ON THE VARIATION OF THE SPECIFIC PHASE OF *SALMONELLA AMERSFOORT* N.SP.

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Bruce White (1929) described three forms of antigenic variation occurring in the genus *Salmonella*: (1) the "H" form—"O" form variation of Weil & Felix (1920), (2) the Smooth form—Rough form variation of Arkwright (1921), and (3) the specific phase—non-specific phase variation of Andrewes (1922). Later Kauffmann & Mitsui (1930) described a form of dissociation of the specific phase itself which they regarded as different from the phenomenon of Andrewes (1922); they called the variations of the specific phase α and β phases. Recently Bernard (1935), Tesdal (1936) and Kauffmann (1936 *a*) studied analogous variations. Bernard found that the "O" antigen of his *Salmonella* coincided with factor IV of the Kauffmann-White scheme, while the "H" antigen, which occurs only in the specific phase, contained factors **e n** plus another new component. The organism (*S. hvittingfoss*) of Tesdal is described as having a new "O" antigen and an "H" antigen, present only in the specific phase, composed of factors **b** and **e n**. The bacterium (*S. bispebjerg*) studied by Kauffmann was found to possess the following antigenic components: "O" antigen IV, XII and "H" antigen **a**, **e n**. Moreover, Kauffmann (1936 *b*) has found that the specific "H" antigen of *S. typhi* is also capable of dissociating into two variants, factors **d** and **j**, but he was not able to demonstrate a similar variation in the specific phase (factor **d**) of *S. stanley*.

The inagglutinable (containing Vi antigen) and agglutinable forms of *S. typhi* described by Felix and his co-workers (1934, 1935, 1936) may be regarded as another type of variation. Kauffmann (1935) introduced the terms "V-form" and "W-form" to denote, respectively, the variant containing Vi-antigen and that devoid of it. Craigie & Brandon (1936), Brown (1936), Scholtens (1936) and others showed the effect of bacteriophage on the V → W degradation.

While making an antigenic analysis of a new *Salmonella* obtained from a fowl the author noticed well-marked flocculation occurring between this organism and the sera of organisms that are generally regarded to be not even remotely related antigenically. It is the study of the antigenic structure of this organism that forms the basis of the discussion below.

About a year ago a farmer at Amersfoort in the Transvaal sustained serious losses amongst his chickens from what appeared to be an infectious

disease. The disease was not investigated and the cause of the mortality remained unknown until a few months ago when the malady reappeared and a few affected birds were sent to Onderstepoort for examination. An apparently pure culture, obtained by Mr J. D. W. A. Coles, head of the Poultry Diseases Section, from the heart blood of a 7-day-old chick, was handed over to the author for identification. The culture was plated and a few isolated colonies were picked. The cultures obtained from these were now tested against various agglutinating sera. It was found that the antigenic structure of the organisms exhibited an entirely new combination of antigenic components, and that it should, therefore, be admitted to species rank in compliance with the recommendations of the Salmonella Subcommittee of the Nomenclature Committee of the International Society of Microbiology (1934). The name *Salmonella amersfoort* is proposed for the organisms—after the place of its origin.

Morphology and cultural characters. Morphologically, *S. amersfoort* resembles a typical *Salmonella*, and, like it, grows readily on ordinary laboratory media. It is Gram-negative and is actively motile. Saline and thermo-agglutination tests as well as the shape of individual colonies show that it is smooth.

Biochemical characters. S. amersfoort forms acid and gas in glucose, dulcite, mannite, maltose, arabinose, rhamnose, and sorbite; it forms hydrogen sulphide and renders litmus milk alkaline; it does not produce indol.

Pathogenicity. S. amersfoort is pathogenic for chickens and mice; 0·25 c.c. of a 24-hour-old broth culture, given intraperitoneally, kills a 6-week-old chicken in 4 days and 0·05 c.c. kills an adult mouse in 36 hours. *S. amersfoort* was recovered from the heart blood and spleen in each case. But chickens dosed with 1 c.c. of the virulent broth culture remained apparently healthy.

Serology. For the study of the antigenic structure of *S. amersfoort* "O" sera, "H" specific and non-specific sera, and mixed "O" and "H" sera, prepared against a number of representative strains of *Salmonella*, were used. Sera prepared against *S. amersfoort* were also used. The sera were prepared according to the method described by Henning (1936) and the agglutinating suspensions by Lovell's (1932) technique. For absorption tests the germ was grown on Mason tubes (Mason, 1933), the growth from one tube being more than sufficient for the complete absorption of about 3 c.c. of 1 : 50 dilution of serum. By using Mason tubes instead of Petri dishes aerial contamination was completely avoided. All "H" suspensions were killed by addition of formalin to a concentration of 0·25 per cent and heating at 57° C. for 2 hours.

The tubes containing the serum dilutions plus the antigen were placed in the water-bath at 55° C. and read after 2 hours, and again after 18 hours. In "H" agglutination the two readings usually corresponded but in "O" agglutination the second reading generally gave a much higher titre.

Preliminary tests showed that *amersfoort* gave a well-marked fine granular agglutination with "O" sera containing factors VI and VII of the Kauffmann-

White schema (*cholerae-suis*, *newport*, *potsdam* and others) while a distinctly coarse floccular agglutination was produced not only with "H" sera containing factors **en** or **e** (*abortus equi*, *brandenburg*, *potsdam*, *dar-es-salaam* or *onderstepoort*, *newport*, *reading* or *anatum*) but also with those containing factor **d** (*stanley*, *muenchen* and *typhi*). However, a much stronger agglutination was produced by sera containing factors **en** than with those containing factor **e** but not **n**.

The culture was again plated on Mason tubes to obtain a number of separate colonies for independent study. After 5 hours' incubation at 37° C. broth cultures of these colonies were tested against *Kunzendorf* and *Binns* group sera as well as against the type sera of *onderstepoort*, *newport*, *potsdam* and *typhi*. The results are given in Table I. It will be noticed that the majority of the cultures agglutinated with *typhi* serum (factor **d**), a number agglutinated with *potsdam* (factors **enlv**), *newport* (factors **eh**) and *onderstepoort* (factors **eh**) sera, a few agglutinated incompletely with all four sera, being apparently intermediate forms, but no agglutination whatsoever was effected with *Kunzendorf* and *Binns* sera.

Table I. *Thirty colonies grown in broth for* 5 *hours and tested against* 5 *different sera*

No. of colony	*Typhi* s.	*Newport* or *Onderstepoort* s.	*Potsdam* s.	*Kunzendorf* s.	*Binns* s.
1–16	+ + + +	0	0	0	0
17–25	0	+ + + +	+ + + +	0	0
26–30	+	+	+	0	0

+ + + + = complete flocculation within 30 min.
+ = partial flocculation after 1 hour.
0 = no flocculation after 18 hours.
In headings to table s. = serum.

These results indicated (1) that the organism occurred only in the type phase and (2) that the culture used was either a mixed one or that it exhibited properties that have hitherto not been described in a member of the *Salmonella* group. In order to settle the matter of the purity of the strain Dr J. H. Mason kindly single-celled fresh cultures derived from a colony of each of the two types—i.e. from one colony agglutinating only with sera made against specific factor **d** and from another that flocculated solely with the anti-sera of specific factors **en** and **eh**. After plating the primary cultures obtained from the single cells a number of well-isolated colonies were again picked into broth tubes and incubated at 37° C. for 5 hours—in order to reduce the lag phase in the growth the broth tubes were placed in a water-bath at 40° C. for 10 min. before transferring them to the incubator.

Four single cells (*a b c* and *d*) obtained from colony 1, Table III, were now cultivated separately in broth and plated. A number of colonies from each plate were picked into broth, incubated and tested against both **d** and **en** sera. The results are given in Table IV.

Table II. *Twenty-two colonies picked from the plate seeded with growth from the single cell obtained from colony* 1, *Table I*

No. of colony	*Typhi* serum	*Onderstepoort* or *potsdam* serum
1 to 21	+ + + +	0
22	0	+ + + +

Table III. *Thirty colonies picked from the plate seeded with the broth culture from single cell of colony* 17, *Table I*

No. of colony	*Typhi* serum	*Onderstepoort* or *potsdam* serum	Saline control
1 to 28	0	+ + + +	0
29 and 30	+ + + +	0	0

Table IV

Single cell	No. of colony	*Typhi* serum	*Onderstepoort* or *potsdam* serum
a	1 to 4	+ + + +	0
a	5 to 12	0	+ + + +
b	1 to 14	0	+ + + +
b	15	+ + + +	0
c	1 to 14	+ + + +	0
c	15	0	+ + + +
d	1 to 10	+ + + +	0

+ + + + = complete flocculation after 30 min.
0 = no flocculation after 18 hours.

Therefore, these results clearly show that *S. amersfoort* is composed of two distinct "H" antigenic complexes, both of which occur in the specific phase; the second (**d**-) antigen, apparently corresponding to the β phase of Kauffmann & Mitsui (1930), is agglutinated with the "H" serum of *typhi* and, as will be shown below, also with specific sera of other Salmonellas, *stanley* and *muenchen*, containing specific factor **d**, while the other component, the first (**en**-) antigen, apparently corresponding to the α phase of Kauffmann & Mitsui (1930), is agglutinated solely with *potsdam*, *onderstepoort*, and other sera containing agglutinins for the type factors **en** and **eh** (*vide infra*). Sera containing agglutinins for factors **en** always give a much stronger flocculation than the anti-sera of factors **eh**. It has also been shown that single cells composed of either the one or other complex constantly give rise to daughter cells some of which resemble the parent cell antigenically, while others have adopted a new antigenic structure entirely different from that present in the parent. The latter daughter cells again give rise to offspring some of which resemble themselves, while others are like their parent. These mutations constantly proceed and cells containing either the one or other antigenic complex continually produce cells of both types, and neither the one nor the other type of cell has been found to breed entirely true.

On single-celling the growth obtained from each of the two types of colonies serially for three successive times, both variants constantly appeared in the cultures arising from the single cells.

The purity of the culture is therefore beyond dispute; it is the property of the bacterium of giving rise to two distinct types of variants in the specific phase that is responsible for the uncommon behaviour of the culture. The organism apparently does not occur in the non-specific phase.

"O" agglutination. Cross-agglutination tests were carried out with the heat-stable "O" antigens and "O" sera of the different *Salmonella* types of the Kauffmann-White schema; also with *S. aberdeen* (Smith, 1934), *S. poonae* (Bridges & Scott, 1935) and *S. onderstepoort* (Henning, 1936). The reactions obtained are given in Table V.

Table V. *"O" agglutination*

	"O" antigen				
	Amers-foort	*Potsdam*	*Muenchen*	*Onderste-poort*	*Branden-burg*
Unabsorbed sera:					
Amersfoort s.	800	800	200	100	0
Potsdam s.	800	800	—	—	—
Muenchen s.	200	—	1600	—	—
Brandenburg s.	0	—	—	—	1600
Onderstepoort s.	50	—	—	800	—
Absorbed sera:					
Amersfoort s.a.b. *amersf.*	0	0	—	—	—
Amersfoort s.a.b. *potsdam*	0	0	—	—	—
Amersfoort s.a.b. *muenchen*	200	—	0	—	—
Amersfoort s.a.b. *brandenburg*	800	—	—	—	0
Potsdam s.a.b. *potsdam*	0	0	—	—	—
Potsdam s.a.b. *amersf.*	0	0	—	—	—
Muenchen s.a.b. *amersf.*	0	—	800	—	

0 = less than 1 : 50. — = not tested

In this table s. = serum, s.a.b. = serum absorbed by.

The results show that *amersfoort* "O" as well as *potsdam* "O" sera are completely exhausted for the homologous "O" antigen by *amersfoort.* In the same way both sera are exhausted by *potsdam.* The somatic "O" antigen of *amersfoort* must, therefore, be regarded as identical with that of *potsdam*, i.e. it is composed of factors VI, VII.

"H" agglutination. Flocculation, approximately equivalent in titre to that produced with the homologous antigen, was obtained with the specific sera of *abortus equi*, *potsdam*, *brandenburg*, *dar-es-salaam*, *stanley*, *muenchen* and *typhi*, but a much weaker agglutination resulted when the type sera of *onderstepoort*, *newport*, *reading* or *anatum* were used for the test. In the same way *amersfoort* "H" serum agglutinated the specific antigens of *abortus equi*, *potsdam*, *brandenburg*, *dar-es-salaam*, *stanley*, *muenchen* and *typhi* almost up to full titre, while its titre for type antigens containing factors **eh** was much lower.

On absorbing *amersfoort* "H" serum with the specific phase of either *potsdam* (factors **enlv**), *brandenburg* (factors **enlv**) or *dar-es-salaam* (factors **enlw**) the titre of the serum for one of the homologous specific antigens (**en**-), α phase, was reduced from 6400 to approximately 800, while the titre for the

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Table VI

	"H"-specific antigen														
	Amers-foort **d**	*Amers-foort* **en**	*Typhi*	*Stanley*	*Muen-chen*	*Potsdam*	*Branden-burg*	*Dar-es-Salaam*	*Abortus-equi*	*Onder-stepoort*	*Newport*	*Reading*	*Anatum*	*Panama*	*London*
Unabsorbed sera:															
Amersfoort **d** s.	12,800	6400	12,800	12,800	12,800	1600	3,200	3200	3200	200	—	—	—	0	0
Amersfoort **en** s.	6,400	6400	6,400	6,400	6,400	3200	6,400	3200	6400	400	400	400	400	0	0
Typhi s.	12,800	0	12,800	12,800	12,800	—	—	—	—	—	—	—	—	—	—
Stanley type s.	25,600	400	25,600	25,600	25,600	—	—	—	—	—	—	—	—	—	—
Muenchen type s.	6,400	0	6,400	6,400	12,800	—	—	—	—	—	—	—	—	—	—
Potsdam s.	0	6400	—	—	—	6400	6,400	6400	3200	800	—	—	—	6400	1600
Brandenburg s.	0	6400	—	—	—	6400	12,800	3200	3200	400	—	—	—	6400	3200
Dar-es-salaam s.	0	1600	—	—	—	—	—	3200	—	—	—	—	—	1600	—
Onderstepoort s.	0	400	—	—	—	200	—	200	200	1600	—	—	—	—	—
Abortus-equi s.	—	6400	—	—	—	1600	3,200	6400	3200	—	—	—	—	—	—
Absorbed serum:															
Amersfoort **d** s.a.b. *amersf.*	0	0	0	0	0	0	—	—	0	—	—	—	—	—	—
Amersfoort **d** s.a.b. *typhi*	400	6400	0	—	—	—	—	—	—	—	—	—	—	—	—
Amersfoort **d** s.a.b. *stanley*	400	3200	—	0	—	—	—	—	—	—	—	—	—	—	—
Amersfoort **d** s.a.b. *muenchen*	400	3200	—	0	0	—	3,200	3200	—	—	—	—	—	—	—
Amersfoort **en** s.a.b. *potsdam*	6,400	800*	—	—	—	0	—	0	800	—	—	—	—	—	—
Amersfoort **en** s.a.b. *brandenburg*	6,400	800*	6,400	—	—	—	0	—	—	—	—	—	—	—	—

Amersfoort **en** s.a.b. *dar-es-salaam*	6,400	1600*	—	6,400	—	—	—	0	—	—	—	—	—	—
Amersfoort **en** s.a.b. *abortus equi*	6,400	200	—	—	—	—	0	—	0	—	—	—	—	—
Typhi s.a.b. *amersf.*	0	0	800	—	—	—	—	—	—	—	—	—	—	—
Typhi s.a.b. *typhi*	0	0	0	—	—	—	—	—	—	—	—	—	—	—
Stanley s.a.b. *amersf.*	0	0	—	400	400	—	—	—	—	—	—	—	—	—
Stanley s.a.b. *stanley*	0	0	—	0	—	—	—	—	—	—	—	—	—	—
Muenchen s.a.b. *amersf.*	0	0	—	0	100	—	—	—	—	—	—	—	—	—
Potsdam s.a.b. *amersf.*	—	0	—	—	—	6400*	6,400	6400	0	—	—	—	6400	3200
Brandenburg s.a.b. *amersf.*	—	0	—	—	—	—	6,400	—	—	—	—	—	6400	3200
Dar-es-salaam s.a.b. *amersf.*	—	0	—	—	—	800	—	1600	—	—	—	—	1600	800
Abortus equi s.a.b. *amersf.*	0	0	—	—	—	0	0	—	0	—	—	—	—	—
Potsdam s.a.b. *amersf.*,* then by *panama*	—	—	—	—	—	100	100	—	—	—	—	—	0	0
Brandenburg s.a.b. *amersf.*, then by *london*	—	—	—	—	—	—	200	—	—	—	—	—	0	0
Abortus equi s.a.b. *potsdam*	—	400	—	—	—	—	0	—	200	—	—	—	—	—

0 = less than 1 : 50. * = partial flocculation, fluid remaining turbid. In this table s. = serum, s.a.b. = serum absorbed by.
— = not tested

other homologous specific antigen (**d**-), β phase, as well as for *stanley*, *muenchen* and *typhi* (factor **d**) remained unaltered. When *abortus equi* (factors **enx**) was used for the absorption the reduction in titre for the homologous **en** antigen (α phase) was almost complete, but still no noticeable decrease in agglutinins, for the homologous **d** antigen (β phase) was effected; a small residue, however, remained which caused an incomplete agglutination with the **en**-variant (α phase) of *amersfoort*. The cause of this flocculation is discussed below.

On the other hand, when *amersfoort* "H" serum was absorbed with either *stanley*, *muenchen* or *typhi* (factor **d**) most of the agglutinins for the second variant (**d**-), β phase, of *amersfoort* were removed, while the titre for the other homologous antigen (**en**-), α phase, remained unaltered (Table VI).

When either *potsdam* or *brandenburg* serum was absorbed with *amersfoort* all agglutinins for *amersfoort* were removed, but the titre of the serum for *panama* (factors **lv**) and *london* (factors **lv**) was not affected. Moreover, the treated serum still agglutinated the homologous antigen although the flocculation was incomplete and the fluid remained turbid, due, no doubt, to the persistence of **lv** agglutinins in the serum. *Dar-es-salaam* serum behaved in practically the same way, but *amersfoort* is apparently capable of removing all the agglutinins for the homologous antigen from *abortus-equi* serum.

On absorbing either *stanley*, *muenchen* or *typhi* serum with *amersfoort* most of the agglutinins for the homologous "H" specific antigen were exhausted, *muenchen* serum being exhausted much more completely than either *stanley* or *typhi* serum, while all the agglutinins for the second variant of amersfoort (factor **d**-) were removed.

Discussion

These results show that *Salmonella amersfoort* contains two distinct antigenic complexes, the one, α phase, corresponding to factors **en** of *abortus-equi*, *potsdam*, *brandenburg* and *dar-es-salaam* plus an additional factor, part of which apparently corresponds to factor **x** of *abortus-equi*; the other complex, β phase, coincides largely with factor **d** of *stanley*, *muenchen* and *typhi*. The additional factor is probably responsible for the residue of agglutinins left for the first (**en**-) antigen, α phase, after absorbing *amersfoort* serum with *potsdam*, *brandenburg* or *dar-es-salaam*; but, although factor **x** of *abortus-equi* apparently forms a part of this additional factor, there may be another component which is not present in *abortus-equi*. The fact that *amersfoort* exhausts all agglutinins from *abortus-equi* serum for itself as well as for the homologous specific antigen indicates that *amersfoort* contains all the specific antigenic components of *abortus-equi* i.e. factors **enx**; but since *abortus-equi* fails to exhaust *amersfoort* serum completely for the homologous first (**en**-) antigen it is possible that this antigen of *amersfoort* contains a minor factor in addition to the **enx** of *abortus-equi*.

After absorbing *amersfoort* serum with either *stanley*, *muenchen* or *typhi* a small residue is left which still agglutinates the homologous second (**d**-) antigen,

β phase, but not the specific antigen (factor **d**) of either *stanley*, *muenchen* or *typhi*. It is not quite clear to what this residue can be ascribed; whether it should be regarded as an extra factor in the second (**d**-) antigen, β phase, in addition to factor **d** of *stanley*, *muenchen* and *typhi*, or whether it can be attributed to a trace of the first (**en**-) antigen, α phase, present in the emulsion of the second (**d**-) antigen, β phase, of *amersfoort* used for the test is not certain. If the latter explanation holds it is likely that the agglutination occurring in *amersfoort* serum absorbed with *abortus-equi* is likewise due to an overflow of the second (**d**-) antigen, β phase, in the emulsion of the first (**en**-) antigen, α phase, of *amersfoort*.

Neither *abortus-equi*, *potsdam*, *brandenburg*, nor *dar-es-salaam* effected any reduction in the titre of *amersfoort* serum for the homologous second (**d**-) antigenic complex, β phase, or for the type phases of *stanley*, *muenchen* and *typhi*. In the same way neither *stanley*, *muenchen* nor *typhi* absorbed an appreciable amount of agglutinins from *amersfoort* serum for the homologous first (**en**-) antigen, α phase, or for *abortus-equi*, *potsdam*, *brandenburg* and *dar-es-salaam*.

When *potsdam* serum was absorbed by *amersfoort* all agglutinins for both *amersfoort* and *abortus-equi* were completely exhausted, but flocculation to nearly full titre was still effected with the specific phases of *potsdam*, *brandenburg*, *panama*, and *london*. On reabsorbing the partly absorbed *potsdam* serum with *panama* (factors **lv**) no appreciable agglutination resulted when specific antigens of *potsdam*, *brandenburg*, *panama* and *london* were used. *Amersfoort*, therefore, removed only the agglutinins of factors **en** from the *potsdam* serum, leaving the agglutinins of factors **lv** to be absorbed by *panama*.

The fact that *amersfoort* almost completely exhausted *muenchen* serum for the homologous specific antigen suggests that the second (**d**-) factor, β phase, is similar to the specific phase (factor **d**) of *muenchen*; the small residues of agglutinins left in *stanley* and *typhi* sera for their homologous specific antigens after absorption with *amersfoort* cannot be explained at present.

SUMMARY AND CONCLUSIONS

A new type of pathogenic *Salmonella* for the fowl has been described. Its somatic "O" antigen corresponds with factors VI, VII of *potsdam*. It occurs only in the specific phase, but its flagellar "H" antigen contains at least two distinct and separate antigenic complexes, which commonly occur in organisms that are not even remotely related. The one complex (the first, **en**-, antigen, α phase of Kauffmann and Mitsui) contains factors **enx**, which represent also the factors of the specific phase of *abortus-equi*. The other complex (the second, **d**-, antigen, β phase of Kauffmann and Mitsui) contains factor **d**, which comprises the type phase of *stanley*, *muenchen* and *typhi*.

Single cells containing factors **enx** on multiplying constantly yield variants containing factor **d** as well as offspring that retain antigenic complex **enx**. In the same way single cells containing apparently only specific factor **d** will bring

forth new cells, most of which retain the parental antigenic structure, but a small proportion of the progeny will acquire specific factors **enx** instead of **d**.

When a broth culture of *amersfoort* in either the **enx** or **d** phase in an apparently pure form, as judged by the agglutination test, using heterologous sera which contain agglutinins either against factors **enx** (or **en**) or **d**, is used for the preparation of sera, agglutinins of approximately the same titre for both variants are produced in the sera.

As a result of the information given above the following antigenic structure is proposed for *Salmonella amersfoort*:

Somatic "O" antigen—VI, VII.

Flagellar "H" antigen—

(1) α phase of Kauffmann and Mitsui—**enx**.

(2) β phase of Kauffmann and Mitsui—**d**.

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