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A comparative study of a plaque and quantal method for assaying the neutralizing activity of antisera to type 1 poliovirus*

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INTRODUCTION

The plaque neutralization test was originally developed for assaying antisera to bacteriophage, and was later adapted to the measurement of the neutralizing antibody content of sera against various animal viruses, such as poliovirus (Dulbecco, Vogt & Strickland, 1956). Before this, except for those viruses which produce pocks on the chorio-allantoic membrane of the developing chick embryo, neutralizing activity had been estimated by the classical 'quantal' method, using either eggs, animals, or tubes of tissue culture.

While it is intuitively recognizable that the plaque neutralization method is probably more sensitive than the quantal in all circumstances, the quantitative relationship between the results obtained by these two methods, for any one system, has never been fully elucidated. Moreover, it has always been tacitly assumed that either of these two assay techniques gives a linear response with respect to the concentration of neutralizing antibody. This latter assumption could only be tested rigorously in the case of either method, provided that some entirely independent means of measuring antibody concentration was available. At the same time, it can be shown that if both the quantal and plaque methods independently yield linear estimates of the neutralizing antibody concentration, then the two assays themselves will be linearly related.

During the study of the antibody response of the rabbit to type 1 poliovirus, the neutralizing antibody content of a number of sera from sample bleedings was estimated in parallel by the quantal and plaque neutralization techniques. The two techniques were compared for accuracy, sensitivity and facility of use under ordinary laboratory conditions. In addition, a comparison between the antibody estimates yielded by the two methods was carried out in order to confirm that the relationship between them is of the general linear form

$$p = Rq + c,$$

where p is the plaque neutralizing titre, and q the quantal neutralizing titre, for each serum in the series, R and c being constants.

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MATERIALS AND METHODS

Materials

Virus

The virus challenge used in both neutralization tests was the Brunhilde strain of type 1 poliovirus grown in the ERK line of HeLa cells (Westwood, Macpherson & Titmuss, 1957), by the technique of Churcher, Sheffield & Smith (1959). The tissue culture fluid produced was stored at $-10°$ C., except for the sample in current use which was stored at 4° C.

Sera

Two rabbits, 1/59 and 2/59, were immunized with the Brunhilde strain of type 1 poliovirus grown in ERK, concentrated and partially purified by high-speed centrifugation, so that it contained approximately $10^{9\cdot5}$ plaque-forming units (pfu) per ml. Each rabbit received 0·5 ml. of this antigen intravenously on day 0, 5 ml. on day 20 and 2·5 ml. on day 127. A total of thirty blood samples from each rabbit was taken at intervals over a period of 217 days after the initial inoculation. The serum was separated, inactivated by heating at 56° C. for 30 min., and stored at $-10°$ C. A pool of sera from the primary and secondary responses of rabbits similarly immunized was used as a reference standard serum.

Cell cultures and media

The ERK line was used to estimate both virus and residual virus concentration. The cells were cultured in a growth medium consisting of Earle's balanced salt solution with 0·25 % lactalbumin hydrolysate, 5 % tryptic digest meat broth, and 10 % calf serum. To each 100 ml. of medium, 2 ml. of 2·78 % sodium carbonate was added and the pH adjusted to 7·4 by gassing the medium with CO_2 immediately before use. Penicillin and streptomycin were added to the final medium to give a concentration of 100 units/ml. and 100 μg./ml. respectively.

For plaque production, the cells from a confluent cell sheet on one side of a 250 ml. 6-sided Pyrex bottle (approximately 4×10^6 cells) stripped by versene, were seeded into three bottles in 10 ml. of growth medium per bottle, and usually produced confluent monolayers in 48 hr.

For tube cultures, the cells from a similar confluent cell sheet (approximately 4×10^6 cells) were seeded into twenty $6 \times \frac{5}{8}$ in. tubes, using 1 ml. of growth medium per tube. Seeding at this concentration gave rise to confluent growth in 48 hr.

Methods

All dilutions were made in physiological saline with 10 % calf serum added. Dilutions and inoculations were made with mercury calibrated pipettes, using volumes of 0·1 ml. or greater.

Virus titration by the plaque method

The technique employed was based on that described by Hsiung & Melnick (1957), 0·3 or 0·5 $\log_{10}$ dilutions of virus suspension were made and 0·1 ml. of each dilution delivered on to each of three cell monolayers previously drained of growth medium. One hour at 37° C. was allowed for adsorption of the virus and then

10 ml. of overlay, held at 43° C., was added to each bottle, mixed with the inoculum and allowed to cover the cell monolayer. This overlay consisted of equal parts of 2·4 % Difco 'Bacto' agar and double strength medium with increased sodium carbonate. The sodium carbonate concentration of the complete overlay was 0·11 %, the double-strength medium which was not gassed contained 0·22 % sodium carbonate. An hour at room temperature permitted the agar to harden, after which time the bottles were inverted and the cultures incubated at 37° C. After 42 hr. 0·25 ml. of 1/1000 neutral red was flooded over the surface of the agar and the bottles immediately inverted and re-incubated at 37° C. Plaques could be discerned 5 hr. after the addition of the dye, when the site of each plaque was marked on the bottle. The bottles were examined on each of the following 2 days when plaques making their first appearance were marked and the total number of plaques present counted, a final count of the plaques being recorded on the 4th day. The mean of the plaque counts of three bottles at any one dilution gave an estimate of the number of plaque-forming units per ml. of the original virus suspension when corrected for the dilution and volume of the inoculum.

Plaque neutralization test

Dilutions of serum at 0·3 $\log_{10}$ intervals were made in 0·5 ml. amounts on either side of the dilution which gave a 50 % reduction in plaque count of the virus challenge, as assessed in a pilot experiment using 0·5 $\log_{10}$ dilutions. Each dilution was mixed with an equal volume of virus suspension containing 40 pfu/0·1 ml.; one tube containing diluent in place of the serum dilution was employed to assay the virus challenge. After 1 hr. incubation at 37° C., the virus-serum mixture for each serum dilution and the virus challenge was inoculated into three bottles, 0·2 ml. per bottle. The plaque count at each serum dilution was determined by the procedure described under virus titration and the neutralizing titre of the serum was taken as the reciprocal of the initial serum dilution which reduced the plaque count to 50 % of the virus challenge. Percentage neutralization at each serum dilution, calculated as a mean of the three bottles, was plotted against $\log_{10}$ of the serum dilution, and the 50 % neutralization end-point calculated from a straight line fitted to the resulting experimental points by the method of least squares.

Virus titration by the quantal method

A volume of 0·1 ml. of each 0·3 $\log_{10}$ or 0·5 $\log_{10}$ dilution of virus suspension was inoculated into each of six tube cultures containing 2 ml. of maintenance medium. Maintenance medium differed from growth medium only in an increase of sodium carbonate from 2 to 3 ml./100 ml. of medium and the omission of gassing. The tubes were rolled at 37° C., read at 24 hr. to detect any toxic effects, and subsequently at 3, 4, and 5 days, the results recorded being those read on the 5th day. The titre, expressed in TCD 50's, was taken as the reciprocal of that dilution of virus suspension which produced a cytopathogenic effect in half the tubes inoculated, and was interpolated where necessary by the moving average method of Thomson (1947).

Quantal neutralization test

Dilutions of serum at 0·3 $\log_{10}$ intervals were made in 0·5 ml. amounts straddling the dilution which would protect 50 % of the tube cultures exposed to a virus challenge of approximately 100 TCD 50. The relevant dilution interval in which the end-point would be expected had been estimated previously as in the plaque neutralization method. Each serum dilution was mixed with an equal volume of virus suspension containing approximately 200 TCD 50 per 0·1 ml. and then incubated for 1 hr. at 37° C. The virus challenge was titrated by $\log_{10}$ steps under the same conditions as the serum dilutions. After neutralization had been allowed to proceed for 1 hr. at 37° C., 0·1 ml. of virus-serum mixture was inoculated into each tissue culture tube, six tubes being used per serum dilution. The presence of infective virus at each serum dilution was determined in the manner described under virus titration by the quantal method. The neutralizing titre of the serum was taken as the reciprocal of the initial dilution of the serum which would protect half the tissue cultures against the cytopathic effect of the virus challenge and was calculated by the moving average method. The standard rabbit type 1 antiserum was titrated in the above manner with each batch of serum assays.

RESULTS

The plaque neutralization test

The neutralizing titre of a serum has been defined as the reciprocal of that dilution which will reduce the mean plaque count to half its expected value as determined by the mean plaque count in the absence of serum. This means that the variation of plaque number in individual monolayers is the factor governing the reproducibility of the test.

It will be apparent that the factors limiting the accurate estimation of plaque-forming units per bottle will be: (1) the number of plaques present, (2) their rate of growth, (3) the time for which they are allowed to grow, and (4) the distribution of the infective foci over the cell monolayer. Thus, should two adjacent plaques be in contact when first observed, difficulty will be experienced in distinguishing them subsequently when they enlarge. The following experiments were designed to demonstrate the influence of such coalescence upon the results obtained.

Study of plaque development

Presence of small and large plaques

Although the predominant plaque type was large in both virus titrations and serum assays, small plaques were also present. The possibility that these small plaques arose from a small plaque variant was investigated, in case they represented a genetically stable variant with a different antibody sensitivity. A large and a small plaque were 'picked' and passed in tissue culture. When the cultures showed complete degeneration, each fluid was titrated by the plaque method. The proportion of large to small plaques arising from the large plaque parent was 3·96 to 1, while from the small plaque parent the ratio was 3·63 to 1. As the two ratios

are of the same order of magnitude, the difference in size was not due to genetically different particles, but probably to asynchrony of infection, maturation or release of the virus from the infected cells.

Coalescence of plaques

The inclusion of neutral red in the overlay in virus titrations by the plaque method was found by Darnell, Lockhart & Sawyer (1958) to reduce the plaque count of type 1 poliovirus by 50 %, when this virus was titrated in HeLa cells. It was decided, therefore, to delay the addition of the vital stain until 48 hr. after the inoculation of the tissue culture. It was then possible to mark the plaques as they appeared and to reduce the number which would be lost, through coalescence of enlarging adjacent foci, if counting was delayed until the 3rd or 4th day. To

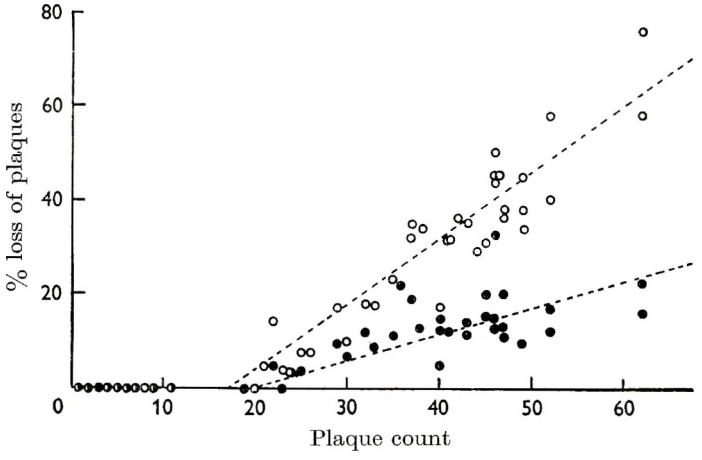

Fig. 1. Percentage loss of plaques due to coalescence as a function of the plaque count. ●, Between 2 and 3 days; ○, between 2 and 4 days.

determine the extent of this loss, several titrations of the standard serum and virus challenge were performed, the plaques being marked on the 2nd, 3rd and 4th days. The number of plaques so estimated (a_2, a_3, and a_4) were then compared with the apparent number of plaques (b_3, b_4) present on the 3rd and 4th days as estimated by ignoring the markings. The percentage loss of plaques at 3 and 4 days (e.g. at 3 days, $[(a_3 - b_3)/a_3] \times 100$) was plotted against the plaque count on the corresponding day as shown in Fig. 1. It will be noted that for a plaque count of 20 or less there is little or no loss. Above this figure, for every increase of 10 in the plaque count, there is an approximate increase of 5 % in the loss due to a coalescence between the 2nd and 3rd days, and this is increased to approximately 14 % for the interval between 2 and 4 days. Despite this early marking of infected foci, it is still possible that some plaques were being overlooked because they occurred under pre-existing lesions, or multiple infection of a cell had occurred.

To determine the extent of this loss, several virus titrations were undertaken, and the mean plaque count at each dilution recorded. A loss due to the above-mentioned factors would be expected to follow a similar pattern to that of Fig. 1,

i.e. with a progressive increase in the concentration of virus there would be increased likelihood of overlaying or multiplicity of infection of cells, and a corresponding decrease in the plaque count below that expected. Fig. 2 shows the relationship between virus concentration and the plaque count for four such virus titrations, and there is no evidence that there is any departure from linearity. As the virus challenge seldom exceeded 50 plaques per bottle in the neutralization tests, loss due to this cause can be ignored in this study.

Further evidence that this loss is negligible is found in the effect of the size of the virus challenge upon the titre of an immune serum. If with increasing plaque counts there is loss due to 'overlaying' of plaques, or multiple infection of cells, then with a small number of plaques, i.e. a high degree of neutralization, there will

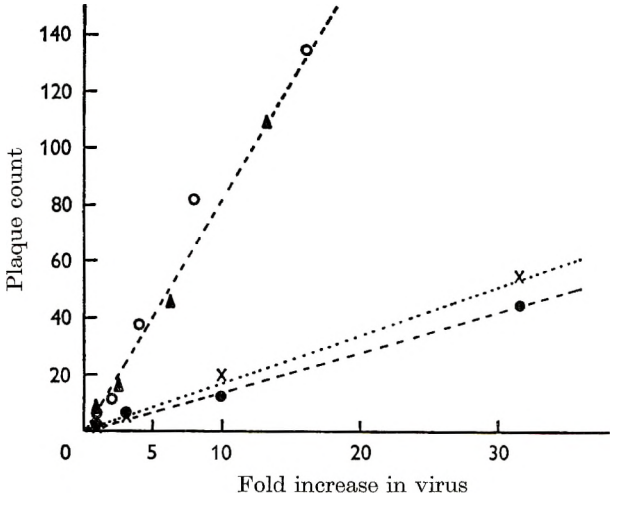

Fig. 2. The relationship between plaque count and virus concentration. ○, ▲, Two titrations of type I poliovirus preparation, 29/59; ×, ●, titrations of two other type I poliovirus preparations, 3/59 and 17/59.

be little or no loss of plaques. However, as the plaque count increases with decreasing neutralization, the plaque loss would correspondingly increase, and be maximal in the virus control. This will obviously not affect each serum dilution equally. Thus not only will the titre be changed, but also the slope of the line relating percentage neutralization to $\log_{10}$ serum dilution. The exact manner in which this affects the neutralization curve can be seen from an experiment where the plaque counts on the 4th day, obtained by daily markings, were compared with those estimated ignoring the previous markings (see Table 1). It can be seen that the titre of the serum is reduced by $0{\cdot}2 \log_{10}$, owing to the significant ($0{\cdot}05 > P > 0{\cdot}02$) increase in the slope of the line relating percentage neutralization to $\log_{10}$ serum dilution.

In the example shown, the loss of plaques is overcome by early marking, but should further loss of plaques occur which is not detected by this method, then a reduction in titre with increased virus challenge would be expected. It can be shown, however (see 'Effect of the size of the virus challenge', p. 319) that no such

relationship is demonstrable. Further evidence in favour of such a loss would be a significant increase in the slope of the line relating percentage neutralization to $\log_{10}$ serum dilution, b, with increase in virus challenge. In a series of ten titrations it was shown that b was not correlated with the size of virus challenge ($0{\cdot}2 > P > 0{\cdot}1$) so that there is no evidence for a residual, significant loss of plaque number when marking at 48 hr.

The reduction in titre and change in slope which are associated with loss of plaques due to coalescence is of considerable importance in those tests where the addition of vital dye is delayed until the 4th day, for then the effect of the size of the virus challenge will be appreciable.

Table 1. *The effect of coalescence of adjacent plaques on the slope of the neutralization curve and the serum titre*

	4th day counts observing daily markings				4th day counts ignoring markings			
Serum dilution	Mean plaque count	Neutralization (%)	'b'	Neutralizing titre	Mean plaque count	Neutralization (%)	'b'	Neutralizing titre
$10^{-3.7}$	1·33	97·5	87	$10^{4.31}$	1·33	95	111·67	$10^{4.1}$
$10^{-4.0}$	8·33	84·5			8·33	68		
$10^{-4.3}$	23·33	56·5			21·33	18		
$10^{-4.6}$	46	14·5			31·33	0		
$10^{-4.9}$	52·67	2			25·67	1		
V.C.	53·67	—			26	—		

'b' = slope of the line relating % neutralization to $\log_{10}$ serum dilution. V.C. = virus challenge.

Error of plaque counting

Many workers have shown that the pock counts on the chorio-allantoic membrane of the developing chick embryo, when titrating pox virus suspensions (Fenner & McIntyre, 1956; Kaplan & Belyavin, 1957; Armitage, 1957; Westwood, Phipps & Boulter, 1957), or virus-serum mixtures (McCarthy, Downie & Armitage, 1958) have a variation greater than that expected if the distribution of the pocks between the membranes was poissonian.

If plaque counts have a Poisson distribution, then the variance between replicate plaque counts would approximate to their means. A mean was calculated for each group of three counts in the plaque neutralization tests on 61 sera. Each mean count was plotted against its own standard deviation, s, in Fig. 3. It will be seen that there appear to be two populations, one tending to a Poisson distribution, inasmuch as the standard deviation approximates to the square root of the mean plaque count, the other having a greater variance. On studying the nature of the sera showing the higher variance, it became apparent that the majority of them were derived from the rabbits which had received only one inoculation of antigen. When the data derived from the sera of the primary responses are plotted independently, the same bimodal distribution is seen, and no further division can be made on the basis of individual rabbits, or on the subculture of the cell line on which the results were obtained. The data from the remaining sera approximate

to a Poisson distribution, as indicated by the relationship between the variance and the mean plaque count. The points obtained from three bottle estimates in virus titrations were also shown to follow the same pattern. From this it can be assumed that the distribution of the plaque-forming elements is the same whether they are taken from virus or serum-virus mixtures. The large degree of scatter of the points around the line predicted by a Poisson distribution could be attributed to the small number of samples from which the statistics have been calculated. As the mean plaque count increases, it would be expected that the distribution would approximate to a normal one, but it is not possible, owing to the scatter of the points, to decide whether the variance is dependent on the mean or not, at higher plaque counts.

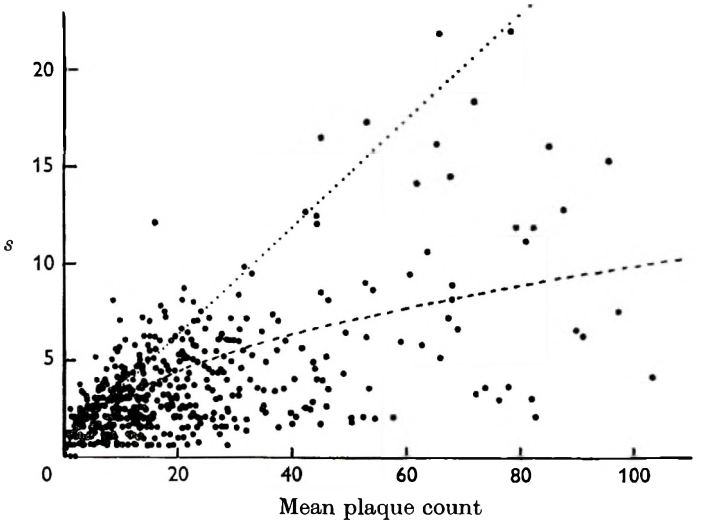

Fig. 3. The relationship between the calculated standard deviation, s, and the mean plaque count for all serum titrations. - - - -, Theoretical relationship for a Poisson distribution; ....., relationship of a group of points with greater values for s than expected from a Poisson distribution.

Error of the plaque neutralization test

In measuring neutralizing antibody titres of the sera from rabbits immunized with poliovirus, it is important to know the error of estimation. As has already been intimated, this will arise directly from the actual variation in plaque count between replicates, and the magnitude of its effect on the calculated neutralizing titres can be directly estimated by repeated titrations.

The standard serum was estimated on one occasion by three replicate titrations, using the same virus challenge, and on seven other occasions with an independent virus challenge (Table 2). This was because the number of replicate titrations that can be accomplished on one occasion is limited by technical factors. A smaller number of estimates were made on the test sera, a primary bleeding 'D' and a tertiary bleeding 'V' and these are also recorded and analysed in this table.

From simple inspection of the data in Table 2, it will be seen that the variation in titre of the standard serum is approximately the same whether the serum is

estimated in three replicate titrations with a common virus challenge (first three rows), or on separate occasions with an independent virus challenge. From this it can be assumed that there was no gross change in the quantity or nature of the antibody, or the behaviour of the tissue culture cells over the period during which these estimations were made, any error being due to the manipulations of the technique itself, e.g. sampling, dilution, counting, etc.

Table 2. *The error of estimating the neutralizing titre of three sera by the plaque method*

Serum	Estimate	Virus challenge (pfu's)	Titre	Mean titre	Standard deviation	Coefficient of variation	Fiducial limits ($P = 0{\cdot}05$)
'Standard'	1	21·33	21,380	22,811	± 3,280	14·38	14,398–30,224
	2	21·33	29,854				
	3	21·33	22,856				
	4	23·67	20,989				
	5	25	20,701				
	6	32·33	28,445				
	7	46·33	22,131				
	8	48·67	21,380				
	9	53·67	20,417				
	10	58·67	19,953				
'D'	1	—	169	206·75	± 26·3	12·72	123–290·5
	2	—	219				
	3	—	227				
	4	—	212				
'V'	1	—	153,780	150,598	± 19,690	13·2	100,298–200,898
	2	—	153,640				
	3	—	162,180				
	4	—	169,820				
	5	—	151,710				
	6	—	112,460				

pfu = plaque-forming unit.

Reference to Table 2 shows that the three sera, having titres between 207 and 150,598 and derived from primary, secondary and tertiary responses, yield a variance between replicates proportional to their mean titre, the coefficient of variation being approximately constant. The overall data show that 95 % of estimates of the titre of any one serum could be expected to fall within 0·4 $\log_{10}$ of each other. These findings are in agreement with French, Armstrong & Nagler (1959), who, using their plaque neutralization technique, determined a coefficient of variation for type 1 poliovirus neutralization of 12·3 %.

Effect of the size of the virus challenge

It would be expected that with an increase in the virus challenge the titre of the serum would be reduced. However, the range of virus challenges that can be studied has strict limitations. A virus challenge of less than 20 pfu results in an unsatisfactory neutralization curve, and in excess of 60 pfu difficulty is experienced in counting the plaques formed. The ten estimates of the standard serum recorded

in Table 2 have been accomplished with virus challenges in this range. The slope of the straight line of best fit, as calculated by the method of least squares, to the plot of the titre versus the virus challenge does not differ significantly from zero ($P = 0{\cdot}2$). These observations, therefore, show that the virus challenge and the serum titre appear to be unrelated over the range employed. In other words, the change in serum titre with virus challenge is no greater than the variation inherent in the neutralization test itself over the narrow range of virus challenge capable of being studied.

The quantal neutralization test

As each tube in this type of neutralization test does not give an individual estimate, the error of this test and the influence of the size of the virus challenge could be studied directly.

Error of the quantal neutralization test

Twenty-two estimates of the neutralizing activity of the standard serum were made on separate occasions by this technique and the results are recorded in Table 3. Unlike the plaque test, many replicate titrations could be performed by

Table 3. *The error of estimating the neutralizing titre of two sera by the quantal method*

Serum	Estimate	Titre	Mean titre	Standard deviation	Coefficient of variation	Fiducial limits ($P = 0{\cdot}05$)
'Standard'	1	5,370	5,197	± 875·2	16·84	3,377–7,017
	2	6,310				
	3	5,012				
	4	4,571				
	5	5,623				
	6	4,571				
	7	5,012				
	8	6,310				
	9	5,012				
	10	4,571				
	11	6,310				
	12	6.310				
	13	5,012				
	14	4,571				
	15	3,981				
	16	3,350				
	17	5,012				
	18	4,467				
	19	5,012				
	20	5,623				
	21	7,079				
	22	5,248				
'V'	1	28,150	31,058	± 5,254	16·92	14,351–47,766
	2	25,119				
	3	35,481				
	4	35,481				

the quantal method at any one time. However, to obtain an estimate of the error of this test, which was comparable with that already determined for the plaque test, repeated titrations on different occasions were undertaken. The standard deviation, coefficient of variation and the fiducial limits of the titre of the standard serum calculated from these titrations are also included in Table 3, as is a similar analysis on a serum from a tertiary response, bleeding 'V'. The coefficients of variation are of the same order of magnitude for the standard serum and the high titred serum from the tertiary response, showing that, as in the plaque neutralization test, the error is proportional to the titre.

Effect of the size of the virus challenge on the serum titre

It was possible in the quantal technique to determine the effect of the size of the virus challenge on the neutralizing titre of a serum over a larger range of values than in the plaque neutralization test. The limiting factors operative in the plaque test were no longer applicable, provided that the virus challenge was of such magnitude that the possibility of a tube containing no antibody being uninfected, owing to sampling error, was exceedingly small. The plot of the percentage neutralization against $\log_{10}$ serum dilution, for three titrations of the standard serum against fourfold increase in virus challenge (42, 169 and 676 TCD 50) showed that the slope differed only slightly, but a reduction in the titre with increase in the virus challenge was observed. The relationship between the size of the virus challenge and the corresponding neutralizing titre was such that for every increase of 100 TCD 50 in the virus challenge, within the range studied, the titre of the serum fell by approximately 0·1 $\log_{10}$.

Comparison of the titres of immune sera measured by the plaque and quantal neutralization tests

Each of the neutralization methods used in these experiments yields an estimate of the neutralizing antibody content of a serum in terms of certain numerical parameters p and q, p being the neutralization titre as measured by the plaque method, and q the corresponding neutralizing titre as estimated by the quantal titration. If both p and q are linear functions of the same antibody concentration, then these parameters should themselves be linearly correlated. However, reference to Fig. 4 shows that there is a non-linear relationship between the titres obtained by the two tests, for the bleedings from the secondary and tertiary responses of rabbit 1/59. A good fit to the points obtained has been accomplished by using an arbitrary quadratic equation having the form $p = 8{\cdot}2q - 0{\cdot}12q^2$. A similar good fit has been obtained in the case of the primary bleedings from both rabbits using the equation $p = 9q - 0{\cdot}05q^2$ (Fig. 5) and to the secondary and tertiary responses of rabbit 2/59 (Fig. 6) by the line $p = 6q - 0{\cdot}1q^2$. Sera having titres less than 60,000 when measured by the plaque neutralization method, approximate to a straight line and it is only with the higher titres obtained from sera collected during the tertiary response of one rabbit that the curvilinear relationship between the two becomes evident. It must be stressed that the quadratic function fitted to the points obtained is entirely empirical, and cannot be given any fundamental

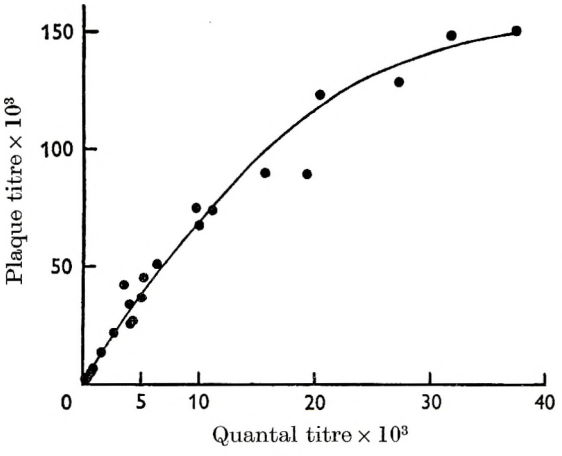

Fig 4

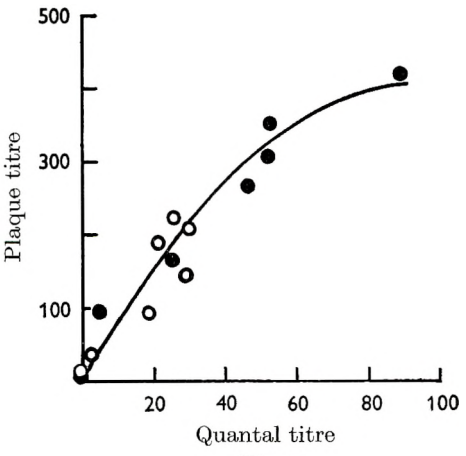

Fig 5

Fig. 4. The relationship between the neutralizing titres determined by the plaque and quantal methods for sera from the secondary and tertiary responses of rabbit 1/59. ——, $p = 8{\cdot}2q - 0{\cdot}12q^2$.

Fig. 5. The relationship between the neutralizing titres determined by the plaque and quantal methods for sera from the primary responses of rabbits 1/59 and 2/59. ●, Sera from rabbit 1/59; ○, sera from rabbit 2/59; ——, $p = 9q - 0{\cdot}05q^2$.

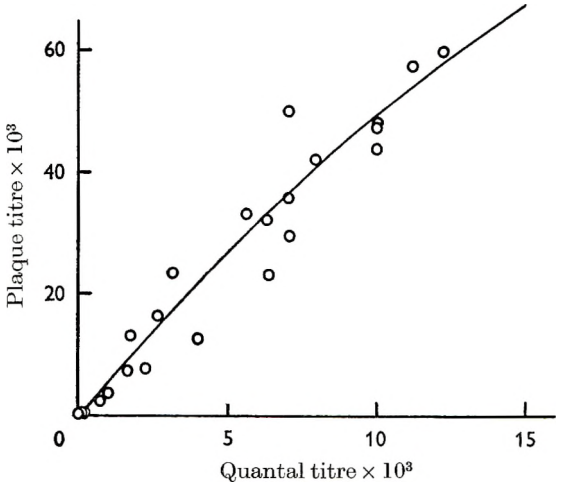

Fig. 6. The relationship between the neutralizing titres determined by the plaque and quantal methods for sera from the secondary and tertiary responses of rabbit 2/59. ——, $p = 6q - 0{\cdot}1q^2$.

significance at this stage; the non-linearity of the correlation between the two antibody estimates is, however, made clear thereby.

The non-linear relationship can be attributed to one of two possibilities:

(1) That the relationship between the absolute antibody concentration and the titre obtained is not linear for one or both tests.

(2) There is a mixture of antibodies in the sera, the proportion of which varies with the state of immunization, each antibody component having a different straight line relationship between absolute antibody concentration and titre when measured by the two tests.

These two possibilities can be differentiated by titrating a series of dilutions of one of the high titred sera in Fig. 4 by both methods, and plotting the titres so obtained one against the other. If the correlation illustrated in Fig. 4 is a function of the tests themselves, then the titres of the dilutions will follow the same relationship. If, however, it is a reflexion of varying proportions of different antibodies in the sera, then, providing p and q are linear functions of the antibody concentrations, the titres of the dilutions as estimated by the two techniques will lie on a straight line passing through the origin. This will be so because the proportions of different antibodies in a single serum will be constant irrespective of

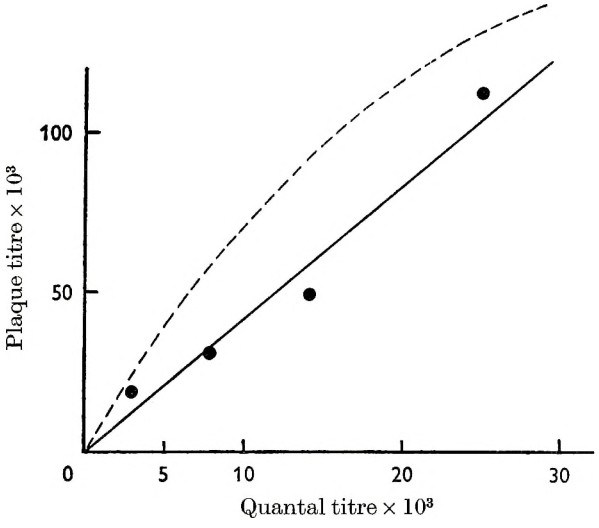

Fig. 7. The relationship between the neutralizing titres determined by the plaque and quantal methods for dilutions of serum 'V' of rabbit 1/59. - - - -, Relationship exhibited in Fig. 4.

dilution. The results of such an experiment (Fig. 7) clearly demonstrate that the two tests are linearly and proportionally related, and that departures from such a relationship are most probably due to varying proportions of different antibodies in the high titred sera.

DISCUSSION

It has been stated that the accuracy of the plaque technique should be far greater than a quantal method using a practicable number of tubes, (Dulbecco, 1955, Lorenz, 1962). In this study the error of the two tests, performed as described here, was very similar and this was due primarily to the large variance of the plaque count at a given dilution. It may be that this could be reduced by removing the inoculum before overlaying, but this, of course, would no longer be the technique accepted at the onset of the study. Cooper (1961) has pointed out that the day-to-day variation in plaque count in the suspended cell method can be reduced by various techniques. These include the use of galactose in place of glucose-carbonate, treating virus with sodium deoxycholate before dilution, and

the inclusion of various supplementary components in the medium. He also advocates the use of tetrazolium salts for the differentiation of plaques in preference to neutral red. However, as has already been demonstrated, it is important to detect plaques as early as possible, and tetrazolium salts can only be used at the time of the final count. It should be added that Cooper (1961) recommends the use of larger plates as the plaque count increases. Even so, considering the area available for the number of plaques present, it is probable, arguing on the basis of this study, that the loss of plaques due to overlaying and coalescence would be of measurable proportions. A further obvious method to reduce the variance of the plaque count at a given virus concentration would be to increase the number of estimates at that concentration, but this would impose severe restrictions on the number of sera that could be assayed at a given time. The relative cost, in time and materials, of these two tests, as employed in this study, is illustrated by the fact that, for every three sera estimated by the plaque technique, 12–15 sera can be titrated by the quantal method. Considering the slight increase in accuracy of the plaque neutralization test over the quantal, in relation to its cost, there seems little justification for the use of the former as a basic routine method. It is obvious, however, that owing to its greater sensitivity, which has also been demonstrated by Ginsberg, Kasymow & Al'tshtein (1960), it has a role to fulfil in the titration of low titred sera.

Comparison of the antibody titres determined by the two methods shows a non-linear relationship despite the absolute antibody concentration and titre being linearly related in both tests. The two sets of values for antibody titres are approximately proportional at titres of less than 60,000 as determined by the plaque method but with increasing stimulation, especially in the tertiary response, the antibody produced differs from the earlier antibody; i.e. there is evidence of inhomogeneity of the antibody produced in response to multiple inoculations of type 1 poliovirus. These findings agree with those of Svehag & Mandel (1964*a*, *b*), who showed that differing proportions of 7*s* and 19*s* antibody are produced in rabbits according to the state of immunization when inoculated with the Brunhilde strain of type 1 poliovirus.

SUMMARY

Sera from two rabbits bled after one, two and three inoculations of concentrated type 1 poliovirus tissue culture fluid were assayed for neutralizing activity by a quantal and a plaque method. The plaque counts in these tests have been shown to approximate to a Poisson distribution as determined by the relationship between the variance and the mean of replicate counts. However, there is a considerable scatter of the points in such a comparison and this accounts for the absence of the expected increase in accuracy of this plaque method over the quantal.

The relationship between the antibody content of the sera determined by the two methods is non-linear. This can be attributed to inhomogeneity of the antibody produced which increases with the number of antigenic stimuli given.

I wish to acknowledge the helpful advice and criticism of Prof. G. Belyavin and Prof. Wilson Smith and also to thank Dr J. C. N. Westwood for supplying the virus suspensions from which the concentrates were prepared.

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Experiments on the spread of colds

II. Studies in volunteers with coxsackievirus A21

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In the first paper of this series (Buckland & Tyrrell, 1964) we described experiments in which tracers were used to follow the dispersal of secretions from the upper respiratory tract of volunteers without colds. To amplify these studies we have performed further experiments in volunteers who had been given colds, and these experiments are described in the present report.

Coxsackievirus A 21 has now been isolated in a number of different outbreaks of disease, first of all in California by Lennette, Fox, Schmidt & Culver (1958), later in Great Britain by Pereira & Pereira (1959) and more recently in North Carolina by Johnson, Bloom, Mufson & Chanock (1962). In each outbreak it was associated with mild upper respiratory infections diagnosed as common colds or pharyngitis. Volunteers at this Unit were successfully infected by intranasal inoculation and developed typical common colds (Parsons, Bynoe, Pereira & Tyrrell, 1960); more severe illnesses were also observed by Spickard, Evans, Knight & Johnson (1963) in volunteers to whom they gave larger doses of virus. Coxsackievirus A 21 can therefore be regarded as a 'cold virus', although it is an uncommon cause of the disease.

We chose to use coxsackievirus A 21 in these experiments because it has several practical advantages over other viruses which also cause colds. For example, it may be readily and accurately titrated in human diploid cells, and it is stable on storage at $-20°$ C. Since antibodies are uncommon in the general population few volunteers are immune to infection.

We recognize that this virus does not seem to spread as readily as do other agents which cause the common cold—this is the apparent explanation for its being usually found in the congested conditions of training camps for the Services rather than among civilians. Nevertheless, we chose it for our initial experiments in preference to viruses such as the myxoviruses, which are unstable and therefore difficult to titrate accurately, and to the rhinoviruses to which a large number of our volunteers possess antibodies.

This paper reports experiments in which we attempted to find: (*a*) where the concentration of virus was highest in infected volunteers and therefore by what route virus was most likely to leave the body, (*b*) the area of the upper respiratory tract which was most susceptible to infection, and (*c*) the means by which the virus might most readily initiate an infection under natural conditions.

MATERIALS AND METHODS

The virus used was obtained from a swab collected from a patient in the R.A.F. (Parsons *et al.* 1960). This virus was given to volunteers, and pooled nasal secretion from them was used as inoculum in some experiments. In others, the virus was passed into human amnion cultures and then into human embryo kidney cultures and the fluids from the latter were used as the inoculum.

Titration of viruses

The specimens obtained from volunteers were titrated by inoculating serial dilutions of virus into tube cultures of human diploid cell strains, which were highly sensitive to coxsackievirus A21 (Brown & Tyrrell, 1964). The cells were maintained in Eagle's medium containing 2% calf serum, and were rotated in a roller drum. Tenfold dilutions of virus were inoculated into groups of three tubes in most instances. When more accurate results were required 3·16-fold dilutions were inoculated into groups of five tubes. Fifty per cent infectivity end points (TCD 50) were estimated by the method of Reed & Muench (1938).

Antibody titrations

Antibody against coxsackievirus A21 was estimated by haemagglutination inhibition tests using the techniques of Takátsy (1955). In most experiments the red cells used were human group O cells from cord blood and were selected because they were highly sensitive to the virus agglutinin. To obtain the highest titre the virus was usually prepared by the inoculation of tube or bottle cultures of human embryo kidney cells. The fluids were harvested when an advanced cytopathic effect was observed and were stored at $-70°$ C. until required. Serial dilutions of inactivated serum were made in phosphate buffered saline pH 7·1. To each dilution were added, in an equal volume of saline, four haemagglutinating units of virus. After incubation at room temperature for 30 min. a further volume of 1% red cells was added and the mixtures were allowed to settle at 4° C. The titre was taken as the highest dilution giving complete inhibition of haemagglutination.

The antibody levels determined by haemagglutination inhibition were checked by neutralization tests on fifteen sera, using about 100 TCD 50 of virus, and holding the mixture of serum and virus at 37° C. for 2 hr. and then at 4° C. for 12 hr. Of eleven sera with a titre of four or less by the H.I. test, only one contained detectable neutralizing antibody at a titre of 4. Neutralizing antibody was found in all four sera with an H.I. titre greater than 4.

Inoculation of volunteers

The standard method of inoculation at this Unit was used in the early experiments. Volunteers lay on their backs with the neck extended, and 0·5 ml of inoculum was instilled into each nostril. The patient remained on his back for 1 min. before getting up and refrained from blowing his nose for the next hour. Other methods of inoculation will be described later in the text.

Selection and observation of volunteers

Volunteers of both sexes aged between 18 and 50 years were observed and cared for in isolation as described by Andrewes (1948). Nasal washings were collected daily from each volunteer for at least 4 days after inoculation and a specimen of serum was collected just before inoculation and again 3 weeks later, after the volunteer had left the Unit. In certain experiments swabs were collected instead of nasal washings. A final concentration of 2% bovine plasma albumin was added to all clinical specimens before they were frozen. Volunteers were classified as 'infected' if virus was recovered from one or more nasal washings; in many of these subjects a rising antibody titre was also detected.

RESULTS

The first experiments, summarized in Table 1 and Fig. 1, were performed in order to measure the concentration of virus excreted during an experimental infection. One group of volunteers received tissue-culture-passed virus and the

Table 1. *Recovery of virus during a period of* 6 *days from ten volunteers who were inoculated with nasal drops*

Source of virus given	Dose TCD 50	Proportion of tests positive: Nasal washings	Garglings	Saliva	Faeces
Tissue culture	750	6/6*	4/6	2/6	5/6
		21/36	7/36	5/36	6/36

Source of virus given	Dose TCD 50	Nose swab	Throat swab	Saliva
Nasal washing	8	4/4	4/4	2/4
		15/24	14/24	4/24

* The upper row of fractions shows the proportion of volunteers giving a positive result and the lower row shows the proportion of specimens giving a positive result

other group received nasal washings of virus which had never been passed through tissue culture. All the volunteers became infected, but it may be seen that virus was recovered most frequently from the nasal washings and least frequently and in the lowest concentration from the saliva and faeces (Table 2 and Fig. 1). The mean titre of virus in nasal secretions was at least 100-fold higher than that in the throat secretion or saliva, and this was consistent with its being produced in the nose and overflowing into the throat. The concentration ratios were similar to those seen in experiments in which spores were slowly discharged into the nose, and their concentrations in the throat and saliva were followed (Buckland & Tyrrell, 1964). It can be seen from Fig. 1 that there were wide variations in the amount of virus excreted by different volunteers. These differences were not closely correlated with the severity of the disease from which they suffered. The upper and lower halves of Table 1 and the right and left halves of Fig. 1 also show

that the results were very similar, whether the virus administered had been passed in tissue cultures or not. In one set of experiments the concentration of respiratory secretions in a washing was assumed, and in the other the weight of secretion taken up by the swab was measured. It was possible that a swab might have irritated the nose and changed the character of the secretion or retained virus; on the other hand, the estimated amount of secretion in the washing might have been wrong. Since the results were so similar despite the variations in procedure we do not believe that serious technical errors occurred.

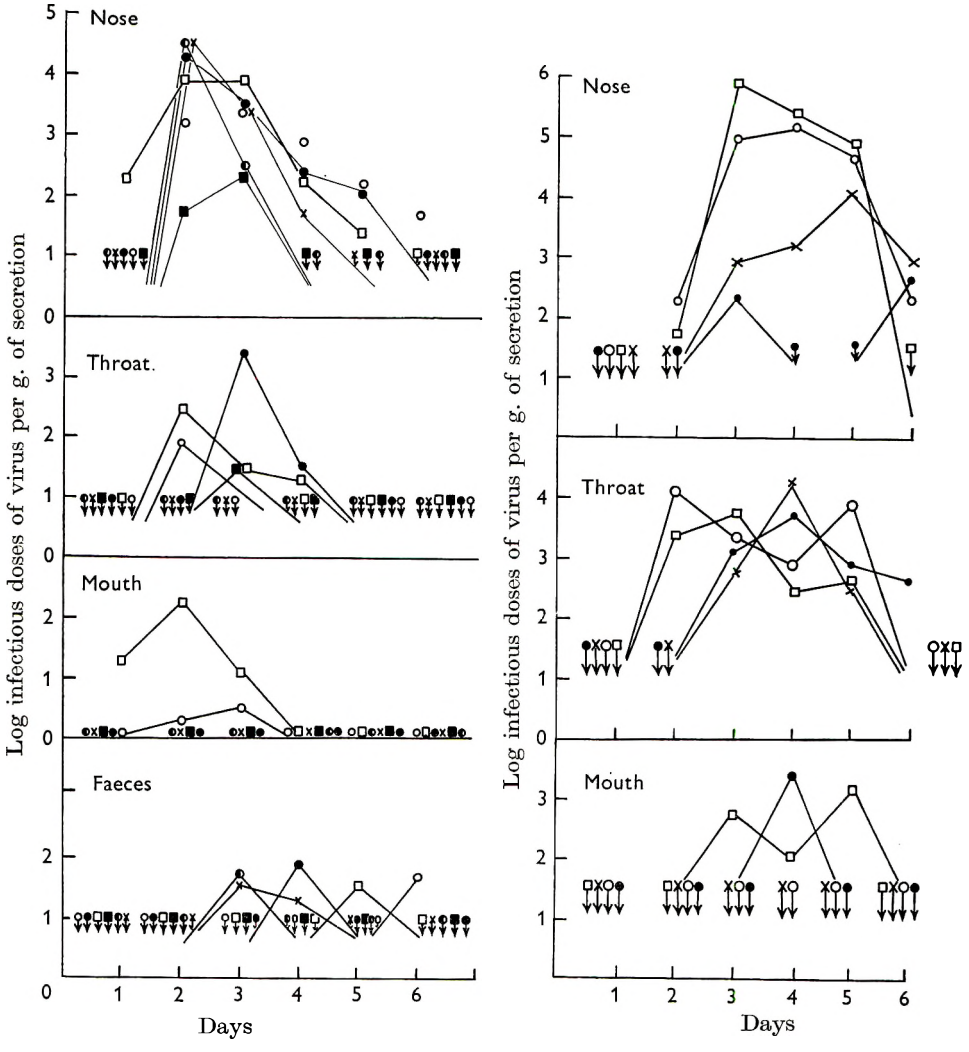

Fig. 1. Two experiments to measure the concentration of virus in subjects with colds due to coxsackievirus A 21. The left-hand panel summarizes the results with six volunteers in whom the virus was recovered from the nose and throat by washings. Virus was only occasionally found in the saliva and faeces. Most virus was found in the nose (see also Table 2). The right-hand panel shows the results with four volunteers from whom the virus was collected by swabbing. The results are similar but one volunteer had little virus in the nose and a relatively large amount in the throat and saliva.

The inoculation of virus by various routes

When virus is administered in 1 ml. of saline it rapidly spreads over all the nasal mucous membranes and the pharynx, and one cannot be sure in what area infection is initiated. We therefore prepared experiments in which the virus was administered in various ways.

Table 2. *Estimated mean concentration of virus during infection of six volunteers inoculated with 750 TCD 50 of virus passed in tissue culture*

Day after inoculation	Log_{10} TCD 50/g. of Nasal secretion*	Throat secretion*	Saliva	Faeces
1	2·2	<0·5	0·9	<0·5
2	4·7	2·4	1·3	<0·5
3	4·6	2·5	1·0	1·9
4	3·0	2·3	<0·5	2·0
5	2·6	<0·5	<0·5	1·8
6	2·1	<0·5	<0·5	1·8

* Estimated from the titre of nasal washings and garglings assuming that nasal washings contained 10 % nasal secretion and garglings 10 % of throat secretion.

Table 3. *Infectivity of virus given by various routes*

Method	Route	Dose TCD 50	Infected	With colds
Swab	Hand/nose	1500	0/4	0/4
Swab 0·01 ml.	Nasal septum	16	4/4	4/4
	Conjunctiva	16	4/4	3/4
	Oropharynx	16	0/6	0/6
	Oropharynx	280	1/6	0/6
	Nasopharynx	280	2/8	0/8
Drop 0·05 ml.	Nasal septum	8	5/6	5/6
	Nasal septum	0·8	3/4	4/4
	Nasal septum	0·08	0/5	0/5

With the exception of one subject in row 8 of this table the volunteers classified in Tables 3 and 5 as 'with colds' were always also 'infected'.

Table 3 shows that no volunteers became infected when a large dose of virus was rubbed on to their index fingers and thence on to the outside of the nose and the external nares. The volunteers carried on with their normal day's activities for 4 or 5 hr. without washing their hands and ate at least one meal, but nevertheless none of them became infected. We also studied the effect of placing the virus in different parts of the upper respiratory tract by gently rubbing a swab soaked in virus inoculum on to the posterior wall of the pharynx, the nasopharynx, the nose and the inferior fornix of the conjunctiva. It was estimated that roughly 0·01 ml. of the inoculum was rubbed off on the mucous membranes. Virus put in this manner on to the nasal septum or the conjunctiva infected in low dosage in every instance, whereas the same or even larger doses were practically non-infectious when placed on the pharynx, and only slightly more infectious when put on to the the membranes of the naso-pharynx. The virus used in these experiments was

'natural', that is, it had not been passed through tissue culture, but it was felt that the rubbing of the swab was rather unnatural, since viruses probably reach the mucous membrane in small droplets or dry particles; the trauma of rubbing might have increased the susceptibility of the mucosa as it apparently does for adenovirus (Huebner *et al.* 1955; Bell *et al.* 1956). Therefore, in a further series of experiments an attempt was made to estimate the minimum amount of virus required to infect the nasal mucous membrane as a small drop. In these experiments 0·05 ml. of virus suspension was placed under direct vision in the nose, half on each side of the anterior part of the nasal septum, using a fine capillary pipette. It can be seen from Table 3 that rather less than one tissue culture infectious dose infected most of the volunteers when administered in this way.

Exposure of volunteers to airborne virus

It is probable that, unlike the small drops used in this experiment, inhaled particles are not moist, are smaller than 0·025 ml. in volume and are trapped not so much on the anterior nasal septum as on the anterior end of the turbinates

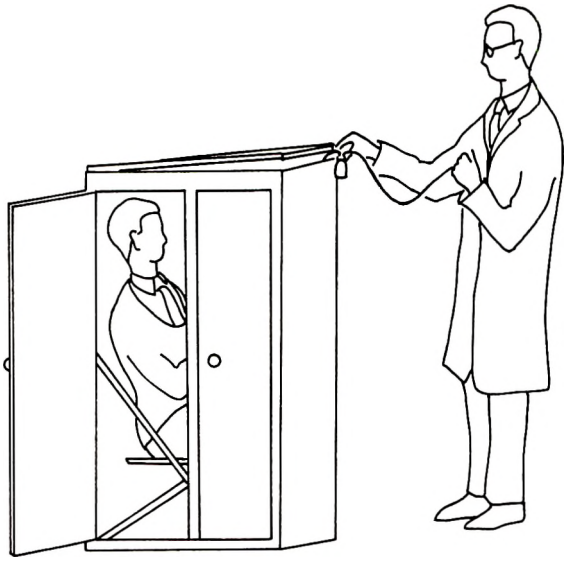

Fig. 2. The cupboard used for inoculating volunteers with sprayed virus. The volunteer sat inside with his eyes closed. In an actual experiment the door would be closed. The lid has been slightly lifted for the moment so that the spray nozzle is in position to deliver a 'puff'. The impinger or preimpinger samplers are placed by the subjects left cheek.

(Negus, 1958). We therefore felt that these experiments should be completed by trying to measure the minimum infectious dose of virus administered as an aerosol which resembled a natural sneeze. The method used was developed from one described in an earlier paper (Buckland & Tyrrell, 1964). A simple apparatus, a 'sneeze cupboard', was constructed, the essentials of which are shown in Fig. 2. The subject sat in a chair inside a wardrobe of which the top had been replaced by a transparent lid which would admit light and allow observation of the volunteer. During an experimental exposure both doors of the wardrobe were closed.

The virus suspension was sprayed in as shown using a special spray which produced droplets of the same size range as those produced by a sneeze (Buckland & Tyrrell, 1964). An impinger and preimpinger containing 2 % calf serum in Eagle's medium as a collecting fluid stood alongside the volunteer's head and sampled air at 11 l./min. while he was sitting there. Experiments were first done using a suspension of *Bacillus mycoides* spores as an inert tracer (Buckland & Tyrrell, 1964); these showed that the level of exposure could be kept to within a range of about two-fold. The persistence of spores in the air of the apparatus is shown in Table 4; clearly most of the coarser droplets are collected within the first few minutes of sampling. In order to administer virus and measure its inactivation in the aerosol, mixtures of virus and spore suspension were sprayed and the volume of spray fluid collected in the sampler was estimated by the spore count.

Table 4. *Recovery of spores after spraying about* 10^6 *spores into 'sneeze' cupboard*

Time of sample after spray	Spores recovered in 1 min. from 11 l. of air by Preimpinger	%	Impinger	%
1st min.	12,000	47	7,400	34
2nd min.	5,000	19	4,000	18
3rd min.	3,600	14	4,600	21
4th min.	2,700	11	2,800	13
5th min.	2,200	9	3,000	14
Total	25,500		21,800	

The preimpinger sampler collects particles of the size range trapped in the upper respiratory tract while the impinger collects smaller particles which are trapped in the lower respiratory tract.

Tissue culture fluid containing 2 % bovine plasma albumin was sprayed in two experiments and nasal washings were sprayed in one. Both virus and spores were recovered on the impinger and preimpinger. The results were consistent. The titre of virus recovered in the preimpinger was lower than that expected from the spore count by 0·44 to 1·12 $\log_{10}$ units and the mean reduction in five estimates was 0·6 unit (fourfold). In impingers the titres were 1·45 units lower than expected. Control experiments showed that no virus was inactivated in the sprayer or the samplers. We therefore concluded that 75 % of the virus sprayed as coarse droplets likely to be trapped in the nose was inactivated in the spray, and that 97 % of the virus in the finer droplets was so inactivated.

In order to infect a volunteer the spray was projected into the 'sneeze' cupboard in which he sat for 5 min. following one or more puffs. The volunteer breathed through the nose in most experiments and always kept the eyes closed just as the spray was blown in. A preimpinger sampler containing 2 % calf serum in Hanks's saline was running at about the level of the volunteer's head throughout the time of exposure, and the fluid was assayed both for the virus and for spores of *B. mycoides* which were added to the inoculum as a tracer. The dose of virus administered to each volunteer was calculated from the titre of virus in the impinger fluid.

In cases where only spores were detected because the concentration of virus was too low the dosage was calculated from the concentration of spores found allowing for inactivation of the virus at the same rate as was found at higher concentrations. The results of several experiments of this type are shown in Table 5. They indicate that airborne virus was infective. The rate of infection was significantly higher if air was drawn in through the nose rather than through the mouth; it is probable

Table 5. *Infection of volunteers with virus administered as a spray*

Dilution sprayed	Amount sprayed	Volunteer's type of breathing	Dose collected in pre-impinger TCD 50	Proportion of volunteers	
				Infected	With colds
Undiluted	10 puffs	Mouth closed	40	6/6	6/6
Undiluted	10 puffs	Nose closed	40	3/6	2/6
Undiluted	1 puff	Normal breathing	4	3/6	3/6
1/5	1 puff	Normal breathing	0·8	3/6	1/6

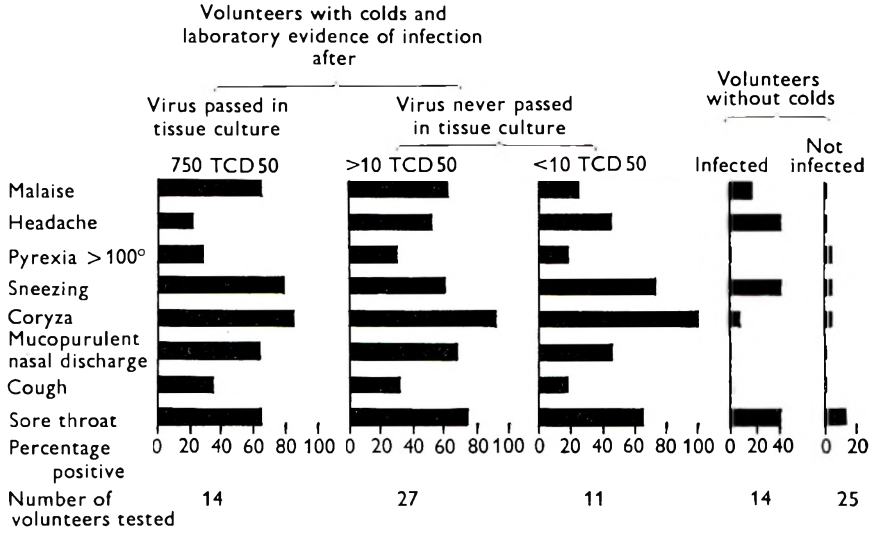

Fig. 3. Proportion of volunteers showing certain symptoms and clinical signs after administration of virus as drops, on swabs or as an aerosol. There were, in addition, three volunteers who developed colds although there was no laboratory evidence of infection.

that some of the aerosol drawn in through the mouth eddied up into the nasal cavities and therefore the result is consistent with the earlier finding that the pharynx is highly resistant to infection. It can also be seen that the amount of airborne virus required to infect a volunteer was about the same as that required when the virus was administered as a drop onto the nasal septum.

Clinical and serological responses to infection

It can be seen from Tables 3 and 5 that almost all those volunteers who received large or moderate doses of virus (8–160 TCD 50) on the nasal mucous membrane

became infected. A small proportion of those who were infected with small doses of virus became ill.

The main clinical features of the illnesses produced by various doses of virus are shown in Fig. 3. The clinical symptoms were those of a common cold. They were the same in volunteers who received large doses of virus and those who received small ones, although the former had rather higher fever and more mucopurulent nasal discharge and cough than the latter. The clinical picture was the same by whatever route the virus was administered and the data include the records of volunteers who acted as 'donors' in transmission experiments (see below).

Antibody responses

Of nineteen volunteers who were infected with small doses of virus ten developed antibody, while of those given larger doses of virus thirty out of thirty-six did so. Eight out of eleven volunteers who had significant antibody levels, i.e. H.I. titre

Table 6. *Relation of antibody to infectivity when virus is given by the nasal route*

		Proportion of volunteers with antibody at time of inoculation H.I > 4			
			Infected		
Virus given	Dose	Not infected	No cold	Cold	Total
Washing	0·8–10	4/6	0/6	2/8	2/14
Washing	11–160	—	1/4	0/16	1/20
Tissue culture fluid	750	—	3/5	3/13	6/18

over 4, developed a rising titre, while twenty out of thirty-six without previous antibody did so. It was concluded that antibody responses to infection did occur but not in every volunteer, and that responses were more frequent in those given large doses of virus and in those who already possessed antibody.

It was thought that antibody might protect against infection. Spickard *et al.* (1963), using 3000 TCD 50 of virus, found such an effect only when antibody levels estimated by the haemagglutination inhibition test were equal to or greater than 40. Table 6 shows that when a small dose of virus was given only two out of fourteen volunteers who were successfully infected had antibody to a titre of 4 or more, while four out of six who resisted infection had antibody. When larger doses of virus, i.e. 750 TCD 50, were given all the volunteers were infected but none had a titre as high as 40. Similar results were obtained here earlier by Parsons *et al.* (1960). Spikard *et al.* observed six subjects with antibody levels comparable with ours who became infected, but whose illnesses appeared milder than those of subjects without antibody. In our studies thirty-seven volunteers without antibody became infected and developed colds, 62 % mild, 32 % moderate and 6 % severe, while the percentage figures for seventeen with antibody were 60, 25 and 15 %. It was concluded that antibody did protect volunteers against infection with small doses of virus but did not ameliorate the illness if they were infected with large doses.

This was supported by further experiments in which volunteers were challenged on more than one occasion at intervals of 6 months or more. The results of these experiments are shown in Table 7. Volunteers A–D were all infected on the first occasion and resisted re-infection with 8 TCD 50 of virus. Volunteers E and F first

Table 7. *Immunity to challenge after inoculation*

First exposure

Name	H.I. titre before	TCD 50	Cold	Virus recovered	H.I. titre after
A	6	750	+	+	48
B	8	750	0	+	8
C	< 4	750	0	+	32
D	< 4	750	+	+	16
E	4	160*	0	0	4
F	< 4	160	0	0	< 4
G	< 4	2500	+	+	4

Second exposure

Name	Interval months	H.I. titre before	Dose	Cold	Virus recovered	H.I. titre after
A	12	32	8	0	0	32
B	12	8	8	0	0	8
C	6	8	8	0	0	8
D	6	8	8	0	0	8
E	6	4	8	0	0	4
F	6	< 4	8	+	+	8
G	10	< 4	8	0	+	< 4

Third exposure

Name	Interval months	H.I. titre before	Dose	Cold	Virus recovered	H.I. titre after
A	—	—	—	—	—	—
B	—	—	—	—	—	—
C	—	—	—	—	—	—
D	—	—	—	—	—	—
E	6	< 4	8	0	+	8
F	6	< 4	8	0	+	< 4
G	—	—	—	—	—	—

* The virus was swabbed on to the tonsil.

received virus on the pharynx and were not infected; thereafter they were both successfully infected, one of them on two successive occasions, but F showed no rise of antibody or any symptoms. Volunteer G developed an insignificant antibody response after infection with a large dose of unpassaged virus and was successfully reinfected, also without rise of antibody or symptoms. These experiments show that if there is an antibody response to infection with coxsackievirus A 21 immunity to reinfection usually, but not invariably, develops, and that reinfection with the virus is also possible.

The shedding of virus by volunteers

Colds were induced in volunteers by administering to the nasal septum 30 TCD 50 of virus unpassaged in tissue cultures. When symptoms of a cold developed the volunteers carried out the following manoeuvres:

(*a*) They coughed deeply into a large plastic bag, 120 × 60 cm.

(*b*) They talked for 5 min. into a small plastic bag, 33 × 19 cm.

(*c*) They blew the nose vigorously three times with the head in a large plastic bag as in (*a*). The handkerchief was a 27 × 27 cm. square of cotton and was held through the wall of the plastic bag with the hand outside.

(*d*) They snorted out ten times with the head in a large bag—it was found in control experiments that this dispersed spores from the nose in a similar fashion to an uninhibited sneeze which many volunteers were unable to produce with the head in the bag.

Table 8. *Dispersal of virus by volunteers with colds*

Volunteer	Day after inoculation	Titre of nasal washings/ml.	Amount of virus TCD 50 recovered from volunteers after indicated procedure. Cough	Talk	Blowing nose: Air	Blowing nose: Wall of bag	Blowing nose: Handkerchief	Snorting or sneezing: Air	Snorting or sneezing: Side of bag
A*	3	280,000	1500	—	0	16,000	9,000,000	—	—
	4	28,000	—	10	—	—	—	64	28,000
B	3	1,600	0	0	0	0	28,000	40	16,000
	4	160	—	0	0	9,000	30,000	0	1,600
	5	16,000	—	—	—	—	—	0	2,800
C	3	1,600	0	—	0	800	30,000	—	—
	4	160	—	0	—	—	—	0	500
D	3	280	0	—	0	8,000	90,000	—	—
	4	160	—	0	—	—	—	0	9,000
E	3	160	0	0	0	8,000	280,000	0	300
F	3	160	0	0	0	200	16,000	0	500

* In order of peak concentration of virus.

The air was sampled with a preimpinger containing culture medium and the wall of the polythene bag into which the volunteer talked, sneezed, etc., was washed with culture medium.

The assay of nasal secretion is given as particles per ml. Virus recovered is given as number of particles.

Virus was recovered from the air of the bags with a preimpinger sampler; the wall of the bag was washed with 100 ml. medium and the handkerchief was shaken in a flask with 100 ml. of medium. The washings and sampler fluid were assayed for virus. The results are shown in Table 8. This indicates that no virus was shed on coughing or talking except by one volunteer whose nasal secretions contained an exceptionally high concentration of virus. All six volunteers excreted virus on blowing the nose, and the virus could be recovered from the handkerchief and bag but none could be recovered from the air. Finally, after snorting, virus was recovered in relatively large amounts in the washings of the bag and twice from

the air also. It was concluded that only a sneeze or snort unmuffled by a handkerchief produced detectable amounts of airborne virus under the experimental conditions used. More important than this, it appeared that one volunteer was probably far more efficient than any of the others as a potential spreader of infection, and this was apparently due to the high concentration of virus in the respiratory secretions; the cold from which she suffered was graded as mild and was not more severe than other illnesses in which virus was not shed. We noted that the amount of virus expelled into the handkerchief and bag was more than expected from the titre of the nasal washings (Table 8). This might have occurred if the nasal washings contained about 1 % of nasal secretion rather than the 10 % assumed earlier.

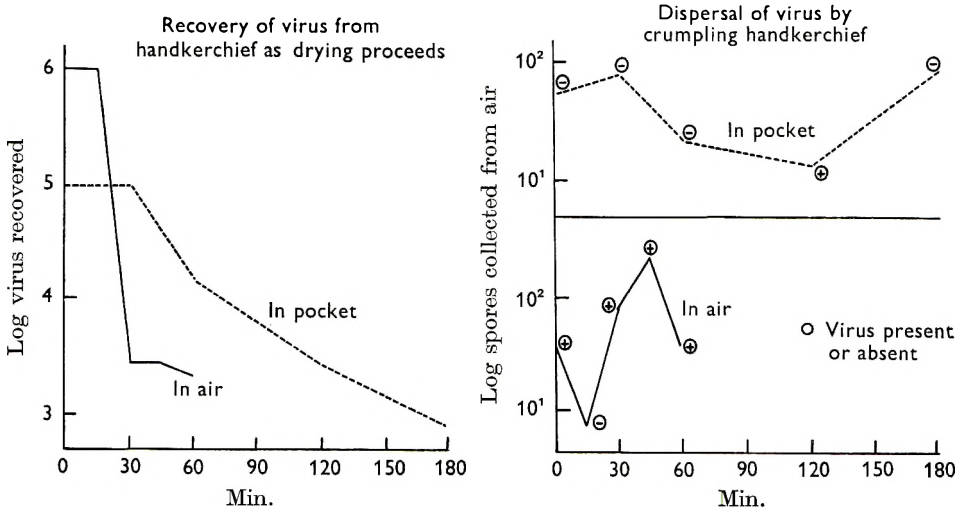

Fig. 4. The fate of coxsackievirus A 21 in tissue culture fluid containing 2 % bovine plasma albumen added to a handkerchief. The virus died off as the fabric dried, either in air or in a pocket. $10^{6 \cdot 45}$ spores were added with the virus as a tracer. Very few of these could be recovered by vigorously crumpling and shaking the handkerchief inside a plastic bag and sampling the air with a preimpinger. However, traces of virus were recovered though in amounts too small to titrate.

We wondered if virus sneezed into a handkerchief or clothing might subsequently become airborne, and one of the experiments performed to test this hypothesis is summarized in Fig. 4. Virus produced in tissue culture ($10^{7 \cdot 5}$ TCD 50/ml.) was mixed with spores and a few drops with a total volume of 0·02 ml. were placed on each of a series of new cotton handkerchiefs. These were then allowed to dry either by hanging on a wire in the laboratory (temperature about 70° F., R.H. about 60 %) or by being folded and placed in the coat pocket of a laboratory worker. At intervals handkerchiefs were assayed for virus and spores, or crumpled and shaken by hand for 45 sec. in a plastic bag, which was then evacuated through a preimpinger in the usual way. The results (Fig. 4) show that virus infectivity was rapidly lost, although the spores could be recovered from the fabric almost quantitatively. A few spores and traces of virus were recovered from the air. The experiment was repeated using nasal secretion containing a fairly high titre ($10^{4 \cdot 5}$ TCD 50/ml.) of

virus which had been collected as it dropped from the volunteer's nose and had not been diluted with saline or supplemented with any preservative. Infectivity was lost equally rapidly, and few spores and no virus were recovered from the air.

We noted that there was not a strict parallel between the titre of virus in a nasal secretion, and the amount which the patient sneezed or blew out. We therefore placed 0·05 ml. volumes of a tracer suspension of *B. mycoides* spores in the anterior nasal septum of volunteers without colds and studied the dispersal of the spores when they blew the nose 2 min. later. The results summarized in Table 9 show what a variable effect a nose-blow had. Most of what was expelled was trapped in the handkerchief, but up to 35 % might be found in drops attached to the side of the bag. In some cases no spores were recovered from the air while in others there were appreciable numbers. The original data suggested that there was a tendency for certain individuals rather than others to produce airborne spores.

Table 9. *Summary of twelve experiments with six subjects on the expulsion of spores by blowing the nose*

	Absolute values		Proportion of total expelled (%)	
	Mean	Range	Mean	Range
Total spores expelled	14,000,000	1,600,000–34,000,000	100	—
No. trapped in handkerchief	13,000,000	1,600,000–32,000,000	93	66–99·7
No. trapped in impinger	790	0–2,900	0·006	0–0·13
No. trapped in preimpinger	290	0–1,600	0·002	0–0·09
No. trapped on side of bag	910,000	400–440,000	6·5	0·004–35

It also seemed possible that the quantity of virus secreted might continually be changing. Three washings were therefore taken from four volunteers at intervals of from 5 min. to 3 hr. Virus was found in all specimens. In one case a second nasal washing taken 5 min. after the first showed a 60-fold lower titre, but apart from this instance the titre of washings taken at this or longer intervals fell within a tenfold range, and did not show a downward trend. It was impossible to decide whether the variation was due to variation in the amount of secretion removed or in the concentration of virus, or to the error of the titrations. It was concluded that virus was being produced in a fairly steady stream and that more virus might be expelled into a handkerchief than could be recovered from a nasal washing.

Transmission of virus from one volunteer to another

Although very little virus was shed from a volunteer at each sneeze it was possible that another volunteer who was living with him and inhaling air throughout the course of a cold might pick up a live virus particle and become infected. Attempts were therefore made to transmit infection from volunteer to volunteer.

In the successful attempts 'donor' volunteers were inoculated shortly after arriving at the Unit and lived with an uninoculated partner for the next 9 days. Nasal washings were collected on each day from each partner. Table 10 shows that tissue-culture-passed virus and unpassaged virus were transmitted and that about one in five volunteers exposed became infected. Nevertheless, it was not possible

to transmit infection to eleven volunteers each of whom inhaled the air from a plastic bag into which an infected volunteer had just sneezed or snorted. This was reasonable since virus was recovered only from the walls of the bag and not from the air (see above). Unfortunately, no volunteers were exposed to the one donor subject who apparently transmitted a cold to his partner. He was excluded from this experiment because his cold seemed to be so mild!

Table 10. *Transmission of coxsackievirus A* 21 *between volunteers with various types of contact*

Donor volunteers			
Dose given	No. infected	Type of contact	Result in receptor volunteers. No. infected
750*	12/12	Lived in flat with 1 or 2 partners	2/12
30	6/6	Lived in flat with 1 or 2 partners	1/5
30	6/6	Breathing in air from sneeze or three snorts into a bag	0/11
0·8	2/6	Lived in flat with 1 or 2 partners	0/3
0·8	2/6	Went to 'sneeze parties' for 2 hr. a day on 3 days	0/8

The three infected recipients had antibody titres of 4 or less as did twenty-eight of the thirty-six uninfected recipients.

* The 750 TCD 50 were of tissue-culture-passed virus. The other doses were of virus passed only in man.

It seemed unlikely that volunteers would be infected in nature with as much as 30 TCD 50 of virus; also coxsackievirus tends to spread under conditions of communal living. We therefore infected two volunteers with a small dose of virus (0·8 TCD 50). They had very mild symptoms but spent 1 hr. twice a day playing card games with eight other volunteers in a room with closed windows and doors and were encouraged to snort or sneeze several times during each session. No virus was transmitted.

It was concluded that virus could be transmitted from one volunteer to another by regular personal contact, but that the rate of transmission was too low to support an epidemic.

DISCUSSION

These experiments were designed to test experimentally various common assumptions about the way colds spread by making semiquantitative measurements of virus concentration and clinical response in a convenient model disease. Having considered these new data our present conception of the spread of colds due to this virus is as follows:

Because of the high concentration of virus in nasal secretion it seems that the nose is the most likely origin of infectious virus, although rare individuals with virus in the saliva might disperse it on talking. As earlier tracer experiments led us to expect, we now have evidence that virus is expelled most easily by sneezing or blowing the nose. However, the experiments on infection of volunteers indicate that the only virus of epidemiological importance is that which reaches the nasal

mucous membrane, since we have shown earlier that relatively few infectious particles were likely to be taken up by the conjunctiva (Buckland & Tyrrell, 1964). Our experiments there described, using spore tracers and a variety of sampling methods, together with the present analysis of virus sneezed into polythene bags, indicate that only a tiny fraction of what is sneezed out is in the form of infectious airborne droplets. Furthermore, most of the virus which is caught in the handkerchief or falls on to the clothes or floor becomes inactivated, unlike those bacteria which resist drying and can therefore be readily resuspended in small particles after they have dried on to fabrics (Dumbell & Lovelock, 1949). As virus is very rapidly inactivated in droplets under 4 μ in size and fairly rapidly inactivated in droplets larger than this, which in any case will tend to settle out, it is probable that there is a high risk of inhaling an infectious particle only if the recipient is near a cold sufferer who has just sneezed. Fine droplets are unlikely to be important; they might travel far but would probably be non-infectious when inhaled and in any case would be trapped in the lower respiratory tract where they would presumably cause bronchitis or a similar disease (Knight *et al.* 1963): such is not in fact seen in natural infection with this virus.

At first sight our laboratory data may be suspect because they suggest that viruses of this sort are unlikely to spread and that in fact we could not have maintained the chain of infection required in order to produce an epidemic; after all twenty experimental infections were followed by only three secondary ones and one clinically recognizable cold. The average secondary attack rate based on clinical observations of colds is about 1 in 5 (Lidwell & Sommerville, 1951), although less than half the adult population develops colds after infection with most cold-producing viruses (Andrewes, 1948; Jackson *et al.* 1960). On the other hand, when we planned our experiments we did not realize how variable was the excretion of virus by volunteers. It is possible that only one of every ten subjects infected is really likely to pass this infection on, and in this case it would be necessary to infect a group of about ten subjects and expose them all to each of another group of ten or more susceptibles in order to have a chance of propagating the infection continuously from man to man. Furthermore, the living conditions were very spacious. Even though a human being samples around 10 l. of air per minute throughout the day it is obvious that in large well-ventilated rooms the probability of inhaling a virus particle was much lower than in smaller ill-ventilated ones. In fact, our data may well explain adequately why this virus seems to spread successfully only under the conditions of the barrack room or in crowded oriental cities (Fukumi, Nishikawa, Sonoguchi & Shimizu, 1962).

The clinical picture observed in volunteers is identical with that observed in our own and other trials in which large doses of tissue-culture-passed virus were given to volunteers (Parsons *et al.* 1960; Spickard *et al.* 1963; Patel, Buthala & Walker, 1964). However, it is important to note that a similar disease was induced in the present studies by small doses of virus which had not been passed in tissue cultures. In addition, since the disease was indistinguishable from that observed in the field (McDonald, Miller, Zuckerman & Pereira, 1962; Johnson *et al.* 1962; Forsyth, Bloom, Johnson & Chanock, 1963) we believe that our experimental results may

be assumed to apply to the spread of virus in the field. The concept that one patient who appears on clinical examination to be like many others may be a particularly potent 'spreader' of infection is not new. Hamburger, Green & Hamburger (1945) showed that nasal carriers of streptococci shed far more organisms than those who carried them elsewhere in the respiratory tract. The experiments of Lovelock *et al.* (1952) with an undefined cold-producing agent gave results which were in many ways similar to those in the present trial; in particular they concluded that it was very difficult to transmit colds from volunteer to volunteer, that virus produced colds only if introduced into the nasal cavity and that virus was probably transmitted from infected children in the form of 'short-range' airborne droplets.

It is likely that certain findings in this series of experiments, such as the proportion of virus expelled by sneezing, will apply also to colds produced by other viruses, and we wonder whether it will be possible in future experiments using this approach to find reasons for the apparently more efficient spread of viruses such as influenza or parainfluenza. It might also be possible to define one or other link in the epidemiological chain which is readily modified by seasonal changes. Much further work is needed on these problems.

SUMMARY

The amount of virus in nasal and other secretions after infection with coxsackievirus A 21 has been measured daily in ten volunteers. Most virus was found in nasal secretion, less in throat secretion and small amounts were found intermittently in the saliva and faeces.

Virus administered as small drops or on a swab was more infectious for man if put on to the nasal mucosa than on to the throat or outside the nose. It was also infectious by the conjunctival route.

Virus was sprayed in droplets of about the same size range as those found in a natural sneeze. Virus survived better in larger ($> 4\ \mu$) than in smaller droplets. About one tissue culture infectious dose of virus in such droplets also infected volunteers.

The symptoms produced by these experimental infections have been analysed. The disease produced was largely independent of the dosage and route of infection. Those with pre-existing antibody resisted infection better than those with no antibody. Antibody rises were detected in about two-thirds of infected volunteers.

Volunteers with colds shed virus in large drops on sneezing, or into the handkerchief on blowing the nose, but virus was recovered from the air only after simulated sneezes by volunteers with high concentrations of virus in their nasal secretions. Virus died off rapidly on fabric at room temperature and humidity, and was only resuspended as airborne droplets when large doses such as 0·02 ml. of virus of high titre ($10^{7.5}$ TCD 50/ml.) were used.

Infection was transmitted from an infected volunteer to an uninfected partner living in the same flat in three out of twenty tests. Infection was not transmitted in experiments when volunteers mixed for a few hours with subjects with colds, or inhaled air into which a subject with a cold had just sneezed.

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Combined enteric and cholera vaccination by the intradermal route

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INTRODUCTION

The value of immunization with a combined typhoid, paratyphoid A and B (TAB) vaccine, in the prevention of enteric fevers, has been accepted for many years by the majority of workers in this field.

Tuft (1931) and (1940) advocated the administration of TAB vaccine by the intradermal route for both primary immunization and for subsequent reinforcement injections. Valentine, Park, Falk & McGuire (1935) reported that appreciably higher agglutinating antibody titres were produced when TAB vaccine was administered by the intradermal rather than by the subcutaneous route. Kamp (1943) noted a marked absence of both general and local reactions in 946 children inoculated intradermally with a combined enteric vaccine and Luippold (1944) reported favourably upon this method of inoculation in volunteer medical students.

Barr, Sayers & Stamm (1959) investigated both the clinical reactions produced, and the protection afforded by a combined enteric vaccine administered to Royal Air Force volunteers either subcutaneously or intradermally. These authors concluded that, whereas the protection afforded by the enteric component was as good when the vaccine was given intradermally as when it was injected subcutaneously, the reactions which occurred with the former technique were significantly reduced, both in number and severity.

As a result of this work, intradermal vaccination for both combined enteric and tetanus (TABT), and enteric (TAB), was introduced at first into the British Army and later into the Royal Navy and the Royal Air Force.

Noble (1963) reviewed all the reactions that occurred during the first 4 years of intradermal inoculation in the British Army, and which were of sufficient severity to warrant the suggestion that the vaccine may have been unduly toxic. He found the incidence of reactions to be negligible.

Ferrán (1885) is accepted, by most modern writers, as the first worker to demonstrate the possibility of an actively induced immunity against cholera. During the 1884 outbreak of cholera in Spain, he was able to demonstrate that guinea-pigs, which survived inoculation with a bile-broth culture of live cholera vibrios, were resistant to the administration of further doses lethal to unprotected animals. Because of impure cultures, Ferrán's vaccination technique gave unsatisfactory results in man and, more often than not, produced excessively severe reactions.

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Camaleia (1888) was the earliest worker to show that protection against cholera could be afforded by the administration of heat-killed suspensions of *Vibrio cholerae*. Further developed by Haffkine (1892), and with only minor refinements since, heat-killed suspensions are to this day the antigen of choice for immunization of non-immune persons against cholera.

Although some workers have from time to time doubted that cholera vaccine provides protection, there seems little doubt that such protection, though of limited duration, is afforded to persons at risk to infection by *V. cholerae* (Pandit, 1948; Mukerjee & Guha Roy, 1962; Archer, 1963) and by the El Tor vibrio (Vella, 1963; Noble, 1964*b* and 1965*a*).

Noble (1964*a*) has reviewed the literature and presented the case for the administration of cholera vaccine by the intradermal route. He concludes that not only is the protection afforded by intradermal inoculation as good as that given by the subcutaneous route, but that a significant reduction in vaccination reactions would occur if the former route were adopted. He goes on to suggest that in order to reduce the number of inoculations required by individuals proceeding overseas, it should be possible to combine intradermal combined enteric prophylactic (TAB) with intradermal cholera vaccine and still retain the freedom from undesirable inoculation reactions, shown separately by the two preparations. This article is a description of laboratory experiments carried out to test this hypothesis.

MATERIALS AND METHODS

Vaccines

Three vaccines were prepared:

(*a*) TAB vaccine, identical with that issued for administration to Service personnel and consisting of *Salmonella typhi* 2000 million, *S. paratyphi* A 1500 million and *S. paratyphi* B 1500 million organisms per ml. suspended in 0·5% phenol-saline.

(*b*) Cholera vaccine (Noble, 1964*a*) consisting of a heat-killed and phenol-preserved suspension of 4000 million *V. cholerae* strain Inaba and 4000 million *V. cholerae* strain Ogawa organisms per ml. in 0·5% phenol-saline.

(*c*) Combined TAB and cholera vaccine (TAB/Ch) containing 5000 million enteric organisms and 8000 million cholera organisms per ml. suspended in 0·5% phenol-saline and obtained by adding equal quantities of double strength TAB and cholera vaccines.

Mice

Because of limitation in supply, mouse strain C/57/Black was used for those experiments in which the challenge organism was *V. cholerae* and mouse strain Ajax albino (Porton) was used for experiments in which an enteric organism was used for challenge purposes. Preliminary experiments suggested that there was little to choose between the mouse strains except that the lethal dose for 100% of animals inoculated (LD 100) in Ajax albino mice was slightly higher (1500 million orgs.) than the LD 100 for C/57/Black mice (1000–1250 million orgs.) when both strains of mice were challenged intraperitoneally with *V. cholerae*.

Active mouse-protection test

This experiment was devised to compare the protection afforded to mice by the administration of each of the three vaccines described above against challenge by *V. cholerae*, *S. typhi*, *S. paratyphi A* and *S. paratyphi B*.

Groups of mice, of average weight 25 g. and with sexes approximately equal in each group, were given two 0·1 ml. intradermal immunizing inoculations of one of the vaccines at an interval of 7 days as described by Noble (1964*a*). Seven days after the second inoculation the mice, together with unprotected mice as controls, were challenged with a predetermined lethal dose of either *V. cholerae* or one of the enteric organisms. Challenge doses were suspended in sterile isotonic saline and administered in a volume of 0·5 ml. injected intraperitoneally. The mice were observed for 72 hr. and survivors noted.

The challenge organisms used in this test and the passive mouse-protection tests which follow were the most virulent of their type available in these laboratories at the time of the experiments. They were: *V. cholerae* Inaba, N.C.T.C. no.7260 (LD 100 approximately 1000×10^6 organisms); *S. typhi*, Ty 2 (LD 100 approximately 20×10^6 organisms); *S. paratyphi A*, Kasauli Institute no. B 31/4 (LD 100 approximately 80×10^6 organisms) and *S. paratyphi B*, Millard, recently isolated from a case of paratyphoid meningitis (LD 100 approximately 15×10^6 organisms).

Antiserum

Pooled antiserum was obtained from both rabbits and human volunteers by inoculating them with two intradermal inoculations of 0·1 ml. of the combined enteric and cholera vaccine (TAB/Ch) at an interval of 21 days.

(*a*) Six rabbits (David Bruce Laboratory Lop) were bled to establish a base-line titre of agglutination. No agglutinins for *S. typhi* H, O and Vi or *V. cholerae* O suspensions were detected. Each rabbit was given two intradermal inoculations of 0·1 ml. of the TAB/Ch vaccine, into the skin of the shaved abdominal wall, at an interval of 21 days. The inoculation sites were carefully observed hourly for 12 hr. after injection and thereafter daily, for evidence of untoward reactions. The rabbits were bled 7 days after the second injection. As a check for the effectiveness of the immunizing inoculations agglutination tests (Felix & Bensted, 1954) were performed on each of the six sera separately and upon a Seitz-filtered serum obtained by pooling the six specimens. Agglutinating antibodies to *S. typhi* H, O and Vi and *V. cholerae* O suspensions were detected and their titres expressed as the reciprocal of the highest dilution of serum in the test tube which showed granularity of deposit to the naked eye.

(*b*) Eight human volunteers, from the staff of the David Bruce Laboratories, were found not to have had either enteric or cholera inoculations during the previous 12 months. These volunteers were bled to establish base-line *S. typhi* O and *V. cholerae* O antibody titres. Two 0·1 ml. intradermal inoculations of TAB/Ch vaccine, at an interval of 21 days, were administered to each volunteer into the skin of the outer aspect of the arm behind the posterior border of the distal portion

of the deltoid muscle. The inoculation sites were observed for evidence of undue reactions. Three weeks and three months after the second injection the volunteers were bled again. Serum specimens obtained from the earlier blood specimens were stored in the refrigerator at 4° C. until the later specimens became available. *S. typhi* O and *V. cholerae* O agglutination titres were determined for each serum separately. Finally all the sera were pooled, Seitz filtered and titrated for both *S. typhi* O and *V. cholerae* O agglutinins.

Passive mouse-protection tests

These experiments were devised to demonstrate the protection afforded to mice by either the pooled rabbit or the pooled human antiserum described above against a challenge by a predetermined lethal dose of *V. cholerae* and each of the enteric organisms.

(*a*) Groups of mice, of average weight 25 g. and with the sexes approximately equal in each group, were given one 0·5 ml. subcutaneous injection of pooled rabbit antiserum. Twenty-four hours after the protective inoculation the mice, together with groups receiving serum from unprotected rabbits and unprotected groups as controls, were challenged with a lethal dose of *V. cholerae* (1250×10^6 organisms), *S. typhi* (15×10^6 organisms), *S. paratyphi A* (80×10^6 organisms) and *S. paratyphi B* (15×10^6 organisms). Challenge doses were suspended in sterile isotonic saline and administered in a volume of 0·5 ml. injected intraperitoneally. The mice were observed for 72 hr. and survivors noted.

(*b*) The passive mouse-protection test using human antiserum was performed in exactly the same way as in (*a*) above in which rabbit antiserum was used.

Intradermal skin tests

This group of experiments was designed to study the effects of the combined cholera and enteric vaccine (TAB/Ch) when inoculated into the skin of guinea-pigs, rabbits and human volunteers.

(*a*) TAB/Ch vaccine was inoculated intradermally in two doses of 0·1 ml. at an interval of 21 days. Guinea-pigs and rabbits were injected into the skin of the shaved abdominal wall, and twenty-four human volunteers were inoculated into the skin of the arm, behind the posterior border of the distal portion of the deltoid muscle. The inoculation site and any skin lesions produced were observed and measurements taken at frequent intervals.

(*b*) The effect of repeated intradermal inoculations of TAB/Ch vaccine was studied in both guinea-pigs and rabbits by injecting these animals with doses of 0·1 ml. intradermally at weekly intervals for 9 successive weeks. The injections were all placed into a 1 in. diameter shaved area of abdominal skin but not into the exact site of previous inoculations. The inoculation sites were examined daily for evidence of ulceration, and the presence or absence of indurated nodules assessed by palpation. Daily records of these observations were kept.

Table 1. *Active protection tests in mice inoculated with TAB, cholera or combined TAB/cholera vaccines and challenged with LD* 100 *doses of* Salmonella typhi, S. paratyphi A, S. paratyphi B, *or* V. cholerae

(Active mouse protection test. Average mouse weight 25 g. Sexes approx. equal in each group.)

Mouse strain...	C/57/Black				Ajax albino							
Challenge at 7 days, 0·5 ml. intraperitoneally	*V. cholerae* (Inaba), NCTC 7620 1000×10^6 orgs.				*S. typhi* (Ty 2) 20×10^6 orgs.				*S. paratyphi A* (B 31/4 Kasauli) 80×10^6 orgs.		*S. paratyphi B* (Millard) 15×10^6 orgs.	
Protection 0·1 ml. intradermally × 2 vaccine at 7 days interval	Cholera	TAB	TAB plus cholera	Nil. Unprotected controls	Cholera	TAB	TAB plus cholera	Nil. Unprotected controls	TAB plus cholera	Nil. Unprotected controls	TAB plus cholera	Nil. Unprotected controls
Survivors at 72 hr.	38/40	3/20	37/40	1/20	1/20	39/40	40/40	0/20	40/40	0/20	40/40	0/20

Control virulence tests

Organism...	*V. cholerae* (NCTC 7260)			*S. typhi* (Ty 2)				*S. paratyphi A* (B 31/4)				*S. paratyphi B* (Millard)			
Dose in 0·5 ml. intraperitoneally × 10^6	500	1000	1500	5	10	20	30	40	60	80	100	5	10	15	20
Survivors at 72 hr.	12/20	1/20	0/20	18/20	4/20	0/20	0/20	12/20	3/20	0/20	1/20	12/20	6/20	0/20	0/20
Plate count viability	58 %			62 %				51 %				62 %			

Numerators represent the number of animals to survive. Denominators represent the number of animals inoculated.

RESULTS

Active mouse-protection test

The results obtained in this test are shown in Table 1.

Cholera vaccine and TAB/Ch vaccine, administered intradermally, protected groups of C/57/Black mice equally and well against challenge with live virulent *V. cholerae*, whereas no significant protection was afforded, against a similar challenge, in mice receiving TAB vaccine and in unprotected control mice.

This experiment also showed that groups of Ajax albino mice were almost completely protected against a challenge with virulent *S. typhi* after either TAB vaccine or TAB/Ch vaccine. Those which received cholera vaccine and an unimmunized control group showed no protection against the same challenge.

Finally all the Ajax albino mice in the groups protected with TAB/Ch vaccine survived challenge with either *S. paratyphi A* or *S. paratyphi B* and unvaccinated mice failed to survive a similar challenge.

Antiserum

Agglutination tests performed as a check for the effectiveness of the immunizing inoculation upon the serum of both rabbits and human volunteers gave satisfactory agglutinin titres. The cholera O titres were comparable to the results

Table 2. *Agglutinin titres produced in rabbits after two intradermal inoculations of TAB/Ch vaccine at* 21 *days interval*

	Titres at 7 days			
Rabbit	T 'H'	T 'O'	T 'Vi'	Cholera 'O'
A	640	160	10	320
B	1280	160	5	640
C	640	160	10	1280
D	640	320	10	640
E	320	160	0*	320
F	1280	320	0*	320
Pool A–F and filter	640	160	5	640

Titres expressed as the reciprocal of the highest dilution of serum in the test tube showing granularity of deposit to the naked eye.

* Less than 1:5, the lowest dilution used.

obtained by Noble (1964*a*) when intradermal cholera vaccine was given alone and not in combination. The titres for *S. typhi* antigens were of the same order as those obtained in these laboratories when TAB vaccine was inoculated alone and not in combination with cholera vaccine.

The results obtained for rabbits are shown in Table 2 and for human volunteers in Table 3.

Passive mouse-protection tests

In these experiments most of the mice receiving serum from either TAB/Ch vaccinated rabbits or volunteer humans survived challenge with *V. cholerae* and

Table 3. *Agglutinin responses in human volunteers before, three weeks and three months after the intradermal administration of combined TAB/cholera vaccine*

	S. typhi 'O'			*V. cholerae* 'O'		
Volunteer	Before	3 weeks	3 months	Before	3 weeks	3 months
PF	40	40	320	40	160	320
SO	80	80	160	80	160	80
JH	0*	40	80	80	160	160
CL	20	40	160	40	160	80
TP	20	20	40	0*	20	0*
CM	0*	20	160	0*	20	80
GC	0*	40	320	40	160	320
NH	0*	20	80	20	80	40
Pool and filter		80			160	

Titres expressed as the reciprocal of the highest dilution of serum in the test tube showing granularity of deposit to the naked eye.

* Less than 1:5, the lowest dilution used.

each of the enteric organisms. In contrast, nearly all the mice in the control groups, which received serum from unprotected rabbits and humans or were not given serum, died as a consequence of a similar challenge dose of each of these organisms.

Tables 4 and 5 show the results obtained.

Intradermal skin tests

Combined enteric and cholera vaccine, inoculated intradermally in two doses of 0·1 ml. of a vaccine containing a total of 13,000 million organisms per ml., at an interval of 21 days, failed to produce a reaction of any kind in rabbits and guinea-pigs. The inoculum was absorbed entirely within 24 hr.

In human volunteers similar vaccinations produced local skin responses which were strikingly consistent in appearance, size and duration. At 6 hr. the site of the skin puncture showed a 5–10 mm. firm indurated nodule surrounded by an erythematous flare of a further 5–10 mm. At 12 hr. the central nodule was less well defined, softer to palpation and measured from 25–30 mm. in diameter. The total diameter of the lesion at this time was never greater than 80 mm. From this time onward the reaction gradually resolved, the erythema lessened in intensity and the demarcation between the central and peripheral parts became decreasingly apparent, producing at about 24 hr. an erythematous, soft, brawny, intracutaneous oedema. Resolution rapidly took place, the erythema faded in 48 hr. and by 72 hr. only a small intradermal nodule about 5 mm. in diameter remained. No pyrexia was encountered in any of the twenty-four volunteers. Two subjects complained of slight axillary tenderness but no adenitis was palpable and deep axillary palpation was possible without pain. No volunteer complained of a painful reaction and tenderness to touch was minimal throughout. A series of exercises, including slow and rapid movements, weight lifting and fine movements, such as performing serological agglutinations, showed that in no case was there any loss or impairment of normal function of the vaccinated arm. Several volunteers played

Table 4. *Passive mouse-protection test using pooled serum from rabbits inoculated with TAB/Ch vaccine by the intradermal route*

(Mice of average weight 25 g. Sexes approx. equal in each group.)

Mouse strain...	C/57/Black			Ajax albino								
Challenge at 24 hr., 0·5 ml. intraperitoneally	*V. cholerae* (Inaba) NCTC 7260 1250×10^6 orgs.			*S. typhi* (Ty 2) 15×10^6 orgs.			*S. paratyphi A* (B 31/4 Kasauli) 80×10^6 orgs.			*S. paratyphi B* (Millard) 15×10^6 orgs.		
Protection 0·5 ml. serum subcutaneously	Pooled immune rabbit serum	Normal rabbit serum	No serum	Pooled immune rabbit serum	Normal rabbit serum	No serum	Pooled immune rabbit serum	Normal rabbit serum	No serum	Pooled immune rabbit serum	Normal rabbit serum	No serum
Survivors at 72 hr.	37/50	8/50	2/50	45/50	9/50	1/50	40/40	4/40	1/40	40/40	5/40	2/40

Numerators represent the number of animals to survive. Denominators represent the number of animals inoculated.

Table 5. *Passive mouse-protection test using pooled serum from human volunteers inoculated with TAB/Ch. vaccine by the intradermal route*

(Mice of average weight 25 g. Sexes approx. equal in each group.)

Mouse strain...	C/57/Black			Ajax albino								
Challenge at 24 hr., 0·5 ml. intraperitoneally	*V. cholerae* (Inaba) NCTC 7260 1000×10^6 orgs.			*S. typhi* (Ty 2) 20×10^6 orgs.			*S. paratyphi A* (B 31/4 Kasauli) 80×10^6 orgs.			*S. paratyphi B* (Millard) 15×10^6 orgs.		
Protection 0·5 ml. serum subcutaneously	Pooled immune human serum	Normal human serum	No serum	Pooled immune human serum	Normal human serum	No serum	Pooled immune human serum	Normal human serum	No serum	Pooled immune human serum	Normal human serum	No serum
Survivors at 72 hr.	22/30	1/20	0/20	28/30	0/20	0/20	20/20	1/20	0/20	20/20	0/20	1/20

Numerators represent the number of animals to survive. Denominators represent the number of animals inoculated.

golf or rugby football 18 hr. after vaccination and when questioned reported no adverse effects either generally or locally at the injection site.

Repeated intradermal inoculation of 0·1 ml. of the 13,000 million organisms per ml., combined cholera and enteric vaccine, at weekly intervals for 9 weeks into a limited area of both rabbit and guinea-pig skins, failed to produce ulceration due to local tissue hypersensitivity. No reactions of any type occurred, except that early in the series it was noted that the inoculum was completely absorbed in approximately 8 hr., whereas after the sixth injection the inoculum tended to require a little longer time, which never exceeded 24 hr., to absorb.

DISCUSSION

Máté, Joó & Pusztai (1964) and Joó *et al.* (1964) have described a combined tetanus toxoid, cholera and typhoid antigen vaccine which showed excellent protection in laboratory tests, including human volunteers, similar to that shown in the experiments described above. These authors inoculated test animals either subcutaneously or intraperitoneally and human volunteers intramuscularly. Although some reduction in reactions was achieved in humans by employing an antigenic extract rather than a bacillary suspension of *S. typhi*, a significant number of both general and local reactions was provoked. Furthermore, at least one drug house in Great Britain is marketing, at this time, a combined enteric and cholera vaccine for subcutaneous or intramuscular administration.

It has been shown that the incidence of severe reactions following the administration of intradermal TAB to Service personnel is negligible (Noble, 1963). An acceptable, painless, local lesion of short duration however occurs (Zuckerman, 1964; Noble, 1965*b*). Noble (1964*a*) has suggested that cholera vaccine administered by the intradermal route is as effective an immunizing agent as when it is given subcutaneously, and reactions are decreased both in numbers and severity when the former route is adopted. The author of a leading article in *Lancet* (1964) appears to support this view.

In the foregoing experiments, it has been shown that in laboratory animals combined TAB and cholera vaccine administered intradermally gives excellent protection against both *V. cholerae* and enteric organisms, while at the same time retaining, in human volunteers, the freedom from severe reactions which would occur if the combined vaccine were administered by the subcutaneous or intramuscular routes.

Many of the reservations held at the time of the adoption of the intradermal technique (Barr *et al.* 1959) have been found to be groundless and vaccination by this method is now a routine procedure in the Services. It is suggested that in view of the increasing number of vaccinations necessary, for example in travellers, following natural disasters and for the rapid mobilization of armies, combined enteric and cholera vaccine (TAB/Ch) by the intradermal route is, where appropriate, preferable to the administration of the two components separately. The intradermal technique rather than either the subcutaneous or intramuscular routes is advocated, not only because the protection afforded appears to be as

good, but also because the incidence and severity of both local and general reactions is considerably less where the former technique is adopted.

Where large numbers are concerned, economy of time and medical manpower become important. Needle-less injectors such as the Microbiological Research Establishment (Porton) 'Hypospray' instrument, already in use for subcutaneous inoculations, have been adapted and are under trial for use with the intradermal technique (Darlow, personal communication). Once the injector has been shown to be satisfactory, the advantages of speed, economy and freedom from reactions obtained by vaccinating, where appropriate, large numbers of non-immune persons with the combined enteric and cholera vaccine by the intradermal route become obvious.

SUMMARY

The protection afforded by a combined enteric and cholera vaccine administered by the intradermal route, and demonstrated by active and passive mouse protection tests and agglutination titres, is excellent and equal to that given individually by the component TAB and cholera vaccines.

In order to reduce the number of injections required and the unpleasant reactions provoked it is suggested that combined enteric and cholera vaccine administered by the intradermal route is of value, where appropriate, for the vaccination of travellers, and for the rapid protection of non-immune persons in the event of natural disasters or mobilization.

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Isolation of variants during passage of a strain of foot-and-mouth disease virus in partly immunized cattle

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Subtype antigenic differences exist between strains of the virus of foot-and-mouth disease (FMD) within the seven immunologically distinct types. Reports of effects of this diversity were reviewed briefly in a recent paper (Hyslop, Davie & Carter, 1963). Although it has been suspected that the antigenic changes leading to the appearance of new subtype strains may result from spread of the virus through a partially immune population, direct experimental evidence has been lacking. Recently we have demonstrated subtype changes in strains of FMD virus propagated by serial passage in monolayer cultures of pig kidney cells (PKTC) in which increasing concentrations of strain specific antiserum were incorporated in the medium.

The present report records the serial passage of FMD virus of Type SAT 1 (strain Turkey 323/62) in partially immunized cattle, and the isolation of an immunologically distinct variant after the 34th serial passage.

MATERIALS AND METHODS

Virus

FMD virus of strain Turkey 323/62 (Type SAT 1), originally isolated from a field outbreak near Istanbul, was inoculated into susceptible cattle at Pirbright. A 1/10 suspension (in M/25 phosphate buffer) of infected tongue epithelium from one of these animals, steer DY 90, was clarified by centrifugation and was then inoculated into the lingual mucosa of steer EH 6, a fully susceptible animal. Virus from the resultant vesicles was passaged serially by inoculating clarified suspensions of epithelium into the mucosa of the tongues of partially immunized cattle. In the early passages a 1/10 suspension was employed, but later the dilution was increased to 1/1000, then to 1/10,000 and finally to 1/100,000. Vesicular material was usually harvested from tongue lesions, but on two occasions epithelium was harvested from secondary lesions on the feet, so that only virus capable of generalizing in the host in the presence of circulating antibody was selected on these occasions.

Virus of substrains was propagated also in secondary monolayers of pig kidney cells (PKTC) which were cultured in EYL medium (Earle's saline containing 0·01 % yeast extract and 0·5 % lactalbumin hydrolysate) for 48 hr. before inoculation of the virus.

Virus titration

Infectivity was titrated by intraperitoneal inoculation of serial 0·5 log dilutions of the virus into five groups of eight unweaned mice of the Pirbright 'P' strain (Skinner, 1951); 50 % end-points were estimated by the method of Kärber (1931). The litters were randomized before inoculation.

Cattle

All the cattle were Devon or Devon-cross steers, about 18 months old, which were purchased through a dealer who collected them from various parts of south-west England. During the experiment the animals were housed, usually in pairs, in an isolation compound.

Vaccine

Groups of cattle were vaccinated with one or more graduated doses of inactivated (formalin-treated, aluminium hydroxide adsorbed) vaccine prepared by a modification of the method of Frenkel (1949, 1951, 1953). The vaccine (batch no. 6215), of strain Turkey 323/62, had protected 10/10 cattle against generalized infection when given in the standard 15 ml. dose.

Serum-virus neutralization tests

Cattle were bled immediately before inoculation with virus and their sera were titrated for neutralizing antibody by the tissue culture metabolic inhibition test described by Martin & Chapman (1961), in serial two-fold dilutions against 100 tissue culture ID 50 of virus of each of the substrains isolated during the experiment.

Virus and sera derived from guinea-pigs

Young guinea-pigs, bred in the colony at this Institute, were infected by intradermal inoculation into the left tarsal pad of FMD virus suspensions containing 0·05 % (w/v) saponin. Virus of each of the substrains was passaged 2–3 times to adapt it to the guinea-pig. Groups of guinea-pigs were immunized by two doses of virus at 21-day intervals and were exsanguinated 10 days later.

Complement-fixation test (CFT)

Virus from lesions which developed at various stages of the experiment was tested, by the method described by Brooksby (1952), against subtype-specific serum produced in guinea-pigs and also against stock-strain sera of the seven main immunological types supplied by the World Reference Laboratory (WRL). Results were assessed in accordance with the principles recorded by Bradish, Brooksby & Tsubahara (1960) and by Bradish & Brooksby (1960). The cross-fixation ratios (CFR) of the various systems were determined, i.e

$$\frac{\text{Amount of complement fixed in the heterologous serum-virus system.}}{\text{Amount of complement fixed in the homologous serum-virus system}}$$

Calculation of the product of these ratios (CFP) excluded differences attributable to the relative concentrations of individual reagents, and pairs of strains were considered to be antigenically distinct when their CFP was less than 0·5 (Bradish & Brooksby, 1960).

RESULTS

Clinical results in cattle

Passage of virus in vaccinated cattle

Only one of the vaccinated animals in which the virus was passaged (steer EH 7) was without previous experience of FMD virus. Some animals had been used for innocuity tests before being vaccinated and so had received multiple inoculations of 0·1 ml. of inactivated FMD vaccine into the epithelium of the tongue, i.e. 'sensitizing inoculations' (SI), which possibly might have increased their ability to respond to the subsequent vaccination, although none of the animals tested possessed virus-neutralizing antibody titres (SVNT) exceeding 1/6 at the time of vaccination. Other animals had been vaccinated on one or more occasions with inactivated vaccine of strain Turkey 323/62 and possessed considerable levels of strain-specific antibody before the experiment commenced. On the 21st day before the start of serial passage, the animals were grouped on the basis of their known or expected degree of resistance to infection and all were vaccinated subcutaneously with graded doses (0·5–15·0 ml.) of inactivated vaccine. Animals possessing low resistance received smaller doses than those with high resistance and, in the group expected to have the highest resistance, five animals were vaccinated yet again after the start of the experiment. The vaccination history and the immune status of individual animals, as demonstrated by their SVNT immediately before passage of the virus, are shown in Table 1.

The field strain Turkey 323/62 had been inoculated into susceptible cattle 3 times before the experiment commenced. A suspension of infected tongue epithelium (titre $10^{6·9}$ mouse ID 50/ml.) was inoculated into the lingual mucosa of a fully susceptible animal, steer EH 6, and also by the same route into steer ED 89, fully immunized with inactivated vaccine of strain Turkey 323/62. In the susceptible steer, infection generalized from the primary tongue vesicles, part of which was harvested, to produce lesions on the dental pad, gums, muzzle and on all four feet; no lesions developed in the immunized animal. Virus from the lesions of steer EH 6 failed to fix complement with specific sera of types other than Type SAT 1.

The first serial passage in partly immunized cattle was made with a 1/10 suspension of vesicular material from steer EH 6. The recipient animal, steer EH 7, developed a generalized infection but its SVNT was 1/4 and the animal must be considered to have remained fully susceptible at the time of infection. Thereafter, serial passages were made in cattle which possessed detectable antibody against virus of strain Turkey 323/62. The clinical results of infection are summarized for individual animals in Table 1.

Infection generalized in each of the first ten animals; at the 11th passage, generalization ceased but extensive primary lesions continued to occur in every animal. At the 14th passage, secondary lesions appeared again and all succeeding

Table 1. *Passage of FMD virus of strain Turkey* 323/62 *in partly immunized cattle*

Virus passage no.	Animal no.	History: S.I.	History: Vaccine dose	History: V–I interval (days)	1/SVNT of sera (pre-infection) against virus of: EC 83 strain 1	EC 83 strain 2	Virus inoculated: Source	Virus inoculated: Dilution	Clinical result of inoculation
—	EH 6	0	0	—	⩽ 3	⩽ 3	Tongue DY 90	10^{-1}	T D G M 4F
—	ED 89	0	3 × 15 ml.	21	355	⩽ 3	Tongue DY 90	10^{-1}	Negative
1	EH 7	0	1 × 0·5 ml.	24	4	⩽ 3	Tongue EH 6	10^{-1}	T D 4F
2	EC 83	1	1 × 0·5 ml.	25	16	⩽ 3	Tongue EH 7	10^{-1}	T M 4F
3	EC 84	1	1 × 0·5 ml.	26	45	⩽ 3	Tongue EC 83	10^{-1}	T D 4F
4	EC 87	1	1 × 0·5 ml.	27	90	⩽ 3	Tongue EC 84	10^{-1}	T 1F
5	EC 89	1	1 × 0·5 ml.	31	256	6	Tongue EC 87	10^{-1}	T 2F
6	EC 90	1	1 × 0·5 ml.	32	178	4	Tongue EC 89	10^{-1}	T 1F
7	EC 91	1	1 × 1·0 ml.	33	355	16	Tongue EC 90	10^{-1}	T D M 2F
8	EC 92	1	1 × 1·0 ml.	34	32	⩽ 3	Tongue EC 91	10^{-1}	T D 1F
9	EC 93	1	1 × 1·0 ml.	35	178	⩽ 3	Tongue EC 92	10^{-1}	T 1F
10	ED 50	1	1 × 1·0 ml.	38	45	⩽ 3	Tongue EC 93	10^{-1}	T D 4F
11	ED 51	1	1 × 1·0 ml.	39	128	4	Tongue ED 50	10^{-1}	T
12	ED 52	1	1 × 2·0 ml.	40	45	⩽ 3	Tongue ED 51	10^{-1}	T
13	ED 53	1	1 × 2·0 ml.	41	90	⩽ 3	Tongue ED 52	10^{-1}	T
14	ED 54	1	1 × 2·0 ml.	42	22	⩽ 3	Tongue ED 53	10^{-1}	T 2F
15	ED 55	1	1 × 2·0 ml.	45	32	⩽ 3	Tongue ED 54	10^{-1}	T 4F
15	ED 56	1	1 × 2·0 ml.	46	32	⩽ 3	Tongue ED 55	10^{-1}	T 1F
15	EG 84	1	1 × 5·0 ml.	47	N.A.	⩽ 3	Tongue ED 56	10^{-1}	T 2F
16	EG 85	1	1 × 5·0 ml.	48	45	⩽ 3	Foot ED 55	10^{-1}	T D 4F
17	EG 86	1	1 × 5·0 ml.	49	90	⩽ 3	Tongue EG 85	10^{-1}	T D 2F
18	EG 69	1	1 × 5·0 ml.	52	128	⩽ 3	Tongue EG 86	10^{-1}	T 3F
19	EG 88	1	1 × 5·0 ml.	53	512	16	Tongue EG 69	10^{-1}	T 1F
20	EG 89	1	1 × 10 ml.	54	32	⩽ 3	Tongue EG 88	10^{-1}	T 3F
20	EG 90	1	1 × 10 ml.	59	178	8	Tongue EG 89	10^{-1}	T D 1F
21	EG 91	1	1 × 10 ml.	60	N.A.	⩽ 3	Foot EG 89	10^{-1}	T D 1F
22	EG 92	1	1 × 10 ml.	61	45	4	Tongue EG 91	10^{-1}	T 2F
23	EG 93	1	1 × 10 ml.	62	N.A.	⩽ 3	Tongue EG 92	10^{-1}	T 4F
24	EG 94	1	1 × 15 ml.	63	32	⩽ 3	Tongue EG 93	10^{-1}	T D 2F
25	EG 95	1	1 × 15 ml.	66	90	⩽ 3	Tongue EG 94	10^{-1}	T D 4F
26	EG 96	1	1 × 15 ml.	67	32	4	Tongue EG 95	10^{-1}	T 3F
27	EG 97	1	1 × 15 ml.	68	32	⩽ 3	Tongue EG 96	10^{-3}	T 3F
28	EG 98	1	1 × 15 ml.	70	32	⩽ 3	Tongue EG 97	10^{-3}	T 1F
28	EG 99	1	2 × 15 ml.	—	N.A.	N.A.	Contact EG 98	—	T 1F
29	EG 68	1	2 × 15 ml.	5	64	4	Tongue EG 98	10^{-3}	T 1F
30	EH 1	1	2 × 15 ml.	6	178	4	Tongue EG 68	10^{-4}	T 3F
31	ED 85	0	3 × 15 ml.	80	178	6	Tongue EH 1	10^{-5}	T 3F
32	ED 86	0	3 × 15 ml.	81	256	6	Tongue ED 85	10^{-5}	T 1F
33	ED 87	0	4 × 15 ml.	12	256	⩽ 3	Tongue ED 86	10^{-5}	T 1F
34	ED 88	0	4 × 15 ml.	13	708	32	Tongue ED 87	10^{-5}	T Died
35a	EH 7	Recovered 77 days after Passage 1			355	11	Tongue ED 88	$10^{-1.3}$	T D 1F
35b	EC 83	Recovered 76 days after Passage 2			512	11	Tongue ED 88	$10^{-1.3}$	T D 2F

S.I. Sensitizing inoculation (20 × 0·1 ml. vaccine i.d. tongue).
V–I Interval between last vaccination and inoculation of virus.
SVNT Virus neutralizing titre of serum.
T Primary vesicles on tongue.
D Secondary vesicles on dental pad.
G Secondary vesicles on gums.
M Secondary vesicles on muzzle.
1–4F Secondary vesicles on 1 or more feet.

animals showed some degree of generalization, though occasionally (e.g. at the 18th passage) both primary and secondary lesions were delayed.

After the 15th and 20th serial passages, infected epithelium was harvested from secondary lesions on the feet instead of from primary lesions, and filtrates prepared from this material were inoculated into the tongues of the next animals. The 21st and all subsequent passages were made in cattle vaccinated with the normal field dose (15 ml.) on at least one occasion.

The inoculum was diluted 1/1000 after the 26th passage and the dilution was increased to 1/10,000 for the 30th passage, then to 1/100,000 for the 31st and subsequent passages. Although satisfactory primary lesions developed, the secondary lesions were sometimes small and their development was often delayed until somewhat later than might have been expected as a result of the inoculation of virus into fully susceptible cattle.

At the 28th passage, steer EG 98 developed extensive primary lesions and a secondary lesion on one foot. Steer EG 99 was not infected by inoculation but was allowed to remain in contact with steer EG 98 and to drink from the same water bowl. On the 4th day after the appearance of primary lesions on the tongue of steer EG 98, two primary vesicles were observed on the tongue of steer EG 99 and infection generalized later to one foot; CF tests showed that the virus present in these lesions was of Type SAT 1.

The penultimate animal of the series had been vaccinated with 15 ml. doses on four occasions before infection. Nevertheless, three primary lesions and a single secondary lesion resulted. The last animal, steer ED 88, which had been vaccinated similarly 199, 164, 83 and 13 days before infection showed primary lesions at two of the ten inoculation sites despite a SVNT of 1/708 against virus of strain Turkey 323/62. This steer died under general anaesthesia, during harvesting of the primary vesicles, before development of secondary lesions was possible. CF tests employing the harvested material indicated that virus of Type SAT 1 only was present.

Passage of virus in recovered cattle

On the 76th day after primary infection, steer EH 6, the 1st (unvaccinated) animal in the experiment, was reinoculated at ten sites on the tongue with a 1/10 suspension of epithelium from its own original reaction. At this time all lesions had healed completely and the SVNT was 1/1024. No lesions resulted from the second attempt to infect the animal and thus strain Turkey 323/62 produced complete immunity against challenge with homologous virus.

On the same day, the vaccinated steer ED 89, which had been immune when challenged with strain Turkey 323/62 at the start of the experiment, was inoculated at ten sites with a 1/20 suspension of vesicular epithelium from steer ED 88, the last animal in the passage series. By contrast with the homologous virus challenge, although steer ED 89 also possessed SVNT of 1/1024 against virus of strain Turkey 323/62, the 'heterologous' strain produced extensive lesions on the tongue but generalization did not occur. Similarly, steers EH 7 and EC 83, the 1st and 2nd vaccinated animals in the passage series, which had recovered from generalized

infections with virus from the unvaccinated steer EH 6 some 10 weeks previously, were both inoculated at ten sites with a 1/20 suspension of vesicular epithelium from steer ED 88. Steer EH 7, whose SVNT against strain Turkey 323/62 virus was 1/355 at the time of the second infection, developed two primary vesicles and a secondary vesicle appeared on one foot. Steer EC 83, whose SVNT against virus of strain Turkey 323/62 was 1/512, developed a single large vesicle on the tongue and secondary vesicles occurred on the dental pad and on both hind feet.

The fluid and epithelium from the primary vesicle of steer EC 83 were harvested under anaesthesia. CFT on this material demonstrated some fixation of complement in the presence of WRL stock antiserum of Type SAT 1 but no fixation occurred with stock reference sera of FMD virus of the remaining six types. By virtue of its ability to break through the immunity of recovered cattle, this strain, from steer EC 83 was considered to be an extreme subtype variant of Type SAT 1 and was designated strain $C^{i}34.C^{I}1$.

In an attempt to determine whether variation had occurred early or later in the passage series, a 1/10 suspension of the vesicular epithelium from the second infection of steer EC 83 was inoculated into steers EG 94, 95, 96 and 97, which were respectively the 24th–27th cattle in the passage series. No clinical reactions resulted.

Passage and contact infection in cattle possessing waning immunity

To simulate conditions which might arise after vaccination campaigns in the field, four new steers, ED 81–84, were all vaccinated with 15 ml. doses of strain Turkey 323/62 vaccine on 6 May 1963; steers ED 83 and 84 were revaccinated on 27 September 1963. The immunity of the group was allowed to regress naturally until 2 December 1963 and on this date their SVNT against virus of strain Turkey 323/62 were 1/22, 1/22, 1/178 and 1/90 respectively.

Steer ED 81 was inoculated on the tongue with 10–100 ID 50 of virus from steer EH 6 (strain Turkey 323/62). Steer ED 82 was not inoculated but was housed in contact with steer ED 81. Steer ED 83 was inoculated with 10–100 ID 50 of virus from steer ED 88 (strain $C^{i}34$). Steer ED 84 was allowed to remain in contact with steer ED 83.

Both of the inoculated steers developed generalized lesions, although steer ED 83 possessed a SVNT of 1/178 against virus of strain Turkey 323/62. Whilst residual immunity (SVNT 1/22) protected steer ED 82 against contact infection with the homologous strain Turkey 323/62, the heterologous strain $C^{i}34$ was able to spread by contact from steer ED 83 to steer ED 84 and generalized later to two feet, despite a SVNT of 1/90 against virus of the original strain Turkey 323/62. Subsequently, steer ED 82 proved to be fully susceptible to parenteral challenge with strain $C^{i}34$.

Virus titres in the lesions of immunized cattle

It appeared possible that the infectivity titre of virus present in the vesicular epithelium of cattle possessing high antibody titres might be much less than the virus titres of susceptible cattle or of cattle only incompletely immunized. Consequently, fragments of the epithelium were harvested during passage at the 24th

hour after inoculation and the infectivity of samples collected at several passage levels was titrated in mice. The results shown in Table 2 indicate that, even during the early passages, humoral antibody titres exerted little influence on the titre of virus in the vesicles. The virus titres of the primary vesicles of steers ED 51, 52 and 53, in which infection failed to generalize, were $10^{5.9}$, $10^{6.1}$ and $10^{6.2}$ mouse ID 50/ml. respectively. These were not significantly different from those found in the primary lesions of animals in which generalization occurred.

In the later passages, by which time adaptation was well advanced, the virus titre of the epithelium was also independent of the amount of virus inoculated.

Table 2. *FMD virus in tongue epithelium fragments harvested 24 hr. after the infection of partly immunized cattle*

Animal no.	SVNT for strain Turkey 323/62	Dilution of inoculum	Virus titre of epithelium harvested (mouse ID 50/g.)
EH 6	≤ 1/3	1/10	$10^{6.125}$
EC 83	1/45	1/10	$10^{6\,7}$
EC 89	1/256	1/10	$10^{6.8}$
ED 50	1/45	1/10	$10^{6.4}$
ED 55	1/32	1/10	$10^{7.2}$
EG 69	1/128	1/10	$10^{7.1}$
EG 89	1/32	1/10	$10^{5.9}$
EG 95	1/90	1/10	$10^{6.6}$
EG 97	N.A.	1/1,000	$10^{6.1}$
EG 68	1/64	1/1,000	$10^{5.3}$
ED 87	1/256	1/100,000	$10^{8.0}$
ED 88	1/708	1/100,000	$10^{7.0}$

N.A. = not available.

Serum-virus neutralization tests

Each animal was bled for serum immediately before the inoculation of virus. Virus in filtrates of tongue epithelium from steer EC 83, the 2nd vaccinated animal in the cattle passage series and the 1st in which a significant antibody titre was detectable before infection, was passaged 6 times in pig kidney monolayer cultures and was then used in serum-virus neutralization tests. Virus in filtrates of the 2nd crop of vesicles from steer EC 83 (strain $C^{i}34.C^{I}1$) was passaged similarly in pig kidney monolayers 6 times and was then employed in further serum-virus neutralization tests with all the cattle sera tested previously against the virus isolated from the 1st infection of this animal. As a further check, all sera were also tested against a stock (high PKTC passage) substrain of strain Turkey 323/62.

All except one of the thirty-four serum samples collected from cattle infected later than the 1st infection of steer EC 83 possessed SVNT to virus of strain Turkey 323/62 which were equal to or greater than 1/32, but only the serum of steer ED 88 had an antibody titre of 1/32 against virus of strain $C^{i}34.C^{I}1$ and the sera from thirty-one animals had titres equal to or less than 1/6.

The SVNT of all sera when tested against the stock (high PKTC passage) virus of strain Turkey 323/62 was usually one serial dilution lower than the titre against the 6th PKTC passage. The geometric mean titres of all the serum samples tested against the three virus strains were:

Turkey 323/62 stock	1/40
Turkey 323/62 6th PKTC	1/80
$C^{i}34.C^{I}1$ 6th PKTC	1/4.

The very great difference in the neutralizing titres of the sera of individual cattle against the two strains isolated from steer EC 83 (Table 1, cols. 6 and 7), after both strains had experienced the same number of passages in PKTC, suggests that a considerable degree of subtype variation had occurred during passage in immunized cattle; and the SVNT are consistent with the clinical results only if such variation had occurred.

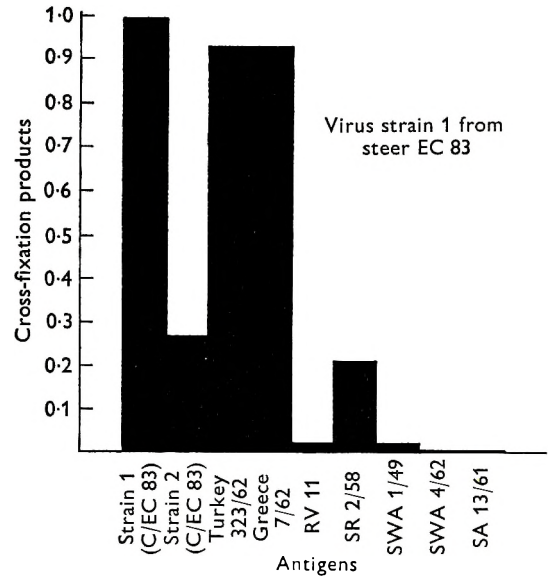
Reinfection of convalescent guinea-pigs

Virus of the 1st and 2nd strains from steer EC 83 was passaged 3 times in groups of guinea-pigs which were housed in separate rooms. The guinea-pig passaged strains were each inoculated into further groups of guinea-pigs and generalization of infection resulted. On the 28th day after primary infection, the immunity of eight guinea-pigs convalescent from infection with strain 1 was challenged by intradermal inoculation of virus of the guinea-pig passaged homologous strain; no lesions resulted. On the same day, ten guinea-pigs convalescent from infection with strain 2, together with five susceptible controls, were subjected to challenge with virus of the guinea-pig passaged strain 1; all developed generalized lesions.

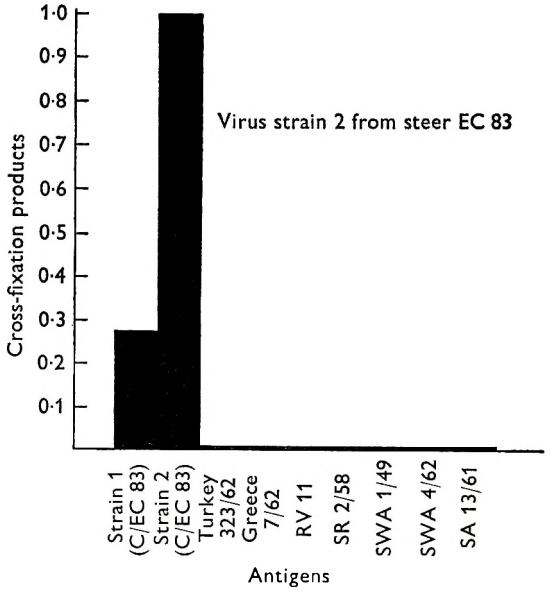
Complement-fixation tests

Virus of the 6th PKTC passages of the two strains isolated from steer EC 83 were each passaged twice on the plantar pads of guinea-pigs. The 'adapted' viruses were used to immunize further groups of guinea-pigs, and pooled sera from these groups were employed in CFT against the homologous and heterologous viruses derived from the respective 6th PKTC. The complement-fixation ratios were determined and the CFP for the two strains was found to be 0·28. Because the CFP of the two strains was less than 0·5 they may be regarded as antigenically different.

Similarly, CFP with the viruses of strains 1 and 2 from steer EC 83 were determined for all available stock strains of Type SAT 1. The results, which are expressed as histograms in Fig. 1, indicate that strain 1 was identical with the WRL stock strains Turkey 323/62 and Greece 7/62, and it also possessed minor antigenic relationships with several other strains. Strain 2, however, possessed only a minor antigenic relationship with strain 1 and was devoid of significant relationship to the other strains.



(*a*)



(*b*)

Fig. 1. Histogram of cross-fixation products with SAT 1 strains of FMD.

DISCUSSION

During a major epizootic in a fully susceptible population, FMD appears often to infect very large numbers of animals without showing marked modification of the characteristics of the causal strain of virus; thus, in 1962–63, although FMD of Type SAT 1 spread from Bahrein in the southern part of the Persian Gulf through Israel and Turkey to the borders of Greece, the serological characteristics

of the late strains from Greece resemble closely those of the early strains from Israel. It is not possible to state unequivocally that FMD of Type SAT 1 had never occurred in the Middle East before 1962 but it is very probable that few, if any, of the cattle in the area had previously experienced a virus of this type.

By contrast, a number of markedly *different* strains have been isolated in recent years in Southern Africa, where FMD of Type SAT 1 is endemic. For example, strains SA 13/61 and SWA 40/61, both isolated in the same year, though showing some antigenic resemblance were nevertheless demonstrably different from one another and differed to a much greater extent from the 'older' strain, RV11 (Hyslop *et al.* 1963), and also from the Middle East strain Isr. 4/62 (Hyslop, unpublished result). These observations are consistent with a widely accepted belief that variant strains of many viruses pathogenic for animals and man tend to become dominant when either vaccination or previous exposure to endemic infection causes resistance to increase in the general 'host' population.

Whether in the case of FMD this effect results *in vivo* solely from the presence of high serum antibody titres in the animal population or is caused by the interaction of several factors remains unknown; but the influence of serum antibody was illustrated *in vitro* by the emergence of immunologically distinct substrains from strains of FMD virus propagated in PKTC monolayers maintained in media containing progressively increasing concentrations of homologous cattle or guinea-pig antiserum (unpublished results).

In the present experiment, the antibody titres of successive cattle did not increase uniformly. Although at the start of the experiment the graded doses of vaccine probably produced an 'immunity gradient' between the 1st animal of the series and the last, the natural regression in SVNT which occurred during the period of passage largely offset the increase in dosage. Nevertheless, after the 1st passage, SVNT always indicated a marginal or clearly protective degree of immunity against the original strain of virus, and the last eight animals possessed SVNT great enough to have protected them against generalization of virus of the 'parent' strain.

The experimental data do not reveal clearly the stage at which the emergence of the variant strain was most rapid. It is particularly noteworthy, however, that by the 5th passage the virus was able to generalize despite a SVNT of 1/256, and generalization occurred at the 7th passage although the animal possessed a SVNT of 1/355—a titre which is very frequently sufficient to protect against any clinical response to challenge whatsoever. The probability that some degree of adaptation continued throughout the greater part of the passage series is indicated by the CFP of the virus samples isolated at the 2nd, 10th and 35th passages; thus, although the CFP had fallen to 0·47 by the 10th passage, a further decrease to 0·27 occurred during the subsequent twenty-five passages. Furthermore, the inability of the strain of virus isolated from the 35th animal of the passage series to reinfect the 25th, 26th or the 27th animals suggests that variation between the 1st and the 25th passages may have been very much greater than that between the 25th and 35th passages.

It seems possible that the change in antigenic structure might have been more

rapid if epithelium had been harvested routinely from lesions which had generalized to the feet instead of principally from lesions on the tongue. However, if the former method had been adopted it would have been necessary to delay each passage until foot lesions appeared and to accept the risk, at each passage, that generalization might not occur; also the recovery of adequate amounts of material from small and often ruptured foot vesicles presents obvious difficulties. The possible danger of dissemination of variant strains from the foot lesions of naturally infected cattle was demonstrated by titrating epithelium harvested from the 15th serial passage (steer ED 55), by which passage demonstrable variation had already occurred; the titre of the tongue epithelium of this animal was $10^{7\cdot2}$ mouse ID 50/g. and that of the foot epithelium was $\geqslant 10^{6\cdot75}$ mouse ID 50/g.

The results shown in Table 2 indicate that, irrespective of titres of humoral antibody against the 'parent' strain of virus, cattle with primary vesicles caused by emergent variants were likely to be highly infective; as much virus was detected in such lesions at the 24th hour as was present in 24 hr. samples collected in this and in other experiments from the lesions of fully susceptible cattle. No attempt was made to transmit the emergent strain by contact until it had been passaged in immunized cattle 28 times but by this passage the strain was able, by contact infection, to overcome the resistance of steer ED 99, which had been inoculated twice with the standard 15 ml. dose of vaccine; two other animals having the same vaccination history possessed SVNT which were high enough to protect against the 'parent' strain. Contact infection also occurred between vaccinated steers ED 83 and ED 84, when the strain had been passaged 34 times in vaccinated cattle.

The capacity of the variant strain to infect cattle possessing SVNT as high as 1/355, 1/512 and 1/708 against virus of the 'parent' strain suggests a very considerable degree of difference in antigenic structure between the two strains, and the ability of the 'parent' strain to reinfect guinea-pigs convalescent from infection with virus of the variant strain provides additional evidence of a dissimilarity of significant magnitude.

The mean SVNT of the sera of all cattle tested, 1/80 against the 6th PKTC of virus of strain 1 of steer EC 83 but only 1/4 against the 6th PKTC of virus of strain 2, provided supplementary evidence of change during serial passage and confirmed the difference in antigenic constitution revealed by the CF tests.

Whether the antigenic change was a gradual process throughout the experiment or was a stepwise progression during certain phases is not revealed by the CFP. Nevertheless, the CFP of two strains from steer EC 83 shows an obvious difference between the strains. It appeared possible that the influence of humoral antibody might have caused the strain to change until it resembled one of the older field strains of Type SAT 1. Fig. 1 demonstrates that a change of this nature did not result; indeed, in this particular instance, a well-marked loss of antigenic components common to strain Turkey 323/62 and the other stock strains of Type SAT 1 appears to have occurred during passage. CF tests with sera of the WRL stock strains of types other than SAT 1 failed to reveal a significant degree of fixation of complement. The possibility that additional passages in highly resistant

cattle might result in the emergence of a strain possessing the characteristics either of a different serological type or of an entirely new type cannot be excluded without further investigation. The present observations suggest one way in which subtype variant strains may arise in areas where the disease is endemic or where the herd immunity induced by vaccination is allowed to become dangerously low.

SUMMARY

Foot-and-mouth disease virus of Type SAT 1 (strain Turkey 323/62) was passaged serially 34 times in cattle previously vaccinated with increasing doses of formol-treated vaccine of the homologous strain. Primary vesicles developed in all the partly immunized animals and secondary lesions occurred in the majority. Virus from the 34th passage was capable of reinfecting a steer only 76 days after primary infection early in the passage series. Virus isolated from the second infection of this animal differed from that isolated from the primary infection in complement-fixing properties and in sensitivity to antiserum, and these differences were of a degree indicative of subtype variation.

The variant strain was transmissible by contact, and virus titres in tongue and foot lesions of partly immunized animals were of the same order as those encountered in susceptible cattle.

These observations suggest one way in which variant strains may arise in the field.

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Description and analysis of a simple micro-titration immune cytolytic test

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INTRODUCTION

Immune cytolytic tests so far described (Gorer & O'Gorman, 1956; Hanks & Wallace, 1958; Reif & Norris, 1960; Boyse, Old & Thomas, 1962), all involve separate diluting and reading stages in their performance. The technique of Boyse *et al.* (1962) still requires master dilutions of antiserum, although incubation and reading are done on the same slide.

This communication describes a development of the test described by Boyse *et al.* (1962) in which titration of antiserum, incubation with cells and complement, and reading can all be carried out on one slide. The lack of bias and the reproducibility of this method of titration were tested and the reliability of rapid grading of the tests was assessed.

The paper consists of two parts. The first part is a general description of the materials and methods of the modified cytolytic test, and the second part is the analysis of variance of this method of testing in one clearly defined cytolytic system.

EXPERIMENTAL METHODS AND MATERIALS

Preparation of slides

Eleven rings were ground on the surface of a 7·6 cm. × 5·0 cm. glass microscope slide, the size of each ring being 12·5 mm. internal diameter and 18·7 mm. external diameter. The function of these ground circles was to provide a base to which araldite resin would adhere. The glass was then thoroughly defatted with detergent, rinsed and dried in a 60° C. incubator. Araldite resin was prepared by mixing 100 parts by weight of resin X 83/93 with eight parts by weight of hardener HY 951. The mixture of resin and hardener was applied to the ground rings on the slides from a pasteur pipette; this was best done at 37° C. to obtain a dry slide. To facilitate the application of resin, the slide was clamped to a revolving table and gently rotated whilst the pipette was held in a clamp. After 24 h. at 37° C. the resin had hardened sufficiently for the slides to be washed again and then siliconed after which they were ready for use. Slides prepared in this way can be washed and reused many times.

Since this experiment was performed slides have been obtained from Clay-Adams Inc. New York, U.S.A., which are very similar to the ones described above

having twelve ceramic rings on a 3 in. × 2 in. slide. These when siliconed are quite adequate for this test.

Loops and droppers

Standard droppers to deliver and wire loops to hold 0·025 ml. of saline were obtained from Cooke Engineering Co. Alexandria, Virginia, U.S.A. These were normally supplied for use with a plate micro-titration system developed by Sever (1962).

General description of test

One standard drop (0·025 ml.) of diluent, usually tissue culture medium 199 (Morgan, Morton & Parker, 1950) was placed in each well of the slide. A wire loop (holding 0·025 ml.) was filled with serum and dipped successively into each drop of diluent. Standard mixing was obtained by rotating the loop five times in each drop. The loop and slide are shown in Pl. 1. At the end of a titration the loop was rinsed in a stream of diluent, dried on clean filter paper, and was then ready for the next titration.

The washed cell suspension to be tested was diluted to a concentration of between 5×10^4 and 5×10^5 cells per ml. With large cells the lower concentration was preferable, whereas with small cells such as lymphocytes a concentration slightly in excess of 10^5 cells per ml. proved most suitable. The dilution of complement used varied with the test system, but when using rabbit complement it was usually in the range 1/10 to 1/20. Equal volumes of diluted cell suspension and diluted complement were mixed and one standard drop added to each well. The slide was then incubated in a moist chamber at 37° C. for 90 min.

At the end of incubation the slide was removed from the chamber and a 12·5 mm. diameter round cover-slip was placed on each drop. The slide was then examined under phase-contrast illumination at a magnification of 100 or 250 times. The titration end-point of 50 % cell lysis (CL50) was estimated from gradings made by assigning each dilution into one of seven grades. Viability was determined by phase-contrast microscopy alone without the use of dyes. This simplified the technique whilst giving the same accuracy as dye exclusion methods. The observation of Goldberg & Green (1959) that cells treated with antibody and complement swelled and their contents became visible under phase microscopy, at the same time as they were losing most of their soluble cytoplasmic contents (Green, Fleischer, Barrow & Goldberg, 1959), provided very adequate differentiation of live and dead cells. Examples of a negative control of cells in the presence of complement but no antibody and one of maximum lysis where cells have been treated with antibody diluted 1/128 and complement are shown in Pl. 2*a* and *b*.

REPRODUCIBILITY EXPERIMENT

General considerations

Errors in the micro-titration system could accumulate in a variety of ways, e.g. from variation in the size of the drops dispensed or from inadequate mixing. If such errors are systematic the results of the titration may be biased. In particular

if mixing is incomplete it will tend to raise the apparent number of dilutions required to reach a given end-point. Errors of all forms might be expected to accumulate as the number of titrations carried out on the slides increased.

In principle, the bias of the micro-titration system may be investigated by testing the same cell suspension with, for example, a 1/2 and 1/32 dilution of the same antiserum made up using standard diluting techniques. If the micro-titration method is unbiased the 1/2 dilution of antiserum should require exactly four more dilution steps to reach the CL50 than the 1/32 antiserum dilution. The magnitude of other errors involved may be estimated by experimental replication.

Detailed considerations

Using these principles an experiment using a standard statistical design was planned as follows:

Five master dilutions (1/2 to 1/32) of an antiserum were prepared from a single source on each of 5 days. Each of these master dilutions was titrated by the micro-titration method described above on five slides. Each day's testing was replicated using a second set of five slides.

The experimental design allowed for the possibility of four factors each at five levels affecting the results, namely: (*a*) the five dilutions of antiserum; (*b*) the individual slides 1–5 or 6–10; (*c*) days; (*d*) order of reading.

These four factors were arranged in a balanced design, namely a Graeco–Latin square (Fisher & Yates, 1953), so that the effect of each factor can be studied independently of the others, The square used for slides 1–5 was exactly replicated using slides 6–10.

Cells and reagents

Cells. A continuous line of mouse thyroid cells C3H-T1 (Franks, Daniel, Gurner & Coombs, 1964) were washed free of growth medium with medium 199 and suspended in a preserving medium of methyl cellulose and dimethyl sulphoxide (Spooner, 1965). Seven drops of cell suspension and preserving medium were distributed to each of ten vials from a single pipette giving a final volume in each of approximately 0·2 ml. These were numbered, and stored in liquid nitrogen. The initial cooling to $-100°$ C. was carried out slowly in an expanded polystyrene plug of the Linde flask (Nagington & Greaves, 1962). In this way standard uniform cell suspensions were available for each set of slides tested each day.

Antiserum. Rabbit antiserum prepared against mouse red cells (with a cytolytic titre of 512 against C3H-T1 cells) was pipetted accurately in 0·5 ml. volumes into each of five siliconed 2·0 ml. tubes. Each was numbered and stored at $-23°$ C. One day's testing with all ten slides was carried out initially to check the system, and one of the five antiserum samples was used up. A further 0·5 ml. of antiserum had to be measured out to replace it. This was done without thawing, pooling and remeasuring the original four. An error led to serum from a different bleeding of the particular rabbit used for antiserum production being used, and the titre of this was higher than in the remaining four antiserum samples. Since a balanced experimental design was used, however, this did not invalidate the results.

Complement. Rabbit serum as a source of complement was pooled and placed in five 0·5 ml. amounts, numbered and stored in liquid nitrogen. A sample of cells and a sample of serum and complement were each removed from storage in random order on each day of testing.

The antiserum was thawed and doubling dilutions of this serum were made in 0·5 ml. amounts using a clean graduated 1 ml. pipette at each dilution step. Dilution was carried out in siliconed tubes so that any fluid on the side of the tube could be included in the titration by brief centrifugation before mixing.

When the master dilutions of antiserum had been made up they were relabelled with numbers 1–5 in accordance with the experimental design and the original labels were removed. The relabelling was done by a member of the laboratory staff not otherwise involved in the experiment, and the procedure was such that those subsequently titrating the antiserum and reading the tests had no idea what the results were likely to be.

The vial of cell suspension was thawed rapidly in a 37° C. water bath and diluted to 2 ml. with medium 199. It was then centrifuged for exactly 10 min. at 60*g* in a 5 cm. × 1 cm. tube, the supernatant was removed and the deposited cells resuspended in exactly 1·5 ml. of medium. The concentration of the final cell suspension from one vial was counted with a Coulter Electronic Cell Counter Model B, using a 100 μ aperture tube, and was found to be $6{\cdot}7 \times 10^4$ cells per ml.

Complement was thawed and diluted 1/12 with medium 199. Complement and antiserum dilutions were kept at 4° C. until required.

Details of the test

The master dilutions of antiserum which had been relabelled with the numbers 1–5 at the beginning of each day's testing were titrated in numerical order on slides 1–5 respectively. Thus because the order of these titrations was the same as that of the slide numbers each day, any effects due to carry-over from one slide to the next were confounded with effects due to different slides. For simplicity of experimental arrangements this shortcoming of the design was accepted. It was expected that neither slides nor effects of carry-over from slide to slide would appreciably affect the results. Then one drop of cell suspension mixed with the complement dilution was applied to each well of the five slides followed by incubation as already described. Half an hour after the first five slides began incubation the test was repeated on slides 6–10. A fresh sample of cell suspension from the original thawed sample was mixed with complement for these repeat titrations. Thus on each day the test procedure was repeated exactly except for a slightly longer time between thawing and testing and a shorter time between the end of incubation and reading in the second group of slides.

After incubation the slides were graded into seven grades by two observers. The grades were as follows: 6, > 99·5 % dead; 5, 99·5 to 90 % dead; 4, 90 to 60 % dead; 3, 60 to 40 % dead; 2, 40 to 10 % dead; 1, 10 to 0·5 % dead; 0, < 0·5 % dead. A third person counted the percentage of dead cells in 100 cells.

The order in which the slides were read within a group of five was determined by the experimental design, and the results of each slide were recorded on separate

slips of paper, stapled together in the order of reading for the particular day. This was a simple way of ensuring that the prescribed order of reading was adhered to and that the observer was minimally affected by earlier readings.

The time and order of grading and counting varied irregularly during the experiment. On occasions the slides stood for up to 2 hr. at room temperature after incubation before they were graded.

Calculation of the end-point of 50 % *cell lysis* (*CL*50)

Cytolytic tests are a form of bioassay, and the number of wells through which an antiserum is titrated before the CL 50 is reached, X, is directly proportional to the $\log_2$ concentration of antiserum in the well. When the percentage of cells killed is

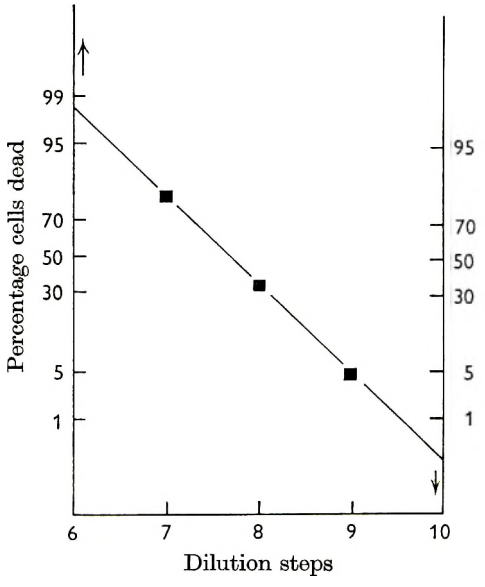

Fig. 1. Results from the first slide encountered with three counts between 95 and 5 % plotted against the number of micro-titration dilution steps on arithmetic probability paper.

plotted on arithmetical probability paper against the $\log_2$ concentration, or equivalently X, the relationship may be expected to be approximately linear from bioassay studies in other fields (Finney, 1947). This is borne out by Fig. 1, which shows the results of the first slide encountered with counts of between 1 and 99 % in three successive wells. The majority of slides gave countable results in only two wells, the remainder of the wells containing 99 % or more live or dead cells.

The CL 50's were estimated by plotting the two counts, on either side of 50 % as in Fig. 2 and reading off the results as the point where the line joining these two points intersects the 50 % line. Similarly, the grades 6 to 0 were taken as corresponding to counts of 99·99, 95, 75, 50, 25, 5 and 0·01 % dead cells respectively. Plotting these percentages on arithmetic probability paper (Fig. 2) and joining all possible pairs of successive grades either side of the 50 % count we find that the grading system groups the result of a titration as a whole number or one of five intermediate values.

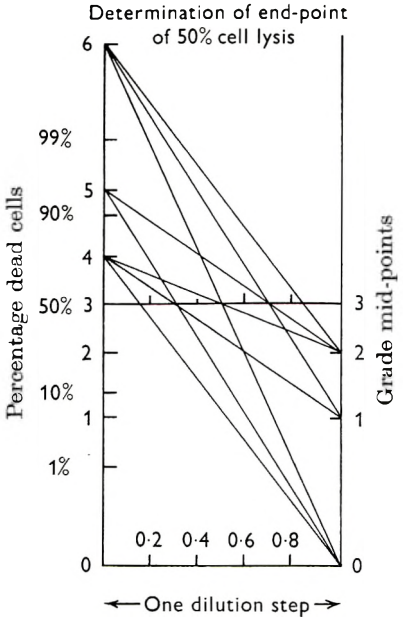

Fig. 2. How the CL 50 is derived. The diagram shows the joining of the mid-points of each grade. To derive the end-point from counted results the exact counts on each side of 50 % are joined.

RESULTS

The CL50's determined from cell counts were used to analyse the reliability, bias and other factors affecting the micro-titration system.

Reliability

The standard deviation (S.D.) of a CL50 estimated by a single micro-titration may be determined from standard analysis of the data. Two estimates of this S.D. were calculated, one from each replication of the experiment. They were 0·14 and 0·3 wells respectively, and are statistically significantly different ($0{\cdot}05 > P > 0{\cdot}025$). Subsequent detailed examination of the data suggested that there might have been two recording errors and that these had inflated the second estimate of the S.D. None of the observations, however, differed very greatly from expectation, and it was felt that if there were any recording errors they were of the kind that might easily occur occasionally in routine experimental work. We therefore pooled all our data and obtained the overall estimate of the S.D. of a single micro-titration as 0·23 dilution step.

In titrating an antiserum experimental errors carry over from one dilution to the next. It seemed important, therefore, to investigate whether the reliability of micro-titration deteriorated with the number of dilutions performed.

Table 1 shows the S.D.'s for a single titration with each master dilution of antiserum, and the nearest whole number of micro-dilutions used to reach the CL50. These S.D.'s were calculated by differencing the two results for the same master dilution obtained each day, and taking out average differences and the overall

effect of slides. As a result the S.D.'s shown in Table 1 are comparable with the overall S.D. shown, but each have only 3 degrees of freedom. They do not differ significantly from one another, nor from the overall S.D. There is no evidence in Table 1 that experimental errors increase significantly with the number of micro-dilutions, and it is concluded that the random experimental errors introduced by this method of making doubling dilutions are small relative to the other errors involved, for example making up the master dilutions, dispensing the drops and counting the cells, etc.

Table 1. *Effect of the number of micro-dilutions on experimental errors*

Concentration of antiserum tested...	1/32	1/16	1/8	1/4	1/2	All concentrations
No. of dilutions to 50 %, end-point = X	4	5	6	7	8	—
S.D. (of X)	0·26	0·14	0·39	0·16	0·29	0·23

Lack of bias

Although the micro-titration method of doubling dilutions may not introduce large experimental errors, unless there is satisfactory mixing in the wells at each step the method will be biased.

Table 2 shows the mean CL50 for each of the five master dilutions of antiserum together with their standard errors. As already explained, if the micro-titration method was unbiased the number of wells to reach the end-point should decrease

Table 2. *The mean no. of micro-dilutions to reach a* 50 % *end-point for each master dilution, and their standard errors*

Master dilution	No. of micro-dilutions to 50 % end-point	
	Mean	S.E.
1/2	8·12	0·023
1/4	7·07	0·023
1/8	6·02	0·023
1/16	5·12	0·023
1/32	3·99	0·023

by exactly 1 each time the master dilution was doubled. The results shown in Table 2 conform closely to this pattern. There is no suggestion that the means are non-linear (see Table 3(*c*)) and the estimated reduction in the number of micro-titrations required for each doubling dilution of the master antiserum is $-1{\cdot}022$ with 95 % confidence interval ($-0{\cdot}975$ to $-1{\cdot}069$) (Fig. 3). Thus the observed relationship is not significantly different from -1, and there is no evidence that the micro-titration method is significantly biased in relation to the method of preparing doubling dilutions of master antiserum.

Differences between slides and between days

Table 3 sets out the analysis of variance of the experimental results. In addition to the significant linear differences between dilutions the table shows that there

Table 3. *The analysis of variance of the experimental observations*

Measured variable: the number of micro-dilutions required to reach a 50 % end-point as determined from counted results.

Source of variation	Sum of squares	Degrees of freedom	Mean square	Significance of variance ratio
Replications	1·02	1	(*a*) 1·02	*a*/*g* insignificant
Master dilutions:				
Line fitted to means	104·35	1	(*b*) 104·35	*b*/*r* 0·001 > *P*
Variation of means about fitted line	0·10	3	(*c*) 0·03	*c*/*r* insignificant
Days	15·61	4	(*d*) 3·90	*d*/*r* 0·001 > *P*
Order of reading	0·41	4	(*e*) 0·10	*c*/*r* insignificant
Slides:				
Linear trend, ? due to carry-over effects from one titration to next	0·43	1	(*f*) 0·43	*f*/*g* insignificant
Variations about linear trend	1·50	7	(*g*) 0·21	*g*/*r* 0·001 > *P* > 0·001
Interactions of master dilutions, days and order of reading with replication	0·7	12	(*h*) 0·06	*h*/*r* insignificant
Residual	0·87	16	(*r*) 0·0542	—
Total	124·99	49		

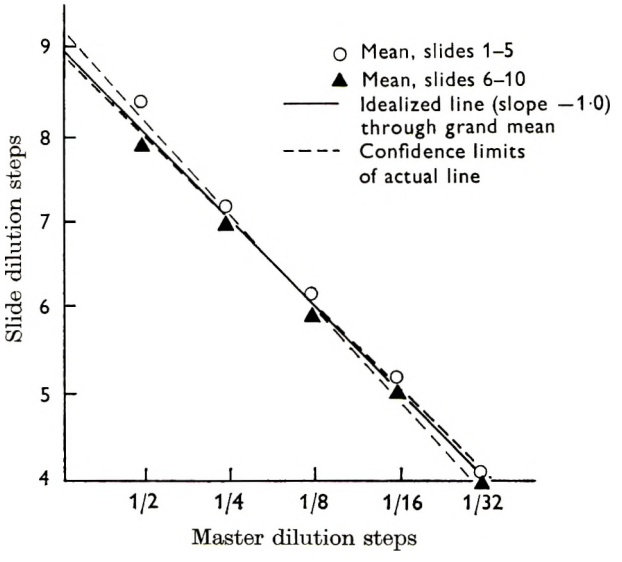

Fig. 3. The means of the CL 50's for each master dilution, i.e. the average end-point over the 5 days for each master dilution when titrated on slides 1–5 and 6–10 plotted against the master dilutions.

were significant differences between slides and differences between days. The order of counting the slides did not affect the results, and the differences between days, between master dilutions and order of reading effects were consistent in both replications of the experiment.

Slides. The differences between slides is illustrated in Fig. 4, in which the average CL 50 over the five master dilutions is shown for each slide. It will be observed

that the average for slides 1–5 is higher than for slides 6–10. This difference corresponds to the sum of squares for replications which, compared with differences between the slides, is not significant. A probable explanation, discussed in the next section, is that the second set of slides stood for a shorter time on the bench after incubation.

It will be noted from Fig. 4 that the CL50's for slide 10 were on average 0·25 dilution higher than those of slides 1–5 and 0·5 dilution higher than those of slides 6–9. Before the experiment began it was noted that slide 10 was not as well siliconed as the others. It was included to see what effect this might have on the results. The higher result for slide 10 shown in Fig. 5 could be explained by the fact that poor siliconing caused incomplete mixing and consequently a higher end-point.

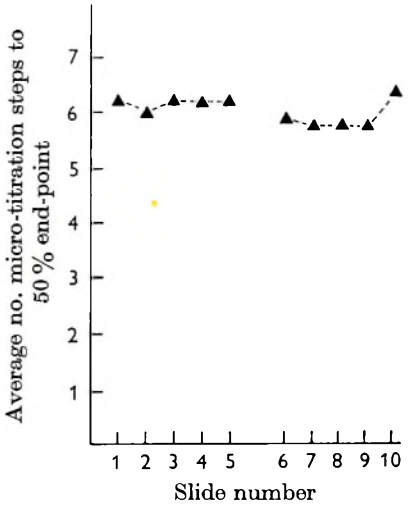

Fig. 4. The mean of the results for each slide, to show the overall effect of each slide.

Relative to the variation from slide to slide, there is no evidence of a trend within a set of 5 suggesting a carry-over effect from one micro-titration to the next.

Days. The highly significant difference between days shown in Table 3 largely results from the fact that the average results on the last day were more than a dilution higher than on any of the previous four days. The averages for the first 4 days also vary significantly, $0{\cdot}05 > P > 0{\cdot}01$ compared with residual variation, but the range of their variation is 0·33 dilution and, compared with the variation in the day-to-day means, the results for the 5th day are statistically significantly different from the average of the other four, $0{\cdot}05 > P > 0{\cdot}01$.

The fact that there are day effects which are consistent in both replications suggests that the master antiserum may have been made up slightly differently on each of the first 4 days rather than that there were differences between the stored cell samples or complement, of which different samples were used for slides 1–5 and 6–10 each day. Another source of day-to-day variation was the length of time the slides stood after incubation before they were counted. This period though shorter for slides 6–10 because they were read immediately after 1–5

varied similarly from day to day for both sets of slides. Variation in standing time cannot explain the generally high results on the last day.

Estimating the CL50 by grading

At an early stage of the experiment it was noticed that the end-point changed after the slide had been standing on the laboratory bench for some while. The time interval between grading and counting the results was then deliberately varied to study this.

The time taken to grade or count a set of five slides is negligible. All slides were graded by observers I and II and counted by observer III. Fig. 5*a* shows, for each set of five slides, the average end-point difference between observer I's graded

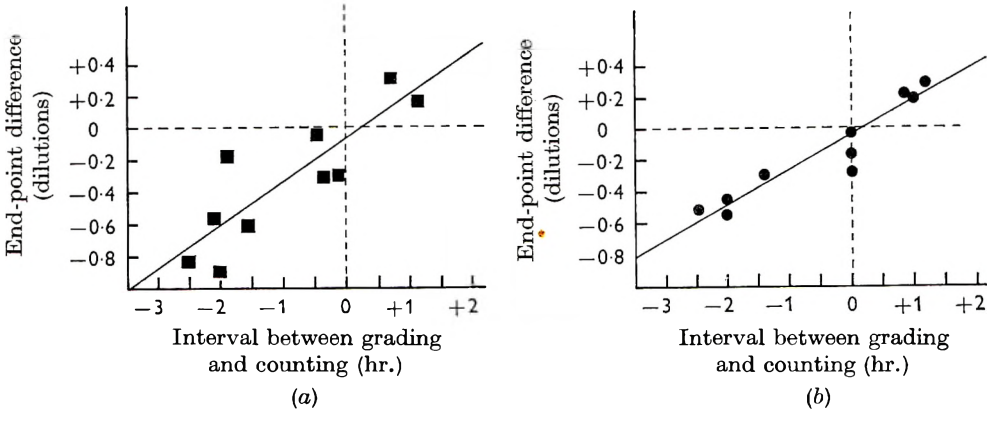

Fig. 5. Difference between graded results and counted results plotted again time interval between grading and counting. (*a*) Observer I. (*b*) Observer II.

results and observer III's counted results plotted against the time the slides stood between grading and counting. Similarly, Fig. 5*b* is a plot of the average end-point difference between observer II's grading and observer III's counting, against the time interval between grading and counting.

Both sets of results show that the end-point advanced with time, the rates of advance were similar in both cases and were of the order of a dilution every 4 or 5 hr.

The regression lines shown in Fig. 5*a* and *b* indicate the estimated relationship between the end-point difference between grading and counting on the one hand and the time interval between grading and counting on the other. The bias of grading as compared with counting is the end-point difference when the time interval between grading and counting is zero. The fitted regression lines are not significantly different from zero at this point, from which we conclude that there is no evidence that assessing the end-point by grading gives rise to biased results.

The standard deviation of a CL50 determined by counting was estimated to be 0·23 dilution. Using this estimate, we obtain from regression analysis to fit the lines shown in Figs. 5*a* and 5*b*, that the S.D. of a CL50 estimated by grading is 0·25 for observer I and 0·17 for observer II. The reliability of grading is, therefore, very similar to that of counting.

DISCUSSION

The use of standard micro-titration equipment for the performance of cytolytic tests on a siliconed glass slide provides considerable simplification of technique from previous methods.

In the test described here incubation was carried out on the slide without a cover-slip and the cells settled within the restricted area of the base of the drop. When, before reading, a cover-slip was applied very few of the cells were disturbed, whether live or dead, and hence most of the cells in the test could be seen under one field of the $\times 10$ objective. This meant that tests could be carried out with as few as 200 to 300 cells per drop, although usually about 500 cells per drop were employed. Before the test could be accepted it was felt that the reliability and bias of the method should be investigated.

Reliability. The standard deviation of a single estimate of a CL50 which was found to be 0·23 dilution, was derived from cell counts of more than 100 cells in at least two wells. Although only a sample of cells was counted, calculations indicate that random sampling errors could account for less than half the observed S.D.

In grading the results there will be classification errors. It is possible, however, that the method admits of a scan of a larger number of cells, and hence a more accurate impression of the mortality than might be obtained by counting cells in a limited field. In addition, the graded results allow only six intermediate end-points between whole dilutions, but random errors introduced by this grouping can be shown to be relatively small.

The estimates given for the S.D. for a single titration all assume that repeated estimates are made on the same day with the same slide and using the same cells and antiserum. It will be noted that the slides vary slightly one from another, that the results appear to vary from day to day, and that the time the slides are left standing affects the end-point.

It may be concluded, therefore, that in making any comparison involving differences of less than one dilution, experimental replication is essential, preferably on different days, and also that it is essential to standardize the amount of time slides are left standing before they are counted and to see that the slides themselves are constantly maintained fully siliconed.

Bias. From the results of this experiment no evidence was found that the micro-titration method is significantly biased, compared with doubling dilutions made in 0·5 ml. amounts with graduated pipettes. The observed results predict a bias of 0·2 dilution in 10 but do not exclude the possibility that the bias could vary between $-0{\cdot}25$ and $+0{\cdot}79$ dilution in 10. Thus whilst the limits of bias at each step are fairly small the possibility of some bias building up by the last well on the slide has not been excluded. The experiment could have been more sensitive in this respect if a wider range of master dilutions had been used.

Suitability of grading. It can be seen that CL50 estimated by grading is not biased relative to the counted results, and also that the S.D. of graded results is very similar to that of counted results; from this it is concluded that grading is an efficient

method of assessing a CL50. Because grading is very much quicker than counting it has been adopted for all subsequent cytolytic tests.

The micro-titration method described is simple, reliable and unbiased. It has made it possible to do much more extensive cytolytic tests with known accuracy than was possible with previous methods of testing. The ability to use stored cells in cytolytic tests (Spooner, 1965), together with this method of titration, means that a standard cell suspension can be available for the comparison of antisera on different days. Standardization of incubation and reading coupled with repetition of the test if small differences are to be detected, is necessary.

SUMMARY

A method of micro-titration for cytolytic antibodies is described. Standard drops of diluent are placed on a subdivided siliconed slide and dilutions are made by rotating a wire loop holding 1 drop of serum in each drop sequentially. One drop of a mixture of cells and complement is then added. The slide is incubated in a moist chamber for 90 min at 37° C. after which a small cover-slip is placed on each drop and the amount of cell lysis, judged by phase-contrast microscopy, is graded.

An analysis of variance of the method has been performed and the method has been found to be reliable, accurate and unbiased.

It allows accurate estimates of cytolytic activity in sera using cell concentrations of 10^4 to 10^5 cells per ml. Eight ten-dilution titrations can be carried out with 1 ml. of such a suspension.

We would like to thank Miss Lynn L. Flory and Dr David Franks for most valuable assistance in the grading and counting of the slides. Also we are grateful to Dr M. R. Daniel for supplying the C3H-T1 cells.

One of us (R.L.S.) was a Research Training Scholar of the Animal Health Trust whilst this work was done.

We would also like to thank Dr R. R. A. Coombs for his constant help and encouragement.

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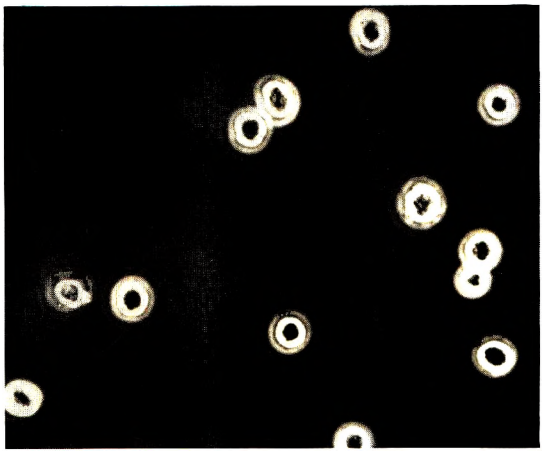
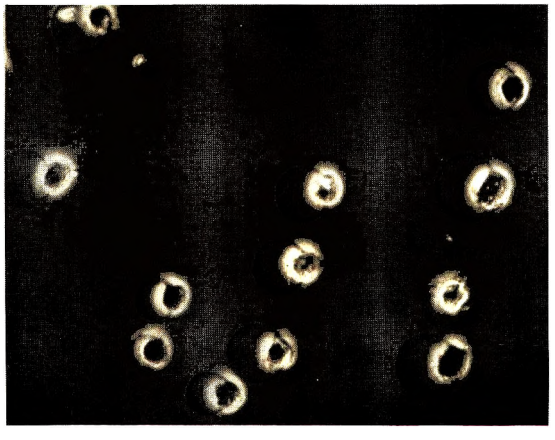


(*a*)



(*b*)

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EXPLANATION OF PLATES

PLATE 1

Subdivided slide with drops of diluent in place and loop as held during titration.

PLATE 2

(*a*) Preserved C3H–T1 cells; control suspension after 90 min. incubation with 1/24 rabbit complement.

(*b*) Preserved C3H–T1 cells; complete lysis in presence of 1/24 rabbit complement and 1/128 dilution of a rabbit antiserum prepared against BALB/c red cells.

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An association between adenoviruses isolated from simian tonsils and episodes of illness in captive monkeys

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During recent years considerable interest has been shown in the presence of 'latent' adenoviruses in human tonsils and adenoids (Rowe, Huebner, Gilmore, Parrott & Ward, 1953; Evans, 1958; Israel, 1962). These viruses have been shown to exist in the tonsil without multiplying to any extent and to become active only under certain conditions such as culture of the infected organs. Certain types, for example 1, 2 and 5, predominated in tonsils and adenoids of young people up to puberty.

The investigation now described was done primarily to see whether the simian tonsil had a similar reservoir of 'latent' virus. Techniques selective for simian adenoviruses, the group I simian viruses (SV) of Hull, Minner & Smith (1956), were used. No illness was observed during the period of investigation of the tonsils, but 2 months after it ended acute febrile respiratory manifestations occurred in two fresh batches of monkeys. These showed nasal discharge, sometimes accompanied by conjunctivitis and bleeding gums. They refused to eat and sat listlessly in their cages. Nine out of twenty monkeys died and at autopsy were found to have pneumonia. Nose and throat swabs were taken from all the monkeys and virus isolation in tissue culture was attempted.

Some simian adenoviruses had been isolated from monkey tonsils by Pille, Yermakova, Zuyeva & Nadaichik (1961). With one exception (Tyrrell, Buckland, Lancaster & Valentine, 1959) these viruses have not been shown to cause any disease in the monkey. They have been found in various organs including the pancreas, kidney, intestines and nervous tissue and occasionally in nose or throat swabs.

MATERIALS AND METHODS

For 4 months, from October 1962, tonsils were collected from rhesus monkeys at the time of kidney excision for the preparation of cell cultures. Tonsils were placed in 10 ml. of phosphate-buffered saline (Dulbecco & Vogt, 1954) at 37° C. containing 200 units of penicillin per ml. and 200 μg. of streptomycin per ml. This concentration of antibiotics was used in all solutions unless otherwise stated. After the tonsils had been in this fluid for approximately 2 hr., they were minced with sharp scissors in fresh fluid to give small fragments, large ones being treated separately and small ones in pairs. The fragments were washed thoroughly in

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Hanks's balanced salt solution containing 0·15 % $NaHCO_3$ and antibiotics. Each tissue suspension was then placed in a universal container with 2 ml. of warmed medium containing 1·8 ml. of bovine amniotic fluid, 0·1 ml. of 5 % lactalbumin hydrolysate and 0·1 ml. of horse serum with antibiotics. This medium had been used successfully by Pereira (1960) for the culture of 'latent' adenoviruses in human tonsils. The containers were tightly stoppered with rubber bungs, sloped at 5° in a 37° C. incubator and the medium changed at weekly intervals.

Table 1. *Simian adenovirus antiserum pools*

Pool I	Pool II	Pool III
SV 1, 11, 15, 17, 20	SV 23, 25, 27, 30, 31	SV 32, 33, 34, 36

Fluids removed from the cultures were frozen immediately and stored at − 70° C until tested. They were then thawed rapidly in a water bath at 37° C. and 0·5 ml. was inoculated into each of three primary rhesus monkey kidney cell culture tubes. These tubes, maintained on synthetic medium 199 containing 0·15 % $NaHCO_3$ and antibiotics, were incubated at 37° C. on a roller apparatus as long as possible, the maintenance medium being changed when necessary. Any tubes with cell sheets showing an advanced stage of the characteristic cytopathic effect of simian adenoviruses were frozen and thawed rapidly (using alcohol and dry ice as a freezing mixture). The harvested fluid obtained was inoculated into two parallel series of tubes, three of monkey kidney cells and three of the continuous HeLa cell line. If satisfactory evidence of growth was found after the second passage, virus pools were made in primary vervet kidney cells for further study.

Nose and throat swabs from sick monkeys were placed in 2 ml. of phosphate-buffered saline containing antibiotics as before and also 5 μg. per ml. Amphotericin B. After contact for 2–3 hr., the fluid from each swab was used to inoculate three rhesus monkey kidney culture tubes. Tubes showing an adenovirus-like cytopathic effect were further studied in the same way as those from the tonsil cultures.

To identify the viruses isolated, complement-fixation tests were done by the method of Bradstreet & Taylor (1962) to determine whether adenovirus group complement-fixing antigen was present. Each virus isolated was tested against the serum of a patient convalescent from an adenovirus infection and against a rabbit antiserum prepared with SV 15. Neutralization tests in two stages were done in primary rhesus monkey kidney culture tubes maintained as before. Each strain was first titrated against three pools of antiserum prepared as shown in Table 1 with the serum diluted to 1/32 in each virus-serum mixture. For final identification, arising from the results of the pool tests, titrations were done with individual antisera.

In each instance 0·2 ml. of the virus at 10-fold dilutions ranging from 10^{-1} to 10^{-7} was mixed with 0·2 ml. of the antiserum pool previously diluted in medium 199 to 1/32; 0·2 ml. of the virus-serum mixture was then inoculated into each of two monkey kidney culture tubes which were incubated at 37° C. on a roller apparatus. After 6 days the tubes were examined for cytopathic effect and evi-

dence of inhibition by the antiserum pools. Control tubes of virus titrations and uninfected cells were included in all tests.

RESULTS

The necessity of washing the excised tonsils carefully is made apparent by the fact that, despite our precautions, two pairs of tonsils had to be discarded because of bacterial or fungal contamination. One pair of tonsils stored overnight at 4° C. before washing yielded no virus, but as the tonsil cells were still viable it seemed

Table 2. *Isolation of adenovirus from tonsils of healthy monkeys*

Date tonsils removed	Virus present (weeks) 1	2	3	4	5	6	Strain no.	Identification of strains. Complement-fixation test: Human convalesent serum	Rabbit SV 15 antiserum
4. x. 62 (1)	–	–	–	–	–	–	.	.	.
4. x. 62 (2)	–	–	–	–	–	–	.	.	.
10 x. 62	–	–	–	–	–	–	.	.	.
16. x. 62	–	–	–	–	–	+	1	+	+
14. xi. 62	–	–	+	–	–	–	3	+	+
17. xi. 62	–	+	+	+	–	–	2, 4, 5	+ – +	+ – +
20. xi. 62 (1)	–	–	–	–	–	–	.	.	.
20. xi. 62 (2)	–	–	–	–	–	–	.	.	.
27. xi. 62	–	–	–	–	–	–	.	.	.
29. xi. 62	Yeast contaminant								
4. xii. 62	–	–	+	–	–	–	6	+	+
3. i. 63	–	–	–	–	–	–	.	.	.
17. i. 63	–	–	–	+	–	–	7	+	+
14. ii. 63	Bacterial contaminant								

Table 3. *Isolation of adenovirus from nose and throat swabs taken from sick monkeys*

Batch no.	Virus isolated	Strain no.	Identification of strains. Complement-fixation test: Human	Rabbit SV 15 antiserum	Comments
11/63	+	8	+	+	.
12/63	+	9	+	+	.
13/63	–	.	.	.	Died of pneumonia
14/63	–	.	.	.	.
15/63	–	.	.	.	Died of pneumonia
16/63	+	10	+	+	.
17/63	+	11	+	+	Killed for kidneys
18/63	–	.	.	.	.
19/63	+	12	+	+	Ill but recovered
M 93	+	13	+	+	Diarrhoea and pneumonia; seven of this batch died

Table 4. *Results of pool tests*

Strain no.	Pool I	Pool II	Pool III
1	Neut.	–	–
2	Neut.	–	–
3	Neut.	–	–
5	Neut.	–	–
6	–	–	Neut.
7	–	–	Neut.
8	Neut.	–	–
9	–	–	Neut.
10	–	–	Neut.
11	–	–	Neut.
12	–	–	Neut.
13	–	–	Neut.

Table 5. *Specific antiserum neutralization test*

Strain no.	Type	Strain no.	Type
1	SV 15	8	SV 17
2	SV 17	9	SV 32
3	SV 17	10	SV 32
5	SV 17	11	SV 32
6	SV 32	12	SV 32
7	SV 32	13	SV 32

unlikely that release of any virus present in the cells had been affected by the treatment. The earliest release of virus appeared after 21 days incubation in one instance but usually 28 days or more elapsed. The first subculture of released virus was slow, taking 2 weeks or more before a cytopathic effect appeared, but later passages produced cell degeneration in about 5 or 6 days.

As can be seen in Table 2, seven strains obtained from the tonsil cultures all showed the group complement-fixing antigen characteristic of adenoviruses. One negative result on one occasion was preceded and followed by a virus isolation from tissue from the same tonsil. Both strains were identical and proved to be SV 17 virus. Of the other viruses isolated, one was SV 17, two were SV 32 and the remaining one was SV 15 (Tables 4 and 5).

From the nose and throat swabs six viruses were obtained (Table 3); five, from a group of monkeys received on the same date, were SV 32; one from a second group of monkeys received on another date proved to be SV 17 (Tables 4 and 5).

There was complete failure to isolate any viruses in HeLa cells.

DISCUSSION AND CONCLUSIONS

The presence of a 'latent' viral flora in the tonsils of rhesus monkeys indicates a further similarity between the simian and human adenoviruses. Both groups require a long period of incubation before release of viable particles from the tonsillar material. A further period of incubation is necessary for simian adenoviruses before cytopathic effects appear. The amount of virus liberated from the tonsils

was very small. Incubation of inoculated monkey kidney cultures for several weeks was needed before negative results could be accepted. Parallel use of HeLa cell cultures for testing the infectivity of tonsillar material from monkeys was disappointing but, unlike human adenoviruses, most simian adenoviruses failed to multiply in these cells even after subculture.

An interesting feature was the isolation of the same adenovirus serotype from the tonsils of healthy monkeys and from a monkey with acute respiratory illness. SV 17 had been previously isolated from patas monkeys with conjunctivitis and rhinorrhoea by Tyrrell *et al.* (1959) and was here implicated in one outbreak in rhesus monkeys. SV 32 was isolated from five members of one batch of monkeys suffering from a similar illness and would appear to be associated with the same type of disease. SV 15, although existing as a latent virus, was not isolated from sick monkeys.

SUMMARY

A study was made of the 'latent' viral flora of simian tonsils. This was followed by isolation of viruses from two batches of sick monkeys whose illness is described. Simian viruses 15, 17 and 32 were isolated from nose and throat swabs obtained from the sick monkeys. A connexion between human and simian adenoviruses is suggested.

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Toxoplasmosis and Tristan da Cunha

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In October 1961 the volcano on Tristan da Cunha erupted driving the Islanders from Tristan. They eventually arrived in Britain thus providing interesting opportunities to study this, until recently, isolated community. Thanks to the work of Drs C. K. Thacker (at that time at the Hospital for Tropical Diseases) and Dr C. E. D. Taylor (of the Central Public Health Laboratory, Colindale) and others who helped in the collection of sera and clinical assessment of the Tristan population (Black, Thacker, Lewis & Thould, 1963), a survey of toxoplasma antibody was carried out on them. Reference has been made to this (Thacker, 1963), but it was felt that a more detailed analysis of the results would be of interest.

METHODS

Of the 267 Islanders who landed in Britain, the sera from 220 were tested with the dye test (Sabin & Feldman, 1948). The method of performing this test is similar to that previously described (Fleck & Payne, 1963).

The results of the dye tests were classified according to the age and sex of the Islanders and compared with a survey made in South Wales (Fleck, 1963).

A full clinical examination of the Islanders, including examination of the ocular fundi, was carried out. The intelligence, educational attainment and personalities of the school children were also tested (Keir, 1965).

RESULTS

Tables 1 (*a*) and (*b*) show the results of dye tests made on sera from blood donors and children admitted to hospital with afebrile illnesses in South Wales, classified according to age and sex. There is little difference in antibody incidence between the sexes, but there is a difference between those under 20 years of age and those over 20. The percentage positive at 1/16 or more is 25·8 %.

Tables 2 (*a*) and (*b*) show the results of dye tests made on sera from Tristan da Cunhans. Again there is little difference in incidence between the sexes but there is a difference between those under 10 years of age and those over 10. The Tristan community has a significantly higher overall incidence of toxoplasma antibody, when compared with people living in Wales. The percentage positive at 1/16 or more is 80 %. The only sera showing a dye test titre of 1/1024 were from children under 10. Although the figures are too small for any definite conclusions Table 3 shows that children's sera were less likely to be positive in the dye test, but when

Table 1*a*. *Dye tests titres of males in South Wales*

Dye test titres	Age in years 0–10	11–20	21–30	31–40	41–50	51–60	Totals
1/256–1/512	0	1	1	0	0	0	2
1/16–1/128	2	4	4	5	5	6	26
< 1/16	18	15	15	15	15	14	92
Totals positive at 1/16 or more	2/20	5/20	5/20	5/20	5/20	6/20	= 28/120 = 23 %

Table 1*b*. *Dye test titres of females in South Wales*

Dye test titres	Age in years 0–10	11–20	21–30	31–40	41–50	51–60	Totals
1/256–1/512	0	0	0	0	0	0	0
1/16–1/128	1	3	5	9	9	7	34
< 1/16	19	17	15	11	11	13	86
Totals positive at 1/16 or more	1/20	3/20	5/20	9/20	9/20	7/20	= 34/120 = 28 %

Total male and female positive at 1/16 or more = 62/240 = 25·8 %.

Table 2*a*. *Dye test titres of males on Tristan*

Dye test titres	Age in years 0–10	11–20	21–30	31–40	41–50	51–60	60+	Totals
1/1024	2	0	0	0	0	0	0	2
1/256–1/512	0	2	0	2	0	1	0	5
1/16–1/128	2	14	16	13	10	13	12	80
< 1/16	5	6	2	6	3	1	3	26
Totals positive at 1/16 or more	4/9	16/22	16/18	15/21	10/13	14/15	12/15	= 87/113 = 77 %

Table 2*b*. *Dye test titres of females on Tristan*

Dye test titres	Age in years 0–10	11–20	21–30	31–40	41–50	51–60	60+	Totals
1/1024	1	0	0	0	0	0	0	1
1/256–1/512	3	2	0	0	1	0	0	6
1/16–1/128	4	12	19	18	11	6	12	82
< 1/16	6	1	4	2	2	1	2	18
Totals positive at 1/16 or more	8/14	14/15	19/23	18/20	12/14	6/7	12/14	= 89/107 = 83 %

Total male and female positive at 1/16 or more = 176/220 = 80 %.

they were positive, they had higher titres than their elders. Unfortunately sera from children under 3 years were not collected, it is therefore not possible to say how much infection is congenital and how much acquired.

Of the twelve members of the community showing posterior segment lesions of the eye other than retinitis pigmentosa Mr D. P. Choyce examined seven. He considered three were clinical cases of toxoplasmic retinitis and two others were

probable cases of this disease. The incidence of this disease in the whole community is therefore at least 1 %. This is very much higher (probably tenfold) than the incidence in Britain or in U.S.A. (Prof. E. S. Perkins, personal communication).

Dr G. Keir (1965), in her survey of the Tristan children, found one possible and four definite mentally defective children. Of these two were negative at 1/16 in the dye test, and one was positive at 1/32. The other two were not tested. One of the mentally defective children, with a dye test negative at 1/16, was a case of arrested hydrocephalus. Toxoplasmosis as a cause of mental deficiency on the Island was unconvincing.

Table 3*a*. *Dye test titres of males in Tristan children*

Dye test titres	Age in years			
	3–5	6–8	9–11	Totals
1/1024	1	1	0	2
1/256–1/512	0	0	0	0
1/16–1/128	1	1	0	2
< 1/16	3	1	1	5
Totals positive at 1/16 or more	2/5	2/3	0/1	= 4/9 = 44·5 %

Table 3*b*. *Dye test titres of females in Tristan children*

Dye test titres	Age in years			
	3–5	6–8	9–11	Totals
1/1024	1	0	0	1
1/256–1/512	1	1	1	3
1/16–1/128	3	0	2	5
< 1/16	0	4	1	5
Totals positive at 1/16 or more	5/5	1/5	3/4	= 9/14 = 64·5 %

Total male and female positive at 1/16 or more = 13/23 = 56·7 %

Many authors have found the incidence of abortions to be increased among women infected with *Toxoplasma gondii*. This problem has been well reviewed (Remington, 1963). Information supplied by Dr E. J. S. Wooley, Medical Officer on the Island 1942–44, showed that two abortions occurred. During the same period there were sixteen live births. Drs M. Samuels, L. Schrire and S. Gooch were not asked to attend any cases of abortion while they were on the Island.

To avoid the possibility that toxoplasma infection occurred after the Tristan community moved to Britain, Dr J. H. S. Gear of the South African Institute for Medical Research sent 55 sera collected from the Islanders in 1961 before they left the Island. All except seven showed the same dye test titres as those obtained in Britain. Of the seven sera five differed by only a twofold dilution, one differed by a fourfold dilution and in one case, a boy aged 4, the titre rose from less than 1/16 to 1/1024. Nothing of note was found on clinical examination of this boy on reaching Britain. Only this last case was considered to show a significant rise in

titre, since, because of the technical difficulties of the dye test, Sabin, Feldman & Jacobs (1952) consider only an eightfold change in titre to be significant. The high incidence of antibody was therefore present in the island population while still on Tristan.

DISCUSSION

Numerous serological surveys in human populations of five continents have shown the widespread incidence of toxoplasma antibody. Usually the sex distribution has been equal and there is often an increasing prevalence with increasing age. The pattern is however, still too complicated to give a clear indication of the mode of transmission of the organism.

Feldman & Miller (1956) have suggested that the infection is less prevalent in temperate and more prevalent in tropical areas in the world. Certainly the highest incidence is in the wet Central American countries. Tristan da Cunha, although wet, is not tropical and is devoid of the forests common in Central America. Easter Island, in contrast, is dry and tropical and devoid of forests yet shows an incidence of 92% positive dye tests in its population (Morales *et al.* 1961).

The high incidence of toxoplasma antibody suggests a high prevalence of infection, but the organism has yet to be isolated from the Islanders. It is hoped that sera from various animals on the Island may be tested, and that attempts at isolation of the organism will be made.

SUMMARY

Sera from most of the Tristan da Cunha community were tested for toxoplasma antibody. In a comparison with sera from 'normal' inhabitants of South Wales there was a significantly higher incidence of dye tests positive at 1/16 or more in the Tristan community at all age groups. The incidence of retinitis was also found to be much higher than in Britain. It appears unlikely that toxoplasmosis caused any cases of mental defect or abortion on the Island.

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Observations on photodynamic inactivation of vaccinia virus and its effect on immunogenicity*

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INTRODUCTION

Raab (1900) first showed that living organisms could be sensitized to visible light in the presence of certain dyes. This dye-sensitized photodynamic action, or photoinactivation, was extensively discussed by Blum (1941). Perdrau & Todd (1933*a*) and Herzberg (1933) demonstrated dye-sensitized photoinactivation of several animal viruses. There are few studies on the antigenicity of photoinactivated viruses. Perdrau & Todd (1933*b*) and Dempsey & Mayer (1934) used photoinactivation for the experimental production of effective canine distemper vaccine. Burnet, Keogh & Lush (1937) showed that bacteriophages retained antigenicity after photodynamic inactivation with methylene blue. Galloway (1934) produced good immunity in rabbits with photoinactivated fixed rabies virus. After the start of the present work, Wallis, Sakurada & Melnick (1963) showed that both photoinactivated and formalin-inactivated influenza vaccines produced good immunity in mice. We have investigated some of the factors influencing the photoinactivation of the Lister Institute strain of vaccinia virus and the immunity of animals inoculated with photoinactivated material.

MATERIALS AND METHODS

Virus

For most of the experiments the Lister Institute strain of vaccinia virus was used. The virus was extracted from sheep dermal pulp in 0·004 M-McIlvaine buffer pH 7·2, and partially purified by treatment with 'Arcton 113' (trifluorotrichloroethane, I.C.I.), extraction with diethyl ether at 0° C., and differential centrifugation. The material usually had titres of 10^7–10^8 plaque-forming units per ml. and a total N_2 content of $< 200\ \mu g$/ml. The virus was stored in small volumes at −70° C. and was rapidly thawed for use. Unused material was discarded. For some experiments the mouse neurotropic strain WR was used. This was propagated in chorioallantoic membranes (CAM) from dried mouse brain stocks, purified by a modification of the method of Joklik (1962*a*) and stored in small volumes at −70° C.

Virus assay

Virus was usually assayed by plaque counts in chick cell monolayer cultures in Leighton tubes without cover-slips or agar overlay. Some titrations were made

* Much of this work is incorporated in a Thesis to be presented to The University of London by G. S. T. for the degree of Ph.D.

by pock counts in CAM or by cytopathic end-point in rolled-tube cultures of monkey kidney cells (*Cercopithecus aethiops*). Titres are expressed as plaque-forming units (p.f.u.)/ml., or pock-forming units (po.f.u.)/ml., or 50 % tissue culture infective doses (TCD 50)/ml. The chorioallantoic membrane and monkey kidney cells are sensitive indicators of vaccinia virus infectivity; both were used for the detection of very small amounts of virus.

Buffers

McIlvaine's buffer mixtures were used in most experiments. Virus dilutions for assay were made in diluted McIlvaine buffer at pH 7·2. Sørenson's phosphate buffer, Clark & Lubs borate + KCl + NaOH buffer and 0·01 M-tris buffer (1,3-propanediol,2-amino-2-hydroxymethyl) were used in some experiments. All pH values were determined with a Model 23A pH meter (Electronic Industries Ltd.).

Methylene blue

Tetramethylthionine chloride (Methylene blue, May and Baker Ltd., 96 % pure) was kept at room temperature as a 3×10^{-3}M aqueous stock solution. It was diluted for use to a concentration of 3×10^{-5}M, unless otherwise stated.

Illumination

The illuminant for photoinactivation was a Philips Photolita bulb connected to the mains through a variable resistance. Light was measured at the object level with a model S85 Weston photometer graduated in foot-candles (ft.-c.). All necessary manipulations were done under a safelight consisting of a domestic 40 W. bulb immersed in methylene blue solution of sufficient strength to give no meter reading at zero distance.

Photoinactivation

Reaction mixtures of methylene blue and suitably diluted virus in buffer were exposed in 9 cm. diameter glass Petri dishes with lids. The dishes stood upon a reflecting surface of aluminium foil. Ten ml. volumes gave a fluid depth of 1·5–1·7 mm. Occasionally larger volumes were used, giving depths up to 3 mm. After exposure for the required time, 0·5 ml. samples were removed and titrated. The removal of up to 3 ml. decreased fluid depth by $< 0{\cdot}5$ mm. and increased light transmission by $< 2\%$. In most experiments stock virus was diluted 100-fold giving an initial virus titre of 10^5–10^6 p.f.u./ml. and a total N_2 content of less than 2 μg./ml. Mixtures of diluted virus + methylene blue were limpid. In all experiments controls of unilluminated reaction mixtures (dark controls) and illuminated samples with dye omitted (light controls) were included.

Inhibition of photoinactivation

Inhibition was tested by including in the reaction mixtures suitable amounts of several substances.

Deoxyribose nucleic acid (*DNA*) and *ribose nucleic acid* (*RNA*) were commercial samples (British Drug Houses laboratory reagents) derived respectively from calf thymus and yeast.

Bases. Adenosine, thymidine, cytosine and guanylic acid (B.D.H.).

Proteins and derivatives tested were bovine crystalline albumin (BCA) Fraction V (Armour); a highly purified sample of human albumin kindly supplied by Mr L. Vallet of the Blood Products Laboratory at the Lister Institute; histidine monohydrochloride (L. Light and Co.); and calf serum (Oxoid Ltd.).

Enzymes

Peroxidase consisting of crude horse-radish extract, and a highly purified sample containing 102 units/mg., kindly given by Seravac Laboratories Ltd., were incorporated into the reaction mixtures in suitable amounts.

Catalase. Two samples were used, one a crude beef liver extract (L. Light and Co.); the other a more highly purified preparation containing 3000 units/mg. (Nutritional Biochemicals Corporation).

Ion exchange resin

Dowex A.G. 50 W.— × 8 200–400-mesh H form analytical grade (Bio Rad Laboratories) was converted to the Na form and equilibrated to the required pH by repeatedly washing with the appropriate buffer. Ten ml. volumes of virus + dye were shaken with 1·0 ml. volumes of packed resin in 50 ml. centrifuge tubes. This treatment rapidly removed visible dye without decreasing the virus titre of the sample.

Virus nucleic acid

Photoinactivated and untreated samples of vaccinia virus were concentrated by ultracentrifugation. The DNA was extracted by the method of Joklik (1962*b*), and estimated in a Unicam SP700 recording spectrophotometer, against a solution of thymus DNA 10 μg./ml. as standard.

Reactivation experiments

The methods described by Joklik, Woodroofe, Holmes & Fenner (1960) were closely followed. The WR strain of vaccinia virus has a large distinctive pock and is mouse neurovirulent. The Lister Institute strain of vaccinia virus produces compact well demarcated pocks and is *not* virulent for mice. These characters were used as easily distinguishable markers in reactivation experiments. WR had an initial titre of $5{\cdot}6 \times 10^8$ po.f.u./ml., and 5×10^7 LD50/ml. for mice by the intracerebral route. The Lister Institute strain had an initial titre of 10^9 po.f.u./ml. Samples of the viruses were thawed from $-70°$ C., given brief ultrasonic treatment to disperse aggregates, then either heated in sealed ampoules at 60° C. for 12 min., or photoinactivated, either minimally (7 min. at 50 ft.-c.) or for 20 min. at 50 ft.-c. After inactivation, excess dye was removed with ion exchange resin. Some samples were inactivated by brief exposure to ultra-violet light. The inactivated material was

used immediately. Its inactivity was tested in mice, CAM, or tissue culture. Reactivation experiments were done with combinations of undiluted variously inactivated material and suitable doses of live Lister virus. They were controlled with a mixture of undiluted heated WR (reactivable particles) and live Lister virus as reactivating agent. For the CAM, 30–50 po.f.u./dose of live Lister virus were used; for mice, the reactivating agent was increased to 10^4 po.f.u./dose. Reactivation in the CAM with the control material was readily observable. The identity of any doubtful pocks was checked by subinoculation to CAM or mice. Reactivation of control material in the mouse brain was readily detected by subinoculation to CAM and mice. Direct detection was not possible, probably because of interference by live Lister virus with the multiplication of WR in the mouse brain (Joklik *et al.* 1960). Brains were harvested for subculture on the fifth day after inoculation, a period adequate for the reproduction of infectious WR inoculated alone. In all experiments controls of the initial live virus of both strains were titrated. Ten 20 g. mice were used per group, with an intracerebral inoculum of 0·02 ml. In the CAM 6–8 embryos were used per group and the dose was 0·1 ml.

Preparation of experimental vaccines

Vaccinia virus was extracted from the dermal pulp of sheep, and bacterial contamination eliminated by treatment with phenol (0·6 %, w/v). The virus was partially purified by treatment with Arcton 113 followed by differential centrifugation.

Vaccines A–C. Ten ml. volumes of vaccinia virus suspension with a titre of 10^7 TCD50/ml. were mixed with methylene blue in a concentration of 3×10^{-5}M. They were then exposed in 6 in. Petri dishes to a light intensity of 50 ft.-c. for 10, 20 and 30 min. respectively. The methylene blue was not removed. The vaccines, labelled A, B and C, were stored at 4° C in bottles completely covered with aluminium foil to exclude light. Each vaccine was tested for residual infectivity by inoculating ten rolled-tube cultures of monkey kidney cells. All tubes were examined for cytopathic change after 1 week. With vaccine A 2/9 tubes showed cytopathic change. The culture fluid from these tubes was subinoculated, when 20/20 tubes were clearly positive. Though no cytopathic change was seen in the tubes inoculated with vaccines B and C, 20 % of the pooled fluids of each group was subinoculated, with negative results. Unlike vaccines B and C, vaccine A, therefore, was not completely inactivated.

Vaccines D–F. These vaccines with initial titre of 5×10^8 po.f.u./ml. were inactivated the same way as vaccines A–C, except that exposures were for 20, 40 and 60 min. After illumination, the suspensions were treated with ion exchange resin and then centrifuged in an International Model PR-2 at 2000 rev./min. for 10 min. The dye-free supernatants were pipetted into separate bottles and stored at 4° C. Vaccine D was inoculated into 20 rolled-tube cultures of monkey kidney cells which were incubated for a week at 37° C., when 20 % of the pooled culture fluid was subinoculated into fresh cultures and again incubated for a week; no cytopathic change was seen. Since no residual infectivity was demonstrable in vaccine D it could be safely assumed that vaccines E and F were similarly free of infectivity.

Immunization of rabbits

Vaccines A–C were each tested in three rabbits. The animals were bled and given an intramuscular injection of 1·0 ml. of vaccine. Fourteen days later they were bled again and given a second intramuscular injection of 1·0 ml.; 11 days later they were bled for the third time.

Vaccines D–F were each tested in five rabbits. The animals were bled and given an intramuscular injection of 1·0 ml. of vaccine. Nineteen days later they were bled again and given a second intramuscular injection of 1·0 ml. Thirteen days after the second dose they were bled for the third time. Serum samples separated from all the bleedings were inactivated at 56° C. for 30 min. and stored at 4° C. until neutralizing antibody was assayed.

Antibody assay

Neutralizing antibody was assayed by inhibition of plaque formation in monolayers of chick embryo cells. Dilutions of serum + standard challenge virus calculated to give 80–100 plaques per tube were incubated at 37° C. for 2 hr. in 10% skimmed milk in dilute McIlvaine buffer pH 7·2 (Boulter, 1957). The mixtures were chilled at the end of the incubation period and inoculated each into four cultures of chick embryo cells as for infectivity assays. A standard preparation of antivaccinial γ globulin made from pooled sera of hyperimmunized sheep was included in each group of assays. The standard was assigned an arbitrary potency of 100 units/ml. Dose-response curves of unknowns and standard were constructed in the usual way and the neutralizing potencies of immune rabbit sera were calculated in units.

Dermal challenge of immunized rabbits

Six weeks after the second dose of vaccine all the animals immunized with vaccines D–F were challenged by the application to the scarified skin of dilutions of a known potent smallpox vaccine. The animals were inspected daily and the extent of the lesions noted. The state of the lesions on the sixth day was taken as indicating the titre attained, as in potency assays of smallpox vaccine by rabbit dermal scarification. Observations, however, were continued until the eighth day, when any modification of lesions present (e.g. the presence of an eschar) were unequivocal.

RESULTS

Photoinactivation

A typical inactivation curve is shown in Fig. 1 where time was varied and intensity of illumination kept constant. When intensity was varied with time constant, similar curves were obtained. Data from such experiments plotted as logarithms of surviving fractions gave points falling closely about the same straight line (Fig. 2). The results indicate that: (1) the survival curve is exponential; (2) the rate of inactivation is determined by the intensity of illumination, and dose (intensity × time) controls the degree of inactivation; (3) the reaction is probably of the 'single hit' type described by Lea (1955); and (4) at 50 ft.-c. very little virus

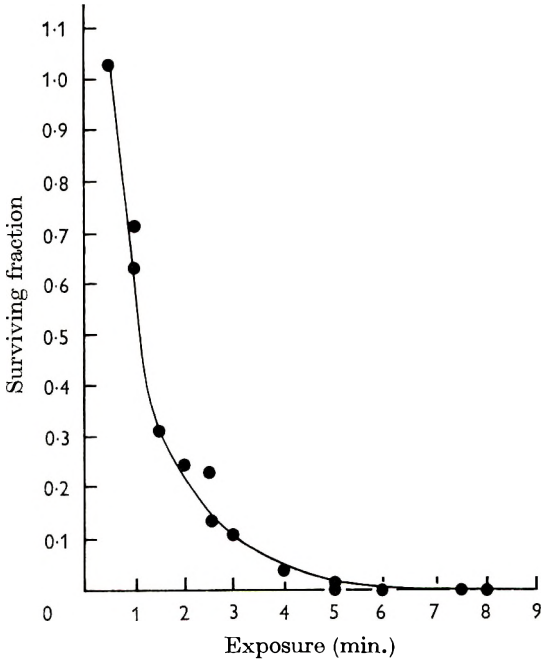

Fig. 1. Photoinactivation of vaccinia virus. Light intensity 50 ft.-c. $5{\cdot}5 \times 10^5$ p.f.u./ml. virus in pH 7·0 buffer containing 3×10^{-5} M-methylene blue.

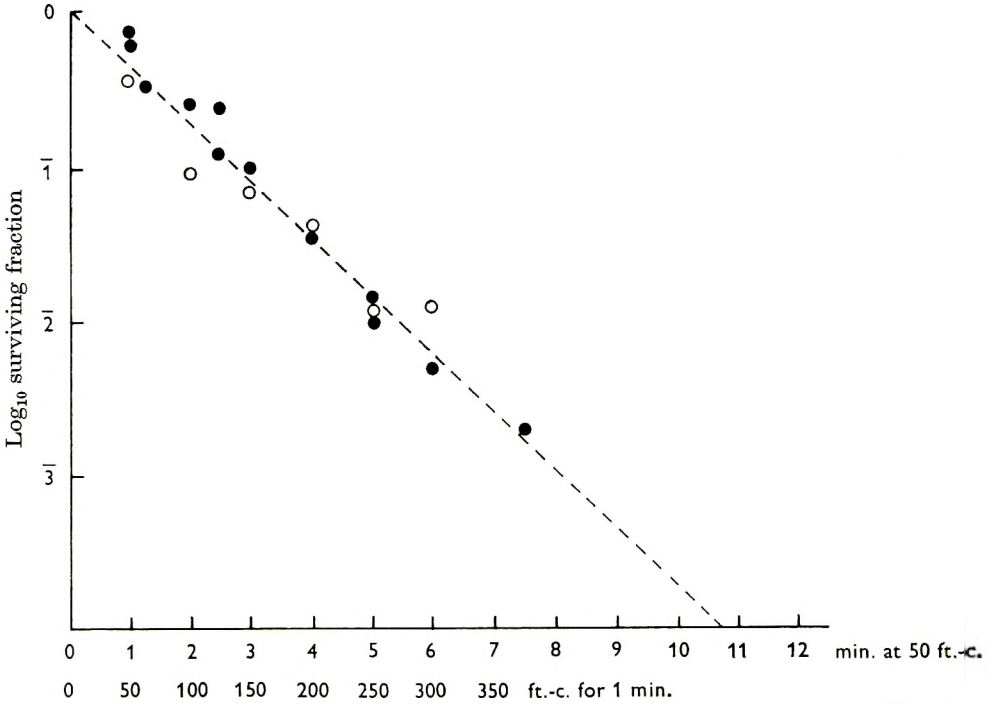

Fig. 2. Photinactivation of vaccinia virus. Composite mean destruction curve. ● = 50 ft.-c. for various times. ○ = 1 min. at various intensities. $5{\cdot}0 \times 10^5$ p.f.u./ml. virus in pH 7·0 buffer containing 3×10^{-5} M-methylene blue.

survived 10 min. exposure and none was detectable after 15 min. 'Blind' passage of undiluted photoinactivated material indicated that no multiplicity reactivation occurred. Nor did subsequent removal of methylene blue by dialysis, centrifugation or ion exchange resin reactivate already inactivated material.

Influence of methylene blue concentration

Inactivation was complete at approximately 10^{-5}M-methylene blue (Fig. 3). The standard concentration in most experiments was 3×10^{-5}M. Very high concentrations are said to mask photoinactivation in the deeper layers of a reaction mixture.

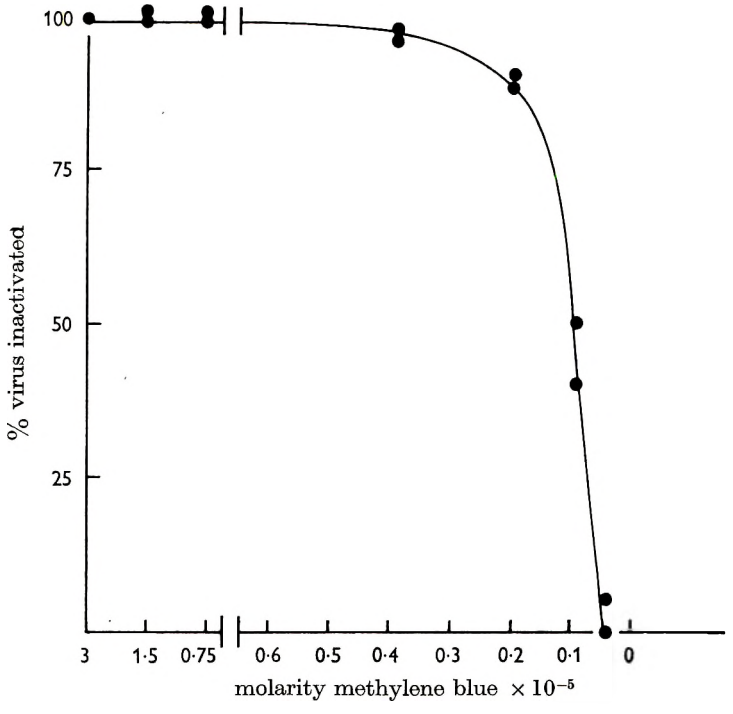

Fig. 3. Influence of methylene blue concentration on photoinactivation of vaccinia virus. 6×10^3 p.f.u./ml. virus in pH 7·0 buffer + various concentrations of methylene blue. Samples at each concentration exposed 10 min. at 50 ft.-c.

Influence of temperature

Most investigators have found photoinactivation to be virtually independent of temperature. Since low temperature might decrease reactivity we illuminated reaction mixtures at temperatures down to $-20°$ C. (Table 1). Even at these temperatures reaction rates were not significantly affected. The presence of glycerol, used as an antifreeze agent, did however reduce the rates by approximately tenfold over the whole temperature range investigated, a phenomenon which is being examined further.

Influence of hydrogen-ion concentration

The photoinactivation of bacteriophages and some animal viruses depends on hydrogen-ion concentration (Welsh & Adams, 1954; Yamamoto, 1958; Wallis &

Melnick, 1963). Vaccinia is no exception; the rate increases directly with pH (Fig. 4). The pH affects the adsorption of dye to virus. Reaction mixtures were made in McIlvaine buffer at different pH values well within the stability limits of the virus (pH 5·0–9·0). After standing them at room temperature for 20 min. the mixtures were either centrifuged at 25,000 *g* for 30 min. and washed free of dye,

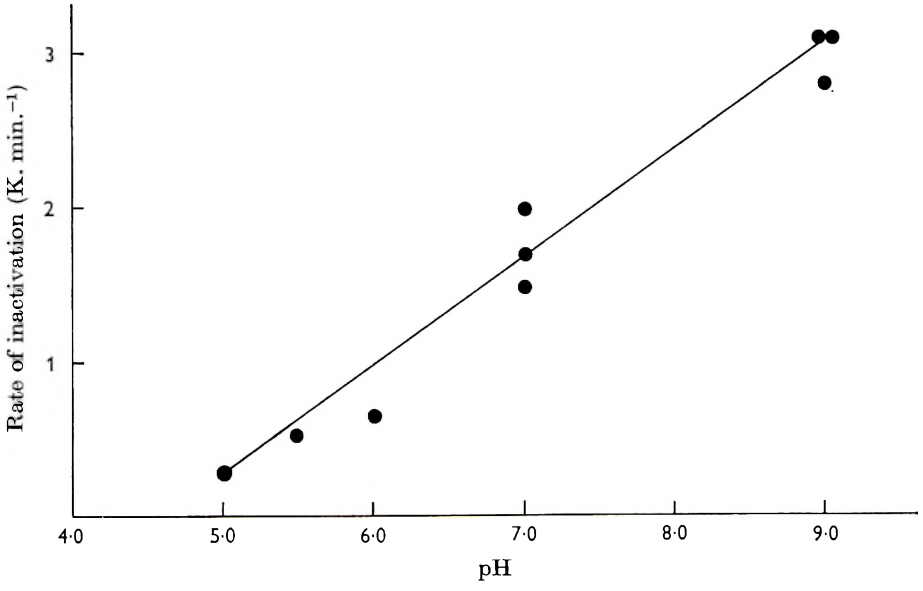

Fig. 4. Influence of pH on rate of photoinactivation of vaccinia virus. 10^5 p.f.u./ml. virus + 3×10^{-5} M-methylene blue, illuminated 50 ft.-c. for various times at different H ion concentrations.

Table 1. *The influence of temperature on photoinactivation of vaccinia virus in* pH 7 *buffer by* 50 *ft.-c.*

Temp. of Photoinactivation (°C.)	Velocity constant (K min.$^{-1}$)	
	Buffer alone	Buffer + 40 % glycerol
−20	—	0·13
−10	—	0·11
0	1·46	0·11
20	1·42	0·12
37	1·55	—

or treated with ion-exchange resin equilibrated at the required pH values, all these manipulations being done in the safelight. On subsequent exposure to 50 ft.-c. for 30 min. photosensitivity was found to increase directly with pH (Fig. 5).

Methylene blue + virus mixtures were made at pH 9·0 and allowed to stand at room temperature. Free dye was removed by either ion-exchange resin or high-speed centrifugation or both and the virus resuspended in buffer at pH 5·0 and 7·0. During exposure to 50 ft.-c. the photoinactivation rates were slower than those of samples maintained at pH 9·0, but photosensitivity was not completely

abolished. Similar results were obtained by varying the test as follows. Methylene blue + virus mixtures were made at pH 9·0; free dye was removed with ion exchanger and virus sedimented by high-speed centrifugation. The sedimented virus together with suitable controls was resuspended in buffers at pH 5·0 and 6·0, shaken and treated again with ion-exchange resin. High-speed centrifugation was repeated, all the samples were resuspended at pH 9·0 and exposed to 50 ft.-c.

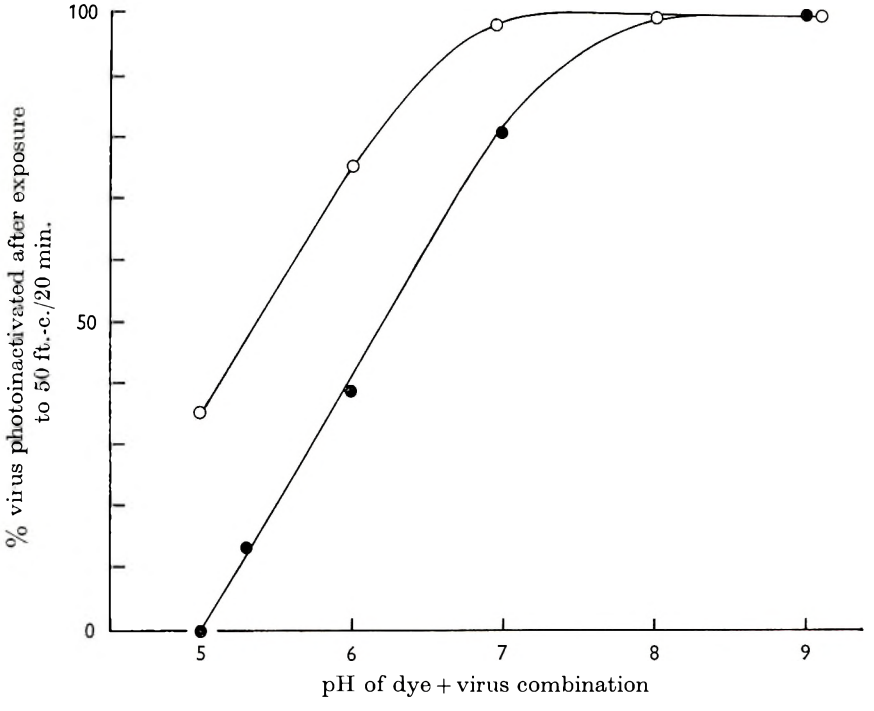

Fig. 5. Influence of pH on dye + virus combination. 3×10^{-5} M-methylene blue + 10^5 p.f.u./ml. virus allowed to combine at room temperature in dark at the pH values indicated. ○ = free dye removed with cationic exchange resin. ● = free dye removed by centrifugation. Colourless samples then exposed at 50 ft.-c. for 20 min.

The results indicate that the slow inactivation rate at low pH values is due to minimal combination of dye with virus; the attachment of dye to virus at low pH values is reversible; reversibility diminishes with increasing pH. No differences in inactivation rates were observed when reaction mixtures were prepared at the same pH in the three different buffers described under Methods.

Preincubation of methylene blue + virus mixtures

Mixtures of methylene blue + virus were made in pH 9·0 tris buffer and incubated at 0° C. or 37° C. for 1 hr. before removal of excess dye and exposure to 50 ft.-c. No significant differences in inactivation rates were observed. Since the temperature coefficient of methylene blue + vaccinia virus combination appeared to be negligibly small, mixtures were held at 0° C. for 10, 30, 60 min. and 24 hr. before removal of excess dye and exposure to 50 ft.-c. Again there were no differences in inactivation rates. Inactivation was always fastest in the presence of excess dye. At pH 9·0 removal of excess dye by ion exchange resin decreased the rate tenfold.

Influence of added nucleic acid and bases

Photodynamic action upon DNA and its guanine derivatives was reported by Simon & van Vunakis (1962, 1964). Since viral DNA is thought to be involved in photoinactivation, indirect evidence of DNA participation was sought by the addition of DNA and RNA solutions to vaccinia + methylene blue reaction mixtures. Both DNA and RNA inhibited photoinactivation, DNA more strongly than RNA (Fig. 6). Depolymerized samples of nucleic acid did not inhibit, nor did

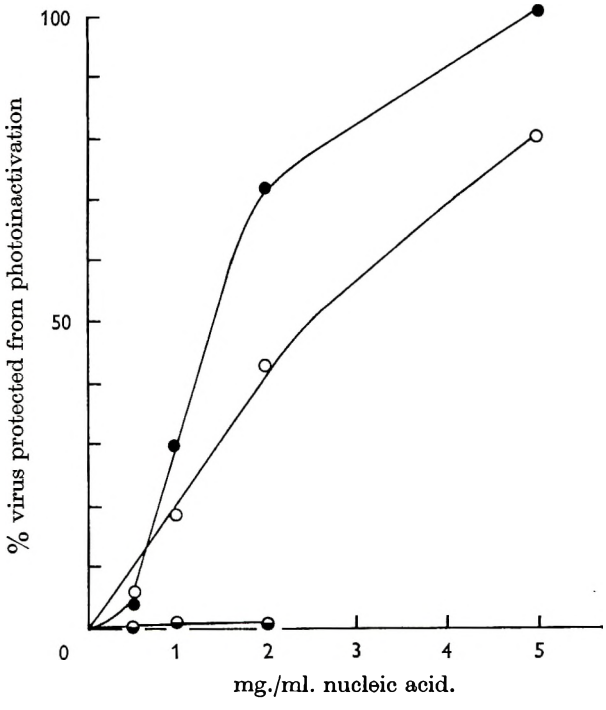

Fig. 6. Inhibition of photoinactivation by nucleic acids. ● = DNA; ○ = RNA; ◒ = depolymerized RNA.

the same concentrations of the individual bases or pentoses. Methylene blue has a peak absorption wavelength at approximately 700 mμ. Addition of nucleic acid to methylene blue reduced the total absorption and shifted the absorption maximum towards the red. The optical changes, however, occurred at DNA concentrations much lower than those with any measurable effect on photoinactivation. Methylene blue remained effective, and light at the altered wavelengths was still lethal in our reaction mixtures in the absence of added DNA. Experiments similar to those described for determining the effect of pH indicated that DNA too exerted its inhibitory effect by preventing dye–virus combination.

Influence of added protein

Since extraneous proteins often contaminate virus suspensions we incorporated into reaction mixtures bovine crystalline albumin, human serum albumin and whole calf serum. No inhibition of photoinactivation was observed when protein

was added in the same concentration as DNA; amounts in excess of 3% were required before the reaction rate was halved. Histidine (5 mg./ml.) did not prevent photoinactivation. The inhibitory effects of nucleic acid and large amounts of protein were not dependent on pH.

Influence of peroxidases

To test the possibility that photoinactivation might be mediated by transient peroxides formed during illumination of methylene blue + virus mixtures, we incorporated different samples of peroxidase and catalase into our reaction mixtures with suitable controls. Catalase up to concentrations of 6000 units/ml. and peroxidase up to 400 units/ml. were without effect.

Effect on viral nucleic acid

In attempts to determine whether photoinactivation is due to attack on viral DNA, nucleic acid was extracted from the virus and examined spectrophotometrically. In a typical experiment two 20 ml. portions of virus purified from rabbit dermal pulp were used. To one sample methylene blue was added. Both were exposed at 50 ft.-c. for 1 hr. The sample without dye had a titre of 3×10^8 p.f.u./ml.; there was no live virus in the photoinactivated sample. Nucleic acid was extracted from both samples by the method of Joklik (1962*b*). The photoinactivated sample yielded 100 μg./ml. and the control 67 μg./ml. The photoinactivated sample was diluted to the same DNA content as the control and both were scanned in the recording spectrophotometer. There were no spectral differences between the samples; nor were there any changes referable to a particular base when solutions of guanylic acid, adenosine, cytosine and thymine 10 μg./ml. were scanned and recorded on the same plot.

Reactivation experiments

Cross-reactivation among suitably inactivated pox viruses is well established (Joklik *et al.* 1960; Fenner, 1962). The reactivation process requires that the

Table 2. *The influence of photoinactivation on reactivation of infectivity*

	Reactivating agent		
Potential reactivable material	Live Lister	Photo-inactivated Lister	Ultra-violet inactivated Lister
Heated WR	+	0	0*
Photoinactivated WR	0	nd	nd

+ = reactivation; 0 = no reactivation; nd = not done.
(Results summarized from four experiments in mice and CAM)
* In one experiment in CAM reactivation may have occurred, but could not be confirmed.

participating virus particles should have either protein or nucleic acid intact. We utilized this requirement in an attempt to determine the site of action of photoinactivation. The results are summarized in Table 2. In the control system of

heated WR and live Lister virus, reactivation readily occurred, both in mouse brain and CAM. Photoinactivated virus was not reactivable by live virus, nor was it capable of reactivating heated virus under similar conditions.

Immunogenicity of photoinactivated vaccinia virus

Vaccines A–C

Despite the presence of a small amount of residual infectivity in vaccine A the mean antibody concentrations in the sera of the animals immunized with it were only about twice those given by the non-infective vaccines B and C (Table 3).

Table 3. *Influence of duration of exposure on immunogenicity of photoinactivated vaccinia virus*

Vaccine	Exposure (min.)	No. of rabbits	Mean neutralizing antibody (units)		
			Initial	After 1 dose	After 2 doses
A	10	3*	0†	0·41	54·05
B	20	3	0	0·3	22·5
C	30	3	0	0·28	23·0

Titre of virus before inactivation = 10^7 TCD 50.
* One animal died before second bleed.
† 0 = no antibody detected in lowest dilution tested, i.e. < 0·25 unit.

Vaccines D–F

The antibody concentrations attained by the animals immunized with these vaccines (Table 4) indicate that apparent differences in immunogenicity between them are not due to exposure time. When the rabbits of the three groups were later

Table 4. *Influence of duration of exposure on immunogenicity of photoinactivated vaccinia virus*

Vaccine	Exposure (min.)	No. of rabbits	Mean neutralizing antibody (units)		
			Initial	After 1 dose	After 2 doses
D	20	5	0*	2·3	185·6
E	40	5	0	2·2	81·9
F	60	5	0	8·9	183·2

Titre of virus before inactivation = 5×10^8 p.f.u./ml.
* 0 = no antibody detected in lowest dilution tested, i.e. < 0·25 unit.

challenged by dermal scarification with potent vaccine a considerable degree of skin immunity was apparent (Tables 5–7), but it was not possible to distinguish between the three groups. Compared with the control animals, in all the immunized rabbits there was, by the 8th day, considerable acceleration in the evolution of the vaccinial lesions.

The large difference in the mean antibody response to the two groups of vaccines can be ascribed to their differences in virus content. Vaccines D–F had approximately 50 times the virus content of vaccines A–C.

Table 5. *Response to dermal challenge of rabbits immunized with vaccine D (20 min. illumination)*

	Dilution of challenge vaccine $\times 10^{-3}$					
Animal no.	1	3	9	27	81	243
Control	c	c	c	c	c	sc −
IR 10	sc	5	2	1	0	0
IR 11	7	6	1	0	0	0
IR 12	10	7	2	0	1	0
IR 13			Died before challenge			
IR 14	11	7	3	0	0	0

c, sc+, sc, sc− = respectively 100, 75, 50 and < 50% confluent lesion. Numerals = number of discrete pocks.

Table 6. *Response to dermal challenge of rabbits immunized with vaccine E (40 min. illumination)*

	Dilution of challenge vaccine $\times 10^{-3}$					
Animal no.	1	3	9	27	81	243
Control	c	c	c	c	sc −	1
IR 15	c	sc	1	0	0	0
IR 16	12	4	1	1	1	0
IR 17	4	3	1	0	0	0
IR 18	sc	4	2	0	1	0
IR 19	5	3	0	0	0	0

c, sc+, sc, sc− = respectively 100, 75, 50 and < 50% confluent lesion. Numerals = number of discrete pocks.

Table 7. *Response to dermal challenge of rabbits immunized with vaccine F (60 min. illumination)*

	Dilution of challenge vaccine $\times 10^{-3}$					
Animal no.	1	3	9	27	81	243
Control	c	c	c	c	sc	sc −
IR 20	sc+	3	2	3	0	0
IR 21	c	sc	1	0	1	0
IR 22			Died of intercurrent infection			
IR 23	6	5	1	0	0	0
IR 24	4	3	0	2	0	0

c, sc+, sc, sc− = respectively 100, 75, 50 and < 50% confluent lesion. Numerals = number of discrete pocks.

DISCUSSION

The dye-sensitized photoinactivation of vaccinia virus demonstrated here is similar to that of many other mammalian and bacterial viruses. It would be unwise however, to extend all the findings of one system to another. After Perdrau & Todd (1933*a*) first applied the method to animal viruses, Burnet (1933) recorded differing sensitivities to photoinactivation among serologically different members of the same group of bacteriophages. The external structure of the virus protein coat probably determines susceptibility to a particular dye by controlling either adsorption or permeability. The striking effects of pH on photosensitization may be concerned with virus protein in a manner similar to that reported by Stearn & Stearn

(1923) for bacteria. Our results follow from the basicity of methylene blue (pK < 1·0) and the increasing net negative charge of vaccinia virus protein with increasing pH (isoelectric point *ca.* 4·5) (Smadel & Hoagland, 1942). The binding of dye to virus around neutrality and below is probably ionic, since what little dye unites is readily detachable by cationic exchange resins. The irreversibility at high pH values may be due to firmer electrostatic bonding; or to modification of the virus protein facilitating dye penetration, a phenomenon utilized by Müller & Peters (1963) in the negative staining of subsurface structures in vaccinia virus for electron microscopy. Wallis & Melnick (1963) showed that vaccinia virus could be reversibly photosensitized. Subsequently they demonstrated irreversible photosensitization (Wallis & Melnick, 1964). Our experiments confirm both these findings and show that the photosensitization of vaccinia virus by methylene blue and its reversibility is determined largely by H ion concentration. We were unable to show an influence of time or temperature on the uptake or tenacity of binding of dye to virus before photoinactivation, unlike Helprin & Hiatt (1959) with coliphage and Wallis & Melnick (1964) with herpes virus. The removal of excess dye by ion exchange resin, centrifugation or dilution was always followed by a reduction in inactivation rate, suggesting the reversibility of combination of some dye and virus (Yamamoto, 1958).

The inhibition of photoinactivation by nucleic acid can be readily explained as the result of formation of complexes with dye (see Northland, De Bruyn & Smith, 1954; Peacocke & Skerette, 1956; Kay, Walwick & Gifford, 1964*a*, *b*). The protective effects of cellular material reported by Perdrau & Todd (1933*a*) may also have been due to nucleic acid. The inhibition of photoinactivation of poliovirus by cell fractions (Wallis & Melnick, 1963) also appears to be due to inhibition of the combination of dye with virus.

The amino acid histidine is principally attacked by photo-oxidation of protein (Weil, James & Butchert, 1953; Sajgó Mihály, 1963). At the concentration tested added histidine was without effect in our system. Whole protein was a less effective inhibitor than intact nucleic acid. The inhibition by nucleic acid, and possibly protein, could probably be effected non-specifically by many substances whose affinity for dye is greater than that of the specific receptor on the virus. Perdrau & Todd (1933*a*) discussed the oxygen dependence of photoinactivation and its possible mediation by a transient organic peroxide. However, Welsh & Adams (1954) could not correlate the oxidative effects of dye-sensitized photoinactivation with inactivation by agents like hydrogen peroxide. Our negative results with peroxidase and catalase also indicate that organic peroxide is not involved. It is generally assumed that photoinactivation is mediated solely through the modification of viral nucleic acid since the photosensitizing dyes are basic and have strong affinity for nuclear material. McLaren & Shugar (1964) emphasized the desirability of some direct evidence of the participation of nucleic acid in photoinactivation. Our attempts to provide such evidence were unsuccessful. Simon & van Vunakis (1962, 1964) and Sussenbach & Berends (1963) produced spectral changes in solutions of nucleic acid or bases by dye-sensitized photodynamic action, with light intensities that were much higher than ours. Under our

conditions, which inactivated vaccinia virus in a few minutes, 3 days were required to produce significant changes in a solution of guanylic acid, suggesting that the damage needed to inactivate intact virus and the quantities of material involved are probably far too small to be detected by conventional techniques.

Under controlled conditions of protein denaturation pox viruses are inactivated in a manner which leaves them reactivable. They may be reactivated by other active viruses of the same group or by virus inactivated by agents which selectively modify viral nucleic acid leaving protein intact (Joklik, 1964). Our results with photoinactivated material indicate strongly that vaccinia viral DNA is not the sole participant in photoinactivation but that protein too is involved. The results substantiate those quoted and demonstrated by Joklik (1964) although our experiments were not designed to support a hypothesis of inducer protein. McLaren & Shugar (1964) reviewed the ample evidence for photo-oxidation of enzymes and proteins sensitized by methylene blue. The cyclic amino acids are usually attacked and the effects are local in character leaving the general properties of the protein intact. It may well be for this reason that the antigenicity of vaccinia virus is spared.

SUMMARY

Photoinactivation of vaccinia virus sensitized by methylene blue had parameters similar to those observed with other viruses. Thus inactivation proceeded exponentially to completion, was irreversible, independent of temperature and the intensity of illumination. Inactivation was dependent on the dose of illumination and the concentrations of both methylene blue and hydrogen ions. The effects of pH appeared to be primarily concerned with the tenacity of dye-virus binding. Inactivation was inhibited by small amounts of nucleic acid but not by their bases or pentoses. Inactivation was only affected by the presence of extraneous protein in relatively high concentration: it was not affected by the enzymes catalase or peroxidase. Attempts to obtain direct chemical evidence of the participation of viral nucleic acid in photoinactivation were unsuccessful.

Recombination experiments strongly indicated the involvement of viral protein in photoinactivation. Immunogenicity was not impaired since good responses of neutralizing antibody were obtained in rabbits immunized with vaccines photoinactivated over a wide range of exposure times.

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Printed in Great Britain

The transmission of bacteria and viruses on gummed paper

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The widespread practice of using the tongue to moisten envelope flaps, gummed labels and postage stamps not only is unattractive but could involve a danger to health. In order to assess the possible risk of infection associated with this practice, some simple studies were undertaken.

MATERIALS AND METHODS

Samples

A visit was paid to a local factory which produces envelopes and other gummed stationery. Samples of three representative adhesives were collected in sterile jars from the machines and from the stock barrels. Gum 'A' was a white plastic product ('433–0553', National Adhesives Ltd.); gums 'B' and 'C' were of the dextrin type ('Golden Grain R. 655', Alfred Adams Products; and 'Tragacine 2568', Tragacine Adhesives Ltd., respectively). Envelopes were taken from the output of several machines, and other samples were obtained from the stationery store of this Department.

Samples of commercial gum arabic as used in the manufacture of British postage stamps were obtained from a local chemical supplier. The solid gum was dissolved in Seitz-filtered tap water (30 g. in 100 ml.—'gum D').

Growth studies

Each gum was dispensed into eight 30 ml. bottles. Pairs of bottles were inoculated with approximately 100 cells, respectively, of *Staphylococcus aureus* (bacteriophage propagating strain '75'), *Escherichia coli*, *Salmonella paratyphi B* (both freshly isolated in the diagnostic laboratory of this Department) and *Klebsiella aerogenes* (isolated from the roller used to moisten gummed labels in the diagnostic laboratory). After thoroughly mixing the contents, samples were removed from each bottle for initial viable counts (by the method of Miles & Misra, 1938). One of each pair of bottles was incubated at 37° C. and one at room temperature (18–22° C.). Subsequent counts were made each day for one week, and thereafter at less frequent intervals.

Survival tests on bacteria and viruses

Bottles of the various gums were inoculated, in pairs, with approximately 10^8 cells of *Staph. aureus*, and *Salm. paratyphi B*, respectively. Other bottles were inoculated with echovirus Type 1 and adenovirus Type 3, respectively (10^7 tissue culture doses per bottle). Bottles were incubated at room temperature.

Streaks of these artificially infected gums were made on note paper (equivalent to the films produced on envelope flaps). They were stored in closed containers at room temperature.

Bacterial survival in gums was tested by inoculating a loopful on horse-blood-agar plates. Tests for survival in films and contamination of envelope flaps were made by rubbing the streaks with swabs moistened in nutrient broth. Gums containing virus were examined by inoculating 0·1 ml. of the gum solution into roller tube tissue cultures (HEp-2 cell line for the adenovirus, and primary monkey kidney cells for the echovirus). Dried films containing virus, and gummed flaps of envelopes, were cut away, and each strip was dropped into 2 ml. of Hanks's balanced saline. After the gum had completely dissolved, 0·2 ml. samples were transferred to tissue culture tubes. The Hanks's solution contained 500 units of penicillin and 500 μg. each of streptomycin and nystatin per ml., while the tissue culture media contained one-tenth of these concentrations.

Identification of organisms

Representative colonies of coagulase-positive staphylococci were subcultured and tested for susceptibility to bacteriophages of group III (including '75'). The other bacteria were subcultured on MacConkey's agar and tested biochemically, and by the slide agglutination test in the case of *Salm. paratyphi B*. If tissue cultures showed cytopathic effects, the presence of virus was confirmed by the neutralization technique.

RESULTS

Micro-organisms on envelopes and in gums

Neither pathogenic bacteria nor viruses were isolated from any of the 50 envelopes examined. Scanty growths of *Bacillus* spp. and saprophytic micrococci were obtained from the gummed flaps and from other parts of the envelopes. Similar cultures were obtained from the liquid gums, and one sample of gum arabic also yielded a moderate growth of *K. aerogenes*. This sample was not used in any further tests, but no attempt was made to sterilize any of the gums used.

Bacterial multiplication in gums

The test organisms did not grow in gums A, B or C, although within 2 weeks moulds and *Bacillus* spp. appeared as surface growths in B and C. The pH meter readings were 6·1, 3·8 and 7·6 in gums A, B and C, respectively. These values were unchanged after storage.

Gum arabic solution (D) had a pH of 4·6, but it nevertheless supported bacterial growth, as shown in Table 1. Following multiplication and death of the bacteria at 37° C., the pH fell to 4·0 in the case of *Staph. aureus*, and 3·8 in bottles containing the Gram-negative bacilli. The drop in pH was less marked in bottles kept at room temperature. To minimize the overgrowth of indigenous bacteria on subculture, it was found helpful to perform viable counts in parallel on plates which had been incubated anaerobically.

Table 1. *Growth of bacteria in gum arabic solution*

Organism	Temp. (° C.)	Log. of viable count per ml. after (days) 0	1	2	3	4	5	6	7	10	20	30
Staph. aureus	37	1·7	3·6	5·0	4·8	3·9	3·6	2·0	0·9	–	–	–
	20	1·9	2·1	2·7	5·6	6·1	7·0	7·2	5·6	4·0	1·3	–
Salm. paratyphi B	37	1·8	5·5	6·7	3·2	1·3	0·7	–	–	–	–	–
	20	1·7	2·3	2·3	4·2	6·2	4·0	2·3	2·4	2·0	1·6	1·4
Esch. coli	37	1·9	6·6	7·5	4·3	4·0	2·3	0·5	–	–	–	–
	20	1·9	2·6	3·2	4·8	4·6	6·3	7·6	6·2	5·8	4·6	1·8
K. aerogenes	37	2·0	5·6	7·8	7·0	7·2	5·8	3·6	3·0	1·6	–	–
	20	2·1	3·6	4·3	6·2	8·0	8·6	6·3	6·6	5·3	4·0	2·3

– = no growth.

Survival of bacteria and viruses

Dried films. As shown in Table 2, *Staph. aureus* was still viable after 6 months in dried films of C and D. *Salm. paratyphi B* survived for 4 months in C, and 3 months in D. The echovirus could not be recovered after a month, and the adenovirus not after 10 days.

Table 2. *Survival of bacteria and viruses in gums*

	Survival period (days) in: Dried films A	B	C	D	Liquid gums A	B	C	D
Staph. aureus	21	10	180+	180+	10	6	(21+)	30
Salm. paratyphi B	21	18	120	90	18	21	(21+)	48
Adenovirus 3	3	1	10	3	0*	0*	6	3
Echovirus 1	18	6	30	21	3	1	10	7

* Virus was isolated from samples examined after 30 min. but not after 1 day.

Liquid gums. Organisms usually remained viable for considerably shorter periods than in dried films (Table 2). Survival in C could not be investigated after 21 days because of overgrowth by moulds.

Adhesion of bacteria to gummed paper

The efficiency of gummed paper in the transfer of *Staph. aureus* from a solid surface was compared with that of self-adhesive cellulose tape (Sellotape). This kind of tape has been used for several years in hospital to study the bacterial contamination of surfaces (Selwyn, Maccabe & Gould, 1964).

By means of a glass rod, 0·5 ml. of an overnight broth culture of *Staph. aureus* diluted 1/10,000 in broth was spread over a sterile ceramic tile. After the broth had dried, the surface of the tile was sampled by pressing alternate strips of Sellotape and gummed paper on to it for 1 sec. (The gum arabic film on the paper had been slightly moistened with a damp swab.) The strips of tape and paper were then pressed on to blood-agar in Petri dishes for a few seconds. Fresh strips were

placed on the tile, and transferred to blood-agar in the same way. After incubation, the growth of *Staph. aureus* was almost confluent on all the plates that had been inoculated during the first series of transfers. Relatively low colony counts were obtained on all the plates inoculated during the second series—thus demonstrating the comparable efficiency of slightly moist gummed paper and Sellotape in the original transfer process.

DISCUSSION

The quantitative studies of Heller (1941) defined the conditions necessary for the long-term survival of *Streptococcus pyogenes* and *Esch. coli* in the dried state. The most favourable suspending media were shown to be either hydrophilic colloids or crystalloids that are water-soluble and utilizable by the bacteria; moreover, the two types of substance together exert an additive protective effect. Applying these principles, Stamp (1947) reported the preservation of various bacteria for over 4 years in dried disks containing gelatine and ascorbic acid. Lyophilization was found to be unnecessary. Naylor & Smith (1946) preferred dextrin to gelatine as the colloidal component of the suspending medium; and more recently McCracken (1964) has re-explored Stamp's method of preserving bacteria without lyophilization.

The present work demonstrates that certain commercially important gums also provide a favourable environment for the preservation of bacteria and viruses under normal atmospheric conditions. The relatively short periods during which the adenovirus and the echovirus could be recovered might have been due to the insensitivity of the system used for viral isolation.

The ability of gum arabic (or 'gum acacia') to support the growth of the four strains of bacteria was perhaps surprising on account of both the acidity and the chemical nature of the material. Data on pH minima for the growth of bacteria are scanty, and are mainly derived from early work in which neither standardized media nor accurate methods of pH determination were used. Porter (1946*a*) and Thimann (1963) both cite various minima which range from 4·0 to 4·5 for the species of enterobacteria used in the present work. *Staph. aureus* is not included by these authors, though Dernby (1921) recorded a minimum of 5·6 for growth, and Wilson & Miles (1964) state that growth is possible down to a pH of 4·0. A consideration of the effect of pH on bacterial growth in gum arabic is further complicated by the intrinsic protection afforded by such substances (Porter, 1946*b*). Gum arabic consists mainly of salts of arabic acid which may hydrolyse spontaneously to give various sugars, but it is usually contaminated also with other plant products (Dispensatory, 1955). The growth of saprophytic organisms in the gum further increases its biochemical complexity. In the samples examined, the material evidently contained all the nutrients necessary for the growth of widely differing bacteria.

During the manufacture of gummed paper and related products, contamination of the liquid gums by pathogenic bacteria and viruses can readily occur. This could be of particular importance in the production of postage stamps, for if relatively small numbers of pathogenic bacteria are introduced into the gum arabic adhesive,

they may multiply and subsequently retain their viability in the dried gum for several months. The use by the stamp manufacturer of unchlorinated spring water for the preparation of gum solution raises additional possibilities of contamination. Furthermore, postage stamps are often handled very carelessly when issued over the counter, and yet the purchaser will usually lick them without hesitation. The present work shows how readily bacteria can adhere to the surface of gummed paper which has been slightly moistened; and the finger is a suitable source both of moisture and of bacterial contamination.

There is an interesting parallelism between the probable consequences of contamination as revealed in this study, and the hazards associated with the entry of small doses of intestinal pathogens into cans of meat. Despite the marked differences in the vehicle, the experiments of Meers & Goode (1965) on the growth of typhoid bacilli in tins of corned beef produced similar results to those of the present tests on paratyphoid bacilli in gum. The initial growth phase is comparable following the introduction of small numbers of bacilli, which may be waterborne. Thereafter, at room temperature, bacterial survival in the acid gum is not very greatly inferior to survival in a can of meat. Moreover, the dried and uniformly infected gum shows as little evidence of danger as did the tins of meat which Meers and Goode infected with typhoid bacilli. Finally, the direct route of infection of the individual is the same in each case. However, the person who licks stamps runs the risk of acquiring respiratory tract as well as alimentary tract infection.

As long as this insanitary habit persists, adhesives used in the production of gummed paper should contain non-toxic agents which are active against the common pathogenic micro-organisms.

SUMMARY

The possible risks of infection associated with the practice of licking envelopes, stamps and labels were investigated. Although pathogenic bacteria and viruses were not isolated from sample envelopes obtained from various sources, the gums used in manufacture were found to exert a protective effect against death from desiccation on the bacteria and viruses which had been introduced into them. *Staph. aureus* and *Salm. paratyphi B* remained viable for several months in dried films of two out of four gums tested. An echovirus could be recovered from similar films for up to 30 days, and an adenovirus for up to 10 days.

Bacterial multiplication occurred in the gum used for the manufacture of postage stamps. The comparison is drawn between the possible consequences of minimal contamination of this gum and of cans of corned beef in the light of recent studies.

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Toxicity tests on suspected warfarin resistant house mice (*Mus musculus* L.)*

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INTRODUCTION

Infestations of house mice (*Mus musculus* L.) are generally more difficult to control with warfarin [3-(α-acetonyl)-4-hydroxycoumarin] than those of common rats, *Rattus norvegicus* Berk. This is not surprising for two reasons. First, although warfarin is included in bait against mice at a concentration of 0·025% (or five times the strength normally employed in the control of rats), it is still comparably less toxic to *Mus musculus*. Secondly, the mouse is a more diffuse and sporadic feeder than the rat and thus has a greater tendency to feed irregularly on warfarin baits. Prolonged warfarin treatments are therefore sometimes necessary before the effective control of mice is obtained.

Recently, however, Dodsworth (1961) reported mouse infestations that had proved impossible to control with warfarin even with treatments lasting several months. These treatment failures occurred despite the use of adequate numbers of baiting points and continuously high levels of poison bait consumption. As a result of these reports and others made later elsewhere, live-trapping campaigns were carried out in the premises concerned. The results of laboratory tests on the toxicity of 0·025% warfarin to the trapped animals are presented below and compared with similar information obtained on mice drawn from habitats not previously treated with warfarin (Rowe & Redfern, 1964).

MATERIALS AND METHODS

In all, 108 mice (49 males, 59 females) caught in Longworth live-traps at fifteen different localities were tested in the laboratory. For a minimum period of 2 weeks before it was tested each animal was isolated in a cage measuring 14 in. × 11 in. × 6 in. and supplied with a mixed diet of whole wheat and pinhead oatmeal, a wooden nesting-box and water *ad lib.*

The toxicity tests were conducted in the same manner as those reported earlier (Rowe & Redfern, 1964). Each mouse was fed on a sugar/oil/coarse oatmeal bait (hereafter referred to as SOCO) for 4 days before poisoning, when an excess amount of the bait-base containing 0·025% warfarin was offered to it for a limited number of days. Except for occasional 2- or 3-day periods, the amount of warfarin bait eaten was recorded daily.

The first mice tested were offered warfarin bait for 10 days and, after a recovery period, the survivors were re-tested over 12 days. In later trials mice were offered warfarin bait for a single 21-day period. The time of death and the weight of the mice that died were recorded and dead animals were autopsied. Animals still alive at the end of a test were given the wheat/oatmeal diet again and kept under observation.

Further toxicity studies were undertaken with mice reared under laboratory conditions. Six pairs of mice that had survived 21 days feeding on 0·025 % warfarin bait were allowed to breed in metal pens (1–6) supplied with nesting boxes, whole wheat, diet 41 B and water *ad lib.* The litters were isolated from their parents when they were between 5 and 6 weeks old. The young (F_1) mice were then caged individually and raised on the same diet as the parent animals until they were 12 weeks old, when the diet 41 B was removed. Four weeks later, each mouse was fed SOCO for 4 days and then offered the same bait-base containing 0·025 % warfarin for 21 days.

After a recovery period lasting several months, the four surviving males and four of the eight surviving females from one of the pens (pen 3) were paired; their progeny (F_2) were isolated, maintained on the same diet and tested in the same manner as the F_1 mice reported above.

In a third breeding experiment, a further five F_1 male mice (one from each of pens 1, 2, 4, 5 and 6) that had survived feeding on 0·025 % warfarin bait for 21 days, were each mated with several L.A.C. Grey (non-inbred laboratory strain) female mice. Earlier work had indicated that this strain was susceptible to warfarin poisoning (thirty-five males fed 0·025 % warfarin bait from 4 to 10 days all died and twenty-seven out of thirty females were killed after feeding on 0·005 % warfarin bait for from 5 to 10 days). Each breeding pair was maintained on diet 41 B and water *ad lib.* The F_1 hybrid litters produced from these crosses were removed from their parents when they were about 4 weeks old and the sexes separated. One week before they reached the age of 16 weeks the mice were housed separately; at the end of this period, the diet 41 B was withdrawn and the SOCO bait-base was offered to each mouse for 4 days, followed by the bait-base containing 0·025 % warfarin for 21 days. The fourteen parent L.A.C. females were similarly tested several weeks after their last litters had been removed.

RESULTS

Toxicity tests with Mus musculus *caught in the field*

The data from the feeding experiments are summarized in Tables 1–4. Fifty-two mice (26 males, 26 females) were offered warfarin bait for 10 days (Table 1). After 30 days, the period during which mice tested in earlier work had been kept under observation (Rowe & Redfern, 1964), the mortality was 22/52 (13/26 males and 9/26 females). Ten mice died between day 31 and day 64 (no. 466), three (nos. 426, 446 and 447) without normal warfarin symptoms. The death of some animals with poison symptoms several weeks after the end of a single test period showed that the effects of warfarin in mice can be extremely prolonged. Jaques (personal

Table 1. *Effect of feeding warfarin to* Mus musculus *for* 10 *days*

Mouse no.	Sex	Locality	Weight (g.)	Amount of bait eaten (g.)	Warfarin dose (mg./kg.)	Day of death
422	M	Leeds (A)	10·4	20·5	492·8	28
423	M		13·9	14·0	251·8	Survived
424	F		10·1	24·8	613·9	Survived
425	F		10·6	14·0	330·2	9
426	M		8·3	20·4	614·5	35*
428	F		15·3	19·0	310·5	Survived
440	F		16·4	22·5	343·0	Survived
441	M		8·3	18·7	563·3	34
462	M		9·8	8·7	221·9	7
443	F		8·1	16·8	518·5	Survived
444	F		9·4	20·7	550·5	Survived
434	M	Leeds (B)	12·5	23·4	468·0	27
445	F		7·5	2·9	96·7	5
446	M		8·3	17·4	524·1	42*
447	F		11·6	22·6	487·1	49*
429	M	Harrogate (A)	12·7	29·0	570·9	Survived
430	M		14·6	9·9	169·5	6
431	M		13·6	26·0	477·9	25
432	F		11·6	9·3	200·4	5
433	M		15·4	13·8	224·0	7
450	M		11·4	14·9	326·8	10
451	M		10·4	14·7	353·4	14
452	F		12·0	13·8	287·5	Survived
453	F		12·0	20·5	427·1	35
454	F		12·2	25·8	528·7	Survived
455	F		10·5	24·1	573·8	Survived
476	F		18·3	11·1	151·6	7
435	F	Harrogate (B)	15·1	20·8	344·4	9
471	F		19·9	32·1	403·3	Survived
472	F		14·9	15·2	255·0	Survived
473	M		11·4	23·3	511·0	Survived
474	M		9·9	22·9	578·3	49
475	M		17·7	17·7	250·0	Survived
439	F	Harrogate (C)	8·0	17·6	550·0	9
448	F		19·2	25·9	337·2	12
449	F		9·3	9·0	241·9	6
436	F	Harrogate (D)	11·6	25·2	543·1	Survived
437	M		13·8	18·8	340·6	13
456	M		8·7	23·1	663·8	27
457	M	Norwich (A)	12·7	30·0	590·6	Survived
458	F	Huddersfield (A)	15·9	19·0	298·7	7
459	M		11·3	10·7	236·7	7
460	F		21·7	32·7	376·7	Survived
461	M		19·5	27·7	355·1	Survived
463	M		23·5	37·7	401·1	Survived
464	M		20·8	15·8	189·9	7
465	F		16·4	20·2	307·9	Survived
466	F		19·0	30·6	402·6	64
467	M		22·4	36·1	402·9	59
468	M	Huddersfield (B)	11·8	18·6	394·1	49
469	M		16·9	16·6	245·6	10
470	F		11·6	18·0	387·9	32

* No warfarin symptoms.

communication) observed similar delayed toxic effects in rabbits treated with dicumarol.

The lowest fatal dosage was 96·7 mg./kg. of body weight ingested by a female (no. 445) that weighed 7·5 g. and which ate 2·9 g. of 0·025 % warfarin bait, and died on the fifth day. The highest total dose survived by any animal was 613·9 mg./kg. of body weight consumed by a female (no. 424) that weighed 10·1 g. and which ate 24·8 g. of poison bait.

The mortality among mice from Leeds (A) [5/11] and Harrogate (B) [2/6] was particularly low. The twelve mice from Leeds (A), Harrogate (A), Harrogate (D), and Norwich (A) that survived the 10-day warfarin test were re-tested (after a recovery period of 39 days) over a 12-day feeding period (Table 2). Ten of the twelve mice died; one (no. 444) from Leeds (A) died after only one day but without

Table 2. *Effect of feeding warfarin for* 12 *days to* Mus musculus *that had survived a* 10*-day test*

Mouse no.	Sex	Locality	Weight (g.)	Amount of bait eaten (g.)	Warfarin dose (mg./kg.)	Day of death
423	M	Leeds (A)	16·1	17·5	271·7	Survived
424	F		12·5	33·0	660·0	56
428	F		15·0	13·5	225·0	7
440	F		16·3	33·7	516·9	Survived
443	F		9·1	2·9	79·7	4
444	F		9·3	0·8	21·5	1*
429	M	Harrogate (A)	13·2	11·4	215·9	7
452	F		11·5	10·6	230·4	7
454	F		11·2	33·4	745·5	35
455	F		9·5	18·4	484·2	11
436	F	Harrogate (D)	10·9	2·5	57·3	3
457	M	Norwich (A)	12·5	35·7	714·0	14

*No warfarin symptoms.

warfarin symptoms. Five of the nine mice with symptoms died within 10 days, the time of the test period that they had survived previously. The last death (no. 424) occurred on day 56; both survivors (nos. 423, 440) came from Leeds (A).

The results of feeding 0·025 % warfarin bait for a 21-day period to the latter two animals and to the mice from Harrogate (B) and Huddersfield (A) that had survived a single 10-day poisoning period are shown in Table 3. One of the two mice from Leeds (A) died on the 20th day of the test period; the other died, apparently from natural causes, 10 weeks after the end of the test period. All eight mice from Harrogate (B) and Huddersfield (A) died, but only two did so within 30 days; the last death of a mouse showing symptoms of warfarin poisoning occurred on day 75. Evidence of warfarin poisoning was not found in the one animal that died later (no. 475; day 126).

The toxicity of warfarin to the remaining fifty-six mice captured (23 males, 33 females) was determined over a 21-day feeding period. The data in Table 4

show that there was considerable individual variation in susceptibility to warfarin poisoning. The death of some mice occurred as early as the fourth day. However, the mortality at the end of 30 days was only 32/56 (14 males, 18 females). As in the earlier tests, some mice died a considerable time after the end of the test period. Of eight animals that died after the 30th day, three had no visible warfarin symptoms. The highest dose of warfarin survived by any animal was 1129·1 mg./kg. of body weight consumed by a female (no. A 124) that ate 55·1 g. of poison bait.

Table 3. *Effect of feeding warfarin to* Mus musculus *(survivors of* 10- *and* 12-*day tests) for* 21 *days*

Mouse no.	Sex	Locality	Weight (g.)	Amount of bait eaten (g.)	Warfarin dose (mg./kg.)	Day of death
423	M	Leeds (A)	15·1	19·8	327·8	20
440	F		17·4	36·2	520·1	Survived
460	F	Huddersfield (A)	19·2	68·6	893·2	49
461	M		16·4	23·8	362·8	10
463	M		20·9	53·7	642·3	21
465	F		15·3	48·5	792·5	71
471	F	Harrogate (B)	16·8	61·3	912·2	75
472	F		12·8	37·7	736·3	60
473	M		12·1	51·1	1055·8	35
475	M		14·8	58·2	983·1	126*

* No warfarin symptoms.

Toxicity tests with Mus musculus *bred in the laboratory*

Each of the six breeding pairs of mice produced three or more litters. In all eighty-nine F_1 mice were fed 0·025 % warfarin bait over a 21-day period. After 30 days, the combined litter mortalities obtained were 10/16 (3 litters), 4/15 (4), 4/16 (3), 1/8 (3), 5/18 (3) and 9/16 (4) in pens 1–6 respectively giving a mortality of 33/89 (37·1 %). A further four mice died between days 41 and 80. More males (20/38, 52·6 %) were killed than females (13/51, 25·5 %), the difference being significant at the 0·05 level [$\chi^2 = 5{\cdot}76$; $P = 0{\cdot}01$–$0{\cdot}02$].

The four established breeding pairs of F_1 survivors (mice that were born in pen 3) produced a total of eighty-nine young. The mortality obtained after feeding these F_2 mice 0·025 % warfarin bait for 21 days was 48/89 (53·9 %). The kills obtained with mice from each of the four pens were similar—21/37 (5 litters), 9/19 (4), 8/17 (4) and 10/16 (4) respectively. No mice died after day 30. Although, as with the F_1's, more males (34/55, 61·8 %) than females (14/34, 41·2 %) were killed, the difference is not significant [$\chi^2 = 2{\cdot}82$; $P = 0{\cdot}05$–$0{\cdot}10$].

Of the 176 hybrid F_1 progeny (87 males, 89 females) produced by crossing five wild male and fourteen L.A.C. Grey female mice, all but two (one male, one female) died after feeding on 0·025 % warfarin bait in a 21-day test (days to death 2–23). All fourteen parent L.A.C. females, similarly tested, were dead by day 9.

Table 4. *Effect of feeding warfarin to* Mus musculus *for* 21 *days*

Mouse no.	Sex	Locality	Weight (g.)	Amount of bait eaten (g.)	Warfarin dose (mg./kg.)	Day of death
558	M		16·0	64·3	1004·7	62
559	M		17·9	55·4	773·7	23
560	M		15·6	17·4	278·8	8
561	M		15·3	63·3	1034·3	82*
563	F		13·6	50·5	928·3	40
565	F		12·5	47·2	944·0	82*
567	F		13·4	40·4	753·7	62
568	F	Norwich (A)	9·4	20·1	534·6	16
570	M		12·5	18·9	378·0	12
572	M		16·3	47·9	734·7	27
573	F		19·0	22·6	297·4	10
574	M		14·9	18·6	312·1	8
575	F		11·5	40·1	871·7	19
576	F		8·6	5·3	154·1	7
577	F		15·2	17·1	281·3	13
578	F	Norwich (B)	17·9	49·6	692·7	36*
579	F		13·4	57·8	1078·4	23
580	F		14·1	49·8	883·0	Survived
582	F	Norwich (C)	12·2	44·3	907·8	56
583	M		15·0	14·0	233·3	8
A 120	F		18·9	32·9	435·2	15
A 121	M		17·0	12·9	189·7	7
A 122	M		20·0	11·3	141·3	6
A 123	F		22·4	27·4	305·8	13
A 124	F	Leeds (C)	12·2	55·1	1129·1	Survived
A 125	M		14·4	47·8	829·9	Survived
A 126	M		14·7	10·2	173·5	7
A 127	F		11·1	43·1	970·7	Survived
A 129	M		10·1	46·3	1146·0	39
A 130	F		14·3	8·9	155·6	6
A 572	M		16·4	46·0	701·2	Survived
A 573	F		16·7	52·6	787·4	Survived
A 574	M		13·2	50·4	954·5	Survived
A 575	M		16·4	47·3	721·0	Survived
A 576	F		16·9	24·0	355·0	15
A 601	F		10·0	43·3	1082·5	Survived
A 602	M	Kidbrooke (A)	11·8	8·8	186·4	4
A 603	F		14·4	23·1	401·0	15
A 604	F		14·0	47·6	850·0	Survived
A 605	F		13·4	8·2	153·0	5
A 612	M		8·7	32·3	928·2	20
A 613	F		16·5	22·1	334·8	14
A 614	F		10·4	18·0	432·7	10
A 838	F		19·9	3·9	49·0	11
A 839	F		10·8	42·3	979·2	Survived
A 840	F		14·6	27·3	467·5	14
A 841	M		16·4	6·1	93·0	5
A 842	F	Kidbrooke (B)	14·9	44·9	753·4	Survived
A 843	F		18·2	31·8	436·8	Survived
A 844	M		13·8	0·5	9·1	4
A 845	F		8·7	34·8	1000·0	Survived
A 846	F		11·0	3·2	72·7	4
B 166	M		13·4	33·0	615·7	Survived
B 167	M		15·7	52·7	839·2	Survived
B 168	M	Wakefield	12·5	12·8	256·0	14
B 169	F		12·7	30·1	592·5	17

* No warfarin symptoms.

DISCUSSION

In an earlier laboratory investigation of the toxicity of 0·025 % warfarin to so-called 'normal' mice (animals that had not been subjected to warfarin previously), the most significant aspect was the high poison dosage that some animals could withstand (Rowe & Redfern, 1964). Even after 21 days' continuous feeding on warfarin bait, five of the fifty-three mice tested (9·4 %) survived. As it is likely that these animals would have been equally difficult to kill with warfarin in the field, it could be said that from the control viewpoint there are probably some mice 'resistant' to 0·025 % warfarin in any sizeable population.

With this in mind, the degree of resistance to warfarin of the mice tested in the present study can be assessed by comparing the data on mortality and days to death given in Tables 1 and 4 with the information obtained previously for 'normal' mice over the same two test periods. At the end of 30 days, the mortality figures of the mice fed for 10 and 21 days were 22/52 and 32/56 respectively compared with 31/37 and 48/53 for 'normal' mice (Rowe & Redfern, 1964). These differences in mortality are highly significant ($\chi^2 = 13{\cdot}8$; $P = 0{\cdot}001$ for 10 days, and $\chi^2 = 13{\cdot}9$; $P = 0{\cdot}001$ for 21 days). In both test periods, furthermore, the mean time to death of the mice killed by warfarin was considerably longer for allegedly resistant mice than it was for normal animals. In the 10-day test shown in Table 1 the mean time of death of twenty-two mice killed within 30 days was $11{\cdot}7 \pm 7{\cdot}7$ days compared with the $6{\cdot}3 \pm 2{\cdot}2$ days for thirty-one 'normal' mice. Student's '*t*' test applied to the data gives a probability of less than 0·001 that this was due to chance. The difference between the mean time of death of $11{\cdot}9 \pm 6{\cdot}1$ days for the thirty-two mice shown in Table 4 and the $8{\cdot}9 \pm 3{\cdot}9$ days of forty-eight 'normal' mice killed in 21-day tests is also significant ($P < 0{\cdot}01$).

Most of the samples of trapped mice used in the present study are small (and it is for this reason that the information on them has been combined), but it was noticeable that in all samples some individuals succumbed readily to warfarin poisoning and in one at least (Harrogate C) the mice were all fairly easily killed. It must be presumed that these mice survived the field treatments because of inadequate feeding on the poison bait. In general, however, it is clear that the proportion of mice resistant to warfarin in the premises concerned was higher than would normally be expected.

As stated above, the house mouse is rather more prone than the rat to the ingestion of sublethal doses of poison during control treatments. One possible explanation therefore for the increased resistance to warfarin found in some mouse populations is that resistance has been 'acquired' following sublethal dosing with warfarin. Direct observation and examination of the daily bait consumption figures of a few of the mice tested over 21 days in the laboratory suggested that these individuals may in fact have developed some sort of tolerance to warfarin. Mouse no. A 843, for example, fed normally on warfarin bait for 7 days, but in the next 3 days it ate only 0·3 g. The bloody droppings voided during this time indicated that in this animal at least the prothrombin level was lowered, but on the eleventh day it fed normally again and continued to do so for the remainder of

the test period. In contrast, however, some of the mice re-tested with warfarin succumbed within the time of the test period that they had survived previously, suggesting that the earlier sublethal feeding period had rendered them more rather than less susceptible to subsequent poisoning.

While the possibility that mice may become more tolerant to warfarin following the repeated ingestion of sublethal doses of the poison is being investigated further, present evidence suggests that resistant populations are more likely to have arisen as a result of selection, susceptible animals being killed off during poison treatments, leaving the more resistant forms to reproduce. If this is so, resistant populations would be most likely to develop after long or frequent warfarin treatments. Unfortunately there is little detailed information available concerning the history of most of the infestations from which the samples were trapped. However, according to the rodent control workers concerned, several of the treatments extended over a period of months and all appeared to become less effective the longer they were continued, although the amount of poison bait consumed increased.

Laboratory support for the genetical control of warfarin resistance in mouse populations is to be found in the low kills obtained with the progeny of resistant animals. Only 33/89 F_1's died after being fed with 0·025 % warfarin bait for 21 days compared with 48/53 'normal' mice treated in the same manner [$\chi^2 = 36{\cdot}63$, $P = 0{\cdot}001$]. Although proportionally more F_2's were killed (48/89) compared with the F_1 stock from which they were derived (mortality 4/16), the difference is not significant ($\chi^2 = 3{\cdot}46$; $P = 0{\cdot}05$–$0{\cdot}10$) and the mortality of the F_2 mice was still much lower than that obtained with 'normal' animals [$\chi^2 = 18{\cdot}72$; $P = 0{\cdot}001$].

The absence of clear-cut monofactorial ratios and the failure to maintain resistance in crosses with the warfarin susceptible L.A.C. strain, suggest two possible explanations of the inheritance of warfarin resistance in house mice. Either resistance is under polygenic control, determined by the interaction between genes showing no dominance—in which case it differs from the resistance shown by *Rattus norvegicus*, for in the two areas in Britain where resistant rat populations are widespread a single dominant mutant gene is believed to be responsible (Sheppard & Drummond, personal communications)—or it is controlled by a single major gene whose expression is strongly affected by the rest of the genotype, that is, influenced by modifiers.

There is as yet no satisfactory explanation of the physiological mechanism or mechanisms involved in the resistance to 0·025 % warfarin in the house mouse. Abnormal supplies or requirements of vitamin K, poor absorption of warfarin from the gastro-intestinal tract or its rapid excretion or metabolic transformation, and altered enzymic activity resulting in a general decrease in sensitivity to warfarin may all play some role in determining resistance.

In more recent tests, the comparative response of the progeny of warfarin resistant mice to 0·025 % warfarin, 0·10 % warfarin, 0·025 % pival (2-pivalyl-1,3-indandione), 0·025 % chloradione (2-bis (para-chlorophenyl) acetylindane-1-3-dione), and 0·025 % warfarin plus 0·025 % sulphaquinoxaline has been determined. Sulphaquinoxaline has been reported to potentiate the action of warfarin

by reducing the bacterial flora in the gut and thereby diminishing vitamin K synthesis (Derse, 1963). The mortalities obtained (Table 5) indicate that mice unresponsive to warfarin at 0·025 % are likely to be little if any more responsive to warfarin at 0·10 %, to other anti-coagulant poisons, and to the potentiating effects of sulphaquinoxaline.

Table 5. *Mortality of* Mus musculus *after unrestricted feeding on anti-coagulants for* 21 *days*

Poison	Concentration (%)	Mortality	Dosage range that killed (mg./kg.)	Highest dosage survived (mg./kg.)	Range of days until death
Warfarin	0·025	9/23	164–361	1022	7–15
Warfarin	0·10	7/11	255–1282	2702	6–13
Pival	0·025	6/10	94–448	802	7–14
Chloradione	0·025	5/10	127–502	718	9–21
Warfarin Sulphaquinoxaline	0·025 0·025	5/10	294–607	964	14–23

SUMMARY

1. Residual populations of wild house mice (*Mus musculus* L.) were trapped alive in premises where poison treatments with 0·025 % warfarin bait had been reported to be ineffective.

2. In the laboratory the individually caged mice were fed 0·025 % warfarin in a sugar/oil/coarse oatmeal base. In toxicity tests lasting 10 and 21 days, the total mortality was low and the rate of death slow compared with mice drawn from habitats not treated with warfarin.

3. It is suggested that tolerance to warfarin in *M. musculus* either is polygenically based or is controlled by a single major gene influenced by modifiers and that these hard-to-kill populations have arisen through selection during successive control treatments.

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The epidemiology of the common cold
IV. The effect of weather

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INTRODUCTION

It is a challenging fact that the most obvious feature of the epidemiology of the common cold, the seasonal variation in incidence, remains without any satisfactory explanation. Many attempts have been made to explain why these infections are so much more frequent in the winter months of the year in temperate climates. A general discussion of these arguments has been given recently by Andrewes (1964) but, as the subtitle of his review—'We do not yet understand how seasonal and other factors affect the incidence of colds and influenza'—shows, none of them is convincing. One difficulty in establishing any relation between the seasonal variation of an infection and climatic factors is that these factors, together with many aspects of human behaviour dependent on them, are all highly intercorrelated. It is, therefore, necessary to employ relatively complex methods of analysis in exploring such relationships.

We have accumulated a considerable series of records of the incidence of the common cold, over a period of 6 years in a group of offices in Newcastle upon Tyne, a total of over 2000 person-years of observations, and over a period of 4 years in offices in London, nearly 3000 further person-years (Lidwell & Williams, 1961*a*, *b*; Kingston, Lidwell & Williams, 1962). The availability of electronic computer methods now makes it possible to apply appropriate statistical procedures to this body of data.

METHODS OF ANALYSIS

In analyses of time series containing a number of intercorrelated variables it is usually helpful to eliminate the dominant trends, e.g. seasonal or yearly trends, and to investigate the association between the residual deviations from fitted trends. The data we are concerned with here, the incidence of colds and the meteorological variables, might show yearly differences, i.e. a given year might be consistently wetter or sunnier than usual, and they certainly exhibit seasonal

variation, i.e. there are more colds in January than in May. By eliminating these trends we mean deducing a value for the variable in question, for example temperature, which can be regarded as the expected value for the particular day concerned allowing for the date in the year and the character of that year as warmer or cooler than usual in the set of data involved. These expected values can be derived from the daily records in many ways, in particular we might take the average values for each calendar date in the year and adjust these by adding the difference of the year's average from the overall average or we might fit a smoothed curve, using some suitable algebraic function, to the data. We adopted the second of these methods. Having arrived at the expected values in one way or other the differences between the actual values observed on a given day and the expected values can be obtained and the inter-relationships among these differences explored, e.g. are more colds than expected for the time of year recorded when the temperature is below expectation for that date?

This form of procedure is discussed by Quenouille (1952) and has been applied by Spicer (1959) to examine the relationship between meteorological factors and the incidence of poliomyelitis in England and Wales. An interesting feature of Spicer's analysis is that the regressions found for poliomyelitis incidence on temperature and relative humidity, based on the residuals of these quantities after the elimination of seasonal and annual trends and including correlations lagged by 1 or 2 months, are such as to predict remarkably closely the actual monthly incidence of the disease when applied to the raw weather data.

It appeared then that it might be useful to try a similar approach for exploring the relationship between the incidence of the common cold and weather. We had available daily records of the incidence of the common cold obtained in the offices by the methods described in the earlier papers in this series (Lidwell & Williams, 1961*a*, *b*; Kingston *et al.* 1962). These covered the 9 months from September to May inclusive for the 6 years 1951–57 at the offices at Newcastle upon Tyne and for the 4 years 1951–55 at the London offices.

The weather data were taken from the records of the nearest meteorological stations and included values for the month of August so that it was possible to compute correlations of disease incidence on the weather variables up to a lag of 30 days. The seasonal weather trends appeared to be adequately represented by fitting simple sine curves, one for London and one for Newcastle, to the averaged figures for each one-third of a month (10 days $\pm$ 1 day) for each of the variables recorded. There did not appear to be any significant systematic differences between the years. It was not possible to fit the data for the incidence of the common cold, averaged similarly for each one-third of a month, to such a simple sine curve over the whole year owing to the occurrence of a substantial peak in the incidence in the early autumn. A sine curve could, however, reasonably be fitted to the data for the months November to May inclusive. This was done and the autumn peak was fitted to a normal distribution superimposed on the calculated curve for November to May extrapolated back through October and September. The constants of the fitted curves are given in Table 1, which also shows the meteorological variables studied. The average seasonal variation in the cold incidence over the year

is shown in Fig. 1. The differences between these smoothed curves and the actual day-to-day observed values were then tabulated to be used in the statistical analysis.

In addition we attempted to make use of an index of 'spreading' for the common cold in the form

$$r_i = \frac{3n_i}{n_{i-1}+n_{i-2}+n_{i-3}}$$

where n_i is the cold incidence observed on day i, i.e. the index is a way of representing the rate of increase or decrease of the disease in the community. In order to avoid infinite values and to produce a symmetrical function the quantity $r^* = (\tan^{-1} r)/45$ was computed. r^* is then unity if the incidence of new colds remains

Table 1. *Averaged values of cold incidence and weather data fitted to smooth curves*

Variable	Mean value	Swing ±	Trough or peak date
Newcastle 1951–57			
*Mean day temperature (° F.)	49·4	10·3	30 Jan., T
Day max.–night min. (° F.)	8·5	1·9	28 Dec., T
Water-vapour pressure, 9 a.m. (mb.)	9·1	3·1	3 Feb., T
Relative humidity, 9 a.m. (%)	79·3	6·8	12 Dec., P
Sunshine, for day (hr.)	3·8	2·7	25 Dec., T
Rainfall, 9 a.m.–9 p.m. (mm.)	0·90	0·21	7 Mar., T
Pollution index (smoke and SO_2)	0·17	0·08	6 Jan., P
†Chance of cyclonic or westerly weather	0·37	0·10	1 Apr., T
†Chance of anticyclonic or easterly weather	0·23	0·08	13 Apr., P
New colds/1000 at risk/day	6·9	3·8	22 Dec., P
Autumn peak	(Maximum value 4·5, S.D. 0·36 months)		26 Sept., P
London 1951–55			
*Mean day temperature (° F.)	52·4	13·8	21 Jan., T
Day max.–night min. (° F.)	13·8	5·0	25 Dec., T
Water-vapour pressure, 9 a.m. (mb.)	10·2	3·7	4 Feb., T
Relative humidity, 9 a.m. (%)	81·3	9·9	9 Dec., P
Sunshine, for day (hr.)	4·4	3·0	25 Dec., T
Rainfall, 9 a.m.–9 p.m. (mm.)	0·89	0·37	10 Mar., T
Pollution index (smoke and SO_2)	0·24	0·18	8 Jan., P
†Chance of cyclonic or westerly weather	0·37	0·10	1 Apr., T
†Chance of anticyclonic or easterly weather	0·23	0·08	13 Apr., P
New colds/1000 at risk/day	5·3	5·5	14 Jan., P
Autumn peak	(Maximum value 14·4, S.D. 0·44 months)		28 Sept., P

* The mean day temperature was taken as
½[maximum temperature (9 a.m.–9 p.m.) + minimum temperature (9 a.m.–9 p.m.)].

† The synoptic weather patterns were assessed for England as a whole.

Weather data fitted to sine curve, $y = a + d \sin x$.

Colds, Nov.–May, fitted to similar curve; end of Aug.–Oct. fitted to normal distribution superimposed on Nov.–May sine curve extrapolated back.

steady, with a minimum value of 0 and a maximum of 2. There were no apparent seasonal or annual trends in r^* so this quantity itself was used in the preliminary analyses, but as it was clearly less closely associated with the weather differences than the actual difference between the numbers of colds expected and observed on a given day it was omitted from the final computations and will not be referred to further in this paper.

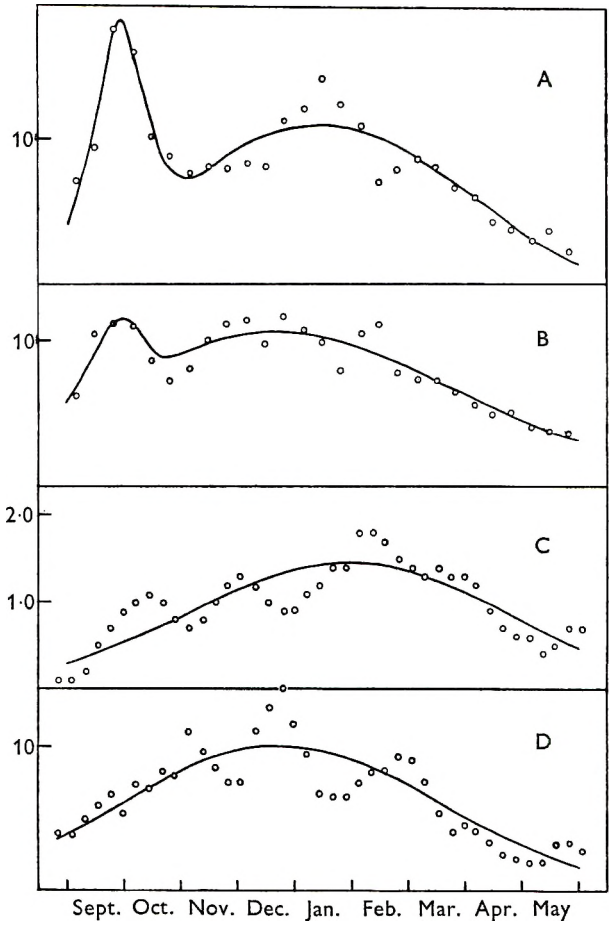

Fig. 1. Seasonal variation in incidence of colds. A. London offices: average one-third of a month (10 days ± 1 day) values for years 1951–55. Full line drawn according to fitted constants given in Table 1. B. Newcastle offices: average one-third of a month (10 days ± 1 day) values for years 1951–57. Full line drawn according to fitted constants given in Table 1. C. Cirencester: all upper respiratory symptoms, average weekly values for years 1954–56. Taken from Hope-Simpson (1958). Full line best fitted sine curve for values over the whole year. D. Chalke valley: average weekly values for colds in families for years 1948 and 1949. Taken from Lidwell & Sommerville (1951). Full line best fitted sine curve for values over the whole year. For A, B and D the ordinate represents the numbers of infections reported per 1000 person-days exposure. For C the figures are given as a fraction of the median weekly incidence.

CORRELATION ANALYSIS ON THE DIFFERENCES

We thus had available for analysis values of the difference between the expected weather and that observed for some 1200 days of observation spread over 4 years in London and for more than 1800 days over 6 years in Newcastle upon Tyne. In addition, we had values of the difference between the expected and recorded numbers of colds for about 1100 days in the London offices and for more than 1600 days in the Newcastle offices.

The analysis of the differences was carried out at the Unit of Biometry, Oxford, using an Elliott 803 computer. This analysis fell into two parts, a correlation study, described in this section, and a regression analysis, which is described in the next section.

A correlation coefficient is a measure of the degree of association of two series of numbers. The methods of calculating correlation coefficients for time series are explained in Quenouille (1952), chapter 11, and several of the methods described in that chapter were used in the analysis. First, all eleven series of differences were tested for serial correlation and the effects eliminated in subsequent analyses by using partial correlation coefficients.

Initially the analysis was confined to two portions of the data, Newcastle 1951/2 and London 1951/2. Because of the irregular occurrence of colds in autumn each of these was split up into three periods of approximately equal length, August–October, November–January and February–May. For each period, each of the nine weather variable differences was compared with the expected–observed cold difference. Thirty correlation coefficients were calculated every time, on the weather for the day and each of the 29 preceding days.

Thus for any period we had a measure of the degree of association between colds and the weather on the same day, on any of the preceding 29 days and on the following 5 days. The lags were chosen to cover such a wide period because of the large difference in phase of the variables. An examination of the results revealed that four of the variables were clearly not important factors in relation to the incidence of colds. These were sunshine, pollution, day maximum minus night minimum temperature and anticyclonic weather. The analysis was then repeated including three additional portions of the data, Newcastle 1952/3, London 1952/3 and London 1953/4, omitting the above four variables. The extra results allowed us to eliminate relative humidity, rainfall and cyclonic weather, leaving mean day temperature and vapour pressure, both having highly significant association with colds.

Temperature and vapour pressure are highly correlated variables and this is true even when the main trend has been removed, i.e. there is still a high degree of correlation between the values of the differences between the observed and expected values of these two quantities on each day. The analysis for the above-mentioned five portions of the data was therefore repeated, using partial correlation to estimate the degree of association between colds and temperature and between colds and water-vapour pressure each independently of the other. These results showed clearly that the main association was related to temperature. Since almost all the significant contribution to the correlation was derived from the data for the periods November–January and February–May the final analysis for the 6 years 1951–57 at Newcastle and the 4 years 1951–55 in London made use of the colds reported between the beginning of November and the end of May only.

Owing to limitations in computer storage the serial correlations were not eliminated in these calculations. This will tend to depress the peak values in the series and will also exert a general smoothing effect. The values obtained, however, confirm

the findings of the analysis based on the years 1951–53 at Newcastle and 1951–54 in London, and the two together show that similar effects are present in both cities over the whole period of the study. Table 2 gives the values of the first-order and of the partial correlation coefficients for the whole period. Smoothed curves derived from these are given in Fig. 2. On the same figure are also plotted curves for the

Table 2. *Correlation coefficients*

	Colds and temperature		Colds and vapour pressure	
Lag (days)	Vapour pressure not eliminated	Vapour pressure eliminated	Temp. not eliminated	Temp. eliminated
−7	−0·0251	−0·0058	−0·0339	−0·0235
−6	−0·0425	0·0138	−0·0883	−0·0787
−5	−0·0646	−0·0286	−0·0695	−0·0384
−4	−0·0894	−0·0261	−0·1144	−0·0763
−3	−0·0989	−0·0402	−0·1118	−0·0660
−2	−0·0810	−0·0342	−0·0900	−0·0521
−1	−0·0842	−0·0359	−0·0931	−0·0536
0	−0·1087	−0·0401	−0·1286	−0·0798
1	−0·1397	−0·0774	−0·1308	−0·0596
2	−0·1616	−0·0969	−0·1419	−0·0575
3	−0·1468	−0·0861	−0·1313	−0·0551
4	−0·1573	−0·0936	−0·1391	−0·0573
5	−0·1327	−0·0668	−0·1337	−0·0688
6	−0·1219	−0·0673	−0·1146	−0·0528
7	−0·1340	−0·0813	−0·1164	−0·0463
8	−0·1341	−0·0778	−0·1214	−0·0527
9	−0·1151	−0·0645	−0·1074	−0·0494
10	−0·1083	−0·0673	−0·0921	−0·0354
11	−0·0874	−0·0468	−0·0844	−0·0409
12	−0·0697	−0·0275	−0·0806	−0·0490
13	−0·0740	−0·0494	−0·0579	−0·0176
14	−0·0411	−0·0109	−0·0547	−0·0377
15	−0·0542	−0·0365	−0·0420	−0·0124
16	−0·0468	−0·0360	−0·0301	−0·0031
17	−0·0185	−0·0181	−0·0665	0·0051
18	−0·0101	0·0035	−0·0221	−0·0200

The lag is given as the number of days by which the weather correlated antedated the reporting of the colds.

The 95 % confidence limits for the individual values in this table are approximately ± 0·05.

distribution of incubation times following nasal inoculations with common cold viruses and the distribution of serial intervals between colds in families. The correlations with temperature (Fig. 2A) rise to a peak value for a time interval of about 3 days after the weather difference. This is true both of the first-order coefficients and of the partial coefficients from which the effect of water-vapour pressure has been eliminated. As the coefficients are negative this means that an increased number of colds follow colder weather. The lag of about 3 days is very close to the median incubation period following nasal inoculation, namely 2–4 days, and to the median interval between presumed cross-infection in families, namely 2·8 days.

The absolute values of the correlation coefficients are not large, probably, in part, owing to the considerable amount of random variation in the data. This aspect of the analyses is discussed later in connexion with the results of the regression analysis. The relative vertical scale of the correlation curve depends on the relationship

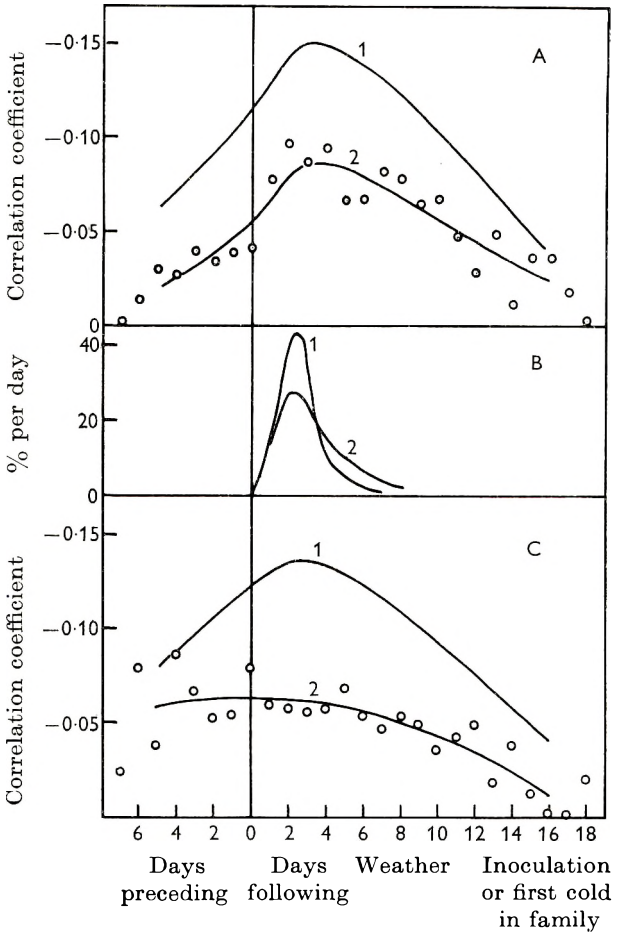

Fig. 2. Correlation between colds and the outside temperature, or the outside water-vapour pressure, on the days preceding and following the first reported day of symptoms. A. Correlation with outside mean day temperature. 1, 1st-order coefficients; 2, partial coefficients with outside water-vapour pressure eliminated. B. 1, The distribution of intervals between nasal inoculation and the appearance of symptoms (Sartwell (1950), based on the data of C. H. Andrewes); 2, the distribution of intervals between the onset of colds presumed transmitted from one member of a family to another (Lidwell & Williams, 1961 *b*). C. Correlation with outside water-vapour pressure. 1, 1st-order coefficients; 2, partial coefficients with outside temperature eliminated. The actual values of the 2nd-order partial correlation coefficients only are shown as points on graphs A 2 and C 2.

assumed between a correlation coefficient and the percentage of infections falling within a given day. Any assumption is more or less arbitrary so that no deductions can be based on comparisons of this aspect of the curves. The wider temporal

spread of the curve of correlation coefficients is, to some extent, accounted for by the effects of serial correlation but this seems likely to be responsible for only a small part of it. Among other possible causes, the consequences of any increased number of infections at a particular time will persist through a series of person-to-person transmissions over, possibly, several generations or many days. The apparently random effects of a multiplicity of unrecorded influences operating on a relatively small number of events are, however, possibly the principal causes of this. The correlations with water-vapour pressure (Fig. 2C) show a different picture. As this variable is highly correlated with mean outdoor temperature, the first-order correlation coefficients are similar to those with temperature. The elimination of the effect of temperature, however, removes the peak at about a 3-day lag. The values of the partial correlation coefficients themselves are somewhat smaller and more irregular than the corresponding cold-temperature correlations. The smoothed values show a tendency to a broad maximum value at or about the same day as the day of reputed onset of colds. These coefficients are also negative, i.e. increased numbers of colds are associated with low water-vapour pressures.

REGRESSION ANALYSIS

The correlation analyses demonstrate the extent to which the incidence of colds, or rather the difference between the observed and expected number of colds, on a given day was associated with the differences between the observed weather and that expected on that day or on the preceding days. It is of interest to explore further the quantitative aspects of this association, e.g. how many more colds are reported for each degree difference between the observed and the expected mean day temperature, and to see how far the actual differences between summer and winter weather can account for the observed difference between the summer and winter incidence of colds. Limitations of storage space made it inconvenient to apply the standard methods of multiple regression analysis and the method of Woolf was employed (Woolf, 1951).

All the analyses were performed on the differences between the observed values on the day in question and the expected value for that day derived from the curves fitted to the averaged values.

A preliminary analysis was carried out for a single year using temperature and water-vapour pressure on the day and on each of the 16 previous days as independent variables with colds reported as the dependent variable. This showed that almost all the variation that could be accounted for by the regression was accounted for by the same day and the 7 preceding days.

With this result and those of the correlation analyses described in the previous section in mind, full analyses were carried out for the 4 years in the London offices and the 6 years at Newcastle omitting the autumn period, namely the period up to the end of October, in each year. The analyses each included forty-one variables; one cold variable and the value on the day of reporting and on each of the preceding 7 days, of mean day temperature, water-vapour pressure, relative humidity, cyclonic weather and atmospheric pollution. Temperature and vapour pressure were included as the variables showing the highest correlation with colds. Relative

humidity was also included as being closely related to these. Air pollution was added in view of the considerable current interest in its relation to respiratory disease and finally cyclonic weather as a check since it had shown negligible correlation in the previous analyses.

The effects of relative humidity and cyclonic weather were not significant. A significant positive regression was found, both for London and for Newcastle, with values of atmospheric pollution on the same day only, although closer examination showed that this almost entirely derived from the data recorded for the year 1952/53 in both London and Newcastle. The regressions with mean day

Table 3. *Predicted seasonal swings derived from regression coefficients (reported colds/1000 person-days)*

Variables included		Newcastle, 1951–57	London, 1951–55
Mean day temperature on day	2 only	2·26	1·64
Mean day temperature on day	0	1·46	1·29
	0 and 1	2·02	1·60
	0–2	2·54	1·93
	0–3	2·81	2·02
	0–4	3·05	2·52
	0–5	3·14	2·67
	0–6	3·25	2·86
	0–7	3·53	3·10
Including also water-vapour pressure on day	0	3·71	2·94
	0 and 1	3·74	2·90
	0–2	3·74	2·87
	0–3	3·75	2·93
	0–4	3·76	2·84
	0–5	3·78	2·87
	0–6	3·78	2·98
	0–7	3·78	3·11
Including also atmospheric pollution on day	0	4·11	3·26

The 95 % confidence limits for the figures in the final row of the table are approximately ± 0·7 for Newcastle and ± 1·0 for London.

temperature and water-vapour pressure were negative and highly significant in both sets of data. As non-significant results were obtained with some of the variables this could not be attributed to the large number of degrees of freedom involved. The regression accounted for only about 10 % of the variance. The limited size of the populations studied, about 675 in London and 350 in Newcastle, with a daily average of about five colds reported in London and less than three in Newcastle, leads of itself to a substantial variance equal to at least one half of the gross variance. The variance accounted for by the regression represents therefore at least 20–25 % of the variance potentially explicable in terms of the independent variables.

Using the regression coefficients obtained in these analyses and applying to them the range of variation of the smoothed meteorological variables as given in Table 1, a predicted swing, or half-range of variation, for the seasonal variation in cold

incidence was obtained. The figures are given in Table 3. With the method of analysis employed it is not possible to distinguish the contributions of the several variables independently. Table 3, therefore, serves only to demonstrate that the total seasonal effect accounted for by the regression appears to have reached a stabilized value with that number of variables which have been included in the computations.

For the Newcastle data the seasonal swing predicted in this way, $\pm 4{\cdot}11$, slightly exceeds that of the curve fitted to the averaged weather data, $\pm 3{\cdot}8$ (Table 1). For the London data the predicted value of $\pm 3{\cdot}26$ is appreciably less than that of the weather data curve, $\pm 5{\cdot}5$, but as this curve actually leads to negative values of incidence for the summer minimum the indicated swing of 5·5/1000 person-days would seem to be too large in any case. Taking the two sets of data together the average predicted swing is of the order of 80 % of that of the fitted curves, which represents very reasonable agreement, probably within the statistical errors of the calculations.

DISCUSSION

The analysis of time series is rarely straightforward and when, as in this case, we have no clear idea of the underlying model it is impossible to put the validity of the method beyond question. It is generally necessary to remove the trends from such series in order to minimize the association due to trends common to the several series. There is, however, no way of deciding what the real trends are so that the method of elimination used is a matter of judgement and once the main component of trend is removed it is impossible to distinguish between the remaining trend and serial correlation in the series. All we can say in this case is that the methods of trend elimination used leave residuals having no obvious trends.

Spicer (1959), in his discussion on the poliomyelitis analysis, had two main statistical worries, serial correlation which we have been able to allow for, and the fact that the strength of the relation between the meteorological data and the poliomyelitis incidence varied from year to year, even to the extent that the correlation in one year was negative. We have found some variation in the magnitude of the correlations from year to year but to a much smaller extent; the correlations are identical in sign for each year and, despite the variation, suggest a stable underlying relationship.

There is obviously a strong negative association between the numbers of colds reported as starting on a given day and the mean outdoor day temperature about that time. Our analyses show that this is present not only between the actual values of these quantities but also between their differences from their expected values for that particular day in the year and that this association with temperature is independent of the intercorrelation of temperature with the other meteorological variables studied, i.e. water-vapour pressure, relative humidity, the difference between the maximum day and the minimum night temperature, rain, hours of sunlight, atmospheric pollution, cyclonic or anticyclonic weather. Of these only water-vapour pressure shows a small but perhaps significant independent association with colds. The correlation reaches a maximum value between the colds caught and the temperature difference, i.e. its fall below the expected value for the time

of year, about 3 days before. This time interval is so close to the median interval between inoculation and the development of symptoms as to suggest that some consequence of lowered external temperature exerts a direct influence on the transmission of the infection. This association is independent of any effect of water-vapour pressure and does not, therefore, support the suggestion that the seasonal variation in the numbers of colds is a consequence of change in indoor humidity which might exert drying effects on the mucous membranes of the upper respiratory tract.

If there is any independent association between colds and reduced water-vapour content of the air then it is probably maximal for water-vapour content and colds arising on the same day. There is also some evidence of an association between colds and increased atmospheric pollution on the same day. If this is real, the effect was largely confined to one winter although present in both places at that time. These two effects may reflect some exacerbation of symptoms which influences the day on which the disease becomes apparent.

The regression analysis shows that these associations are sufficient to account quantitatively for most, if not all, of the difference between the summer and winter incidence of colds. This analysis was carried out using the differences of the observed values, both of the numbers of reported colds and of the meteorological variables, from those expected for the time of year. When, however, the regression coefficients obtained in this way are applied to the actual range of temperature, humidity and atmospheric pollution values found over the year, then the predicted range for the numbers of colds reported corresponds remarkably closely to the observed seasonal variation.

The exception to this is the autumn peak in cold incidence. This was very marked in the results from London and moderate, but unmistakable, at Newcastle. Figure 1 shows the average seasonal course in these two places together with data from Cirencester and the Chalke valley, Salisbury. There is little evidence of any such autumn peak in the Cirencester records and none at all from the Chalke valley. The records from both these places, however, show apparent cyclic variations, with a period of about 6–8 weeks, superimposed on the seasonal movement. If real, these may indicate small distinct epidemics each tending to die out with exhaustion of the limited number of susceptible individuals in these small populations. It is interesting and, possibly, of significance that the magnitude of this autumn peak follows the size of the community concerned, which ranges from around 10^7 for London down to a few hundred only in the Chalke valley. No weather variable has a distribution remotely resembling this autumn peak in colds and this, together with its apparent association with community size, suggests strongly that it is related to the immunity state of the population at this time of the year.

The proportion of variation absorbed by the regression analysis is limited and might lead to suspicion that the effects revealed by the analysis are, themselves, of limited importance. Since, however, such a large proportion of the variance is certainly irremovable it may be unrealistic to expect to be able to absorb a much greater fraction from data of this kind.

The striking success of the correlation analysis in reproducing the general shape of the incubation curve and of the regression analysis in predicting the magnitude of the seasonal variation in the incidence of the disease is, however, the main justification for the whole procedure. At no point where we might doubt the validity of the analysis should we expect it to produce effects of this kind unless they were really due to strong association between the variables. The nature and force of this association are probably dependent on the community concerned so that it should occasion no surprise if studies in other climatic regions or in populations with different social habits should lead to results quantitatively or qualitatively different.

We referred in the introduction to the many, but unconvincing, explanations adduced for the seasonal variation in upper-respiratory infections in temperate climates. Our analysis does not, of itself, solve this problem. In so far as the indications of the correlation analysis are a reliable guide we have to look for some effect of low outdoor temperature which promotes transmission of the virus from person to person, or the development of overt disease. A number of possible ways in which cold weather might induce colds are discussed by Andrewes (1964). It has often been suggested that changes in room ventilation consequent upon the seasonal climatic changes might be responsible for a more widespread dissemination of the disease in the colder months of the year when windows are more usually closed. Experimental studies of the effect of artificially increased ventilation (Kingston *et al.* 1962) suggest that this could not produce the observed effects.

The virus might survive better in cold environments, but it seems very unlikely that outdoor survival plays any part in the transmission of the infection. Indoor temperatures on the other hand remain relatively constant.

The low indoor humidities found in cold weather are a consequence of the low absolute outdoor humidity associated with cold weather. This analysis has shown that it is the low outdoor temperature, independent of the humidity, which is associated with the increased number of winter colds. This contradicts any arguments based on virus survival in relation to indoor humidity or on a postulated damaging effect, due to drying, on the mucous membranes, predisposing to the initiation of infection when the indoor humidity falls in cold weather. It is possible that exposure to cold outdoor conditions produces physiological changes, in the membranes of the respiratory tract or elsewhere, which promote the transmission of the infection. This might take place either through increased dispersion of infected secretion from an infected individual, caused by increased volume of secretion, or increased tendency to sneezing, etc., or through some increase in susceptibility of the relevant sites so that infection is more easily initiated or perceived.

SUMMARY

An investigation has been made of the association between weather and the numbers of colds reported on a given day. The seasonal trends were eliminated by working with the differences between the observed values on any day and the expected values derived from smooth curves fitted to the averages for the time of year.

Examination of nine weather variables for the day on which the colds were reported and for each of the 29 preceding days showed that only two, mean day temperature and water-vapour pressure at 9 a.m., were significantly correlated with the numbers of colds. Partial correlation studies showed that the strongest association was with lowered mean day temperature between 2 and 4 days before the reported onset of symptoms.

Regression analysis demonstrated that the magnitudes of the associations were sufficient to account for the greater part of the seasonal variation in the incidence of the common cold in both London and Newcastle. A small effect of atmospheric pollution appeared in this analysis.

These results suggest that some effect of low outdoor temperature promotes transmission of the virus or the development of disease.

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