our studies, the collagen content of 10-day embryonic skin dropped to 60% of that on day 9. This is because hydroxyproline values were determined for *whole* skin, not for the dermis alone. On day 9, the epidermal component grew much more rapidly, due to feather formation, than the dermis. The *relative* amount of the collagen is therefore reduced without a decline in the absolute values. The shift in the ratio of insoluble to neutral salt soluble collagen, in the developing dermis, parallels a similar change observed in whole chick embryos (13). A marked increase in insoluble collagen was also found in wound healing of mammals between 8 and 12 days after wounding, especially around day 10 (11). According to Gillman *et al.* (14) and Lindsay and Birch (15), this coincides with the time when the wound epidermis stops its infiltrative downgrowth and begins to cover the wound surface.

The epidermal wound infiltration observed by Weiss and Matoltsy in chick embryos less than 10 days old could be related to the poor collagen polymerization at these early stages. The close time relationship between an increase in collagen polymerization and the first appearance of wound healing suggests that the collagen structure of the dermis strongly influences the behavior of epidermal cells.

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Serologic Grouping of *Cryptococcus neoformans* (32812)

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Evans (1,2) divided the species *Cryptococcus neoformans,* a pathogenic fungus, into three antigenic types, A. B, and C by agglutination, precipitation, and capsular reactions. Agglutinins in the sera of rabbits immunized with killed cryptococcal cells have appeared to be directed against both type specific (2) and species specific (3) antigens. The antigens concerned with type specificity have been associated with the capsular polysaccharide (4). The purpose of this study was to examine further the antigenic variation among isolates of *C. neoformans* and to determine whether serotyping would have value as an epidemiological tool.

Materials and Methods. Cultures tested. All cultures were designated *C. neoformans* by previously published criteria (5). In this scheme, *C. neoformans* was separated from

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other members of the genus Cryptococcus by growth at 37°C, mouse pathogenicity, and brownish colonies on Guizotia seed agar. One hundred and six isolates of *C. neoformans* were used. Strains A [16],[1] A [111], B [17], C [18], and C [113] were donated by Dr. E. E. Evans.[2]

Strain B [88] was a dry variant isolated from B [17]. Evans' L2 strain, our B [112], was a mutant of the strain 1523 that he used in his original studies. Six isolates were obtained from soil or avian droppings and sent to us by Dr. C. W. Emmons.[3] One strain was isolated from mesquite bark in Arizona and one strain was a poorly encapsulated variant of the Arizona strain.[4] Fifteen strains were obtained from California.[5] The remainder were from patients at this institution. Of the 106 isolates, 86 were from clinically infected patients and of the latter, 74 were from cerebrospinal fluid, 8 from sputum, 2 from skin, 1 from lymph node and 1 from brain tissue.

Immunization of rabbits. Cryptococcus strains for immunization were grown on 2% glucose, 1% neopeptone agar at room temperature for 72 hours. The cells were harvested in 0.85% saline, heated in a water bath at 100°C for 1 hour, washed twice in sterile 0.85% saline and preserved with 0.1% sodium azide. Rabbits weighing 2 kg were given intravenous injections of 2×10^8 cells (determined by hemocytometer count) in sterile buffered 0.85% saline. Cultures of the vaccines for bacteria and fungi after each series of injections showed no growth. Rabbits received either 10 daily injections or two 5-day series, 2 days apart. One week after the last injection, titers against the immunizing strain were measured by slide agglutination. Courses of injections were repeated up to four times until minimum titers of 1:128 were reached with the homologous cells. Rabbits were exsanguinated and the antisera, preserved with 0.1% sodium azide, were stored at 10°C when in use or −20°C for prolonged storage.

Agglutination and absorption. Serum was tested by slide agglutination with suspensions of cells in a concentration corresponding to McFarland tube no. 5. Cells to be serotyped were either taken directly from slant cultures or treated by heat killing and washing. They were then suspended in 0.85% buffered saline with 0.1% sodium azide. The serotyping results of both methods were identical. The tests were run using 0.05 ml of cells and 0.05 ml of antisera. Saline controls were used. Optimal agglutination was seen after 10 min on a horizontal platform rotating 160 times/min. Titers were expressed as final dilutions of antiserum with agglutinating cells.

Antisera used for final serotyping were first absorbed with a mixture of cells of the other serotypes, the total volume of heat-killed packed cells being equal to the volume of antiserum. Antiserum and cells were incubated at 37°C for 30 min. In a second absorption two volumes of packed cells were incubated similarly with three volumes of antiserum. After the two absorptions, titers against the absorbing strains were negative at 1:2 dilution. Absorbed antisera used for serotyping were diluted 1:4 prior to use.

Results. Immunization with strains B [112] and C [18] produced antisera of sufficient titer to withstand absorption by heterologous cells (Table I). For this reason, antisera were prepared in rabbits using six isolates which did not type B or C. With two of these isolates, [68] and [52], antisera were produced which retained a homologous titer of 1:32 or greater after absorption with both types B [112] and C [18] cells. In addition, antiserum to either isolate, [68] or [52], retained its homologous titer after absorption

[1] Upper case letters refer to the serotype, the bracketed numbers to the NIH collection number.

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TABLE I. Absorption of Antiserum for Serotyping.

	Antiserum			
	Type A	Type B	Type C	Type D
Immunizing strain	68	112	18	52
Absorbing strains	52 (128)[a]	68 (1024)	68 (128)	68 (256)
	112 (8)	52 (512)	52 (16)	112 (256)
	18 (4)	18 (256)	112 (64)	18 (32)
Initial titer vs immunizing strain	1024	2048	128	256
Final titer vs immunizing strain	32	64	32	64
Final titer vs all absorbing strains	<2	<2	<2	<2

[a] Figures in parentheses represent original titers to the absorbing strain. Titers are expressed as reciprocal serum dilutions.

by the other isolate, indicating a difference in serotype. The antiserum to isolate [68] lost its titer after absorption with Evans' A [16] cells and was considered to be type A. Antiserum to isolate [52] withstood absorption with cells of all three of Evans' serotypes and was considered to represent a new serotype, type D.

Unabsorbed antisera reacted with the three heterologous cell types in varying degrees (Table I) and one absorption did not remove all heterologous titers. In order to make specific typing sera, it was necessary to absorb first with the other three types and then with cells of the serotypes to which the antisera still retained heterologous titer. That is, anti-A serum was absorbed with D cells, anti-B serum with C and D cells, anti-C serum with A and B cells and anti-D serum with A cells. After the two absorptions, titers to the heterologous cells were no longer demonstrable at 1:2 dilution and their homologous titers were at least 1:32.

Of the 106 Cryptococcus isolates tested, 101 could be typed with absorbed antisera diluted 1:4. Titers of antisera to various isolates ranged from 1:8 to 1:256, indicating quantitative differences in antigenic reactivity. In an attempt to improve the sensitivity of the typing sera, undiluted antisera were used to type the 5 untypable isolates as well as representative isolates of the other serotypes. However, with undiluted antisera, cross reactivity between the A, B, and D types was detectable when cells other than the prototype isolates were used. Further absorptions of the typing sera removed the cross reactivity but the final undiluted material was not as reactive with cells of the same serotype as the original absorbed antisera diluted 1:4. An attempt was made to prepare antisera from each of the untypable isolates but none was sufficiently antigenic.

Table II lists the results of typing the 106 isolates using absorbed antisera diluted 1:4. The isolates obtained from Dr. Evans gave the same serotype as they did in his original studies. The isolates obtained from California showed a high prevalence of type B and C with no type A's. Isolates obtained elsewhere showed few type B and C with a high percentage of type A's. Four isolates, 1 from

TABLE II. Serotyping of 106 Isolates of *C. neoformans* Obtained from Man and Nature.

	Source of isolate		Total	
Serotype	California (No. of isolates)	Elsewhere (No. of isolates)	No. of isolates	Isolates (%)
A[a]	0	74	74	69.8
B	9	2[b]	11	10.4
C	4	3[b]	7	6.6
D	0	5	5	4.7
A & D	1	3	4	3.8
Untypable	3	2	5[c]	4.7

[a] All isolates typed with antisera diluted 1:4 prior to use, except as indicated.

[b] Origin of one type B and two type C isolates was unknown.

[c] Using more concentrated antiserum, 3 isolates cross-reacted, 1 typed A and 1 typed D.

California and 3 from elsewhere, cross-reacted with both A and D antisera at the 1:4 dilution. Two additional absorptions of the typing serum with the prototype of the cross-reacting strain did not eliminate this cross reactivity.

Discussion. The results of previous serological studies (1,2) have suggested that *C. neoformans* could be separated into three antigenic types. Several of the strains used in the original studies were available to us. These strains were used whenever possible for absorption, immunization, and for confirmation of the specificity of typing sera in order to maintain uniformity in the nomenclature of the serotypes. Five isolates were antigenically distinct from the isolates received from Dr. Evans and were designated as a fourth serotype, type D. Another discrepancy was encountered. Evans (2) concluded that there was a nonreciprocal relationship between A and B cell types; that is, type B antisera could not be absorbed with type A cells without removing all, or nearly all, agglutinin activity. This was not true with the B typing serum and A cells used here.

In typing the 106 strains, 91.5% fell into one of the four serotypes. Five isolates were untypable when the antiserum was diluted 1:4 prior to use and 4 isolates reacted with both A and D typing sera. In dilutions of antiserum less than 1:4, cross reactions were encountered. Further absorption by heterologous cells overcame this problem. With the improvement in specificity, however, there was a concurrent loss of ability to agglutinate some of the more weakly reacting homologous isolates. Techniques for improving the immunogenicity of the prototype strains may, in future work, permit more exhaustive absorption of typing antisera without prohibitive losses in titer against the more weakly reacting isolates of their homologous serotype. The five isolates which did not agglutinate in any of the typing antisera may have failed to do so because they were antigenically deficient or because they represented one or more distinct but poorly immunogenic serotypes.

In the original reports of Evans (1,2) six strains were type B and C. All but one of these are known to have originated in California. The exception came from the American Type Culture Collection and the original source is unknown to us. In the initial phase of the work reported here, 3 isolates only were type B or type C and one of these came from California. The overwhelming majority of the isolates from elsewhere were type A. This observation raised the possibility that there were geographical differences in the prevalence of the various serotypes. Additional evidence supporting such an hypothesis was noted when the 15 isolates from California were typed. The majority typed B or C and none typed A. The few isolates from nature in California were either type B or type C, types which were not found in nature elsewhere. Of the 18 type B and type C isolates in our collection the source of 3 isolates was unknown. Of the remaining 15 isolates, 13 (86.6%) originated in California. On the basis of these results, investigation of the epidemiological distribution of *C. neoformans* serotypes would seem warranted.

Summary. One hundred and six isolates of *Cryptococcus neoformans* were investigated by agglutination and absorption studies with anticryptococcal sera. In addition to the three previously reported serotypes, a fourth serotype was distinguished and designated type D. Of the 106 isolates from man and nature, 69.8% were type A, 10.4% were type B, 6.6% were type C, and 4.7% were type D, 3.8% reacted with more than one serum, and 4.7% were untypable. The majority of type B and C strains were obtained from California, suggesting the possibility of major differences in the distribution of cryptococcal serotypes.

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