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Trachoma Viruses Isolated in the United States* 2. Assay of Egg Infectivity and Stability. (26056)

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Current virologic activity in the field of trachoma was started by T'ang *et al.*(1) who succeeded in growing Chinese strains of trachoma virus and studying their characteristics. The methods of these investigators were rapidly confirmed and trachoma viruses have now been isolated from patients in many parts of the world(2,3,4,5,6,7). However, there have been discrepant reports on the reliability of egg infectivity assays. T'ang(1) obtained regular and reproducible egg LD_{50} values, but Collier and his associates(8,9) stated that "the infectivity of the inocula used cannot be given in terms of 50% end-points" because egg titrations gave very variable results.

When we began to isolate trachoma viruses from patients in the United States(6,7) it seemed important to evaluate the reproducibility of infectivity titrations of our isolates. We wish to record here such observations, together with some stability characteristics of these viruses.

Materials and methods. Viruses: Three representative strains of trachoma virus isolated in the United States(7) were employed. To evaluate possible strain-specific characteristics, pools from 2 yolk sac passage levels (YS) of each isolate were employed, with the following egg LD_{50} titers: Strain BOUR, YS 10 ($10^{-6.4}$), YS 12 ($10^{-4.4}$), strain ASGH, YS 7 ($10^{-7.4}$), YS 8 ($10^{-6.7}$), strain APACHE #1, YS 7 ($10^{-6.7}$), YS 8 ($10^{-5.8}$).

Preparation of stock virus: Virus dilutions estimated to kill 50% of eggs in 6 or 7 days were inoculated in 0.5 ml volume into yolk sacs of 7-day embryonated eggs. Eggs were incubated at 35°C and candled twice daily. Deaths occurring during the first 48 hours of incubation were discarded. When 50% of the inoculated eggs had died, remaining live eggs were refrigerated for 3 hours. Yolk sacs were then harvested aseptically, pooled, weighed, vigorously shaken with glass beads for 5 minutes, and diluted with sterile broth

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TABLE I. Sample of Egg Infectivity Titration: Trachoma Strain Asgh YS 7; Jan. 27, 1960.

Virus dilution	No. eggs inoc.	No. non-specific deaths (48 hr)	Specific deaths on day	Specific deaths Total
10^{-3}	5	1	5, 6, 6, 6	4/4
10^{-4}	5	0	6, 7, 7, 7, 8	5/5
10^{-5}	5	2	8, 9, 9	3/3
10^{-6}	5	0	8, 9, 10, 11, 12	5/5
10^{-7}	5	1	9, 9, 9	3/4
10^{-8}	5	0	11	1/5

$LD_{50} = 10^{-7.1}$.

(Difco proteose-peptone #3) containing 4 mg/ml streptomycin to make a 50% suspension. This stock suspension was distributed in screw-capped vials and stored at –40°C in a mechanical deep freeze.

Infectivity titrations: A vial of stock virus was rapidly thawed, 10-fold dilutions were prepared in broth and kept in an ice bath until inoculated. Five-tenths ml of each dilution was injected by the yolk sac route into groups of 7-day embryonated eggs, incubated at 35°C and candled twice daily. Deaths occurring during the first 48 hours were discarded as non-specific. All deaths from the third to the twelfth days were recorded. Random yolk sacs from dead eggs in each group were smeared and stained by Macchiavello's method to confirm microscopically presence of virus. Such virus was seen regularly in eggs dying on or after the 6th day of incubation. Deaths between day 3 and 5 of incubation were exceedingly rare, and in them virus was seen infrequently. Fifty percent death endpoints were calculated by accepted methods(10) and expressed as the infective titer per g of yolk sac.

Results. After initial isolation in eggs, our strains required several additional passages before infective titers were uniformly high. After being "established" in eggs, infectivity titrations appeared to be reproducible. A representative titration (Table I) shows that the distribution of deaths is proportional to dose. In groups of eggs receiving more than 50 LD_{50} there were rarely any survivors. Repeated assays were performed with a single pool of virus stored at –40°C for 5 months as a 50% yolk sac suspension in broth (Table II). Using only 5-6 eggs per 10-fold dilution, the range of LD_{50} values ($10^{-6.7}$ to $10^{-7.5}$) and range of average days of death for the 10^{-5} dilution (7.0 to 8.7 days) was quite constant for the 8 titrations performed between Jan. 20 and April 6. Subsequently slightly lower titers were found, perhaps because of true loss of infectivity upon prolonged storage at –40°C. Pools of other trachoma viruses have given similarly constant results.

When psittacosis viruses are titrated in eggs, time of death is inversely related to the infective virus inoculum and if sufficiently large groups of eggs are employed, infectivity assays may be performed by observing the average day of death for a single virus dilution(11). When groups of 50 to 100 eggs were inoculated with 10^3 to $10^{4.4}$ egg LD_{50}/ml of stock trachoma virus in preparation of pools, 6-7 days elapsed before 50% of the eggs had died, with variation up to 24 hours. We have not used sufficiently large groups of eggs to establish the "average day of death" for trachoma viruses with great precision. Fig. 1 shows average day of death of small groups (5-6 eggs) inoculated with varying doses of 3 strains of trachoma viruses. It is apparent that in general the linear inverse relationship between amount of virus and time of death applies to trachoma viruses. When average day of death for different virus concentrations was calculated separately for the 2 strains used in most tests the averages fell on straight lines (Fig. 1). It is remarkable that at any dose level, eggs inoculated with strain BOUR died, on the average, one day

TABLE II. Repeated Assay of Egg Infectivity of Trachoma Virus Stored at –40°C.

Date	Egg LD_{50} of stock	Avg day of death of eggs inj. with 10^{-5} dilution
Jan. 20	7.1*	7.5
" 27	7.4	8.6
Feb. 15	7.3	8.2
" 25	6.8	7.9
March 9	7.4	8.7
" 22	6.7	7.6
" 30	7.5	8.4
April 6	7.5	7.0
" 28	6.3	10.4
May 18	6.2	8.6

* Neg. log of dilution of 1 g YS which resulted in death of 50% eggs.

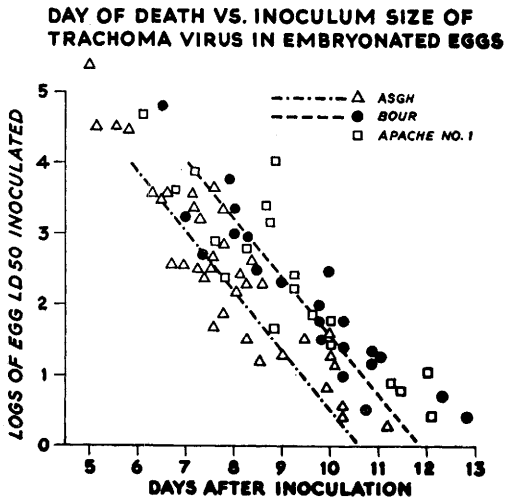

FIG. 1. Relationship of amount of trachoma virus to time of death of inoculated eggs. Each point is observed avg day of death in a group of 5-6 eggs, inoculated with a given amount of one of 3 strains. Lines are fitted to avg day of death calculated from many experiments with each of 2 strains.

later than those inoculated with strain ASGH. This suggests that strain ASGH grows more rapidly to reach a lethal concentration of virus.

Stability. Temperature: Julianelle observed marked temperature lability of infective trachoma virus in the form of fresh conjunctival scrapings(12). T'ang found that in 10% yolk sac suspensions virus was inactivated by incubation at 50°C for 30 minutes or at 37°C for 24 hours, but survived at 4°C for at least 9 days. We stored aliquots of pools of 2 strains of trachoma virus (50% yolk sac suspension in broth, pH 7.1) for various periods at 4, 22 and 37°C and compared the titer by simultaneous titration with that of the same pools stored at –40°C. Loss of infectivity is shown in Table III. Some virus activity survived heating at 60°C for 1 minute, but not for 5 minutes. Inactivation of virus proceeded rapidly at 37°C but there was surprisingly little loss of infectivity at room and refrigerator temperatures. At 4°C titers declined by 0.8 to 1.7 logs in 4 days, and by 1.0 to more than 2 logs in 7 days. Storage at –40°C yielded constant titers for at least 3 months (Table II). Two cycles of freezing and thawing 50% suspensions did not reduce infectivity any more than a single cycle. Virus freeze-dried as 50% suspension of yolk sac in skim milk maintained viability when stored at 4°C for at least 5 months, or when mailed without refrigeration to other parts of the world. A large proportion of viable virus was lost during lyophilization and one blind passage of the freeze-dried material was often required before eggs died with abundant virus in smears.

Ether. When yolk sac suspensions (50% in broth) containing $10^{6.2}$ egg infective LD_{50} per ml were mixed with diethyl ether (30% final concentration) and stored overnight at 4°C no viable virus could be recovered in repeated tests.

Sodium desoxycholate: It has been said that bile salts inactivate trachoma virus(12). Sodium desoxycholate 40 mg/ml was dissolved in 0.25 M sucrose and sterilized by autoclaving(13). Various dilutions of this stock solution were mixed with equal parts untreated 50% suspension of infected yolk sac and the mixture incubated with frequent agitation in a 37°C waterbath for 1 hour. The mixture was then chilled, stained smears prepared, and serial dilutions in broth titrated for infectivity. Up to a final concentration of 20 mg/ml, sodium desoxycholate reduced the infective titer from $10^{7.4}$ to $10^{5.8}$. Whereas Macchiavello or Giemsa stained smears of controls or of mixtures containing 1.2 mg/ml desoxycholate (infective titer $10^{-6.5}$) contained abundant elementary bodies none were seen with concentrations of 2.5 mg/ml desoxycholate or higher. Thus there was gross loss of tinctorial properties at concentrations of desoxycholate which interfered relatively little with infectivity(13).

Discussion. The 3 strains of trachoma viruses used in the present work permitted reasonably precise and reproducible

TABLE III. Loss of Egg Infectivity at Various Temperatures. Avg logs egg LD_{50} inactivated.*

	Hr of storage			
°C	2	7	24	48
4	<.5	<.5	.5	.7
22	.5	.5	.6	.7
37	.8	1.9	3.7	5.1

* Difference in infectivity titer (negative log of dilution) of material kept at –40°C and material stored at indicated temperature.

titration of egg infectivity. This work was performed when egg susceptibility was uniform and could not have been done during the season of partial egg resistance which seems to occur during the late summer or fall months(7). Similar partial egg insusceptibility has been noted by others working with psittacosis viruses(14). Apart from this seasonal variation, the mechanism of which is not understood, we were able to obtain reliable 50% endpoints in defining the infectivity of inocula in apparent contrast to the experience of the Lister Institute workers (8,9). The general inverse linear relationship between infective inoculum and time of egg death applied to our trachoma viruses. However, small but definite variations were observed in time of death from a constant inoculum. In spite of this slight heterogeneity in egg response, it was shown clearly that strain ASGH tended to kill eggs more rapidly than strain BOUR, at all inoculum levels. This difference is interesting because these 2 strains differ markedly in other respects, such as severity of experimental disease in monkeys and behavior toward antibiotics. In view of the heterogenous clinical picture of trachoma in various parts of the world such strain differences deserve to be explored further. Maintenance of viability by lyophilization should permit ready exchange of representative strains between different laboratories.

The relative stability of trachoma viruses at 4°C confirms the use of "wet ice" as possible transport medium for specimens(2,8). The rapid loss of infectivity at 37°C parallels other viruses of the psittacosis group. The destruction of our trachoma viruses by ether is in keeping with the ether lability of other psittacosis-like viruses but contrasts with T'ang's statement that "ether . . . possesses no effect on the virus"(1). Sodium desoxycholate interfered with tinctorial properties of the virus far more than with infectivity, in the presence of massive amounts of yolk sac components. While the presence of these protective materials does not permit conclusions concerning the anti-viral effects of desoxycholate, the differential action merits further study with purified virus suspensions(13).

Summary. Except for the seasonal insusceptibility of eggs during late summer and fall, reliable and reproducible infectivity assays were performed on 3 representative strains of trachoma virus isolated in United States. In general the linear inverse relationship between amount of virus and time of death applied to these trachoma viruses. One strain (ASGH) tended to kill eggs one day earlier than another strain (BOUR) at all inoculum levels. The strains were ether labile, and rapidly lost infectivity at 37°C or above. Sodium desoxycholate interfered with staining properties of virus far more than with infectivity.

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