THE PROPERTIES OF DIFFERENT *SALMONELLA* Vi ANTIGENS

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INTRODUCTION

The important role played by the Vi antigen in the pathogenic and immunogenic activities of the typhoid bacillus was generally recognized soon after the existence of this antigenic constituent of *Salmonella typhi* was discovered (Felix & Pitt, 1934*a*, *b*). The physical, chemical and biological properties of this antigen have been the subject of extensive investigations during the intervening years, and a most useful review of the numerous relevant papers has been published recently by Jude (1950).

On the other hand, the Vi antigens of other members of the *Salmonella* group have, so far, attracted but little attention. The presence in *Salm. paratyphi A* and *B* and in *Salm. typhi-murium* of labile somatic antigens similar to the Vi antigen of *Salm. typhi* was first reported by Felix & Pitt in 1936, who found that *Salm. paratyphi A* possesses a specific Vi antigen of its own, whereas the Vi antigen of *Salm. paratyphi B* is identical with that of *Salm. typhi-murium*. Felix (1941)

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

stressed the necessity, in selecting strains for T.A.B. vaccine, of using only those cultures of *Salm. paratyphi A* and *B* that develop their Vi antigens in maximum amount, strains analogous to the well-known strain Ty2 of *Salm. typhi*, which represents the type of O+Vi bacillus of maximum virulence, widely used in experimental work and in the preparation of typhoid vaccine. The knowledge of the existence of these different *Salmonella* Vi antigens also served as the theoretical basis of investigations that led to another development of considerable practical importance, namely, the recognition of specific Vi bacteriophages acting on *Salm. paratyphi B* and *Salm. typhi-murium* (Felix & Callow, 1943) and more recently also on *Salm. paratyphi A* (Felix, 1951*b*).

Little attention has been paid to these findings and most authors of text-books ignore them. The demonstration of Vi antigens in *Salmonella* species is difficult, and because their effects on virulence, O-inagglutinability and immunizing properties are less conspicuous than those of the Vi antigen of *Salm. typhi*, attention has been focused on those species that share with *Salm. typhi* its particular Vi antigen, namely, *Salm. paratyphi C* (Kauffmann, 1935), *Salm. ballerup* (Kauffmann & Møller, 1940) and *Salm. hormaechei* (Monteverde, 1944). It is not surprising, therefore, that erroneous conclusions arrived at many years ago by Kauffmann (1936*a*, *b*), and since repeated in many of his publications, have been accepted.

This paper is the first of a series written to show the different Vi antigens in their true perspective, and it includes experiments reported briefly in the *Annual Report of the Lister Institute* for 1937, but not previously published in detail.

INACTIVATION OF Vi ANTIGENS BY DILUTE ACID

Evidence of the existence of the Vi antigens of *Salm. paratyphi A* and *B* and *Salm. typhi-murium* was first obtained from experiments in which sera from rabbits immunized with living bacilli were absorbed with suspensions of these organisms that had been treated with dilute hydrochloric acid (Felix & Pitt, 1936). Earlier attempts to demonstrate a hypothetical Vi antibody in these *Salmonella* antisera by the use of absorbing suspensions heated for $2\frac{1}{2}$ hr. at 100° C. had failed. This was not surprising in view of the fact that the Vi antigen of *Salm. typhi*, which in some respects is even less resistant to heat than the H antigen, was nevertheless found to be capable of specifically absorbing Vi antibodies after the suspensions had been heated for 1 hr. at 100° C. (Felix, Bhatnagar & Pitt, 1934).

It is known from earlier work on the antigenic analysis of this group of organisms that the O antigens are highly resistant to the action of hydrochloric acid even in relatively strong concentrations, whereas the H antigens are readily inactivated (Weil & Felix, 1920). It seemed probable that this method could be used to separate the hypothetical labile Vi antigens from the stable O antigens.

(*a*) *Treatment with* 0·5 N-HCl *for* 48 *hr. at room temperature*

Sera from rabbits immunized with living *Salm. paratyphi A* and *B* and *Salm. typhi-murium* were absorbed with suspensions of the homologous strains that had been exposed to the action of 0·5 N-HCl for 48 hr. at room temperature. These experiments clearly indicated that the sera contained Vi agglutinins of relatively

low titre, in addition to high-titre O agglutinins. The titres of Vi agglutination were of the same order as those previously found in antisera to *Salm. typhi*. The maximum titres recorded with some of the *Salmonella* sera were 1 in 1000 or 1 in 2000, when estimated against those strains that showed the highest degree of agglutinability by Vi antibody. The O titres were on the average ten times higher.

It was soon found, however, that the results obtained by this technique were irregular. When the same serum was absorbed with acid-treated suspensions under what appeared to be standard conditions of experiment, the absorbed sera showed very wide variations in titre, and occasionally Vi agglutinins were not demonstrable at all. This suggested that the acid-treated suspensions of *Salm. paratyphi A* and *B* and *Salm. typhi-murium* still contained some residual Vi antigen in active form. The effects of acid treatment on the classical Vi antigen, i.e. that of *Salm. typhi*, were, therefore, carefully investigated.

The strains of *Salm. typhi* employed in these and the following experiments were the two virulent Vi + O strains Ty 2 and 'Watson', and the 'pure' Vi variant 'Ty 6S', which is completely devoid of the smooth O antigen. The antigenic properties of these strains, which are known from earlier work, have been redescribed in a recent paper, together with details of the mode of maintenance of the cultures and the technique followed in the agglutination and agglutinin-absorption tests (Felix & Pitt, 1951).

The technique of the acid treatment was as follows: Cultures grown on trypsin-digest agar, on slopes or in Roux bottles, for 24 hr. at 37° C., were suspended in saline and standardized by opacity to contain 8000×10^6 organisms per ml.; an equal volume of N-HCl was added, thus making the final concentrations 4000×10^6 organisms per ml. and 0·5 N-HCl. The suspensions were kept in screw-capped bottles, with or without glass beads, in the dark at room temperature for 48 hr. and were repeatedly shaken; they were then neutralized with the equivalent volume of N-NaOH, adjusted to pH 7·4, centrifuged and the sediments were washed twice with fresh saline.

Acid-treated suspensions of *Salm. typhi*, *Salm. paratyphi A* and *B* and *Salm. typhi-murium* have an opacity approximately 10 % less than that of suspensions equivalent in numbers but killed and preserved with 0·5 % phenol or 0·2 % formalin. For comparison it may be stated that treatment with 75 % alcohol, or heating for $2\frac{1}{2}$ hr. at 100° C., results in a loss of approximately 20 % as estimated by opacity. It was originally thought that the difference in opacity was due partly to destruction of the flagella and partly to some alteration in the physical condition of the surface of the bacteria. Since, however, the reduction in opacity is of the same order with motile and non-motile strains of these organisms, it cannot be accounted for by the destruction of the H antigen, but appears to be caused mainly, or at least partly, by alteration in, or extraction of, the Vi and O antigens. When agglutination and agglutinin-absorption tests are carried out in a strictly quantitative manner, as required in this kind of antigenic analysis, it is obviously essential to pay due attention to the apparent loss in some of the components of the bacterial cell that has occurred in the course of the various treatments to which the organisms have been subjected.

The results of the experiments with the three *Salm. typhi* strains Ty 2, Watson and Ty 6S may be summarized as follows:

(1) The inagglutinability by O antiserum of the two Vi + O strains Ty 2 and Watson was almost annulled by the acid treatment, but their agglutinability by Vi antiserum was maintained. With all three strains the acid-treated suspensions, when tested against a pure Vi serum, usually gave titres approximately two or three times higher than those obtained with the corresponding formolized suspensions that served as controls. In each instance control suspensions were prepared by treating a portion of the same suspension of live organisms with 0·2 % formalin for 48 hr. at room temperature. It is known from earlier work that the titre of the Vi-agglutination reaction shows, within a certain range, an inverse ratio to the Vi-antigen content of the agglutinated bacteria (Felix & Pitt, 1934*b*; Felix *et al.* 1934; Felix, 1938); thus the agglutination tests indicated that the acid treatment only partially inactivated the TVi* antigen, leaving enough of it intact to be demonstrable even by direct agglutination.

Acid-treated suspensions of the two Vi + O strains Ty 2 and Watson were, as a rule, perfectly stable in physiological saline and in 2·5 and 5 % solutions of NaCl. On the other hand, the 'pure' Vi variant Ty 6S after acid treatment often yielded suspensions that were salt-agglutinable and could not be employed in agglutination tests. The acid-treated suspensions containing not more than 4000×10^6 organisms per ml. were always found to be sterile; if the density of the suspension was much greater, a few of the organisms occasionally survived.

(2) Strictly quantitative agglutinin-absorption tests, carried out by the technique recently described (Felix & Pitt, 1951), confirmed that the amount of residual Vi antigen left intact in the acid-treated bacteria was approximately one-third or one-half of that present in the corresponding formolized or alcohol-treated organisms.

(3) Rabbits immunized with acid-treated suspensions invariably elaborated Vi agglutinins in significant titres. Eleven rabbits were immunized by the intravenous route with acid-treated suspensions of the three strains of *Salm. typhi* and none failed to give a significant Vi-antibody response. The following 'standard' TVi-agglutinin titres, based on the 'Provisional Standard Anti-typhoid Serum' (Felix, 1938), were recorded in this series: 1 in 50, once; 1 in 100, once; 1 in 200, four times; 1 in 400, three times; 1 in 800, once; and 1 in 1000, once.

Some rabbits also developed TH agglutinins in fairly high titres. It is known from earlier work that a small amount of the labile H antigen may escape inactivation when relatively heavy suspensions of bacteria are exposed to treatment with HCl or alcohol or to heating at 100° C. (Weil & Felix, 1920; Felix & Robertson, 1928). The quantity of residual undamaged H antigen contained in such suspensions is usually too small to be detected by the agglutination reaction, but it is often demonstrable in agglutinin-absorption tests and more readily still by the immunization of rabbits.

* The symbols TVi, AVi and BVi are used throughout this and the following papers of this series to denote, respectively, the Vi antigens of *Salm. typhi*, *Salm. paratyphi A* and *Salm. paratyphi B*, and the corresponding antibodies.

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In the light of this experience with the acid treatment of *Salm. typhi* it was not surprising that absorption tests with acid-treated organisms of *Salm. paratyphi A* and *B* and *Salm. typhi-murium* had given irregular results.

(*b*) *Treatment with* N-HCl *for* 20 *hr. at* 37° C.

Kauffmann (1936*a*, *b*), following a suggestion by the present writer, also treated various members of the *Salmonella* group with dilute HCl in an attempt to identify other labile somatic antigens similar to the Vi antigen of *Salm. typhi*. In order to accelerate the process Kauffmann (1936*a*) doubled the concentration of HCl and allowed it to act for 20 hr. at 37° C. By this procedure he established that the O-factor V (five) of the Kauffmann-White schema was acid-labile, whereas the O-factor IV withstood the treatment. The Vi antigen of *Salm. typhi* was, according to Kauffmann (1936*a*), also completely destroyed by the stronger acid treatment, as indicated by the results of agglutination and agglutinin-absorption tests and immunization of rabbits.

In view of the discrepancy between Kauffmann's findings and those described in the preceding section, it appeared desirable to repeat the experiments with the various *Salmonella* species following exactly Kauffmann's modified procedure. These experiments showed that the Vi antigen of *Salm. typhi* was the most resistant to treatment with N-HCl at 37° C., the Vi antigen of *Salm. paratyphi B* and *Salm. typhi-murium* was the most readily inactivated, whereas that of *Salm. paratyphi A* held an intermediate position. Tables 1, 2 and 3 illustrate these findings.

Table 1 shows the varying degrees of acid resistance of the different *Salmonella* Vi antigens as revealed by agglutination tests with the corresponding pure Vi sera. For comparison the Vi-agglutinability of live organisms and of suspensions treated with 0·05 N-NaOH, 0·2 % formalin, 75 % alcohol or heated for 2½ hr. at 100° C. is also shown in the table. The effects of these various treatments are more fully discussed in later sections of this paper. On the other hand, the results of the agglutination tests with pure O antisera, which were in each instance carried out simultaneously with the Vi sera, are for the sake of simplicity omitted from the table.

Table 2 records examples of quantitative agglutinin-absorption tests with organisms treated with N-HCl at 37° C. It is seen from the table that the B Vi antigen is completely inactivated by this treatment, the A Vi antigen is greatly impaired, whereas the T Vi antigen suffers only a certain diminution in its capacity of absorbing the corresponding antibody.

Table 3 summarizes the results of the immunization of rabbits. None of the rabbits immunized with *Salm. paratyphi B* or *Salm. typhi-murium* treated with N-HCl at 37° C. produced any demonstrable Vi antibody. With *Salm. paratyphi A* four of six animals showed a low-titre Vi-agglutinin response. With *Salm. typhi*, however, all the rabbits tested developed Vi agglutinins. Table 3 also shows that exposure to N-HCl for 2 hr. at 37° C. often failed to inactivate the H antigens completely, as evidenced by the development of H agglutinins in significant titres. This point has been discussed already in the preceding section.

In view of the abundant production of TVi antibody in the rabbits recorded in Table 3 it was of interest to establish whether the Vi antibody elaborated in response to immunization with acid-treated TVi antigen possesses full 'functional efficacy' in passive-protection tests in mice. It is known that treatment of *Salm.*

Table 1. *Vi agglutination of differently treated suspensions of* Salmonella typhi, Salm. paratyphi A *and* B *and* Salm. typhi-murium

Pure Vi sera: From rabbits immunized with alcohol-treated suspensions of strains	Dilutions	Titre of agglutination: Saline suspensions of cultures from trypsin-digest agar. Treated with: Fresh live	0·2% formalin 48 hr. at room temperature	75% ethyl alcohol 48 hr. at room temperature	0·5N-HCl 48 hr. at room temperature	N-HCl 48 hr. at room temperature	N-HCl 20 hr. at 37° C.	0·05N-NaOH 4 hr. at room temperature	He 2½
Salm. typhi, strain Watson [Vi+O+H variant]									
Salm. typhi strain Ty 6S [unabsorbed]	1:500	+++	+++	++	+++	+++	+++	−	
	1:1000	+±	++	(±)	+++	+++	+++	−	
	1:2000	(±)	±	−	++	++±	±	−	
	1:4000	−	−	−	±	+	(±)	−	
	1:8000	−	−	−	−	−	−	−	
Salm. paratyphi A, strain HA 1 (Durazzo) [Vi+O+H variant]									
Salm. paratyphi A strain HA 6 [absorbed with HA 6 treated with 0·05N-NaOH]	1:100	+++	+++	+++	++	+	−	−	
	1:200	++	++	++	+	(±)	−	−	
	1:400	+	+	+	−	−	−	−	
	1:800	±	±	(±)	−	−	−	−	
	1:1600	−	−	−	−	−	−	−	
Salm. paratyphi B, strain HB 3 [Vi+O+H variant]									
Salm. paratyphi B strain HB 3 [absorbed with HB 3 treated with 0·05N-NaOH]	1:200	+++	++	+++	++	−	−	−	
	1:400	++±	±	+++	+	−	−	−	((
	1:800	±	((±))	+	(±)	−	−	−	
	1:1600	−	−	−	−	−	−	−	
	1:3200	−	−	−	−	−	−	−	
Salm. typhi-murium, strain 'Glasgow' (Schütze) [Vi+O variant]									
Salm. typhi-murium strain 'Glasgow' [absorbed with strain 'Glasgow' treated with 0·05N-NaOH]	1:200	+++	+++	+++	++±	−	−	−	
	1:400	+++	+++	+++	++	−	−	−	(
	1:800	++	++	+±	±	−	−	−	
	1:1600	(±)	±	((±))	−	−	−	−	
	1:3200	−	−	−	−	−	−	−	

Notes. Saline controls and O-agglutination reactions with all suspensions are omitted from the table.

+++ = 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.

typhi with formalin (Felix & Bhatnagar, 1935) or extraction with anhydrous diethylene-glycol (Henderson & Morgan, 1938) leads to a peculiar 'functional deficiency' of the altered TVi antibody, which possesses only a fraction of the protective power of the antibody stimulated by the 'natural' Vi antigen contained in living bacilli. Sera from three of the rabbits immunized with acid-treated *Salm.*

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Table 2. *Agglutinin-absorption tests with acid-treated and with formolized bacteria*

Agglutination of strain	Serum	Serum dilutions	Control serum un-absorbed	Absorption with suspensions treated for 20 hr. at 37° C. with: 0·2% formalin; N-HCl. Absorbing dose per 1 ml. of serum dilution in millions of organisms of strains *Salm. typhi*, strain Watson: 2000	2000	4000	6000
Salm. typhi strain Vi I [almost pure Vi variant] [formolized]	Pure T Vi serum [diluted 1:400]	1:400	+ + +	((±))	+ +	±	((±))
		1:800	+ + +	−	±	−	−
		1:1200	+ +	−	−	−	−
		1:1600	+	−	−	−	−
		1:2000	(±)	−	−	−	−
		1:2400	−	−	−	−	−
				Salm. paratyphi A, strain HA 6: 2000	2000	6000	18,000
Salm. paratyphi A strain 17689 [Vi + O variant] [formolized]	Pure A Vi serum [diluted 1:100]	1:100	+ + ±	(±)	+ + ±	+ + ±	+ + ±
		1:200	+	−	+	+	±
		1:300	±	−	±	±	(±)
		1:400	(±)	−	(±)	((±))	−
		1:500	((±))	−	((±))	−	−
		1:1000	−	−	−	−	−
				Salm. paratyphi B, strain HB 3: 500	2000	10,000	20,000
Salm. paratyphi B strain HB 3 [Vi + O + H variant] [alcohol-treated]	Pure B Vi serum [v. HB 3] [diluted 1:800]	1:800	+ +	−	+ +	+ +	+ +
		1:1000	+ ±	−	+ ±	+ ±	+ ±
		1:1200	+	−	+	+	+
		1:1600	(±)	−	(±)	(±)	(±)
		1:2000	((±))	−	((±))	((±))	((±))
		1:3000	−	−	−	−	−
				Salm. typhi-murium, strain 'Glasgow': 500	2000	10,000	20,000
Salm. typhi-murium strain 'Glasgow' [Vi + O variant] [formolized]	Pure B Vi serum [v. 'Glasgow'] [diluted 1:800]	1:800	+ + +	−	+ + +	+ + +	+ + +
		1:1000	+ +	−	+ +	+ +	+ +
		1:1200	+	−	+	+	+
		1:1600	(±)	−	(±)	(±)	(±)
		1:2000	((±))	−	((±))	((±))	((±))
		1:3000	−	−	−	−	−

Note. The formolized and acid-treated absorbing suspensions had been washed twice with equal quantities of saline.

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Table 3. *Agglutinin titres of sera from rabbits immunized with acid-treated bacteria*

	Rabbits immunized by four intravenous injections [total approximately $10{,}000 \times 10^6$ organisms]		Antigens present in the strains	Titres of agglutinins present in the sera			
				TVi	TO	TH	
1	*Salm. typhi* treated with N-HCl for 20 hr. at 37° C.	Strain Ty2	Vi O H	160	10,000	0	
2				100	5,000	20	
3				1,400	10,000	50	
4		Strain Watson	Vi O H	100	10,000	0	
5				400	20,000	50	
6		Strain Ty6S	Vi . H	800	0	0	
				AVi	AO	AH	
1	*Salm. paratyphi A* treated with N-HCl for 20 hr. at 37° C.	Strain HA6	Vi O H	0	2,000	0	
2				20	5,000	1,000	
3				40	10,000	500	
4		Strain A2035	Vi O .	0	5,000	0	
5				40	10,000	0	
6				80	5,000	0	
				BVi	BO	BH spec.	H non-spec.
1	*Salm. paratyphi B* strain HB3	Treated with 0·5N-HCl for 48 hr. at room temperature	Vi O H	300	10,000	1,000	500
2				500	10,000	2,000	500
3		Treated with N-HCl for 20 hr. at 37° C.	Vi O H	0	2,000	500	100
4				0	5,000	2,000	500
5				0	5,000	100	0
				BVi	BO	H spec.	H non-spec.
1	*Salm. typhimurium* strain 'Glasgow'	Treated with 0·5N-HCl for 48 hr. at room temperature	Vi O .	150	5,000	0	0
2				500	10,000	0	0
3		Treated with N-HCl for 20 hr. at 37° C.	Vi O .	0	5,000	0	0
4				0	2,000	0	0
5				0	5,000	0	0

'Standard' TVi- and TO-agglutinin titres are based on the 'Provisional Standard Anti-typhoid Serum' (Felix, 1938).

Vi-agglutinin titre O = a negative result in a dilution 1 in 10.

O-agglutinin titre O = a negative result in a dilution 1 in 500.

H-agglutinin titre O = a negative result in a dilution 1 in 20.

typhi listed in Table 3 were, therefore, compared with the 'Provisional Standard Anti-typhoid Serum' (Felix, 1938) which had been produced by immunization with living bacilli. The mouse-protection tests showed that the three sera possessed unaltered Vi antibody of full 'functional efficacy'.

The results of the experiments recorded in this paper are in general accord with those originally described by Felix & Pitt (1936). Those early experiments were concerned only with the problem of demonstrating the existence of the Vi antigens

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of the various *Salmonella* species other than *Salm. typhi*. That aim was achieved by means of the acid treatment. Since this technique was not needed for the separation of the TVi and TO antigens and their corresponding antibodies, it did not at first appear necessary to investigate the sensitivity to acid treatment of the TVi antigen in greater detail. On the other hand, it is obvious that the results are in disagreement with Kauffmann's often repeated statement that the Vi antigen of *Salm. typhi* is completely destroyed by treatment with N-HCl at 37° C. (Kauffmann, 1936*a*, *b*, 1941*b*, 1951).

INACTIVATION OF Vi ANTIGENS BY DILUTE ALKALI

When it was established that some of the *Salmonella* Vi antigens possess a considerable degree of resistance to dilute acid it was thought possible that treatment with alkali might be a better method of separating the 'labile' Vi antigens from the 'stable' O antigens. From the early work on the 'double type of receptors' in the *Salmonella* group it was known that the O antigens withstood even the action of 1% NaOH when applied for 24 hr. at room temperature, whereas the H antigens after this treatment were no longer demonstrable by means of the agglutinin-absorption technique (Weil & Felix, 1920). It was hoped, therefore, that alkali treatment might completely inactivate the various *Salmonella* Vi antigens and thus provide 'pure' O-antigen suspensions suitable for absorption of antisera that contain O agglutinins in high titre but Vi agglutinins only in much lower titre. This expectation proved to be correct.

Preliminary experiments with the three strains of *Salm. typhi* and a number of strains of *Salm. paratyphi A* and *B* showed that exposure to 0·02 N-NaOH for 1 hr. at room temperature was often sufficient to destroy the Vi-agglutinability of the organisms almost completely, or at least to reduce it very considerably. The capacity to absorb the corresponding Vi antibody was also markedly impaired by this brief action of 0·02 N-NaOH. However, suspensions so treated usually contained a considerable number of viable organisms, which on culture developed their full quota of the Vi antigen. The concentration of NaOH was, therefore, gradually raised to 0·05 N-NaOH and the time of exposure extended to 4 hr. at room temperature.

(*a*) *Preparation of alkali-treated suspensions*

The technique of the alkali treatment was as follows: Cultures grown in Roux bottles on trypsin-digest agar for 24 hr. at 37° C. were suspended in saline and standardized by opacity to contain $20{,}000 \times 10^6$ organisms per ml. An equal volume of 0·1 N-NaOH was added, thus making the final concentrations $10{,}000 \times 10^6$ organisms per ml. and 0·05 N-NaOH. The mixtures were kept in screw-capped bottles containing glass beads at room temperature for 4 hr. and were repeatedly shaken; they were then neutralized with the equivalent volume of N-HCl, adjusted to pH 7·4 and washed on the centrifuge twice with fresh saline.

The addition of NaOH to the heavy bacterial suspension turns it almost instantaneously into a gelatinous mass. It is, therefore, necessary to have glass beads in the flasks in order to facilitate thorough mixing. After neutralization with HCl sticky flakes are formed which cannot be resuspended completely by repeated

shaking and washing on the centrifuge. These flakes are finally discarded, and only that portion of the alkali-treated suspension which remains stable in physiological saline is employed in agglutination and agglutinin-absorption tests, or for the immunization of rabbits. This portion usually represents not more than approximately 25 % of the total number of organisms originally submitted to treatment with alkali.

An attempt was made to estimate what proportion of the total loss, approximately 75 % of the original bacterial content, was accounted for by the residual flaky material that was discarded. The alkali treatment was usually carried out on the growth from batches of twenty-four Roux bottles, corresponding on the average to a total of approximately $20{,}000{,}000 \times 10^6$ organisms. On nine occasions the flakes resulting from the manipulation were carefully collected, repeatedly washed on the centrifuge, dried *in vacuo* over P_2O_5 at 37° C. and the dry weight determined. The weights varied from 0·74 to 1·44 g., with an average of 1·03 g. The figures were of the same order with strains of *Salm. typhi*, *Salm. paratyphi A* and *B* and *Salm. typhi-murium*. There was also no significant difference between Vi + O forms that contained H antigen and those that were devoid of it. The estimation of the dry weight of the original number of bacilli submitted to treatment with alkali was based on the figures published some years ago by Raistrick & Topley (1934), who found in experiments with *Salm. typhi-murium* that 0·017 mg. of dried bacterial cells, prepared from growth on nutrient agar in Roux bottles, corresponded approximately to 50×10^6 bacilli. If these figures are accepted as a basis for comparison, the dry weight of $20{,}000{,}000 \times 10^6$ bacilli will be approximately 6·8 g. and the flaked residue from the alkali-treated organisms will represent only 15 % of the dry weight of the original number of bacilli.

Obviously the major part of the 75 % loss in the original bacterial content is due to the fact that the various constituents of the cell, antigenic or non-antigenic, are gradually dissolved by dilute alkali at room temperature. Control tests with the *Salm. typhi* strain O 901, which is entirely devoid of the Vi antigen, showed that in this instance, too, the loss was approximately 75 % as estimated by opacity. The 'pure' Vi variant Ty 6S was not included in this series because alkali-treated suspensions of this strain are not stable in normal saline.

Long experience of this technique has shown that it is essential to allow the solution of the bacterial cells to progress sufficiently far in order to ensure complete inactivation, or extraction, of the Vi antigen. This stage is always reached when the yield of alkali-treated suspension represents no more than 25 % of the original bacterial count as estimated by opacity.

The suspensions of *Salm. typhi* and *Salm. paratyphi A* prepared in this way were always found to be sterile, but with *Salm. paratyphi B* and *Salm. typhi-murium* a few organisms usually survived the treatment. The addition of NaOH to the saline suspension causes rapid coagulation of the bacteria into clumps, which include undamaged individuals protected by the envelope of coagulated bacillary bodies. In the re-emulsification these undamaged bacteria are set free. This difficulty may be overcome by the use of a mechanical shaker during the treatment with alkali or by prior sterilization of the suspensions by heating to 58° C.

(*b*) *Summary of experiments with alkali-treated bacilli*

It has been shown in the preceding section that the Vi antigens of the various *Salmonella* species vary considerably in the degree of their resistance to treatment with dilute acid. On the other hand, all the Vi antigens so far tested were found to be equally susceptible to the action of dilute alkali. Treatment with 0·05 N-NaOH for 4 hr. at room temperature invariably resulted in complete inactivation, or extraction, of the Vi antigens, as judged by agglutination, agglutinin-absorption and immunization of rabbits.

(1) *Agglutination tests.* Alkali-treated cultures of O + Vi strains of the various *Salmonella* species usually form 'smooth' suspensions in normal saline and in 2·5 and 5 % solutions of NaCl. They may, therefore, be safely employed in agglutination tests, with adequate controls for inagglutinability by normal serum and salt solutions included in the test. These suspensions are always completely inagglutinable by pure Vi sera (see Table 1). Their O-agglutinability is usually equal to that of acid-treated organisms, somewhat higher than that of suspensions heated for $2\frac{1}{2}$ hr. at 100° C., but inferior to alcohol-treated or live organisms.

(2) *Agglutinin-absorption tests.* Alkali-treated suspensions, prepared in the manner described, were never found to be capable of absorbing the corresponding Vi antibody. Enormous numbers of bacillary bodies had often to be employed in order to remove completely the high-titre O antibody from the serum. Nevertheless, when a given Vi + O serum was absorbed on different occasions the resulting 'pure' Vi serum showed in each instance the same Vi-agglutinin titre.

(3) *Immunization of rabbits.* In the course of these experiments the following numbers of rabbits were immunized by intravenous injections of large doses of alkali-treated organisms:

7 rabbits with *Salm. typhi*,
4 rabbits with *Salm. paratyphi A*,
4 rabbits with *Salm. paratyphi B*,
4 rabbits with *Salm. typhi-murium*.

Vi agglutinins could not be detected in the serum of any of these animals, though all elaborated high-titre O agglutinins, often reaching titres of 1 in 10,000 or 1 in 20,000. Most of the sera also contained H agglutinins in relatively low titre.

(*c*) *Control tests with the Vi-negative variant strains* Salmonella typhi H 901 *and* O 901

The objection may be raised that the residual agglutinins present in the sera after absorption with alkali-treated bacilli are not Vi antibodies but O agglutinins which correspond to certain of the components of the complex O antigens of the various *Salmonella* species. It was known from the early work on this group of organisms that their respective O antigens consist of a number of different components, some of which are common to many members of the group (Weil & Felix, 1920), and that for this reason O-agglutination tests were unsuitable for the differential diagnosis between typhoid and paratyphoid infections (Felix, 1924, 1930). This fact was at first neglected in the Kauffmann-White scheme (Salmonella

Subcommittee, 1934) and caused considerable confusion. Subsequently certain of these common O-antigen components were determined in minute detail by Kauffmann and his collaborators and were included in the diagnostic scheme.

It was, therefore, necessary to determine whether serological differences could be detected in the O antigens as a result of treatment with dilute acid or alkali. Two kinds of alteration in the complex O antigens might occur, namely:

(i) some of the various O components might be completely inactivated;

(ii) some of the O components might be so modified that immunization would lead to the elaboration of a modified O antibody, different from that stimulated by the 'natural' O antigen contained in living bacilli.

The two points were carefully investigated in experiments with the O-antigen complex of *Salm. typhi*. High-titre O antisera were prepared by immunizing rabbits with alkali-treated, acid-treated and living bacilli of the two Vi-negative variants H 901 and O 901. In addition antisera were available against similarly treated cultures of strains Ty 2 and Watson, and also against live organisms of two further Vi-negative variant strains, 'Ty 8' and 'Rawlings', that had been employed in earlier work (Felix & Pitt, 1934*a*, *b*). These sera were examined in cross-absorption tests with the Vi-negative variants H 901 and O 901, using suspensions of alkali-treated, acid-treated and living bacilli. No serological differences were detected in any of these experiments that could be attributed to a differential action of the two chemicals on some of the O-antigen components of *Salm. typhi*. The relative sensitivity to O agglutinins of the differently treated suspensions was, of course, carefully checked in these tests.

The O-antigen complex of *Salm. typhi* is usually described as consisting of the four components IX, XII_1, XII_2 and XII_3 (Kauffmann, 1941*a*; Hayes, 1947*a*, *b*). There is, however, good reason for believing that this formula by no means indicates the actual degree of complexity of this particular O-antigen complex. For instance, the recent paper on this subject by Schmid & Kauffmann (1952) clearly shows (in their Table 1) that the strain O 901 contains at least one further O-antigen component, although this point is not emphasized by the authors. It may, therefore, be concluded from the experiments here summarized that all the partial O antigens of *Salm. typhi*, those already listed in the Kauffmann-White scheme and those not yet listed, are resistant to treatment with dilute alkali or acid. Consequently, absorption with an adequate quantity of alkali-treated organisms removes all the corresponding O antibodies from the serum.

It was not considered necessary to carry out equally elaborate experiments with the O-antigen complexes of *Salm. paratyphi A* and *B* or *Salm. typhi-murium*. The evidence available from numerous experiments with these three species proved conclusively that their respective O-antigen components behaved in the same way as did those of *Salm. typhi*. The residual agglutinins present in the various *Salmonella* antisera that had been adequately absorbed with homologous alkali-treated bacilli could, therefore, be confidently regarded as Vi antibodies. As a general rule it is essential, at any rate in the first instance, to use for absorption the same strain that has been employed in the immunization, in order to ensure that the various partial O antigens are all present in the absorbing suspension. Once the particular

Vi antibody has been identified by this most exacting method, it is in some instances possible to replace the alkali-treated suspension, wholly or in part, by a suitable pure O-antigen suspension prepared from a Vi-negative variant by one of the simpler techniques.

INACTIVATION OF Vi ANTIGENS BY HEAT

The effects of heat on the Vi antigen of *Salm. typhi* have been studied more extensively than those of any other physical or chemical treatment, but the results published by different workers have been most confusing. Those aspects of this puzzling problem that have a direct bearing on the preparation of typhoid vaccine have been discussed in a previous paper (Felix, 1951*a*), and a more general review of the published data has been ably presented by Jude (1950).

The contradictory results obtained in the investigation of this subject are due to a number of different factors. First, when the typhoid Vi antigen, which, by comparison with the O antigen, is undoubtedly 'heat-labile', is exposed to heat, its various serological properties are not inactivated according to the pattern familiar from the H antigens, which for a long time represented the prototype of a 'heat-labile' bacterial antigen. In the inactivation by heat of H antigens agglutinability is damaged most readily; next follows their capacity to absorb agglutinins; and the last to be destroyed is their agglutinogenic property (Felix & Mitzenmacher, 1918; Weil & Felix, 1920). With the typhoid Vi antigen the order was found to be different; its agglutinogenic activity disappeared almost as readily as its agglutinability, whereas the agglutinin-binding capacity withstood even heating at 100° C. for 1 hr., though it was considerably lower than that of unheated suspensions (Felix *et al.* 1934).

Secondly, the TVi antigen is heat-labile only in aqueous suspensions but is highly resistant to heat when the bacilli are suspended in absolute alcohol or acetone (Peluffo, 1941). In a remarkable series of experiments Peluffo (1941) showed that bacilli dehydrated *in vacuo* retained their Vi-agglutinability and their relative O-inagglutinability even after heating for 2 hr. at 100° C. or for 1 hr. at 150° C.

Another factor influencing the outcome of exposure to heat was found during early experiments in which the 'pure' Vi variant Ty6S was compared with the Vi + O strains Ty2 and Watson.

(*a*) *TVi antigen in a 'pure' Vi variant and in Vi + O strains of* Salmonella typhi

(1) *Agglutination and agglutinin-absorption tests.* Part I of Table 4 shows that suspensions of the Vi + O strains Ty2 and Watson heated for 2 hr. at 100° C. are no longer agglutinable by the Vi antibody, whereas similarly treated suspensions of the 'pure' Vi variant Ty6S are still agglutinated. The latter strain, which was extensively employed in earlier work, is completely devoid of the O antigen but, nevertheless, shows most of the characters of 'smoothness' (Felix & Petrie, 1938; Felix, 1938; Felix & Pitt, 1951). Suspensions of this strain, living or heated at 100° C., are stable in salt solutions up to 5% NaCl, and such saline controls were,

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of course, included in every test although they are not shown in Table 4. The normal serum no. 118I was from the rabbit that served for the preparation of the pure Vi serum no. 118II (immunization with live organisms of strain Ty2, absorption with strain H901 heated for 1 hr. at 58° C.). There was no doubt whatsoever that the agglutination of the suspensions of strain Ty6S heated for 2 hr. at 100° C. was due to the interaction of the Vi antigen and its corresponding antibody.

Part II of Table 4 shows that the Vi + O strains and the 'pure' Vi variant did not differ with regard to the effects of heat on their capacities to absorb the Vi antibody. The absorbing power was in each instance preserved after heating for 2 hr.

Table 4. *Agglutination and agglutinin-absorption tests with 'pure' Vi and with Vi + O variants of* Salmonella typhi

Part I. Agglutination tests

Saline suspensions from agar-slope cultures of strains

Serum	Dilution	Ty2 [Vi+O variant] Fresh living not washed	Ty2 [Vi+O variant] Heated 2 hr. at 100° C. Not washed	Ty2 [Vi+O variant] Heated 2 hr. at 100° C. Washed twice	Watson [Vi+O variant] Fresh living not washed	Watson [Vi+O variant] Heated 2 hr. at 100° C. Not washed	Watson [Vi+O variant] Heated 2 hr. at 100° C. Washed twice	Ty6S ['Pure' Vi variant] Fresh living not washed	Ty6S ['Pure' Vi variant] Heated 2 hr. at 100° C. Not washed	Ty6S ['Pure' Vi variant] Heated 2 hr. at 100° C. Washed twi
Pure O serum no. 20 II	1:1000	–	+++	+++	–	+++	+++	–	–	–
	1:2000	–	+++	+++	–	+++	+++	–	–	–
Pure Vi serum no. 118 II (v. Ty2 live)	1:100	+++	–	–	+++	–	–	+++	+±	+
	1:400	±	–	–	++±	–	–	±	(±)	(±
Normal rabbit serum no. 118 I	1:100	–	–	–	–	–	–	–	–	–

Part II. Agglutinin-absorption tests

Pure Vi serum from horse M immunized with strain Ty2

	Serum dilutions	Control serum un-absorbed	Absorbing dose per 1 ml. of serum dilution 1:100 in millions of organisms: 300	500	1000	300	500	2000	300	500	100
Agglutination of strain Watson (live)	1:100	+++	(±)	+++	+++	++	+++	+++	(±)	+++	++
	1:200	+++	((±))	+	+++	(±)	+±	+++	–	+	++
	1:400	+++	–	(±)	+++	–	(±)	+++	–	(±)	++
	1:600	+±	–	–	+	–	((±))	+	–	–	+
	1:1000	±	–	–	(±)	–	–	(±)	–	–	(±
	1:2000	–	–	–	–	–	–	–	–	–	–

at 100° C., though it was considerably reduced. It is also seen from the table that 'washing' with saline removed a large proportion of the Vi antigen from the heated suspensions. In this respect, too, there was no difference between the 'pure' Vi variant and the Vi + O strains.

(2) *Precipitation tests.* In view of the unexpected result of the agglutination tests illustrated in Table 4 it was desirable to examine the heat resistance of the TVi antigen in the two varieties of Vi strain by means of the precipitation reaction. It was known from earlier work that saline extracts of Vi strains of *Salm. typhi* contain Vi antigen precipitable by pure Vi antiserum (Felix *et al.* 1934). It is even possible to estimate approximately the relative Vi-antigen content of different

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strains by comparing the precipitation titres of saline extracts made from the cultures (Schütze, 1936).

Table 5 shows that successive extraction with saline of very dense suspensions, containing $80{,}000 \times 10^6$ organisms per ml., first for a few minutes at room temperature, next for 2 hr. at 37° C. and finally for 2 hr. at 100° C., yielded extracts of approximately equal precipitation titres. According to expectation the extracts

Table 5. *Precipitation tests*

Saline extracts [$80{,}000 \times 10^6$ organisms/ml.] filtered through Chamberland L5 candles			Pure Vi serum [rabbit No. 169, *versus* Ty2 live] 0·05 ml. of dilution 1:5				Pure O serum [rabbit No. 18, *versus* 0901] 0·05 ml. of dilution 1:5		
Extracts			0·1 ml. of extract in dilution						
From strains	No.	Mode of preparation	1:2	1:10	1:40	1:80	1:2	1:10	1:40
Ty2	I	Supernatant from first suspending fluid centrifuged immediately	+++	+	(±)	−	+	(±)	−
	II	Sediment from I extracted in equal volume of saline 2 hr. at 37° C.	+++	+	(±)	((±))	++	(±)	−
	III	Sediment from II extracted in equal volume of saline 2 hr. at 100° C.	+++	+±	±	(±)	++	(±)	−
Ty6S	I	Supernatant from first suspending fluid centrifuged immediately	+±	+++	+±	±	−	−	−
	II	Sediment from I extracted in equal volume of saline 2 hr. at 37° C.	+++	+	±	(±)	−	−	−
	III	Sediment from II extracted in equal volume of saline 2 hr. at 100° C.	++	+++	++	+	−	−	−

Reading after 24 hr. (2 hr. at 37° C., then in the cold room).
± = weakest degree of precipitation which could be estimated with the naked eye.
(±) = trace, ((±)) = faint trace } estimated by means of magnifying lens.

prepared from the 'pure' Vi strain Ty6S gave precipitation only with the Vi antiserum, whereas those from the Vi+O strain Ty2 were also precipitated by pure O serum. No damaging effect of heating at 100° C. was demonstrable, irrespective of whether the Vi antigen was present in the extract alone or together with the O antigen.

The extracts were also tested for their capacity to inhibit the agglutination reaction between the Vi-negative variant strain O901 and pure O serum. No

inhibition of O agglutination was observed with these fairly potent Vi extracts, whereas the presence of the Vi antigen in the bacterial cell, as is well known, invariably inhibits O agglutination either partly or completely.

It would appear, therefore, from the experiments summarized in Tables 4 and 5 that the physico-chemical behaviour of an antigenic substance, in this case the TVi antigen, may vary as the result of the presence in, or absence from, the bacterial cell of some other substance, which itself may be either antigenic or non-antigenic in nature.

It would be tempting to speculate on the bearing of this observation on the generally accepted conception of the spatial arrangement of different antigenic constituents in a bacterial cell. According to the surface theory in its extreme form, as postulated by Bruce White (1933) and Topley (1933), the dominant position on the surface of the cell of the virulent Vi + O form of *Salm. typhi* would be assigned to the Vi antigen. The latter thus inhibits O agglutination by protecting the O antigen which is implicitly placed beneath the surface. The observations illustrated in Tables 4 and 5, however, cannot be explained in this way. In order to explain why the heated Vi + O bacilli are inagglutinable by Vi antibody, whereas the heated 'pure' Vi bacilli are agglutinated, it would be necessary to postulate a protective action by the O antigen that originally lay beneath the surface. The observations here recorded obviously do not conform with the theory, already referred to, of the spatial arrangement of antigens contained in a bacterial cell (see discussion by Spooner, 1949).

(*b*) *Heat resistance of TVi, AVi and BVi antigens*

The data assembled in Table 6 have been derived from experiments carried out over many years and summarized in the preceding tables and from the results of the immunization of rabbits. In the course of this latter work forty-eight rabbits were immunized with organisms killed by heating for 1 hr. at 60° C. or

Table 6. *Heat resistance of different* Salmonella *Vi antigens in saline suspensions*

		TVi antigen		AVi antigen	BVi antigen	
	Heating	*Salm. typhi* Vi + O strains	*Salm. typhi* 'Pure' Vi variant	*Salm. paratyphi A*	*Salm. paratyphi B*	*Salm. typhi-murium*
Agglutinability	1 hr. at 60° C.	−	+	±	+	+
	2 hr. at 100° C.	−	±	−	±	±
Agglutinogenic activity	1 hr. at 60° C.	(±)	±	+	+	+
	2 hr. at 100° C.	−	−	−	±	±
Agglutinin-binding capacity	2 hr. at 100° C.	+	+	+	+	+

\+ = preserved, though reduced.
± = greatly impaired.
(±) = almost annulled.
− = completely inactivated.

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2 hr. at 100° C.; nearly half this number received injections of the two varieties of Vi strain of *Salm. typhi*; the remainder were divided between *Salm. paratyphi A* and *B* and *Salm. typhi-murium*. Although the three latter groups were rather small, each comprising only three to five rabbits, results in each group were clear-cut and permitted a definite conclusion to be drawn.

Table 6 shows the degree of deterioration of the various *Salmonella* Vi antigens due to heat, as reflected by agglutination and absorption tests and by antibody formation in the rabbit. It is seen that the three different Vi antigens possess slightly different degrees of heat resistance. The TVi antigen is the most readily inactivated, the BVi antigen is the most resistant and the AVi antigen holds an intermediate position. It is, however, evident that these differences are only of minor significance, since similar differences have been established for the TVi antigen itself when it is contained in the two varieties of Vi strain of *Salm. typhi*.

It is difficult to indicate the observed differences in an accurate manner without reproducing the actual titres of the sera in the various groups, but these are omitted for the sake of economy in space. The following figures may serve to illustrate this point. In rabbits immunized with Vi+O strains of *Salm. typhi* heated for 1 hr. at 60° C. the 'standard' TVi titres very rarely exceeded a dilution of 1 in 10, but reached 1 in 50 in two of three rabbits when the immunizing suspension was made from the 'pure' Vi variant. Similarly heated suspensions of *Salm. paratyphi A* and *B* and *Salm. typhi-murium* often induced AVi or BVi titres as high as those resulting from immunization with living bacilli, i.e. reaching or exceeding a dilution of 1 in 1000. On the other hand, the BVi titres observed after immunization with suspensions heated for 2 hr. at 100° C. were much lower, usually not exceeding a dilution of 1 in 200. For the reduction in agglutinin-binding capacity reference should be made to Table 4. It is thus necessary to emphasize that the few symbols employed in Table 6 indicate rather inadequately the diminution in the degree of reactivity of the different *Salmonella* Vi antigens resulting from exposure to heat.

It should be noted here that Stuart & Kennedy (1948) recorded experiments in which saline extracts heated at 100° C. for 3 hr., prepared from a Vi+O strain of *Salm. typhi*, induced formation of high-titre Vi antibody in the rabbit. Those extracts, heated and filtered, that had been employed in the precipitation tests now described in Table 5 were not tested for their capacity to induce formation of Vi agglutinins in the rabbit, since immunization with 100° C. suspensions of whole organisms of the same strains of *Salm. typhi* had given uniformly negative results. Such suspensions were always injected without 'washing' and contained, therefore, a certain amount of 'extract'. It must, however, be admitted that the concentration of extracted Vi antigen in these suspensions was much lower than that in the extracts employed by Stuart & Kennedy (1948).

RESISTANCE TO THE ACTION OF ETHYL ALCOHOL

Alcohol-treated suspensions of flagellated bacteria have been widely employed as reagents for O agglutinins, since it was shown by Weil & Felix (1920) that H antigens are readily inactivated by treatment with alcohol, whereas O antigens are

highly resistant. When this treatment was first applied to the investigation of the various *Salmonella* Vi antigens the findings were summed up as follows: '*S. typhi* also differs from the other salmonella species with regard to the agglutinability by the Vi antibody of suspensions treated with alcohol. The Vi agglutinability of suspensions of *S. typhi* treated with 75% alcohol is very much reduced, whereas that of suspensions of *S. paratyphi A* and *B* and *S. aertrycke* is not impaired. High-titre Vi antisera can, however, be obtained by immunizing rabbits with alcohol-treated suspensions of *S. typhi*. Should further investigation show that the Vi antibody elaborated in response to immunization with alcohol-treated suspensions is not deficient in protective power (Felix & Bhatnagar, 1935), the use of such suspensions may prove to be of practical value in the preparation of vaccines and therapeutic sera' (Felix & Pitt, 1936, p. 85). It is now known that this prediction proved to be correct (Felix & Petrie, 1938; Felix, 1941).

The original statement about the reduction in Vi agglutinability of alcohol-treated suspensions of *Salm. typhi* was later restated in the following words: '...the agglutinogenic properties of the Vi antigen of *S. typhi* are not impaired by treatment with alcohol although the Vi agglutinability of alcohol-treated suspensions is much reduced and for practical purposes almost annulled' (Felix & Petrie, 1938, p. 675). These statements are often referred to in a misleading manner. Kauffmann (1941*b*), in his first monograph on *Salmonella* (see p. 89, Table 7), transcribed this finding by means of the symbol '—' = 'is destroyed' and reprinted this mistake in numerous publications, including his most recent monograph (Kauffmann, 1951). Other workers have been led astray by relying on this inaccurate statement (Edwards, 1951; Bader & Kleinmaier, 1952).

Table 1 shows that exposure to the action of 75% alcohol for 48 hr. at room temperature does not 'destroy' the Vi agglutinability of suspensions of *Salm. typhi*, though it reduces it very much. Since Vi agglutinins in the sera of typhoid patients and carriers are as a rule only of low titre, it is obvious that a sensitive reagent is needed for their demonstration and that alcohol-treated T Vi suspensions are unsuitable for this purpose. On the other hand, alcohol-treated suspensions of *Salm. paratyphi A* and *B* and *Salm. typhi-murium* have approximately the same degree of sensitiveness to Vi agglutinins as live or formolized suspensions and are therefore useful reagents for the demonstration of A Vi and B Vi agglutinins.

Comparative tests of the two Vi + O strains of *Salm. typhi* and the 'pure' Vi variant Ty 6S showed that treatment with 75% alcohol also has different effects on the Vi agglutinability of the two varieties of Vi strain, i.e. the agglutinability of the 'pure' Vi variant is not impaired, but that of the Vi + O strains is greatly reduced. This is an exact analogy to the effects produced by exposure to heat, described in the preceding section, and indicates once more that the physico-chemical behaviour of the altered T Vi antigen is conditioned by the presence of other constituents of the bacterial cell. It is evident, therefore, that the difference between the effects of alcohol on the Vi agglutinability of *Salm. paratyphi A* and *B* and *Salm. typhi-murium*, on the one hand, and *Salm. typhi* on the other hand, is of no greater significance than that found in the two varieties of Vi strain of *Salm. typhi*.

Table 1 also shows that the 'pure' Vi sera that served in the experiments re-

corded in the table were all derived from rabbits immunized with alcohol-treated organisms. As soon as it had been established that alcohol had no damaging effect on the agglutinogenic and immunogenic properties of the TVi antigen, it became clear that alcohol-killed bacilli were destined to replace living bacilli in the preparation of Vi antisera. Suspensions of *Salm. paratyphi A* and *B* and *Salm. typhi-murium* prepared by this method soon proved to be the most satisfactory immunizing agents for the production of antisera with high Vi-agglutinin content.

The great importance which alcohol treatment has attained in the preparation of the alcohol-killed and alcohol-preserved T.A.B. vaccine is well known and need not be discussed here (Felix, 1941, 1951*a*; Felix & Anderson, 1951).

The technique employed for the preparation of alcohol-killed bacilli remained unchanged throughout the years, i.e. treatment with 75% ethyl alcohol for 48 hr. at room temperature. The suspensions are then centrifuged, the sediments washed on the centrifuge twice and resuspended in fresh saline. Suspensions containing 8000×10^6 organisms per ml. treated in this way are invariably found to be sterile. Batches of alcohol-treated suspensions of *Salm. paratyphi B* and *Salm. typhi-murium*, resuspended in buffered saline containing 0·2% formalin (Felix & Gardner, 1937), have retained their original Vi agglutinability for periods of several years.

Kauffmann (1941*b*) modified the alcohol treatment first by exposing the bacteria to 96% alcohol for 20 hr. at 37° C. and later to 50% alcohol for 20 hr. at 37° C. (Kauffmann, 1951), and recorded in each instance the Vi agglutinability of *Salm. typhi* as 'destroyed'. Comparative tests with the two Vi + O strains and with the 'pure' Vi variant Ty6S showed that treatment with 50% alcohol for 20 hr. at 37° C. produces essentially the same effects as those resulting from the action of 75% alcohol for 48 hr. at room temperature.

EFFECTS OF TREATMENT WITH FORMALDEHYDE

(*a*) *Preservation of O inagglutinability*

All the different chemical and physical treatments so far discussed suppress most readily that remarkable property of the Vi antigen of *Salm. typhi* which led to its discovery, i.e. the inhibition of O agglutination. Formalin, however, leaves this property intact. Suspensions of Vi + O strains of *Salm. typhi*, preserved with 0·2% formalin, maintain their inagglutinability by O antibody and their sensitiveness to Vi antibody for about six months when stored in the cold room at 2° C. (Felix & Bhatnagar, 1935; Felix, 1938). Similarly prepared suspensions of the 'pure' Vi variant Ty6S and of the strain ViI of Bhatnagar (1938) tend to deteriorate rather sooner, that is, after storage for about four months. Since the Vi-agglutinin titres against the formolized suspensions are equal to or slightly higher than those against the corresponding fresh suspension of the living bacteria, it was found practicable to adopt formolized suspensions of Bhatnagar's strain ViI as the most suitable reagent for the estimation of the Vi-agglutinin titre in the serum of typhoid patients and carriers. This reagent is standardized by means of a 'provisional standard serum for Vi agglutination' (Felix, 1950; Bensted, 1951).

The preservation of O inagglutinability by treatment with formalin is of no practical importance with *Salm. paratyphi A* and *B* and *Salm. typhi-murium*, since

the presence in these organisms of their respective Vi antigens does not confer on them a high degree of O inagglutinability. Reference to Table 2 will show that formolized suspensions are employed as reagents for the demonstration of A Vi and B Vi agglutinins when non-motile variants, that are completely devoid of H antigen, are available; when the agglutinable suspension is to be made from a strain containing H antigen, alcohol treatment is the method of choice.

(*b*) '*Functional deficiency*' *of the altered Vi antibody*

Although the undamaged O inagglutinability of formolized suspensions seemed to indicate that this treatment was the most innocuous method of preserving the Vi antigen of *Salm. typhi*, it was, nevertheless, found at an early stage that formaldehyde produced a chemical alteration in the Vi antigen which had important immunological consequences. The corresponding altered Vi antibody displays what has been termed a 'functional deficiency'. This is characterized by a reduced power of promoting phagocytosis and of protecting mice against infection with virulent Vi + O strains of *Salm. typhi* and is measured by the ratio mouse-protective value/Vi-agglutinin titre. The ratio is constant for sera containing Vi antibody elaborated in response to immunization with the 'natural' Vi antigen as contained in the living bacilli. The ratio is quite different, in fact many times smaller, when a formolized suspension or extract from a Vi strain has been employed in the immunization (Felix & Bhatnagar, 1935; Felix, 1938). The damaging effect is evident with suspensions of the intact bacterial cells and with saline extracts irrespective of whether these are made from a Vi + O strain or from a 'pure' Vi variant, and renders such antigens unsuitable for use as immunizing agents.

On the other hand, cross-absorption tests with living and formolized Vi strains and the corresponding antisera show that complete mutual absorption of the two varieties of Vi antibody is obtained.

It was concluded from these experiments that the altered Vi antibody, though fully effective as an agglutinin or precipitin in combination with the 'natural' Vi antigen, is deficient in the capacity for inducing all those immunity reactions that depend on the active participation of complement. This conclusion was confirmed by Henderson (1939*b*) in complement-fixation tests with the Vi-antigen fraction prepared by Morgan's (1937) method of extraction with anhydrous diethyleneglycol, which causes the same type of alteration in the Vi antigen (Henderson & Morgan, 1938; Henderson, 1939*a*).

Whether the Vi antigens of *Salm. paratyphi A* and *B* and *Salm. typhi-murium* also suffer a similar alteration through the action of formalin has not yet been determined.

EFFECTS OF TREATMENT WITH PHENOL

(*a*) *Loss of O inagglutinability*

Exposure to phenol of O + Vi strains of *Salm. typhi* damages almost immediately the inhibiting effect which the Vi antigen in its 'natural' state exerts on O agglutinability. Freshly prepared suspensions of O-inagglutinable cultures killed by treatment with 0·5 % phenol for 48 hr. at room temperature are no longer fully

resistant to O agglutinin. Such suspensions also readily absorb O antibody, whereas the living or formolized organisms do so only very feebly.

The Vi agglutinability of phenolized suspensions of *Salm. typhi* is maintained for a short time almost unimpaired; after storage for three months at 2° C. it was found to be reduced to about half of the original titres.

(*b*) *Reversible inactivation of the Vi antigen of* Salmonella typhi

The peculiar susceptibility to the action of phenol of the Vi antigen of *Salm. typhi* is often erroneously referred to as 'destruction' of the antigen. Felix & Bhatnagar (1935) found that sera from rabbits immunized with suspensions sterilized by 0·5% phenol contained no Vi antibody, and consequently had no protective action in passive immunization of mice. In later experiments it was shown that the loss of agglutinogenic activity was not a permanent one, but was due to a reversible reaction between phenol and the Vi antigen. When the phenol was removed from the suspension and replaced by fresh saline, such phenol-treated bacilli invariably stimulated the production of circulating Vi antibody in the rabbit, and this antibody was not found to be deficient in protective power (Felix & Petrie, 1938).

In the course of this work nine rabbits were immunized intravenously with freshly prepared phenolized suspensions of Vi strains and only two produced circulating Vi antibody; of six rabbits immunized with phenol-killed organisms that had been washed twice with saline, all developed Vi antibody in significant titres. Absorption tests with phenolized suspensions that had been stored for periods up to 10 months, and were tested washed and unwashed, showed that the absorbing capacity of the TVi antigen had remained unimpaired.

Groups of three rabbits were immunized with Vi strains of *Salm. paratyphi A* and *B* and *Salm. typhi-murium* sterilized by 0·5% phenol. All the rabbits elaborated the corresponding Vi antibody. The reversible inactivation by phenol of the agglutinogenic activity is therefore a peculiarity of the Vi antigen of *Salm. typhi*.

DISCUSSION

The main interest of the experiments recorded in this paper lies in the demonstration that the Vi antigens of *Salm. typhi*, *Salm. paratyphi A* and *B* and *Salm. typhi-murium* have a number of properties in common that characterize them as 'labile' somatic antigens. Each of the chemical and physical treatments that have been applied to them has some deleterious effect on the Vi antigens, while leaving the O antigens undamaged. The degree of deterioration produced, as reflected by the various *in vitro* and *in vivo* activities, varies with the three different *Salmonella* Vi antigens. But there can be little doubt, on the evidence presented, that these antigens are 'labile' substances and are to be separated from the 'stable' O antigens. This view has been the guiding principle throughout the writer's work on prophylactic T.A.B. inoculation (Felix, 1941, 1951*a*) and on the Vi-phage typing of *Salm. paratyphi A* and *B* and *Salm. typhi-murium* (Felix & Callow, 1943, 1951;

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Felix, 1951*b*) according to the technique originally evolved by Craigie & Yen (1938*a*, *b*) for the typing of *Salm. typhi*.

Inhibition of O agglutination is the most distinctive and also the most vulnerable property of the Vi antigen of *Salm. typhi*. Of the six different chemical and physical agents investigated only one, namely, formaldehyde, leaves this property intact. The peculiar reversible inactivation by phenol of the agglutinogenic activity of the Vi antigen of *Salm. typhi*, to which there is no analogy in the other two *Salmonella* Vi antigens, also points to the extreme lability, i.e. chemical reactivity, of this substance. On the other hand, the effects of exposure to HCl, NaOH, ethyl alcohol and heat of the Vi antigens of *Salm. paratyphi A* and *B* and *Salm. typhi-murium* are essentially similar to those they produce in the classical Vi antigen of *Salm. typhi*.

Undue emphasis should not be laid on the differences in the degree of alteration resulting in the TVi, AVi and BVi antigens, respectively, from the action of any one of the different agents. The original designation of the *Salmonella* H antigens as 'labile' and of the O antigens as 'stable' was also based on the fact that the former were found to be generally susceptible to the various chemical and physical treatments, whereas the latter were resistant (Weil & Felix, 1920). The properties of the three *Salmonella* Vi antigens clearly indicate that their chemical structures differ profoundly from those of the O and H antigens. The fact that the different Vi antigens react less uniformly to the various chemical and physical treatments than do the H or the O antigens is apparently due to the greater complexity of the Vi-antigen molecule. While a considerable body of data is available regarding the basic chemical structure of the O antigens (Boivin & Mesrobeanu, 1933, 1935; Raistrick & Topley, 1934; Morgan, 1937; Morgan & Partridge, 1942), and more recently attention has also been paid to the chemistry of the H antigens (Weibull, 1948, 1949), very little, so far, is known about the chemical nature of the Vi antigens (Ashida, 1949; Grabar & Corvazier, 1951).

Considerable importance is attached to the finding that heating and treatment with alcohol produce different effects on the Vi-agglutinability of O + Vi strains of *Salm. typhi* and on that of the 'pure' Vi variant Ty6S. So far as is known the TVi antigen present in the two varieties of Vi strain is serologically, and presumably chemically, identical. Its different physico-chemical behaviour in the two variants is, therefore, conditioned by the presence or absence of another constituent of the bacterial cell, in this instance the TO antigen. No mutual interference between the two antigens could be detected by means of the precipitation reaction. These experiments clearly show the fallacy of adopting resistance to heat as the sole characteristic on which the differentiation of supposedly different kinds of antigen is based (Kauffmann, 1943, 1947*a*, 1951).

A comparison of Tables 1, 2 and 6 shows how difficult it is to indicate accurately the changes in the degree of reactivity of an antigen that has been subjected to different treatments. The fashion of expressing these changes by a few symbols compressed into a small table (Kauffmann, 1936*a*, 1951) is an over-simplification. Such tables convey the impression of a degree of accuracy which, in fact, they do not possess.

Observations recorded in this paper are at variance with those published on many occasions by Kauffmann (1936*a*, *b*, 1941*b*, 1947*b*, 1950, 1951) and endorsed by the Salmonella Sub-Committee (1949) and the Enterobacteriaceae Sub-Committee of the Nomenclature Committee of the International Association of Microbiologists (1952). Kauffmann's erroneous conclusions will be discussed in the two following papers, dealing separately with the Vi antigens of *Salm. paratyphi A* and *Salm. paratyphi B*. In the concluding paper of this series the bearing of these sources of error on the nomenclature employed in the serological classification of Bacteriaceae will be discussed.

SUMMARY

The labile Vi antigens of *Salmonella typhi*, *Salm. paratyphi A* and *B*, and of *Salm. typhi-murium* were compared, especially in their response to various chemical and physical agents.

1. Inactivation by dilute acid:

(*a*) The TVi antigen is the most resistant to acid treatment, the BVi antigen is most readily inactivated and the AVi antigen holds an intermediate position.

(*b*) Pure Vi sera may be obtained by absorption with acid-treated bacilli, but this technique gives irregular results.

(*c*) Contrary to Kauffmann's statements, the TVi antigen is not 'destroyed' by acid treatment.

2. Inactivation by dilute alkali:

(*a*) All the Vi antigens so far tested are susceptible to dilute alkali.

(*b*) Complete inactivation, or extraction, of the Vi antigens is ensured when the yield of alkali-treated bacteria represents no more than 25% of the original bacterial count.

(*c*) The most reliable method of preparing a pure Vi serum is by absorption of the Vi+O serum with alkali-treated organisms of the same strain as that employed in immunization.

(*d*) Immunization with alkali-treated bacilli results in the elaboration of pure O antisera entirely devoid of Vi antibody.

3. Inactivation by heat (in aqueous suspensions):

(*a*) The TVi antigen is the one most readily inactivated, the BVi antigen is the one most resistant and the AVi antigen holds an intermediate position.

(*b*) These differences are only of minor significance, since similar differences have been established for the TVi antigen itself when it is contained in the two varieties of Vi strain of *Salm. typhi*.

4. Treatment with alcohol:

(*a*) Most of the properties of the three *Salmonella* Vi antigens remain unimpaired by alcohol treatment.

(*b*) The physico-chemical behaviour of alcohol-treated or heated bacilli, as exemplified by agglutinability by pure Vi antiserum, depends on the presence of other constituents of the bacterial cell.

5. Treatment with formalin:

(*a*) The Vi agglutinability of the different *Salmonella* is preserved undamaged for long periods of time. The O inagglutinability of *Salm. typhi* is also well maintained.

(*b*) It is not yet known whether formolized AVi and BVi antigens undergo an alteration that leads to 'functional deficiency' of the corresponding antibody similar to that of the altered TVi antibody.

6. Treatment with phenol produces a reversible inactivation of the agglutinogenic activity of the TVi antigen but not of the AVi and BVi antigens.

7. The inadequacy of the symbols employed by Kauffmann for expressing the changed reactivity of differently treated antigens is emphasized.

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