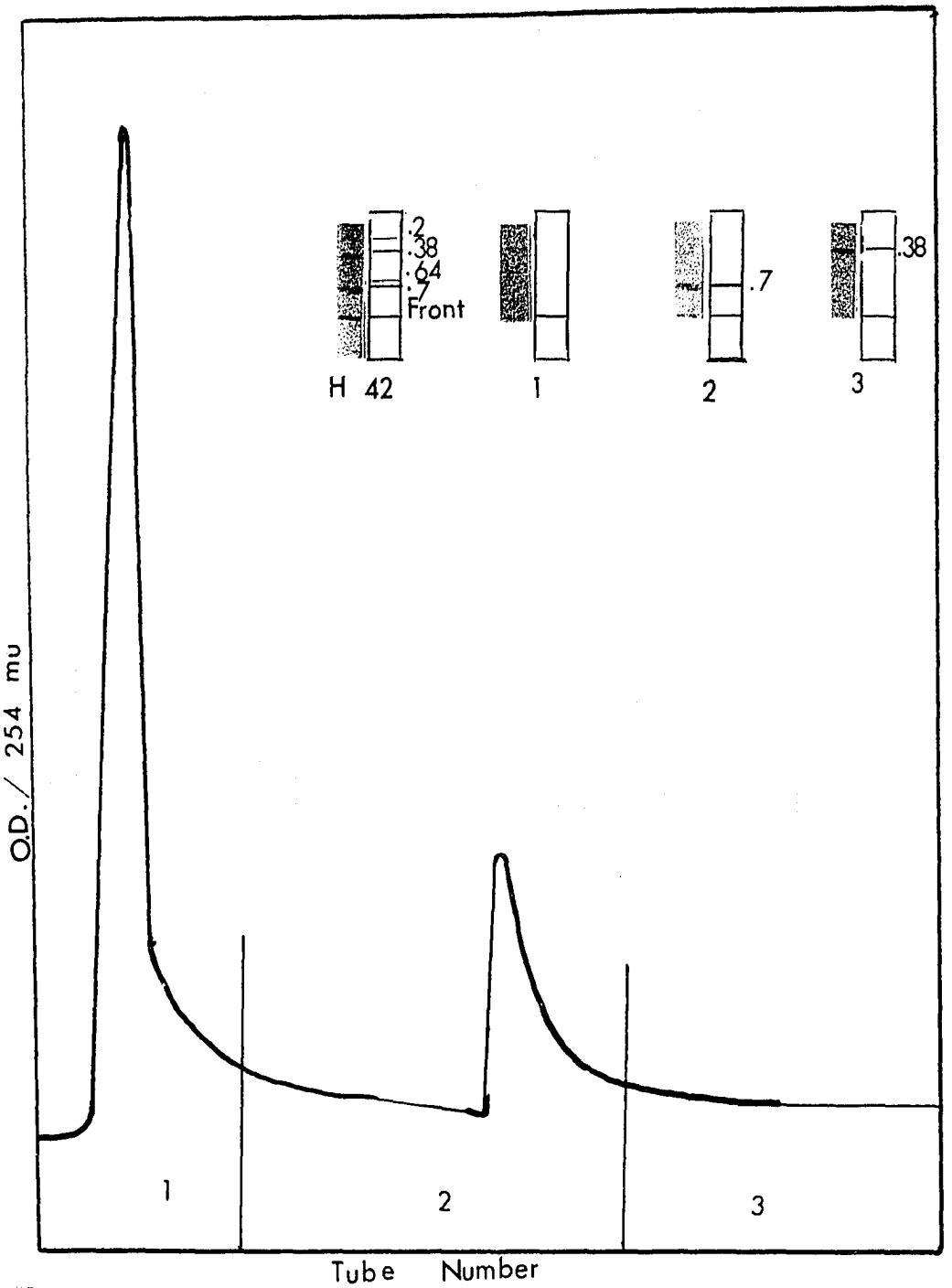


Fig. 22.--Electrophoretic elution pattern of histoplasmin H-42 on 10% separator gel.
Left: Elution pattern from preparative column.
Upper right: Analytical gel pattern of H-42 and its fractions 1, 2, 3.

TABLE 5

CHEMICAL ANALYSIS OF HISTOPLASMIN H-42 FRACTIONS ELUTED FROM PREPARATIVE ACRYLAMIDE GEL COLUMN (10% SEPARATOR)

	Fraction number				
	1	2	3	Total	% Recovery
Total protein ug/pool	1100	500	600	2200/2800	78%
Total carb. ug/pool	400	200	310	910/2320	39.2%
Reducing sugar	+	+	+		
Skin test dilution	100	100	100		
Skin test					
Erythema	6.8	11.6	9.0		
Induration	3.2	9.4	6.4		

H-42 showed at least 6 spots of carbohydrate. Mannose, galactose and glucose were identified. Three spots were very close to the origin suggesting that they represent disaccharide or trisaccharide from incomplete hydrolysis. A characteristic spot with RF 0.04 on descending and 0.75 on ascending chromatography, giving brown color with phthalate spray and pink with benzidine spray was found in all fractions. It was the only spot given by fraction A-1 and S-1. A-2 and S-2 (protein band with RF 0.7) gave mannose and galactose in addition to this spot. Hexosamine was identified in all fractions by eluting the ion exchange columns with 1 N HCl and testing by descending paper chromatography

including glucosamine as standard. One hexosamine common to all fractions was detected.

Table 6 shows the summary of the results of carbohydrate hydrolysis.

Amino acid analysis

One milligram H-42 was hydrolyzed in 1 ml of HCl. Samples of S-1 and S-2, each containing 250 ug protein, estimated by Lowry method, were hydrolyzed in 0.2 ml of acid. A-1 and A-2, 500 ug per sample, were hydrolyzed in 0.5 ml acid. The following amino acids were identified in H-42: glutamic acid, aspartic acid, serine, glycine, alanine, lysine, ornithine, valine, tyrosine, leucine, isoleucine, proline, histidine, arginine, phenylalanine, cysteine, threonine and methionine. The same picture was obtained from A-2 and S-2. Hydrolysates of A-1 and S-1 showed almost no reaction with ninhydrin.

End group analysis

N-terminal amino acid analysis.--Only A-2 was chosen for this experiment on the assumption that it is a pure fraction, having one protein with RF 0.7 and giving a positive skin test. To 300 ug of protein in 10 ml of 0.05 N aqueous KOH, 0.03 ml of DNFB was added. The ether extract of the hydrolysate in two TLC systems showed only one spot with RF 0.22 and 1.00 which corresponds to none of the amino acid DNP derivatives. It was identified as a dinitrophenol

TABLE 6

CARBOHYDRATE CONTENTS OF H-42 AND ITS FRACTIONS

Fraction	ug hydrolyzed	Descending Chromatography			Ascending Chromatography			Hexosamine
		ug used	No. of spots	Sugar identified	ug used	No. of spots	Sugar identified	
H-42	800	400	6	Mannose Glucose Galactose Pink spot RF 0.04	400	5	Mannose Glucose, Galactose** Spot RF 0.75	+
S-1	40	40	1	Pink spot RF 0.04	-	-	-	+
S-2	90	90	3	Mannose Galactose* Pink spot RF 0.04	-	-	-	+
A-1	290	145	1	Pink spot RF 0.04	145	1	Spot RF 0.75	+
A-2	390	195	3	Mannose Galactose* Pink spot RF 0.04	195	2	Spot RF 0.75 Mannose	+

*very light spot.

**appeared as one spot due to poor separation of these two sugars in ascending chromatography.

spot. Water soluble DNP fraction showed one bright yellow spot and two faint yellow spots. The first spot ran parallel to the di-DNP-histidine derivative. Upon spraying with ninhydrin the di-DNP-histidine remained yellow while the other two spots one turned purple and the other turned brown suggesting that they may be imidazole DNP, and ε-DNP-lysine or O-DNP-tyrosine or both since they have the same RF.

C-terminal amino acids

360 ug of protein as estimated by Lowry method was incubated with 1 ul of the carboxypeptidase suspension containing 7.6 ug of enzyme protein in a total volume of 5 ml. Aliquots of 0.4 ml were transferred to 0.2 ml HCl at the following intervals of time: 1, 15, 45, 60, 90, 240 minutes. Chromatography showed two pink spots with ninhydrin with RF 0.21 and 0.18 after 1 min. The spots have been identified as serine RF 0.18 and either threonine or ornithine. It was difficult to determine which amino acid was split off first.

Quantitative amino acid determination

Results are shown in Fig. 23. The unknown amino acid was estimated as ornithine but identification should be confirmed. The protein showed a high percentage of proline and low values for histidine and amino acids containing sulfur (Table 7).

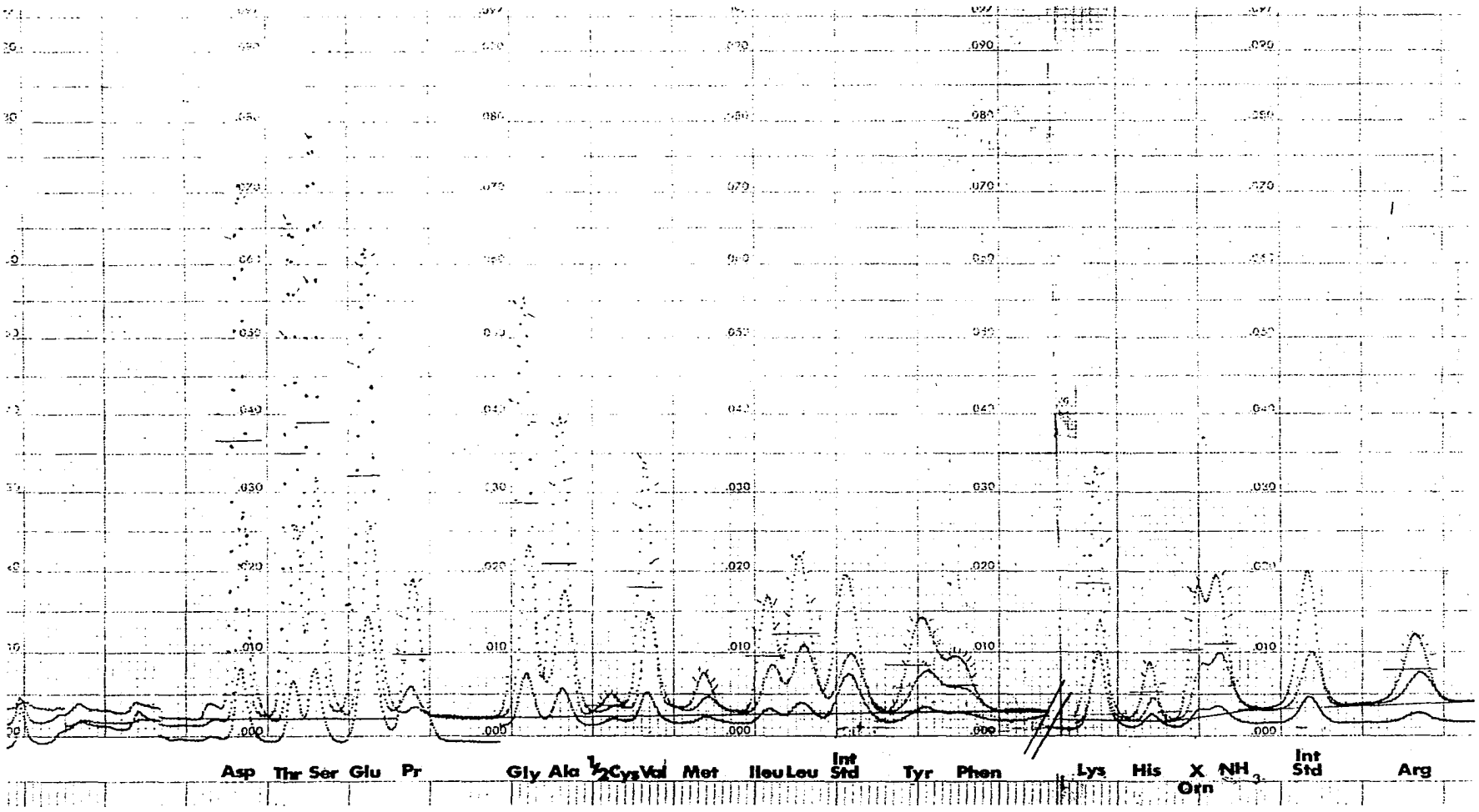


Fig. 23.--Amino acids obtained from hydrolysate of protein fraction with RF 0.7.

TABLE 7

AMINO ACIDS COMPOSITION FROM HYDROLYSATE OF PROTEIN FRACTION WITH RF 0.7

Amino acid	Micro moles x 100	Residues/ histadine
Lysine	2.2	3.7
Histidine	0.6	1
Ammonia	1.3	2.1
Arginine	1.5	2.5
Unknown	1.0	1.7
Aspartic Acid	6.1	10
Threonine	5.6	9.3
Serine	5.2	8.7
Glutamic Acid	7.8	13
Proline	9.6	16
Glycine	4.8	8.0
Alanine	4.3	7.1
Half Cystine	0.9	1.5
Valine	2.9	4.8
Methionine	0.6	4.3
Isoleucine	1.8	3
Leucine	0.6	1.0
Tyrosine	2.4	4.0
Phenylalanine	1.2	2.0

Discussion

Histoplasmin H-42 contains at least 5 components as indicated in analytical gel electrophoresis. All the bands stained with Amido Schwarz. The bands with RF 0.7 and 0.38 stained with Schiff's indicating the presence of carbohydrate associated with these bands. The protein with RF 0.7 appears consistently in analytical gels of the crude filtrate, dialyzed material and fractions eluted from preparative electrophoresis. It has been recovered from both 7% stacker and 10% separator preparative columns. This protein has antigenic properties as indicated by the positive skin test. It is isolated in a relatively pure homogeneous form as indicated by the presence of one band on the analytical gel electrophoresis. The chemical analysis indicates that it is a glycoprotein containing mannose, galactose, hexosamine and nineteen amino acids with histidine as N-terminus and a hydroxyamino acid or ornithine as C-terminus.

It is known that histoplasmin H-42 is a mixture of crude filtrate of three organisms. A glycoprotein with RF 0.7 was found in the crude filtrate of Scritchfield and Grand Island cultures. The proteins from District culture filtrate and NCDC #267 have RF 0.38 and 0.63 respectively.

A combination of the culture filtrates or pure preparation of the glycoprotein might result in high potency skin test material.

Further chemical studies on these glycoproteins and comparison with those obtained from H-42 will result in a better preparation of skin test material by choosing the specific antigen and eliminating the unspecific proteins.

It was noticed that the concentrations of the major components of H-42 are related to each other. If the solution is freshly prepared and applied to columns with a good cooling system, the 0.38 band will be dominant, with less care the 0.7 band becomes the more dominant, otherwise the bands are usually light. This behavior could be explained if the two bands have the same protein moiety but differ in the carbohydrate residue which can be split off easily with a change in electrophoretic mobility.

Separation on polyacrylamide gel helped in the isolation of some relatively pure fractions. The 7% stacker gel gave closely spaced bands yielding poor separation but fast. The 10% separatory gel gave pure fractions such as the protein with RF 0.7 but was slow that spreading of some bands such as that of RF 0.38 occurred.

Prep-disc electrophoresis is promising, a combination of the two gel systems, or the use of intermediate gels such as 5% separatory preparative column will give better resolution of the antigen components by allowing proteins with large molecular size to pass more easily than 10% separator gel.

CHAPTER VI

SUMMARY

Histoplasmin has been subjected to various fractionation and characterization procedures. The following conclusions can be drawn:

1. The Histoplasma isolates are not the same; they differ in their growth rates.

2. Analytical polyacrylamide gel electrophoresis of culture filtrates showed that each isolate has its characteristic protein pattern which is different from that of culture filtrates of other fungi.

3. Age of culture is very important in preparing histoplasmin.

4. A very small amount of the antigen, as low as 0.05 ug of protein, is sufficient to give a good skin reaction.

5. Preparative polyacrylamide gel electrophoresis gave good separation of culture filtrate components although the resolution was more efficient with some isolates than with others.

6. H-42 histoplasmin is a mixture of different protein components of which one is a glycoprotein.

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