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# Observations on antigenic variation in a strain of *Trypanosoma brucei* growing in mice

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## INTRODUCTION

The phenomenon of antigenic variation in trypanosomes has been known for many years. Earlier workers obtained variants in the form of relapse strains in infected experimental animals inadequately treated by chemotherapy (Ritz, 1914; Lowrie & O'Connor, 1937) and, although the existence of as many as twenty-two variants could be demonstrated by this means (Ritz, 1914), the experimental system made it difficult to investigate adequately the mechanism underlying variation.

Gray (1962) infected rabbits intravenously with *Trypanosoma brucei*, and at intervals injected infected blood from the rabbits into mice, in which strains of trypanosomes grew which were found by agglutination tests to be antigenically different from the strain originally injected into the rabbits. He studied five variants and observed some serological cross-reaction between them. It seemed possible that a modification of Gray's technique, in which variants would be produced in mice passively immunized with rabbit antisera, might clarify the problem further by enabling cross-protection tests to be carried out in addition to agglutination tests.

## MATERIALS AND METHODS

### Trypanosoma brucei

The strain used was supplied by Dr Hoare of the Wellcome Laboratories of Tropical Medicine, London. It was isolated in 1936 from a cow in Pong Tamale, Ghana, and had undergone more than 3000 passages in mice before being used in these experiments.

#### *Preservation of trypanosomes*

Mice under ether anaesthesia were bled from the axillary artery shortly before death. The blood was added to a drop of heparin (about 200 i.u.) and diluted 1 in 10 in fluid containing 10% (v/v) horse serum and 20% (v/v) glycerol in Hanks's balanced salts solution (BSS). The suspension was distributed in ampoules and kept at $-70°$ C. after rapid freezing in $CO_2$/alcohol. In these conditions trypanosomes remained viable for at least 3 months. Stock preparations contained about $10^8$ trypanosomes per ml.

*Counting of trypanosomes*

Trypanosome suspensions were diluted in a white blood cell pipette in white blood cell counting fluid containing 0·1 % gentian violet, and counts were performed in a conventional counting chamber with an improved Neubauer ruling. As a routine, one side of the chamber was filled, and the trypanosomes in five of the central small squares (0·2 mm. × 0·2 mm.) were counted.

*Rabbit antisera*

Trypanosome suspension was diluted in 5 % horse serum in Hanks's BSS to contain $10^8$ trypanosomes/ml. and 0·1 ml. was injected subcutaneously. The rabbits were bled out from the heart 7 days later and the serum was stored at −20° C. Sera were labelled AT 1, AT 2, AT 3 and so on (AT = anti-trypanosome; number = variant).

*Protection tests and development of variants*

Two groups of five Swiss albino mice received 0·2 ml. of diluted trypanosome suspension ($10^6$ trypanosomes) intra-peritoneally. One hour later five of the mice received 0·2 ml. of undiluted antiserum intra-peritoneally. The mice were examined twice a day, and deaths recorded. A mouse was assumed to have died half-way between the time it was last seen alive and the time at which it was found dead. The mean survival time (M.S.T.) was determined for each group and the protection conferred by the antiserum expressed as the difference in M.S.T. between the protected and unprotected groups. Variants were recovered by bleeding a mouse in the protected group which showed symptoms (lethargy, paralysis). Mice invariably died within 12 hr. of the onset of symptoms.

The parent strain (designated T 1) was derived from one of fifty mice which had received 0·1 ml. of a trypanosome suspension diluted to contain approximately 10 trypanosomes per ml. Forty-seven of the fifty mice died with an M.S.T. of 6·7 days. The mouse from which the parent strain was recovered was killed 6 days after inoculation. Since this was the sixth passage of *T. brucei* in mice in this laboratory, the parent strain was designated T 1/6. It was passed seven more times in mice, $10^7$ trypanosomes being injected intra-peritoneally at each passage. The first variant, T 2/1, was derived from the thirteenth passage of the parent strain, T 1/13. For all variants except T 1 and T 2, agglutination and cross-protection tests were carried out on trypanosomes from the first passage.

*Agglutination tests*

Doubling dilutions of antisera were made in 0·5 ml. amounts in 5 % horse serum in Hanks's solution, from 1/20 to 1/10,240. Drops of 0·01 ml. of stock trypanosome suspension ($10^6$ trypanosomes) were delivered from an 'Agla' micro-syringe (Burroughs Wellcome) on to a Perspex plate 1 mm. thick and 0·05 ml. of antiserum dilution was placed on each drop of suspension, using a dropping pipette. The plate was kept at room temperature in a moist atmosphere for 1 hr. Drops were then examined for agglutination by dark-field microscopy with a 5 × objective and

20 × eyepieces (magnification 150 × ). The end-point was taken as the last dilution in which any clumps of trypanosomes were seen. Pl. 1 shows a large clump of agglutinated trypanosomes from a preparation which was dried, fixed with methyl alcohol and stained with May–Grünwald/Giemsa (George T. Gurr, London). The trypanosomes in the agglutinated masses remained actively motile for at least an hour.

*Absorption of agglutinins*

For the preparation of absorbing suspensions 0·1 ml. of stock suspension (about $10^7$ trypanosomes) was injected intra-peritoneally into three mice. Two days later, when the blood contained more than $10^8$ trypanosomes per ml., the mice were bled as described above. The trypanosomes in the heparinized blood were counted and the volume adjusted so that 0·5 ml. contained $10^8$ trypanosomes. For absorption, 1 ml. of 5 % horse serum in Hanks's solution, 0·5 ml. of antiserum diluted 1 in 20, and 0·5 ml. of trypanosome suspension, 2000 $\mu$g. streptomycin, and 200 i.u. of heparin were placed in a 50 ml. conical flask. The flasks were stoppered and shaken gently overnight in a water-bath at 37° C. Next day the contents of the flask were centrifuged and the clear supernatant was stored at −20° C. Agglutination tests with absorbed sera were carried out with dilutions from 1 in 2 to 1 in 128 (1/160 to 1/10,240 in terms of original serum).

## RESULTS

### *Relationship of mean survival time to number of trypanosomes inoculated*

Two experiments were performed in which tenfold dilutions of trypanosome suspension were inoculated intraperitoneally into groups of mice, and the mean survival times determined. Text-fig. 1 shows that the M.S.T. was inversely proportional to the number of trypanosomes injected. In one experiment all the mice were killed after receiving a $10^{-6}$ dilution (5·6 trypanosomes per dose) and in the other after receiving a $10^{-7}$ dilution (*c.* 1·3 trypanosomes per dose). The minimal lethal dose of this strain of *Trypanosoma brucei* was therefore probably one trypanosome.

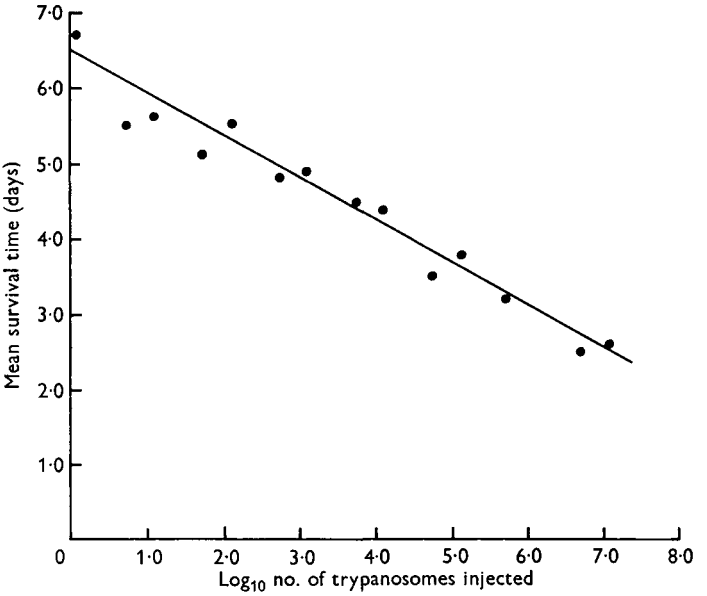
The best straight line drawn by eye through the points shows that a tenfold decrease in the number of trypanosomes inoculated extended the mean survival time by about half a day.

*Cross-protection tests*

Calculation of the standard errors of the mean survival times of eight randomly selected control groups of mice gave values ranging from 0·1 to 0·4 days. The means of these groups ranged from 3·2 to 4·1 days. A difference of 1 day or more between the mean survival times of the control group and the passively immunized group was therefore considered to have shown a highly significant level of protection by the antiserum.

Variant strains were derived sequentially from the parent strain. For example,

T2 was recovered from mice injected with T1 and protected by AT1; T3 from mice injected with T2 and protected by AT2, and so on. Sixty-seven cross-protection tests were carried out, in which antisera were tested against homologous and heterologous strains. Table 1 shows that the homologous serum conferred significant protection in every case. Protection against several strains was given only by the homologous serum, but some sera protected the mice against heterologous strains as well. The pattern of protection by antisera was different for every




Text-fig. 1. Regression of mean survival time of groups of five mice on $\log_{10}$ dose of *T. brucei* injected.

strain. With the exception of antiserum AT8, which gave 1 day's prolongation of M.S.T. against strain T9, antiserum against a given strain never gave protection against the next strain in the series, nor did it protect against the preceding strain in the series.

It is clear that in infected mice protected with a homologous serum death occurred 1–3 days later than in the unprotected controls. During this lag of 1–3 days the antigenic constitution of the protective antigen in the trypanosome population in the protected mice changed. This antigenic change could have arisen either by selection of an antigenically different mutant, or by adaptive alteration in the antigenic structure of the injected population. That the latter possibility is unlikely is shown by the following experiment:

Tenfold dilutions of trypanosomes were injected into groups of five mice, all of which received also 0·2 ml. of homologous antiserum. Table 2 shows that the serum gave complete protection against approximately 10,000 trypanosomes, or 10,000 minimal lethal doses, five of five surviving 14 days in one experiment and three of five surviving in the other. In the first experiment three of five mice were protected against about 100,000 trypanosomes, and in the second there were no

survivors after the same dose. The significance of these results is dealt with in the discussion section.

All the survivors of these experiments were challenged with the same strain 14 days after the original inoculation, each mouse receiving $10^6$ trypanosomes. All the mice died at the same time as the controls, so that the mice had failed to develop immunity.

Table 1. *Cross-protection tests*

(Extension (days) of mean survival time by antiserum).

| Anti-serum against strain | Trypanosome strain | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| 1 | 2·0 | 0 | 0·18 | 0 | 1·0 | 0·8 | 1·2 | 3·1 | 1·5 |
| 2 | 0·5 | 1·8 | 0·6 | 0 | 1·0 | 0·4 | 0·4 | 0·7 | 0·1 |
| 3 | 2·0 | 0·3 | 1·4 | 0 | 1·1 | 0·3 | 0·6 | 0·4 | 0·5 |
| 4 | 0 | – | 0 | 1·3 | 0 | 0·3 | 0·2 | 0 | 0·4 |
| 5 | 0·8 | – | 0·6 | 0 | 2·0 | 0·3 | 0·6 | 0 | 0·2 |
| 6 | 0 | – | – | 0 | 0·2 | 2·4 | 0·7 | 0·1 | 0·2 |
| 7 | 0·2 | – | – | 0 | 0 | 0 | 1·4 | 0 | 0·2 |
| 8 | 0·3 | – | – | 0 | 1·3 | 0 | 0 | 1·9 | 1·0 |
| 9 | 0 | – | – | – | – | 0·4 | – | – | 1·1 |

– = not done.

Table 2. *Effect of dose of trypanosomes on protection by antiserum*

| T 1/17 | | T 1/20 | |
|---|---|---|---|
| $Log_{10}$ dose | Survivors | $Log_{10}$ dose | Survivors* |
| 4·95 | 3/5 | 5·11 | 0/5 |
| 3·95 | 5/5 | 4·11 | 3/5 |
| 2·95 | 5/5 | 3·11 | 5/5 |
| 1·95 | 5/5 | 2·11 | 5/5 |
| 0·95 | 5/5 | 1·11 | 5/5 |

* All survivors challenged with $10^6$ T 1/20 i.p. 2 weeks after first injection; all died at same time as normal controls.

*Cross-agglutination tests*

All strains were agglutinated to high titre by the homologous serum. An antiserum against a given strain generally failed to agglutinate the preceding and succeeding strains in the series. Cross-reactions were more frequent than in the protection tests (Table 3). Three strains (T 2, T 4 and T 6) gave rise to highly specific antisera.

*Relation between agglutination and protection*

The agglutinating and protective powers of the antisera are compared in the correlation diagram (Text-fig. 2) which is derived from Tables 1 and 3. In this diagram the agglutinating titre and extension of M.S.T. are charted for every combination of serum and strain on which both kinds of observation were made. The diagram shows that a serum with an agglutinating titre of less than 1/320 for a given strain failed to protect against that strain. Of thirty-six combinations of

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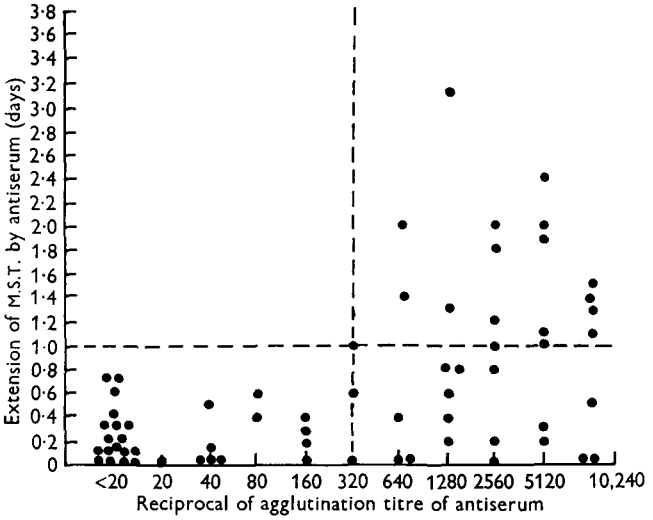
serum and trypanosome strain in which the agglutinating titre of the serum was 1/320 or greater, eighteen showed extension of the mean survival time of 1 day or more. Thus, all the sera which protected mice had high agglutination titres for the strain against which they protected, and none of the sera with low agglutination titres for a particular strain conferred protection against that strain.

Table 3. *Cross-agglutination by antisera*

| Anti-serum against strain | Trypanosome strain | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 2a | 9a |
| 1 | 2,560 | 20 | 1,280 | 160 | 5,120 | 2,560 | 2,580 | 1,280 | 10,240 | 1,280 | 20 |
| 2 | 40 | 2,560 | < 20 | < 20 | 320 | 160 | 80 | < 20 | < 20 | < 20 | < 20 |
| 3 | 640 | < 20 | 640 | < 20 | 5,120 | 160 | 1,280 | 1,280 | 10,240 | < 20 | < 20 |
| 4 | < 20 | < 20 | < 20 | 1,280 | < 20 | < 20 | < 20 | < 20 | < 20 | < 20 | < 20 |
| 5 | 1,280 | – | 320 | < 20 | 5,120 | < 20 | 80 | 2,560 | 2,560 | < 20 | < 20 |
| 6 | 40 | – | < 20 | < 20 | < 20 | 5,120 | < 20 | 40 | 160 | < 20 | < 20 |
| 7 | 1,280 | – | – | 640 | 10,240 | < 20 | 10,240 | 40 | 5,120 | < 20 | 80 |
| 8 | 5,120 | – | – | 320 | 10,240 | 640 | 40 | 5,120 | 2,560 | < 20 | 40 |
| 5 | 10,240 | – | – | – | 5,120 | 640 | 2,560 | – | 10,240 | < 20 | < 20 |
| 2a | < 20 | – | – | – | < 20 | – | – | – | – | 10,240 | – |
| 9a | < 20 | – | – | – | < 20 | – | – | – | – | < 20 | 5,120 |

*Note.* Strain 2a from mice given T 1 and protected with AT 1. Strain 9a from mice given T 8 and protected with AT 1.
– = not done.




Text-fig. 2. Correlation diagram showing the relationship between the agglutinating titre of an antiserum for a given strain, and its ability to protect against that strain.

*Cross-absorption tests*

The results of these tests were erratic, in that a given strain sometimes failed to reduce the agglutination titre against itself. Some reactions in which an unequivocal result was obtained are set out in Table 4. It is clear that the antibodies in AT 1 which agglutinated T 1 and T 3 were removed by T 1 and T 3 and the

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antibodies in AT3 which agglutinated T1 and T3 were removed by T3. The antibody in AT1 which agglutinated T1 is therefore identical with the antibody agglutinating T3. This would suggest that T1 and T3 were antigenically identical were it not for the fact that AT1 agglutinated strain T2a, while AT3 did not. Furthermore, T3 removed from AT3 the antibody agglutinating T9, but did not remove it from AT1, indicating that the antibody in AT1 which agglutinated T9 differed from the antibody in AT3 which agglutinated T9. It is also apparent that the antibodies agglutinating T1 and T3 differed from the antibodies agglutinating T5, T8 and T9. The cross-agglutination reactions were therefore not due to antigenic identity or even to the possession of common major (i.e. absorbing) antigens. Population T1, for example, must have contained several different antigens of which some were minor (i.e. giving sera of high agglutination titre but failing to absorb) and some were major (i.e. giving sera of high agglutination titre and absorbing).

Table 4. *Agglutination titres of absorbed antisera*

| Antiserum | Absorbed with | Titrated against | Agglutination titre: Before absorption | Agglutination titre: After absorption |
|---|---|---|---|---|
| AT1 | T1 | T1 | 2,560 | 160 |
| | | T3 | 1,280 | 160 |
| | | T5 | 2,560 | 2,560 |
| | | T8 | 1,280 | 1,280 |
| | | T9 | 10,240 | 10,240 |
| AT1 | T3 | T1 | 2,560 | 160 |
| | | T3 | 1,280 | 160 |
| | | T5 | 2,560 | 1,280 |
| | | T8 | 1,280 | 1,280 |
| | | T9 | 10,240 | 10,240 |
| AT3 | T3 | T1 | 640 | 160 |
| | | T3 | 640 | 160 |
| | | T5 | 5,120 | 2,560 |
| | | T8 | 1,280 | 1,280 |
| | | T9 | 10,240 | 640 |

*Antigenic variation in the absence of antiserum*

Mice failed to develop immunity to *Trypanosoma brucei*. The development of new antigens in a trypanosome population growing in unprotected mice would thus provide evidence that the variation was caused by random mutation. In order to test this possibility a highly specific strain was used, designated T2a. This strain was isolated from mice protected with AT1 and challenged with T1, and differed from strain T2, which was derived from the same mixture of AT1 and T1 on another occasion. The antigenic structure was specified by the agglutination pattern, using all available antisera at a dilution of 1/320. The parent strain was agglutinated only by AT1 and AT2a (Table 5). Approximately $10^6$ trypanosomes of this strain were injected into each of three mice. Three days later, when the mice were sick, blood from each mouse was diluted 1/10 in heparinized storage medium. The three mice had approximately $10^9$ trypanosomes per ml. of blood at

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this time. The agglutination reactions of each trypanosome population were then determined. One population ($T2a_1$) gave the same pattern as the parent. The second population ($T2a_2$) had developed a new antigen and was agglutinated by AT3. The third population ($T2a_3$) was agglutinated by every antiserum except AT8, so that it too had developed a new antigen, or new antigens. It should be noted that none of the three populations had lost their agglutinability with AT2a.

Table 5. *Development of new antigens in the absence of antiserum*

| | Antiserum (1/320) against strains | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 2a | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 9a |
| Parent strain | + | − | + | − | − | − | − | − | − | − | − |
| Strains derived after a single passage in a mouse 1 | + | − | + | − | − | − | − | − | − | − | − |
| Strains derived after a single passage in a mouse 2 | + | − | + | + | − | − | − | − | − | − | − |
| Strains derived after a single passage in a mouse 3 | + | + | + | + | + | + | + | + | − | + | + |

*Note.* + = agglutination; − = no agglutination.

*Estimation of 'mutation rate' of antigenic structure*

It has been shown that if mice are injected with a trypanosome strain and homologous antiserum the trypanosomes present in the mouse at death are antigenically different from the parent strain in that the original antiserum will no longer protect against them or agglutinate them. An attractive explanation for this would be that the original population contained a small proportion of trypanosomes carrying protective antigens (i.e. antigens giving rise to protecting antibodies) for which there was no corresponding antibody in the homologous antiserum. Suppression of the parent population by homologous antiserum would cause these variants to be selected to kill the mouse. The M.S.T. of a group of protected mice would, on this model, be determined by the number of variants present in the inoculum, and an estimate of that number could be obtained from the dose–response curve (Text-fig. 1). It will be argued in the discussion section that this model, of mutation and selection, is more likely to be correct than the alternative, of adaptational change in antigenic structure under the influence of antiserum.

An experiment was devised to give an estimate of the 'mutation rate per organism per division cycle' (Luria & Delbrück, 1943) according to the proposed model. The assumptions underlying the experiment were as follows:

(1) Mutants are selected by antiserum in protected mice and are responsible for death of these mice.

(2) Under the experimental conditions of development of clones, no back-mutation occurs in the mutant clones.

(3) The growth rates of parent and mutant trypanosomes are the same.

Armitage (1952), on a deterministic model of bacterial mutation, derived the following equation for the proportion of mutants present at time '$t$' in a population showing single mutation, in which the growth rates of mutant and parent are the same:

$$\Psi = 1 - \frac{x_0}{x_0 + y_0} e^{-gt}, \qquad (1)$$

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where $\Psi$ = proportion of mutants at time $t$, $x_0$ = number of parent organisms at time 0, $y_0$ = number of mutants at time 0, $g$ = constant representing rate of mutation, $t$ = time. If the probability of a mutation is evenly distributed throughout the division cycle the 'mutation rate per organism per division cycle' (Luria & Delbrück, 1943) is

$$\Lambda g = \frac{g \ln 2}{a+g} \quad \text{per organism (Armitage, 1952),} \tag{2}$$

where $a$ = constant representing the division rate, $\Lambda$ = generation time of the organisms.

In the experiment 50 mice were injected with 0·1 ml. of a trypanosome suspension (T 1/17) diluted to contain approximately 10 organisms per ml. Six days later, when most of the mice were sick or dead, ten sick mice were bled separately into tubes containing 200 i.u. of heparin. The trypanosomes in each of the ten samples of blood were counted, and an estimate of the total number of trypanosomes in the whole mouse was obtained by multiplying the count per ml. by an arbitrary factor of 4. The remaining 40 mice were allowed to die, and their M.S.T. was 6·3 days. This corresponds to a dose of about 3 trypanosomes in the dose–response curve (Text-fig. 1). The calculation of $a$ was carried out on the assumption that each of the ten trypanosome populations was derived from a single organism, using the equation

$$x = x_0 e^{at}, \tag{3}$$

where $x$ = number of trypanosomes at time $t$ in the mouse in which the clone developed, $x_0 = 1$, $t = 144$ hr. (6 days). Each clone was then diluted so that 0·1 ml. contained approximately $10^6$ trypanosomes, and 0·1 ml. of the diluted clone was injected into each of ten mice. Thirty minutes later, five mice in each group were passively immunized with 0·2 ml. of antiserum (AT 1). The M.S.T.'s of the protected and unprotected mice were determined, and the $\log_{10}$ doses corresponding to the M.S.T. determined from Text-fig. 1.

The proportion of mutants, $\Psi$, present in the original clone was calculated as shown in Table 6. The value of $g$ was determined from equation (1), by taking $y_0 = 0$, and rearranging, and the mutation rate per trypanosome per division cycle was calculated from equation (2). The generation time, $\Lambda$, was also calculated, using equation (2), and the values obtained ranged from 4·4 to 5·4 hr. The mean value of the 'mutation rate' was found to be $10^{-5\cdot17}$ mutations per trypanosome per division cycle, or about 1 in 150,000.

## DISCUSSION

Two alternative explanations are possible for the change in protective antigen under the influence of antiserum. The first is mutation and selection, and the second adaptation. In the experiments summarized in Table 2, populations of $10^4$, and, in three of five cases, of $10^5$ trypanosomes, failed to modify their antigens so as to evade the protective action of the antiserum. Since the minimal lethal dose of trypanosomes was of the order of one trypanosome it is clear that, in the mice surviving the larger dose, less than 1 in $10^5$ trypanosomes modified their

Table 6. *Estimation of 'mutation rate' of protective antigen in* Trypanosoma brucei

| Clone no. | No. of trypanosomes/mouse in the parent population $= x$ ($= 4\times$ number of tryps./ml. of blood) | Rate of division $= a = \frac{\log x}{144 \log e}$ | M.S.T. of unprotected mice (days) | $\text{Log}_{10}$ trypanosomes inoculated into unprotected mice (from Text-Fig. 2) $= p$ | M.S.T. of protected mice (days) | $\text{Log}_{10}$ 'mutants' inoculated into protected mice (from Text-fig. 2) $= q$ | $\text{Log}_{10}$ proportion of 'mutants' in original population $= q - p = \log \Psi$ | $\Psi$ | $= -\frac{g}{\log (1-\Psi)}{144 \log e}$ | Mutation rate per trypanosome per division cycle $= \frac{g \ln 2}{a+g}$ | Generation time in hr. $= \Lambda$ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | $10^9$ | 0·144 | 3·1 | 6·1 | 4·8 | 3·1 | −3·0 | 0·001 | $6{\cdot}9\times10^{-6}$ | $3{\cdot}3\times10^{-5}$ | 4·8 |
| 2 | $4{\cdot}8\times10^9$ | 0·155 | 2·7 | 6·8 | 5·3 | 2·2 | −4·6 | 0·00003 | $3{\cdot}2\times10^{-7}$ | $1{\cdot}4\times10^{-6}$ | 4·4 |
| 3 | $3{\cdot}8\times10^9$ | 0·154 | 3·1 | 6·1 | 5·3 | 2·2 | −3·9 | 0·00013 | $9{\cdot}6\times10^{-7}$ | $4{\cdot}3\times10^{-6}$ | 4·5 |
| 4 | $9{\cdot}2\times10^8$ | 0·144 | 2·9 | 6·5 | 5·2 | 2·4 | −4·1 | 0·00008 | $6{\cdot}4\times10^{-7}$ | $3{\cdot}1\times10^{-6}$ | 4·8 |
| 5 | $3{\cdot}0\times10^9$ | 0·150 | 3·3 | 5·7 | 5·6 | 1·6 | −4·1 | 0·00008 | $6{\cdot}4\times10^{-7}$ | $3{\cdot}1\times10^{-6}$ | 4·8 |
| 6 | $3{\cdot}6\times10^8$ | 0·137 | 3·3 | 5·7 | 5·3 | 2·2 | −3·5 | 0·00031 | $2{\cdot}1\times10^{-6}$ | $1{\cdot}1\times10^{-5}$ | 5·4 |
| 7 | $4{\cdot}8\times10^9$ | 0·155 | 3·1 | 6·1 | 5·3 | 2·2 | −3·9 | 0·00013 | $9{\cdot}6\times10^{-7}$ | $4{\cdot}3\times10^{-6}$ | 4·5 |
| 8 | $2{\cdot}4\times10^9$ | 0·150 | 2·7 | 6·8 | 5·3 | 2·2 | −4·6 | 0·00003 | $3{\cdot}2\times10^{-7}$ | $1{\cdot}4\times10^{-6}$ | 4·4 |
| 9 | $5{\cdot}2\times10^9$ | 0·156 | 2·7 | 6·8 | 5·3 | 2·2 | −4·6 | 0·00003 | $3{\cdot}2\times10^{-7}$ | $1{\cdot}4\times10^{-6}$ | 4·4 |
| 10 | $2{\cdot}9\times10^9$ | 0·151 | 3·1 | 6·1 | 5·3 | 2·2 | −3·9 | 0·00013 | $9{\cdot}6\times10^{-7}$ | $4{\cdot}3\times10^{-6}$ | 4·5 |

Mean value of mutation rate per trypanosome per division cycle $= 10^{-5{\cdot}7}$

protective antigen. The frequency of 'adaptation', in these trypanosomes, was less than $10^{-5}$. In Table 2, column 9, it is seen that, when about $10^6$ trypanosomes were injected into mice, the frequency of adaptation (on the adaptational hypothesis) was of the order of $10^{-3}$ to $10^{-4}$. Thus, if the hypothesis of adaptation is correct, the frequency of adaptation in a trypanosome population in the presence of antiserum is a function of the number of trypanosomes in the population, which means that the probability that a single trypanosome will adapt is not independent of adaptation in other trypanosomes. It seems unlikely that the process of adaptation in a single trypanosome could influence the ability of other trypanosomes to adapt. An 'adaptation-producing factor' would have to be postulated, which would be released as a result of adaptation, to confer the ability to adapt on other trypanosomes. The adaptation hypothesis therefore requires complex subsidiary hypotheses in order to explain the experimental findings. Furthermore, in Table 5 it is seen that variation in the agglutinating antigen can occur in the absence of antiserum. If the agglutinating antigen is the same as the protective antigen, as will be argued below, then variation in protective antigen occurs in the absence of antiserum. The balance of evidence therefore strongly favours the mutation and selection hypothesis.

Variation in the protective antigen was accompanied by variation in the agglutinating antigen, and the simplest explanation of this is that both antigens are the same. However, the correlation diagram (Text-fig. 2) shows that, while high protective capacity of an antiserum is associated with high agglutinating titre, the inverse correlation of high agglutinating titre with high protective capacity is less marked, in that only half the sera with agglutinating titres greater than 1/320 gave significant protection against the corresponding strain. Reference to Tables 1 and 3 will show that high agglutination titre and high protective capacity were associated in the nine paired reactions between a trypanosome strain and the homologous antiserum. A plausible explanation (assuming that protective and agglutinating antigens are the same) for the failure of some agglutinating antisera to protect in heterologous reactions can readily be devised. For successful performance of agglutination tests it was repeatedly observed that not much less than $10^6$ trypanosomes were required for each drop of antiserum dilution; when $10^5$ were used, no agglutination occurred because collisions were too infrequent. Suppose 90 % of the members of a trypanosome population contained an antigen A, and 10 % an antigen B, and the paired reaction was carried out with an antiserum containing only antibodies to A. Agglutination would occur, since very nearly $10^6$ trypanosomes carrying antigen A would be present in the test. In the protected mice trypanosomes with antigen A would be destroyed and the effective dose of trypanosomes in these mice would have been reduced tenfold. Text-fig. 1 shows that a tenfold reduction of the inoculum extended the M.S.T. by about half a day. It is clear that in such a situation a serum would show high agglutinating power and low protective capacity. If the two antigens are not the same one would expect many sera to show low agglutinating power and high protective capacity, but in thirty-one tests this did not occur once. The results therefore support the view that the protective antigen is the same as the agglutinating antigen.

What is the mechanism underlying antigenic variation? The value of the mutation rate required to account for the experimental findings was $10^{-5.17}$ per trypanosome per division cycle, which is a high but not extraordinary rate. The number of possible variants is high, eleven having been produced in these experiments and twenty-two by Ritz (1914). It is reasonable to suppose that there must be a limit to the possible number of antigenic variants, but the determination of this number could be a thankless task. For example, proof of the existence of 100 different variants, using the methods described in this paper, would necessitate at least 10,000 cross-protection tests, using at least 100,000 mice. If to this is added the fact, deduced from the data in Table 4, that a given trypanosome population contains several different antigens, the prospect of the investigations required is sufficiently awesome to terrify even the most dedicated research worker. It should be borne in mind that a large number of different antigens could, in theory, be produced by a fairly simple underlying genetic mechanism. Suppose, for example, that in the gene locus determining the structure of the surface antigen there are $n$ 'unstable' sites each of which determines a different portion of the antigen, and each of which may exist in one of two forms such that the two forms of the determinant at a given site differ from the two forms at all the other sites. Alternation between the two forms of determinant (i.e. mutation) at a site might occur randomly, with a frequency of about $10^{-5}$ mutations per trypanosome per division cycle, and independently of mutations at the other sites. The surface antigen of a single trypanosome could then exist in $2^n$ different forms. Thus, 1024 variants could be produced from as few as ten unstable determinants.

## SUMMARY

Variation in agglutinating and protective antigens was studied in a strain of *Trypanosoma brucei* growing in mice protected with anti-trypanosomal rabbit serum. It was concluded that the agglutinating and protective antigens were the same, and that variation in the antigenic structure of trypanosome populations exposed to antiserum was due to mutation and selection rather than to adaptation. The mutation rate per organism per division cycle required to account for the experimental findings on the mutational hypothesis was found to have a mean value of $10^{-5.17}$.

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## EXPLANATION OF PLATE

Clump of agglutinated trypanosomes. (May–Grünwald/Giemsa. × 200.)