# THE DEMONSTRATION OF NON-SPECIFIC COMPONENTS IN SALMONELLA PARATYPHI A BY INDUCED VARIATION[1]

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Phases induced by growth in immune serum have been demonstrated in a number of enteric bacilli. Kauffmann (1936) found a second phase in *Eberthella typhosa* after the organism was cultivated in agglutinating serum derived from the Muenchen type. While Kauffmann found this variant to be irreversible, Edwards and Bruner (1939) reverted similar forms to the original phase by cultivation in serum derived from the variants. Induced phases were described by Kauffmann and Tesdal (1937) in the Schleissheim type and by Gard (1938) in *Salmonella abortus-canis*. Both the latter types are monophasic under ordinary conditions of culture. Gnosspelius (1939) induced variants with altered flagellar antigens in the naturally diphasic types Stanley and Hvittingfoss. These forms also were irreversible even when cultivated in homologous immune serums. It is significant that all the phases described above were "artificial" phases; that is, they possessed no antigenic relationships to the naturally occurring antigens of the genus *Salmonella*.

The cultivation of *Salmonella* strains in immune serum does not always lead to the isolation of artificial phases, as witness the results obtained in the study of the "totally and permanently non-specific" *Salmonella* types. Thus, through cultivation in immune serum, Scott (1926) isolated a specific phase from a non-specific culture of the Thompson type and Gard isolated specific

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phases identical with that of *Salmonella cholerae-suis* from cultures of *S. cholerae-suis* var. *kunzendorf*. Bruner and Edwards (1939) isolated specific phases from *Salmonella typhi-murium* var. *binns*, *Salmonella typhi-suis* var. *voldagsen*, *S. cholerae-suis* var. *kunzendorf* and the Berlin and Puerto Rico types by cultivation in immune serums. In each instance the specific phases so isolated were identical with those that occur naturally in the diphasic counterparts of the variants. Edwards and Bruner (1939a) working with the naturally monophasic *Salmonella abortus-equi* isolated two additional phases by cultivation of the organism in various immune serums. One of these was an "artificial" phase closely related to the induced phase of the Schleissheim type described by Kauffman and Tesdal. The second induced phase, on the contrary, was the antigen a of the Kauffmann-White classification and was identical with the flocculating antigen of *Salmonella paratyphi A*.

The purpose of the present work is to describe the isolation of phases closely related to the naturally occurring non-specific phases of the genus *Salmonella* from *S. paratyphi A*. It is realized that the serology of the Salmonellas is already sufficiently complicated and that work such as that presented here may seem on first thought only to confuse the classification of the bacilli. However, the demonstration of inapparent components of the bacilli adds to our knowledge of the genetic relationships and hereditary tendencies in the genus.

## MATERIALS AND METHODS

Sixteen cultures of *S. paratyphi A* were used in the study. The sources and designations of the strains were as follows:

1015, Marta, HA1, HA6, Durazzo—From Dr. F. Kauffmann, International Salmonella Center, Copenhagen.

38250, 37407—From Dr. Ruth Gilbert, New York State Department of Health.

Fried, Boyd, 17, W, WB39—From Dr. Ralph Muckenfuss, New York City Department of Health.

228, HR, GV—From Dr. L. F. Rettger, Yale University.

Cal. 49—From Dr. W. R. Hinshaw. Stock culture from Medical School, University of California.

All the cultures possessed the biochemical and serological properties generally attributed to *S. paratyphi A* with the following exceptions: The strain Durazzo lacked antigen I of the Kauffmann-White classification and produced large amounts of hydrogen sulphide. In these respects it conformed to the description of Kauffmann (1937). The remainder of the strains produced smaller amounts of hydrogen sulphide, as evidenced by the blackening of lead acetate papers suspended over cultures in 2 per cent Bacto-peptone water. In this connection it should be stated that the observations of the writers confirm the conclusions of Hunter and Crecelius (1938) that differences observed in hydrogen sulphide production by different members of the genus *Salmonella* are quantitative and not qualitative. It is probable that all *Salmonella* strains produce hydrogen sulphide and that the results obtained in testing for its production depend upon the methods employed for the detection of the substance.

The methods employed to induce variation were largely the same as those used by Edwards and Bruner (1939a) in the study of *S. abortus-equi*. The organisms were grown in semi-solid agar to which was added sufficient agglutinating serum to immobilize the bacilli. The medium was inoculated by stabbing at one side of the tube. Outgrowths from the line of stab occurred only when the flagellar antigens were altered. The spreading growth was transferred successively in tubes of the same medium until the serological reactions indicated that the induced phase was pure. The culture was then plated and isolations made from single colonies for further study. Serum derived from phase 1 of the Bispebjerg type was used to induce variations in the normal phase of the cultures. As additional phases were isolated agglutinating serums were prepared from them. These were used in the study of induced phases and in forcing the bacilli to produce further alterations in the flagellar antigens. Serum derived from *S. cholerae-suis* var. *kunzendorf* was used in the reversion of non-specific phases. In many instances, it was found necessary to use combinations of serums to force variation in the desired direction or to revert induced phases to the original phase. When the serums contained O or H agglutinins which might interfere with

the migration of the organisms, such agglutinins were removed by absorption with appropriate bacilli. The serums were preserved by the addition of small amounts of chloroform. The chloroform effectively sterilized the serums and did not affect the growth or motility of the bacilli.

## RESULTS

It should be emphasized that the changes described below involved only the H or flagellar antigens; that is, the antigens which are affected in the phase variation of normally diphasic types. The heat-stable O antigens remained unchanged throughout the experiments. On the whole the cultures were quite stable and it was more difficult to induce variation in *S. paratyphi A* than in *Eberthella typhosa*, *S. abortus-equi* or the "totally and permanently" non-specific types. In some instances it was necessary to transfer the organisms repeatedly in the presence of agglutinating serum before variation was observed.

From cultures GV and W no induced phases were isolated. Strains 17 and 37407 each yielded two induced phases (phases 3 and 4) neither of which was closely related to any of the naturally occurring antigens of the genus. All the remaining cultures yielded three induced phases. The normal phase of the bacilli (antigen a of the Kauffmann-White classification) was designated as phase 1. The induced antigens were denoted as phases 2, 3 and 4. Phase 2 is closely related to the non-specific phases of the diphasic *Salmonella* types. Phases 3 and 4 are "artificial" phases which closely resemble none of the natural antigens. The relationships of the phases to each other and to the antigens of other species are given in table 1. Only one culture of *S. paratyphi A* is included in the table since the phases isolated from other strains reacted similarly. Phase 2 is agglutinated in high dilution by Kunzendorf and non-specific Sendai serums. Likewise serum derived from phase 2 agglutinates Kunzendorf and the non-specific phase of Sendai to the titre of the serum. Phase 3 is slightly related to the normal phase 1, but has little relationship to the other phases or to the natural antigens of the genus. It is agglutinated in low dilution by all the serum of all types that

contain antigen a. This agglutination probably represents a slight residue of the normal flagellar antigens of the species. Phase 4 shows evidence of a slight serological relationship to phase 3 and to the non-specific phases of other types, otherwise it is unrelated to the known antigens of the *Salmonella*.

The phases were further examined by agglutinin absorption. The results of these tests confirmed the agglutination tests and need not be given in detail. When absorbed serums were used the cross reactions between the four phases were no longer evident.

TABLE 1

*Agglutination tests with phases of S. paratyphi A*

| ANTIGENS | SERUMS | | | | | | |
|---|---|---|---|---|---|---|---|
| | Paratyphi A 228 Phase 1 | Bispebjerg Phase 1 | Paratyphi A 228 Phase 2 | Paratyphi A 228 Phase 3 | Paratyphi A 228 Phase 4 | Sendai Phase 2 | Kunzendorf |
| Paratyphi A 228: | | | | | | | |
| Phase 1 | 20,000 | 40,000 | 0 | 2,000 | 0 | 0 | 0 |
| Phase 2 | 0 | 0 | 40,000 | 0 | 1,000 | 10,000 | 20,000 |
| Phase 3 | 500 | 500 | 0 | 10,000 | 200 | 0 | 0 |
| Phase 4 | 0 | 0 | 1,000 | 0 | 20,000 | 0 | 0 |
| Abortus equi—Phase 1 | 20,000 | 40,000 | 0 | 1,000 | 0 | 0 | 0 |
| Bispebjerg—Phase 1 | 20,000 | 40,000 | 0 | 1,000 | 0 | 0 | 0 |
| Sendai—Phase 2 | 0 | 0 | 40,000 | 0 | 500 | 10,000 | 20,000 |
| Kunzendorf | 0 | 0 | 40,000 | 0 | 500 | 10,000 | 20,000 |

Figures indicate highest dilution at which agglutination occurred.
"0" indicates no agglutination at dilution of 1 to 200.

Furthermore, the absorption of serum derived from one phase by organisms of another did not result in an appreciable reduction of the titre of the serum for the homologous phase. Absorption of serum derived from phase 3 of one strain by phase 3 organisms of a second strain resulted in a complete removal of agglutinins from the serum. This was also true of phase 4. The non-specific components (phase 2) derived from all the cultures were closely related but not identical. This will be discussed below.

While the variants isolated from the various cultures of *S. paratyphi A* were similar, the variational tendencies of the cultures differed. When the organisms were inoculated into semi-solid

agar which contained serum derived from phase 1 of the Bispebjerg type, culture HR yielded phase 2, 228 yielded a mixture of phases 2 and 4, while the remainder of the organisms yielded phase 3 or a mixture of phases 3 and 4. For this reason mixtures of serums were used to force variation in the desired direction. The variations induced in one culture, strain 228, are given in

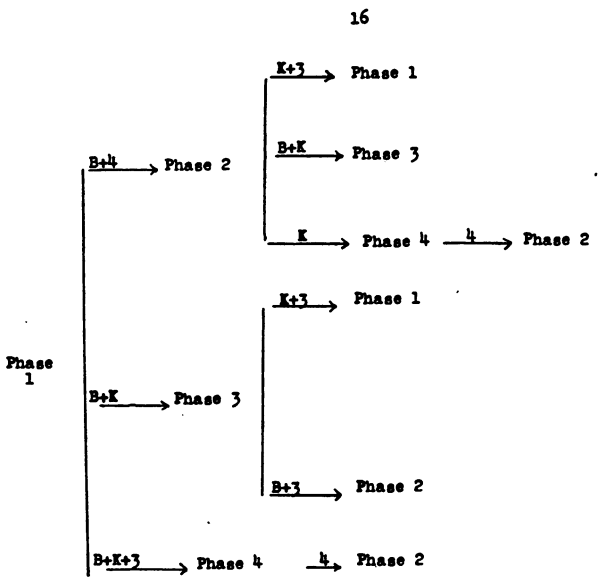


FIG. 1. CHANGES INDUCED IN S. PARATYPHI A 228

Symbols on arrows indicate what serums were added to medium.
B, serum derived from phase 1 of Bispebjerg type.
K, serum derived from *S. cholerae suis* var. *kunzendorf*.
3, serum derived from *S. paratyphi A*, phase 3. The serum was absorbed with phase 1 before use.
4, serum derived from *S. paratyphi A*, phase 4. The serum was absorbed with phase 3 before use.

figure 1. The changes produced in other cultures differed slightly but they were all quite similar.

When once isolated the induced phases of *S. paratyphi A* are quite stable. No changes in the phases were noted during the two years they were maintained in the laboratory. In this respect they resemble the induced phases of *E. typhosa* and *S. abortus-equi* and differ from the normal phases of the diphasic *Salmonella*

types. The latter display phase variation under normal conditions of culture. As shown in figure 1, it is possible to produce changes in the induced phases of *S. paratyphi A* by cultivation in appropriate immune serums. The reversibility of the phases is illustrated in figure 2. Phases 1, 2 and 3 were completely interchangeable and reversible. The changes illustrated in these phases were accomplished repeatedly with cultures isolated from single colonies. Phase 4, on the contrary, was more stable. It was readily converted to phase 2 but not to phase 1 or phase 3. Efforts to convert phase 4 to phase 1 or phase 3 resulted in the

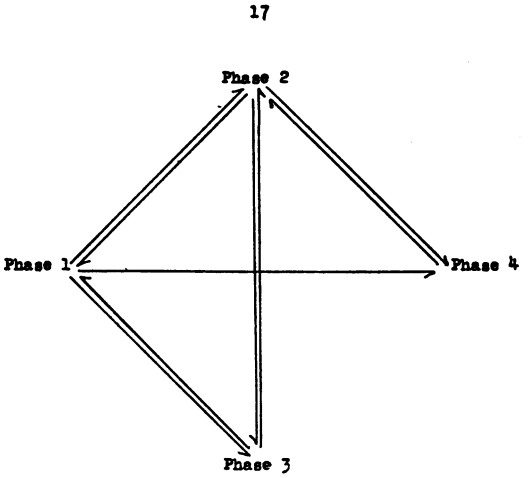


FIG. 2. REVERSIBILITY OF PHASES OF S. PARATYPHI A 228
Arrows indicate direction in which variation occurred

production of a series of ill-defined, serologically-related variants. This observation confirms the work of Gnosspelius (1939), who concluded that through proper manipulation it was possible to isolate an endless number of antigenic components from *Salmonella* strains.

It is known that "artificial" phases that display little or no relationships to the normal antigens of the genus can be isolated from a number of *Salmonella* types by induced variation. The isolation of components closely related to the naturally occurring antigens is much more unusual. The isolation of non-specific

components from a normally monophasic-specific species has not been reported previously. For that reason the characteristics of the non-specific components (phase 2) of *S. paratyphi A* are given in more detail than are the characteristics of the "artificial" phases 3 and 4. The agglutinative relationships between the non-specific components of *S. paratyphi A* and the non-specific phases of diphasic types are given in table 2.

It is apparent that the non-specific phases of *S. paratyphi A* are more closely related to the 1, 5... phases than to the 1, 2..., 1, 6... or 1, 7... phases. The organisms were tested with ab-

TABLE 2

*Agglutination of non-specific phases of S. paratyphi A by non-specific serums of other types*

| ANTIGENS | SERUMS | | | | | | |
|---|---|---|---|---|---|---|---|
| | Paratyphi B (1, 2...) | Newport (1, 2...) | Kunzendorf (1, 5...) | Paratyphi A 228 Phase 2 | Paratyphi AWB39 Phase 2 | Anatum (1, 6...) | Nyborg (1, 7...) |
| Paratyphi B (1, 2...) | 10,000 | 10,000 | 5,000 | 1,000 | 500 | 2,000 | 2,000 |
| Newport (1, 2...) | 5,000 | 40,000 | 5,000 | 1,000 | 1,000 | 2,000 | 2,000 |
| Kunzendorf (1, 5...) | 2,000 | 10,000 | 20,000 | 40,000 | 40,000 | 5,000 | 2,000 |
| Paratyphi A 228 | 2,000 | 2,000 | 10,000 | 40,000 | 20,000 | 5,000 | 1,000 |
| Paratyphi A WB39 | 2,000 | 2,000 | 10,000 | 40,000 | 40,000 | 5,000 | 1,000 |
| Sendai (1, 5...) | 2,000 | 5,000 | 10,000 | 40,000 | 20,000 | 5,000 | 1,000 |
| Anatum (1, 6...) | 2,000 | 2,000 | 5,000 | 10,000 | 5,000 | 10,000 | 1,000 |
| Nyborg (1, 7...) | 5,000 | 5,000 | 2,000 | 200 | 200 | 2,000 | 10,000 |

Figures indicate highest dilution at which agglutination occurred.

sorbed serums containing agglutinins for non-specific factors 2, 3, 5, 6 and 7, respectively. The preparation of these serums was described by Bruner and Edwards (1941). The non-specific components of *S. paratyphi A* were flocculated only by serums containing agglutinins for factors 3 and 5. In this respect they resembled the Kunzendorf, Berlin and Sendai types. Phase 2 of *S. paratyphi A*, therefore, may be expressed as 1, 5....

As mentioned above, the non-specific phases derived from cultures of *S. paratyphi A* were not identical. The differences in the phases were discernible only in agglutinin absorption tests. It was possible to divide the phases into two groups whose non-

specific components were identical. In one group were cultures 228, HR and Fried; while 38250, Marta, Cal. 49, 1015, HA1, HA6, WB39, Boyd and Durazzo were included in the second. The differences between these two groups were evidenced only by a slight residue of agglutinins remaining when the serum derived from a member of the second group was absorbed with a member of the first group. These residues of agglutinins did not exceed 2 per cent of the original titres of the serums. The differences in the non-specific components of *S. paratyphi A* were no more pronounced than those that exist in the non-specific phases of different diphasic species in which the second phase is expressed as 1, 5.... The phases denoted as 1, 5... in the Kauffmann-White classification are quite complex and their exact relationships cannot be expressed without the use of further symbols. In this connection it should be remembered that the classification was designed by Kauffmann as a diagnostic schema and that it does not give a complete antigenic delineation of the bacilli.

The relationships of the non-specific components of *S. paratyphi A* to the naturally occurring 1, 5... phases were studied by agglutinin absorption tests. It was found that serums derived from 228, HR or Fried were completely exhausted of agglutinins by absorption with the non-specific components of 38250, Marta, Cal. 49, 1015, HA1, HA6, WB39, Boyd, Durazzo or Sendai. The serums of the latter group were not completely absorbed by 228, HR, Fried or Sendai. None of the non-specific phases of *S. paratyphi A* removed all the agglutinins from Sendai serum. The relationships of the non-specific components of *S. paratyphi A* and Sendai were appreciably closer than were their relationships with the non-specific phases of any other type.

## DISCUSSION

While earlier workers realized that the *Salmonella* group was composed of a mosaic of interrelated antigens, Bruce White (1926) was the first to identify these antigens by symbols and thus express the relationship and divergences of different species in graphic form. In addition he was the first to discuss the

genetic relations of the various forms with reference to antigenic composition and to propose a theory of *Salmonella* phylogeny. White believed that the *Salmonella* species as we know them today arose from a primitive diphasic ancestral stock by variation in O and H antigens. He regarded the monophasic state, as seen in *S. paratyphi A*, *S. abortus-equi* and *E. typhosa*, as an acquired characteristic which developed through loss variation. The demonstration by Edwards and Bruner that antigen a could be isolated from *S. abortus-equi* revealed that the organism was more closely related to diphasic forms having the antigens a-enx than was previously realized. They suggested that in monophasic types, the antigens present in the original diphasic state were not lost but merely suppressed. The isolation of non-specific components from *S. paratyphi A* confirms this view. Both observations strengthen the theory of White that monophasic types have evolved from a diphasic ancestry.

White emphasized the close relationship that existed between *S. paratyphi A* and Sendai, the only two types then known which possessed antigen a. He used these two types to illustrate the identity of the H antigens of monophasic types and the specific antigens of diphasic types. The isolation of non-specific phases from *S. paratyphi A* which are all but identical with the non-specific phase of Sendai reinforces the relationship between the specific phases of the two types and demonstrates the keen appreciation of White of the evolutionary tendencies in the genus.

With the exception of the recovery of specific phases from "totally and permanently" non-specific types, the isolation of antigen a from *S. abortus-equi* and of non-specific components from *S. paratyphi A* constitute the only instances in which antigens resembling the naturally occurring phases have been induced in monophasic cultures. The stability of these induced antigens has been mentioned. It is in direct contrast to the instability of the phases of the diphasic types. These results indicate that once an organism has lost the power of phase variation, it cannot resume this function, even when suppressed components become dominant. Only under the influence of a stimulant, such as specific immune serum, does phase variation occur.

It has been stated that the third and fourth phases induced in *S. paratyphi A* showed little relationship to the natural antigens of the genus. However, they do display relationships to the induced phases of several other *Salmonella* types. As mentioned by Edwards and Bruner (1939a) these induced phases follow a recurring pattern, just as do the naturally occurring antigens. Whether they are artifacts produced under the influence of immune serum or whether they represent components that have been suppressed in the evolution of known species is not clear at present. The writers are inclined to the latter view.

## SUMMARY

1. Through cultivation in agglutinating serums three induced phases were isolated from cultures of *Salmonella paratyphi A*. One of the phases was closely related to the non-specific phases of diphasic types. The other two bore little resemblance to the normal *Salmonella* antigens.

2. Non-specific components were isolated from 12 of 16 cultures studied. These non-specific antigens were expressed as 1, 5... and were more closely related to the non-specific phase of the Sendai type than to any of the other non-specific phases.

3. The induced phases were stable under ordinary conditions of culture and could be reverted only by the addition of appropriate serums to the medium.

4. The isolation of non-specific antigens from *S. paratyphi A* was cited as additional evidence that monophasic *Salmonella* types are derived from multiphasic types through suppression of phases.

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