

VARIATION IN BACTERIA IN RELATION TO AGGLUTINATION BOTH BY SALTS AND BY SPECIFIC SERUM.¹

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(PLATE II.)

THE varieties of bacteria with which the present paper is mainly concerned belong to the category commonly called "spontaneously agglutinating" strains. They have been obtained from cultures of *B. typhosus*, *B. paratyphosus* B., *B. enteritidis*, and *B. dysenteriae*. Most of the observations here recorded have been made on Shiga's bacillus, but the writer has been working with similar variants of *B. typhosus* and *B. enteritidis* since 1912, and has already recorded some observations on a spontaneously agglutinable strain of *B. typhosus* (Arkwright, 1914¹).

TWO FORMS OF VARIANT.

The chief variants now to be described are two in number, which for reasons given subsequently have been designated the "S" form, which makes good stable emulsions in 0.85 per cent. solution of sodium chloride, and the "R" form, which agglutinates spontaneously in salt solution of this concentration.

Both these forms differ in some degree from the original parent cultures which may be regarded as the normal. The relation of the two variants to the normal is such that the latter appears to contain representatives of them both, or to be composed wholly or in part of the characters of the two forms which can, as it were, be split off from the normal. The normal, at any rate superficially, resembles more nearly the "S" form. The observations which show the "S" form to be distinctly different from the normal and those which concern agglutination by specific serum have reference almost entirely to *B. dysenteriae* (Shiga). These variants have proved constant in their characters when subcultured in ordinary nutrient broth at intervals of about one week and cannot therefore be classed as mere modifications due to the environment. The expressions "Variant" and "Variation"

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are here used in respect of any change which is not merely due to temporary external conditions. What kind of variation is manifested by the forms "S" and "R" will be discussed later.

Cultures examined for "S" and "R" Forms.

Micro-organism.	Ref. No.	Source.	Forms obtained.
<i>B. dysenteriae</i> (Shiga)	47	Capt. Gilmour, Italy.	Agar, 3 mths. "S" and "R"
	190	" "	" " "S" " "R"
	236	" "	" " "S" " "R"
	177	" "	" " "S" " "R"
	550	" "	" " "S" " "R"
	862	" "	" " "R" only.
	"L.I.P.M."	Lister Institute.	" " 76 days. "S" and "R"
	"Parker"	" "	" " 1 mth. "S" " "R"
	"Wynne"	" "	" " 47 days. "S" " "R"
	215	Capt. Gilmour, Italy.	" " 95 " "S" only.
<i>B. dysenteriae</i> (Flexner-Y)	573	" "	" " 99 " "S" and "R"
	577	" "	" " 99 " "S" only.
	"Simpson"	Lister Institute.	" " 80 " "S" only.
<i>B. typhosus</i>	"Guy"	" "	" " 53 " "S" and "R"
	"Lincoln"	" "	" " "S" " "R"
	"F"	Dr J. C. G. Ledingham,	" " "S" " "R"
	"Schottmüller"	Lister Institute.	" " 75 " "R" only.
<i>B. paratyphosus B.</i>	"Tidy"	" "	" " 82 " "R" only.
	"Van Ermenghem"	" "	" " "S" only.
	"Lab"	" "	" " "S" only.
	"Rat"	" "	" " "S" and "R"

The strains of *B. dysenteriae* obtained from Captain Walter Gilmour, R.A.M.C., were isolated by him in Italy in 1918, and very kindly sent to me in the autumn of that year. They were examined by me in December 1919 and subsequently.

The strain of *B. typhosus*, "F," was isolated by Dr Ledingham from a carrier. The "R" form was obtained by him by plating on MacConkey's medium after growth in immune typhoid rabbit serum.

All the original cultures mentioned above and the "S" and "R" forms derived from them gave the typical cultural reactions in carbohydrate media and in milk; they also behaved normally as regards the production of indol and motility, except that the "R" forms of bacteria which were normally motile showed little or no motility.

Both the "S" and "R" forms and the sub-variant "RV" of *B. dysenteriae* (Shiga) 550 were fatal to rabbits when injected subcutaneously or intravenously in very small doses, but no attempt was made to find the M.L.D.

CHARACTERS OF THE VARIANTS.

The most obvious distinguishing characteristic of the "R" form is the property which emulsions possess of forming clumps and precipitating in solutions of sodium chloride. The percentage of salt which leads to agglutination in the case of different strains varies considerably. Often emulsions of the "R" form are stable in saline solution of half or one-quarter the usual strength (*i.e.*, in solutions containing 0.42 or 0.21 per cent. NaCl); but sometimes clumping and precipitation occur in saline diluted eight times (0.1 per cent.), or even in weaker solutions. Agglutination occurs in much weaker solutions of calcium chloride (Table I.) as is usually the case with

emulsions or colloidal solutions which are precipitable by weak electrolytes (*e.g.*, Tulloch, 1914³⁸).

In liquid culture media the "R" form is distinguished from the "S" form and from the normal by the appearance of the growth. Cultures of the "R" form in ordinary nutrient broth or peptone water produce a deposit at the bottom leaving the liquid above clear, instead of making it turbid, as do cultures of the normal or the "S" form. This precipitation of the bacteria in the culture tube is due to the salt content of the medium, for if the broth is diluted with distilled water to one-half or one-quarter of its original strength, or if the medium is made with less salt, the growth of the "R" form also causes uniform turbidity with little or no deposit.

On solid media the differences between the colonies of the two forms are often very characteristic. (Plate II., Figs. 1, 2, 3, and 4.)

The main characters given below apply to all the kinds of bacteria mentioned, though there are individual differences and the descriptions given are not intended to be exhaustive. The appearances to be described refer to discrete colonies on plates of ordinary nutrient agar which have been at 37° C. for about twenty-four hours. Similar appearances are seen on plates of MacConkey's medium, and the peculiarities of the "R" colonies may then be rather exaggerated.

Colonies of the "S" form have a smooth and glistening surface, are raised, dome-shaped, and round, with smooth, regular, well-defined margins: they are translucent, and when examined with a low power of the microscope (1 inch) appear quite smooth or only very finely granular.

The shape of colonies of the "R" form is more varied, but their appearance is often quite distinctive. In the most characteristic form they are larger than the "S" colonies, flat and thin, with a slight central boss and have a jagged or irregular, waved or indented margin; the surface is coarsely granular, recalling the appearance of very finely-grained morocco leather, and may be marked by irregular branching lines. By transmitted light they appear slightly opaque, or have a frosted appearance, especially when seen through a hand lens: they are seen to be coarsely granular under a low power of the microscope. The irregularity of the surface has led to this variant being called the Rough ("R") form in distinction from the Smooth ("S") form. The irregular shape of the "R" colonies is probably due to the way in which the individual bacilli cohere in the presence of sodium chloride, forming many centres of growth instead of spreading evenly from a single centre.

The variety of colonies met with is so great and the differences between them often so indefinite that attention has purposely been directed only to the more obvious characters which are definitely associated with especial behaviour in broth cultures and in emulsions in salt solution. It is not, however, intended to convey the impression that colonies of the "S" and "R" types can always be readily distinguished. The behaviour on agar and in broth must be examined before a variant is definitely regarded as of the "S" or "R" form.

Some varieties of colonies have been met with which corresponded

with those described by Baerthlein (1912,³ 1918⁴), but they appeared for the most part to be intermediate between the "S" and "R" forms and to yield uncertain results when tested in respect to their agglutinability by salt.

Besides the colonies which appear to be definitely "S" or "R" in character, others of a mixed type are often seen which at first are smooth and later become irregular in outline and more or less uneven on the surface. Colonies in part translucent and in part opaque or granular are also seen which appear to be composed of bacilli of both "S" and "R" form. These latter colonies look very much like mixed colonies of two entirely different kinds of bacteria, whereas they are really composed of the two forms imperfectly blended.

Different colonies of the "R" form of the same kind of bacillus may be very unlike each other in appearance. (See Plate II., Fig. 4.)

When the "R" form is replated, marked differences often appear in the colonies which seem to indicate degrees of specialisation of this variety, *e.g.*, some colonies may definitely be larger, thinner, and much rougher on the surface, and this difference is apparently associated with a tendency to agglutinate and precipitate in weaker salt solution.

In the case of *B. dysenteriae* (Shiga) I have found one sort of colony commonly associated with the characters of the "S" form and two with the "R" form. The "S" colonies are round, shiny, smooth, sharply defined, raised, domed, and translucent; the "R" colonies may be (1) flat and thin with very irregular outlines and rough, granular, or wrinkled surface; they resemble the "R" colonies of *B. typhosus*. These are larger than the "S" colonies and may be 4 to 6 mm. in diameter; (2) more commonly they are raised and moderately thick, but flattened, rather larger than the "S" form, and have indented or slightly jagged margins; and are also somewhat opaque. They are seen to be granular under a low power of the microscope, and may appear finely granular or frosted with a hand lens; the surface is usually dull. These latter colonies are associated with a sliminess which is apparent in emulsions.

One other type of colony, "RV" (= "R" variant), must be described, however, since it was definitely associated with the property of forming clear cultures with a deposit in broth and of agglutinating spontaneously in salt solution. These colonies were very small, round, smooth, and of an unusually coherent or sticky consistency. They appeared on agar plates inoculated from a culture of *B. dysenteriae* (Shiga) 550 form "R," which had been growing in pure horse-serum at 37° C. for seven days. Only this type of colony was present on the plates inoculated on three different occasions, *i.e.*, the seventh, fourteenth, and twenty-first days of growth in horse-serum. These colonies were difficult to subculture, since when picked off to broth they almost invariably failed

to grow. Subcultures were, however, obtained on agar or in glucose peptone water, but these at first died after two or three days. It was not till after several subcultures on agar at short intervals that growth could be obtained in broth or on agar which was viable after seven days. The resulting free growth on agar formed emulsions in distilled water which were slimy and agglutinated in 0.1 per cent. sodium chloride solution. The results of agglutination experiments with specific sera prepared with this variant are recorded later in this paper.

CHANGES IN OTHER CULTURAL CHARACTERS AND MORPHOLOGY ASSOCIATED WITH AGGLUTINABILITY IN SALT SOLUTION.

(1) A surface film in broth cultures is very often formed by the "R" form whether of *B. typhosus* or *B. dysenteriae*, but the "S" form occasionally also forms a film.

(2) Sliminess of emulsions from agar slopes is very often present in the "R" form of Shiga strains, especially in cultures from flat, thick "R" colonies, and has also been noticed to a smaller degree in the "R" form of *B. dysenteriae* (Flexner-Y), and of *B. typhosus*. It has not always been noticeable in my Shiga "R" forms, and certainly may be absent in the "R" forms of *B. typhosus* and *paratyphosus*. This character appeared to be associated with large swollen forms of the bacilli, but capsules could not be demonstrated and no large swollen mucoid colonies appeared on agar as in the case of the "mucoids" described by Revis (1910³⁰) and Fletcher (1918¹⁴).

(3) The morphology of the bacteria varied very much in different one-day old agar cultures, and to a large extent varied independently of the "S" and "R" characters. The individuals of the "R" type were usually rather wider and had rounded ends. Occasionally the papillated or shagreened surface of rough colonies was associated with very long thread-like bacteria.

When the slimy character was well marked in the "R" form, some of the bacteria were usually very much swollen, *e.g.*, two or three times the diameter of normal individuals and were often very irregular in shape. Sometimes they were forked or had knob-like protrusions on their side, and many ill-stained forms varying very greatly in size and shape were also seen. In these cultures there were also very great differences in the size and shape of the well-stained bacilli, both as regards breadth, length, and the uniformity in width of single bacilli, and these irregularities were often not confined to the "swollen" individuals. (See Plate II, Figs. 5 and 6.)

Source and Frequency of Occurrence.—The source of the "R" variants has been almost invariably old broth or agar cultures. They may be obtained from most strains if a culture which has been kept at room temperature or in the incubator for a month or more is plated out, but

the proportion of such colonies varies very much in different strains, and some strains have not so far been found to yield the "R" form. Of nine agar cultures of *B. dysenteriae* (Shiga) over a month old all yielded this variety; eight did so at once when plated, and the other strain (Wynne) which did not on the first plating, did so on a subsequent occasion.

Degree to which "R" Characters are manifested by Different Cultures.—The agglutinability by salt is to a certain extent a matter of degree. Most "R" variants obtained from *B. typhosus*, *B. paratyphosus* B., and *B. enteritidis* (Gaertner) were agglutinated in 0·85 per cent. but not in 0·42 per cent. NaCl. Variants of *B. dysenteriae* (Shiga) were usually not quite stable in 0·42 per cent., but did not agglutinate in 0·21 per cent. NaCl; again a variant obtained from *B. typhosus* (Guy), one from *B. dysenteriae* (Flexner-Y) 573, and the variant "RV" obtained from *B. dysenteriae* (Shiga) 550 were stable in 0·05 per cent. but not in 0·1 per cent. (i.e., in 1/16 but not 1/8 saline). (Table I.)

TABLE I.—Agglutination of "S" and "R" Forms of Bacteria by Salts.

	NaCl.					CaCl ₂ .									D.W.
	M 1	M 2	M 4	M 8	M 16	M 2	M 4	M 8	M 16	M 32	M 64	M 128	M 256		
<i>B. typhosus</i> Strain "Guy"															
"S" form	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
"R" form	+++	+++	+++	++	-	++	+++	+++	+++	+++	+++	+++	+++	-	
<i>B. enteritidis</i> (Gaertner)															
"S" form	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
"R" form		-	++	+	-		+++	+++	+++	+++	+++	+++	+	-	

M/1, M/2, M/4, etc. = Molar 1/1, 1/2, 1/4, etc.

+++, ++, + = complete, well marked, and slight agglutination.

NaCl M/1 = 5·8%. CaCl₂ M/2 = 5·5%. D.W. = Distilled Water.

Reversion.—When after selection of the colonies one of the variants "S" or "R" has been obtained apparently quite pure, a change into the other form is not so easy to bring about as when unselected cultures have shown the "S" or "R" characters. The reason of this would appear to be that in the latter case the character of the culture is not "fixed," i.e., the culture does not consist of uniform members. Thus *B. typhosus* (Guy) was separated into two forms "S" and "R" by plating twice from an old culture. When subcultured weekly the two forms have remained distinct, but when subcultured daily the "R" form showed some turbidity at the 4th subculture, and at the 10th subculture showed much turbidity and hardly any deposit, in this way indicating that the culture contained the "S" form in a pure or nearly pure state. A culture of the "R" form of *B. paratyphosus* B. (Tidy) became slightly turbid at the 2nd or 3rd daily subculture in broth,

and at the 18th subculture the turbidity was uniform and the deposit only very slight. The estimation of this change by the characters of broth cultures alone is not always quite easy, because the "R" variants often form a surface film in broth, and this readily breaks up and causes slight temporary turbidity. The presence of a film is, however, shown by the clearing of the broth and the appearance of a deposit on the following day. The changes found in broth cultures need to be corroborated by plating out and observing the shape and appearances of the colonies. When a culture is changing from "R" to "S" or from "S" to "R," but perhaps less so in the latter case, the colonies are often of a mixed or indefinite character till a late stage in the change, when pure "S" or "R" forms of colony can be picked off plates.

B. dysenteriae (Shiga) 550 form "R" when subcultured daily showed no sign of changing into the "S" form either by production of turbidity in broth or by the appearance of "S" colonies on plates when subcultured daily forty-eight times, but the 49th, 50th, 51st, 52nd, and 53rd subcultures were slightly turbid and some smooth or partly smooth colonies appeared on plating. These, however, did not behave towards salt solution and specific serum like the "S" form though culturally like the original stock culture.

B. dysenteriae (Shiga) 862 "R" showed slight turbidity in broth at the 18th, 19th, 20th, and 21st subcultures, but no distinctly smooth colony could be found after plating.

Similar results were obtained with other strains of *B. dysenteriae* (Shiga). On the other hand, a broth culture of "Shiga" 550 "S" at the 25th daily subculture was plated when a month old and still gave all smooth colonies; the 40th subculture when plated after nineteen days at 37° C. gave a few rather opaque colonies which in broth formed a large deposit with slight turbidity, and on replating this broth some definitely "R" colonies appeared which were semi-opaque and irregular in outline. These observations may be summarised by saying that the reversion from pure variants of *B. dysenteriae* (Shiga) is slow, and the first colonies of changing type are apt not to be pure. The mixed type of colony is more often seen when a culture is changing from "R" to "S" than when the reverse change is proceeding. The "R" form can almost always be obtained from the "S" form, but the "S" form cannot so uniformly be obtained from "R" cultures especially in the case of *B. dysenteriae*.

Changes observed as regards Specific Agglutination.

Method of Agglutination.—The macroscopic method was used with the assistance of microscopical examination of the deposit in some cases. The emulsions were always made in distilled water and the serum was diluted with physiological saline (0.85 per cent. NaCl), or a weaker solution if the emulsions were very readily agglutinated by salt. The emulsions and dilutions of serum all contained 0.5 per cent. phenol.

The agglutination was carried out in a 37° C. incubator for four hours or sometimes longer. Agglutination was considered to be complete if the liquid was quite clear and if there was a well-defined deposit distributed over the concavity at the bottom of the test-tube.

Examination of the "Shiga" strains was first made by means of a stock agglutinating serum which had been prepared from three strains of Shiga's bacillus and had a titre for stock strains of 1/600 to 1/800.

In order to test the agglutination of the "R" form, it was necessary to use 0.2 per cent. NaCl solution, since emulsions in stronger saline were not always stable. This strength was therefore used for all strains and forms of *B. dysenteriae* (Shiga). (Table II.)

TABLE II.—*Agglutination of B. typhosus Emulsions of Normal, "S" and "R" Forms, with Specific Serum $\frac{1}{500}$ and Varying Salt Content.*

NaCl.	M 6 0.97%	M 12 0.48%	M 24 0.24%	M 48 0.12%	M 96 0.06%	M 192 0.03%	D.W.
<i>B. typhosus</i> , Normal .	+	+++	+++	+++	++	+	-
"S" .	++	+++	+++	+++	+++	++	-
"R" .	-	+++	+++	+++	+++	+	-

It is well known that the strength of salt required to produce agglutination of sensitised bacteria is much less than that commonly used. With a moderately strong dilution of serum the optimum concentration of salt is sometimes less than 0.85 per cent. and a concentration of about 1/25 of the ordinary strength (*i.e.*, 0.034 per cent. NaCl) is quite sufficient. It has, however, been shown that when the serum is highly diluted, rather more salt is required than when the serum is stronger.

Type of Agglutination: Size of Clumps.—It has often been noticed that the type of agglutination is not the same for all kinds of bacteria nor even for different strains of the same kind. For instance, the agglutination of some strains of *B. typhosus* (*e.g.*, "Simpson," *vide* p. 37) takes place in large loose clumps which settle near the bottom of the tube in a loosely flocculent mass like a "cumulus," whilst other strains such as "Guy" settle in a dense concave layer and the individual clumps are smaller. Dysentery bacilli as a rule agglutinate in small dense clumps and form a thin granular layer distributed over the concave surface at the bottom of the tube, but the size of the individual clumps may vary very much. In some strains, on shaking the tube, the deposit breaks up into large pieces which are with difficulty shaken into a finely granular emulsion; in other strains, the deposit shakes up more readily and a few large clumps are then seen, but the resulting emulsion consists chiefly of small clumps. In still other strains (especially perhaps of *B. dysenteriae*, Shiga) the deposit when shaken up forms a

fine muddy emulsion in which no clumps can be seen with the naked eye, or even a hand lens ($2\frac{1}{2}$ -inch focus); at least this has been the experience of the writer during routine examination of the faeces of dysentery patients. A drop of this resulting emulsion put under the microscope is seen to consist of a suspension of small dense clumps containing four to twenty bacilli each. When an emulsion of the "S" form of *B. dysenteriae* (Shiga) is agglutinated the deposit is dense, and when shaken gives a suspension of large clumps. The deposit formed by agglutinating the "R" form, however, when shaken up forms an emulsion appearing uniformly turbid, the clumps being too small for recognition until a low power ($1/6$ inch) of the microscope is used. Even in the case of "R" emulsions, however, the appearance of the agglutinated deposit is quite characteristic of true agglutination as regards its distribution over the concave bottom of the tube, and as distinguished from the deposit in control tubes in which the un-agglutinated bacteria are collected in a very small compass at the centre of the concavity. These differences between the agglutination of "S" and "R" forms occur whether the agglutination has taken place at 37° or at 55° C. When the precaution is taken of using weak salt solution (0.2 per cent. NaCl), and the different types of agglutination by the two forms are taken into account, it is found that both forms "S" and "R" of *B. dysenteriae* (Shiga) 550 agglutinate to approximately the same titre, $1/640$, as the parent strain with a stock serum, prepared from three strains of Shiga.

Absorption of Stock Serum.—These experiments were carried out by mixing 1 c.c. of serum diluted $1/10$ in 0.85 per cent. salt solution with 1 c.c. of a strong emulsion of bacilli in distilled water; the emulsion was made by adding the growth on one agar slope to 1 c.c. of water. After incubation at 37° for two hours the mixtures were put in the cold room till the next day and then centrifuged. The mixtures contained 0.5 per cent. of phenol. (Tables III., IV.)

TABLE III.—*Agglutination by Stock Specific Serum, before and after Absorption by B. dysenteriae (Shiga), Strain 550, Forms "S" and "R." The Serum diluted with Solution of NaCl 0.42 per cent.*

Stock Serum v. <i>B. dysenteriae</i> (Shiga).	$\frac{1}{200}$	$\frac{1}{400}$	$\frac{1}{800}$				NaCl. 0.42%
<i>Unabsorbed</i> —							
Emulsion 550 "S" .	+++	+++	+				—
„ 550 "R" .	++	++	+				—
<i>Absorbed by 550 "S"</i> .	$\frac{1}{40}$	$\frac{1}{80}$	$\frac{1}{160}$	$\frac{1}{820}$	$\frac{1}{640}$	$\frac{1}{1280}$	
Emulsion 550 "S" .	+++	—	—	—			—
„ 550 "R" .	+++	+++	+++	++			—
<i>Absorbed by 550 "R"</i> .							
Emulsion 550 "S" .	+++	+++	+++	+++	++	+	—
„ 550 "R" .	+++	++	+	—	—	—	—

TABLE IV.—*Agglutination of "S" and "R" Forms of Heterologous Strains of B. dysenteriae (Shiga) by Stock Specific Serum before and after Absorption with B. dysenteriae (Shiga), Strain 550, "S" and "R" Forms. Diluent 0.21 per cent. NaCl.*

Stock Serum.	$\frac{1}{80}$	$\frac{1}{160}$	$\frac{1}{320}$	$\frac{1}{640}$	NaCl. 0.21%
<i>Unabsorbed—</i>					
<i>B. dys.</i> 47 III. "S" .	+++	+++	+++	+++	—
" 47 V. "R" .	+++	+++	++	+	—
" 177 II. "S" .	+++	+++	+++	++	—
" 177 IV. "R" .	+++	+++	+++	+	—
<i>Absorbed by 550 I. "S"—</i>					
<i>B. dys.</i> 47 III. "S" .	+++	—	—	—	—
" 47 V. "R" .	+++	+++	+++	++	—
" 177 II. "S" .	+++	—	—	—	—
" 177 IV. "R" .	+++	+++	+++	+	—
<i>Absorbed by 550 II. "R"—</i>					
<i>B. dys.</i> 47 III. "S" .	+++	+++	+++	+++	—
" 47 V. "R" .	+	+	—	—	—
" 177 II. "S" .	+++	+++	+++	+++	—
" 177 IV. "R" .	++	—	—	—	—

The results of the absorption experiments showed that serum absorbed with the "S" form of Shiga 550 had lost the greater part of its power of agglutinating form "S," whether this was derived from strains 550, 47, or 177, but that it still agglutinated the "R" form derived from these strains nearly as well as before. After absorption with form "R" derived from 550 this form, whether derived from 550, 47, or 177 was subsequently very feebly agglutinated, whereas form "S" from all these three strains was agglutinated as well as before absorption of the serum.

It was also found that absorption with the "R" form of 550 much diminished the agglutinating action on form "R" of strain 236, but absorption with the "S" form of 550 did not do so; and conversely the "R" form of 236 absorbed the agglutinins for the "R" form of 550 but not for the "S" form of 550.

The "S" form of strain 236 of Shiga has not so far been examined.

Agglutination with Special Single Sera.—Single sera were prepared from rabbits by inoculating intravenously emulsions of "S" and "R" forms of *B. dysenteriae* (Shiga) which had been killed by heat. The exceptional form "RV" was also inoculated into a rabbit. All three forms were found to be very fatal to rabbits, but after two or three inoculations of very small doses sufficiently good agglutinating sera were obtained.

The "S" serum obtained by two inoculations agglutinated 550 "S" up to a titre of about 1/320 but 550 "R" scarcely above 1/80, whereas the "R" serum agglutinated 550 "R" in a dilution of 1/320, but did not affect 550 "S" above 1/80. Six other strains of Shiga (Parker, L.I.P.M., 190, 47, 177 and Wynne) which had also been split into "S" and "R" forms were also tested with both sera "S" and "R."

Similar results were obtained with these heterologous strains. The 550 "S" serum agglutinated all the "S" forms and the 550 "R" serum all the "R" forms, but there was very little cross-agglutination between the "S" and "R" forms. (Table V.)

TABLE V.—*Agglutination of the "S" and "R" Forms of Different Strains of B. dysenteriae (Shiga) by Sera made with the "S" and "R" Forms of Strain 550. The Serum diluted with NaCl 0·2 per cent.*

Serum v. <i>B. dys.</i> 550 I. "S."	$\frac{1}{80}$	$\frac{1}{160}$	$\frac{1}{320}$	$\frac{1}{640}$	$\frac{1}{1280}$	NaCl. 0·2%	NaCl. 0·42%	NaCl. 0·85%
Emulsion 550 I. "S" . . .	+++	+++	++	+	—	—	—	—
" 550 II. "R" . . .	++	+	—	—	—	—	—	++
" Parker I. "S" . . .	+++	+++	+++	+++	—	—	—	—
" II. "R" . . .	++	—	—	—	—	—	—	+++
" L.I.P.M. I. "S" . . .	+++	+++	+++	+++	+++	—	—	++
" v. "R" . . .	+++	++	++	—	—	—	—	++
" 190 I. "S" . . .	+++	+++	+++	+++	—	—	—	+
" 190 III. "R" . . .	++	—	—	—	—	—	—	—
" 47 III. "S" . . .	+++	+++	+++	+++	+	—	—	—
" 47 v. "R" . . .	—	++	+	—	—	—	—	—
" 177 II. "S" . . .	+++	+++	+++	++	+	—	—	—
" 177 IV. "R" . . .	—	++	++	—	—	—	—	—
" Wynne I. "S" . . .	+++	+++	+++	+++	+++	—	—	—
" III. "R" . . .	+++	+++	+	++	—	—	—	—
Serum v. <i>B. dys.</i> 550 II. "R."								
Emulsion 550 I. "S" . . .	++	—	—	—	—	—	—	—
" 550 II. "R" . . .	+++	+++	+++	—	—	—	—	—
" Parker I. "S" . . .	++	++	—	—	—	—	—	—
" II. "R" . . .	+++	+++	++	++	—	++	+++	—
" L.I.P.M. I. "S" . . .	+++	++	++	—	—	—	—	++
" II. "R" . . .	+++	+++	+++	++	+	—	—	++
" 190 I. "S" . . .	+++	+++	++	++	—	—	—	—
" 190 III. "R" . . .	+++	++	+	++	—	—	—	+
" 47 III. "S" . . .	—	++	—	—	—	—	—	—
" 47 v. "R" . . .	—	+++	+++	++	++	—	—	—
" 177 II. "S" . . .	—	+++	+++	+++	+	—	—	—
" 177 IV. "R" . . .	—	+++	+++	+++	+	—	—	—
" Wynne I. "S" . . .	—	+++	+++	+++	+	—	—	—
" III. "R" . . .	—	+++	++	+	—	—	—	+

+++ = complete agglutination.
++ = good

+ = distinct agglutination.
++ = very slight

The rabbit inoculated with 550 "RV," a sub-variant of 550 "R," gave a serum which agglutinated 550 "R" but not 550 "S." The emulsion of "RV" was partially agglutinated in the strength of saline (NaCl 0·2 per cent.) used for the test. (Table VI.)

TABLE VI.—*Agglutination by Serum made with B. dysenteriae (Shiga), Strain 550, Form "RV." The Serum diluted with Solution of NaCl 0·2 per cent.*

Serum v. <i>B. dys.</i> 550 "RV."	$\frac{1}{80}$	$\frac{1}{160}$	$\frac{1}{320}$	$\frac{1}{640}$	NaCl. 0·21%	NaCl. 0·42%
Emulsion 550 I. "S" . . .	+	—	—	—	—	—
" 550 II. "R" . . .	+++	+++	+++	++	++	++
" 550 RV" . . .	+++	+++	++	+	++	++

Sera prepared with "S" and "R" forms of a strain of *B. dysenteriae*, Y, No. 573 have given similar results so far as they have been tested. The two forms apparently had quite distinct agglutinins as shown by

direct agglutination and by absorption, and there was only slight cross-agglutination. The "S" serum was of much higher titre (1/5120) than the "R" serum (1/1280).

It was found necessary to use 0.1 per cent. NaCl for this test, since the "R" emulsion was not quite stable in 0.2 per cent. An experiment made with 0.2 per cent. NaCl gave almost exactly the same titre of agglutination with both sera for both strains, but the control with 0.2 per cent. NaCl of the "R" emulsion was not quite negative. (Table VII.)

TABLE VII.—*Agglutination of the "S" and "R" Forms of B. dysenteriae (Flexner-Y), with Sera made with the same Two Forms, before and after Absorption with Homologous Cultures.*

Serum v. <i>B. dys.</i> 573 "S."	1 80	1 160	1 320	1 640	1 1280	1 2560	1 5120	1 10240	1 20480	NaCl. 0.1%	NaCl. 0.2%	NaCl. 0.4%
Unabsorbed— Emulsion 573 "S"	+++	+++	+++	+++	+++	+++	+++	++	+	-	-	-
„ 573 "R"	+++	++	+	-	-	-	++	-	-	-	-	+
Absorbed 573 "S"— Emulsion 573 "S"	+++	+++	+++	+++	++	-	-	-	-	-	-	-
„ 573 "R"	++	++	-	-	-	-	-	-	-	-	-	-
Absorbed 573 "R"— Emulsion 573 "S"	+++	+++	+++	+++	+++	+++	+++	+	-	-	-	-
„ 573 "R"	++	+	-	-	-	-	-	-	-	-	-	-
Serum v. <i>B. dys.</i> 573 "R."												
Unabsorbed— Emulsion 573 "S"	++	+	-	-	-	-	-	-	-	-	-	-
„ 573 "R"	+++	+++	+++	+++	+++	+	-	-	-	-	-	-
Absorbed 573 "S"— Emulsion 573 "S"	-	-	-	-	-	-	-	-	-	-	-	-
„ 573 "R"	+++	+++	+++	+++	+	-	-	-	-	-	-	-
Absorbed 573 "R"— Emulsion 573 "S"	-	-	-	-	-	-	-	-	-	-	-	-
„ 573 "R"	++	+	-	-	-	-	-	-	-	-	-	-

DISCUSSION.

Variation in the Morphology of Colonies.

I. The method of obtaining variants by selecting colonies of different morphology in plate cultures has been adopted by many writers.

Cobbett and Phillips (1897¹¹) described colonies of two distinct types in gelatine plate cultures of *B. diphtheriae*. They obtained them from six different strains. On gelatine both types remained unchanged through many subcultures, though occasionally some of the larger type appeared on plating out subcultures of the smaller type.

Slawyk and Manacatide (1898³³) described permanent or very persistent variants obtained by the selection of colonies on agar plates of *B. diphtheriae*. The strains obtained differed in the vigour of their growth and in the size and form of their colonies. The present writer has also confirmed these observations. Baerthlein (1912³ and 1918⁴) has published a large amount of work in recent years on the subject of colony variation, and though other workers have not

always been able to confirm his descriptions of colonies, or to find all his variants stable, this may in part be due to the difficulty in satisfactorily describing colonies and to differences in strains, culture-media, etc. Working with *B. typhosus*, *B. paratyphosus*, *B. dysenteriae*, and *V. cholerae* he obtained many variants which were constant in subculture as regards the appearance of the colonies and in some cases as regards the morphology of the bacilli and other characters.

II. It is interesting to try and form an opinion as to the relation of the "S" and "R" forms to the stock cultures which are usually met with in the laboratory. Most strains when examined by plating old cultures can be made to yield both "S" and "R" forms.

If young stock cultures which have been frequently subcultured are plated, the colonies are not of two distinct types but often show intermediate characters, or the colonies may appear to belong for the most part to the "S" form. When inoculated into broth the culture is usually turbid. These stock cultures may agglutinate with both "S" and "R" form sera. It would seem that the colonies from these cultures are made up of bacteria capable of producing both "S" and "R" forms, but that the two forms are not necessarily actually present. In old cultures, however, the "S" and "R" forms appear separately and form separate colonies.

III. No attempt has yet been made to grow the two forms from a single cell. However, since the individuals of the "S" form do not tend to cohere in salt solution emulsions nor in broth cultures, it seems unlikely that isolated colonies of the "S" form are often formed from more than one bacillus; and the "R" form can almost always be derived from old cultures of the "S" form.

If it is assumed that single colonies represent single bacilli, then it seems probable that the two characters are potentially present in all or most of the individual bacilli in young cultures which have been subcultured fairly frequently, and that these characters to a certain extent become segregated in different individuals in old cultures.

This hypothesis of the segregation of the characters in old cultures is also suggested by Ledingham's (1918¹⁶) experience with a reverting strain of *B. dysenteriae* (Flexner-Y). Starting with an isodulcitate fermenting culture he found that a subculture when plated on an isodulcitate neutral-red agar gave (1) red colonies (2) white or pale red colonies which after forty-eight hours became deep red and later produced white papillae. This behaviour suggests that in some of the individual bacilli plated the character of fermentation was feebly represented and did not suffice to make the colony deep red till the second day, by which time more acid had been produced. As the culture got older the character of "non-fermentation" became segregated into individual bacilli which produced white papillae composed of "non-fermenters." Some of the colonies which formed papillae described by Penfold (1910²⁶) suggest the same interpretation.

IV. The third form of Shiga 550 ("RV") met with in the course of these observations presents another problem. It appears to be a further development of the "R" form, since it was obtained in apparently pure culture from a flask inoculated with form "R" of strain 550, and it is normal in its other characters (sugars, indole, motility). Emulsions were agglutinated and precipitated readily in very weak salt solution (0.1 per cent. NaCl). A serum prepared with it agglutinated form "R" of 550 but not form "S."

V. Spontaneous or pseudo-agglutination was noticed and commented on in the years which immediately followed the discovery of specific agglutination by Grubler and Durham, and variant cultures, which showed spontaneous clumping and consequently presented difficulties in the way of the specific agglutination test, have been often described.

Nicolle (1898²⁴) described spontaneous agglutination in broth cultures of *B. typhosus*. Cultures twenty-four hours old were quite clear with a small granular deposit. He found that these cultures had only a slight sensitiveness to serum.

Savage (1901³²) examined the phenomenon of pseudo-clumping in cultures of *B. typhosus*, but found no cause or remedy except that it occurred more frequently in old laboratory cultures, and he did not encounter it in salt solution emulsions from agar nor in peptone-water cultures but only in the presence of broth or meat extract.

Neisser and Friedemann (1904²³) and Bechold (1904⁵) made numerous experiments on the agglutination of sensitised and unsensitised bacteria in different solutions of salts, with a view to studying the relation of specific agglutination to the aggregation and precipitation of particles in inorganic suspensions. However, neither they nor subsequent writers appear to have used a salt solution of diminished concentration to dilute the serum as a means of overcoming the difficulty of testing the serum-agglutinability of spontaneously agglutinating cultures.

Porges and Prantschoff (1906²⁰) attempted to explain the cause of spontaneous agglutinability, and showed that heating an emulsion of *B. typhosus* to 80° C. (or 65° C. sometimes) removed spontaneous agglutinability, but agglutination by specific serum was also diminished or abolished. In the case of cholera vibrios, heating to 80° in some instances made the emulsion stable in salt solution without preventing agglutination by specific serum.

Paltauf (1912²⁵) in a review of the whole subject of agglutination says that a tendency to spontaneous agglutination by strains of bacteria may be due to the culture medium having been of an unusual or unsuitable reaction, or to the culture being an old one. He also says that if on subculturing on to a suitable medium the emulsion is not stable, and heating the emulsion to 80° C. does not improve it, then the culture must be considered unsuitable for the usual direct agglutination test.

Von Lingelsheim (1913¹⁷) and Sachs-Mücke (1913³¹) described as the "Q" form a variant obtained by plating old cultures of *B. typhosus*. Emulsions in salt solution agglutinated spontaneously. In broth cultures the growth formed a deposit and a surface film leaving the liquid clear. Colonies on plates were large and flat, irregular in outline, dry and coarsely granular on the surface. The emulsions were slimy and the bacilli cohered and showed hardly any motility; otherwise the cultural characters were those of normal *B. typhosus*. Direct agglutination was not tested on account of the spontaneous clumping,

and no attempt at absorption of agglutinins is recorded, but a serum made by immunising with the "Q" form agglutinated other strains of *B. typhosus*. This form persisted in cultures for five years and its peculiar characters survived passage through a mouse. Occasionally some colonies occurred which showed reversion to the normal. Similar forms, but less persistent, were obtained from *B. paratyphosus* B. and *B. enteritidis* cultures. V. Lingelsheim considered that all the characters of the "Q" form might be explained by the presence of a sticky inter-bacillary substance due to a kind of capsule formation.

Baerthlein (1918⁴) states that he obtained inagglutinable variants from all groups of bacteria with which he was working except *V. cholerae*, but he was usually able to obtain sera which agglutinated the original strains by inoculating the inagglutinable variants into rabbits. He does not describe changes of agglutinability such as to suggest a change in antigenic structure of the variant, except in the case of (1) a varying *B. paratyphosus* B. strain and (2) a variant of *B. dysenteriae* "Y."

The "R" form described in this paper corresponds very closely as regards the appearance of the colonies with some of the "inagglutinable" variants of *B. typhosus*, *B. paratyphosus*, and *B. dysenteriae* "Y" described by Baerthlein (1918). One of his *B. paratyphosus* variants was sometimes inagglutinable, and sometimes spontaneously agglutinable and did not absorb agglutinins for the other variants of the same strain. It seems probable that these feebly agglutinating and "inagglutinable" variants described by the above writers were in many cases more or less pure cultures of the "R" form, whose agglutination was not tested in sufficiently weak salt solution. If saline which is too strong is used, the difference between the tubes containing specific serum and the control may be scarcely appreciable. The smallness of the clumps formed by the "R" form has probably confirmed the view taken by these observers that any agglutination which took place was not of a specific nature. This also was the conclusion arrived at by Benians (1920⁷) in the case of an "inagglutinable" strain of Shiga's bacillus, which he described. From his account it appears probable that it was an example of the "R" form.

The "Q" forms described by v. Lingelsheim are undoubtedly variations of the same kind as the "R" forms described in this paper.

Variation in Serological Properties.

VI. In spite of a number of recorded observations on changes in bacteria in respect of the property of agglutination by specific serum, it has usually been held that serological properties are constant and never vary, although variability has been accepted as regards other properties. The antigen peculiar to one kind of bacterium has come to be regarded as an almost unchangeable constituent of the bacterium which always produces the same antibody response, as in the case of a pure protein, such as a particular kind of egg albumen.

Although some bacteria are remarkably constant as regards their antigenic constituents and the corresponding antibodies, e.g., *B. typhosus*,

the generalisation is perhaps hardly justified in so complete a form on experimental grounds. Loss of agglutinability by a culture has perhaps too exclusively been ascribed to a failure in the second non-specific part of the reaction.

The changes described in respect of serum properties have usually been in the direction of loss of agglutinability, but some instances of the additional loss of antigenic power and of ability to fix complement have been recorded. Records of changes such that a variant produces an antibody which does not react with the parent culture or with other variants from the same stock are rare.

Bordet and Sleswyk (1910⁹) described the manifestation of entirely different serological properties by the whooping-cough bacillus according to whether it was grown on blood-agar or on ordinary agar. Although the difference which they record is simply ascribed to the change in culture medium, the need for gradually accustoming the cultures to the new medium suggests the selection out of a variant.

Sobernheim and Seligmann (1910^{34,35}, 1911³⁶) gave evidence of interchange of characters between members of the *B. paratyphosus* and *B. enteritidis* groups of bacteria and of the occurrence of intermediate forms. These changes concerned agglutination, absorption of agglutinins, and antigenic properties.

Baerthlein (1918⁴) described (A) a mutant of *B. dysenteriae* Y, which was "inagglutinable" and did not absorb specific agglutinins nor fix complement with a serum (titre 1/10,000) derived from a normal strain; (B) a strain of *B. paratyphosus* B. which underwent still more extraordinary changes, since it was altered eventually in almost all its characters. The changes are stated to have taken place by intermediate steps. This strain first produced six varieties of colony. Two of these (1) and (4) varied as regards agglutinability and they did not absorb from a specific serum the agglutinins for the other variants. Variant (4) formed very large colonies with jagged outline and was inagglutinable; a serum made from it agglutinated the homologous and all the other variants. Variant (1) also formed large, jagged colonies; it was non-motile and sometimes inagglutinable, and at other times agglutinated spontaneously. When inoculated into a rabbit it produced a serum which did not agglutinate itself nor the other variants. In respect of sugar reactions it agreed with the parent strain. Variant (1) was very constant and irreversible for three and a half months, but after five and a half months a further variation made its appearance. This sub-variant fermented glucose and mannite without gas, but it still turned litmus milk blue in forty-eight hours after a preliminary acid stage. With paratyphoid B. serum (1/3000 titre) it agglutinated to 1/500 or slightly higher, and with *B. typhosus* serum (titre 1/20,000) to 1/10,000. Baerthlein appears to consider these changes as almost equivalent to a transmutation from *B. paratyphosus* to *B. typhosus*.

Van Loghem (1919¹⁸) describes variations which occurred in a culture of *B. paratyphosus* B. after it had been used as a type culture for nine years. The first changes noticed were that it formed indole and agglutinated irregularly. After three more years of subculture it was found that a large proportion of the colonies formed indole and that gas production was diminished. Further variants from the same strain agglutinated feebly with paratyphoid serum and up to half titre with typhoid serum. V. Loghem looks on variability as a characteristic of *B. paratyphosus* B., and does not consider his observations as evidence that one species is being transformed into another.

Mellon and Anderson (1919²²) claim to have made separate sera from the bacillary and spore forms respectively of *B. subtilis* which have quite distinct

serological properties, and state that cross agglutination does not occur. If this is confirmed, the results may have some analogy to the different properties of the "S" and "R" forms described in this paper.

Hort (1920¹⁵) describes remarkable forms of *B. typhosus* which he believes to have a relation to a special method of reproduction. He finds these forms sometimes associated with a different behaviour towards agglutinating sera.

VII. The correlation of the variations in agglutination by salt and by serum is of great assistance in enabling one to select colonies of serological variants. The coincident change in both characters (agglutination by salt and by serum) is perhaps not fortuitous.

It has been shown by Beintker (1912⁶), Beniasch (1912⁸) (and others) that these two properties (agglutinability by electrolytes and by specific serum) are to some extent related. Moreover, they considered that the substance in the bacteria which was concerned with agglutination by electrolytes and by specific serum was the same in both cases since, they found, that emulsions which are inagglutinable by acid are also inagglutinable by serum.

Arkwright (1914²) confirmed the close association of the properties of agglutinability by serum and by acid but considered the identity of the agglutinable substances unproven.

The two phenomena do not run exactly parallel, but there is a large amount of evidence tending to support this view.

Eisenberg (1919¹³) examined a long series of cultures of different kinds of bacteria, and again called attention to the close connection between agglutinability by specific serum and by electrolytes.

Other Variations.

VIII. Other variations as regards the fermentation of sugars, etc., pigment formation, hæmolytic action, toxin-production, virulence, etc., have been frequently recorded and are of importance in connection with the subject of this paper, in so far as they have been carefully observed, because they indicate the wide range of the tendency to variation.

Variations in Fermentation.—The occurrence of "mutation" in respect of the production of acid and gas from carbohydrates and alcohols has been worked at very carefully and the processes much elucidated by Massini, Twort, Müller, Penfold, Revis, Ledingham, and other writers who have observed and discussed the degree of permanence of the mutations and the occurrence of reversion. For reference to papers on this subject, see Penfold (1910²⁶ and 1912²⁷).

IX. *Relation of Sliminess of Cultures to Morphology.*—The swollen-looking, large, broad, irregularly shaped bacteria sometimes showing bud-like and branch-like processes which I have met with in films from some 24-hour agar cultures of the "R" form of *B. dysenteriae* (Shiga) are very like the forms of *B. typhosus* figured by Hort (1920¹⁵), who considers them to be of special reproductive importance. In my cultures these peculiar forms have always been associated with sliminess of the emulsions. The latter property appears to be due to a change in the internal composition of the bacilli and not to a capsule formation as was the case in the variant of *B. coli*, which formed jelly-like colonies

described by Revis (1910³⁰), and the "mucoid" forms of *B. paratyphosus* B. and *B. dysenteriae* described by Fletcher (1918¹⁴).

The special agglutination properties of the "R" form may have some relationship to the swollen forms and the presence of slimy material.

Ledingham has suggested that the mucus-like substance in the cultures may be the explanation of the peculiar type of agglutination exhibited by the "R" form both when under the influence of specific serum and of salt alone. V. Lingelsheim (1913¹⁷) considered the peculiarities of his "Q" form to be entirely due to the sliminess of the cultures. In my cultures, however, the special kind of agglutination of the "R" form by serum appears to be more nearly associated with agglutinability by salt than with the presence of slime which seems to be an independently variable character.

X. Variations of bacteria which have arisen in the animal body have also been recorded. Sørensen (1912³⁷) and Arkwright (1913¹) both relate instances in which organisms of the *B. coli* group lost the power of forming gas in the human bladder. The inagglutinable variant of *B. dysenteriae* (Shiga) described by Benians (1920⁷), was obtained from an inoculation abscess in a guinea-pig.

Nature of the Variation into "S" and "R" Forms.

XI. The views of biologists as to the nature of variation are very unsettled at the present time, but since it seems quite justifiable to consider bacteria as asexual throughout their life history, the explanation of variation as due to cross fertilisation need not be considered. The accounts of conjugation and of the union of several individuals by Löhnis and Smith (1916²¹) at present need confirmation.

There are several possible explanations of the origin of the "S" and "R" forms which may eventually be partially settled by single cell culture.

(1) The "R" form might be due to a contamination with an entirely different micro-organism. This seems unlikely, since it would involve the hypothesis that two entirely distinct bacteria were constantly present in stock cultures of different strains and were practically inseparable.

(2) It may be held that the "S" and "R" forms pre-exist in all cultures as different strains or elementary forms of the same "species," and that many such strains habitually live side by side. This hypothesis would be available to account for "variations" of the same organism in other directions, *e.g.*, pigment production, fermentations, sliminess, agglutination by salts and by specific serum, etc. Such an interpretation would be consistent with the hypothesis of multiple antigens put forward by Durham (1901¹²).

A somewhat analogous explanation of polymorphism in species of higher plants, *e.g.*, cereals, is stated by de Vries (1909³⁹). According to

this view certain apparently new mutants or variants, which when selected by the plant-breeder prove constant and breed true, have in reality been for long present amongst the many elementary forms which together make up the Linnæan species.

This explanation has been advocated by Brierley (1919¹⁰) when dealing with the meaning and limits to be attached to the idea of species in fungi and bacteria. He considers that the claim that species are constantly shifting and changing whilst under observation is *a priori* highly improbable. This line of reasoning appears to prejudge the question of the origin of new hereditary forms or species in unicellular non-sexual organisms, like bacteria, whilst under observation.

It appears to have been settled as far as is possible at present by methods of single cell culture that some forms of variants can arise from single bacteria and are not explicable on the theory of multiple strains. Experiments with *B. coli mutabilis* by Müller (1909), Penfold (1912²⁷), and others, by Burri's method are among the most important evidence on this point; and other forms of variation may be tested in this way.

(3) The two forms "S" and "R" might be merely "modifications" due to the environment, but in this case a change of environment, *e.g.*, a different culture medium, should soon re-establish the normal; and the two forms when grown on identical medium should at once approximate. This, however, does not occur.

(4) The different characters of the "S" and "R" forms might be due to "fluctuation" or "variation within the limits of the species." By this is meant such changes as are limited in kind and degree, are obtainable from all strains of the organism, and have a very strong tendency to reversion.

This notion of variation is of the same kind as that put forward by R. Müller (1909) and Penfold (1912²⁷), as applicable to certain "mutants" observed by them. They held that these variations did not afford evidence of a tendency to transmutation from one species to another, but were really special changes which were characteristic of the species and might aid in its identification.

It is difficult to decide whether this is a suitable category in which to place the "S" and "R" forms, since the dividing line between "fluctuations" and "mutations" (the two chief kinds of variations described) appears to be a very narrow and uncertain one. The permanence of the "S" and "R" forms and their definite characters are opposed to the view that they are fluctuating variants, but on the other hand the constancy with which they can be obtained from different strains shows a strong inherent tendency of the "species" to vary in this way. Van Loghem (1919¹⁸) appears to attach a very wide significance to the term "variation within the species." When discussing his *B. typhosus*-like variant of *B. paratyphosus* B. he seems to imply that all the characters of a member of the *B. paratyphosus* must

change, till it is absolutely indistinguishable from the members of another species and has become fixed, before he would admit it as a case of mutation. Such a change would more aptly be called a transmutation.

In the category of "fluctuations" should probably be included the kind of change described by Löhnis and Hanzawa (1914²⁰), Löhnis and Smith (1916²¹), and Hort (1920¹⁵), if the hypothesis of these writers were to be accepted that such changes were of a cyclical nature. The further question as to whether the processes concerned include sexual phenomena as they suggest, may be left for the present till further confirmation is forthcoming.

Löhnis and Smith's paper suggests, however, that the changes with which they are dealing are in part persistent with no very strong tendency to revert, and in this they agree with the "S" and "R" forms.

(5) The term "mutant" may perhaps be used appropriately for the "S" and "R" forms. By a "mutant" is meant a decided and persistent variation which may be progressive, whether by loss or acquirement, *i.e.*, may lead to further changes such as are held on the Darwinian hypothesis to lead to new species by selection. There does not seem to be anything definitely against these forms being classed as "mutants." Still the fact that they arise from so many strains must make one hesitate unless further evidence of progressive change is forthcoming, than is afforded by the "RV" form referred to above.

(6) The manner of origin of the "S" and "R" forms does not affect the fact that they are both present in many cultures which are believed to be pure, and that their special characters concern the agglutinating, absorbing, and serum-producing properties of these strains.

XII. Whatever view is taken regarding the origin of the "S" and "R" forms they undoubtedly readily arise under artificial surroundings. It is clear, therefore, that selection must play a very important part in explaining the normally uniform characters of such well-known bacteria as *B. typhosus* and *B. dysenteriae* (Shiga). The human body infected with dysentery may be considered a selective environment which keeps such pathogenic bacteria to the forms in which they are usually encountered.

Occasionally, however, colonies suggesting the "R" form occur on the first plates used for isolating from the faeces, and Benians (1920⁷) has recently described a spontaneously agglutinating strain of *B. dysenteriae* (Shiga) which was isolated from an abscess in a guinea-pig produced by inoculating a normal strain.

It must, moreover, be remembered that strains which do not agglutinate spontaneously, and which consequently form smooth colonies and are agglutinated in easily visible clumps by serum, are sought and

selected by bacteriologists, and that colonies of unusual appearance and cultures which make unstable emulsions are as a rule discarded and neglected. In this way the strains most commonly studied and held to be normal and typical have been artificially selected by man.

That variants and mutants do not become fixed more frequently is perhaps due to the environment (whether in the body in the case of pathogenic bacteria, or in the test-tube in the case of artificial cultures) being rather rigid and stereotyped, so that "natural" selection tends to keep the strain to the form in which we know it. In the case of the "S" and "R" forms (1) the shape and appearance of the colonies on agar and (2) the manner of growth in broth are prominent features acting as indicators of the changes in agglutinability by salt which enable artificial intentional selection to act, and to obtain apparently pure cultures of the two forms.

Whether varieties of bacteria in any direction can be detected, obtained pure and fixed in the laboratory, probably depends largely on whether the variation is such that it is accompanied and revealed by some readily noticed change, which acts as an indicator of the variation and assists intentional selection. Such an indicator is most useful for the purpose if it can be seen in colonies on a plate culture. For instance, the pigment variations of cocci, of *B. prodigiosus* or *B. pyocyaneus*, the red and white colonies on MacConkey's medium indicating fermentation properties, or the smooth and rough colonies associated with variations in agglutination.

XIII. Variation of the kind under discussion appears to be liable to occur in many directions, but there seems to be no reason why it should occur in more than one direction at once, and transmutation in artificial culture of one pathogenic organism into another as regards all its characters seems therefore to be highly improbable. It seems likely that such a change if it were possible would require the complicated environment of the animal body reacting to a more or less pathogenic organism, in fact the conditions under which all well-defined strains of pathogenic organisms may be supposed to have risen.

XIV. It seems probable that even considerable changes may be liable to occur in cultures without disturbing the ordinary conclusions arrived at in the routine practice of bacteriology, so long as well-known methods are used and new media and technique are not introduced without due caution and careful study of their effects. The characters of fairly frequently subcultured cultures tend to be ill-marked and indefinite as regards the commoner variations, at least so far as the "S" and "R" characters are concerned. That is, the stock cultures usually exhibit mixed characters, and a mixture of forms may actually be present.

XV. It is obvious that before accepting large changes which affect simultaneously important properties such as agglutination by specific

serum, absorption of agglutinins and complement fixation, such as Baerthlein (1918⁴) claims for one of his variants of *B. dysenteriae*, great care in using the technique of isolation and subculture is required of the observer and in applying tests with regard to the other properties of the variants. Indeed, if many characters are said to have varied at the same time such as gas-production, indole-formation and relative agglutinability with typhoid and paratyphoid sera, as in the case of the "mutant" of *B. paratyphosus* B. described by van Loghem (1918¹⁸), the conclusion must remain in doubt unless the variation can be obtained repeatedly.

CONCLUSIONS.

(1) Eight out of the nine strains of *B. dysenteriae* (Shiga) examined have been made to yield two forms ("S" and "R"), which remain constant in broth when subcultured weekly; they have different cultural characters in broth and on agar, but are identical and resemble the stock culture as regards sugar reactions and the absence of indole production, and of motility. The remaining strain only grew in the "R" form.

(2) The "S" form makes a good stable emulsion in salt solution, and in broth cultures causes uniform turbidity. The "R" form agglutinates in 0.85 per cent. solution of sodium chloride, and in broth cultures it forms a deposit leaving the liquid clear. Weaker salt solution must therefore be used for agglutination experiments with specific sera.

(3) Many cultures of bacteria which agglutinate spontaneously when emulsified in 0.85 per cent. solution of sodium chloride form good stable emulsions in weaker salt solution, e.g., 0.42 per cent., 0.21 per cent., or 0.1 per cent.

(4) Agglutination tests with specific sera and "spontaneously agglutinating" strains can be carried out perfectly satisfactorily in salt solution of 1/2 to 1/8 the usual concentration (i.e., 0.42 per cent. to 0.1 per cent. sodium chloride). A normal (not spontaneously agglutinating) culture should, if possible, be also tested in the same strength of salt solution, so as to ascertain the titre of the serum in this concentration of salt.

(5) The "S" form, when agglutinated by specific serum, forms large clumps. The "R" form makes small clumps and the deposit is readily shaken up into a turbid suspension.

(6) The two forms differ very decidedly in their agglutinating, antigenic, and absorbing properties with specific sera.

(7) The "S" forms obtained from different strains of *B. dysenteriae* (Shiga) resemble each other serologically, but are distinct from all the "R" forms, and the "R" forms of different strains appear to have little relation to any "S" forms, but to be closely related to each other.

(8) "S" and "R" forms showing similar properties have been obtained from one strain of *B. dysenteriae* (Flexner-Y).

(9) Similar "S" and "R" forms have been obtained from several strains of *B. typhosus*, but the serological differences between the two forms were less distinct than in the case of *B. dysenteriae*, in the case of the one strain of *B. typhosus*, in which specific serum reactions were tested.

(10) Forms resembling the "S" and "R" forms as regards cultural characters and "spontaneous" agglutination have also been obtained from cultures of *B. paratyphosus* B., *B. enteritidis* (Gaertner), and other members of the *B. coli-typhosus* group, but the serological characters have not been examined by means of specially made sera.

I wish to express my indebtedness to Dr Duncan Reid for the beautiful photographs from which the plates have been made.

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DESCRIPTION OF PLATE II.

- FIG. 1.—Agarplate showing colonies of *B. dysenteriae* (Shiga) of two kinds: (1) smooth round domed ("S" form) and (2) flattened with irregular surface and margins ("R" form). (× 6 diams.)
- FIG. 2.—Agar plate showing colonies of the "S" form of *B. dysenteriae* (Shiga) No. 550 I. 24-hours old culture. Typical smooth colonies. (× 6 diams.)
- FIG. 3.—Agar plate showing the commonest kind of colonies of the "R" form of *B. dysenteriae* (Shiga) No. 550 II. which gives a slimy emulsion. 24-hours old culture. Typical flattened semi-opaque colonies with irregular margins and slightly dull surface. (× 6 diams.)
- FIG. 4.—Culture on agar plate of *B. dysenteriae* (Y) No. 573 III. 24 hours old, showing two kinds of colonies of the "R" form: (1) flattened with irregular margins and slightly irregular surface; semi-opaque. (2) Larger colonies, flat, very thin, with very uneven coarsely granular surface, and thin edges.
- FIG. 5.—Film from 24-hours culture on agar of the "S" form of *B. dysenteriae* (Shiga) showing uniform short bacteria. (× 2000.)
- FIG. 6.—Film from 24-hours culture on agar of the "R" form of *B. dysenteriae* (Shiga) from colonies like those in Fig. 3, which give a slimy emulsion. Very irregularly shaped, wide bacteria sometimes with lateral bud-like knobs; mostly deeply stained, but some ill-stained and shadowy. (× 2000.)

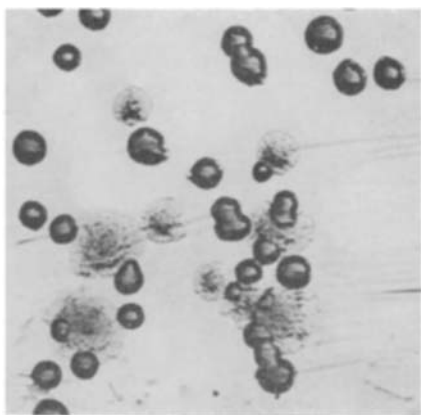


FIG. 1.

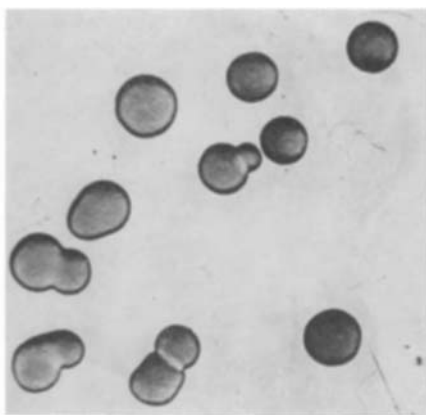


FIG. 2.

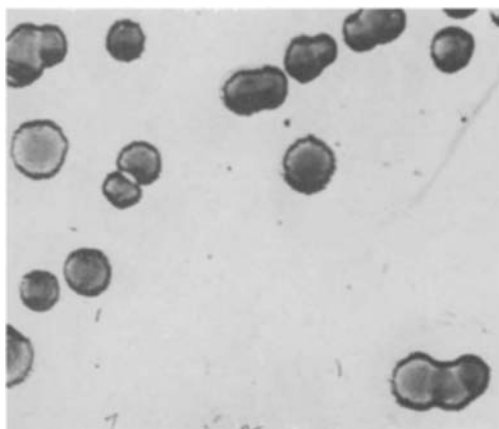


FIG. 3.

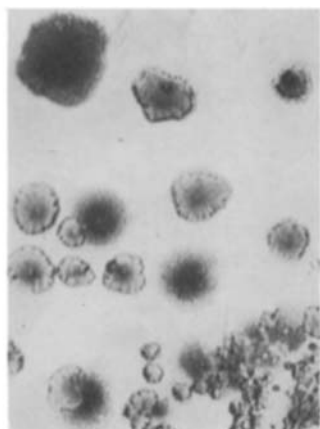


FIG. 4.

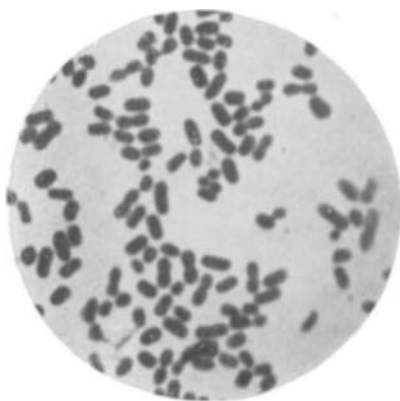


FIG. 5.

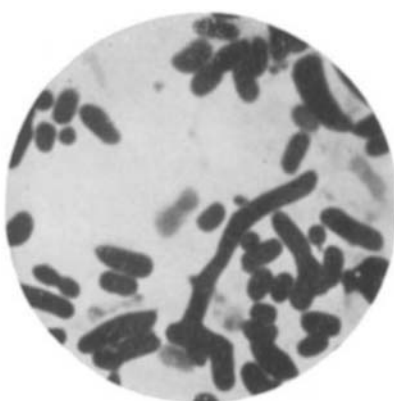


FIG. 6.