# A NEW TYPE OF ANTIGENIC VARIATION OCCURRING IN THE FLEXNER GROUP OF DYSENTERY BACILLI

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(With 1 Figure in the Text)

In studying the antigenic structure of a bacterial species, type, or variant, by means of the agglutination reaction, the problem can be approached either from the qualitative or quantitative aspect. So far as the former is concerned, the method of agglutinin absorption, as ordinarily practised, enables us to differentiate between two bacteria that share one or more antigens in common, but possess one or more additional antigens that are peculiar to each type, or to one of them. It is clear that bacteria may differ in another way. Two species, or types, may, for instance, be qualitatively similar, in that both possess the antigens *a* and *b*, but they may differ quantitatively, in that one possesses a large amount of *a*, in association with a small amount of *b*, while in the other the proportions of the two components may be reversed. As Wilson & Miles (1932) have shown in the case of *Brucella melitensis* and *Br. abortus*, a difference of this kind can be demonstrated by quantitative absorption tests, or by the use of sera that have been absorbed with a suitable dose of the heterologous organism.

The problem at issue may, however, be attacked in other ways. Duncan (1934) has discussed the relative values of four different methods of estimating the agglutinin content of serum by agglutination of bacteria. These methods comprised (*a*) reading the highest serum titre at which visible agglutination occurred in a constant and measured mass of bacteria (the method in common use); (*b*) reading the highest serum titre at which the suspension was *completely* agglutinated, leaving a clear supernatant liquid; and (*c*) and (*d*) two different methods of determining the optimal proportions of serum and bacterial suspension for the most rapid agglutination. In (*c*) a uniform dose of bacteria is titrated against a series of falling concentrations of serum, as in method (*a*) above, which is analogous to Ramon's (1922) method of measuring the antitoxic value of antidiphtheritic sera. In (*d*) a uniform dose of serum is titrated against a series of falling concentrations of the bacterial suspension, as in the Dean & Webb (1926) method of measuring the antibody content of precipitating sera.

In agglutination, as opposed to precipitin, tests, method (*c*) has the advantage that the density of the bacterial suspension is kept constant. In

https://doi.org/10.1017/S0022172400034975 Published online by Cambridge University Press

method (*d*) it must be varied widely; and it is by no means easy to detect the first trace of agglutination in a series of tubes containing bacterial suspensions of widely different turbidities. Da Costa Cruz (1929) believes that optimal or primary agglutination determined by method (*c*) indicates a balanced mixture of agglutinin and bacterial suspension, but Duncan (1932) found that the mixture giving optimal agglutination contained a considerable excess of agglutinin.

In the present study methods (*a*), (*b*) and (*c*) have been applied in investigating the antigenic structure of various strains of Flexner dysentery bacilli.

Since each of these species, or types, contains several different antigens, and each antiserum contains several different agglutinins, it is clear that an optimal-proportions titration might reveal multiple zones, each zone corresponding to a particular antigen-antibody system. Miles (1933) has recorded observations suggesting that this method of analysis may be of service in determining antigenic structure; but its utility will obviously depend, in any particular case, on the extent to which the different zones overlap, or merge into each other. In the experiments here described, the spacing of the antiserum dilutions was not such as would have revealed the presence of different optima, unless these had been widely separated, and multiple zones of agglutination were not, in fact, observed.

After the work had been in progress for some months, an antigenic variation of an interesting kind was observed in the strains under study, and attention was thereafter confined to this particular phenomenon.

## Materials and technique

The following strains of Flexner dysentery bacilli were used: V, *Lentz*; W, *Cable*; X, *Hughes*; Y, *Hiss* and *Russell* and Z, *Whittington* from the National Collection of Type Cultures, and two freshly isolated strains, W, *Park William* and Z, *Leech Staveley* obtained from Dr W. M. Scott. It was subsequently discovered that the strain labelled Y was, in fact, a degraded W strain, and it is referred to in the rest of this paper as $W_3$, the other two strains being labelled $W_1$ and $W_2$, and the two Z strains $Z_1$ and $Z_2$. No Y strain is, therefore, included in this series.

Suspensions used for immune serum production and for agglutination tests were made from 18-hour cultures on agar at 37° C., washed into isotonic saline and sterilized by 0·25 per cent formol, without heating.

For immune serum production, rabbits were inoculated intravenously with four doses of suspension equal to 0·1, 0·2, 0·6 and 1·0 mg. of dried culture at 1 week intervals, and the serum was collected 1 week after the final inoculation.

In the agglutination tests the density of the suspension was set to equal 0·2 mg. of dried culture in 1·0 c.c. of isotonic saline, equivalent to about $600 \times 10^{\circ}$ bacilli per c.c. Suspensions very much lighter or very much denser than this were less suitable for accurate reading of the optimal agglutination point.

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### DETERMINATION OF THE OPTIMAL RATIO OF SERUM TO BACTERIA

A series of falling concentrations of immune serum in isotonic saline, as in the usual form of the agglutination test, were titrated against a uniform dose of the bacterial suspension. The tubes were incubated at 50° C. on the water bath and the mixture in the series showing earliest agglutination was recorded as optimal. Readings of the end-titre of agglutination (*a*), and the end-titre of *complete* agglutination (*b*), were taken after standing overnight.

### EXPERIMENTAL RESULTS

Table I shows the results of agglutination of the seven stock strains by V, W, $W_3$, X and Z sera.

Table I reveals several points of interest. In regard to the relative value of the different readings taken, it will be seen that the findings are in accord with the observations of Duncan (1934). If we judge the antigenic relationship of the various strains by method (*b*)—the highest serum dilution giving complete agglutination with a clear supernatant fluid—or by method (*c*)—the serum dilution giving optimal agglutination—we shall arrive at very similar conclusions. But if we took as our criterion the readings obtained by method (*a*)—the end-point of visible agglutination after prolonged incubation—we should obtain a different picture. In a few instances the final end-point of visible agglutination coincides with the end-point of complete agglutination. More commonly the final end-point of visible agglutination occurs at a serum dilution two to eight times greater than the end-point of complete agglutination. Sometimes no tube shows complete agglutination, and no optimal reading is obtained, though partial agglutination may occur to a dilution as high as 1 : 800. It is not possible, in our present state of ignorance, to hazard any opinion as to why the relation of these two end-points varies so widely. An obvious possibility is that the antigens concerned in incomplete agglutination are present at the surface of only some of the bacteria in a given suspension, while the antigens responsible for complete agglutination are present in all. We cannot, of course, exclude quantitative, as opposed to qualitative, differences; but the fact that a particular suspension, when tested against a particular antiserum, may show incomplete agglutination at dilutions varying from 1 : 25 to 1 : 800, without the occurrence of complete flocculation in any tube, suggests rather strongly that we are dealing either with qualitative differences, or with quantitative differences so great as to have qualitative effects.

Turning to the question of the relation of the serum dilution giving optimal agglutination to the highest serum dilution giving complete agglutination, it may be noted that Duncan (1934) found that, in the system he was studying, the mixture giving optimal agglutination contained about eight times as much antiserum as the mixture corresponding to the complete flocculation

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Table I

| Dilution of sera | V serum | | | | | | | W serum | | | | | | | $W_3$ serum | | | | | | X serum | | | | | | | Z serum | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Bacterial suspensions | V | $W_1$ | $W_2$ | $W_3$ | X | $Z_1$ | $Z_2$ | V | $W_1$ | $W_2$ | $W_3$ | X | $Z_1$ | $Z_2$ | V | $W_1$ | $W_2$ | $W_3$ | X | Z | V | $W_1$ | $W_2$ | $W_3$ | X | $Z_1$ | $Z_2$ | V | $W_1$ | $W_2$ | $W_3$ | X | Z |
| 1/102,400 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | + | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| 1/51,200 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | c | . | . | . | . | . | . | . | + | . | . | . | . | . | . | + |
| 1/25,600 | + | . | . | . | . | . | . | . | + | + | . | . | . | . | . | . | . | c | . | . | . | . | . | . | + | c | . | . | . | . | . | . | + |
| 1/12,800 | + | . | . | . | . | . | . | . | c | + | . | . | + | . | . | . | . | c | . | . | . | . | . | . | + | c | + | . | . | . | . | . | c |
| 1/6,400 | c | . | . | . | . | . | . | + | c | c | . | . | c | + | . | . | . | c | . | . | . | . | . | . | c | c | + | . | . | . | . | . | c |
| 1/3,200 | c | . | . | . | . | . | . | c | c | c | . | . | c | + | . | . | . | c | . | . | c | . | . | . | c | c | c | . | . | . | . | . | c |
| 1/1,600 | c | . | + | . | . | + | . | c | **c** | **c** | . | . | c | c | . | . | . | **c** | . | . | c | . | . | . | c | **c** | c | + | . | . | . | . | **c** |
| 1/800 | c | . | + | . | . | c | + | c | c | c | . | c | c | c | . | . | . | c | . | . | c | . | + | . | **c** | c | **c** | c | . | . | . | c | c |
| 1/400 | **c** | + | c | . | + | c | c | c | c | c | . | c | **c** | c | . | . | . | c | . | . | c | . | + | . | c | c | c | c | . | . | . | c | c |
| 1/200 | c | + | c | . | + | c | c | **c** | c | c | . | c | c | **c** | . | . | . | c | . | . | **c** | . | + | . | c | c | c | c | . | . | . | c | c |
| 1/100 | c | c | c | . | c | **c** | c | c | c | c | . | **c** | c | c | . | . | . | c | . | c | c | + | + | . | c | c | c | c | . | . | . | **c** | c |
| 1/50 | c | c | **c** | . | c | c | **c** | c | c | c | . | c | c | c | + | . | . | c | . | c | c | + | + | . | c | c | c | c | . | . | . | c | c |
| 1/25 | c | c | c | + | c | c | c | c | c | c | c | c | c | c | c | + | . | c | . | c | c | + | + | . | c | c | c | **c** | + | . | + | c | c |

c = agglutination complete. The position of primary agglutination (optimal ration by method (*c*)) is shown in heavy type.
+ = agglutination not complete.

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end-point. It is clear that this ratio may vary from one system to another; and a study of Table I will show that it appears to be determined largely by the nature of the cells in any given bacterial strain. In this particular series of tests, for instance, strain V shows a high ratio, the optimal mixture usually containing about sixteen times as much antiserum as the "complete" end-point mixture; while with strains $W_2$ and $Z_2$ this ratio is approximately 4 or 8.

Taking the findings as a whole, the following suggestions in regard to quantitative differences in the antigenic structure of the various strains would seem to be afforded. The results with the X antiserum indicate that the strains X, $Z_1$ and $Z_2$ contain antigenic components corresponding to the antibodies in these sera in amounts that do not differ very widely, since the optimal mixtures contain approximately the same proportions of serum. In the case of V rather more serum is required to give optimal proportions. The most probable explanation would seem to be that the major antigen of V corresponds to an antibody that is present in relatively lower concentration in the X antiserum. The antigens that are prominent in strains X, $Z_1$, $Z_2$ and V are clearly present in very small amount in $W_1$ and $W_2$, and apparently absent in the degraded strain $W_3$. The results with the W antiserum show that the strains $W_1$ and $W_2$ have the same optimum, and this probably indicates that they have the same quantitative antigenic structure. The strains X, V, $Z_1$ and $Z_2$ require more serum to give optimal agglutination; but there is less difference between these strains, and the two W strains, than in the tests carried out with the X antiserum. The obvious suggestion is that, while $W_1$ and $W_2$ contain very little of the antigens that dominate the behaviour of the X, V and Z strains, the latter strains possess a larger proportion of the antigens that are dominant in $W_1$ and $W_2$. The degraded strain $W_3$ clearly contains very little of the characteristic W component, or components; and this conclusion is confirmed by the results obtained with the $W_3$ antiserum. The results with the V and Z antisera are in accord with the other findings.

It would clearly be possible to study these quantitative differences in antigenic structure more closely by carrying out similar tests with the various antisera, after partial absorption with heterologous strains; and it was with this intention that the present study was undertaken. During the early stages of the investigation, however, while the results outlined above were being controlled by testing many subcultures of each strain under study to determine the constancy of its antigenic behaviour, a type of variation was encountered that seemed to deserve more detailed investigation.

## ANTIGENIC VARIATION IN THE COLONIES DEVELOPING FROM A SINGLE STRAIN

The different strains were plated out on agar and from every plate ten colonies were picked off and cultivated. Suspensions from these ten cultures were tested against the homologous serum for the agglutination optimal ratio and end-points. In the case of Flexner V, the results are given in Table II.

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The ten substrains fall into two groups of five each, most clearly differentiated by their optimal ratios; the lower serum ratio in cultures 1, 2, 3, 9 and 10, which for convenience may be designated "group *a*", and the higher serum ratio in 4, 5, 6, 7 and 8, designated "group *b*".

Table II

| Dilutions of V serum | Suspensions. Colony number | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| 1/51,200 | . | . | . | . | . | . | . | . | . | . |
| 1/25,600 | + | + | . | . | . | . | . | . | + | . |
| 1/12,800 | + | *c* | + | . | . | . | . | . | + | + |
| 1/6,400 | *c* | *c* | *c* | + | + | . | + | . | *c* | *c* |
| 1/3,200 | *c* | *c* | *c* | + | + | + | + | *c* | *c* | *c* |
| 1/1,600 | *c* | *c* | *c* | *c* | *c* | *c* | *c* | *c* | *c* | *c* |
| 1/800 | *c* | *c* | *c* | *c* | *c* | *c* | *c* | *c* | *c* | *c* |
| 1/400 | **c** | **c** | **c** | *c* | *c* | *c* | *c* | *c* | **c** | **c** |
| 1/200 | *c* | *c* | *c* | **c** | **c** | **c** | **c** | **c** | *c* | *c* |
| 1/100 | *c* | *c* | *c* | *c* | *c* | *c* | *c* | *c* | *c* | *c* |
| 1/50 | *c* | *c* | *c* | *c* | *c* | *c* | *c* | *c* | *c* | *c* |

*c* = agglutination complete. The position of optimal agglutination is shown in heavy type.
\+ = agglutination not complete.

Cultures 1 and 2 of "group *a*" and 5 and 6 of "group *b*" were tested against the heterologous sera X, Z and W and the results, recorded in Table III, show a striking difference between the two groups. The "group *a*" strains were agglutinated completely at titres nearly as high as those shown for the parent V suspension and the X, Z and W antisera in Table I, while the *b* group were agglutinated to relatively low titre. Optimal agglutination was easily determined in the case of the *a* group, and the ratio was the same for the three sera, X, Z and W.

Table III

| Dilutions of sera | Serum $W_1$ Suspension group | | | | Serum X Suspension group | | | | Serum $Z_1$ Suspension group | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $A_1$ | $A_2$ | $B_5$ | $B_6$ | $A_1$ | $A_2$ | $B_5$ | $B_6$ | $A_1$ | $A_2$ | $B_5$ | $B_6$ |
| 1/12,800 | + | + | . | . | . | . | . | . | . | . | . | . |
| 1/6,400 | *c* | *c* | . | . | . | . | . | . | . | . | . | . |
| 1/3,200 | *c* | *c* | . | . | + | + | . | . | . | . | . | . |
| 1/1,600 | *c* | *c* | . | . | + | *c* | . | . | + | *c* | . | . |
| 1/800 | *c* | *c* | . | . | *c* | *c* | . | . | *c* | *c* | . | . |
| 1/400 | *c* | *c* | . | + | *c* | *c* | . | . | *c* | *c* | . | . |
| 1/200 | **c** | **c** | + | + | **c** | **c** | . | + | **c** | **c** | . | + |
| 1/100 | *c* | *c* | + | + | *c* | *c* | + | + | *c* | *c* | . | + |
| 1/50 | *c* | *c* | + | + | *c* | *c* | + | + | *c* | *c* | + | + |
| 1/25 | *c* | *c* | + | + | *c* | *c* | + | + | *c* | *c* | + | + |

The group number refers to colony culture (see Table II).

The suggestion offered by these findings is clearly that the *b* variant of the V strain had lost, completely or almost completely, the antigenic component that it shared with the X, Z and W strains. Since the behaviour of the parent strain, when tested against its homologous serum, is dominated by the antigen peculiar to itself, the loss of the shared antigen will result in a relatively small shift in the optimal ratio; but this same loss will entirely change the behaviour

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of the variant when tested against an antiserum which contains little, if any, of the specific V antibody.

An attempt was then made to determine the permanence, or otherwise, of the *a* and *b* characters through several generations by examination of colonies isolated by plating out. The original V strain "Lentz" was plated out on agar and ten colonies were picked off: five of these were of the *a* type and five of the *b* type. One of the *a* cultures and one of the *b* cultures were plated out and ten colonies from each picked off and examined. In this second generation all of the ten colonies from the *a* culture presented the characters of the *a* form, but of the ten colonies from the *b* culture, three showed the *a* and seven the *b* characters. One culture from the second generation in the *a* line was plated out and five colonies examined; these were all of the *a* form. From the second

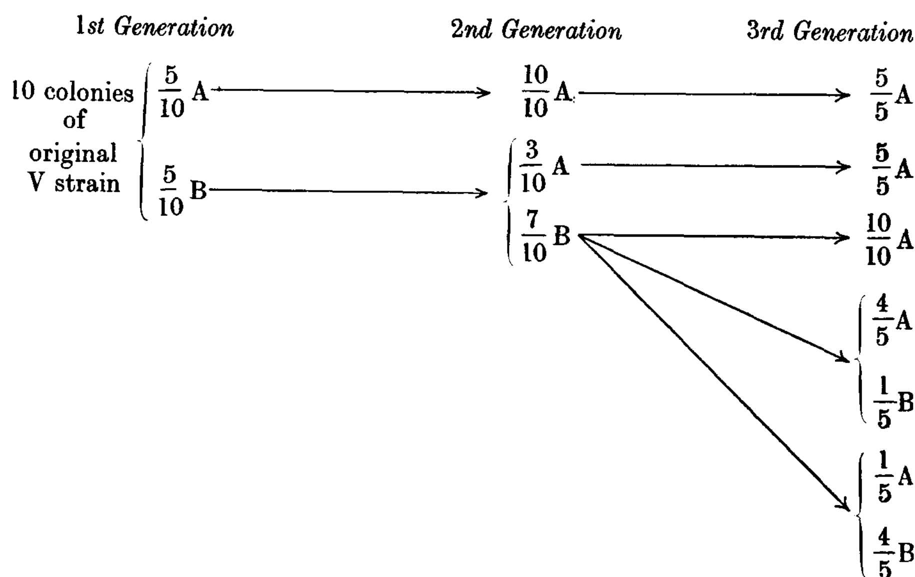


Fig. 1. An arrow indicates the plating of a culture derived from a single colony.

generation in the *b* line, one of the three *a* cultures was plated out and the five colonies examined were all of the *a* form; three of the *b* cultures from this generation were plated out and from the first of these the five colonies examined were made up of one *a* and four *b*; five colonies from the second *b* culture were four *a* and one *b*, while ten colonies from the third *b* culture were all *a*. The examination of these three generations from the parent culture was made over a period of 105 days and the result is illustrated in Fig. 1.

As far as the examination has gone, the *a* line appears to be stable, while the *b* line subdivides into *a* and *b* forms, with a tendency to revert to the *a* form. Since the parent strain shows a constant tendency to give rise to *b* variants, while the *a* substrains do not, the two must be supposed to differ in some way. A comparison of Tables I and III will show that, so far as these results are concerned, a possible explanation would be that the *b* substrains had

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lost, partially or completely, the antigenic component that they shared with X, Z and W.

To test this hypothesis, rabbits were immunized against suspensions of the *a* and *b* substrains of strain V, and their sera were tested against substrains V*a* and V*b*, and against strains X, Z and W 7 days after the first injection of bacterial suspension, and again 7 days after the third injection of the same suspension. The results obtained were not easy to interpret, and were in no sense decisive. The V*a* antiserum, tested after the rabbit had had a single immunizing injection, gave complete agglutination, to varying titre, with strains V*a*, Z, W, X and Y, but only incomplete agglutination with V*b*. This would be compatible with the view that V*a* had lost the specific V antigen. But after three injections this rabbit developed agglutinins that gave complete agglutination with V*b*. Either, therefore, the V*a* suspension contained a proportion of V*b* bacilli, or any loss of the specific V antigen by the V*a* substrain must have been incomplete. The serum of the rabbit injected with the V*b* strain never gave any clear-cut differentiation. This is in no way surprising, since we have noted the constant tendency of the V*b* variant to revert to the V*a* form, and it is more than likely that the V*b* suspension used for immunization may have contained a proportion of V*a* bacilli. In view of the minute amount of antigenic substance that may induce an antibody response, it is by no means easy to determine the antigenic structure of a given species or variant by examining the nature of the antibodies developed in response to relatively massive injections of bacterial cells.

An attempt was then made to answer the same problem by absorption experiments. The V serum, prepared against the parent strain, was absorbed separately with the V*a* and V*b* substrains, and the unabsorbed and absorbed sera were then tested against V*a*, V*b* and Z suspensions. The results are set out in Table IV, the titres given being the end-point of complete agglutination.

Table IV. *Showing the end-point (complete agglutination) given by a* V *antiserum, unabsorbed and after absorption with substrains* V*a and* V*b*

| | Serum V | | |
|---|---|---|---|
| Suspensions | Unabsorbed | Absorbed with V*a* | Absorbed with V*b* |
| V*a* | 6400 | 0* | 200 |
| V*b* | 1600 | 0 | 0 |
| Z | 800 | 50 | 100 |

*0 = no agglutination at 1 : 25.

These findings clearly suggest that the substrain V*a* retains a considerable proportion of the specific V antigen, since it completely removes the agglutinins acting on V*b*. The results with the serum absorbed with V*b* are quite compatible with the view that this variant has lost all or most of the antigen shared with Z; since, in view of its tendency to revert to the V*a* form, it will

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be almost impossible to prepare a thick bacterial suspension suitable for absorption that contains no bacteria of the V*a* type.

It would seem, then, that the main difference between the parent V strain and the V*a* substrain examined, lies in the antigenic instability of the former and the stability of the latter; though we cannot exclude the possibility that there is a quantitative difference in the proportion of specific and group antigens, or that the original culture, contained both *a* and *b* forms. The V*b* variant, on the other hand, appears to have lost the specific antigen, but to have retained the capacity to produce it, as shown by its marked tendency to revert to the V*a* form.

Variants showing analogous properties were obtained from W and Z strains, but, to economize space, these are not given in detail.

## Summary and conclusions

The Flexner dysentery bacilli are non-flagellated, and their agglutination is determined by somatic antigens situated at the cell surface. So far as the author is aware, phasic variations, of the kind that Andrewes (1922) demonstrated in the flagellar antigens of the bacilli of the typhoid-paratyphoid group, have not yet been observed in the case of O antigens; but the variations described above would appear to be of this type. The V, W and Z strains examined gave rise to variants, such as V*b*, that had lost, completely or almost completely, the group somatic antigen, or antigens, but showed a consistent tendency to revert to the form in which the group antigens were present.

No evidence could be obtained of variants in which the group antigens were present, while the specific antigen was lacking; but the substrains, such as V*a*, that showed the presence of both group and specific antigens, appeared to differ from the parent strain in showing no tendency to give rise to variants of the V*b* type.

I am indebted to Dr J. T. Duncan for his help and advice during the course of the work.

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(*MS. received for publication* 17. xi. 1936.—Ed.)

https://doi.org/10.1017/S0022172400034975 Published online by Cambridge University Press