THE PATHOGENIC AND IMMUNOGENIC ACTIVITIES OF *SALMONELLA TYPHI* IN RELATION TO ITS ANTIGENIC CONSTITUENTS

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INTRODUCTION

The so-called Vi antigen of the typhoid bacillus has been the subject of extensive investigations during the past 15 years. There is fairly general recognition that, among the antigens so far identified, the O and Vi antigens of the typhoid bacillus are the two most essential in determining its pathogenic and immunogenic characters. Only strains that contain maximum amounts of these two antigens possess the highest degree of pathogenicity of which the typhoid bacillus is capable when tested in the mouse, the guinea-pig or the rabbit. On the other hand, the Vi antibody alone, without accompanying O antibody, is sufficient to protect mice effectively against infection with strains of the highly virulent O + Vi type (Felix & Pitt, 1935). The latter finding at first appeared to be rather puzzling, but it was subsequently fully corroborated by Kauffmann & Møller (1940), Longfellow & Luippold (1943) and Luippold (1946), who showed that *Salmonella ballerup* and certain coliform organisms, which share with the typhoid bacillus the Vi antigen but none of its O or H antigens, are also capable of inducing in the mouse a state of effective immunity against highly virulent O + Vi strains of *Salm. typhi*. Thus the outstanding importance of the Vi antigen and its corresponding antibody was again emphasized.

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The exact mechanism by means of which the two antigenic components exert their effect on the virulence of the typhoid bacillus is still, however, in question. According to Ørskov & Kauffmann (1936) and other workers, the Vi antigen in the living bacillus acts as a toxin, and the so-called virulence test in the mouse is in reality a toxicity test. To examine the correctness of this opinion, experiments were carried out from which it was concluded that the lethal effects of O + Vi bacilli in the mouse could not be ascribed solely to a summation of the toxic effect of the two antigens. This finding was briefly recorded in the *Proceedings of the Third International Congress for Microbiology* in New York (Felix, 1940), but the experiments are now published in detail for the first time.

In two papers recently published from the United States Army Medical Department (Washington), Batson, Landy & Brown (1950*a*, *b*) reached the conclusion that the Vi antigen of *Salm. typhi* plays a role neither in virulence nor in active or passive protection against infection with this organism when tested in the mouse. These startling findings have prompted us now to publish the experiments carried out more than 10 years ago at the Lister Institute, London. It is hoped that the full description of the technical details may help workers to avoid some of the pitfalls inherent in the preparation of T.A.B. vaccine and in the testing of its immunizing potency.

SELECTION OF VIRULENT AND AVIRULENT STRAINS

All the strains selected for the following experiments had been employed in earlier work and their properties were known from long experience, extending in some instances over periods of many years. The cultures were preserved in waxed Lemco-agar stabs and were always grown on a trypsin-digest agar of good quality. The precautions to be taken in the preservation and maintenance of the cultures have been described in detail in a previous paper (Felix, 1938). When these precautions are carefully observed it is possible to maintain the antigenic composition of the different strains intact almost indefinitely.

Table 1 shows that the strains selected were representative of nearly all the types of antigenic variants of *Salm. typhi* that have been described hitherto. In addition to their content of the O and Vi antigens the table also shows the H-antigen content of the different variants, though this is listed merely for the sake of completeness, in view of the agglutination tests to be discussed later. It is well known that H antigens play no significant part in producing disease and in stimulating immunity (Felix, 1924; Felix & Olitzki, 1926). The so-called R and ρ antigens, characteristic of completely or partly rough typhoid bacilli and other members of the Salmonella group (Bruce White, 1931, 1932; Henderson, 1939*b*), are omitted from the table, since they are not known to have any significance in infection with, and immunity to, the typhoid bacillus.

(1) The strain Ty 2 represents the type of O + Vi bacilli of maximum virulence and has been widely used in experimental work on *Salm. typhi* and in the preparation of typhoid vaccine and therapeutic anti-typhoid serum. The strain has been chosen for its outstanding ability to produce Vi antigen in maximum amount when grown on plain nutrient agar for periods of many years.

Table 1. *Details of strains of* Salmonella typhi *used in virulence and toxicity tests*

Strain	Year of isolation	Antigens present in the strains			Parent strain from which variant was derived	Isolated		References
						Locality	Year	
Ty 2	1918	Vi	O	H	.	Cherson	1918	Weil & Felix (1920)
Watson	1932	Vi	O	H	.	Yorkshire	1932	Perry, Findlay & Bensted (1933*a*)
O 901	1925	.	O	.	H 901	Cherson	1918	Weil & Felix (1920); Felix (1930)
Ty 2 Rough	1935	.	.	H	Ty 2	Cherson	1918	Felix & Pitt (1935)
Ty 6S	1936	Vi	.	H trace	Ty 441	Palestine	1923	Felix & Olitzki (1926); Felix & Petrie (1938); Henderson & Morgan (1938)
Vi I	Prior to 1938	Vi trace	O	H trace	From a urinary carrier (Kauffmann)			Bhatnagar, Speechly & Singh (1938; Felix (1938)

(2) The strain 'Watson' is employed as a control strain in the examination of the strain Ty 2, to ensure that the culture Ty 2 used on a given occasion does in fact possess the Vi antigen in maximum amount. Like strain Ty 2 the Watson strain, too, retains its O inagglutinability in a remarkably constant manner, provided it is preserved and grown under standard conditions. Cultures of this strain are, however, decidedly less virulent to mice than those of the strain Ty 2, and their content of the Vi antigen is correspondingly smaller (Felix, 1938).

(3) The strain O 901, which is generally used as a 'pure' and sensitive reagent for typhoid O agglutinins, is the only permanent non-motile variant of *Salm. typhi* that has so far been described. Since its isolation in 1925 by Felix & Olitzki this variant has maintained its antigenic properties unchanged and no reversion to the motile O + H form has yet been observed. Similarly, no one has yet induced this Vi-negative variant of low virulence to revert to the virulent O + Vi form.

It is, however, of considerable interest to relate here the antecedents of the parent strain H 901, and its subsequent history. The strains H 901 and Ty 2 were isolated at the same time and place during a typhoid outbreak in 1918 in Cherson, Russia (Weil & Felix, 1920). Almost from the time of isolation the strain H 901 was highly sensitive to O agglutinins whereas Ty 2 was resistant; a similar difference was later found in the sensitiveness of these two strains to bactericidal serum action (Felix & Olitzki, 1926). They maintained their distinctive characters throughout the years, and in 1934 the strain Ty 2 was found to be highly virulent for the mouse and the strain H 901 relatively avirulent (Felix & Pitt, 1934*a*). In 1936 Kauffmann succeeded in 'rejuvenating' the H 901 strain by mouse passage (Kauffmann, 1936) in the same way as Perry, Findlay & Bensted (1933*b*) had accomplished the transformation of the avirulent Rawlings strain into one of high virulence. In this process the Vi-negative H 901 strain, after an interval of 18 years, again developed its Vi antigen. Two years later Craigie & Yen (1938)

typed the rejuvenated H 901 strain and the strain Ty 2 by means of their Vi-phage technique and found that both belonged to the same Vi-phage type, namely, Type E 1. Neither Kauffmann nor Craigie knew at the time that the two original cultures had been isolated during the same outbreak.

This finding is not only a striking example of the remarkable constancy of Vi-phage type, but it also has a bearing on the assessment of comparative virulence tests carried out with the strains Ty 2, H 901 and O 901. The two strains H 901 and O 901 have now to be regarded as antigenic variants derived from strain Ty 2.

(4) The strain 'Ty 2 Rough' is identical with the rough variant of Ty 2 described in Table 1, on p. 429, in the paper by Felix & Pitt (1935). This variant induced in the rabbit the formation of H antibody, but no trace of O and Vi antibody.

(5) The origin of the strain 'Ty 6S' has been recorded in a previous paper (Felix & Petrie, 1938). It was derived from the variant strain 'Ty 441 R5', described as a typically rough variant according to the accepted criteria, but which, nevertheless, contained the Vi antigen (Felix & Pitt, 1935). Like 'Ty 441 R5', the strain 'Ty 6S' is an antigenically 'rough' variant, that is, devoid of O antigen, but otherwise it resembles the 'smooth' type in broth and on agar. Suspensions of this culture are stable even in 5% saline and after heating at 100° C., and give reliable readings in agglutination tests. Scholtens (1937), who used this strain in experimental work, suggested the name 'Vi half-smooth form' for this type of antigenic variant. The strain 'Ty 6S' and its precursor 'Ty 441 R5' have been extensively employed in the preparation of purified antigenic fractions representing the 'complete' Vi antigen of *Salm. typhi* (Henderson & Morgan, 1938; Henderson, 1939*a*; Boivin & Mesrobeanu, 1938; Boivin, 1939). It would appear that these two variants, both derived from the same parent strain Ty 441, originally a strain of intermediate virulence and O agglutinability, are the only carefully studied examples of a permanent 'pure' Vi variant, that is to say, Vi strains that are completely devoid of the smooth O antigen. Strain 'Ty 6S' contains a trace of the H antigen, demonstrable by the immunization of rabbits.

(6) Bhatnagar's strain Vi I, now widely used as a 'pure' and sensitive reagent for the demonstration of Vi agglutinins, is not at all affected by the presence in the serum of H or O agglutinins (Bhatnagar, Speechly & Singh, 1938). Nevertheless, cultures of this strain always contain a very small quantity of the H and the O antigen, readily detectable by the immunization of rabbits (Felix, 1938).

TECHNIQUE

(*a*) *The maintenance of the cultures*

The cultures employed in the various tests were grown on a trypsin-digest agar of a pH of 7·4, transplanted daily, including Sunday, and kept in the incubator at 37° C. The necessity for carefully watching the quality of the agar medium and the constancy of the temperature at which the cultures are grown cannot be over-emphasized. It is also essential to reduce to a minimum the time taken for the preparation at room temperature of the suspensions to be used in the virulence or agglutination tests (Felix, Bhatnagar & Pitt, 1934).

Throughout this investigation cultures of all the strains used were plated out on trypsin-digest agar at least once a week, at some periods even daily. When colonies of suspected rough or translucent appearance were detected, the cultures were replaced by others derived from carefully selected colonies of perfect appearance, of which not less than ten or twelve were seeded together on an agar slope.

(*b*) *Agglutination tests*

Unless otherwise stated, fresh saline suspensions of living bacteria from 18 hr. agar-slope cultures were used (see Felix & Pitt, 1934*a*). The tubes were incubated for 2 hr. at 37° C. and the readings taken after a further 22 hr. at room temperature.

Every agglutination and agglutinin absorption test included controls for salt-agglutinability, made with the customary normal saline and with 2·5 and 5% solutions of NaCl (see Felix, 1938). The controls were put up for each strain with the living suspension and also with a suspension heated for 2 hr. at 100° C. None of the strains listed in Table 1, except the strain 'Ty 2 Rough', was considered to be in proper condition unless the two sets of controls gave clear-cut negative readings.

(*c*) *Quantitative agglutinin-absorption tests*

(1) *For the estimation of the Vi antigen in living bacilli.* Agar-slope cultures grown for 18 hr. (unless otherwise specified) were suspended in saline and the bacterial content was estimated by opacity. A mixture of 2 ml. of the suspension containing the required number of organisms and 2 ml. of a 'pure' Vi serum of the appropriate concentration was incubated for 1 hr. at 37° C. The bacilli, agglutinated or not as the case might be, were centrifuged down and the supernatant was titrated for residual Vi agglutinin. Three or four different doses of each of the absorbing organisms were required to allow of a reliable estimation of their relative Vi-antigen content. The results discussed in this paper have been derived from agglutination tests performed with live suspensions of the strain Watson. In later experiments a preserved suspension of the strain Vi I was employed.

(2) *For the estimation of the O antigen in heat-killed bacilli.* A 'pure' O serum was similarly absorbed in a strictly quantitative manner using suspensions of the different strains that had been heated for 2 hr. at 100° C. The absorbing suspensions were not washed and thus corresponded to the suspensions used in the toxicity tests. The absorbed sera were titrated against the strain O 901. Again, the experiments recorded in this paper were carried out with live suspensions of strain O 901, though in later experiments formolized suspensions of this strain were employed.

(*d*) *Virulence tests*

Male mice only were used, all from one stock and weighing 16–20 g. The technique was the same as employed in earlier work (Felix, 1938). Cultures grown on trypsin-digest agar slopes for not more than 16 hr., or for periods stated in the tables, were suspended in and further diluted with freshly prepared Ringer solution of

a pH of from 7·6 to 7·4, the temperature of which had been brought up to 37° C. The bacterial content was determined by opacity, estimating each suspension three times in succession. The required number of organisms was always contained in 0·5 ml. and injected intraperitoneally with the least possible delay.

The mice were shaved on the abdomen and occasional accidents, such as loss of a fraction of the volume injected or injury to the animal, were recorded. Most animals that succumbed to the infection died within 48 hr. The survivors, nevertheless, were kept under observation for 5 days.

(*e*) *Toxicity tests*

These, too, were carried out according to the technique previously described (Felix, 1938). The test suspensions consisted of the growth from Roux bottles containing trypsin-digest agar, washed off with saline and sterilized by heating for 2 hr. at 58° C. The quantitative estimation of the O-antigen content of the different strains was invariably carried out on the same suspensions as were being tested for toxicity to mice. For this reason the bacterial content was standardized in both tests on the basis of the density of the live suspension immediately the organisms were washed off the agar medium. A few ml. of each of the standardized suspensions were removed and steamed for 2 hr. at 100° C. for use in the absorption tests with the 'pure' O serum, while the bulk of the suspensions was heated for 2 hr. at 58° C. and served in the toxicity tests. Both suspensions remained in their first suspending fluid, since washing with saline considerably reduces the toxicity for mice.

The test dose, always contained in 0·5 ml. volumes, was injected intraperitoneally into male mice weighing not more than 14–16 g. Deaths resulting from doses of killed bacilli may occur as late as 5–6 days after the injection. The survivors, therefore, were kept under observation for 8 days.

(*f*) *Viability tests and estimation by opacity*

In many of the experiments the estimations by opacity were checked by viable counts. Three plates were counted for each strain and the average calculated.

QUANTITATIVE ESTIMATION OF THE Vi- AND O-ANTIGEN CONTENT OF THE CULTURES

It was observed early in the work on the Vi antigen that the most virulent strains of *Salm. typhi* not only are O-inagglutinable but also exhibit a peculiar behaviour in the Vi-agglutination reaction. The higher the virulence of a strain the lower is the titre of the Vi-agglutination reaction obtained with the culture (Felix & Pitt, 1934*b*; Felix *et al.* 1934). The most plausible explanation of this phenomenon that suggested itself was that strains of highest virulence contain in them the greatest amount of Vi antigen, requiring for its neutralization the greatest quantity of Vi antibody, and that consequently the titre of the reaction is lowered. Quantitative absorption tests (unpublished) soon showed that this explanation was correct.

Because of this constant relationship between degree of virulence and agglutinability it is possible to use agglutination tests with pure O and pure Vi sera as a means of detecting even slight variation in the Vi-antigen content of the cultures (Felix, 1938). This simple procedure has in fact been followed for many years as a reliable control measure in the maintenance of the cultures of virulent Vi + O bacilli that are employed in preparing typhoid vaccine and therapeutic anti-typhoid serum. Virulence tests in mice were found invariably to agree with the results of the agglutination tests. Similar observations have been published by Bensted (1937).

As a preliminary to the virulence and toxicity tests described in this paper the Vi- and the O-antigen content of the strains was determined by quantitative absorption tests with pure Vi and pure O sera. A portion of each of the suspensions used in the absorption tests was simultaneously examined for its Vi and O agglutinability.

Part I of Table 2 shows that equal numbers of organisms of the strains Ty 2 and Ty 6S absorbed the same amount of Vi antibody, whereas nearly three times this number was needed in order to produce the same effect with strain Watson. No reduction in the Vi-agglutinin titre could be demonstrated with 100-fold or still greater doses of strain O 901.

The absorption test of the pure O serum shows that the heat-killed suspensions of the strains Ty 2, Watson and O 901 removed about the same quantity of the O antibody, that is to say, the three cultures contained about the same amount of the O antigen. On the other hand, a 100-fold greater number of organisms from strain Ty 6S did not absorb any appreciable amount of O antibody.

When part I of the table is read in conjunction with part II it is evident that agglutination tests of the living bacteria do in fact permit of a reliable estimation of the Vi content of the cultures. The agglutinin titre of the pure Vi serum is the same when tested against the cultures Ty 2 and Ty 6S, but appears to be nearly three times higher against strain Watson. These results of the agglutination tests are almost identical with those published in a previous paper (Felix, 1938). It follows that the strain Ty 2 is in optimum condition in regard to its virulence and its Vi-antigen content so long as its Vi and O agglutinability, in relation to the control strains Watson and O 901, are similar to those shown in the table.

Strictly quantitative absorption tests of the kind described in Table 2 were carried out regularly throughout this investigation. The absorption of the pure Vi serum, for which living bacilli are used, had to be completed the same day the virulence tests were done. The absorption of the pure O serum with heat-killed bacilli was conveniently postponed till the next day.

Table 3 shows the relative amount of each of the two antigenic components contained in cultures of the different strains used. The Vi-antigen content of the most virulent strain Ty 2 was taken as equivalent to 100 units of this substance per 10^8 bacilli, and the O-antigen content of strain O 901 as 100 units of this antigenic component per 10^8 bacilli. The figures in Table 3 are the averages from many estimations carried out simultaneously with the virulence and toxicity tests, and also in the course of earlier and subsequent work.

Table 2. *Estimation of the Vi- and O-antigen content of the cultures by agglutination and agglutinin-absorption tests*

Part I. Agglutinin-absorption tests

Absorption of pure Vi serum. 4 ml. of serum dilution 1 : 200 absorbed with live organisms.
Absorbing dose per 1 ml. of serum in millions of organisms of strains

	Serum dilutions	Ty 2				Watson				Ty 6S				O 901				Control serum unabsorbed
		240	160	80	40	240	160	80	40	240	160	80	40	24,000	16,000	8000	4000	
Agglutination of strain Watson	1 : 200	+	+±	+±±	+++	++±	+++	+++	+++	+	+±	++±	+++	+++	+++	+++	+++	+++
	1 : 400	(±)	±	+±	+++	+	++	+++	+++	((±))	±	+±	+++	+++	+++	+++	+++	+++
	1 : 800	–	((±))	(±)	++	((±))	+	++	++	–	((±))	((±))	++	++	++	++	++	++
	1 : 1400	–	–	((±))	±	–	(±)	±	±	–	–	((±))	±	±	±	±	±	±
	1 : 2000	–	–	–	((±))	–	–	((±))	((±))	–	–	–	((±))	((±))	((±))	((±))	((±))	((±))
	1 : 3000	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

Absorption of pure O serum. 4 ml. of serum dilution 1 : 8000 absorbed with organisms heated 2 hr. at 100° C.
Absorbing dose per 1 ml. of serum in millions of organisms of strains

	Serum dilutions	Ty 2				Watson				Ty 6S				O 901				Control serum unabsorbed
		4000	2000	1000	500	4000	2000	1000	500	400,000	200,000	100,000	50,000	4000	2000	1000	500	
Agglutination of strain O 901	1 : 8,000	(±)	±	++	++	(±)	±	++	++	++±	++±	++±	++±	((±))	±	+±	++	++±
	1 : 12,000	–	(±)	±	+	–	(±)	±	+	++	++	++	++	–	((±))	±	+	++
	1 : 16,000	–	((±))	(±)	(±)	–	((±))	(±)	(±)	+	+	+	+	–	–	((±))	(±)	+
	1 : 20,000	–	–	((±))	(±)	–	–	–	(±)	±	±	±	±	–	–	–	((±))	±
	1 : 40,000	–	–	–	((±))	–	–	–	((±))	(±)	(±)	(±)	(±)	–	–	–	–	(±)
	1 : 80,000	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

Table 2 (*cont.*)

Part II. Agglutination tests

Serum	Dilution	Ty 2	Watson	Ty 6S	O 901	
Pure H serum	1 : 1,000	+ ±	+ ±	−	−	
Pure O serum	1 : 200	((±))	((±))	−	.	Suspensions of living organisms
	1 : 500	((±))	((±))	−	.	
	1 : 1,000	−	−	−	.	
	1 : 2,000	−	−	−	+ + +	
	1 : 5,000	−	−	−	+ + +	
	1 : 10,000	−	−	−	+ + +	
	1 : 20,000	−	−	−	+ +	
	1 : 50,000	.	.	.	±	
	1 : 100,000	.	.	.	((±))	
	1 : 200,000	.	.	.	−	
Pure Vi serum	1 : 400	+ + ±	+ + +	+ + ±	.	
	1 : 600	+	+ + +	+	.	
	1 : 700	±	+ + +	(±)	.	
	1 : 800	((±))	+ + ±	−	.	
	1 : 1,000	−	+ +	−	.	
	1 : 1,400	−	+	−	.	
	1 : 2,000	−	(±)	−	.	
	1 : 3,000	−	−	−	.	
Saline controls (% NaCl) 0·04		−	−	−	−	
0·85		−	−	−	−	
2·5		−	−	−	−	
5·0		−	−	−	−	
Saline controls (% NaCl) 0·04		−	−	−	−	Suspensions heated 2 hr. at 100° C.
0·85		−	−	−	−	
2·5		−	−	−	−	
5·0		−	−	−	−	
Pure O serum	1 : 2,000	+ + +	+ + +	−	+ + +	
	1 : 5,000	+ + +	+ + +	−	+ + +	
	1 : 10,000	+ +	+ +	−	+ +	
	1 : 20,000	+	±	−	+	
	1 : 50,000	((±))	((±))	−	(±)	
	1 : 100,000	−	−	−	−	

+ + + = strongest degree of agglutination; supernatant fluid completely clear.
+ + ± to + = degrees of incomplete agglutination; supernatant fluid turbid.
± = weakest degree of agglutination which could be estimated with the naked eye.
(±) = trace, ((±)) = faint trace } estimated by means of magnifying lens.

Table 3. *Relative Vi- and O-antigen content of selected variants of* Salmonella typhi

	Approximate antigen content of strains in units per 10^6 bacilli					
	Ty 2	Watson	Ty 6S	O 901	Ty 2 Rough	ViI
Vi antigen	100	40	100	0	0	40
O antigen	100	100	0	100	0	Trace

Note. By definition, strain Ty 2 contains 100 units of Vi antigen, and strain O 901 100 units of O antigen per 10^8 bacilli.

The deviation in the readings of the absorption tests was only slight, provided the cultures were maintained under standard conditions. It should be borne in mind, however, that the degree of accuracy that can be attained in quantitative agglutinin-absorption tests is not greater than that which can be achieved in agglutination tests generally. It has been stated previously (Felix, 1938) that the deviation in the readings of agglutination tests is not less than ± 20%, even in the hands of experienced investigators.

VIRULENCE AND TOXICITY OF 'PURE' O, 'PURE' Vi AND O+Vi VARIANTS

The relative virulence and toxicity for mice of the six variant strains listed in Table 1 are shown in Table 4. Again, the figures represent the averages from numerous tests carried out over long periods of time.

Table 4. *Virulence and toxicity for mice of selected variants of* Salmonella typhi

	Approximate antigen content in units per 10^6 bacilli		Lethal effects following intraperitoneal inoculation of mice with (dose in millions of organisms)					
			Living bacilli					Bacilli heated 2 hr. at 58° C.
Strain	Vi	O	40	100	200	400	800	16,000
Ty 2	100	100	5/10	10/10	10/10	.	.	18/20
Watson	40	100	3/10	6/10	10/10	.	.	19/20
O 901	0	100	.	0/10	0/10	2/10	6/10	19/20
Ty 6S	100	0	.	0/10	0/10	1/10	4/10	7/20
Ty 2 Rough	0	0	.	0/10	0/10	0/10	2/10	8/20
ViI	40	Trace	.	3/10	8/10	10/10	.	8/20

Note. The numerator indicates the number of mice that died, the denominator the number inoculated.

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The results shown in Table 4 confirm what is known from earlier work, namely, that virulence for mice depends on the presence in the bacilli of both the Vi and the O antigen. The pure O variant O 901, which contains the maximum quantity (100 %) of the O antigen, and the pure Vi variant Ty 6S, which possesses the maximum amount (100 %) of the Vi antigen, both exhibit a low degree of virulence to mice, similar to that of the typically rough variant Ty 2 Rough. That is to say, the presence in a culture of only one of the two antigens, even in maximum quantity, does not cause a great increase in virulence over that of a rough variant which is entirely devoid of the two antigenic constituents. On the other hand, the combination of only 40 % of the possible maximum quantity of Vi antigen with a very small amount (trace) of the O antigen, as instanced in strain Vi I, is reflected by a considerably higher degree of virulence. When strain Vi I is compared with strain Watson, both of which contain the same quantity of the Vi antigen but differ greatly in their O-antigen content, it is seen again that there is a corresponding difference in the virulence of the two strains.

An entirely different picture presents itself when the six strains are compared for their relative toxicity to mice, as measured in customary manner by the lethal effects of heat-killed bacilli. In this test the variant Ty 6S, which contains no O antigen, and the variant Vi I, which contains only a trace, produce lethal effects of the same low order as that of the completely rough variant Ty 2 Rough. The three strains Ty 2, Watson and O 901, which possess an equal quantity of the O antigen, all exhibit the same high degree of toxicity, irrespective of whether the Vi antigen is present in different quantities, or is entirely absent from the cultures. These experiments, therefore, confirm the earlier finding that virulence and toxigenicity do not run parallel in different antigenic variants of *Salm. typhi* (Felix & Pitt, 1934*a*, *b*).

The results of the toxicity tests are the same whether the typhoid bacilli are killed by heating at 58 or at 100° C., or by treatment with alcohol. The toxicity of the suspensions is, however, reduced by any treatment requiring centrifugation and washing with saline, since an appreciable quantity of the O antigen is extracted from the bacilli in the process. It has been mentioned before that the quantitative absorption tests of O agglutinins were carried out with suspensions of bacilli heated for 2 hr. at 100° C., whereas the toxicity tests in mice were done with bacilli heated for 2 hr. at 58° C. This is necessary because it is known from earlier work that heating at 58° C. inactivates the Vi antigen only partially and its presence in Vi + O bacilli that have been heated for 2 hr. at 58° C. still partially inhibits the absorption of the O antibody. When washing with saline is avoided it is found that the toxicity of the suspensions is practically the same after heating for 2 hr. at 58 or at 100° C.

The fact that alcohol-treated suspensions of bacilli which possess both the O and Vi antigens are not more toxic than those containing only the O antigen is of particular interest, since it is known that treatment with alcohol does not destroy the agglutinogenic and immunogenic properties of the Vi antigen (Felix & Pitt, 1936; Felix & Petrie, 1938).

These findings are in general agreement with the observations made on the

antigenic fractions that have been extracted from these variants by different chemical methods. Partly purified antigens have been prepared from our 'pure' Vi and 'pure' O variants by extraction with trichloracetic acid; the 'pure' Vi-antigen fraction was found to be about five times less toxic for mice than the 'pure' O-antigen fraction (Boivin & Mesrobeanu, 1938). Extraction with anhydrous diethylene-glycol, according to the method described by Morgan (1937), yielded a partly purified Vi substance of a toxicity only one-fiftieth of that exhibited by the corresponding O substance (Henderson & Morgan, 1938). In later experiments Henderson (1939*b*) found that the partly purified Vi extracts from strain Ty 6S were not more toxic than the corresponding extracts from the completely rough strain Ty 2 Rough. It must be remembered, however, that each of these chemical treatments, including sterilization with alcohol, probably reduces the toxicity of the labile Vi antigen to a much greater extent than that of the stable O antigen.

SUMMATION OF TOXIC EFFECTS OF 'PURE' O AND 'PURE' Vi VARIANTS

If the lethal effects of the living bacilli were due solely to the toxic action of their antigenic constituents, as has been suggested by Ørskov & Kauffmann (1936) and other workers, one could not expect to find the differences between lethal effects of O + Vi variants, on the one hand, and those of the 'pure' O and 'pure' Vi variants, on the other hand, to be as great as, in fact, they are. A glance at Table 4 shows that the approximate LD 50 value for the O + Vi strain Ty 2 is about 20 times that of strains O 901 and Ty 6S. If the lethal effects of the living bacilli were due to the summation of the toxic action of the two antigens, one would expect the LD 50 of strain Ty 2 to be only about twice that of the 'pure' O or the 'pure' Vi variant, each of which possesses the full quota (100 %) of its respective antigen. Similarly, the live organisms of strain Vi I show a degree of a hypothetical 'toxicity' which is quite out of proportion to the quantities of the two antigens contained in the culture.

It was thought possible that the injection into mice of a mixture of the 'pure' O and the 'pure' Vi variants would produce lethal effects similar to those caused by the O + Vi strains. Table 5, which summarizes one of the experiments devised to test this point, shows however that this was not the case.

Table 5. *Summation of toxic effects of 'pure' O and 'pure' Vi variants*

		Lethal effects following intraperitoneal inoculation of mice with living bacilli				
			Antigen units in the dose		Numbers of mice	
Strain	Type of antigenic variant	Dose in millions of organisms	Vi	O	Tested	Died
O 901	O	300	0	300	20	4
Ty 6S	Vi	300	300	0	20	2
Mixture of O 901 and Ty 6S	Mixture of O and Vi	300 and 300	300	300	20	8
Ty 2	O + Vi	75	75	75	20	19
		60	60	60	20	15

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Table 5 shows that the lethal effect of the mixture of the two variant strains was approximately that which one would expect it to be as a result of the summation of the effects of the two strains. But a dose of the virulent O + Vi strain Ty 2 which contained only a quarter, or a fifth, of the quantities of each of the two antigens had a killing power of a much higher order.

It does not appear possible, therefore, to ascribe the lethal effects for mice of living O + Vi bacilli solely to the summation of the toxic actions of the two antigens. The two substances, when combined in the bacterial cell, obviously exert some other effect in addition to their direct toxic action.

COMPARISON OF YOUNG AND OLD CULTURES

At an early stage of the work on the Vi antigen Craigie & Brandon (1936) made the observation that the majority of recently isolated strains of *Salm. typhi* are completely O inagglutinable, i.e. they contain the Vi antigen in maximum amount, when grown in broth culture for not more than 4 hr., but lose this property on incubation for 18 hr. It was thought, therefore, that very young cultures might prove more lethal to mice than older cultures given in similar dose. This point was investigated.

It is well known that in nearly all bacterial species the young organisms in cultures from 4 to 9 hr. are much larger than those in cultures 24 hr. old (Clark & Ruehl, 1919; Henrici, 1926). In the case of *Salm. typhi-murium* Wilson (1926) compared the average size of the cells in a 4 hr. agar culture with that in the same culture after 26 hr., and he estimated from micrometer measurements that the cells from the young culture were nearly six times the volume of those from the older culture. We did not take measurements of the different variant strains of *Salm. typhi*, but stained preparations from 4 and 24 hr. agar-slope cultures invariably showed that the former were much larger and more deeply stained than the latter.

In comparing young and old cultures for their virulence for mice the estimation of the total number of organisms by the customary opacity method was checked in every instance by the plating method for viable count. Table 6 gives a few examples of the results obtained.

It is seen from Table 6 that in four out of the five strains examined the viable counts of the 18–20 hr. cultures were approximately half the respective total counts as estimated by opacity. The exception was strain Ty 6S, which also differs morphologically from the other variants in that most of the cells are shorter and thicker, approaching coccoid forms. The viable counts of all the variant strains, however, were smaller in the 4 hr. than in the 24 hr. cultures, although their densities had been matched very carefully. As stated before, the opacity of each suspension was estimated three times in succession, and the viable counts also represent averages from three counts. Evidently the smaller numbers of live organisms in the 4 hr. cultures are due to the larger size of the younger cells.

The figures for the first three strains listed in Table 6 show that the lethal effects of a smaller number of younger cells were appreciably greater than those of larger

numbers of the corresponding older cells. The differences were not great enough to warrant elaborate absorption tests in order to determine whether or not they were associated with a corresponding increase in the Vi- or O-antigen content of the organisms. It is obvious, however, that there is a definite advantage in the employment of younger cultures in the mouse test. In our early experiments in this series the customary 24 hr. cultures had been used (Felix & Pitt, 1934*a*, *b*). In a later paper incubation 'for not more than 16 hr.' was recommended (Felix, 1938). As a result of the experiments described in the present paper the incubation period of cultures grown for use as a challenge dose in actively or passively immunized mice was subsequently reduced to 12 hr.

Table 6. *Comparative virulence tests on cultures grown for* 4 *hr. and* 18–20 *hr.*

	Lethal effects following intraperitoneal inoculation of mice with living bacilli			Viable count of dose in millions of organisms grown at 37° C. for	
	Dose in millions of organisms estimated by	Grown at 37° C. for			
Strain	opacity	18–20 hr.	4 hr.	18–20 hr.	4 hr.
Ty 2	40	$\frac{3}{10}$	$\frac{8}{10}$	21	13
	40	$\frac{4}{10}$	$\frac{8}{10}$	25	15
ViI	100	$\frac{3}{10}$	$\frac{5}{10}$	46	31
	100	$\frac{3}{14}$	$\frac{8}{14}$	50	34
O 901	400	$\frac{2}{10}$	$\frac{5}{10}$	260	190
	400	$\frac{3}{14}$	$\frac{8}{14}$	210	160
Ty 6S	400	$\frac{1}{10}$	$\frac{1}{10}$	110	90
	400	$\frac{2}{14}$	$\frac{1}{14}$	120	90
Ty 2 Rough	600	$\frac{3}{16}$	$\frac{3}{16}$	350	230

Note. The numerator indicates the number of mice that died, the denominator the number inoculated.

DISCUSSION

The observations recorded in this paper are in full agreement with our earlier findings. The most virulent strains of the typhoid bacillus are those that develop the Vi and the O antigen in maximum amounts. The presence in the cell of either of the two antigens alone is associated with a very low degree of virulence, approaching that of a completely rough variant which is devoid of the two antigens. The combination in a variant strain of a certain quantity of the Vi antigen

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with even a very small quantity of the O antigen is sufficient to endow the organism with a considerable degree of virulence.

There is nothing in our own observations, or in those of other workers, to indicate that the Vi antigen is a peculiar kind of endotoxin, as Ørskov & Kauffmann (1936) have assumed. It is true that the evidence against this assumption, which has been derived from experiments with chemically purified antigenic fractions, does not carry much weight. The 'pure' Vi fraction extracted with trichloracetic acid was found to be five times less toxic for mice than the corresponding 'pure' O fraction (Boivin & Mesrobeanu, 1938), and the purified Vi antigen extracted with anhydrous diethylene-glycol had a toxicity of only one-fiftieth of that of the purified O antigen (Henderson & Morgan, 1938). In both instances, however, it had to be admitted that the rather brutal chemical procedures to which the bacilli were subjected during fractionation might have profoundly altered the labile Vi antigen and thus reduced its toxicity, while leaving the stable O antigen unchanged. Only experiments with living bacilli could serve as the crucial test.

It has now been shown by a strictly quantitative technique that the Vi antigen as it exists in the living cell is less toxic than the O antigen. This is evident from Tables 4 and 5, which show that the 'pure' Vi strain Ty 6S, which develops the Vi antigen in maximum quantity, is not more but rather less virulent for mice than the 'pure' O variant O 901. Table 5 further shows that the relatively high lethal effects of the virulent Vi + O strain Ty 2 cannot be attributed solely to summation of the toxic effects of its two antigenic components.

It has been shown in previous work that the Vi antigen protects the O antigen against the action of the natural or immune O antibody and thereby protects the bacterial cell as a whole. This has been clearly demonstrated in opsonic experiments (Felix & Bhatnagar, 1935), and is equally true of the bactericidal action of normal and immune serum, which also is due to the O antibody (Felix & Olitzki, 1926). The resistance to the O antibody that the fully O-inagglutinable O + Vi variant enjoys provides a powerful defence mechanism for the bacterial cell and undoubtedly plays an important part in determining the virulence of the typhoid bacillus. The present experiments indicate that this protective function of the Vi antigen is of greater consequence than its direct toxic action.

There is, of course, nothing surprising in the fact that an antigenic constituent of the cell, besides being a toxic substance, also exerts some particular effect on another cell constituent and thereby on the bacterial cell as a whole. This dual function has its counterpart in the roles which the corresponding antibodies play. It has been established beyond any reasonable doubt that the O antibody functions in the dual role of an anti-endotoxin and an antibacterial immune body, which is responsible for the bactericidal and opsonizing activities of the serum (Felix & Olitzki, 1926; Felix & Pitt, 1934*b*; Felix & Bhatnagar, 1935).

Henderson (1939*a*) considered that the results of passive-protection experiments in the mouse, carried out in parallel tests with and without the use of mucin, seemed to support the view expressed by Ørskov & Kauffmann that the Vi antigen acted as a toxin. Henderson's experiments, however, merely established the fact that the amount of Vi antibody required for passive protection is determined by

the quantity of the Vi antigen contained in the bacilli that are injected as the challenge dose. In the presence of mucin the challenge dose is small and so is the quantity of antibody required, and vice versa. These observations are analogous to those we made in active-protection tests with highly virulent and relatively avirulent strains (see Table V of Felix & Pitt, 1934*b*). The conclusion we drew from those early experiments was that the Vi and the O antigens play quite different roles in the pathogenic and immunogenic activities of the typhoid bacillus, and this conclusion has been amply corroborated by subsequent work.

Two further points appear to be relevant to this discussion. First, the clinical picture observed in the mice that receive the large dose of killed bacilli differs altogether from that which follows the injection of the comparatively small doses of living virulent bacilli. In the latter case the mice do not show any symptoms of illness during the first few hours after the injection, whereas practically every mouse that is given the large dose of killed bacilli shows symptoms of illness within the first few hours, irrespective of whether the animal ultimately succumbs or survives. Secondly, the difference between the lethal doses of virulent and relatively avirulent strains is often underestimated. With strains Ty 2 and O 901 the ratio of the approximate LD 50 doses is not 4 to 1, as has been stated in the useful review by Weil, Gall & Wieder (1939), but is 20:1. The ratio of the approximate M.L.D. of live and killed organisms of strain Ty 2 is 1 to 160, as has been shown in Table 4. It is reasonable to assume that the protection against the bactericidal and phagocytic defence mechanisms which the Vi antigen confers upon the O antigen enables the Vi + O variant to multiply, whereas the Vi-negative O 901 strain is readily disposed of.

The question is often asked whether conclusions drawn from the experimental disease in the mouse can be justifiably applied to the natural disease in man. Since experiments in man cannot be carried out it is, of course, impossible to ascertain whether the different variants of *Salm. typhi* possess the same comparative virulence for man and for the mouse. Nevertheless, a certain amount of evidence on this point has been collected during the past few years. Findlay (1951) compared five strains isolated from a fairly mild outbreak and five from a rather severe outbreak for their virulence to mice. Each of the strains from the severe outbreak was more mouse-virulent than any of the strains from the mild outbreak. Felix & Anderson (1951) examined a number of cultures immediately after isolation by blood culture from patients concerned in an unusually mild outbreak of typhoid fever. All the cultures from this outbreak were less mouse-virulent than old laboratory cultures representing strains of average virulence. It can thus be stated that the relative virulence for mice of strains of *Salm. typhi* does in fact reflect their virulence for man.

The most recent contribution to this discussion, that contained in two papers by Batson *et al.* (1950*a*, *b*), calls for some comments. These workers employed four strains which had all been derived from a chronic carrier, known as the 'Panama Carrier'; they included the strain '58', which has been used for the preparation of United States Army vaccine since 1936 (Siler *et al.* 1941). Two of the strains were considered to be virulent and two avirulent. The strains were

tested in mice by intracerebral inoculation and by intraperitoneal injection of suspensions in saline and in mucin, and the conclusion was reached that their relative virulence was independent of the presence or absence of Vi antigen. No attempt was made to carry out a proper antigenic analysis of the four strains. They were classified on the basis of the following tests: 'All strains were tested for the presence of Vi antigen by direct slide agglutination with *Salmonella ballerup* (Vi) antiserum (Kauffmann & Møller, 1940). Also, the characteristic V or W form colonial morphology (Kauffmann, 1935) of each strain was determined by examination under low power magnification employing oblique illumination' (Batson *et al.* 1950*a*, see p. 221).

It is obvious that the American workers have been led astray by relying on these crude and inaccurate techniques. Although slide agglutination has its legitimate place in the preliminary testing of organisms isolated in the course of routine work, it is entirely inadequate for antigenic analysis of the kind required in experimental work such as that under discussion.

The following is an English version of Kauffmann's (1941, see p. 123) suggestion for subdividing strains of the typhoid bacillus:

	Development of Vi antigen	Vi agglutination	O agglutination	
(1) Vi + + form	Optimal	+ +	−	= V form
(2) Vi + form	Moderate	+	+	= V W form
(3) Vi ± form	Weak	− or ±	+ +	= W form
(4) Vi − form	No trace	−	+ +	

Kauffmann's own comment on this table was: 'In slide agglutination no. 1 therefore corresponds to the V form, no. 2 to the V W form, whereas nos. 3 and 4 correspond in slide agglutination to the W form, which is thus subdivided into two sub-forms' (Kauffmann, 1941, see p. 124).

This is not the place to discuss the question whether there was ever any necessity for introducing these symbols into typhoid serology. They certainly lost their meaning as soon as the 'pure' Vi variant was described, which is devoid of the O antigen (Felix & Pitt, 1935). It is, however, evident to anyone who has had experience of Vi and O agglutination that this classification is misleading. The strains that show the strongest Vi agglutination are not those richest in the Vi antigen, but the reverse is the case. Variant strains differing in their antigenic composition and mouse-virulence as widely as, for instance, strains Ty 2, Ty 6S and Vi I cannot be distinguished by slide agglutination and still less by determining by oblique illumination whether the colonies appear opaque or translucent. From the description of strain '58' given by Batson, Landy & Abrams (1949) and by Batson *et al.* (1950*a*), it must be concluded that this strain, throughout the experiments recorded in their papers, never possessed maximum mouse-virulence, such as that exhibited by strain Ty 2.

It is not surprising that Batson *et al.* (1950*b*), having failed to establish what the antigenic constitution of their four variant strains was, were unable to detect any essential relationship between antigens and immunizing potency. In addition, their mouse-protection tests reveal a number of other pitfalls, namely: the use as

the challenge organism of a strain ('63') which was even 'markedly less virulent than is strain 58'; the inoculation of the immunizing and challenge doses by the same route (intraperitoneally) after an interval of only 6 days; and the employment of the mucin technique. The bearing of these sources of error on the outcome of mouse-protection tests is discussed in another paper (Felix, 1951).

SUMMARY

1. The most virulent strains of *Salmonella typhi* are those that develop both the Vi and the O antigens in maximum quantities.

2. The Vi antigen is not a particularly toxic substance; it is less toxic for the mouse than the O antigen.

3. The main biological function of the Vi antigen is to protect the O antigen against the action of the natural or immune O antibody, thereby protecting the bacterial cell against phagocytosis and the bactericidal action of the serum.

4. The relative virulence for mice of freshly isolated strains of *Salmonella typhi* reflects their virulence for man.

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