CAPSULAR REACTIONS AND SOLUBLE ANTIGENS OF TORULA HISTOLYTICA AND OF SPOROTRICHUM SCHENCKII*

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This paper deals with capsular reactions and soluble antigens in two species of pathogenic fungi, *Torula histolytica* (*Cryptococcus hominis* or *Torulopsis neoformans*) and *Sporotrichum schenckii*. Although capsular reactions (1) of bacteria have been utilized for some years both in practical diagnosis and in various fundamental studies, a survey of the accessible literature did not reveal any report of serological reactions on the capsules of fungi. The demonstration of capsular reactions for *Torula histolytica* and *Sporotrichum schenckii* seems of general microbiological interest because it establishes the occurrence of a fundamental immunological phenomenon in another biological group (fungi), and at the same time contributes new information on the agents of two diseases. Human infections with these fungi are not of high incidence. However, *Torula* infections of the central nervous system have a high case mortality, and a new aspect of interest in *Sporotrichum* infections has been introduced by the magnitude and the unusual epidemiology of outbreaks of over 2800 cases of sporotrichosis among mine workers in South Africa (2, 3).

There is a considerable literature on both *Torula histolytica* and *Sporotrichum schenckii*, but the immunological information is not systematic nor far advanced. The papers on *Torula* (4–10) give the general impression that most strains have poor immunogenic capacity, although in a few instances serum with reasonably good agglutinating titres have been gotten. Precipitation reactions, when obtained, have been only with undiluted serum; the test antigens usually were filtrates of broth cultures or various extracts of the cells. Kligman (9) prepared a product, presumably of capsular origin, but concluded that it lacked serological properties. We could find no reports of a purified polysaccharide from *Torula histolytica*, which was proved to be serologically reactive, and indeed there has been a rather general belief that the capsular material of this fungus probably is not serologically reactive.

In the case of *Sporotrichum*, agglutination and complement fixation with patients' serum were reported as early as 1908 (11). Some reports have also been made on the agglutination

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reactions of antiserums produced by immunization of laboratory animals; the most comprehensive of these is the recent paper of Lurie (12), who included sixteen strains of *Sporotrichum* of widely different origin and also carried out absorption experiments. However, we found no reports of tests of *Sporotrichum* soluble antigens of even the partial degree of purity of those used in the present study.

Materials and Methods

Strains of Torula and Sporotrichum.—All the strains of both the fungi had been isolated from human infections. *Torula* strain 1 was cultured from spinal fluid in 1940 by Miss Mary E. Hopper at the New York Hospital; No. 2, obtained from Dr. N. F. Conant, is believed to have come originally from the classical Busse (13) case; No. 3, obtained from Centraalbureau voor Schimmelcultures, was isolated before 1912 at the Institut Pasteur in Paris; No. 4, obtained from Dr. F. D. Weidmann, was isolated in 1921. No. 2 was the most highly encapsulated and its cultures on broth and agar were very viscous and mucoid. *Sporotrichum* strain 1 was obtained from Dr. Weidmann; No. 2 from Dr. Rhoda Benham; No. 3, from Dr. Charlotte Campbell; No. 4, from Miss Hopper. None of the strains gave observable capsules on any of the culture media we tested.

Antiserums.—All of the antiserums were from rabbits. The fungi represented were *Torula* strains 1 and 4, *Sporotrichum* strains 1, 2, and 3, two strains of saprophytic *Oospora*, and six others of various species. *Oospora* strain 1936, which will be shown in the experiments to have the capacity of evoking *Torula*-reactive antibodies, was isolated by us in 1936 from the surface of sour cream that had been stored in a household refrigerator. From its source and its morphological properties we considered it to be *Oospora lactis*. Unfortunately, the culture was lost before we discovered its serological relationship to *Torula*, and was not sent to an expert for exact identification. The loss of the culture has prevented experiments on the reactions of antigens of this *Oospora* with antiserums of *Torula* and of other *Oospora*. However, the antiserums produced by immunization with it are useful in the present investigation as a means of demonstrating the *Torula* capsular phenomenon. *Oospora* strain 1940 was similar to strain 1936 in morphology and in source, but it did not produce *Torula*-reactive antibodies in the two rabbits we immunized with it. (Definite conclusions that strain 1940 is entirely lacking in anti-*Torula* immunogenic capacity would require immunization of a larger number of rabbits over a long period of time.)[1]

The immunization routine consisted of courses of five or six intravenous injections at 1 or 2 day intervals with a rest period of about 10 days between each course. Six or more courses were given to all the rabbits, except those immunized with *Torula* and with *Oospora* 1940, which received only four courses. The strain of fungus and the medium for growth of the culture from which the immunizing materials were obtained will be indicated in the presentation of the data in Table I.

Capsular Tests.—Suspensions of the fungus cells both from mice infected by intraperitoneal

[1] The serological properties of the *Oospora* group of fungi have never been systematically investigated. Perhaps a number of different varieties have some immunological relationship with *Torula histolytica*, and would, like strain 1936, produce *Torula*-reactive antibodies if rabbits were immunized with them. On the other hand, it is possible that the possession of a *Torula*-related antigen, comparable to the one apparently possessed by strain 1936, is an uncommon property among the saprophytic *Oospora*. That point is suggested, but not proved, by the fact that antiserums of *Oospora* 1936 failed to agglutinate either *Oospora* strain 1940, or two other strains isolated by us, which were similar to strain 1936 in morphology and in source; and failed also to react with three strains of *Oospora lactis* or *Geotrichum candidum* obtained from the Centraalbureau voor Schimmelcultures.

injection, and from agar cultures, were studied. The suspensions from infected mice were obtained by gently washing the peritoneal cavity with 2 to 3 ml. of sterile salt solution, with particular care to avoid rubbing the peritoneal surfaces; they were made as free as possible from extraneous material by a series of low speed centrifugations with successive removals of the supernatant fluids. The suspensions from cultures were prepared in formalinized or merthiolated salt solution from peptone-yeast extract-glucose agar plate colonies of *Torula* and from blood agar slants of *Sporotrichum*, and were washed several times. The suspensions were stored in the ice box. Since the presence of free soluble antigen decreased the reactivity of the suspensions in capsular tests, the stored suspensions were centrifuged and the supernatant fluid replaced with new formalinized or merthiolated salt solution before each experiment.

Preliminary tests were made upon all the suspensions with a series of dilutions of *Torula*-reactive and of *Sporotrichum*-reactive antiserums. In the case of the suspensions prepared from mice, the cells of all strains of *Torula* and the cells of all strains of *Sporotrichum* reacted with reasonably high dilutions of antiserum. In the case of the suspensions prepared from cultures, none of the *Sporotrichum* gave more than equivocal reactions, even with undiluted serums; all the *Torula* strains gave definite reactions, but, with the exception of strain 2, the capsules were much smaller, and higher concentrations of antiserum were required to produce the reactions than sufficed for the *Torula* cells from mice.

The procedure in the tests was about as in the usual *Quellung* tests on penumococci. In the case of *Sporotrichum*, a loopful of an appropriately dilute suspension of the fungus cells was placed on the center of a coverslip and allowed to dry in the air; a loopful of serum or of serum dilution was placed directly on the dried film; a small amount of stain (dilute aqueous eosin Y or methylene blue) was added, and the coverslip, previously edged with vaseline, was mounted on a slide. The procedure in the *Torula* tests was the same, except that the suspension of fungus cells was not allowed to dry before addition of the serum. In experiments that included comparisons of the degree of reactivity of different antiserums, care was taken to have the number of fungus cells approximately the same in all the test preparations. In the case of *Torula* cells prepared from mice, capsular reactions were recognized about as easily with the low power objective as capsular reactions of pneumococci are with the high power objective. This feature, which is indicative of the size of encapsulated *Torula* cells, was a technical advantage, and a scanning of the entire preparation with the lower power objective was adopted as the first step in the examination of all *Torula* test mixtures.

Agglutination Tests.—The *Torula* suspensions were from cultures on glucose agar slants, and were formalinized and washed several times. The *Sporotrichum* suspensions were from glucose-cystine–rabbit blood agar slants grown at 37°C.; under those conditions, the cultures were less mycelial (14) and gave better suspensions for agglutination than when grown at 25–30°C. on ordinary agar.

In the tests, the cell suspensions were mixed with equal volumes of serum or serum dilution, and incubated for 2 hours, at 55°C. in the case of *Torula* and at 37°C. in the case of *Sporotrichum*. Observations were made then and again after 3 days' storage in the ice box. In the *Sporotrichum* tests, the lowest serum dilution was 1:5, since we had found in previous experiments that agglutination was common with undiluted normal rabbit serums.

Precipitation Tests.—Two products from different strains were used to represent the soluble antigens of each of the two fungi. The first *Torula* product was a polysaccharide material[2]

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[2] This product was kindly supplied by Dr. Edward J. Hehre and Mr. Arthur S. Carlson. It was prepared by further purification of the non-amylose portion, called fraction B by Hehre, Carlson, and Hamilton (16), of the hot-water–soluble material contained in the alcohol precipitate of the culture. The final product contained 0.34 per cent N and 3.0 per cent ash (as

from strain 1 grown, in a continuously aerated flask, in a synthetic medium (15) in which the culture became very acid. The second was an unpurified product from the highly encapsulated strain 2 grown, without aeration, in a medium containing per 100 cc., the dialysate from 2.0 gm. of Bacto peptone and 0.2 gm. of Bacto yeast extract, plus 0.5 gm. sodium acetate added before sterilization and 1.0 gm. glucose after sterilization. The supernatant fluid of the grown culture, which was very mucoid, was precipitated with 2.0 volumes of alcohol; the precipitate was extracted in water, centrifuged to remove insoluble material, reprecipitated with alcohol, and finally taken up in sterile salt solution in a volume equivalent to the original culture.

The first *Sporotrichum* product was from the supernatant fluid of strain 2 grown in the same lot of dialyzed peptone-yeast extract broth utilized in the preparation of the second *Torula* product, and was prepared similarly except that it was subjected to repeated chloroform treatments and several reprecipitations with alcohol. The final solution represented a 50-times concentration of the original culture. The other *Sporotrichum* product was from mice infected with strain 3. During the 3rd week following their intraperitoneal injection, mice that appeared gravely ill were sacrificed; the peritoneal surfaces were rubbed and washed with about 10 ml. of sterile distilled water per mouse. Twenty samples, selected because they were the most viscous and contained abundant *Sporotrichum* (some of them obviously encapsulated) but no other microscopically detectable microorganisms, were combined. After removal of the larger particles and of the *Sporotrichum* cells by centrifugation, the fluid was treated with 2.5 volumes of alcohol. The aqueous solution of the precipitate was given successive shaking treatments with chloroform until no protein-emulsion layer was formed. The water-clear solution was reprecipitated with alcohol several times. The final precipitate, which was gummy and sticky, was taken up in 10 ml. of sterile salt solution, so that each 1 ml. represented the material from two infected mice.

Description of the Capsular Reactions

Torula Cells.—The capsular reaction of this fungus was studied in preparations containing India ink (17) as well as in unstained and in methylene blue preparations. The results of an experiment with these three types of mounts are illustrated in Figs. 1, 2, and 3.

In the unstained and methylene blue preparations (Figs. 1 and 2), the *Torula* mixed with a reactive serum showed prominent capsules of considerable area with a relatively dark and sharply outlined border; whereas, the *Torula* mixed with a control antiserum or with salt solution showed either no capsules or only indistinct halos. The marked difference in the capsular areas apparent in these preparations might suggest that the reactive serum had caused a tremendous swelling of an originally small capsule. That this was not the case was shown by the India ink preparations, in which the capsular areas of the *Torula* cells mixed with a control serum (Fig. 3 *B*) or with salt solution (Fig. 3 *C*) appeared to be about the same size as the capsular areas shown by the *Torula* cells mixed with reactive antiserum in the preparations containing no India ink (Figs. 1 *A*

Na), and upon acid hydrolysis, yielded 65 per cent reducing sugar if calculated as glucose; it gave a strongly positive Bial test indicative of the presence of pentoses; tests for starch were negative. It gave positive precipitation reactions in dilutions of one to two million with the *Torula* and the *Oospora* 1936 serums.

and 2 *A*). This comparison showed that the major part of the phenomenon seen in the preparations not containing India ink depended, not upon increase in actual size of the capsule, but upon some change which made the capsular structure more visible. The experiment could not determine the exact effect produced by the action of the antiserum. However, the increase in visibility may have involved both an increase in the optical density of the outer part of the capsular substance, which was indicated by the darkening of the capsular substance in the preparations containing no India ink (Figs. 1 *A* and 2 *A*), and also an increase in the "stickiness" of the capsular surface, which was indicated by the rapid piling up of ink particles on the capsules in the India ink mixtures containing reactive serum (Fig. 3 *A*).[3] An increased "stickiness" of the capsular surface would facilitate the peripheral accumulation of any extraneous material, and in that way, contribute to the darkness and distinctness of the capsular borders seen in preparations containing no India ink. Some increase in size of the capsule probably did occur, but the fact that it was not great enough to be visually prominent is of interest, since swelling is a prominent visual feature in the capsular reactions of bacteria. The much greater volume or depth of the capsular substance of *Torula* cells is a factor to take into account in comparing the phenomena visible in the capsular reactions of this fungus with the phenomena seen in the capsular reactions of bacteria. However, many other factors complicate the comparison, and without data from experiments planned for that purpose, no explanation can be attempted.

Although positive capsular reactions were given by suspensions of the cells of all strains whether obtained from cultures or from infected mice, the microscopic picture varied, due to differences in the size both of the capsule and of the endocapsular portion of the cells. The reactions of cells with moderately large capsules and relatively small endocapsular portions are shown in Figs. 1 and 2. The reactions of cells with relatively small capsules and large endocapsular portions are shown in Fig. 4. The reactions of irregularly shaped cells, which accompanied the usual round cells in some of the suspensions from mice, are shown in Figs. 5 and 6.

Sporotrichum Cells.—When *Sporotrichum* cells prepared from mice were mixed with *Sporotrichum*-reactive antiserums (Fig. 7 *A*), capsules with a distinctly outlined and relatively dark outer border were clearly evident;

[3] In Fig. 3 *A*, the ink particles cover such a large part of the capsules that the borders are hidden. When mounts of similar mixtures of *Torula* cells plus reactive serum are prepared with more dilute India ink and examined immediately, the attachment of the ink particles to the capsular borders is more apparent. As the preparations stand, the particles collect at a rapid rate, and soon the borders are completely covered as in Fig. 3 *A*. Ink particles also collect on the periphery of the capsules in mixtures of *Torula* cells plus non-reactive serum, but they collect much more slowly, and even in preparations left standing overnight fewer ink particles are on the periphery of the capsules than are present within a few minutes in mixtures of *Torula* plus a reactive serum.

whereas, when mixed with a control antiserum (Fig. 7 *B*) or with salt solution (Fig. 7 *C*), no definite capsules were apparent. However, it should be noted that *Sporotrichum* cells which showed capsules in ordinary wet mounts without serum were frequent in some of the materials (obtained by vigorous rubbing of the peritoneal surfaces) that were utilized for preparing the *Sporotrichum* soluble antigen, described under "Methods." The materials in these instances were noticeably mucoid and viscous, and the encapsulated fungus cells were embedded in gelatinous masses. Capsules on *Sporotrichum* organisms were prominent also in a number of Hastings' stained impression-preparations made from the livers of infected mice.

Suspensions of *Sporotrichum* cells prepared from cultures did not give satisfactory capsular reactions in the preliminary tests and hence were not used in any of the reported experiments.

Association of the Capsular Reactivity of Serums with Capacity to Agglutinate Suspensions and to Precipitate Soluble Antigens of the Fungi

In the following experiment, a collection of *Torula*, *Oospora*, *Sporotrichum*, and other fungus antiserums were tested for their reactivity with the capsules of *Torula* and of *Sporotrichum*. In order to relate the capsular reactions to specific antibodies, the serums were tested also for capacity to agglutinate suspensions and to precipitate soluble antigens of *Torula* and *Sporotrichum*. The results are summarized in Table I. In the case of the *Torula* and *Oospora* serums, data for all the available bleeding samples are included to aid the interpretation of the *Torula*-cross reaction that was given by the *Oospora* 1936 serums. In the case of the *Sporotrichum* serums, data are presented only for sufficient samples to show the relative ease of producing capsular-reactive antiserums by immunization with that fungus.

The tests for capsular reactivity were made with a suspension of *Torula* No. 3 and a suspension of *Sporotrichum* No. 3, prepared from mice. The agglutination tests were made with a suspension of *Torula* No. 1 and a suspension of *Sporotrichum* No. 3, prepared from cultures. The soluble antigens described under "Methods" were employed in the precipitation tests; in the case of the two antigens from dialyzed peptone broth cultures, the dilutions used contained about 80 times the minimal precipitating dose found in preliminary titrations against potent samples of *Torula*-reactive and of *Sporotrichum*-reactive serums; in the case of the purified *Torula* polysaccharide and the *Sporotrichum* antigen from mice, the dilutions used contained about 20 and 10 times the minimal precipitating dose.

The capsular reactivity is presented by symbols: ++++ = strong or moderate with 1:9 or higher dilutions; +++ = strong or moderate with 1:3, weak or negative with 1:9; ++ = moderate to weak with undiluted, negative with 1:3 and 1:9; 0 = negative with undiluted serum. The agglutinating and precipitating titres are presented in terms of the highest dilution of serum that gave positive reactions; 0 = no reaction with undiluted serum; U = reactions only with undiluted serum; the figures given represent the initial dilutions of the serums, and are based upon the 3 day observations, which, particularly in the case of the *Torula* agglutinins, were at least one dilution higher than the titres for the 2 hour observations.

TABLE I

Comparison between the Capacities of Antiserums to React with Torula and Sporotrichum Capsules and Their Capacities to Agglutinate Suspensions and to Precipitate Soluble Antigens of the Same Fungi

Serum			Capsular reactions		Agglutinating and precipitating titers					
					Torula			*Sporotrichum*		
Rabbit No.	Immunized with	Bleeding sample*	*Torula*	*Sporotrichum*	Suspension of cells	Soluble antigens AT‡	Soluble antigens BT‡	Suspension of cells	Soluble antigens AS‡	Soluble antigens MS‡
1	*Torula*, strain 1§	3d, 4th	++	0	3, 9	U, 3	3	<5	0	0
	" " "	1st, 2d	0	0	3	0	0	<5	0	0
2	" " 4§	1st, 2d, 3d, 4th	0	0	0–U	0	0	<5	0	0
3	*Oospora*, strain 1936‖	1st	0	0	3	0	0	<5	0	0
	" " "	2d, 3d, 5th, 6th	++	0	20–40	U–9	3–9	<5	0	0
	" " "	4th, 7th	+++	0	80, 160	9, 20	9, 20	<5	0	0
4	" " "	1st	++	0	9	U	3	<5	0	0
	" " "	2d, 3d, 4th, 6th	+++	0	20–80	9–20	9–20	<5	0	0
	" " "	5th, 7th, 8th, 9th	++++	0	160–640	20–80	20–80	<5	0	0
5, 6	*Oospora*, " 1940¶	1st, 2d, 3d, 4th	0	0	0	0	0	<5	0	0
7	*Sporotrichum*, strain 1**	1st, 2d, 3d	0	0	0	0	0	9	U–3	U
	" " "	4th	0	++	0	0	0	20	9	3
	" " "	9th	0	+++	0	0	0	80	20	20
8	" " 2	1st, 2d	0	+++	0	0	0	80	40	20
9	" " "	5th, 6th, 7th, 8th	0	++++	0	0	0	320–640	40–160	40–80
10–12	" " 3	1st	0	+++	0	0	0	80–160	20	20
13, 14	*Penicillium*‡‡		0	0	0	0	0	<5	U, 3	U, 0
15–26	Other fungi‡‡		0	0	0	0	0	<5	0	0
1–14	Normal serums	Pre-immunization	0	0	0	0	0	<5	0	0

* Bleedings were taken about 10 days after each course of injections. The numbers refer to the courses that had been given at the time the sample was taken.

‡ AT and AS = *Torula* and *Sporotrichum* antigens obtained from cultures grown in dialyzed peptone broth; BT = purified polysaccharide obtained from *Torula* culture grown in synthetic medium; MS = antigen from *Sporotrichum*-infected mice.

§ Heated suspensions prepared from cultures grown on glucose-peptone-yeast extract agar at 30°C.

‖ Heated suspensions prepared from cultures grown on glucose-peptone agar at 25°C.; the suspensions were repeatedly frozen and thawed before being heated.

¶ Suspensions similar to those prepared from strain 1936, but not frozen and thawed before being heated.

** The suspensions used for rabbit 7 were prepared from cultures grown on glucose-peptone agar at 25°C., and consisted largely of the mycelial form of the fungus; the suspensions used for rabbits 8 to 12 were from cultures grown on blood agar at 37°C., and consisted almost entirely of unicellular forms.

‡‡ Two rabbits were immunized with each of the six following fungi: *Candida albicans* (two strains), *Trichophyton mentagrophytes*, *Penicillium spinulosum*, *Aspergillus amstelodami*, *Mucor circinelloides*. The suspensions used for the injections were from cultures grown on the same medium and had been subjected to the same freezing and thawing treatment as the suspensions of *Oospora* 1936. These antiserums had been proved, in supplementary experiments, to be reactive with soluble antigens of the homologous fungus prepared by the same method as described, under "Methods," for the AT and AS antigens used in this experiment.

It is evident (Table I) that the antiserums of *Torula* and of *Oospora* strain 1936 reacted with the *Torula* capsules and that the *Sporotrichum* antiserums

reacted with the *Sporotrichum* capsules. The specificity of these reactions is shown by the negative results obtained with the other antifungus serums. Moreover, the negative reactions of the *Oospora* 1940 antiserums indicate that the capacity to produce *Torula*-reactive antibodies is not a regular occurrence among *Oospora* strains.[1]

Evidence that the reactions of the *Torula* and of the *Sporotrichum* capsules were due to the action of immunologically specific antibodies is furnished by the direct relationship, apparent throughout the data, between the degree of capsular reactivity of the serums and the capacities of the serums to agglutinate suspensions and to precipitate soluble antigens of the respective fungi. For example, the antiserums of *Oospora* 1936, which had a higher degree of *Torula* capsular reactivity than the *Torula* antiserums, exceeded them also in capacity to agglutinate *Torula* suspensions and to precipitate *Torula* soluble antigens;[4] likewise, the samples of *Sporotrichum* antiserum from rabbit 7, which had the lowest degree of *Sporotrichum* capsular reactivity, also showed the lowest content of *Sporotrichum* antibodies in the agglutination and precipitation tests.

It is evident in the data that the most reactive samples among the antiserums obtained by immunization with *Torula* cells[4] gave *Torula* capsular reactions only when tested undiluted. However, some of the antiserums obtained by immunization with *Oospora* 1936 gave moderately strong *Torula* capsular reactions when diluted 1:40, which shows that *Torula* capsules in spite of their relatively great size, are potentially capable of giving observable reactions with reasonably high serum dilutions, provided the antiserums are reasonably high in antibody content. Antiserums of sufficient potency to give strong *Sporotrichum* capsular reactions are apparently easy to obtain by immunization with *Sporotrichum* cells, since a single series of injections sufficed with the five rabbits (Nos. 8 to 12) immunized with the unicellular forms of that fungus.

Although their chief use in the present paper is to establish the immunological nature of the capsular reaction, the data on the soluble antigens of both the fungi include new information of independent value. For example, the *Torula* polysaccharide,[2] prepared from a culture grown in a synthetic medium, gave precipitation in dilutions of one or two million, which, we believe, presents the first evidence of serological reactivity of a purified polysaccharide from *Torula histolytica*. The data on the *Sporotrichum* soluble antigens are the first information on the serological reactivity of even moderately purified products of this species in tests controlled by antiserums of other fungi; the antigen from infected mice has special interest because serological tests with soluble antigens obtained directly from animals have not previously been reported for *Sporotrichum* and have rarely if ever been reported for any species of fungus.

[4] The experiment was not planned to compare the immunogenic capacities of *Torula* and *Oospora* strain 1936, and we do not regard the low potencies of the *Torula* antiserums in Table I as evidence of a consistently poor immunogenic capacity on the part of *Torula* cells. We are now immunizing rabbits with other strains of *Torula* in an attempt to get more reactive antiserums.

Absorption of the Serums with the Soluble Antigens

In the preceding experiment (Table I), the degree of reactivity of serums with *Torula* and *Sporotrichum* capsules was shown to be directly related to the capacities of the serums to precipitate soluble antigens of the respective fungi. The question of whether or not these soluble antigens would absorb the capacity of serums to react with the capsules of the same fungi, was tested in the following experiment.

Two series of 10 tubes, each containing 0.3 cc. of 1:2 dilution of potent samples of *Oospora* 1936 or of *Sporotrichum* antiserum, were prepared; 0.1 cc. of one of the following solutions was added to a separate tube of each series: (1) salt solution; (2) and (3), undiluted stock solutions of the *Torula* or of the *Sporotrichum* antigens from cultures grown in dialyzed peptone; (3) and (4), 1:1,000 and 1:5,000 dilutions of the purified *Torula* polysaccharide; (5) undiluted stock solution of the *Sporotrichum* antigen from mice; and (6 to 10), for controls, 1:1,000 dilutions of the following polysaccharides: Pneumococcus C, *Leuconostoc mesenteroides* B dextran, *Streptococcus salivarius* levan, Bacillus N9 levan, and 1:5,000 dilution of type 2 pneumococcus S. After incubation for 2 hours at 37°C., storage overnight in the ice box, and centrifugation, the supernatant fluids were transferred to new tubes. The same amount (0.1 cc.) of the same material as in the first absorption was added to each tube, and the mixtures incubated, stored in the ice box, and centrifuged as before.

The supernatants of the final mixtures, containing about 1:3 dilution of the serums, were then tested for capsular reactivity against suspensions of *Torula* and of *Sporotrichum*. In the case of *Torula*, the capsular tests were made with suspensions of each of the four different strains (Nos. 1, 3, and 4 from mice, and No. 2 from cultures); in the case of *Sporotrichum*, suspensions from only two strains were available at the time of the experiment. The results, which were the same for the different strains of each fungus, are summarized in Table II, and illustrated in Figs. 8 and 9.

As shown in Table II, absorption with the *Torula* soluble antigens removed the capacity of the *Oospora* antiserums to react with the *Torula* capsules but did not diminish the capacity of the *Sporotrichum* antiserum to react with the *Sporotrichum* capsules; whereas, absorption with the soluble antigens of *Sporotrichum* removed the *Sporotrichum* capsular reactivity from the *Sporotrichum* antiserum but did not appreciably affect the *Torula* capsular reactivity of the *Oospora* antiserum. Further evidence of the specificity of the absorption is that the bacterial polysaccharides, which were included as controls on the possibility that a considerable concentration of any polysaccharide material might inhibit the capsular reactions, had no detectable influence upon the capsular reactions of either of the fungi. The negative results obtained with the Pneumococcus C substance deserve separate mention because that substance has been found (18) to absorb the capacity of serums to give non-specific capsular swelling of pneumococci. The sum of the data in Table II confirms the previous evidence that the reactions of the capsules with unabsorbed serums (Table I) were caused by immunologically specific antibodies.

The ability of these soluble antigens to absorb the capsular reactivity of the *Oospora* and of the *Sporotrichum* antiserums has another significant aspect.

That is, this ability is a strong indication that these products, prepared from cultures of *Torula* and of *Sporotrichum* and from *Sporotrichum*-infected mice, are related to the antigens involved in the capsular reactions observed on the cells of the fungi. That point is particularly important in the case of the purified *Torula* polysaccharide, because it indicates that the major antigens involved in the capsular reactions of *Torula* cells are polysaccharides, identical with or closely similar to the product used in the present study. Other substances probably accompany it in the large *Torula* capsule, but apparently polysaccharides like the present product occur on the capsular surfaces in sufficient amount to account for the microscopically observable changes that

TABLE II

Tests with Absorbed Serums

Antigen* used for absorption of the antiserums	Capsular reactions‡	
	Torula cells *vs.* *Oospora* antiserum	*Sporotrichum* cells *vs.* *Sporotrichum* antiserum
Salt solution (unabsorbed serum)	+++	+++
Torula purified polysaccharide	0	+++
Torula antigen from broth culture	0	+++
Sporotrichum antigen from broth culture	+++	0
Sporotrichum antigen from infected mice	+++	0
Pneumococcus C substance	+++	+++
Various other bacterial polysaccharides§	+++	+++

* The two *Torula* antigens and the two *Sporotrichum* antigens used for absorption were prepared from different strains of the respective species.

‡ The capsular tests included cells from four strains of *Torula* and cells from two strains of *Sporotrichum*.

§ *Streptococcus salivarius* levan, Bacillus N9 levan, *Leuconostoc mesenteroides* B dextran, type 2 pneumococcus capsular polysaccharide.

occur when antiserums react with the cells. This point is emphasized because there has been a common belief that the capsules of *Torula histolytica* do not include serologically reactive constituents.

Capsular Reactions with Oxalated Serums and with Concentrated Serums

Following Abernethy and Avery's (19) report on the dependence of anti-C reactivity upon the presence of calcium in the serum, Carlens (20) and Lofstrom (18) found that binding of calcium by addition of potassium oxalate would remove the capacity of serums to give non-specific capsular swelling of pneumococci. We applied the oxalate treatment used by Lofstrom (18) to samples of *Oospora* and of *Sporotrichum* antiserum, and found it to have no appreciable influence upon their capacity to give capsular reactions with *Torula* and *Spo-*

rotichum cells. The results with these oxalated serums, together with the previously demonstrated failure of Pneumococcus C substance to absorb or to inhibit the capsular reactivity, clearly distinguish the capsular reactions described in this paper from the class of reactions represented by Lofstrom's (21, 18) non-specific capsular swelling of pneumococci.

We have observed capsular reactions with *Torula histolytica* when mixed with commercially concentrated therapeutic antiserums (especially horse but also rabbit to some extent), which at present seem to us to be immunologically non-specific phenomena, perhaps somewhat similar to though not identical with the non-specific capsular swelling of pneumococci by various protein solutions reported by Jacox (22). This phenomenon with *Torula* cells is being studied further.

DISCUSSION

The reactions between antiserums and the capsules of *Torula histolytica* and of *Sporotrichum schenckii*, which are described in this paper, are evidence that the capsules of these fungi contain serologically reactive substances. The data demonstrate that the capacity of serums to produce the capsular reactions is related to their capacities to agglutinate suspensions and to precipitate soluble antigens of the respective fungi; they also show that the capsular reactivity is removed from the serums, in a specific way, by absorption with soluble antigens derived from the particular fungus. These points establish the immunological nature of the capsular reactions of *Torula* and of *Sporotrichum*, and place them in the same class of serological reactions as the commonly recognized capsular swelling of bacteria. In view of the proved usefulness of the capsular reactions of bacteria in both practical and fundamental studies, the demonstration of this phenomenon among the fungi seems to us of general interest. In the first place, there is great need for the accumulation of new immunological information on this microbiological group. In the second place, some of the unicellular forms of fungi, because of their larger size and more complex structure, might perhaps lend themselves better than bacteria to the study of certain fundamental aspects of the capsular reaction.

Although identification of the microorganism is the usual aim in its practical application, the *Quellung* test was utilized also, in Neufeld's laboratory (23), as an aid in demonstrating the existence of capsular structures in various bacterial species. Its usefulness for demonstrating the capsular structures of unicellular fungi was evident in our observations. In the case of *Sporotrichum schenckii*, which has not commonly been considered to be an encapsulated species, halos were frequently seen around the fungus cells in ordinary wet mounts (no antiserum or India ink) of materials from the peritoneal surfaces, liver, and testes of infected mice; but, these halos did not furnish as clear evidence for a capsular structure as did the definitely outlined capsular borders seen in the serological

test mixtures (Figs. 7 and 9). In the case of *Torula histolytica*, either India ink preparations without antiserum (17) or serological preparations without India ink sufficed to demonstrate the large capsules of highly encapsulated forms, like those illustrated in Figs. 1, 2, and 3. The advantage of the serological preparations was that they revealed definite, although relatively narrow, capsular structures on weakly encapsulated forms (cells from agar cultures of some *Torula* strains) which gave negative or only equivocal signs of capsules in India ink preparations without antiserum.

The question of the practical application of the capsular reactions of these fungi requires further study. The visual prominence of the *Torula* capsule in preparations containing a reactive antiserum would be likely to aid in the microscopic detection of *Torula* cells in spinal fluids, with less complication from artefacts than might be encountered with India ink. However, in order to apply the capsular reaction for identification of *Torula histolytica*, more data are needed on the species-specificity, particularly whether or not other encapsulated yeast-like fungi (various species of *Torula*, *Torulopsis*, *Rhodotorula*) would react with the same serums. With that information at hand, the capsular reaction might be used in the study of strains isolated from patients by culture. But, the application to materials direct from patients would be limited, because specimens containing microorganisms in sufficient numbers to be seen in microscopic examination are by no means always obtainable in *Torula* infections of human beings and are apparently rare in human sporotrichosis.

The antiserums obtained by immunization with *Oospora* strain 1936,[1] which were highly reactive with the *Torula* capsules, also precipitated solutions of the purified *Torula histolytica* capsular polysaccharide[2] and agglutinated suspensions of all strains of that species that were tested. These cross-reactions are the first evidence of serological relationship between saprophytic *Oospora* and *Torula histolytica*. Since this relationship involved a capsular polysaccharide antigen of *Torula histolytica*, the reactions of the *Oospora* antiserums with the capsules of *Torula* cells are similar, in principle, to the capsular cross-reactions reported for type 2 pneumococcus and type B *Bact. friedlaenderi* (24), and for other serologically related species of bacteria (25–27).

SUMMARY

The present paper deals with reactions between antiserums and the capsules of *Torula histolytica* (*Cryptococcus hominis* or *Torulopsis neoformans*) and of *Sporotrichum schenckii*. The reactions, although similar in principle to the capsular swelling seen in *Quellung* tests of bacteria, present a special interest because the microorganisms are larger in size and more complex in structure than bacteria.

That the reactions on the capsules were caused by immunologically specific

antibodies was shown by the fact that the capsular reactivity of the serums was directly related to their capacities to agglutinate suspensions and to precipitate soluble antigens of *Torula* and of *Sporotrichum*, and by the fact that the capsular reactivity was removed from the serums by absorption with soluble antigens of the respective fungi.

The soluble antigens included partially purified products from broth cultures of *Torula* and of *Sporotrichum*, a partially purified product from *Sporotrichum*-infected mice, and a purified polysaccharide from a culture of *Torula* grown in a synthetic medium. The purified polysaccharide was highly reactive in precipitation tests with unabsorbed serum, and its ability to absorb the *Torula* capsular reactivity from the serums indicates that the major antigens on the surface of encapsulated *Torula* cells are polysaccharides, identical with or similar to the product used in the present study.

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EXPLANATION OF PLATES

The lighting conditions and diaphragm openings were the same in photographing the positive and negative reactions comprised in each of the figures presented. The photographs are by Dr. Smith and Mr. Carlson.

PLATE 8

FIG. 1 to 3. Experiment with *Torula histolytica* in the presence and in the absence of India ink, described in the text. The cells are strain 2 from agar culture. × 700.

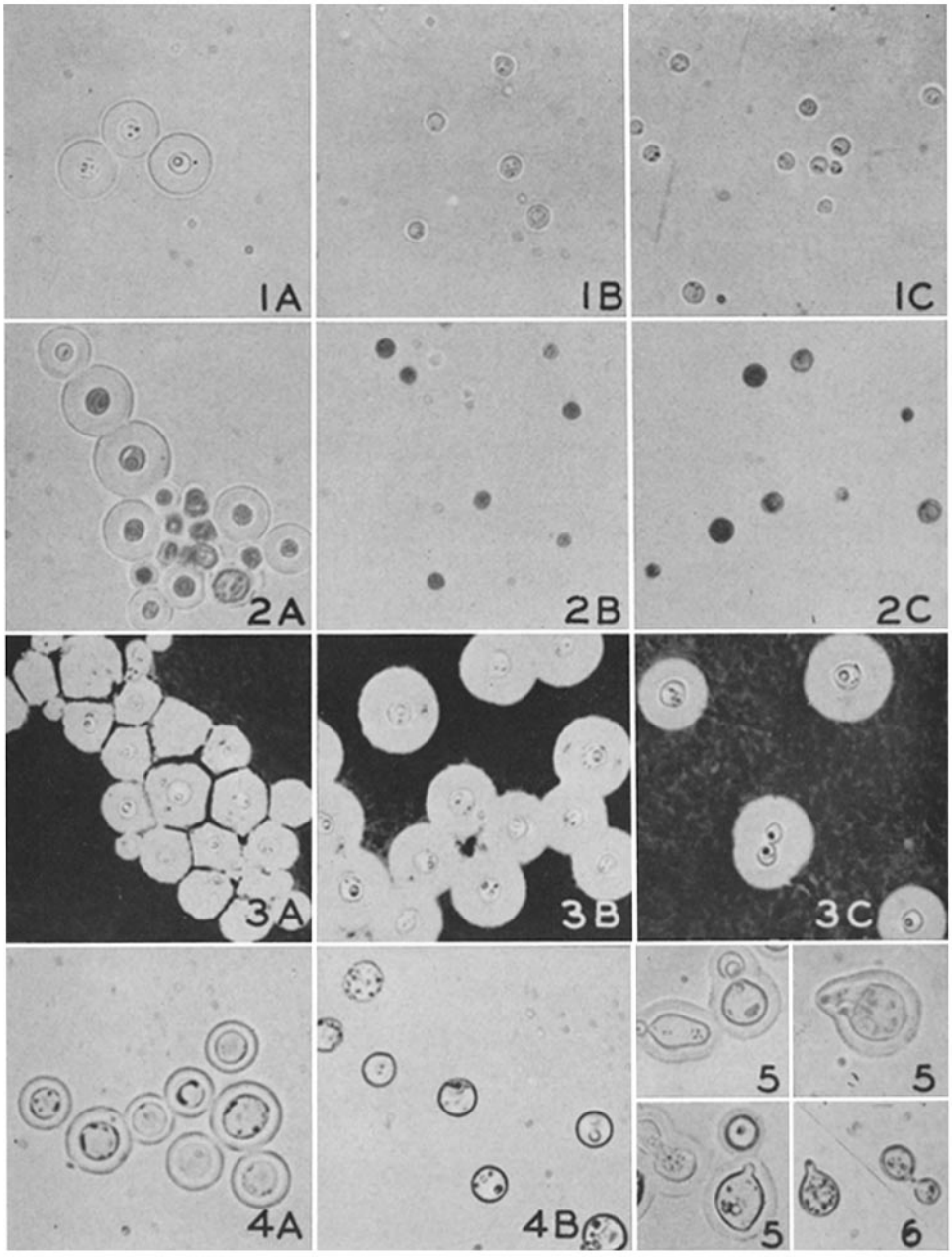
1 *A*. *Torula* cells plus *Oospora* 1936 serum, unstained.
1 *B*. " " " *Sporotrichum* " " .
1 *C*. " " " salt solution, " .
2 *A*. " " " *Oospora* 1936 serum plus methylene blue.
2 *B*. " " " *Sporotrichum* " " " " .
2 *C*. " " " Salt solution " " " .
3 *A*. " " " *Oospora* 1936 serum " India ink.
3 *B*. " " " *Sporotrichum* " " " " .
3 *C*. " " " salt solution " " " .

FIG. 4. Capsular reactions of large cells of *Torula histolytica*. The cells are strain 3 from mice; this strain regularly had a smaller capsule than did the other strains. × 700.

A. *Torula* cells plus *Oospora* 1936 serum.
B. " " " *Sporotrichum* " .

FIG. 5. Capsular reactions of irregularly shaped cells of *Torula histolytica* strain 3, when mixed with *Oospora* 1936 serum. (A small number of irregular forms accompanied the usual round forms in some suspensions obtained from mice.) × 700.

FIG. 6. Irregularly shaped cells of *Torula histolytica* from the same suspension as those in Fig. 5, plus *Sporotrichum* serum. × 700.



(Neill *et al.*: Capsules of pathogenic fungi)

PLATE 9

FIG. 7. Capsular reactions of *Sporotrichum schenckii*. The cells are strain 2 from mice. × 1400.

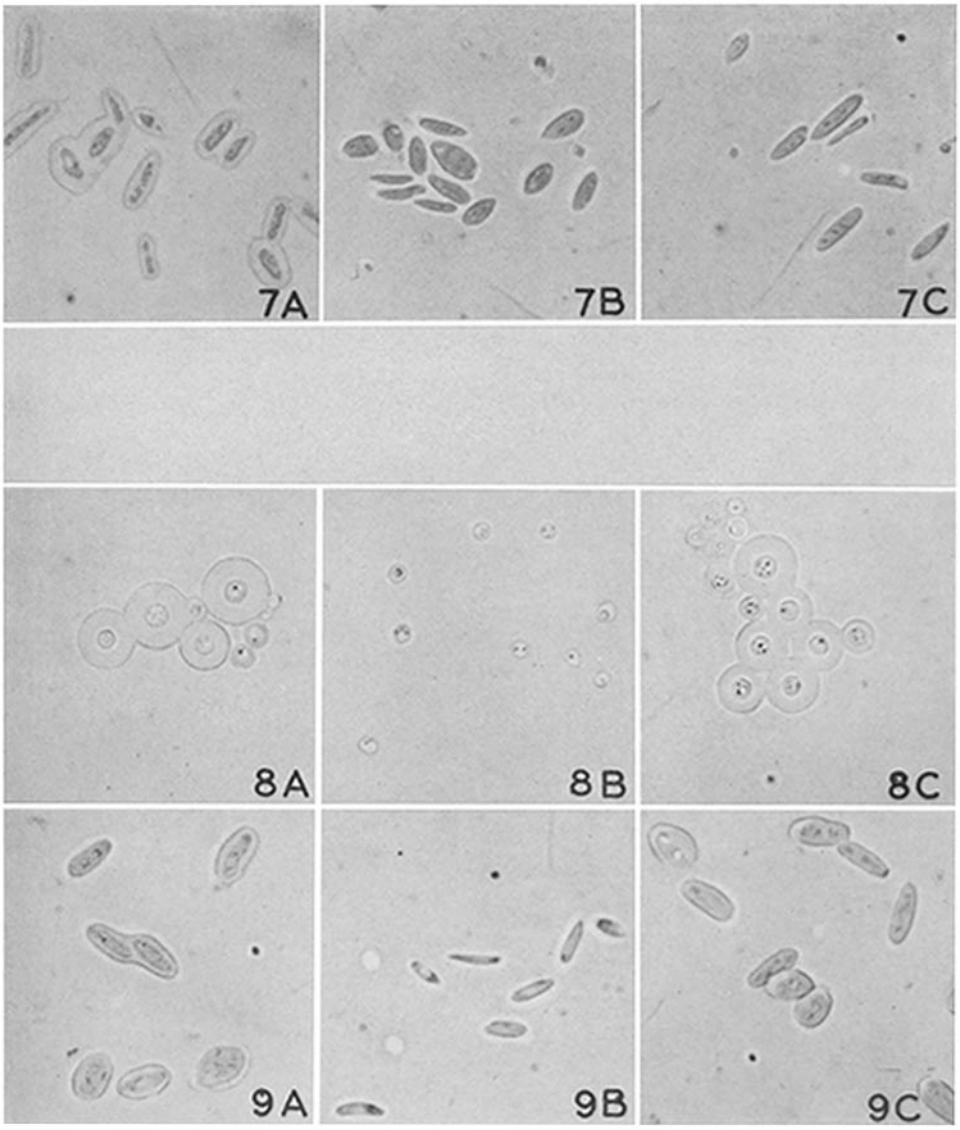
A. *Sporotrichum* cells plus *Sporotrichum* serum.
B. " " " *Oospora* 1936 "
C. " " " salt solution.

FIG. 8. Inhibition of the capsular reactions of *Torula histolytica* by absorption of the serum with *Torula* antigen, but not by absorption with *Sporotrichum* antigen. The preparations are from the experiment presented in Table II. The *Torula* cells in *A*, *B*, *C* are from the same suspension. × 700.

A. *Torula* cells plus *Oospora* 1936 serum, unabsorbed.
B. " " " the same serum absorbed with the *Torula* purified polysaccharide.
C. *Torula* cells plus the same serum absorbed with the *Sporotrichum* soluble antigen prepared from broth cultures.

FIG. 9. Inhibition of the capsular reaction of *Sporotrichum schenckii* by absorption of the serum with *Sporotrichum* antigen, but not by absorption with *Torula* antigen. The preparations are from the experiment presented in Table II. The *Sporotrichum* cells in *A*, *B*, *C* are from the same suspension. × 1400.

A. *Sporotrichum* cells plus *Sporotrichum* serum, unabsorbed.
B. " " " the same serum absorbed with the *Sporotrichum* soluble antigen prepared from broth cultures.
C. *Sporotrichum* cells plus the same serum absorbed with the *Torula* purified polysaccharide.



(Neill *et al.*: Capsules of pathogenic fungi)