

## SEROLOGY OF THE SOLUBLE ANTIGENS OF THE PATHOGENIC CLOSTRIDIA

PAUL D. ELLNER<sup>1</sup> AND STANLEY S. GREEN

*Department of Medical Microbiology, College of Medicine, University of Vermont, Burlington, Vermont*

Received for publication 27 June 1963

### ABSTRACT

ELLNER, PAUL D. (University of Vermont, Burlington), AND STANLEY S. GREEN. Serology of the soluble antigens of the pathogenic clostridia. *J. Bacteriol.* **86**:1084-1097. 1963.—Soluble antigens of 42 strains, representing nine species of clostridia commonly occurring in human infections, were prepared by growing the organisms in a nonantigenic medium. Serological studies demonstrated the occurrence of considerable strain variation within each species. Interactions among the nine species, as well as with the previously characterized *Clostridium perfringens*, were also investigated. Extreme heterogeneity was observed among the species studied, with many cross-reactions due to common antigens, although species-specific antigens were also found in some cases. Occasional weak reactions were also demonstrated between certain clostridial antisera and the soluble antigens of three of the four species of *Bacillus* studied.

The 90 or more species comprising the genus *Clostridium* represent an extremely heterogeneous collection, with the common characteristics of being gram-positive, anaerobic, catalase-negative, spore-forming rods. Included in this genus are a wide variety of saprophytic forms normally occurring in soil and in the intestinal tracts of man and animals. These species vary widely in their biochemical activities, and include thermophilic species and nitrogen-fixing species, as well as a small number of species pathogenic for man. Identification of the pathogenic anaerobes has been accomplished by biochemical reactions. Spray (1936) set up a "Tentative Key" for the sporulating anaerobes by the use of physiological tests and morphological characteristics. Other classifications by biochemical reactions have been

<sup>1</sup> Present address: Department of Microbiology, College of Physicians and Surgeons of Columbia University, New York, N.Y.

formulated by Duffett (1935), Reed and Orr (1941), Marshal, Wetzler, and Kawatomari (1956), Brooks and Epps (1959), and Kaufman and Weaver (1960).

The initial serological grouping of clostridia was attempted by Robertson (1920). In his study of *Vibrion septique* (*Clostridium septicum*), Robertson found three agglutinating types. By use of washed cultures as antigens, three serological races were found, and toxin could be demonstrated for all strains tested.

Clark and Hall (1937) demonstrated cross-reactions of *C. sordellii* and *C. bifermentans*, using the agglutination of cells by specific antisera. They also observed that animals protected with *C. bifermentans* antiserum resisted infection with *C. sordellii*. Studies by McCoy and McClung (1938) indicated that cross-agglutination occurs among some species of clostridia, and serological investigations of individual or small groups of species of clostridia have also been done (Coleman and Gunnison, 1928; Gunnison, 1937, 1947; Smith, 1937; Miles and Miles, 1947, 1950; Oakley, Warrack, and Clarke, 1947; Mandia and Bruner, 1951). Double diffusion in agar gel was utilized by Bjorkland and Berengo (1954) to study the interactions of seven species of clostridia. This was the first attempt at an overall study of the pathogenic clostridia. Ellner and Bohan (1962) used the same technique to study the soluble antigens of *C. perfringens* types A-F. This study showed marked heterogeneity among the types, as well as strain variation within each type. Walker (1963), with spore antigens of the *C. sordellii*-*C. bifermentans* complex, found cross-agglutination between the two species. In a preliminary study with *C. perfringens*, *C. sporogenes*, and *C. novyi* types A and B, Ellner and Green (1963a) showed strain variation and species interactions among these species by utilizing the soluble antigens in an agar gel system.

The present study represents an attempt to

elucidate the serological relationships among the ten clostridial species most frequently involved in human disease.

#### MATERIALS AND METHODS

**Cultures.** Cultures were obtained from various sources as indicated in Table 1. All strains were streaked for purity on plates of lactose-egg yolk-milk agar (Willis and Hobbs, 1959) and blood agar. Sucrose, maltose, and salicin fermentations were determined by the rapid method of Kaufman and Weaver (1960). Trypticase Lactose Iron Agar (BBL) was used to determine H<sub>2</sub>S production. Indole production was determined with Kovack's reagent in Indole-Nitrite Medium (BBL). The glucose-gelatin medium of Willis and Hobbs (1959) was used to measure fermentation and liquefaction. Urea hydrolysis was tested for in the medium of Brooks and Epps (1959), with phenol red substituted for the universal indicator. All tubed media were boiled for 10 min and cooled prior to use, and all cultures except *Bacillus* species were incubated anaerobically, either by the addition of a reducing substance or by the incubation of plates in a Brewer jar.

Pathogenicity of *C. bifermentans* and *C. sordellii* was assayed by the method of Brooks and Epps (1959).

**Production of antigen.** The medium employed was that of Ellner and Bohan (1962), modified by the use of dialyzed portions of NZ Amine type A (Sheffield Chemical, Norwich, N.Y.) and Thiotone (BBL) rather than the peptones themselves. A 10-ml portion of antigen medium was inoculated from the appropriate stock culture and incubated at 37 C until active growth was evident. This served as the inoculum for the 1-liter batches used for antigen production. The time of incubation and the glucose concentration were varied with the species (Table 2).

Cells were removed by centrifugation at 12,000 × *g* for 15 min, and the supernatant fluid was filtered under positive pressure through a type G filter (Gelman Instrument Co., Chelsea, Mich.) to insure the removal of all bacterial cells. The soluble antigens were precipitated from the cell-free supernatant fluid by saturation with ammonium sulfate to 70%. The material that precipitated was collected by centrifugation at 12,000 × *g* for 15 min, and the precipitate was dissolved and dialyzed against 0.85% NaCl.

TABLE 1. Sources of *Clostridium* and *Bacillus* cultures

Species	No.	Strain	Source*
<i>C. perfringens</i> type A		16 strains	g
<i>C. sporogenes</i>	1	176	a
	2	47-R-2	b
	3	3057	c
	4	4824	c
	5	2	d
<i>C. novyi</i> type A	1	360	c
	2	40	d
	3	1739	c
	4	17	d
	5	898	b
	6	2	a
<i>C. novi</i> type B	1	1789	c
	2	1025	c
<i>C. histolyticum</i>	1	948	c
	2	919	c
	3	250	a
	4	UVM	e
<i>C. bifermentans</i>	1	596	b
	2	1618	c
	3	127	a
	4	1617	c
<i>C. sordellii</i>	1	CN 1733 (P)†	c
	2	CN 1732 (P)	c
	3	CN 1694 (P)	c
	4	1621 (P)	c
	5	Weaver (NP)	d
	6	640 (NP)	c
	7	CN 1619 (NP)	c
	8	CN 1620 (NP)	c
	9	CN 1621 (NP)	c
<i>C. septicum</i>	1	362	c
	2	832	a
	3	3610	c
	4	6009	b
<i>C. fallax</i>	1	1890	c
	2	1747	a
<i>C. tetani</i>	1	8033	f
	2	10709	f
	3	454	f
	4	10779	f
	5	453	f
	6	9441	f
<i>B. subtilis</i>		9858	f
<i>B. cereus</i>		10987	f
<i>B. cereus</i> var. <i>mycoides</i>		10206	f
<i>B. anthracis</i>		6605	f

\* (a) L. S. McClung, Indiana University; (b) H. Noyes, Walter Reed Army Institute of Research, Washington, D.C.; (c) M. Sterne, Wellcome Research Laboratories, Kent, England; (d) R. H. Weaver, University of Kentucky, Lexington; (e) University of Vermont, Burlington; (f) American Type Culture Collection, Washington, D.C.; (g) cited in Ellner and Bohan (1962).

† P = pathogenic; NP = nonpathogenic.

TABLE 2. *Period of incubation and glucose concentration employed for antigen production*

Organism	Time	Glucose
	hr	%
<i>Clostridium histolyticum</i>	24	0
<i>C. septicum</i> .....	24	1
<i>C. sporogenes</i> .....	72	1
<i>C. fallax</i> .....	72	1
<i>Bacillus anthracis</i> .....	72	1
<i>B. cereus</i> .....	72	1
<i>B. cereus var. mycoides</i> ...	72	1
<i>B. subtilis</i> .....	72	1
<i>C. novyi</i> type A.....	96	0.25
<i>C. novyi</i> type B.....	96	0.25
<i>C. tetani</i> .....	144	1
<i>C. bifermentans</i> .....	168	0
<i>C. sordellii</i> .....	168	0

The protein concentration was estimated by the method of Warburg and Christian (1942). Thimerosal was added to a final concentration of 1:10,000, and the antigens were stored at 5 C.

*Immunization of animals.* The soluble antigenic preparations characteristic of each strain within a species were pooled, and the protein concentration of each pool was adjusted to 10 to 20 mg of protein per ml. A portion of each pool was treated with 0.15% formalin (Fulthorpe and Thomson, 1960), incubated at 37 C for 72 hr, and stored at 5 C for at least 10 days to destroy toxicity. Rabbits were immunized as previously described by Ellner and Bohan (1962). Three rabbits were employed for each antigen pool. The sera of the three rabbits were pooled, and an equal volume of saturated ammonium sulfate was added. The resulting globulin precipitate was collected by centrifugation, dissolved in a volume of 0.85% NaCl equivalent to half the original volume of the pooled sera, and dialyzed against 0.85% NaCl. Thimerosal was added to a final concentration of 1:10,000, and the pooled globulin was stored at 5 C until used.

*Agar gel diffusion.* The technique employed was that of Ellner and Bohan (1962) except that plates were incubated at 5 C.

## RESULTS

*Biochemical reactions.* All strains gave typical reactions, with the following exceptions: strain 1 of *C. novyi* type B fermented sucrose and salicin, and gave a positive proteolytic reaction on lactose-egg yolk-milk agar; strains 3 and 4 of

*C. septicum* failed to ferment salicin or liquefy gelatin. The identity of strain 1 of *C. novyi* type B was confirmed by animal protection tests with guinea pigs and an antiserum specific for this organism; the two aberrant strains of *C. septicum* were identified by their microscopic appearance in infected guinea pigs and their colonial morphology.

*Strain variation within species.* The strain variation among the 16 type A strains of *C. perfringens* was described in a previous report (Ellner and Bohan, 1962).

The soluble antigens of the five strains of *C. sporogenes* were tested individually with the pooled globulin produced against the *C. sporogenes* antigen pool. The number of lines obtained varied from two to four, depending on the strain (Fig. 1). The total number of antigens represented by the pool was five. Of these, two were common to all strains, one was unique to strains 1 and 4, another was unique to strains 2 and 4, and a third antigen was present only in strain 5.

By the same procedure, a total of seven antigens were found among the six strains of *C. novyi* type A. Six of these lines were shared by all strains, and the seventh was present only in strains 4 and 5 (Fig. 2).

With the two strains of *C. novyi* type B, a total of four antigens were found. Three of these antigens were common to both strains, and an additional antigen was unique to strain 2 (Fig. 3).

With the four strains of *C. bifermentans*, a total of seven lines were observed. Of these, five were common to all strains, an additional antigen was shared by strains 1 and 3, and another antigen was unique to strain 4 (Fig. 4).

Among the nine strains of *C. sordellii*, a total of seven antigens could be demonstrated. Five of these were common to all strains, one was unique to strain 3, and one was unique to strain 4 (Fig. 5 and 6). No antigenic differences could be observed between pathogenic and nonpathogenic strains.

A total of six antigens were found to be present among the four strains of *C. histolyticum*. Four of these were common to all strains, and an additional two were shared by strains 2, 3, and 4 (Fig. 7).

With the six strains of *C. tetani*, no fewer than 12 antigens were found. None of these antigens was common to all strains. Two antigens were

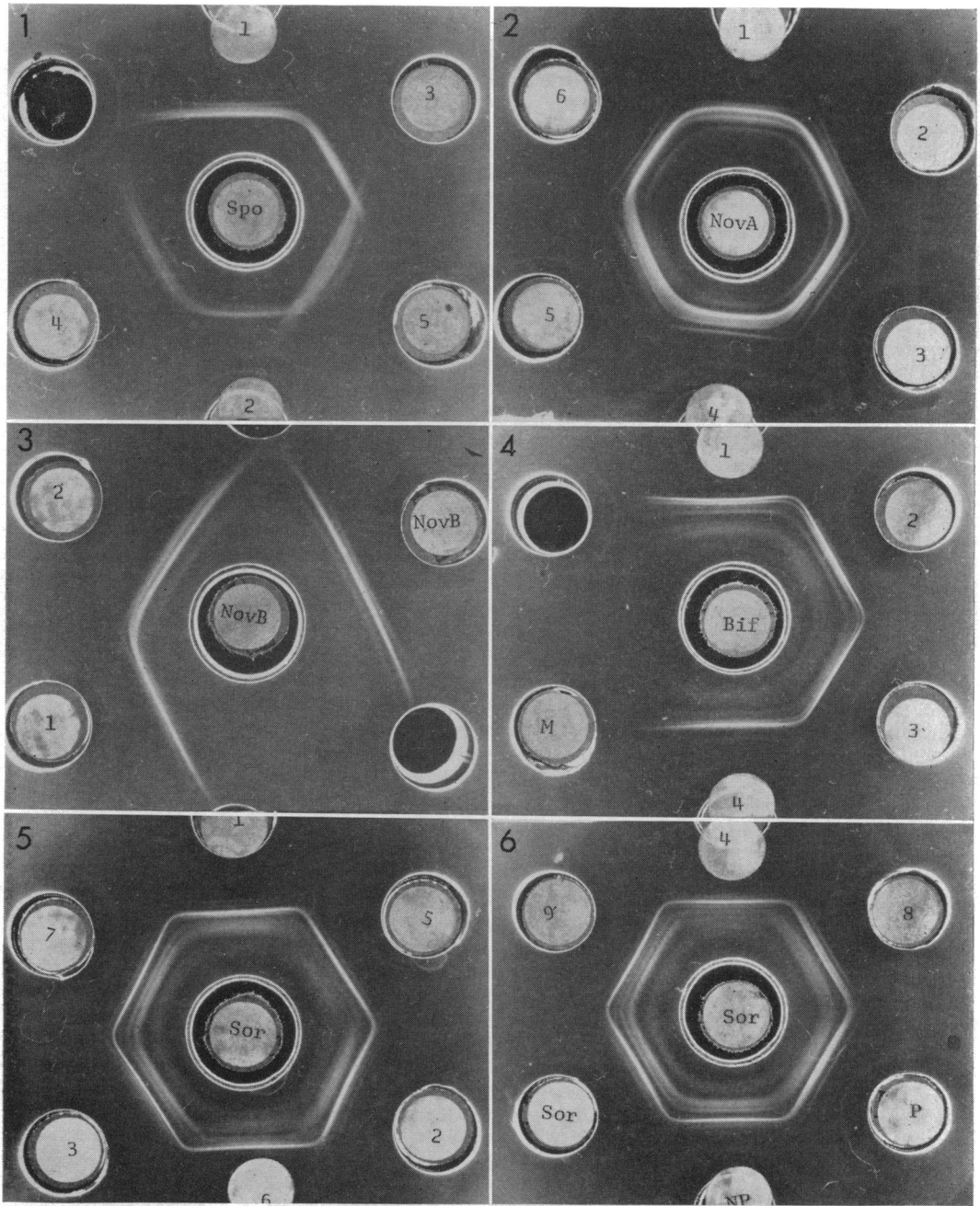


FIG. 1. Antigens of the five individual strains of *Clostridium sporogenes* against pooled homologous globulin.

FIG. 2. Antigens of the six individual strains of *Clostridium novyi* type A against pooled homologous globulin.

FIG. 3. Antigens of the two individual strains of *Clostridium novyi* type B and the antigen pool (NovB) against pooled homologous globulin.

FIG. 4. Antigens of the four individual strains of *Clostridium bifermentans* and uninoculated medium (M) against pooled homologous globulin.

FIG. 5. Antigens of strains 1, 2, 3, 5, 6, and 7 of *Clostridium sordellii* against pooled homologous globulin.

FIG. 6. Antigens of *Clostridium sordellii* strains 4, 8, and 9, pooled P strains (P), pooled NP strains (NP), and total pool (Sor) against pooled homologous globulin.

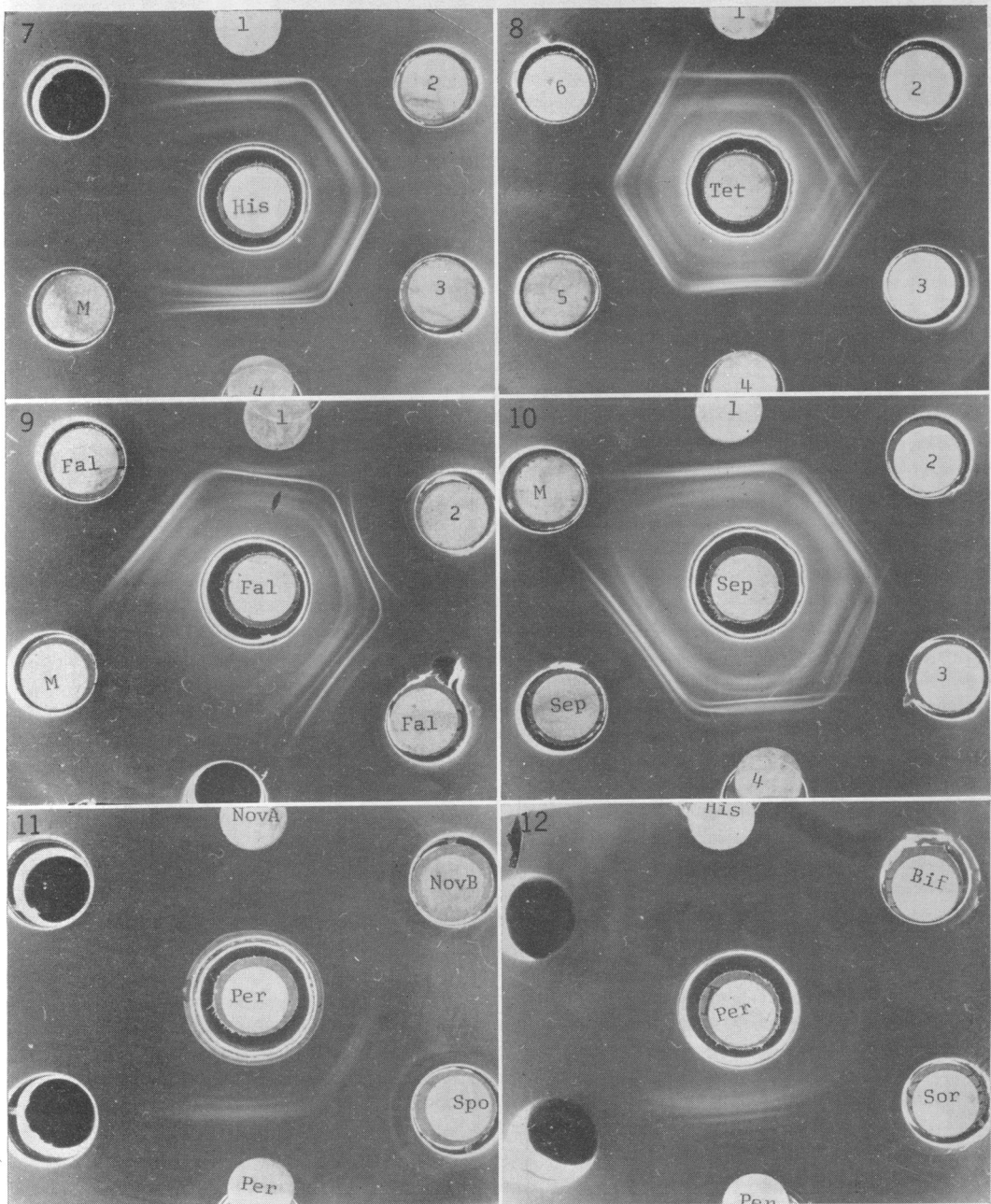


FIG. 7. Antigens of the four individual strains of *Clostridium histolyticum* and uninoculated medium (M) against pooled homologous globulin.

FIG. 8. Antigens of the six individual strains of *Clostridium tetani* against pooled homologous globulin.

FIG. 9. Antigens of the two individual strains of *Clostridium fallax* and the antigen pool (Fal) and uninoculated medium (M) against pooled homologous globulin.

FIG. 10. Antigens of the four individual strains of *Clostridium septicum* and the antigen pool (Sep) and uninoculated medium (M) against pooled homologous globulin.

FIG. 11. Antigen pools of *Clostridium novyi* type A (NovA), *C. novyi* type B (NovB), *C. sporogenes* (Spo), and *C. perfringens* type A (Per) against pooled *C. perfringens* globulin (Per).

FIG. 12. Antigen pools of *Clostridium histolyticum* (His), *C. bifermentans* (Bif), *C. sordellii* (Sor), and *C. perfringens* (Per) against pooled *C. perfringens* globulin (Per).

unique to strain 1; four antigens to strains 1 and 2; another four antigens were shared by strains 3, 4, 5, and 6; a single antigen was shared by strains 4, 5, and 6; and an additional antigen was shared by strains 1, 3, 4, 5, and 6 (Fig. 8).

With the two strains of *C. fallax*, a total of seven antigens were found, all of which were common to both strains (Fig. 9).

Among the four strains of *C. septicum*, a total of nine antigens were detected, three of which were common to all strains. Two additional antigens were shared by strains 1 and 2; another antigen was shared by strains 1, 2, and 3; two antigens were unique to strain 3; and one antigen was unique to strain 4 (Fig. 10).

*Relationship between species.* Globulin prepared against pooled *C. perfringens* antigens reacted with the antigens of all the species tested (Fig. 11, 12, and 13). However, *C. perfringens* appeared to have one antigen unique to this species.

*C. sporogenes* globulin reacted only with the antigens of *C. novyi* types A and B (Fig. 14, 15, and 16). One of the antigens of *C. sporogenes* was found to be species-specific.

Globulin prepared against *C. novyi* type A reacted only with the antigens of *C. sporogenes*, *C. novyi* type B, *C. bifermentans*, and *C. sordellii* (Fig. 17, 18, 19, and 20). At least one species-specific antigen was detected.

Globulin prepared against *C. novyi* type B reacted with the pooled antigens of all species tested with the exception of *C. perfringens* (Fig. 21, 22, and 23). No antigens were found in *C. novyi* type B that were not also present in one or more other species.

*C. bifermentans* globulin reacted with the antigens of all species tested except *C. perfringens* and *C. sporogenes* (Fig. 24 and 25). Although no antigens were found to be unique to *C. bifermentans*, this species can be distinguished from *C. sordellii* by the use of absorbed sera (Ellner and Green, 1963b).

*C. sordellii* globulin reacted with the antigens of all species tested except *C. novyi* types A and B (Fig. 26 and 27). One unique antigen was present in this species.

*C. histolyticum* globulin reacted with the antigens of all species tested (Fig. 28 and 29), as did globulin prepared against *C. fallax* (Fig. 30 and 31). Neither of these species was found to contain unique antigens.

Although *C. tetani* globulin disclosed 12 lines when tested against individual strains of this

species, the pooled antigens disclosed only 6 lines. *C. tetani* globulin reacted with the antigens of all species tested except *C. sporogenes* and *C. novyi* type A (Fig. 32 and 33). Only one of the *C. tetani* antigens appeared to be species-specific.

*C. septicum* globulin reacted with the antigens of all species tested except *C. perfringens* and *C. sporogenes* (Fig. 34 and 35). Two antigens are unique to this species.

The cross-reactions of all species are summarized in Table 3.

*Reactions with Bacillus antigens.* The soluble antigens prepared with the four species of *Bacillus* were each tested individually against the globulins prepared against the ten clostridial species. *B. anthracis* reacted with the globulins of *C. bifermentans*, *C. sordellii*, *C. fallax*, and *C. septicum*. *B. cereus* var. *mycoides* cross-reacted with the same globulins as *B. anthracis* plus the globulin prepared against *C. novyi* type A.

*B. cereus* only reacted with *C. septicum* globulin, and *B. subtilis* failed to react with any of the globulins. All reactions between *Bacillus* antigens and clostridial globulins were extremely faint. The results of these interactions are summarized in Table 4.

*Control studies.* Results obtained when uninoculated medium was tested against various pooled globulins indicated that the medium itself was nonantigenic (Fig. 4, 7, 9, and 10).

## DISCUSSION

It is apparent from these studies that the soluble antigens of the clostridial species examined are extremely heterogeneous. *C. perfringens*, *C. sporogenes*, *C. tetani*, and *C. septicum* strains varied considerably, while the strains of the remaining species were remarkably constant. Species-unique antigens were found in *C. perfringens*, *C. sporogenes*, *C. novyi* type A, *C. sordellii*, *C. tetani*, and *C. septicum*.

Although no attempt has been made to examine the soluble antigenic preparations for toxigenicity or other biological activity, it seems reasonable to assume, in view of the method of preparation, that many such materials are present. Furthermore, while efforts were made to exclude cellular antigens, the possibility exists that materials such as lipopolysaccharides, flagellar antigens, or other antigenic materials normally associated with structural components of the cell may have become solubilized through autolysis and could also be present in our preparations.

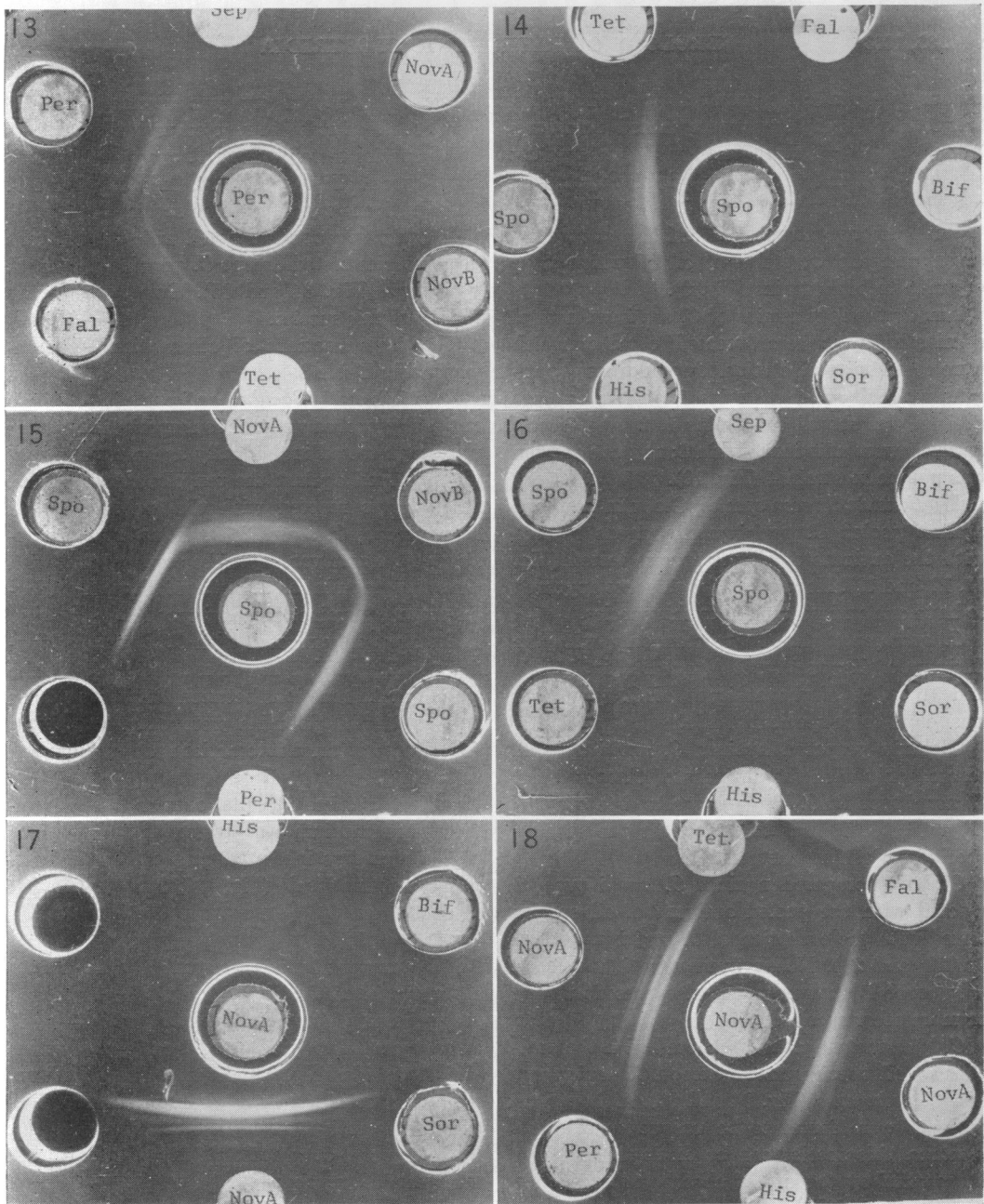


FIG. 13. Antigen pools of *Clostridium septicum* (Sep), *C. novyi* type A (NovA), *C. novyi* type B (NovB), *C. tetani* (Tet), *C. fallax* (Fal), and *C. perfringens* (Per) against pooled *C. perfringens* globulin (Per).

FIG. 14. Antigen pools of *Clostridium tetani* (Tet), *C. fallax* (Fal), *C. bifermentans* (Bif), *C. sordellii* (Sor), *C. histolyticum* (His), and *C. sporogenes* (Spo) against pooled *C. sporogenes* globulin (Spo).

FIG. 15. Antigen pools of *Clostridium novyi* type A (NovA), *C. novyi* type B (NovB), *C. sporogenes*, (Spo), and *C. perfringens* (Per) against pooled *C. sporogenes* globulin (Spo).

FIG. 16. Antigen pools of *Clostridium septicum* (Sep), *C. bifermentans* (Bif), *C. sordellii* (Sor), *C. histolyticum* (His), *C. tetani* (Tet), and *C. sporogenes* against pooled *C. sporogenes* globulin (Spo).

FIG. 17. Antigen pools of *Clostridium histolyticum* (His), *C. bifermentans* (Bif), *C. sordellii* (Sor), and *C. novyi* type A (NovA) against *C. novyi* type A globulin (NovA).

FIG. 18. Antigen pools of *Clostridium tetani* (Tet), *C. fallax* (Fal), *C. novyi* type A (NovA), *C. histolyticum* (His), and *C. perfringens* (Per) against pooled *C. novyi* type A globulin (NovA).

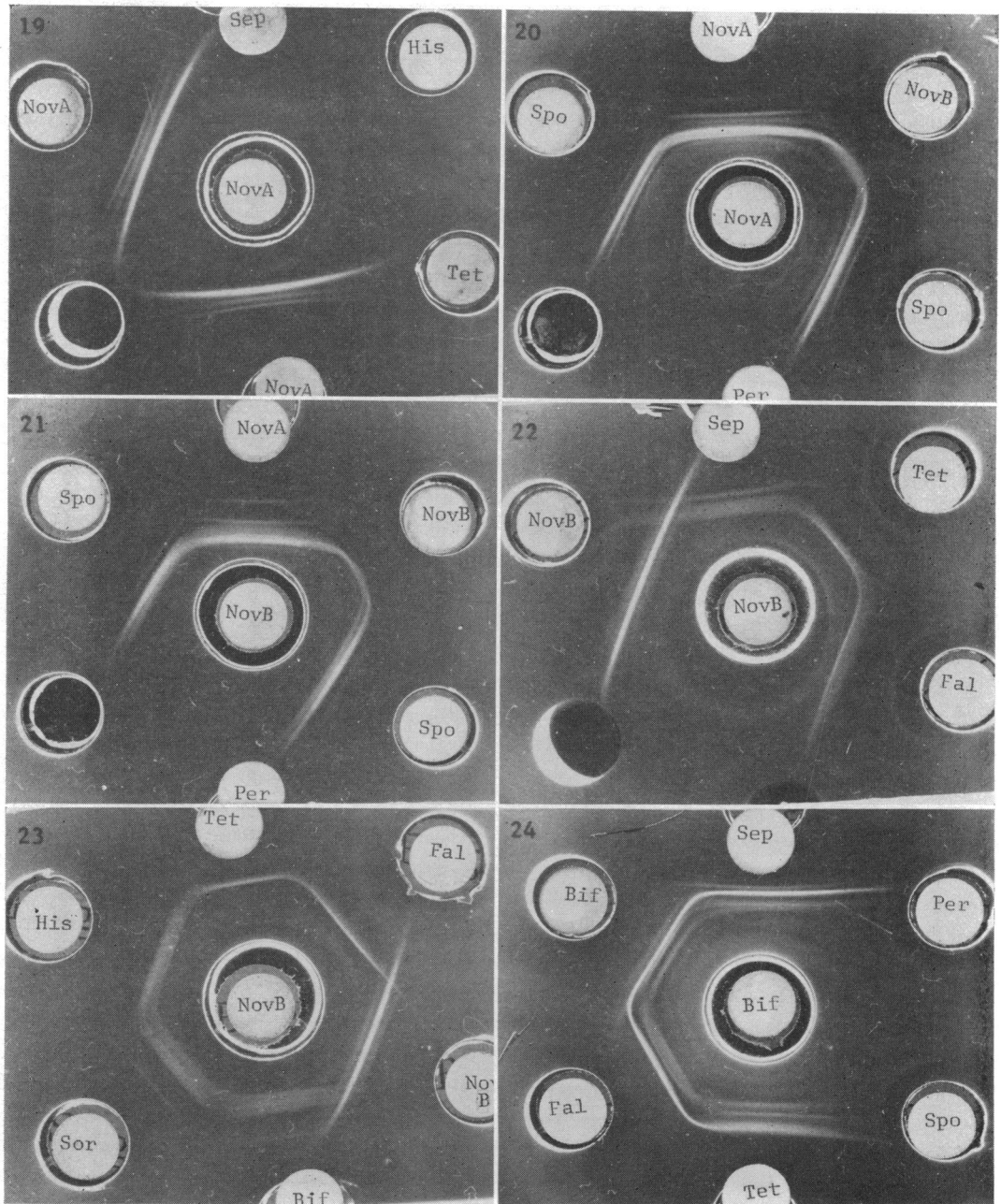


FIG. 19. Antigen pools of *Clostridium septicum* (Sep), *C. histolyticum* (His), *C. tetani* (Tet), and *C. novyi* type A (NovA) against pooled *C. novyi* type A globulin (NovA).

FIG. 20. Antigen pools of *Clostridium novyi* type A (NovA), *C. novyi* type B (NovB), *C. sporogenes* (Spo), and *C. perfringens* (Per) against pooled *C. novyi* type A globulin (NovA).

FIG. 21. Antigen pools of *Clostridium novyi* type A (NovA), *C. novyi* type B (NovB), *C. sporogenes* (Spo), and *C. perfringens* (Per) against pooled *C. novyi* type B globulin (NovB).

FIG. 22. Antigen pools of *Clostridium septicum* (Sep), *C. tetani* (Tet), *C. fallax* (Fal), and *C. novyi* type B (NovB) against pooled *C. novyi* type B globulin (NovB).

FIG. 23. Antigen pools of *Clostridium tetani* (Tet), *C. fallax* (Fal), *C. novyi* type B (NovB), *C. bifermentans* (Bif), *C. sordellii* (Sor), and *C. histolyticum* (His) against pooled *C. novyi* type B globulin (NovB).

FIG. 24. Antigen pools of *Clostridium septicum* (Sep), *C. perfringens* (Per), *C. sporogenes* (Spo), *C. tetani* (Tet), *C. fallax* (Fal), and *C. bifermentans* (Bif) against pooled *C. bifermentans* globulin (Bif).



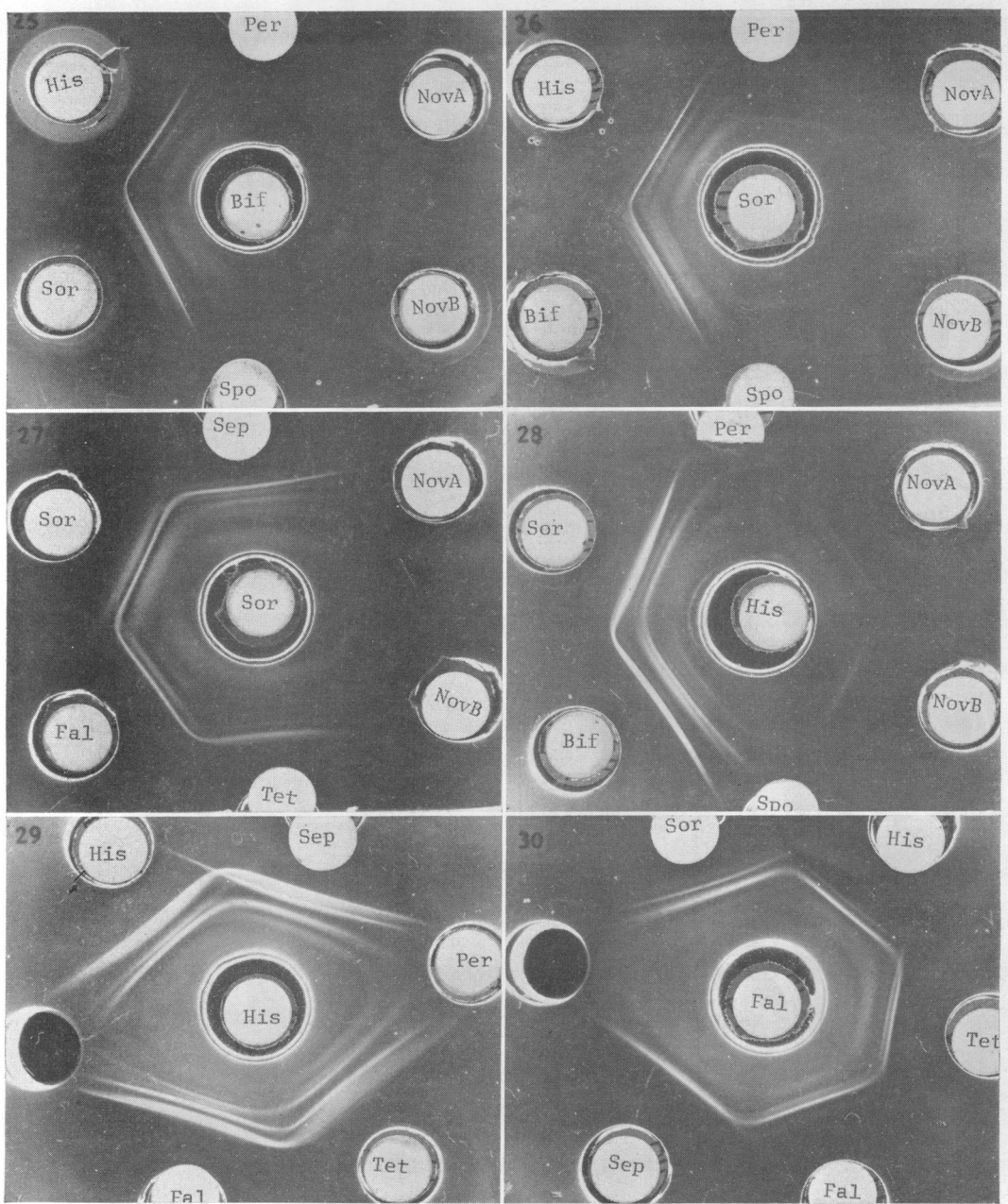


FIG. 25. Antigen pools of *Clostridium perfringens* (Per), *C. novyi* type A (NovA), *C. novyi* type B (NovB), *C. sporogenes* (Spo), *C. sordellii* (Sor), and *C. histolyticum* (His) against pooled *C. bifermentans* globulin (Bif).

FIG. 26. Antigen pools of *Clostridium perfringens* (Per), *C. novyi* type A (NovA), *C. novyi* type B (NovB), *C. sporogenes* (Spo), *C. bifermentans* (Bif), and *C. histolyticum* (His) against pooled *C. sordellii* globulin (Sor).

FIG. 27. Antigen pools of *Clostridium septicum* (Sep), *C. novyi* type A (NovA), *C. novyi* type B (NovB), *C. tetani* (Tet), *C. fallax* (Fal), and *C. sordellii* (Sor) against pooled *C. sordellii* globulin (Sor).

FIG. 28. Antigen pools of *Clostridium perfringens* (Per), *C. novyi* type A (NovA), *C. novyi* type B (NovB), *C. sporogenes* (Spo), *C. bifermentans* (Bif), and *C. sordellii* (Sor) against pooled *C. histolyticum* globulin (His).

FIG. 29. Antigen pools of *Clostridium septicum* (Sep), *C. perfringens* (Per), *C. tetani* (Tet), *C. fallax* (Fal), and *C. histolyticum* (His) against pooled *C. histolyticum* globulin (His).

FIG. 30. Antigen pools of *Clostridium sordellii* (Sor), *C. histolyticum* (His), *C. tetani* (Tet), *C. fallax* (Fal), and *C. septicum* (Sep) against pooled *C. fallax* globulin (Fal).

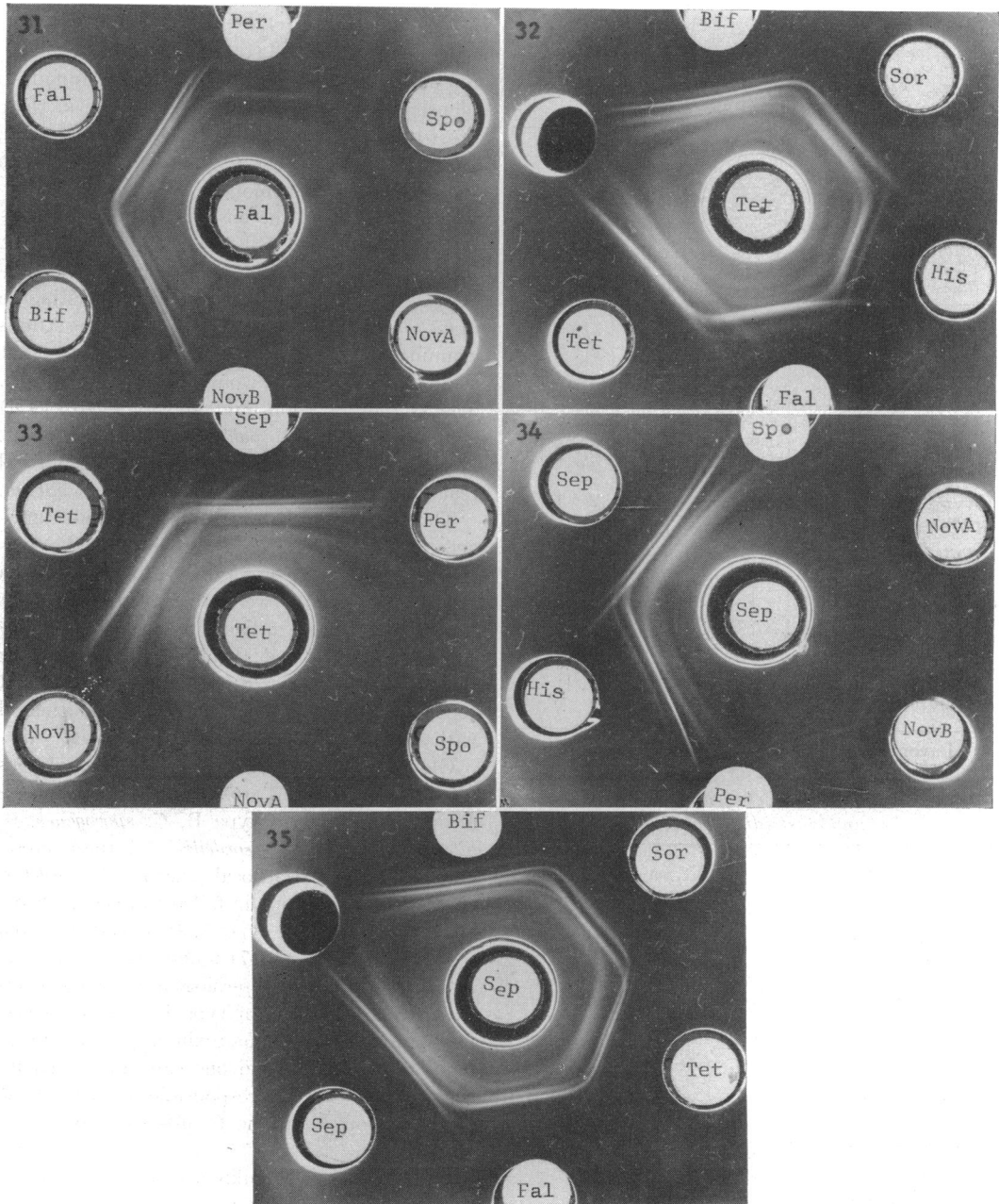


FIG. 31. Antigen pools of *Clostridium perfringens* (Per), *C. sporogenes* (Spo), *C. novyi* type A (NovA), *C. novyi* type B (NovB), *C. bifermentans* (Bif), and *C. fallax* (Fal) against pooled *C. fallax* globulin (Fal).

FIG. 32. Antigen pools of *Clostridium bifermentans* (Bif), *C. sordellii* (Sor), *C. histolyticum* (His), *C. fallax* (Fal), and *C. tetani* (Tet) against pooled *C. tetani* globulin (Tet).

FIG. 33. Antigen pools of *Clostridium septicum* (Sep), *C. perfringens* (Per), *C. sporogenes* (Spo), *C. novyi* type A (NovA), *C. novyi* type B (NovB), and *C. tetani* (Tet) against pooled *C. tetani* globulin (Tet).

FIG. 34. Antigen pools of *Clostridium sporogenes* (Spo), *C. novyi* type A (NovA), *C. novyi* type B (NovB), *C. perfringens* (Per), *C. histolyticum* (His), and *C. septicum* (Sep) against pooled *C. septicum* globulin (Sep).

FIG. 35. Antigen pools of *Clostridium bifermentans* (Bif), *C. sordellii* (Sor), *C. tetani* (Tet), *C. fallax* (Fal), and *C. septicum* (Sep) against pooled *C. septicum* globulin (Sep).

TABLE 3. Cross-reactions between clostridial species

Globulin prepared against	Antigens									
	<i>C. perfringens</i>	<i>C. sporogenes</i>	<i>C. novyi</i> type A	<i>C. novyi</i> type B	<i>C. bifermentans</i>	<i>C. sordellii</i>	<i>C. histolyticum</i>	<i>C. tetani</i>	<i>C. fallax</i>	<i>C. septicum</i>
<i>C. perfringens</i> .....	(3)*	2	2	2	2	2	1	2	2	1
<i>C. sporogenes</i> .....	0	(5)	4	2	0	0	0	0	0	0
<i>C. novyi</i> type A...	0	5	(7)	4	1	2	0	0	0	0
<i>C. novyi</i> type B...	0	3	6	(4)	4	4	2	2	1	2
<i>C. bifermentans</i> .....	0	0	1	1	(7)	7	5	4	5	5
<i>C. sordellii</i> .....	1	1	0	0	6	(7)	5	6	4	6
<i>C. histolyticum</i> .....	2	1	3	2	7	5	(6)	6	6	5
<i>C. tetani</i> .....	1	0	0	1	7	5	4	(6)	5	5
<i>C. fallax</i> .....	2	1	1	1	7	5	4	4	(7)	4
<i>C. septicum</i> .....	0	0	1	1	7	5	5	5	5	(9)

\* Parentheses indicate homologous reactions.

TABLE 4. Reactions between *Bacillus* antigens and clostridial globulins

Globulin prepared against	Antigens			
	<i>B. subtilis</i>	<i>B. cereus</i>	<i>B. cereus</i> var. <i>mycoides</i>	<i>B. anthracis</i>
<i>C. perfringens</i> .....	-	-	-	-
<i>C. sporogenes</i> .....	-	-	-	-
<i>C. novyi</i> type A.....	-	-	+	-
<i>C. novyi</i> type B.....	-	-	-	-
<i>C. bifermentans</i> .....	-	-	+	+
<i>C. sordellii</i> .....	-	-	+	+
<i>C. histolyticum</i> .....	-	-	-	-
<i>C. tetani</i> .....	-	-	-	-
<i>C. fallax</i> .....	-	-	+	+
<i>C. septicum</i> .....	-	+	+	+

The cross-reactions observed between *C. perfringens*, *C. sordellii*, *C. histolyticum*, *C. tetani*, and *C. fallax* are reciprocal. *C. perfringens* globulin also reacted with the soluble antigens of *C. sporogenes*, *C. novyi* types A and B, *C. bifermentans*, and *C. septicum*, although the globulins of these species failed to react with *C. perfringens*

antigen. Although we have not attempted to identify lines of precipitation occurring in these cross-reactions, it should be noted that the theta toxin of *C. perfringens* is serologically related to the oxygen-labile hemolysin of *C. tetani* (MacLennan, 1962) and the epsilon toxin of *C. histolyticum* (Howard, 1953). Miles and Miles (1947, 1950) showed that the lecithinase of *C. perfringens* was serologically related to the lecithinase of *C. bifermentans*, and we assume that the strains examined by these workers included strains that some current workers would class as *C. sordellii*. The studies of Bjorklund and Berengo (1954) confirmed the reciprocal cross-reaction of *C. perfringens* and *C. tetani* and the nonreciprocal reactions between *C. perfringens* and *C. septicum*.

The globulin prepared against the soluble antigens of *C. sporogenes* cross-reacted with the soluble antigens of *C. novyi* types A and B. Mandia and Bruner (1951) were able to identify four heat-labile somatic antigens associated with this species and classified them in group 1 of the proteolytic clostridia. Smith (1937) found that rough variants of *C. histolyticum* agglutinated with sera prepared against *C. sporogenes*.

Globulin prepared against the soluble antigens of *C. novyi* type A cross-reacted with soluble antigens of *C. novyi* type B, *C. sporogenes*, *C. bifermentans*, and *C. sordellii*. All these cross-reactions were reciprocal except *C. sordellii*, since *C. sordellii* globulin failed to react with the antigens of *C. novyi* type A. It should be noted that Oakley et al. (1947) found the alpha toxin of *C. novyi* type A to be serologically homogeneous with the alpha toxin of type B of this species. Furthermore, the gamma toxin of type A appears to be a lecithinase, and one can only speculate whether this factor is responsible in part for the cross-reactions with the *C. bifermentans*-*C. sordellii* group.

*C. novyi* type B globulin cross-reacted with the soluble antigens of *C. novyi* type A, *C. histolyticum*, *C. sporogenes*, *C. tetani*, *C. fallax*, *C. septicum*, *C. bifermentans*, and *C. sordellii*. As with *C. novyi* type A, all reactions were reciprocal, except that with *C. sordellii* globulin which failed to react with the antigens of *C. novyi* type B. Again, as in the case of *C. novyi* type A, it should be noted that the alpha toxin of the two types is homogeneous, and one might also speculate on the significance of the beta-lecithinase of type B with respect to the cross-reaction with the *C. bifermentans*-*C. sordellii* group.

Globulins prepared against *C. bifermentans* and *C. sordellii* both cross-reacted with soluble antigens of *C. histolyticum*, *C. tetani*, *C. fallax*, and *C. septicum*. In addition, *C. bifermentans* globulin cross-reacted with the antigens of *C. novyi* types A and B as well as *C. sordellii*, while *C. sordellii* globulin reacted with the antigens of *C. perfringens*, *C. bifermentans*, and *C. sporogenes*. *C. sporogenes* globulin, however, failed to react with the antigens of *C. sordellii*.

Cross-reactions occurred between *C. histolyticum* globulin and the soluble antigens of *C. perfringens*, *C. novyi* types A and B, *C. bifermentans*, *C. sordellii*, *C. tetani*, *C. fallax*, *C. septicum*, and *C. sporogenes*. The globulins of *C. sporogenes* and *C. novyi* type A failed to react with *C. histolyticum* antigen. Of the five toxins ascribed to *C. histolyticum*, the lethal toxin has been found to cross-react with the alpha toxin of *C. septicum* (Sterne and Warrack, 1962), and, as mentioned previously, the oxygen-labile epsilon hemolysin appears to be related to hemolysins of *C. perfringens*, *C. novyi*, and *C. septicum*. Mandia (1952) classified *C. histolyticum* in group III of the proteolytic clostridia and observed some cross-reactions with *C. sporogenes*. Cross-reactions between rough strains of this organism and *C. sporogenes* were also observed by Smith (1937).

Bjorklund and Berengo (1954) found that *C. tetani* antitoxin gave 8 to 12 lines when reacted with the homologous toxin. This finding correlates very well with the 12 lines we observed among the various strains of *C. tetani* and the 6 lines produced by the *C. tetani* pool when reacted with the homologous globulin. Ten types of *C. tetani* have been described (Coleman and Gunnison, 1928; Gunnison, 1937, 1947) based upon heat-stable somatic antigens. One such antigen was found to be common to all strains, and an additional heat-stable antigen was common to types II, IV, V, and IX. Type-specific reactions, however, were due to flagellar antigens and were present in types I, III, VII, and VIII. Type VI (a nonmotile strain) was found to be devoid of flagellar antigens. Of the six strains of *C. tetani* employed in our studies, strain 1 was type II, strain 3 was type VI, and strain 5 was type V. The type designation of the remaining three strains was unknown. Our studies confirmed Bjorklund and Berengo's (1954) findings that *C. tetani* serum cross-reacts with the antigens of *C. perfringens*, *C. histolyticum*, and *C. septicum*. However, we also found that *C. tetani* globulin

cross-reacts with the soluble antigens of *C. novyi* type B, *C. bifermentans*, *C. sordellii*, and *C. septicum*.

Cross-reactions were observed between the soluble antigens of *C. perfringens*, *C. novyi* types A and B, *C. bifermentans*, *C. sordellii*, *C. histolyticum*, *C. tetani*, *C. septicum*, and *C. sporogenes* with the globulin prepared against *C. fallax*. Globulins prepared against *C. novyi* type A and *C. sporogenes* failed to react with antigens of *C. fallax*. The unusual curved lines obtained with *C. fallax* globulin and homologous antigen is suggestive of a disparity between the molecular weights of the antigen and of the antibody (Kabat and Mayer, 1961).

*C. septicum* globulin cross-reacted with the soluble antigens of *C. novyi* types A and B, *C. bifermentans*, *C. sordellii*, *C. histolyticum*, *C. tetani*, and *C. fallax*. *C. novyi* type A globulin failed to react with *C. septicum* antigens. Of the four toxins associated with this organism, the alpha toxin has been found to cross-react with the alpha toxin of *C. histolyticum* (Sterne and Warrack, 1962). Bjorklund and Berengo (1954) failed to observe any cross-reactions between *C. septicum* and *C. histolyticum*, but Sterne and Warrack's finding of such a cross-reaction confirms our observations of a reaction between these two species.

Reactions between globulins of *C. septicum*, *C. fallax*, *C. sordellii*, *C. bifermentans*, and *C. novyi* type A and antigens of one or more species of *Bacillus* were observed. Most reactions occurred with *B. cereus* var. *mycoides*, some with *B. anthracis* and *B. cereus*, and none with *B. subtilis*.

It should be emphasized that these reactions were all extremely faint and only appeared after prolonged incubation. Since comparative studies were not done, it is not known whether these reactions indicate antigens common to both genera, or distantly related antigens.

Two phenomena deserve comment, namely, the occurrence of nonreciprocal reactions, and the occasional observation that heterologous reactions may give rise to a greater number of lines than homologous reactions. Indeed, both observations may well be manifestations of the same phenomenon. Nonreciprocal reactions were also observed by Bjorklund and Berengo (1954), who suggested that they might be due to differences in immune responses of individual animals. We must, however, reject this postulated explanation, since our studies employed globulin pools.

On the other hand, while no ready evidence is available, we do concur with Bjorklund's alternate postulate that some antigens might occur in low concentrations in some preparations insufficient to cause visible precipitation.

Despite the extreme heterogeneity and the great number of cross-reactions observed, preliminary experiments with absorbed sera suggest that it may be possible in some cases to produce group-specific or even species-specific sera. Should it be possible to produce such sera, the implications are that they could prove extremely useful as an additional adjunct in the identification of the pathogenic clostridia, perhaps by means of the fluorescent-antibody technique. The utility of such procedures, however, must be determined by additional studies.

#### ACKNOWLEDGMENT

The authors are indebted to Louis D. S. Smith for his generous gift of *C. novyi* type B antiserum.

#### LITERATURE CITED

- BJORKLUND, B., AND A. BERENGO. 1954. Studies on the antigenic composition of some clostridia toxins with the aid of gel diffusion. *Acta Pathol. Microbiol. Scand.* **34**:79-86.
- BROOKS, M. E., AND H. B. G. EPPS. 1959. Taxonomic studies of the genus *Clostridium*: *Clostridium bifermentans* and *C. sordellii*. *J. Gen. Microbiol.* **21**:144-155.
- CLARK, F. E., AND I. C. HALL. 1937. A comparative study of *Bacillus bifermentans* (Tissier and Martelly), *Bacillus centrosporogenes* (Hall) and certain closely related proteolytic anaerobes. *J. Bacteriol.* **33**:23-24.
- COLEMAN, G. E., AND J. B. GUNNISON. 1928. Serologic types of *Clostridium tetani*. *J. Infect. Diseases* **43**:184-188.
- DUFFETT, N. D. 1935. The differentiation of *Bacillus fallax* (Weinberg and Séguin) from *Bacillus carnis* (Klein). *J. Bacteriol.* **29**:573-582.
- ELLNER, P. D., AND C. D. BOHAN. 1962. Serology of the soluble antigens of *Clostridium perfringens* types A-F by agar-gel diffusion. *J. Bacteriol.* **83**:284-296.
- ELLNER, P. D., AND S. S. GREEN. 1963a. Serologic relationships between clostridia based on soluble antigens. *Bacteriol. Proc.*, p. 64.
- ELLNER, P. D., AND S. S. GREEN. 1963b. Serological grouping of the pathogenic clostridia. *J. Bacteriol.* **86**:1098-1100.
- FULTHORPE, A. J., AND R. O. THOMSON. 1960. Antigenic efficiency of tetanus toxoids modified by excess formalin or by heat and phenol. *Immunology* **3**:126-134.
- GUNNISON, J. B. 1937. Agglutination reactions of the heat stable antigens of *Clostridium tetani*. *J. Immunol.* **62**:63-74.
- GUNNISON, J. B. 1947. Complement fixation reactions of the heat labile and the heat stable antigens of *Clostridium tetani*. *J. Immunol.* **57**:67-70.
- HOWARD, J. G. 1953. The hemolysin of *Clostridium histolyticum*. *Brit. J. Exptl. Pathol.* **34**:564-567.
- KABAT, E. A., AND M. M. MAYER. 1961. Experimental immunochemistry. Charles C Thomas, Publisher, Springfield, Ill.
- KAUFMAN, L., AND R. H. WEAVER. 1960. Rapid methods for the identification of clostridia. *J. Bacteriol.* **79**:119-121.
- MCCOY, E., AND L. S. MCCCLUNG. 1938. Serological relations among spore-forming anaerobic bacteria. *Bacteriol. Rev.* **2**:47-97.
- MACLENNAN, J. D. 1962. The histotoxic clostridial infections of man. *Bacteriol. Rev.* **26**:177-276.
- MANDIA, J. W. 1952. Serological identification of cultures of *Clostridium histolyticum* as group III of the proteolytic clostridia. *J. Infect. Diseases* **90**:48-50.
- MANDIA, J. W., AND D. W. BRUNER. 1951. The serological identification of cultures of *C. sporogenes*. *J. Immunol.* **66**:497-505.
- MARSHAL, J. D., T. WETZLER, AND T. KAWATOMARI. 1956. Differentiation of the Clostridia associated with medical specimens. *U.S. Armed Forces Med. J.* **7**:1445-1455.
- MILES, E. M., AND A. A. MILES. 1947. The lecithinase of *C. bifermentans* and its relationship to the alpha toxin of *C. welchii*. *J. Gen. Microbiol.* **1**:385-389.
- MILES, E. M., AND A. A. MILES. 1950. The relation of toxicity and enzyme activity in the lecithinases of *C. bifermentans* and *C. welchii*. *J. Gen. Microbiol.* **4**:22-35.
- OAKLEY, C. L., G. H. WARRACK, AND P. H. CLARKE. 1947. The toxins of *Clostridium oedematis* (*C. novyi*). *J. Gen. Microbiol.* **1**:91-107.
- REED, G. B., AND J. H. ORR. 1941. Rapid identification of gas gangrene anaerobes. *War Med.* **1**:493-510.
- ROBERTSON, M. 1920. Serological grouping of *Vibrio septique* and their relation to the production of toxin. *J. Pathol. Bacteriol.* **23**:153-170.
- SMITH, L. 1937. Some serological aspects of the S-R change in *Clostridium histolyticum*. *J. Bacteriol.* **34**:409-417.
- SPRAY, R. S. 1936. Semisolid media for cultivation and identification of the sporulating anaerobes. *J. Bacteriol.* **32**:135-155.
- STERNE, M., AND H. G. WARRACK. 1962. The inter-

- actions between *C. septicum* and *C. histolyticum* toxins and antitoxins. *J. Pathol. Bacteriol.* **84**:277-288.
- WALKER, P. D. 1963. The spore antigens of *Clostridium sporogenes*, *C. bifermentans* and *C. sordellii*. *J. Pathol. Bacteriol.* **85**:41-49.
- WARBURG, O., AND W. CHRISTIAN. 1942. Isolierung und Krystallisation des Garungsferments Enolase. *Naturwissenschaften* **29**:589-590.
- WILLIS, A. T., AND G. HOBBS. 1959. Some new media for the isolation and identification of Clostridia. *J. Pathol. Bacteriol.* **77**:511-521.