# THE SEROLOGICAL CLASSIFICATION OF BACTERIACEAE

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CONTENTS



## INTRODUCTION

In the first paper of this series (Felix, 1952*a*) it was shown that heating and treatment with alcohol do not produce the same changes in the Vi antigen when it is contained in the two varieties of Vi strain of *Salmonella typhi*, namely, the O + Vi form and the 'pure' Vi variant. It was concluded from the results of agglutination and precipitation tests that the physico-chemical behaviour of the Vi antigen after exposure to heat or alcohol was conditioned by the presence or absence of another constituent of the bacterial cell, in this instance the TO antigen. On the other hand, the chemical changes resulting from treatment with dilute acid or dilute alkali had the same effect on the antigenic properties of the TVi antigen, irrespective of whether it was contained in an O + Vi strain or in the 'pure' Vi variant.

The particular Vi antigen of *Salm. typhi* also occurs in *Salm. paratyphi* C (Kauffmann, 1935), *Salm. ballerup* (Kauffmann & Møller, 1940) and in certain strains of *Bacterium coli* (Kauffmann, 1941*a*, *b*). It seemed, therefore, to be of interest to determine how the TVi antigen present in these different species of Bacteriaceae would respond to the various chemical and physical agents.

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## SECTION 1. A COMPARATIVE STUDY OF THE BEHAVIOUR OF TVi ANTIGEN IN DIFFERENT BACTERIACEAE

### (*a*) *Strains employed in the experiments*

The following strains of the four different Bacteriaceae were used in the experiments described in this paper:

*Salm. typhi*—strains Ty 2, 'Watson' and Ty 6S (Felix & Pitt, 1951).

*Salm. paratyphi* C—strain Baghdad 782, received in 1940 from the National Collection of Type Cultures, London.

*Salm. ballerup*—original type strain (Kauffmann & Møller, 1940); culture received in 1943 from Major G. F. Luippold, United States Army Medical Centre, Washington.

*Bact. coli* 5396/38—one of three strains of *Bact. coli* described by Gard & Erikson (1939) as containing *Salmonella* non-specific H antigen. Kauffmann (1941*a*, *b*) confirmed this finding and also established the presence of the typhoid Vi antigen in these cultures which he designated as '*Salmonella coli* 1'. Strain 5396/38 has been employed extensively in experimental work on the preparation of typhoid vaccine at the United States Army Medical Centre, Washington, by Longfellow & Luippold (1943), Luippold (1944, 1946) and more recently by Landy (1952*a*), Webster, Landy & Freeman (1952) and Landy & Webster (1952). The culture of *Bact. coli* 5396/38 was received in 1943 from Major G. F. Luippold.

### (*b*) *Effects of treatment with dilute* HCl *and dilute* NaOH

The cultures of the different strains were exposed to the action of the various chemical and physical agents listed in Table 1. The technical details of the various procedures were exactly the same as those previously described. The results illustrated in Table 1 are, therefore, directly comparable with those shown in the corresponding table in the previous paper (Felix, 1952*a*).

Table 1 shows that acid-treated organisms of the four different Bacteriaceae were still agglutinated by TVi antiserum, whereas alkali-treated bacteria were completely Vi-inagglutinable. The acid-treated and alkali-treated cultures of all the Vi + O strains tested formed perfectly stable suspensions in normal saline and in 2·5 and 5 % solutions of NaCl. Only the 'pure' Vi variant Ty 6S yielded, after treatment with acid or alkali, suspensions that were salt-agglutinable and could not be used in agglutination tests. This was mentioned in the previous paper (Felix, 1952*a*). The alkali-treated organisms were fully agglutinable by homologous pure O serum; the results of the O-agglutination tests are, however, omitted from the table for the sake of simplicity.

Quantitative agglutinin-absorption tests confirmed that the acid-treated organisms had lost only part of their capacity of absorbing TVi antibody. On the other hand, a 400-fold greater number of alkali-treated organisms did not absorb any appreciable amount of Vi antibody.

Rabbits immunized with acid-treated *Salm. paratyphi* C, *Salm. ballerup* and *Bact. coli* 5396/38 elaborated TVi agglutinins in significant titres; rabbits injected with the corresponding alkali-treated suspensions failed to produce any detectable TVi antibody, though all gave a good O-antibody response.

### (c) *Effects of heat*

Exposure to heat did not produce the same effects on the TVi antigen contained in the four different Bacteriaceae. Table 1 shows that the Vi + O strains of *Salm. typhi* and *Salm. paratyphi* C after heating for 1 hr. at 60° C. were completely, or almost completely, inagglutinable by TVi serum. On the other hand, 60° C.

Table 1. *Vi agglutination of differently treated suspensions of various Bacteriaceae containing T Vi antigen*

| | | Saline suspensions of cultures from Difco agar | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Treated with | | | | | | | | |
| | | | | Ethyl alcohol | | | | Heated | | | |
| | | | 0·2 % formalin, 48 hr. at room | 75 %, 48 hr. at room | 50 %, 20 hr. at | N-HCl, 20 hr. at | 0·05 N-NaOH, 4 hr. at room | 1 hr. at 60° C. | | 2½ hr. at 100° C. | |
| Serum | Dilution | Fresh live | tem- perature | tem- perature | 37° C. | 37° C. | tem- perature | Not washed | Washed* | Not washed | Washed* |
| | | *Salm. typhi*, strain Watson [Vi + O + H variant] | | | | | | | | | |
| Pure Vi serum [rabbit no. 803, v. *Salm. typhi*, strain Ty 6S, treated with N-HCl] | 1:200 | +++ | +++ | +++ | +++ | +++ | − | − | − | − | − |
| | 1:500 | +++ | ++ | +± | +± | +++ | − | − | − | − | − |
| | 1:1000 | ++ | (±) | (±) | ± | ++ | − | − | − | − | − |
| | 1:2000 | ((±)) | − | − | ((±)) | (±) | − | − | − | − | − |
| | 1:4000 | − | − | − | − | − | − | − | − | − | − |
| | | *Salm. paratyphi* C, strain Baghdad 782 [Vi + O + H variant] | | | | | | | | | |
| | 1:200 | +++ | ++ | ± | + | + | − | − | (±) | − | − |
| | 1:500 | +++ | ++ | (±) | ± | (±) | − | − | ((±)) | − | − |
| | 1:1000 | ++± | + | − | ((±)) | ((±)) | − | − | ((±)) | − | − |
| | 1:2000 | (±) | (±) | − | − | − | − | − | − | − | − |
| | 1:4000 | − | − | − | − | − | − | − | − | − | − |
| | | *Salm. ballerup* [Vi + O + H variant] | | | | | | | | | |
| Vi + O serum [rabbit no. 836, v. *Salm. typhi*, strain Ty 2, treated with N-HCl] | 1:200 | +++ | +++ | +++ | +++ | +++ | − | +++ | +++ | − | (±) |
| | 1:500 | ++ | ± | +± | +++ | ++ | − | +± | +++ | − | − |
| | 1:1000 | + | − | ± | +++ | + | − | (±) | ++± | − | − |
| | 1:2000 | − | − | − | + | − | − | − | ± | − | − |
| | 1:4000 | − | − | − | − | − | − | − | − | − | − |
| | | *Bact. coli*, strain 5396/38 [Vi + O + H variant] | | | | | | | | | |
| | 1:200 | +++ | +++ | +++ | − | +++ | − | +++ | +++ | − | (±) |
| | 1:500 | +± | +± | ++ | − | +++ | − | +± | +++ | − | − |
| | 1:1000 | ± | ± | + | − | +± | − | (±) | ++ | − | − |
| | 1:2000 | − | − | (±) | − | (±) | − | − | ± | − | − |
| | 1:4000 | − | − | − | − | − | − | − | − | − | − |
| | | *Salm. typhi*, strain Ty 6S ['pure' Vi variant] | | | | | | | | | |
| | 1:200 | +++ | +++ | +++ | +++ | § | § | ++ | +++ | + | ++ |
| | 1:500 | ++ | ++ | ++ | +++ | · | · | − | ++± | − | + |
| | 1:1000 | + | + | ± | +++ | · | · | − | + | − | (±) |
| | 1:2000 | − | − | − | +++ | · | · | − | − | − | − |
| | 1:4000 | − | − | − | + | · | · | − | − | − | − |

*Notes.* The technique of the agglutination test has been described in a previous paper (Felix & Pitt, 1951).

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

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

*Washed = suspension centrifuged, supernatant removed, and sediment re-suspended in equal volume of saline.

§ = not examined because suspensions were salt-agglutinable.

suspensions of *Salm. ballerup* and *Bact. coli* 5396/38, both Vi + O strains, were still Vi-agglutinable, as was also the 60° C. suspension of the 'pure' Vi variant of *Salm. typhi*. *Salm. ballerup* and *Bact. coli* 5396/38 showed detectable Vi agglutination even after heating for 2½ hr. at 100° C., although these reactions were much weaker than that of the 'pure' Vi variant of *Salm. typhi*.

Table 1 also shows that 'washed' organisms from 60° C. suspensions were agglutinated to a higher titre by the TVi serum than the 'not washed' 60° C.

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suspensions, or those of live or formolized organisms. This is due to the well-established fact 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, Bhatnagar & Pitt, 1934). It was shown by agglutinin-absorption and precipitation tests that the TVi antigen is readily extracted in saline suspensions of *Salm. typhi*, and that washing with saline removes a large proportion of the Vi antigen (Felix, 1952*a*). With 'washed' and 'not washed' suspensions heated for $2\frac{1}{2}$ hr. at 100° C. the effect of removing the extracted and precipitable Vi antigen from the suspension is as noticeable as with 60° C. suspensions.

In view of the tendency to adopt resistance to heat as the most important, or even the sole, characteristic on which the differentiation of supposedly different antigens is based (Kauffmann, 1943, 1947*a*, 1951) it appeared desirable to examine the effects of heat in greater detail. Table 2 illustrates the results obtained in a number of experiments.

In the experiment recorded in Table 2 only 'washed' suspensions were employed, i.e. the suspensions were centrifuged, the supernatants removed and the sediments re-suspended in an equal volume of saline. Thus, the disturbing effect on the reaction between bacterial cell and Vi antibody, caused by precipitation of the extracted Vi antigen present in 'not washed' suspensions, was eliminated.

It is seen from Table 2 that *Salm. ballerup* and *Bact. coli* 5396/38 heated for 1 hr. at 60° C. were not only fully agglutinable by TVi antibody but also remained highly resistant to homologous pure O serum. This observation was particularly impressive since inhibition of O agglutination is the most vulnerable property of the TVi antigen in *Salm. typhi*. The strain of *Salm. paratyphi* C employed throughout these experiments was fully sensitive to homologous pure O serum even in the living state, although its Vi-antigen content, as judged by quantitative absorption tests, was quite considerable, i.e. approximately 25 % of the amount contained in strain Watson.

An unexpected result was that obtained with suspensions heated for 1 hr. at 75° C. This treatment rendered the cultures of all the strains tested completely Vi-inagglutinable and, at the same time, also partly O-inagglutinable. It is seen from Table 2 that the suspensions of *Salm. typhi* (strain Watson) and *Salm. paratyphi* C, which were fully O-agglutinable after heating at 60° C., became partly resistant to O agglutinin by heating at 75° C. As these two phenomena are known to be mutually exclusive it was obvious that the apparently paradoxical result observed was due to some peculiar physical or physico-chemical condition of the heated bacteria. The suspensions heated at 75° C. did not differ in appearance from those heated at 60 or 100° C. However, it was found on centrifugation that the 75° C. bacteria had become extremely viscous and formed, instead of the usual sediment, a gelatinous mass which could not be completely re-suspended in fresh saline. After prolonged mixing with a pipette the flakes eventually yielded salt-stable suspensions which were resistant to both the Vi and the O agglutinins. Heating at 100° C. restored full O-agglutinability of all the Vi+O strains; simultaneously weak Vi-agglutinability reappeared in the 100° C. suspensions of

Table 2. *Effects of heat on Vi and O agglutination of various Bacteriaceae containing TVi antigen*

| Strain | Serum | Dilution | Agglutination of saline suspensions of agar-slope cultures: Fresh live | Heated: 1 hr. at 60° C. | Heated: 1 hr. at 75° C. | Heated: 2½ hr. at 100° C. |
|---|---|---|---|---|---|---|
| *Salm. typhi*, strain Watson | Pure Vi serum [rabbit no. 803, v. *Salm. typhi*, strain Ty 6S, treated with N-HCl] | 1:200 | + + + | − | − | − |
| | | 1:500 | + + + | − | − | − |
| | | 1:1000 | + + | − | − | − |
| | | 1:2000 | (( ± )) | − | − | − |
| | Pure O serum [rabbit no. 288, v. *Salm. typhi*, strain O 901, treated with 0·05 N-NaOH] | 1:500 | − | + + ± | ( ± ) | + + + |
| | | 1:1000 | − | + + ± | (( ± )) | + + + |
| | | 1:2000 | − | + + | (( ± )) | + + + |
| | | 1:5000 | − | + ± | (( ± )) | + + + |
| | | 1:10000 | − | ± | (( ± )) | + ± |
| | | 1:20000 | − | ( ± ) | − | ( ± ) |
| *Salm. paratyphi* C, strain Baghdad 782 | Pure Vi serum [rabbit no. 803, see above] | 1:200 | + + + | ( ± ) | − | − |
| | | 1:500 | + + + | (( ± )) | − | − |
| | | 1:1000 | + + ± | (( ± )) | − | − |
| | | 1:2000 | ( ± ) | − | − | − |
| | Pure O serum [rabbit no. 859, v. *Salm. paratyphi* C, strain Baghdad 782, heated 2½ hr. at 100° C.] | 1:100 | + + + | + + + | + | + + + |
| | | 1:200 | + + + | + + + | + | + + + |
| | | 1:500 | + + + | + + + | + | + + + |
| | | 1:1000 | + + + | + + + | + | + + + |
| | | 1:2000 | + + | + + | ± | + ± |
| | | 1:5000 | ± | ± | ( ± ) | ( ± ) |
| *Salm. ballerup* | Vi + O serum [rabbit no. 836, v. *Salm. typhi*, strain Ty 2, treated with N-HCl] | 1:200 | + + + | + + + | − | ( ± ) |
| | | 1:500 | + + | + + + | − | − |
| | | 1:1000 | + | + + ± | − | − |
| | | 1:2000 | − | ± | − | − |
| | Pure O serum [rabbit no. 870, v. *Salm. ballerup*, treated with 0·05 N-NaOH] | 1:500 | − | ( ± ) | + | + + + |
| | | 1:1000 | − | ( ± ) | + | + + + |
| | | 1:2000 | − | ( ± ) | + | + + + |
| | | 1:5000 | − | (( ± )) | + | + + + |
| | | 1:10000 | − | (( ± )) | + | + + + |
| | | 1:20000 | − | (( ± )) | + | + + |
| *Bact. coli*, strain 5396/38 | Vi + O serum [rabbit no. 836, see above] | 1:200 | + + + | + + + | − | ( ± ) |
| | | 1:500 | + ± | + + + | − | − |
| | | 1:1000 | ± | + + | − | − |
| | | 1:2000 | − | ± | − | − |
| | Pure O serum [rabbit no. 876, v. *Bact. coli*, strain 5396/38, heated 2½ hr. at 100° C.] | 1:100 | (( ± )) | ( ± ) | + | + + + |
| | | 1:200 | − | ( ± ) | + | + + + |
| | | 1:500 | − | ( ± ) | + | + + + |
| | | 1:1000 | − | (( ± )) | + | + + + |
| | | 1:2000 | − | − | ± | + + |
| | | 1:5000 | − | − | − | (( ± )) |
| *Salm. typhi*, strain Ty 6S ['pure' Vi variant] | Vi + O serum [rabbit no. 836, see above] | 1:200 | + + + | + + + | − | + + |
| | | 1:500 | + + | + + ± | − | + |
| | | 1:1000 | + | + | − | ( ± ) |
| | | 1:2000 | − | − | − | − |

*Note:* All the suspensions had been centrifuged, the supernatants removed, and the sediments re-suspended in an equal volume of saline.

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*Salm. ballerup* and *Bact. coli* 5396/38 and strong Vi-agglutinability with the 'pure' Vi variant of *Salm. typhi*.

It was first thought possible that the phenomenon was due to interference by the H antigen, altered through heating, although most of the cultures gave only a moderate degree of H agglutination when tested in the living state, and the strain Ty6S gave none at all. Control tests showed that the two Vi-negative variants of *Salm. typhi*, strains H901 and O901, underwent the same change and so also did a strain of *Salm. paratyphi* A. On the other hand, a culture of *Salm. paratyphi* B and two strains of *Salm. typhi-murium* did not become viscous on heating at 75° C. The changed physico-chemical condition of the 75° C. bacteria does not appear, therefore, to show any special relationship to one of the three antigens, the H, O or Vi.

Quantitative absorption tests carried out with 60 and 100° C. suspensions proved that the agglutinin-binding property of the TVi antigen had been preserved equally well in the four different Bacteriaceae.

On the other hand, certain differences were observed in the agglutinogenic properties of the heated organisms, though it is difficult to indicate the differences accurately without reproducing elaborate tables. A more abundant TVi-antibody response to 60° C. suspensions of the 'pure' Vi variant, as compared with that to Vi+O strains of *Salm. typhi*, was mentioned in a previous paper (Felix, 1952*a*). The contrast was still greater with 60° C. suspensions of *Salm. paratyphi* C and *Bact. coli* 5396/38, both of which induced in the rabbits formation of TVi-agglutinins in fairly high titres. With these two species TVi-agglutinin responses were detectable even in rabbits immunized with suspensions heated for $2\frac{1}{2}$ hr. at 100° C. In this respect *Bact. coli* 5396/38 excelled the other three species. Of five rabbits immunized with 100° C. suspensions of this organism two responded with traces of TVi antibody, and one rabbit showed a 'standard' TVi-agglutinin titre of 1:30.

In view of the report by Stuart & Kennedy (1948) on the formation of high-titre TVi antibody in rabbits immunized with saline extracts of *Salm. typhi* heated at 100° C. for 3 hr., the following experiment was carried out. Very dense suspensions of *Salm. typhi* strain Ty2 and of the other three Bacteriaceae, each containing $80{,}000 \times 10^6$ organisms/ml., were extracted with saline for $2\frac{1}{2}$ hr. at 100° C. The extracts were filtered through Seitz disks and increasing doses, suitably diluted with saline, were injected intravenously into groups of three rabbits. Each rabbit received a total dosage equivalent to 4 ml. of undiluted extract, corresponding to $320{,}000 \times 10^6$ organisms in the original suspension. None of the twelve rabbits produced any detectable TVi agglutinin, though high-titre O-antibodies were present in the sera. The extracts gave strong precipitation reactions with Vi antisera, but evidently contained only Vi hapten and no complete Vi antigen.

### (*d*) *Effects of treatment with ethyl alcohol*

The treatment with alcohol was carried out in two series: the one according to the original technique employed throughout the writer's work, i.e. treatment with 75 % alcohol for 48 hr. at room temperature; the other according to Kauffmann's modification, i.e. treatment with 50 % alcohol for 20 hr. at 37° C. On the basis of

comparative tests with *Salm. typhi* it was previously stated that the two techniques produced essentially identical effects. In no instance was the Vi-agglutinability of *Salm. typhi* 'destroyed', as has been claimed by Kauffmann (1941*b*, 1951). The same was found to be true with the Vi antigen of *Salm. paratyphi* A and the Vi antigen of *Salm. paratyphi* B and *Salm. typhi-murium*, although these comparative tests were not specifically mentioned in the previous paper (Felix, 1952*a*).

When the two modifications of alcohol treatment were applied to the different Bacteriaceae under investigation it was found that *Bact. coli* 5396/38 behaved differently from the other three species. Treatment with 50 % alcohol for 20 hr. at 37° C. rendered *Bact. coli* 5396/38 completely inagglutinable by TVi serum, whereas exposure to 75 % alcohol for 48 hr. at room temperature did not affect the Vi-agglutinability of this organism. With the other three species of Bacteriaceae the two techniques produced uniform effects. It is seen from Table 1 that the suspensions treated at 37° C. gave even higher readings in the Vi-agglutination test than those treated at room temperature. Repeat tests showed that these results were readily reproducible.

The altered physico-chemical state of the TVi antigen in *Bact. coli* 5396/38, resulting from treatment with alcohol at 37° C., was also reflected in the results of quantitative absorption tests. With *Salm. typhi*, *Salm. paratyphi* C and *Salm. ballerup* there was only a slight diminution in the absorbing power of bacteria treated with alcohol at 37° C. as compared with those treated at room temperature, the ratio being approximately 1 to 2 or 1 to 4. With *Bact. coli* 5396/38 the ratio was 1 to 50.

The same batch of suspension of *Bact. coli* 5396/38 treated with alcohol at 37° C. that had been found inactive in TVi-agglutination and absorption tests was also used for the immunization of two rabbits. Both rabbits responded with abundant production of TVi antibody.

(*e*) *Serological identity of the TVi antigen in the four different Bacteriaceae*

There was no reason for suspecting that the TVi antigen contained in the four species of Bacteriaceae was qualitatively different, thus accounting for the differences observed after heating or treatment with alcohol. Nevertheless, this point was examined by cross-absorption of agglutinins and by cross-precipitation tests.

(1) *Cross-agglutination and agglutinin-absorption tests.* The results of these tests may be summarized as follows:

(i) In cross-agglutination tests with living or formolized bacteria the relative titres observed with homologous and heterologous sera always conformed to the well-established rule already referred to, i.e. the greater the Vi-antigen content of a strain the lower was the titre of the Vi-agglutination reaction obtained with the culture. This indicated that the end-titres recorded with the homologous and heterologous sera were due to the same antibody.

(ii) Quantitative absorption tests showed that *Bact. coli* 5396/38 contained the greatest quantity of TVi antigen, *Salm. ballerup* was a close second, and the other strains followed in decreasing order: Ty 6S, Ty 2, 'Watson' and *Salm. paratyphi* C (strain Baghdad 782). This is in good agreement with the early observation by

Luippold (1944, 1946) that *Bact. coli* 5396/38 offered 'an appreciably more abundant source' of TVi antigen than *Salm. typhi*.* It should be mentioned that careful colony selection at very short intervals is essential in tests of this kind, to ensure that the cultures employed contain TVi antigen in their respective maximum amounts. With *Salm. ballerup* and *Bact. coli* 5396/38, both of which readily split off the Vi-negative variant, colony selection was practised daily.

(iii) Cross-absorption of agglutinins showed that the Vi antibody produced by organisms of each of the four species could be completely absorbed by those of the other three Bacteriaceae. The Vi sera used in these tests were all from rabbits immunized with alcohol-treated bacteria. The serum against *Salm. typhi* was employed unabsorbed, since the rabbit had been immunized with the 'pure' Vi variant Ty6S. The sera against *Salm. paratyphi* C, *Salm. ballerup* and *Bact. coli* 5396/38 were first absorbed with alkali-treated homologous bacteria. Each of the 'pure' Vi sera was further absorbed with living organisms of the three heterologous species and was tested against live bacteria of the homologous strain. When the sera employed were first completely freed of O agglutinins the subsequent absorption with the heterologous strains invariably removed all residual agglutinins for the homologous living bacteria.

In cross-agglutination and cross-absorption tests with *Salm. paratyphi* C and *Bact. coli* 5396/38 due attention must be paid to the presence in the latter organism of *Salmonella* non-specific H antigen (Gard & Erikson, 1939). The corresponding H agglutinins were found even in the serum of rabbits immunized with suspensions treated with 50 % alcohol for 20 hr. at 37° C. and gave rise at first to confusing results.

(2) *Cross-precipitation tests.* The four saline extracts prepared by heating dense suspensions for $2\frac{1}{2}$ hr. at 100° C. and filtering through Seitz disks, which were used for the immunization of rabbits, were also examined in cross-precipitation tests. Serial dilutions of the four extracts were tested against a constant dose of each of the three heterologous unabsorbed Vi + O sera. High-titre pure O sera against the heterologous organisms served as controls. These tests did not give any indication of qualitative antigenic differences in the four TVi extracts.

### (*f*) *Summary of Section* 1

The results of the experiments described in section 1 confirm the conclusion previously arrived at that the physico-chemical behaviour of Vi-positive strains of *Salm. typhi*, after heating or treatment with alcohol, is conditioned by the presence or absence of the O antigen. It has now been shown that the effect of treatment by heat or alcohol on the TVi antigen varies according to the species of organism, but that the reaction to dilute acid or alkali is the same in all species. The observed differences in the response to exposure to heat are summarized,

* While this paper was being written Dr M. Landy kindly informed me of his recent finding that the yield of extracted and purified TVi antigen he obtained from *Bact. coli* 5396/38 was approximately 3 % of the dry weight, from *Salm. ballerup* 2 % and from strain Ty2 of *Salm. typhi* 0·25 % (Landy, 1952*b*, personal communication). At the same time Jude & Nicolle (1952*a*, *b*) and Nicolle & Jude (1952*a*, *b*) published interesting observations on the conditions governing the development of the TVi antigen in the four species of Bacteriaceae.

though rather inadequately, in Table 3. It is necessary again to emphasize that this kind of presentation does not permit of more than a very rough comparison of the diminution in the degree of reactivity observed.

When Table 3 is compared with the corresponding Table 6 in the previous paper (Felix, 1952*a*), it will be seen that the differences in the changes produced in the heated TVi antigen itself, when present in the four different Bacteriaceae, are of the same order of magnitude as those observed in the AVi and BVi antigens on the one hand and the TVi antigen on the other. The available evidence indicates that

Table 3. *Heat-resistance of TVi antigen in saline suspensions of different Bacteriaceae*

| | | *Salm. typhi* | | | | |
|---|---|---|---|---|---|---|
| | Heating | Vi+O strains | 'Pure' Vi variant | *Salm. paratyphi* C | *Salm. ballerup* | *Bact. coli* 5396/38 |
| Agglutinability | 1 hr. at 60° C. | − | + | (±) | + | + |
| | 2½ hr. at 100° C. | − | ± | − | (±) | (±) |
| Agglutinogenic activity | 1 hr. at 60° C. | (±) | ± | + | ± | + |
| | 2½ hr. at 100° C. | − | − | (±) | − | ± |
| Agglutinin-binding capacity | 2½ hr. at 100° C. | + | + | + | + | + |
| Inhibition of O-agglutination | 1 hr. at 60° C. | − | Not applicable | | + | + |
| | 2½ hr. at 100° C. | − | Not applicable | | − | − |

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

the TVi antigen contained in the four species of Bacteriaceae is serologically, and presumably chemically, identical. It is obvious, therefore, that the different behaviour of this substance in the various organisms is due to the interaction of other constituents of the bacterial cell. Consequently, it is not possible to accept resistance to heat as the sole, or even the most important, criterion for distinguishing supposedly different kinds of antigen.

## SECTION 2. REVIEW OF WORK ON LABILE SOMATIC ANTIGENS OF BACTERIACEAE

### (*a*) *The Vi antigens of different* Salmonella *species*

Kauffmann (1935) was one of the first workers to confirm the early observations by Felix & Pitt (1934*a*, *b*) and Felix *et al.* (1934) on the properties of the Vi antigen of *Salm. typhi*. Kauffmann (1935) reported at the same time that strains of *Salm. paratyphi* C also contained the typhoid Vi antigen. His conclusions, translated from the original German, were as follows: 'From this finding it is to-day already clear that we are facing a new and large field in *Salmonella* serology which it will take years of work to explore' (Kauffmann, 1935, p. 641). 'In the interest of uniform nomenclature I would like to suggest already to-day that all such thermolabile somatic antigens be designated as "Vi antigens", following the procedure of Felix and Pitt, and be labelled with capital Latin letters A, B, C, etc. in order to distinguish those that are found to differ serologically. The typhoid Vi-antigen and

also the paratyphoid-C Vi-antigen would receive the symbol A and the next Vi-antigen to be found would be designated B. We therefore add a new "Vi" column, in which the Vi antigens are placed, to the present Kauffmann-White schema of *Salmonella* antigens, of the incompleteness of which we have always been convinced' (Kauffmann, 1935, p. 642 and Table 15).

For some years Kauffmann adhered to his suggestion in regard to the particular Vi antigen of *Salm. typhi*. Table 4, reprinted from Kauffmann (1941*a*, p. 240 and 1941*b*, p. 17), shows that the symbol A was employed to designate the Vi antigen common to *Salm. typhi*, *Salm. paratyphi* C, *Salm. ballerup* and the so-called *Salm. coli* 1. The table served to support Kauffmann's recommendation that *Bact. coli* strains possessing one of the known *Salmonella* H, O or Vi antigens should be recognized as *Salmonella* types.

Table 4

[This is an English version of the table published as Table 5 by Kauffmann (1941*a*, p. 240) and reprinted as Table 1 by Kauffmann (1941*b*, p. 17).]

| Type | O antigen | H antigen | | Vi antigen |
|---|---|---|---|---|
| | | 1 phase | 2 phase | |
| *S. paratyphi* B | [I], IV, V, XII ... | *b* | 1, 2 ... | – |
| *S. coli* 3 | IV, V, XII ... | $z_{20}$ | . | – |
| *S. schleissheim* | IV, XXVII, XII ... | *b*, $z_{12}$ | – | – |
| *S. coli* 4 | IV, [XXVII], XII ... | $z_{21}$ | . | – |
| *S. paratyphi* C | VI, VII, XII ... | *c* | 1, 5 ... | A |
| *S. typhi* | IX, XII ... | *d* | – | A |
| *S. onderstepoort* | (I), VI, XIV, XXV | *e*, *h* | 1, 5 ... | – |
| *S. coli* 5 | (I), VI, XIV, XXV | $z_{22}$ | . | – |
| *S. ballerup* | XXIX | $z_{14}$ | – | A |
| *S. coli* 1 | XXXI | . | 1, 5 ... | A |
| *S. coli* 2 | XXXII | . | 1, 5 ... | – |

*Key*

[] = these antigens may be missing.
(I) = only a part of I antigen is present.
XII ... = the partial antigens of the XII antigen are not considered here.
. = not investigated.
– = missing.
1, 5 ... = much more complex than indicated.

On the other hand, Kauffmann did not follow his own suggestion in regard to the labile somatic antigen of *Salm. paratyphi* B and *Salm. typhi-murium* which Felix & Pitt (1936) described as the Vi antigen of these organisms. Kauffmann (1936*a*, *b*) found that this antigen was identical with the O-factor V of the Kauffmann-White schema, but he failed to draw the logical conclusion from this finding. Instead of acknowledging that O-factor V could not be classified as an O antigen according to the definition derived from the work of Weil & Felix (1920), Kauffmann has attempted in numerous papers published during the past 15 years to maintain the position of the V antigen ($V_1$ and $V_2$) as an O antigen in the diagnostic scheme. This has been discussed in the preceding paper (Felix, 1952*c*). In his latest monograph Kauffmann (1951, p. 40) removed the V antigen from the group of *Salmonella* O antigens, because it 'has a special position and differs from other

somatic antigens'. However, contrary to Kauffmann's own earlier recommendation (Kauffmann, 1935, p. 642) this labile somatic antigen was not assigned to its rightful place among the *Salmonella* Vi antigens, but a new class of antigens was created for it, namely, the K antigens.

### (*b*) *The K antigens of the* coli *group*

Kauffmann (1943) described the presence in *Bact. coli* of a heat-labile somatic antigen and designated it as L antigen (L derived from labile). The starting-point of this work was Kauffmann's observation that living cultures of *Bact. coli*, especially of strains isolated from infections of the urinary tract or from pus, bile or blood, were inagglutinable by homologous antiserum whereas suspensions heated at 100° C. were readily agglutinated. He stated: 'We were dealing therefore with a phenomenon known from typhoid serology, namely, a clear-cut "O-inagglutinability". Heating for 1–2 hr. at 100° C. destroyed the binding property of the L antigen, therefore the assumption that a Vi antigen was the cause of the O-inagglutinability had to be abandoned. It should be recalled, however, that I have earlier demonstrated the Vi antigen in several strains of *Salmonella coli* 1' (Kauffmann, 1943, p. 22).

Kauffmann (1943) summarized the antigenic differences between this 'new' L antigen and the various kinds of *Salmonella* antigens shown in Table 5.

Some of Kauffmann's (1943) own comments on the L antigen were: 'In strains of *Bact. coli* we have therefore to distinguish between three different heat-labile somatic antigens, namely, the Vi antigen, the $V_2$ antigen and the L antigens' (p. 24). 'Though *coli* O-inagglutinability and its cause, the L antigens, appear to resemble outwardly the typhoid O-inagglutinability and the Vi antigen, the two antigens, Vi and L, differ nevertheless in regard to resistance and serology. The L antigen is also unrelated to the $V_2$ antigen (antigen no. five) of the *Salmonella* group. The main difference between the Vi antigen and the L antigens is that the binding capacity of the L antigens is destroyed by heating for 1–2 hr. at 100° C., so that it is possible to prepare pure L antiserum by absorption with boiled *Bact. coli*' (pp. 39–40).

Kauffmann's (1943) paper enumerated the following points of similarity in the properties of L and Vi antigens:

(1) The occurrence of a type of variation in the L antigen, analogous to that observed in the typhoid Vi antigen; *coli* cultures with well-developed L antigen, the L-plus forms, are opaque, like Vi-forms of *Salm. typhi*; colonies without L antigen, the L-minus forms, are translucent, similar to Vi-negative variants of *Salm. typhi*.

(2) The pathogenic effects of *Bact. coli* in the mouse are determined by the combined activity of the O and L antigens.

(3) The L antigens are of importance in active and passive immunization of mice since the L antibody exerts a special protective action.

(4) The 'qualitative receptor analysis' has to distinguish not only between O and H antigens but also between the various heat-labile somatic antigens which are of diagnostic and immunological importance.

Subsequently, Kauffmann (1944*a*, *b*) found that the O-inagglutinable strains of *Bact. coli* did not all behave identically when tested against the two kinds of homologous antisera he employed routinely, i.e. one prepared by immunization with living bacteria (O + L serum) and the other with bacteria heated for 2½ hr. at 100° C. (O serum). The great majority of strains became fully O-agglutinable after heating at 100° C. and in these Kauffmann (1944*a*, *b*) identified seventeen serologically different L antigens. A few strains, designated by Kauffmann as 'A forms', were not rendered fully O-agglutinable after heating, and Knipschildt (1945) showed that the O-inagglutinability of these cultures was due to an antigen

Table 5. *Antigenic differences*

[This is an English version of Kauffmann's Table 2 published in *Acta path. microbiol. scand.* 1943, **20**, 25.]

| | | L | Vi | $V_2$ | $V_1$ | IV | H | M |
|---|---|---|---|---|---|---|---|---|
| Living or 0·5 % formalin | 1 | + | + | + | + | + | + | + |
| | 2 | + | + | + | + | + | + | + |
| | 3 | + | + | + | + | + | + | + |
| 1 hr. at 60° C. | 1 | − | − | + | + | + | + | + |
| | 2 | (−) | + | + | + | + | + | + |
| | 3 | − | − | + | + | + | + | + |
| 2½ hr. at 100° C. | 1 | − | − | − | + | + | − | − |
| | 2 | − | + | − | + | + | − | + |
| | 3 | − | − | − | + | + | − | − |
| 50 % alcohol, 20 hr. at 37° C. | 1 | − | − | + | + | + | − | − |
| | 2 | (−) | + | + | + | + | (−) | . |
| | 3 | + | + | + | + | + | + | − |
| N-HCl, 20 hr. at 37° C. | 1 | − | − | − | − | + | − | − |
| | 2 | − | − | − | − | + | − | − |
| | 3 | − | − | − | − | + | − | − |

*Key*

L, Vi, $V_2$, $V_1$ and IV = somatic antigens.
H = flagellar antigens.
M = slime antigens.
1 = agglutinability.
2 = agglutinin-binding capacity.
3 = agglutinogenic capacity.
\+ = preserved.
− = destroyed.
(−) = not completely destroyed in dense suspension.
. = not investigated.

considerably more heat-resistant than the L antigen. Accordingly, this antigen was called 'A' antigen and Knipschildt demonstrated thirteen serologically different 'A' antigens amongst the twenty strains of *Bact. coli* he examined. Knipschildt also found that these 'A' forms were capsulated and showed capsule-swelling when tested against the corresponding 'A' antiserum. Yet another 'new' heat-labile somatic antigen was described by Knipschildt (1946) in three O-in-agglutinable strains of *Bact. coli*. As this antigen was somewhat less heat-resistant than the 'A' but more resistant than the 'L' antigen it was accepted as a new variety and named 'B' antigen. Each of the three strains tested was found to

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possess a serologically different 'B' antigen. Serum containing 'B' agglutinin did not produce capsule-swelling of the homologous living culture. Table 6, first published by Knipschildt (1946) and reprinted by Kauffmann (1947*a*, 1951), shows the suggested criteria for the identification of the L, A and B antigens of *Bact. coli* and their differentiation from the known antigens of the *Salmonella* group. For some obscure reason the V antigen (antigen no. five), shown in Kauffmann's preceding two tables, has not been included in this table.

Table 6. *Antigenic differences (according to Kauffmann & Knipschildt)*

[This table is reprinted from Knipschildt (1946, Table 4, p. 182), Kauffmann (1947*a*, Table 1, p. 74), Kauffmann (1951, Table 25, p. 157).]

| | | L | Vi | H | M | IV | A | B |
|---|---|---|---|---|---|---|---|---|
| Living or 0·5 % formalin | 1 | + | + | + | + | + | + | + |
| | 2 | + | + | + | + | + | + | + |
| | 3 | + | + | + | + | + | + | + |
| 1 hr. at 60° C. | 1 | − | − | + | + | + | + | + |
| | 2 | (−) | + | + | + | + | + | + |
| | 3 | − | − | + | + | + | + | + |
| 2½ hr. at 100° C. | 1 | − | − | − | − | + | + | − |
| | 2 | − | + | − | + | + | + | + |
| | 3 | − | − | − | − | + | (+) | − |
| 50 % alcohol, 20 hr. at 37° C. | 1 | − | − | − | − | + | + | + |
| | 2 | (−) | + | (−) | . | + | . | + |
| | 3 | + | + | + | − | + | + | + |
| N-HCl, 20 hr. at 37° C. | 1 | − | − | − | − | + | + | + |
| | 2 | − | − | − | − | + | . | + |
| | 3 | − | − | − | − | + | (+) | − |

*Key*

L = L antigen.
Vi = Vi antigen.
H = flagellar antigen.
M = mucous antigen.
IV = O antigen.
A = A antigen.
B = B antigen.
1 = agglutinability.
2 = agglutinin-binding capacity.
3 = agglutinogenic capacity.
+ = preserved.
(+) = somewhat weakened.
− = destroyed.
(−) = not completely destroyed in dense suspension.
. = not examined.

Kauffmann & Vahlne (1945) introduced a collective designation of the L, A and B antigens and named the whole group 'K' antigens (K derived from the German word 'Kapsel', meaning capsule). The reasons for doing so were given in the following statements, translated from the original German: 'Although these three antigens represent substances differing one from another in several respects, they nevertheless belong to one group of envelope or capsular antigens, and shall be designated, therefore, uniformly and given successive arabic numbers. The definition of the L antigens as heat-labile somatic antigens, originally given by Kauffmann, has been shown by further work to be too narrow since we now recognize heat-labile as well as heat-stable envelope or capsular antigens. It should be mentioned that heat-labile L antigens may also be capsular antigens, as shown by strain 316 of group 9, whose L antigen was demonstrated by Kauffmann and

whose capsular nature was proved by Knipschildt. It would be possible, therefore, to call all these antigens (L, A and B) uniformly L antigens, but this might cause confusion in view of the earlier designation "L-antigen" according to Kauffmann. The same also applies to the designation "Vi-antigen" introduced by Felix and Pitt; this antigen undoubtedly also belongs to this group of envelope or capsular antigens. For this reason we do not wish to call these antigens "Vi-antigens", but we suggest that all envelope or capsular antigens should be designated uniformly as "K-antigens"' (pp. 121–2).

'There is no objection to subdividing the K antigens into L, A, B and Vi antigens in purely experimental work; in the diagnostic antigen schema, however, they are all to be labelled with consecutive arabic numbers. So far as the nature of these antigens has been more closely determined, this may be indicated by adding to the numbers the letters L, A, B or Vi. The heat-labile O 1 antigens of dysentery bacilli described by H. Braun and E. K. Unat undoubtedly also belong to the envelope or capsular antigens, although we have not yet had an opportunity of investigating this point.'

'The term "K-antigens" is therefore not to be restricted to the *coli* group alone, but such envelope or capsular antigens are quite generally to be designated by the letter K, analogous to the O and H antigens. It should be emphasized that it is unnecessary to demonstrate a definite capsular swelling or staining, as this is often difficult and not always possible in routine work. Moreover, there are intermediate stages between swelling of the bacteria and definite capsular swelling, and it is often impossible to decide whether or not one is dealing with genuine capsular swelling.'

'The most important criteria of K-antigens are therefore the demonstration of O-inagglutinability of living bacteria (smooth forms) and their agglutinability by K-sera (prepared with living bacteria), irrespective of whether the antigens concerned are heat-stable or heat-labile' (Kauffmann & Vahlne, 1945, p. 123).

These statements have since been reprinted by Kauffmann, verbatim or with slight modifications, in many of his publications (e.g. Kauffmann, 1947*a*, 1951).

### (c) *The M (mucoid) antigens*

In the course of his early attempts to demonstrate the existence of a 'Vi antigen' in *Salm. paratyphi* B, Kauffmann (1935) found that mucoid forms of this organism, and the slime-wall of colonies grown at room temperature, contain a special 'slime antigen'. This antigen Kauffmann (1936*a*) designated as M antigen (M derived from mucoid). He found at the same time that twelve different *Salmonella* types that formed mucoid colonies or slime-walls all contained the same M antigen. The change from the normal, non-mucoid 'N form' to the mucoid 'M form' was termed N-M form variation (Kauffmann, 1936*a*). The M antigen was listed throughout the years in Kauffmann's tables showing the various *Salmonella* antigens but it played no role in *Salmonella* serology, either from the theoretical or from the practical point of view.

More recently the term M antigen has come to be employed in the serology of other Bacteriaceae, and the result is complete confusion. Kauffmann (1949*a*)

assigned to the *Klebsiella* group (Friedländer's bacillus, *Bacterium aerogenes*) four antigens: M in the mucoid envelope, K in the capsule, and the O and R antigens in the soma. Later he stated: 'Probably, however, the mucoid envelope is serologically identical with the capsule, even though this has not been proven experimentally' (Kauffmann, 1951, p. 205). Nevertheless, the different forms of *Klebsiella* are designated by their antigens as MKO form, KO form, MO form, etc.

Henriksen (1949*a–c*) examined twelve different strains of mucoid *Bact. coli* isolated from pathological processes, and found that all contained the same M antigen, although the strains belonged to at least four different O-antigen groups. 'The relationship between the M antigen and the K antigens of Kauffmann, Vahlne and others has not been established' (Henriksen, 1949*c*, p. 915). In carefully controlled agglutination and precipitation tests the M antigen of mucoid *Bact. coli* was found to give cross-reactions with mucoid strains of *Salm. paratyphi* B and three other *Salmonella* types (Henriksen, 1950; Josephsen & Henriksen, 1951).

Kauffmann and his co-workers did not pay much attention to the occurrence of M antigen, or antigens, in *Bact. coli*. In one of the early papers Kauffmann (1944*a*, p. 30) described two mucoid (M) forms of *Bact. coli* as possessing M antigens. These antigens differed serologically one from the other and also from the L antigens of *Bact coli*. Later, however, he stated: 'Apart from the A forms and one L form, they (i.e. the *Escherichia* group) possess no capsule, and as a rule, they form no "mucus"' (Kauffmann, 1949*a*, p. 404). On the other hand, Henriksen (1949*d*, *e*) found that mucoid *Bact. coli* are quite common and possess the same M antigen.

More recently, Perch (1950) reported on antigenic relationships between M forms of *Salm. paratyphi* B and *Klebsiella*. In Kauffmann's latest monograph this finding is summarized as follows: 'A capsular serum of *Klebsiella* 13 gave typical capsular swelling with the M form of *S. paratyphi* B (grown in broth for 8 hr. at about 20° C.), while an M serum of *S. paratyphi* B gave capsular swelling with a *Klebsiella* Type 13 culture. By cross-absorption it was shown that the capsules are not identical, but contain a common partial antigen' (Kauffmann, 1951, p. 215).

### (*d*) *The α antigen* (*Stamp & Stone*)

Stamp & Stone (1944) described the presence of a heat-labile somatic antigen, called the α antigen, in certain strains of coliform bacteria, both lactose fermenters and non-fermenters. These organisms, α strains, were isolated from the faeces of patients and of healthy individuals, from normal rabbit and guinea-pig faeces and from milk. The practical importance of this finding lay in the fact that α agglutinins were found in the serum of normal rabbits and in certain diagnostic sera obtained from the most reputable sources. This proved to be a possible source of error in routine diagnosis, particularly if slide agglutination was relied on for identification. Francis & Buckland (1945) soon confirmed this observation.

Stamp & Stone (1944) noted that the α antigen resembled in certain respects the Vi antigen of *Salm. typhi*, particularly in the inhibitory effect on O agglutination. They wrote (p. 271): 'α antigen was obviously not of the nature of a capsular antigen, as only a small proportion of these α strains showed capsular formation or

a mucoid type of growth.' Kauffmann (1947*b*, p. 584), however, stated: 'As a heat-labile somatic antigen it may be placed among the K antigens (Kauffmann & Vahlne).'

It may be recalled here that 'normal' rabbits also occasionally harbour *Bact. coli* strains containing the typhoid Vi antigen and consequently show a raised titre of TVi agglutinin. Such rabbits do not respond to immunization with TVi antigen and are a serious source of error in the testing of typhoid vaccine (Felix, 1951).

### (*e*) *Labile somatic antigens of* Shigella

Archer (1942) described a heat-labile factor responsible for the inagglutinability of strains of *Bact. alkalescens*, but was unable to show that the inhibiting factor was an antigen, analogous to the Vi antigen of *Salm. typhi*. About the same time Braun & Unat (1942, 1943) clearly demonstrated the presence of a labile somatic antigen in cultures they designated as *Sh. flexneri*. These strains were later assigned to the *alkalescens* group (Kauffmann, 1949*b*).

The observations of Braun & Unat had a stimulating effect on subsequent work on *Shigella*. Schütze (1944) found that living cultures of *Sh. shigae* that were inagglutinable by homologous O antiserum became readily agglutinable after heating at 100° C. He did not succeed, however, in demonstrating that the heat-labile agent involved was an antigen. That this was so was proved by Olitzki, Schelubsky & Koch (1946) and Schelubsky & Olitzki (1947, 1948). Using the methods employed in the qualitative receptor analysis of H, O and Vi antigens these workers showed, first in precipitation and then in agglutination tests, that the O-inagglutinability of *Sh. shigae* is due to the presence of a heat-labile somatic antigen. Similar results were obtained by Madsen (1949) with a number of O-inagglutinable strains of *Sh. flexneri* and *Sh. boydii*. Szejnberg (1948) and Ewing (1950) recommended agglutination tests of living and 100° C. suspensions as a routine procedure in the examination of O-inagglutinable *Shigella* cultures.

None of these workers observed capsules in the O-inagglutinable cultures possessing labile somatic antigens. Nevertheless, Kauffmann (1951), referred to these antigens as 'envelope antigens, which may, as in the *Escherichia* group, be designated as K antigens' (p. 233). Recently Ewing, Edwards & Hucks (1951) described an encapsulated culture of *Sh. boydii* possessing a capsular antigen related to those of some *Klebsiella* types.

## DISCUSSION

The experiments described in this paper show that exposure to heat or alcohol produces different changes in the TVi antigen contained in four species of Bacteriaceae. In *Salm. ballerup* and *Bact. coli* 5396/38 heating alters the agglutinability of the organisms according to a pattern similar to that observed in the 'pure' Vi variant of *Salm. typhi*, which is devoid of the O antigen. On the other hand, in *Salm. paratyphi* C the changes follow the pattern known from the O + Vi strains of *Salm. typhi*. The greatest differences in the changed reactivity of the heated bacteria are seen in agglutination tests with pure Vi and pure O sera; the differences

in the changes in agglutinogenic activity are less conspicuous and those in the agglutinin-binding capacity are hardly discernible.

Treatment with alcohol at 37° C. revealed another difference in the degree of alteration in the TVi antigen. *Bact. coli* 5396/38 so treated was completely Vi-inagglutinable and its capacity to absorb Vi antibody was reduced to a much greater extent than that of any of the other three Bacteriaceae.

On the other hand, dilute acid or dilute alkali acted uniformly on the TVi antigen of the four species. The customary cross-agglutination and precipitation tests, and cross-absorption of agglutinins, failed to show any qualitative differences in the TVi antigen of the four different Bacteriaceae.

These results confirm the earlier observation that other constituents of the bacterial cell, which may or may not be antigenic, interact in the changes which the TVi antigen undergoes when the bacteria are exposed to a relatively mild chemical solvent such as alcohol, or a physical procedure such as heating. The great lability, i.e. chemical reactivity, of the TVi-antigen complex apparently offers the requisite condition for such interaction.

The extent to which changes in the colloidal state of the various cell components may influence the agglutinative behaviour of the cell as a whole is revealed by the results of heating at 75° C. The almost complete inagglutinability by O and Vi antisera after this treatment is not specifically related to any one of the known *Salmonella* antigens. This finding is reminiscent of an early observation by Porges & Prantschoff (1906), who found that suspensions of various Gram-negative organisms that were spontaneously agglutinable became salt-stable after heating at 80° C. but reverted to the original condition after heating at 100° C.

The L antigen of *Bact. coli* has been separated by Kauffmann from the Vi antigen and designated as a 'new' kind of labile somatic antigen because of its greater susceptibility to heat. The A antigen was introduced on account of its greater resistance to heat. Finally, the B antigen had to be postulated as a third kind of labile antigen because its resistance to heat was intermediate between those of L and A, that is to say, it corresponded approximately to that of the typhoid Vi antigen. The experiments described in this and the preceding paper of this series (Felix, 1952*a*) clearly show the fallacy of this procedure. By analogy with what is known about the TVi antigen of the four different Bacteriaceae, it may be concluded that one and the same labile antigenic substance, giving qualitatively identical serological reactions, may present itself as L antigen in one strain of *Bact. coli*, as B antigen in another and as A antigen in the third. There can be little doubt that this would be found to be true if a representative collection of strains of *Bact. coli*, including Kauffmann's type strains, were submitted to a critical examination.

There is also no firm evidence on which to base the claim that these various antigens of *Bact. coli* are most appropriately called K antigens collectively (Kauffmann & Vahlne, 1945). From the review given in the preceding section it is obvious that all these differently labelled antigens have the general characters of the Vi antigen of *Salm. typhi* and have been identified by methods developed in the study of this antigen. The demonstration of a capsule or envelope was not considered necessary, or practicable, or even possible. The criteria of K antigens, as

formulated by Kauffmann & Vahlne (1945, p. 123) (see section 2 of this paper, p. 571), are exactly the same as those of the classical Vi antigen of *Salm. typhi*.

Kauffmann's latest innovation in *Salmonella* serology, namely, the suggestion that the various *Salmonella* Vi antigens be included in a 'new' class of K antigens embracing all Bacteriaceae (Kauffmann, 1951, p. 40), is little less than grotesque. It is all the more unfortunate therefore that it has received the endorsement of the Enterobacteriaceae Sub-Committee of the Nomenclature Committee of the International Association of Microbiologists (1952). So far, the 'new' class of K antigens comprises the following three *Salmonella* antigens: V antigen (the former O-factor V), Vi antigen and M antigen.

The position of the V antigen ($V_1$ and $V_2$) has been discussed in a preceding paper (Felix, 1952*c*) and requires no further comment.

'Vi antigen', according to Kauffmann, denotes solely the particular labile somatic antigen possessed by *Salm. typhi*, *Salm. paratyphi* C, *Salm. ballerup* and *Salm. coli* 1 (*Bact. coli* 5396/38). The existence of further special Vi antigens in *Salm. paratyphi* A and *Salm. paratyphi* B is not recognized by Kauffmann. For reasons that are difficult to appreciate Kauffmann, for the past fifteen years, has persistently adhered to the view that the typhoid Vi antigen need not be regarded as the first recognized member of a class of labile somatic antigens but merely as one definite, serologically defined, substance. When the first 'L' antigen of *Bact. coli* was identified it was immediately proclaimed the representative of a 'new' class of antigens, in spite of the fact that its characters were strikingly similar to those of the Vi antigen of *Salm. typhi*. The same process was repeated with the 'A' and 'B' antigens of *Bact. coli*. By this method the Vi antigen of *Salm. typhi*, which is the classical labile somatic antigen of one of the Bacteriaceae, was relegated to a minor position in a class of antigens of which, in reality, it is the prototype. The review given in the preceding section makes it abundantly clear that the whole of the modern work on labile somatic antigens of Bacteriaceae, with the exception of that on the $\alpha$ antigen of Stamp & Stone (1944), sprang in a sense from the typhoid Vi antigen.

The proposed third member of the new class of *Salmonella* K antigens is the one that is being given the most remarkable promotion from its present inferior position. The M (mucoid) antigen, which has remained in a state of obscurity for the past fifteen years, is thus elevated to a status out of all proportion to its comparative unimportance. It is well known that the production of mucus by *Salmonella* bacteria is favoured by growth at 20° C. instead of at 37° C., and that this substance plays no role in the pathogenic and immunogenic activities of the organisms. Bruce White (1929) very aptly stated that 'in mucoid growth of *Salmonella* the bacilli are not strictly capsulate but merely suspended in a communal mass of secreted slime' and that 'this growth habit is not in any sense homologous with encapsulation as seen in the pneumococcus' (Bruce White, 1929, pp. 90–1). The force of this argument has not in the least been diminished by the recent observation by Perch (1950), prominently re-stated by Kauffmann (1951), that capsular swelling can be demonstrated in *Salm. paratyphi* B cultures 'grown

in broth for 8 hr. at about 20° C.' It is also known that the mucus has a very weak and uncertain antigenic effect in rabbits (Josephsen & Henriksen, 1951). The M antigen is common to all *Salmonella* species (Kauffmann, 1936*a*; Josephsen & Henriksen, 1951) and has its counterpart in another, serologically partly related, M antigen which is common to all mucoid strains of *Bact. coli* (Henriksen, 1949*a–c*; Josephsen & Henriksen, 1951). Nothing, therefore, could be more superficial than the suggestion that a substance such as the M antigen be grouped in a class together with the Vi antigens.

The objection may be raised that the M antigen, though unimportant in the pathology and serology of *Salmonella* infections, is nevertheless essential to the ecology of the organisms and deserves special consideration. It is probable that the mucus is secreted in order to protect the bacteria against desiccation and thus assists their survival in nature, as does the mucinous substance described in the so-called rugose cultures of *Vibrio cholerae* (Bruce White, 1940). But the suggestion that a substance of this kind be classified together with the *Salmonella* Vi antigens is ill-conceived and is based on a purely mechanistic approach to the problem.

A glance at Tables 5 and 6, which are reprinted from Kauffmann's publications, shows that the M antigen is listed as behaving very much like some of the labile somatic antigens (L, Vi, $V_1$ and $V_2$). The misleading effect of these tables has been emphasized in a previous paper (Felix, 1952*a*), and it has been shown that the tables contain inadequate information. For instance, under the heading Vi, which in Kauffmann's tables denotes the typhoid Vi antigen, four of the fifteen attributes listed are wrong, namely, the three '–' (minus) indicating inactivation by N-HCl and one '–' indicating destruction of Vi-agglutinability by treatment with alcohol. However, through endless repetition these tables, and the wrong information they contain, have come to be accepted by many workers.

If Kauffmann's suggestion were adopted *Salm. paratyphi* B would be endowed with two different K antigens, the M and the V (no. five). By analogy with the *Klebsiella* group Kauffmann would presumably assign the M antigen to a mucoid envelope and the V antigen (K) to a capsule, in addition to the O and R antigens in the soma. A mucoid strain of *Salm. paratyphi* B would then be given the same formula as one of the *Klebsiella* organisms, namely, MKO form. It is surprising therefore to find in the same monograph (Kauffmann, 1951) that the V antigen is still listed as one of the somatic antigens in the Kauffmann-White schema.

Much of this and the preceding papers (Felix, 1952*a–c*) has been critical because Kauffmann's general approach to the problem, some of his technical procedures, and his attitude to the work of other investigators call for criticism. The exaggerated claim for the dominant place of serology in the classification of Bacteriaceae has been criticized by other workers (Borman, Stuart & Wheeler, 1944; Wilson & Miles, 1946). Nevertheless, the hope 'that in the future it will be possible to set up one antigenic schema covering all enteric bacteria' has not been given up by Kauffmann (1949*a*, p. 404). This idea, if carried to the extreme, might well discredit the earlier achievements of qualitative receptor analysis, when the

method was so successfully applied to the *Salmonella* group by Bruce White (1926) and later by Kauffmann.

It is beyond the scope of this paper to make comprehensive recommendations on the serological classification of Bacteriaceae. A few further comments, however may not be out of place.

First, on the negative side. It would be unfortunate if the detailed description in this series of papers of the Vi antigens of *Salm. paratyphi* A and *Salm. paratyphi* B had the effect of stimulating an attempt to demonstrate Vi antigens in all, or in most of the known *Salmonella* types or subtypes. In a few types of special importance as pathogens of man or animals such attempts are certainly called for. For instance, *Salm. enteritidis* var. *chaco*, which causes continuous fever in man, gave in preliminary experiments by Hayes & Freeman (1945) suggestive results and deserves further investigation. But the wholesale re-examination of all known *Salmonella* types, numbering well over 200, already contemplated by Kauffmann (1951, p. 47), would obviously be a futile undertaking.

So far, the question of nomenclature has caused no difficulty. The symbols TVi, AVi and BVi have long been applied to the three antigens, the corresponding antibodies and the related bacteriophages. These symbols are intelligible to the laboratory worker and to the clinician and epidemiologist. The few additional *Salmonella* Vi antigens which it may be necessary or profitable to recognize in the future may also be designated in this simple fashion.

In the antigenic analysis of strains of *Bact. coli* suspected of being the cause of infections in man or animals, it is also unnecessary to employ a battery of hundreds of typing sera. To answer the question whether the suspected strains are antigenically homogeneous, what is needed is a reliable method of separating their labile somatic antigens from the stable O antigens. According to Kauffmann (1951) the two serological types of *Bact. coli* isolated from cases of infantile enteritis (Bray, 1945; Giles & Sangster, 1948; Giles, Sangster & Smith, 1949; Smith, 1949; Taylor, Powell & Wright, 1949) possess the so-called 'B' variety of labile antigen, and it has so far not been possible to obtain these 'B' agglutinins in pure form. Since the resistance of the 'B' antigen to heat and to dilute HCl seems to be very similar to that of the TVi antigen it is probable that absorption with alkali-treated organisms may help to solve the difficulty. Treatment with dilute NaOH of the very large quantities of bacterial growth required for absorption of high-titre O sera is a tedious process. Nevertheless, it appears to be the technique generally applicable in Bacteriaceae when the simpler methods fail to accomplish separation of the Vi from the O antigens.

With regard to the M antigen the main point of interest seems to be the necessity of avoiding this substance in routine diagnostic work. That is to say, cultures that have stood and grown for some time at room temperature should not be employed as antigens in agglutination tests or in the preparation of diagnostic sera. Since the titre of M antibody is very low the danger of confusing results caused by this antibody is greatest in slide agglutination. This is an additional reason for refusing to place too much reliance on the slide test.

SUMMARY

1. Four different Bacteriaceae possessing the same Vi antigen showed, after exposure to heat, striking differences in the physico-chemical behaviour of the Vi antigen. The most noticeable differences observed were those in the changes in Vi-agglutinability and O-inagglutinability of the bacteria; lesser differences were noted in the agglutinogenic activity of the Vi antigen, and none in its agglutinin-binding capacity.

2. Treatment with alcohol altered the TVi antigen of one of the species (*Bact. coli* 5396/38) in a way different from that seen in the other three species.

3. On the other hand, dilute acid or alkali produced the same chemical changes in the TVi antigen of all four Bacteriaceae.

4. The TVi antigen present in the four Bacteriaceae appears to be one and the same substance; it cannot be differentiated by the customary serological methods. Its different physico-chemical state after exposure to heat or alcohol is, therefore, conditioned by other constituents of the bacterial cell, which may, or may not, be antigenic.

5. The simultaneous O- and Vi-inagglutinability resulting from heating at 75° C. is particularly impressive since it does not appear to be specially related to any one of the known antigenic components.

6. These findings invalidate the basis on which the L, A and B antigens of *Bact. coli* have been differentiated.

7. There is also no valid reason for designating the labile somatic antigens of *Salmonella* and other Bacteriaceae as K antigens. These antigens have the general characters of the Vi antigen of *Salm. typhi*, are demonstrated by methods developed in the study of the typhoid Vi antigen, and are not associated with typical capsules.

8. The M (mucoid) antigens of *Salmonella* and of *Bact. coli* are in many respects different from the Vi antigens and should be classified separately.

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