THE DEMONSTRATION OF PHASE VARIATION IN SALMONELLA ABORTUS-EQUI[1]

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The discovery by Andrewes (1922) that certain *Salmonella* species could be divided into specific and nonspecific phases by selection of colonies offered an entirely new approach to the differentiation of types within the paratyphoid group. This work was followed by the observation of Kauffmann and Mitsui (1930) that some types which did not display nonspecific components could be separated into two phases which exhibited different antigenic properties. This phenomenon was shown to be comparable to the specific-nonspecific variation of Andrewes and was called alpha-beta phase variation. While it has been demonstrated that many *Salmonella* types are diphasic, a number are still considered to be monophasic species which are not subject to phase variation.

The value of antiserums in inducing phase variation has been recognized for several years. Scott (1926) was able to isolate the specific phase from nonspecific cultures of the Thompson type by transferring them in broth which contained serum derived from the nonspecific phase. Using the same method Kauffmann (1936) was able to demonstrate a second phase in the typhoid bacillus, which had hitherto been considered monophasic. Wassén (1935) originated a method for the separation of phases which was based on the immobilizing effect of agglutinating serums. The details of this method were described by Bruner and Edwards

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(1939) who used it successfully for the isolation of specific phases from all of the so-called totally and permanently nonspecific varieties of *Salmonella*. Since the Wassén technique is so greatly superior to other methods for the isolation of phases which are suppressed under ordinary methods of culture, the writers have used it to investigate phase variation in the supposedly monophasic specific types. *Salmonella abortus-equi* was chosen as the first species to be examined. The reason for this choice is obvious. The flocculating antigens of *S. abortus-equi* (*enx* of the Kauffmann-White schema) are those present in the beta phase of almost all the types which possess alpha-beta phase variation. It would thus appear that *S. abortus-equi* is a type in which the alpha phase is suppressed. This hypothesis was advanced by Kauffmann (1938), who examined several cultures of this species but could demonstrate no variation.

MATERIALS AND METHODS

From the collection of Professor E. S. Good, who very generously supplied cultures, and from specimens isolated in this laboratory, 24 strains of *S. abortus-equi* were available for study. Of these 24 cultures, 6 were non-motile or only very feebly motile and 3 were rough. The remaining 15 strains of smooth, motile bacilli were typical representatives of the species. Phase variation was noted in 6 cultures, whose designations and histories are as follows: M1, From aborted fetus, isolated 1924. M5, Isolated from aborted fetus by Dr. K. F. Meyer in 1916. McC, From aborted fetus, isolated 1923. WH2, From aborted fetus, isolated 1931. 4K88, Received from Army Medical School. Isolated from aborted fetus in 1933. P, From aborted fetus, isolated 1913.

These cultures were inoculated into semi-solid agar containing various antiserums by stabbing along one side of the tube. In the original experiments, *S. abortus-equi* antiserum which had been freed of somatic agglutinins by absorption with the Reading type was added to the agar. In later trials serum derived from the beta phase of the Minnesota type was substituted. The antigenic formula of this type was established by Edwards and

Bruner (1938) as XXI: $b \rightleftharpoons enx$. Thus the flocculating antigens of the beta phase of Minnesota are similar to those naturally occurring in *S. abortus-equi*. These serums were used in amount sufficient to confine the growth of the normal phase of the bacilli to the line of inoculation. Outgrowths from the line of stab represented the appearance of hitherto masked phases. As these phases appeared they were purified by continued transfer and agglutinating serums were prepared for them. These serums, after absorption with appropriate bacilli, and *Salmonella paratyphi A* serum were in turn added to semi-solid agar for use in attempts to revert the induced phases.

RESULTS

Before the results of the experiments can be given, some designation must be assigned the phases isolated. Kauffmann (1938a) has revised the Kauffmann-White schema so that the phases of diphasic organisms are referred to as phase 1 or phase 2. In the case of the types which exhibit specific-nonspecific variation the specific antigens are designated as phase 1, the nonspecific as phase 2. In organisms which display alpha-beta variation, the alpha components are called phase 1, the beta are denoted as phase 2. Since the normal antigens of *S. abortus-equi* (*enx*) are those found in phase 2 of the revised schema, they will be designated as phase 2. Two other phases which were found will be designated as phase 1 and phase 3. As will be shown later, phase 1 is identical with the normal flocculating antigen of *S. paratyphi A* (*a*), while phase 3 is closely related to the antigen which has been known as the beta phase of the Schleissheim type (*z*5 of Kauffmann and Tesdal, 1937).

Phase 1 was recovered from each of the six cultures listed above. Phase 3 was recovered from four cultures; M5, McC, WH2, and P. No special effort was made to recover phase 3 from cultures M1 and 4K88. Not only was it possible to recover these hitherto suppressed antigens from the cultures, but by using a suitable serum or combination of serums the phases could be transformed from one into another at will. The degree of control which it was possible to exercise over phase variation in these

cultures was surprising. It is unnecessary to give in detail the results obtained with all the cultures. The results obtained with one culture are presented in figure 1.

From figure 1 it can be seen that either phase 1 or phase 3 can be obtained from the naturally-occurring phase 2 and that both of these artificially induced phases may be reverted to the original phase or transformed to the other induced phase as desired.

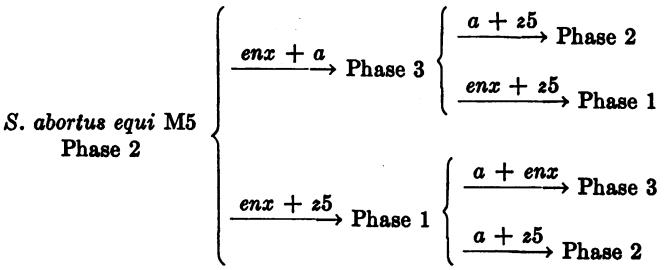

FIG. 1. PHASE VARIATION INDUCED IN S. ABORTUS EQUI

The symbols on the arrows indicate the serums added to the medium.

enx—antiserum derived of the beta phase of the Minnesota type or antiserum derived from phase 2 of *S. abortus equi*. The latter serum was absorbed by the Reading type before use.

z5—antiserum derived from phase 3 of *S. abortus equi*. This serum was absorbed with phase 2 of *S. abortus equi* before use.

a—antiserum derived from *S. paratyphi* A.

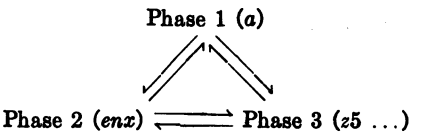

FIG. 2. THE REVERSIBILITY OF THE PHASES OF S. ABORTUS EQUI

The symbols accompanying the phases indicate the antigens which they contain. These are designated in accordance with the Kauffmann-White schema.

Unless the proper combination of serums is used a mixture of phases may result. For instance, if phase 1 is grown in *S. paratyphi A* serum alone, both phase 2 and phase 3 may be obtained from it. The phases of *S. abortus-equi* and the changes which have been induced in them are indicated in figure 2.

The serological relationships of the phases were examined by agglutination and agglutinin absorption. It should be emphasized that the changes described in the organisms apply only

to the heat-labile or flocculating antigens. The heat-stable or somatic antigens remained unchanged throughout the course of the work. Agglutination and agglutinin absorption tests proved that the somatic antigens of phases 1, 2 and 3 were identical.

TABLE 1

Agglutination tests

ANTIGENS	ANTISERUMS					
	Abortus equi M1, phase 1	*Abortus equi* M5, phase 2	*Abortus equi* M5, phase 3	*Paratyphi A*	Minnesota-beta	Schleissheim-beta
Abortus equi M5—phase 1....	20,000	500	200	20,000	0	0
Abortus equi M5—phase 2....	0	10,000	500	0	20,000	0
Abortus equi M5—phase 3....	0	1,000	5,000	0	0	2,000
Paratyphi A.....	20,000	0	0	20,000	0	0
Minnesota-beta.....	0	10,000	0	0	20,000	0
Schleissheim-beta.....	0	0	2,000	0	0	5,000

0 indicates no agglutination at 1:200.

TABLE 2

Agglutinin absorption tests

ANTIGENS	ANTISERUMS											
	Abortus equi M1, phase 1, absorbed by			*Abortus equi* M5, phase 2, absorbed by			*Abortus equi* M5, phase 3, absorbed by				*Paratyphi A* absorbed by *Abortus equi* M5, phase 1	Schleissheim-beta absorbed by *Abortus equi* M5, phase 3
	Abortus equi M5, phase 2	*Abortus equi* M5, phase 3	*Paratyphi A*	*Abortus equi* M5, phase 1	*Abortus equi* M5, phase 3	Minnesota-beta	*Abortus equi* M5, phase 1	*Abortus equi* M5, phase 2	Schleissheim-beta	Schleissheim-beta and *Abortus equi* M5, phase 2		
Abortus equi M5—phase 1...	10,000	10,000	0	0	0		0	0	0			
Abortus equi M5—phase 2...	0	0		10,000	10,000	0	200	0	500	0	0	
Abortus equi M5—phase 3...	0	0		200	0		5,000	5,000	2,000	1,000		0
Paratyphi A.....	10,000	10,000	0								0	
Minnesota-beta.....				10,000	10,000	0						
Schleissheim-beta.....							2,000	2,000	0	0		2,000

0 indicates no agglutination at 1:200.

The relationships of the flocculating antigens are given in tables 1 and 2. Except for the relationships shown in table 1, the 3 phases of *S. abortus-equi* failed to react in significant degree with any of the antigens of the Kauffmann-White classification.

Naturally, they reacted with all types containing the antigens *a*, *e*, *n* or *x*. Although the three phases show more or less relationship to each other they are easily distinguished. While serum derived from phase 1 fails to agglutinate phase 2 or phase 3, serums derived from both phases 2 and 3 agglutinate phase 1 in low dilution. The closest relationship is between phase 2 and phase 3. The agglutination and absorption tests demonstrate the identity of phase 1 with the flocculating antigen of *S. paratyphi A*. Phase 2, the only phase of the bacillus which is known to occur naturally, is identical with the flocculating antigens of the beta phase of the Minnesota type. It is surprising that while the flocculating antigens of phase 2 and of Minnesota beta are identical, phase 3 is agglutinated by phase 2 serum but not by Minnesota beta serum. Phase 3 is closely related to the beta phase of the Schleissheim type but, as demonstrated by absorption tests, the two are not identical. The phases, when once isolated, are quite stable. It is obvious that phase 2, which occurs naturally, is quite stable since it is the only form in which the organism has been found previously. Phase 1 and phase 3 are apparently equally constant. No variation has been observed in these phases except when they were cultivated in semisolid agar containing their respective antiserums. This stability of the phases under ordinary conditions of culture supports the view that changes observed when they were placed in contact with appropriate serums were actually due to reversion and not to impurity of the phases. The behavior of the phases in semisolid agar containing immune serum also supports the view that reversion actually occurred. A species which is naturally diphasic spreads readily through semisolid agar containing serum which acts upon the predominant phase. Even the Binns type, whose stubborn non-specificity is notorious, migrates rapidly through a medium containing serum which immobilizes the non-specific phase. The phases of *S. abortus-equi*, on the contrary, act in an entirely different manner. The initial cultures show only one or two small filmy protrusions from the line of stab, and these usually appear after the tubes have been incubated for 48 hours or more. Such behavior strongly indicates actual reversion.

DISCUSSION

It is difficult to discuss the facts presented above because the mechanism upon which phase variation rests is not understood. Nevertheless several points call for comment. First, there is the demonstration of more than two phases in a single bacterial culture. So far as the writers are aware, no more than two phases have been reported previously. The demonstration of a third phase suggests that eventually a still larger number may be found. The significance of this observation is not clear. It may be said to add support to the view of White (1926) that the paratyphoid bacilli as we know them today are loss variants of primitive types which had wide antigenic relationships and little or no host specificity. White further postulated that, as these primitive types became adapted to specific hosts, certain antigens were lost and that the types most specialized in their host relationships were likely to exhibit restricted antigenic components. The monophasic typhoid bacillus, *S. paratyphi A* and *S. abortus-equi* were cited as examples. If this theory of *Salmonella* phylogeny is accepted it must be realized that the phases which have disappeared are not actually lost, but are merely suppressed. In further support of this view it may be mentioned that the writers have isolated nonspecific phases from *S. paratyphi A*, which has hitherto been considered monophasic. The work on this species is not sufficiently advanced to warrant a detailed report.

Kauffmann (1936) demonstrated a beta phase in one culture of the typhoid bacillus by cultivation in broth containing immune serum for the Muenchen type. Efforts to isolate the beta phase from other cultures resulted in failure. Further, the induced phase could not be reverted to the normal alpha phase. Attempts to induce phase variation in *S. paratyphi A* were unsuccessful. In commenting on these results Kauffmann expressed the view that *S. paratyphi A*, like the typhoid bacillus, was capable of phase variation and that possibly all *Salmonella* types were diphasic or multiphasic. He also pointed out the possibility that diphasic strains such as *S. paratyphi B* or *S. paratyphi C* could be induced to yield beta variants of the specific

antigens by cultivation in suitable immune serums. The present work is partial proof of that hypothesis.

Kauffmann (1938a) has also advanced the opinion that the flocculating components of any *Salmonella* type are complex and consist of a number of partial antigens, one or two of which predominate and determine the character of the phases. He believes that it is possible by cultivation in certain immune serums to subordinate the dominant antigens and promote those which ordinarily are of minor importance. Thus, it would be possible to obtain a number of phases from a given type, corresponding to the partial antigens of that type.

While immune serums were used to isolate beta phases from the typhoid bacillus by Kauffmann (1936), from the Schleissheim type by Kauffmann and Tesdal (1937) and from *Salmonella abortus-canis* by Gard (1938), the work reported here constitutes the first instance in which phases obtained from apparently monophasic specific types have been demonstrated to be reversible. The reversibility of the induced antigens indicates that the phenomenon being dealt with is a true example of phase variation and not merely a mutation or degeneration produced through cultivation in immune serum. The fact that phase 1 is identical with the normal flocculating antigens of *S. paratyphi A*, and that this component frequently occurs in diphasic cultures whose beta phase is identical with phase 2 of *S. abortus-equi* is a further indication that a true phase variation has been discovered.

The relationship of phase 3 of *S. abortus-equi* and the beta phase of the Schleissheim type holds added interest when it is considered that both are induced phases. Likewise the beta phase of *S. abortus-canis* of Gard (1938) and the beta phase of the Schleissheim type are induced phases which are more or less related to each other. Similarly the writers have been able to isolate a beta phase from *S. paratyphi A* and an induced phase from the Kentucky type which are closely related. After more work has been done on this problem it is possible that phases isolated with the aid of immune serums will be found to follow a recurring pattern, as do the naturally occuring antigens of the Kauffmann-White schema.

The discussion would not be complete without some reference to the relation of this work to transformation of types within the genus. The demonstration of phase 1 in *S. abortus-equi* endows this species with all the major antigenic components of the naturally diphasic Bispebjerg type, whose antigenic formula is IV: $a \rightleftharpoons enx$. All efforts to demonstrate a third phase in the one available culture of the latter type have failed. The two types differ in their biochemical activity and while closely related serologically, they are not identical. Although it is entirely probable that further unsuspected antigenic relationships between different types will be established by work similar to that reported here, it seems improbable that actual transformation of types will be accomplished in this manner.

CONCLUSIONS

Study of the supposedly monophasic *Salmonella abortus-equi* by the Wassén technique revealed that this organism contained three phases. Phase 1 is identical with the flocculating antigens of *Salmonella paratyphi A*. Phase 2 is the form in which the species naturally occurs. Phase 3 is related to, but not identical with, the beta phase of the Schleissheim type. The phases are reversible and by use of suitable immune serums, one phase may be changed to either of the others. This is the first instance in which more than two phases have been demonstrated in a *Salmonella*. It is also the first instance in which induced phases isolated from monophasic specific types have been proved to be reversible.

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