mate, availability of food and presence of predatory animals, have been instrumental in producing cyclic habits of reproduction(5). In animals with restricted breeding seasons it is known that there is a seasonal variation in sensitivity response of the reproductive organs to sex hormones(6). In the domesticated laboratory rat, however, which is protected from many variables besetting the wild rodent and is thus able to breed year-round, it would be anticipated that sensitivity response of sex accessory glands to hormones would be relatively uniform throughout the year. On the contrary, our experiments using Zn^{65} uptake of DLP, as indicator of glandular function, have revealed variations in sensitivity response of the gland to sex hormones at different times of year, correlating with the pattern of the archaic reproductive cycle(4). Furthermore, our data demonstrated that these variations in sensitivity response of DLP are inherent in the gland itself, since the same seasonal variations were noted even in the absence of pituitary. In use of hormones experimentally and therapeutically, it appears that cognizance of seasonal variations in sensitivity response would do much to reduce variability of results.

Summary. Studies using Zn^{65} uptake by DLP as indicator of glandular function have demonstrated seasonal variations in sensitivity response of castrated and hypophysectomized rats to administered sex hormones. These variations in sensitivity response coincide with the pattern of the archaic reproductive cycle found in intact non-breeding male laboratory rat.

1. Gunn, S. A., Gould, T. C., Ginori, S. S., Morse, J. G., PROC. SOC. EXP. BIOL. AND MED., 1955, v88, 556.
2. Gunn, S. A., Gould, T. C., *Endocrinol.*, 1956, v58, 443.
3. Gunn, S. A., *J. Endocrinol.*, 1957, v16, 18.
4. ———, *ibid.*, 1958, v17, 344.
5. Bullough, W. S., *Vertebrate sexual cycles,* London: Methuen, 1951, 18.
6. Burrows, H., *Biological actions of sex hormones,* Cambridge University Press, 1949, 2nd Ed., 50.

Received January 26, 1959. P.S.E.B.M., 1959, v100.

Role of Vi Antigen in *Salmonella paratyphi* C Infections. (24733)

ADRIAN D. MANDEL, L. S. BARON AND CHARLES E. BUCKLER
(Introduced by O. Felsenfeld)
Division of Immunology, Walter Reed Army Inst. of Research, Walter Reed Army Medical Center, Washington, D.C.

The dominant role of Vi antigen in determining virulence of *Salmonella typhosa* for mice has been demonstrated by Felix and Pitt(1). This antigen is also highly effective in affording mice active and passive protection against *S. typhosa*(1-3). However, whether Vi antigen of *Salmonella paratyphi* C bears the same relationship to infection by and protection against *S. paratyphi* C in mice has not been fully determined. Kaufmann(4) has shown that an antiserum produced against living Vi-containing *S. paratyphi* C passively protected mice against the mouse-virulent Ty2 strain of *S. typhosa,* thus indicating the close immunological identity of the Vi antigen of *S. typhosa* and *S. paratyphi* C. In addition, Kaufmann(4) assumed that Vi antigen was of no importance in determining virulence of *S. paratyphi C,* since *S. paratyphi* C and *Salmonella hirschfeldi,* which differ antigenically only in absence of the Vi antigen in the latter organism, were of equal virulence. This assumption, nevertheless, did not take into account the fact that *S. hirschfeldi* and *S. paratyphi* C studied by Kaufmann, were obtained from different sources. A more critical evaluation of the role of Vi antigen as virulence factor in *S. paratyphi* C infections of mice is best accomplished by comparison of a Vi negative variant with its Vi containing parent strain. It is our purpose to determine relationship of the Vi antigen of *S. paratyphi* C

TABLE I. Mouse Virulence of Vi and Non-Vi Strains of *S. paratyphi* C.

	Challenge dose					
	5×10^3	5×10^4	5×10^5	5×10^6	5×10^7	5×10^8
Vi form	9/12*	12/20	15/20	16/20	20/20	20/20
Non-Vi form	0/20	2/20	5/20	4/20	17/20	"

* No. of deaths in 21 days/No. of mice inj.

to virulence and immunogenicity in mice, by comparing these 2 activities in a Vi-containing strain of *S. paratyphi* C and in a non-Vi containing variant selected from the Vi culture.

Materials and methods. Cultures. The East Africa strain of *S. paratyphi* C used, was the strain originally studied by Kaufmann. This organism contains the Vi antigen but is O agglutinable. The Vi-negative variant was obtained by selection from a population of Vi forms. *S. typhosa* Ty2, used as a control in certain experiments, is well characterized by its high Vi antigen content. All cultures were maintained in the lyophilized state until ready for use and were checked for their Vi antigen content immediately prior to use. A new ampule was used for each experiment. *Virulence tests.* In all virulence titration, and protection experiments, number of deaths was recorded daily for 3 weeks following challenge. For virulence titrations white female Bagg mice weighing 10 to 12 g were injected intraperitoneally with graded doses of a 6-hour meat extract agar culture suspended in 0.5 ml of saline. Viable counts were performed at time of challenge. Twenty mice were used at each dose level. *Immune sera* were prepared in rabbits by successive daily graded intravenous injections of 18-hour-old suspension of live organisms. Each rabbit received a total of 4 x 10^9 organisms in 5 injections. Ten days following last injection, blood was taken from the heart, the serum removed and stored frozen until used. The antiserum against the Vi form had an O agglutination titer of 1:2560 and a Vi hemagglutination titer of 1:10,240(5). The antiserum against the non-Vi form had an O titer of 1:10,240, and was negative for Vi hemagglutinins. The *S. typhosa* Ty2 antiserum had a Vi hemagglutination titer of 1:2560. *Purified Vi antigen* used was prepared from *Escherichia coli* 5396/38 and was kindly supplied by Doctor M. Webster of Division of Immunology, Walter Reed Army Inst. of Research. Its preparation has been described by Webster, Landy and Freeman(6). *Heat-killed vaccines* were prepared from 18-hour meat extract agar cultures suspended in saline and standardized to contain 1 x 10^9 bacteria/ml. Bacterial suspensions were heated at 56°C for one hour. Acetone-killed and dehydrated vaccine was prepared from *S. paratyphi* C and *S. typhosa* Ty2, according to the method described by Landy(7). The vaccine was rehydrated with distilled water immediately prior to use.

Results. Prior to virulence and protection tests, Vi and non-Vi forms of *S. paratyphi* C were examined in morphological, biochemical and nutritional characteristics. The original Vi form required thymine but mutated to thymine independence at a rate of $1x10^{-6}$. There was, however, no difference in mouse virulence of the thymine-requiring and thymine-independent Vi forms. The Vi-negative form as isolated was thymine-independent. No other difference could be detected.

Virulence of Vi and non-Vi strains. Virulence titrations were repeated on several occasions with similar results. The results of a typical titration of virulence of Vi and non-Vi forms of *S. paratyphi* C are shown in Table I. At challenge levels below 10^5 organisms, the response of groups of mice was flat and very irregular, as reported by Archer and Whitby (8). Above the 10^5 level there was rather sharp response over a 2-3 log range although even at these levels the response was somewhat irregular. Therefore it was not possible by usual graphic methods to determine the LD_{50} of each strain with any degree of accuracy. However, repeated titrations indicated decreased virulence of the non-Vi forms, roughly estimated about one hundred fold, in those titrations amenable to graphic representation. In all subsequent experiments, the challenge doses were adjusted to anticipate a

TABLE II. Active Immunization of Mice against *S. paratyphi* C.

Immunizing agent	Challenge dose: *S. paratyphi* C, Vi form, 3×10^6	*S. paratyphi* C, Non-Vi form, 4×10^8	*S. typhosa* Ty2 1×10^8
S. paratyphi C, Vi form, heat-killed	20/20*	19/20	2/19
S. paratyphi C, non-Vi form, heat-killed	"	"	14/20
Control	16/20	20/20	19/20

* No. of deaths in 21 days/No. of mice inj. Intraper. challenge 10 days following immunization.

mortality of 80-100% of control mice.

Active immunization. The comparative effectiveness of Vi and non-Vi forms of *S. paratyphi* C as active immunizing agents was studied, using heat-killed vaccine of each form. Groups of mice were immunized by intraperitoneal injection of 5 x 10^8 heat-killed cells of either form of *S. paratyphi* C. Ten days later, an equal number of mice from each group was challenged by intraperitoneal injections of Vi or non-Vi forms of *S. paratyphi* C. The data presented in Table II demonstrate lack of protection afforded mice by either heat-killed vaccine.

Passive Immunization. Passive immunity to *S. paratyphi* C infections in mice was studied employing rabbit antisera prepared against the Vi and non-Vi strains of *S. paratyphi* C and *S. typhosa* Ty2 as control. Groups of 60 mice were injected intraperitoneally with 0.1 ml of one of these antisera. One hour later 20 mice from each group were injected intraperitoneally with saline suspension of each strain of *S. paratyphi* C and of *S. typhosa* Ty2. The data shown in Table III demonstrate failure of specific antiserum to protect against either organism. In contrast, when Vi antiserum was present, protection against *S. typhosa* Ty2 was good. This protection of mice against *S. typhosa* Ty2 given by the Vi form *S. paratyphi* C antiserum is in agreement with findings of Kaufmann, and is additional evidence of the presence of Vi antibody in the Vi-form antiserum.

Immunization with acetone-killed vaccine. Since it is known that heat reduces Vi antigen content of bacterial cells, active immunity was studied with acetone-killed and dehydrated vaccine of the Vi form. One half ml amounts of a reconstituted acetone-killed and dehydrated vaccine of the Vi form containing 5 x 10^8 cells were injected intraperitoneally into 20 mice. Ten days later, one half of the mice were injected with the Vi form of *S. partyphi* C and the remainder with *S. typhosa* Ty2 as a control for Vi antigenicity of the *S. paratyphi* C vaccine. The results (Table IV) fail to show better protection of mice by vaccine of maximal Vi antigen content.

Immunization with purified Vi antigen. To provide more definitive data on the role of Vi antigen in active immunity of mice against *S. paratyphi* C, purified Vi antigen was used as immunizing agent. Sixty mice were immunized intraperitoneally with 50 μg of purified Vi antigen derived from *E. coli* 5396/38. Seven days later one group of immunized mice was challenged intraperitoneally with the Vi form of *S. paratyphi* C; one group with non-Vi form, and as a control of immunological activity of the purified Vi antigen, the remaining mice were challenged with *S. typhosa* Ty2.

TABLE III. Passive Immunization of Mice against *S. paratyphi* C.

Rabbit antiserum	Challenge dose: *S. paratyphi* C, Vi form, 5×10^5	*S. paratyphi* C, Non-Vi form, 5×10^8	*S. typhosa* Ty2 1×10^8
S. paratyphi C, Vi form	13/20*	19/20	1/20
S. paratyphi C, non-Vi form	15/20	"	12/20
S. typhosa Ty2	"	20/20	1/20
Control	"	19/20	19/20

* No. of deaths in 21 days/No. of mice inj. 0.1 ml antiserum intraper. followed in 1 hr by intraper. challenge.

TABLE IV. Active Immunization with AKD Cells and Purified Vi Antigen.

Immunizing agent	Challenge dose: *S. paratyphi* C, Vi form, 5×10^6	*S. paratyphi* C, Non-Vi form, 5×10^8	*S. typhosa* Ty2 1×10^8	
S. paratyphi C, Vi form, 5×10^8 AKD	7/10*		1/10	
Purified Vi† antigen, 50 μg	8/10	9/10		1/10
Control‡	9/10	10/10	9/10	9/10

* No. of deaths in 21 days/No. of mice inj. Intraper. challenge 7 days following immunization.

† From *E. coli* 5396/38.

‡ This table includes 2 separately controlled experiments.

Suitable numbers of non-immunized control mice were challenged at the same time. The results demonstrate that a purified Vi antigen failed to provide active protection of mice against infection with either Vi or non-Vi strain of *S. paratyphi C* (Table IV).

Discussion. Since *S. paratyphi* C (Vi+) and *S. hirschfeldi* (Vi–) have been reported of equal virulence for mice(4), it has been assumed that the Vi antigen plays no part in determining virulence of *S. paratyphi* C. In these experiments, when a Vi-positive and a Vi-negative form of the same strain of *S. paratyphi* C were compared in the mouse, the Vi-negative mutant was of reduced virulence. Unlike *S. typhosa,* loss of Vi antigen did not result in an avirulent form. It must be recognized, nevertheless, that the mutant strain may have suffered a loss of factors other than the Vi antigen, which may also be closely associated with the observed decrease in virulence.

The failure of immunizing agents employed, to protect mice against *S. paratyphi* C is evidence that the Vi antigen was not protective under conditions of these experiments. The protection of mice against *S. typhosa* Ty2, given by the Vi form of *S. paratyphi* C, shows that the Vi antigen of these 2 organisms, qualitatively at least, has the same immunological activity. Passive protection against *S. typhosa* Ty2 by antiserum against the Vi form of *S. paratyphi* C indicates that lack of protection against homologous challenge could not have been due to production of a non-protective or "inactive" Vi antiserum by *S. paratyphi* C. It is evident that the Vi antigen does not play the same role in *S. paratyphi* C infection of mice as in *S. typhosa.*

Archer and Whitby(8) have reported that a Vi vaccine prepared from either living or alcoholized cells afforded mice temporary significant protection against toxic (high) challenge doses of *S. paratyphi* C, and that specific O antigen is similarly effective against a fatal (low) challenge dose. These results, which are at variance with the findings reported here using heat-killed and acetone-killed and dehydrated vaccines or purified Vi antigen, may reflect an effect of the method of vaccine preparation on protection. Experiments designed to furnish more information on this point are presently in progress.

Summary. A comparison was made of virulence and immunogenicity of the Vi form of *S. paratyphi* C and of a non-Vi form selected from the Vi form. Repeated virulence titrations in mice indicate that the Vi-negative mutant is of reduced virulence but does not become avirulent. We have been unable to demonstrate that antibody directed against the Vi antigen plays any role in protecting mice against *S. paratyphi* C infection.

1. Felix, A., Pitt, R. M., *J. Path. Bact.,* 1934, v38, 409.

2. Longfellow, D., Luippold, G. F., *Am. J. Hyg.,* 1943, v37, 206.

3. Landy, M., Webster, M. E., *J. Immun.,* 1952, v69, 143.

4. Kaufmann, F., *Z. Hyg.,* 1936, v117, 779.

5. Landy, M., Lamb, E., PROC. SOC. EXP. BIOL. AND MED., 1953, v82, 593.

6. Webster, M., Landy, M., Freeman, M., *J. Immun.,* 1952, v69, 135.

7. Landy, M., PROC. SOC. EXP. BIOL. AND MED., 1952, v80, 55.

8. Archer, G. T. L., Whitby, J. L., *J. Hyg.,* 1957, v55, 513.

Received January 5, 1959. P.S.E.B.M., 1959, v100.