Linkage Between 9 and 12 Antigens of *Salmonella typhosa.** (27287)

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During an investigation of the antigenic structure of *Salmonella typhosa* and related microorganisms(1), certain preliminary data suggested that the 9 and 12 somatic antigens of *S. typhosa* were present in unpurified water extracts as separate entities. Cohen(2) presented direct evidence that in a purified lipopolysaccharide of *S. typhosa,* prepared by the method of Webster *et al.*(3), these antigenic components are part of the same molecule. He recovered antigen from washed specific precipitates, prepared with either 9 or 12 antibody and *S. typhosa* lipopolysaccharide, by denaturing the antibody with 0.1 N sodium hydroxide. Similar conclusions have been reached by Staub and Pon(4). These investigators used lipopolysaccharides purified after acetic acid hydrolysis.

The present communication presents experiments to reconcile the preliminary data mentioned above with the results of Cohen(2) and Staub and Pon(4). In these experiments, only 9 and 12 antigens present in unpurified water extracts of *S. typhosa* without treatment with either acid or alkali were studied.

Materials and methods. The antigens used were water extracts of acetone-killed bacilli prepared as previously described(1). Antisera were prepared in rabbits by a series of 6 intravenous injections at 3-day intervals, of acetone-killed bacilli. The organisms used included *S. typhosa,* strain Ty2, *S. typhosa,* strain 0-901 and *S. pullorum* which contain both 9 and 12 O antigens, *S. haarlem* and *S. strasbourg* to provide the 9 antigen and *S. paratyphi A* and *S. paratyphi B* to represent the 12 O antigen component.

Specific precipitates were prepared by addition of 5 ml of the appropriate antiserum to *S. typhosa* water extracts in amounts sufficient to provide a slight antibody excess as determined by preliminary precipitin tests. Sterile technic was used throughout the procedure. The mixtures were allowed to stand at refrigerator temperature for 48 hours with intermittent stirring. Precipitates were collected by centrifugation and washed 5 times with sterile saline. The washed precipitates were suspended in 1 ml of sterile saline and emulsified with an equal portion of complete Freund adjuvant(5). The entire precipitate-adjuvant emulsions were injected into the hind foot pads, and intracutaneously into the skin of the back and ears of rabbits.

Booster injections consisting of washed specific precipitates suspended in saline were given intravenously a month after the initial specific precipitate-adjuvant injection. The amount of specific precipitate in the booster injection varied due to different degrees of toxicity among the preparations. All rabbits were bled by cardiac puncture a week after the last injection. Thus, antiserum to the specific precipitate of *S. typhosa,* 0-901 antigen and *S. haarlem* antiserum was prepared in one rabbit. One rabbit was inoculated with the specific precipitate of *S. typhosa,* 0-901 antigen and *S. strasbourg* antiserum and 2 rabbits were injected with the specific precipitate of *S. typhosa,* 0-901 antigen and *S. paratyphi B* antiserum. One rabbit was immunized with the specific precipitate of *S. typhosa,* Ty2 antigen and *S. paratyphi B* antiserum.

Results. Serum obtained from the rabbit immunized with specific precipitate of *S. typhosa,* 0-901 water extract and *S. haarlem* antiserum agglutinated *S. haarlem, S. typhosa,* 0-901, *S. paratyphi B, S. paratyphi A, S. strasbourg* and *S. pullorum.* Agglutination titers of this serum may be seen in Table I. This serum did not agglutinate a salmonella serotype *(S. virginia)* which does not contain either the 9 or 12 antigen specificities nor does this serum agglutinate *Shigella flexneri* organisms. These organisms have been found(1) to possess antigens, which have been called group specific antigens, in common with some salmonellas although they do not agglutinate in presence of salmonella anti-O serum. The

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TABLE I. Agglutination Reactions of Sera from Rabbits Injected with Specific Precipitates and Various Salmonellae.

Antigen	Antisera prepared by inj. of specific precipitate of *S. typhosa*, 0-901 antigen and				Antiserum prepared by inj. of specific precipitate of *S. typhosa*, Ty2 antigen and *S. paratyphi B* antiserum
	S. haarlem antiserum	*S. strasbourg* antiserum	*S. paratyphi B* antiserum		
			Rabbit #21	Rabbit #22	
S. paratyphi B	50	200*	3200	200	25
S. paratyphi A	25	400*	1600	400	
S. typhosa, 0-901	3200	200	1600	50	200
S. typhosa, Ty2					200
S. haarlem	50	100	1600	50	12.5
S. strasbourg	50	200	800	100	25
S. pullorum	400	25	100	50	

Numbers refer to the reciprocal of the highest dilution of antiserum producing 3+ or 4+ agglutination.

* Agglutination was read as 1+ throughout and indicates highest dilution of positive agglutination.

pre-immunization sera of all rabbits immunized with specific precipitates or with whole organisms did not contain antibody to any of the antigens of the salmonellas when tested by the methods employed here.

The antiserum prepared against the specific precipitate from *S. typhosa*, 0-901 antigen and *S. haarlem* antiserum also was examined by agar gel precipitin tests. Salmonella group specific antibody as well as antibody reactive with *Sh. flexneri* antigen could be detected in the antiserum. Apparently, in preparation of the specific precipitate, the salmonella group antigen and the antigen which can be also found in *Sh. flexneri* were precipitated from the water extract of *S. typhosa*, 0-901 by *S. haarlem* antiserum. These antigens have been shown to be identical in *S. typhosa* and *S. haarlem*(1). As stated above(1), these antigen-antibody systems were not detectable by the classical agglutination reaction and consequently were not responsible for the agglutination reactions reported in Table I. Therefore, the reactions recorded in Table I must be due to the 9 and 12 antigens and their antibodies. In addition, another zone of precipitate formed in agar gel plates when the specific precipitate antiserum reacted with *S. typhosa* water extract. This was a single zone of precipitate which coalesced with the zone of precipitate due to O antigen of the *S. typhosa* system. Dilution of the antigen or antibody and subsequent testing still produced a single zone of precipitate due to O antigen, indicating no superimposition of another zone (Fig. 1). The *S. typhosa*, Ty2 antiserum used in the agar gel plate shown in Fig. 1 was the same used in experiments reported previously(1) in which *S. typhosa* antigens were identified. Zones 1 and 3 were formed by group specific antigens and their respective antibodies. Zone 2 was formed by an antigen which is also found in *Sh. flexneri*, type 3. Zone 4 was due to the reaction of H antigen and its antibody. Zone 5 was due to Vi antigen and its antibody. Zone 6 was formed by O antigen and its antibody. The zone of precipitate which formed between the 2 antisera (specific pre-

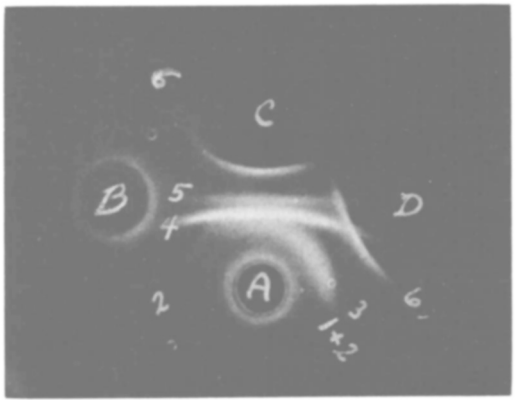

FIG. 1. Reaction of *S. typhosa* water extracts with specific precipitate antiserum and *S. typhosa*, Ty2 antiserum. A = *S. typhosa*, Ty2 antiserum. B = Antiserum to specific precipitate of *S. typhosa*, 0-901 antigen and *S. haarlem* antiserum absorbed with *Sh. flexneri*. C = *S. typhosa*, Ty2 water extract, 20 mg/ml. D = *S. typhosa*, 0-901 water extract, 20 mg/ml.

cipitate antiserum and *S. typhosa,* Ty2 antiserum) is due to excess *Sh. flexneri* used to absorb this antibody from the specific precipitate antiserum. Antibody could also be demonstrated by agar gel precipitin tests in the specific precipitate antisera which reacted with the mycobacteria used in the adjuvant. However, there was no cross reaction with any of the systems found in the salmonellas.

Serum from a rabbit immunized with specific precipitate containing the 9 antigen-antibody complex from *S. typhosa,* 0-901 water extract and *S. strasbourg* antiserum agglutinated organisms containing either or both of the 9 and 12 antigen specificities (Table I).

Specific precipitates which contained the 12 antigen-antibody complex were prepared from *S. typhosa,* 0-901 water extract and *S. paratyphi B* antiserum. After 2 small booster doses were given, sera collected from these rabbits contained antibody to both the 9 and 12 antigens as demonstrated by agglutination. In agar gel precipitin tests with these immune sera, no zone of precipitate was formed when tested with O antigens containing either or both 9 and 12. It was thought that this might be due to insufficient antibody to form a visible precipitate although one of the antisera (Table I, Rabbit #21) had generally higher agglutination titers than the other antiserum.

To determine whether the 9 and 12 antigens of *S. typhosa,* Ty2 exist in the same state as in *S. typhosa,* 0-901, an experiment was carried out in which water extract of *S. typhosa,* Ty2 was precipitated with *S. paratyphi B* antiserum. The results of agglutination reactions of serum from a rabbit immunized with this precipitate indicate that the precipitate contained both the 9 and 12 specificities.

Although the above results indicate that the 9 and 12 antigens are closely linked, the possibility still remained that some free 9 and/or 12 antigens may also be present in the preparations. Therefore, serologic tests were performed on the supernatants from the specific precipitates and no free antigen could be demonstrated. Therefore, the supernatants were concentrated 5-fold by pervaporation. Even after concentration, no free 9 or 12 antigen could be demonstrated.

Discussion. The 9 and 12 specificities of the O antigen of *S. typhosa* have been studied by immunization of rabbits with specific precipitates prepared by precipitation of either the 9 or 12 antigens in a water extract of *S. typhosa* with anti-9 or anti-12 sera. Agglutination tests and agar gel precipitin tests were used to test the immunologic responses. Rabbits so immunized produced antibody against both the 9 and 12 components.

Agglutination of organisms containing either the 9 or 12 or both antigens could be demonstrated with sera prepared by injection of rabbits with O antigen precipitated by either an anti-9 or anti-12 serum. When *S. typhosa,* Ty2 was used as the source of O antigen in preparation of the specific precipitate with *S. paratyphi B* antiserum, agglutination titers were quite low. Terres and Wolins(6) reported a decrease in intensity of the immune response when antigen-antibody complexes prepared in antibody excess were used for immunization. The precipitates used in the present studies were prepared in the region of antibody excess because of the necessity of precipitating all O antigen capable of reacting with the antiserum. Perhaps the deceleration of the immune response described by Terres and Wolins was responsible for the low antibody titers obtained in some instances.

In agar gel precipitin tests, a single zone of precipitate formed by the reaction of O antigen with its antibody. Antibody produced against the specific precipitates reacted with O antigen to form a zone of precipitate which coalesced with (reaction of identity) the zone formed by the reaction of O antigen and its antibody. It may be concluded that the 9 and 12 specificities of O antigen are incorporated on a single molecule.

These findings are consistent with those of Cohen(2) who found the 9 and 12 specificities to be closely linked in a purified O-lipopolysaccharide preparation. This was done by analysis of the supernatant and precipitate formed in the quantitative precipitin test when anti-9 or anti-12 sera were used to precipitate the O antigen. Staub and Pon(4) also were unable to demonstrate either 9 or 12 antigens in the supernatant of a precipitate of an extract from *S. typhosa,* 0-901 (after acid hydrolysis) with *S. paratyphi B* antiserum.

Summary. The 9 and 12 antigens of *S. typhosa* have been specifically precipitated by

either anti-9 or anti-12 sera and used to immunize rabbits. The antisera obtained contained both 9 and 12 antibodies indicating that the 9 and 12 antigens of *S. typhosa* are on the same molecule.

1. Whiteside, R. E., Baker, E. E., *J. Immunol.*, in press.
2. Cohen, H. H., *ibid.*, 1958, v81, 445.
3. Webster, M. E., Sagin, J. F., Landy, M., Johnson, A. G., *ibid.*, 1955, v74, 455.
4. Staub, A. M., Pon, G., *Ann. Inst. Pasteur*, 1956, v90, 441.
5. Leskowitz, S., Waksman, B., *J. Immunol.*, 1960, v84, 58.
6. Terres, G., Wolins, W., *ibid.*, 1961, v86, 361.

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Crystals and Concretions in the Vaginae of Persistent-Estrous Mice.* (27288)

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Transplantation of the testis of newborn male rats into littermate females induces persistent estrus by the time of puberty(1). Later investigators have found that androgen (2,3,4) or estrogen(5,6,7,8,9) treatment induces persistent estrus in rats if injections are started shortly after birth. The exogenous hormone presumably causes an irreversible disturbance of pituitary gonadotropin secretion. Induction of persistent estrus by sex steroid injections has not been reported in other mammals, although Perrotta has accomplished this in mice (personal comm.). During our successful attempts to accomplish this in various strains of mice, peculiar vaginal concretions were encountered, which are reported herein.

Materials and methods. 40 A/Crgl, 16 BALB/cCrgl, 15 C3H/Crgl, 16 RIII/Crgl and 24 C57BL/Crgl female mice were injected with 5 μg estradiol-17β in 0.02 ml aqueous suspension for 5 days starting within 24 hours after birth. 20 A, 5 BALB/c, 6 C3H, 4 RIII and 5 C57BL mice served as controls, being injected with 0.02 ml of 0.5% aqueous phenol (the vehicle for the estradiol). The mice were weaned at about 30 days of age. Vaginal smears were recorded daily for various periods of time.

Results. In all estrogen-injected mice, the vagina opened at 5-7 days of age, and clitoridal hypospadias occurred as in the female rat treated with estrogen in prenatal or early postnatal life(10,11,12). Persistent estrus began at 15 to 40 days of age in almost all mice; occasionally, the estrus was interrupted by a diestrous period in about 30% of the mice, not extending for more than 12 days. All control mice generally had normal estrous cycles; however, slightly prolonged estrus or diestrus occasionally occurred in all mice. The controls never showed uninterrupted cornified smears for longer than 9 days. Abnormal estrous cycles and interruption of persistent estrus appear to be more frequent in mice than in rats.

From about 50 days of age, prismatic crystals were noted in vaginal smears of the persistent-estrous mice of every strain (Fig. 1). These crystals resemble the so-called triple phosphates of human urinary sediments. In C57BL mice, numerous crystals were found regularly; about 50% of the daily vaginal smears taken showed crystals (over a 24-day period). About 10% of the vaginal smears from BALB/c mice and about 20% of those from A and RIII mice showed crystals. Crystals were found only rarely in C3H mice; only 2% of the vaginal smears had crystals. No crystals appeared in the control mice.

Large white concretions or clumps of concretions were found in the vaginal lumens of 21 of 40 persistent-estrous A mice at 6-7 months of age (Fig. 2); stones appeared later in an additional 9 mice at about 9 months of age. Many crystals and concretion frag-

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