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Fractionation and Composition Studies of Skin Test-Active Components of Sensitins from *Coccidioides immitis*

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Coccidioidin skin-test activities from mycelial culture filtrates and autolysates were partially purified. Major chemical constituents included 3-*O*-methylmannose, mannose, and amino acids.

Coccidioidin is the filtrate of liquid cultures of *Coccidioides immitis* which is used as a skin test and serological antigen. Cooke, in 1915 (1), reported equivocal results with several "emulsions" and heat-concentrated glucose broth filtrates. The first successful coccidioidin, prepared by Davis in 1924 (3), was a suspension of the fungus rather than a filtrate. However, Hirsch and D'Andrea, in 1927 (7), reported skin reactions of the tuberculin type with culture filtrates from complex and synthetic medium. This material withstood heating, was precipitated by alcohol, and appeared to be a polysaccharide with 3 to 4% protein. Stewart and Kimura (17) reported that the skin-test sensitin was both dialyzable and heat stable, withstanding autoclaving for 30 min at 15 lb of pressure. Hassid et al. (6) reported the major component of the alcohol-precipitated fraction of coccidioidin to be glucose. These authors observed total nitrogen values from 0.95 to 3.23%, depending on the preparation, and reported the occurrence of galacturonic acid, glucose, and an unknown sugar. Pappagianis et al. (11), on the other hand, found mannose to be the major hexose, along with small amounts of galactose and an unidentified sugar, but no uronic acids were observed. Pappagianis et al. (11, 12) also reported serological activity of coccidioidins prepared by the toluene autolysis of young mycelia and obtained positive skin-test responses with 5 μg of the nondialyzable materials. Ethanol fractionation resulted in active polysaccharide fractions which contained 3 to 4% nondialyzable nitrogen present as amino acids. Huppert and Bailey (8) prepared complement fixing and precipitin coccidioidins by a modification of the Pappagianis et al. (11) procedure, which could be used in immunodiffusion assays. Wallraff and co-workers (18-20) used Huppert's coccidioidin preparation as a skin-test antigen and found that it elicited positive skin tests in humans and guinea pigs. We have extended the study of the composition and purification of skin-test antigens for *C. immitis* and report here some of our findings.

MATERIALS AND METHODS

Organism. *C. immitis* strain Silveira was obtained as an arthrospore suspension from H. B. Levine (Naval Biological Laboratory, University of California, Oakland). *Histoplasma capsulatum* strain 6651 and *Blastomyces dermatitidis* strain 410 were obtained from The University of Utah culture collection of S. Marcus.

Culture. Yeast-form cells of *H. capsulatum* and *B. dermatitidis* were grown on antibiotic-blood-agar for use as infecting agents in skin-test animals. Mycelia of *C. immitis* were grown in liquid culture at 37 C for 2 to 3 days on an incubator-shaker (G-25, New Brunswick Scientific Co.). The liquid medium used in this study was glucose and yeast extract dialysate (8).

After checking for contamination by both culture and microscope examination, mycelia and liquids were separated by filtration (8). The culture filtrate was passed through a Selas Candle, porosity 03 (Selas Corp. of America, Spring House, Pa.), and evaporated under reduced pressure (20 mm or less) and temperature (40 C or less). After concentration, the culture filtrate was dialyzed against deionized water for 24 to 48 hr, lyophilized, weighed, and stored in a desiccator. The mycelium was allowed to autolyze in aqueous 3% toluene for 7 days at 37 C and harvested by Büchner funnel filtration on Whatman no. 1 paper to separate the particulate residue from the toluene-autolyzed soluble materials. The autolysate was then passed through a Selas Candle.

The results of the investigation reported in this study are of the two soluble sensitins obtained as the culture filtrate and toluene autolysate.

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Dialysis. Dialysis of the sensitins was first carried out at room temperature with constant stirring against multiple changes of deionized water. Later, the procedure was changed to dialysis against hot, running tap water followed by dialysis against several changes of deionized water at room temperature. Dialysis tubing (Visking Corp., Chicago, Ill.) used was number 27, except where number 8 is specifically mentioned.

Ultrafiltration. Ultrafiltration cell model 401 (Amicon Corp., Lexington, Mass.) and a 2.75-liter liquid reservoir were used at room temperature with constant stirring. The membranes were used in increasing number and pore size, beginning with UM-2 and ending with XM-100. Culture filtrate, protected with toluene, was dialyzed for 48 hr against cold, running tap water at 15 to 18 C, and the solution was centrifuged to remove particulate material. Approximately 4 liters of culture filtrate was passed through membrane no. UM-2 at approximately 50 psi. The ultrafiltrate was collected, concentrated, centrifuged, and lyophilized for use in skin-test experiments. The material retained, after the initial concentration, was centrifuged, diluted to 400 ml, and passed through the next membrane. The procedure was then repeated for the next size membrane. Membrane SM-50 filtration was tested twice in this series. The final retained material, after XM-100 ultrafiltration, was centrifuged and lyophilized.

Gel filtration. Samples of culture filtrate and toluene autolysate were fractionated on a column (2.5 by 40 cm) of Sephadex G-25 (Pharmacia, Uppsala, Sweden). The column effluent was collected in 2.4-ml portions. Flow rate used was 14 to 16 ml/hr with Merthiolate (100 mg/liter) containing neutral (*p*H 7.0) saline (0.15 M NaCl) as eluant. Fractions were collected and pooled by their protein content by using the modified Folin phenol method (9). Carbohydrate was determined by the phenol-sulfuric acid procedure (4). Nucleic acid content was estimated from ultraviolet (UV) absorption (21). Pooled fractions were dialyzed against deionized water, to remove salts, and lyophilized.

Skin-test assays. Albino guinea pigs (Hartley strain) were infected with 500 to 1,000 *C. immitis* arthrospores strain Silveira injected intraperitoneally (ip) in 0.1 ml of saline. Guinea pigs were also infected with *H. capsulatum* 5% (v/v) yeast-phase cell suspension injected ip or infected with *B. dermatitidis* 1% (v/v) yeast-phase cell suspension injected ip. Infected guinea pigs were injected intracutaneously with 0.1-ml amounts of various saline dilutions of test material, and positive and negative (saline) controls at sites were randomized by the use of 6 by 6 Latin square statistical tables (5). Groups of three to six animals were used to obtain each value reported in text and figures with the exceptions that three animals per group were used in Table 1 and two animals per group in Table 2. One-milliliter disposable tuberculin syringes and 26-gauge disposable needles were used. Skin-test sites were read for induration at 24 and 48 hr, but only 48-hr induration readings are reported in the present work.

Lipid extraction. Lipids were removed by the following solvents and extraction sequence: methanol, chloroform-methanol (2:1), acetone, and ether. Materials were extracted at room temperature for 30 min with occasional stirring. Lipid content was determined by dry weight loss after solvent extraction.

Hydrolysis. Samples of sensitins or authentic compounds were sealed in glass ampoules at reduced pressure and hydrolyzed in 1 ml of 6 N HCl/18 hr for amino acid analysis or 1 N HCl/4 hr for carbohydrates. After hydrolysis, acids were removed in vacuo in a rotary evaporator and the residues were dissolved in deionized water.

Amino acid analysis. The separation of amino acids and amino sugars was performed on a Beckman-Spinco 120-C amino acid analyzer modified as described by Steele et al. (16). Concentrations of compounds were determined by the ½ peak × height

TABLE 1. *Results of delayed hypersensitivity skin test cross-reactions of coccidioidin fractions in Histoplasma capsulatum-, Blastomyces dermatitidis-, and Coccidioides immitis-infected guinea pigs*

Sensitin	Skin-test (mm reaction of induration)											
	C. immitis-infected animals				*H. capsulatum*-infected animals				*B. dermatitidis*-infected animals			
	Animal no.			Avg	Animal no.			Avg	Animal no.			Avg
	1	2	3		1	2	3		1	2	3	
Dialyzed culture filtrate (5 μg/dose)	3	6	10	6.3	0	1	0	0.3	0	0	0	0
Dialyzed autolysate (5 μg/dose)	2	6	6	4.7	2	1	0	1.0	0	0	0	0
Standards[a]												
Coccidioidin	5	10	4	6.3	—	—	—	—	—	—	—	—
Histoplasmin	—[b]	—	—	—	2	4	6	4.0	—	—	—	—
Blastomycin	—	—	—	—	—	—	—	—	4	3	6	4.3

[a] Standards were from this laboratory (N.F.C.).

[b] Symbols: —, not done; 0, no reaction.

TABLE 2. *Results of ultrafiltration with various membranes and crude culture filtrate*

Membrane no.[a]	Membrane[b] molecular weight retention range	Amt (ml)	Pressure[c] (lb)	Time required	Filtrate skin[d] test activity (10 μg/dose)
UM 2+	1,000–16,000	4,000	55	7 days	—
PM 10	10,000–100,000	400	55	1 day	—
XM 20E	10,000–16,000	400	35	6 hr	—
XM 50	64,000–200,000	400	10	6 hr	+(10.5 mm)
XM 100	100,000–1,000,000	400	10	4 hr	+(5.0 mm)

[a] Carried out at 4 C by using membranes in ascending numbers.
[b] Amicon designation, publication 400-A.
[c] Compressed helium used.
[d] Average skin test results on two *Coccidioides immitis*-infected guinea pigs for each fraction tested.

method by using calibration factors obtained with standard amino acid mixtures.

GLC. An F and M Hewlett Packard (model 400) apparatus was used for gas-liquid chromatography (GLC). Sugars were separated as alditol acetates, prepared by following the borohydride reduction and peracetylation procedure of Sawardeker et al. (13) on 3% ECNSS-M on Gas Chrom Q (Applied Science Lab, State College, Pa.) at 185 to 190 C, He flow rate 70 ml/min, column length of 6 feet.

Standards. Galactose, glucose, mannose, and glucosamine were obtained from Pfanstiehl Laboratories, Waukegan, Ill.

RESULTS

Dialysis. Extensive dialysis of the crude culture filtrates and toluene autolysates consistently yielded 5 to 12% of the pooled crude material in the retained material. For example, a 39.4-g batch of pooled culture filtrate yielded 4.8 g of retentate, whereas 2.6 g of retentate was recovered from a 27.7-g batch of autolysate. The dialysates of both culture filtrates and autolysates were negative for skin-test activity when lyophilized and tested at the 100-μg level. The retentates, however, gave positive skin tests at 10 μg or less. Results in Table 1 show the reactivity of culture filtrate and autolysate when skin tested on *C. immitis*-, *B. dermatitidis*-, and *H. capsulatum*-infected guinea pigs. Normal animals were also skin tested at 100- and 50-μg dose levels, and no specific reactions were observed.

The effect of hot-water dialysis on skin-test activity in *C. immitis*-infected animals was checked by using the culture filtrate antigen and a medium sized (no. 27) dialysis tubing. Comparison of hot-running-water dialysis (78 C) with room-temperature (26 C) dialysis for 48 hr by skin test of 10-μg amounts of the respective lyophilized retentates revealed little difference; i.e., hot-water-dialyzed sensitin yielded 6.5 mm and room-temperature-dialyzed sensitin, 7.7 mm of induration. Similarly, the room-temperature dialysate was skin tested at the 100-μg dose level, again with negative results.

By using smaller dialysis tubing (no. 8, about 0.25 inch, ca. 0.636 cm diameter), the previously exhaustively dialyzed (in no. 27 dialysis tubing) culture filtrate preparation was further dialyzed against deionized water at room temperature for 48 hr. The retentate and the dialysate were separately lyophilized and made up to 10 ml each. Skin-test results, with 0.1 ml of each of these solutions, yielded 27.5 mm of induration for retentate and 5.0 mm for dialysate, indicating some loss of material by dialysis in the no. 8 dialysis tubing.

Ultrafiltration. The results of the ultrafiltration studies on crude culture filtrate are shown in Table 2. Shown in the table are the membranes used, molecular weight retention range for that membrane as given by the manufacturer, and results of skin tests. The ultrafiltrate from membranes UM-2, PM-10, and XM-20E were all negative, whereas the XM-50 and XM-100 ultrafiltrates were both positive in skin-test activity. The XM-100 retentate also was negative in its skin-test activity.

Gel filtration. Sensitins were subjected to Sephadex G-25 fractionation at various stages of purification. Figure 1 illustrates the Sephadex G-25 elution patterns of an autolysate preparation before and after dialysis. Dialysis obviously removed large amounts of small-molecular-weight carbohydrates and peptides. Protein concentration was used as the index for pooling the fractions from the dialyzed preparation, labeled I, II, III, and IV and indicated by the vertical lines. Skin-test activities are also indicated. Although the void volume fraction contained most of the activity, the following fraction also contained activity. This is in contrast to the culture filtrate antigen (Fig. 2) where only the void volume contained significant activity. Merthiolate, used in the gel filtration buffer to avoid bacterial contamination, appeared to have little or no effect on skin-test activity since fractions I, II, and III of another culture filtrate preparation elicited 3-, 14-, and 2-mm responses after dialysis com-

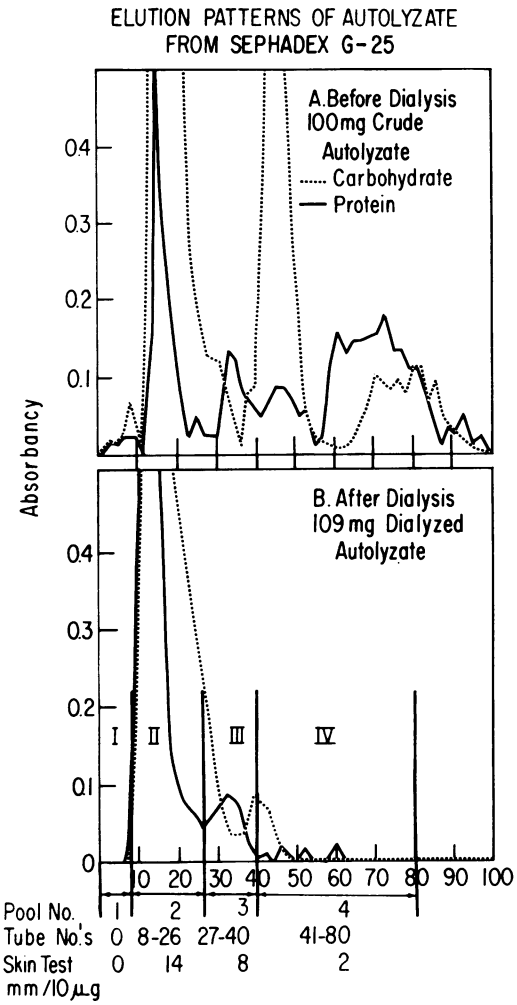

FIG. 1. *Sephadex G-25 profile of 100 mg each of crude autolysate before and after dialysis. Since 90 to 95% weight loss occurs by dialysis, B is the 10- to 20-fold concentrated nondialyzable fraction of A. Fractions were pooled as indicated, dialyzed against deionized water to remove salts, and lyophilized. Six guinea pigs were used for skin tests of 10 μg of each pooled fraction dissolved in saline.*

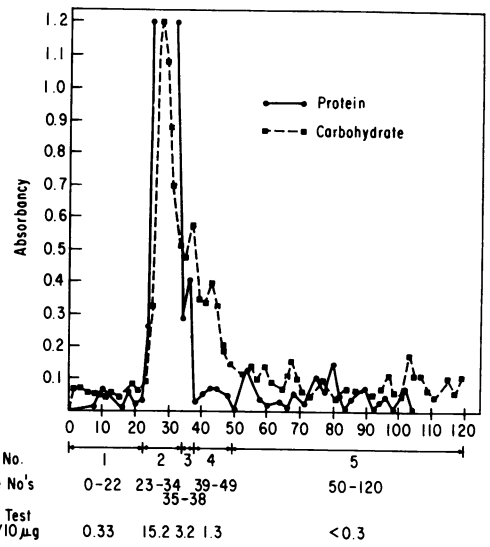

FIG. 2. *Elution pattern from Sephadex G-25 of 100 mg of dialyzed culture filtrate. Fractions were recovered and tested as in Fig. 1.*

pared to 4-, 16-, and 3-mm responses before extensive dialysis to remove the Merthiolate.

The Sephadex fraction II of both toluene autolysates and culture filtrates contained between 60 and 70% of the original sample weight. For example, 72 mg was recovered in fraction II from the G-25 column fractionation of 105 mg of the extensively dialyzed culture filtrate. Overall recovery was 75 to 85% of the material added to the columns.

Composition studies. The high carbohydrate and protein content of the antigens used is obvious from Fig. 1 and 2. The dialyzed culture filtrates and autolysates contained 1 to 3% nucleic acid as estimated by 260-nm absorption (21). Lipid content of one dialyzed autolysate preparation was 12%. The data shown in Table 3 are the results of amino acid analyses of the dialyzed sensitins before and after fractionation by Sephadex G-25. After Sephadex treatment, culture filtrate lacked methionine, whereas autolysate contained only traces of methionine and no, or insignificant amounts of, leucine, tyrosine, or phenylalanine. Of some interest is the enrichment of proline compared to the other amino acids. The amino acid content of culture filtrate was 39% before Sephadex treatment and 8% after. The autolysate antigen contained 26% amino acids before Sephadex fractionation and 6% after. Both dialyzed fractions contained approximately 5% glucosamine.

The results of carbohydrate analysis by GLC are shown in Table 4. Four hexoses and three other peaks were observed. The hexoses were identified as 3-*O*-methylmannose, mannose, glucose, and galactose. Other peaks were present in small or trace amounts. These were presumed to be the acetyl alditols of the pentoses, ribose, xylose, and 2-deoxyribose, which varied in concentration after different procedures, and were not enriched in proportion to skin-test activity upon fractionation. Therefore, the identity of these components was not pursued.

DISCUSSION

Findings recorded here essentially corroborate and extend the composition of skin test-active

material of coccidioidin as given by earlier workers (6, 7, 10, 12). Mannose was the main hexose found along with small amounts of galactose and glucose. The unknown reducing sugar reported by Hassid et al. (6) and Pappagianis (12) and identified as a methylmannose by Goldschmidt and Taylor (Bacteriol. Proc., p. 127, 1958) was recently shown to be 3-*O*-methylmannose (14). McNall et al. (10) and Hassid et al. (6) reported uronic acids from *C. immitis*, but Pappagianis et al. (12) and we, in the present study, were unable to confirm the presence of uronic acid.

TABLE 3. *Amino acid content of Coccidioides immitis sensitins*

Amino acid	Content culture filtrate[a]		Content in autolysate[a]	
	Dialyzed	Sephadex peak II	Dialyzed	Sephadex peak II
Aspartic acid	0.160	0.080	0.140	0.030
Threonine	0.110	0.050	0.200	0.050
Serine	0.120	0.004	0.180	0.042
Glutamic acid	0.120	0.051	0.170	0.031
Proline	0.270	0.200	0.310	0.200
Glycine	0.012	0.056	0.016	0.031
Alanine	0.008	0.035	0.013	0.025
Cystine	0.063	0.028	0.000	0.027
Valine	0.038	0.004	0.073	0.020
Methionine	0.001	0.000	0.000	0.008
Isoleucine	0.039	0.013	0.063	0.017
Leucine	0.053	0.023	0.068	0.000
Tyrosine	0.035	0.016	0.023	0.000
Phenylalanine	0.035	0.013	0.058	0.000
Ornithine	Trace	0.014	Trace	0.004
Lysine	0.031	0.023	0.120	0.016
Ethanolamine	0.054	0.010	0.083	0.010
NH_3	0.040	0.035	0.029	0.029
Histidine	0.014	0.010	0.009	0.009
Arginine	0.013	0.014	0.013	0.014
Per cent	39	8	26	6

[a] Micromoles of amino acid per milligram of sample.

Our findings to date indicate that 3-*O*-methylmannose and mannose are consistently present in our delayed hypersensitivity skin test-active fractions. Moreover, these two constituents appear to be enriched by the simple purification steps reported here, whereas glucose and galactose were consistently decreased in concentration. The ratio of 3-*O*-methylmannose to mannose varied. In most culture filtrate preparations examined, 3-*O*-methylmannose has been present in lower concentration than mannose, but this ratio was reversed in the case of the autolysate Sephadex fraction shown in Table 4.

Hirsch and D'Andrea (7) reported 3 to 4% nitrogen by nesslerization. Hassid et al. (6) reported similar data and suggested that the nitrogen component was not protein since no biuret reaction could be demonstrated. Pappagianis et al. (11, 12) reported amino acids in their dialyzed autolysate preparations. We also find significant amounts of amino acids in hydrolysates of both autolysates and culture filtrates after dialysis and Sephadex treatment. Only proline was significantly enriched in autolysates and Sephadex G-25 treatment. It may be that proline, along with 3-*O*-methylmannose and mannose, constitute major components of the skin-test activity, but the true significance of these observations must await isolation of a purified antigen.

The question of dialyzable skin-test activity is answered in part from the present work. Pappagianis et al. (12) and Wallraff et al. (18–20) reported skin test activity in retained material of dialyzed autolysates, whereas Stewart and Kimura (17) reported a loss of skin-test potency upon dialysis of coccidioidin through cellophane. It should be pointed out that Stewart and Kimura used long-term culture filtrate preparations which had presumably undergone considerable autolysis, whereas Pappagianis et al. and Wallraff and Snow were using materials from short-term autolysates. It could therefore well be that the amount of dialyzable material correlates with the

TABLE 4. *Major monosaccharide components of coccidioidin fractions*

Determination	Amt of component (μg/100 μg)[a]					
	3-*O*-Methylmannose	Mannose	Glucose	Galactose	Ribose	2-Deoxyribose
Culture filtrate						
Dialyzed	7.9	8.5	5.1	1.1		
Sephadex peak II	4.7	12.5	1.1	0.7		
Autolysate						
Dialyzed	3.9	10.5	2.2	1.7		
Sephadex peak II	38.4	23.1	3.0	1.9	4.1	1.7

[a] Determined by gas chromatography by using internal standard calibration techniques.

degree of autolysis. Other factors such as strain variations and media could also influence autolysis. Our own results showed that, by using no. 8 dialysis tubing and short-term culture filtrates (i.e., 7 days rather than several months), a small amount of skin-test activity was found in the dialysate although most of the activity was retained within the dialysis bag. On the other hand, all of the activity was retained when no. 27 dialysis tubing was used. These results correlate with the observations (2) that no. 8 dialysis tubing retains proteins above 15,000 to 20,000 molecular weight. Unfortunately, no data are available as to the molecular weight cut-off range of no. 27 dialysis tubing, but since exhaustive dialysis was carried out with it the pore size must be smaller than that of no. 8. It follows, then, that the dialyzable active fragments may range in size up to 20,000 daltons. Since the active coccidioidin fractions also contain polysaccharide, we must also consider that the correlation of molecular size ranges with proteins by Sephadex fractionation may be only approximate and that, if a glycopeptide or polysaccharide is involved, the molecular weight range would probably be lower than for a discrete globular protein. However, ultrafiltration results also indicate that the molecular weight of skin-test activity found in culture filtrate lies between 10,000 and 60,000 since it passed the Amicon Diaflo XM-100 and the XM-50 membranes but not the XM-20E membrane. The material retained on the XM-100 membrane did not exhibit skin-test activity, indicating that use of such membranes may offer a possibly useful purification step.

Sephadex G-25 fractionation and purification of histoplasmin have been reported previously by Sprouse et al. (15). In our hands, the Sephadex G-25 gel filtration of coccidioidin preparations yielded an antigen containing less extraneous materials and of slightly higher potency. Perhaps of most significance was the observation that gel filtration demonstrated that the autolysate preparation used contained at least two clearly separable activities as compared to the culture filtrate. Preliminary disc electrophoresis studies yielded only three protein bands from both culture filtrates and autolysates. In summary, our data indicate that coccidioidin skin-test activity may correlate with a heterodisperse mixture of antigens rather than with a single discrete antigen.

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LITERATURE CITED

1. Cooke, J. V. 1915. Immunity test in coccidioidal granuloma. Arch. Inst. Med. 15:479–486.
2. Craig, L. 1967. Techniques for the study of peptides and proteins by dialysis and diffusion, p. 870–905. *In* C. H. W. Hirs (ed.), Methods in enzymology, vol. XI. Academic Press Inc., New York.
3. Davis, D. J. 1924. Coccidioidal granuloma with certain serological observations. Arch. Dermatol. 9:577–588.
4. Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith. 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28:350–356.
5. Fisher, R. A., and F. Yates. 1953. Statistical tables for biological agricultural and medical research, vol. 15, p. 18. Hafner Publishing Co., New York.
6. Hassid, W. Z., E. E. Baker, and R. M. McCready. 1943. An immunologically active polysaccharide produced by *Coccidioides immitis*, Rixford and Gilchrist. J. Biol. Chem. 149:303–311.
7. Hirsch, E. F., and D. D'Andrea. 1927. The specific substance of *Coccidioides immitis*. J. Infec. Dis. 40:634–637.
8. Huppert, M., and J. W. Bailey. 1963. Immunodiffusion as a screening test for coccidioidomycosis serology. Sabouraudia 2:284–291.
9. Lowry, O. H., A. L. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265–275.
10. McNall, E. G., L. J. Sorensen, V. D. Newcomer, and T. H. Sternberg. 1960. The role of specific antibodies and properdin in coccidioidomycosis. J. Invest. Dermatol. 34:213–216.
11. Pappagianis, D., E. W. Putnam, and G. S. Kobayashi. 1961. Polysaccharide of *Coccidioides immitis*. J. Bacteriol. 82:714–723.
12. Pappagianis, D., C. E. Smith, G. S. Kobayashi, and M. T. Saito. 1961. Studies of antigens from young mycelia of *Coccidioides immitis*. J. Infec. Dis. 108:35–44.
13. Sawardeker, J. S., J. H. Sloneker, and A. Jeanes. 1965. Qualitative determination of monosaccharides as their alditol acetates by gas liquid chromatography. Anal. Chem. 37: 1602–1604.
14. Scheer, E., T. Terai, S. Kulkarni, N. F. Conant, R. Wheat, and E. P. Lowe. 1970. An unusual reducing sugar from *Coccidioides immitis*. J. Bacteriol. 103:525.
15. Sprouse, R. F., N. L. Goodman, and H. W. Larsh. 1969. Fractionation, isolation, and chemical characterization of skin test active components of histoplasmin. Sabouraudia 7:1–12.
16. Steele, R. S., K. Brendel, E. Scheer, and R. W. Wheat. 1970. Ion-exchange separation and automated assay of complex mixtures of amino acids and hexosamines. Anal. Biochem. 34:206–225.
17. Stewart, R. A., and F. Kimura. 1940. Studies in the skin test for coccidioidal infection. J. Infec. Dis. 66:212–217.
18. Wallraff, E. B., and I. B. Snow. 1964. Coccidioidin skin reactions. J. Immunol. 93:625–628.
19. Wallraff, E. B., R. M. Van Liew, and S. Waite. 1967. Skin reactivity and serological response to coccidioidin skin tests. J. Invest. Dermatol. 48:553–559.
20. Wallraff, E. B., and S. A. Wilson. 1965. Skin test activity of dialyzed coccidioidin. Amer. Rev. Resp. Dis. 92:121–123.
21. Warburg, O., and W. Christian. 1941. Isolierung und Kristallisation des Garungsferment enolase. Biochem. Z. 310:384–421.