THE Vi ANTIGEN OF *SALMONELLA PARATYPHI A*

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CONTENTS

SELECTION OF O-SENSITIVE AND O-INSENSITIVE STRAINS

The starting-point of the work on Vi antigens in *Salmonella* species other than *Salm. typhi* was the observation that different strains of *Salm. paratyphi A* showed a different degree of sensitiveness to O antibody. It was found in earlier work (Felix & Olitzki, 1926) that the strain HA 1 of *Salm. paratyphi A* which was highly sensitive to O agglutinins was also highly susceptible to the bactericidal action of serum, whereas the strain HA 6 was resistant to both. Normal rabbit sera killed the strain HA 1 so readily that it was quite unsuitable for experiments on the bactericidal action of immune sera. In agglutination tests with pure O antisera the difference in the titres observed with the two strains was approximately tenfold. This was much less striking than the difference in sensitiveness of an O-agglutinable and an O-inagglutinable strain of *Salm. typhi*, which may be 100-fold or even 1000-fold. However, the property of being either O-sensitive or O-insensitive was as constant in the two strains of *Salm. paratyphi A* as it was in certain of the classical strains of *Salm. typhi*. The two strains HA 1 and HA 6 were therefore employed side by side in the estimation of O agglutinins to *Salm. paratyphi A* during the early work on 'qualitative receptor analysis' (Felix & Olitzki, 1926; Felix, 1930).

By analogy with what was known about the correlation between O agglutinability and virulence of *Salm. typhi* (Felix & Pitt, 1934*a*, *b*), it was decided to select for the experiments on the hypothetical Vi antigen of *Salm. paratyphi A* mainly strains that belonged to the two extreme types. Only one strain of the type of intermediate agglutinability, comprising the majority of old laboratory strains of *Salm. paratyphi A*, was included in this study. The strains were selected by preliminary agglutination tests with a pure O antiserum; their antecedents are shown in Table 1.

* Miss R. Margaret Pitt collaborated in some of the early experiments described in this paper.

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Table 1. *Details of strains of* Salmonella paratyphi A *used in the present investigation*

Strain	Sensitiveness to O agglutinins	Isolated: Locality	Isolated: Year	Reference
HA 6	Non-sensitive	Palestine	1925	Felix & Olitzki (1926)
20606	Non-sensitive	Palestine	1934	Culture received in 1934 from Dr K. S. Krikorian, Jerusalem
2035	Intermediate	Unknown	Before 1924	Culture received in 1934 from the National Collection of Type Cultures, London
17689	Sensitive	Transjordan	1934	Culture received in 1934 from Dr K. S. Krikorian, Jerusalem
HA 1	Sensitive	Durazzo	1917	Weil & Felix (1920)

VIRULENCE AND AGGLUTINATION WITH O AND Vi SERA

According to expectation it was found that virulence for mice of different strains of *Salm. paratyphi A* was correlated with their behaviour towards the action of the O antibody; strains of low agglutinability are relatively virulent, whereas highly agglutinable strains are avirulent. This is illustrated in Table 2.

Table 2. *Agglutination reactions and virulence of strains of* Salmonella paratyphi A

Serum	Dilution	Strains: HA 6	20606	2035	17689	HA 1
Agglutination of living organisms						
H serum	1:20,000	+ +	+ +	+	+	+ +
O serum	1:500	+ + +	+ + +	+ + +	+ + +	+ + +
	1:1000	+ +	+ +	+ + +	+ + +	+ + +
	1:2000	±	+	+ +	+ + +	+ + +
	1:5000	–	–	+	+ + +	+ + +
	1:10000	–	–	–	+ +	+ +
	1:20000	–	–	–	±	(±)
Vi serum	1:100	+ +	+ +	+ + ±	+ + +	+ + +
(rabbit no. 97 *versus* strain 20606	1:200	±	±	+	+ +	+ +
live, absorbed with acid-treated	1:400	–	–	(±)	+	+
organisms of strain 20606)	1:800	–	–	–	±	±
	1:1600	–	–	–	(±)	((±))
Virulence for mice						
Dose 400×10^6 organisms intraperitoneally		7/8	6/8	4/8	0/8	0/8
Dose 200×10^6 organisms intraperitoneally		2/8	2/8	0/8	0/8	0/8
Degree of virulence		High	High	Inter-mediate	Low	Low

The technique of the agglutination and virulence tests has been described in a previous paper (Felix & Pitt, 1951).

Reading of agglutination tests after 24 hr. (2 hr. at 37° C., then in the cold room).

± = weakest degree of agglutination which could be estimated with the naked eye.

(±) = trace, ((±)) = faint trace } estimated by means of magnifying lens.

The numerators of the fractions indicate the number of mice that died, the denominators the number inoculated.

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Table 2 shows that the difference in O agglutinability between the two non-sensitive strains HA 6 and 20606 and the two fully sensitive strains 17689 and HA 1 is approximately tenfold. This is about 10–100 times less than the corresponding difference between the inagglutinable virulent Vi + O form of *Salm. typhi* and the fully agglutinable avirulent O variant which is completely devoid of the Vi antigen.

It was not surprising, therefore, to find that the difference between the lethal doses of the O-insensitive and the O-sensitive strains of *Salm. paratyphi A* was also of lesser magnitude than that existing between the two extreme types of *Salm. typhi*. With the strains Ty 2 and O 901 of *Salm. typhi* the ratio of the approximate LD 50 is 20:1 (see Felix & Pitt, 1934*a*, 1951). No attempt was made to determine the ratio accurately with the various strains of *Salm. paratyphi A*. Their virulence for mice is known to be much lower than that of *Salm. typhi*. To obtain regularly reproducible results in virulence tests with *Salm. paratyphi A* it is essential to employ only male mice weighing not more than 14–16 g., to use agar medium of the best quality and to test the cultures after incubation for not more, and preferably less, than 12 hr. at 37·5° C. (Felix & Pitt, 1951; Felix & Anderson, 1951).

Contrary to the view still widely held, virulence is not necessarily a property of recently isolated strains. The experiment recorded in Table 2 was carried out during 1935, and it was found that the strain 17689, isolated the preceding year, possessed the lowest degree of virulence, whereas the old laboratory strain HA 6 was the most virulent of the five strains tested. Thus, the experiment clearly indicated that it was resistance to the action of the O antibody that determined the degree of virulence of the cultures.

Table 2 also shows that the factor responsible for the relative insensitivity to O antibody is the Vi antigen which is present in the two virulent strains HA 6 and 20606 in greater quantity than in the O-sensitive strains 17689 and HA 1. This is indicated by the titres of Vi agglutination determined with the different cultures. In *Salm. typhi* it was observed early in the work on the Vi antigen that the higher the virulence of the strain the lower was the titre of the Vi-agglutination reaction obtained with the culture (Felix & Pitt, 1934*b*; Felix, Bhatnagar & Pitt, 1934). Quantitative absorption tests confirmed that there was an inverse ratio between Vi-agglutinability and the Vi-antigen content of the culture. This relationship is, within a certain range, so constant, that it is employed as a simple and reliable means of estimating the Vi-antigen content of cultures of different strains of *Salm. typhi* (Felix, 1938).

It was concluded, therefore, from the Vi-agglutinin titres exhibited by the five strains of *Salm. paratyphi A* that the strains HA 6 and 20606 possessed the greatest amount of Vi antigen, the strains 17689 and HA 1 the smallest amount, and that the strain 2035 held an intermediate position.

CONTENT OF Vi ANTIGEN AND VIRULENCE OF STRAINS

To confirm the conclusion drawn from the O- and Vi-agglutination tests illustrated in Table 2 quantitative absorption tests were carried out with Vi antiserum, analogous to those recently described for strains of *Salm. typhi* (Felix & Pitt, 1951).

Table 3 shows that equal numbers of organisms of the strains HA 6 and 20606 absorbed the same amount of Vi antibody, whereas approximately four times this number was needed in order to produce the same effect with the strains 17689 and HA 1. Again, the strain 2035 held an intermediate position. The relative virulence of the five cultures employed in the absorption tests was the same as in the previous experiment.

Table 3. *Virulence of strains of* Salmonella paratyphi A *in relation to their content of Vi antigen*

Vi serum (rabbit no. 96 *versus* strain 17689 live, absorbed with acid-treated organisms of strain 17689)

2·5 ml. of serum dilution 1:40 absorbed with live organisms. Absorbing dose per 1 ml. of serum in millions of organisms of strains

	Serum dilutions	Control serum unabsorbed	HA 6 4000	HA 6 1000	20606 4000	20606 1000	2035 4000	2035 1000	17689 4000	17689 1000	HA 1 4000	HA 1 1000
			Absorption of Vi antibody									
glutination of	1:40	+++	−	±	−	+	(±)	++	+	+++	+	+++
ring organisms of	1:80	+++	−	(±)	−	(±)	−	±	(±)	+++	±	+++
rain HA 1	1:160	+++	−	−	−	−	−	(±)	−	+	((±))	+
	1:400	++±	−	−	−	−	−	−	−	((±))	−	(±)
	1:800	+	−	−	−	−	−	−	−	−	−	−
	1:1600	−	−	−	−	−	−	−	−	−	−	−
			Virulence for mice									
se 400×10^6 organisms intraperitoneally			5/6		5/6		3/6		0/6		0/6	

Note. The technique of quantitative agglutinin-absorption tests has been described in a previous paper (Felix & Pitt 51).

Strictly quantitative absorption tests of the kind described in Table 3 were carried out on many occasions in parallel with agglutination tests with pure O and pure Vi sera. The two sets of tests invariably confirmed one another. At the beginning of the investigation the Vi sera employed were prepared by immunizing rabbits with living bacilli and absorbing the Vi + O + H sera with acid-treated organisms of the same strain (see Tables 2 and 3). Such sera contained, of necessity, also H agglutinins in high titre. However, the floccular H agglutination did not cause any real source of error in the correct reading of the granular Vi agglutination. In later experiments pure Vi sera were prepared by using alcohol-treated bacilli for immunization and alkali-treated suspensions for absorption, as described in the preceding paper of this series (Felix, 1952).

LOSS OF VIRULENCE ON PROLONGED SUBCULTURE

Numerous authors, working in various parts of the world, have described strains of *Salm. typhi* which, at the time of their isolation, possessed maximal virulence and maximal O inagglutinability but gradually lost these properties on prolonged subculture on plain agar. Thanks to the co-operation of Lt.-Col. (then Major) H. J. Bensted it was possible to observe the same process in a freshly isolated strain of *Salm. paratyphi A*.

The strain, labelled 170/37, was isolated by Lt.-Col. Bensted in Kasauli, India,

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and the first subculture on an agar slope, posted on 2 November 1937, was received by the present writer in London on 3 December 1937.

On 8 December 1937 the second subculture on agar of strain 170/37 and the old laboratory strain HA 6 were compared for their agglutinability by pure O and pure Vi serum, and for their virulence for mice. The O- and Vi-agglutinability of strain 170/37 were only about a quarter that of the strain HA 6; a dose of 200×10^6 organisms of strain 170/37 killed eight of eight mice, whereas a similar dose of strain HA 6 killed only two of eight mice.

On 15 December 1937 the ninth subculture of strain 170/37 was re-examined; its O- and Vi-agglutinability were now only half that of the strain HA 6; the results of the comparative virulence tests were still the same as a week earlier.

Subcultures were made from the second agar-slope culture of strain 170/37 into Lemco-agar stabs, and re-examined after storage for one year at room temperature; the O- and Vi-agglutinability of the cultures were now considerably higher than those of the strain HA 6, and the mouse-virulence of the strain 170/37 had fallen below that of the strain HA 6.

These observations represent an exact analogy with what is known about Vi-antigen content and virulence of different strains of *Salm. typhi* after prolonged subculture on laboratory media. Similar observations on a freshly isolated strain of *Salm. paratyphi A* were reported from India by Bhatnagar, Freeman & Gera (1937).

The ability to produce Vi antigen in maximum amount when maintained on plain nutrient agar is an inherent property of certain strains, a hereditary trait of remarkable stability. The strain HA 6 of *Salm. paratyphi A* resembles in this respect the well-known strain Ty 2 of *Salm. typhi*, both of which have served for many years as much valued strains for T.A.B. vaccine.

EFFECTS OF VARYING CONDITIONS OF GROWTH

(*a*) *Effect of growth on phenol agar*

Growth on phenol agar (Braun, 1918), even for periods as short as 24 hr., completely suppresses resistance to O agglutinins of virulent strains of *Salm. typhi* and reduces their virulence for mice very considerably (Felix & Pitt, 1934*a*). Several attempts were made to demonstrate a similar phenomenon with the O-insensitive strains of *Salm. paratyphi A*, but all failed.

The two strains HA 6 and 20606 grew rather poorly on agar containing 1 in 900 phenol, but were maintained without difficulty on this medium for at least seven daily subcultures. Agglutination tests with pure O and pure Vi sera, and quantitative absorption of Vi antibody, did not disclose any diminution in the development of Vi antigen in the two strains of *Salm. paratyphi A*, whereas control tests with the strains Ty 2 and Watson of *Salm. typhi*, carried out on the same phenol-agar medium, invariably showed the phenomenon to occur after a few passages.

This observation has a parallel in the one recorded in the preceding paper (Felix, 1952), namely, that phenol produces a peculiar reversible inactivation of the T Vi antigen but not of the A Vi antigen.

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(*b*) *Effect of growth at various temperatures*

The development of the TVi antigen is suppressed by growing virulent strains of *Salm. typhi* on a good trypsin-digest agar at temperatures between 20 and 25° C., and also between 40 and 44·5° C. (Felix, *et al.* 1934). On the other hand, cultures of *Salm. paratyphi A* grown at 21° C. for 4 days were found to contain as much of the AVi antigen as the controls grown at 37·5° C. The virulent strain 20606 and the intermediate strain 2035 were grown on trypsin-digest agar at 21° C. for 100 days. Although the cultures had become partially rough, as judged by their salt-agglutinability, their capacity to absorb AVi agglutinin was still undiminished.

(*c*) *Effect of growth on serum agar and ascitic agar and in* CO_2 *atmosphere*

Because of the rapid decrease in the Vi-antigen content observed in cultures of freshly isolated strains of *Salm. paratyphi A*, it was thought possible that media of a nutritive value higher than that of plain trypsin-digest agar might enhance development of the Vi antigen. Agar media containing 20% normal rabbit serum or 20% ascitic fluid were used, and the cultures were grown aerobically under normal atmospheric conditions and also in an atmosphere of 5% CO_2 (see Huddleson, 1921; Wilson, 1930, 1931*a*, *b*). After seventeen subcultures on these media the two O-sensitive strains 17689 and HA 1 did not show any increase in their Vi-antigen content.

DISCUSSION

The results of the experiments described in this paper indicate beyond any reasonable doubt that the virulence of *Salm. paratyphi A* for mice is closely associated with resistance to the action of the O antibody; that the factor responsible for this resistance is the Vi antigen; that this antigen is present in virulent strains in greater quantity than in avirulent strains; and that freshly isolated strains are usually rich in Vi antigen but lose it gradually on subculture on laboratory media.

The analogy between these observations and the facts known from the work on the Vi antigen of *Salm. typhi* is evident. Taken in conjunction with the experiments recorded in the preceding paper of this series (Felix, 1952), it is clear that the labile somatic antigen of *Salm. paratyphi A* is to be classified as a Vi antigen, together with the Vi antigen of *Salm. typhi*. This conclusion appears to be inescapable on the evidence presented, the essential part of which was summarized in the original paper on this subject (Felix & Pitt, 1936).

It is of interest to inquire how the Vi antigen of *Salm. paratyphi A* was disposed of in Kauffmann's writings, and consequently has no place in the Kauffmann-White schema.

In 1930 the two strains HA 6 and HA 1 were recommended for use in the 'qualitative serum diagnosis of enteric fevers' (Felix, 1930), and at Kauffmann's request the two cultures were sent to him.

Kauffmann & Silberstein (1934) reported that the strain HA 1 did not contain the O-factor I which, up to that time, had been considered to be characteristic for *Salm. paratyphi A*. On the strength of this finding a special type or subtype was created, *Salm. paratyphi A* var. *durazzo*.

Kauffmann (1936, see p. 326) assumed *a priori*, without any experimental evidence, that the antigen described by Felix & Pitt (1936) as the Vi antigen of *Salm. paratyphi A* might be the O-factor I.

Kauffmann (1940, 1941*b*) searched for the presence in *Salm. paratyphi A* of an antigen similar to the Vi antigen of *Salm. typhi*. The following is a translation of Kauffmann's statement: 'Various attempts were made to demonstrate the presence of a "Vi antigen", but all failed' (Kauffmann, 1940, see p. 139). Instead, he described in the course of this work a new 'form variation', namely, I variation (Kauffmann). and established that twenty-one of the different *Salmonella* types recognized at that time contained the O-antigen I and were liable to the new 'form variation'.

In many of his subsequent publications, including the latest monograph on Enterobacteriaceae, Kauffmann was satisfied with stating simply that '...he could not confirm the occurrence of a Vi antigen in *Salm. paratyphi A*...maintained by Felix and Pitt...' (Kauffmann, 1951, see p. 44).

Kauffmann's most recent contribution to the serology of *Salm. paratyphi A* concerns another case of 'form variation', this time the XII_2 form variation (Kauffmann, 1941*a*; Schmid & Kauffmann, 1952). Although Schmid & Kauffmann referred to the strains HA 6 and HA 1, though not mentioning their origin, they again managed to overlook the labile Vi antigen and concentrated on the analysis of minute details concerning a minor fraction (XII_2) of one of the many components of the O-antigen complex.

The futility of this kind of antigenic analysis is obvious. A glance at the latest edition of the Kauffmann-White schema (Kauffmann, 1951) shows that 47 of the 209 recognized *Salmonella* types contain the O-factor I. According to Saxholm (1950) factor I, or some antigen related to factor I, also occurs in other *Salmonella* types so far listed as devoid of factor I in the Kauffmann-White schema. Similarly, O-factor XII is stated to be present in 62 of the 209 recognized *Salmonella* types. If the O-factor XII of these 62 *Salmonella* types were to be further split into its components XII_1, XII_2 and XII_3 (Kauffmann, 1941*a*) and 'form variation' of factor XII_2 were allowed to raise further strains to the rank of new types or subtypes, it is not difficult to foresee the confusion that would result.

It has been recognized from the outset that the presence of the Vi antigen in *Salm. paratyphi A* does not cause inhibition of the interaction between the O antigen and its antibody in the same degree as it does in the most virulent strains of *Salm. typhi*. In this respect the most virulent O-insensitive strains of *Salm. paratyphi A* may well be compared with the common type of *Salm. typhi*, namely, the type of intermediate virulence that is agglutinable by both the O and the Vi antibody. Nevertheless, the Vi antigen of *Salm. paratyphi A* plays an important role in infection and immunity.

Reference has been made in previous papers to the outstanding ability of the strain HA 6 to develop the Vi and the O antigens in maximum quantities when grown on plain nutrient agar (Felix, 1941, 1951*a*). Because of this property the strain HA 6 has been selected as the most suitable strain for the preparation of T.A.B. vaccine. The criteria for selection, described in this paper, are precisely the same as those applied to the well-known vaccine strain Ty 2 of *Salm. typhi*.

Kauffmann's erroneous conclusion on the non-existence of the A Vi antigen and his over-estimation of the value of slide-agglutination tests as a means of antigenic analysis have had a most misleading effect on workers engaged in the preparation of T.A.B. vaccine. It may be of interest to recall the difficulties experienced by many workers in the selection and maintenance of a suitable vaccine strain of *Salm. paratyphi A*. For instance, Luippold (1942) and Longfellow & Luippold (1943), following Kauffmann's methods, were unable to select a satisfactory vaccine strain and were led to believe that 'antigenic completeness' of a strain, as determined by typing according to Kauffmann's technique, is not synonymous with immunogenic effectiveness. The difficulties experienced by these workers were solved by the adoption of the strain HA 6 as the paratyphoid-*A* vaccine strain for the United States Army vaccine (Callender & Luippold, 1943, p. 320). This is reminiscent of the inadequate characterization of the typhoid vaccine strains employed by Batson, Landy & Brown (1950*a*, *b*), which was also based on Kauffmann's technique.

Another important gap the A Vi antigen and A Vi antibody are likely to close in the future is in the diagnostic field. The A Vi antigen is remarkably specific. Pure A Vi serum was tested against a representative collection of those *Salmonella* types that possess the O-factor I. The following twenty *Salmonella* types were examined:

S. saint-paul	*S. dublin*	*S. claibornei*	*S. simsbury*
S. budapest	*S. rostock*	*S. sendai*	*S. worthington*
S. bispebjerg	*S. onarimon*	*S. taksony*	*S. wichita*
S. schwarzengrund	*S. dar-es-salam*	*S. senftenberg*	*S. habana*
S. bredeney	*S. panama*	*S. niloese*	*S. mississipi*

The cultures were obtained through the co-operation of Dr Joan Taylor, of the Salmonella Reference Laboratory, London, who very kindly re-examined the cultures and confirmed that all of them contained the O-factor I. Tube agglutination tests were set up with dilutions 1:200, 1:500 and 1:1000 of a pure A Vi serum which in the last dilution gave a naked-eye reading of + with the sensitive strain 17689 of *Salm. paratyphi A*. None of the twenty *Salmonella* types showed any trace of agglutination with the A Vi serum.

In view of the fact that the classical Vi antigen of *Salm. typhi* is shared by a number of different *Salmonella* species and is not rare even amongst *Bact. coli* strains (Kauffmann, 1941*c*; Marmion, 1944), it is to be expected that the A Vi antigen will also be found in organisms other than *Salm. paratyphi A*. So far the complete set of 209 recognized *Salmonella* types have not been tested against pure A Vi serum.

On the other hand, no strain of *Salm. paratyphi A* devoid of the A Vi antigen has yet been encountered. This was found in experiments on the phage typing of *Salm. paratyphi A* by means of an adapted A Vi-phage, carried out in collaboration with Prof. R. G. Dhayagude and Dr D. D. Banker of Bombay (Felix, 1951*b*). Of 132 strains of *Salm. paratyphi A* isolated in distant parts of the world all were agglutinated by pure A Vi serum, except those cultures that were typically

'rough'. It must be stated, however, that no special effort has been made to detect Vi-negative variants in fresh or old laboratory cultures, or to induce their formation by the action of Vi antibody or Vi bacteriophage, the two procedures known to favour development of Vi-negative variants of *Salm. typhi*.

The H antigen *a*, characteristic of *Salm. paratyphi A*, is already listed in twelve other *Salmonella* types specified in the latest Kauffmann-White schema (Kauffmann, 1951), and another *Salmonella* that contains factor *a* has since been added to this list (Jebb, Douglas & Taylor, 1951). Coliform organisms possessing the H antigen *a* have also been mentioned (West, Edwards & Bruner, 1947; Edwards & West, 1950), and Luippold (1942) and Longfellow & Luippold (1943) referred to coliform organisms containing the O-factors I and II. It seems probable, therefore, that a highly specific reagent for *Salm. paratyphi A*, such as a pure AVi serum, may yet become a useful diagnostic tool.

SUMMARY

1. *Salmonella paratyphi A* possesses a specific Vi antigen essentially similar to that of the Vi antigen of *Salm. typhi*.

2. O-insensitive strains of *Salm. paratyphi A* contain more Vi antigen, and are more virulent for mice, than O-sensitive strains.

3. The most suitable strains for the preparation of paratyphoid-*A* vaccine are those that develop both the Vi and the O antigens in maximum quantities. The methods of selecting the vaccine strain of *Salm. paratyphi A* and routinely examining the cultures are essentially the same as those applied to the virulent Vi + O form of *Salm. typhi*.

4. Pure AVi antiserum is destined to assume importance in the identification of *Salm. paratyphi A* strains.

5. Kauffmann's erroneous conclusion that *Salm. paratyphi A* does not develop Vi antigen is critically reviewed.

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