Antigens from Yeast Phase of *Histoplasma capsulatum*. II. Immunologic Properties of Protoplasm vs. Cell Walls. (22012)

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Antigens from the yeast phase of *Histoplasma capsulatum* have been shown to enter into several immunologic or serologic reactions. In complement-fixation tests for detection of antibodies in experimental and naturally occurring histoplasmosis, antigens have included whole cells(1,2) and supernatant fluid from ground cells(3). In precipitation tests, a filtrate from the growth of cells of the yeast phase in broth has been used extensively(4). These complex antigens are of definite value as aids in diagnosis, althqugh some cross-reactions occur with sera from other mycoses, primarily blastomycosis and coccidioidomycosis(5-7). Recently, attempts have been made to improve the specificity of the yeast-phase antigens(8,9). An endotoxin was demonstrated in formalin-killed whole cells of the yeast phase by intraperitoneal injection into 21-day-old white Swiss male mice (10). Inoculation of similarly prepared cells of the yeast phase in smaller quantities induced the development of resistance against subsequent lethal intracerebral or intraperitoneal challenges(11,12). Reactions of tuberculin or delayed type in sensitized individuals after injection of yeast-phase antigens are well known(13). Recently, it was also reported that hypersensitive mice could be killed in less than 48 hours by injection of dead whole cells in 5% mucin(14). Hemolysins that were most active against hen and guinea pig erythrocytes were demonstrated in soluble extracts from the yeast phase(15). Until recently, it had been believed that each cell of the yeast phase was enveloped in a capsule(16). Studies with the electron microscope, however, showed that these cells did not have a capsule(17). Since whole cells had been proven to be useful and active as antigens, the question arose as to where in the cell each antigen was located.

In this paper, methods are described for the separation of cell walls from the protoplasm and data are presented illustrating some of the antigenic properties of these two fractions.

Materials and methods. White Swiss male mice, 21 days old and raised at the Rocky Mountain Laboratory, were employed. One isolate of *H. capsulatum* was used throughout, namely #6515, which originally had been isolated from a dog. It was maintained at 37°C on an agar medium consisting of 51 g/1 Difco cystine heart agar and 21 g/1 Difco bactohemoglobin. The yeastlike cells for preparation of antigen and for injection into animals were grown for 3 days under constant rotation at 37°C in a liquid medium containing salts, dextrose, and amino acids(12). The immunologic and serologic tests were similar to those previously described(1,18). *Preparation of Antigens.* Antigens were prepared by one of two methods, both of which included vibration with glass beads (#1241 glass beads, obtained from Cataphote Corp., Toledo, O.) in a container of a Mickle tissue disintegrator (manufactured by H. Mickle Co., Hampton, England). The time necessary for exposure of cells of the yeast phase to vibration was determined with the aid of an electron microscope. Varying concentrations of cells were exposed for varying periods of time in the tissue disintegrator. That time and concentration were used for separation of protoplasm from the cell wall wherein the cells were shattered sufficiently for the protoplasmic contents to escape but not enough for the cell walls themselves to be fragmented. For example, many of the ether-killed cells were suspended in 8 ml quantities (10^{10} cells/ml) with 8 g glass beads and vibrated for 10 minutes at 4°C.

Method 1. A cell suspension of the yeast phase was shaken with approximately 3 volumes of ethyl ether in large separatory funnels. At first, the cells sank to the bottom of

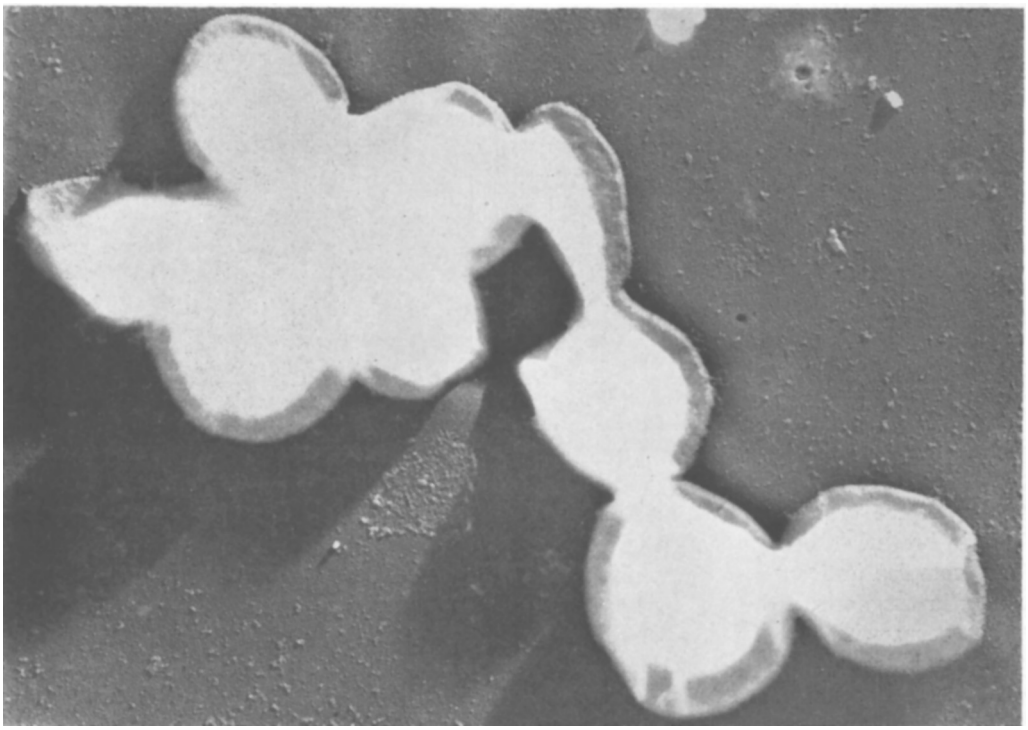

FIG. 1. Water-washed cells of yeast phase of *H. capsulatum*. Magnification 20000 ×.

the aqueous portion of this mixture. However, after several shakings over a period of approximately 24 hours, the cells rose to the water-ether interface. Cells were then harvested, washed by centrifugation in distilled water and reshaken with 3 volumes of ether. When cells of the yeast phase once again rose to the interface, they were washed and treated again in ether. After a third washing in distilled water, the cells were exposed to vibration in the Mickle tissue disintegrator. Subsequent washings by centrifugation in distilled water at 2500 g at 4°C produced two fractions: (a) the cell walls and (b) the supernatant protoplasm. The second fraction was filtered through a fine sintered glass filter to remove possible cell-wall fragments. Both fractions were subsequently lyophylized. *Method 2.* Cell suspensions from individual flasks were pooled and the cells washed by centrifugation in distilled water. Cells were then suspended in 1:5000 merthiolate for 5 days at 37°C, then the cells showed no signs of growth when cultured on Sabouraud's agar slants. The cells were washed in distilled water and exposed to vibration in the Mickle disintegrator. After washing of resulting cell residue and filtration of protoplasm, the two fractions were lyophylized. Similar results were obtained in various immunologic tests described below with fractions prepared by these two methods.

Results. Morphology. The morphology of cells of the yeast phase of *H. capsulatum* was examined under the electron microscope after each step in the fractionation procedure. This examination was of great importance in procedures associated with disruption of cells by mechanical agitation and the separation of disrupted products by centrifugation. Representative pictures are included to illustrate changes in morphology (Fig. 1-4).

Fig. 1 shows the original morphology of the yeastlike cell as seen in preparations of cells suspended in water. A purified cell-wall preparation obtained from ether-killed cells is shown in Fig. 2; the corresponding filtered protoplasm, in Fig. 3. Fig. 4 demonstrates the unfiltered supernatant material after merthiolate-killed cells had been cracked in

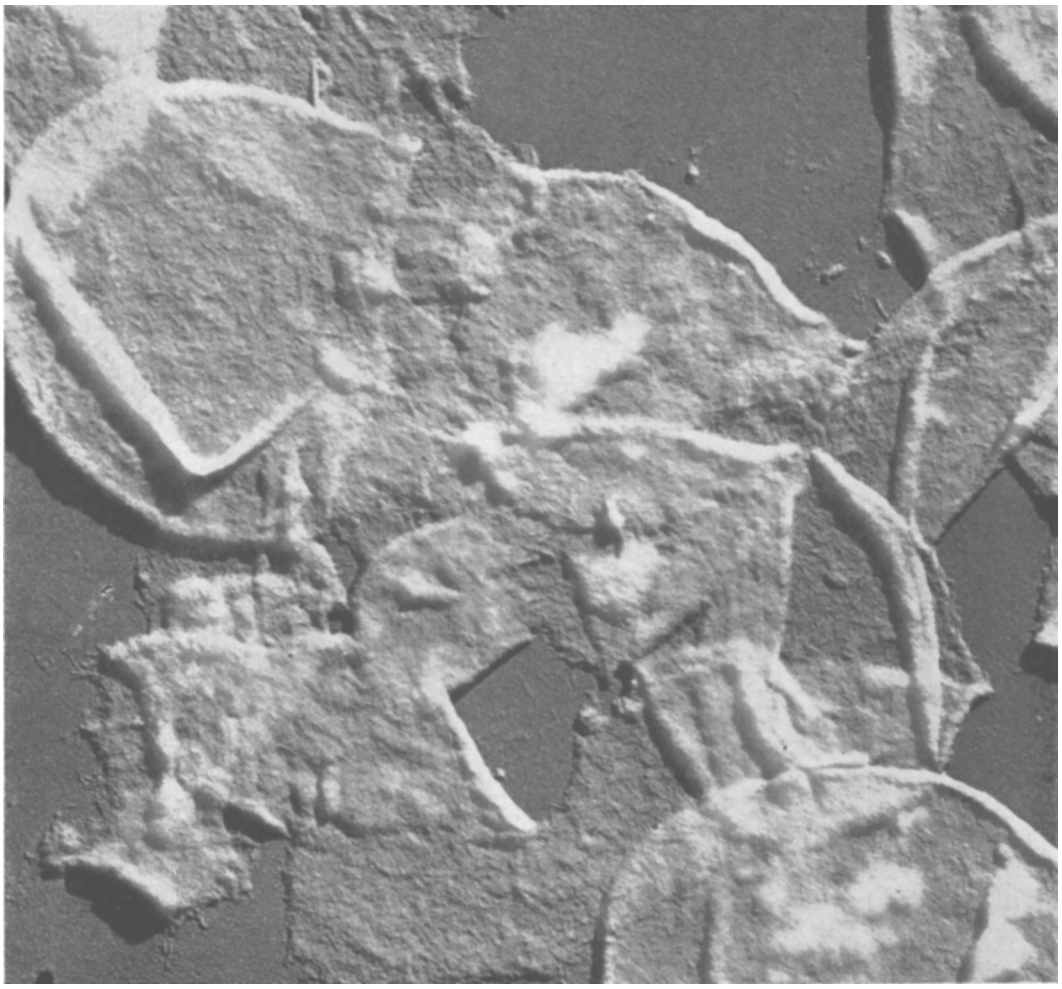

FIG. 2. Cell walls of ether-killed yeastlike cells of *H. capsulatum*. Magnification 44000 ×.

the Mickle disintegrator and centrifuged at 2500 g at 4°C for 30 minutes. Some cell-wall material was invariably present with the soluble protoplasm. The picture demonstrates the striking contrast in morphology between cell wall and protoplasm, and as a result the ease with which the two fractions may be distinguished under the electron microscope.

Examination of the protoplasm in Fig. 3 and 4 indicates that protoplasm of merthiolate-killed cells is released in a very fine, uniform, dispersed state, which condition permits easy separation of cell walls by centrifugation and filtration. In contrast, the protoplasm of ether-killed cells tends to contain more insoluble nondispersible granules, the larger of which are separated from cell walls by centrifugation only with difficulty.

Complement Fixation. The complement-fixing activities of cell walls and protoplasm from ether-killed or merthiolate-killed cells were compared with the activity of heat-killed, lyophilized whole cells in the presence of human sera from proved or suspected cases of histoplasmosis and in the presence of sera from experimentally infected guinea pigs and rabbits. The cell walls had much more complement-fixing activity against human sera than an equal weight of whole cells. The filtered protoplasm, on the other hand, had much less activity than an equal weight of whole cells. The optimum level for whole

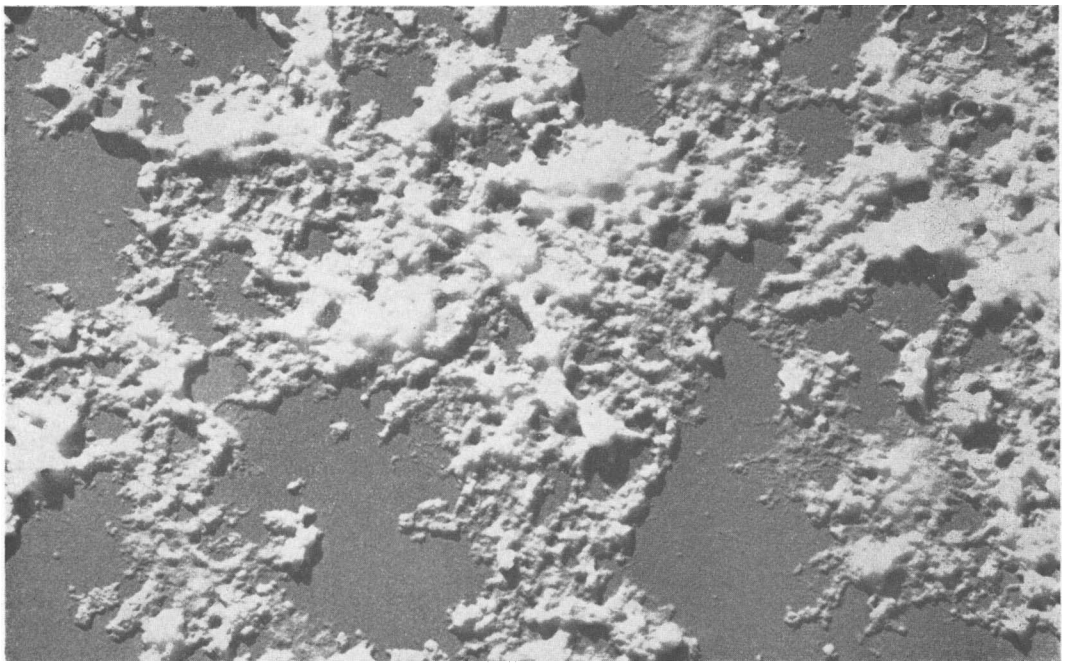

FIG. 3. Filtered protoplasmic fraction from ether-killed yeastlike cells of *H. capsulatum*. Magnification 33000 ×.

FIG. 4. Unfiltered protoplasmic fraction from merthiolate-killed cells of yeast phase of *H. capsulatum*. Magnification 29000 ×.

cells against human or experimentally infected animal sera was approximately 0.5 mg/ml, that for cell walls 0.125 mg/ml, and that for filtered protoplasm 4 to 8 mg/ml.

The possibility existed that some active antigen was washed out of the cell wall into the supernatant liquid in the process of separating the protoplasm. Accordingly, both the sediment and the supernatant fluid were assayed as complement-fixing antigens before initial washing in distilled water, and individually after each successful washing by centrifugation. The results indicated there was a marked increase in complement-fixing powers in both sediment and supernatant liquid as the cell walls were washed free of adherent protoplasm. The increase in activity of the cell-wall fraction may be attributed to gradual separation of the inactive protoplasm from the antigenically active cell wall. The increase in activity of protoplasm was attributed to leaching out of the active complement-fixing antigens from the cell wall during the washing process.

Intraperitoneal injection of the filtered soluble protoplasm or of the cell wall from merthiolate-killed or ether-killed cells induced the formation of complement-fixing antibodies in normal guinea pigs and rabbits, when heat-killed whole cells of the yeast phase were used as complement-fixing antigen. However, cell walls proved to be a much stronger antigen (Table I). When another group of rabbits was similarly injected and subsequently infected with an intravenous dose of 10^7 live cells of *H. capsulatum*, a much greater booster response was noted in the cell-wall fraction as compared with either whole cells or the soluble supernatant liquid (Fig. 5 a and b).

Further exposure of the cell wall to vibration in the Mickle disintegrator resulted in a suspension of minute fragments of cell wall. This suspension, which was clear to the naked eye, was very active antigenically in fixing complement in the presence of sera from cases of human histoplasmosis.

Protection. Mice were protected against lethal intracerebral or intraperitoneal challenge by prior injection of formalin-killed acetone-dried cells of the yeast phase, administered in one or 2 doses. When a single dose was used, 5 mg were administered intraperitoneally and 2 weeks later the animals were challenged. When 2 immunizing doses were employed, 2½ mg injections were given one week apart, and one week after the second dose the mice were challenged. Five mg of either cell-wall material or filtered protoplasm were injected intraperitoneally and the mice subsequently challenged intracerebrally with a lethal dose. In all cases, mice immunized with whole cells and non-immunized mice were included as controls. The protoplasm showed little or no protective powers in contrast to cell-wall material which did have good protective properties. Thus, only 22 of 69 mice (32%) died when first injected with cell walls, and subsequently challenged, in contrast to 53 of 60 mice (89%) that died when first inoculated with the protoplasm and subsequently challenged. Only 32 of 90 mice (35%) immunized with whole cells succumbed to the lethal challenge, while 87 of 93 (93%) of controls died. The cell wall was effective in quantities of approximately 0.5 to 5.0 mg of dried material per mouse, while the optimum dose for whole cells was approximately 5 mg per mouse.

TABLE I. Complement-Fixation Titers of Sera from 40 Rabbits Injected with 30 mg of Cell Walls, Protoplasm, or Whole Cells of *H. capsulatum.**

	Protoplasm	Cell walls	Whole cells	Controls
Range	0–16	128–2048	16–64	0
Median	4	256	32	0
Mode	0	256–512	32	0

* Heat-killed whole cells of the yeast phase were used as the complement-fixing antigen. Ten rabbits were included in each of 4 groups.

Toxin. The toxic action of protoplasm and cell walls was examined in mice, with 2 mg of dried tubercle bacilli incorporated into each mouse dose. When 5 mg or 10 mg of antigen were injected per mouse (Table II), most of the deaths within 48 hours occurred as a result of the action of the cell walls and not of the soluble protoplasm. None of 160 mice receiving adjuvant alone died.

Hypersensitivity. Mice were inoculated intraperitoneally with sublethal dose of 10^6 72 hour cells of the yeast phase. Fourteen days later, these mice were challenged with equal

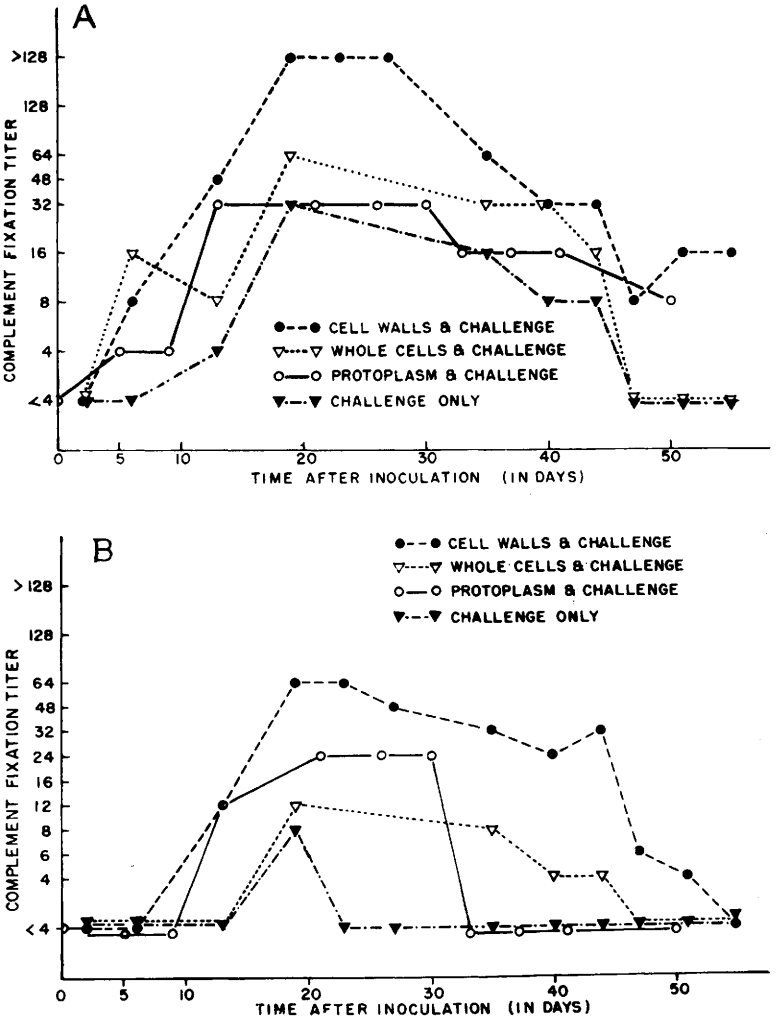

FIG. 5. Influence of cell walls or protoplasm on development of complement-fixing antibodies in experimentally infected rabbits. A. Peak titers. B. Median titers.

weights of whole cells, cell walls, or protoplasm, suspended in 5% mucin. Death rates were high and similar in mice that had been inoculated with cell-wall material or protoplasm during 48 hours post challenge. Either of these fractions produced a higher mortality than whole cells. For example, when 7.5 mg in 5% mucin were injected intraperitoneally per mouse, 40 of 50 mice (80%) succumbed to cell walls, 31 of 35 (89%) to protoplasm, and 12 of 50 (24%) to whole cells. Five of 148 mice (3%) sensitized but not further inoculated, died during this period. None of 50 sensitized mice succumbed after intraperitoneal inoculation of 5% mucin alone. Antigen, or antigens, active in initiating a response in sensitized mice seems to be present in both cell wall and protoplasm. Thirty-five guinea pigs which had been infected with a sublethal dose of the yeast phase were subsequently skin tested with the fractions previously described, as well as with histoplasmin. Edema developed 24 to 48 hours after intradermal injection, both with the cell-wall fraction and with filtered protoplasm. When the fractions were diluted to a similar extent, a slightly greater response was initiated by the soluble protoplasm. Five uninfected guinea pigs showed

TABLE II. Comparison of Toxic Action of Protoplasm and Cell Walls in 21-Day-Old Male Mice.

	Mortality			
	Protoplasm	Cell walls	Whole cells	Adjuvant controls
5 mg	2/70* (3%)	11/70 (16%)	3/70 (4%)	0/70 (0%)
10	10/90 (11%)	55/90 (61%)	16/90 (18%)	0/90 (0%)

* Numerator represents No. of mice that died; denominator represents total No. of mice inoculated. These figures are totals from 3 separate experiments.

no skin reactions at all. Results in sensitized guinea pigs were, therefore, similar to those obtained in sensitized mice.

Hemolysis. A 0.5% suspension of guinea pig erythrocytes in 0.85% saline underwent hemolysis when exposed to 0.125 to 0.25 mg whole cells/ml. Protoplasm showed about as much activity over a similar weight range. The cell wall in comparison showed little hemolytic activity with concentration as high as 8 mg/ml not producing complete hemolysis.

Discussion. With the methods of preparation used in the foregoing experiments, the cell wall of the yeastlike cells of *H. capsulatum* contained most of the antigenic activity responsible for the fixation of complement in the presence of specific antiserum. This fixation of complement occurred with human sera from proven or suspected cases of histoplasmosis and with animal sera from experimentally infected rabbits or guinea pigs. The antigen associated with complement fixation is soluble in water, since extensive washing caused it to pass gradually into the supernatant liquid. Attempts, therefore, to prepare a soluble, purified, specific antigen from the yeast-like cells should be directed toward the cell wall.

The cell wall can be readily separated from the internal protoplasm by vibration and subsequent centrifugation. Mechanical agitation with the Mickle disintegrator apparently cracked the cell walls and released the protoplasm in a dispersed state. The products of disruption, cell walls and protoplasm, were then readily separated by centrifugation. The amount and type of vibration should be carefully controlled, in combination with electron-microscope examinations, in order to permit ready separation of the two fractions. If the cells are exposed to excessive vibration or grinding, then the resulting cell-wall fragments are too small to separate from the coarse granular protoplasm. Cell disintegrators like the Raytheon ultra-sonic oscillator were accordingly found not to be useful, since they tended to fragment the cell wall excessively rather than merely to crack it (19), and, therefore, to make impossible the recovery of purified cell-wall fractions. Excessive ether or merthiolate treatment tended to coagulate the protoplasm and prevent its release in a dispersed state, to such an extent that separation of the protoplasm by washing and centrifugation became impossible.

Similarly, most of the activity responsible for the protection of mice or the toxic death of mice was situated in the cell wall. The identity of or relationship between these 3 antigenic actions is still in doubt. It is of interest, however, to note that complement-fixing antibodies were not readily detected in immunized mice which had marked resistance to reinfection or to lethal challenge. Rabbits and guinea pigs, on the other hand, readily developed complement-fixing antibodies in high titers, but showed little tendency to develop marked resistance to lethal challenge (19).

A delayed reaction in sensitized animals was produced by both cell walls and protoplasm. This result may mean that parts of either fraction are capable of initiating a response, or that the particular antigen involved is highly soluble and readily dissolves out of the cell wall into the surrounding fluid.

Since the tissue phase of *H. capsulatum* is yeastlike in some of its properties, the possibility has presented itself that zymosan may be present in the cell wall (20). If zymosan is present, then it would tend to reduce the bactericidal, and possibly mycocidal, properties of serum. However, the cell walls of *H. capsulatum* make the mice more resistant to subsequent challenge, rather than less resistant. Hence, the zymosan-properdin system, if present, has no obvious influence on the immunization of mice against *H. capsulatum*.

Summary. A technic, involving the use of

the Mickle tissue disintegrator and the electron microscope, was described for the separation of the cell wall and the internal protoplasm from the cells of the yeast phase of *Histoplasma capsulatum*. The cell wall contained the antigenic activity associated with complement fixation, protection and toxins. Sensitized animals, on the other hand, showed a delayed type of reaction to either fraction. The protoplasm was more active than the cell wall in the hemolysis of guinea pig erythrocytes.

1. Salvin, S. B., PROC. SOC. EXP. BIOL. AND MED., 1947, v66, 342.
2. Grayston, J. T., *J. Lab. and Clin. Med.*, 1952, v40, 90.
3. Saslaw, S., and Campbell, C. C., *ibid.*, 1948, v33, 1207.
4. Salvin, S. B., and Hottle, G. A., *J. Immunol.*, 1948, v60, 57.
5. Salvin, S. B., *J. Lab. and Clin. Med.*, 1949, v34, 1096.
6. ———, *J. Immunol.*, 1950, v65, 617.
7. Campbell, C. C., and Binkley, G. E., *J. Lab. and Clin. Med.*, 1954, v42, 896.
8. Campbell, C. C., *Am. J. Pub. Health*, 1953, v43, 712.
9. Sorenson, L. J., and Evans, E. E., PROC. SOC. EXP. BIOL. AND MED., 1954, v87, 339.
10. Salvin, S. B., *J. Immunol.*, 1952, v69, 89.
11. ———, *ibid.*, 1953, v70, 267.
12. ———, *Am. J. Hyg.*, 1955, v61, 72.
13. Zarafonetis, C. J. D., and Lindberg, R. B., *Univ. Hosp. Bull.*, Ann Arbor, 1941, v7, 47.
14. Salvin, S. B., *J. Immunol.*, 1955, in press.
15. ———, PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 852.
16. Skinner, C. E., Emmons, C. W., and Tsuchiya, H. M., *Molds, Yeasts, and Actinomycetes*. John Wiley and Sons, N. Y., 1947.
17. Ribi, E., and Salvin, S. B., *Experimental Cell Research* (in press).
18. Salvin, S. B., and Furcolow, M. L., *J. Lab. and Clin. Med.*, 1954, v43, 259.
19. Salvin, S. B., and Ribi, E., unpublished results.
20. Pillemer, L., Blum, L., Lepow, I. H., Ross, O. A., Todd, E. W., and Wardlaw, A. C., *Science*, 1954, v120, 279.

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Reproduction in Rats Fed *Lathyrus* Peas or Aminonitriles. (22013)

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Growth disturbances produced by feeding *Lathyrus* peas vary inversely with age and stage of development of the experimental animal(1,2). Profound effects might be expected, therefore, during rapid growth and development of the fetus. Turney, Salmon, and Copeland reported that feeding *Lathyrus hirsutus* peas prevented completion of pregnancy in the rat, but they did not completely describe the nature of the disturbance(3). They were unable to prevent the effect of peas by addition of alpha-tocopherol to the diet(4), although it has since been reported that large doses of that compound give partial protection against lathyrism(5). For these reasons the effect of *Lathyrus odoratus* peas was studied in regard to reproduction. During this investigation, isolation of the active principle of *Lathyrus pusillus* was announced(6). A similar isolation product of *Lathyrus odoratus* was identified as (β-L-glutamyl-amino) propionitrile(7). The characteristic activity in producing skeletal lesions in rats was also shown by β-amino-propionitrile(1). This compound also profoundly influenced development of larval frogs(2). Aminoacetonitrile was later found to be more toxic to weanling rats than any compound previously tested(8). A number of aminonitrile compounds were consequently included in this study.

Methods. Albino rats of Sprague-Dawley strain were used predominantly. The few hooded rats of Long-Evans strain used gave identical results. The basic diet was ground (Rockland) rat pellets. *Lathyrus odoratus* seeds were finely ground, mixed with this diet in various proportions, refrigerated at 5°C, and fed *ad libitum*. Diets containing syn-