Influence of Amount of Test Virus on Quantitative *in ovo* Canine Distemper Virus Neutralization Test. (27397)

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The *in ovo* method of Baker, Gorham and Leader(1) for quantitatively determining canine distemper virus (CD) neutralizing antibodies has been used to measure the immune reaction of dogs after natural infection(2) and after immunization with different types of modified live virus and inactivated vaccines (2-9). It has also been used to measure the antibody content of hyperimmune canine serums and globulin concentrates(10-13), and the distemper virus neutralizing ability of human serums(11,14).

The CD titers reported by different investigators are not always directly comparable because of variables in the test, use of modified technics, and variations in method of interpretation.

Baker, Gorham and Leader(1) found that a 10-fold decrease in 50% egg infectious dose of virus (EID_{50} 1300 and 130) produced an 8-fold increase in CD neutralization titer; however, results with intermediate levels of test virus did not show this correlation. They concluded that for reproducibility of serum titrations within a narrow range the virus titer must be controlled within closer limits. Subsequently, Karzon(11) and Gillespie *et al.*(5) reported an inverse linear relationship with a slope of 1 between log serum titer and log EID_{50} virus. Thus, each 10-fold increase in virus EID_{50} would give a corresponding 10-fold decrease in serum ND_{50}. On the basis of this inverse linear relationship, it has been proposed(15) that results of CD neutralization tests might be made directly comparable by relating them to a common denominator, for example, 100 EID_{50} of virus. Thus if the amount of test virus either exceeded or was less than 100 EID_{50}, the recalculated ND_{50} of the serum would be proportionately increased or decreased.

Another method recently was introduced (13) whereby CD antibody content is expressed in terms of "antibody units" per ml. This method also is based on the reported inverse linear relationship, and the unit is defined as the least amount of antibody that will neutralize 1 EID_{50} of virus. The number of "antibody units" is calculated by the formula: serum neutralization endpoint $\times$ EID_{50} of virus neutralized. For example, if the neutralization endpoint of a serum is 450 per 0.1 ml against 300 EID_{50} virus, the "antibody units" per ml would be 1,350,000. However, we had previously applied this formula to neutralizing antibody titers that had been obtained in this laboratory with several pooled and individual serums at 2 or more levels of virus EID_{50}, and in no case did the mathematical calculations yield a common result.

The present report concerns an investigation of the influence of virus concentration and incubation time of serum-virus mixtures on the results of the *in ovo* CD neutralization test. The work was directed toward the establishment of standards for performance and evaluation of the test which might permit more direct comparison with the results reported by different laboratories.

Materials and methods. Three stock lots of a purified hyperimmune canine globulin concentrate(12) were used for serum titrations. All tests were made with the same stock virus.† The geometric mean titer (4.81 EID_{50}/0.1 ml) of this virus as determined from 40 titrations was used to test the validity of experimental results.

The original technic(1) for the *in ovo* test was modified as follows: phosphate buffered saline diluent, pH 7.2, containing 200 units of penicillin and 200 μg of streptomycin was substituted for 10% inactivated horse serum; serum-virus mixtures and virus concentrations used for neutralization were held in an

* A Division of Dow Chemical Co.

† Thirty-first egg passage of Lederle Encephalitis Strain, received from J. A. Baker, Cornell University as a pool of egg passages 40-45.

ice bath (0°C) until inoculation of eggs; virus concentrations were diluted for titration just prior to inoculation of eggs; and eggs were inoculated by the chorioallantoic membrane (CAM) inoculation technic of Gorham (16) and incubated at 35°C.

The concentrations of test virus were mixed in equal volume with 1 ml amounts of serial 5-fold dilutions of the 3 serum samples. Serum-virus mixtures and virus concentrations were held 18 hours at 0°C; 8- to 9-day fertile hen's eggs were then inoculated with 0.1 ml amounts of the various serum-virus mixtures and dilutions of the various virus concentrations which were made at this time. Egg membranes were examined after 6 days incubation, and titers calculated by the method of Reed and Muench(17).

To determine whether length of incubation of virus and serum-virus mixtures might influence results, these tests were repeated except that titrations in eggs were also made after 2 hours incubation at 0°C.

Results. Serum neutralization endpoints tended to increase with each 10-fold decrease in virus concentration, but not proportionately. In the first experiment, the 3 serums were tested simultaneously with the same virus suspensions, and their neutralization endpoints were similar at equivalent levels of virus. Calculated "antibody units" for these serums varied from approximately 50,000,000 with the highest concentration (8,600 EID_{50}) of virus to approximately 1,000,000 with the lowest (8.6 EID_{50}).

In the second experiment (Table I), the 3 serums were tested independently; thus the EID_{50} of virus are not the same. Serum titers tended to be somewhat higher after 18 hours than after 2 hours at 0°C.

Log virus EID_{50} *vs.* log serum ND_{50} did not follow the hypothetical slope 1 inverse linear relationship after either 18 hours or 2 hours incubation of serum-virus mixtures (Fig. 1 and 2).

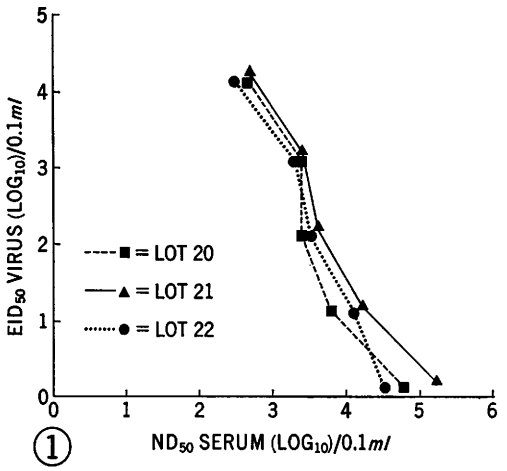
Results of repeated tests have been in accordance with those reported here.

Discussion. No ready explanation is apparent for the conflicting results between our tests and the reported inverse slope 1 relationship between virus EID_{50} and serum ND_{50}. The fact that this relationship was determined(5,11) after a shorter period of incubation for virus and serum-virus mixtures than the 24-hour period in the test as originally described(1) was considered. But the shorter period of incubation in the present experiments also did not give a slope of 1 (Fig. 2). The calculated results in Table I indicate that a mathematical interpolation cannot be accurately applied to standardization of test results.

In some instances we noted a difference that was significant statistically (*t*-test for paired samples(17)), between the 2-hour and the 18-hour titration of a given virus sample. For example, virus titrations for serum samples 20 and 22 (Table I) showed some loss of virus after 18 hours incubation as compared with the 2-hour determination. Although this loss of virus may be a factor in the higher neutralization titers at 18 hours, it apparently

TABLE I. Serum Titers with Various Virus Concentrations after 2-Hour and 18-Hour Incubation of Serum Virus Mixtures at 0°C.

Serum lot	Holding time, hr	ND_{50}/ml serum	EID_{50} virus	Calculated "antibody units"/ml
20	2	4,600	25,000	120,000,000
		5,600	2,500	14,000,000
		6,600	250	1,700,000
		63,000	25	1,600,000
		310,000	2.5	780,000
20	18	5,600	11,000	62,000,000
		28,000	1,100	31,000,000
		28,000	110	3,100,000
		63,000	11	690,000
		570,000	1.1	630,000
21	2	5,600	14,000	78,000,000
		5,600	1,400	7,800,000
		6,600	140	920,000
		6,600	14	92,000
		400,000	1.4	560,000
21	18	5,600	17,000	95,000,000
		28,000	1,700	48,000,000
		42,000	170	7,100,000
		160,000	17	2,700,000
		1,600,000	1.7	2,700,000
22	2	1,100	24,000	26,000,000
		4,600	2,400	11,000,000
		5,600	240	1,300,000
		48,000	24	1,200,000
		310,000	2.4	740,000
22	18	3,300	13,000	43,000,000
		19,000	1,300	23,000,000
		29,000	130	3,800,000
		140,000	13	1,800,000
		310,000	1.3	400,000



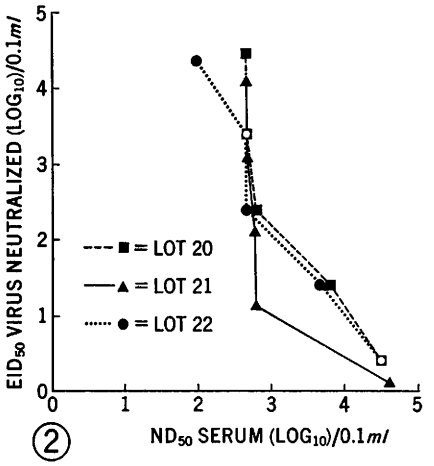


FIG. 1. Serum-virus mixtures incubated 18 hr at 0°C.

FIG. 2. Serum-virus mixtures incubated 2 hr at 0°C.

is a minor one, since the effect would be expected to be more pronounced with higher dilutions of virus, with an increase in the calculated "antibody units," rather than the decreases that actually occurred.

Standardized procedures that would permit greater reproducibility of results, and therefore more direct comparison of titrations by different laboratories, would be desirable. The present results indicate that any attempt to standardize the *in ovo* serum neutralization test would need to be made within a rather limited range of variation in amount of virus.

Virus EID_{50} ranges that might be used without materially affecting the observed titers are indicated by the vertical plateaus in Fig. 1 and 2. The 18-hour determinations showed little difference between ND_{50} values for any one serum between approximately 100 to 1,000 virus EID_{50}. For the 2-hour determinations, this range was between 100 to 10,000 EID_{50}. Use of these ranges would appear to assure a sufficient dose for infection without destroying the sensitivity of the test.

The following attempts, which we feel have definite advantages, have been made in our laboratories to control variation in the results of the test: (1) The phosphate-buffered saline diluent, which can be reproduced exactly, has been substituted for horse serum, greatly reducing the possibility of introducing variations in the test. (2) Test samples are incubated in an ice bath because this quickly brings them to a constant holding temperature, and little or no loss of virus occurs. (3) Only those tests are accepted as valid in which the virus dose falls within 100 to 1,000 EID_{50} per egg. (4) Only those tests are accepted as valid in which the results of the virus titration are within 2 standard deviations of the geometric mean titer. Repeated titrations of our test virus indicate that a sufficient number of titrations will permit the calculation of a geometric mean and standard deviation; for example, 95% of 190 titrations were within 2 standard deviations of the geometric mean of the first 40 titrations.

The use of a common standard test virus would contribute to reproducibility of test results by different laboratories. The inclusion of a standard reference serum in each test, as has been previously suggested(15), would be a step in the same direction. Either or both such standards might be maintained and supplied by the Agricultural Research Service of the USDA.

Summary. In experiments under precisely controlled conditions, an inverse linear slope 1 relationship between log canine distemper (CD) virus concentration and log CD neutralizing antibody titer was not demonstrated. The data indicate that a method of mathematical interpolation whereby serum titers are converted to "antibody units" will not be

in accord with experimentally determined *in ovo* test results. Possible standard procedures that might make possible direct comparison of test results reported by different laboratories are discussed.

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Renal Electrolyte Effects of Synthetic 18-Hydroxylated Steroids in Adrenalectomized Rats. (27398)

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The ability of the adrenal cortex to synthesize steroids with oxygen functions at carbon 18 has been recognized only in the past decade. The first evidence for such a mechanism was found in the structure of aldosterone (1,2), a corticosteroid oxygenated at both carbon 11 and 18(3). Additional evidence was derived from data of various *in vitro* studies using adrenal tissue, which demonstrated biosynthesis of other corticosteroids similarly oxygenated as in aldosterone(4,5) and of others with oxygen at carbon 18, but not at 11(6-13). The data have indicated, therefore, presence of a distinctive adrenal mechanism for oxidation at carbon 18.

Recently, Pappo reported on the synthesis of 18-hydroxylated derivatives of deoxycorticosterone and progesterone, to provide supplies for assay in laboratory animals. The data concerning renal electrolyte properties of 18-hydroxylated steroids, reported herein, are believed to be of interest as only limited biological data have appeared to date for this class of steroids(13).

Materials and methods. Mineralocorticoid and anti-mineralocorticoid activities of the steroids were investigated in acute experiments with adrenalectomized male rats (150-200 g body weight), which were maintained postoperatively for about 24 hours with sucrose cubes and tap water. In the assay for mineralocorticoid activity, groups of animals were injected subcutaneously with 2.0 ml isotonic saline and with deoxycorticosterone acetate (DCA) or test compounds suspended in 0.5 ml saline. The animals were placed in circular metabolism cages (4 rats/cage) after treatment for collection of a 4-hour sample of urine. Voiding was induced by application of pressure to the bladder at the beginning and end of the collection period to insure recovery of a complete urinary specimen. Each sample from a cage, including rinse water, was diluted to 50 ml for analyses of Na and K concentration. In the assay DCA produces Na retention, K loss and a reduction of the ratio of Na/K in the urine. All compounds were tested at several dosage levels in groups