THE SEGREGATION OF BIOLOGICAL FACTORS IN *B. ENTERITIDIS* (AERTRYCKE)

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(With Plate III.)

In the preceding report (Topley and Ayrton, 1923 *b*) it has been shown that, if various strains of *B. aertrycke* be fed to mice and the subsequent events observed over a period of 42 days, and if all strains fed to the mice or isolated from their faeces or tissues be examined as regards their type of growth and their agglutination reactions, then the following associations are found to exist between the characters studied. The presence of group antigen is associated with persistent faecal excretion, whether the strain be rough or smooth. Roughness is associated with decreased faecal excretion and a decrease in the percentage mortality, when rough strains are compared with smooth.

In view of these facts it seemed desirable to attempt to define, somewhat more exactly, the relations existing between the various biological factors concerned.

The data already available for the study of this question are not limited to the organisms of the enteric group; and it appears probable that any associations which can be demonstrated within this group will have their counterparts among widely separated groups of bacteria, although there will, of course, be no justification for assuming that associations will always be found between the same biological factors in the different groups.

Arkwright (1921), in his study of smooth and rough strains, was concerned mainly with *B. shiga*, although several other species were studied less completely. All of these belonged to the typhoid-paratyphoid-dysentery group. His studies established the association of a particular kind of colony-formation, "roughness," with flocculation of the bacilli in normal saline; or in salt solutions of lower concentration. This sensitiveness to a relatively low salt-content, when in suspension, was exhibited in another way by the character of the growth in nutrient broth, which was marked by granularity, leading to a heavy deposit with a relatively clear supernatant fluid, often accompanied by the formation of a surface film. In the case of *B. shiga*, Arkwright also found that roughness and smoothness were associated with antigenic differences, as tested by agglutination.

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Schütze (1921), working with organisms of the Salmonella group, records observations of a similar kind. He emphasises, however, the lack of correlation between the degree of colonial roughness, the saline instability and the differences in agglutinability when tested by various specific sera. He regards the rough strains as being definitely more cosmopolitan in their serological relationships than the smooth strains.

De Kruif (1921) working with a strain of pasteurella, demonstrated the dissociation of this species into a granular and non-granular form, as judged by growth in fluid medium. These differences in the characters of the growth in broth were associated with differences in colony-formation. Far more important, however, was his demonstration that these differences in mode of growth were related to differences in virulence as tested by inoculation into rabbits. The non-granular form was highly virulent, the granular form was almost without effect. De Kruif noted certain small differences between the two types, as regards their agglutination reactions, but these were not very definite and the question was not investigated in detail.

Cowan (1922), working with streptococci, and using rabbits and mice as her test-animals, demonstrated a similar association between smoothness and virulence; and roughness and lack of virulence. Roughness was exhibited by the colonial form and by the character of the growth in broth.

Griffith (1923) demonstrated the existence of the same association between smoothness and virulence, and roughness and non-virulence in the case of the pneumococcus, using mice as his test-animals. Here again the distinction between the character of the growth of the two varieties could be demonstrated either with liquid or solid media. As regards serological relationship, Griffith's findings suggest that the rough form was antigenically a simpler or less complete variant derived from the smooth form. He brought about the change from smoothness to roughness by growing strains of pneumococci in immune serum.

The study by Andrewes (1922) of the antigenic structure of the Salmonella group has been referred to in a previous report. It may here be noted that he specifically states that the antigenic differences which he observed were in no way related to roughness or smoothness of the growths.

Both Arkwright and Schütze state that considerable difficulty may be met with in deciding whether a given colony is rough or smooth, and that the association between roughness and a granular growth in broth, and smoothness and a diffuse growth in broth, is not entirely constant. Our own work has dealt only with *B. aertrycke*. With this species we have met with no real difficulty in differentiating between rough and smooth colonies; and the association between roughness on solid media and granularity in nutrient broth has been absolute. This is, however, the case only if we rely entirely on the structure of the surface of the colony, and pay no attention to its border. A smooth colony may vary from a lenticular form, with sharply defined margins and a high degree of translucency, to a much flatter form with deeply

serrated edges, and much more opaque, but in every case the surface is smooth or very slightly granular. The surface of the rough colony is always coarsely granular, giving much the appearance of morocco leather, when viewed with a low-power objective. Plate I shows two smooth and two rough colonies as viewed with a two inch objective and No. 2 ocular.

We have already recorded the fact that rough strains of *B. aertrycke*, when fed to mice, produce a lower percentage mortality than do smooth strains under the same conditions. It was, however, clearly desirable to discover whether the sharp differences between the virulence of rough and smooth strains as tested by direct inoculation into the tissues, which had been demonstrated for pasteurella by De Kruif, for streptococci by Cowan, and for pneumococci by Griffith, held true in the case of *B. aertrycke*. It was also necessary to determine whether there was any association between the presence or absence of type or group antigen and different degrees of virulence.

We have tested the effect of intraperitoneal inoculation into mice of smooth and rough strains of *B. aertrycke*, using in each case some strains which contained type antigen alone, and others which contained group antigen, alone or combined with type antigen. In the case of the smooth strains, we tested type, group, and mixed varieties. In the case of the rough variants we had not, at the time when these experiments were made, succeeded in isolating pure group strains, so that we compared type with mixed strains.

Table I.

Showing time to death, in days, of mice inoculated intraperitoneally with different strains of B. aertrycke (*mutton*).

		Strains								
		Smooth					Rough			
		Type		Group		Mixed	Type		Mixed	
No. of mouse in each series	Dose in c.c. of 18 hours broth culture	*E*	*F*	*G*	*H*	*S*	*K*	*O*	*N*	*R*
1	0·25	0·75	1	0·75	0·75	0·75	13	1	4	0·75
2	0·25	0·75	0·75	0·75	0·75	0·75	6	3	–	4
3	0·025	1	2·5	0·75	1·5	0·75	12	9	–	–
4	0·025	6	0·75	0·75	0·75	0·75	–	6	14	–
5	0·0025	2·5	3·5	3·5	–	4	–	–	–	–
6	0·0025	3·5	3·5	0·75	1·5	1	–	–	19	–
7	0·00025	5	6	2·5	12	5	–	–	10	–
8	0·00025	3·5	2	2	7	11	–	–	–	–
9	0·000025	3·5	–	11	10	6	–	–	–	–
10	0·000025	9	3·5	2	–	7	–	–	–	–

– = lived for 21 days.

Table I shows the results obtained in testing nine strains, and they are quite definite. Smoothness is associated with high virulence. Roughness is associated with low virulence. There is no obvious association between the presence or absence of type or group antigen and high or low virulence. As regards strain *N*, the two deaths with small doses are offset by the two survivals with larger doses. They occurred late and cannot be regarded as significant.

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One point may be noted in passing, since we are not aware that similar observations have been reported, and the results in question confirm a previous finding in connection with the persistence of *B. aertrycke* in the tissues after feeding. All inoculated mice were kept under observation for 21 days. At the end of this period all survivors were killed, a post-mortem examination was carried out in each case: and cultures were prepared from the spleen. Of 90 mice inoculated intraperitoneally 30 lived for 21 days. Of these, 27 had been inoculated with rough strains, 3 with smooth. Spleen cultures from these 30 surviving mice yielded pure growths of *B. aertrycke* in 24 cases.

We may also note here that we have never observed a change from smooth to rough, or *vice versa*, in comparing the strains fed or inoculated into mice with the strains isolated from their faeces during life, or from their tissues after death.

Table II.

Showing results of absorbing "type" and "group" sera with "type" and "group" bacteria (B. aertrycke).

Serum	Absorbed with	Titre against			
		Smooth (Type)	Rough (Type)	Smooth (Group)	Rough (Group)
A. Type (Absorbed)	–	3,200	3,200	–	–
,,	Smooth (Type)	400	400	–	–
,,	Rough (Type)	600	600	–	–
B. Group (Newport)	–	–	–	1,600	6,400
,,	Smooth (Group)	–	–	0	0
,,	Rough (Group)	–	–	0	0
C. Smooth (Type)	–	25,600	25,600	1,600	6,400
,,	Smooth (Type)	400	400	800	1,600
,,	Rough (Type)	400	200	200	400
D. Smooth (Group)	–	1,600	1,600	3,200	12,800
,,	Smooth (Group)	0	0	0	0
,,	Rough (Group)	0	0	0	0
E. Rough (Type)	–	51,200	51,200	100	200
,,	Rough (Type)	800	800	0	–
,,	Smooth (Type)	800	800	0	100
F. Rough (Mixed)	–	25,600	25,600	1,600	1,600
,,	Rough (Group)	6,400	6,400	0	0
,,	Smooth (Group)	6,400	6,400	0	0

0 = no agglutination at 1/100. – = not tested.
Incubation at 55° C. for 2 hours.

It has been noted above that Schütze, in recording his observations on the Salmonella group, drew attention to the lack of correlation between the degree of roughness, the saline stability and differences in agglutinability by specific sera. It appeared from our results that, as regards *B. aertrycke*, smoothness and roughness were associated respectively with high and low virulence; while presence or absence of group or type antigen were not associated with different degrees of virulence. It was clearly necessary to determine whether or no variations in antigenic structure were associated with smoothness or roughness. It was already perfectly clear that type and group antigen were present in both smooth and rough varieties. It remained only to determine whether the type and group antigens of the smooth strains were

respectively identical with the type and group antigens of the rough strains. The only difference we had so far noted between the antigenic structure of the smooth and rough varieties was that, while it was quite easy to separate pure type or pure group strains of the smooth variety, we were for many months unsuccessful in isolating a rough strain containing group antigen alone, whereas apparently pure type strains of the rough variant were readily obtained. More recently we have obtained rough strains containing group antigen alone; and we cannot say whether our previous lack of success was due to chance, or to an actual rarity of this combination of biological characters.

In order to determine the identity or otherwise of the antigens of the rough and smooth strains, we carried out the series of absorption tests, the results of which are given in Table II.

It will be noted that, by the time the actual tests were carried out, we had isolated a rough group strain, which was used as one of the test bacterial suspensions. The serum containing group agglutinin, elaborated in response to the inoculation of the group antigen of a rough strain, contained type agglutinin in addition, since at the time the rabbit was inoculated we had only a mixed rough strain at our disposal. The test suspensions used were in all cases formalinised broth cultures, and the same suspension was used throughout for each of the four strains. All essential facts are recorded in the table. Serum A was an absorbed aertrycke serum. Serum B was a newport serum containing a large amount of group agglutinin. Both had been prepared against smooth strains, and were known to differentiate sharply between type and group strains. Sera C, D, E and F were obtained, in each case, by inoculating a rabbit with the antigen specified. As the table indicates, these sera were not absolutely specific, nor were the bacterial suspensions employed for the absorptions strictly specific in their action. The difficulty of obtaining a mass of bacteria sufficient for absorption and containing only one variety of antigen is very great, and there is little doubt that the lack of sharpness in some of the results is due to failure in this respect, and to the inclusion in the suspensions used for immunisation of a proportion of bacteria containing the variety of antigen which we had intended to exclude.

The question at issue is, however, quite clearly answered. The type and the group antigens of the smooth variety are respectively identical with the type and the group antigens of the rough variety.

Taking the results so far obtained, we are faced with the following facts in regard to *B. aertrycke*.

Smoothness is associated with high virulence; as tested by intraperitoneal inoculation into mice, roughness with low virulence.

The presence of group antigen is associated with the phenomenon of persistent excretion in the faeces, its absence with the absence of such excretion.

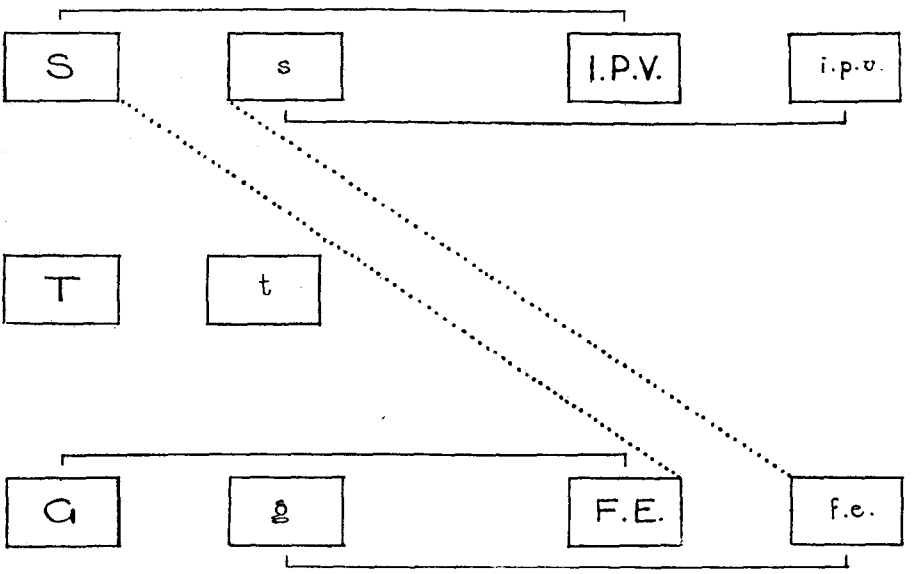
Smoothness and roughness vary independently of the presence or absence of type or group antigen.

Although the association of group antigen with persistent faecal excretion is as close among rough strains as among smooth, yet there appears to be some other factor which determines a diminished faecal excretion, when rough strains as a whole are compared with smooth strains as a whole.

It seems difficult to avoid the conclusion that a segregation of biological factors may occur, when *B. aertrycke* undergoes division; that, when such segregation takes place, there is a definite association or linkage between certain of these factors; that the factor which determines smoothness is either identical with, or is inseparably linked to, the factor which determines high intraperitoneal virulence; and that the factor for group agglutinin is similarly associated with the factor which determines persistent excretion in the faeces.

Diagram I indicates the linkages which we may suppose to exist. There appears to be some reason for believing that roughness is due to the loss of

Diagram I.



G = Presence of Group Antigen
g = Absence „ „
T = Presence of Type Antigen
t = Absence „ „
S = Smoothness
s = Roughness

I.P.V. = High Intraperitoneal Virulence
i.p.v. = Low „ „ „
F.E. = Faecal Excretion—Persistent
f.e. = „ „ Rare and Transient

some character which is present in the smooth strains. We have indicated this probability in the diagram by using a symbol for absence or decrease, as in the case of the other factors considered. We have indicated the linkage between smoothness and faecal excretion by a dotted line, in place of the continuous line used in the other cases, in order to emphasise that the association is independent of that existing between the other factors, and appears

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to be of a different order. It will be noted that we have so far obtained no evidence of any linkage affecting the type antigen.

It remains to express some opinion as to the number of different varieties of *B. aertrycke*, which may result from such segregation of factors. We have mentioned, in the preceding report, that we are disposed to recognise a variety of *B. aertrycke* in which both the type and group antigen are fully developed in each bacillus. If we are correct in this view, such a strain would clearly represent the complete organism from which the other variants were derived by loss of certain factors.

The evidence which leads us to support this view is largely based on the frequency with which we have encountered subcultures from single colonies, which agglutinate with both type and group sera. It is frequently the case that 20 or more colonies from one plate will show this result, while, when pure type and pure group strains have been isolated from the same plate, the proportion of mixed colonies has often been very small.

There are, however, two observations which appeal to us with particular force. One concerns the agglutination results obtained with strains isolated from the tissues of mice which have been inoculated with group or type strains, or fed on such strains.

Of 382 strains isolated from mice inoculated with type strains, 330 reacted as type, 44 as mixed, and 8 as group. Of 313 strains isolated from mice inoculated with group strains, 231 reacted as group, 81 as mixed, and 1 as type. Of 159 strains isolated from mice fed on type strains, 107 reacted as type, 50 as mixed and 2 as group. Of 143 strains isolated from mice fed on group strains, 83 reacted as group, 60 as mixed and none as type. This is not the distribution which would be expected on the assumption that the strains which reacted as mixed were derived from colonies which owed their mixed character to the chance association, in the formation of the colony, of a group and a type bacillus.

The other piece of evidence is gathered from a consideration of the results obtained by applying agglutination tests to considerable numbers of strains isolated from the faeces of individual mice on repeated examination.

In one experiment (*H*), in which a series of mice were fed on a smooth group strain of *B. aertrycke*, 20 strains isolated from six specimens of faeces, collected from one mouse, over a period of 16 days, were tested by agglutination. All reacted as pure group strains. In the case of another mouse of the same batch, 18 of 23 strains isolated from the faeces reacted to both group and type sera. Specimens from this mouse were examined over a period of 27 days, and while the first two specimens gave pure group strains, these were later replaced by mixed strains, and mixed strains alone were isolated from the last three specimens examined. In the whole experiment no type strain was ever isolated.

In another experiment (*S*) in which a batch of mice were fed on a strain which agglutinated with both type and group sera, 23 positive specimens of

faeces were obtained from 5 mice. From these 23 specimens 88 strains were isolated and tested by agglutination: 86 of these were agglutinated by both type and group sera: 2 reacted as pure group strains, none as pure type strains. Many other observations of a similar kind could be cited.

It seems impossible to explain such figures as the result of chance admixture of type and group bacilli in the same colony[1]. To do so in the case of Exp. *S* we must accept the view that chance selection has resulted, on 86 occasions, in our picking colonies which have been formed from the development of a type and a group bacillus lying in accidental juxtaposition, while we have only twice picked a pure group colony, and have not once picked a pure type colony. We cannot invoke any theory of symbiosis, for

Diagram II.

Smooth	Type +	Group +	Behaves as Type − Group +
	Type +	Group −	Intraperitoneal virulence high. Excretion in faeces after feeding rare and transient
	Type −	Group +	Intraperitoneal virulence high. Excretion in faeces after feeding frequent and persistent
	[Type −	Group −]?	
Rough	Type +	Group +	Behaves as Type − Group +
	Type +	Group −	Intraperitoneal virulence low. Excretion in faeces after feeding rare and transient
	Type +	Group +	Intraperitoneal virulence low. Excretion in faeces after feeding frequent and persistent but less so than with the corresponding smooth variety
	[Type −	Group −]?	

we know (*a*) that pure group strains are readily excreted as such in the faeces, (*b*) that, although pure type strains are seldom excreted, yet, when such excretion occurs, they are not accompanied by group or mixed forms, and (*c*) that when we purposely feed to mice a mixed culture, known to contain both type and group bacilli, strains of both of these types are isolated from the faeces, though the group strains greatly predominate.

Finally, we have, in the preceding paper (Topley and Ayrton, 1923 *b*) brought forward evidence which shows that pure type strains are seldom excreted in the faeces. The results of Exp. *S* could only be explained as due to the chance admixture of type and group bacilli, if the two varieties were excreted in the faeces with equal readiness.

Consideration of these results would lead on naturally to a discussion of the extent to which variations in antigenic structure occur in the intestine or in the tissues. This question has been briefly referred to in the preceding report and we hope to consider it in more detail in future communications.

[1] "It will clearly be impossible to distinguish, with the technique employed, between a colony, in which the individual bacilli contain both type and group antigens, and another colony, in which the bacilli contain only one kind of antigen, but are particularly liable to give rise to bacilli containing the other kind of antigen, in both subcultures. In both cases we should obtain agglutination with both group and type antisera."

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Fig. 1

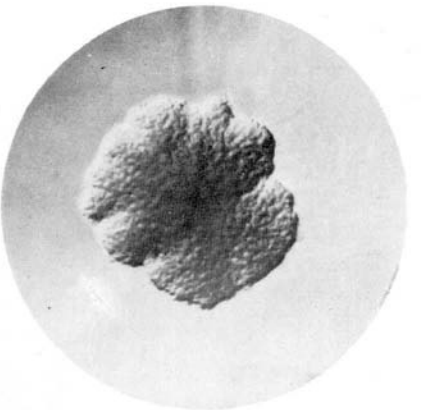

Fig. 2

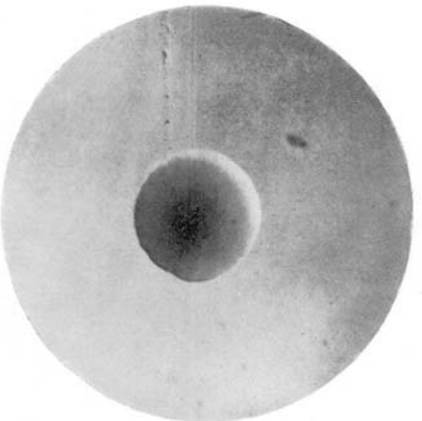

Fig. 3

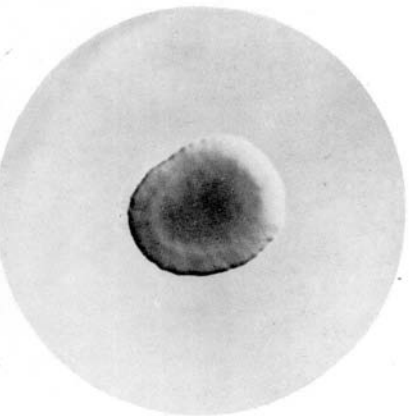

Fig. 4

Figs. 1 and 2. Rough colonies (2 inch obj. and No. 2 ocular).
Figs. 3 and 4. Smooth ,, ,, ,, ,,

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At the moment we are concerned only with the question of the existence of a complete or mixed antigenic variety of *B. aertrycke*.

We should, for the reasons given, be disposed to recognise at least six varieties of this organism, and probably eight. We have indicated the nature of these varieties in Diagram II, and have noted in each case the characteristics which distinguish the behaviour of the six varieties we have studied, when functioning as parasites with the mouse as host. The two varieties of *B. aertrycke* which contain neither type nor group antigen, if such varieties exist, have not been studied as regards their behaviour as parasites. As noted elsewhere, we have, on several occasions, met with strains of *B. aertrycke* which react neither to type nor to group sera; but whether their inagglutinability is due to absence or marked deficiency of the corresponding antigens, or to some quite different cause, it is impossible to say until we have more facts at our disposal.

DISCUSSION.

There is little to add to what has been said above, but we should wish to emphasise one point. We have purposely employed terms drawn from Mendelian sources. The biological factors discussed must have a material basis, and the suggestion that the variations observed are due to an unequal distribution of the substances concerned, at the moment when division of the bacillus occurs, is so obvious, that it may well be provisionally accepted as a working hypothesis. Such acceptance does not imply any specific view as regards the structure or mode of reproduction of the bacilli. We know that multiplication by binary fission occurs, and this form of growth gives ample opportunity for such a segregation as we have suggested.

The conclusions arrived at have been set out in the body of the report and need not be repeated.

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