

THE NATURE OF SOMATIC PHASE VARIATION AND ITS IMPORTANCE IN THE SEROLOGICAL STANDARDIZATION OF O-SUSPENSIONS OF SALMONELLAS FOR USE IN THE WIDAL REACTION

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Dreyer introduced his system of serological standardization in order to ensure uniformity of results and to enable valid comparison to be made of titres obtained by different workers or by the same worker using different batches of suspension in the quantitative Widal Reaction. The principle of this procedure is simple and is widely used in the standardization of biological products. The sensitivity to agglutination of a new suspension is compared with that of a standard suspension by titrating both against an homologous antiserum. Depending on the result obtained, a factor is given to the new suspension so that titres obtained by its use may be expressed in terms of standard agglutination. Thus if the new suspension proves half or twice as sensitive to agglutination as the standard, all titres, expressed as reciprocals, subsequently deriving from it should be multiplied by or divided respectively, by 2. The demonstration by Weil & Felix in 1917 that the agglutinable properties of Proteus X strains were not due to a single substance but to distinct flagellar H and somatic O antigens and the subsequent finding that the agglutinable properties of Salmonellas were similarly complex, upset the theoretical basis of Dreyer's method, but not its practical value in the standardization of H-suspensions since the H-titre of antisera to flagellated organisms usually greatly exceeds the O while the flagella themselves, in formalized suspension, tend to mask somatic agglutinability. The complicated serological responses which followed the introduction of T.A.B. Vaccine and the finding that the sera of a considerable proportion of cases of proven enteric fever might show no H-agglutinin (Felix & Olitzki, 1928-9) led to the adoption of the qualitative Widal Reaction (Felix, 1924) in which the titre of H and O-agglutinins in the patients' sera were estimated separately. The occurrence of anamnestic H responses in T.A.B. inoculated persons resulted in O-type agglutination only being used when dealing with immunized populations (e.g. the Army). Moreover, the undoubted presence of specific O-agglutinins in the sera of immunized people stressed the importance not only of accurate estimation of absolute titre but also the importance of a rising O titre, assessed by means of successive comparative estimations, in the Widal Reaction.

Variability in the sensitivity to O-antisera of different strains of *Bact. typhosum* was known to be especially marked (Weil & Felix, 1920) due, as was later discovered, to variation in the content of the Vi antigen. Serological standardization was therefore applied to Salmonella O-suspensions (Gardner, 1928-9, Felix & Gardner, 1937) and has since been widely practised in one form or another. Gardner, in criticizing the principles governing Dreyer's original procedure, states as an underlying principle of standardization, that 'an agglutinability standardization can only deal with a single agglutinable substance'. Although the antigenic complexity of the body fraction of Salmonellas had long been recognized it was thought that, apart from S→R variation, and the rare occurrence of loss of a somatic factor by loss variation, the complex remained stable in smooth forms of the same organism and, therefore, could be regarded as a 'single agglutinable substance' for purposes such as serological standardization. Recently, however, Kauffmann (1941) has described a new type of qualitative variation which occurs independently in Salmonella somatic antigens I and XII. If a strong *Bact. seftenberg* (I, III, XIX) antiserum is tested by the slide agglutination method against colonies derived from a Salmonella containing the factor I, strong, medium or weak agglutination may be observed. On similarly testing colonies resulting from the plating of one colony, it is found that although the majority show the degree of agglutinability of the parent colony, a few display the variant type of agglutinability. On plating these variant colonies the contrary state of affairs is observed. Rabbits immunized with strong agglutinators produce a high anti-I titre, those with weak agglutinators a low anti-I titre. *Bact. seftenberg* and *Bact. niloese* possess antigen I in the strongly agglutinable and antigenic form only. The same type of variation can be demonstrated in antigen XII by testing colonies of *Bact. typhosum* or *Bact. enteritidis* (IX, XII) with a *Bact. paratyphosum* B (I, IV, V, XII) O-antiserum and *vice versa*. Colonies of *Bact. paratyphosum* A (I, II, XII), however, do not show variation in their XII antigen. On the basis of such results, Kauffmann has subdivided antigen XII into three components, XII₁, XII₂ and XII₃. Only

component XII₂ undergoes variation. When it is dominant it is highly agglutinable and antigenic, at the same time partially masking the reactivity of XII₁, XII₃; when recessive it is but poorly agglutinable and of low antigenicity while XII₁, XII₃ become unmasked and dominant. *Bact. paratyphosum* A contains only XII₁, XII₃ and *Bact. paratyphosum* B and *Bact. typhimurium* only XII₁, XII₂ thus enabling component-specific sera against XII₂ and XII₃ to be prepared. *Bact. typhosum* and *Bact. enteritidis* possess all three components.

It is clear that this work undermines at least the theoretical basis of serological standardization of Salmonella O-suspensions containing antigens I or XII₂ since the somatic complexes of these organisms can no longer be considered as a stable 'single agglutinable substance' but as a combination of antigens whose quantitative interrelationship is constantly changing. While engaged primarily in analysing the somatic structure of strains of *Bact. enteritidis* (Gaertner) isolated from cases of continuous fever in India, I had the opportunity of studying this type of variation and the degree of effect which it might have on serological standardization. Observations relative to this matter are recorded below.

METHODS EMPLOYED

Agglutinable suspensions. A smooth colony was picked and seeded to broth. After 4–6 hr. growth the broth culture was poured over a nutrient agar surface and then drained off. After overnight incubation at 37° C. the growth was harvested in a small volume of saline and alcohol added to 75–80%. 1 hr. later the suspension was centrifuged, the supernatant alcohol removed as completely as possible with a pipette and the deposited bacteria resuspended to an opacity equivalent to approximately 6000 million *Bact. coli* per ml. in 1/2500 HgI₂—0.45% saline solution, buffered at pH 7.4. Suspensions were tested for H-inagglutinability before use. Suspensions of the permanently non-motile *Bact. typhosum* strain 901/O (T/901/O) were not alcoholized.

Antisera. Antisera were prepared by the intravenous injection of rabbits with suspensions prepared as described above. Suspensions of flagellated organisms were not boiled prior to injection. Since only H-inagglutinable, alcoholized suspensions and suspensions of non-motile strains were used as antigen in serum titrations, the presence of H-agglutinins in sera was ignored. Four injections of 100–250, 500, 1000 and 2000 million organisms were given at intervals of 3–5 days, the animal being bled 5–7 days after the last injection.

The agglutination test. Serial dilutions of serum were made in 1.0 ml. volumes in wide bore (10 mm. approximately) round-bottomed tubes. The diluting

fluid was 1/2500 HgI₂—0.45% saline solution, buffered at pH 7.4. One drop of suspension was added to each tube. After shaking, the rack was incubated at 37° C. for 2 hr. and then left overnight on the bench. The end-point of agglutination was taken as the highest dilution of serum not showing a discrete spot of unagglutinated bacteria at the bottom of the tube. When the end-point appeared equivocal, each tube was shaken and its magnified image examined in a concave microscope mirror, the titre being taken as the highest dilution of serum showing visible particulation.

EXPERIMENTAL FINDINGS

Kauffmann's observations on phase variation in the somatic factors I and XII were confirmed for several freshly isolated strains of *Bact. paratyphosum* A, for T/901/O and for several recent strains of *Bact. enteritidis* (Gaertner). Factor I antiserum was prepared by absorbing *Bact. seftenberg* (I, III, XIX) antiserum with T/901/O to remove the *Bact. typhosum* O agglutinin normally present in rabbit serum: components XII₂ and XII₃ antisera by absorbing *Bact. reading* (IV, XII₁, XII₂) and *Bact. durazzo* (II, XII₁, XII₃) antisera respectively with suspensions of the heterologous type. At a later stage in the work it was recognized that the majority of Indian strains of *Bact. enteritidis* appeared to be totally deficient in either XII₂ or XII₃, one-third of approximately 50 strains analysed having the somatic formula IX, XII₁, XII₃ and the other two-thirds the formula IX, XII₁, XII₂ (Hayes & Freeman, 1945).^{*} Cross-absorption of antisera to these types enabled pure XII₂ and XII₃ antisera to be obtained which proved of value in subsequent antigenic analysis.

Observations on the quality of agglutination. When colonies from an organism subject to active variation are tested on a slide against specific serum to the antigen under study, some show rapid and clear-cut agglutination (strong agglutinators) while with others (weak agglutinators) clumping is appreciably slower and much less marked. Although the difference in agglutinability of the two types of colony is obvious, a colony failing to show any agglutination at all has never been observed in the absence of S→R variation. Observation of the results of slide agglutination shows that, when the reaction is complete, strong agglutinators appear as numerous large granules dispersed in a clear watery medium. With weak agglutinators, on the other hand, the appearance is that of a few granules, individually not

^{*} In this paper the strains of *Bact. enteritidis* possessing component XII₃ were also provisionally credited with antigen I. This opinion was incorrect and was due, for reasons too involved to mention here, to the presence of XII₃ antibody in the factor I antiserum used.

obviously smaller than those of strong agglutinators, dispersed in a milky fluid of unagglutinated bacteria. Similar findings were obtained when agglutination was carried out in tubes. Suspensions of strong agglutinators were agglutinated cleanly to the titre of the serum leaving no residual turbidity of the supernatant fluid.

Suspensions of weak agglutinators appeared, on superficial examination, not to have been agglutinated at all for in every serum dilution there was a well defined and unequivocal spot with no clearing of the supernatant fluid. On shaking the tubes, however, well marked granules of agglutinated bacteria were seen to the titre of the serum.

colonies were not performed in view of the continuous nature of the variation and the demonstration of the same phenomenon in T/901/O, an organism devoid of Vi antigen. Nevertheless, it has been shown that weak agglutinators are not transformed into strong agglutinators by boiling.

It is clearly important to decide upon the nature of this type of variation. Three types of change are possible:

(1) A quantitative variation in each organism of a colony; that is, that the antigen concerned retains its immunological specificity but varies its proportion relative to the other antigens on the cell surface.

Table 1. *Agglutination of selected colonies from one plating of Bact. paratyphosum A (strain Beamish) by factor I antiserum*

Colony no.	Slide agglutination (1 in 5 serum)	Agglutination in tubes (serum dilution 1 in)						
		20	40	80	160	320	640	1280
7	Rapid. Many granules in clear fluid	++	++	++	++	++	+	-
		N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	S.
		Com.Cl.	Com.Cl.	Com.Cl.	Com.Cl.	Com.Cl.	Com.Cl.	No.Cl.
10	Rapid. Many granules in clear fluid	++	++	++	++	+	-	-
		N.S.	N.S.	N.S.	N.S.	Sl.S.	S.	S.
		Com.Cl.	Com.Cl.	Com.Cl.	Com.Cl.	P.Cl.	No.Cl.	No.Cl.
8	Slow. Few granules in milky fluid	+	+	+	+	+	+	-
		S.	S.	S.	S.	S.	S.	S.
		No.Cl.	No.Cl.	No.Cl.	No.Cl.	No.Cl.	No.Cl.	No.Cl.
9	Slow. Few granules in milky fluid	+	+	+	+	+	+	-
		S.	S.	S.	S.	S.	S.	S.
		No.Cl.	No.Cl.	No.Cl.	No.Cl.	No.Cl.	No.Cl.	No.Cl.

All tubes first examined for the presence of a spot and clearing; then shaken and examined without magnification for agglutination.

++ and + = degrees of agglutination.
 - = no agglutination.
 N.S. = no spot.
 S. = definite spot.
 Sl.S. = slight, indefinite spot.

Com.Cl. = complete clearing } Compared with
 P.Cl. = partial clearing } saline control.
 No.Cl. = no clearing }

An example of this type of reaction, showing the behaviour of colonies from a culture of *Bact. paratyphosum A*, and suspensions prepared from them, towards factor I antiserum is given in Table 1. Similar results can be demonstrated with factor XII₂ antiserum and strains of *Bact. typhosum* and *Bact. enteritidis* undergoing variation.

When such an experiment is put up in Dreyer tubes in a water bath at 52° C. and examined the following morning, comparable results are obtained. Strong agglutinators are clumped completely to a sharp end-point with clearing of the supernatant fluid. With suspensions of weak agglutinators, every tube appears as turbid as the control but contains a small deposit of agglutinated bacteria.

Experiments to exclude the possibility of a masking effect of an antigen of Vi type (Felix & Pitt, 1936) being responsible for the weakly agglutinating

(2) A qualitative variation; that is, that the antigen remains quantitatively the same in each organism but alters its immunological specificity so that it becomes less reactive.

(3) A quantitative variation affecting, not each individual cell in a colony, but the proportion of cells in the colonial population possessing an antigen which is both qualitatively and quantitatively normal. Variation in accordance with theories (1) or (2) should result in the agglutination of all the organisms of a weakly agglutinating colony, with the production of fine granules more slowly formed than in the case of strong agglutinators, since a demonstrable decrease or alteration in the quantity or specificity of the sensitized antigen must lead to a decrease in the attractive force between organisms possessing it. This form of reaction has not been seen to occur. The observations outlined above lend

strong support to theory (3). One of the advantages of the tube agglutination technique used lies in its sensitivity to extremely fine degrees of agglutination which may not be visible with the magnification provided by a hand lens or microscope mirror. It is considered that, with this technique, the presence of a discrete spot in any serum dilution indicates complete absence of agglutination. If this is so, then the greater proportion of organisms in weakly agglutinating colonies are not agglutinated at all and, therefore, are devoid of the antigen at least in demonstrable amounts. The clumping of the remaining cells to form granules of approximately the same size as those of strong agglutinators suggests that these possess a qualitatively normal antigen in normal amount. Since the velocity of agglutination depends on the incidence of effective contacts between the agglutinable particles and varies directly with the concentration of these particles (Duncan, 1938), weakly agglutinating colonies of the composition suggested above would, as has been observed, also be slow agglutinators. The ultimate size of the particles formed might be as great as those of strong agglutinators, though fewer in number. Moreover, the titres of antibodies produced by injection of rabbits with bacterial suspensions is, within limits, dependent on the number of organisms injected (Topley & Wilson, 1936) so that the lower titres produced by immunization with weak agglutinators are at least as readily explainable by theory (3), as by theories (1) and (2). It seems probable, therefore, that strong agglutinators comprise a bacterial population each member of which possesses the antigen under examination, whereas with weak agglutinators such organisms form only a small minority of the population but are always present.

Effect of the variation on the interpretation of anti-serum titrations. Routine titrations of human or rabbit sera for agglutinins against organisms of the Salmonella group are usually examined somewhat superficially. With Dreyer's technique the criteria used for assessing the end-point are clearing of the supernatant fluid, accompanied by an obvious deposit of clumped bacteria or the presence of visible granules in suspension. Similarly, when the technique described here (Felix's method) is used, the end-point is simply taken as the highest dilution of serum not showing a definite spot at the bottom of the tube. It has frequently been observed with *O*-suspensions of *Bact. typhosum* or *Bact. paratyphosum* A or B that the end-point of agglutination is indefinite and spread over several tubes. With Dreyer's technique, following a series of tubes showing clearing and obvious agglutination there may be one or more tubes containing particles in suspension in a turbid fluid. With Felix's method, one or more tubes showing a spot together with aggregates may follow a series showing normal agglutination. It is

thus impossible to say whether the true end-point should be judged by the spot or by the last tube showing agglutination, the titre varying by 2, 4 or more times depending on which of these now mutually exclusive criteria is adopted. This difficulty has not been encountered with suspensions of *Bact. paratyphosum* C, none of whose somatic antigens has been shown to undergo somatic phase variation. Rabbit antisera prepared against T/901/O in the XII₂-dominant phase have repeatedly been found, by titration against *Bact. enteritidis* strains having somatic structures IX, XII₁, XII₂ and IX, XII₁, XII₃ respectively, to possess a much higher titre against component XII₂ than against any other homologous somatic antigen. This has also been confirmed by absorption with IX, XII₁, XII₃ strains. When such antisera are titrated against a *Bact. enteritidis* strain undergoing active variation, suspensions of XII₂-dominant colonies are agglutinated cleanly to a well-defined end-point whereas suspensions of XII₂-recessive colonies show the indefinite type of end-point described above.

It will be seen from what has been said in the preceding section that while suspensions prepared from strongly agglutinating colonies are antigenically homogeneous, those from colonies showing weak or medium agglutination are composed, in effect, of organisms of two distinct antigenic types. If, then, such a suspension is titrated against an antiserum having a higher titre for the variant antigen than for the other antigens of the type, paradoxical agglutination will occur in those tubes covering the difference between the two titres. Although this may not be the only cause of indefinite end-points in agglutinin titrations, it is considered to be at least one of the most important where Salmonella types possessing antigen I or component XII₂, or both, are concerned.

Effects of the variation on serological standardization. Somatic phase variation affects not only those bacterial cultures subject to it, but also the quantitative relationships between the various antibodies of a serum produced by immunization with such cultures. It has already been mentioned that a rabbit antiserum against T/901/O in the XII₂-dominant phase possesses a much higher titre against component XII₂ than against the other antigens of the strain. It will therefore agglutinate suspensions of *Bact. enteritidis* in the same phase to a much higher titre than suspensions of the same strain in the XII₂-recessive phase, if we ignore the few granules of clumped XII₂-containing cells in the suspension. On the other hand, antigen IX and components XII₁, XII₃ usually appear to be less antigenic in rabbits than XII₂, so that in antisera produced against XII₂-recessive suspensions no single agglutinin is pre-eminent. Such sera will tend to agglutinate both XII₂-dominant and recessive

suspensions to approximately the same titre. Thus, when tested against one antiserum the difference in sensitivity between two suspensions prepared from different colonies of the same culture may be marked, while with another antiserum they may appear equally sensitive. Occasionally the titres given by two such suspensions with an antiserum to one phase are transposed when serum to the other phase is used as the basis of comparison. Table 2 shows the sensitivity of suspensions of T/901/O and *Bact. enteritidis* strain 12316/JT (kindly supplied to me, with other strains, by Dr Joan Taylor) to agglutination by their homologous and heterologous antisera. Both organisms can be shown by agglutination with factor-specific antisera to possess the antigens IX,

obviously to interfere with the reading of titration end-points.

Similar, though rather less marked results have been obtained with suspensions of strong and weak agglutinators picked, by testing colonies with factor I antiserum, from the same plating of a strain of *Bact. paratyphosum* A, and rabbit sera against each suspension. While antiserum against the weak agglutinators gave the same titre with both suspensions, that against the strong agglutinators clumped the homologous suspension to between two and four times the heterologous titre.

It is clear that when dealing with *Salmonellas* undergoing somatic phase variation, the results of serological standardization may vary widely de-

Table 2. *The relative sensitivity to agglutination of a XII₂-dominant strain of Bact. typhosum and a XII₂-recessive strain of Bact. enteritidis by their homologous and heterologous antisera*

Rabbit antiserum against	*Titres shown by suspension of		Sensitivity ratio T/901/O/E:12316/JT
	T/901/O (XII ₂ -dominant)	E/12316/JT (XII ₂ -recessive)	
T/901/O (XII ₂ -dominant)	20,480	1,280	16:1
E/12316/JT (XII ₂ -recessive)	6,400	3,200	2:1

* Titres expressed as the reciprocal of the serum dilution concerned.

Table 3. *The relative sensitivity to agglutination of two strains of Bact. enteritidis by their homologous and heterologous antisera and by human sera from a case of proven typhoid fever*

Serum	Titres shown by suspension of			Sensitivity ratio EO/511:EO/570
	T/901/O (IX, XII ₁ , XII ₂ , XII ₃) (XII ₂ -dominant)	EO/511/44 (IX, XII ₁ , XII ₃)	EO/570/44 (IX, XII ₁ , XII ₂)	
E/511/44	6,400	6,400 ±	1,600	Between 2:1 and 4:1
E/570/44	51,200	800	12,800	1:16
Human typhoid fever 20th day	5,120	640	160	4:1
Human typhoid fever 26th day	5,120 ±	320	160	2:1

XII₂, XII₃ and, when in the same phase, will each absorb all agglutinin from the heterologous antiserum. They may, therefore, be regarded as qualitatively identical. Both are subject to phase variation of component XII₂. The stock culture of T/901/O used, however, is strongly in the XII₂-dominant phase and the number of weakly agglutinating colonies thrown off from it is very small. Moreover, despite the dominance of XII₂, component XII₃ appears to remain markedly reactive in this strain and capable readily of absorbing agglutinin from an anti-XII₃ serum. *Bact. enteritidis* strain 12316/JT, on the other hand, tends to cling to the XII₂-recessive phase and when in this phase will only with difficulty exhaust XII₂ antibody from a serum. The proportion of XII₂-dominant organisms in XII₂-recessive colonies of this strain is so small as not

pending on the particular colony picked both for serum production and for the preparation of agglutinable suspensions. This difficulty could to a large extent be overcome by selecting, with the aid of factor I and XII₂ antisera, only strong agglutinators for both purposes, a procedure which would, at the same time, ensure the clarity of agglutination end-points. Whether standardization against rabbit antisera by this or any other method has any real significance in relation to antibody response in human infection is another matter. The antigenic composition of a small number of recently isolated strains of *Bact. typhosum* has been investigated and all have proved as agglutinable as T/901/O by XII₂ antiserum after destruction of Vi antigen by treatment with saturated chloroform-saline. Sera from cases of typhoid fever and from persons

recently inoculated with T.A.B. Vaccine have been examined for agglutinins against two strains of *Bact. enteritidis*, E/511/44 with the somatic structure IX, XII₁, XII₃ and E/570/44 with the structure IX, XII₁, XII₂. Unlike rabbits, which usually respond in the most marked way to antigen XII₂, all the human sera tested have shown a higher titre against E/511/44 than against E/570/44. Table 3 shows the relative sensitivities of these two *Bact. enteritidis* strains as estimated by means of two rabbit antisera, one rich and the other deficient in anti-XII₂ agglutinin, and two samples of human serum from a case of typhoid fever showing an unusually high titre against T/901/O. It demonstrates clearly that standardization against a serum rich in XII₂ antibody bears no relation whatsoever to the agglutinin response in human infection, whereas the results given by rabbit antiserum deficient in XII₂ antibody accord reasonably well.

DISCUSSION

Despite the many assertions in the literature that *Bact. typhosum* and *Bact. enteritidis* possess identical O-antigens, and differ only in their H-antigens and in the possession of Vi antigen by the former organism, the work outlined here shows beyond doubt that their apparent identity is dependent on the colonies selected for comparison. Two colonies in opposite phase, even when picked from the plating of a single colony, can be shown to behave agglutinogenically like related but antigenically dissimilar types. It has been noticed that strains of *Bact. paratyphosum* A and *Bact. enteritidis* differ considerably in the ease with which variation can be demonstrated in them and that the tendency of any one strain to dissociate may alter under laboratory conditions. For example, *Bact. paratyphosum* A strain Beamish and *Bact. enteritidis* strain 12316/JT were subject to active variation when first examined shortly after isolation. Now, after many months of laboratory culture, both have become much more stable, one in the I-dominant and the other in the XII₂-recessive phase, so that a large number of colonies must be picked in order to demonstrate the alternate phase. Variation has only been demonstrated in British strains of *Bact. enteritidis*. All the Indian strains of this type which have been tested appear to fall into two distinct antigenic types depending on absence of one of the two components XII₂ or XII₃. It is interesting that these strains, in contradistinction to the usual pathogenic behaviour of European strains, have without exception been isolated by blood culture from invasive disease in man (Hayes & Freeman, 1945). The absence of demonstrable variation in them does not necessarily mean that variation does not occur, but simply implies that they display an abnormal degree of

antigenic stability. It is possible that variation does occur but that the number of organisms showing it comprise such a minute proportion of any culture that it cannot be demonstrated by the ordinary method of colony selection. An analogy can be found in the behaviour of the flagellar antigens of *Bact. typhosum* and *Bact. paratyphosum* A. These organisms are normally monophasic, but second, and even third and fourth flagellar phases in the case of *Bact. paratyphosum* A, can be induced by passage through antiserum against the prevalent phase (Edwards & Bruner, 1939, Bruner & Edwards, 1941). It is generally assumed that this is not the result of a simple adaptation to an unfavourable environment but is due to selection of the very small number of organisms in the culture which are capable of displaying the variation. By the use of this selective technique, transformation of one *Salmonella* type (*Bact. salinatis*) into another (*Bact. sandiego*) containing the same somatic but different flagellar antigens, has been effected (Dubos, 1945). So far as flagellar alternations are concerned, it thus appears that truly monophasic *Salmonellas* do not exist but represent diphasic types which have become to a large extent stabilized in one phase. From the biological point of view it seems not improbable that the behaviour of types showing somatic phase variation should be similarly interpreted. Referring to flagellar variability, Dubos suggests that 'the immunological complexity of *Salmonella* is due to the fact that the members of this bacterial group are in an unstable state and are undergoing evolution at a rapid rate'. Evidence that the somatic complex which, unlike the flagella, bears a close correlation with virulence, is similarly unstable opens up interesting possibilities in the field of *Salmonella* epidemiology. Much work remains to be done on the relationship between somatic variation and human infection. It is possible that this type of variation represents a means of bacterial defence and that a comparative inability of the human body to respond to antigen XII₂ might have some significance in the natural history of enteric fever.

Somatic phase variation might lend itself readily to the study of the mechanism of bacterial variation in general. Most observed continuous variations have been concerned with the ability of cultures biochemically to attack specific substrates, so that quantitative estimations of the numbers of individual cells in a colony or culture capable of exhibiting a certain change cannot be made. It has been shown that in a culture undergoing active variation in one of its antigens, no colonial population is devoid of cells possessing the antigen although in weak agglutinators the number of such cells is small and overshadowed by negative reactors. If it is allowed that single, well isolated colonies

spring from the growth of one organism, then it is clear that negatively reacting cells produce, on division, a certain number of positive reactors. The proportions of these two types of cell should be capable of quantitative assessment by relatively simple methods. The occurrence of a paradoxical end-point in agglutinin titrations, as described above, is probably indicative of active variation in a strain.

This type of variation and its immunological reflexion in the agglutinin content of antisera removes the theoretical basis for the standardization of the majority of O-suspensions used in the Widal Reaction. That such standardization seems to have worked well in the past is probably due to the relative stability of the laboratory strains used for serum and suspension production, so that markedly aberrant batches would only rarely be encountered and discarded. The examples chosen to demonstrate this fallacy in standardization represent, perhaps, the extremes of error which may be found. Nevertheless, the fact that these and other equally divergent results were obtained in the course of a largely routine investigation and caused considerable confusion until the significance of Kauffmann's observations was realized, shows that the problem is by no means only a theoretical one. In the absence of more knowledge of the antibody response to Salmonella infection in man it is difficult to decide whether the use of uniform suspensions, as assessed by titration against rabbit antisera, is of any value. In the case of T/901/O suspensions it is probable that past standardization has been based largely on the sensitivity to agglutination of antigen XII₂. It

seems that, in human infection, either this antigen is recessive in the organism or else the body responds weakly to it, since XII₂ antibody, if developed, is of lower than average titre. On the other hand, the recognition of phase variation has increased the need for standardization of some sort. In view of the reasonable uniformity shown by human sera both in typhoid fever and following T.A.B. Vaccine, these might more rationally be used as a basis for standardization until more information is forthcoming.

SUMMARY

1. Phase variation of the somatic antigen I in *Bact. paratyphosum* A and of the component XII₂ in *Bact. typhosum* and British strains of *Bact. enteritidis* has been confirmed.

2. Strains of *Bact. enteritidis* isolated in India by blood culture from cases of invasive disease in man fall into two serological types, neither of which undergoes demonstrable somatic phase variation.

3. The probable mechanism of this type of variation is discussed in the light of experimental findings.

4. The effect which phase variation may have on the clarity of end-points in agglutinin titrations and on the serological standardization of Salmonella O-suspensions is demonstrated.

5. The variation is briefly discussed from the points of view of Salmonella phylogeny and of human Salmonella infection.

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