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Relationship of Plaque Size and Virulence for Chickens of 14 Representative Newcastle Disease Virus Strains[1]

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Ability of 14 Newcastle disease virus strains to produce large plaques was related to virulence for chickens. Plaque-size comparisons were made under standard conditions in chick embryo cell monolayers. All plaque-producing strains showed a range of plaque sizes modified to a degree by the overlay medium used. An increase in size was found for most strains under methyl-cellulose overlay medium. Markedly larger plaques were found under this medium for both Calif-RO and Calif-CG strains. Heterogeneity in plaque size was most pronounced in velogenic (high virulence) strains. Only populations of small plaques were found in mesogenic (intermediate virulence) strains, and plaques were rarely found in lentogenic (low virulence) strains. Statistical analysis showed that the plaque size of velogenic strains differed significantly from mesogenic strains. None of the 11 plaque-producing strains had a normal distribution of plaque sizes, owing primarily to the presence of different genotypes within the plaquing population of a strain. This was demonstrated by derivation of clones from two of the strains. The populations of the large (Herts L) and small (Herts S) clear plaque clones derived from Eng-Herts were homogenous and distinct from one another on the basis of plaque size. Herts L was more virulent than Herts S. Although Herts L became more heterogenous in respect to plaque size upon repeated passage in embryonated eggs, no decrease in virulence of the strain was observed.

Strains of Newcastle disease virus (NDV) have been grouped on the basis of virulence for chick embryos for 1-day-old chicks and for adult chickens (6). Velogenic strains rapidly kill adult chickens, irrespective of route and dose, and cause death in chick embryos within 40 to 60 hr. Mesogenic strains produce mild or severe signs of disease but cause deaths in adult birds only when massive inocula are given. Lentogenic strains produce only slight respiratory signs in chickens and require 100 or more hr to kill chick embryos.

We have shown previously that the plaque composition of NDV varies with the strain. Plaques may differ in size and type or degree of clarity. Greater plaque diversity (2 to 4 types) was found in velogenic strains than in mesogenic strains, which often had only a single plaque type (Schloer and Hanson, Am. J. Vet. Res., *in press*).

[1] Part of a dissertation submitted by the senior author in partial fulfillment of the requirement for a Ph.D. degree from the University of Wisconsin.

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The virulence of clones of representative plaques derived from two velogenic strains was studied. Whereas no difference in virulence was found between red and clear type clones of the Italy-Milano strain (Schloer and Hanson, Bacteriol. Proc., p. 132, 1966) the large and small size clones from Eng-Herts were found to differ in virulence (Schloer, Bacteriol. Proc., p. 140, 1964).

Variation in plaque size of NDV strains has been reported previously (3, 5), and Granoff has described a degree of correlation between virulence for chick embryos and size of plaque (6, 7; A. Granoff, Bacteriol. Proc., p. 74, 1955). This paper examines the distribution of plaque sizes in a group of strains representing different degrees of virulence and the properties of clones that differed on the basis of plaque size.

MATERIALS AND METHODS

Viruses. Strains used in this study were obtained from the NDV repository at the University of Wisconsin. Passage history, mean death time, and average titers of these strains are listed in Table 1. The preparation of virus stocks in 10- to 12-day-old

TABLE 1. *Passage history, mean death time, and PFU/EIU (plaque-forming units/egg-infectious units) ratios of representative NDV strains*

NDV strain	Year of isolation	No. of passages at Univ. of Wisconsin	Mean death time[a]	No. of determinations[b]	Avg titer (EIU/ml)	Avg titer (PFU/ml)	EIU/PFU[c]
Velogenic							
Calif-CG	1946	15	54	3	2.8×10^9	5.8×10^8	4.8
Calif-RO	1944	15	51	5	8.9×10^9	3.0×10^8	29.7
Eng-Herts	1933	5	45	4	1.6×10^9	2.9×10^8	5.5
Italy-Milano	1945	12	57	6	5.5×10^9	8.6×10^8	6.4
Kan-Man	1948	11	59	4	4.7×10^9	4.1×10^8	11.5
Texas-GB	1948	8	56	4	5.0×10^9	5.0×10^8	10.0
Mesogenic							
Mass-MK	1945	12	66	4	1.7×10^9	1.4×10^9	1.2
NY-Jones	1948	9	67	3	1.3×10^9	1.1×10^9	1.2
NJ-Roakin	1946	16	75	4	3.5×10^9	5.5×10^8	6.4
Texas-DK	1958	4	63	4	—[d]	3.2×10^8	—
Eng-P3R10	1945	4	79	3	1.6×10^9	1.2×10^9	1.3
Lentogenic							
B1	1949	14	111		2.4×10^9	—[e]	
Eng-F	1949	5	101		6.9×10^8	—	
NJ-LaSota	1946	5	104		2.2×10^8	—	

[a] Mean death time in embryonated eggs; figures represent an average of three or more determinations.
[b] Number of replicates used in calculation of EIU/PFU ratio.
[c] Ratios represent an average of ratios obtained from individual virus stocks.
[d] Not tested.
[e] Plaques not found at 96 hr.

chick embryos was described previously (Schloer, Ph.D. Thesis, Univ. of Wisconsin, Madison, 1965). Virus was stored in 1-ml amounts at −20 C.

Embryonated egg assay. Titrations were done in 10- to 12-day-old embryonated eggs. The LD_{50} (median lethal dose) end point was determined from groups of eggs given 10-fold virus dilutions, with five eggs per group. The egg-infectious units (EIU) were calculated from the LD_{50} titer. Both the LD_{50} and mean death time (MDT) of the minimal lethal dose (10) were estimated from the same titration. The MDT for lentogenic strains is 90 to 150 hr, for mesogenic strains, 60 to 90 hr, and for velogenic strains, 40 to 60 hr (6).

Cell cultures. Primary chick embryo cell (CEC) cultures were prepared from 9- to 10-day-old embryos as described by Schloer (Ph.D. Thesis, Univ. of Wisconsin, Madison, 1965). Monolayers for estimation of plaque titers were inoculated 24 hr after seeding. For studies on plaque morphology, they were inoculated 48 hr after seeding.

Plaque assay. Fivefold dilutions of virus were made in cold phosphate-buffered saline. Monolayers were inoculated with 0.1 ml of virus by using two dilutions with three replicates per dilution. After adsorption for 30 min at 37 C, they were washed with Medium 199. Each washed plate was overlaid with 5 ml of Medium 199 containing 5% calf serum and 0.9% purified agar. At 3 days postinoculation, each monolayer again received 5 ml of medium containing 2.5% serum and 0.001% neutral red. Titers were determined from an average count of the six plates.

Isolation of virus clones. Details of the isolation procedure have been described (16; Schloer and Hanson, Am. J. Vet. Res., *in press*). Only well-separated plaques were picked. If morphology was maintained after three successive steps of purification, then plaques were again picked and a sample was injected into 10-day-old embryonated eggs. Fluids were harvested, clarified by low-speed centrifugation, and stored at −20 C. Clones were obtained from two NDV strains. Herts L and Herts S were derived from large and small, clear plaques, respectively, from Eng-Herts; Milano Cl was derived from a large, clear plaque of Italy-Milano.

Plaque size determination under agar-overlay medium. For experiments on plaque size, 0.1 ml containing 20 to 80 plaque-forming units (PFU) of the respective virus was inoculated onto each of five monolayers. After adsorption for 60 min at 37 C, 5 ml of modified (formula obtained from H. Temin, McArdle Laboratories, Univ. of Wisconsin) Eagle's medium containing 5% bovine serum and antibiotics in 0.9% purified agar was added to each culture. At 3 days postinoculation, each plate was given 5 ml of this overlay medium containing 2.5% serum and 0.001% neutral red.

Plaque sizes were measured 4 days after inoculation, when maximal titers are attained. Since some variation in plaque size occurs from one experiment to another, the size of 10 strains and three clones was compared in one experiment. The Calif-RO strain was examined separately. The five replicates of each virus were photographed at 96 hr postinoculation, and black and white prints enlarged twofold were made of all photographs. Measurements of the

diameter of all plaques on a print were made, to the nearest 0.5 mm, with a millimeter ruler under the lens of a Quebec colony counter.

Plaque size of the different egg passages of the Herts L and S clones were determined in another single experiment. Measurements were made at 88 to 90 hr postinoculation directly from stained monolayers.

Histograms of the plaque size distribution were made for all of the strains and clones. Calculations were made of the mean and standard deviation of plaque size for each virus.

Effect of overlay medium on plaque size. Three different overlay media were compared in the same experiment. For each NDV strain, 10 to 20 PFU of virus were inoculated into each of 12 monolayers. After 60-min adsorption at 37 C, three groups of four monolayers each were overlaid with 5 ml of medium for each group. The control group received the modified Eagle's medium, the second group received 200 μg/ml of diethylaminoethyl dextran in the stock medium, and the third group received the stock medium in which 1.5% methyl cellulose was substituted for the 0.9% agar present in the previous medium.

Cultures with the two agar overlays were stained at 3 days as described previously. Cultures with methyl cellulose were fixed at 96 hr postinoculation with Formalin-acetic acid-alcohol and stained with a dilute solution of crystal violet. Plaques were measured directly from the stained petri dishes. In some instances, they were photographed and measured as described previously. Since plaques tend to be less symmetrical under methyl-cellulose medium, the smallest diameter was measured. Plaque size was therefore underestimated, compared to the size obtained under an agar-overlay medium.

RESULTS

Comparison of EIU and PFU titers. For velogenic strains, plaque titers in CEC monolayers

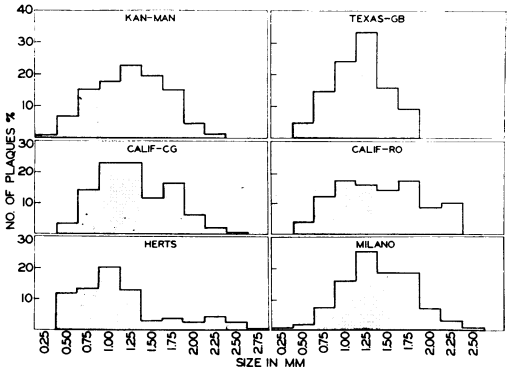

FIG. 1. *Distribution of plaque sizes found within the populations of representative velogenic strains of NDV. Note the diversity and range in size of plaques within each strain. Plaque diameters were measured with a millimeter ruler from photographs enlarged ×2. Data for Fig. 2, 5, and 6 were obtained in the same manner.*

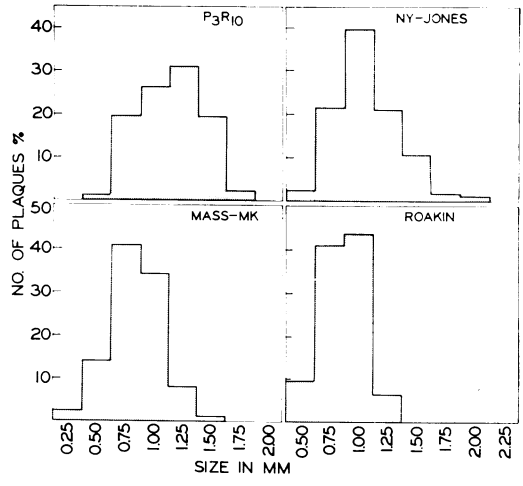

FIG. 2. *Distribution of plaque sizes found within the populations of representative mesogenic strains of NDV. Note the decrease in size and diversity of plaques within the strains as compared to velogenic strains in Fig. 1.*

were always lower than the titers in embryonated eggs. This is expressed in an elevated EIU/PFU ratio. Both EIU and PFU titers of the more virulent strains were reproducible for a given stock of virus. In contrast, titers could not be calculated for lentogenic strains, because plaques were seldom found in CEC monolayers, and marked fluctuations in egg titers sometimes occurred owing to the erratic pattern of death in chick embryos. In most instances the ratios for mesogenic strains approached unity.

Plaque size under agar-overlay medium. Velogenic strains had a greater mean plaque size compared to mesogenic strains (Table 2); large plaques were absent in mesogenic strains (Fig. 1 and 2). Large plaques under the conditions of these experiments were those with a diameter of 1.75 mm or greater. None of the mesogenic strains had a significantly large class of plaques of this size, whereas most had a predominant class of 0.75 mm. The distribution and principal class of P3R10 more closely resembled that of the virulent strains. On the rare occasions when lentogenic strains produced plaques in chick embryo monolayers, the plaques were less than 1 mm at 5 days postinoculation.

Lack of symmetry in the plaque-size distribution indicated the presence of subpopulations within a strain. Irregularity within Eng-Herts and Calif-CG suggested the presence of two subpopulations and of three subpopulations within Calif-RO. Plaque distributions in Italy-Milano and Texas-GB appeared symmetrical.

Plaque size under agar containing diethylamino-

TABLE 2. *Comparison of plaque size of representative NDV strains under agar and methyl-cellulose media*

NDV strain	Plaques under agar[a]				Plaques under methyl cellulose[b]				Significance of size under methyl cellulose and agar		
	No. of plaques (n_1)	Mean size (x_1)	Variance (s_1^2)	Standard deviation (s)	No. of plaques (n_2)	Mean size (x_2)	Variance (s_2^2)	Standard deviation (s)	$(\bar{d})^d$	(f^e)	$(t')^f$
Velogenic											
Calif-CG	160	1.285	0.177	0.421	16	2.376	0.562	0.750	0.890	3.18**[g]	5.73**
Calif-RO	50[c]	1.35	0.242	0.492	27	2.667	0.520	0.721	1.317	2.15**	8.48**
Eng-Herts	310	1.27	0.248	0.498	46[a]	2.093	0.492	0.701	1.223	1.98**	7.67**
Italy-Milano	196	1.42	0.203	0.450	39[a]	1.705	0.266	0.515	0.185	1.31[nsg]	3.51
Kan-Man	299	1.235	0.105	0.324	—	—	—	—	—	—	—
Texas-GB	113	1.16	0.100	0.315	55	1.60	0.249	0.499	0.34	2.50**	5.96**
Mesogenic											
Mass-MK	197	0.836	0.055	0.234	21	1.643	0.324	0.569	0.807	5.92**	6.45**
NY-Jones	282	0.82	0.026	0.163	56	1.625	0.261	0.511	0.805	9.88**	11.69**
NJ-Roakin	132	0.614	0.035	0.188	33	1.152	0.113	0.336	0.538	1.79[ns]	12.29**
Eng-P3R10	77	1.14	0.025	0.157	42	1.357	0.280	0.525	0.217	3.34**	0.26[ns]

[a] Plaque diameter measured from photographs enlarged × 2.
[b] Plaque diameter measured directly from stained petri dishes.
[c] Plaque diameter obtained from separate experiment.
[d] $x_1 - x_2 = \bar{d}$.
[e] F test of larger variance/smaller variance.
[f] $t' = \bar{d}/s_{\bar{d}}$, where F is significant then $s_2 = (s_1^2/n_1) + (s_2^2/n_2)$; where F is not significant $s_2 = s^2(n_1 + n_2)/(n_1 n_2)$ and $s^2 = [(n_1 - 1)s_1^2 + (n_2 - 1)s_2^2]/[(n_1 - 1) + (n_2 - 1)]$.
[g] Highly significant = **; not significant = ns.

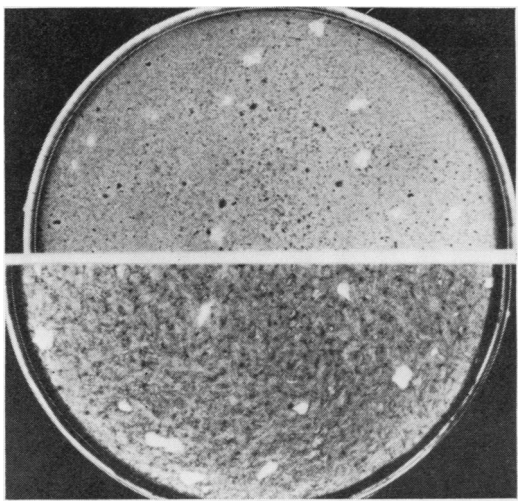

FIG. 3. *Texas-GB strain at 96 hr postinoculation. Enlarged ×2. (Top) Plaques under agar-overlay medium; (bottom) plaques under methyl-cellulose overlay medium. Note that the plaque diameter is nearly the same under both overlay media.*

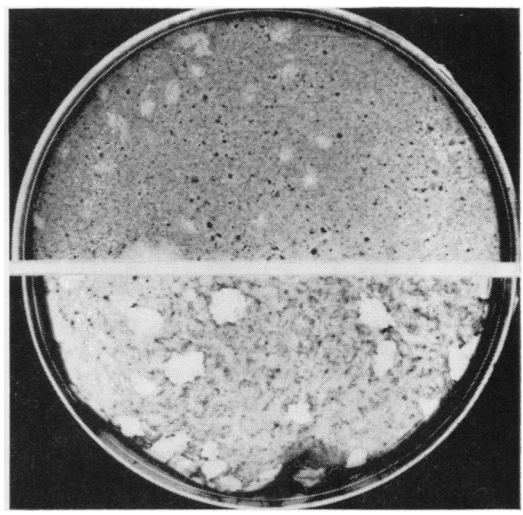

FIG. 4. *Calif-RO strain at 96 hr postinoculation. Enlarged ×2. (Top) Plaques under agar-overlay medium; (bottom) plaques under methyl-cellulose overlay medium. Note the marked increase in the diameter of some of the plaques under the methyl-cellulose overlay medium.*

ethyl dextran. No change in plaque size or type was found when 200 μg/ml of diethylaminoethyl dextran was added to the agar-overlay medium, nor did it increase the plaquing ability of lentogenic strains.

Plaque size under methyl-cellulose overlay. Plaques of all strains were found to be larger under methyl-cellulose than under agar-overlay medium. Some strains, like Texas-GB, showed

TABLE 3. *Comparison of plaque size of velogenic and mesogenic strains under two kinds of overlay medium*

	Plaques under agar		Plaques under methyl cellulose	
	Velogenic (1)	Mesogenic (2)	Velogenic (1)	Mesogenic (2)
No. of plaques measured (n)	1,122	684	178	149
Pooled mean ($\bar{x}$)	1.287	0.8523	2.852	1.444
Pooled variance (s^2)	0.1769	0.036	0.3742	0.2430
F^a	4.90**[e]		1.54**	
$\bar{d}^b$	0.4347		1.4074	
$s_{\bar{d}}{}^c$	0.1445		0.735	
t'^d	30.06**		19.15**	

[a] F test of larger variance/smaller variance.

[b] Difference (d) between means where $\bar{d} = \bar{x}_1 - x_2$.

[c] Standard deviation ($s_{\bar{d}}$) of the difference between two means where $s_{\bar{d}} = s_1^2/n_1 + s_2^2/n_2$. The symbols used are listed in the body of the table. Subscripts 1 and 2 refer to velogenic and mesogenic values, respectively.

[d] t prime (t') test for significant difference where $t' = d/s_{\bar{d}}$

[e] Highly significant difference**.

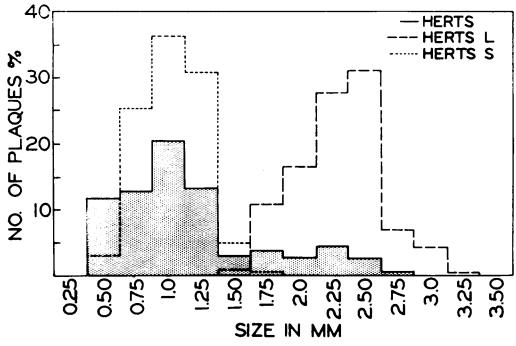

FIG. 5. *Distribution of plaque sizes of the parental Eng-Herts strain as well as the Herts L and Herts S clones derived from this strain. Note that the two clonal plaque populations are distinct from each other and that each contains but a portion of the plaques of the parental population.*

only a slight increase in size (Fig. 3), whereas others, like Calif-RO and Calif-CG, had many plaques as large as 3.5 mm, compared to a maximum of 2.5 mm in the presence of agar medium (Fig. 4). In addition to size, methyl-cellulose medium also affected plaque type. Eng-Herts and Italy-Milano strains, which had many red and clear plaques under agar overlay, appeared to have only clear plaques in this medium.

Statistical anlaysis of plaque size distribution under agar and methyl-cellulose overlay media. The distribution of plaque sizes can be tested for normality by calculating the accuracy of fit of a population to the normal curve (14). Mesogenic strains had too few classes of plaque size for comparison. All of the velogenic strains were found to have a non-normal distribution of plaque size. Both Eng-Herts and Calif-RO deviated greatly from normal, whereas statistical tests showed, as do the histograms, that the Italy-Milano and Texas-GB strains were most nearly normal. However, Kan-Man, which appears visually to have a symmetrical distribution, is not at all normal statistically.

The degree of variability found in the mean plaque-size measurements is expressed in the variance. Thus, Calif-RO and the Eng-Herts, with the greatest diversity of size, have the greatest variance (Table 2). Heterogeneity of size increased in the presence of methyl-cellulose medium, but the increase in variance was not significant for Italy-Milano and NJ-Roakin. This may indicate a uniform response of these two strains to the methyl-cellulose medium and to the agar medium. The increased variability of most strains suggests that they have plaques which differ in response to agar inhibition. This was evident in the Calif-RO and Calif-CG strains.

A comparison of average plaque diameters

TABLE 4. *Comparison of the distribution of plaque sizes of two clones derived from the Eng-Herts strain with the distribution of the parental strain*[a]

Virus stock	No. of plaques counted	Mean size	Variance	Standard deviation	Coefficient of variability
		mm			
Eng-Herts	310	1.270	0.248	0.498	39.2
Herts L	126	2.291	0.112	0.335	14.6
Herts S, 126	182	1.030	0.080	0.238	23.1

[a] Plaque diameter measured from photographs enlarged × 2.

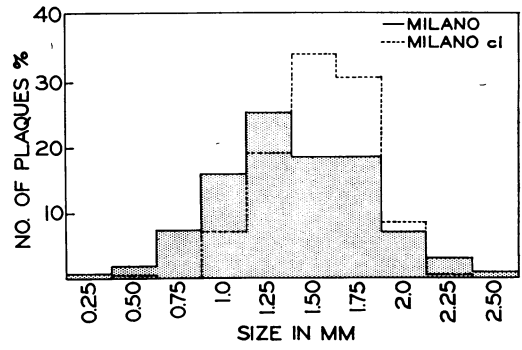

FIG. 6. *Plaque population of the Italy-Milano strains and the Cl clone derived from this strain. Note that the Cl clone is more homogeneous and has larger plaques than does the parental strain.*

TABLE 5. *Comparison of the plaque size in chick embryo cell monolayers and the mean death time in chick embryos of various embryonated egg passages of the Herts L clone*

No. of passages	No. of plaques measured	Mean size	Variance	Standard deviation	No. of determinations of MDT[a]	Average MDT
2	35	1.95	0.0421	0.205	3	39.8
3	56	2.01	0.2260	0.475	1	44.0
4	124	1.65	0.1498	0.3897	1	42.5
5	78	1.77	0.1427	0.378	3	42.4

[a] MDT = mean death time.

TABLE 6. *Comparison of plaque size in chick embryo cell monolayers of various passages and isolates of the Herts S clone*

No. of plaque purifications	No. of egg passages	No. of plaques counted	Mean size ($\bar{x}$)	Variance (S^2)	Standard deviation (S)	Coefficient[a] variability
						%
3	3	80	0.88	0.0913	0.30	34.1
3	4	37	1.04	0.1693	0.25	24.0
3	6	133	1.19	0.1464	0.27	22.7
6	0	78	1.03	0.0954	0.31	30.0
2	0	112	1.17	0.1154	0.34	29.0
2	0	103	1.26	0.1136	0.34	27.0

[a] Coefficient of variability = $100\ S/\bar{x}$.

showed that velogenic strains were significantly larger than mesogenic strains, when tested under either agar or methyl-cellulose overlay medium (Table 3).

Plaque size of clones compared to strains. Figure 5 illustrates that Herts L and S each contains only a part of the population present in the parent Eng-Herts strain. In the parental strain, the most frequent class of the small plaque subpopulation was a 1.0-mm plaque, whereas, in the large plaque subpopulation, it was a 2.25-mm plaque. A parallel was found in the clones. Herts S had a peak at 1.0 mm, whereas Herts L had a peak at 2.25 mm. There was no overlap in the mean plaque size and standard deviation of the two clones. Thus, a clear separation of two populations was achieved by plaque isolation.

Table 4 shows that there was a decrease in the variance in the two clones as compared to the Eng-Herts population. In addition, the Milano Cl clones had a larger plaque size and lower variance than did the parental strain (Fig. 6). The latter population was statistically nearly normal.

Stability of plaque-size character upon passage in chick embryos. Some change in plaque size occurred upon serial passage of Herts L in embryonated eggs (Table 5). Increased variance owing to the presence of small-plaque mutants was random. Variance in a third passage was higher than that found in a fourth and fifth passage. However, passage 4 and 5 originated from a third passage that was different from the one examined. The population of plaque sizes of all these passages of Herts L were distinctly different from the parent strains.

The variation in mean-plaque size of different egg-passage populations of the Herts S clones was not appreciably different from that of the population of plaque-purified virus without egg passage (Table 6). Numerous attempts failed to isolate large-plaque revertants from this clone.

Comparison of virulence in embryonated eggs and plaque size in CEC monolayers. The embryo MDT found for these strains confirmed their classification as velogenic, mesogenic, and lentogenic strains (Table 1). Increased plaque size was associated with embryo virulence, but no direct correlation could be made owing to the diversity of size within most strains (Fig. 1 and 2). Strains of low virulence seldom produced plaques.

Plaque size and mean death time can be compared in clones. Embryo death with Herts L was 20 hr faster than with Herts S, and the average plaque size of Herts L was more than twice that of Herts S (Table 4). The presence of small plaques within the Herts L clone did not affect MDT. Moreover, there was only a slight difference in MDT of the large plaque clones, compared to the parent strain (Tables 1 and 5).

DISCUSSION

In our previous report, we showed that NDV strains may contain several types of plaques ranging from red to clear, the greatest plaque

diversity being found in velogenic strains (Schloer and Hanson, Am. J. Vet. Res., *in press*). This was further substantiated in this study of six velogenic strains and four mesogenic strains. The latter had populations of small plaques. It may be argued that the heterogeneity is caused by accumulation of mutants during laboratory passage. However, two or three types of plaques have been found in the second and third passage of virus after recovery from field cases (*unpublished data*). This suggests that plaque heterogeneity is not peculiar to laboratory cultures.

The presence of subpopulations within strains was readily seen by examination of histograms showing plaque-size distribution. The reality of these subpopulations was shown by the isolation of large and small plaque clones from Eng-Herts. Plaque-size diversity is thus a reflection of genetic differences within this strain. These results indicate that all of the velogenic strains examined are genetically mixed populations.

Analysis for normality is dependent on a sufficient number of classes and accurate measurement (14), both of which were readily obtained for the large but not for the small plaque. As a result, tests for homogeneity could not be made within clones or strains with populations of small-sized plaques. Analysis of large-plaque populations for normal distribution was useful for the detection of heterogeneity not evident by visual inspection of histograms.

Increased uniformity of the population was found for NDV clones as compared to their parent strains. Similar results have been reported for foot-and-mouth disease virus (13). A normal distribution of plaque size (8) was found for a clone of vaccinia virus IV; this was true of both Milano Cl and Herts L populations. This suggests that, under ideal conditions of measurement, a normal distribution may be evidence for genetic similarity of the population.

Homogeneity of plaques may not always be an indication that a strain consists of a single genotype. Inhibitors may obscure the expression of differences. The unusually large plaques of Calif-CG and Calif-RO seen under methyl cellulose overlay were not evident under agar medium. Sulfated polysaccharides present in agar have been shown to inhibit encephalomyocarditis (15), polio (1), and dengue viruses (12). This inhibition has been reversed by the use of diethylaminoethyl dextran added to the agar medium (2, 7, 11). However, no detectable reversal of the Calif-CG and Calif-RO was found by the addition of this compound. Inability to find plaques of lentogenic strains in the presence of diethylaminoethyl dextran suggested that the sulfated polysaccharide inhibitor was responsible only partly or not at all. Subsequent work has shown that Mg^{++} as well as other substances in addition to diethylaminoethyl dextran are needed for a reproducible plaque assay for lentogenic strains (Barahona and Hanson, Avian Diseases, *in press*).

The range in plaque size which Granoff found for three NDV strains (5) does not differ materially from what we have observed. But, in contrast to what he reported, we could readily isolate clones which differed in size distribution from the parental strains. Presumably, both his RO and Milano and our Calif-RO and Italy-Milano originated from the same source, but it is possible that passage conditions which preceded his work and ours may have selected different populations.

Cloning will not decrease size distribution or variance if the original population was uniform, as was evident in the recloned lines of the Herts S clone. Large plaques were not seen or isolated from any passage of the Herts S clone or from any of the mesogenic strains. On the other hand, small-plaque mutants were regularly found in Herts L and were readily isolated. These results differ from those of Durand and Eisenstark (3), who found that after a single egg passage their plaque clones reverted to the heterogeneity of the parent strains. Other workers have also isolated large-plaque revertants from NDV clones of small plaque size (4, 5, 16). However, small-plaque clones were stable if they had been derived from virus after mutagenic treatment. Our Herts S clone population was either unusually stable or more readily separable from the parent population than were clones obtained by others. Part of the difficulty in obtaining stable clones may have been due to the failure to recognize size as a quantitative character expressable in terms of a mean and standard deviation.

The high EIU/PFU ratios found in most velogenic strains may be caused by the presence of red plaques which are difficult to detect and by differences in maximal adsorption time of subpopulations, as has been revealed by study of clones (Schloer and Hanson, Bacteriol. Proc., p. 132, 1966). Presumably, under optimal conditions for the plaque assay, EIU/PFU ratios of velogenic strains would be expected to approach unity as found in other strains (9) and clones (Schloer, Ph.D. Thesis, Univ. of Wisconsin, 1965).

Virulence of the strains studied was related to the presence of large plaques within the population, even when these plaques were a minor component of the population as shown in Eng-Herts. The presence of small-plaque mutants did not appreciably reduce virulence, as could be seen in the passages of Herts L. But absence of large plaques, either in clones or strains, was associated with a reduction in virulence for the chick embryo.

Embryo virulence of these clones was also correlated with virulence for adult chickens (Schloer, Bacteriol. Proc., p. 140, 1964), a relationship that has been previously established for strains (Hanson, Ph.D. Thesis, Univ. of Wisconsin, 1949).

An association between embryo virulence and plaque size was also observed by Granoff (4, 5), but he found two exceptions in the I-125 strains and in some clones from the Milano strain (5). Viruses in both instances were virulent for chick embryos, although plaque size was small. It is tempting to speculate that these plaques were small owing to agar inhibitors. Nonetheless, in the majority of cases studied by Granoff, and in our studies (Schloer, Ph.D. Thesis, Univ. of Wisconsin, 1965), plaque size and virulence appear to be related.

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